1

Basic Cell Culture

Jeffrey W. Pollard

1. Introduction

This article describes the basic techniques required for successful cell culture. It also acts to introduce some of the other chapters in this volume. It is not intended to describe the establishment of a tissue culture laboratory, nor to provide a historical or theoretical survey of cell culture. There are several books that adequately cover these areas, including the now somewhat dated, but still valuable volume by Paul *(1)*, that of Freshney *(2)*, and the multi-authored volumes edited by Jakoby and Pastan *(3)*, Davis *(4)*, and Celis *(5)*. Instead, this chapter focuses on the techniques for establishing primary rodent cell cultures from embryos and adult skin, maintaining and subculturing these fibroblasts and their transformed derivatives, and the isolation of genetically pure strains. The cells described are all derived from Chinese hamsters since to date, these cells have proved to be the most useful for somatic cell genetics *(6,7)*. The techniques, however, are generally applicable to most fibroblast cell types.

I only discuss growing fibroblastic cells in semidefined media. A very detailed consideration of serum-free culture and the maintenance of epithelial cells can be found in Chapter 4. Methods for culturing many other non-fibroblastic cell types are described in Chapters 2 through 24.

2. Materials

1. Alpha minimum essential medium (α-MEM) containing penicillin and streptomycin: for economy, we buy prepared medium as powder in 20-L aliquots. A 44-g quantity of sodium bicarbonate is added, the powder is made up to 20 L in deionized distilled water, the pH adjusted to 7 4, and the media sterile-filtered through a 0.22-μ*M* filter using a pressure vessel coupled to a filtration apparatus and driven by a pressurized 95.5% air CO_2 gas mix This gas mix maintains pH

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

on preparation and storage The 500-mL bottles are stored at 4°C in the dark until use (*see* Notes 1 and 2). Prepared media can also be purchased from many suppliers.

2 Growth medium: α-MEM plus 15 or 7 5% (v/v) fetal calf serum This is made up as required and stored at 4°C.

3. Fetal calf serum (FCS) should be pretested to ensure it supports optimal growth (*see* Section 3.6.). It can also be heated at 56°C for 30 min to destroy complement if it is to be used for cultures where the presence of complement can cause complications. Sera should be aliquoted and stored at –20°C

4 $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (Dulbecco's PBS). 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 2.16 g/L Na_2HPO_4 $7H_2O$), pH 7 2.

5 PBS citrate· PBS + sodium citrate at 5.88 g/L.

6. Trypsin· One vial of lyophilized Difco (Detroit, MI) Bacto-trypsin in 400 mL of PBS citrate (0 125% trypsin) or 10 times this concentration for the isolation of embryonic fibroblasts (*see* Note 3)

7 Counting fluid: PBS + 0 2% (v/v) FCS.

8. Formalin fixative: 10% (v/v) commercial formaldehyde (comes as a 40% [v/v] solution).

9. Methylene blue stain: 0.1% (w/v) methylene blue in distilled water filtered through a Whatman No. 1 filter.

10 Trypan blue: 0.5% (w/v) in PBS

11 Colcemid: 10 μg/mL, store at 4°C

12. Karyotype fix· Methanol acetic acid (3:1) made up on the day of use and kept on ice in a tightly stoppered bottle

13 Giemsa stain: Use commercial Giemsa concentrate diluted 3.47 parts in commercial Gurr's buffer (one tablet to 1 L distilled water). Alternatively, 10 m*M* potassium phosphate, pH 6.8, can be used as the buffer. The diluted stain is only stable for 2–3 mo.

3. Methods

3.1. Establishment of Primary Chinese Hamster Fibroblast Cultures

3.1.1. Embryo Culture

1. Kill a 12-d old pregnant Chinese hamster with ether.
2 Wash the animal in tap water and then with 70% ethanol
3. Make a surgical incision on the dorsal side to expose the uterus using sterile instruments (these can be dipped in ethanol and flamed to maintain sterility during the operation)
4 Remove the uterus *in toto*, and transfer it to a sterile Petri dish. Dissect the embryos, and place them in a new sterile Petri dish (*see* Note 4).
5. Mince the embryos very finely, and while still in the Petri dish, wash the pieces with 5 mL of 0.125% Bacto-trypsin at 37°C.
6. Tilt the Petri dish so that embryo pieces go to the side Remove the pieces into a 50-mL centrifuge tube using a wide-bore pipet.

7. Add 40 mL of fresh 1.25% Bacto-trypsin, and incubate at 37°C for 5 min
8 Regain the embryo pieces by centrifugation at 100*g* for 3 5 min, and discard the supernatant.
9 Resuspend the pieces in 40 mL of fresh 0 125% Bacto-trypsin, and incubate at 37°C for 25 min (this can be performed in a roller apparatus).
10. Neutralize the trypsin with 4 mL of FCS
11. Deposit the supernatant through a 100-μm sterile mesh into another centrifuge tube.
12 Centrifuge the supernatant for 5 min at 300*g* at room temperature
13 Resuspend the pellet in 10 mL α-MEM plus 15% FCS, and count the cells in a hemocytometer (*see* Section 3.3.) at about 1/100 dilution.
14 Lay down 1.5×10^7cells in 40 mL of a-MEM plus 15% FCS into a 75 cm^2 flask, and place it in a 37°C tissue-culture incubator
15 The next day, replace the medium with an equal volume of α-MEM plus 15% FCS.
16 Forty-eight to 72 h later, the monolayer should be confluent, and at this point, the cells are ready for subculture. This is performed by incubating the monolayer with 4.5 mL of 0.125% Bacto-trypsin at 37°C until the cells detach. Cell detachment can be visualized either by observing the cell monolayer in oblique light or directly under the microscope. When the cells have detached (~80%), add 0.5 mL FCS (10%), pipet up and down five times, and transfer contents to a 15 mL centrifuge tube.
17. Centrifuge the cells at 300*g* for 3.5 min at room temperature.
18. Remove the supernatant, resuspend the cell pellet in 5 mL α-MEM plus 15% FCS, and determine the cell concentration.
19 Resuspend the cells at 4×10^6/vial in α-MEM plus 15% FCS plus 10% (v/v) sterile dimethyl sulfoxide (DMSO), and freeze at –135 or –176°C The cells will remain viable for several years
20. The cells may also be subcultured at one-third to one-tenth dilutions They have doubling times of approx 36 h At this point, start to calculate the number of mean population doublings by keeping careful records of subculture number and split ratio (*see* Chapter 3 for details).

3.1.2. Skin Fibroblasts

See Chapter 2 of this volume for human explants.

1. Kill and wash an animal as described for the isolation of embryonic fibroblasts (*see* Section 3 1.1., steps 1–3). In fact, it is often convenient to prepare skin fibroblasts from the same animal as the one from which the embryos were obtained.
2. Cut small pieces (1–2 mm^2) of dermis from the exposed skin flaps using sterile instruments, avoiding any fur.
3. Place several (5–10) small pieces (*see* Note 4) into a 25-cm^2 flask, and allow them to adhere for 30 min in a very thin film of medium (0.5 mL) at 37°C.
4. Once adhered, add 5 mL of growth medium to the opposite surface (i.e., top surface) of the flask to avoid washing off the skin pieces. Place the flask in the incubator in the upside-down position for 24 h (the surface tension holds a thin film of medium to the upper surface and stick the explants to the flask surface).

5 Once the explants are firmly stuck, gently invert the flask and return to the incubator
6 The next day, it is often advisable to change the medium to remove any debris and unattached explants.
7. After several days, first "epithelial" type cells and then fibroblast will grow out of the explants (*see* Chapter 2 for details) Let this process continue until most of the surface is covered with fibroblasts or until obvious necrosis is observed in the explant. It may be necessary to change the medium every week until substantial outgrowth is observed.
8. Remove the explanted material (*see* Note 5) with a Pasteur pipet attached to a vacuum line leaving the adherent fibroblasts
9 At this stage, depending on the density, the fibroblasts can either be trypsinized (>50% confluent) or allowed to continue to grow to form a monolayer before they are trypsinized, subcultured, and frozen as described in Section 3 1 1

3.2. Maintenance and Subculture of Transformed Cell Lines

Many transformed cell lines will grow both as monolayers and in suspension culture. The CHO-S cell line is one such line having been selected for suspension growth by Thompson from the original K1 CHO cell line isolated by Puck (*see* Note 6, ref. *7*). Because CHO cells are transformed, they do not require as much serum as normal diploid fibroblasts, and we routinely culture them in 7.5% (v/v) FCS. Despite the relative ease with which transformed cells can be cultured, however, unlike normal diploid fibroblasts, they do not enter a stationary phase of long-term viability *(8,9)*. In this phase, they rapidly lose viability, and therefore must be subcultured during the exponential phase of growth and cannot be maintained as arrested cultures in reduced serum

1 CHO cells are stored frozen at ~4 × 10^6 cells/mL at –135 or at –176°C (liquid nitrogen) in growth medium containing 7 5% FCS and 10% (v/v) DMSO A single vial is removed from the frozen stock, rapidly defrosted in a 37°C water bath, and the cells regained by centrifugation at 300*g* at room temperature for 3.5 min
2 The supernatant is discarded and the pellet resuspended in 1 mL of prewarmed medium and placed into a 25-cm^2 flask or a 60-mm diameter dish containing 4 mL of growth medium
3 Approximately 2 d later, the cells should be almost confluent and ready for subculture (*see* Note 7) They are trypsinized as described for the primary diploid fibroblasts. After cell detachment, FCS is added to 10% and the cells resuspended as single cells by pipeting up and down about five times with a 5 mL pipet An aliquot of this cell suspension (up to a total of 10% of the recipient volume of the medium) can be added directly to a new tissue culture vessel containing growth medium and returned to the incubator until the next subculture Alternatively, the cells may be regained by centrifugation, resuspended, and the concentration/mL determined (*see* Section 3.3). Known concentrations of cells may then be subcultured by appropriate dilution. In a 25-cm^2 flask with 5 mL of growth medium,

CHO cells should yield about 2 5 × 10^5/cm^2 but yields are variable depending on serum batch and media used

4 At this stage, cells maybe transferred to a magnetically stirred spinner flask (commercially available) containing pregassed (95% air/5% CO_2) growth medium; usually a 250-mL spinner flask is seeded to give a density of ~8 × 10^4 cells/mL. These flasks are then placed in a warm room or in a temperature-regulated water bath (Heto), and stirred at 100 rpm (full details of spinner culture and scale up are described in Chapter 5, *see also* Note 8) CHO cells grown in suspension should give ~10^6 cells/mL at saturation density, at which point the medium will be very yellow (acid).

