Cell Cycle Checkpoint Control Protocols

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Cell Cycle Checkpoint Control Protocols

Edited by

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Preface

The field of cell cycle regulation is based on the observation that the life cycle of a cell progresses through several distinct phases, G1, M, S, and G2, occurring in a well-defined temporal order. Details of the mechanisms involved are rapidly emerging and appear extraordinarily complex. Furthermore, not only is the order of the phases important, but in normal eukaryotic cells one phase will not begin unless the prior phase is completed successfully. Checkpoint control mechanisms are essentially surveillance systems that monitor the events in each phase, and assure that the cell does not progress prematurely to the next phase. If conditions are such that the cell is not ready to progress—for example, because of incomplete DNA replication in S or DNA damage that may interfere with chromosome segregation in M—a transient delay in cell cycle progression will occur. Once the inducing event is properly handled—for example, DNA replication is no longer blocked or damaged DNA is repaired—cell cycle progression continues. Checkpoint controls have recently been the focus of intense study by investigators interested in mechanisms that regulate the cell cycle. Furthermore, the relationship between checkpoint control and carcinogenesis has additionally enhanced interest in these cell cycle regulatory pathways. It is clear that cancer cells often lack these checkpoints and exhibit genomic instability as a result. Moreover, several tumor suppressor genes participate in checkpoint control, and alterations in these genes are associated with genomic instability as well as the development of cancer.

Cell Cycle Checkpoint Control Protocols is designed to augment the growing field and, through detailed descriptions of cell cycle-related methodologies using mammalian, yeast, and frog model systems, aid in the performance of experiments that bear on furthering the understanding of cell cycle checkpoint control. Chapters include descriptions of methods to induce cell cycle checkpoints, detect changes in cell cycle progression, identify and analyze genes and proteins that regulate the process, and characterize chromosomal status as a function of cell cycle phase and progression. The list of protocols is by no means complete, yet is comprehensive enough to at a minimum describe major methodologies used by investigators in the field.

The editor wishes to thank colleagues and friends in the Center for Radiological Research at Columbia University for their valuable support and input, and the authors of all the chapters whose contributions are really the foundation of this volume. In addition, a special thanks is extended to Dr. John Walker for his valuable guidance and encouragement during every facet of this project, from its inception to the final publication of this book.

Howard B. Lieberman

Dedication

This book is dedicated to three very special people in my life, my wife, Jaime, and two kids, Emma and Jared, whose encouragement, patience, understanding, occasional light-heartedness, and love were sustaining forces that helped carry this book to completion.

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I

INDUCTION AND DETECTION
OF CHANGES IN CELL CYCLE PROGRESSION

1

Methods to Induce Cell Cycle Checkpoints

Howard B. Lieberman and Kevin M. Hopkins

1. Introduction

The way cells respond to radiation or chemical exposure that damages deoxyribonucleic acid (DNA) is important because induced lesions left unrepaired, or those that are misrepaired, can lead to mutation, cancer, or lethality. Prokaryotic and eukaryotic cells have evolved mechanisms that repair damaged DNA directly, such as nucleotide excision repair, base excision repair, homology-based recombinational repair, or nonhomologous end joining, which promote survival and reduce potential deleterious effects *(1)*. However, at least eukaryotic cells also have cell cycle checkpoints capable of sensing DNA damage or blocks in DNA replication, signaling the cell cycle machinery, and causing transient delays in progression at specific phases of the cell cycle *(2*; *see* **ref. 3** for a review). A related but more primitive system may exist in prokaryotes *(4–7)*. These delays are thought to provide cells with extra time for mending DNA lesions before entry into critical phases of the cell cycle, such as S or M, events that could be lethal with damaged DNA.

The precise mechanisms by which checkpoints function are under intensive investigation, and details of the molecular events involved are being pursued vigorously. This is owing not only to the complexity and the intellectually and technically challenging aspects of the process (*see* **ref. 3** for a review) but also to the relevance of these pathways to the stabilization of the genome and carcinogenesis *(8)*. Nevertheless, it is clear that checkpoint mechanisms are very sensitive and can be induced by the presence of relatively small amounts of DNA damage. For example, in the yeast *Saccharomyces cerevisiae*, as little as a single double-strand break in DNA can cause a delay in cell cycle progression *(9,10)*. One important aspect of studying cell cycle checkpoint mechanisms is an understanding of how to induce the process.

   This chapter focuses on the application of radiations, such as gamma rays and ultraviolet (UV) light, that are capable of causing DNA damage, and thus leading to the induction of cell cycle checkpoints. Certain chemicals, or the use of temperature-sensitive mutants to disrupt DNA replication, are also used routinely to induce checkpoints, but related protocols are not described in this chapter. Gamma rays cause primarily single- and double-strand breaks in DNA but can infrequently induce nitrogenous base damage as well. In contrast, UV light (i.e., 254 nm) causes a preponderance of bulky lesions, such as pyrimidine dimers, although single-base damage and strand breaks are a smaller part of the array of lesions that can be produced. Regulation of cell cycle checkpoints induced by ionizing radiation versus UV light is mediated by overlapping but not identical genetic elements *(11–13)*. Although the protocols described in this chapter concern the treatment of mammalian cells, the same general principles can apply to irradiation of yeast and other types of nonmammalian cells as well.

2. Materials

2.1. Supplies

   1. Cells: Any mammalian cell type is appropriate for exposure to gamma rays, but those that can grow attached to a Petri dish surface (glass slide or any other open surface) as a monolayer, such as fibroblasts, are ideal for UV-related experiments because this nonionizing radiation does not efficiently penetrate medium or reach one cell "shielded" by another.
   2. Growth medium: standard mammalian medium appropriate for the cells of interest (i.e., Dulbecco's modified Eagle's medium [DMEM], Roswell Park Memorial Institute-1640 [RPMI-1640], McCoy's, etc.), available commercially from several companies: Atlanta Biologicals (Norcross, GA), Invitrogen (Carlsbad, CA), Mediatech (Herndon, VA), Sigma-Aldrich (St. Louis, MO), Specialty Media (Phillipsburg, NJ).
   3. Sterile phosphate buffered saline (PBS) made up as 0.144 g/L KH_2PO_4, 9 gm/L NaCl, 0.795 g/L $Na_2HPO_4·7H_2O$ in distilled H_2O, pH adjusted to 7.0 and autoclaved, or purchased commercially from Atlanta Biologicals (Norcross, GA) Invitrogen (Carlsbad, CA), Mediatech (Herndon, VA), Sigma-Aldrich (St. Louis, MO), Specialty Media (Phillipsburg, NJ).
   4. Petri dishes or flasks (*see* **Note 1**): Any size and shape Petri dish, multiwell dish, or flask will be fine, and it should be chosen based on the number of cells needed to irradiate, as well as any particular requirements posttreatment. A large selection of tissue-culture ware is available from numerous commercial suppliers (e.g., BD Falcon (Bedford, MA), Corning (Corning, NY), Nunc [Naperville, IL]).

2.2. Equipment

2.2.1. Source of Ionizing Radiation

   Several different types of equipment are used, and various manufacturers provide the needed sources. The following are some examples:

1. X-rays: Siemens Stabilipan (Siemens, Iselin, NJ)
2. Gamma rays: Based on the decay of ^{60}Co, such as a Gammacell 220 (Nordion, Alberta, Canada) for a high dose rate, or based on the decay of ^{137}Cs, such as a Gammacell 40 (Nordion, Alberta, Canada) for a lower dose rate (*see* **Notes 2** and **3**).
3. Source of UV light: Usually a germicidal bulb is used to produce 254 nm UV light as an inducer of cell cycle checkpoints (*see* **Fig. 1** and **Note 4** for details).
4. Voltage stabilizer: Constant Voltage Transformer, Catalog number 30806 (Sola Electric, Chicago, IL; *see* **Note 5**).
5. UV meter and probe (Model UVX Digital Radiometer, Probe Model UVX-25, UVP Inc., Upland, CA; *see* **Note 6**).

3. Methods

3.1. Preparation of Cells

1. All procedures involving cell culturing should follow standard sterile techniques and optimum conditions for growth of the specific cells of interest.
2. For cell cycle studies, our laboratory has routinely employed mouse embryonic stem cells, so their use will serve as an example. Other mammalian cells can easily be adapted, with modifications, to essentially the same procedures.
3. Cells are grown in DMEM.
4. Add 0.1 m*M* nonessential amino acids.
5. Add 1 m*M* sodium pyruvate.
6. Add 10^{-4} *M* β-mercaptoethanol.
7. Add 2 m*M* L-glutamine.
8. Add 15% fetal bovine serum (FBS) ES cell qualified (heat inactivated, 56°C, 30 min).
9. Add 50 µg/mL penicillin.
10. Add 50 µg/mL streptomycin.
11. Add 1000 U/mL Leukemia Inhibitory Factor (LIF).
12. The cells are seeded into 6 well or 10-cm dishes at a concentration of 1×10^5 cells per mL or 1×10^6 cells per mL, respectively.
13. Cells should be plated and allowed to attach as well as grow for 1 d prior to irradiation.
14. At this same time, an equal number of cells and dishes should be prepared to provide conditioned medium for the experimental cells postirradiation.
15. Control cells should be prepared separately from the cells that will be irradiated if multiwell dishes are being used.
16. Cells are grown in a 37°C incubator with a 5% CO_2 humidified atmosphere.
17. At the time of irradiation, cells should be actively growing and in log phase.
18. Cells should not be confluent at the time of irradiation, unless studies on a quiescent population are specifically planned.
19. In addition, for UV-light-related experiments, cells should be plated at least 0.25 in. from the perimeter of the Petri dishes because the lip can interfere with exposure of cells in the vicinity.

3.2. Exposure to Ionizing Radiation

1. To expose cells to gamma rays, dishes or flasks are transferred from the 37°C incubator to the irradiator.
2. The instructions that accompany each machine should then be followed to ensure accurate and safe operation (*see* **Note 7**).
3. When the irradiation is completed, the dishes are removed from the chamber and transferred back to the 37°C incubator for further incubation (*see* **Note 8**).

3.3. Exposure to UV Light (254 nm)

1. The UV light apparatus must be turned on for at least 10 min prior to the irradiation of cells. This will ensure that the UV light is emitted at a stable, constant dose rate, and the chamber is sterilized.
2. The dose of UV light can be determined by using a radiometer, in conjunction with the appropriate probe for detecting 254 nm wavelength light. We typically expose cells at a dose rate of 1.0 J/m^2 (*see* **Note 9**).
3. Before exposing cells to UV light, the cell growth medium needs to be removed. This is achieved by either aspiration or pipetting.
4. The cells are then washed twice with sterile PBS to remove residual medium. The PBS must be completely removed before exposing the cells to UV light.
5. Place the covered dishes in the UV chamber, making sure that the dishes will be directly underneath the UV bulb. Remove the lids from the dishes, close the chamber door, then simultaneously fully pull open the shutter and start timing the exposure.
6. When the appropriate time has been reached, push the shutter to the completely closed position. Open the chamber door, replace the lids, then remove the dishes from the chamber.
7. Immediately add conditioned medium to the irradiated cells equivalent to the amount of medium present prior to irradiation.
8. The dishes should then be returned to their appropriate incubating apparatus.
9. This wavelength of light is carcinogenic and cataractogenic. Therefore, proper precautions should be taken to avoid investigator exposure (*see* **Note 10**).
10. Furthermore, manipulations during and soon after irradiation should be performed in very dim light or under yellow lights to ensure exposure occurs without the neutralizing effects of repair by photoreactivation (if potentially active in the cells being exposed) or photorepair *(1)*. These repair processes usually need intense light for proper function, so even a dimly lit room should be appropriate for avoiding unwanted repair by these activities that could reduce a checkpoint inducing DNA damage signal. Mammalian cells, in general though, have weak photoreactivation capability. This coupled with the usual presence of other more active repair mechanisms makes this issue, however, essentially not a significant concern.
11. For mammalian cells (or yeast and other microorganisms for that matter) that must be in liquid culture, resuspend in a minimum amount of PBS or sterile water if cells will remain viable, then irradiate while swirling the liquid to optimize for even exposure of samples. Circular movement of the dishes to cause swirling can

be performed manually or by use of an electric gyrating platform available commercially (Lab Rotator Model 1304, Lab-Line Instruments, Melrose Park, IL). If performed manually, remember to follow the precautions outlined in **Note 10**.

4. Notes

1. Gamma rays and X-rays are highly energetic and can penetrate as well as pass through cells, Petri dishes, and flasks. UV light cannot pass through these objects efficiently. Therefore, for UV irradiation, cells should be plated onto Petri dishes such that the lids can be removed for proper exposure.
2. Gamma rays and X-rays are both forms of ionizing radiation, with slightly different energies. However, they produce essentially comparable biological effects when applied at the same doses and similar dose rates.
3. Although we use equipment manufactured by Siemens and Nordion, as listed, comparable devices are available from other commercial sources, such as Shepherd Model 280, JL Shepherd & Associates, San Fernando, CA.
4. The main component for production of UV light is a germicidal bulb capable of emitting 254 nm UV light (Model X-15B, bulb number 34000801, UVP Inc., Upland, CA). An apparatus illustrated in **Fig. 1** is most convenient for exposing cells to UV, but other perhaps simpler systems are just as valid.
5. It is important to have a stable, constant voltage delivered to the UV light fixture. This will ensure a uniform, constant, reproducible dose rate during the exposure of samples.
6. The dose rate emitted from a germicidal bulb usually remains fairly constant for many years. However, when a new bulb is first set up, a UV meter should be used to determine the dose rate, and this parameter should be checked periodically. Be sure to use a probe for the meter that is capable of measuring 254 nm UV light, as probes are available for detecting different wavelengths of light.
7. Consult the manufacturer of the equipment, as well as the local Radiation Safety Department, to ensure that the equipment is monitored, maintained, and used properly.
8. Dose and dose rate are important parameters to consider when using gamma rays to induce cell cycle checkpoints. We typically expose mammalian cells to between 8 and 20 gy (800 to 2000 rads) of gamma rays, although even lower doses may be sufficient to induce a cell cycle checkpoint or the desired effect. Even though the high dose range kills 99.99% of the cells, we use this high dose when long-term viability is not an issue. This dose is fine when using flow cytometry to study delays in cell cycle progression, within 24 h posttreatment, because even this high dose range will not immediately kill cells and will allow them to cycle long enough to be able to express a transient delay. This high dose is also reasonable if cell extracts will be isolated, and intact reproductive capacity is not a relevant issue. Some published papers have reported the use of doses as high as 50 or more gy, but usually such levels are not necessary to observe a cell cycle effect. We typically use a dose rate of approximately 1 gy/min

Fig. 1. Source of 254 nm UV light. **(A)** Photograph of UV light box closed. **(B)** Same light box opened with Petri dishes inside. **(C)** *(next page)* Diagram of light box depicting dimensions and side view. Germicidal bulb serving as the source of UV is on top of makeshift shutter system. A voltage stabilizer connecting light fixture to an A/C socket is also presented. The inside walls are painted black, and black material is used for the bottom surface as well. This reduces reflection of light.

C
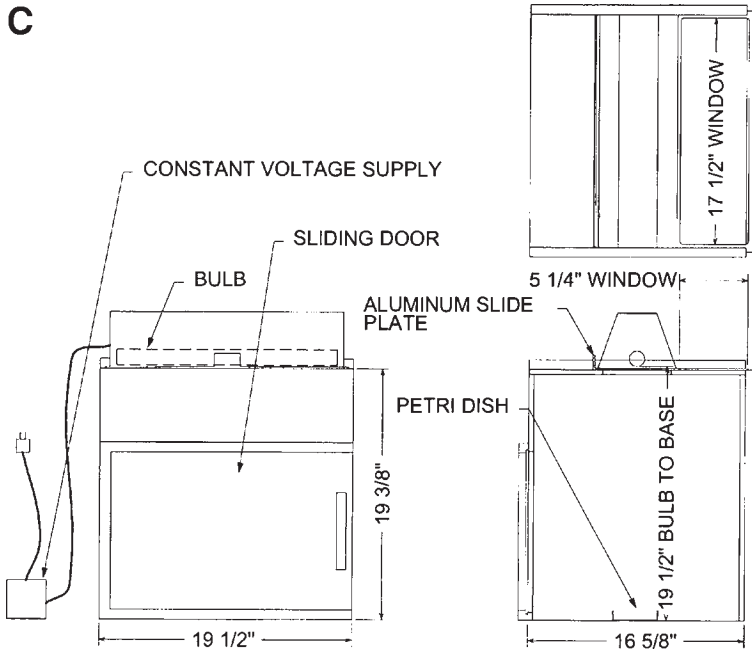
Fig. 1. *(continued).*

(For yeast, we typically use a Gammacell 220 irradiator with a dose rate of 30 gy/min.) Higher dose rates are probably fine, but significantly lower dose rates should be avoided. The problem involves DNA repair and the elimination of the potential checkpoint-inducing signal. Low dose rates will allow repair to occur efficiently, resulting in the rapid removal of damage and, thus, the cell cycle checkpoint signal. If equipment constraints will only allow the application of ionizing radiation at low dose rates, cells can be kept on ice during exposure. However, this is not ideal because such incubation can by itself potentially perturb cell cycle kinetics and add an additional experimental variable that should really be avoided.

9. Dose rate can be altered by changing the distance between the germicidal bulb and the sample. The dose rate changes as the inverse square of the distance, such that for example if the distance between the sample and the bulb is halved, then the dose rate increases fourfold.
10. Do not look directly or indirectly at the light emitted from the bulb. Wear a long-sleeved shirt or a lab coat. Protective eyewear would also be helpful.

Acknowledgments

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2

Methods for Synchronizing Mammalian Cells

Michael H. Fox

1. Introduction

When studying cell cycle checkpoints, it is often very useful to have large numbers of cells that are synchronized in various stages of the cell cycle. A variety of methods have been developed to obtain synchronous (or partially synchronous) cells, all of which have some drawbacks. Many cell types that attach to plastic culture dishes round up in mitosis and can then be dislodged by agitation. This mitotic shake-off method, originally discovered by Terasima and Tolmach *(1)*, is useful for cells synchronized in metaphase, which on plating into culture dishes move into G1 phase in a synchronous manner. A drawback to the mitotic shake-off method is that only a small percentage (2–4%) of cells are in mitosis at any given time, so the yield is very small. Also, cells rapidly become asynchronous as they progress through G1 phase, so the synchronization in S phase and especially G2 phase is not very good. The first limitation can be overcome by plating multiple T150 flasks with cells, using roller bottles, or blocking cells in mitosis by inhibitors such as Colcemid or nocodazole *(2)*. Mitotic cells that are collected can be held on ice for an hour or so while multiple collections are done to obtain larger numbers of cells.

To obtain more highly synchronous populations of cells in S phase, the mitotic shake-off procedure can be combined with the use of deoxyribonucleic acid (DNA) synthesis inhibitors, such as hydroxyurea (HU) or aphidicolin (APH), to block cells at the G1/S border (but probably past the G1 checkpoint). APH inhibits DNA polymerase α *(3–5)*, whereas HU inhibits the enzyme ribonucleotide reductase *(6)*, though it may operate by other mechanisms also *(7)*. On release from the block, cells move in a highly synchronized fashion through S phase and into G2 phase *(8)*. In terms of number of synchronized cells, this method has the same limitation as discussed above, because the starting cell population

derives from the mitotic shake-off procedure. In addition, the block of cells with drugs can cause unbalanced cell growth, so one cannot necessarily conclude that all biochemical processes are also synchronized.

Large numbers of synchronous cells can be obtained using centrifugal elutriation *(9)*. This method requires the use of a special rotor in a large floor centrifuge and separates cells into the cell cycle based on cell size. Cells may be obtained in early or late G1 phase, or primarily in S phase. However, the cell populations are not highly synchronous in S phase but instead have significant populations of G1- and G2-phase cells included. Nevertheless, it is possible to synchronize very large numbers of cells using this method, and biochemical processes are not perturbed.

Another method that results in highly synchronous populations is based on labeling cells with a viable dye for DNA (Hoechst 33342) *(10)*. Cells stained with this dye can then be sorted by cell cycle phase. Sorted G1 cells will be distributed throughout G1, however. Cells in S phase can be sorted into a small window in S phase and thus will be highly synchronized, but only a small number of cells can be obtained. G2 phase cells will be contaminated with late S phase cells. Furthermore, some cell types do not stain well with Hoechst 33342, so sufficiently good DNA histograms cannot be obtained.

The protocols presented in this chapter are based on the mitotic shake-off procedure optimized to obtain large numbers of cells. Procedures for obtaining highly synchronized cells in G1 phase, various stages in S phase, and G2 phase are described, along with DNA histograms demonstrating the quality of results that can be obtained.

2. Materials

2.1. Cell Culture

1. Attached cell lines, such as Chinese hamster ovary (CHO) or HeLa.
2. Appropriate medium, such as Ham's F12 or minimum essential medium (MEM).
3. Fetal bovine serum (10–15%).
4. T75 or T150 tissue-culture flasks, or both.

2.2. Stock Solutions and Reagents

1. HU (2 m*M* in medium).
2. APH (1–3 µg/mL in medium; *see* **Note 1**).
3. Trypsin (*see* **Note 2**).

2.3. Equipment

1. Variable speed shaker with platform to hold T75 or T150 tissue-culture flasks (available from Fisher Scientific, VWR, Daigger, ISC Bioexpress, etc.).
2. Flow cytometer for analysis of synchronized cell populations.
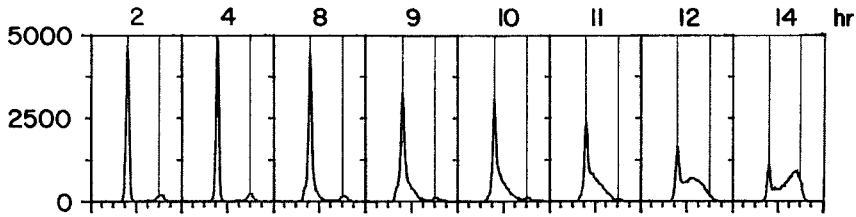
Fig. 1. Flow cytometry histograms at various times after mitotic selection synchrony procedure. The vertical lines show locations of the G1 and G2 peaks. The time after mitotic selection is shown at top of histograms. This figure is reproduced in part from **ref. 8**.

3. Floor centrifuge to spin down large volumes of cells and medium or tabletop centrifuge that can handle 50-mL centrifuge tubes for smaller experiments.

3. Methods

3.1. Mitotic Shake-Off for G1 Synchrony

1. Culture cells in T75 or T150 flasks (*see* **Note 3**).
2. Trypsinize and replate $3–5 \times 10^6$ cells in 25 mL medium in T150 flasks.
3. Grow cells in incubator for 24–48 h to increase cell numbers (*see* **Note 4**).
4. Select mitotic cells by placing flasks on shaker tray suitable for holding 96-well trays and shake for 30 s to 1 min at 150–200 rpm (*see* **Note 5**).
5. Collect the medium containing mitotic cells in 500-mL centrifuge bottles and put on ice.
6. Add 25 mL prewarmed medium to flasks and incubate for 10 min.
7. Select mitotic cells by repeating **steps 4–6**. This can be done sequentially for 1–2 h to collect sufficient numbers of mitotic cells (*see* **Note 6**).
8. After sufficient numbers of cells have been collected and held on ice, pool the collections and centrifuge them in a floor-model centrifuge to concentrate the mitotic cells.
9. Plate appropriate numbers of cells ($1–5 \times 10^5$) into T25 flasks.
10. Add 4 mL prewarmed medium.
11. Incubate flasks in a 37°C incubator for desired time to get cells in early-, mid-, or late-G1 phase (*see* **Note 7** and **Fig. 1**).
12. Process parallel samples for cell cycle analysis to monitor cell cycle progression. Fix cells with 70% ethanol on ice for 20–30 min, stain with propidium iodide for 5–10 min, and analyze by flow cytometry (*see* Chapter 4 in this book for details on flow cytometry cell cycle analysis).

3.2. Mitotic Shake-Off Plus HU for S- and G2-Phase Synchrony

1. Follow **steps 1–8** in **Subheading 3.1**.
2. Plate appropriate numbers of cells ($1–5 \times 10^5$) into T25 flasks containing 4 mL medium with 2 m*M* HU.
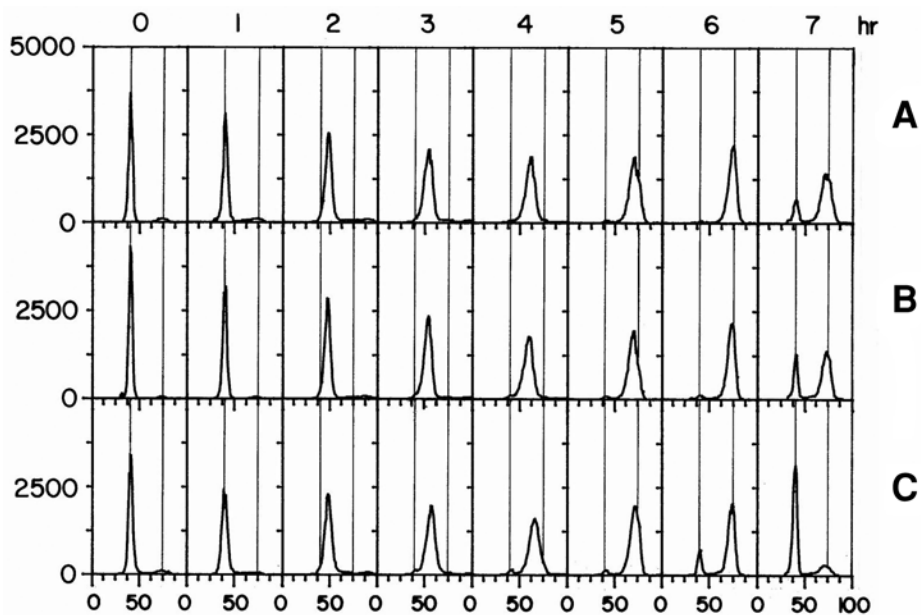
Fig. 2. Flow cytometry histograms obtained after releasing cells from mitotic selection plus 12 h of drug. **(A)** 2 m*M* HU. **(B)** 3 µg/mL APH. **(C)** 1 µg/mL APH. This figure is reproduced from **ref. 8**.

3. Incubate for approx 1 cell cycle time (approx 12 h for CHO cells, 24 h for human cells; *see* **Note 8**).
4. Aspirate off medium; rinse once with 4 mL of warm (37°C) medium.
5. Add 4 mL of warm medium and put flasks in incubator.
6. Remove flasks at various times after removing medium to get cells synchronized at various points in S phase or G2 phase (*see* **Note 9** and **Fig. 2A**).
7. Process parallel samples for flow cytometry analysis to determine the exact location of cells in S phase. To determine the G2 phase, it is best to use BrdU uptake and dual-parameter flow cytometry analysis as described in **Chapter XX**.

3.3. Mitotic Shake-Off Plus APH for S and G2 Synchrony

1. Follow **steps 1–10** in **Subheading 3.1.**
2. Add 4 µL APH from 10 mg/mL dimethyl sulfoxide (DMSO) stock to flasks for a final concentration of 1 µg/mL.
3. Follow **steps 3–7** in **Subheading 3.2.** (*see* **Note 10**, and refer to **Fig. 2B,C** for examples of synchronized populations obtained by this method).

4. Notes

1. APH is made in a stock solution of 10 mg/mL DMSO because it is not water soluble. At a final concentration of 3 µg/mL APH, the DMSO concentration is only 0.03% and should have little effect on cells.

2. Optimum trypsin concentration can vary for different cell types. We typically use 0.03% but sometimes use 0.25%. One m*M* ethylenediaminetetraacetic acid (EDTA) can also be used.

3. Make sure that cells are in exponential growth, not approaching confluence, so that the mitotic index will be as high as possible. The limiting concentration of cells in the flask will depend on cell type. The number of plates needed will depend on how many cells need to be synchronized.

4. Because about 3–4% of cells are in mitosis at any given time, the number of synchronized cells needed will govern how many flasks and how many cells are needed. The time for incubation to increase cell numbers will also depend on the cycle time for the cells. CHO cells have a cycle time of 12–14 h, whereas human cells have a cycle time of approx 24 h. The final concentration in a T150 should not exceed 1×10^7 cells to assure a high mitotic index. This will yield about 3–4 $\times 10^5$ mitotic cells in a shake-off.

5. The exact conditions to shake the flasks will depend on the cell type. Typical conditions would be 1 min at 200 rpm. If shaking is too vigorous, the mitotic selection window will not be as narrow. In the absence of a mechanical shaker, it is possible to manually shake the cells off by firmly banging the flasks against your hand. This will work for a small number of cells but is not practical for a large synchrony experiment.

6. Discard the first 3–5 shakes to eliminate loosely attached cells that are not in mitosis. It is a good idea to quickly make a slide of collected cells and get a mitotic index. This can be done by swelling the cells in water for a minute, spinning them down, resuspending and adding a few drops of ice-cold methanol:acetic acid (3:1), then dropping the cells onto a microscope slide. The mitotic index should be above 95% to get highly synchronized cells.

7. It will take about 1 h for cells to attach to the plastic and move into G1 phase. Different stages of G1 can be studied by waiting different time periods before analyzing or treating the cells. Cells will become desynchronized as they move through G1, however, because this is a heterogeneous phase for transit time. Cells can also be allowed to move into S phase and G2 phase, but the synchronization is degraded substantially (*see* **Fig. 1A**).

8. It is important to hold cells at the G1/S border with HU for approx 1 cell cycle time because some cells take much longer to traverse G1 than others. One cell cycle time will be sufficient for >95% of the cells to block at the G1/S border. HU may become toxic to cells after about 12 h, however *(8)*. This is not the G1 checkpoint because HU allows cells to initiate DNA synthesis *(7)*.

9. There will be a slight delay for cells to begin progression into S phase. However, by 1 h about 98% of cells should be in early S phase in a tight distribution (*see* **Fig. 2A**). It is hard to predict the time when the maximum population will be in G2 phase. It is possible to quickly fix and analyze a sample of cells by flow cytometry as they progress through S phase and then predict more accurately when the maximal concentration will be in G2 phase.

10. APH at 1 µg/mL is not toxic to G1 cells and is not very toxic to S-phase cells *(8)*. One µg/mL APH does not delay cells in moving through S phase, but 3 µg/mL causes a slight delay.

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3

Enrichment of Cells in Different Phases of the Cell Cycle by Centrifugal Elutriation

Tej K. Pandita

1. Introduction

Understanding the molecular and biochemical basis of cellular functions involved in growth and proliferation requires the investigation of regulatory events that most often occur in a cell cycle phase-dependent fashion. Studies involving cell cycle regulatory mechanisms and progression invariably require cell cycle synchronization of cell populations. Several methods are employed for obtaining and examining synchronized cells as they pass through one or more rounds of the cell cycle. Most of these methods involve pharmacological agents that act at various points throughout the cell cycle. Because of adverse cellular perturbations resulting from many of the synchronizing drugs used, other synchrony methods, such as serum deprivation and contact inhibition, have been exploited. Although such procedures allow synchronization of cells in a particular phase of the cell cycle, these approaches do not allow enrichment of cells, simultaneously in various phases of the cell cycle, from exponentially growing cell populations. Centrifugal elutriation described for the first time by Lindahl *(1)* is used to enrich cells in different phases of the cell cycle simultaneously with minimum changes in conditions during cell culture. Centrifugal elutriation can be used to obtain samples of uniformly sized cells, and because cell size is correlated with cell cycle stage, these cells are synchronized with respect to their position in the cycle.

Centrifugal elutriation has been applied, with variable degrees of success, to the separation of hemopoietic cells, mouse tumor cells, testicular cells, and a variety of other specialized cells as well as lymphoblastoid cells in particular phases of the cell cycle. The capacity of the elutriator to separate large numbers of cells is its major advantage. The technique of centrifugal elutriation

exploits differences in sedimentation velocity of different cell types, to enrich or isolate various types of cells from a heterogeneous population. In this technique, cell populations are subjected to two opposing forces to facilitate their fractionation into subpopulations based on cell size. Therefore, the process is also known as counterflow centrifugation. This has been used successfully to separate a wide variety of cell types from suspension and substrate-dependent cultures and to separate mixed cell populations liberated directly from tissues or body fluids *(2–6)*. The technology has proved to be effective in fractionating cells, based on very small differences in cell size, with nominal cross-contamination and in numbers unmatched by other methods of cell separation. In addition, the methodology of centrifugal elutriation is rapid, and cell separation can be achieved in less time (0.5–2 h) and with very little physiological stress to the cells, which are maintained in isotonic media, such as tissue-culture media, phosphate-buffered saline (PBS), or balanced salt solution (BSS).

2. Materials
2.1. Equipment

1. J6-MC centrifuge equipped with a JE-5.0 rotor and Sanderson chamber *(7)*.
2. Masterflex peristaltic pump, Cole-Palmer Instruments.
3. Electronic Coulter counter.
4. Flow cytometer.
5. 23G needle.
6. Nylon mesh.

2.2. Reagents

1. Growth and elutriation media: minimum essential medium (MEM) with 10% fetal bovine serum (FBS) for growth and 5% deoxyhypusine synthase (DHS) for elutriation.
2. 0.5 *M* HEPES.
3. 0.5 *M* Na$_2$ ethylenediaminetetraacetic acid (EDTA).
4. Trypsin solution.
5. 70% ethanol.
6. 1X Hank's balanced salt solution (BSS).
7. 2-naphthol-6,8-disulfonic acid dipotassium salt (NDA) from Eastman Kodak, Rochester, NY.
8. Deoxyribonuclease (DNase) I type IV (Sigma).
9. Ribonuclease (RNase).
10. Propidium iodide.
11. FACS Vantage™ Flow Cytometry System.

3. Methods
3.1. Preparation of Cells for Loading on Centrifugal Elutriator

1. Culture the cells in appropriate medium at 37°C with 5% CO$_2$ and 100% humidity.

2. Suspension cells (lymphoblastoids) or adhered cells (fibroblasts) after trypsinization are suspended at $1.3–1.5 \times 10^8$ cells in 50 mL of elutriation buffer (1X Hanks' BSS containing 3.3% heat-inactivated FBS and 5 m*M* NDA).
3. Add 4 mL of 0.02% (w/v) of DNase I type IV, dissolved in RPMI-1640 medium. Place the cells on ice and pass through 23G needle and nylon mesh to remove clumped cells.
4. Monitor the cell viability by trypan blue exclusion.
5. Concentrate the cells by low-speed centrifugation (3000*g* for 5 min) at 4°C and resuspend in 5 mL of ice-cold MEM with 5% DHS for each plate. Alternatively, 0.15 *M* PBS supplemented with 1% D-glucose, EDTA (0.3 m*M*) and 0.5% human serum albumin (HSA) pH 7.2 and osmolarity 285 ± 5 mosM can also be used throughout the elutriation procedure as the elutriation medium. Maintain the cells on ice until they are loaded into the elutriator.

3.2. Setting up the Centrifugal Elutriation

1. Arrange the elutriation system and assemble the elutriator rotor, elutriator chamber, and the elutriator centrifuge according to manufacturers' directions. Assemble the Sanderson chamber, which is used because it allows work with small numbers of cells (range: 10,000 to 10 million cells). The elutriator rotor is assembled in the centrifuge, which is attached to a peristaltic pump and tubing that feeds fluid into the centrifuge rotor as it is spun.
2. Sterilize the apparatus by running 500 mL of 70% ethanol through the rotor at about 10 mL/min without turning on the centrifuge at this treatment step.
3. Thoroughly wash the elutriator-loading chamber (rotor) with 2 L of cold Millipore filtered water by running through the rotor at 40 mL/min. Prevent and remove all bubbles during the process of washing and loading cells.
4. Pretreat the rotor with 200 mL of Dulbecco's PBS at 10 mL/min. At this pretreatment step, run the centrifuge at 500*g* to monitor and correct leaks, bubbles, or other problems.

3.3. Loading of Cells in the Elutriator Chamber

1. Run the centrifuge at 500*g* at 4°C. Shift the valve to the open position and load the cells into a 10-mL syringe connected to the tubing running through the peristaltic pump. Load the cells into the running centrifuge at the loading flow rate of 10 mL/min at 4°C.
2. After the cells are loaded, shift the valve to load 100 mL of PBS at the same flow rate. Do not allow any bubbles to form or enter the system. This loading step allows the cells to settle into the Sanderson chamber with largest cells at the bottom and layers of smaller cells at the top. It usually takes about 5 min to make the gradient on the basis of cell size and mass. The loading fraction can be collected, as it contains some of the smallest cells.
3. Turn the flow rate up by stepwise increments of the pump speed in order to collect larger cells in the fraction. Start with a flow rate of 12–14 mL/min and collect 50-mL fractions in 50-mL tubes on ice.

4. Several 50-mL fractions are then collected at each stepwise increment from 14 to 35 mL/min. A fraction is collected as the centrifuge is slowed to a stop, which helps to push the largest cells out. Cells remaining in the chamber and the tubing are collected, after the centrifuge is stopped, by removing the rotor and emptying the fluid that remains in the tubing.

5. Maintain the fractions on ice to prevent cell cycle progression and monitor the viability by trypan blue exclusion.

3.4. Determination of Purity of Cells in Each Fraction by Flow Cytometry (see *Notes 1–4*)

1. The cell cycle distribution of the fractionated samples is determined using flow cytometry to measure deoxyribonucleic acid (DNA) content.

2. Aliquots of each fraction are washed twice in PBS, fixed in 70% ethanol: 30% PBS.

3. Samples are treated with 0.5% RNase for 5 min and stained with propidium iodide.

4. DNA content is determined by quantitative flow cytometry using the FACS Vantage Flow Cytometry System. The accuracy of the analyzer is checked with calibrated fluorescent beads and chicken erythrocytes.

5. The quality of cell cycle enrichment can also be monitored by premature chromosome condensation *(5)*.

4. Notes

1. Depending on the frequency of cells at different phases of the cell cycle in asynchronously dividing cell populations, enrichment by centrifugal elutriation in different phases of the cell cycle may vary *(7)*. For human lymphoblastoid cell lines, G1-phase enriched populations contain greater than 98% G1-phase cells. The S-phase and G2/M-enriched populations are about 88 and 80% pure, respectively *(5)*.

2. Centrifugal elutriation does not influence the physiology or reproductive capability of the cells. Cells elutriated or not elutriated have similar cell viability and cell survival after ionizing radiation treatment *(6)*.

3. Centrifugal elutriation allows enrichment of cells in different phases of the cell cycle within a period of 2 h. Enriched cells in different phases can be simultaneously treated and examined for biochemical as well as biological function.

4. Cells enriched in different phases of the cell cycle allowed examination of the cycle's age-related radiation sensitivity, DNA repair, and kinase activity of ataxia telangiectasia mutant (ATM) protein after ionizing radiation treatment throughout the cell cycle *(5,6)* and telomere–nuclear matrix interactions *(8)*.

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4

Analysis of the Mammalian Cell Cycle
by Flow Cytometry

Haiying Hang and Michael H. Fox

1. Introduction

One of the most common uses of flow cytometry is to analyze the cell cycle
of mammalian cells. Flow cytometry can measure the deoxyribonucleic acid
(DNA) content of individual cells at a rate of several thousand cells per second
and thus conveniently reveals the distribution of cells through the cell cycle.
The DNA-content distribution of a typical exponentially growing cell popula-
tion is composed of two peaks (cells in G1/G0 and G2/M phases) and a valley
of cells in S phase (*see* **Fig. 1**). G2/M-phase cells have twice the amount of
DNA as G1/G0-phase cells, and S-phase cells contain varying amounts of DNA
between that found in G1 and G2 cells. Most flow-cytometric methods of cell
cycle analysis cannot distinguish between G1 and G0 cells or G2 and M cells,
so they are grouped together as G1/G0 and G2/M. However, there are flow-
cytometric methods that can distinguish four or even all five cell cycle sub-
populations: G0, G1, S, G2, and M *(1–3)*. Furthermore, each subpopulation
can be quantified *(4)*. Obviously, flow cytometry with these unique features is
irreplaceable for monitoring the cell cycle status and its regulation.

Cell cycle checkpoint genes are key elements in cell cycle regulation. Check-
point gene mutation can lead to defects in one or more cell cycle checkpoint
controls, which can then result in cell death or cancer. Many of the cell cycle
checkpoint genes are tumor suppressors, such as *p53,* ataxia-telangiectasia
mutant *(ATM)*, ataxia-telangiectasia and *Rad3 (ATR)*, and *BRCA1 (5,6)*.

In mammalian cells, the cell cycle checkpoint controls that can be analyzed
by flow cytometry are G1 arrest, suppression of DNA replication, and *ATM*-
dependent as well as independent G2 arrest. Exposure to a genotoxic agent can
activate some or all of the checkpoints. The flow cytometry methods to analyze
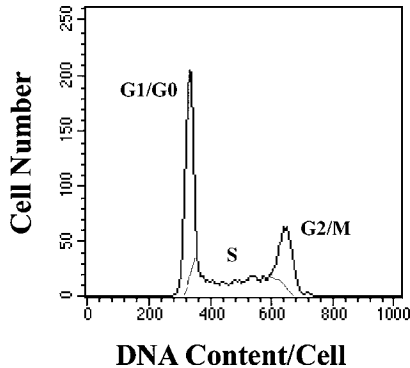
Fig. 1. A typical cell cycle distribution of DNA content. Based on DNA content in individual cells, a cell population in exponential growth status can be divided into three subpopulations: G1/G0, S, and G2/M.

the status of the different checkpoints are described here. A typical S/M checkpoint similar to those in the fission and budding yeasts *(7,8)* has not been reported in mammalian cells; thus the protocol to monitor it is not included in this chapter.

2. Materials

2.1. Cells

Examples of cell lines used for demonstrating the four cell cycle checkpoints with flow cytometry:

1. Human fibroblasts from an ataxia-telengiectasia (AT) patient (GM05823, Coriel Institute for Medical Research, Camden, NJ) and normal human fibroblasts (BJ1, Clontech, Palo Alto, CA) are used for analyzing G1 and *ATM*-independent G2 checkpoints. GM05823 AT cells are deficient in all three checkpoint controls.
2. *Rad9*$^{+/+}$ and *Rad9*$^{-/-}$ mouse embryonic stem (ES) cells (K. M. Hopkins, W. Auerbach, X. Y. Wang, M. P. Hande, H. Hang, D. J. Wolgemuth, A. L. Joyner, and H. B. Lieberman, unpublished) are used in the protocol for the analysis of the S-phase checkpoint control.
3. Human fibroblasts GM847 and GM847/*ATR*kd are used for illustrating radiation-dose-dependent G2-checkpoint control. GM847 is an SV40-transformed human fibroblast line from a healthy individual. The GM847/*ATR*kd cells were derived from GM847 cells and express a kinase-inactive allele of *ATR* in doxycycline-free medium. GM847 lacks the G1-checkpoint control, and GM847/ *ATR*kd are deficient in G2-checkpoint control *(9)*.

2.2. Media, Reagents, and Solutions

*2.2.1. Cell Culture (*see *Note 1)*

1. Medium for AT patient and normal human fibroblasts: Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 15% heat-inacti-

vated fetal bovine serum (FBS); Mediatech, Herndon, VA), 1% minimum essential medium (MEM) nonessential amino acids solution (Gibco), and 10 U/mL penicillin and streptomycin (Gibco).

2. Medium for *Rad*$^{+/+}$ and *Rad9*$^{-/-}$ mouse ES cells: knockout DMEM (cat. no. 10829-018, Gibco) containing 15% FBS (Cell and Molecular Technologies, Phillipsburg, NJ), 1% MEM nonessential amino acids solution (Gibco), 200-mM 1% L-glutamine solution (Gibco), 10-U/mL penicillin and streptomycin (Gibco), 0.0007% 2-mercaptoethanol (Sigma, St. Louis, MO), 1000-U/mL leukemia inhibitor factor (Chemicon, Temekula, CA).

3. Medium for human fibroblasts GM847 and GM847/ATRkd: the same as used for AT cells except that it contains only 10% FBS.

4. Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS; Gibco).

5. Trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco[MHF1]).

6. BrdU (5-bromo-2'-deoxyuridine; Sigma). Add distilled H$_2$O to make 10-mM BrdU stock solution and store at –20°C.

2.2.2. Cell Processing and Staining

1. 100% ethanol stored at –20°C.

2. Ca^{++}- and Mg^{++}-free PBS (Gibco).

3. Propidium iodide (PI) solution for staining DNA in fixed cells: PBS containing 20 or 50 µg/mL PI (Sigma) and 40 U/mL ribonuclease (RNase) A (Sigma). Store in the dark at 4°C. RNase is added to the PI solution before staining cells.

4. 2 N HCl containing 0.2 mg/mL pepsin (Sigma). It is used to partially denature genomic DNA and expose incorporated BrdU for detection.

5. 1M Tris-HCl buffer at pH 8.0.

6. PBS-TxBF solution: PBS containing 0.05% Triton X-100, 0.5 % bovine serum albumin (BSA), and 0.5% FBS.

7. PBS-TwBF solution: PBS containing 0.1% Tween-20, 1% BSA, and 1% FBS.

8. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-BrdU IgG1 antibody (cat. no. 23614L, Pharmingen, San Diego, CA).

9. Rabbit polyclonal antibody against phosphorylated form of histone H3 and (FITC)-conjugated antirabbit IgG2 antibody (Upstate Biotechnology, Lake Placid, NY).

10. FITC-conjugated goat antirabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

2.3. Equipment

1. A flow cytometer equipped with a 488-nm argon laser line is suitable for all the analyses of cell cycle checkpoint controls described in this chapter. A FACSCalibur flow cytometer connected with a FACSStation from Becton Dickinson was actually used for collecting and analyzing the data presented in this chapter.

2. A sterile hood to manipulate cells.

3. A CO$_2$ incubator at 37°C.

4. A tabletop centrifuge and an Eppendorf microcentrifuge.

5. A nutator to keep cells in solutions from precipitation (Adams Clay Inc, Sparks, MD).
6. An ultraviolet (UV) light chamber (*see* **Chapter 1** for details).
7. A ^{137}Cs irradiator (*see* **Chapter 1** for details).

3. Methods

3.1. G1-Phase Checkpoint Control

The G1-phase checkpoint, when activated, arrests cells in late G1 phase. The activity of the G1 checkpoint is regulated by the *p53/p21* pathway, and mutations in *p53, p21*, and the other factors (e.g., *ATM* gene, mutated in AT patient cells) that modify *p53, p21*, or both can result in G1-checkpoint control defects. Two methods can be used to analyze G1-checkpoint deficiency: (a) DNA-content measurement and (b) simultaneous measurement of DNA content and BrdU uptake.

3.1.1. DNA-Content Measurement

1. Inoculate AT and normal (BJ1) cells in 10-cm dishes containing 10-mL DMEM. Incubate at 37°C with 5% CO_2 overnight. Adjust the seeded cell numbers so that they reach 50 to 70% confluence levels the next day. 5×10^5 to 2×10^6 cells are needed to conveniently carry out the steps in this protocol.
2. Irradiate cells in a ^{137}Cs γ-ray irradiator at 4 Gy, followed by incubating cells for 8, 12, and 16 h (*see* **Note 2**). Use unirradiated cells as controls.
3. Trypsinize and harvest cells by centrifugation at $200g$, and then rinse once with 2 mL cold PBS. Suspend cells with 0.5-mL cold PBS. Make sure that the suspensions contain single cells with no cell clumps. Slowly drop 1.5 mL ice-cold 100% ethanol into suspended cells while mildly vortexing them. Keep the cells at 4°C or –20°C for at least 30 min.
4. Collect cells by centrifugation and rinse once with cold PBS. Suspend cells in 1-mL PBS containing 50-µg/mL PI and 40-U/mL RNase A, and stain at 4°C for at least 30 min.
5. Use a 488-nm argon laser line to excite PI and measure fluorescence at wavelengths >600 nm (*see* **Note 3**). Measure at least 10,000 cells.
6. Determine the G1-checkpoint status of cell lines by inspection, quantification, or both of the cell cycle distribution. A cell line with normal cell cycle checkpoint control will have an increased number of cells in G1 phase and a decreased number of cells in S phase compared to unirradiated cells at about 12 h after irradiation (*see* **Fig. 2** and **Note 2**). At the same time-point, cell populations with a G1-checkpoint defect contain fewer cells in G1 phase and significantly more cells in S phase than normal cells. Numbers of G1/G0, S, and G2/M cells can be quantified to give more precise estimation of defective extent of G1 checkpoint using commercially available programs such as MultiCycle (Phoenix Flow Systems) and ModFit (Verity Software). A quick and simple method to assess a checkpoint block is to measure the cells in a window in early S phase. For example, at 8, 12, and 16 h after irradiation, the number of normal cells in early S phase is
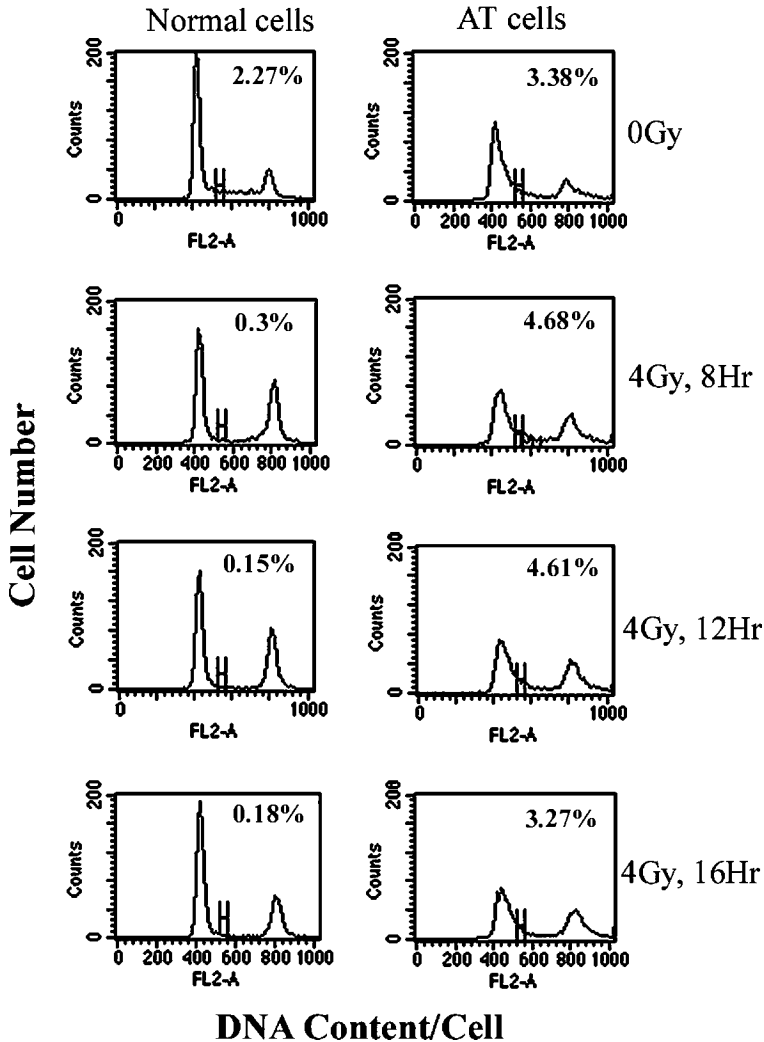
Fig. 2. γ radiation induces G1-phase checkpoint control in normal cells but not in AT cells. Incubation in a CO_2 incubator at 37°C for a few hours (usually 6 h or longer) after irradiation reveals G1-phase checkpoint activity in normal cells: S-phase cells during irradiation have moved out of S phase, and new G1 cells have not been able to enter S phase, and number of early S-phase cells (gated area) is reduced to very low levels. AT cells do not have a normal G1 checkpoint, and the number of cells in early S phase does not vary much with or without irradiation.

reduced dramatically (less than 10%) because no G1-phase cells move into S phase, but the number of AT S-phase cells does not change much (**Fig. 2**). Several other quantification methods were clearly and concisely described by Ormerod *(4)*.

3.1.2. Quantification of BrdU Uptake

Quantification of BrdU uptake by S-phase cells is another way to determine whether a cell line is defective in the G1 checkpoint. Because early S-phase cells are judged not only by their DNA content but also by BrdU incorporation, this method is more precise than the previous one.

1. Inoculate AT and normal cells in 10-cm dishes containing 10-mL DMEM. Incubate at 37°C with 5% CO_2 overnight. Adjust the seeded cell numbers so that they reach 50 to 70% confluence levels the next day. 5×10^5 to 2×10^6 cells are needed to conveniently carry out the steps in this protocol.
2. Irradiate cells in a ^{137}Cs γ-ray irradiator at 4 Gy, incubate at 37°C for 12 h, and then pulse-label cells with 10-μM BrdU for 20 min at 37°C.
3. Harvest and fix cells as in **step 3** in **Subheading 3.1.1.**
4. Collect cells by centrifugation at 200*g*. Suspend cells in 100-μL PBS, and add 2 mL of 2 *N* HCl with 0.2 mg/mL pepsin. Incubate at 37°C for 20 min, and add 3 mL Tris-HCl buffer. Centrifuge, decant, vortex pellet, and rinse with 2 mL PBS once.
5. Suspend pellet in 200-μL PBS-TxBF and transfer cells to Eppendorf tubes. Incubate for 20 min at room temperature (RT) while agitating cells on a rolling nutator.
6. Collect cells by centrifugation at 200*g* in an Eppendorf centrifuge and incubate in 200 μL PBS-TxBF containing 1.2 μL FITC-conjugated anti-BrdU antibody (Pharmingen) in the dark at RT on a rolling nutator for 30 min. From this step on, avoid exposing cells to strong light.
7. Collect cells and rinse them once in 1 mL PBS-TxBF.
8. Resuspend cells in 1 mL PBS containing 20 μg/mL PI and 40 U/mL RNase, and stain at 4°C for at least 1 h.
9. Use a 488-nm argon laser line to excite PI and FITC; measure fluorescence at 530/30 nm and 585/42 nm emitted from FITC and PI, respectively (*see* **Note 3**). Measure at least 10,000 cells.
10. Determine the G1-checkpoint status of cell lines by inspection, quantification of the cell cycle distribution, or both. Unirradiated cells that incorporated BrdU (synthesized DNA) during the 20-min labeling appear as an archlike subpopulation on a bivariate histogram of BrdU vs PI (*see* **Fig. 3**). Irradiation significantly reduces the size of the BrdU-positive subpopulation in normal cells, compared to that of AT cells, because a normal G1 checkpoint prevents G1 cells from entering S phase, whereas a defective G1 checkpoint cannot stop G1 cells from moving into S phase. The number of BrdU-positive cells can be quantified with analysis regions to give a more precise estimation of the extent of the G1-checkpoint defect (*see* **Fig. 3**).

3.2. S-Phase Checkpoint Control

S-phase checkpoint control by definition is the suppression of DNA synthesis induced by genotoxic stress in S-phase cells. Replicative DNA synthesis
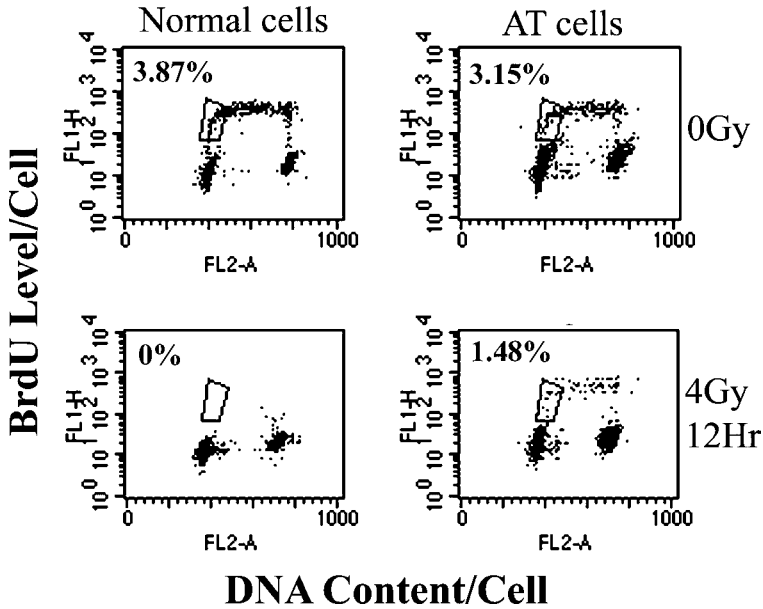
DNA Content/Cell

Fig. 3. Assessment of G1-checkpoint control by simultaneous measurement of DNA content and BrdU uptake. Early S-phase cells are gated based on both DNA content and BrdU incorporation, and therefore their percentage is more precisely quantified. At 12 h after irradiation, no normal G1 cells move into S phase (normal G1 checkpoint), whereas AT G1 cells continue entering S phase, though at a reduced rate (abnormal G1 checkpoint).

suppression can be detected by bivariate distributions of BrdU incorporation vs DNA content as described in **Subheading 3.1.2.**, except that the timing for labeling and sampling cells is arranged differently to evaluate the DNA synthesis rate of S-phase cells, instead of estimating the efficiency of blocking cells in G1 phase after irradiation. Therefore, the details for common steps are omitted.

1. Grow *Rad9*$^{+/+}$ and *Rad9*$^{-/-}$ ES cells in knockout DMEM overnight.
2. Remove medium completely. Irradiate cells with 20-J/m^2 ultraviolet (UV) light, and add prewarmed conditioned medium (50% fresh medium and 50% medium from cell culture) back to cell culture dishes. Incubate for 40 min and pulse-label cells with 10-μ*M* BrdU for 20 min.
3. Process and measure the samples as described in **Subheading 3.1.2.**, **steps 3–9**.
4. Determine the S-phase checkpoint status of the cell lines by inspection, quantification, or both. After UV light irradiation, both *Rad9*$^{+/+}$ and *Rad9*$^{-/-}$ ES cells reduce the incorporation of BrdU (measured by geometric mean of intensities of green fluorescence from BrdU-positive cells, gated area A across S phase), indicating a lower replicative DNA synthesis (*see* **Fig. 4**). However, the reduction level for
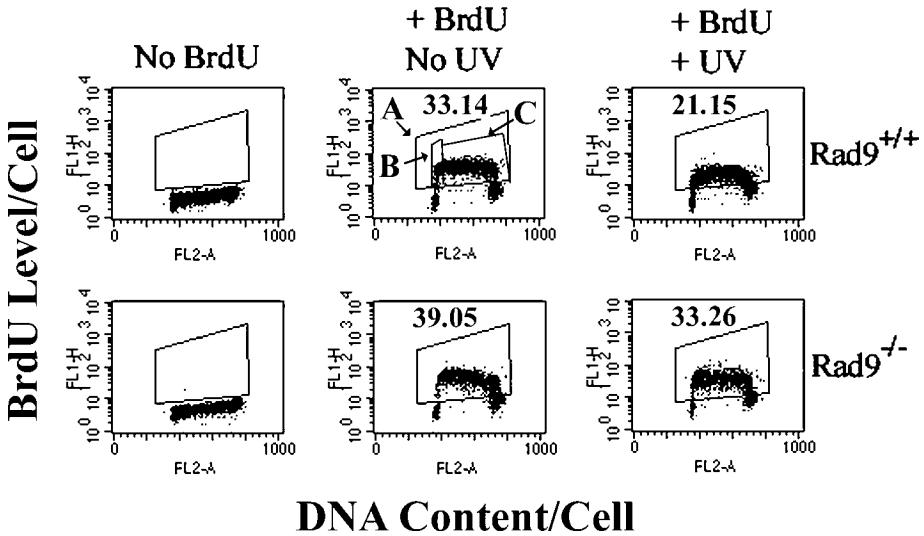
DNA Content/Cell

Fig. 4. Determination of S-phase checkpoint activity by simultaneous measurement of DNA content and BrdU uptake. The rate of BrdU uptake can be quantified (gated area A) and can be used to evaluate the ability of cells to suppress replicative DNA synthesis induced by genotoxic stress (e.g., UV light). This ability of mouse ES cells is compromised by deleting both copies of the *Rad9* gene.

Rad9$^{-/-}$ cells is significantly less than that for *Rad9$^{+/+}$* cells; this is the indication that *Rad9$^{-/-}$* cells are defective in S-phase checkpoint control. The change in the rate of DNA synthesis after irradiation can be calculated as the ratio of the geometric mean of green fluorescence (FL1 in FACSCalibur) from irradiated BrdU-positive cells to that of unirradiated BrdU-positive cells ($R_{geo-mean}$). In this example the ratios for *Rad9$^{-/-}$* and normal cells are 85% (33.26/39.05) and 65% (21.15/33.14), respectively. Instead of including all the BrdU positive cells (area A), one can chose the cells in later stages of S phase (area C) to calculate the $R_{geo-mean}$ that measures the S-phase checkpoint status of the cells already in S phase when exposed to UV light (*see* **Fig. 4**).

3.3. G2-Phase Checkpoint Control

3.3.1. Dose-Dependent G2-Phase Accumulation

G2-checkpoint accumulation does not appear right after cells are exposed to genotoxic stresses; it takes time for the cells that were in S and G1 phases during irradiation to accumulate in G2 phase (*1,9*). The percentage of accumulated cells in G2 phase and the length of the delay are proportional to the radiation dose given to the cells. The accumulation of cells in G2 lasts 16 h or longer depending on dose and cells. GM847 is an SV40-transformed human fibroblast line from a healthy individual, and it has normal G2-checkpoint control.
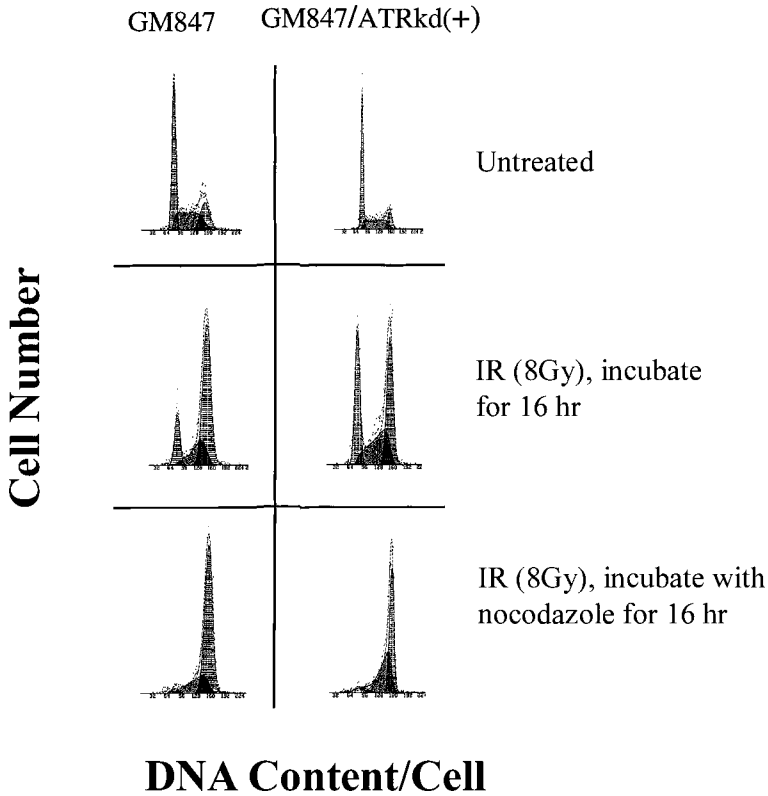
DNA Content/Cell

Fig. 5. Assessment of G2-checkpoint activity. The ability of cells to arrest in G2 phase can be judged by measuring DNA content in individual cells in combination with utilization of nocodazole or other microtubule inhibitors. This figure is modified from Cliby et al. with permission from the *EMBO Journal (10)*.

The GM847 cells that overexpress kinase-inactive *ATR* (GM847*ATR*kd) are deficient in this checkpoint control *(10)* and are used to help describe the assay for G2-checkpoint status.

1. Inoculate two sets of normal and kinase-inactive *ATR* expressing GM847 as in **Subheading 3.1.1.**, **step 1**.
2. Irradiate both sets of cells in a γ-ray irradiator at 8 Gy. Add nocodazole (at the final concentration of 100-ng/mL) to one set of cells right after radiation exposure to block the cells in mitosis.
3. Harvest cells at 16 h after irradiation. Process, stain, and measure cells as in **Subheading 3.1.1.**, **steps 3–5**.
4. Evaluate the G2 checkpoint by inspection, quantification, or both with a cell cycle model. **Figure 5** (modified from Cliby et al. with permission from the *EMBO*

Journal, **10**) is used to explain the analysis of the G2 checkpoint. At 16 h after irradiation, normal cells have a small G1 peak and a large G2/M peak (the G2-phase checkpoint accumulation), whereas cells overexpressing kinase-inactive *ATR* have G1 and G2/M peaks that are about equal in size. Addition of nocodazole (to block cells between late G2 and early M phase) suppresses the G1 peak in both cells lines, indicating (a) that the G1 cells came from G2 phase and (b) that a larger G1 peak in the cells overexpressing kinase-inactive *ATR* stem from partial loss of checkpoint control in G2 phase after irradiation (*see mid-right panel*, **Fig. 5**).

3.3.2. G2-Checkpoint Block Measured by Histone H3 Phosphorylation

Normal cells stop entering mitosis within the first hour after irradiation. By 12 h after irradiation, these cells are released from G2 and begin to reenter mitosis *(1,9)*. AT cells lack this brief block in G2 phase after irradiation; the number of AT cells in mitotic phase does not vary much following radiation exposure. Cells with mutated *BRCA1* are also deficient in the brief G2 block. The assay is based on the fact that mitotic cells contain a high level of phosphorylated histone H3 molecules, but the rest of the cells contain few *(11)*.

1. Prepare both normal and AT cells as in **step 1** in **Subheading 3.1.1.**
2. Irradiate cells with 4 Gy γ-radiation.
3. Harvest cells at various times after irradiation (1, 2, and 12 h are used here, but other times can be used). Fix cells as in **step 3** in **Subheading 3.1.1.**
4. Collect cells by centrifugation at 200g, and rinse once in PBS.
5. After centrifugation, resuspend cells in 200 μL PBS-TwBF. and transfer cells to Eppendorf tubes followed by incubation on a rolling nutator at RT for 30 min.
6. Collect cells by centrifuging at 200g for 3 min. Suspend cells in 200 PBS-TwBF containing 1 μg of anti-phospho-histone H3 rabbit polyclonal antibody and incubate for 2 h at RT on a rolling nutator.
7. Rinse cells once in 1 mL PBS-TwBS. Resuspend cells in 200 μl PBS-TwBS containing 1 μg FITC-conjugated goat antirabbit antibody. Incubate for 30 min at RT in the dark on a rolling nutator.
8. After centrifuging and rinsing twice in 1 mL PBS-TwBS, resuspend cells in 1 mL PBS containing 20 μg/mL PI and 40 U/mL RNase, and stain in the dark at 4°C for at least 1 h.
9. Use a 488-nm argon laser line to excite PI and FITC; measure fluorescence using a 530/30-nm band-pass filter and a 650 long-pass filter for FITC and PI, respectively. Measure at least 10,000 cells. The combination of the two antibodies yields a fluorescent light from FITC so strong that it interferes with the detection of signals from PI when using 585/42 channel, and compensation cannot get rid of the interference (*see* **Note 3**). Using a 650LP (FL3 in FACSCalibur) channel to collect signals from PI solves the problem properly.
10. Examine G2 status by inspection, quantification, or both (*see* **Fig. 6**). One and 2 h after irradiation, normal cells that contain high levels of phosphorylated H3 almost completely disappear, but they reappear at 12 h. In contrast, AT cells
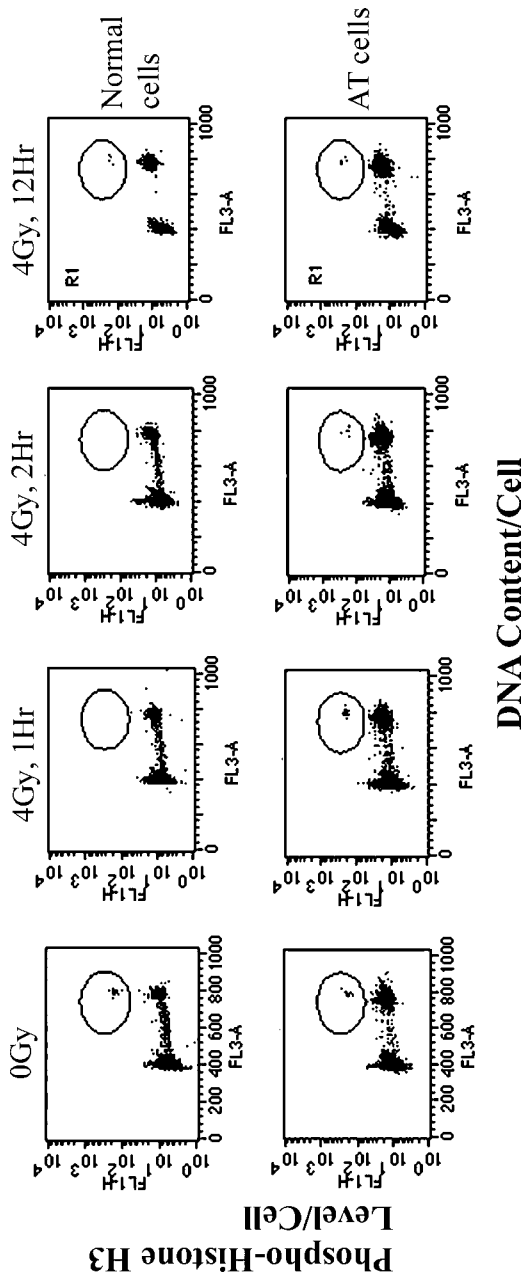
Fig. 6. Assessment of G2-checkpoint activity by histone phosphorylation. Cells with 4n DNA and a high level of phosphorylated histone H3 are mitotic cells, and their detection can be used to assess G2-checkpoint activity induced by radiation. Normal G2 cells stop moving into M phase within 1 h after irradiation, but mutation of the *ATM* gene, as in AT cells, allows cells to continue to move into mitosis.

always contain this subpopulation with a high level of phosphorylated H3, and the number of cells in this category only vary slightly before and after irradiation.

4. Notes

1. Different cell lines need different media to grow properly. To accurately compare checkpoint controls in two or more cell lines, a medium should be chosen to minimize the differences in their growth rates because growth rates have significant effects on the results of checkpoint control analysis.
2. Doses and types of genotoxic agents determine the timing for harvesting cells, and the proper timing should be tested in each case.
3. A standard filter for PI on a FACSCalibur is a 585/42 band-pass. This is fine when only PI is measured but not optimal for PI when used in combination with FITC or other dyes. A better filter choice is to use a long-pass at 600-nm or longer.

Acknowledgments

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5

Methods for Detecting Cells in S Phase

Wei-Hsin Sun and Melvin L. DePamphilis

1. Introduction

1.1. S Phase vs Mitochondria DNA Replication and DNA Repair

S phase is that period of time in the cell-division cycle during which nuclear chromosomal deoxyribonucleic acid (DNA) is replicated *(1,2)*. The time required for S phase depends on the size of the genome, the organism, and its developmental state. DNA replication requires only 15 to 20 min in budding yeast, but 6 to 7 h in mammalian cells. In organisms, such as frogs, fish, echinoderms, and flies that undergo rapid cell cleavage events at the beginning of their development, S phase takes only a few minutes during these initial cell cleavage events, but it takes several hours in late-stage embryos, adult animals, or cells cultured in vitro.

Both mitochondrial DNA (mtDNA) replication, which occurs in the cytoplasm, and DNA repair, which occurs in the nucleus, take place throughout the cell-division cycle *(1,2)* and can contribute significantly to the amount of DNA synthesis observed when looking for cells in S phase. These problems can be avoided in two ways. First, measure only DNA synthesis that is localized to the nucleus. Second, take advantage of the differences between chromosomal DNA replication and DNA repair *(1,2)*. DNA replication is a semiconservative process that produces long DNA molecules in which only one of the two strands is newly synthesized. At replication forks, DNA synthesis occurs continuously on one arm and discontinuously on the other through repeated synthesis and joining of ribonucleic acid (RNA)-primed nascent DNA chains called Okazaki fragments; methods for their identification and characterization have been described *(3)*.

Chromosomal DNA replication is an adenosine triphosphate (ATP)-dependent process that is sensitive to aphidicolin (a specific inhibitor of replicative

DNA polymerases α, δ, and ε, but not to dideoxythymidine triphosphate (ddTTP), a selective inhibitor of DNA polymerases β and γ. Finally, chromosomal DNA replication begins at discrete foci distributed throughout the nucleus, and methods for identification of DNA replication origins have been described *(4)*.

1.2. Applications for S-Phase Assays

Methods for detecting cells in S phase have three purposes: determining the fraction of cells undergoing DNA replication, determining when cells begin DNA replication after cell division is complete, and determining how long DNA replication takes. We have grouped the methods in terms of their utility. They also can be grouped in terms of their applications:

1. Living cells (*see* **Subheadings 3.2.**, **3.4.2.**, **3.5.1.**, **3.6.**).
2. Permeabilized cells or isolated nuclei (*see* **Subheadings 3.3.**, **3.4.1.**, **3.5.2.**).
3. Fraction of cells in S phase (*see* **Subheadings 3.2.2.**, **3.2.3.**, **3.3.**, **3.5.1.**, **3.6.**).
4. Fraction of DNA replicated (*see* **Subheading 3.3.5.**).
5. Timing and length of S phase (most protocols when synchronized cells are used).
6. Distinguishing nuclei with active DNA replication forks (S phase) from nuclei with functional prereplication complexes that have not initiated DNA synthesis (late G1 phase; *see* **Subheadings 3.3.3.** and **3.3.4.**).

2. Materials

Can be obtained from Sigma, unless stated otherwise.

1. Buffer A: 0.15 *M* NaCl, 0.01 *M* ethylenediaminetetraacetic acid (EDTA), 0.01 *M* Tris-HCl, pH 9.5.
2. Chrome alum/gelatin: 0.5 g chrome alum and 5 g gelatin per liter.
3. Coverslips: 13-mm diameter, grade 1 (0.15-mm thick) glass coverslips, clean, and then sterilize with dry heat.
4. CsCl Solution: 109% (w/v) CsCl, 10 m*M* Tris-HCl (pH 8.0), and 1 m*M* EDTA (adjust refractive index to 1.4093).
5. Cytoskeleton (CSK) buffer: 10 m*M* PIPES (pH 7.0), 100 m*M* NaCl, 300 m*M* sucrose, 3 m*M* $MgCl_2$.
6. 1,4-diazabicyclo[2.2.2]octane (DABCO): Dissolve 250 mg DABCO in 9 mL glycerol, incubate at 37°C overnight, and then mix with 1-mL phosphate-buffered saline (PBS). Store at –20°C.
7. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche): Dissolve 10 mg DAPI in 5 mL H_2O (5-mg/mL stock solution), aliquot and store at –20°C. The diluted working solution (1-μg/mL) can be stored at 4°C for 1 mo.
8. Digitonin: Stock solution of 40 mg/mL in dimethyl sulfoxide.
9. Formaldehyde, 4% (v/v): Dilute concentrated HCHO in PBS.
10. Giemsa stain: Dilute Giemsa stain (Fisher Scientific) 25-fold in 10 m*M* phosphate buffer (pH 6.8) before using.
11. Glycerol, 25% (v/v): Dilute concentrated glycerol in PBS.

12. Hypotonic buffer: 20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 1.5 mM MgCl$_2$, 0.1 mM dithiothreitol.
13. Lysing buffer: 0.15 M NaCl, 0.015 M EDTA, 0.05 M Tris-HCl (pH 9.5), 0.25% sodium dodecyl sulfate (SDS), and 1-mg/mL freshly added pronase.
14. Nuclear isolation buffer (NIB): 50 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM MgCl$_2$, 0.5 mM spermidine, 0.15 mM spermine, 2 mM β-mercaptoethanol, 1 μg/mL each of aprotinin, leupeptin, and pepstatin (β-mercaptoethanol and protease inhibitors, fresh added).
15. Nucleotide mix: 40 mM K-HEPES (pH 7.8), 7 mM MgCl$_2$, 3 mM ATP, 0.1 mM each guanosine 5'-triphosphate (GTP), cytidine-5'- triphosphate (CTP), and uridine-5'-triphosphate (UTP), 0.1 mM each dATP, dGTP, and dCTP, 0.5 mM dithiothreitol, 40 mM phosphocreatine (PC), and 5 μg creatinine phosphokinase (CPK).
16. Paraformaldehyde, 4% (w/v): Dissolve 40 g paraformaldehyde in 900-mL deionized water, stir at 60°C, and then add 150 μL NaOH and 100 mL PBS.
17. Dulbecco's phosphate-buffered saline (Dulbecco's PBS): 2.7 mM KCl, 1.4 mM KH$_2$PO$_4$, 137 mM NaCl, and 8 mM Na$_2$HPO$_4$ 7H$_2$O, adjust pH to 7.2.
18. PBS-T: PBS containing 0.1% Tween-20.
19. Poly-L-lysine-coated coverslips: 1 mg/mL poly-L-lysine (average mol wt 400,000) filter sterilize through 0.45 μM filter and store at –20°C. Apply 25-μL 1 mg/mL poly-L-lysine to each sterilized coverslip, allow to stand 10 min, then rinse coverslips three times with water and air-dry.
20. Standard sodium citrate (SSC): 0.15 M NaCl, 0.015 M sodium citrate, adjusted to pH 7.0.
21. Stop C: 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.5% SDS.
22. SuNaSP/BSA: 0.25 M sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, and 3% bovine serum albumin (BSA).
23. TCA solution, 10%: 10% (w/v) TCA containing 2% (w/v) sodium pyrophosphate.
24. TE buffer: 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.
25. Transport buffer: 20 mM HEPES (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, and 1 mM EDTA.

3. Methods

3.1. Synchronizing Cells

Described in Chapters 2 and 3 and in *(5)*.

3.2. Measuring DNA Replication In Vivo by Incorporation of Labeled Precursors

Intact cells proliferating in culture or in tissues can incorporate labeled deoxyribonucleosides from the surrounding medium, convert them into deoxyribonucleoside triphosphates, and then incorporate these DNA precursors into newly synthesized DNA. Therefore, S-phase cells can be detected using cells in culture or in tissues either by incorporation of ^{3}H-thymidine

([^{3}H]TdR) followed by acid precipitation of DNA or autoradiography, or by incorporation of 5-bromo-2'-deoxyuridine (BUdR) followed by immuno-staining with antibodies against BUdR. The onset of S phase after cell division and the length of S phase is measured as the ^{3}H-DNA/total DNA ratio as a function of time elapsed after cell division. The fraction of cells in S phase is determined by counting the fraction of ^{3}H- or fluorescent-labeled nuclei by microscopy.

One should score the number of *unlabeled* cells per field rather than the number of labeled cells, because cell division increases the number of labeled cells. Therefore, the longer the labeling period, the greater the error becomes when labeled cells are scored. The initial number of cells per field is determined by fixation of a parallel culture of cells at the time when the [^{3}H]TdR incubation begins.

The length of the labeling period depends on the purpose of the experiment. The shorter the pulse, the greater the resolution with which S phase can be determined, and with which the sites of nucleotide incorporation can be identified. The longer the pulse, the more sensitive the assay. The amount of nucleotide incorporation will depend on the number of cells used, the concentration of their nucleotide pools, the specific activity of the labeled compound, and the length of time cells are in exposure to the labeled precursor. Mammalian cells, which replicate their genome in 6 to 7 h, are usually labeled for 10 min to 1 h.

3.2.1. Incorporation of [^{3}H]TdR Followed by DNA Precipitation (6)

The simplest assay for detecting newly synthesized DNA is to culture cells in the presence of [^{3}H]TdR, lyse the cells, precipitate total DNA with acid, and then quantify the amount of acid-insoluble ^{3}H with a liquid scintillation counter. DNA or RNA molecules greater than 20 nucleotides in length are quantitatively precipitated in strong acids, whereas dNTP or NTP precursors remain in solution. This method is most accurate when total cellular DNA is prelabeled with [^{14}C]TdR to allow newly synthesized DNA to be quantified as the [^{3}H]TdR/[^{14}C]TdR ratio. Alternatively, separate dishes of unlabeled cells can be cultured in parallel, isolated at the appropriate time by trypsinization, and counted using a hemocytometer to provide the ratio of [^{3}H]TdR/cell. This assay is generally used with synchronized cells to determine when they enter S phase after cell division or after release from a metabolic block. Caution: This assay assumes that the bulk of the acid-insoluble, radio-labeled material represents nuclear DNA replication.

1. Transfer $8{-}10 \times 10^6$ mammalian cells in exponential growth to 225-mL tissue-culture flasks.
2. Add [^{14}C]TdR (0.05 μCi/mL, 50 mCi/mmole; Amersham Pharmacia Biotech) and allow cells to double in number (approx 16 h).

3. Collect dividing cells from two flasks by vigorous shaking ("mitotic shake-off" protocol *[5]*). Larger numbers of cells can be synchronized at metaphase by addition of nocodazole *(5,7)*.

4. Add fresh medium without radioisotope after two harvests of cells. The first harvest is frequently contaminated with nondividing cells.

5. Transfer dividing cells to Leighton tubes in conditioned medium (*see* **Note 1**). More than 90% will divide within 20 to 30 min.

6. At 3 h after collection, add [^{3}H]TdR (1 µCi/mL, 25-mCi/mmole; Amersham Pharmacia Biotech). Culture cells at 37°C for 10 min.

7. Rinse cells briefly with TE buffer and then add lysing buffer (1 mL/1–10 × 10^6 cells) at 37°C for 16–24 h (*see* **Note 2**).

8. Extract the lysate gently over chloroform-isoamyl alcohol (25:1) to remove detergent and residual protein.

9. Dilute an aliquot of the aqueous (top) layer with water, and add an equal volume of ice-cold 10% TCA solution and allow to stand on ice for 10 min.

10. Collect the precipitates on glass microfiber filters using a multiwell vacuum manifold such as the Millipore 1225 Sampling Vacuum Manifold for 25-mm discs. Whatman GF/A or GF/C filters are commonly used; GF/C retains more low molecular weight DNA.

11. Wash filters twice with 5 mL 5% TCA to get rid of unincorporated nucleotides. Check background using a control sample.

12. Wash filters with 70% ethanol to get rid of acid and then dry them under a heat lamp.

13. Determine ratio of ^{3}H to ^{14}C on each filter by placing it in a scintillation vial with 5-mL scintillation fluid (Ecoscint H, National Diagnostics). Measure radioactivity in a liquid scintillation system (*see* **Note 3**). Correct overlap of ^{3}H and ^{14}C isotopes using samples of ^{3}H and ^{14}C prepared in exactly the same way (*see* **Note 4**).

3.2.2. Incorporation of [^{3}H]TdR Followed by Autoradiography (8,9)

Sites of [^{3}H]TdR incorporation require several days to visualize by autoradiography, whereas sites of BUdR incorporation require only a few hours to visualize by immunostaining. However, [^{3}H]TdR autoradiography is a more sensitive and reliable assay.

1. Culture mammalian cells on 13-mm diameter glass coverslips (PGC Scientifics) in 4-well plates (Nunc) containing 0.75-mL culture medium at 37°C (*see* **Note 5**). If cells have difficulty adhering, try poly-L-lysine-coated coverslips. Centrifuge suspension cells onto glass microscope slides using a StatSpin Cytofuge (PGC Scientifics, <www.statspin.com>), or simply incubate a 10^5 cell/mL suspension in PBS with poly-L-lysine coverslips for 10 min at 37°C.

2. Label DNA by replacing fresh medium with medium that contains [^{3}H]TdR (1 µCi/mL, 25 Ci/mmole; Amersham Pharmacia Biotech) and culture them at 37°C for 0.25 to 2 h.

3. Rinse coverslips three times with 5 mL PBS each time. Remove excess PBS. Cover them with 4% formaldehyde and incubate for 10 min at room temperature (RT).

4. Remove unincorporated [³H]TdR by washing cells twice with cold 5% (w/v) trichloroacetic acid.
5. Rinse cells with ethanol and air-dry.
6. Mount coverslips (cells on top) in Cytoseal (PGC Scientifics) onto glass microscope slides.
7. Coat slides with chrome alum/gelatin and air-dry.
8. In a dark room, prepare Ilford G5 photographic emulsion by diluting 1 part emulsion in 2 parts distilled water, or use Kodak NTB-2 autoradiographic emulsion *(10)*, and pour emulsion into clean slide mailer (PGC Scientifics) or dipping chamber.
9. Dip slides slowly and smoothly into a slide mailer. Withdraw them slowly and place them vertically in a test-tube rack to dry for 2 h.
10. Place thoroughly dry slides in a light-tight slide box containing desiccant. Seal box with black tape, cover with foil, and expose 7–10 d at 4°C.
11. Put developer (Kodak D-19), water, and Kodak fixer in slide jars and bring to 15 to 20°C in a water bath. The exact temperature is not critical, but lowering the temperature reduces the silver grain size; 15°C appears optimal.
12. Remove slides from refrigerator and allow them to warm to the same temperature as the developing solution.
13. Transfer slides to slide racks and develop them in a light-tight darkroom as follows: 2.5 min in developer, 30 s in water, and 3 min in fixer.
14. Rinse slides 10 to 15 min under gently running, cool tap water in the light, then once in cool distilled water. While slides are still wet, scrape off emulsion on the back of the slides with a razor, and then allow them to dry in a dust-free location.
15. Counterstain nuclei with Giemsa stain for a few seconds to a few minutes, depending on how intense a staining is desired.
16. Rinse slides in water three times, 2 min each time.
17. Dehydrate slides through the ethanol series (3 staining dishes or jars each filled with 50, 70, and 95% ethanol, and 2 dishes filled with 100% ethanol), 2 min in each dish. Transfer to Xylenes (98.5% xylene isomers plus ethyl benzene, Sigma) and do three changes, 2 min each time (*see* **Note 6**).
18. Place a drop of Permount (Fisher Scientific) onto the cells followed by a new coverslip. This improves viewing.
19. Determine the fraction of cells with labeled nuclei using a light microscope (without phase contrast).

3.2.3. Incorporation of BUdR Followed by Immunostaining (9)

5-bromo-2'-deoxyuridine (BUdR) is a TdR analog and, as such, is incorporated into cellular DNA through the same pathway. Inhibition of the *de novo* biosynthesis of TdR allows complete replacement of BUdR for TdR. To achieve this, 5-fluoro-2'-deoxyuridine (FUdR), which forms 5-F-UMP, an irreversible inhibitor of thymidylate synthase, is sometimes used in conjunction with BUdR *(11,12)*. Replication domains can be visualized *in situ* by indirect immunofluo-

rescence using antibody against BUdR, followed by a secondary fluorescent-labeled antibody that recognizes the primary antibody *(13,14)*.

1. Culture cells on coverslips (*see* **Subheading 3.2.2.**, **step 1**).
2. Transfer coverslips to a 3-cm tissue-culture dish containing 2-mL culture medium supplemented with 25 μ*M* BUdR and 2 μ*M* FUdR.
3. Incubate cells for the required time at 37°C (15 min to several hours).
4. Rinse cells in 5 mL PBS (3 times) at RT and drain excess liquid from coverslips by touching their edges to an absorbent tissue (Kimberly-Clarke tissue wipers).
5. Fix cells by immersing them in 100% methanol at –20°C for 10 min (*see* **Note 7**).
6. Drain excess liquid from coverslips and air-dry.
7. Denature DNA by placing coverslips either in 2 *M* HCl containing 15 μg/mL pepsin for 15 min at RT or in 4 *M* HCl for 30 min at RT.
8. Rinse coverslips with PBS, and then soak them in PBS for 5 min. Rinse again and drain excess liquid, but do not allow samples to dry until staining procedure is completed or nonspecific background staining will appear.
9. Add 20 μL mouse anti-BUdR antibody (Becton Dickinson) per coverslip, and incubate for 60 min at RT in a humidified chamber (to prevent sample drying), or incubate overnight at 4°C. The anti-BUdR should be diluted to a suitable concentration (usually 1:100) in PBS containing 1% BSA.
10. Rinse coverslips with PBS, soak in PBS for 5 min, rinse again, drain excess liquid, but do not allow to dry.
11. Add 20 μL fluorescein isothiocyanate (FITC) or tetramethyl-rhodamine isothiocyanate (TRITC) conjugated goat (or rabbit) antimouse-IgG antibody per coverslip. This antibody should be diluted to a suitable concentration (usually 1:100) in PBS containing 1% BSA. Incubate for 60 min at 37°C in a humidified chamber in the dark (to prevent bleaching of fluorescence).
12. Rinse coverslips with PBS, soak in PBS for 5 min, and rinse again.
13. Rinse the back side of coverslips (not the cells) once with distilled water, drain excess liquid (*see* **Note 8**), and mount coverslips in DABCO (*see* **Note 9**) on a glass microscope slide.
14. Count labeled nuclei using fluorescence microscopy *(15)*.

