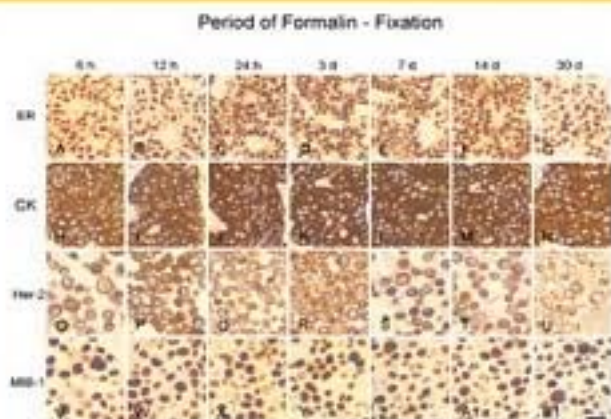


Wiley Series in Biomedical Engineering and Multi-Disciplinary Integrated Systems
Kai Chang, Series Editor

Antigen Retrieval Immunohistochemistry Based Research and Diagnostics



$$R\% = \int(P_{fpe}) / \int(P_f) \times 100\%$$

Edited by

SHAN-RONG SHI • CLIVE R. TAYLOR

ANTIGEN RETRIEVAL IMMUNOHISTOCHEMISTRY BASED RESEARCH AND DIAGNOSTICS

WILEY SERIES IN BIOMEDICAL ENGINEERING AND MULTI-DISCIPLINARY INTEGRATED SYSTEMS

Kai Chang, Series Editor

Advances in Optical Imaging for Clinical Medicine *Nicusor Iftimia,
William R. Brugge, and Daniel X. Hammer*

Antigen Retrieval Immunohistochemistry Based Research and
Diagnostics *Shan-Rong Shi and Clive R. Taylor*

ANTIGEN RETRIEVAL IMMUNOHISTOCHEMISTRY BASED RESEARCH AND DIAGNOSTICS

Edited by

SHAN-RONG SHI

CLIVE R. TAYLOR

 **WILEY**

A JOHN WILEY & SONS, INC., PUBLICATION

No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, scanning, or otherwise, except as permitted under Section 107 or 108 of the 1976 United States Copyright Act, without either the prior written permission of the Publisher, or authorization through payment of the appropriate per-copy fee to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, (978) 750-8400, fax (978) 750-4470, or on the web at www.copyright.com. Requests to the Publisher for permission should be addressed to the Permissions Department, John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, (201) 748-6011, fax (201) 748-6008, or online at <http://www.wiley.com/go/permission>.

Limit of Liability/Disclaimer of Warranty: While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. No warranty may be created or extended by sales representatives or written sales materials. The advice and strategies contained herein may not be suitable for your situation. You should consult with a professional where appropriate. Neither the publisher nor author shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages.

For general information on our other products and services or for technical support, please contact our Customer Care Department within the United States at (800) 762-2974, outside the United States at (317) 572-3993 or fax (317) 572-4002.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic formats. For more information about Wiley products, visit our web site at www.wiley.com.

Library of Congress Cataloging-in-Publication Data:

Antigen retrieval immunohistochemistry based research and diagnostics / [edited by] Shan-Rong Shi, Clive R. Taylor.

p. cm.—(Wiley series in biomedical engineering and multi-disciplinary integrated systems ; 1)

Summary: “An antigen is a substance that prompts the generation of antibodies and can cause an immune response. The antigen retrieval (AR) technique is used worldwide and has resulted in a revolution in immunohistochemistry (IHC). Featuring contributors who are distinguished experts and researchers in the field, this book discusses several scientific approaches to the standardization of quantifiable IHC. It summarizes the key problems in the four fields of antigen retrieval and provides practical methods and protocols in AR-IHC. Clinical pathologists, molecular cell biologists, basic research scientists, technicians, and graduate students, will benefit from this fully up-to-date work”—Provided by publisher.

Summary: “This book is based on the development and application of AR by the editors, one of whom is the inventor of AR, together with members of a world-leading research center of AR”—Provided by publisher.

ISBN 978-0-470-62452-4 (hardback)

1. Immunohistochemistry. 2. Antigens. I. Shi, Shan-Rong, 1936– II. Taylor, C. R. (Clive Roy)

QR183.6.A577 2010

616.07'56—dc22

2010024561

Printed in Singapore

10 9 8 7 6 5 4 3 2 1

PREFACE ix

CONTRIBUTORS xv

1

Shan-Rong Shi and Clive R. Taylor

Shan-Rong Shi and Clive R. Taylor

47

Shan-Rong Shi and Clive R. Taylor

75

Shan-Rong Shi, Kevin A. Roth, and Clive R. Taylor

5	STANDARDIZATION OF IMMUNOHISTOCHEMISTRY BASED ON ANTIGEN RETRIEVAL TECHNIQUE	87
	<i>Shan-Rong Shi and Clive R. Taylor</i>	
6	STANDARD REFERENCE MATERIAL: CELL LINE DEVELOPMENT AND USE OF REFERENCE CELL LINES AS STANDARDS FOR EXTERNAL QUALITY ASSURANCE OF HER2 IHC AND ISH TESTING	101
	<i>Bharat Jasani, Vicky Reid, Colin Tristram, Jeremy Walker, Paul Scorer, Michael Morgan, John Bartlett, Merdol Ibrahim, and Keith Miller</i>	
7	PEPTIDES AS IMMUNOHISTOCHEMISTRY CONTROLS	123
	<i>Steven A. Bogen and Seshi R. Sompuram</i>	
8	STANDARD REFERENCE MATERIAL: PROTEIN- EMBEDDING TECHNIQUE AND DESIGN OF BAR CODE	141
	<i>Shan-Rong Shi, Jiang Gu, and Clive R. Taylor</i>	
9	THE PROS AND CONS OF AUTOMATION FOR IMMUNOHISTOCHEMISTRY FROM THE PROSPECTIVE OF THE PATHOLOGY LABORATORY	151
	<i>David G. Hicks and Lorelee McMahon</i>	
10	IMAGE ANALYSIS IN IMMUNOHISTOCHEMISTRY	165
	<i>Alton D. Floyd</i>	
PART III	TISSUE/CELL SAMPLE PREPARATION	187
11	TISSUE CELL SAMPLE PREPARATION: LESSONS FROM THE ANTIGEN RETRIEVAL TECHNIQUE	189
	<i>Shan-Rong Shi and Clive R. Taylor</i>	
12	MECHANISMS OF ACTION AND PROPER USE OF COMMON FIXATIVES	195
	<i>Richard W. Dapson</i>	
13	CELL SAMPLE PREPARATION FOR CLINICAL CYTOPATHOLOGY: CURRENT STATUS AND FUTURE DEVELOPMENT	219
	<i>Yan Shi and Patricia G. Wasserman</i>	
14	DESIGN OF A TISSUE SURROGATE TO EXAMINE ACCURACY OF PROTEOMIC ANALYSIS	235
	<i>Carol B. Fowler, Jeffrey T. Mason, and Timothy J. O'leary</i>	

PART IV	MOLECULAR MECHANISM OF ANTIGEN RETRIEVAL TECHNIQUE	251
15	STUDY OF FORMALIN FIXATION AND HEAT-INDUCED ANTIGEN RETRIEVAL	253
	<i>Jeffrey T. Mason, Carol B. Fowler, and Timothy J. O'leary</i>	
16	A LINEAR EPITOPES MODEL OF ANTIGEN RETRIEVAL	287
	<i>Steven A. Bogen and Seshi R. Sompuram</i>	
17	pH OR IONIC STRENGTH OF ANTIGEN RETRIEVAL SOLUTION: A POTENTIAL ROLE FOR REFOLDING DURING HEAT TREATMENT	303
	<i>Shuji Yamashita</i>	
18	COMMENTARY: FUTURE DIRECTIONS	323
	<i>Timothy J. O'Leary, Carol B. Fowler, David L. Evers, Robert E. Cunningham, and Jeffrey T. Mason</i>	
PART V	PROTEOMIC ANALYSIS OF PROTEIN EXTRACTED FROM TISSUE/CELLS	333
19	TECHNIQUES OF PROTEIN EXTRACTION FROM FFPE TISSUE/CELLS FOR MASS SPECTROMETRY	335
	<i>Carol B. Fowler, Timothy J. O'Leary, and Jeffrey T. Mason</i>	
20	APPLICATION OF SHOTGUN PROTEOMICS TO FORMALIN-FIXED AND PARAFFIN-EMBEDDED TISSUES	347
	<i>Brian M. Balgley</i>	
21	VISUALIZING PROTEIN MAPS IN TISSUE	369
	<i>Masahiro Mukai and Mitsutoshi Setou</i>	
22	SYMBIOSIS OF IMMUNOHISTOCHEMISTRY AND PROTEOMICS: MARCHING TO A NEW ERA	391
	<i>Shan-Rong Shi, Brian M. Balgley, and Clive R. Taylor</i>	
APPENDIX	RELATED LABORATORY PROTOCOLS	399
INDEX		413

PREFACE

The purpose of this collection of contributions by experts in the field is to set forth current knowledge with respect to antigen retrieval (AR) and immunohistochemistry (IHC). In so doing, we hope to contribute to the ongoing evolution of these methods, and the development of greater reliability and reproducibility of IHC. Effective standardization of AR and IHC would lend improved capabilities to IHC when employed in a “special stain” capacity. In addition, effective standardization would allow the development of IHC methods into tissue-based immunoassays, having true quantitative capabilities, analogous to the ELISA method. In order to attain this latter capability, quantifiable reference standards are required to calibrate the IHC method and assessment of proper tissue preparation. This book deals with all of these complex issues in a manner designed both to inform and to stimulate further research, particularly with respect to how AR methods might be employed for improved test performance.

The two of us (Shan-Rong Shi and Clive Taylor) have worked towards these goals, together for two decades, coming to the problem from different directions, but walking down a common path.

I (Shi) have been asked many times the same question: “What made you think of boiling a slide in a microwave oven before doing immunostaining?” There is no short answer for this question. I would like to share my story of AR to honor those people who touched my life and helped me meet my career goals.

My interest in IHC began in 1981 when I went to Massachusetts Eye and Ear Infirmary (MEEI) and Massachusetts General Hospital in Boston as a research fellow under the guidance of Drs. Harold F. Schuknecht, Max L. Goodman, and Atul K. Bhan. One of my projects was focused on IHC staining using archival formalin-fixed paraffin-embedded (FFPE) tissue sections of nasopharyngeal carcinoma obtained from China. I was deeply impressed by the sharp staining contrast between the cancer cells and the background inflammatory cells highlighted by a series of cytokeratin markers. Without IHC, not a single malignant cell would be identified. Because of the great diagnostic potential of IHC demonstrated by this project, I decided to exploit the application of this technique on thousands of valuable samples of human temporal bone collected by Professor Schuknecht, a world-renowned Otologist

at MEEI. Although I tried many different IHC protocols with enzyme digestion for these archival formalin-fixed celloidin-embedded temporal bone sections, only moderate positive results were achieved with one antibody tested. This experience made me realize that the key point for successful IHC on archival formalin-fixed tissue sections was to find a method for the recovery of formalin-masked antigenicity, in the search for an AR approach.

In 1987, I had a research opportunity for a newly developed monoclonal antibody at InTek Laboratories, Inc., in Burlingame, California. This antibody was effective only on frozen sections, and I was asked to try to adapt it to FFPE tissue. At that time, enzyme digestion was the only option of choice, and it was not successful. As a result, I lost my job. I moved to a small room close to San Jose State University (SJSU), and in order to make a living, I started to work at a Chinese supermarket. I was insulted regularly by the sales manager, but these poor working conditions in a way inspired a strong feeling that I have never had before. I spent days and nights searching the literature at the library of SJSU, in order to answer what had become an obsession: “was formalin-masked antigenicity reversible or irreversible?” At that time online searching was not available. I read numerous volumes of the “index” page by page, taking notes line by line. I then looked for the journals one by one. In this way I searched all related literature regarding formalin and proteins starting from the most recent year back to 1940s. Finally, I found key clues to the answer in a series of studies published by Fraenkel-Conrat in the 1940s.¹⁻³ Their studies indicated that cross-linkages between formalin and protein could be disrupted by heating above 100°C or by strong alkaline treatment. However, I did not think of using high-temperature heating of FFPE tissue sections because I believed so strongly that high temperature denatures the protein.

In 1989, after much trying, I obtained a job interview at BioGenex Laboratories, San Ramon. That was a sunny afternoon. I met Dr. Marc E. Key, Director of Research, in his office. As soon as I sat down, he asked me: “What can you do for BioGenex?” I answered: “I intend to develop a new method which enables IHC to be performed on archival FFPE tissues.” He was interested in my answer, and told me: “Many people have tried to find such a way but they all failed. If you could succeed, you would become world-famous.” I was hired. Today, when I look back, I appreciated Marc and Dr. Krishan L. Kalra, President of BioGenex, for giving me the opportunity that made it possible for my dream to come true.

Shortly thereafter, Marc gave me an abstract⁴, and suggested that I drop zinc sulfate solution on FFPE tissue sections prior to IHC staining for enhancing IHC staining results. After multiple attempts following the reported protocol, I did not observe any improvement. At this most frustrating moment, a microwave oven sitting at the table near my desk caught my attention and reminded me of those long forgotten studies performed by Fraenkel-Conrat. Even though I still doubted their conclusions and worried that high temperature might destroy all the antigens on the tissue sections, I decided to give it

a try. I covered the FFPE sections with a few drops of zinc solution and heated them in the microwave oven for a few minutes. Unfortunately this attempt was not successful, because the solution evaporated. I decided to immerse the slides in a Coplin jar containing zinc solution and heated them twice in the oven for five minutes, in order to avoid drying the artifact during the boiling process. To my great surprise, I observed a significantly improved IHC staining signal with a clean background. I could not believe my eyes! I repeated the same experiment several times with similar results. This was “antigen retrieval (AR).”

The President of BioGenex, Dr. Kalra, invited three distinguished experts of IHC, Drs. Clive R. Taylor, Ronald A. DeLellis, and Hector Battifora to evaluate AR. They repeated this heat-induced AR protocol at their labs, and were all impressed by the great effects of this simple method. The first landmark article of AR was quickly accepted by Dr. Paul Anderson, Editor of the *Journal of Histochemistry and Cytochemistry* and published in 1991.⁵

At that time I started to work with Dr. Clive R. Taylor, Professor and Chairman of Pathology at the University of Southern California, Keck School of Medicine. Clive is a world renowned pioneer in archival IHC used for pathology since the early 1970s. With his kind help and support, I have been conducting a series of research projects on basic principles, further development, standardization and mechanisms of the AR technique. This work has yielded more than 40 peer reviewed articles and a book. Our AR research has been funded by NIH grant since 2001.

In 2000, we published *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology* attempting to summarize major achievements in this interesting field with a wish to stimulate further development of AR-IHC.⁶ Since then, the AR technique has been accepted not only by pathologists who routinely apply AR-IHC for daily pathologic diagnosis in surgical pathology, but also by all scientists who work with cell/tissue morphology worldwide. Because of the expanded application of AR-IHC, the philosophy embedded in this simple technique has created several approaches for further study. For this second AR-IHC book, we categorize the recent literature concerning the AR technique into five sections: recent advances of AR techniques and their application, standardization of IHC, tissue/cell sample preparation, molecular mechanism of the AR technique, and proteomic analysis of proteins extracted from tissue/cells. Our goal is to summarize current key issues in these five fields, to stimulate future studies. It is our intention to initiate research projects addressing several critical issues such as standardization and quantifiable IHC, a desired topic for targeted cancer treatment as emphasized by the American Society of Clinical Oncology/College of American Pathologists Guideline for human epidermal growth factor receptor 2 testing in breast cancer documented in 2007.

Our plan for editing this book was enhanced by the Histochemical Society Annual Meeting held at the Experimental Biology 2007 Meeting in Washington, DC. Several interesting workshops with respect to tissue fixation for molecular

analysis in pathology and cell biology, as well as tissue banking and sample preparation, were presented by world-renowned experts from Europe, the United States and Japan. We greatly appreciated all valuable presentations at these workshops that have been driving us in editing this book.

I (Taylor) find Shan-Rong Shi's story to be interesting in many ways, not least because during its course the conventional scientific dogma of the day, was overturned, by experimental evidence. When Shan-Rong first spoke to me, in his early days at BioGenex, of the notion of boiling deparaffinized sections in buffer, I assured him that, based on what I know of proteins (which turned out to be remarkably little) the method was unlikely to work. After all if one heats complement to just 56 degrees, it is inactivated. But lurking in the back of my mind there was just enough of my own experience, to temper that initial judgment. Almost two decades earlier, when I had first tried to "stain" immunoglobulins in formalin fixed paraffin embedded tissues, I too had been assured by those senior to me that it would not work. Examination of the literature also supported the view that it was doomed to failure, but with just a few glimmers of hope. Cold alcohol processing of paraffin embedded tissues (Sainte-Marie) did allow demonstration of some antigens by immunofluorescence.

I was then working on my D. Phil thesis in Oxford, under the mentorship of Alistair Robb-Smith, murine models of lymphoma and Hodgkin's disease. And I had problems. Already after just a year in the pathology department I was disconcerted to find that histopathology was not the definitive discipline that I had imagined, that it was subjective and that senior experienced pathologists could disagree vehemently with the diagnosis of a single slide. Recognition of the individual cells contributing to the development of "reticulum cells sarcomas" in my murine models was even more of a challenge, with differing criteria offered by almost every expert whom I consulted, or every paper that I read. I resolved to try immunologic identification of cells using the specificity of antibodies. Like Shan-Rong, I was inspired by the literature of the 1940s, Albert Coons, and Astrid Fagraeus, and the genesis of the immunofluorescence method. A long story, cut short, by switching from fluorescein to peroxidase labeled antibodies we circumvented the problem of "background" fluorescence in FFPE sections, greatly simplifying the task. With Ian Burns, we obtained our first positive results. The late Dr. David Mason joined me in Oxford shortly thereafter. With his healthy disbelief of most of what was written, we did, what I encouraged Dr. Shi to do 20 years later, we did the experiments, and they worked. This was "immunoperoxidase."⁷

In an exhilarating 2-year period we multiplied the world literature in the field, and then watched it grow exponentially. With the distant collaboration of Ludwig Sternberger we improved the "sensitivity." All that was left then, was to try multitudes of new anti-sera (polyclonal antibodies) and the new monoclonal antibodies that began to pour from labs worldwide. Some of these gave results on FFPE tissue sections, most did not, or at least gave poor or inconsistent results after prolonged tissue manipulations. Thus the world of

IHC was ripe for Dr. Shi's equally unconventional idea, and the time was ripe to perform the experiment. The outcome we all now know. Many antigens can be "retrieved." I have come to think of AR as "unfixation," and by the use of AR, IHC has become more straightforward and more widespread.

The very success of AR has, however, added to the problems of performing IHC in a reliable and reproducible manner. Less care is taken, than once it was, with fixation, processing, antibody selection and titration, because with AR the stain "works." In addition, many different labs perform IHC, treating it much like an H&E stain, without fully controlling the method, all because AR allows that to happen. Then the AR protocol itself has inevitably changed as others have sought to improve upon Shan-Rong's original formula. The result has been a proliferation of different AR methods, that allow the staining of many antigens, in diverse ways that certainly are not standard, and are difficult to reproduce exactly. While AR unarguably has improved the overall qualitative results of IHC, it has in some ways hindered the development of more quantitative methods that are necessary for "measuring" prognostic or predictive markers. For example ER or HER2 results can be converted from negative to positive, from weak to strong and back again, by different AR protocols. Thus for any particular analyte, where the goal is measurement, AR also must be standardized. This book presents the views of many experts with broad and diverse experience in AR and IHC, about how to consolidate the gains that have been made, and how to extend them for diagnosis and research.

Antigen Retrieval Immunohistochemistry Based Research & Diagnostics is intended for clinical pathologists, molecular cell biologists, basic research scientists, technicians, and graduate students who undertake tissue/cell morphologic and molecular analysis and wish to use and extend the power of immunohistochemistry. It is our hope that the readers will find it informative and useful.

ACKNOWLEDGMENTS

We greatly appreciate those people who have contributed to or are working on the development of the AR technique. We express our sincere appreciation to all contributors for writing excellent chapters for this book. Our appreciation also goes to Dr. Richard J. Cote, for his support and collaboration of research, and to Chen Liu, Lillian Young, Leslie K. Garcia, Carmela Villajin, and William M. Win for their technical assistance. The editors wish to express our deep gratitude for the active support of George J. Telecki, Lucy Hitz, Kellsee Chu, Stephanie Sakson, and the production and sales teams at John Wiley & Sons, and Best-Set Premedia. We also appreciate Lindsey Gendall and Wayne Yuhasz of Artech House, Inc. We are grateful for permission to reproduce illustrations and data of published materials from all publishers appearing in every chapter of this book.

I (Shi) greatly appreciate valuable clinical and research training in Sichuan Medical College (currently Huaxi Medical School of Sichuan University, Chengdu, China), and I also would like to thank those who have helped me during the most difficult time in my life, especially Drs. Iwao Ohtani, Masahiro Fujuta, Andrew C. Wong, Jimmy J. Lin, as well as Susan Price, and Victor Jang. It would have been impossible for me to develop this technique without their kindness.

Shan-Rong Shi, MD
Clive R. Taylor, MD, PhD

REFERENCES

1. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins. IV. Participation of indole groups. *J. Biol. Chem.* 1947; 168: 99–118.
2. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. *J. Biol. Chem.* 1948; 174: 827–843.
3. Fraenkel-Conrat H, Olcott HS. The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups. *J. Am. Chem. Soc.* 1948; 70: 2673–2684.
4. Abbondanzo SL, Allred DC, Lampkin S, et al. Enhancement of immunoreactivity in paraffin embedded tissues by refixation in zinc sulfate-formalin. Proc. Annual Meeting US and Canadian Acad. Pathol. Boston: March 4–9, 1990. *Lab. Invest.* 1990; 62: 2A.
5. Shi SR, Key ME, Kalra KL. AR in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
6. Shi S-R, Gu, J, Taylor CR. *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, Natick, MA: Eaton, 2000.
7. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*, 3rd Edition. Philadelphia: Elsevier Saunders, 2006.

CONTRIBUTORS

BRIAN M. BALGLEY, Chief Scientific Officer, Bioproximity, LLC, Annandale, VA

JOHN M.S. BARTLETT, Professor of Molecular Pathology, Edinburgh University Cancer Research Centre, Edinburgh, UK

STEVEN A. BOGEN, Medical Director, Clinical Chemistry, Tufts Medical Center, Boston, MA

ROBERT E. CUNNINGHAM, Histologist, Department of Biophysics, Armed Forces Institute of Pathology, Rockville, MD

RICHARD W. DAPSON, Dapson & Dapson, LLC, Richland, MI

DAVID L. EVERS, Armed Forces Institute of Pathology, Rockville, MD, and Veterans Health Administration, Washington, DC

ALTON D. FLOYD, ImagePath Systems, Inc., Edwardsburg, MI

CAROL B. FOWLER, Research Associate and Technical Director, Proteomics Facility, Department of Biophysics, Armed Forces Institute of Pathology, Rockville, MD, and Veterans Health Administration, Washington, DC

JIANG GU, Professor of Pathology, Dean, Shantou University Medical College, Shantou, and Professor, School of Basic Medical Sciences, Peking University, Beijing, China

DAVID G. HICKS, Professor and Director, Surgical Pathology Unit, Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY

MERDOL IBRAHIM, Manager, United Kingdom National External Quality Assessment Service Immunocytochemistry & In situ Hybridization, London, UK

BHARAT JASANI, Professor of Oncological Pathology, Head of Pathology, School of Medicine, Cardiff University, Cardiff, Wales, UK

JEFFREY T. MASON, Chairman, Department of Biophysics, Armed Forces Institute of Pathology, Rockville, MD

LORALEE McMAHON, Supervisor, IHC Laboratory, University of Rochester Medical Center, Rochester, NY

KEITH D. MILLER, Chief Scientific Officer, UCL-Advanced Diagnostics, Cancer Institute, Director of the UK National External Quality Assessment Scheme for Immunocytochemistry & In-situ Hybridisation and, Fellow of the Institute of Biomedical Science, London, UK

MICHAEL M. MORGAN, Department of Histopathology, University Hospital of Wales, Wales, UK

MASAHIRO MUKAI, Research Associate, Department of Frontier Bioscience, Hosei University, Tokyo, Japan

TIMOTHY J. O'LEARY, Deputy Chief Research and Development Officer and Director, Clinical Science R&D Service, Veterans Health Administration, Washington, DC

VICKY REID, R&D Programme Manager, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK

KEVIN A. ROTH, Robert and Ruth Anderson Professor and Chair, Department of Pathology, University of Alabama at Birmingham, Director of Alabama Neuroscience Blueprint Core Center, and Editor-in-Chief of *Journal of Histochemistry and Cytochemistry*, Birmingham, AL

PAUL SCORER, Senior Project Leader, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK

MITSUTOSHI SETOU, Professor, Department of Molecular Anatomy, Hamamatsu University School of Medicine, Shizuoka, Japan

SHAN-RONG SHI, Professor of Clinical Pathology, University of Southern California Keck School of Medicine, and Associate Editor of *Journal of Histochemistry and Cytochemistry*, Los Angeles, CA

YAN SHI, Clinical Assistant Professor, and Attending Cytopathologist, New York University, Langone Medical Center, New York, NY

SESHI R. SOMPURAM, V.P. Research, Medical Discovery Partners LLC c/o Tufts Medical Center, Boston, MA

CHIARA SUGRUE, Director, Clinical Laboratory Operations, Division of Cytopathology and Assistant Professor, Hofstra School of Medicine, North Shore-Long Island Jewish Health System, New Hyde Park, NY

CLIVE R. TAYLOR, Professor of Pathology, University of Southern California Keck School of Medicine and Editor-in-Chief, *Applied Immunohistochemistry and Molecular Morphology*, Los Angeles, CA

COLIN TRISTRAM, Innovations Manager, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK

JEREMY WALKER, Senior Research Scientist, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK

PATRICIA G. WASSERMAN, Senior Director, Division of Cytopathology, and Director, Cytopathology Fellowship Program, North Shore—Long Island Jewish Health System, Albert Einstein College of Medicine, New Hyde Park, NY

SHUJI YAMASHITA, Assistant Professor, Electron Microscope Laboratory, Keio University School of Medicine, Tokyo, Japan

PART I

RECENT ADVANCES IN ANTIGEN RETRIEVAL TECHNIQUES AND ITS APPLICATION

CHAPTER 1

STANDARDIZATION OF ANTIGEN RETRIEVAL TECHNIQUES BASED ON THE TEST BATTERY APPROACH

SHAN-RONG SHI and CLIVE R. TAYLOR

Following the development of the antigen retrieval (AR) technique in 1991,¹ hundreds of articles have been published worldwide that document its application in immunohistochemistry (IHC) for archival formalin-fixed, paraffin-embedded (FFPE) tissue sections. In addition, there are numerous articles that focus on standardization of the AR technique, stimulated by the current demand for a more quantitative method of IHC.²⁻⁶ The critical importance of standardization of antigen retrieval immunohistochemistry (AR-IHC) has been emphasized by the American Society of Clinical Oncology and the College of American Pathologists in their Guideline Recommendations for human epidermal growth factor receptor 2 (HER2) testing in breast cancer.⁷ The problem was, however, recognized and addressed to some degree much earlier. To optimize the results of AR-IHC in formalin paraffin sections, a “test battery” approach was proposed in 1996.⁸ The basic principle of this approach is based on the fact that two major factors influence the achievement of a satisfactory result of AR-IHC, namely, the heating condition (heating temperature $\times$ heating time) and the pH value of the AR solution (in which the FFPE tissue sections are immersed during heating).⁸⁻¹² In practice, it suffices to test the (new) primary antibody using three different pH values, ranging from low (acidic), moderate (neutral), and high (basic) buffer solutions (or other comparable commercial AR solutions) under three heating temperatures: low (below boiling), moderate (boiling), and high (pressure cooker or autoclave), to establish an optimal AR protocol for tested antibodies (Table 1.1). Subsequently, numerous investigators have demonstrated the advantages of using this simple test battery method. As emphasized by O’Leary,² the use of a “test battery” provides a rapid way to optimize AR for a particular antibody/antigen pair.

TABLE 1.1 Test Battery Suggested for Screening an Optimal Antigen Retrieval Protocol

	Tris–HCl Buffer		
	1.0–2.0	7.0–8.0	10.0–11.0
	(Slide #) ^a	(Slide #) ^a	(Slide #) ^a
Super-high (120°C) ^b	1	4	7
High (100°C), 10 min	2	5	8
Mid-high (90°C), 10 min ^c	3	6	9

^a One more slide may be used for control without AR treatment. Citrate buffer of pH 6.0 may be used to replace Tris–HCl buffer, pH 7.0–8.0, as the results are the same.

^b The temperature of super-high at 120°C may be reached by either autoclaving or microwave heating at a longer time.

^c The temperature of mid-high at 90°C may be obtained by either a water bath or a microwave oven monitored with a thermometer.

Reprinted with permission from Shi et al., *J. Histochem. Cytochem.* 1997; 45: 327–343.

Recent studies have further extended the application of this approach to establish and validate the optimal AR protocol for various antibodies (exemplified in Table 1.2) with different detection systems, employing a multi-tissue microarray (TMA) to achieve a rapid and accurate evaluation.^{26,27} It has become apparent that significant differences can be found in IHC staining results among various primary antibodies and different detection systems with the use of different AR protocols. For example, Pan et al.²⁷ evaluated the consistency of IHC staining for four antibodies to thyroid transcription factor (TTF)-1, manufactured by Dako, Zymed, Novocastra, and Santa Cruz, employing TMA blocks of 77 hepatocellular carcinomas and 334 nonhepatic epithelial tumors, using two solutions for AR treatment. Significantly different cytoplasmic IHC staining results were observed among different antibodies, as well as different AR solutions (e.g., Dako Target Retrieval Solution vs. ethylenediaminetetraacetic acid [EDTA] buffer at pH 8.0). In another study, Gill et al.²¹ standardized an AR method for IHC staining using antibody to a neuronal nuclear protein, NeuN, as the outcome measure. They compared three different pH values of AR solutions including low, middle, and high pH, with heating at three temperatures of 95, 100, or 105°C, for 15 or 20 min. They found that heating FFPE tissue sections in an alkaline pH buffer at high temperature gave the best results. The utility of the test battery approach used to establish optimal AR protocols has been demonstrated by abundant literature as summarized in Table 1.2.

The increasing attention directed to the adverse effects of variation in sample preparation upon the quality of IHC staining of FFPE tissues has served to reinforce the importance of determining the optimal AR method for each antibody/detection system/antigen to achieve optimal retrieval and optimal staining of tissues that may have been processed and stored in different and unknown ways (see Chapter 5 for details). Practically, in considering

TABLE 1.2 Randomly Selected Examples of Test Battery Approach Documented in Abundant Literature

Reference	Sample	Purpose and AR Method	Conclusion
Shi et al. ⁸	FFPE tissues of normal spleen, small cell lung ca. bladder ca. with comparable frozen tissues of bladder ca.	To establish an optimal AR protocol for poly- and monoclonal antibodies to retinoblastoma protein (pRB). Tris buffer at three pH values of 1, 6, and 10, heating at autoclave 120°C, MW 100°C, and 90°C for 10 min	An optimal AR protocol of boiling FFPE tissue sections in low pH (1–2) buffer for 10 min was established to achieve a maximal retrieval result.
Ferrier et al. ¹³	FFPE tissues of several tumor specimens with matched frozen tissues as comparison	To validate AR-IHC staining protocols for plasminogen activation system testing citrate buffer of pH 2.5, 4.5, and 6.0, 3 M urea, and Tris–HCl of pH 10.0, with MW heating at 97°C for 10–20 min	A pretest based on three different pH value (low, middle, and high) as a test battery is helpful to determine an optimal AR protocol.
Rocken and Roessner ¹⁴	Aldehyde-fixed and Epon-embedded autopsy tissues	To establish an optimal AR protocol for post-embedding IEM of amyloid detection, testing water, citrate buffer of pH 6.0, EDTA of pH 8 as AR solution heating at 91°C, 30 min, and combining etching	Application of test battery proved valuable in assessing appropriate AR protocol.
Shi et al. ¹⁵	FFPE tissues of bladder ca. and cell lines	To establish an optimal AR protocol for a polyclonal antibody to COX-2 (PG-27) using above-mentioned test battery approach	A reduced temperature AR protocol was established.

TABLE 1.2 *Continued*

Reference	Sample	Purpose and AR Method	Conclusion
Yano et al. ¹⁶	Tissues of insulinoma fixed in 2% glutaraldehyde, postfixed in 1% OsO ₄ , embedded in Epon	To establish an optimal AR protocol for detection of chromogranin A in ultrathin sections, testing three AR solutions of citrate buffer pH 6.0, EDTA buffer pH 8.0, alkaline solution pH 10.	Considerably improved efficiency of IHC was achieved by MW heating in pH 10 solution with IHC staining at 60°C.
Saito et al. ¹⁷	Aldehyde-fixed cultured <i>Helicobacter pylori</i> , embedded in Lowicryl K4M	Using the cultured bacteria as a model to establish optimal AR protocol for post-embedding IEM, based on comparison of heating conditions and various AR solutions: water, phosphate buffer pH 7.4, EDTA pH 7.2, Tris pH 10.0, urea pH 7.2, citric acid pH 6.0, commercial fluid pH 6.0, with heating at 121°C, 99°C, or 65°C	AR in Tris buffer solution of pH 10 showed better IHC staining results for ultrathin sections. AR method should be applied for routine use for post-embedding IEM.
Naito et al. ¹⁸	FFPE tissues of Alport's syndrome and normal portion from resected renal tumor	To establish optimal heating conditions for AR-IHC of mAb to α chains of collagen IV, testing autoclave heating at 105, 110, 115, 121, or 127°C for 6 min, or 127°C for 8 min with buffers of pH 3.5, 6, and 7.4	Heating at two or three different temperatures could be helpful for diagnosis; AR method extends the IHC diagnosis for Alport's syndrome.

TABLE 1.2 *Continued*

Reference	Sample	Purpose and AR Method	Conclusion
Kim et al. ¹⁹	Archival FFPE tissues of pathology	To investigate optimal AR protocols for 29 antibodies commonly used in pathology, testing 7 different buffers with variable pH value ranging from 2 to 9 under 2 heating conditions	Borate pH 8.0 or Tris pH 9.5 buffer combining with pressure-cooking heating method yielded the best results.
Choi et al. ²⁰	FFPE tissues of invasive aspergillosis from 16 pediatric cases, fixed in formalin for 6–72 h	To establish an optimal AR protocol for mAb WF-AF-1 (Dako), testing three different retrieval solutions of pH 6.0, 8.0, and 10.0 with MW heating for 10 min	Satisfactory IHC results are achieved using AR with high pH.
Gill et al. ²¹	Archival FFPE spinal cord tissue; both paraformaldehyde-fixed frozen rat spinal cord tissue and paraffin-embedded same tissue	To establish an optimal protocol for detection of low-abundance protein (NeuN) in human spinal cord FFPE tissue sections, testing three AR solutions of pH 6, alkaline, and acidic buffer, with three heating conditions: 95, 100, and 105°C	Heating FFPE tissue sections in an alkaline buffer yields most effective AR-IHC staining results.
Du et al. ²²	FFPE tissues of prostate ca., benign prostate hyperplasia, and breast disease	To find optimal AR protocols for IHC staining of p504s, p63, CD10, and Ki-67, testing citrate buffer pH 6.0, EDTA buffer pH 8.0, and 9.0 with MW heating at 700W for 12, 20, 25, 30 min	Different antigens require variable AR protocols. In general, most antibodies tested showed better results for pH 9.0.

TABLE 1.2 *Continued*

Reference	Sample	Purpose and AR Method	Conclusion
Luo et al. ²³	Archival FFPE tissues of normal or tumors	To establish optimal AR protocols for 30 commonly used antibodies, testing 9 AR protocols	Different antigens require variable AR protocols to reach the best IHC staining results.
Ge et al. ²⁴	Murine pancreas and other organs fixed in 10% neutral buffered formalin (NBF) for 6–24h, embedded in paraffin	Searching for an AR protocol that works with a variety of tissues and antigens, testing AR solutions of Vector buffer pH 6, Tris buffer pH 7.5 (+0.1% Tween-20) with low-and high-power MW heating	Low-power heating AR protocol provides a successful IHC detection for several key antigens in the pancreas.
Slater and Murphy ²⁵	FFPE prostate cancer and benign tissue sections from pathology	To establish optimal AR protocol for studying relationship of IL-6 and growth hormone, testing three AR solutions of pH 10.0, 7.0, and 2.0, with four heating temperatures of 100, 90, 80, and 70°C	No positive IHC results using AR solutions of pH 7 or 10, but good result was obtained at pH 2, with heating at 80°C for 50min.
Lyck et al. ²⁶	Two tissue arrays of predominantly aldehyde-fixed, paraffin-embedded brain tissues, fixed in variable times ranging from 1 day to 10 years	To identify antibodies and protocols that could visualize neurons and glia for quantitative studies, testing 29 antibodies, 4 AR buffers: Tris–EGTA pH 9.0, citrate buffer pH 6.0, and 2 commercial solutions with several heating conditions of MW heating	Application of IHC for quantitative studies of human brain tissue is possible with careful selection of staining method in well-preserved specimens.

Note: All tissue samples are human source unless otherwise noticed.

Ca., carcinoma; MW, microwave.

the busy workload in a clinical service laboratory, we recommend a two-step procedure based on the typical design of a test battery (Table 1.1): in the first step, test three AR solutions at different pH values under one heating condition (100°C for 10 min) to find the optimal pH value; in the second step, test optimal heating conditions based on the optimal pH identified in step 1.²⁸ Similarly, Hsi²⁹ recommended using microwave pressure cooker as the standard heating condition for testing two commonly used AR solutions, citrate buffer of pH 6.0 and EDTA solution at pH 8.0, along with protease digestion. With the goal of identifying the optimal AR protocol for a new primary antibody, they used five different concentrations of the antibody, including the manufacturer's recommended dilution, plus two serial twofold dilutions above and below this concentration. As seen in Table 1.2, many investigators have already accepted the basic principle of test battery, incorporating three levels of pH values and three heating conditions (Table 1.1). However, within this model, different investigators have used different heating methods and different AR methods to achieve optimal results for their individual laboratories. With this broad variety of approaches, clearly, we are a long way from achieving a universal method, even if such is possible.

1.1 SEARCHING FOR NOVEL CHEMICAL SOLUTIONS

Namimatsu et al.³⁰ reported a novel AR solution containing 0.05% citraconic anhydride, pH 7.4, for heating FFPE tissue sections at 98°C for 45 min. They compared the IHC staining results using 62 commonly used antibodies and other conventional AR protocols (0.01 M citrate buffer, pH 6.0 in a pressure cooker; or 0.1 M Tris-HCl buffer containing 5% urea, pH 9.0 microwave heating for 10 min), and found that most antibodies showed stronger intensity with the new method. In particular, some difficult-to-detect antigens such as CD4, cyclin D1, granzyme β , bcl-6, and CD25 gave distinct IHC staining signals only by using the new protocol, leading to a claim that the method might be a candidate for the “universal” approach.

We therefore tested Namimatsu's protocol and also obtained satisfactory results.³¹ Among 30 antibodies tested, more than half (53%) showed a stronger intensity of IHC when using the citraconic anhydride for AR, as compared to citric acid buffer, whereas 12 antibodies (43%) gave equivalent results. There was only one antibody (OC-125) that, in our hands, gave a stronger intensity using conventional citric buffer for AR. When using citraconic anhydride for AR, the heating conditions of boiling (100°C) or less than boiling (98°C) temperature yielded identical results for most antibodies tested (90%). However, 3 of 30 antibodies showed lower intensity at 100°C. In addition, some antibodies showed nonspecific background staining at 100°C. In particular, we demonstrated that when using antibody to retinoblastoma protein (pRB), the new protocol had advantages over a previously published low pH

TABLE 1.3 Comparison of pRB-IHC between Frozen and Paraffin Sections Using Four Protocols of AR

Sample	Frozen Section	FFPE Section with Antigen Retrieval			
		Acetic Buffer pH 1–2, 100°C	Citraconic Anhydride 100°C	Citraconic Anhydride 98°C	Citrate Buffer pH 6.0, 100°C
T24	+++	+++,>50%	+++,>50%	+++,>50%	+++,>50%
J82	+	+,>10%	+,>10%	+,>10%	±,<10%
Case 1	—	—	—	—	—
Case 2	Nuclear +++, >50%	+++,>50%	+++,>50%	+++,>50%	++,<50%
Case 3	Perinuclear++, >50%	+,>50%	++,>50%	++,>50%	+,<50% ^a
Case 4	Nuclear +++, >50%	++,>50%	+++,>50%	+++,>50%	++,<50%

Notes: T24 and J82 are cell lines of bladder cancer. Cases 1 to 4 are specimens of human bladder cancer.

^aAlthough peripheral area of the slide showed a percentage of positive staining about 50%, the central area of the slide showed significantly weak positive result.

Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

protocol,⁸ including superior morphologic preservation, greater reproducibility, and more intense staining signal.

As a further motivation, there is evidence that establishing the optimal AR protocol will also contribute to standardization of IHC, through “equalizing” variable IHC staining results obtained following different times of formalin fixation. In the light of the studies described above, further studies were conducted as to the utility of the citraconic anhydride method.

First Step: A comparative study of IHC staining for pRB was carried out using paired sections of frozen versus FFPE cell/tissue samples, comparing citraconic anhydride as the AR solution under two different temperatures (98°C vs. 100°C), with solutions of low pH buffer (acetate buffer, pH 1–2) and citrate buffer (pH 6.0). Findings are summarized in Table 1.3. Conventional citrate buffer yielded inconsistent and weaker signals for all specimens, except the cell line T24 (Table 1.3, Fig. 1.1). Stronger intensity was found in pRB-positive cases, while using the citraconic anhydride for AR (Fig. 1.1), although more nonspecific background staining was observed using citraconic anhydride under boiling condition (Fig. 1.1, C vs. D, and R vs. S).

Second Step: For further evaluation, a comparative IHC study was performed using citraconic anhydride and conventional AR protocols with a TMA of 31 cases of bladder cancer. Findings are summarized in Table 1.4. Only 27 cases were available for evaluation due to loss of tissue cores for four cases. Among 27 cases, there were 6, 8, and 13 cases for strong, moderate

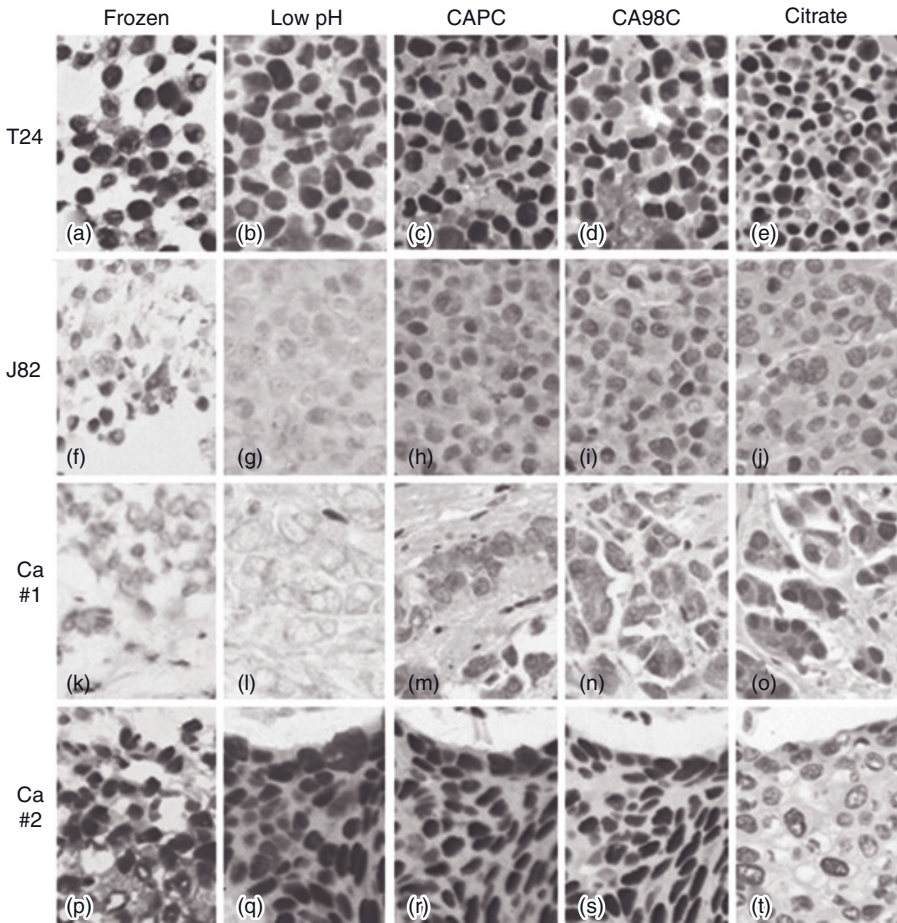


Figure 1.1 Comparison of pRB-IHC staining results for frozen and FFPE tissue sections using four AR protocols. All images are arranged in the same order as given in Table 1.3, indicating all scores indicated in the table. T24 and J82 are two cell lines, Ca #1 and Ca #2 are specimens of human bladder cancer, frozen means frozen cells or tissues fixed in acetone, other terms listed in the top line represent FFPE tissue sections after various AR treatments: Low pH, AR solution at low pH value; CAPC, citraconic anhydride solution with boiling; CA98C, citraconic anhydride solution with heating at 98°C; citrate, conventional boiling heating with citrate acid buffer at pH 6.0. Original magnification $\times 200$. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309. See color insert.

positive, and negative pRB-IHC, respectively. Identical percentages of pRB-positive nuclei were found in all cases, using either of the two protocols for citraconic anhydride or the low pH solution for AR. Inconsistent and significantly weaker nuclear pRB staining results were found when using citrate buffer of pH 6.0 for AR (Table 1.4; Fig. 1.2).

TABLE 1.4 Comparison of pRB-IHC in 27 Cases of FFPE Tissues of Bladder Cancer Using Four Protocols of AR

Cases	AR Protocols	IHC Results			Cases	AR Protocols	IHC Results		
		Intensity	%	Conclusion			Intensity	%	Conclusion
1	CA 98°C	+++	>10	+	12	CA 98°C	++	>10	+
	CA PC	+++	>10	+		CA PC	+++	>10	+
	Low pH	++	>10	+		Low pH	++	>10	+
	Citrate	+	<10	–		Citrate	+	>10	+
2	CA 98°C	+++	>50	++	13	CA 98°C	–	<10	–
	CA PC	+++	>50	++		CA PC	–	<10	–
	Low pH	++	>50	++		Low pH	–	<10	–
	Citrate	+	>10	+		Citrate	–	<10	–
3	CA 98°C	–	<10	–	14	CA 98°C	+	>10	+
	CA PC	–	<10	–		CA PC	++	>10	+
	Low pH	–	<10	–		Low pH	+	>10	+
	Citrate	–	<10	–		Citrate	–	<10	–
4	CA 98°C	–	<10	–	15	CA 98°C	–	<10	–
	CA PC	–	<10	–		CA PC	–	<10	–
	Low pH	–	<10	–		Low pH	–	<10	–
	Citrate	–	<10	–		Citrate	–	<10	–
5	CA 98°C	–	<10	–	16	CA 98°C	–	<10	–
	CA PC	–	<10	–		CA PC	–	<10	–
	Low pH	–	<10	–		Low pH	–	<10	–
	Citrate	–	<10	–		Citrate	–	<10	–
6	CA 98°C	–	<10	–	17	CA 98°C	+++	>50	++
	CA PC	–	<10	–		CA PC	+++	>50	++
	Low pH	–	<10	–		Low pH	+++	>50	++
	Citrate	–	<10	–		Citrate	++	>50	++
7	CA 98°C	++	>10	+	18	CA 98°C	–	<10	–
	CA PC	+++	>10	+		CA PC	–	<10	–
	Low pH	++	>10	+		Low pH	–	<10	–
	Citrate	+	>10	+		Citrate	–	<10	–
8	CA 98°C	–	<10	–	19	CA 98°C	++	>50	++
	CA PC	–	<10	–		CA PC	+++	>50	++
	Low pH	–	<10	–		Low pH	+++	>50	++
	Citrate	–	<10	–		Citrate	++	>10	+
9	CA 98°C	–	<10	–	20	CA 98°C	++	>10	+
	CA PC	–	<10	–		CA PC	+++	>10	+
	Low pH	–	<10	–		Low pH	++	>10	+
	Citrate	–	<10	–		Citrate	+	>10	+
10	CA 98°C	++	>50	++	21	CA 98°C	++	>50	++
	CA PC	+++	>50	++		CA PC	+++	>50	++
	Low pH	+	>50	++		Low pH	++	>50	++
	Citrate	+	>10	+		Citrate	+	>10	+
11	CA 98°C	++	>10	+	22	CA 98°C	–	<10	–
	CA PC	++	>10	+		CA PC	–	<10	–
	Low pH	+	>10	+		Low pH	–	<10	–
	Citrate	±	<10	–		Citrate	–	<10	–

TABLE 1.4 Continued

Cases	AR Protocols	IHC Results			Cases	AR Protocols	IHC Results		
		Intensity	%	Conclusion			Intensity	%	Conclusion
23	CA 98°C	++	>10	+	26	CA 98°C	–	<10	–
	CA PC	++	>10	+		CA PC	–	<10	–
	Low pH	+	>10	+		Low pH	–	<10	–
	Citrate	+	<10	–		Citrate	–	<10	–
24	CA 98°C	–	<10	–	27	CA 98°C	++	>10	+
	CA PC	–	<10	–		CA PC	+++	>10	+
	Low pH	–	<10	–		Low pH	++	>10	+
	Citrate	–	<10	–		Citrate	+	>10	+
25	CA 98°C	++	>50	++					
	CA PC	+++	>50	++					
	Low pH	++	>50	++					
	Citrate	+	>10	+					

Notes: CA98°C, heating tissue sections in 0.05% citraconic anhydride at 98°C for 45 min; CAPC, heating tissue sections in 0.05% citraconic anhydride in a plastic pressure cooker using microwave oven for 30min; Low pH, heating tissue sections in acetic buffer of pH 1–2 for shorter time as described in the text; Citrate, conventional citrate acid buffer 0.01 M at pH 6.0 with same heating condition of a plastic pressure cooker described above.

Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

Third Step: The Western blotting technique, applied to cell extracts, was used to confirm the pRB immunostaining results in two bladder cancer cell lines of T24 and J82, giving quantitative results for pRB in the two cell lines, comparable with that demonstrated by IHC (Fig. 1.3).

Although the novel AR protocol using citraconic anhydride improved the intensity of IHC on FFPE tissue sections for more than half of the antibodies tested, compared to that achieved by other conventional AR protocols, not all antibodies benefitted, which would argue that the citraconic anhydride method does not serve as a truly universal AR protocol. Indeed, many investigators (Table 1.2) have concluded that different antigens may require different “specific” AR protocols. In this respect, the “test battery” is a convenient and cost-effective method for assessing the appropriate AR protocol.^{2,8} Nevertheless, the present data certainly support inclusion of the citraconic anhydride AR method in such a “test battery.” With respect to the two heating temperatures for citraconic anhydride, the ultimate choice of method for any laboratory may depend on the equipment available.

In a study involving decalcified FFPE rat joint tissue sections and a variety of AR methods, Wilson et al.³² reported successful application of 0.2M boric acid at pH 7.0 as the AR solution combining a low-temperature incubation (60°C for 17h). The principal advantage of this AR protocol was that it minimized lifting or loss of decalcified hard tissue sections from charged slides. Their basic approach for establishing an optimal AR protocol was a “test battery” as described above. In a separate series of studies, based upon prior

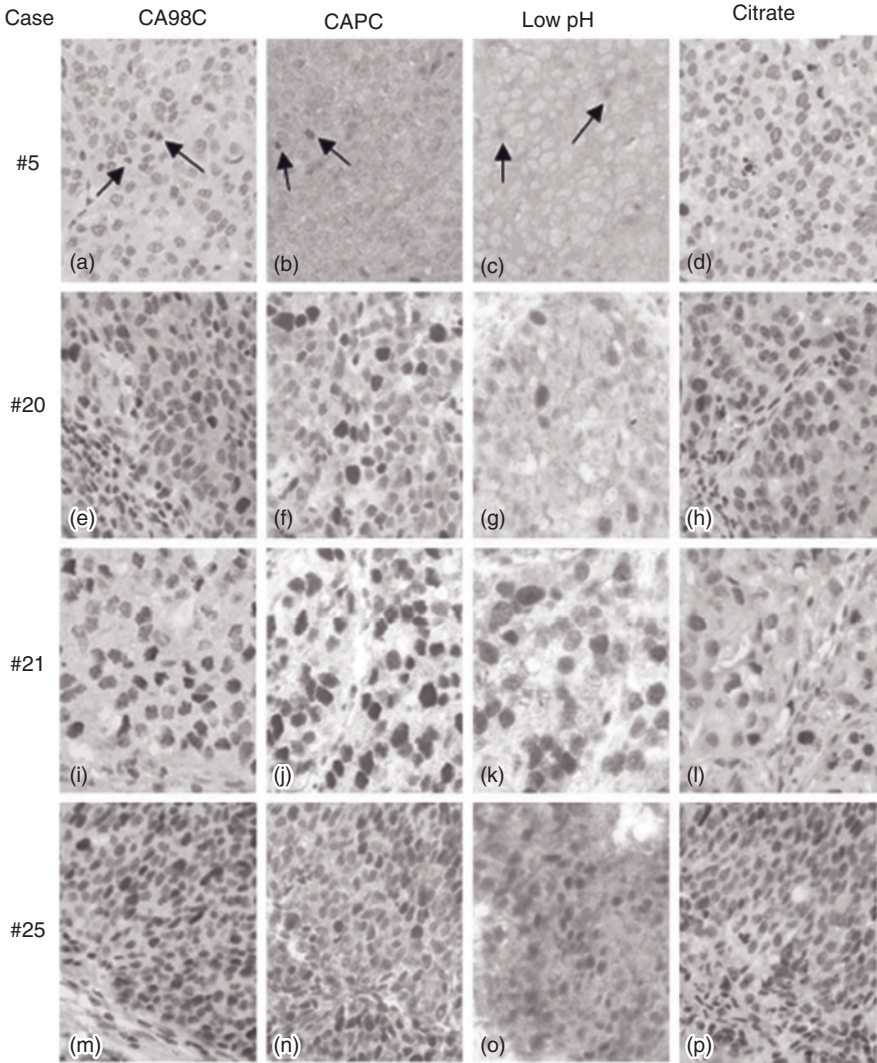


Figure 1.2 Examples of immunostaining intensity from comparison of pRB-IHC in 27 cases of FFPE tissues of bladder cancer (Table 1.4). (A–D) Negative (<10%) showing a few weak positive nuclei (arrows); (E–H) moderate positive (>10%); (I–P) strong positive (>50%). Arrows indicate positive nuclear staining for some lymphocytes or other stromal cells as an internal control. Note the lack of nuclear hematoxylin counterstaining due to low pH AR treatment. The order of cases are indicated in Table 1.4. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309. See color insert.

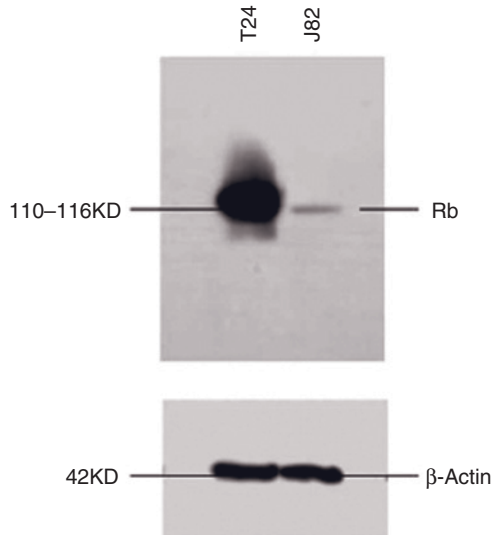


Figure 1.3 Western blotting of pRB protein extracted from two fresh cell lines, T24 and J82. The pRB proteins in fresh T24 cell line showed a stronger band than that obtained from J82 cell line. The Western blotting results correlated well with IHC staining intensity (Table 1.3 and Fig. 1.1). Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

literature,^{33,34} and with the goal of reducing tissue damage due to boiling during AR, Frost et al.³⁵ compared a microwave boiling AR protocol and a combination AR protocol that included predigestion in 0.1% trypsin in phosphate-buffered saline (PBS) for 15min, followed by low-temperature heating in a water bath at 80°C for 2h. Although tissue damage was reduced by using the low-temperature AR protocol, not all antigens could be recovered equally by this method. They concluded that prior to setting up a new IHC stain, it is critical to assess AR protocols, and primary antibody concentrations as well as detection systems, their standard end point was that method giving the strongest IHC staining signal (maximal retrieval level). In addition, Frost and colleagues also emphasized that the IHC results should be correlated with clinical behavior of diseases in order to provide data that are directly useful for treatment. With a similar principle in mind, Umemura et al.³⁶ undertook a comparative study of IHC evaluation of hormone receptor status for 861 breast cancer samples with data from IHC and biochemical methods. They demonstrated that optimizing the AR treatment, primary antibodies, and detection systems significantly affects technical validation of IHC data for hormone receptors. They emphasized that the cutoff point should be set higher to reflect the increasing IHC “scores” achieved by more sensitive IHC method, based on the correlation of biochemistry and IHC, as well as clinical follow-up data.

1.2 ANTIBODY AND DETECTION SYSTEM -DEPENDENT TEST BATTERY

Numerous recent articles have emphasized that the application of test battery for establishing an optimal AR protocol is also dependent on the primary antibody and the subsequent detection system. In other words, if an optimal AR protocol is good for antibody clone “1” to protein “A” employing detection system “B,” it is not necessarily good for antibody clone 2 to protein A, using the same or different detection systems, but a different AR protocol might give acceptable results. In this respect AR, while in some respects “leveling the playing field” so that many antigens may be detected, in some instances does add yet another variable to achieving consistency among different laboratories. For example, Pan et al.²⁷ found variable cytoplasmic IHC staining results of TTF-1 for hepatocellular carcinoma, which depended on different sources of the primary antibody and different AR methods. However, they only tested two conditions of AR. Similarly, Slater and Murphy²⁵ showed great variation in the effectiveness of different AR protocols for IHC staining of an anti-mouse IL-6 antibody (purchased from R&D System, MN, USA) using three AR solutions (pH values of 10.0, 7.0, and 2.0) and four heating conditions (100°C for 10 min, 90°C for 30 min, 80°C for 50 min, 70°C for 1 h). They finally found that there was no staining for IL-6 when using AR solution at pH 10.0 or 7.0 but obtained positive IHC staining at pH 2.0 heated at 80°C for 50 min. Higher temperature heating of 100°C resulted in damage of tissue sections, while lower temperature of 70°C resulted in weak IHC staining.

Again using the test battery principle, Kim et al.³⁷ compared IHC staining results of two monoclonal antibodies to CD4 (clone: YG23 and 1F6) and three monoclonal antibodies to CD8 (clone: YG20, DN17, and 1A5) on archival FFPE tissue sections using eight different AR solutions at pH values ranging from 2 to 10, combining two heating conditions (heating in a microwave oven vs. heating in a pressure cooker). They found that among five monoclonal antibodies tested, only 1F6 (CD4), and 1A5 (CD8) worked on FFPE tissue sections, and that an AR solution of borate at pH 8.0, containing 1 mM EDTA, and 1 mM NaCl yielded the best IHC staining results for CD4 and CD8. Note, however, that according to their data, it is clear that the use of Tris buffer at higher pH (9–10) also provides satisfactory IHC staining intensity for these two antibodies, a finding having extensive support in the published literature.^{14,16,20,21,38–43} Kim et al.¹⁹ also studied seven AR solutions at pH ranging from 2 to 10 for 29 commonly used antibodies and concluded that the optimal AR protocol depends on the particular antibody tested; therefore, the best AR solution should be sought for each antibody, and there is no “universal” approach, nor does AR add reproducibility among laboratories in this context.

Vassallo et al.⁴⁴ compared two routinely used antibodies of estrogen receptor (ER), 1D5 (Dakopatts [Carpinteria, CA], code E7101) and 6F11 (Newmarker [Fremont, CA], code MS391-S1) by using two AR protocols,

citrate buffer at pH 6.0 and Tris-EDTA at pH 8.9. For IHC staining, they adopted three different detection systems, EnVision, EnVision Plus, and labeled streptavidin-biotin (LSAB) peroxidase complex (all three systems purchased from Dakopatts). In their study, antibody 6F11, using the citrate AR protocol with EnVision, yielded a poorer IHC signal than that obtained by using Tris-EDTA solution for AR treatment. Kan et al.⁴⁵ did a similar comparative study to evaluate the efficacy of different AR protocols, using sodium citrate, citric acid, Tris-HCl, and EDTA buffers of pH 4, 6, and 8, with four different clones of monoclonal antibodies for microtubule-associated protein (MAP)-2-IHC. Staining on FFPE guinea pig brain tissue sections, they found that satisfactory IHC staining was obtained only when MAP-2 antibody clone AP18 was used with the use of AR heating in citric acid buffer of pH 6.0. Gutierrez et al.⁴⁶ tested the immunoreactivity of 25 monoclonal antibodies to different leucocyte antigens on FFPE tissue sections, with differing fixation conditions. Employing the test battery approach and the biotin-tyramide amplification system, they concluded that all 25 antibodies tested were readily detectable using an appropriate combination of antibody, AR method, and signal amplification system. Again, no method was optimal for all.

1.3 APPLICATION OF TMA TECHNIQUE FOR TEST BATTERY

Multi-tissue technique has been used for many years in IHC staining to screen numerous samples on one single slide.⁴⁷⁻⁴⁹ Based on these early observations, TMAs were introduced in IHC for rapid study and to economize in the use of expensive reagents.⁵⁰ The TMA technique has the advantage of collecting hundreds of tissue samples on one single slide and provides the additional advantage of increasing the uniformity of staining across the TMA tissues, by reducing diversity of staining signals that result from separate staining of hundreds slides, perhaps on different days, by different technologists. Recent cooperative studies among multiple research centers, such as the BrainNet Europe Consortium, demonstrated the possibility of using the TMA technique in standardization of AR-IHC to achieve reliable results between different laboratories.⁵¹ A multi-tissue “spring-roll” section provided a foundation for standardization of AR-IHC based on giving improved reproducibility and performance of AR-IHC staining results.⁵² Camp et al.⁵³ validated the availability of TMA using three common antigens (ER, progesterone receptor [PR], and HER2) in FFPE tissue sections of invasive breast carcinoma and demonstrated that many proteins retained antigenicity for longer than 60 years using optimal AR pretreatment. Based on numerous studies, a combination of tissue array with AR technique provides an approach to optimize the use of archival FFPE tissue sections with a variety of fields.⁵⁴ The advantages are further enhanced by the application of recently developed image analysis software (AQUA) that is designed for quantitative IHC in TMA using an automatic scan.⁵⁵

1.4 SCIENTIFIC ACCURACY OF IHC RELYING ON OPTIMAL AR PROTOCOL

As described above, an optimal AR protocol established by test battery approach produces the best IHC result, defined as the maximal retrieval level (see Chapter 5). It is worthy to note, although not surprising, that not only is “intensity” of staining affected by the choice of the AR method, but also in some cases the distribution and pattern of staining. For example, Mighell et al.⁵⁶ demonstrated that fibronectin protein expression pattern, using a polyclonal antibody, was dependent on methods of AR. They used archival FFPE specimens of oral pyogenic granuloma and fibroepithelial polyp, and compared four AR protocols: combinations of enzyme digestion, microwave boiling in citrate buffer, or Tris–HCl buffer at pH 6 or 7.8, and autoclave. They found that after enzyme digestion, there was intense IHC staining in vascular endothelial cells but no staining or minimal staining in connective tissue; in contrast, microwave AR yielded IHC positive staining in connective tissue but no specific vascular staining, while autoclave AR showed positive staining in connective tissue and epithelial nuclei. Comparing these findings with the patterns obtained on frozen tissue sections, there was positive labeling in both vascular endothelial cells and connective tissue. They postulated that different protocols might expose different epitopes. The findings again emphasize the need for optimizing AR for IHC staining in FFPE tissue, while highlighting the concern that AR, when applied without rigorous validation, in fact increases variability observed in IHC staining. Potential causes of these diverse IHC patterns were discussed, including such possibilities as cross-reactivity of the different antibody species within the polyclonal antibody. It is critical to emphasize the fact that variable protein expression patterns may result from different AR protocols, and caution must be taken to avoid misinterpretation. Subsequent published studies obtained somewhat contrasting results.^{57,58} Yamashita and Okada⁵⁸ studied the mechanism of heat-induced AR employing SDS-PAGE, Western blotting, and IHC. They adopted the same rabbit polyclonal antibody to fibronectin (F-3648, Sigma [St. Louis, MO]) as used by Mighell et al.⁵⁶ and found that heating FFPE tissue sections in pH 9.0 buffer solution yielded strong positive fibronectin staining along the basal lamina in the hepatic sinusoid of mouse liver tissue, but no staining when using pH 6.0 buffer. Moreover, they found that boiling FFPE tissue sections in pH 9.0 buffer, followed by heating in pH 6.0 buffer also gave absent or minimal staining. However, boiling the same FFPE slide in pH 9.0 buffer could achieve strong positive staining of fibronectin, suggesting that the pH of AR solution may be an essential factor for proper refolding of epitopes to react with antibodies (see Part IV for details on the study of mechanism of AR).

The generation of artifacts has also been an intermittent concern. Hayashi et al.⁵⁹ reported a heat-induced artifact for conversion of Amadori products of the Maillard reaction to *N*^ε-(carboxymethyl) lysine that had the potential to affect IHC staining. However, among thousands of articles pertaining to

numerous antigen/antibody combinations based on AR-IHC in FFPE tissue sections, “false-positive staining” has not been convincingly demonstrated. Nevertheless, caution must be exercised when evaluating a new antibody using AR-IHC staining procedure for FFPE tissue sections. The following issues should be kept in mind to minimize unexpected or spurious staining results: (1) understanding the specificity of the antigen/antibody under test and the distribution in cells/tissues based on information provided by biochemical research; (2) examination of previous IHC staining reports in fresh cell/tissue samples pertaining to this antibody; (3) staining of negative control FFPE tissue section under identical AR treatment but omitting the primary antibody; (4) critical morphological analysis to confirm that observed patterns of distribution are consistent with other known information relating to pathology, molecular biology, and clinical outcome; and (5) in suspicious cases, further confirmation should be sought by using other methods such as Western blotting to confirm the IHC result as emphasized by Wick and Mills.⁶⁰

1.5 ACCURACY OF AR-IHC AS DEMONSTRATED BY IEM AND OTHERS

In recent years, with more accurate quantitative methods, numerous immunoelectron microscopic (IEM) studies have validated the application of AR in archival Epon or other plastic material embedded tissues fixed in aldehyde, plus other fixatives such as osmium tetroxide.^{16,57,61–63} Ramandeep et al.⁶² designed an interesting study using *Escherichia coli* DH5 α cells as a test model, based on quantitative measurements of immunogold labeling IEM, compared to enzyme-linked immunosorbent assay (ELISA) data, to optimize various tissue processing and IEM procedures including AR. They demonstrated that AR can achieve approximately 90–100% retrieval efficiency for osmium-postfixed material, a very interesting finding because cell/tissue samples postfixed with osmium provide the best preservation of ultrastructural morphology for IEM study. Hann et al.⁵⁷ carried out a quantitative IEM study based on carefully counting gold labeling particles of collagen IV and fibronectin in the basement membrane underlying the cells of Schlemm’s canal from archival aldehyde-fixed LRWhite-embedded eye tissue and found that duration of storage time for archival tissues did not affect AR results. AR did not change the components of the extracellular matrix labeled, and no artifacts were found after AR. They concluded that heat-induced AR can be used on selected extracellular matrix antigens to achieve positive label that would otherwise be lost due to fixation and storage. The test battery approach has also been evaluated by quantitative IEM using gold labeling techniques.^{16,17,61} Based on comparison of two polyclonal anti-nestin antibodies, Almqvist et al.⁶⁴ demonstrated precise localization of nestin in pediatric brain tumors, previously a controversial issue in the IHC literature. To confirm the reproducibility of counting neurons and glia in human brain tissue sections

by IHC staining, Lyck et al.²⁶ compared 29 different antibodies with various AR protocols using four buffers (Table 1.2). They reported that it is possible to use IHC staining for reproducible cell counting in brain tissue sections, based on optimal AR protocols, with well-preserved sample materials.

1.6 SUMMARY

- Standardization of AR technique should be based on the test battery principle. Achieving the “maximal retrieval level” of IHC staining intensity is a guideline for standardization.
- Three pH values (acidic, neutral, and basic AR solution), and three heating conditions (under boiling, boiling, and pressure heating) are recommended for the basic “test battery.” However, alternative procedures may be applied according to laboratory facilities and routine protocols as described above. Currently, citrate buffer pH 6.0, Tris–EDTA buffer pH 8–9, and certain AR solutions at lower pH, such as boric acid pH 2–3, or acidic acid buffer pH 2, as well as 0.05% citraconic anhydride pH 7.4, may be used to evaluate the optimal AR protocol.
- TMAs are valuable in rapid and cost-effective evaluation of new antibodies, in determining optimal AR methods.

REFERENCES

1. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
2. O’Leary TJ. Standardization in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 3–8.
3. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 2006; 49: 411–424.
4. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.
5. Vani K, Sompuram SR, Fitzgibbons P, et al. National HER2 proficiency test results using standardized quantitative controls. *Arch. Pathol. Lab. Med.* 2008; 132: 211–216.
6. Yaziji H, Taylor CR. Begin at the beginning, with the tissue! The key message underlying the ASCO/CAP task-force guideline recommendations for HER2 testing. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 239–241.
7. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *Arch. Pathol. Lab. Med.* 2007; 131: 18–43.

8. Shi SR, Cote RJ, Yang C, et al. Development of an optimal protocol for antigen retrieval: a "test battery" approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. *J. Pathol.* 1996; 179: 347–352.
9. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
10. Shi S-R, Cote RJ, Chaiwun B, et al. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl. Immunohistochem.* 1998; 6: 89–96.
11. Shi S-R, Cote RJ, Taylor CR. Standardization and further development of antigen retrieval immunohistochemistry: strategies and future goals. *J. Histotechnol.* 1999; 22: 177–192.
12. Shi S-R, Gu J, Cote RJ, et al. Standardization of routine immunohistochemistry: where to begin? In *Antigen Retrieval Technique: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 255–272. Natick, MA: Eaton, 2000.
13. Ferrier CM, van Geloof WL, de Witte HH, et al. Epitopes of components of the plasminogen activation system are re-exposed in formalin-fixed paraffin sections by different retrieval techniques. *J. Histochem. Cytochem.* 1998; 46: 469–476.
14. Rocken C, Roessner A. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. *J. Histochem. Cytochem.* 1999; 47: 1385–1394.
15. Shi S-R, Cote RJ, Liu C, et al. A modified reduced temperature antigen retrieval protocol effective for use with a polyclonal antibody to cyclooxygenase-2 (PG 27). *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 368–373.
16. Yano S, Kashima K, Daa T, et al. An antigen retrieval method using an alkaline solution allows immunoelectron microscopic identification of secretory granules in conventional epoxy-embedded tissue sections. *J. Histochem. Cytochem.* 2003; 51: 199–204.
17. Saito N, Konishi K, Takeda H, et al. Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. *J. Histochem. Cytochem.* 2003; 51: 989–994.
18. Naito I, Ninomiya Y, Nomura S. Immunohistochemical diagnosis of Alport's syndrome in paraffin-embedded renal sections: antigen retrieval with autoclave heating. *Med. Electron Microsc.* 2003; 36: 1–7.
19. Kim SH. Evaluation of antigen retrieval buffer systems. *J. Mol. Histol.* 2004; 35: 409–416.
20. Choi JK, Mauger J, McGowan KL. Immunohistochemical detection of *Aspergillus* species in pediatric tissue samples. *Am. J. Clin. Pathol.* 2004; 121: 18–25.
21. Gill SK, Ishak M, Rylett RJ. Exposure of nuclear antigens in formalin-fixed, paraffin-embedded necropsy human spinal cord tissue: detection of NeuN. *J. Neurosci. Meth.* 2005; 148: 26–35.
22. Du J, Shi XY, Zheng J, et al. Antigen retrieval immunohistochemistry under the influence of pH value and time. *Beijing da Xue Xue Bao. Yi Xue Ban/J. Peking Univ. Health Sci.* 2005; 37: 195–197.
23. Luo X-L, Cai X-L, Liu Y-H, et al. Influence of different antigen retrieval on the immunohistochemistry. *Chinese J. Pathol.* 2005; 34: 52–54.

24. Ge S, Crooks GM, McNamara G, et al. Fluorescent immunohistochemistry and in situ hybridization analysis of mouse pancreas using low-power antigen-retrieval technique. *J. Histochem. Cytochem.* 2006; 54: 843–847.
25. Slater MD, Murphy CR. Co-expression of interleukin-6 and human growth hormone in apparently normal prostate biopsies that ultimately progress to prostate cancer using low pH, high temperature antigen retrieval. *J. Mol. Histol.* 2006; 37: 37–41.
26. Lyck L, Dalmau I, Chemnitz J, et al. Immunohistochemical markers for quantitative studies of neurons and glia in human neocortex. *J. Histochem. Cytochem.* 2008; 56: 201–221.
27. Pan CC, Chen PC, Tsay SH, et al. Cytoplasmic immunoreactivity for thyroid transcription factor-1 in hepatocellular carcinoma: a comparative immunohistochemical analysis of four commercial antibodies using a tissue array technique. *Am. J. Clin. Pathol.* 2004; 121: 343–349.
28. Shi S-R, Cote RJ, Shi Y, et al. Antigen retrieval technique. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 311–333. Natick, MA: Eaton, 2000.
29. Hsi ED. A practical approach for evaluating new antibodies in the clinical immunohistochemistry laboratory. *Arch. Pathol. Lab. Med.* 2001; 125: 289–294.
30. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.
31. Shi S-R, Liu C, Young L, et al. Development of an optimal antigen retrieval protocol for immunohistochemistry of retinoblastoma protein (pRB) in formalin fixed, paraffin sections based on comparison of different methods. *Biotech. Histochem.* 2007; 82: 301–309.
32. Wilson E, Jackson S, Cruwys S, et al. An evaluation of the immunohistochemistry benefits of boric acid antigen retrieval on rat decalcified joint tissues. *J. Immunol. Methods* 2007; 322: 137–142.
33. Elias JM, Margiotta M. Low temperature antigen restoration of steroid hormone receptor proteins in routine paraffin sections. *J. Histotechnol.* 1997; 20: 155–158.
34. Elias JM, Rosenberg B, Margiotta M, et al. Antigen restoration of MIB-1 immunoreactivity in breast cancer: combined use of enzyme predigestion and low temperature for improved measurement of proliferation indexes. *J. Histotechnol.* 1999; 22: 103–106.
35. Frost AR, Sparks D, Grizzle WE. Methods of antigen recovery vary in their usefulness in unmasking specific antigens in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2000; 8: 236–243.
36. Umemura S, Itoh H, Ohta M, et al. Immunohistochemical evaluation of hormone receptor for routine practice of breast cancer: highly sensitive procedures significantly contribute to the correlation with biochemical assays. *Appl. Immunohistochem. Mol. Morphol.* 2003; 11: 62–72.
37. Kim SH, Kook MC, Song HG. Optimal conditions for the retrieval of CD4 and CD8 antigens in formalin-fixed, paraffin-embedded tissues. *J. Mol. Histol.* 2004; 35: 403–408.
38. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J. Pathol.* 1997; 183: 116–123.

39. Evers P, Uylings HB. An optimal antigen retrieval method suitable for different antibodies on human brain tissue stored for several years in formaldehyde fixative. *J. Neurosci. Methods* 1997; 72: 197–207.
40. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.
41. Shi SR, Cote RJ, Young L, et al. Use of pH 9.5 Tris-HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. *Biotech. Histochem.* 1996; 71: 190–196.
42. Evers P, Uylings HB, Suurmeijer AJ. Antigen retrieval in formaldehyde-fixed human brain tissue. *Methods* 1998; 15: 133–140.
43. Jiao Y, Sun Z, Lee T, et al. A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *J. Neurosci. Methods* 1999; 93: 149–162.
44. Vassallo J, Pinto GA, Alvarenga JM, et al. Comparison of immunoexpression of 2 antibodies for estrogen receptors (1D5 and 6F11) in breast carcinomas using different antigen retrieval and detection methods. *Appl. Immunohistochem. Mol. Morphol.* 2004; 12: 177–182.
45. Kan RK, Pleva CM, Hamilton TA, et al. Immunolocalization of MAP-2 in routinely formalin-fixed, paraffin-embedded guinea pig brain sections using microwave irradiation: a comparison of different combinations of antibody clones and antigen retrieval buffer solutions. *Microsc. Microanal.* 2005; 11: 175–180.
46. Gutierrez M, Forster FI, McConnell SA, et al. The detection of CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin embedded bovine tissue using a range of antigen recovery and signal amplification techniques. *Vet. Immunol. Immunopathol.* 1999; 71: 321–334.
47. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab. Invest.* 1986; 55: 244–248.
48. Wan WH, Fortuna MB, Furmanski P. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. *J. Immunol. Methods* 1987; 103: 121–129.
49. Lampkin SR, Allred DC. Preparation of paraffin blocks and sections containing multiple tissue samples using a skin biopsy punch. *J. Histotechnol.* 1990; 13: 121–123.
50. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.* 1998; 4: 844–847.
51. Alafuzoff I, Parkkinen L, Al-Sarraj S, et al. Assessment of [alpha]-synuclein pathology: a study of the BrainNet Europe Consortium. *J. Neuropathol. Exp. Neurol.* 2008; 67: 125–143.
52. Wong SC, Chan JK, Lo ES, et al. The contribution of bifunctional SkipDewax pretreatment solution, rabbit monoclonal antibodies, and polymer detection systems in immunohistochemistry. *Arch. Pathol. Lab. Med.* 2007; 131: 1047–1055.
53. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. *Lab. Invest.* 2000; 80: 1943–1949.
54. Eguíluz C, Viguera E, Millán L, et al. Multitissue array review: a chronological description of tissue array techniques, applications and procedures. *Pathol. Res. Pract.* 2006; 202: 561–568.

55. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch. Pathol. Lab. Med.* 2006; 130: 1026–1030.
56. Mighell AJ, Robinson PA, Hume WJ. Patterns of immunoreactivity to an anti-fibronectin polyclonal antibody in formalin-fixed, paraffin-embedded oral tissues are dependent on methods of antigen retrieval. *J. Histochem. Cytochem.* 1995; 43: 1107–1114.
57. Hann CR, Springett MJ, Johnson DH. Antigen retrieval of basement membrane proteins from archival eye tissues. *J. Histochem. Cytochem.* 2001; 49: 475–482.
58. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
59. Miki Hayashi C, Nagai R, Miyazaki K, et al. Conversion of Amadori products of the Maillard reaction to N(epsilon)-(carboxymethyl)lysine by short-term heating: possible detection of artifacts by immunohistochemistry. *Lab. Invest.* 2002; 82: 795–808.
60. Wick MR, Mills S. Consensual interpretive guidelines for diagnostic immunohistochemistry. *Am. J. Surg. Pathol.* 2001; 25: 1208–1210.
61. Röcken C, Roessner A. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. *J. Histochem. Cytochem.* 1999; 47: 1385–1394.
62. Ramandeep, Dikshit KL, Raje M. Optimization of immunogold labeling TEM: an ELISA-based method for rapid and convenient simulation of processing conditions for quantitative detection of antigen. *J. Histochem. Cytochem.* 2001; 49: 355–367.
63. Saito N, Konishi K, Takeda H, et al. Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. *J. Histochem. Cytochem.* 2003; 51: 989–994.
64. Almqvist PM, Mah R, Lendahl U, et al. Immunohistochemical detection of nestin in pediatric brain tumors. *J. Histochem. Cytochem.* 2002; 50: 147–158.

CHAPTER 2

EXTENDED APPLICATION OF ANTIGEN RETRIEVAL TECHNIQUE IN IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDIZATION

SHAN-RONG SHI and CLIVE R. TAYLOR

2.1 BRIEF SUMMARY OF PREVIOUS APPLICATIONS OF AR

The heat-induced antigen retrieval (AR) technique has been adopted world-wide since it was developed in 1991.¹ Thousands of articles have been published in a wide spectrum of clinical and research fields relevant to pathology and other fields of morphology. The AR technique has been applied predominantly for immunohistochemical (IHC) staining on archival formalin-fixed, paraffin-embedded (FFPE) tissue sections for diagnostic surgical pathology.²⁻⁶ In addition, the AR technique has also been used in the following related applications: plastic-embedded tissue samples for immunostaining both by light and electron microscopy; as a blocking procedure to avoid cross antigen/antibody reaction during multiple IHC staining procedures; enhancement of DNA/RNA *in situ* hybridization (ISH); terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) of apoptotic cells in FFPE tissue sections; as well as in flow cytometry to achieve stronger positive signals while reducing nonspecific background noise. For a review of these and other applications, see our first AR book,⁷ and two recent review articles by Yamashita⁸ and D'Amico et al.⁹

Recently, the use of AR has extended into several other areas, yielding interesting information for cytology, fresh cell/tissue sections, and fluorescence IHC (fluorescence *in situ* hybridization [FISH]), in addition to adaptations of the method for extraction of nucleic acids and proteins from FFPE tissues for use with modern methods of molecular analysis. In this chapter, the emphasis is on expanded applications in diagnostic cytology, fresh frozen cell/

tissue sections fixed in formalin, and immunofluorescence (IF) methods applied to FFPE tissue sections, as well as other novel applications. Application of the AR method to DNA/RNA and protein extraction from FFPE tissues are presented in Chapters 3 and 19, respectively.

2.2 DIAGNOSTIC CYTOPATHOLOGY

Widespread application of IHC in histopathology has revolutionized diagnostic pathology, transforming it from something of an “art” into a “science.”^{5,6} There is little argument that the use of numerous panels of markers in the differential diagnosis of various lesions has greatly improved the accuracy of diagnosis in surgical pathology, with particular reference to the diagnosis and classification of tumors. Furthermore the recent growth of molecularly oriented cancer research has led to personalized and targeted treatment as exemplified by human epidermal growth factor receptor 2 (HER2) antibody (Herceptin) for breast cancer, coupled with the development of specific IHC tests to qualify patients for clinical trials or as candidates for such therapies. Searching for and subsequently using various biomarkers to meet the goal of targeted therapies is a major focus of current research in the field of cancer.

Following the introduction of the Pap smear for early detection of cervical cancer, clinical cytology has grown steadily in range of application and in importance. While IHC (or “immunocytochemistry” or ICC as some time deferred to in the context of cytological analysis) has been used in cytopathology, its application has lagged behind the level of use in histopathology, in part due to differences in specimen availability and in cell sample preparation, which is quite different from that employed for FFPE tissues in surgical pathology. In contrast to typical surgical tissue specimens, in cytopathology, the cell sample is usually limited to a small amount that allows for only a few “smear” or imprint slides for cytologic evaluation in order to make a diagnosis. This circumstance alone renders it difficult, if not impossible, to undertake a panel of IHC (or ICC) stains as is frequently required by histopathology. In a minority of instances, when a larger amount of cell sample is obtained, it is possible to utilize the cell block technique, which does allow for the cutting of serial sections for a panel of IHC stains that is comparable with histology. Based on these conditions, Fowler and Lachar¹⁰ pointed out that several challenges exist in application of ICC to cytopathology. One of the major and most common issues is unavailability of proper control samples. Another is the frequent use of inappropriate antibody concentrations, due to lack of appropriate cell samples for titration studies, and the resultant frequent use of dilutions established as suitable primarily for FFPE sections. Indeed, it should be emphasized that ICC controls for cytology specimens must be of similarly prepared cell specimens for accurate comparison. The use of FFPE tissue section as positive control for cytology sample is not appropriate and is likely

to result in misinterpretation. This problem represents a major practical obstacle because most pathology laboratories lack the resources and expertise to establish appropriate cell line control systems.

Additional difficulties arise from the fact that numerous protocols of cell sample preparation, as well as fixation, are used in cytopathology, with lack of standardization across laboratories. More than a decade ago, Suthipintawong et al.¹¹ performed a comparative study for 23 fixation protocols, including acetone, acetone/methanol, acetone/formalin, glutaraldehyde, ethanol, methanol, and formal saline in immunostaining of cell preparations, and indicated that fixation in 0.1% formal saline overnight at 27°C followed by 10-min fixation in 100% ethanol gave the best results with the use of microwave AR pretreatment for further enhancement of ICC staining. Shidham et al.¹² carried out a comparative study to identify the most suitable method of cytological sample preparation and fixation protocols, including wet-fixed in 95% ethanol, air-dried saline rehydrated smears fixed in alcoholic formalin, and air-dried smears fixed in 95% ethanol with 5% acetic acid, for ICC of cytology. They employed seven commonly used markers and demonstrated that the ICC staining pattern with each tested marker is greatly affected by the protocol of sample preparation and fixation. In general, all markers, except vimentin, showed stronger IHC signals with use of preparations “wet-fixed in alcohol,” or air-dried and fixed in alcoholic formalin. Vimentin showed the best results for “wet-fixed in alcohol” samples. Specimens “air-dried” and then fixed in alcohol showed poor results of ICC.¹²

Heat-induced AR was successfully applied for archival Pap-stained cytological slides more than 10 years ago.^{13,14} Since then, numerous articles have been published that demonstrate the value of AR methods in the application of ICC staining on stored cytological slides (Table 2.1). Boon et al.¹³ noticed that the Papanicolaou stain was “removed” from the smear after AR (boiling slides in water solution), and the smear slides could be then be stained for MIB-1 antibody following a routine immunoperoxidase protocol. Interestingly, they indicated that MIB-1-stained Pap smear slides provided a “fine-tuning” approach as an aid for visualizing normal and abnormal proliferating reserve cells, especially in smears that were otherwise judged as unsatisfactory because they showed abundant folding or overlay of dense epithelial cells with blood cells. Gong et al.²¹ carefully compared ICC staining results of estrogen receptor (ER) between cell smears and corresponding tissue sections using AR technique for several fixatives, including formaldehyde and Carnoy’s fixative, and demonstrated that the use of the AR technique in cytological smears greatly improved ER immunodetectability and staining intensity in both formalin-fixed and Carnoy’s-Pap smears, raising the final concordance rate from 31% of formalin-fixed, and 29.4% of Canoy’s smears without AR to 93% between cell samples and corresponding tissue sections for ER immunostaining results.

It follows that several technical issues require further study to improve the immunocytological staining result, in terms of increasing signal intensity while reducing nonspecific background staining. In general, the principles for IHC

TABLE 2.1 Antigen Retrieval Used for Non-Formalin-Fixed Cytological Slides

Reference	Sample Condition	Antigen Retrieval	IHC (IP)	Results
Boon et al. ¹⁵	Archival stained (Pap or HE) smears Not de-stained	MW oven 100°C in citrate buffer pH 6.0 for 20 min	MIB-1	Valuable for quantifying analysis for proliferation.
Liu and Farhood ¹⁶	43 FNA specimens paired in Pap smear and cell block for comparison Not de-stained	1 mM EDTA solution, pH 8, steamed for 40 min	TTF-1 (clone 8G7G3/1, Dako)	Alcohol-fixed, Pap-stained smears gave identical positive rate as that obtained by cell block, but air-dried slides were unreliable.
Shtilbans et al. ¹⁷	60 archival smears of bronchoscopic or FNA samples De-stained	Postfixed in 10% formalin 8–10 min, MW oven for 6 min	Monoclonal antibody to p63 and TTF-1	Satisfactory results indicated that p63 may be of value in differential diagnosis.
Goel et al. ¹⁸	49 archival stained Pap smears De-stained	Microwave AR	Monoclonal antibodies to MIB-1 and PCNA	Simple, reliable, and easily applicable.
Shroyer et al. ¹⁹	330 unstained cervical cytology slides made on TriPath Imaging PrepStain Slide Processor	95°C, 15 min in Slide Preparation Buffer	ProEx C including two antibodies: topoisoemerase II- α and minichromosome maintenance protein 2	Achieved intense nuclear staining results with no variable score. Staining reproducibility was consistent.
Yoshida et al. ²⁰	63 cervical liquid-based Pap-stained slides	Boiling in 0.01 M citric acid phosphate buffer at pH 8.0 for 20 min	Monoclonal antibodies to p16 and HPV L1 capsid protein	Satisfactory results to indicate that combination of p16 and L1 is useful for diagnosis.

Note: References are randomly selected by online search.

PCNA, proliferating cell nuclear antigen; HE, hematoxylin and eosin.

used in tissue sections should be adopted in ICC, such as the test battery approach to establish an optimal AR protocol, as well as titration of optimal concentrations for primary antibody and all detection systems used as mentioned in Chapter 1. As noted, the problem with advocating this approach is the limitation of the number of specimen slides available for performance of these types of studies. Cell block studies, while informative to a degree, do not entirely solve the problem, in that the findings are not directly applicable to non-formalin-fixed cell smears. For example, recently, Fetsch and Abati²² reported HER2 immunostaining in 54 FFPE cell block sections of metastatic breast cancer using three primary antibodies to HER2 with one single heat-induced AR protocol (boiling sections in citric acid buffer of pH 6.0 for 20 min) as pretreatment prior to ICC staining. They reported variable results among three antibodies tested with increased background of cytoplasmic staining results, while achieving enhanced membrane staining signal in two antibodies (CB-11 and A0485), but destroying ICC staining of another antibody TAB250 after boiling treatment. They emphasized standardization of AR-ICC staining procedures as being imperative for the optimal interpretation of patient samples, particularly for cytopathology due to variation in sample size, fixative, and preparation method. It is likely that establishing an optimal AR protocol, based on the test battery approach for each primary antibody tested, instead of using one single AR protocol, should be the best choice in this case.

For fine-needle aspiration (FNA) samples, an air-dried preparation stained with Diff-Quik (modified Romanowsky stain) is traditionally used in conjunction with wet fixation in 95% alcohol, stained by Papanicolaou stain. The air-dried method, particularly modified by rehydration step and fixed in an aqueous or alcoholic weak formalin solution immediately before the staining procedure, provides the advantages of rapid cell sample preparation and better morphology, with clearer definition of some cytoplasmic as well as nuclear details, such as ground glass nuclei in papillary thyroid carcinoma, nucleolar features in Reed Sternberg cells, and cytoplasmic or nuclear structures in breast cancers. However, Liu and Farhood¹⁶ questioned the use of ICC using previously air-dried and Diff-Quik stained smears, when using an antibody to thyroid transcription factor-1 (TTF-1, clone 8G7G3/1, Dako, Carpinteria, CA). They compared TTF-1-ICC staining results in 43 FNA samples of lung tumors, using the heat-induced AR as pretreatment prior to ICC staining, and found that TTF-ICC staining was feasible in both wet-fixed Pap-stained and cell block slides, showing satisfactory positive results, but most air-dried slides were nonreactive for TTF-1. They postulated that methanol, used as fixative for air-dried smears, might adversely affect TTF-1 immunoreactivity.¹⁶ On the other hand, several articles have documented successful application of ICC in air-dried smears, particularly with a postfixation of formaldehyde or paraformaldehyde accompanying the use of heat-induced AR treatment prior to ICC staining.²³⁻²⁵

In conclusion, a series of technical issues remain to be addressed with respect to successful application of ICC in diagnostic cytopathology. Future

studies with respect to the role of AR and sample preparation in achieving improved standardization are likely to be fruitful.

2.3 IMMUNOFLOUORESCENT STAINING OF FFPE TISSUE SECTIONS

In recent years, following advances in immunofluorescent dye technology, including “non-quenching” labels, as exemplified by quantum dots (review by Resch-Genger),²⁶ and imaging analysis digital imaging methods, there has been a rapid increase of application of IF in FFPE tissue sections. In part, this growth has also been fueled by the long-recognized limitations of frozen tissue sections with conventional IF or immunoperoxidase methods, as well as several drawbacks of sample preparation, availability, and storage of frozen tissues. IF staining provides clear contrast and potentially offers a means of precise quantification of positive signal. In combining use of modern image analysis approaches, such as spectral imaging, IF may provide a powerful multiplex color labeling approach to the localization of multiple analytes in the same or adjacent subcellular compartments. Rimm²⁷ pointed out that IF appears to be a better method for multiple labeling than conventional IHC, and is also potentially more accurate for quantitative tissue-based assays. Although IF staining method has often been used in FFPE tissue sections for retrospective study of skin diseases,²⁸ it has not commonly been employed in FFPE tissue section, in large part due to inherent autofluorescence that is manifest as a diffuse and often strong fluorescence background.

2.4 METHODS OF REDUCING AUTOFLUORESCENCE

Autofluorescence is considered to result from inherent properties of specific tissue constituents, such as lipofuscin granules, flavins, reticulin, elastin, and collagen fibers, plus fixative-induced or tissue processing-induced fluorescence at emission wavelength 450–650 nm. Different strategies have been advanced to combat this problem, including extraction of the autofluorescent constituents, chemical modification of the fluorochrome, photo-bleaching methods, and “blocking” the autofluorescent structures.^{29,30} Several methods have been reported in the literature, often combining more than one of these approaches, such as ammonia-ethanol treatment (purporting to reduce autofluorescence by extraction of fluorescent molecules and by inactivating pH-sensitive fluorochromes); sodium borohydride (reducing aldehyde and keto groups, thus changing the fluorescence of tissue constituents); and various dyes, such as Trypan blue, Sky blue, and Sudan Black B (serving to mask fluorescent tissue components). Baschong et al.²⁹ performed a careful comparative study to evaluate the effects of three reagents, ammonia-ethanol, borohydride, and Sudan Black B on autofluorescence in FFPE tissue sections based on matched

frozen and FFPE tissues. The protocols for these studies are summarized briefly: (1) ammonia-ethanol—during rehydration of deparaffinized FFPE tissue sections in graded alcohol, the slides were immersed in 70% ethanol mixed with 0.25% NH_3 for 1 h, and followed by immersion in 50% ethanol for 10 min, then in modified Hanks' buffer; (2) borohydride—deparaffinized FFPE tissue sections were immersed in ice-cooled freshly prepared Hanks' buffer including 10 mg/mL sodium borohydride for 40 min, followed by three washes in Hanks' buffer; (3) Sudan Black B—after the fluorescence staining procedure, the slides were immersed in 70% ethanol containing 0.1% Sudan Black B for 30 min, followed by thorough washes of excess black dye with Hanks' buffer to avoid remaining black color in tissue sections. These investigators were unable to identify a universal protocol for control of autofluorescence because any observed “blocking effects” depended on tissue type and the method of processing. A combination of several blocking methods has been recommended for improving the overall blocking effect. Viegas et al.³¹ compared ammonia/ethanol, Sudan Black B, and photobleaching methods using murine kidney, liver, and pancreas FFPE tissue sections, and found that ammonia/ethanol alone had no value; Sudan Black B alone reduced autofluorescent staining at 488 nm only; combination of both ammonia and Sudan Black B showed reduced autofluorescence, but in pancreas only. They concluded that a combination of short-duration, high-intensity UV irradiation (2 h at 30 W) and Sudan Black B was the best protocol to reduce autofluorescence in both highly vascularized, high lipofuscin content tissues, and in poorly vascularized, low lipofuscin content tissues, showing complete elimination of autofluorescent background in kidney, liver, and pancreas FFPE tissue sections. Gill et al.³² also demonstrated the effectiveness of immersing FFPE tissue sections in Sudan Black B solution for blocking autofluorescence associated with FFPE tissue section. However, this chemical blocking treatment has been reported to reduce the IF positive signal when it is used after IF labeling procedure.

A different approach, using red fluorescence on FFPE tissue sections was documented by Niki et al.³³ These investigators selected an albuminous dye called peridinin chlorophyll α protein (PerCP) for IF staining of FFPE human tumor tissue sections, using AR treatment prior to the IF procedure, and achieved a satisfactory result, showing the red fluorescence of PerCP clearly distinguished the tumor region from the yellow-green autofluorescence background.

More recently, Robertson et al.³⁴ reported a complex combined approach to multiple IF labeling of FFPE tissue, employing AR pretreatment, an indirect IF staining method, and the use of confocal laser scanning microscopy to circumvent autofluorescence background. It is noteworthy that the use of AR as a procedure to enhance antigenicity for IHC in FFPE tissue appears to have overlooked the advantage it offers for reducing nonspecific autofluorescence background. The significance of AR pretreatment used for IF staining of FFPE tissue sections has been emphasized by numerous publications from

more than a decade ago.²⁸ The test battery approach has also been employed in IF to establish an optimal protocol for IF staining in FFPE tissue sections. For example, Long and Buggs³⁵ tested three buffer solutions, 100mM Tris at pH 10, 0.05% citraconic anhydride, and 10mM citrate with 2mM ethylenediamine tetraacetic acid (EDTA), and 0.05% Tween 20, pH 6.2, for AR heating in microwave oven, and achieved satisfactory IF staining results for all three AR solutions tested. Bataille et al.³⁶ successfully performed multiple IF staining in FFPE tissue sections by using 0.1M sodium citrate buffer at pH 7.2 heating at 90°C in a water bath for 40min. They emphasized some critical points for reducing background staining, including selecting primary antibodies derived from different species, incubation of slides at 4°C overnight, optimizing the concentration of antibodies carefully, and establishing optimal AR conditions. In another study, the AR method, pretreating glutaraldehyde-fixed, epoxy-embedded tissue sections in TEG buffer (10mM Tris, 0.5mM ethylene glycol bis [α -aminoethylether]-*N,N,N',N'*-tetraacetic acid, pH 9) contributed to satisfactory double IF staining results with well-preserved tissue morphology.³⁷ IF staining has also been used on pronase-digested FFPE renal tissue sections to achieve good results, comparable with those obtained by using frozen tissue sections.³⁸

Numerous articles pertaining to multiple IF labeling or combining IF and FISH labeling for FFPE tissue sections have appeared in recent years based on AR technique. Ge et al.³⁹ established a low-power AR technique for combining IF and FISH in FFPE tissue sections using microwave oven at power level 4 (40%) for 3 cycles $\times$ 5 min, with a 1-min break between each cycle. They set one slide jar in the microwave oven for each time, and there was no bubbling or overflow during heating process, although the temperature reached 100°C in the jar. Their results showed satisfactory IF and FISH staining signals with clean background when viewing slides under Leica DMRXA microscope. Similarly, Xia et al.⁴⁰ reported combining FISH, using a monkey Y chromosome-specific probe with IF staining of epithelial cell markers, with AR pretreatment at 96°C in citrate buffer pH 6.0, in FFPE monkey tissue sections, and achieved satisfactory results. They emphasized the use of AR as effective pretreatment to replace enzyme digestion to make combination of FISH and IF accessible.

With its unique advantages of emitting bright green fluorescence, without any exogenous substrates, green fluorescent protein (GFP) has widely been applied in experimental biology for visualizing cell activity as well as monitoring gene and protein expression. A newly developed technique of affinity-purified antibodies to heat-denatured GFP may provide a useful approach for application of AR heating treatment on tissue sections that have been labeled by GFP.⁴¹ Nakamura et al.⁴¹ successfully performed IF labeling for GFP on heated formalin-fixed mouse brain tissue sections by using their novel polyclonal antibody to heat-denatured GFP. This method allows the use of a heating process such as AR treatment in GFP-labeled tissue sections for multiplexed detection of IF signals in GFP-labeled experimental samples.

2.5 ALDEHYDE-FIXED FROZEN CELL/TISSUE SECTIONS

Fresh cell smears or tissue section were used for IHC during the early stages of development of IF methods since the 1940s. Subsequently, major efforts were employed to application of IF for archival FFPE tissue sections, in view of the worldwide collection of FFPE tissue blocks forming invaluable resources of specimens for translational studies of cancer and various other diseases. Despite the fact that the AR technique has widely been applied in pathology to create a new era of IHC used for archival FFPE tissue sections,^{4,42,43} fresh cell/tissue samples are still recognized as “gold standards” of IF and IHC, especially when evaluating new markers, as well as new reagents, to represent the “true” findings as compared to results obtained by other tissue samples such as FFPE tissue sections.

As a “gold standard,” fresh tissue prepared by snap-frozen method, cut by cryostat, and fixed in acetone, ethanol, or other non-cross-linking fixatives, has been generally accepted as reliable.

However, worldwide application of the AR technique in IHC found some discrepancies of IHC results between frozen tissue and FFPE tissue sections. Recently, Yamashita and Okada⁴⁴ compared immunostaining results of 22 antibodies between acetone-fixed and aldehyde-fixed frozen tissue sections and found that most antibodies showed stronger intensity of IHC for aldehyde-fixed frozen tissue sections, after the AR treatment, than obtained in acetone-fixed tissues. In particular, a total of 11 (50%) antibodies showing negative IHC staining results using acetone-fixed frozen tissue sections achieved positive staining by using aldehyde-fixed frozen tissue sections with the use of AR.

Recently, we also experienced weak or absent immunostaining for some antibodies tested on acetone-fixed fresh cell/tissue sections. For example, a newly developed monoclonal antibody to GRP78 showed negative result in acetone-fixed fresh cell line specimens, but gave a clear positive staining result in formalin-fixed preparations of the same fresh cell sample after the AR treatment. All these data challenge the reliability of acetone, or alcohol-fixed fresh frozen tissue section, used as the “gold standard” for IHC staining.

Our research group at USC has recently conducted a study evaluating frozen sections prepared under various conditions of fixation and AR treatment. Fresh human tissues were frozen in OCT Compound (Miles Laboratories, Elkhart, IN). An adjacent block of tissue was fixed routinely in 10% neutral buffered formalin (NBF) and paraffin-embedded (FFPE). Preparations of human cell lines (LNCaP and C42B of prostate cancer, MCF-7 of breast cancer) were also processed into frozen and FFPE cell blocks in parallel to confirm the IHC results of tissues. Frozen tissue/cell sections were fixed by six different protocols: acetone 10min; ethanol 10min; NBF 30min, and 24h; NBF + CaCl₂ 30min, and 24h. The AR technique was used for all NBF-fixed tissues sections. A total of 26 antibodies were tested. The ABC kit, with diaminobenzidine (DAB), was used to generate the IHC signal. In summarizing our

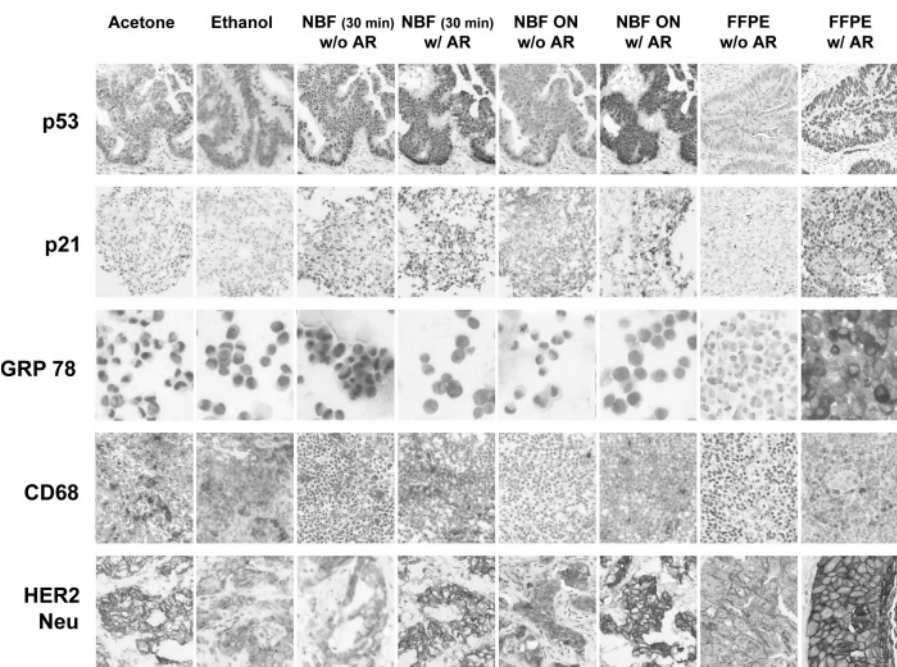


Figure 2.1 Comparison of IHC staining intensity among various protocols of fixation, AR pretreatment for frozen and FFPE cell/tissue sections. Five markers are selected as examples: p53 stained colon cancer tissue (1st row); p21 stained bladder cancer tissue (2nd row); GRP78 stained cell line C42B (3rd row); CD68 stained lymph node tissue (4th row); and HER2 stained breast cancer tissue (5th row). In general, neutral buffered formalin (NBF)-fixed frozen cell/tissue with AR treatment showed identical or stronger IHC staining intensity when compared with that obtained by acetone/ethanol-fixed cell/tissue, except CD68. FFPE cell/tissue sections yield the strongest IHC signals and the best morphology consistently. w/o AR, without use of the AR pretreatment; w/ AR, use of the AR pretreatment prior to IHC staining procedure. (All figures, $\times 200$.) Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology. See color insert.

results, more than half of the antibodies (16/26, 61.5%) showed identical IHC staining results between acetone-fixed and NBF-fixed tissue sections. Among the remaining antibodies, eight (30.8%) showed better IHC signals following NBF and AR, while only two antibodies gave better IHC staining results for acetone-fixed frozen tissue sections. Most cytoplasmic proteins (10/13) showed comparable IHC signal between acetone and NBF-fixed tissue sections. For nuclear proteins, NBF-fixed tissue sections gave better IHC signals than those obtained by acetone-fixed sections. In most cases, NBF yielded a stronger signal with less background and better morphology. Overall, FFPE tissue sections yielded the best results of IHC staining of all antibodies tested (Fig. 2.1). In addition, we found that in some instances, the nuclear IHC staining patterns, such as p21 or p27, was changed in the frozen tissue section after acetone or

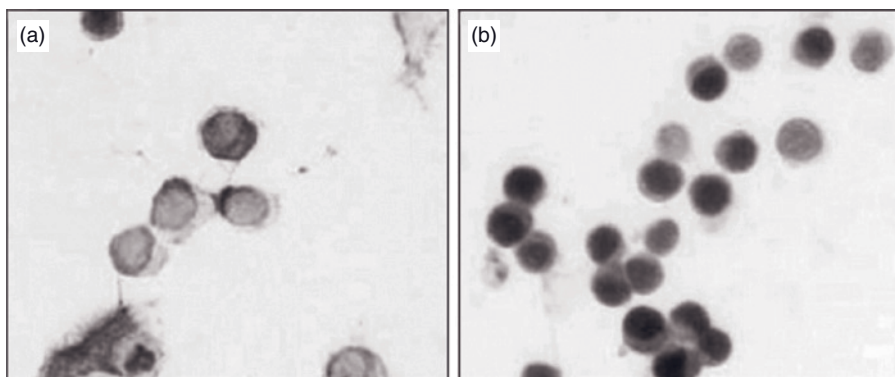


Figure 2.2 Comparison of p21 IHC staining results using fresh cell line MCF-7. (a) Acetone-fixed cells showing an irregular positive staining pattern indicating dislocated p21 protein from nuclei to cytoplasm and outside of cells ($\times 400$). (b) NBF-fixed cells with the use of AR treatment before IHC staining showing an intact nuclear p21 staining pattern ($\times 400$). Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology. See color insert.

alcohol fixation, showing dislocation of nuclear into cytoplasmic perinuclear area, or even “leaking out” from cells into the tissue space (Fig. 2.2, a vs. b). In reviewing the literature, it has been recognized that some proteins of low molecular weight, and certain lipoproteins, are readily extracted by coagulant fixatives (alcohol), and about 13% of total protein may be lost following acetone fixation.^{45,46} Comparison of the p21-nuclear staining patterns between acetone and formalin-fixed cells indicates a distinct, intense nuclear staining pattern for formalin-fixed cells in contrast with acetone-fixed pattern showing low intensity or a dislocated staining pattern (Fig. 2.2 a vs. b).

Based on numerous publications, it is apparent that most of the proteins in tissues are preserved very well by formalin-fixation, as demonstrated by an abundance of IHC studies with use of the AR technique, including recent increasing experimental reports by mass spectrometry.^{47–51} Although the molecular mechanism of formalin fixation and AR technique is unclear, it is accepted that formaldehyde is a cross-linking fixative characterized by fixing proteins *in situ* through the formation of extensive intramolecular and intermolecular covalent cross-links.^{52,53} Therefore, it is formalin fixation, a “historical” routine tissue preparation method, that provides an effective approach to the preservation of proteins *in situ* of the tissues, while preserving excellent morphology for diagnostic. On the other hand, it is the simple heat-induced AR technique that provides an effective approach to reversal of the formaldehyde-induced chemical modification of proteins for IHC, as well as other techniques. In fact, the worldwide application of the antigen retrieval immunohistochemical (AR-IHC) staining on FFPE tissue sections created “pre” and “post” AR eras in the literature.^{4,5} Several investigators adopted the results of AR-IHC staining on FFPE tissue sections as the “gold standard”

to evaluate IHC staining results achieved by other protocols of sample preparation, as exemplified by Shidham et al., who evaluated three different protocols of cell sample preparation, based on IHC staining of FFPE tissue sections with AR used as the standard positive control. Their results demonstrated that FFPE tissue sections with AR yielded the best IHC staining signals.¹²

In addition to the advantages of preservation of both morphology and total proteins in FFPE tissue, archival tissue has other advantages, in terms of availability, storage, transportation, well-sterilized tissue samples, and the ability to yield adequate qualities of nucleic acids for molecular analysis, and so on.⁵⁴ In contrast, stored frozen tissues give inferior cell morphology due to problems in technical handling of frozen tissue samples and tissue heating by compression during the preparation of frozen tissue sections.⁵⁵ Also, small proteins and lipoproteins may be lost by diffusion. Frozen tissue is difficult to transport to other laboratories and must be regarded as a non-sterilized biohazard specimen due to inconsistent frozen storage condition.

In the course of these studies, a further critical issue emerged, to the effect that it is necessary to use independent objective biochemical methods, such as Western blot analysis, to validate variations in IHC or IF staining results for those proteins under different conditions of sample preparation. This independent validation is of particular importance for cases showing negative IHC staining when using coagulate fixatives for frozen cell/tissue sections, but giving positive staining results when using formalin-fixed samples with AR. Lacking validation by independent methods, as pointed out by Wick and Mills, "there is a real risk that artifacts may become 'facts.'"⁵⁶ We followed this practice in our laboratory, applying Western blot methods to protein extracts in order to evaluate the IHC results of four proteins that gave discrepant IHC staining results between alcohol/acetone and formalin-fixed cell/tissue samples.⁵⁵

As noted above, it is of interest that the nonspecific background staining frequently found in frozen tissue sections during IF or IHC staining is significantly reduced after AR treatment (Fig. 2.3). Although the underlying mechanism is unclear, it may be caused by AR-induced alteration of the overall electrostatic charge of the tissue leading to reduced nonspecific binding, or by other potential mechanisms as discussed by Tom Boenisch.⁵⁷ A potential application of AR-reduced background staining is in IHC detection of disseminated tumor cells in bone marrow or blood, serving to reduce the abundant nonspecific background staining that renders interpretation difficult when using acetone/alcohol-fixed samples. In this instance, a formalin-fixed cell sample may provide significantly improved IHC staining result.⁵⁸ Particularly, 9/26 markers, including 3 keratin antibodies tested, showed strong IHC positive signals after NBF fixation for 30 min. Therefore, routinely 10-min NBF-fixed cell smear slides can be used even without AR for detection of disseminated tumor cells.⁵⁵

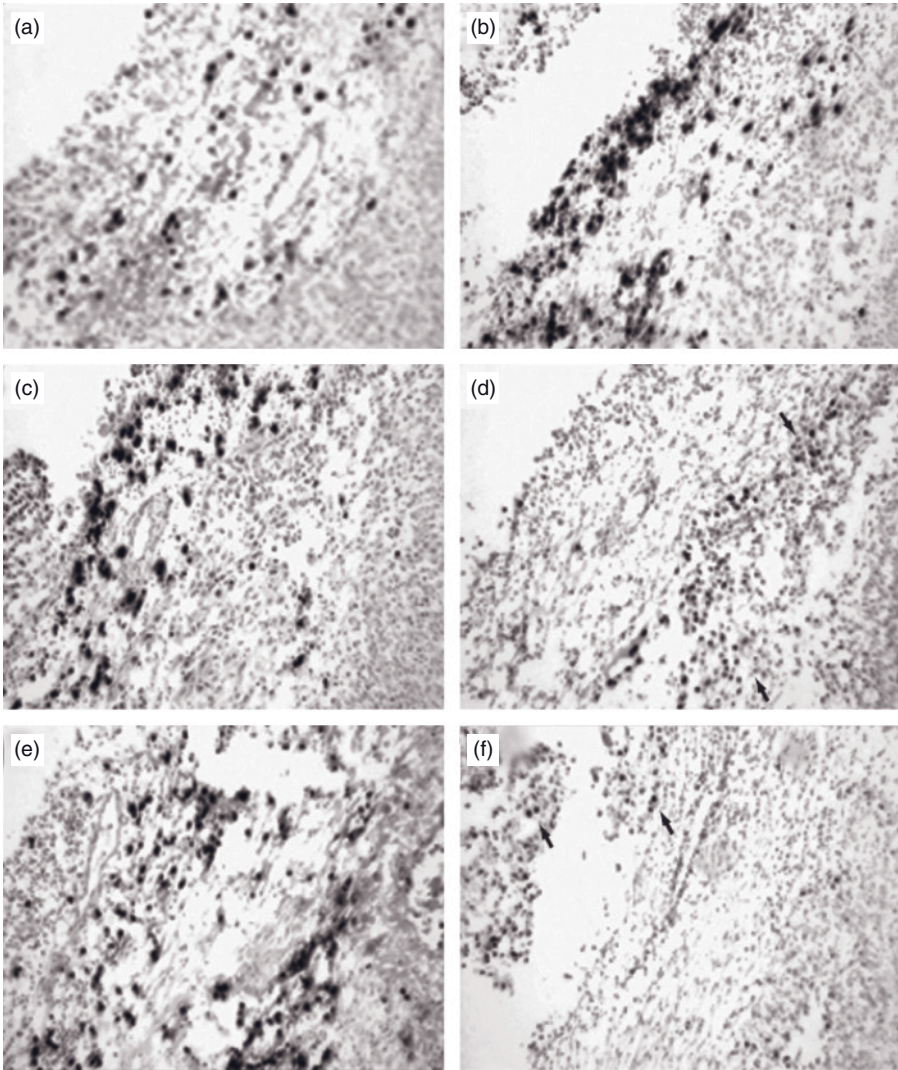


Figure 2.3 Comparison of nonspecific background IHC staining results among various fixation of frozen tissue sections, and antigen retrieval immunohistochemical (AR-IHC) staining protocols. Human bladder cancer tissue samples were used for p21 staining procedure. Significant strong, nonspecific background staining results can be found in acetone-fixed (a), ethanol-fixed (b), NBF-fixed 30 min (c), and NBF-fixed overnight (e) samples showing irregular large dots stained positively. In contrast, the same kinds of NBF-fixed frozen tissue sections after AR treatment before IHC staining (d and f) showing clear background. Arrows indicate the p21-positive nuclear staining results. (a–f, $\times 100$.) Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology. See color insert.

In conclusion, based on the literature and our data, the traditional use of acetone-fixed frozen tissue sections as the “gold standard” for IHC is not justified for all antigen/antibody pairs. For validating any new antibody, it would be prudent to employ a combination of both acetone and NBF-fixed frozen sections. From our experience, FFPE tissue sections may serve as the standard for most antigens for IHC.

A recent study has adopted AR pretreated frozen tissue sections for IHC detection of micrometastasis in sentinel lymph nodes in breast cancer obtaining improved morphology and clean background, resulting in significant improvement of detection rate of micrometastasis. The pretreatment prior to IHC staining is simple: frozen tissue section was fixed in NBF for 1 min and for 1 min in Lillies AAF (acetic acid, alcohol, formalin), followed by microwave heating in 60°C-preheated Tris-ethylene glycol tetraacetic acid (EGTA) buffer, pH 9.0 until boiling point (about 30s) for simultaneous blocking of endogenous peroxidase and AR.⁵⁹

2.6 OTHER APPLICATIONS

2.6.1 FISH

The high temperature heating “retrieval” principle has been successfully used for ISH in FFPE tissue sections based on theoretical and practical analogies between formalin-induced chemical modification of nucleic acids and proteins.^{60,61} We also have directly experienced successful application of FISH and chromogenic *in situ* hybridization (CISH) using heat-induced retrieval protocol in FFPE tissue sections.^{60–62} Recently, as part of a study of human carcinogenesis and potential biomarkers for cancer treatment, Sugimura⁶³ reviewed the use of “microwave (MW)-assisted” FISH for detection of chromosomal alterations in archival FFPE tissue sections, obtaining a much higher success rate (of >90%) compared with the conventional protocol (40% or less). The MW-assisted protocol is particularly useful for tissue microarrays containing samples fixed under variable conditions, including both short and prolonged formalin fixation, to provide comparable FISH results, while preserving excellent morphology. The protocol of intermittent MW irradiation consists of boiling the deparaffinized FFPE tissue sections in 0.01 citrate buffer solution at pH 6.0 for 15 min in a microwave, followed by 0.3% pepsin/0.01 N HCl for 10 min at 37°C, and by the FISH procedure, involving 4% paraformaldehyde for 5 min, and DNA denaturation at 85°C for 5 min, and so on.⁶³ Application of this MW-FISH protocol provides a useful approach to comparing comparative genomic hybridization (CGH), or single-nucleotide polymorphism (SNP) microarrays, with FISH analysis, to validate the areas of gain and loss in the genome.⁶³ Another variation of the method, combining the use of heating FFPE tissue sections in 8% sodium thiocyanate solution at 80°C for 30 min, followed by 0.5% pepsin in 0.2 N HCl at 37°C for 26–32 min, also gave satisfactory result for FISH.⁶⁴

2.6.2 IHC Detection of Bromodeoxyuridine (BrdU)

The utility of AR for IHC detection of proliferation cell markers was discussed in detail in our previous AR book.⁶⁵ Recently, several publications have documented the use of the AR technique for enhancement of IHC detection of BrdU. More than two decades ago, IHC detection using an antibody to BrdU was used to identify sites of BrdU incorporation into S-phase cells during the cell cycle, as a measure of active cell proliferation. The conventional IHC protocol adopted a pretreatment step of incubation of slides in warm 2.0 M HCl solution, prior to the IHC staining procedure. A drawback of HCl treatment was loss of nuclear counterstaining that rendered impossible attempts to count total nuclei. In addition, HCl pretreatment also appeared to reduce IHC detection for some other proteins, a problem when attempting multiple IHC stain. Tang et al.⁶⁶ reported their success in IHC detection of BrdU by using high temperature AR method, and emphasized the critical issues in terms of heating conditions (higher temperature of around 99°C) and the ionic strength of the AR buffer solution. They found that increasing the citrate concentration reduced significantly the IHC labeling results; the use of 10 mM Tris buffer as the AR solution yielded excellent BrdU labeling, but the use of 100 mM Tris gave poor results.

2.6.3 AR by Heating En Bloc for Paraformaldehyde -Fixed Frozen Tissue

Ino⁶⁷ reported a simple AR method that involved heating 4% paraformaldehyde-fixed tissue, followed by the OCT-embedding procedure, and then cutting frozen tissue sections for IHC staining. A somewhat similar application of AR treatment in fixed floating brain tissue had been documented previously.^{68,69} Ino⁶⁷ emphasized the critical importance of establishing an optimal AR protocol, based on the AR principle—heating condition and pH value of the AR solution—in developing this new method. The advantages of the “en bloc” heating procedure gave remarkable enhancement of IHC staining for most antibodies tested, as well as unexpectedly lower background staining, which may be attributable to denaturation of endogenous IgG by heating treatment.⁶⁷ This “en bloc” heating AR method was subsequently used for double IF labeling brain tissue sections, using soluble immune complexes of the second primary antibody mixed with a second monovalent fluorescence-labeled secondary antibody from identical species, to avoid cross-reaction between these two IHC detection systems. Satisfactory IF results were reported.⁷⁰

2.6.4 Boiling Unfixed Frozen Tissue Sections for Background Reduction

Mundegar et al.⁷¹ applied a modified AR protocol to unfixed frozen sections with the goal of reducing background. Their study was designed to detect a 427 kD subsarcolemmal protein dystrophin in mdx mouse skeletal muscle

tissue that had been implanted with stem cells or muscle progenitor cells, as an experimental model for study of Duchenne muscular dystrophy. They immersed unfixed frozen muscle tissue sections in a boiling PBS solution for 15 s to 5 min, followed by a wash in either PBS or 1% Triton X-100 in PBS for 15 min. They obtained satisfactory immunofluorescence staining for dystrophin and several other thermostable proteins, but recognized that boiling unfixed tissue sections might induce denaturation of labile proteins. As a result, some antibodies might not give good IHC results, whereas others, capable of reacting with their denatured target proteins, could give better IHC staining. Dr. Yoshiyuki Osamura's research group presented similar experimental data with respect to heating unfixed fresh tissue sections in buffer solution, giving stronger IHC staining results for several antigen/antibody reactions at the Antigen Retrieval Workshop during the 12th International Congress of Histochemistry and Cytochemistry held in San Diego, California on July 28, 2004. Their group also discussed a potential mechanism of the heat-induced AR technique.

2.6.5 AR Used for Tissue Samples Subject to Autolysis

Monleón et al.⁷² documented an interesting IHC result of detecting the abnormal isoform of prion protein (PrP^{sc}) in cattle brain tissues that had been subjected to very advanced autolysis (liquid state) using the AR treatment. They took the liquid-like animal brain tissue by a swab, and made smear onto Vectabond (Vector Laboratories, Burlingame, CA)-pretreated glass slides after dilution of the liquid tissue, followed by drying at 56°C for 24 h, and fixed in 10% formalin for 1 h. Prior to IHC staining for PrP^{sc} detection using a monoclonal antibody, they performed a combined AR protocol by pretreating slides with 98% formic acid and hydrated autoclaving for AR, followed by proteinase K digestion. They achieved a satisfactory result in all cases, including a control autolysis experimental sample that was left for environmental exposure after 80 days.

To evaluate the value of IHC used for autopsy tissues that had been degraded in variable conditions, Maleszewski et al.⁷³ collected eight surgical specimens of placenta, kidney, coronary artery, and dorsalis pedis artery, which were allowed to autolyze at variable time schedule: 12, 24, and 48 h under two conditions of room temperature (20°C) and 4°C in refrigerator. Tissues were fixed in formalin and embedded in paraffin as two multiple tissue blocks. They carried out IHC staining using 18 antibodies of matrix metalloproteinases (MMPs), and their inhibitors (tissue inhibitor of metalloproteinases [TIMPs]), scavenger receptors, and advanced glycation end products with AR pretreatment for most antibodies tested. Western blotting technique was used to confirm the IHC results. They found that their tested proteins degraded slowly and faithfully maintain IHC staining patterns over 24 h after tissue removal from living bodies, and supported the use of autopsy tissues with short post-mortem intervals for IHC studies.

In our research group at USC, we also did an IHC study to evaluate vimentin expression in autopsy tissues by comparing vimentin-IHC staining intensity between matched frozen and variable formalin-fixed autopsy tissues of kidney, liver, adrenal, lymph node, lung, myocardium, and liver cancer, and found that the IHC staining intensity and pattern obtained in FFPE autopsy tissue after the use of AR treatment were comparable with that obtained using matched frozen autopsy tissues fixed by acetone. In general, the AR technique has increasingly been applied in various fields other than FFPE tissue sections for IHC, where it began, based on universal advantages, in terms of simplicity, effectiveness, and facilitation of the use of archival accumulated tissue samples with a variety of other molecular techniques.

REFERENCES

1. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
2. Gown AM, de Wever N, Battifora H. Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* 1993; 1: 256–266.
3. Boon ME, Kok LP. Breakthrough in pathology due to antigen retrieval. *Mal. J. Med. Lab. Sci.* 1995; 12: 1–9.
4. Gown AM. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* 2004; 121: 172–174.
5. Taylor CR, Cote RJ. *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2006.
6. Jagirdar J. Immunohistochemistry, then and now. *Arch. Pathol. Lab. Med.* 2008; 132: 323–325.
7. Shi S-R, Gu J, Taylor CR. *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, 1st edition. Natick, MA: Eaton, 2000.
8. Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. *Prog. Histochem. Cytochem.* 2007; 41: 141–200.
9. D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. *J. Immunol. Methods* 2009; 341: 1–18.
10. Fowler LJ, Lachar WA. Application of immunohistochemistry to cytology. *Arch. Pathol. Lab. Med.* 2008; 132: 373–383.
11. Suthipintawong C, Leong AS-Y, Vinyuvat S. Immunostaining of cell preparations: a comparative evaluation of common fixatives and protocols. *Diagn. Cytopathol.* 1996; 15: 167–174.
12. Shidham VB, Chang C-C, Rao RN, et al. Immunostaining of cytology smears: a comparative study to identify the most suitable method of smear preparation and fixation with reference to commonly used immunomarkers. *Diagn. Cytopathol.* 2003; 29: 217–221.

13. Boon ME, Kleinschmidt-Guy ED, Ouwerkerk-Noordam E. PAPNET for analysis of proliferating (MIB-1 positive) cell populations in cervical smears. *Eur. J. Morphol.* 1994; 32: 78–85.
14. Abendroth CS, Dabbs DJ. Immunocytochemical staining of unstained versus previously stained cytologic preparations. *Acta Cytol.* 1995; 39: 379–386.
15. Boon ME, Beck S, Kok LP. Semiautomatic PAPNET analysis of proliferating (MiB-1-positive) cells in cervical cytology and histology. *Diagn. Cytopathol.* 1995; 13: 423–428.
16. Liu J, Farhood A. Immunostaining for thyroid transcription factor-1 on fine-needle aspiration specimens of lung tumors: a comparison of direct smears and cell block preparations. *Cancer* 2004; 102: 109–114.
17. Shtilbans V, Szporn AH, Wu M, et al. p63 immunostaining in destined bronchoscopic cytological specimens. *Diagn. Cytopathol.* 2005; 32: 198–203.
18. Goel MM, Mehrotra A, Singh U, et al. MIB-1 and PCNA immunostaining as a diagnostic adjunct to cervical Pap smear. *Diagn. Cytopathol.* 2005; 33: 15–19.
19. Shroyer KR, Homer P, Heinz D, et al. Validation of a novel immunocytochemical assay for topoisomerase II- and minichromosome maintenance protein 2 expression in cervical cytology. *Cancer* 2006; 108: 324–330.
20. Yoshida T, Sano T, Kanuma T, et al. Immunochemical analysis of HPV L1 capsid protein and p16 protein in liquid-based cytology samples from uterine cervical lesions. *Cancer (Cancer Cytopathol.)* 2008; 114: 83–88.
21. Gong Y, Symmans WF, Krishnamurthy S, et al. Optimal fixation conditions for immunocytochemical analysis of estrogen receptor in cytologic specimens of breast carcinoma. *Cancer (Cancer Cytopathol.)* 2004; 102: 34–40.
22. Fetsch PA, Abati A. The effects of antibody clone and pretreatment method on the results of HER2 immunostaining in cytologic samples of metastatic breast cancer: a query and a review of the literature. *Diagn. Cytopathol.* 2007; 35: 319–328.
23. Suthipintawong C, Leong AS-Y, Chan KW, et al. Immunostaining of estrogen receptor, progesterone receptor, MIB1 antigen, and c-erbB-2 oncoprotein in cytologic specimens: a simplified method with formalin fixation. *Diagn. Cytopathol.* 1997; 17: 127–133.
24. Shidham VB, Lindholm PF, Kajdacsy-Balla A, et al. Methods of cytologic smear preparation and fixation. Effect on the immunoreactivity of commonly used anti-cytokeratin antibody AE1/AE3. *Acta Cytol.* 2000; 44: 1015–1022.
25. Fulciniti F, Frangella C, Staiano M, et al. Air-dried smears for optimal diagnostic immunocytochemistry. *Acta Cytol.* 2008; 52: 178–186.
26. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, et al. Quantum dots versus organic dyes as fluorescent labels (Review). *Nat. Methods* 2008; 5: 763–775.
27. Rimm DL. What brown cannot do for you. *Nat. Biotechnol.* 2006; 24: 914–916.
28. D'Ambra-Cabry K, Deng DH, Flynn KL, et al. Antigen retrieval in immunofluorescent testing of bullous pemphigoid. *Am. J. Dermatopathol.* 1995; 17: 560–563.
29. Baschong W, Suetterlin R, Laeng RH. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). *J. Histochem. Cytochem.* 2001; 49: 1565–1571.

30. Billinton N, Knight AW. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. *Anal. Biochem.* 2001; 291: 175–197.
31. Viegas MS, Martins TC, Seco F, et al. An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues. *Eur. J. Histochem.* 2007; 51: 59–66.
32. Gill SK, Ishak M, Rylett RJ. Exposure of nuclear antigens in formalin-fixed, paraffin-embedded necropsy human spinal cord tissue: detection of NeuN. *J. Neurosci. Methods* 2005; 148: 26–35.
33. Niki H, Hosokawa S, Nagaike K, et al. A new immunofluorostaining method using red fluorescence of PerCP on formalin-fixed paraffin-embedded tissues. *J. Immunol. Methods* 2004; 293: 143–151.
34. Robertson D, Savage K, Reis-Filho JS, et al. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biol.* 2008; 9: 13. doi: 10.1186/1471-2121-9-13.
35. Long DJ, Buggs C. Microwave oven-based technique for immunofluorescent staining of paraffin-embedded tissues. *J. Mol. Histol.* 2008; 39: 1–4.
36. Bataille F, Troppmann S, Klebl F, et al. Multiparameter immunofluorescence on paraffin-embedded tissue sections. *Appl. Immunohistochem. Mol. Morphol.* 2006; 14: 225–228.
37. Zhai XY, Kristoffersen IB, Christensen EI. Immunocytochemistry of renal membrane proteins on epoxy sections. *Kidney Int.* 2007; 72: 731–735.
38. Nasr SH, Galgano SJ, Markowitz GS, et al. Immunofluorescence on pronase-digested paraffin sections: a valuable salvage technique for renal biopsies. *Kidney Int.* 2006; 70: 2148–2151.
39. Ge S, Crooks GM, McNamara G, et al. Fluorescent immunohistochemistry and in situ hybridization analysis of mouse pancreas using low-power antigen-retrieval technique. *J. Histochem. Cytochem.* 2006; 54: 843–847.
40. Xia X, Rasmussen T, Alvarez X, et al. Fluorescence in situ hybridization using an old world monkey Y chromosome-specific probe combined with immunofluorescence staining on rhesus monkey tissues. *J. Histochem. Cytochem.* 2007; 55: 1115–1121.
41. Nakamura KC, Kameda H, Koshimizu Y, et al. Production and histological application of affinity-purified antibodies to heat-denatured green fluorescent protein. *J. Histochem. Cytochem.* 2008; 56: 647–657.
42. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
43. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.
44. Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. *J. Histochem. Cytochem.* 2005; 53: 1421–1432.
45. Larsson L-I. *Immunocytochemistry: Theory and Practice*. Boca Raton, FL: CRC Press, 1988.
46. Eltoum I, Fredenburgh J, Myers RB, et al. Introduction to the theory and practice of fixation of tissues. *J. Histotechnol.* 2001; 24: 173–190.

47. Prieto DA, Hood BL, Darfler MM, et al. Liquid Tissue™: proteomic profiling of formalin-fixed tissues. *BioTechniques* 2005; 38: S32–S35.
48. Chu W-S, Liang Q, Liu J, et al. A nondestructive molecule extraction method allowing morphological and molecular analyses using a single tissue section. *Lab. Invest.* 2005; 85: 1416–1428.
49. Crockett DK, Lin Z, Vaughn CP, et al. Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Lab. Invest.* 2005; 85: 1405–1415.
50. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
51. Rahimi F, Shepherd CE, Halliday GM, et al. Antigen-epitope retrieval to facilitate proteomic analysis of formalin-fixed archival brain tissue. *Anal. Chem.* 2006; 78: 7216–7221.
52. Rait VK, Xu L, O’Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RBase A II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
53. Sompuram AR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
54. Taylor CR, Shi S-R. Practical issues: fixation, processing and antigen retrieval. In *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist*, 3rd edition, ed. CR Taylor and RJ Cote, pp. 47–74. Philadelphia: Elsevier Saunders, 2006.
55. Shi S-R, Liu C, Pootrakul L, et al. Evaluation of the value of frozen tissue section used as “gold standard” for immunohistochemistry. *Am. J. Clin. Pathol.* 2008; 129: 358–366.
56. Wick MR, Mills S. Consensual interpretive guidelines for diagnostic immunohistochemistry. *Am. J. Surg. Pathol.* 2001; 25: 1208–1210.
57. Boenisch T. Heat-induced antigen retrieval: what are we retrieving? *J. Histochem. Cytochem.* 2006; 54: 961–964.
58. Swerts K, Ambros PF, Brouzes C, et al. Standardization of the immunocytochemical detection of neuroblastoma cells in bone marrow. *J. Histochem. Cytochem.* 2005; 53: 1433–1440.
59. Jylling AMB, Lindebjerg J, Nielsen L, et al. Immunohistochemistry on frozen section of sentinel lymph nodes in breast cancer with improved morphology and blocking of endogenous peroxidase. *Appl. Immunohistochem. Mol. Morphol.* 2008; 16: 482–484.
60. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
61. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 107–116.
62. Shi Y, Chatterjee S, Brands FH, et al. Coordinate molecular alterations in the development of androgen resistance in prostate cancer: an in vitro model that corroborates clinical observations. *BJU Int.* 2006; 97: 170–178.
63. Sugimura H. Detection of chromosome changes in pathology archives: an application of microwave-assisted fluorescence in situ hybridization to human carcinogenesis studies. *Carcinogenesis* 2008; 29: 681–687.