3.3. Determination of Cell Number

This can be performed either using an electronic particle counter (e.g., Coulter Electronics Inc.) or a hemocytometer. The former is the more accurate and can be used to count low concentrations of cells (~10^3 cells/mL); the latter requires higher density and is more prone to sampling error, but allows a visual estimation of the "health" of the cells and, combined with Trypan blue exclusion, can be used to estimate cell viability.

1 Resuspend cells to give a uniform cell suspension by pipeting up and down against the side of the plastic centrifuge tube
2 If the cells have been trypsinized, as described in Section 3 1 1 , 0 2 mL of the cell suspension to 7.8 mL of counting fluid in a 15-mL Falcon snap-cap tube will give a statistically reliable cell count (1000–14,000 particles/0 5 mL counted). Count three aliquots with the Coulter counter set to count 0 5 mL, sum the three counts, divide by 3, and multiply by 40 (for dilution) and 2 to calculate the cells/mL (*see* Note 9)
3 The cells can then be appropriately diluted for the experimental setup or subculture
4 Alternatively, the cells can be counted on a hemocytometer. The cells need to be resuspended at 3–5 × 10^5 cells/mL A drop of a cell suspension is added to either side of the hemocytometer, taking care not to overfill it and making sure that the coverslip is firmly in place
5. Each large square on the hemocytometer (improved Neubauer type) gives an area of 1 mm^2 and a depth of 0.1 mm (i e , the volume is 10^{-4} mL). Count the cells in the square (usually using the one bounded on each side with triple lines) on either side of the counter, average the counts, and divide by 2 and multiply by 10^4 to give the number of cells/mL If there are too many cells (>1000), just count the 5 diagonal squares and multiply by 5 to give the number to be multiplied by 10^4 If there are too few cells, count more than one complete square on each side of the chamber, and divide the total cell number accordingly.
6 This procedure can also be used to determine cell viability, since prior to placing the cells in the hemocytometer, they can be diluted 1 1 with 0 5% Trypan blue

The number of cells that can then exclude the stain (i e , have intact cell membrane) can be determined by counting the cells as described in steps 3–5

3.4. Isolation of Genetically Pure Cell Lines

The isolation of somatic cell mutants is outside the scope of this chapter, and the reader is referred to Thompson *(10)* for the considerations necessary to isolate such mutants successfully. All cell lines will genetically alter over time, however, and periodically the parental type will need to be purified from variants or revertants. The easiest way to do this is to isolate a single clone. This causes some potential problems, however, since a clone may itself be a variant, and thus several clones will need to be isolated and tested to ensure the phenotype selected is the required one. To overcome this problem of clonal variability, it is usually better to contract the cell population to about 100 cells and then expand this to the mass culture. This contraction should statistically remove any variants from the population. It is worth remembering, however, that any variant that has a growth advantage over the parental type will soon overgrow the whole culture. Once a mass culture is obtained, it should be frozen in a large number of vials (20–50) to provide a base for future experiments. This enables the investigator to grow a culture for approx 3 mo before discarding it, and then to return to the frozen stock for the next set of experiments. This protocol reduces the genetic drift in the culture and avoids the necessity of frequent genetic purification using the following methods.

1 Trypsinize a culture, recover the cells, and determine the cell number as described in Section 3 1 1
2 Dilute to 2.5 cells/mL with 20 mL of growth medium.
3 Plate out 0 2 mL/well into a 96-well tissue-culture plate
4. Incubate plates at 37°C in an humidified incubator for 10–12 d Do not move or disturb the plates, mitotic cells will float off and form satellite colonies.
5 Examine every well with a microscope, and ring those that have a single clone. These may be pure clones but a second cloning ensures that you end up with populations derived from a single cell
6. Trypsinize two to three of these individual clones with 0.2 mL of trypsin and, once detached, transfer the well's contents into 4 mL of growth medium in a snapcap tube.
7. Pipet this up and down to ensure a single cell suspension, and then plate it again at one-tenth serial dilutions (i e., 0.4–3 6 mL) and 0 2 mL/well into a 96-well tissue-culture dish (*see* Note 10).
8. Return these new plates to the incubators Add medium from a different batch to the trypsinized wells of the old plates, and also return this to the incubator This provides a backup in case the new plates are contaminated Again, do not move the plates.

9. After 10–12 d, select individual clones in the new plates, and expand them up to mass culture (remember to always keep a backup culture).
10. Freeze a large stock (20–50 vials) as described in Section 3.1 1 , since at this stage, you will have a genetically pure line (except for the mutations that may have occurred during the clone's expansion). Split the frozen stock between a liquid N_2 store (long-term) and a –70 or –35°C store (short-term experimental stock)
11 Alternatively, the mass culture that needs to be genetically cleansed can be plated into 60-mm dishes containing 5 mL growth at 100 cells/dish
12 Leave these to grow for approx 10 d. Trypsinize the ~100 clones from each plate and expand them together to a mass culture in the normal way.
13 Freeze 20–50 vials of these cultures as described in step 10.

3.5. Karyotyping

It is often desirable to karyotype your cells. Full details for banding and identifying karyotypes are given in Chapter 27. This chapter, therefore, deals with a simple method, derived from Deaven and Petersen *(11)* for producing karyotypes of Chinese hamster cells.

1 A culture growing in the exponential phase of growth (i e., having a high mitotic index) in a 10-mL suspension culture (2×10^5 cells/mL) or as a monolayer (10^6 cells/60-mm plate) is treated with colcemid at 0 06 μg/mL for 2 h to accumulate cells in mitosis
2. For the monolayer culture, tap the plate and remove the medium containing detached mitotic cells Trypsinize the remaining monolayer, pool with the medium, and proceed
3 Regain cells by centrifugation at 300*g* for 3.5 min at room temperature.
4. Resuspend cells in 1 mL of growth medium, add 3 mL of distilled water, and invert to mix (do not pipet because the cells are fragile)
5. Leave for 7 min to allow the cells to swell (this time can be altered if satisfactory spreads are not obtained)
6. Add 4 mL of freshly prepared ice-cold fixative (methanol:acetic acid, 3:1) directly to the hypertonic solution to avoid clumping
7. Regain the cells by centrifugation at 300*g* for 3.5 min
8. Disperse the pellet gently by agitation (do not pipet) in 10 mL of fixative
9 Repeat this procedure three times. At this point, the fixed cells can be stored for a week at 4°C or slides can be made immediately.
10. Using a Pasteur pipet, drop two to three drops of the resuspended cells onto a chilled slide from about 20 cm. Blow gently onto the surface, and place the slide onto a hot plate at 60–65°C (just too hot to keep the palm of one's hand on the plate).
11. Leave the slide to dry for 5 min and then place in a staining chamber (a Coplin jar) ensuring that the surfaces do not touch (*see* Note 11).
12 Stain the karyotypes with Giemsa for 3 min.

13 Wash the slides by dipping the slides through three additional Coplin jars each containing 50 mL of water
14 Dry the slides and count the chromosome number under the microscope, or process for banding (*see* Chapter 27)

3.6. Serum and Media Testing

Before a new batch of serum or media is purchased, it is advisable to obtain a sample from the manufacturer and test its growth-supporting characteristics. This is particularly important for serum. I usually select two of the most used cell types in the lab—currently these are a human diploid fibroblast strain and CHO cells—to test their growth and plating efficiencies (*see* Note 12).

1 Make up individual aliquots of growth media, all containing the same media batch, but with the different test sera and including the serum batch currently being used (or vice versa if you are testing media batches)
2. Plate the cells into 15 dishes for each test media at 5×10^5 cells/60 mm tissue-culture dish and containing 5 mL of the media
3 Every day for 5 d thereafter, trypsinize the cells from triplicate plates and determine the cell number/plate
4 Plot a growth curve (log cell number vs time), and calculate the doubling time and saturation density
5 At the same time as setting up the growth curves, seed in triplicate 60-mm dishes containing 5 mL of the appropriate media with 100 and 200 cells (6 plates/test)
6 After 10–12 d fix the culture for 15 min by flooding with formalin
7 Tip the media and formalin down the drain, and stain the clones with methylene blue
8 Leave the stain for 15 min, and then wash it away with water
9 Leave the plates stacked up against each other to dry in a 37°C room
10 Count the colonies
11. The three parameters of doubling time, saturation density, and plating efficiency should allow the section of a serum (or media) that gives optimal growth (*see* Note 13)

4. Notes

1 The shelf-life of a powdered medium is several years Once reconstituted, however, this is reduced to 2–3 mo, mainly because glutamine is unstable If older medium is used, the glutamine should be replenished (292 µg/mL) The pH of a medium, on storage, should not be allowed to rise, and to achieve this, good plastic caps with close-fitting rubber inserts should be used I also find it useful to seal the caps with a strip of Parafilm®, since this prevents condensation around the cap rim and, thus, minimizes the risk of fungal contamination. Medium containing HEPES can also be used to avoid bicarbonate buffering. I have never been entirely happy, however, with the cell's long-term growth characteristics in HEPES-containing medium.