3.3. Measuring DNA Replication In Vitro by Incorporation of Labeled Precursors

Labeled deoxyribonucleoside triphosphates can be incorporated directly into nascent DNA by incubating them either with permeabilized cells or with isolated nuclei instead of with whole cells. This strategy has several advantages. It can be applied to most cells and tissues. Higher specific activities of the labeled compound can be obtained. [α-^{32}P] dNTPs can be used to increase the sensitivity of the assay. Shorter labeling times can be used. Nonradioactive DNA precursors labeled with fluorescein, digoxigenin, biotin, or rhodamine can be used. Like nucleoside triphosphates, these molecules cannot cross plasma membranes and therefore must either be injected into cells *(16)* or the cells

must be permeabilized. However, they have three advantages over BUdR. The DNA does not have to be denatured. The background staining is generally lower, and there are fewer and shorter incubation steps. Moreover, whereas digoxigenin-dUTP and biotin-dUTP incorporation are detected by fluorescein-conjugated antibodies, fluorescein-dUTP and rhodamine-dUTP are visualized directly by fluorescence microscopy.

Another important advantage is that S-phase cells can be distinguished clearly from G1-phase cells, because when cells are permeabilized or nuclei are isolated by methods that release nuclear proteins, then only those nuclei that contain active replication forks (i.e., S-phase nuclei) continue to synthesize DNA. The ability of replication forks to continue under these conditions depends on the amounts of various replication enzymes that are present. Therefore, amount of nucleotide incorporation is markedly increased by incubating the nuclei either in a *Xenopus* egg extract *(7,17)* or a mammalian cell extract *(18–20)*. Methods have been described for isolating nuclei from cultured cells *(18–21)*, tissues *(21)*, *Drosophila* cells and tissues *(22,23)*, and yeast *(24)*.

In contrast, when mammalian cells are lysed with digitonin under conditions that leave nuclei impermeable to large molecules *(17)*, then nuclei from G1-phase cells can initiate site-specific DNA replication *de novo* when incubated in a *Xenopus* egg extract *(7)* or in a human cell extract *(20)*. Under these conditions, S-phase nuclei can be distinguished from G1-phase nuclei by inhibiting cyclin-dependent and DNA-dependent protein kinase activities *(7,25)*.

The protocols described below for incorporation of fluorescein-12-dUTP can also be used for incorporation of biotin-16-dUTP, digoxigenin-11-dUTP, or rhodamine-5-dUTP (Roche Molecular Biochemicals), as well as [^{3}H]dNTP, [α-^{32}P]dNTP or BrdUTP using the detection methods described above.

3.3.1. DNA Replication in Permeabilized Cells (26)

1. Culture cells on coverslips (*see* **Subheading 3.2.2.**, **step 1**).
2. Rinse coverslips with PBS and then with ice-cold transport buffer.
3. Permeabilize cells for 5 min on ice by soaking coverslips with transport buffer containing 40 µg/mL digitonin, 2 m*M* dithiothreitol, and 1 µg/mL each of protease inhibitors aprotinin, leupeptin, and pepstatin A.
4. Stop reaction by adding BSA to final concentration at 1%.
5. Rinse coverslips three times with transport buffer.
6. Place coverslips in SuNaSP/BSA.
7. Incubate coverslips for 60 min at 37°C in a humidified chamber with 40-µL incubation buffer consisting of 30 µL SuNaSP/BSA with protease inhibitors (1 µg/mL each of aprotinin, leupeptin, and pepstatin A) and 10 µL nucleotide mix containing 10 µ*M* fluorescein-12-dUTP.
8. Wash out the unincorporated fluorescein-12-dUTP with excess PBS, and then fix cells in 4% paraformaldehyde for 10 min at RT.

9. Wash out formaldehyde with PBS and then incubate with PBS for 5 min.
10. Mount coverslips (cells facing down) in a drop of DABCO containing 1 µg/mL DAPI (*see* **Note 10**) on glass microscope slides and view by fluorescence microscopy *(15)*.

3.3.2. DNA Replication in Frozen Tissue Sections

The same protocol (*see* **Subheading 3.3.1.**) used to determine the fraction of cultured cells in S phase can be applied to sections of frozen tissues, with some modifications *(26)*.

3.3.3. DNA Replication in Isolated Nuclei

Only nuclei already in S phase will synthesize DNA under these conditions *(18,19)*.

1. Wash mammalian cells free of culture medium with PBS. For attached cells, simply wash the surface of the dish with excess solution. For suspension cells, wash cells by sedimentation.
2. Rinse cells once with hypotonic buffer.
3. In the cold room, scrape cells from the dish into ice-cold hypotonic buffer (approx 4×10^6 cells/mL) using a rubber policeman. Cell concentration is not critical, but they must be cold to prevent inactivation of replication forks.
4. Use three strokes in a Dounce homogenizer with the tight pestle to create a uniform suspension. Check extent of cell lysis by light microscopy. If cells are difficult to break, then adjust sample to 0.05% Triton X-100.
5. Recover nuclei by sedimentation. A swinging bucket rotor is ideal (e.g., Sorvall HB4, 17,500g, 10 min, 4°C), but for large numbers of samples an angle rotor will do nicely.
6. Resuspend nuclei (approx 5×10^5 nuclei/µL) in a final concentration of 30 mM HEPES-KOH (pH 7.5), 5 mM MgCl$_2$, 0.8 mM dithiothreitol, 0.1 mM each dATP, dGTP, and dCTP, 20 µM fluorescein-12-dUTP, 200 µM each CTP, GTP, and UTP, 2 mM ATP, 40 mM creatine phosphate and 20 µg CPK.
7. Incubate for 1 h at 37°C.
8. Proceed as in **steps 2–5**, **Subheading 3.3.4.**

3.3.4. DNA Replication in Xenopus *Egg Extract* **(9,27)**

G2- and M-phase nuclei will not initiate DNA replication under these conditions, but G1-phase nuclei will (in addition to S-phase nuclei) unless 3 mM 6-dimethylaminopurine is included *(7)*.

1. Incubate digitonin-prepared nuclei (1000 to 5000 nuclei/µL extract) in *Xenopus* egg extract *(7,25,28)* supplemented with 24 mM PC, 30 µg/mL CPK, 250 µg/mL cycloheximide (CHX), and 10 µM fluorescein-12-dUTP for 60 min at 22°C.
2. Fix 10 µL aliquots of samples in 100 µL 4% formaldehyde for 10 min at RT.

3. Layer sample over 300 µL of 25% glycerol and centrifuge nuclei onto microscope slides.
4. Use a StatSpin Cytofuge (PGC Scientifics, or http://www.statspin.com) to attach nuclei to specific sites on a clean microscope slide. Attach the three-chamber sample loading device onto a standard slide using two clips to seal the chamber slide. Load 25% glycerol into each chamber, and then layer samples over the glycerol. Centrifuge samples at 500*g* for 15 min. Remove glycerol using a pipet, remove the sample loading chamber, and air-dry the slide. Alternatively, nuclei can be sedimented onto coverslips (*see* **Note 11**).
5. Mount coverslips on microscope slide (**step 10** in **Subheading 3.3.1.**).

3.3.5. Quantifying the Amount
of DNA Synthesis Using a Xenopus Egg Extract (27)

The actual amount of radio-labeled DNA can be quantified either as acid-precipitable radioactivity, or by fractionating the DNA by gel electrophoresis and then using densitometry to quantify the intensity of the radio-labeled DNA bands in autoradiographs of the gels *(28,29)*. The first procedure is simpler and faster.

The dATP *(28)* and dCTP *(29)* pools in *Xenopus* egg extracts are approx 50 µ*M* each. Therefore, if one knows the specific activity (disintegration per minute/mole) of the radio-labeled dNTP purchased and the total amount of the dNTP present, one can calculate the number of moles dNTP incorporated into acid-insoluble DNA. Alternatively, this can be calculated from the *fraction* of radio-labeled dNTP incorporated into the acid insoluble fraction. The accuracy of the first method relies on the specific radioactivity stated by the manufacturer. The accuracy of the second method requires that a significant fraction of the isotope is incorporated into DNA.

1. Place [^{3}H]dATP (0.5 µCi/10 µL reaction, approx 30 to 60 Ci/mmol, Amersham Pharmacia Biotech) into 0.5-mL Eppendorf-type tubes.
2. Take to dryness under vacuum.
3. Add DNA template, *Xenopus* egg extract, supplement with PC, CPK, and CHX (*see* **step 1**, **Subheading 3.3.3.**), and incubate at 22°C (usually 1 to 2 h).
4. Add 200 µL of Stop C containing 0.5-mg/mL proteinase K to 10 µL samples (total volume of each sample is 210 µL).
5. Incubate either at 37°C for 30 min or at RT overnight.
6. Run a sonication bath for 15 min to remove air bubbles, and then sonicate samples for 15 min at RT to break up DNA.
7. To determine the total amount of [^{3}H]dATP/µL, spot 15 µL of each sample onto a 2.5-cm GF/C Whatman filter and dry.
8. To determine the amount of acid-insoluble [^{3}H]dATP (i.e., [^{3}H]DNA), spot 60 µL of each sample onto a 2.5-cm GF/C filter and dry.

9. Place a multiwell tray (Fisher or 6-well plates) on ice and add sufficient ice-cold 10% TCA solution (approx 3 mL) to each well to immerse the filter.
10. Place one of the 60 µL filters in each well and incubate for 30 min.
11. Remove TCA by aspiration and then soak each filter three times in cold 5% (w/v) TCA, allowing 10 min for each wash.
12. Soak each filter three times for 10 min in 100% methanol to remove acid and salts.
13. Dry filters. Place each filter in a scintillation vial with 5 mL scintillation fluid (Ecoscint H, National Diagnostics) and record radiation in a scintillation counter (Beckman, LS 5801). Percentage [^{3}H]dATP incorporated = [(cpm from 60 µL) × (210/60)] ÷ [(cpm from 15 µL) × (210/15)] × 100.
14. ng DNA synthesized/µL extract = [65.4 ng dNTPs/µL extract] × [fraction of [^{3}H]dATP incorporated] (*see* **Note 12**).
15. Percentage DNA template replicated = [ng DNA synthesized/µL extract] ÷ [ng input DNA/µL extract] × 100 (*see* **Note 13**).

3.4. Semiconservative DNA Replication

DNA replication can be distinguished from nuclear DNA repair and mtDNA replication by measuring the amount of DNA synthesis that takes place in nuclei by a semiconservative mechanism: each DNA strand is a template for newly synthesized DNA. Therefore, newly replicated DNA contains one newly synthesized strand and one old strand. Semiconservative DNA replication can be detected by incorporating BUdR, a nucleotide analog of TdR whose density is greater than the natural nucleotide it replaces. If all of the cells replicate their DNA, then all of the DNA in the population will change from its normal density (light:light; approx 1.70 gm/cm^3) to the density observed after one round of replication (light:heavy; approx 1.75 gm/cm^3). This change can be detected by density equilibrium sedimentation at neutral pH. If the cells continue into a second division cycle, then light:heavy DNA is converted into equal portions of heavy:heavy DNA (approx 1.78 gm/cm^3) and heavy:light DNA. Confirmation of semiconservative replication is made by isolating the heavy:light peak of Br-DNA and fractionating it by density equilibrium sedimentation at alkaline pH to separate it into two peaks, one containing the light strand and one containing the heavy strand. DNA that has been damaged and then repaired at randomly selected sites will appear as partially substituted DNA (at neutral pH, the leading edge of the light:light DNA peak will smear toward the heavy:light density).

This analysis is usually applied to monitoring the progression of a synchronous population of cells into S phase after their release from synchrony. It is also useful to monitor DNA replication in vitro. mtDNA can be eliminated from consideration by first isolating the nuclei and then extracting Br-DNA.

3.4.1. Density Substitution Using a Xenopus Egg Extract (9)

1. Incubate permeabilized cells or isolated nuclei with *Xenopus* egg extract and supplements (*see* **Subheading 3.3.3.**, **step 1**) containing 0.5 m*M* BUdR and [^{3}H]dATP (1 µCi/10-µL; approx 30 to 60 Ci/mmol) at 22°C for 1 to 2 h.
2. Stop reaction by adding 180 µL Stop C containing 0.5-mg/mL proteinase K to 20 µL aliquots from each sample (total sample volume = 200 µL), and incubate either at 37°C for 30 min, or at RT overnight.
3. Extract DNA by adding 200 µL phenol/sample and shake vigorously.
4. Separate layers by centrifugation. Save the aqueous (top) layer.
5. Add aqueous layer to equal volume of phenol/chloroform (1:1), and repeat **steps 3–4**.
6. After adding 6 µL 5 *M* NaCl to the aqueous layer, precipitate DNA with 375 µL ethanol, spin down, and resuspend samples in 50 µL TE with 1-µL phenol red.
7. Separate DNA from free label on a 2.4-mL disposable column (Pierce) packed with Whatman G50 medium Sephadex and prequilibrated with TE.
8. Collect the elute as 4-drop fractions and 2.5 µL aliquots of each sample are removed for scintillation counting in 5-mL of Ecoscint H.
9. Pool the fractions from the first peak of radioactivity and adjust samples to 0.5 mL with TE.
10. Mix with 2.5 mL of CsCl solution.
11. Layer over 3 mL of CsCl solution in a Beckman 50 Ti centrifuge tube.
12. Centrifuge in a Beckman 50 Ti rotor at 140,000*g* at 20°C for 60 h (*see* **Note 14**).
13. Collect fractions with a capillary tube lowered slowly to the bottom of the tube using a jack and by pumping 30 timed fractions. Measure radioactivity by scintillation counting in 90% Aquasol (New England Nuclear Corporation)/10% distilled water.
14. Determine the density of each fraction from its refractive index using a refractometer.

3.4.2. Density Substitution in Cultured Cells (11)

1. Prelabel DNA by culturing cells with [^{14}C]TdR (0.25 µCi/mL; 33 mCi/mmole) for 18 h.
2. Synchronize cells (*see* **Subheading 3.1.**).
3. Release synchronized cells into culture medium containing 50-µg/mL BUdR and 0.1 µg/mL FUdR at 37°C.
4. At various times, isolate cells by trypsinization and resuspend in buffer A (10^6 cells/mL).
5. Lyse cells in 1% Sarkosyl and incubate samples for 30 min at 60°C.
6. Extract DNA twice by mixing samples with an equal volume of chloroform-isoamyl alcohol (24:1).
7. Centrifuge the mixture and save the aqueous (top) layer.
8. The following steps are the same as **steps 7–14** in **Subheading 3.4.1.**

3.5. S-Phase Specific Proteins

Proliferating cell nuclear antigen [PCNA, *(30,31)*] is a required co-factor for DNA polymerase-δ. PCNA consists of two subpopulations; one is soluble

in the nucleoplasm throughout the cell cycle; the other is insoluble, tightly associated with DNA replication sites, and present only during S phase *(30,31)*. Replication protein A [RP-A, *(32,33)*] is a single-stranded DNA-binding protein that stimulates both DNA polymerase and DNA helicase activities. RP-A is associated with chromatin throughout the cell cycle, but during S phase it is tightly bound to DNA replication forks. Therefore, either protein can be used to identify cells in S phase by permeabilizing cells with a nonionic detergent directly before fixation to release proteins not tightly associated with chromatin, so that only chromatin-bound nuclear proteins are detected by the antibody *(32,34)*.

3.5.1. Measuring the Fraction of Cells With Chromatin-Bound PCNA

1. Culture cells on coverslips as described in **Subheading 3.2.2.**, **step 1**.
2. Wash cells once with PBS and once with CSK buffer.
3. Extract cells for 5 min at RT with CSK buffer containing 0.5% Triton X-100, 0.5 m*M* phenylmethylsulfonyl fluoride PMSF, and 10 µg/mL each of leupeptin and aprotinin.
4. Rinse cells twice with CSK buffer.
5. Fix cells in 4% paraformaldehyde at 4°C for 30 min (or in 4% formaldehyde for 10 min at RT).
6. Stain cells with anti-PCNA antibody (Santa Cruz Biotechnology; dilute to a suitable concentration, usually 1:100, in PBS containing 1% BSA).
7. Wash cells with PBS for 5 min.
8. Stain cells with fluorescein-conjugated (FITC or TRITC) goat (or rabbit) antimouse-IgG that has been diluted to a suitable concentration (usually 1:100) in PBS containing 1% BSA.
9. Wash cells with PBS for 5 min.
10. Visualize cells by fluorescence microscopy *(15)*.

3.5.2. Detecting Chromatin-Bound PCNA
During DNA Replication in Xenopus *Egg Extract*

1. Incubate permeabilized cells or isolated nuclei with *Xenopus* egg extracts as in **Subheading 3.3.3.**, **step 1**.
2. After incubation, dilute 10 µL of each sample in 4 volumes of NIB, and then centrifuge at 6000*g* (microfuge) for 5 min through an equal volume of NIB containing 15% sucrose.
3. Resuspend nuclear pellet in 10 µL NIB containing 0.5% Triton X-100 and incubate for 5 min.
4. Fix nuclei with 4% paraformaldehyde at 4°C for 30 min (or in 4% formaldehyde for 10 min at RT), and centrifuge nuclei onto coverslips as in **Subheading 3.3.3.**, **steps 3–6**.
5. Stain nuclei with anti-PCNA antibodies and visualize stained nuclei as in **Subheading 3.5.1.**, **steps 6–10**.

3.6. Flow Cytometry Analysis (35,36)

Flow cytometry measures the amount of DNA per cell and records the fraction of cells with a specified amount of DNA. Therefore, the fraction of S-phase cells (>2N < 4N DNA content) can be estimated by subtracting the number of G1 (2N) and G2 +M (4N) phase cells from the total number of cells in the population. Analysis of DNA content by flow cytometry requires that the cells first be stained with a fluorochrome that binds to DNA. Propidium iodide (PI) is commonly used for this purpose, but because it also binds RNA, cellular RNA must first be eliminated. DAPI can also be used to stain DNA, in which case the RNase A treatment is omitted because DAPI stains only double-stranded DNA. However, scoring DAPI stained cells requires a flow cytometry equipped with ultraviolet (UV) light. The following is a protocol for cells cultured in vitro. A similar protocol for yeast cells is found at http://www.bio.uva.nl/pombe/handbook/. Furthermore, Chapters 4 and 6 in this volume address flow cytometry in great detail (*see* Chapters 4, 7, 8).

1. Grow cells on 9-cm Petri dishes (Nunc) (*see* **Note 15**).
2. Trypsinize cells. Recover them by centrifugation at 200*g* (Benchtop centrifuge) for 10 min at RT.
3. Wash cells once with culture medium and once with PBS.
4. Resuspend approx 5×10^5 to 1×10^7 cells in 0.5-mL PBS.
5. Fix cells (0.5 mL) in 4.5-mL cold 70% (v/v) ethanol for 2 h on ice (*see* **Note 16**).
6. Recover cells by centrifugation (4°C) and remove ethanol.
7. Wash cells once with PBS and resuspend them in 5-mL PBS.
8. Treat cells with 50 mg/mL PI and 1 mg/mL RNase A for 10 min at RT.
9. Measure cell fluorescence by flow cytometry *(37)* using a Becton Dickinson FACScan with an appropriate computer program for curve and area analysis (provided with the instrument). Use of the FACScan is best learned from a demonstration and by reading the manual. Again, *see* Chapters 4 and 6 in this volume for more detail (*see* Chapters 4, 7, 8).

4. Notes

1. Conditioned medium is obtained by removing aliquots 2 to 3 h after adding fresh medium to cells of the same age and population density as those used in the experimental cells.
2. Cells are typically lysed while attached as monolayers or suspended in SSC (cell pellets).
3. Set one channel to count high energy β radiation, another to count the low energy β radiation, and a third to count the total spectrum.
4. The calculation of the two isotopes in a mixture:

$$C^3H = [C^T \times 1/(1 - xy)] - [A^T \times x/(1 - xy)] \tag{1}$$

$$A^{14}C = [A^T \times 1/(1 - xy)] - [C^T \times y/(1 - xy)] \tag{2}$$

C^3H is the number of scaler C counts owing to ^3H. $A^{14}C$ is the number of scaler A counts owing to ^{14}C. C^T is the total cpm in scaler C. A^T is the total cpm in scaler A; x is the ratio [scaler C cpm/scaler A cpm] when a standard containing only ^{14}C is counted at the setting used for both isotopes; y is the ratio [scaler A cpm/scaler C cpm] when a standard containing only ^3H is counted at the setting used for both isotopes.

5. Cells can be grown directly on Lab-Tek chamber slides (Nunc).
6. It is important to perform the dehydration and equilibration in Xylenes (98.5% xylene isomers plus ethyl benzene, Sigma) carefully, because Permount will not mix well with water or ethanol.
7. It is crucial to prechill methanol to –20°C before fixation. Cells will get better fixation under prechilled methanol.
8. Rinse the back side of coverslips with water to remove PBS, which interferes with visualization under the microscope.
9. DABCO can be replaced by Vectashield mounting medium (Vector).
10. DAPI and DABCO mixture can be replaced by Vectashield mounting medium containing DAPI (Vector).
11. Using bath wax (Fisher Scientific), attach 22-mm diameter glass coverslips to the bottoms of acrylic rings (depth 10 mm, outer diameter 28 mm, inner diameter 8 mm), which are constructed to fit into the bottom of a round-bottom centrifuge tube (in this case, an MSE Super Minor benchtop centrifuge, *[9]*). Load 300 µL of 25% glycerol into the ring with the coverslip attached and then layer nuclei over the glycerol. Stack 3 or 4 of these devices into a single centrifuge tube and spin at 500*g*, 15 min, 4°C. Aspirate off glycerol. Detach coverslips and air-dry.
12. Using 327 as the average molecular weight of the four dNTP DNA precursors, 327×10^{-6} µg/µL = 1 µM dNTP. Therefore, the concentration of all four dNTPs = (4 dNTPs)(50 µM/dNTP)(327×10^{-6} µg/µL)(10^3 ng/µg) = 65.4 ng dNTP/µL extract.
13. Input DNA ng/µL extract = (number of nuclei added) × (ng DNA/per mammalian nucleus) × 100. Based on the amounts of DNA in sperm, mammals contain approx 6.6 pg DNA/nucleus and frogs contain approx 6.3 pg DNA/nucleus.
14. Caution: Crystallization of CsCl during centrifugation can result from overfilling tubes or centrifuging at low temperatures.
15. A convenient number of cells to use is 1×10^7, although 5×10^5 to approx 1×10^6 cells are sufficient.
16. Cell suspension in ethanol can be stored at –20°C until needed.

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6

Yeast Cell Synchronization

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1. Introduction

Saccharomyces cerevisiae, the budding yeast, is widely used as a model eukaryote to study a large number of cellular processes including cell cycle regulation *(1–4)*. Extensive genetic research in the last two decades has revealed that the basic mechanism of cell cycle control is highly conserved in all eukaryotic cells *(4,6)*. These observations combined with powerful genetic, molecular, and biochemical tools have frequently made *S. cerevisiae* the experimental organism of choice for studying the mechanisms that regulate cell cycle progression *(1,4,5)*.

The propagation and maintenance of yeast are simple and economical *(3,6,7)*. Because of this and the fact that yeast grow very rapidly (doubling time approx 90 min in rich media), large numbers of cells can easily be obtained for genetic, molecular, and biochemical studies. To study cell cycle processes, it is frequently necessary to use synchronized cultures *(1,8–15)*. There are essentially two methods to obtain synchronized yeast populations: centrifugal elutriation and block-and-release methods. Each method has specific advantages and disadvantages, and it is recommended that multiple approaches be tried before generalizing a result.

The design and type of experiment usually dictates the method for synchronizing cells. Centrifugal elutriation is generally regarded as the best method for obtaining homogenous populations of relatively unperturbed synchronously dividing yeast cells *(10–13,15)*. However, in comparison with other block-and-release protocols, moderately small numbers of synchronous cells are obtained. Sufficient numbers of cells are obtained for small expression analysis experiments (e.g., northern or western analysis and flow cytometry). In contrast, block-and-release protocols can easily generate the large numbers of synchronized

cells that biochemical experiments often necessitate *(8,11,12)*. In this chapter, centrifugal elutriation and several types of block-and-release methods are discussed in detail. Most important, each method described is only as good as the synchrony that it produces. The methods described here provide a good degree of synchrony through one to two rounds of cell division, but it is highly recommended that the investigator carefully assess the degree of synchrony throughout the experiment (*see* **Subheading 3.6.**).

2. Materials

2.1. Media

1. Yeast Extract Peptone (YEP): Yeast extract peptone (YEP) is a rich, complex medium without a carbon source (*see* **Note 1**).

    a. 10 g yeast extract.
    b. 20 g peptone.
    c. 1000 mL distilled water.
    d. Sterilize by autoclaving.

2. 5X Yeast Extract Peptone (YEP): A 5X stock of YEP is made as a stock for elutriation experiments (*see* **Note 2**).

    a. 50 g yeast extract.
    b. 100 g peptone.
    c. 1000 mL distilled water.

3. YEP with 2% Acetate (YPA)
4. YEP with 2% Glucose (YEPD)
5. Synthetic Dextrose Medium (SD): SD is a synthetic minimal medium containing yeast nitrogen base (YNB), vitamins, trace elements, salts, and glucose (*see* **Note 3**).

    a. 1.7 g YNB without amino acids and without ammonium sulfate.
    b. 5 g ammonium sulfate.
    c. 20 g glucose.
    d. 1000 mL distilled water.

6. SC (Synthetic Complete Medium): SC is a completely synthetic medium made by supplementing SD with 22 amino acids, Na_2HPO_4, and para-aminobenzoic acid (PABA; *see* **Note 3**).

    a. 1.7 g YNB without amino acids and with ammonium sulfate.
    b. 5 g ammonium sulfate.
    c. 0.65 g primary amino acids mix.
    d. 0.35 g secondary amino acids mix.
    e. 0.2 g "drop-out" amino acid mix.
    f. 0.45 g Na_2HPO_4.
    g. 20 g glucose.
    h. 1000 mL distilled water.

7. Primary Amino Acids Mix: This mix contains 6 g each of the following amino acids: alanine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, isoleucine, valine, phenylalanine, proline, serine, threonine, glycine, and 0.6 g of PABA (*see* **Note 3**).

8. Secondary Amino Acids Mix: This mix contains 6 g each of the following amino acids: arginine, lysine, methionine, and tyrosine and 4 g of adenine (*see* **Note 3**).

9. "Drop-Out" Amino Acid Mix: This mix contains 1 g each of the following amino acids: histidine, leucine, tryptophan, or uracil (*see* **Note 3**).

10. "Drop-Out" Medium: This is SC medium where one or more supplemented amino acids have been omitted ("dropped-out"). Most frequently these are histidine, leucine, tryptophan, or uracil but could include any amino acid in the primary or secondary mixes. For the sake of simplicity, individual "drop-out" powders can be made for each medium. As an example, the recipe for -URA "drop-out" medium is given.

    a. 1.7 g YNB without amino acids and with ammonium sulfate.
    b. 5 g ammonium sulfate.
    c. 0.65 g primary amino acids mix.
    d. 0.35 g secondary amino acids mix.
    e. 0.05 g leucine.
    f. 0.05 g histidine.
    g. 0.05 g tryptophan.
    h. 0.45 g Na_2HPO_4.
    i. 20 g glucose.
    j. 1000-mL distilled water.

11. Sporulation Medium (SPM):

    a. 10 g potassium acetate.
    b. 0.05 g zinc acetate.
    c. Supplement with required amino acids (*see* **Note 4**).
    d. 1000 mL distilled water.

2.2. Strains

BY4741 *MATa his3Δ leu2Δ met15Δ ura3Δ*

BY4743 *MATa/MATα his3Δ/his3Δ leu2Δ/leu2Δ met15Δ/MET15 lys2Δ/LYS2ura3Δ/ura3Δ*

W303 *MATa ade2 his3 leu2 trp1 ura3 can1*

W303 *MATαade2 his3 leu2 trp1 ura3 can1 cdc15-2*

Cell cycle drugs (*see* **Note 5**).

α factor is available from Sigma Chemicals (cat. no. T6901) and U.S. Biological (cat. no. Y2016).

Nocodazole is available from Sigma Chemicals (cat. no. M1404) and U.S. Biological (cat. no. N3000).

Hydroxyurea is available from Sigma Chemicals (cat. no. H8627) and U.S. Biological (cat. no. H9120).

2.3. Equipment

1. Standard Yeast Culture: Access to the following standard yeast culture equipment, supplies, and knowledge of general yeast manipulation are a presumed prerequisite *(3,5,7)*: variable temperature incubators, shaking water baths, standard culture flasks, tubes, glassware, midspeed centrifuge, spectrophotometer, centrifuge tubes and bottles, filter apparatus, and a standard microscope. Although not required, access to a Z2 Coulter Counter/Channelyzer (for monitoring cell size and cell number) and a benchtop flow cytometer are highly recommended.
2. Centrifugal Elutriator: Complete elutriation systems are available from Beckman Instruments (2500 Harbor Blvd., Fullerton, CA 92834 USA) (**Fig. 1**). The JE-5.0 rotor is recommended and can be used in Beckman J6 series or Avanti J-20 series centrifuges. Three sizes of elutriation chambers are available: a large 40-mL chamber, a standard 4-mL chamber, and a Sanderson 5.5-mL chamber. Researchers are encouraged to examine the Beckman manual for detailed equipment specifications. The complete elutriator system includes a midspeed centrifuge, rotor, one or two elutriation chambers, pressure gage, stroboscope, control unit, tubing, injection ports, and spare parts. In addition, a variable-speed peristaltic pump (Cole-Parmer Masterflex drive 7520-20 and pump head 7016-20) and a sonicator (Fisher cat. no. 15-338-53) are required.

3. Methods

3.1. Synchronization by Centrifugal Elutriation

Centrifugal elutriation is a process for separating cells on the basis of their size, mass, and shape *(10–13,15)*. This process uses centrifugal force to sediment cells in the presence of counterflowing media (**Fig. 2**). In this manner, large budded cells or irregularly shaped cells sediment toward the outer edge of the elutriation chamber (**Figs. 2B** and **3**). In contrast, smaller cells are less affected by centrifugal force (**Figs. 2B** and **3**). In addition, owing to their small volumes, small cells have large surface-area-to-volume ratios. Thus, they tend to be pushed toward the top of the elutriation chamber by the counterflowing media (**Figs. 2B** and **3**). This process sets up a cell-size gradient across the elutriation chamber (**Figs. 2B** and **3**). By maintaining the rotor at a constant speed and by increasing the rate with which counterflowing media enters the elutriation chamber, the smallest cells can be elutriated out of the chamber and collected (**Fig. 3**).

Centrifugal elutriation is probably the best method for obtaining pure populations of synchronously dividing cells *(10–13,15)*. There are three main advantages to this technique. First, it generates synchrony in the most physiologically relevant manner. Because this is a size selection technique, it does not perturb the normal coordination between cell growth and cell division (*see* **Note 6**). Second, it is the most universally adaptable of the protocols because any yeast strain can be used (*see* **Note 7**). Finally, elutriation is the only method for studying the earliest events in cell cycle regulation in small G1-phase daughter cells.

Fig. 1. Complete elutriation system setup.

A Centrifugal Force→

Small and large yeast begin to enter the elutriation chamber

B

Centrifugal elutriation creates a size gradient with smaller cells towards the top and larger cells towards the bottom of the elutriation chamber.

C ← Counter flow

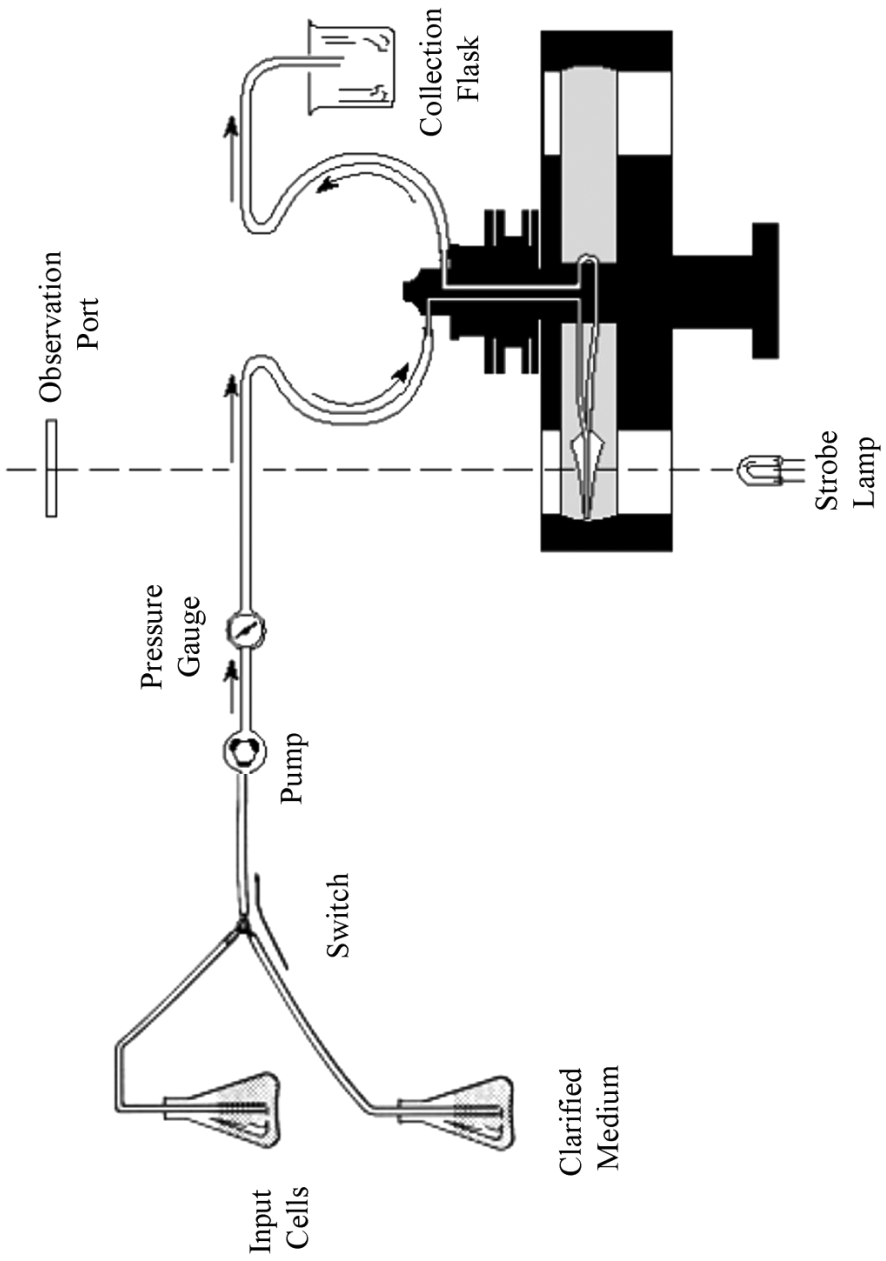Increasing the counter flow elutes the smallest cells out of the elutriation chamber.
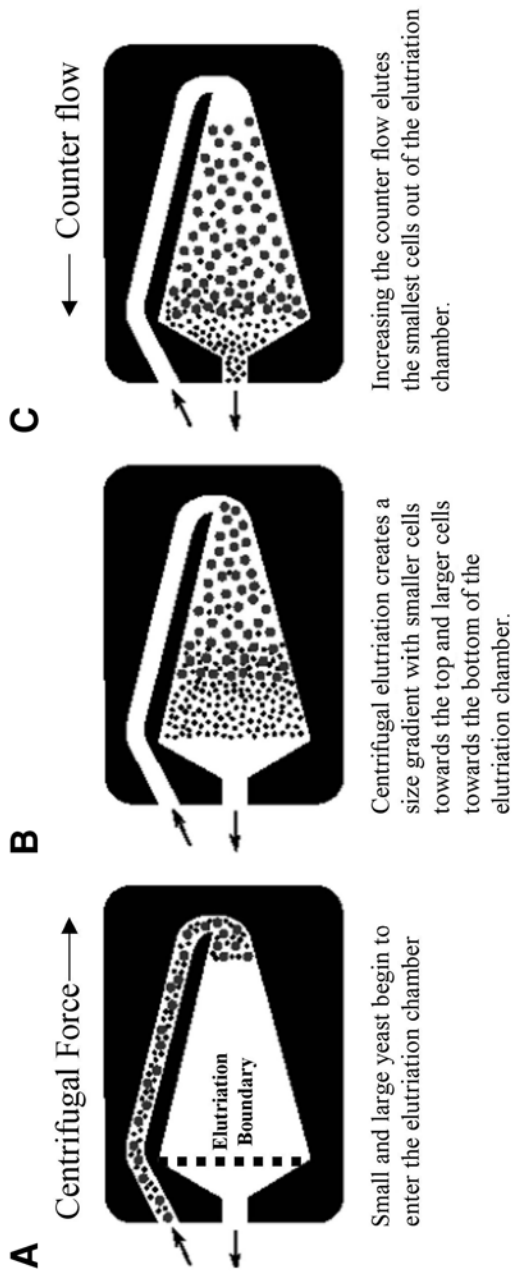
Fig. 2. (**A**) Small and large yeast begin to enter the elutriation chamber. (**B**) Centrifugal elutriation creates a size gradient with smaller cells toward the top and larger cells toward the bottom of the elutriation chamber. (**C**) Increasing the counter flow elutes the smallest cells out of the elutriation chamber.

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Small cells accumulate here, and are collected first

Large cells accumulate here, and are collected last

Cells exit the elutriation chamber here, and enter the collection tubing

Elutriation boundary is illustrated as a dotted line.

Fig. 3. Bird's eye view of the elutriation chamber as seen from observation port.

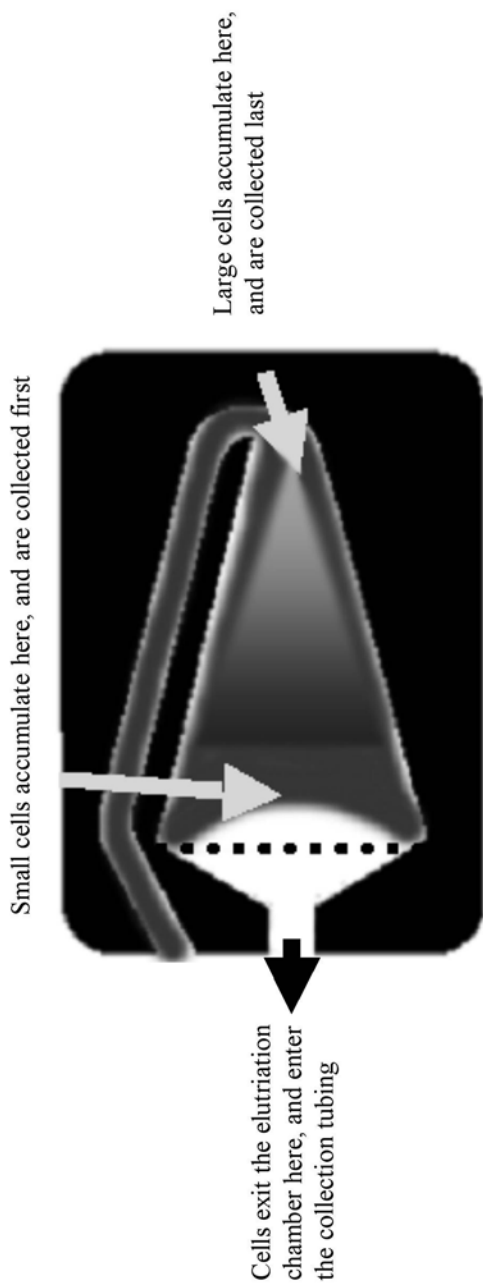The major disadvantage to elutriation is the requirement for large and expensive centrifugation equipment. In addition, the manipulation and centrifugation of yeast cells may induce stress responses *(10–13,15)*. Therefore, it is always advisable to compare results from elutriation protocols to at least one other synchronizing protocol (*see* **Note 8**).

Centrifugal elutriation can be difficult the first few times it is attempted. This is because a number of tasks need to be performed in rapid succession. For this reason, it is recommended that for the first few elutriations investigators work in pairs. There are two ways to use centrifugal elutriation to obtain synchronized cell populations: synchronized outgrowth of size-fractionated cells and chilled incremental fractionation *(10–13,15)*.

3.1.1. Synchronized Outgrowth of Size-Fractionated Cells

The best synchrony of minimally perturbed cultures is obtained by loading large numbers of midlog cells onto a rotor prewarmed to growth temperature. The basic centrifugal elutriation protocol, for use with a single 40-mL elutriation chamber, is described. Depending on the growth conditions, this protocol will generate approx 3×10^9 equivalently sized G1-phase unbudded cells (*see* **Note 9**).

1. Determine growth conditions (*see* **Note 10**). By using conditions that support rapid cell division, the cells obtained by centrifugal elutriation will represent equivalently sized mother and daughter cells. These cells will proceed through two cell cycles relatively synchronously. In contrast, by using conditions that support slow cell division, the cells obtained by centrifugal elutriation will represent a nearly pure population of small daughter cells. These cells will proceed through only one cell cycle synchronously (*see* **Note 11**).
2. Growth of cells. One of the most difficult aspects of elutriation is ensuring that cells are at the appropriate cell density at the correct time. It is highly recommended that a starter culture be pregrown in the medium of choice for 6–12 population doublings (approx 18 h depending on the growth rate of the cells; see **Note 12**).
3. From the starter culture, a 2-L elutriation culture in a 4-L flask should be started at a density such that after approx 18 h of growth, the culture will be approx 2–3×10^7 cells/mL (*see* **Note 13**).
4. Assemble the elutriator. Rinse all parts and tubing with sterile distilled water, and then drain completely (*see* **Note 14**). It is not necessary to install a bubble trap, as the pressure gage will fulfill this role (*see* **Note 15**).
5. Set the elutriator centrifuge to the correct temperature (*see* **Note 16**).
6. Determine cell density and harvest cells by centrifugation at cell growth temperature (*see* **Note 17**). Pour off supernatant being careful not to transfer cells to clarified medium. Resuspend cell pellet in 100–200 mL of clarified medium and save the remaining clarified medium to use as a counterflow medium. At this point, the best results are achieved by minimizing the time cells spend in **steps 7–12** (*see* **Note 18**).

7. Sonicate cells (setting 7) twice for 30 s. Swirl cells continuously to ensure even sonication and to disperse heat (*see* **Note 19**).

8. Fill the elutriator chamber, tubing, and pressure gage with clarified medium. Invert the pressure gage to ensure filling it completely. Tap the pressure gage to remove any bubbles. Make sure that there are no air bubbles in the tubing or elutriation chamber. Collect the flowthrough in a sterile flask (*see* **Note 20**).

9. Set the centrifuge to 2400 rpm, place the centrifuge timer on" hold," and start the centrifuge (*see* **Note 21**). Begin pumping cells into the centrifuge at a rate of approx 20 mL/min. Load no more than 6×10^{10} cells, and do not fill the chamber past the elutriation boundary (**Fig. 2A**). Cells can be visualized loading into the chamber using the strobe lamp and observation port (**Fig. 1**). At first the cells will appear cloudy and turbulent, but as the remainder of cells load into the elutriation chamber, they will appear as a compact, sharp crescent front (**Fig. 3**).

10. After cells have been loaded into the elutriation chamber, begin pumping in clarified medium (**Fig. 1**; *see* **Note 22**). Gradually increase pump speed every 1 to 2 min until the cell front reaches the top of the elutriation chamber (*see* **Note 23**). Depending on the number and size of cells, this should take between 10–20 min at a pump rate of approx 30–40 mL/min.

11. As the cell front reaches the top of the elutriation chamber, the smallest cells, debris and cell ghosts (cells broken by sonication), will begin to elute off the centrifuge (**Figs. 2C** and **3**). When this occurs, the flowthrough medium will become slightly cloudy. At this point examine 3 µL of flow with a phase-contrast microscope with an ×40 objective. Begin collecting 200- to 300-mL fractions. Increase the pump speed in small increments between fractions. Check 3 µL of the flowthrough after every 100 mL with a phase-contrast microscope to determine the morphology and density of the cells that are eluting in each fraction. Roughly assess the number of budded cells. The goal is to collect a high-density fraction of cells that are <5% budded. It is very useful at this time to measure the cell number and size with a Z2 Coulter Counter/Channelyzer (*see* **Note 24**). Continue collecting fractions until the percentage of budded cells approaches 10%.

12. Depending on the experimental design and the number of cells needed, choose either the best fraction (e.g., the highest concentration of cells with a budding percentage (<5%) or pool fractions. The final cell concentration should be approx 1×10^7 cells/mL (*see* **Note 25**).

13. Remove a time-zero fraction to assess synchrony and for experimental purposes. Return the synchronized population to the original incubator. Begin taking timepoints every 15 to 30 min. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.1.2. Chilled Incremental Fractionation

1. Follow **steps 1–4** from **Subheading 3.1.1.**
2. Set the elutriator centrifuge to 4°C.
3. Chill a midspeed centrifuge and centrifuge bottles to 4°C.

4. Ensure that cell cultures are in midlog phase (e.g., $2–3 \times 10^7$ cells/mL). Place the flask on ice. Add an equal volume of ice to the flask to chill the culture rapidly. When the culture temperature is 0–4°C, aliquot into centrifuge bottles and centrifuge (*see* **Note 17**).

5. Pour off the supernatant being careful to not transfer cells to the clarified medium. Resuspend the cell pellet in 100–200 mL of ice-cold water.

6. Sonicate cells (setting 7) twice for 30 s. Swirl cells continuously to ensure even sonication and to disperse heat (*see* **Note 19**).

7. Fill the elutriator chamber, tubing, and pressure gage with ice-cold water. Invert the pressure gage to fill it completely. Tap the pressure gage to remove any bubbles. Make sure that there are no air bubbles in the tubing or elutriation chamber.

8. Set the centrifuge to 2400 rpm, place the centrifuge timer on "hold," and start the centrifuge (*see* **Note 21**). Begin pumping cells into the centrifuge at a rate of approx 20 mL/min. Load no more than 6×10^{10} cells, and do not fill the chamber past the elutriation boundary (**Fig. 2A**). Cells can be visualized loading into the chamber using the strobe lamp and observation port (**Fig. 1**). At first the cells will appear cloudy and turbulent, but as the remainder of cells load into the elutriation chamber, they will appear as a compact, sharp crescent front (**Figs. 2C** and **3**).

9. After the cells have been loaded into the elutriation chamber, switch the stopcock from input cells to ice-cold water (**Fig. 1**). Begin pumping in ice-cold water (*see* **Note 22**). Gradually increase the pump speed every 1 to 2 min until the cell front reaches the top of the elutriation chamber (*see* **Note 23**). Depending on the number and size of cells, this should take between 10–20 min at a pump rate of approx 30–40 mL/min.

10. As the cell front reaches the top of the elutriation chamber, the smallest cells, debris and cell ghosts (cells broken by sonication), will begin to elute off the centrifuge (**Figs. 2C** and **3**). When this occurs, the flowthrough medium will become slightly cloudy. At this point examine 3 µL of flow with a phase-contrast microscope with an ×40 objective. Begin collecting 200- to 300-mL fractions. Increase the pump speed in small increments between fractions. Check 3 µL of the flowthrough every 100 mL with a phase-contrast microscope to determine the nature and density of the cells that are eluting in each fraction. Roughly assess the number of budded cells. The goal is to collect fractions that span the entire cell cycle (e.g., from fractions that are <5% budded to >95% budded). This should be accomplished in ten to fifteen 200- to 300-mL fractions. It is useful at this time to measure the cell number and size with a Z2 Coulter Counter/Channelyzer (*see* **Note 24**).

11. Remove aliquots from each fraction to assess synchrony (*see* **Note 26** and **Subheading 3.6.**).

12. Centrifuge the fractions and pellet cells. Pour off supernatant and resuspend cells in approx 1.0 mL of ice-cold water. Transfer cells to prechilled 1.5- to 2.0-mL microcentrifuge tubes. Centrifuge for 30 s to pellet cells. Pour off the supernatant and freeze cells at –80°C (*see* **Note 27**).

3.2 Synchronization by Cell Cycle Block-and-Release Protocol

Synchronization of cells by block-and-release protocols has several advantages over centrifugal elutriation *(8,12)*. First, all block-and-release protocols can be planned and completed much more rapidly than elutriation experiments. Second, they are the protocol of choice when large numbers of synchronized cells are required *(8)*. In addition, in contrast to elutriation, these protocols work very well with small cultures (e.g., 5 mL). Finally, there are a number of good block-and-release protocols and none require specific large or expensive equipment *(8,12)*. However, block-and-release protocols are also prone to generating artifacts because they block cell cycle progression but not cell growth *(8,12)*. Nonetheless, block-and-release protocols are an essential part of any yeast cell cycle laboratory.

There are essentially two ways to use cell cycle blocks for cell cycle studies. The first is to use a panel of agents to block cells at different stages of the cell cycle. In this manner, cells arrested at different stages can be compared. A second approach involves blocking the cell cycle for a short time and then releasing arrested cells synchronously into fresh medium. Three common protocols for block-and-release experiments are provided.

3.2.1. Alpha Factor

Alpha factor is produced by MATα cells and arrests MATa cells at START, the G1/S-phase boundary, by inhibiting Cln–Cdc28 activity *(16)* (**Fig. 4**). Cells arrest in G1 phase with a 1N deoxyribonucleic acid (DNA) content, and with an enlarged "schmoo" morphology (*see* **Note 28**). Alpha factor is particularly useful because cells recover rapidly and progress very synchronously through two or three cell cycles *(8)*.

1. Grow yeast cultures in YEPD at 30°C to early log phase (0.2–0.5×10^7 cells/mL; *see* **Note 29**).
2. Add α factor to 5–10 μg/mL (*see* **Note 30**).
3. To arrest cells in G1 phase, incubate cultures at 30°C for 1.5 to 3 h.
4. Begin assessing cell cycle arrest at 1.5 h. Examine 3 μL under the microscope. Count and size 100 μL with a Coulter counter. Cells prior to START will arrest at the G1/S-phase boundary, adopt a "schmoo" morphology, and continue to get larger. Cells past START will divide and arrest at the G1/S-phase boundary in the subsequent cell cycle. Thus, the cell number should not increase after 2 h. Examine cells every half hour until they are <5% budded (*see* **Note 28**).
5. Release cells from α factor arrest by centrifugation (4000*g* for 3–5 min). Wash cultures twice by resuspending cell pellets in large volumes of prewarmed YEPD. Pellet cells by centrifugation and resuspend cultures in prewarmed YEPD to a final concentration of approx 0.5×10^7 cells/mL (*see* **Note 31**).
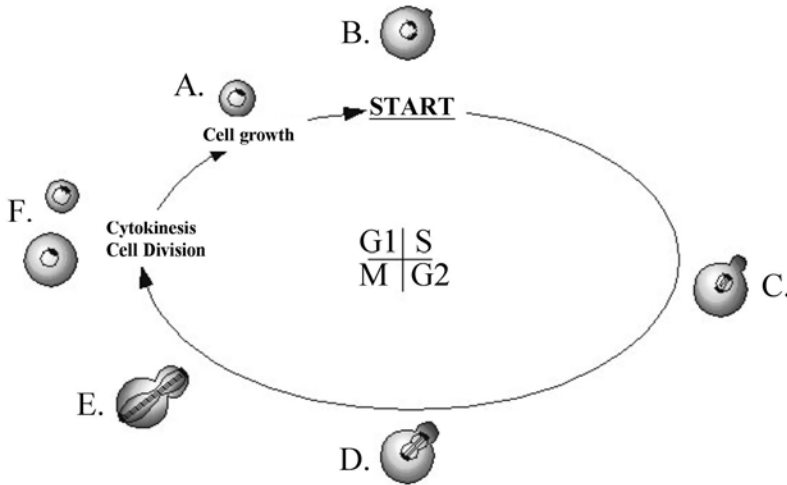6. Remove a time-zero fraction to assess synchrony and for experimental purposes.

Fig. 4. Yeast cell cycle.

Return the synchronized population to the original incubation temperature. Begin taking time-points every 15 to 30 min. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.2.2. Hydroxyurea

Hydroxyurea blocks cells in S phase by inhibiting the DNA metabolism enzyme, ribonucleotide reductase *(12)*. Cells arrest with medium-sized buds and a DNA content between 1 and 2N (**Fig. 4**).

1. Grow yeast cultures in YEPD to early log phase ($0.2–0.5 \times 10^7$ cells/mL).
2. Add hydroxyurea to a final concentration of 0.2 *M*.
3. To arrest cells in S phase, incubate cultures at 30°C for 1.5 to 3 h.
4. Begin assessing cell cycle arrest at 1.5 h. Examine 3 µL under the microscope. Count and size 100 µL with a Coulter counter. Cells that have not yet entered S phase will bud but should not divide. Cells that have completed S phase will divide and arrest in S phase in the subsequent cycle. Thus, the cell number should not increase after 2 h. Examine cells every half hour until they are >95% budded.
5. Release cells from hydroxyurea arrest by centrifugation (4000*g* for 3–5 min). Wash cultures twice by resuspending cell pellets in large volumes of prewarmed YEPD. Pellet cells by centrifugation and resuspend cultures in prewarmed YEPD to a final concentration of approx 0.5×10^7 cells/mL (*see* **Note 31**).
6. Remove a time-zero fraction to assess synchrony and for experimental purposes. Return the synchronized population to the original incubation temperature. Begin taking time-points every 15 to 30 min. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.2.3. Nocodazole

Nocodazole inhibits microtubule polymerization and blocks cells in G2/M phase *(11)*. Cells arrest with large buds and 2N DNA content (**Fig. 4**).

1. Grow yeast cultures in YEPD + 1% dimethyl sulfoxide (DMSO) to early log phase (0.2–0.5×10^7 cells/mL; *see* **Note 32**).
2. Add nocodazole to a final concentration of 15 µg/mL (*see* **Note 33**).
3. To arrest in G2/M phase, incubate cultures at 30°C for 1.5 to 3 h.
4. Begin assessing cell cycle arrest at 1.5 h. Examine 3 µL under the microscope. Count and size 100 µL with a Coulter counter. Cells will arrest with buds nearly the same size as mother cells. Examine cells every half hour until they are >95% budded.
5. Release cells from nocodazole arrest by centrifugation (4000*g* for 3–5 min). Wash cultures by resuspending cell pellets in at least two volumes of prewarmed YEPD. Pellet cells by centrifugation and resuspend cultures in prewarmed YEPD to a final concentration of approx 0.5×10^7 cells/mL (*see* **Note 31**).
6. Remove a time-zero fraction to assess synchrony and for experimental purposes. Return your synchronized population to the original incubation temperature. Begin taking time-points every 15 to 30 min. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.2.4. Cdc15-2

The *cdc15-2* allele has a temperature sensitive mutation in a protein kinase required for exit from mitosis *(12)*. At the restrictive temperature (e.g., 37°C), cells arrest in late anaphase/telophase with characteristic "dumbbell" morphology.

1. Grow the appropriate strain (e.g., W303 *MATα cdc15-2*) in 0.6 to 1.0 L YEPD in a 2-L flask at 23°C to 2×10^6 cells/mL (*see* **Note 34**).
2. Transfer the culture to a 37°C air incubator (*see* **Note 35**).
3. Incubate cultures at 37°C for 3.0–3.5 h to arrest.
4. Begin assessing cell cycle arrest at 3 h. Examine 3 µL under the microscope. Count and size 100 µL with a Coulter counter. Cells will bud but should not divide. Thus, the cell number should not increase after 3 h. Examine cells every half hour until they are >95% budded.
5. Release cells from the *cdc 15-2* arrest by transferring the flask to a 23°C water bath.
6. Remove a time-zero fraction to assess synchrony and for experimental purposes. Return your synchronized population to the original incubator. Begin taking time-points every 15 to 30 min. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.3. Meiotic Cell Cycle Synchronization

As in mitosis, to study specific meiotic processes, it is useful and often essential to synchronize entry into meiosis *(9,17–19)*. For most strains, syn-

chronization is not as tight as in mitosis. However, two commonly used proto-
cols are provided *(9,17–19)* (*see* **Note 36**).

1. Inoculate diploid strains into YEPD cultures (5–50 mL) and incubate overnight
   at 30°C.
2. Centrifuge cultures (4000g for 3–5 min) and resuspend cell pellets in YPA to an
   optical density (OD$_{600}$) of approx 0.3. Grow cultures 24–30 h at 30°C to arrest
   cells in G1 phase (*see* **Note 37**).
3. Centrifuge cultures (4000g for 3–5 min) and wash cell pellets twice in sterile
   water. Resuspend cells in SPM to an OD$_{600}$ of approx 1.0. Sporulate cultures
   at 30°C.
4. Remove a time-zero fraction to assess synchrony and for experimental purposes.
   Return the synchronized population to the original incubator. Take time-points at
   1, 3, 5, 9, 12, 24, 48, and 72 h. Monitor synchrony and cell cycle progression as
   discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.4. Centrifugal Elutriation
to Induce Meiotic Cell Cycle Synchronization

Centrifugal elutriation is an excellent method to promote the synchronous
entry into meiosis of large numbers of cells *(21)*. By obtaining populations
enriched for cells >90 fL, maximal sporulation frequencies are obtained.

1. Follow **Subheading 3.1.1.**, **steps 1–3**, use a volume of 1–2 L.
2. Follow **Subheading 3.1.1.**, **steps 4–9**.
3. After cells have been loaded into the elutriation chamber, switch the stopcock
   from input cells to clarified medium (**Fig. 1**). Begin pumping in clarified medium
   (*see* **Note 22**). Incrementally increase pump speed every 1 to 2 min until the cell
   front reaches the top of the elutriation chamber (*see* **Note 23**). Depending on the
   number and size of cells, this should take between 15–30 min at a pump rate of
   approx 30–50 mL/min.
4. As the cell front reaches the top of the elutriation chamber, the smallest cells,
   debris and cell ghosts (cells broken by sonication), will begin eluting off the
   centrifuge (**Figs. 2C** and **3**). When this occurs, the flowthrough medium will
   become slightly cloudy. Begin collecting 200-mL fractions. Continue incremen-
   tally increasing the pump speed after every fraction. Measure the cell number
   and size with a Z2 Coulter Counter/Channelyzer. Continue collecting fractions
   until the median cell size is >90 fL (*see* **Note 24**).
5. Depending on the experimental design and the number of cells needed, choose
   either the best fraction (e.g., the highest concentration of cells with a median cell
   size of >90 fL) or pool fractions.
6. Centrifuge fractions (4000g for 3–5 min) and wash cell pellets twice in sterile
   water; resuspend cultures in SPM to an OD$_{600}$ of approx 1.0, and sporulate
   at 30°C.
7. Remove a time-zero fraction to assess synchrony and for experimental purposes.
   Return the synchronized population to the original incubator. Take time-points at

1, 3, 5, 9, 12, 24, 48, and 72 h. Monitor synchrony and cell cycle progression as discussed below (*see* **Note 26** and **Subheading 3.6.**).

3.5. Age Synchronization

In addition to the protocols provided here, there are several good protocols available for synchronizing cultures based on the relative age of the cells *(10,14,21–24)*.

3.6. Monitoring Synchrony

Accurately assessing the synchrony of each experiment is critical to its success. A graphic representation of the *S. cerevisiae* cell cycle is shown in **Fig. 4**. Using phase-contrast and fluorescence microscopy, it is relatively easy to assign cell cycle stages based on morphology. Cells in G1 phase are unbudded (**Fig. 4A**). The appearance of a small bud signifies progression past START, a commitment point at the G1/S phase boundary (**Fig. 4B**). The size of the bud relative to the mother helps stage cells between START and mitosis (**Figs. 4C–E**). Fluorescent staining of DNA and microtubules can be used to visualize nuclear and spindle morphologies. The shape and location of the nucleus and spindle are excellent criteria for cell cycle staging in *S. cerevisiae* (**Fig. 4**). Five basic protocols for assaying cell synchrony are given *(8,11,12)*.

3.6.1. Percentage of Budded Cells

In wild-type cells, budding closely correlates with entry into S phase. Buds grow more than mother cells during cell cycle progression. Thus, cell cycle phase can be reasonably deduced by comparing the relative sizes of buds to mother cells (**Fig. 4**). Samples should be sonicated briefly to ensure separation of daughters from mother cells. To avoid bias, samples should be coded before counting. To accurately determine the percentage of budded cells, the number of budded and unbudded cells should be counted from a minimum of 200 cells. Samples stored in growth medium can be stored on ice or at 4°C for several hours. Transfer samples to water for longer storage times. The percentage of budded cells is a good indicator of synchrony in the first cycle after synchronization but is less useful in subsequent cycles.

3.6.2. Flow Cytometry

Flow cytometry is an excellent technique for measuring cell cycle progression *(11,25)*. Commonly, cells are stained with propidium iodide to measure DNA content (*see* Chapter 7 in this volume for protocols).

As discussed above, the DNA content of wild-type cells is usually closely correlated to the budding index of cells.

3.6.3. Gene Expression

Elegant microarray experiments have demonstrated that mitotic and meiotic cells express cell cycle phase-specific genes. Thus, cultures can be staged by examining the expression levels of standard mitotic or meiotic genes. Standard mitotic cell cycle genes for staging cell cycle phase include *CLN2* (highest at G1/S-phase boundary), *H2A* (highest in S phase), *CLB4* (highest in G2 phase), *CLB2* (highest in M phase), and *EGT2* (highest at M/G1-phase boundary) *(12,26)*. Standard meiotic genes for staging sporulation include: *DMC1* (early sporulation, approx 2 h), *SPS1* (middle sporulation, approx 5 h), *DIT1* (middle-to-late sporulation, approx 7 h), and *SPS100* (late sporulation, approx 9 h) *(10)*. Meiotic gene expression patterns are strain specific *(18)*.

3.6.4. Nuclear and Spindle Morphology

The preceding three techniques (*see* **Subheadings 3.6.1.–3.6.3.**) are sufficient for assessing cell synchrony in most experiments. However, immuno-fluorescent staining of the DNA and microtubules is the best technique for definitively assessing cell cycle position. Because this technique is more time consuming and requires an immunofluorescence microscope, readers are referred to several excellent published protocols *(11,12,27)*.

3.6.5. Spore Formation (Meiosis)

Like budding index for cells in mitosis, the frequency of spore formation can be used as a semiquantitative method to assess the degree with which meiosis has been completed. A minimum of 200 total cells is counted, and cells are classified as either sporulated or unsporulated (*see* **Note 38**).

4. Notes

1. Autoclaving can break down complex carbon sources into simple sugars like glucose. It is highly recommended that all carbon sources be filter sterilized and added after autoclaved media has cooled. Reagents are: yeast extract (Fisher/Difco cat. no. BP 1422-500), peptone (Fisher/Difco cat. no. DF048170), glucose (U.S. Biological cat. no. G1030), raffinose (U.S. Biological cat. no. R1030), and potassium acetate (Fisher cat. no. BP364-500). (Final concentration is 2% except for ethanol and glycerol, which are added to 3% after autoclaving.)
2. Filtration of 5X YEP removes a large percentage of clumps and yeast cell debris. This prevents this debris from collecting in the smallest elutriated fractions. Because of the time-consuming process of filtering 5x YEP, it is usually made in large batches and filtered with Whatman filter paper (Fisher cat. no. 09-805F). 5X YEP is stored at –20°C prior to dilution to 1X and autoclaving.
3. Recipes are given for the most common primary, secondary, and "drop-out" amino acid mixes. These recipes will need to be modified for complex (e.g., lacking one or more amino acids) "drop-out" mixes. Amino acids are from Sigma

(cat. no.): alanine (A7627), aspartic acid (A9256), asparagine (A0884), cysteine (C7755), glutamic acid (G1626), glutamine (G3126), isoleucine (I2752), valine (V0500), phenylalanine (P2126), proline (P0380), serine (S4500), threonine (T8625), glycine (G7126), PABA (A9878), arginine (A5131), lysine (L5626), methionine (M6039), tyrosine (T3754), adenine (A9126), histidine (H8125), leucine (L8000), tryptophan (T0254), and uracil (U0750). Additional reagents are: YNB (Fisher/Difco cat. no. DF0335159), ammonium sulfate (U.S. Biological A1450), and sodium phosphate dibasic (Fisher cat. no. BP 332-1).

4. Sporulation medium should be supplemented with nutritional supplements (10–120 µg/mL) to complement diploid auxotrophies. 10 µg/mL ampicillin can be added to SPM to reduce bacterial growth after elutriations.

5. The α factor is a 13-amino-acid peptide (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr; MW 1684). Stocks are made up at 1–50 mg/mL in ethanol and stored at –20°C. When using large quantities of α factor, it is worthwhile to chemically synthesize α factor *(9)*. Stocks of nocodazole are made up at 15 mg/mL in DMSO and stored at –20°C. Stocks of hydroxyurea are made up at 2.0 *M* in sterile water and stored at –20°C.

6. A major disadvantage of block-and-release synchrony experiments is that they disrupt the normal coordination between cell growth and cell division. Block-and-release protocols block cell cycle progression but not cell growth. Thus, on release from the cell cycle block, cells are abnormally large. The abnormally large cell size is the main reason why block-and-release protocols yield highly synchronous cultures for two divisions following release. This is because mother and daughter cells are similar in size and are significantly larger than the "critical cell size" required for cell cycle progression. However, the abnormally large cell size can lead to nonphysiologically relevant results and other artifacts.

7. Unlike block-and-release experiments, which often require a particular genotype of strain (e.g., haploid MATa cells for α factor arrests, or *cdc 15-2* strains), centrifugal elutriation can be used to obtain synchronous populations from any type or yeast strain. Most important, both haploid and diploid strains can be used. Moreover, elutriation is also more universal because certain strain backgrounds recover poorly from block-and-release protocols.

8. Direct comparisons of results from synchronization methods frequently yield slightly different results *(26)*. Although it is generally accepted that elutriation experiments perturb cell cycle progression less than block-and-release experiments, it should be noted that elutriated populations are subject to abnormal centrifugal stresses. In addition, cells may be briefly stressed by anaerobic or mild nutrient starvation conditions.

9. A typical elutriation experiment will generate approx 300 mL of synchronized G1-phase cells at 1×10^7 cells/mL. It is recommended that approx $2.5–5.0 \times 10^9$ cells (e.g., 25–50 mL of cells at approx 1×10^7 cells/mL) be harvested for protein or ribonucleic acid (RNA) expression-analysis experiments. This is sufficient for 12 samples at 25 mL each or 6 at 50 mL each. Using a second 40-mL elutriation chamber can double the number of cells obtained. When using two chambers, approx $1–2 \times 10^{11}$ initial cells can be used. The use of two chambers

slightly increases the synchrony. However, this protocol is significantly harder than the use of a single chamber because both elutriation chambers must be carefully observed during the separation process.

10. Appropriate growth conditions are a key aspect in determining the success of elutriations. Although good G1-phase fractions can be obtained from diploid cells grown in YEPD at room temperature, it is nearly impossible to obtain pure (>95% unbudded) synchronous populations of haploid cells grown in YEPD. Reducing the growth rate of cultures increases the ease with which small unbudded G1-phase cells can be obtained. Common suggested media given in order of the growth rate that they support are (from most rapid to slowest growth): YEP plus 2% raffinose, SC plus 2% raffinose, YEP plus 3% ethanol, and SC plus 3% ethanol. Because elutriation can produce anaerobic stresses, YEP plus 2% raffinose is recommended. However, ethanol-grown cultures will also produce very good synchrony.

11. Because *S. cerevisiae* has a size requirement for cell cycle progression, the synchrony achieved by elutriation is directly related to the uniformity of cell size in the fractions obtained *(28,29)*.

12. Specifically, it is recommended that a 50- to 100-mL starter culture be grown such that after approx 18 h the culture will be approx $2–3 \times 10^7$ cells/mL. It is strongly recommended that the approximate doubling time of this culture be determined.

13. Perhaps the hardest aspect of successful elutriation experiments is ensuring that the initial cultures are in midlog phase (e.g., $2–3 \times 10^7$ cells/mL). Cells grown to higher densities generate artifacts and yield irreproducible results. Knowledge of the doubling time of the strain under appropriate growth conditions is essential. Begin checking the cell density at least two doublings prior to predicted start time of the experiment. Allow a sufficient amount of time to complete the experiment. When working with slowly growing cultures, it is sometimes helpful to start two initial cultures; one culture having two to three times more cells. This ensures that at least one of the cultures will be at the correct density on time. Approx 2 L of cells at $2–3 \times 10^7$ cells/mL can be loaded into the 40-mL elutriation chamber. It is recommended that a single 4-L flask be used rather than two 2-L flasks. For proper aeration, do not fill flasks more than 40% of capacity. Baffled-bottom flasks increase aeration and growth rate and can be filled to 50% of capacity. The use of foam stoppers in the necks of flasks helps decrease contamination.

14. Sterility only becomes an issue if synchronized cultures will be maintained more than 12 h after elutriation. The entire elutriator system can be cleaned and sterilized by flushing with 70% ethanol. Follow this with sterile water and allow to air-dry. The addition of ampicillin to 100 μg/mL greatly reduces bacterial contaminations.

15. Having large air bubbles can disrupt the sensitivity of separation or cause the rotor to become unbalanced. During normal operating conditions, the pressure gage will act as a bubble trap. Do not allow the pressure to rise above 10 psi

or the tubing will pop out of the rotor. If the elutriation chamber is overloaded, gas bubbles can frequently be seen in the collection tubing.

16. If possible, turn the refrigeration unit off as it generates vibrations that can disrupt the cell-size gradient in the elutriation chamber.

17. Cell density is most accurately determined with a Z2 Coulter Counter Channelyzer. However, a spectrophotometer and OD_{600} absorbance readings can be substituted. One OD_{600} is equivalent to approx 3×10^7 cells/mL *(3)*. However, OD_{600} absorbance readings are sensitive to the size of cells. In block-and-release protocols, where cell size increases dramatically, OD_{600} absorbance readings will increase whereas the cell number does not. Centrifuge cells in polypropylene bottles (250–1000 mL) at 4000g for 5 min.

18. Minimizing the time spent manipulating the cells increases synchrony and decreases artifacts.

19. Sonication is essential to ensure maximal separation of daughters from mother cells. In addition, after centrifugation, yeast will sometimes clump. Clumps can clog tubing and disrupt cell-size separation. Proper sonication will disperse clumps.

20. To achieve maximum synchrony, it is essential to elute in clarified medium.

21. If the rotor has any balancing problems, they nearly always present before the rotor attains maximum speed.

22. The easiest way to achieve this is to switch the stopcock from input cells to clarified medium (or ice-cold water). Be careful not to allow bubbles into the tubing or run the flasks dry.

23. A number of variables (e.g., cell size, cell number, length of tubing, density of the medium, etc.) affect the rate at which cells move in the elutriation chamber. Thus, the rate at which the pump speed is incrementally increased needs to be determined empirically.

24. Because centrifugal elutriation separates cells largely on the basis of cell size, a determination of the uniformity of cell size with a Coulter Counter/Channelyzer gives a very good estimation of the synchrony achieved.

25. Centrifuging fractions and resuspending in the correct volume of fresh medium is the easiest way to achieve this.

26. It is also a good practice to save samples of asynchronous midlog-phase cells for comparison. Depending on the design of the experiment, it is typical to save three samples at each time-point: a 100-μL sample to determine cell number and size, a 200-μL sample to determine the percentage of budded cells, and an approx 500-μL sample for cell cycle analysis by flow cytometry (*see* Chapter 7 in this volume).

27. Cell pellets are stable at –80°C for years. RNA and protein can easily and efficiently be isolated from frozen cell pellets.

28. Cells arrested with α factor will first appear as unbudded cells. After time, these cells will enlarge, elongate, and form comma-shaped "schmoos." A fully arrested culture will contain unbudded cells, "schmoos," and some unbudded doublets *(8)*.

29. Buffering YEPD to pH 5.0 with 50 m*M* sodium succinate may inhibit the Bar1 protease and improve α arrests *(12)*.
30. The Bar1 protease helps yeast recover from α factor arrests by degrading α factor. Yeast lacking the Bar1 protease can be arrested with 100- to 1000-fold less α factor. Addition of the filter-sterilized protease pronase E (Sigma P-6911) to a final concentration of 0.1 mg/mL can help yeast recover from α factor arrests *(8)*.
31. Alternatively, arrested cells can be collected by vacuum filtration and washed. In either case, the key aspect is ensuring that the cell-cycle-arresting drug is washed away with copious amounts of medium. Because arrested cells are increasing in volume, they should be resuspended in fresh medium at a concentration $<5 \times 10^6$ cells/mL *(8,12)*.
32. Adding DMSO helps nocodazole to more efficiently arrest cells.
33. Some strains respond differently to nocodazole, and the amount to add may need to be determined empirically. Too little or too much nocodazole can result in poor arrests or artifacts.
34. Smaller volumes increase in temperature too rapidly *(12)*.
35. Shifting yeast cultures rapidly from room temp (e.g., 23°C) to 37°C induces a "heat shock" reaction, which results in poor synchrony. The use of an air incubator slows the rate of temperature increase and lessens the induction of "heat shock *(12)*."
36. Synchronous entry into meiosis is strain dependent. The SK1 strain enters more synchronously than W303 or BY4743.
37. Cultures are incubated until the percentage of budded cells is less than 5%.
38. The appearance of a spore is interpreted to signal the completion of sporulation. However, it is not assumed that all spores are viable.

Acknowledgments

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7

Analysis of the Budding Yeast *Saccharomyces cerevisiae* Cell Cycle by Morphological Criteria and Flow Cytometry

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1. Introduction

The budding yeast *Saccharomyces cerevisiae* represents the eukaryotic model system in which the checkpoint concepts were initially developed *(1)*. Whereas many arguments can be made in favor of the continued use of *S. cerevisiae* as a model organism for checkpoint studies, the ease of distinguishing cell cycle stages is usually not put forward. Indeed, peculiar features of the yeast cell cycle can be regarded as a disadvantage. For instance, there is no discernible G2 stage, and the intranuclear mitotic spindle is assembled already during S phase. Also, the degree of chromosome compaction during mitosis is insufficient to allow visualization of individual chromosomes or definitive detection of mitotic stages by conventional light microscopy. On the other hand, there are a number of morphological cell cycle landmarks available that are easily traceable with very little experience and experimental manipulation. Even such a complex methodology as fluorescence-activated cell sorting (FACS) can be readily adapted with additional calibration for the small cell size and the relatively low deoxyribonucleic acid (DNA) content of diploid or haploid yeast. In the following, we have compiled protocols for some of the most common methods of yeast cell cycle analysis. In general, we have assumed time-course experiments, following the activation of checkpoint arrest responses (e.g., by irradiation of synchronized cells). Individually, some of these protocols will only suffice for preliminary studies, and a combination of methods is highly recommended to monitor certain cell cycle transitions unequivocally. Other useful protocols can be found, for example, in Guthrie and Fink *(2)* or the most recent edition of the *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual (3)*.

2. Budding Analysis

2.1. Introduction

In *S. cerevisiae*, bud emergence is a landmark of early S phase that can easily be observed with a well-adjusted phase contrast microscope. Although uncoupling of bud emergence and S-phase initiation is known to occur in certain mutants, determination of the frequency of *small*-budded cells over time can serve as a fairly reliable initial measurement of S-phase entry of synchronized cells and of its possible delay, for example, in response to DNA damaging agents. In the case of DNA damage, such budding delay (e.g., after ultraviolet [UV] treatment) frequently does not last for more than 1 h, and for unknown reasons, its extent is very much dependent on the genetic background.

In contrast to small-budded cells, the exact cell cycle stage of a *large*-budded yeast cells cannot be determined by light microscopy alone. Such cells could be in late S, G2/M, various stages of M, or even in G1, immediately following telophase. The last type of cells is still held together by the cell wall but can most likely be separated by mild sonication. This effect is strain dependent. Nevertheless, many agents that introduce spindle damage (such as nocodazole) or DNA double-strand breaks (such as γ-irradiation, streptonigrin, or bleomycin and its derivatives) cause a prominent arrest response at the metaphase-to-anaphase transition that can last for many hours. Accumulation of enlarged budding cells of typical dumbbell shape can easily be observed even in initially asynchronous logarithmic-phase cultures. Thus, determination of the fraction of large-budded cells can quickly provide useful preliminary information—for instance, if an unknown agent triggers a predicted checkpoint response or if a strain behaves abnormally toward a well-characterized agent.

The following protocol is designed for a determination of UV-induced budding delay. Similar protocols can easily be established for a variety of different synchronization and treatment regimens.

2.2. Materials

1. Haploid yeast cells (MATa strain)
2. α Factor (Sigma or U.S. Biological) resuspended at 1 mg/mL in deionized water and frozen in aliquots at –20°C.
3. Yeast extract-peptone-dextrose (YPD) growth medium (2% peptone, 1% yeast extract, 2% dextrose). Dextrose should be autoclaved separately as a 20% stock solution.
4. Calibrated germicidal UV lamp (254 nm).
5. Sonicator.

2.3. Protocol and Experimental Example

1. An early logarithmic-phase culture of a *MAT*a haploid yeast strain (2×10^6–1×10^7 cells/mL) is synchronized in G1 by α-factor. Low-density cultures can be concentrated by centrifugation (1500g, 3 min) and resuspension in YPD at not more than 2×10^7 cells/mL. Typically, α-factor is added to a final concentration of 20 μg/mL in two aliquots, separated by 75 min. The total time required for synchronization is strain dependent and will be between 135 and 205 min. (*see* **Note 1** and Chapter 3).

2. Cells are harvested in a low-speed centrifuge (1500*g*, 3 min), washed in the same volume of sterile deionized water, resuspended in 2–5 mL deionized water, and sonicated (5–10 s at low setting) (*see* **Note 2**).

3. The culture is expanded with deionized water and a titer of 2.5×10^7 cells/mL is adjusted. The cell suspension is transferred to a disposable plastic Petri dish, and a magnetic stir bar is added. A volume of 4–6 mL can be treated in a Petri dish of 6-cm diameter, whereas treatment of 15-mL samples requires a Petri dish of 10-cm diameter. The cell suspension is irradiated under constant stirring with 254 nm UV radiation. An aliquot is mock treated and serves as control (*see* Chapter 1).

4. Cells are collected by aspiration with an automatic pipet, spun down as above and resuspended in fresh YPD. A culture density is chosen that is convenient for microscopic analysis, but no more than 1×10^7 cells/mL. Cells are incubated with shaking at 30°C (*see* **Note 3**).

5. The fraction of small-budded cells among all cells is determined microscopically in a hemacytometer at a magnification level of at least ×600 and plotted as a function of time after release from α factor arrest (**Fig. 1**) (*see* **Note 4**). Typically, a bud that is larger than one-third of the mother cell is counted as a separate cell (*see* **Note 5**). At least 200 cells should be classified per time-point with the aid of a mechanical multichannel cell counter.

3. Microcolony Assay

3.1. Introduction

Ultimately, a synchronized control population will traverse the cell cycle and reenter the cell cycle stage in which the treated portion has arrested. Thus, a comparison between treated and untreated cultures will be difficult at later time-points if just based on cell cycle distributions. Also, all cells of the treated population may not have received identical amounts of damage, and those with low levels may have reentered the same stage following passage through the cell cycle. If an arrest phenomenon lasts for a significant amount of time in a majority of the treated cells, it is highly desirable to "trap" all cycling cells in a stage downstream of the arrest point and thus avoid confusion by preventing cell cycle reentry. This can be achieved by the use of chemical inhibitors or thermoconditional cell cycle mutations; see, for example, Gardner, Putnam, and Weinert *(4)*. More generally applicable (but less specific) is the use of a
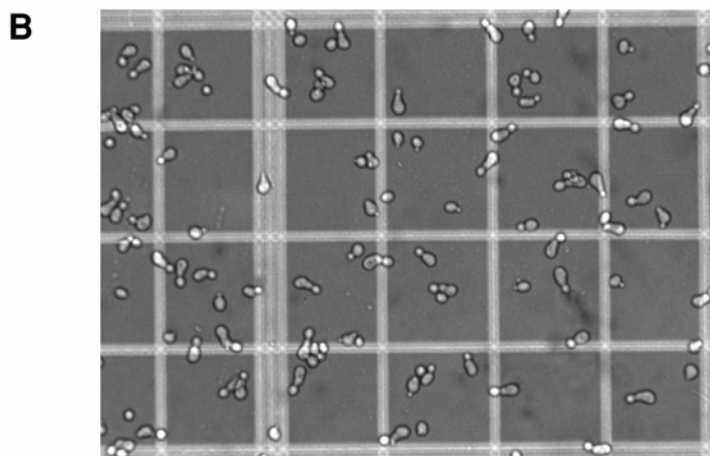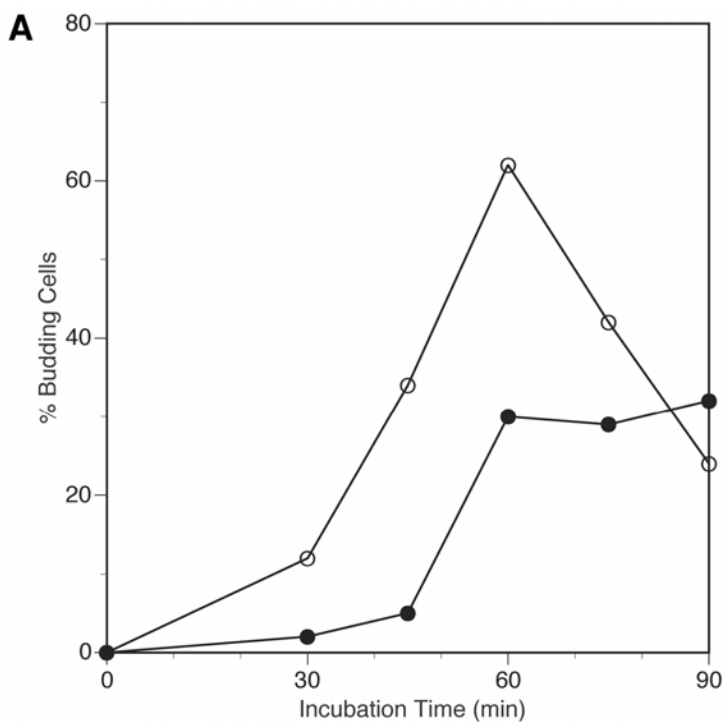
Fig. 1. **(A)** Budding delay following UV treatment (80 J/m^2). G1 cells of strain SX46A were irradiated in suspension following release from α factor arrest (closed circles). The fraction of small-budded cells was determined as a function of time and compared to unirradiated control (open circles). **(B)** Population of unirradiated yeast cells traversing S phase with high synchronicity 60 min after release from α-factor arrest. At this point, majority of cells have formed a small-to-medium-sized bud.

microcolony assay. Basically, treated and untreated cells are placed on solid media and the number of cell "bodies" (separated cells or large buds) per colony is analyzed over time. Thus, cells that have progressed through the cell cycle can be easily distinguished from those that stay arrested.

The following example illustrates the general technique by using hydroxyurea as a synchronizing agent and γ-irradiation as the checkpoint trigger (**Fig. 2**).

3.2. Materials

1. YPD broth and plates.
2. Hydroxyurea: Because of the required high concentration, preparation of a stock solution is very difficult. Therefore, one can add the powder to YPD at the working concentration and filter-sterilize.
3. ^{137}Cs-irradiator.

3.3. Protocol and Experimental Example

1. A small volume of an early logarithmic-phase culture of haploid or diploid yeast cells is synchronized in S phase by incubation in YPD with hydroxyurea (200 m*M* for 150 min). The majority of cells will accumulate in a large-budded stage. A portion of the culture is then treated with γ-irradiation (e.g., 70 Gy in a ^{137}Cs-irradiator for a haploid strain) (*see* Chapters 1 and 6 for more details).
2. Aliquots of cells of the irradiated and unirradiated culture are spun down (1500g, 3 min), washed free of the inhibitor with an equal volume of YPD, spun down again, and resuspended in YPD at a convenient density (about 1×10^7 cells/mL).
3. A loop full of the cell suspensions is streaked out on a YPD plate that has been prewarmed to 30°C. The plate is incubated at 30°C.
4. Periodically, the cells on the plate are examined microscopically (at least at ×160 magnification). In each microscopic field, they are classified as being in the single-cell (very few), in the double-cell, or in the microcolony stage, that is, containing more than two cell bodies. At least 200 cells or microcolonies should be classified for each time-point. Care should be taken to count within regions of comparable cell density (*see* **Notes 6–9**).

4. Visualization of the Mitotic Spindle

4.1. Introduction

To further classify yeast cells in the budded stage, the mitotic spindle can easily be visualized by indirect immunofluorescence using tubulin-specific antibodies. Although emerging during S phase, the intranuclear rodlike spindle is formed by tubulin bundles that undergo a very characteristic elongation at the metaphase-to-anaphase transition (**Fig. 3**). The following procedure is adopted from David Amberg's protocol, available on the World Wide Web (http://genome-www.stanford.edu/group/botlab/protocols/non_MeOH_IF.
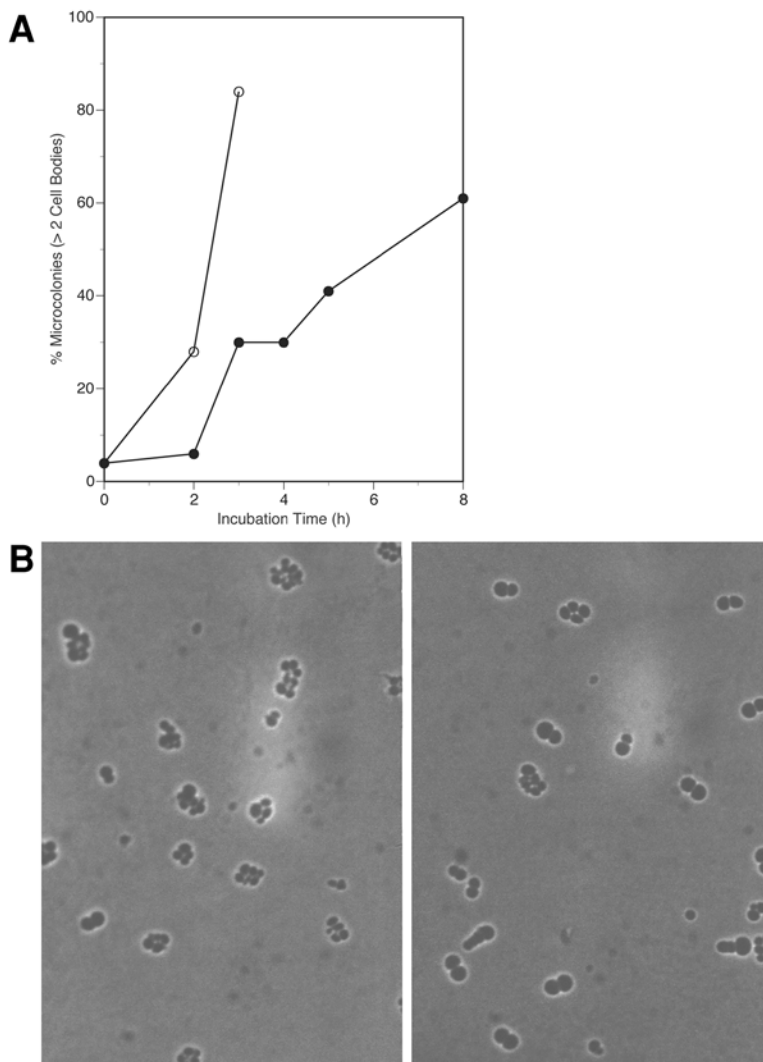
Fig. 2. **(A)** Delayed formation of microcolonies following treatment with γ-irradiation (50 gy, administered with a ^{137}Cs source). Strain BY4741 was synchronized in S phase with hydroxyurea; control (*open circles*) and irradiated sample (*closed circles*) were streaked out on a YPD plate, and the fraction of microcolonies with more than two cell bodies was determined as a function of time. **(B)** An example of microcolony formation in the control (*left*) as compared to predominant arrest in two-cell body stage (G2/M arrest) in treated sample (*right*). Pictures were taken 3 h after plating.
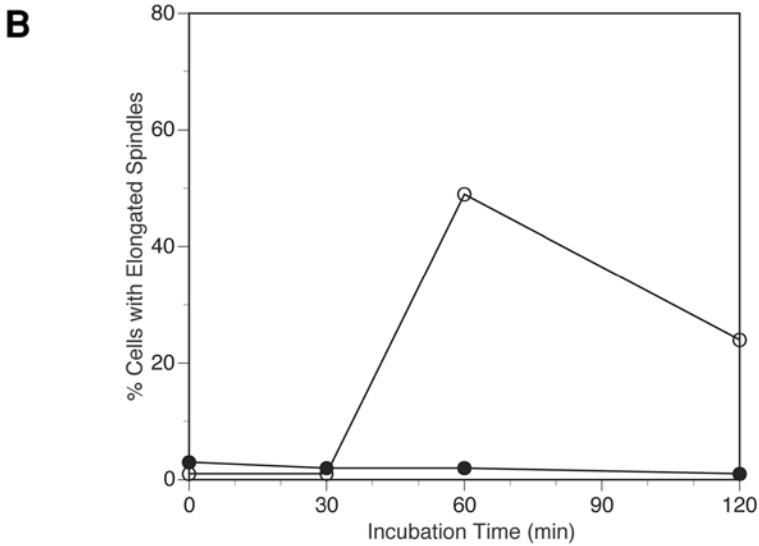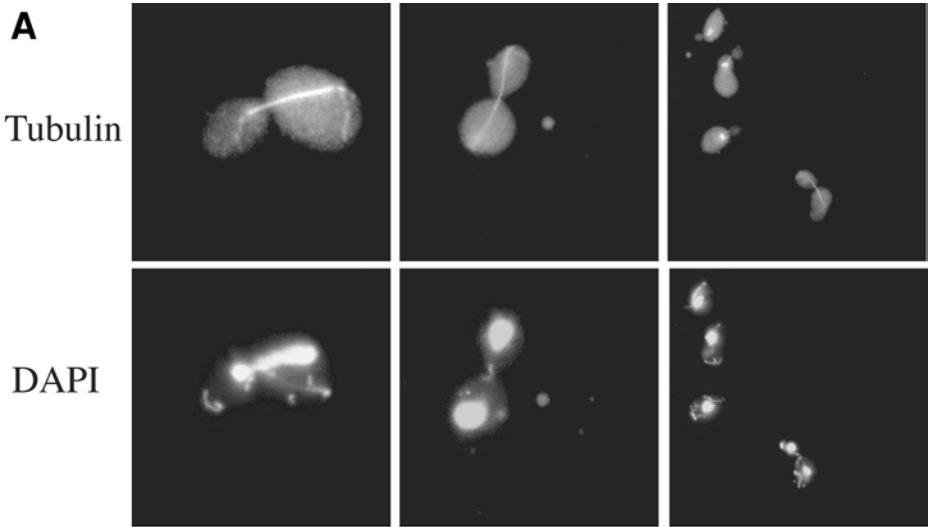
Fig. 3. **(A)** Various examples of elongated mitotic and short premitotic spindles (*top row*) compared to nuclear staining pattern with DAPI of the same cells (*bottom row*). **(B)** Spindle elongation is inhibited by hydroxyurea. Haploid cells were released from G1 arrest into YPD medium with (*closed circles*) or without HU (*open circles*).

html), a modification of a protocol from M. Rose in the Cold Spring Harbor yeast genetics manual *(3)*. A few examples of stained cells are shown in **Fig. 3A**; refer to Kilmartin and Adams *(5)* for further illustrations (*see* **Note 10**).