64. Watters AD, Bartlett MS. Fluorescence in situ hybridization in paraffin tissue sections. *Mol. Biotechnol.* 2002; 21: 217–220.
65. Shi S-R, Cote RJ, Shi Y, et al. Antigen retrieval technique. In *Immunohistochemistry and Molecular Morphology*, 1st edition, ed. S-R Shi, J Gu, and CR Taylor, pp. 311–333. Natick, MA: Eaton, 2000.
66. Tang X, Falls DL, Li X, et al. Antigen-retrieval procedure for bromodeoxyuridine immunolabeling with concurrent labeling of nuclear DNA and antigens damaged by HCl pretreatment. *J. Neurosci.* 2007; 27: 5837–5844.
67. Ino H. Antigen retrieval by heating en bloc for pre-fixed frozen material. *J. Histochem. Cytochem.* 2003; 51: 995–1003.
68. Evers P, Uylings HB. Effects of microwave pretreatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. *J. Neurosci. Methods* 1994; 55: 163–172.
69. Shiurba RA, Spooner ET, Ishiguro K, et al. Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections. *Brain Res. Brain Res. Protoc.* 1998; 2: 109–119.
70. Ino H. Application of antigen retrieval by heating for double-label fluorescent immunohistochemistry with identical species-derived primary antibodies. *J. Histochem. Cytochem.* 2004; 52: 1219–1230.
71. Mundegar RR, Franke E, Schafer R, et al. Reduction of high background staining by heating unfixed mouse skeletal muscle tissue sections allows for detection of thermostable antigens with murine monoclonal antibodies. *J. Histochem. Cytochem.* 2008; 56: 969–975.
72. Monleón E, Monzón M, Hortells P, et al. Detection of PrPsc in samples presenting a very advanced degree of autolysis (BSE liquid state) by immunocytochemistry. *J. Histochem. Cytochem.* 2003; 51: 15–18.
73. Maleszewski J, Lu J, Fox-Talbot K, et al. Robust immunohistochemical staining of several classes of proteins in tissues subjected to autolysis. *J. Histochem. Cytochem.* 2007; 55: 597–606.

CHAPTER 3

EXTRACTION OF DNA/RNA FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE BASED ON THE ANTIGEN RETRIEVAL PRINCIPLE

SHAN-RONG SHI and CLIVE R. TAYLOR

Recognizing the critical importance of formalin-fixed, paraffin-embedded (FFPE) tissue sections as a resource for translational research, extraction of DNA/RNA from archival FFPE tissue sections has become a major priority to meet the need of rapid development of molecular morphology. The technical procedure of DNA/RNA extraction from archival paraffin-embedded tissue sections includes many steps, such as dewaxing in xylene, enzyme digestion, or other chemical treatment incorporated with phenol-chloroform purification and alcohol precipitation. Extraction of DNA from archival FFPE tissue was accomplished as early as 1985 using proteinase K and sodium dodecyl sulfate (SDS) as the major reagents.^{1,2} RNA extraction from FFPE tissues was carried out a few years later.³ High-temperature heating of the paraffin-embedded tissue was not applied for extraction of DNA, although heating the dewaxed paraffin sections at 100°C was used as an initial step of polymerase chain reaction (PCR) after regular DNA extraction.⁴ Subsequently, the heating antigen retrieval (AR) technique was used for enhancement of *in situ* hybridization (ISH) methods applied to archival paraffin-embedded tissue sections in 1995.⁵ Numerous heating retrieval protocols have been documented producing remarkable improvement of signal by chromogenic ISH (CISH), and fluorescence ISH (FISH).⁶⁻⁹ Successful application of heat-induced retrieval method for CISH and FISH has already demonstrated that the high-temperature heating AR approach may behave in a similar manner for “retrieval” of nucleotides as for “retrieval” of antigens in the enhancement of immunohistochemistry (IHC). On the other hand, high-temperature heating methods have also been used to simplify and improve extraction of nucleic acids from

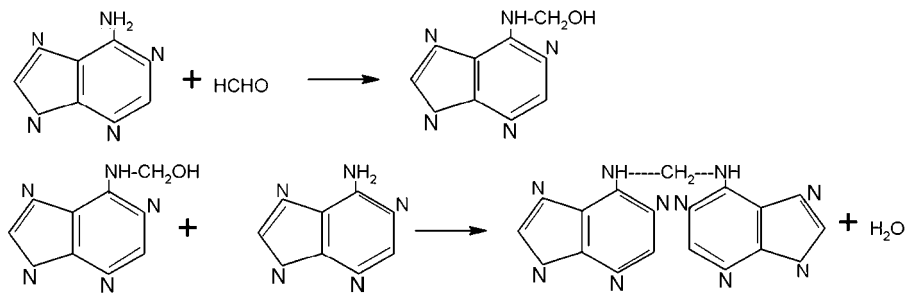


Figure 3.1 Two essential steps of chemical reaction of formaldehyde (HCHO) with nucleic acid exemplified by adenine that are similar to formaldehyde-protein reactions. (a) Addition reaction as the first step, resulting in a methylol derivative, methylol adenylic acid; (b) Second step is a condensation reaction, a stable product methylene-bis-adenylic acid is derived between the methylol derivative and another adenine. Reproduced with permission from Shi et al., *AJMM* 2001; 9: 107–116.

paraffin-embedded tissues.^{10,11} In contrast to the AR technique, heat was not used as the primary retrieval procedure in DNA/RNA extraction from archival paraffin-embedded tissue. Initially, it was not clear that the heating effect in DNA/RNA extraction could be used instead of enzyme digestion, until careful comparative studies have demonstrated this potential in recent years.^{12–14} Frank et al.¹³ pointed out that simple boiling was comparable in outcome, with proteinase K digestion with detergents followed by phenol-chloroform extraction of DNA from paraffin-embedded tissue. Coombs et al.¹⁴ reported a study that sought to optimize DNA/RNA extraction by comparing 10 protocols. They concluded that heating treatments at 90–99°C by thermal cycler, microwave, and simple boiling in solution (0.5% Tween-20, Tris-EDTA, and Chelex-100 from Bio-Rad Laboratories, Hercules, CA) significantly increased the efficiency of extraction of nucleic acids for use in molecular analysis.

There are a number of various commercial products designed for DNA/RNA extraction, but their use may lead to variable results. Based on the evidence that formalin-induced modification of protein is similar to that of nucleic acid modification by formalin (Fig. 3.1),^{15–19} our research group at the University of Southern California has conducted a serial study of AR based heating protocols for DNA/RNA extraction from FFPE tissues following our experience of the AR principle as applied to IHC on tissue sections: heating under the influence of pH.^{19–21}

3.1 DEVELOPMENT OF SIMPLE AND EFFECTIVE PROTOCOL OF DNA EXTRACTION

A simple and effective AR technique of boiling archival paraffin-embedded tissue sections in water to enhance the signal of IHC was developed to circumvent the deleterious effects of formalin fixation, which had previously

proved to be a great challenge to pathologists attempting to apply IHC staining to archival FFPE tissue.²² Successful application of this heat-induced AR method has led to a dramatic change in the attitude of users of IHC, resulting in an exponential increase in articles related to AR-IHC published worldwide.^{21,23} To our understanding, a similar situation may exist in extraction of DNA/RNA from archival paraffin-embedded tissue sections based on basic principles of the AR technique: heating the tissue under the influence of pH as previously documented.²⁰

Our early studies demonstrated that the efficiency of DNA extraction from archival paraffin-embedded tissue sections is correlated with the heating temperature and the pH value of the retrieval solution used during heating,²⁴ just as for AR applied to tissue sections for IHC. It was concluded that higher temperature and higher pH produce better results in terms of quality of DNA extracted from archival tissue. Based on quantitative analysis of three exons of human p53 gene kinetic thermocycling (KTC)-PCR products, it was readily demonstrated that a high-temperature heating protocol at higher pH solution yields much better results than those obtained by nonheating protocols (Fig. 3.2). It appears that high-temperature heating of FFPE tissue may play a critical role in retrieval of nucleic acids in a way mimicking retrieval of antigen, possibly based upon a similar formaldehyde-induced chemical modification between nucleic acids and protein structures.¹⁹ Traditionally, for the first step of DNA extraction from cell or tissue, it is critical to have an effective treatment to dissociate DNA from other cellular constituents and separate it from associated proteins. The use of enzymes and other lytic chemical agents is the usual procedure, in addition to mechanical methods to disrupt cell walls. Although the improved quality of DNA extracted from archival paraffin-embedded tissue sections by “heat-induced retrieval” has been revealed by KTC-PCR and PCR, the quantity of total DNA yields still is lower than obtained by regular nonheating method.²⁴ In contrast to enzyme digestion method, we found that tissue sections, after heating in conventional buffer solutions, such as the Britton and Robinson type of buffer solution (BR buffer), remained substantially intact. However, in the case of enzyme treatment for DNA extraction, a critical point for controlling the incubation time of enzyme digestion is based on complete dissolution of the tissue, as long as 2 days if necessary. Additional enzyme also may be added to maintain the effective concentration during DNA extraction procedure to reach the goal of solubilization of tissue.^{1,2} Several chemicals such as SDS, guanidine isothiocyanate (GITC) or Tween-20 have been used in addition to enzyme (proteinase K), to enhance the effectiveness of nucleic acid extraction from tissue.

In order to develop a more efficient, and practical protocol of DNA extraction based on heating procedure, we performed a serial study to test various chemicals in a combination with heating, in order to identify an optimal protocol. As a result, we developed a simple protocol of boiling FFPE tissue sections in a solution of sodium hydroxide (NaOH) or potassium hydroxide

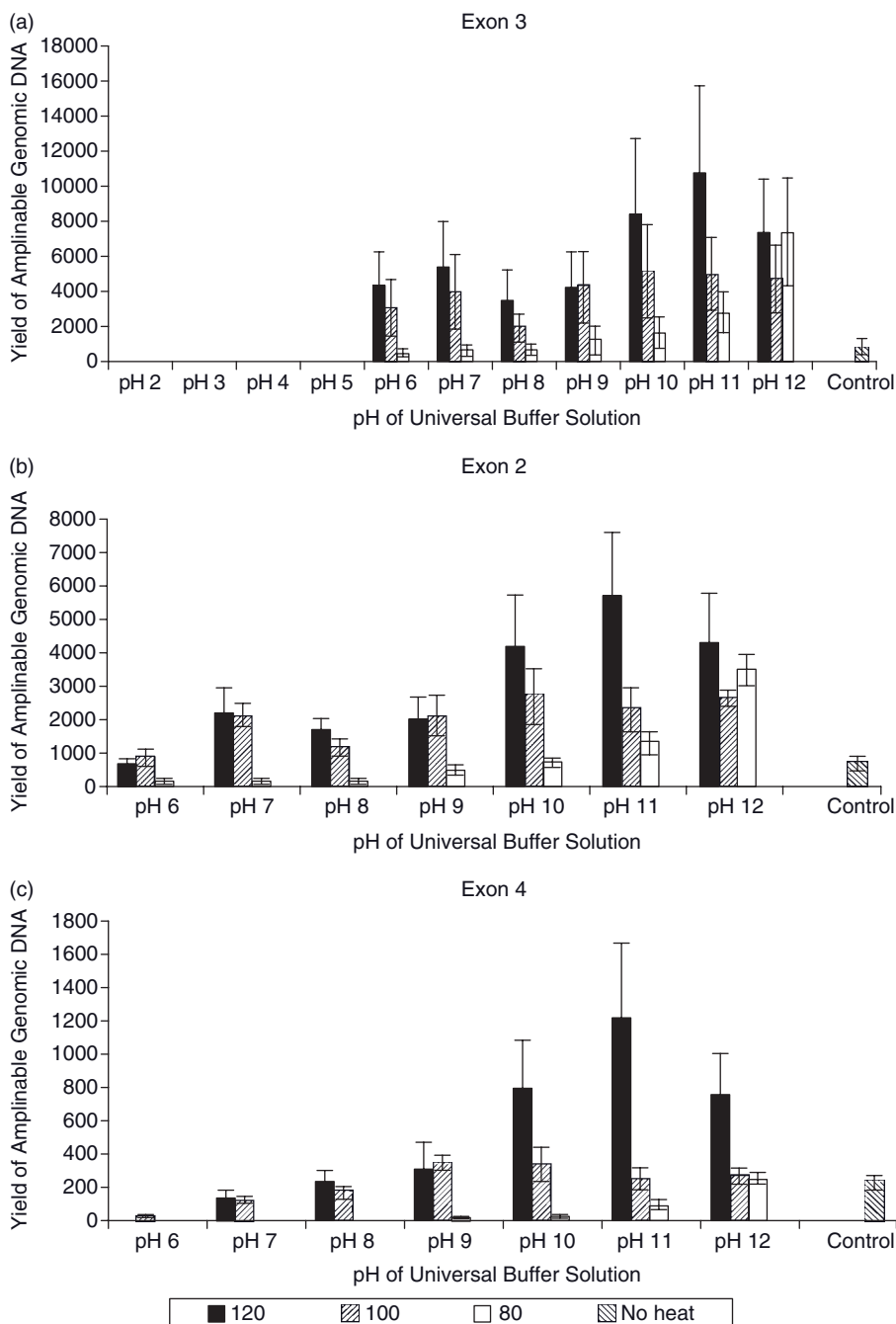


Figure 3.2 Amplifiable genomic DNA measured by KTC-PCR using Exon 3 (a), Exon 2 (b), and Exon 4 (c) of human p53 gene. There is no amplifiable DNA that can be recovered under pH 5 as shown in a (omitted in b and c). Higher yields of amplifiable genomic DNA are achieved by higher temperature (120°C) heating tissues at higher pH with a peak at pH 11. Compared to amplifiable genomic DNA obtained by nonheating protocol (control), the yields of genomic DNA achieved by heating protocol are much higher, particularly at higher temperature heating conditions. Reproduced with permission from Shi et al., *J. Histochem. Cytochem.* 2002; 50: 1005–1011.

(KOH) as the basic retrieval solution. This experiment was based on previous studies in which a higher pH solution provides superior DNA extraction when used as the retrieval solution for heating treatment.²⁵ Previously, NaOH solution has been applied for DNA extraction from bacteria,²⁶ plant tissue,²⁷ whole blood,²⁸ or blood culture fluids and other fresh/frozen tissues.^{29–31} However, alkaline solution has not been used as retrieval solution for high-temperature heating approach to extract DNA from archival FFPE tissue. It appears that sodium hydroxide plays a critical role in disruption of cell membranes for solubilization of proteins through ionization of aspartic, glutamic, cysteic, and tyrosine residues, while the DNA structure is relatively stable in alkaline condition.^{28,30} Tissue sections after heating in NaOH shows full tissue dissolution that is comparable to that achieved by enzyme digestion.

The optimal concentration of NaOH or KOH is 0.1 M. Lower concentrations, such as 0.01 M NaOH or KOH, gave obviously poorer results. Rudbeck and Dissing³⁰ extracted DNA from whole blood by heating the sample in NaOH solution in order to dissolve the blood pellets and found that ≥ 0.1 M NaOH at $\geq 70^\circ\text{C}$ completely dissolved the pellet in 5 min, whereas 0.02 M NaOH had no effect even after incubation for 24 h. Our results indicated satisfactory dissolution of archival tissue sections when using 0.1 M NaOH as retrieval solution.

In general, the mechanism of heat and alkaline solution for DNA extraction may be based upon a hypothesis, previously proposed for the AR technique.³² Strong alkaline solution may denature and hydrolyze proteins, resulting in breaking cell and nuclear membranes as well as disrupting cross-linkages due to formalin fixation. It is no surprise to observe the similarity between retrieval of nucleic acid and retrieval of protein (antigen) based on a similar chemical reaction of formaldehyde with these two kinds of macromolecules (Fig. 3.1).^{15–19}

Sato et al.³³ compared several DNA extraction methods and found that a microwave heating protocol was superior to traditional organic chemical reagent-based extraction method. Their heating protocol was a modification of that used by Banerjee et al.¹⁰; it consisted of a first phase of microwave heating paraffin sections in digestion buffer for a total of 60 s, split in four 15 s increments to dewax, followed by proteinase K digestion overnight, and finally by boiling the sample for 10 min. They concluded that the microwave-based DNA extraction method has the advantages of simplicity, lower contamination risk, and better quality of DNA, yielding longer fragments by PCR. However, they emphasized that “the microwave irradiation is beneficial only at the dissolution of paraffin by heating,” differing in this respect from the approach based directly on the AR principle, where high-temperature heating retrieval technique is the key element. Recently, Ferrari et al.³⁴ also used an AR type of approach and documented detection of bovine herpes virus type 5 in FFPE bovine brain by PCR technique using DNA samples extracted from FFPE cattle brain tissues using the protocol of heating FFPE tissue sections in 0.1 M NaOH solution at 121°C , and achieved satisfactory results.

3.2 DNA QUALITY EVALUATED BY ARRAY -BASED COMPARATIVE GENOMIC HYBRIDIZATION

Array-based comparative genomic hybridization (a-CGH) is a genomic analysis system that allows identification of alterations in DNA sequence and copy number, with a potential goal of individualized diagnosis and targeted cancer therapy.^{35,36} A number of reports have described application of a-CGH to DNA extracted from FFPE tissues, with variable results.^{35,37,38}

We performed a study to apply the a-CGH technique to test the quality of DNA extracted from FFPE tissues by different methods, using a nonheating protocol, a heat-induced extraction protocol, based on AR as applied to IHC, and comparing the findings to extracts from paired fresh frozen tissue samples (unpublished data). The study was conducted in two stages.

First, a limited study was performed using breast cancer tissue to establish an optimal protocol of DNA extraction for a-CGH analysis that would allow comparison of a-CGH results after boiling in different solutions: three pH values of 7, 9, and 12 of Britton and Robinson buffer solution, and a 0.1M sodium hydroxide solution. DNA samples extracted from frozen and from FFPE tissue sections by a nonheating protocol were employed, and the results were compared: a protocol of boiling samples in 0.1 M sodium hydroxide gave optimal results.

Based on these findings, 0.1 M sodium hydroxide solution was used for the second stage to test nine paired samples of various human cancer tissues from lymph nodes, breast, kidney, adrenal gland, bladder, colon, and ovary, in each instance comparing to the heat extracted DNA with samples extracted from frozen and from FFPE tissue sections using the usual nonheating protocol. Array-based CGH was performed in Dr. F. Waldman's Lab at UCSF for all samples in triplicate fashion. To avoid bias, a double-blind principle was used to compare CGH results for the nine samples tested. The a-CGH tests were carried out using protocols previously documented.³⁵ Array results were compared visually, and subjective scores were assigned from 1–4, with 1 being excellent and 4 being the worst. A good hybridization was defined as one where the ratios for a region (such as a chromosome arm) had a low standard deviation for gains (>0.2), normal (0.0) or losses (<-0.2). The study was designed such that samples were de-identified prior to a-CGH such that scoring was done in blinded fashion. Results demonstrated that DNA extracted from FFPE tissue sections by using heat-induced retrieval protocol yielded satisfactory a-CGH results, essentially identical with that obtained by using the conventional nonheating protocol (Table 3.1). Based on a-CGH analysis, DNA extracted from unfixed frozen tissue sections always showed better scores than DNA extracted from FFPE tissue sections (Table 3.1). However, while the yields are less, it is feasible to achieve comparable a-CGH data by using DNA extracted from FFPE tissue sections, using a simple heating retrieval based protocol. Several articles published in recent years have examined the utilization of DNA samples extracted from archival FFPE tissues

TABLE 3.1 Comparison of CGH Scores of DNA Samples Extracted from FFPE Tissue Sections between Heating and without Heating Protocols

CGH test	Non-Heating = Heating	Heating > Nonheating	Nonheating > Heating
First step	DNA extracted from FFPE tissue by heating showed the best score (better than fresh tissue section)		
Second step	2	3	2
Third step	3	3	3

used for a-CGH analysis, even with limited amount of DNA, such as 10 to 20 ng, extracted from microdissection of 2000 cells.³⁷ Our study has also proved the efficiency of a previously documented protocol of random-primed amplification of 50 ng FFPE DNA that has advantages compared to conventionally used degenerate oligonucleotide-primed (DOP) PCR method.³⁵

The accuracy of a-CGH array generated from DNA samples extracted from FFPE tissue sections using our heat-induced retrieval protocol was shown by careful comparison of three groups of samples as indicated by Figure 3.3 and Table 3.1.

It is because a-CGH provides such a powerful approach to identification of genomic imbalances associated with cancer and other diseases,³⁶ that the application of a-CGH on archival FFPE tissue sections is demanded. Not surprisingly, therefore, the search for optimal protocols for extraction and amplification of DNA from FFPE tissues has attracted the particular attention of numerous investigators and has led to a positive conclusion in terms of satisfactory reproducibility of a-CGH analyses using DNA extracted from FFPE tissues.^{35,37,39–41} A review of our experience and the literature reveals two major approaches to improving the efficiency of a-CGH, performed on DNA samples extracted from archival FFPE tissue sections: (1) Development of novel protocols for DNA amplification: Klein and co-workers^{42,43} reported a protocol, which they named SCOMP (single cell comparative genomic hybridization), based on DNA digestion combined with adaptor ligation to amplify whole genome PCR when using low-molecular weight DNA preparations, such as “fixative-damaged” DNA extracted from FFPE tissue sections. Subsequently, Wang et al.⁴⁰ developed a balanced-PCR method in order to remove biases associated with PCR saturation and impurities on the basis of SCOMP. In addition, the aforementioned random prime amplification showed superior a-CGH results than those obtained by DOP amplification³⁵; (2) As already described, a second potential approach may be “retrieval” of formaldehyde-induced modification of DNA by AR based methods. High-temperature heating treatment on FFPE tissue sections significantly improves the “positive signal” of *in situ* hybridization,^{5,6,9,21} and several investigators have demonstrated that heating the FFPE tissue sample may improve reverse transcription polymerase chain reaction (RT-PCR) yields for RNA extracted from FFPE tissue sections.^{14,44}

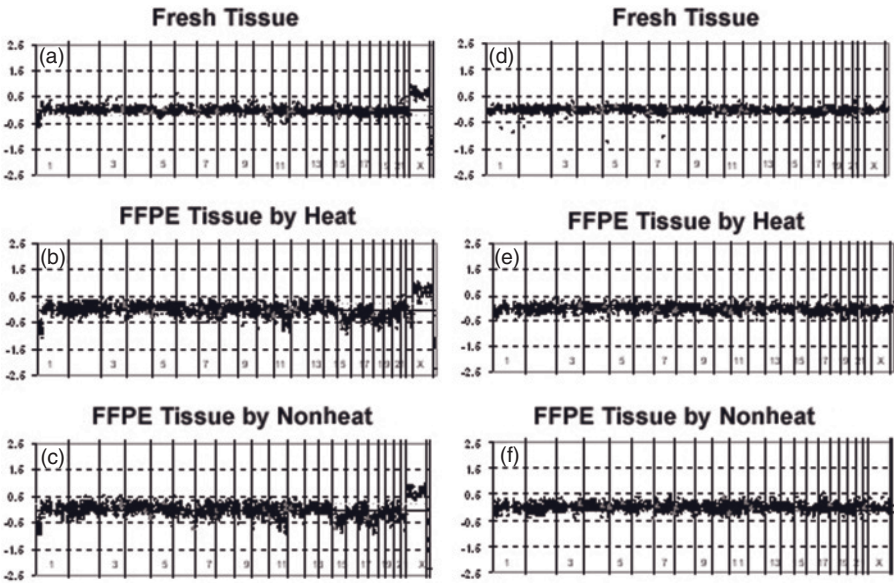


Figure 3.3 Comparison of array CGH among DNA extracted from fresh tissue, FFPE tissue by heating protocol or nonheating protocol for two human tissue samples of metastatic carcinoma in lymph node (a–c), and undifferentiated non-small cell carcinoma (d–f). Array CGH hybridization genomic profiles show ratio values representing relative copy number of single BACs. A good result is scored as 1.0 that indicates a low standard deviation for gains (>0.2), normal (0.0), or losses (<-0.2). In these two cases, fresh samples show best score as 2, both FFPE tissue samples show identical score of 3. Each spot represents the average of three replicates. Clones are ordered by chromosomal position as numbers at the bottom (x axis) of each picture. The y axis is the $\log_2$ ratio of test:reference intensity. Provided by Sandy DeVries from Dr. Frederic Waldman’s Lab at UCSF.

The heat-induced retrieval protocol for extraction of formaldehyde-modified DNA from FFPE tissue sections provides a simple and effective method of DNA extraction from archival tissue samples.^{25,45} Based on PCR using three primer pairs ranging from 152–541 bp and a real time KTC-PCR analysis, the heat-induced retrieval protocol yields a better quality and quantity of DNA samples extracted from FFPE tissue sections than conventional methods of extraction.²⁴ In addition, this heating protocol may provide an alternative approach for DNA extraction in some cases such as a recent publication by Ferrari et al. mentioned above.³⁴

In conclusion, the data described demonstrate the reproducible quality of DNA samples extracted by using a heat-induced retrieval protocol from FFPE tissue sections, based on careful comparison of a-CGH analysis data. This simple and effective DNA extraction protocol may provide an alternative technique for DNA analysis for CGH as well as for other methods of DNA

analysis. Although the mechanism of heat-induced retrieval for protein and nucleic acids remains unknown, the reliable retrieval efficiency for formalin-modified protein has been demonstrated by numerous publications.^{19,21}

3.3 ARTIFACTUAL DNA SEQUENCE ALTERATIONS OF FFPE TISSUE AND RETRIEVAL STRATEGY

Some investigators described artifactual DNA sequence alterations after formalin fixation, when testing DNA samples extracted from FFPE tissues. Williams et al.⁴⁶ reported that up to one mutation artifact per 500 bases was found in FFPE tissue. They also found that the chance of artificial mutations in FFPE tissue sample was inversely correlated with the number of cells used for DNA extraction; that is, the fewer cells, the more the artifacts. However, they mentioned that these artifacts can be distinguished from true mutations by confirmational sequencing of independent amplification products, in essence comparing the product of different batches. Quach et al.⁴⁷ documented that damaged bases can be found in DNA extracted from FFPE tissues, but are still “readable” after *in vitro* translesion synthesis by Taq DNA polymerase. They pointed out that appropriate caution should be exercised when analyzing small numbers of templates or cloned PCR products derived from FFPE tissue samples.

One potentially fruitful research direction is further evaluation of the quality of DNA extracted from FFPE, in search of a retrieval approach that may eliminate these types of errors, based on experiments using model systems as a designed project to increase quantity of higher molecular weight of DNA. Koshiba et al.⁴⁸ studied the effect of different fixatives under variable conditions, using a model system of Lambda phage DNA and FFPE tissues, and found that tissue fixation by buffered formalin at 4°C allowed extraction of high quality of DNA for Southern blot analysis. In addition, they demonstrated that application of modified tissue-lysing buffer, containing 4M urea, enabled extraction of high-molecular-weight DNA. Fang et al.⁴⁹ developed a technique using gradual dehydration and critical point drying for high-quality DNA extraction from old formalin-fixed tissue specimens. Their method allows successful extraction of DNA from animal tissues overfixed in formalin for as long as 70 years, yielding high-quality DNA (>194kb). These pioneer studies provide encouragement for ongoing attempts on retrieval of nucleic acids that have been modified by formalin fixation.

3.4 DEVELOPMENT OF HEAT-INDUCED PROTOCOL FOR RNA EXTRACTION FROM FFPE TISSUE

Following the development of successful heating protocols for DNA extraction from archival FFPE tissue sections as described, it required no great leap of imagination to evaluate similar methods for RNA extraction. Analysis of

gene expression at the mRNA level is critical for molecular profiling, with extensive applications in cancer research. Retrospective studies correlating molecular features with therapeutic response and clinical outcome based upon efficient methods of RNA extraction from FFPE tissues would be expected to yield interesting findings. However, RNA extracted from FFPE tissues is usually considered to be poor material for molecular analysis, due to degradation and RNA fragmentation.⁵⁰ However, several recent articles have demonstrated that FFPE tissue and other processed tissues may be amenable to RT-PCR (Table 3.2).^{3,51–53}

Masuda et al.¹⁸ utilized a model system to examine formalin-induced modification of synthetic oligo RNA, as well as cellular RNA, and complementary DNA synthesized from RNA. The heating procedure employing low temperature (70°C) was used after extraction of RNA by routine commercial kit. They calculated the modification rate and compared different methods of RNA extraction from formalin-fixed tissue, with the following conclusion: “The majority of RNA can be extracted from properly processed archival samples. Although chemical modification by formalin does not allow the direct application of extracted RNA to cDNA synthesis and RT-PCR, more than half of the modification is simple methylol addition, which is reversed by simply heating in TE buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA).” In this context, it is important to note that extraction of RNA may be compromised due to degradation of RNA in the tissue, before fixation. Thus, delayed or inadequate fixation may result in degradation of RNA, which is irreversible, rendering any subsequent attempts at retrieval futile. This period prior to fixation has recently been termed “warm ischemic time” and is recognized of importance in attempts to standardize sample preparation for quantitative IHC methods. It is just as critical, or more so, for RNA extraction, due to rapid enzymatic degradation, unless quickly fixed. Following Masuda et al., Hamatani et al.⁴⁴ recently reported that preheating in citrate buffer (pH 4.0) of RNA extracted from long-term preserved FFPE tissues resulted in significantly increased efficiency of RT-PCR. They demonstrated that RNA extracted from archival FFPE tissues stored for a long period as up to 21 years with fragment sizes of smaller than 60bp could be amplified successfully at a rate of greater than 80% by RT-PCR.

3.5 A DETAILED EXAMPLE OF RNA EXTRACTION FROM FFPE CELLS/TISSUES PERFORMED AT OUR LABORATORY

We recently conducted experiments to extract mRNA from a cell model (MBA-MB-486 cell line of human breast cancer) processed in both frozen and FFPE blocks in a comparable fashion (unpublished data). The cell model system was prepared in three ways for comparison: (1) Positive Control Fresh Cell Pellet: Two flasks of cells were collected in a pellet, and stored at –70°C until use; (2) Frozen Cell Block: Two flasks of cells was embedded in OCT

TABLE 3.2 RNA Extraction from FFPE Tissues Documented in Literature

Reference	FFPE Tissue/ Cells	Extraction Method	RT-PCR	Conclusion
Rupp and Locker ³	Rat liver	Homogenized in a solution containing SDS, and proteinase K	Not available, using hybridization including dot-blot method	RNA purified from FFPE tissue is suitable for hybridization.
Finke et al. ⁵⁴	BJAB cells (B-lymphocytic cell line)	1% SDS + proteinase K	Housekeeping gene PBGD (porphobilinogen deaminase)	Most routine FFPE tissues will be available for RNA analysis.
Mies ⁵⁵	Breast cancer	RNAzol (Biotecx, Huston, TX)	ER gene (exons 1 and 2)	150bp amplicon is feasible for clinical and research studies.
Svoboda-Newman et al. ⁵⁶	Liver from transplant patients with hepatitis C virus infection	TRIzol reagent (Gibco BRL)	HCV using nested PCR	RT-PCR is a sensitive and specific method to detect HCV in routine FFPE tissue.
Krafft et al. ⁵²	All types of FFPE tissues	Proteinase K	HCV, morbillivirus, and influenza virus	Amplifiable influenza RNA is possible using FFPE blocks stored 79 years.
Godfrey et al. ⁵⁷	Liver after variable time of delay fixation (refixation time)	Proteinase K plus digestion buffer	Real time, for 15 genes with amplicon size of 72–291 bp	It should be feasible to analyze gene expression in FFPE tissue despite variable prefixation time.
Lehmann et al. ⁵⁸	Microdissected CD68 (+) cell from liver section	N/A	Real time, for oncogenes with amplicon size of 150–300 bp	It may provide a powerful tool to study gene alterations in FFPE tissue.
Lahr et al. ⁵⁹	Microdissected thyroid tissues	PUREscript kit (BIOzym, Germany)	Nested PCR, RET oncogene with amplicon size of 141–383 bp	Expressed genes can be analyzed from routine FFPE tissue slides or pooled single cells.
Specht et al. ⁶⁰	Microdissected cancer tissues	RNA lysis buffer containing SDS and proteinase K	Real time, for seven cancer-relevant genes	Reproducible quantitation of specific mRNAs can be achieved with only 50 cells.

TABLE 3.2 *Continued*

Reference	FFPE Tissue/ Cells	Extraction Method	RT-PCR	Conclusion
Macabeo- Ong et al. ⁶¹	Oral tissues of normal, dysplasia, and cancer	RNA Isolation kit (Ambion, Austin, TX) and proteinase K	Real time quantitative for EGFR, p21, MMP-1, and VEGF mRNA	RNA can be reliably isolated from FFPE tissue sections for reliable qRT-PCR data, but results for some markers are adversely affected by prolonged fixation.
Abrahamsen et al. ⁶²	Lymph nodes dissected from patients of melanoma	RNA isolation kit (Ambion) with proteinase K	Real-time, for β -actin (99 bp), β_2 microglobulin (85 bp), and MART- 1(497/439 bp) mRNAs	All three markers were consistently detected even after 3 weeks of fixation. Quantitative analysis of mRNA is possible for FFPE tissues.
Beaulieux et al. ⁶³	Spinal cord of amyotrophic lateral sclerosis (ALS)	Trireagent (Sigma Aldrich, France) with proteinase K	Enterovirus	FFPE tissue is comparable with frozen tissue for detection of enterovirus.
Jin et al. ⁶⁴	Microdissected small round cell sarcoma tissues	TRIzol reagent (Life Technologies, Grand Island, NY)	20 specific chimeric fusion gene transcripts	Positive signal could be detected using 200–1000 cells.
Benchekroun et al. ⁶⁵	Normal colon and ovarian tumor tissues; rat liver	Proteinase K plus digestion buffer	Real time, for both human, and rat β -actin	RNA extracted from FFPE tissue only contains fragments of 200 bp or less.
Bibikova et al. ⁶⁶	Colon and breast cancers archived up to 11 years	High Pure RNA Paraffin Kit (Roche, Basel, Switzerland) with proteinase K	Real time, for RPL 13A transcript to amplify 90–155 bp. DASL assay	DASL assay system should prove useful for high-throughput expression proofing of archival FFPE tissues.

TABLE 3.2 *Continued*

Reference	FFPE Tissue/ Cells	Extraction Method	RT-PCR	Conclusion
Cronin et al. ⁶⁷	Breast cancer	MasterPure Purification kit (Epicenter, Madison, WI)	TaqMan reactions 92 genes	RT-PCR analysis of FFPE tissue RNA is feasible for clinical tests.
Mikhitarian et al. ⁵⁰	Breast, colon, and lung cancers	RNA lysis/ isolation buffer and proteinase K	Real time for a panel of truncated gene-specific primers	C _t values from FFPE tissues are comparable to matched fresh tissues.
Byers et al. ⁶⁸	Lymph node, nasopharynx, prostate. Lung, bone marrow, and thyroid	Paraffin Block RNA Isolation Kit (Ambion) with proteinase K	AolyA RT-PCR for GAPDH, CD33, C-myb, and SNF2	PolyA RT-PCR enables globally amplifying RNA extracted from FFPE tissues that can be probed for any cDNA species.
Hunter et al. ⁶⁹	Brain tumors and non-neoplastic tissues	Lysis buffer containing SDS with proteinase K	Real time, for apolipoprotein D and GAPDH	Successful in FFPE sample due to use of small amplicons (<100 bp), and prolonged enzyme digestion.
Chung et al. ⁷⁰	Tumor samples stored over 5 years. Rat tissues	Dewax at 95°C, denaturing/lysis solution	Multiplex RT-PCR by MPCR kit (Maxim Biotech, San Francisco, CA)	Extensive dewax is critical to enhance the use of RNA extracted from FFPE tissues.
Mangham et al. ⁷¹	Decalcified and non-decalcified Ewing's sarcoma of bone	Heating tissue section based on AR, Ambion Paraffin Block RNA Isolation kit (Huntingdon, UK) with proteinase K	A panel of Ewing's sarcoma-specific transcript genes	Use of heating extraction and primers to generate amplicons within 150 bp, high sensitivity and specificity can be achieved for archival FFPE tissues.
Hamatani et al. ⁴⁴	Archival thyroid cancer tissues stored up to 21 years	High Pure RNA Paraffin Kit (Roche) with preheating RNA at 70°C in citrate buffer (pH 4.0)	Breakpoint cluster region (BCR) and N-ras genes	Preheating method of RNA significantly increases efficiency of RT-PCR.

TABLE 3.2 *Continued*

Reference	FFPE Tissue/ Cells	Extraction Method	RT-PCR	Conclusion
Hamoud et al. ⁷²	Bursal tissue of chicken	Trizol (Life Technologies, Gaithersburg, MD) with proteinase K	Real time, for IBDV VP2 gene (400bp)	Optimal tissue processing condition is recommended as 10% formalin, pH 7.0, at 4°C, stored at 4–25°C for 7 days.
Castiglione et al. ⁷³	Colon tissue 10cm away from cancer	RNasy Fibrous Tissue Mini Kit-Qiagen (Hilden, Germany), and PCR Tissue Homogenizing Kit (PBI, Milan, Italy)	Real time, for GAPDH and COX-2	To achieve reliable results, optima formalin-fixation time is 24h, at least not longer than 72h.
Linton et al. ⁷⁴	Leiomyosarcoma, liposarcoma, and synovial sarcoma, stored 1–8 years	Optimum FFPE extraction protocol (Ambion Diagnostics, TX), with proteinase K at 50°C for 2–4h	Real time, for 24 prognostic genes of sarcoma	Reliable, clinically relevant data can be obtained from FFPE tissue, but protocol amendments are needed.

Note: Literature selected randomly by online search.

DASL, cDNA-mediated annealing, selection, extension, and ligation; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IBDV, infectious bursal disease virus; VEGF, vascular endothelial growth factor.

Compound (Miles Laboratories, Elkhart, IN), snap-frozen, and cut into sections for comparison with paraffin-embedded cell sections; (3) FFPE Cell Blocks: Six cell pellets were fixed in 10% neutral buffered formalin immediately after harvest, at room temperature for 6, 12, 24 h, 3, 7, and 30 days, respectively. For further comparison with the cell model system, recently collected sample of human breast cancer tissues were processed by OCT-embedding and snap-freezing; the corresponding routine FFPE block that was obtained from the Norris Cancer Hospital and Research Institute at the University of Southern California Keck School of Medicine (USC). This tissue block was processed routinely (formalin-fixed 24 h and processed by automatic equipment).

For the frozen cell/tissue samples, RNA extraction was carried out by using the TRIzol reagent kit. For the paraffin-embedded cell/tissue, RNA extraction was carried out by two methods: heating and nonheating using enzyme digestion for comparison. RT-PCR was performed to compare the results. A

TABLE 3.3 Primers of 10 Genes Used for RT-PCR

Tested Genes	Primer Sequences	Amplicon Sizes (bp)
B2M	AGT ATG CCT GCC GTG TGA AC CTA AGT TGC CAG CCC TCC TA	309
Mucin	TGG AGA CGC AGT TCA ATC AG CAG CTG CCC GTA GTT CTT TC	233
CK 19	AGG TGG ATT CCG CTC CGG GCA ATC TTC CTG TCC CTC GAG CA	461
P53	AGA CCG GCG CAC AGA GGA AG CTT TTT GGA CTT CAG GTG GC	280
P27	CGG CTA ACT CTG AGG ACA CG GTC TGC TCC ACA GAA CCG GC	198
Maspin (F)	TCA AGC GGC TCT ACG TAG AG CCT CCA CAT CCT TGG GTA GT	447
Maspin (N)	GAT CTC ACA GAT GGC CAC TT GCA CTG GTT TGG TGT CTG TC	175
HMAM (F)	CAG CGG CTT CCT TGA TAA TTG ATA AGA AAG AGA AGG TGT GG	402
HMAM (N)	TGA ACA CCG ACA GCA GCA G TCC GTA GTT GGT TTC TCA CC	367
EGFR	CAG CTG CCA AAA GTG TGA TC TCC ATC TCA TAC CTG TCG GC	402
HER2	CCC TCA TCC ACC ATA ACA CC CAT TCC TCC ACG CAC TCC T	235
CK7	CCA GTT TGC CTC CTT CAT CG GCA ATC TGG GCC TCA AAG ATG	138

F, first primer used for nested PCR; N, nested PCR.

panel of primers designed to amplify mRNA sequences of 10 genes was used to demonstrate the efficiency of heat-induced RNA retrieval technique (Table 3.3).

3.5.1 Nonheating RNA Extraction Protocol

For frozen cell/tissue sections, RNA was extracted using the TRIzol reagent kit (Invitrogen Co., Carlsbad, CA) following the manufacturer's instructions (protocol found in Appendix). For the FFPE cell blocks, deparaffinization was carried out using Octane (Sigma, St. Louis, MO), and enzyme digestion was performed with a lysis buffer containing proteinase K at 56°C overnight, using a rotation incubator. Further steps of extraction by using the TRIzol reagent kit to extract and purification were by the recommended protocol.

3.5.2 Heating RNA Extraction Protocol

The heating method of RNA extraction was carried out using the same technique as used for DNA extraction from archival tissue sections documented

previously.²⁴ To establish an optimal heating protocol for RNA extraction from FFPE tissue/cells, a pilot study was carried out using serial retrieval solutions of various pH values, ranging from pH 1 to pH 12, under three heating conditions, following the AR principle (80°C by water bath, 100°C by a heat block, or 120°C by an autoclave). The heating time was 20 min for all temperatures; elongated heating times were explored in preliminary studies for establishing an optimal protocol, but showed no advantage. Both highly acidic or strongly basic buffer solutions produced poor results. Based on a comparison of various buffer solutions and heating conditions, an optimal heating protocol was established, using a neutral BR buffer solution with boiling heating condition, and the method was adopted for all subsequent tests of RNA extraction from FFPE tissue sections. Briefly, a total of 500 µL of BR buffer at pH 7.4 was added to each microtube containing a 20-µm tissue sections, and was heated at boiling condition. After heating treatment, the microtube was allowed to cool for 5 min at room temperature. Further steps of extraction were as described above for the nonheating protocol using TRIzol reagent kit (see protocol in Appendix).

Combining both heating and nonheating protocols employed in a sequential order were evaluated, but without any advantage (Fig. 3.4). RT-PCR was performed by standard methods, RNA extracted from fresh MDA cells and human tissue of breast cancer with known tested genes was used as positive control, and pure water was used to replace template (cDNA) as negative control for every experiment of PCR. To assure the accuracy of PCR tests, all reactions were performed in triplicate.

The quantity of RNA extracted from FFPE cell/tissue sections by the heating and nonheating methods, and extracted from fresh cell/tissue embedded in OCT without fixation, was comparable, showing no significant difference for all yields of RNA by Student's *t*-test, with the exception of one sample, MDA cells fixed in formalin for 24 h ($p < 0.05$).

The quality of RNA was evaluated by RT-PCR. Table 3.4 and Figure 3.4 showed comparative results of RT-PCR between the heating and nonheating methods. In general, for the 10 pairs of intron spanning primers tested, comparable products of RT-PCR were demonstrated in most markers, except five bands that were only found by the heating protocol in the following samples: MDA cells, B2M 7 days, p53 30 days, Maspin 24h, Her2/neu 30 days, and tissue, EGFR 24h. Examples of gel electrophoresis of RT-PCR products with (some markers) were shown in Figure 3.4. All negative control samples revealed absence of bands as expected.

Based on this study, it is apparent that RNA extracted from FFPE tissue by either heating or nonheating protocol is sufficient in quantity and quality for successful application for RT-PCR tests, to achieve amplicons up to larger size of 461 bp in 7-day-fixed FFPE tissue using heating protocol (Fig. 3.4). This conclusion is supported by most publications in recent years (Table 3.2). Nevertheless, there is one gene (hMAM) that showed negative results for all samples in contrast to the positive control. The exact reason is not clear. It may be caused by technical issues or degradation of certain RNA.

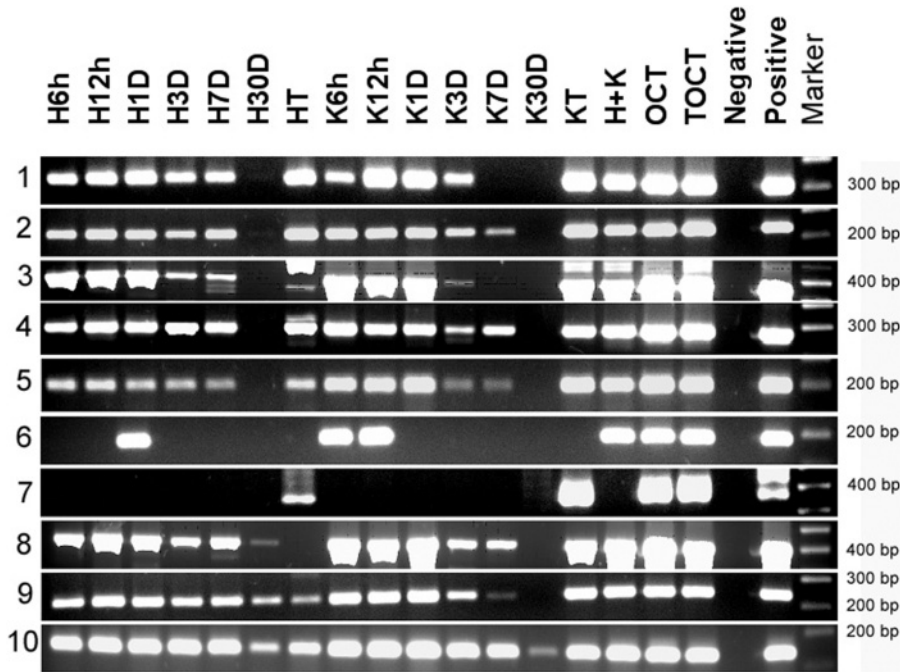


Figure 3.4 Comparison of RT-PCR products of 10 genes among RNA samples extracted from FFPE cells (MBA-MB-486 cell line of human breast cancer) with variable time of fixation by heating or nonheating protocols, and fresh cell/tissue. Primers are listed in Table 3.3, with the same order numbers: 1, B2M; 2, mucine; 3, CK19; 4, p53; 5, p27; 6, maspin; 7, hMAM; 8, EGFR; 9, HER-2; 10, CK7. Sample preparation conditions are labeled in the top as: H6h, cell sample fixed in 10% neutral buffered formalin (NBF) for 6h using the heating protocol for RNA extraction; H12h, cell fixed in NBF for 12h by heating protocol; H1D, H3D, H7D, H30D, cells fixed for 1 day, 3 days, 7 days, and 30 days, respectively, by heating protocol for RNA extraction; HT, RNA extracted from archival FFPE tissue by heating protocol; K6h, K12h, K1D, K3D, K7D, and K30D are representing the same sample condition mentioned above for variable time of NBF fixation; K, nonheating protocol using proteinase K; KT, RNA extracted from FFPE tissue by nonheating protocol; H + K, combining both heating and nonheating protocol sequentially; OCT, fresh cell sample embedded in OCT; TOCT, fresh tissue sample embedded in OCT; Negative, negative control (without primers); Positive, positive control with known gene positive samples. Commercially available marker was used for labeling molecular weight of RNA products on the right side.

Numerous articles have demonstrated the availability of RNA extracted from a few cells or even a single cell taken by laser captured microdissection (LCM) system from archival FFPE tissue sections that had been previously stained by IHC. Using a combination of pre-immunostained FFPE tissue section with LCM, a sensitive real-time quantitative RT-PCR can be achieved based on a few immuno-detected cells, creating a way to study pathophysiological gene regulation in a cell-specific manner in archival tissues housed in

TABLE 3.4 Summary of RT-PCR Results of 10 Genes for All Samples

Samples	<i>PBGD</i>	<i>MUC</i>	<i>CK19</i>	<i>P53</i>	<i>P27</i>	<i>MAS</i>	<i>HMAM</i>	<i>EGFR</i>	<i>HER</i>	<i>CK7</i>
MDA	339	233	461	280	198	175	367	402	235	138
6h S	■	■	■	■	■			■	■	■
12h S	■	■	■	■	■			■	■	■
1D S	■	■	■	■	■			■	■	■
3D S	■	■	■	■	■			■	■	■
7D S		■	■	■	■			■	■	■
30D S										■
1D H + S	■	■	■	■	■	■		■	■	■
6h H	■	■	■	■	■			■	■	■
12h H	■	■	■	■	■	■		■	■	■
1D H	■	■	■	■	■	■		■	■	■
3D H	■	■	■	■	■			■	■	■
7D H	■	■	■	■	■			■	■	■
30D H				■					■	■
Positive	■	■	■	■	■	■	■	■	■	■

Notes: MDA cell line fixed for variable times: h, hour; D, day; H, heating; S, nonheating protocol; Positive, positive control. All terms of genes are comparable with Figure 3.4, all figures under each gene are the size of band (bp). Black column (■), showing band of PCR product with correlated bp (see Fig. 3.4). Empty column, no band.

pathologic file worldwide.^{58,60,75,76} The heat-induced retrieval protocol also provides an effective method to extract RNA from FFPE tissue/cells in LCM applications. Based on comparison of RT-PCR, the efficiency of heat-induced retrieval protocol has been demonstrated to be a comparable method of conventional nonheating protocol for RNA extraction from FFPE tissue (Table 3.4, Fig. 3.4). The authenticity of RNA isolated by the heating protocol is indicated by 10 markers showing comparable bands of PCR with that obtained by fresh cell/tissue. In addition, there are three unique bands showing products of RT-PCR for MDA cell model fixed in formalin at longer times as 7 days (B2M), 30 days (p53, and Her2/neu).

According to previous reports, most authors recognize that tissues fixed in formalin over 5 days⁷⁷ or 1 week cannot be used to extract available RNA for RT-PCR. However, our data show that cells fixed in formalin for 7 days, or even over 1 month could still yield suitable RNA for RT-PCR as indicated in Table 3.4 and Figures 3.4. Despite the fact that the size of RNA extracted by heating protocol is smaller than that extracted by nonheating method, the size of amplicon of RT-PCR generated by heating protocol is larger than 400bp (Table 3.4, Fig. 3.4) and appears to lend itself to analysis. Previously, it has been suggested that amplicon size of RNA extracted from FFPE tissue is smaller than 400bp.^{60,78} Also, Benchekroun et al.⁶⁵ documented recently that RNA extracted from FFPE tissue only had fragments of 200bp or less, and did not contain templates for a (250bp) amplicon. However, our results in

part contradict these findings and demonstrate a much larger size of RNA fragments by both nonheating and heating protocols of RNA extraction from the FFPE tissue sections, with amplicons over 450bp derived by the heating protocol of RNA extraction. Other aspects of the tissue preparation should not be forgotten in evaluating these findings; as already noted, “warm ischemic time” prior to fixation is critical because of degradation or “autolysis,”⁶² and postfixation steps, including wax embedment, may also be deleterious. For example, Chung et al.⁷⁰ extracted RNA from FFPE tissue using 95°C deparaffinization for 15 min in Autodewaxer prior to lysis extraction procedure and found that the increase of RNA yield was correlated to an increase of dewax temperature, 95°C > 80°C, with RT-PCR amplicon size of up to 300bp.

The role of these other factors may be reflected in the fact that during our studies, we found that not all archival tissue blocks could be utilized to extract RNA efficiently, by the same heating or nonheating protocol. Despite the fact that gene expression tests can be reliably conducted from FFPE tissues, as described in multiple publications mentioned above, it is necessary to conduct further studies to evaluate, confirm, and develop techniques, with the goal of extending and standardizing the use of archival tissues for molecular medicine worldwide.⁷⁹ Several recent studies have demonstrated that reliable microRNA profiling may be achieved by using routinely processed FFPE breast cancer specimens using fluorescence-labeled bead technology,⁸⁰ and some factors in tissue handling and processing that may influence the quality of RNA extracted from FFPE tissue.^{81,82}

CONCLUSION

Based on the heat-induced AR principle, DNA/RNA extraction from FFPE tissues can be successfully achieved by a simple heating protocol that allows satisfactory application of molecular analysis using FFPE tissue samples housed in pathology laboratories worldwide. By a combination of improved extraction methods with various innovative techniques of molecular biology, more reliable results of molecular profiling for archival tissue are anticipated.

ACKNOWLEDGMENT

DNA array-CGH data were provided by Sandy DeVries at Dr. Fredric Waldman’s Lab at UCSF. RNA extraction experiments were technically performed by Cheng Liu and Kelly Smith. Both studies were supported by NIH grant 1 R33 CA103455. Use of human tissues has been exempted under 45 CFR 46.101 (b) and was approved by the Institutional Review Board (IRB #009071) at USC.

REFERENCES

1. Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde-fixed and paraffin-embedded tissue. *Biochem. Biophys. Res. Commun.* 1985; 130: 118–126.
2. Dubeau L, Chandler LA, Gralow JR, et al. Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. *Cancer Res.* 1986; 46: 2964–2969.
3. Rupp GM, Locker J. Purification and analysis of RNA from paraffin-embedded tissues. *BioTechniques* 1988; 6: 56–60.
4. Shibata D. Extraction of DNA from paraffin-embedded tissue for analysis by polymerase chain reaction: new tricks from an old friend. *Hum. Pathol.* 1994; 25: 561–563.
5. Sibony M, Commo F, Callard P, et al. Enhancement of mRNA in situ hybridization signal by microwave heating. *Lab. Invest.* 1995; 73: 586–591.
6. Oliver KR, Heavens RP, Sirinathsinghi DJS. Quantitative comparison of pretreatment regimens used to sensitive in situ hybridization using oligonucleotide probes on paraffin-embedded brain tissue. *J. Histochem. Cytochem.* 1997; 45: 1707–1713.
7. Kitayama Y, Igarashi H, Sugimura H. Amplification of FISH signals using intermittent microwave irradiation for analysis of chromosomal instability in gastric cancer. *Mol. Pathol.* 1999; 52: 357–359.
8. Bull JH, Harnden P. Efficient nuclear FISH on paraffin-embedded tissue sections using microwave pretreatment. *BioTechniques* 1999; 26: 416–422.
9. Gu J, Farley R, Shi S-R, et al. Target retrieval for in situ hybridization. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, 1st edition, ed. S-R Shi, J Gu, and CR Taylor, pp. 115–128. Natick, MA: Eaton, 2000.
10. Banerjee SK, Makdisi WF, Weston AP, et al. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. *BioTechniques* 1995; 18: 768–770, 772–773.
11. Faulkner SW, Leigh DA. Universal amplification of DNA isolated from small regions of paraffin-embedded, formalin-fixed tissue. *BioTechniques* 1998; 24: 47–50.
12. Sepp R, Szabo I, Uda H, et al. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J. Clin. Pathol.* 1994; 47: 318–323.
13. Frank TS, Svoboda-Newman SM, Hsi ED. Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. *Diagn. Mol. Pathol.* 1996; 5: 220–224.
14. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res.* 1999; 27: e12–e17.
15. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. 3. Equilibrium denaturation of DNA and synthetic polynucleotides. *Biochemistry* 1977; 16: 3267–3275.
16. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. 4. Mechanism of the initial reaction of formaldehyde with DNA. *Biochemistry* 1977; 16: 3276–3293.

17. Auerbach C, Moutschen-Dahmen M, Moutschen J. Genetic and cytogenetical effects of formaldehyde and related compounds. *Mutat. Res.* 1977; 39: 317–362.
18. Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* 1999; 27: 4436–4443.
19. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 107–116.
20. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.
21. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
22. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
23. Shi S-R, Gu J, Taylor CR. *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, 1st edition. Natick, MA: Eaton, 2000.
24. Shi S-R, Cote RJ, Wu L, et al. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. *J. Histochem. Cytochem.* 2002; 50: 1005–1011.
25. Shi S-R, Datar R, Liu C, et al. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. *Histochem. Cell Biol.* 2004; 122: 211–218.
26. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 1979; 7: 1513–1523.
27. Wang H, Qi M, Cutler AJ. A simple method of preparing plant samples for PCR. *Nucleic Acids Res.* 1993; 21: 4153–4154.
28. Klintschar M, Neuhuber F. Evaluation of an alkaline lysis method for the extraction of DNA from whole blood and forensic stains for STR analysis. *J. Forensic Sci.* 2000; 45: 669–673.
29. Kulski JK, Pryce T. Preparation of mycobacterial DNA from blood culture fluids by simple alkali wash and heat lysis method for PCR detection. *J. Clin. Microbiol.* 1996; 34: 1985–1991.
30. Rudbeck L, Dissing J. Rapid, simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. *BioTechniques* 1998; 25: 588–592.
31. Valsecchi E. Tissue boiling: a short-cut in DNA extraction for large-scale population screenings. *Mol. Ecology* 1998; 7: 1243–1245.
32. Shi S-R, Gu J, Turrens F, et al. Development of the antigen retrieval technique: philosophy and theoretical base. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 17–39. Natick, MA: Eaton, 2000.
33. Sato Y, Sugie R, Tsuchiya B, et al. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. *Diagn. Mol. Pathol.* 2001; 10: 265–271.

34. Ferrari HF, Luvizottob MCR, Rahald P, et al. Detection of bovine Herpesvirus type 5 in formalin-fixed, paraffin-embedded bovine brain by PCR: a useful adjunct to conventional tissue-based diagnostic test of bovine encephalitis. *J. Virol. Methods* 2007; 146: 335–340.
35. DeVries S, Nyante S, Korkola J, et al. Array-based comparative genomic hybridization from formalin-fixed, paraffin-embedded breast tumors. *J. Mol. Diagn.* 2005; 7: 65–71.
36. Pinkel D, Albertson DG. Comparative genomic hybridization [Review]. *Annual Review of Genomics and Human Genetics* 2005; 6: 331–354.
37. Johnson NA, Hamoudi RA, Ichimura K, et al. Application of array CGH on archival formalin-fixed paraffin-embedded tissues including small numbers of microdissected cells. *Lab. Invest.* 2006; 86: 968–978.
38. Ylstra B, Van den IJssel P, Carvalho B, et al. BAC to the future! or oligonucleotides: a perspective for micro array comparative genomic hybridization (array CGH). *Nucleic Acids Res.* 2006; 34: 445–450.
39. Daigo Y, Chin S-F, Gorringer KL, et al. Degenerate oligonucleotide primed-polymerase chain reaction-based array comparative genomic hybridization for extensive Amplicon profiling of breast cancers. A new approach for the molecular analysis of paraffin-embedded cancer tissue. *Am. J. Pathol.* 2001; 158: 1623–1631.
40. Wang G, Brennan C, Rook M, et al. Balanced-PCR amplification allows unbiased identification of genomic copy changes in minute cell and tissue samples. *Nucleic Acids Res.* 2004; 32 (e76): 1–10.
41. Ghazani AA, Arneson NC, Warren K, et al. Limited tissue fixation times and whole genomic amplification do not impact array CGH profiles. *J. Clin. Pathol.* 2006; 59: 311–315.
42. Klein CA, Schmidt-Kittler O, Schardt JA, et al. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. *Proc. Natl. Acad. Sci. U S A* 1999; 96: 4494–4499.
43. Stoecklein NH, Erbersdobler A, Schmidt-Kittler O, et al. SCOMP is superior to degenerated oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. *Am. J. Pathol.* 2002; 161: 43–51.
44. Hamatani K, Eguchi H, Takahashi K, et al. Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. *J. Histochem. Cytochem.* 2006; 54: 773–780.
45. Shi S-R, Cote RJ, Wu L, et al. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. *J. Histochem. Cytochem.* 2002; 50: 1005–1011.
46. Williams C, Pontén F, Moberg C, et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am. J. Pathol.* 1999; 155: 1467–1471.
47. Quach N, Myron GF, Shibata D. In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR. *BioMed Central* 2004; 4: 1–11. <http://www.biomedcentral.com/1472-6890/4/1>.
48. Koshiba M, Ogawa K, Hamazaki S, et al. The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded tissues. *Pathol. Res. Pract.* 1993; 189: 66–72.

49. Fang S-G, Wan Q-H, Fujihara N. Formalin removal from archival tissue by critical point drying. *BioTechniques* 2002; 33: 604–611.
50. Mikhitarian K, Reott S, Hoover L, et al. Enhanced detection of RNA from paraffin-embedded tissue using a panel of truncated gene-specific primers for reverse transcription. *BioTechniques* 2004; 36: 474–478.
51. Jackson DP, Lewis FA, Taylor GR, et al. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J. Clin. Pathol.* 1990; 43: 499–504.
52. Krafft AE, Duncan BW, Bijwaard KE, et al. Optimization of the isolation and amplification of RNA from formalin-fixed, paraffin-embedded tissue: The Armed Force Institute of Pathology experience and literature review. *Mol. Diagn.* 1997; 2: 217–230.
53. Liu H, Huang X, Zhang Y, et al. Archival fixed histologic and cytologic specimens including stained and unstained materials are amenable to RT-PCR. *Diagn. Mol. Pathol.* 2002; 11: 222–227.
54. Finke J, Fritzen R, Ternes P, et al. An improved strategy and a useful house-keeping gene for RNA analysis from formalin-fixed, paraffin-embedded tissues by PCR. *BioTechniques* 1993; 14: 448–453.
55. Mies C. A simple, rapid method for isolating RNA from paraffin-embedded tissues for reverse transcription-polymerase chain reaction (RT-PCR). *J. Histochem. Cytochem.* 1994; 42: 811–813.
56. Svoboda-Newman SM, Greenson JK, Singleton TP, et al. Detection of hepatitis C by RT-PCR in formalin-fixed paraffin-embedded tissue from liver transplant patients. *Diagn. Mol. Pathol.* 1997; 6: 123–129.
57. Godfrey TE, Kim S-H, Chavira M, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. *J. Mol. Diagn.* 2000; 2: 84–91.
58. Lehmann U, Bock O, Glockner S, et al. Quantitative molecular analysis of laser-microdissected paraffin-embedded human tissues. *Pathobiology* 2000; 68: 202–208.
59. Lahr G, Sticha M, Schützea K, et al. Diagnosis of papillary thyroid carcinoma is facilitated by using an RT-PCR approach on laser-microdissected archival material to detect RET oncogene activation. *Pathobiology* 2000; 68: 218–226.
60. Specht K, Richter T, Muller U, et al. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am. J. Pathol.* 2001; 158: 419–429.
61. Macabeo-Ong M, Ginzinger DG, Dekker N, et al. Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analyses. *Mod. Pathol.* 2002; 15: 979–987.
62. Abrahamsen HN, Steiniche T, Nexø E, et al. Towards quantitative mRNA analysis in paraffin-embedded tissues using real-time reverse transcriptase-polymerase chain reaction. A methodological study on lymph nodes from melanoma patients. *J. Mol. Diagn.* 2003; 5: 34–41.
63. Beaulieux F, Berger MM, Tchong R, et al. RNA extraction and RT-PCR procedures adapted for the detection of enterovirus sequences from frozen and paraffin-embedded formalin-fixed spinal cord samples. *J. Virol. Methods* 2003; 107: 115–120.

64. Jin L, Majerus J, Oliveira A, et al. Detection of fusion gene transcripts in fresh-frozen and formalin-fixed paraffin-embedded tissue sections of soft-tissue sarcomas after laser capture microdissection and RT-PCR. *Diagn. Mol. Pathol.* 2003; 12: 224–230.
65. Benckekroun M, DeGraw J, Gao J, et al. Impact of fixative on recovery of mRNA from paraffin-embedded tissue. *Diagn. Mol. Pathol.* 2004; 13: 116–125.
66. Bibikova M, Talantov D, Chudin E, et al. Quantitative gene expression profiling in formalin-fixed, paraffin-embedded tissues using universal bead arrays. *Am. J. Pathol.* 2004; 165: 1799–1807.
67. Cronin M, Pho M, Dutta D, et al. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. *Am. J. Pathol.* 2004; 164: 35–42.
68. Byers R, Roebuck J, Sakhinia E, et al. PolyA PCR amplification of cDNA from RNA extracted from formalin-fixed paraffin-embedded tissue. *Diagn. Mol. Pathol.* 2004; 13: 144–150.
69. Hunter SB, Varma V, Shehata B, et al. Apolipoprotein D expression in primary brain tumors: analysis by quantitative RT-PCR in formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* 2005; 53: 963–969.
70. Chung J-Y, Braunschweig T, Hewitt SM. Optimization of recovery of RNA from formalin-fixed, paraffin-embedded tissue. *Diagn. Mol. Pathol.* 2006; 15: 229–236.
71. Mangham DC, Williams A, McMullan DJ, et al. Ewing's sarcoma of bone: the detection of specific transcripts in a large, consecutive series of formalin-fixed, decalcified, paraffin-embedded tissue samples using the reverse transcriptase-polymerase chain reaction. *Histopathology* 2006; 48: 363–376.
72. Hamoud MM, Villegas P, Williams SM. Detection of infectious bursal disease virus from formalin-fixed paraffin-embedded tissue by immunohistochemistry and real-time reverse transcription-polymerase chain reaction. *J. Vet. Diagn. Invest.* 2007; 19: 35–42.
73. Castiglione F, Degl'Innocenti DR, Taddei A, et al. Real-time PCR analysis of RNA extracted from formalin-fixed and paraffin-embedded tissues: effects of the fixation on outcome reliability. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 338–342.
74. Linton KM, Hey Y, Saunders E, et al. Acquisition of biologically relevant gene expression data by Affymetrix microarray analysis of archival formalin-fixed paraffin-embedded tumours. *Br. J. Cancer* 2008; 98: 1398–1402.
75. Bernsen MR, Dijkman HB, de Vries E, et al. Identification of multiple mRNA and DNA sequences from small tissue samples isolated by laser-assisted microdissection. *Lab. Invest.* 1998; 78: 1267–1273.
76. Lindeman N, Waltregny D, Signoretti S, et al. Gene transcript quantitation by real-time RT-PCR in cells selected by immunohistochemistry-laser capture microdissection. *Diagn. Mol. Pathol.* 2002; 11: 187–192.
77. Tanji N, Ross MD, Cara A, et al. Effect of tissue processing on the ability to recover nucleic acid from specific renal tissue compartments by laser capture microdissection. *Exp. Nephrol.* 2001; 9: 229–234.
78. Tyrrell L, Elias J, Longley J. Detection of specific mRNAs in routinely processed dermatopathology specimens. *Am. J. Dermatopathol.* 1995; 17: 476–483.

79. Farragher SM, Tanney A, Kennedy R, et al. RNA expression analysis from formalin fixed paraffin embedded tissues. *Histochem. Cell Biol.* 2008; 130: 435–445.
80. Hasemeier B, Christgen M, Kreipe H, et al. Reliable microRNA profiling in routinely processed formalin-fixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology. *BMC Biotechnology* 2008; 8: 90, doi:10.1186/1472-6750-8-90.
81. Chung J-Y, Braunschweig T, Williams R, et al. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* 2008; 56: 1033–1042.
82. von Ahlhen S, Missel A, Bendrat K, et al. Determinants of RNA quality from FFPE samples. *PLoS ONE* 2007; 2: e1261.

PART II

STANDARDIZATION OF IMMUNOHISTOCHEMISTRY

CHAPTER 4

KEY ISSUES AND STRATEGIES OF STANDARDIZATION FOR QUANTIFIABLE IMMUNOHISTOCHEMISTRY

SHAN-RONG SHI, KEVIN A. ROTH, and CLIVE R. TAYLOR

Although fluorescein labeled antibodies were developed in 1940s,¹ the related method of immunohistochemistry (IHC) was not applied to archival formalin-fixed, paraffin-embedded (FFPE) tissue until 1974.²⁻⁵ Subsequently, a series of technical advances, including several more sensitive detection systems such as PAP, ABC, etc, based on the enzyme labeled antibody technique, plus monoclonal antibodies and enzyme digestion pre-treatment provided approaches for improved and diverse IHC staining in FFPE tissue sections. With these improvements IHC found an increasingly important role in biomedical research and diagnostic pathology.

In the early 1990s, an antigen retrieval (AR) technique based on boiling FFPE tissue sections in water was described.⁶ Application of this simple and effective technique produced a dramatic increase in the applicability of IHC to surgical pathology as well as other fields of morphology. In the past decade thousands of articles have been published using AR-IHC and FFPE sections for retrospective translational studies, resulting in the creation of a new field of molecular morphology. The AR technique has been credited as a revolutionary breakthrough in IHC which divides the history of IHC into two eras: pre- and post-AR.⁷⁻⁹ In the daily practice of diagnostic surgical pathology, IHC has become an essential tool for classification of many cancers, and for prognostic and predictive information that can be used to select markers of value selecting patients for individualized cancer treatment.⁷

However, the recent rapid development of targeted cancer treatment based on IHC results raised a new issue, the need for quantitative IHC. Previously, assessment of patients was based upon “characteristic patterns of IHC stained

protein markers,” followed by semi-quantitative methods of IHC assessment, as exemplified by successful application of anti-HER2 therapy. However, semi-quantitative methods leave much to be desired in terms of reproducibility and consistency among and between laboratories, with the realization that IHC standardization is a critical issue that must be addressed prior to discussing quantitative IHC techniques.^{10,11}

4.1 A TOTAL TEST APPROACH FOR STANDARDIZATION OF IHC

It has long been recognized that standardization is the key point for successful IHC staining technique.^{7,12} Considerable, but intermittent, efforts to improve IHC standardization have focused on three principal areas: antibodies and reagents, technical procedures, and the interpretation of immunohistochemical findings for use in diagnostic pathology. A “total test” approach was advocated by Taylor in terms of pre-analytical, analytical, and post-analytical issue (Table 4.1).^{10,11,13} A call for taking a definite position on

TABLE 4.1 Immunohistochemistry: The Total Test

Elements of Testing Process	Quality Assurance Issues	Responsibility
Pre-analytical		
1. Test selection: the clinical question	Indications for IHC. Selection of stain(s)	Surgical pathologist; sometimes clinician
2. Specimen acquisition and management	Specimen collection, fixation, processing, sectioning, antigen retrieval	Pathologist/technologist
Analytical		
3. Technology/methodology	Validation of reagents and protocols	Pathologist/technologist
4. Analytical issues	Sensitivity and specificity. Automation Quantifications of staff intra- and inter-laboratory testing Performance of controls	
Post-analytical		
5. Results: validation/reporting	Criteria for positivity/negativity in relation to controls. Content and organization of report. Turnaround time.	Pathologist/technologist
6. Interpretation, significance, final report	Experience/qualifications of pathologist. Proficiency testing of interpretational aspects. Diagnostic, prognostic significance. Appropriateness/correlation with other data.	Surgical pathologist and/or clinician

Reproduced with permission from Taylor, *Biotech. Histochem.* 2006; 81: 3–12.

methodology in diagnostic IHC by certifying and accrediting organizations in pathology has been raised in recent years.¹⁴ Most recently, an Ad-Hoc Committee was formed to discuss standardization of IHC, and recommended 14 critical issues ranging from pre-analytical, analytical and post-analytical phases.¹⁵

4.2 STANDARDIZATION OF IHC: CURRENT STRATEGIES AND THE LONG RUN

The recent publication of “American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer” highlighted the significance, strategies, and practical approach of standardization of IHC based upon current knowledge,¹⁶ although there is still a long way to go to reach the goal of satisfactory standardization, as a necessary prerequisite for quantitative IHC. The pressing need for standardization of IHC is illustrated by the fact that, according to a literature survey, about 20% of current HER2 IHC testing may be inaccurate.¹⁶ Considering the poor outcome of many breast cancer patients, that at least in part may be attributed to an inaccurate IHC test, in addition to the high medical cost of inappropriate and failed therapies, standardization of IHC has rightly received growing emphasis. Practical recognition of this problem by the NIH has resulted in several new funding proposals, though the amount and number of related awards remain inadequate.

From a practical point of view, one of the most difficult issues in the standardization of IHC on FFPE tissues is the adverse and variable influence of formalin-fixation on IHC detection, resulting in a major uncontrollable (and unknown) intrinsic factor. Based on our, and other investigators’ studies, we proposed to minimize the variable IHC staining signals among hundreds and thousands of FFPE tissue sections by using optimal AR protocols. Briefly, the hypothesis is that use of optimized AR protocols may provide a potential approach to reach a comparable (if not identical) level of IHC staining following variable conditions of FFPE tissue processing treatment.^{17–21} While this is an empirical approach that ignores the finer points of the diverse effects of fixation and processing upon different proteins, it has the advantage of being the simplest, and most practical approach to reach the goal of standardization of IHC for archival FFPE tissue sections. A detailed discussion of this hypothesis is provided in Chapter 5.

Indeed, there are potentially several different levels to approach standardization of IHC: For the greatest scientific rigor, it is required to perform serial experiments based upon the hypothesis mentioned above to investigate and validate, if possible, standardization of IHC based on the AR technique (Chapter 5 in detail). Another approach, that may be combined with AR, is the systematic development of Quantifiable Internal Reference Standards (QIRS) that will allow assessment of the degree of protein degradation

(antigen loss) present in a tissue sections by measurement of defined internal standards (QIRS).^{11,22}

However, the immediacy of current needs for standardization of IHC also requires some practical short term action as work proceeds to a scientifically based and practical solution. As indicated by the guideline of ASCO/CAP, some practical approaches to standardization of IHC for HER2 detection have been documented. This guideline emphasized not only scientific concerns but also recommended formation of a national monitoring control system that would act as a “task force.”²³ It is critical to establish an authorized administration to monitor standardization of IHC, possibly similar to that introduced in the UK NEQAS external evaluation program.²⁴ Rhodes et al.^{25,26} demonstrated improved standardization of IHC for HER2-IHC detection in a multi-laboratory study through stringent quality control, use of standard reference material and an ongoing quality assurance program. As a corollary, it should be a requirement in the US that HER2 testing be done in a CAP-accredited laboratory as emphasized by the ASCO/CAP guideline.¹⁶

On the other hand, the “guideline” also identified several unresolved issues. There is no satisfactory “universal” reference material for standardization of IHC, or to monitor the reliability of the AR-IHC procedure, absent which true quantitative IHC is not possible, as described below. Currently, cell lines are used as external reference control materials, as exemplified by HER2-IHC detection. Rhodes et al.²⁶ established a cell standard control using 4 cell lines to represent variable HER2 expression, ranging from negative to strongly HER2-IHC positive staining (Chapter 6 in detail). Recently, we observed that one of the four cell lines, MCF-7 might not consistently express HER2, when comparing cell culture samples, a known but real issue in the preparation of these types of controls (unpublished data). Similarly it has been recognized that the prostate cancer cell line, LNCaP, after 80 passages may be altered from androgen-dependent, into androgen-independent cancer cells that also show variable protein expression of prostatic acid phosphatase (PACp).²⁷ Thus, it is necessary to frequently monitor the culture conditions and check the related protein expression in order to maintain any given cell line as a reliable reference material. With this issue in mind, other potential approaches have been explored including a protein-embedding technique.²⁸ The advantages of this protein-embedding technique include the fact that it is simple, reliable and quantifiable with known amount of proteins embedded in the FFPE block. Furthermore, it may be possible to establish a “barcode- like” quantitative standard reference material, based on different serial concentrations of the control protein, which can be read by computer simultaneously with the tested FFPE tissue section (Chapter 8 in detail). Recently, Sompuram et al. documented synthetic peptides, identified from phage-displayed combinatorial libraries, that could be used as positive standard controls for IHC^{29,30} (Chapter 7 in detail). It is likely that synthetic peptide reference material may be further developed based on protein-embedding techniques to more closely mimic FFPE tissue sections.³¹

4.3 CUTOFF POINT: HOW TO DEFINE OPTIMAL SCORE?

Determining the scoring or the cutoff point for prognostic and predictive markers also is problematic. Based on clinical trials and international studies, the newly documented guideline of ASCO/CAP has changed the cutoff point of HER2-IHC assay from 10% to 30%, emphasizing a strong circumferential “chicken wire” like staining pattern.¹⁶ Currently, many cutoff points with respect to IHC assays for prognostic or predictive biomarkers have not been well defined and have not followed scientific principles as recommended in the ASCO/CAP guidelines. This deficiency is a critical issue for many commonly used biomarkers such as ER, PR, and p53, where there still is a need to establish more accurate and widely accepted scoring systems or cutoff points. There are still no universal accepted criteria for establishing an optimal cutoff point for most individual biomarkers, and even with HER2, which has received the most intense focus, significant deficiencies and disagreements remain. The recommended adjustment of the HER2 cutoff point relied upon an expert-panel discussion and an extensive literature search, as well as numerous clinical trials and experiments¹⁶ in an attempt to provide a model of establishing scientific cutoff point. Previously, 10% of “positive” cells with IHC staining has been used for many markers although the choice of this 10% value has no scientific basis. In the future, scoring and cut off points should be validated against clinical follow-up data, as difficult as this may be, absent efficient and uniform data collection and sharing.

Many have argued that automation of IHC staining procedure coupled with image analysis (IA) would be expected to improve reproducibility of IHC results (Chapters 9 and 10 in detail). Many of the leading commercial suppliers have several types of automated IHC staining and IA equipment that are in development and testing. Clearly today, there is no universal IA equipment that has been adopted in facilities nationwide, let alone worldwide, and given the “competitive” nature of the business, there probably never will be. It is imperative, therefore, that some standardization of these various commercial approaches takes place, with the most likely option being the availability of commonly used reference materials (QIRS). In addition, current IA approaches have some drawbacks; they are time consuming and expensive, technical maintenance is complex, with no conclusive evidence indicating its clinical value. However, times change, and great strides are being made in terms of IA hardware, software and cost effective protocols.¹¹ The guideline of ASCO/CAP also fall short in this regard, in that quantitative IA was not put forward as a prerequisite for HER2 evaluation, but was merely “encouraged” to improve consistency, particularly for cases with weak membrane staining results.¹⁶ Therefore, we remain in much the same place as before: whether IA is used, or the naked tutored eye, it is essential to validate existing and novel IHC staining procedures, including reagents and scoring methods, based on stringent comparative studies to meet the criteria of 95% concordance rate with some “gold standard” method or external reference.¹⁶

Much of the preceding has been written in the context of current IHC methods, which are qualitative, producing “staining patterns” only, but which are sometimes manipulated to yield a contrived “semi-quantitative” score, that in effect compares patterns, with or without use of a cell line control. True quantification of IHC, defined as the measurement of quantity by weight, is clearly desirable, and may be achievable.¹¹ It is obvious that standardization of IHC must be the first step for true quantification as described above. There are two major issues that must be addressed: (1) The “total test” must be standardized.^{13,32} Factors intrinsic to “tissue preparation,” including variable “pre-fixation periods” (ischemic time), different conditions of tissue fixation, processing and embedding, plus storage of paraffin blocks and cut sections, strongly influence IHC staining results and must be standardized and “controlled,” in addition to reagents, protocols, and reading/scoring methods^{11,33}; (2) Biological heterogeneity of biomarkers certainly exists within different patients, and also within the same tumor (or different tumors) from a single patient, and even within different cells within a single section (or specimen). Further studies are required to better understand this heterogeneity; but such studies of themselves may require more precise quantitative methods in order to recognize differences that the current cruder semi-quantitative methods cannot identify.³⁴

With respect to “tissue preparation,” it is important to recognize that the practical details of “tissue preparation” are so dependent on the local environment in different hospitals that achieving uniformity is never going to be possible. Recognizing, therefore, that formalin is likely to remain as the fixative of choice in most hospital laboratories for the foreseeable future, and that not only will it be differently prepared, but fixation times will differ and be largely unknown, an alternative simple and practical approach is to attempt to minimize the effects of variable preparation (fixation) through the use of optimal AR treatment²⁰; to level the “playing field” as it were. Such a simple and pragmatic approach does yield improvements in the ability to detect (stain) many antigens, but still falls short when precise measurement by weight (quantification) is required.

4.4 STANDARD REFERENCE MATERIAL

In order to measure the exact amount of a specific protein (analyte) by IHC signal intensity, a critical requirement is the availability of a standard reference material (present in a known amount by weight) that can be used to calibrate the assay (IHC stain). It is then possible to determine the amount of test analyte (protein) by a translation process from the intensity of IHC signals. In this respect it is helpful to consider the IHC stain as a tissue based ELISA assay (Enzyme Linked ImmunoSorbent Assay), noting that ELISA is used in the clinical laboratory as a standard quantitative method for measuring protein by weight in fluids, by reference to a calibrating reference standard.

In the context of IHC, the reference material should contain a known amount of the reference protein, and must be treated exactly the same way as the test FFPE tissue sections.¹¹ Furthermore, the effect of fixation/processing on the ability of IHC to detect the reference protein (“loss of antigenicity”), also should be known, to allow for calculation of the original amount present of both reference protein and test analyte.

Two different and possibly complementary approaches have been explored. One utilizes a panel of “quantifiable internal reference standards” (QIRS), which are common proteins present widely in tissues in relatively consistent amounts.^{11,22} In this instance because the reference proteins are intrinsic to the tissue they are necessarily subjected to identical fixation and processing, and incur no additional handling or cost, other than synchronous performance of a second IHC assay (stain), such that the intensity of reaction for the QIRS and the test analyte can be compared by IA, allowing calculation of the amount of test analyte (protein) present on a formulaic standard curve basis. The other approach seeks to identify external reference materials and to introduce these into each step of tissue preparation for cases where IHC studies are anticipated; in this instance the logistical issues of production, distribution, and inclusion of the reference standard into all phases of tissue processing also must be considered, along with attendant costs.

Some investigators raised questions challenging housekeeping genes or proteins that were recommended as internal controls due to the variable gene/protein expression that exists in various tissue or organs.^{35,36}

To reach this goal using an external standard, such as protein-embedded reference material, it is necessary to identify a suitable matrix material to carry the protein through the multiple stages of fixation and processing, in parallel with the test tissue. Based on experiments, one method that shows promise is to coat protein onto the surface of plastic “beads” that can be pelleted, fixed in formalin, embedded and finally easily cut by a microtome either separately, or incorporated with the tissue into a single block.²⁸ In addition to beads, the development of thin protein plastic plates, coated with graduated concentrations of protein, may create a practical reference material in the form of a “barcode” that can be read by computer, as described briefly above (Chapter 8 in detail). Using this barcode reference material, serial studies indicate the effect of fixation and processing can be measured experimentally for the reference material, which then allows for calculation of the amount of unknown protein (test analyte) by quantitative comparison of a double IHC stain; a side-by-side IHC “staining” of the FFPE protein reference material with the FFPE tissue section, which will permit accurate measurement of protein in cells and tissues, in a manner analogous to the use of QIRS.

The second issue of heterogeneity of proteins within cells and tissues, especially within tumors, also must be addressed, as emphasized recently by Chung et al.³⁴ Currently, many of the most commonly used biomarkers such as HER2, ER, p53, etc, are assessed, in both clinical and research settings, based on a single FFPE tissue section. Much more data is required to

determine the extent to which this practice may lead to errors in outcome for individual patients, but in many respects this work cannot be undertaken until an effective standardized quantitative IHC method is available. Therefore, we do not currently know whether heterogeneity of protein distribution in tissue is common or important from a clinical point of view. But we do need to find out by further study, when accurate methods are available.

A final issue in this context, relates to interpretation or scoring of IHC results, especially for prognostic markers where some quantification is desirable. In order to accurately and reproducibly assess intensity of staining and numbers of positive cells, comparing reference standard with test protein, IA will be essential. In particular, to compare relative intensities of staining in tissue section, computer assisted IA is essential to avoid subjective estimation by the naked eye. Although IA systems have been used for more than 20 years, in many ways their use is still more in the research mode, and routine clinical applications are relatively few. The accurate counting of rare events (IHC stained metastatic cells for example) has been one success for IA, with FDA approved instruments and tests. The use of IA for automated analysis of IHC stained tissue microarrays has been another success, where individual cores may be selected and arrayed so as to consist of relatively “pure” critical cell populations eg the AQUA (automated quantitative analysis) system.³⁷ In most other circumstances the lack of standardized protocols as to what should be counted and the necessity to involve the pathologist in area selection has meant that IA has not replaced naked eye evaluation in most situations.

Recently, spectral imaging microscopy coupled with IA has shown promise in overcoming some of the “weak points” of conventional RGB camera-based IA systems. Based on its determination of accurate optical spectra at every pixel location, it may be possible to measure and compare several IHC staining signals in a standard way.¹¹ Furthermore, spectral imaging has the unique advantage of “un-mixing” multiple spatially co-localized chromogens that provides a strong tool for multiple IHC labeling techniques.^{11,37} Finally, it has been suggested that immunofluorescence signals can replace or be combined with immunoenzyme labels for quantitative multiplexed assays and a direct quantitative comparison of the signals.³⁷

4.5 OTHER POTENTIAL APPROACHES FOR QUANTIFIABLE IHC

Is there any other approach or concept that can directly measure protein amount in the tissue section? Ten years ago, Roth et al.³⁸ documented a novel method, named the “Midwestern assay.” This method is based on using two chromogens, soluble and insoluble, for the IHC staining process, to produce sequential production of soluble and insoluble reaction products. The soluble IHC product is used to measure the amount of antigen (protein) by spectrophotometry, while insoluble product indicates the localization of protein in the tissue section. Their experimental results demonstrated that soluble reac-

tion product was proportional to antigen concentration in the sample, and correlated well with IHC labeling index.³⁸ Recently, another new technique, named layered peptide array (LPA), for multiplex IHC enables the quantitative measurement of proteins on a tissue section.³⁹ Although the LPA is still prototypic, it may provide a simple and inexpensive technique for molecular measurements on tissue sections, while retaining the histological structure for morphologic study on the same tissue section. Egorina et al.⁴⁰ reported a new method of “in-cell” Western assay to visualize protein in cultured mononuclear cells. A similar concept of quantitative cellular protein detection has been documented in the literature, under the term of cellular or in situ ELISA as long ago as 1982.^{41–45} This method is a quantitative ELISA assay processed directly on cultured cell monolayers growing in a 96-wells of a flat-bottomed microtiter plate. After fixation, an ELISA test is subsequently carried out, followed by quantitative detection of the correlated antigen/antibody reaction as well as microscopic reading to approve both results using the same cell sample in the wells. In fact, the above mentioned “barcode” technique and the QIRS method both are based on the same concept of a quantitative tissue based ELISA with IA reading of the result against a calibrated standard. Rapid advances of proteomics, based on mass spectrometry (MS), with a quantifiable imaging MS platform, provides yet another potential approach to protein measurement on tissue sections (see Chapters 20 and 21 for detail).

In summary – IHC should no longer be regarded a “just a special stain” but rather as a standardized controlled quantitative tissue based ELISA assay – quantification of IHC is coming, it is just a matter of how and when.^{11,22}

REFERENCES

1. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* 1941; 47: 200–202.
2. Taylor CR. The nature of Reed-Sternberg cells and other malignant “reticulum” cells. *Lancet* 1974; 2 (7884): 802–807.
3. Taylor CR, Burns J. The demonstration of plasma cells and other immunoglobulin containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase labelled antibody. *J. Clin. Pathol.* 1974; 27: 14–20.
4. Taylor CR, Mason DY. The immunohistological detection of intracellular immunoglobulin in formalin-paraffin sections from multiple myeloma and related conditions using the immunoperoxidase technique. *Clin. Exp. Immunol.* 1974; 18: 417–429.
5. Burns J, Hambridge M, Taylor CR. Intracellular immunoglobulins. A comparative study of three standard tissue processing methods using horseradish peroxidase and fluorochrome conjugates. *J. Clin. Pathol.* 1974; 27: 548–557.
6. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.

7. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2006.
8. Gown AM. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* 2004; 121: 172–174.
9. Gown AM, de Wever N, Battifora H. Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* 1993; 1: 256–266.
10. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.
11. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 2006; 49: 411–424.
12. DeLellis RA, Sternberger LA, Mann RB, et al. Immunoperoxidase technics in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute. *Am. J. Clin. Pathol.* 1979; 71: 483–488.
13. Taylor CR. Quality assurance and standardization in immunohistochemistry. A proposal for the annual meeting of the Biological Stain Commission. *Biotech. Histochem.* 1992; 67: 110–117.
14. Wick MR, Mills S. Consensual interpretive guidelines for diagnostic immunohistochemistry. *Am. J. Surg. Pathol.* 2001; 25: 1208–1210.
15. Goldstein NS, Hewitt SM, Taylor CR, et al. Recommendations for improved standardization of immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 124–133.
16. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med.* 2007; 131: 18–43.
17. Shi S-R, Cote RJ, Chaiwun B, et al. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl. Immunohistochem.* 1998; 6: 89–96.
18. Shi S-R, Cote RJ, Taylor CR. Standardization and further development of antigen retrieval immunohistochemistry: strategies and future goals. *J. Histotechnol.* 1999; 22: 177–192.
19. Shi S-R, Gu J, Cote RJ, et al. Standardization of routine immunohistochemistry: where to begin? In *Antigen Retrieval Technique: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 255–272. Natick, MA: Eaton, 2000.
20. Shi S-R, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval technique: from experiments to hypothesis. *J. Histochem. Cytochem.* 2007; 55: 105–109.
21. Boenisch T. Effect of heat-induced antigen retrieval following inconsistent formalin fixation. *Appl. Immunohistochem. Mol. Morphol.* 2005; 13: 283–286.
22. Taylor CR. Quantifiable internal reference standards for immunohistochemistry: the measurement of quantity by weight. *Appl. Immunohistochem. Mol. Morphol.* 2006; 14: 253–259.

23. Yaziji H, Taylor CR. Begin at the beginning, with the tissue! The key message underlying the ASCO/CAP task-force guideline recommendations for HER2 testing. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 239–241.
24. Rhodes A, Jasani B, Anderson E, et al. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. *Am. J. Clin. Pathol.* 2002; 118: 408–417.
25. Rhodes A, Jasani B, Balaton AJ, et al. Study of interlaboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe: documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays. *Am. J. Clin. Pathol.* 2001; 115: 44–58.
26. Rhodes A, Jasani B, Anderson E, et al. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. *Am. J. Clin. Pathol.* 2002; 118: 408–417.
27. Lin M-F, Meng T-C, Rao PS, et al. Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. *J. Biol. Chem.* 1998; 273: 5939–5947.
28. Shi S-R, Liu C, Perez J, et al. Protein-embedding technique: a potential approach to standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 2005; 53: 1167–1170.
29. Sompuram SR, Kodela V, Zhang K, et al. A novel quality control slide for quantitative immunohistochemistry testing. *J. Histochem. Cytochem.* 2002; 50: 1425–1434.
30. Sompuram SR, Kodela V, Ramanathan H, et al. Synthetic peptides identified from phage-displayed combinatorial libraries as immunodiagnostic assay surrogate quality-control targets. *Clin. Chem.* 2002; 48: 410–420.
31. Riera J, Simpson JF, Tamayo R, et al. Use of cultured cells as a control for quantitative immunocytochemical analysis of estrogen receptor in breast cancer. The Quicgel method. *Am. J. Clin. Pathol.* 1999; 111: 329–335.
32. Taylor CR. An exaltation of experts: concerted efforts in the standardization of immunohistochemistry. *Hum. Pathol.* 1994; 25: 2–11.
33. Leong AS-Y. Quantitation in immunohistology: fact or fiction? A discussion of variables that influence results. *Appl. Immunohistochem. Mol. Morphol.* 2004; 12: 1–7.
34. Chung GG, Zerkowski MP, Ghosh S, et al. Quantitative analysis of estrogen receptor heterogeneity in breast cancer. *Lab. Invest.* 2007; 87: 662–669.
35. Pusztaszeri M, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand Factor, and Fli-1 in normal human tissues. *J. Histochem. Cytochem.* 2006; 54: 385–395.
36. True LD. Quality control in molecular immunohistochemistry. *Histochem. Cell. Biol.* 2008; 130: 473–480.
37. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch. Pathol. Lab. Med.* 2006; 130: 1026–1030.

38. Roth KA, Brenner JW, Selznick LA, et al. Enzyme-based antigen localization and quantitation in cell and tissue samples (Midwestern assay). *J. Histochem. Cytochem.* 1997; 45: 1629–1642.
39. Gannot G, Tangrea MA, Erickson HS, et al. Layered peptide array for multiplex immunohistochemistry. *J. Mol. Diagn.* 2007; 9: 297–304.
40. Egorina EM, Sovershaev MA, Osterud B. In-cell Western assay: a new approach to visualize tissue factor in human monocytes. *J. Thromb. Haemost.* 2006; 4: 614–620.
41. Anderson J, Rowe LW. The use of an enzyme-labelled assay as an aid to reading micro virus-neutralisation tests. *J. Immunol. Methods* 1982; 53: 183–186.
42. Berkowitz FE, Levin MJ. Use of an enzyme-linked immunosorbent assay performed directly on fixed infected cell monolayers for evaluating drugs against varicella-zoster virus. *Antimicrob. Agents Chemother.* 1985; 28: 207–210.
43. Myc A, Anderson MJ, Baker JRJ. Optimization of in situ cellular ELISA performed on influenza A virus-infected monolayers for screening of antiviral agents. *J. Virol. Methods* 1999; 77: 165–177.
44. Frahm SO, Rudolph P, Dworeck C, et al. Immunoenzymatic detection of the new proliferation associated protein p100 by means of a cellular ELISA: specific detection of cells in cell cycle phases S, G2 and M. *J. Immunol. Methods* 1999; 223: 147–153.
45. Fan X, Tyerman K, Ang A, et al. A novel tool for B-cell tolerance research: characterization of mouse alloantibody development using a simple and reliable cellular ELISA technique. *Transplant. Proc.* 2005; 37: 29–31.

CHAPTER 5

STANDARDIZATION OF IMMUNOHISTOCHEMISTRY BASED ON ANTIGEN RETRIEVAL TECHNIQUE

SHAN-RONG SHI and CLIVE R. TAYLOR

Standardization of immunohistochemistry (IHC) has been emphasized as an issue of great importance since 1977 when the First National Cancer Institute Workshop on the standardization of IHC reagents took place.¹ Subsequently, there have been many attempts to improve the reproducibility of IHC, including several other workshops and numerous articles published worldwide.² Nevertheless, still today, standardization remains a great challenge, which is easier said than done, due in large part to the presence of uncontrollable factors that are not intrinsic to the staining method itself, but are more a reflection of inconsistent tissue preparation. These pre-analytic issues include variable conditions of fixation and tissue processing, which result in levels of antigen preservation and loss that are unknown for the thousands of formalin-fixed, paraffin-embedded (FFPE) tissues housed in pathology department archives throughout the world.³

As described earlier in this book (Chapter 4), one of the most difficult obstacles for the standardization of IHC on FFPE tissues is the adverse influence of formalin, a major uncontrollable factor intrinsic to the most commonly used method of tissue preparation. The use of formalin, dating from the late 1800s, is not new, but some of the disadvantages came more to the forefront with the growth of IHC, when quality of fixation was judged by the ability to perform satisfactory immunohistochemical stains, not just by the provision of satisfactory morphologic detail. As noted in the chapter relating to antigen retrieval (AR), many attempts were made to “reverse” the deleterious effects of formalin fixation. While successful to a degree, these “retrieval” approaches did not automatically resolve issues relating variable fixation, and did not produce uniform staining of all antigens, in all tissues. In 1991, Battifora⁴ recommended the use of vimentin as an internal control, using it as a marker

for assessment of antigen damage in FFPE tissues, as a surrogate marker to estimate “loss” of other tested proteins, in an attempt to achieve more accurate interpretation of IHC staining results. In the pre-AR era, the vimentin internal control was useful in rejecting certain sections (and the blocks from which they were derived) as being unsuited for IHC, because no staining for vimentin could be elicited. There was also value in identifying the “best” areas for better grading IHC staining results, within large sections taken from large blocks subject to internal variation in the penetration of the fixative. It should be noted that the use of “vimentin controls” was highly subjective and was itself poorly controlled as no attempt was made to optimize the density (intensity) of vimentin staining within individual laboratories or between laboratories. Moreover, the introduction and widespread application of the heat-induced AR dramatically revolutionized the practical approach to IHC, such that almost every section could be successfully stained for vimentin post-AR, and its utility as an indicator of “over fixation” was to a large degree lost. Arber⁵ also challenged the rationale of vimentin internal control, and performed a comparative IHC study in 33 FFPE breast cancer tissues, with variable times of formalin fixation ranging from 24 h to 154 days, using antibodies to ER, PR, HER2, Ki-67, p27, and vimentin, with AR treatment prior to IHC staining. Arber reported that vimentin, as well as Ki-67 and p27, were “too easily unmasked” by AR technique, in that these antigens could be detected satisfactorily by AR-IHC even following prolonged formalin fixation up to 154 days. The conclusion was that vimentin is not a suitable internal control marker for assessment of formalin-induced antigen damage, simply because with use of the AR treatment, there are few detectable differences among FFPE tissue sections, with a wide range of formalin-fixation times as long as a 5 months. In addition, Arber observed that most other antigen/antibody reactivity was also retrieved satisfactorily with prolonged formalin-fixed times: HER2, 20 days; ER/PR, 57 days.⁵ A recent study in our laboratory, based on human autopsy tissues and animal tissue samples fixed in formalin for variable times ranging from 6 h to 7 plus days also demonstrated a similar result (unpublished data). It is our belief that most, but not all, antigens (proteins) fixed in formalin from 24 to 72 h can be “recovered,” to the point of giving detectable IHC staining by using the optimal AR protocol, as described in Chapter 1; however, while subjective reproducibility, as judged by detectable staining is thereby markedly improved, a level of strict reproducibility that would allow quantification is not achieved, because the degree to which an antigen (protein) is recovered is not known, with reference to the amount present in the fresh tissue, nor is it likely to be uniform for different proteins. These issues warrant further consideration.

5.1 INTERNAL REFERENCE STANDARDS (IRS)

Leong³ postulated that internal controls were required to optimize the variable influences resulting from aspects of tissue preparation and factors intrinsic to the staining method. Various controls have been employed, includ-

ing the use of parallel sections, prepared in the same laboratory, having undergone the same (at best similar) fixation process. Taking this notion one step further, many laboratories mount a separate section of “control” tissue on the same glass slide as the test section, so that both are subjected to near identical staining protocols. There is some confusion here, in that in the literature, and in casual conversation, these types of controls are often referred to as “internal controls.” In reality, while these controls are “internal” to the laboratory in question, they are not internal to the test tissue, and unless processed exactly in parallel through every stage of tissue preparation, will not have been exposed to identical conditions of processing or fixation. In order to function as an exact internal control for both specimen preparation (fixation) and the AR and IHC staining protocols, the selected control should be some tissue component (antigen/protein) that exists in the same tissue section as the target antigen, when tested by IHC. For the purposes of this discussion, we choose to refer to such controls as IRS, where internal means internal or intrinsic to the tissue section being tested, meaning that the selected control protein will have undergone specimen preparation and fixation in a manner as near identical to the test antigen (present in an adjacent cell in the same tissue). Extending this idea to its ultimate conclusion, the goal would be to identify internal reference standards that exist within the tissue after specimen preparation (and fixation) in known amounts, as quantified by an independent method. We choose to refer to such rigorously defined standards as Quantifiable Internal Reference Standards (QIRS).⁶⁻⁸

To date, it has proven difficult to identify a QIRS in FFPE tissue sections. That we have not successfully identified QIRS does not mean that it is impossible to do so. More likely, it is that we have not looked for them, or looked in the right way, for several reasons, including the following: (1) There have been no systemic attempts to identify and quantify tissue components that may also be found within the test sample, other than the antigen (protein or analyte) being tested; (2). Protein extraction studies of formalin fixed tissues that may provide data as to the range of proteins detectable, in reasonably intact form, in FFPE tissues, are in their infancy. Nevertheless, such studies do show the reproducible detection of more than 2000 recognizable proteins by mass spectrometry, proving some encouragement that many candidate molecules do exist (for a review see Chapter 20); (3) While ubiquitous candidate proteins do exist, the distribution of proteins (antigens) in different tissues may be variable⁹; this is unknown and requires investigation; (4) The IHC staining methods employed must be strictly controlled to yield reproducible intensity of staining; in essence this means that carefully controlled automated systems will be essential in the performance of these studies. (5) The human eye and brain is capable of complex pattern interpretation and recall, but is poor on reproducibility, within even a single observer over time, and falls far short in detecting variations in intensity of IHC signal, as opposed to “simple” positive or negative interpretations. Computer-assisted image analysis therefore will be necessary to measure and compare intensity of the reference standard versus the test antigen. Each of these requirements is

demanding; taken together they require a level of detailed application not yet attempted in a large study where sample preparations also are closely controlled and monitored, but unless taken together the results are unlikely to be meaningful.

It seems likely that “external tissue controls” will continue to be used until such a time as a system of qualified internal reference standards is established. Approaches have been proposed toward ensuring that external control tissue, matches as closely as possible the test tissue, during all phases of sample preparation, as well as during testing. One proposal seeking to address this difficult issue was the Quicgel method, using a breast cancer cell line mixed in agar gel, processed, and incorporated into the FFPE tissue block side by side with the tissue specimen, under exactly the same condition in order to establish an “artificial internal” control. The Quicgel method was claimed to allow accurate calculation of the amount of protein (estrogen receptor [ER]) in the tested sample tissue based on biochemical quantitative analysis.¹⁰ The Quicgel method, however, has proven not to be practical for routine use due to logistical issues, and of course is not applicable for retrospective studies on archival tissue. In recent years, several other proposals have been described for the development of standard control material, such as cell lines (see Chapter 6), synthetic peptides on-slide used for quality control (see Chapter 7), protein-embedding using beads (see Chapter 8), and mouse spleen tissue used as a staining intensity reference.¹¹ In addition, as alluded to earlier, the notion has been advanced of using ubiquitous internal proteins present in essentially all tissues as internal reference standards that potentially could be quantified as used both to “measure” the adverse impact of sample preparation upon classes of proteins, and also potentially to calibrate the IHC method for accurate quantification of key analytes (antigens/proteins).

5.2 AN INTERIM APPROACH TO IMPROVED REPRODUCIBILITY BASED ON AR

Numerous reports that AR-IHC gives excellent results for many of the markers used in diagnostic pathology¹² raised the possibility of improving the reproducibility of IHC and achieving some measure of standardization through the use of AR technique.^{13,14} Furthermore, the use of a “test battery” approach was advocated in order to identify an optimal protocol of AR-IHC,^{15,16} based on monitoring the two major factors that influence the effectiveness of AR-IHC, namely the heating condition (temperature and duration of heating) and the pH value of the AR solution. As described elsewhere (see Chapter 1), a consistent “maximal retrieval” level, showing the strongest intensity of AR-IHC, may be obtained by using this “test battery” approach.¹⁵

Ten years ago, we conducted an experiment using AR-IHC on FFPE tissues fixed in formalin for different periods ranging from 4 h to 30 days to explore

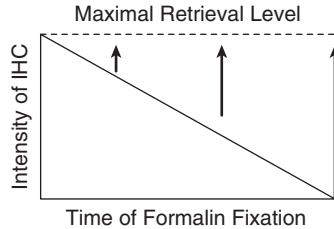


Figure 5.1 Diagrammatic explanation of standardization of IHC via AR and test battery to achieve a maximal retrieval level by an optimal protocol of AR. The intensity of IHC (axis y) is inversely correlated with the time of formalin fixation (axis x) as indicated by a reduced slope. Three arrows indicate a potential maximal retrieval level that may equalize the intensity of IHC to a comparable result for routinely processed, paraffin-embedded tissues with various time of fixation. Reproduced with permission from Shi et al., *J. Histotechnol.* 1999; 22: 177–192.

the possibility of obtaining equivalent IHC staining following “maximal retrieval” for selected antigen/antibody combinations. In a pilot study, five antibodies were tested with results that support the notion that it is possible to achieve equivalent maximal immunostaining levels in FFPE tissue sections, following fixation for as long as 1 month.¹⁷ As described above, the rationale for this approach was drawn from the maximal retrieval concept, indicated diagrammatically in Figure 5.1.¹⁸

In reviewing literature, several investigators also reported similar findings. For example, Boenisch¹⁹ described a study using human tonsil tissue fixed in 10% neutral buffered formalin (NBF) for 12 h, 1, 2, 4 and 8 days, in order to determine whether AR could be applied to equalize variable immunostaining results resulting from inconsistent formalin fixation. Among 30 antibodies tested, 26 showed consistent optimal staining by using one single AR protocol of 0.01 M citrate buffer of pH 6.0 with heating at 97°C for 20–60 min. Boenisch concluded that “Application of a given method for heat retrieval can compensate for variable formalin-induced damages resulting from inconsistencies in the length of formalin fixation and thus equally restore the immunoreactivity of a wider range of antigens.”

More recently, we have compared IHC staining results between frozen human tissue sections, fixed in coagulant fixatives (acetone and ethanol) and formalin, and found that frozen tissue sections fixed in formalin, with use of AR, always gave stronger IHC signal than that obtained by using acetone or ethanol-fixed sections.²⁰ In addition, we have demonstrated that FFPE tissue sections after AR treatment give the best IHC signals for all 26 commonly used antibodies that were tested.²⁰ Based on our study, we recommended that, in practice, FFPE tissue sections may serve as the standard for most antigens for IHC. Based on experiments, van der Loos²¹ concluded that the concept of an acetone-fixed cryostat tissue section serving as “golden standard” no longer exists, and recommended that FFPE cell blocks coupled with optimal AR

protocols should be used as controls for IHC. In fact, a number of other articles also have adopted IHC results generated from FFPE tissue sections after AR treatment as reliable standards for both clinical and research purposes, maximizing the advantages of FFPE tissue sections over frozen tissue sections.²² The HER2-IHC guidelines put forward by ASCO/CAP represent an example of use of FFPE tissue plus AR for standardization of IHC.²³ Shidham et al.²⁴ performed a comparative study of fresh cell samples, fixed in different fixatives, compared with FFPE tissue sections and heat-induced AR treatment used as standard positive control (“gold standard”), to identify the most suitable method of smear preparation and fixation by IHC. They concluded that FFPE tissue sections with use of AR showed the best results of IHC staining. Much of the recent published literature, with respect to new antibodies used as prognostic markers, such as p21 and p27, is solely based on FFPE tissue sections with the use of AR technique.

Nevertheless, caution must be taken to avoid any false IHC negative or false positive results after AR treatment. It should be emphasized that additional biochemical assays other than IHC need to be adopted in order to validate the AR-IHC results whenever necessary. Otherwise, as pointed out by Wick and Mills, “there is a real risk that artifacts may become ‘facts.’”²⁵

5.3 HYPOTHESIS

On this basis of our recent study,²⁶ the following hypothesis is proposed. The use of optimized AR protocols permits maximal retrieval of specific proteins (antigens) from FFPE tissues to a defined and reproducible degree (the retrieved rate of AR, expressed as R%), with reference to the amount of protein present in the original fresh/unfixed tissue.

This hypothesis may also be presented mathematically: the protein amount in a fresh cell/tissue expressed as Pf produces an IHC signal in fresh tissue of $\int$ (Pf). When the identical IHC staining plus AR treatment is applied to FFPE tissue section, the IHC signal is $\int$ (Pffpe). The degree of retrieval after AR (R%) is calculated as: $R\% = \int (Pffpe) / \int (Pf) \times 100\%$. The amount of protein in the FFPE tissue may then be derived as follows: $Pffpe = Pf \times R\%$. In a situation where optimized AR is 100% effective, then the IHC signal would be of equal strength in fresh tissue and FFPE tissue, and $Pffpe = Pf$.

Based on this hypothesis, it is possible to measure the adverse influence of formalin fixation and tissue-embedding processing for certain ubiquitous antigens. Having derived these data experimentally, such antigens may then serve as QIRS for other antigens of interest (that are being tested), for which data are not available as to loss or degree of retrieval when compared to fresh frozen tissue. It is envisioned that it will be necessary to establish a panel of QIRS representative of the different protein classes, which are likely to show different degrees of loss and retrieval efficiency, using model systems as described below.

5.4 PRELIMINARY TEST TO CONFIRM PREVIOUS STUDIES

Our laboratory has conducted pilot studies to explore the validity of this approach. Routinely processed FFPE tissue/cell sections of human breast cancer obtained from the Norris Cancer Hospital and Research Institute, Los Angeles, California, plus cultured cell pellets of human breast cancer cell line MCF-7, with variable periods of fixation in 10% NBF ranging from 6h to 30 days, were collected for analysis, using a protocol adapted from an earlier study of fixation as a variable.¹⁷ The study of human archival tissue specimens was exempted under 45 CFR 46.101 (b), and was approved by the Institutional Review Board (IRB #009071) at the University of Southern California. All cell/tissue sections were processed for AR-IHC routinely, using 0.05% citraconic anhydride (Sigma Chemical Co. St. Louis, MO, USA) at pH 7.5 as the AR solution, with a plastic pressure cooker heated in microwave oven (Sharp Carousel, 1100W, 60Hz, Thailand) as previously reported.^{27,28} To compare the results of IHC more accurately, all staining procedures were performed in the same side-by-side run in an identical manner. Four representative monoclonal antibodies were utilized; for ER (NeoMarkers, Fremont, CA, 1:100), for MIB-1 (DAKO, Denmark, 1:500), for cytokeratin (cocktail of AE-1 from Signet Laboratories, Dedham, MA, 1:500, and for CAM 5.2, Becton Dickinson, San Jose, CA, 1:50), and Her2/neu (BioGenex Laboratories, San Ramon, CA, 1:200) were used as the primary antibodies. The avidin-biotin detection system of Elite (Vector Laboratories, Burlingame, CA) was used for IHC staining following the manufacturer's instruction; 3,3'-diaminobenzidine was used as chromogen, and hematoxylin was used as counterstain. Evaluation of IHC staining results were conducted by two observers independently by light microscopy. The intensity of positive immunostaining was graded as +++, ++, +, or - for strong, moderate, weak, and negative, respectively.

Results: All four markers tested showed comparable positive IHC staining results among FFPE tissue sections fixed for various time periods ranging from 6h to 30 days, although the immunostaining intensity of 30-day-fixed FFPE tissue sections for Her2/neu and ER was slightly weaker than that observed with shorter fixation times. Both intensity and positive staining populations of all FFPE tissue sections achieved strong ("+++") level (Fig. 5.2), supporting the contention that standardization of IHC staining results may be feasible among variable periods of formalin fixation and that certain proteins may behave in a repeatable predictable manner under different fixation conditions.¹⁷

To test further this hypothesis, a simulated cell/tissue model system has been devised using quantitatively comparable cell lines, in which the amount of selected antigen (potential reference standard) can be measured accurately on a cell-to-cell basis in fresh and FFPE specimens that are processed under clearly defined but variable conditions, including periods of formalin fixation, delay times of fixation (prefixation time or warm ischemic time), storage conditions, and other technical issues such as thickness of each tissue section, in

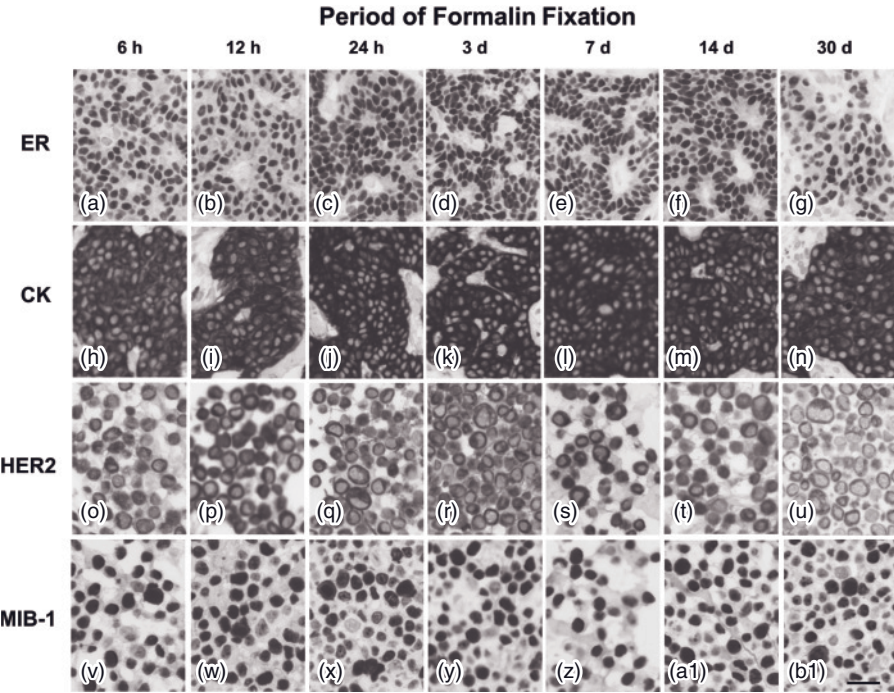


Figure 5.2 Comparison of immunohistochemical staining results among variable periods, 6 h to 30 days, of formalin-fixed, paraffin-embedded human breast cancer tissue (A–N), and cell line MCF-7 sections (O–B1). All four markers, estrogen receptor (ER) (A–G), CK (cytokeratin cocktail, H–N), Her2/neu (O–U), and MIB-1 (V–B1), showed comparable positive immunostaining results at “+++” level after antigen retrieval. Original magnification $\times 200$. Bar = $50\mu\text{m}$. Reproduced with permission from Shi et al., *J. Histochem. Cytochem.* 2007; 55: 105–109. See color insert.

order to simulate all possible fixation and processing schedules in the histopathology laboratories worldwide. This model system provides the basis for performing serial experiments to examine this hypothesis in a multidimensional approach as illustrated in the accompanying diagram (Fig. 5.3).

The technical requirements are quite exacting. IHC staining must be performed in a side-by-side fashion for accurate comparison of immunohistochemical staining intensity, cell numbers, and cell types, including appropriate standard positive controls. Also, the goal is to evaluate differences in loss/retrieval, based upon differences in the intensity of the immunohistochemical stain result, which often will be too subtle for naked eye evaluation, requiring routine use of computer-assisted image analysis. Use of a tissue microarray method is proposed to simplify these tests, employing either a series of algorithms called AQUA technology for quantitative assessment,²⁹ or comparative quantitative spectral imaging, using the Nuance System (Cambridge Research

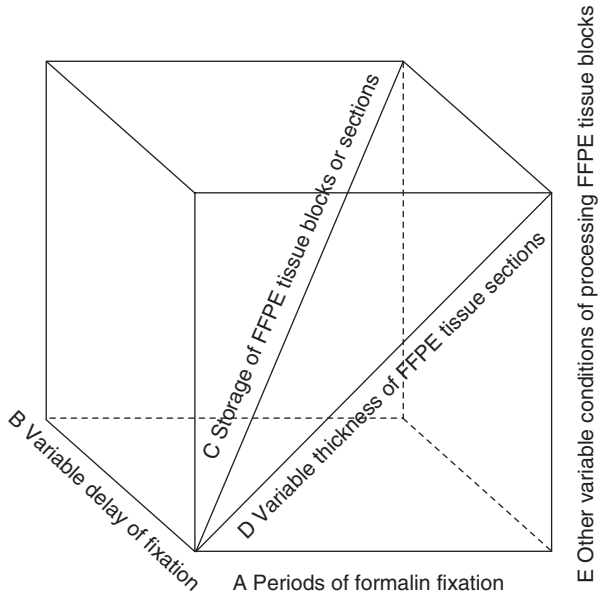


Figure 5.3 Diagram depicts the further-designed studies to test our hypothesis with respect to standardization of immunohistochemistry based on the antigen retrieval technique exemplified in a multiple direction to draw a conclusion. (a) Periods of formalin fixation. (b) Variable delay of fixation. (c) Storage of FFPE tissue blocks or sections. (d) Variable thickness of FFPE tissue sections. (e) Other variable conditions of processing FFPE tissue blocks. The stereoscopic frame of a cube represents the reliable limitation of quantitative IHC demonstrated by serial studies as recommended in the text. Reproduced with permission from Shi et al., *J. Histochem. Cytochem.* 2007; 55: 105–109.

Instruments). The antibody panel will be selected to include ubiquitous cytoplasmic, nuclear, and surface markers. Accurate biochemical quantification of proteins in the cell/tissue model will be undertaken for validation of the IHC findings.

A research design using the cell/tissue model is presented to encourage examination of the limitations of this hypothesis, based on correlation of accurate quantitative biochemical measurements and precisely measured IHC staining results.

5.5 AN EXPECTED FULL RETRIEVAL RATE AMONG MOST ANTIGENS

As mentioned above, our studies and other articles demonstrate substantially complete restoration of immunoreactivity (AR rate = 100%) for many antigens (proteins) exemplified by ER, progesterone receptor (PR), HER2/neu,

Ki-67 (MIB-1), and so on, for FFPE tissues, with the literature supporting high concordance between IHC and biochemical positive results.^{30,31} In addition, more than a dozen articles have demonstrated comparable IHC staining results between frozen and FFPE tissue sections following AR. For example, Von Boguslawsky³² performed IHC detection of PR for 25 paired frozen and FFPE tissue sections of breast cancer to compare the percentage of positively stained nuclei between frozen and FFPE tissue sections, demonstrating that with the AR treatment, 84% (21/25) of FFPE tissue sections showed identical positive nuclei compared with frozen tissue sections. Among the four discrepant cases, comparing to the frozen tissue sections, only one case showed a lower percentage of positive cancer cells in the FFPE tissue section; higher percentages of positive staining were found in the other three cases for FFPE tissue sections.

One area that has been neglected, but has potential impact, is the prefixation time (warm ischemia time). Time to complete fixation represents an unknown and uncontrollable factor in most institutions, and may contribute to inconsistent IHC staining results. Srinivasan et al.³³ analyzed prefixation parameters in detail, including constant factors such as nature and duration of anesthesia, as well as anoxic injury during surgical clamping vessels, and variable factors in terms of differing time lapse from the surgical excision of the specimen to fixation. All these variables may result in warm ischemia cell/tissue damage, which has been demonstrated in altered quality of RNA extracted from such delayed fixed samples. For example, a recent study demonstrated that RNA degradation was observed after a 4-h incubation at room temperature (25°C),³⁴ and significant loss may occur in even shorter periods. For proteins, the findings may differ, though studies are limited in number and extent. According to available publications, as judged by IHC staining results in FFPE tissue sections, most proteins retain their antigenicity during the usual prefixation time lapse (although “usual” is not well defined). Gudmundsdottir et al.³⁵ reported that cold ischemia time of up to 60 min did not influence protein expression in the human renal cortex and in renal cell carcinoma tissues, based on surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI). In our own studies, many proteins extracted from FFPE tissue sections were preserved well as determined by mass spectrometry.^{36,37} However, studies are limited in range of proteins and range of conditions that have been studied, leaving ample room for further study.

These principles are supported by a related set of studies that showed reliable “inter-laboratory” IHC staining for Her2/neu, ER, and so on could be achieved based on optimal AR-IHC protocols and stringent quality control using standard reference materials, even though ischemia time itself was uncontrolled and unknown.^{38–40}

In conclusion, broad-based experimental data from multiple investigators support the motion that AR-IHC, coupled with use of stringent controls, has the potential greatly to improve the reliability of IHC staining. However, more robust control systems are needed, which have general application, and

ultimately may allow for quantifiable results of IHC. Theory supports this last possibility, in that the widely used enzyme linked immunosorbent assay (ELISA) method employs identical immunologic principles and essentially the same reagents, and delivers strictly quantitative results, in the presence of appropriate reference standards for assessment of tissue preparation and calibration of the assay.

REFERENCES

1. DeLellis RA, Sternberger LA, Mann RB, et al. Immunoperoxidase technics in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute. *Am. J. Clin. Pathol.* 1979; 71: 483–488.
2. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2005.
3. Leong AS-Y. Quantitation in immunohistology: fact or fiction? A discussion of variables that influence results. *Appl. Immunohistochem. Mol. Morphol.* 2004; 12: 1–7.
4. Battifora H. Assessment of antigen damage in immunohistochemistry. The vimentin internal control. *Am. J. Clin. Pathol.* 1991; 96: 669–671.
5. Arber DA. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 183–186.
6. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 2006; 49: 411–424.
7. Taylor CR. Quantifiable internal reference standards for immunohistochemistry; the measurement of quantity by weight. *Appl. Immunohistochem. Mol. Morphol.* 2006; 14: 253–259.
8. Taylor CR. Editorial—A personal perspective. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 121–123.
9. Pusztaszeri M, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand Factor, and Fli-1 in normal human tissues. *J. Histochem. Cytochem.* 2006; 54: 385–395.
10. Riera J, Simpson JF, Tamayo R, et al. Use of cultured cells as a control for quantitative immunocytochemical analysis of estrogen receptor in breast cancer. The Quicgel method. *Am. J. Clin. Pathol.* 1999; 111: 329–335.
11. Moon Y, Park G, Han K, et al. Mouse spleen tissue as a staining intensity reference for immunohistochemistry. *Ann. Clin. Lab. Sci.* 2008; 38: 215–220.
12. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
13. Taylor CR. An exaltation of experts: concerted efforts in the standardization of immunohistochemistry. *Hum. Pathol.* 1994; 25: 2–11.
14. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.

15. Shi SR, Cote RJ, Yang C, et al. Development of an optimal protocol for antigen retrieval: a "test battery" approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. *J. Pathol.* 1996; 179: 347–352.
16. O'Leary TJ. Standardization in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 3–8.
17. Shi S-R, Cote RJ, Chaiwun B, et al. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl. Immunohistochem.* 1998; 6: 89–96.
18. Shi S-R, Cote RJ, Taylor CR. Standardization and further development of antigen retrieval immunohistochemistry: strategies and future goals. *J. Histotechnol.* 1999; 22: 177–192.
19. Boenisch T. Effect of heat-induced antigen retrieval following inconsistent formalin fixation. *Appl. Immunohistochem. Mol. Morphol.* 2005; 13: 283–286.
20. Shi S-R, Liu C, Pootrakul L, et al. Evaluation of the value of frozen tissue section used as "gold standard" for immunohistochemistry. *Am. J. Clin. Pathol.* 2008; 129: 358–366.
21. van der Loos CM. A focus on fixation. *Biotech. Histochem.* 2007; 82: 141–154.
22. Press MF, Spaulding B, Groshen S, et al. Comparison of different antibodies for detection of progesterone receptor in breast cancer. *Steroids* 2002; 67: 799–813.
23. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med.* 2007; 131: 18–43.
24. Shidham VB, Chang C-C, Rao RN, et al. Immunostaining of cytology smears: a comparative study to identify the most suitable method of smear preparation and fixation with reference to commonly used immunomarkers. *Diagn. Cytopathol.* 2003; 29: 217–221.
25. Wick MR, Mills S. Consensual interpretive guidelines for diagnostic immunohistochemistry. *Am. J. Surg. Pathol.* 2001; 25: 1208–1210.
26. Shi S-R, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval technique: from experiments to hypothesis. *J. Histochem. Cytochem.* 2007; 55: 105–109.
27. Shi S-R, Cote RJ, Shi Y, et al. Antigen retrieval technique. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 311–333. Natick, MA: Eaton, 2000.
28. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.
29. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch. Pathol. Lab. Med.* 2006; 130: 1026–1030.
30. Pertschuk LP, Axiotis CA. Antigen retrieval for detection of steroid hormone receptors. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 153–164. Natick, MA: Eaton, 2000.

31. MacGrogan G, Soubeyran I, De Mascarel I, et al. Immunohistochemical detection of progesterone receptors in breast invasive ductal carcinomas. *Appl. Immunohistochem.* 1996; 4: 219–227.
32. Von Boguslawsky K. Immunohistochemical detection of progesterone receptors in paraffin sections. *APMIS* 1994; 102: 641–646.
33. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am. J. Pathol.* 2002; 161: 1961–1971.
34. Chung J-Y, Braunschweig T, Williams R, et al. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* 2008; 56: 1033–1042.
35. Gudmundsdottir H, Haraldsdottir F, Baldursdottir A, et al. Protein expression within the human renal cortex and renal cell carcinoma: the implication of cold ischemia. *Cell Preserv. Technol.* 2007; 5: 85–92.
36. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
37. Xu H, Yang L, Wang W, et al. Antigen retrieval for proteomic characterization of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2008; 7: 1098–1108.
38. Rhodes A, Jasani B, Balaton AJ, et al. Immunohistochemical demonstration of oestrogen and progesterone receptors: correlation of standards achieved on in house tumours with that achieved on external quality assessment material in over 150 laboratories from 26 countries. *J. Clin. Pathol.* 2000; 53: 292–301.
39. Rhodes A, Jasani B, Anderson E, et al. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. *Am. J. Clin. Pathol.* 2002; 118: 408–417.
40. Jacobs TW, Gown AM, Yaziji H, et al. HER-2/neu protein expression in breast cancer evaluated by immunohistochemistry. A study of interlaboratory agreement. *Am. J. Clin. Pathol.* 2000; 113: 251–258.

CHAPTER 6

STANDARD REFERENCE MATERIAL: CELL LINE DEVELOPMENT AND USE OF REFERENCE CELL LINES AS STANDARDS FOR EXTERNAL QUALITY ASSURANCE OF HER2 IHC AND ISH TESTING

BHARAT JASANI, VICKY REID, COLIN TRISTRAM, JEREMY WALKER,
PAUL SCORER, MICHAEL MORGAN, JOHN BARTLETT,
MERDOL IBRAHIM, and KEITH MILLER

6.1 INTRODUCTION

The aim of this chapter is four-fold. First, to outline the historical rationale for development of reference cell lines as UK National External Quality Assessment Scheme (UK NEQAS) standards for external quality assessment (EQA) of HER2 immunohistochemistry (IHC) testing; second, to provide an overview of the procedures used for the commercial preparation of these cell lines, paying particular attention to the key manufacturing quality control checkpoints that are implemented to ensure that the control cell lines maintain the highest standards of consistency; third, to emphasize the correct interpretation of the control cell lines as standards for HER2 IHC testing; and fourth, to give a brief account of the applications and the educational and research value accruing from the use of these cell lines as UK NEQAS standards for EQA of HER2 IHC and *in situ* hybridization (ISH) testing.

6.2 HISTORICAL RATIONALE

Testing for protein marker expression by IHC is used in routine clinical diagnostics and has increasingly been used for semiquantitative analysis of both predictive and prognostic markers, especially in breast cancer diagnostics. Improved analytical stage standardization can be achieved using a combination of (1) IHC cell line controls, (2) automated IHC platforms, and (3) involvement of EQA.¹ EQA services, such as College of American Pathologists (CAP), the UK National External Quality Assessment Scheme for immunocytochemistry and *in situ* hybridization (UK NEQAS ICC & ISH), Quality Management Program-Laboratory Services, Canada (QMP-LS), and NordiQC, provide independent, objective data on individual laboratory and test method performance. This can help laboratories identify problems by comparing their performance with others using the same or different methodology.²

The utility of cell line controls in IHC testing has long been established as a tool for monitoring assay performance; however, their use has become ever more prominent due to their application as system controls for highly regulated prognostic/predictive assays such as the determination of HER2 status for breast cancer patients for whom Herceptin® is being considered. Testing for HER2 protein expression by IHC relies on the consistent interpretation of a semiquantitative assay. An IHC control that could reflect variations in section thickness, section storage, and staining protocols would provide a useful tool for improved standardization.³ Control cell lines may offer a practically reliable solution to this need. However, in order to meet the increasing demand for control slides in histology, there also remains a need for control slides that can be manufactured in a consistent and uniform fashion so that they may be utilized widely as more reliable controls compared with in-house tissue controls which may vary more significantly from preparation to preparation and laboratory to laboratory as a consequence of variations in tissue content and quality as well as fixation and processing methodologies.⁴ Commercially supplied cell line controls are manufactured using a standardized protocol and therefore represent a continuous supply of uniformly produced material. In contrast with other commercially manufactured IHC controls, such as synthetic peptide spots, cell lines also provide the appropriate medium and profile for demonstrating protein and therefore antibody localization within cellular context.

While we recommend the application of control cell lines as reagent, assay, and EQA monitoring tools, it is important to emphasize that appropriate tissue controls are also continued to be used in parallel, as tissue is still considered the gold standard in laboratory assay control. For reliable results, it is also important that tissue or cell line controls are fixed and processed in the same manner as diagnostic material submitted for evaluation.

6.3 DEVELOPMENT AND PREPARATION OF CELL LINES AS STANDARD REFERENCE MATERIAL

HER2 IHC testing presented a new challenge to the utility of reference controls in a routine in-house setting as well as for EQA purpose, as tissue sections of breast cancers with different levels of HER2 protein expression are not so readily available. Also, heterogeneity within the cancers, which may arise either due to a biological phenomenon or possibly caused by uneven penetration of a fixative, or a combination of the two, can undermine the reliability of assessment. With this in mind, the UK NEQAS for HER2 IHC chose to develop formalin-fixed, paraffin-embedded cell lines. The prototype cell line controls for pilot and initial studies were developed and prepared in a National Health Service laboratory in Cardiff.^{2,4} They were further modified and developed to include more robust cell lines, including replacement of an ovarian cell line SK-OV-3 at Leica Biosystems, Newcastle, UK (formerly Novocastra Laboratories, Newcastle, UK). Subsequently, all manufacturing occurred here due to the capacity for mass production and greater batch-to-batch reproducibility.

6.3.1 Importance of Using Validated Cell Lines

Contamination of cell cultures with fast growing cell lines such as HeLa has led to the emergence of incorrectly classified cell lines. These robust cells are capable of outcompeting slower growing cell lines and consequently take over. A study performed by MacLeod⁵ determined that 18% of the cell lines investigated harbored cross-contaminants. These cell lines were obtained from the source of origin. When working with cell lines it is of paramount importance to use those that have been authenticated.

Authentic cell lines can be obtained from the following certified cell banks:

- European Collection of Cell Cultures (ECACC)
- Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
- Interlab Cell Line Collection (ICLC)
- American Type Culture Collection (ATCC).

In addition, there are commercial suppliers such as Coriell Cell Repositories, who provide essential research reagents to the scientific community by establishing, verifying, maintaining, and distributing cells, cultures, and DNA derived from cell cultures. These collections, supported by funds from the National Institutes of Health (NIH) and other foundations, are extensively utilized by research scientists around the world. There are new cell lines being established and cultured all of the time, and therefore, the ensuing collections are ever expanding.

6.3.2 Cell Culture

The term “cell culture” refers to the growth of isolated cells *in vitro*, whereas tissue culture is a term used to describe the growth of not only isolated cells, but also isolated tissues or organs. Both these terms are often used to describe the growth of animal and human cells in culture. Advances in medical research have largely driven animal cell culture development.

6.3.3 Essential Requirements of Growing Cells in Culture

Cells grown in culture (*in vitro*) must exist in an environment that replicates as closely as possible that found *in vivo*. This environment must also be sterile. Growth medium provides many of the nutrients required for cell growth. The cell type dictates the composition of the growth medium. Placing the culture in a humidified incubator set at 37°C, 5% CO₂ will serve the temperature and gas mixture requirements of most mammalian cells and help to maintain the culture medium osmolarity. Cell phenotype expression may change if cells *in vitro* are not placed in their optimal environment. Media is also routinely supplemented with calf serum, which is a source of essential growth factors. However, serum may harbor viruses or even prions, and the usefulness of contaminated cultured cells may be limited. It is important to obtain serum from a reputable source. It is becoming common to use serum-free medium to initiate and establish a cell line culture in order to avoid any potential contamination.

Cells in culture must be maintained in an aseptic (sterile) environment as the growth rate of microorganisms is far faster than that of animal cells. Bacteria will thrive and soon outgrow animal cells if they contaminate an animal cell culture. Sterility is also essential to prevent the undetectable changes in the properties of a cell that may arise following infection of cells with virus or mycoplasma. The sterility of cultures is maintained by performing manipulations in a vertical laminar-flow hood (see Fig. 6.1). A sterile work area is created by drawing air through a high-efficiency particulate air (HEPA) filter at the top of the hood and blowing the filtered air down toward the work surface.

Cells *in vivo* exist either attached to a surface or free in suspension. Adherent cell lines originate from cells of solid tissue. Breast carcinoma cell lines (such as MCF7, T47D, and SK-BR-3) are adherent cultures, and these cells are grown on the surface of plastic flasks that have been treated to facilitate adhesion (see Fig. 6.2). Suspension culture cell lines originate from cells that exist in suspension, such as those cells present in the blood and the lymphatic system (see Fig. 6.3).

6.3.4 Passaging of Cells

When a fraction of the cells from an existing culture is placed in a new flask these transferred cells are said to have advanced in “passage number.” This action is called passaging, splitting cells, or subculture, and each cell line has



Figure 6.1 Vertical Laminar-flow Class II hood.

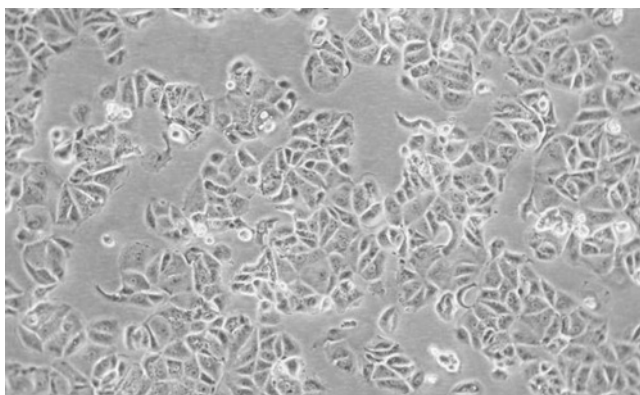


Figure 6.2 MDA-MB-175-VII monolayer cell growth on the surface of a flask.

a recommended split/subcultivation ratio. In order for the cells of an adherent culture to advance in passage number they must first be removed from the surface of the flask. This is achieved mechanically via the use of a cell scraper or chemically via the use of dissociation reagents such as trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA).

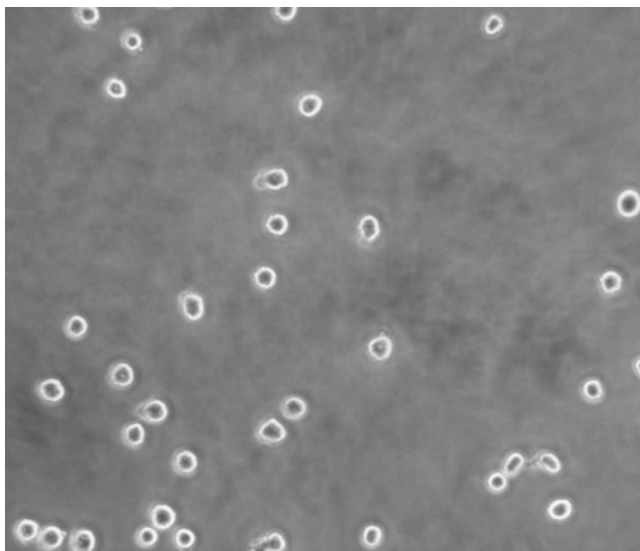


Figure 6.3 Cells in suspension culture.

6.3.5 Harvesting Cells

It is important that the method used to detach cells from their growing surface is compatible with end use. For final use as cell control material, it is important to use a methodology that preserves structural integrity and membrane protein localization. Enzymatic-based reagents may affect proteins on the surface of cells.