2. α-MEM is a rich, multipurpose medium developed by Stanners et al *(12)* to grow hamster cells I have not had the experience of any mammalian cell type that will not grow in this medium, including hybridomas It is slightly more expensive than most media, however, and many cells will tolerate less rich and, therefore, cheaper media
3 Purified trypsin can also be used and is sometimes necessary, e.g , for macrophage cell lines *(13)*, but it is much more expensive and usually not necessary The citrate chelates Mg^{2+} and Ca^{2+} and replaces EDTA (Versene) in the buffer
4 It is advisable to keep fibroblast cultures from individual animals distinct, since it may be required to distinguish between individuals genetically
5 If the explant is not necrotic, it is possible to remove it with sterile forceps and transfer it to a new culture flask for further outgrowth of cells.
6 The detailed derivation of the various CHO stains is given in Gottesman *(7)*. It should be noted that CHO is a proline auxotroph and should always be maintained in proline-containing medium.
7. CHO cells can maintain viability, providing the medium pH does not become alkali, at 4°C for extended periods of time (7–10 d). Cultures in capped bottles can therefore be moved to the cold room to avoid subculture under desperate circumstances
8 Primary cell cultures may also be grown on microcarriers in suspension culture Full details of this technology are given in Chapter 5
9 The Coulter counter should have a 140-μ*M* aperture and the thresholds set as described in the machine's Instruction Manual. Serum in the PBS prevents cells from aggregating and giving unreliable counts The counter sometimes gets partially blocked, only experience of the time taken for each count and for the cell's particular display on the spectroscope will indicate problems with counting. Gentle brushing of the orifice with a camel-hair brush will unblock the counter. The Coulter counter can also give a visual display of cell volumes This, when combined with a pulse height analyzer, can be used quantitatively to measure cell volume or to determine cell viability by estimating the amount of cell debris in a sample.
10 To maintain genetically pure cell lines, it is absolutely essential not to cross-contaminate cultures To ensure this, fresh pipets must *always* be used at every step. Do not re-enter a media bottle with a pipet that has been near a culture Similarly, *never* pour from a media bottle into a culture. Splash-backs can occur. If you have more than one culture at a time in a tissue culture hood, only one of these should be opened at any one time. Meticulous attention to these small details will prevent the cross-contamination scandals (e.g., HeLa cells in all cultures!) that one so often reads about
11 It is usual to prepare one slide and check it with phase contrast microscopy so that adjustments can be made on subsequent slides. If there are many nuclei without cytoplasm and a few metaphases, reduce the swelling time. If there are many scattered chromosomes, blow less vigorously. If the metaphase spreads are overlapping, either swell for a longer time (up to 40 min) or blow more vigorously

All these parameters need to be adjusted according to the local environment conditions and cell type (*see* Chapter 27 for greater detail and ref. *14*).

12. If many cell lines are being used, it is often impractical to test the serum out on all the cell types. Usually the most difficult to grow are chosen for the test, but caution needs to be exercised since I once had a batch of serum that supported the cloning and growth of primary diploid fibroblasts but failed to allow cloning of CHO cells!
13. This procedure need only be performed about once every year Enough serum can then be ordered for the next year, since the serum is stable at –20°C for at least 2 yr We used to check our serum using [^{3}H]-thymidine incorporation 1 d after seeding the cells, but given the hazard of using radioactive thymidine, we abandoned this procedure. It is less labor-intensive, however, than measuring growth curves and gives perfectly adequate results. Details of measuring radioactive isotope incorporation into acid insoluble material may be found in Chapter 9, Volume 1 of the *Methods in Molecular Biology* series

Acknowledgments

I acknowledge the training given to me by Cliff Stanners, many of whose methods are represented in this chapter. This chapter was written while my research was supported by the National Institutes of Health, grants HD/AI 30280, DK/CA 48960, and P30 13330, J. W. Pollard is a Monique Weill-Caulier Scholar.

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2

Establishment, Maintenance, and Cloning of Human Dermal Fibroblasts

Gareth E. Jones and Clare J. Wise

1. Introduction

The widespread use of human diploid fibroblasts in many tissue-culture-based systems has its origins in the pioneering work on cellular senescence by Hayflick *(1)*. He established a reliable protocol for the maintenance of fibroblast strains that was also favorable for stimulating cell proliferation. The reproducibility of the cell cultivation system he developed allowed the realization that normal diploid cells exhibit a limited proliferative potential in vitro. Depending on the age of the donor and the biopsy site, human dermal fibroblasts can reach over 50 population doublings before senescence sets in *(2)*. This high value is of great advantage to laboratories wishing to study some aspect of cell function in a normal diploid cell population. With the development of reliable cryopreservation protocols, it is possible to build up a substantial bank of early to mid passage fibroblasts from a single primary culture derived from a biopsy. This bank of stable diploid cells thus has many attributes usually only found in transformed cell lines where homogeneity of cell population over a prolonged period of time is often crucial to the experimenter. Unlike a transformed line however, human fibroblasts maintained, as described here, generally retain the normal diploid karyotype.

A second consequence of the proliferative potential of human fibroblasts is that they can be successfully cloned. In most other species, and especially in rodents, a culture crisis will arise after some 5–7 passages in which diploid cells will cease undergoing mitosis. Subsequent random events will often throw up a spontaneously transformed clone that will take over the culture dish *(3)*. Although this transformant may well be useful, it is no longer a normal diploid cell and it is thus unsuitable for many purposes. Given such behavior, cell clon-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

ing with the aim of using stable diploids is out of the question, but human fibroblasts have been found to be quite agreeable to the rigours of cloning. This allows the experimenter to derive clonal populations of cells, as for example in the cases of females who are heterozygous for an X-chromosome linked phenotype of interest *(4)*.

This chapter describes the basic procedures that enable successful establishment of human diploid fibroblasts and gives two methods by which early passage cells may be cloned. The success of our cloning method is largely the result of the use of adsorbed fibronectin as a seeding substratum; fibroblastic cells bind avidly to fibronectin via cell surface integrins resulting in high cell seeding efficiency.

There are a few problems unique to collecting human biopsy material. First, the tissue sample is usually taken for sound clinical reasons, such as aiding diagnosis. The needs of the pathologist must be met first, and it is essential that your wishes should have been communicated to the clinician in charge well in advance of the biopsy. Second, there is the problem of repeat sampling; multiple biopsy of one human is rare since it often cannot be clinically justified. Similarly, one cannot always be confident that the site of biopsy is consistent between patients. For these and other reasons, it is virtually obligatory to obtain the active participation of clinical colleagues who are well versed in the requirements of a prolonged research program plus the backing of the hospital ethical committee for your project. Lack of attention to these early considerations will probably render your research efforts useless.

2. Materials

1 Basal salt solution, with Hank's balanced salt solution (HBSS, ICN Flow, Thame, Oxfordshire, UK) (*see* Appendix)
2. Growth medium containing: 10% fetal calf serum (heat inactivated) (Globepharm), 1% penicillin/streptomycin (ICN Flow), 1% L-glutamine (ICN Flow) (*see* Note 1).
3 Calcium- and magnesium-free phosphate buffered saline (PBS-A) (Oxoid, Basingstoke, Hampshire, UK)
4 0.04% EDTA (Sigma, St Louis, MO) in PBS-A.
5. 0 25% Trypsin/0 02% EDTA solution in HBSS (Gibco BRL, Paisley, Scotland, UK).
6 Stock solution of human plasma fibronectin (Sigma, St Louis, MO) prepared following the instructions of the supplier, diluted to 10 µg/mL in PBS-ABC (with calcium and magnesium salts).

3. Methods

3.1. Collection of Biopsy

1 Provide a labeled container of medium to your clinical collaborator. A 20-mL sterile universal tube, with leak proof cap is ideal, about half full of complete culture medium.

2 If it will not be possible to transfer the biopsy directly to the tissue culture laboratory, provide a Thermos flask filled with ice (or a polystyrene box filled with ice). If kept at 4°C, biopsy samples will survive for periods up to 2 or 3 d, though tissue degeneration may be expected
3 It should not be necessary to ensure that the biopsy will be taken under controlled sterile conditions The site of collection will be sterilized (usually with 70% ethanol), perhaps following shaving to remove hair, and many patients will ask for a local anesthetic (*see* Note 2)

3.2. Primary Explant Culture

1. Transfer the biopsy to fresh sterile HBSS in a centrifuge tube, and rinse by gentle hand agitation for a few minutes Transfer to a Petri dish in a sterile cabinet.
2. Dissect off unwanted fat and necrotic material. Using scalpels, chop up the material into 2-mm cubes
3. Wet the inside of a few wide-bore sterile Pasteur pipets to stop subsequent sticking of biopsy fragments to the glass. Transfer fragments using sterile Pasteur pipets to a sterile centrifuge tube containing 1 mL HBSS, and allow the pieces to settle
4 Wash twice in fresh HBSS, aspirate off the HBSS, rinse using a sterile Pasteur pipet and replace with fresh buffer. Allow the pieces to settle under gravity
5. Rinse the culture surface of a 25-cm^2 culture flask with 1 mL of complete growth medium. There should be just enough to cover the whole surface with a film of liquid after a vigorous shake or two. This will aid the attachment of the biopsy fragments to the culture surface.
6 Wet a clean sterile Pasteur pipet. Using this pipet, transfer pieces of tissue to the culture flask, placing them evenly over the whole available surface. Ideally, you should aim to have some 20 fragments of material per flask, but less is practicable and often inevitable with human material.
7. Cap the flask and invert the culture vessel so that tissue fragments will be in a "hanging drop" configuration. Surface tension properties will retain a pool of medium around and over the explants, gravity will drain excess medium to the "upper" surface of the flask.
8 Transfer the flask to a tissue culture incubator set at 37°C and 5% CO_2/air mixture (*see* Note 3) A 37°C hot room is sufficient if media are HEPES buffered Leave the flasks in a "hanging drop" configuration for up to 8 h The tissue fragments should stick to the culture surface within this time.
9. Return the culture flasks to a sterile cabinet, still inverted Uncap and remove excess medium (containing any explant material that failed to adhere to the culture surface) using a Pasteur pipet attached to a vacuum pump. Reorient the culture flask, add 1–2 mL of growth medium, cap the flask, and return to the culture incubator. Leave for 18–24 h.
10. Examine the cultures. If the explants have adhered, make up the medium volume to 5 mL (*see* Note 4)
11. Leave for 1 d, then remove the explants. These may either be picked off with a pair of sterile fine curved (watchmakers) forceps or dislodged with suction pressure generated down the capillary column of a fine-bore Pasteur pipet.