4.2. Materials

1. Teflon-coated slides (e.g., Medical Packaging Corp., Fisher Scientific cat. no. NC9706026).
2. YOL1/34 rat monoclonal antibody to yeast tubulin (Genetex).
3. Fluorescein-conjugated goat-antirat IgG (Pierce).
4. 40 mM KH$_2$PO$_4$ (pH 6.5)/500 µM MgCl$_2$, with or without 1.2 M sorbitol ("sorbitol buffer").
5. Zymolyase 100T (U.S. Biological) dissolved at 10 mg/mL in sorbitol buffer and frozen in aliquots at –80°C.
6. Blocking solution: phosphate-buffered saline (PBS) (pH 7.4)/0.5% bovine serum albumin (BSA)/0.5% Tween-20.
7. Polylysine (Sigma, >400,000 MW) dissolved as a 1% stock in water and frozen in aliquots at –80°C
8. Mount: 100 mg *p*-phenylenediamine is dissolved in 10 mL PBS, the pH is adjusted to above 8.0 with 0.5 M sodium carbonate buffer (pH 9.0), and the volume is brought to 100 mL with glycerol. From a 1 mg/mL stock solution in water, 4',6-diamidino-2-phenylindole (DAPI) is added to 50 ng/mL. After mixing, the solution is stored at –20°C. Solutions that have turned brown are discarded.

4.3. Protocol

1. Between 5×10^6 and 5×10^7 cells are harvested and resuspended in 5 mL 40 mM KPO$_4$ (pH 6.5)/500 µM MgCl$_2$. Add 0.5 mL 37% formaldehyde (best quality) and store cells for at least 4 h at 4°C. During a time-course experiment, cells are kept in this stage until all samples have been collected (*see* **Note 11**).
2. The fixed cells are washed two times in the same buffer, once in the same buffer containing 1.2 M sorbitol and gently resuspended in 0.5 mL sorbitol buffer.
3. 30 µL Zymolyase 100T is added and cells are typically incubated between 10–30 min at 30°C. Microscopically, cells should look transparent but intact, not misshapen or dark, which would indicate overdigestion. Cells are spun down (3 min, 1500g), carefully washed once with sorbitol buffer, very gently resuspended in 100–500 µL sorbitol buffer, and placed on ice.
4. The wells of a Teflon-coated slide are coated with 0.1% polylysine for 10 min at room temperature (RT). It is recommended that all solutions that go on wells be spun free of particle matter just before using (10 min, microfuge). Slides are incubated in a moist chamber (*see* **Note 12**).
5. The wells are washed 4–5 times with water and dried. A 20 µL cell suspension is spotted on the wells and incubated at RT for 10 min. Most of the liquid is aspirated, without letting the slides dry.
6. Blocking solution is layered on top of the cells and incubated for 15 min at RT.

7. Next, cells are incubated with the primary tubulin antibody at a dilution of 1:300 in blocking solution for 1 h at RT. Cells are washed 4–5 min with blocking solution. Do not let dry at any point.

8. Cells are incubated with secondary antibody at a dilution of 1:500 in blocking solution for 1 h at RT, then washed as before.

9. Mount is applied to the slide next to the cells and a coverslip is carefully put down, thus overlaying the cells with the mount. Paper towels are used to clean up the excess mount that is squeezed out. The slide is cleaned, and the edges of the coverslip are sealed with nail polish. When dry, slides can be stored at –20°C.

10. Cells are examined on a fluorescence microscope at high magnification with appropriate filter sets, e.g., Nikon B-2A (EX 450-490, DM 505, BA 520) for fluorescein or Nikon DAPI (EX 360/40, DM 400, BA 460/50) (*see* **Notes 13** and **14**).

5. Nuclear Staining With DAPI

5.1. Introduction

Especially if large-budded yeast cells are concerned, further information on the cell cycle stage can be gained from the staining of nuclear DNA. Cells in mitosis are characterized by an elongated nucleus in between mother and daughter cell (**Fig. 3A**). Cells that have undergone anaphase are clearly identified by their divided nuclei. Nuclear staining is routinely done with the fluorescent dye DAPI. Cells can be stained with or without fixation and a variety of protocols will give excellent results. However, a time-course experiment will normally involve collection of many samples and thus fixation of cells before going on. Because microscopic analysis may take time, we prefer to stain fixed cells sealed in mounting medium, essentially as described in **Subheading 4.3.** (*see* **Fig. 4** and **Note 15**).

5.2. Materials

1. DAPI (Sigma), a stock solution in water (1 mg/mL), can be stored at –20°C.
2. Polylysine (Sigma, >400,000 MW) dissolved as a 1% stock in water and frozen in aliquots at –80°C.
3. Mount: 100 mg *p*-phenylenediamine is dissolved in 10 mL PBS, the pH is adjusted to above 8.0 with 0.5 *M* sodium carbonate buffer (pH 9.0), and the volume is brought to 100 mL with glycerol. From a 1 mg/mL stock solution in water, DAPI is added to 50 ng/mL. After mixing, the solution is stored at –20°C. Solutions that have turned brown should be discarded.

5.3. Protocol

1. Between 5×10^6 and 5×10^7 cells are harvested and resuspended in 5 mL deionized water. Add 0.5 mL 37% formaldehyde, and store cells for at least 4 h at 4°C. During a time-course experiment, cells are kept in this stage until all samples have been collected (*see* **Note 16**).

2. The fixed cells are washed two times and resuspended in 0.5 mL deionized water.
3. The wells of a Teflon-coated slide are coated with 0.1% polylysine for 10 min at RT. Slides are incubated in a moist chamber.
4. The wells are washed four to five times with water and dried. A 20-µL cell suspension is spotted on the wells and incubated at RT for 10 min. Most of the liquid is aspirated, without letting the slides dry.
5. Mount is applied to the slide next to the cells, and a coverslip is carefully put down, thus overlaying the cells with the mount. Paper towels are used to clean up the excess mount that is squeezed out. The slide is cleaned, and the edges of the coverslip are sealed with nail polish. When dry, slides can be stored at –20°C.
6. Cells are examined on a fluorescence microscope at high magnification with an appropriate filter set, e.g., Nikon DAPI (EX 360/40, DM 400, BA 460/50).

6. Flow Cytometry

6.1. Introduction

In this method, DNA of fixed cells is stained with a fluorescent dye and a histogram of cells sorted by DNA content is established by laser flow cytometry *(6)*. With careful detector adjustments, the method is suitable for the analysis of cell cycle distributions within a yeast cell sample, and satisfactory results can easily be obtained. Although simpler staining procedures can be found in the literature, we have achieved the most consistent results with the following protocol, adopted from Paulovitch and Hartwell *(7)*. The protocol described below assumes the use of Becton-Dickinson FACScan® or FACSort® sorter with CELLquest® software. The manuals should be consulted extensively for details about using the instrument and its software (*see* **Fig. 5** and **Note 17**).

6.2. Materials

1. FACS analysis tubes (Falcon 2054, 12×75 mm).
2. 50 m*M* sodium citrate, pH 7.0.
3. 16 µg/mL propidium iodide (PI; Sigma) in 50 m*M* sodium citrate (pH 7.0). Make up in a large volume or as a 10X stock solution.
4. 10 mg/mL RNase A in STE (100 m*M* NaCl, 10 m*M* Tris-HCl, 1 m*M* ethylenediaminetetraacetic acid [EDTA, pH 8.0]), made DNase free by boiling for 10 min *(8)*.
5. 10 mg/mL proteinase K, in water
6. Sonicator.

6.3. Protocol

1. About 4×10^6–2×10^7 cells are spun down in 1.5 mL Eppendorf tubes for a few seconds at 14,000*g*. Cells are resuspended in 1 mL deionized water and spun down again.
2. The fluid is poured off, and cells are resuspended in the remaining traces of water by vortexing (this prevents excessive clumping during the following fixation
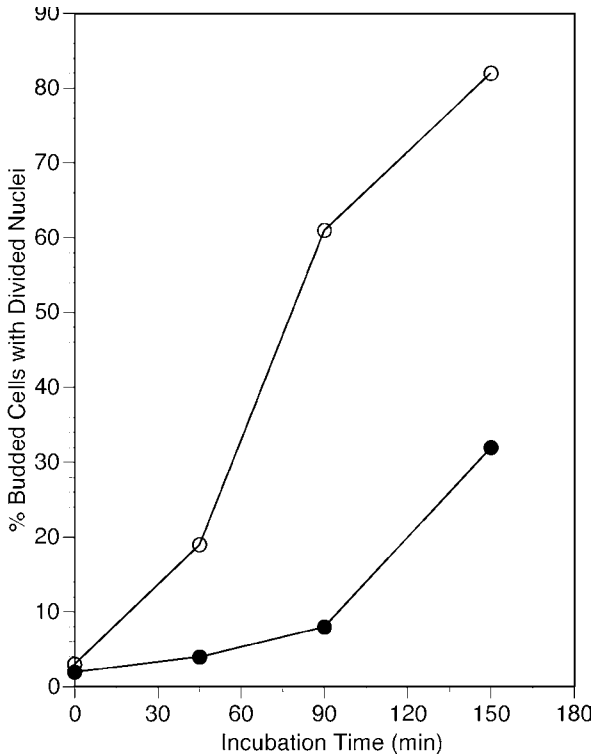
Fig. 4. Delayed progression of cells past mitosis following treatment with γ-irradiation (70 Gy, closed circles; untreated, open circles). Haploid cells (strain BY4741) were synchronized in S phase with hydroxyurea resulting in mostly large-budded cells containing an undivided nucleus. Using nuclear staining with DAPI, premitotic and postmitotic cells (with divided nuclei) were distinguished (*see also* **Fig. 3A**).

    step). 1 mL of absolute ethanol is added and the sample is mixed well. In this stage, cells can be stored at 4°C. During a time-course experiment, cells are kept in this stage until all samples have been collected (*see* **Note 18**).

3. The samples are vortexed extensively, cells are spun down for 1 min at 14,000*g*, washed with 1 mL of water, and spun down again.

4. The cell pellet is resuspended with 1 mL 50 m*M* sodium citrate (pH 7.0) and transferred to appropriate FACS analysis tubes (*see* **Note 19**).

5. 8 μL of 10 mg/mL DNase-free RNase A is added and samples are incubated for 1 h at 50°C.

6. 25 μL of 10 mg/mL proteinase K is added and incubation at 50°C is continued for another hour.

7. 1 mL of 16 μg/mL PI in 50 m*M* sodium citrate (pH 7.0) is added. Protect from light! Briefly sonicate all samples just before analysis. Filtration is usually not required (*see* **Note 20**).

Fig. 5. (**A**) Example of detector settings used for FACS of PI-stained yeast cells. (**B**) Dot-plot of side scatter (SSC-H) vs PI fluorescence (FL2-A) of asynchronously dividing yeast cells. (**C**) Histogram of PI fluorescence of the same sample. (**D**) Cell cycle progression of a UV-irradiated culture (SX46A, 40 J/m^2) following release from α-factor arrest as compared to untreated control (left).

8. Vortex briefly just before mounting the tube, and run each sample in the cell sorter set to low speed.
9. The detector setup is critical and may vary somewhat from experiment to experiment. It is suggested to adjust the settings with an asynchronous logarithmic-phase cell sample taken from the same experiment before synchronization.

First, the forward-scatter threshold is set to 24. Second, a dot plot of PI fluorescence signal (FL2-A) vs. side scatter (SSC-H) or forward scatter (FSC-H) is created. By manipulating detector voltage and amplifier gain, using values similar to those in **Fig. 5A** as approximate settings at the outset, a diagram resembling the one shown in **Fig. 5B** is created during real-time analysis. Two cell populations (G1, G2) that are distinguished by the FL2-A signal intensity should be clearly visible. Lastly, the FL2-A histogram is created (**Fig. 5C**) and fine-tuned by adjusting FL2 voltage and FL2-A amplifier gain (*see* **Note 21**).

10. At least 10,000 cells are analyzed for each time-point. Gating is unnecessary with well-stained samples.

11. Because yeast has a tendency to clog the fluidics of the sorter, the (institute-specific) cleanup and shutdown procedure should be followed meticulously.

7. Notes

1. Synchronization with α-factor results in characteristically shaped, elongated cells ("shmoo" phenotype). We recommend to carefully monitor the duration of α-factor treatment because the bud typically emerges at the elongated cell tip (**Fig. 1B**) and can be difficult to identify if excessive "shmooing" occurs. For the same reason, we do not recommend the use of α-factor hypersensitive bar1 mutant strains.

2. If synchronization conditions have been firmly established (*see* Chapter 6), a starting population containing virtually no background of budding cells can be generated. For this and other protocols, the sonication step is critical. In a typical case, we found that sonication of a 5-mL cell sample at a low setting for 10 s will separate the vast majority of G1 cells (e.g., by use of a Fisher Scientific Sonic Dismembrator 60, equipped with a microtip and set to "5"). Even a sonication period of 6×10 s with intermittent cooling on ice will not cause any lethality.

3. The kinetics of cell cycle reentry appears to be dependent on culture density. A series of experiments should always be performed at the same density.

4. The given example (**Fig. 1A**) shows a typical result for budding delay following UV treatment. **Figure 1B** illustrates the synchronous "burst" of small buds in an untreated culture typically found 20–40 min after release from α-factor arrest.

5. It should be noted that the fraction of budded cells diminishes following a maximum fraction of up to 80%, depending on the degree of synchronization (**Fig. 1A**). The shape of the curve will depend on the preset size limit required to classify a large bud as a separate cell. Thus, there is an element of subjectivity in establishing budding curves. However, the initial burst of small buds can be determined quite accurately, and analysis by different individuals will yield very similar numbers.

6. **Figure 2A,B** shows a typical result for γ-irradiation. Depending on the dose, the arrest can last for several hours. Microscopic examination after 24 h of incubation permits an estimate of the fraction of surviving macrocolony-forming cells.

7. Small buds are difficult to identify in cells on a plate and a discrimination of separated cells or large buds is impossible. Consequently, the detected arrest stage

is not well defined. The technique is only recommended for agents whose effects on cell cycle progression have been well established.

8. If desired, the number of cells per microcolony can be categorized in more detail—see, for example, Yamamoto et al. *(9)*. For instance, accumulation of cells in a 4-cell stage following initial arrest in a double-cell stage could indicate adaptation to the initial checkpoint-triggering event followed by repeated arrest owing to residual damage.

9. Although the random analysis outlined here can provide satisfactory results, more accurate data can be generated if cells of a desired stage are micromanipulated and distributed along a grid on solid YPD. This ensures that identical populations of cells are analyzed at each time-point, and individual pedigrees can be established. (*See* Lee et al. *[10]* and Bennett et al. *[11]* for examples.)

10. Spindle elongation is prevented by agents that inhibit replication, such as hydroxyurea. The example shown in **Fig. 3B** illustrates such checkpoint arrest.

11. Cells can be stored overnight after **step 1** or **2**. The blocking, **step 6**, can be extended to overnight incubation.

12. Coated slides can be prepared in advance and stored dust free.

13. Note that the mount contains DAPI, and by switching to a different filter set during fluorescence microscopy, the status of the nuclear DNA and the mitotic spindle can be determined in the same cell (**Fig. 3A**). Refer to **Subheading 5** for simplified protocols if just nuclear staining is required.

14. The use of a strain containing GFP-tagged tubulin provides another simple way of spindle detection *(12)*.

15. The example in **Fig. 4** shows delayed progression through mitosis following γ-irradiation of S-phase cells, as detected by nuclear staining followed by determination of the fraction of budded cells with divided nuclei.

16. Alternatively, cells can be fixed in ethanol as described in **Subheading 6.3.**, **steps 1** and **2**. Without fixation, cells can be stained with 50 ng/mL DAPI in deionized water.

17. The example in **Fig. 6D** shows the cell cycle response induced by UV irradiation in a population released from α-factor arrest. Following a short G1 arrest, a slow S phase and the beginning of G2/M arrest is shown.

18. Cells can be stored indefinitely at 4°C after **step 2**. Without problems, we have also stored cells overnight at 4°C after **step 5** or **7**.

19. Volumes in **steps 4** and **7** can be adjusted to accommodate samples of unusually low or high cell density.

20. SYTOX® Green (Molecular Probes) can be used as an alternative to PI to stain yeast DNA *(12)*.

21. Although FACS analysis can be used to detect arrest stages successfully, treatment of cells with certain inhibitors, such as nocodazole, may negatively affect the histogram quality by broadening peaks and overstaining cellular DNA.

Acknowledgment

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8

Analysis of the Fission Yeast *Schizosaccharomyces pombe* Cell Cycle

Eliana B. Gómez and Susan L. Forsburg

1. Introduction

1.1. Fission Yeast Cell Cycle

Fission yeast is a popular model organism for the study of the cell cycle. It grows quickly compared with other eukaryotic species; under normal conditions, a wild-type cell takes about 2.5–3 h to complete a cell cycle. A wild-type *S. pombe* cell has a rod shape, grows by elongation, and divides by medial fission. These characteristics allow the position of a cell in the cell cycle to be estimated simply by visualization of cell morphology. The organization of the fission yeast cell cycle is similar to that in larger cells. Because fission yeast has only three chromosomes, it is also a convenient organism for the cytological study of chromosome dynamics. Thus, fission yeast has become a popular system for studies of cell growth and division *(1–4)*.

Wild-type *S. pombe* is haploid during most of its life cycle, and divides asexually through mitosis. The cell cycle consists of distinct G1, S (deoxyribonucleic acid [DNA] synthesis), G2, and M (mitosis) phases. In contrast to *Saccharomyces cerevisiae*, fission yeast spends most of its time in G2 (about three-quarters of the cycle) and controls most of its cell cycle by regulating the G2/M transition. The remaining cell cycle time is equally divided among G1, S, and M phases. A diagram of the *S. pombe* cell cycle is depicted in **Fig. 1**. At mitosis, the replicated chromosomes segregate, forming two nuclei. During this process, chromosomes condense, but the nuclear envelope does not break down. A peculiar feature of the *S. pombe* cell cycle is that the timing of cytokinesis and nuclear division cycle is out of synchrony. The septum is not formed

A

Stationary phase

N-starvation

S

G1

M

G2

B

2C DNA content

No 1C cells in exponential wild type cultures

Cells with more than a 2C DNA content: S-phase or G2 cells stuck together.

FL2-A
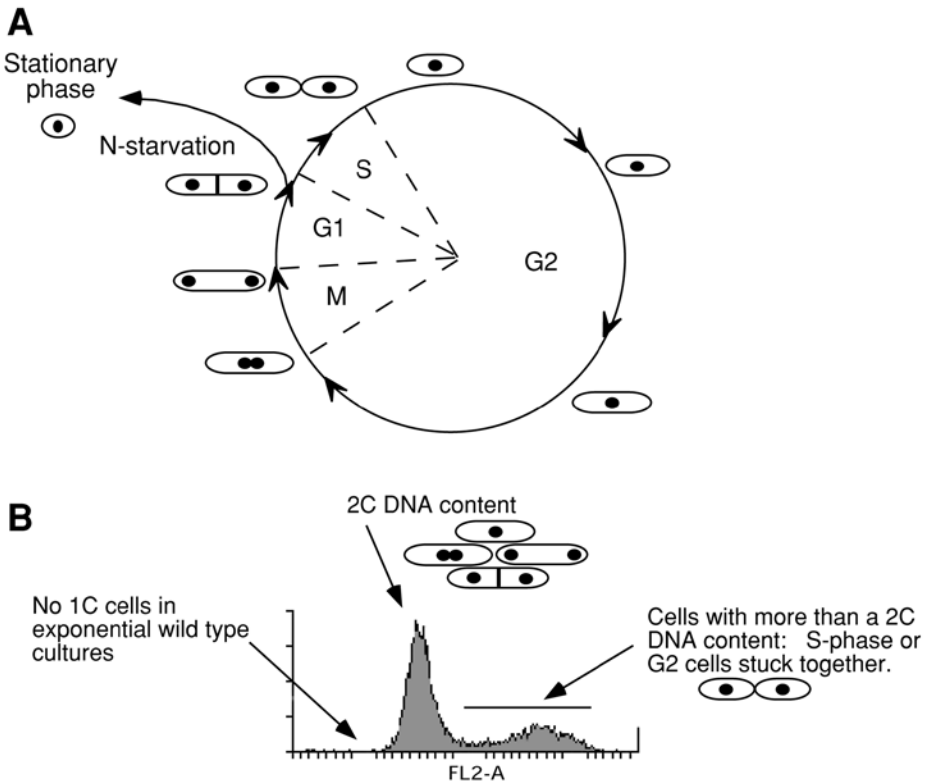
Fig. 1. **(A)** Cartoon of the fission yeast cell cycle. Exponentially growing cells spend approx 70% of cell cycle in G2 phase and 10% in each of other phases. Note delay of cell separation relative to S phase; *see* Introduction for details. **(B)** Typical flow cytometry histogram profile of an exponentially growing population of wild-type fission yeast cells stained with propidium iodine (PI). The amount of signal, proportional to DNA content, is along the *x*-axis, and the number of cells is along the *y*-axis.

until G1/S, with cytokinesis occurring during S phase. This means that after nuclear division (mitosis), the nuclei go through G1 and enter S phase before the cells complete cytokinesis. Combined with the very short G1 phase, this means that most *S. pombe* cells have 2C DNA content, either with a single G2 nucleus or two G1 nuclei in the same cell particle. Thus, in a normal cycling population, cells with a G1 or 1C DNA content are not detected by flow cytometry. Furthermore, there is a very short period during the cell cycle where cells will have more than a 2C DNA content (one cell with two late S-phase nuclei) that again is not detected by flow cytometry. When cells are cycling normally, septum formation can be used as a morphological marker for S phase.

However, when studies are carried out with mutant strains, or when using drugs to arrest the cell cycle, the formation of the septum does not necessarily coincide with S phase because timing of these events may be perturbed.

Starvation of haploid cells causes exit from the mitotic cycle and entry into either a stationary phase (**Fig. 1**) or the alternative pathway of sexual differentiation if a partner of opposite mating type is present. When cultures are depleted for nitrogen, cells enter stationary phase from G1. In contrast, if glucose is limiting, the majority of the cells arrest in G2 *(5)*; this is the usual situation if cells are grown in stationary phase in normal growth medium. Under starvation conditions, the size control mechanism is reset and, as a result, cells are smaller and acquire a rounded shape *(6)*. Nitrogen-starved cells are smaller than glucose-starved cells.

1.2. Cell Cycle Mutants

The cellular morphology of fission yeast cell cycle mutants can be readily characterized by visible light and fluorescence microscopy and their DNA content measured by flow cytometry. The tight correlation between length and cell cycle progression has been exploited to identify mutants in which nuclear division was prevented, delayed, or advanced. Fission yeast cell cycle mutants were first isolated by Paul Nurse and colleagues in the 1970s *(7–9)*. They identified the so-called *cdc*, or cell division cycle mutants. The main criterion for isolating these mutants was the formation of elongated cells, indicating cell cycle delay, although a few mutants that divided at unusually small sizes were also isolated.

The *cdc* phenotype proved to be fairly common. Different *cdc* mutants were shown to arrest cells at different points in the cell cycle as determined by nuclear and spindle morphology, DNA content, and genetic interactions. For example, mutants arrested in mitosis can be identified because of their condensed nuclei and the presence of a mitotic spindle. Defects in septation generally result in multiple nuclei or multiple septa. However, most *cdc* mutants arrest in interphase, with a single nucleus. A few mutants, such as *cdc10* and *cdc22*, arrest as elongated cells with a 1C DNA content. However, most other DNA-replication mutants synthesize a considerable amount of DNA, arresting with a 2C DNA content. They are thus indistinguishable from cells arrested in G2. Subsequent work revealed that the S-phase *cdc* mutants arrest because a checkpoint is activated that stops cell cycle progression *(10)*. For example, *cdc21 (mcm4)* mutants at the restrictive temperature undergo defective DNA synthesis *(11)*. By flow cytometry, they appear to have a 2C DNA content, although compared to wild type, the peak is shifted to the right (**Fig. 2**) *(12)*. This changed profile is caused by their elongated shape. However, in combina-
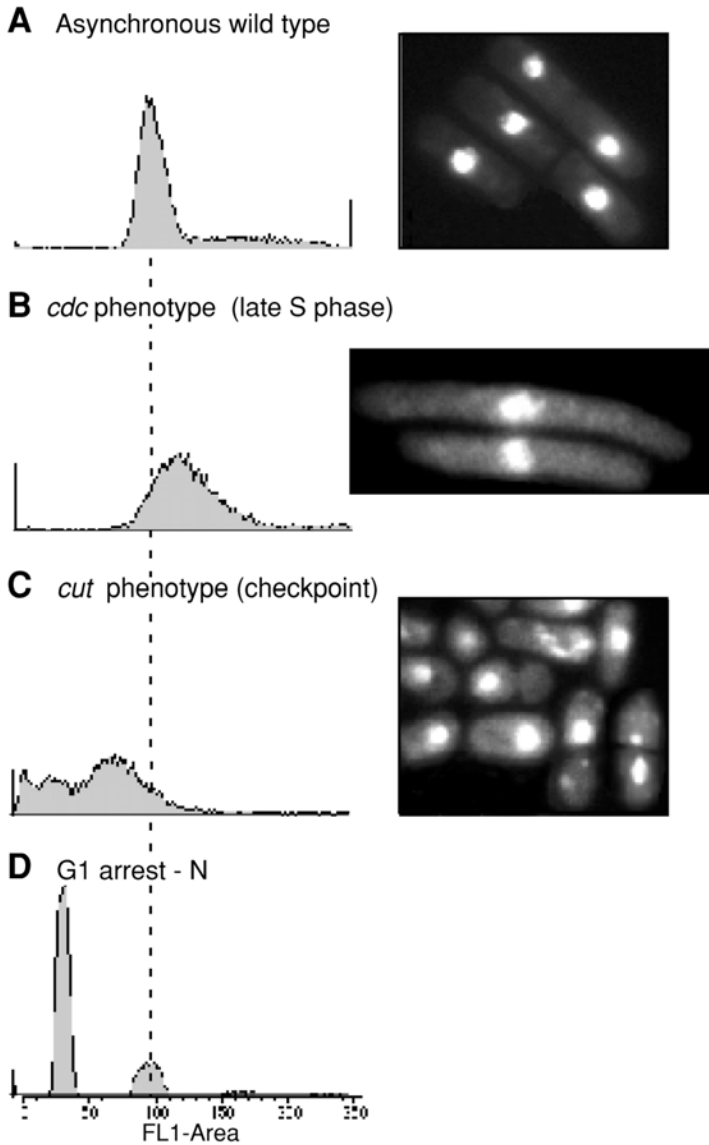
Fig. 2. Representatives of cell cycle phenotypes. Flow cytometry profiles from cells stained with SYTOX green (left). The dotted line indicates position of 2C peak in wild type. Note the shifts in the shape of the peak reflecting both DNA content (as with the cut phenotype) and cell size (as with *cdc* mutant). DAPI staining (right). Note abnormal segregation apparent in cut strain. Nitrogen-starved cells (not shown) are small and uninucleate. **(A)** Asynchronous wild-type cells. **(B)** A *cdc* mutant with late S-phase arrest. **(C)** Cut cells from a replication checkpoint-defective strain. **(D)** Nitrogen-starved wild-type cells.

tion with a checkpoint mutation, *cdc21* mutants do not arrest at the restrictive temperature but undergo lethal mitosis (a cell untimely torn [*cut*] phenotype; *see* below) *(13)*. Thus, an essential method to distinguish S-phase *cdc* mutants from G2-arrested cells is to see whether their phenotype is changed by deletion of a checkpoint gene.

Not all defects in cell cycle genes cause a *cdc* phenotype, and thus the original *cdc* screens did not identify all cell cycle genes. Another important morphology is the *cut* phenotype; this results from cells that cannot arrest mitosis in response to cell cycle defects or damage *(14)*. They attempt to divide often bisecting the nucleus with their septum, resulting in uneven DNA segregation and lethality (**Fig. 2**). The population is generally somewhat mixed, with cut, aneuploid, and normal-looking cells apparent in a given field. A wide variety of cell cycle defects can cause a cut phenotype, ranging from checkpoint mutants to spindle abnormalities. Often the cells are rather small, and by flow cytometry, they show a flattened peak shifted far to the left (**Fig. 2**). This shift is caused by cells that have less than 1C DNA content, owing to abnormal segregation.

There is a range of chromosome segregation defects that also become apparent on detailed analysis. For example, a commonly described chromosome-missegregation phenotype is a defect called lagging chromosomes (**Fig. 3**) *(15)*. This phenotype is very common in mutants that affect chromosome segregation, including cells with defects in kinetochore or centromere structure. Lagging chromosomes or chromatids result from merotelic attachment to the spindle; they appear as prominent bulges of DNA separated or semiseparated from the two main masses of the separating daughter nuclei associated with mitotic spindles *(16)*. In fission yeast, live analysis of lagging chromosomes has been carried out *(16)*. Some mutants have lagging chromosomes that eventually catch up and segregate properly; others have lagging chromosomes that stay behind on the metaphase plate (**Fig. 3**). Thus, many lagging mutants maintain high viability. Interestingly, the rate of spindle elongation is approx 50% slower in mutants with lagging chromosomes than in wild-type cells *(16)*.

Fragmented nuclei are also observed in other mutants, most notably in cut cells. In this case, more than three 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-staining bodies may be observed, and they may appear irregular and abnormally stretched out. These fragments are generally not associated with spindles and are usually associated with loss of viability. More important, individual chromosomes can be fragmented without affecting nuclear morphology. For example, *cdc24* is an S-phase mutant that accumulates DNA fragments visualized by pulsed-field gel electrophoresis *(17)*. How-
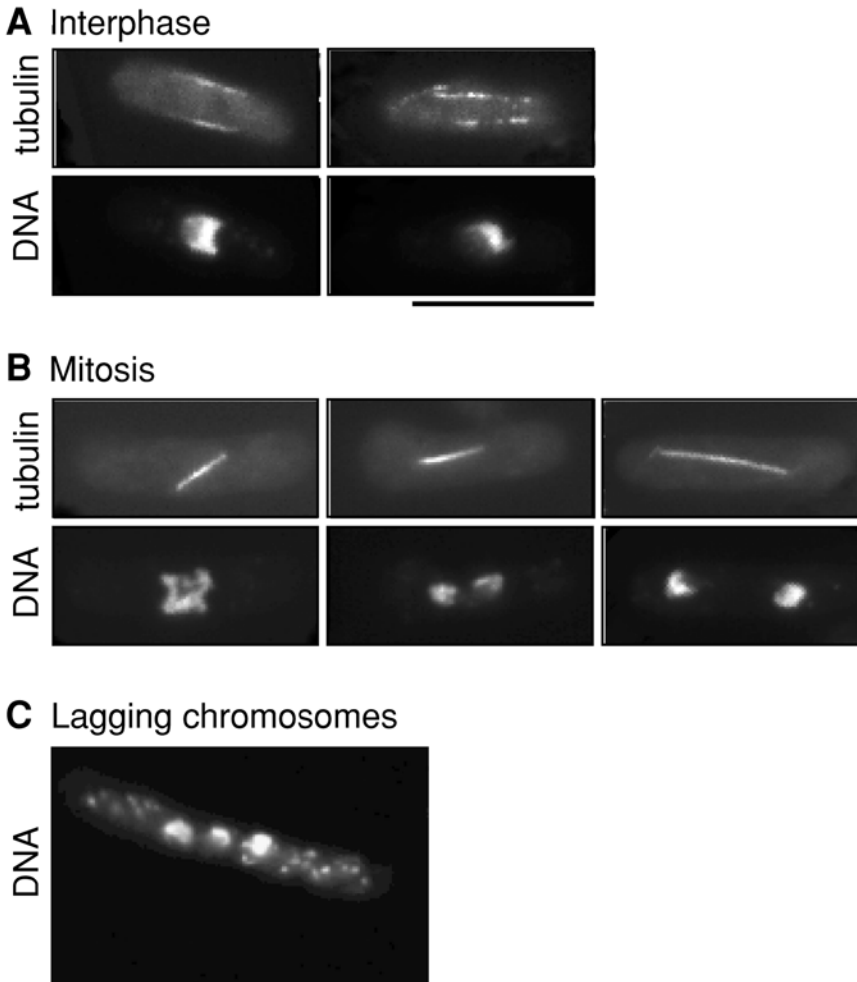
Fig. 3. Nuclear and spindle dynamics during cell cycle. **(A)** interphase cells. Note characteristic weakly staining tubulin baskets along periphery of cell, and weak DAPI staining of nucleolus, which appears as a bite taken out of nucleus. **(B)** Mitotic cells from early, mid, and late anaphase. The spindle is a single, brightly staining bar, and condensed nuclei are somewhat granular. Separated nuclei are noticeably compact. **(C)** Lagging chromosome phenotype shows additional DAPI staining body in cell, associated with anaphase spindle (not shown).

ever, the cells have a classic *cdc* phenotype with a single intact nucleus. Thus, the investigator needs to be cautious in concluding too much from morphological analysis.

1.3. Protocols

This chapter describes procedures for the analyses of *S. pombe* cell morphology using fluorescence microscopy and DNA content by flow cytometry. Protocols using fluorescent probes to label the chromatin and cell wall, as well as immunofluorescence to label α-tubulin are described. The behavior of wild-type compared to mutant cells is discussed. Not discussed here is resolution of the chromosomes on pulsed-field gels, which is useful to monitor chromosome integrity *(18)*. The reader is also referred to several excellent sources of fission yeast methods *(19,20)*.

2. Protocol, Materials, Methods, and Notes

2.1. Protocol 1: DAPI Staining of DNA

DAPI is used to stain DNA and, thus, analyze nuclear morphology. The shape, position, and number of nuclei in a cell can indicate the stage of the cell cycle as well as the integrity of the chromosomes. DAPI staining can be combined with other stains, including Calcofluor for septa, anti-tubulin antibodies or green fluorescent protein (GFP)-tubulin for microtubules, and specific antibodies to proteins of interest. Nuclear DAPI staining is the most sensitive of the fluorescent DNA-binding probes, quick, and the easiest to do. It gives better results when used to stain fixed cells, although unlike some stains, it can also be used with live cells *(21)*.

DAPI selectively forms fluorescent complexes with double-stranded DNA but not with double-stranded ribonucleic acid (RNA) *(22)*. When complexed to DNA, it fluoresces about 20 times more than DAPI alone, and the fluorescence is directly proportional to the amount of DNA present. Cations (divalent or heavy metal) substantially quench its blue fluorescence; however, the fluorescence is unchanged over a pH range of 4.0 to 11.0. The DNA-DAPI complex is stable at room temperature (RT) and does not photodissociate during the procedure *(23)*. It exhibits an excitation wavelength around 370 nm and emission peak around 450 nm. These spectral characteristics make it particularly useful for fluorescence microscopy because DAPI staining can be used in combination with indirect immunofluorescence using both red and green fluorochromes.

2.1.1. Materials

1. DAPI powder (Sigma, St. Louis, MO; cat. no. D9542).
2. *p*-Phenylenediamine (PPD) powder (Sigma, cat. no. P1519); antifade. Caution: very harmful if swallowed, inhaled, or absorbed through the skin. Use gloves when handling.

3. n-Propyl gallate powder (Sigma, cat. no. P3130); antifade.
4. DAPI stock (1000X): 1 mg/mL in dimethyl sulfoxide (DMSO). Keep at –20°C.
5. PPD stock (10X): 10 mg/mL in 0.1 M Tris-HCl pH 8. Photosensitive; keep in the dark at –20°C.
6. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4. Adjust pH with HCl.
7. DAPI staining solution I: Prepare 20 μL aliquots of 10X DAPI working solution, (10 μg/mL DAPI, 10 mg/mL PPD) and keep at –20°C (e.g., 1 μL DAPI stock in 100 μL PPD stock). When about to use, thaw and add 180 μL 50% glycerol (100% can be used); this gives a 1X working solution (1 μg/mL DAPI, 1 mg/mL PPD, 50% glycerol) that should be kept at –20°C in the dark. This solution has a clear gray color; if it turns dark brown, do not use and discard.
8. DAPI staining solution II (1X): Dissolve 50 mg n-propyl gallate and 50 mg PPD in 5 mL PBS, then add 100% glycerol to 50 mL. Aliquot in 1.5 mL volume and store at –20°C in the dark. This solution is colorless; if it turns dark, discard. When about to use, add 1 μL DAPI stock per 1.5 mL (final concentration is 0.66 μg/mL), and store frozen in the dark. This solution lasts at least 2–3 mo when stored under these conditions. This solution gives better results than solution I because it contains two antifade agents.
9. Ethanol-cleaned 22 × 75 mm × 1-mm microscope slides and 22 × 22-mm coverslips.
10. 96-well plates.
11. Poly-L-lysine (Sigma cat. no. P8920).

2.1.2. Methods

DAPI can be used on live cells or on cells fixed by a variety of methods. The choice of method depends on convenience and the requirements of any additional probe to be employed. Cells can be heat fixed on a hot microscope slide or fixed in ethanol, methanol, or methanol/formaldehyde. The crucial variable is to ensure that the cells are in a monolayer on the slide. This can be accomplished using a sufficiently diluted culture and heat fixing, or by treating a coverslip or slide with poly-L-lysine and dribbling the cell suspension across it (*see* **Subheading 2.1.6.**, **Note 3**). Although DAPI is generally added in the mounting medium, cells can also be prestained with DAPI prior to mounting (*see* **Subheading 2.1.6.**, **Note 1**).

2.1.3. Heat Fixation

1. Take a little of a colony with a sterile toothpick and mix in 5–50 μL of dH$_2$O; alternatively, take up to 20 μL from a liquid culture (these can be washed in sterile dH$_2$O if desired). Pipet 5 μL onto the slide.
2. Pipet off excess liquid. A layer of cells will stay adhered to the slide. Heat fix the cells by putting the slide on a hot plate (approx 70°C) for 1 min or by passing the slide several times by a Bunsen burner flame.

3. Check that the slide is completely dry. Let it cool for a few seconds and add 3–4 μL of 1X DAPI staining solution I or II. Cover carefully with a 22 × 22-mm cover-slip avoiding air bubbles (*see* **Subheading 2.1.6.**, **Note 4**).

2.1.4. Ethanol Fixation

This fixation method is used for cells collected for flow cytometry. It preserves nuclear morphology quite well.

1. Spin down 1 mL culture, optical density (OD) $_{595}$ nm approx 0.5–1, in a microfuge for 15 s. Remove the supernatant by aspiration.
2. Resuspend the cells in 1 mL ice-cold 70% ethanol while vortexing. Cells can be immediately stained or stored at 4°C indefinitely.
3. Before staining, rehydrate by adding 30 μL of fixed cells (any small volume will do) to 1 mL dH$_2$O in Eppendorf tube. Vortex 5 s and spin down 15 s.
4. Discard the liquid and resuspend in 20 μL dH$_2$O. Pipet 5 μL onto a slide.
5. Proceed as from **step 2** above. In this case the heat step is unnecessary for fixation but serves to provide a monolayer of adhered cells. Alternatively, cells can be air-dried without heat, although it will take longer.

When analyzing many samples, the "micromethod" can be used as from **step 3** described below. Ninety-six well plates are used instead of Eppendorf tubes, making the manipulation much faster and easier.

3. Rehydrate by adding 5 μL of fixed, settled cells to 50 μL dH$_2$O in 96-well plate.
4. Mix on a rotary shaker for 5 min.
5. Pipet 5 μL of settled cells onto a slide and proceed as from **Subheading 2.1.3.**, **step 2**.

2.1.5. Visualization

To visualize cells that have been DAPI stained, a microscope with a mercury lamp and an adequate filter is needed. The filter should allow ultraviolet excitation at 240–380 nm. The microscope should have a film or charge-coupled device (CCD) camera attached to record the images. Numerous packages combining digital cameras with computer software are available; more sophisticated software allows false color, overlays, and complex image analysis. Slides containing DAPI-stained cells can be stored in the dark in a freezer for several months.

Figures 2 and **3** show DAPI staining of wild-type and representative fission yeast mutants. The interphase nucleus typically appears as a round structure with a bite taken out of it (the weakly staining nucleolus). Mitotic nuclei are generally condensed and somewhat granular in appearance. Roughly 10% of cells in a wild-type culture are in mitosis. It is important to remember that in binucleate cells, the nuclei are a mixture of late-mitotic and interphase morphologies. Septated cells that have not completed cell separation contain inter-

phase (S phase) nuclei. A wild-type control strain is thus essential for comparison to the experimental sample.

2.1.6. Notes

1. Sometimes a better signal and lower background is obtained by prestaining the cells with DAPI before mounting on the slide. The cells are incubated with 1 mL of a 1 µg/mL DAPI in PBS or PEM (*see* **Subheading 2.2.1.**, **Note 7**) for 10 min at RT in the dark and rinsed in the buffer lacking DAPI. They are applied to the slide with a mount solution containing the antifade agents but lacking DAPI.
2. It is very important to adjust the number of cells to get a monolayer. When too many cells are mounted on the slide it is very difficult to have them in the same plane of focus, making the visualization and photographing very difficult. If cells are not well adhered to the slide, they will start moving (floating) when covered with the coverslip. Make sure that the cells are dry before adding the mounting solution and the coverslip.
3. To facilitate a monolayer, slides or coverslips can be treated with poly-L-lysine, which creates a positively charged surface to which cells adhere. Cover the area of the slide, or coverslip where cells will be mounted, with poly-L-lysine. Leave for 2–5 min and tip the slides on one end to let the poly-L-lysine run off. Air-dry before mounting the cells. If the cell volume is large, the coverslip or slide can be held at an angle and the drop of cell suspension allowed to run across it. The excess liquid is removed and the cells allowed to air-dry, before adding the mounting medium and the coverslip. This can be used for fixed cells in lieu of heat treating the slides.
4. The coverslip should be gently laid over the cells starting at one side to avoid air bubbles. Make sure that the slides are on an even surface.
5. The edges of the coverslip may be sealed with clear nail polish to preserve the sample of fixed cells.
6. Several samples can be accommodated on a single slide.
7. The final volumes of cell suspension added to the slide can be scaled up to allow the use of larger coverslips.
8. Ethanol-fixed cells maintain nuclear structures very well and are preferred for photography. Unfixed or heat-fixed cells are useful for immediate evaluation, or time-course analysis. They are adequate for photography.

2.2. Protocol 2: Staining the S. pombe *Septum With Calcofluor*

Calcofluor white can be used to stain fission yeast cell wall and septum *(24)*. This well-known fluorochrome is widely used as an optical brightener. It stains 1,4-linked polymers (cellulose, chitin). An advantage of Calcofluor is its stable fluorescence. Slides stained with Calcofluor can be stored for months at –20°C. It exhibits an excitation wavelength between 340 and 360 nm and emission peak around 400–440 nm; thus, the same filter can be used as for detection of DAPI staining. The use of this probe in fission yeast is useful particularly to

monitor septation. In a wild-type culture, approx 10% of the cells will have a single septum. Septation mutants may have multiple nuclei and no septa; some mutants have multiple septa. Although Calcofluor staining is very useful, the septum can also be visualized by light microscopy and in DAPI-stained cells with background cytoplasmic fluorescence.

2.2.1. Materials

1. Calcofluor powder: fluorescent brightener 28 (Sigma, cat. no. F-3397). Avoid contact and inhalation. Use gloves when handling.
2. Calcofluor stock: 1 mg/mL in 50 m*M* sodium citrate, 100 m*M* sodium phosphate pH 6.0. Calcofluor can be difficult to dissolve and may require several hours to overnight of stirring at RT in darkness. Store in the dark at 4°C. This stock remains good for several months.
3. *p*-Phenylenediamine (PPD) powder (Sigma, cat. no. P1519); antifade. Very harmful if swallowed, inhaled, or absorbed through the skin. Use gloves when handling.
4. PPD stock: 10 mg/mL in 0.1 *M* Tris-HCl pH 8.0. Photosensitive; keep in the dark at –20°C.
5. PPD mount solution: 1 mg/mL PPD in 50% glycerol.
6. Calcofluor 1X mounting solution (prepare fresh each time): Dilute the Calcofluor stock to 50 μg/mL with 50% glycerol containing 0.3 mg/mL PPD.
7. 10X PEM: 1 *M* PIPES pH 6.9, 10 m*M* EGTA, 10 m*M* MgSO$_4$ (adjust the pH with 5 *N* NaOH).
8. 1X PEM: dilute 10X PEM to 1X with sterile dH$_2$O.

2.2.2. Methods

Calcofluor can be used to stain cells along with any of the methods described for DAPI, using the Calcofluor 1X mounting solution. Alternatively, cells can be Calcofluor stained before mounting on the microscope slide:

1. Spin down 1 mL culture (OD$_{595}$ nm approx 0.5–1) in a microfuge for 15 s. Remove the supernatant by aspiration.
2. Resuspend the cells in 100 μL of Calcofluor stock solution for 5 min at RT.
3. Wash the cells three to five times with 1X PEM. Resuspend in approx 1 cell volume with 1X PEM (e.g., if volume of cells is 50 μL, resuspend in 50 μL PEM).
4. Pipet 5 μL onto a microscope slide.
5. Pipet off excess liquid. A layer of cells will stay adhered to the slide. Heat fix the cells by putting the slide on a hot plate (approx 70°C) for 1 min, by passing the slide several times by a Bunsen burner flame, or just let air-dry.
6. Check that the slide is completely dry. Add 3–4 μL of PPD mounting solution, and cover with a coverslip.

2.2.3. Notes

1. *See* **Subheading 2.1.6.**
2. Sometimes it is necessary to adjust the Calcofluor concentration and/or incubation time to get a good staining.

3. To stain cell wall/septum and nuclei simultaneously, follow the Calcofluor stain-ing protocol and add 1 µg/mL DAPI to the PPD mount solution. The level of Calcofluor may need to be adjusted so that the signal intensity is similar to the DAPI.
4. PBS can be used instead of PEM.

2.3. Protocol 3: Visualizing S. pombe *Microtubules* by Immunofluorescence Microscopy

The use of anti-α-tubulin antibodies can be very useful to visualize fission yeast microtubules. The length and shape of spindles can be very informative when analyzing mutants that affect normal progression of mitosis or meiosis *(25)*. About 10% of wild-type cells will contain a spindle, which is a bright bar-shaped structure quite different from the basket array seen in metazoan cells (**Fig. 3**). Interphase cells contain visible baskets of microtubules that run the length of the cell along the edges. Antibodies raised against tubulins from different species have been used successfully to stain fission yeast α-tubulin. Recently, a GFP α-tubulin fusion has been used to analyze in vivo microtubule structures and dynamics *(26)*. The immunofluorescence protocol described in this section was modified from *(27)* and *(28)*.

2.3.1. Materials

1. 10X PEM: 1 M PIPES pH 6.9, 10 mM EGTA, 10 mM MgSO4 (adjust the pH with 5 N NaOH).
2. 1X PEM: dilute 10X PEM to 1X with sterile dH$_2$O.
3. 1X PEMS: 1X PEM, 1.2 M sorbitol.
4. PEMBAL: 1X PEM, 1% bovine serum albumin (BSA, globulin free, Sigma, cat. no. A 7638), 0.1% sodium azide, 100 mM lysine hydrochloride. Dilute in sterile H$_2$O using sterile solutions. Keep at RT. It remains stable for several months. Sodium azide is extremely toxic, and gloves should be worn at all times.
5. FIX solution: 0.1 M potassium phosphate pH 6.5, 10% methanol, 3.7% formalde-hyde. Keep at RT for several months. Formaldehyde is very toxic; handle in the fume hood, and use gloves.
6. Enzyme mix: 0.2 mg Lysing enzymes (Sigma, cat. no. L 1393), 0.5 mg Zymolyase 20T (Seikagaku, Tokyo; cat. no. 120491)/per mL PEMS. It must be diluted just before use.
7. DAPI/PEMBAL solution: 1 µg DAPI/mL PEMBAL. It must be prepared just before use.
8. PPD stock: 10 mg/mL in 0.1 M Tris-HCl pH 8. Photosensitive; keep in the dark at –20°C.
9. PPD mount solution: 1 mg/mL PPD in 50% glycerol.
10. α-tubulin antibody: a good commercial antibody is the monoclonal rat anti-tubu-lin from Immunologicals Direct, cat. no. OBT1062.

11. Secondary antibody coupled to a fluorochrome such as the anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA) (cat. no. 715-165-150). Other secondary antibodies coupled to alternative fluorochromes such as fluorescein isothiocyanate (FITC) or Texas Red are also suitable.

2.3.2. Methods

The immunofluorescence protocol described in this section was modified from *(27)* and *(28)*.

1. Grow cells to 2–4×10^6 cells/ mL (OD_{595} nm approx 0.5–0.8).
2. Filter 50 mL of culture onto 5-cm GF/C (Whatman) disk.
3. Fix cells by placing the filter in a 50-mL polypropylene tube containing 25 mL of FIX solution for 30 min at RT.
4. Remove filter and spin cells down at 1500*g* for 5 min at RT.
5. Wash cells three times with 5 mL of PEM. Pellet cells at 2000*g* for 5 min at RT in between washes.
6. Resuspend cells in 3 mL enzyme mix, and digest at 30°C until 80–90% of cells lose cell-wall integrity, as judged by a loss of birefringence in 1% sodium dodecyl sulfate (SDS). The incubation time varies depending on the activity of the enzymes.
7. Spin down the cells and wash three times in 1 mL PEMS. Use 1.5-mL tubes and centrifuge at 3000*g* for 1 min at RT. At this point the cells are very delicate; use a pipet to resuspend them in between washes; do not vortex.
8. Incubate in 1 mL PEMBAL for at least 30 min at RT. A 50-mL cell pellet can be split into two or three tubes for treatment with different antibodies. Cell pellet volume per antibody treatment should be approx 50 µL.
9. Incubate cells in 200–500 µL α-tubulin antibody diluted in PEMBAL, overnight on rotator at RT.
10. Wash three times in 1 mL PEMBAL. Each wash should be in rotator for 10 min. Spin cells at 5000*g* for 1 min in between washes, and discard each wash solution.
11. Add secondary antibody (200–500µL) diluted in PEMBAL and incubate at RT for 1 h in the dark. The dilution of the secondary antibody must be determined empirically; use 1:500 initially.
12. Wash two times in 1 mL PEMBAL. Mix on rotator for 10 min in darkness in between washes. Spin cells at 5000*g* for 1 min in between washes.
13. Wash once in 1 mL DAPI-PEMBAL solution. Mix on rotator for 10 min in darkness.
14. Spin cells and resuspend in PEMBAL using 1 volume of cells (e.g., if cell pellet is approx 50 µL, use 50 µL of PEMBAL). Keep cells in darkness.
15. Pipet 5 µL of cells onto a clean glass microscope slide. Pipet off excess liquid. A layer of cells will stay adhered to the slide. Let the cells air-dry, always keeping the microscope slide in darkness. Alternatively, cells can be heat fixed by putting the slide on a hot plate (70°C) for 1 min or by passing the slide several times by a Bunsen burner flame.

16. When cells are completely dry, add 3 μL PPD mount solution. Cover very gently with a 22 × 22-mm coverslip avoiding air bubbles.

2.3.3. Visualization

See **Subheading 2.1.5.** for visualizing DAPI stained cells. Cy3 exhibits an excitation wavelength around 550 nm and emission peak around 570 nm.

2.3.4. Notes

1. *See* **Subheading 2.1.6.** to get a monolayer of cells.
2. Cells can be collected by centrifugation at low speed. In some cases, either filtration or centrifugation of cells may disrupt their intracellular architecture.
3. If background staining is high, use PEMS with 3% BSA or PBS with 5% dry milk to block cells and provide a staining buffer.
4. As Cy3 is very photolabile, first photograph the immunofluorescence stain and then the DAPI nuclear stain. A newer generation of fluorochromes, including for example, Molecular Probes' Oregon Green, is reportedly more resistant to photobleaching.
5. In the last wash (**step 13**), instead of incubating the cells with DAPI, wash them once more with 1 mL PEMBAL, and add DAPI to the mount solution (*see* DAPI staining solutions in **Subheading 2.1.1.**).
6. Other anti-tubulin antibodies raised to different species have been used successfully, including a mouse monoclonal antibody raised against *Drosophila* (4A1), or trypanosome antibody (anti-TAT1) *(25,32)*.

2.4. Procedure to Determine Fission Yeast Cells DNA Content by Flow Cytometry

Flow cytometry is a method for separating cells based on a fluorescence signal. The signal can be generated by labeling some feature of the cell (typically the DNA) with a fluorescent dye. The signal generated is proportional to the amount of dye and the shape of the cell. Although the machine makes measurements on one cell at a time, it can process thousands of cells in a few seconds. The term *FACS* is Becton Dickinson's registered trademark and is an acronym for fluorescence-activated cell sorter. Another major vendor of flow cytometers is Coulter Electronics.

In yeast cells, flow cytometry has been used almost exclusively for measures of DNA content, although in principle GFP staining and other labels are also suitable for this analysis. For DNA staining, the nucleic acid is stained by propidium iodine (PI, a red fluorochrome) or SYTOX Green (a green fluorochrome). Both dyes are intercalation agents and only fluoresce when bound to nucleic acids. However, for these stains to gain access to the DNA, the cells must be permeabilized by ethanol fixation. An essential step is ribonuclease (RNase) treatment to eliminate any background from the extensive RNA in the yeast cell because both dyes also bind RNA.

PI is extremely photostable and is very robust even under the confocal microscope. It exhibits an excitation wavelength around 530 nm and emission peak around 615 nm. SYTOX Green nucleic acid stain easily penetrates compromised cell membranes, but it is completely excluded from live eukaryotic and bacterial cells. After a brief incubation with SYTOX Green stain, fixed, dead cells fluoresce bright green (570 nm emission peak) when excited with any 450 to 490 nm source. SYTOX Green is quite bright, and anecdotally, it is reported to be more sensitive than PI for DNA staining in FACS. However, it may not be as photostable as PI for other uses.

2.4.1. Materials

1. SYTOX Green powder (Molecular Probes, Eugene, OR; cat. no. S-7020).
2. PI powder (Sigma, cat. no. P4170).
3. Sodium citrate stock (10X): 500 m*M*, filter and store at RT.
4. Sodium citrate working solution (1X): 50 m*M*, diluted stock 1/10 with sterile dH$_2$O. Keep at RT for several months.
5. RNase A: 10 mg/mL deoxyribonuclease (DNase) free.
6. PI: 4 mg/mL, filter and store in darkness at –20°C. Harmful if swallowed or absorbed through the skin. Use gloves to handle.
7. SYTOX Green stock: 5 m*M* in DMSO; store in darkness at –20°C.
8. SYTOX Green working solution: 2 µ*M* in 50 m*M* sodium citrate.
9. Cold (4°C) 70% ethanol.

2.4.2. Methods

The following method was adapted from **ref. *30***.

1. Spin down 10^7 cells from an exponentially growing culture at approx 2000*g* for 1 min. Pour off supernatant.
2. Vortex tube while adding 1 mL cold 70% ethanol. Invert tube several times and store at 4°C (cells keep indefinitely).
3. To process the cells, vortex and take 0.3 mL (this will be $2–3 \times 10^6$ cells, assuming a little loss in the washing), and add to 3 mL 50 m*M* sodium citrate in a 5-mL FACS tube (when using a Becton Dickinson FACScan, use Falcon 352052 tubes). Mix and spin at 2000*g* for 5 min.
4. Carefully aspirate the supernatant and resuspend pellet in 0.5 mL 50 m*M* sodium citrate containing 0.1 mg/mL RNase A. Incubate at 37°C for 1–2 h.
5. For staining:
   PI: Add 0.5 mL 50 m*M* sodium citrate containing 8 µg/mL PI, so that final concentration in the sample is 4 µg/mL. There can be nonspecific staining of *S. pombe* ends at higher concentrations if cells are starved, or with spores. Cells can be processed immediately or conveniently stored overnight at 4°C in the dark before processing the next day. If necessary, stained cells can be stored for a maximum of 1 wk at 4°C in the dark.

SYTOX Green: Add 0.5 mL 50 m*M* sodium citrate containing 2 μ*M* SYTOX Green, so that the final concentration in the sample is 1 μ*M*. Cells can be stored at 4°C in the dark for at least 1 wk before processing.

6. Data should be collected according to procedures for the individual machine and software package. If the machine is run by a technician not used to yeast, be sure to provide the settings, if possible, and a sample profile of the wild-type distribution to help adjust the machine. Approximate settings on the Becton Dickinson FACScan for PI are detector FSC E00, gain 3; detector FL2-A, voltage: 890, gain: 2. Approximate settings on the Becton Dickinson FACScan for SYTOX Green are detector FSC E00, gain 2; detector FL1-A, voltage 400, gain 4.

7. Values can be plotted using a linear or logarithmic scale. Generally the linear scale is used to distinguish 1C, 2C, and 4C cells. However, when higher DNA contents are possible (for example, in re-replicating strains *[30,31]*), the logarithmic scale can be more informative. A common software used to analyze flow cytometry profiles is Becton Dickinson's Cellquest.

An example of a wild-type strain flow cytometry profile is shown in **Figs. 1B** and **2**. Profiles of mutant strains are shown in **Fig. 2**.

2.4.3. Notes

1. Staining of cells can be verified with a fluorescence microscope using the appropriate filters.
2. When the cells are dilute, and a cell pellet is not visible after the spin in **step 3**, do not aspirate all the liquid. Leave approx 100 μL to ensure that the cells are retained in the tube.
3. Just before processing the cells, samples should be sonicated for 5–10 s at a low power setting in the 5-mL FACS tubes. Sonication prevents doublets of cells, which give spurious peaks, and is particularly useful if analyzing starved cells, spores, "wee" mutants, or cells having heterogeneous DNA contents. Make sure not to oversonicate to avoid breakage of the cells.
4. More than 10^7 cells can be fixed but do not stain more than 5×10^6 fixed cells. Using too many cells can lead to incomplete staining and artifacts.
5. It is essential to include control samples representing 1C- and 2C-DNA contents. A 4C-control is also useful. Nitrogen-starved haploid cells, exponentially growing haploids, and exponentially growing diploid cells, respectively, can generate the appropriate signals. A large number of cells can be fixed and used over many months.
6. Cells that are to be compared to one another should be stained and analyzed at the same time. Variations in the staining from day to day makes it difficult to compare independently stained samples.
7. Ethanol-fixed cells can be sent in the post at RT without coming to any harm.
8. If dealing with particularly fragile cells (e.g., very elongated cells) there may be a problem with lysis. This can be avoided by fixing the cells in 70% ethanol, 30% 1 *M* sorbitol; 1.2 *M* sorbitol can be included in growth media as well. It is impor-

tant to wash out the sorbitol before flow-cytometric analysis because it destabilizes the sample stream.

9. Learn how to use the live-gate option, which provides a way to eliminate spurious signals, allowing a reduction in the background in the samples (this may be caused by anything from particles of medium to bacteria or other contaminants). It also gives the option of focusing on a particular subpopulation of interest. Similarly, it can be useful to set a size threshold below the size of a fission yeast cell to reduce background from extraneous particles or bacteria.

10. Samples should be examined quickly prior to actual data collection to optimize gates, gains, and amplifier for the experiment. The peaks should be adjusted to maximize separation between 1C and 2C peaks on a linear scale. This will facilitate observation of sub-1C- or S-phase populations. However, if the cells are elongated, the peaks shift to the right, and care must be taken to ensure they do not fall off the distribution. Settings should not be changed once data collection begins.

11. If the flow cytometry gives a profile that is flattened and shifted to the right, it could be owing to RNA contamination. Make sure that the RNase A is active and that the incubation time is long enough.

Acknowledgments

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II

ANALYSIS OF GENES INVOLVED IN CHECKPOINT CONTROL

9

Strategies to Isolate Evolutionarily Conserved Cell Cycle Regulatory Genes

Scott Davey

1. Introduction

Unlike many of the other topics covered in this book, the difficulty in identifying conserved checkpoint genes lies more in choosing an appropriate method than in carrying out the work. Thus, this chapter concentrates more on the alternative strategies and under which circumstances the choice of a given method is appropriate. There are essentially three types of strategies for isolating conserved genes of any sort: through deoxyribonucleic acid (DNA)-sequence conservation, protein-structure conservation, and through functional conservation. Each of these approaches can be further subdivided into different techniques that could be applied, and the choice of technique is dependent on the system in which one is working and on the gene one is attempting to isolate.

Isolation of genes by DNA-sequence conservation has gone through several phases over the last 20 yr. This technique originally was synonymous with low-stringency hybridization. To this was added the polymerase chain reaction (PCR), particularly using short, highly conserved regions in a protein family. More recently, with genomic-sequencing projects yielding large databases of expressed sequences, one is very likely to find a conserved gene of interest by low-stringency hybridization *in silico* (i.e., via a Basic Local Alignment Search Tool [BLAST]).

Identification of a protein via conserved structure is a viable alternative approach and can work where other methods fail. Historically, this meant having both an expression library from the target organism and an antibody directed against the protein of interest. One would screen for conserved epitopes, isolate the clone expressing the epitope, and sequence the gene

encoding it. Alternatively, one can look for conserved protein–protein interactions, either across species or within species, depending on the reagents available. A protein purified in this way can be identified by protein microsequencing or by mass spectrometry. Finally, the gene encoding the protein may be identified by reverting to the DNA-sequence-based methods described above—primarily by determining if an expressed-sequence tag (EST) encoding the protein exists, by studying it, or by degenerate PCR (one need not have conserved regions, as one knows actual protein sequence, so this task is made simpler).

Finally, arguably both the most elegant and coarse of the methods fall into the functional conservation category. First, if a mutant version of a gene is known in an organism for which one can perform a functional complementation screen—typically one of the yeasts—and if an appropriate complementary deoxyribonucleic acid (cDNA) expression library exists or can be constructed, one can attempt to complement the defect in the model organism with a gene from a different species. This leads to direct isolation of the gene and a strong suggestion of true conservation. However, functional complementation is absolutely dependent on the availability of a suitable assay. Alternatively, when observing a known protein activity, one can try the time-tested approach of purifying the activity. Although this does not lead directly to the gene of interest, it does give material that, by use of previously described techniques, will lead to identification of the gene; an added bonus is that the protein will also be in hand.

Given that one is attempting to identify a conserved gene, there must be a minimum amount of information available about the nature of the gene or its gene product. The Materials section (*see* **Subheading 2.**) contains a list of potentially available information one can have at his or her disposal at the time of initiating a search for a conserved cell cycle gene. The Methods section (*see* **Subheading 3.**) is a flowchart, with dependencies on some of the information at hand.

2. Materials

As a starting point, ask the following questions regarding a gene of interest, and take the answers through the methodological flowchart presented in **Fig. 1**.

2.1. Gene-Based Information

1. Is it a single gene sequence, or a family of genes?
2. Is the level of sequence conservation high, low, or restricted to specific domains?
3. Are there defined consensus domains that characterize the gene family?

2.2. Protein-Based Information

1. Is a biochemical assay for the gene product available?
2. Is purified protein or antibodies directed against the protein available?
3. Are conserved proteins known to interact with this protein?

2.3. Cellular Information

1. Has the mutant phenotype associated with loss of this gene of interest been characterized?
2. Is a functional complementation screen available?

3. Methods

The approach to choose for identifying a conserved cell cycle protein depends on the information available. Typically, one will have at least some, but not all, of the information listed under Materials (*see* **Subheading 2.**). Use the following chart (*see* **Fig. 1**) to choose one or more methods that are appropriate for identifying the gene. It is almost certainly a good idea to try more than one approach to maximize chances for success. Although each method has strengths and weaknesses, these may vary from situation to situation. Consult the Notes section (*see* **Subheading 4.**) to decide which approach may best suit one's needs.

4. Notes

1. Functional complementation: One of the easiest and most convincing methods for identifying a conserved protein is via a functional complementation screen. The major issue in attempting this type of screen is the availability of a suitable assay. This means having a mutant with a sufficiently pronounced phenotype such that it can be used to readily identify a rare, wild-type clone among many mutants. For practical reasons, doing a screen of this type has two limitations. First, even with a good cDNA expression library, one will have to screen a minimum of 10^6 transformants to have a reasonable chance to identify a complementing gene. In practical terms, this means one will be performing the complementation in a single-celled organism. Yeasts are the most often-used models for this type of work, although bacteria are also possible, depending on the gene one is interested in. The second, and even more limiting restriction, is that because one is screening such a large number of cDNAs, one needs a very low false-positive rate. In practical terms, this translates to an assay for viable vs inviable cells and one that has very good discriminatory power. Ideally, a temperature-sensitive lethality screen is either available or can be designed. For example, one can screen directly for complementation of a *cdc* gene deficiency, as was done for fission yeast *cdc2 (1)*. Alternatively, if, for example, one is looking for a checkpoint gene that is not lethal on its own, one could consider screening for restoration of the intermediate temperature sensitivity of a double mutant in the checkpoint gene plus a cell cycle gene whose viability is modulated by the checkpoint. A nice example of such a screen made use of the synthetic lethality of a *wee1-50 cdc25$^+$ OP* strain to clone the human homolog of fission yeast *wee1$^+$ (2)*; without the *cdc25$^+$ OP* in this strain, *wee1-50* mutants would be viable on their own, making a complementation screen impractical. Previous experience has shown that screens relying on an exogenous agent (e.g., ultravio-
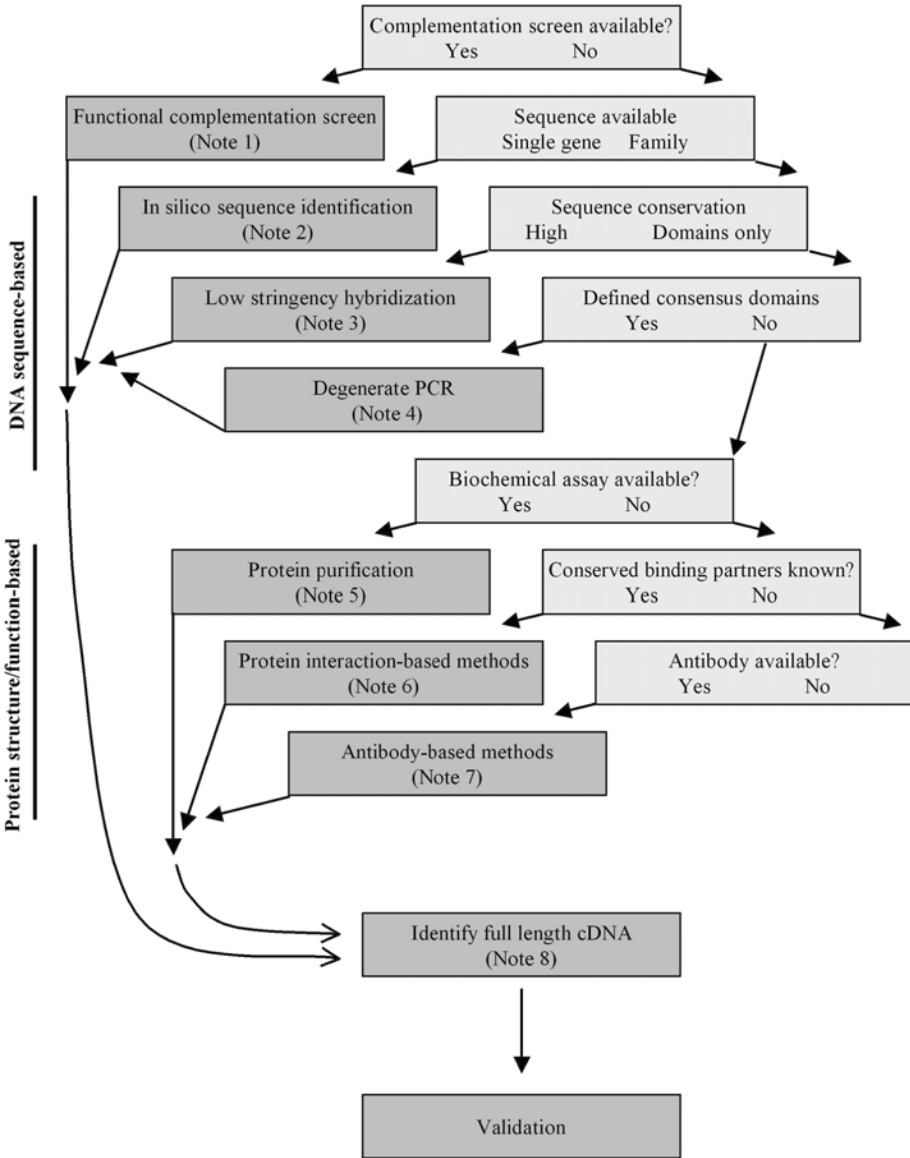
Fig. 1. Flowchart for selection of methods for identifying conserved cell cycle genes.

let [UV] light)-induced killing are extremely challenging, as the difference between the sensitive and rescued phenotypes is slight. In a screen of 10^6 transformants, the level of false positives can quickly become prohibitively high.

Choice of cDNA library is obviously the other extremely important aspect of this type of screen. The source of ribonucleic acid (RNA) for generating the

library will be screen specific, as well as other more mundane things, particularly the choice of promoter from which expression of the cDNA will be driven.

Transformants are generally selected in two stages, first by complementation of a nutritional requirement (to select for transformants and to score transformation efficiency), and second for complementation of the mutation being studied. In most cases, transformants are given time to recover prior to shifting to restrictive conditions. Depending on the organism, the time involved would be from an hour to overnight. Colonies are then allowed to form, and primary positives are isolated. Normally, one should do a second colony purification under restrictive conditions to test for quality of rescue—colony formation is actually a very stringent assay for complementation. Then plasmids can be recovered from the positive transformants and subjected to DNA sequencing for direct identification of the functionally homologous gene.

2. *In silico* sequence identification: Undoubtedly the easiest way to clone conserved genes these days is via a BLAST search *(3,4)*. With many whole genomes now available for searching, and many more on the way, one often can find at least an expressed piece of the desired gene without so much as hefting a pipet. BLAST services require a DNA- or protein-sequence input, which can be compared to DNA- or protein-based libraries of information. One popular BLAST service is provided by the National Center for Biotechnology Information (NCBI). The URL for their Web site is (http://www.ncbi.nlm.nih.gov/BLAST/).

In practical terms, one searches a gene or protein sequence against the entire nonredundant database or, specifically, against the preferred organism. The human homolog of the fission yeast *rad9* checkpoint gene, *hRad9*, was first identified in this manner *(5)*. Variants of BLAST will search either nucleotide or protein sequences against various databases, and the search can be adjusted to target either large domains with moderate conservation or short, highly conserved regions. Extensive descriptions of how to maximize the efficacy of these searches are provided on the NCBI Web site (http://www.ncbi.nlm.nih.gov/BLAST/producttable.html).

BLAST searches almost inevitably identify something, and it is important to confirm that what is identified is truly a homolog of a protein. While the ultimate determination requires validation of the candidate gene, as described in **Note 9** below, a quick shortcut that is helpful is to take the results of a BLAST search, and perform a BLAST search on them. If other members of a family are found, then the research is likely on the right track. If, for example, ribosomal protein #@* turns up, then one should strongly consider modifying the BLAST search parameters, or going on to the next potential method for identifying the gene.

3. Low stringency hybridization: Adjusting Southern or Northern blotting conditions for low stringency has the advantage of relying only on having a prototype gene (probe) and a suitable library from the organism of choice. Ideally, the library would be a cDNA library, but genomic libraries can also be used. Also, the library would preferably be constructed in a bacteriophage vector, although plasmid-based vectors can also be used. This approach is rarely necessary any

more, with the abundance of sequenced genomes, but there may be special cases in which one might use this technique. Primarily, this would be useful for identifying a homologous gene where one already has the prototype gene identified in a highly conserved organism. The human, murine, and fission yeast homologs of *Rad51*, a highly conserved RecA-like protein, were all cloned in this manner *(6)*. Even then, degenerate PCR (*see* **Note 4**) is probably a more generally applicable technique today.

4. Degenerate PCR: This technique has some notable disadvantages. First, one requires sufficient examples of the gene from which one is attempting to identify conserved protein regions. Second, at least two regions are required—separated by some distance—that are highly conserved between protein members of the family. Finally, the conserved protein regions must contain amino acids encoded by a minimal number of codons to keep degeneracy to a minimum. Two contrasting approaches here are to use short, less degenerate primers vs long, more degenerate primers. Short in this case is 12–15 residues, and long is more than 20 residues; less degenerate is defined as approximately less than 64-fold, and more is defined as greater than 64-fold. Obviously, if a protein contains long, characteristic stretches of methionines mixed with tryptophanes, this is good. If the conserved region contains amino acid residues that are only encoded by two codons (tyr, cys, asp, glu, phe, his, gln, asn, lys), it may still be a good target. If it contains even one of the amino acids encoded by six codons (leu, ser, arg), it may be useless. A detailed consideration of the factors involved in this process has been published *(7)*.

   One way of getting around the problem is to make multiple oligonucleotides of less degeneracy—thus, if one had a conserved YCSKF as an upstream sequence, one might be better off attempting two oligonucleotides, one TA[TC]TG[TC]TC[ACGT]AA[AG]TT[TC] and the other TA[TC]TG[TC]AG[AC]AA[AG]TT[TC], rather than trying to roll all the combinations of the central serine together into TA[TC]TG[TC][AT][CG][ACGT]AA[AG]TT[TC]. These oligonucleotides are all 15-mers, with 64X, 64X, and 256X degeneracy.

   Balancing these disadvantages is the fact that this is the quickest of the techniques available—it is not impossible to go from a DNA sequence to a cloned homolog in a week if this technique works right away. This technique has been applied to the cloning of the Erk1 kinase *(8)* and various members of the minichromosome maintenance (MCM) family of replication-initiation proteins *(9)*.

5. Protein purification: Protein purification has the advantage of yielding functional protein for biochemical analysis. The primary concern with this method is simple—the time and work involved in the process. However, the benefits are significant, and although it should be considered as an alternative method for identification of conserved genes, it does have some notable advantages. Most important, this is the only approach that, in addition to allowing relatively easy cloning of the gene of interest, also intrinsically provides protein for future biochemical analysis. This method relies entirely on the availability of a biochemical assay for protein and on the presence of such a conserved activity in an organism

of choice. Protein purification can be performed by a variety of standard methods, including ion exchange, gel filtration, and affinity chromatography. A variety of semiautomated systems are available to assist in the process, and any of them would certainly decrease the work required in the end. As with all methods that rely on identification of a protein, one needs to subject the protein to microsequence analysis or to mass spectrometry followed, usually, by degenerate PCR to ultimately identify the gene that encodes it. The Cdk activating kinase (CAK) MO15 was cloned by purification of the activity from *Xenopus* extracts *(10,11)*. Of course this technique works in reverse as well; purifying a protein for its own sake can ultimately lead to the identification of a conserved gene. The classic example of this was realization that Cdc2-cyclin-dependent kinase and *Xenopus* maturation-promoting factor (MPF) are conserved entities *(12)*.

6. Protein-interaction based: A technique that has been used successfully in recent times involves identifying a protein by one of its functional characteristics, namely via conserved interactions with proteins. If a protein is not only conserved but also a member of a conserved pathway (likely), then one may know proteins with which it interacts in one or more organisms. If the proteins with which a particular protein interacts are themselves conserved, it is likely that a conserved version of this protein also interacts with such a protein. In practical terms, this means that one may be able to identify the homolog of a protein via its interaction with another characterized (conserved) protein. Coimmunoprecipitation and copurification are the most straightforward ways to assess such an interaction. This technique was used to identify ATRIP, a homolog of the fission yeast *rad26*+ checkpoint gene *(13)*. Once a candidate protein is identified, it is analyzed as described in **Note 5.** One of the clear advantages of this approach is that it intrinsically generates data confirming the conservation of function of a protein, at least as far as interaction with another protein member of a pathway is concerned.

7. Antibody/epitope-based methods: Several years ago, one might have screened an expression library (e.g., in λ-gt11) for epitopes recognized by an antibody directed against a protein of interest. This is still a reasonable approach in limited cases, where one has a limited number of gene family members but also has antibodies directed against the gene product. Technically, this is similar to a DNA-DNA-based hybridization screen, but one that is using an immunoblot protocol, rather than a Southern-type procedure. A practical application of this technique was used to clone a murine cell cycle regulated gene, that is homologous to both fission yeast and *Caenorhabditis elegans* genes *(14)*.

A more recent approach, made possible by the proliferation of protein microsequencing and mass spectrometry, is to simply attempt to immunoprecipitate a protein, hopefully of similar molecular weight or showing some other property similar to one's protein of interest. Such an immunoprecipitation can be scaled up to yield sufficient quantities for protein characterization and fed back into the degenerate PCR-based identification approach. Both microsequencing *(10)* and mass spectrometry *(13)* have been used successfully for

identification of conserved cell cycle genes. Here, the advantage of the prior protein immunoprecipitation is that degenerate oligonucleotides would be generated against the actual gene that one is attempting to clone, so degeneracy is not as high as when using a consensus sequence across various members of a gene family.

8. Identifying the full-length cDNA: Following any of the above approaches, one may be left with a partial fragment of the cDNA for a gene, so the sequence likely needs to be subcloned and, in some cases, sequenced using standard techniques. Once a sequence has been identified, it can be compared against the prototypical sequence to determine if it is indeed a member of the desired gene family. Finally, one requires the full-length cDNA. Such full-length cDNAs have been used to identify conserved cell cycle proteins via screening a phage cDNA library at high stringency *(5,8)* via a PCR or rapid amplification of complementary DNA ends (RACE)-PCR-based approaches *(9*,*13)*. The genomic region containing a gene can be isolated by similar hybridization methods, using appropriate libraries.

9. Validation: In the end, one is left with the process of validation. The choice of validation method is as varied as the methods of identification described above. Is the ultimately identified gene expressed in a manner similar to the prototype? Does the encoded protein interact with the same partners and localize to the same subcellular area? Does elimination of the homolog lead to a phenotype similar to what was characterized in the original organism? Years of work can go into finally deciding whether the "homolog" one has identified is a true "ortholog." Certainly functional studies are required in the long run to arrive at this conclusion, but many things of interest can be learned en route.

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10

Microarray Approaches for Analysis of Cell Cycle Regulatory Genes

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1. Introduction

Cell cycle phase-specific regulation of transcription is a major mechanism for the regulation of progress through the eukaryotic cell cycle. Hundreds of genes are known to be cell cycle regulated, including histones, cyclins, transcription factors, and genes for such cycle-specific processes as initiation of replication and cytokinesis. Many of the transcription factors involved in cell cycle regulation have been identified (**Table 1**). Many of these transcription factors and their downstream effector genes also play important roles outside of normal cell cycle progression, such as in induction of cell cycle arrest or apoptosis in response to deoxyribonucleic acid (DNA) damage or other cellular stresses. Loss of control of cell cycle-regulated genes is also known to play an important role in disease states such as genomic instability and cancer. For instance, p53, a cell cycle regulatory transcription factor, is also one of the most commonly mutated tumor-suppressor genes in human cancer *(1)*, and hence one of the most exhaustively studied. Estimates based on a survey of p53 binding sites in the genome put the number of p53 regulated genes at several hundred *(2)*, whereas the finding that p53 can affect the expression of some genes in the absence of direct DNA binding may increase this number *(3)*. Genes known to be regulated by p53 are not restricted to roles in cell cycle progression, but are involved in other important cellular processes, such as DNA repair and apoptosis (**Table 2**). As this is an example of just one transcription factor, it is obvious that cell cycle dependent transcription involves many layers of complexity. Comparative analysis of complex patterns of gene expression in both normal and disease states can therefore provide a powerful

Table 1
Representative Transcription Factors Involved in Cell Cycle Regulation

Cell cycle phase	Mammalian	Yeast
G0/G1	AP-1 family (FOS, JUN)	
	MYC	
	MYB	
G1/S	pRB	SBF (Swi4, Swi6)
	E2F-1, E2F-2	MBF (Mbp1, Swi6)
	TP53 (Tumor protein p53)	
G2/M	Forkhead proteins	
	(AFX, FKHR-L1)	Fkh1, Fkh2, Ndd1
M/G1		Mcm1, Ace2, Swi5

Table 2
Examples of Tp53 Effector Genes With Roles in Cellular Stress Response Processes

Cell cycle control	Apoptosis	DNA repair	Other
CIP1/WAF1	*BAX*	*XPC*	*MDM2*
ClnG	*BCL-X*	*DDB2*	*FRA1*
ClnD1	*PAG608*	*GADD45A*	*ATF3*
WIP1	*FAS/APO1*	*PCNA*	*14-3-3σ*
EGF-R	*KILLER/DR5*	*P53A2*	*Rb*
TGF-α	*TRUNDD*		*c-MYC*
Rb	*TRID*		*MMP2*
PCNA	*Seven in absentia*		*MAP4*
GADD45A	*IGF-BP3*		*TSP1 & 2*
14-3-3σ	*PIG1 to PIG14*		*BAI-1*
BTG2			*WIG1*
Seven in absentia			*Amyloid*
IGF-			*GML*
PIG1 to PIG14			*bFGF*
inhibin-β			*PIR121*

tool to develop insight into mechanisms of cell cycle regulation by gene transcription.

Techniques for simultaneously comparing expression levels of thousands of genes, or even the entire expressed genome, have become widely accessible in recent years. Sequencing-based methods of large-scale highly parallel gene-expression studies, including serial analysis of gene expression (SAGE) **(4)**, can be biased toward detection of highly expressed or strongly induced

transcripts. Multigene nylon filter arrays hybridized to radioactive probes provide a useful method of screening for genes with alterations in expression levels, although such differential hybridization screening has its own limitations *(5)*. The use of fluorescent probes labeled with different fluorochromes and cohybridized to the same microarray can circumvent some of these problems.

Microarrays can be constructed either by direct printing of complementary DNAs (cDNAs) on a glass surface *(6)*, or photolithographic synthesis of oligonucleotides in situ *(7)*. Both types of microarray are currently in wide use. Newer methods of array production, involving the use of bubble jet *(8)* and ink jet *(9,10)* printing technology to produce either oligonucleotide or cDNA microarrays, are also under development. Such refinements may increase the flexibility of the technology while making microarrays even more widely accessible.

The protocols in this chapter are for use with cDNA microarrays for fluorescent hybridization, and require access to such arrays, either from a core printing facility, or from a commercial source, and to an appropriate scanning device and data analysis software. The specific methods covered, therefore, focus on preparation and labeling of the ribonucleic acid (RNA) samples to be compared, and hybridization of the labeled samples to the arrays. A general discussion of array preparation is included to provide context, and references to detailed methodology are given to assist readers interested in construction of their own printed arrays. Scanning and data extraction steps are discussed generally, but owing to the wide variety of platforms available for these tasks, specific protocols will depend on the system used. Finally, some examples of informatic approaches that have been applied to analysis of gene-expression data are discussed. Again, this section does not detail specific methods, but is intended to serve as an introduction to the type of data analyses that are being actively developed in tandem with our ability to obtain vast gene-expression data sets.

1.2. Microarray Production

The cDNAs used in the printing of microarrays are generally prepared by polymerase chain reaction (PCR) amplification from purified plasmid DNA. A common strategy used for human and mouse sequences is amplification of cloned expressed sequence tags (ESTs) using primers to the vector sequence adjacent to the cloning site. Selection of ESTs representing the 3' ends of the genes to be screened on the array enhances hybridization when an Oligo dT primer is later used for reverse transcription of the cellular messenger RNA (mRNA) pools to be compared.

The purified PCR products are resuspended in 3X sodium chloride–sodium citrate buffer (SSC) at 100–500 µg/mL and specialized pens moved by a highly

accurate industrial robot are used to deposit several nanoliters of this solution onto poly-L-lysine coated glass microscope slides. The use of a poly-L-lysine coating on the slides increases their hydrophobicity, reduces the spreading out of the printed DNA spots, and allows ultraviolet (UV) cross-linking of the printed DNA to the slide. After the slides have been cross-linked, the poly-L-lysine coating leaves charged amines on the slide surface. These can cause nonspecific electrostatic binding of the labeled cDNA during the hybridization step, resulting in high fluorescent background. The charged amines can be reacted with succinic anhydride in a buffer with a high organic solvent content *(11)* in a chemical passivation step that circumvents this problem. Detailed protocols for the handling and amplification of EST clones and the printing and preparation of slides can be found at (http://www.nhgri.nih.gov/DIR/Micro-array/main.html).

2. Materials

2.1. Preparation of RNA Samples for Microarray Analysis

1. Liquid nitrogen.
2. TRIzol reagent (Life Technologies, Rockville, MD).
3. Chloroform.
4. Dulbecco's phosphate-buffered saline (DPBS).
5. GTC lysis buffer: 4 M guanidine thiocyanate, 10 mg/mL Sarkosyl, 50 mM Tris-HCl (pH 7.5). Immediately before use, add 10 μL β-mercaptoethanol per milliliter lysis solution.
6. 2 M sodium acetate (pH 4.0).
7. Phenol, water-saturated.
8. Chloroform:isoamyl alcohol (24:1 v:v).
9. Ethanol (200-proof, USP ethyl alcohol).
10. RNeasy Maxi Kit (Qiagen, Valencia, CA).
11. Tissue homogenizer.
12. Sonicator.