6.3.6 Growth Conditions and Characteristics of Some Breast Cancer Cell Lines

A variety of different growth conditions are employed in cell line growth, as indicated by the following examples:

SK-BR-3: This is an adherent breast carcinoma cell line. This cell line is cultured in McCoy's 5A medium with 2mM glutamine and 10% Fetal Bovine Serum (FBS) at 37°C in a 5% CO₂ in air atmosphere.

MDA-MB-453: This represents an adherent breast carcinoma cell line which is cultured in Leibovitz's L-15 medium with 2mM glutamine and 15% FBS at 37°C in atmospheric air.

MDA-MB-175-VII: This is an adherent breast carcinoma cell line. It is cultured in Leibovitz's L-15 medium with 2mM glutamine and 15% FBS at 37°C in atmospheric air.

MDA-MB-231: This represents an adherent breast carcinoma cell line, which is cultured in Leibovitz's L-15 medium with 2 mM glutamine and 15% FBS at 37°C in atmospheric air.

6.3.7 Cell Line Fixation and Processing

There are a number of different cell fixation and process methods used in laboratories worldwide. Fixation time in tissue will reflect a commonly accepted fixation time such as that seen in pre-analytical guidelines published in the package inserts for commercially available kits. Regarding cell lines points to note are: how soon are the cells fixed after harvesting, are the cells fixed in suspension, or when are they in a suspension matrix such as agarose.

This is important when considering your subsequent period of fixation. For example, suspended cells in fixative for 24 h are not being subjected to the same effects as a 5-mm biopsy for the same period. The formalin penetration and fixation effects are greater across a 50–100 μ cell versus a 5-mm piece of tissue. Penetration and the actual fixation of the tissue, cells, and protein are two different things.

In the 1940s, Medawar created a formula to demonstrate the coefficient for diffusibility for a number of fixatives and created the following:

$$d = K\sqrt{t}$$

where d , is the distance equal to the Medawar's constant times the square root of time.⁶ Using plasma clots, he was able to demonstrate that formaldehyde has a $K = 5.5$.

Although formaldehyde penetrates very quickly, its protein cross-linking fixative effects are not as immediate. Modifications to this and improvements on the model used originally in studies by Baker et al.,⁷ Fox et al.,⁸ and Helander⁹ have demonstrated that in tissue and in these more complex models, the penetration and therefore Medawar's constant for formaldehyde is not as great as 5.5 and is probably more like 3.6.⁷

Finally temperature and tissue type do have an effect on the penetration and fixation. This, combined with a failure to appreciate protein cross-linking time or actual fixation time required for formaldehyde, is probably the main reason for intra- and interlaboratory variation.

6.3.8 Processing

For illustrative purposes, the method used by Morgan⁴ is described below. In this example, cells are re-suspended in agarose gel that is taken up in a 1 mL syringe (see Fig. 6.4), generating a "cylinder" of cells. The cylinder of cells can be treated like tissue and placed in wax which, in turn, can be cut and embedded in paraffin wax blocks for cutting on a microtome.

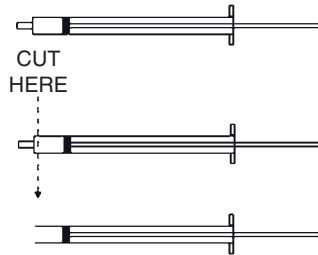


Figure 6.4 A sharp blade is used to slice tip off a 1 mL syringe as indicated.

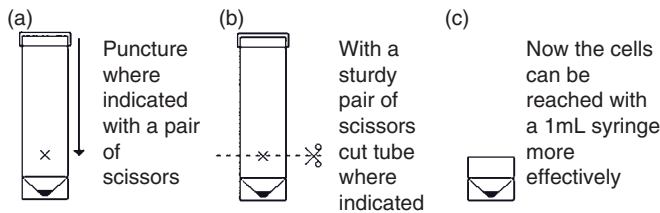


Figure 6.5 After formalin fixation the centrifuge tube is cut open to gain access to the cells with the syringe.

The desired quantity of cells are centrifuged in a 50 mL centrifuge tube for 1 min at 2000rpm (726g) in a centrifuge. The cells are then resuspended in 5 mL of neutral buffered formalin (NBF) and left at room temperature to fix. Although cell line protein expression will always show some degree of cell-to-cell variation within a single harvest, due to individual cell expression phases, fixation in suspension enables the cells to fix in a relatively homogeneous manner allowing for a more uniform IHC profile. This is a luxury not often observed in real-life tissue controls where staining heterogeneity may be as a result of genuine variable tumor profile or uneven/incomplete tissue fixation. Cells are then centrifuged again and the excess NBF is discarded. The top three-fourths of the centrifuge tube is then cut off (see Fig. 6.5).

The pellet is resuspended by gently flicking the remaining cut tube. Approximately 0.5 mL of molten agarose gel is taken up in an adapted syringe, transferred to the centrifuge tube and mixed with the cells by gently “pumping” the syringe fully two to three times (see Fig. 6.6). A “cloudy” appearance results, indicating that the cells have been sufficiently mixed. They are then left to set (approximately 10–20 min).

Once set, the “cylinder” of cells is placed in a 50 mL centrifuge tube with sufficient 70% alcohol and left overnight at room temperature. The cells are then processed through graded alcohols, cleared in xylene through to paraffin wax. Lastly, the cylinder of cells can be cut into approximately 6 mm segments

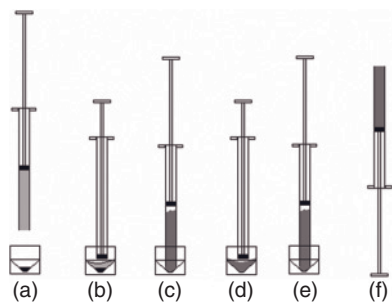


Figure 6.6 (a) Syringe is filled with 2% molten agarose, (b) agarose is extruded from syringe into cells, (c–e) cells and agarose are mixed together, (f) syringe is inverted and allowed to set.

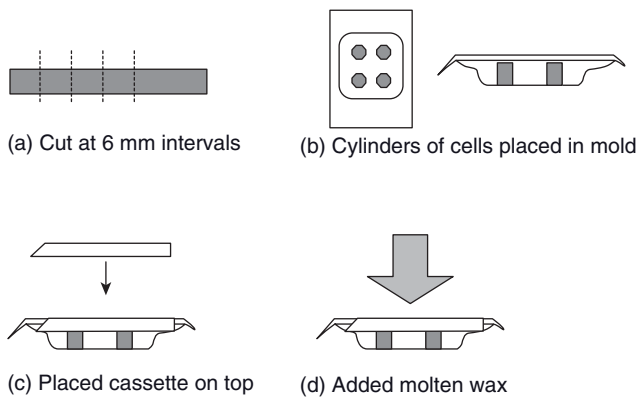


Figure 6.7 Overview of the embedding process.

and placed in appropriate cell line combinations to produce the desired cell blocks (see Fig. 6.7).

The 6 mm cylindrical segments can then be embedded by placing them into an appropriate-sized embedding mold, on top of which is placed a prewarmed plastic embedding cassette. Molten wax is then added to the mold and cassette before transferring onto ice and allowed to set. The complete blocks are removed from the mold when sufficiently solidified.

6.3.9 Section Preparation

Cutting sections at 3 μm facilitates single cell adherence to charged slides. The presence of nonadhered cells sloughing off between cell spots is a key characteristic of thick cell line sections (see Fig. 6.8). An awareness of actual

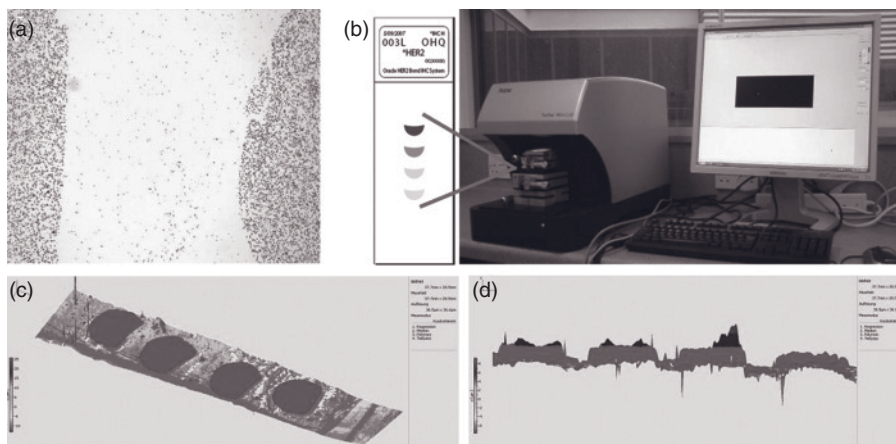


Figure 6.8 (a) Unattached SK-BR3 cells in between two cell spots on a thick section ($\times 4$ magnification), (b) interferometer used at Leica Biosystems Newcastle Ltd. to measure thickness of unstained control cell line section, (c) 3D image of cell cores on the surface of a glass slide, generated by the interferometer, and (d) 2D image of cell cores showing thickness of cores relative to surface of glass slide.

section thickness is crucial when assessing control cell lines and in certain cases may be the cause of false positive/negative results¹⁰.

Indeed, Barker et al.¹⁰ have generated data that has led to the design of a methodology that can be employed estimate the thickness of control cell line sections in a nondestructive manner. In the process of establishing this methodology, we have also shown both qualitatively and quantitatively that section thickness plays a significant role in IHC interpretation of protein profiles in tissue sections. Prior to this method, the only technique available for performing quality control check on section thickness of cut sections was staining by immunohistochemistry. Testing in this manner is destructive, as once control slides have been stained; they can no longer be used as unstained controls. Further, while destructive testing in this manner can indicate the quality of a particular section, it does not provide information as to the quality of all unstained sections, especially since the process of preparing numerous cut sections is variable.

6.3.10 Quality Control

At Leica Biosystems Newcastle Ltd., all control cell lines undergo strict quality control evaluation using haematoxylin & eosin (H&E) and Oracle™ HER2 Bond™ IHC System (Leica Microsystems Newcastle, UK) stained sections. This allows for evaluation of the three main cell line characteristics: *cellular morphology*, *IHC profile*, and *core density* (see Table 6.1)

TABLE 6.1 IHC Profile, Cellular Morphology, and Core Density Quality Control Procedures employed at Leica Microsystems

Score	Staining Pattern
0	No staining at all or very slight partial membrane staining in less than 10% of tumor cells.
1+	Faint, barely perceptible membrane staining in more than 10% of tumor cells; the cells are only stained in part of their membrane.
2+	Weak to moderate complete membrane staining observed in more than 10% of tumor cells.
3+	Strong, intense, and complete cytoplasmic membrane staining in more than 10% of cells.

- (1) Cellular Morphology. Satisfactory cellular morphology is the cornerstone of any good cell line preparation. For purposes of EQA utilizing IHC/ISH methodologies, this ensures that suboptimal morphology, as a consequence of poor starting material, can be distinguished from the effects of participant assay conditions. Any present, but distinguishable, dead cell population is excluded from interpretation.
- (2) IHC Profile. Due to strictly controlled commercial processing methodology, a relatively homogeneous HER2 protein profile should be observed in all cell lines. Greatest variation in HER2 expression may be seen in the MDA-MB-453 (2+) cell line due to subtle variations of natural protein expression levels of this cell within its given population. When assessing homogenous control cell lines, one should not employ the percentage cutoff criteria that are designed to facilitate HER2 interpretation in tissue cases.
- (3) Core Density. At Leica Biosystems Newcastle Ltd., the density of “viable” cell numbers within each core is strictly regulated, yielding consistent and reliable material for EQA assessment.

At Leica Biosystems Newcastle Ltd., invasive breast cancer tissue controls, demonstrating HER2 expression levels at 3+, 2+, 1+, and 0, are incorporated into all Oracle™ HER2 Bond™ IHC System cell line quality control runs. This ensures that control cell lines are validated as a viable assay control. The evaluation of control cell lines should always be performed within the context of appropriate tolerance limits. Subtle changes from batch to batch may occur, and it is the correct evaluation of the cell line staining patterns within appropriate tolerance limits that enables control cell lines to be utilized both in a commercial setting and as an EQA monitoring device.

Commercially processed HER2 expressing cell lines offer opportunities for enhanced standardization to both laboratories and companies participating in

laboratory quality assurance, for example, UK NEQAS ICC & ISH.^{11,12} In 2006, Novocastra Laboratories (now Leica Biosystems Newcastle Ltd) and UK NEQAS ICC & ISH collaborated in the development of cell lines for use in HER2 IHC quality control assessments. Primarily, this was to meet the increasing demand placed upon UK NEQAS to provide control materials and also to provide a reproducible quality control service for their Breast HER2 module (247 participants in 2008). Below are some examples of participants' staining on control cell lines obtained using different methodologies and assay platforms (see Figs. 6.9–6.12).

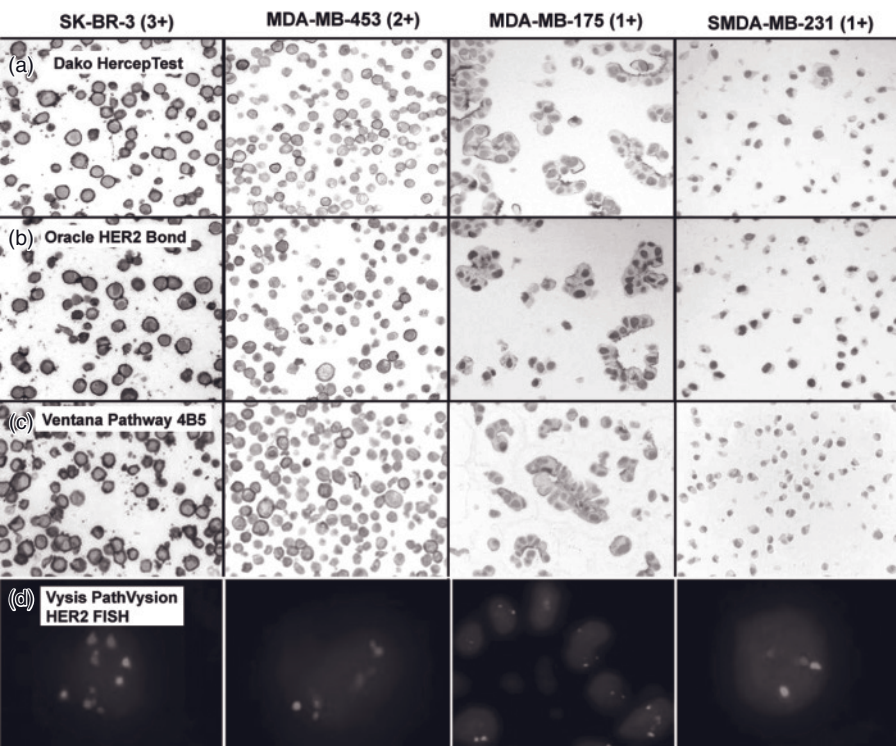


Figure 6.9 The HER2 antigen/gene correctly demonstrated in the UK NEQAS cell line control slides (indicated top to bottom) stained with four commercially validated systems (running left to right) (a) Dako HercepTest™, (b) Leica Microsystems Oracle™ HER2 Bond IHC System, (c) Ventana Medical Systems Pathway™ 4B5, and (d) Vysis PathVysion™ HER2 FISH. See color insert.

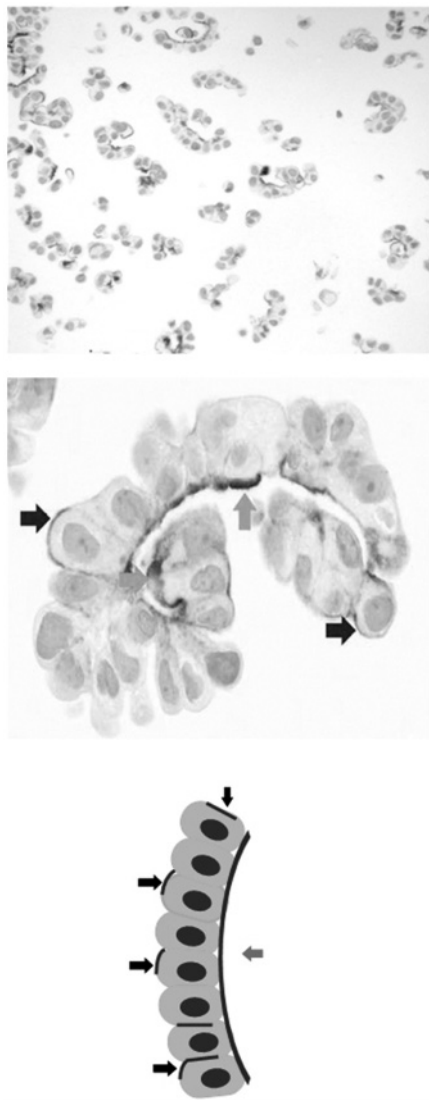


Figure 6.10 MDA-MB-175 (1+) cell line demonstrating unique glandular-like luminal formation with correct HER2 IHC staining pattern highlighted. Stained with Dako HercepTest™ K5204. Blue arrows indicate specific weak incomplete 1+ membrane staining, whereas the green arrows illustrate nonspecific moderate luminal surface staining, which is not interpreted. See color insert.

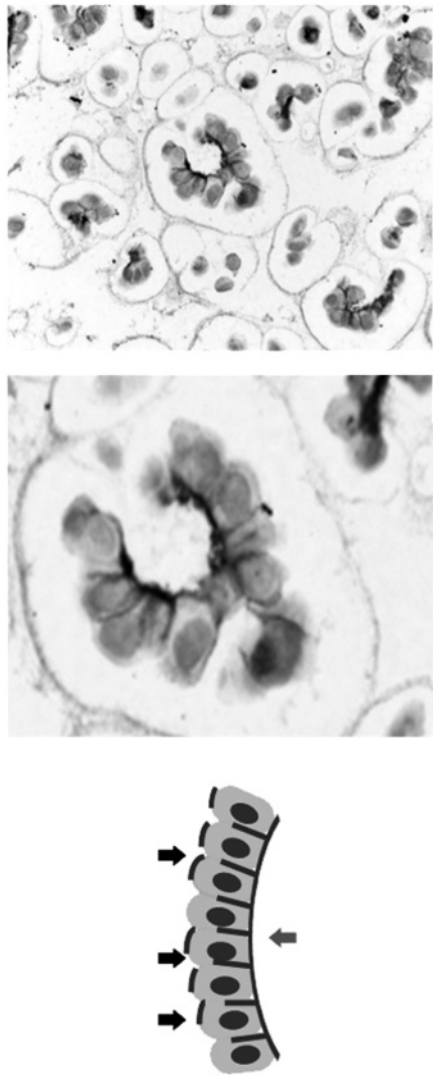


Figure 6.11 MDA-MB-175 (1+) cell line demonstrating unique glandular-like luminal formation with incorrect over stained HER2 IHC pattern. This sections was stained using the Dako Polyclonal (A0485) antibody using pressure cooker antigen retrieval. Blue arrows show specific incomplete staining of moderate intensity, however becoming complete in part, therefore interpreted as being overstained. The red arrows show overstained luminal surface staining. See color insert.

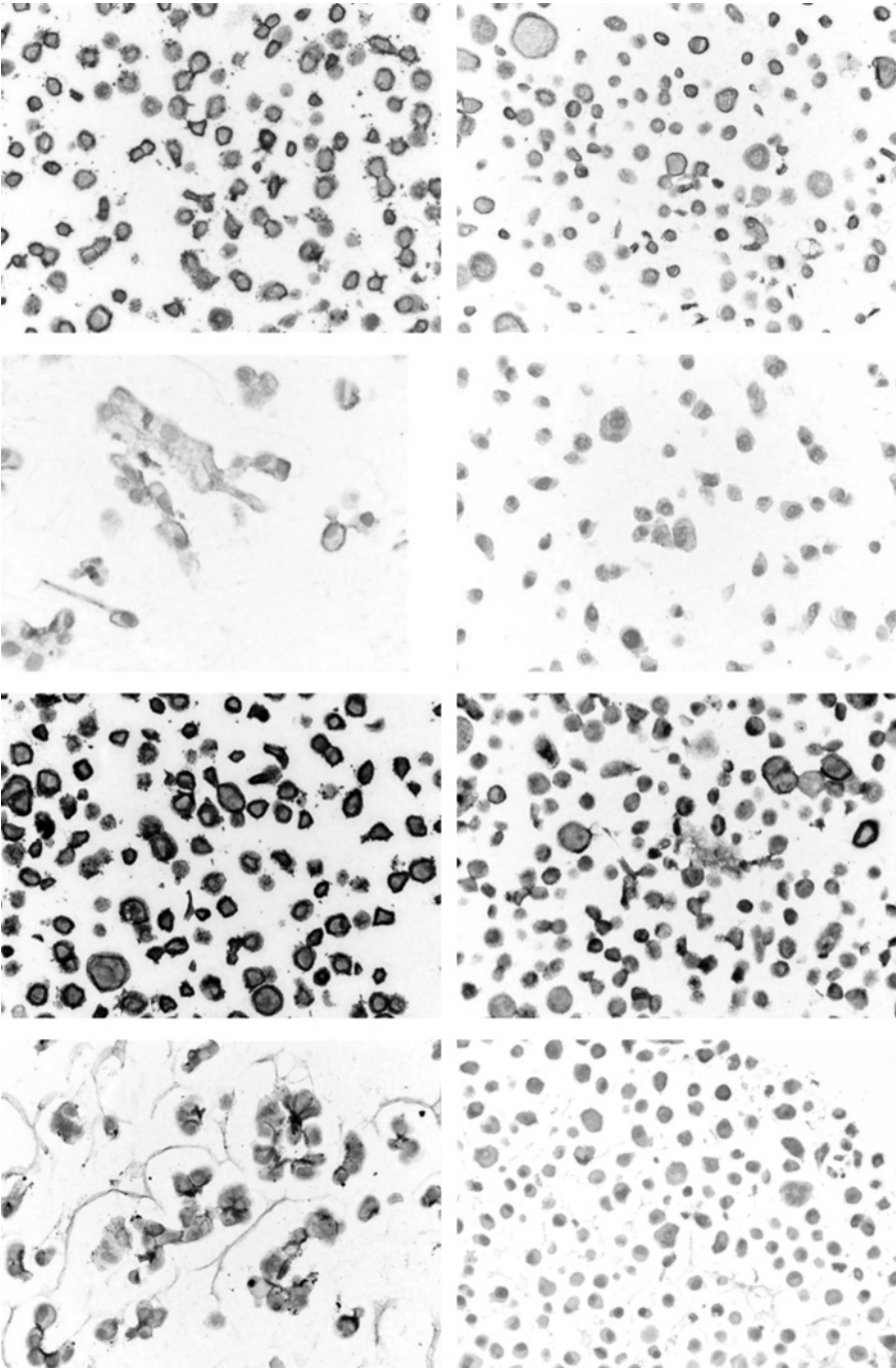


Figure 6.12 Examples of the UK NEQAS Control Cell Lines showing damaged morphology and incorrect IHC profile due to over retrieval. See color insert.

6.4 APPLICATIONS AND EDUCATIONAL FEEDBACK VALUE

6.4.1 EQA of HER2 IHC

Formalin-fixed and paraffin-processed sections from commercially prepared composite cell line block comprising the human breast carcinoma cell lines SK-BR-3, MDA-MB-453, MDA-MB-175, and MDA-MB-231, have been used consistently for the past 5 years for HER2 IHC EQA evaluation. More recently, the same composite cell line block reference material has been introduced for EQA evaluation of HER2 ISH testing of breast cancer. The principles and protocols developed and established for running of these two schemes have been recently described by Miller et al.¹³ and Bartlett et al.¹⁴

The HER2 IHC cell line based results are assessed independently by four experts using a multi-head microscope using the HercepTest™ (Dako, Glostrup, Denmark) criteria depicted in Table 6.2. The results are scored as showing acceptable or unacceptable quality of staining, with any features of suboptimal immunostaining such as:

- Damaged cell morphology due to excessive antigen retrieval
- Excessive cytoplasmic staining, making membrane staining difficult to interpret.
- Excessive counterstaining, masking membrane staining being indicated in the feedback comments supplied with the results.

Since this module began in 2001, the data have previously shown that the Dako HercepTest™ generally produced the most appropriate results. However, two more recently introduced kits, namely the Ventana Pathway™ 4B5 and the Leica Oracle™ HER2 Bond™ IHC System appear to show that HER2 antibodies in conjunction with more fully automated staining systems may produce more reproducible results. Her2 antibody/assay platform combination versus results of UK participants from Run 85 (2009) of the UK NEQAS ICC & ISH HER2 assessments are shown in Table 6.3.

Table 6.4 shows the heat-mediated retrieval methods used in conjunction with the HER2 antibodies. Previous UK NEQAS data showed that the water bath retrieval method at 98°C was by far the most efficient method, but with the introduction of new Her2 IHC kits (as discussed above) retrieval methods are being further standardized on automated systems, which have on slide retrieval systems. Irrespective of retrieval method used, the antigen retrieval solution of choice appears to be 0.01 M citrate buffer at pH 6.0. Although the data below show one participant using (and passing) with a pressure cooker retrieval method, UK NEQAS ICC & ISH data have shown that this method can be quite harsh and damaging to the cell membranes with a loss of quality.

Feedback of such information to the UK participants has helped to improve the pass rate steadily over the period 2003–2008, as depicted in Figure 6.13.

TABLE 6.2 Scoring Criteria Used to Assess the UKNEQASHER2 Cell Line

IHC Profile	Score	Cellular Morphology	Result	Core Density	Result
No staining in observed or membrane staining is observed in less than 10% tumor cells.	0	>75% of cells appear rounded with central nuclei. No visible distortion of nuclei or cell membranes.	Pass	>75% cell coverage	Pass
Faint, barely perceptible membrane staining is detected in 10% of tumor cells. The cells are only stained in part of the membrane.	1+	51–75% of cells appear rounded with central nuclei. No visible distortion of nuclei or cell membranes.	Pass	51–75% cell coverage	Pass
Weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.	2+	50–75% of cells appear damaged and distorted. Cell nuclei and membranes appear severely damaged and distorted.	Fail	25–50% cell coverage	Fail
Strong, complete membrane staining is seen in more than 10% or 30% of the tumor cells.	3+	>75% of cells appear damaged and distorted. Cell nuclei and membranes appear severely damaged and distorted.	Fail	<25% cell coverage	Fail

TABLE 6.3 Choice of HER2 Antibody from Run 72 of the UKNEQASICC and ISH Assessments

Antibody Details (UK Participants Only)	N	%
Dako A0485 C-erbB-2	5	80
Dako HercepTest™ K5204	8	63
Dako HercepTest™ K5206	2	0
Dako HercepTest™ K5207	26	88
Dako Link HercepTest™ SK001	2	100
Leica Oracle™ HER2 Bond™ IHC (CB11)	4	100
Novocastra NCL-CB11 (CB11)	2	50
Ventana Pathway™ 790-100 (4B5)	12	92

Note: The table shows the number of participants (N) using a particular antibody along with the percentage (%) which have achieved an acceptable level of staining.

TABLE 6.4 Choice of Pretreatment from Run 85 of the UKNEQASICC and ISH Assessments, Showing only the UK Participants of the HER2 Assessments

Heat-Mediated Retrieval (UK Participants Only)	<i>N</i>	%
Dako PTLink	12	83
Lab vision PT Module	2	100
Leica BondMax ER1	9	89
Pressure Cooker	1	100
Pressure Cooker in Microwave Oven	1	0
Ventana Benchmark CC1 (Mild)	1	100
Ventana Benchmark XT CC1 (Mild)	10	100
Ventana Benchmark XT CC1 (Standard)	1	0
Ventana Benchmark XT CC1# (8min)	1	100
Water bath 95-98 OC	25	72

Note: The table shows the number of participants (*N*) using a particular retrieval method along with the percentage (%) which have achieved an acceptable level of staining.

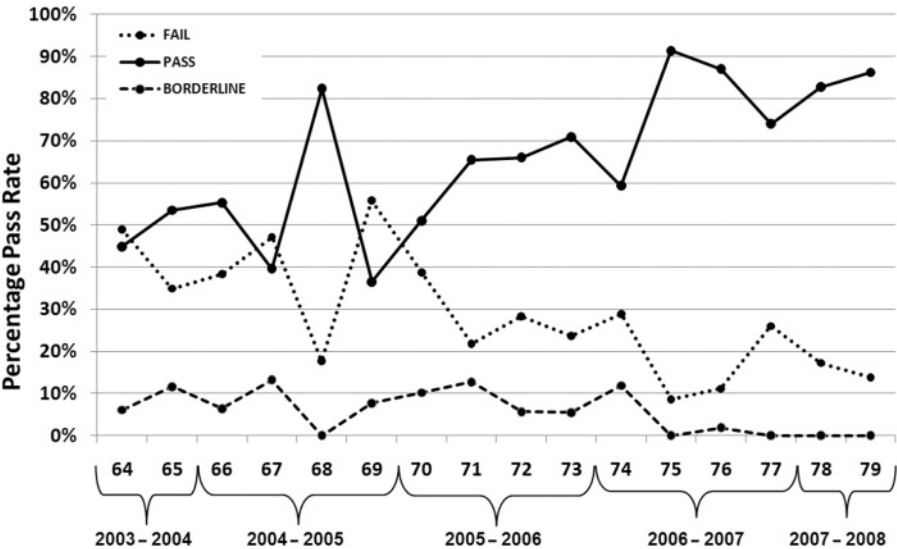


Figure 6.13 With assessor feedback to individual labs (UK data only shown), pass rate has steadily increased over a 5-year period.

6.4.2 EQA of HER2 ISH

The UK NEQAS pilot scheme for FISH was established in 2004/2005. The main aim of the HER2 FISH module is to provide results scored as being appropriate or inappropriate with respect to the set standards, as well as any

feedback information on methods and reagents judged to be accountable for high-quality analysis of HER2 gene status in breast cancer.

Cell lines were selected to cover the critical diagnostic threshold interval for the HER2 FISH test, where gene amplification is recognized if the ratio of HER2 signals to chromosome 17 signals exceeds 2.0. Previous evidence suggests that inter-observer error in scoring FISH signals can be controlled within 10% for both HER2 and other solid tissue analyses (see Bartlett et al.¹⁵ and Bartlett¹⁶ for review). This results in a small population of cases (<2.0% of cases, unpublished observations; FISH used as frontline test) where the result reported lies within a 10% range of the diagnostic interval, that is, between 1.80 and 2.20. While this compares favorably with the approximately 15% of cases reported as borderline by immunohistochemistry, it highlights the need to rigorously control scoring approaches. Data suggest that allowing variation to increase by 5% (to 15%) would double the number of equivocal cases (to 4%), and allowing an increase to 20% variation at the diagnostic interval would lead to a fourfold increase in equivocal cases (Bartlett unpublished observations). These observations underpin the approach taken in assessing participants' results. For each cell line, participants scored three points if the result reported fell within the range observed by the reference laboratories (see Fig. 6.14). These reference laboratory results already reflect the acceptable observer variation of 10% for inter-observer variation in scoring discussed above since they are collated from seven different laboratories, each of which presents two scores (up to 14 observations per cell line per assessment). Thus, results must fall within this range to be regarded as appropriate. For each cell line, participants scored two points if results lay within a further 10% of the range of results observed by the reference centers. These results reflect a band approximating to the 20% variation described above. Such results reflect acceptable performance with the potential for improvement. For results, which lay outside this second band of variation, one point was given, representing inappropriate performance. For such specimens, it is important to note that despite the correct diagnosis being given in many cases, a degree of variation >20% from the accepted result is indicative of a high degree of potential for error in diagnosis. In cases where results were not only deviating to this degree from the accepted result, but were also misdiagnosed (e.g., where a nonamplified case is reported as amplified), a score of 0 was recorded in recognition of the negative impact this has on the laboratories' likely performance on diagnostic samples. For the MDA-MB-453 cell line, where "borderline" results are often obtained and lie within the 20% margin-for-error described above, such results are *not* regarded as misdiagnoses for the purpose of this scheme.

Comparison of UK NEQAS ICC & ISH, HER2/CEP17 ratio ranges from Runs 4–6 indicate a much closer range of results between the reference centers when compared to the participating laboratories (Fig. 6.15). These data further indicate that "reference laboratories/centers," with greater experience, provide more consistent analysis of HER2 status over time.^{14,17}

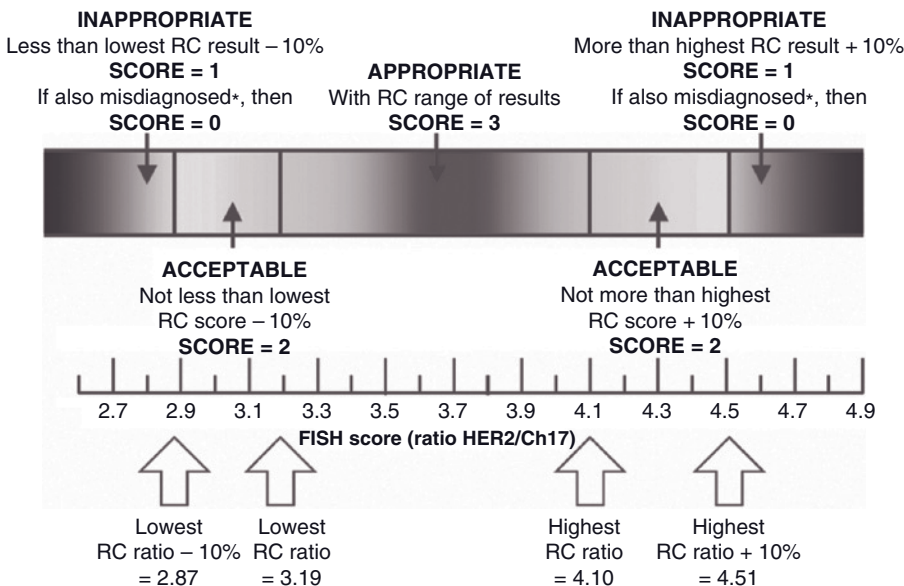


Figure 6.14 Schematic representation of the scoring system; the example illustrated uses the Reference Center set of HER2/Ch17 ratios obtained for the SK-BR3 cell line at Run 4. In this case the lowest ratio obtained by a Reference Center was 3.19, and the highest was 4.10; participants submitting ratios within this range were judged to have achieved an appropriate result (score = 3). The lower cutoff for acceptable ratios (score = 2) was calculated as 3.19 minus 10% of 3.19, that is 2.87; and the upper cutoff was calculated as 4.10 plus 10% of 4.10, that is 4.51. Participants who submitted ratios outside these 10% cutoffs were judged to have achieved an inappropriate result and received a score of 1. Except in the case of the MDA-MB-453 cell line, misdiagnosis (amplified reported as nonamplified, and vice versa) resulted in a score of 0. Superscript notation and abbreviation used in figure: * Does not apply to results obtained for MDA-MB-453 cell line; RC, Reference Center. See color insert.

6.5 CONCLUSION

The use of cell line-based reference standard material has helped to achieve reliable and meaningful EQA monitoring of quality of diagnostic HER2 IHC testing of breast cancers across the UK. It has also allowed monitoring of standards of performance across Europe and worldwide. The consistency of the cell line preparations as assured by monitoring both cell line protein and gene expression over extended time periods has ensured that long-term trends in variation in quality of HER2 testing can be assessed effectively and sensitively. This is a unique property of cell line control materials which is not achievable through assessment of tissue-based control material. However, when coupled with the use of in-house tissue control material, this partnership ensures that QA monitoring is reflective of the individual, the laboratory, and

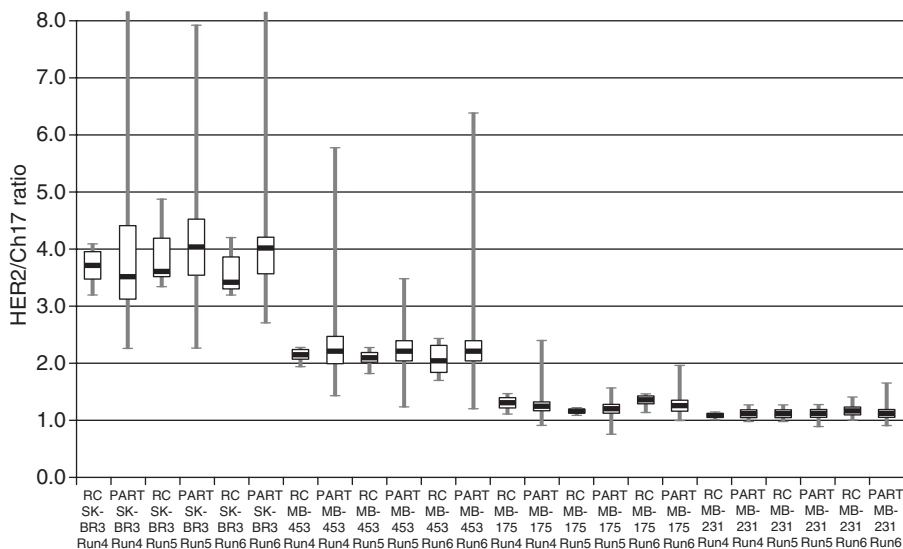


Figure 6.15 Chart in which the Reference Center (RC) and participant (PART) data sets are compared. This chart shows HER2/Ch17 ratio results from Runs 4–6 of the UK NEQAS ICC and ISH assessments. The heavy bar indicates the median, the limits of the shaded box the inter-quartiles, and the extending lines the minimum and maximum for the range.

the overall testing population. The stability of EQA test platform afforded by the cell line-based reference material has provided the additional benefit of allowing reliable feedback to individual laboratories to improve their performance from run to run, and thereby also help to raise the national standards of HER2 IHC performance.

ACKNOWLEDGMENT

All reproduced figures of this chapter have been approved for permission from the original publishers. Authors appreciated their kindness.

REFERENCES

1. Wester K, Andersson A, Ranefall P, et al. Cultured human fibroblasts in agarose gel as a multi-functional control for immunohistochemistry. Standardisation of Ki67 (MIB1) assessment in routinely processed urinary bladder carcinoma tissue. *J. Pathol.* 2000; 190: 503–511.
2. Rhodes A, Jasani B, Anderson E, et al. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin fixed and paraffin processed cell lines and breast tumours. *Am. J. Clin. Pathol.* 2002; 118: 408–417.

3. Hicks D, Kulkarni S. Review of biologic relevance and optimal use of diagnostic tools. *Am. J. Clin. Pathol.* 2008; 129: 263–273.
4. Morgan JM. A protocol for preparing cell suspensions with formalin fixation and paraffin embedding which minimises the formation of cell aggregates. *J Cell. Pathol.* 2001; 5: 171–180.
5. MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* 1999; 83: 555–563.
6. Medawar PB. The rate of penetration of fixatives. *J. R. Microsc. Soc.* 1941; 61: 46.
7. Baker JR, Hew H, Fishman WH. The use of a chloral hydrate formaldehyde fixative solution in enzyme histochemistry. *J. Histochem. Cytochem.* 1958; 6: 244–250.
8. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.
9. Helander KG. Kinetic studies of formaldehyde binding in tissue. *Biotech. Histochem.* 1994; 69: 177–179.
10. Barker C, et al. Non-destructive quality control of HER2 control cell line sections: the use of interferometry for measuring section thickness and implications for HER2 interpretation on breast tissue. *Appl. Immunohistochem. Mol. Morphol.* 2009; 17(6): 536–542.
11. Hammond M, Barker P, Taube S, et al. Standard reference material for Her2 testing. *Appl. Immunohistochem. Mol. Morphol.* 2003; 11: 103–106.
12. Dowsett M, Hanby AM, Laing R, et al. HER2 testing in the UK: consensus from a national consultation. *J Clin. Pathol.* 2007; 60: 685–689.
13. Miller K, Ibrahim M, Barnett S, et al. Technical aspects of predictive and prognostic markers in breast cancer: what UK NEQAS data shows. *Curr. Diagn. Pathol.* 2007; 13, 135–149.
14. Bartlett JMS, Ibrahim M, Jasani B, et al. External quality assurance of HER2 FISH testing: results of a UK NEQAS pilot scheme. *J. Clin. Pathol.* 2007; 60: 816–819.
15. Bartlett JMS, Going JJ, Mallon EA, et al. Evaluating HER2 amplification and overexpression in breast cancer. *J. Pathol.* 2001; 195: 422–428.
16. Bartlett JM. Pharmacodiagnostic testing in breast cancer: focus on HER2 and trastuzumab therapy. *Am. J. Pharmacogenomics* 2005; 5: 303–315.
17. Bartlett JM, Ibrahim M, Jasani B, et al. External quality assurance of HER2 FISH and ISH testing: three years of the UK national external quality assurance scheme. *Am. J. Clin. Pathol.* 2009; 131: 106–111.

CHAPTER 7

PEPTIDES AS IMMUNOHISTOCHEMISTRY CONTROLS

STEVEN A. BOGEN and SESHU R. SOMPURAM

7.1 INTRODUCTION

In this chapter, we introduce a new method for quality control of immunohistochemistry (IHC) testing, one that is expected to be first introduced commercially in 2011. Quality control (QC) is a process of examining a product, service, or process for certain minimum levels of quality. In this instance, the QC we refer to is a new IHC positive control. This new type of QC is a supplement to existing quality assurance procedures, which include already-existing protocols fostering adequate tissue fixation, minimizing tissue autolysis, and checks on equipment and reagents (as described in Chapter 4). The IHC positive control is a final check on the entire procedure, since it produces a measurable signal that is affected by nearly all of the components comprising the IHC stain.

The peptide IHC positive control concept is illustrated in Figure 7.1. A peptide that is immunoreactive with the primary antibody is covalently attached to a glass microscope slide, as a small round spot. The patient's tissue sample is mounted on the same slide. When the primary antibody is applied, it binds to its target in the tissue sample as well as the peptide on the glass, in parallel. During the succeeding detection steps, the detection reagents bind to their respective targets on both the tissue sample and the peptide. The immunohistochemical stain results in the deposition of color in both locations. Thus, the peptide serves as a check on the proper performance of the immunohistochemical assay. Depending on the specific clinical need, control peptides can be printed in various formats. For example, Figure 7.1 depicts eight separate peptide spots, which can be comprised of various concentrations of the same

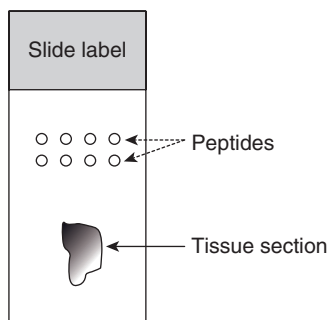


Figure 7.1 Schematic illustration of eight peptide controls, in a 2×4 array, on a glass microscope slide that also contains the patient's tissue sample.

peptide, or constant concentrations of different peptides, or duplicates, or a combination of these.

Until now, the composition of positive IHC controls resemble that of the patient sample. Pathologic discard tissue sections that express the biomarker in question are almost universally used as positive controls. Clinical laboratories bear the responsibility for identifying and validating the controls, typically from previous cases. An alternative QC material, which is especially popular for HER2 testing, is to use cell lines expressing the biomarker in question (as described in Chapter 6). Positive controls comprised of tissue sections or cell lines resemble patient samples in that they are cellular in nature.

In the context of historical practice, using peptides as positive controls seems counterintuitive. An acellular, synthetic protein fragment is a different matrix than patient samples. The final IHC stain result with a peptide control spot also appears different and may be initially disconcerting to some pathologists or histotechnologists. Whereas patient samples are examined under the microscope, a stained peptide control is readily visible without magnification. Despite these differences, peptides offer advantages that are unmatched by traditional biological controls, especially in the area of standardization, precision, and reproducibility. Moreover, a careful consideration of the various pre-analytical, analytical, and post-analytical components of an IHC stain will reveal that peptides offer a similar range of control as tissue sections or cell lines. In this chapter, we review this new QC modality.

7.2 WHY USE PEPTIDES AS IHC CONTROLS?

The advent of personalized therapies that are dependent on the outcome of an immunohistochemical stain has increased the need for quantitative positive controls. When IHC was first introduced as an adjunct in surgical pathology diagnosis, the interpretation was largely qualitative. Specific markers were present or absent, thereby characterizing a tumor cell's lineage. The fact that IHC interpretation was qualitative, rather than semiquantitative, minimized

the impact of many known testing inconsistencies. As the need for quantitative immunohistochemical testing grew, especially in response to widely publicized inconsistencies among laboratories,¹⁻⁴ so too did the need for better, more precise positive IHC assay controls.

The statistical tools for generating greater precision and reproducibility in immunohistochemistry testing are readily available from other areas of clinical laboratory medicine. For example, the science of quality control is well established from the clinical chemistry laboratory. For each quantitative blood analyte, there is at least one quantitative control that is tested on a regular basis. Often, two or three controls are used for each analyte, corresponding to varying levels of analyte concentration. A series of guidelines have evolved into routine practice articulating a prescribed method for analyzing the data from these positive controls.⁵ A tenet of current quality methods is to measure controls on a regular predefined basis. This generates data bearing on the quantitative value for a particular control, over a period of time. Sudden erratic deviations in a control value as well as slow trends in one direction signal the likely presence of a problem with the assay, requiring investigation. The answer for quantitative IHC testing may be found with similar methods that are adapted to the context of IHC. Positive controls for immunohistochemical staining are performed in conjunction either with each batch of slides, or alternatively, on each slide. Performing a control on each slide addresses the risk that a spurious error (e.g., failed reagent dispense) only occurred to a single patient sample, resulting in a false negative that would not be detected on a batch positive control.

It may be helpful to consider the following criteria when evaluating a proposed positive control for immunohistochemistry. A positive control is ideally: (1) reproducible and quantitative, (2) available in unlimited quantities, (3) antigen-specific, (4) inexpensive, and thereby adaptable for mass production, and (5) stable over time. Judged against these criteria, current practice falls short of the ideal. Biopsy materials are variable, both from patient to patient and even within a single tumor sample. Biopsy or resection materials are also obviously not available in unlimited quantities. The need for (microtome) sectioning of each positive control also creates a labor cost that is often underappreciated by many clinical IHC laboratories.

Cell lines solve some of these problems (Chapter 6). To serve as controls for IHC staining, cell lines can be grown *in vitro*, fixed in formalin, and embedded in paraffin for sectioning and mounting on glass microscope slides. Cell lines offer the advantage of being potentially available in unlimited quantities, addressing the second criterion. In addition, if the cell line exhibits stable expression of the analyte in question, then it meets the first criterion. Some of the best characterized cell line controls have been developed for HER2 testing. They are used to foster inter-laboratory standardization by several proficiency survey agencies, including UK National External Quality Assessment Scheme (UK NEQAS)-Immunocytochemistry, Nordic-QC, and QMPLS (Canada), for periodic assessments of laboratory staining performance.

Although cell line controls offer unique advantages for proficiency survey testing, they have not met the need for routine clinical IHC QC. One significant limitation is that they are only commercially available for the breast cancer panel of markers. This limitation is largely due to the challenges in producing such cell lines at a commercially practical price. Heterogeneity and consistency of protein expression are often challenges in generating such cell lines for use as controls. The amount of an analyte in a transformed cell line can vary from cell to cell. Different batches of cells may not express consistent levels of the analyte. In addition, the amount of any particular analyte expressed by the cells can drift over time. This phenomenon has been attributed to the outgrowth of sub-clones expressing higher or lower amounts of the analyte. Therefore, a manufacturer may need to periodically sub-clone the cells so as to select for cells with a predetermined level of analyte expression. This further complicates manufacture and increases the cost of production. If a manufacturer were to section the blocks and provide them pre-mounted on slides, costs would rapidly escalate because of the labor-intensive nature of histological sectioning. Leaving the sectioning and mounting to customers simply shifts the labor cost burden to the laboratories, many of which are already understaffed. All of these technical challenges can be overcome. So far, however, it has not been commercially practical to do so, except for the breast cancer markers (i.e., HER2, estrogen and progesterone receptor).

The absence of commercial IHC controls that meet the minimum requirements previously mentioned led to the concept of using antigen as an IHC control. By “antigen,” we mean the protein to which an antibody binds in a high affinity and specific manner. Antigen spots, unlike cells or tissues, can be printed onto slides in an assembly line fashion. Similar printing techniques had already been developed for creating arrays of nucleic acids, mounted on microscope slides. Printing antigen on slides avoids the labor cost for embedding and sectioning of paraffin blocks. It is also possible to print a predefined level of antigen on the slide, ensuring a high degree of reproducibility. Conceptually, printing antigen on slides creates a highly quantitative immunoassay control, similar to the kind used for conventional serum immunoassays. The color intensity of the antigen spots after immunohistochemical staining reflects the efficacy of the antibodies, detection reagents, and protocol.

For many IHC assays, the antigen is a cell-associated, oftentimes multi-subunit, complex glycoprotein. Such antigens are usually in short supply or expensive to manufacture, challenging a manufacturer’s ability to generate reproducible, low-cost quality control material. It is possible to produce such antigens in recombinant form, or by purification from natural sources. The costs associated with such manufacture, however, results in a product price that most customers will find excessive for a QC product. By contrast, short peptides can be manufactured relatively inexpensively and to high standards of reproducibility, both of which are important features. If a peptide can be designed to mimic the antibody binding site of the native antigen (the

“epitope”), then it could serve as a QC target in lieu of the native antigen. This technology is therefore potentially an ideal source of QC material for antigens that are scarce or expensive to manufacture.

Having explained the evolution of the peptide controls concept, the remainder of this chapter will describe the technology for creating peptide controls and review the data regarding their performance and applicability to IHC laboratory testing controls.

7.3 IDENTIFYING PEPTIDE EPITOPES

The creation of standardized, reproducible IHC peptide controls requires the identification of antibody binding targets that are inexpensive, reproducible, and available in unlimited supply. Peptide epitopes can have these properties, as illustrated in Figure 7.2. Antibodies can bind equally well to the native protein as to a peptide that mimics the native antigen (the “peptide epitope”). The major challenge with using peptide controls is in identifying a peptide sequence that will be recognized in the immunoassay with an affinity similar to that of the native antigen.

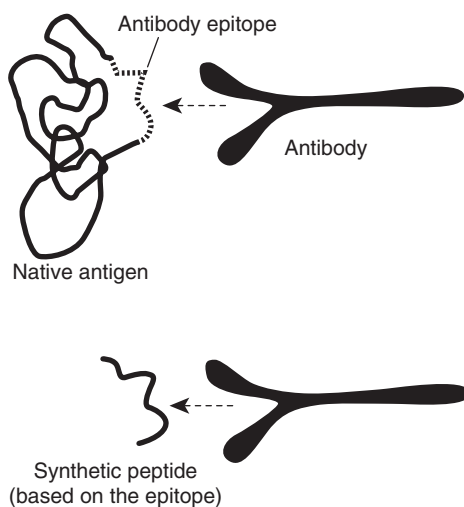


Figure 7.2 Schematic showing the relationship of the native antigen to the peptide mimic. The native antigen (a protein) is shown as a winding, twisted line, so as to represent a hypothetical three-dimensional structure. The peptide represents the antibody-binding epitope (shown in dotted lines) of the native antigen. The epitope can represent a linear sequence of the native protein. Alternatively, the epitope can be formed by amino acids that are not immediately adjacent to each other in the primary sequence but brought together by the three-dimensional folding of the protein. Adapted with permission from Sompuram et al.⁶

There are two general methods for identifying antibody epitopes: (1) evaluate peptides whose composition is based on the sequence of the native protein, or (2) evaluate peptides that are selected from a random combinatorial peptide library. The former is only effective if the epitope is composed of a linear sequence of amino acids in the native protein sequence. If it is, then analysis of overlapping peptides from the native protein is a simple method for identifying antibody epitopes. Each peptide is tested for immunoreactivity to the antibody. Those peptides that are immunoreactive contain the epitope. The other method of epitope identification, selection from a random combinatorial library, will identify peptides that represent both linear and conformationally dependent epitopes. The drawback of this method is that biopanning from a random combinatorial peptide library is more time consuming. Phage-displayed peptide libraries have previously been used for epitope identification in this context.^{6,7}

Choosing which method to use also depends on how the antibody was generated. Many widely used antibodies for clinical IHC laboratories bind to linear epitopes because the original immunogen was a peptide. Generating antibodies using peptide immunogens is often a preferred method when the native protein is complex and difficult to isolate. For example, the widely used antibodies to HER2 (Herceptest, CB11) were both generated to peptides. If the antibody was originally generated by immunization with a linear peptide, then the antibody epitopes will of necessity be linear and not dependent on the three-dimensional conformation of the native protein. In such a circumstance, it is easiest to use the same peptide for the positive control target.

If a monoclonal antibody was generated by immunization with a full-length native protein rather than a peptide, then the immunized mouse will generate antibodies that recognize both linear and conformationally dependent epitopes. Only a small subset of these monoclonal antibodies will likely be useful for clinical use on formalin-fixed, paraffin-embedded tissue (FFPE) samples. Those that are useful tend to have epitopes that are linear; the epitopes are not dependent on the protein's three-dimensional conformation (see Chapter 16). Therefore, for antibodies generated in response to immunization with full-length proteins, the peptides that serve as positive controls will be linear stretches of amino acids derived from the native protein sequence, as listed in protein databases.

There are two types of specificity checks that may be warranted when choosing a specific peptide. The first is to demonstrate that the peptide is bound by the desired antibody and not by other, antigenically irrelevant antibodies. An example of this kind of specificity check is shown in Figure 7.3. A peptide that is immunoreactive with the 1D5 estrogen receptor (ER) mAb was covalently bound as a 1 μ L spot to the center of each grid location. Various antibodies and controls were subsequently applied to the different grid locations. The bottom panel describes each of the antibodies that were applied to each grid location. The ability of the various antibodies to bind to the peptide was tested by immunohistochemistry. The presence of antibody bound to a

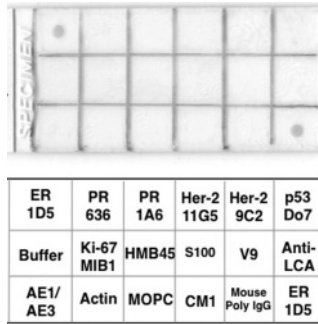


Figure 7.3 The ER 1D5 peptide only binds to the ER 1D5 mAb (upper left and lower right). Other mAbs do not bind. The antibody abbreviations in the lower panel are: *Her2 11G5*, and *9C2*, human epidermal growth factor receptor type 2 clones 11G5 and 9C2; *Mela-HMB45*, melanocyte-specific antibody clone HMB45; *Vimen V9*, vimentin clone V9; *Anti-LCA*, anti-leukocyte common antigen clones PD7/26 and 2B11, combined as a cocktail; *Mouse poly IgG*, mouse polyclonal IgG. Reproduced with permission from Sompuram et al.⁷

peptide spot is therefore revealed as a colored dot. The peptide is immunoreactive only with the ER 1D5 mAb.

Another method to confirm that the peptide is specific is to test for specific inhibition of the binding of the antibody to its native protein target (i.e., in a tissue section) using soluble peptide. This tests whether the peptide was binding at or very close to the antigen binding site on an antibody. For each of the peptides described in this chapter, there was specific inhibition by soluble peptide but not by other, antigenically irrelevant peptides.^{6,7}

A drawback of using peptide controls is that they are useful only for antibodies that bind to a particular epitope. Other antibodies to the same protein, but which bind to different epitopes, will require their own peptides as positive control targets. This may raise a concern that peptide positive controls will not be broadly applicable for clinical use. Clinical practice by IHC laboratories suggests that this is not a significant problem. For any single target, there is relatively little diversity in mAb clone selection among clinical immunohistochemistry laboratories. This is a result of the relatively stringent performance requirements for mAbs in clinical IHC testing. Not only do the mAbs need to recognize their target analyte, they must further react with the target after formalin fixation and paraffin embedding. Relatively few mAbs have that capability, even with the use of antigen retrieval techniques. Therefore, a small number of peptides for each analyte will have broad applicability for the overwhelming majority of clinical immunohistochemistry laboratories. For example, one HER2 peptide accommodated more than 95% of the clinical laboratories' HER2 tests in a 2006 clinical trial of almost 200 laboratories.⁸ Another method for addressing the market need, accommodating the use of several different mAbs, is to combine multiple peptides into a single spot on a glass microscope slide.

7.4 REPRODUCIBILITY OF PEPTIDE CONTROLS

The most compelling advantage of peptide controls is that they can be printed in an automated fashion, resulting in a high degree of slide-to-slide reproducibility. Unlike biological controls, such as tissues or cells, peptide IHC controls provide for the application of a precise molar amount of analyte to the glass slide, thereby creating a defined and reproducible quantitative standard. Because the patient's tissue sample is mounted on the same slide as the control indicators, both are treated in an identical fashion. As the tissue sample is stained, so too is the control. The precise amount of color that develops on the peptide control directly reflects the efficacy of the IHC stain's analytic components.

Printing on glass microscope slides in a reproducible manner is an important challenge. Manual pipetting one microliter droplets (using appropriately sized micropipettors) is relatively imprecise and tedious. In contrast, automatic printing of liquids onto glass microscope slides is an established technology in the context of creating microarrays. However, there are limitations of microarray printers that preclude their use for this purpose. In developing the technology, the authors found that contact spotting (using pins that make contact with the glass surface) often produced inconsistent spots due to variability in the transfer of liquid from the pin to the glass surface (data not shown). Therefore, a noncontact printing method was developed, which ejects small droplets of peptide onto the glass slide surface. Another important problem with using microarray printers is that they are designed to print hundreds (or thousands) of spots on a relatively small number of slides. For printing peptide controls, there is an opposite need, namely, a small number of spots per slide need to be printed, but on thousands of slides. To solve this problem, a custom-designed printer was developed.

Figure 7.4 is a photograph of a portion of the peptide controls slide printer. A stack of microscope slides, ready for printing, is at the far left. The slides are automatically ejected from the stack, one at a time. The slides are moved on a conveyer to the right, positioning them under the print head. The print head has eight nozzles, out of which microliter-sized droplets are ejected onto an underlying slide. The slide conveyer then places a new slide under the nozzles, and the process repeats.

A high level of printer reproducibility is important if peptide controls are to be helpful in assessing IHC laboratory stain variability. To assess the reproducibility of printing, the intensity of 96 sequential replicate peptide spots was measured. The peptide control spots were then immunostained as per a standard IHC protocol, as described elsewhere.⁸ In order to minimize the variability associated with the IHC staining process, slides were dipped as a batch (of 24 slides) into staining buckets that contained the appropriate primary, secondary, or tertiary staining reagents. Although this uses more reagent, it fosters better staining reproducibility. Therefore, any residual variability is more likely to be due to printing rather than staining. After immunostaining,

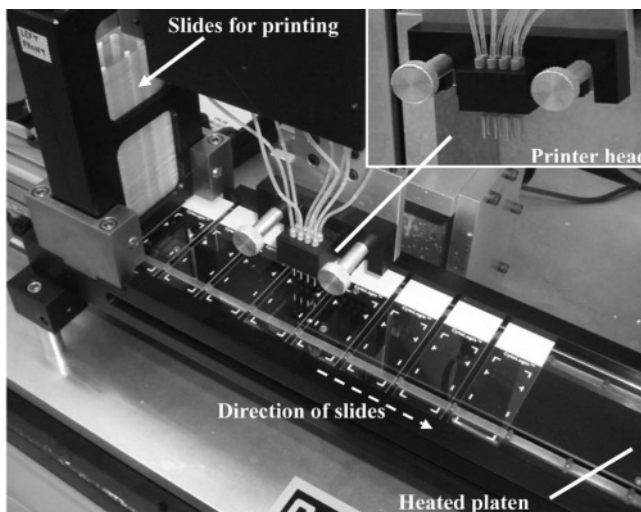


Figure 7.4 Photograph of the prototype slide printer, with a higher magnification of the printer head (inset), showing eight nozzles, each of which dispense microliter-sized droplets of peptide onto passing slides. As the slides proceed toward the right, they pass onto a heated platen, which accelerates the peptide coupling reaction to the activated glass surface. Copied with permission from Bogen et al.⁹

the spot stain intensity was quantified using a flatbed document scanner and an image analysis software program. The program measures the color intensity over a defined number of pixels and calculates the average among them (mean pixel intensity). The coefficient of variation (CV) of the peptide positive controls was $<7.5\%$.⁹ This measurement includes any variability in the dispensing of peptide droplets, attachment of the peptides to the slide, and measurement by IHC. This high degree of reproducibility rivals that of some of the best immunoassays in clinical chemistry and is unparalleled in the field of IHC. In summary, automated printing of peptide spots results in a highly reproducible positive IHC control.

7.5 STABILITY OF PEPTIDE CONTROLS

Another important requirement is that the peptide controls must be stable. This includes stability over time as well as stability after treatment with organic solvents (alcohol, xylene) or heat. Without stability, inter- or intra-laboratory variability could instead be ascribed to differences in the peptide controls' length of storage or susceptibility to treatment conditions. Instability could obscure differences in the analytical component of immunohistochemical staining.

A series of tests were conducted, measuring immunoreactivity after a variety of treatment conditions.⁹ Figure 7.5 illustrates the data from a stability

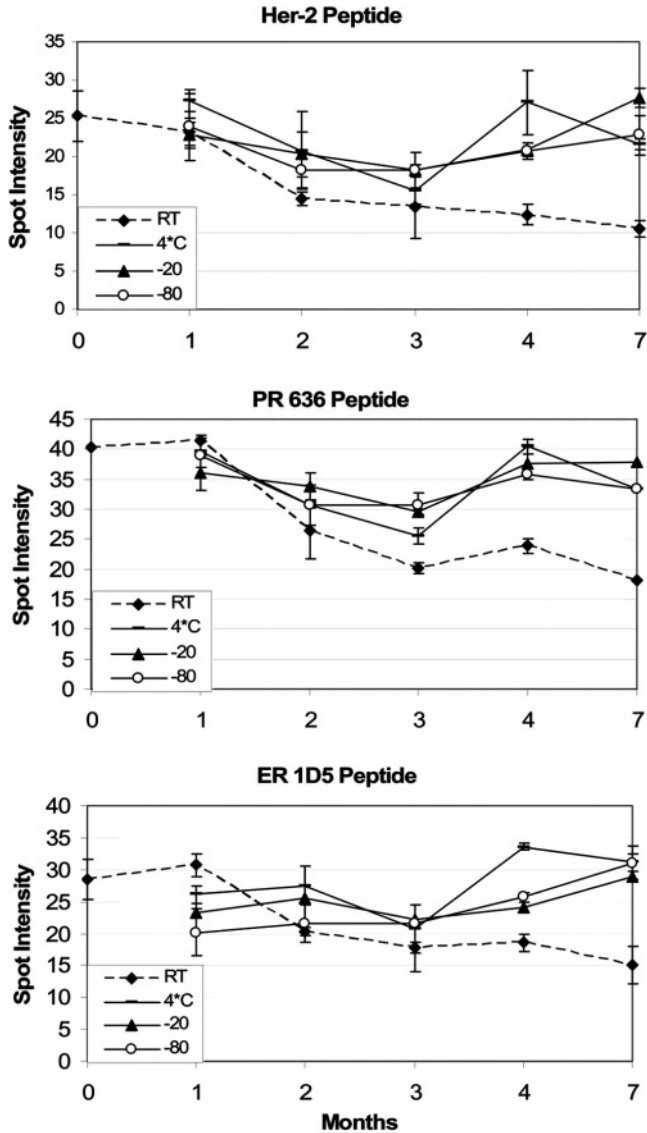


Figure 7.5 Stability of peptide controls, for HER-2 (top panel), progesterone receptor (PR) 636 monoclonal antibody (middle panel), and estrogen receptor (ER) 1D5 monoclonal antibody (bottom panel). The dotted line represents the immunoreactivity of peptide controls stored at room temperature. The slight dip in immunoreactivity at the 3-month time interval is related to a slight inconsistency in the staining protocol (immunohistochemical detection) rather than an actual decrease in stability. Each time point is the mean + SD of four replicate peptide controls. Copied with permission from Bogen et al.⁹

study over time. Four different temperatures were evaluated: room temperature, refrigeration (4–7°C), a conventional freezer (–20°C), and an ultra-cold freezer (–80°C). Peptide spot intensity was measured (by immunohistochemistry) at periodic intervals. Since the immunohistochemical stains were, of necessity, performed at different times, the IHC reagents were aliquoted and stored at –80°C until they were needed.

Figure 7.5 illustrates the results from the stability study, for peptides specific for HER2, ER (1D5 clone), and PR (636 clone) peptide controls. Despite aliquoting and freezing a common set of reagents for each immunostain time interval, there is still month-to-month variability in IHC staining. The variability is not due to degradation of the peptide controls, since there is no consistent trend. Despite the variability, the data illustrate that storage at room temperature lowers staining intensity after 2 months. For the slides stored at 4–7°C, –20°C, and –80°C, there was no difference in any of the peptide controls; no degradation of staining intensity is apparent after 7 months.

The peptide controls' resilience to dry and wet heat, as well as organic solvents, was also evaluated. This is important because the controls need to withstand "baking" of tissue sections (dry heat) as well as deparaffinization (organic solvents) and boiling in a pressure cooker (wet heat), as per many antigen retrieval protocols. In a first set of experiments testing dry heat similar to that of "baking" tissue sections, the peptides (bound to glass) were exposed to 60°C for 1 h. Figure 7.6 (left side, solid bars) demonstrates representative

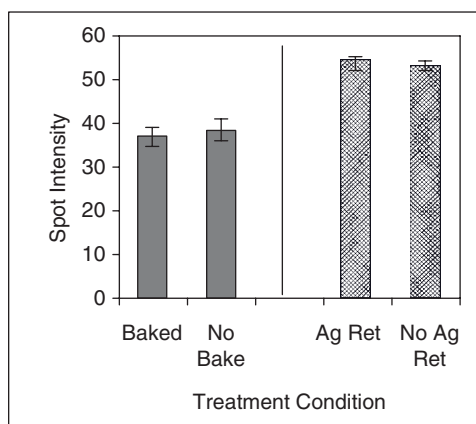


Figure 7.6 Peptides do not denature after baking (dry heat) or deparaffinization and antigen retrieval (wet heat). Peptide-coupled slides were treated as indicated on the x-axis and then immunohistochemically stained. In this particular example, an ER peptide with an ER MAb was used. The resulting peptide spot intensity (mean pixel intensity on a 1–256 scale) was measured and is shown on the y-axis. The data represent the means and SD or triplicate measurements. The experiments on the left (solid bars) and the right (hatched bars) were conducted at different times and have no connection to one another. Adapted with permission from Sompuram et al.⁶

data for IHC staining of peptide spots with and without baking (60°C dry heat, 1 h) of the peptide-coated slides. There was not any noticeable decrement in the staining signal after baking.

Figure 7.6 also illustrates the findings with respect to deparaffinization and antigen retrieval (right side, hatched bars). This experiment was unconnected to that on the left of Figure 7.6 and conducted at a different time. The data show that the ER peptide is not affected by the deparaffinization and antigen retrieval processes (“Ag Ret” group). The peptide spot intensity is essentially unchanged after the treatment. This was true for all of the peptides tested (data not shown). In summary, the peptide controls are remarkably stable over time and resilient to the usual processes to which tissues are exposed. In retrospect, this resilience may not be surprising. The peptides are covalently attached to the glass surface, and the treatments are not capable of dislodging them. Also, as short (approximately 20-mer) peptides, there is no higher-order protein structure to denature. The antibodies all recognize the linear sequence of amino acids, which is unchanged after these treatments.

7.6 PEPTIDE CONTROLS ARE SENSITIVE INDICATORS OF IHC STAINING PROBLEMS

Ideally, a positive control will provide an early warning of newly developing problems, such as reagent deterioration and instrument malfunction. For example, if an antibody deteriorates, then an abnormally low amount of *functional* antibody is applied to the patient’s tissue section. Instrument malfunctions can also affect stain performance, such as through insufficient reagent dispense volume, inappropriate temperature, or evaporation of reagent. The purpose of the positive control is to detect the problem early on, so that a correction can be implemented before it affects patient care. Applying suboptimal concentrations of a reagent in an IHC stain can simulate many of these failure modes.

The peptide controls’ ability to detect subtle levels of IHC stain failure was evaluated by diluting out each of the three components of the IHC assay, that is, primary antibody, secondary antibody, and streptavidin–peroxidase conjugate. The results are similar regardless of which reagent is diluted. We applied serial dilutions of reagent to microscope slides bearing both a peptide control and a PR+ breast carcinoma. The two (peptide control and tissue section) were positioned side by side, on the same slide. Representative data are shown in Figure 7.7, for the dilution of the primary antibody. Each data point represents a triplicate measurement of IHC stain intensity, as measured on the tissue section (by image analysis) or on the peptide spot (by scanning). There is a near-linear decrement in stain intensity. As shown in Figure 7.7, the decrement is equivalently reflected in both tissue and peptide spot optical density. These findings are representative of those found with other peptides and antibodies as well.

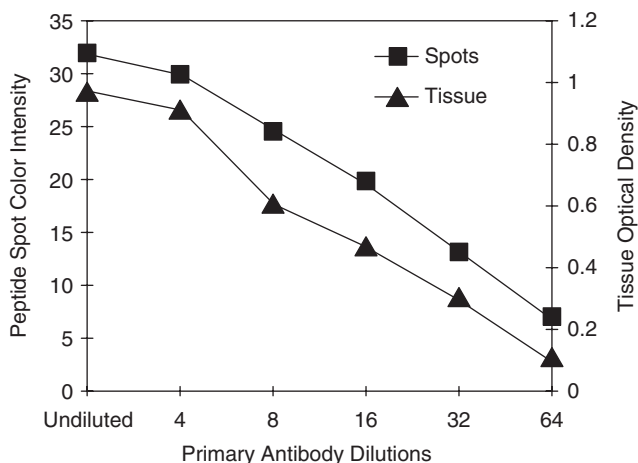


Figure 7.7 Peptide spot color intensity as a function of doubling dilutions of primary (PR) antibody. PR peptides and PR+ tissue sections were both placed on the same slides and stained with various dilutions of the PR mAb. Color intensity of the peptide spots (square symbols) or tumor cells (triangle symbols) was measured and plotted on the y-axis. The figure shows a linear decline in intensity with decreasing antibody concentrations for both the peptide spots and the tissue sections. Tissue color intensity is measured as optical density on a 0–2 scale. Peptide spot color is measured as mean pixel intensity on a 1–256 scale. Copied with permission from Sompuram et al.⁶

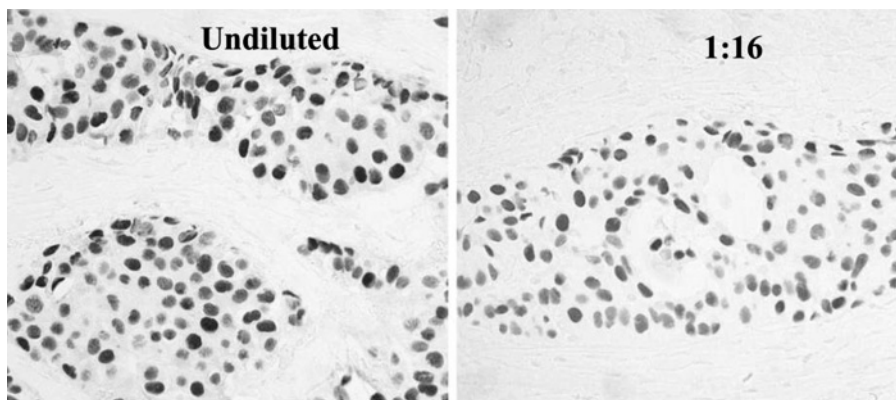


Figure 7.8 PR+ tumor tissue photomicrographs after staining for PR, using the primary antibody at the optimal concentration or a 1:16 dilution. Adapted with permission from Sompuram et al.⁶

Although tissue sections and peptide controls behaved equivalently in detecting decreasing concentrations of reagent, fine decrements are often difficult to detect by eye. Figure 7.8 shows a representative image of two tissue sections that were quantified in Figure 7.7. The image marked “undiluted”

was created using the primary antibody (PR 636) at the optimal concentration, based on the manufacturer's recommendation ("undiluted"). The image marked "1:16" was created by diluting the primary antibody sixteen-fold. There is a difference in staining intensity that is detectable when the two images are held side by side, and quantifiable using image analysis (as per Figure 7.7).⁶ However, the decrement might be missed if the 1:16 dilution image were examined by eye, independent of the other image. Subtle differences in staining intensity, such as twofold or fourfold dilutions of antibody, are often difficult to evaluate without the aid of image analysis. Peptide controls, on the other hand, are easy to quantify using an inexpensive flatbed document scanner that cost approximately one hundred dollars at an office supply store. Even more important is that the concentration of the analyte (peptide) can be standardized to a constant molar concentration, facilitating intra-laboratory staining comparisons over time. The standardization also facilitates inter-laboratory comparisons.⁸

7.7 PEPTIDE CONTROLS CAN DETECT PROBLEMS WITH ANTIGEN RETRIEVAL

It is important that an IHC laboratory positive control be able to identify problems with antigen retrieval. An unfixed peptide control will not do this; it will be insensitive to antigen retrieval treatment, as shown in Figure 7.6. With unfixed peptides, the peptide control spot is immunoreactive and appears at the same color intensity after immunostaining, regardless of whether antigen retrieval was performed. Since antigen retrieval is a denaturing process for reversing the effect of formalin fixation, the peptides must be first fixed in formalin if they are to serve as a positive control for antigen retrieval. Short (approximately 20-mer) peptides can sometimes react with formaldehyde and lose immunoreactivity, if formaldehyde-reactive amino acid side chains are present at or near the epitope. A method of fixing peptides with formalin was developed. It results in the loss of immunoreactivity, regardless of amino acid composition. This topic is reviewed in Chapter 16. For this chapter, it suffices to point out the unique QC opportunity with peptide controls. By using both fixed and unfixed controls, in a paired analysis, it is possible to analyze antigen retrieval conditions separate from the other IHC staining components.

In Chapter 16 and as previously published,¹⁰ there is a description of formalin fixation of peptide controls, mimicking fixation of tissue biopsies. Such formalin-fixed peptide controls require antigen retrieval for IHC staining. Without antigen retrieval, formalin-fixed peptide controls cannot be immunostained. Alternatively, peptide controls can be printed on slides without formalin fixation. Unfixed peptide controls stain regardless of whether or not antigen retrieval was performed. Comparing the staining results of both fixed and unfixed peptide analyte controls from the same laboratory can



Figure 7.9 Appropriate antigen retrieval and immunostaining of peptide controls and tissue sections, stained for HER2. The tissue section on the left has an island of 3+ HER2 tumor, toward the top of the tissue section. The tissue section on the right does not express HER2. Identifying information on the label was removed. See color insert.

help identify the cause of immunostaining failure. Unfixed controls are sensitive to variables relating to antibody and detection reagents. Formalin-fixed controls are sensitive to those same experimental variables as well as antigen retrieval.

The utility of paired (fixed and unfixed) peptide controls were tested in a national study, conducted with the cooperation of the College of American Pathologists. Each peptide was printed in duplicate, at four different concentrations (50, 10, 2, and 0.4 μM), yielding a total of eight peptide spots per slide. These concentrations were determined empirically, as providing a high degree of discrimination for both the Herceptest kit as well as the CB11 monoclonal antibody. Each participating clinical laboratory received two slides—one fixed in formalin and a second that was unfixed. On each slide was also a tissue section of a breast carcinoma, one that was 3+ for HER2, and the other that did not express the HER2 glycoprotein. Each clinical IHC laboratory was asked to stain both slides as per their normal routine and send them back to us. Figure 7.9 illustrates an expected result, whereby both the fixed and unfixed controls stain approximately equally. Peptide spots that were printed at 50 and 10 $\mu\text{g}/\text{mL}$ were positive.

Figure 7.10 shows the result from a clinical laboratory that did not perform antigen retrieval correctly. The unfixed peptides at 50 and 10 $\mu\text{g}/\text{mL}$ stained correctly, but the fixed controls did not. The tissue section (left slide, labeled “HER2-99”) also did not stain well, since it requires antigen retrieval.

Figure 7.11 shows another variation, associated with a lower sensitivity. Both fixed and unfixed peptide controls stain approximately equivalently, but both only stain the 50 $\mu\text{g}/\text{mL}$ peptide control. This lower sensitivity can be due to a suboptimal reagent concentration, reagent degradation, improper procedure, or partial instrument failure.

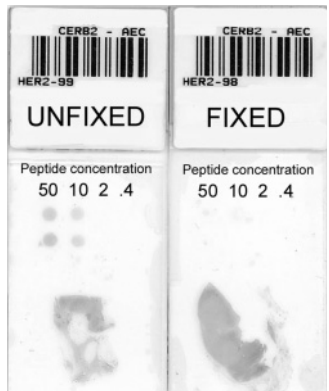


Figure 7.10 Immunostain result with inadequate antigen retrieval, resulting in staining of unfixed but not fixed peptide controls. The HER2+ tumor (left slide) is largely unstained as well. See color insert.

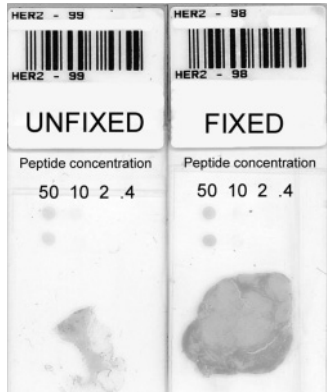


Figure 7.11 Immunostain result that demonstrates a lower level of sensitivity, in that only the highest peptide concentration is stained. The HER2+ tumor tissue (left slide) is also relatively unstained. See color insert.

Figure 7.12 shows as yet another variation, associated with a higher than average immunohistochemical staining sensitivity for HER2. This particular laboratory has its HER2 stain optimized so that it detects even the 0.2 μ g/mL peptide control spot. Only a small percentage of clinical laboratories in our national clinical trial achieved this highest degree of staining sensitivity. There are currently no standards for HER2 staining that would mandate a specific sensitivity in terms of molar concentration limit of detection. It is likely that as the controls enter the regular use, practice guidelines will evolve.

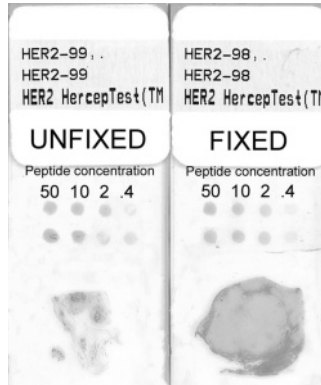


Figure 7.12 Immunostain result that demonstrates a higher than average sensitivity, in that all peptide controls are stained, down to $0.2\mu\text{g/mL}$. The HER2+ tumor tissue (left slide) is also intensely stained. See color insert.

7.8 SUMMARY

Peptide controls represent a new method for quantitatively measuring the efficacy of immunohistochemical staining. The peptide controls are stable, reproducible, and sensitive indicators of problems associated with the immunohistochemical stain. In addition, the peptide controls can be fixed with formalin so that they will require antigen retrieval for visualization, just like fixed tissue sections. By comparing fixed and unfixed controls, the laboratory obtains a unique insight into the performance of different parameters of the IHC staining process. The peptide controls technology was transferred to ThermoFisher Corporation, who will be introducing them commercially in 2009. The availability of reproducible and quantifiable positive controls creates the opportunity to apply quantitative statistical control methods, such as by using Levy–Jennings charting and Westgard rules. These QC methods were previously foreign to clinical IHC testing but are widely used for other quantitative clinical laboratory tests. Now that IHC testing is becoming more quantitative in nature, it seems reasonable to expect that quantitative QC measures will be adopted in this field as well.

ACKNOWLEDGMENTS

We are grateful for the financial support provided by the National Institutes of Health, who supported this work through NIH grants CA106847 and CA094557.

CONFLICT OF INTEREST DISCLOSURE

The authors disclose a financial conflict of interest, as inventors of the peptide controls technology, now licensed to ThermoFisher Corporation.

REFERENCES

1. Roche P, Suman V, Jenkins R, et al. Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. *J. Natl. Cancer Inst.* 2002; 94: 855–857.
2. Paik S, Bryant J, Tan-Chiu E, et al. Real-world performance of HER2 testing—National Surgical Adjuvant Breast and Bowel Project experience. *J. Natl. Cancer Inst.* 2002; 94: 852–854.
3. Perez E, Suman V, Davidson N, et al. HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 Intergroup adjuvant trial. *J. Clin. Oncol.* 2006; 24: 3032–3038.
4. von Wasielewski R, Hasselmann S, Ruschoff J, et al. Proficiency testing of immunohistochemical biomarker assays in breast cancer. *Virchows Arch.* 2008; 453: 537–543.
5. Westgard J, Barry P, Hunt M, et al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem.* 1981; 27: 493–501.
6. Sompuram S, Vani K, Zhang K, et al. A novel quality control slide for quantitative immunohistochemistry testing. *J. Histochem. Cytochem.* 2002; 50: 1425–1434.
7. Sompuram S, Vani K, Ramanathan H, et al. Synthetic peptides identified from phage-displayed combinatorial libraries as immunodiagnostic assay surrogate quality control targets. *Clin. Chem.* 2002; 48: 410–420.
8. Vani K, Sompuram S, Fitzgibbons P, et al. National HER2 proficiency test results using standardized quantitative controls: characterization of laboratory failures. *Arch. Pathol. Lab. Med.* 2008; 132: 211–216.
9. Bogen S, Vani K, McGraw B, et al. Experimental validation of peptide immunohistochemistry controls. *Appl. Immunohistochem. Mol. Morphol.* 2009; 17: 239–246.
10. Sompuram S, Vani K, Bogen S. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.

CHAPTER 8

STANDARD REFERENCE MATERIAL: PROTEIN-EMBEDDING TECHNIQUE AND DESIGN OF BAR CODE

SHAN-RONG SHI, JIANG GU, and CLIVE R. TAYLOR

The demand for quantitative immunohistochemistry (IHC) continues to escalate, as a direct result of the widespread utilization of IHC in clinical diagnosis and in translational cancer research, with particular reference to the current development of targeted cancer treatment. This demand in large part stems from the growing emphasis on prognostic markers and therapeutic indicators, as exemplified by the clinical application of Herceptin (anti-Her2 antibody) for breast cancer treatment.¹ Several key issues of standardization for quantitative IHC have been discussed in previous chapters. There are two critical issues that must be addressed in order to provide a practical way to reach the goal of standardization and quantitative IHC. These issues are (1) controlling the variable IHC staining results that result from inconsistent formalin fixation, possibly by use of an optimal antigen retrieval (AR) protocol (see Chapters 1 and 5); and (2) establishing a standard reference material that can serve both to assess the quality of sample preparation and as a “calibration standard for quantification” (see Chapters 6 and 7). In this chapter, a protein-embedding matrix technique is discussed as a possible reference standard, together with the design of bar code protein matrix display as a calibration standard.

The application of purified protein incorporated within various matrices was used as a model system for cytochemistry more than half a century ago (reviewed by van der Ploeg and Duijndam²). Beginning in 1943, Coujard smeared a gelatin solution or egg white containing the “test” substances in variable concentrations on glass slides to determine the sensitivity of certain cytochemical reactions. Subsequently, the use of other types of artificial substances was documented, such as a polymerized normal rabbit serum containing various amount of specified antigen by diffusion, employed to determine

specificity and optimal working titer of fluorescent antibodies, or used as a measure of the influence of different fixatives, or even used in early studies as standards for quantification of tissue antigens.^{3,4} Camargo and Ferreira⁵ adopted cellulose powder activated by cyanogen bromide and coupled to protein that was subsequently smeared on glass slides for immunofluorescence staining. In 1975, Streefkerk et al.⁶ reported a model utilizing agarose beads covalently linked to protein (either antibody or antigen) that was used for quantitative direct immunoperoxidase procedures. The advantages of a protein-embedding technique include consistency in quantity and quality of incorporated protein, allowing for easier and more accurate measurement of protein. Because standardization and quantification of IHC are desired, the protein-embedded matrix model must be subjected to identical conditions as the test specimen, including phases of sample preparation and any AR process. This requirement represents a major logistical problem, in addition to a considerable technical challenge. To date, there is no published report of a satisfactory protein-embedding technique that lends itself to all of the identical processing steps employed for routine formalin-fixed, paraffin-embedded (FFPE) tissues.

Recently, we have conducted an extensive search for an optimal matrix medium in which to embed proteins for establishing a model reference control system.⁷ At the beginning, we followed previous literature, mixing a protein in a supporting matrix.⁴ To reach the goal of identical treatment of FFPE tissue sample, an optimal matrix must have several properties: (1) it must be capable of existing in two phases, liquid and solid; (2) the liquid phase must allow even mixing of a protein and should then easily be converted into the solid phase; (3) the solid phase should be amenable to fixation with formalin and embedding in paraffin, without excessive hardening or brittleness; that is, it must be suited to sectioning by a microtome after embedding in paraffin; (4) sections of this embedding material must remain adherent on glass slides after boiling AR treatment; (5) it must be nonreactive and must not interfere with subsequent AR, or IHC methods. With these requirements in mind, a variety of materials and methods have been evaluated.

8.1 PROTEIN ABSORPTION METHOD

Small pieces of different solidified matrix media were immersed in a solution containing known amount of proteins for defined periods of time at 4°C as documented previously.⁴ In one example, normal rabbit serum (DAKO) was polymerized with glutaraldehyde to form a gel. The gel was stored at 4°C in several changes of phosphate buffer saline (PBS, pH 7.6) for at least 3 days and then sliced into small pieces (about 1.5 × 1.5 × 5 mm). These fragments were soaked in serial dilutions of protein, and thereafter transferred to 10% neutral buffered formalin (NBF) for fixation, and subsequent paraffin embedding (along with a gel fragment that had not been exposed to antigen [negative

control]). Thin sections were cut for IHC staining. It was expected that a positive color would be detected in the protein impregnated gel matrix. However, there were no significant observed differences of staining among positive and negative protein sections, due in large part to cross reactions and apparent nonspecific staining of the rabbit serum gel. Another drawback of this method was an apparent uneven distribution of the protein in this medium (the peripheral area showed much stronger intensity than the center). Finally, the exact amount of protein absorbed into the medium was difficult to calculate. In addition to this classical polymerized rabbit serum method, other support gels were evaluated, including egg white, duck salted egg white (purchased from a Chinese supermarket), and plastic sponges (e.g., regular sponge used for packaging). None were satisfactory due to similar issues as described for the rabbit serum gel. The sponge failed to retain protein adequately due to the fact that the holes were in fact very large in “microscopic” terms, and due to lack of covalent coupling to protein.

8.2 DIRECT MIXING PROTEIN INTO MATRIX MEDIA

Having learned the drawbacks of the protein absorption methods described above, a direct protein mixing method was tested, by adding known concentrations of protein solution into the liquid phase of the matrix medium (polymerized rabbit serum, agarose gel, alginate beads, gelatine, etc), and then attempting to induce a solid phase by fixation. Some materials such as agarose and alginate were unable to withstand the boiling condition of AR; that is, sections made of agarose or alginate were totally lost after the heating AR procedure. Materials such as polyacrylamide (material used for gel electrophoresis) were excessively hardened by formalin fixation and/or other treatments such as dehydration or paraffin embedding. Other materials, such as gelatine and polymerized rabbit serum, did not allow an even distribution of protein in the medium, and showed nonspecific background staining as a further complication.

8.3 COATING PROTEIN ON SURFACE OF BEADS

Although several types of fluorescent beads were proposed as a microscopic fluorescence standard 30 years ago,² beads have not been used as a protein-embedding matrix for routine IHC on FFPE tissue. We recently tested primary coated beads (“Dynabeads,” Dynal, New York) that are coated with a goat anti-mouse antibody on the surface of the beads. In the first experiment, a monoclonal antibody to cytokeratin 7 (DAKO, 50 μ L/34.5 μ g) was bound to the beads by incubating with the beads (150 μ L at a concentration of 10^9 beads/1 μ L) at 4°C in a cold room with an automatic shaker for overnight. Incubation was followed by three phosphate-buffered saline (PBS) washes,

then the addition of biotinylated horse anti-mouse antibody (20 μ L of concentrated reagent purchased from Vector Lab, Burlingame, CA), and further incubation under the same conditions for 3 h. The beads, now coated with biotin-conjugated protein, were then fixed in 10% NBF for 20 min, mixed into 1% agarose gel in a small tube, and fixed in 10% NBF overnight. The blocks of agarose gel, containing biotinylated protein-coated beads, were subjected to the routine tissue-embedding procedure. The AR technique was performed routinely using a microwave oven as previously documented.⁸ Both AR-treated and untreated slides were then incubated with the avidin–biotin–peroxidase complex (ABC) reagent, and compared. For the second experiment, a purified S-100 protein was bound to the beads by the following two steps: (1) a monoclonal mouse antibody to S-100 (30 μ L of concentrated reagent from Sigma, St. Louis, MO) was incubated with beads for 1 h at the same conditions described above. (2) After three PBS washes, purified S-100 protein (Sigma, 5 μ L of a concentration of 10 mg/mL), was then incubated at the same conditions for 1 h, followed by three PBS washes, and all of the subsequent procedures of fixation, paraffin-embedding, and AR treatment described above. Slides were then incubated with the primary monoclonal antibody to S-100, followed by biotinylated anti-mouse antibody and the ABC incubation. Sections of a human melanoma were used as positive control for S-100 (Fig. 8.1a). Biotinylated anti-mouse antibody, precipitated onto a slide, was employed as a baseline positive control. One section of beads-gel that was treated by AR heating was used for a negative control by omitting the ABC, or primary antibody incubation for first and second experiments, respectively. Amino-ethyl carbazole was employed as the chromogen, yielding a red color in a positive staining result.

Figure 8.1 shows the results of IHC staining using Dynabeads coated with biotinylated anti-mouse IgG and protein S-100, for the first and second experiments, respectively. Strong positive staining results were obtained after AR, appearing as red circles surrounding beads (Fig. 8.1b,c). Slides without AR treatment also showed positive results, but staining was much less intense. Negative control slides showed clean background and no evidence of positive staining (Fig. 8.1d).

Identification of a suitable matrix to carry protein is a key issue in attempting to create a protein-embedding reference material for standardization of IHC. It appears that the Dynabeads tested in these experiments have the potential to serve as the matrix based on the following results: (1) Beads that are able to bind a variety of mouse monoclonal antibodies and their corresponding protein antigens are commercially available. (2) These beads are suitable for formalin fixation and all subsequent processes of dehydration, clearing, and embedding in paraffin. (3) Various proteins (antigens) can be applied consistently to coat polymer beads uniformly. (4) Cut sections of embedded beads can be boiled in water for the AR treatment. (5) IHC staining demonstrates specificity and sensitivity comparable to staining of human tissue sections. (6) In one example, the quantitative IHC stain for certain surface

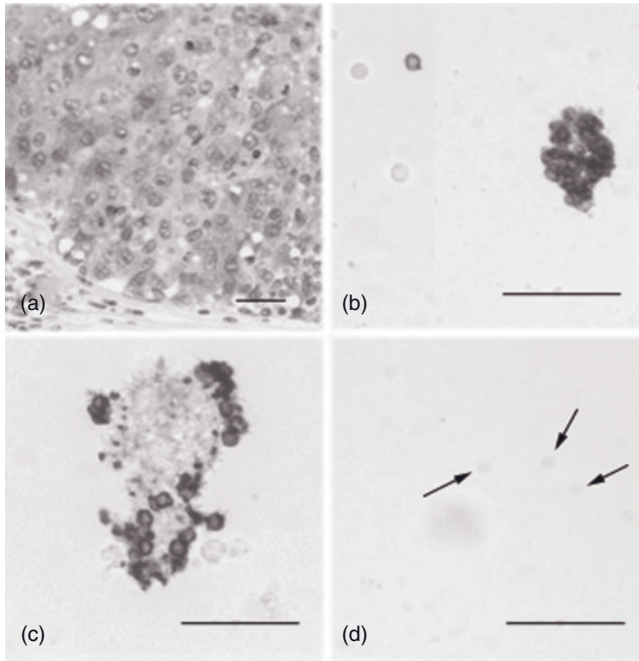


Figure 8.1 The results of IHC of two experiments using Dynabeads (Dyna, New York, NY) coated with biotinylated anti-mouse IgG (first experiment) and protein S-100 (second experiment). (a) Positive control showing red color (S-100) localized in the melanoma cells. (b) Strong positive red color circles all beads coated with biotinylated anti-mouse antibody after the heating AR treatment (first experiment). (c) Using the heating AR treatment, S-100-coated polymer beads show positive red color around the beads as circles (second experiment). (d) Negative control of the first experiment. No red color could be seen for polymer beads (arrows) that had been treated with exactly the same protocol as that of slide (b), but omitting the avidin–biotin–peroxidase (label). Bar = 50 μ m. Reproduced with permission from Shi et al., *J. Histochem. Cytochem.* 2005; 53: 1167–1170. See color insert.

markers such as Her2/neu appear particularly suited to this method in that the surface positive label on the beads mimics Her2/neu cell surface marking.

8.4 A DESIGN OF BAR CODE

A bar code is a computer or machine-readable representation of information. It is usually made of dark ink on a light background to create high and low reflectance which is converted to 1s and 0s, when read by the computer program. A similar result may be achieved by patterns of dots, concentric circles, or text codes hidden within images. A bar code containing stored data in the widths and spacings of printed parallel lines or other patterns as

mentioned above can be used for a variety of purposes, particularly, if it has widely been adopted in commercial markets worldwide.

The initial idea using a barcode for measurement of IHC staining intensity was proposed by a young master's degree student, Guotao Wang (under the guidance of Professor Jiang Gu), at the School of Basic Science, Peking University. This idea in turn came from our collaborative work together on the protein-embedding technique.

First of all, to reach the goal of this bar code measurement system, it is critical to find a suitable material based on the above-mentioned criteria, in order to coat proteins on the surface of a thin layer of suitable matrix material. By using a serial application of protein-coated thin layer sheets with known variable graded concentrations of specific protein, it is possible to establish a bar code as illustrated in Figure 8.2. Following routine formalin-fixation and paraffin-embedding procedure, a comparable FFPE protein-coated bar code is available as a standard material. Having constructed an FFPE bar code, containing known graded concentration of a specific protein, it was

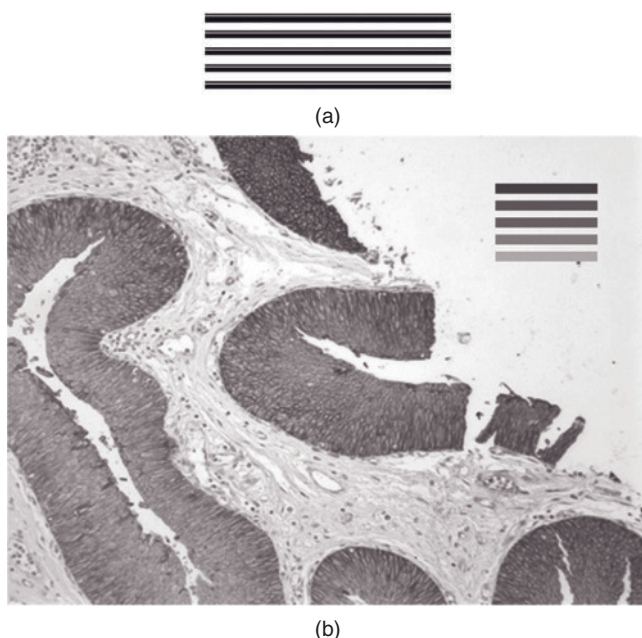


Figure 8.2 Design of protein-embedding barcode is depicted in (a) five thin layers of matrix (the thicker lines) coated with variable concentration of tested protein (thinner lines located above the matrix). (b) A FFPE tissue section of bladder cancer IHC-stained by monoclonal antibody to E-cadherine showing variable intensity of positive staining results which is compared with a protein-embedding bar code as designed in this chapter. Using computer-assisted image analysis with a special software, an automatic quantitative measurement of protein is performed. See color insert.

possible to compare intensity of staining of the same protein in FFPE tissue sections following the IHC staining procedure. Using computer-assisted image analysis, it was then possible to convert quantitative information from the barcode to measure the amount of the same protein in tissue as illustrated by Figure 8.2.

While these preliminary data are encouraging, before it is possible to accept this bar code design as a standard reference material, it must be tested extensively and validated under different laboratory conditions for a range of different protein analytes commonly examined by IHC, following principles discussed in previous chapters (Chapters 1 and 5).

One possible approach is to combine the use of protein matrix barcodes with efforts to improve reproducibility of IHC staining by the use of optimized AR methods that were discussed in Chapter 5. In this design, the protein matrix bar codes, following formalin fixation and paraffin embedding, could in theory provide accurate quantitative IHC measurement information with respect to the degree of “antigen loss” following FFPE, and the extent of recovery of “antigenicity” following AR. Antigen loss and AR in the protein bar code could be assessed side-by-side with the results (intensity) of IHC staining in cell/tissue model systems. In this way, the protein-embedding bar code reference material, containing as it does linear deposits of different concentrations of protein, could be developed to provide objective data as to the degree of loss and retrieval of specific proteins. It is recognized that while simple “naked eye” examination of the bar codes following FFPE and AR and subsequent IHC staining may allow determination of the net extent of protein retention by the degree to which linear bands containing lesser concentrations of protein can be detected, that for accurate assessment image analysis techniques must be employed. Also different classes of proteins, and different individual proteins, behave differently following FFPE and AR, requiring the construction of several bar codes composed representing these different protein classes. Because of the many variables present, experimental design is complex, but nonetheless logical, lending itself to a step-by-step approach. (Figure 8.3):

From Figure 8.3, several major steps include the following:

1. Establish a cell/tissue model that is reproducible; Western blot or mass spec for 6–10 different proteins present, at range of concentrations from low to high.
2. Attempt to identify same Identity 6–10 proteins by IHC, including range in amount/intensity.
3. Make protein matrix bar codes for each of the selected proteins.
4. Test for consistency of bar code production for each 6–10 types of protein bar code.
5. Test each type of bar code for apparent antigen loss with different periods of fixation; test for reproducibility run to run.

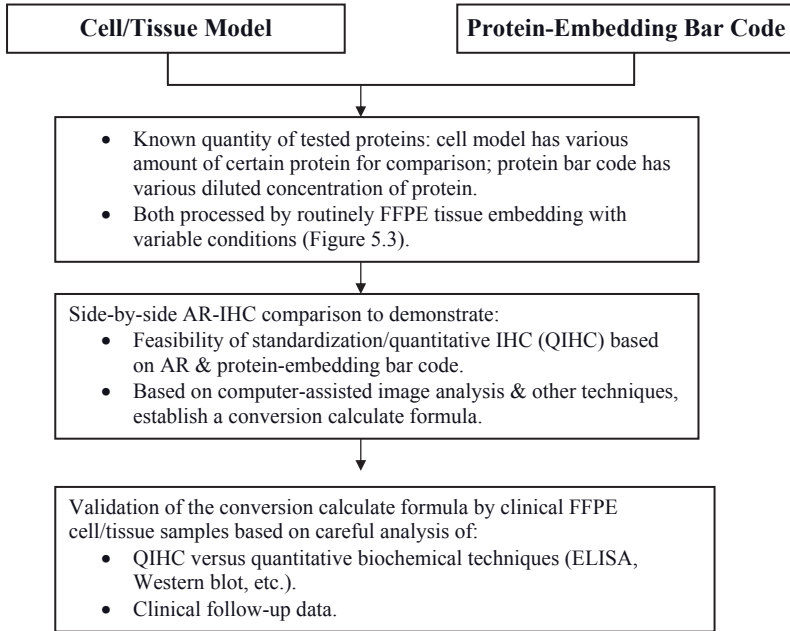


Figure 8.3 Diagram depicts major experimental steps for validation of the protein-embedding barcode used as standard reference material for standardization/quantitative immunohistochemistry.

6. Test effect of AR on FFPE protein bar codes following different periods of fixation; compare bar codes made of different types of protein, do they perform similarly or differently on FFPE and on AR?
7. Compare bar codes FFPE/AR with cell/tissue model FFPE/AR for each protein type.
8. Evaluate absence, presence, and intensity in each case by naked eye—according to number of bar code markers still detectable, and compare naked eye analysis with image analysis.
9. Compare findings with Western blot or mass spectrometry analysis to validate the quantitative measurement using protein-embedded bar code-based image analysis.
10. Validate the protein-embedding bar code based quantitative IHC method by clinical samples with known clinical follow-up data.

The use of independent methods, other than IHC, for quantitative demonstration of proteins is particularly important. Both enzyme-linked immunosorbent assay (ELISA) and Western blot may be employed to confirm the amount of protein in a cell/tissue model, and in the protein-embedding bar code model under both comparable fresh and FFPE samples for accurate

comparative analysis. Data obtained by these independent methods may then be used as the basis for developing mathematical formulae for conversion of intensity of IHC signal in a tissue section to absolute amounts of protein per unit area (or cell) by reference to the extent of stain detected in the bar code.

While logical in theory and in construct, there are significant challenges to successful completion of this type of experiment. Pilot experiments have shown that current protein matrices are difficult to produce uniformly and consistently and are not sufficiently robust to provide the basis for the serial studies described. Additional work is necessary to develop more sophisticated materials for a protein matrix, so that protein can be coated on the surface of a thin layer of matrix reliably, meeting all requirements described above. To accomplish this goal, it will be necessary to develop active collaborations with chemical engineers and biochemists, in addition to those working primarily in IHC. Improved methods must be identified to assure a firm covalent bond, coupling protein on the surface of the matrix, similar perhaps to the protected isocyanate microscope slide-coating technology proposed by Sompuram.⁹

It is important to recognize that establishing a model reference material, such as the protein-embedding model described above, while essential, is just the first step for standardization of IHC. Further studies will be required to develop mathematically conversion factors, and to explore the potential utility and limitations of this approach for different proteins that are of clinical interest, as diagnostic, prognostic, or predictive markers, as described above.¹⁰⁻¹²

In summary, the protein-embedding bar code design for quantitative IHC has the unique advantage of representing a known quantity of selected protein for FFPE/AR and IHC staining, thus providing a calibration standard that may allow direct measurement of protein by IHC. However, further experimental work is demanded in order to create such a technique. Probably, the synthetic peptides used as IHC control^{13,14} may be combined with this protein-embedding bar code design (see Chapter 7).

ACKNOWLEDGMENTS

Part of contents pertaining to the protein-coated beads is reproduced with permission from our article entitled "Protein-embedding technique: a potential approach to standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections" published in *J. Histochem. Cytochem.* 2005, 53(9):1167-1170.

REFERENCES

1. O'Leary TJ. Standardization in immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 3-8.
2. van der Ploeg M, Duijndam WAL. Matrix models: essential tools for microscopic cytochemical research. *Histochemistry* 1986; 84: 283-300.

3. Brandtzaeg P. Evaluation of immuno fluorescence with artificial sections of selected antigenicity. *Immunology* 1972; 22: 177–183.
4. Brandtzaeg P, Rognum TO. Evaluation of nine different fixatives. 2. Preservation of IgG, IgA and secretory component in an artificial immunohistochemical test substrate. *Histochemistry* 1984; 81: 213–219.
5. Camargo ME, Ferreira AW. A microscopic immunofluorescence technique with soluble protein antigens fixed to cellulose particles. *Int. Arch. Allergy* 1970; 39: 292–300.
6. Streefkerk JG, van der Ploeg M, van Duijn P. Agarose beads as matrices for proteins in cytophotometric investigations of immunohistoperoxidase procedures. *J. Histochem. Cytochem.* 1975; 23: 243–250.
7. Shi S-R, Liu C, Perez J, et al. Protein-embedding technique: a potential approach to standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 2005; 53: 1167–1170.
8. Shi S-R, Cote RJ, Shi Y, et al. Antigen retrieval technique. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 311–333. Natick, MA: Eaton, 2000.
9. Sompuram SR, McMahon D, Vani K, et al. A novel microscope slide adhesive for poorly adherent tissue sections. *J. Histotechnol.* 2003; 26: 117–123.
10. Taylor CR. An exaltation of experts: concerted efforts in the standardization of immunohistochemistry. *Hum. Pathol.* 1994; 25: 2–11.
11. Shi S-R, Cote RJ, Chaiwun B, et al. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. *Appl. Immunohistochem.* 1998; 6: 89–96.
12. Shi S-R, Gu J, Cote RJ, et al. Standardization of routine immunohistochemistry: where to begin? In *Antigen Retrieval Technique: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and R Taylor, pp. 255–272. Natick, MA: Eaton, 2000.
13. Sompuram SR, Kodela V, Zhang K, et al. A novel quality control slide for quantitative immunohistochemistry testing. *J. Histochem. Cytochem.* 2002; 50: 1425–1434.
14. Sompuram SR, Kodela V, Ramanathan H, et al. Synthetic peptides identified from phage-displayed combinatorial libraries as immunodiagnostic assay surrogate quality-control targets. *Clin. Chem.* 2002; 48: 410–420.

CHAPTER 9

THE PROS AND CONS OF AUTOMATION FOR IMMUNOHISTOCHEMISTRY FROM THE PROSPECTIVE OF THE PATHOLOGY LABORATORY

DAVID G. HICKS and LORALEE MCMAHON

9.1 INTRODUCTION

The traditional diagnostic evaluation performed by anatomic pathologists involves the critical analysis and interpretation of morphologic features from routinely prepared hematoxylin and eosin (H&E)-stained tissue sections. Armed with his or her microscope and training, the pathologist's task is to scrutinize the morphologic features of these stained sections in an attempt to determine whether a specific disease process is present and, if so, to further classify and comment on the nature of that disease. Backed by over 100 years of clinical experience, these morphologic techniques are quite remarkable, allowing for the accurate and reproducible classification and diagnosis of many disease states within pathologically altered tissue samples.

The appearance of cells and their architectural organization within tissues, while informative, can often be quite complex, subtle, confusing, and at times maddeningly difficult, even in the hands of the most experienced observer. In addition, these morphologic features represent only a fraction of the information contained within H&E stained histologic samples of human tissues. These tissue sections also contain all of the cellular proteins and expressed genes, which help ultimately to determine the biology and clinical behavior of abnormal cells, as well as provide clues to the origins and pathogenesis of disease states.¹ What was needed was a way to unlock this deeper information so that it could be used in diagnostic evaluation.

A number of important technical advances have provided pathologists with just such tools that can probe beyond pure morphology into the cellular abnormalities in both the protein and gene expression that underlies human disease. These tools, which include immunohistochemistry (IHC) as well as other molecular applications to tissue sections, have dramatically changed the field of pathology and continue to play an increasingly important role in the diagnosis of disease in biopsy samples and subsequent clinical decisions.

9.2 DEVELOPMENT OF IHC AS AN ADJUNCT TO PATHOLOGIC DIAGNOSIS

The IHC methodology has become firmly established as an important supplement in the diagnostic armamentarium of the pathologist. The development of IHC gave pathologists a powerful new tool for the localization of protein antigens within tissue sections while preserving the underlying morphology. This capability provided additional new information, and allowed for further discrimination among cells and tumor types and provided objective confirmation of morphologic impressions determined during a careful review of H&E stained slides. A more recent application of IHC has been the development of assays for important new cancer protein biomarkers. These biomarkers have prognostic and predictive utility in the evaluation of some malignancies and have demonstrated the potential to help determine the most appropriate therapy for the newly diagnosed cancer patient. This is particularly true in breast cancer, where the evaluation of estrogen receptor, progesterone receptor, and the tyrosine kinase growth factor receptor HER2 is now a part of the standard initial workup for any newly diagnosed tumor.^{2,3}

While IHC has become an increasingly important adjunct to pathologic diagnosis, it should not be considered a substitute for a careful morphologic evaluation of clinical biopsy samples. A wise pathologist once said, “if you do not have a pretty good idea of what a lesion is before you stain it, IHC will only turn what you do not know brown, and in all likelihood, you still will not know what the lesion is.”

The technical developments that led to the widespread clinical application of IHC in pathology laboratories grew out of the initial research applications on frozen tissue sections and cells which can be dated back more than 50 years ago. By the late 1970s and early 1980s, refinement of detection chemistry, assay methodologies, and improvements in assay procedure led to the application of IHC methods in routinely processed clinical samples.⁴ During this era of discovery, immunohistochemical techniques were increasingly becoming a part of the clinical diagnostic pathology laboratory as an explosion of literature documented the utility and potential of this technology to expand the capabilities of the pathologist in diagnostic procedures.

9.3 MANUAL METHODS FOR PERFORMING IHC

In the early days of IHC, assays were performed only at large university-based hospitals and were done manually by trained and experienced technical personnel. The manual assays were complex and were labor intensive, requiring multiple incubation steps, including the manual dispensing of appropriate concentrations of reagents onto tissue sections for specific periods of time, followed by rinsing.^{5,6} The interpretation of the finished product (an immunohistochemically stained slide) was then performed by an experienced pathologist who evaluated these sections both for the presence and the pattern of staining of protein antigens. These results could then be interpreted in the context of the morphologic changes seen in the tissue.

It soon became clear that the usefulness of IHC in clinical diagnosis was going to be directly dependent upon the quality, reliability, and reproducibility of these staining techniques.⁷ A number of critical steps in manual immunohistochemical methods are operator-dependent and are essential for the quality of the final results. These steps include reagent preparation, antigen retrieval, antibody and solution application, incubation times, washing and wiping—all of which need to be performed reliably and consistently in precisely the same sequence from sample to sample.⁵ The large number of steps involved in these manual hand-staining procedures increased the likelihood of technical errors, and had the potential for considerable technologist-to-technologist and laboratory-to-laboratory variability in the quality, consistency, and reliability of assay results.⁸

As the clinical applications and demand for IHC grew, it became widely recognized that IHC assay standardization was going to be vital in order to achieve reliable and reproducible results that would be comparable between the different laboratories performing these stains. A major step toward improvement came from the efforts of a number of agencies, including the Biologic Stain Commission, the Clinical and Laboratory Standards Institute (CLSI), the FDA, and the manufacturing sector.⁹ These agencies established guidelines, standards, and recommendations for reagent manufacturing along with detailed package inserts. These efforts represented a major advance forward and led to widespread commercial availability of high-quality IHC reagents. The goal was to improve the consistency of these assays through the use of better reagents.⁹ However, despite the considerable progress that had been made, many authors continued to note considerable variability in the overall quality and consistency of IHC assays.¹⁰ This recognition led to the development of the IHC assay “total test concept,” which called for the rigorous standardization of all aspects of IHC testing, including all pre-analytic variables (tissue handling and fixation), analytic variables (assay reagents and performance), and post-analytic variables (assay evaluation and reporting) involved in performing these assays.¹¹

The considerable issues facing pathology laboratories related to the increasing diagnostic reliance on IHC, laboratory workloads with expanding demand,

and shortages of trained technical personnel, and the need for improvements in quality, consistency, and reproducibility along with technical advances were the driving forces for the development of automation in IHC.⁸

9.4 DEVELOPMENT OF AUTOMATION FOR IHC

The development of machine automation for IHC assays represented another major advance and innovation in diagnostic surgical pathology. An automated instrument's control over and standardization of all the critical steps (reagent application, washing, kinetics optimization, control of evaporation, temperature, and humidity) led to improvements in the accuracy, consistency, reproducibility, and reliability of staining by reducing the possibility of human and technique-dependent errors.^{7,12} Automation not only made it possible to control all aspects of the staining procedure, it also enabled IHC assays to be performed in smaller community hospital laboratories that previously did not have the technical expertise or personnel to perform manual IHC techniques.

In essence, an automated IHC stainer is nothing more than a robot that mirrors the steps that a skilled technician would follow when performing an IHC stain on a slide. However, it is important to point out that automation will not make a stain work that cannot be made to work by manual methods.¹³

Because of continuing improvements in computer software design, the use of bar coding to help identify and track patient slides and reagents, as well as other innovations in hardware,^{8,12} automated immunostaining technology is now widely used in most IHC laboratories throughout the United States. While there have been tremendous advancements in the technology platforms and a proliferation of different vendors offering different types of instrumentation, there is in fact no system that is perfect.^{14,15} Each of the systems that are commercially available have their specific strengths and weaknesses that should be considered during any objective evaluation into which system would be best suited for the need of a particular laboratory. However, the ideal automated staining system ought to have the following requirements and functionalities¹³:

- Relative ease of use and maintenance by the technical staff of the laboratory
- Ease of integration into the laboratory work flow
- User-friendly computer interfaces and work stations that do not require considerable amounts of technical background to run the instrument
- Comparable or superior turnaround time compared with manual staining
- Flexibility in terms of selection of reagents, setup, and run times

- Cost-effectiveness both in terms of savings in technician time and in reagent costs
- Consistent, reliable, and reproducible high-quality stained slides that are comparable to or superior to manually stained slides

9.5 OPEN VERSUS CLOSED IHC AUTOMATED STAINING INSTRUMENTS

At a basic level, all IHC automated instruments function similarly to provide a suitable environment for reagents to react with tissues or cells during an incubation period, applying unique reagents onto slides in a prespecified manner, and applying appropriate rinse solutions onto the tissue sections at specific intervals.¹² However, the various available systems can vary considerably in terms of the freedom of the operator to choose the source of their reagents.^{14,15} Flexibility and freedom to choose reagents from any source allows for the creation of individualized staining protocols (open system) and is at one end of the spectrum. In contrast, systems that require the use of proprietary reagents obtained only from the instrument vendor would lack such flexibility and would constitute a closed system. Different instruments vary considerably in the amount of “openness” that is allowable to the laboratory operator. In many respects, a more flexible, open platform is by necessity more complex. Open systems allow the technician to dictate the staining protocols being used, while closed system staining protocols are dictated by the machine.⁶ In general, the proper operation of an open system requires far greater technical expertise and background in IHC on the part of the technical staff than does the operation of a closed system. Evaluating the daily activities and personnel of the lab will help to determine which system will best fit the needs of your laboratory.¹⁵

Some manufacturers supply proprietary reagents that must be used in conjunction with their instrument. Protocols requiring the strict use of proprietary reagents are not as flexible, and these reagents tend to be more costly. However, the use of proprietary reagents (which are bar-coded) permits computer-driven tracking and monitoring of reagent volumes, lot number, and expiration dates, which will assist with standardization of protocols and quality control of laboratory procedures.⁸

The closed system offers the most standardized and reproducible protocols for a laboratory, but all antibodies that are needed for clinical diagnosis may not be available from these closed system vendors. In the same respect, antibodies manufactured by other suppliers may or may not work properly with the reagents and buffers supplied by the closed system vendors. Antibody vendors test their antibodies with their own diluents, pretreatment buffers, and detection kits, so when optimizing these antibodies using closed systems reagents, good quality staining may not be possible. In contrast, open automated systems are more flexible and allow for the use of other reagents and

protocols. These protocols can be customized and stored for use at any time.¹⁵ However, the details related to monitoring, standardization, and quality control, as well as tracking to ensure that reagents are used within appropriate expiration dates, fall upon the technical staff of the laboratory. Also, the open automated system allows for so much flexibility that this may open the door to inconsistent staining, unless the technical staff is extremely diligent with reagent and protocol tracking (see Table 9.1).

TABLE 9.1 Open versus Closed Automated IHC Systems

	Open System	Closed System
Detection kit flexibility	Allows for a mix and match of detection kits with antibodies, to optimize antibodies. Open systems good for research applications where other species of antibodies may be in use	Must use the vendor's detection kit with all antibodies
Antibody selection	Can use almost any antibody from any vendor in any species due to the ability to use various detection kits	Can use only the antibodies that work with the vendor's detection system This may limit the number of antibodies a lab can offer.
Cost	Because of the flexibility, labs are able to shop around for the best pricing.	Pricing is set by the vendor.
Protocol flexibility	Each antibody protocol can be adjusted and saved in the computer system to achieve the best staining.	Most protocols are preprogrammed and are not very adjustable. Can change some incubation times from preset times
Technical staff	Staff must be highly trained in IHC and diligent in record keeping. Staff must have some knowledge of staining to be able to have flexibility in staining protocols.	Staff may be less trained in IHC. A closed machine can be run by loading slides and solutions, and pressing the start button.
Staining consistency	Good—very dependent on the diligence of the technical staff. They must pay close attention to consistency in the performance of the staining protocols, and also maintain accurate logs of lot numbers and expiration dates of solutions.	Excellent—computer keeps track of all solution expiration dates and does not allow use of outdated solutions. Staining protocols are mostly preprogrammed, so there is no variation from run to run.
Antigen retrieval	Off-line retrieval	Can choose between online or offline

9.6 PRINCIPLES OF IHC AUTOMATION

A number of significant technical challenges had to be overcome in order for IHC automation to be introduced into the routine clinical operation of the anatomic pathology laboratory. Many of these technical challenges are related to the following^{6,12,13}:

- Performing appropriate antigen retrieval before initiating the staining protocol.
- Identification and tracking of individual slides and matching them to the proper staining protocol.
- Dispensing proper volumes of the appropriate reagents in the correct sequence onto the correct slide.
- Incubation of reagents on the slides for the proper length of time, followed by appropriate rinsing.
- Optimization of reaction kinetics for staining.
- Prevention of evaporation, which would cause the slides to dry out during the staining protocol.

One of the first technologies employed for the automated staining of slides was based on the capillary gap principle, which resulted from the pioneering work of Dr. David Brigati.^{16,17} The essence of this technology was to place paired slides close together and vertically in the instrument, with the tissue side of each pair of slides facing each other. A gap of a defined width (typically 50 μ) is formed between each slide pair. A similar method would use just one slide having tissue, paired with a cover plate, also to form a gap. During the staining protocol, the ends of these two slides are partially immersed in a reagent well, resulting in the gap between them being filled by capillary action.^{16,17} The efficiency of this process is dependent to some extent on the surface tension of the liquid being used, so that there is some potential for insufficient delivery of reagents to the tissues. After incubation, the gap between the slides is emptied of fluid by touching the slides onto blotting pads. Refilling of the capillary gaps and blotting is repeated for each reagent and wash step. In systems employing a cover plate in place of one slide, reagents can be dispensed from the top using gravity and top-to-bottom capillary action to displace the liquid that is contained between the vertically positioned slides and cover plate. The capillary gap will retain a defined amount of fluid by surface tension. Washing is then performed by the flow of buffer, dispensed from the top through the gap. A drawback of both of these types of capillary gap techniques is the ease of filling or emptying the capillary gap, which is somewhat dependent on the surface tension of the liquid reagents and buffers. Other problems can be created by the presence of thick, loose, or folded tissue sections that may affect the proper spacing of the gap and therefore result in poor staining due to incomplete tissue coverage by reagents or entrapment of air bubbles.¹⁸

Other automatic staining instruments place individual slides in a horizontal position with the reagents and buffers dispensed onto the tissue from above, using a dispenser, Teflon-coated pipettes or disposable pipette tips. The physical layout or format of such instruments determines their size and shape as well as their slide and reagent capacities.¹⁴ The instruments either arrange slides in linear rows (rectangular shape or array-format stainer) or in a circular pattern (carousel-format stainer). In both of these layouts, reagents and buffers are dispensed from above the slides.¹⁵

Using the linear row design, the slides are placed on a removable support rack forming an array or matrix which arranges the slides in parallel rows, and utilizes a robotic arm that moves over the top to dispense reagents and buffers. One advantage of an array-format stainer is that each rack of slides can be easily removed as the staining procedures are completed. Some models may even allow the operator to add a new rack of slides to a run in progress, a feature referred to as continuous throughput.¹⁴ The circular design would arrange the slides in a circular pattern on a platter-like support, which has been described as a carousel or rotary format, with reagents and buffers also being dispensed from above.¹⁴ In this format, the reagents can circle around above to be dispensed to the appropriate slide, the slides can be moved underneath to the appropriate reagent dispenser, or a combination of both may be used.

9.7 HEAT-INDUCED ANTIGEN RETRIEVAL (HIAR) METHODS: ONLINE VERSUS OFF-LINE

During the tissue fixation process, proteins are cross-linked, causing some epitopes to become undetectable by the staining protocols.¹⁰ HIAR reverses this effect, allowing these epitopes to be stained, and therefore has become increasingly important for many IHC staining protocols.¹⁹⁻²² However, the available automated IHC platforms vary in their ability to perform online HIAR.

For systems that do not offer online HIAR, these procedures must be performed manually or off-line prior to loading slides on the instrument. Online HIAR methods are usually found as part of the closed-type automated staining systems, and are therefore less flexible in terms of what a technician can do to change the HIAR portion of a staining protocol to “help” optimize staining for particularly difficult antibodies. In spite of this, the ability to perform online HIAR is advantageous for many antibodies because it is extremely consistent and frees up technician time to complete other laboratory tasks.

Another aspect to consider is that performance of HIAR within an automated staining instrument can add considerably to laboratory cost compared to performing manual HIAR off-line with devices such as pressure cookers, vegetable steamers, or microwave ovens.¹⁹ In addition, off-line HIAR methods

allow for greater flexibility in retrieval protocols in terms of time, temperature, and buffer selection. However, such laboratory procedures again require a greater degree of experience with IHC techniques on the part of the technical staff.

In general, online versus off-line HIAR offers similar pros and cons as does the open versus the closed automated staining systems. Closed systems and online HIAR not only produce extreme consistency and require less technical knowledge, but it can also limit the number of antibodies that can be used successfully. Open systems and off-line HIAR allow the flexibility to use many more types of antibodies, but technicians need to be well trained and diligent in their record keeping in order to insure consistent staining.

9.8 CONCLUSIONS

There is a general consensus throughout the anatomic pathology laboratory community that with machine automation, the quality and reproducibility of IHC staining are vastly improved.⁸ With automation, technicians are more efficient and productive, and are freed to perform more important and interesting tasks, such as quality control review of stained tissue sections, while still providing the necessary throughput for today's busy IHC laboratories (Table 9.2). Because the acquisition of instrumentation for automated IHC is usually a major capital investment, the individual laboratory should proceed with the decision-making process only after a careful consideration of a number of important factors.^{23,24}

The goal of this chapter is not to build a case for one sort of automated system versus another (or favoring one vendor over another) but rather to point out the relative strengths and weakness of a number of different format designs and platforms that are commercially available, and that may or may not meet the needs of a particular laboratory. To this end, we have chosen not to mention specific vendors or their platforms by name. We have sought rather to represent the perspective of the pathologist laboratory director and the technical supervisor (because that is who we are) regarding important principles and operational differences that are part and parcel of the various automated staining platforms. We have over our careers worked with and are experienced with a number of different commercially available automated systems, and they all perform reasonably well with good consistency and quality when properly maintained and operated according to the manufacturer's instructions and standard laboratory practices.

When considering the purchase of an automated IHC staining system, the challenge is that there are so many options from which to choose. How does one find the right system that best meets the needs (and the budget) for your unique laboratory? Potential buyers in the market for an IHC automated staining system are encouraged to contact the individual vendors and ask them for an on-site laboratory demonstration of their equipment. The volume of

TABLE 9.2 Manual Staining versus Automated Staining

Features	Manual Staining	Automated Staining
Requires highly skilled and knowledgeable technical staff	Yes	(1) Not as much is needed (2) Little or no technical background required for closed staining system
Flexibility with reagent use and incubation times	Yes	(1) Less flexibility with open systems (2) Very little flexibility with closed systems
Consistent incubation time per slide from run to run	No consistency, variable incubation times based on number of slides, etc.	Better consistency of incubation time but can still vary based on number of slides in platform robotic stainers
Turn-around-time	Usually longer, with limitation being number of technicians	Usually much shorter, particularly with closed systems
Slide capacity and laboratory volume of work	More limited, dependent on the number of skilled technicians	Higher throughput with fewer technicians, particularly with closed systems
Consistency of staining	Variable, usually technician dependent	Much more consistent and usually technician independent
Cost	Usually lower, not considering labor and technician time	Usually higher, particularly for closed systems, which use proprietary reagents
Antigen retrieval	Very flexible with options to use different buffers and times	Less flexible when done online with limited buffer selection
Dispensing appropriate reagent volumes	More difficult and technician dependent	Much easier to dispense smaller volumes of expensive reagents
Laboratory safety	Increased risk of exposure to toxic reagents	Minimal risk of exposure to toxic reagents

the laboratory, complexity of its testing menu, experience of the technical staff, and the laboratory operating budget as discussed above are all important considerations when making this important decision. Potential customers should respectfully request a complete cost breakdown from the vendor, including the cost of the hardware, software, and all reagents. This will be particularly important when considering a closed system in which consumables and proprietary reagent costs may be considerably higher than with a more open system.

In summary, the following items bear mentioning and should be carefully considered by the Anatomic Pathology Laboratory when trying to fill the laboratory demands and needs with an automated IHC staining system.

Laboratory Safety: From a laboratory safety standpoint, both open and closed systems have mechanisms for the disposal of hazardous wastes, thus reducing the technician's exposure to potentially toxic materials. Performing HIAR online (closed system) as opposed to off-line mechanisms (microwave ovens or steamers) removes the possibility of being burned while handling hot containers or boiling liquids.

Error Reduction: Both open and closed systems have bar coding capabilities to reduce errors. The closed system has a second level of error reduction by tracking reagent expiration dates, and by not allowing the technician to run slides with expired reagents. Also, computer interfaces have been developed that allow IHC tests to be ordered online within the laboratory information system, and transmitted to the staining instrument, thus eliminating the need for redundant data entry and decreasing the potential for transcriptional errors.

Slide Capacity and Clinical Volume: A careful evaluation of clinical workflow should be done in order to determine what instrument best meets the needs of the laboratory. Slide capacity, clinical volume, turnaround time, staffing, and future needs are examples of parameters that should be considered. The number of antibodies on the laboratory's menu should also be considered. A small lab with a limited number of antibodies can benefit from the closed type system, while larger labs with a larger antibody menu may find the open system more beneficial.

Personnel and Instrument Flexibility: A careful consideration of the background, experience, knowledge, and expertise of the current technical staff, as well as the impact of automation on the staff, should be carefully considered. The level of experience and technical background of the laboratory staff will influence the degree of flexibility that the laboratory director will be able to utilize when using an automated staining system. Some laboratories will require more flexibility in protocols and reagents than others, especially if the testing menu is very complex or the instruments will be utilized in a research capacity. These factors must be also considered when choosing between an open versus a closed system.

Customer Service and Service Contracts: It is important to choose a vendor that has a good customer service track record. Contact other laboratory directors and supervisors to ascertain their experience with the vendors that service their laboratories. Also, the cost of a service contract will need to be factored into the laboratory operating budget after the acquisition of an automated staining platform.

ACKNOWLEDGMENT

The authors wish to acknowledge the excellent help provided by Mary Jackson in the preparation of this chapter.

REFERENCES

1. Hicks DG, Longoria G, Pettay J, et al. In situ hybridization in the pathology laboratory: general principles, automation, and emerging research applications for tissue-based studies of gene expression. *J. Mol. Histol.* 2004; 35: 595–601.
2. Layfield LJ, Saria EA, Conlon DH, et al. Estrogen and progesterone receptor status determined by the Ventana ES 320 automated immunohistochemical stainer and the CAS 200 image analyzer in 236 early-stage breast carcinomas: prognostic significance. *J. Surg. Oncol.* 1996; 61: 177–184.
3. Hicks DG, Kulkarni S. HER2-positive breast cancer: review of biologic relevance and optimal use of diagnostic tools. *Am. J. Clin. Pathol.* 2008; 129: 263–273.
4. Rahman S, Leong AS. Diagnostic immunohistochemistry: current applications and future directions. *Malays J. Pathol.* 1991; 13: 17–28.
5. Moreau A, LeNeel T, Joubert M, et al. Approach to automation in immunohistochemistry. *Clin. Chim. Acta.* 1998; 278: 177–184.
6. Le Neel T, Moreau A, Laboisie C, et al. Comparative evaluation of automated systems in immunohistochemistry. *Clin. Chim. Acta.* 1998; 278: 185–192.
7. Bankfalvi A, Boecker W, Reiner A. Comparison of automated and manual determination of HER2 status in breast cancer for diagnostic use: a comparative methodological study using the Ventana BenchMark automated staining system and manual tests. *Int. J. Oncol.* 2004; 25: 929–935.
8. Taylor CR. Principles of immunomicroscopy. In *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist*, 3rd edition, ed. CR Taylor and RJ Cote, pp. 28–29. Philadelphia: Saunders Elsevier, 2006.
9. Taylor CR. Editorial—A personal perspective. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 121–123.
10. Goldstein NS, Hewitt SM, Taylor CR, et al. Recommendations for improved standardization of immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2007; 15: 124–133.
11. Taylor CR. The total test approach to standardization of immunohistochemistry. *Arch. Pathol. Lab. Med.* 2000; 124: 945–951.
12. Grogan TM. Automated immunohistochemical analysis. *Am. J. Clin. Pathol.* 1992; 98 (Suppl. 1): S35–S38.
13. Herman GE, Elfont EA, Floyd AD. Overview of automated immunostainers. *Methods Mol. Biol.* 1994; 34: 383–403.
14. Myers J. Automated slide stainers for SS, IHC, and ISH: a review of current technologies and commercially available systems. *MLO Med. Lab. Obs.* 2004; 36: 28–30.
15. Zeheb R. Automating immunohistochemistry. In *Immunohistochemical Staining Methods*, 4th edition, ed. ME Key, pp. 103–106. Carpinteria, CA: Dako, 2006.
16. Montone KT, Brigati DJ, Budgeon LR. Anatomic viral detection is automated: the application of a robotic molecular pathology system for the detection of DNA viruses in anatomic pathology substrates, using immunocytochemical and nucleic acid hybridization techniques. *Yale J. Biol. Med.* 1989; 62: 141–158.
17. Unger ER, Brigate DJ. Colorimetric in-situ hybridization in clinical virology: development of automated technology. *Curr. Top. Microbiol. Immunol.* 1989; 143: 21–31.

18. Taylor CR, Shi S-R, Barr NJ, et al. Techniques of immunohistochemistry: principles, pitfalls, and standardization. In *Diagnostic Immunohistochemistry*, ed. DJ Dabbs, pp. 27–29. Philadelphia: Churchill Livingstone, 2002.
19. Myers J. Antigen retrieval: A review of commonly used methods and commercially available devices. *MLO Med. Lab. Obs.* 2006; 38: 10–15.
20. Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
21. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 107–116.
22. Shi SR, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. *J. Histochem. Cytochem.* 2007; 55: 105–109.
23. Myers J. Reducing immunohistochemistry expense—part 2. *Adv. Adm. Lab.* 2004; 13: 18–22.
24. Myers J. Primer for selecting lab equipment. *MLO Med. Lab. Obs.* 2007; 39: 26–27.

CHAPTER 10

IMAGE ANALYSIS IN IMMUNOHISTOCHEMISTRY

ALTON D. FLOYD

Deriving analytical results from stained, microscope slides has been a goal since the demonstration by Caspersson¹ of the ability to accurately measure the extinction of dyes in spheres 4–60 μ in diameter. The availability of a defined histochemical procedure, the Feulgen Rossenbeck procedure for DNA,² provided a way to directly visualize DNA in fixed, stained microscopic preparations, in a stoichiometric manner. With advances in electronics and quantitative microscopes, Swift³ used this procedure to demonstrate the DNA constancy hypothesis in animal cell nuclei. The development of quantitative cytochemistry, both staining methods and instrumentation, was documented in texts by Wied⁴ and by Wied and Bahr.⁵ These texts clearly describe the requirements for instrumentation, stain performance, and methods controls required for precision and repeatability of results. It should be understood that in this time frame, the electronic detection devices were photomultipliers, and computers were not laboratory devices. The only “images” that were available were photographs, and using these for quantitative purposes was difficult. However, it was shown that accurate measurements of nuclear DNA content could provide a wealth of diagnostic information. The downside was that obtaining such information could entail weeks of effort per specimen, and in the clinical environment, pre-analytical factors were exceptionally difficult to control.

Image analysis is the result of two developments: digital image acquisition devices (charge-coupled device [CCD] and complimentary metal oxide semiconductor [CMOS] detectors) and computers with image analytic software. Image acquisition devices are available from many suppliers and are still in a rapid improvement phase. Suffice it to say, it is now almost trivial to collect images of stained specimens as digital images. Likewise, there are many image analysis software packages available, and many of these contain large libraries

of routines that can perform a multitude of analytic analyses of an image. However, neither the capture device nor the analytic software can insure the fundamental requirement for the analytic result—*does the image that is being analyzed faithfully represent the element of the original specimen that is the object of the analysis?* Because of the ease with which images can be collected, and the power of modern software algorithms to extract numerical data from these images, much image analytic data are published which cannot be repeated, simply because the investigator did not insure that the element being analyzed was a true representation of the original specimen.

10.1 IMAGE ACQUISITION

Manufacturers of microscopes provide integrated cameras for image acquisition, as well as adapters for cameras with standardized mounting, such as “C” mounts. In most cases, an image capture software program will be provided. As a consequence, acquiring of images is very easy, and too often, the user does not understand that simply because an image “looks good” it may not faithfully represent the specimen.

Image analysis is most often used to answer two questions: how much material of interest is present and where is it located. Location can be used to determine the percentage of a specimen that is “positive,” and it can also be used to determine if the component of interest coincides with the location of a second component of interest (colocalization). To determine the amount of material present, the specimen must be stained in a stoichiometric fashion. That is, the stain density must be directly proportional to the amount of the specimen component that is being stained. Additionally, the image must have been obtained to insure photometric accuracy. These requirements are absolute, and if not met, any numbers generated from the image will simply be useless and be possibly misleading. Image analysis can analyze any image and generate a result. It is the responsibility of the user to insure that the result is valid. This can only be achieved by understanding the requirements for specimen preparation and for acquiring of the image.

Historically, stained specimens have been evaluated subjectively. This has been true from the beginning of histopathology and remains true today. That subjective evaluation has been successful and useful in diagnosis of specimens is a given, yet there is no guarantee that a subjective evaluation of an image insures it is adequate for image analysis. Stains used in histopathology have been developed based on the properties of the human eye and in general exploit the ability of the eye to detect many different hues of color. The issue is that no two individuals see identical hues, when looking at the same specimen. This sensitivity to specific hues of colors (mixtures of two or more dyes) complicates staining of specimens, as individual pathologists often request different “color balance” in routine stains such as hematoxylin and eosin.

Image analysis does not depend on color hue in general. The majority of image analytic routines work on the individual color planes of the image (typically three planes, RGB, or another color space). For analytic purposes, each image plane is simply an array of numerical values, the values representing the image density at each array point. The image points are referred to as pixels. The simplest detectors provide a minimum of eight bit detection; that is, the digitized values range from 0 to 256. Contrast this with the performance of the human eye: for most individuals, only about 30 different levels of “intensity” can be seen. There are two other significant differences in the performance of the eye versus the electronic detector. The electronic detector (camera) is essentially a linear detector, while the eye is a logarithmic detector. The eye is also an adaptive sensor, with the detectable range varying depending on the total brightness. The electronic detector is not adaptive, with a fixed gain for a given acquisition. Since the eye and the electronic camera “see” such different aspects of the specimen, it is clear that one cannot depend on visual assessment of an image for suitability for image analysis.