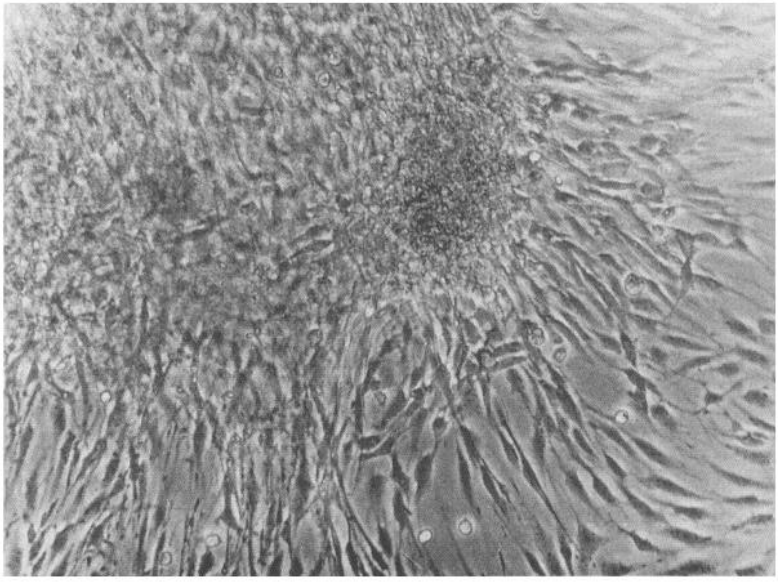

Fig. 1. A primary culture showing a skin explant, rounded epithelial cells, and stellate fibroblasts. The epithelial cells are the first to appear from the explant, but after 5 d, fibroblasts have migrated out to the substratum and are proliferating rapidly (magnification ×250).

12. Replace the medium in the culture flask, and monitor the outgrowth of cells over the next few days (Fig. 1). Once some 75% of the culture surface has been covered by cells, the culture is ready for passage (*see* Notes 5 and 6).

3.3. Subculture and Maintenance (see Note 7)

1. Remove the medium by aspirating into a medium trap, placing the culture flask on end, and allowing full drainage.
2. Add a wash of 5 mL of HBSS or PBS-A to rinse the cells and remove traces of serum. Remove the wash as in step 1 (*see* Note 8).
3. Add 1 mL of trypsin/EDTA in HBSS, and swirl the flask to cover the monolayer with solution (*see* Note 9). Incubate in trypsin until the cells begin to round up. Do not force the cells to detach by trituration, since this will cause cell clumping. Gentle rocking of the flask is acceptable. It usually takes some 10 min to detach 90% of the monolayer. Do not leave the cells in trypsin longer than is necessary (*see* Note 10).
4. Add 4 mL of growth medium to inactivate the trypsin. Disperse the cells with gentle and repeated pipeting to provide a single-cell suspension (*see* Note 11).
5. Count the cells by hemocytometer (*see* Note 12) or electronic particle counter. Dilute to the appropriate seeding concentration by simply adding the cell suspen-

sion to the total volume of medium required for distribution to fresh culture flasks. This ensures that each flask will contain the same concentration of cells (*see* Note 13)

6 Add 4 mL of cell suspension to each flask, cap the flasks, and return to the incubator. If needed, gas with sterile 5% CO_2 Leave in an incubator for 2 h with caps left slightly open (vented) to allow for gas exchange Recap the flasks and leave for at least 24 h.

7. To determine when a medium change is required, check the culture every day for a drop in pH (*see* Note 14). This is usually evidence of depletion of medium by the growing culture Examine cells under an inverted microscope for signs of cytoplasmic vacuolation, and estimate cell density

8 If only feeding is required, remove and discard medium Add an equal volume of fresh medium that has been prewarmed to 37°C Return the culture to the incubator (*see* Note 15). Continue to feed cultures until the culture surface is completely covered with cells (a confluent culture) Then repeat the subculture protocol (*see* Note 16).

3.4. Cell Cloning (see Note 17)

1 Clean glass coverslips in an acid wash (0.1*M* HCl for 10 min), rinse repeatedly in distilled water, and sterilize at 120°C for 2 h in a dry oven, or use a microwave oven if preferred

2. Snap the coverslips to produce small shards of glass The size will be very variable, and you should select shards of approximately 4 mm^2

3 Place the glass fragments into wells of a 24-multiwell plate. Aim to cover about 75% of the bottom surface of each well with glass

4 Add a solution of plasma fibronectin to just cover the glass fragments Leave for 2 h at room temperature or 30 min at 37°C

5 Prepare a cell suspension by trypsinization, following the routine methods given for cell subculture

6 Aspirate off the fibronectin solution (this can be reused for up to 5 h if kept cold). Wash the wells in medium Add the cells in complete medium, plating at population densities of about 25 cells/32 mm diameter plate. Keep the depth of the medium to a minimum

7. Place the culture in a 37°C incubator, and leave for 2–3 h to allow adhesion and spreading of cells to fibronectin-coated glass

8 Examine the culture with an inverted microscope. Identify those glass shards with one cell attached Using sterile forceps, remove selected pieces to 96-well tissue culture multiwell plates

9 Place a single fragment of glass into each well before adding medium to the well. The glass will stick firmly to the underlying plastic as a result of surface tension (*see* Note 18)

10. Add complete medium to the wells, again keeping the total volume to a minimum (50 µL) Place the multiwell plate in a tissue culture incubator after checking again that each well contains only a single cell, and leave for 18–24 h.

11 Examine cultures for signs of colony formation. At this early stage, it is possible to check that each well contains a single colony. Remove glass shards having multiple colonies using forceps (*see* Note 19)
12 Allow the cells to proliferate until the base of the well is covered with fibroblasts. Use standard trypsin detachment procedure to isolate cells Seed clones into a 6-well tissue-culture plate in complete medium and allow to grow up On the next passage, transfer the cells into a standard 25cm^2 tissue-culture flask (*see* Note 20) Use the standard subculture protocol to passage cell clones (*see* Notes 21 and 22) (For a protocol on dilution cloning, *see* Note 23)

4. Notes

1 L-Glutamine is usually present in commercially obtained media, but it is degraded if the media is stored for a long time For this reason we usually supplement media with a further 1% L-glutamine
2 This chapter deals with the culture of cells from dermal biopsy, but in practice, fibroblastic cells can be grown from virtually any biopsy material following this protocol.
3 If cells are grown in a sodium bicarbonate containing medium, then the cultures need to be gased, with 5% CO_2, by venting the flasks This will allow the pH to adjust. If media are HEPES buffered, then no pH adjustment is required and the flasks should not be vented.
4 You are likely to be provided with very small amounts of biopsy with which to begin your culture. Explant culture is particularly suited to this restriction, since there is a great risk of losing cells during enzymic disaggregation There are two major disadvantages to this technique. Some tissue has very poor adhesiveness of explant to culture surface This is not a problem for skin or muscle biopsy. Two variations can be tried to overcome this problem if need be. First, try to make your explant size smaller; this is harder than it sounds and may call for the use of a good binocular dissecting microscope. Second, increase the percentage of serum in the growth medium from 10–20% or more to aid the attachment of the explants to the culture surface The second disadvantage relates to cell selection. The technique will only allow culture of those cell populations with good migratory powers; it does not provide a culture that represents the mixture of cell types to be found in the biopsy. Skin biopsy explants will show an initial migration of epithelial cells from the explants over the first few days, these cells are then overgrown by fibroblasts (Fig. 1)
5. Always use fetal bovine serum in the culture medium of primary explant cultures This gives better survival figures than serum from nonfetal sources. Some materials proliferate better on culture plastic ware that has been precoated with denatured collagen (Gelatin) in PBS-ABC
6 Despite the rarity of good biopsy material, I always discard explants that have been removed from the established primary culture Many sources suggest that these explants can be reused to seed a second round of cultures. Although this is true in theory, I feel that problems over toxins released as a consequence of tissue necrosis within the explant do not justify the effort.

7 Once a primary culture is subcultured, it becomes known as a cell strain, partially reflecting the heterogeneous lineages of the explant. As the cell lines proliferate and are subcultured, a selection process will usually occur that narrows the range of cell phenotype within the strain. For consistency between experiments and/or between biopsied samples, it is essential to give a code to each cell line established in your laboratory and a record of the number of subcultures performed For finite cell strains that are derived from biopsies, it has been a routine to reduce the cell concentration at each subculture by two- or fourfold. Under these circumstances, each passage is accompanied by either a one or two times population doubling prior to the next subculture. A record of the number of passages and population doublings must be kept with each cell culture For example, a cell strain will be designated by normal human skin fibroblast (NHSF) on the first subculture, together with a number to indicate the source of the cell strain (NHSF5 will indicate the fifth biopsy taken). We will also add a note of the population doublings, so this will become NHSF5/1. This last number will increase by one for a split ratio of 1·2 (NHSF5/2, NHSF5/3, and so on) or by a two for a split ratio of 1·4 (NHSF5/2, NHSF5/4, and so on).

8 All solutions and media should be prewarmed to 37°C before use

9 If cells adhere strongly to the tissue-culture plastic, they may take some time to dissociate in trypsin/EDTA. The length of time that the cells are exposed to trypsin should be kept to a minimum to prevent damage to the cells Addition of a prewash of 0.04% EDTA in PBS-A should be added for up to 30 s, removed, and then trypsin solution added This will help the trypsin to act more quickly and minimise damage to cells

10 Many proteolytic enzymes are available in varying degrees of purity Crude trypsin (e g., Difco 1:250) contain other proteases that may be toxic to some cells. Start with the crude preparation, and move on to purer grades only if necessary You will also need much lower concentrations for shorter exposure periods than those given here for crude trypsin

11. If cells have formed a confluent monolayer, it is possible that when exposed to trypsin, detachment of the monolayer as a sheet will occur, rather than as a single cell suspension. By passing the cell suspension through a 1-mL syringe with a 25-gage hypodermic needle, while still in trypsin, a single-cell suspension can be achieved without shearing of the cells.