2.2. Preparation of Labeled cDNA: Direct Incorporation of Fluorochrome

1. Whole cell RNA.
2. Ribonuclease (RNase)-free water.
3. 3.3% v/v 3 M sodium acetate in ethanol.
4. 2.0 μg/μL Oligo dT$_{20}$ (Life Technologies, Rockville, MD).
5. 5X first-strand synthesis buffer for Superscript II (Life Technologies, Rockville, MD).
6. 0.1 M dithiothreitol (DTT).
7. 10X low T NTP mix: 5 mM dGTP, 5 mM dATP, 5 mM dCTP, 2 mM dTTP (Pharmacia).

8. RNasin RNase inhibitor (Promega).
9. Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD).
10. 1 m*M* Cy3-dUTP and 1 m*M* Cy5-dUTP (NEN Life Sciences, Boston, MA).
11. 0.5 *M* ethylenediaminetetraacetic acid (EDTA) (pH 8.0).
12. 1 *N* NaOH.
13. 1 *M* Tris-HCl (pH 7.5).
14. PCR cleanup kit (Qiagen, Valencia, CA).
15. 35% guanidine hydrochloride.
16. SpeedVac.
17. Agarose.
18. Tris-HCl acetate buffer (pH 7.8).
19. PCR thermal cycler.
20. Fluorescence scanner (e.g., Molecular Dynamics Storm).

2.3. Alternate Labeling Protocol: Amino-Allyl Coupling of Fluorochromes

1. Whole cell RNA.
2. 5.0 µg/µL Oligo dT_{20} (Life Technologies, Rockville, MD).
3. RNase-free water.
4. 5X first-strand synthesis buffer for Superscript II (Life Technologies, Rockville, MD).
5. 0.1 *M* DTT.
6. Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD).
7. 40X amino-allyl dNTP mix: 20 m*M* dATP, 20 m*M* dCTP, 20 m*M* dGTP, 12 m*M*. dTTP (100 m*M* stocks from Pharmacia), 8 m*M* amino-allyl-dUTP (Sigma).
8. 0.5 *M* EDTA (pH 8.0).
9. 1 *N* NaOH.
10. 1 *M* Tris-HCl (pH 7.5).
11. TE (pH 7.4).
12. MicroCon 30 (Millipore, Bedford, MA).
13. Monofunctional Cy3 and Cy5 dye (Amersham Pharmacia Biotech, Piscataway, NJ).
14. 0.1 *M* sodium bicarbonate (pH 9.0).
15. 4 *M* hydroxylamine.
16. SpeedVac.
17. PCR thermal cycler.

2.4. Microarray Hybridization

1. cDNA microarrays.
2. Fluorescently labeled cDNA probes.
3. 20X SSC: 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.4.
4. 8 mg/mL poly d(A).
5. 4 mg/mL yeast transfer RNA (tRNA).
6. 10 mg/mL C_0t-1 DNA, human or mouse (Life Technologies, Rockville, MD).

   7. 50X Denhardt's Blocking Solution: 10 mg/mL Ficoll, 10 mg/mL bovine serum albumin (BSA) (Pentax Fraction V), 10 mg/mL polyvinylpyrrolidone.
   8. 10% sodium dodecyl sulfate (SDS).
   9. Microarray hybridization chambers.
  10. Water bath.
  11. Wash buffer: 0.5X SSC, 0.01% SDS.
  12. 0.06X SSC.
  13. Clinical centrifuge.
  14. Fluorescent microarray imaging device and data-extraction software.

3. Methods

3.1. Preparation of RNA Samples for Microarray Analysis

   1. For RNA extraction from tissues, fresh tissue samples should be flash frozen in liquid nitrogen, or immediately homogenized in TRIzol solution (*see* **Note 1**). To a 15-mL polypropylene centrifuge tube containing 4 mL TRIzol, add 100 mg frozen or fresh tissue. Dissociate thoroughly using a rotating-blade tissue homogenizer.
   2. Add 800 µL chloroform and shake vigorously for 15–20 s. Incubate at room temperature (RT) for 3 min.
   3. Centrifuge at 12,000g for 15 min at 4°C. Transfer the aqueous (top) phase into a clean polypropylene tube.
   4. Cells from tissue culture can be prepared similarly up to this point, first washing cells or cell pellets twice with DPBS and adding 1 mL TRIzol per 2×10^7 cells. Alternatively, lysis in GTC and subsequent ϕ extraction can be used. GTC lysates may be stored conveniently at –80°C to collect samples throughout the course of an experiment.
   5. Cells growing in suspension must be pelleted by centrifugation, washed in DPBS, then up to 10^8 lymphoid cells can be resuspended in 5 mL GTC lysis buffer. (Add 10 µL/mL β-mercaptoethanol immediately before use.) Cells growing as monolayers may be rinsed in DPBS, then lysed *in situ*. For larger cells containing greater amounts of cytoplasm, it is advisable to use a higher proportion of GTC lysis buffer: up to 5×10^7 cells per 5 mL lysis buffer.
   6. Tissue culture lysates, whether in GTC or TRIzol, should be disrupted using several 5 to 10 s bursts of sonication to reduce the viscosity of the solution.
   7. To each 5 mL GTC lysate, add 0.5 mL 2 *M* NaOAc. Invert to mix.
   8. Add 1 volume water-saturated phenol. Invert to mix, then add 2 mL chloroform:isoamyl alcohol 24:1. Shake vigorously for at least 20 s, and incubate on ice for at least 20 min. Centrifuge and reserve the aqueous phase as in **step 3** above.
   9. To the recovered aqueous phase, add 0.53 vol of 200-proof ethanol dropwise while vortexing the tube (*see* **Note 2**).
  10. Immediately add this mixture to an RNeasy maxi column and centrifuge in a clinical centrifuge at 3000g for 5 min at RT.
  11. To increase recovery of RNA, pour the flowthrough back over the column and repeat the centrifugation.

12. Discard the flowthrough, wash with 15 mL of RW1 buffer (from Qiagen RNeasy kit) and centrifuge again at 3000*g* for 5 min.
13. Discard the flowthrough, wash with 10 mL of RPE buffer (from Qiagen RNeasy kit), centrifuging for 2 min at 3000*g*.
14. Repeat with a fresh 10 mL of RPE buffer (Qiagen), this time centrifuging for 10 min to dry the column.
15. Place the column in a clean 50-mL tube, and elute RNA with 1 mL Rnase-free water (included in RNeasy kit). Let stand for 1 min, then centrifuge at 3000*g* for 3 min.
16. Repeat the elution in **step 15** with another 1 mL water. Quantitate the RNA and store at –80°C. The ratio of absorbance at 260nm/280nm should be very near to 2.0 (*see* **Note 3**).

3.2. Preparation of Labeled cDNA: Direct Incorporation of Fluorochrome

In this approach, the cDNA to be hybridized to the microarray is synthesized from the RNA isolated in **Subheading 3.1.** Reverse transcription is carried out in the presence of dUTP conjugated to either Cy3 or Cy5 fluorescent dyes (**Fig. 1.**). Despite a relatively low incorporation rate of dUTP containing these bulky fluorescent groups, consistently good results have been obtained using such protocols.

1. Aliquot the RNA to be labeled, usually between 50 and 100 µg per sample. Add two volumes of 3.3% sodium acetate in ethanol. Precipitate on ice for 20 min, then centrifuge at 16,000*g* at 4°C for 20 min, and discard the supernatant (*see* **Note 4**).
2. Wash the RNA pellet twice with 0.5–1 mL 70% ethanol, centrifuging for 5 min before pouring off supernatant.
3. Allow the RNA pellets to dry for approx 20 min, then remove any residual liquid with a pipet tip. Resuspend each pellet in 16 µL Rnase-free water in a 0.2 mL thin-wall PCR tube. Add 1 µL Oligo dT primer (2 µg/µL), and preanneal for 10 min at 65°C in a PCR thermal-cycler block.
4. While the primer is annealing, make a reaction mix containing 8 µL 5X first-strand buffer, 4 µL 0.1 *M* DTT, 4 µL 10X low T NTP mix, 1 µL RNasin (30 U/µL), 2 µL Superscript II enzyme (200 U/µL) per reaction. (It is generally advisable to make enough reaction mix for an extra reaction to ensure sufficient volume for all reactions.)
5. After preannealing, cool the reactions on ice for 2 min. Then add 4 µL either Cy5-dUTP or Cy3-dUTP, and 19 µL of the reaction mix from **step 4** to each tube. Mix well, and incubate at 42°C (*see* **Note 5**).
6. After 30 min, add another 2 µL Superscript II, mix well, and continue incubating the reaction at 42°C for an additional 30 to 60 min.
7. Remove from the heat block and stop the reaction by adding 5 µL of 0.5 *M* EDTA.
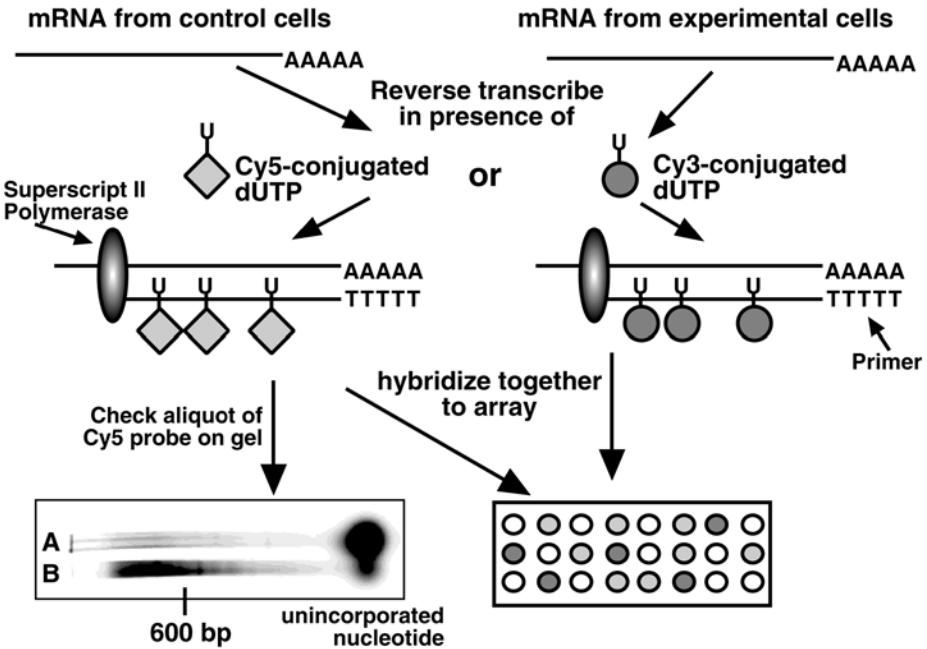
Fig. 1. Overview of direct approach for making fluorescent-labeled cDNA for microarray hybridization (Methods, *see* **Subheading 3.2.**). A single round of reverse transcription is used to make cDNA from mRNA samples to be compared. Cy5 (green diamonds) or Cy3 (red circles) conjugated to dUTP will be incorporated by Superscript reverse transcriptase. The probes are then hybridized together to the microarray, washed and scanned using a confocal laser scanning microscope. Transcripts present in greater proportion in the control sample, here labeled in green, will produce a green spot on the final microarray image, but transcripts increased in the experimental sample, here labeled in red, will yield a red spot. Equal representation in both original mRNA pools results in a yellow spot. If a sample of the labeled probe is run on an agarose gel (*see* **Subheading 3.2.**), incorporated Cy5 can be easily detected as in the example in the lower left of this figure. Lane A is the result of poor incorporation, with most of the Cy5 being present as unincorporated nucleotide, whereas lane B shows good incorporation and a strong signal in the region of high molecular weight transcripts.

8. To hydrolyze the remaining RNA, add 10 μL 1N NaOH and incubate at 65°C for 30–60 min, then cool to RT (*see* **Note 6**).
9. Add 25 μL 1 M Tris-HCl (pH 7.5) to neutralize the solution.
10. Use the Qiagen PCR product cleanup kit to clean up the reactions and remove any unincorporated fluorochromes. Mix each labeling reaction with 400 μL Qiagen binding buffer PB, apply to Qiagen spin column, and microfuge 1 min on maximum speed.

11. Discard flowthrough. To more efficiently remove unincorporated fluorochromes, wash the columns with 750 µL 35% guanidine hydrochloride. Microfuge for 1 min at maximum speed.

12. Discard the flowthrough and wash the column again with 750 µL Qiagen wash buffer PE (with ethanol added). Microfuge for 1 min at maximum speed.

13. Discard the flowthrough, and microfuge one more minute to remove any traces of ethanol.

14. Place each column in a clean 1.5-mL tube. Add 30 µL Qiagen elution buffer EB, incubate for 1 min, then microfuge 1 min at maximum speed. Repeat elution with a second 30 µL buffer EB. The probe will then have to be dried down in a SpeedVac to achieve the final volume for hybridization. Alternatively, the initial flowthrough can be used for the second elution to reduce the final volume and minimize drying time. This may also result in some loss of label, however.

15. Incorporation of Cy5 may be checked by running a 2–3 µL aliquot of the Cy5-labeled cDNA on a 2% agarose (TAE) gel without ethidium bromide. Scan the gel on a Molecular Dynamics Storm fluorescence scanner set to detect red fluorescence, 200 micron resolution and 1000 volts on the photomultiplier tube (PMT). Successful labeling should yield a dense smear of probe in the range of 400 to 1000 bp. An excess of low molecular weight transcripts and unincorporated nucleotides indicates weak labeling (*see* **Fig. 1**).

3.3. Alternate Labeling Protocol: Amino-Allyl Coupling of Fluorochromes

Incorporation of an amino-allyl modified dUTP in the cDNA synthesis step, with subsequent coupling of monofunctional Cy3 or Cy5 dyes may circumvent problems associated with unequal incorporation rates of dUTP coupled to the two different fluorochromes (**Fig. 2**).

1. Prepare RNA samples for labeling as in **steps 1–3** of the preceding protocol (*see* **Subheading 3.2.**), but resuspend in 14.5 µL RNase-free water, then transfer to a 0.2-mL thin-wall PCR tube. Add 1 µL of Oligo dT primer (5 µg/µL). Incubate for 10 min at 70°C to preanneal, then put on ice for 10 min.

2. Prepare a reaction mix containing 6 µL 5X first-strand buffer, 1 µL 40X amino-allyl NTP mix, 3 µL 0.1 *M* DTT, 3 µL water, and 2 µL Superscript II per reaction.

3. Add 15 µL of reaction mix from **step 2** to each reaction. Mix and incubate at 42°C for 2 h. A second aliquot of Superscript II enzyme may be added midway through the reaction.

4. Stop the reaction with 10 µL 0.5 *M* EDTA. Add 10 µL 1*N* NaOH, mix well, and hydrolyze remaining RNA for 20–30 min at 65°C.

5. Cool to RT and add 25 µL 1 *M* Tris-HCl (pH 7.5) to neutralize.

6. Add each reaction to a MicroCon 30 cartridge containing 450 µL water. Centrifuge at 16,000*g* for 6–8 min. Wash two more times with 450 µL water, followed by centrifugation, and collect RNA in a clean tube by centrifuging for 3 min at 500*g*. The target volume at this step is 4.5 µL. The samples can be further concentrated using a SpeedVac if necessary.
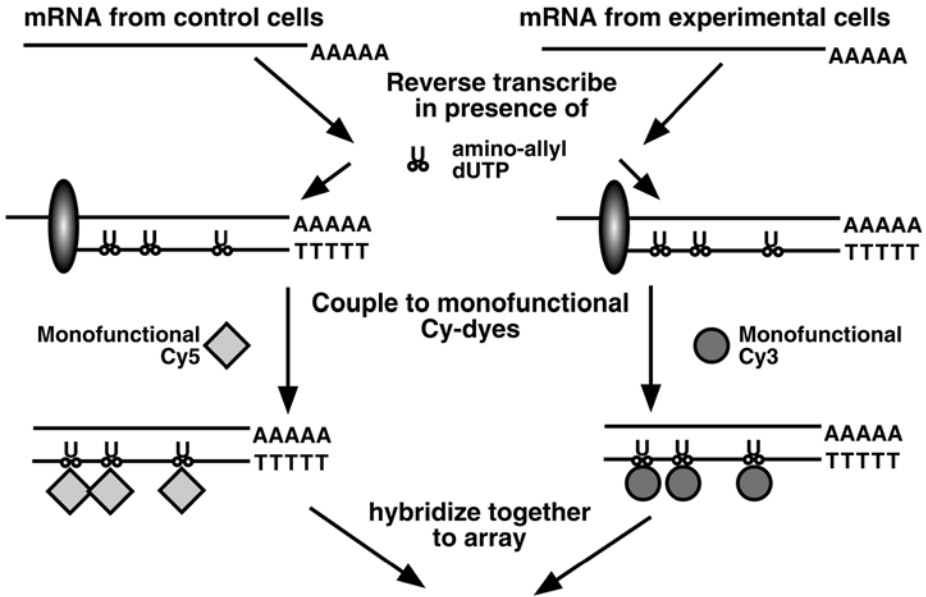
Fig. 2. Overview of alternate labeling by amino-allyl coupling of fluorochromes (*see* **Subheading 3.3.**). In this case, the reverse transcription reaction is carried out in the presence of dUTP modified only with an amino-allyl site. This creates much less steric hindrance than the presence of the bulky cyanine dyes during this step. Chemical coupling is then used to label the amino-allyl sites within newly synthesized cDNA with appropriate reactive cyanine fluorescent molecule. Hybridization and scanning are then carried out as with standard labeling technique.

7. Resuspend 1 dye vial each of monofunctional Cy3 and Cy5 in 72 μL RNase-free water. Use a SpeedVac to dry down individual aliquots of 4.5 μL. Store these at 4°C until needed.
8. Prepare a fresh aliquot of monofunctional Cy3 and Cy5 for each reaction by resuspending in 4.5 μL 0.1 *M* sodium bicarbonate (pH 9.0). Mix the appropriate fluorochrome with each RNA sample, and incubate at RT for 1 h in the dark.
9. Add 4.5 μL 4 *M* hydroxylamine to each reaction. Incubate for 15 min at RT in the dark.
10. Pool the Cy3 and Cy5 reactions for each set of samples being compared and clean on a MicroCon 30 cartridge as in **step 6** above. The total volume should be concentrated to around 30–34 μL. Incorporation of Cy5 may be checked by running 4–5 μL on a gel as described in **Subheading 3.2.**, **step 15**.

3.4. Microarray Hybridization

Blocking conditions may need to be individually adjusted, as arrays and samples may vary somewhat. The conditions given in **steps 1–9** below for a

40-µL hybridization may be used as a starting point for hybridization under a 24 × 50 mm coverslip. If a smaller area is to be covered by the hybridization, the volume should be adjusted proportionately.

1. Combine the two labeled samples to be compared in a thin-wall PCR tube in a total volume of 30 µL. To this, add 6 µL 20X SSC, 1 µL poly d(A) (8 mg/mL), 1 µL yeast tRNA (4 mg/mL), 1 µL human C_0t-1 DNA (10 mg/mL) (mouse C_0t-1 DNA should be used for blocking of mouse arrays), and 1 µL 50X Denhardt's blocking solution. Mix well and incubate at 98°C for 2 min.
2. Cool rapidly to 25°C, then add 0.6 µL 10% SDS. Mix well and centrifuge for 5 min at 14,000*g* (*see* **Note 7**).
3. Apply the hybridization mixture to a glass coverslip. Apply the inverted microarray and allow surface tension to draw the coverslip gently onto the array. A very delicate touch is required in this step to prevent the formation of bubbles under the coverslip.
4. Transfer the slide to a microarray hybridization chamber. Add 20–40 µL 3X SSC to the chamber to prevent drying of the hybridization solution (**Fig. 3A**), seal the chamber and submerge in a 65°C water bath for 16–20 h.
5. Remove the chamber from the water bath and dry carefully, especially around the seals, before unsealing it and removing the slide. Leaking of the chamber, or introducing water when unsealing the chamber, can spoil the hybridization (**Fig. 3B**).
6. Place the slide in a jar filled with 0.5X SSC/0.01% SDS wash buffer. Allow the coverslip to float off, remove it from the jar, and allow the slide to wash for 3–5 min.
7. Transfer the slide to fresh wash buffer for another 3–5 min.
8. Wash the slide in 0.06X SSC for 3–5 min. Immediately spin dry by centrifuging in a slide rack in a clinical centrifuge for 3 min at 200*g* (*see* **Note 8**).
9. Acquire a fluorescent image of the microarray (**Fig. 3C**) and extract the signal data following the recommendations of the manufacturer of the microarray imaging device and data-extraction software used.

3.5. Data Analysis and Examples of Informatic Approaches

Although the specific protocols involved in adjusting a microarray scanning device to obtain the best possible image differ widely depending on the manufacturer, the basic principles are more or less the same. Whatever the device used, the scan should ideally make use of as much of the scanner's linear detection range as possible, and both signals should be scaled to occupy close to the same range. The laser power and voltage of the photomultiplier detectors can generally be adjusted to achieve this goal. Once a satisfactory image has been captured, it can be loaded into a variety of data-extraction software packages for analysis. Custom analysis tools are available and have different algorithms for identifying the signals within the image, subtracting background fluorescence levels, and normalizing the ratios of the signals in the two chan-
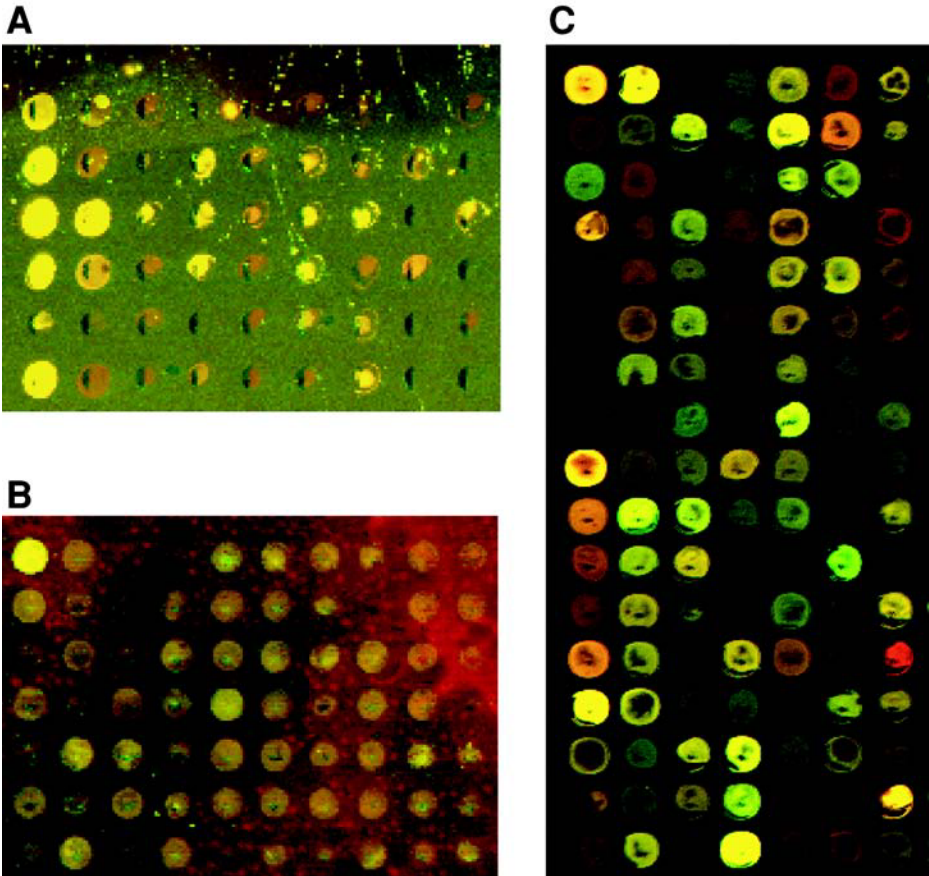
Fig. 3. The good, the bad, the ugly: Examples of microarray hybridization out-
comes. **(A)** Typical example of microarray dehydrating during hybridization or subse-
quent processing. Note high degree of overall fluorescent haze and poor signal quality.
(B) Example of chamber leakage during hybridization. This tends to degrade portions
of the image, creating variable signal quality and areas of high local background.
(C) Hybridization showing uniformly low background and good signal quality. Some
features will show low or absent signal with even the best hybridization, as not all
genes are expressed in all samples. This should not be considered a sign of failure.

nels to compensate for any labeling or detection bias in an individual experi-
ment. One straightforward method of testing the performance of array hybridiza-
tions is to compare samples from the same source in the two channels.
A scatterplot of the signal intensities in the two channels will give an idea of
the amount of error in the system when measuring identical signals. Similarly,
repeating hybridization of two different samples after switching the fluoro-

chrome with which each is labeled and then comparing the resulting ratios will give an idea of the accuracy of detection of different expression levels.

It is also advisable to verify a selection of the results obtained by microarray analysis. Quantitative hybridization *(12)* or real-time PCR are the preferable methods, but a general confirmation of relative differences between two samples may also be obtained from Northern blot analysis. It is important for such verification purposes to use the same EST sequence that served as the detector of interest on the array and to test the same RNA samples that were used for the array. Such testing should be applied to ESTs showing a broad range of signal intensities and relative expression ratios.

The extremely complex nature of the large data sets obtained from microarray experiments demands advanced approaches for meaningful analysis. Numerous groups are devising relational databases and search tools specifically tailored for the management of microarray expression data, and many are already available commercially. One of the most widely used tools for organizing, sorting, and visualizing microarray data is cluster analysis. This includes a number of approaches for arranging expression patterns so that genes behaving most similarly in a series of experiments are ordered closest together. Clustering algorithms applied successfully to gene-expression data include agglomerative hierarchical clustering *(13)* and divisive clustering, with two-dimensional sorting, to order both genes and experiments *(14)*. Self-organizing maps and k-means clustering have also been applied to microarray data. For a recent review and discussion of these approaches see Alizadeh et al. *(15)*.

Such cluster analysis of microarray gene-expression patterns has already been used to reveal signatures indicative of different stages of immune cell function and physiology *(15)* and to identify subclassifications of diffuse large B-cell lymphoma correlating with patient survival *(15)*. A similar approach with clustering of expression data from melanomas has revealed a set of genes correlating with aggressively metastatic tumors *(16)*. Such use of expression profiling to define molecular phenotypes of cancer correlating with clinical outcome has great potential for improvements in therapy. Distinct expression profiles have also been defined recently in human breast cancers with mutation in either the *BRCA1* or *BRCA2* tumor-suppressor gene *(17)*. Not only does this allow more precise classification of nonhereditary breast cancers, but the availability of information on differential regulation of a large number of genes on inactivation of these tumor suppressors may provide insight into their mechanisms of action.

Given the great amount of cell cycle research accomplished in yeast in the pregenomic era, and the availability of the entire yeast genome, surveys of cell cycle responsive genes in yeast were among the earliest applications of microarray research. One such study, using temperature-sensitive mutants of

cdc15 or *cdc28* to arrest *Saccharomyces cerevisiae* in G2 or G1, respectively, identified 416 transcripts with periodic fluctuations consistent with cell cycle progression *(18)*. Of these genes, over 60% had previously been identified with cell cycle-specific functions. In another study, yeast synchronized by three independent methods (α-factor arrest, elutriation, and a *cdc15* temperature-sensitive mutant) yielded 800 genes consistent with cell cycle regulation *(19)*. Analysis of this data leads to identification of common promoter elements that may predict cell cycle regulation.

Building on such studies, more recent work has focused on the regulation of cell cycle-specific genes by specific transcription factors. For instance, chromatin immunoprecipitation was used in conjunction with microarray analysis to identify about 200 genes that bound the G1/S transcription factors SBF and MBF in their upstream promoter regions *(20)*. Interestingly, a division of function was noted in this study, in that genes activated by SBF functioned in budding and formation of cell walls and membranes, whereas genes activated by MBF mostly had roles in DNA replication and repair. A similar approach applied to all nine known yeast cell cycle regulatory-transcription-factor subunits has revealed that the transcription factors active during one stage of the cell cycle activate the transcription factors that will regulate the next phase, creating an autoregulatory circuit *(21)*. Identifying the subsets of genes activated by each of the transcription factors will help shape understanding of the transcriptional and posttranscriptional networks that regulate the cell cycle.

Although the characterization of the human genome has lagged behind that of yeast, surveys of cell cycle-regulated genes have also been undertaken in human cell lines. For instance, microarray analysis of a cell line expressing inducible E2F1 revealed 470 genes showing regulation by this cell cycle regulatory transcription factor *(22)*. In another study using human fibroblasts synchronized by double thymidine block, about 700 genes were identified as cell cycle regulated *(23)*. Such studies can form a framework for understanding human cell cycle progression at the transcriptional level and for studying relationships between the mammalian and yeast cell cycles. Some caution is, however, warranted. In a statistical reanalysis of the human fibroblast experiment mentioned in this section, no significant difference was found between the identified cyclic genes and random fluctuations *(24)*. This is in contrast to the experiments with synchronized yeast cells, where the same analysis did indicate a large excess of cyclically regulated genes over that predicted by chance. As the availability of large gene-expression data sets increases and informatic tools are refined, refinements of studies such as these are likely to have a profound impact on the study of cell cycle regulation.

4. Notes

1. Any delay in the initial lysis of samples can result in gene-expression changes not associated with the parameters under study, thus adding large amounts of noise to the final data. Extremely high-quality RNA is a prime requirement for successful microarray analysis. Impurities in the RNA can inhibit fluorochrome incorporation in subsequent steps, and cellular contaminants, such as carbohydrates, may produce high background fluorescence on the hybridized arrays. Experience has shown that including precipitation steps at an early stage of extraction tends to exacerbate these problems. Degradation of the RNA will obviously also lead to decreased hybridization specificity, and all standard RNA handling procedures should be observed.

2. The slow addition of ethanol at this step is necessary to prevent precipitation of the RNA at areas of high local-ethanol concentration. Such local precipitation can reduce RNA yields.

3. Although microarray analysis is extremely sensitive to many factors and can often be difficult to troubleshoot, poor quality RNA is a major cause of poor results. Even RNA with a good A$260/280$ ratio, which appears intact by Northern analysis, can label poorly, and in cases of poor hybridization results, starting over with a fresh preparation of RNA is often advisable. Care should be taken not to exceed the binding capacity of the columns. For some samples, improved results can be obtained by doing a second round of TRIzol extraction before proceeding to the Qiagen columns, or by adding a DNase digestion step.

4. In contrast to earlier steps in RNA isolation, precipitation does not seem to be harmful at this stage, as carbohydrates and other contaminants that can pose problems at earlier stages in the isolation should no longer be present.

5. Although relatively stable, the cyanine dyes used in this protocol are photosensitive, and once they have been added to the reactions, it is advisable to protect them from light exposure as much as possible. Care should also be taken in the storage of these dyes to avoid photo bleaching, especially if they are not used very rapidly.

6. This is a relatively sensitive step, and care should be taken to avoid any impurities in the NaOH used. Make fresh NaOH if there is any sign of discoloration, and avoid storage in glass. If a color change is visible in the Cy5 reactions when the NaOH is added, it will likely result in a weak signal, and fresh NaOH should be made. Hydrolyzing for excessive time can result in a weakened signal. Insufficient hydrolysis, however, will also produce a weak signal owing to hybridization with unlabeled cDNA when this is not removed from the reaction.

7. Excessive cooling at this step will cause the SDS to precipitate when it is added, contributing to hybridization background. Occasionally, when the reactions have been microfuged, some debris will be visible at the bottom of the tube. Care should be taken to avoid pipetting this onto the array.

8. If a rotor for microtiter plates is not available, good results can also be obtained by centrifuging individual slides in uncapped 50-mL polypropylene tubes in a standard rotor, or by washing for 2 min in 100% isopropanol and then air-drying.

If the slides are allowed to air-dry slowly, this frequently will contribute to high background.

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11

Using the Yeast Genome-Wide Gene-Deletion Collection for Systematic Genetic Screens

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1. Introduction

The generation and analysis of mutants has had an essential role in the identification of genes involved in cell cycle control *(1–3)*. In this regard, the genetic analysis of mutations has helped reveal the normal function of wild-type gene products as well as provided powerful insights into the intricate relationships between large numbers of gene products. The use of genetic screens and straightforward selection techniques in simple eukaryotes, like the yeast *Saccharomyces cerevisiae*, has greatly facilitated the ability to study and understand complex processes like cell cycle regulation *(1–3)*.

S. cerevisiae is one of the most powerful and popular genetic model systems *(4–12)*. It is a free-living yeast that can reproduce sexually to produce spores or divide asexually by budding off daughter cells *(4–6,10)*. Yeast is inexpensive and simple to propagate *(4–6,10)*. Moreover, facile genetics and a multitude of excellent genetic, molecular, and biochemical tools have allowed yeast to emerge as a driving force in the development of functional genomic methodologies *(7,8,13,14)*.

The genome of the yeast *S. cerevisiae* is very gene dense and relatively small. At approx 12-Mb, the yeast genome is less than four times larger than the *Escherichia coli* genome and is >200 times smaller than the human genome *(6,8,14)*. The yeast genome is encompassed by 16 linear chromosomes *(6)*. On average, one yeast open reading frame (ORF) is found every 2 kb *(8)*. In total, the yeast genome is predicted to encode approx 6200 genes *(15)*. In contrast to higher eukaryotes, only a very small number (approx 4%) of genes have introns (*[8]* or see [http://www.cse.ucsc.edu/research/

compbio/yeast_introns.html]). Because yeast can be propagated as haploids, recessive mutations can be easily recovered and analyzed. The powerful genetics available in yeast allows for epistatic, complementation, and suppressor analysis of mutations (*see* **Note 1**). These characteristics have helped yeast usher in the era of functional genomics reviewed previously *(8,13,14)*.

In 1996, the yeast genome became the first eukaryotic genome to be completely sequenced *(15)*. Then, several years later in a remarkable tour de force, an international consortium assembled a nearly complete collection of single-gene deletions *(16,17)*. Today, these collections contain approx 6000 single gene deletions that encompass approx 97% of the genome. More important, they can be purchased commercially at a reasonable cost (*see* **Note 2**). The diploid collection contains approx 4800 homozygous diploids and approx 1200 heterozygous diploids (*see* **Note 3**). In addition, haploid collections can also be obtained. Another excellent resource is TRIPLES, a database of **TR**ansposon-**I**nsertion **P**henotypes, **L**ocalization, and **E**xpression in *Saccharomyces*. Deletion strains and data can be obtained from TRIPLES at (http://ygac.med.yale.edu/triples/triples.htm). These deletion collections are incredibly powerful genetic tools that are likely to have a large number of applications in the next few years. Five general and conceptual applications are detailed below. These methods can be used to specifically study cell cycle processes or, with ingenuity and imagination, these techniques can be easily applied to study almost any simple or complex process. The observation that nearly 50% of human disease genes have yeast homologues demonstrates the medical and biological relevance of using yeast genetics to study gene function *(18–20)*.

2. Materials

1. Media: *See* Chapter 6 for a general listing of basic yeast media.
2. Deletion Parental Strains:
   BY4741 *MATa his3Δ leu2Δ met15Δ ura3Δ*
   BY4742 *MATα his3Δ leu2Δ lys2Δ ura3Δ*
   BY4743 *MATa/MATα his3Δ/his3Δ leu2Δ/leu2Δ met15Δ/MET15 lys2Δ/LYS2 ura3Δ/ura3Δ*
3. Equipment: Access to the following standard yeast culture equipment, supplies, and knowledge of general yeast manipulation is a presumed prerequisite *(4–6,10)*: variable temperature incubators, shaking water baths, standard culture flasks, tubes, glassware, midspeed centrifuge, spectrophotometer, fluorometer, centrifuge tubes or bottles, filter apparatus, and a standard microscope. Although not required, access to a Z2 Coulter Counter/Channelyzer (for monitoring cell size and cell number) and a benchtop flow cytometer is highly recommended.

3. Methods

The following section briefly details four methods for using the entire yeast gene collection to conduct basic genetic studies. These methods represent examples of how the deletion collection is yielding new technologies for the genetic dissection of basic cell processes (e.g., the cell cycle) *(7,8,14)*. References are provided for basic and common techniques essential for each method discussed.

3.1. Deletion Collection As a Source of "Your Favorite Gene (Yfg) Knockouts"

Currently, the yeast-deletion collection is only available in a single genetic background *(16,17)*. Nonetheless, this collection can be used as a source of approx 6000 individual deletions. Thus, for researchers who are interested in a particular gene or subset of genes (e.g., cyclins), there is a high degree of likelihood that their deletions of interest are in this collection. In this manner, the deletion collection can be used as a source for transferring or introducing these deletions into different backgrounds or in creating combinations of deletion strains. A basic polymerase chain reaction (PCR)-based cloning and transformation protocol is detailed.

3.1.1. PCR-Based Cloning and Transformation of yfg Deletion

This method briefly describes how to PCR amplify and clone *yfg* deletion with 1 kb of upstream and downstream flanking sequence. This creates a renewable plasmid-based source of *yfg* deletion allowing investigators to easily transfer this deletion to any strain of choice.

1. Use Web-based search engines to identify the strain containing yfg deletion (*see* **Note 4**).
2. Download the deoxyribonucleic acid (DNA) sequence of *yfg* with 1 kb of upstream and downstream flanking sequence from the Stanford Genome Database (*see* **Note 5**), and load the sequence into the preferred bioinformatics software program (*see* **Note 6**).
3. The goal is to clone an amplified PCR product with the intent of using the clone as a source of DNA for yeast transformations. Therefore, it is essential to identify the plasmid vector and examine its sequence before designing primers for PCR amplification (*see* **Note 7**). Using the sequence of the vector and *yfg*, design primers such that a cloned *yfg*-deletion product can be cut from the plasmid vector with a simple one-(or two)-enzyme restriction digestion (*see* **Note 8**).
4. Design PCR primers approx 1 kb upstream and downstream of *yfg* (*see* **Note 9** and **Fig. 1**). Primers are 18–20 bp in length and are designed with a C or a G in the final two 3' base positions (*see* **Note 10**).
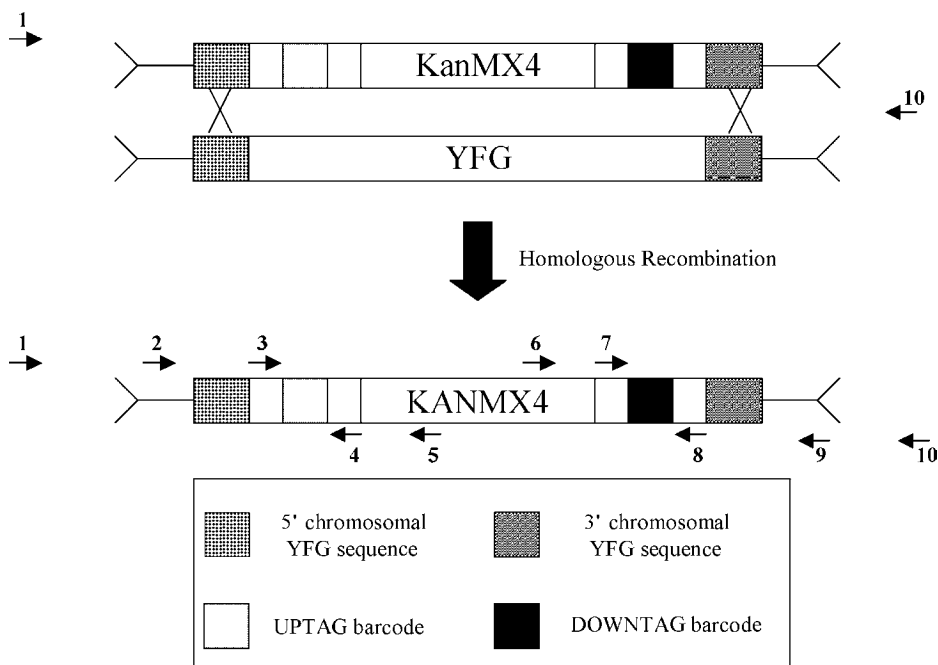
Fig. 1. This figure illustrates the basic strategy undertaken to systematically delete every yeast ORF. Oligonucleotides mentioned in text are represented as arrows (*see also* **Notes 9**, **16**, **28**, **31**, and **33**).

5. A number of approaches can be used to obtain template DNA for PCR amplification. The simplest approach is to use a small amount of a yeast colony from a strain containing *yfg* deletion (*see* **Note 11**). Alternatively, for more reproducible results, genomic DNA can be isolated from liquid cultures (*see* **Note 11**).

6. Remove 10 µL of each PCR reaction and resolve with ethidium bromide agarose gel electrophoresis to ensure the amplification of a single discrete band of the appropriate size.

7. Clone amplified product using a kit commercially available for the cloning of PCR fragments (*see* **Note 12**). After having created and identified the correct clone (*see* **Note 13**), prepare a glycerol stock for long-term storage (*see* **Note 14**).

8. Use *yfg*-deletion clone in a restriction-enzyme digestion as a source of DNA for yeast transformation (*see* **Note 13**).

9. Transform yeast with DNA from a *yfg*-deletion clone restriction-enzyme digestion (*see* **Note 15**). Select for *yfg* deletion on a yeast extract-peptone-dextrose (YPD) plus kanamycin plate, and confirm that the deletion is correct (*see* **Fig. 1** and **Note 16**).

3.2. Classical Genetic Screens Using the Entire Deletion Collection

Classical genetics relies on the isolation of mutations to identify and elucidate the function of wild-type gene products. The first step in the design of a genetic screen is the identification of the process of interest (e.g., cell cycle control). The next step is to choose a mutagenesis scheme followed by a selection scheme (*see* **Note 17**). Traditionally, genetic screens have been used to identify genetic alterations that convey either phenotypic advantages or disadvantages to the parental strain. In this manner, it is possible to select for (gain of function) or against (loss of function) a given trait. This type of genetic screening is called suppressor analysis *(7,21)*. As an example, suppressor analyses have been used to identify gain and loss of function genetic alterations that restore viability to yeast strains lacking G1-phase cyclins *(22–24)*. Traditionally, suppressors have been identified with two basic techniques: mutagenesis or transformation with high-copy plasmid libraries (*see* **Note 18**). Mutagenesis of yeast strains has proved to be a highly productive method of identifying both gain and loss of function genetic alterations. However, traditional techniques have several disadvantages. First, although the initial identification of a genetic suppressor is simple and straightforward, identification and cloning of the mutant can be difficult (*see* **Note 19**). Second, the completeness of these screens is always somewhat in doubt (*see* **Note 20**). The use of pooled yeast-deletion strains greatly simplifies loss of function suppressor screens by largely alleviating both of these disadvantages *(25,26)*. Using pooled-deletion strains, the completeness of the screen is known a priori and it is straightforward to identify the mutant responsible for the selected phenotype (*see* **Notes 21** and **22**). Several examples of classical genetic suppressor screens using pooled yeast-deletion strains are provided.

3.2.1. Using Deletion Collections
in Systematic Quantitative Phenotypic Screens

The genome-wide deletion collection is an extremely useful genetic tool for assessing the phenotypic effect of each and every gene deletion. In the simplest sense, these types of analyses can be used to determine if a specific gene is required for a given phenotype (e.g., the ability to metabolize galactose). The main requirement for these types of experiments is a defined end point. Because gene deletions most frequently correlate with the loss of a biochemical function, it is often necessary to design replica-plating schemes to screen for mutants. The example provided in the list below describes a scheme to identify mutants that are unable to metabolize galactose. However, virtually any phenotype could be used as an end point. The entire yeast-deletion collection can be obtained from a number of commercial vendors (*see* **Note 2**). Both pooled

and individual deletion-strain collections can be obtained. The method below describes how pooled-deletion strains can be used in a systematic phenotypic screen.

1. Obtain deletion collections (*see* **Note 2**).
2. If deletion collections are not already pooled, combine deletion collections as follows: Plate 3–5 µL of each deletion from a single 96-well plate onto a 150-mm YPD plus kanamycin plate. Incubate plates for 3 d at 30°C. Using sterile technique, wash the colonies from the plate with 3–5 mL of YPD plus 15% glycerol (*see* **Note 23**). Freeze 1-mL subpool aliquots at –80°C. Genome-wide pools are obtained by combining 1-mL subpool aliquots from each deletion plate. Genome-wide pools should also be frozen at –80°C in 1-mL aliquots.
3. To begin the screen, thaw frozen aliquots and add 100–500 µL of the pooled aliquots to 50 mL YPD. Grow pooled dilutions in a 250–500-mL flask. Incubate flasks in a shaking incubator at 30°C until culture reaches midlog phase (1–4×10^7 cells per milliliter).
4. Use a spectrophotometer or Coulter counter to determine the cell density (*see* **Note 24**).
5. Dilute cells in sterile H_2O to a concentration of 2–4×10^3 cells per milliliter in sterile H_2O. Plate 100 µL of the diluted culture to permissive conditions, that is, to YPD plates (*see* **Note 25**).
6. Incubate plates 2–3 d at 30°C.
7. Using velvets and a replica-plating tool, replica plate colonies to the nonpermissive condition, that is, yeast extract peptone (YEP) galactose plates (*see* **Note 26**).
8. Incubate both the new and original plates 2–3 d at 30°C.
9. By comparing the permissive plates to the nonpermissive plates, determine if any colonies that grew under permissive conditions failed to grow under nonpermissive conditions. Select these colonies and repeat the selection conditions to ensure that the chosen mutant is unable to proliferate under nonpermissive conditions (*see* **Note 27**).
10. Confirm that a single deletion is responsible for the observed mutant phenotype (*see* **Notes 28** and **29**).

3.2.2. Microarray Analysis As an End Point for Quantitative Phenotypic Analysis: Functional Profiling

The preceding section detailed a protocol for screening the genome-wide deletion collection for the identification of a specific phenotype (e.g., the inability to metabolize galactose). Recent studies indicate that the majority of yeast mutants have milder phenotypes that affect fitness less severely *(16,17,27)*. A number of excellent manuscripts have taken advantage of these observations and used microarray analyses to quantitatively assess the fitness of each deletion mutant under a large spectrum of environmental conditions *(16,17,27)*.

This technique is referred to as *functional profiling (17)*. To facilitate functional profiling, each of the yeast-deletion strains created was marked with one or two unique sequences (uptag or downtag) known as bar codes (**Fig. 1**) *(17)*. PCR-amplified bar codes from single colonies can be used for the identification of individual deletion strains (*see* **Fig. 1** and **Note 28**). In addition, because each yeast deletion was designed identically (with the exception of the unique bar codes), PCR can be used to amplify bar codes from pooled-deletion strains and hybridized to bar-code-specific microarray chips (*see* **Fig. 1** and **Note 30**) *(17)*. In this manner, it is possible to simultaneously determine how every deletion in a collection responds to a given condition (e.g., the fitness of individual strains can be determined after a drug treatment). Microarray analysis of yeast deletions has been used to (1) identify genes acting in networks or pathways, (2) assess the fitness of a given gene deletion in a mixed population, (3) assign function to unknown genes, and (4) identify drug targets *(8,16,17,28–30)*. The basic protocol for using the genome-wide deletion collection and microarray analysis as an end point for quantitative phenotypic analysis is given in the next paragraph.

The following protocol uses the example of comparing growth on rich glucose (YPD) to growth on galactose (YEP galactose). Previous studies have indicated that this type of protocol can detect quantitative fitness differences after 15–18 doublings *(16,17,27)*. However, better and more significant results are achieved with more population doublings (*see* **Note 31**). The protocol listed below is an abridged modification of methods reported elsewhere (*see* **Note 30**) *(16,17,27)*.

1. Obtain deletion collections (*see* **Note 2**).
2. If deletion collections are not already pooled, combine deletion collections as follows: Plate 3–5 µL of each deletion from a single 96-well plate onto a 150-mm YPD plus kanamycin plate. Incubate plates for 3 d at 30°C. Using sterile technique, wash the colonies from the plate with 3–5 mL of YPD plus 15% glycerol (*see* **Note 23**). Freeze 1-mL subpool aliquots at –80°C. Genome-wide pools are obtained by combining 1-mL subpool aliquots from each deletion plate. Genome-wide pools should also be frozen at –80°C in 1-mL aliquots.
3. To begin the screen, thaw frozen aliquots and add 100–500 µL of the pooled aliquots to 100 mL YPD and another 100–500 µL to 100 mL YEP galactose. Grow pooled dilutions in a 500–1000-mL flask.
4. Prior to incubating at 30°C, use a spectrophotometer or Coulter counter to determine the cell density (*see* **Note 24**).
5. Incubate flasks in a shaking incubator at 30°C until culture reaches midlog phase ($1–4 \times 10^7$ cells per milliliter). Use a spectrophotometer or Coulter counter to determine the cell density and determine the number of population doublings that each culture has undergone. Harvest $5–10 \times 10^7$ cells from each culture for an initial time-point.

6. Remove $0.5–1 \times 10^6$ cells and dilute into 500 mL of fresh prewarmed YPD medium. Repeat with YEP galactose medium.

7. Repeats **steps 5** and **6** until a minimum of 20 population doublings have been achieved (*see* **Note 31**). Harvest $5–10 \times 10^7$ cells from each culture for time-points that evenly span the number of population doublings achieved.

8. Isolate genomic DNA from harvested cells (*see* **Note 32**).

9. To generate probes for microarray hybridizations, uptag and downtag bar codes are amplified separately from genomic DNA samples (*see* **Note 33**).

10. Amplified PCR products are combined in 200 µL of hybridization buffer (*see* **Note 34**). Probes are heated to 100°C for several minutes and cooled on ice before hybridizing on high-density oligonucleotide microarrays (Affymetrix tag3 arrays) at 42°C for 1 h.

11. Arrays should be washed 4–6 times with hybridization buffer plus 0.005% Triton X-100 and then stained at 42°C for 10–15 min with hybridization buffer containing 2 µg/mL phycoerythrin-streptavidin (Molecular Probes) and 1 µg/mL bovine serum albumin (BSA).

12. Arrays should be scanned and analyzed as recommended by the manufacturer (Affymetrix) (*see* **Note 35**).

3.2.3. Using Deletion Collections
to Identify Suppressors of Dominant Negative Alleles

Dominant negative alleles are mutants that interfere with the function of the coexpressed normal gene. In many cases, the expression of dominant negative alleles yields a phenotype that can be selected for or selected against. For example, alleles of the cell cycle control gene *sic1*, which lack a number of consensus *cdc28* phosphorylation sites, act as dominant negative mutants that prevent cell division (*see* **Note 36**) *(31)*. As opposed to the genetic screen detailed in **Subheading 3.2.1.**, the following protocol details a genetic selection scheme whereby only the desired mutants survive the restrictive conditions.

1. Obtain deletion collections (*see* **Note 2**).

2. If deletion collections are not already pooled, combine deletion collections as follows: Plate 3–5 µL of each deletion from a single 96-well plate onto a 150-mm YPD plus kanamycin plate. Incubate plates for 3 d at 30°C. Using sterile technique, wash the colonies from the plate with 3–5 mL of YPD plus 15% glycerol (*see* **Note 23**). Freeze 1-mL subpool aliquots at –80°C. Genome-wide pools are obtained by combining 1-mL subpool aliquots from each deletion plate. Genome-wide pools should also be frozen at –80°C in 1-mL aliquots.

3. To begin the screen, thaw frozen aliquots and add 100–500 µL of the pooled aliquots to 50 mL YPD. Grow pooled dilutions in a 250–500 mL flask. Incubate flasks in a shaking incubator at 30°C until culture reaches midlog phase ($1–4 \times 10^7$ cells per milliliter).

4. Harvest cultures and transform with the desired inducible dominant negative allele (*see* **Note 37**).

5. Plate pools of transformed deletion strains to restrictive conditions that allow for selection and induction of the dominant negative allele (*see* **Note 38**).
6. Incubate plates 2–3 d at 30°C or until robust colonies begin to form.
7. Confirm that a single deletion is responsible for the observed selectable phenotype (*see* **Notes 28** and **39**).

3.3. Brute-Force Genetic Screens

The previous protocol takes advantage of a selection scheme to identify mutants. In contrast, mutants with the desired phenotype can be identified by systematically analyzing the deletion-mutant collection one at a time. This type of screen is considerably more time consuming. However, it has recently been used to successfully identify mutants involved in processes, including the cell cycle *(32,33)*. *Brute-force* screens are a powerful mechanism for the identification of mutants or phenotypes that cannot be selected for *(32–40)*. Because the deletion strains are analyzed one at a time, virtually any phenotype can be identified. Most screens have strict plus/minus (e.g., viability) end points. However, this type of screen allows researchers to quantify the contribution of the loss of any specific gene product to a given phenotype (*see* **Note 40**). Although labor intensive, these types of screens are very powerful and are likely to increase in popularity in the near future.

1. Obtain deletion collections (*see* **Note 2**).
2. Plate strains under appropriate experimental conditions (*see* **Note 41**).
3. Examine and score mutant strains one at a time for the desired phenotype (*see* **Note 42**).

3.4. Complex Genetic Screens

In addition to the techniques described above, the complete yeast-deletion collection can also be used to conduct complex genetic screens. The assembly of the yeast-deletion collection has revealed that only approx 19% of all genes are essential *(16,41)*. The remaining 81% of genes are nonessential. This observation can be interpreted in two ways: (1) A large number of protein functions may be nonessential for viability or (2), more likely, a large percentage of protein functions are essential, but functional redundancy frequently prevents loss-of-function mutations from displaying essential phenotypes (*see* **Note 43**). Recently, an elegant and innovative approach was published to identify and evaluate genetic redundancies *(41)*. Tong et al. have developed a technique called *systematic genetic analysis* that allows for the methodical identification of synthetic interactions *(16,41)*. Synthetic interactions arise when double mutants display different phenotypes than either single mutant alone *(7,42)*. These interactions can result in synthetic lethality (*see* **Note 44**) or synthetic viability (*see* **Note 45**). Adaptation of techniques like this will allow for the

quantitative examination of multigenic phenotypes *(7,41,42)*. Frequently, a thorough understanding of a complex process like the cell cycle requires understanding of how large, overlapping, and redundant networks of gene products interact (*see* **Note 46**). Systematic genetic analysis provides the conceptual framework for methodically evaluating and assessing gene products in virtually any genetic background and illustrates how systematic genetic analysis can be used to identify the complex genetic networks that create functional redundancies *(41)*. In this manner, and for the first time, it is becoming relatively straightforward to analyze how combinatorial pairs of deletions will affect a given process or produce a specific phenotype (*see* **Note 47**). A simplified protocol for identifying synthetic lethal interactions is briefly detailed (*see* **Note 48**) *(41)*.

1. Obtain *MATa* deletion collection (*see* **Note 2**).
2. Obtain parental query strain (*see* **Note 49**). This is a *MATα* strain that has a unique reporter fusion, *MFA-pr-HIS3*. The *MFA-pr-HIS3* reporter fusion is only expressed in *MATa* haploid cells.
3. Transform the parental query strain with a *yfg*-deletion construct marked with a dominant selectable marker (*see* **Note 50**). Select for transformants and confirm the deletion of *yfg* by PCR (*see* **Note 51**).
4. Mate the parental query strain containing a marked deletion of *yfg* to either a specific *MATa* deletion strain or ordered arrays of these strains. Select for heterozygous diploids (e.g., on YPD plates containing kanamycin and nourseothricin) (*see* **Note 50**).
5. Transfer heterozygous diploids to sporulation medium and incubate at 30°C for 3–5 d or until a high percentage of sporulated cells are observed.
6. Transfer sporulated diploids to a synthetic medium lacking histidine to select for the germination and outgrowth of *MATa* meiotic progeny.
7. Transfer *MATa* meiotic progeny to medium that allows for selection of double mutants (e.g., a medium containing kanamycin to select for a mutant from the deletion collection and nourseothricin to select for *yfg*).
8. Analyze the phenotype of double mutants (*see* **Note 52**).

3.5. Summary

The use of a genome-wide gene-deletion collection for systematic screens is likely to be invaluable in the future genetic dissection of cell cycle control and is likely to greatly alter the basic design of many prospective genetic screens. To date, approx 30% of *S. cerevisiae* genes encode proteins of unknown function *(8)*. Because of genetic redundancies, it is highly likely that techniques such as synthetic genetic analysis will be essential for the elucidation of the function of these gene products *(41)*. Further, the introduction of genomic-scale RNA interference in higher eukaryotes (e.g., *Caenorhabditis elegans*) for the analysis of gene function demonstrates that systematic genetic approaches are adaptable to and feasible in metazoans *(43,44)*.

4. Notes

1. Epistasis is a genetic interaction in which the phenotype caused by a mutation in gene y is masked by a mutation in gene x. If the phenotypes caused by mutations in gene x and gene y are discernible, then epistatic analysis can be used to establish the order of gene function. If the phenotype of the double mutant xy resembles the phenotype of the mutant x (but not the phenotype of y), then x is said to be epistatic to y. That is, gene x acts upstream of gene y. Complementation analysis is used to determine if two recessive mutations are in the same or different genes. Suppressor analysis is a genetic technique for the isolation of genetic alterations that rescue or suppress the original phenotype. Common ways to isolate suppressors include mutagenesis or altering gene dosage by transformation with high-copy libraries.

2. Deletions in this collection are available from Research Genetics, (http://www.resgen.com/products/YEASTD.php3), American Type Culture Collection (http://www.atcc.org/cydac/cydac.cfm), Open Biosystems (http://www.open biosystems.com/productPage.php?pageType=yeast.knockout&q= 0&PHPSESSID=52b2ea2dc5ba77b3aafca2a954ad5183), and Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/data/by.html).

3. Heterozygous diploids are provided for genes that are essential for viability. It has been reported that some deletion strains are aneuploid *(45)*.

4. Technical information, protocols, and deletion strains available can be obtained at (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3. html and http://www-deletion.stanford.edu/cgi-bin/deletion/search3.pl).

5. Basic information, sequences and annotations for every yeast ORF can be obtained at the *Saccharomyces* Genome Database (http://genome-www. stanford.edu/Saccharomyces/). The database can be searched by ORF, gene name, or key words. ORF sequences can be obtained from the drop-down menu entitled *retrieve sequences*. Choose the option "DNA + 1kb up/downstream."

6. Sequences can be copied and pasted as text or saved in GCG or FASTA format. Any suitable bioinformatics can be used. Vector Nti suite 8.0 is recommended.

7. Load vector sequence into a bioinformatics program and identify unique restriction sites.

8. Based on the vector sequence, design primers for the amplification of *yfg* deletion such that the amplified product does not contain the unique restriction sites identified in **Note 7**.

9. The inclusion of a large amount of upstream and downstream sequence flanking *yfg* deletion greatly increases the probability that the amplified yfg-deletion product will integrate at the correct chromosomal position (*see* **Fig. 1** *primers 1* and *10*).

10. Tools for the design of appropriate oligonucleotides can also be obtained at the *Saccharomyces* Genome Database. Primers should be designed with approx 50% G + C content. Designing primers such that the final two 3' base positions are G or C increases the specificity of the PCR reaction.

11. PCR reactions can be conducted with DNA obtained directly from yeast colonies or with purified genomic DNA. Protocols for colony PCR or for purification of genomic DNA and subsequent PCR are available in *Molecular Genetics of Yeast: A Practical Approach (11)* and *Methods in Yeast Genetics: A Laboratory Course Manual (10)*. Excellent protocols are also available at or within the following Web sites: (http://www.fhcrc.org/labs/gottschling/yeast/), (http://www.umanitoba.ca/faculties/medicine/units/biochem/gietz/Trafo.html), and (http://genome-www.stanford.edu/Saccharomyces/VL-yeast.html).

12. Kits for the cloning of PCR-amplified products are available from a number of commercial vendors.

13. Correct clones can be identified by PCR amplification or restriction digestion. Simple protocols for PCR and restriction-enzyme digestion can be found in *Current Protocols in Molecular Biology (6)* and *Molecular Cloning: A Laboratory Manual (47)*.

14. Simple protocols for freezing bacterial stocks can be found in *Current Protocols in Molecular Biology (6)* and *Molecular Cloning: A Laboratory Manual (47)*.

15. Protocols for the transformation of yeast and selection of transformants are available in *Molecular Genetics of Yeast: A Practical Approach (11)* and *Methods in Yeast Genetics: A Laboratory Course Manual (10)*. Excellent protocols are also available at or within the following Web sites (http://www.fhcrc.org/labs/gottschling/yeast/), (http://www.umanitoba.ca/faculties/medicine/units/biochem/gietz/Trafo.html), and (http://genome-www.stanford.edu/Saccharomyces/VL-yeast.html).

16. A straightforward PCR approach exists to confirm that *yfg* has been correctly deleted. In this approach, primers specific to 5' flanking and 3' flanking regions (**Fig. 1**, *primers 2* and *9*, respectively) of yfg are required. In addition, primers internal to the kanMX sequences are also required (**Fig. 1**, *primers 5* and *6*, respectively). Subsequent PCR amplifications with *primers 2* and *5* and *primers 6* and *9* will yield bands only in strains where *yfg* has been correctly deleted (**Fig. 1**). Similarly, use of *primers 2* and *9* will give different-size bands in wild-type strains as compared to *yfg*-deletion strains (**Fig. 1**). The details of this approach are given at (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html#delconfirm). However, this approach has two significant drawbacks. First, separate primers must be made for individual deletions to be checked. Second, this approach cannot be used to identify unknown deletions. A novel approach for the identification of unknown *yfg* deletions involves the direct sequencing of PCR-amplified products (*see* **Note 28**).

17. Frequently, the relative informative value of mutants obtained can be predetermined by the innovation and significance of the selection scheme. Protocols for mutagenesis of yeast are available in *Molecular Genetics of Yeast: A Practical Approach (11)*, *Methods in Yeast Genetics: A Laboratory Course Manual (10)*, *Current Protocols in Molecular Biology (6)*, and *Methods in Enzymology, vol. 194: Guide to Yeast Genetics and Molecular Biology (4)*. Readers are referred to an excellent review discussing the design of yeast genetic screens *(7)*.

18. At least four different types of genetic alterations can act as suppressors in genetic screens: (1) bypass suppressors, (2) dosage suppressors, (3) allele-specific suppressors, and (4) nonsense suppressors. Bypass suppressors function by activating or inactivating an alternative pathway (e.g., high-copy *BCK2* and deletion of *sic1*; both bypass the phenotype of strains lacking G1-phase cyclins). Dosage suppressors are genes that rescue the parental phenotype when they are expressed at high levels (e.g., high-copy *CLN2* rescues the temperature-sensitive phenotype of *cdc28ts* mutants). Finally, if the phenotype of the initial mutant is owing to a mutation causing a premature stop codon, then this phenotype can be nonspecifically rescued by nonsense suppressors (e.g., a mutant transfer RNA (tRNA) that introduces a random amino acid to prevent premature translation termination).

19. Steps required for the cloning of suppressors are discussed in these recent reviews *(7,21)*.

20. For any given selection, it is difficult to assess how many genetic alterations are capable of producing the desired phenotype. In general, a screen is thought to be saturated when several mutations are recovered more than once. However, this criterion is subjective, and it is not really possible to objectively determine the completeness of classic genetic screens.

21. If all of the available deletions were pooled, the pool would contain approx 97% of the genome as individual single-gene deletions.

22. An enormous advantage to the approach of screening pooled-deletion strains as compared to classical genetic screens is the ability to rapidly identify and clone the isolated mutant (*see* **Note 28**).

23. The genome-wide deletion collections are usually shipped from commercial vendors in 70–75 96-well plates. Pooled-deletion strains may be obtained from Research Genetics (http://www.resgen.com/products/YEASTD.php3). The best results are achieved when pools are made with roughly equivalent numbers of mutants. If, in the initial pool, individual mutants are represented unequally, then final results can be skewed. Creating the initial pooled-deletion collections is rather time intensive, so it is usually worthwhile to scale up the process to generate a large number of pooled aliquots for future use. If the cells are frozen at very high densities, then glycerol stocks can be thawed and refrozen at least 10 times without any obvious deleterious effects.

24. Coulter counters can be used to directly determine cell concentrations. Using the spectrophotometer, 1 mL of culture with an OD_{600} of 1.0 corresponds to approx 3 $\times 10^7$ cells per milliliter.

25. Ideally, there should be 200–400 colonies per 100-mm plate or 400–800 colonies per 150-mm plate.

26. Because the mutants of interest will fail to grow under the nonpermissive conditions, the only way these colonies can be identified is by a replica-plating protocol.

27. Repeat the selection criteria to ensure that the mutant reproducibly generates the same phenotype. When selecting for mutants that fail to divide under restrictive

conditions, it may be necessary to replica plate to the restrictive conditions more than once. Nondividing cells frequently retain the ability to grow in volume and mass. In this manner, replicated colonies from mutants sometimes appear to be proliferating when they are not.

28. A new approach for the definitive identification of unknown deletions has been developed. This method makes use of four primers designed to amplify sequences present in every deletion (**Fig. 1**). Using **primers 3** and **5**, the sequence containing the uptag bar code can be amplified with PCR (**Fig. 1**). Similarly, using **primers 6** and **8**, the sequence containing the downtag bar code can be amplified (**Fig. 1**). Amplified PCR products are purified and sequenced with internal primers. Sequences obtained are compared to a bar-code-tag database. Searching the database with the uptag and downtag bar code sequence can definitively identify the deletion isolated. The wild-type gene can easily be cloned by the straightforward PCR amplification of the gene from nonmutant strains. Detailed specifics of this protocol and an advanced bar-code-tag database can be obtained from the authors via e-mail (brondt.schneider@ttmc.ttuhsc.edu).

29. At least two approaches can be used to confirm that a single deletion is responsible for the mutant phenotype. The first approach is to determine if introduction of the identified deletion alone into the mutant strain can rescue the original phenotype. A second approach is to complement the identified deletion with a copy of the wild gene on a plasmid. If the plasmid complements and reverses the mutant phenotype, then it is likely that a single deletion is responsible for the mutant phenotype.

30. Readers are strongly recommended to consult the primary references where these basic methods were first described and elegantly put to use *(16,17)*. Microarrays for this protocol can be ordered on a made-per-order basis from Affymetrix (http://www.affymetrix.com/index.affx). The relatively high cost of these arrays and this technology should be factored into the decision to pursue this protocol.

31. It has been reported that in cultures grown for at least 60 population doublings, mutants with only a 5% growth-rate defect could be readily identified *(27)*. In contrast, when cultures were grown for 15–18 population doublings, a 10% growth-rate defect or greater was required to be identified *(16,17,27)*.

32. A number of commercial kits are available, or excellent protocols can be found at (http://genome-www.stanford.edu/Saccharomyces/VL-yeast.html).

33. Pairs of primers identical to sequences contained in every deletion (e.g., **Fig. 1** *primers 3* and *4* or *primers 7* and *8*) can be used to amplify the uptag and downtag sequences unique to each deletion construct. Uptag bar codes are amplified with the following biotinylated primers: 5' GTCGACCTGCAGCGTACG-3' and 5'-GATGTCCACGAGGTCTCT-3'; downtag bar codes are amplified with the following biotinylated primers: 5'-CGAGCTCGAATTCATCG-3' and 5'-CGGTGTCGGTCTCGTAG-3'.

34. A number of published buffers can be used, but the buffer used in the published protocol is as follows: 6X SSPE (1 *M* NaCl, 66 m*M* NaH$_2$PO^4–, 6.6 m*M* ethylenediaminetetraacetic acid (EDTA, pH 7.4). It has been reported that inclu-

sion of 200 pmol of the following four primers decreases background: 5'-GATGTCCACGAGGTCTCT-3', 5'-CGTACGCTGCAGGTCGAC-3', 5'-CGGTGTCGGTCTCGTAG-3', and 5'-CGATGAATTCGAGCTCG-3' *(16,17,27)*.

35. Notes on scanning and analysis are available in the primary references and at (http://genomics.lbl.gov/YeastFitnessData) and (http://www.affymetrix.com/index) *(16,17,27)*.

36. In principle, this protocol could be used to select for genetic suppressors of any gene whose induced expression produced a phenotype that could be selected against. In the example given, induced expression of an unphosphorylatable form of *sic1* prevents cells from dividing *(31)*. In this case, one could screen for suppressors that allow cells expressing this allele to divide.

37. The most current yeast transformation protocols are available at (http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html) and (http://www-sequence.stanford.edu/group/yeast_deletion_project/transprot.html).

38. In the specific example of *URA3*⁺ plasmid containing a galactose-inducible unphosphorylatable form of *sic1–URA* plates containing galactose would be required.

39. The best approach is to determine if introduction of the identified deletion alone into a strain containing the dominant negative allele can rescue the phenotype owing to induction of the dominant negative allele.

40. As an example of a brute-force screen, the entire yeast-deletion collection was recently screened for cell-size mutants *(32,33)*. In addition to the isolation of frank cell-size mutants, this screen also demonstrated the degree with which every yeast gene deletion affected cell size *(32,33)*.

41. Depending on the experimental condition chosen, systematic genomic-wide genetic screening of this type can be used to quantify the contribution of every known gene to a given phenotype.

42. As discussed in the previous note, virtually any phenotype can be chosen for study, and a quantitative assessment of the contribution of each deletion in the collection can be made. Readers are referred to the primary references *(32,33)*.

43. A number of nonessential yeast genes have a very closely related homologue in the genome. This suggests that genetic redundancies prevent sporadic genetic alterations from having lethal phenotypes.

44. In synthetic lethal interactions, each single mutant alone is viable, but the double mutant is inviable.

45. In synthetic viable interactions, each single mutant alone is inviable, but the double mutant is viable.

46. The power of the analysis of synthetic interactions lies in the observation that many genetic interactions translate into physical interactions or have biochemical or physiological mechanistic significance.

47. The technique developed by Tong et al. allows for the rapid, systematic, and automated construction of combinatorial mutants. Also as noted by the authors, systematic genetic analysis could be used to back-cross the entire yeast-deletion collection into another yeast background *(41)*.

48. Readers are strongly recommended to consult the primary reference where systematic genetic analysis was first described and elegantly put to use *(41)*.
49. Corresponding authors of systematic genetic analysis can be e-mailed at Brenda.andrews@utoronto.ca, tyers@mshri.on.ca, or charlie.boone@utoronto.ca.
50. In addition to kanamycin resistance, dominant selectable markers for resistance to hygromycin B, nourseothricin, and bialaphos are now available at (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/data/Del_plas.html) *(46)*.
51. The correct integration of deletions can be easily determined by PCR amplification. By designing an upstream primer that is outside the deleted region and using a primer specific to the dominant selectable marker, a PCR product of the correct size will only be obtained when the deletion integrates at the correct place in the genome (*see also* **Note 16**).
52. In the primary reference, systematic genetic analysis makes use of robotics to automate the methodical screening of the entire deletion collection. Although considerably more time consuming, this process can be done manually. Regardless, it is essential to confirm observed phenotypes (e.g., synthetic lethality) by tetrad analysis *(41)*.

Acknowledgments

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12

Gene Targeting in Cultured Human Cells

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1. Introduction

The technique described in this chapter—gene targeting in cultured human cancer cells—brings a powerful tool to scientists studying the function of cell cycle control genes *(1)*. This technology allows scientists to knock out genes in cultured human cells in an analogous fashion to the creation of knockout mice. This approach brings the power of genetics (the comparison of cells or organisms that are genetically identical except for a single, well-defined mutation) to the study of human genes in cultured human cells. Gene targeting is a particularly valuable approach for the study of cell cycle control genes because ectopic expression of these genes frequently results in cell cycle arrest or apoptosis. To date, several cell cycle control genes have been studied using human somatic-cell gene targeting, including p21$^{\text{WAF1/CIP1}}$, p53, 14-3-3σ, and (ataxia-telangiectasia and *Rad3 [ATR]*) *(2–5)*.

Once this "molecular scalpel" has been employed, a researcher has at his or her disposal a set of cultured human cells that are genetically identical to each other except for a specific, targeted change—the presence (in the parental cells) or absence (in the knockout cells) of a functional gene. Because the cells are otherwise genetically identical, any biological or biochemical difference between the cells sheds light on the function of the targeted gene. This type of classical genetic analysis—the study of gene function by comparing cells or organisms that are genetically identical except for a single, well-defined genetic change—has been a mainstay of genetics in model systems for decades and has yielded innumerable critical discoveries. Until fairly recently, this capability has been restricted to model organisms and was not applicable to human cells.

The biological and biochemical characteristics of such isogenic sets of human cells can be analyzed in many different ways. The growth properties of

the cells can be studied in vitro to assess differences in their morphology and growth characteristics. The cells can also be analyzed using flow cytometry to assess their cell cycle profile during exponential growth, during cell cycle arrest, or after treatment with growth factors, small molecule therapeutics, and lead compounds. In addition, the propensity of the cells to undergo apoptosis in vitro can be assessed using a variety of assays.

The cells can also be studied in vivo. Because many human cancer-derived cell lines will recapitulate a tumor if injected subcutaneously into immunodeficient mice, it is possible to recapitulate so-called isogenic tumors. By comparing isogenic tumors it is possible to examine the role of the targeted gene in a wide variety of processes including, for example, the sensitivity of tumors to anticancer agents, and angiogenesis.

Creating an isogenic set of gene-targeted human cells is a technically demanding process. Because the ratio of homologous to nonhomologous recombination events is very unfavorable in human cells, *promoterless* targeting vectors are routinely employed to reduce the background of colonies with random integrations and therefore enrich for knockouts. It generally takes approx 1 yr to build and test a new targeting vector. Once the targeting vector has been tested and proved effective at homologous recombination, it generally takes another 6 to 12 mo to create a variety of isogenic sets of homozygous knockout human cell lines suitable for experimentation. There are multiple, detailed, and often alternative procedures involved in making such cell lines. Therefore, instead of describing them in great detail, in this chapter I will attempt to provide a general protocol, set of strategies, important issues to consider, and a variety of references to guide the creation of human somatic-cell knockouts.

2. Materials

Described as needed throughout the text, or in references cited.

3. Methods

3.1. Choice of an Appropriate Gene

There are two criteria to consider when choosing a gene to target in human somatic cells.

First, the gene must be expressed in the cell line or lines used for gene targeting. There are both practical and biological reasons for this requirement. Because the ratio of homologous to nonhomologous recombination is very unfavorable for gene targeting in human cultured cells (i.e., virtually all integration events are random, not homologous), promoterless gene-targeting vectors are used to enrich for knockouts (6). In such promoterless targeting vectors,

the drug resistance gene (generally neo^R) is inserted in-frame into the open reading frame of the gene being targeted and, therefore, lacks its own heterologous promoter. Consequently, the resistance gene is only expressed when the targeting vector is integrated into a transcriptionally active region, such as the gene being targeted. However, in order for this promoterless strategy to work, the locus being targeted must be expressed. In addition to this practical requirement, there is no obvious reason to target nonexpressed genes in human somatic cells because the gene-targeted derivatives would likely be functionally identical to their parental precursors. This is one situation in which gene targeting in human cells differs from gene targeting in mouse embryonic stem (ES) cells. In the case of ES cell gene targeting, investigators routinely knock out genes that are not expressed, with the expectation that the genes will eventually be expressed either during embryonic development or in the adult mouse. This scenario is obviously irrelevant in the case of gene targeting in human somatic cells.

Second, the gene of interest should not be an essential gene in human somatic cells. This is frequently impossible to predict and as such represents a risk in any gene-targeting project. Because of this risk, in our laboratory we focus, in part, on targeting wild-type tumor-suppressor genes because their disruption is predicted to be beneficial to the growth of cultured human cells.

One does not learn that a given gene is essential until well over a year into a gene-targeting project, generally when attempting to convert a heterozygous knockout cell line to a homozygous knockout cell line via a second round of gene targeting. In the case of an essential gene, knockouts will be obtained in the second round of gene targeting; however, in every case the targeting vector will have integrated into the already-targeted allele, not the remaining wild-type allele. In this circumstance, it is possible to modify the second allele-targeting vector to create a conditional knockout allele *(5)*. By doing this, the remaining wild-type allele can be converted to a so-called "floxed" allele (*f*lanked by *lox* sites), in which the gene remains wild type but can be deleted by transient introduction of Cre protein. However, creation of such floxed alleles is complex and represents an added element of uncertainty.

3.2. Choice of an Appropriate Cell Line

Most human somatic-cell knockouts have been performed in one of two human cancer cell lines—HCT116 and DLD1 cells *(7–9)*. Both cell lines were initially derived from invasive colonic adenocarcinomas and have a number of biological features making them suitable for gene targeting. First, HCT116 and DLD1 cells are diploid, unlike the vast majority of cultured human cancer cell lines, which are aneuploid *(10)*. Because the cell lines have two (and only two) copies of most human genes, it is feasible to obtain homozygous knockouts via sequential transfection of two targeting vectors. Second, HCT116 and

DLD1 cells grow unusually quickly in tissue culture (with a doubling time of approx 22 h) and grow clonally in 96-well plates. These characteristics make it possible to generate hundreds of individual stably transfected clones in 2 to 4 wk. Third, both HCT116 and DLD1 cells transfect with high efficiency. Transfection efficiency is a critical parameter because transfection of high-efficiency promoterless targeting vectors results in the formation of relatively few drug-resistant colonies. Formation of only a small number of colonies is desirable because it suggests that the promoterless enrichment is working to limit the formation of drug-resistant colonies derived from random integration events. Practically, however, it also makes it difficult to obtain large numbers of transfected, drug-resistant colonies when using cell lines with low transfection efficiencies. Because HCT116 and DLD1 cells have proved to be suitable for gene targeting, we generally first validate our targeting strategy by creating knockouts in one of these cell lines. Only then do we attempt to create knockouts in other cell lines whose utility in gene targeting is unproved.

Though HCT116 and DLD1 cells are invaluable for human somatic-cell gene targeting, there are potential disadvantages in using cell lines derived from human cancers, such as the presence of uncharacterized mutations in oncogenes and tumor suppressor genes. To circumvent such potential problems with cancer-derived cell lines, John Sedivy's group has created human somatic-cell knockouts in primary human fibroblasts *(11)*. The major challenge in creating knockouts in primary human cells is the fact that the cells will inevitably senesce, perhaps before both rounds of targeting can be completed. In an effort to circumvent this limitation, Sedivy's group has focused on targeting genes whose absence might be predicted to alleviate senescence (i.e., $p21^{WAF1/CIP1}$ and p53). Another theoretical way to circumvent the problem of senescence is to use telomerase-immortalized primary cell lines. However, gene targeting in telomerase-immortalized primary cells has not yet been reported in the literature.

3.3. Targeting Vector Construction

Promoterless targeting vectors consist of two homology arms (generally 2–5 kb each) flanking a drug resistance gene (generally neo^R) inserted in-frame into the locus being targeted. Several additional sequence features are frequently added flanking the drug resistance gene to simplify identification of knockouts in human cells, such as polymerase chain reaction (PCR) priming sites and restriction sites for diagnostic Southern blots. Lox sites are frequently also added flanking the neo^R gene, making it possible to delete the neo^R gene from heterozygous knockout cell lines and recycle the targeting vector for a second round of targeting. The sequence features of one such promoterless targeting vector are depicted in **Fig. 1A**.
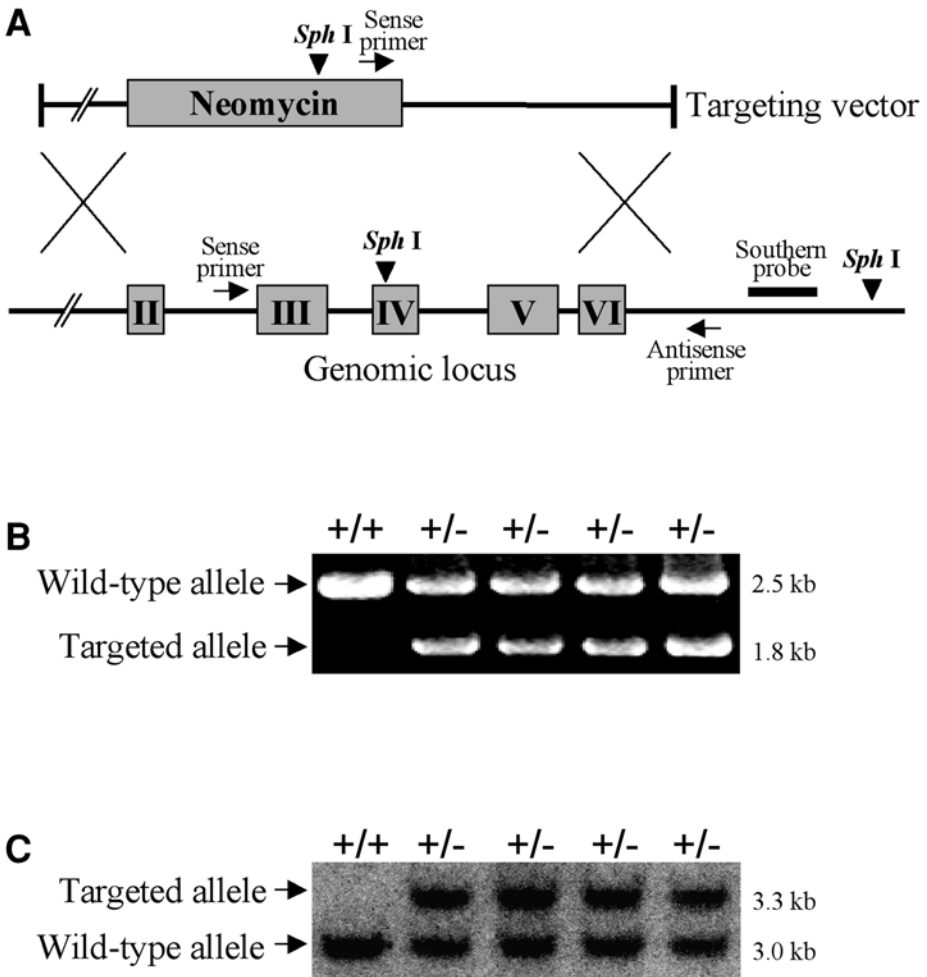
Fig. 1. Strategy for human somatic-cell gene targeting. (**A**) Homologous recombination between the genomic locus and targeting vector deletes critical exons and replaces them with a promoterless neo^R gene. PCR primers used for identification of knockouts are indicated, as are restriction-enzyme cleavage sites and a probe used for Southern blot-based confirmation of knockouts. (**B**) PCR-based identification of gene-targeted clones. Parental cells have a single 2.5-kb band derived from two untargeted alleles. Heterozygous knockout clones have a 2.5-kb band derived from the untargeted allele and a 1.8-kb band from the targeted allele. PCR priming sites are depicted in A. (**C**) Southern blot-based confirmation of gene-targeted clones. Parental cells have a single 3.0-kb band derived from two untargeted alleles. Heterozygous knockout clones have a 3.0-kb allele derived from the untargeted allele and a 3.3-kb band derived from the targeted allele. The locations of the restriction sites and probe are depicted in A.

Several different cloning strategies are commonly employed for the creation of human promoterless targeting vectors. Generally, the most challenging step is engineering the junction of the left homology arm and the *neo^R* gene such that the drug resistance gene is in-frame with the open reading frame of the gene being targeted. Because these types of precise manipulations are frequently impossible using conventional recombinant deoxyribonucleic acid (DNA) approaches (generally limited by the absence of perfectly placed unique restriction sites), we generally use homologous recombination in *Saccharomyces cerevisiae* to create these precise junctions *(12)*. Here I will describe the strategy most commonly employed in our laboratory, and briefly describe alternative strategies. The strategy involves the following steps:

1. Obtaining human genomic DNA for the gene of interest.
2. Subcloning an appropriate fragment into a yeast shuttle vector.
3. Identifying and validating a PCR primer pair for use in eventual identification of knockouts.
4. Identification and validation of critical restriction sites.
5. PCR amplification of the *neo^R* gene with appropriate flanking sequence features.
6. Homologous recombination in yeast.

3.3.1. Obtaining Human Genomic DNA for the Gene of Interest

The first step in building a targeting vector is to obtain the genomic DNA that will form the left and right homology arms. To do this, we obtain bacterial artificial chromosome (BAC) clones containing human genomic DNA inserts corresponding to the locus of interest. One simple way to obtain needed BACs is to create a PCR-generated probe corresponding to the gene of interest, and provide it to a company such as Research Genetics for screening an arrayed BAC library. Bacteria harboring positive BACs are supplied and confirmed using whole cell PCR, and BAC DNA is prepared using standard protocols (as provided by the company).

Alternatively, it is possible to derive the raw material for the homology arms using PCR from a genomic DNA template. This has several advantages, including the ability to design the homology arms exactly as desired and the ability to add useful restriction sites to the ends of the PCR products. However, this approach also has several disadvantages, including the inherent difficulty in creating long PCR products from genomic DNA templates and the possibility of introducing PCR-generated mutations into the homology arms.

3.3.2. Subcloning an Appropriate Fragment into a Yeast Shuttle Vector

Next, a single relatively large fragment (generally 6–10 kb) that will form the basis of both the left and right homology arms is subcloned from a BAC into a yeast shuttle vector. The term *yeast shuttle vector* refers to the fact that

these vectors have origins of replication and selectable markers for propagation and selection in both *S. cerevisiae* and *Escherichia coli.* As such, they can be used to exploit useful features of both organisms (e.g., high efficiency of homologous recombination in *S. cerevisiae* and ease of DNA propagation in *E. coli*). The genomic DNA fragment cloned into the yeast shuttle vector will ultimately be modified by homologous recombination to delete critical exons and replace them with an in-frame *neo^R* gene with appropriate sequence features on each end.

The availability of the complete sequence of the human genome has greatly simplified the identification of appropriate restriction enzymes for this subcloning step. After identifying candidate restriction enzymes based on the genome sequence, it is prudent to confirm the presence of the restriction fragment in the cloned BAC DNAs using a Southern blot approach.

Numerous yeast shuttle vector systems are available to form the backbone of the targeting vector. We generally use one of the series pRS423-6, which are high-copy-number vectors with different auxotrophic markers and useful polylinkers. We have also used YEp24, a classic low-copy-number yeast shuttle vector, with success. To perform the subcloning, the BAC is digested with the enzyme of interest and shotgun cloned into a linearized, phosphatase-treated vector backbone; 500–1000 colonies are then transferred to a grid plate, and colony hybridizations are performed with a radiolabeled oligonucleotide specific for the insert of interest. After identification of positive clones, the DNA is prepared using standard techniques and the integrity of the insert confirmed by restriction digestion and sequencing of critical junctions.

3.3.3. Identifying and Validating a PCR Primer Pair for Use in Eventual Identification of Knockouts

Most knockouts, whether performed in human somatic cells or in mouse ES cells, are identified with a PCR-based screen and confirmed with a Southern blot. In a conventional PCR screen, one PCR primer is located in the *neo^R* gene, and the other PCR primer is located in the genomic region adjacent to one of the homology arms of the targeting vector. Only clones in which the targeting vector has integrated by homologous recombination are expected to serve as effective templates for PCR.

This PCR strategy has proved extremely valuable and has been used to identify hundreds (if not thousands) of different knockout mouse ES cell lines. However, it has several fundamental limitations. First, it is virtually impossible to optimize PCR conditions for the chosen primer pair because by definition the only suitable template available for such an optimization is genomic DNA derived from a gene-targeted cell line itself. Although it is possible to use recombinant DNA approaches to create a plasmid-based template for PCR

optimization (and even integrate it into the genome of cultured cells for the preparation of genomic DNA templates), these approaches are labor intensive and virtually never performed. Second, the lack of a positive control template, coupled with the inability to optimize the PCR reaction, makes it difficult to interpret a negative result. Put another way, it is difficult to definitely interpret a negative result when the negative result is the absence of a PCR product, and the PCR has never been optimized and lacks a positive control.

Chan et. al. have recently published a PCR-based strategy for identification of knockouts that overcomes both of these problems and forms the basis of all PCR screens performed in our laboratory *(4)*. The strategy is virtually identical to the conventional PCR screen described above, with one simple difference. Instead of using a PCR priming site located in the *neo^R* gene, a PCR priming site located in the genomic region to be deleted by the targeting vector is used. More important, this priming site is built into the targeting vector itself, at the junction of the drug resistance gene and a homology arm. Homologous integration of the targeting vector will delete the endogenous priming site and move it, changing the size of the PCR product. *See* **Fig. 1A**,**B** for an example of this strategy.

The fundamental advantage of this strategy is the presence of an internal positive control for PCR because even untargeted clones can serve as a template for PCR. This advantage makes it possible to *(1)* optimize the PCR screen with genomic DNA derived from the parental cells prior to screening actual clones and *(2)* definitively interpret a negative result because there is an internal positive control for PCR in each reaction.

The only clear disadvantage of this screening strategy is that the PCR primer pair must be designed and optimized prior to completing construction of the targeting vector because one of the priming sites must be built into the targeting vector itself. As such, it is necessary to optimize a suitable primer pair at this stage in targeting vector construction.