Digital cameras attached to microscopes must compensate for the effect of microscope optics and illumination systems. The camera adapter must provide a high-quality image to the camera, and this image should not contain optical distortion. The systems available from manufactures of microscopes and of scientific cameras meet this requirement, but caution should be observed if using a consumer camera with an ocular adapter. These simple systems often contribute considerable optical distortion, particularly at the edge of the image, and often do not provide the user with control of exposure or white balance. Even with the best of systems, there is often a decrease in illumination at the edges or corners of an image. This may be hidden from the user, as many capture programs automatically compensate for this with some type of “field flattening” computation. It is imperative that the user understand the mathematics behind this flattening, to determine if it has any adverse effect on the validity of the image in accurate representation of the specimen. White balance is another consideration. For a microscopic image, any area that does not contain specimen should have a constant numerical value. A “raw” uncorrected image will never achieve this, as the illumination will always decrease toward the edges of the image (a consequence of microscope optics). Most capture programs will correct the image for white balance, and again, the user must understand how this is done. Often, the algorithm used for this correction does not faithfully mirror the specimen itself. One way to check the validity of such corrections is to image the same specimen at different locations (focusing, light intensity, and magnification must not change) and then compare the same specific pixel in the two images. Since the two images will have that pixel at different locations, any nonlinearity contributed by the flattening and white balance computations will be evident as a different numerical result. The more different “positions” used, that is, the more images compared, the better the confidence in the flattening and white balance algorithms.

10.2 CAMERA AND OPTICS SELECTION

There are two main types of electronic cameras, CCD and CMOS. CCD is the more mature technology and is often cited as superior for scientific image analysis. This is because many CCD devices have larger physical pixels and can hold a larger signal. This becomes important when the image being collected is fluorescence, and the image may be collected over a significant time frame. CMOS devices originally produced images with significant noise, particularly with the long exposures in some fluorescence work. For brightfield microscopy, this is not an issue, and CMOS devices can produce high-quality images. Sensitivity of the camera is not an issue for brightfield microscopy, as all modern cameras are sensitive enough that some means of reducing illumination is generally required. This can take the form of a neutral density filter inserted in the light path when taking an image, but a different filter will be required for each objective. Two other options are individual neutral density filters fitted to each objective lens, or a gain change in the camera itself as the illumination level changes. These type of corrections are often found in integrated camera solutions provided by microscope manufacturers. If automatic gain algorithms or devices are fitted to a camera, the user will need to determine if this addition affects the results of an image analytic result. As an example, if one measures the density of a cell nucleus (after a stoichiometric stain such as Feulgen), one should expect to get the same result from the same nucleus at two different magnifications, assuming both magnifications can meet photometric requirements.

Cameras are available in many different pixel densities. It is generally assumed that the higher the pixel number, the better the images produced. This is not always the case if the camera is a color camera rather than a monochrome camera. There are two basic ways to get color images using a digital camera. The most common implementation is the single-chip color camera. In these devices, a single detector chip is used. To obtain color, each group of four pixels (in a square pattern) are covered with a set of colored filters. Two of the pixels will have a green filter, one will have red, and one will have blue. Such an arrangement is referred to as a Bayer filter pattern. To obtain a color image, the internal electronics of the camera average the results from each of the color filtered pixels and then use this average to set the value for the color plane under the other colors. In other words, the “red” value will be placed in the red plane under the “blue” and the two “green” pixels, even though only one of the four pixels was used to detect “red.” The end result is that for a Bayer-filtered, single-chip camera, color resolution is essentially one quarter of the number of total pixels. In practical terms, a Bayer-filtered camera of 1000×1000 pixels will have the approximate resolution of a standard television image (640×480). For additional details of Bayer-filtered cameras, see Floyd⁶ or Russ.⁷ While it may be possible to utilize a single-chip color camera for determination of geometric properties of an image (assuming the manufacturer will reveal details of the internal color plane manipulations), these type of cameras are entirely unsuitable for photometric studies.

Photometry requires either a monochrome camera or a three-chip color camera. Both are capable of generating color images. For a three-chip camera, the image is split using a prism into three identical images. Each is then directed to a detector chip that is covered by either a red, a blue, or a green filter. While this type of camera can generate excellent color images, there may be issues in using such a camera for photometric determinations. That is because a separate detector is being used for each individual color, and the pixel that sees a single spot on the specimen may not have identical gain or identical linearity in all three of the chips. For photometric accuracy, by far the better solution is to use a monochrome camera in the “three-shot” mode. This means that three separate images are collected, each through a separate filter. The camera then can combine these three separate color planes into a composite color image. Because the same pixel is used to detect each individual spot in the specimen for each color, issues of gain and linearity between color planes are no longer of concern. The three images can be collected quickly, using either a mechanical filter wheel in the light path or in the camera itself, or using a switching liquid crystal display (LCD) to switch colors. LCD filters are typically built into cameras, but can also be used in the microscope light path.

The number of pixels required in a camera is also a function of the magnification required in the image to define the image elements of interest. Photometric accuracy requires that a detection system obey the Beer–Lambert law of photometry. Basically, this law states that one can determine the amount of material in a solution (read specimen) if the material is a true absorber of light and is homogeneously distributed. This statement should cause any microscopist pause, as the reason we can see detail in a specimen is because it is not homogeneous! After all, homogeneity would mandate that the entire specimen would have zero detail. Since we have already stated that many have generated detailed photometric data from stained specimens, the question is how is this done? The simple answer relates to something that microscopists know, yet do not think about. If the detector element (i.e., a pixel) sees an area of the specimen that is smaller than the resolving power of the optics, then by definition, the area is homogenous. In practical terms, this means that the magnification used to collect an image for photometric analysis is determined by the number of pixels in the camera detector. For a camera of 1000×1000 pixels, reasonable data can be collected using a $20\times$, $40\times$, or higher magnification lens. These combinations give a pixel resolution of approximately 0.45μ , and with normal microscope use with white light, higher point resolution cannot be achieved. While it would seem that it would be easier to use low magnifications with a given camera, such is not the case. For an objective of $4\times$, one would need a camera target of approximately 4000×4000 pixels. While it is possible to subject an image collected at lower pixel resolution to image analysis, and obtain a result, the result will not be photometrically valid. If the requirement for homogeneity at the detector (individual pixel) is not met, an error of up to 40% can result. Rather than demonstrate this mathematically, it is easier to describe this error with a

simple, intuitive example. Imagine a container filled with water, with a light meter on one side and a light source on the other. Now place a small, stoppered bottle of ink in the container. Note the reading on the light meter. Now unstopper the bottle of ink, and allow the ink to diffuse through the container of water, and note the light reading. It will be significantly lower than when the ink was contained in a small volume, yet in both cases, the amount of ink in the larger container was the same. The only difference in the two instances is the *distribution* of the ink. In microscope photometry, this is referred to as distributional error and is the reason for the requirement for homogeneity at the pixel level. Details of the requirements for accurate microscope photometry can be found in Pillar.⁸

10.3 IMAGE FORMAT

There are numerous formats for storage of images in computers. Many microscope manufactures who supply integrated cameras have some type of proprietary format. It is always best to store images in a generic format that can be used with a variety of image analytic and image display systems. The single most important element in image format is that the format must not alter the data in any way. It is perfectly permissible for the “header” or information portion of the image format to vary, but the actual pixel data in each image plane must not differ from the original data collected. This means that one should not use any image formats that compress image data unless it can be clearly demonstrated that the original data is not changed. One example of image compression that does not change the original data is called run-length encoding. Essentially, this gives a pixel value, and then a number indicating how many times that particular value is repeated before there is a change. While this type of encoding can be very effective in certain types of images, such as line illustrations, it generally results in larger images for microscopic images, simply because there are so many changes from one pixel to the next (except for holes, there are seldom long stretches of identical pixel values).

The format that is most often used for storage of images for quantitative purposes is the TIFF format. This format does not alter the pixel data, and permits storage of information about the image in the “header” portion of the file. Common image formats such as JPEG should not be used for quantitative purposes. JPEG is a “lossy” compression technique. The original JPEG used a complex cosign transform to compress the image, and the newer JPEG 2000 uses a wavelet transform. While the newer version yields better images, it is still not suitable for quantitative photometric results. With the decreasing cost of data storage, reducing size of images is no longer a major concern. If one must reduce image sizes, and still perform photometric assessment of images, then the image should simply be cropped to the area of interest. Smaller images require less storage space than large images.

10.4 IMAGE DISPLAY

Much is made of color display of images. With modern computer systems, colors can be altered to almost anything desired. The unfortunate truth is that monitors used to display images all have different characteristics. While it is possible to calibrate monitors (as in the graphics arts industry), the cost of doing this is simply too high for routine use in the laboratory. Even if every monitor were calibrated, the specimens themselves, due to either deliberate stain variation or to pre-analytic factors, will have significant color variation. Due to decisions made by programmers of capture software, few systems duplicate the same colors as others, even when the identical specimen is imaged on each different system and displayed on the same monitor. These same factors also affect achieved density of stain and must be controlled if photometric data are to be meaningful (Fig. 10.1).

Many modern image capture cameras have very high pixel density, and the general perception is that more pixels translate to better images. While this may be true, it does not necessarily mean that the *displayed* image will be better. If the image itself has more pixels than the display resolution, the computer operating system will automatically scale the image for the monitor. This scaling may compromise the displayed image. If the user is aware of this, and turns off the scaling, then the entire image cannot be displayed at once, and the user will need to “scroll” the display to see all of the image. While the display of an image should not affect the information content of the image, nor the ability to extract data from the image, often the user will judge image quality based on the displayed image, and may reject an image based on that display, even though the image itself is perfectly suitable for analysis. This is an example of where a subjective evaluation of the displayed image may be misleading, since the displayed image may not be a true representation of the actual image.

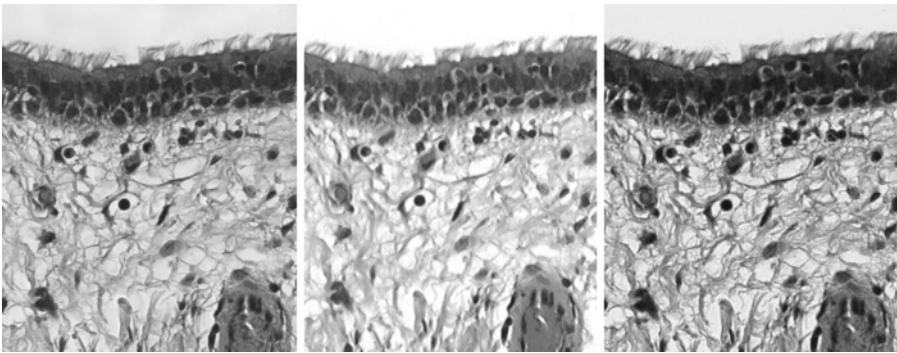


Figure 10.1 The same specimen imaged by three different commercial imaging systems. Note the significant color variations in each image, as well as the shading of the background. There are also visible differences in resolution between the systems, even though total magnification is the same in all images. See color insert.

10.5 IMAGE ANALYSIS

Image analysis routines can operate on individual pixels (point processes) or arrays of pixels (geometric processes). Extraction of data such as areas or intensities are examples of point processes. Geometric processes are used to correct for distortions such as those created by lenses, or to correct two image areas that are going to be stitched together into a larger image. Point processes often involve image transforms or convolutions, which are used to perform operations such as sharpen, define edges, and integrate areas. An explanation of image transforms can be found in any text on image analysis.^{9,10}

A key event in analysis of an image is the isolation of the portions of the image of interest. For example, if the area of interest is in cell nuclei, then a first step of analysis would be to isolate the cell nuclei. Such an operation is referred to as *segmentation*, and after such an operation, a new image would be created that would have only cell nuclei present. In this new image, one could then determine the total number of nuclei present, and assuming the image had been calibrated to a magnification standard, the actual area of the nuclei (for microscopic objects, in microns). The accuracy of such area measurements depends on the accuracy of the calibration and on the accuracy of the segmentation. It should be well understood that many segmentation algorithms are based on edge detection of objects within the image, and that this edge detection is highly dependant on determination of a particular gray level (threshold). In studies depending on segmentation, great care must be taken to insure that identical parameters are used to analyze each image, and that each image has identical properties (threshold) at the same location with respect to objects being segmented. Another consideration is whether the specimen being imaged is a cytological specimen, containing intact cells, or is a histological section. Sectioned nuclei will display a distribution depending on how much of the nucleus is included in the section. While many authors have stressed that one can simply measure sufficient numbers of sectioned objects and obtain a statistically valid result, this assumption is based on a random distribution of the objects being measured. One reason we can recognize one tissue from another in microscopic specimens is because tissue elements are not randomly distributed. Any data generated from sectioned material should be carefully examined to recognize the limitations of the specimen itself (Fig. 10.2).

Another complication of segmentation is the typical stains used in histopathology. As mentioned previously, the traditional stains used in histology were developed for the human eye. Unfortunately, many of these stains create significant problems for segmentation algorithms. A specific example is the hematoxylin and eosin (H&E) stain. The red and the blue colors of this stain overlap for approximately one-half of their total absorbance spectra, and their spectra are very broad.⁶ When such overlapping spectra are collected as a monochrome image, there are a large number of “gray” values that are

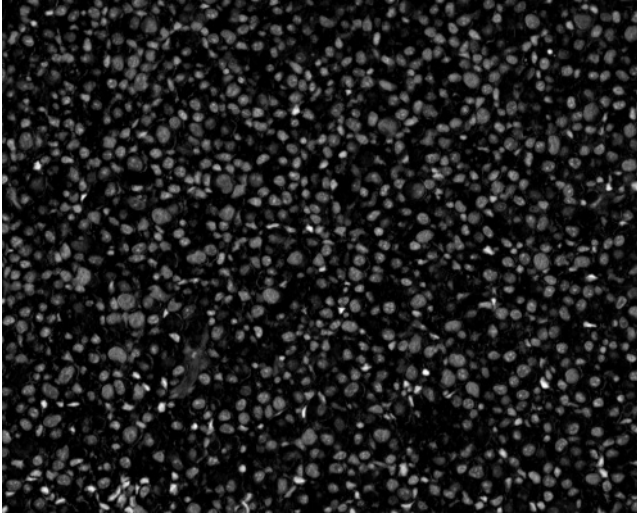


Figure 10.2 An immunostained specimen, after segmentation for the stained cell nuclei. In this specimen, one can either count the nuclei or divide the total segmented white area by an “average” nuclear area (taking into account few nuclei are full size).

difficult to assign to either “blue” or “red” areas of the image, and this complicates segmentation. This is not a new problem, as it was often seen in H&E photomicrographs when these were reproduced as black and white illustrations. An old trick that was used by some to avoid this limitation was to substitute another dye for the eosin, such as Orange G. This dye has less overlap with hematoxylin than does eosin, and the result is a black and white photograph with the appearance of sharper detail because there are fewer “muddy gray” values over much of the image. The same approach can be used in electronic imaging, but it is not necessary to alter the actual stain used. What is needed is to collect the images themselves through very narrow band filters, in areas of the spectra where the dyes within the specimen do not overlap. For a typical H&E stain, this would mean collecting an image through a narrow band blue, a narrow band red, and a narrow band green filter. These three images can then be used to generate a full color display image, but the individual images will be much easier to analyze using image analysis, simply because segmentation will be much more precise. Such images, when displayed as full color, will also appear “sharper” to the eye, simply because there are fewer overlapping color values in the image.

Assuming a calibrated, segmented image, it is easy to determine the area of segmented portions of the image. For reasons already stated, photometric (intensity) measurements must be made at magnifications that provide homogeneity at the pixel level of the detector. However, areas can be determined at any magnification. Caution must be observed in that the objects being measured for area should be of sufficient size to contain enough pixels that

the measurements are not compromised. Remember that a pixel is generally a square, and if the object being measured does not contain enough pixels, then the actual area determined may have a significant error. This is because the area that is determined is made of total number of segmented pixels, and the outline of this area will follow the square pixel boundaries. Ideally, one wants enough pixels in the object that approximately the same number of pixel corners stick past the segmented edge as just reach the segmented edge from within. Since most image display programs permit "zooming" of the image, it is easy to zoom the image until the individual square pixels can be seen. This can give confidence that sufficient pixels overlay the object(s) of interest to generate reliable area data.

Photometric accuracy is an absolute requirement for any measure of amount of material staining. There are also significant specimen requirements. The selection of the stain to be used is critical if one wishes to measure the amount of material staining. As already stated, any stain used for photometric quantitation must be a true absorber, and the stain must have some constant relationship (stoichiometric) to the material being measured. For immunohistochemistry (IHC), the most common stain used in U.S. laboratories is diaminobenzidine (DAB). DAB has a long history in histochemistry and was an early substrate used for demonstration of the peroxidase enzyme. Subsequently, it was employed in electron microscopy because it provides an electron dense *particle*. Since it is a particulate, DAB cannot be used as a quantitative photometric stain, even though the pathology literature is replete with papers claiming it can be so used. In fact, because the end product of DAB staining is a particulate, as the concentration of deposited DAB increases, the amount of light scattering increases. As light scatter increases, more and more of the light is scattered *outside* the capture cone of the microscope objective. So any signal is by default very nonlinear. It is true that there are techniques that can be used to measure light scattering particles, and this technology was well developed for grain counting in autoradiographs. However, these techniques fail with DAB because DAB particle size seems to change, either by change of particle size or by aggregation of particles, with age of the DAB solution, and with source of DAB. Reflection measurements require a constant relationship between number of particles and amount of reflected light. In the case of DAB, this relationship is constantly changing as the solution ages, which may account for the change in color of DAB solutions with time.

IHC stains using peroxidase as a detection system can be subjected to photometric quantitation if a true absorbing chromogen is used. Aminoethylcarbazole (AEC) is one example, and there are a few others. The chromogens normally used with alkaline phosphatase enzyme systems are also true absorbing dyes, and can be used for photometric quantitation. The issue of sectioning geometry still applies however, and because of the size of either cell nuclei, or cells, measuring the total amount of stained material within the entire structure is exceedingly difficult. A better strategy is to determine the

concentration per unit volume, and this can be done by segmenting the stained object, determining the total area, then performing a photometric measurement on a number of small areas of the segmented object. By averaging the photometric measurements, a concentration per unit area can be determined. Still, numbers must be reported with caution. Such area concentrations are based on sectioning assumptions, most of which are invalid. The major assumption is that the section is a particular thickness, and that thickness is determined by the setting of the microtome used to produce the section. This is not true in practice. Almost all paraffin blocks are cooled prior to cutting, and if one examines a “ribbon” of sections on a water bath with a critical eye, it will be seen that every ribbon is actually a very long wedge, with each section increasing slightly in thickness over the one just before. From one end to another of the ribbon, there may be a variation of up to a micron or more in thickness. This degree of variation will have a significant impact on calculations of concentration per unit area. There are currently no embeddable and sectionable fiducials that could be included in each paraffin block that would permit correction for this error.

It is much easier to use IHC stained specimens to determine numbers of stained objects, and this is particularly the case for those antibodies targeting cell nuclei. One reason this is an easier task is that the question generally asked is: “What percentage of nuclei are positive?” The problem is resolved to doing two segmentations: first, nuclei that are stained with the IHC stain are segmented, and the total number of objects are counted. Second, all nuclei are segmented and counted based on a general counterstain such as hematoxylin. The “percent positive” is simply the percentage of IHC positive nuclei (number) compared to the total number of nuclei seen by the counterstain. While this sounds simple, there are a number of specific decisions that must be made, and these can affect the result. Examples include how dense must the immunostain be to count a nucleus as positive, and how much of a nuclear profile must be included in the section to be counted as a “nucleus.” In some specimens, nuclear profiles overlap to the extent that it is difficult to separate one nucleus from another. As long as the same decisions are used for every specimen, the data will provide excellent comparisons between specimens. It must be understood that the data is not absolute, and may not transfer to another laboratory using different decision points, and more particularly, different staining parameters.

General cytoplasmic stains are much more problematic in that a single section never contains an entire cell, even for very small cells. The only strategy that can be used for these stains is to determine a concentration per area, given that there will be variation due to the variation in section thickness and perhaps also to cytoplasmic localization of stained product. And there is simply no accurate way to determine the total amount of material within a single cell cytoplasm, unless one has a cytological (intact) specimen.

There is considerable interest in measuring the concentration of cell surface markers, such as *Her2/neu*. For image analysis, this is a very difficult problem.

Very little of the cell is contained within a single section, and cells are cut at all orientations. The only area of such preparations that can be used for even a simple concentration per area type measurement are those areas of the cell surface where the stained material is the most narrow and is the darkest. This geometrically is the part of the cell membrane surface that is cut at right angles to the surface. Any other area is cut at some angle, and this will decrease the signal and give variable results. Because these various factors have seldom been taken into account in published studies, the data in the literature are highly suspect.

10.6 MULTIPLE STAINS AND COLOCALIZATION

Performing immunohistochemical staining using multiple primary antibodies and different chromogen detection systems has always been a goal. The work of van der Loos¹¹ provided reliable, systematic approaches to achieving this. Commercial detection kits are now available for two-color immunostaining. Recently, van der Loos and Teeling¹² have published a simplified method for multiple staining, which may encourage widespread adoption of these techniques.

The choice of chromogens is crucial in multiple stain protocols. One needs sufficient contrast between the two final colors to allow discrimination of the different colored signals. When one uses the two most common chromogen pairs, with peroxidase and alkaline phosphatase, the two colors are generally brown and red. The result may be obvious if the two epitopes being evaluated are expressed in high concentration. However, if one of the two epitopes is significantly lower in concentration than the other, it may be impossible to visually evaluate the stained result. If the two chromogens are of distinctly different colors, such as brown (or red) and blue, then the eye may be able to evaluate the result. Even with a high color contrast between the two chromogens, subjective evaluation may be very difficult when one epitope is expressed at high concentrations and the other at low concentrations.

Spectral imaging provides a way to separate multiple colors in a stained specimen reliably and accurately. Spectral imaging is also referred to as multispectral imaging and in some cases hyperspectral imaging. The approach is to collect sufficient information from the specimen that one can reconstruct the absorption profile for each colored material in the specimen. Using this information, each of these stained materials can be separated (segmented) into a separate image. Using a spectral approach results in extremely efficient segmentation and high accuracy, assuming the stain is very specific and is performed in a manner that does not generate nonspecific background staining. The ease and precision of segmentation with spectral approaches simplifies image analysis sufficiently that one can often perform accurate segmentation at lower magnifications than can be achieved using standard color or monochrome images.

Once an image is segmented into the various colors seen in the specimen, the image can then be reconstructed using false colors or pseudocolors. As an example, assume a specimen has been stained for some epitope using a DAB chromogen. Ordinarily, such specimens are counterstained using a light hematoxylin so as not to obscure any potential light positive immunohistochemical staining. Using a spectral approach, the IHC stain can be followed with a standard H&E stain and still not obscure any of the IHC stain detail. This is accomplished by separating the stained image into three separate images, DAB, hematoxylin, and eosin. By choosing specific pseudocolors for the reconstructed image, one can generate a result that looks like a routine H&E stain, and then display the DAB signal in a highly contrasting color, such as green. The result is a specimen that is easy to interpret based on traditional morphology and yet has an easily seen IHC stain signal. There is no worry that the red stain of eosin will hide any of the brown DAB staining (Fig. 10.3).

Spectral imaging also makes multiple stain signals easy to separate and also makes cases of colocalization easy to identify. This capability comes from the ability to pseudocolor the individual stain results and then overlay them. If the user chooses pseudocolors such as red and green, when overlain in a composite image, any areas of colocalization will appear in a contrasting color—yellow (this is a consequence of the way color is generated on display monitors). This simple but powerful technique makes image analysis quite easy, as one can determine the areas of the specimen occupied by each of the positive signals, and also the area of the specimen that expresses both signals simultaneously. Spectral imaging can easily separate four or more colors in brightfield microscopy and has been used to separate up to seven specific fluorescence markers.

10.7 SPECIMEN PREPARATION

It is clear that pre-analytic factors greatly influence results obtained in histopathology laboratories. Among the factors that have significant impact are time from removal to beginning of fixation, type of fixation, and length of fixation.¹³ For specimens intended for image analysis, every aspect of specimen handling must be rigidly controlled if the results are to be compared from one specimen to another. Other chapters in this book provide additional details on some of these variables.

10.8 STAINING

Immunohistochemical staining is very different from routine chromogenic stains such as H&E. To utilize IHC stains for quantitative purposes, the user must understand these differences and perform the stain in both a standardized and repeatable manner. As has been described, image analysis

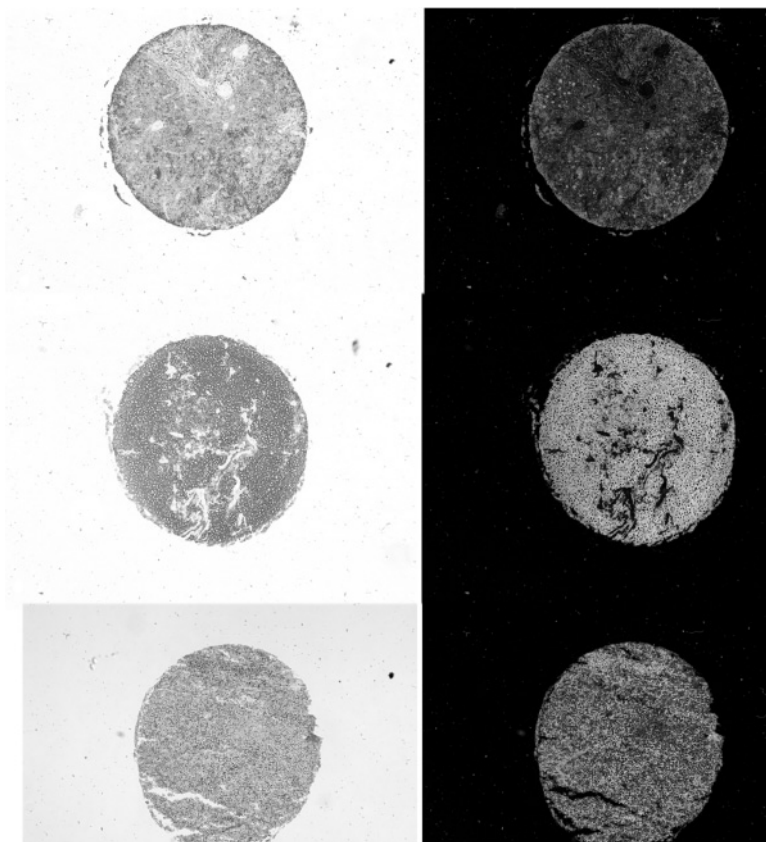


Figure 10.3 Three pairs of images from a single tissue microarray (TMA) block, stained for CD20 using peroxidase-DAB and CD43 using alkaline phosphatase-fast red. In the brightfield images, it is difficult to distinguish the red staining from the brown staining, particularly when one or the other is at low concentration. After spectral imaging and pseudocolor display, the distinction is easy. This example also illustrates the use of pseudocolor to show areas of colocalization as yellow. Specimen courtesy of Dr. C. Taylor. See color insert.

is exquisitely sensitive as compared to the human eye, and minor variations in technique that would never be recognized in visual assessment can provide analytical results that are so variable as to be impossible to evaluate.

IHC stains as used for brightfield microscopy are “sandwich” procedures, consisting of sequential staining steps, each of which contributes amplification of the original signal. *Specificity* of the stain is provided by the primary antibody. The primary antibody also contributes to the *sensitivity* of the stain, but sensitivity is also the combination of each of the other steps of the stain protocol. It is critical that the steps used to amplify the signal in the stain process are not overdone, as these amplification steps can easily produce excess back-

ground that significantly alters image analysis segmentation routines, preventing repeatable results.

Image analysis is often used to evaluate IHC stained specimens, but the results are highly variable, because the control systems used in IHC staining do not monitor the many variables in the stain process. Stain controls have been used in image analysis for many years in histochemical staining. These controls are similar to the “known positive” controls used in IHC staining but generally are constructed using known biological materials such as isolated cells, cell nuclei, or purified materials deposited onto control slides to provide a range of reactivities for each control. An example of this approach is the deposition of cell nuclei of differing DNA content which are used as standards in ploidy analysis studies.

Another potential variable in IHC staining is the use of polyvalent secondary antibodies in the detection process. Polyvalent secondary antibodies were introduced as a way to avoid a common laboratory mistake, which was using an inappropriate secondary antibody for a particular primary antibody in the detection process. Polyvalent secondary antibodies are simply mixtures of two or more antibodies directed against different *species* used to generate the primary antibody. In current practice (2008), this generally means that a secondary antibody cocktail consists of a mixture of goat anti-rabbit and goat anti-mouse. The user *assumes* the manufacturer of the detection system has constructed this cocktail to provide essentially identical sensitivity and specificity to primary antibodies of either species. The user also generally assumes that there are no lot-to-lot variations in these cocktails when obtained from the same source.

All antibodies age and therefore have a specific shelf life. Aging may be different for different antibodies, and real aging may be quite different from the expiry dates printed on containers of antibodies. Mixtures of antibodies as are found in secondary antibody cocktails may show distinct aging differences. In other words, over time, one of the species in a secondary cocktail may age at a more rapid rate than the other(s). This would result in a significant decrease in *sensitivity* for that particular species of primary antibody. A user performing IHC stain runs with multiple tissues, and using primary antibodies from more than one species, must utilize primary controls for each species of primary antibody to detect a change in one of the components of the secondary antibody cocktail.

The significant difference in sensitivity of evaluation between subjective visual assessment and image analysis compounds recognition of procedural stain issues. The relative insensitivity of the eye to density changes and the accommodation of the eye to intensity obscure this major source of stain variation. The routine use of “positive” controls derived from actual specimens may also be misleading, as the user in general has no way to determine how positive a “positive” specimen is. If the positive specimen is highly positive, does this bias the procedure toward highly expressing specimens, perhaps at the expense of sensitivity of the procedure for low expressing specimens?

10.9 IHC STAIN CONTROLS

The concept of a control is a device or mechanism to detect variations in the monitored procedure that are significant enough to produce a detectable result. In the case of IHC stains, the inability of subjective visual assessment to detect significant stain results has obscured this major source of variation for most users. This author has worked with quantitative evaluations of histochemical stains for many years. Initial attempts at quantitative evaluation of IHC stains clearly indicated that current practice does not provide adequate control of IHC stain protocols.

The IHC stain procedure is a multistep staining protocol, the various steps intended to provide amplification of stain results. Therefore, a control system must include elements to control each step of the stain *process*. Such a control should also include a range of reactivities, and that range ideally would encompass the total expression range expected for the measured component. The control should also monitor each step of the multistep protocol. This author has devoted a number of years to this concept, resulting in a patented control for multistep staining processes.¹⁴ Such a control provides sufficient information to monitor every IHC stain run, and when the control is evaluated quantitatively, normalization of data from one stain run to another within the same laboratory, and even between laboratories. A process control is a measure of the stain protocol and does not take the place of a control for the primary antibody. While the primary antibody control should include range of expression level detection, a different primary control must be present for every primary antibody used in a stain run (Fig. 10.4).

The utility of a process control was demonstrated by constructing a series of slides containing mouse and rabbit serum. Five rows of a series of dilutions of each species were applied to slides, and these were then stained in mixed cocktails of goat anti-rabbit and goat anti-mouse secondary antibody, followed by routine peroxidase-DAB visualization steps. The mixed cocktails were constructed as variable mixtures to demonstrate the ability to detect lack of sensitivity of either of the cocktail components. The results of this study were published in abstract form.¹⁵ Clearly, this approach can provide a way to monitor changes in secondary antibody cocktails, either due to manufacturing variables or differential aging of components within the cocktail (Fig. 10.5).

The process control is constructed by printing of various concentrations of defined biological materials onto glass slides. While printing of materials onto glass slides has been done for a number of years, particularly in the field of DNA and protein arrays, the technology is not as mature as might be assumed. DNA and protein arrays are invariably assessed using fluorescence technology, and this means that spot morphology is not of concern. Reproducibility of spots is an issue, and because there are variations in these spots, DNA and protein arrays typically replicate the same spot multiple times. During analysis, these replicated spots are all measured, and then averaged, as a way to

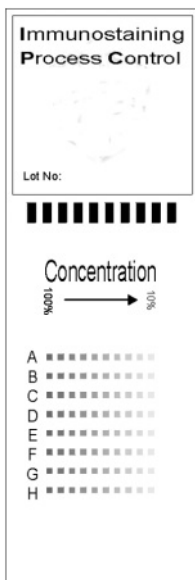


Figure 10.4 Schematic concept for a process control slide. The number of rows of analytes printed onto the slide depend on the number of process steps which need to be monitored.

compensate for variable spot content. The spot sizes used in DNA and protein arrays are also very small and cannot be evaluated by eye.

In the case of a process control for IHC stains, the spots should be large enough to be seen by eye, and subjectively evaluated by the unaided eye. This places severe constraints on the required size of spots and the morphology of the spots. For easy visual interpretation, the spots should be at least 1 mm in diameter. The spots also must be visually homogeneous to the user. Producing spots of this size and consistency is technically challenging, as is reproducibility of printing control spots. Not only must a consistent dilution gradient be achieved, but variations from one control slide to another must be very low if the control is to be useful as an image analytic control. As a routine control device, a process control slide would need to be evaluated photometrically rather than visually. Such quantitative evaluation would allow normalization of achieved stain density from run to run and from laboratory to laboratory, assuming both used the same process control and the same evaluation methodology.

As a test of reproducibility of spot printing and staining, a 10 spot by 10 row array (100 spots total) were printed on three slides. All slides were stained in a single staining run, using the same secondary detection chemistry with DAB as a chromogen. While DAB cannot provide accurate photometric measurements, it can provide relative comparisons from one spot to another on the same slide. When the stained control slides were analyzed in this

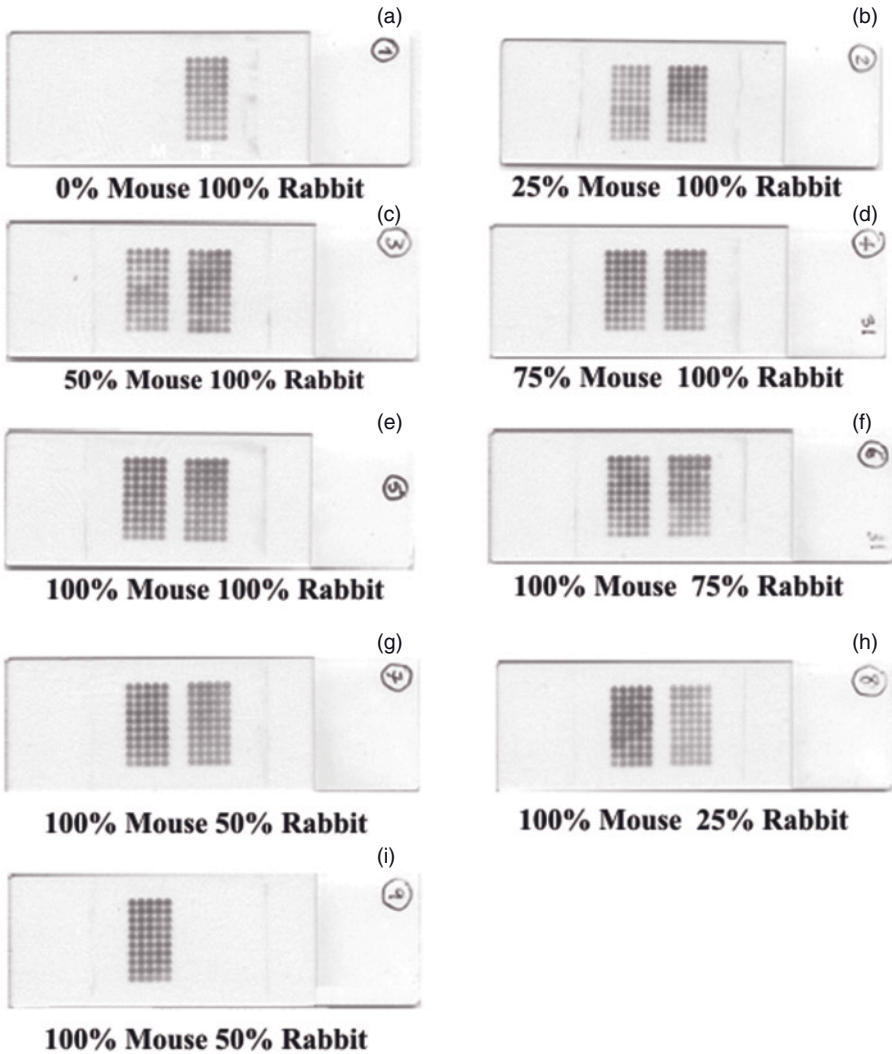


Figure 10.5 A series of slides printed with dilutions of mouse serum and rabbit serum. There are five replicates of each species, and all slides have identical printing. The secondary antibody cocktail was constructed according to the labels on each slide. This demonstrates that a control slide can easily detect sensitivity changes in secondary antibodies.

manner, a variation of less than 1% (out of 256 gray levels) was seen from spot to spot on each slide (Fig. 10.6).

A process control is just that, a control for the stain process. While the concept can be extended to the primary epitope also, this may not be practical in all cases. Purified protein epitopes are very expensive, and adding a dilution series of primary epitope to a control slide would create a unique and possibly

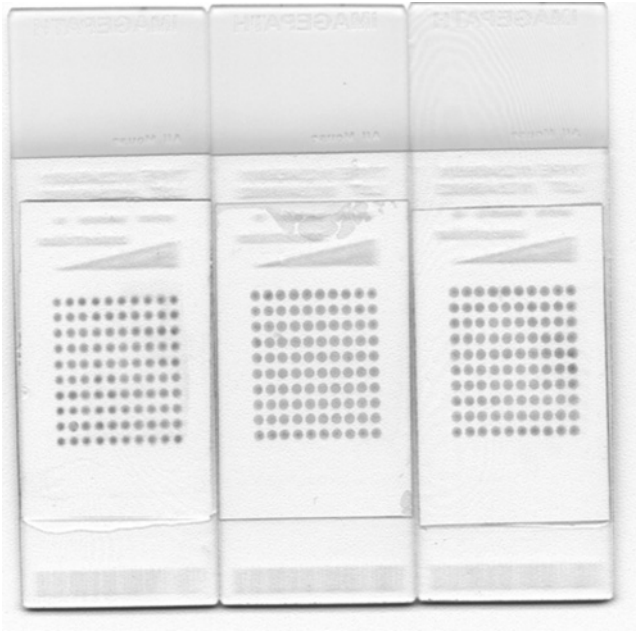


Figure 10.6 Three slides printed with the same concentration of protein in each dot, with 100 dots per slide to demonstrate consistency of printing and homogeneity of printed dots.

expensive control. A primary control has been created and used in one of the College of American Pathologists proficiency trials.¹⁶ The number of dilutions used was quite low (four), and the results of the proficiency trial was ambivalent.

Another approach to primary control would be to place a series of small microarray fragments obtained from specimens of known but varying degrees of positivity for the marker of interest. These could be added to the process control so all elements of the stain process could be controlled with one control device. The issue here is that for many epitopes of interest, the specimens must be subjected to antigen retrieval procedures prior to staining. This means that the process control must also withstand the antigen retrieval process; that is, the applied protein dots must be resistant to both the temperature and the pH used in the retrieval process. Preliminary results suggest that this can be achieved, and under some conditions, we have achieved spot stability using pH 6 buffers and high temperatures for up to 1 h. We are currently investigating the stability of printed proteins under a variety of antigen retrieval conditions (Fig. 10.7).

Our use of the process control slide during its development has already surprised us by demonstrating significant variations in lot-to-lot commercial detection systems. While we have yet to perform a systematic evaluation of

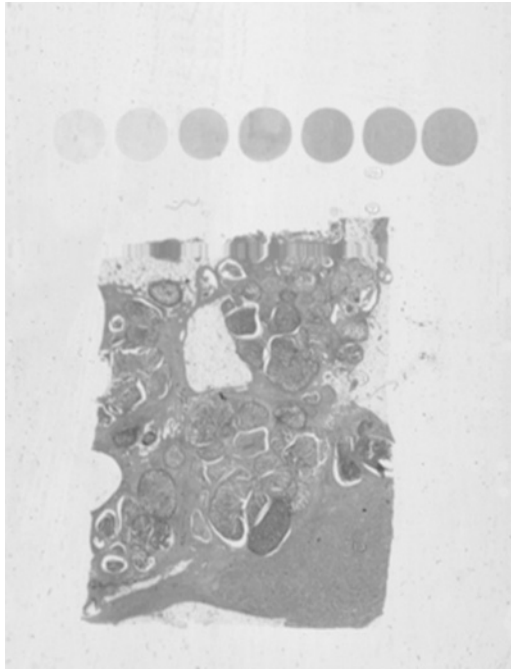


Figure 10.7 Demonstration of protein dots and tissue on single slide. This specimen was subjected to antigen retrieval prior to immunostaining, and a complete hematoxylin and eosin counterstain.

all detection systems, it is clear this should be done. We have also observed curious aging effects in secondary antibodies. These observations need additional study and confirmation, but users of secondary antibodies should watch for inappropriate species cross-reactivity as these antibodies age.

10.10 CONCLUSIONS

Image analysis offers the potential to significantly reduce or eliminate the variation seen in IHC staining and evaluation. The use of image analysis must be standardized, and the actual process of staining must be standardized. Selection of appropriate image acquisition techniques can materially assist in the actual process of analysis. Because of the unique nature of IHC stains (internal amplification steps), controls for the stain process must be included if the quantitative result is to be meaningful. As quantitative procedures are developed, users will need to reach consensus on how data are collected and interpreted, as well as the clinical significance of the result.

REFERENCES

1. Caspersson T. Über den chemischen Aufbau der Strukturen des Zellkernes. *Skand. Arch. Physiol.* 1936; 7 (Suppl. 8): 1–151.
2. Feulgen R, Rossenbeck H. Mikroskopisch-chemischer Nachweis einer Nucleinsäure von Typus der Thymonucleinsäure und die darauf beruhende Elektiv Färbung von Zellkernen in mikroskopischen Präparaten. *Z. Physiol. Chem.* 1924; 135: 203–248.
3. Swift H. The deoxyribose nucleic acid content of animal nuclei. *Physiol. Zool.* 1950; 23: 169–198.
4. Wied GL. *Introduction to Quantitative Cytochemistry*. New York: Academic Press, 1966.
5. Wied GL, Bahr GF. *Introduction to Quantitative Cytochemistry II*. New York: Academic Press, 1970.
6. Floyd AD. Quantitative data from microscopic specimens. In *Theory and Practice of Histological Techniques*, 6th edition, ed. J Bancroft and M Gamble, pp. 641–659. Philadelphia: Churchill Livingstone, 2008.
7. Russ JC. *The Image Processing Handbook*. Boca Raton, FL: CRC Press, 1995.
8. Pillar H. *Microscope Photometry*. New York: Springer-Verlag, 1977.
9. Baxes GA. *Digital Image Processing: Principles and Applications*. New York: John Wiley and Sons, 1994.
10. Gonzalez RC, Woods RE. *Digital Image Processing*. Reading, MA: Addison Wesley, 1993.
11. Van der Loos CM. *Immunoenzyme Multiple Staining Methods*. Oxford, UK: Bios Scientific Publishers, 1999.
12. Van der Loos CM, Teeling P. A generally applicable sequential alkaline phosphatase immunohistochemical double staining. *J. Histotechnol.* 2008; 31: 119–127.
13. Yaziji H, Taylor CR, Goldstein NS, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* 2008; 16: 513–520.
14. Floyd AD. Quality control of assays. U.S. Patent 7,271,008 B2, September 18, 2007.
15. Floyd AD, Yaziji H. Process control for standardization of multiple step staining protocols. *Arch. Pathol. Lab. Med.* 2008; 132: 870–871.
16. Vani K, Sompuram SR, Fitzgibbons P, et al. National HER2 proficiency test results using standardized quantitative controls: characterization of laboratory failures. *Arch. Pathol. Lab. Med.* 2008; 132: 211–216.

PART III

TISSUE/CELL SAMPLE PREPARATION

CHAPTER 11

TISSUE CELL SAMPLE PREPARATION: LESSONS FROM THE ANTIGEN RETRIEVAL TECHNIQUE

SHAN-RONG SHI and CLIVE R. TAYLOR

11.1 ANTIGEN RETRIEVAL (AR) DEVELOPMENT BACKGROUND— A CONTINUUM OF PAST, PRESENT, AND FUTURE

Development of the AR technique in 1991¹ was based on efforts of earlier pioneers who were searching for new approaches to render the immunohistochemical (IHC) staining technique more suitable for use with routine formalin-fixed, paraffin-embedded (FFPE) tissue sections.²⁻⁸ The major reasons that FFPE tissue sections are so important for IHC relate to several factors: the long history of use of formalin as the standard fixative; the fact that pathologists have honed their morphologic skills by morphologic examination of FFPE tissues; and not least, that all stored or archival tissue is in the form of FFPE blocks. Because of the long history of the use of FFPE tissue sections in histopathology, most of the criteria for pathological diagnosis have been established by the observation of FFPE tissue sections stained by hematoxylin and eosin. Additionally, a great number of FFPE tissue blocks, accompanied by known patients' follow-up data, have been accumulated worldwide, providing an extremely valuable resource for translational clinical research and basic research that cannot easily be reproduced. The possible advantages of FFPE tissues in terms of preservation of both morphology and molecules in cell/tissue sample are therefore under active exploration in comparison to other fixatives, and there is a growing body of literature demonstrating successful application of FFPE tissue samples for molecular analysis (see Chapters 3 and 19 for detail).

Over the last three decades, multiple strategies have evolved to address this critical issue, all having the goal of rendering IHC staining more readily and more universally applicable to FFPE tissues. In the beginning, the

development of more sensitive detection reagents, coupled with the generation of antibodies against formalin-resistant epitopes, made some limited progress, but the range of antigens that could be demonstrated reliably and with acceptable reproducibility remained very limited. One result was an intense search for alternative optimal fixatives, including numerous commercial fixatives, to replace formalin. However, to date, no ideal fixative has been found that is broadly effective for IHC. In addition, those new fixatives that have been offered do not closely reproduce the morphologic detail achieved by formalin fixation. One major consequence of this last observation is that changing fixatives, away from formalin, may also mean changing the histopathological criteria employed for diagnosis. While not impossible, such a massive “retraining” represents a serious logistical obstacle. A corollary, not often recognized, is that changing to a new fixative, and retraining to a “new morphology,” eventually devalues the utility of the huge collection of archival FFPE tissues that are formalin-fixed, and display “traditional morphology.” This problem has greater significance in some areas of pathology than others but is not insignificant.

In addition, in spite of extensive experimentation, it has proven difficult, if not impossible, to find a universal fixative that can preserve all proteins and at the same time preserve nucleic acids.

As a result, quite early on, some investigators concluded that a more productive approach might be to attempt to undo, or reverse, some of the effects of formalin fixation, that is, to “unfix” the tissues by additional manipulations of the FFPE sections. Enzymatic digestion was one of the first approaches to encounter some success.^{7,9} The advantages of this approach, as opposed to attempts to develop an entirely new fixative, are significant, and include maintaining access to archival “banks” of FFPE tissues and associated clinical outcomes data. The common goal was to identify a method that retained the utility and validity of 100 years of accumulated morphologic criteria at the same time bridging across to a variety of modern techniques. Along these lines, the authors and others expended considerable thought and effort in searching for a “retrieval” technique that would improve the applicability of immunohistochemical, and other techniques, when applied to routinely processed formalin paraffin tissues.^{2-5,9}

There were a number of practical and theoretical issues to be addressed. A key scientific question was whether fixation in formalin modified antigens in a reversible or irreversible manner. To be more specific, was there any theoretical or prior scientific evidence that the effects of formalin fixation on proteins could be reversed, and if reversed, was the structure of protein restored to a sufficient degree for recovery of antigenicity? With these key questions in mind, one of the authors (Shi) spent many days and nights in 1988 searching the chemical literature under somewhat adverse conditions, with a second job as an apprentice in a supermarket, and prior to the increased efficiency of such searches that is afforded today by the Internet and online databases. The answer was finally found in a series of studies of the chemical

reactions between protein and formalin, published in the 1940s.^{10–12} The genesis of this work related to preparation of anti-tetanus sera by injection of “formalinized” tetanus toxin (toxoid) into horses. The tetanus toxin was rendered biologically inactive (nontoxic, i.e., a “toxoid”) for injection by pretreatment with formalin. It was observed that different batches of toxoid had differing efficiencies in induction of antitoxin titers because of overtreatment with formalin, and furthermore, that efficiency could be restored by relatively simple empirical methods, such as boiling. Read antigenicity for efficiency in producing antitoxin and the basis for AR becomes evident.

Specifically, the work of Fraenkel-Conrat and colleagues indicated that hydrolysis of cross-linkages that form between formalin and protein during the fixation process may be prevented under usual laboratory conditions by certain amino acid side chains, such as imidazol and indol. Nevertheless, these cross-linkages could be disrupted by high-temperature heating or strong alkaline treatment. These observations gave rise to the initial experiments leading to what today is known as AR, encompassing both heating and nonheating methods.^{1,13} The basic, simple but effective technique of boiling FFPE tissue sections in water, later in buffer solutions, provided the first AR method and was widely recognized as a revolutionary breakthrough, a milestone, in immunohistochemistry.^{9,14–16} With the rapid worldwide adoption of the AR method, the quest for an alternative new fixative became less urgent; indeed it became possible to argue, or at least consider, the notion that formalin may be an optimal fixative to preserve not only morphology, but also to preserve and extract nucleic acids and proteins from FFPE tissue sections for use in a variety of modern analytic methods (see Chapters 3 and 20 for details).

11.2 TWO PHILOSOPHICALLY DIFFERENT APPROACHES FOR CELL/TISSUE SAMPLE PREPARATION

In a sense, therefore, there have been two conflicting views with respect to the suitability of formalin as a fixative, in the face of demands that biopsy tissues may be examined not only by traditional morphologic methods, but also by IHC, *in situ* hybridization (ISH) and, following extraction procedures, by other molecular methods. Both views recognize that these newer methods do not perform well, or at all, on routinely processed FFPE tissues. One view advocates the development of new fixatives that are “molecular friendly,” the other view holds that AR-based methods may be employed to achieve accurate valid results of IHC, ISH, and other molecular methods using FFPE tissues.

The “new fixative” approach presents very large logistical issues, including a long “transition” period when both the new fixative and formalin would be in use in different laboratories across the world. In addition, archival banks for FFPE tissues would diminish in value and become worthless as techniques adapted to the “new fixative tissues.” On the other hand, data are

TABLE 11.1 Comparison between Two Philosophically Different Approaches for Cell/Tissue Sample Preparation

Two approaches	Alternative approach replacing formalin with new fixative	Retrieval approach, with ongoing use of formalin under controlled conditions
Continuation of tissue banks worldwide	Existing FFPE archives become “obsolete,” and not comparable with new fixative	Continued use of archival FFPE tissue banks
Retrospective study	Not possible; characteristics of “new fixatives” differ from FFPE	Possible with optimized AR
Morphology for surgical pathology	Not satisfactory for most alternative fixatives	Satisfactory; FFPE continues to be the morphologic standard
Maintain most proteins in cell/ tissue samples	Possible, may be superior, but data are limited at present	Possible with optimized AR for most proteins to remain <i>in situ</i>
Maintain nucleic acids in cell/tissue samples for analysis	Possible	Possible, using AR-based recovery methods (see Chapter 3)
Sterilize virus in cell/ tissue samples	Possible theoretically, but few data at present	Possible ²⁴
Practical issues and cost	Logistics of replacing formalin worldwide are daunting	Continue to use formalin, with better documentation of fixation times, and so on

accumulating that strongly support the utilization of AR with FFPE tissues for many molecular applications, justifying, at least for the present, the use of formalin as the standard preservation method of histopathology. Actually, the development of AR technique gives a new, or at least extended, life to the traditional formalin tissue fixation method created more than a hundred years ago (see Chapters 2 and 12). Conversely, the long-established and entrenched use of formalin provides a strong incentive for continuing studies to refine further the AR method as a means of enhancing the applicability of molecular techniques to routinely processed FFPE tissues.^{17–23}

The pros and cons of these two philosophically different approaches are summarized in Table 11.1.

Prior to beginning of IHC on formalin paraffin tissues more than 30 years ago, fresh cell smears or frozen tissue sections were used for immunofluorescence studies on tissues sections, and in a more limited way for immunoperoxidase studies, now generally known as immunohistochemistry, prior to adaptation of the method to FFPE in 1974. The traditional viewpoint that

acetone or ethanol-fixed frozen tissue sections represent the “gold standard” for IHC has been accepted for many years but recently was challenged by experimental work from our group and others.^{25,26} Today, the successful and widespread use of AR techniques in fact favors continuation of the routine use of FFPE (with AR) for most diagnostic work and for many research studies, including the evaluation of new markers and new reagents for IHC.^{15,27,28} Similarly, the success of AR has reduced the incentives for identifying alternatives to formalin, with the goal of replacing it in routine use. At the same time, AR has permitted the demonstration of many molecules in FFPE sections, which otherwise cannot reliably be shown, such as estrogen receptor (ER), Her2, and a variety of oncogene products and cell surface receptors, some of which provide the basis for new prognostic and predictive “stains.” The rapid progress of “personalized medicine” in the form of biomarker-based targeted treatments that rely upon these new predictive IHC stains has placed new demands upon standardization and quantification of IHC methods, and has drawn attention to major inconsistencies in the use of formalin, and to the importance of cell/tissue sample preparation overall.

REFERENCES

1. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
2. Taylor CR, Burns J. The demonstration of plasma cells and other immunoglobulin containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase labelled antibody. *J. Clin. Pathol.* 1974; 27: 14–20.
3. Taylor CR. Immunohistologic studies of lymphomas: new methodology yields new information and poses new problems. *J. Histochem. Cytochem.* 1979; 27: 1189–1191.
4. Taylor CR. Immunohistologic studies of lymphoma: past, present and future. *J. Histochem. Cytochem.* 1980; 28: 777–787.
5. Taylor CR, Kledzik G. Immunohistologic techniques in surgical pathology—a spectrum of new special stains. *Hum. Pathol.* 1981; 12: 590–596.
6. DeLellis RA, Sternberger LA, Mann RB, et al. Immunoperoxidase technics in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute. *Am. J. Clin. Pathol.* 1979; 71: 483–488.
7. Huang S-N. Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. *Lab. Invest.* 1975; 33: 88–95.
8. Colvin RB, Bhan AK, McCluskey RT. *Diagnostic Immunopathology*, 2nd edition. New York: Raven Press, 1995.
9. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2005.
10. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins. IV. Participation of indole groups. *J. Biol. Chem.* 1947; 168: 99–118.

11. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. *J. Biol. Chem.* 1948; 174: 827–843.
12. Fraenkel-Conrat H, Olcott HS. The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups. *J. Am. Chem. Soc.* 1948; 70: 2673–2684.
13. Shi SR, Cote C, Kalra KL, et al. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. *J. Histochem. Cytochem.* 1992; 40:787–792.
14. Boon ME, Kok LP. Breakthrough in pathology due to antigen retrieval. *Mal. J. Med. Lab. Sci.* 1995; 12: 1–9.
15. Gown AM. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* 2004; 121: 172–174.
16. Jagirdar J. Immunohistochemistry, then and now. *Arch. Pathol. Lab. Med.* 2008; 132: 323–325.
17. Mason JT, O’Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
18. Rait VK, O’Leary TJ, Mason JT. Modeling formalin fixatin and antigen retrieval with bovine pancreatic ribonuclease A: I—structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
19. Rait VK, Xu L, O’Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RBase A II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
20. Sompuram AR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
21. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
22. Sompuram SR, Vani K, Bogen SA. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.
23. Yamashita S. Heat-induced antigen retrieval: Mechanisms and application to histochemistry. *Progress in Histochemistry and Cytochemistry* 2007; 41: 141–200.
24. Laman JD, Kors N, Heeney JL, et al. Fixation of cryo-sections under HIV-1 inactivating conditions: integrity of antigen binding sites and cell surface antigens. *Histochemistry* 1991; 96: 177–183.
25. Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. *J. Histochem. Cytochem.* 2005; 53: 1421–1432.
26. Shi S-R, Liu C, Pootrakul L, et al. Evaluation of the value of frozen tissue section used as “gold standard” for immunohistochemistry. *Am. J. Clin. Pathol.* 2008; 129: 358–366.
27. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
28. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.

CHAPTER 12

MECHANISMS OF ACTION AND PROPER USE OF COMMON FIXATIVES

RICHARD W. DAPSON

12.1 INTRODUCTION

Immunohistochemistry (IHC) depends upon molecular shape. When a target molecule has been conformationally altered, antibodies can no longer recognize it. Ideally, we would be working with fresh tissue that has never encountered the shape-changing events inherent in the preparation of a histological section or cytological specimen. Preservation of some sort must occur, however, and we rely on specimens that appear familiar and react predictably. That requires a series of chemical and physical treatments to render fresh tissue into something visually recognizable and permanent. These requirements are at odds with the need to keep molecules as natural as possible. Reconciling those opposing forces is one of the greatest challenges of IHC; antigen retrieval (AR) has served as the bridge between the two. The restoration of immunoreactivity through enzymatic digestion or treatment with heat is a great milestone in the evolution of immunohistochemical techniques, and not just because it expands the scope of IHC to archival and conventional surgical specimens. Additionally, it has toppled a long-held belief that the effects of fixation and tissue processing are irreversible. The present chapter covers some basic tenets of fixation that are usually glossed over or lacking in other reviews: denaturation, penetration, specimen preparation, and chemical action of important fixatives in common use. As such, it updates and builds upon a comprehensive, innovative collection of papers from 1991.¹⁻⁴

12.2 DENATURATION

Fixation is denaturation: changing the shape of tissue molecules. This is accomplished in a variety of ways by different denaturing agents and the resultant shapes differ markedly, but the consequences are much the same: endogenous and microbial enzymes can no longer attack the tissue (it is preserved), and the structure of tissue molecules is stabilized. Whether we like what we see from this depends upon what we *want* to see and what we are *used* to seeing.

It is important to realize that *not all denaturing events are considered to be fixation*. Many of the things we do to tissue after fixing it have the potential to cause further changes in molecular structure. We are only now beginning to realize how important those other denaturing events are. As the extent of denaturation increases, immunoreactivity tends to decrease (changes in shape reduce the chance for immunorecognition), and the ability to restore it also declines (the molecule becomes too stabilized).

The concepts of coagulation and precipitation, common in the classical literature of fixation, are outmoded and confusing: a coagulant fixative gels some, but not all proteins, while a precipitant fixative causes only certain proteins to fall out of solution. Instead, we will use terms that actually describe the chemical and physical reality of fixation at the molecular level.

Some fixatives work by combining with tissue molecules, hence the term *addition* reactions. This may continue as *cross-linking*, whereby the original *adducted* (added-onto) molecule attaches to another portion of the same molecule or to an adjacent molecule. A small branched polymer is thus created. Formaldehyde is the prime example of an additive and cross-linking fixative.

Other fixatives and denaturing agents cause shape changes but do not actually attach to tissue molecules. Some remove water in various ways. Others change the environment, causing molecules to twist about to lower their overall energy (molecules always go to their lowest possible energy state).

12.3 PENETRATION

Penetration of a fixative into the specimen is not usually given more than a cursory acknowledgment in reviews or in actual practice, yet it is of paramount importance and is worth emphasizing.

If fixative molecules are not present within a tissue, fixation will not occur.

It is that simple. Understanding penetration is vital to seeing why the preparation of specimens sometimes (often) turns out other than what we expect or desire. Penetration of all other fluids after the fixative also must be considered. One of the purported consequences of fixation is to make the tissue more

permeable so that processing fluids move more readily in and out. New concepts of fixation and processing are starting to shed doubt on this claim, however. As adducts and cross-links fill intermolecular spaces, it is only logical to realize that pathways for subsequent penetration are constricted.

All steps in fixation and tissue processing involve exchange of fluids in the three-dimensional space of the specimen. At the start of fixation, tissue fluid (mostly water) is inside the specimen, while fixative molecules are on the outside. Ignoring for the moment the actual structure of the tissue and the effect a fixative may have upon it, assume that the specimen is like a porous sponge filled with water. To enter this system, a fixative molecule must replace a molecule of water. Diffusion is the driving force: when two different liquids meet, there is a gradual equalization in the distribution of their molecules. Ideally, at the end of the process, the concentration of one in the other will be the same both inside and outside the specimen.

Diffusion operates along well-defined physical principles first described in 1855 by Adolf Fick and now widely known as Fick's Laws of Diffusion. Philbert⁵ provides a detailed explanation of the laws and a historical account of Fick. While they were designed to describe the behavior of gas molecules under ideal theoretical conditions, Fick's Laws serve reasonably well to describe a wide variety of real diffusion events. Fick wrote the laws as a set of equations in the language of calculus but these can be rephrased in plain English.

The rate of diffusion

- increases as a function of
 - the diffusion coefficient and
 - the square of the difference in concentration; but
- decreases as a function of the square of depth to be penetrated.

The foundation of fixation in particular as well as the dynamics of tissue processing can be summarized by four points drawn from this relationship. Anyone experienced in histotechnology can attest to the veracity of each, even if they had not thought about it in these terms.

12.3.1 Each Chemical Has a Characteristic Diffusion Coefficient

A high diffusion coefficient increases the rate of diffusion, all else being the same. The diffusion coefficient is determined in part by molecular size and shape. Small molecules tend to have high diffusion coefficients, which is one reason why formaldehyde penetrates faster than glutaraldehyde. In addition, interactions between the chemical and its environment will influence the diffusion coefficient. Thus, if the chemical hydrogen bonds to the water around it, the diffusion coefficient will be lower and the rate of diffusion will be reduced.

The diffusion coefficient is also dependent upon the rate at which molecules vibrate, a fact not evident in my simplified equation but vitally important nonetheless. Higher temperatures cause greater vibrational movement, so having heat on the processor accelerates fluid exchange (this may be advantageous for decreasing turnaround time but may be harmful to the specimen because heat itself is a denaturing agent). Microwave energy also causes greater vibrational movement, which generates intermolecular friction that creates heat and causes temperature to rise; hence, microwave fixation and processing works (at least in part) by speeding penetration.

12.3.2 Rate of Diffusion Is Proportional to the Difference in Concentration of the Two Types of Molecules, Specifically to the Square of that Difference

The greater the concentration of the fixative, the faster it will diffuse into the specimen. This is not a linear function, and the ramifications of this are important. If the fixative concentration is doubled, the rate of diffusion will be fourfold greater (double squared). Tripling the concentration will increase the rate of diffusion ninefold (triple squared). Increasing the fixative's concentration may help compensate for slow diffusion through "difficult" tissue like brain. Neuroanatomists have long appreciated this, using 15–20% formalin instead of the more usual 10%. Taken out of context, this relationship between concentration and rate of diffusion might be carried to an extreme, as in using concentrated formaldehyde. Other factors come into play, however, that may negate and actually reverse any theoretical gains. Concentrated formaldehyde is so aggressive that tissue shrinks as cross-links form, blocking penetration beyond the surface.

A common practice is to fix tissues in a small volume of fluid, much to the detriment of quality. If the fluid volume of the fixative is not adequate, water from the specimen will reduce its concentration sufficiently to slow diffusion. A ratio of 20:1 in the volume of fixative to the volume of the specimen will prevent that from happening.

Graded series of alcohols will diffuse more slowly than an abrupt change in alcohol strength; thus, diffusion currents will act more gently within delicate tissues. This is good if speed is not important, but must be considered if fast processing is attempted. Properly stabilized specimens can withstand more abrupt changes in solvent concentration.

12.3.3 Rate of Diffusion Is Inversely Proportional to the Square of Distance

The deeper the fixative has to go into the specimen, the slower it moves inward (and disproportionately so): in other words, the longer it takes to get there. Because penetration time is inversely proportional to rate of diffusion, point (4) is a direct corollary of (3).

12.3.4 Penetration Time Is Proportional to the Square of Distance

The thicker the specimen, the longer it takes for penetration to be complete. A 1-mm thick piece of tissue will take a certain length of time to become penetrated. A 2-mm piece of the same tissue will take four times as long (double squared), and a 3-mm piece will take nine times as long (triple squared). For very thick gross specimens, complete penetration may not occur in any reasonable period of time. If you want very rapid fixation and processing, specimen thickness must be kept as thin as possible.

So much for the theoretical basis of diffusion: a reality check is now in order. Chemicals used in fixation and tissue processing do not behave as theoretically ideal entities, so the complexity of the system increases. This and other factors discussed below make quantification implausible, although it has been attempted⁶; however, this should not prevent us from gaining a strong understanding of the forces driving the inflow of fixative and subsequent exchange of processing fluids. One such nonideal factor is the bonding behavior of formaldehyde. It bonds to water, which slows it down, and then it may bond to tissue molecules, which stops it from going further. As unbound formaldehyde molecules try to slide past, their way may be blocked by the reaction products just formed. Think of it as closing down the pores through which fluid exchange takes place. Solvents also have an effect (described below) that similarly alters the rate of diffusion. It should be obvious that diffusion past a certain point eventually becomes problematic, as evidenced by spleen and liver pieces fixed for a month but still raw inside.

Thus far, we have considered the specimen only as an inert sponge through which diffusing molecules travel. In the real world, the specimen itself plays an active role in determining rate of penetration. Consider cell and organelle membranes that present bilayers of hydrophilic and hydrophobic substances. Each membrane is a barrier to penetration of aqueous substances, slowing the inflow of fixative. There are larger hydrophobic things, like fat, that must be circumnavigated. Dense tissue elements are more of an impediment than loosely arranged ones. Thus, in a way, tissue elements have diffusion coefficients of their own and should be factored into the general.

12.4 MANAGING SPECIMEN QUALITY

The production of quality specimens is critically dependent upon the initial steps in the process: treatment before transport, transport, grossing, and fixation before processing.⁴ Nothing about this is profound or even new, yet many specimens, perhaps a large majority of them, show evidence that some basic rule has been ignored. The quest for ever-shorter turnaround time has taken us down a very undesirable path. It has become such a hot topic that an acronym for turnaround time, TAT, has been coined for it. Specimen quality has suffered, putting diagnostic conclusions in doubt. This is well-known, but

few laboratories have tried to deal with it realistically. There is a finite limit to reducing TAT with standard protocols and equipment. A significant reduction can be achieved by grossing at about 1.5 mm and using a conventional tissue processor. Even greater reduction can be had through microwave processing. Faster fixatives can pare a few hours off the time needed. Regardless of the strategies chosen for improving efficiency, specimens must be fixed and therefore *must be given time to be fixed*.

12.4.1 Treatment Before Transport

Specimens should never be allowed to air dry nor should they be placed on a dry, absorbent surface. Both actions will seal the surface and jeopardize penetration. Instead, specimens should be placed in a fixative immediately if at all possible, or kept moist for a short period until fixation can be started. The volume of fixative should be about 20 times greater than the size of the specimen. Specimen containers should not be prone to tipping over, or should be held in a carrier to prevent tipping. Vials are particularly susceptible to tipping over, and tend to leave the specimen clinging to the inside of the cap when placed upright. Fixation cannot occur if the specimen is not in the fixative.

12.4.2 Transport

If small specimens are truly immersed in a proper amount of fixative, there is no technical reason to rush them to the laboratory. For biopsies at least, longer transport times usually result in better quality.

12.4.3 Grossing

Tissue cassettes have an internal clear depth of less than 5 mm. Specimens grossed thicker than that will be compressed by reinforcing ridges or the mesh itself. We joke about specimens so thick that fat oozes out the holes, but that happens on a more frequent basis than we care to admit. Any subsequently applied fluid will not infiltrate compressed tissue. Specimens grossed slightly thinner than this may swell and be compressed. Any of these specimens will show the telltale marks of the cassette when the block is being rough cut. All specimens should be cut no thicker than 3 mm, and preferably less than that if fixation time is minimal. If levels are needed, make multiple cassettes rather than placing a thick piece into one cassette. Having a few good blocks to cut is far better than having one whose interior is not processed. Lack of time is no excuse for good specimen management.

Biopsies present challenges of their own. Many are so small they would pass through the holes of standard cassettes, necessitating some kind of restraining device, most of which simply replace one problem with another. Special cassettes with very small holes tend to inhibit fluid flow in and out of

the cassette due to surface tension between the plastic and the more aqueous fluids. Foam sponges may impede diffusion, especially if they become compressed. Neither device should be used with very short processing times. Paper wraps made from lens paper or tea bags are better options if they are not layered excessively, but they are messy during embedding. Nylon mesh bags, if sized properly for the cassette, are more reliable. New technologies that entrap specimens within the cassette are promising but have been used so little that conclusions cannot be drawn at this time.

12.4.4 Fixation Before Processing

Except for biopsies that really do start to fix when first placed into fixative, specimens generally do not achieve any significant amount of fixation until after they have been grossed. Fixative can then penetrate from at least two sides and has only half the depth of tissue to travel. If grossed less than 2 mm, this should take only a few hours, not 24–36 h, using formalin.

12.5 FIXATION WITH FORMALDEHYDE

Many fixatives have been created for histology over the years, but few of them are in routine use today. We will discuss formaldehyde in some detail, and will later deal more briefly with alcoholic formalin, zinc formalin, zinc salt solutions, glyoxal, and solvent-based fixatives. Formaldehyde offers a good platform for discussing subsequent denaturing events during tissue processing and how all of that interrelates with time given to fixation. Formaldehyde fixation, when done properly, serves as the standard for what high-quality specimens should look like structurally (Fig. 12.1).

12.5.1 The Myths of Formaldehyde Fixation

Formaldehyde has been in use as a fixative since the late 1800s when Ferdinand Blum introduced it to the fields of microbiology and histology.⁷ It has been studied extensively with model systems and sophisticated analytical tools, yet we continue to hear that the mechanism of tissue fixation is not well understood. Worse, the conclusion of Underhill⁸ that formaldehyde penetrates rapidly but fixes slowly is simply not true. People who work with formalin on a daily basis can readily attest that the skin of a finger exposed to formalin for far less than a minute will soon feel different, as Blum himself discovered.⁷ Formalin splashed into an eye will cause almost immediate damage to the cornea, which is why governmental safety regulations require the use of splash proof goggles and readily accessible eyewash stations. Gross and microscopic examination of specimens clearly reveals that penetration is slow: outer areas are different from the interior. If formaldehyde were fast to penetrate and slow to fix, the quality of fixation would be uniform throughout. It is not and

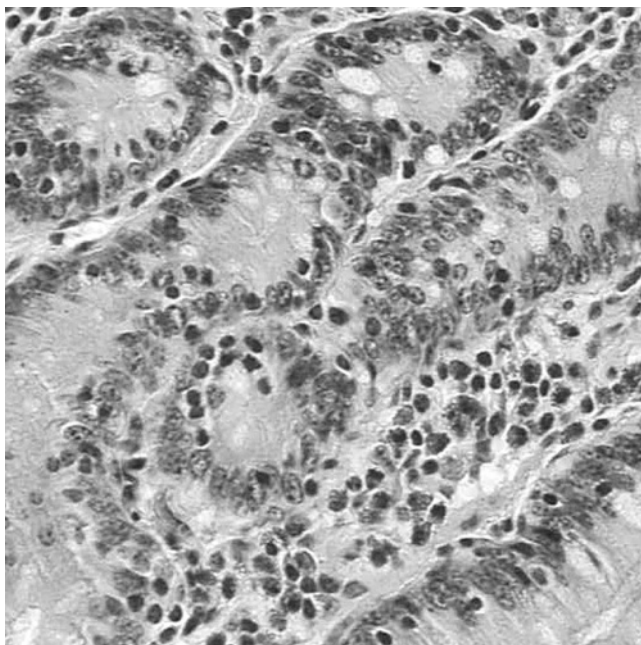


Figure 12.1 Typical good quality specimen. Small intestine fixed for 24h in neutral buffered formalin after grossing at 2mm. Most nuclei show good chromatin patterns, but cell membranes are indistinct.

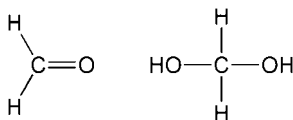


Figure 12.2 Structural formulas of formaldehyde and its hydrate, methylene glycol.

never has been regardless of the time allotted to fixation. We now need to reexamine the foundation equation that led to all this confusion.

Formaldehyde is usually described as a gas, but it also exists dissolved in water or other solvents. Because of very strong tendencies to hydrogen-bond, both formaldehyde and water combine avidly to make a hydrated compound called methylene glycol (Fig. 12.2).

Much has been made about methylene glycol being the cause for formaldehyde's slow rate of fixation, succinctly expressed by Fox et al.⁷: "Equilibrium between formaldehyde as carbonyl formaldehyde and methylene glycol explains most of the mystery of why formaldehyde penetrates rapidly (as methylene glycol) and fixes slowly (as carbonyl formaldehyde)." However, the equilibrium equation indicates the *proportional amounts* of carbonyl formaldehyde and methylene glycol, not the *rate of conversion* between the two

species. The equation might favor the presence of methylene glycol, but when formaldehyde is removed as tissue is fixed, the equilibrium is rapidly restored. A continuous supply, however tiny, of highly reactive formaldehyde is always present. Fixation with formaldehyde is slow because penetration, not chemical reactivity or rate of conversion from methylene glycol, is slow. Various authors⁹⁻¹¹ agree that this process takes 24–48 h or more to occur throughout a specimen that is 2–3 mm thick.

12.5.2 General Fixation Reactions

Most of the following discussion on formaldehyde is described in greater detail elsewhere.^{1,12} Formaldehyde combines with tissue molecules in an *addition reaction*, and all atoms of the fixative molecule become part of the tissue molecule (Table 12.1). The CH₂OH group on the end of each of these molecules is called an *adduct* (add-on product), and may be referred to as methylol or a hydroxymethyl adduct. The latter term is preferred because it is more descriptive. While formaldehyde eventually will add onto any group containing a reactive hydrogen atom, the rate of reaction varies considerably. Amine reactivity is generally high, and the other examples given are slower. Carboxyl groups are so slow to react that they are not considered to be important in normal fixation times.¹³

Once addition has occurred, a second, *cross-linking*, reaction *may* occur if a potentially reactive group on a neighboring molecular strand is present and is at the right distance for conjugation with the initial adduct. The CH₂ in the middle of this cross-linked structure comes from the formaldehyde in the original addition reaction and is now called a *methylene bridge*. This reaction is slower and may not become noticeable for another 12 h or more. If specimens are left in formalin, cross-linking continues for months or years, tying up more functional groups as time progresses, which is why museum specimens are so difficult to use for histological study. They become so densely cross-linked that sectioning is very difficult, and all once-reactive groups have been consumed, leaving no opportunity for dyes or other reagents to bind.

TABLE 12.1 Important Functional Groups that Are Likely to React with Formaldehyde Under Routine Histological Conditions

	Formaldehyde		Cross-Linked
	Group	Structure	Adduct Product
Primary amine	R–NH ₂	R–NH–CH ₂ OH	R–NH–CH ₂ O–Z
Secondary amine	R–NH–R′	R–NR′–CH ₂ OH	R–NR′–CH ₂ O–Z
Hydroxyl	R–OH	R–O–CH ₂ OH	R–O–CH ₂ O–Z
Sulfhydryl	R–SH	R–S–CH ₂ OH	R–S–CH ₂ O–Z

Note: Cross-linked products are shown with Z representing any of the functional groups in the first column.

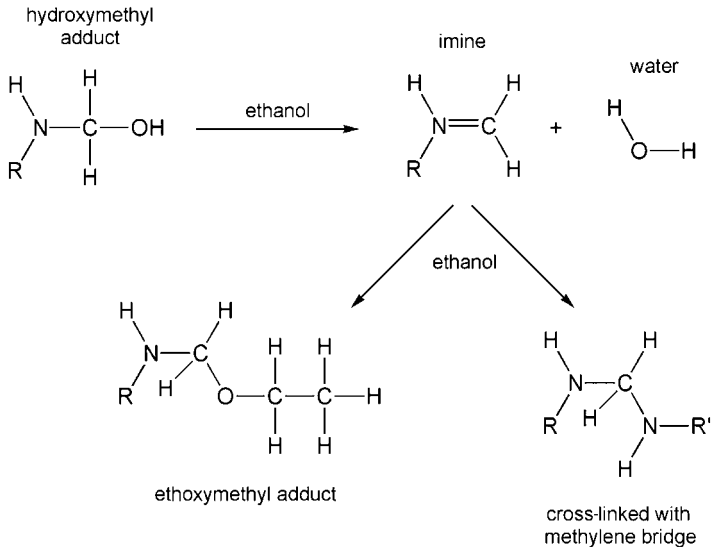


Figure 12.3 Hydroxymethyl adducts from formaldehyde fixation form highly reactive imines in the presence of ethanol during tissue processing, giving off a molecule of water (upper). While still in alcohol, imines may either form more complex ethoxymethyl adducts or will cross-link to neighboring reactive groups (lower).

12.5.3 Cross-Linking during Tissue Processing

Alcohol in the dehydration station of a tissue processor removes water from specimens in three ways. Obviously, it replaces free water in the spaces of the specimen. It will also remove bound water, water that is hydrogen-bonded to macromolecules. If the tissue has not been sufficiently stabilized by fixation, it will shrink with the removal of bound water. Another type of molecular dehydration may also occur, in which a hydrogen atom and a hydroxyl group are removed from non-cross-linked hydroxymethyl adducts,¹⁴ creating a highly reactive imine (Fig. 12.3, upper). The imine is then able to react with ethanol to form a more complex ethoxymethyl adduct (Fig. 12.3, lower left) which further changes the shape of the molecule.¹⁵ Alternatively, the imine can cross-link to another functional group nearby (Fig. 12.3, lower right). This occurs faster than the direct cross-linking by formaldehyde. Bonds within the methylene bridge $\text{-N-CH}_2\text{-N-}$ are much weaker than bonds between adjacent carbon atoms $\text{-C-CH}_2\text{-C-}$. The same is true for many of the other cross-linking bonds involving adducts to hydroxyl, sulfhydryl, and other original end groups. It is these bonds that will break during heat-induced antigen retrieval (HIAR).

12.5.4 Formaldehyde and Nucleic Acids

Srinivasan et al.¹⁶ provide a comprehensive review of how fixation and tissue processing affect DNA and RNA. Formaldehyde attacks the exocyclic nitro-

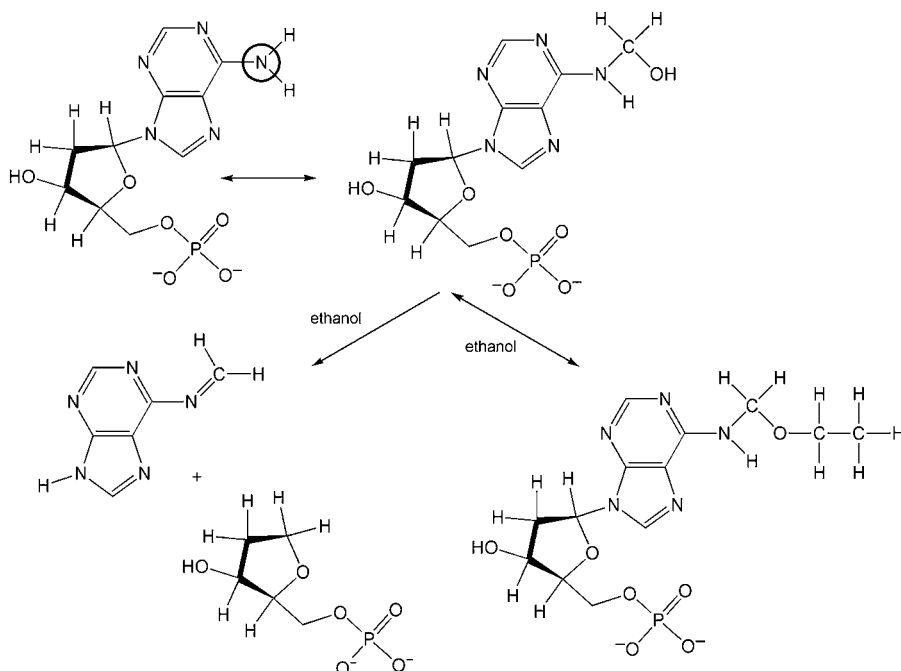


Figure 12.4 In the presence of formaldehyde, the exocyclic nitrogen (encircled, upper left) on a nucleotide forms an *N*-hydroxymethyl adduct (upper center). Further denaturing reactions of formalin-fixed nucleic acids during tissue processing lead to a compound ethoxymethyl adduct (lower right), fragments from depurination (lower left), cross-links to associated proteins (not shown), and hydrolysis of phosphodiester bonds (not shown).

gen atom to form a hydroxymethyl adduct (Fig. 12.4). This is fairly stable in formalin and forms few cross-links.¹⁷ If the specimen is washed, adducts are readily removed, but few labs wash tissues today (this was routine decades ago). In high concentrations of alcohol, however, several events may occur. *N*-ethoxymethyl derivatives form from adducts. The derivatized base may split off (depurination). If spatial conditions are favorable, adducted nucleic acids may cross-link to associated proteins. Formaldehyde may also slowly hydrolyze phosphodiester bonds.¹⁶ Obviously, depurination and phosphodiester hydrolysis are catastrophic events, while adduction and cross-linking may be reversible. One of the most urgent challenges is to learn the precise conditions that favor the less destructive denaturation over the catastrophic, and then optimize fixation and processing accordingly.

12.5.5 Hydrophobic Inversions

Biological molecules generally exist in an aqueous (hydrophilic) environment and must be physically compatible with it. What makes a molecule hydrophobic

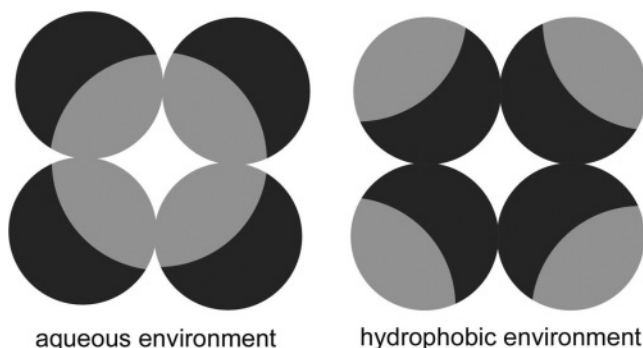


Figure 12.5 Hypothetical protein with four parallel helical strands, viewed from the end looking down the axis. Each circle represents a cross section through a helix. Black regions are hydrophilic, gray areas are hydrophobic. Left: protein in an aqueous environment such as body fluid or aqueous fixative. Right: protein in hydrophobic solvent such as alcohol or clearing agent. Each helix has rotated around its own axis to bring hydrophobic realms outward.

or hydrophilic depends upon its component makeup and how those components are arrayed relative to the environment. With mammalian proteins, there are 20 common amino acids differing by the character of their side chains. Four bear negative charges, three have positive charges, and another four have polar but nonionized side chains. Together, these are the hydrophilic amino acids. The remaining nine are hydrophobic. Regardless of amino acid sequence, all proteins (and other macromolecules) have one thing in common: the exterior of the molecule tends to be hydrophilic while hydrophobic realms occupy the interior. Globular proteins have solid hydrophobic interiors; barrel-like proteins have hydrophobic walls lining the interior.¹⁸ Proteins comprised of several helices lying parallel to one another also keep their hydrophobic areas away from hydrophilic tissue fluid (Fig. 12.5, left).

Adequate fixation stabilizes macromolecules against conformational changes during processing. Infrared spectroscopic studies suggest that this is accomplished at least in part by “locking in” the secondary structure after fixation with formaldehyde.¹⁹ Stabilizing interactions at the tertiary and higher levels are certainly likely as well.

Inadequate fixation leaves macromolecules open to further alteration, as when we process poorly fixed tissue. Alcohol is a strong denaturing agent and will do its job if the tissue molecules are susceptible. Even clearing agents can influence tertiary structure of macromolecules. Alcohol is far less polar than water, and clearing agents are nonpolar. When a poorly stabilized protein with an exterior composed of hydrophilic amino acids comes into contact with a weakly polar or nonpolar solvent, the molecule comes under a great deal of tension (its energy level rises). To reduce this tensional energy, tertiary structure is shifted so that hydrophobic areas orient outward and hydrophilic

regions face inward. This is called *hydrophobic inversion*. Barrel-shaped proteins may literally turn inside out. Proteins made up of a bundle of parallel helices, with hydrophilic realms facing outward in native conformation, experience a curious shift. Each helix rotates 180° about its own axis, bringing hydrophobic realms outward and turning hydrophilic areas inward (Fig. 12.5, right). Globular proteins may unwind and rearrange the relative positions of hydrophilic and hydrophobic realms so that tensional energy is minimized.

12.5.6 A Unified Model of Formaldehyde Fixation

Any model of fixation must account for why specimen quality and immunostaining are so variable across specimens within a day's batch, from day to day and from laboratory to laboratory, especially when the same fixative and processing reagents are used. We now have the mechanistic understanding, laid out in the preceding discussion, to construct a satisfactory model encompassing fixation and the events immediately following which collectively comprise tissue processing. Later we can even extend this model to other fixatives.

Addition and cross-linking by formaldehyde are distinct processes that proceed at their own rates, the latter being much slower. With "long" fixation times (48 h or more), many adducts are formed and cross-linking is well underway. Immunoreactivity for many antigens is reduced or even eliminated. Macromolecules are stabilized sufficiently to prevent formation of microscopically visible structural artifacts like nuclear bubbling and tinctorial aberrations. Alcohol in the tissue processor may create some new secondary adducts that may contribute to further diminution of immunoreactivity. Hydrophobic inversions may be inhibited if enough cross-links hold macromolecules rigid (Table 12.2). With thin specimens, there should be little heterogeneity from the surface to the interior. In summary, longer fixation (within reason) produces better morphology and conventional staining while impairing most immunostaining even when combined with vigorous AR. Specimens are remarkably uniform in how they look and react, are ideally suited for classical diagnostic tests but are poor subjects for modern immunohistochemical and molecular procedures.

Shorter fixation times produce substantially greater variability across specimens in the same laboratory and even more so across laboratories. At the

TABLE 12.2 Chemical Consequences of "Long" and "Very Short" Fixation Time

	Properly Fixed (24 h)	Poorly Fixed 0–Few Hours
Cross-links from formaldehyde	Some	Essentially none
Cross-links from tissue processing	Some	Few
DNA/RNA fragments	Yes	Fewer
Hydrophobic inversions	Few	Many

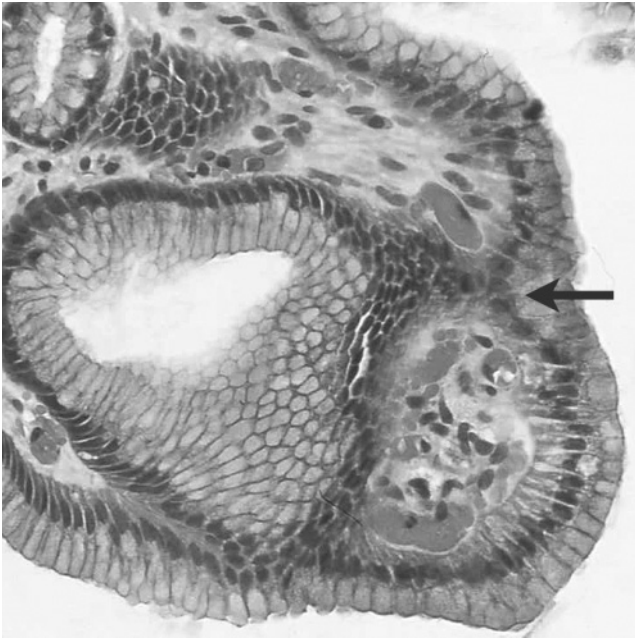


Figure 12.6 Fixation in formalin for less than 6 h produces blurry detail and loss of chromatin patterns. Some nuclei appear faded (arrow).

extreme with little or essentially no fixation (Table 12.2), denaturation occurs on the tissue processor. Formalin has little chance to penetrate, much less react except at the very surface of specimens. Alcohol strips insulating layers of bound water off macromolecules, causing new ionic interactions and hydrogen bonding across molecular strands. These are not sufficient to afford much protection, however. Hydrophobic inversions begin in the higher alcohol stations and become more severe in the nonpolar environment of the clearing agent. The result is that morphology is distorted (Fig. 12.6), conventional and special stains are compromised, but immunostaining is more likely to be strong or more readily restored to strong reactivity with AR. This is radically different from the scenario culminating from extensive fixation.

Between these two extremes is a bewildering array of possibilities, and this is the most common set of circumstances to be found in surgical pathology today. In other words, we have the worst of all options as the foundation of specimen management. The effects of slow penetration are obvious when comparing surface and interior regions. Primary adducts become more numerous as fixation time increases, allowing more ethanol-induced secondary adducts to form. The visual effects of hydrophobic inversions slowly diminish as macromolecules are gradually stabilized by primary (formalin-induced) and secondary (imine-induced) cross-links. This is accompanied by a concomitant reduction in responsiveness to immunoreagents and AR. The critical point

here, however, is that in this intermediate time period for fixation (e.g., 6–18 h), a lot is happening in cascading fashion. A difference of a very few hours in the fixative will make a significant difference in staining. Standardization of fixation time is the first step in gaining control of specimen management.

The model is in good accord with what we know of the chemistry of fixation and from what we observe coming out of our laboratories. We now have recent experimental evidence from several independent sources that provides scientific validation for the model.^{3,21,22} To circumvent the problems of penetration and to simplify the system, surrogate “specimens” were used in place of histological sections. That shortcoming aside, the common thread emerging from these studies is that while fixation in formalin causes problems with detection of some antibodies, the events in tissue processing are even more damaging. One study²² will be described briefly here because it was most similar to real histological specimens (cell cultures expressing various antigens, grown on microscope slides, instead of model peptides or proteins).

Slides were sequentially removed from the normal progression of steps in fixation and processing, then immunostained for the expressed antigen. Fixation time varied from 0 to 72 h. With Ki-67, immunoreactivity was greatest in the fresh state and got progressively weaker as fixation time increased. In fact, there was visible diminution of staining after only 5 min in formalin. This is further evidence that the rate of conversion from methylene glycol to formaldehyde, and the rate of chemical reactivity of formaldehyde, are rapid. In all cases that had not been processed, HIAR recovered full staining intensity compared to the unfixed control, although results were more consistent with longer fixation times.

When subjected to the solvents used in tissue processing, immunoreactivity got progressively weaker after each successive exposure to alcohol and xylene, with little change noted after the paraffin. The greatest change in immunoreactivity came at the transition to the completely hydrophobic environment of xylene, probably because it makes it harder to get water back into the protein prior to staining. By the time specimens reached paraffin, heat did little further damage because macromolecules were so drastically modified.

Other cell lineages and antigens produced minor variations on this general theme, as did the other model specimens used by Bogan²⁰ and O’Leary.²¹ Provided that modification of protein structure was not too drastic (e.g., fixation only), full immunoreactivity could be restored. With each successive transition in processing (aqueous to aqueous alcoholic to anhydrous to hydrophobic), the chances for full demodification of certain proteins dwindled. These studies are at odds with work from other authors who found little impairment of immunoreactivity after processing, especially with Ki-67,^{23,24} although the procedures were hardly comparable. One important point to be made here is that variations in protocol may be critical to success and that lab-specific optimization of AR is essential. Furthermore, antigens are highly variable in their response to fixation and the events encountered during tissue processing.

Clearly, however, processing *can* have a profound effect on the specimen, especially with the short fixation times common to surgical pathology. There are several mechanisms to explain this: cross-linking by formalin, cross-linking in the presence of ethanol, and hydrophobic inversions. None of these chemical events necessarily destroys reactive sites (except for nucleic acids that may be permanently split apart). Instead, they may directly change epitopes with adducts and subsequent cross-links. Alternatively, cross-linking elsewhere along the macromolecule may hinder access to epitopes by large immunoreagents. Adducts are readily reversed, cross-links less so. Modifications from hydrophobic inversions may be reversible if the molecule is not too tightly condensed, the degree of which is dependent upon any prior stabilization by the fixative. Thus, we can see why fixed but not processed tissue is very suitable for IHC, whereas processed specimens present challenges. Further complexity is introduced into the system by varying specimen thickness and composition, fixation time, and antigen to be detected, but it is now all explainable.

12.6 OTHER POPULAR FIXATIVES

12.6.1 Alcoholic Formalin

Formaldehyde in 70% alcohol acts similarly to formaldehyde in water, except it is better able to penetrate membranes and other fatty structures. Lillie⁹ claimed that fixation time could be reduced by 50% by using alcoholic formalin, but that seems overly optimistic with fixation times in vogue today. However, because penetration is faster, the degree of fixation after a given period of time is greater. Hydrophobic inversions are minimized so morphology is generally improved. Customary practice is to use aqueous formalin as the primary fixative and to follow that with alcoholic formalin in either the second station on the tissue processor or in the first two stations. Both plain and buffered alcoholic formalin are available commercially, the latter offering advantages for bloody tissues. If alcohol concentration is higher than 70%, tissue may show a combination of formalin and alcohol patterns of fixation. Studies paralleling those described above have not been done and are needed.

Despite occasional admonitions to avoid alcoholic formalin for certain IHC procedures, there is no reason to think that this fixative would do more harm than a functionally equivalent degree of fixation with aqueous formalin since tissues go through 70% alcohol during processing. The degree of fixation, not the type of fixation, is the major difference if alcoholic formalin is used. When trying to standardize IHC procedures, keep this in mind if some specimens have been exposed to alcoholic formalin and others have not.

12.6.2 Zinc Fixatives

Zinc formalin arose out of a need to prevent nuclear bubbling artifact,²⁵ for which it was ideally suited²⁶ (Fig. 12.7). Only later was it discovered that it

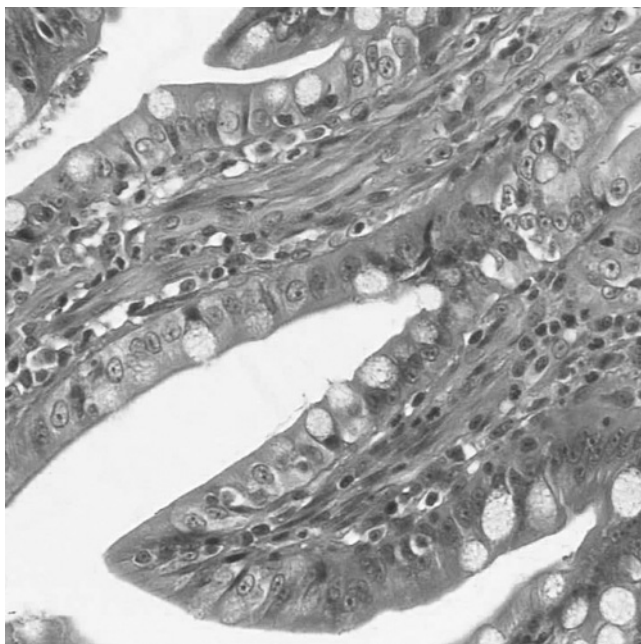


Figure 12.7 Colon fixed in buffered zinc formalin (Z-Fix, Anatech Ltd., Battle Creek, MI) for only 6 h. Cellular detail is very prominent, nuclear chromatin is unusually well displayed and cytoplasmic staining is intensified.

preserved immunoreactivity of many antigens^{23,27} and subsequently became quite popular. The need for AR is almost eliminated, even after prolonged exposure (30 days) to zinc formalin.²⁸

The mechanism of fixation is straightforward.²⁸ Remember that formaldehyde first forms adducts which stabilize secondary structure. With short fixation times, adducts are converted to cross-links during dehydration, but not before some disruption of tertiary structure occurs. Zinc intercedes in this process during initial fixation by quickly forming metal chelation complexes with a variety of ligands (cysteine, histidine, tryptophan, arginine, guanine, cytosine, and phosphate groups). These coordination complexes can have 2–4 associated ligands, effectively acting as staples holding native tertiary structure in place through tissue processing. Hydrophobic inversions cannot occur.

Other zinc solutions, free of formaldehyde, have been proposed.^{29–31} All of these simple buffered salt solutions preserve immunoreactivity well and are suitable for DNA, RNA, and proteomics research. Judging by published photomicrographs of hematoxylin and eosin-stained specimens, cytological detail is inferior to that achieved with standard formalin. Nuclei are condensed to the point where many lack chromatin patterns.^{31,32} Such zinc salt solutions may be good for specialized purposes but are best used as special fixatives. To get good structural detail as well, specimens should be split so that a portion can

be fixed in formalin, zinc formalin, or glyoxal. The management of this protocol is too cumbersome to be practical. A better strategy would be to use one fixative in a program that optimizes preservation of all molecules, coupled with select demodification techniques for the analyte under study.

12.6.3 Glyoxal

The use and reactions of glyoxal as a fixative have been reviewed extensively.^{33,34} Glyoxal is the second smallest aldehyde, being like two formaldehyde molecules arranged back-to-back (Fig. 12.8, left). It too forms hydrates with water, the most common of which is 1,3-dioxolane (Fig. 12.8, right).

Glyoxal shares some of the characteristics of formaldehyde but has some peculiarities as well. It is so tightly hydrated that it cannot form a gas, so it does not evaporate and hence presents no risk of inhalation. It undergoes addition and cross-linking reactions only under highly specific conditions, usually with the aid of a catalyst or other reaction accelerator. By itself, glyoxal will react only with the side chains of arginine, lysine, and cysteine, as well as with the alpha amine at the end of each protein chain. Reaction rate with all other groups is simply too slow to have significant meaning, either industrially or as a fixative. With a catalyst or other reaction accelerator, reactions which were too slow to be practical occur rapidly. Most industrial applications involve cross-linking, but control of pH is critical. Amines and amides are selectively cross-linked at one pH (depending upon the catalyst), hydroxyls are selectively cross-linked at a different pH. In contrast, most glyoxal fixatives are designed to inhibit any type of cross-linking, again through careful control of pH (3.75–4.25). Ethanol is used as the reaction accelerator, but the concentration is not enough to act as a fixative in itself.

Glyoxal-based fixatives work faster than formalin. Small biopsies may be ready to process after only an hour while properly grossed larger specimens are ready in about 6h. Structural detail is remarkable in its clarity (Fig. 12.9). Red blood cells are lysed, but that rarely presents a problem. Eosinophilic granules are reduced in prominence (see below). Special stains work well, except for tests for iron (the mildly acidic pH is detrimental) and the silver detection methods for *Helicobacter pylori*. Most notably, glyoxal-fixed tissues retain strong immunoreactivity for most antigens. The chemistry behind most of this is known.

Under the right conditions, glyoxal fixatives form adducts with the same groups listed for formaldehyde (Table 12.1). We do not know if compound

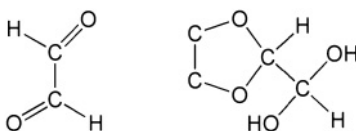


Figure 12.8 Structure of glyoxal and its hydrate, 1,3-dioxolane.

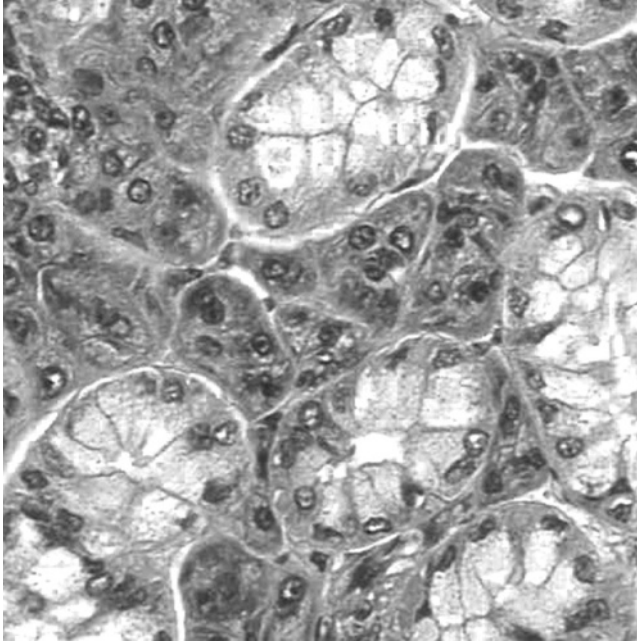


Figure 12.9 Salivary gland fixed in a glyoxal solution (Prefer, Anatech Ltd.). Membranes around mucus cells are unusually clear and nuclear chromatin is sharply defined.

adducts arise from exposure to ethanol, but adduct formation of either type is responsible for stabilizing macromolecules against hydrophobic inversions during the later stages of tissue processing. Fine structural detail is evidence that stabilization was accomplished while strong immunoreactivity confirms that cross-linking did not occur.

Glyoxal has a unique reaction with arginine (Fig. 12.10) that creates cyclic compounds called dihydroimidazolidines.³⁵ This imidazole reaction is so specific that it has been used in histochemistry to block arginine and was thought to be irreversible under conditions encountered in histochemistry.³⁶ The reaction has important implications for routine and immunohistochemical staining on glyoxal-fixed specimens. With cyclization, arginine loses its strong positive charge, so arginine-rich sites will not stain prominently with acid dyes. Obviously, arginine changes its shape so arginine-rich epitopes may not be recognized by antibodies. Fortunately, the cyclic adduct can be demodified by a glyoxal-specific AR protocol (pH 8.5 buffer at approximately 120°C in a pressure cooker) that restores strong immunoreactivity. The original charge site characteristic of arginine is not recovered. The imidazole reaction is probably behind the failure of silver detection methods for *H. pylori*, although that has not been verified.

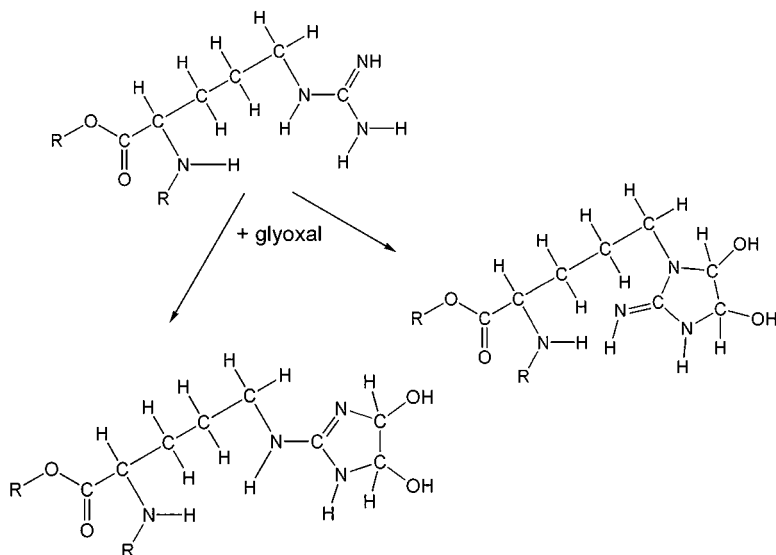


Figure 12.10 The imidazole reaction between glyoxal and arginine creates cyclic addition compounds lacking positive charge.

AR techniques commonly used for formalin-fixed specimens do not work on glyoxal-fixed tissues and often damage the sections beyond use. Many of the problems involving immunohistochemistry with glyoxal-fixed tissues stem from trying to treat them and formalin-fixed specimens alike. They are different and must be handled accordingly.

12.6.4 Nonadditive Fixatives

Frozen sections fixed in acetone have been a favored alternative to formalin-fixed, paraffin-embedded (FFPE) material in critical IHC work, to the point where this was considered the standard against which other fixatives were compared. Recent studies^{37,38} have now cast doubt on that status, showing that many antigens perform equally well after FFPE (with or without AR) and that nuclear antigens in particular fare better, perhaps because some proteins may be extracted by solvent fixatives.

Nonadditive fixatives followed by paraffin processing avoid some of the problems with formaldehyde. These gained popularity with microwave fixation^{39,40} and have since been used for molecular studies.⁴¹ The primary agent is alcohol (methanol or ethanol), with at least one other ingredient (usually a low molecular weight polymer of ethylene glycol) designed to prevent the severe shrinkage associated with simple alcohol fixation. The mode of fixation undoubtedly is hydrophobic inversion without adducts or cross-links, as neither alcohol is capable of forming adducts (except in the unlikely reaction

with carboxylic acids). Near-native conformations apparently are reestablished upon hydration of sections, as AR reportedly is not necessary. Without formaldehyde adducts, nucleic acids will not form higher order (ethoxymethyl) adducts, depurination products, or cross-links to associated proteins. While all of this sounds ideal, the problem with these fixatives has been that they fail to produce morphological patterns familiar to, and desired by most pathologists. Nuclei tend to lack chromatin, general cellular detail is suboptimal, and collagen takes on a harsh, glassy appearance. The addition of acetic acid and/or zinc salts, attempted in some failed commercial products, did little to improve the situation.

REFERENCES

1. Eltoun I, Fredenburgh J, Myers RB, et al. Introduction to the theory and practice of fixation of tissues. *J. Histochemol.* 2001; 24: 173–190.
2. Eltoun I, Fredenburgh J, Grizzle WE. Advanced concepts in fixation. *J. Histochemol.* 2001; 24: 201–210.
3. Grizzle WE. Models of fixation (Guest Editorial). *Biotech. Histochem.* 2009; 84: 185–193.
4. Grizzle WE, Stockard CR, Billings PE. The effects of tissue processing variables other than fixation on histochemical staining and immunohistochemical detection of antigens. *J. Histochemol.* 2001; 24: 213–219.
5. Philibert J. One and a half century of diffusion: Fick, Einstein, before and beyond. *Diffus. Fundam.* 2006; 4: 1–19.
6. Medivan PB. The rate of penetration of fixative. *J. R. Microsc. Soc.* 1941; 61: 46–57.
7. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.
8. Underhill BML. The rate of penetration of fixatives. *J. R. Microsc. Soc.* 1932; 52: 113–120.
9. Lillie RD, Fullmer HM. *Histopathologic Technic and Practical Histochemistry*, 3rd edition. New York: McGraw-Hill Book Company, 1976.
10. Pearse AGE. *Histochemistry: Theoretical and Applied*, 3rd edition, Vol. 1. Boston: Little Brown, 1968.
11. Helander KG. Kinetic studies of formaldehyde binding in tissue. *Biotech. Histochem.* 64: 177–179.
12. Dapson RW. Macromolecular changes caused by formalin fixation and antigen retrieval. *Biotech. Histochem.* 2007; 82: 133–140.
13. French D, Edsall JT. The reactions of formaldehyde with amino acids and proteins. *Adv. Protein Chem.* 1945; 2: 277–335.
14. Namimatsu S, Ghazizadeh M, Sugiski Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.

15. Rait VK, Zhang Q, Fabris D, et al. Conversions of formaldehyde-modified 2'-deoxy-adenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. *J. Histochem. Cytochem.* 2006; 54: 301–310.
16. Srinivasan M, Dedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nuclei acids. *Am. J. Pathol.* 2002; 161: 1961–1971.
17. Solomon MJ, Varshavsky A. Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. *Proc. Natl. Acad. Sci. U S A* 1985; 82: 6470–6474.
18. Brandon C, Tooze J. *Introduction to Protein Structure*. New York: Garland, 1991.
19. Mason JT, O'Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
20. O'Leary TJ, Fowler CB, Evers DL, et al. Protein fixation and antigen retrieval: chemical studies. *Biotech. Histochem.* 2009; 84: 217–221.
21. Otali D, Stockard CR, Oelschlager DK, et al. The combined effects of formalin fixation and individual steps in tissue processing on immunorecognition. *Biotech. Histochem.* 2009; 84: 223–247.
22. Bogan SA, Vani K, Sompuram SR. Molecular mechanisms of antigen retrieval: antigen retrieval reverses steric interference caused by formalin-induced crosslinks. *Biotech. Histochem.* 2009; 84: 207–215.
23. Williams JH, Mephram BL, Wright DH. Tissue preparation for immunocytochemistry. *J. Clin. Pathol.* 1997; 50: 422–428.
24. Arber DA. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 183–186.
25. Jones MD, Banks PM, Caron, BL. Transition metal salts as adjuncts to formalin for tissue fixation (Abstract). *Lab. Invest.* 1981; 44: 32A.
26. Banks, PM. Technical aspects of specimen preparation and special studies. In *Surgical Pathology of the Lymph Nodes and Related Organs*, ed. ES Jaffe, pp. 1–21. Philadelphia: W. B. Saunders, 1985.
27. Herman GE, Elfont EA, Chlipala EA, et al. Zinc formalin fixative for automated tissue processing. *J. Histotechnol.* 1988; 11: 85–89.
28. Dapson RW. Fixation for the 1990's: a review of needs and accomplishments. *Biotech. Histochem.* 1993; 68: 75–82.
29. Beckstead JH. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J. Histochem. Cytochem.* 1994; 42: 1127–1134.
30. Lykidis D, Van Noorden S, Armstrong A, et al. Novel zinc-based fixative for high quality DNA, RNA and protein analysis. *Nucleic Acids Res.* 2007; 35: 1–10.
31. Webster K, Asplund A, Bäckvall H, et al. Zinc-based fixative improves preservation of genomic DNA and proteins in histoprocessing of human tissues. *Lab. Invest.* 2003; 83: 889–899.
32. Hicks DJ, Johnson L, Mitchell SM, et al. Evaluation of zinc salt based fixatives for preserving antigenic determinants for immunohistochemical demonstration of murine immune system cell markers. *Biotech. Histochem.* 2006; 81: 23–30.
33. Dapson RW, Feldman AT, Wolfe D. Glyoxal fixation and its relationship to immunohistochemistry. *J. Histotechnol.* 2006; 29: 65–76.

34. Dapson RW. Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. *Biotech. Histochem.* 2007; 82: 161–166.
35. Cotham WE, Metz TO, Ferguson PL, et al. Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease. *Mol. Cell. Proteomics* 2004; 3: 1145–1153.
36. Lillie RD, Pizzolato P, Dessauer HC, et al. Histochemical reactions at tissue arginine sites with alkaline solutions of β -naphthoquinone-4-sodium sulfonate and other *o*-quinones and oxidized *o*-diphenols. *J. Histochem. Cytochem.* 1971; 19: 487–497.
37. van der Loos CM. A focus on fixation. *Biotech. Histochem.* 2007; 82: 141–154.
38. Shi S-R, Liu C, Pootrakul L, et al. Evaluation of the value of frozen tissue section used as “gold standard” for immunohistochemistry. *Am. J. Clin. Pathol.* 2008; 129: 358–366.
39. Kok LP, Visser PE, Boon ME. Histoprocessing with the microwave oven: an update. *Histochem. J.* 1988; 20: 323–328.
40. Boon ME, Ouwerkerk-Noordam E, Suurmeijer AJH, et al. Diagnostic parameters in liquid-based cervical cytology using a coagulant suspension fixative. *Acta Cytol.* 2005; 49: 513–519.
41. Vincek V, Nassiri M, Nadji M, et al. A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. *Lab. Invest.* 2003; 83: 1427–1435.

CELL SAMPLE PREPARATION FOR CLINICAL CYTOPATHOLOGY: CURRENT STATUS AND FUTURE DEVELOPMENT

YAN SHI and PATRICIA G. WASSERMAN

An ancient Chinese proverb says: “Great achievements must rely on advanced tools.” In review of the development of immunohistochemistry (IHC) in diagnostic pathology, it is obvious that the success of IHC in this field is accomplished by a series of technical innovations.^{1,2} In her recent article pertaining to the development of IHC during the last three decades, Jagirdar listed all major milestones in the history of IHC, including the development of monoclonal antibody, avidin–biotin detection system, and antigen retrieval (AR).² In particular, the author emphasized that the heat-induced AR technique is a revolutionary breakthrough that divides IHC into two eras: pre-AR and post-AR eras.^{1,3–6}

The application of AR-assisted IHC (AR-IHC) has demonstrated the following advantages: (1) It is simple, safe, and cost-effective.^{7,8} (2) It revolutionized the manner in which pathologists practice. It makes numerous antibodies working reliably on formalin-fixed, paraffin-embedded (FFPE) tissue sections. Otherwise, IHC has to rely largely on frozen tissue.^{3,7,8} AR-IHC has become one of the most important tools for diagnostic pathology and retrospective pathological research. (3) It highlights the scientific mechanism of formalin fixation. The philosophy of AR sheds light on the future development of AR-assisted molecular techniques for FFPE.⁷ (4) It highlights the immunoprofiles of numerous biomarkers, which advances our knowledge of carcinogenesis, facilitating the clinical management and prognosis prediction. It serves as a bridge between traditional morphology and molecular biology. (5) It may contribute to IHC standardization as well as the development of quantitative IHC.^{4,9} Therefore, AR-IHC has proved to be extremely helpful

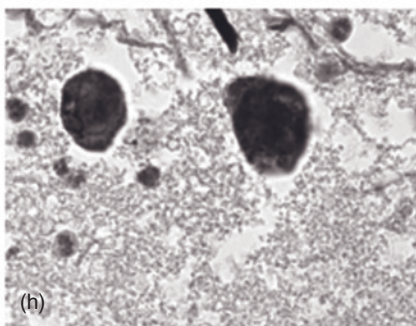
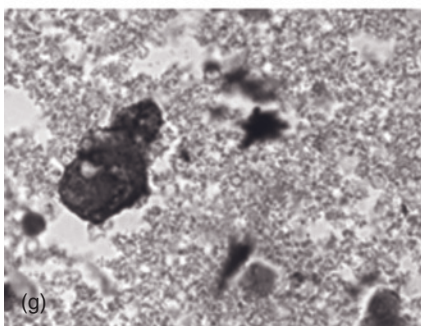
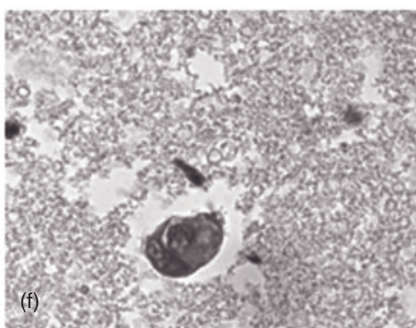
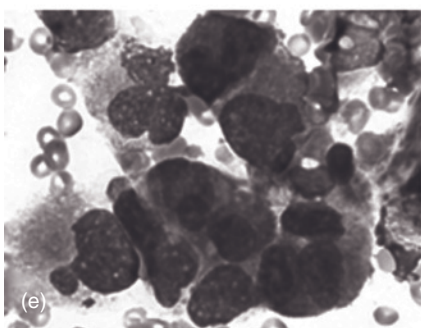
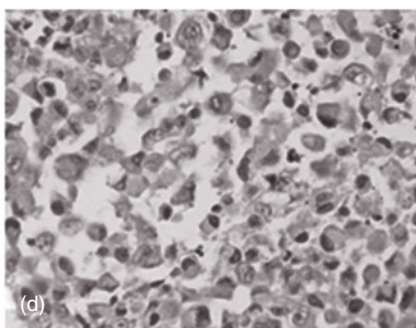
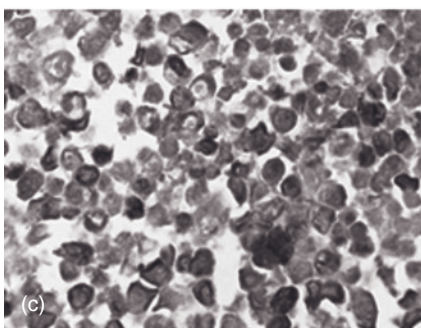
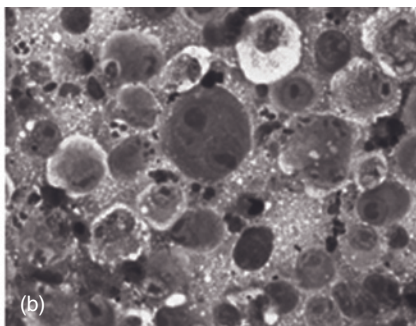
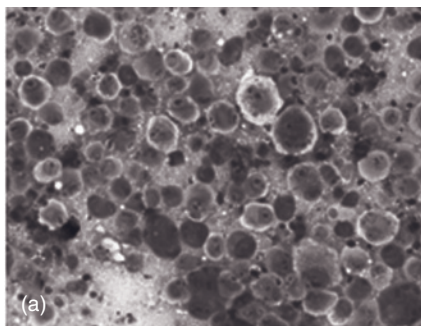
not only for arriving to accurate diagnoses, but also for aiding in the contemporary personalized target treatment of cancer.^{10–13}

In light of the above-mentioned advantages of AR-IHC achieved in surgical pathology, cytopathologists quickly adopted this technique for cytology specimens. Cell blocks, smears, and cytospins are all excellent sources for immunocytochemistry (ICC). Despite technical limitations, ICC is proved to be a valuable ancillary study for identification and classification of tumor cells, especially when dealing with a tumor of unknown origin. Based on our experience at Long Island Jewish Medical Center in the last 5 years, ICC contributed to the diagnosis of more than 90% of the cases in which ICC was performed, supplying either additional or essential information for confirmation or histogenesis identification. This was especially true for cases of fine-needle aspiration (FNA) and serous effusions (Fig. 13.1). With the application of ICC, cytopathology has emerged as a powerful diagnostic tool which is able to provide important therapeutic and prognostic information with a limited pool of cells, many times retrieved from deep-seated lesions, obviating the need for major surgery.^{14–17}

However, ICC is not used as frequently in cytology as IHC in surgical pathology due to some technical issues, such as scanty or lack of diagnostic material in cell blocks or cytospins, and questionable reliability of immunostaining on smears.

Flens et al. conducted a study comparing ICC on smears of 64 patients with their corresponding IHC on the histological specimens. They found that although ICC was a valuable technique with good concordance with IHC, there was still a 10% discrepancy in marker expression. The major issues

Figure 13.1 (a) and (b) showing an endobronchial ultrasound (EBUS)-FNA biopsy of an enlarged mediastinal lymph node from a 63-year-old male with a left lower lobe lung mass and a remote history of melanoma. The smear was composed of sheets of large atypical cells with prominent nucleoli. Bi- and multinucleated cells were present. The differential diagnosis included metastatic anaplastic large cell carcinoma versus melanoma. ICC analysis found the tumor cells were positive for pancytokeratin (c) and negative for S100 (d). Therefore, the final diagnosis was metastatic anaplastic large cell carcinoma. (e) CT-guided FNA biopsy of a liver mass from a 51-year-old male with multiple hepatic lesions and a large right atrial mass. The smear was composed of loosely cohesive aggregates and singly dispersed large atypical cells with high nuclear-cytoplasmic ratio and irregular nuclear membranes. Bi- and multinucleated cells were present. The differential diagnosis included metastatic anaplastic carcinoma versus high-grade sarcoma. ICC analysis revealed the tumor cells were negative for pancytokeratin (f). They were positive for CD34 (g) and CD31 (h). Therefore, the final diagnosis was metastatic angiosarcoma, favor cardiac origin. (a: Diff-Quik stain, $\times 200$; b: Diff-Quik stain, $\times 400$; c and d: Immunostaining, $\times 200$; e: Diff-Quik stain, $\times 400$; f–h: Immunostaining, $\times 400$). See color insert.



contributing to these discrepancies included (1) sampling error due to heterogeneous protein expression observed in tumor cells; (2) different protein expression profiles between the tumor cells suspended in serous effusion and those fixed in tissue; (3) difference in fixation and sample preparation between ICC and IHC; and (4) misinterpretation. The first two causes for discrepancy are inherent to the clinical samples and are largely beyond control. To avoid misinterpretation, the author recommended using several independent antibodies for the diagnosis.¹⁸ This approach has also been suggested by a number of other investigators.¹⁹

The amount of diagnostic cells is limited in cytological preparations. Among all technical issues concerning cell sample preparation, the most important one is how to make a limited cell sample available for multiple biomarkers (a panel of antibodies) in order to reach the diagnostic goal. Strategies to produce multiple cytological slides from one single specimen include monolayer preparation (ThinPrep and Sure Path), cell block techniques, and tissue transfer. Numerous research projects on cell sample preparation, optimal fixation, and tissue transfer have been conducted in the recent two decades. In this chapter, authors try to focus on four critical points: (1) cell block preparation; (2) multiple markers on cytologic smear slides; (3) AR and ICC on smear slides; and (4) standardization of ICC. We wish to improve practical application of ICC in cytopathology and minimize the gap between ICC and IHC.

13.1 CELL BLOCK TECHNIQUE

Cell block technique is one of the oldest preparation methods in cytology, tracking back as early as 1896.²⁰ With this method, all residual cytologic material is processed using histological techniques after completing routine cytologic preparations. It has been recognized as a useful adjunct in diagnostic cytology, revealing valuable diagnostic tissue fragments and additional diagnostic evidence which may not be appreciated on cytology preparation.^{21,22} For the purpose of ICC, FFPE cell blocks are considered the most ideal samples. Cell blocks simulate surgical samples by providing the following advantages: (1) It can be handled like routine surgical pathology samples in a busy immunohistochemical laboratory.^{15,19} No additional positive or negative controls are needed for immunostaining. IHC protocols for surgical specimens including AR, antibody titration, and incubation time can be applied safely on cell blocks. (2) Basic technical principles of AR and IHC can be applied on FFPE cell blocks, including test battery approach of AR (see Chapter 1). (3) It is easy to test a given ICC panel by cutting multiple sections from the same cell block. (4) It provides a clean background, superior to most ICC results on cytospins or smears.^{23,24} (5) Cell blocks can be easily stored for future molecular studies (see Part I, and Chapters 11 and 21). (6) It is the most cost-effective for ICC analysis compared to ThinPrep and cytospin.²⁴ Several investigators have demonstrated the effectiveness of cell block for cytological

diagnosis and research.^{15,23,25,26} Therefore, this technique is highly recommended as the first choice for ICC analysis whenever enough cell samples are available.^{15,23,27}

In essence, the basic steps of making cell blocks consist of fixation, centrifugation to make cell pellet, transfer the pellet to a labeled tissue cassette which then is processed and embedded in paraffin. The most challenging component of this technique is the methods to harden the cell pellet so it can be easily picked up from the tube without losing precious material. With only a simple sedimentation technique, the cell pellet is usually small and friable. In order to harden the cell pellet, several technical modifications have been reported. The most popular methodology includes plasma–thrombin clot technique, agar technique, and fixation with Bouin's solution.

Plasma–thrombin clot methodology is widely used in many cytology laboratories. It can improve the quality of cell blocks, especially ideal for collecting limited cell samples.^{28,29} The major steps for this simple method are: (1) Mix the cell pellet with a few drops of blood plasma after centrifugation. (2) Add the same number of drops of thrombin into cell–plasma mixture. (3) The clot usually forms in 1–2 min. (4) Remove the clot, wrap it with lens paper, and transfer it into a cassette for further processing. The best sample for this method should be unfixed cellular material, collected in either RPMI medium, normal saline, or fresh serous fluid. The clotting action of plasma and thrombin is inhibited by fixatives including alcohol and formalin. If the sample is fixed, the cell pellet needs to be washed with normal saline or phosphate buffered saline (PBS) several times before proceeding to plasma–thrombin. According to the experience of Miller and Kubier, thrombin and plasma can be placed in dropper bottles and carried to the patient's bedside.²⁷ They found it is far more effective for cell block preparation by adding thrombin–plasma mixture to a small drop of bloody samples on a slide. Nigro et al. compared four cell block methods, including inverted filter sedimentation (IFS) method, thrombin method, albumin method, and simple sedimentation using 12 cases of nongynecologic specimens. The thrombin technique was deemed the best method because it produced the highest cellularity, optimal cytomorphology, and a clean background for ICC. In contrast, the IFS method was less ideal due to technical difficulty and less cellular specimen with artifactual crowding. Albumin cell blocks displayed distracting high backgrounds. Simple sedimentation is the worst, and most cell blocks made with this method contained insufficient cellularity.³⁰ Numerous ICC reports published in recent years adopted this thrombin-enriched cell block methodology.^{26,31}

Agar technique is also a well-known method for cell block. It takes the advantage of the special nature of agar which can be solidified when the temperature drops under 50°C. The major steps are: (1) Heat 3% agar gel using hotplate or waterbath which converts the gel into a liquid state. (2) Fix cells in formalin. (3) After centrifugation, mix the prefixed cell pellet with a few drops of liquefied agar gel. (4) Cool down the agar in room temperature or cold water. (5) When the agar hardens, remove it from the tube, wrap it

with lens paper, and then transfer it into a cassette for further processing. According to our experience, the key technical issue of this method is to avoid overheating the specimen. Therefore, we emphasize to prefix the cells with formalin before proceeding to agar method. Formalin-fixed tissue is resistant to high temperatures as exemplified by AR techniques. Mayall et al. investigated 28 cases of ICC using cell blocks made with agar method. They found it was a reliable and technically simple aid in diagnostic cytology. Unfortunately, they did not provide any surgical pathology correlation or follow-up studies.³² Fowler and Lachar reported a poor correlation of ICC results from cell blocks made with agar methods in comparison with IHC results from surgical specimens of the same tumor. They suggested that plasma-thrombin method is better than agar in terms of better correlation with surgical specimens.¹⁹ We suspect that their observation may be caused by overheating the unfixed tissue by hot agar.

Bouin's solution is one of the traditional ways to harden cell pellet. Some cytologists believe it provides the best cellular details, especially nuclear features in cell blocks.²⁸ The major steps are: (1) After centrifugation, fix the cell pellet with Bouin's solution. (2) After 2 h, discard the solution. (3) Remove the hardened cell pellet from the tube, wrap it with lens paper, and transfer it into a cassette for further processing. We have been using this method for many years. In our experience, most of the time, ICC results are consistent with IHC from the surgical specimen. The biggest drawback of this method is the toxicity of Bouin's fixative which creates biohazard and safety issues for the laboratory. We also found cell blocks gave poor fluorescence *in situ* hybridization (FISH) results after Bouin's fixation.

Cytoscape is a relatively new approach for cell block preparation characterized by scraping off darkly stained tissue fragments from smeared slides. It is especially helpful when there are not enough samples for conventional cell block and re-aspiration is impossible. This novel technique was first introduced by Verbeek et al. in 1996. The basic steps of their method are: (1) Remove the coverslip with xylene. (2) Cover the darkly stained tissue fragments with a thick layer of mounting medium and leave to dry for 45 min. (3) Cut and remove the mounting medium from the slide, which contains tissue fragments. (4) Dissolve mounting medium in xylene. (5) Process and embed the tissue fragment as a surgical specimen. Using this method, Verbeek archived beautiful morphology for all three cases in their study, and performed successful ICC for two of those cases.³³ In 2000, Kulkarni et al.³⁴ developed this method by scraping thick cell clusters directly from Papanicolaou or Romanowsky-stained smear slides using a scalpel blade. They mixed those scraped cells with 3% molten agar, and then followed routine agar method for cell block. Using this technique, they studied 27 cases in which the routine cytological preparations could not offer a definitive diagnosis, primarily due to thick cell clusters. They were able to obtain additional diagnostic information in 12 cases. Unfortunately, they only performed immunostaining for one case. Their conclusions were similar to Verbeek et al., except they found that

destaining before scraping gave better nuclear details. In 2007, Bhatia et al. further validated this method by compared cytomorphology preservation and ICC results between conventional cell blocks and cytosrape cell blocks.³¹ Different from Kulkarni's study, plasma–thrombin clot method was used to make cell block. They tested ICC in seven cases. They found cytosrape cell blocks were able to provide not only equally good cytomorphology as conventional cell blocks, but also opportunities to test a diagnostic panel for ICC.³¹ A similar experience with this method has also been reported by Nga et al.³⁵ Cytosrape cell block opens up a unique way to make use of nondiagnostic thick cell clusters and blood clot-covered cell aggregates on smear slides. However, the studies regarding this methodology were all small with only three to seven cases for ICC analysis. In addition, they all lacked correlation with IHC results from surgical specimens.

There are some other cell block methods described in the literature. For example, Nordgren et al.³⁶ designed a funnel-shaped filtration device in order to collect sample materials for cell block preparation. It was made of plexiglass with a polycarbonate membrane attached beneath the funnel. Immediately after FNA, the sample was fixed in 4% formaldehyde solution which was subsequently allowed to pass through the filtration device, whereas the cells were retained on the membrane. The membrane was then folded and embedded into paraffin block. Musso et al. described “cotton block method” for cell block preparation. They introduced the cotton wool tip of a commercial cotton bud into the plastic hub of a disposable 23–25-gauge needle. During FNA, the cotton wool tip served as mesh network for specimen collection, which could be removed and embedded into paraffin blocks. According to their experience, this method was easy to perform, yielding cell blocks of high quality for further study.³⁷ Krogerus and Anderson³⁸ introduced a simplified cell block method that minimizes cell loss by carrying out all procedures, including embedding in the same conical tube. However, this technique uses acetone for postfixation. Probably because of either technical difficulty or fixation issue in these methods, they were not widely adopted.

Recently, Hologic, Inc. developed a fully automated cell block system, Cellient™ system (Bedford, MA), expecting to improve capture, presentation, consistency, and efficiency of cell block preparation. This system is built on ThinPrep technology with vacuum-assisted filtration to maximize cell collection. The cell block can be produced in less than an hour.³⁹ However, the biggest concern of this methodology is the fixation issue. Cellient system adopts alcohol instead of formalin for fixation, which unfortunately creates problems for ICC analysis.

In summary, it is recognized that cell block technique provides a valuable ancillary cytopreparation for diagnostic cytopathology and ICC. There are many ways to prepare cell blocks. So far, there is no universally accepted method. As emphasized by Fowler and Lachar, it is advisable to validate any new cell block methodology by comparing its immunostaining results with IHC results from surgical samples in order to avoid misinterpretation.¹⁹

13.2 MULTIPLE MARKERS ON CYTOLOGIC SMEARS

Even though cell block is the first choice for ICC, there is not always sufficient cell sample for cell block preparation. Therefore, it is necessary to develop techniques that allow multiple immunostaining on limited smears.

The answer to this problem is to divide a smear into several sections by either a diamond pen, a crayon, or by breaking the slide and gluing each piece to a different slide.⁴⁰ Weintraub et al.⁴¹ reported a successful ICC study using three antibodies (AE1/AE3, CAM5.2, and leukocyte common antigen) on one single smear. These antibodies were carefully dropped at three different circled areas on the slides. They tested 15 cases and all demonstrated satisfying immunostaining results. Nevertheless, this kind of “separation cell zone” method is largely limited by the number of antibodies one can test on one single slide. In addition, there is a high risk for antibodies to interfere with each other.

In 1998, Dabbs and Wang reported “repeat ICC” for cytologic specimens of limited quantity.⁴⁰ The principle of this method is ICC can be performed more than once on the same cytologic specimen if the initial test is negative. The key points for their method are the following: (1) Formulate a differential diagnosis based on cytomorphology, such as carcinoma versus lymphoma. (2) Choose one negative/positive antibody pair which can favor one diagnosis over the other, such as cytokeratin and leukocyte common antigen, for the above differential diagnosis. (3) Perform the expected negative antibody first. (4) Perform the positive antibody to confirm the diagnosis. They found that this method could be helpful in situations where more than one antibody is needed on limited cytologic smear slides.⁴⁰ However, this method is also largely restricted by the number of antibody one can apply on a single slide. According to their experience, more than three tests may result in tissue loss and potential antigen loss through leakage.⁴⁰

Double-label ICC is another approach using a similar principle. It pairs a nuclear antibody with a cytoplasmic antibody on the same sample, such as keratin and estrogen receptor. At present, this method is mostly used in the research field.

Cell transfer technique is another important method when in need of multiple ICC tests on limited smears.^{27,40,42-44} With the application of mounting media, diagnostic cells can be transferred from a single smear to multiple slides, which allow a battery of ICC to be performed. Briefly, the cell transfer technique includes the following major steps: (1) Mark the interested areas on the reverse side of a Papanicolaou-stained smear with a diamond pen. The number of marked area depends on the antibodies one would like to test. (2) Remove the coverslip with xylene. (3) Cover the slide with a thick layer of mounting media. (4) Bake the slide in a 60°C oven overnight in order to harden the mounting media. (5) Remove the slide from the oven and re-mark the selected areas on the surface of the mounting media by a water-resistant marker pen. (6) Soak the slide in 45°C water bath for 30 min. (7) Peel off the cell-containing mounting media from the slide with a scalpel blade. (8) Divide

the lifted membrane into several pieces along the marked lines. (9) Transfer each piece to a new adhesive glass slide. (10) Perform ICC following regular protocols.^{27,43–46} Gong et al.⁴⁴ investigated the reliability of ICC results from transferred cytologic samples. They prepared 100 transfer slides from 22 cytologic cases with unequivocal diagnosis. Twenty-one commonly used ICC markers were tested. They found that 97% showed comparable staining results with previous ICC from cell blocks. No false positive results were identified. False negativity was found in three cases. Two of these false negative cases were due probably to sample error because the tested immunomarkers were only focally positive in the tumor cells. Based on their findings, they concluded that ICC could be reliably conducted on cell-transferred cytologic samples for most immunomarkers commonly applied in diagnostic cytology.⁴⁴ Zu et al. reported their successful experience with cell transfer technique using ultrafast Papanicolaou-stained cell slides of Hodgkin lymphoma.⁴⁶ For each case, they tested CD15, CD20, CD30, EMA on five transferred cell samples. They demonstrated that cell transfer technique provided the possibility to further analyze immunophenotypes of those tumor cells even though they were sparsely present on smears.⁴⁶ Because of the impressive ICC results achieved through cell transfer technique, Miller and Kubier suggested to use non-adhesive slides for routine clinical cell sample preparation in order to apply cell transfer technique when necessary.²⁷

A recent technical development named “multiplex-immnostain chip” (MI chip) has been reported by Furuya et al.⁴⁷ Their novel method allows testing as many as 50 markers in one single tissue section. The key point of this method is a unique 5-mm-thick silicon rubber plate containing 50 small wells. Each well can be filled with various primary antibodies. The major steps of immunostaining are the following: (1) Place a tissue section on the top of the silicon plate, and tighten these two with a specially designed clamp in order to maintain each antibody in one area during the subsequent procedures. (2) Turn the silicon plate upside down to allow the tissue section contact with antibodies later. (3) Add different antibodies into 50 small wells. (4) Follow by regular IHC protocols. Since 50 different immunomarkers can be tested at the same time on one single slide, this novel method obviously saves time, effort, and expense. In addition, pathologists can compare different protein expression on the same slide. However, it is only suitable for cases where sheets of target cells are distributed evenly on the slide. In clinical practice, especially in cytology, most of the target cells are distributed irregularly and mixed with benign cells. Thus, “MI chip” technique need to be further modified in order to be applied in diagnostic cytology.