12 A Trypan blue (Sigma) (0 2% in PBS-B [ICN Flow]) exclusion test can be carried out by mixing 1 1 with a cell suspension to be counted by hemocytometer The dye will be excluded from viable cells, and present in dead or dying cells. This allows calculation of cell concentration including only viable cells

13 Once a routine of medium change and subculture is underway, you may wish to replace the 25-cm^2 culture flasks with 75-cm^2 versions Do this if the cells proliferate rapidly, and do it early in the life of the cell line

14. Most commercial media contain Phenol Red, which is a pH indicator. When at the correct pH, the dye is orange/red When media is depleted the color will change to straw yellow, indicating that a subculture or feed is needed. When cells

have just been fed or cultured the media will often be pink and flasks should be vented to allow pH adjustment by CO_2 until they are orange/red

15 It is good practice to pretest a batch of serum for the capacity to support your cell cultures and, once satisfied, to use the same batch in all your subculture work for a particular experimental program In this way, you will avoid problems associated with the considerable batch variation between seemingly identical serum

16 The subculture method given here will eventually provide a series of replicate samples for setting up your experiment You should not necessarily adhere to this method when preparing a cell population for an actual experimental assay

17 Cloning of most cell strains is a difficult and tedious procedure that is only to be undertaken for strong scientific reasons. Normal tissue cells only have a limited number of generations, and by the time that sufficient cells have been produced from a clone, they may be already near to senescence Low plating densities are needed to ensure successful isolation of clones, but his results in low survival levels in all but a few cell lines This method attempts to overcome these difficulties by choosing a rich medium, by seeding cells at high densities (some five to six times the recommended concentrations) onto small fragments of easily manipulated glass, and by pretreatment of the seeding surface with fibronectin to improve the adhesiveness of the substratum It is possible to use feeder layers of irradiated cell monolayers, but we find that the extra work involved offers no improvement in the success rate over fibronectin coating

18 We normally do not take much care over checking the orientation of the glass fragment when it is taken to the well of the 96-well dish, the cell can either be face up to the medium or sandwiched against the surface of the well Sometimes it seems that cells caught in the latter orientation survive better than those facing up The confines of the microenvironment between the glass and plastic surfaces may allow the cell to create a locally enriched environment that mimics a higher cell density state If you have problems with plating efficiency, it would be worth experimenting with this observation, perhaps taking care to place the cloning medium into the well *before* adding the glass fragment in this case.

19 The visual identification of an isolated cell mass should not be taken as conclusive evidence for its origin from a single progenitor cell With females, it is possible to use individuals who are heterozygous for some X-chromosome linked gene such as G6PD, clone their cells, and then test individual clones for G6PD phenotype. You should either follow this suggestion to check against genetic contamination of a clone or find another gene that you can assay for in some way. If all you need is a clonal population of fibroblasts, you should use females with a well-known allelic polymorphism of an X-chromosome gene

20 Most cells proliferate better when the cell density is not too low By gradually increasing the size of the culture vessel when subculturing you avoid the risk of losing the cells

21. If no cultures appear in the isolates by 3 wk, it is very unlikely that they will do so. We suggest that if you get this result more than once, you should consider using conditioned media in order to establish the clones Collect the medium

from cultures of fibroblasts that have been growing for at least 4 d, centrifuge at 10,000*g* for 30 min at 4°C to clarify, and filter the supernatant through a 0 2-μm low protein binding sterilizing filter. Add this conditioned medium as a 40% (v/v) fraction to your cloning medium.

22 We have used cloning rings in combination with Petri dishes to generate clones Isolated cells are plated at densities of between 25 and 50 cells/60-mm diameter Petri dish in Ham's F10 medium and 15% foetal calf serum Individual clones were isolated using glass cylinders that are dipped in silicone grease prior to pressing onto the base of the Petri dish to encompass a growing clone and isolate it. Trypsin solution is then added by pipet to the clone, and the dissociated cells are removed to a culture flask Production of clones was less efficient using this technique, and this led to the development of the method we have described here

23 Another method of cell cloning is dilution cloning which should be carried out in 96-well plates Dilute cells down to a concentration of 10 cells/mL To be accurate, this concentration should be reached by serial dilution. Seed cells out at 100 μL/well in complete growth medium and incubate for 3–4 d Check to see that some wells contain proliferating cells and return to the incubator until clones have reached confluence If cells need to be fed during this time, do this by removing 50 μL media and adding 50 μL fresh media Repeat dilution cloning once again Transfer clones to a 24-well plate, then to a 12-well plate, at which point clones can be characterized and expanded further.

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3

Aging of Cultured Human Skin Fibroblasts

Calvin B. Harley and Steven W. Sherwood

1. Introduction

Since the seminal observations of Hayflick and Moorhead *(1)* that cultured human fibroblasts have a finite replicative lifespan, study of in vitro cellular senescence has provided a foundation for understanding organismal aging.

Recent studies have shown that molecular and biochemical markers of in vitro senescence can be demonstrated in vivo providing direct evidence that aging in tissues in vivo involves accumulation of replicatively senescent cells *(2)*. Similarly, the finding that in vitro cellular senescence is correlated with the progressive loss of telomere sequences from chromosome ends and that this process represents a significant change accompanying organismal aging has provided new impetus for examination of the role of replicative senescence in aging and age-related disorders, including cancer (for review, *see* ref. *3*).

The role of cellular senescence in gerontological research has utilized in vitro cultured cells in three ways. First, longitudinal studies have utilized cells isolated from donors of various ages, or a single donor sampled at different times during their lifespan, to compare structural, biochemical, and molecular features of cells derived from organisms of increasing age. Second, cells cultured from individuals with accelerated aging syndromes such as progeria or Werner's syndrome have been compared with age-matched cells from normal donors. The third method uses continuously propagated cell cultures established from normal donors to compare cell function between early and late passages as cells proceed to replicative senescence in vitro. In all three cases, it is a goal to relate in vitro differences in cell function to age-related organismal changes. No one method is without limitation, however, and therefore it is ideal to utilize more than a single approach *(4)*.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

Because in vitro culture lifespan and cell "age" (population doubling level [PDL]) are operationally defined by culture conditions and procedures, experimentally reproducible comparisons between cultures of different "age'" demand strict adherence to standardized culture procedures and use of well-characterized reagents. Careful attention to the establishment of primary cultures and ongoing estimation of population doublings are points emphasized in this chapter. Other chapters in this and other volumes provide greater detail on maintenance of cell strains and special techniques relevant to specific cell types, some of which have been used to examine aging in cultured cells *(5)*.

Rigorous evaluation of population doublings requires measurement of

1. The number of viable cells used as culture inoculum, N_o,
2. The fraction of cells that attach, f_a,
3. The fraction of cells that divide, f_d, and
4. The number of cells in the culture at confluence, N_c

Provided that there are not dramatic changes in other parameters such as cell size and interdivision time, these factors determine the incrememental cell doublings during one passage according to the equation:

$$\text{Doublings} = \log_2 \{[N_c - (1 - f_d) f_a N_o)/f_a f_d N_o\} \quad (1)$$

Because in practice the fraction of normal skin fibroblasts that attach and divide is close to 1 for the most of the lifespan of the culture, these effects are ignored or assumed in the term MPD (mean population doublings) according to the equation:

$$\text{MPD} = \log_2 (N_c/N_o) = \log_2 (1/\text{split ratio}) \quad (2)$$

Thus, if confluent cells are split at a 1:4 or 1:8 ratio, for example, the number of MPD required to repopulate a dish to confluence is simply 2 or 3, respectively. It should be emphasized, however, that this estimate of cell generations can be significantly in error toward the end of culture lifespan when the fraction of cells attaching and dividing becomes significantly less than 1, and the cell number at confluence declines. The methods described here for determining population doublings can be applied to any system of cells growing on a solid matrix *(6–8)*.

2. Materials

1. Regular growth medium (RGM) Media of varying degrees of complexity and "definedness" have been used to culture primary fibroblasts. Highly specialized medium is not, however, required for skin fibroblasts We currently use a mixture of DMEM and M199 media (GibcoBRL, Life Technologies, Grand Island, NY) as a basal medium. Bottles of RGM are stored at 4°C without additives (glutamine 5 m*M* and fetal bovine serum [*see* Note 1], 10% by volume). The latter are added

at the time of use, and once medium is made, it should be used within 2 mo Medium is warmed to 37°C at time of use but otherwise kept at 4°C

Medium composition and/or vendors should not be changed without adequate growth testing Rapid testing can be done by simply growing cells for several doublings, comparing the rates of growth in different media Subtle effects of medium may require prolonged culture and may only be detectable as a reduced rate of cumulative growth over the proliferative lifetime of the cells Serum represents the medium component most subject to variation in quality and very careful attention must be paid to characterizing specific lots prior to use (*see* Chapters 1 and 4) At a minimum, cells should be grown for several doublings at high and low density with test serum to insure the reproducibility of cell growth rate. To insure sustained consistency in cell growth for long periods (6–12 mo), samples of different lots of sera can be obtained, tested, and satisfactory lots purchased in adequate quantities to avoid changing lots

2. Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). 0 14*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.4.
3. Trypsin: 0.125% solution is obtained from Gibco BRL, Life Technologies (Grand Island, NY). Aliquots (5–10 mL) are stored frozen at –20°C Thaw as needed Unused portions should be kept at 4°C The presence of EDTA in the trypsin solution can have an impact on cell behavior and should be evaluated for specific experiments (*see* Note 2).