3.3.4. Identification and Validation of Critical Restriction Sites

Prior to completing construction of the targeting vector, it is necessary to identify two unique restriction sites—one in the cloned genomic region to perform homologous recombination in yeast, and the other in the vector backbone for linearization of the completed targeting vector prior to transfection into human cells. There are two reasons that it is useful to identify these restriction sites prior to completion of the targeting vector. First, the location of the site used in the yeast step will determine the placement of the recombination junctions. Second, if the restriction site that will be used to linearize the completed targeting vector has been predetermined, one can avoid inadvertently adding extra sites during final construction of the targeting vector.

3.3.5. PCR Amplification of the Neo^R Gene
With Appropriate Flanking Sequence Features

Next, a PCR-generated *neo^R* gene is created, using high-fidelity PCR and long primers, to engineer various sequences on each end of the *neo^R* gene. These features may include *(1)* approx 50 nucleotides of homology on each end for directing homologous recombination in yeast with the cloned genomic region, *(2)* the PCR-priming site, described in **Subheading 3.3.3.**, for identification of knockouts in human cells, *(3)* any restriction sites needed to simplify Southern blot-based diagnosis of gene-targeted human cells, and *(4)* lox sites for eventual excision of the *neo^R* gene.

3.3.6. Homologous Recombination in Yeast

In the final step in targeting vector construction, the PCR product generated as described above is cotransformed into *S. cerevisiae* with the linearized yeast shuttle vector containing the genomic region of interest. Individual colonies expressing the appropriate auxotrophic marker are tested by whole-cell PCR to identify those in which the desired recombination event has occurred. Generally, recombination has occurred in 80–100% of colonies tested. The completed targeting vectors are then transferred into *E. coli*, DNA is prepared using standard techniques, and the integrity of the finished targeting vector is confirmed by restriction digestion and sequencing of critical junctions.

3.4. Creation of Heterozygous Knockout Cell Lines

The completed targeting vector is then linearized and transfected into cultured human cells. It is prudent to first conduct a pilot transfection experiment to determine the number of drug-resistant colonies formed per transfected T25. This information is useful because the number of colonies can be used for a rough prediction of the homologous integration efficiency of the targeting vector (*see* **Notes 1–4**). Also, if one knows the number of colonies formed per transfected T25, it is possible to determine the most appropriate dilutions to use when plating transfected cells in 96-well plates for the formation of single colonies.

Next, the transfection is repeated using larger numbers of T25s. Approx 12 h after transfection, the cells are trypsinized and transferred at various dilutions, in selective media, to 96-well plates. We generally transfect 20–50 T25s in this step and create 60–100 of the 96-well plates. The plates are wrapped in plastic to minimize evaporation, and allowed to incubate for 10–14 d.

Several hundred single colonies are then identified, trypsinized, and transferred to 24-well plates. When confluent, the wells are again trypsinized and transferred to a T25. When confluent, the contents of the T25s are trypsinized.

Approximately half of the cells are cryopreserved and the other half used for preparation of genomic DNA, using standard techniques.

Genomic DNA is then tested by PCR to identify heterozygous knockout clones, as depicted in **Fig. 1B**. Positive clones are then thawed, expanded, retested by PCR, and refrozen in greater numbers. The integrity of the heterozygous knockouts is then confirmed by Southern blot, as depicted in **Fig. 1C.**

3.5. Removal of the Neo^R Gene by Cre-Mediated Recombination (Optional)

If the *neo^R* is flanked by lox sites, it is possible to remove it from the heterozygous knockout cells created in **Subheading 3.4.** via Cre-mediated recombination. To do this, heterozygous knockout cells are infected with a Cre-expressing adenovirus for 24 h, then plated out at limiting dilution in 96-well plates. After 10–14 d of growth in nonselective media, single colonies are expanded, cryopreserved, and tested for G418 sensitivity. G418-sensitive clones are thawed, expanded, and refrozen for disruption of the remaining allele.

3.6. Modification of the Targeting Vector by Changing the Drug Resistance Gene (Optional)

If the *neo^R* gene is not flanked by lox sites, it is possible to disrupt the remaining allele by changing the drug resistance gene in the targeting vector and transfecting this modified targeting vector into heterozygous knockout cells. There are numerous selectable markers available for use in a second allele-targeting vector, including *hyg^R* (most commonly used), *puro^R*, *zeo,^{R}*, and others.

To swap selectable markers, PCR is employed to create a modified *hyg^R* gene (for example) with the same flanking-sequence features as were added to the *neo^R* gene. Homologous recombination in yeast is employed to integrate it into the cloned genomic region, as described in **Subheading 3.3.6.**

3.7. Creation of Homozygous Knockout Cell Lines

The second allele-targeting vector is then transfected into heterozygous knockout cell lines, and homozygous knockout clones are selected and identified with PCR and Southern blots, as described in **Subheading 3.4.**

Generally, disruption of the remaining allele is less efficient than targeting the first allele because half of the homologous integration events will disrupt the already-targeted allele. After confirmation of homozygous knockout clones by PCR and Southern blot, the absence of protein is confirmed by Western blot.

4. Notes

1. The neo^R gene is the "gold-standard" selectable-marker cassette for human somatic-cell gene targeting; it seems to be more reliable at forming drug-resistant colonies and knockouts than other resistance genes (notably, hyg^R). The reason for this is unknown.
2. Ideally, promoterless targeting vectors are designed such that the initiating methionine of the selectable-marker gene is inserted precisely into the initiating methionine of the gene being targeted. However, this is not possible if the initiating methionine of the targeted gene is in the first exon because the left homology arm of the targeting vector would then contain the promoter of the gene being targeted, thus reducing the efficiency of the promoterless enrichment. If the initiating methionine is in the first exon, the best alternative is to target downstream exons using an internal ribosome entry site (IRES)-neo. Do not attempt to create neo^R fusion proteins because the neo^R protein is sensitive to the presence of extra amino acids and can easily be rendered nonfunctional.
3. Lipofectamine (from Invitrogen) is used to transfect HCT116 and DLD1 cells. A higher transient transfection efficiency is generally achieved in HCT116 cells (20–40%) than in DLD1 cells (10–20%), though the number of drug-resistant colonies formed after stable transfection of targeting vectors is usually comparable.
4. With high-efficiency promoterless targeting vectors (homologous integration efficiencies of greater than 10%), approx 50–100 drug-resistant colonies are generally formed per transfected T25. When the same cells are transfected with a drug resistance gene whose expression is driven by a heterologous promoter such as cytomegalovirus (CMV), several thousand drug-resistant colonies are formed per T25. This difference in colony number points out the utility of the promoterless enrichment.

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13

Use of In Vivo Gap Repair for Isolation of Mutant Alleles of a Checkpoint Gene

Migdalisel Colòn and Nancy C. Walworth

1. Introduction

Genetic screens have been extraordinarily useful for the identification of protein components that function in a variety of cellular processes. For example, a number of proteins that are necessary for responding to deoxyribonucleic acid (DNA) damage were found in screens for loss-of-function mutants that confer hypersensitivity to DNA-damaging agents *(1)*. Genetic screens that report a loss of function can also be used to discern structure-function relationships within a particular protein by providing an assay for loss of function. Combining such an assay with a method that generates mutant alleles of a particular gene can aid in the identification of domains within the encoded protein that are functionally important for the process under investigation. Such approaches are particularly straightforward in unicellular eukaryotes such as *Schizosaccharomyces pombe*.

To identify and screen for random mutations in a specific gene, we have combined an approach whereby random mutations targeted to a particular region of a gene are generated in vitro with the polymerase chain reaction (PCR), and then production of the "library" of mutants is carried out in vivo by a process known as gap repair (*see* **Fig. 1** and **ref. *2***). Gap repair describes the ability of yeast cells to insert into a vector a region of DNA that possesses homology to the vector at both ends (*see* **Fig. 2A** and **ref. *3***). The event is selected for because the gapped vector is a linear molecule and only circular plasmids are autonomously stable in yeast. Yeast can repair a gapped vector using chromosomal sequences that are homologous to the ends. Alternatively, if an exogenous source of homologous sequence is provided, such as a linear
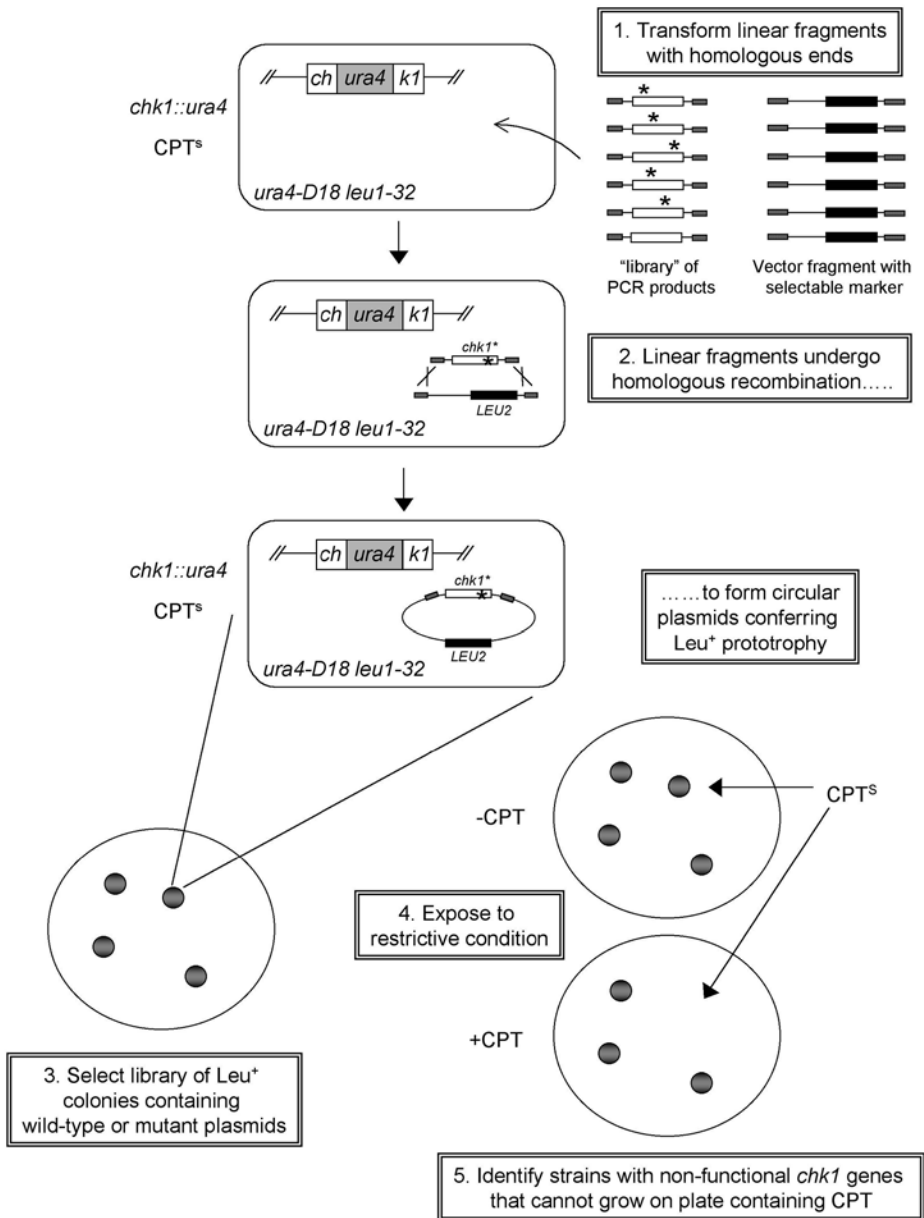
Fig. 1. Schematic of mutagenesis coupled with in vivo gap repair for isolation of mutant alleles of *chk1*. The screen is carried out in a strain that has a deletion of the *chk1* gene on the chromosome and is, therefore, hypersensitive to DNA-damaging agents such as camptothecin (CPT). A library of PCR products prepared as described in the text is cotransformed with a vector that has homology to the PCR products at

PCR product, then the vector may be repaired with the PCR product. Thus, by amplifying a PCR product that overlaps the ends of the gapped vector, a "library" of mutants can be generated (**Fig. 1**). The utility of this approach is that a whole gene or specific regions of a gene can be targeted for mutagenesis. One advantage of this approach is that the "library" never passages through *Escherichia coli (E. coli)*, a process that can lead to selection for or against particular alleles of genes on plasmids if they are expressed in bacteria. Another advantage is that there are no cloning steps: The yeast carries out the work of ligating the mutagenized inserts into the vector. In the screen described here as an example, we included a region of the vector's selectable marker as part of our PCR product to ensure that all viable colonies represented ones in which the insert had been incorporated into the vector (**Fig. 2B**). Without such a selection, it is possible for yeast to ligate an open vector (*see* **Fig. 2A** and **ref. 4**).

Our laboratory has been interested in dissecting the function of the *chk1* gene, which encodes a protein kinase important for delaying cell cycle progression in response to DNA damage, a phenomenon known as the DNA damage checkpoint *(5)*. To dissect the role of *chk1* in the checkpoint pathway, we wanted to identify domains of the protein that may be important for its function. We accomplished this by generating and identifying random mutations that conferred a defect in the checkpoint response. *chk1* is a nonessential gene unless cells are exposed to DNA-damaging agents *(6)*. Thus, a strain harboring a null allele of *chk1* in the chromosome is viable. However, such a strain is sensitive to DNA-damaging agents *(6,7)* and will not grow if plated in the presence of a topoisomerase I poison called camptothecin (CPT) *(8)*. A plasmid bearing a wild-type copy of the *chk1* gene will grow under such conditions. Thus, loss-of-function alleles carried on a plasmid could be identified in the *chk1* null background by screening for strains that were unable to form colonies in the presence of CPT *(2)*. The principles of the screen are generally applicable *(4)*, and we have successfully used it with gene-specific variations to identify conditional, temperature-sensitive, loss-of-function alleles of another fission yeast gene that encodes an essential protein (Colòn and Walworth, unpublished).

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each end. Fission yeast cells take up the DNA fragments. By homologous recombination, a PCR product and vector fragment will recombine to generate a circular plasmid that is stable in yeast cells. The vector fragment includes a marker gene for selection in yeast. In the example shown, the *S. cerevisiae LEU2* gene that complements the *S. pombe leu1-32* mutant is used. Individual Leu⁺ colonies are then screened for those that are still sensitive to CPT and, therefore, represent a loss of *chk1* function.
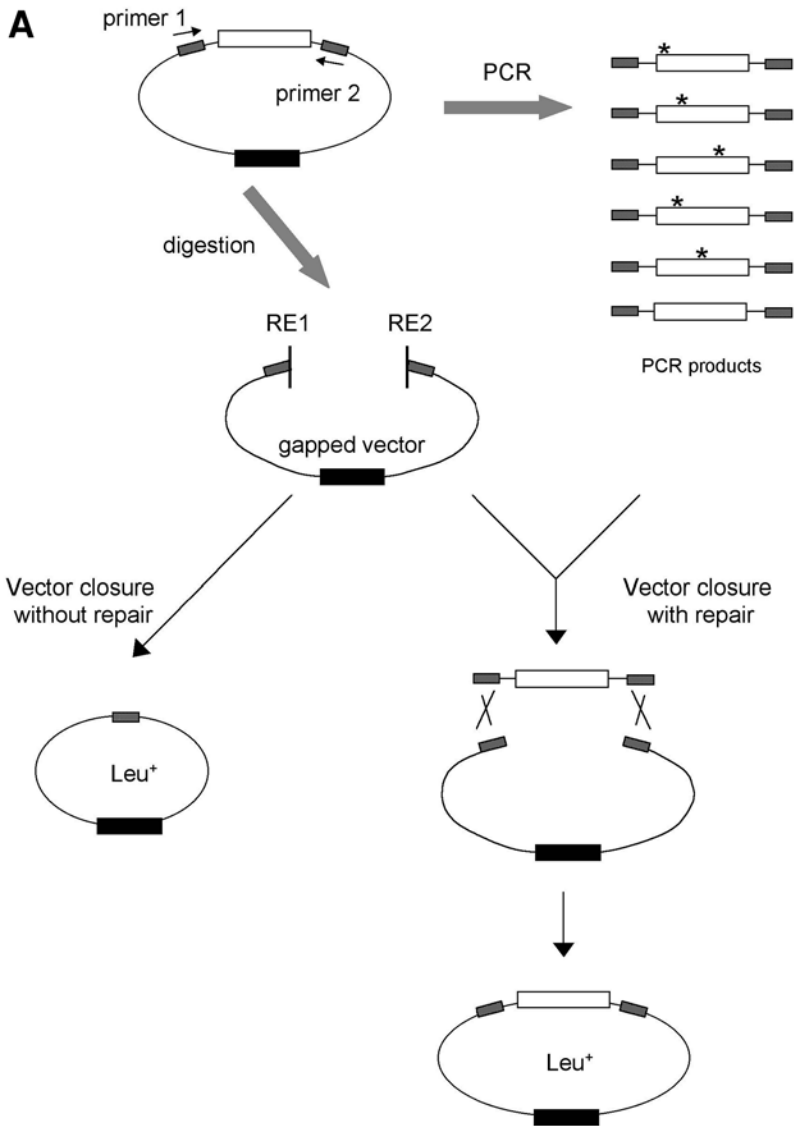
Fig. 2. Mutagenesis and gap repair. **(A)** Primers are designed for PCR amplification such that the region amplified will overlap the ends of the vector by approx 200 base pairs. The circular plasmid is used as template for amplification using Taq polymerase. Restriction enzymes (RE1 and RE2) are used to remove the fragment of the vector that has been amplified in the first step. The PCR products and the vector recombine within the region of overlap. Some vectors may religate without incorporating an insert (closure without repair), which will confer a Leu+, Chk1−– phenotype and thus, masquerade as a loss-of-function allele. This background may be alleviated as described in **(B)**.
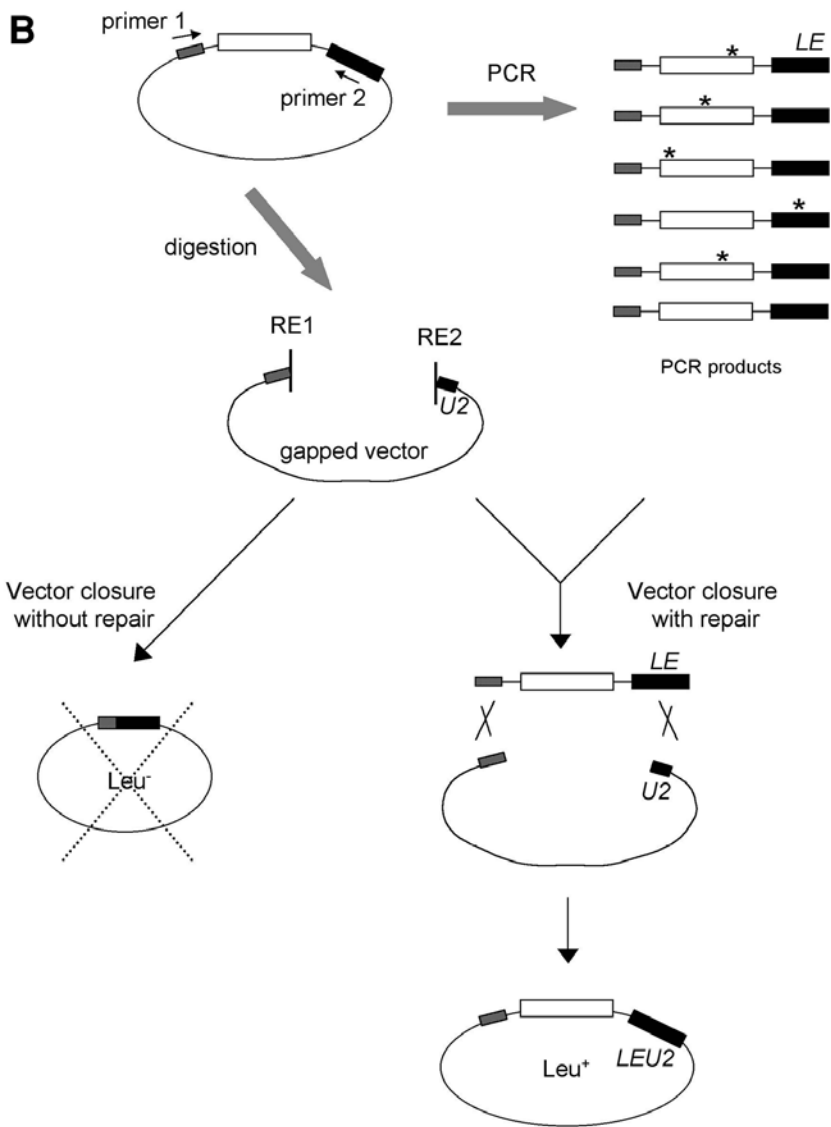
(B) To circumvent the problem of religation without incorporation of an insert, the primers may be designed such that the amplified fragment includes a portion of a selectable marker, in this case, *LEU2*. Restriction enzymes must be chosen appropriately, as well, to generate the gapped vector. If the vector religates without incorporating an insert, then the plasmid will not have a selectable marker and, therefore, will be unable to support growth on media lacking leucine (in this example). Thus, only vectors that have recombined with a PCR-amplified insert will generate Leu+ colonies.

2. Materials

2.1. Introduction of Random Mutations by PCR

1. Taq polymerase buffer.
2. $MgCl_2$.
3. $MnCl_2$ (optional).
4. dCTP, dATP, dGTP, dTTP.
5. Template DNA.
6. Taq polymerase.

2.2. Preparation of Gapped Vector

1. Vector DNA.
2. Restriction endonucleases to generate the gapped vector.
3. Agarose gel and kit for fragment purification.

2.3. Transformation of S. pombe

1. Yeast.
2. Media.
3. Lithium acetate (LiAc), pH 4.9; keep a 1 M stock solution. Prepare a fresh 100 mM solution for every transformation.
4. 50% PEG3350; must be filter sterilized. Prepare in advance because slow to filter.
5. 5-fluoro-orotic acid (5-FOA).

2.4. Screen for Loss-of-Function Alleles

1. Transformed yeast colonies.
2. Media.
3. Camptothecin lactone (CPT), stock of 40 mM prepared in dimethyl sulfoxide (DMSO) and stored at –20°C.

2.5. Recovery of Plasmid DNA from Yeast

1. SP#1 for 100 mL: Dissolve 21.8 g sorbitol, 0.71 g Na_2HPO_4, 1.15 g citric acid, 1.48 g ethylenediaminetetraacetic acid (EDTA) in 80 mL of ddH_2O. Adjust pH to 5.6 and then complete to a 100 mL volume with ddH_2O. Autoclave.
2. Zymolyase; store powder at 4°C and prepare fresh at a concentration of 0.2 mg/mL with SP#1 before use.
3. NaCl.
4. EDTA.
5. Tris-HCl pH 7.5.
6. 10% sodium dodecyl sulfate (SDS).
7. 10% Triton X-100.
8. Proteinase K.
9. ϕ/chloroform/isoamyl alcohol.
10. 70% ethanol.
11. TE.

12. DH5α electrocompetent cells.
13. SOC media.
14. LB liquid media and plates.
15. Ampicillin; keep powder at –20°C. Prepare 1000X (50 mg/mL) stock solution; aliquot and store at –20°C.

3. Methods

The general scheme for mutagenesis and isolation of loss-of-function alleles is depicted in **Fig. 1**. Selection of CPT-sensitive *chk1* alleles was conducted in a strain in which about half of the *chk1* coding sequence is replaced with the *ura4* gene (*chk1::ura4*) *(6)*. This null allele confers sensitivity to agents that damage DNA. This strain also harbors a mutation in the *leu1* gene that can be complemented by the *S. cerevisiae LEU2* gene encoded on the plasmid. Gap repair of the vector with the PCR products generates a "library" of cells bearing various mutant alleles, which is then screened for those mutants that confer a loss of function. Loss-of-function alleles that encode full-length proteins are identified, and the DNA is recovered for integration at the *chk1::ura4* locus. Cells that replaced the *ura4* gene with the loss-of-function allele of *chk1* were selected on minimal media plates *(9)* containing 5-FOA, which selects for cells that have lost the *ura4* gene product *(10)*.

3.1. Design of Gapped Vector and Selection of Primers for PCR Products

The region to be mutagenized must be removed from the vector; therefore, it is useful to have restriction enzyme sites close to the region of interest (**Fig. 2A**). Primers for the PCR reaction are then designed such that they will overlap the ends of the vector once the region of interest is removed. We chose to design the oligonucleotide primers such that there were approx 200 bp of overlap at each end. To ensure the selection of circular plasmids that incorporated a PCR product, the region of the vector that was removed included a portion of the *LEU2* gene (*see* **Note 2** and **Fig. 2B**). Thus, one of the PCR primers was designed to anneal within the *LEU2* gene. To facilitate the detection of proteins produced by the mutant alleles, the template for the PCR reaction included a fragment encoding 3 HA epitopes at the C-terminal end of the gene (*see* **Note 3**). This would not be necessary if a specific antibody for the protein of interest were readily available.

3.2. Introduction of Random Mutations by PCR

The oligonucleotides (oligos) used for this PCR reaction were 20 bases long, with a melting temperature of approx 55°C. The product amplified was 3.8 kb.

Taq polymerase is sufficiently error prone to allow for the introduction of random mutations during amplification with standard manufacturer-defined Taq polymerase conditions. Cycle times and temperature may need to be adjusted depending on the primers and the length of the desired product:

3.2.1. PCR Reaction Components

1. 1X Taq buffer.
2. 2 mM MgCl$_2$.
3. 50 ng template.
4. 600 ng oligo one.
5. 600 ng oligo two.
6. 0.2 mM dCTP.
7. 0.2 mM dATP.
8. 0.2 mM dGTP.
9. 0.2 mM dTTP.
10. 10 U of Taq.

3.2.2. PCR Reaction Cycles

1. 94°C 3 min.
2. 94°C 30 s.
3. 55°C 1 min/kb.
4. 72°C 4 min.

Repeat **steps 2–4** 30 times. PCR product is purified using the Qiagen QIAquick PCR purification kit. The concentration of the purified product is determined by ethidium bromide staining of an agarose gel containing the purified PCR product by comparison to a DNA standard.

3.3. Preparation of Gapped Vector

1. Digest 1 µg of DNA.
2. Separate fragments by agarose gel electrophoresis.
3. Purify desired linear fragment using Qiagen QIAquick gel extraction kit.
4. Determine concentration of the fragment as above (*see* **Subheading 3.2.2.**).

3.4. Transformation of S. pombe.

Approx 500 ng each of the gapped vector and PCR product are cotransformed into yeast using the following LiAc procedure *(11)*:

1. Grow a 50-mL culture to a density of 5×10^6 to 1×10^7 cells/mL.
2. Harvest cells by centrifugation and wash with 20 mL of sterile ddH$_2$0.
3. Wash cells with 20 mL of 100 mM LiAc pH 4.9.
4. Resuspend cells in 100 mM LiAc pH 4.9 to a density of 1×10^9 cells/mL.
5. Incubate at permissive temperature (30°C for non-temperature-sensitive strains) for 30 min.

6. During incubation, aliquot 500 ng of PCR product and 500 ng of gapped vector DNA in a total volume of 10 µL TE into 5-mL sterile 12 ×75 mm polypropylene tubes.
7. After **step 5** is completed, add 100 µL of cells to the aliquots of DNA prepared in **step 6**. Mix gently.
8. Incubate at permissive temperature for 30 min (no shaking).
9. Add 290 µL of 50% PEG3350. Mix gently with vortex on low speed.
10. Incubate at permissive temperature for 30 min (no shaking).
11. Heat shock cells by incubating at 42°C for 15 min (no shaking).
12. Spin down cells in tabletop centrifuge. Remove supernatant by aspiration.
13. Resuspend in 200 µL of minimal media and spread on minimal media plates that select for Leu⁺ colonies.

3.5. Screen for Loss-of-Function Alleles

Leu⁺ colonies should either be replica plated or patched to restrictive conditions to screen for the loss of function of the mutagenized gene *(9)*. For isolation of *chk1* alleles sensitive to DNA damage caused by CPT, colonies were patched to plates containing 5 µM CPT.

To check that loss-of-function phenotype is conferred by a point mutation in the full-length gene Western blot analysis should be performed. The preparation of protein lysates for Western blot of Chk1p consists of the following steps:

1. Harvest midlog-phase cells (5×10^6 cells/mL) by centrifugation.
2. Resuspend cell pellet in 100 µL of RIPA buffer.
3. Transfer to microcentrifuge tube.
4. Add acid-washed glass beads to the meniscus.
5. Break in FastPrep©, setting of 6.5, 1×20 s.
6. Pellet insoluble debris and unbroken cells by centrifugation at 735*g* (3000 rpm) for 5 min.
7. Determine protein concentration of supernatant using Bradford assay.
8. Run 100–200 µg of total protein in an 8% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE).
9. Transfer to nitrocellulose filter.
10. Western blot using anti-HA antibodies.

3.6. Identification of Mutation That Confers the Phenotype

3.6.1. Recovery of Plasmid DNA from Yeast

1. Grow a 10-mL culture to stationary phase.
2. Harvest cells and wash in 1 mL of SP#1. Transfer to a microfuge tube. Pellet the cells by spinning for 5 s at top speed in microfuge.
3. Resuspend the pellet with 1 mL of SP#1 and Zymolyase (0.2 mg/mL).
4. Incubate at 36.5°C for 45–60 min. Check that cell wall removal is sufficient by attempting lysis with SDS treatment. Cells with an intact cell wall are impervious to SDS. If the integrity of the cell wall has been compromised, then the sphero-

plasts will burst when exposed to SDS. This can be monitored by mixing 10 µL of cells with 1 µL of 10% SDS and examining under the microscope. Debris and cell wall ghosts will be apparent if cells have experienced sufficient cell wall removal.

5. Spin 2 min at 735g (3000 rpm) in a microcentrifuge to pellet spheroplasts.
6. Resuspend in 300 µL of 50 mM EDTA, 50 mM Tris-HCl pH 7.5, add 33 µL of 10% Triton X-100 to lyse cells.
7. Spin 10 min at 325g (2000 rpm) to remove unlysed cells. Transfer supernatant to a fresh tube.
8. Spin for 10 min at 8160g (10,000 rpm) to pellet nuclei. Discard supernatant.
9. Resuspend pellet in 500 µL of 0.3 M NaCl, 50 mM EDTA, 50 mM Tris-HCl pH 7.5; add 55 µL of 10% SDS (sample can be stored at –20°C).
10. Add 5.5 µL of 10 mg/mL proteinase K. Incubate at 50°C for 3 h.
11. Extract with φ/chloroform 3 times until aqueous phase is clear.
12. Add 1 mL of 100% ethanol to the aqueous phase. Mix gently and centrifuge at top speed in a microcentrifuge for 15 min to precipitate DNA (sample can be stored at –20°C).
13. Rinse the pellet with 70% ethanol. Air-dry the pellet.
14. Resuspend the pellet in 200 µL of TE (sample can be stored at 4°C as DNA goes into solution).
15. Add 4 µL of 10 mg/mL ribonuclease A (RNase A); incubate at 37°C for 1 h.
16. Extract twice with φ/chloroform.
17. Add 20 µL of 3 M sodium acetate and 440 µL of ethanol to the aqueous phase and mix gently (sample can be stored at –20°C).
18. Pellet DNA by centrifugation for 15 min at top speed in a microcentrifuge.
19. Rinse the pellet with 70% ethanol. Air-dry.
20. Resuspend the pellet in 20 µL of H$_2$0. Use 2 µL of DNA for electroporation of DH5α electrocompetent cells.

3.7. Transformation of Electrocompetent Cells

1. Thaw DH5α electrocompetent cells on ice.
2. Combine 40 µL of competent cells and 2 µL of DNA in a microfuge tube. Transfer to an electroporation cuvette.
3. Place cuvette in the electroporation chamber. Electroporate using 1500 V, 25 mA, 25 W (conditions may vary with instrument).
4. Immediately add 1 mL of SOC to the cuvette and transfer to a culture tube.
5. Recover for 1 h at 37°C.
6. Harvest cells by centrifugation, resuspend in 100 µL of LB, and spread on LB plates containing ampicillin.

3.8. Plasmid DNA Isolation for Sequencing

Plasmid DNA is isolated from ampicillin-resistant DH5α cells using Qiagen miniprep according to the manufacturer's protocol.

3.9. Integration of the Mutant Allele into the Genomic Locus

1. Linearize the DNA containing the mutant allele.
2. Transform DNA using the protocol described in **Subheading 3.4.**, **step 4**.
3. Spread transformants in media that selects for loss of the disrupting gene (*see* **Note 4**).

4. Notes

1. Controlling the degree of mutagenesis: The goal of random mutagenesis is to identify residues that are singly important for the function of a particular gene. Therefore, if multiple mutations are present within the gene, then the determination of which one is actually critical for the function of the protein becomes difficult to determine, requiring the generation of single mutants by site-directed mutagenesis. At the same time, it is theoretically and even practically possible that every now and then an allele may be picked up for which there are two mutations, both of which are required for the phenotype. The mutation frequency can be controlled to some extent by varying the conditions under which the PCR is performed. Several protocols exist for deliberately mutagenizing a gene by PCR by varying the level of a single nucleotide or including $MnCl_2$ in the reaction buffer. In our experience, varying the level of a single nucleotide resulted in multiple mutations in the 3.8 kb fragment of *chk1/LEU2* that we amplified. Therefore, we used the manufacturer's recommended conditions for amplification with Taq polymerase. Of the alleles that encoded full-length Chk1 proteins, 4 had single mutations and two had double mutations *(2)*. For a second gene under investigation in our laboratory, we have found it necessary to add $MnCl_2$ and vary the concentration of a single nucleotide. In that case we increased the concentration of dGTP in the reaction mix by 10-fold.
2. Inclusion of a selectable marker in the gapped region: To guard against vector closure without incorporation of an insert, we included in our PCR product approximately half of the *LEU2* selectable marker. Thus, only gapped vectors that had recombined appropriately with the PCR product would generate plasmids that gave rise to Leu[+] colonies on plates lacking leucine. We did not test whether this step was strictly necessary. However, based on experience attempting to gap repair a plasmid from genomic sequence (as a means of determining the identity of a genomic mutation), vector religation without repair is not uncommon. The cotransformation of the gapped vector with PCR product may circumvent this problem by providing significantly more recombination target than is present in single copy in the genome.
3. Full-length vs premature termination mutants: An easy way to inactivate any protein is to insert stop codons midway through the coding sequence resulting in a truncated protein (or incomplete and unstable message). With this in mind, we amplified by PCR a version of the gene that encoded a triple HA epitope at its C-terminus to permit the detection of full-length proteins by immunoblot with anti-HA antibodies. Only about 1 in 6 clones encoded full-length proteins. Thus, a number of mutants were discarded from further analysis with one round of immunoblotting.

4. Integration of mutant alleles: In our experience, it is essential to integrate mutant alleles into the genome to assess effectively the requirement of any particular amino acid for the function of the protein in vivo. For example, several *chk1* alleles when expressed from multicopy plasmids that probably result in, at most, 10-fold overexpression of the protein, were partially functional. However, on integration, the alleles were found to be phenotypically equivalent to the null allele. Integration is achieved by homologous recombination of a linear fragment with homology to the gene destined for replacement. If the chromosomal copy of the gene is disrupted with the *ura4* gene, then the gene replacement event can be selected in a positive fashion. The product of the *ura4* gene will convert 5-FOA into a toxic compound. Successful gene replacement causes cells to become *ura4⁻* and, therefore, resistant to 5-FOA. If the gene is disrupted with a different selectable marker, such a positive selection is not possible.

Acknowledgments

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In Vitro Mutagenesis to Define Functional Domains

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1. Introduction

The identification of protein domains required for function is an important means of defining biochemical roles for a polypeptide. Our studies on regulatory proteins that function during the transition between mitosis and meiosis have extensively relied on targeted in vitro mutagenesis coupled with in vivo genetic assays *(1,2)*. For example, fission yeast Mei3p is a meiotic activator. It functions by binding to and inhibiting a protein kinase that prevents meiosis *(3,4)*. To perform structure and function studies, we developed a genetic assay to discriminate between active and inactive *mei3* alleles. Systematic deletion mutations were created to define a region of Mei3p that was required for function in vivo *(1,3)*. Next, site-specific alanine scanning mutagenesis was used to further define important functional residues in the active region of the protein *(1)*. This approach led to the identification of a kinase-binding site and has been instrumental in the identification of potential substrates for the kinase. Here, one of the methods used for obtaining site-specific mutations is described.

Mutations created in a gene of interest are usually produced in vitro using a cloned gene. Plasmid deoxyribonucleic acid (DNA) containing the mutated version of the gene is amplified in bacteria. The mutated alleles are frequently transferred to other plasmid vectors suitable for protein expression and purification or in vivo analysis in the experimental organism of choice. We use a plasmid-based mutagenesis system. The system is a modification of the unique site-elimination system originally designed by Deng and Nickoloff *(5)*. In this technique, two mutagenic oligonucleotides are used. One carries the desired mutation and the other, referred to as the selection primer, repairs a small deletion in the β-lactamase gene. A specific plasmid is used for this procedure, BH95NN *(6)*. Major features of BH95NN are illustrated in **Fig. 1**. The gene of
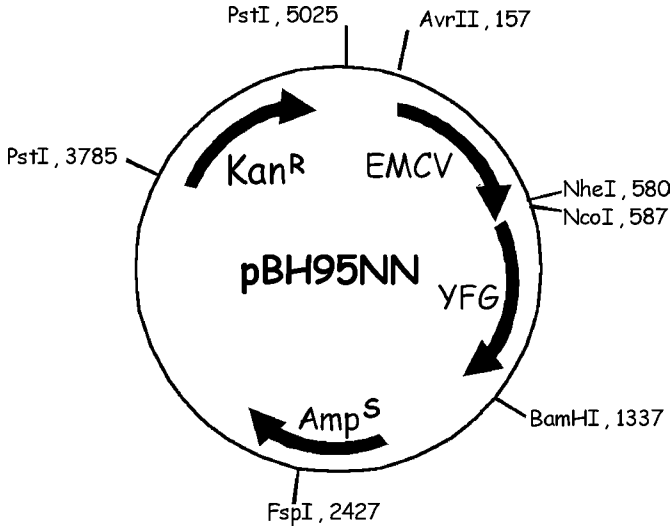
Fig. 1. Diagram of BH95NN.

interest (your favorite gene [*yfg*]) is inserted into the plasmid as a cassette con-
tained on an *Nhe* I to *Bam*H I or an *Nco* I to *Bam*H I DNA fragment. In
addition to YFG, the plasmid carries a kanamycin resistance gene for selection
in *Escherichia coli (E. coli)*.

The plasmid also contains a β-lactamase gene (responsible for the ampicill-
in-resistant phenotype) that has been inactivated by deletion of four base pairs.
The mutagenesis scheme relies on simultaneous annealing of both primers to
the same strand of denatured plasmid DNA. The primed plasmid is elongated
in vitro and used to transform a repair-defective strain of *E. coli*. Ampicillin-
resistant colonies result from transformation by DNA to which the ampicillin
selection primer has annealed and which has been filled in in vitro. This popu-
lation of plasmid DNA is greatly enriched for the mutation in *yfg* that was
specified by the second oligonucleotide.

2. Materials

2.1. Plasmids and Strains

1. The BH95NN plasmid *(6)* was derived from a plasmid constructed in the labora-
   tory of W. T. McAllister (SUNY Downstate Medical School). It has been modi-
   fied to contain unique *Nco* I and *Nhe* I restriction enzyme sites for insertion of
   DNA cassettes bordered on one end by either *Nco* I or *Nhe* I and on the other end
   by *Bam*H I restriction enzyme sites.
2. All plasmid DNA was purified using Qiagen columns (Qiagen, Valencia, CA).
3. The mutagenic reaction mixture is transformed into *E. coli* strain NM522 mutS
   [*supE, thi-1, Δ(lac-proAB), Δ(hsdMS-mcrB-)5*, [*mut*S::Tn10], [F *proAB lacIq,*D

(*lacZ*)*M15*]. This strain is available from our laboratory. It was originally obtained from Pharmacia Biotech (now Amersham Pharmacia Biotech).

2.2. Microbiological Media

1. 1 L SOB Medium: 20 g bacto-tryptone, 5 g yeast extract, 0.584 g NaCl, 0.186 g KCl. Mix components and adjust pH to 7.0 with NaOH and autoclave.
2. 2 *M* Mg^{++} Stock: 20.33 g MgCl$_2$ 6H$_2$O, 24.65 g MgSO$_4$ 7H$_2$O, dH$_2$O to 100 mL. Autoclave or filter sterilize.
3. 2 *M* glucose: 36.04 g glucose. dH$_2$O to 100 mL. Filter sterilize.
4. SOC Medium: Add 1 mL 2 *M* Mg^{++} stock and 1 mL 2 *M* glucose to 98 mL SOB.
5. 1 L LB + Ampicillin Plates: 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Mix components and adjust pH to 7.0 with NaOH and autoclave. Add ampicillin as a powder to a final concentration of 80 mg/L.
6. For LB – NaCl + Tetracycline: Omit the NaCl and add tetracycline as a powder to 15 mg/L.

2.3. Buffers and Reagents for Mutagenesis

1. 10X polynucleotide kinase buffer: 500 m*M* Tris-HCl (pH 7.6 at 25°C), 100 m*M* MgCl$_2$, 50 m*M* dithiothreitol (DTT), 1 m*M* spermidine, 1 m*M* ethylenediamine-tetraacetic acid (EDTA).
2. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
3. 5 *M* ammonium acetate: 38.54 g ammonium acetate dissolved in 50 mL deionized H$_2$O. Use NaOH to bring to pH 7.5 and add H$_2$O to a final volume of 100 mL.

2.4. Synthesis and Purification of Oligonucleotides

All oligonucleotides were obtained from a commercial source and purified using 12% denaturing acrylamide/urea gels followed by desalting through a Sep-Pac™ C18 column (ABI-Perkin Elmer). The sequence of the BH 44 oligonucleotide, which is used to repair the β-lactamase gene is:

5' CGTGACACCACGATGCCCGCGGCAATGGCAACAACGTT-3'

3. Methods

3.1. Mutagenesis

1. Prepare plasmid DNA (pBH95NN/YFG) for mutagenesis by resuspending 25 pmol DNA in 1.0 mL H$_2$O. Oligonucleotides supplied in lyophilized form from the supplier are at 100 μ*M* in H$_2$O.
2. Phosphorylate the mutagenic oligonucleotide and the BH44 oligonucleotide in separate tubes (*see* **Note 1**). Set up kinase reactions according to the following table:

Oligonucleotide	3.0 μL (300 pmol)
10 × T4 polynucleotide kinase buffer	3.0 μL
10 m*M* ATP	3.0 μL
T4 polynucleotide kinase (10 U/μL)	1.0 μL
H$_2$O	20.0 μL
Total reaction volume	30.0 μL

3. Incubate reaction at 37°C for 30 min. Stop the reaction by incubation at 65°C for 10 min. The phosphorylated oligonucleotides are used directly for the following step.

4. Anneal the phosphorylated oligonucleotides to 0.025 pmol plasmid according to the following table:

pBH95NN/YFG	1.0 μL (0.025 pmol)
10X T4 polynucleotide kinase buffer	2.0 μL
Mutagenic oligonucleotide (1.25 nmol/mL) (*see* **Note 2**)	1.0 μL
BH44 oligonucleotide (1.25 nmol/mL)	1.0 μL
H₂O	15.0 μL
Total reaction volume	20.0 μL

5. Incubate the reaction mixture at 100°C for 5 min to denature the plasmid DNA. Chill on ice for 5 min (*see* **Note 3**). Centrifuge at 10,000*g* briefly to collect the mixture in the bottom of the tube. Incubate at room temperature for 30 min.

6. Set up the polymerization reaction by adding the following reagents to the test tube:

10X T4 ligase buffer	3.2 μL
2.5 m*M* each dNTP	7.0 μL
T4 DNA polymerase	1.0 μL (1 U)
T4 DNA ligase	0.5 μL (2.5 U)
Total reaction volume	31.7 μL

7. Incubate the reaction mixture at 37°C for 1 h. Stop the reaction by incubation at 85°C for 15 min. Centrifuge the reaction mixture briefly and incubate on ice. Alternatively, the mixture may be frozen for storage prior to transformation.

3.2. Precipitate DNA for Electrotransformation (see Note 4)

1. Add 10 μg of transfer RNA (tRNA) to a 40-μL ligation reaction. Add 20 μL 5 *M* ammonium acetate. Mix well.

2. Add 100 μL absolute ethanol. Mix well and incubate on ice for 15 min.

3. Centrifuge at 12,000*g* for 15 min at 4°C. Carefully decant the supernatant.

4. Wash the pellet with 60 μL of 70% ethanol. Centrifuge at 12,000*g* for 15 min at room temperature. Remove the supernatant.

5. Air-dry the pellet by inverting the tube over a piece of absorbent paper.

6. Resuspend the DNA in 20 μL 0.5X TE buffer (5 m*M* Tris-HCl, 0.5 m*M* EDTA, pH 8.0). Use 1 μL per transformation of 2 μL of cell suspension.

3.3. Preparation of Electroporation Cells

1. Use a fresh colony of NM522mutS (or other appropriate host strain) to inoculate 50 mL of LB – NaCl + tetracycline medium in a 500-mL flask. Grow cells with vigorous aeration overnight at 37°C.

2. Dilute 10 mL of cells into 0.5 L LB – NaCl + tetracycline in a 2-L flask. Grow for 2 to 3 h with vigorous aeration at 37°C until the cells reach an A_{600} 0.5–0.8.
3. Harvest cells by centrifugation in sterile bottles at 2600g in a GSA rotor for 10 min. at 4°C.
4. Wash cells in 1 L ice-cold H_2O and pellet again.
5. Wash cells in 500 mL ice-cold H_2O and pellet again.
6. Wash cells in 20 mL ice-cold 10% glycerol (in H_2O) and pellet. (*see* **Note 5**).
7. Resuspend cells in a final volume of 2 mL 10% glycerol.
8. Use immediately or freeze in 40-µL aliquots in microcentrifuge tubes using a dry ice-ethanol bath. Store the frozen cells at –70°C.

3.4. Electroporation

1. Chill cuvette (0.2-cm gap) on ice.
2. Thaw an aliquot of cells. Add 1 µL DNA prepared as described in **Subheading 2.1.**
3. Using a micropipet, pipet the cell-DNA mixture into the electroporation cuvette. (*see* **Note 6**).
4. Electroporate at 200 ohm, 25 µF and 2.5 kV (*see* **Note 7**).
5. Immediately add 1 mL SOC medium to the cells and transfer the mixture to a culture tube (*see* **Note 8**).
6. Transfer the cell suspension to a Falcon 2059 tube and incubate at 37°C. Shake slowly at about 225 rpm, and continue incubation for 30–60 min.
7. Plate out on LB medium supplemented with 80 µg/mL ampicillin.

4. Notes

1. Oligonucleotides can be purchased already phosphorylated from the supplier. Purification of oligonucleotides by denaturing polyacrylamide gel electrophoresis (PAGE) is highly recommended and is a supplier-provided service. For a discussion of oligonucleotide design, see the technical literature on Altered Sites® ll in vitro mutagenesis system from Promega. It can be obtained via the Internet at (www.promega.com).
2. The frequency of obtaining mutations in yfg varies, most likely owing to the specific sequence of the primer or of the target DNA. One method that may increase the frequency of obtaining mutations is to increase the amount of mutagenic oligonucleotide added to the reaction (up to 20-fold) but keeping the amount of BH44 oligonucleotide constant.
3. Chill tubes by incubation in an H_2O/ice bath to quickly lower the temperature of the reaction mixture.
4. DNA for electrotransformation must have a very low ionic strength and a high resistance. The success of this mutagenesis method is very dependent on obtaining a high transformation frequency.
5. Cells washed in 10% glycerol do not pellet well. If the supernatant is turbid, increase the centrifugation time.

6. Do not leave an air bubble in the droplet of cells; the pressure of a bubble may cause arcing and loss of the sample.
7. The time constant should be in the 3–5 ms range.
8. It is most important to immediately add 1 mL of SOC media to the cuvet directly. Mix SOC with the cells quickly, but gently.

Acknowledgments

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Use of Gene Overexpression to Assess Function in Cell Cycle Control

Erik K. Flemington and Antonio Rodriguez

1. Introduction
1.1. General Introduction

Transient overexpression of wild-type or mutant cell cycle regulatory factors is a quick and relatively easy means to derive important information regarding the function of the respective factor in regulating cell cycle checkpoint pathways. The general approach for these studies is to introduce an expression vector for the gene of interest into a relevant cell type and assess alterations in cell cycle progression. There are numerous means for introducing expression vectors into cells, including a number of different transfection methods as well as various viral vector technologies. Because it is impossible to detail the protocol for each of these different methods in this chapter, the use of the calcium phosphate method will be discussed as a prototype. The calcium phosphate method is relatively easy, it is efficient in a number of cell lines, and it is inexpensive.

Typically, less than 100% transduction efficiency is obtained by most transfection methods. To specifically address cell cycle alterations in cells expressing the gene of interest, cotransfections are carried out with a small amount of a vector-driving expression of the green fluorescent protein (GFP). A control transfection must also be done in which a control plasmid (empty vector) is cotransfected with the GFP vector. After a certain amount of time following transfection, cells are harvested and subjected to fluorescence-activated cell sorting (FACS). The percentage of cells in G0/G1, S, or G2/M phase can specifically be determined for both the GFP-positive population and for the

GFP-negative population. Analysis of the GFP-negative population is not necessary, but it can serve as an additional control to show that an observed cell cycle alteration in the test cells is specific for cells that successfully took up the deoxyribonucleic acid (DNA).

1.2. Application of Method for Cell Cycle Studies

The most basic application of this method is to assess effectors that activate cell cycle checkpoints. This could take the form of simply testing whether a factor of interest has the capacity to elicit growth arrest, or it could take the form of carrying out genetic studies with factors known to have growth-arrest properties. For example, in proliferating cells, the tumor-suppressor protein, p53, is typically expressed at low levels or is in a functionally inactive form (either through posttranslational modification or through mutation). Transfection of proliferating cells with a plasmid driving the expression of wild-type p53 results in overexpression of functional p53, which typically results in a G1 arrest (or, in some settings, a G2 arrest or both a G1 and G2 arrest), which is not observed in cells transfected with a control plasmid (i.e., empty vector). Genetic studies could then be carried out using the same system. Growth arrest observed by transfecting with p53 mutants can be compared to growth arrest observed with transfection of wild-type p53 to quickly determine the functional consequences of respective p53 mutants in inducing the G1 or the G2 checkpoint.

Transient overexpression experiments can also be used to address the function of factors that mediate progression through a cell cycle checkpoint or activate cell cycle progression. One approach is to assess whether a dominant negative mutant of the respective factor leads to growth arrest. From a practical standpoint, this is probably the best approach and will typically yield the most easily interpretable data. Alternatively, overexpression of the wild-type protein can sometimes lead to shortening of the G1 phase of the cell cycle (if the factor of interest is involved in mediating G1–S-phase transit). The FACS profile would then show a lower percentage of cells in G1 with a corresponding increase in the percentage of cells in S and G2/M. This approach may not always yield significant results, however. First, even though a factor is involved in mediating progression through a G1 checkpoint, it may not shorten G1 because the checkpoint may not be limiting in the particular cell system being used. Further, even if G1 is indeed shortened, it will typically only be shortened by a few hours, which will only be a small fraction of the time it takes to transit the entire cell cycle. Therefore, these studies must be repeated and statistical analysis should be carried out.

2. Materials

1. Membrane-localized green fluorescence protein expression vectors, Us9-GFP *(1)*, or GFP-SP *(2)*.
2. Carrier plasmid.
3. Effector plasmids of interest.
4. 100 m*M* tissue-culture plates (Fisher, cat. no. 08 772E).
5. Dulbecco's modified Eagle's medium (DMEM; Life Technologies cat. no. 11995065).
6. Pen/Strep (Life Technologies cat. no. 15140122).
7. Fetal bovine serum (FBS).
8. 12 × 75 FACS tubes (Fisher cat. no. 14 959 2A).
9. 155-µL pipet tips, filtered (USA Scientific cat. no. 1124-5810).
10. 20-µL pipet tips, filtered (USA Scientific cat. no. 1120-1810).
11. High-pressure (performance) liquid chromatography (HPLC)-purified H_2O (Aldrich cat. no. 32,007-2).
12. 1X HBS (*see* **Note 1** for recipe).
13. 500 mL 0.2-µ filter system (Fisher cat. no. 09761 5).
14. 50-mL centrifuge tube (Fisher cat. no. 14 432 22).
15. 2.5 *M* $CaCl_2$ (*see* **Note 2** for recipe).
16. Rubber policeman (Fisher cat. no. 07 200 366).
17. 15-mL centrifuge tubes (Fisher cat. no. 05 527 90).
18. 1X phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.4.
19. 1X PBS +0.1% glucose; store at 4°C.
20. 70% ethanol (store at –20°C).
21. 69 µ*M* propidium iodide (PI) solution (Sigma, cat. no. P4170) in 38 m*M* sodium citrate (pH 7.4).
22. 10 mg/mL ribonuclease (RNase; high-quality deoxyribonuclease [DNase]-free RNase should be used, e.g., Sigma, cat. no. R6513).

3. Methods

3.1. Transfection

The method described here is a modification of the original calcium phosphate method but is simpler (i.e., the "drip" technique is not required) and is more reliable and consistent. The calcium phosphate transfection method is a very efficient means of introducing DNA into a variety of adherent cell types. In many transformed cell lines, transfection efficiencies between 20 and 90% are common. In other cells, however, different transfection procedures, such as liposome-mediated transduction will have to be used. Nevertheless, the principles discussed here should carry over to these other methods and should yield similar results.

3.1.1. Effector-Expression Plasmid

Most transfection methods are quite sensitive to the amount of total-input DNA and this is the case for calcium phosphate transfection. The optimal amount of total DNA that should be used per transfection is 30 µg (for a 100-mm culture plate). This amount will yield the greatest percentage of cells that successfully take up DNA.

Plasmids that contain strong promoters can signal cell-growth-arrest pathways and can induce transient G0/G1 growth arrest as well as apoptosis *(3)*. In general, as little expression plasmid as possible should be added to the transfection to try to minimize any possible influences of this type of signaling on the outcome of the experiment. In addition, minimizing the amount of effector plasmid will help avoid superphysiological levels of any protein that could have a nonphysiological impact on the cells. Typically between 100 ng and 1 µg of a cytomegalovirus early promoter/enhancer-based expression vector will yield significant levels of expression of the gene of interest and will yield physiological effects on the cell cycle control machinery. On the other hand, weaker promoters, such as the SV40 promoter/enhancer have a smaller effect on cell cycle signaling and yield lower expression. If a weaker promoter is to be used, higher amounts of the plasmid can be added without significantly affecting the quality of the results.

3.1.2. GFP-Expression Plasmid

In addition to the expression vector driving the gene of interest, an expression vector containing the GFP must also be added to the transfection to allow selection of successfully transfected cells for DNA-content analysis. Again, the amount of the GFP-expression vector used in a transfection should be low to minimize promoter effects. Further, high levels of GFP expression are known to be toxic to many cells *(4)*.

The fixation method that gives the highest quality FACS profiles (ethanol fixation, *see* **Subheading 3.2.**) allows the release of significant amounts of GFP into the supernatant. To avoid having to compensate for this problem by simply adding excess GFP-expression vector, Kalejta et al. have generated GFP-expression vectors, Us9-GFP *(1)* and GFP-SP *(2)*, that contain a GFP open reading cloned in-frame with a membrane localization signal. This keeps GFP tightly bound to intracellular membranes, and little is released from the cell during mild fixation procedures. Using one of these expression vectors, only 50 ng to 250 ng are required for a typical transfection, and these amounts typically yield excellent FACS profiles. The use of these or a related vector is strongly advised for these experiments.

3.1.3. Carrier Plasmid

For transfections using 100-mm plates, a total of 30 μg of DNA should be used. As discussed previously, the effector and GFP plasmids should be minimized and the bulk of the input DNA should be carrier. As a result, the selection of carrier DNA is an important consideration. Typically, plasmid DNA gives higher transfection efficiencies (i.e., a higher percentage of cells that take up DNA) than genomic DNA. The carrier DNA should not contain any known eukaryotic promoters to minimize nonspecific toxic effects. Another consideration is that it is helpful to use a plasmid that grows to high-copy number because large amounts can be consumed quickly in these experiments. Typically, empty cloning plasmids that do not contain promoters should be used.

3.1.4. Transfection Procedure (100-mm Plates)

1. The day before transfection, split cell cultures that are near, but not at, confluency (70–90% confluency). Cells should be split between 1:10 and 1:15 depending on the growth rate of the cells (*see* **Note 3**).
2. The next morning, replace the media on each plate with 8 mL of fresh media, usually DMEM (+10% FBS, +Pen/Strep; *see* **Notes 4** and **5**).
3. Later in the day, cells can be transfected; 30 μg of total DNA should be used for each transfection and the amounts of effector, carrier, and GFP plasmids should be added based on the considerations discussed under **Subheadings 3.1.1–3.1.3.**
4. For each transfection, put 0.5 mL of 1X HBS in a sterile FACS tube. (Whenever possible, the total amount of 1X HBS needed for the experiment should first be mixed with the total amount of GFP and carrier plasmids needed for the experiment; 0.5 mL of this mixture should then be doled out to each FACS tube. In some cases, different amounts of carrier plasmid may be required for different samples. In this case, add a common amount for all samples, and then make up the difference by adding additional DNA to samples that need it after the 1X HBS/DNA mix has been added to the FACS tubes [*see* **step 5** below].)
5. Add appropriate additional DNA to each tube. After adding DNA to all tubes, mix contents of each tube (*see* **Note 6**).
6. Add 30 μL of 2.5 *M* CaCl$_2$ to each tube (mix each tube immediately after adding 2.5 *M* CaCl$_2$ to a tube, then add 2.5 *M* CaCl$_2$ to the next tube, etc.; *see* **Note 7**).
7. Let sit for 20 min in tissue-culture hood. It is best not to go longer or shorter than 20 min.
8. Add transfection mix to plates in a dropwise fashion, attempting to distribute throughout entire plate. Immediately after adding precipitate to a plate, the plate should be rocked back and forth a couple of times to mix. Avoid circular motions because this results in concentration of the precipitate in the center of the plate.
9. After adding precipitate, put plates in a 5% CO$_2$, 37°C tissue-culture incubator and incubate overnight.

10. The next morning (typically, 16 h later), replace media with fresh media containing 10% FBS plus Pen/Strep.
11. Harvest cells 48–72 h later (*see* **Subheading 3.2.**).

3.2. Harvesting and Fixing Cells

1. Harvest cells by gentle scraping with a rubber policeman and transfer cells plus media to a 15-mL centrifuge tube.
2. Spin cells for 5 min at 500*g* and pour off supernatant.
3. Resuspend cells in 5 mL 1X PBS by pipetting up and down several times (*see* **Note 8**).
4. Spin cells for 5 min at 500*g* and pour off supernatant.
5. Suspend cells in 500 µL 1X PBS (+0.1% glucose, stored at 4°C; *see* **Note 9**).
6. Add 5 mL of cold 70% ethanol (keep at –20°C) and mix immediately by rocking back and forth a few times.
7. Keep at 4°C for at least 1 h prior to processing for FACS analysis. At this stage, however, the samples are very stable and can be kept at 4°C for up to 1 mo prior to analysis.

3.3. Preparing Cells for FACS Analysis

In this step, the cellular DNA is stained with PI. The level of PI staining in each cell corresponds to the relative amount of DNA in the respective cell. The number of cells with a 2*N* DNA content (G0/G1 cells), a 4*N* DNA content (G2/M cells), or cells with a DNA content in between 2*N* and 4*N* (S-phase cells) can then be determined by fluorescence-activated cell sorting. More important, PI also stains RNA. Because there is a high abundance of RNA in the cell, RNA staining significantly overrides the signal from DNA. An RNase digestion step is used to diminish the RNA signal so that the DNA profile can be accurately determined.

Chapters 4 and 7 in this book describe flow cytometry in detail.

1. Approximately 2–3 h prior to carrying out FACS analysis, spin down fixed cells for 5 min at 500*g*.
2. Add 1 mL 1X PBS to each tube and resuspend by pipetting up and down.
3. Add an additional 10 mL 1X PBS and mix by rocking back and forth a few times.
4. Spin cells for 5 min at 500*g* and pour off supernatant.
5. Without adding anything else to tubes, spin again for 2 min at 500*g*.
6. Aspirate off residual liquid at the bottom of the tube (*see* **Note 10**).
7. Add 300–500 µL PI solution (stored at 4°C) to each tube and resuspend with a 1-mL pipetman.
8. Add 20 µL of 10 mg/mL RNase solution and vortex briefly (high-quality DNase-free RNase should be used, e.g., Sigma, cat. no. R6513).
9. Incubate tubes at 37°C for 45 min.
10. Transfer to FACS tubes and analyze by FACS.

3.4. FACS Analysis and Interpretation

Running samples in a fluorescence-activated cell sorter is discussed in several other chapters (4 and 7) in this book and is therefore not specifically addressed here. In addition, this procedure is highly machine specific. Therefore, the instructions manual should be used as a reference to carry out this process. Nevertheless, there are a number of points that are important to keep in mind when interpreting data obtained by flow cytometry following transient expression or overexpression of cell cycle regulatory genes. These points stem largely from cell cycle issues intrinsic to DNA transfection. First, during transfection, plasmid DNA can enter the nucleus only during mitosis where nuclear membrane breakdown occurs *(3,5,6)*. In addition, no protein translation occurs during mitosis, so the first expression of the selectable marker (e.g., GFP) and the gene of interest following transfection occurs as the transfected cell enters G1. This issue is illustrated in **Fig. 1** where an extreme bias for cells in G0/G1 is observed in the GFP-expressing population but not in the GFP-negative population immediately after transfection.

A second issue that should be considered in carrying out cell cycle studies using transient transfection is that the introduction of DNA into cells generally results in a transient G1 arrest during the transfection procedure *(3)*. As shown in **Fig. 1**, a greater percentage of G0/G1 cells are observed in cultures subjected to transfection compared to cells treated identically with the exception that they are not exposed to the precipitate. This is not owing to the transfection procedure itself but is instead the result of uptake of DNA into the cells *(3)*. The extent to which this signaling occurs varies in different cell lines but clearly occurs in tumor cells and in p53-positive and p53-negative contexts *(3)*. More important, however, this growth arrest is usually transient, and cells typically reenter the cell cycle at or before the media is changed following transfection.

The combined effect of the cell cycle-specific entry of DNA into cells and the transient growth arrest results in a significantly synchronized population of GFP-positive cells at early times following transfection. In fact, this property may be useful as a synchronization method to follow cell cycle-dependent events that occur specifically in the transfected cell populations *(7)*. From the perspective of the experimental approaches discussed here, these observations have practical importance. In most cases it is necessary to allow this synchrony to resolve into an asynchronous population prior to harvesting cells. Typically, the transfected cell population becomes fairly asynchronous by 30 h following transfection (*see* **Fig. 1**). Therefore, a reasonable harvest point is 48 h. As shown in **Fig. 2**, a distinct difference in the cell cycle profile can be observed at 24 h posttransfection in cells transfected with a gene, *Zta* (the Epstein-Barr

Non-Transfected

Total Cell Population

G0/G1=54%
S=27
G2/M=27

PI Staining

Number of Cells

Immediately After Transfection

Total Cell Population

G0/G1=63%
S=20%
G2/M=17%

PI Staining

Number of Cells

GFP Negative Population

G0/G1=45%
S=26%
G2/M=29%

PI Staining

Number of Cells

GFP Positive Population

G0/G1=89%
S=11%
G2/M=1%

PI Staining

Number of Cells

30hrs Post-Transfection

GFP Negative Population

G0/G1=55%
S=32%
G2/M=13%

PI Staining

Number of Cells

GFP Positive Population

G0/G1=59%
S=28%
G2/M=13%

PI Staining

Number of Cells
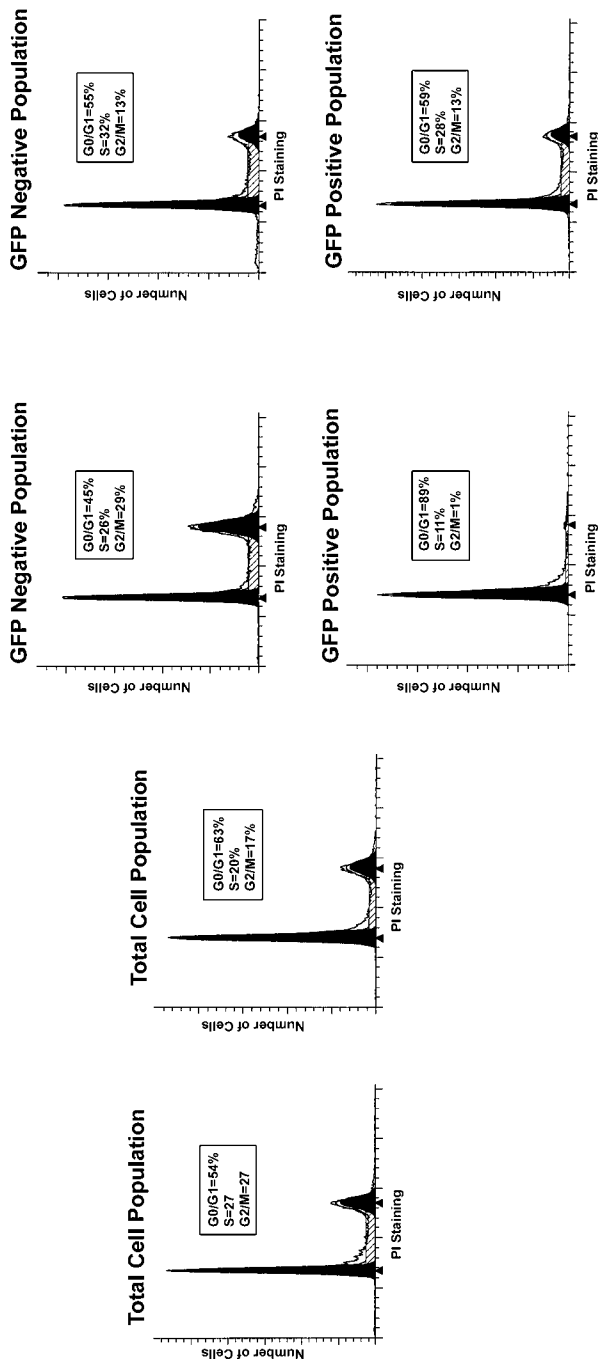
Fig. 1. Hela cells were transfected with 30 µg of the carrier plasmid, pGL3Basic, plus 50 ng of the membrane-localized GFP-expression plasmid, pGFP-SP. Precipitates were left on cells for 16 h, and cells were either harvested immediately or harvested 30 h after changing media. Nontransfected cells were treated identically to transfected cells except that they were not exposed to the precipitate. Nontransfected cells were harvested at a time corresponding to 0 h posttransfection.
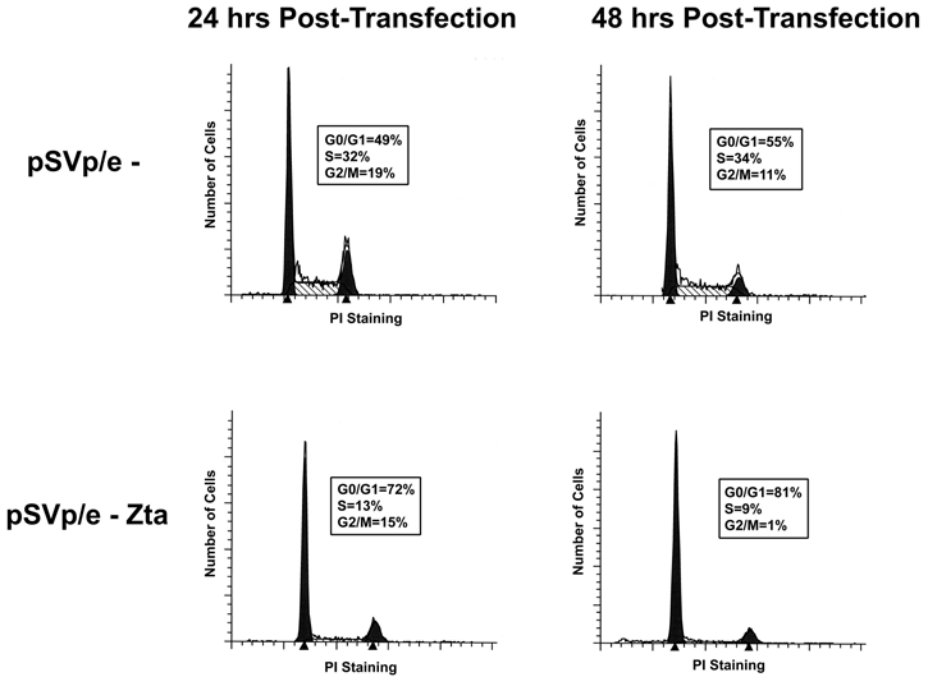
Fig. 2. Cell cycle analysis of a factor that induces G0/G1 cell-growth arrest using transient overexpression. Hela cells were transfected with 1.5 µg of the SV40 promoter/enhancer-based expression vector, pSVp/e-, or pSVp/e-Zta plus 50 ng pGFP-SP and 28.5 µg pGL3Basic. Cells were harvested at 24 h or 48 h posttransfection, and the DNA content of the GFP-positive cells was analyzed.

virus immediate early transactivator), that induces G0/G1 arrest compared to cells transfected with a control plasmid. Nevertheless, better results can frequently be obtained by allowing cultures to go for 48 h prior to harvesting.

Transient transfection analysis allows the analysis of cell cycle factors that induce arrest in a single point in the cell cycle. However, for genes such as p53, which can in some cases induce a G0/G1 or a G2 arrest, the synchronous nature of the transfection method will typically yield synchrony only in the first checkpoint that is encountered following mitosis. For example, if p53 were transfected into cells where it could induce both a G1 and a G2 checkpoint, it is likely that an increase in the percentage of cells in G2 would not be observed using a transient transfection approach because cells would be trapped in the p53-dependent G1 checkpoint. This issue should be taken into consideration when exploring factors known to induce multiple checkpoints or when exploring the properties of previously uncharacterized checkpoint proteins.

4. Notes

1. The pH of the 1X HBS is crucial for high transfection efficiencies. Typically, three batches should be made: one that is pH 7.0, one that is pH 7.1, and one that is pH 7.2. Each of these batches should be tested in transfections with a GFP-expression vector, and the batch that results in the highest percentage of GFP-positive cells (as analyzed by fluorescence microscopy) should be used.

   To make 1 L 1X HBS:
   5 g HEPES (acid).
   8 g NaCl.
   1 g dextrose.
   3.7 g KCl.
   *10 mL $Na_2HPO_4(7H_2O)$ stock solution (*see* directions at end of this section).
   Add HPLC-purified H_2O up to approx 900 mL.
   Adjust pH to exactly 7.0, 7.1, or 7.2 for each batch.
   Add HPLC-purified H_2O up to 1 L.
   Filter through a 0.2 μ sterile filter system (carry this step out in a sterile tissue-culture hood).
   Aliquot into 50-mL sterile centrifuge tubes and keep frozen at –20°C. Keep one working tube at 4°C.
   *$Na_2HPO_4(7H_2O)$ stock solution = 0.94 g of $Na_2HPO_4 \cdot 7H_2O$ in 50 mL HPLC-purified H_2O.

2. Recipe for 2.5 *M* $CaCl_2$:
   To make 100 mL 2.5 *M* $CaCl_2$:
   27.75 g anhydrous $CaCl_2$.
   or
   36.75 g $CaCl_2 \cdot 2H_2O$.
   Add HPLC-purified H_2O up to 100 mL. Sterile operating procedures should be used to prepare 2.5 *M* $CaCl_2$. However, because of the high salt concentration, it is not essential to sterilize this solution. If sterilization is desired, the solution should be autoclaved because the high salt concentration inhibits filtration.

3. It is important to have a relatively high number of cells at the time of harvest for the FACS procedure. On the other hand, the cultures should not be confluent at the time of harvest (although they should be close) because this can result in density-dependent cell cycle effects. Fast-growing cells can generally be split near 1:15 and slower growing cells can be split near 1:10. However, it is helpful to carry out a test transfection with a few different dilutions to determine the optimal conditions that result in high but subconfluent cell numbers. It should also be noted here that the cells will typically slow down temporarily during the period of time that they are incubated with the precipitate. Therefore, it is important to carry out a transfection and not simply test cell growth in the time period of a typical transfection experiment.

4. Unless sterile DNA is used, Pen/Strep must be used to suppress growth of trace amounts of contaminating bacteria in the plasmid DNA.

5. Addition of media a few hours prior to transfection helps keep the cells cycling optimally. This tends to increase transfection efficiency in part because cells only take up DNA into the nucleus during mitosis, when nuclear membrane break-down occurs *(3,5,6)*. Faster cycling cells typically have shorter G1 phases resulting in a higher percentage of cells in mitosis.

6. Mixing of DNA at this stage is important.

7. Mixing immediately after the addition of $CaCl_2$ to each sample is crucial. The high molarity of the 2.5 *M* $CaCl_2$ helps keep it in a separate phase unless sample is mixed. If the sample is left unmixed, unequal precipitate formation occurs with only portions of the DNA sample. This issue is not fully resolved by mixing later.

8. At this time, a portion of cells can be set aside for Western blot analysis to assess expression of the effector. In addition, if reasonably high transfection efficiencies are obtained, it is possible to address changes in the level of cell cycle regulatory proteins that result from overexpression of the effector. Transfer 1.25 mL of cells (from the total of 5 mL) to an Eppendorf tube, spin at 500*g* in a microfuge, and remove the supernatant. Samples at this stage can be prepared as desired for Western blot analysis; however the following procedure typically works reasonably well: Suspend cells in 10 vol 1X sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE)-loading buffer. It is important to pipet up and down vigorously as soon as the buffer is added. The sample will be thick, but make sure that there are no clumps of cells that are not suspended. Poke a hole in the top of the Eppendorf tube with a 20-gage needle and incubate tube for 20 min in a 95°C dry block with occasional vortexing (a double-gloved finger should be used over the hole to prevent leakage of sample during vortexing—double gloving helps protect finger from the heat of the sample). Samples can either be loaded directly onto an SDS-PAGE gel or can be stored at –20°C until used.

9. It is important to distribute cells well at this step by pipetting up and down 10–20 times because the ethanol-fixation step, which is next, tends to make clumps of cells difficult to disrupt. Clusters of two or more cells are gated out during FACS analysis so that they are not interpreted as single cells that contain an abnormally high DNA content.

10. **Steps 5** and **6** are essential because it is important to take off as much residual ethanol as possible. The RNase step that follows is crucial for obtaining an interpretable FACS profile. Residual ethanol may inhibit the RNase digestion and result in obtaining poor FACS data.

Acknowledgments

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Histone Acetylation/Deacetylation
As a Regulator of Cell Cycle Gene Expression

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1. Introduction

Chromatin structure, which plays a key role in the regulation of gene expression, is dynamically regulated by diverse posttranslational modifications. Histone-modifying proteins alter chromatin proteins through phosphorylation, adenosine 5'-diphosphate (ADP)-ribosylation, methylation, ubiquitination, and acetylation *(1,2)*. Histone acetylation involves the transfer of an acetyl group from acetyl-coenzyme A (CoA) to the ε-amino group of lysine side chains within the substrate by histone acetyl transferase enzymes (HATs). Deoxyribonucleic acid (DNA) in eukaryotes is typically packaged as repeating arrays of nucleosomes, in which 146 bp of DNA are wrapped around a histone octamer consisting of four histone proteins (H2A, H2B, H3, and H4). As the charged NH_2-terminal histone tail protrudes from the nucleosome, posttranslational modification of the histone tails results in substantial changes in charge and electrostatic forces within the chromatin and between the chromatin and DNA. Thus acetylation of specific lysines of amino-terminal histone tails changes the interaction with other proteins within the chromatin structure and facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene, leading to transcriptional activation *(3,4)*. Acetylation of lysine residues requires energy and is reversible through histone deacetylases (HDACs), which may be either nicotinamide adenine dinucleotide (NAD)-dependent or NAD-independent. The modification of the lysine groups of core histones by multiple posttranslational events, including phosphorylation and acetylation coincident with activation of mitogenic signaling, has led to a model in which the N-terminal tail of the core histone is considered to function as a "signaling platform" *(5)*.

Multiple signaling pathways converge on these signaling platforms providing a dynamic epigenetic mechanism-regulating gene expression.

Recent studies have identified multiple distinct substrates for HATs, including transcription factors, nuclear transport proteins, and cytoskeletal proteins *(3,6)*. Nonhistone proteins that are directly acetylated include a subset of transcription factors and coactivators (p53, the Kruppel-like factor (EKLF), HMG1 (Y), GATA-1, E2F-1, androgen receptor, estrogen receptor, and a p160 coactivator, ACTR) *(7–12)*. Acetylation of transcription factors may either enhance or inhibit transcriptional activity. p300/CBP-dependent acetylation enhanced the activity of the tumor suppressor p53, EKLF, and the erythroid cell-differentiation factor, GATA-1 *(13)*. In contrast, acetylation of the coactivator ACTR or ERα contributed to an inhibition of hormone-induced nuclear receptor signaling *(14)*. Although the mechanisms by which acetylation regulates transcription-factor function remain unclear even for p53, the first transcription factor demonstrated to be a target of acetylation *(15)*, the possibilities include alterations in DNA binding, chromatin access, coactivator recruitment, or as recently shown, disengagement of corepressors, including HDAC/N-CoR *(16)*.

In addition to changes in chromatin organization that occur during cell cycle transition, specific physical interactions occur between components of the cell cycle regulatory apparatus and proteins regulating histone acetylation *(6)*. Transition through the G1 phase of the cell cycle is regulated by cyclin-dependent kinase holoenzymes consisting of a regulatory subunit (cyclin D or cyclin E) and a catalytic subunit (cdk4/6). The HAT P/CAF physically interacts with cyclin D1 to regulate activity of both the estrogen and androgen receptor *(17,18)*. The application of methods such as the in-gel HAT assay will be important in the identification of new HATs. The use of in vivo HAT assays to understand the signal-transduction pathways regulating HAT activity for specific substrates is also pivotal in understanding the biological significance of acetylation and its role in human disease.

2. Materials

2.1. Cell Culture

The 293T cells are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin and streptomycin (10,000 U/mL, GIBCO-BRL). Cells were transfected by Superfect.

2.2. Reagents

1. Histone H2A, H2B, H3, H4 (Boehringer Mannheim) are dissolved in ddH$_2$O, distributed into 10-mL aliquots, and stored at –80°C.

2. Antibody against p300 (C20; Santa Cruz Biotechnology).
3. p300 can be generated in baculovirus and purified.
4. [^{14}C] acetyl-CoA, [^{3}H] acetyl-CoA, [^{3}H] acetic acid, sodium salt (Amersham Pharmacia Biotech).

2.3. Equipment

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.
2. Liquid scintillation counter.
3. Orbital shaker.
4. Phosphor image system.
5. Centrifuge.
6. Cell culture incubator.
7. Water bath.

2.4. Buffers

1. Phosphate-buffered saline (PBS).
2. New RIPA buffer: 50 m*M* HEPES [N- (2-hydroxyethyl) piperazine-*N*'- (2-ethane-sulfonic acid), pH 7.2], 150 m*M* NaCl, 1 m*M* ethylene glycol *bis* (2-aminoethyl ether)-*N,N,N*'*N*'-tetraacetic acid (EGTA), 1 m*M* ethylenediaminetetraacetic acid (EDTA). Buffer containing the above compounds can be made as a large volume, filtered through a 0.22 µm filter, and stored at room temperature (RT). Prior to use, the appropriate volume is aliquoted and chilled on ice. Tween-20 is added to a final concentration of 0.1% (v/v), 1 m*M* dithiothreitol (DTT), 1 m*M* phenyl-methylsulfonyl fluoride (PMSF), 0.1 m*M* sodium orthovanadate, and 2.5 m*M* leupeptin just prior to use.
3. IB buffer: 10 m*M* Tris-HCl pH 7.4, 2 m*M* MgCl$_2$, 3 m*M* CaCl$_2$, 10 m*M* sodium butyrate, and 1 m*M* PMSF.
4. NIB buffer: IB buffer supplemented with 1% Nonidet P-40.
5. In vitro HAT assay buffer (for 2X stock buffer): 100 m*M* Tris-HCl pH 8.0, 20% glycerol, 100 m*M* NaCl, 20 m*M* butyric acid, 0.2 m*M* EDTA, 2 m*M* DTT, 2 m*M* PMSF.
6. HDA buffer: 50 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, 10% glycerol.

3. Methods

3.1. Purification of Core Histone Substrate

3.1.1. Cell Labeling *(see **Note 1**)*

1. Six liters of HeLa cells are grown to a density of 5×10^5 cells/mL in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% newborn calf serum, 1% fetal calf serum (FCS), 0.1 g/L streptomycin, and 0.06 g/L penicillin.
2. The cells are centrifuged at 500*g* and resuspended in 120 mL of cold PBS.
3. The solution is resuspended to a final concentration of 100 µg/mL cyclohexim-ide, 10 m*M* sodium butyrate, and 0.2 mCi/mL [^{3}H] acetic acid and incubated for 1 h at 37°C with gentle stirring.

3.1.2. Isolation of Core Histone Substrates

1. Cells are chilled on ice and centrifuged at 500*g* for 5 min.
2. The cells are washed three times in 50 mL of PBS supplemented with 10 m*M* sodium butyrate.
3. Cells are then lysed in 40 mL of NIB buffer.
4. Nuclei are collected (500*g*) and washed twice in 40 mL of NIB buffer followed by one wash with NIB buffer supplemented with 100 m*M* NaCl.
5. One additional wash is performed in 40 mL of 100 m*M* NaCl and IB buffer.
6. The nuclei are then extracted twice in high salt, 40 mL of 400 m*M* NaCl and IB buffer, followed by centrifugation.
7. The nuclear pellet is extracted twice in 10 pellet volumes of 0.2 *M* H_2SO_4 for 90 min on ice and centrifuged at 30,000*g* for 25 min.
8. The supernatants are pooled and dialyzed extensively at 4°C against 100 m*M* acetic acid.
9. The extracted histones are lyophilized and resuspended in H_2O to a concentration of 4 mg/mL (*see* **Note 2**).

3.2. Preparation of Nonhistone Substrates (**see** *Note 3*)

1. The bacteria are grown in 2X YT medium supplemented with 100 µg/mL ampicillin. The cells are grown to an optical density of $OD_{600} = 0.5$.
2. 1 µL/mL of 100 m*M* isopropylbeta-D-thiogalactopyranoside (IPTG) is added to induce glutathione-*S* transferase (GST) fusion protein expression. For 500 mL medium, 500 µL of IPTG is added.
3. The cells are grown at 37°C for 2 h, or temperature is reduced to 30°C and cells grown for 4 h.
4. The bacteria are pelleted using a Beckman 10,500 rotor, and washed once with PBS.
5. Resuspend in 15 mL of ice-cold buffer A (PBS supplemented with 2 m*M* DTT, 1 m*M* PMSF, 1 mg/mL pepstatin, 1 mg/mL leupeptin, 1 mg/mL aprotinin).
6. The sample is kept on ice with 2 mg of lysozyme for 10 min.
7. Sonicate on ice until the solution appears clear.
8. The sample is mixed with 1 mL of 15 % Triton X-100 in PBS and centrifuged at 100,000*g* for 15 min at 4°C.
9. At the same time, 500 µL of Sepharose 4B beads are washed three times with buffer B (PBS supplemented with 0.5% Tween-20, 0.5% Triton X-100, 1 m*M* PMSF, 1 mg/mL leupeptin).
10. The washing buffer is removed after the last wash, and the supernatant from **step 8** is placed in the beads.
11. The sample is shaken and stored at 4°C for 6 h to overnight.
12. The supernatant is removed and the beads washed with buffer C (PBS supplemented with 2 m*M* DTT, 0.5% Tween-20) three times. After the last wash, transfer the beads into an Eppendorf tube and remove supernatant.

13. The beads are washed with buffer D (20 m*M* Tris-HCl, pH 8.0, 2 m*M* DTT) three times. After the last wash, add back 400 µL buffer D.
14. The beads are incubated with 50 µL of glutathione (GSH) (100 m*M* in stock, Tris-HCl pH 7.8) at RT for 30 min. The sample is centrifuged at 3000*g* in a benchtop centrifuge, and the supernatant is collected.
15. The GSH is dialyzed from the sample with dialysis buffer (20 m*M* Tris-HCl pH 8.0, 25 m*M* NaCl, 2 m*M* EDTA) using three changes of buffer.

3.3. In Vitro-Immunoprecipitation (IP) HAT Assay

3.3.1. Cell Extract Preparation

1. Cells are grown in 150-mm culture dishes.
2. Approximately 24–48 h after transfection, cells are collected by scraping them into 1 mL of ice-cold PBS and pelleted by centrifugation.
3. The PBS is aspirated and the cells are resuspended in 300 µL of new RIPA buffer. The sample of lysis mixture is placed in a microfuge tube on dry ice to freeze the sample and then thawed to release the cellular-soluble protein.
4. Samples are centrifuged at 25,000*g* at 4°C for 10 min. and the supernatant is transferred to a new tube.
5. Check the concentration using Bradford assay reagent (Bio-Rad).

3.3.2. Immunoprecipitation

1. Adjust the protein concentration to 1 mg/mL in 500 µL.
2. Relevant antibodies are added (2 µg per 500 µg extract) and incubated at 4°C for 2 h.
3. Protein A-Sepharose or G-Sepharose beads (1:1 mix, 30 µL) are added. The mixture is rotated at 4°C overnight. Pellet the beads-Ab complexes and wash with New RIPA buffer three times.

3.3.3. IP-HAT Assay

1. Wash the beads with HAT assay buffer.
2. Add back 30 µL HAT buffer, 1 µL of 5 mg/mL histones or nonhistone substrates, enzyme and 1 µL (6 pmol) of ^{3}H-acetyl-CoA.
3. The mixture is incubated at 30°C for 30 min. Flick the tube several times during the incubation.
4. ^{14}C-acetyl incorporation into the substrates is determined by liquid scintillation counting (*see* **Note 4**).

3.4. In Vitro Filter HAT Assay

1. The reaction mixture (*see* **Subheading 3.2.3.**) is spotted onto Whatman P-81 phosphocellulose filter paper.
2. The filter paper is air-dried for 2–5 min. and washed with 0.2 *M* sodium carbonate buffer (pH 9.2) at RT with five changes of the buffer for a total of 30 min.
3. The dried filter paper is counted in a liquid scintillation counter.
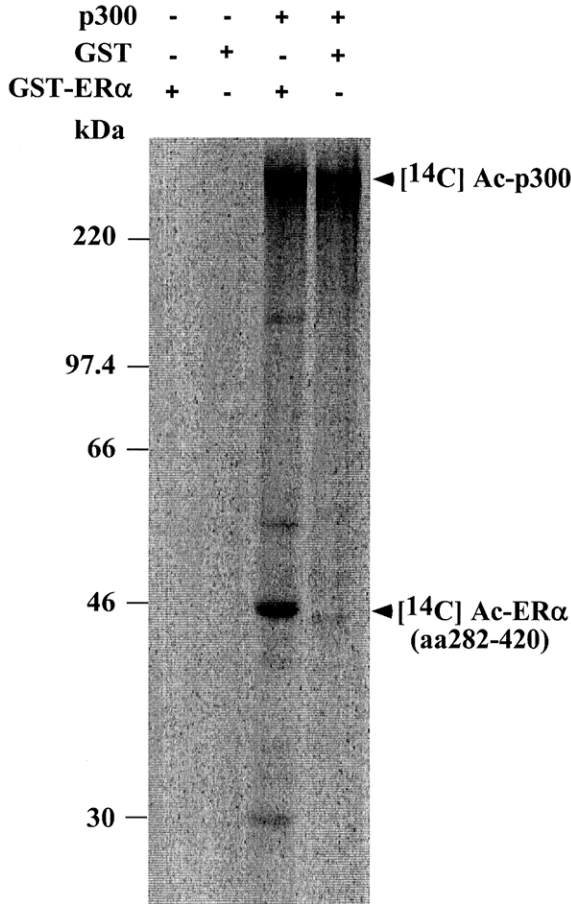
Fig. 1. p300 acetylates the ERα C-terminal to the zinc finger DNA binding domain (reprinted with permission from *[12]*). Equal amounts of either the GST-ERα fusion protein or GST protein were incubated with p300 and [14C] acetyl-CoA. The *arrow* indicates the autoradiogram of the acetylated ERα fusion protein and autoacetylated p300. The autoradiogram of the electrophoresed products demonstrates equal amounts of autoacetylated p300 in both lanes and the presence of acetylated ERα.