13.3 AR AND ICC ON SMEARS

Even though AR was originally designed for FFPE, several investigators found it can improve immunostaining on smears fixed in alcohol, formalin, Carnoy’s Pap, and ThinPrep fixative.^{27,48} Gong et al. compared a number of

fixation conditions for ICC analysis of estrogen receptor in cytologic specimens. They found that without AR, traditional Abbott fixation correlated best with results from tissue samples. The overall correlation was 91.5%. The smears fixed in formalin and Carnoy's Pap performed the worst, with only 30% correlation. However, after AR, the concordance of estrogen receptor between smears and tissue sections was improved to 93% for both formalin and Carnoy fixatives. In addition, they found that AR also increased the staining intensity without producing any false positivity. Similar experience with archival alcohol-fixed cell smears has also been reported in the literature (Please see Chapter 2 for details).

Air-dried slides have been considered less desirable for ICC.⁴⁹ Wet-fixed cytologic slides represent a better resource for immunostaining. Fulciniti et al. developed a unique method to make ICC work on air-dried smears.⁵⁰ Their staining protocol was derived from the idea behind ultrafast Papanicolaou staining method and consisted of three major steps: (1) Rehydrate air-dried slides with normal saline. (2) Postfix the slides with 10% formalin for 3 min. (3) Apply AR and then follow the routine immunostaining protocol for the rest of the procedures. With this technique, they tested a variety of antibodies, including epithelial, mesenchymal, lymphoma, neuroendocrine, and prognostic markers. They found formalin-postfixed air-dried smears could produce reliable ICC results after AR treatment, comparable with those archived on wet-fixed slides. Furthermore, they suggested that the interpretation of ICC results may be easier with air-dried slides, because the visualization of ICC signals was better due to larger and flatter cells.⁵⁰ This is an interesting study; however, it did not compare the ICC results of air-dried smears with IHC from the corresponding surgical specimens. Although the author tested an extensive panel of various markers, half of them were only performed on a couple of cases. Thus, the reliability of ICC on air-dried slides with postfixation needs to be further evaluated.

13.4 STANDARDIZATION OF ICC

Standardization of IHC/ICC has been a critical issue for more than three decades, especially with the advances in targeted therapy such as the development of trastuzumab (Herceptin) for advanced breast cancer.⁵¹ Nevertheless, standardization is a difficult issue because numerous factors may influence the consistency and reliability of immunostaining results, including fixatives, fixation time, AR, antibody clones, detection system, and interpretation (see Part II). In cytopathology, the situation is even worse due to its variable cell sample preparation techniques. "Cytopreparation is ... the foundation of cytomorphology."⁵² We believe it is also the foundation of ICC. Therefore, standardization of ICC needs to start with uniform and reliable cytopreparation.

As we emphasized before, the optimal resource for ICC is FFPE cell block. Cytopathologists and cytotechnologists should make an effort to obtain enough material for cell block, especially when dealing with a complicated case of FNA or body fluid. Even though there is no universal methodology for cell block preparation, through a well-designed validation test comparing ICC results with IHC results from surgical specimen of the same case, various preparation methods can be standardized, producing reliable and reproducible ICC results for accurate diagnosis.

On the other hand, because of the nature of cytologic specimen, ICC still needs to be performed on other type of preparations (i.e., cytopsins, smears, and monolayer preparations). Since these specimens are fixed and processed differently as surgical samples, we recommend using a series of validation studies to establish optimal ICC protocol for each antibody, including titration, incubation time, and pretreatment, as necessary. Control materials should be prepared with the same methodology as the tested specimen. Nadji and Gangi suggested setting up an imprint file from normal and tumor tissues with known antigens for cell sample positive control.⁵³ IHC results from surgical specimens of the same cases can also be included in the validation studies to verify the immunostaining results. We hope through these attempts we can minimize the chance of misleading ICC results and move to the goal of standardization.

Recently, Her2 testing in breast cancer is a hot topic in the field of immunohistochemistry. American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) guidelines for Her2 testing were published simultaneously in two medical journals.^{51,54} Aiming to improve the accuracy of Her2 testing in invasive breast cancer, the guidelines discuss in great detail issues regarding tissue fixation, assay validation, and interpretation criteria. However, most recommendations are based on tissue sections, not cytologic specimens. With the development of radiologically guided FNA, the role of cytology in breast cancer management is expanding. There are many occasions where FNA samples of metastatic lesions are the only diagnostic material available for study.¹⁵ As we discussed before, cell blocks are the optimal choice for Her2 testing. Shin et al. investigated Her2 status by ICC and FISH using cell blocks from 25 cases of breast cancer.¹⁵ They found 17 cases showed no protein overexpression or gene amplification. Five cases were positive for both ICC and FISH. The remaining three cases showed some positivity by ICC but negative for amplification. Their data demonstrated that there was good correlation between ICC results and FISH. Thus, they concluded that ICC performed on cell block is a reliable method to evaluate Her2 status in breast cancer.¹⁵ It would be more convincing if this study could include the comparison of Her2 results from cell blocks with those from the corresponding surgical excisions or core biopsies. The reliability of Her2 testing on smear slides has also been investigated by several groups.^{16,17,55} These studies have shown a good correlation of Her2 results between ICC on FNA smears and IHC on the corresponding tissue samples. However, different investigators used different fixation

protocols, various antibodies, and different interpretation criteria. For example, Troncone et al. evaluated Her2 expression in 54 breast aspirates and corresponding surgical specimens. They found Her2 was positive in 26 (48%) smears and 23 (43%) matched surgical specimens. The data suggested a higher incidence of Her2 positivity on the smears. Thus, the author postulated that the higher Her2 expression may be due to better antigen preservation in fresh cytological preparation.¹⁷ However, this study tested Her2 on acetone-fixed air-dried cytopsin preparations, which is not a routine cytopreparation method for breast FNA specimens. In addition, they used enzyme digestion with trypsin as the pretreatment procedure instead of AR for IHC analysis on the surgical specimens, which may not be an optimal protocol for Her2 detection on histological sections. Based on the literature and our past experience, AR usually produces better immunostaining results than protease digestion.⁵⁶ In order to avoid all potential pitfalls as in previous investigations, Beatty et al. published a study using FDA-approved HercepTest for immunostaining and PathVysion Kit for FISH study.⁵⁵ They evaluated Her2 status by both methods in 51 FNA smears and corresponding surgical specimens. The FNA smears were fixed in three different solutions including ethanol, Cytolyt, and formalin. They found FISH results from the smears, and the surgical specimens correlated very well; however, there were discrepancies with immunostaining results. Moderate to poor concordance was observed between ICC from FNA smears and IHC from tissue specimens. Ethanol-fixed smear slides showed the worst correlation. Of eight cases with amplified Her2 gene, protein overexpression was only detected by ICC in two (25%) ethanol-, four (50%) CytoLyt-, and five (63%) formalin-fixed FNA specimens. Therefore, they concluded that FISH is more reliable than ICC to assess Her2 status in FNA smears.⁵⁵ However, we need to keep in mind that the HercepTest was designed to detect Her2 expression in formalin-fixed tissue, not in ethanol-fixed specimens. Thus, the staining protocol may not be optimal for ethanol-fixed smears. The ideal way to compare Her2 results between these two different kinds of specimens should first establish the optimal ICC protocol for ethanol-fixed slides, including pretreatment procedure, antibody titers, and incubation time. Further investigation is needed to clarify all these issues in Her2 testing using cytologic specimens.

13.5 CONCLUSIONS

It has been recognized that increased application of ICC as well as other molecular techniques has transformed cytology from a screening tool to a reliable diagnostic approach. However, compared with the success of IHC in surgical pathology, ICC still lags behind due to a series of technical issues as discussed in this chapter. It is essential to develop standardized protocols pertaining to cell block preparation and ICC on smears to prepare diagnostic cytology for advanced molecular techniques of the future.

REFERENCES

1. Taylor CR, Cote RJ. *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2006.
2. Jagirdar J. Immunohistochemistry, then and now. *Arch. Pathol. Lab. Med.* 2008; 132: 323–325.
3. Gown A, de Wever N, Battifora H. Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* 1993; 1: 256–266.
4. Taylor CR. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. *Biotech. Histochem.* 2006; 81: 3–12.
5. Boon M, Kok L. Breakthrough in pathology due to antigen retrieval. *Mal. J. Med. Lab. Sci.* 1995; 12: 1–9.
6. Gown A. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* 2004; 121: 172–174.
7. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
8. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
9. Shi S-R, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval technique: from experiments to hypothesis. *J. Histochem. Cytochem.* 2007; 55: 105–109.
10. Pritchard K, Shepherd L, O'Malley F, et al. HER2 and responsiveness of breast cancer to adjuvant chemotherapy. *N. Engl. J. Med.* 2006; 354: 2103–2111.
11. Konecny G, Thomssen C, Luck H, et al. Her-2/neu gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J. Natl. Cancer Inst.* 2004; 96: 1141–1151.
12. Nogi H, Kobayashi T, Suzuki M, et al. EGFR as paradoxical predictor of chemosensitivity and outcome among triple-negative breast cancer. *Oncol. Rep.* 2009; 21: 413–417.
13. Bergmann F, Breinig M, Hopfner M, et al. Expression pattern and functional relevance of epidermal growth factor receptor and cyclooxygenase-2: novel chemotherapeutic targets in pancreatic endocrine tumors. *Am. J. Gastroenterol.* 2009; 104: 171–181.
14. Barr N, Wu, N-Y. Cytopathology/FNA. In *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist*, 3rd edition, ed. CR Taylor and RJ Cote, pp. 397–416. Philadelphia: Elsevier Saunders, 2006.
15. Shin SJ, Chen B, Hyjek E, et al. Immunocytochemistry and fluorescence in situ hybridization in Her-2/neu status in cell block preparations. *Acta Cytol.* 2007; 51: 552–557.
16. Nizzoli R, Bozzetti C, Crafa P, et al. Immunocytochemical evaluation of Her-2/neu on fine-needle aspirates from primary breast carcinomas. *Diagn. Cytopathol.* 2003; 28: 142–146.
17. Troncone G, Panico L, Vetrani A, et al. c-erbB-2 expression in FNAB smears and matched surgical specimens of breast cancer. *Diagn. Cytopathol.* 1996; 14: 135–139.

18. Flens MJ, Valk Pvd, Tadema TM, et al. The contribution of immunocytochemistry in diagnostic cytology. *Cancer (Cancer Cytopathol.)* 1990; 65: 2704–2711.
19. Fowler L, Lachar W. Application of immunohistochemistry to cytology. *Arch. Pathol. Lab. Med.* 2008; 132: 373–383.
20. Bahrenburg L. On the diagnostic results of the microscopical examination of the ascitic fluid in two cases of carcinoma involving the peritoneum. *Cleve. Med. Gaz.* 1896; 11: 274–278.
21. Richardson H, Koss L, Simon T. An evaluation of the concomitant use of cytological and histocytological techniques in the recognition of cancer in exfoliated material from various sources. *Cancer* 1955; 8: 948–950.
22. Harris M. Cell block preparation: three percent bacterial agar and plasmathrombin clot methods. *Cytotech. Bull.* 1979; 15: 25–27.
23. Fetsch PA, Abati A. Immunocytochemistry in effusion cytology. *Cancer (Cancer Cytopathol.)* 2001; 93: 293–308.
24. Fetsch PA, Simsir A, Brosky K, et al. Comparison of three commonly used cytologic preparations in effusion immunocytochemistry. *Diagn. Cytopathol.* 2002; 26: 61–66.
25. DeLellis R, Hoda R. Immunocytochemistry and molecular biology in cytological diagnosis. In *Koss' Diagnostic Cytology and Its Histopathologic Bases*, 5th edition, ed. L. Koss and M. Melamed, pp. 1635–1680. Philadelphia: Lippincott Williams and Wilkins, 2006.
26. Liu H, Shi J, Wilkerson M, et al. Immunohistochemical detection of p16INK4a in liquid-based cytology specimens on cell block sections. *Cancer (Cancer Cytopathol.)* 2007; 111: 74–82.
27. Miller R, Kubier P. Immunohistochemistry on cytologic specimens and previously stained slides (when no paraffin block is available). *J. Histotechnol.* 2002; 25: 251–257.
28. Bales C. Techniques in diagnostic cytology: laboratory techniques. In *Koss' Diagnostic Cytology and Its Histopathologic Bases*, 5th edition, ed. L. Koss and M. Melamed, pp. 1569–1634. Philadelphia: Lippincott Williams and Wilkins, 2006.
29. Kulkarni M, Desai S, Ajit D, et al. Utility of the thromboplastin-plasma cell-block technique for fine-needle aspiration and serous effusions. *Diagn. Cytopathol.* 2009; 37: 86–90.
30. Nigro K, Tynski Z, Wasman J, et al. Comparison of cell block preparation methods for nongynecologic ThinPrep specimens. *Diagn. Cytopathol.* 2007; 35: 640–643.
31. Bhatia P, Dey P, Uppal R, et al. Cell blocks from scraping of cytology smear: comparison with conventional cell block. *Acta Cytol.* 2008; 52: 329–333.
32. Mayall F, Chang B, Darlington A. A review of 50 consecutive cytology cell block preparations in large general hospital. *J. Clin. Pathol.* 1997; 50: 985–990.
33. Verbeek DH, Smedts F, Wijnen-Dubbers CW, et al. Histologic processing of thick tissue specimens from cytology slides. *Acta Cytol.* 1996; 40: 1198–1204.
34. Kulkarni M, Prabhudesai N, Desai S, et al. Scrape cell-block technique for fine needle aspiration cytology smears. *Cytopathol.* 2000; 11: 179–184.
35. Nga ME, Lim G-L, Barbro N, et al. Successful retrieval of fine-needle aspiration biopsy material from previously stained smears for immunocytochemistry: a novel technique applied to three soft tissue tumors. *Mod. Pathol.* 2005; 18: 728–732.

36. Nordgren H, Nilsson S, Runn A-C, et al. Histopathological and immunohistochemical analysis of lung tumors: description of a convenient technique for use with fine needle biopsy. *APMIS* 1989; 97: 136–142.
37. Musso C, Silva-Santos M, Pereira F. Cotton block method: one-step method of cell block preparation after fine needle aspiration. *Acta Cytol.* 2005; 49: 22–26.
38. Krogerus L, Anderson L. A simple method for the preparation of paraffin-embedded cell blocks from fine needle aspirates, effusions and brushings. *Acta Cytol.* 1988; 32: 585–587.
39. Cellient: automated cell block system. Hologic, Inc., Bedford, MA, USA. <http://www.cellientsystem.com/>.
40. Dabbs D, Wang X. Immunocytochemistry on cytologic specimens of limited quantity. *Diagn Cytopathol.* 1998; 18: 166–169.
41. Weintraub J, Redard M, Wenger D, et al. The application of immunocytochemical techniques to routinely fixed and stained cytologic specimens. *Pathol. Res. Pract.* 1990; 186: 658–665.
42. Brown G, Tao L. Restoration of broken cytology slides and creation of multiple slides from a single smear preparation. *Acta Cytol.* 1992; 36: 259–268.
43. Sherman M, Jimenez-Joseph D, Gangi M, et al. Immunostaining of small cytologic specimens. Facilitation with cell transfer. *Acta Cytol.* 1994; 38: 18–22.
44. Gong Y, Joseph T, Sneige N. Validation of commonly used immunostains on cell-transferred cytologic specimens. *Cancer (Cancer Cytopathol.)* 2005; 105: 158–164.
45. Jimenez-Joseph D, Gangi M. Application of diatex compound in cytology: use in preparing multiple slides from a single routine smear. *Acta Cytol.* 1986; 30: 446–447.
46. Zu Y, Gangi M, Yang G. Ultrafast Papnicolaou stain and cell-transfer technique enhance cytologic diagnosis of Hodgkin lymphoma. *Diagn. Cytopathol.* 2002; 27: 308–311.
47. Furuya T, Ikemoto K, Kawauchi S, et al. A novel technology allowing immunohistochemical staining of a tissue section with 50 different antibodies in a single experiment. *J. Histochem. Cytochem.* 2004; 52: 205–210.
48. Gong Y, Symmans W, Krishnamurthy S, et al. Optimal fixation conditions for immunocytochemical analysis of estrogen receptor in cytologic specimens of breast carcinoma. *Cancer (Cancer Cytopathol.)* 2004; 102: 34–40.
49. Ganjei-Azar P, Nadji M. *Color Atlas of Immunocytochemistry in Diagnostic Cytology*. New York: Springer, 2007.
50. Fulciniti F, Fangella C, Staiano M, et al. Air-dried smears for optimal diagnostic immunocytochemistry. *Acta Cytol.* 2007; 52: 178–186.
51. Wolff A, Hammond M, Schwartz J, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J. Clin. Oncol.* 2007; 25: 118–145.
52. Gatscha R. Selection of techniques. In *A Guide to Cytopreparation*, ed. KA Allen, pp. 43–57. Raleigh, NC: American Society for Cytotechnology (ASCT), 1995.
53. Nadji M, Gangi M. Immunocytochemistry in diagnostic cytology: a 12-year perspective. *Am. J. Clin. Pathol.* 1990; 94: 470–475.

54. Wolff A, Hammond M, Schwartz J, et al. American Society of Clinical Oncology/ College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med.* 2007; 131: 18–43.
55. Beatty BG, Bryant R, Wang W, et al. Her-2/neu detection in fine-needle aspirates of breast cancer: fluorescence in situ hybridization and immunocytochemical analysis. *Am. J. Clin. Pathol.* 2004; 122: 246–255.
56. Shi S-R, Key M, Kalra K. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.

CHAPTER 14

DESIGN OF A TISSUE SURROGATE TO EXAMINE ACCURACY OF PROTEOMIC ANALYSIS

CAROL B. FOWLER, JEFFREY T. MASON, and TIMOTHY J. O'LEARY

14.1 INTRODUCTION

Many diseases are characterized by the expression of specific proteins¹; in some cases, malignant cells yield unique “protein profiles” when total cellular protein extracts are analyzed by proteomic methods such as two-dimensional gel electrophoresis or matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS).² High-throughput proteomic studies may be useful to differentiate normal cells from cancer cells, to identify and define the use of biomarkers for specific cancers, and to characterize the clinical course of disease. Proteomics can also be used to isolate and characterize potential drug targets and to evaluate the efficacy of treatments.

When fresh or frozen tissue is used for proteomic analyses, the results cannot be related directly to the clinical course of diseases in a timely manner. Instead, researchers frequently reduce the number of “interesting” proteins to a manageable number and then attempt to use immunohistochemistry to understand the implications of proteomic changes in archival formalin-fixed, paraffin-embedded (FFPE) tissue for which the clinical course has been established.³ Unfortunately, immunohistochemistry is a semiquantitative proteomic method, and the choice of “interesting” proteins must occur without advance knowledge of the clinical course of the disease or the response to therapy. If routinely fixed and embedded archival tissues could be used for standard proteomic methods such as 2-D gel electrophoresis and mass spectrometry (MS), these powerful techniques could be used to both qualitatively and quantitatively analyze large numbers of tissues for which the clinical course has been established. However, analysis of archival FFPE tissues by

high-throughput proteomic methods is hampered by inefficient methods to extract proteins from archival tissue and by the adverse effects of formalin fixation.⁴

Formaldehyde fixes proteins in tissue by reacting with basic amino acids—such as lysine,^{5–7}—to form methylol adducts. These adducts can form cross-links through Schiff base formation. Both intra- and intermolecular cross-links are formed,⁸ which may destroy enzymatic activity and often immunoreactivity. These formaldehyde-induced modifications reduce protein extraction efficiency and may also lead to the misidentification of proteins during proteomic analysis.

Several proteomic studies using archival FFPE tissues were reported in recent years, and two review articles on this subject were published in 2008.^{9,10} The majority of the proteomic studies on FFPE tissues employ protein extraction methods that are derived from heat-induced antigen retrieval (HAR) techniques originally developed for immunohistochemistry.^{11–17} A number of groups have successfully used a variation of the protocol first reported by Ikeda et al.¹⁸ and Shi et al.,¹² in which tissue is extracted at 100°C for 20 min, followed by 60°C for 2 h in an sodium dodecyl sulfate (SDS)-containing buffer. Other published extraction techniques utilize heating in 6M guanidine HCl,¹³ direct digestion of the tissue without an intermediate heating step,¹³ or digestion of the tissue after heating.¹⁹ These studies, though encouraging, highlight a number of challenges, such as incomplete or selective recovery of proteins and the incomplete reversal of formaldehyde modifications that must be addressed to develop better and more reproducible protocols for performing proteomics analysis on archival tissues. The ability to rapidly evaluate sample preparation protocols for their efficacy would be beneficial to the development of improved methods for the extraction of proteins from archival tissue. However, proteomic studies involving whole tissues require time-consuming methods, such as MS, to identify their constituent proteins and thus are inefficient as a format for screening recovery conditions. A simplified model system for FFPE tissue would streamline sample analysis and allow the identification of optimal recovery conditions and the identification of remaining formalin-induced protein modifications.

14.2 STUDIES WITH FFPE CELL BLOCKS AND GEL-EMBEDDED PROTEINS

Studies of formalin-fixed proteins generally fall into three categories: (1) studies of proteins or peptides in solution^{6,7,20,21}; (2) model peptides attached to solid matrices for antigenic epitope studies²²; (3) or proteomic studies on whole FFPE tissue. Another approach, reported by Ronci et al.,²³ utilized a tissue surrogate formed by embedding a 10% (w/v) bovine serum albumin (BSA) solution in agarose or polyacrylamide gels. The gels were fixed in 10% buffered formalin overnight, dehydrated through a series of ethanols, and

paraffin-embedded. The FFPE gels were either left untreated, or were or incubated with 0.1 M EDTA, pH 8.4 for 30 min at 98°C prior to trypsin digestion and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging-MS analysis. The total number of signals obtained from the analysis of the fixed, gel-embedded BSA was slightly lower than that obtained for the unfixed BSA surrogate (~70 signals vs. 80 for the acrylamide gel surrogate, respectively). Subjecting the FFPE gel to the HIAR step in the EDTA solution increased the total number of signals to ~120, an improvement over even the unfixed BSA sample. The mascot scores for the unfixed and HIAR unlocked samples were comparable, as was total sequence coverage (percent theoretical tryptic peptides identified).

Crockett et al.,²⁴ utilized an FFPE cell block in an attempt to develop an improved approach for tandem mass spectrometry (MS/MS)-based identification of proteins in FFPE material. Lymphoma cell lines were mixed with thromboplastin-DS, and then formalin-fixed and paraffin embedded. The cell blocks were stored at room temperature for up to 3 years before further analysis. The cell blocks were cleared of paraffin in xylenes, rehydrated through a series of graded alcohols, and then resuspended in RIPA lysis buffer on ice. The cell lysates were digested overnight at 37°C with sequencing grade trypsin. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to compare proteins identified in a fresh cell lysate from an equal number of cells with those from the same cells processed as the FFPE cell plug. There were 514 proteins identified in the fresh cell lysate, and 324 proteins were identified in the FFPE cell block. There were 263 proteins common to both samples. However, 251 proteins (48.8%) identified in the fresh cell lysate were not seen in the FFPE cells, and 61 proteins (18.8%) were only found in the FFPE cell lysate. This result suggests incomplete, and possibly selective, protein recovery from the FFPE cells and misidentification of proteins, possibly due to the failure to completely reverse formaldehyde–protein modifications.

A comparative study of published protein extraction methods for FFPE tissue also suggests that incomplete protein recovery may hinder the analysis of archival material.²⁵ HeLa cells fixed in 10% buffered formalin were mixed with an equal volume of 2% agarose. The agarose plugs were gelled at 4°C overnight to complete the fixation process, dehydrated through a series of graded alcohols, and embedded in paraffin. After storage at room temperature for several days, the cell pellets were incubated through two changes of xylenes to remove the paraffin and then rehydrated through graded alcohols prior to resuspension in an array of protein retrieval buffers (Table 14.1). The most effective protein extraction protocol studied was heating at 20mM Tris–HCl containing 2% SDS, with 35% total cellular protein solubilized at pH 6,¹² relative to a fresh cell lysate. RIPA buffer containing 2% SDS extracted 15% of total cellular protein.¹⁸ HeLa–agarose cell plugs extracted with a commercially available FFPE tissue extraction buffer¹⁹ recovered 5% of the total protein.

TABLE 14.1 Extraction of Proteins from HeLa Cells Fixed in a 1% Agarose Plug

Recovery Buffer	Temperature/Time	% Recovery (<i>n</i> =3)
20mM Tris–HCl+2% SDS, pH4 ¹²	100°C for 20min/60°C for 2h	15 ± 3.3
20mM Tris–HCl+2% SDS, pH6 ¹²	100°C for 20min/60°C for 2h	35 ± 2.9
20mM Tris–HCl+2% SDS, pH9 ¹²	100°C for 20min/60°C for 2h	18 ± 1.4
Liquid tissue buffer ¹⁹	95°C for 90min	5.3 ± 1.5
RIPA ¹⁸	100°C for 20min/60°C for 2h	15 ± 2.0

Notes: HeLa cells (1×10^6) were formalin-fixed in an equal volume of 1% agarose. After histological processing and paraffin embedding, the cell plugs were rehydrated and resuspended in the indicated buffer. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean, $\pm$ the standard deviation and relative to a fresh cell lysate from the sample number of cells (for more detail, see Reference 25).

14.3 STUDIES WITH TISSUE SURROGATES

As the above studies suggest, even a block composed of a single cell line can be too complex for the rapid evaluation of protein extraction methods for FFPE material, requiring LC-MS/MS or 2-D gel electrophoresis for analysis. We have recently developed a procedure for the formation of a “tissue surrogate” as a model system for studying protein recovery from archival FFPE tissues, which can be used to efficiently evaluate the efficacy of tissue extraction protocols for proteomic studies.²⁵ High concentrations of cytoplasmic proteins, such as lysozyme or ribonuclease A, are fixed with 10% neutral buffered formalin (NBF). These protein plugs have sufficient physical integrity to be processed through graded alcohols, xylene, paraffin embedding, and “tissue” sectioning according to standard histological procedures, and unlike gel-embedded cells plugs or proteins, more closely mimic tissue in physical properties. Using tissue surrogates formed in this manner, it is possible to quickly evaluate tissue extraction protocols for efficiency by routine 1-D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and to more easily identify formaldehyde adducts since the tissue surrogates are comprised of only one, or at most a few, proteins.

We found that solutions of hen egg white lysozyme, bovine ribonuclease A (RNase A), or a 1:2 mol ratio of bovine carbonic anhydrase:lysozyme formed opaque gels within 2min when mixed with an equal volume of 20% NBF.^{25,26} Multi-protein tissue surrogates comprised of 50% w/v lysozyme and up to four additional proteins have also been formed (Fowler et al., unpublished results). After overnight fixation, the surrogates were firm and sliced easily with a razor blade for sampling. To determine the optimal

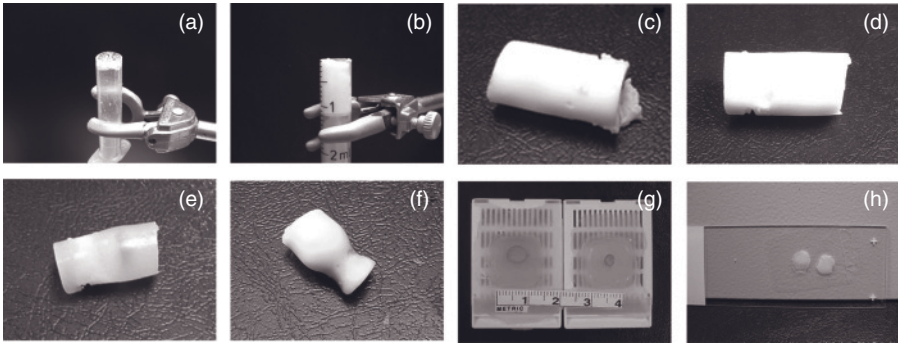


Figure 14.1 Illustration of the tissue surrogate method. (a) Solution of lysozyme (150 mg/mL) immediately after mixing with an equal volume of 20% formalin. (b) The mixture after 24 h of fixation. (c) The tissue surrogate extruded from the syringe. (d) The tissue surrogate after processing through a series of graded alcohols. (e) The tissue surrogate after processing in xylene. (f) The tissue surrogate after incubation in hot liquid paraffin. (g) Side-by-side comparison of surrogates incubated for 30 min (left) and overnight (right) in 100% ethanol. The tissue surrogates were stained in a 0.001% Eosin Y solution for 30 min prior to paraffin embedding. (h) Pair of tissue surrogates after 50 μ sectioning and mounting on a glass slide. For more detail, see Reference 25.

histological processing conditions, lysozyme tissue surrogates were formed, as shown in Figure 14.1a–c, and processed through a series of graded alcohols (Fig. 14.1d) and xylene (Fig. 14.1e) before paraffin embedding. When we passed lysozyme tissue surrogates through graded alcohols and xylene for 10 min per treatment, they shrank by over 66%, hardened noticeably upon paraffin embedding, and continued to shrink further following paraffin embedding (Fig. 14.1f). The surrogates also took on a waxy, semi-transparent appearance after treatment with xylene. Increasing the processing time to a 30-min incubation through each of the graded alcohols and xylene did not prevent the FFPE tissue surrogate from shrinking. However, extending the final 100% ethanol step to an overnight incubation reduced shrinkage considerably, and the surrogate was of a more uniform consistency. Figure 14.1g illustrates the differences between the surrogates processed through ethanol overnight or just 30 min. The reason for this behavior is not clear. It is possible that prolonged incubation in ethanol is required to completely dehydrate the protein plug which, in turn, prevents shrinkage during the remaining histological steps. Samples of the tissue surrogates can be stored indefinitely or prepared for protein recovery by clearing the paraffin through two changes of xylenes and rehydrating the surrogates through a series of graded alcohols (100%, 85%, and 70%, followed by incubation in water).

14.4 EVALUATION OF THE EFFECTS OF HISTOLOGICAL PROCESSING ON TISSUE SURROGATES

To determine the effects of fixation and histological processing on the components of the tissue surrogate, samples of the lysozyme surrogate processed through to formalin only, 100% ethanol overnight, xylenes, or paraffin embedding were rehydrated and resuspended in a solution of 20mM Tris-HCl, supplemented with 2% SDS, as described by Shi and Taylor.¹² After heating at 100°C for 20min, followed by 60°C for 2h, the solubilized lysozyme surrogates were analyzed by SDS-PAGE, and the total protein content was assessed colorimetrically. At pH 4, 84–95% of the total protein was successfully solubilized. All samples showed intermolecular cross-links, with the formalin-only treated surrogate composed of a mixture of monomer, dimer, trimer, and tetramer species (Fig. 14.2, lane 1). Pentameric and hexameric lysozyme was present in the samples processed through 100% ethanol and xylene, while processing through to the paraffin-embedding stage resulted in highly cross-linked species, with oligomers in excess of 100kDa (Fig. 14.2, lane 4).²⁵ As demonstrated by SDS-PAGE, the formaldehyde-induced cross-links

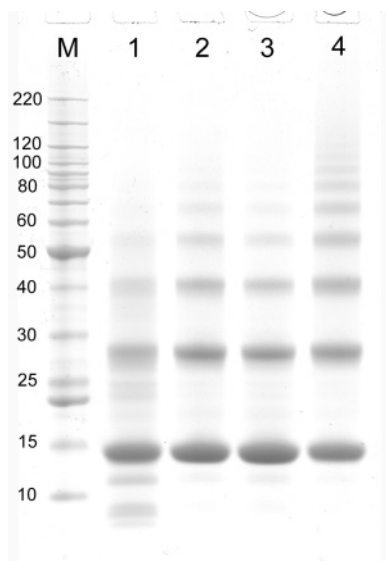


Figure 14.2 SDS-PAGE of proteins extracted from lysozyme tissue surrogates processed to different points. Lane M, molecular weight marker; lane 1, surrogate after formalin fixation; lane 2, surrogate after processing through a graded alcohol series; lane 3, surrogate after processing in xylene; lane 4, surrogate after paraffin embedding. The surrogates were rehydrated and subjected to a protocol of heating at 100°C for 20min followed by a cycle of heating at 60°C for 2h, in 20mM Tris-HCl, pH 4.0, with 2% SDS. For more detail, see Reference 25.

in the formalin-fixed surrogate were not reversed after heating, which is contrary to previous findings in studies of proteins that were fixed at lower concentrations.^{20,21}

14.5 EFFECTS OF DETERGENT AND TEMPERATURE ON RECOVERY EFFICIENCY

To optimize protein recovery from the FFPE tissue surrogates, a number of variables can be examined, including pH, the use of detergent, the use of protein denaturants and reducing agents, and temperature. We evaluated a number of HIAR techniques that have been applied in immunohistochemistry for FFPE tissues.^{12,20} These results are summarized in Table 14.2. Heating deparaffinized lysozyme tissue surrogates in 20mM Tris–HCl, at pH 4, 6, or 9, at 100°C for 20min, followed by incubation 60°C for 2h, resulted in the solubilization of only 2–6% of the total protein. The addition of 2% SDS to the above protocol improved protein solubilization by more than 15-fold, with >80% of total lysozyme recovered from the tissue surrogate. At pH 4, a further increase in recovery efficiency of ~10% was observed for surrogates retrieved in 20mM Tris–HCl, pH 4 with 2% SDS supplemented with 0.2M glycine. These results are consistent with previous surveys of protein retrieval techniques from archival FFPE human tissues.¹² Significant proteolysis was evident in tissue surrogate sections recovered at pH values less than 3 or greater than 9 (data not shown). It should be noted that pH values of 4 and 6 are outside the optimal buffering range of Tris–HCl. These conditions were analyzed because they have been used in HIAR methods reported in the literature.¹²

TABLE 14.2 Effects of Detergent and pH on the Recovery of Protein from FFPE Lysozyme Tissue Surrogates

Recovery Buffer	% Recovery (n=3)
20mM Tris–HCl, pH4	5.8 ± 0.50
20mM Tris–HCl, pH6	3.1 ± 0.50
20mM Tris–HCl, pH9	2.3 ± 0.40
20mM Tris–HCl+2% SDS, pH4	83 ± 10
20mM Tris–HCl+2% SDS, pH6	88 ± 4.1
20mM Tris–HCl+2% SDS, pH9	84 ± 5.8
20mM Tris–HCl+2% SDS+0.2M glycine, pH4	95 ± 6.7
20mM Tris–HCl+2% SDS+0.2M glycine, pH6	81 ± 9.3
20mM Tris–HCl+2% SDS+0.2M glycine, pH9	79 ± 7.2

Notes: Lysozyme tissue surrogate samples (1.5mg) histologically processed to paraffin embedding were rehydrated and resuspended in the indicated recovery buffer. Total protein in the supernatants was assessed colorimetrically after heating at 100C for 20min, followed by 60C for 2h. The % recovery values are the mean, ± the standard deviation. For more detail, see Reference 25.

TABLE 14.3 Effect of Temperature on the Recovery of Protein from FFPE Lysozyme Tissue Surrogates

Temperature/Time	% Recovery (n=3)
60°C for 2 h	26 ± 2.7
80°C for 2 h	64 ± 2.4
100°C for 30 min	67 ± 3.7
100°C for 20 min/60°C for 2 h	83 ± 10

Notes: Lysozyme tissue surrogate samples (1.5 mg) histologically processed to paraffin-embedding were rehydrated and resuspended in 20 mM Tris-HCl+2% SDS, pH4. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean, ± the standard deviation. For more detail, see Reference 25.

Heating time and temperature also affect protein recovery efficiency as shown in Table 14.3. Heating lysozyme tissue surrogate samples for 2 h at 60–65°C in 20 mM Tris-HCl, pH 4 with 2% SDS solubilized only ~25% of the total protein. Increasing the recovery temperature to 80–100°C improved the extent of protein recovery to >60%. Optimal protein solubilization was achieved using a thermal program that consisted of incubating the tissue surrogates at 100°C for 20 min, followed by a cycle of heating at 60°C for 2 h.^{12,18} This resulted in >80% of total protein recovered from the surrogate samples.

14.6 EFFECTS OF OTHER BUFFER FORMULATIONS ON RECOVERY EFFICIENCY

The surrogates can also be used to rapidly evaluate additional conditions from published antigen retrieval and FFPE proteomic tissue studies for efficacy. These results are shown in Table 14.4. Namimatsu et al.²⁷ reported improved immunohistochemical staining of FFPE tissue sections heated in solutions of citraconic anhydride at pH 7.4. Heating lysozyme tissue surrogate samples in freshly prepared 0.05–0.1% (w/v) citraconic anhydride, pH 1–2, resulted in excellent protein recovery, with >90% of the lysozyme being solubilized. Adjusting the pH of the citraconic anhydride solutions to 7.4 decreased the protein recovery by >13-fold. SDS-PAGE of the surrogates treated with citraconic anhydride indicated the presence of ~15% monomeric protein, with ~85% of the protein remaining in the form of higher-order oligomers that were not reversed during treatment (data not shown).

Tissue surrogates heated in 6M guanidine HCl supplemented with 0.5M β-mercaptoethanol (BME), a disulfide-reducing agent, resulted in a protein

TABLE 14.4 Effect of Buffer Formulation the on Recovery of Protein from FFPE Lysozyme Tissue Surrogates

Recovery Buffer	Temperature/Time	% Recovery ($n=3$)
0.05% Citraconic anhydride, pH 2 ²⁷	100°C for 20 min/60°C for 2 h	91 $\pm$ 5.7
0.05% Citraconic anhydride, pH 7.4 ²⁷	100°C for 20 min/60°C for 2 h	6.6 $\pm$ 0.40
6M Guanidine+0.5M BME, pH 4 ²⁸	100°C for 20 min/60°C for 2 h	58 $\pm$ 2.5
20mM Tris-HCl+2% SDS+0.5M BME, pH 4	100°C for 20 min/60°C for 2 h	74 $\pm$ 2.4
RIPA buffer ¹⁸	100°C for 20 min/60°C for 2 h	2.0 $\pm$ 0.20
Liquid tissue buffer ¹⁹	95°C for 90 min	17 $\pm$ 6.3

Notes: Lysozyme tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in the indicated recovery buffer. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean, $\pm$ the standard deviation (for more detail, see Reference 25).

recovery of 58%. Recovery efficiency increased to >70% in solutions of 20 mM Tris-HCl with 2% SDS and 0.5M BME (Table 14.4). Addition of a protein denaturant such as guanidine or SDS was found to improve tissue surrogate solubility. However, reduction of disulfide bonds did not improve either protein recovery or reversal of formaldehyde cross-linkages. In heat-coagulated lysozyme, reduction of scrambled disulfide linkages is required for regeneration of native protein.²⁸ Figure 14.3 compares formalin-fixed lysozyme tissues surrogates heated in the presence of BME with a lysozyme solution that was boiled for 10 min to coagulate the protein prior to treatment with BME. After treatment with BME (Fig. 14.3, lane 2), monomeric protein, and peptide fragments resulting from protein hydrolysis were present in the heat-coagulated lysozyme sample. In contrast, oligomeric protein remained in the FFPE tissue surrogate after treatment with the reducing agent (Fig. 14.3, lane 1). Thus, any increased protein flexibility brought about by the elimination of disulfide linkages did not facilitate the reversal of the formaldehyde cross-linkages.

Several methods for extracting soluble protein from archival FFPE tissue for proteomic studies were reported in recent years. Results obtained from applying these methods to lysozyme tissue surrogates are shown in Table 14.4. Heating samples of the lysozyme tissue surrogate in RIPA buffer at 100°C for 20 min, followed by a 2-h incubation at 60°C recovered only 2% of the surrogate protein.¹⁸ Extraction of the FFPE tissue surrogate using a commercially available FFPE tissue extraction buffer¹⁹ yielded only about 17% solubilized lysozyme.

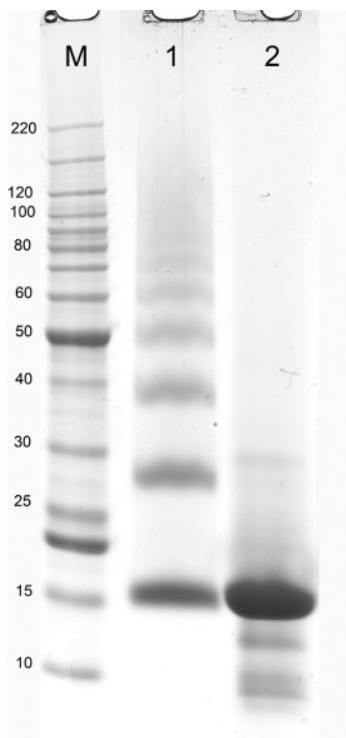


Figure 14.3 SDS-PAGE of recovery of lysozyme in the presence of BME. Lane M, molecular weight marker; lane 1, FFPE lysozyme tissue surrogate; lane 2, a 75 mg/mL solution of lysozyme heat coagulated for 10 min at 100°C in 10 mM sodium phosphate buffer, pH 7.4. Both preparations were resuspended in 20 mM Tris-HCl, pH 4, with 2% SDS and 0.5 M BME, and heated at 100°C for 20 min followed by a cycle of heating at 60°C for 2 h. For more detail, see Reference 25.

14.7 STUDIES WITH TISSUE SURROGATES FORMED FROM OTHER PROTEINS

Comparative extraction studies on tissue surrogates formed from one or more other proteins were performed to further evaluate the utility of tissue surrogates as a model for FFPE tissues. The results of these studies are shown in Table 14.5. Tissue surrogates produced from 75 mg/mL solutions of RNase A formed oligomeric complexes similar to the fixed lysozyme solutions, and formed solid tissue surrogates after a 1–2 min fixation in buffered formalin. Heating the deparaffinized surrogate sections in 20 mM Tris-HCl, pH 4, with 2% SDS for 20 min at 100°C, with a subsequent heating cycle at 60°C for 2 h, recovered the greatest amount of protein (81%). The RNase A or lysozyme surrogates were of similar consistency and, after deparaffinization and recovery, exhibited similar gel banding patterns²⁵ (data not shown). In both surro-

TABLE 14.5 Protein Extraction Efficiency from Tissue Surrogates Formed from Other Proteins

Tissue Surrogate	pH of Recovery Buffer	% Recovery ($n=3$)
RNase A	4	81 $\pm$ 12
RNase A	6	79 $\pm$ 2.3
RNase A	9	65 $\pm$ 12
Carbonic anhydrase:lysozyme	4	81 $\pm$ 7.5
Carbonic anhydrase:lysozyme	6	68 $\pm$ 3.1
Carbonic anhydrase:agarose	4	30 $\pm$ 2.5
Carbonic anhydrase:agarose	6	46 $\pm$ 12

Notes: Tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in recovery buffer (20 mM Tris-HCl+2% SDS) at the indicated pH. Total protein in the supernatants was assessed colorimetrically after heating at 100°C for 20 min, followed by 60°C for 2 h. The two-protein tissue surrogates were composed of carbonic anhydrase:lysozyme (2:1 mol/mol). The % recovery values are the mean, $\pm$ the standard deviation. For more detail, see Reference 25.

gates retrieved at pH 4.0, there was ~15% monomeric protein and ~85% higher order oligomers, indicating the presence of intermolecular formaldehyde-induced cross-links.

While aqueous lysozyme ($pI = 11.0$) or RNase A ($pI = 9.45$) solutions at 75 mg/mL formed solid gels upon formalin fixation, a solution of carbonic anhydrase ($pI = 6.0$) did not gel after 24 h. This suggested that the isoelectric point of the protein may affect its ability to form tissue surrogates. However, a surrogate consisting of 33 mol % carbonic anhydrase and 66 mol % lysozyme formed a solid gel within 1–2 min. In the mixed carbonic anhydrase:lysozyme tissue surrogate, analysis of the surrogate was complicated by the presence of two proteins, indicating that further analysis by 2-D gel electrophoresis or MS may be necessary to fully identify all of the protein components (Fig. 14.4). In samples extracted at pH 4.0, ~72% of total protein corresponded to monomeric lysozyme, while monomeric carbonic anhydrase and a band of the correct size for a lysozyme:carbonic anhydrase heterodimer accounted for 19% and 3.5%, respectively. In the mixed surrogate extracted at pH 6.0, there was a relatively greater concentration of heterodimeric protein, as well as possible minor higher-order oligomers.

For the mixed surrogate, 82% of the total protein was recovered in the 20 mM Tris-HCl buffer with 2% SDS at pH 4, but the total protein recovery decreased to 68% when the pH was increased to 6. A greater percentage of carbonic anhydrase was recovered at pH 6 than at pH 4, indicating that the recovery of individual proteins may be dependent upon pH. The pH-dependent recovery from a single-protein-agarose plug formed by fixing carbonic anhydrase in a 1% agarose matrix supports this hypothesis. In recovery trials with the carbonic anhydrase:agarose tissue surrogate, 46% of total carbonic anhydrase was recovered from the agarose plug at pH 6, as opposed to only ~30% recovery observed at pH 4.

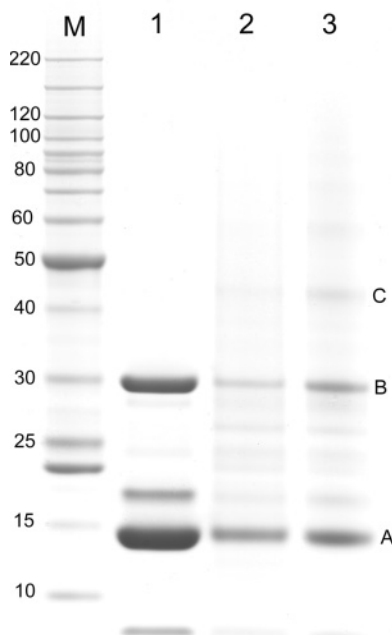


Figure 14.4 Gel image of proteins extracted from a mixed carbonic anhydrase:lysozyme tissue surrogate. Lane M, molecular weight marker; lane 1, a 1:2 mol ratio mixture of native, non-formalin-treated carbonic anhydrase and lysozyme; lane 2, mixed surrogate with 1:2 mol ratio carbonic anhydrase:lysozyme, solubilized and retrieved in 20mM Tris-HCl, pH 4.0, with 2% SDS; lane 3, mixed surrogate with 1:2 mol ratio carbonic anhydrase:lysozyme, solubilized and retrieved in 20mM Tris-HCl, pH 6.0, with 2% SDS. Protein bands corresponding to lysozyme monomer (a), carbonic anhydrase monomer (b), and the putative lysozyme-carbonic anhydrase heterodimer (c) are indicated. For more detail, see Reference 25.

14.8 CONCLUSION

In summary, studies carried out with tissue surrogates²⁵ highlight some of the problems that must be overcome before proteins extracted from FFPE tissues can be used for routine proteomic studies. First, these studies demonstrate that reversal of protein-formaldehyde adducts does not assure quantitative extraction of proteins from FFPE tissues or vice-versa. It may ultimately turn out that there is no one “universal” method that can accomplish both tasks, but that instead, each step will need to be optimized separately. Studies with tissue surrogates also suggest that failure to quantitatively extract the entire protein component from FFPE tissues may result in sampling bias due to the preferential extraction of certain proteins. This behavior may be linked to protein physical properties, such as the isoelectric point. The results of our

comparative extraction studies particularly highlight this point. Tissue surrogates composed of highly basic proteins like lysozyme (pI = 11.0) or RNase A (pI = 9.45) were most successfully extracted at low pH, while FFPE agarose cell plugs or gel-embedded carbonic anhydrase were more efficiently extracted at pH 6. Multiple extraction steps (perhaps using a range of pH values) may be necessary to achieve quantitative, or at least representative, extraction of proteins from complex protein tissue surrogates and FFPE tissues.

Based on the results with tissue surrogates and FFPE cell plugs, it is clear that reversal of protein–formaldehyde modifications in the systems that we have examined requires heating at high temperatures ($\geq 100^{\circ}\text{C}$) in acidic ($\text{pH} < 5$) buffers. Indeed, this approach appears to substantially reverse formaldehyde-induced protein modifications (data not shown).^{25,26} Unfortunately, these conditions also promote deleterious protein modifications. These pH-dependent modifications include beta-elimination of cysteine residues, deamidation of asparagine and glutamine residues, and hydrolysis of peptide bonds at aspartic acid residues.^{29,30} The hydrolysis products running below the lysozyme monomer in Figure 14.3 (lane 2) represent lysozyme peptide fragments produced by such aspartic acid hydrolysis.²⁶ Accordingly, conditions will need to be developed to abrogate these modifications during reversal of the fixation process,³¹ or it will be necessary to account for them during proteomic analysis.³²

The tissue surrogates described here clearly represent a simplification of real FFPE tissues. However, they represent a useful and efficient construct for the evaluation and optimization of tissue extraction conditions for proteomic studies. More informative studies will likely be realized by using more complex tissue surrogates, which can be created by incorporating additional proteins into lysozyme solutions. Tissue surrogates comprised of up to five proteins have been successfully analyzed by MS (Fowler, unpublished data). Additionally, RNA, DNA, lipids, or carbohydrates can be added at nanomolar to millimolar concentrations to increase the complexity of the model system to better mimic whole tissue. The use of these more complex tissue surrogates should facilitate the development of protein recovery protocols optimal for proteomic investigation.

REFERENCES

1. Chong BE, Lubman DM, Miller FR, et al. Rapid screening of protein profiles of human breast cancer cell lines using non-porous reversed-phase high performance liquid chromatography separation with matrix-assisted laser desorption/ionization time-of-flight mass spectral analysis. *Rapid Commun. Mass Spectrom.* 1999; 13: 1808–1812.
2. Chaurand P, Stoeckli M, Caprioli RM. Direct profiling of proteins in biological tissue sections by MALDI mass spectrometry. *Anal. Chem.* 1999; 71: 5263–5270.
3. Becker KF, Schott C, Hipp S, et al. Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J. Pathol.* 2007; 211: 370–378.

4. Conti CJ, Larcher F, Chesner J, et al. Polyacrylamide gel electrophoresis and immunoblotting of proteins extracted from paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 1988; 36: 547–550.
5. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.
6. Metz B, Kersten GFA, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
7. Metz B, Kersten GFA, Baart GJ, et al. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug. Chem.* 2006; 17: 815–822.
8. Mason JT, O’Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
9. Stewart NA, Veenstra TD. Sample preparation for mass spectrometry analysis of formalin-fixed paraffin-embedded tissue: proteomic analysis of formalin-fixed tissue. *Methods Mol. Biol.* 2008; 425: 131–138.
10. Nirmalan NJ, Harnden P, Selby PJ, et al. Mining the archival formalin-fixed paraffin-embedded tissue proteome: opportunities and challenges. *Mol. Biosyst.* 2008; 4: 712–720.
11. Hood BL, Darfler MM, Guiel TG, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics* 2005; 4: 1741–1753.
12. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
13. Jiang X, Jiang X, Feng S, et al. Development of efficient protein extraction methods for shotgun proteome analysis of formalin-fixed tissues. *J. Proteome Res.* 2007; 6: 1038–1047.
14. Palmer-Toy DE, Krastins B, Sarracino DA, et al. Efficient method for the proteomic analysis of fixed and embedded tissues. *J. Proteome Res.* 2005; 4: 2404–2411.
15. Guo T, Wang W, Rudnick PA, et al. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. *J. Histochem. Cytochem.* 2007; 55: 763–772.
16. Hwang SI, Thumar J, Lundgren DH, et al. Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. *Oncogene* 2006; 26: 65–76.
17. Patel V, Hood BL, Molinolo AA, et al. Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. *Clin. Cancer Res.* 2008; 14: 1002–1014.
18. Ikeda K, Monden T, Kanoh T, et al. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 1998; 46: 397–403.
19. Prieto DA, Hood BL, Darfler MM, et al. Liquid tissue: proteomic profiling of formalin-fixed tissues. *Biotechniques* 2005; 38 (Supl.): 32–35.

20. Rait VK, O'Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
21. Rait VK, Xu L, O'Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A: II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
22. Sompuram SR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
23. Ronci M, Bonanno E, Colantoni A, et al. Protein unlocking procedures of formalin-fixed paraffin-embedded tissues: application to MALDI-TOF imaging MS investigations. *Proteomics* 2008; 8: 3702–3714.
24. Crockett DK, Lin Z, Vaughn CP, et al. Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Lab. Invest.* 2005; 85: 1405–1414.
25. Fowler CB, Cunningham RE, O'Leary TJ, et al. “Tissue surrogates” as a model for archival formalin-fixed paraffin-embedded tissues. *Lab. Invest.* 2007; 87: 836–846.
26. Fowler CB, Cunningham RE, Waybright TJ, et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. *Lab. Invest.* 2008; 88: 185–195.
27. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.
28. Buttkus H. On the nature of the chemical and physical bonds which contribute to some structural properties of protein foods: a hypothesis. *J. Food Sci.* 1974; 39: 484–489.
29. Tomizawa H, Yamada H, Imoto T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 degrees C. *Biochemistry* 1994; 33: 13032–13037.
30. Zale SE, Klibanov AM. Why does ribonuclease irreversibly inactivate at high temperatures? *Biochemistry* 1986; 25: 5432–5444.
31. Tomizawa H, Yamada H, Wada K, et al. Stabilization of lysozyme against irreversible inactivation by suppression of chemical reactions. *J. Biochem. (Tokyo)* 1995; 117: 635–640.
32. de Koning LJ, Kasper PT, Back JW, et al. Computer-assisted mass spectrometric analysis of naturally occurring and artificially introduced cross-links in proteins and protein complexes. *Eur. J. Biochem.* 2006; 273: 281–291.

PART IV

MOLECULAR MECHANISM OF ANTIGEN RETRIEVAL TECHNIQUE

CHAPTER 15

STUDY OF FORMALIN FIXATION AND HEAT-INDUCED ANTIGEN RETRIEVAL

JEFFREY T. MASON, CAROL B. FOWLER, and TIMOTHY J. O'LEARY

15.1 INTRODUCTION

In 1991 Shi et al. published their seminal observation that high-temperature incubation of formalin-fixed, paraffin-embedded (FFPE) tissue sections in buffers for short periods led to improved immunohistochemical staining.¹ However, more than 15 years later, heat-induced antigen retrieval (AR) remains largely an empirical procedure, requiring the optimization of several critical parameters by trial and error.^{2,3} Further improvements in AR will require an in-depth understanding of the chemistry of formaldehyde fixation and the molecular mechanism(s) underlying the AR method.

It is commonly assumed that decreased immunoreactivity in fixed tissues results from formaldehyde-induced cross-linking. However, cross-linking can affect protein immunoreactivity on many levels. The extremely high concentration of proteins and other solutes within tissues^{4,5} leads to the formation of a dense irregular network of cross-links (gelation) that can prevent antibody penetration to the location of its antigen within the tissue.⁶ Even if this primary barrier is partially destroyed by enzymatic or chemical treatment, additional effects of formaldehyde can impede antigen–antibody interactions. Antibody binding can be inhibited by steric hindrance (excluded volume effect) arising from the shielding of epitopes on the target antigen due to the close proximity of adjacent molecules to which the antigen is cross-linked. Immunoreactivity can be further compromised by formalin-induced chemical modifications of the amino acid residues within the epitope, by neutralization of protein electrostatic charge, and by alterations in protein secondary or tertiary structure.

Although numerous mechanisms have been put forth to explain antigen unmasking by AR methods^{1,7,8} they remain speculative because intact tissue

sections are too complex to allow for a detailed understanding of the behavior of individual protein antigens. Our laboratory has taken a reductionist approach to understanding the mechanisms of heat-induced AR by studying the effect of formalin fixation and AR methods on the chemical and conformational properties of isolated proteins and relating these findings to the immunoreactivity of the proteins.⁹⁻¹² In this chapter, we summarize our studies on the effects of formalin fixation, ethanol dehydration, and heat treatment on the structural, thermotropic, functional, and immunoreactive properties of bovine pancreatic ribonuclease A (RNase A). This approach is used as a framework to offer fundamental chemical explanations for many phenomenological observations that have been reported regarding the mechanisms of heat-induced AR. The strength of this approach is in relating the behavior of complex tissues subjected to AR methods to the easily understood properties of single proteins that have been treated with formalin. The weakness in this approach is that it almost certainly represents an oversimplification of tissue fixation and AR methods, particularly as it is based on the study of a single protein. However, these studies do present direct evidence that the reversal of formaldehyde adducts and cross-links is correlated with the restoration of immunoreactivity in a formalin-treated antigen. This suggests that the core mechanism of heat-induced AR lies in the restoration of normal protein chemical composition and electrostatic charge coupled with the removal of steric barriers to antigen-antibody binding. The protein denaturation that accompanies heat-induced AR also likely serves to enhance the presentation of linear epitopes to their target antibodies.

15.2 REACTION OF FORMALDEHYDE WITH PROTEINS

The early work of Fraenkel-Conrat and colleagues¹³⁻¹⁵ and the more recent work of Metz et al.^{16,17} have identified four types of chemical modifications following treatment of peptides or amino acids with aqueous formaldehyde. As shown in Figure 15.1, these modifications are methylol (hydroxymethyl) adducts (reaction 15.1), Schiff-bases (reaction 15.2), 4-imidazolidinone adducts (reaction 15.3), and methylene bridges [cross-links] (reaction 15.4). The hydroxymethyl and Schiff-base adducts form rapidly upon reaction of formaldehyde with primary amino or thiol groups (such as lysine and cysteine). These adducts are readily reversible, and it is not known what role, if any, they play in epitope masking or AR. The 4-imidazolidinone adduct can form at the *N*-terminal site of a protein, likely by way of a Schiff-base intermediate,¹⁸ but would appear unlikely to play a significant role in epitope masking. With regard to immunoreactivity, the most important modification of proteins induced by formaldehyde is the formation of methylene bridges (cross-links). The cross-linking process is initiated by a very fast reaction of formaldehyde with the ϵ -amino group of lysine or the β -thiol group of cysteine. In the case of lysine, the resulting hydroxymethyl group exists in equilibrium with trace

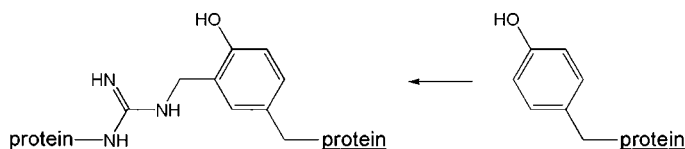
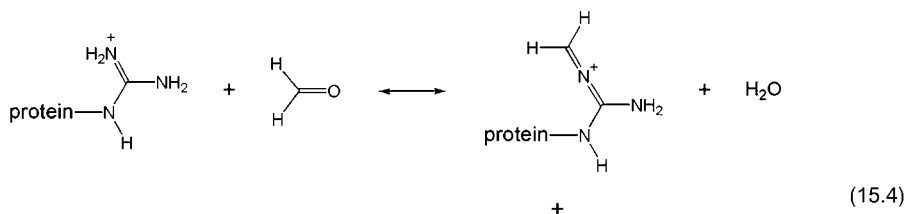
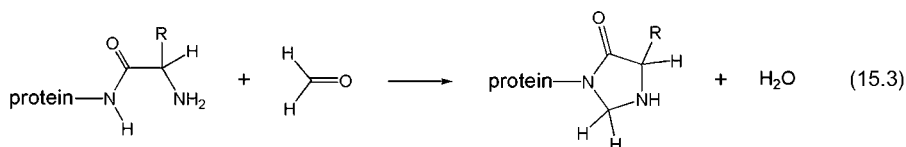
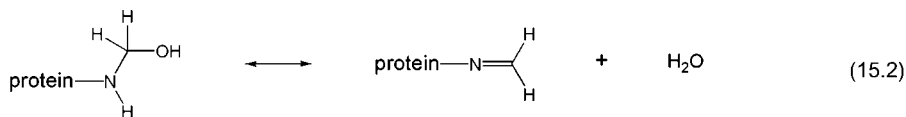
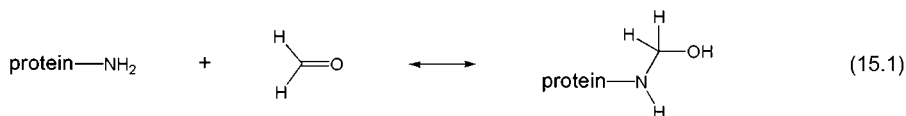


Figure 15.1 Reactions of formaldehyde with peptides and amino acids. Shown are the four types of reaction products seen when peptides or amino acids are treated with formaldehyde in aqueous solution. These reaction products are: methylol (hydroxy-methyl) adduct (reaction 15.1), Schiff-base (reaction 15.2), 4-imidazolidinone adduct (reaction 15.3), and one type of methylene bridge [cross-link] (reaction 15.4).

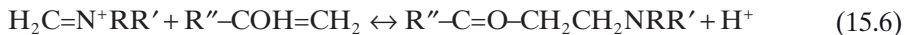
amounts of protonated Schiff-base, $-\text{N}^+\text{H}=\text{CH}_2$, and the highly reactive electrophile, $-\text{NH}-\text{C}^+\text{H}_2$, which then forms methylene bridges ($-\text{CH}_2-$) in a second reaction by attacking available nucleophiles.¹⁹ The amino acids that can serve as nucleophiles for this second reaction are tyrosine, arginine, asparagine, glutamine, histidine, and tryptophan. No methylene bridges between primary amino groups were identified in the comprehensive study by Metz et al.¹⁶

A second type of protein cross-link has been proposed to occur between a secondary amine and a carbonyl compound through the Mannich reaction²⁰

as shown in Figure 15.1 (reaction 15.4). This process starts with the reaction of formaldehyde with a secondary amine (such as the side chain of arginine) to form an iminium ion (reaction 15.5).



The iminium ion then reacts with an enol (vinyl alcohol) compound (such as the side chain of tyrosine) to form a cross-link (reaction 15.6).



This cross-link is reversible upon heating at alkaline pH²¹. Lysine and cysteine can also form a cross-link at the ortho positions of the phenolic group of tyrosine through a Schiff-base intermediate.¹⁶ In aqueous formalin solution, <1% of the solute exists as formaldehyde^{22,23} with the remainder consisting predominantly of hydrated methylene glycol, HOCH₂OH, with modest levels of divalent reactive polyacetals of variable length, HO(CH₂O)_nH, where $n = 2-8$. These polyacetals can react with the ϵ -amino group of lysine to form polyether cross-links that are stable to heat but depolymerize in acidic media.²⁴ Additional examples of protein cross-links are discussed in Metz et al.¹⁶

Most of the above reactions were identified through studies with short peptides and amino acids. Metz et al.¹⁷ investigated the reaction of formaldehyde with an intact protein by incubating insulin with aqueous formalin and examining the reaction products by liquid-chromatography-mass spectrometry of the peptide fragments obtained by proteolytic digestion of the modified protein. Four intra-peptide cross-links were identified, two between cysteine and tyrosine residues, one between a lysine and a tyrosine residue, and one between a glutamine and cysteine residue. In addition, the *N*-terminal residues of the A and B fragments were partially converted to 4-imidazolidinone adducts. When insulin was treated with formaldehyde in the presence of glycine, only 8 of the 16 reactive amino acids were modified, indicating that protein conformation likely plays an important role in determining the reactivity of specific amino acid residues and the formation of cross-links. It was further shown that the formaldehyde-treated insulin monomers formed a heterogeneous population with regard to the number and location of the formaldehyde-induced cross-links in the proteins.

There have been no comprehensive studies of how exposure to ethanol, xylene, or paraffin affects proteins following their treatment with aqueous formaldehyde. However, in a related study, Rait et al.²⁵ examined the effect of ethanol incubation on 2'-deoxyadenosine that had been treated with aqueous formaldehyde. Mass spectrometry revealed the presence of *N*⁶-ethoxymethyl adducts in addition to hydroxymethyl adducts. This led to the suggestion that tissue dehydration can result in molecular dehydration, transforming hydroxymethyl groups into Schiff-bases. In such a scheme, the bulk anhydrous ethanol acts as a medium to effectively absorb the water of the

molecular dehydration and, additionally, as an abundant nucleophile to react with the Schiff-base to form the ethoxymethyl derivative. In addition, the methylene *bis*-nucleotide was identified, which is a cross-link between primary amino groups. As stated above, such methylene bridges are not formed in aqueous formaldehyde solutions.

15.3 FORMATION OF INTRA- AND INTERMOLECULAR CROSS-LINKS IN FORMALIN-TREATED RNASE A

Bovine pancreatic RNase A is a single polypeptide composed of 124 amino acid residues²⁶ of which eight different residues—arginine, asparagine, glutamine, histidine, lysine, serine, threonine, and tyrosine—can, in principle, be modified by reaction with formaldehyde. All 10 lysine residues of RNase A are accessible for reaction with formaldehyde.²⁷ The progressive formation of RNase A intra- and intermolecular cross-links with time in neutral 10% formalin was followed by gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Figure 15.2. As shown in lane 2, RNase A dimers, trimers, and tetramers appeared after just 20 min of incubation with formalin. Lanes 3–7 reveal a steady accumulation of oligomers consisting of 2–9 cross-linked proteins over a period of 9 days and an increase in the high molecular

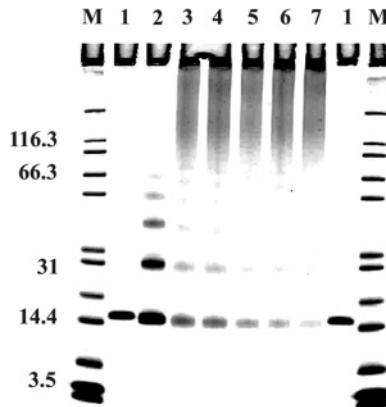


Figure 15.2 SDS-PAGE of RNase A before (lane 1) and after incubation in 10% buffered formalin for 20 min (lane 2), and 1 (lane 3), 2 (lane 4), 3 (lane 5), 6 (lane 6), and 9 days (lane 7) at 23°C. Lane M, molecular weight markers, kDa. RNase A samples at 6.5 mg/mL were freed from formaldehyde by fast thin-layer dialysis against phosphate buffered saline (PBS), denatured at 70°C for 10 min in the presence of dithiothreitol and lithium dodecyl sulfate, and fractionated according to the size of the RNase oligomers on precast Bis-Tris gradient (4–12%) polyacrylamide gels. Each gel lane was loaded with sufficient protein to allow the high molecular weight oligomers to be visible; therefore, spot intensities between lanes are not normalized to a common scale. See Rait et al.¹⁰ for details.

weight oligomeric fraction (≥ 10 cross-linked proteins) retained at the top of the gel. Simultaneously, the fraction of monomer decreases as shown by a decrease in the intensity of the band of approximately 14.4 kDa in lanes 3–7 of Figure 15.2. The only consequence of intramolecular cross-linking was an increase in the mobility of formaldehyde-modified monomeric RNase A (lanes 2–7) relative to untreated enzyme (lane 1). In the absence of disulfide bridges (due to the reducing conditions of the SDS-PAGE), the formaldehyde-treated enzyme remains more compact than the native enzyme due to the presence of intramolecular formaldehyde cross-links, and thus it runs slightly below the native protein on the gel. These findings confirm that formalin treatment induces both intra- and intermolecular protein cross-links and that the degree of cross-linking increases with time of exposure to formalin.^{10,28}

15.4 EFFECT OF FORMALIN ON THE THERMAL PROPERTIES OF RNASE A

Cross-linking constrains the conformational flexibility of biopolymers and, as a rule, stabilizes their secondary, tertiary, and quaternary structures against the denaturing effects of high temperatures.²⁹ We used differential scanning calorimetry (DSC) to compare the heat-induced conformational transitions of selected RNase A samples that were characterized in Figure 15.2. A brief introduction to DSC is provided in Section 15.15.1 for those readers unfamiliar with this biophysical method. Trace 1 in Figure 15.3a is the heat absorption

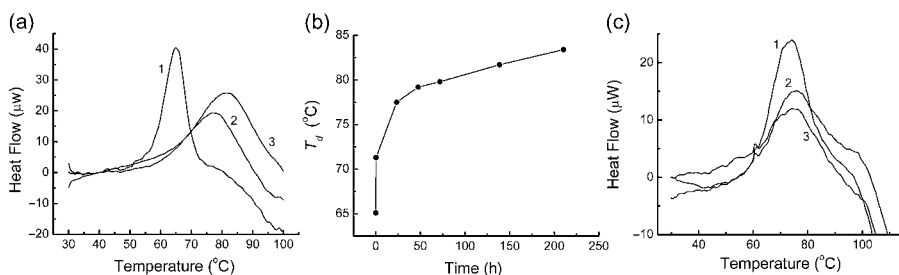


Figure 15.3 (a) Heat absorption in solutions of native RNase A (trace 1) and RNase A kept in 10% buffered formalin for 2 days (trace 2) and 6 days (trace 3) at pH 7.4 and 23°C. All samples were dialyzed against 75 mM potassium phosphate buffer (pH 7.4) prior to DSC. (b) Dependence of T_d of the dialyzed RNase A samples on time of incubation in 10% buffered formalin at pH 7.4 and 23°C. (c) Heat absorption of solutions of formalin-treated RNase A fractions isolated by size-exclusion gel chromatography: monomer (trace 1), dimer (trace 2), and a mixture of oligomers with ≥ 5 cross-linked proteins (trace 3). Protein concentrations were ~ 0.5 mg/mL. The thermal denaturation transition temperature (T_d) is defined as the temperature of the maximum in the excess heat absorption trace associated with the protein's endothermic denaturation transition. See Rait et al.¹⁰ for details.

profile of native RNase A, which exhibits a thermal denaturation transition at a temperature (T_d) of 65.1°C in neutral phosphate buffer. Traces 2 and 3 show the thermal denaturation of RNase A that was kept in 10% formalin for 2 and 6 days, respectively, and then freed from formaldehyde by dialysis. As shown, cross-linking shifts the denaturation profiles toward higher temperatures. The data shown in Figure 15.3b summarize the time course of the T_d increase over the entire period of incubation. The main increase in thermostability occurred during the first 24 h of reaction with formaldehyde.

The effect of intra- versus intermolecular cross-links on the thermal properties of formalin-treated RNase was investigated by fractionating a mixture of oligomers formed by incubating RNase A with formalin using size-exclusion gel chromatography. This resulted in a fully resolved monomer and dimer, as well as a fraction consisting of a mixture of oligomers with ≥ 5 cross-linked molecules as shown in the SDS-PAGE profile in Figure 15.4 (lanes 1–3). Fractions corresponding to the monomer, dimer, and higher oligomers were then analyzed individually by DSC as shown in Figure 15.3c. The difference in the T_d values of the modified RNase monomer (trace 1) and the higher oligomers (traces 2 and 3) was only 2–3°C. This suggests that intramolecular cross-linking is primarily responsible for the increase in protein thermostability. This idea is supported by the fact that RNase A already possesses four intrinsic disulfide cross-links: Cys26-Cys84, Cys40-Cys95, Cys58-Cys110, and Cys65-Cys72. Experiments with enzyme variants³⁰ where each cysteine was independently replaced with a pair of Ala residues showed that removing just one cross-link, either Cys26-Cys84 or Cys58-Cys110, resulted in a decrease of

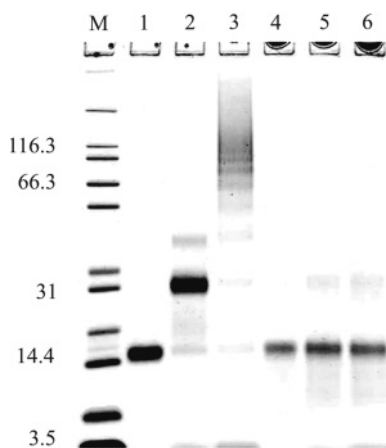


Figure 15.4 SDS-PAGE of formalin-treated RNase A fractions taken before (lanes 1–3) and after (lanes 4–6) the DSC scans shown in Figure 15.3c. Lanes 1 and 4, monomer; lanes 2 and 5, dimer; lanes 3 and 6, mixture of oligomers with ≥ 5 cross-linked proteins; M, molecular mass markers as in Figure 15.2. See Rait et al.¹⁰ for details.

the T_d by almost 40°C. SDS-PAGE of the samples prior to the DSC experiment (lanes 1–3 in Fig. 15.4) and after the DSC scans (lanes 4–6) demonstrated that heating to ~100°C almost completely reversed the cross-linking in the RNase oligomers. The lack of any bands running below monomeric RNase A indicate that heating to 100°C did not cause hydrolysis of peptide bonds resulting in chain scission.

These thermal analysis studies serve to establish a direct relationship between a heat-induced AR method and the reversal of formalin-induced intra- and intermolecular protein cross-links.^{10,28,31} Further, while formalin-treatment provides thermal stability to RNase A, this stabilization is not sufficient to prevent thermally induced protein denaturation at temperatures ($\geq 100^\circ\text{C}$) typically used in heat-induced AR methods.^{32–34} The implications of this finding for the mechanism of AR will be discussed further in Section 15.6.

15.5 EFFECT OF FORMALIN ON THE IONIZATION STATE OF RNASE A

RNase A is a basic protein with an isoelectric point (pI) of 9.45 and a net positive charge in neutral solution.³⁵ However, the conversion of positively charged lysine side chains to polar, but neutrally charged, methylol adducts would be expected to lower the pI of formalin-treated RNase A. To explore this further, RNase A was treated with 5% formalin and analyzed by isoelectric focusing (IEF) gel electrophoresis. Figure 15.5a shows that the pI values were shifted into the pH 6.0–7.4 range. Figure 15.5b shows the results of IEF

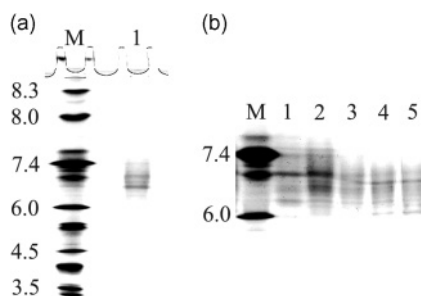


Figure 15.5 IEF gel electrophoresis of formalin-treated RNase A (a) and its fractions (b) separated by gel filtration. (a) M, IEF markers (pI); lane 1, unfractionated formalin-treated RNase A. (b) lane 1, monomer; lane 2, dimer; lane 3, trimer; lane 4, tetramer; lane 5, pentamer. IEF gel electrophoresis was performed using precast 5% polyacrylamide gels with a pI range of 3–10. The pI range for the individual oligomers in 15.5b appears greater than that for the unfractionated sample because a higher concentration of protein was used for the lanes in 15.5b. This results in an increased staining intensity for the oligomers at the pI extremes, which are minor components of the unfractionated sample in 15.5a. See Rait et al.¹⁰ for details.

gel electrophoresis on the RNase A fractions isolated by gel chromatography. The RNase oligomers with ≥ 3 protein molecules are grouped at the lower limit of this pH range, indicating the further involvement of basic amino acid residues in intermolecular cross-linking. The abundance of discrete bands seen in Figure 15.5b reflects the microheterogeneity within the fractions, analogous to the results described in Section 15.2 for formalin-treated insulin.¹⁷ Thus, formalin treatment results in protein charge neutralization through the reaction of charged amino acid side chains, such as lysine, with formaldehyde to form cross-links or adducts (methylol moieties).

Such charge neutralization may play an important role in epitope masking and the recovery of immunoreactivity by heat-induced AR as pointed out by the studies of Boenisch.^{36–38} The kinetics of antigen–antibody binding is derived largely from the complimentary surface charges of the two proteins, including the electrostatic attraction between complimentary charged amino acid side chains on the epitope of the antigen and the paratope of the antibody.³⁷ Accordingly, the mechanism of heat-induced AR may be coupled, in part, to the restoration of the protein electrostatic charges through the reversal of formaldehyde cross-links and adducts.³⁸ Because electrostatic charge appears to predominately affect the kinetics, but not the thermodynamics, of antigen–antibody binding,³⁸ the deleterious effect of charge neutralization can, in some cases, be overcome by increasing the antibody incubation time or antibody concentration resulting in staining sensitivity equivalent to that of heat-induced AR methods.^{38–41} In a related observation, the reduced staining intensity observed when using high ionic strength buffers as the antibody diluent likely results from shielding (such as by Na^+ ions) of the negative electrostatic charges on the target antigen.^{36,42,43} Although restoration of normal protein charges by AR methods plays an important role in the recovery of antigenicity, the presence of cross-links in or near the epitope will deny access to the target antibody by steric blocking as demonstrated by Sompuram et al.²⁰ Accordingly, charge restoration and cross-link reversal must work in concert to restore protein antigenicity.

15.6 EFFECT OF FORMALIN ON THE SECONDARY AND TERTIARY STRUCTURE OF RNASE A

The effect of formalin-treatment on the structural properties of RNase A was examined using circular dichroism (CD) spectropolarimetry. A brief introduction to CD spectropolarimetry is provided in Section 15.15.2 for those readers unfamiliar with this biophysical method. The secondary structure of RNase A consists of one long four-stranded anti-parallel β -sheet and three short α -helices,⁴⁴ which places RNase A in the $\alpha + \beta$ structural class of proteins. The effect of a 9-day incubation of RNase A (6.5 mg/mL) in 10% formalin on the protein secondary structure was examined with CD spectropolarimetry in the far-UV region (170–240 nm) as shown in Figure 15.6a. The resulting

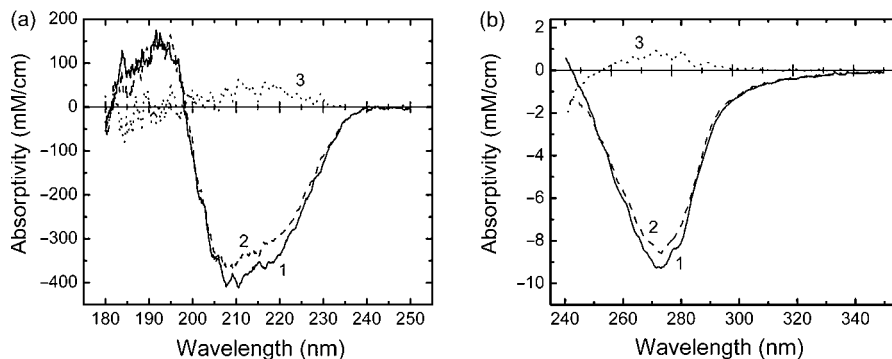


Figure 15.6 (a) Far-UV CD spectra of native RNase A (trace 1) and RNase A (6.5 mg/mL) kept in 10% buffered formalin (pH 7.4) at 23°C for 9 days and then dialyzed (trace 2), and their difference spectrum (trace 3). (b) Near-UV CD spectra of native RNase A (trace 1) and RNase A (6.5 mg/mL) kept in 10% buffered formalin (pH 7.4) at 23°C for 9 days and then dialyzed (trace 2), and their difference spectrum (trace 3). See Rait et al.¹⁰ for details.

changes are shown by the difference spectrum (trace 3) derived by subtraction of the native enzyme spectrum (trace 1) from the spectrum of the formalin-treated RNase A (trace 2). The decrease in the negative band intensity and the associated decrease in the positive band intensity are both indicative of a subtle secondary structure perturbation. Similar small changes, seen at the very beginning of the native enzyme disorder transition, are likely associated with a loosening of the interaction of helix I (residues 3–13) with the major hydrophobic protein core.⁴⁵ The effect of formalin treatment on the tertiary structure of RNase A was examined by CD spectropolarimetry in the near-UV region (240–350 nm) as shown in Figure 15.6b. The partially resolved band at 283 nm is attributed to the exposed Tyr residue(s) of the enzyme.⁴⁶ Therefore, the 10% decrease in the intensity of this band caused by the 9-day incubation with 10% formalin likely reflects changes affecting the Tyr residues brought about by minor perturbations in the secondary and/or tertiary structure of the enzyme.^{46,47} Overall, the changes to the secondary and tertiary structure of RNase A that result from formalin treatment are subtle. The central conclusion that can be drawn from these optical spectroscopic studies is that formalin treatment leaves the secondary and tertiary structure of RNase A essentially intact.¹⁰ This is in agreement with earlier infrared spectroscopy studies on concentrated solutions of formalin-treated proteins.⁹

The effect of heating on the structure of formalin-treated RNase A is shown in Figure 15.7a (far-UV region) and 15.7b (near-UV region). In both Figures 15.7a,b, trace 1 is the spectrum of RNase A (6.5 mg/mL) kept in 10% buffered formalin (pH 7.4) for 9 days and then analyzed at 23°C following removal of excess formaldehyde by fast dialysis. Trace 2 is the same sample after heated to 95°C at a rate of 5°C/min and allowing 10 min for temperature equilibration.

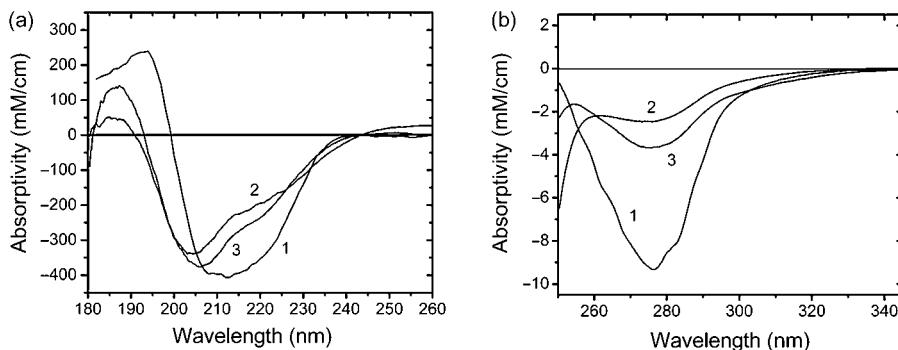


Figure 15.7 (a) Far-UV CD region and (b) near-UV CD region. Trace 1, spectra of RNase A (6.5 mg/mL) kept in 10% buffered formalin (pH 7.4) for 9 days and then analyzed at 23°C following removal of excess formaldehyde by fast dialysis. Trace 2, spectra of the samples from trace 1 after heating to 95°C at a rate of 5°C/min and allowing 10 min for temperature equilibration. Trace 3, spectra of the samples from trace 2 after cooling to 23°C at a rate of 5°C/min and allowing 10 min for temperature equilibration.

These spectra are similar to that of native RNase A at 95°C (not shown). The far-UV spectrum at 95°C indicates a retention of substantial β -sheet secondary structure, but a significant loss of the α -helix conformation as indicated by the decrease of intensity at 222 nm.⁴⁸ The near-UV spectrum at 95°C indicates a complete collapse of tertiary structure as is seen in molten globule proteins.⁴⁹ Trace 3 is the sample from trace 2 after cooling the protein to 23°C. Both spectra reveal little recovery of either secondary or tertiary protein structure.

In summary, formalin-treated does not significantly perturb the native structure of RNase A at room temperature. It also serves to stabilize the protein against the denaturing effects of heating as revealed by the increase in the denaturation temperature of the protein. However, formalin-treatment does not stabilize RNase A sufficiently to prevent the thermal denaturation of the protein at temperatures used in heat-induced AR methods as shown by both DSC and CD spectropolarimetry. This denaturation likely arises from the heat-induced reversal of formaldehyde cross-links and adducts, as shown in Figure 15.4 of Section 15.4. Further, cooling formalin-treated RNase A that had been heated to 95°C for 10 min does not result in the restoration of the native structure of the protein, particularly in regard to protein tertiary structure.

These structural findings have important implications for the mechanism of heat-induced AR. Heating formalin-treated proteins at elevated temperatures ($\geq 100^\circ\text{C}$) results in the reversal of formaldehyde adducts and cross-links regenerating the native protein chemical composition without causing a loss of primary structure (chain scission). However, this reversal is accompanied

by a partial loss of secondary structure, and a nearly complete loss of tertiary protein structure, which does not regenerate upon cooling of the protein. Several studies have suggested that formalin treatment leads to protein denaturation and that heat-induced reversal of formaldehyde adducts and cross-links improves immunoreactivity by partially restoring the native conformation of proteins.^{29,32} The above results¹⁰ argue against this interpretation. Instead, they support the model proposed by the Bogen laboratory, which suggests that antibodies chosen for their immunoreactivity for FFPE tissue sections recognize contiguous linear epitopes rather than the more common conformational epitopes.²¹ Antibodies developed against denatured proteins are also likely to consist of paratopes that recognize short contiguous amino acid epitopes.⁵⁰ In this model, heat-induced AR is only required to regenerate and expose the primary amino acid sequence of the protein in order to restore immunoreactivity. Since protein refolding is not required, this model is compatible with AR methods using heat or denaturants (such as urea or guanidine hydrochloride) that irreversibly denature proteins. It has been further suggested^{28,51} that protein denaturation may also enhance immunoreactivity through the exposure of immunogenic linear peptide sequences that are normally buried within the protein interior. This concept is supported by the observation that heat-induced AR methods can enhance staining intensity in unfixed fresh-frozen tissue sections.⁵² Finally, it has been noted that for optimal heat-induced AR, there is an inverse relationship between heating temperature and time, such that the higher the heating temperature, the shorter the time required to restore immunoreactivity.³² This relationship derives directly from the temperature-dependence of the kinetic rate constants for the formaldehyde reversal reactions and the requirement for the energy that must be supplied to drive these reactions.

15.7 RECOVERY OF ENZYMATIC ACTIVITY FROM FORMALIN-TREATED RNASE A

The DSC and SDS-PAGE studies described above demonstrated that the protein modifications produced by formalin treatment could be significantly reversed by heating the RNase A at elevated temperatures. This suggested that protein immunoreactivity and function could be recovered by prolonged heating of formalin-treated proteins, particularly if the incubation temperature is kept below the T_d of the native protein ($\sim 65^\circ\text{C}$). To test this hypothesis, formalin-treated RNase A that had been freed of excess formaldehyde by dialysis was incubated at elevated temperatures, and the recovery of enzymatic activity was assayed with the minimal substrate, cytidine 2',3'-cyclophosphate. As shown in Figure 15.8a, incubation in tris-acetate ethylenediamine tetraacetic acid (TAE) buffer, pH 7.0, at 50°C does lead to a recovery of activity, but at a rate of only 1.2% per hour. When the temperature was raised to 65°C , the rate of activity recovery increased to 3.5% per hour. The

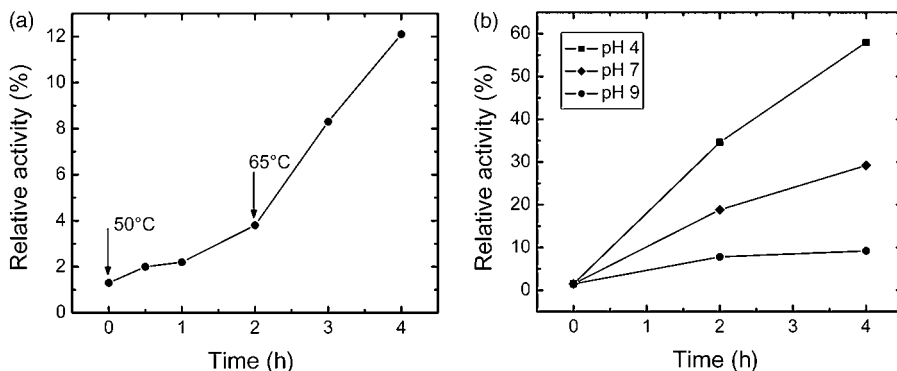


Figure 15.8 (a) Time course of the activity restoration of formalin-treated RNase A during incubation at 50°C (0–2 h) and 65°C (2–4 h) in TAE buffer, pH 7.0. (b) Time course of the activity restoration of formalin-treated RNase A during incubation at 65°C in TAE buffers of various pH values. All RNase A preparations were freed of excess formaldehyde by dialysis prior to the assay. The RNase A activity was determined with a colorimetric assay using cytidine 2',3'-cyclophosphate as the substrate as described by Crook et al.⁵⁴ Note that the slopes of the curves decrease with incubation time at 65°C, which is near the denaturation temperature of native RNase A. This loss of activity is likely due to the competing effect of protein denaturation of the recovered RNase A at this temperature. See Rait et al.¹⁰ for details.

effect of pH on the recovery of enzymatic activity at 65°C is shown in Figure 15.8b. An acidic medium provided a faster and more complete enzymatic recovery than a neutral or basic medium.¹⁰ In this context, it is interesting to note that AR methods using alkaline buffers generally yield a stronger immunogenic response than do acidic buffers.⁵³ It is possible to reconcile these observations by suggesting that some formaldehyde-modified antigens tend to denature in acidic medium, thus masking their more efficient demodification at low pH. This possibility is discussed in greater detail in Section 15.14. The recovery of RNase A enzymatic activity is likely to be correlated with the cross-link reversal that occurs when the formalin-treated enzyme is incubated at elevated temperatures in formaldehyde-free buffers.

15.8 THE EFFECT OF FORMALIN TREATMENT ON THE IMMUNOREACTIVITY OF RNASE A

The successful of recovery of RNase A functional activity by a heat-induced AR method suggested the possibility of recovering RNase A immunoreactivity as well. The immunoreactivity of native RNase A and RNase A that was incubated at a concentration of 4 mg/mL in 10% neutral buffered formalin for 1 day and then freed of formaldehyde by dialysis against PBS was compared using capture enzyme-linked immunosorbent assay (ELISA). Selected fractions that

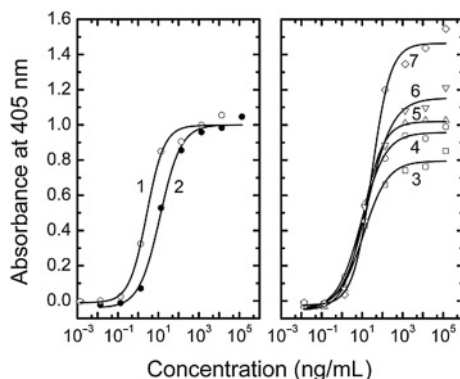


Figure 15.9 The results of capture ELISA on native RNase A and formalin-treated RNase A. Right panel, native RNase A (curve 1) and unfractionated formalin-treated RNase A (curve 2). Left panel, individual fractions of formalin-treated RNase A: monomer (curve 3), dimer (curve 4), trimer (curve 5), tetramer (curve 6), and a mixture of oligomers with ≥ 5 cross-linked proteins (curve 7). The ELISA plate wells were coated with monoclonal antibody against bovine pancreatic RNase A ($1 \mu\text{g/mL}$) overnight at 4°C and then blocked with bovine serum albumin. The wells were incubated for 1 h at 37°C in the presence of various concentrations of antigen in $100 \mu\text{L}$ of PBS. After washing, each plate well received a 1:4000 dilution of horseradish peroxidase conjugated rabbit polyclonal anti-RNase A antibody followed by incubation at ambient temperature for 1 h. After washing, detection was achieved using a mixture of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate) and hydrogen peroxide. Absorbance was monitored at 405 nm. See Rait et al.¹¹ for details.

were isolated from the formalin-treated RNase A preparation by size-exclusion gel chromatography were also analyzed by ELISA.¹¹ The results of these studies are shown in Figure 15.9. Curves 1, 2, and 3 in Figure 15.9 correspond to the titration of native RNase A ($OD_{50} = 2.7 \text{ ng/mL}$), formalin-treated unfractionated RNase A ($OD_{50} = 12.2 \text{ ng/mL}$), and the monomeric fraction of the latter ($OD_{50} = 11.5 \text{ ng/mL}$), respectively. The 4.3-fold difference in the OD_{50} values derived from curves 1 and 3 are interpreted as a measure of the disruption to the RNase A immunoreactivity that results from the formation of intramolecular cross-links. This occurs even though the antigen secondary and tertiary structures remain essentially unperturbed as reported in Section 15.6. The OD_{50} value obtained for the titration of cross-linked dimer is 9.3 ng/mL (curve 4) and that for the cross-linked trimer is 11.2 ng/mL (curve 5). The non-linear pattern of the OD_{50} values observed for the monomer through the cross-linked trimer results from changes in the balance between the number of epitopes per oligomer and the steric blocking of the antibodies that arises from intermolecular cross-links. The fact that these changes are small indicates that the effect of the physically excluded volume due to intermolecular cross-linking is negligible for short oligomers. However, curve 6 ($OD_{50} = 18.3 \text{ ng/mL}$) and curve 7 ($OD_{50} = 29.6 \text{ ng/mL}$), which correspond to tetramer and a mixture of

oligomers containing >5 cross-linked molecules, respectively, are shifted toward significantly higher concentrations. This indicates that the shielding of epitopes due to an excluded volume effect becomes more significant as the number of cross-linked proteins per oligomer increases above four.

Capture ELISA on selected oligomeric fractions of formalin-treated RNase A (see curves 3–7 in Fig. 15.9) also reveal that the plateau values increase with an increase in the number of cross-linked molecules in the fractions. This is due to an increasing proportion of bound epitopes per binding site or, in other words, epitope density on the surface. Thus, the nearly identical plateau values for the titration of native RNase A and formalin-treated unfractionated RNase A (curves 1 and 2 in Fig. 15.9) are fortuitous, being caused by the particular composition of oligomers present in the formalin-treated RNase A preparations that was analyzed.

The above results demonstrate that formalin-induced intramolecular protein modifications significantly reduce, but do not eliminate, protein immunoreactivity. A greater impairment of immunoreactivity occurs upon forming intermolecular cross-links leading to the creation of large protein aggregates consisting of ≥ 4 protein molecules. These observations are consistent with the hypothesis that formaldehyde fixation can reduce the accessibility of antibodies to their antigens, regardless of the effect it has on epitope structure.^{55,56} They are also consistent with the observation by Morgan et al.^{7,57} that calcium ions, together with formaldehyde, form cage-like structures that inhibit access of antibodies to their target epitopes through steric blocking. Although calcium chelation by ethylenediamine tetraacetic acid (EDTA) improves immunostaining for some antigens,⁵⁷ it is ineffective for many others,^{58,59} suggesting that the calcium effect is selective for a limited number of antibodies and antigens.⁵⁹ Finally, steric blocking of epitopes also explains the success of AR methods using proteolytic enzymes, such as trypsin, proteinase K, and pronase,⁶⁰ either alone or in combination with heat-induced AR. Proteolytic enzyme digestion can lead to “debulking” of the tissue, removing proteins that block access of the antibody to its target antigen. Proteolytic digestion can also reduce steric hindrance by removing peptides fragments containing cross-links.³³ However, this approach is not universally applicable as the target epitope itself can be digested.^{61,62} It is likely that oxidizing agents, such as hydrogen peroxide and sodium *meta* periodate,⁶³ and etching agents, such as sodium hydroxide in methanol,⁶⁴ also act as debulking agents through their chemical reactivity with proteins and other tissue constituents.

15.9 RESTORATION OF IMMUNOREACTIVITY IN FORMALIN-TREATED RNASE A

Capture ELISA was used to follow the restoration of immunoreactivity in formalin-treated RNase A preparations after incubation at elevated temperatures in TAE buffer at pH 4.¹¹ Two different formulations of formalin-treated

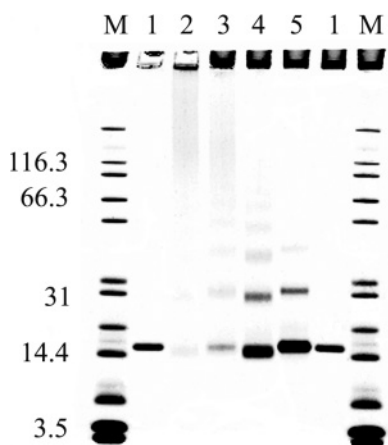


Figure 15.10 SDS-PAGE of native RNase A (lane 1) and RNase A incubated in 10% neutral buffered formalin for 9 days (lane 2) or in 5% neutral buffered formalin for 1 day (lane 4). The formalin-treated samples were then demodified for 4 h in TAE buffer (pH 4) at 65°C; 10% formalin oligomers (lane 3) and 5% formalin oligomers (lane 5). M, molecular mass markers in kDa. See Rait et al.¹¹ for details.

RNase A oligomers were prepared. One preparation was enriched in RNase oligomers consisting of five or more intermolecular cross-linked proteins (10% formalin oligomers), and the other was enriched in monomer and RNase A oligomers consisting of fewer than five intermolecular cross-linked proteins (5% formalin oligomers). The 10% formalin cross-linked RNase A oligomers were prepared by incubation of the enzyme (6.5 mg/mL) for 9 days in 10% neutral buffered formalin. To obtain the 5% formalin cross-linked RNase A oligomers, the concentrations of the enzyme and formaldehyde were reduced to 1 mg/mL and 5%, respectively, and the incubation time was decreased to 1 day. Following incubation, the protein samples were freed from excess formaldehyde by dialysis against PBS. As shown in Figure 15.10, the 10% formalin oligomers consist mostly of oligomers with seven or more cross-linked proteins (lane 2), whereas the 5% formalin oligomers have a higher content of modified monomers, dimers, and trimers (lane 4). A 4-h incubation in TAE buffer (pH 4) at 65°C resulted in a significant destruction of intermolecular cross-links in both the 10% formalin oligomers (lanes 2 vs. 3) and 5% formalin oligomers (lanes 4 vs. 5). The incubation also restored the mobility of modified monomer to that of the untreated enzyme (lane 1).

The structural changes that occurred upon incubation at 65°C resulted in the partial restoration of immunoreactivity of the samples as shown in Figure 15.11a for the 5% formalin oligomers and Figure 15.11b for 10% formalin oligomers. In both figures, curve 1 corresponds to unheated native enzyme, curve 2 corresponds to unheated formalin-treated RNase A, and curve 3 corresponds to samples heated at 65°C for 4 h in TAE buffer (pH 4). The

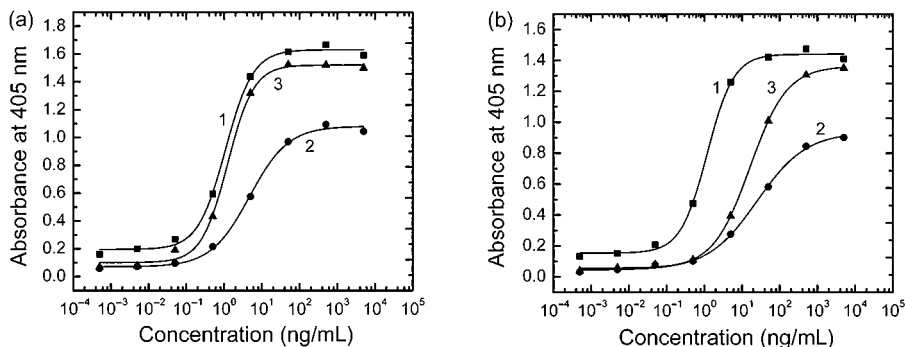


Figure 15.11 (a) The results of capture ELISA on native RNase A (curve 1) and RNase A incubated in 5% neutral buffered formalin at a concentration of 1 mg/mL for 1 day (curve 2) and then demodified for 4 h in TAE buffer (pH 4) at 65°C (curve 3). (b) The results of capture ELISA on native RNase A (curve 1) and RNase A incubated in 10% neutral buffered formalin at a concentration of 6.5 mg/mL for 9 days (curve 2) and then demodified for 4 h in TAE buffer (pH 4) at 65°C (curve 3). See Rait et al.¹¹ for details.

restoration of RNase A immunoreactivity upon heating at 65°C is clearly correlated with the destruction of formaldehyde cross-links as shown in Figure 15.10. The data in Figures 15.10 and 15.11 represent direct evidence of a relationship between formaldehyde cross-link reversal and immunoreactivity restoration in a formalin-treated antigen resulting from a heat-induced AR procedure.^{1,11} By comparing the results obtained with the 10% and 5% formalin oligomers, it can be seen that the higher the extent of initial cross-link formation in the RNase A preparation, the less restoration of RNase immunoreactivity is obtained from a 4-h incubation at 65°C. This leads to the conclusion that efforts to standardize immunohistochemical staining³¹ must begin with standardization of fixation time, which determines the extent of cross-linking. However, this is cold comfort to those using archival FFPE tissues. The Bogen model²⁰ argues that antibodies used in immunohistochemistry predominately recognize contiguous linear epitopes and are thus insensitivity to protein denaturation. If true, this supports the argument and experimental observations of Shi et al.⁶⁵ that carefully optimized heat-induced AR can be used as an approach to overcome the variable effects of fixation on immunostaining intensity.

15.10 EFFECT OF FIXATION ON THE RECOVERY OF RNASE A ACTIVITY FROM TISSUE

The above studies suggest that significant enzymatic activity and protein immunoreactivity can be recovered from formalin-treated RNase A when the protein remains in an aqueous medium. To explore this observation further,

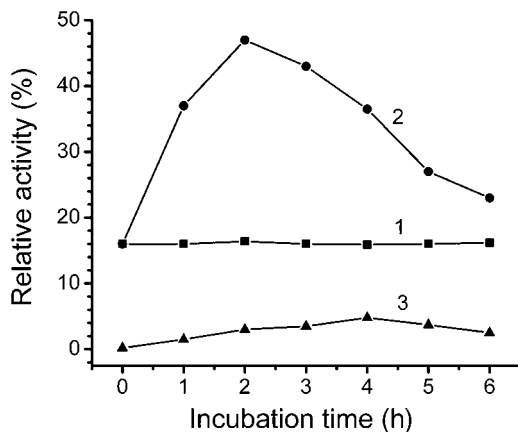


Figure 15.12 Relative recovery of RNase A activity from porcine pancreatic tissue. Curve 1, formalin-treated tissue homogenate dialyzed against excess buffer; curve 2, formalin-treated tissue homogenate heated at 65°C after removal of excess formaldehyde by dialysis; curve 3, FFPE pancreatic tissue heated at 65°C following reversal of the histological process, homogenization of the tissue in ice-cold buffer, and dialysis against two exchanges of a 100-fold excess of ice-cold buffer for 2h. Dialysis buffer was 10mM potassium phosphate, 10mM potassium chloride, 100mM glycine, pH 4. Pancreatic tissue was homogenized by passage through a tissue press followed by processing using a high-shear tissue homogenizer. All tissue homogenates were centrifuged for 5min at 2,000rpm in 1-mL plastic vials before absorbance readings. The RNase A activity was determined with a colorimetric assay using cytidine 2',3'-cyclophosphate as the substrate.⁵⁴ All results were normalized to 500mg of tissue.

the ability to recover RNase A enzymatic activity from porcine pancreatic tissue was studied using several approaches. Fresh porcine pancreas (500mg) was homogenized in ice-cold buffer and immediately assayed for RNase A activity with a colorimetric assay using cytidine 2',3'-cyclophosphate as the substrate as described by Crook et al.⁵⁴ This assay served to define 100% enzymatic activity on the relative activity scale (y-axis) of Figure 15.12. In a second experiment, 500mg of pancreatic tissue that had been fixed in 10% neutral buffered formalin for 24h was assayed for RNase A activity and, as expected, gave no colorimetric reading relative to a 10% formalin blank. This assay served to define 0% on the relative activity scale in Figure 15.12. The fixed pancreatic tissue was then dialyzed for 2h against two exchanges of a 100-fold excess of ice-cold buffer. Following dialysis the RNase A activity was assayed and found to have a relative activity of 17%, which did not change with further dialysis as shown in curve 1. The fixed and dialyzed tissue was also heated at 65°C (pH 4) for 6h and the relative RNase A activity was assayed every hour as shown in curve 2. The relative activity increased to a maximum of 47% at 2h, after which the relative activity progressively decreased and reached a value of ~22% after 6h. The reason for this biphasic

behavior is not clear, but could involve protein denaturation, as discussed in Section 15.6, or possibly the degradation of RNase A by reactivated proteases in the tissue.

In the above studies, the pancreatic tissue homogenate was always suspended in excess aqueous solution. Formalin-fixed pancreatic tissue was also subjected to normal histological processing to form FFPE tissue plugs. Following reversal of the histological process, the hydrated tissue was homogenized in ice-cold buffer, dialyzed for 2 h against two exchanges of a 100-fold excess of ice-cold buffer, and then heated at 65°C (pH 4) for 6 h with the relative RNase A activity assayed every 2 h as shown in Figure 15.12, curve 3. The curve shows the same biphasic trend as was seen for curve 2, with a maximum activity recovery at 4 h. However, only a very low level of activity (~5%) was recovered. This result suggests that the initial chemical changes that occur in the reaction of formaldehyde with proteins in aqueous solution do not represent the chemical state of these molecules following histological tissue processing. In the presence of organic solvents and under anhydrous conditions, formaldehyde-protein adducts are certain to undergo further reactions that are not exhibited in aqueous solution.

15.11 EFFECT OF ETHANOL DEHYDRATION ON THE REVERSAL OF FORMALDEHYDE CROSS-LINKS

As a first step toward understanding the additional modifications alluded to above, a comparative evaluation was performed of RNase A following fixation in 10% neutral buffered formalin solution alone, and after subsequent dehydration in 100% ethanol.¹² After fixation in 10% buffered formalin, SDS-PAGE of 1 mg/mL solutions of RNase A showed a mixture of intermolecular cross-linked proteins composed of monomeric (25%), dimeric (21%), trimeric (18%), tetrameric (15%), pentameric (10%), and hexameric (11%) species (Fig. 15.13, lane 2). Removal of the formaldehyde by rapid dialysis did not reduce the level of cross-linking (data not shown); however, heating the formalin-treated sample in 20 mM Tris-HCl with 2% SDS (pH 4) at 100°C for 20 min, followed by 60°C for 2 h resulted in an almost fourfold increase in monomeric protein, with ~92% of total protein comprised of monomers (lane 3). To mimic the ethanol-dehydration step typically performed during the histological processing of FFPE tissues,⁶⁶ the formalin-treated RNase A was precipitated and incubated in 100% ethanol for 1 h, 24 h, or 1 week. SDS-PAGE revealed that the formalin-fixed, ethanol-treated samples were as highly cross-linked as those treated with formalin (lanes 4, 6, and 8). However, after heating in the Tris-SDS recovery buffer at 100°C for 20 min, followed by 60°C for 2 h, little reversal of the formaldehyde-induced cross-links was observed (lanes 5, 7, and 9), with the total protein content corresponding to 26% monomeric, 23% dimeric, 18% trimeric, 14% tetrameric, 11% pentameric, and 8% hexameric species.

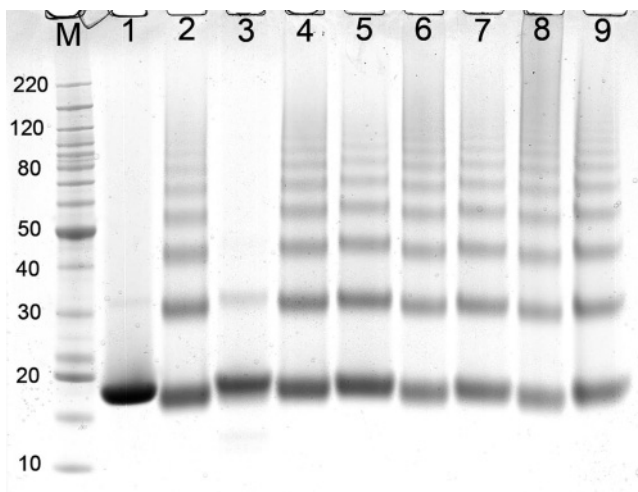


Figure 15.13 SDS-PAGE of formalin-fixed RNase A before and after protein retrieval. Lane M: molecular weight marker; lane 1: native RNase A; lane 2: formalin-treated RNase A after the removal of excess formaldehyde by rapid dialysis; lane 3: formalin-treated RNase A sample from lane 2 after retrieval in 20mM Tris-HCl, pH 4.0, with 2% SDS; lanes 4, 6, and 8: formalin-treated RNase A after incubation in 100% ethanol for 1h, 24h, or 1 week, respectively; lanes 5, 7, and 9: 1-h, 24-h, or 1-week formalin-fixed, ethanol-treated RNase A after retrieval in 20mM Tris-HCl, pH 4.0, with 2% SDS. All RNase A samples were heated at 100°C for 20 min, followed by 60°C for 2 h. See Fowler et al.¹² for details.

15.12 EFFECT OF FIXATION AND ETHANOL DEHYDRATION ON PROTEIN STRUCTURE

The structural properties of native, formalin-fixed, and formalin-fixed plus ethanol-treated RNase A were examined using CD spectropolarimetry. The solvent-corrected far-UV spectrum of native RNase A in PBS is shown in curve 1 of Figure 15.14a. The spectrum exhibits a minimum at ~212 nm, with a broad shoulder centered at ~220 nm, which is characteristic of an $\alpha + \beta$ protein conformation.⁴⁸ Incubation of native RNase A in 10% formalin (curve 3) for 1 week did not significantly alter the secondary structure of the protein, as was also demonstrated in Section 15.6. In addition, native RNase A incubated under ethanol for 1 week recovered its native structure after the ethanol was removed and the RNase A was reconstituted in PBS (curve 2). However, when the formalin-fixed RNase A was incubated under ethanol for 1 week and then rehydrated in PBS (curve 4), there was a significant decrease in band intensity, and the profile changed to one with a single negative peak around 215 nm, which is characteristic of an all- β protein conformation.⁶⁷ This spectrum was also observed for native, formalin-fixed, and formalin-fixed plus ethanol-treated RNase A in 80% aqueous ethanol solution in which all three

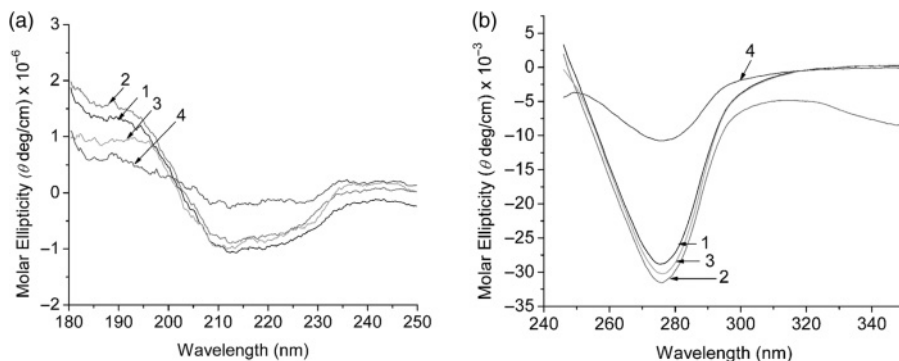


Figure 15.14 The effect of ethanol on RNase A structure: (a) far-UV and (b) near-UV CD spectra of 0.65 mg/mL solutions of RNase A. Curve 1, native RNase A in PBS; curve 2, native RNase A incubated under 100% ethanol for 1 week and then rehydrated in PBS; curve 3, RNase A kept in 10% formalin for 1 week; curve 4, RNase A fixed in 10% formalin, incubated under 100% ethanol for 1 week, and then rehydrated in PBS. See Fowler et al.¹² for details.

proteins were minimally soluble (data not shown). The solvent-corrected near-UV spectrum of native RNase A incubated in ethanol (Fig. 15.14b, curve 2) or treated with 10% formalin (curve 3) for 1 week exhibited no significant changes in their near-UV CD spectra, as compared to native RNase A in PBS (curve 1). However, there was a 60% decrease in negative band intensity for the formalin-treated protein after 1 week of incubation in ethanol followed by rehydration in PBS (curve 4). These changes likely result from the collapse of tertiary structure as the spectrum is analogous to those seen in molten-globule proteins.⁴⁹

15.13 THE ROLE OF ETHANOL DEHYDRATION IN AR

Both native and formalin-fixed RNase A undergo a structural transition from the native $\alpha + \beta$ to a nearly all- β conformation as ethanol concentration is increased to $\geq 80\%$. The transition from the native to an all- β conformation at high ethanol concentrations is characteristic of most soluble proteins^{68–70} and is driven by the disruption of water structure by ethanol and the associated energetically unfavorable interaction of ethanol with the peptide backbone.⁷¹ The response of most proteins to this situation is to form β -sheets to sequester the peptide bonds away from the solvent while exposing nonpolar side chains to the alcohol.⁶⁷ This secondary structural transformation is typically accompanied by a significant disruption (collapse) of tertiary structure⁷² as was observed for RNase A in the above studies. This new protein conformation is further stabilized by the formation of intermolecular hydrogen bonds between geometrically compatible hydrophobic β -sheets,⁷³ which then leads

to extensive protein aggregation.^{69,74} Such β -sheet aggregates can form in response to a number of protein structural perturbants, in addition to alcohol, and are intermediates in the formation of amyloid fibrils by some proteins.⁶⁸ Consequently, our results demonstrate that the presence of formaldehyde adducts and cross-links do not inhibit the conformational changes exhibited by RNase A in the presence of ethanol. An important implication of the above observations is that formalin-fixed RNase A maintains a high degree of conformational flexibility despite the presence of intra- and intermolecular formaldehyde cross-links.

The protein aggregates formed by exposure of native RNase A to ethanol were reversible using the protein recovery conditions described in this study, while those that are formed by formalin-fixed, ethanol-treated RNase A were only partially reversible.¹² There are several possible explanations for this observation. One possible explanation is that the neutralization of charged amino acids by the formation of formaldehyde adducts contributes to aggregate formation by increasing the hydrophobicity of the protein surface. We previously demonstrated that formaldehyde treatment lowers the pI of RNase A from 9.2 to $\sim$ 7.4 (Section 15.5). To investigate this possibility,¹² we treated formalin-fixed RNase A solutions with a 50-M excess of sodium cyanoborohydride (NaBH_3CN), which has been shown to reduce a wide variety of organic functional groups, including Schiff-bases and hydroxymethyl groups, at neutral pH.^{75–77} SDS-PAGE analysis revealed that formalin-fixed RNase A reduced with NaBH_3CN and then incubated in ethanol exhibited significantly fewer intermolecular cross-links than its nonreduced counterparts (not shown), with only monomeric, dimeric, and trimeric species present. After heating at 100°C for 20 min, followed by 60°C for 2 h in 20 mM Tris-HCl with 2% SDS at pH 4, only monomeric protein ($\sim$ 99%) was recovered. Treatment of formalin-fixed RNase A with NaBH_3CN reduces formaldehyde adducts to methyl groups, which further increases protein hydrophobicity. Thus, the recovery of mostly RNase A monomer following NaBH_3CN treatment of formalin-fixed RNase A is the opposite of what would be expected if the protein aggregates were stabilized predominantly by strong hydrophobic bonding.

Taken together, the above findings suggest that the cross-links in formalin-treated RNase A in ethanol are difficult to reverse because they are largely sequestered within geometrically compatible intermolecular hydrophobic β -sheets present in the protein aggregates.¹² These cross-links may result from the rearrangement of existing cross-links or by the formation of new cross-links from latent formaldehyde adducts and formaldehyde-reactive amino acid side chains present in the protein's interior. The latter may occur because the co-planar orientation of the side chains in β -sheets may provide a more favorable geometry for forming formaldehyde cross-links than the α -helix conformation (unpublished experiments). This conclusion is further supported by previous findings in which intermolecular cross-linking was increased by heating formalin-treated RNase A above its unfolding transition temperature in the presence of formaldehyde, indicating that additional cross-links were

formed by previously buried formaldehyde-adducts or formaldehyde-reactive amino acid side chains.¹⁰ An additional contributing factor is that the dehydrating effects of ethanol may promote cross-link formation by the conversion of methylol adducts to reactive Schiff's-base intermediates, a reaction that involves the loss of a water molecule,²⁵ as discussed in Section 15.2.

In summary, the above body of work suggests that the ethanol dehydration step in tissue histology plays a critical role in confounding the recovery of immunoreactivity from FFPE tissues.¹² This observation has also been inferred from immunohistochemical studies using FFPE tissue sections.^{23,78} In addition, these studies demonstrated that xylene and paraffin were less deleterious toward immunoreactivity than was ethanol. Ethanol-induced rearrangement of protein conformation can lead to protein aggregation through the formation of large geometrically compatible hydrophobic β -sheets that are stabilized by hydrogen bonds, and Van der Waals interactions in addition to formaldehyde cross-links. Such β -sheets would require substantial energy to induce sufficient rehydration to reverse the formaldehyde cross-links within these sheets and regenerate protein monomers free of formaldehyde modifications. The required energy can be introduced using extremely high temperatures $\geq 100^\circ\text{C}$ ^{79,80} or by the application of elevated hydrostatic pressure at moderate temperatures.⁸¹

15.14 GENERAL COMMENTS ON THE MECHANISM OF AR

Since its introduction nearly 20 years ago,¹ the AR method has evolved to include many different techniques mediated by a number of chemical and physical factors, such as heat, ionic strength, buffer pH, detergents, chaotropic agents, metal ions, chelators, proteolytic enzymes, oxidizers, and detergents.^{31–33,82} Although several mechanisms have been proposed³³; a consensus has yet to emerge on exactly how and why AR works.⁸³ It would seem unlikely that a unitary model of AR will be identified. Instead, certain mediators are likely universal to all AR methods, while others are restricted to certain combinations of antigens, antibodies, and tissues. The reversal of protein formaldehyde adducts and cross-links is almost certainly fundamental to the success of the AR technique. This reversal restores the normal electrostatic charge of the protein and removes steric barriers to antigen–antibody binding. While this may occur slowly in excess buffer at room temperature,³⁸ heat serves as a superior mediator to provide the energy required for the chemical reversal of these formaldehyde modifications. Other mediators likely act synergistically with heat to promote recovery of immunoreactivity through such mechanisms as the chemical or proteolytic digestion of proteins,^{84,85} precipitation or diffusion of proteins out of the tissue,⁸² rehydration of tissues¹² allowing better penetration of antibodies,⁸⁵ removal of divalent ion complexes,^{7,57} opening physical pores within the tissues,^{9,31} and heat-induced mobilization of residual paraffin.⁸⁶

The following series of events likely form the core of the heat-induced AR method. First, the tissue must be rehydrated to allow the formaldehyde adducts and cross-links access to water.^{12,85} This is necessary (1) to provide the water molecules required to participate in the chemical reversal of the formaldehyde modifications, (2) to allow the acid or base catalyst in the buffer access to the formaldehyde modifications, and (3) to allow access of the antibody to the target protein epitope by aqueous diffusion. Second, a significant fraction of the formaldehyde adducts and cross-links must be chemically reversed. The reversal process accomplishes three important goals, which are (1) to reestablish the chemical composition of the epitope, (2) to reestablish the electrostatic charge of the protein, including the epitope, and (3) to remove steric barriers that block access of the antibody to its target epitope. As stated above, heat is the most efficient mediator to drive this reversal and also to induce tissue rehydration, although elevated hydrostatic pressure has recently been shown to act synergistically with heat in this regard.⁸¹

The temperature used for AR is dictated by the nature of the antibody-antigen interaction. For contiguous linear epitopes, high temperature ($\geq 100^{\circ}\text{C}$) will promote protein denaturation, which is likely to facilitate the presentation of such linear epitopes to their antibodies.^{20,87} The treatment of the tissue sections with chaotropic agents (such as urea or guanidinium hydrochloride) promotes protein denaturation and hydration by breaking hydrogen bonds, and disrupting hydrophobic and Van der Waals interactions.³³ Analogously, detergents (such as SDS) promote protein denaturation and hydration and may also promote solubilization and removal of diffusible proteins from the tissue.⁸⁸⁻⁹⁰ In contrast, for antibodies that recognize conformational epitopes heating the tissue sections at lower temperatures ($\leq 60^{\circ}\text{C}$) in the absence of protein denaturants or detergents is likely to produce superior results.¹⁰

Other AR mediators, such as divalent ion chelators,⁷ formaldehyde scavengers, such as citraconic anhydride,⁹¹ metal ions,¹ or proteolytic enzymes⁶⁰ can enhance AR in certain cases; however, their applications are not universal and, in some cases, may even inhibit immunostaining. As noted above, the removal of steric barriers that restrict access of the antibody to its target epitope is a key component of AR.^{11,55,56} In this context, heating may serve to promote the extraction of diffusible proteins out of the tissue sections following cross-link reversal or proteolytic treatment, opening physical holes or channels in the tissue sections that allow better penetration of antibodies.^{9,31} The physical process of opening holes or channels within the tissue section also likely explains the modest success of ultrasonics as an AR method.⁹²

Successful application of heat-induced AR requires regulation of the pH of the recovery buffer. Shi et al.^{31,93} found three patterns of pH dependence as judged by immunostaining: (1) antigen recovery is independent of pH (stable type), (2) antigen recovery improves with increasing pH (ascending type), and (3) optimal antigen recovery occurs at low or high pH (V-type). In

another study, Emoto et al.⁴² demonstrated that the majority of the antigens they studied showed maximal staining when AR was carried out at either acidic (~3.0) or basic (~9.5) pH. The remaining antigens exhibited optimal recovery at alkaline pH (~9.5). They argued that the denatured proteins exhibited maximal electrostatic charge at the two pH extremes and that this serves to minimize random entanglement, and thus epitope shielding, of the polypeptides caused by hydrophobic attractive forces. This model was supported by studies showing that immunostaining was reduced when heat-induced AR was carried out using high ionic strength buffers (such as NaCl) as this reduced electrostatic repulsion of the polypeptides through ionic shielding.

However, there are two important complications regarding the pH of buffers used in heat-induced AR methods that are typically overlooked, which can significantly influence the pH-dependent recovery of immunostaining. The first is the temperature dependence of pH for many buffer solutions, particularly Tris-HCl, used for heat-induced AR.^{94,95} For example, a buffer that is pH 6.0 at room temperature will, at 120°C, be reduced to pH 3.06 (Tris),⁹⁴ pH 5.24 (phosphate),⁹⁶ or pH 5.69 (citrate).⁹⁵ Temperatures of 120°C, referred to as superheating AR,⁸⁰ are frequently attained during heat-induced AR using microwave,⁹⁷ pressure cooker,⁹⁸ or steamer⁹⁹ methods. The second complication is temperature-induced protein degradation reactions that can occur at temperatures $\geq 100^\circ\text{C}$.¹⁰⁰⁻¹⁰² At acidic pH, these degradation reactions are deamination of glutamine or asparagine residues, and peptide bond hydrolysis on the carboxylate side of aspartic acid residues that results from the formation of an internal anhydride.¹⁰¹ At neutral pH, the deamination reactions and thiol-catalyzed disulfide exchange constitute the degradation reactions, and at alkaline pH, the disulfide exchange reaction and β -elimination of cystine residues dominate the degradation reactions. We have observed aspartic acid cleavage reactions in tissue surrogates consisting of model proteins that were heated at 80–100°C in AR buffers at pH 4.0.^{81,90} Such thermally induced degradation reactions would adversely affect the ability to recover antigens if the epitope were to contain one of these heat-labile amino acid residues. Cleavage at aspartic acid residues would be particularly deleterious as this could also lead to a loss of epitopes due to extraction of diffusible peptide fragments during the heat-induced AR process. This could explain the ascending pH dependence of recovered immunoreactivity in the heat-induced AR of some antigens⁹³ as was noted in Section 15.7. Consequently, the pH dependence of heat-induced AR can be complicated by the presence of thermally labile amino acid residues in, or near, the epitope of the protein.

In conclusion, the core components that constitute the mechanism of AR are beginning to come into focus. Further work will be required to solidify these core components and to understand the variations that arise for specific tissues and antigen-antibody pairs. This knowledge, in turn, will lead to improved AR methods, improved ways to overcome the variable effects of tissue fixation, and improved standardization between clinical laboratories.

15.15 BIOPHYSICAL METHODS

15.15.1 Differential Scanning Calorimetry

A scanning calorimeter measures the heat flow into (endothermic) or out of (exothermic) a substance as it is heated or cooled. In differential scanning calorimetry, this heat flow is measured relative to a reference, which for a protein typically consists of the dialysis buffer used to prepare the protein solution. When analyzing a protein, differential scanning calorimetry (DSC) measures the change in heat capacity (C_p) that results from temperature-induced conformational changes, such as protein unfolding (protein denaturation). In DSC, the heat capacity of the protein is given in units of calories/degree/mole. Protein unfolding produces a peak in the plot of heat capacity versus temperature as no corresponding thermal event takes place in the reference solution. The area under this peak is the enthalpy (ΔH) of unfolding, which has the units of calories/mole. During the unfolding transition, the native and denatured protein states are in thermodynamic equilibrium. The temperature where equal amounts of native and denatured protein are present is called the denaturation transition temperature (T_d). For a two-state protein unfolding transition (the native and unfolded states are the only forms present), T_d is typically taken to be the temperature corresponding to the maximum of the transition peak. The factors that contribute to the heat absorbed during protein thermal denaturation include hydrophobic interactions, van der Waal's interactions, changes in hydrogen bonding, changes in ion binding, and changes in conformational entropy. Protein cross-links, such as disulfide bonds or formaldehyde cross-links, require energy to break, thus they increase the denaturation temperature of the protein. Accordingly, they serve to stabilize the native state against the disordering effects of increasing temperature. See Splink,¹⁰³ Stelea et al.,¹⁰⁴ and Klink et al.³⁰ for reviews on calorimetry and protein denaturation.

15.15.2 Circular Dichroism Spectropolarimetry

Circularly polarized light is characterized by an electric field vector that is constant in length, but rotates about the direction of propagation forming either a right- or left-handed helix. An optically active substance, such as a protein, will show a difference in absorption between left and right circularly polarized light. This difference in absorption, which is wavelength dependent, is called circular dichroism (CD). An instrument that measured the CD of a protein as a function of wavelength is called a CD spectropolarimeter. CD is given in units of absorptivity ($\text{mM}^{-1}\text{cm}^{-1}$) or molar ellipticity ($\text{degrees cm}^2/\text{decimole}^{-1}$). For proteins, the CD spectrum is typically divided into two regions referred to as the far-UV region (170–240 nm) and the near-UV region (240–350 nm). The far-UV region is sensitive to the secondary structure of the protein, with each secondary structural element giving rise to

a unique far-UV CD spectrum. The α -helix conformation has strong negative absorption bands at 208 and 222 nm and a strong positive absorption band at 191 nm. The β -sheet conformation has a single moderate-intensity negative absorption band at 217 nm and a single moderate-intensity positive absorption band at 195 nm. A random coil has a single strong negative absorption band at 197 nm. Most proteins will contain a combination of secondary conformational domains, which give rise to a unique far-UV CD spectrum. Mathematical algorithms can be used to deconvolve a protein's far-UV CD spectrum to give an estimate of the percentage of α -helix, β -sheet, and random conformational elements present in the protein.

The near-UV region, which arises from chromophoric side chains, such as tyrosine, provides an indirect assessment of protein tertiary structure. The tyrosine ring is symmetric and thus has very weak optical activity. However, in the native protein, most tyrosine residues will be spatially oriented in an environment where the electric field gradients surrounding the tyrosine side chain are asymmetric. This distorts the electron displacement in the tyrosine ring giving rise to a strong negative absorption band in the near-UV region. When the protein unfolds due to thermal denaturation, the fixed orientation of the tyrosine side chains is lost, and there is sufficient conformational freedom to average any external electric field gradients surrounding the tyrosine residues. As a result, the far-UV CD band intensity is dramatically diminished. The near-UV CD region of a protein can thus be used to distinguish between the native and denatured states of the protein. CD spectropolarimetry of proteins is reviewed in Kelly et al.,¹⁰⁵ Kelly and Price,¹⁰⁶ and Woody.¹⁰⁷

REFERENCES

1. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
2. Shi SR, Cote RJ, Yang C, et al. Development of an optimal protocol for antigen retrieval: a “test battery” approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. *J. Pathol.* 1996; 179: 347–352.
3. Miller RT, Swanson PE, Wick MR. Fixation and epitope retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. *Appl. Immunohistochem. Mol. Morphol.* 2000; 8: 228–235.
4. Ellis RJ. Macromolecular crowding: obvious but underappreciated. *Trends Biochem. Sci.* 2001; 26: 597–604.
5. Verkman AS. Solute and macromolecule diffusion in cellular aqueous compartments. *Trends Biochem. Sci.* 2002; 27: 27–33.
6. Hopwood D. Microwaves and heat in aldehyde fixation: model experiments with bovine serum albumin. *Methods* 1998; 15: 119–122.

7. Morgan JM, Navabi H, Schmid KW, et al. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. *J. Pathol.* 1994; 174: 301–307.
8. Shi SR, Jiang G, Turrens JF, et al. Development of the AR technique: philosophical and theoretical basis. In *The Antigen Retrieval Techniques: Applications in Immunohistochemistry*, ed. SR Shi, G Jiang, and CR Taylor. Westborough, MA: BioTechniques Press, 2000.
9. Mason JT, O’Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
10. Rait VK, O’Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
11. Rait VK, Xu L, O’Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A: II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
12. Fowler CB, O’Leary TJ, Mason JT. Modeling formalin fixation and histological processing with bovine ribonuclease A: effects of ethanol dehydration on reversal of formaldehyde-induced cross-links. *Lab. Invest.* 2008; 88: 785–791.
13. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins: cross-linking of amino groups with phenol, imidazole, or indole groups. *J. Biol. Chem.* 1948; 174: 827–843.
14. Fraenkel-Conrat H, Mecham DK. The reaction of formaldehyde with proteins; demonstration of intermolecular cross-linking by means of osmotic pressure measurements. *J. Biol. Chem.* 1949; 177: 477–486.
15. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins. IV. Participation in indole groups, Gramicidin. *J. Biol. Chem.* 1947; 168: 99–118.
16. Metz B, Kersten GFA, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
17. Metz B, Kersten GFA, Baart GJ, et al. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug. Chem.* 2006; 17: 815–822.
18. Fowles LF, Beck E, Worrall S, et al. The formation and stability of imidazolidinone adducts from acetaldehyde and model peptides. A kinetic study with implications for protein modification in alcohol abuse. *Biochem. Pharmacol.* 1996; 51: 1259–1267.
19. Kunkel GR, Mehrabian M, Martinson HG. Contact-site cross-linking agents. *Mol. Cell. Biochem.* 1981; 34: 3–13.
20. Sompuram SR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
21. Sompuram SR, Vani K, Hafer LJ, et al. Antibodies immunoreactive with formalin-fixed tissue antigens recognize linear protein epitopes. *Am. J. Clin. Pathol.* 2006; 125: 82–90.
22. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.

23. Guhl B, Ziak M, Roth J. Unconventional antigen retrieval for carbohydrate and protein antigens. *Histochem. Cell Biol.* 1998; 110: 603–611.
24. Tome D, Naulet N, Martin GJ. Application de la RMN à l'étude des réactions du formaldéhyde avec les fonctions aminées de l'alanine et de la lysine en fonction du pH du milieu. *J. Chim. Phys.* 1982; 79: 361–368.
25. Rait VK, Zhang Q, Fabris D, et al. Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. *J. Histochem. Cytochem.* 2006; 54: 301–310.
26. Raines RT. Ribonuclease A. *Chem Rev.* 1998; 98: 1045–1066.
27. Brown LR, Bradbury JH. Proton-magnetic-resonance studies of the lysine residues of ribonuclease A. *Eur. J. Biochem.* 1975; 54: 219–227.
28. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
29. Volkin DB, Klibanov AM. Minimizing protein inactivation. In *Protein Function: A Practical Approach*, ed. TE Creighton. New York: IRL Press, 1989.
30. Klink TA, Woycechowsky KJ, Taylor KM, et al. Contribution of disulfide bonds to the conformational stability and catalytic activity of ribonuclease A. *Eur. J. Biochem.* 2000; 267: 566–572.
31. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 1997; 45: 327–343.
32. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
33. D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. *J. Immunol. Methods* 2009; 341: 1–18.
34. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 107–116.
35. Tanford C, Hauenstein JD. Hydrogen ion equilibria of ribonuclease. *J. Am. Chem. Soc.* 1956; 78: 5287–5291.
36. Boenisch T. Formalin-fixed and heat-retrieved tissue antigens: a comparison of their immunoreactivity in experimental antibody diluents. *Appl. Immunohistochem. Mol. Morphol.* 2001; 9: 176–179.
37. Boenisch T. Heat-induced antigen retrieval: what are we retrieving? *J. Histochem. Cytochem.* 2006; 54: 961–964.
38. Boenisch T. Heat-induced antigen retrieval restores electrostatic forces: prolonging the antibody incubation as an alternative. *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 363–367.
39. Pearse AGE. *Histochemistry: Theoretical and Applied*. Livingstone, Edinburgh: Churchill, 1980.
40. Puchtler H, Meloan SN. On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry* 1985; 82: 201–204.
41. Elias JM. *Immunohistopathology: A Practical Approach to Diagnosis*. Chicago, IL: ASCP Press, 1990.
42. Emoto K, Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: does pH or ionic strength of the solution play a role for refolding antigens? *J. Histochem. Cytochem.* 2005; 53: 1311–1321.

43. van Oss CJ, Absolom DR. Nature and thermodynamics of antigen-antibody interactions. In *Molecular Immunology*, ed. MZ Atassi, CJ van Oss, and DR Absalom. New York: Marcel Decker, 1984.
44. Wlodawer A, Bott R, Sjölin L. The refined crystal structure of ribonuclease A at 2.0 Å resolution. *J. Biol. Chem.* 1982; 257: 1325–1332.
45. Navon A, Ittah V, Laity JH, et al. Local and long-range interactions in the thermal unfolding transition of bovine pancreatic ribonuclease A. *Biochemistry* 2001; 40: 93–104.
46. Woody RW, Dunker AK. Aromatic and cysteine sidechain circular dichroism in proteins. In *Circular Dichroism and the Conformational Analysis of Biomolecules*, ed. GD Fasman. New York: Plenum Press, 1996.
47. Katakura Y, Kumamoto T, Iwai Y, et al. Fluorescence polarization study of a salt bridge between a single-chain Fv and its antigen ribonuclease A. *Mol. Immunol.* 1997; 34: 731–734.
48. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. *Biochim. Biophys. Acta* 2005; 1751: 119–139.
49. Peng Z, Kim PS. A protein dissection study of a molten globule. *Biochemistry* 1994; 33: 2136–2141.
50. Brown D, Lyndon J, Tilley SA, et al. Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem. Cell Biol.* 1996; 105: 261–267.
51. Yasuda K, Yamashita S, Aiso S, et al. Immunohistochemical study of gamma-glutamyl transpeptidase with monoclonal antibodies. I. Preparation and characterization of monoclonal antibodies to gamma-glutamyl transpeptidase. *Acta Histochem. Cytochem.* 1986; 19: 589–592.
52. Kakimoto K, Takekoshi S, Miyajima K, et al. Hypothesis for the mechanism for heat-induced antigen retrieval occurring on fresh frozen sections without formalin-fixation in immunohistochemistry. *J. Mol. Histol.* 2008; 39: 389–399.
53. Taylor CR, Shi S-R, Chen C, et al. Comparative study of antigen retrieval heating methods: microwave, microwave and pressure cooker, autoclave, and steamer. *Biotech. Histochem.* 1996; 71: 263–270.
54. Crook EM, Mathias AP, Rabin BR. Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2',3'-phosphate. *Biochem. J.* 1960; 74: 234–238.
55. Bell PB Jr., Rundquist I, Svensson I, et al. Formaldehyde sensitivity of a GFAP epitope, removed by extraction of the cytoskeleton with high salt. *J. Histochem. Cytochem.* 1987; 35: 1375–1380.
56. Montero C. The antigen-antibody reaction in immunohistochemistry. *J. Histochem. Cytochem.* 2003; 51: 1–4.
57. Morgan JM, Navabi H, Jasani B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. *J. Pathol.* 1997; 182: 233–237.
58. Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. *J. Histochem. Cytochem.* 2005; 53: 1421–1432.
59. Shi SR, Cote RJ, Hawes D, et al. Calcium-induced modification of protein conformation demonstrated by immunohistochemistry: What is the signal? *J. Histochem. Cytochem.* 1999; 47: 463–470.