3. Methods

3.1. Primary Culture

1 A standard 2–4-mm punch biopsy of epidermis plus dermis is taken from the abdomen or inner forearm by a qualified person using sterile technique (*see* Notes 3 and 4).
2 Place the tissue into a 100-mm tissue-culture dish containing a small amount of RGM Cut tissue in half and place one-half in a vial containing 10 mL of RGM. Place at 4°C as a backup sample It will retain viable cells for many days
3. Mince remaining piece of tissue into pieces 1-mm^3 or less and place three pieces each (dermis side down, if possible) into 35-mm dishes (*see* Note 5) Use a siliconized Pasteur pipet to transfer tissue Prepare a sterile 25-mm coverslip by putting sterile silicone grease on each corner to permit coverslip to adhere to dish. Place coverslip over pieces of tissue, add 2 mL RGM under coverslip, and incubate at 37°C in humidified 5% CO_2 atmosphere.
4 Monitor explants for appearance of cells along the edges of tissue pieces every 2 d and refeed cultures weekly Outgrowth of spindle-shaped fibroblasts will occur within several days Irregularly shaped loosely packed keratinocytes will appear prior to fibroblasts but will differentiate and die in RGM
5 When fibroblast outgrowth reaches a size of about 1 cm^2 (2–4 wk), gently remove coverslip, remaining tissue, and RGM and rinse dish twice with PBS. Add 0.3 mL trypsin making sure that cells are covered. Return dish to incubator

Table 1
Estimated MPD During Primary Culture[a]

Cell number/dish	MPD
30,000	6
60,000	7
120,000	8
250,000	9
500,000	10

[a]This estimation assumes that about 500 cells initiate outgrowths from the tissue fragments in one 35-mm dish. Some heterogeneity in population doublings is introduced into the culture because of density-dependent inhibiton of growth *(4)*

Table 2
Tissue-Culture Dishes

Parameter	35 mm	60 mm	100 mm
Surface area, cm^2	8	21	55
Cells at confluence, N_c	5×10^5	1.3×10^6	3×10^6
Trypsin, mL	0.3	0.6	1.0
RGM, mL	1.7	3.4	9.0

for 10 min or until cells begin to detach from dish (monitor with inverted microscope). Gentle agitation of dish may be required to loosen cells

6. Add 1.5 mL RGM, and triturate cells to suspend. Remove debris and count an aliquot of cells. Pool cells from several dishes
7. Estimate MPD according to Table 1
8. Place remaining cells into fresh medium in 35- or 60-mm dishes. Record date, strain designation, and expected MPD at confluence on lid of dish. Expected MPD is obtained by adding to the calculated MPD of the primary culture 3.3 log (N_c/N_o), where N_c is the expected number at confluence (Table 2) and N_o is the initial number of cells (*see* Notes 6 and 7).

3.2. Secondary Cuture and Subsequent Passages

1. As culture reaches confluence, aspirate medium, wash cells once with PBS, add trypsin (Table 2), and return dish to incubator for about 10 min or until cells begin to detach. Gentle agitation may be necessary to loosen cells
2. Add RGM and triturate cells to suspend. Count an aliquot of cells and inoculate a fresh dish with cells in an appropriate volume of RGM. The inoculum should be 1/8 to 1/2 the number of cells required to form a confluent monolayer. Uniform

and reproducible growth requires even dispersion of cells over surface. Record date, strain designation and expected MPD at confluence.

3. Refeed culture at least once a week until culture attains confluence. Passage cells at confluence making sure that total cell number is determined and split ratio is appropriate (*see* Notes 7 and 8).

3.3. Senescence (Phase III)

1. Visually monitor cells using inverted microscope at each passage and estimate growth rate as days to confluence from a standard inoculum (e.g., 1/8 of number at confluence) When the growth rate declines, the culture is approaching its terminal passage. Cells will become larger and more irregular in shape (Fig. 1).
2 Senescence (also known as MPD max, Phase III, or terminal passage) is the point at which one MPD requires longer than 1 wk to attain (*see* Note 9). For example, if the split ratio is 1.8, a culture not reaching confluence in 3 wk may be considered terminal. Senescence cells will become more difficult to trypsinize if they are maintained for prolonged periods without subcultivation (*see* Note 10)

4. Notes

1 Do not use dialyzed or heat-inactivated serum for routine cultivation of normal human fibroblasts. Fibroblasts are tolerant of short-term medium changes and biochemical studies often require exposure to media lacking (or containing) particular components Conducting a growth curve in special media will serve to ensure that cells are not adversely affected by medium. If less than one population doubling is involved in studies, metabolic assay of the rate of protein synthesis or DNA synthesis using radiolabeled precursors can be used to measure the impact of specialized medium on cell growth
2 Crude preparations of trypsin may contain other proteases. Variaiblity in activity may also occur Standardization of trypsin exposure can be achieved by varying trypsin concentration up or down if cells require longer than 15 or shorter than 5 min for release from plates. The use of EDTA in trypsin solutions may represent an important factor in the lifespan of cultured cells and should be evaluated as a potential source of variabiltiy in cell lifespan
3. Rigorous use of good sterile technique should obviate the need for antibiotics and antimycotics, which may have unpredictable effects on cells
4. Fibroblast cultures can be initiated from skin taken from essentially anywhere on the body Anatomical location of sample including exposure to sun and environmental factors should be considered as factors potentially affecting the cultures established.
5. Disaggregation of biopsies can be done mechanically or with proteases, resulting in cell suspensions rather than outgrowth from explants. The effects of the proteases on cells are, however, somewhat unpredictable and it is difficult to estimate the number of founder cells for the culture. The advantage of using proteases is that it eliminates heterogeneity in doublings within the primary population that arises from density-dependent arrest of cells in the outgrowth technique *(7)*. By

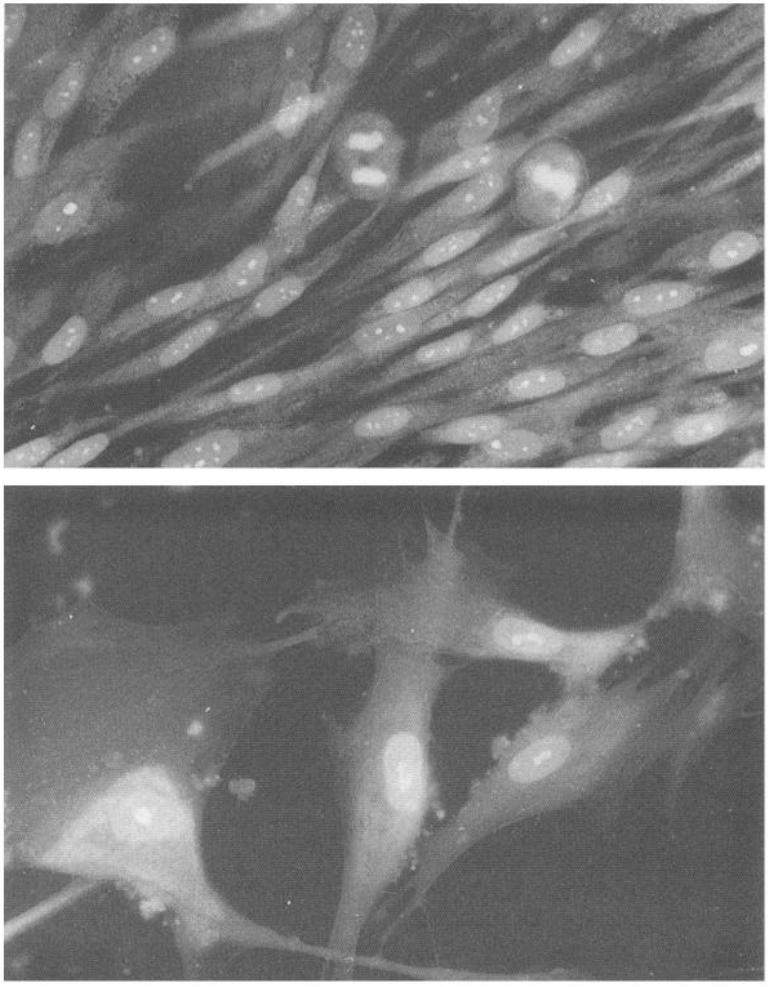

Fig. 1. Senescence-related change in cell and nuclear size in human diploid skin fibroblasts (cell line BJ). **(A)** Proliferating PDL 30 cells. **(B)** Senescent PDL 78 cells. A large difference in both cytoplasmic and nuclear size (but decrease in nucleocytoplasmic ratio) is apparent in senescent cells. Fluorescent micrograph of viable cells stained with acridine orange.

harvesting the primary culture before outgrowths become large and heterogeneous, this problem can be minimized.

6. Proper evaluation of the "age" of cultures established from secondary sources (cultures or frozen ampules) requires estimation of MPD. Assume 9 MPD for primary culture and 1–3 MPD for each passage depending on the split ratios

Table 3
Labeling Schedule for Estimating S-Phase Fraction Using Immunodetection of DNA Synthesis (Bromodoeoxyuridine Incorporation)

	Time, h					
Culture phenotype	0	24	48	72	96	120
Young	Split 1.8, 10% FBS	BrdU	Harvest, fix, stain	—	—	—
Quiescent	Split 1 8, change to 0 5% FBS 6 h after plating	—	—	Refeed 0 5% FBS	BrdU	Harvest fix, stain
Senescent	Split 1 4 or 1 2, change to 0.5% FBS after 6 h	—	—	Refeed 0.5% FBS	BrdU	Harvest, fix, stain

previously used (1 2, 1.4, or 1:8). On initiating cultures from such sources, subculture cells at a split ratio of 1 2. After 6–12 h, estimate the proportion of cells attached and add this to the number of MPDs estimated initially For example, if one-half of the cells in the first 1 2 split attach, this first spilt is approx 2 MPD This number is then added to the initial estimate of MPD

7. The values of N_c (Table 2) reflect an average value for forearm skin fibroblasts N_c and N_o should be checked periodically throughout the lifespan of each strain and these values used for MPD calculations
8. In reporting comparisons of early and late passage cells, it is useful to report culture age as "percent lifespan completed," i.e , MPD/MPD_{max} together with MPD_{max}
9. Various criteria have been used to define senescence or Phase III. Our experience is that terminal cells will fail to reach confluence after 3 wk following a 1:8 split
10. It is important in comparisons of young vs old cells to distinguish between quiescent young cells and senescent cells. The latter two are distinguished by capacity of quiescent cells to re-enter the cell cycle when stimulated by mitogens (e.g., serum) We utilize a standard procedure for serum-starving cells followed by readdition of serum and labeling of S phase cells using either radiolabeled DNA precursors or bromodeoxyuridine to assess the mitogenic potential of cultures and hence the degree of culture senescence (*see* Chapter 29 for details) Table 3 presents a schedule of cell plating, serum withdrawal, refeeding, and bromodeoxyuridine labeling. In this protocol, cells are plated onto sterile 22-mm^2 coverslips for labeling. Detection of incoporated label (BrdU) is done by

immunohistochemical staining with commercially available antibodies to BrdU-substituted DNA (we utilize monoclonal anti-brdU-sibstituted DNA, clone IU-4 available from Caltag Laboratories, South San Francisco, CA).