3.5. In-Gel HAT Assay

This protocol is derived from the method of Dr. Brownell *(19,20)*.

1. Preparations containing Acetyltransferase are separated by SDS-PAGE gel containing 1 mg/mL of core histones.
2. The gel is washed with 50 mM HEPES pH 7.9, 25% isopropanol for 30 min at RT.
3. The proteins are denatured by incubation with 6 M guanidine-HCl in 50 mM HEPES pH 7.9, 1 mM DTT, 5 mM PMSF at RT for 30 min.
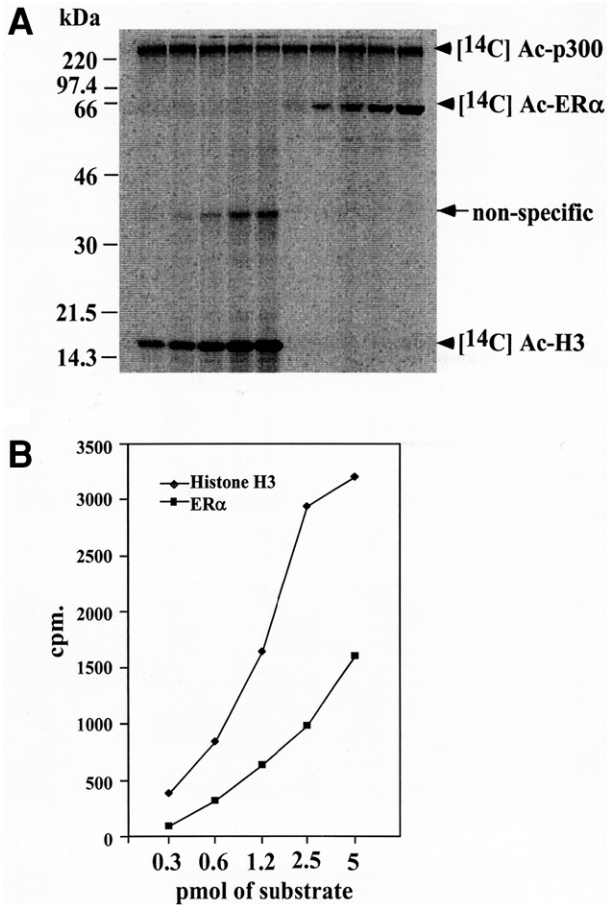
Fig. 2. ERα is an efficient substrate for p300 acetylation (reprinted with permission from *[12]*). **(A)** and **(B)**, HAT assays were performed using a constant amount of enzyme and equimolar amounts of either ERα or histone H3 substrate. **(B)** The acetylated bands were excised and counted by liquid scintillation counter.

4. The proteins on the gel are renatured by incubation with chilled 50 m*M* HEPES pH 7.9, 1 m*M* DTT, 5 m*M* PMSF and 0.05% Tween-20 at 4°C for 12 h.
5. After renaturation of acetyltransferase in the gel, the gel is incubated with [^{14}C] acetyl CoA in in-gel reaction buffer at 30°C for 1 h.
6. Acetylated histones are visualized by autoradiography.

3.6. *In Vivo HAT Assay*

1. The experiment can be conducted using either endogenous "target protein" or protein expressed in cells, using a mammalian expression vector. In the case of

transfected cells, 293T cells are transfected by Superfect Transfection reagent (Qiagen, Valencia, Calif.) on a 150-mm plate with the expression vector encoding the protein of interest.

2. Twenty-four hours after transfection, the cells are transferred to the fresh DMEM medium containing 1 mCi/mL of [^{3}H]-sodium acetate (Amersham) for 1 h before lysis.
3. Cells are washed twice with cold PBS and lysed in New RIPA buffer.
4. The lysates are centrifuged at 100,000g for 30 min at 4°C.
5. Supernatants collected from 5 plates are immunoprecipitated, with the antibody to the relevant target protein that had been conjugated to protein A agarose beads, for 6 h to overnight at 4°C.
6. The beads are washed five times with 1 mL of New RIPA buffer supplemented with 0.5% Tween-20.
7. Immunoprecipitates are solubilized with SDS-PAGE sample buffer and resolved on 8% SDS-PAGE gel.
8. Gels containing [^{3}H]-acetate-labeled "target" protein are fixed with 10% glacial acetic acid and 40% methanol for 1 h and enhanced by fluorography enhancing solution (Amplify, Amersham) for 30 min.
9. Gels are then dried and subjected to autoradiography at –70°C for 15 d.

3.7. Histone Deacetylase Assay

1. Histone deacetylases were prepared from tissue or cultured cells by immunoprecipitation with antibody against histone deacetylase.
2. Incubate beads/extract in between 50 to 100 µL of HDA buffer.
3. Add 10,000–30,000 dpm of tritiated substrate histones or peptide. Incubate at 37°C for 2 h.
4. Acidify reaction by the addition of 50 µL of 0.1 *M* HCl, 0.16 *M* AcOH.
5. Add 600 µL of EtOAc, vortex, and separate layers by brief centrifugation.
6. Determine specific activity through scintillation counting 400 µL of organic extract.

4. Notes

1. If core histones are made as substrates for acetyltransferase assay, HeLa cells will not be labeled with [^{3}H] acetic acid.
2. Check radioactivity of labeled core histones by liquid scintillation counting. Typically, a specific activity of 2.5×10^6 cpm/mg of protein is obtained.
3. The fusion protein can be made either as a GST fusion in bacterial cells or from mammalian or insect cell-expression systems using other tags (Flag, Myc, His, HA). These systems are commercially available.
4. Alternatively, reaction mixtures can also be resolved by SDS-PAGE gel. Gels are dried, enhanced, and subjected to autoradiography (*see* **Figs. 1** and **2**).
5. Several protease inhibitors are also potent inhibitors of HATs. Samples assessed for HAT activity should not be exposed to Hg-containing compounds, *N*-ethylmaleimide or iodoacetamide. Other inhibitors including PMSF, leupeptin, aprotonin, bestatin, pepstatin, and benzamidine are reported to not affect HAT activity and can be used to protect against proteolysis *(21)*.

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III

ANALYSIS OF PROTEINS INVOLVED IN CHECKPOINT CONTROL

17

Cataloging Proteins in Cell Cycle Control

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1. Introduction
1.1. No Direct Path From Sequence Similarity to Biological Similarity

How can bioinformatics tools help to identify particular types of proteins? In general, the answer depends on the type of protein. Alignment methods can identify similarities between two proteins. However, although database search tools are optimized to finding the best possible superposition between two proteins, they fail in answering questions such as: Does the query protein Q perform the same function as the protein in the database H for which we have some experimental data about function? In fact, alignment methods typically provide some statistical score evaluating the probability that the similarity between Q and H happened by chance *(1,2)*. The precise function relating such a statistical score for sequence similarity to the actual *biological* similarity of two proteins, that is, similarity in terms of their three-dimensional (3D) structure and/or function depends on the problem. For example, if the Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST *[2]*) expectation value for the similarity between Q and H is below 10^{-5}, then this typically implies that H and Q have similar local 3D structure *(3)*. However, less than 70% of all pairs of enzymes that have this level of sequence similarity have exactly the same enzymatic activity *(4)*, and over 90% of all pairs with so similar sequences are observed in the same subcellular compartment *(5)*. Establishing these estimates typically requires solving three different tasks: (a) defining biological similarity (3D, enzyme activity, subcellular localization); (b) building unbiased data sets of experimentally reliable information; and (c) establishing thresholds that relate sequence to biological similarity. These steps have been completed for a variety of biological features such as structure *(3,6–9)*, enzymatic activity *(4,10–14)*, active sites *(11)*, binding sites

(11), functional key words *(11)*, functional classes *(11,13)*, and subcellular localization *(5)*. However, there is no way to infer from these results at which level of sequence similarity we can conclude that two homologous proteins play the same role in processes such as cell cycle control.

The field of proteomics has evolved into various levels of biological and computational techniques that identify and classify proteins in the context of entire genomes and proteomes. These techniques include a broad spectrum of approaches; from detailed literature searches *(15,16)* or text analysis of database annotations *(17)*, database mining *(16,18–24)*, multiple-sequence alignments *(2,25–29)*, protein-family clustering *(30–35)*, methods predicting aspects of protein function and structure *(36–42)*, and computational modeling of the cell cycle *(43)* to gene microarray or "chip" expression techniques *(44–48)*, yeast two-hybrid systems *(49,50)*, and recently mass spectroscopy of protein complexes *(51,52)*. The process of unifying these techniques from an assortment of cataloging tools into a more eloquent analysis of the cell cycle and, specifically, cell cycle control proteins is only beginning to take shape. Here, we present a first step for this process using database mining and literature searches to evaluate the current status of cell cycle control proteins present in various databases, combined with sequence-alignment evaluation to identify cell cycle control proteins in various proteomes. We began by archiving proteins known to be involved in cell cycle control through database and literature searches. Then we established levels of sequence similarity that imply similarity in function. Finally, we attempted to identify cell cycle control proteins through homology in entirely sequenced eukaryotic proteomes.

2. Materials

2.1. Public Databases

Curated, well-formatted, and annotated databases are one of the most important resources for bioinformatics. A few public databases contain information about cell cycle proteins (*see* **Table 1**); from these we built a resource that identifies the general register of cell cycle information currently available. To create this repository, we collected about 3811 records from MEDLINE (www.ncbi.nlm.nih.gov/entrez/). Using Sequence Retrieval System (SRS) *(53)*, we retrieved about 364 proteins from SWISS-PROT *(54)* and 98 proteins of known structure from the Protein Data Bank (PDB) *(55)*. Only 7 of these 98 were classified as "cell cycle control" proteins. A closer inspection of the SWISS-PROT data set revealed 534 proteins with the key word *cell cycle* and 940 with the key word *cell division.* ProtoNet *(32,56)* is a tool that clusters all proteins from SWISS-PROT into somehow-related families. ProtoNet identified 1476 clusters with a total of 512 proteins for the SWISS-PROT key word *cell cycle* and 887 proteins in 1983 clusters with the key word *cell division.*

Table 1
Public Resources for Cell Cycle Proteins[a]

Databases

The Suiseki Information Extraction System	(www.pdg.cnb.uam.es/suiseki/) and (www.pdg.cnb.uam.es/suiseki/system/Start_cellCycle_new.html)
Yeast Cell Cycle Analysis Project	(http://genome-www.stanford.edu/cellcycle/data/rawdata/)
SCPD: Promoter Database of *Saccharomyces cerevisiae*	(http://cgsigma.cshl.org/jian/)
Mouse Genome Informatics	(www.informatics.jax.org)
The Interactive Fly-Cell Cycle in *Drosophila*	(http://sdb.bio.purdue.edu/fly/aimain/aadevinx.htm)
Transfac & Transpath	(http://transfac.gbf.de/TRANSFAC/)
Mitosis World	(www.bio.unc.edu/faculty/salmon/lab/mitosis/mitosis.html)
TRRD—Transcription Regulatory Regions Database	(www.bionet.nsc.ru/trrd/ and (http://wwwmgs.bionet.nsc.ru/mgs/papers/kel_ov/celcyc/)
The Ubiquitin System for Protein Modification and Degradation	(www.nottingham.ac.uk/biochemcourses/students/ub/ubindex.html)
KEGG: Kyoto Encyclopedia of Genes and Genomes	(www.genome.ad.jp/kegg/) and (www.genome.ad.jp/kegg/pathway/hsa/hsa04110.html)
The p53 Web site	(http://p53.curie.fr/)
The Kinesin Home Page	(www.proweb.org/kinesin/) and (www.proweb.org/kinesin//KinesinTree.html)
The Database for Interacting Proteins	(http://dip.doe-mbi.ucla.edu/)
The Forsburg Lab pombe Pages	(http://pingu.salk.edu/~forsburg/lab.html)
Protonet—Automatic Hierarchical Classification of Proteins	(www.protonet.cs.huji.ac.il/protonet/index.php)
MIPS—Comprehensive Yeast Genome Database	(http://mips.gsf.de/proj/yeast/CYGD/db/index.html)
Protein Information Resource	(http://pir.georgetown.edu/)
PDB: database of protein structures	(www.rcsb.org/pdb/)
SWISS-PROT (annotated proteins)	(www.expasy.ch/sprot/sprot-top.html)

Tools

PSI-BLAST(database search)	(www.ncbi.nlm.nih.gov/BLAST/)
Predictions of posttranslational modifications	(www.cbs.dtu.dk/services/)
PredictProtein (sequence analysis + structure prediction)	(http://cubic.bioc.columbia.edu/predictprotein)
META-PP (interface to variety of tools)	(http://cubic.bioc.columbia.edu/predictprotein/submit_meta.html)
ExPasy (tools, databases, links)	(www.expasy.ch/)
WWW links for molecular biology	(http://cubic.bioc.columbia.edu/doc/links_index.html)

[a]Our data along with a novel cell cycle-specific database is available through our Web site (http://cubic.bioc.columbia.edu).

The obvious next task was to retrieve a catalog of unique families of proteins related to the cell cycle (*see* **Subheading 3**).

2.2. Sources of Sequences for Entire Proteomes

All human sequences were extracted from SWISS-PROT and TrEMBL *(54)*. We retrieved all other proteome sequences from the respective public sites: *Drosophila melanogaster*: (http://www.fruitfly.org/), *Caenorhabditis elegans*: (ftp://ncbi.nlm.nih.gov/genbank/genomes/), *Saccharomyces cerevisiae* from the Yeast Genome Directory *(57)*, *Arabidopsis thaliana*: (http://www.arabidopsis.org/), and *Mus musculus*: (http://www.ensembl.org).

3. Methods

3.1. Cell Cycle and Cell Cycle Control Proteins in Public Databases

3.1.1. Keyword Search in SWISS-PROT

First, we searched for proteins of trusted experimental information about cell cycle control in SWISS-PROT. Most proteins retrieved thus control the G1/S and G2/M transitions or are related to the M and S phases. In total, we found 361 proteins (**Table 2**) that were distributed among various species. Next, we clustered these proteins into families.

3.1.2. Sequence-Unique Data Sets

To reduce the bias from too-similar sequences, we generated sequence-unique subsets for all types of proteins under consideration. *Sequence-unique* was defined as no pair in the set having more than 33% identical residues over more than 100 residues aligned (homology-derived secondary structure of proteins [HSSP]-threshold of zero) *(3)*. Given an all-against-all pairwise alignment for the biased set, we simply used a greedy search to find the largest subset that fulfilled the above condition. This reduced the entire set of 361 to 42 unique proteins or protein families.

3.1.3. Extending Simple Keyword-Based Search

Forty-two unique proteins did not suffice to develop any statistical criteria for determining levels of significant sequence similarity and also implying similarity in the cell cycle process. We expanded our original data set by including searches for other cell cycle controlling factors, such as ubiquitin and those in the ras super-family plus other proteins annotated for cell-division control. This extensive search for cell cycle control proteins increased the list to a total of 595 proteins; 97 of these had multiple, conflicting annotations (**Table 2**); 113 were sequence-unique, that is, we increased the numbers of families from 42 to 113 through the extended keyword-based search. The entire data set of cell cycle control proteins is in preparation for availability on-line at the CUBIC Web site: (http://cubic.bioc.columbia.edu).

Table 2
Numbers of Cell Cycle Control Proteins Found in SWISS-PROT[a]

Species	Cell Cycle Control	G1/S	G2/M	M Phase	S Phase	Other	Multiple
Eukaryotes	582	135	86	66	156	229	90
Homo sapiens	99	28	11	23	41	24	28
Mus musculus	68	25	8	10	30	18	23
Drosophila melanogaster	15	5	3	2	4	3	2
Caenorhabditis elegans	10	1	4	1	2	2	0
Arabidopsis thaliana	5	0	1	0	0	4	0
Saccharomyces cerevisiae	87	20	11	5	19	46	14

[a]Eukaryotic proteins presented; the remainder of proteins in the set of 595 cell cycle proteins are involved in the prokaryotic cell cycle process.

3.2. Cell Cycle Control Protein Identification Through Sequence Similarity

3.2.1. Establishing Threshold for Significant Sequence Similarity

If we want to find proteins that have similar roles in the cell cycle as the proteins for which we have experimental information in public databases, we have to first establish a threshold for "significant sequence similarity," that is, we have to address the question: At which level of sequence similarity can we infer similarity in the specific functional role of that protein? Obviously, such thresholds have to find a balance between accuracy and coverage, in other words, we have to navigate between the Skylla of "high selectivity/low sensitivity," that is, finding very few homologues all of which are right, and the Charibdis of "low selectivity/high sensitivity," that is, finding many putative homologues, most of which are wrong. Cumulative accuracy and coverage were calculated as:

$$\text{Cumulative accuracy} = 100 \cdot \frac{\text{Number of true pairs found above threshold}}{\text{Number of all pairs above threshold}} \quad (1)$$

$$\text{Cumulative coverage} = 100 \cdot \frac{\text{Number of true pairs found above threshold}}{\text{Number of all true pairs}} \quad (2)$$

with the thresholds for sequence similarity specified in **Subheading 3.2.3.**

3.2.2. Aligning Proteins

We generated alignments for all sequences from the cell cycle unique data set (595) against a set of nonnuclear (but including cytoplasmic) proteins of known function other than those functions in cell cycle control (total of 6728 proteins) using pairwise BLAST *(1)*. To refine the analysis, we also generated PSI-BLAST profiles using a filtered version of all currently known sequences with three iterations *(58)*. These profiles were then aligned against our "cell cycle control plus all other proteins" data set. Sequence similarity was defined by percentage identity, BLAST E-values, and the distance from the HSSP-threshold, which relates percentage sequence identity to alignment length, thus accounting for the fact that 80% pairwise identity is not significant when achieved over a stretch of 15 consecutive residues; however, it is highly informative when achieved over entire proteins *(59)*.

3.2.3. Accuracy and Coverage of Inferring Cell Cycle Role by Homology

When we aligned all trusted cell cycle proteins (595) against all true negatives (6116 non-cell-cycle proteins), we found that at HSSP distances of 15 (corresponding to 48% pairwise-sequence identity for more than 100 aligned residues), we could seemingly infer the role in the cell cycle at an accuracy of 95% (**Fig. 1**). However, when using the unbiased, sequence-unique subset of
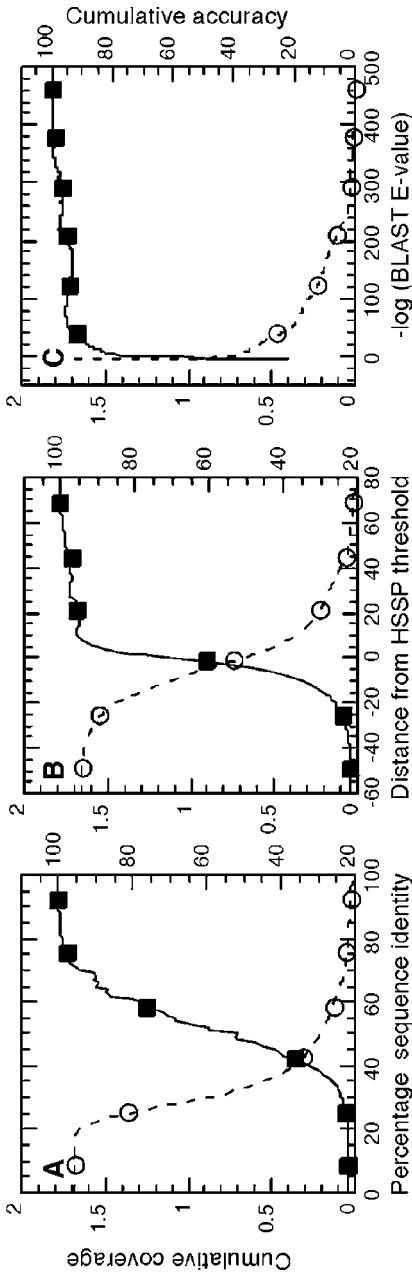
Fig. 1. Sequence conservation of all trusted cell cycle control proteins. We aligned all trusted cell cycle proteins (595) against all true negatives (6116 non-cell-cycle proteins) using BLAST. Solid lines with filled squares describe cumulative accuracy (percentage of correctly identified cell cycle proteins at given threshold; *see* **Eq. 1**); dotted lines with open circles describe cumulative coverage (cell cycle proteins found at threshold/all cell-cycle proteins; *see* **Eq. 2**). We measured sequence similarity in three different ways: (**A**) by the percentage pair-wise-sequence identity (*left graph*), (**B**) the distance from the HSSP threshold accounting for the length of the alignment (*central graph*), and (**C**) by the negative logarithm of the BLAST E-values (note: log to the base of 10) (*right graph*). For example, the accuracy exceeded 80% for levels >60% pair-wise sequence identity (*left*), HSSP distances above 3 (*center*), and BLAST expectation values below 10^{-12} (*right*). At all levels of accuracy ≥80%, the HSSP distance performed best in terms of coverage. Note that these estimates were based on large data sets; however, they constituted overestimates because the bias in the data sets was not removed.

225
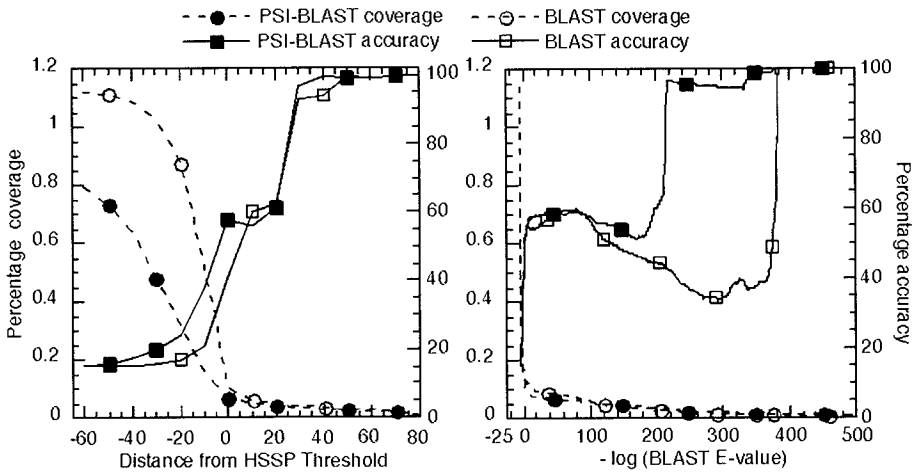
Fig. 2. Estimating accuracy and coverage for BLAST and PSI-BLAST. To correctly estimate the likely accuracy and coverage, we had to remove the bias from our initial data sets by aligning the subset of 113 sequence-unique trusted cell cycle proteins against all trusted cell-cycle proteins and against all true negatives. For this, we compared the performance of pairwise BLAST *(open symbols)* to that of PSI-BLAST *(filled symbols)*. Accuracy *(solid lines)* and coverage *(dashed lines with circles)* were as in **Fig. 1.** In general, PSI-BLAST clearly outperformed BLAST. For example, at HSSP distances >40 the accuracy of PSI-BLAST searches was above 95%. Note that these estimates were sufficiently lower than those that would have been obtained using the biased data (**Fig. 1**). Using only the E-values taken from PSI-BLAST and BLAST alignments required very high cutoff thresholds—even at levels of 10^{-10}—implying that only one in 10 million hits occurred by chance, and less than 70% of the inferences were correct. The residual problem with the data resulted from the small set sizes *(rigged curves)*.

113 cell cycle proteins to evaluate accuracy, we found levels of only 60% accuracy. To reach a level of 95% accuracy, we had to increase the HSSP distance from 15 to 40 (**Fig. 2**), that is, we had to require over 70% pairwise-sequence identity. Replacing the HSSP distance by the expectation values from BLAST or PSI-BLAST (E-values) did not yield a more accurate distinction between true and false positives. This finding confirmed our previous results on establishing thresholds for sequence similarity, implying similarity in 3D structure and subcellular localization *(4,5)*.

3.2.4. Identifying Cell Cycle Control Proteins From Entirely Sequenced Proteomes

We used a variety of thresholds for inferring the role of cell cycle control proteins by homology so as to confer the annotations about these roles from

Table 3
Cell Cycle Control Proteins Predicted by Homology in Entire Proteomes[a]

		Predicted cell cycle control proteins			
Proteome	Known cell cycle control proteins[b]	D = 0 (55%)	D = 15 (65%)	D = 25 (90%)	D = 40 (95%)
Homo sapiens	99	3073	782	476	299
Mus musculus	68	3162	574	310	203
Drosophila melanogaster	15	970	181	96	50
Caenorhabditis elegans	10	1005	185	87	32
Arabidopsis thaliana	5	1888	303	148	63
Saccharomyces cerevisiae	87	513	148	119	100
Sum	284	10,611	2173	1236	747

[a]Distance from HSSP threshold chosen, as seen in **Fig. 2** for various levels of percentage accuracy using the PSI-BLAST curve. Levels of accuracy are estimated according to **Fig. 2**, that is, at a threshold of D = 40 more than 95% of the proteins for which we infer the involvement in cell cycle control by homology are supposedly correctly inferred.
[b]The number of previously known annotated cell cycle control proteins represented in each specific proteome as used in our trusted data set is given for comparison.

our trusted data set to homologues in entirely sequenced eukaryotes. In particular, we scanned the proteomes of human (*Homo sapiens*), mouse (*Mus musculus*), fly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*), weed (*Arabidopsis thaliana*), and yeast (*S. cerevisiae*). At levels of around 95% accuracy, we could extend the number of proteins known to be involved in cell cycle control from 284 for the six completely sequenced organisms to about 747 (**Table 3**). Our analysis also pulled out about 500–1300 additional proteins (difference between columns D = 40 and D = 25 and D = 15 in **Table 3**) that may constitute candidates for unknown cell cycle control proteins. On the other extreme end, our data illustrated that over 10,000 proteins in any of these six proteomes have similar 3D structures to one of the known cell cycle proteins. Supposedly most of these are not related to cell cycle control, illustrating the variety of functions that can be adopted by proteins of similar structure (*see* **Note 1**).

4. Notes

1. Every day biologists are searching with their protein Q of interest by standard alignment methods to uncover putative homologies to their protein. Owing to large-scale sequencing efforts, these database searches retrieve more and more often proteins without any annotation other than "hypothetical protein." To initiate hypotheses about function, such results are obviously not very

informative. More difficult are the "helpful" cases when a protein with experimental annotation about function H is similar to Q. The number of pitfalls that can lead to incorrect hypotheses based on database searches are manifold *(11,24,60–63)*. Nevertheless, an increasing number of publications in modern biology are based on some beneficial hints obtained from database searches. How can we separate the chaff from the wheat? Certainly, it is a sine qua non to establish thoroughly evaluated, statistically significant estimates for which level of sequence similarity implies what *(4,10,11,13,14,64)*. In the context of cell cycle proteins, our approach aims at identifying commonalities in the evolutionary conservation of a selected group of functions. On the one hand, it appears evident that all proteins involved in cell cycle and cell cycle control have common evolutionary constraints. If true, we can infer the involvement of a protein in the cell cycle process based on sequence similarity. On the other hand, we may suspect that two kinases such as pyruvate dehydrogenase kinase and Cdk1 are more similar than the two cell cycle proteins Cdk1 kinase and the E2F transcription factor. If true, we have to define all types of function related to cell cycle and have to establish thresholds for each functional type; in other words, our inference of cell cycle roles based on homology is rather limited. Arguably, reality falls between these two extremes. Therefore, our ability to discover new proteins in cell cycle control through homology works to some extent but is rather restricted.

2. Jones and Sgouros *(65)* studied cohesion complex proteins through sequence motifs and database searches. They used PSI-BLAST to identify all homologues of the structural maintenance of chromosomes (SMC) proteins and the sister chromatid cohesion (SCC) proteins from yeast (Smc1, Smc3, Scc1, Scc2, Scc3, and Scc4), as well as four proteins interacting with cohesion proteins (Trf4, Prp11, Tid3, Esp1). Next, the authors aligned the putative homologues identified by PSI-BLAST, using the dynamic programming-based method ClustalX *(28,29)*, and constructed putative evolutionary trees from these ClustalX alignments using the program PHYLIP *(66)*. Finally, the study identified possible binding partners from the complete two-hybrid screens available through the Yeast Proteome Database and putative sequence motifs through the program Teiresias *(67)*. The study resulted in the establishment of five families of SMC proteins, a cohesion interaction network of 17 proteins, and the identification of possible common sequence motifs for binding and a kinase-active site.

Kel and colleagues *(68)* combined experimental and theoretical techniques in a comprehensive study identifying the 5' regulatory regions of cell cycle-related genes. First, the group developed a program that identifies context-specific binding sites for the E2F transcription factors. All these sites were identified in entirely sequenced genomes with the aim to identify new genes that play a role in controlling cell proliferation, differentiation, and apoptosis. Finally, the predictions were verified by chromatin immunoprecipitation assays. The study resulted in finding a total of 313 new potential E2F targets, 8 of which were verified through the in vivo experimentation.

Blaschke and Valencia *(16)* developed a text analysis system: System for Information Extraction on Interactions (SUISEKI) that automatically identifies cellcycle-related protein–protein interactions from scientific literature, that is, from MEDLINE abstracts. At the heart of the system, text searches are defined into frames that capture the various language constructs used to convey protein interactions. The authors selected 5283 abstracts that included the word "cell cycle"; the system detected 6778 protein interactions from all of the abstracts, resulting finally in 4657 distinct interactions from a total of 1471 abstracts. The data is currently available at (www.pdg.cnb. uam.es/suiseki/).

Acknowledgments

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Multidimensional Proteomic Analysis of Proteolytic Pathways Involved in Cell Cycle Control

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1. Introduction

Many cell cycle transitions are controlled by ubiquitin-mediated proteolysis of key cell cycle regulators *(1)*. The ubiquitin system targets substrates to the proteasome by attaching a polyubiquitin chain *(2)*. The traditional ubiquitin transfer reaction involves a minimum of three enzymes: E1, which mediates the ATP-dependent activation of ubiquitin, and the E2 ubiquitin-conjugating enzyme (UBC), which, together with an E3 ubiquitin ligase, transfers ubiquitin to the target protein. Despite the successful identification of many ubiquitin ligases, only a few of their substrates are known. This is because ubiquitin ligases share conserved motifs, whereas substrates seem to have little in common other than critical lysine residues.

Whereas systematic approaches to identifying components of ubiquitin ligases have been fruitful *(3–5)*, to our knowledge, no systematic approaches have been employed for revealing their substrates. Here we describe a protocol for multidimensional proteomic analysis to systematically exploit a conserved biochemical rather than structural feature of ubiquitin ligase substrates: their delayed degradation and, hence, accumulation in fission yeast ubiquitin ligase mutants.

In a simplified description, protein lysates prepared from wild-type cells and from proteolysis mutants are separated by two-dimensional gel electrophoresis (2DGE), digital gel images are obtained, and proteins of higher abundance in proteolysis mutants are identified by image analysis and liquid chromatography coupled with tandem mass spectrometry. The identified proteins can then be further validated biochemically with in vitro ubiquitination assays employing purified ubiquitin ligases.

Our initial studies have shown that the dynamic range of protein abundance is far too wide, even in a simple organism such as fission yeast, to enable a comprehensive proteome analysis by comparing total cell lysates by 2DGE (*see*, for example, **Fig. 2C**). We have therefore established a highly reproducible chromatographic prefractionation scheme that is outlined in **Fig. 1**. Total cell lysate is prepared from *Schizosaccharomyces pombe* cells by bead lysis and chromatographed on an anion-exchange column. Three pooled fractions resulting from elution with a linear salt gradient and containing equal amounts of protein (approx 3.5 mg) are collected. Fractions are then precipitated with 10% trichloroacetic acid (TCA) in acetone, resuspended, and analyzed by 2DGE. Gels are stained and analyzed with specialized 2D imaging software to reveal differentially accumulating proteins.

Fig. 2 shows results for a sample taken through the entire fractionation (**Fig. 2A**) and 2D analysis (**Fig. 2C**). A largely nonoverlapping protein pattern can be revealed (**Fig. 2B**, **C**). In addition, many low-abundance proteins not visible in the total cell lysate (**Fig. 2C**) are revealed after sample prefractionation. Comparative analysis of averaged gels from independent duplicate preparations of wild-type and mutant cell lysates enables the reliable identification of differentially accumulating proteins (**Fig. 3**).

Although 2DGE has continuously improved over the past several years, there are numerous frequently cited limitations, many of which have been successfully circumvented in the described protocol:

1. The problem of the wide dynamic range of protein abundance that notoriously obscures low-abundance proteins is addressed by chromatographic prefractionation. This procedure currently allows us to detect a minimum of 1800 independent features on our gels, which are spread over a wide range of molecular weights and ionic strengths (*see* **Fig. 2C**). This number represents approx 40% of the theoretical fission yeast proteome. Although no other techniques currently afford a greater coverage, penetrance of the fission yeast proteome can be further increased by using narrow-range pH gradient strips or further chromatographic fractionation of the flowthrough fraction.
2. Protein losses during sample preparation can result from inefficient extraction, precipitation, and resolubilization. Although the standard extraction buffer,

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Fig. 1. *(opposite page)* Flowchart of multidimensional proteome analysis of a fission yeast proteolysis mutant. Crude cell lysate from wild-type and mutant cells is fractionated by ion-exchange chromatography, and the eluate is pooled into three fractions. The three fractions are analyzed by two-dimensional gel electrophoresis (2DGE), and corresponding gels derived from wild-type and mutant fractions are compared by image analysis. Differentially accumulating proteins are identified by tandem mass spectrometry.

Fig. 1.

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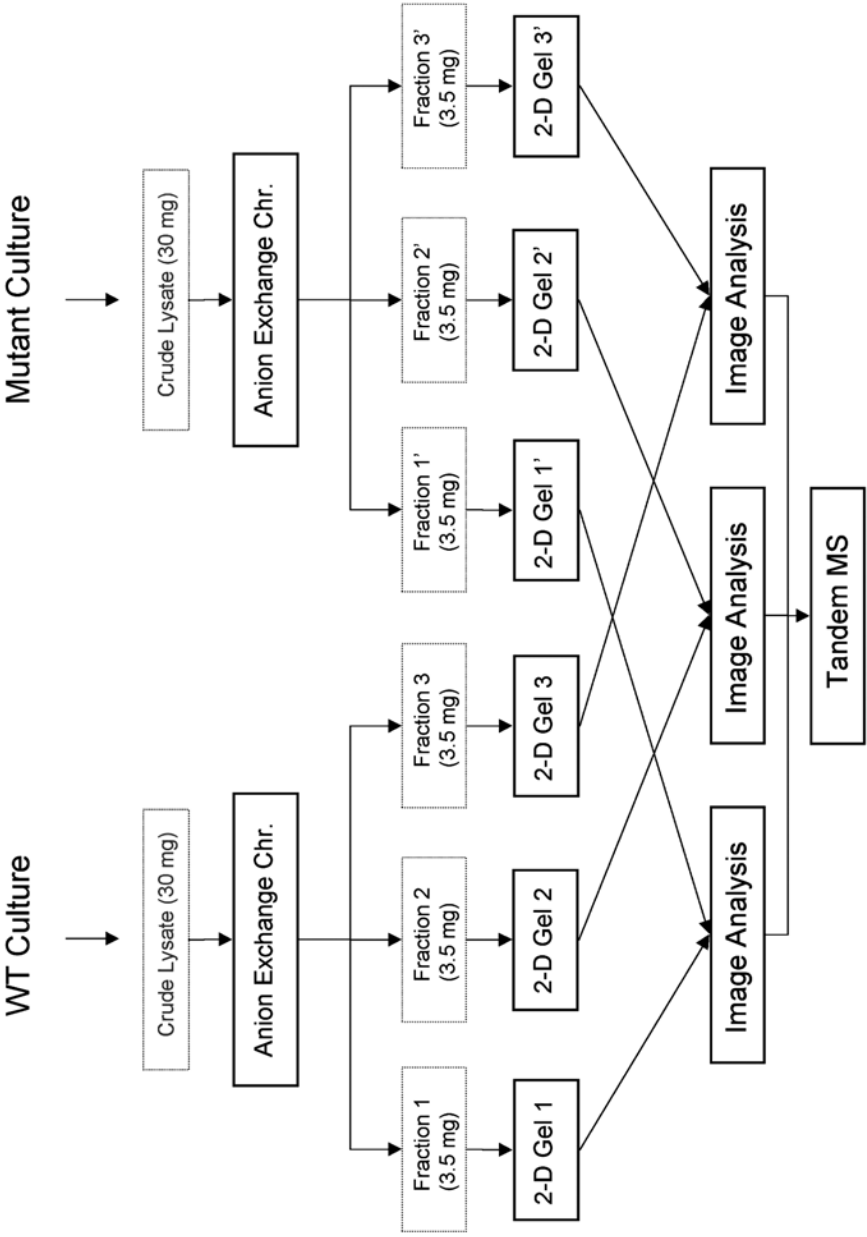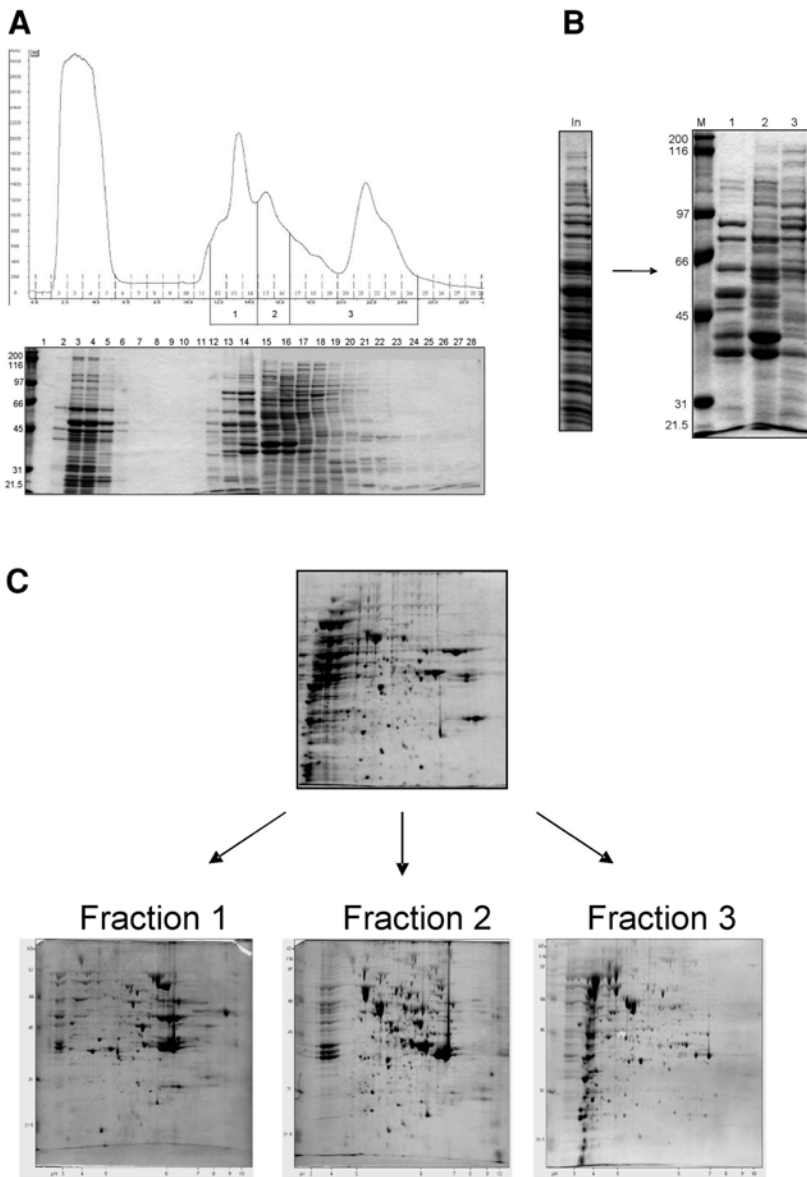
Fig. 2. Chromatographic fractionation. **(A)** Total *S. pombe* lysate was fractionated by anion-exchange chromatography. Fractions eluting with a linear salt gradient were pooled into three fractions of equal protein content. **(B)** Fractions were precipitated with trichloroacetic acid (TCA) in acetone, resuspended in 2D sample buffer, and analyzed by one-dimensional gel electrophoresis to document sample quantity and quality. Total cell lysate is shown as a comparison. **(C)** The three fractions prepared in **(B)** were analyzed by 2DGE on nonlinear pH 3–10 strips. Gels were stained with Coomassie Brilliant Blue and scanned.
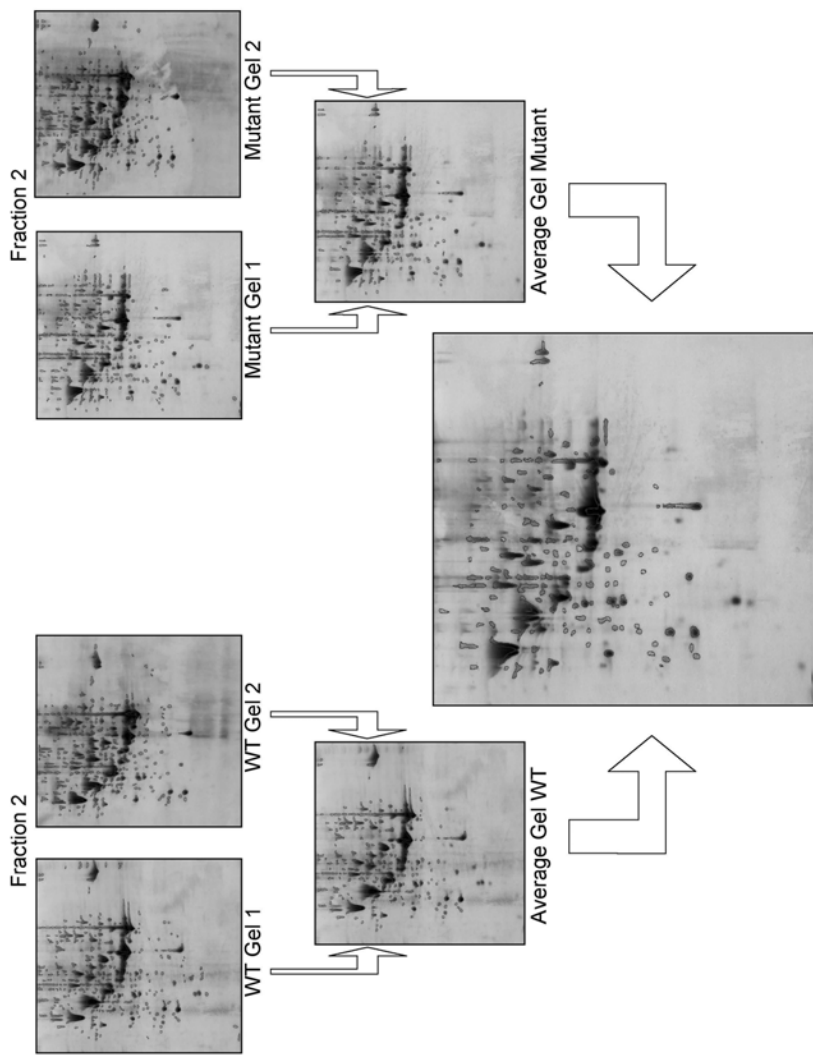
Fig. 3. Multidimensional proteome analysis. Two independently prepared fractions #2 from wild-type and mutant *S. pombe* were analyzed by 2DGE. Replicate gels were scanned, averaged with imaging software, and compared by spot matching and spot densitometry. Proteins accumulating to at least threefold higher levels in either wild-type or the mutant yeast are indicated.

containing nonionic detergent, is difficult to improve, we have optimized the conditions for protein precipitation and resolubilization by testing numerous different agents and methods. Although losses are impossible to avoid completely, we estimate that protein recovery with the described protocol is greater than 90% (data not shown).

3. The problem of reproducibility: We have found in numerous experiments that owing to the use of an automated fast-protein liquid chromatography (FPLC) system, the reproducibility of the chromatographic steps is extremely high. Using the same lot of immobilized pH gradient strips for isoelectric focusing (IF), consistent protein patterns have been obtained when running samples in duplicates (**Fig. 3**). In addition, averaging several gels from independently prepared samples further reduces gel-dependent variability.

2. Materials

2.1. Lysate Preparation

1. Yeast YE media (5 g/L Bacto-yeast extract, 30 g/L dextrose). Autoclave.
2. 7.5 g/L Adenine (50X stock solution).
3. 7.5 g/L Uracil (100X stock solution).
4. Incubator.
5. Yeast lysis buffer (prepare fresh): 25 mM Tris-HCl pH 8.8, 0.5% Triton X-100, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 15 µg/mL aprotinin, 1 mM phenyl-methylsulfonyl fluoride (PMSF).
6. Bead beater and cup set (Biospec Products).
7. 0.5 mm silica beads (Biospec Products).
8. Centrifuge RC 5B Plus (Sorvall).
9. Fixed-angle rotor SS-34 (Sorvall).
10. 1-L centrifuge buckets (Sorvall).
11. 50-mL oak ridge screw cap polypropylene centrifuge tubes (Nalgene).
12. D_c-protein assay kit (Bio-Rad).

2.2. Prefractionation

1. Ätka FPLC system (Amersham Biosciences).
2. HiTrap Q HP 1-mL column (Amersham Biosciences).
3. Buffer A: 25 mM Tris-HCl pH 8.8, 1 mM DTT.
4. Buffer B: 25 mM Tris-HCl pH 8.8, 1 M NaCl, 1 mM DTT.
5. 1.5-mL microcentrifuge tubes (VWR).
6. 30-mL glass centrifuge tubes (Corex).
7. 10X deoxyribonuclease (DNAse)/ribonuclease (RNAse) mix: 1 mg/mL DNAse 1, 0.25 mg/mL RNAse A, 50 mM MgCl$_2$. Freeze in aliquots.
8. Acetone/13.3% TCA/0.093% β-mercaptoethanol.
9. Sample loading buffer *(6)*: 7 M urea (Genomic Solutions), 2 M thiourea (Sigma-Aldrich), 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate

      (CHAPS) (EM Science), 2% ampholytes pH 3–10 (Genomic Solutions), 65 mM DTT (Research Products Int.), Serdolit MB-1 (Serva/Crescent Chemical Co.), 0.01% bromophenol blue (Sigma-Aldrich).

10. 10% sodium dodecyl sulfate (SDS) mini gel.
11. 5X SDS sample buffer: 2 M Tris-HCl pH 6.8, 25% β-mercaptoethanol, 10% SDS, 50% glycerol, 10% H$_2$O, 5% bromophenol blue (0.1% solution).
12. SDS gel running buffer (Tris-glycine pH 8.3, 10% SDS).

2.3. Two-Dimensional Analysis

1. 18-cm immobilized dry strips NL, pH 3–10, (Amersham Biosciences).
2. 20-cm × 20-cm Whatman filter paper.
3. 30-cm × 30-cm plastic bag (Ziplock).
4. Investigator pHaser isoelectric focusing system (Genomic Solutions).
5. Investigator 5000 programmable power supply (Genomic Solutions).
6. Nonconducting oil for IPG (Genomic Solutions).
7. pHaser electrode wicks (Genomic Solutions).
8. Equilibration trays (Genomic Solutions).
9. Equilibration buffer 1: 6 M urea (Genomic Solutions), 375 mM Tris-HCl pH 7.4 (ICN Biomedicals), 2% SDS (Sigma-Aldrich), 2% glycerol (ICN Biomedicals), 2% DTT (Research Products International).
10. Equilibration buffer 2: 6 M urea (Genomic Solutions). 375 mM Tris-HCl pH 7.4 (ICN Biomedicals), 2% SDS (Sigma-Aldrich), 2% glycerol (ICN Biomedicals), 2.5% iodoacetamide (Sigma-Aldrich).
11. Upper (cathode) gel running buffer: 200 mM tricine (Sigma-Aldrich), 200 mM Tris-Base (Sigma-Aldrich), 0.4% SDS (Sigma-Aldrich).
12. Lower (anode) gel running buffer: 25 mM Tris-acetate pH 8.3 (Sigma-Aldrich/ EM Science).
13. Investigator 2D running system (Genomic Solutions).
14. Precast tricine gels (Genomic Solutions).
15. Gel gaskets (Genomic Solutions).
16. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standard broad range markers (Bio-Rad).
17. Gel fixing solution: 40% methanol (EM Science), 10% glacial acetic acid (EM Science).
18. Staining solution: 10% Coomassie Brilliant Blue G-250 (Baker), 50% methanol (EM Science), 10% glacial acetic acid (EM Science).
19. Destaining solution: 25% methanol (EM Science), 5% glacial acetic acid (EM Science).
20. Glass container.
21. Rocking device.
22. Flat bed scanner (Hewlett Packard).
23. Software (Phoretix2D Professional, Nonlinear Dynamics).

3. Methods

3.1. Sample Preparation

3.1.1. Yeast Culture

1. Autoclave 1 L yeast extract sucrose (YES) medium in a 2-L glass flask and add adenine and uracil when medium is cooled down.
2. Inoculate a single colony from an agarose plate (using a sterile loop) in 10 mL YES medium (YE medium plus supplements) and incubate for 24 h at 30°C under agitation to produce a preculture.
3. Inoculate 2.5 mL of the preculture in 1 L YES and grow at 30°C under agitation until OD_{595} 1.5 is reached.
4. Spin culture for 15 min at 2000g in 1-L buckets and discard supernatant.
5. Resuspend pellet in 25 mL Tris-HCl pH 7.5 and transfer to 40-mL tubes.
6. Spin 5 min at 2000g and remove supernatant.
7. Freeze pellet immediately at –80°C.

3.1.2. Lysate Preparation (see **Note 1**)

1. Thaw pellet of 1 L culture quickly (use warm water bath).
2. Add 25 mL chilled yeast lysis buffer (*see* **Subheading 2.1.**).
3. Transfer to bead beater medium cup and grind approx 6 × 1 min (*see* **Note 2**).
4. Transfer bead-cell homogenate in chilled glass beaker, and separate cell homogenate from beads by aspiration with a 5-mL pipet.
5. Spin lysate in SS-34 rotor in prechilled Sorvall centrifuge at 20,000 rpm (48,000g) for 40 min (*see* **Note 3**).
6. Determine protein concentration immediately using the D_c-protein assay.

3.1.3. Prefractionation (see **Note 4**)

1. Equilibrate the HiTrap Q 1-mL column with 5 column volumes (CV) buffer A.
2. Apply 30 mg of cell lysate to a column and collect flowthrough in 1-mL fractions.
3. Wash out unbound sample with 5 CV buffer A.
4. Elute bound sample with a linear gradient (0 to 100% Buffer B) over 20 CV and collect 1-mL fractions (*see* **Fig. 2A**).
5. Clean column with 5 CV buffer B.
6. Reequilibrate with 5 CV buffer A.
7. Determine protein concentration in each 1-mL fraction and pool in larger fractions containing 3.5 mg each into 30-mL Corex tubes.
8. DNAse/RNAse treatment: Add 0.1 vol of the 10X DNAse/RNAse mix and incubate for 10 min at 4°C.

3.1.4. Precipitation in Trichloracetic Acid in Acetone (see **Note 5**)

1. Add 3 vol of the chilled (–20°C) stock solution (*see* **Subheading 2.2.**) to the sample (final concentration is 10% TCA and 0.07% β-mercaptoethanol in acetone) and incubate for 1.5 h or overnight at –20°C.

2. Spin samples 15 min at 2500*g* (Sorvall SS-34) at –20°C.
3. Discard as much of the supernatant as possible (to avoid TCA contamination) and wash pellet in chilled (–20°C) acetone containing 0.07% β-mercaptoethanol.
4. Spin samples 15 min at 2500*g* (Sorvall SS-34) at –20°C.
5. Remove all acetone and dry pellets at room temperature (approx 1.5 h).
6. Resuspend pellets in 1 mL sample loading buffer (*see* **Subheading 2.2.** and **Note 6**).
7. Store sample at –80°C.

3.2. Two-Dimensional Gel Electrophoresis

For the 2D analysis, it is very convenient to process several samples in parallel.

3.2.1. Isoelectric Focusing

1. Thaw the samples to 30°C, vortex, then briefly spin down any insoluble particles.
2. Apply a 400-µL (approx 1.5-mg) sample to a focusing tray and place an IPG-strip with the gel side facing down on the sample film (*see* **Note 7**).
3. Place a moist Whatman filter paper *under* the focusing tray and put it in a Ziplock plastic bag and incubate until the sample is completely absorbed by the strip (usually overnight).
4. Focus the strip until 100,000 Vh are reached (*see* **Note 7**). Running parameters: run-time 24 h, maximum voltage 5000 V, holding voltage 125 V, current per gel 80 µA.

3.2.2. Second Dimension

Before starting the equilibration, it is convenient to set up the gel running tanks, the gels, and the gaskets as described in the product instruction manual. To speed up the process, the upper and the lower buffers can be prepared and cooled to 4°C in advance.

1. Equilibrate the IPG strip (gel side down) in 10 mL equilibration buffer 1 (*see* **Subheading 2.3**) at room temperature for 10 min under gentle agitation. Remove all buffer 1 before continuing.
2. Equilibrate the IPG strip (gel side down) in 10 mL equilibration buffer 2 (*see* **Subheading 2.3.**) at room temperature for 10 min under gentle agitation. Remove all buffer 2.
3. Place the strips, plastic side facing backward to the glass of the gel without trapping any air between the gel surface and the IPG strip.
4. Running parameters: run-time 8 h, maximum voltage 500 V, max power/gel 20,000 mW.
5. Carefully take the gel out and place it in fixing solution for 2 h under agitation.
6. Remove the gel from fixing solution and place it in staining solution for 2 h under agitation (*see* **Note 8**).
7. Place the gel in destaining solution until gel is completely transparent.

3.2.3. Image Acquisition

To analyze the protein spots in a quantitative manner, it is necessary to obtain digital images. This is done in a regular flat bed scanner, which generates Tagged Image File Format (TIFF) files that can then be analyzed using spot detection and quantitation software (Phoretix 2D).

1. Carefully remove the gel from the destaining solution, and place it between two layers of transparent plastic sheet protectors. Make sure not to trap any air bubbles between the gel and the plastic foil. The use of a light box can be helpful.
2. Scan the gel and make sure that no liquid gets on the scanner surface or between the gel and the scanner lid because this may lead to artifacts.
3. Save the image as a TIFF.

3.3. Data Analysis

For increased accuracy, the sample preparation and the 2D gel electrophoresis should be repeated two to three times, each time starting with an independent colony. Using specialized imaging software (2D Phoretix from Nonlinear Dynamics), independent replicate gels can be averaged, resulting in a virtual 2D gel (**Fig. 3**). Averaged gels from wild-type and mutants strains can then be compared for expression differences (**Fig. 3**).

4. Notes

1. Lysate preparation: To minimize protein degradation, it is important to chill the sample at all steps prior to denaturation in sample-loading buffer. In case the sample warms up (e.g., during bead lysis), stop and chill before continuing. Try to do the sample preparation in one continuous work flow without any interruptions. In our experience, consistent expeditious sample preparation during all steps before isoelectric focusing is key to ensuring reproducibility of the procedure. Repeated 2D gel analyses of the same sample never revealed major variations, whereas comparative analysis of independently prepared samples showed variability, mainly owing to differences in sample handling before isoelectric focusing.
2. Bead lysis: Check state of lysis by phase-contrast microscopy during a chilling cycle. Broken cells lose their typical bright halo and appear dark (simultaneously compare to a sample of unlysed cells, if in doubt). Lysis is complete, when approx 60 to 80% of cells are broken.
3. Protein quantitation: It is convenient to perform the protein quantitation during the centrifugation step, to ensure quick processing.
4. Anion-exchange chromatography: The flow rate for the entire run is 1 mL/min. To be able to exactly compare several individually prepared samples, always normalize the protein concentration *and* the sample volume before application to the column. After the start of the elution, make sure the fraction collector is set to collect all fractions to the end of the entire run. The void volume of tubing may

lead to a shift of eluting proteins toward later fractions relative to the ultraviolet (UV) trace of protein elution.

5. TCA/acetone precipitation: Add β-mercaptoethanol to the chilled (–20°C) solution fresh. Do not use any plastic devices (tubes, pipets) in each of the following steps, because this may result in background peaks during subsequent mass-spectrometric protein identification.

6. 2D sample loading buffer: It is important to resuspend the protein pellet completely. Any insoluble protein causes problems and low reproducibility. To achieve complete resuspension, place the samples in a 30°C incubator under agitation. Do not heat the sample above 37°C as urea can cause protein modifications. At this point, it is recommended to analyze 5 μL of the samples on a regular SDS mini gel before continuing (*see* **Fig. 2B**). This step helps to monitor the quality and quantity of the sample prior to 2D analysis.

7. Isoelectric focusing: Avoid air bubbles under the IPG gel strip. Work expeditiously because evaporating buffer can lead to urea or protein precipitation. The isoelectric focusing is a very critical step. Poor focusing is a major reason for "bad" gels and can be caused by the presence of salt in the sample. Make sure 100,000 Vh are reached before continuing the second dimension.

8. Protein quantities in this protocol are adjusted such that proteins can be visualized efficiently with Coomassie Brilliant Blue. Silver staining is not recommended for this protocol because of its nonquantitative characteristics. Sypro Ruby can be used instead of Coomassie Brilliant Blue but requires a laser imager and a robotic spot picker to excise protein spots from the gels.

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Purification and Identification
of Protein Complexes That Control the Cell Cycle

Matthew A. Burtelow, Vladimir N. Podust, and Larry M. Karnitz

1. Introduction

The human Rad9-Hus1-Rad1 (9-1-1) and Rad17-RFC40-RFC38-RFC37-RFC36 (hRad17-RFC) protein complexes are important components of the mammalian deoxyribonucleic acid (DNA) damage response. Accumulating evidence from biochemical and molecular modeling studies indicate that these two complexes function as a clamp-clamp loader pair *(1)*. The 9-1-1 complex is converted to a less-extractable, DNA-bound form after DNA damage *(2)*. DNA damage-inducible binding of the 9-1-1 complex to chromatin is dependent on hRad17, but independent of ataxia-telangiectasia and *Rad3* (ATR) *(3)*. These data support a model in which hRad17-RFC and 9-1-1 are essential components of a DNA damage sensor that functions early in the DNA damage checkpoint pathway.

The baculovirus-expression system is a versatile and proved method to generate ample amounts of recombinant, multiprotein complexes. Recombinant baculoviruses are generated by inserting a gene of interest into viral DNA under the control of a strong polyhedrin promoter. These recombinant viruses can be amplified in culture to produce high-titer viral stocks on the order of 1×10^9 plaque-forming units (pfu)/mL. An advantage of the baculovirus-expression system is the ability to infect a single cell with multiple viruses encoding different recombinant proteins. This is a critical advantage when expressing multi-sub-unit proteins such as the 9-1-1 and hRad17-RFC complexes.

Optimization of protein expression using the baculovirus-expression system requires testing different insect cell lines, and varying multiplicities of infection (MOI) and infection times. Reports in the literature show that the choice of insect cell line is important because a protein that is expressed well in

one cell line may be expressed poorly in another line. Hi-5 cells are generally regarded as a superior protein-producing cell line whereas Sf9 cells are preferred for viral amplification. MOI is another important parameter that must be optimized. Low-MOI infections typically yield limited amounts of protein in the initial phases of the infection (1–2 d) and large amounts of protein later in the infection (3–5 d). In contrast, high-MOI infections are more reproducible, and large amounts of protein are consistently produced within 48 h of infection. An optimal MOI must be empirically determined for each recombinant baculovirus. When combinations of baculoviruses are used to infect insect cells, the relative amounts of each virus included in the infection must be optimized. In addition, in multivirus infections, the virus encoding the affinity-tagged subunit should be present in limiting amounts.

This chapter describes expression of the 9-1-1 and hRad17-RFC checkpoint complexes in Hi-5 insect cells and introduces a novel one-step affinity-purification scheme. The insect cell expression protocols were adapted from the methods used by Podust et al. to express recombinant, functional RFC complexes *(4,5)*. The affinity-purification method is a peptide-elution strategy that uses a 15-amino acid S-tag sequence. Elution of S-tag fusion proteins from an S-protein resin using competitor peptide was used to purify the 9-1-1 and hRad17-RFC protein complexes to near-homogeneity in a single step. Moreover, for these two protein complexes, S-tag purification was superior to both glutathione-*S*-transferase (GST)-tag and His-tag-affinity-purification schemes.

2. Materials

2.1. Insect Cell Culture and Baculovirus Reagents

1. Supplemented Grace's insect cell media (GIBCO cat. no. 11605-094) to which has been added 1 m*M* L-glutamine (Bio-Whittaker), 10% fetal bovine serum (Bio-Fluids), 100 U/mL penicillin (GIBCO), and 100 U/mL streptomycin (GIBCO).
2. 150 × 20-mm sterile tissue-culture dishes (Sarstedt).
3. Sf9 cells are derived from the fall army worm, *Spodoptera frugiperda*. Healthy cultures consist of spherical, regular-sized yellow cells that attach firmly to surfaces. The doubling time is 20–24 h.
4. Hi-5 insect cells are derived from the cabbage looper, *Trichoplusia ni*. A heterogeneous appearance of cultured cells is normal. These cells are seen in a light microscope as a mixture of spherical yellowish cells and blue spindle-shaped cells. Normally, Hi-5 cells are loosely attached to surfaces. The doubling time is 20–24 h.
5. Baculoviruses encoding S-tagged hRad9, hHus1, and hRad1 *(6)*.
6. Baculoviruses encoding S-tagged hRad17 (M.A.B. and L.M.K., unpublished data), RFC36, RFC37, RFC38, and RFC40-His *(5)*

2.2. S-Tag Purification Reagents

1. Hi-5 Lysis Buffer: 20 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl$_2$, 0.1% Nonidet P-40 [NP-40] freshly supplemented with 40 µg/mL Pepstatin (Sigma), 40 µg/mL Leupeptin (Sigma), 20 µg/mL Aprotinin (Sigma), 20 ng/mL Microcystin LR (Fisher), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1 mM dithiothreitol (DTT) (Sigma), and 10 mM β-glycerophosphate (Sigma).
2. S-tag wash buffer: 20 mM HEPES, pH 7.4, 250 mM NaCl, 10 mM MgCl$_2$, 0.1% NP-40 supplemented with 1 mM DTT, 10 mM β-glycerophosphate, and 1 mM PMSF.
3. S-tag elution buffer: 20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM MgCl$_2$, 0.01% NP-40 supplemented with the same inhibitors used in the lysis buffer and with 1.0 mg/mL S-peptide (KETAAAKFERQHMDS).
4. S-protein agarose (Novagen).
5. S-peptide, lyophilized, KETAAAKFERQHMDS (Mayo Protein Synthesis Core)
6. Bio-Rad protein assay reagent, cat. no. 500-0006.

3. Methods

3.1. Amplification and Functional Titering of Baculovirus Stocks

1. Produce high-titer baculoviral stocks by amplifying each parental virus three times in Sf9 cells. For the initial amplification step, add 1 mL of viral supernatant obtained from CellFECTIN (GIBCO)-mediated transfection of Sf9 cells to a 100×20-mm tissue-culture dish containing 3×10^6 freshly plated Sf9 cells in 7 mL of supplemented Grace's insect cell media. After 4–6 d, collect the medium and remove cell debris by centrifugation at 500g.
2. For the second amplification, add 1 mL of the initial amplification stock to a fresh 100×20-mm tissue-culture dish containing 3×10^6 Sf9 cells in 7 mL of media. Again, allow the infection to proceed for 4–6 d. Collect the viral stock and clarify by centrifugation.
3. Perform the third and final amplification by repeating the steps outlined for the second amplification. For routine use, store supernatants at 4°C. Freeze 1-mL fractions of baculovirus stocks in cryovials at –80°C for long-term storage (*see* **Note 1**).
4. Rather than precisely determining viral titers through tedious agar plaque assays, the viruses are "functionally titered." Inoculate Hi-5 cells with increasing amounts of viral supernatants (e.g., 10 µL, 100 µL, and 1000 µL). Harvest cells 48 h later and purify the recombinant S-tagged fusion protein using S-protein agarose (*see* **Subheading 3.2.**). Analyze the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. This method identifies the smallest volume of viral stock that produces the greatest amounts of S-tagged hRad9 protein. Once an optimal amount of the baculovirus encoding S-tagged hRad9 is determined, coinfect increasing volumes of viruses encoding untagged hRad1 and hHus1 with the empirically determined optimal volume of S-tagged hRad9 virus. This optimization identifies the rela-

tive volumes of baculovirus stocks that yield the maximal amount of the three-subunit 9-1-1 complex. Because hRad9 is the least expressed of the three proteins, the affinity-purification tag is affixed to this polypeptide.

3.2. Expression and Purification of the 9-1-1 Complex in Insect Cells

1. Suspend Hi-5 cells (2×10^7) in 20 mL of supplemented Grace's insect cell media and plate into a 150×20-mm tissue-culture dish (*see* **Note 2**).
2. Add the empirically optimized volumes of S-tagged hRad9, hRad1, and hHus1 viral stocks to each culture dish. Gently swirl dishes to distribute the viruses in the media (*see* **Note 3**).
3. Harvest cells 48 h postinfection. Collect infected Hi-5 cells by dislodging cells from the bottom of the dish. In contrast to uninfected cells, infected cells are easily detached from the dish when a gentle pressure stream is applied using a pipet.
4. Place the cell suspension in a 50-mL conical tube and collect cells by centrifugation at 500g for 5 min. To avoid cell rupture, do not subject the cells to higher centrifugal forces. Do not wash the cell pellet with phosphate-buffered saline (PBS); this will increase cell lysis.
5. Decant or aspirate the media from the insect cell pellet. The insect cell pellet can be frozen at –80°C for future use or immediately processed for purification. Freezing of cell pellets does not affect subsequent purification of protein complexes.
6. Resuspend the cell pellets from three 150-mm dishes (6×10^7 Hi-5 cells) in 5 mL of Hi-5 lysis buffer. Lyse cells for 10 min at 4°C.
7. Aliquot the cell lysate into six 1.5-mL microcentrifuge tubes (approx 1.1 mL per tube) and centrifuge (20,000g) for 10 min at 4°C.
8. Transfer the supernatant from the six microcentrifuge tubes to clean, prechilled 1.5-mL microcentrifuge tubes. Adjust the cell lysate to a final NaCl concentration of 250 mM by adding 1/25 the volume of 5 M NaCl. This will increase the salt concentration from 50 mM to 250 mM. Mix well and incubate on ice for 5 min. Centrifuge (20,000g) for 10 min.
9. Collect the supernatants from each tube and transfer them to fresh 1.5-mL microcentrifuge tubes that each contain 15 µL (packed volume) of S-protein-agarose beads suspended in 100 µL of Hi-5 wash buffer.
10. Gently rotate the tubes for 60 min at 4°C.
11. While the proteins are binding to the S-protein agarose, prepare a solution of S-tag peptide by dissolving 1.0 mg of lyophilized S-peptide (KETAAA KFERQHMDS) in 1.0 mL of S-tag elution buffer. Aliquots can be frozen at –80°C. Addition of the S-peptide does not alter the pH of the elution buffer.
12. Wash the S-protein-agarose beads in each microcentrifuge tube three times with 1 mL Hi-5 wash buffer. For each wash, centrifuge (5800g) for 30 s. Next, wash the beads once with 1 mL of S-tag elution buffer. During the second wash with elution buffer, pool the S-protein-agarose beads into a single 1.5-mL microcentrifuge tube.

13. Centrifuge (5800g) the pooled S-protein-agarose beads for 30 s and remove all traces of buffer with a 27-gage needle connected to an aspirator. Add 120 µL of S-peptide-containing elution buffer. Mix the beads well and incubate for 15 min in a 37°C water bath. Flick the tube every 2–3 min.
14. Centrifuge (9000g) the S-protein-agarose beads for 1 min. Transfer the supernatant to a clean, prechilled microcentrifuge tube. This peptide elution strategy elutes approx 50% of the fusion protein bound to the beads in a single extraction. If desired, perform additional peptide elutions; however, the yield is significantly diminished.
15. Analyze the purity of the eluted protein complexes by SDS-PAGE followed by Coomassie blue staining. Determine protein yield using the Bio-Rad protein assay reagent. An infection of three 150-mm plates yields about 50 µg of the 9-1-1 complex.

3.3. Purification of hRad17-RFC Complex From Insect Cells

1. Generate high-titer baculoviral stocks of S-tagged hRad17, RFC40, RFC38, RFC37, and RFC36. Amplify all stock viruses three times as described previously for the 9-1-1 complex methods. Functionally titer S-tagged Rad17 by infecting Hi-5 cells with 10-fold increments of viral supernatants as described for S-tagged Rad9. Optimize multiple infections using the empirical method described for purification of the 9-1-1 complex (*see* **Note 2**).
2. Plate 2×10^7 Hi-5 cells suspended in 20 mL of Grace's insect cell media into a 150×20-mm tissue-culture dish.
3. Add the empirically determined ratios of the five baculoviruses encoding components of the hRad17-RFC complex to a 150-mm tissue-culture dish. Swirl to mix the viruses and incubate for 48 h.
4. Follow **steps 3–14** in the previous 9-1-1 purification protocol described in **Subheading 3.2.** In this case, lyse pellets from four dishes (instead of three dishes for 9-1-1) in 5 mL of lysis buffer. Because the insect cell lysate is very concentrated, we double the concentrations of protease and phosphatase inhibitors in the initial lysis buffer. Despite the increased quantity of protease inhibitors, we still observed some degradation of Rad17 (*see* band below Rad17 in **Fig. 1B** and *see* **Note 4**). Infection of four 150-mm tissue-culture dishes yields 25–100 µg of the hRad17-RFC protein complex.

4. Notes

1. For routine use, store baculoviral stocks at 4°C. Store long-term at –80°C. Reamplify baculoviral stocks every 12 mo.
2. Hi-5 and Sf9 cells age and lose viability with time. Thaw fresh stocks of frozen insect cells when increases in doubling time or morphologic changes are observed. Subclones of insect cells acquired from different suppliers or laboratories may differ greatly in protein expression. If available, test several cell subclones for protein expression.
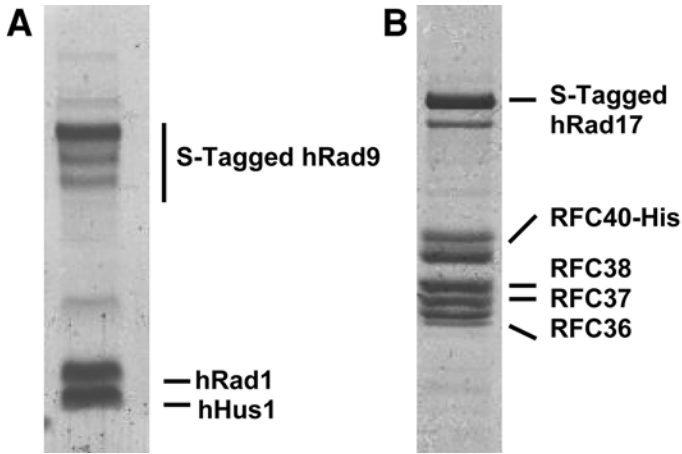
Fig. 1. (**A**) Hi-5 cells (2×10^7) were infected with the following baculoviruses: 150 μL S-tag hRad9, 150 μL hRad1, and 75 μL hHus1. Cells were harvested 48 h later and lysed in 1 mL of Hi-5 lysis buffer. The 9-1-1 complex was purified using 15 μL (packed volume) of S-protein agarose. The resin with bound proteins was washed three times. Bound proteins were eluted with 125 μL of 1 mg/mL S-peptide elution buffer. Eluted proteins were analyzed by 10% SDS-PAGE followed by Coomassie blue staining. (**B**) Hi-5 cells (4×10^7) were infected with the following baculoviruses: 200 μL S-tag hRad17, 90 μL RFC40-His, 90 μL RFC38, 90 μL RFC37, and 90 μL RFC36. Cells were harvested 48 h later and lysed in 1 mL Hi-5 lysis buffer. The hRad17-RFC complex was purified using 15 μL of S-protein-agarose beads. Bound proteins were eluted with 125 μL of 1 mg/mL S-peptide elution buffer. Eluted proteins were analyzed by 10% SDS-PAGE followed by Coomassie blue staining.

3. Every protein is expressed with varying efficiency in insect cells. One protein may be expressed poorly regardless of multiplicity of infection, whereas another protein may be expressed to high levels even in a low-MOI infection.
4. Proteolysis of hRad17 was consistently observed during purification of the hRad17-RFC complex. Affinity chromatography of the His-tagged Rad17-RFC complex using Ni^{+2} resin resulted in copurification of a protease that degraded hRad17, producing a distinct 45-kDa fragment. We determined that one-step affinity-purification techniques, including S-tag and GST, minimize hRad17 proteolysis.

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Xenopus Cell-Free Extracts to Study DNA Damage Checkpoints

Vincenzo Costanzo and Jean Gautier

1. Introduction

Surveillance mechanisms monitoring genomic integrity operate through signal transduction pathways called checkpoints. Checkpoints are essential to delay cell cycle progression in response to deoxyribonucleic acid (DNA) damage *(1,2)*. These pathways require the coordinated monitoring and sensing of the damaged DNA with downstream signaling ultimately leading to cell cycle arrest. Traditionally, the DNA damage response and its checkpoint's component have been studied mostly using yeast and mammalian cells. Budding and fission yeasts have been instrumental to identify mutations in genes impaired in DNA damage cell cycle checkpoints. Genetic screens in yeast have been a successful approach to identify radiation-sensitive (*rad* mutants) and checkpoint genes (*hus*, *mad*, *bub* mutants) participating in the maintenance of genomic integrity *(3)*. However, this approach has several limitations. Essential genes may never be isolated in standard genetic screens. DNA damage response is more complex in vertebrates than it is in yeast and critical regulators of the DNA damage response, such as p53 and *BRCA1*, are found only in vertebrates *(2)*. Mammalian cell lines from diverse origins, including some derived from patients harboring defects in the DNA damage response, have also been used extensively to study the DNA damage response *(4–6)*. However, these cell-based systems do not allow using specific biochemical readouts as they are based on phenotypes resulting from complex outputs such as cell growth or survival.

DNA replication can be studied in vitro in cell-free extracts derived from *Xenopus* eggs. Chromosomal DNA added to these extracts undergoes a complete round of semiconservative replication *(7)*. Cell-free systems derived from

Xenopus eggs have also been widely used to elucidate the biochemical bases of cell cycle transitions. They have been especially powerful in probing the regulation of entry into S phase and into mitosis *(8–13)*. Finally, *Xenopus* extracts have been used to study checkpoint signaling following the experimental inhibition of DNA replication. In such systems, G2 cell cycle checkpoint activity is monitored by the inhibition of nuclear envelope breakdown following experimental interference with DNA replication *(14–18)*. These cell-free systems allow extensive biochemical analysis and are dispensable for both transcription and protein synthesis.

Here we describe our recent technical advances in designing cell-free extracts derived from *Xenopus* eggs that recapitulate several aspects of the DNA damage response, including DNA damage cell cycle checkpoints. To date, these cell-free systems have been instrumental to analyze the DNA damage cell cycle checkpoints that prevent initiation of DNA replication *(19)* as well as the coordination between DNA replication, DNA recombination, and DNA repair *(20)*. We anticipate that these cell-free systems will allow for the analysis of poorly understood aspects of the DNA damage response, such as the characterization of the aberrant DNA structure(s) that can elicit a DNA damage response and the study of the early steps of the response during which such structures are sensed.

2. Materials

2.1. Animals

Xenopus laevis, females and males.

2.2. Buffers

1. MMR buffer: 5 mM HEPES, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$.
2. XB buffer: 10 mM HEPES, pH 7.7, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 100 mM KCl, 50 mM sucrose.
3. Cytostatic factor (CSF)-XB buffer: 10 mM HEPES, pH 7.7, 2 mM MgCl$_2$, 0.1 mM CaCl$_2$, 100 mM KCl , 5 mM ethylene glycol *bis* (2-aminoethyl ether)-*N*,*N*,*N'N'*-tetraacetic acid (EGTA), 50 mM sucrose.
4. Energy mix: 50 mM creatine phosphate, 20 mM adenosine triphosphate (ATP), 2 mM EGTA, 20 mM MgCl$_2$.
5. Chromatin isolation buffer: 50 mM KCl, 5 mM MgCl$_2$, 2 mM dithiothreitol (DTT), 50 mM HEPES, 0.5 mM spermine 3 HCl, 0.15 mM spermidine 4HCl, 1 μg/μL aprotinin, pepstatin, leupeptin, and 0.125% Triton X-100.
6. Stop solution: 8 mM EDTA, 80 mM Tris-HCl, pH 8.0, 2.5% sodium dodecyl sulfate (SDS).
7. LFB buffer: 50 mM KCl, 40 mM HEPES-KOH, pH 8.0, 20 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 8.0, 2 mM DTT, 2 mM MgCl$_2$, 1 mM EGTA, 10% sucrose, 1 μg/μL of aprotitin, pepstatin, and leupeptin.

8. NPB buffer: 250 m*M* sucrose, 15 m*M* HEPES, pH 7.4, 1 m*M* EDTA, pH 8.0, 0.5 m*M* spermidine, 0.2 m*M* spermine, 1 m*M* DTT, 10µg/mL leupeptin, and 0.3 m*M* phenylmethylsulfonyl fluoride (PMSF), made fresh each time.

2.3. Other Reagents

Bovine serum albumin (BSA); 6-dimethylaminopurine (6-DMAP), (Sigma); pBR322 plasmid; HAEIII restriction enzyme (NEB); etoposide (Sigma); caffeine (Sigma), lysolecithin (Sigma); tautomycin (BioMol); wortmannin (Sigma); hormones: human chorionic gonadotropin (HCG), pregnant mare's serum gonadotropin (PMSG) (Sigma, Calbiochem); terminal deoxynucleotidyl transferase (TdT) (GIBCO-BRL); antiphospho histone H2AX antibodies (Upstate Biotech); α ^{32}P-dGTP (Amersham); dATP, dGTP (Sigma).

3. Methods

The methods described below outline (a) the preparation of different types of extracts, (b) the preparation of the templates, (c) the assays' protocols, and (d) related assays for the DNA damage response.

3.1. Extracts

The description of extracts preparation includes (a) CSF extracts, arrested in M-phase; (b) activated egg extracts; and (c) extracts treated with 6-DMAP to inhibit protein kinases required for initiation of DNA replication. These extracts can be further fractionated for checkpoint studies into (d) fractionated membrane-free cytosol (*see* **Fig. 1** and **Note 1**).

3.1.1. CSF Extract

1. CSF-arrested extracts are freshly prepared as previously described *(21)*.
2. *Xenopus* females are induced to lay eggs with 500 IU of HCG injected the night prior to egg collection.
3. The eggs are collected overnight in MMR solution.
4. The eggs are washed in MMR, and as much MMR as possible is removed before dejellying the eggs in 2% cysteine in H$_2$O, pH 7.8.
5. Eggs are then incubated in cysteine with occasional stirring until they are packed. All cysteine is then removed. The eggs are washed three times with XB in a gelatin-coated Petri dish; remove all XB.
6. The eggs are washed three times in CSF-XB, and as much buffer as possible is removed.
7. The eggs are then washed two times in XB containing protease inhibitors (10 µg/mL leupeptin, pepstatin, and aprotinin).
8. The eggs are transferred to 1 mL of CSF-XB with protease inhibitors and 100 µg/mL cytochalasin B and then into 1.5-mL Eppendorf tubes.
9. The eggs are packed by spinning for a few seconds at 1000*g*, excess buffer is removed, and the eggs are crushed at 16°C for 15 min at 16,000*g*.

10. The extract is collected with an 18-gage needle by puncturing the side of the tube and gently sucking out the cloudy intermediate cytoplasmic layer. This layer varies slightly in color from batch to batch and is located between the superficial opaque lipid yellow layer and the solid pellet of pigments and egg debris.
11. The cytosolic extract is supplemented with 1/20 volume of cytochalasin B, 1/20 vol of energy mix, and 1/40 vol of 2 *M* sucrose.
12. The cytosolic extract is further clarified by centrifugation for 30 min at 13,000*g* in an Eppendorf centrifuge at 4°C. The clear cytoplasmic layer is then collected for immediate use.

3.1.2. Activated CSF Extract

For most assays, including DNA replication, chromatin binding (*see* **Subheading 3.4.1.**) and checkpoint assays (*see* **Subheading 3.3.1.**), extracts are supplemented with 100 µg/µL of cycloheximide and 0.4 m*M* CaCl$_2$, then incubated for 15 min at 23°C. This treatment mimics the wave of calcium normally taking place following fertilization and triggers the degradation of mitotic cyclins, the inactivation of Cdc2/Cyclin B and the subsequent exit from mitosis.

3.1.3. 6-DMAP Extract

1. Double-stranded breaks (DSBs)-induced checkpoint assay (*see* **Subheading 3.3.2.**) requires extract treated with 6-DMAP.
2. CSF extract (*see* **Subheading 3.1.1.**) is incubated for 30 min with 3 m*M* 6-DMAP at 23°C. The extract is then supplemented with 0.4 m*M* CaCl$_2$ and incubated for 15 min at 23°C.

3.1.4. Fractionation of Membrane-Free Egg Cytosol

1. Fractions are prepared as originally described *(22)* to study DNA replication initiation.
2. Interphase extracts (*see* **Subheading 3.1.2.**) are subjected to fractionation by stepwise PEG precipitation to yield fractions M and B *(23)*.
3. Interphase extracts (*see* **Subheading 3.1.2.**) are diluted fourfold with cold LFB buffer.
4. Diluted extracts are then subjected to an ultracentrifugation step at 80,000*g* for 40 min at 4°C in a Beckmann TL100 tabletop ultracentrifuge, using a TLS55 rotor.
5. Supernatants are carefully pipetted and transferred to avoid pellet contamination, then supplemented with 0.075 vol of a 50% PEG solution to give a final concentration of 3.5%.
6. Samples are incubated on ice for 30 min and spun for 10 min at 10,000*g* at 4°C.
7. Pellets are resuspended in a volume of LFB containing 2.5 m*M* Mg-ATP corresponding to one-fifth of the starting vol of extract. This yields to fraction B.
8. The corresponding supernatant is adjusted to 9% PEG, then incubated on ice for 30 min, and spun for 10 min at 10,000*g* at 4°C to yield fraction M (*see* **Note 2**).
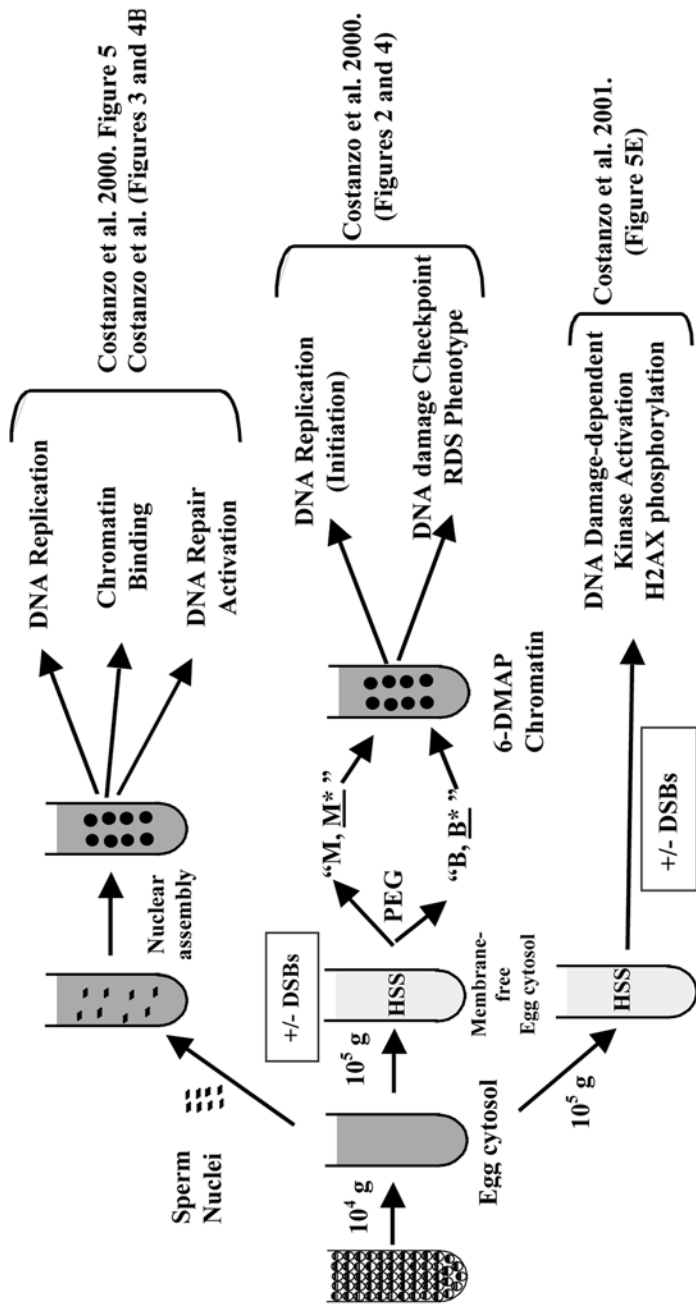
Fig. 1. Schematic representation of the different types of extracts described. For each type of extract, some examples of the assays relevant to the study of the DNA damage responses are given.

3.2. Templates

Here we describe the preparation of (a) the chromosomal templates used for DNA replication assay, (b) the 6-DMAP chromatin template used for checkpoint assay, and (c) the damage DNA templates used to induce a checkpoint response (*see* **Note 3**).

3.2.1. Chromatin Templates for DNA Replication

1. Chromatin templates for DNA replication are prepared from demembraned *Xenopus* sperm nuclei as previously described *(21)*.
2. *Xenopus* males are injected with 50 U of PMSG 3 d before the experiments.
3. The day prior to nuclei preparation each male is injected with 500 IU HCG.
4. Males are anesthetized in 1% tricaine in H_2O.
5. Testes are removed using surgical scissors and placed in a 35-mm tissue-culture dish containing cold 1X MMR.
6. The testis are rinsed three times in cold 1X MMR and transferred to another 35-mm tissue-culture dish with 5 mL of cold 1X NPB for 2 to 5 min.
7. The testes are transferred to a clean 35-mm tissue-culture dish with 5 mL of cold 1X NPB and macerated thoroughly with Dumont #5 forceps.
8. The sperm suspension is then centrifuged at 2500*g* for 10 min in microfuge tubes.
9. During this step, 1 mg of L-α-lysophosphatidylcholine (lysolecithin) is dissolved in 100 μL of H_2O (10mg/mL) at room temperature (RT). Lysolecithin will not remain in solution below RT.
10. The sperm pellet is resuspended in 1 mL of 1X NPB that has been equilibrated at RT, and 50 μL of lysolecithin solution is added. The suspension is mixed and incubated for 5 min at RT.
11. 10 mL cold 1X NPB containing 3% BSA is added to the suspension that is then centrifuged for 10 min at 2500*g*.
12. The pellet is resuspended in 5 mL cold 1X NPB containing 0.3% BSA and centrifuged for 10 min at 2500*g*.
13. The supernatant is decanted and the pellet resuspended in 500 μL of 1X NPB containing 30% (w/v) glycerol and 0.3% BSA (sperm storage buffer).
14. Aliquots (50-μL) are frozen in liquid nitrogen and kept for up to 6 mo at –80°C.
15. The concentration of demembraned sperm nuclei is calculated by diluting a small aliquot of the preparation in NPB containing 1 μg/mL Hoechst 33342 and by counting the fluorescent nuclei with a hemocytometer. Normal yield should range between 3 and 10×10^7 nuclei/frog.

3.2.2. 6-DMAP Chromatin

6-DMAP chromatin is prepared as follows:

1. Nuclei (*see* **Subheading 3.2.1.**) at a concentration of 40,000 nuclei/μL are incubated for 20 min at 23°C in 6-DMAP extract (*see* **Subheading 3.1.3.**).

2. The chromatin-containing extract (50 μL) is then diluted 10-fold in chromatin isolation buffer.
3. The suspension is then carefully layered on top of 1.2 mL of chromatin isolation buffer containing 30% sucrose.
4. 6-DMAP chromatin is then pelleted at 6000*g* for 15 min at 4°C and is resuspended in chromatin isolation buffer supplemented with 2.5 m*M* Mg-ATP.
5. 6-DMAP chromatin is used immediately (*see* **Note 4**).