60. Ward JM, Erexson CR, Faucette LJ, et al. Immunohistochemical markers for the rodent immune system. *Toxicol. Pathol.* 2006; 34: 616–630.
61. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J. Pathol.* 1997; 183: 116–123.
62. van Hecke D. Routine immunohistochemical staining today: choices to make, challenges to take. *J. Histotechnol.* 2002; 25: 45–47.
63. Holm R, Farrants GW, Nesland JM, et al. Ultrastructural and electron immunohistochemical features of medullary thyroid carcinoma. *Virchows Arch. A Pathol. Anat. Histopathol.* 1989; 414: 375–384.
64. Shi SR, Cote C, Kalra KL, et al. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. *J. Histochem. Cytochem.* 1992; 40: 787–792.
65. Shi SR, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. *J. Histochem. Cytochem.* 2007; 55: 105–109.
66. Bratthauer GL. Processing of tissue specimens. *Methods Mol. Biol.* 1999; 115: 77–84.
67. Cao A, Hu D, Lai L. Formation of amyloid fibrils from fully reduced hen egg white lysozyme. *Protein Sci.* 2004; 13: 319–324.
68. Dobson CM. Protein misfolding, evolution, and disease. *Trends Biochem. Sci.* 1999; 24: 329–332.
69. Tanaka S, Oda Y, Ataka M, et al. Denaturation and aggregation of hen egg lysozyme in aqueous ethanol solution studies by dynamic light scattering. *Biopolymers* 2001; 59: 370–379.
70. Yan YB, Zhang J, He HW, et al. Oligomerization and aggregation of bovine pancreatic ribonuclease A: characteristic events observed by FTIR spectroscopy. *Biophys. J.* 2006; 90: 2525–2533.
71. Pace CN, Trevino S, Prabhakaran E, et al. Protein structure, stability and solubility in water and other solvents. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 2004; 359: 1225–1235.
72. Kamatari YO, Konno T, Kataoka M, et al. The methanol-induced transition and the expanded helical conformation in hen lysozyme. *Protein Sci.* 1998; 7: 681–688.
73. Herskovits TT, Jaullet H. On the structural stability and solvent denaturation of proteins. *J. Biol. Chem.* 1970; 245: 2588–2598.
74. Goda S, Takano K, Yamagata Y, et al. Amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution. *Protein Sci.* 2000; 9: 369–375.
75. Watkins NG, Thorpe SR, Baynes JW. Glycation of amino groups in protein. Studies on the specificity of modification of RNase by glucose. *J. Biol. Chem.* 1985; 260: 10629–10636.
76. Mason JR, Leong FC, Plaxco KW, et al. Two-step covalent modification of proteins. Selective labeling of Schiff base-forming sites and selective blockade of the sense of smell in vivo. *J. Am. Chem. Soc.* 1985; 107: 6075–6084.
77. Borch RF, Bernstein MD, Durst HD. Cyanohydridoborate anion as a selective reducing agent. *J. Am. Chem. Soc.* 1971; 93: 2897–2904.

78. Stein H, Gatter K, Asbahr H, et al. Use of freeze-dried paraffin-embedded sections for immunohistologic staining with monoclonal antibodies. *Lab. Invest.* 1985; 52: 676–683.
79. Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J. Histochem. Cytochem.* 2001; 49: 931–937.
80. Leong AS, Lee ES, Yin H, et al. Superheating antigen retrieval. *Appl. Immunohistochem. Mol. Morphol.* 2002; 10: 263–268.
81. Fowler CB, Cunningham RE, Waybright TJ, et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. *Lab. Invest.* 2008; 88: 185–195.
82. Leong TY, Leong AS. How does antigen retrieval work? *Adv. Anat. Pathol.* 2007; 14: 129–131.
83. Gown AM. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* 2004; 121: 172–174.
84. Rojo F, Tabernero J, Albanell J, et al. Pharmacodynamic studies of gefitinib in tumor biopsy specimens from patients with advanced gastric carcinoma. *J. Clin. Oncol.* 2006; 24: 4309–4316.
85. Suurmeijer AJ, Boon ME. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. *Eur. J. Morphol.* 1993; 31: 144–150.
86. Gown A, Weaver ND, Battifora H. Microwave-based antigenic unmasking: a revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* 1993; 1: 256–266.
87. Sompuram SR, Vani K, Bogen SA. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.
88. Robinson JM, Vandre DD. Antigen retrieval in cells and tissues: enhancement with sodium dodecyl sulfate. *Histochem. Cell. Biol.* 2001; 116: 119–130.
89. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
90. Fowler CB, Cunningham RE, O’Leary TJ, et al. “Tissue surrogates” as a model for archival formalin-fixed paraffin-embedded tissues. *Lab. Invest.* 2007; 87: 836–846.
91. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.
92. Podkletnova I, Alho H. Ultrasound-amplified immunohistochemistry. *J. Histochem. Cytochem.* 1993; 41: 51–56.
93. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.
94. Good NE, Winget GD, Winter W, et al. Hydrogen ion buffers for biological research. *Biochemistry* 1966; 5: 467–477.
95. Novalic S, Jagschits F, Okwor J, et al. Behavior of citric acid during electrodialysis. *J. Memb. Sci.* 1995; 108: 201–205.

96. Reijenga JC, Gagliardi LG, Kennidler E. Temperature dependence of acidity constants, a tool to affect separation selectivity in capillary electrophoresis. *J. Chromatogr. A* 2007; 1155: 142–145.
97. Shin RW, Iwaki T, Kitamoto T, et al. Hydrated autoclave pretreatment enhances tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. *Lab. Invest.* 1991; 64: 693–702.
98. Igarashi H, Sugimura H, Maruyama K, et al. Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *APMIS* 1994; 102: 295–307.
99. Suurmeijer AJH, Boon ME. Optimizing keratin and vimentin retrieval in formalin-fixed, paraffin-embedded tissue with the use of heat and metal salts. *Appl. Immunohistochem.* 1993; 31: 144–146.
100. Tomizawa H, Yamada H, Imoto T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 degrees C. *Biochemistry* 1994; 33: 13032–13037.
101. Zale SE, Klibanov AM. Why does ribonuclease irreversibly inactivate at high temperatures? *Biochemistry* 1986; 25: 5432–5444.
102. Joshi AB, Sawai M, Kearney WR, et al. Studies on the mechanism of aspartic acid cleavage and glutamine deamidation in the acidic degradation of glucagon. *J. Pharm. Sci.* 2005; 94: 1912–1927.
103. Spink CH. Differential scanning calorimetry. *Methods Cell Biol.* 2008; 84: 115–141.
104. Stelea SD, Pancoska P, Benight AS, et al. Thermal unfolding of ribonuclease A in phosphate at neutral pH: deviations from the two-state model. *Protein Sci.* 2001; 10: 970–978.
105. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. *Biochim. Biophys. Act.* 2005; 1751: 119–139.
106. Kelly SM, Price NC. The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* 2000; 1: 349–384.
107. Woody RW. Circular dichroism. *Methods Enzymol.* 1995; 246: 34–71.

CHAPTER 16

A LINEAR EPITOPES MODEL OF ANTIGEN RETRIEVAL

STEVEN A. BOGEN and SESHU R. SOMPURAM

16.1 INTRODUCTION

From the standpoint of the practicing histotechnologist or surgical pathologist, formalin fixation is like a double-edged sword. Formalin is a time-honored fixative for preserving tissue samples and is deeply entrenched for routine diagnostic use. Formalin's disadvantage, however, is the deleterious effect it has on signal detection for immunohistochemistry (IHC) and *in situ* hybridization. Fixation in a cross-linking fixative such as formalin alters proteins in tissue sections, resulting in a loss of immunoreactivity. Formaldehyde reacts with tissue proteins in a sequence of reactions to ultimately create protein cross-links.¹⁻⁴ The specific types of cross-links depend on which amino acid side chains are involved. Such protein cross-links can result in protein denaturation, accounting for the loss of immunoreactivity after formalin treatment.

Historically, formalin fixation was not generally compatible with immunohistochemical detection of proteins, with a few exceptions. This problem was solved by Shi et al., when they found that boiling tissue sections ("antigen retrieval") restores immunoreactivity for many antibodies.⁵ The subsequent integration of antigen retrieval techniques into the histopathology laboratory facilitated explosive growth in the clinical application of IHC. The overwhelming majority of tissue samples that are processed in clinical IHC laboratories are treated with an antigen retrieval method.

Conceptually, the finding that boiling restores immunoreactivity after formalin fixation is surprising and counterintuitive. Like boiling an egg, exposure of proteins to high temperature should irreversibly denature those proteins, further degrading any possible signal that might be generated by an immunohistochemical stain. The finding that boiling did the opposite begged for an

explanation. A variety of mechanisms, including reversal of formalin-induced protein cross-links, have been proposed and reviewed to explain why boiling tissue sections restores immunoreactivity.^{1,6-12} Even if formalin-induced protein cross-links are broken during antigen retrieval, boiling is a highly denaturing treatment in its own right. It is not clear how proteins might regain their native conformation so as to restore immunoreactivity after such a harsh treatment.

This chapter focuses on the findings and conclusions from studies using peptide epitopes as a model system to study formalin fixation. Prior studies²⁻⁴ indicate that formaldehyde is capable of many different types of chemical reactions with proteins. However, from a practical diagnostic laboratory standpoint, we will focus in this chapter on those chemical reactions that explain why immunoreactivity is lost after fixation and restored following antigen retrieval. Therefore, the assay described in this chapter directly addresses this question. The peptide epitope model system examines how fixation and antigen retrieval affect the ability of antibodies to bind to their epitopes. The antibodies used for this study are all in common use by diagnostic IHC laboratories. A unique aspect of this study is the use of peptide epitopes rather than cells, tissue sections, or whole proteins. Studying only the epitope is a reductionist approach for deciphering the mechanism of antigen retrieval.

16.2 PEPTIDE ARRAY EXPERIMENTAL MODEL

The model immunostaining system uses short (approximately 20-mer) synthetic peptides as staining targets.¹³⁻¹⁵ Each peptide mimics or is identical to the antibody-combining site (epitope) of a particular antigen, such as estrogen receptor (ER), progesterone receptor (PR), Ki-67, p53, or human epidermal growth factor receptor type 2 (HER2). Some peptides are identified using phage display of a random peptide library (expressed in M13 bacteriophage), a combinatorial display technique in which an antibody selects the best peptide binders from billions of possible random peptides. The selected peptides represent the highest affinity binders to the antibody and closely match the epitope of the protein to which the antibody binds. Some studies use a peptide that precisely matches the native sequence (as described in the protein database). The peptides are covalently conjugated to a microscope glass slide. After the peptides attach, the slides are incubated briefly in a solution of irrelevant proteins (e.g., bovine gamma globulins) to quench remaining reactive sites on the glass surface. The glass slides, with attached peptide spots (as described in Chapter 7), are then stained by any desired immunohistochemical method. The primary antibody binds to the peptide epitope in the same manner as if it were binding to the native protein in a cell or tissue section. The sequential application of detection reagents results in a color that appears on the glass microscope slide wherever the peptide is attached. Various

peptides can be attached to the glass slide, in an array, facilitating the testing of different antigens or experimental conditions. Relevant negative controls include irrelevant peptides as well as the proteins on the glass slide outside of the small 3-mm diameter peptide epitope spot.

16.3 SOME PEPTIDES ARE DIRECTLY SUSCEPTIBLE TO FORMALIN FIXATION

The first series of experiments examined whether peptide epitopes can be fixed with formalin, resulting in a loss of immunoreactivity. The peptide epitopes’ sensitivity to formalin fixation depends on the amino acid composition. The first step was to identify peptides that mimic the epitopes of the following monoclonal antibodies: p53 (DO7), HER2 (9C2), ER (1D5), PR (636), and Ki-67 (MIB-1). Each peptide was attached to glass microscope slides as a 3-mm diameter spot, in duplicate. The peptide spots were exposed to formalin and then immunostained with the appropriate antibodies. Like a tissue section, the spots turn brown at the end of the immunohistochemical stain. The findings are illustrated in Figure 16.1, which is a montage of the scanned images of the stained peptide spots.

The far left column of Figure 16.1 shows a scanned image of the duplicate immunostained peptide spots before formalin fixation. The fact that the spots are stained demonstrates that each of the peptides (p53, HER2, ER, PR) binds

Peptide	Not Fixed	Formalin Fixed	
p53			
HER2			
ER			
PR			
	No Antigen Retrieval		Antigen Retrieved

Figure 16.1 Montage of images, after immunostaining of peptides. The antibody clones for these analytes are DO7 (p53), 9C2 (HER2), 1D5 (ER), and 636 (PR). The peptides were spotted in duplicate, adjacent to each other. The left-hand column (“Not Fixed”) illustrates stained peptide spots that were not fixed, representing a baseline condition. The middle column was fixed in formalin and not antigen retrieved. The peptides for p53 and HER2 lost immunoreactivity whereas the peptides for ER and PR continued to be immunoreactive. The right-hand column of peptide spots were both formalin fixed and antigen retrieved. Adapted with permission from Reference 16, © 2004 American Society for Clinical Pathology.

<u>Susceptible to formalin fixation</u>																						
p53 D07:	S	S	3	4	<u>5</u>	<u>6</u>	<u>Y</u>	8	9	10	<u>Y</u>	R	13	14	15	16	17	18	19	20		
Her 9C2:	1	S	N	4	5	6	R	<u>S</u>	<u>Y</u>	<u>10</u>	11	12	13	14	15	16	17	18	19	20	21	22
<u>Not susceptible to formalin fixation</u>																						
ER 1D5:	H	S	H	4	<u>Q</u>	<u>6</u>	<u>7</u>	<u>Y</u>	9	10	11	12	13	14	15	16	17	18	19	20	21	
PR 636:	S	R	3	<u>4</u>	<u>5</u>	<u>6</u>	7	8	9	10	H	12	13	14	S	16	17	18	19	20	21	
Ki-67 MIB-1:	S	2	N	4	5	6	7	8	<u>9</u>	<u>10</u>	11	S	13	14	N	H	N	18	19	20	21	22

Figure 16.2 Amino acid sequence of peptides showing only the amino acid residues that can react with formaldehyde. The peptides are aligned, starting with the first amino acid on the amino terminus. Amino acids that do not react with formalin are not indicated, except for a number denoting the position in the sequence. Underlined residues are epitopes, or at least part of them. The single-letter amino acid codes are as follows: R, arginine; Y, tyrosine; N, asparagine; H, histidine; Q, glutamine; and S, serine. Peptides that lost immunoreactivity after formalin fixation (“susceptible” group) have a tyrosine at the epitope and an arginine elsewhere. Adapted with permission from Reference 16, © 2004 American Society for Clinical Pathology.

to its antibody. For those peptides that were fixed in formalin, some lost their immunoreactivity whereas others did not. The middle column of Figure 16.1 represents images of the replicate spots that were fixed in formalin. The peptides matching the antibodies for p53 (D07) and HER2 (9C2) do not stain. This indicates that formalin fixation somehow denatured the peptides and caused a loss of immunoreactivity. For those peptides that lost immunoreactivity in response to formalin fixation, antigen retrieval reversed the effect and restored antibody binding, mimicking the antigen retrieval process in the tissue. This resulted in a reappearance of the colored peptide spots (right-hand column, Fig. 16.1). For the ER and PR peptides, formalin fixation had no effect on immunoreactivity. Even after overnight immersion in formalin (middle column), those peptides continued to bind to their antibodies, resulting in a positive immunoreaction.

Since we know the amino acid composition of the peptides, it was possible to correlate the presence of specific amino acids with the loss of immunoreactivity following formalin fixation (Fig. 16.2). In Figure 16.2, each amino acid is designated by the standard single letter code. Analysis of the amino acid sequences revealed a pattern that potentially accounts for their susceptibility to formalin fixation. For the peptides susceptible to formalin fixation (i.e., that lost immunoreactivity after fixation), there is a tyrosine (Y) at the antibody binding site and an arginine (R) elsewhere in the peptide. Other peptides lacking this combination of amino acids maintain immunoreactivity after formalin fixation. The amino acids tyrosine (Y) and arginine (R), in the context of a chemical reaction with formaldehyde, suggest that the Mannich condensation reaction is responsible, at least with these particular antibody epitopes.¹⁶

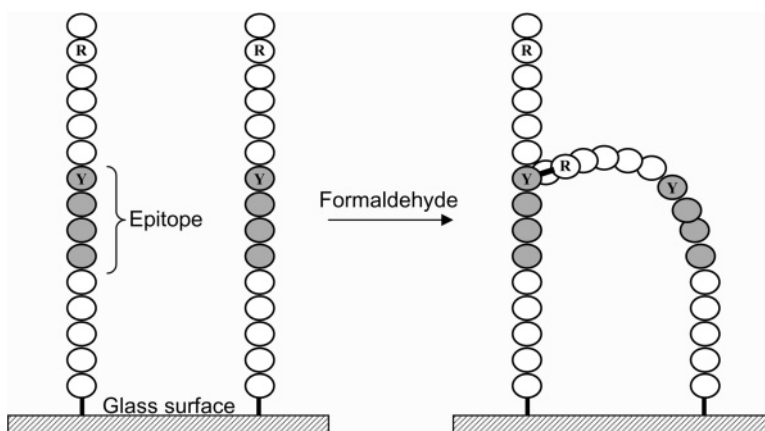


Figure 16.3 Conceptual illustration of two peptides before (left) and after (right) a chemical reaction with formaldehyde. The amino acids are represented as circles. In this particular peptide, a tyrosine (Y) is located within the epitope (shaded circles). An arginine (R) is located elsewhere in the peptide. Formaldehyde results in the formation of a covalent bond between the two residues, due to a Mannich condensation reaction, as shown on the right. The new configuration prevents antibodies from binding to the epitope on the left.

A likely explanation of these findings is that peptides immobilized on glass can form formaldehyde-induced protein cross-links, conceptually illustrated in Fig. 16.3. Each amino acid is represented as a circle, with a tyrosine (Y) at the epitope and an arginine (R) elsewhere. The shaded amino acids represent the epitope. The point of the illustration is that the formaldehyde-induced cross-link may position another adjacent peptide close to the epitope, sterically blocking the antibody from binding. This would account for the loss of immunoreactivity after formalin fixation. Antigen retrieval likely disrupts the tyrosine–arginine cross-link, thereby undoing the steric interference. It is also possible that a tyrosine–arginine cross-link forms within a single peptide, creating a loop structure, and thereby distorting or blocking the epitope. Either way, formalin fixation results in changes at the epitope that directly prevent an antibody from binding.

These initial findings do not exclude other possible formaldehyde-induced reactions with tissue proteins. Notably, this first model system was not designed to detect the role of lysine residues. Lysine has a propensity to react with and form a variety of different types of cross-links with other amino acids in the presence of formaldehyde.^{1,3,4,17} Therefore, it is likely to also be important in reactions with formaldehyde. In fact, peptides with internal lysine residues were purposefully excluded from this initial study for technical reasons. To explore the importance of lysine residues in antigen retrieval, an alternative method was employed.

To study the role of lysine residues in susceptibility to formalin fixation, the amino acid composition of immunoreactive peptides (to various monoclonal antibodies) was studied. Each peptide was evaluated to determine if immunoreactivity was lost after formalin fixation. Formalin sensitivity was correlated with the peptides' amino acid composition. The first step in the method is biopanning from a peptide combinatorial library with a monoclonal antibody. The peptides that bind to the antibody were tested for their sensitivity to formalin fixation. Some peptides remain immunoreactive whereas others do not. The peptides were then sequenced to look for differences between those that were sensitive to formaldehyde versus those that were not. The goal was to find whether there is a particular amino acid that is present in formalin-sensitive epitopes but absent in formalin-resistant epitopes, or vice versa. An advantage of this approach is that it is open-ended, without excluding any amino acids.

Figure 16.4 illustrates the important finding from the amino acid analysis. Four separate antibody clones were studied (for PR, 1A6 and 636; for HER2, CB11; and for ER, 1D5). For each antibody, up to 50 different peptides were sequenced. Each peptide was capable of binding to the cognate antibody even though their amino acid sequences varied. The epitope variants are often called "mimotopes." After sequencing the peptides, we found that lysine was the only amino acid that consistently correlated with sensitivity to formaldehyde.¹⁸ For each antibody, the formalin-sensitive group of peptides had more lysines than the formalin-insensitive group. This finding is consistent with the view that protein cross-linking is an important reason for the loss of immunoreactivity after formalin fixation. Moreover, the cross-linking includes chemical reactions that operate via the epsilon amino group of lysines.

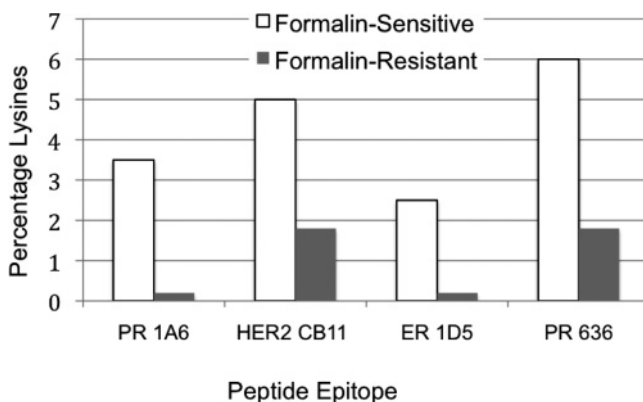


Figure 16.4 Graph depicting the percentage of lysine residues among peptides that bind to the indicated monoclonal antibodies. The peptides were isolated after affinity selection (biopanning) from a phage-displayed combinatorial peptide library. The peptides are grouped as to whether they are susceptible to formalin fixation, resulting in a loss of immunoreactivity.

16.4 IHC ANTIBODIES BIND TO LINEAR EPITOPES

Antibody epitopes can be classified as either linear or conformational. The linear epitopes comprise a stretch of approximately 5–7 amino acids that are contiguous. Although other amino acids may lend additional strength to the noncovalent interactions associated with antibody binding, these 5–7 amino acids are responsible for the majority of the binding energy. Conformationally dependent (or “discontinuous”) epitopes, on the other hand, are formed as a result of distantly and spatially distributed small groups of amino acids brought together by protein folding. It is generally believed that most of the antibodies generated in the course of an immune response to conventional protein antigens are conformationally dependent.^{19,20}

The monoclonal antibodies commonly used for clinical immunohistochemistry are a select group and not necessarily representative of a typical immune response. These antibodies were selected and commercialized because of their ability to bind to their antigens even after formalin fixation (and antigen retrieval). An important distinguishing characteristic of the monoclonal antibodies useful for formalin-fixed tissues is that the epitopes to which they bind are linear. This assertion is based on the analysis of nine antibody epitopes that are widely used for clinical IHC, directed to the human ER, PR, HER2, and Ki-67. In other words, the peptide epitopes for each of the nine monoclonal antibodies are derived from contiguous amino acids as found in the native protein.¹⁵ A series of experiments using peptide epitopes illustrate why linear epitopes are conducive to recovering immunoreactivity after antigen retrieval.

16.5 ADJACENT PROTEINS ARE IMPORTANT IN UNDERSTANDING ANTIGEN RETRIEVAL

In order to model the effect of formalin fixation and antigen retrieval on antibody immunoreactivity, we used a peptide epitope array (Fig. 16.5). The peptide epitopes are derived from the exact sequences in the native proteins. In this experiment, the initial findings are similar to those shown in Figure 16.1, but they are then extended by allowing for the role of adjacent proteins.

Column A (Fig. 16.5) shows the baseline binding capability of each antibody for its peptide epitope. Prior experiments had established that each antibody only binds to its specific peptide and not the others.¹⁵ The presence of a colored spot on the glass slide indicates immunoreactivity. Each row contains a different peptide and antibody combination, as indicated by the legend to the left. The legend at the top of Figure 16.5 indicates that the peptides in column A are not treated in any way.

Similar to the earlier findings illustrated in Figure 16.1, some but not all peptides lose immunoreactivity after formalin fixation. In this instance, two of the peptides partially PR(1A6) or completely Ki-67(MIB-1) lose immunoreactivity after formalin fixation (Fig. 16.5, column B). The other peptides in the

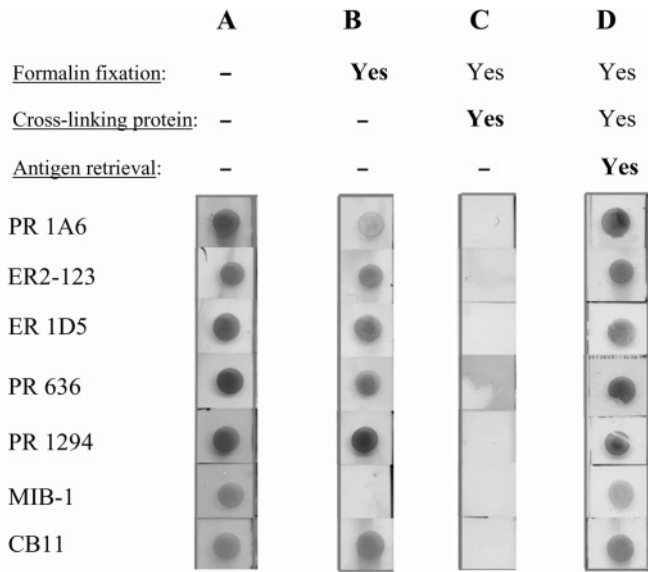


Figure 16.5 Immunostained peptide arrays after various treatments of fixation, protein cross-linking, and antigen retrieval, as indicated at the top. Each row has a different peptide that is immunoreactive for the antibody denoted to the left. Column A represents the baseline condition, without any treatment whatsoever. Column B shows immunoreactivity of each peptide after overnight formalin fixation. Column C shows the immunoreactivity after first coating the array with an irrelevant protein (casein) followed by overnight formalin fixation. Column D illustrates the immunoreactivity of the peptides after the treatment of column C, and then antigen retrieval. Reproduced with permission from Reference 15, © 2006 American Society for Clinical Pathology.

array do not lose immunoreactivity after formalin fixation (Fig. 16.5, column B). The reason why only some peptides lose immunoreactivity was already discussed in Section 16.3. Presumably, these other peptides retain their immunoreactivity because of their small size and limited number of amino acid residues that can form cross-links in the presence of formaldehyde.

Based on the amino acid composition of the various peptides, it is not surprising that the Ki-67(MIB-1) and PR(636) peptides are sensitive to formalin fixation. The Ki-67(MIB-1) peptide is the only one with a lysine at the antibody epitope, facilitating formaldehyde-induced cross-links at the lysine’s epsilon amino group. Such cross-linking could alter the site at which the antibody binds, rendering the epitope inaccessible to antibody binding. The PR(1A6) peptide is also moderately formalin-sensitive but lacks a lysine. However, it is the only peptide in this group with a tyrosine at the epitope and a nearby arginine. This combination of amino acids suggests the possibility that formalin fixation creates cross-links between adjacent peptides through a Mannich condensation reaction, as previously described (Section 16.3).

The fact that most of the peptide epitopes do not lose immunoreactivity after exposure to formalin demonstrates that something in addition to the epitope is important. These epitopes normally exist as part of a larger protein and in a cellular milieu admixed with thousands of other proteins. The presence of other proteins can be simulated by dipping the slides (on which the peptides are attached) into a dilute protein solution (e.g., 0.2% casein). This creates an initial weak, noncovalent physical adsorption of casein onto the array surface. The array is then incubated overnight in formalin vapor at 37°C. The use of formalin vapor, rather than formalin liquid, helps retain the casein on the peptide array surface during the formaldehyde cross-linking reaction. Formaldehyde cross-linking requires hours. If the slides had been dipped in liquid formalin (rather than formalin vapor), the casein would have been rinsed off before it could be cross-linked. Cross-linking of another protein (such as casein) onto the peptides results in complete abrogation of immunoreactivity (Fig. 16.5, column C). If the peptide array (cross-linked to an irrelevant protein) is then treated with an antigen retrieval protocol, immunoreactivity is restored (Fig. 16.5, column D). Therefore, column C represents an analogous condition to fixed tissues before antigen retrieval and column D is analogous to fixed tissues after antigen retrieval.

These data suggest that the loss of immunoreactivity after formalin fixation involves a protein cross-linking reaction. The first amino acid is at or near the antibody epitope. The second can be on the same protein or on another nearby protein. A variety of different chemical reactions with formaldehyde can be occurring. The common theme is that regardless of the details of the formaldehyde-induced cross-linking reaction, steric interference prevents antibodies from gaining access to the epitope.

16.6 A MODEL OF ANTIGEN RETRIEVAL REQUIRING LINEAR EPITOPES

These findings suggest a reason as to why antigen retrieval, a highly denaturing procedure, restores immunoreactivity of antibody epitopes after formalin fixation. In tissue biopsies, formalin fixation creates cross-links between adjacent proteins. Some of those cross-links are at or near the antibody epitope, resulting in steric interference to antibody binding. Antigen retrieval causes hydrolytic cleavage of intra- or intermolecular cross-links, dissociating proteins that are sterically interfering with antibody binding. Since the epitope is linear and not dependent on protein conformation, its immunoreactivity is preserved despite the denaturing conditions associated with antigen retrieval. Protein refolding to a native conformation is irrelevant in the context of linear epitopes.

Figure 16.6 illustrates the proposed sequence of events during immunohistochemistry staining with the peptide array. Amino acids are represented as circles. Each peptide is a string of amino acids that are covalently

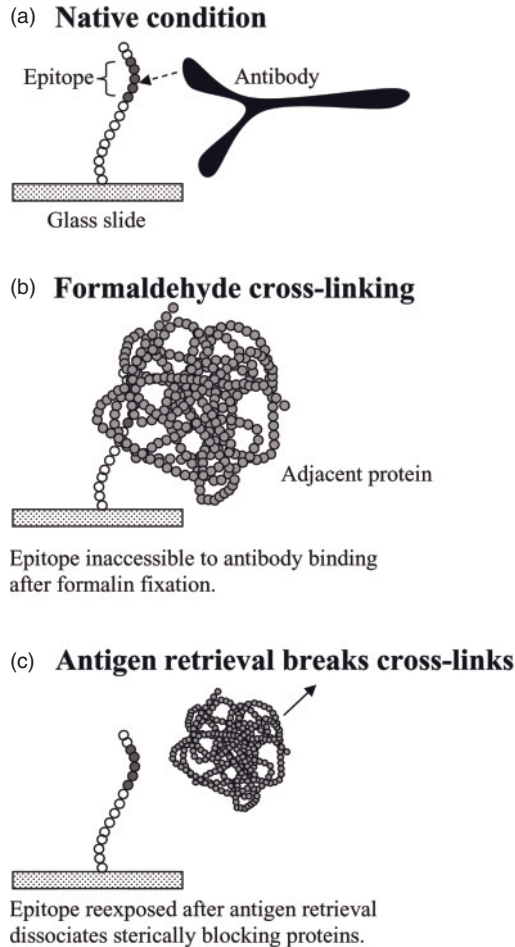


Figure 16.6 Schematic molecular model accounting for the loss and subsequent recovery of immunoreactivity after formalin fixation and antigen retrieval. Adapted from Reference 15, © 2006 American Society for Clinical Pathology.

anchored to the glass substrate at one end. At the uppermost row, labeled “a. Native condition,” the antibody is immunoreactive with a peptide containing the epitope (shaded circles). The figure also illustrates that the antibody recognizes a linear epitope, that is, a contiguous series of 5–7 amino acids. Although this model is for the peptide array, there is an analogous situation in surgical biopsy samples. In a tissue section, the peptide epitope is part of a larger protein, with many adjacent proteins in the normal cellular milieu.

In the middle row of Figure 16.6, labeled “b. Formaldehyde cross-linking,” the peptide is represented after formalin-induced cross-linking to an irrelevant

nearby protein. Cross-linking to an irrelevant protein (e.g., casein) blocks antibody access to the linear epitope, accounting for a loss of immunoreactivity. The cross-linking reaction can include any formaldehyde-reactive amino acid side chain. Various formaldehyde-induced reactions probably occur. The chemical reaction that is responsible for blocking the epitope will likely depend on which formaldehyde-reactive amino acids are near the epitope. In the analogous situation for cells and tissues, formaldehyde-induced protein cross-linking leads to cross-links forming within the protein itself or with other adjacent proteins.

Chemical treatment with formaldehyde may not necessarily result in significant denaturation. A recent study of RNase A indicated that treatment with formaldehyde does not significantly alter secondary structure.^{21,22} Although formalin treatment may induce subtle changes in secondary structure, alpha helices and beta-pleated sheets are left essentially intact after formalin treatment. It is reasonable to assume, however, that boiling (as per antigen retrieval protocols) will significantly alter secondary structure.

Figure 16.6c (the lowermost row) illustrates the likely outcome of antigen retrieval in our peptide epitope array. Antigen retrieval breaks formalin-induced cross-links, resulting in the dissociation of sterically interfering proteins from the peptide epitope. Since the peptide epitope is reexposed, immunoreactivity is restored. In a tissue section, the temperature associated with antigen retrieval (approaching 120°C, if a pressure cooker is used) exceeds the denaturation temperature of most proteins. Consequently, secondary structure is irreversibly altered. Regardless of the loss of secondary structure, antigen retrieval facilitates antibody binding by reexposing the linear epitope. Since the epitope is linear, antibody binding is not dependent on the secondary (or higher level) structures and are therefore not abrogated by the heat associated with antigen retrieval.

16.7 EVALUATION OF THE MODEL

A reasonable objection to any *in vitro* model is whether it accurately mirrors the actual process. A strength of this model is that the peptides in the array, mounted on the microscope glass slide, are the very same as the antibody epitopes in the native proteins. Therefore, the types of formaldehyde-induced chemical reactions at or near the epitope are the same as would likely occur in a tissue sample. An additional strength of the model is that the experimental data using the peptide array completely account for the loss of immunoreactivity after formalin fixation and the recovery of immunoreactivity after antigen retrieval (Fig. 16.5). Nonetheless, our data do not prove that the model accurately represents formaldehyde reactions in tissue specimens. For example, our data do not exclude other causes of steric interference.

An alternative hypothesis for antigen retrieval is that steric interference to antibody binding occurs via the formation of calcium coordinate compounds with neighboring hydroxymethyl, carboxyl, and phosphoryl moieties.^{8,23} This concept was initially proposed because of the observation that, at least for some antibodies, antigen retrieval was more effective if a calcium chelator (e.g., citrate or EDTA) is included in the antigen retrieval buffer. According to this alternative hypothesis, calcium cage-like complexes may also contribute to steric interference of epitopes. Calcium chelation associated with antigen retrieval might then destroy the complexes, reestablishing immunoreactivity.

Chelation is not required in this peptide epitope model system, as divalent cations are not even added to the buffers during formalin fixation. In tissue biopsies, chelation in the absence of high temperatures is not effective for antigen retrieval. On the other hand, heating without chelation is usually effective, even if not always optimal, in reestablishing immunoreactivity. This observation suggests that reversal of formaldehyde-induced protein cross-links (i.e., methylene bridges) is required for antigen retrieval to restore immunoreactivity. Using an *in vitro* model of formaldehyde fixation with intact proteins (instead of peptides), Yamashita and Okada arrived at a similar conclusion.²⁴

16.8 HETEROGENEITY OF ANTIGEN RETRIEVAL REACTIONS

After the initial description of antigen retrieval by Shi et al.,⁵ numerous investigators examined the optimal antigen retrieval conditions associated with many different monoclonal antibodies.^{25–27} The variables that were examined included pH, temperature/type of heating, molarity, as well as chelation and the presence or absence of metal ions. Various antibodies and proteins displayed different optimal antigen retrieval requirements. Adding further to the complexity, immunoreactivity with some proteins is recovered adequately with enzymatic cleavage of proteins instead of heat treatment. In the context of the model presented in this chapter, the observations suggest that the protein cross-links are heterogeneous. The common denominator among them all may be steric interference with antibody binding, but the precise amino acids involved in the reaction can vary. A variety of amino acids are known to participate in protein cross-linking after exposure to formaldehyde, including arginine, cysteine, histidine, lysine, tryptophan, tyrosine, serine, asparagine, and glutamine.^{1–4,28} Cross-links involving amino acids at or near the epitope have the greatest likelihood to affect immunoreactivity. Therefore, the optimal conditions for antigen retrieval will be expected to vary, depending on the chemical requirements for reversal of different protein cross-links. The variability in optimal antigen retrieval conditions is consistent with a model involving linear epitopes.

Even among the group of peptide epitopes, there was a striking variability in the speed of formalin fixation and antigen retrieval, suggesting hetero-

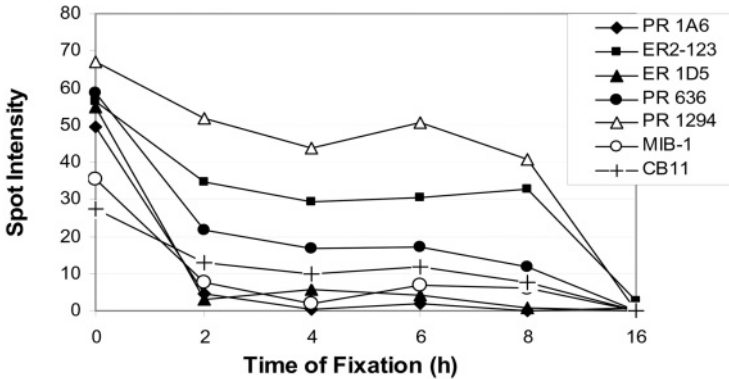


Figure 16.7 Immunohistochemical staining intensity as a function of the duration of formalin fixation. Immunostaining intensity was measured in triplicate. The coefficients of variation (CVs) were almost all less than 10% and, for purposes of clarity, are not shown. Staining intensity is measured as mean pixel intensity units on a 1–256 scale. ER(1D5), ER(2-123), PR(636), PR(1294), and Ki-67(MIB-1), DakoCytomation, Carpinteria, CA; PR(1A6) and HER2(CB11), Vision Biosystems, Norwell, MA. ER, estrogen receptor; PR, progesterone receptor. Reproduced with permission from Reference 15, © 2006 American Society for Clinical Pathology.

geneity of cross-linking reactions. The fact that peptide epitopes are short (approximately 20 amino acids) probably limits the range of reactions that can occur, since each peptide only has 1–3 formaldehyde-reactive amino acids. Figure 16.7 illustrates the variability in the kinetics of formalin fixation among the peptide epitopes. For example, the peptide epitope for the progesterone receptor PR(1A6) antibody lost all immunoreactivity within 2 h. Since the reaction occurs on a single plane of glass, formalin penetration is not relevant in this model system. The reaction kinetics can be completely attributed to the rate of the formaldehyde reaction itself. In contrast to the rapid loss of immunoreactivity for the PR(1A6) antibody, the peptide epitope for the PR(1294) antibody retained almost all of its immunoreactivity at the same time point. Eventually, by 16 h, all of the peptide epitopes lost their immunoreactivity. The same variability is true for antigen retrieval (Fig. 16.8). In general, the peptides that took the longest for formaldehyde fixation were the first to recover immunoreactivity after antigen retrieval. These findings suggest that the precise amino acid composition and the spatial relationship of formaldehyde-reactive amino acids to the epitope are important determinants affecting the optimal conditions for antigen retrieval. This interpretation is also consistent with the observation that the amino acidic residues arginine, tyrosine, and lysine residues are highly reactive with formaldehyde whereas asparagine, glutamine, and histidine residues show a weaker reaction.²⁹

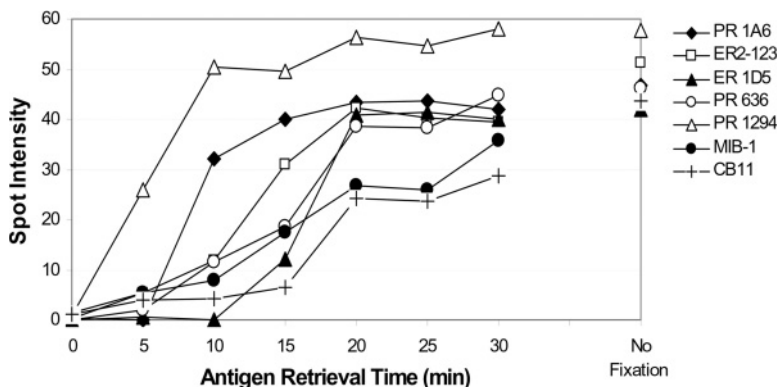


Figure 16.8 Intensity of immunohistochemical staining as a function of the length of antigen retrieval time. All values represent the mean of triplicate measurements. The staining intensity of a peptide array that was not formalin fixed is shown at the far right of the graph, as a control. Reproduced with permission from Reference 15, © 2006 American Society for Clinical Pathology.

16.9 SUMMARY

Antigen retrieval can be explained with a model whereby antibodies commonly used for clinical IHC are immunoreactive to epitopes that are linear. Epitope mapping of nine monoclonal antibodies support this view. In each instance, there was a contiguous sequence of amino acids derived from the native protein sequence that represented the antibody's binding site. This model predicts that antibodies recognizing discontinuous epitopes may not be useful for formalin-fixed paraffin-embedded tissue samples. This study is different from others in using a highly reductionist approach. Instead of using tissues, cells, or even proteins, the effects of formalin fixation and antigen retrieval were studied using isolated peptide epitopes. The peptide epitopes were incorporated into a model system for simulating the loss of immunoreactivity after fixation. Most peptide epitopes did not lose immunoreactivity after treatment with formalin. However, they did lose immunoreactivity if an irrelevant protein was added, blocking the peptide epitope after formaldehyde-induced protein cross-linking. Antigen retrieval restored immunoreactivity. The precise amino acid composition of an epitope appears to be an important determinant of the precise conditions that are required during antigen retrieval.

CONFLICT OF INTEREST DISCLOSURE

The authors disclose inventorship of patents or patent applications bearing on the topic of formalin fixation (of peptides), now transferred to ThermoFisher Corp.

ACKNOWLEDGMENT

We are grateful for the financial support provided by the National Institutes of Health, who supported this work through NIH grants CA106847 and CA094557.

REFERENCES

1. Shi S-R, Gu J, Turrens J, et al. Development of the antigen retrieval technique: philosophical and theoretical bases. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 17–40. Natick, MA: Eaton, 2000.
2. Fraenkel-Conrat H, Brandon B, Olcott H. The reaction of formaldehyde with proteins. IV. Participation of indole groups. Gramicidin. *J. Biol. Chem.* 1947; 168: 99–118.
3. Fraenkel-Conrat H, Olcott H. Reaction of formaldehyde with proteins. VI. Crosslinking of amino groups with phenol, imidazole, or indole groups. *J. Biol. Chem.* 1948; 174: 827–843.
4. Fraenkel-Conrat H, Olcott H. The reaction of formaldehyde with proteins. V. Crosslinking between amino and primary amide or guanidyl groups. *J. Am. Chem. Soc.* 1948; 70: 2673–2684.
5. Shi S, Key M, Kalra K. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
6. Gown A, Wever ND, Battifora H. Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* 1993; 1: 256–266.
7. Suurmeijer A, Boon M. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. *Eur. J. Morphol.* 1993; 31: 144–150.
8. Morgan J, Navabi H, Schmid K, et al. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. *J. Pathol.* 1994; 174: 301–307.
9. Leong T, Leong A. How does antigen retrieval work? [Review]. *Adv. Anat. Pathol.* 2007; 14: 129–131.
10. Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. [Review]. *Prog. Histochem. Cytochem.* 2007; 41: 141–200.
11. Boenisch T. Heat-induced antigen retrieval: what are we retrieving? [Review]. *J. Histochem. Cytochem.* 2006; 54: 961–964.
12. Dapson R. Macromolecular changes caused by formalin fixation and antigen retrieval. *Biotech. Histochem.* 2007; 82: 133–140.
13. Sompuram S, Vani K, Ramanathan H, et al. Synthetic peptides identified from phage-displayed combinatorial libraries as immunodiagnostic assay surrogate quality control targets. *Clin. Chem.* 2002; 48: 410–420.

14. Sompuram S, Vani K, Zhang K, et al. A novel quality control slide for quantitative immunohistochemistry testing. *J. Histochem. Cytochem.* 2002; 50: 1425–1434.
15. Sompuram S, Vani K, Bogen S. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.
16. Sompuram S, Vani K, Messana E, Bogen S. A molecular mechanism of formalin-fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
17. Hua C, Langlet C, Buferne M, et al. Selective destruction by formaldehyde fixation of an H-2Kb serological determinant involving lysine 89 without loss of T-cell reactivity. *Immunogenetics* 1985; 21: 227–234.
18. Vani K, Bogen S, Sompuram S. A high throughput combinatorial library technique for identifying formalin-sensitive epitopes. *J. Immunol. Methods* 2006; 317: 80–89.
19. Kubly J. *Immunology*, 2nd edition. New York: W.H. Freeman, 1994.
20. Huebner J. Antibody-antigen interactions and measurements of immunologic reactions. In *Immunology, Infection, and Immunity*, 1st edition, ed. GB Pier, JB Lyczak, and LM Wetzler, pp. 209–210. Washington, DC: American Society for Microbiology, 2004.
21. Rait V, O’Leary T, Mason J. Modelling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
22. Rait V, Xu L, O’Leary T, et al. Modelling formalin fixation and antigen retrieval with bovine pancreatic RNase A: II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
23. Morgan J, Navabi H, Jasani B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. *J. Pathol.* 1997; 182: 233–237.
24. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
25. Hsi E. A practical approach for evaluating new antibodies in the clinical immunohistochemistry laboratory. *Arch. Pathol. Lab. Med.* 2001; 125: 289–294.
26. Pileri S, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J. Pathol.* 1997; 183: 116–123.
27. Ramos-Vara J, Beissenherz M. Optimization of immunohistochemical methods using two different antigen retrieval methods on formalin-fixed, paraffin-embedded tissues: experience with 63 markers. *J. Vet. Diagn. Invest.* 2000; 12: 307–311.
28. D’Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. *J. Immunol. Methods* 2000; 341 (1–2): 1–18.
29. Metz B, Kersten G, Baart G, et al. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug. Chem.* 2006; 17: 815–822.

PH OR IONIC STRENGTH OF ANTIGEN RETRIEVAL SOLUTION: A POTENTIAL ROLE FOR REFOLDING DURING HEAT TREATMENT

SHUJI YAMASHITA

17.1 INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) materials are widely used for histological studies because they provide good morphology and are easy to store for long periods and because FFPE specimens are commonly collected as pathological, surgical, and autopsy specimens in many laboratories. To prepare FFPE specimens, tissues are fixed with formalin, dehydrated with ethanol, cleared in xylene, and infiltrated and embedded in paraffin at 55–60°C. Formaldehyde treatment is shown to add methylol groups to amino groups of proteins, RNA and single-strand DNA, and then to cross-link them by forming methylene bridges. Mason and O’Leary (1991) found that formaldehyde-fixed proteins retain their secondary protein structure using a model system, while fixation in glutaraldehyde causes a loss of up to 30% of a protein’s α helix structure because of glutaraldehyde’s strong fixing ability.¹ Fowler et al. further examined the effect of dehydration on formaldehyde-treated proteins and demonstrated that ethanol treatment causes the rearrangement of β -sheets in formaldehyde-treated proteins, severely destroying the secondary and tertiary structures of the proteins and further advancing intra- and intermolecular cross-linkage.² Since the protein concentration is high in cell organelles and cytoplasm and FFPE specimens are dehydrated and heated during embedding, the proteins in the FFPE materials are likely to be highly cross-linked and denatured, compared with those in frozen sections.

Therefore, FFPE materials have been regarded as unsuitable, or at least not ideal, for enzyme histochemical and immunohistochemical studies, and antigen retrieval techniques including heating, enzyme digestion, and treatment with protein denaturants have been developed for immunohistochemistry. Shi et al. reported that the antigenicity of some antigens is restored after heat treatment.³ Since the publication of Shi et al.'s report, heat-induced antigen retrieval (HIAR) has become a common immunohistochemistry technique.⁴⁻⁸ Investigators have tried to select the most suitable conditions (heating devices, temperatures, kinds of solutions, pH of solutions, and additives) for heating each antigen and to elucidate the mechanisms of antigen retrieval. Recent studies show that HIAR is also applicable to formaldehyde-fixed frozen sections⁹ and tissues unexposed to chemical fixatives.^{7,10} Furthermore, HIAR is also used for immunoelectron microscopy in conjunction with pre-embedding and post-embedding methods.^{8,11,12}

Although antigen retrieval techniques including HIAR continue to be regarded as enigmatic techniques, recent studies are rapidly clarifying the mechanisms of HIAR. In this article, the effects of pH and the ion concentrations of retrieval solutions on HIAR will be reviewed, and the probable mechanisms of HIAR and other antigen retrieval techniques will be discussed based on these results.

17.2 EFFECTS OF pH

Since heating and metal ions in solution were initially speculated to be important for HIAR, many additives and metal ions have been assessed and reported to be effective.^{3,13,14} Subsequent studies, however, have demonstrated that heating is the essential factor for HIAR, and have shown the importance of pH of retrieval solutions.

17.2.1 pH Dependency of HIAR in FFPE Sections

Many investigators have demonstrated that the efficiency of HIAR is highly dependent on the pH of the retrieval solution. Shi et al. was the first group to study systematically the effects of the antigen retrieval solution pH on HIAR.¹⁵ They used nine monoclonal antibodies to examine the pH-dependent retrieval of nine antigens: three nuclear antigens (including proliferating cell nuclear antigen [PCNA] and estrogen receptor [ER] α), three cytoplasmic antigens (including neuron-specific enolase), and three cell-surface antigens. They classified the pH-influenced HIAR immunostaining patterns as follows: type A (five antibodies), in which staining was almost the same at any pH, with a slight decrease in intensity between pH 3.0 and pH 6.0; type B (two antibodies), in which a dramatic decrease in staining intensity occurred between pH 3.0 and pH 6.0; and type C (two antibodies), in which the immunostaining

intensity increased with the pH value of the HIAR solution. Based on these results, they recommended using a basic solution for HIAR.

Kim et al. studied the pH dependency of immunostaining using pH values ranging from pH 2.0 to pH 9.5 and 29 monoclonal antibodies commonly used for diagnosis.¹⁶ They reported that three antibodies exhibited a type-A pattern, four antibodies showed a type-B pattern, and two antibodies exhibited a type-C pattern, whereas the other antibodies did not exhibit any of these three patterns. Their results showed that 50mM borate buffer (pH 8.0) or 50mM Tris-HCl buffer (TB) (pH 9.5) yielded the strongest immunostaining for most of the antibodies, and that 50mM glycine-HCl buffer (pH 2.0) was also an excellent buffer for antigen retrieval, although it destroyed the tissue structures; in particular, the nuclei of the epithelial cells were damaged.^{16,17}

Emoto et al. reexamined the pH dependency of HIAR by evaluating the immunostaining intensity of nuclear, cytoplasmic, cell membrane, and extracellular matrix antigens with 17 different antibodies in FFPE mouse and human tissues, and showed two immunostaining patterns for the pH dependency of HIAR.¹⁷ Deparaffinized sections from tissues fixed with 4% formaldehyde or 10% formalin were autoclaved for 10 min in buffers with pH values ranging from 3.0 to 10.5. The majority of the antibodies (13 antibodies) produced the first immunostaining pattern independent of the isoelectric point (pI) of the antigen proteins and the antigen localization; that is, they yielded a positive immunoreaction when heated in buffers that had either an acidic pH (20mM glycine-HCl buffer, pH 3.0) or a basic pH (20mM TB, pH 9.0 and pH 10.5) (Fig. 17.1). This HIAR pattern may correspond to the type-B pattern (V-form type) of the classification by Shi et al.¹⁵ Four antibodies produced the second immunostaining pattern, which consisted of strong immunostaining when heated in TB (pH 9.0 and pH 10.5) (Fig. 17.1), corresponding to the type-C pattern (ascending type) described by Shi et al.¹⁵ The α -amylase immunostaining pattern was almost constant regardless of the pH value of the buffers used for HIAR when the antiserum was diluted 5,000-fold,¹⁸ but the type-B pattern was observed when the antiserum was diluted 50,000-fold.¹⁷ If highly diluted antibodies were to be used in the immunohistochemical studies described by Shi et al.,¹⁵ the type-A pattern (stable type) might become nearly equivalent to the type-B pattern. These results were summarized in Table 17.1.

Similar results have been reported by several investigators. Pileri et al. compared three antigen retrieval solutions and found that 1mM EDTA-NaOH solution (pH 8.0) was the best, 100mM M TB (pH 8.0) was fair, and 10mM citrate buffer (CB) (pH 6.0) was the poorest.⁷ Of the 61 antibodies, 55 yielded a positive reaction after heating, and 53 of the 55 antibodies displayed a stronger immunoreaction when heated in 1mM EDTA-NaOH (pH 8.0) than when heated in 10mM CB (pH 6.0). Imam et al. compared the efficacy of two buffers, 50mM glycine-HCl (pH 3.6) containing 0.01% EDTA and 100mM CB (pH 6.0).¹⁹ The glycine-HCl buffer (pH 3.6) containing EDTA yielded stronger immunostaining for six nuclear antigens (including

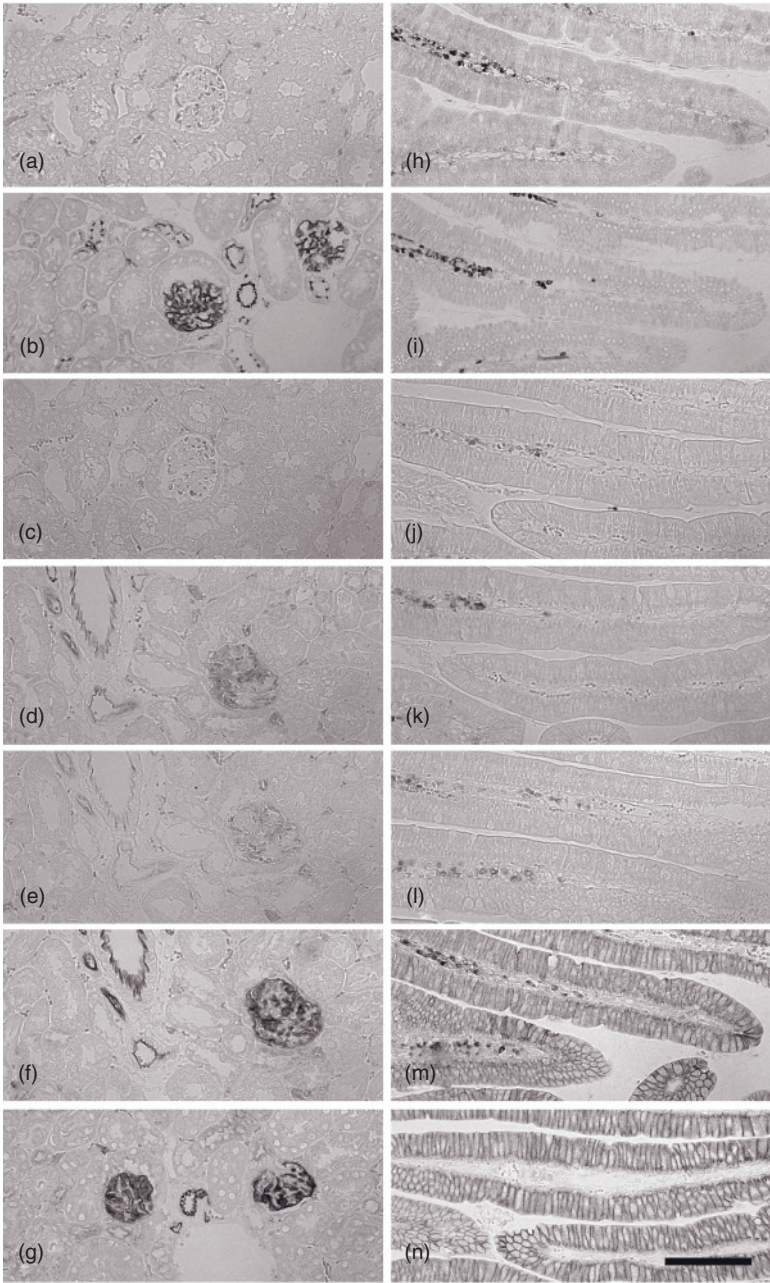


TABLE 17.1 pH Dependency in HIAR

Antigens	pIs	Nonheating	pH 3.0	pH 4.5	pH 6.0	pH 7.5	pH 9.0	pH 10.5
ER α	8.3	+	^a	+	++	+	+++	++
ER β	8.8	–	$\pm$	–	+	$\pm$	++	+
AnR	6.0	–	^a	–	+	$\pm$	+++	++
PR	6.7	–	^a	+	++	+	+++	++
GR	6.0	$\pm$	^a	+	+	+	+++	++
p300	8.8	+	$\pm$ ^a	$\pm$	++	+	++	+
SRC-1	5.7	–	^a	$\pm$	+	$\pm$	+++	$\pm$
PCNA	4.6	–	^a	$\pm$	+	$\pm$	++	++
β -Actin	5.2	++	+++	+	+	+	++	++
Tubulin	4.9	+	+	$\pm$	++	+	+++	++
GFAP	5.3	+	++	$\pm$	++	$\pm$	+++	++
Claudin-5	8.3	–	+++	–	$\pm$	–	++	++
Amylase	7.0	+	+++	+	+	+	++	+++
Lysozyme	9.6	+++	–	$\pm$	+	+	++	++
E-Cadherin	9.9	–	–	–	–	–	++	+++
Fibronectin	5.9	–	+	–	$\pm$	–	+++	+
Laminin	5.4	++	++	–	+	+	++	++

Notes: Mouse tissues were fixed with 10% formalin for 24h. Estrogen receptor (ER) α , progesterone receptor (PR), p300, steroid receptor coactivator-1 (SRC-1) were localized in the uterus. ER β and androgen receptor (AnR) were detected in the ovary and epididymis, respectively. Glucocorticoid receptor (GR) and fibronectin were localized in the liver, and immunoreaction for proliferating cell nuclear antigen (PCNA), β -actin, tubulin, glia fibrillary acidic protein (GFAP), lysozyme, and E-cadherin was examined in the small intestine. Claudin-5 and laminin were detected in the kidney, and amylase was localized in the pancreas. Immunostaining was scored as follows: +++, strong; ++, moderate; +, weak; $\pm$, faint; –, negative.

^aImmunostaining was decreased in some cell types that the nuclear components were extracted. pIs, isoelectric points of antigen proteins.

Figure 17.1 pH-Dependency of antigen retrieval. Sections from mouse tissues fixed with 4% paraformaldehyde for 24h were autoclaved at 120°C for 10min in the following buffers: 20mM glycine-HCl buffer (pH 3.0; b and i); 20mM citrate buffer (CB) (pH 4.5; c and j); 20mM CB (pH 6.0; d and k); 20mM Tris-HCl buffer (TB) (pH 7.5; e and l); 20mM TB (pH 9.0; f and m); 20mM TB (pH 10.5; g and n). Sections without autoclaving were used as control staining (a and h). Claudin-5 was localized in the kidney (a–g), and E-cadherin was detected in the small intestine. Claudin-5 is present in the glomeruli and arterioles. The junctional region of distal tubules shows weak claudin-5 immunoreaction. Claudin-5 immunoreaction is strong when heated at pH 3.0, pH 9.0, or pH 10.5, which may correspond to type-B pattern (V-form type) classified by Shi et al.¹⁵ E-cadherin is localized along the lateral membrane of epithelial cells in the small intestine; junctional region exhibits strong immunostaining. E-cadherin immunoreaction is detected when heated at pH 9.0 or pH 10.5, corresponding to the type-C pattern (ascending type).¹⁵ Bar = 100 μ m.

androgen receptor [AnR]), ER α , and Ki-67), two cytoplasmic antigens, and three cell-surface antigens, compared with CB (pH 6.0), whereas CB exhibited superior immunostaining for vimentin and two cell-surface antigens. Ferrier et al. reported that immunostaining for tissue-type plasminogen activator and plasminogen activator inhibitor type 1 was positive only after heating in retrieval solution at pH 2.5 or pH 10.0.²⁰ TB at pH 9.0–9.5 was recommended by Evers and Uylings for HIAR for several neuronal antigens.²¹ Effects of EDTA, a chelating agent of metallic ions used in the reports by Pileri et al.⁷ and Imam et al.¹⁹ will be discussed in Section 17.4.

These results clearly demonstrate that an acidic or basic solution is effective for HIAR of most antigens, although examinations have been performed by different heating devices, temperatures, heating durations, and buffer concentrations. Simply evaluating the effects of pH on antigen retrieval is difficult because a single buffer system cannot cover a wide range of pH values; thus, different buffer systems composed of various chemical reagents must be used to examine the effect of pH on HIAR. Different chemical reagents and buffering capacities may influence the results of HIAR. For example, CB around neutral pH yielded a stronger immunoreaction than TB for most antigens. Since pH values are highly dependent on temperatures, buffers prepared and adjusted pH value at room temperature should show different pH values at a high temperature. To examine the effect of chemical composition of buffers on HIAR, Shi et al. used three different buffer systems with wide-range buffer capacities, that is, sodium diethylbarbiturate-HCl, sodium phosphate-citrate acid buffer, or dimethylglutaric acid-NaOH buffer, and demonstrated that the buffers provided almost the same pH-dependent pattern for nine antigens examined.¹⁵ These results strongly suggest that the pH value of retrieval solutions is more important than the chemical composition of buffers.

17.2.2 HIAR Using Citraconic Anhydride at a Neutral pH

Namimatsu et al. reported that heating in citraconic anhydride solution at a neutral pH was useful as a universal antigen retrieval method.²² They compared the effectiveness of 0.05% citraconic anhydride solution (pH 7.4) and 50mM CB (pH 6.0). Although the exact effects of the two solutions were difficult to evaluate since different heating devices, heating durations, and methods were used, they demonstrated that 62 antibodies yielded stronger immunostaining when the deparaffinized sections were kept in the citraconic anhydride solution (pH 7.4) for 45min at 98°C than when the sections were heated in CB (pH 6.0) for 10min in a microwave oven, and that 10 antibodies exhibited similar staining intensities with or without heating in the citraconic anhydride solution. In the other HIAR solutions described above, the reagents contained in the solutions do not form chemical bindings with macromolecules in the tissues, whereas citraconic anhydride modifies the ϵ -amino groups of lysine residues and places numerous negative charges

on proteins at a neutral pH, and causes conformational changes in the proteins.²³

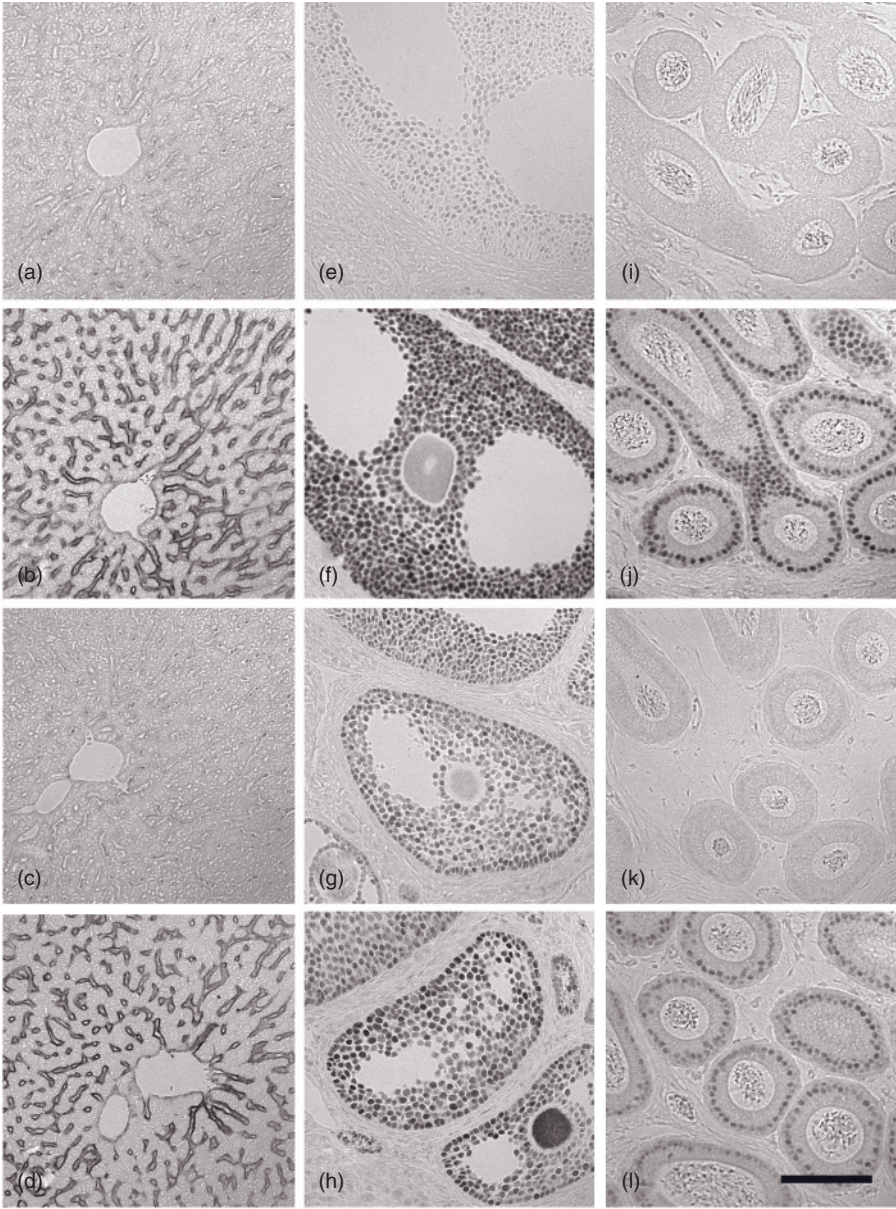
17.2.3 pH-Dependent Reversibility of HIAR Efficiency

Yamashita and Okada demonstrated that the intensities of the immunoreactions obtained by heating in a buffer are reversibly altered by successive heating in another buffer with a different pH.¹⁸ Specimens fixed with 4% paraformaldehyde for 6 h (Table 17.2) or fixed with buffered 10% formalin for 24 h (Fig. 17.2) yielded almost similar results. Nine of the ten antigens were immunostained much more strongly in the sections that were heated in 20mM TB at pH 9.0 than in the sections that were heated in 20mM TB at pH 6.0: ER α , ER β , AnR, glucocorticoid receptor (GR), p300, steroid receptor coactivator (SRC)-1, β -actin, fibronectin, and laminin. Only anti-amylase antibody showed a similar staining intensity independent of the pH when the antibody was used at a 5,000-fold dilution (Table 17.2). A second heating at pH 6.0 significantly decreased the immunostaining of the antigens that had been heated at pH 9.0, but the immunostaining was restored after a third heating at pH 9.0 (Fig. 17.2). These results indicate that the degradation or extraction of antigens or epitopes is a minor factor in the pH dependency of HIAR and that the pH of the solution is a critical factor for HIAR, and acidic or basic pH is suitable for the exposure of epitopes to enable interactions with antibodies.

TABLE 17.2 pH Dependency of HIAR: Reversibility of Immunoreactivity

Antigens	Heating Procedures						
	No Heating	pH 6	pH 6-9	pH 6-9-6	pH 9	pH 6	pH 9-6-9
ER α	±	—	+	±	+++	+	+++
ER β	—	—	+	±	++	—	++
AnR	—	—	++	+	+++	+	+++
GR	—	—	+	+	+++	++	+++
P300	±	+	++	+	+++	++	+++
SRC-1	—	±	++	+	+++	+	+++
Amylase	+++	++	++	++	++	++	++
β -actin	+	+	++	++	+++	++	+++
Fibronectin	+	—	++	—	++	±	+++
Laminin	++	—	+	—	+	—	+

Notes: Mouse tissues were fixed with 4% formaldehyde for 6 h. Paraffin sections were boiled in 20mM Tris-HCl buffers (TB), at pH 6.0 or at pH 9.0, for 10 min. After cooling, the sections were briefly washed with distilled water and heated in another buffer (e.g., pH 9.0 and then 6.0) for 5 min. Some specimens treated in the second buffer were heated in the first buffer (e.g., pH 9.0, then pH 6.0, and finally pH 9.0; pH 9-6-9) for 5 min. The antigens were localized in respective tissues described in Table 17.1. Immunostaining was scored as follows: +++, strong; ++, moderate; +, weak; ±, faint; —, negative.



17.2.4 Effect of pH on Formaldehyde Treated -Proteins

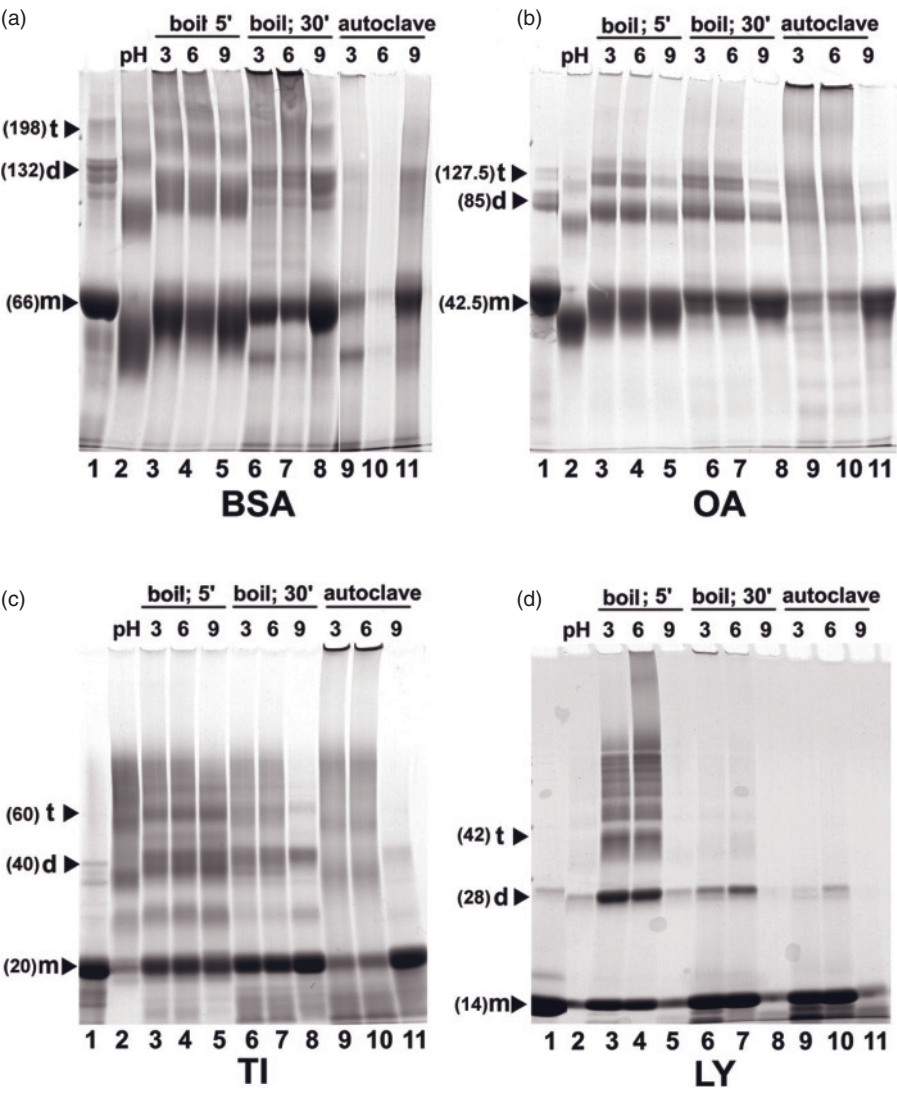
Efficiency of antigen retrieval has been shown to be highly dependent on the pH in immunohistochemical studies as described above. Yamashita and Okada treated five purified proteins with formaldehyde followed by heating in buffers with different pH values, and then analyzed them using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to examine whether these phenomena are based on the pH-dependent cleaving of cross-links formed by formaldehyde.¹⁸ SDS-PAGE is the most popular biochemical technique to evaluate the molecular size of proteins.²⁴ Formaldehyde produces intra- and intermolecular cross-links in proteins. Intermolecular cross-links form protein oligomers/polymer. Intramolecular cross-links cause the protein to migrate more rapidly (resulting in a smaller apparent molecular mass) than unmodified native proteins when examined using SDS-PAGE because the intramolecular cross-links probably prevent the protein molecule from extending in the presence of SDS.^{18,25,26} (Fig. 17.3, lanes 2). The cleaving efficiency was almost the same when the formaldehyde-treated proteins were heated for 5 min at 100°C in 10mM TB at pH 3.0, pH 6.0, pH 7.5, or pH 9.0 (Fig. 17.3). However, when the proteins were drastically heated, such as by autoclaving for 10 min at 120°C, at a pH close to their respective pI, they tended to produce insoluble protein precipitates in SDS solution at 37°C (Fig. 17.3).

When formaldehyde-treated proteins were heated in solutions with the same pH values, that is, in 20mM CB (pH 6.0), 20mM phosphate buffer (PB) (pH 6.0) or 20mM TB (pH 6.0), and in 20mM CB (pH 7.5) or 20mM PB (pH 7.5), and in 20mM TB (pH 9.0) or 20mM PB (pH 9.0), different kinds of buffers with the same pH value yielded similar effects on formaldehyde-treated proteins. Heating in the buffers showing the same pH value cleaved methylene bridges with almost the same efficiency and precipitated proteins at pH close to their pIs.



Figure 17.2 Effect of pH: reversibility of immunoreaction. Mouse tissues were fixed with buffered 10% formalin for 24h and embedded in paraffin. Fibronectin and estrogen receptor (ER) β were localized in the liver (a–d) and the ovary (e–h), respectively. Androgen receptor (AnR) was detected in the caput epididymis (i–l). Antigens were localized without preheating (a, e, and i). Sections were boiled in 20mM TB (pH 9.0) for 20 min (b, f, and j), and further boiled in 20mM TB (pH 6.0) for 15 min (c, g, and k). The sections were boiled again in 20mM TB (pH 9.0) for 15 min (d, h, and l). Fibronectin is localized along the sinusoid. ER β immunoreaction is seen in the nucleus of granulosa cells and AnR immunoreaction is present in the nucleus of epithelial cells of ductus epididymis. Heating at pH 9.0 enhances the immunoreaction of these antigens, but the second heating at pH 6.0 significantly decreases the immunoreaction. The third heating at pH 9.0 recovers immunostaining for three antigens. Bar = 100 μ m.

Masuda et al., using time-of-flight (TOF) mass spectrometry, demonstrated that the majority of methylol groups and methylene bridges in formaldehyde-treated RNA are removed after heating in 10mM TB (pH 7.0) containing 1mM EDTA at 70°C for 1 h.²⁷ Heating has also been shown to cleave the cross-links in FFPF materials, since solubilization of proteins from FFPF sections is demonstrated by using SDS-PAGE and Western blot analysis and by mass spectrometry, while proteins from unheated sections are exceedingly difficult to extract proteins.^{18,28,29}



17.3 EFFECT OF IONIC STRENGTH

Although a few systematic studies have been performed to elucidate the effect of ionic strength on HIAR, ionic strength of retrieval solution is shown to be important for the efficiency of HIAR. Yamashita and associates examined the effects of ionic strength on HIAR using three buffer systems with different pH values. When FFPE specimens were autoclaved for 10 min at 120°C in 20mM TB (pH 9.0), 0.05% citroconic anhydride solution (pH 7.4), or 10mM CB (pH 6.0) containing 0, 50, 100, or 200mM NaCl and then immunostained with 10 antibodies; ER α , AnR, PCNA, vascular endothelial cell growth factor (VEGF), β -catenin, claudin-5, translocase of mitochondrial outer membrane (Tom) 20, fibronectin, and laminin, all antibodies showed the strongest immunostaining results when the sections were autoclaved in the NaCl-free solutions.¹⁷ The staining intensity decreased as the NaCl concentration increased (Table 17.3, Fig. 17.4). This phenomenon was independent not only of the pH of the retrieval solution but also of the pIs of the antigen proteins and the intracellular localization of the antigens. Polyclonal antibodies and monoclonal antibodies showed the same results. When the sections were treated with TB (pH 10.5), on the other hand, all antibodies examined (ER α , AnR, GR, β -actin, tubulin, claudin-5, fibronectin, and laminin) yielded stronger reactions in the buffers containing NaCl than in the buffer without NaCl, and five antibodies exhibited their strongest immunoreactions at concentrations from 25 to 50mM.

Yamashita further examined whether the intensity of the immunoreactions obtained by heating in 20mM TB (pH 9.0) containing 200mM of NaCl was reversibly altered by successive heating in 20mM TB (pH 9.0) without NaCl.



Figure 17.3 Effects of heating and pH on formaldehyde-treated proteins. Bovine serum albumin (BSA) (a), ovalbumin (OA) (b), and soybean trypsin inhibitor (TI) (c), 10mg/mL, dissolved in distilled water solutions were mixed with an equal volume of 8% formaldehyde in 0.2M phosphate buffer (pH 7.2) for 25h at room temperature. Egg white lysozyme (LY) (d) was incubated with the formaldehyde solution for 30min. After dialysis, the proteins were boiled in a water bath for 5min (lanes 3–5) or 30min (lanes 6–8), or autoclaved at 120°C for 10min (lanes 9–11) in 10mM TB, at pH 3.0 (lanes 3, 6, and 9), at pH 6.0 (lanes 4, 7, and 10), and at pH 9.0 (lanes 5, 8, and 11) and then precipitated by adding 4 vol of acetone. The precipitated proteins were dissolved in sample buffer containing 2-mercaptoethanol, incubated for 1h at 37°C and then analyzed on SDS-PAGE. BSA and OA were run on 7.5% and 10% gels, respectively. TI and LY were analyzed on 12.5% gels. Lane 1 of each gel shows formaldehyde-untreated native proteins and lane 2 demonstrates the formaldehyde-treated proteins without heating. A: m, native monomer (66kD); d, dimer (132kD); t, trimer (198kD). B: m, native monomer (42.5kD); d, dimer (85kD); t, trimer (127.5kD). C: m, native monomer (20kD); d, dimmer (40kD); t, trimer (60kD). D: m, native monomer (14kD); d, dimer (28kD); t, trimer (42kD). pI of each proteins is as follows: BSA, 5.6; OA, 5.2; TI, 4.6; LY, 9.7. From Yamashita and Okada,¹⁸ with permission.

TABLE 17.3 Effect of Ionic Strength on HIAR

Antigens	Buffers	NaCl Concentration (mM)				
		0	50	100	200	200-0
ER α	TB	++++	+++	++	+	++
	CA	++	++	++/+	+	
	CB	+++	++	++/+	+	
AnR	TB	+++	++/+	$\pm$	—	$\pm$
	CA	+	+	$\pm$	—	
	CB	++	+	$\pm$	—	
PCNA	TB	+++	++/+	+	$\pm$	+
	CA	++	+	+	$\pm$	
	CB	++	+	+	$\pm$	
VEGF	TB	+++	++/+	+	$\pm$	+
	CA	++	+	$\pm$	—	
	CB	++	+	$\pm$	$\pm$	
β -Catenin	TB	+++	++	$\pm$	—	+
	CA	+++	++	+	—	
	CB	++	+	$\pm$	—	
β -Actin	TB	+++	++	+	$\pm$	$\pm$
	CA	+++	+++	+	$\pm$	
	CB	++	$\pm$	—	—	
Claudin-5	TB	+++	++	+	—	++/+
	CA	+++	++	+	$\pm$	
	CB	++	$\pm$	—	—	
Tom20	TB	+++	++	++/+	++/+	++
	CA	+++	++	+	+	
	CB	++	++/+	+	+	
Fibronectin	TB	+++	+	$\pm$	$\pm$ /—	+
	CA	+++	+	$\pm$	—	
	CB	+	—	—	—	
Laminin	TB	+++	+	$\pm$	$\pm$	++/+
	CA	+++	++	+	+	
	CB	++	+	$\pm$	$\pm$	

Notes: Mouse tissues fixed with 4% formaldehyde for 24h were embedded in paraffin. Sections were autoclaved in the following antigen retrieval solutions containing 0, 50, 100, or 200mM of NaCl for 10min at 120°C: 20mM Tris-HCl buffer (TB) (pH 9.0), 0.05% citraconic anhydride solution (CA) (pH 7.4) or 20mM citrate buffer (CB) (pH 6.0). Some sections were autoclaved in 20mM TB (pH 9.0) containing 200mM NaCl for 10min, cooled and heated in NaCl-free 20mM TB (pH 9.0) for 15min at 95–100°C: 200-0. Vascular endothelial cell growth factor (VEGF) was detected in the uterus. β -Catenin and translocase of mitochondrial outer membrane (Tom) 20 were localized in the small intestine and the kidney, respectively. Other antigens were localized in respective tissues described in Table 17.1. Immunostaining was scored as follows: +++, strong; ++, moderate; +, weak; $\pm$, faint; —, negative.

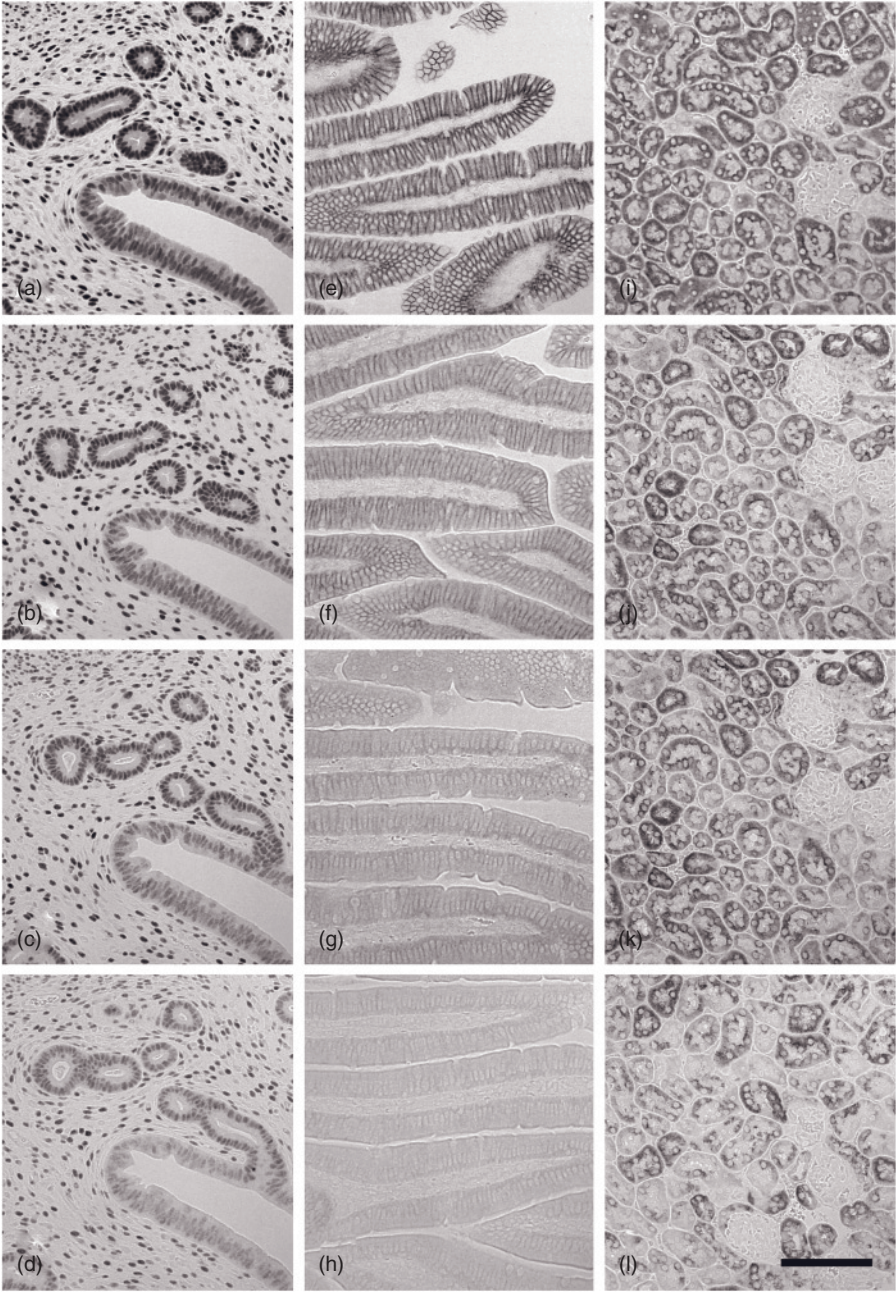
After autoclaving in TB containing 200mM NaCl for 10 min, the sections were further treated with TB for 15 min at 95–98°C. All antibodies partially recovered their immunostaining after the second heating (Table 17.3). These results demonstrate that the ionic strength of the solution is one of the critical factors for HIAR, and that a high concentration of salt inhibits the exposure of epitopes, preventing their reaction with antibodies.

17.4 MECHANISMS OF HIAR

Several mechanisms have been proposed to explain HIAR: the cleavage of protein–protein cross-links,^{3,6} the disruption of cross-links involving calcium ions,⁵ an increase in tissue permeability for antibodies, and the removal of trace amounts of paraffin in the tissues. However, recent studies have indicated that the fundamental mechanism of HIAR is based on the cleavage of protein–protein cross-links.

Biochemical studies have demonstrated that unfolded polypeptides treated with denaturants, such as urea or guanidine hydrochloride, readily self-associate or randomly associate with other proteins in the solution upon the removal of the denaturants. The hydrophobic force is thought to be the principal driving force responsible for protein aggregation.^{30,31} Similarly, thermal denaturation also aggregates proteins. Heating at around the pI of the proteins coagulates them even under mild heating conditions, and increasing the ionic strength promotes aggregation.³²

The biochemical and immunohistochemical results described above strongly suggest that the mechanisms of HIAR are as follows. One of the main reasons for the utility of HIAR is that heating destroys the gel-like structure formed by the cross-links in the FFPE specimens and partially extracts the macromolecules, enabling the antibodies to easily penetrate the tissues and interact with the antigens; this process is similar to the effects of digestion with proteinases or nucleases. When the methylene bridges are cleaved by heating, the higher-order structure of the protein antigens is destroyed, and the polypeptide chains extend, exposing both hydrophobic and hydrophilic regions. During the cooling process, the polypeptide chains rapidly refold. In tissues, many kinds of proteins with different pIs and molecular weights are tightly packed and the neighboring polypeptides can come in contact with each other. Therefore, epitopes should be concealed during the refolding of the proteins at around a neutral pH because a strong hydrophobic attractive force would randomly entangle the neighboring polypeptides: an electrostatic force may act locally as an attractive or repulsive force. At basic or acidic pH values, however, the majority of the extended polypeptides would be charged negatively or positively, and the electrostatic repulsive force would act to prevent random aggregation and entanglement with neighboring polypeptides by the hydrophobic force, thereby maintaining a suitable conformation for antigen–antibody interactions. In sections that are heated in a



solution containing citraconic anhydride, citraconylated proteins have a net negative charge, and the electrostatic repulsive force might balance the hydrophobic attractive force, leading to the exposure of the epitopes even at a neutral pH.

The mechanisms described above are further supported by the results concerning effects of ionic strength on HIAR. When salt is added to the retrieval solutions, the electrostatic force between neighboring polypeptides is canceled, and the hydrophobic attractive force may cause the antigen proteins and neighboring proteins to aggregate, masking the epitopes. At pH 10.5, on the other hand, the immunoreaction was stronger in the sections treated with TB containing NaCl than in those heated in TB without NaCl, and five out of eight antibodies showed the strongest immunostaining results when the sections were heated in TB containing 25–50 mM NaCl. The lower electrostatic forces in the buffers containing NaCl may moderate polypeptide denaturation as a result of the highly negatively charged polypeptide chains, yielding stronger immunoreactions than NaCl-free buffer buffers.

Since purified proteins in solutions are very heat-sensitive and rapidly aggregate around their pIs, the antigen–antibody reaction is assumed to be severely inhibited when the specimens are heated at around the pIs of the protein antigens. In FFPE specimens, however, the immunoreaction of most of the antibodies was strong when heated in an acidic or basic solution, independent of the pI of each antigen. These results suggest that the pI of the antigen molecule is not a critical factor for HIAR in tissues and that the total net charge of the microenvironment is important, since the antigens are surrounded by different kinds of macromolecules with different pIs that interact with each other.

Morgan et al. reported that Ki-67 immunostaining was completely suppressed when FFPE sections were heated in a solution containing CaCl_2 , but recovered when heated in buffers containing EDTA or EGTA, and proposed

Figure 17.4 Effects of ionic strength of retrieval solution. Mouse tissues were fixed with 4% formaldehyde for 24 h. Deparaffinized sections were autoclaved at 120°C for 10 min in the following antigen retrieval solutions: 20 mM TB (pH 9.0) (a–d); 0.05% citraconic anhydride (pH 7.4) (e–h); 20 mM CB (pH 6.0) (i–l). NaCl was added to these retrieval solutions to examine the effects of ionic strength on HIAR: 0 mM (a, e, and i); 50 mM (b, f, and j); 100 mM (c, g, and k); 200 mM (d, h, and l). ER α was localized in the uterus (a–d). β -Catenin was detected in the small intestine (e–h) and translocase of mitochondrial outer membrane (Tom) 20 immunoreaction was demonstrated in the kidney (i–l). ER α immunostaining is present in the nucleus of epithelial cells and stromal cells. β -Catenin locates along the lateral cell membrane and Tom20 distributes in the basal cytoplasm of proximal and distal tubular cells. Staining intensity for ER α , β -catenin, and Tom20 decreases in proportion to the increase of NaCl concentration of antigen retrieval solutions. Bar = 100 μm .

the hypothesis that coordinate bonds between calcium ions and the methylol groups in proteins form a cage-like structure and prevent antigen-antibody interactions.^{5,33} However, the following results indicate that the calcium cage-like structure is not formed in formaldehyde-fixed tissues, and that the effect of calcium ions on immunoreactions is selective for certain antigens that calcium ions may elicit conformational changes and inhibit antibody binding. (1) Heating in buffers removes the majority of methylol groups and methylene bridges in formaldehyde-treated proteins and nucleic acid.^{18,27} (2) Shi et al. prepared fresh-frozen sections and treated them with CaCl_2 or CaCl_2 followed by EDTA, and then fixed them with acetone, and reported that immunostaining of Ki-67 with MIB1 and five of the seven anti-thrombospondin monoclonal antibodies was severely suppressed after CaCl_2 treatment, but the suppression could be reversed by sequential incubation with EDTA, whereas immunostaining with two anti-thrombospondin monoclonal antibodies was unaffected by CaCl_2 pretreatment.³⁴ (3) When FFPE sections were boiled in TB (pH 9.0) with or without 1 mM EDTA, EDTA had no effect on immunostaining with eight antibodies examined (α -amylase, laminin, ER α , ER β , p300, AnR, GR, SRC-1).⁸ (4) When fresh frozen sections were fixed with 10% formalin containing 25 mM CaCl_2 for 30 min, and then incubated in 20 mM TB (pH 9.0) with or without 1 mM EDTA at room temperature for 2 h or autoclaved in the same solutions for 10 min at 120°C, EDTA treatment had no effect on the immunostaining patterns of 12 of the 16 antibodies, but the immunostaining of four antigens decreased slightly when the sections were autoclaved in TB containing 1 mM EDTA.⁸

The reason why some epitopes lose their immunoreactivity after heating is assumed to be as follows. In protein antigens, epitopes are generally composed of 5–20 amino acids; there are two types of epitopes, that is, conformational and linear epitopes. Conformational epitopes are composed of amino acids that are located far apart in their linear sequence but become juxtaposed when the protein is folded in its native shape. Since these epitopes bind to antibodies in their native or formalin-fixed state but lose their binding activity when the proteins are denatured or unfolded,^{35–37} enzyme digestion or mild heating may be preferred for the antigen retrieval of these epitopes. Linear epitopes are composed of a particular stretch of consecutive amino acids and are located on the surface of the proteins or in an internal portion of the proteins. Surface linear epitopes may react with antibodies independent of whether the protein is in its native or denatured state. In polymerized proteins, such as cytoskeleton or proteins composed of subunits, the epitopes may be hidden by their adjacent subunits even when the epitopes are present on the surface. Heating and treatment with protein denaturants are presumably effective for exposing this type of epitopes. By contrast, internal linear epitopes may not bind to antibodies when the antigen proteins are in their native or fixed states but can bind to antibodies when the proteins are denatured and extended. Therefore, HIAR should be effective for immunohistochemistry using most antibodies generated for linear epitopes.

17.5 CONCLUSION

Antigen retrieval in FFPE specimens involves the exposure of epitopes by treating sections with heat, enzymes, or protein denaturants. HIAR is most commonly used because of its simplicity and effectiveness. The pH and ionic strength of the retrieval solution are critical factors for HIAR; that is, basic or acidic solutions with low ionic strength yield strong immunoreactions for most antigens. These results suggest the following mechanisms of HIAR: heating treatment cleaves the cross-links and methylol groups in polypeptides and nucleic acids, and extends the polypeptides in the tissues. During the cooling process, the polypeptide chains rapidly refold; the epitopes are presumably concealed in solutions with a neutral pH or high ionic strength because strong hydrophobic attractive forces would entangle the neighboring polypeptides randomly. In solutions with basic or acidic pH values and low ionic strength, however, the majority of the extended polypeptides would be charged negatively or positively, and the electrostatic repulsive force would act to prevent random aggregation and the entanglement of neighboring polypeptides by the hydrophobic force, thereby exposing the epitopes for interaction with antibodies.

Recent studies have shown that HIAR is also effective for physically fixed materials, un-embedded and plastic-embedded specimens, and immunoelectron microscopy. Heating has also been shown to be useful for the extraction of proteins and nucleic acids from FFPE materials. The elucidation of the mechanisms described above should support the usefulness of such applications and lead to the establishment of standardized protocols for immunohistochemical staining.

REFERENCES

1. Mason JT, O'Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
2. Fowler CB, O'Leary TJ, Mason JT: Modeling formalin fixation and histological processing with ribonuclease A: effects of ethanol dehydration on reversal of formaldehyde cross-links. *Lab. Invest.* 2008; 88: 785–791.
3. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
4. Cattoretto G, Pileri S, Parravicini C, et al. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J. Pathol.* 1993; 171: 83–98.
5. Morgan JM, Navabi H, Schmid KW, et al. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. *J. Pathol.* 1994; 174: 301–307.

6. Werner M, Von Wasielewski R, Komminoth P. Antigen retrieval, signal amplification and intensification in immunohistochemistry. *Histochem. Cell Biol.* 1996; 105: 253–260.
7. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J. Pathol.* 1997; 183: 116–123.
8. Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. *Prog. Histochem. Cytochem.* 2007; 41: 141–200.
9. Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. *J. Histochem. Cytochem.* 2005; 53: 1421–1432.
10. Itoh H, Miyajima Y, Osamura RY. Immunohistochemistry of intranuclear antigens. *Jpn. J. Breast Cancer* 1995; 10: 3–10.
11. Brorson SH. Heat-induced antigen retrieval of epoxy sections for electron microscopy. *Histol. Histopathol.* 2001; 16: 923–930.
12. Goode NP, Shires M, Crellin DM, et al. Post-embedding double-labeling of antigen-retrieved ultrathin sections using a silver enhancement-controlled sequential immunogold (SECSI) technique. *J. Histochem. Cytochem.* 2004; 52: 141–144.
13. Suurmeijer AJH, Boon ME. Optimizing keratin and vimentin retrieval in formalin-fixed, paraffin-embedded tissue with the use of heat and metal salt. *Appl. Immunohistochem.* 1993; 1: 143–148.
14. Shin HJ, Shin DM, Shah T, et al. Methods in pathology. Optimization of proliferating cell nuclear antigen immunohistochemical staining by microwave heating in zinc sulfate solution. *Mod. Pathol.* 1994; 7: 242–248.
15. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43:193–201.
16. Kim SH, Kook MC, Shin YK, et al. Evaluation of antigen retrieval buffer systems. *J. Mol. Histol.* 2004; 35: 409–416.
17. Emoto K, Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: does pH or ionic strength of the solution play a role for refolding antigens? *J. Histochem. Cytochem.* 2005; 53: 1311–1321.
18. Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J. Histochem. Cytochem.* 2005; 53: 13–21.
19. Imam SA, Young L, Chaiwun B, et al. Comparison of two microwave based antigen-retrieval solutions in unmasking epitopes in formalin-fixed tissue for immunostaining. *Anticancer Res.* 1995; 15: 1153–1158.
20. Ferrier CM, van Geloof WL, de Witte HH, et al. Epitopes of components of the plasminogen activation system are re-exposed in formalin-fixed paraffin sections by different retrieval techniques. *J. Histochem. Cytochem.* 1998; 46: 469–476.
21. Evers P, Uylings HB. An optimal antigen retrieval method suitable for different antibodies on human brain tissue stored for several years in formaldehyde fixative. *J. Neurosci. Methods* 1997; 72: 197–207.
22. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J. Histochem. Cytochem.* 2005; 53: 3–11.

23. Mir MM, Fazili KM, Abul Qasim M. Chemical modification of buried lysine residues of bovine serum albumin and its influence on protein conformation and bilirubin binding. *Biochim. Biophys. Acta* 1992; 1119: 261–267.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680–685.
25. Hopwood D, Slidders W, Yeaman GR. Tissue fixation with phenol-formaldehyde for routine histopathology. *Histochem. J.* 1989; 21: 228–234.
26. Rait VK, O'Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
27. Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* 1999; 27: 4436–4443.
28. Chu WS, Liang Q, Liu J, et al. A nondestructive molecule extraction method allowing morphological and molecular analyses using a single tissue section. *Lab. Invest.* 2005; 85: 1416–1428.
29. Shi SR, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
30. Jaenicke R, Seckler R. Protein misassembly in vitro. *Adv. Protein Chem.* 1997; 50: 1–59.
31. Fink A. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold. Des.* 1998; 3: R9–23.
32. Saito M, Taira H. Heat denaturation and emulsifying of plasma protein. *Agric. Biol. Chem.* 1987; 51: 2787–2792.
33. Morgan JM, Navabi H, Jasani B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. *J. Pathol.* 1997; 182: 233–237.
34. Shi SR, Cote RJ, Hawes D, et al. Calcium-induced modification of protein conformation demonstrated by immunohistochemistry: What is the signal? *J Histochem Cytochem.* 1999; 47:463–470.
35. Yasuda K, Yamashita S, Aiso S, et al. Immunohistochemical study of gamma-glutamyl transpeptidase with monoclonal antibodies. I. Preparation and characteristics of monoclonal antibodies to gamma-glutamyl transpeptidase. *Acta Histochem. Cytochem.* 1986; 19: 589–600.
36. Augstein P, Ziegler B, Schlosser M, et al. Immunohistochemical differentiation of monoclonal GAD antibodies recognizing linear or conformational epitope regions. *Pancreas* 1997; 15: 139–146.
37. Sompuram SR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. Clin. Pathol.* 2004; 121: 190–199.

CHAPTER 18

COMMENTARY: FUTURE DIRECTIONS

TIMOTHY J. O'LEARY, CAROL B. FOWLER, DAVID L. EVERS,
ROBERT E. CUNNINGHAM, and JEFFREY T. MASON

18.1 INTRODUCTION

To suggest a set of directions for future research in antigen retrieval requires that one first state the objective(s) toward which that research is directed. There are a number of important research directions that are deserving of attention, two of which we will consider here—(1) the recovery of unmodified proteins in native conformation from formalin-fixed, paraffin-embedded (FFPE) tissue and (2) the development of new techniques for assessing the quantity and functional state of tissue proteins recovered from FFPE tissue.

18.2 RECOVERING UNMODIFIED PROTEINS FROM FFPE TISSUE

If the objective of recovering completely unmodified proteins, in native conformation, from FFPE tissue can be achieved, it seems likely that such objectives as maximizing immunoreactivity of a specific protein or set of proteins within a tissue section should be relatively straightforward. However, given our current state of knowledge, recovery of such unmodified proteins is a very tall order indeed. To fully appreciate the tasks which must be accomplished, it is perhaps useful to first enumerate some of the things that we know about fixation with formaldehyde, dehydration, and embedding in paraffin wax, and what we know about reversing or ameliorating some of the effects of this process.

Although fixation with formaldehyde has been a mainstay of tissue preservation for well over 100 years, the chemical basis for this effect has been elucidated very slowly over that time. Exposing tissue to formaldehyde rather rapidly stops the enzymatic degradation and bacterial growth that begin immediately after tissue is excised or an organism dies, and causes the tissue to shrink and become hard relative to the unfixed state. This results in a tissue

that can be dehydrated and embedded in paraffin, sectioned, mounted, and stained. Staining of paraffin-embedded tissue with hematoxylin and eosin reveals crisply presented cellular structures, including nuclei, cell membranes, and certain intracellular structures. It is these characteristics that make formaldehyde the mostly widely used fixative in histology. Although most of these aspects of formaldehyde fixation are useful to the practicing pathologist, the advent of newer techniques, including immunomicroscopy and molecular diagnosis, have revealed some less desirable characteristics of formaldehyde fixation, including loss of immunoreactivity by comparison with unfixed tissues and greater difficulty in carrying out analyses of nucleic acids.

These functional characteristics reflect what is known about the chemistry of formaldehyde fixation, much of which has previously been reviewed by Fox and by Shi et al.^{1,2} Some significant facts about formaldehyde fixation of proteins include:

1. In dilute aqueous solution, formaldehyde exists primarily in the hydrated form known as methylol. Only an insignificant fraction of the compound exists in the unhydrated aldehyde form.¹
2. In dilute aqueous solution, formaldehyde can react with a variety of amino acids. However, the primary initial targets are lysine and cysteine. The primary amine moiety of lysine can accept two methylol adducts. Other amino acids with which formaldehyde reacts include arginine and tyrosine, which are particularly reactive, as well as histidine, serine, tryptophan, glutamine, and asparagine.²⁻⁴
3. In either dilute or concentrated solutions, additional reactions occur that result in both intra- and intermolecular cross-linking of proteins. There is little direct chemical information from such techniques as nuclear magnetic resonance spectroscopy or mass spectrometry to detail the precise nature of these cross-links.^{5,6}
4. This process of cross-linking does not appear have a major effect on protein secondary structure at room temperature. However, cross-links formed by reactions of formaldehyde with proteins retard, but do not eliminate, protein denaturation that occurs when proteins are heated to a temperature of approximately 70°C or above.⁷
5. The combination of methylol adducts and intermolecular cross-links significantly reduces the reactivity of certain proteins with antibodies that react strongly with unmodified proteins. The steric exclusion that results from intermolecular cross-linking generally has a significantly greater effect than that of intramolecular cross-linking or adduction by methylol.^{6,8-11}
6. Fixation by formaldehyde virtually eliminates enzymatic activity in a process that is sometimes reversible (*vide infra*). Dehydration and embedding significantly reduces the reversibility of this process, suggesting that dehydration and embedding facilitate additional chemical reactions that are not observed in aqueous solution.

To these facts regarding formaldehyde fixation we can then enumerate some of the things that we know about antigen retrieval techniques:

1. It is possible to restore antigenicity in tissue sections by digesting the rehydrated sections with any of several proteolytic enzymes, or by incubating the tissue section at elevated temperatures in any of several liquids, including water and various buffers (antigen retrieval).¹²
2. Incubation of proteins in aqueous media at elevated temperatures significantly reduces the number of methylol adducts and intermolecular cross-links. Increasing pressure not only seems to accentuate this process significantly, but also appears to be accompanied by protein cleavage at aspartate residues.¹³
3. The effectiveness of antigen retrieval depends on the specifics of fixation, dehydration and embedding, the nature of the protein to be detected, and the pH, ionic strength, and temperature of the incubation buffer, as well as the length of incubation.
4. As yet, there is not a method for recovering completely unmodified proteins in native conformation from FFPE tissues.

18.3 QUESTIONS ABOUT THE CHEMISTRY OF FIXATION AND DEMODIFICATION

Studies to date have given a great deal of information about the adduction and cross-linking of proteins by formaldehyde in aqueous solution, but fundamental questions about the chemistry remain. Aqueous fixation represents only the first of a number of steps in tissue processing that occur prior to immunohistochemical assessment. This step is followed by dehydration in graded alcohols and (typically) acetone, exposure to a completely nonpolar solvent (typically xylene), and impregnation with paraffin wax at an elevated temperature. Clearly, these steps provide opportunities for further reactions, and there is ample evidence that such actions occur. Indeed, a number of questions regarding the chemistry of fixation and “defixation” are raised by simple examination of the relatively small number of questions for which we already have answers. Some of these questions, and suggestions as to how they might be attacked, follow:

18.3.1 What Is the Strength of the Chemical Bond Formed When Formaldehyde Reacts with an Amino Acid Molecule to Form a Methylol Adduct?

This is a deceptively simple question to ask, but one which is quite hard to answer. We have performed titration calorimetry experiments (unpublished) intended to determine the enthalpy of reaction for the reaction of aqueous formaldehyde with polypeptides or proteins, without success. In our experiments, the enthalpy of mixing was much larger than that which could be

attributed to any chemical reaction. This suggests that the bond formed when methylene glycol reacts with any of the reactive protein moieties is quite weak. More evidence that this is a very weak bond comes from the fact that methylol adducts that can be observed by mass spectrometry of ribonuclease A are, for the most part, not present after the protein has been heated to temperatures of $\sim 75\text{--}80^\circ\text{C}$ for 1–2 h, suggesting that the enthalpy of this reaction is on the order of 0.1 kcal/mol or less—clearly too small to accurately estimate using titration calorimetry. One possible approach to the problem is to measure the equilibrium concentration of methylol which is bound to protein as a function of temperature, thus obtaining the enthalpy of reaction from the temperature dependence of the equilibrium constant. Clearly this too will be a difficult task. Raman spectroscopy might be capable of assessing this equilibrium in the right hands, since methylol binding events are expected to increase the intensity of C-N symmetric ($\sim 710\text{cm}^{-1}$) and asymmetric ($\sim 960\text{--}970\text{cm}^{-1}$) bands.¹⁴

18.3.2 What Chemical Moieties Are Formed When Methylol - Adducted Proteins Are Exposed to Alcohols?

Following fixation, during which aqueous proteins have the opportunity to react with formaldehyde, it is customary to dehydrate tissue through graded alcohols and xylene. Thus, the formaldehyde–protein reaction products have the opportunity to undergo further chemical reactions resulting from the presence of the reactive alcohol group, the reduction in water content of the solution (dehydration), and the reduced polarity of the solution. Each of these factors may, in principle, contribute to reactions in which methylol-adducted proteins undergo further reactions with the alcohol solvent itself. As yet, it is unknown which chemical moieties, if any, are formed in this first state of tissue dehydration. While nontrivial, this question is easier to answer than determining the enthalpy of reaction. Rait et al. have used electrospray mass spectrometry to investigate the reactions of 2'-deoxyadenosine 5'-monophosphate with formaldehyde, demonstrating ethoxymethyl derivatives, as well as relatively unstable cross-links.¹⁵ It seems highly likely that similar experiments can be performed using single amino acids or short polypeptides.

18.3.3 Are Additional Reaction Products Formed during the Final Steps of Dehydration or during the Process of Paraffin Embedding? What Is the Temperature Dependence of the Reactions that Lead to These Products?

One might expect that dehydration of a methylol-adducted protein would lead to the formation of Schiff bases, which might be expected to serve as highly reactive groups capable of undergoing further reactions—particularly cross-linking reactions, as the water content of the surrounding solution is reduced. As yet, however, there is little direct evidence that such intermediates are

produced. Experiments in our own laboratory, using spectroscopic and chromatographic techniques, have failed to provide evidence for a dramatic increase in Schiff base formation in dehydrated nucleic acids. It should be straightforward to look for Schiff base formation in methylol-adducted lysine using the infrared and Raman-active C=N stretching band. This intermediate, if stable, should also be observable using either nuclear magnetic resonance spectroscopy or mass spectrometry.

1. Can the adducts and cross-links formed when formaldehyde reacts with proteins in solution be completely reversed under any circumstances? Can they be reversed while preserving primary, secondary, tertiary, and quarternary structures?
2. In principle, there are a variety of additional reactions which methylol adducts can undergo when exposed to alcohol, some of which have been identified in isolated nucleic acid and protein molecules.^{2,15}

18.3.4 Assuming that the Final Protein–Formaldehyde Reactions Found After Paraffin-Embedding Are Different from Those Found After Aqueous Fixation, Will Careful Heating in Nonpolar Solvents Assist in the Reversal of These Reactions?

The enthalpy, entropy, and free energy of an adducted protein dissolved in a given solvent, such as an aqueous buffer, do not depend upon the pathway by which the adducts are formed (assuming the chemical conformation is maintained), since these properties are thermodynamic “state functions.” Nevertheless, the ease with which particular reaction products are formed (reaction kinetics) is very much pathway dependent. Certain reactions which proceed quickly in aqueous solvents go slowly, or not at all, in nonpolar solvents (and vice versa). It is, therefore, by no means obvious that a one-step attempt to reverse formaldehyde cross-links in an aqueous medium is an optimal approach by which to reverse reactions that may occur as protein–formaldehyde adducts are exposed to increasingly nonpolar environments during tissue processing. Obviously, a more detailed understanding of what additional reactions occur during tissue processing would facilitate developing better approaches to antigen retrieval, but empirical experiments in which, for example, the deparaffinization of tissue specimens occurs not at room temperature, which is the normal practice, but at temperatures similar to those used in embedding should be straightforward. Care must be taken, of course, to conduct these experiments in a manner that eliminates risk of explosion, given the high volatility and low flash points of these solvents.

18.3.5 Is There an Optimal Heating Strategy for Removing Adducts for a Given Protein in Aqueous Media?

Calorimetric experiments on fixed proteins demonstrate with certainty that the process of fixation both elevates the mean denaturation temperature of

the protein and spreads out the transition so that denaturation takes place over a temperature range of tens of degrees, rather than 2–3 degrees. At a temperature of around 85°C or above, removal of formaldehyde-induced cross-links is likely to be accompanied by immediate denaturation. This may, for some systems, reduce immunological reactivity. It may be possible, at least in some cases, to carry out the defixation/antigen retrieval process at a lower temperature—perhaps even as low as the melting temperature of the unfixed protein. This would, if complete, facilitate recovery of these proteins in their native conformations. Alternatively, it is possible that a procedure in which specimens are heated to a temperature well above the melting temperature and then programmatically cooled would improve removal of cross-links and adducts while preserving some or all of the proteins in their native conformations.

Although it is likely that lower temperature antigen retrieval will be more time consuming than that undertaken at more conventional temperatures, it is worth exploring whether the results obtained are better, worse, or comparable to those obtained near or above the boiling point of water. One might expect a temperature of 65°C or so to be reasonable, given that this is near the melting temperature of most proteins. Both immunohistochemical experiments and mass spectrometry should be considered as analytical techniques since they serve complementary roles in the research laboratory, and because this complementarity may eventually extend to the clinical setting as well.

18.3.6 What Is the Optimal Way in which to use High Hydrostatic Pressures to Enhance Antigen Retrieval Techniques?

Experiments by Fowler et al. suggest that very high hydrostatic pressures may improve the extraction of proteins for mass spectrometry.¹³ It seems highly likely that this occurs by enhancing the removal of formaldehyde-induced adducts, and perhaps by causing additional cleavage within peptide backbones. It is worth exploring whether this or similar techniques can also be used to improve immunohistochemical staining in tissue sections.

18.4 NEW TECHNIQUES FOR ASSESSING THE QUANTITY AND FUNCTIONAL STATE OF TISSUE PROTEINS

Although mass spectrometry is taking an increasingly important role as a research method, it is a low-throughput technique which is not currently appropriate for clinical diagnosis. Nevertheless, the combination of antigen retrieval techniques and surface-enhanced laser desorption/ionization (SELDI) provides promise for high-throughput protein mapping.^{16,17} Currently, such mapping requires tissue microdissection prior to preparing the tissue for analysis. By significantly improving protein availability through improved

antigen retrieval techniques, it may be possible to avoid the microdissection step, instead scanning the entire tissue specimen for mass spectroscopic signatures characteristic of the proteins of interest. Some progress has been made in this approach using matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging with FFPE tissue sections.^{18–22} A combination of high-temperature antigen retrieval followed by *in situ* trypsin digestion has allowed the identification and spatial mapping of cancer biomarkers in FFPE tissue sections²² and tissue microarrays.¹⁹

A second area which deserves investigation is the expanded use of enzyme histochemistry, particularly in tissue sections that have undergone antigen retrieval at non-denaturing temperatures. Enzyme histochemistry retains a small but important research role, but, except for esterase detection in granulocytic tumors,^{23–25} has almost no applications in the routine diagnostic lab. If low-temperature antigen retrieval can restore significant enzyme activity in FFPE sections, then the utility of enzyme histochemistry in research would unquestionable be facilitated, as might its utility in routine diagnosis.

Finally, a third area worthy of investigation is the use of new detection systems. Binding histochemistry is currently carried out using only two classes of detection reagents—lectins, which are in limited use for identification of carbohydrate moieties, and immunoglobulins, which have broad application in protein detection. Nevertheless, it remains difficult to raise antibodies to some proteins, and only a fraction of the antibodies available are useful in FFPE tissues. Techniques are available for creating short DNA or RNA segments, known as aptamers, which are capable of selectively binding to almost any protein or carbohydrate, differentiating among phosphorylated and non-phosphorylated forms, and so on. Indeed, one investigator has used FFPE tissue sections to develop an aptamer that selectively bound to a highly expressed target in breast cancer tissues.²⁶ The use of this technique, together with antigen retrieval techniques, is potentially very powerful and is highly worthy of further exploration.

18.5 CONCLUSIONS

Antigen retrieval techniques provide powerful methods by which to improve immunohistochemical staining and increase the availability of proteins for mass spectrometric analysis. Research that results in an improved understanding of the chemical changes that accompany fixation, dehydration, and embedding may result in further improvements that increase the already substantial clinical utility of immunohistochemistry. Further explorations that consider the use of antigen retrieval with newer diagnostic modalities, including tissue SELDI and detection of antigens using nucleic acid aptamers may result in new approaches to diagnosis that further expand our capabilities.

REFERENCES

1. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.
2. Shi S-R, Gu J, Tuttle RM, et al. Development of the antigen retrieval technique: philosophical and theoretical bases. In *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, ed. S-R Shi, J Gu, and CR Taylor, pp. 17–39. Natick, MA: Eaton, 2000.
3. Metz B, Kersten GF, Baart GJ, et al. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug. Chem.* 2006; 17: 815–822.
4. Metz B, Kersten GF, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
5. Rait VK, O'Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
6. Rait VK, Xu L, O'Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A: II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
7. Mason JT, O'Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
8. Sompuram SR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
9. Sompuram SR, Vani K, Hafer LJ, et al. Antibodies immunoreactive with formalin-fixed tissue antigens recognize linear protein epitopes. *Am. J. Clin. Pathol.* 2006; 125: 82–90.
10. Sompuram SR, Vani K, Bogen SA. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.
11. O'Leary TJ, Mason JT. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 122: 154–155.
12. Taylor CR, Chen C, Shi SR, et al. A comparative study of antigen retrieval methods. *CAP Today* 1995; 9: 16–22.
13. Fowler CB, Cunningham RE, Waybright TJ, et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. *Lab. Invest.* 2008; 88: 185–195.
14. O'Leary TJ and Levin IW: Raman spectroscopic study of the melting behavior of anhydrous dipalmitoylphosphatidylcholine bilayers. *J. Phys. Chem.* 1984; 88: 1790–1796.
15. Rait VK, Zhang Q, Fabris D, et al. Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. *J. Histochem. Cytochem.* 2006; 54: 301–310.
16. Diamond DL, Zhang Y, Gaiger A, et al. Use of ProteinChip array surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify thymosin beta-4, a differentially secreted protein from lymphoblastoid cell lines. *J. Am. Soc. Mass Spectrom.* 2003; 14: 760–765.

17. Fetsch PA, Simone NL, Bryant-Greenwood PK, et al. Proteomic evaluation of archival cytologic material using SELDI affinity mass spectrometry: potential for diagnostic applications. *Am. J. Clin. Pathol.* 2002; 118: 870–876.
18. Chaurand P, Stoeckli M, Caprioli RM. Direct profiling of proteins in biological tissue sections by MALDI mass spectrometry. *Anal. Chem.* 1999; 71: 5263–5270.
19. Groseclose MR, Massion PP, Chaurand P, et al. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. *Proteomics* 2008; 8: 3715–3724.
20. Lemaire R, Desmons A, Tabet JC, et al. Direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections. *J. Proteome Res.* 2007; 6: 1295–1305.
21. Ronci M, Bonanno E, Colantoni A, et al. Protein unlocking procedures of formalin-fixed paraffin-embedded tissues: application to MALDI-TOF imaging MS investigations. *Proteomics* 2008; 8: 3702–3714.
22. Stauber J, Lemaire R, Franck J, et al. MALDI imaging of formalin-fixed paraffin-embedded tissues: application to model animals of Parkinson disease for biomarker hunting. *J. Proteome Res.* 2008; 7: 969–978.
23. Dunphy CH, Polski JM, Johns G, et al. Acute promyelocytic leukemia, hypogranular variant, with uncharacteristic staining with chloroacetate esterase. *Leuk. Lymphoma* 2001; 42: 215–219.
24. Lam KW, Li CY, Siemens M, et al. Immunohistochemical detection of monocytes by the antiserum specific to monocytic esterase. *J. Histochem. Cytochem.* 1985; 33: 379–383.
25. Long JC, Mihm MC. Multiple granulocytic tumors of the skin: report of six cases of myelogenous leukemia with initial manifestations in the skin. *Cancer* 1977; 39: 2004–2016.
26. Li S, Xu H, Ding H, et al. Identification of an aptamer targeting hnRNP A1 by tissue slide-based SELEX. *J. Pathol.* 2009; 218: 327–336.

PART V

PROTEOMIC ANALYSIS OF PROTEIN EXTRACTED FROM TISSUE/CELLS

TECHNIQUES OF PROTEIN EXTRACTION FROM FFPE TISSUE/ CELLS FOR MASS SPECTROMETRY

CAROL B. FOWLER, TIMOTHY J. O'LEARY, and JEFFREY T. MASON

19.1 INTRODUCTION

High-throughput proteomic methods hold great promise for the discovery of novel protein biomarkers that can be translated into practical interventions for the diagnosis, treatment, and prevention of disease. These approaches may also facilitate the development of therapeutic agents that are targeted to specific molecular alterations in diseases such as cancer. In many cases, malignant cells yield unique “protein profiles” when total protein extracts from such cells are analyzed by 2-D gel electrophoresis or mass spectrometry (MS) methods. Such proteomic studies have the potential to provide an important complement to the analysis of DNA and mRNA extracts from these tissues.¹

When fresh or frozen tissue is used for proteomic analyses, the results cannot be related directly to the clinical course of diseases. If routinely fixed and embedded archival tissues could be used for standard proteomic methods such as MS, these powerful proteomic techniques could be used to both qualitatively and quantitatively analyze large numbers of tissues for which the clinical course has been established. However, analysis of archival formalin-fixed, paraffin-embedded (FFPE) tissues by high-throughput proteomic methods has been hampered by the adverse effects of formalin fixation,² specifically formaldehyde-induced protein adducts and cross-links that are formed during tissue fixation and subsequent histological processing.³ If the adverse effects of formalin fixation could be overcome, the use of high-throughput proteomic methods to analyze existing archival FFPE tissues could reduce health-care costs, lives lost, and human suffering.

Several proteomic studies using archival FFPE tissues have been reported in recent years. Some involve the analysis of very small tissue samples prepared

by laser-capture microdissection, for example, the study by Patel et al.⁴ This method is limited to the analysis of the protein expression profiles of only a small number of cells. Another method of performing proteomic profiling on FFPE tissue sections employs matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI imaging MS),^{5–7} which is outside the scope of this chapter. The majority of the more robust proteomic studies on FFPE tissues employ tissue extraction methods that are derived from heat-induced antigen retrieval (HIAR) techniques originally developed for immunohistochemistry. A systematic comparison of published extraction techniques reiterates the importance of heat, detergent, and a protein denaturant for efficient protein extraction from FFPE tissues.⁸ However, while several studies report improved identification of proteins by MS using these HIAR-based methods,^{4,9–14} there are a number of challenges that must be addressed to develop more efficient and reproducible techniques for extracting and identifying proteins from archival FFPE tissue.

19.2 REACTION OF FORMALDEHYDE FIXATIVES WITH PROTEINS

Formaldehyde reacts with proteins to form adducts and cross-links.^{3,15,16} Metz et al.³ have identified three types of chemical modifications after treatment of proteins with formaldehyde: (a) methylol (hydroxymethyl) adducts, (b) Schiff bases, and (c) methylene bridges. The reaction of formaldehyde with proteins is summarized in Figure 19.1, but briefly, formaldehyde reacts primarily with lysine and cysteine to form methylol adducts. The methylol adduct can subsequently undergo a dehydration reaction to form a Schiff base. Adducted primary amine and thiol groups can undergo a second reaction with arginine,

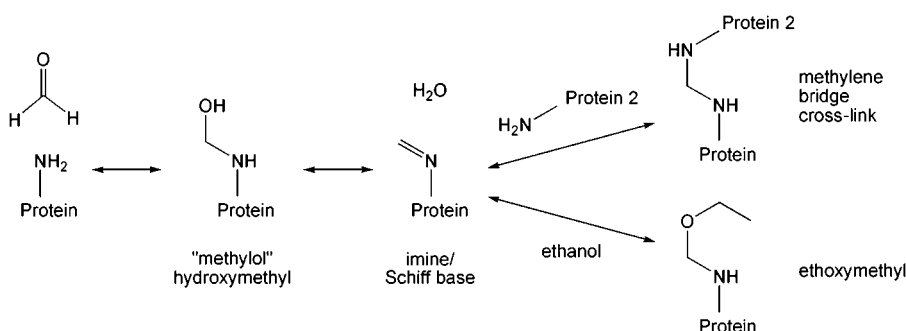


Figure 19.1 A schematic view of the common formaldehyde-induced modifications in proteins. Reactive methylol adducts are formed in the initial reaction between formaldehyde and cysteine or the amino groups of basic amino acid residues. The methylol adduct can subsequently undergo a dehydration reaction to form a Schiff's base. Adducted residues can undergo a second reaction to form methylene bridges or can convert to the ethoxymethyl adduct after the ethanol dehydration step.

asparagine, glutamine, histidine, tryptophan, and tyrosine residues to form methylene bridges, or can convert to the ethoxymethyl adduct after the ethanol dehydration step.¹⁷ All of the above formaldehyde-induced protein modifications exhibit protein sequence dependence.

Formaldehyde-induced protein modifications can complicate the proteomic analysis of FFPE tissues in several ways. The formation of either methylol adducts or methylene cross-links results in the neutralization of lysine or arginine residues, both of which are formaldehyde-reactive. Thus, the modification of these reactive residues can interfere with trypsin cleavage, leading to large peptides with many missed internal cleavage sites, leading to unidentified or misidentified peptides. Even if the expected peptide fragments can be generated, any incomplete reversal of formaldehyde-induced protein modifications can lead to unidentified or misidentified peptides. To compensate for this effect, researchers typically use stringent identification criteria to limit peptide misidentification,¹⁸ but this approach can significantly reduce the number of proteins identified during the proteomic analysis of FFPE tissues.

19.3 HEAT-INDUCED PROTEIN EXTRACTION

For a number of years, HIAR techniques have been used to increase the reactivity of immunohistochemical staining in FFPE tissue.^{19–21} These methods require heating tissue sections in buffers, such as Tris–HCl or citrate at temperatures ranging from 60°C to 121°C. Though the mechanisms of antigen retrieval are unclear, it is hypothesized that heating the tissue sections “unmasks” the antigenic epitope by at least partially reversing the protein–formaldehyde cross-links and adducts. Many recent proteomic studies of archival tissues have relied upon HIAR techniques, but their adaptation for tissue extraction has been largely an empirical process. The rest of this chapter focuses on several successful approaches to extracting proteins from FFPE tissues. The work flow for the majority of published protocols has a number of common elements and is represented graphically in Figure 19.2.

An early published protocol by Ikeda et al.²² using a detergent containing buffer (RIPA) for heat-induced protein extraction of FFPE tissues for Western blots has since been modified by a number of groups. For example, Shi et al.¹⁰ performed a comparative proteomic study using frozen and FFPE tissue sections from the same human renal cancer biopsy. In that study, protein extraction was performed by heating the tissue specimens in 10 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS) buffer, pH 7 or 9, at 100°C for 20 min followed by incubation at 60°C for 2 h. The protein extracts were dialyzed to remove excess SDS and digested with trypsin. Liquid chromatography–tandem MS (LC-MS/MS) identified 2404 and 3263 total proteins in the frozen and FFPE specimens, respectively (Table 19.1). There were 1720 proteins common to both specimens, while 595 proteins (25%) were unique to the frozen tissue, and 1448 proteins (45%) were unique to the FFPE tissue. Xu et al.²³ reported

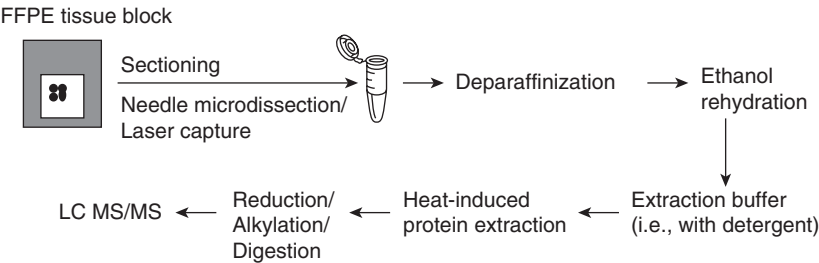


Figure 19.2 A generalized proteomics work flow for the extraction and identification of proteins in FFPE tissue. Formalin-fixed tissues acquired by sectioning, needle dissection, or laser capture are deparaffinized in xylenes and are rehydrated in graded alcohols. The material is resuspended in buffer which generally contains a detergent/protein denaturant and the sample is heated to complete the extraction process. The protein extract is reduced, alkylated, and digested with trypsin before protein profiling.

TABLE 19.1 Protein Extraction Protocols from FFPE Tissues Documented in the Literature

Reference	FFPE Tissue Source	FPPE Tissue Extraction Method	Fresh Tissue		FFPE Tissue	
			Distinct Peptide IDS	Protein IDs	Distinct Peptide IDS	Protein IDs
Shi et al. ¹⁰	Human renal cancer	Tris + SDS, pH 7 ^a	3305	2404	3336	3263
Shi et al. ¹⁰	Human renal cancer	No AR ^b	3305	2404	1714	1883
Guo et al. ¹³	Glio-blastoma	Tris + SDS, pH 9 ^a	12517 ^c / 16023 ^d	2380 ^c / 3110 ^d	14748	2733
Xu et al. ²³	Mouse liver	Tris + SDS, pH 9 ^a	n/a	n/a	—	3895
Palmer-Toy et al. ¹²	Canalplasty	2% SDS/ammonium bicarbonate/70°C/1h	266	94	412	123
Jiang, et al. ¹¹	Mouse liver	1. 6M guanidine-HCl/ no heating	3207	480	352	57
		2. 6M guanidine/ 100°C, 30 min			3005	470
		3. CNBr treatment of cell pellet from #2			1129	202
		4. Tris + SDS, pH 8.2 ^a			2540	395
		5. Direct tryptic digest			589	106

^aSamples were heated at 100°C for 20 min, followed by 60°C for 2 h.

^bNo heat-induced protein extraction step performed.

^cFresh-frozen soluble fraction.

^dFresh-frozen cell pellet fraction.

n/a, not available; IDS, identifications.

a total of 3895 nonredundant proteins identified from three FFPE liver samples extracted in an SDS solution using the method of Shi et al.,¹⁰ with 76% (2544) proteins common to all samples (Table 19.1).

Guo et al.¹³ published a similar comparison of fresh-frozen and FFPE glioblastoma tissues. FFPE tissue sections were microdissected, and the isolated cells were extracted in a Tris buffer (pH 9) containing 2% SDS by heating at 100°C for 20 min, followed by 60°C for 2 h. Microdissected fresh-frozen tissue was placed first in an 8M urea solution to collect the soluble proteins, and the cell pellet was then extracted in a 1% SDS solution. Combined capillary isoelectric focusing/nano-reversed-phase LC-MS/MS analysis was performed on the FFPE tissue extracts and the fresh-frozen tissue extracts from the same patient. The results are summarized in Table 19.1. A total of 14,478 distinct peptides and 2845 nonredundant proteins were identified from the FFPE tissue. For the corresponding fresh-frozen samples, 12,517 and 16,023 peptides were identified from the soluble and cell pellet fractions, respectively, for a total of 3902 unique proteins found in the fresh sample. Of these, 1882 proteins were common to the FFPE tissue and the soluble and pellet fractions of the fresh tissue, with an additional 232 proteins common to the FFPE and fresh soluble fraction and 488 proteins common to the FFPE and fresh cell pellet fraction. This large degree of overlap (83%) between the FFPE and cell pellet fraction of the fresh tissue was due to the application of a detergent-based extraction protocol for both samples.

More recently, Balgley et al.²⁴ evaluated the effects of time in storage on archival tissue proteome analysis across 10 uterine mesenchymal tumor tissue blocks. Ten micrometer sections from nine uterine leiomyomas from 1990 to 2002 and a single case of alveolar soft part sarcoma dating from 1980 were deparaffinized, treated in a 20mM Tris buffer containing 2% SDS and heated at 100°C for 20 min, followed by 60°C as previously described.¹⁰ The extracted proteins were dialyzed to remove excess SDS, and then denatured, reduced, and alkylated prior to digestion with trypsin overnight at 37°C. After analysis by combined capillary isoelectric focusing/LC-MS/MS, it was shown that all 10 tumor sections shared over 1800 common proteins in a core set, with 80 proteins unique to the sarcoma case. Additionally, the expression levels of actin, desmin, and progesterone, three commonly used protein markers for leiomyomas, were consistent between all nine cases archived between 1990 and 2002. Finally, k-means clustering was performed on all proteins across the average of archival years to evaluate the effects of archival time on individual proteins or groups of proteins. There was little difference in protein expression among the average proteomes of the 1997 and 2002 cases. However, there was an increase in average protein expression of 23% from the 1990 to 2002 leiomyoma cases suggesting an archival effect that may influence protein retrieval, particularly for low-abundance proteins.

A comparative study of extraction methods from formalin-fixed mouse liver by Jiang et al.¹¹ highlights the importance of sample preparation technique. Formalin-fixed mouse tissue was extracted using five different protocols: (1) 6M guanidine-HCl without heating; (2) 6M guanidine-HCl

with heating at 100°C for 30 min; (3) cyanogen bromide (CNBr) treatment overnight of the tissue pellet from method 2; (4) 2% SDS with heating at 100°C for 20 min, followed by 60°C for 2 h; and (5) direct digestion of the tissue homogenate with trypsin at 37°C for 20 h. For methods 1–4, the resulting extracts were diluted, alkylated, and treated with trypsin at 37°C for 20 h prior to LC-MS/MS. The results are summarized in Table 19.1. For the formalin-fixed tissue extracted using protocol 1 (no heating), only 130 proteins were identified, with 57 identified with at least 2 unique tryptic peptides, representing a significant decrease from fresh tissue extracted using the same protocol (976 total proteins and 480 confident proteins identified). Extraction method 2 (6 M guanidine with heating) and method 4 (SDS with heating) yielded the greatest improvements with 827 and 820 total proteins (or 470 and 395 confident proteins) identified, respectively. Direct digestion of the fixed tissue yielded 331 total protein identifications and 106 confident proteins. CNBr treatment of the pellet from method 2, followed by lyophilization resulted in 202 proteins identified with at least 2 peptide hits, out of 526 total proteins.¹¹

A study of by Palmer-Toy et al.,¹² summarized in Table 19.1, provides further empirical evidence of the utility of techniques coupling heating with efficient protein extraction for the proteomic analysis of FFPE tissue. A specimen from a patient with chronic stenosing external otitis was divided in half and preserved as fresh-frozen tissue or FFPE. Ten micromolar sections of the FFPE tissue were vortexed in heptane to deparaffinize the tissue and were then co-extracted with methanol. The methanol layer was evaporated, and the protein residue was resuspended in 2% SDS/100 mM ammonium bicarbonate/20 mM dithiothreitol (DTT), pH 8.5 and heated at 70°C for 1 h. After tryptic digestion, 123 total confident proteins were identified in the FFPE tissue, compared to 94 proteins identified from the fresh-frozen tissue. Hwang et al. also reported up to a fivefold increase in protein extraction efficiency for samples extracted in a Tris-HCl/2% SDS/1% Triton X-100/1% deoxycholate solution at 94°C for 30 min versus samples extracted in 100 mM ammonium bicarbonate/30% acetonitrile at the same temperature.¹⁴

19.4 LIQUID TISSUE™ METHOD FOR PROTEIN FROM FFPE TISSUE

A number of proteomic studies on archival material have utilized Liquid Tissue™ (Expression Pathology, Inc., Gaithersburg, MD), a commercial protein extraction kit for FFPE tissue.^{4,9,25–28} This kit is also based upon HIAR techniques and shares a similar work flow to the methods already discussed. Thin, typically 5–10 μm, sections are cut from paraffin tissue blocks, the paraffin is removed, and the tissue deparaffinized and rehydrated in alcohols and distilled water before microdissection. The cellular material is then suspended in Liquid Tissue buffer and heated at 95°C for 90 min. Trypsin is added, and the material is digested overnight at 37°C prior to reduction with DTT and analysis by LC-MS/MS.²⁶

In one study by Hood et al., 282 of 1153 identified proteins were identified by at least 2 unique tryptic peptides from FFPE prostate cancer (PCa) tissue.⁹ According to the gene ontology classification of the proteins identified, ~65% of proteins were predicted to be intracellular proteins, while ~50% of the total human proteome is predicted to be located in the intracellular compartment. Additionally, 20% of the proteins identified in the PCa tissue were classified as membrane proteins, which is significantly less than the predicted 40% for the human proteome. This relative disparity is not unexpected, considering the Liquid Tissue sample preparation kit lacks specific protocols for membrane protein extraction. The Liquid Tissue method has also been used for proteomics studies of a variety of FFPE tissue samples, including pancreatic tumors,²⁸ squamous cell carcinoma,⁴ and oral human papillomavirus lesions.²⁷

19.5 OTHER TISSUE EXTRACTION METHODOLOGIES FOR FFPE TISSUE

While the Liquid Tissue kit yields tryptic peptides for direct LC-MS/MS analysis, there are instances when it is desirable to isolate full-length proteins and proteins with posttranslational modifications intact. The Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany), like the other protocols discussed, utilizes a heating step (100°C for 20 min, followed by 80°C for 2 h) for protein extraction. Qiagen has reported extraction of full-length, intact proteins from a variety of tissues, such as liver or brain and has validated the presence of a variety of protein types, including membrane proteins. To date, published studies have only utilized the Qproteome kit for Western blots and reverse phase protein microarrays.^{29–32} However, such a work flow would also allow analysis of the FFPE tissue by LC-MS/MS.