Acknowledgments

C. B. Harley thanks Samuel Goldstein and Elena Moerman for their introduction to tissue culture techniques. The authors thank their colleagues at Geron for critical feedback and sharing their experiences with primary cells.

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4

Ex Vivo Maintenance of Differentiated Mammalian Cells

Lola M. Reid and Thomas L. Luntz

1. Introduction

Maintenance of differentiated epithelia cells including ex vivo can be achieved now to a surprising degree through recognition of two complimentary and dynamically interacting sets of mechanisms: Mechanisms associated with epithelial–mesenchymal interactions, a relationship that is universal and that constitutes the organizational basis for all metazoan tissues; and mechanisms associated with stem cell biology and with stem cell-fed maturational lineages.

In this chapter we provide a summary of these principles and with some details in altered conditions for maintenance of cells ex vivo that offer an update on this review as previously published in the first edition of this book.

2. Concepts and Principles

Studies on molecular mechanisms governing differentiation in mammalian cells are rapidly progressing to elucidate key principles. Among these is the recognition that stem cell-fed maturational lineages occur in most, if not all tissues, persisting into adulthood. The maturational process results in cells with a gradient in expression of phenotypic markers dictated in part by restriction of genetic potential through physical and biochemical changes in the chromatin and, secondarily, by signal transduction mechanisms activated by gradients of hormonal and extracellular matrix signals. The ability experimentally to dissect the dynamic interactions between maturational effects on chromatin versus signal transduction has been made possible in recent years by the methods to identify and isolate purified populations of stem cells and early progenitors and by methods to maintain them ex vivo using precisely defined hormonal, nutritional, and extracellular matrix components. This chapter is intended to

From. *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

highlight some of the general themes discovered by recent studies but is not intended to be comprehensive. Within the reference list are given a number of excellent recent comprehensive reviews in the fields of stem cells, apoptosis, extracellular matrix, signal transduction, and ex vivo maintenance of cells.

2.1. Stem Cells and Lineage Biology

Stem cells are pluripotent cells capable of producing daughter cells with more than one fate *(1–28)* The only stem cells with capacity to produce all known cell types are the zygote and germ cells. During embryogenesis, pluripotency is narrowed in somatic stem cells to the restricted pluripotent state in determined stem cells—cells with ability to generate daughter cells maturing into all the cell types for a specific tissue. The determined stem cells produce a lineage of daughter cells undergoing a unidirectional, terminal differentiation process. In all well-characterized mammalian lineage systems (e.g., hemopoiesis, gut and epidermis), stem cells have been identified by entirely empirical assays in which they prove capable of producing the full range of descendants *(1–9)*. Although there are suggestions now of molecular markers for stem cells (*see below*), these markers have been defined for only one or two tissues *(29–35)*. Therefore, at present, there are no known markers that uniquely identity all stem cells, and no molecular mechanisms are known that result in the conversion from a stem cell phenotype to a commitment to differentiation in a unique pathway.

Despite the lack of general molecular markers, stem cells can be defined by two general features: pluripotency, the ability to produce daughter cells of more than one fate, and expandability, the ability to produce a large number of differentiated progeny *(1–16)*. They are usually slow-cycling cells, with long ^{3}H-thymidine-retention times and usually in a G_0 state, indicative of a quiescent state but having the potential to respond to appropriate signals by increasing their rate of growth *(2,3,11–16)*. Stem cells are also smaller than the mature cells, have higher nuclear to cytoplasmic ratios (blast-like cells), are diploid, and have a fetal phenotype that includes expression of fetal isoforms of tissue-specific genes *(2,3,7,8,19,27,28)*. Stem cells are bound to a form of extracellular matrix with a chemistry extensively overlapping with that found in embryonic tissues and consisting, in part, of type IV collagen, fetal isoforms of laminin, fetal cell adhesion molecules (CAMs), fetal forms of proteoglycans, and hyaluronates *(16,17)*. Other markers, recently identified for some classes of stem cells include:

1. Expression of the multidrug resistance gene, MDR1 *(32)*,
2. Having poor uptake of mitochondrial dyes such as rhodamine and referred to as being "rhodamine dull" *(20,32)*;

3 Paracrine signaling between embryonic, tissue-specific forms of mesenchyme and epithelia *(9,11,12,17,21)*, and
4 Relatively stable telomere lengths and expression of telomerase, an enzyme that maintains telomere length *(33–35)*

Stem cell compartments are usually located in protected, well-vascularized areas, such as the base of intestinal crypts *(4–6)*, the limbus of the cornea *(3)*, the bulges of the hair follicle *(2)*, the deep troughs of the epidermal/stromal interface in the palm *(1–3)*, among small ductules within glandular tissues such as lungs *(10)* or pancreas *(25,26)*, among the small ductules, the canals of Hering, around the portal triads in the liver *(14–24)*, and in small cells lining the ventricles of the brain *(27)* Within the stem cell compartment are typically a layer of epithelial stem cells, glued onto a layer of mesenchymal progenitor cells via an embryonic form of extracellular matrix, and in close association with one or more support cells *(1–28)*. In Table 1 the known cellular constituents for the stem cell compartment of a number of representative tissues are listed, and in Table 2 some of the classic features of stem cells are summarized.

Pluripotentiality, a defining feature of stem cells, consists of the ability of a stem cell to generate a range of daughter cells having distinctly different morphological, cytological, or functional phenotypes. For example, intestinal stem cells give rise to enterocytes, goblet cells, chromaffin cells, and endocrine cells of the gut. There is an assumption that each tissue will have a determined stem cell capable of producing only the cell types for that tissue (e g., brain stem cells can produce cells with phenotypes of mature brain cells but are not capable of producing skin cells or cell types of any other tissue). However, there are several examples in which the determined stem cell may have broader plasticity that encompasses multiple tissues with a common embryological origin. For example, there may be a common precursor between liver and pancreas *(14–26)* with induction of liver or pancreas phenotypes depending on the microenvironment. This complicates the assessment of pluripotentiality, since the extent of plasticity of gene expression within specific stem cell types is not known Thus, although the principle of how to define a determined stem cell is generally accepted among investigators, how to apply that principle to all known examples of "plasticity" within tissues is not yet known.

The concept of "self-replicative ability," that is, the presumption that stem cells can generate daughter cells that are phenotypically identical to the parent cell, is a concept now thought to apply only to germ cells, but is being hotly debated for determined stem cells *(13)*. Striking evidence, published recently, strongly suggests that pluripotent hemopoietic stem cells have a finite lifespan and are undergoing aging both functionally and physically *(33–36)*. Hemopoietic stem cells, defined rigorously by antigenic profile and derived from older

Table 1
Known Cellular Constitutents of Stem Cell Compartments in Representative Tissues

Tissue	Stem Cell	Mesenchymal cell	Support cells	Location
Skin	Epidermal stem cell	Stromal progenitor cells	Melanocytes	Deep troughs of epidermal/dermal interface; limbus of the cornea
Intestine	Intestinal stem cell	Stromal progenitor cells	Paneth cells	Intestinal crypts
Hemopoietic tissue	Hemopoietic stem cell	Stromal/endothelial progenitor cells	Adipocyte	Bone marrow near the vascular input for the plates (the lineages) of maturing hemopoietic cells
Testis	Spermatagonia	Stromal progenitor cells	Leydig cell, Sertoli cell	Periphery of seminiferous tubules
Lung	Clara cell	Stromal/endothelial progenitor cells	?	Bronchiole–alveolar junction
Brain	CNS neuronal stem cells	Endothelial progenitor cells	Glial cells (glial progenitors?)	Small cells lining the ventricles
Prostate	Basal cell	Stromal progenitors	?	Periphery of prostate
Liver	Hepatoblasts (canals of Hering)	Stromal/endothelial progenitor cells	Ito cell, hemopoietic progenitor cells	Near portal triads of liver acinus

animals, have been shown to have less regenerative capacity than antigenically identical cells derived from embryonic or neonatal tissue. Thus, aging is thought to result in a decline in the number of determined stem cells and/or in a decline in the regenerative capacity of those determined stem cells even though not resulting in loss of pluripotency. In addition, the telomeres on chromosomes from the cells are shortening with age, albeit at a dramatically slower rate than for somatic cells *(33–36)*. Parallel reports indicate a decline in the number of mesenchymal stem cells with age *(11–12)*, and preliminary studies suggesting a similar phenomenon have been described in hepatic stem cells (*20*; Reid and associates, unpublished data). However, rigorous studies defining even the phenomenology, much less molecular mechanisms explaining it,

Table 2
Relevance of Lineage Paradigms to Ploidy, Regulation of Growth, and Tissue-Specific Gene Expression[a]

Maturational stage	Matrix chemistry[b]	Ploidy (cell cycle stage)	Hormonal regulation of growth	Tissue-specific genes available for regulation
Stem Cells	Embryonic	Diploid (mostly G_0)	Governed primarily, if not entirely, by local, paracrine signaling within the stem cell compartment. epithelial and age- and tissue-specific stroma plus support cells (slow growth)	Early (fetal) genes
Committed Progenitors	Embryonic	Diploid (mostly G_1)	Primarily governed by epithelial–stromal interactions, but there is also a significant sensitivity to systemic signaling (rapid regenerative responses)	Early (fetal) genes
Early Adult cells	Mixture of embryonic and mature	Diploid (G_1)	Governed by a mixture of paracrine signaling and systemic signaling (growth can be fast)	Early, adult-onset genes
Adult cells	Increasing proportion of mature matrix components	Mixture of diploid and tetraploid (cells in either G_1 or G_2)	Governed predominantly by systemic signaling (growth limited)	Intermediate, adult-onset genes
Terminally differentiated adult cells (apoptotic)	Mature matrix chemistry	Tetraploid/ octaploid (G_2)	Little or no growth; hypertropic responses responses to regenerative stimuli	Late, adult-onset genes

[a]The data in the table are based on studies from skin, hemopoietic tissues, gut, liver, prostate, and brain. The description of the patterns in matrix chemistry are based on studies in the gut and liver. Similar maturational processes in matrix chemistry occur in all tissues, however, there are important variations with respect to matrix chemistry described in the text and summarized in Table 3

[b]*See* Table 3 Embryonic matrix chemistry· type IV collagen, fetal laminins, fetal cell adhesion molecules, fetal proteoglycans, hyaluronates. Mature matrix chemistry. fibrillar collagens, fibronectins, adult cell adhesion moelcules, adult proteoglycans

have yet to be done. Therefore, the existence and/or extent of self-replication among classes of stem cells remains to be elucidated.