3.2.3. Damaged DNA Templates

DNA molecules containing DSBs are generated from plasmid DNA. Circular pBR322 plasmid is digested to completion with restriction endonucleases to yield DNA fragments containing DSBs. We tested different enzymes generating different types of DNA ends (blunt, 3' overhang, or 5' overhang) and did not observe differences in the DNA damage response *(19)*.

1. 0.5 mg of pBR322 is digested with HaeIII (NEB). HaeIII cuts pBR322 plasmid 25 times, thus generating 26 fragments containing 2 DSBs each.
2. Digested DNA is then extracted twice in ϕ-chloroform and then precipitated in ethanol and sodium acetate.
3. DSBs-containing DNA is resuspended in H_2O at a concentration of 1 mg/mL.
4. The DSBs DNA stock solution is then diluted into the extracts to the desired concentration.
   Alternatively, we have used λ-DNA that was digested with a series of restriction enzymes giving rise to different numbers of restriction fragments. λ-DNA is digested with *Xbe*I, *Nco*I, *Hin*dIII, and *Bst*EI enzymes that generate 2, 5, 7, and 14 fragments, respectively. This latter approach allows us to increase the concentration of DSBs in the extracts but keep the mass of added DNA constant.

3.3. Checkpoint Assays

We have reconstituted two kinds of DNA damage checkpoint responses in cell-free systems. In the first assay *(1)*, the damage is taking place on the template during DNA replication, in *cis*. In the second type of assay *(2)*, the template used for DNA replication is not damaged, and DNA damage signaling is induced in *trans* by exogenous DSBs-containing DNA.

3.3.1. DNA Damage Checkpoint Induced by Etoposide

Cytosolic extracts derived from *Xenopus* eggs can support semiconservative DNA replication of genomic DNA when chromatin templates are added to extracts *(7)*. We have reconstituted a cell-free system that recapitulates the inhibition of DNA replication once early firing origins have been blocked.

We use etoposide, an inhibitor of topoisomerase II, to generate DNA lesions during DNA replication and to trigger a DNA damage checkpoint response. Etoposide generates lesions in the chromatin templates that are undergoing

DNA replication by blocking the activity of DNA topoisomerase II covalently linked to DNA 5' termini *(24)*.

1. 20 µL of activated extract (*see* **Subheading 3.1.2.**) is incubated with 5000 demembraned sperm nuclei/µL in the presence of etoposide at 23°C for 90 min.
2. Concentrations of etoposide ranging from 10 to 50µ*M* are effective to induce a checkpoint response as seen by the inhibition of genomic DNA replication.
3. Etoposide-induced inhibition of DNA replication is rescued by addition of 5 m*M* caffeine, a known inhibitor of checkpoint signaling kinases, including ATM and ataxia-telangiectasia and *Rad3* (ATR) *(25)*.
4. DNA replication is monitored by incorporation of ^{32}P-dATP into the chromatin.
5. 0.2 µCi of α-^{32}P-dATP is added to each replication reaction.
6. DNA replication reactions are stopped by diluting the samples in 200 µL of stop solution.
7. Diluted samples are incubated with 1 mg/mL of proteinase K for 30 min at 37°C.
8. DNA is extracted with one vol of φ-chlorophorm.
9. The samples are then centrifuged for 10 min at RT.
10. The aqueous phase is separated and precipitated with two vol of ethanol and 10 m*M* ammonium acetate.
11. The pellet is resuspended in DNA loading buffer and run on a 0.8% agarose gel in TBE.
12. The gel is fixed in 7% tricarboxylic acid (TCA). The gel is then positioned between two layers of Whatman 3MM paper and stacks of filter paper and dried overnight. The dried gel exposes X-ray film for autoradiography.

3.3.2. Cell-Free System to Study DNA Replication in the Presence of DSB

To recapitulate the cell cycle response to DNA damage at the onset of S phase, we modified a cell-free system designed to study initiation of DNA replication *(22)*. Activated extracts (*see* **Subheading 3.1.2.**) are treated with either circular plasmid DNA, plasmid DNA containing DSBs, or λ-DNA containing DSBs (*see* **Subheading 3.2.3.**). Treatment of the cytosolic extracts with DSBs-containing DNA activates a checkpoint in *trans*. In this protocol the damaged DNA that triggers the checkpoint is not carried over during the replication reaction, and the extract is tested for its ability to replicate intact chromatin templates (*see* **Subheading 3.2.2.**). The damaged template is removed to avoid any interference with genomic DNA replication, such as titration of essential factors required in the elongation step of genomic DNA replication.

1. 100 µL of activated extract (*see* **Subheading 3.1.2.**) is incubated at 23°C in the presence of 50 ng/mL of circular plasmid DNA or digested plasmid (DSB) for 15 min to activate the checkpoint.
2. In rescue experiments, extracts are pretreated for 15 min at 23°C with 5 m*M* caffeine, 200 n*M* wortmannin, or affinity-purified anti-X-ATM antibodies *(26)* and then incubated with damaged DNA.

M and B fractions are prepared as originally described *(23)* to study DNA replication initiation. M and B fractions are prepared from cytosolic extracts treated as described above (*see* **Subheading 3.1.4.**), except that the fractions are prepared from extracts in which the checkpoint response has been activated by DSBs. Fractions derived from extract treated with DSBs are called M* and B*.

1. Replication assays are performed by mixing 0.5 µL of 6-DMAP chromatin (10,000 nuclei/µL) with 1 µL each of either M and B or M* and B* fraction obtained from extract treated with different types of DNA molecules and/or caffeine, wortmannin, and ATM-neutralizing antibodies.
2. The reactions are incubated for 15 min at 23°C. 10 µL of 6-DMAP extract (*see* **Subheading 3.1.3.**) is then added.
3. DNA synthesis is monitored by the incorporation of α-^{32}P-dATP for 90 min at 23°C, followed by agarose gel electrophoresis (*see* **Subheading 3.3.1.**).

3.4. Other DNA Damage Responses

3.4.1. Chromatin Binding

One critical aspect of the DNA damage response is the damage-dependent localization of a variety of proteins to the chromatin. This is exemplified by the formation of damage-induced foci within the nuclei of mammalian cells. Cell-free systems allow for the rapid mixing and subsequent separation of chromatin, nuclear, and cytoplasmic fractions. To analyze the status of the proteins that bind the chromatin in a replication or checkpoint-dependent manner, we routinely perform chromatin-binding assays.

1. Chromatin-binding assays are performed in activated extracts (*see* **Subheading 3.1.2.**) or in fractionated extracts (*see* **Subheading 3.1.4.**). In the case of activated extracts, chromatin is assembled in 50 µL of interphase extracts in which a checkpoint has either been activated or not activated.
2. 10,000 nuclei/µL are incubated for 60 min, and the extract is diluted with up to 800 µL of chromatin isolation buffer (*see* **Subheading 2.2.**).
3. In the case of fractionated extracts (*see* **Subheading 3.1.4.**), replication reactions are assembled as above (*see* **Subheading 3.3.2.**) with the following modifications. Reactions are scaled up 10-fold. 10 µL of M and B fractions are incubated for 15 min with 5 µL of 6-DMAP chromatin (10,000 nuclei/µL).
4. Following incubation, each reaction is diluted in 200 µL of chromatin isolation buffer supplemented with 0.1% Triton X-100.
5. The chromatin is then layered onto the same buffer containing 30% sucrose.
6. The chromatin is centrifuged at 6000*g* for 15 min at 4°C.
7. The pellet is resuspended in Laemmli loading buffer.
8. The samples were run on 10% SDS-PAGE and analyzed by Western blotting with specific antibodies.

3.4.2. Monitoring the Generation
of Double-Stranded Breaks in Cell-Free Systems

DNA DSBs can arise as a consequence of normal physiological processes such as during normal DNA replication *(20)*. DSBs can also occur as a primary or a secondary consequence of damage inflicted to cells. Therefore, it is important to have sensitive assays to monitor the occurrence of DNA DSBs when studying checkpoint signaling.

We have designed two techniques that monitor the formation of DSBs, and we have used them successfully to demonstrate that DSBs arise during DNA replication in the absence of X-Mre11. The first technique (a) is based on the direct labeling of DNA containing DSBs by terminal transferase, whereas the second technique (b) uses indirect labeling of chromatin protein using an antibody against phosphorylated histone H2AX. Terminal transferase covalently adds dNTP to 3-OH of deoxynucleotides. H2AX is a histone variant that specifically becomes phosphorylated in the presence of DSB. Phospho-H2AX is detected in nucleosomes that are in proximity to the breaks *(27,28)*. Details of these protocols follow.

3.4.2.1. TUNEL ASSAY

1. 50 μL of control interphase extract (*see* **Subheading 3.1.2.**) or extract in which the occurrence of DSBs will be assessed are incubated with 10,000 nuclei/μL for 120 min at 20°C.
2. Extracts are diluted in 1 mL of a buffer consisting of 100 mM KCl, 25 mM HEPES, pH 7.8, 2.5 MgCl$_2$ and 0.4% Triton X-100.
3. Samples are layered onto the same buffer containing 30% sucrose without Triton and spun for 20 min at 6000g in a HB-6 rotor (Sorvall).
4. Pellets are washed and incubated at 37°C for 4 h in a buffer containing 90 U of terminal transferase, 100 mM potassium cacodylate, pH 7.0, 1 mM CoCl$_2$, 0.2 mM DTT, 25 μCi dGTP, 3000 Ci/mM, and 50 μM dGTP.
5. Control reactions are incubated in the same buffer without TdT.
6. Reaction mixtures are then treated with 0.1 mg/mL proteinase K and the DNA was φ-chloroform extracted and electrophoresed on a 0.5% agarose gel at 100 V for 60 min.
7. The gel is fixed in 20% TCA, dried, and then exposed for autoradiography.
8. The labeled band are excised from the gel and quantified by scintillation counting (*see* **Note 5**).

3.4.2.2. PHOSPHORYLATED HISTONE H2AX DETECTION

1. 50 μL of control interphase extract (*see* **Subheading 3.1.2.**) or extract in which the occurrence of DSBs will be assessed are incubated with 10,000 nuclei/μL for 90 min at 23°C.
2. Postreplicative chromatin is isolated by diluting the extracts in chromatin isolation buffer containing 1 mM NaF, 1 mM sodium vanadate, and 0.125% Triton X-100.

3. Samples are layered onto chromatin isolation buffer (*see* **Subheading 2.2.**) containing 30% sucrose and lacking Triton X-100, then spun at 6000*g* for 20 min at 4°C.
4. A positive control is prepared by incubating sperm nuclei for 30 min in interphase extract to decondense chromatin.
5. The chromatin is then isolated and digested for 4 h with *Not*I.
6. Digested chromatin is isolated through a sucrose cushion and incubated in interphase extract for 60 min.
7. Chromatin is boiled in Laemmli buffer and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
8. Antiphosphorylated H2AX antibody is used for Western blotting at 1/6000 dilution.

4. Notes

1. The homogeneity of cytosolic extracts is critical to the success of all procedures; therefore, extracts need to be mixed several times by pipetting very gently, but very thoroughly, to avoid formation of aggregates.
2. For M/B fractionation, the quality of the eggs and the timing of the preparation are critical to get functional fractions. We perform the fractionation quickly, as soon as possible following the preparation of the extract. The complete procedure should not take more than 3 h to recover functional fractions. The quality of M and B fractions can also be tested in pilot experiments. M or B fractions do not support DNA replication by themselves but only in combination. If background replication is observed with either M or B fraction alone, the concentration of PEG used for fractionation can be modified with a 1% window: $3.5 \pm 0.5\%$ for B and $9 \pm 0.5\%$ for M.
3. The stability of damaged DNA templates can be evaluated following incubation in cell-free extracts. 5' DNA termini and 3' DNA termini are labeled with T4 kinase and TdT, respectively (GIBCO labeling kits).
4. Some antigens tested for chromatin binding can be very abundant in the cytosolic fraction in addition to the chromatin-bound fraction. It is therefore critical to avoid cytoplasmic contamination during the chromatin isolation step. To reduce background owing to cytoplasmic contamination of the chromatin pellets, we further centrifuge interphase extract for 30 min at 13,000*g* at 4°C. Furthermore, the chromatin pellets are isolated after freezing the bottom of the Eppendorf tube in liquid nitrogen by cutting the tip of the tube with scissors. We recover the pellet from the tip of the tube, resuspending it in Laemmli buffer.
5. For the TdT-mediated dUTP nick end-labeling (TUNEL) assay, it is critical to check whether the extract has supported DNA replication. An aliquot of replicating extract can be incubated with α-^{32}P-dATP, processed, and run on the gel to monitor the DNA replication.

Acknowledgments

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21

Protein–Protein Interactions

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1. Introduction

Most proteins need to interact physically with other proteins to be able to perform their biological functions; the identification of new protein-binding partners for a protein of interest may therefore be a key strategy in defining the mechanism of action for a particular protein. Many different approaches to studying protein–protein interactions exist, but identifying which interactions are biologically significant is not trivial. Thus, once an interaction is found, the first step is to validate the suggested interactions using genetic, biochemical, and cell–biological approaches. Here we will focus on three complementary protein interaction approaches: (a) the yeast two-hybrid (YTH) system, a genetic assay performed in living yeast cells, which can be used both to identify proteins that bind to a protein of interest and to determine domains or residues critical for an interaction; and two biochemical approaches, (b) the coimmuno-precipitation of interacting proteins with a specific antibody; and (c) the far-Western method that can show direct physical interaction of two proteins.

We have successfully used the combination of these methods to study the interaction between two proteins, BLM and hMLH1 *(1)*. Here, we give an experimental set-up for these three methods as we have used them in our laboratory (*see* **Fig. 1**).

2. Materials

2.1. Yeast Two-Hybrid System

1. Yeast strain L40 [Mata *trp1 leu2 his3LYS2::4 lexAop-HIS3 URA3::*8 *lexAop-lacZ*].
2. pBTM116: LexA deoxyribonucleic acid (DNA)-binding domain vector. LexA, *TRP1, ADH1* promoter (truncated) and terminator, 2 μ, 5.4 kb, MCS: *Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I.
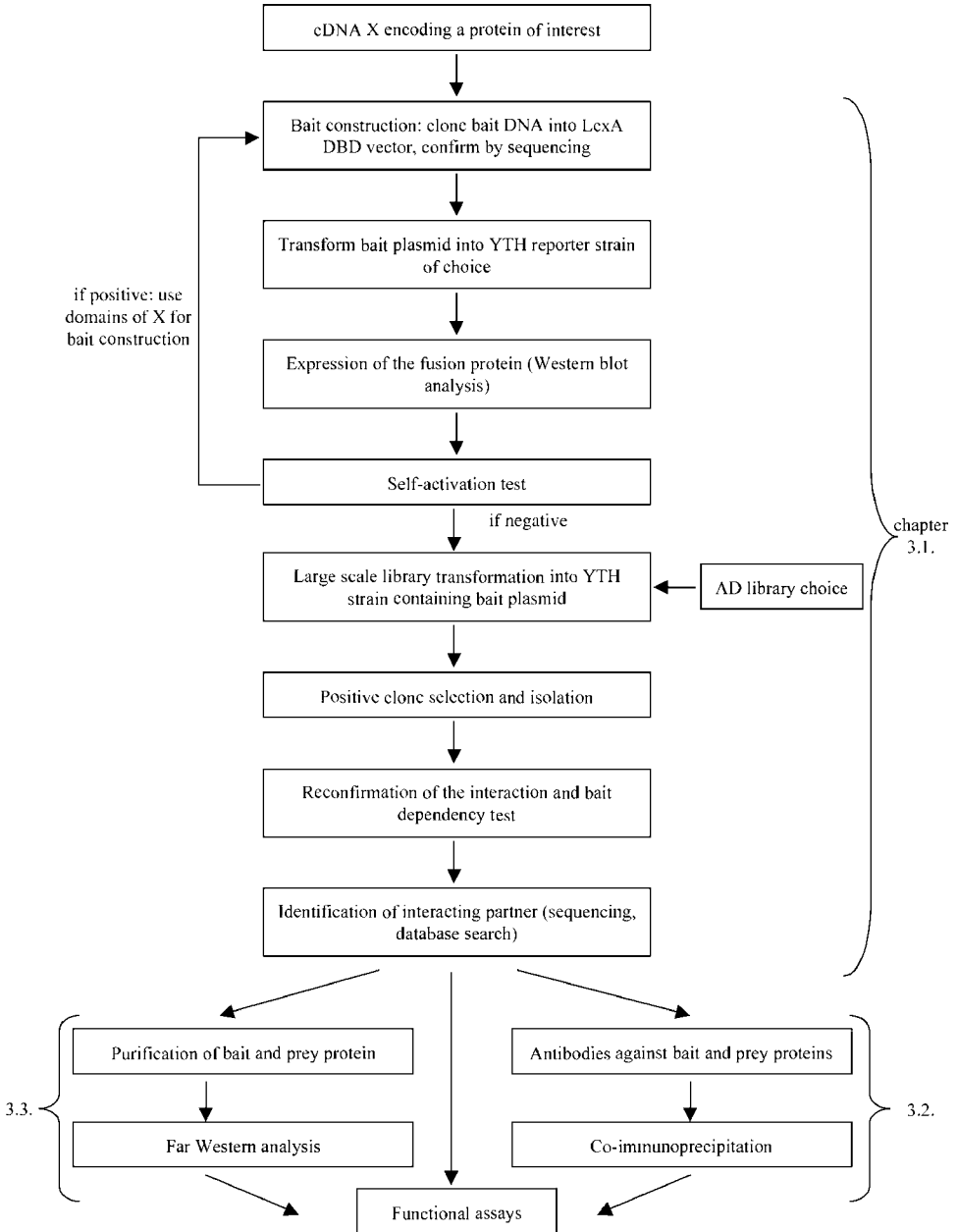
Fig. 1. Methods for detecting protein–protein interactions.

3. pGAD424: *GAL4* AD fusion vector. *GAL4*AD (768-881), *LEU2*, *ADH1* promoter (truncated) and terminator, 2 μ, 6.6 kb, MCS: *Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I.
4. Complementary DNA (cDNA) of gene of interest (or yeast genomic DNA).
5. AD library from relevant organism/tissue source.
6. Sequencing primers.
7. Yeast extract-peptone-dextrose medium (YPD): 1% yeast extract, 2% peptone, 2% glucose, 2% agar (plates). Autoclave 15 min at 121°C.
8. 50% PEG 4000.
9. 1 *M* lithium acetate (LiAc).
10. 10 mg/mL carrier DNA (sheared salmon sperm DNA).
11. Synthetic drop-out medium (SD): 0.67% yeast nitrogen base (without amino acids), 2% glucose, 1X drop-out mix, 2% agar (plates); autoclave at 121°C for 15 min.
12. 1 L 10X drop-out mix: 0.2 g adenine, 0.2 g arginine, 0.2 g histidine, 0.3 g isoleucine, 1 g leucine, 0.3 g lysine, 0.2 g methionine, 0.5 g phenylalanine, 2 g threonine, 0.2 g tryptophan, 0.3 g tyrosine, 0.2 g uracil, 1.5 g valine. Leave out appropriate components for drop-out medium (SD-Trp-Leu-His, SD-Trp-Leu, SD-Trp, SD-His).
13. Extraction buffer: 100 m*M* Tris-HCl pH 7.5, 200 m*M* NaCl, 20% (v/v) glycerol, 5 m*M* ethylenediaminetetraacetic acid (EDTA); add before use: 14 m*M* β-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF).
14. 450–550 μm acid-washed glass beads.
15. 1 *M* 3-amino-1,2,4-triazole (3-AT); store at –20°C.
16. *Escherichia coli* strains DH5α and KC8 (*leuB⁻*) competent for electroporation *(2)*.
17. LB-Amp plates (1 L): 10 g tryptone, 5 g yeast extract, 10 g NaCl, 20 g agar (plates). After autoclaving, add ampicillin (Amp) to a final concentration of 50 μg/mL.
18. Saline solution: 150 m*M* NaCl.
19. Qiagen Miniprep Kit and Qiagen Maxiprep Kit.
20. YPD with 15% glycerol.
21. Whatman paper.
22. Liquid nitrogen.
23. Tris-buffered saline (TBS): 20 m*M* Tris-HCl pH 7.5, 200 m*M* NaCl.
24. 10 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside): Dissolve 1.0 g of X-Gal in 100 mL of dimethyl formamide (DMF) and store at –20°C.
25. TBS/agarose/X-Gal: Prepare fresh. Dissolve agarose (0.5% w/v) in TBS and cool to 50°C. Add 1 mL X-Gal solution per 100 mL of TBS/agarose.
26. Yeast lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate (SDS).
27. Phenol:chloroform:isoamylalcohol (25:24:1).
28. Isopropanol.
29. 3 *M* NaAc, pH 6.0.
30. 70% ethanol.
31. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

32. SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose.
33. M9 minimal plates: 1L (in order): 750 mL ddH$_2$O, 200 mL 5X M9 salts, 15 g agar, H$_2$O up to 980 mL, then 2 mL 1 M MgSO$_4$, 0.1 mL 1 M CaCl$_2$. After autoclaving, add 20 mL 20% glucose (4°C).
34. 1 L 5X M9 salts: 64 g Na$_2$HPO$_4$·7H$_2$O, 15 g KH$_2$PO$_4$, 2.5 g NaCl, and 5 g NH$_4$Cl.

2.2. Coimmunoprecipitation

1. Protein extract.
2. Antibodies for immunoprecipitation and Western blot analysis (for both proteins of interest).
3. Protein A/G beads.
4. Immunoprecipitation (IP) buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40.
5. Protease inhibitor cocktail (100X): 50 µg/mL PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin in 100% ethanol; store at –20°C.
6. Sample loading buffer: 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2% SDS, 0.2% bromphenol blue, 20% glycerol.

2.3. Far-Western Analysis

1. Purified recombinant proteins.
2. Nitrocellulose membrane.
3. Denaturation buffer: 6 M guanidine-HCl in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$).
4. TBS with 0.3% Tween-20 and 10% milk (blocking) or 0.25% milk (washing/incubation).
5. 1 M DTT.
6. 200 mM PMSF.
7. Glutaraldehyde.
8. Antibodies specific for both proteins of interest for Western blot analysis.

3. Methods

3.1. Yeast Two-Hybrid Assay

The YTH system is based on a property that is shared among many transcription factors: They have a modular structure consisting of two separable domains, a DNA-binding domain (DBD) and an activation domain (AD) *(3)*. A protein of interest, X, is fused to the DBD of a transcription factor (for instance, the *E. coli* LexA or the *Saccharomyces cerevisiae* Ga14 DBD); this fusion protein is used to search for interacting partners and is therefore often referred to as the "bait." A second protein, Y, is fused to the AD of, for example, the Ga14 protein (called the "prey"). The X-DBD and Y-AD hybrids are transformed into a yeast strain that contains the binding site for the DBD

upstream of reporter genes. Alternatively, instead of using a defined protein Y as the "prey," an entire collection of cDNAs fused to the AD can be transformed into yeast containing the X-DBD bait. If X and Y physically interact, they will bring the DBB and AD into close proximity, resulting in, for example, positive growth selection using a prototroph-restoring reporter gene such as *HIS3*, or a colorimetric reporter, such as *lacZ*. In other words, a positive interaction of a prey with the bait will result in yeast prototrophy for histidine and its subsequent blue coloration on X-Gal-containing plates. The ability to quickly screen large cDNA libraries for proteins that interact with a given protein of interest is the major advantage of the YTH system over biochemical methods used for the identification of protein–protein interactions. However, the YTH assay is often limited by the frequent appearance of either false positives—when the bait acts as an activator—or false negatives—when the bait acts as a repressor in yeast. False negatives can also be caused by the failure of fusion proteins to enter the nucleus, by steric hindrance resulting from the fusion, or by the absence of posttranslational modifications.

The procedure outlined here shows how to perform the screening of a library to quickly identify interacting partners for a protein of interest. The same approach can also be used to study the interaction between two known proteins. In our laboratory, we have been using the prokaryotic LexA DBD and the yeast *GAL4* AD. This approach combines the advantages of the LexA-based YTH system with the ability to use any of a large variety of commercially available libraries for the *GAL4*-based YTH system. For more detailed information and alternative YTH methods see these recently published reviews *(4,5)* and **Note 1**.

3.1.1. Choosing the Appropriate YTH Strain and Vectors

The yeast reporter strain and vectors are chosen to match the requirements of the vector-selectable markers and the reporter genes present in the strain. The most useful YTH vectors are shuttle vectors able to replicate autonomously in *E. coli or S. cerevisiae* by virtue of the yeast 2 μ and *E. coli* Col E1 replication origins. The presence of the β-lactamase gene responsible for ampicillin resistance allows selection for the plasmid in bacteria, whereas a nutritional gene permits selection for the plasmid in yeast. The transcription of the hybrid genes–fusions to the LexA DBD in pBTM116 and the *GAL4* AD in pGAD424 is driven by a truncated *ADH1* promoter.

A YTH reporter strain should contain at least two different reporter genes. Commonly these are the bacterial *lacZ* gene that encodes β-galactosidase and the *HIS3* gene. The *HIS3* reporter allows one to directly select for positives based on the resulting histidine prototrophy. The *lacZ* reporter allows verification of the positives as well as "quantification" of the interaction strength by measuring the β-galactosidase activity *(6)*.

3.1.1.1. Construction and Characterization of the Bait Plasmid

The construct of a hybrid protein consisting of the LexA DBD fused to the protein of interest is termed bait as it will be used to "fish" new interacting partners. Ideally, it is expressed at a detectable level and does not self-activate the transcription of the reporter genes.

Either the entire open reading frame (ORF) of a bait protein of interest or domains of it are cloned in-frame to the LexA DBD domain. The more sequence of the ORF used to create the fusion protein, the more potential interacting regions will be included. Confirm the in-frame fusion by sequencing and check expression in the YTH yeast strain by Western blot analysis (good antibodies against LexA DBD are commercially available, e.g., LexA mab from BD Biosciences Clontech).

Protocol for yeast transformation:

1. Grow cells (20 mL) in YPD to optical density $(OD)_{600} = 1$.
2. Spin down cells at $700g$ for 5 min, wash pellet twice with dH_2O.
3. Resuspend pellet in 1 mL dH_2O. For each plasmid to be transformed, take 100 μL of cells, add 240 μL 50% PEG 4000, 36 μL 1 *M* LiAc, 5 μL carrier DNA (10 mg/mL), 1 μg plasmid DNA, and dH_2O up to 360 μL.
4. Vortex strongly for 1 min and heat shock at 42°C for 15 min.
5. Aspirate supernatant, resuspend pellet in 100 μL dH_2O, and plate on appropriate selective plate (for plasmid pBTM116 use a SD-Trp plate).
6. Incubate at 30°C for 2–4 d.

Protocol for yeast protein extraction:

1. Grow cells (25 mL) in appropriate selective medium to $OD_{600} \geq 1$.
2. Spin down cells at $700g$ for 5 min, discard supernatant.
3. Add 2.5 mL extraction buffer; resuspend and spin down at $700g$ for 5 min, discard supernatant.
4. Resuspend pellet in 200 μL of extraction buffer, and add 200 μL of glass beads.
5. Vortex strongly 10 s at room temperature, and cool for approx 1 min in ice H_2O; repeat seven times.
6. Spin at 4°C $20,000g$ for 15 min.
7. Transfer supernatant to a new tube and spin again for 20 min.
8. Transfer to a new tube and freeze immediately in liquid nitrogen.
9. Perform Western blot analysis using standard protocols *(2)*.

Before any screen, each bait plasmid construct should be tested for self-activation. This is activation of the reporter genes in the absence of an interacting AD fusion protein.

Protocol to test for self-activation:

1. Plate yeast cells containing the bait plasmid onto SD-His medium supplemented with increasing concentrations of 3-AT (1, 5, 10, 25, and 50 m*M*), to quench

possible background expression of the *HIS3* gene product, and onto SD-Trp medium. Incubate at 30°C for 4–5 d.

2. To check LexAop–*HIS3* reporter activation, compare growth on SD-Trp (growth control) and SD-His + 3-AT plates (self-activation).

3. If growth occurs, even at high concentrations of 3-AT, generate a different gene fragment (hoping to create one devoid of the region responsible for self-activation) in the bait plasmid.

4. Use the SD-Trp plate to test for LexAop–*lacZ* self-activation following the protocol for the filter-lift assay described below (*see* **Subheading 3.1.2.2.**).

3.1.1.2. CONSTRUCTION OF THE PREY PLASMID

The other hybrid in the YTH is a Ga14 AD fusion to a known protein or proteins encoded by a cDNA or genomic library (called AD library hereafter). As these are the potential interacting partners to be fished with a certain bait, the constructs are referred to as "prey" plasmids. For cloning a defined prey construct, follow the same guidelines as for the bait plasmid, and use an antibody against the Gal4 AD to monitor its expression (e.g., from Santa Cruz Biotechnology). As many AD libraries are commercially available (e.g., from BD Biosciences), we will focus on the use of a library rather than on its construction *(2)*. For a library screen, large amounts of plasmid DNA are needed, therefore an amplification step may be necessary.

Protocol for library amplification:

1. Transform the library plasmid DNA into *E. coli*.

2. To determine the titer, plate 2, 20, and 200 µL of a 10^{-2} dilution of the *E. coli* library culture onto LB-Amp plates (in duplicate) and incubate overnight at 37°C. The remaining culture can be stored at 4°C.

3. Plate a total of about 10 times the library complexity onto large LB-Amp plates (2×10^5 cells per plate). Incubate for 16–24 h to allow full formation of the colonies.

4. To harvest the colonies, add 10 mL of sterile saline solution to the plates (the same solution can be used for 5 plates), and carefully scrape the colonies from the agar surface with a bent glass rod.

5. Combine all the bacteria-saturated aliquots, and collect the cells by centrifugation at 10,000*g* for 10 min.

6. Extract the plasmid DNA using a kit (e.g., Qiagen Maxiprep Kit).

3.1.2. YTH Screen

3.1.2.1. LARGE-SCALE YEAST TRANSFORMATION

To efficiently use the library plasmid DNA, and to target a specific number of transformants, the transformation efficiency should be determined before starting a large-scale screen. Be aware that the transformation yield increases with increasing DNA concentration, but the transformation efficiency

decreases. Therefore it is better to scale up the transformation reaction rather than to increase the amount of transformed DNA, thus avoiding transformation of multiple prey plasmids into a single cell.

Protocol for determining library transformation yield and efficiency:

1. Transform increasing amounts of the library plasmid DNA (e.g., 0.1 µg, 1 µg, 2 µg, 5 µg, and 10 µg) into the YTH strain containing the bait, using the large-scale transformation protocol (see the list that follows) at 1X scale.
2. Plate 10 µL and 100 µL of a 10^{-1} dilution for each reaction onto SD-Trp-Leu plates (in duplicate).
3. Incubate the plates for 3–4 d at 30°C.
4. Determine transformation yield (total number of transformants) and transformation efficiency (transformants/µg) for each transformation by counting the colonies.

Protocol for large-scale transformation (50X scale):

1. Grow YTH strain containing the bait plasmid overnight in SD-Trp.
2. Inoculate 1 L of YPD with an overnight culture (to a starting $OD_{600} = 0.25$) and grow to $OD_{600} = 1$.
3. Spin down cells in four 250-mL beakers at 4200g for 10 min in a GS3 rotor. Wash pellets twice with 250 mL dH$_2$O.
4. Resuspend each pellet in 20 mL dH$_2$O, and transfer to a Falcon tube. Centrifuge (700g, 5 min) and decant supernatant.
5. To each Falcon tube add: 12 mL 50% PEG 4000, 1.8 mL 1 M LiAc, 250 µL carrier DNA (10 mg/mL), 50 µg plasmid DNA and dH$_2$O up to 18 mL.
6. Vortex strongly for 5 min and shake at 180 rpm. at 30°C for 30 min.
7. Heat shock at 42°C for 30 min in a shaker at 180 rpm.
8. Spin down at 1170g for 5 min at 4°C, carefully discard supernatant, wash the pellet with 20 mL dH$_2$O, and resuspend it in 5 mL dH$_2$O.
9. Plate 250–300 µL onto an SD-Trp-Leu-His + 3-AT plate (use 60–80 large plates to avoid dense plating, which would allow growth of false positives from a heavy inoculum), and incubate at 30°C for approx 5–15 d. As a transformation control, plate several dilutions of the transformation reaction on SD-Trp-Leu plates.

3.1.2.2. SELECTION OF POSITIVES

Protocol for picking positives:

1. Pick positives from the screening plates after 4–5 d. Check for slower growing colonies after 12–15 d. Often bacterial and fungal contaminants grow on the screening plates. Immediately discard heavily contaminated plates.
2. To maintain the plasmids with the interacting proteins, patch the colonies in a grid pattern to fresh selection plates (SD-Trp-Leu-His + 3-AT).
3. Make freezer stocks (resuspend an inoculation loop full of cells in 1 mL of sterile YPD supplemented with 15% glycerol in a 1.5-mL cryotube and store at –70°C) of the colonies as soon as they grow on the patched plate (reduced viability on

3-AT medium), and always use the original freezer stock for further tests. Eliminate colonies that do not grow after 5–7 d.

A good indication of a true YTH positive is activation of all the reporter genes. When assaying *lacZ* gene activation on the patched positives, it is important to include positive and negative controls as references. Activation of the *lacZ* gene can give blue color in an X-Gal filter-lift assay after 30 min to overnight incubation. Best results are obtained with freshly growing yeast colonies.

Protocol for filter-lift assay:

1. Replicate the colonies growing on SD-Trp-Leu-His + 3-AT plates onto a sterile Whatman filter paper (carefully place filter on top of the colonies, ensuring it lies flat. Remove it with sterile forceps). Mark filter and plate in an asymmetrical way to make the orientation obvious.
2. Freeze the filter in liquid nitrogen for approx 10 s and let it thaw (colony-side-up). Repeat the freeze–thaw cycle twice.
3. Place the filter (colony-side-up) in an empty Petri dish and cover it with a TBS/ agarose/ X-Gal solution.
4. Incubate the filter at 30°C. Record the time required to observe a blue color change.

3.1.2.3. CHARACTERIZATION AND IDENTIFICATION OF THE POSITIVES

YTH positives that activate both the *HIS3* and the *lacZ* reporter genes can now be subjected to further analysis. The first step is to recover the prey plasmids from the yeast cells of each YTH positive. As the nucleic acid preparations will include both *TRP1* and *LEU2* plasmids, they will be electroporated into an *E. coli* host containing a *leuB*-mutation that allows selection for the *LEU2*-containing prey plasmid.

Protocol for plasmid recovery from yeast:

1. Inoculate individual YTH positives into 2 mL selective SD-Leu medium and incubate at 30°C overnight.
2. Spin down the yeast cells from the liquid culture for 5 s in a microcentrifuge.
3. Decant the supernatant and resuspend the pellet in the residual liquid by vortexing.
4. Add 200 μL of yeast lysis buffer, 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 g of glass beads (0.45–0.55 mm diameter).
5. Vortex each sample vigorously for 1 min and then place on ice. Repeat twice, leaving samples 1 min on ice between treatments.
6. Centrifuge tubes for 5 min in a microcentrifuge.
7. Transfer the aqueous phase (approx 200 μL) to a fresh tube and precipitate the nucleic acids by adding an equal volume (approx 200 μL) of isopropanol and 20 μL of 3 *M* NaAc (pH 6.0).
8. Collect the precipitate by centrifugation at 13,000*g* for 20 min at 4°C. Wash the pellet with 100 mL of 70% ethanol, and dry the pellet for 5 min at RT.
9. Dissolve the pellet in 25 μL of TE (10 m*M* Tris-Cl pH 8.0, 1 m*M* EDTA).

Protocol for electroporation of *E. coli*:

1. Add 2 μL of DNA to 25 μL of electrocompetent KC8 (or another leuB-containing *E. coli* strain), mix gently, and transfer carefully to the bottom of a chilled electroporation cuvet.
2. Place the cuvet into the electroporation device (Bio-Rad) and electroporate at 200 Ω, 25 mF, and 2.5 kV. The time constant should be around 5 ms.
3. Immediately add 1 mL of prewarmed SOC medium and transfer to a sterile tube.
4. Incubate at 37°C for up to 30 min (shake gently).
5. Plate samples (25–100 μL) onto LB-Amp plates and incubate overnight at 37°C.

Protocol for selection of colonies containing the prey plasmid:

1. Replica plate the colonies growing on LB-Amp plates onto M9-Leu to select for the prey plasmid. Incubate overnight at 37°C.
2. For every positive, inoculate 4–5 colonies (as multiple prey plasmids could be present in a single yeast cell) into 2 mL LB-Amp medium and incubate at 37°C overnight, with shaking.
3. Extract the plasmid DNA using a miniprep kit (e.g., from Qiagen).

The isolated AD library plasmids (4–5 minipreparations for each YTH positive) can be characterized by restriction-enzyme digestion using restriction enzymes cutting at the 5' and 3' ends of the cDNA (vector- and library-specific). This allows one to group them according to insert size and restriction pattern to identify potentially identical isolates. A representative of each different group should be analyzed separately.

Protocol for identification of true positives (bait-dependency test):

1. Transform a member of each plasmid group into the YTH strain containing the original bait plasmid, and check the activation of both reporter genes, as described previously, to reconfirm the interaction.
2. In parallel, transform the prey plasmid into the YTH strain alone, with the empty LexA DBD vector, or with an unrelated bait, and examine the activation of the two reporter genes (bait-dependency test).
3. Isolates that reconfirm activation of both the *HIS3* and *lacZ* reporter genes in combination with the original bait, and not in its absence or with an unrelated bait, are considered true positives. Transformants activating both reporter genes independent of the original bait can be discarded as false positives. A potential mechanism for the failure to reconfirm an interaction can be the presence of multiple AD library plasmids in the original YTH positive (in this case more colonies have to be analyzed) or—very rarely—alteration of the bait plasmid in the yeast strain resulting in self-activation.
4. Sequence representative members of each group of bait-dependent AD library plasmids to identify those positives containing ORFs in-frame with the *GAL4* AD. As DNA isolated from the *E. coli* KC8 strain is usually not suitable for DNA sequencing, it is best to isolate the plasmids from a strain such as DH5α.

5. Analyze the sequences from the AD library plasmids using the Basic Local Alignment Search Tool (BLAST) algorithm to identify the inserts and classify them according to known or unknown genes, potential biological relevance, and so forth.

3.2. Coimmunoprecipitation

Interactions between two proteins detected in the YTH system are identified using fusion proteins and overexpression. Therefore, these interactions have to be confirmed under more physiological conditions, ideally using endogenous proteins. The coimmunoprecipitation approach fulfills these criteria. This biochemical method uses extracts from cells expressing both proteins (at endogenous levels) to monitor their interaction by capturing the protein of interest with a specific antibody and analyzing the proteins that are in complex with it. A main difficulty arises from the fact that suitable antibodies are not always available. It is a very useful method for confirming a potential interaction, and in combination with other techniques such as mass-spectrometry, it can also be used to screen for interacting partners. The power of this approach was demonstrated recently in that standard affinity-tagging methods combined with high-throughput coimmunoprecipitations and mass spectrometric analysis have been applied to several yeast protein complexes on a genomewide scale *(7,8)*.

3.2.1. Extract Preparation

There are many different methods to prepare extracts depending on the model organism, tissue, and subcellular localization of the proteins of interest. For every protein conditions (salt, detergents, mechanical processes) have to be established empirically to solubilize it without disrupting its protein–protein interactions. Before attempting an immunoprecipitation experiment, be sure to check for expression of the proteins of interest by Western blot analysis. Where possible, prepare also extracts from cells lacking one of the two proteins. These can be used as control because a coprecipitating protein should not be precipitated in the absence of the primary target of the antibody. For additional helpful hints *see* **Note 2**.

3.2.2. Antibodies and Beads

A very crucial step is the choice of the appropriate antibody—the success of the immunoprecipitation depends very much on its affinity for the antigen as well as the affinity of Protein A or G for the specific immunoglobulin class of the antibody. Furthermore, the epitope for a specific antibody could be masked by interacting proteins: as different antibodies recognize different epitopes, not all of them are expected to coprecipitate the same associated proteins. The ability to coprecipitate the same protein, Y, using different antibodies directed

against one specific protein, X, increases the confidence for an in vivo association of X and Y. Cross-reactivity and nonspecific binding to cellular proteins are major causes for false positives. In general, polyclonal antibodies work best, but also purified monoclonal antibodies, ascites fluid, or hybridoma supernatant are used. It is worthwhile to attempt the conditions recommended by the manufacturer or found in the literature; however, optimization is often necessary. An antibody from the same subclass should always be included as a control antibody. The beads selected to bind the antibodies depend on the species and subclass of the antibody used (e.g., Protein A beads for rabbit, mouse IgG2a, IgG2b, and IgG3; and Protein G beads for mouse IgG1 and most subclasses of rat IgGs *[2]*). Furthermore, there are various insoluble matrices (Sepharose, magnetic beads, etc.), all of which may require different handling (follow manufacturer's conditions).

3.2.3. Immunoprecipitation

1. Add 5–10 µg (the optimal amount must be titrated for each antibody) of either the specific antibody or the control antibody to the Eppendorf tube containing the cold extract (approx 300 µg, depending on the expression level of the proteins to be studied). Adjust the volume to 500 µL with IP buffer and add 5 µL of protease inhibitor cocktail.
2. Incubate at 4°C for 1–4 h.
3. Wash 50 µL of Protein G slurry by adding 450 µL cold IP buffer and spinning for 5 s in a microcentrifuge. Remove supernatant and repeat the washing step twice. Resuspend the beads in 50 µL of cold IP buffer.
4. Add the prewashed 50 µL of Protein G slurry to the extract.
5. Incubate for 1 h at 4°C on a rolling wheel.
6. Spin for 5 s at 4°C.
7. Carefully remove the supernatant completely, and wash the beads three to five times with 500 µL of IP buffer.
8. After the last wash, aspirate supernatant and add 50 µL of sample loading buffer to bead pellet. Vortex and heat to 100°C for 5 min.
9. Spin at 10,000*g* for 5 min, collect the supernatant and load onto an SDS-polyacrylamide gel.

3.2.4. SDS-Page/Western Blot

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis use standard protocols *(2)*. For the Western blot analysis use antibodies specific to the partner proteins that are from a different species to prevent cross-detection of the IP antibody on the blot. (An alternative is to covalently couple IP antibody to beads and then no antibody should be carried through the rest of the experiments).

3.3. Far-Western Analysis

Both the YTH system and the coimmunoprecipitation approaches do not answer the question of whether two proteins interact directly, or whether the interaction is mediated by bridging proteins. However, this question can be answered using the far-Western method. Here, a purified protein of interest is loaded on a gel, renatured on the membrane after blotting, and probed with either another purified protein or with a labeled in vitro transcribed and translated protein (*see also* **Note 3**; the conditions presented there are also useful when working with one of the many proteins that fail to renature under the conditions described below). The interaction can then be monitored using suitable antibodies or directly by autoradiography in the case of labeled proteins.

Protocol:

1. 0.2–1.0 µg of each polypeptide to be tested and a control protein (e.g., bovine serum albumin [BSA]) are subjected to SDS-PAGE and transferred onto a nitrocellulose filter.
2. For the denaturation/renaturation steps (at 4°C), immerse the filter twice in cold denaturation buffer (6 *M* guanidine-HCl in PBS) for 10 min and then incubate it six times for 10 min each time in serial dilutions (1:1) of denaturation buffer supplemented with 1 m*M* DTT.
3. Block the filter in TBS supplemented with 10% powdered milk and 0.3% Tween-20 for 30 min at 4°C.
4. Add a purified protein (0.5 µg/mL) in TBS supplemented with 0.25% milk, 0.3% Tween-20, 1 m*M* DTT, and 1 m*M* PMSF and incubate for 60 min at 4°C.
5. Wash four times for 10 min each time in TBS containing 0.3% Tween-20 and 0.25% milk. Add 0.0001% glutaraldehyde to the second wash.
6. Perform conventional Western blot analysis to detect the presence of bound proteins using an antibody specific to the second purified protein at the recommended dilution and enhanced chemiluminescence (ECL) according to the manufacturer's instructions.

4. Notes

In this section, some additional steps and modifications of the presented methods are given that allow further characterization of the interaction of interest.

1. Mapping the interaction domain using YTH. Instead of screening a library, different fragments of both the prey and bait genes can be generated in the YTH vectors using restriction sites, or by polymerase chain reaction (PCR), and tested for interaction on selective plates and in an X-Gal filter test. This approach allows identification of the region of the protein directly involved in the interaction (interaction domain mapping). Alternatively, a high-throughput YTH method for selection of the prey-interaction domain can be used *(9)*. Sonicated fragments of the prey cDNA are cloned into an AD plasmid, thus generating a library of

random prey fragments to be used in a YTH screen performed with the original bait plasmid.

2. Preclearing step in immunoprecipitation process. Nonspecific background resulting from proteins with high affinities for Protein G or Protein A can be removed using a preclearing step.
   a. Wash beads as in **Subheading 3.2.2.**
   b. Add this 50 µL of Protein G slurry to 300 µg of extract in an Eppendorf tube. Bring volume to 500 µL and incubate on ice for 30–60 min.
   c. Spin for 5 s in a microcentrifuge and transfer the supernatant to a fresh Eppendorf tube. If any bead has been transferred, spin again and carefully transfer the supernatant to a fresh Eppendorf tube.
   d. Proceed in the protocol using this precleared extract.

3. In vitro binding assay. If one of the proteins of interest is not available in purified form, it can be transcribed and translated in vitro using a commercially available kit (e.g., Promega TNT Reticulocyte Lysate kit). As proteins generated with this kit can be ^{35}S-labeled, interactions with the protein immobilized on the membrane can be easily detected by autoradiography. This approach is also very useful to map the interaction domains of the interacting proteins using mutants.
   a. Dot increasing amounts (normally from 0 to 4 pmoles) of purified protein onto a nitrocellulose membrane (volume should not exceed 5 µL).
   b. Dry for 10 min at RT.
   c. Block for 1 h in TBS with 0.5% Tween-20 (TBST) and 5% (w/v) milk at RT.
   d. Wash twice with TBST/BSA (0.1% w/v).
   e. Add 1 mL of TBST/BSA (0.1% w/v) to tubes.
   f. Add 20–40 µL of ^{35}S-labeled protein; mix well.
   g. Incubate at 4°C for 3 h on a rolling wheel.
   h. Take out the membrane and wash with 30 mL of TBST for 10 min at RT. Repeat two to four times.
   i. Dry the membrane completely (5 min).
   j. Expose membrane to phosphorimager or film.

Acknowledgments

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Detection of Kinase and Phosphatase Activities

Sean M. Post and Eva Y.-H.P. Lee

1. Introduction

The eukaryotic cell cycle is a process in which cells grow and then divide into two genetically identical cells. The cell cycle is divided into four discrete phases allowing for the orderly transition from deoxyribonucleic acid (DNA) replication to chromosomal condensation, spindle formation, and cytokinesis. These phases are G1, in which a cell prepares for DNA replication; S, in which DNA synthesis takes place; G2, in which the cell ensures its genetic information has been faithfully replicated; and M, in which the homologous chromosomes are separated and cell division takes place.

To ensure normal progression of the cell cycle, two systems have evolved in eukaryotes. The first identified was the cyclin-dependent kinase (Cdk)/cyclin system; these initial findings, by Leland Hartwell, Tim Hunt, and Paul Nurse, were rewarded with the Nobel Prize for Medicine in 2001. Cdks are normally inactive protein kinases that become activated on complex formation with their regulatory partners called *cyclins*. Throughout the cell cycle, Cdk protein levels are relatively constant, whereas cyclin levels fluctuate. For example, Cdks 2, 4, 6, and Cdc2 are constitutively expressed during the cell cycle, but cyclin D is expressed in early G1 and is present throughout S and G2/M phases; cyclin E is present at the G1/S transition, cyclin A during S phase, and cyclin B in late S phase and at the G2/M transition; each one is rapidly degraded at different times during the cell cycle (**Fig. 1**). The cell cycle-dependent flux in protein levels of cyclins regulates the kinase activities of the Cdks. These active Cdk/cyclin kinase complexes phosphorylate proteins at conserved substrate motifs containing a serine or threonine residue followed by a proline and a lysine or an arginine. These phosphorylations result in cell cycle progression by driving the transition from one phase to the next.
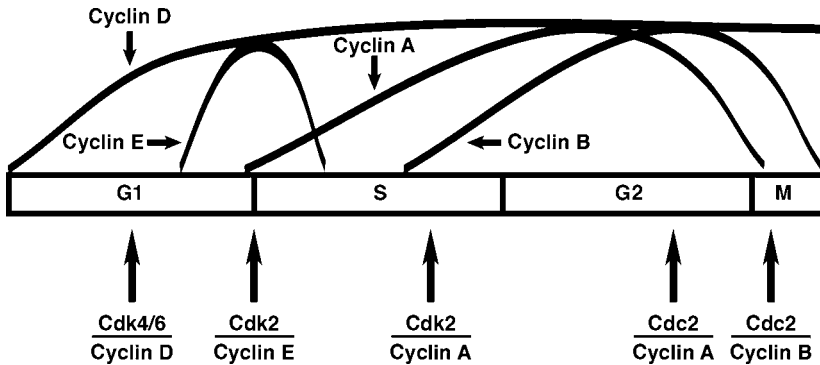
Fig. 1. A representation of the cell cycle demonstrating protein levels of various cyclins and when individual Cdk/cyclin complexes function.

The mechanisms of Cdk/cyclin activation and inactivation have been widely studied. In addition to changes in protein levels of the cyclins, the kinase activities of the Cdks are also controlled by association with cyclin-dependent kinase inhibitors (Ckis) and posttranslational modifications. The Cdc2/cyclin complexes are inactivated by phosphorylation of Cdc2 on threonine14 (Thr14) and tyrosine15 (Tyr15) by the cell cycle inhibitory kinases Myt1 and Wee1, respectively *(1–4)*. The dual phosphatase, Cdc25, relieves the inhibitory constraints of the Thr14 and Tyr15 phosphorylations and, thus, activates the Cdc2/cyclin kinase complex *(5)*.

The second system controlling cell cycle progression is called checkpoint. Checkpoints negatively regulate cell cycle progression by inhibiting the Cdk/cyclin pathways. Hartwell and Weinert first coined the term *checkpoint* in 1989 when they observed that a repair-deficient yeast strain maintained the ability to arrest at the G2/M transition following exposure to γ-irradiation *(6,7)*. This early finding led to the identification of many DNA damage-dependent cell cycle-checkpoint kinases and phosphatases. Protein complexes, called *sensors*, first recognize aberrant DNA structures caused by damaging agents or incomplete replication and then initiate a signaling cascade, which activates these kinases and phosphatases, ultimately resulting in checkpoint activation (**Fig. 2**); this is reviewed in *(8–10)*. This signal-transduction cascade results in the amplification of the signal from a single DNA break, leading to cell cycle arrest.

ATM (ataxia-telangiectasia mutant) and ATR (ataxia-telangiectasia and *Rad3*-related) are two of the most widely studied serine/threonine-directed checkpoint protein kinases. Activation of the ATR and ATM checkpoint-signaling pathways results in cell cycle arrest by inactivating Cdk/cyclin kinase complexes. Both ATR and ATM phosphorylate proteins at a consensus motif,

IR/ UV/ Replication Block
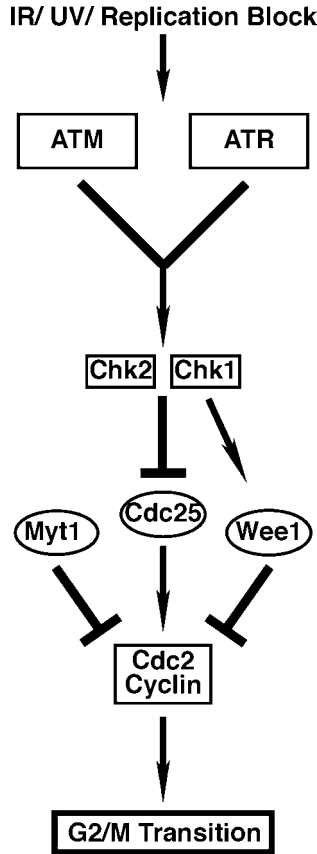
Fig. 2. A schematic representation of the DNA damage and replication block-induced checkpoint-signaling cascade. On DNA damage, ATR and ATM act as sensors/signal transducers to modulate downstream checkpoint kinases and phosphatases, whose activities inhibit cell cycle progression.

which contains a serine or threonine residue followed by a glutamine *(11)*. In response to DNA damage or replication block, these two kinases phosphorylate and activate two downstream checkpoint protein kinases, Chk1 and Chk2 *(12,13)*. Chk1 and Chk2 further transduce the checkpoint-signaling cascade by phosphorylation of and subsequent inactivation and relocalization of the phosphatase, Cdc25 *(14–17)*. In addition, Chk1 phosphorylates and stabilizes the inhibitory cell cycle kinase Wee1 after DNA damage *(13)*. This signal-transduction cascade prevents cell cycle progression once DNA damage is sensed. The loss of these checkpoint-signaling processes can be disastrous to cells, resulting in cell death (apoptosis) or genetic instability (a hallmark of cancer).

This chapter describes procedures to detect in vitro kinase activities of the DNA damage-induced checkpoint kinases ATR and ATM, as well as a Cdk/cyclin complex (Cdc2/cyclinB). We also describe basic protocols to determine the phosphatase activities of Cdc25. The procedures discussed here provide guidelines for detection of kinase/substrate relationships and the detection of phosphatase activities. These protocols may be modified to test the kinase/substrate relationships of interest.

2. Materials

2.1. Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) (GIBCO, cat. no. 1330-032).
2. Fetal bovine serum (GIBCO, cat. no. 10099-141).
3. Penicillin/streptomycin (GIBCO, cat. no. 15070-063).
4. Phosphate-buffered saline (PBS) (GIBCO, cat. no. 70011-044).
5. 2 mM thymidine (Sigma, cat. no. T-9250). Make fresh in 1 M NaOH.

2.2. Preparation of Cell Lysates and Immunoprecipitation

All lysis and kinase buffers are made at the time of use by dilution of stock solutions.

1. TGN lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20, 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 100 µM Na$_3$VO$_4$, 1 mM benzamidine, 5 µg/mL pepstatin.
2. NP-40 lysis buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM NaF, 100 µM Na$_3$VO$_4$, 1 mM benzamidine, 5 µg/mL pepstatin.
3. Protein G-Sepharose beads (Amersham Biosciences, cat. no. 17-0618-02).
4. Antibodies: mouse-α-ATR *(18)* (GeneTex, cat. no. MS-ATRII-PX1), mouse-α-ATM *(19)*, (GeneTex, cat. no. MS-ATMII-PX1), and mouse-α-Cdc2 (Santa Cruz or Upstate Biotechnology).
5. ATR wash buffers: TGN containing 0.5 M LiCl, and TGN containing 1 M NaCl.
6. ATM wash buffer: TGN containing 0.5 M LiCl.

2.3. Kinase Assays

1. ATR kinase buffer: 10 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 5% glycerol, 1 mM dithiothreitol (DTT), 50 µM Na$_3$VO$_4$.
2. ATM kinase buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 6 mM MgCl$_2$, 4 mM MnCl$_2$, 10% glycerol, 1 mM DTT, 100 µM Na$_3$VO$_4$.
3. Histone H1 kinase buffer: 20 mM Tris-HCl, pH 7.4, 15 mM MgCl$_2$, 1 mM DTT, 80 mM β-glycerol phosphate.
4. Histone H1 (GIBCO, cat. no. 13221-015).
5. 10mCi/mL γ-^{32}P ATP (NEN, cat. no. NEG502Z).
6. 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.4% bromophenol blue, 4% β-mercaptoethanol. Add the β-mercaptoethanol at the time of use.

2.4. SDS-PAGE

1. SDS-PAGE stacking gel (based on 10-mL total volume): 7.0 mL H$_2$O, 1.25 mL 1M Tris-HCl, pH 6.8, 1.5 mL 30% acrylamide solution, 100 µL 10% SDS, 100 µL 10% APS, 10 µL TEMED.
2. 8% SDS-PAGE separating gel (based on 10-mL total volume): 4.6 mL H$_2$O, 2.5 mL 1.5 *M* Tris-HCl, pH 8.8, 2.7 mL 30% acrylamide solution, 100 µL 10% SDS, 100 µL 10% adenosine 5'-phosphosulfate (APS), 6 µL TEMED.
3. 12% SDS-PAGE separating gel (based on 10-mL total volume): 3.3 mL H$_2$O, 2.5 mL 1.5 *M* Tris-HCl, pH 8.8, 4.0 mL 30% acrylamide solution, 100 µL 10% SDS, 100 µL 10% APS, 4 µL TEMED.
4. SDS-PAGE running buffer (for 1 L of a 10X solution): 30.3 g Tris-HCl, 144 g glycine, 10 g SDS, pH to 8.3.
5. Coomassie stain solution (for 1 L total volume): 1.25 g Coomassie Brilliant Blue (R250), 500 mL methanol, 100 mL glacial acetic acid, 400 mL H$_2$O.
6. Coomassie destain solution (for 1 L total vol): 100 mL methanol, 100 mL glacial acetic acid, 800 mL H$_2$O.

2.5. Phosphatase Analysis

1. Cdc25 phosphatase buffer: 20 m*M* Tris-HCl, pH 8.3, 150 m*M* NaCl, 2 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* DTT, 0.1% Triton X-100, pH to 8.3.
2. 4-nitrophenyl phosphate tablets, (Sigma, cat. no.N-9389). Once made, protect from light.
3. 2 *M* NaOH (Sigma, cat. no. S-8045).

3. Methods

3.1. ATR and ATM Kinase Assays

The first step in determining the activity of a kinase on a given substrate is to have a rational idea regarding the relationship between the two proteins. One of the most important properties of a potential substrate is that it must be a phosphoprotein. This can be rapidly determined by [γ-^{32}P]-orthophosphate-labeling of cells, followed by immunoprecipitation with an antibody against the protein of interest. The immunoprecipitate can then be separated by SDS-PAGE, dried on Whatman paper, and exposed to X-ray film. In addition, under most circumstances, a kinase and the substrate in question should typically lie in a related biological response; that is, mutations in one or the other protein result in a similar phenotype. Also, as mentioned in the Introduction, kinases generally phosphorylate conserved motifs; therefore, possible ATR and ATM substrates should contain a serine-glutamine site.

Generally, these rules are good beginning points in identifying potential substrates. A caveat to these rules is that kinases have been shown to phosphorylate proteins at nonconserved motif sites; making it necessary to determine which residue is specifically phosphorylated. This can be determined by

mutating the suspected serine or threonine residue of the substrate to a nonphosphorylatable alanine residue. In addition, these in vitro phosphorylation sites should be confirmed in vivo. Two approaches to address the in vivo phosphorylation are two-dimensional gel analysis and Western blot analysis using antibodies that specifically recognize the phosphorylated residues of the substrate.

The actual relationship between a kinase and the substrate in question must be determined experimentally; therefore, positive and negative controls should be used when available. For that reason, we describe the detection of a known substrate of ATM and ATR, a glutathione-*S*-transferase (GST) fusion protein with the first 106 amino acids of p53. Procedures to purify GST-substrates are not the focus of this work and are, therefore, not discussed here. Detailed GST-purification procedures are available from Amersham Biosciences.

The ATM kinase assay was originally developed in Dr. Yosef Shiloh's laboratory (Tel Aviv University, Israel) and has been slightly modified in our laboratory. We have successfully used these modified protocols to determine the unique substrate specificities of the ATR and ATM protein kinases (*see* **Fig. 3**) and *(18,20,21)*.

1. Grow log-phase HeLa cells on 10-cm dishes in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (*see* **Note 1**).
2. Treat log-phase HeLa cells with 10 Gy γ-irradiation.
3. One hour posttreatment, aspirate the media and wash cells twice with ice-cold PBS.
4. Add 1 mL PBS; scrape cells off the tissue-culture plates, and aliquot into 1.5-mL microfuge tubes (*see* **Note 2**).
5. The remaining steps are at 4°C unless stated otherwise.
6. Centrifuge for 5 min at 3 K.
7. Aspirate the supernatant and lyse the cells by adding 1 mL of TGN buffer and place on ice for 30 min.
8. Centrifuge the lysate for 10 min at 14 K.
9. Remove the supernatant (soluble protein) and aliquot into a new 1.5-mL microfuge tube.
10. Determine the protein concentration and dilute lysate to 1 mg/mL (*see* **Note 3**).
11. For each kinase reaction, aliquot 1 mL of lysate into new microfuge tubes (*see* **Note 4**).
12. Preclean the lysate by adding 5 µg of mouse IgG along with 20 µL bed volume of equilibrated protein G-Sepharose beads, followed by rotation for 1 h (*see* **Note 5**).
13. Centrifuge for 5 min at 8 K.
14. Aliquot supernatant into a new microfuge tube.
15. Add 5 µg of IgG purified m-α-ATR or m-α-ATM antibody and rotate for 2 h.
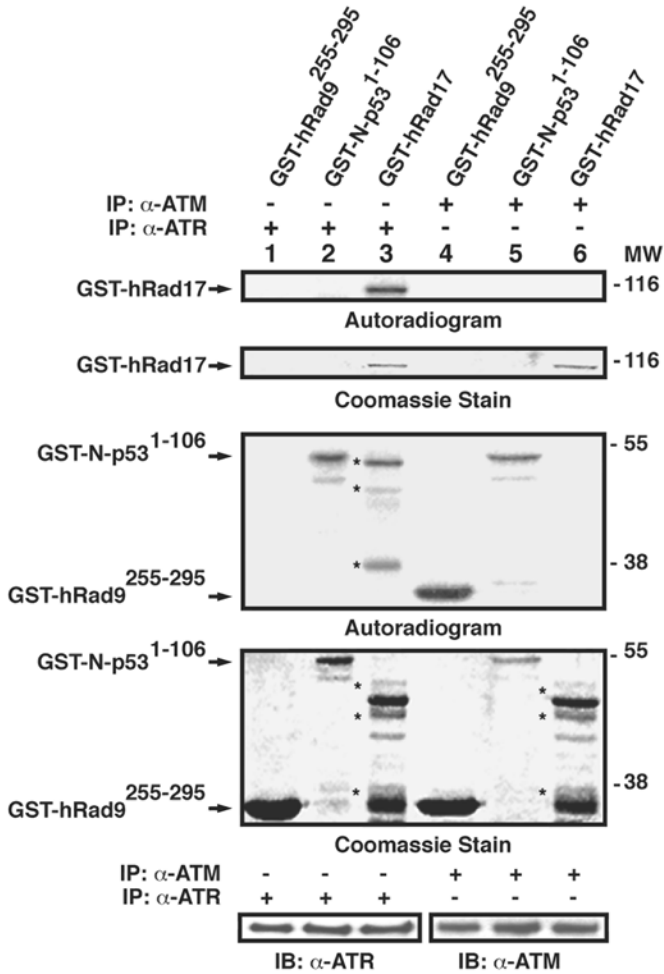
Fig. 3. Kinase assays using immunoprecipitated ATM and ATR. GST-hRad17 was incubated with ATM *(top two panels, lane 6)* and ATR *(top two panels, lane 3)* immunoprecipitated from HeLa cells treated with 10 Gy irradiation. GST-N-p53$^{1-106}$, a known substrate of the two kinases, was incubated with ATM *(bottom two panels, lane 5)* or ATR *(bottom two panels, lane 2)*. GST-hRad9$^{255-295}$, a known substrate of ATM, was incubated with ATM *(bottom two panels, lane 4)* or ATR *(bottom two panels, lane 1)*. Three µg of substrate was used in each reaction. The kinase reaction products were separated by SDS-PAGE and analyzed by Coomassie staining and autoradiograph. The * indicates degraded GST-hRad17 products. Levels of ATR and ATM in the kinase reactions were determined by immunoprecipitation followed by Western blotting.

16. Add 20 µL bed volume of equilibrated protein G-Sepharose beads and rotate an additional hour.
17. Centrifuge for 5 min at 8 K.
18. Aspirate; then wash the ATR-protein-G complex with TGN-LiCl buffer, and the ATM-protein-G complex with TGN buffer. Repeat **steps 17** and **18** one additional time.
19. Aspirate; then wash the ATR-protein-G complex with TGN-NaCl buffer, and the ATM-protein-G complex with TGN-NaCl buffer. Repeat **steps 17** and **19** one additional time.
20. Aspirate; then wash the ATR-protein-G complex with ATR kinase buffer, and the ATM-protein-G complex with ATM kinase buffer. Repeat **steps 17** and **20** one additional time (*see* **Note 9**).
21. Prepare the substrate by adding 3 µg of substrate (1 µg/mL GST-p53N$^{1-106}$), 1 µL of γ-^{32}P ATP (6000mCi/mol) to 16 µL of ATR or ATM kinase buffer containing 20 µM ATP (*see* **Note 10**).
22. Add the substrate to the IgG-immunocomplex and incubate 15 min at 30°C.
23. Stop the reaction by adding 6 µL of 4X SDS-PAGE sample buffer, and boil for 5 min followed by separation of the proteins by 8.0% SDS-PAGE (*see* **Note 11**).
24. Visualize the proteins by Coomassie staining for 1 h followed by overnight destaining. Dry the gel on 3M Whatman paper and expose to X-ray film (*see* **Fig. 3**) and (*see* **Notes 12** and **13**).

3.2. Cdc2/CyclinB Kinase Assay

To study the kinase activity of Cdc2/cyclin B, it is helpful to obtain this complex by immunoprecipitation from cells synchronized in late G2 and early M phases, as cyclin B is expressed during these phases of the cell cycle. This can be accomplished by using HeLa cells synchronized by a double-thymidine block *(22)*.

Although a variety of substrates can be tested, we will focus on a known substrate of Cdc2/cyclinB, histone H1. The in vitro kinase protocols described here have been modified from several laboratories *(23,24)* and from Dr. P. Renee Yew (personal communication) and have been used successfully in our laboratory.

1. Plate log-phase HeLa cells at 30% confluence onto 10-cm dishes in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM thymidine. Place cells in a 37°C incubator for 16 h.
2. Aspirate media, wash cells twice with PBS, and refeed with DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Place cells in a 37°C incubator for 9 h.
3. Aspirate media, wash cells twice with PBS, and refeed with DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM thymidine. Place cells in a 37°C incubator for 16 h.

4. Aspirate media, wash cells twice with PBS, and refeed with DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Place cells in a 37°C incubator for 6–9 h.

5. Aspirate media and wash cells twice with ice-cold PBS.

6. Add 1mL PBS; scrape cells off the tissue-culture plates and aliquot into 1.5-mL microfuge tubes (*see* **Note 2**).

7. The remaining steps are at 4°C unless stated otherwise.

8. Centrifuge for 5 min at 3 K.

9. Aspirate the supernatant and lyse the cells by adding 1 mL of NP-40 lysis buffer, and place on ice for 30 min.

10. Centrifuge for 10 min at 14 K.

11. Remove the supernatant (soluble protein) and transfer to a new 1.5-mL microfuge tube.

12. Determine the protein concentration and dilute lysate to 1 mg/mL (*see* **Note 3**).

13. Aliquot 1 mL of lysate for each kinase reaction to new microfuge tubes (*see* **Note 4**).

14. Preclean the lysate by adding 5 µg of mouse IgG along with 20 µL bed volume of equilibrated protein G-Sepharose beads, followed by rotation for 1 h (*see* **Note 5**).

15. Centrifuge for 5 min at 8 K.

16. Aliquot supernatant into new microfuge tube (*see* **Note 6**).

17. Add 5 µg of IgG-purified m-α-Cdc2 antibody and rotate for 2 h (*see* **Note 7**).

18. Add 20 µL bed volume of equilibrated protein G-Sepharose beads and rotate an additional hour (*see* **Note 8**).

19. Centrifuge for 5 min at 8 K.

20. Aspirate and wash the Cdc2-protein G complex with NP-40 lysis buffer. Repeat **steps 19** and **20** three additional times.

21. Aspirate and wash the Cdc2-protein G complex with histone H1 kinase buffer. Repeat **steps 19** and **21** one additional time (*see* **Note 9**).

22. Prepare the substrate for the kinase reaction by adding 1.25 µg of substrate (1 mg/mL histone H1) and 1 µL of γ-^{32}P ATP (6000mCi/mol) to 17.75 µL of histone H1 kinase buffer containing 50 µM ATP (*see* **Note 10**).

23. Add the substrate to the Cdc2-protein G complex and incubate for 10 min at 30°C.

24. Stop the reaction by adding 6 µL of 4X SDS-PAGE sample buffer and boil 5 min followed by separation of the protein by 12% SDS-PAGE (*see* **Note 11**).

25. Visualize the proteins by Coomassie staining for 1 h followed by overnight destaining. Dry the gel on 3M Whatman paper and expose to X-ray film (*see* **Notes 12–14**).

3.3. Cdc25 Phosphatase Activity

The specific activities of cell cycle phosphatases have not been as well described as those of the kinases, partly owing to difficulty in obtaining purified phosphosubstrates. To determine a specific phosphatase/substrate relation-

ship, one must obtain a purified phosphatase and a purified substrate that has been specifically phosphorylated at the residues to be tested. These tasks can be very challenging; therefore, we will describe the phosphatase activity of purified recombinant GST-Cdc25 on a well-known artificial substrate, 4-nitrophenyl phosphate (p-NPP). The use of p-NPP allows for the rapid detection of phosphatase activity, as this is a colorimetric assay. Briefly, addition of a phosphatase removes the phosphate group from the p-NPP, generating a yellow color. The absorbance of this resulting color change can be measured by spectroscopy at 410 nm.

Although three isoforms of Cdc25 have been identified, Cdc25A (inhibits G1/S transition), Cdc25B (inhibits S-phase progression), and Cdc25C (inhibits G2/M transition), we will focus on GST-Cdc25B. The protocols discussed here will give basic techniques and procedures for detection of the phosphatase activity of recombinant GST-Cdc25B. Detailed procedures for purification of endogenous and recombinant Cdc25 as well as purification of phospho-specific targets, for example, Cdc2 phospho-Thr14 and Tyr15, have been eloquently described elsewhere *(25–27)*.

1. Add purified GST-Cdc25B to Cdc25 phosphatase buffer, containing p-NPP at a final concentration of 20 m*M*, to a 96-well microplate. The total volume should be 200 μL.
2. When starting out, serial dilutions of purified proteins and various time-points should be tested to obtain optimal results.
3. Cover the 96-well plate with Parafilm.
4. Place at 37°C for 1 h and protect the reaction from light.
5. Stop the reaction by adding 100 μL of 2 *M* NaOH.
6. Measure the absorbance by using a microplate reader at 410 nm. A blank (without the addition of the phosphatase) should serve as a negative control.

4. Notes

1. One 10-cm dish of 80% confluent HeLa cells will yield approx 3 mg of soluble protein.
2. Unirradiated and γ-irradiated cells may be harvested, then flash frozen in liquid nitrogen and stored at –70°C without a noticeable loss of in vitro kinase activities.
3. Protein concentration can be determined by using the Bradford assay. We normally use 1, 2, 4, and 6 μg of bovine serum albumin (BSA) at 1 mg/mL as a protein standard. Bradford reagent is available from Bio-Rad, cat. no. 500-0006.
4. We have found 1 mg of soluble extract per kinase reaction yields excellent results.
5. Equilibration of Protein G-Sepharose beads is accomplished by washing the beads three times in the appropriate lysis buffer.
6. The amount of kinase used in the reactions should be determined for each experiment. Therefore, we recommend immunoprecipitating an additional 1 mg of

lysate under the same conditions used in the kinase assay, followed by SDS-PAGE and Western blotting with the appropriate antibodies (**Fig. 3**).

7. The choice of antibody used to precipitate the kinase source should be determined by the availability of the antibody. Antibodies used in the kinase reactions may be purchased from commercial sources or generated by individual laboratories. An alternative approach is to epitope tag the kinase, overexpress the protein in cells, and then immunoprecipitate the kinase using antibodies raised against the epitope.

8. We normally elute GST substrates in the appropriate kinase buffer containing reduced glutathione; that is, a potential ATR substrate should be eluted in ATR kinase buffer containing glutathione. Reduced glutathione can be purchased from Sigma, cat. no. G-4251.

9. When performing the final wash of the kinase-IgG immunocomplex, carefully remove all liquid, but do not disturb the IgG beads.

10. We routinely determine the concentration of the GST substrates by SDS-PAGE followed by Coomassie staining using 1, 3, and 5 μg BSA as a protein standard.

11. Carefully load the kinase-substrate reaction into the wells of the polyacrylamide gel. We have seen high, nonspecific radioactive background in our autoradiographs when boiled IgG beads are inadvertently loaded into the wells.

12. When destaining, we find that repeated changes of the destaining buffer and addition of Kimwipes rapidly assists in the removal of the Coomassie stain from the gel.

13. We can normally visualize the phosphorylated GST-p53[1–106] in the autoradiograph within 5 h and the histone H1 within 30 min after exposure to X-ray film.

14. The length of the second thymidine release will need to be tested for each cell line. Fluorescence-activated cell sorting (FACS) analysis should be performed each time to ensure the cells are in the appropriate cell cycle phase. The laboratory manual, *Current Protocols in Cell Biology (22)*, provides detailed procedures for various methods of cell synchronization as well as FACS analysis. Chapters 2, 3, 4, 6, 7, and 8 in this book also address these issues.

Acknowledgments

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23

Monitoring Changes in the Subcellular Location of Proteins in *S. cerevisiae*

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1. Introduction

Immunofluorescence (IF) microscopy is indisputably a key tool to study the location of proteins and to visualize intracellular structures in immobilized cells and in spread nuclei. In yeasts, much has been learned using fluorescent tags as labels employing the original methods described, which continue to be quite valuable *(1)*. Fixation and staining of whole cells allows for visualization of the subcellular distribution of the protein of interest relative to the phase of the cell cycle. Spreading and staining yeast nuclei are useful steps to determine subnuclear distribution of chromatin-associated proteins and to detect changes in chromosome morphology through the cell cycle. Overtime, these methods have been optimized to promote the preservation of cellular or nuclear structures and/or the antigen of interest, the visualization, or the blocking of nonspecific staining.

IF remains far from trivial and this section is meant to guide researchers through the initial trial-and-error steps. Here we discuss multiple techniques for fixing whole yeast cells (*see* **Subheading 2.1.**) and spreading yeast nuclei onto slides (*see* **Subheading 2.2.**) as well as methods of immunostaining to visualize cellular and nuclear structures.

2. Materials

2.1. Protocol 1: Preparation and Immunostaining of Whole Yeast Cells

The yeast model system has considerably expanded the knowledge regarding controls of cell cycle events at the molecular level. Instrumental in this

effort has been a combination of IF with techniques to synchronize yeast cell populations such that all cells in the culture are in the same cell cycle phase until the study is complete. As a result, enormous insight has been gained on the function of many proteins based on their redistribution according to cell cycle phase. Researchers typically induce synchrony in yeast cultures by blocking the cells in a particular stage of the cell cycle and releasing them into fresh media under growth-permissive conditions, or by collecting cells in one particular cell cycle phase by centrifugal elutriation and releasing the gathered cells into fresh media. After release, time-points are taken at chosen intervals by harvesting part of the culture each time. Harvested cells are immediately fixed, as described in **Subheading 3.1.**, **steps 3–7**, such that samples are "frozen" permanently until all the necessary samples are ready to be processed simultaneously. The end result is visualization of the subcellular distribution of the protein of interest relative to deoxyribonucleic acid (DNA) and/or actin or tubulin stains that indicate the phase of the cell cycle at the time of fixation. DNA staining with 4,6-diamidino 2-phenylindole (DAPI) is commonly used as counterstain in preparations probed to detect a protein of interest, but it is valuable to double-stain for marker proteins as well. This is particularly feasible if the primary antibody was raised in a different species than the antibodies to the marker proteins. For example, if the antibody of interest is raised in a rabbit, double-staining with tubulin is straightforward using commercially available rat or mouse monoclonal antibodies. Alternatively, different cell aliquots can be stained in parallel to get a rough idea of the fraction of a population at a particular cell stage. Staining actin and tubulin are also recommended as valuable controls for antibody accessibility and cell integrity after the fixation and permeabilization steps.

For an example of results obtained with this method, *see* Rossanese et al. *(2)*.

2.1.1. Formaldehyde Fixation and Spheroplasting

1. Formaldehyde fixative (16% solution, EM grade; Ted Pella cat. no. 18505).
2. 50 mM KPO$_4$ pH 6.5, 1 mM MgCl$_2$, 4% formaldehyde.
3. PM buffer: 100 mM phosphate buffer pH 7.5, 1 mM MgCl$_2$, 0.25 mM phenyl-methylsulfonyl fluoride (PMSF), 1 µM pepstatin.
4. β-mercaptoethanol (Sigma cat. no. M6250).
5. Yeast lytic enzyme (ICN cat. no. 153529) or zymolyase 100T (ICN cat. no. 320932) or glusulase (Sigma cat. no. G-1512).

2.1.2. Immobilization of Cells

1. 22 × 22-mm coverslips or 22 × 50-mm coverslips.
2. Poly-L-lysine (Sigma cat. no. P1524).
3. Poly-Prep slides (Sigma cat. no. P0425).
4. Pap pen (Sanford Expo dry-erase marker).

2.1.3. Solvent Fixation/Permeabilization and Blocking

1. Phosphate-buffered saline (PBS)-Block:PBS, pH 7.4, 1% dried milk, 0.5% bovine serum albumin (BSA).
2. Methanol or acetone prechilled to –20°C.

2.1.4. Staining Cells With Antibodies

1. PBS-Block.
2. 0.2 µg/mL DAPI (Sigma cat. no. D-9564).
3. Anti-actin monoclonal antibody (C4, ICN Biomedicals).
4. Anti-tubulin monoclonal antibody (YOL1/34, Accurate Chemical & Scientific Corp).
5. Alexa 594 goat anti-mouse IgG conjugate (A-11032, Molecular Probes).
6. Alexa 594 goat anti-rat IgG conjugate (A-11007, Molecular Probes).
7. 1 mg/mL phenylenediamine (dissolved in 90% glycerol plus 10% PBS).

2.2. Protocol 2: Preparation and Immunostaining of Spread Nuclei

Nuclear proteins are often nonuniformly distributed and their distribution can be regulated during the cell cycle. Given the small size of the yeast nucleus, it can be difficult to visualize subnuclear distribution of chromatin-bound proteins in intact cells. The problem can be overcome by "spreading" nuclei. Spreading involves removal of the cell wall, as well as cellular and nuclear membranes. This causes the insoluble contents of the nucleus, the "nucleoid," to expand before settling on to the surface of a microscope slide. This expansion makes it possible to detect subnuclear complexes of proteins such as those involved in recombination and replication *(3–6)*.

The nucleoid consists of the nuclear scaffold, the chromosomes, and chromosome-associated proteins. The amount of nucleoid expansion depends on the conditions used. Spread nucleoids have diameters on the order of fivefold greater than those of intact cells (3–5 microns as compared to 0.5 microns). The method is particularly valuable for studies of meiosis as chromosomes undergo dramatic morphological changes. These changes include dramatic chromosome condensation that makes it possible to visualize individual chromosomes. As a result, meiotic prophase provides a unique opportunity to examine the distribution of chromosome-associated proteins given that chromosome condensation is limited in mitotic yeast cells. Meiotic spreads have been used to localize telomeric and centromeric proteins *(7)* as well as correlate changes in chromatin-associated proteins, such as modification of histones, with respect to the condensation state of chromosomes *(8)*. Furthermore, a number of the proteins that drive meiotic chromosome morphogenesis have been localized on spread nucleoids *(9)*.

An additional feature of spread nucleoids is that association between the nucleoid and the spindle is largely retained during the spreading procedure,

allowing nucleoids to be staged with respect to spindle development and also localization of spindle associated proteins *(10,11)*. A commercially available anti-tubulin antibody detects microtubule organizing centers in up to 80–90% of spread nuclei (Accurate Chemicals cat. no. YOL1/34).

Two methods for preparing nuclear spreads have been described; one was developed by Franz Klein and Josef Loidl *(12)* and the other by Mike Dresser and Craig Giroux *(13)*. Although both methods were developed for visualizing meiotic chromosome ultrastructure by electron microscopy, they are equally suitable for light microscopy studies and can be used to examine mitotic as well as meiotic cells. The key difference between the two methods is that the Klein/Loidl method uses a detergent during fixation, whereas the Dresser/ Giroux method does not. Each method has advantages. The Klein/Loidl method yields a large number of uniformly spread nuclei. This method is particularly valuable for time-course studies in which analysis of large numbers of unselected nuclei are needed to accurately determine the timing of changes in nuclear distribution of proteins *(14)*. The quality of nuclei spread by the Dresser/Giroux method tends to vary dramatically from one area of the slide to another, with many nuclei remaining "underspread." However, chromosome morphology can be particularly exquisite in "well-spread" regions owing to better preservation of chromatin structure. The following protocols contain modest modifications of the original versions. The reader is encouraged to consult the original articles for comparison.

2.2.1. Klein/Loidl Spreads

1. High quality glass slides washed with 95% ethanol and polished with lens tissue.
2. 1 *M* dithiothreitol (DTT) (Fisher cat. no. BP172-5).
3. Zymolyase solution (make fresh on the day of the experiment and keep at 4°C until use; gentle agitation with a pipettor is needed to dissolve zymolyase powder): 20 mg/mL zymolyase 100T (US Biological cat. no. Z1004), 2% glucose, 50 m*M* Tris-HCl, pH 7.5.
4. 3% paraformaldehyde (PFA)/3.4% sucrose solution. (make fresh on the day of the experiment; it is convenient to make about 50 mL; the solution should be prepared with gloved hands in a fume hood): 1.5 g PFA (VWR/JT Baker cat. no. JTS898-4) in a 125-mL flask. Add about 45 mL dH$_2$O at 60°C and stir bar. Add 150 µL 1N NaOH. Stir on an unheated stir plate until fully dissolved (Wait 15 min. If not completely dissolved add an additional 50 µL NaOH, and stir for an additional 15 min). Filter with a 0.2 micron filter unit, and transfer to a clean flask. Adjust to pH 7.0 with 1N HCl. Add 1.7 g sucrose, stir until in solution, and bring to final vol of 50 mL. Keep solution on ice until used.
5. Although the 3% solution is usually optimal, different lots of PFA may vary. Furthermore, optimal fixation conditions vary depending on the protein of interest. It may prove useful to make a set of PFA solutions that vary from 2 to 4%.

6. MES/sorbitol wash buffer (0.2 micron sterilize; chill to 4°C before use): 1.0 *M* sorbitol, 0.1 *M* MES (Sigma cat. no. 2933) pH 6.5, 1 m*M* ethylenediamine-tetraacetic acid (EDTA), 0.5 m*M* MgCl$_2$.
7. ZK buffer (filter through 0.2 micron; store at room temperature [RT]): 25 m*M* Tris-HCl, pH 7.5, 0.8 *M* KCl.
8. 1% lipsol (L.I.P. Ltd, England cat. no. 40022): Dilute in dH$_2$O; store diluted aliquots at 4°C.

2.2.2. Dresser/Giroux Spreads

1. DTT solution, zymolyase solution, MES/sorbitol solution, ZK Buffer (*see* **Subheading 2.2.1.**).
2. PFA solution prepared as described above, but at a concentration of 4% rather than 3% and with no sucrose added (*see* **Subheading 2.2.1.**).
3. MES lysis solution (0.2 micron sterilize and chill to 4°C before use): 0.1 *M* MES pH 6.5, 1 m*M* EDTA, 0.5 m*M* MgCl$_2$.
4. 0.2% Kodak Photo-Flo 200 (Sigma cat. no. P 7417).
5. Poly-L-lysine coated slides.

2.2.3. Immunostaining Spread Nuclei

1. Slides containing spread nuclei.
2. 22 × 22 or 22 × 50-mm coverslips.
3. Slide staining dishes—at least two, more depending on the scale of the experiment, as each dish holds about 10 slides, and it is convenient to have two for each set of slides (Fisher cat. no. 08-812).
4. Moist chambers prepared by placing several H$_2$O-saturated paper towels in a sealable plastic box (e.g., Tupperware with approximate dimensions of 12 in. × 8 in. and then placing a glass plate on top of the wet towels).
5. 0.2% Kodak Photo-Flo 200.
6. 1000 mL Tris-buffered saline (TBS; 20X stock): 160 g NaCl, 4 g KCl, 60 g Tris Base; adjust pH to 8.0 with 1 *M* HCl; dilute to 1X for protocol.
7. TBS/BSA (10 mL): 1% BSA (Sigma cat. no. A-2153) in 1X TBS filtered through 0.2 μ filter unit.
8. Vectashield mounting medium for fluorescence with DAPI (Vector Labs, Burlingame, CA cat. no. H1200).
9. Primary antibody: We have found that whole serum often works as well as purified antisera because only a fraction of total protein is present after spreading. When possible, deletion mutants are used as controls to determine if signal is specific to the protein of interest. For whole serum, a dilution of between 1/50 and 1/500 is often optimal. If a deletion control is not possible, affinity-purified serum should be used.
10. Secondary antibody: Fluorophore-conjugated anti-IgG antibodies are used. Secondary antibodies must be specific to the animal used to raise the primary antibody (Alexa Fluor Anti-IgG antibodies, Molecular Probes). A 1:1000 dilution of these antibodies in TBS/BSA (2μg/mL) is often optimal or near optimal.

3. Methods

3.1. Protocol 1

As with all protocols, the conditions necessary to give optimal results will vary in each case. In the following generic protocol, those steps that frequently require optimization have been italicized, and some helpful considerations are discussed in the Notes section (*see* **Subheading 4**).

3.1.1. Formaldehyde Fixation and Spheroplasting

1. A yeast culture is grown overnight in the appropriate medium to an optical density (OD)$_{600}$ of between 0.25 and 1.0.
2. Pipet 8 mL of the culture onto a 150-mL Nalgene bottle-top filter (45-mm diameter, 0.2 μ*M* pores). Remove the growth media by applying vacuum.
3. Resuspend cells with 5 mL of freshly prepared 50 m*M* KPO$_4$ pH 6.5, 1 m*M* MgCl$_2$, *4% formaldehyde*, and transfer the suspension to a 15-mL Falcon tube.
   Alternatively, the fixative can be added straight to the overnight culture.
4. Incubate at RT for *2 h*, vortexing once, briefly, after 1 h.
5. Spin cells for 3 min at 400*g* in a low-speed centrifuge. Remove supernatant carefully with a Pasteur pipet.
6. Resuspend the cells in 5 mL fresh PM buffer (100 m*M* KPO$_4$, pH 7.5, 1 m*M* MgCl$_2$, 0.25 m*M* PMSF, 1 μ*M* pepstatin) and then spin as in **step 5**. Remove the supernatant.
7. Resuspend cells in PM buffer to a final OD$_{600}$ of 10.
8. Add 0.6 μL of β-mercaptoethanol followed by 20 μL of recombinant yeast lytic enzyme (20,000 U/mL). Mix end-over-end at RT for 15 min.
   Alternatively, zymolyase or glusulase can be used for digestion, but be aware that proteases are present in the commercially available stocks of these enzymes. *Sorbitol can be added to 1.2* M *concentration*.
9. Harvest the spheroplasts by spinning 2 min at 400*g* in a microfuge. Resuspend in 100 μL of PM buffer.
10. Repeat **step 9** once more.

3.1.2. Immobilization of Cells

1. Use a glass coverslip of 1.5 thickness. For 1–4 samples use a 22 × 22-mm coverslip; for 5–10 samples use a 22 × 50-mm coverslip.
2. To create wells, cut out a suitable rectangular piece from the adhesive backing of a FedEx document pouch, and use a hole puncher to punch holes. Attach the perforated plastic to the surface of the coverslip. Make sure that the plastic around the wells is smooth and flat.
3. Add 10 μL 0.1% polylysine to each well. Leave 30 s, then aspirate off the polylysine. Wash each well three times by adding a drop of dH$_2$O and then aspirating off the drop. Let the slide air-dry.

Alternatively, microscope slides coated with poly-ʟ-lysine can be used and hydrophobic barriers made on the slides with a Pap pen.

4. About 10 μL of the spheroplast suspension is added to each well.
5. After 10 min, aspirate the excess liquid off using a Pasteur pipet linked to a vacuum.