We recently reported the extraction of full-length proteins and reversal of formaldehyde-induced protein adducts and cross-links when lysozyme tissue surrogates were extracted under elevated pressure.³³ Tissue surrogates are model tissue plugs that can be used to rapidly evaluate the efficacy of FFPE tissue extraction protocols. Cytoplasmic proteins, such as lysozyme, are fixed with 10% buffered formalin, and the resulting opaque gel is then dehydrated through graded alcohols, and embedded in paraffin.⁸ Proteins extracted from tissue surrogates at atmospheric pressure and 80–121°C remained highly cross-linked, with total protein content consisting of ~20% monomeric protein and 80% multimeric protein by SDS-polyacrylamide electrophoresis. However, when the lysozyme surrogate suspension was heated at 80°C at pH 4 for 2 h at 43,500 psi, 100% of the protein was recovered in the soluble phase, and almost complete reversal of the formaldehyde-induced protein adducts and cross-links was observed. Of the total protein content, 42% corresponded to monomeric protein, and only 1% oligomeric protein (mostly dimer) was present.

The addition of pressure to the heat-induced protein retrieval technique has a sound thermodynamic basis. Under elevated pressure, cavities in proteins become filled with water molecules, which leads to the hydration of the protein interior.^{34,35} Hydration of the buried hydrophobic residues induces protein unfolding because the change in molar volume associated with this unfolding is negative.³⁶ Further, formaldehyde-induced protein modifications increase protein stability³⁷ and can raise the thermal denaturation temperature of fixed proteins to temperatures above 100°C.³⁸ Because the change in molar volume associated with unfolding is negative, the thermal transition temperature decreases with increasing pressure,^{35,39} thus counteracting the stabilizing effect of the formaldehyde modifications.

The total protein extract from the lysozyme tissue surrogate heated at 80°C in Tris buffer, pH 4, for 2 h at 43,500 psi was gel-purified, digested with trypsin, and the resulting peptides extracted from the gel were analyzed by MS. Native, non-formaldehyde-fixed lysozyme was analyzed in tandem with the pressure-extracted FFPE surrogate. A comparison of the peptides observed in the analysis of native and high-pressure FFPE lysozyme tissue surrogates by both MALDI and electrospray ionization (ESI)-MS showed a high degree of overlap. Ten tryptic lysozyme peptides, representing >70% overall sequence coverage, were identified within each sample. Eight of these peptides were in common, with each of the samples containing two uniquely identified peptides.³³ The two peptides unique to the native lysozyme sample, GYSLGNMMVCAAK (22–33) and NLCNIPCSALLSSDITASVNCAK (74–96), had very low signal intensity when the digested protein was analyzed using MALDI; therefore, it is not surprising that they were not observed in the analysis of the FFPE sample. The peptides identified by MS in the high-pressure extracted FFPE sample are shown in Table 19.2.

TABLE 19.2 Recovery of Protein from a Lysozyme Tissue Surrogate

Peptide	MALDI	ESI
R.CELAAAMK.R		✓
R.HGLDNYR.G	✓	✓
K.FESNFNTQATNR.N	✓	✓
R.NTDGSTDYGILQINSR.W	✓	✓
R.WWCNDGR.T	✓	
R.TPGSR.N	✓	
K.GTDVQAWIR.G	✓	✓
R.GCR.L	✓	
K.KIVSDGNGMNAWVAWR.N		✓
K.IVSDGNGMNAWVAWR.N		✓

Notes: Peptide fragments detected using MALDI and electrospray ionization (ESI)-MS. For more detail, please see Reference 33.

19.6 CONCLUSION

In summary, a review of the literature reveals that a majority of extraction techniques employ a combination of heat and a detergent/protein denaturant for efficient protein extraction from FFPE tissues. A number of groups have successfully used a variation of the protocol first reported by Ikeda et al.²² and Shi et al.,¹⁰ in which tissue is extracted at 100°C for 20 min, followed by 60°C for 2 h in an SDS-containing buffer. Other published extraction techniques utilize heating in 6M guanidine-HCl,¹¹ direct digestion of the tissue without an intermediate heating step,¹¹ or digestion of the tissue after heating.²⁶ Our laboratory has also demonstrated that an increase in hydrostatic pressure to augment heat treatment dramatically improves the protein extraction efficiency (from 60% to 100%) and the reversal of formaldehyde-induced protein modifications (from 20% to 100%) in a model tissue surrogate.³³

These studies clearly demonstrate the number of concerns that still must be addressed when performing proteomic studies on archival FFPE tissue. One such concern is clearly poor recovery of protein, demonstrated most succinctly in the comparative study by Jiang et al.¹¹ in which the addition of heat to the extraction protocol markedly increased the number of protein identifications over protocols with no heat-induced extraction step. The equal importance of a detergent or protein denaturant was also highlighted in a study by Hwang et al.¹⁴ in which heating samples in an SDS-containing buffer markedly improved protein extraction efficiency over samples extracted at the same temperature in a detergent-free buffer. Charge neutralization of basic amino acids from reactions with formaldehyde may be a determining factor in protein insolubility, thus making the reversal of these adducts an all-important step in sample preparation. Another concern is selective recovery of protein, such as the disproportionate recovery of cytoplasmic proteins relative to membrane proteins reported by Hood et al.⁹ Also, though most extraction protocols are carried out at pH 7–9, there is evidence that proteins may extract preferentially under different conditions and at different pH's, which may be linked to protein physical properties, such as the isoelectric point.⁸ Multiple extractions steps (perhaps using a range of pH values or conditions) may be necessary to achieve quantitative, or at least representative, extraction of proteins from FFPE tissues. Finally, a concern when analyzing proteins extracted from FFPE tissue is the existence of covalent modifications resulting from formalin fixation and long-term storage in paraffin blocks. A search of the results from the LC-MS/MS of FFPE tissue done by Hood et al.⁹ indicated that ~6.5% of the peptides identified contained formylated lysyl residues, ~53% of the methionine residues were oxidized, and approximately 25% of the identified peptides contained an internal missed cleavage site. Since the chemistry of formaldehyde fixation is not well understood and it is clear that any incomplete reversal of formaldehyde-induced protein modifications and cross-links can lead to unidentified or misidentified peptides, stringent identification criteria must be used to limit peptide misidentification.

REFERENCES

1. Reymond MA, Schlegel W. Proteomics in cancer. *Adv. Clin. Chem.* 2007; 44: 103–142.
2. Conti CJ, Larcher F, Chesner J, et al. Polyacrylamide gel electrophoresis and immunoblotting of proteins extracted from paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 1988; 36: 547–550.
3. Metz B, Kersten GFA, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
4. Patel V, Hood BL, Molinolo AA, et al. Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. *Clin. Cancer Res.* 2008; 14: 1002–1014.
5. Lemaire R, Desmons A, Tabet JC, et al. Direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections. *J. Proteome Res.* 2007; 6: 1295–1305.
6. Ronci M, Bonanno E, Colantoni A, et al. Protein unlocking procedures of formalin-fixed paraffin-embedded tissues: application to MALDI-TOF imaging MS investigations. *Proteomics* 2008; 8: 3702–3714.
7. Groseclose MR, Massion PP, Chaurand P, et al. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. *Proteomics* 2008; 8: 3715–3724.
8. Fowler CB, Cunningham RE, O’Leary TJ, et al. “Tissue surrogates” as a model for archival formalin-fixed paraffin-embedded tissues. *Lab. Invest.* 2007; 87: 836–846.
9. Hood BL, Darfler MM, Guiel TG, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics* 2005; 4: 1741–1753.
10. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
11. Jiang X, Jiang X, Feng S, et al. Development of efficient protein extraction methods for shotgun proteome analysis of formalin-fixed tissues. *J. Proteome Res.* 2007; 6: 1038–1047.
12. Palmer-Toy DE, Krastins B, Sarracino DA, et al. Efficient method for the proteomic analysis of fixed and embedded tissues. *J. Proteome Res.* 2005; 4: 2404–2411.
13. Guo T, Wang W, Rudnick PA, et al. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. *J. Histochem. Cytochem.* 2007; 55: 763–772.
14. Hwang SI, Thumar J, Lundgren DH, et al. Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. *Oncogene* 2007; 26: 65–76.
15. Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33: 845–853.
16. Metz B, Kersten GFA, Baart GJ, et al. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjug. Chem.* 2006; 17: 815–822.

17. Rait VK, Zhang Q, Fabris D, et al. Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. *J. Histochem. Cytochem.* 2006; 54: 301–310.
18. Sage L. Proteomics gets out of a fix. *J. Proteome. Res.* 2005; 4: 1903–1904.
19. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry used for routinely processed celloidin-embedded human temporal bone sections: standardization and development. *Auris Nasus Larynx* 1998; 25: 425–443.
20. Shi SR, Chaiwun B, Young L, et al. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *J. Histochem. Cytochem.* 1993; 41: 1599–1604.
21. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.
22. Ikeda K, Monden T, Kanoh T, et al. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 1998; 46: 397–403.
23. Xu H, Yang L, Wang W, et al. Antigen retrieval for proteomic characterization of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2008; 7: 1098–1108.
24. Balgley BM, Guo T, Zhao K, et al. Evaluation of archival time on shotgun proteomics of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2009; 8: 917–925.
25. Hood BL, Conrads TP, Veenstra TD. Unravelling the proteome of formalin-fixed paraffin-embedded tissue. *Brief Funct. Genomic. Proteomic.* 2006; 5: 169–175.
26. Prieto DA, Hood BL, Darfler MM, et al. Liquid Tissue: proteomic profiling of formalin-fixed tissues. *Biotechniques* 2005; 38 (Suppl.): 32–35.
27. Jain MR, Liu T, Hu J, et al. Quantitative proteomic analysis of formalin fixed paraffin embedded oral HPV lesions from HIV patients. *Open Proteomics J.* 2008; 1: 40–45.
28. Cheung W, Darfler M, Alvarez H, et al. Application of a global proteomic approach to archival precursor lesions: deleted in malignant brain tumors 1 and tissue transglutaminase 2 are upregulated in pancreatic cancers. *Pancreatolgy* 2008; 8: 608–616.
29. Becker KF, Schott C, Hipp S, et al. Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J. Pathol.* 2007; 211: 370–378.
30. Blechschmidt K, Kremmer E, Hollweck R, et al. The E-cadherin repressor snail plays a role in tumor progression of endometrioid adenocarcinomas. *Diagn. Mol. Pathol.* 2007; 16: 222–228.
31. Hipp S, Walch A, Schuster T, et al. Precise measurement of the E-cadherin repressor Snail in formalin-fixed endometrial carcinoma using protein lysate microarrays. *Clin. Exp. Metastasis* 2008; 25: 679–683.
32. Kroll J, Becker KF, Kuphal S, et al. Isolation of high quality protein samples from punches of formalin fixed and paraffin embedded tissue blocks. *Histol. Histopathol.* 2008; 23: 391–395.

33. Fowler CB, Cunningham RE, Waybright TJ, et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. *Lab. Invest.* 2008; 88: 185–195.
34. Refaee M, Tezuka T, Akasaka K, et al. Pressure-dependent changes in the solution structure of hen egg-white lysozyme. *J. Mol. Biol.* 2003; 327: 857–865.
35. Frye KJ, Royer CA. Probing the contribution of internal cavities to the volume change of protein unfolding under pressure. *Protein Sci.* 1998; 7: 2217–2222.
36. Kobashigawa Y, Sakurai M, Nitta K. Effect of hydrostatic pressure on unfolding of alpha-lactalbumin: volumetric equivalence of the molten globule and unfolded state. *Protein Sci.* 1999; 8: 2765–2772.
37. Mason JT, O’Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J. Histochem. Cytochem.* 1991; 39: 225–229.
38. Rait VK, O’Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
39. Mason JT, O’Leary TJ. Effects of headgroup methylation and acyl chain length on the volume of melting of phosphatidylethanolamines. *Biophys. J.* 1990; 58: 277–281.

CHAPTER 20

APPLICATION OF SHOTGUN PROTEOMICS TO FORMALIN-FIXED AND PARAFFIN-EMBEDDED TISSUES

BRIAN M. BALGLEY

20.1 THE PROMISE AND CHALLENGE OF SHOTGUN PROTEOMICS IN ARCHIVAL, FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUES (FFPE)

Shotgun proteomics of archival FFPE tissue promises, for the first time, to reveal on a large scale the protein constituents of any given histologic morphology. Progress toward this goal will aid in the characterization and elucidation of disease. As diseases become well characterized at the protein level, targeted assays of proteins that these discovery efforts indicate are differentially expressed may be conducted on large numbers of cases. Assays such as liquid chromatography–multiple reaction monitoring–mass spectrometry and tissue microarray immunohistochemistry offer the capability to profile specific proteins at very high throughput. As these targets are validated, they will act, alone or in combination, as molecular markers of disease, refining our definitions of disease and discriminating among cases which, by pathological examination, are otherwise identical. However, many potentially confounding issues could stymie these efforts. These include, but are certainly not limited to, variation in formalin fixation conditions, variation in archival times, variation of antigen retrieval completeness, variation of digestion efficiency, and the impact of all of these to reproducibly perform shotgun proteomic profiling. In the absence of reproducible methods, the results of these efforts will carry little value. Some have been addressed, others remain unanswered.

20.2 DEVELOPMENT OF SHOTGUN PROTEOMICS IN FFPE TISSUES

Shotgun proteomics developed in the 1990s as a method for identifying multiple proteins in complex mixtures. The shotgun adjective refers to the sample preparation process in which proteins are proteolyzed into peptides which are more compatible with the analytical methods utilized, namely liquid-phase separations coupled with mass spectrometry. Many excellent reviews of the method have been written.¹⁻⁴ The method developed coincidentally with the increasing pace of genome sequencing, translations of which the method is dependent on for data interpretation. Partly for this reason, and likely partly because it was a new method, shotgun proteomics was first applied to model organisms. As the genome sequences of higher organisms became available, shotgun proteomics was rapidly applied. The opportunity to study disease in human tissues was met with great efforts to characterize normal human tissues, especially plasma. The first efforts to apply shotgun proteomics to formalin-fixed human tissues was made by Prieto et al.⁵ and then by Hood, et al.⁶ Both used a proprietary formulation to extract peptides from tissue in a method that amounts to conventional antigen retrieval followed by trypsin proteolysis. Hood et al. applied the technique in conjunction with a novel laser microdissection methodology and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify 684 proteins in FFPE prostate tissue. While this was the first application using tissue, the capability to reverse formaldehyde cross-links by heat for the purposes of shotgun proteomics analysis was seemingly first applied by Vasilescu et al.⁷ In this case, formaldehyde was utilized not for its preservation capabilities, but rather as a tool to define protein-protein interactions. Formaldehyde has a long history in this regard, serving to preserve interactions among various molecular complexes and finding use in experiments such as mapping protein-DNA interactions⁸ and characterizing nucleosomal composition and dynamics.^{9,10} Vasilescu et al. used formaldehyde to preserve protein interactions in live cells. They then immunopurified a protein complex, applied heat to reverse the formaldehyde cross-links, ran the resulting mixture by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), performed in-gel digestion of the bands, and sequenced the resulting peptides by LC-MS/MS. The complex was determined to be composed of 16 identifiable proteins. These efforts further demonstrated that formaldehyde cross-linked proteins could be retrieved via heating, making the proteins more amenable to proteolysis, especially by trypsin, a necessary precursor to shotgun proteomic studies.

Our first effort to apply shotgun proteomics to FFPE tissue was conducted in collaboration with experts in the process of antigen retrieval, Drs. Shan-Rong Shi and Clive Taylor.¹¹ Using the method they had optimized for immunohistochemistry,¹² we attempted shotgun proteomics of proteins retrieved from archival FFPE renal carcinoma. The tissue was deparaffinized and then boiled in 2% SDS for 20 min to reverse formaldehyde cross-links followed by incubation at 60°C for 2 h. The resulting solution was dialyzed and digested

with trypsin. The resulting peptide mixture was separated first by capillary isoelectric focusing (cIEF) into 15 fractions, followed by LC-MS/MS of each fraction. Peptide identification was performed using the Open Mass Spectrometry Search Algorithm (OMSSA). The analysis yielded 4811 peptides leading to 1962 protein identifications. This analysis demonstrated the effectiveness of both the antigen retrieval approach using SDS¹³ as well as the multidimensional shotgun proteomics approach using cIEF-LC-MS/MS to identify a large number of proteins from archival FFPE tissue.

20.3 EVALUATION OF LASER CAPTURE MICRODISSECTION OF FFPE TISSUES

Around this same time, we had started to apply shotgun proteomics to microdissected tissues. Microdissection of tissues permits the isolation of morphologically homogeneous cell populations. It is known that gene and protein expression within a seemingly homogeneous tumor may vary significantly. Discrimination of such variations may follow global discovery through the use of targeted assays. However, for the purposes of global discovery via shotgun proteomics, microdissection helps to maximize sample relevance while minimizing sample complexity. We recently evaluated this effect by extracting proteins from a whole tissue section and from laser microdissected portions of an adjacent section. FFPE glioblastoma multiforme (GBM) tissue sections were used. The entirety of one section, following removal of paraffin, was scraped from the slide and placed in a tube. The other section was meticulously microdissected to isolate GBM cells and avoid vascular tissue, extracellular matrix, and so on. Microdissection was performed using a laser capture microscope (Veritas, Molecular Devices, Sunnyvale, CA). The dissected areas were collected on the laser capture microdissection (LCM) cap. The tubes containing the whole section and the dissected areas were then prepared in the same manner. Antigen retrieval and proteolysis was performed by the optimized method described later in this chapter. Ten micrograms of each sample was then analyzed by transient capillary isotachopheresis/capillary zone electrophoresis—reverse phase chromatography coupled to MS/MS. Both analyses identified about the same number of proteins (3349 in the microdissected sample vs. 3153 in the whole section). However, many proteins were uniquely identified in each analysis (Fig. 20.1).

The proteins uniquely identified in the whole section corresponded to proteins typically found in blood or associated with the extracellular matrix. LCM effectively minimized the presence of these proteins in the microdissected sample. The enrichment effect is better illustrated by comparing the relative quantities of proteins identified in each sample (Fig. 20.2).

The bimodal distribution of proteins along the fitted curve is striking. It is immediately obvious that one of the samples has been enriched. Proteins associated with blood are enriched in the whole section while proteins

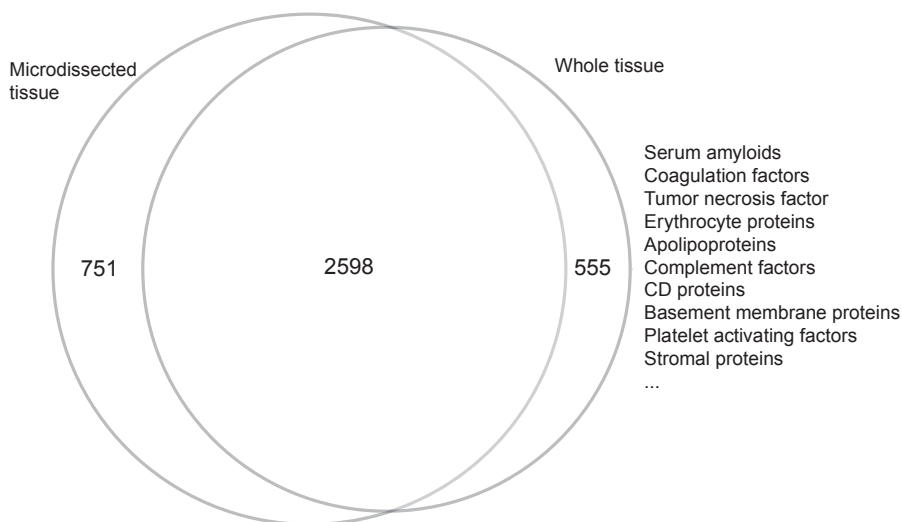


Figure 20.1 Venn diagram of proteins identified from microdissected tissue (left) versus non-microdissected tissue (right).

associated with brain tissue are enriched in the microdissected sample. The level of enrichment varies, from two- to threefold for more abundant proteins to over 10-fold for low abundance proteins. This helps to explain the large number of uniques (~20% of identified proteins) identified and highlights the advantage of using microdissection to maximize the discovery of low-abundance proteins that proteins from other cell types otherwise obscure. The capability to sensitively and quantitatively profile proteins at the level of microdissected cells in the context of complementary histological and pathological evaluations has the potential to greatly impact the study of disease.

20.4 SHOTGUN PROTEOME ANALYSIS OF MICRODISSECTED FORMALIN-FIXED BRAIN TUMOR TISSUE

We subsequently sought to compare the results of shotgun proteomic analysis of archival fresh-frozen tissues versus FFPE tissues.¹⁴ GBM tissue procured at the time of surgery was split equally, with one portion being snap frozen and the other formalin-fixed according to normal protocols. After about 1 year, each portion was sectioned to 6 μ m and microdissected. The fresh-frozen microdissections were processed so as to yield soluble and insoluble fractions. We had earlier developed a method for differential extraction of soluble and insoluble fractions from fresh-frozen tissue using microdissected ovarian carcinoma.¹⁵ Briefly, this consisted of using aqueous solvent for extraction of soluble components and using 1% SDS for extraction of insoluble components. The FFPE sections were deparaffinized prior to microdissection. Following

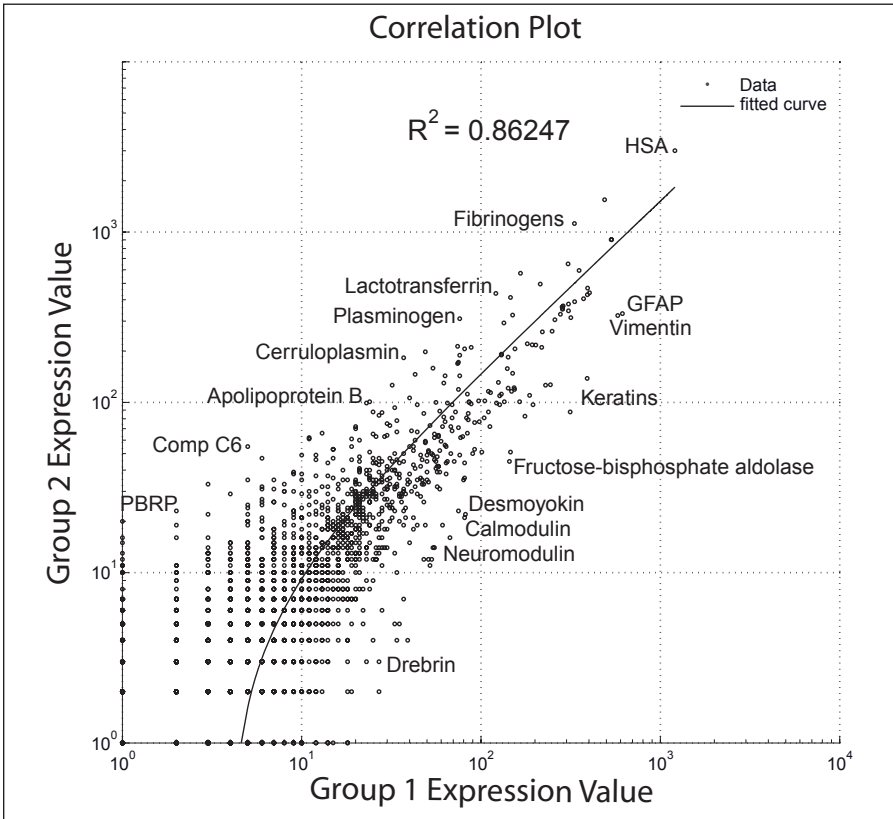


Figure 20.2 Correlation plot of protein identified from microdissected tissue (group 1) versus non-microdissected tissue (group 2).

this, they were subject to antigen retrieval as described above. The cells were placed in 2% SDS, 20mM Tris-HCl, pH 9, and heated at 100°C for 20min then at 60°C for 2h. The resulting solution was centrifuged and the supernatant dialyzed at 4°C overnight against 100mM Tris-HCl, pH 8.2 (Fig. 20.3).

All samples were digested with trypsin and analyzed by cIEF in the first dimension followed by LC-MS/MS as described above. Samples were analyzed in duplicate. Sequence searching was performed using OMSSA. Analysis of the soluble fraction yielded a total of 2856 identified proteins, while the insoluble fraction yielded 3227 proteins. Combined, the fresh-frozen sample yielded 3902 protein identifications. The FFPE portion yielded 2845 protein identifications from 14,178 distinct tryptic peptide sequences, on a par with the fresh-frozen soluble fraction. Combining all identifications gave 4145 proteins. While the soluble fraction and the FFPE extraction yielded similar numbers of protein identification, both found 25% of their respective protein set uniquely (Fig. 20.4).

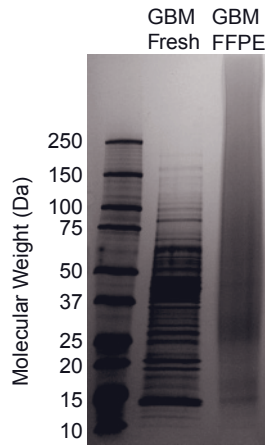


Figure 20.3 SDS-PAGE of proteins extracted from archival fresh-frozen (lane 2) and FFPE (lane 3) GBM tissue using the antigen retrieval method. Reproduced with permission from Reference 14.

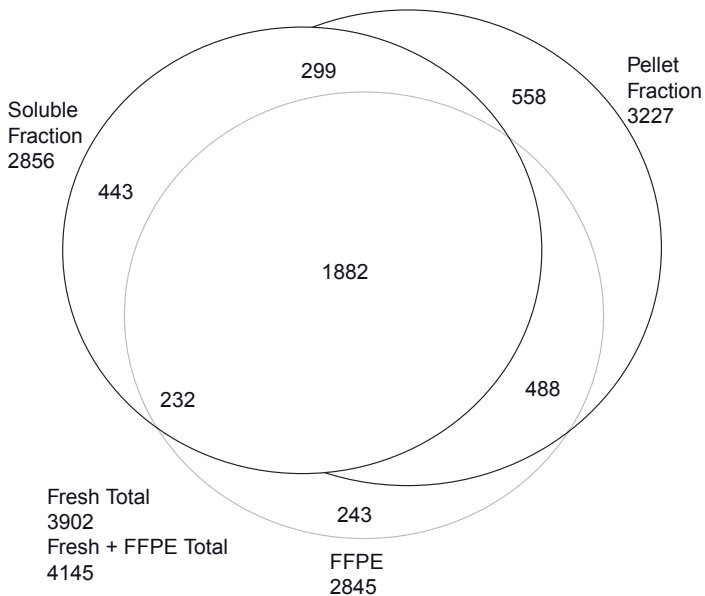


Figure 20.4 Venn diagram of proteins identified in soluble and insoluble extractions from fresh-frozen tissue and from antigen retrieval from FFPE tissue. Reproduced with permission from Reference 14.

This is an unusually high number and at least indicates differences in the extraction method used. The extractions used for the insoluble fraction and the FFPE sample were quite similar, however. In this case, only 16% of the protein set identified by the FFPE sample was unique. This number is consistent with standard variation of shotgun proteomic experiments. The SDS extraction also assists the antigen retrieval method in retrieving proteins containing predicted trans-membrane helices (TMHs). In this experiment the soluble fraction identified 307 TMH-containing proteins, the insoluble fraction 693 and the FFPE sample 488 (Fig. 20.5).

This is an important observation given that plasma membrane proteins are often used as markers of disease. This experiment demonstrated that shotgun proteomic analysis could be successfully performed on microdissected, formalin-fixed tissues using the antigen retrieval method with a sensitivity equal to that of analysis of the soluble fraction of a fresh-frozen sample.

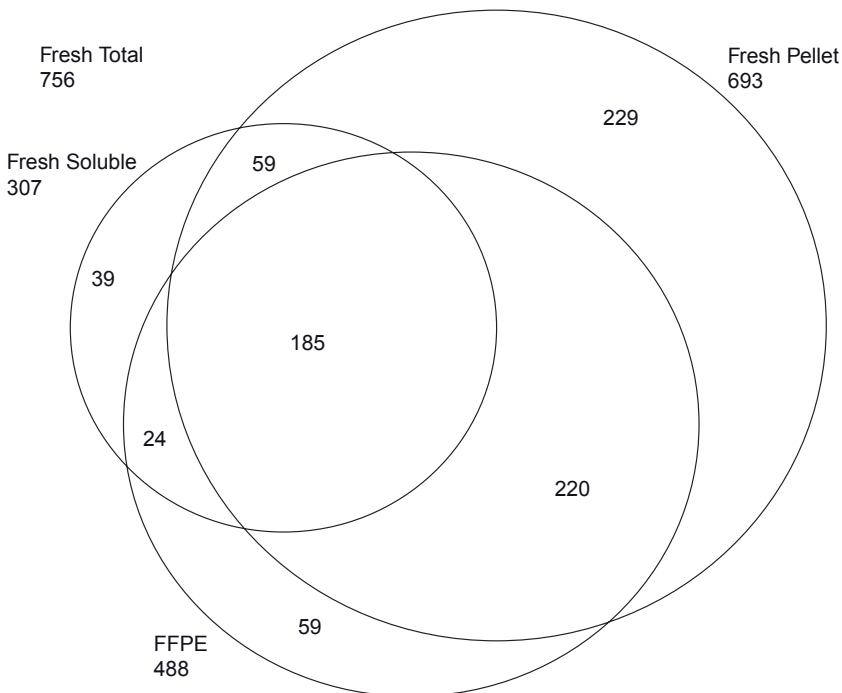


Figure 20.5 Venn diagram of proteins identified containing one or more trans-membrane alpha helices in soluble and insoluble extractions from fresh-frozen tissue and from antigen retrieval from FFPE tissue. Reproduced with permission from Reference 14.

20.5 EVALUATION OF CONFIDENCE AND REPRODUCIBILITY OF QUANTITATIVE SHOTGUN PROTEOMIC ANALYSES

A combination of highly complex samples and the random sampling nature of MS/MS challenges shotgun proteomic experimental reproducibility. To investigate this effect, we conducted analyses using yeast as a model system.¹⁶ Two samples were prepared in which yeast was grown under either aerobic or anaerobic conditions. Each sample was prepared in duplicate to provide a measure of sample preparation variation. Each resulting sample ($n = 4$) was then analyzed in duplicate (8 runs total) using cIEF-LC-MS/MS as described above. It has been reported that shotgun proteomic experiments using strong cation exchange (SCX) chromatography in the first dimension to produce 14 fractions require 10 analyses to reach a predicted 95% protein saturation coverage.¹⁷ Utilizing 15-fraction cIEF in the first dimension, we were able to reach the predicted 95% mark in four analyses (using a newer generation mass spectrometer). In this case, this resulted in the identification of just over 2700 proteins from a predicted saturation level of about 2800 proteins. This is enabled by the higher resolution separation afforded by cIEF relative to SCX which serves to focus more analytes into a single fraction, permitting detection of lower abundance proteins, and relatedly serves to reduce matrix interference effects. More interesting, however, was the result from performing a single 30-fraction cIEF separation in the first dimension. This analysis produced over 2900 protein identifications (more than the 15-fraction cIEF predicted saturation level) using half the mass spectrometer time of the four 15-fraction analyses (30 fractions vs. 60 fractions total) and one-quarter the sample amount (15 mcg vs. 60 mcg total). This result strongly reemphasizes the utility of high-resolution separations and maximizing the number of fractions taken in consideration of the overall peak capacity.

Reproducibility testing demonstrated that protein abundance measured using the spectral counting method exhibited a Pearson correlation R^2 value >0.99 and a coefficient of variance of 14% (Fig. 20.6).

Likewise, the method was able to measure changes in abundance as low as 1.5-fold with confidence ($p < 0.05$), following multiple testing adjustment using the Benjamini–Hochberg method (Fig. 20.7).

This also serves to highlight the search specificity in that an increasing false discovery rate would have an adverse effect on correlation and confidence measures.

While this study did not examine FFPE tissues, it does serve to highlight relevant challenges. Namely, that sensitivity, and to a lesser extent specificity, is driven by a method's ability to inject into the mass spectrometer an analyte as highly concentrated and as highly purified as possible. Microdissection plays a role in this as mentioned earlier, yielding up to 10-fold sample enrichment. However, the multidimensional separations preceding the mass spectrometer perform the bulk of the work, taking an input of tens of thousands of identifiable peptides and separating them such that the mass spectrometer processes

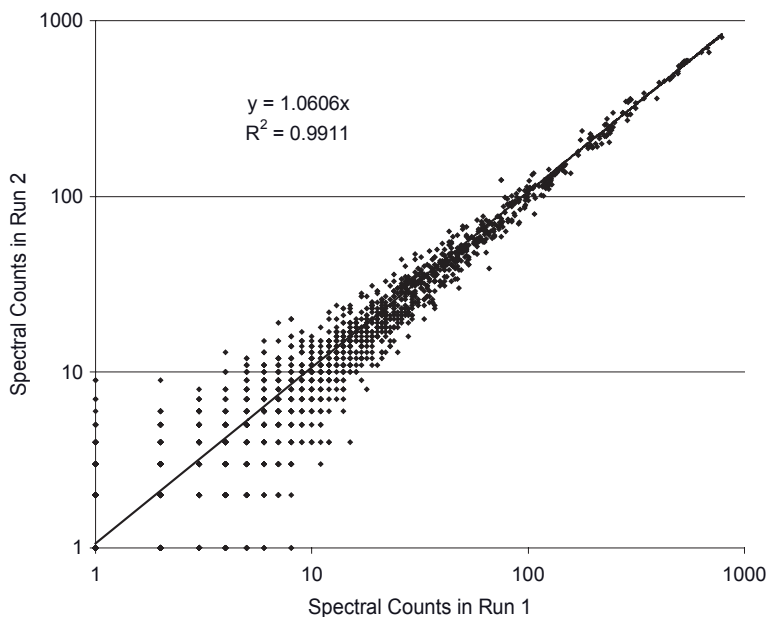


Figure 20.6 Correlation plot of technical replicates of yeast cell extracts. Reproduced with permission from Reference 16.

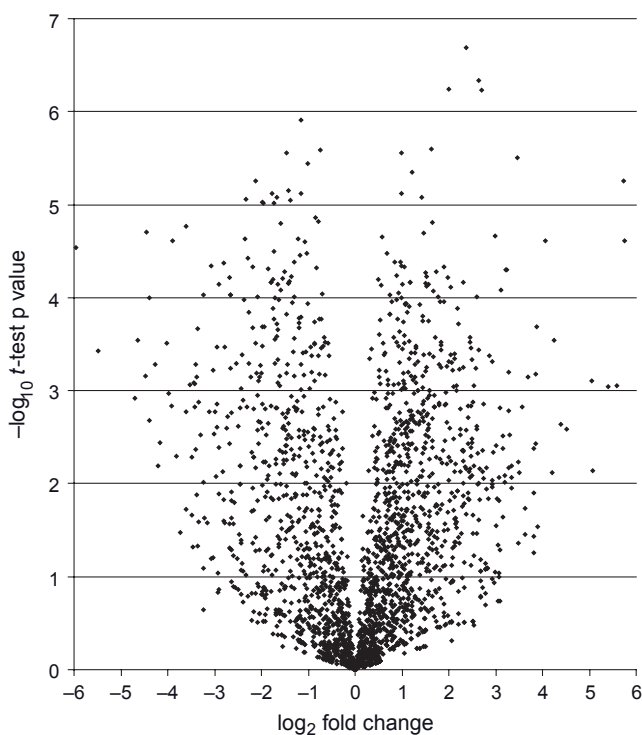


Figure 20.7 Volcano plot showing fold change versus t -test p value of eight runs of yeast cell lysates, four runs of aerobically grown yeast and four runs of anaerobically grown yeast. Reproduced with permission from Reference 16.

only on the order of tens of identifiable peptides per scan. It has been demonstrated that a proteolytic background exists in shotgun experiments.¹⁸ This complex background largely consists of peptides which were partially digested by trypsin or undigested and contributes to matrix effects that lessen sensitivity of the method. Matrix effects impact the method at three “choke points” with finite limits in mass spectrometry: the finite availability of energy for ionization, the finite volume of an ion trap, and the finite dynamic range of the detector(s). Minimizing these effects requires limiting the complexity of analytes at each of these stages. Separations are essential to this, and the result above comparing the single 30-fraction separation to the four 15-fraction separations demonstrates how a high-resolution separation can be used to minimize matrix effects to gain sensitivity while consuming less sample and using less mass spectrometer time.

The proteolytic background in archival FFPE tissues is likely to be much greater. As shown earlier in Figure 20.3, antigen retrieval does not lead to complete reversal of formaldehyde cross-linkages. Further, even in cases in which cross-links have been reversed, one of the cross-linked amino acid residues will retain the carbon atom which had formed the cross-link.¹⁹ Given that formalin fixation of tissue generally occurs over an extended period of time (hours), and that seven amino acids (lysine, tryptophan, cysteine, arginine, histidine, tyrosine, and phenylalanine) plus amino-termini are susceptible to cross-linking under such conditions, there is potential for a much more complex mixture of analytes than found in uncross-linked samples, especially when considering that trypsin digestion will be prevented at modified lysines and arginines.

20.6 EVALUATION OF CONFIDENCE AND REPRODUCIBILITY OF QUANTITATIVE SHOTGUN PROTEOMIC ANALYSES OF FFPE TISSUES

We therefore sought to evaluate reproducibility of shotgun proteomics in studies of archival FFPE tissue. Because FFPE samples are more complex than non-cross-linked samples, we evaluated FFPE human liver for analytical reproducibility and confidence in protein assignments.²⁰ This complexity strengthens the argument for using high-resolution separations to maximize analyte concentration and minimize matrix effects. In this case, we used transient capillary isotachopheresis/capillary zone electrophoresis (cITP/cZE) in place of IEF to help address this effect. cITP/cZE has a resolution superior even to cIEF (90% of identified peptides in 1 fraction, 95% in 2 fractions or less for cITP/cZE, vs. 75% and 80%, respectively, for cIEF).

As part of this work, we evaluated the effect of fixation times, from 0h to 14 days, on shotgun proteomic analyses and found no significant differences (Fig. 20.8).

Three human FFPE liver cases were analyzed and each yielded just under 20,000 distinct peptide sequence identifications and just over 3000 protein

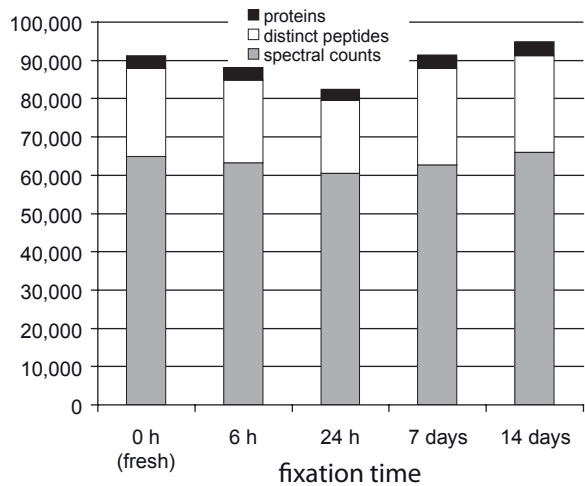


Figure 20.8 Identifications of spectral counts, peptide sequences, and proteins in archival FFPE liver tissue across a time course of increasing fixation time. Reproduced with permission from Reference 20.



Figure 20.9 Venn diagram of proteins identified from three cases of archival FFPE human liver tissue. Reproduced with permission from Reference 20.

identifications. Overlap of the identified proteins was significant, with 10% or fewer proteins uniquely identified by each case, indicating excellent reproducibility of the method (Fig. 20.9).

Correlation between analyses was $R^2 > 0.97$. This is somewhat less than the 0.99 R^2 values obtained in the model yeast system and may be attributable to either case-to-case variation in protein expression or a degradation in the

accuracy of quantitation due to the additional sample complexity. A panel of 17 proteins identified in the analyses, though not necessarily in every sample, was tested by immunohistochemistry of adjacent liver sections. All proteins stained positive, including four proteins identified with single peptide spectral matches. Again, the strong correlation measurement and confirmation by IHC underline the high search specificity. Additionally, this experiment validates the use of cITP/cZE as a very high-resolving first dimension separation mechanism for shotgun proteomics of highly complex samples (Table 20.1, Fig. 20.10).

Another finding of this study was that for some proteins, the peptides which were identified in the FFPE sample often had little to no overlap with

TABLE 20.1 Summary of IHC Staining Results and Comparison with Spectral Counts Measured by Shotgun Proteomics

Protein	IHC Staining Intensity (Number of Spectral Counts)		
	Sample 1	Sample 2	Sample 3
Vimentin	+ (178)	+ (155)	+ (184)
CD74 (LN2)	+ (1)	+ (0)	+ (0)
CD75 (LN1)	+ (3)	± (1)	+ (3)
Villin	+ (19)	+ (24)	+ (21)
Desmin	+ (20)	+ (29)	+ (22)
Lysozyme	+ (1)	+ (5)	+ (4)
CD117	+ (2)	+ (1)	+ (1)
Hemoglobin alpha	+ (500)	+ (384)	+ (314)
CD44	+ (3)	+ (5)	+ (3)
CD45	+ (2)	+ (0)	+ (1)
Cox 2	+ (9)	+ (15)	± (5)
PCNA	+ (2)	+ (2)	+ (2)
S-100	+ (1)	+ (0)	+ (0)
C3	+ (80)	+ (102)	+ (116)
Myeloperoxidase	+ (13)	+ (14)	+ (16)
GRP-78	+ (98)	+ (135)	+ (156)
E-cadherin	+ (2)	+ (3)	+ (6)

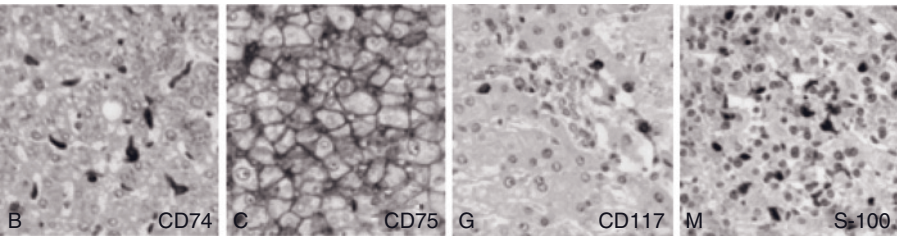


Figure 20.10 Validation by immunohistochemistry of four of the proteins identified in Table 20.1 with single peptide hits. Reproduced with permission from Reference 20. See color insert.

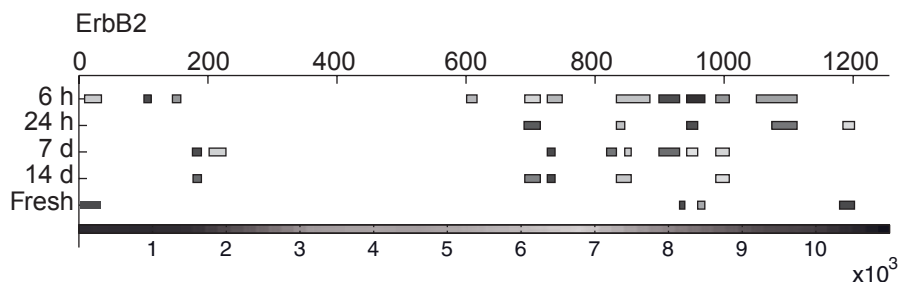


Figure 20.11 Coverage of protein ErbB2 by shotgun proteomic discovery of sample fixed for various times, including fresh. The color gradient represents the increasing abundance of the peptides. All were identified at an FDR <1%. Reproduced with permission from Reference 20. See color insert.

peptides identified in the fresh sample. It is not clear what contributes to this difference in peptide observability. All of the samples, including the fresh sample, were prepared in exactly the same manner using the antigen retrieval plus SDS method (Fig. 20.11).

This discovery has important implications for follow-on studies using mass spectrometry. Typically, follow-on studies such as multiple reaction monitoring mass spectrometry will utilize proteotypic peptides, that is, peptides which uniquely identify a protein and are observable with a given method. It may be that formaldehyde cross-linking followed by antigen retrieval substantially alters the observable population of peptides for any given protein or the likelihood of observation of those peptides. If this is the case, then proteotypic peptide libraries representative of FFPE tissues would need to be defined. This finding also potentially impacts the development of paraffin-compatible antibodies. Antibodies are typically raised to the C-terminus of a protein. Additionally, computational methods may be used to predict immunogenic and surface-accessible regions. However, formalin fixation is known to substantially alter antibody sensitivity and was one of the major drivers in the development of antigen retrieval.²¹ Peptides identified by shotgun proteomics of FFPE tissue may, at the very least, be defined as antigen retrievable given that they were identified. It is possible that some of these peptides, particularly those which are proteotypic, may serve as epitopes for the generation of antibodies compatible with antigen retrieval from FFPE tissues.

20.7 SHOTGUN PROTEOMICS FOR THE ANALYSIS OF ARCHIVAL FFPE TISSUES

Archival tissues stored as FFPE tissue blocks represent the vast majority of tissue archives. To investigate the effect of storage on shotgun proteomic analysis of FFPE tissues, we analyzed 10 samples dating back to 1980.²² Nine leiomyomas (three from 1990, three from 1997, and three from 2002) and one

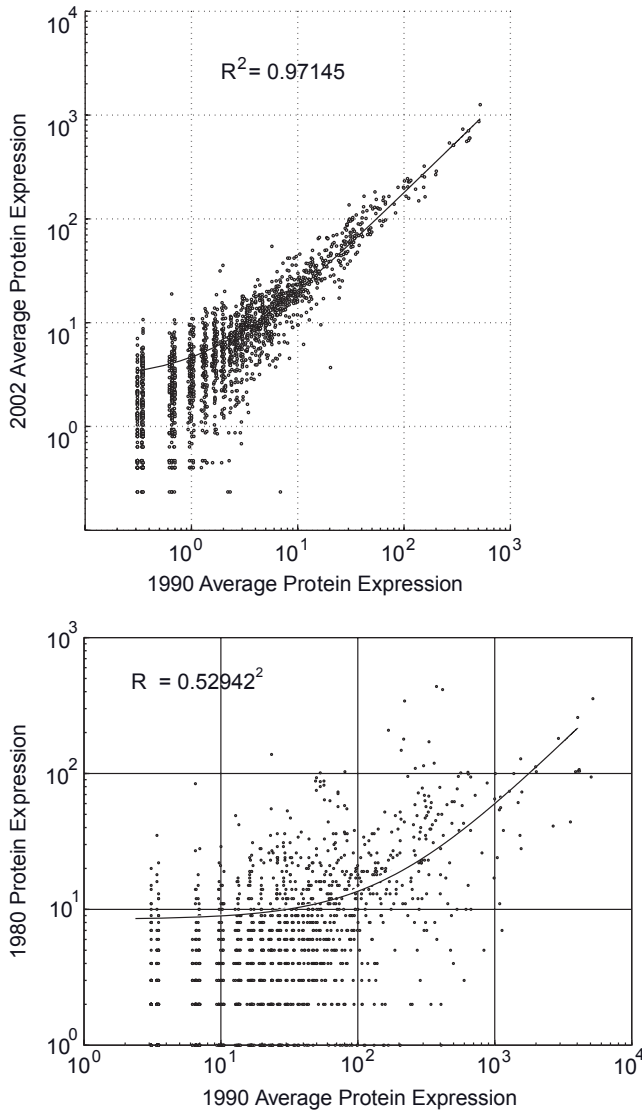


Figure 20.12 Correlation plots of 1990 versus 2002 FFPE leiomyomas (left) and 1990 FFPE leiomyomas versus a 1980 FFPE sarcoma (right). Reproduced with permission from Reference 22.

sarcoma (1980) were analyzed. In this case, transient cITP/ZE was used in place of cIEF in the first dimension. Of 2583 proteins identified across all the cases, 80 were unique to the sarcoma. Correlation of any of the leiomyoma times points gave R^2 values near 0.97, while correlation of any of these time points with the sarcoma gave modest correlations near 0.5, indicating clear differences in protein expression between the two disease types (Fig. 20.12).

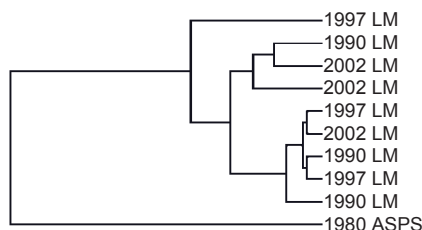


Figure 20.13 Unsupervised hierarchical cluster analysis of nine FFPE leiomyomas from 1990–2002 and one FFPE sarcoma from 1980. Reproduced with permission from Reference 22.

Unsupervised hierarchical cluster analysis showed clear separation between the sarcoma and the leiomyomas but did not reveal associations among the leiomyomas based on storage time, possibly indicating that individual differences exceeded any differences caused by storage (Fig. 20.13).

This study demonstrated that even decades-old archival FFPE tissues are amenable to shotgun proteomic profiling and yield evaluable results.

20.8 FUTURE DIRECTIONS FOR SHOTGUN PROTEOMICS APPLIED TO FFPE TISSUES

Our studies have helped to demonstrate that there is great potential for the characterization and elucidation of disease through proteomic profiling of FFPE tissues. The knowledge gained from selective profiling will pave the way for higher throughput, targeted methods. These methods, such as LC-multiple reaction monitoring-MS and tissue microarray immunohistochemistry, will permit high-throughput evaluation of small numbers of proteins in studies of large numbers of cases. These studies in turn may lead to markers capable of discriminating between morphologically similar disease or defining new disease classifications.

Because formalin fixation is achieved through a cross-linking process, another promising avenue for shotgun proteomic research is the study of protein–protein interactions via formaldehyde cross-linking. Several high-throughput protein–protein interaction prediction methods have been very successful mapping large numbers of potential interactions, often on a proteome-wide basis. These methods include yeast two-hybrid,²³ immunoaffinity purification and affinity tag-based purifications followed by shotgun mass spectrometry,^{24–26} protein arrays,^{27,28} and bioinformatic methods.^{29,30} Each of the methods display various strengths and weaknesses. One shared weakness is their inability to maintain complexes throughout the downstream purification processes. This has been demonstrated to perturb the composition of complexes.³¹ Additionally, these same downstream processes prevent transient protein interactions from being retained. Chemical cross-linking has

been used in association with immunoaffinity and tag-based affinity methods to overcome this limitation.^{1,7} Cross-linking can preserve transient protein interactions in the native environment of the cell and preserve them even through harsh purification methods. Cross-linking presents its own challenges, however. Cross-linked peptides are present at a very low level in the overall sample mixture, and they can be very difficult to identify. Even cleavable cross-links often produce tandem mass spectra in which the cross-linker fragment ions are greatly overrepresented relative to the peptide fragment ions.^{32–34}

Formaldehyde represents a likely alternative to more commonly used chemical cross-linking agents. Due to its small size, it rapidly penetrates cells and tissues. Also, because it maximally spans a very short distance of about 2 Å, the interactions which it preserves are only of very closely associated proteins. The cross-links are very stable under various conditions, permitting isolation of complexes through various protocols. Additionally, the cross-link is reversible via heating, the subject of this book. This permits straightforward identification of the components by mass spectrometry. Finally, formaldehyde is routinely used in the preservation of tissue samples in the form of neutral buffered formalin. That is, formalin-fixed tissues have protein–protein interactions preserved. The strength of these traits is exhibited by formaldehyde's decades-long use in various assays such as chromatin immunoprecipitation⁸ and flow cytometry.³⁵

Indeed, formaldehyde has a long history in the study of protein–protein interactions as well. Jackson described its use in 1978 for the purpose of characterizing the histone complex.⁹ Formaldehyde was used to fix cells at various stages of the cell cycle. Proteins were isolated and analyzed by SDS-PAGE, demonstrating formaldehyde's ability to maintain cross-links in the presence of a strong detergent such as SDS. Following separation lanes were cut and heated to 95°C in 1% SDS, 0.5 M 2-mercaptoethanol for 1 h to reverse the formaldehyde cross-linkages. The lane was then placed at the top of another gel and again electrophoresed by SDS-PAGE. In this manner proteins released from the cross-linked complex could migrate according to their mass (Fig. 20.14).

Recently, Vasilescu et al. demonstrated the use of formaldehyde to preserve protein interactions *in vivo* followed by immunoaffinity purification of a targeted complex, cross-link reversal via heating at 95°C, separation by SDS-PAGE, and identification of bands by LC-MS/MS.⁷ Tagwerker et al. utilized formaldehyde cross-linking in conjunction with a novel tag-based affinity purification method.³⁶

Many recent studies have focused on the mechanisms of formaldehyde modification, cross-linking, and reversal.^{19,37–48} In general, these studies found that formaldehyde is very specific, particularly when reaction times are relatively short. The amino-termini, lysine, tryptophan, and cysteine are the targets of modification in this case. Longer reaction times reveal more extensive modifications, including arginine, histidine, tyrosine, and phenylalanine.

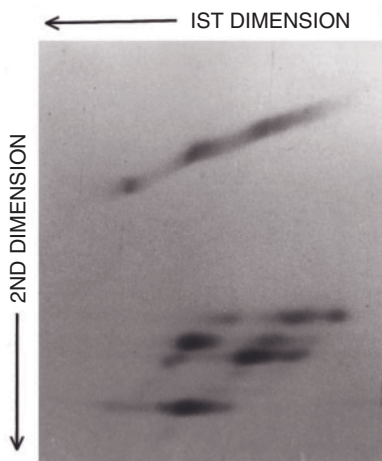


Figure 20.14 2-dimensional SDS-PAGE separation of cross-linked (1st dimension) and then de-cross-linked (2nd dimension) nucleosomes. Reproduced with permission from Reference 10.

However, all of these studies were performed using model components *in vitro*—none have examined formaldehyde-induced modifications *in vivo*. Further, while modification sites have been mapped by MS/MS, intact cross-linked peptide species have not been observed in such experiments.⁴⁹ This possibly indicates that the covalent bonds of the formaldehyde cross-links are not as strong as those of the peptide backbone. The resulting fragment ion spectra are similar to that of the unmodified peptide with the exception of 12 Da or 30 Da additions at modifications sites. Thirty Dalton modifications correspond to the addition of formaldehyde while 12 Da modifications indicate water elimination.

It is noteworthy that following cross-link reversal, the 12 Da modification persists. The reaction is a two-step process, with formaldehyde modification of one residue preceding the cross-link-forming modification of the second. Cross-link reversal results in one residue or the other retaining the 12 Da modification. Given a population of a protein cross-linked to another protein at a given site, cross-link reversal will result in two populations in which the modification is present on one protein or the other. This hypothesis is supported by our own results, described in this chapter. That is, we are able to identify similar numbers of peptide sequences in FFPE tissue as we do in fresh-frozen tissue, although the peptides representing any given protein may vary between the two types of samples. The development of methods for defining protein–protein interactions in FFPE tissue is conceivable. Such a capability would add an additional layer of knowledge to what might already be known for any given FFPE tissue using conventional methods, including those described in this chapter. As critical protein–protein interactions can

often define biological events, their incidences are potentially useful as diagnostics which may be more specific than measurements of individual protein expression. Indeed, it is notable that ErbB2 expression is used as a proxy for ErbB2 dimerization, which is the driver of tumorigenesis, and that numerous studies have shown that ErbB2 expression does not always correlate with response to trastuzumab therapy.^{50,51} An assay for the dimer, for the protein–protein interaction, may provide more specificity than assaying for protein expression alone.

REFERENCES

1. Gingras A, Gstaiger M, Raught B, et al. Analysis of protein complexes using mass spectrometry. *Nat. Rev. Mol. Cell Biol.* 2007; 8: 645–654.
2. Patterson SD, Aebersold RH. Proteomics: the first decade and beyond. *Nat. Genet.* 2003; 33 (Suppl. 3): 11–23.
3. Ong S, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* 2005; 1: 252–262.
4. Lin D, Tabb DL, Yates JR. Large-scale protein identification using mass spectrometry. *Biochim. Biophys. Acta.* 2003; 1646: 1–10.
5. Prieto DA, Hood BL, Darfler MM, et al. Liquid tissue: proteomic profiling of formalin-fixed tissues. *Biotechniques.* 2005; 38 (Suppl.): 32–35.
6. Hood BL, Darfler MM, Guiel TG, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics.* 2005; 4: 1741–1753.
7. Vasilescu J, Guo X, Kast J. Identification of protein-protein interactions using in vivo cross-linking and mass spectrometry. *Proteomics* 2004; 4: 3845–3854.
8. Solomon MJ, Larsen PL, Varshavsky A. Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. *Cell* 1988; 17: 937–947.
9. Jackson V. Studies on histone organization in the nucleosome using formaldehyde as a reversible cross-linking agent. *Cell* 1978; 15: 945–954.
10. Jackson V. Formaldehyde cross-linking for studying nucleosomal dynamics. *Methods* 1999; 17: 125–139.
11. Shi S, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
12. Shi SR, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* 1995; 43: 193–201.
13. Ikeda K, Monden T, Kanoh T, et al. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* 1998; 46: 397–403.
14. Guo T, Wang W, Rudnick PA, et al. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. *J. Histochem. Cytochem.* 2007; 55: 763–772.

15. Wang W, Guo T, Rudnick PA, et al. Membrane proteome analysis of micro-dissected ovarian tumor tissues using capillary isoelectric focusing/reversed-phase liquid chromatography-tandem MS. *Anal. Chem.* 2007; 79: 1002–1009.
16. Balgley BM, Wang W, Song T, et al. Evaluation of confidence and reproducibility in quantitative proteomics performed by a capillary isoelectric focusing-based proteomic platform coupled with a spectral counting approach. *Electrophoresis* 2008; 29: 3047–3054.
17. Liu H, Sadygov RG, Yates JR. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* 2004; 76: 4193–4201.
18. Picotti P, Aebersold R, Domon B. The implications of proteolytic background for shotgun proteomics. *Mol. Cell. Proteomics* 2007; 6: 1589–1598.
19. Toews J, Rogalski JC, Clark TJ, et al. Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. *Anal. Chim. Acta.* 2008; 618: 168–183.
20. Xu H, Yang L, Wang W, et al. Antigen retrieval for proteomic characterization of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2008; 7: 1098–1108.
21. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 1991; 39: 741–748.
22. Balgley M, Guo T, Zhao K, et al. Evaluation of archival time on shotgun proteomics of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2009; 8: 917–925.
23. Fields S, Song O. A novel genetic system to detect protein-protein interactions. *Nature* 1989; 340: 245–246.
24. Ho Y, Gruhler A, Heilbut A, et al. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 2002; 415: 180–183.
25. Rigaut G, Shevchenko A, Rutz B, et al. A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* 1999; 17: 1030–1032.
26. Puig O, Caspary F, Rigaut G, et al. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 2001; 24: 218–229.
27. Zhu H, Snyder M. Protein chip technology. *Curr. Opin. Chem. Biol.* 2003; 7: 55–63.
28. Ramachandran N, Hainsworth E, Bhullar B, et al. Self-assembling protein microarrays. *Science* 2004; 305: 86–90.
29. Schächter V. Bioinformatics of large-scale protein interaction networks. *Biotechniques* 2002; 32(Suppl.): S16–S27.
30. von Mering C, Krause R, Snel B, et al. Comparative assessment of large-scale data sets of protein-protein interactions. *Nature* 2002; 417: 399–403.
31. Zhang H, Tang X, Munske GR, et al. In vivo identification of the outer membrane protein OmcA-MtrC interaction network in *Shewanella oneidensis* MR-1 cells using novel hydrophobic chemical cross-linkers. *J. Proteome Res.* 2008; 7: 1712–1720.

32. Sinz A. Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein-protein interactions. *Mass. Spectrom. Rev.* 25: 663–682.
33. Sinz A. Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. *J. Mass. Spectrom.* 2003; 38: 1225–1237.
34. Trakselis MA, Alley SC, Ishmael FT. Identification and mapping of protein-protein interactions by a combination of cross-linking, cleavage, and proteomics. *Bioconjug. Chem.* 2005; 16: 741–750.
35. Lanier LL, Warner NL. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. *J. Immunol. Methods.* 1981; 47: 25–30.
36. Guerrero C, Tagwerker C, Kaiser P, et al. An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. *Mol. Cell. Proteomics.* 2006; 5: 366–378.
37. Metz B, Kersten GFA, Hoogerhout P, et al.: Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
38. Metz B, Kersten GFA, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* 2004; 279: 6235–6243.
39. Fowler CB, Cunningham RE, O’Leary TJ, et al. “Tissue surrogates” as a model for archival formalin-fixed paraffin-embedded tissues. *Lab. Invest.* 2007; 87: 836–846.
40. Fowler CB, O’Leary TJ, Mason JT. Modeling formalin fixation and histological processing with ribonuclease A: effects of ethanol dehydration on reversal of formaldehyde cross-links. *Lab. Invest.* 2008; 88: 785–791.
41. Rait VK, Xu L, O’Leary TJ, et al. Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A. II: Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Lab. Invest.* 2004; 84: 300–306.
42. Rait VK, O’Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic ribonuclease A: I-structural and functional alterations. *Lab. Invest.* 2004; 84: 292–299.
43. Fowler CB, Cunningham RE, Waybright TJ, et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. *Lab. Invest.* 2008; 88: 185–195.
44. Rait VK, Zhang Q, Fabris D, et al. Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. *J. Histochem. Cytochem.* 2006; 54: 301–310.
45. O’Leary TJ, Mason JT. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 122: 154.
46. Sompuram SR, Vani K, Hafer LJ, et al. Antibodies immunoreactive with formalin-fixed tissue antigens recognize linear protein epitopes. *Am. J. Clin. Pathol.* 2006; 125: 82–90.

47. Sompuram SR, Vani K, Bogen SA. A molecular model of antigen retrieval using a peptide array. *Am. J. Clin. Pathol.* 2006; 125: 91–98.
48. Sompuram SR, Vani K, Messana E, et al. A molecular mechanism of formalin fixation and antigen retrieval. *Am. J. Clin. Pathol.* 2004; 121: 190–199.
49. Sutherland BW, Toews J, Kast J. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *J. Mass. Spectrom.* 2008; 43: 699–715.
50. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Oncologist* 1998; 3: 237–252.
51. Ross JS, Fletcher JA, Bloom KJ, et al. Targeted therapy in breast cancer: the HER-2/neu gene and protein. *Mol. Cell. Proteomics* 2004; 3: 379–398.

VISUALIZING PROTEIN MAPS IN TISSUE

MASAHIRO MUKAI and MITSUTOSHI SETOU

21.1 INTRODUCTION

Mass spectrometry (MS) is a technique that separates ionized particles—such as atoms, molecules, and clusters—using differences in the ratios of their charges to their respective masses, and thus can be used to determine the molecular weights of the particles. In addition, the analysis not only of simple mass but also of molecular architecture is possible. The underlying principle of the measurement is the determination of the molecular weights of the fragments formed by collisions of ionized particles with ionized rare gas molecules. This structural analysis by MS has been called MSⁿ analysis because it employs two or more consecutive MS analyses. This technology made it possible to identify glycosylated proteins and lipids. A wider range of biomolecular species can now be measured. For protein molecules with high molecular weights, amino acid sequencing for molecular identification can be attained by measuring after the molecules are biochemically degraded into peptide fragments. John B. Fenn and Koichi Tanaka won the Nobel Prize in Chemistry for the development of these ionizing techniques for biological macromolecules.

However, the object being analyzed has to be removed from the tissues. Thus, information about the distribution of the target in the organism or in the cells is inevitably lost. What is now needed is a technology to acquire information about the distribution of the biomolecule simultaneously with its identification. The method used for this purpose, called imaging mass spectrometry (IMS), is as follows. The tissue sample is cut into thin slices, and a matrix that assists the ionization of macromolecules is spread onto these slices. The macromolecules are then ionized by a scanning laser, and the generated ions are detected and analyzed by MS.¹

IMS is based on time-of-flight–mass spectrometry (TOF-MS) analyses from the selected locations of thin tissue sections on ionized molecules using matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) or secondary ion mass spectrometry (SIMS).¹ Reconstructed images showing the distribution of any molecules caught in the mass spectrum charts are available by gathering vast amounts of information from thousands of spots on biological tissues and choosing the spectrometric peaks of the selected molecules.

SIMS imaging was theoretically invented in 1949 by Herzog and Viehb of the Vienna University in Austria. The first SIMS device was completed by Liebel and Herzog in 1961 with the support of the National Aeronautics and Space Administration (NASA) and was used to analyze metal surfaces. However, it was not suitable for analyzing biological macromolecules because the second electronic ion beam breaks the molecules into atoms.

Professor Caprioli of Vanderbilt University (US), Professor Heeren's group (Europe), and our group (Japan) independently developed the MALDI-MS imaging and attained the expected resolution.^{1,2}

21.2 THE HISTORY OF OUR IMAGING MS SAMPLE PREPARATION TECHNIQUE

Initially, since the ionization efficiency was remarkably low in tissue section samples, miscellaneous biological molecules such as salt were included in the analysis, which posed a problem. To resolve it, it was necessary to increase the detector sensitivity of the MS device, and improvements in the preparation and pretreatment of tissue samples were very important. Our actions toward solving these problems in the past several years are described below.

First, we examined the correlation between the thickness of the tissue section and ionization efficiency. The highest signal intensity and signal-to-noise ratio during the measurement of high-molecular-mass protein occurred when the thickness of the tissue was $\leq 10\mu\text{m}$.³ Next, to measure proteins larger than 100kDa that constituted biotissues and to identify these proteins simultaneously, we devised a technique to digest proteins in tissue sections, named the on-tissue digestion method.⁴ This method is characterized by infusing a microfluidic solution of trypsin and/or matrix (dozens of picoliters) at the appointed position in the tissue section by a chemical inkjet printer (Shimadzu Corporation, Kyoto, Japan). This method can control the diffusion of proteins and peptides—the objects being measured—to the minutest level in the tissue section because the solution being dropped does not excessively wet the tissue section. Furthermore, the peptide fragments created by on-tissue digestion method can be measured by quadrupole ion-trap TOF-MS (AXIMA-QIT; Shimadzu Corporation) and identified by MS/MS.⁴ To prepare a sample according to this method, a new sample preparation technique was devised to transfer tissue sections from the polyvinylidene fluoride (PVDF) film to a new

PVDF film by using a buffer solution with denaturation/reduction effects.⁵ This technique can denature, reduce, and digest the proteins in the tissue section efficiently and remove the salt from the tissue. Thus, the ionization efficiency for biological molecules is increased.

The above-mentioned method is effective in identifying the molecules of detected ions. However, because PVDF film is not permeable to light, it is difficult to observe tissue sections. To resolve this problem, we developed a method to fix tissue sections on transparent film, and then performed MS on those sections.⁶ We used a conductive film because we expected the ionization efficiency would increase when the electric charge accumulation on the sample was reduced. The film used for this purpose was a polyethylene terephthalate (PET) film with a thickness of 75–125 μm , having a 5–15-nm-thick layer of evaporated oxidation indium tin (ITO) upon it (ITO film). This film is used in touch-panel displays because of its high transparency and superior conductivity. We used it to perform MS/MS for tissue sections and succeeded in identifying multiple proteins from mass spectra.⁶ Therefore, the further development of this method will enable the application of the mass-microscopic method to observe tissue by optical microscope and to perform tandem mass spectrometry (MSⁿ) at the observation part, simultaneously, enabling the identification of molecules included the part.

We also improved the matrix application method. We developed a spray-droplet technique combining the continuous application of the spray-coating and droplet methods combined, although they were originally adopted as separate methods.⁷ In this combined technique, the matrix solution is sprayed and then a matrix spot covered with fine crystals is created by dropping a droplet of a more concentrated matrix solution. When this method was applied to rat brain tissue, the signal peak intensity increased approximately 30-fold and the signal-to-noise ratio improved drastically.⁷ Furthermore, we applied this method to cultured cells. HEK293T cells were easily cultured directly on the above-mentioned ITO film. This culture sample is directly usable for MS by the spray-droplet method.⁸ We performed on-tissue digestion with trypsin in HEK293T cells and MS/MS analysis, and obtained two strong peaks among provided cell-derived protein peaks, identified as histone H2A.2 and nucleophosmin, respectively.⁸

21.3 PRACTICAL SAMPLE PREPARATION FOR IMS MEASUREMENT

In this chapter, we show the method for preparing samples for IMS measurement. First is the process of making a thin-slice section from frozen tissue blocks so that it can be served to the matrix application. The process of making a section for IMS measurement is essentially similar to that of making frozen sections for immunostaining or dye staining. However, since the section created in this case is used for MS, there are some pivotal differences with the section-making processes for other staining methods. By complying with these

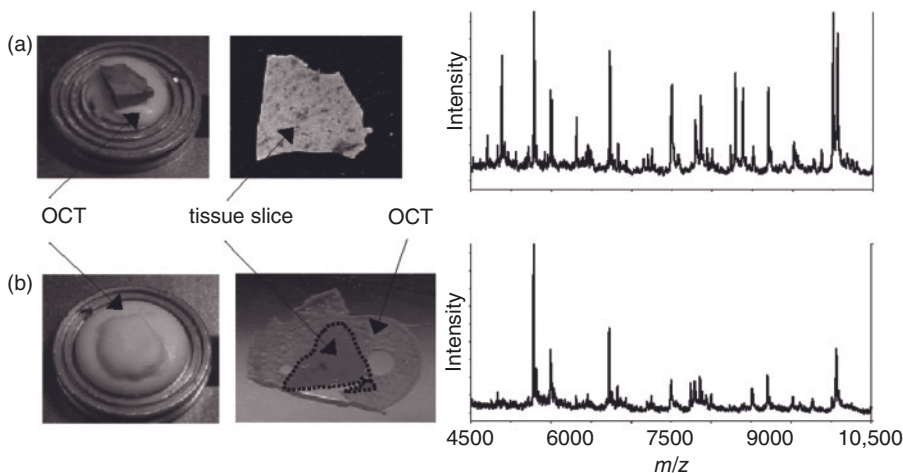


Figure 21.1 Decrease in detection sensitivity of the ions originating from proteins by contamination of OTC. Adhering OTC to the tissue section diminishes the detectable peaks. (a) A case in which OTC was used only to support the tissue block. (b) A case in which the tissue block was completely embedded with OTC. Reprinted with permission from Schwartz et al.⁹

points, one should expect to have signals with high S/N ratios together with a high reproducibility with these sections.

21.3.1 Embedding

In IMS experiments, polymer compounds such as OTC compound (Sakura Seiki, Tokyo, Japan) lead to a deterioration of the signals.⁹ Particularly, the contamination of OTC during the detection of low-molecular-weight compounds with m/z 1000–2000 leads to the emergence of extremely high peaks of polymers in the mass spectra of positive ions, which would virtually hide all the other peaks below them. There is also a decrease in signal sensitivity during the detection of higher-molecular-weight proteins.⁹ For these reasons, when creating the sections for IMS imaging, OTC is used only for “supporting” the tissue blocks as shown in Figure 21.1, so that it does not directly attach to the tissue sections being analyzed. However, some tissues are still difficult to cut into thin slices without the embedding process. For these cases, Stoeckli et al. used sodium carboxymethylcellulose (CMC) as an embedding compound that would not interfere with MS.¹⁰

21.3.2 Excision of a Thin Slice

The most important factor in the excision of a thin slice for IMS measurement is the thickness of the slice. When a slice is thicker than 15 μm , the IMS leads

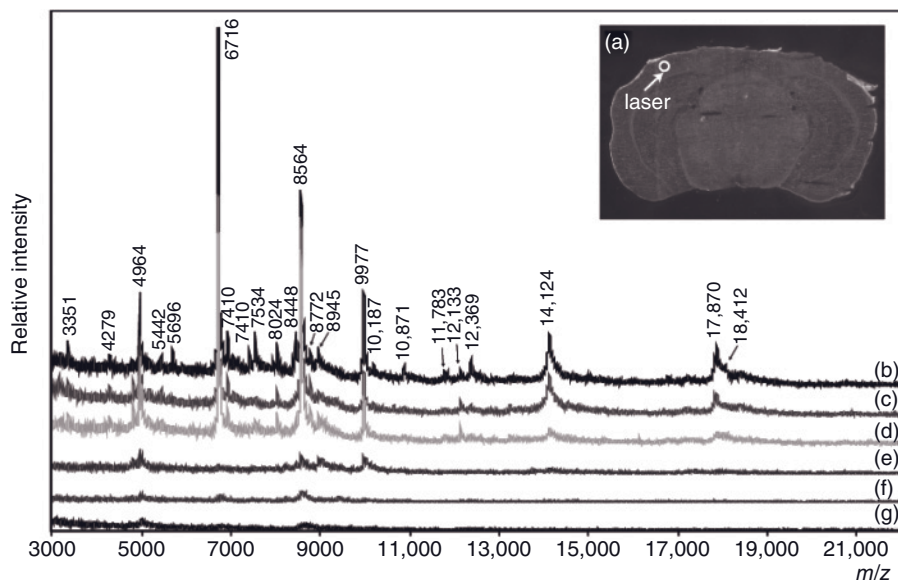


Figure 21.2 Mass spectra obtained from the cerebral cortex region (a) of mouse brain. Signals with good S/N ratios were obtained from thin slices. Modified with permission from Sugiura et al.³

to a deterioration of the detection sensitivity, particularly when analyzing high-molecular-weight proteins.³ Figure 21.2 shows the mass spectra obtained from cerebral cortex regions in mouse brain slices with thicknesses of 2, 5, 10, 15, and 30 μm . A large number of mass peaks with higher S/N ratios were observed in the spectra obtained from sections with 2, 5, and 10 μm thicknesses compared to those with 15 and 30 μm thicknesses.

In the case of frozen sections without fixation, a skillful technology is required to stably create slices that are several micrometers thick. For this reason, in most reports, the samples are prepared with slices that are 10–20 μm thick.¹¹

21.3.3 Section-Supporting Materials

In IMS, supportive materials, whose surfaces are coated with conductive materials, are used in principal. In the simplest way, the tissue slices can be placed on a metal MALDI plate directly.⁹ In this case, however, the target plate must be cleaned carefully after the measurement is over. Currently, the method commonly used is that samples are prepared on a disposable plastic sheet or a glass slide coated with series of conductive materials. In particular, a plastic sheet (ITO sheet) or glass slide (ITO glass slide; available from Bruker Daltonics K.K., Billerica, MA, or Sigma, St. Louis, MO) coated with ITO (indium-tin oxide) is useful because it has superior optical transparency

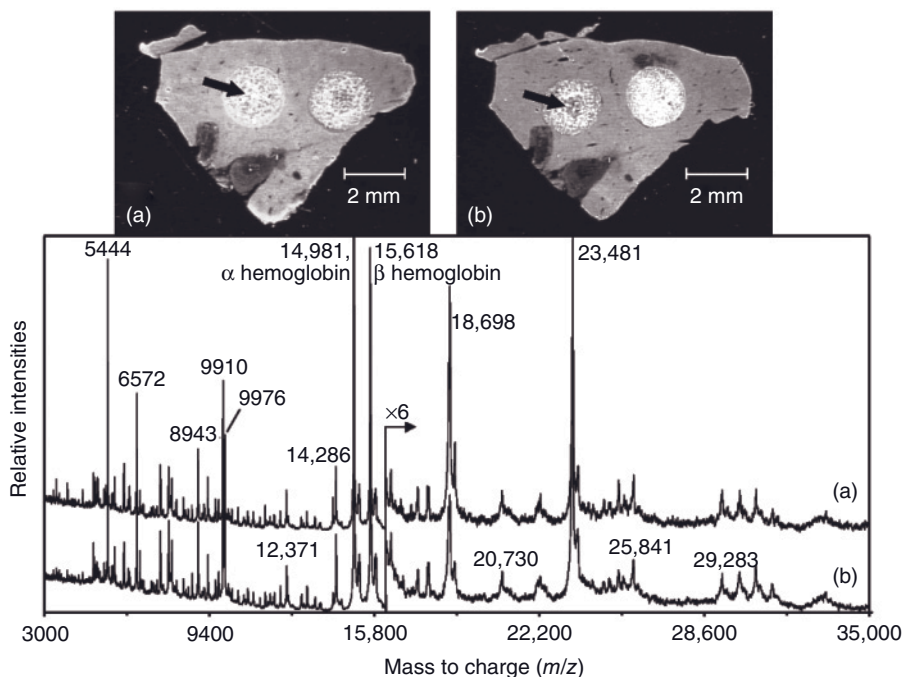


Figure 21.3 Mouse liver sections (upper panel) on glass slides coated with gold (a) and ITO (b), and mass spectra obtained from them (lower panel). Reprinted with permission from Chaurand et al.¹²

sufficient for microscopic observation.^{12,13} In addition, an ITO sheet or glass slide provides mass spectra with high quality, comparable to that obtained with materials coated with gold, the best conductive material (Fig. 21.3).¹²

21.3.4 Washing of the Tissue Sections

The tissue sections must be washed with organic solvents when the detection targets include peptides and proteins. Washing with organic solvents promotes the ionization of peptides and proteins mainly by removing phospholipids from the sections.¹⁴ Washing also flushes out salts that could interfere with the crystallization of the matrix.

Several methods are described in the literature regarding the washing of tissue sections. We describe representative ones here. The authors' impression is that the method in Reference 15, and even more so that in Reference 16, are likely to have a stronger degreasing action than that in Reference 9.

- 70:30%, v/v, ethanol/water (30-s immersion) × 2 times⁹

- 70:30%, v/v, ethanol/water (30-s immersion), 100% ethanol (15-s immersion)¹⁵
- 90:9:1%, v/v, ethanol/water/glacial acetic acid (30-s immersion)¹⁶

21.3.5 Drying the Tissue Sections

If the sections are not washed, they need to be dried well right after excision. On the other hand, sections that are washed need to be dried well before the matrix application. Not drying them sufficiently may cause the samples to peel off in the vacuum chamber of the mass spectrometer.

When there is a possibility that the detection target material can easily be oxidized, particularly when the goal is to detect low-molecular-weight organic compounds that contain many carbon–carbon double bonds (e.g., phospholipids containing highly unsaturated fatty acids), the use of a cold air dryer to dry the samples should be avoided in order to prevent oxidation. Instead, it is better to dry the samples in a vacuum desiccator with reduced pressure or using a nitrogen gas spray.

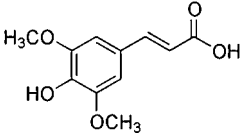
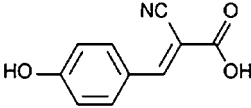
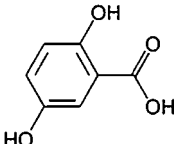
21.3.6 Matrix-Coating Method for Assisted MALDI Imaging

The second method of sample preparation for IMS is a matrix-coating method for MALDI imaging. In this chapter, we review the choices of matrix compound and solvent composition appropriate for IMS of tissue sections. Three kinds of matrix-application methods and examples of their use are illustrated.

21.3.7 Choice of Matrices

The essential functions required for the matrix to measure biological macromolecules in MS are as follows: (1) isolation of analyte molecules by dilution and prevention of analyte aggregation within the preparation; (2) absorption of the laser energy via electronic excitation; (3) disintegration of the condensed phase of co-crystal without excessive destructive heating of the embedded analyte molecules; (4) efficient ionization of analyte molecules; and (5) stabilization of the co-crystal in the high-vacuum chamber of the mass spectrometer. Today, experimenters commonly choose from a relatively small number of established “chemical matrices,” for example, benzoic or cinnamic acid derivatives. A practical choice depends on the type of analyte. For example, 2,5-dihydroxybenzoic (DHB) acid is commonly used for relatively low-molecular-weight analytes of organic compounds, lipids, and peptides. The properties of the three major matrices used for MALDI-IMS of tissue sections are summarized in Table 21.1. The most important factor for obtaining high-quality mass spectra is to ensure that the tissue section is covered uniformly with matrix crystal.

TABLE 21.1 Three Major Matrices Used for MALDI-IMS

Matrix	SA	CHCA	DHB
Other name	sinapinic acid 3,5-dimethoxy-4-hydroxycinnamic acid	α -cyano-4-hydroxycinnamic acid	2,5-dihydroxy benzoic acid
Structural formula			
MW	224.21	189.17	154.12
Chemical formula	C ₁₁ H ₁₂ O ₅	C ₁₀ H ₇ NO ₃	C ₇ H ₆ O ₄
Solubility	low solubility in H ₂ O soluble in methanol/ H ₂ O and polar organic solvents	low solubility in H ₂ O soluble in methanol/ H ₂ O and polar organic solvents	soluble in H ₂ O soluble in methanol/ H ₂ O and polar organic solvents
Feature	high signal-to-noise ratio		The quality of a mass spectrum largely depends on the quality of the matrix's crystal.
Subject	Protein 4-30 kDa	Lipid and peptides 8 kDa	Lipid and peptides 5 kDa

A mixture of equal parts of polar organic solvent (acetonitrile or ethanol) and 0.1–0.5% trifluoroacetic acid (TFA) in water is commonly used as the first choice of solvent for any matrix. It has been reported that a mixture ratio >3 degrades the quality of the spectra.⁹ One cannot categorically describe which solvent is the best since the result of a solvent varies according to the type of tissue. For example, it has been reported that an ethanol mixture is the best solvent for a mouse liver section, whereas an acetonitrile mixture is the best one for a rat brain section.⁹ Further, even for the same type of tissue, certain signal peaks were observed only with an ethanol mixture solvent, whereas certain other signal peaks were detected only when the acetonitrile mixture solvent was used.⁹ Interestingly, those signal peaks could not be measured by using a three-in-one admixture (25:25:50 ethanol/acetonitrile/0.1% TFA in water). It has also been reported that a high concentration (>2%) of TFA can degrade a few signal peaks, which can be detected with a solvent composition with a lower TFA concentration, indicating that one should avoid a high TFA concentration. Therefore, such possibilities should be taken into consideration and solvent composition should be determined with respect to each experimental system. The matrix concentration is generally around 20–30 mg/mL.

One can adjust the thickness of the matrix coat covering a tissue section by varying the number of cycles of coating and drying.

Recently, we developed a new matrix based on nanoparticle technologies, which have a completely different chemical structure from benzoic or cinnamic acid derivatives.¹⁷ Derivatives of these matrices may enhance specific molecules in IMS.

21.3.8 Methods of Matrix Application

Several methods have been used for the matrix application: (1) immersing a tissue section quickly in a matrix solution, (2) spraying matrix solution onto a tissue section with an air brush, (3) putting small droplets of matrix solution onto a tissue section with an automatic pipetting device that can dispense picoliter volumes ($1\text{ pL} = 10^{-6}\mu\text{L}$) of reagents.^{4,5,7,15,16,18,19}

- (1) Immersion method—In this method, the analyte contained in the tissue may pass into the matrix solution, and information regarding the analyte's localization in the complex tissue structure may deteriorate due to the migration of the analyte, which in turn is caused by the excess matrix solution on the surface of a section. For these reasons, the immersion method is rarely used today.
- (2) Spray-coating method—This is the most frequently used method since it can easily coat the entire tissue with a matrix solution. The essence of this method is to maintain an equilibrium between the two rates—the rate at which a fine aerosol of the matrix solution produced by an air brush moisturizes the tissue section and the rate at which the matrix crystallizes as the solvent evaporates. Although the method is convenient, the difficulty of quantitatively controlling the mist size and spraying amount with a hand-operated air brush is undeniable and may cause problems in experimental reproducibility. If the matrix solution is in excess, the degradation of the information regarding the analyte's localization can occur. This is the same problem faced in the immersion method. On the other hand, if the spraying amount is deficient and the matrix solution dehydrates in air without moisturizing the tissue section, it can be difficult to achieve optimal co-crystallization of the analyte and matrix due to a lack of analyte extraction from the tissue section. Such coatings may result in irreproducible mass spectra and imaging. Alternatively, one can use automatic spraying devices such as ImagePrep™ (Bruker Daltonics K.K.), which may achieve the highest reproducibility independent of the experimenter's proficiency.
- (3) Droplet method—This is a pipetting operation that deposits the matrix solution onto a tissue section. It is an important technique along with the spray-coating method since it can give good reproducibility as well as higher ionization efficiency due to better extraction of analyte from a tissue section than is achieved with the spray-coating method.⁷

However, the droplet method has its own drawbacks, such as the degradation of information about the analyte's localization at a spot where the matrix droplet spreads. In general, a dispensed matrix droplet makes a spot of more than 1 mm in diameter on a tissue surface because of the lower limit of a pipetting volume of 500 nL with an ordinary micropipette. For such a large spot, it is insufficient to perform a precise high-resolution IMS. Therefore, technical improvements are needed to dispense the smallest droplets possible.

The Chemical Inkjet Printer (ChIP), an inkjet printer-like device which is equipped with a piezoelectric reagent-dispensing system, has been developed by Shimadzu Corporation. The ChIP can dispense picoliter volumes of matrix solution onto a tissue section, which has four printing heads and nozzles that have no contact with the tissue section surface.

For example, a spot with a diameter of 100–250 μm can be made on a target location of a tissue section by dispensing approximately 10 droplets of matrix solution of 87 pL with a diameter of approximately 55 μm each. Therefore, it can make a two-dimensional matrix-spot array on a surface of tissue sections at intervals of a hundred and several tens of micrometers. The adjacent spots are independent of each other, so there is no possibility of cross-contamination and thus, the distance between them defines the IMS image resolution (Figs. 21.4 and 21.5). On the other hand, the image resolution with the spray-coating method depends on the diameter of the laser beam of the mass spectrometer, which is at present around 10–100 μm . So, the droplet method with the automatic pipetting device can achieve an IMS image resolution that is in no way inferior to that obtained by the spray-coating method. The Acoustic Reagent Multispotter (ARM) is also

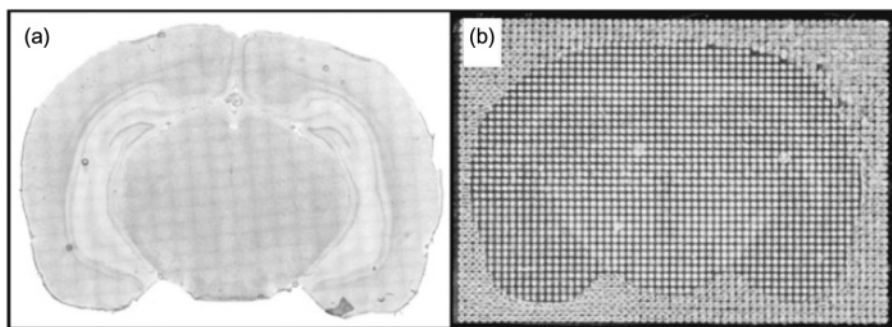


Figure 21.4 (a) Hematoxylin and eosin staining of rat brain coronal section, and (b) tissue section spotted with a synaptic acid matrix solution (250 μm intervals, 2500 spots in all) after the on-tissue trypsin digestion. Reprinted with permission from Groseclose et al.¹⁶

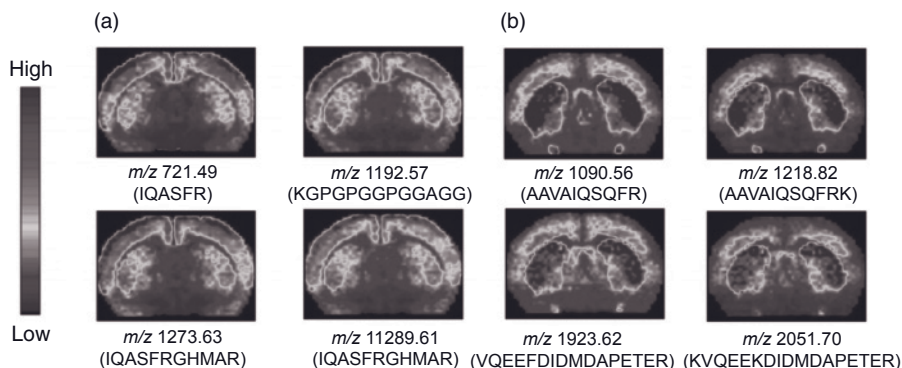


Figure 21.5 (a) Distribution of trypsin-induced peptides generated from the digestion of a 7.5-kDa protein, neurogranin, in a rat brain coronal section. (b) Distribution of trypsin-induced peptides generated from the digestion of the 6.7-kDa protein, PEP-19, in the rat brain coronal section. Reprinted with permission from Groseclose et al.¹⁶ See color insert.

known as an apparatus that has a unique nozzle-free ejector dispensing picoliter volumes of reagent.^{15,19}

- (4) **Spray-droplet method**—We developed a matrix-coating method that combines the two methods described above to spectacularly improve the signal intensity and the signal-to-noise ratio of mass spectra.⁷ The spray-droplet method first forms very fine matrix crystals on the surface of the tissue section by the spray-coating method, which plays the role of crystal nuclei for the subsequent droplet method to generate excellent homogeneous matrix crystals in the spot (Figs. 21.6–21.8).

21.3.9 Transfer Thin-Sliced Tissue Section onto the PVDF Membrane

The last sample preparation method for IMS is the transfer of a tissue section onto the PVDF membrane. Proteins in the section can be transferred onto the PVDF membrane and then analyzed on the membrane. The advantage of this method is that the enzyme can be digested for MSⁿ measurement, because the information on protein localization in the organization is fixed on the membrane.^{5,20} This technique can denature, reduce, and digest the proteins in the tissue section efficiently and remove the salt from the tissue. This increases the efficiency with which biological molecules are ionized, making it possible to obtain sensitive mass imaging spectra.

Another method is the denaturation of a tissue section with denaturant on the membrane. In this method, the frozen section is thawed and mounted on the membrane. The transferred membrane is washed with 70% ethanol to remove salt and lipid in the tissue and to fix the protein on the membrane. After that, denaturation is processed with the denaturant. Another method is

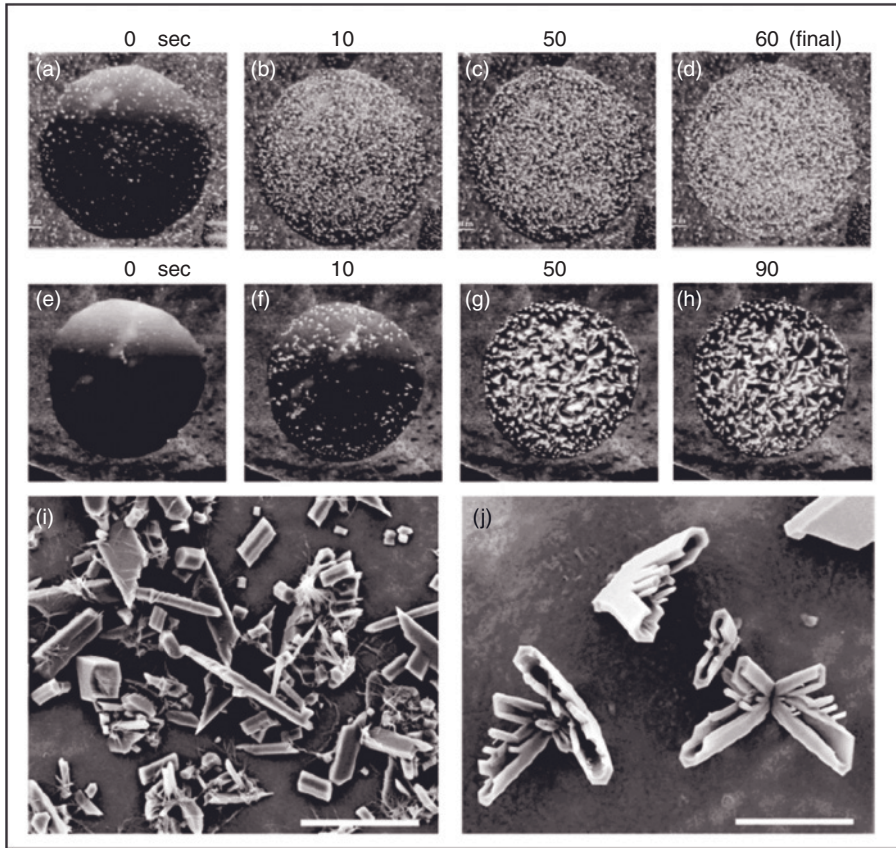


Figure 21.6 Time-lapse observation of synaptic acid crystal formation. The spray-droplet method forms very fine crystals inside and outside of the matrix drop (a–d), so that finer and more homogeneous crystals are generated (d) than those obtained by the droplet method (e–h). Observation with a scanning electron microscope of matrix crystals with the spray-droplet (i) and droplet methods (j). Reprinted with permission from Sugiura et al.⁷

to transfer a section from PVDF membrane to another membrane. Before the denaturation step, this method is the same as the first method. After that, however, proteins are transferred to a new membrane by a blotting technique. This method has the advantage of shortening the time required in the first method, since one can denature and reduce the proteins simultaneously at the time of the transfer. On the other hand, this method has the disadvantage in that it is difficult to retain a good shape of the tissue and to make fine samples, because air bubbles may cut into voided space between the tissue and membrane. Moreover, it is difficult to transfer proteins that have high molecular weights.

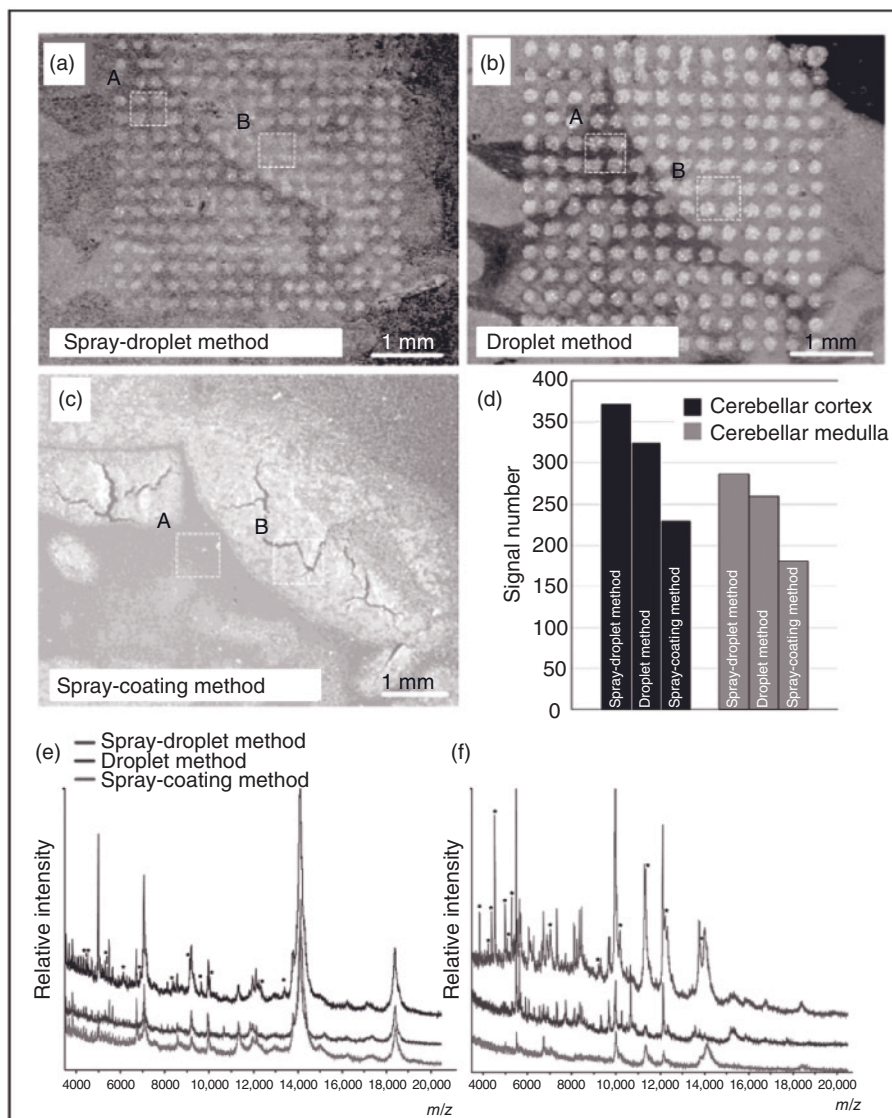


Figure 21.7 Comparison of mass spectra obtained from rat brain. Optical observation of microspotted tissue sections employing spray-droplet (a), droplet (b), and spray-coating (c) methods. Scale bar, 1.0mm. White squares (a–c) represent the cortex (A, d) and the medulla (B, e) of the cerebellum region, respectively. Accumulated mass spectra collected from each region are shown (d, e). In each spectrum, asterisks represent major unique signals for spectra using the spray-droplet method. The number of detected signals in the mass range of $2000 < m/z < 30,000$ from each region is shown (f). Reprinted with permission from Sugiura et al.⁷

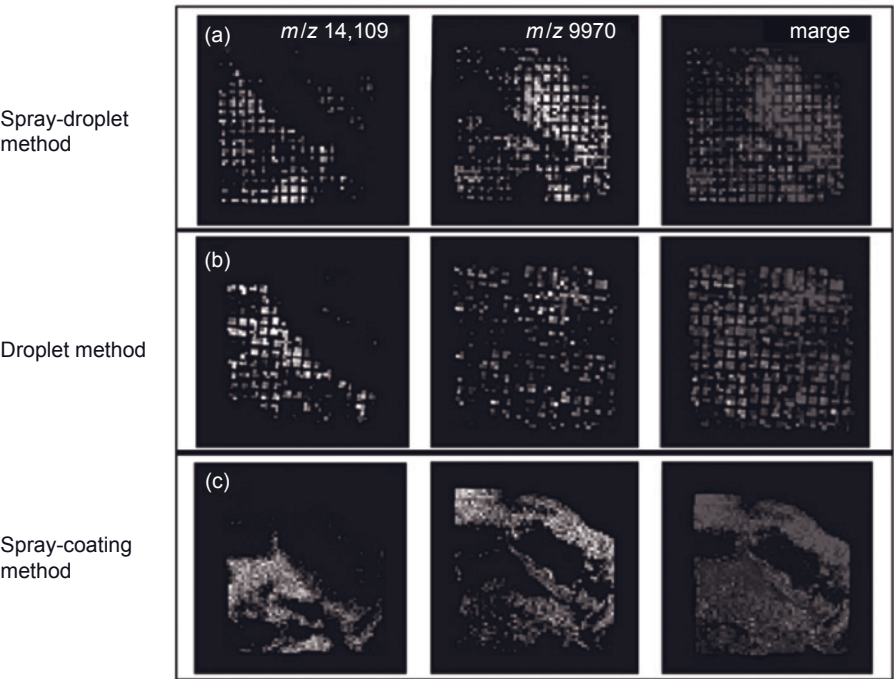


Figure 21.8 Comparison of IMS indicating protein distribution in the rat brain section corresponding to Figure 21.7 (a–c); the spray-droplet (a), droplet (b), and spray-coating (c) methods are used. Reprinted with permission from Sugiura et al.⁷ See color insert.

For the MS^n measurement of proteins, that is, the measurement of peptides, one has to denature and reduce proteins in the tissue samples, followed by enzyme digestion. Therefore, protein samples should be treated with trypsin after membrane transfer. Trypsin can be attached to the membrane and can be performed in the same steps as in the matrix coating method.

21.4 PROTEIN MAPPING ON A TISSUE SECTION BY IMS: SCRAPPER KNOCKOUT (KO) ANALYSIS

We utilized this technique to analyze *Scrapper* gene-deficient (SCR-KO) mice.²¹ SCRAPPER, a protein that we have recently reported, is localized at synapses in neurons. It is a ubiquitin E3 ligase that is involved in the decomposition of RIM (Rab3-interacting molecule) 1, an important regulator of synaptic plasticity, and thus regulates synaptic transmissions.²²

For imaging MS analysis, animals were sacrificed, and the extirpated brains were immediately frozen in powdered dry ice and stored at -80°C until needed. Briefly, fresh-frozen tissues were sliced into $5\text{-}\mu\text{m}$ -thin sections and mounted

on Indium Tin Oxide (ITO)-coated slide glasses (Bruker Daltonics). The dried tissues were then rinsed with 70% ethanol for 30s and were dehydrated using SpeedVac (Thermo Scientific, Yokohama, Japan). Sinapinic acid (SA) (25 mg/mL in 50% CH_3CN , 0.1% TFA) was used as the matrix and was uniformly sprayed over the tissue surface. All solutions were sprayed by a 0.2-mm nozzle caliber airbrush, Procon Boy FWA Platinum (Mr. Hobby, Tokyo, Japan). Tissue sections were analyzed using a MALDI-TOF/TOF-type instrument, Ultraflex II TOF/TOF (Bruker Daltonics), which was equipped with a 355 nm Nd:YAG laser with a 200 Hz repetition rate.

Prior to the comparison of wild-type (WT) and KO mice, we tested our methodology to demonstrate its ability to reveal heterogeneous protein expression patterns among the various brain regions in WT mice. As in Figure 21.9a, the mass spectra obtained from three different brain regions showed distinct protein expression patterns (Fig. 21.9a, arrowheads). To understand the heterogeneous patterns comprehensively, we performed principal component analysis (PCA) of mass spectra from some brain regions of interest, including the cerebral cortex, hypothalamus, and pons/medullary (Fig. 21.9a). PCA is a mathematical procedure that reduces a large set of variables to a small set of variables called principal factors, which are linear combinations of the original variables. PCA is helpful for identifying new meaningful underlying variables and for detecting clusters within multivariate data.^{23–26} We extracted the spectra from the cerebral cortex, hypothalamus, and pons/medullary, ranked the mass peaks in order of size, and determined the expression patterns of the top 210 peaks in total. According to the PCA results, the component scores of the spectra were plotted for PC1 (y-axis) and PC2 (x-axis). For each region, we found that the spectra of one region tended to be separated from those of the other two regions along the PC2 axis (Fig. 21.9b [left]). This means that the protein expression patterns of these three regions were statistically distinct from each other.

Furthermore, we evaluated which protein ions caused the separation on the plot, that is, the uniqueness of expression patterns. Figure 21.9b (right) plots the loading factor of each mass peak for PC1 (y-axis) and PC2 (x-axis). The mass peaks with characteristic loading factor values for PC2 (m/z of 5505, 9918, and 14130) were selected, and their ion images were reconstructed (Fig. 21.9c). As we expected, their distribution patterns were quite different from each other. By checking these images against HE-stained brain sections, it was revealed that substances with m/z of 5005, m/z of 9918, and m/z of 14130 were present in the cerebral cortex, hypothalamus, and pons/medullary, respectively.

Imaging MS and PCA successfully identified which proteins were expressed specifically in the WT-particular brain regions. Next, we applied imaging MS analysis to successfully compare the brains of WT mice and SCR-KO mice and to look for substances differentiating the two genotypes. We focused on several brain regions and extracted the mass spectra (Fig. 21.10). Some mass peaks in the mass spectra directly obtained from these regions were region- and genotype-specific (Fig. 21.10b, arrowheads). Then, using approximately

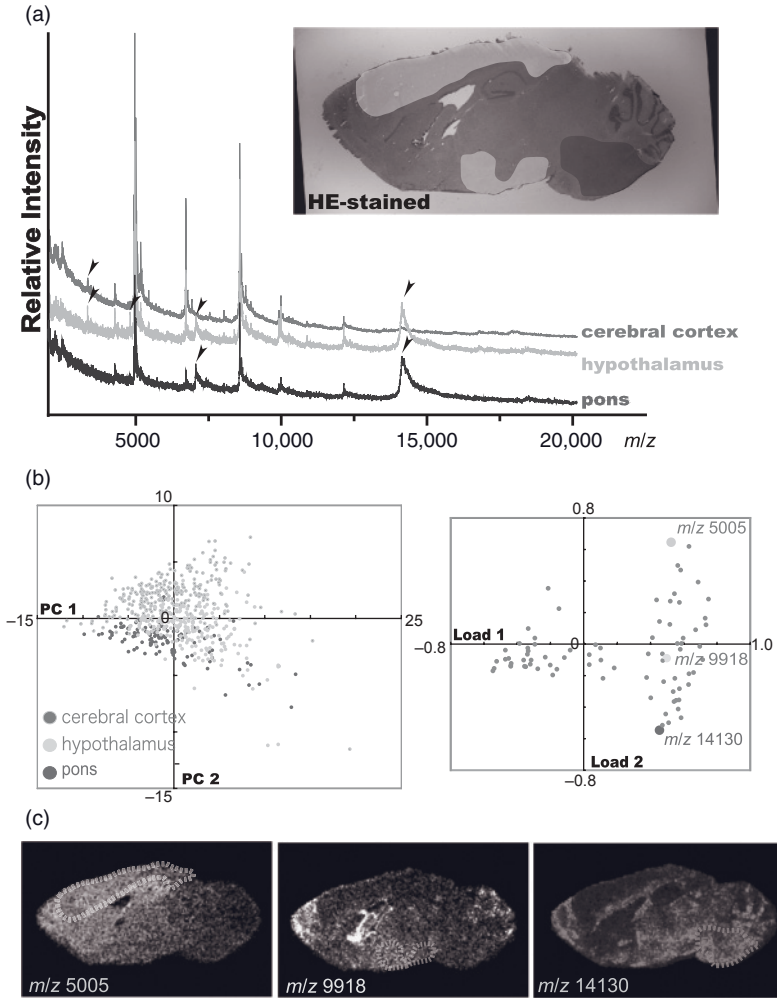


Figure 21.9 *In situ* proteomics and principal component analysis (PCA) of the mouse brain. (a) The mass spectra obtained from each region of mouse brain sections. A sagittal section of the WT mouse brain was analyzed by imaging MS. The observed regions are indicated on the HE-staining images. Signals specific to the regions are indicated by arrowheads. (b) Distribution of various brain components clustered by PCA (left) and the PCA scores plot (right). (c) Reconstructed images of the mouse brain analyzed by imaging MS. The signals show the distribution of substances with indicated m/z . Reprinted with permission from Yao et al.²¹ See color insert.

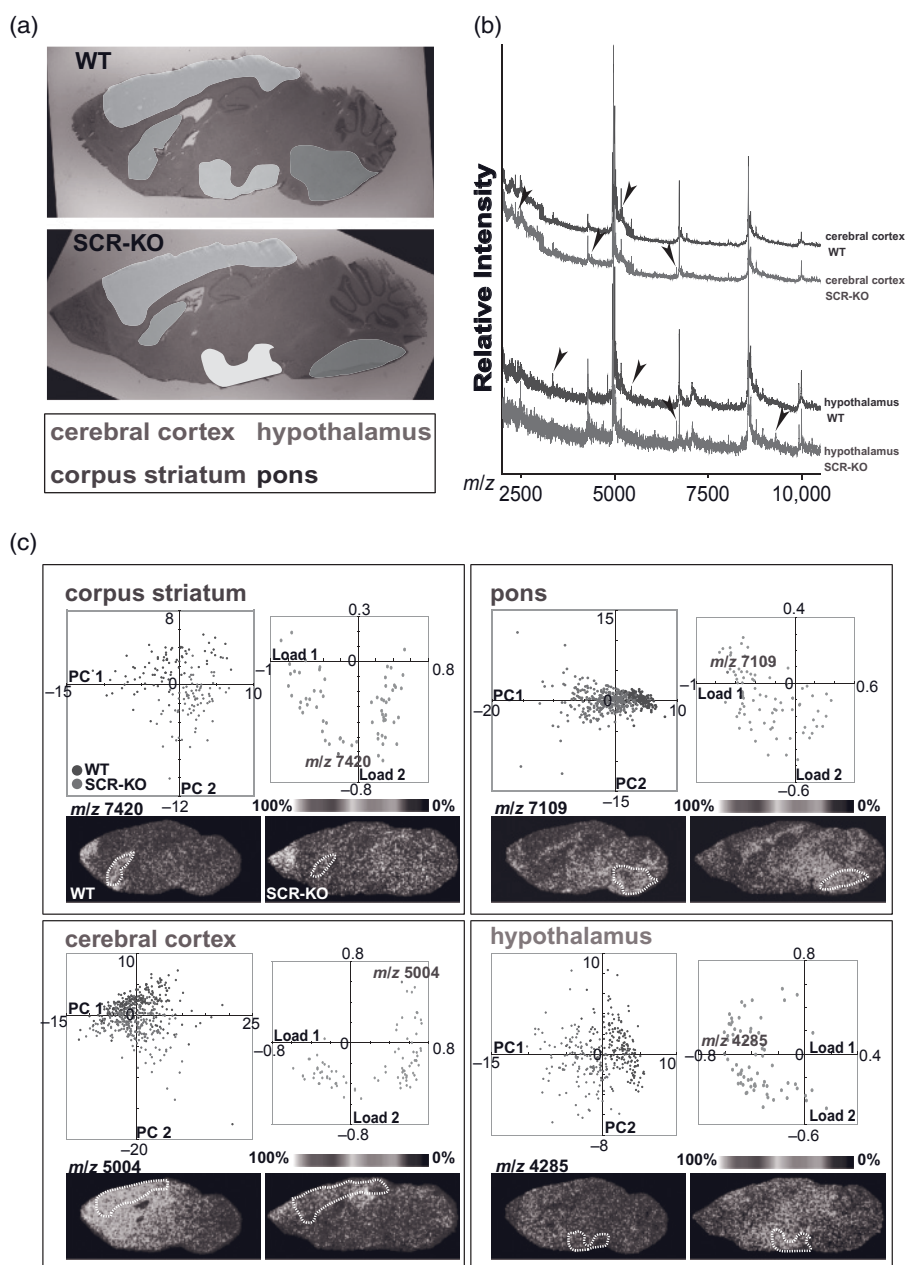


Figure 21.10 *In situ* proteomics of the SCR-KO mouse brain using imaging MS and PCA. (a) HE-staining images of the WT and SCR-KO mouse brain. The regions of focus in imaging MS analyses are indicated in colors. (b) Mass spectra obtained from each region of the WT or SCR-KO mouse brain sections. Specific signals of the regions are indicated by arrowheads. (c) Distributions of various brain components clustered by PCA (left spray graph; WT, blue; KO, red) and the PCA scores plot (right graph). The signal intensities of mass spectra of the substances with indicated m/z are shown in the reconstructed images of the mouse brain analyzed by imaging MS. Reprinted with permission from Yao et al.²¹ See color insert.

80 intense mass peaks detected in WT with KO, we performed PCA on the mass spectra in the four regions presented in color in Figure 21.10a. In each region, the protein expression patterns of two distinct genotypes differed in varying degrees, as seen in the spray graphs in Figure 21.10c. The procedures we employed clearly demonstrated that ions at m/z 7420 were absent in the corpus striatum, while they were commonly detected in the olfactory bulb. Also, ions at m/z of 5004 decreased in the cerebral cortex of the KO mouse. We found not only defects but also increased expression of proteins. PCA of the mass spectra from the pons/medullary and hypothalamus indicated that ions at m/z of 7109 and m/z of 4285, respectively, were increased in the KO mouse brain (Fig. 21.10c).

We conducted proteomic analysis of the KO mouse brain to identify proteins or peptides whose expression levels may change due to a lack of SCRAPPER. Imaging MS allowed us to statistically analyze location and expression intensities of many biomolecules and to extract molecules that exhibited region-specific expression. Groups of molecules whose expression patterns differed between WT mice and KO mice particularly attracted our attention.

21.5 CONCLUDING REMARKS

IMS is a new, developing technique to visualize biomolecule maps in tissue. IMS has opened a new frontier in medicine as well as in clinical applications. Lipids and low-molecular-weight compounds in tissue sections cannot be observed with conventional microscopic or electron microscopic techniques; therefore, no distribution map of these molecules in a tissue structure has been described in the scientific literature or in medical textbooks. However, IMS is bringing to light the characteristic distribution map of lipids (Fig. 21.11); this map made a major impact to lipid research.

Intractable diseases such as cancer and muscular dystrophy require personalized medicine that should consider the patient's individual pathological biochemistry (biochemical state of abnormal tissue), which can indicate the direct cause of disease. Therefore, a microscopic examination of tissue samples removed from the patient is generally performed. However, ordinary techniques of proteomic and metabolomic analyses cannot be applied to biopsies, since most of the samples are extremely small in order to minimize patient burden. When the IMS technique is used, the sample would be first flash-frozen and thick tissue sections would then be obtained in order to perform an accurate metabolomic analysis.^{20,27} Using this method, we have succeeded in discovering a molecular group specific to muscular dystrophy patients (unpublished). IMS is expected to become a standard method of reviewing clinical metabolomes, since its technique is simple and easy. We strongly believe that the mass microscope will be placed together with CT, PET, and MRI in hospitals in the near future.

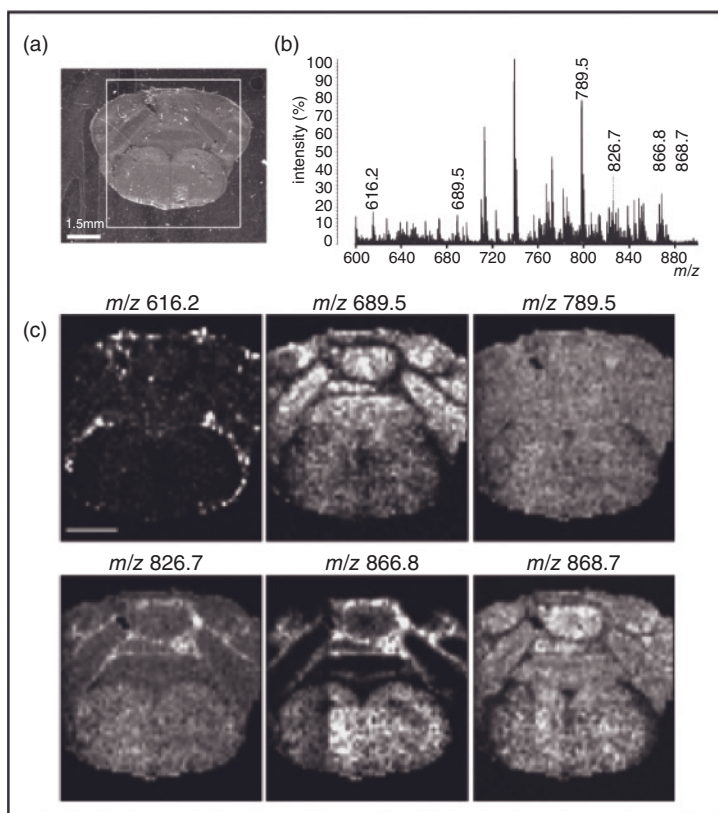


Figure 21.11 Characteristic lipid distribution in a rodent brain. Reprinted with permission from Shimma et al.¹³

REFERENCES

1. Shimma S, Setou M. Review of imaging mass spectrometry. *J. Mass. Spectrom. Soc. Jpn.* 2005; 53: 230–238.
2. Klinkert I, McDonnell LA, Luxembourg SL, et al. Tools and strategies for visualization of large image data sets in high-resolution imaging mass spectrometry. *Rev. Sci. Instrum.* 2007; 78: 053716.
3. Sugiura Y, Shimma S, Setou M. Thin sectioning improves the peak intensity and signal-to-noise ratio in direct tissue mass spectrometry. *J. Mass. Spectrom. Soc. Jpn.* 2006; 54: 45–48.
4. Shimma S, Furuta M, Ichimura K. Direct MS/MS analysis in mammalian tissue sections using MALDI-QIT-TOFMS and chemical inkjet technology. *Surf. Interface Anal.* 2006; 38: 1712–1714.
5. Shimma S, Furuta M, Ichimura K. A novel approach to in situ proteome analysis using a chemical inkjet printing technology and MALDI-QIT-TOF tandem mass spectrometer. *J. Mass. Spectrom. Soc. Jpn.* 2006; 54: 133–140.

6. Shimma S, Sugiura Y, Setou M. Applications of conductive film as a sample support material for direct tissue mass spectrometry (WP336). *J. Mass. Spectrom. Soc. Jpn.* 2006; 54: 210–211.
7. Sugiura Y, Shimma S, Setou M. Two-step matrix application technique to improve ionization efficiency for matrix-assisted laser desorption/ionization in imaging mass spectrometry. *Anal. Chem.* 2006; 78: 8227–8235.
8. Sugiura Y, Shimma S, Moriyama Y, et al. Direct analysis of cultured cells with matrix-assisted laser desorption/ionization on conductive transparent film. *J. Mass. Spectrom. Soc. Jpn.* 2007; 54: 25–31.
9. Schwartz S, Reyzer M, Caprioli R. Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. *J. Mass. Spectrom.* 2003; 38: 699–708.
10. Stoeckli M, Staab D, Schweitzer A. Compound and metabolite distribution measured by MALDI mass spectrometric imaging in whole-body tissue sections. *Int. J. Mass Spectrom.* 2006; 260: 195–202.
11. Altelaar A, Klinkert I, Jalink K, et al. Gold-enhanced biomolecular surface imaging of cells and tissue by SIMS and MALDI mass spectrometry. *Anal. Chem.* 2006; 78: 734–742.
12. Chaurand P, Schwartz S, Billheimer D, et al. Integrating histology and imaging mass spectrometry. *Anal. Chem.* 2004; 76: 1145–1155.
13. Shimma S, Sugiura Y, Hayasaka T, et al. Mass imaging and identification of biomolecules with MALDI-QIT-TOF-based system. *Anal. Chem.* 2008; 80: 878–885.
14. Lemaire R, Wisztorski M, Desmons A, et al. MALDI-MS direct tissue analysis of proteins: improving signal sensitivity using organic treatments. *Anal. Chem.* 2006; 78: 7145–7153.
15. Aerni H, Cornett D, Caprioli R. Automated acoustic matrix deposition for MALDI sample preparation. *Anal. Chem.* 2006; 78: 827–834.
16. Groseclose M, Anderson M, Hardesty W, et al. Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. *J. Mass. Spectrom.* 2007; 42: 254–262.
17. Moritake S, Taira S, Sugiura Y, et al. Magnetic nanoparticle-based mass spectrometry for the detection of biomolecules in cultured cells. *J. Nanosci. Nanotechnol.* 2009; 9: 169–176.
18. Lemaire R, Tabet J, Ducoroy P, et al. Solid ionic matrixes for direct tissue analysis and MALDI imaging. *Anal. Chem.* 2006; 78: 809–819.
19. Meistermann H, Norris J, Aerni H, et al. Biomarker discovery by imaging mass spectrometry: transthyretin is a biomarker for gentamicin-induced nephrotoxicity in rat. *Mol. Cell. Proteomics* 2006; 5: 1876–1886.
20. Shimma S, Sugiura Y, Hayasaka T, et al. MALDI-based imaging mass spectrometry revealed abnormal distribution of phospholipids in colon cancer liver metastasis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2007; 855: 98–103.
21. Yao I, Sugiura Y, Matsumoto M, et al. In situ proteomics with imaging mass spectrometry and principal component analysis in the Scrapper-knockout mouse brain. *Proteomics* 2008; 8: 3692–3701.
22. Yao I, Takagi H, Ageta H, et al. SCRAPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. *Cell* 2007; 130: 943–957.

23. Altelaar A, Luxembourg S, McDonnell L, et al. Imaging mass spectrometry at cellular length scales. *Nat. Protoc.* 2007; 2: 1185–1196.
24. McCombie G, Staab D, Stoeckli M, et al. Spatial and spectral correlations in MALDI mass spectrometry images by clustering and multivariate analysis. *Anal. Chem.* 2005; 77: 6118–6124.
25. Denkert C, Budczies J, Kind T, et al. Mass spectrometry-based metabolic profiling reveals different metabolite patterns in invasive ovarian carcinomas and ovarian borderline tumors. *Cancer Res.* 2006; 66: 10795–10804.
26. Lapolla A, Ragazzi E, Andretta B, et al. Multivariate analysis of matrix-assisted laser desorption/ionization mass spectrometric data related to glycoxidation products of human globins in nephropathic patients. *J. Am. Soc. Mass. Spectrom.* 2007; 18: 1018–1023.
27. Shimma S, Setou M. Mass microscopy revealed the distinct localization of heme B(m/z 616) in colon cancer liver metastasis. *J. Mass. Spectrom. Soc. Jpn.* 2007; 55: 145–148.

SYMBIOSIS OF IMMUNOHISTOCHEMISTRY AND PROTEOMICS: MARCHING TO A NEW ERA

SHAN-RONG SHI, BRIAN M. BALGLEY, and CLIVE R. TAYLOR

Rapid development of mass spectrometry-based proteomic techniques in recent years has contributed to the development of a postgenomic era in biomedical research.¹⁻³ A number of studies of protein extraction from formalin-fixed, paraffin-embedded (FFPE) tissue sections have demonstrated that thousands of proteins are detectable in extracts using mass spectrometry.⁴⁻⁸ Our recent NIH funded research, entitled “Validation and Quantification of FFPE Antigen Retrieval by Proteome Analysis,” has involved close collaboration with chemists specializing in proteomics, and has demonstrated that many of the proteins identified in extracts prepared from FFPE tissue sections, utilizing an antigen retrieval (AR)-based protocol, with Tris-HCl buffer containing 2% SDS under boiling conditions, overlap with those extracted from fresh tissue⁵ (see Chapter 20 for detail). This AR-based protocol has recently been validated by a comparative study with several commercial kits, to be an optimally effective protocol for protein extraction from FFPE tissue.⁹ Ono et al.¹⁰ demonstrated overexpression of heat shock protein 27 in squamous cell carcinoma of the uterine cervix based on a proteomic analysis using proteins extracted from archival FFPE tissues by the heat retrieval protocol developed at our laboratory. Also, based on the heat-induced AR principle, Nirmalan et al.¹¹ heated FFPE tissue sections at 105°C for 20 min, or fresh-frozen tissue sections at 100°C for 5 min in 150 μ L 2% SDS-containing Laemmli buffer, in order to achieve efficient intact protein extraction for Western blotting analysis.

In one experiment, using the AR-based protocol for protein extraction from microdissected FFPE human tissue section, a total of 14,478 distinct

peptides were identified, leading to the identification of 2733 proteins, among which 83% of the proteins overlapped with those obtained from fresh tissue of the same patient.⁶ We also conducted a limited comparative study of proteins identifiable in FFPE sections by IHC, revealing an excellent correlation between IHC analysis and mass spectrometry.⁸ Further studies are ongoing, combining AR technique and proteomics, leading toward a future aim of integration of IHC and proteomics. The peptides identified by mass spectrometry in preparations retrieved from FFPE archived tissues have the potential to form a library of peptide epitopes that are known to be retrievable from “routinely” fixed tissue. Such libraries may help to guide more strictly targeted studies, such as liquid chromatography–multiple reaction monitoring–mass spectrometry (LC-MRM-MS), which track specific peptides in a high-throughput and high-sensitivity approach. The peptide epitopes identifiable in extracts from FFPE tissue may also help to guide the selection of epitopes for the generation of novel antibodies for diagnostic or therapeutic purposes.

Mass spectrometry-based proteomics offers avenues for discovery by virtue of its massive multiplexing capability, facilitating the detection of many thousands of proteins from a single, standard, FFPE tissue section. These studies can guide pathologists in the selection of targets that are expected to be demonstrable by IHC in adjacent FFPE tissue sections of similar cell types. Discovery may be conducted in a prospective or retrospective manner. For example, prospective comparisons may be performed of morphologically defined disease types in an effort to define potential molecular markers to be used for diagnostic purposes. Also, retrospective comparisons may be performed of biopsies collected from clinical trials in an attempt to define differences in protein status or expression that may correlate with therapeutic response. These and other approaches are likely to significantly impact translational medicine.

In 2004, Melle et al.¹² proposed a technical triad of microdissection, proteomic techniques, and IHC that provides an integrated approach for characterization of cancer biomarkers. In recent years, a growing body of literature pertaining to this combined approach has accumulated. Table 22.1 shows a few selected examples under this topic.

In Chapter 21, Mukai and Setou summarize recent achievements of imaging mass spectrometry (IMS), and detail technical issues pertaining to sample preparation for IMS. In other studies, techniques developed for direct tissue profiling by IMS on fresh-frozen tissue sections have visualized 500 to 1000 protein signals, ranging in the molecular weight from 2000 to over 200,000 over a defined tissue area.¹⁶ In 2006, Ernst et al.¹⁷ created a novel term “proteohistography” to describe a surface-enhanced laser desorption/ionization “time-of-flight” (TOF) mass spectrometry method using a ProteinChip System with laser microdissection of specially marked areas on frozen tissue sections. Their findings pointed out a potential way of direct analysis of tissue based on mass spectrometry, having both high sensitivity and spatial resolution,

TABLE 22.1 Some Recent Published Data Pertaining to Combined Use of Proteomics and IHC

Reference	Purpose	Proteomics	IHC	Conclusion
Melle et al. ¹²	Characterization of cancer markers for head and neck tumors	2-D gel isolated S100A8 and S100A9 from LCM dissected frozen tissue sections, identified by MS analysis	Relevance of these biomarkers was evaluated by IHC, positive tissue areas were reanalyzed on ProteinChip arrays to confirm them.	A technical triad: microdissection, proteomics, and IHC opens up the possibility to identify and characterize tumor markers.
Xu et al. ⁸	To validate the quality of proteins extracted from FFPE tissues of livers using AR technique via shotgun-based proteomic analyses	To demonstrate capability of a capillary isotachophoresis (CITP)-based proteomic platform for shotgun proteomic analysis using FFPE tissues	From proteomic-identified a total of 4098 distinct identifications, selected 17 markers for IHC staining for characterization.	Development of an integrated approach to the proper preparation of samples for dual analysis: MS and IHC is critical for cancer management.
Uemura et al. ¹³	To develop a prognostic biomarker for esophageal squamous cell carcinoma (ESCC)	LCM-separated cell populations from frozen tissue sections of ESCC, using 2D-difference gel electrophoresis to find out 22 protein spots, further identified in 18 distinct gene products by MS	Transglutaminase (TGM3) 3 was identified as biomarker candidate, and further demonstrated by IHC in 76 ESCC cases, and found significant difference of survival rate for TGM3 positive and negative cases.	This research approach of combining use of LCM, proteomic analysis, and IHC contributes to accurate protein expression profiling and provides novel strategies for ESCC treatment.

TABLE 22.1 Continued

Reference	Purpose	Proteomics	IHC	Conclusion
Liu et al. ¹⁴	To study the mechanism of adaptation to metabolic stress in gastric cancer cells	2-D PAGE coupled with ESI-Q-TOF MS/MS analysis demonstrated triiodothyronine (T ₃) and overexpression of hypoxia-induced factor (HIF) in gastric cancer.	IHC and electrochemiluminescence immunoassay were used for further demonstration.	This combining study based on proteomics and IHC provides evidence for alteration of metabolic proteins in gastric cancers.
Kraljevic Pavelic et al. ¹⁵	To study the proteomic profiling of Dupuytren's disease (DD)	2D-PAGE and MALDI-TOF/TOF system analysis were used based on comparison between diseased and unaffected patients to create a protein-protein interactions map.	Based on proteomic analysis, IHC was performed for randomly selected four cases of DD using markers of ERBB-2, p53, p-JNK, and IGF-1R in FFPE tissue sections with AR technique.	Using this combining approach revealed the role of oxidative stress, autocrine deregulation, and activation of Akt in DD progression.

LCM, laser captured microdissection; ESI, electrospray ionization; PAGE, polyacrylamide gel electrophoresis; IGF-1R, insulin-like growth factor receptor 1.

although only 215 distinct points could be detected for a fresh tissue sample. The key finding is that the method as described by Ernst and colleagues was able to correlate the “protein map” with the defined spatial areas of tissue, which may be helpful for future combination of IHC methods with proteomics.

In a separate study, a protocol for Matrix-assisted laser desorption-ionization (MALDI) imaging mass spectrometry (IMS) has been proposed.¹⁸ This IMS technique provides a new approach to visualize spatial distribution of thousands of molecular species, including peptides, proteins, and their metabolites in two- or three-dimensional levels. This approach may also provide a straightforward method of determining the tissue distribution of multiple peptides or proteins in a quantitative manner.¹⁸ Chu et al.¹⁹ reported a nondestructive molecular extraction method to obtain proteins from a single FFPE or frozen tissue section, without destroying the tissue morphology, such

that they were able subsequently to perform IHC staining on the same section, in order to correlate both IHC and protein analysis results. This is a very interesting concept, but we have been unable to find evidence of validation of the method by other investigators, following the original publication 3 years ago.

Uhlen et al.²⁰ constructed a comprehensive, antibody-based “protein atlas” of expression and localization profiles in 48 normal human tissues and 20 various cancers. Their research strategy was based on a recombinant DNA technique for developing monospecific antibodies to selected protein epitope signature tags that represent unique regions for each target protein. Using a tissue microarray technique, it was possible to display the findings in a fully comprehensive “protein atlas” of expression in different tissues, again providing a promising basis for future studies.

For more than two decades, IHC has been a critical tool in molecular morphology, and is potentially the most objective approach for diagnostic pathology, although the full potential has yet to be reached, in terms of combining localization with cellular and subcellular quantification of specific analytes. IHC has the unique advantage of sharply localizing certain proteins in an exact cell/tissue component. IHC reveals the distinct morphological distribution of the tested protein *in situ*, thereby providing a scientific demonstration of protein expression in normal or abnormal tissue structures.²¹ Seeing is believing, and for this reason microscopy remains as a very valuable tool to demonstrate cellular and subcellular localization of numerous proteins, for research and for diagnosis.

However, IHC as a practical method continues to evolve with increasing demands for standardization, and for true quantification of protein analytes by weight, in the context of their cellular microenvironment. Further studies combining proteomics by mass spectrometry and IHC are likely to lead to the refinement of both methods in the analysis of FFPE tissues. The end result may be the creation of a broader field that defines and quantifies protein expression at a cellular level, incorporating the advantages of the wide spectrum of proteins demonstrable by mass spectrometry and the precise localization offered by IHC.

ACKNOWLEDGMENT

This article was supported by NIH Grant R41 CA122715.

REFERENCES

1. Washburn MP, Wolters D, Yates JR III. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 2001; 19: 242–247.

2. Chen J, Balgley BM, DeVoe DL, et al. Capillary isoelectric focusing-based multi-dimensional concentration/separation platform for proteome analysis. *Anal. Chem.* 2003; 75: 3145–3152.
3. Fang X, Yang L, Wang W, et al. Comparison of electrokinetics-based multidimensional separations coupled with electrospray ionization-tandem mass spectrometry for characterization of human salivary proteins. *Anal. Chem.* 2007; 79: 5785–5792.
4. Prieto DA, Hood BL, Darfler MM, et al. Liquid Tissue™: proteomic profiling of formalin-fixed tissues. *BioTechniques* 2005; 38: S32–S35.
5. Shi S-R, Liu C, Balgley BM, et al. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. *J. Histochem. Cytochem.* 2006; 54: 739–743.
6. Guo T, Wang W, Rudnick PA, et al. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. *J. Histochem. Cytochem.* 2007; 55: 763–772.
7. Hwang S-I, Thumar J, Lundgren DH, et al. Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. *Oncogene* 2007; 26: 65–76.
8. Xu H, Yang L, Wang W, et al. Antigen retrieval for proteomic characterization of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2008; 7:1098–1108.
9. Fowler CB, Cunningham RE, O’Leary TJ, et al. “Tissue surrogates” as a model for archival formalin-fixed paraffin-embedded tissues. *Lab. Invest.* 2007; 87: 836–846.
10. Ono A, Kumai T, Koizumi H, et al. Overexpression of heat shock protein 27 in squamous cell carcinoma of the uterine cervix: a proteomic analysis using archival formalin-fixed, paraffin-embedded tissues. *Hum. Pathol.* 2009; 40: 41–49.
11. Nirmalan NJ, Harnden P, Selby PJ, et al. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using western blotting. *J. Pathol.* 2009; 217: 497–506.
12. Melle C, Ernst G, Schimmel B, et al. A technical triade for proteomic identification and characterization of cancer biomarkers. *Cancer Res.* 2004; 64: 4099–4104.
13. Uemura N, Nakanishi Y, Kato H, et al. Transglutaminase 3 as a prognostic biomarker in esophageal cancer revealed by proteomics. *Int. J. Cancer* 2009; 124: 2106–2115.
14. Liu R, Li Z, Bai S, et al. Mechanism of cancer cell adaptation to metabolic stress: proteomics identification of a novel thyroid hormone-mediated gastric carcinogenic signaling pathway. *Mol. Cell. Proteomics* 2009; 8: 70–85.
15. Kraljevic Pavelic S, Sedic M, Hock K, et al. An integrated proteomics approach for studying the molecular pathogenesis of Dupuytren’s disease. *J. Pathol.* 2009; 217: 524–533.
16. Chaurand P, Sanders ME, Jensen RA, et al. Proteomics in diagnostic pathology, profiling and imaging proteins directly in tissue sections. *Am. J. Pathol.* 2004; 165: 1057–1068.

17. Ernst G, Melle C, Schimmel B, et al. Proteohistography—direct analysis of tissue with high sensitivity and high spatial resolution using ProteinChip technology. *J. Histochem. Cytochem.* 2006; 54: 13–17.
18. Andersson M, Groseclose MR, Deutch AY, et al. Imaging mass spectrometry of proteins and peptides: 3D volume reconstruction. *Nat. Methods* 2008; 5: 101–108.
19. Chu W-S, Liang Q, Liu J, et al. A nondestructive molecule extraction method allowing morphological and molecular analyses using a single tissue section. *Lab. Invest.* 2005; 85: 1416–1428.
20. Uhlén M, Björling E, Agaton C, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol. Cell. Proteomics* 2005; 4: 1920–1932.
21. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*, 3rd edition. Philadelphia: Elsevier Saunders, 2005.

APPENDIX

RELATED LABORATORY PROTOCOLS

THE “TEST BATTERY” APPROACH OF ANTIGEN RETRIEVAL (AR) TECHNIQUE

Principle

As described in Chapter 1, the “test battery” approach is a pretest to establish an optimal AR protocol, based on the fact that two major factors (heat and pH) influence the achievement of a satisfactory result of AR-immunohistochemistry (IHC). As indicated in Table 1.1, a total of nine slides are required to test three conditions of heating temperature, and three different pH values of the AR solutions. Practically, it may be performed in simpler ways as shown in Table 1.2. The following suggested protocol is based on our experience.

Materials and Reagents

- Microwave (MW) oven. Various domestic MW ovens with an output power around 1000W are commonly used for AR-IHC worldwide, although numerous commercial MW ovens have been designed with controlled temperature.
- Other heating equipment. Autoclave used for sterilization can be used to achieve superheating condition at 120°C. For higher temperature heating, a domestic pressure cooker, or a plastic steamer may be used. Some commercial laboratory pressure cookers have been designed for AR-IHC with controlled temperature. A water bath can be used to achieve lower temperature heating condition.
- Slide container. A plastic Coplin jar was used at the first experiment of AR-IHC in early 1990s. It is still used when testing a few slides. Recently, larger plastic containers are used to contain more slides and AR solutions.

- AR solutions. The AR solution is the solution used to immerse slides during the heating process. It is critical to avoid drying slides when doing AR treatment. The pH value of AR solution is the most important factor that influences the IHC result. Some chemicals may also play a role to improve the result of AR-IHC as described in Chapter 1.

Procedure Suggested

- The “test battery” approach is usually used for a new antibody as the first use in a lab. To begin with this new antibody, it is helpful to read the specification sheet of the manufacturer, and to review literature to ensure its application of IHC, particularly if AR was used previously.
- If this antibody was successfully used, to repeat the protocol of AR-IHC with certain tested tissue slides may be necessary to demonstrate the IHC result. If IHC staining result is not satisfactory, further steps are followed by testing various AR solutions with different pH. For convenience, try to use commonly used solutions such as citrate buffer of pH 6.0, Tris-HCl buffer plus EDTA at pH 8 or 9, 0.05% citraconic anhydride at pH 7.4. In some cases, low pH of 1–2 (acetic buffer or other type of buffer solutions) may be tested in order to find out the optimal pH value that produces the best IHC result.
- In order to achieve the best result: strong positive signal with clean background, it may need to select the best pH value obtained for various heating conditions to establish the optimal AR protocol. Particularly, when weak positive signal or higher background staining result appears, to monitor optimal heating condition is critical. In some situations, to tune up concentration of antibody and other reagents may also be required to reach the best result.
- In very few cases, a combination of heat treatment with other retrieval approach such as enzyme digestion may be helpful.

ANTIGEN RETRIEVAL PROTOCOL WITH USE OF CITRACONIC ANHYDRIDE SOLUTION

Principle

As documented by numerous publications, the key factor of heat-induced AR is high temperature heating formalin-fixed, paraffin-embedded (FFPE) tissue sections in water solution. For some antigens, certain optimized pH value of the AR solution and/or adjusted temperature may be required to reach the best result by the use of the test battery approach mentioned above. In 2005, Namimatsu et al. reported a novel AR solution containing 0.05% citraconic anhydride, pH 7.4, for heating FFPE tissue sections at 98°C for 45 min to

achieve better IHC results than the conventional solutions, including the most commonly used citrate buffer of pH 6.0 (see Chapter 1 for detail). According to Namimatsu et al, a lower temperature of 98°C is recommended to achieve stronger signal and cleaner background. In our hands, not all antigen/antibody pairs show the same satisfactory results of IHC, although most showed better results when using this novel protocol for AR-IHC.

Materials and Reagents

- To maintain a heating condition under boiling point such as 98°C, a laboratory water bath with controlled temperature, or other heating equipment that may provide lower temperature heating condition may be adopted.
- Other equipment as described above, for fewer FFPE tissue slides, a coplin jar may be used; if more slides are tested, a larger container is required.
- A 0.05% citraconic anhydride solution (pH 7.5).

AR Methods

- Set the slide container containing 0.05% citraconic anhydride solution (pH 7.5) in a water bath that has been heated to reach the designed heating temperature (98°C). Keep all tissue sections being immersed in the AR solution to avoid drying for 45 min. Check the heating condition regularly to maintain optimal temperature in the whole AR treatment.
- A boiling that heats FFPE tissue sections in 0.05% citraconic anhydride solution for 10–15 min may achieve identical IHC results as that obtained by heating at 98°C for most antibodies (90%); however, nonspecific background may increase.
- After heating process, wash slides with phosphate buffer solution followed by IHC staining procedure.

DNA/RNA AND PROTEIN EXTRACTION FROM FFPE TISSUE SECTIONS BASED ON AR PRINCIPLE (PROVIDED BY CHENG LIU, HT)

Principle

Based on the similarity of formalin-induced chemical modification between nucleic acids and proteins, the efficiency of heating protocols for DNA/RNA extraction has been demonstrated (see Chapter 3 for detail). Basic AR principle including heating condition and pH value of AR solution as well as certain chemicals may play roles to establish optimal protocols.

Materials and Reagents

- Basic laboratory equipment: centrifuge with cooling condition control at 4°C, heat block, pH meter, eppendorf BioPhotometer, microtube, and so on.
- Buffer solution: Britton and Robinson type of buffer solution is made of 28.6mM of each chemical as follows: citrate acid, KH_2PO_4 , H_3BO_3 , and diethylbarbituric acid that can be monitored into a wide spectrum of pH values ranging from pH 2.0 to 12.0. The optimal pH value for RNA extraction is pH 7.4. For protein extraction, a Tris-HCl buffer containing 2% SDS at pH 7 or pH 9 is optimal heating retrieval solution.
- Other major reagents: TRIzol LS reagent (Invitrogen Co., Carlsbad, CA), a commonly used commercial reagent for RNA extraction from cell/tissue sample that contains phenol and guanidine thiocyanate; 0.1M sodium hydroxide (NaOH) solution is used for DNA extraction.

Protocol for DNA Extraction from FFPE Tissue Sections

1. Cut section $10\mu\text{m} \times 3$ and put into a microtube.
2. Centrifuge the microtube to spin down tissue sections.
3. Add 500 μL 0.1M NaON solution into the microtube.
4. Heat the microtubes at 100°C for 20min.
5. Cool down for 5min.
6. Add 500 μL of phenol:chloroform:isopropanol alcohol (25:24:1), mixed by vortex.
7. Centrifuge at 12,000rpm for 10min.
8. Transfer the supernatant to fresh microtube, add 1 vol of chloroform to each microtube, mixed by vortex.
9. Centrifuge at 12,000rpm for 5min.
10. Transfer the supernatant to a fresh microtube.
11. Add 0.1 vol of 3M sodium acetate, mixed by vortex.
12. Add 1 vol of isopropanol, mixed by vortex, and incubated at -20°C overnight.
13. Centrifuge 12,000rpm for 20min at 4°C.
14. Discard supernatant.
15. Add 75% ethanol 500 μL , mixed by vortex.
16. Centrifuge 12,000rpm for 20min at 4°C.
17. Discard the supernatant.
18. Dry the microtube in a fume hood.
19. Dissolve in 50 μL distilled water.
20. Measure the extracted DNA amount.

Note: A simplified protocol of DNA extraction from FFPE tissue may be modified by using heat treatment alone without subsequent purification steps as a one-step protocol: boiling the FFPE tissue sections in alkalin solution (0.1 M NaOH) for 20 min, cooled down for 15 min, and 0.2 μ L of the retrieval solution is aspirated for PCR test and the remaining sample stored at 4°C for further use.

Protocol for RNA Extraction from FFPE Tissue Sections

1. Cut 10 μ m $\times$ 4 sections and put in microtube.
2. Add 1.0 mL of octane (Sigma, St. Louis, MO), mixed by vortex for 10 s at maximum speed.
3. Add 0.075 mL methanol and mix by vortex.
4. Remove upper layer of octane, then remove methanol.
5. Dry under hood for 2–3 min.
6. Add 500 μ L of Briton and Robinson buffer at pH 7.4.
7. Heat the tube at 100°C for 20 min.
8. Cool down for 5 min.
9. Add 0.3 mL of Trizol LS reagent, mixed by vortex, and incubated at room temperature for 15 min.
10. Add 0.2 mL chloroform, mixed by vortex, incubated at room temperature for 15 min.
11. Centrifuge at 12,000 rpm for 15 min at 4°C.
12. Transfer the aqueous supernatant to a fresh microtube.
13. Add 0.4 mL 2-propanol, mixed by vortex, and incubated at –20°C overnight.
14. Centrifuge at 12,000 rpm for 20 min at 4°C
15. Discard the supernatant. Wash pellet with 75% ethanol (0.8 mL)
16. Centrifuge at 12,000 rpm at 4°C for 15 min.
17. Discard the supernatant.
18. Dry in the hood (20 min)
19. Dissolve in diethylpyrocarbonate (DEPC)-distilled water.
20. Measure the total amount of RNA extracted from FFPE tissue sections.

Protocol for Protein Extraction from FFPE Tissue Sections

1. Cut 5 FFPE tissue sections (10 μ m each), put them in a microtube.
2. Add 1 mL Octane, mixed by vortex for 10 sec, followed by adding 0.075 mL methanol and vortexing once more for deparaffinization.
3. Centrifuge at 12,000 rpm for 10 min.

4. Discard the upper layer of Octane and methanol, leave the residuum dried in a hood for 2–3 min.
5. Add 50 μ L of 20 mM Tris–HCl buffer (pH 7 or 9) containing 2% SDS, heating at 100°C on a heat block for 20 min.
6. Follow by incubation at 60°C in an incubator for 2 h.
7. If visible tissue debris is found in the microtube, a 23G1 Precision Glide Needle (Becton-Dickinson, Franklin Lakes, NJ) with a 1 mL syringe is used to break down the cells through suctioning, until the solution becomes of clear appearance. This procedure may take 15–30 min.
8. Centrifuge at 12,000 rpm for 10 min, transfer the supernatant to a fresh microtube.
9. Measure the total amount of protein.

SELECTED PROTOCOLS OF CELL SAMPLE PREPARATION FOR CYTOPATHOLOGY (DATA PROVIDED BY CHIARA SUGRUE, MBA, MS, SCT [ASCP], LONG ISLAND JEWISH MEDICAL CENTER)

Introduction

With the development of imaging-guided fine needle aspiration, cytopathology laboratories have experienced dramatic increase in the types and amounts of specimens submitted for diagnosis. Different kinds of specimen needs different techniques to handle and process in order to achieve optimal results. Gary W. Gill said “Cytopreparation is a one-time investment that pays multiple dividends.” Accurate cytologic interpretation and utilization of ancillary studies depend on the quality of cytopreparation. As mentioned in Chapter 13, the application of IHC in cytopathology has lagged behind the level of use in histopathology, partially due to differences in cell sample preparation. Currently, numerous protocols of cell sample preparation are used in a variable fashion without a universal standardized protocol. The following selected protocols pertaining to cell sample preparation used in cytopathology are introduced as an example to provide reference materials for brief view from one medical center. It may be helpful for further development and standardization of cell sample preparation.

Materials and Instruments

Centrifuge tubes 50 mL, glass slides and coverslips (size 24 $\times$ 40), and mounting media, standard plastic pipettes, rubber gloves, pencil or permanent marking pen, CytoLyt® (Cytoc Co., Boxborough, MA), PreservCyt® (Cytoc Co., Londonderry, NH), centrifuge, Vortex machine, ThinPrep Processor 2000, Automatic Stainer, Automated Coverslipper, PreservCyt Solution vial, TransCyt Filter for non-gynecologic specimen (blue type), 95% ethyl alcohol for fixation, Cytospin® (ThermoFisher Scientific, Waltham, MA) chamber, Cytospin filter paper, Cytospin slide clip.

BODY FLUID CELL SAMPLE PREPARATION

Principle

Body fluid specimens will be prepared and stained and the morphologic characteristic of the cells and the environment in which these cells are found will be examined by light microscopy. To achieve this, a representative cell sample must be obtained and adequate cell fixation is a prerequisite. Proper identification of the specimen and protection of the specimen's integrity are essential. Finally, pertinent patient clinical history is important for accurate specimen interpretation.

Specimen

Fluid specimens apply to abdominal washings, ascitic fluids, colonic washings, duodenal washings, gastric washings, pleural fluids, pericardial fluids, ovarian cyst fluids, synovial fluids, and sputa.

Procedure

1. The fluid specimen is received at cytopathology laboratory in a plastic container as fresh specimen.
2. The specimen is examined for acceptability according to laboratory procedure.
3. The specimen container is matched against the corresponding cytopathology requisition form by checking at least two items of patient identification:
 - Patient's name
 - Date of birth (DOB)
4. The requisition form is stamped with the date and time.
5. The specimen is accessioned manually in the non-gynecologic specimen logbook: a consecutive number is assigned to the specimen container, requisition, slides, and 50mL-centrifuge tube. In addition, every container and slide is labeled with patient's name. Non-gynecologic specimen identification number starts with prefix "CY."
6. The laboratory assistant provides a gross description of the specimen on the requisition.
7. The specimen is divided in two 50 mL tubes and centrifuged at 2500 rpm for 5 min.
8. The supernatant is discarded and the tube with the specimen is resuspended using a vortex. CytoLyt® Solution is added to specimen at a ratio of 1:2 (specimen:solution). If the specimen is bloody or mucoid, a few more milliliter of CytoLyt are added. The specimen is vortexed again and centrifuged at 2500 rpm for 5 min.

9. The supernatant is discarded and mixed well with the Vortex. A few drop of pellet are placed in a PreservCyt Solution Vial and allowed to sit for no less than 15 min. If the pellet/specimen is too thick or bloody, few more drops of CytoLyt are added, and the specimen is centrifuged for the third time.
10. Using a ThinPrep Processor 2000, select the proper sequence for non-gynecologic specimens (number 2; if mucoid, number 3) to prepare the slide. Please see the next “ThinPrep Non-Gynecologic Preparation” for details.
11. Stain and coverslip slide.
12. For effusion specimens (plural, pericardial, and ascite), in step 8, before adding CytoLyt, a drop of specimen from the pellet is smeared on two slides and stained with Diff-Quick stain.
13. When the fluids are very cellular, the excess pellet is used to prepare a Cellblock.
14. The clerk enters patient and specimen information in the Laboratory Information System.

Results

A thin, uniform layer of cells, stained by Papanicolaou and Diff-Quik methods is prepared on glass slides for microscopic examination.

NON-GYNECOLOGIC SPECIMENS: THINPREP PREPARATION

Principle

Non-gynecologic specimens are prepared with ThinPrep Processor 2000 to obtain a thin layer of cells, well preserved and concentrated in a 20-mm diameter on a glass slide.

Specimen

Abdominal washings, ascitic fluids, bronchial washings, bronchoalveolar lavages, colonic washings, duodenal washings, gastric washings, pleural fluids, pericardial fluids, ovarian cyst fluids, synovial fluids, sputa, and urines.

Procedure

After the specimen is pre-prepared according to procedures, and placed in PreservCyt Vial Solution, follow the following steps:

1. Insert a new TransCyt Filter for non-gynecologic specimens into the filter cap.

2. Load the filter assembly in the filter holder.
3. Insert the slide with the accession number, patient's name, into the slide clamps.
4. Fill the fixative bath vial with 95% ethyl alcohol.
5. Place the fixative bath holder at the left of the instrument.
6. Close the ThinPrep Processor door.
7. Select the proper sequence for non-gynecologic specimen (number 2; for mucoid specimens number 3).
8. Press the Start key to begin the preparation cycle.
9. Remove the fixative bath vial with the prepared slide from its holder when the slide preparation process is complete.
10. Remove the slide from fixative bath vial and deposit in 95% ethyl alcohol for at least 10min prior to staining.
11. Remove the filter assembly and separate the TransCyt Filter from the filter cap.
12. Dispose the TransCyt Filter.
13. Remove the sample vial from the Processor, recap it, and save it for 7 days.
14. Stain slides in the Papanicolaou staining set in the usual manner.
15. Coverslip slides in the usual manner.

Storage

PreservCyt Solution contains methanol, a flammable substance and should be stored in a fire safety cabinet. PreservCyt Solution is stored as follows:

- Without cytology sample, up to 1 year from the date of manufacture at 15°–30°C.
- With cytology sample, up to three weeks at 4°–47°C.

CYTOCENTRIFUGATION PROCEDURE

Principle

Cytocentrifugation is used to prepare additional non-gynecologic specimens containing little or nonvisible sediment after centrifugation. This method is used in addition to the monolayer concentration technique for immunohistochemistry staining.

Specimens

Any non-gynecologic specimen in a fluid state.

Procedure

1. With slide clip in open position, the labeled glass slide and the sample chamber are fit against the slide clip. The spring clip is brought up and secured under the two retaining hooks.
2. The assemblies of slide clip, glass slide, and sample chamber is inserted into the sealed head of the cytocentrifuge.
3. After centrifugation, the supernatant is poured off, leaving few drops of it.
4. The centrifugation tube is recapped and the sediment vortexed on high for a few seconds.
5. Three to five drops of the now homogeneous cell suspension are placed into the sample chamber.
6. Each chamber is capped; the lid is secured on sealed head and placed in the cytocentrifuge.
7. The specimen is centrifuged in the cytocentrifuge for 2 min at 1000rpm.
8. After the spin is completed, the clip assembly is removed holding it in one hand and exerting a slight downward pressure on the slide with the index finger. The spring is released with the other hand and tilted to remove chamber.
9. The slide is removed with care and placed in 70% ethyl alcohol for 10min.
10. The slide is stained and coverslipped following procedure for details.

Results

A thin, rather uniform layer of cells, well preserved is prepared on glass slide for immunohistochemical staining.

GYNECOLOGIC SPECIMENS: THINPREP SLIDE PREPARATION

Principle

The ThinPrep Pap Test is a replacement for conventional method of Pap smear preparation for screening of cervical cancer and its precursor lesions.

The cervical-endocervical sample is collected by the clinician using either a broom or a brush as cervical sampling devices and rinsed in a vial filled with PrecervCyt Solution. The ThinPrep Sample vial is then properly capped, labeled with the patient's name and sent, together with a requisition form, to the laboratory, which is equipped with a ThinPrep 2000 Processor. After processing, the specimen will result in a thin layer of cells concentrated in a 20-mm diameter on a glass slide.

Procedure

1. Open a specimen bag.
2. Examine specimen items for acceptability according to laboratory procedure.
3. Assign a gynecologic accession number to the PreservCyt Sample vial, requisition and clean, new slide.
4. Turn the power switch on, located on the back of the ThinPrep 2000 Processor.
5. Remove the cap from the PreservCyt Sample vial and load it into the sample holder.
6. Insert a new TransCyt Filter for Gynecologic specimens into the Filter Cap.
7. Load the filter assembly in the filter holder.
8. Insert the slide with the accession number into the slide clamps.
9. Fill the fixative bath vial with 95% ethyl alcohol.
10. Place the fixative bath holder at the left of the instrument.
11. Close the ThinPrep Processor door.
12. Select the proper sequence for gynecologic specimen (number 4).
13. Press the Start key to begin the preparation cycle.
14. Remove the fixative bath vial with the prepared slide from its holder when the slide preparation process is complete. The instrument will emit a beeping sound.
15. Remove the slide from fixative bath vial and deposit in 95% ethyl alcohol for at least 10min prior to staining.
16. Remove the filter assembly and separate the TransCyt Filter from the filter cap.
17. Dispose the TransCyt Filter.
18. Remove the sample vial from the Processor, recap it, and save it for 4 weeks.
19. Stain slides in the Papanicolaou staining set, following established procedure.
20. Coverslip slides following established procedure.

BRUSHING SPECIMENS PREPARATION

Principle

Brushing specimens will be stained and the morphologic characteristic of the cells and the environment in which these cells are found will be examined by light microscopy. To achieve a representative, cell sample must be obtained

and adequate cell fixation is a prerequisite. Proper identification of the specimen and protection of the specimen's integrity are essential. Finally, pertinent patient clinical history is important for accurate specimen interpretation.

Specimen

Brushing specimens applies to bronchial brushings, esophageal brushings, gastric brushings, duodenal brushings, and colon brushings that are cytology specimens obtained with a brush device and applied to a glass slide.

Procedure

Cytology brushing specimens are received at the cytopathology laboratory as direct smear, fixed, and unstained. If the slides are received fixed with spray fixative, they are placed in a staining rack and into distilled water for 10 min. If the slides are received in 70% ethyl alcohol, a soaking period is not required. After the soak, the slides are placed in the Sakura Automated Stainer (Sakura Finetek Inc., Torrance, CA) for the Papanicolaou staining procedure for non-gynecologic specimens. Once the staining procedure is completed, the slides are coverslipped.

The total number of slides submitted is recorded on the requisition.

The clerk enters the site of brushing, number of slides submitted, and the patient's information in the laboratory information system.

RESULT

Non-gynecologic directly smeared brushing is prepared, stained by Papanicolaou staining method and coverslipped for microscopic examination.

FINE NEEDLE ASPIRATION (FNA) PREPARATION

Principle

FNA specimens will be prepared and stained, and the morphologic characteristic of the cells and the environment in which these cells are found will be examined by light microscopy. To achieve this, a representative cell sample must be obtained and adequate cell fixation is a prerequisite. Proper identification of the specimen and protection of the specimen's integrity are essential. Finally, pertinent patient clinical history is important for accurate specimen interpretation.

Specimen

FNA specimens consist of abdomen, adrenal glands, breast, bone, brain, chest wall, head and neck, kidney, lymph nodes, liver, lung, mediastinum, pancreas,

retroperitoneum, salivary glands, soft tissues, and thyroid. An FNA can be performed on any palpable mass or within any organ.

Procedure

1. FNA specimens are received at the cytopathology laboratory as direct smear, fixed in 70% ethyl alcohol or air dried or as fluid.
2. The specimen is examined for acceptability according to laboratory procedure.
3. The specimen container is matched against the corresponding cytopathology requisition.
4. The requisition form is stamped with the date and time.
5. The specimen is accessioned manually in the non-gynecologic specimen logbook: a consecutive number is assigned to the specimen container, requisition, slides, and 15 mL-centrifuge tube if needed.
6. In addition, every container and slide is labeled with patient's name. Non-gynecologic specimen identification number starts with prefix "CY."
7. The laboratory assistant provides a gross description of the specimen and records the number of slides received on the requisition.
8. The slides received in alcohol are stained in the Sakura Automated Stainer. Please see Non-Gynecologic staining procedure. The air-dried slides are stained manually in Diff-Quik stain.
9. Once the staining procedure is completed, the slides are coverslipped with the Leica Automated coverslipper.
10. The needle rinse or the fluid retrieved from the FNA is prepared following the cytocentrifugation procedure and stained with Papanicolaou staining method.
11. When the needle rinse or the fluid is very cellular, the excess pellet is used to prepare a Cellblock.
12. The clerk enters patient and specimen information in the Laboratory Information System.

INDEX

- Absorption protocols, protein-embedded reference materials, 142–143
- Accuracy requirements, image analysis, segmentation complications, 174–176
- Acetone-fixed cell/tissue samples: nonadditive fixative compounds, 214–215
- Acoustic Reagent Multispotter (ARM), imaging mass spectrometry, matrix application methods, 378–379
- Addition reactions: fixative compounds, formaldehyde reactions, 203–204 unified model, 207–210 fixative denaturation, 196
- Agar technique, immunocytochemistry, cell block process, 223–225
- Air-dried methods: antigen retrieval protocols, diagnostic cytopathology, 29–30 immunocytochemistry, smear preparation, 228
- Alcoholic formalin, fixative compounds, 210
- Aldehyde-fixed frozen cell-tissue sections, antigen retrieval protocols, 33–38
- American Type Culture Collection (ATCC), 103
- Amino acids: formaldehyde reaction with, methylol adduct formation, 325–326 linear epitope model of antigen retrieval: basic properties, 295–297 peptide formalin susceptibility, 290–292 protein-formaldehyde reactions, 254–257
- Aminoethylcarbazole (AEC), photometric image analysis, 174–176
- Ammonia-ethanol, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 31–32
- Antibody-dependent test battery: antigen retrieval protocol standardization, 16–17 external quality assessment, HER2 IHC, 116–120 heat-induced antigen retrieval mechanisms, 315–318 image analysis, staining protocols, 177–179 immunocytochemistry smear preparation, multiple markers, 226–227 linear epitope model of antigen retrieval: IHC antibody binding, 293 peptide formalin susceptibility, 289–292 positive peptide immunohistochemistry controls: epitope identification, 127–129 stability testing, 131–134 staining failure, 134–136

Antigen retrieval (AR). *See also* Heat-induced antigen retrieval (HIAR) protocols

applications:

aldehyde-fixed frozen cell/tissue sections, 33–38
autofluorescence reduction methods, 30–32

autolysis tissue samples, 40–41

background reduction, unfixed frozen tissue boiling for, 39–40

bromodeoxyuridine

immunohistochemistry, 39

diagnostic cytopathology, 26–30
fluorescence *in situ* hybridization, 38

history of, 25–26

immunofluorescent staining applications, 30

non-formalin-fixed cytology, 27–30
paraformaldehyde-fixed frozen

tissue, en bloc heating, 39

bar code design, 145–149

cell/tissue sample preparation approaches, 191–193

clinical cytopathology, cell sample preparation, research background, 219–222

development background, current and future trends, 189–191

ethanol dehydration and, 273–275

future research issues, 325–331

fixative chemistry and

demodification, 325–328

unmodified protein recovery, FFPE, 323–325

hydrostatic pressure enhancement, 328

immunocytochemistry:

cell sample preparation, 220–222

smear immunostaining, 227–228

immunohistochemistry:

automation:

basic principles, 157–158

heat-induced antigen retrieval

methods, 158–159

standardization, 87–97

retrieval measurement techniques, 92

immunohistochemistry-proteomics

analysis symbiosis, 391–395

linear epitope model:

adjacent proteins, 293–295

applications, 295–297

evaluation, 297–298

formalin fixation, peptide

susceptibility, 289–292

future research issues, 300

heterogeneous reactions, 298–300

immunohistochemistry antibodies,

binding mechanism, 293

peptide array model, 288–289

research background, 287–288

mechanism of, 275–277

positive peptide

immunohistochemistry controls:

epitope identification, 127–129

problem detection using, 136–139

research background, 126–127

stability parameters, 131–134

protein-embedded reference materials,

bead surfaces, protein coatings,

143–145

test battery standardization:

antibody and detection-dependent test system, 16–17

basic procedures, 3–4, 9

immunolectron microscopic studies, 19–20

immunohistochemistry accuracy, 18–19

literature documentation, 5–8

multi-tissue microarray technique, 17

novel chemical solutions, 9–15

tissue protein quantity and

functionality assessment, 328–329

Aqueous fixation, nonpolar solvent

reversal, formaldehyde-protein reactions *vs.*, 327

Archival tissue, shotgun proteomics:

confidence and reproducibility

evaluation, 354–359

current applications, 359–361

Arginine, glyoxal fixative compounds, 213–214

Array-based comparative genomic

hybridization (a-CGH), DNA

extraction evaluation, 52–55

Artifact generation:

antigen retrieval standardization,

accuracy evaluation, 18–19

- DNA extraction, sequence alterations and retrieval strategy, 55
- ASCO/CAP guidelines, immunohistochemistry standardization:
 - current and future strategies, 77–78
 - optimal score definition, 79–80
 - reproducibility improvement, 91–92
- Autofluorescence, reduction techniques, 30–32
- Autolysis of tissue samples:
 - antigen retrieval applications, 40–41
 - RNA extraction, FFPE tissue, 65
- Automated quantitative analysis (AQUA) system, immunohistochemistry standardization, 82
- antigen retrieval testing, 94–97
- Automation protocols:
 - immunohistochemistry standardization, 79–80
 - pathological immunohistochemistry, 151–161
 - basic principles, 157–158
 - development of, 154–155
 - future research issues, 159–161
 - heat-induced antigen retrieval methods, online vs. off-line processes, 158–159
 - manual methods vs., 153–155, 160
 - open vs. closed staining instrumentation, 155–156
 - research background, 151–152
- Avidin-biotin detection system, immunohistochemistry standardization, antigen retrieval testing, 93–97
- Background reduction techniques, antigen retrieval, unfixed frozen tissue boiling, 39–40
- Background staining phenomenon, aldehyde-fixed frozen cell-tissue sections, 36–38
- Bar code design, standard reference materials, 145–149
- Bayer filter pattern, image analysis, camera selection criteria, 168–170
- Bead surfaces, protein coatings, standard reference materials, 143–145
- Beer-Lambert law, image analysis, camera selection criteria, 169–170
- Benjamini-Hochberg method, shotgun proteomics, confidence and reproducibility evaluation, 354–356
- Biomarkers:
 - cytologic smears, 226–227
 - immunohistochemistry standardization, standard reference materials, 81–82
- Biophysical techniques, formalin fixation and heat-induced antigen retrieval, 278–279
- Bladder cancer, antigen retrieval standardization, solution protocol comparisons, 10–15
- Boric acid, antigen retrieval standardization, solution protocol comparisons, 13–15
- Borohydride, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 31–32
- Bouin's solution, immunocytochemistry, cell block process, 224–225
- Brain tissue microdissection, shotgun proteomic analysis, 350–353
- Breast cancer cell lines:
 - growth conditions and characteristics, 106–107
 - immunocytochemistry, standardization, 229–230
- Bromodeoxyuridine (BrdU), immunohistochemistry detection, 39
- Buffer properties:
 - antigen retrieval mechanisms, 276–277
 - heat-induced antigen retrieval protocols:
 - pH-dependent reversibility, 309–310
 - protein extraction, mass spectrometry, 337–340
 - proteomic analysis, tissue surrogate design, recovery efficiency, 242–244
- Calcium ions, heat-induced antigen retrieval mechanisms, 315–318
- Camera device selection criteria, image analysis, 168–170
- Capillary gap principle, immunohistochemistry automation, 157–158

- Capillary isoelectric focusing (cIEF),
 - shotgun proteomics, 349
 - brain tissue microdissection, 350–353
 - confidence and reproducibility evaluation, 354–359
- Capillary isotachopheresis/capillary zone electrophoresis (cITP/cZE):
 - archival tissue, shotgun proteomics, 359–361
 - quantitative shotgun proteomics, confidence and reproducibility evaluation, 356–359
- Carbonyl formaldehyde, fixative compounds, 202–203
- Carnoy's fixative, antigen retrieval protocols, diagnostic cytopathology applications, 27–30
- Cell block techniques:
 - immunocytochemistry and antigen retrieval, 222–225
 - proteomic analysis, tissue surrogate design, 236–238
- Cellient™ system, immunocytochemistry, cell block process, 225
- Cell morphology:
 - antigen retrieval techniques:
 - development background, current and future trends, 189–191
 - sample preparation approaches, 191–193
 - image analysis protocols, segmentation and cell nuclei isolation, 172–176
 - reference cell culture quality evaluation, 110–115
- Cell sample preparation. *See also* Reference cell lines
 - clinical cytopathology:
 - antigen retrieval and immunocytochemistry, 227–228
 - cell block technique, 222–225
 - future research issues, 230
 - immunocytochemistry
 - standardization, 228–230
 - multiple markers, 226–227
 - research background, 219–222
 - defined, 104
 - growth requirements, 104
- Cell transfer technique,
 - immunocytochemistry, 226–227
- Charge-coupled device (CCD), image analysis:
 - development of, 165–166
 - selection criteria, 168–170
- Chelation, linear epitope model of antigen retrieval, 298
- Chemical Inkjet Printer (ChIP), imaging mass spectrometry, matrix application methods, 378
- Chemical moiety formation, methylol-adducted proteins, alcohol exposure, 326
- Chromogenic *in situ* hybridization (CISH):
 - antigen retrieval applications, 38
 - formalin-fixed, paraffin-embedded tissue sections, antigen retrieval, RNA/DNA extraction, 47–48
 - photometric image analysis:
 - multiple staining and colocalization, 176–177
 - particle size, 174–176
- Circular design, immunohistochemistry automation, 157–158
- Circular dichroism spectropolarimetry:
 - formalin fixation and heat-induced antigen retrieval protocols, 278–279
 - formalin-treated RNase A cross-links, secondary and tertiary structures, 261–264
- Citraconic anhydride solution:
 - antigen retrieval standardization, 9–15
 - heat-induced antigen retrieval protocols, neutral pH, 308–309
- Clinical volume, automated pathological protocols, 161
- Closed automated staining protocols, pathological laboratories, 155–156
- Coating techniques:
 - imaging mass spectrometry, matrix-coating method, assisted MALDI imaging, 375
 - protein-embedded reference materials, bead surfaces, 143–145
- Coefficient of variation (CV), positive peptide immunohistochemistry controls, reproducibility improvement, 130–131

- Colocalization protocols, image analysis, 176–177
- Comparative genomic hybridization (CGH):
 - DNA extraction evaluation, 52–55
 - microwave-assisted fluorescence *in situ* hybridization, 38
- Complimentary metal oxide semiconductor (CMOS) detectors, image analysis:
 - development of, 165–166
 - selection criteria, 168–170
- Concentration, fixative penetration, rate of diffusion and, 198
- Confidence evaluation, shotgun proteomics, quantitative analysis, 354–356
- Confocal laser scanning microscopy, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 31–32
- Core density, reference cell culture quality evaluation, 110–115
- Coriell Cell Repositories, 103
- Correlation plot, shotgun proteomic analysis, brain tissue microdissection, 350–353
- Cotton block method, immunocytochemistry, cell block process, 225
- Cross-linking:
 - antigen retrieval mechanisms, 275–277
 - fixative compounds:
 - denaturation, 196
 - formaldehyde reactions, 203–204, 207–210
 - formalin-treated RNase A:
 - ethanol dehydration and, 271–275
 - ethanol dehydration and formaldehyde cross-link reversal, 271–272
 - immunoreactivity restoration, 267–269
 - intra-/intermolecular reactions, 257–258
 - secondary and tertiary structures, 261–264
 - thermal effects, 258–260
 - heat-induced antigen retrieval mechanisms, 315–318
 - linear epitope model of antigen retrieval, 295–297
 - heterogeneity, 298–299
 - protein-formaldehyde reactions, 254–257
 - shotgun proteomics analysis, 361–364
- Customer service, automated pathological protocols, 161
- Cylinder of cells, reference cell cultures, 107–109
- Cytopathology, cell sample preparation:
 - antigen retrieval and immunocytochemistry, 227–228
 - cell block technique, 222–225
 - future research issues, 230
 - immunocytochemistry standardization, 228–230
 - multiple markers, 226–227
 - research background, 219–222
- Cytoplasmic staining, photometric image analysis, segmentation complications, 175–176
- Cytoscape approach, immunocytochemistry, cell block process, 224–225
- Dehydration, methylol-adducted proteins, 326–327
- Denaturation:
 - fixative compounds, 196
 - imaging mass spectrometry, thin-sliced tissue transfer to PVDF, 379–382
- Detection system-dependent test battery:
 - antigen retrieval protocol standardization, 16–17
 - tissue protein quantity and functionality assessment, 328–329
- Detergent effects, proteomic analysis, tissue surrogate design, recovery efficiency, 241–242
- Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), 103
- Diagnostic cytopathology, antigen retrieval applications, 26–30

- Diaminobenzidine (DAB), photometric image analysis, 174–176
 - multiple staining and colocalization, 176–177
 - staining controls, 180–184
- Differential scanning calorimetry (DSC), formalin fixation and heat-induced antigen retrieval, 278
- Diff-Quik stain, antigen retrieval
 - protocols, diagnostic cytopathology, 29–30
- Diffusion coefficient, fixative penetration, 197–199
- Digital image acquisition devices:
 - density and image acquisition, 166–167
 - image analysis, 165–166
- Direct mixing techniques, protein-embedded reference materials, matrix media, 143
- Display protocols, image analysis, 171
- Distributional error, image analysis, camera selection criteria, 169–170
- DNA extraction, formalin-fixed, paraffin-embedded tissue sections, antigen retrieval:
 - array-based comparative genomic hybridization, 52–55
 - artificial sequence alterations, 55
 - protocol development, 48–51
 - research background, 47–48
- Double-label immunocytochemistry, multiple markers, cytologic smears, 226–227
- Droplet matrix application method, imaging mass spectrometry, 377–378
- Drying protocols, tissue sections, imaging mass spectrometry, 375
- Dynabead systems, protein-embedded reference materials, surface coatings, 143–145
- Edge distortion, stain density and image acquisition, 167
- Embedding process:
 - imaging mass spectrometry, 372
 - reference cell cultures, 108–109
- En bloc heating, paraformaldehyde-fixed frozen tissue, antigen retrieval, 39
- Enzyme chemistry:
 - antigen retrieval techniques, development background, current and future trends, 190–191
 - formalin-treated RNase A, recovery of, 264–265
 - tissue protein quantity and functionality assessment, 329
- Enzyme linked immunosorbent assay (ELISA):
 - bar code design, 148–149
 - formalin-treated RNase A immunoreactivity, 265–269
- immunohistochemistry
 - standardization, 80–82
 - antigen retrieval testing, 96–97
- quantifiable immunohistochemistry
 - standardization, 83
- Epitopes:
 - antigen retrieval mechanisms, 275–277
 - formalin-treated RNase A cross-links: immunoreactivity effects, 265–267
 - ionization, 260–261
 - heat-induced antigen retrieval mechanisms, 315–318
 - image analysis, staining controls, 180–184
 - linear model of antigen retrieval:
 - adjacent proteins, 293–295
 - applications, 295–297
 - evaluation, 297–298
 - formalin fixation, peptide susceptibility, 289–292
 - future research issues, 300
 - heterogeneous reactions, 298–300
 - immunohistochemistry antibodies, binding mechanism, 293
 - peptide array model, 288–289
 - research background, 287–288
 - positive peptide immunohistochemistry controls, 127–129
- Error reduction, automated pathological protocols, 161
- Estrogen receptor antibodies:
 - antigen retrieval protocols, diagnostic cytopathology applications, 27–30
 - antigen retrieval protocol standardization, 16–17
 - antigen retrieval techniques, cell/tissue sample preparation, 193

- Ethanol dehydration:
 - antigen retrieval and, 273–275
 - formaldehyde cross-link reversal, 271–272
 - protein structure, fixation effects, 272–273
- Ethanol-fixed cell/tissue samples:
 - antigen retrieval techniques, 192–193
 - fixative compounds, formaldehyde hydrophobic inversions, 206–207
 - nonadditive fixative compounds, 214–215
- European Collection of Cell Cultures (ECACC), 103
- External quality assessment (EQA),
 - reference cell lines:
 - breast cancer cell growth conditions and characteristics, 106–107
 - cell culture techniques and requirements, 104
 - cell passaging, 104–105
 - development and preparation, 103–115
 - fixation, 107
 - harvesting techniques, 106
 - HER2 immunohistochemistry, 116–118
 - HER2 in situ hybridization, 118–120
 - processing, 107–109
 - quality control, 110–115
 - research background, 101–102
 - section preparation, 109–110
 - validated cell lines, 103
- External tissue controls,
 - immunohistochemistry
 - standardization, antigen retrieval techniques, 89–97
- Feulgen Rossenbeck DNA image
 - analysis, research background, 165–166
- Fick's Laws of Diffusion, fixative penetration, 197
- Field flattening computation, stain density and image acquisition, 167
- Fine-needle aspiration (FNA) samples:
 - antigen retrieval protocols, diagnostic cytopathology, 29–30
 - immunocytochemistry and antigen retrieval, 220–222
 - standardization, 230
- Fixation time:
 - image analysis, 177
 - reference cell cultures, 107
- Fixative chemistry:
 - alcoholic formalin, 210
 - antigen retrieval techniques:
 - cell/tissue sample preparation, 191–193
 - development background, current and future trends, 189–191
 - chemistry and demodification, 325–328
 - adduct removal strategy, 327–328
 - alcohol exposure, methylol-adducted protein chemical moieties, 326
 - formaldehyde-amino acid reaction, methylol adduct formation, 325–326
 - hydrostatic pressure-enhanced antigen retrieval, 328
 - protein-formaldehyde reactions, paraffin embedding vs. aqueous fixation, 327
 - reaction product formation during dehydration or paraffin embedding, temperature dependence, 326–327
 - denaturation, 196
 - formaldehyde fixation, 201–210
 - amino acid reaction and methylol adduct formation, 325–326
 - cross-linking during tissue processing, 204
 - general fixation reactions, 203–204
 - hydrophobic inversions, 205–207
 - myths concerning, 201–203
 - nucleic acids and, 204–205
 - unified model, 207–210
 - formalin-treated RNase A, activity recovery effects, 269–271
 - glyoxal, 212–214
 - immunocytochemistry:
 - cell block technique, 222–225
 - smear preparation, 227–228
 - nonadditive fixatives, 214–215
 - penetration, 196–199
 - concentration and diffusion rate, 198
 - diffusion coefficients, 197–198
 - rate of diffusion and square of distance, 198

Fixative chemistry (*cont'd*):

- square of distance proportional to, 199

- protein structure and ethanol dehydration, 272–273

- research background, 195

- specimen quality control, 199–201

- grossing, 199–200

- preprocessing fixation, 201

- pretransport treatment, 199

- transport protocols, 199

- zinc fixatives, 210–212

Fluorescence imaging, camera selection, 168–170

Fluorescence *in situ* hybridization (FISH):

- antigen retrieval applications, 38

- antigen retrieval techniques, cell/tissue sample preparation, 191–193

- external quality assessment, HER2 IHC, 118–120

- formalin-fixed, paraffin-embedded tissue sections:

- autofluorescence reduction, 32

- RNA/DNA extraction, 47–48

- immunocytochemistry, standardization, 229–230

Formaldehyde fixation chemistry.

See also Protein-formaldehyde reactions

- adduct removal, proteins in aqueous media, 327–328

- amino acid reaction, methylol adduct formation, 325–326

- ethanol dehydration, cross-link reversal, 271–272

- fixative compounds, 201–210

- cross-linking during tissue processing, 204

- general fixation reactions, 203–204

- hydrophobic inversions, 205–207

- myths concerning, 201–203

- nucleic acids and, 204–205

- unified model, 207–210

- heat-induced antigen retrieval protocols, pH effects on proteins, 311–312

- methylol-adducted proteins, alcohol exposure, 326

proteins, 254–257

- protein extraction studies, FFPE tissue, mass spectrometry, 336–337

RNA/DNA extraction, 47–48

- array-based comparative genomic hybridization, 54–55

shotgun proteomics, 361–364

unmodified protein recovery, 323–325

Formalin-fixed, paraffin-embedded

(FFPE) tissue sections:

- aldehyde-fixed frozen cell-tissue sections, antigen retrieval protocols, 33–38

antigen retrieval techniques:

- accuracy evaluation, 18–19

- antibody and detection-dependent test battery, 16–17

- basic procedures, 3–4, 9

- cell/tissue sample preparation approaches, 191–193

- development background, current and future trends, 189–191

- mechanisms of, 275–277

- multi-tissue microarray test battery application, 17

- novel chemical solutions, 9–15

- tissue protein quantity and functionality assessment, 328–329

application history, 25–26

autofluorescence reduction, 30–32

biophysical methods, 278–279

circular dichroism spectropolarimetry, 278–279

diagnostic cytopathology limitations, 26–30

differential scanning calorimetry, 278

DNA/RNA extraction:

- array-based comparative genomic hybridization, 52–55

- artifactual sequence alterations, 55

- heat-induced protocol, 55–56

- heating extraction protocol, 61–65

- laboratory example, RNA extraction, 56–61

- nonheating extraction protocol, 61

- protocol development, 48–51

- research background, 47–48

ethanol dehydration:
 antigen retrieval, 273–275
 formaldehyde cross-link reversal,
 271–272
 fluorescence *in situ* hybridization
 (FISH), 38
 formaldehyde-protein reactions,
 254–257
 nonpolar solvent reversal, 327
 heat-induced antigen retrieval:
 current applications, 303–304
 pH effects, 304–312
 citraconic anhydride at neutral
 pH, 308–309
 efficiency reversibility, 309–310
 FFPE dependency, 304–308
 protein-formaldehyde
 interactions, 311–312
 protein extraction, mass
 spectrometry, 337–340
 immunocytochemistry:
 research background, 220–222
 standardization, 228–230
 immunofluorescent staining, 30
 bar code measurement, 145–149
 immunohistochemistry-proteomics
 analysis symbiosis, 391–395
 immunohistochemistry
 standardization, 77–78
 antigen retrieval techniques, 87–97
 linear epitope model of antigen
 retrieval, peptide susceptibility,
 289–292
 protein extraction studies, mass
 spectrometry analysis, 335–343
 Liquid Tissue™ method, 340–341
 methodology comparisons, 341–342
 protein structure, fixation and ethanol
 dehydration effects, 272–273
 proteomic analysis:
 immunohistochemistry-proteomics
 analysis symbiosis, 391–395
 shotgun proteomics, 347–364
 archival tissue analysis, 359–361
 brain tumor tissue
 microdissection, 350–353
 future applications, 361–364
 laser capture microdissection,
 349–350

quantitative analyses, confidence
 and reproducibility, 354–359
 research background, 347–349
 tissue surrogate design:
 cell blocks and embedded
 proteins, 236–238
 protein extraction studies, 236–239
 recovery efficiency
 buffer properties, 242–244
 temperature/detergent effects,
 241–242
 research background, 235–236
 reaction products, temperature and
 dehydration effects, 326–327
 reference cell line development:
 external quality assessment, HER2
 IHC, 116–120
 growth conditions and quality
 control, 103–115
 research background, 253–254
 RNase A effects:
 enzymatic activity recovery, 264–265
 fixation and activity recovery,
 269–271
 immunoreactivity, 265–269
 intra/intermolecular cross-links,
 257–258
 ionization state, 260–261
 secondary/tertiary structure, 261–264
 thermal properties, 258–260
 unmodified protein recovery, 323–325
 Formatted images protocols, image
 analysis, 170
 Frozen tissue sections:
 antigen retrieval:
 background reduction, unfixed
 frozen tissue boiling, 39–40
 paraformaldehyde-fixed frozen
 tissue, en bloc heating, 39
 antigen retrieval techniques, 192–193
 immunohistochemistry
 standardization, reproducibility
 improvement, 91–97
 RNA extraction, FFPE tissue, 56–60
 shotgun proteomics, brain tissue
 microdissection, 350–353
 Funnel filtration systems,
 immunocytochemistry, cell block
 process, 225

- Gain changes, image analysis,
 - camera and optics selection criteria, 168–170
- Gel-embedded proteins, proteomic analysis, tissue surrogate design, 236–238
- Glioblastoma multiforme (GBM) tissue:
 - laser captured microdissection, 349–350
 - shotgun proteomic analysis, 350–353
- Glyoxal fixative compounds, 212–214
- Gold labeling techniques, antigen
 - retrieval standardization, accuracy evaluation, 19–20
- Green fluorescent protein (GFP),
 - formalin-fixed, paraffin-embedded tissue sections, autofluorescence reduction, 32
- Grossing of specimens, fixative compounds, 200–201
- Guanidine buffer, proteomic analysis, tissue surrogate design, recovery efficiency, 242–244
- Harvesting protocols, reference cell cultures, 106
- HCl solution:
 - bromodeoxyuridine
 - immunohistochemistry detection, 39
 - proteomic analysis, tissue surrogate design, recovery efficiency, 242–244
- Heat-induced antigen retrieval (HIAR) protocols:
 - biophysical techniques, 278–279
 - circular dichroism spectropolarimetry, 278–279
 - current applications, 303–304
 - differential scanning calorimetry, 278
 - formalin-fixed, paraffin-embedded tissue sections, research background, 253–254
 - formalin-treated RNase A, thermal effects, 258–260
 - formalin-treated RNase A cross-links:
 - ionization, 260–261
 - secondary and tertiary structures, 261–264
 - future research issues, 319
 - immunohistochemistry automation,
 - online vs. off-line procedures, 158–159
 - ionic strength, 313–315
 - mechanism of, 275–277, 315–318
 - pH effects, 304–312
 - citraconic anhydride at neutral pH, 308–309
 - efficiency reversibility, 309–310
 - FFPE dependency, 304–308
 - protein-formaldehyde interactions, 311–312
 - proteomic analysis:
 - Liquid Tissue™ method, 340–341
 - protein extraction studies, FFPE tissue, mass spectrometry, 335–336
 - tissue surrogate design, 236–238
 - recovery efficiency, temperature/detergent effects, 241–242
- Heating protocols:
 - adduct removal, proteins in aqueous media, 327–328
 - antigen retrieval techniques,
 - development background, current and future trends, 191
 - DNA extraction, 48–51
 - array-based comparative genomic hybridization, 52–55
 - formaldehyde-protein reactions,
 - nonpolar solvent reversal, 327
 - positive peptide immunohistochemistry stability, 133–134
 - RNA extraction, FFPE tissue, 55–56, 61–65
- Hematoxylin and eosin (H&E) tissue staining:
 - automation for pathological diagnosis, 151–152
 - image analysis and segmentation complications, 172–176
 - zinc fixative compounds, 210–212
- HerceptTest™:
 - external quality assessment, HER2 IHC, 116–120
 - immunocytochemistry, standardization, 230
- Heterogeneity, linear epitope model of antigen retrieval, 298–299

- Histological processing, proteomic analysis, tissue surrogate design, 240–241
- hMAM gene, RNA extraction, FFPE tissue, 62–65
- Human epidermal growth factor receptor 2 (HER2):
 - antigen retrieval protocols, diagnostic cytopathology, 26–30
 - Her2/*neu*, image analysis, 175–176
 - immunocytochemistry, standardization, 229–230
 - immunohistochemistry standardization and, 77–78
 - reference cell lines, external quality assessment:
 - breast cancer cell growth conditions and characteristics, 106–107
 - cell culture techniques and requirements, 104
 - cell passaging, 104–105
 - development and preparation, 103–115
 - fixation, 107
 - harvesting techniques, 106
 - immunohistochemistry, 116–118
 - processing, 107–109
 - quality control, 110–115
 - research background, 101–102
 - section preparation, 109–110
 - in situ hybridization, 118–120
 - validated cell lines, 103
- Hydrophobic inversion, fixative compounds, formaldehyde interactions, 205–207
- Hydrostatic pressure, antigen retrieval enhancement, 328
- Image acquisition protocols, development of, 166–167
- Image analysis (IA):
 - acquisition protocols, 166–167
 - camera and optics selection criteria, 168–170
 - image display, 171
 - image format, 170
 - immunohistochemistry standardization, 79–80
 - antigen retrieval techniques, 89–97
 - stain controls, 180–184
 - standard reference materials, 81–82
 - multiple stains and colocalization, 176–177
 - procedures and interpretation, 172–176
 - quantifiable immunohistochemistry standardization, 83
 - research background, 165–166
 - specimen preparation, 177
 - staining protocols, 177–179
- Imaging mass spectrometry (IMS):
 - defined, 369–370
 - immunohistochemistry-proteomics analysis symbiosis, 392–395
 - measurement protocols, 371–382
 - protein mapping, tissue section, *Scraper* knockout analysis, 382–387
 - sample preparation, 370–371
 - drying procedures, 375
 - embedding techniques, 372
 - matrix application methods, 377–379
 - matrix coating, assisted MALDI imaging, 375–376
 - matrix selection, 375–377
 - section-support materials, 373–374
 - thin slice excision, 372–373
 - thin slice tissue transfer, PVDF membrane, 379–382
 - washing procedures, 374–375
- Imidazole reaction, glyoxal fixative compounds, 213–214
- Immersion matrix application method, imaging mass spectrometry, 377
- Immunocytochemistry (ICC):
 - cell block technique, 222–225
 - cell sample preparation:
 - multiple markers, 226–227
 - research background, 220–222
 - standardization of, 228–230
- Immunoelectron microscopy (IEM), antigen retrieval standardization, accuracy evaluation, 19–20
- Immunofluorescent (IF) staining, formalin-fixed, paraffin-embedded tissue sections, 30
- autofluorescence reduction, 31–32

Immunohistochemistry (IHC):

antigen retrieval techniques:

accuracy evaluation, 18–19
aldehyde-fixed frozen cell-tissue
sections, 33–38

diagnostic cytopathology, 26–30

history, 25–26

automation for pathological diagnosis,
151–161

basic principles, 157–158

development of, 154–155

future research issues, 159–161

heat-induced antigen retrieval
methods, online vs. off-line
processes, 158–159

manual methods vs., 153–155, 160

open vs. closed staining

instrumentation, 155–156

research background, 151–152

bromodeoxyuridine detection, 39

image analysis:

acquisition protocols, 166–167

camera and optics selection criteria,
168–170

image display, 171

image format, 170

multiple stains and colocalization,
176–177

procedures and interpretation,
172–176

quantifiable immunohistochemistry
standardization, 83

research background, 165–166

specimen preparation, 177

staining protocols, 177–179

standardization, 79–80

antigen retrieval techniques,
89–97

stain controls, 180–184

standard reference materials,
81–82

linear epitope model of antigen
retrieval, 293

peptide controls:

antigen retrieval techniques,
136–139

applications, 124–127

epitope identification, 127–129

reproducibility, 130–131

research background, 123–124

stability, 131–134

staining problems, 134–136

proteomics analysis and, 391–395

quantitative IHC:

evolution of, 75–76

potential approaches, 82–83

reference cell lines:

breast cancer cell growth conditions
and characteristics, 106–107

cell culture techniques and
requirements, 104

cell passaging, 104–105

development and preparation,
103–115

fixation, 107

harvesting techniques, 106

HER2 external quality assessment,
116–118

HER2 in situ hybridization,
118–120

processing, 107–109

quality control, 110–115

research background, 101–102

section preparation, 109–110

validated cell lines, 103

shotgun proteomics, confidence and
reproducibility evaluation, 357–359

standardization:

antigen retrieval technique, 87–97

current and future strategies, 77–78

optimal score definition, 79–80

reference material, 80–82

total test approach, 76–77

Immunoreactivity testing:

formalin-treated RNase A, 265–269

formalin-treated RNase A cross-links,
ionization, 260–261

heat-induced antigen retrieval
protocols, pH-dependent
reversibility, 309–310

linear epitope model of antigen
retrieval:

adjacent protein properties, 293–295

peptide formalin susceptibility,
290–292

positive peptide

immunohistochemistry stability,
131–134

- Immunostaining intensity:
 - antigen retrieval standardization:
 - accuracy evaluation, 18–19
 - aldehyde-fixed frozen cell-tissue sections, 33–38
 - solution protocol comparisons, 9–15
 - bar code measurement, 145–149
 - heat-induced antigen retrieval protocols:
 - mechanisms of, 317–318
 - pH effects, 304–312
 - image acquisition protocols, 166–167
 - immunocytochemistry, smear preparation, 227–228
 - positive peptide
 - immunohistochemistry controls, 134–136
 - antigen retrieval problem identification, 136–139
 - two-color staining techniques, image analysis, 176–177
- “In-cell” Western assay, quantifiable immunohistochemistry standardization, 83
- Indium tin oxide (ITO), imaging mass spectrometry, section supporting materials, 373–374
- Instrument flexibility, automated pathological protocols, 161
- Insulin, protein-formaldehyde cross-linking, 256–257
- Interlab Cell Line Collection (ICLC), 103
- Intermolecular reactions, formalin-treated RNase A cross-links, 257–258
 - immunoreactivity effects, 267
- Internal controls, immunohistochemistry standardization, antigen retrieval techniques, 89–97
- Internal Reference Standards (IRS), immunohistochemistry standardization, antigen retrieval techniques, 88–97
- Intramolecular reactions, formalin-treated RNase A cross-links, 257–258
 - immunoreactivity effects, 267
- In vitro* cell culture, 104
 - linear epitope model of antigen retrieval, 297–298
- In vivo* cell culture, 104
- Ionic strength:
 - formalin-treated RNase A cross-links, 260–261
 - heat-induced antigen retrieval protocols, 313–315
- Isoelectric focusing (IEF). *See also* Capillary isoelectric focusing
 - formalin-treated RNase A cross-links, 260–261
- JPEG image format, image analysis, 170
- Kinetic thermocycling (KTC)-polymerase chain reaction, DNA extraction, 48–51
- Laser captured microdissection (LCM) system, FFPE tissue:
 - RNA extraction, 63–65
 - shotgun proteomics, 349–350
- Layered peptide array (LPA), quantifiable immunohistochemistry standardization, 83
- Leica Oracle™, external quality assessment, HER2 IHC, 116–120
- Light scattering, photometric image analysis, 174–176
- Linear epitope model of antigen retrieval:
 - adjacent proteins, 293–295
 - applications, 295–297
 - evaluation, 297–298
 - formalin fixation, peptide susceptibility, 289–292
 - future research issues, 300
 - heterogeneous reactions, 298–300
 - immunohistochemistry antibodies, binding mechanism, 293
 - peptide array model, 288–289
 - research background, 287–288
- Linear row design, immunohistochemistry automation, 157–158
- Lipid distribution, imaging mass spectrometry, 386–387

- Liquid crystal display (LCD) technology, image analysis, camera selection criteria, 169–170
- Liquid Tissue™ method, formalin-fixed, paraffin-embedded tissue sections, protein extraction, 340–341
- Literature resources, RNA extraction, FFPE tissue, 57–60
- Lot-to-lot variations, image analysis, staining controls, 182–184
- Low-molecular-weight compounds, imaging mass spectrometry, 386–387
- Lysozyme surrogates:
 - protein extraction studies, mass spectrometry analysis, 341–342
 - proteomic analysis, tissue surrogate design:
 - protein extraction, 244–246
 - recovery efficiency, 241–244
- Macromolecules, fixative compounds, formaldehyde hydrophobic inversions, 205–207
- Mannich reaction, protein-formaldehyde cross-linking, 255–257
- Manual immunohistochemistry assays, automation for pathological diagnosis vs., 153–155, 160
- Mass spectrometry (MS). *See also*
 - Imaging mass spectrometry (IMS)
 - immunohistochemistry-proteomics analysis symbiosis, 392–395
 - ionized particles, 369
 - protein extraction studies, FFPE tissue, 335–343
 - proteomic analysis, tissue surrogate design:
 - cell block and gel-embedded proteins, 236–238
 - research background, 235–236
 - quantifiable immunohistochemistry standardization, 83
 - shotgun proteomics, confidence and reproducibility evaluation, 359
- Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) imaging:
 - imaging mass spectrometry:
 - matrix coating method, 375
 - matrix selection criteria, 375–377
 - section supporting materials, 373–374
 - proteomic analysis, tissue surrogate design:
 - cell block and gel-embedded proteins, 236–238
 - research background, 235–236
 - tissue protein quantity and functionality assessment, 329
- Matrix medium:
 - imaging mass spectrometry:
 - application methods, 377–379
 - selection criteria, 375–377
 - protein-embedded reference materials, direct protein mixing, 143
 - protein-embedding technique, research background, 142–143
 - shotgun proteomics, confidence and reproducibility evaluation, 355–356
- Matrix metalloproteinases (MMPs), autolysis protocols, antigen retrieval applications, 40–41
- MCF-7 cell line, aldehyde-fixed frozen cell-tissue sections, 34–38
- MDA-MB-175-VII breast carcinoma cell line, growth conditions and characteristics, 106, 113–114
- MDA-MB-231 breast carcinoma cell line, growth conditions and characteristics, 107
- MDA-MB-453 breast carcinoma cell line, growth conditions and characteristics, 106, 111
- Medawar's constant, reference cell cultures, 107
- β-Mercaptoethanol (BME), proteomic analysis, tissue surrogate design, recovery efficiency, 242–244
- Methylene bridge:
 - fixative compounds, formaldehyde reactions, 203–204
 - heat-induced antigen retrieval mechanisms, 315–318
- Methylene glycol, formaldehyde chemical reactions, fixative compounds, 202–203
- Methylol adduct formation:
 - alcohol-exposed proteins, chemical moieties, 326

- amino acid-formaldehyde reaction, 325–326
- dehydration and temperature dependence, 326–327
- MIB-1 Papanicolaou stain, antigen retrieval protocols, 27–30
- Micrometastasis detection, aldehyde-fixed frozen cell-tissue sections, 38
- Microwave (MW)-assisted fluorescence *in situ* hybridization (FISH), antigen retrieval applications, 38
- Microwave heating protocols, DNA extraction, 51
- Midwestern assay, quantifiable immunohistochemistry standardization, 82–83
- Molecular friendly fixatives:
 - antigen retrieval techniques, cell/tissue sample preparation, 191–193
 - penetration, 196–199
- Monoclonal antibodies:
 - antigen retrieval protocol standardization, 16–17
 - immunohistochemistry standardization, antigen retrieval testing, 93–97
 - positive peptide immunohistochemistry controls, epitope identification, 128–129
- Monolayer cell preparation, immunocytochemistry and antigen retrieval, 222
- Multiple staining protocols, image analysis, 176–177
- Multiplex immunostain chip (MI chip), immunocytochemistry, 227
- Namimatsu's solution protocol, antigen retrieval standardization, 9–15
- Nestin localization, antigen retrieval standardization, accuracy evaluation, 19–20
- Neutral buffered formalin (NBF):
 - aldehyde-fixed frozen cell-tissue sections, 34–38
 - bead surfaces, protein coatings, 143–145
 - proteomic analysis, tissue surrogate design, protein extraction studies, 238–239
 - reference cell culture processing, 109
- Neutral density filters, image analysis, selection criteria, 168–170
- Nonadditive fixative compounds, 214–215
- Non-formalin-fixed cytology, antigen retrieval protocols, 27–30
- Nonheating protocols:
 - antigen retrieval techniques, development background, current and future trends, 191
 - DNA extraction, array-based comparative genomic hybridization, 52–55
 - RNA extraction, FFPE tissue, 61
- Nonpolar solvents, protein-formaldehyde reaction reversal, heating and, 327
- Nucleic acids, fixative compounds, formaldehyde reactions, 204–205
- Off-line heat-induced antigen retrieval methods, immunohistochemistry automation, 158–159
- Online heat-induced antigen retrieval methods, immunohistochemistry automation, 158–159
- Open automated staining protocols, pathological laboratories, 155–156
- Open Mass Spectrometry Search Algorithm (OMSSA), shotgun proteomics, 349
- Optics selection criteria, image analysis, 168–170
- Optimal scoring definition, immunohistochemistry standardization, 79–80
- p21-nuclear staining patterns, aldehyde-fixed frozen cell-tissue sections, 34–38
- Papanicolaou stain, antigen retrieval protocols, 26–30
- Paraformaldehyde-fixed frozen tissue, en bloc heating, antigen retrieval, 39
- Parallel sections, immunohistochemistry standardization, antigen retrieval techniques, 89–97
- Particle size, photometric image analysis, 174–176
- Passaging technique, reference cell cultures, 104–105

- Pathological diagnosis, automated immunohistochemistry techniques:
 basic principles, 157–158
 development of, 154–155
 future research issues, 159–161
 heat-induced antigen retrieval
 methods, online vs. off-line
 processes, 158–159
 manual methods vs., 153–155, 160
 open vs. closed staining
 instrumentation, 155–156
 research background, 151–152
- PathVysion Kit, immunocytochemistry, 230
- Pelletization, immunocytochemistry, cell block technique, 223–225
- Penetration, fixative compounds, 196–199
 concentration and diffusion rate, 198
 diffusion coefficients, 197–198
 rate of diffusion and square of distance, 198
 square of distance proportional to, 199
- Peptides:
 as immunohistochemistry controls:
 antigen retrieval techniques, 136–139
 applications, 124–127
 epitope identification, 127–129
 reproducibility, 130–131
 research background, 123–124
 stability, 131–134
 staining problems, 134–136
 linear epitope model of antigen retrieval:
 adjacent protein properties, 293–295
 formalin fixation susceptibility, 289–292
 heterogeneity, 298–299
 peptide array experimental model, 288–289
 protein-formaldehyde reactions, 254–257
 shotgun proteomics:
 confidence and reproducibility
 evaluation, 357–359
 development of, 349
- “Percent positive” measurements, photometric image analysis, segmentation complications, 175–176
- Peridinin chlorophyll α -protein, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 31–32
- Personnel flexibility, automated pathological protocols, 161
- pH levels:
 antigen retrieval mechanisms, 276–277
 heat-induced antigen retrieval protocols, 304–312
 citraconic anhydride at neutral pH, 308–309
 efficiency reversibility, 309–310
 FFPE dependency, 304–308
 protein-formaldehyde interactions, 311–312
 test battery standardization, antigen retrieval protocols, 4, 9
- Phosphate buffer saline (PBS):
 antigen retrieval standardization, solution protocol comparisons, 15
 protein-embedded reference materials, bead surfaces, protein coatings, 143–145
 unfixed frozen tissue boiling, antigen retrieval, 39–40
- Photometry protocols, image analysis:
 camera selection criteria, 168–170
 segmentation complications, 173–176
- Pixel densities, image analysis:
 camera and optics selection criteria, 168–170
 display protocols, 171
 image format, 170
 point vs. geometric processes, 172–176
- Plasma-thrombin clot methodology, immunocytochemistry, cell block technique, 223–225
- Polymerase chain reaction (PCR), DNA extraction, 48–51
- Polymer compounds, imaging mass spectrometry, embedding, 372
- Polyvalent secondary antibodies, image analysis, staining protocols, 178–179
- Polyvinylidene fluoride (PVDF) film, imaging mass spectrometry:
 research background, 370–371
 thin-sliced tissue transfer to, 379–382

- Potassium hydroxide (KOH) solution, DNA extraction, 49–51
- Pretreatment protocols:
 - external quality assessment, HER2 IHC, 116–120
 - fixative compounds:
 - preprocessing treatment, 201
 - transport pretreatment, 200
- Principal component analysis (PCA), imaging mass spectrometry, protein mapping, 382–387
- Prion protein tissue samples, antigen retrieval applications, autolysis protocols, 40–41
- Processing techniques:
 - fixative compounds, preprocessing treatment, 201
 - image analysis, staining controls, 180–184
 - reference cell cultures, 107–109
- Protein-embedded reference materials:
 - absorption method, 142–143
 - antigen retrieval techniques,
 - development background, current and future trends, 190–191
 - bead surfaces, protein coating, 143–145
 - direct protein mixing, matrix media, 143
 - fixative compounds, formaldehyde hydrophobic inversions, 205–207
 - immunohistochemistry
 - standardization, 81–82
 - proteomic analysis, tissue surrogate design, 236–238
 - research background, 141–142
- Protein extraction studies:
 - formalin-fixed, paraffin-embedded tissue sections:
 - Liquid Tissue™ method, 340–341
 - mass spectrometry analysis, 335–343
 - methodology comparisons, 341–342
 - heat-induced antigen retrieval
 - mechanisms, 315–318
 - FFPE tissue, mass spectrometry, 337–340
 - immunocytochemistry and antigen retrieval:
 - research background, 220–222
 - standardization, 89–97
 - proteomic analysis, tissue surrogate design, 236–239
 - non-formalin proteins, 244–246
- Protein-formaldehyde reactions, 254–257
 - adduct removal, proteins in aqueous media, 327–328
 - antigen retrieval mechanisms, 275–277
 - fixation and ethanol dehydration effects, 272–273
 - heat-induced antigen retrieval protocols, pH effects, 311–312
 - linear epitope model of antigen retrieval, peptide formalin susceptibility, 290–292
 - methylol-adducted proteins, alcohol exposure, 326
 - nonpolar solvent heating and reversal, 327
 - protein extraction studies, FFPE tissue, mass spectrometry, 336–337
 - shotgun proteomics analysis applications, 362–364
 - unmodified protein recovery, FFPE tissue, 323–325
- Proteomic analysis:
 - immunohistochemistry and, 391–395
 - protein extraction studies, FFPE tissue, mass spectrometry, 335–343
 - shotgun proteomics, FFPE tissue, 347–364
 - archival tissue analysis, 359–361
 - brain tumor tissue microdissection, 350–353
 - future applications, 361–364
 - laser capture microdissection, 349–350
 - quantitative analyses, confidence and reproducibility, 354–359
 - research background, 347–349
 - tissue surrogate design:
 - applications, 238–239
 - FFPE cell blocks and embedded proteins, 236–238
 - future research issues, 246–247
 - histological processing evaluation, 240–241
 - non-FFPE proteins, 244–246

- Proteomic analysis (*cont'd*)
 - recovery efficiency:
 - buffer formulation effects, 242–244
 - detergent and temperature effects, 241–242
 - research background, 235–236
- Proteomics analysis, imaging mass spectrometry, 382–387
- Qproteome FFPE Tissue Kit, protein extraction studies, mass spectrometry analysis, 341–342
- Quality control:
 - fixative compounds, specimen quality management, 199–201
 - peptide positive
 - immunohistochemistry controls:
 - antigen retrieval techniques, 136–139
 - applications, 124–127
 - epitope identification, 127–129
 - reproducibility, 130–131
 - research background, 123–124
 - stability, 131–134
 - staining problems, 134–136
 - reference cell cultures, 110–115
- Quantifiable Internal Reference Standards (QIRS), immunohistochemistry standardization, 77–80
 - antigen retrieval techniques, 88–97
 - standard reference materials, 81–82
- Quantitative immunohistochemistry:
 - evolution of, 75–76
 - image analysis, research background, 165–166
 - positive peptide controls:
 - antigen retrieval techniques, 136–139
 - applications, 124–127
 - epitope identification, 127–129
 - reproducibility, 130–131
 - research background, 123–124
 - stability, 131–134
 - staining problems, 134–136
 - potential approaches to, 82–83
- Quantitative shotgun proteomics, confidence and reproducibility evaluation, 354–359
 - archival tissue, 356–359
- Quicgel method, immunohistochemistry standardization, antigen retrieval techniques, 90–97
- Recovery efficiency:
 - antigen retrieval mechanisms, 275–277
 - formalin-treated RNase A:
 - enzymatic activity, 264–265
 - fixation effects on activity recovery, 269–271
 - proteomic analysis, tissue surrogate design:
 - buffer effects, 242–244
 - detergent/temperature effects, 241–242
- Red fluorescence, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 31–32
- Reference cell lines:
 - breast cancer cell growth conditions and characteristics, 106–107
 - cell culture techniques and requirements, 104
 - cell passaging, 104–105
 - development and preparation, 103–115
 - external quality assessment:
 - HER2 immunohistochemistry, 116–118
 - HER2 in situ hybridization, 118–120
 - fixation, 107
 - harvesting techniques, 106
 - positive peptide immunohistochemistry
 - controls, 125–126
 - processing, 107–109
 - quality control, 110–115
 - research background, 101–102
 - section preparation, 109–110
 - validated cell lines, 103
- “Repeat immunocytochemistry,”
 - multiple markers, cytologic smears, 226–227
- Reproducibility improvement:
 - image analysis, staining controls, 180–184
 - immunohistochemistry
 - standardization, antigen retrieval techniques, 90–92

- positive peptide
 - immunohistochemistry controls, 130–131
- shotgun proteomics, quantitative analysis, 354–356
- Retinoblastoma protein (pRB), antigen retrieval standardization, solution protocol comparisons, 9–15
- Reverse transcriptase-polymerase chain reaction (RT-PCR), RNA extraction, FFPE tissue, 56
 - gene primers, 60–61
 - heating protocols, 62–65
- Ribonuclease A (RNAse A):
 - ethanol dehydration and, 271–275
 - formalin-fixed, paraffin-embedded tissue sections:
 - enzymatic activity recovery, 264–265
 - fixation and activity recovery, 269–271
 - immunoreactivity, 265–269
 - intra/intermolecular cross-links, 257–258
 - ionization state, 260–261
 - secondary/tertiary structure, 261–264
 - thermal properties, 258–260
 - proteomic analysis, tissue surrogate design, 244–246
- RNA extraction, formalin-fixed, paraffin-embedded tissue sections, antigen retrieval:
 - heat-induced protocol, 55–56
 - heating extraction protocol, 61–65
 - laboratory example, RNA extraction, 56–61
 - nonheating extraction protocol, 61
 - protocol development, 48–51
 - research background, 47–48
- Safety issues, automated pathological protocols, 161
- “Sandwich” procedures, image analysis, staining protocols, 178–179
- Schiff bases:
 - methylol adduct formation, dehydration and temperature dependence, 326–327
 - protein-formaldehyde reactions, 254–257
- Scoring criteria, external quality assessment, HER2 IHC, 116–120
- Scraper* gene-deficient (SCR-KO) mice, imaging mass spectrometry, protein mapping, 382–387
- Section preparation technique:
 - imaging mass spectrometry, supporting materials, 373–374
 - reference cell cultures, 109–110
- Segmentation, image analysis protocols, 172–176
- Semi-quantitative immunohistochemistry:
 - limitations of, 75–76
 - optimal scoring definition in, 80
- Sensitivity assessment, image analysis, staining protocols, 178–179
- Service contracts, automated pathological protocols, 161
- Shotgun proteomics, FFPE tissue, 347–364
 - archival tissue analysis, 359–361
 - brain tumor tissue microdissection, 350–353
 - future applications, 361–364
 - laser capture microdissection, 349–350
 - quantitative analyses, confidence and reproducibility, 354–359
 - research background, 347–349
- Single cell comparative genomic hybridization (SCOMP), DNA extraction, 53–55
- Single-chip color camera, image analysis, selection criteria, 168–170
- Single-nucleotide polymorphism (SNP), microwave-assisted fluorescence *in situ* hybridization, 38
- SK-BR-3 breast carcinoma cell line, growth conditions and characteristics, 106, 110
- Slide capacity, automated pathological protocols, 161
- Slide printing techniques:
 - image analysis, staining controls, 180–184
 - immunocytochemistry, smear preparation, 227–228
 - positive peptide immunohistochemistry controls, reproducibility improvement, 130–131

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):
 formalin-treated RNase A cross-links, 257–260
 proteomic analysis, tissue surrogate design:
 histological processing, 240–241
 protein extraction studies, 238–239
 recovery efficiency, buffer properties, 242–244
 shotgun proteomics, brain tissue microdissection, 350–353
 shotgun proteomics analysis applications, 362–364
 Sodium hydroxide (NaOH) solution, DNA extraction, 49–51
 Specificity checks:
 image analysis, staining protocols, 178–179
 positive peptide immunohistochemistry controls, epitope identification, 128–129
 Specimen preparation:
 fixative compounds, quality controls, 199–201
 image analysis, 177
 Spectral imaging microscopy:
 immunohistochemistry standardization, 82
 multiple staining and colocalization, 176–177
 Spray-coated matrix application, imaging mass spectrometry, 377
 Spray-droplet matrix application, imaging mass spectrometry, 379
 Square of distance, fixative penetration:
 rate of diffusion proportional to, 198
 time proportional to, 199
 Stability, positive peptide immunohistochemistry controls, 131–134
 Staining methods:
 automated pathological protocols:
 development of, 154–155
 heat-induced antigen retrieval methods, online vs. off-line, 158–159
 manual methods vs., 153–155, 160
 open vs. closed instrumentation, 155–156

image analysis:
 controls for, 178–180
 density and image acquisition, 166–167
 multiple stains and colocalization, 176–177
 protocols for, 177–179
 segmentation complications, 172–176
 immunocytochemistry, 227–228
 immunohistochemistry
 standardization, antigen retrieval techniques, 89–97
 positive peptide immunohistochemistry controls, failure analysis, 134–136
 Standard reference material:
 bar code design, 145–149
 immunohistochemistry standardization, 80–82
 protein-embedding technique:
 absorption method, 142–143
 bead surfaces, protein coating, 143–145
 direct protein mixing, matrix media, 143
 research background, 141–143
 reference cell lines:
 breast cancer cell growth conditions and characteristics, 106–107
 cell culture techniques and requirements, 104
 cell passaging, 104–105
 development and preparation, 103–115
 external quality assessment:
 HER2 immunohistochemistry, 116–118
 HER2 in situ hybridization, 118–120
 fixation, 107
 harvesting techniques, 106
 processing, 107–109
 quality control, 110–115
 research background, 101–102
 section preparation, 109–110
 validated cell lines, 103
 Steric interference, linear epitope model of antigen retrieval, 298

- Strong cation exchange (SCX)
 - chromatography, shotgun
 - proteomics, confidence and reproducibility evaluation, 354–359
- Sudan Black B dye, autofluorescence reduction, formalin-fixed, paraffin-embedded tissue sections, 30–32
- Surface-enhanced laser desorption/ionization (SELDI), tissue protein quantity and functionality assessment, 328–329
- Test battery approach, antigen retrieval:
 - antibody and detection-dependent test system, 16–17
 - basic procedures, 3–4, 9
 - immunoelectron microscopic studies, 19–20
 - immunohistochemistry accuracy, 18–19
 - literature documentation, 5–8
 - multi-tissue microarray technique, 17
 - novel chemical solutions, 9–15
 - reproducibility improvement, 90–97
- Tetanus toxin, antigen retrieval
 - techniques, development
 - background, current and future trends, 191
- Thermal effects:
 - antigen retrieval mechanisms, 275–277
 - formalin-treated RNase A cross-links, 258–260
 - secondary and tertiary structures, 261–264
 - proteomic analysis, tissue surrogate design, recovery efficiency, 241–242
- ThinPrep technology,
 - immunocytochemistry, 222–225
- Thin-sliced tissue transfer, imaging mass spectrometry, PVDF membrane, 379–382
- Thin slice excision, imaging mass spectrometry, 372–373
- Three-chip cameras, image analysis, selection criteria, 169–170
- Thyroid transcription factor-1 (TTF-1)
 - stain, antigen retrieval protocols, diagnostic cytopathology, 29–30
- TIFF image format, image analysis, 170
- Tissue microarray (TMA) techniques:
 - antigen retrieval standardization:
 - basic procedures, 4, 9
 - test battery application, 17
 - image analysis, staining protocols, 177–179
 - immunohistochemistry
 - standardization, antigen retrieval testing, 94–97
 - shotgun proteomics analysis, 361–364
- Tissue preparation protocols:
 - antigen retrieval techniques:
 - cell/tissue sample preparation approaches, 191–193
 - development background, current and future trends, 189–191
 - imaging mass spectrometry:
 - basic principles, 369–371
 - drying procedures, 375
 - embedding techniques, 372
 - matrix application methods, 377–379
 - matrix coating, assisted MALDI imaging, 375–376
 - matrix selection, 375–377
 - measurement protocols, 371–382
 - protein mapping, tissue section, *Scraper* knockout analysis, 382–387
 - section-support materials, 373–374
 - thin slice excision, 372–373
 - thin slice tissue transfer, PVDF membrane, 379–382
 - washing procedures, 374–375
 - immunohistochemistry
 - standardization:
 - antigen retrieval techniques, 89–97
 - optimal scoring definition, 79–80
 - protein quantity and functionality assessment, 328–329
 - quantity and functionality assessment, 328–329
- Tissue surrogate design, proteomic analysis:
 - applications, 238–239
 - FFPE cell blocks and embedded proteins, 236–238
 - future research issues, 246–247
 - histological processing evaluation, 240–241

- Tissue surrogate design, proteomic analysis (*cont'd*)
 - non-FFPE proteins, 244–246
 - recovery efficiency:
 - buffer formulation effects, 242–244
 - detergent and temperature effects, 241–242
 - research background, 235–236
- Tissue transfer:
 - imaging mass spectrometry, thin-sliced tissue section to PVDF membrane, 379–382
 - immunocytochemistry and antigen retrieval, 222
- Total test approach,
 - immunohistochemistry standardization, 76–77
 - optimal scoring definition, 80
- Transmembrane helices, shotgun proteomics, brain tissue microdissection, 353
- Transport mechanisms, fixative compounds, 200
- Tri-HCl buffer, antigen retrieval standardization, citraconic anhydride solution, 9–15
- Two-step test procedure, antigen retrieval standardization, 4, 9
- Unified model, formaldehyde fixation, 207–210
- Unmodified proteins, formalin-fixed, paraffin-embedded tissue, recovery from, 323–325
- Validated cell lines, sources for, 103
- Venn diagrams, shotgun proteomics:
 - brain tissue microdissection, 350–353
 - laser captured microdissection, FFPE tissue, 349–350
- Ventana Pathway™, external quality assessment, HER2 IHC, 116–120
- Vimentin expression:
 - autolysis protocols, antigen retrieval applications, 41
 - immunohistochemistry standardization, antigen retrieval techniques, 87–97
- Volcano plot, shotgun proteomics, confidence and reproducibility evaluation, 355–356
- Warm ischemic time:
 - immunohistochemistry standardization, antigen retrieval testing, 96–97
 - RNA extraction, FFPE tissue, 65
- Washing protocols, tissue sections, imaging mass spectrometry, 374–375
- Western blotting technique:
 - aldehyde-fixed frozen cell-tissue sections, 36–38
 - antigen retrieval standardization, solution protocol comparisons, 13–15
 - bar code design, 148–149
- White balance algorithms, stain density and image acquisition, 167
- Zinc fixative compounds, 210–212

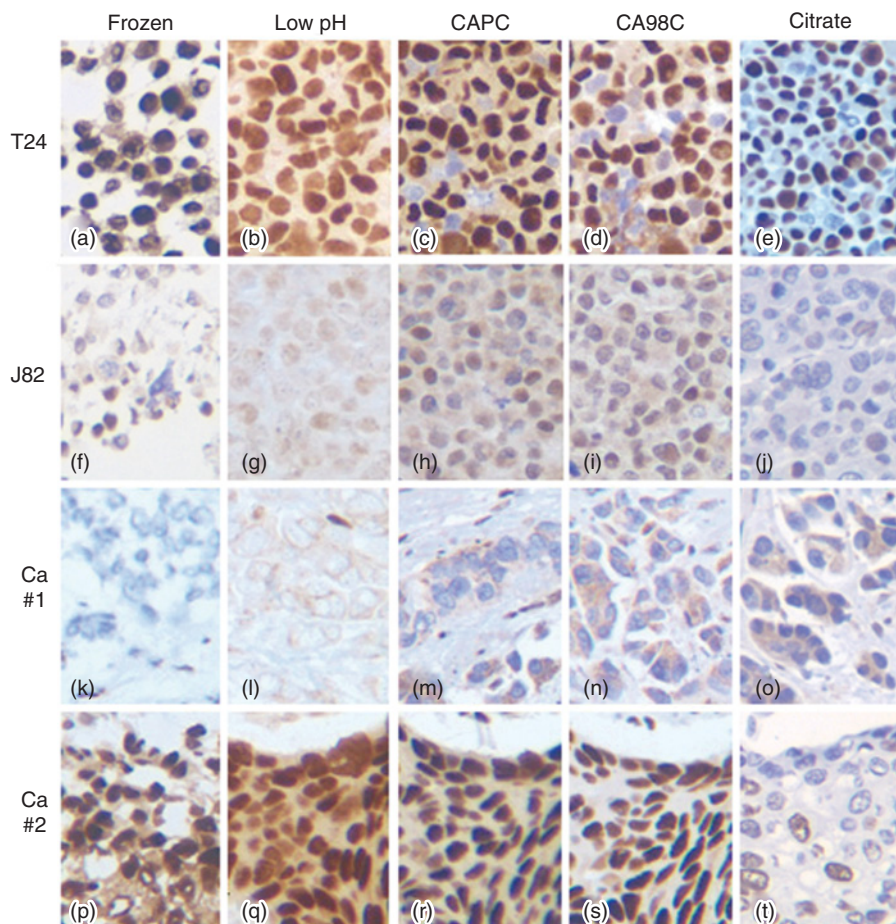


Figure 1.1 Comparison of pRB-IHC staining results for frozen and FFPE tissue sections using four AR protocols. All images are arranged in the same order as given in Table 1.3, indicating all scores indicated in the table. T24 and J82 are two cell lines, Ca #1 and Ca #2 are specimens of human bladder cancer, frozen means frozen cells or tissues fixed in acetone, other terms listed in the top line represent FFPE tissue sections after various AR treatments: Low pH, AR solution at low pH value; CAPC, citraconic anhydride solution with boiling; CA98C, citraconic anhydride solution with heating at 98°C; citrate, conventional boiling heating with citrate acid buffer at pH 6.0. Original magnification $\times 200$. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

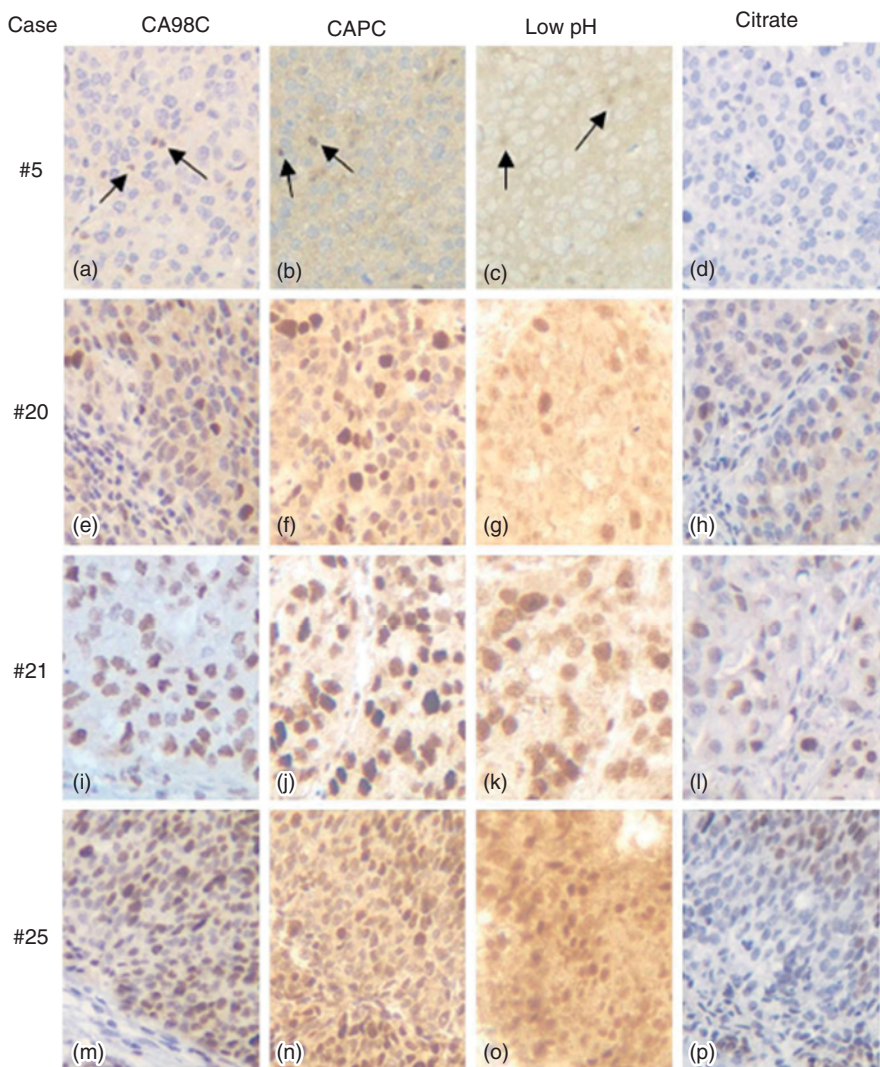


Figure 1.2 Examples of immunostaining intensity from comparison of pRB-IHC in 27 cases of FFPE tissues of bladder cancer (Table 1.4). (A–D) Negative (<10%) showing a few weak positive nuclei (arrows); (E–H) moderate positive (>10%); (I–P) strong positive (>50%). Arrows indicate positive nuclear staining for some lymphocytes or other stromal cells as an internal control. Note the lack of nuclear hematoxylin counterstaining due to low pH AR treatment. The order of cases are indicated in Table 1.4. Reproduced with permission from Shi et al., *Biotech. Histochem.* 2007; 82: 301–309.

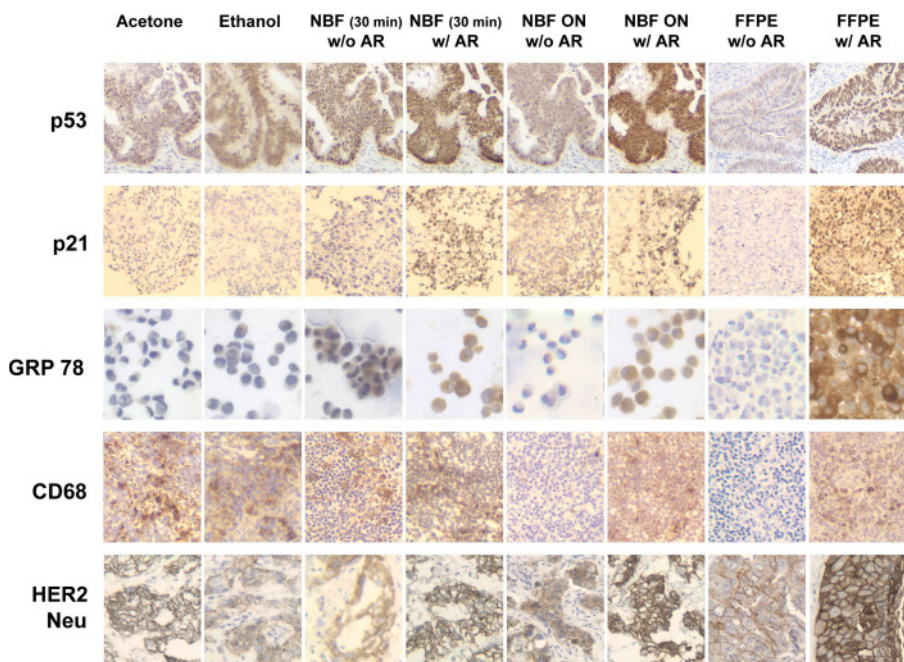


Figure 2.1 Comparison of IHC staining intensity among various protocols of fixation, AR pretreatment for frozen and FFPE cell/tissue sections. Five markers are selected as examples: p53 stained colon cancer tissue (1st row); p21 stained bladder cancer tissue (2nd row); GRP78 stained cell line C42B (3rd row); CD68 stained lymph node tissue (4th row); and HER2 stained breast cancer tissue (5th row). In general, neutral buffered formalin (NBF)-fixed frozen cell/tissue with AR treatment showed identical or stronger IHC staining intensity when compared with that obtained by acetone/ethanol-fixed cell/tissue, except CD68. FFPE cell/tissue sections yield the strongest IHC signals and the best morphology consistently. w/o AR, without use of the AR pretreatment; w/ AR, use of the AR pretreatment prior to IHC staining procedure. (All figures, $\times 200$.) Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology.

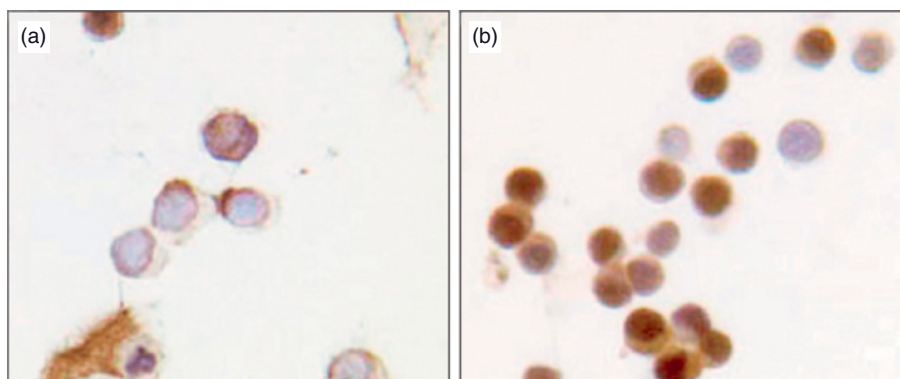


Figure 2.2 Comparison of p21 IHC staining results using fresh cell line MCF-7. (a) Acetone-fixed cells showing an irregular positive staining pattern indicating dislocated p21 protein from nuclei to cytoplasm and outside of cells ($\times 400$). (b) NBF-fixed cells with the use of AR treatment before IHC staining showing an intact nuclear p21 staining pattern ($\times 400$). Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology.

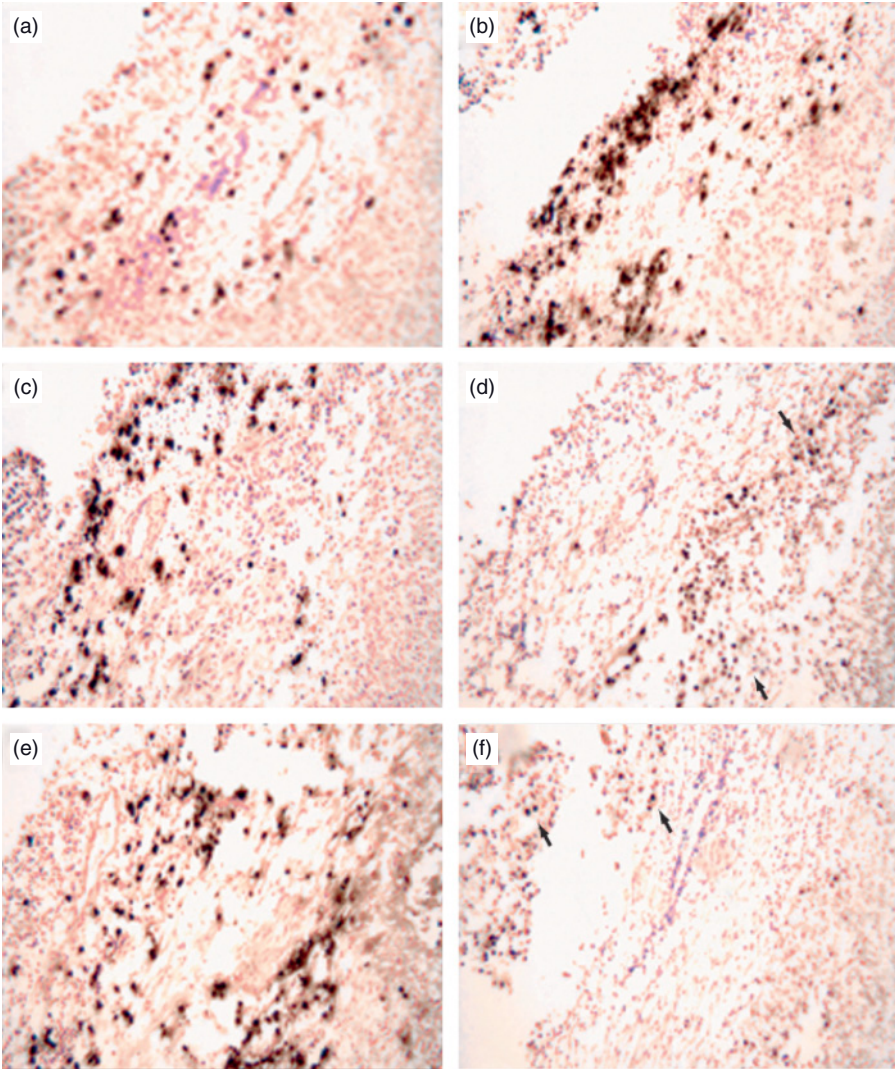


Figure 2.3 Comparison of nonspecific background IHC staining results among various fixation of frozen tissue sections, and antigen retrieval immunohistochemical (AR-IHC) staining protocols. Human bladder cancer tissue samples were used for p21 staining procedure. Significant strong, nonspecific background staining results can be found in acetone-fixed (a), ethanol-fixed (b), NBF-fixed 30 min (c), and NBF-fixed overnight (e) samples showing irregular large dots stained positively. In contrast, the same kinds of NBF-fixed frozen tissue sections after AR treatment before IHC staining (d and f) showing clear background. Arrows indicate the p21-positive nuclear staining results. (a–f, $\times 100$.) Reprinted with permission from Reference 55. © 2008 American Society for Clinical Pathology.

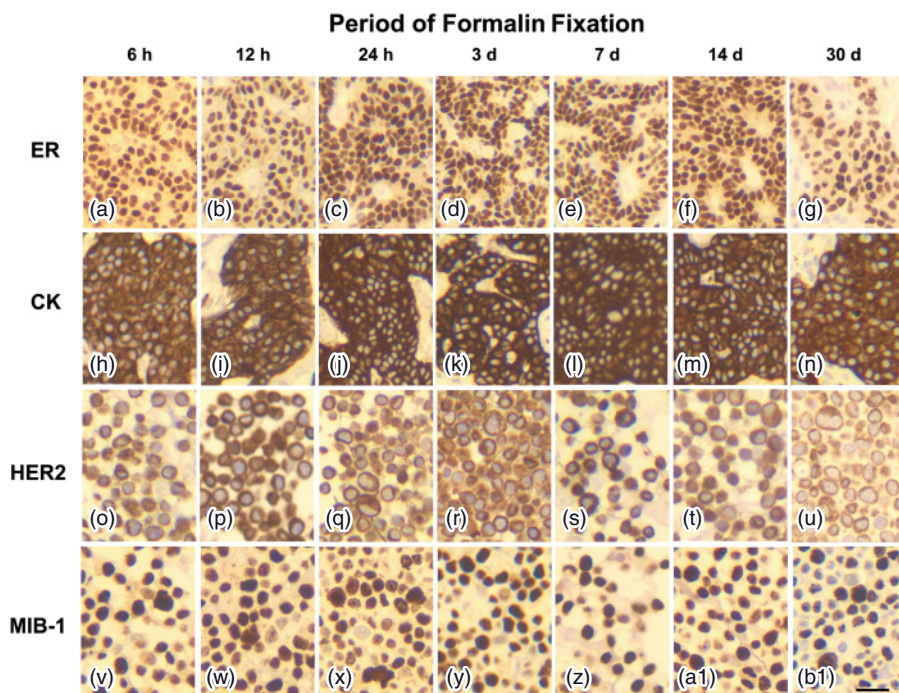


Figure 5.2 Comparison of immunohistochemical staining results among variable periods, 6 h to 30 days, of formalin-fixed, paraffin-embedded human breast cancer tissue (A–N), and cell line MCF-7 sections (O–B1). (See text for full caption).

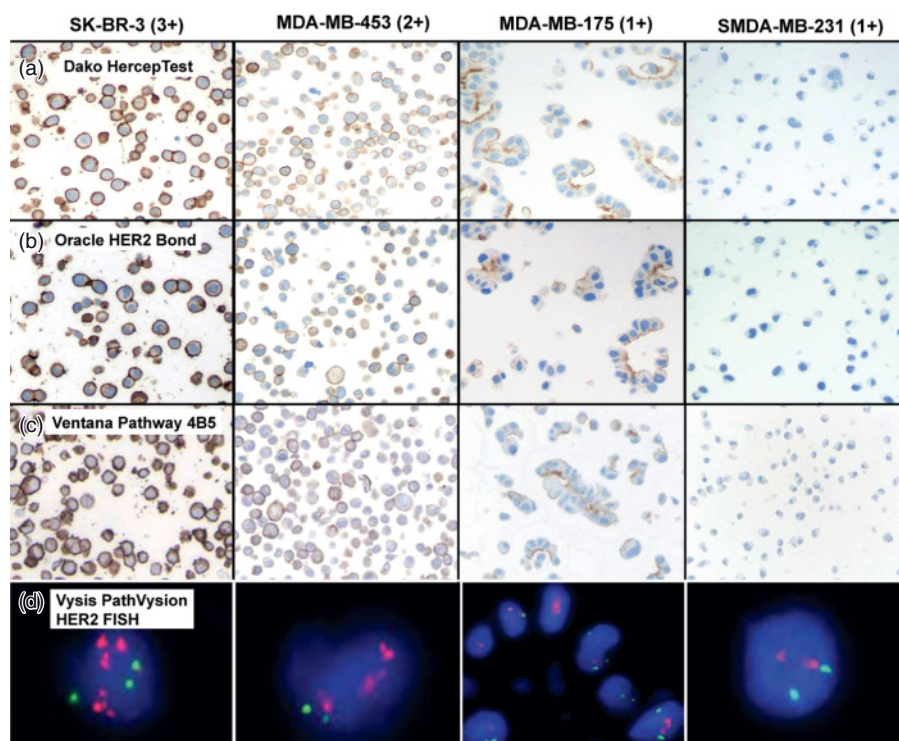


Figure 6.9 The HER2 antigen/gene correctly demonstrated in the UK NEQAS cell line control slides (indicated top to bottom) stained with four commercially validated systems (running left to right) (See text for full caption).

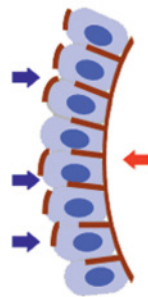
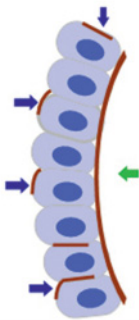
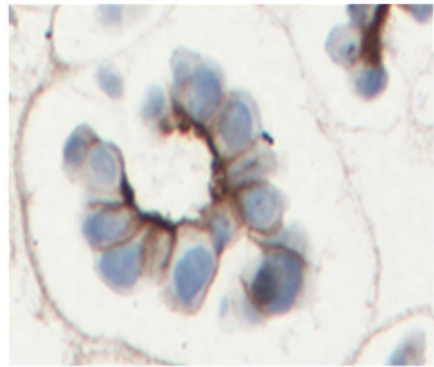
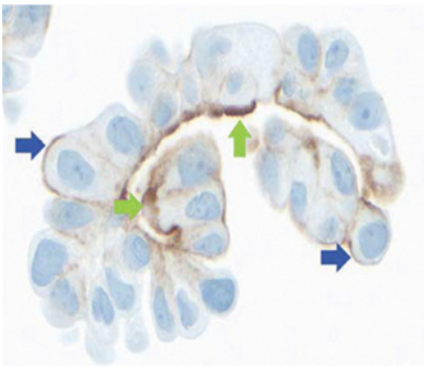
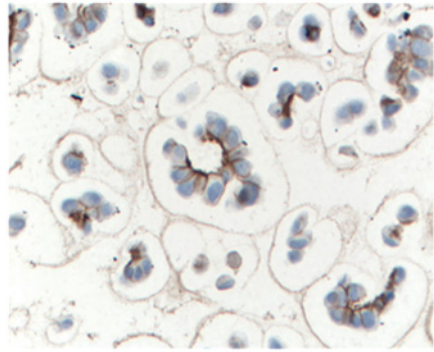
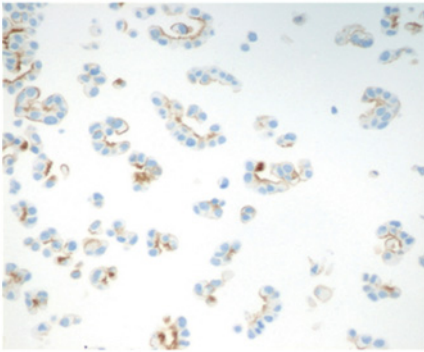


Figure 6.10 MDA-MB-175 (1+) cell line demonstrating unique glandular-like luminal formation with correct HER2 IHC staining pattern highlighted. Stained with Dako HercepTest™ K5204. Blue arrows indicate specific weak incomplete 1+ membrane staining, whereas the green arrows illustrate nonspecific moderate luminal surface staining, which is not interpreted.

Figure 6.11 MDA-MB-175 (1+) cell line demonstrating unique glandular-like luminal formation with incorrect over-stained HER2 IHC pattern. This sections was stained using the Dako Polyclonal (A0485) antibody using pressure cooker antigen retrieval. Blue arrows show specific incomplete staining of moderate intensity, however becoming complete in part, therefore interpreted as being over-stained. The red arrows show overstained luminal surface staining.

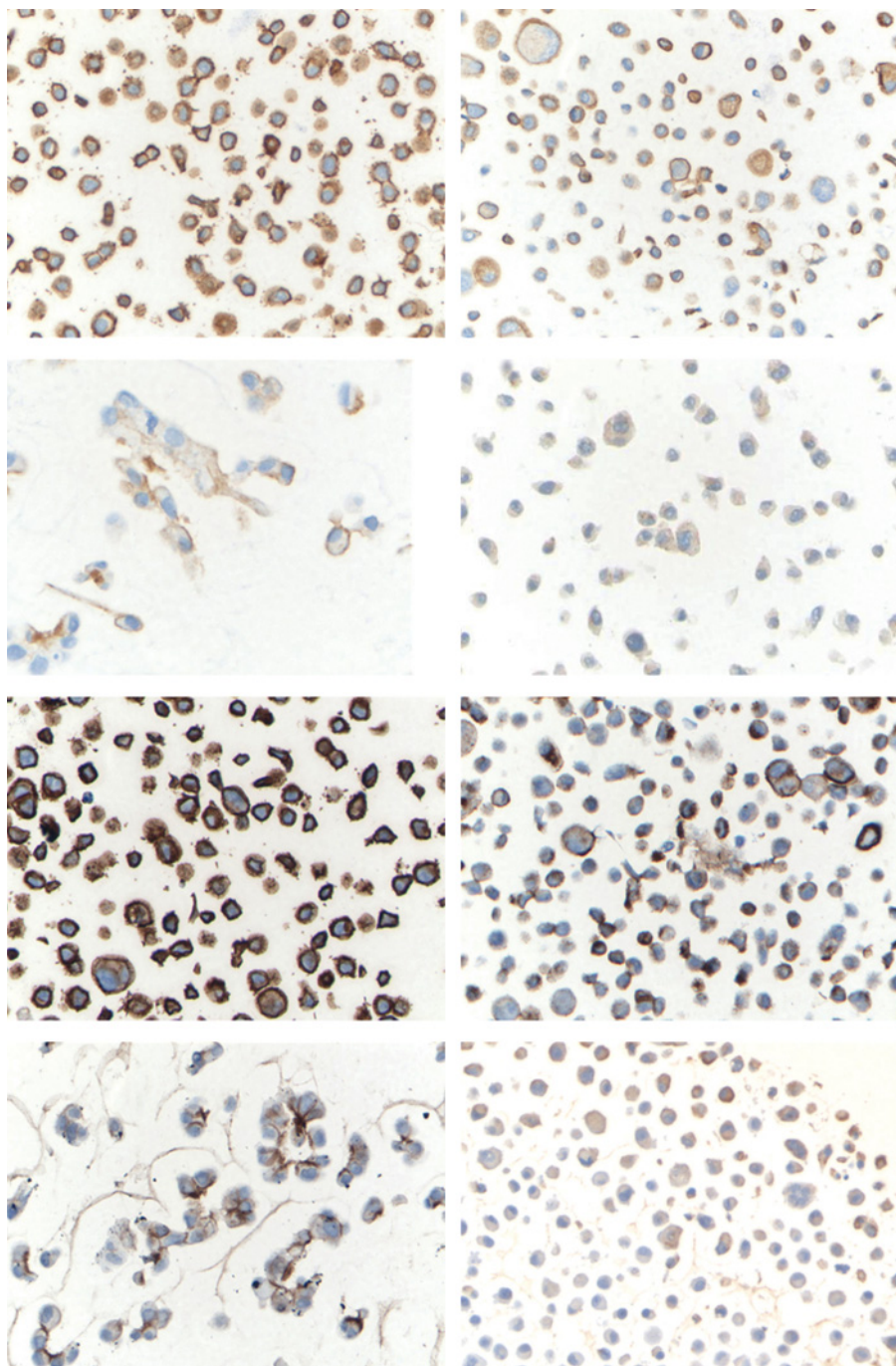


Figure 6.12 Examples of the UK NEQAS Control Cell Lines showing damaged morphology and incorrect IHC profile due to over retrieval.

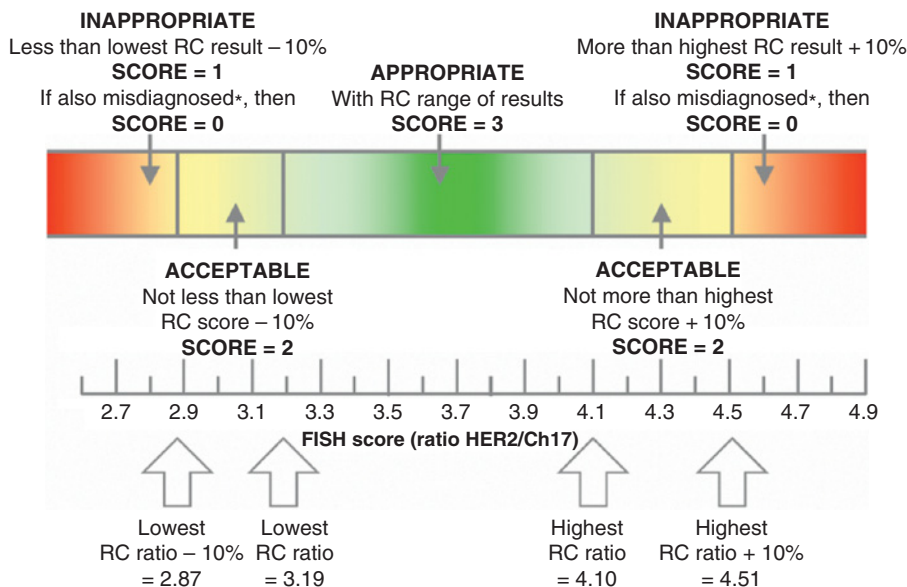


Figure 6.14 Schematic representation of the scoring system; the example illustrated uses the Reference Center set of HER2/Ch17 ratios obtained for the SK-BR3 cell line at Run 4. In this case the lowest ratio obtained by a Reference Center was 3.19, and the highest was 4.10; participants submitting ratios within this range were judged to have achieved an appropriate result (score = 3). The lower cutoff for acceptable ratios (score = 2) was calculated as 3.19 minus 10% of 3.19, that is 2.87; and the upper cutoff was calculated as 4.10 plus 10% of 4.10, that is 4.51. Participants who submitted ratios outside these 10% cutoffs were judged to have achieved an inappropriate result and received a score of 1. Except in the case of the MDA-MB-453 cell line, misdiagnosis (amplified reported as nonamplified, and vice versa) resulted in a score of 0. Superscript notation and abbreviation used in figure: * Does not apply to results obtained for MDA-MB-453 cell line; RC, Reference Center.



Figure 7.9 Appropriate antigen retrieval and immunostaining of peptide controls and tissue sections, stained for HER2. The tissue section on the left has an island of 3+ HER2 tumor, toward the top of the tissue section. The tissue section on the right does not express HER2. Identifying information on the label was removed.



Figure 7.10 Immunostain result with inadequate antigen retrieval, resulting in staining of unfixed but not fixed peptide controls. The HER2+ tumor (left slide) is largely unstained as well.



Figure 7.11 Immunostain result that demonstrates a lower level of sensitivity, in that only the highest peptide concentration is stained. The HER2+ tumor tissue (left slide) is also relatively unstained.

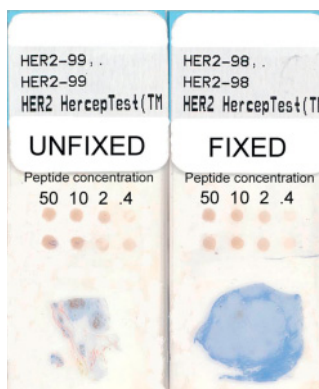


Figure 7.12 Immunostain result that demonstrates a higher than average sensitivity, in that all peptide controls are stained, down to 0.2 µg/mL. The HER2+ tumor tissue (left slide) is also intensely stained.

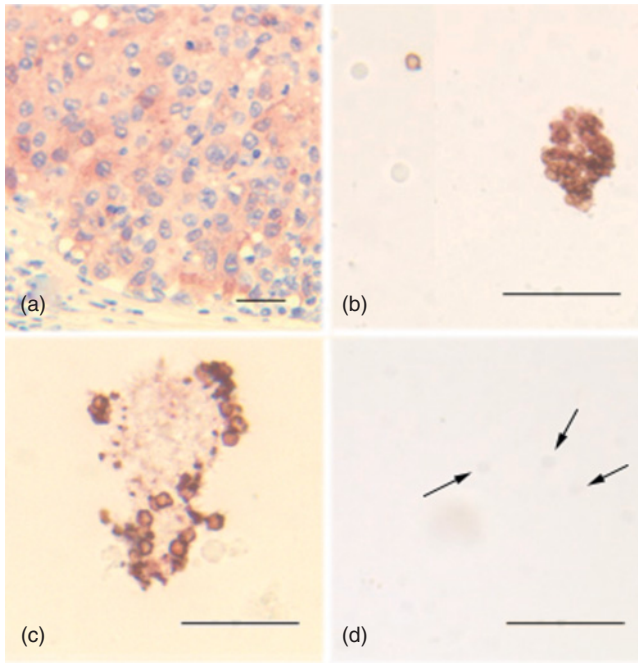


Figure 8.1 The results of IHC of two experiments using Dynabeads (Dynal, New York, NY) coated with biotinylated anti-mouse IgG (first experiment) and protein S-100 (second experiment). (See text for full caption).

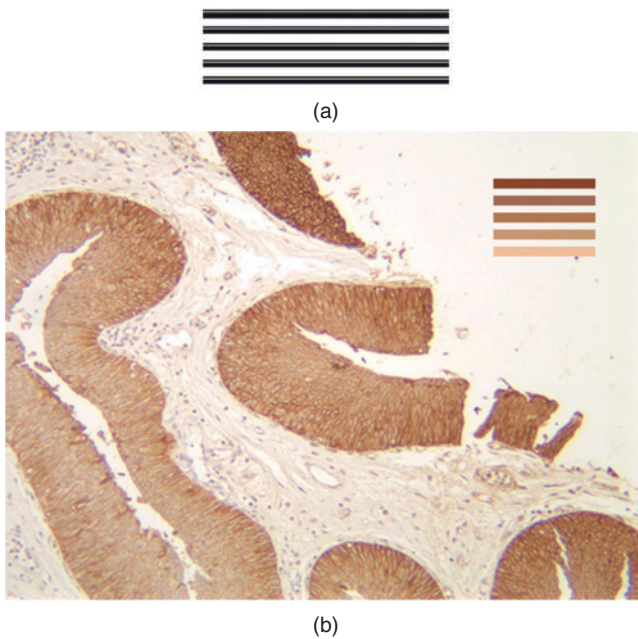


Figure 8.2 Design of protein-embedding barcode is depicted in (a) five thin layers of matrix (the thicker lines) coated with variable concentration of tested protein (thinner lines located above the matrix). (See text for full caption).

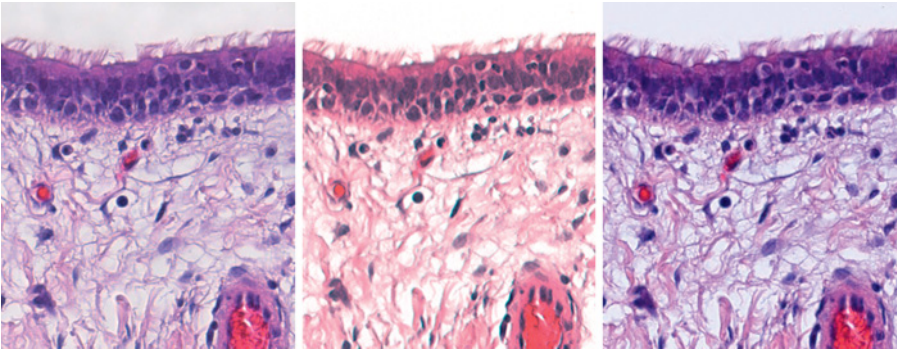


Figure 10.1 The same specimen imaged by three different commercial imaging systems. Note the significant color variations in each image, as well as the shading of the background. There are also visible differences in resolution between the systems, even though total magnification is the same in all images.

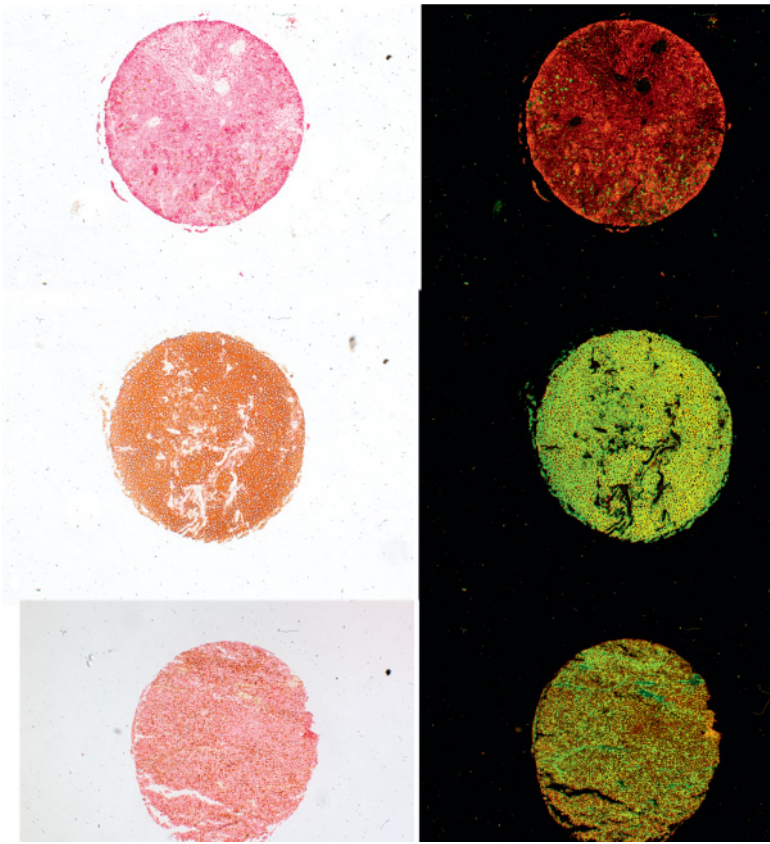


Figure 10.3 Three pairs of images from a single tissue microarray (TMA) block, stained for CD20 using peroxidase-DAB and CD43 using alkaline phosphatase-fast red. In the brightfield images, it is difficult to distinguish the red staining from the brown staining, particularly when one or the other is at low concentration. (See text for full caption).

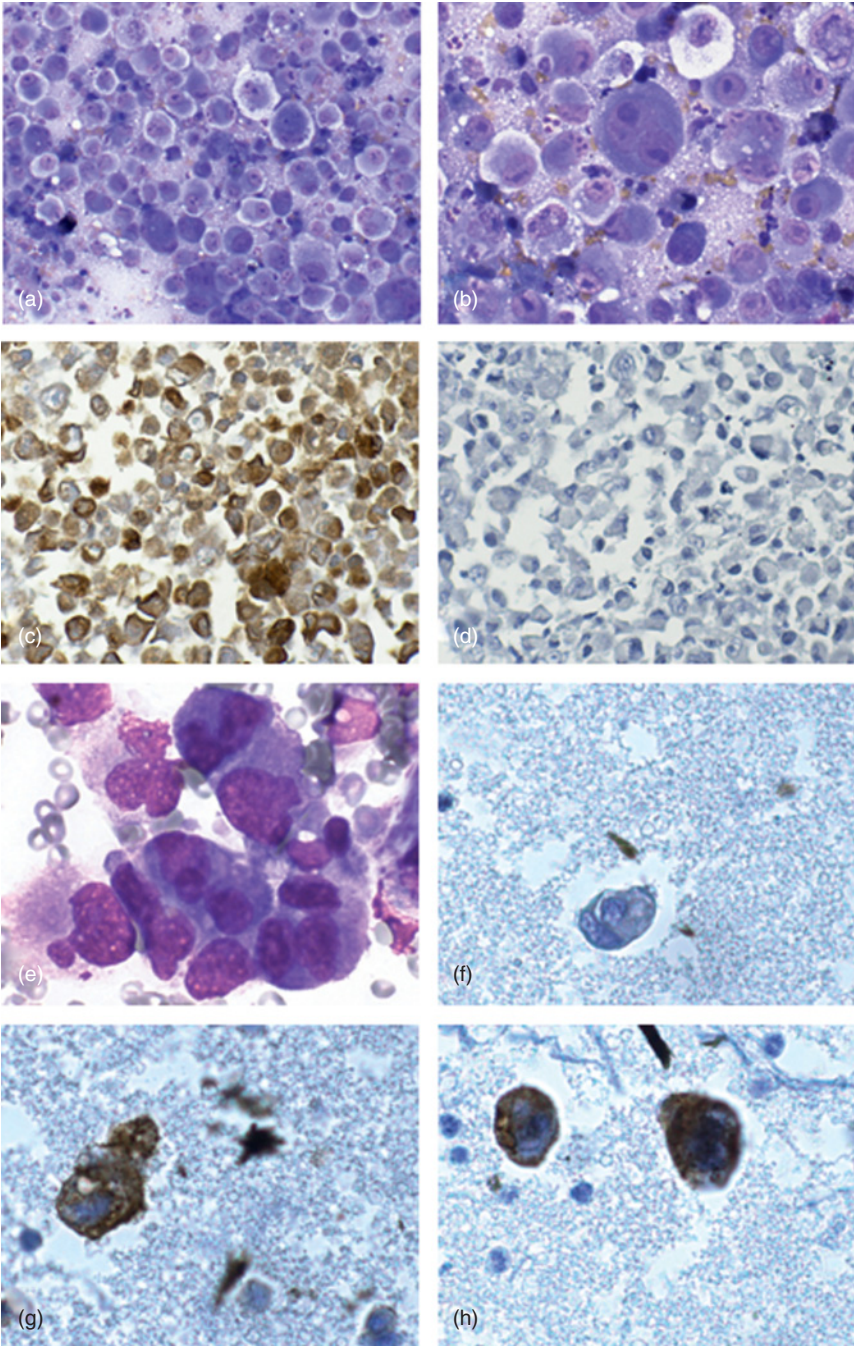


Figure 13.1 (a) and (b) showing an endobronchial ultrasound (EBUS)-FNA biopsy of an enlarged mediastinal lymph node from a 63-year-old male with a left lower lobe lung mass and a remote history of melanoma. (See text for full caption).

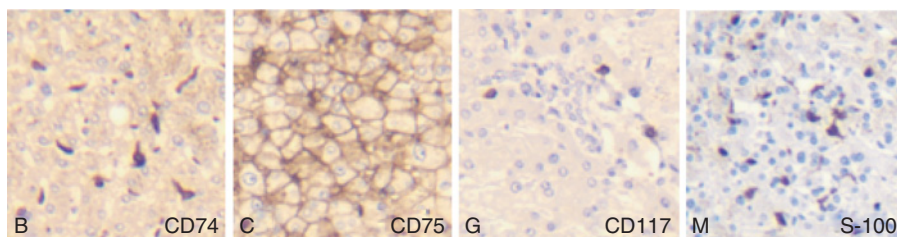


Figure 20.10 Validation by immunohistochemistry of four of the proteins identified in Table 20.1 with single peptide hits. Reproduced with permission from Reference 20.

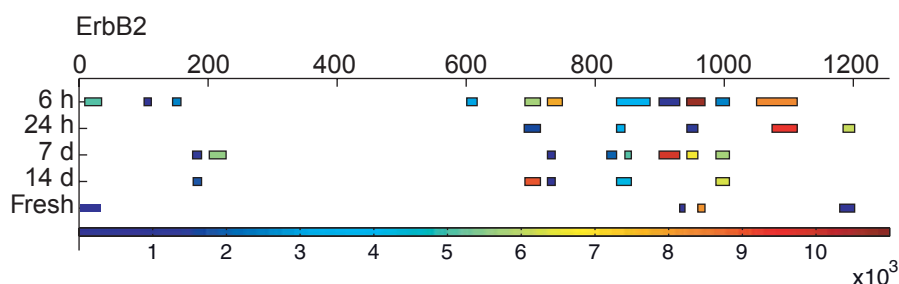


Figure 20.11 Coverage of protein ErbB2 by shotgun proteomic discovery of sample fixed for various times, including fresh. The color gradient represents the increasing abundance of the peptides. All were identified at an FDR < 1%. Reproduced with permission from Reference 20.

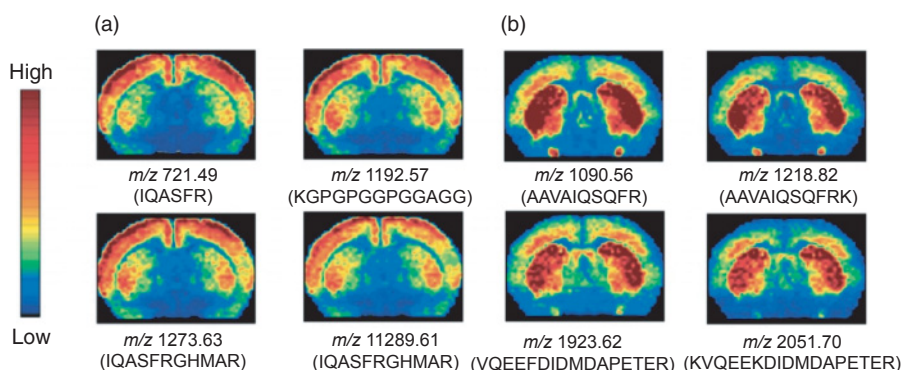


Figure 21.5 (a) Distribution of trypsin-induced peptides generated from the digestion of a 7.5-kDa protein, neurogranin, in a rat brain coronal section. (b) Distribution of trypsin-induced peptides generated from the digestion of the 6.7-kDa protein, PEP-19, in the rat brain coronal section. Reprinted with permission from Groseclose et al.¹⁶

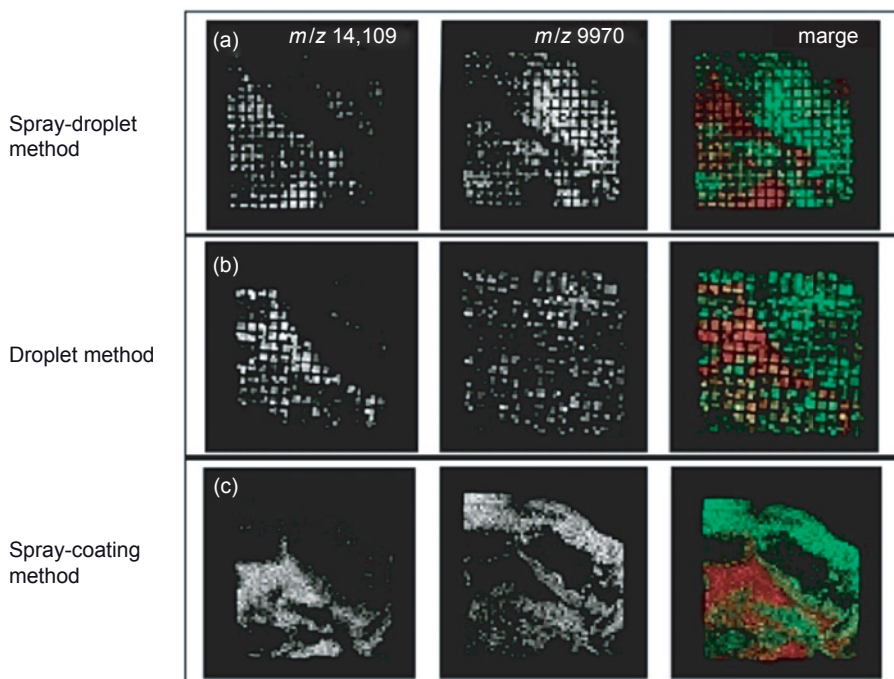


Figure 21.8 Comparison of IMS indicating protein distribution in the rat brain section corresponding to Figure 21.7 (a–c); the spray-droplet (a), droplet (b), and spray-coating (c) methods are used. Reprinted with permission from Sugiura et al.⁷

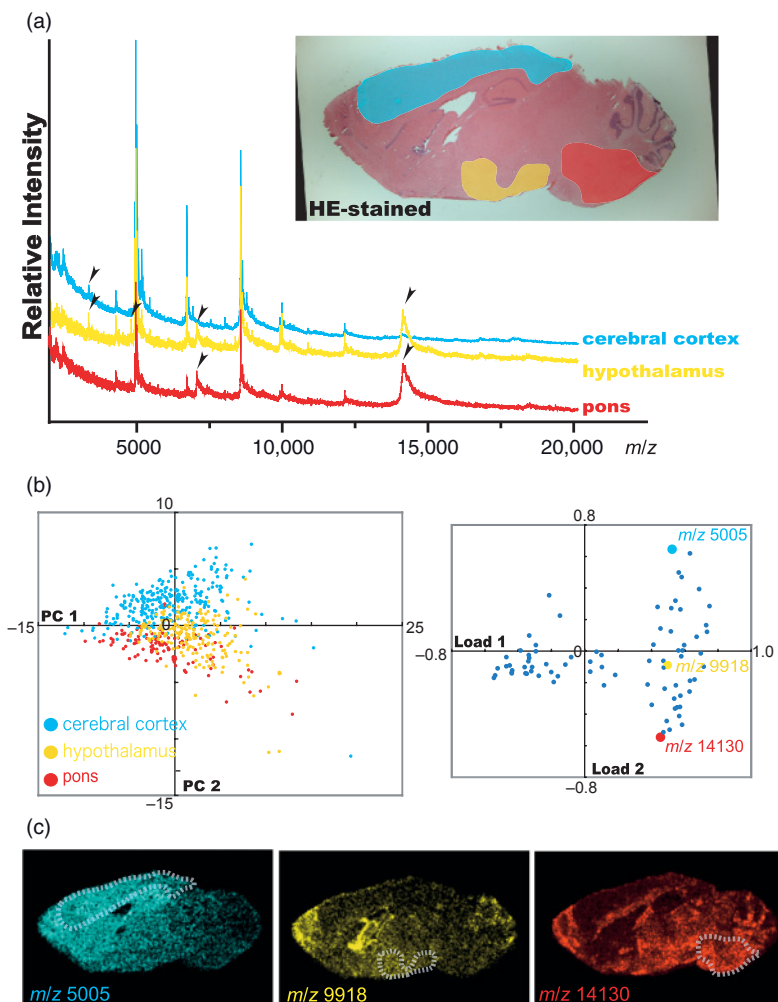


Figure 21.9 *In situ* proteomics and principal component analysis (PCA) of the mouse brain. (a) The mass spectra obtained from each region of mouse brain sections. A sagittal section of the WT mouse brain was analyzed by imaging MS. The observed regions are indicated on the HE-staining images. Signals specific to the regions are indicated by arrowheads. (b) Distribution of various brain components clustered by PCA (left) and the PCA scores plot (right). (c) Reconstructed images of the mouse brain analyzed by imaging MS. The signals show the distribution of substances with indicated m/z . Reprinted with permission from Yao et al.²¹

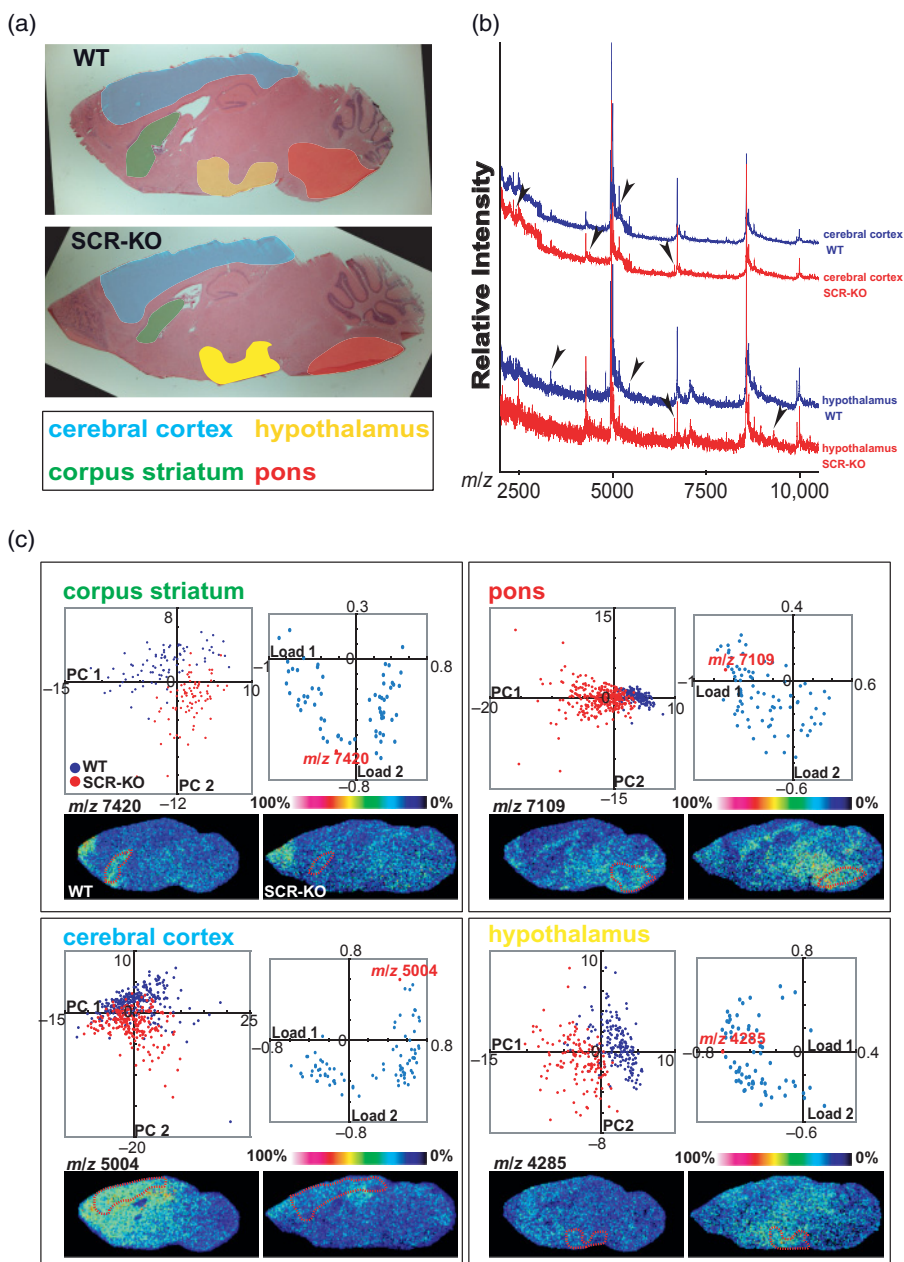


Figure 21.10 *In situ* proteomics of the SCR-KO mouse brain using imaging MS and PCA. (a) HE-staining images of the WT and SCR-KO mouse brain. The regions of focus in imaging MS analyses are indicated in colors. (b) Mass spectra obtained from each region of the WT or SCR-KO mouse brain sections. Specific signals of the regions are indicated by arrowheads. (c) Distributions of various brain components clustered by PCA (left spray graph; WT, blue; KO, red) and the PCA scores plot (right graph). The signal intensities of mass spectra of the substances with indicated m/z are shown in the reconstructed images of the mouse brain analyzed by imaging MS. Reprinted with permission from Yao et al.²¹