3.1.3. Solvent Fixation/Permeabilization and Blocking

1. Immerse the coverslip (or slide) for 5 min in methanol prechilled to –20°C. *Alternatively, immerse the coverslip (or slide) for 5 min in acetone prechilled to –20°C.*
2. Air-dry quickly by placing slides against a warm surface like the lid of a 37°C water bath.
3. Once dried, add to each well a *drop of PBS-Block* (PBS, pH 7.4, 1% dried milk, 0.5% BSA) prespun for 10 min at 800*g*. Incubate for 1 h.
4. Aspirate off the PBS-Block.

3.1.4. Staining Cells With Antibodies

1. Place 10 μL of a primary antibody that has been diluted in PBS-Block and cleared by centrifugation for 5 min at top speed in a microfuge. For actin staining, add anti-actin antibody at 1:250 dilution. For tubulin staining, add anti-tubulin antibody at 1:200 dilution.
2. Incubate in a humid chamber at RT for *1 h*.
3. Wash each well eight times with PBS-Block.
4. Add 10 μL of a secondary antibody mixture in PBS-Block that has been cleared as in **step 1**. For actin staining, use the Alexa 594 antimouse IgG antibody at 1:250 dilution. For tubulin staining, use the Alexa 594 antirat IgG antibody at 1:200 dilution.
   Optional: Include 2 μg/mL Hoechst 33258 or 0.2 μg/mL DAPI in the secondary antibody mixture to stain DNA.
5. Incubate in a dark and preferably humid chamber at RT for *1 h*.
6. Wash each well eight times with PBS-Block.
7. Wick away PBS-Block after the last wash.
8. Add 5 μL of 1 mg/mL phenylenediamine (dissolved in 90% glycerol plus 10% PBS) to each well, and mount the coverslip onto a slide. Phenylenediamine slows photo bleaching. An alternative is to use a prepared "antifade" mounting medium such as Vectashield (Vector Labs, Burlingame, CA cat. no. H1200). Seal the coverslip with nail polish.
9. View immediately with a fluorescent microscope or preserve the slide at –20°C in the dark (good for 2–3 d).

3.2. Protocol 2

3.2.1. Spheroplasting Cells

1. Start with about 2×10^8 cells (e.g., 5 mL of culture at OD_{600} 1.4 = 5×10^7 cells/mL).
2. Pellet cells for 3 min at low speed (2100 rpm, 600*g*) in a clinical centrifuge.

3. Resuspend in 1 mL ZK buffer (about 2×10^8 cells/mL).
4. Add 40 µL 1 *M* DTT.
5. Incubate 2 min at RT, with gentle mixing.
6. Pellet cells as before.
7. Resuspend in 1 mL ZK and add 5 µL zymolyase 100T solution.
8. Incubate for 20–30 min in a 30°C incubator on a rocking platform.
9. Check quality of spheroplasts by phase microscopy after 20 min. Cells should appear round rather than slightly oblong and should be sensitive to lysis when an equal volume of 0.1% sodium dodecyl sulfate (SDS) is added. If cells fail to lyse, incubate for an additional 10 min and repeat the assay.
10. Once spheroplasting is complete, pellet cells as before.
11. Gently resuspend cell pellet in 2.5 mL MES/sorbitol buffer and place suspensions on ice.

3.2.2. Klein/Loidl Spreads

Work in a fume hood to avoid excessive exposure to formaldehyde. It is important to monitor the lysis step in the procedure microscopically. A long-working-distance objective is needed for this purpose because it is not feasible to use coverslips. Most yeast labs have such an objective mounted on the microscope used to dissect tetrads. We use a Zeiss microdissection scope after removing the micromanipulator and plate holder. It is important to set the focal plane of the microscope before starting.

1. Using a P200 Eppendorf pipetter, or equivalent, place 20 µL of cell suspension in MES/sorbitol buffer on a clean slide.
2. Add 40 µL PFA/sucrose solution, and mix by holding the slide and moving your hand in a circular motion taking care not to spill the liquid.
3. Add 80 µL 1% Lispol, swirl briefly, and place the slide under the microscope. Watch the cell lysis occur. Cells appear to deflate, and the remaining material is relatively difficult to see when the membranes rupture. Lysis should take about 20–30 s but may take as long as a minute. Remove slide from scope when about 75% of cells are lysed.
4. Immediately add another 80 µL PFA/sucrose; swirl as before.
5. To spread the droplet over the entire surface of the slide (excluding the frosted edge), pass a glass Pasteur pipet lengthwise along the top of the drop.
6. Leave spread slides overnight in the fume hood. Nucleoids will settle and bind to the glass surface, and the solution will dry to a thick "honey" because of the sucrose it contains.

Optimal results can require immediate use of slides at this stage. However, usually it is possible to store dried slides at –20°C for weeks or even months with only modest reduction in quality. A reasonable approach is to make 2 or 3 duplicate slides, stain one immediately, and freeze the remaining slides for future use or in case of mishap.

3.2.3. Dresser/Giroux Spreads

1. Once cells are resuspended in MES/sorbitol solution, pellet them for 3 min at low speed (2100 rpm, 600*g*) in a clinical centrifuge.
2. Gently resuspend cells in 300 µL MES/lysis solution with a p1000 Eppendorf pipetter or equivalent.
3. Working in a fume hood, add 100 µL of lysed cells to tubes containing 700 µL PFA solution, and mix by inverting the tubes.
4. Transfer fixed cell suspensions to 2 polylysine-coated slides (400 µL/slide) that are resting on paper towels.
5. Allow nucleoids to settle and bind to slides for 10 min.
6. Drain liquid from slides by gently tilting slides until the liquid contacts the paper towel and is absorbed.
7. Add 350 µL of 4% PFA solution and wait 10 min before draining as before.
8. Add 0.8 mL 0.2% Photo-Flo 200, drain as before, and air-dry completely (30 min to 1 h).

It is best to use slides prepared by this method immediately.

3.2.4. Immunostaining Spread Nuclei

Antibody incubation is 80 µL under a 22×50-mm coverslip or 30 µL under a 22×22-mm coverslip. (The larger coverslip is preferable when the antibody is not in short supply.) Avoid bubbles when adding coverslip by slowly lowering from one edge using a small spatula. Remove coverslip by submerging in TBS at a 45° angle in which coverslip is pointing down.

1. For slides prepared by the Klein/Loidl procedure, dip in 0.2% Photo-Flo for 30 s to remove honey. Air-dry slides by resting on an edge in the hood. (This is not necessary for slides prepared by the Dresser/Giroux method).
2. To reduce nonspecific binding, apply 300 µL TBS/BSA to slide with no coverslip. You may need to tilt the slide to ensure even coverage of TBS/BSA on the entire slide. Use care to avoid spilling solution. Incubate in a damp chamber at RT for 10 min.
3. Drain slides by tilting and touching edge to a dry paper towel, but do not allow surface of slide to dry.
4. If the entire slide is to be stained with the same antibody, add 80 µL of TBS/BSA solution containing an appropriate dilution of primary antibody and use a 22×50-mm coverslip. For crude serum dilution ranges are often on the order of 1/50 to 1/500. For purified antibodies, a range of dilutions must be tested. If antibodies are in short supply or if two primary antibodies are to be tested on a single slide, use 30 µL drops and a 22×22-mm coverslip.
5. As in all antibody-staining experiments, control experiments in which primary antibody is omitted should be carried out in parallel.
6. Carefully place coverslip on the slide avoiding bubbles. This can be achieved by resting the edge of the coverslip in a corner of the solution, making use of surface

tension to hold the coverslip in place, then slowly lowering the opposite edge with the help of a small spatula.

7. Seal the slides in the moist chamber, and incubate 4 h to overnight at 4°C.
8. Carefully place slides in the holder supplied with the staining dish, and lower into a dish containing TBS. The coverslips should fall from the slides in a few minutes. Transfer the slides to a second dish containing TBS and incubate 15 min.
9. From this point on, subdued room lighting is used to avoid photo bleaching of the secondary antibody.
10. Drain slides without drying and add TBS solution containing a 1/1000 dilution of an appropriate fluorochrome-conjugated secondary antibody. If the experiment is a double-staining experiment, controls in which only one of the secondary antibodies is added should be carried out. Add coverslips, as before, and seal slides in the moist chamber.
11. Incubate for 2 h at 4°C in the dark.
12. Remove coverslips and wash slides in the dark as in **step 8**.
13. Air-dry slides in the dark (about 1 h).
14. Add three small spots (about 30 µL each) of Vectashield by touching surface with eyedropper supplied. Add a coverslip and seal with nail polish.

View slides with a ×100 objective using an epifluorescence microscope. Confocal microscopes offer little advantage as the spread nuclei are essentially two-dimensional and thus out of focus.

4. Notes

1. *Preparing the tagged protein.*

    Adding an epitope-tag peptide to the target protein is now as simple as one polymerase chain reaction (PCR) *(15,16)*, such that the "brain-involved step" is the decision of which tag to use and where to place it in the protein. Tagging with a green fluorescent protein (GFP) epitope has the enticing advantage of been useful in both living and fixed cells. Two successful GFP-tagged proteins Spc42 and Rpl25 have become wonderful tracking markers for the spindle-pole body and ribosome movement, respectively *(17,18)*. However, GFP and its variants are 25 to 27 kDa proteins that may interfere with the proper localization and function of the tagged protein. When tagging cell-cycle regulated proteins like cyclins with GFP, one need also take in consideration the time required for the tag to achieve its folded state, which is sometimes greater than the time the cyclin is around. Another relatively large tag used is glutathione-*S*-transferase (GST) of approx 26 kDa, which is convenient for both localization by IF and rapid biochemical purification of the tagged protein. A common practice is to add several copies of smaller size tags in tandem to the protein of interest to increase the detection signal. Useful smaller tags include the HA and c-myc epitopes, which are 2 kDa and 1.2 kDa, respectively. However, it is our experience that unequivocal detection by IF of tagged cyclins expressed from their endogenous promoters is facilitated by the addition of ≥ 10 c-myc epitopes per protein, and the detection

is even clearer when using a diploid strain that is homozygous for the tagged protein.

In terms of the choice between N- or C-terminal-protein tagging, the latter is more useful because it confirms the complete translation of the tagged protein. However, the most useful tag is simply one that does not interfere with the function of the protein of interest, and this needs to be tested empirically. Ideally, the functionality of the tagged protein is ascertained by constructing a strain that depends on the tagged protein for life.

2. *IF protocol.*

Formaldehyde fixation (*see* **Subheading 3.1.1.**) is a critical step as slight variations may produce dramatic results. A concentration between 3.7 and 5% formaldehyde is generally used, and the time of fixation should be determined empirically, although longer times appear to work out better for some antibodies. Thus, when taking time-points, samples can be kept in the fixative until all the samples have been taken and are ready to be washed. Although sorbitol is included in the spheroplasting step of many IF protocols, it is completely unnecessary. Formaldehyde-fixed, dead cells do not require sorbitol to keep the cells from rupturing. Moreover, sorbitol is a potent inhibitor of purified yeast lytic enzyme, so leaving it out greatly improves the spheroplasting efficiency of this enzyme.

The rationale for immobilizing cells (*see* **Subheading 3.1.2.**) in wells directly created on the coverslip is that the optics are best when the sample is directly adjacent to the coverslip. When cells are attached to the slide, they are viewed through an intervening layer of mounting medium.

The solvent fixation/permeabilization steps (*see* **Subheading 3.1.3.**) have the purpose of fixing additional antigens, exposing hidden epitopes through dehydration, and improving the antibody permeability of cells. Optimal conditions must be sought empirically. In general, it is best to use either methanol or acetone, but not both. Acetone is better at preserving native structure, whereas methanol is better at denaturing proteins. Some antibodies recognize native epitopes, and other antibodies only recognize denatured proteins; so for a given antibody, one solvent or the other may work better. As for blocking, it turns out that some antigens are masked by milk. For such cases, an alternative blocking agent is PBS with 0.2% Tween-20.

The antibody incubation times and temperature (*see* **Subheading 3.1.4.**) are also prone to optimization. In our experience, the actin and tubulin stains are optimal with a 2 h incubation time for the primary antibody and a 1 h incubation time for the secondary antibody.

Acknowledgments

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IV

CHROMOSOMES AND THE CELL CYCLE

24

Chromosomal Changes and Cell Cycle Checkpoints in Mammalian Cells

Charles R. Geard and Brian Ponnaiya

1. Introduction

The observation of damage to chromosomes and of alterations to normal cell cycle progression were early findings in radiation biology *(1–6)* and provided a strong impetus for the elaboration of the causative basis for understanding deoxyribonucleic acid (DNA) repair in all its manifestations. See Murnane *(7)* for a historical perspective. Ionizing radiations provide a precise tool in this regard, in that they are readily characterized physically, can be delivered with relative precision as well as tight control of timing, and do not require metabolic activation. Such radiations produce discrete energy-deposition events in cells, the intensity and frequency of which depend on the dose and type of radiation. The charged particles that are produced—electrons after photon (X- and γ-ray) irradiation; protons, with heavier charged particles, after neutron irradiation; and α-particles after isotopic or machine irradiation—deposit their energy along relatively short tracks, often of cellular dimensions.

The induction of chromosomal changes by ionizing radiation is probably the most readily discernible early manifestation of radiation damage to proliferating cells. However, the visible changes observed in condensed chromosomes at metaphase are not unique to radiation. On the contrary, whereas frequencies of particular aberration types may differ dramatically, the consequences of cell exposure to a wide variety of DNA-damaging agents produces microscopically similar aberrations. Such observations were made by Bender and colleagues *(8–10)* for ultraviolet (UV) exposure, bromodeoxyuridine plus visible light exposure, a range of chemicals, and ionizing radiation. The combination of a damaging agent and mutant cells shows that the visible consequences of damage induction are not unique but are quantitatively different,

and the spectrum of induced changes may differ. For example, see Cordeiro-Stone et al. *(11)*, for ultraviolet C (UVC) and xeroderma pigmentosum (nucleotide excision repair); Franchitto et al. *(12)* for ionizing radiation and hereditary nonpolyposis colorectal cancer cells (mismatch repair deficient); and Pluth et al. *(13)* for severe combined immunodeficient cells expressing hRAD54 (homologous recombination repair). That is, although the precise type of DNA damage may depend on the damaging agent, and each type of damage may involve specific DNA damage-repair pathways, the consequences of processing lesions can and does manifest as grossly altered chromosomes at metaphase. This may then lead to cell death and prompt the beginnings of a carcinogenic change or mutation, and/or later generation chromosomal changes *(14)*.

1.1. Chromosomal Changes at Mitosis

The spectrum of gross chromosomal changes that can be recorded at mitosis are best exemplified by examining the consequences of cellular exposure to ionizing radiations. The effects of ionizing radiation on chromosomes as seen by the microscope can be largely classified in terms of the stage in the cell cycle when the damage was initiated and the number of chromosomes—or parts thereof—involved. *See* Savage *(15)* for a detailed description and classification of induced chromosomal structural changes.

1.2. G0/G1-Phase Induced Aberrations

A DNA double-stranded break (DSB) disrupting the linear integrity of a chromosome prior to replication in G1 can lead to replication of both elements and to appearance at mitosis as chromosomal breaks involving both chromatids of a chromosome (simple deletions). The rarity of such entities in normal cells, but not in cells derived from some genetic disorders or tumor-derived cells, attests to the notion that an open DNA DSB is not compatible with cellular progression through the cycle. It has often been proposed that a principal purpose of cell cycle checkpoints, along with maintaining an orderly progression through the cell cycle, is to prevent the propagation of damaged DNA *(16–19)*, and the relative paucity of what would be essentially open DNA molecules strongly supports this contention. The vast majority of chromosomal changes that derive from G1 damage and survive into mitosis (**Fig. 1**) involve interactions between pairs of breaks (or lesions), which for ionizing radiations can be induced by one (low doses) or two dissimilar energy deposition events (higher photon doses). Because two spatially distinct lesions are involved, the simplest type of nonhomologous end joining (NHEJ), which rejoins one DNA DSB, cannot apply. The failure of an NHEJ event in G1 would result in a simple terminal deletion as would a failure of rejoining during the formation of dicentric rings/translocations, centric rings/pericentric inversions, and acentric
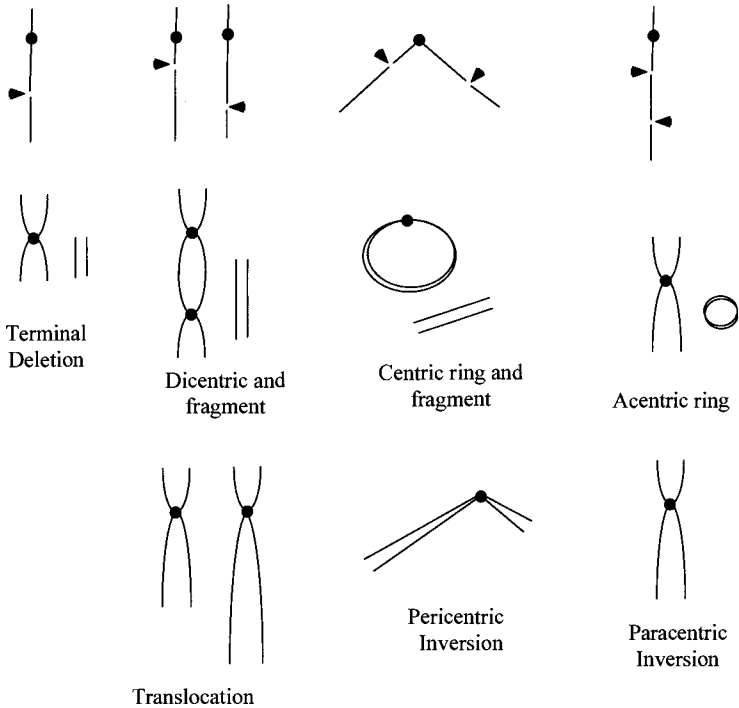
Fig. 1. Types of chromosome aberrations seen at mitosis after irradiation of G0/G1-phase cells. The breaks in the linear integrity of the chromosome are induced while the chromosomes are elongated and individually indistinguishable. Replication during S phase of chromosomes (including aberrants), with continued cellular progression to mitosis results in the appearance in the condensed microscopically visible mitotic chromosomes of deviations from normal. Terminal deletions, dicentrics, centric, and acentric rings are readily distinguishable; however, translocations, pericentric, and paracentric inversions may (if minor) only be seen after refined banding techniques.

rings/paracentric inversions (**Fig. 1**). A failure to complete the inter- or intrachange process results in incomplete exchanges. Interchanges between chromosomes that appear as dicentrics (asymmetric interchanges) or translocations (symmetric interchanges) are readily seen down the microscope—the former either by solid staining or by the use of chromosome-specific fluorescent *in situ* hybridization (FISH) probes. Translocations can only be seen with solid stains if the interchanged parts differ significantly in size, but they are readily seen using FISH probes *(20)* and, with a somewhat lower efficiency of detection, using Giemsa banding techniques. Intrachanges between the arms of a chromosome result in centric rings with accompanying acentric fragments (asymmetric intrachanges) or pericentric inversions (symmetric intrachanges).

Down the microscope, solid stains can detect the asymmetric exchanges but the pericentric inversions are most readily seen as color switches when chromosome arm-specific FISH probes are used, again with a lower efficiency of detection using Giemsa banding. Intra-arm intrachanges—acentric rings/interstitial deletions/double minutes (asymmetric) or paracentric inversions (symmetric)—are probably the most difficult type of aberration to score. If relatively small segments of DNA are deleted from along the length of a chromosome arm, the end-joined piece will still replicate and condense as the cell progresses to mitosis. However, detection depends on visualization, the resolution of the microscope, and the stain used. Solid stains are most useful for detecting small, acentric fragments and whole chromosome region-specific FISH probes (*m*-BAND) for paracentric inversions or the loss of interstitial segments.

Regarding the DNA damage-repair pathway that operates in G1 to produce this range of aberration types, the NHEJ pathway most likely predominates because it is apparent from the configurations seen that lesions are initiated and resolved before replication. However, it is only the consequences of incorrect resolution that are seen at mitosis. After insult, cells are delayed before progressing into S phase in a dose-dependent manner. Although difficult to resolve, the failure of the repair process, in the sense that breaks are left open, may lead to a long-term—even permanent—failure to progress further through the cell cycle or to cell autodestruction in the form of apoptosis. If G1-phase cells are irradiated or treated with a G1-acting clastogen, a finding of aberrations at mitosis that do not fit in the categories described may in and of itself be taken as an indication of a failure of normal cell cycle-checkpoint control.

1.3. S- and G2-Phase-Induced Aberrations

Following DNA replication, the spectrum of aberrations seen at mitosis expands significantly from that seen following irradiation of G1-phase cells. Collectively, these are referred to as chromatid-type aberrations because usually only one chromatid of any individual chromosome is involved *(15)*. If an acentric fragment is associated with, but physically separated from, a chromosome, a chromatid deletion would manifest itself (**Fig. 2**). This category of induced change is most frequently recorded in cells collected close to mitosis, that is, from cells treated in late G2. In contrast to the G1/S checkpoint, which appears oriented to preventing severely damaged DNA from being replicated, the G2/M checkpoint presumably promotes the repair of DNA that was damaged in late S or G2 phase, preventing its segregation into daughter cells. However, proximity to mitosis presumably allows many more damaged cells to progress into mitosis with overt breaks than is observed for chromosome deletions. The frequency of chromatid deletions declines rapidly the further from mitosis cells were damaged, and the relative frequencies of inter- and
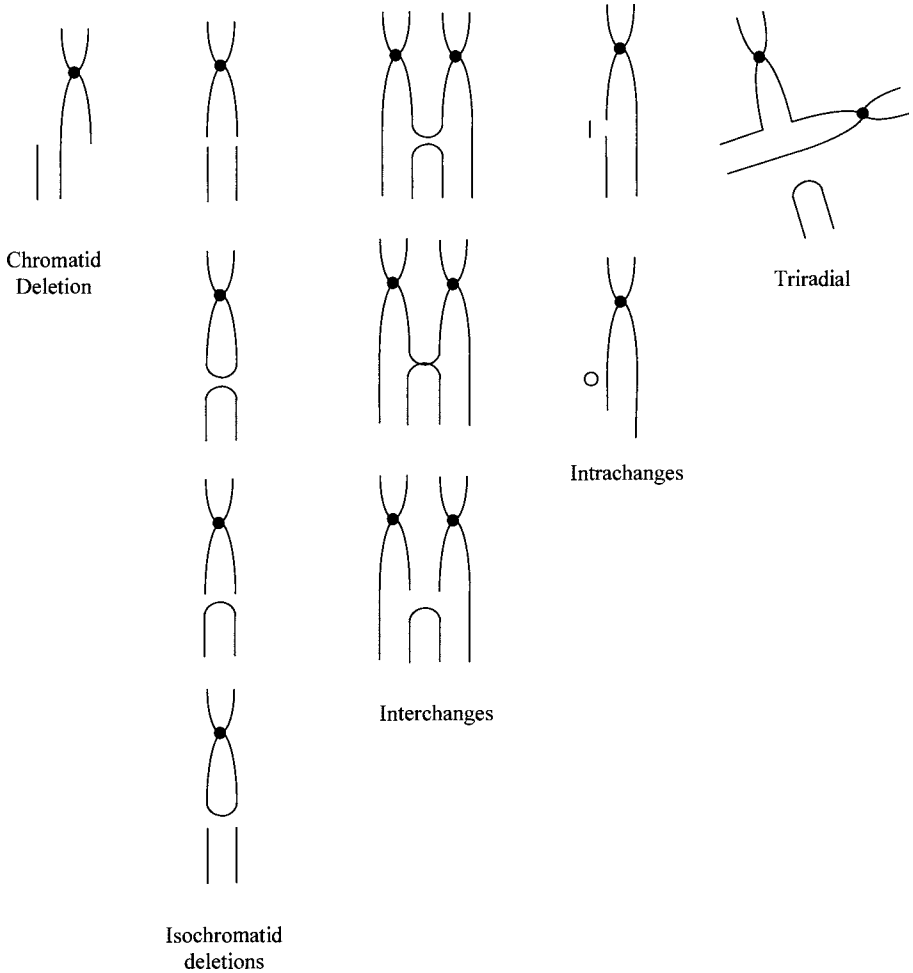
Fig. 2. Types of chromatid aberrations seen at mitosis after irradiation of postreplication (S and G2 cells). Failure of "broken ends" to interact results in incomplete aberrations. Because there are twice the number of DNA molecules in G2 vs G1 cells, the possibilities for interactions between induced lesions are dramatically increased, and only a limited number of discernible aberration types are indicated.

intrachanges along with isochromatid deletions increases rapidly. However, the absolute yields of all aberrations are highest for those cells damaged close to mitosis. Isochromatid deletions most clearly reflect a homologous recombination–repair process, with an interchange between the two newly replicated chromatids being seen as a sister chromatid exchange. This requires a means of distinguishing between the two chromatids, and this is achieved by two rounds

of replication, following incorporation of bromodeoxyuridine, and selective denaturation before staining. Sister chromatid exchanges are efficiently produced by many cytotoxic chemicals and particularly so in some mutant cell lines. They are, however, inefficiently induced by ionizing radiation *(21)*. The cellular consequences of such changes are less than for the asymmetric isochromatid deletions, which are usually seen as U-shaped sister unions (**Fig. 2**). Anaphase separation of such sister unions will, 50% of the time at each cellular division, result in a dicentric chromatid. Hence, at later divisions, complete dicentric chromosomes can be seen at mitosis. These can, however, be distinguished from most G1-induced aberrations because they appear uniform on either side from their midpoint. In contrast to isochromatid changes, which most clearly appear to reflect aberrant homologous recombination–repair, chromatid interchanges and intrachanges reflect interactions between different regions of nonhomologous chromosomes (interchanges) or two separated sites along the length of a chromatid (intrachanges). It is reasonable to assume that there may be similarities between such sites (Alu sequences or the like) but NHEJ may be functioning as the repair pathway, along with homologous recombination (HRR).

The type and frequency of aberrations then is related to the stage in the cell cycle at which DNA is damaged, the appropriate checkpoint to augment the likelihood of damage remediation, and the repair pathways that are used.

1.4. Other Chromosomal Observations

"Gaps" and chromosome end-to-end associations represent other categories of chromosomal change that may be associated with induced change or with cell culture conditions and /or mutant cell phenotypes. Gaps have been a subject of controversy for many years *(15)*. They are seen as achromatic or nonstaining regions of a chromatid or chromosome at mitosis. They can vary in size but do not seem to interfere with the passage of the complete chromatid or chromosome to the poles at anaphase. They are, then, not breaks but can easily be confused with chromatid breaks, particularly in cells observed following treatment close to mitosis. In general, the accepted criterion for a real chromatid break would require a physical movement of the acentric fragment from its colinear position with the chromatid. This approach may result in some true chromatid deletions being incorrectly classified; however, the ubiquitous presence of gaps, and their usual increase with dose, makes them worthy of being recorded as long as it is recognized that their meaning remains uncertain. They probably represent a condensation phenomenon or end product of a nearly successful repair process.

Chromosome end-to-end associations are frequently observed in chromosomal spreads from tumor-derived cells and appear to represent telomeric

associations as part of telomeric erosion and telomerase activity (e.g., **22**). They are useful observational descriptors in telomeric-oriented studies but do not appear to result in true chromosomal aberrations.

Overall, observing chromosomal changes in cells at mitosis or in interphase can provide insights both into the control exhibited by cell cycle checkpoints and into the repair processes that lead to their formation.

2. Materials

2.1. Cells for Metaphase Spreads

Cell growth should be optimal because the intent is to maximize the yield of mitotic cells. Treatment-induced checkpoint activation needs to be taken into consideration. Media requirements for different mammalian cell lines are too divergent to detail but should be optimized for each cell population studied.

2.2. Anaphase Prevention and Accumulation of Mitoses

10 µg/mL Colcemid stock solution. Keep sterile and refrigerated.

2.3. Bromodeoxyuridine to Monitor Number of Cell Divisions

10^{-3} *M* bromodeoxyuridine stock in Dulbecco's phosphate-buffered saline (PBS). Keep sterile, in the dark, and refrigerated.

2.4. Hypotonic Treatment for Cell-Volume Increase

0.075 *M* KCl at room temperature (RT).

2.5. Cell Fixation

1. 500-mL bottles of 3:1 methanol:glacial acetic acid at RT.
2. Fresh fixative is kept in freezer.

2.6. Staining

5% aqueous Giemsa stain pH 6.8 (Invitrogen, Carlsbad, CA)

2.7. Chromosome-Specific Probes

1. Whole chromosome painting (WCP) probes (Vysis, Downer Grove, IL).
2. Multicolored fluorescent *in situ* hybridization (*m*-FISH) (Meta Systems, Belmont, MA).
3. A pool of chromosome painting probes, each labeled with a different combination of 5 fluorochromes, to classify all 24 human chromosomes in distinct colors (22 pairs of autosomes plus the 2 sex chromosomes) (Vysis).
4. Chromosome arm-specific probes (Vysis).
5. Individual chromosome region-specific probes.
6. Centromeric (repetitive sequence).
7. Subtelomeric (repetitive sequence).
8. Single gene.
9. *m*-BAND (multicolored banding) (Meta Systems, Belmont, MA).

2.8. Equipment and Supplies

1. Centrifuge for 15-mL conical tubes.
2. Microcentrifuge for 1.5-mL tubes.
3. Aspirator to remove supernatant.
4. Water baths for hybridization procedures.
5. Dry oven and hot plates.
6. Microscope—dry and oil objectives: high quality upright, equipped with phase contrast to monitor number and quality of mitotic spreads; Tungsten bright field illumination for solid-stain Giemsa or Giemsa banding procedures; fluorescence microscope with mercury burner illumination; filter combinations as necessary for fluorochromes to be examined.
7. Precleaned slides (*see* **Note 1**), tube racks, Coplin jars, slide boxes, fine-tip indelible marker for writing on frosted-end slides, coverslips (22×22 mm to 24×50 mm), sealant, microscope oils, and mountants.

3. Methods

A significant fraction of cytogenetic studies are carried out on chromosome spreads derived from peripheral blood lymphocytes. These are not considered here. (*See* **refs.** *22–27* for details regarding modifications of techniques and applications in diverse circumstances.) Two examples of cell culture and fixation are covered: (a) plateau-phase cells synchronized in G0/G1 for chromosome-type aberrations; (b) exponentially growing asynchronous cells for chromatid-type aberrations.

3.1. Plateau-Phase Cells and Chromosome Aberrations

Cells such as fibroblasts are grown to confluence. Cell synchronization in G1 phase can be assessed by release from dishes into single cells and evaluation by flow cytometry or by monitoring absence of mitotic cells and of uptake of bromodeoxyuridine in S-phase cells.

1. Cells are subcultured by trypsinization, production of a single cell suspension, and replating at a low density.
2. After about 30 h (depending on the length of the cell cycle for each cell type) Colcemid is added (final concentration 1–6 *M*) for 4–6 h to accumulate mitotic cells (*see* **Note 2**).
3. If mitotic yields are high, as assessed by the phase-contrast microscope, mitoses can be collected by shake-off (*see* **Note 3**).
   or
4. If mitotic indexes are to be recorded as indicators of cell cycle delays, all cells are removed by trypsinization or by scraping with a rubber policeman.
5. Cells are collected in conical-bottom 15-mL centrifuge tubes and centrifuged at 100g for 5 min. The supernatant is aspirated, cells washed once in Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}), and repelleted.

6. Supernatant is removed to a covering of the cell pellet and 0.075 *M* KCl (hypotonic buffer) added. Cells are resuspended in hypotonic buffer and incubated at 37°C for 6–18 min (short times for hematopoietic cells and longer times for solid tumor cells). Hypotonic treatment increases cell volume (*see* **Note 4**).

7. Cells are pelleted by centrifugation at 100*g* for 8–10 min. Cell swelling continues. Supernatant is aspirated off completely with care not to dislodge the pellet, and cells resuspended in 1–1.5 mL of ice-cold fixative (3:1 methanol:glacial acetic acid) and transferred with siliconized Pasteur pipets to 1.5–2.0-mL screw-cap vials. Subsequent fixative washes require a microcentrifuge with 1–2 min spins at 3000–4000*g*, which reduces cell loss. The fixative preserves the swollen cells, removes lipids from membranes, and denatures proteins. That is, the cell membrane can be readily disrupted.

3.2. Chromosome Spreading

1. Cells in fixative are diluted to a density that will allow reasonable numbers of noncontacting metaphase spreads in the microscope field (*see* **Note 5**). Phase-contrast observation of cell density is necessary at this point.

2. Precleaned glass slides are washed and rinsed in ddH$_2$O and maintained in ice-cold H$_2$O. The appropriately diluted cell suspension is drawn into the narrow barrel of a glass Pasteur pipet and a few spaced drops of cell suspension placed on the slide from a height of approx 30 cm.

3. Excess liquid is taken off the slide edges with a paper towel prior to placement on a 60–65°C hot plate to dry. The cell membrane is disrupted first in the mitotic cells, and chromosome spreading takes place early during the drying process (*see* **Note 6**).

3.3. Chromosome Staining

1. Light microscopic observations are generally made on chromosomes stained with 5% aqueous Giemsa solution for 10 min. Aceto-Orcein stain (0.2%) also readily provides low background visualization of chromosomes. For the latter, a green interference filter provides better contrast. Stains are rinsed off with PBS and chromosomes can be examined after placing a coverslip on a drop of PBS.

    For observation of many categories of asymmetric chromosomal aberrations, Giemsa or Aceto-Orcein staining is to be preferred. This is particularly true for interstitial deletions or double minutes.

2. If knowledge of individual chromosome involvement in aberration formation is desired, then Giemsa banding procedures can be employed. Here selective denaturation of chromosomal proteins with short trypsin (0.05%) or pepsin (0.005%) treatments followed by Giemsa staining, as described in **Subheading 3.3.1.**, result in a series of light and darker stained bands specific for individual human or rodent chromosomes (G-banding).

Until recently, this has been the approach of choice in clinical cytogenetics. G-banding is best carried out on slides that have been incubated in a dry oven overnight at 65°C (aging). It should be noted that the proteolytic digestion

step, which can be seconds to minutes, has to be closely monitored to ensure that fuzzy chromosomes do not result (*see* **Note 7**).

3.4. Fluorescent In Situ Hybridization (FISH)

Sensitive detection of specific nucleic acid sequences in metaphase chromosomes allows for their ready distinction at both the chromosomal and subchromosomal levels. Obtaining complementary probes by chromosome sorting and/or microdissection are not considered here, nor are modification of probes to allow for tagging with multiple fluorochromes. Whole and partial chromosome painting probes are available from a number of commercial suppliers (e.g., Vysis), as are the probe cocktails for karyotypic analyses. Gene-specific interest would justify in-house preparation of probes for chromosome-localization determination along with loss or gain of such sequences.

3.4.1. Protocol for Directly Fluorochromed Painting Probes

Slides with chromosomal spreads are dry-heat aged.

1. 4 h at 65°C.
2. Protease pretreatment: 0.0025% pepsin in 0.01 N HCl for 1–2 min. Rinse slide in PBS pH 8.0 to inactivate pepsin. Rinse in regular PBS, take through an ethanol series, 70, 90, and 100%—a few seconds in each—and air-dry.
3. Denaturation: The labeled probe is denatured at 73°C for 5 min. Chromosomes are denatured by layering 0.1–0.2 mL of 70% formamide/2X standard sodium citrate (SSC) (1X SSC is 0.15 M NaCl/0.015 M sodium citrate) on the slide, covering with a coverslip, and heating at 73°C for 5 min. This is followed by brief rinsing in 70, 90, and 100% ethanol and air-drying.
4. Hybridization: The probe, at manufacturers' recommendations, is mixed with 50% formamide/2X SSC, 7.5% dextran sulfate, and carrier DNA at 500 µg/mL. Slides are removed from 100% ethanol, excess fluid absorbed, and then placed on a hot plate at 45–50°C to evaporate the remainder. The probe mixture is layered on slides, coverslips of appropriate size are placed on top, and they are sealed with rubber cement. They are then incubated in a humid chamber at 37°C between 2 h and overnight. Slides are then washed with 50% formamide/2X SSC at 45°C three times, then with 2X SSC three times. Slides are then immersed in 0.1 M sodium bicarbonate and Nonidet P-40 buffer before draining and drying in the dark. A thin film (approx 40 µL) of fluorescence antifade solution is placed on the slide. This solution comes preprepared with the blue fluorescing DNA counterstain 4,6-diamidino-2-phenylindole (DAPI).
5. Microscopic observation: The moment of truth. Research-grade fluorescence microscopes from a number of manufacturers (e.g., Nikon, Olympus) can be fitted with filter cubes allowing for simultaneous discrimination between 2 or 3 differently absorbing and emitting fluorochromes. A filter cube fitted for the counterstain DAPI (excitation 367 nm, emission 452 nm) is essential. Other fluo-

rochromes can include spectrum orange (excitation 559 nm, emission 588 nm), spectrum green (excitation 509 nm, emission 538 nm), and spectrum aqua (excitation 433 nm, emission 480 nm). Fluorescent metaphases are observed and chromosomal changes recorded for the specific chromosome painted.

FISH is a relatively straightforward procedure usually yielding good fluorescent signals. However, low hybridization efficiency or low fluorescent signals after hybridization can be associated with poor chromosomal DNA denaturation, high background, and the presence of proteins that inhibit denaturation or hybridization. Individual laboratories have developed a number of protocol variants to cope with such problems, which can be cell type specific.

In general, application of FISH procedures best follows the recommendations of the probe suppliers, with adaptation as necessary. This is particularly true for the more intensive analyses of whole karyotype multicolor FISH and color bar-coded chromosomes.

3.5. Exponentially Growing Asynchronous Cells for Chromatid-Type Aberrations

Exponentially growing cells are subjected to sequential 1 or 2 h Colcemid treatments to accumulate mitotic cells from late G2 back into the cell cycle. All procedures that were described are sequentially applied.

The examination of chromatid-type aberrations is carried out almost exclusively using solid Giemsa staining of chromosomes. Reduced mitotic indexes as a consequence of cellular perturbation reflect G2 delays. The severity of such delays depends on the severity of the treatment.

Overall, observations of the type and frequencies of chromosomal changes at mitosis provide insight regarding the point in the cell cycle at which they are initiated and the controls and pathways that lead to continued cell cycle progression of altered cells.

4. Notes

1. Slides need to be fully wettable. Good-quality, precleaned frosted-end slides, such as Gold Seal (Becton Dickinson) and Super Frost (Menzel Glaser), can be used out of the box. If uncertain, slides can be washed with acetone, then HCl/ethanol, followed by multiple rinses in dH_2O.

2. The time for optimal yield of mitotic cells is dependent not only on the time over which spindle formation is repressed but also on any treatment-induced cell cycle-checkpoint activation. Highly toxic treatments can result in negligible frequencies of mitoses in any reasonable time frame. A rule of thumb for ionizing radiation treatments is 1–2 h delay per gray, up to 8–10 gy.

   There is also a need to strike a balance between the level of damage induced, which can be overwhelming at higher doses of clastogenic agents, and the information desired. Conversely, very low doses of clastogenic/cytostatic agents

require more time-consuming data gathering because of the lower frequency of induced damage that can be detected.

3. Mitoses from cells grown in T25 or T75 flasks can be physically dislodged by a sharp rap of the hand. A paddleball glove helps to prevent bruising. Some hamster cell lines can generate greater than 50% mitoses with prolonged Colcemid treatment. Normal human cells move through the cycle from G1 semisynchronously (baring G1 checkpoint activation), and mitotic yields of approx 20% of the cell population can be achieved.

4. Excess hypotonic treatment can cause cells to burst, whereas inadequate treatment will not allow good chromosome spreading.

5. Cell suspensions in the small microfuge tubes can be stored in fixative for years at <–20°C, which saves freezer space over 15-mL conical tubes.

6. Chromosomal spreads on slides can be stored in sealed boxes in a nitrogen atmosphere (to limit oxidation) or in Coplin jars with screw-top lids, in 100% ethanol at <20°C. Slides stored dry are fine for Giemsa staining procedures but if FISH is desired, slides are best stored in 100% ethanol.

7. Detecting chromosome-specific changes in G-banded chromosomes is labor intensive and requires trained observers because the differences between bands are relatively low contrast. However, the use of distinctive brightly colored probes (FISH) makes the detection of both symmetric and asymmetric aberrations more feasible for nonspecialists. It should be noted, however, that the multicolor FISH procedures for all individual chromosomes and for color bar-coded individual chromosomes require cocktails of probes and dedicated microscopes with image-analysis capabilities. However, the learning curve for *m*-FISH or *m*-BAND analyses is much shorter than for G-banding analyses. Further, these approaches allow for the recognition of a previously unrecorded level of complexity of chromosomal changes, which aid in defining the mechanisms of chromosomal aberration formation and, hence, of DNA damage-repair pathways and their associated checkpoint controls.

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Detecting the Influence of Cell Cycle Regulatory Proteins on Human Telomeres

Tej K. Pandita

1. Introduction

Maintenance of genome stability depends on an appropriate response to deoxyribonucleic acid (DNA) damage. This response is based on a complex network of signaling pathways that activate numerous processes and ultimately lead to damage repair and cellular survival or cell death. Thus, a relationship between telomeres and DNA-damage checkpoints seems inevitable. This is based on the fact that a gene responsible for ataxia telangiectasia (A-T), a master controller of cellular pathways and networks orchestrating the responses to DNA damage, influences telomere metabolism as well as the function of cell cycle regulatory proteins. In mitotic cells, ataxia telangiectasia mutant (ATM) is required for a DNA damage-dependent signal-transduction cascade that activates multiple cell cycle checkpoints. Interestingly, ATM and telomeres influence several common functions. When ATM is missing, several cellular processes are affected, and this results in a variety of disease phenotypes. Some of the common metabolic abnormalities, such as poor growth, have been linked to lack of ATM as well as loss of telomeres.

An increasing body of evidence supports an important role for ATM in regulating telomere metabolism. c-Abl tyrosine kinase, a downstream effector of ATM, associates with telomerase reverse transcriptase (hTERT) in human cell lines and regulates the activity of hTERT *(1)*. The activation of nuclear c-Abl tyrosine kinase by ionizing radiation (IR) requires a functional ATM *(2,3)*. IR induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism *(1)*. The functional significance of the interaction between c-Abl and hTERT was established by examining telomerase activity, which was inhibited

in cells expressing wild-type c-Abl compared to cells expressing a mutant version of c-Abl *(1)*. The role of c-Abl in the regulation of telomerase activity was strengthened by examining the early passage mouse embryo fibroblasts deficient in c-Abl. Such cells have a relatively high telomerase activity as well as long telomeres *(1)*. These findings are consistent with observations in the yeast *Saccharomyces cerevisiae*, wherein the Rap1p protein binds to telomeric DNA and negatively regulates telomere length *(4)*. The function of Rap1p in telomere regulation is mediated by Rap1-interacting factors, known as Rif1 and Rif2 *(5)*. Telomere repeat-binding proteins implicated in the regulation of telomere length have been identified in *Schizosaccharomyces pombe* (Taz1p) *(6)*, in human cells (hTRF1, Tin2) *(7,8)*, and in Chinese hamster cells (chTRF1) *(9)*. Such genes negatively regulate the telomerase activity by limiting the access of telomerase for the extension of G-overhangs and, thus, maintaining the length of the telomeres. Direct evidence for interaction of ATM with telomere-binding factors came from Kishi et al. *(10)* who demonstrated that ATM activated by DNA damage directly phosphorylates Pin2/TRF1 preferentially on SER-219. Interestingly, Kishi and Lu *(11)* demonstrated that inhibition of Pin2/TRF1 in A-T cells is able to bypass the requirement for ATM in restoring telomere length, the G2/M checkpoint defect, and radiosensitivity. These studies suggest that the primary function of ATM in telomere maintenance is to act on the telomeres rather than to activate the enzymatic activity of telomerase. This conclusion is further substantiated by the fact that the downstream effectors of ATM also influence telomere stability. For example, ATM phosphorylates p53 and thereby increases transcription of the cyclin-dependent kinase (Cdk) inhibitor *p21* and the Cdc2 sequester *14-3-3σ*. Dhar et al. *(12)* demonstrated that inactivation of the *14-3-3σ* gene in cells with telomerase activity influences telomere stability. Thus, evidence has been established for the correlation between a dysfunctional G2/M checkpoint control, genomic instability, and the loss of telomeres in human cells mediated by *14-3-3σ*, a gene that is a downstream effector of ATM *(12)*. Interestingly, another human cell cycle gene product Rad9 influences telomere stability (data unpublished). The inactivation of Rad9 specifically influences the G2/M checkpoint as Rad9-deficient cells have an unaltered mitotic index after IR treatment. These cells also have higher G2-type chromatid aberrations after IR treatment. Interestingly, inactivation of Rad9 influences the interactions of telomeres with the nuclear matrix, and such an alteration may influence the function of the telomeres. Although ATM is upstream in signal transduction and may be operating at least in part through telomeres, this chapter focuses on the protocols used to study the influence of cell cycle regulatory proteins on telomere metabolism.

2. Materials

Human cell lines commonly used for the study of telomere biology are HeLa, RKO, HT116, 293, and various others transformed by SV40 or EBV. Most of these cell lines have telomerase activity. Retrovirus preparation and infection of primary human fibroblasts with the hTERT expressing the LTRTNLlox vector, or with pBabepuro–hTERT, is done as described *(13)*. Ectopic expression of hTERT extends the life-span of several fibroblasts. For expression of the mutant version of the gene, standard cloning procedures are adopted. For stable transfection, the retroviral approach is most commonly used. For complete inactivation of the gene, both alleles are inactivated by a somatic knockout approach. For example, both alleles of *14-3-3σ* have been inactivated by somatic recombination. To detect the alteration in telomeres, a telomere-specific probe, TTAGGG, is used for both the Southern analysis and the *in situ* hybridization.

3. Methods

Telomeres are a substructure of all eukaryotic chromosomes and are essential for chromosome stability. The most essential function of telomeres is to prevent chromosomes from fusing with one another. They are involved in nuclear architecture, in chromosome localization, and in repression of the expression of adjacent genes. Human telomeres consist of thousands of base pairs of TTAGGG repeats and an unknown number of proteins. The telomeres of human somatic cells range greatly in length, depending on the type of tissue and the person's age. The TTAGGG repeat array of most human telomeres ranges in size from 5 to 15 kb. Biochemical studies have revealed that termini of human telomeres carry single-stranded TTAGGG repeats, called G-strand overhang, that appear to be present in all cells, irrespective of the presence of telomerase. The average size of the G-rich overhang is 130–210 bases in length. Disruption of the G-strand overhang perturbs telomere function and is therefore important for telomere stability. Because telomeric DNA is rich in G bases, the conformation of telomeric DNA may be different from that of B-DNA. Structural studies of G-rich oligonucleotides have proposed that such molecules can form four-stranded structures called G quartets. Some models for telomere functions have been drawn showing a linear configuration of telomeres at their ends. However, electron microscopic analysis of telomeres has revealed that the chromosome ends form a higher order structure called the T loop (the free 3' end of the G-overhang is tucked back inside the double-stranded DNA forming a T loop). T loops may protect telomeres by physically stitching the potentially vulnerable single-stranded G-strand terminus back into the double-stranded telomere sequence, several kilobases internal to the terminus. Unlike DNA double-stranded breaks, telomeric DNA does not activate DNA damage

checkpoint proteins, such as ATM and its downstream effectors. Several DNA repair-proteins have been found associated with telomeres, although they may not serve as substrates for repair enzymes because of their compaction in a special structure. Because the maintenance of telomeres is essential for long-term cell proliferation, it has been of utmost importance to determine the influence of cell cycle regulatory proteins on telomere stability. Several processes that coordinate to maintain telomeric structure are the protection of the telomere against recombination and degradation, the coordination of leading and lagging strands during DNA replication, and negative and positive regulation of telomerase. Some of the common telomere phenotypes that are influenced by cell cycle checkpoint regulators could be determined by analyzing telomere length, telomere signals, telomere interactions with the nuclear matrix, telomerase activity, karyotypic alterations, and G1 and G2 checkpoints. Protocols used to study the influence of cell cycle regulatory proteins on telomeres are described below.

3.1. Measurement of Mean Telomere Length

It has been proposed that programmed telomere shortening in normal human cells is considered a tumor-suppressor mechanism that limits the growth potential of premalignant cells. Several different mechanisms have been proposed for how telomere shortening may lead to senescence. Telomeres that are too short to mask their ends from the DNA damage-sensing machinery may signal a checkpoint arrest. This results in activation of the p53-dependent damage checkpoint, which induces growth arrest of the cells. Alternatively, silent senescence genes could become activated by the removal of heterochromatic regions. Two methods used to determine the size of telomeres are (a) Southern blot analysis and (b) *in situ* hybridization.

3.1.1. Determination of Terminal Restriction Fragment (TRF) Length by Southern Analysis (see **Note 1**)

1. DNA is digested with restriction enzymes (*Rsa*I and *Hinf*I) and is loaded on 0.8% agarose gels.
2. Gels are run in the presence of ethidium bromide at 2V/cm in TAE buffer (0.04 M Tris-acetate, pH 8.3, 1 mM ethylenediaminetetraacetic acid [EDTA]). Fractionated DNA is depurinated *in situ* by a 20-min incubation in 0.25 N HCl, subsequently denatured with 0.5 M NaOH, 1.5 M NaCl (2X for 20 min) and neutralized in 0.5 M Tris-HCl pH 7.5, 3 M NaCl.
3. DNA is transferred to a nylon membrane in 20X standard sodium citrate (SSC) overnight and baked at 80°C under vacuum for 2 h.
4. A 5'-end-labeled telomere-specific oligonucleotide $(TTAGGG)_5$ probe is used for hybridization.
5. The membrane is prehybridized for 1 h and then incubated in 5X SSC, 0.5% sodium dodecyl sulfate (SDS) at 48°C with ^{32}P-end-labeled $(TTAGGG)_5$ for 12 h.
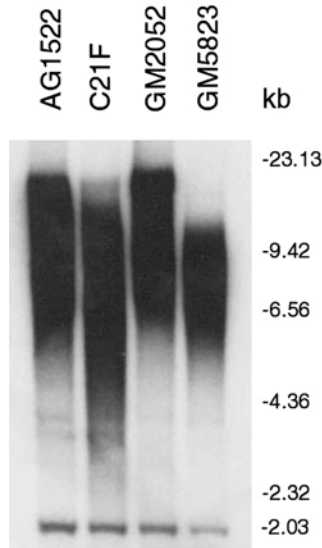
Fig. 1. Genomic blotting analysis to detect size of terminal restriction fragments (mean telomere length, TRF). AG1522 and C21F are primary fibroblasts derived from normal individuals. GM5823 and GM2052 are primary fibroblasts derived from individuals with A-T. DNA was obtained from passage 22, digested with *Rsa*I and *Hinf*I, and analyzed by Southern hybridization using a TTAGGG repeat probe. Molecular sizes are indicated at the right. Note that one of the A-T fibroblasts has a greater telomere length relative to normal fibroblasts.

6. The membrane is washed twice with 2X SSC at 48°C (each wash for 10 min) before exposure to Kodak XAR film for 1–2 d.
7. Mean TRF length is determined from densitometric analysis. The peak of telomere length in kilobases represents the average telomere length (**Fig. 1**).

Southern analysis cannot determine gain or loss of an individual chromosome end. To overcome this problem, quantitative *in situ* hybridization has helped to reveal the actual length of telomeric repeat arrays at individual chromosome ends. Detection of telomeres on metaphases is done by fluorescent *in situ* hybridization (FISH) using a telomere-specific probe (**Fig. 2**).

3.1.2. Detection of Telomeres by FISH Procedure (see **Note 2**)

1. Cultured cells are treated with Colcemid (0.1 μg/mL) for about 3 h and then treated with KCl (75 m*M*) for about 12 min at 37°C.
2. Cells are washed with a freshly prepared fixative of methanol:acetic acid (3:1). Fixed cells can be stored in fixative at 4°C for at least 3 mo.
3. Slides are precleaned, and about 25 μL of cell suspension is dropped on a slide and air-dried.
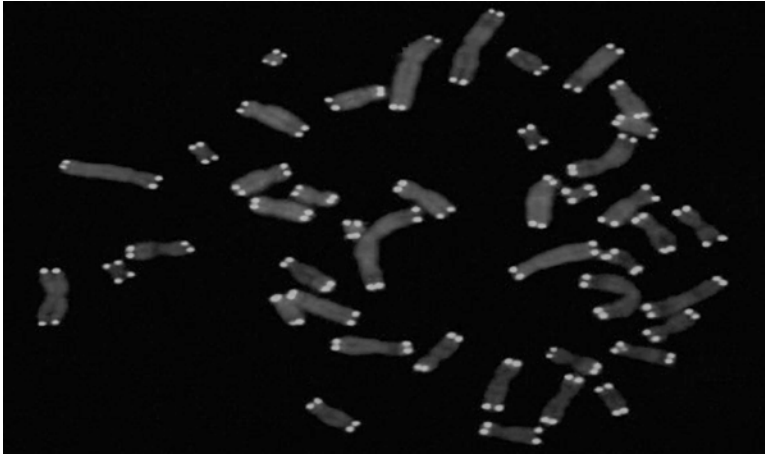
Fig. 2. Human metaphase showing telomeres as detected by fluorescent *in situ* hybridization using a telomeric probe. Telomeres are at the ends of each chromosome.

4. Cells on slides are rehydrated in 1X phosphate-buffered saline (PBS) (pH 7.0–7.5) for 15 min, fixed in 4% formaldehyde in PBS (pH 7.0–7.5) for 2 min, and washed three times in 1X PBS, each wash for 5 min.
5. Cells are treated with pepsin 1 mg/mL at 37°C for 10 min. (Pepsin should be prepared freshly in acidified H_2O, pH 2.) Cells are to be washed twice in PBS, each wash for 2 min.
6. Cells are fixed in 4% formaldehyde in PBS for 2 min, followed by three washes with PBS, each wash for 5 min.
7. Cells are dehydrated in 3 steps: first with 70% ethanol for 5 min, followed by 90% ethanol for 5 min, and 100% ethanol for 5 min. Slides are air-dried.
8. Preparation of hybridization mixture is done as follows:

Stock	Final Concentration	Volume in μL
Formamide (ultrapure, pH 7.0–7.5)	70%	175.0
Blocking reagent in maleic acid (2.5%)	0.25%	25.0
0.2 *M* Tris-HCl	10 m*M*	2.5
PNA Tel-Cy3 or FITC (stock 5 μg/mL)	0.5 μg/mL	25.0
MgCl$_2$ Buffer	5%	21.4
ddH$_2$O	as diluent	1.1

9. Hybridization mixture in a volume of 20 μL is applied carefully on slide and then a coverslip is placed on top.
10. Oven is set at 80°C. Slides are kept for 3 min at 80°C to denature the DNA. Slides are transferred to a humidified chamber at room temperature (RT) for 2 h for hybridization reaction.

11. Coverslip is removed carefully in a wash solution (70% formamide, 10 m*M* Tris-HCl, 0.1% bovine serum albumin (BSA), pH 7.0–7.5), and slides are washed with the same wash solution twice, each wash for 15 min.

12. Slides are again washed three times, each wash for 5 min, with a wash solution of 0.1 *M* Tris-HCl, 0.15 *M* NaCl, 0.08% Tween-20 (pH 7.0–7.5). Cells are dehydrated in three steps: 5 min each in 70% ethanol, 90% ethanol, and 100% ethanol. Slides are air-dried at RT.

13. A volume of 10 µL of Vectashield containing 200 ng of 4,6-diamidino 2-phenylindole (DAPI) is applied on the slide, which is then covered with a glass coverslip. The slide should be kept in a light-protected storage box. Detection of signal should be performed using an appropriate filter on a fluorescent microscope. Any image analysis program can be used to capture the images. Quantitation of individual telomere signals is done as described previously *(1)*.

3.2. Telomerase Activity Assay

Telomeres can be maintained by telomerase because the enzyme is capable of extending the 3' end of the G-rich strand of the telomeric repeats and continue lagging C-rich strand synthesis to complete the replication of chromosomal ends, thus compensating for the shortening of telomeres that otherwise occurs. An in vitro sensitive and simple technique termed *telomeric repeat amplification protocol* (TRAP) is used for the detection of telomerase activity (*see* **Note 3**). After the polymerase chain reaction (PCR) step, reaction products are analyzed either by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography, as described previously *(14,15)*, or by telomeric repeat amplification protocol–enzyme-linked immunosorbent assay (TRAP–ELISA) *(16)*. With the TRAP or TRAP–ELISA assay, telomerase activity has been detected in approx 85% of human cancers but not in most somatic cells, with a few exceptions that include proliferative stem cells. A TRAP–ELISA kit is commercially available from Roche Diagnostic, Germany (cat. no. 1854 666). The manufacturer has provided detailed instructions for the detection and quantitation of telomerase activity.

3.3. Chromosome-Specific Changes

To determine specific changes owing to the influence of cell cycle checkpoint proteins, spectral karyotyping (SKY) can be used to visualize chromosome translocations.

1. SKY analysis is carried out on chromosomal spreads of freshly dropped slides that are less than 2 mo in age. Slides are formalin fixed and denatured in 70% formamide in 2X SSC at 75°C for 2 min.

2. The SKY paints (ASI, Carlsbad, CA) are denatured, preannealed, and hybridized to the denatured chromosome DNA on slides for 48 h at 37°C. Posthybridization washes and detections are carried out according to the manufacturer's instructions (ASI, Carlsbad, CA).

3. Spectral images are acquired and analyzed with an SD 200 spectral bioimaging system (ASI, Migdal Haemek, Israel) attached to a Zeiss microscope (Axioplan 2; Carl Zeiss Canada).
4. The generation of a spectral image is achieved by acquiring approx 100 frames of the same image that differ from one another only in optical path. The images are stored in a computer for further analysis using the SkyView (version 1.2, ASI) software.
5. For every chromosomal region, the identity is determined by measuring the spectral emission at that point. Regions where sites for rearrangement or translocation between different chromosomes occurred are visualized by a change in the display color at the point of transition. Pseudocolor classifications are made to aid in the delineation of specific structural aberrations where the display color of different chromosomes may appear quite similar.

3.4. Assay for G1 and G2 Checkpoints by Examining Chromosome Aberrations

It is now well established that some of the genes capable of influencing checkpoint control also influence telomere stability (*see* **Note 4**). This is evident from studies on *14-3-3σ*, which influences both cell cycle checkpoints, and the telomere stability *(12)*.

1. To determine the G1 checkpoint, cells in plateau phase are irradiated with a dose ranging from 1 to 10 gy of γ-rays, incubated at 37°C for 24 h, subcultured, and then the metaphases are collected.
2. Chromosome spreads are prepared by a standard procedure and G1-type asymmetrical chromosome aberrations are scored. If the cells are defective in the G1 checkpoint, both chromatid- as well as chromosome-type aberrations are found.
3. Presence of higher chromosome aberrations is an indication of the G1-type defect as such cells did not repair damage and entered S phase with the DNA lesions.
4. The efficiency of G2 checkpoint control can be evaluated by comparing mitotic indexes and chromatid-type aberrations at metaphase after irradiation.
5. Chromosomal aberrations are assessed by counting chromatid breaks and gaps per metaphase.
6. Cells in exponential phase are irradiated with 1 or 2 gy of γ-rays, and metaphases are collected at different times postirradiation (0 to 120 min), then examined for chromatid breaks and gaps.

3.5. Determination of Telomere–Nuclear Matrix Associations (see *Notes 5 and 6*)

1. Cells in exponential phase are used to prepare nuclear matrix halos, which are isolated by removing histones and other loosely bound proteins. Cells are trypsinized, washed first with cold PBS, followed by a wash with cold cell wash buffer (CWB): 50 m*M* KCl, 0.5 m*M* EDTA, 0.05 m*M* spermidine, 0.05 m*M* spermine, 0.25 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.5% thiodiglycol,

5 mM Tris-HCl (pH 7.4), pelleted at 1000g for 5 min, and then suspended in 12 mL of CWB containing 0.1% digitonin (Boerhinger Mannheim). Cells are passed through a 20-gage needle, and lysis is monitored by phase-contrast microscopy.

2. The 2-mL suspension is loaded on 3 mL of a 10% glycerol cushion in CWB and is spun for 10 min at 800g. Nuclei are washed with CWB containing 0.1% digitonin, suspended in CWB, and with 0.1% digitonin and 0.5 mM CuSO$_4$, but without EDTA, and incubated for 20 min at 37°C. About 19 volumes of low ionic strength (LIS) solution (10 mM LIS, 100 mM lithium acetate, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, 20 mM HEPES-KOH (pH 7.4) are added, and the mixture is incubated for 10 min at RT.

3. Halos are collected by centrifugation for 10 min at 750g in a benchtop centrifuge and washed three times with matrix wash buffer (20 mM KCl, 70 mM NaCl, 10 mM MgCl$_2$, 10 mM Tris HCl pH 7.4) containing 0.1% digitonin. The nuclear halos are then washed with a restriction enzyme buffer. Halos (6 × 10^6) are cleaved with restriction enzyme *Sty*I and the nuclear matrices are pelleted by centrifugation.

4. To purify released DNA fragments that are attached to the nuclear matrix, both fractions are treated with proteinase K in a solution containing 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 7.4), and are incubated overnight at 37°C. DNA is purified and fractionated by agarose gel electrophoresis.

5. For Southern blot analysis, equal volumes from about 10^6 halos are fractionated on 0.8% agarose gels. Prior to DNA loading, ribonuclease (RNase) is added to a final concentration of 10 µg/mL. Fractionation of DNA, and transfer to a Hybond-N membrane, hybridization with a ^{32}P-labeled (TTAGGG)$_5$ probe are done as described in **Subheading 3.1.**

6. Quantitation and comparison of telomeric DNA, including total, released, and telomeric DNA fragments attached to the nuclear matrix, are achieved by phosphorimaging.

7. For biochemical characterization of telomere–nuclear matrix association, *Bgl*II digested halos from which the unattached (soluble fraction) DNA fragments are removed, are further incubated separately with different concentrations of RNase, proteinase K, SDS, Triton-X, and NaCl, and subsequently centrifuged to separate released *Bgl*II fragments from DNA that remained attached to the nuclear matrix.

4. Notes

1. To determine the influence of cell cycle checkpoint genes on telomere stability, it is important to determine first whether there is a gain or loss of telomere length, which can be examined by measuring TRF length by Southern analysis.

2. To assess the loss or gain of particular telomeres, telomere-specific *in situ* hybridization provides appropriate information.

3. To determine whether the change in telomere metabolism is owing to functional alteration of telomerase, a simple biochemical assay, TRAP-ELISA, can be used to determine telomerase activity.

4. Because there is a link between dysfunctional telomeres and loss of cell cycle checkpoints, the chromosomal assay for G1 and G2 checkpoints will provide the information on whether or not cell cycle regulatory proteins that influence chromosomal stability may be owing to alteration of telomere function.

5. It is possible that cell cycle regulatory proteins may influence the interaction of telomeres with the nuclear matrix and, thus, influence the accessibility of telomerase to telomeres for the elongation of the G-overhang. To determine the influence of cell cycle regulatory proteins on the nuclear matrix, or the interaction of telomeres with the nuclear matrix, biochemical assays described in **Subheading 3.5**. will yield information about the change in associations of telomeres with the nuclear matrix.

6. To determine the influence of cell cycle regulatory proteins on telomeres, it is important to determine telomere length, telomere structure, and telomerase activity.

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26

Monitoring Spindle Assembly and Disassembly in Yeast by Indirect Immunofluorescence

Rita K. Miller

1. Introduction

The budding yeast *Saccharomyces cerevisiae* has become a laboratory favorite for the study of several microtubule-dependent processes, including assembly of the mitotic spindle and spindle positioning. This is owing not only to the pliancy of its genetic system but also to the simplicity of its microtubule structures, which can be viewed by indirect immunofluorescence. *S. cerevisiae* contains two sets of microtubules, the intranuclear microtubules, which comprise the major element of the mitotic spindle, and the cytoplasmic (or astral) microtubules, which play an important role in positioning the mitotic spindle through interactions with the cortex *(1–5)*. In yeast, the spindle is assembled within the nucleus, as the nuclear envelope remains intact at all stages of the cell cycle *(6)*. Cytoplasmic microtubules are attached to the nucleus at the yeast's microtubule organizing center, or spindle-pole body (SPB), which is embedded in the nuclear envelope *(7,8)*.

Microtubules display a series of characteristic patterns at various stages of the cell cycle. With a little practice and a good microscope, the two sets of microtubules can be distinguished morphologically by indirect immunofluorescence with anti-tubulin (**Fig. 1**) *(9–11)*. In the unbudded G1 cell, one or more cytoplasmic microtubules emanate from the SPB, which is located at the center of the array (**Fig. 1**, G1). During early S phase and the formation of a small bud, the SPBs have duplicated but have not separated *(6,12)*, and a spindle is not yet discernible by immunofluorescence. At this stage, cytoplasmic microtubules can sometimes be seen projecting into the bud. However, because microtubules are dynamic structures, undergoing growth and shrinkage at their plus ends, the cytoplasmic microtubules can also be found entirely within the
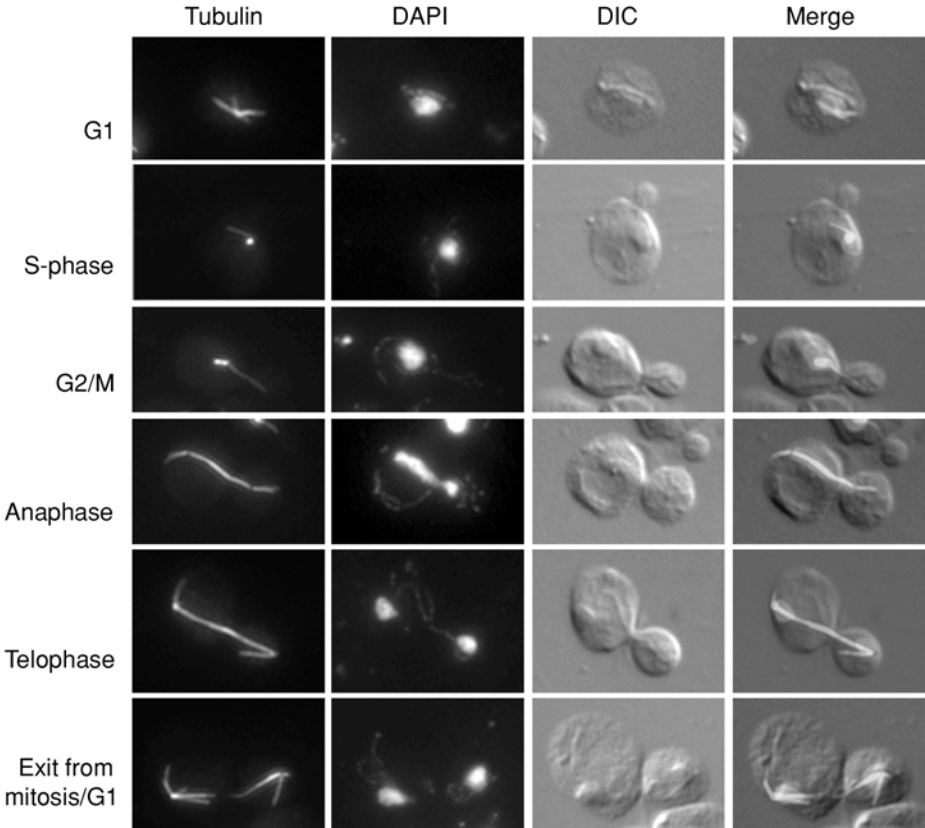
Fig. 1. Indirect immunofluorescence of microtubules. Wild-type cells were fixed and stained using rat anti-tubulin and goat anti-rat conjugated with FITC as described in the protocol. The position of the nucleus was detected using DAPI. Cells representative of the various cell cycle stages were selected.

mother cell, as shown in the S-phase panel of **Fig. 1**. In yeast, the mitotic spindle assembles rapidly near the end of S phase *(12,13)* and is seen as a short bipolar spindle associated with the 4,6-diamidino 2-phenylindole (DAPI) stained nuclear region. The short spindle (**Fig. 1**, G2/M) is characterized by a 1.5–2.0 µ*M* bar of immunofluorescence with a region of lower intensity at its center, thought to correspond to the two halves of the mitotic spindle. The break in fluorescence intensity is usually seen easily under the microscope, but is sometimes lost in published micrographs. Because the spindle contains multiple microtubules *(14)*, and the cytoplasmic microtubule "bundle" is likely to contain fewer microtubules, or perhaps even a single microtubule, cytoplasmic microtubules by immunofluorescence usually appear less intense than the

spindle. At this stage, the cytoplasmic microtubules extend at varying angles from the end of the spindle. With the formation of the short spindle, the spindle begins to be positioned and oriented toward the bud neck through interactions of the plus-ends of the cytoplasmic microtubule with the bud neck (data not shown) *(15,16)* and bud cortex (**Fig. 1**, *G2/M*) *(2, 3–5)*. At anaphase, the nucleus is translocated and elongated through the mother–bud neck *(17,18)*. Using immunofluorescence, the elongated spindle spans the length of the DAPI mass. The cytoplasmic microtubules extend at an angle. At telophase, the spindle reaches the full length of the mother–bud axis, and the two nuclear masses have separated. At this stage, the cytoplasmic microtubules are often seen as short extensions protruding at a distinct angle from the end of the spindle. At the end of mitosis, the spindle breaks down and the cells return to G1 (**Fig. 1**, *exit from mitosis/G1*).

Microtubules are dynamically unstable polymers and the images obtained of microtubules by indirect immunofluorescence represent an inherently different view of microtubule biology than live images of microtubules obtained using green fluorescent protein (GFP)-tubulin. Length measurements obtained by either method represent the average dynamicity of the microtubule, a measurement which is derived from a series of parameters that include the catastrophe frequencies, rescue frequencies, growth and shrinkage rates, and length of time spent in "pause" *(2,19)*. Using live microscopy, the parameter responsible for a difference in microtubule length can be deduced, whereas this information is not obtained from an indirect immunofluorescence image. In addition, live observations of GFP-tagged microtubules can reveal global patterns of microtubule movements such as "sweeping" and "sliding" of microtubules across the bud cortex that are not seen in static images *(20)*. Another apparent difference between time-lapse observations of microtubules in live cells and the appearance of microtubules in fixed cells is the number of cytoplasmic microtubules observed. Live-cell images usually show multiple dynamic cytoplasmic microtubules *(2)*. Fixed populations often show a single microtubule bundle. It may be possible that stable microtubules are uniquely retained during formaldehyde fixation.

Despite the several advantages of live microscopy, there are some important considerations in its use. Setting up a time-lapse video microscopy system requires a substantial financial investment, followed by a significant time commitment in collecting and analyzing live images. Indirect immunofluorescence is a relatively simple technique but, nevertheless, powerful. Although a "snapshot in time," indirect immunofluorescence can provide a wealth of information about microtubules, especially when combined with a cell cycle analysis. As such, this technique has led to numerous advances in our understanding of both nuclear and cytoplasmic microtubules.

This chapter describes an indirect immunofluorescence method designed for the visualization of microtubules in the yeast *S. cerevisiae*. Cells are fixed by treatment with formaldehyde. To allow antibodies access, the cell wall is removed by enzymatic digestion and postfixed with methanol and acetone. Primary antibodies directed against tubulin are bound to microtubules. The signal is enhanced and visualized with secondary antibodies conjugated with a fluorescent moiety. This protocol is a modification of that described previously *(21)*, which was adapted from *(9)* and *(10)*.

2. Materials

1. Anti-tubulin: YOL1/34 is a monoclonal tubulin antibody raised in rat. It is available as ascites fluid or culture supernatant. We have always used the culture supernatant form (Accurate Chemical and Scientific Corp., Westbury, NY, cat. no. YSRT-MCA 78S). Multiple freeze–thaw cycles have an adverse effect on most antibodies. On the initial thawing of the culture supernatant, we recommend aliquoting it 100–150 µL per tube and storing it frozen at –80°C.
2. Beta mercaptoethanol (Fischer Scientific, cat. no. BP176-100).
3. Coplin jars: two, tall enough to hold microscope slides (Fisher cat. no. 08-816).
4. Coverslips. (Corning, No. 1 weight, 22×50 m*M*, VWR cat. no. 48396-068).
5. DAPI. (4', 6-diamidino-2-phenylindole) (Accurate Chemical and Scientific Corp., Westbury, NY, cat. no. 18860). To prepare a stock solution, dissolve 1 mg/mL in H_2O. This can be stored at –20°C for several years.
6. Formaldehyde: 37% solution, available from a number of manufacturers (J. T. Baker, cat. no. 2106-01). Because formaldehyde can oxidize at RT into formic acid, we prefer to use smaller 500-mL bottles. They are more likely to be used up in a shorter time period.
7. Gel-loading pipet tips (Marsh Biomedical Products, Rochester, NY, cat. no. T3000).
8. Nail polish: The use of clear or colored nail polish is a matter of personal preference. We prefer colored polish because it is easily distinguished from the mounting media that may ooze from beneath the coverslip.
9. Microscope slides, Teflon coated (Polysciences, Warrington, PA, cat. no. 18357). We prefer the 10-well slides because we seem to get less leakage of liquid between the wells. However, 14-well slides can also be obtained. If the amount of antibody available is limiting, then slides with more wells may be desirable because the volume per well is less.
10. Mounting media: *p*-phenylenediamine (Sigma, cat. no. P6001) functions as an antibleaching agent. In a 15-mL plastic conical tube, solubilize 10 mg of *p*-phenylenediamine in 1 mL of phosphate-buffered saline (PBS) by vortexing. Bring the volume to 10 mL with glycerol. Mix well. This should be stored wrapped in tinfoil at –20°C. It should be discarded if it has turned light brown or honey colored or if it autofluoresces.
11. PBS: Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of H_2O, pH to 7.4 with HCl, and bring to a final volume of 1 L. This can be sterilized by autoclaving and stored at RT.

12. Poly-L-lysine: (molecular weight >300,000, Sigma cat. no. P-1524). To make a 0.5% stock solution, dissolve 50 mg in 10 mL of H_2O. Aliquot and store frozen at –20°C. Thawed aliquots may be refrozen.
13. Secondary antibody: goat anti-rat conjugated with fluorescein isothiocyanate (FITC) (Jackson Immunoresearch, West Grove, PA, cat. no. 112-096-003). Store as small aliquots at –80°C.
14. 1 *M* sorbitol/PBS: Dissolve 18.2 g of D-sorbitol (Sigma, cat. no. S-7547) in 100 mL PBS. This can be either filter sterilized or autoclaved. Store on a shelf at room temperature (RT).
15. Zymolyase 100T (ICN Pharmaceutical, Costa Mesa, CA, cat. no. 320932). The stock solution should be made fresh. Dissolve 10 mg/mL Zymolyase in 1 *M* sorbitol/PBS. Mix well. The Zymolyase will not dissolve completely. To remove the particulate material, centrifuge at 13,500*g* for 3 min. Use the supernatant.

3. Methods

3.1. Growth and Fixation of Cells

1. Grow a 10-mL culture of the yeast strain of interest to early exponential phase in the appropriate growth media (4×10^5–4×10^6 cells /mL) at 30°C. Yeast extract-peptone-dextrose (YPD) or synthetic complete media work equally well in this protocol.
2. Transfer cells to a plastic conical tube with a screw cap. This decreases potential odor leaks from the formaldehyde fixation step and reduces the risk of broken culture tubes.
3. In a fume hood, fix the cells by adding formaldehyde directly to each culture to a final concentration of 3.7%. Formaldehyde is usually obtained as a 37% liquid solution. Adding one-tenth of the culture volume as formaldehyde is sufficient. Return the cultures to the shaking incubator at 30°C for 2 h. (*see* **Notes 1** and **2**).
4. Wash the cells twice by collecting cells at 2000*g* in a tabletop centrifuge for 3 min. Discard the supernatant and resuspend the cells in PBS. Repeat. Cells can be stored overnight at 4°C at this step.
5. Collect the cells by centrifugation at 2000*g* for 3 min and resuspend the cell pellet in 5 mL of 1 *M* sorbitol/PBS. Collect the cells at 2000*g* for 3 min. Resuspend the cell pellet in 1 mL of 1 *M* sorbitol/PBS.

3.2. Digestion of Cells and Methanol-Acetone Fixation

1. Yeast cells adhere very poorly to untreated microscope slides. To circumvent this problem, microscope slides should be treated with poly-L-lysine. Add 10–15 µL of 0.5% poly-L-lysine to each well of a Teflon-coated microscope slide. Let the poly-L-lysine drop evaporate. A hazy film will form on the slide. Wash the slide by soaking in a beaker of dH_2O for 15 min. Swish the slide back and forth once or twice. Let the slide air-dry. Slides can be prepared several hours in advance.
2. At least 2 to 3 h before they are needed, chill two Coplin jars at –20°C, one containing 100% methanol, and the other 100% acetone. Measure the level of each solvent so that it does not extend above the frosted end of the microscope

Fig. 2. Coplin jars used for postfixation and permeabilization.

slide reserved for writing. The ink of many common laboratory "sharpies" is solubilized by methanol. The "VWR Lab Marker" is alcohol resistant, but smearing of the labeling can still occur.

3. Yeast cells have cell walls that must be digested in order for antibodies to gain access to the interior of the cell. This is accomplished by digestion with Zymolyase, an enzyme that hydrolyzes β-1, 3-glucan linkages of the glucose polymer within the yeast cell wall.

   Add 5 µL of β-mercaptoethanol to the resuspended cells. Add 30 µL of Zymolyase stock (10 mg/mL) at 30°C for 30–90 min. The optimal digestion can be determined by examining the cells under a phase-contrast microscope. Properly digested cells will appear medium to dark gray. Bright, glowing cells are incompletely digested. The pale, translucent cells are overdigested and appear to have lost their internal organelles. These result in poor-quality immunofluorescence. It is helpful to save a 25-µL aliquot of undigested cells for comparison of digested vs undigested cells. Collect the resulting spheroplasts in a tabletop centrifuge at 2000*g* for 3 min and discard the supernatant (*see* **Notes 3** and **4**).

4. Wash the cells by resuspending the pellet in 5 mL of 1 *M* sorbitol/PBS and centrifuging at 2000*g* for 3 min. Resuspend the cells in 1 mL of 1 *M* sorbitol/PBS.

5. Attach the cells to the microscope slide by applying 15 µL of digested cells to each well of a Teflon-coated microscope slide. Let the cells adhere for 10–15 min.

6. Working quickly, pipet off the cells and immerse the slide in methanol (–20°C) for 6 min, as prepared in **step 2** (**Fig. 2**). Remove the slide and very rapidly drain

Fig. 3. Washing the slides with PBS. Work quickly so that the wells do not dry out.

the excess methanol from the back of the slide by laying it on a Kimwipe for 1 s or less. Immediately immerse the slide in acetone (–20°C) for 30 s. Remove the slide and allow it to air-dry. (*see* **Notes 5** and **6**).

3.3. Antibodies

1. Centrifuge the YOL1/34 monoclonal tubulin antibody immediately prior to use at 13,500*g* in a microcentrifuge in the cold for 2 min (*see* **Note 7**).
2. Apply 20–25 µL of the tubulin antibody per well of the microscope slide. We prefer to use this antibody undiluted; however, we have also had success diluting it 1:1 with PBS. Incubate overnight at 4°C (*see* **Notes 8** and **9**). From this point on, it is very important to not let the slides dry out.

   To prevent the antibody from evaporating, incubations should be carried out in a humid chamber. This can be created by wetting several layers of Whatman filter paper or paper towels that have been cut to fit the inverted lid of a Petri dish (**Fig. 3**). Cover the microscope slide and wet blotting paper with the inverted Petri dish.
3. Gently wash the cells four times with PBS by aspirating the antibody and quickly replacing the liquid with a drop of PBS. We use gel-loading pipet tips for the washing steps because they are extra long and narrow. This reduces the force of the flow from the washes, resulting in fewer cells being washed off the slide.
4. Dilute the secondary antibody, in this case goat anti-rat conjugated with FITC, 1:200 in PBS. Perform a clarifying spin at 13,500*g* for 2 min. Remove the last

PBS wash from the slide and apply 20–25 μL of diluted secondary antibody to each well. Incubate for 3–4 h at RT (*see* **Notes 9** and **10**).

5. Remove the secondary antibody and wash the cells four times with PBS, as described in **step 3**.

6. In studying the position of the mitotic spindle, it is useful to identify the position of the nucleus. This is easily done using DAPI, a dye that intercalates into deoxyribonucleic acid (DNA) and fluoresces in the blue range. Thaw the stock solution of DAPI and dilute it 1:1000 in PBS. Perform a clarifying spin at 13,500*g* for 2 min. Apply 20–25 μL to each well and incubate at RT for 15 min. Wash the cells twice with PBS (*see* **Note 11**).

7. The slides are now ready for mounting. Pipet off the excess PBS and apply one drop of mounting media to approximately every other well of the slide. A wooden applicator stick works well for this step. Carefully lay a coverslip over the wells. Using a dull object, such as the reverse end of a yellow pipet tip, squeeze out the excess mounting media. Do not allow bubbles to form. Carefully wipe away the excess mounting media. Seal the edges of the coverslip/slide with nail polish and allow it to dry thoroughly. Rinse any extra mounting media from the surface of the coverslip with cool tap H_2O (*see* **Note 12**).

3.4. Microscopy

The cells are now ready to be viewed under a microscope equipped with fluorescence optics. Most yeast work is done using a ×100 oil objective with a 1.3 or 1.4 numerical aperture (NA). The cells shown in **Fig. 1** were photographed using a Leica DMRA2 fluorescence microscope equipped with a Plan Apo iris lens (1.4-0.7 NA), differential interference contrast optics, and an ORCA-ER CCD camera (Hamamatsu Corp., Hamamatsu City, Japan), also known as a charged-coupled device, which was controlled by Openlab software (Improvision, Inc., Lexington, MA). Image processing of contrast, brightness, and image overlays was performed using Openlab and Phylum-for-free software (Improvision, Inc. Lexington, MA).

3.5. Controls

Indirect immunofluorescence experiments are usually conducted with at least two controls, a no primary antibody control and a no antigen control.

1. For the no primary antibody control, simply omit the primary antibody from one well and add PBS in its place during that incubation period. This controls for the possibility that the observed signal could be owing to an unexpected cross reactivity of the secondary antibody.

2. The no antigen control can take two forms. Yeast strains are now available from Research Genetics, Inc. and Open Biosystems Inc., in which each open reading frame has been deleted. This is an important control that can be used when the protein of interest is nonessential for life. When epitope-tagged proteins (e.g., V5, HA, or myc) are used, the yeast strain containing a non-epitope-tagged version of the protein serves as the no antigen control.

3.6. Preabsorption of Antibodies

For indirect immunofluorescence and other immunologically based applications, the use of monoclonal or affinity-purified antibodies is usually preferred. However, there are instances in which this may not be feasible because the necessary antigen required for purification is not available. In which case preabsorption of the antibodies against a yeast strain lacking the antigen of interest can be a viable alternative, provided the necessary controls with deletion strains are carried out. Improved results for immunofluorescence have been seen when both the primary and secondary antibodies have been preabsorbed against an "antigen-minus" strain (*see* **Note 13**).

This preabsorption protocol has been used successfully on rabbit polyclonal antibodies. Rabbits can often have high background reactivity against yeast lysates because rabbits are susceptible to yeast infections.

1. Grow to saturation a 150–200 mL culture of a yeast strain that lacks the antigen of interest.
2. Fix the cells by the same protocol used for indirect immunofluorescence. In this case, 2 h with formaldehyde and then digest with Zymolyase, as described above in **Subheadings 3.1** and **3.2.** Wash the digested spheroplasts twice in PBS. Resuspend the pellet in approx 3 mL PBS.
3. In microfuge tubes, collect three loose pellets of spheroplasted cells each with a volume of about 150 µL. Use a gentle centrifugation pulse at 850g for 10–20 s. Remove the supernatant. If necessary, add additional spheroplast suspension and repeat the spin to generate a volume of approx 150 µL. Remove the supernatant.
4. Dilute the antibody 1:5 in PBS. More or less dilution may be required depending on conditions.
5. Apply 150 µL of diluted antibody to the first pellet. Gently mix by inverting the tube. Incubate 1 h at RT or overnight at 4°C. Invert the tube occasionally to remix the settled cells. Store the second and third tubes of cells on ice.
6. To compact the pellet to its smallest possible volume, spin the cells at 13,500g for 10–15 min. The pellet of cells will be much smaller, approx 60 µL. Transfer the supernatant to the second Eppendorf tube containing a loose pellet (150 µL) of spheroplast cells. Mix gently. Incubate at RT for 1 h. Invert the tube occasionally.
7. Compact the cells by centrifugation at 13,500g for 10–15 min. Transfer the supernatant to the third Eppendorf tube containing a loose pellet (150 µL) of spheroplast cells. Mix gently. Incubate at RT for 1 h. Invert the tube occasionally.
8. Compact the cells by centrifugation at 13,500g for 10–15 min. Transfer the supernatant to a fresh Eppendorf tube. Perform a clarifying spin at 13,500g for 2 min. Transfer supernatant to a new Eppendorf tube. This volume will be significantly larger than the original 150 µL applied to the first pellet. Calculate the dilution of antibody, typically 2–4X. The antibody is now ready to apply to fixed cells on a slide for indirect immunofluorescence.

4. Notes

1. Formaldehyde is a strong irritant and a carcinogen. Care should be taken to avoid inhalation or contact with the skin. Personal protective equipment (gloves, safety glasses, and lab coat) should be worn. Undiluted formaldehyde should be used in a fume hood. Some universities may require formaldehyde safety training prior to use.

2. For fixation of cold-sensitive strains, we have found that formaldehyde fixation at 18°C for 3–4 h works well.

3. During the enzymatic digestion, 1 M sorbitol in PBS provides osmotic stability to cells that now lack walls. The importance of this can be observed under a microscope. If the osmolarity is inadvertently too high during digestion, the cells will shrink in size. If distilled H_2O is then added back under the microscope coverslip, the cells will quickly swell and burst. Although this can be an amusing aside in demonstrations for high school students, it is neither amusing nor beneficial if applied to one's entire experiment.

4. Once the cell wall has been digested, the resulting spheroplasts are especially fragile. Care should be taken to resuspend and handle them gently. Never vortex cells intended for immunofluorescence. Instead, resuspend them by gentle pipetting.

5. Do not forget to remove the Coplin jars of methanol and acetone from a non-explosion-proof freezer. Otherwise, safety citations can be issued by the appropriate regulatory authorities. If necessary, it is also possible to chill the methanol and acetone in an ice bucket containing a mixture of dry ice and wet ice.

6. If there is a problem with too few cells sticking to the slide, one can determine whether it is occurring at this step by examining the slide under a microscope using a low-power lens. Exercise care to make sure that the lens does not inadvertently contact any residual liquid and/or the cells.

7. For any indirect immunofluorescence application, a clarification spin of both the primary and secondary antibodies can greatly improve background that results from precipitated or tiny aggregates of antibodies.

8. The optimal times of incubation for immunofluorescence can vary depending on the antibody, ranging from 30 min at 37°C to overnight at 4°C. With the YOL1/34 antibody, we have had consistently good results with no background using an overnight incubation at 4°C. Immunofluorescence with other antibodies may require empirical determination for optimal incubation times.

9. For double-label indirect immunofluorescence, application of the two primary antibodies serially is usually successful. Then the two secondary antibodies can be mixed and applied simultaneously to the cells.

10. Secondary antibodies from several other manufacturers can be used successfully. The dilutions for these can be determined empirically.

    If the background is high, we have found that decreasing the incubation time for the secondary antibody often alleviates the problem. Too high a concentration of secondary antibody also contributes to high background. To assure that the secondary antibody concentration is optimal, it is often beneficial to try a range of dilutions the first time that one uses a new secondary antibody.

11. DAPI staining of nuclear DNA appears as a very bright and large staining mass. In addition, DAPI also stains mitochondrial DNA. This staining is usually lighter and punctuate and can occasionally be seen in rows (*see* **Fig. 1**, anaphase stage/ DAPI panel). Distinguishing the two types of DNA staining is usually not a problem.
12. The cytostructure viewed by DIC optics and immunofluorescence is usually best if viewed soon after the nail polish dries. However, excellent results have also been obtained from slides that have been stored overnight at 4°C in the dark (to prevent bleaching of the fluorophore). For longer periods, slides can be stored in the dark at –20°C with only a minimal loss of signal. Extra care should be taken to be sure the nail polish has dried before viewing the slides; removing nail polish from a lens is a very difficult task and could potentially damage the lens.
13. It is not necessary to preabsorb the rat anti-tubulin or the secondary from Jackson Immunoresearch used in this indirect immunofluorescence protocol. Both provide excellent results without preabsorption.

Acknowledgments

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