One of the only tissues in which stem cells do not persist into adulthood is the heart *(28)*. The last evidence for determined stem cells in human cardiac tissue is approx 3 mo in utero. Thus, at birth, the heart tissue is thought to contain only committed progenitors that give rise to all the mature cells of the heart and that remain stably terminally differentiated for the entire adult lifespan. Although stem cells appear not to be present in the adult heart, unquestionably the major factor in the heart's minimal regenerative capacity, it is unknown whether there are significant numbers of committed progenitors in the adult heart, and if so, what proliferative capacity they have.

2.2. Committed Progenitors: The Amplification Compartment, Responding First to Regenerative and Injury Signals

The cells in the "amplification" or "transit" compartment are "committed progenitors," capable of maturing to only one cellular fate *(1–12,15–20,27,28)* They grow rapidly, are marked by short label-retaining times, and are exquisitely sensitive to injury stimuli, which result in a tremendous expansion of the cell population. These transitional cells are diploid, are larger than the stem cells, have lower nuclear/cytoplasmic ratios (thus, more cytoplasm and cytoplasmic organelles), and have a tissue-specific gene expression that is intermediate between the fetal phenotype of the stem cells and the adult phenotype of the mature cells. Successive divisions result in phenotypically more differentiated cells, and result in the displacement and movement of these cells away from the stem cell compartment.

In the epidermis, intestine, liver, and bone marrow, the stem cells and their lineage of daughter cells are positioned in a polarized organization that makes the cellular gradient apparent *(1–21)*. Adjacent to the stem cells are cells of the amplification compartment, the "transit amplification cells" or committed progenitors. These transitional cells move upward in the intestinal crypts *(4–6)*, upward in the epidermis *(1–3)*, and along plates of fenestrated endothelial cells in the liver *(13–24)* and bone marrow *(7–9)* that extend between a peripheral vascular source (e.g., portal triads) toward a vascular exit (e.g , central vein) *(7–9,13–24)*. In addition to the movement away from the stem cell compartment, transit cells (or other forms of committed progenitors) undergo rapid proliferation, allowing for extensive cellular amplification. Lastly, in a few tissues studied it has been found that maturing cells are lacking telomerase and show signs of shortening of telomeres *(30–33)*. Other molecular mechanisms such as chromatin rearrangements (including loss of some chromatin) and highly regulated methylation processes are associated with terminal differentiation *(29,37–48)*. Empirically, commitment has been found to be unidirec-

tional and irreversible. A cell that has restricted its genetic potential to that for one recognizable adult cell type has never been found to be able to revert to another cell type by any condition in vivo or in vitro.

The known regenerative capacity of tissues is hypothesized to be dependent on the proportion of stem cells and committed progenitors *(14,16,17)* Thus, rapidly renewing tissues such as gut, skin, and hemopoietic tissues are assumed to have the highest numbers of stem cells; rapidly regenerating tissues such as liver or breast are thought to have intermediate numbers; and tissues with limited regenerative capacity (e.g., lung and pancreas) are thought to have the fewest or perhaps to have only committed progenitors. Changes in the kinetics of the lineage would define tissues in a quiescent versus a regenerative state.

Another prediction of classic lineage models is for developmental shifts in the relative proportion of cells at distinct maturational stages. Embryonic tissues are assumed to contain a high percentage of determined stem cells and progenitors along with a maturational gradient skewed towards the early stages of the lineage. Adult tissues would be assumed to contain small numbers of determined stem cells and committed progenitors and with a maturational lineage skewed toward cells late in the lineage. These predictions based on lineage models are supported by current evidence *(11,12,20,30–36)* and by the empirical finding of reduced regenerative capacity of tissues with age.

2.3. Lineage Position-Dependent Gene Expression: The Basis for Heterogeneity of Gene Expression in Tissues

The cells in the differentiation or maturation compartment retain the ability to divide slowly in quiescence, or more rapidly during regeneration, and they undergo a gradual maturation process resulting eventually in terminal differentiation *(1–28)*. Terminal differentiation typically results in loss of mitotic ability accompanied by increases in ploidy and hypertrophic growth of the cells. Multinucleated cells with nuclei that are tetraploid or octaploid are common among tissues capable of quiescent states. In addition, maturation is associated with increasing autofluorescence, because of changes in amounts of fluorescent cytoplasmic pigments such as lipofuscins, and with increasing cytoplasmic "complexity" or "granularity," a parameter of flow cytometric analyses and related to maturation-dependent increases in mitochondria, ribosomes, and other cytoplasmic organelles *(1–9,13,20)*.

The determinants of a cell's position within the lineage and its phenotype at that position have been shown to include a combination of.

1 Autonomous intracellular mechanisms that are division number- or time-dependent such as chromatin rearrangements or methylation events *(30–36,40–48)*.

Table 3
Regulation of Signal Transduction by the Synergistic Effects of Hormones and Extracellular Matrix

Process	External signals affecting
Transcription	Extracellular matrix components: heparin proteoglycans, heparins, fibronectins, and laminin have been found active Matrix/hormone synergies: peptide hormones and steroid hormones in the presence of heparin proteoglycans or heparins Steroid hormones· extent of regulation often less than observed with steroid hormones in combination with extracellular matrix components
Processing	Glucocorticoids.
mRNA stability	Collagens; adhesion proteins; all proteoglycans, heparins, dermatan sulfates, peptide hormones Some of these posttranscriptional effects can be mimicked by membrane permeant analogs of cAMP.
Translational efficiency	Hormones + matrix components
Protein secretion	Proteoglycans and glycosaminoglycans through influence on pH and calcium; certain peptide hormones whose effects can be mimicked by second messengers, collagens, adhesion proteins.
Protein removal and turnover	Proteoglycans and glycosaminoglycans via affects on intracellular pH and Ca^{2+}, via phosphorylation mechanisms

2 Signal transduction mechanisms *(49–76)* (summarized in Table 3) activated by·
   a Gradients of soluble signals (autocrine, paracrine, or endocrine factors), of nutrients, and of gases (O_2/CO_2 levels), in multiple combinations, and/or in synergistic effects with extracellular matrix components *(50–54)*
   b. Gradients of extracellular matrix components. a complex, insoluble mixture of proteins and carbohydrate-rich molecules found on the lateral borders (lateral matrix) and on the basal borders (basal matrix) of all cells *(55–76).*

The heterogeneity of gene expression in all tissues is now recognized to be (in a number of tissues) or assumed to be (in all others) the result of a combination of the microenvironmental changes (hormones, nutrients, O_2/CO_2 levels, and extracellular matrix) and lineage position-dependent genotypic changes. In effect, there are early, intermediate and late genes activated in a lineage position-dependent fashion; identical microenvironmental cues can induce distinct genes at different maturational stages.

Some of the questions for future investigations are:

1. What are the distinctions defining the rapidly renewing tissues (skin, gut, and hemopoietic tissues) versus those that can assume a quiescent state?
2. Is this a distinction in the numbers of determined stem cells or committed progenitors?
3. Do rapidly regenerating tissues simply have such high lineage kinetics that we do not appreciate that they have a quiescent versus regenerative state?
4. Are there other mechanisms, missing or diminished in rapidly regenerating tissues, that can alter the kinetics from a quiescent state to a regenerative condition?

2.4. Signal Transduction Mechanisms

Signals from cell–cell interactions maintain and regulate growth and govern gene expression of a restricted set of genes—those defined by the maturational stage of the cells. A summary of the factors and the aspects of gene expression regulated by them are given in Table 3. Therefore, the same soluble and/or insoluble signal can elicit qualitatively or quantitatively distinct biological responses from different cell types or even the same cell type at distinct maturational stages.

Signals from cell–cell interactions consist of a set that are soluble and a set that are insoluble. Soluble signals include autocrine factors, produced by a cell and active on the same cell; paracrine factors, produced by one cell and acting on a neighboring cell(s); and endocrine factors, produced by one cell and acting on a target cell at a distance from the source cell and delivered to the target cell(s) through blood or lymph. All of these soluble signals, including the autocrine ones, are highly regulated and under very strict controls.

Insoluble signals include components of the extracellular matrix, an insoluble material produced by all cells and found on the lateral borders between homotypic cells, the lateral matrix chemistry, and on the basal surface of cells separating them from heterotypic cell types. The soluble and insoluble signals are interregulated and also have multiple synergistic effects. That is, soluble signals can induce synthesis of specific matrix components; matrix substrata can dictate output of specific soluble signals or regulate receptors for these signals, and distinct sets of matrix components and soluble signals cooperate in the regulation of specific physiological processes. Moreover, the protein sequence of some matrix molecules contains concatemers of sequences for biologically active factors interspersed with enzyme-sensitive sequences. Therefore, proteolysis of the matrix molecules results in localized release of those factors.

Extracellular matrix components turn over slowly, on the order of days to weeks, and serve to stabilize cells in specific configurations of adhesion sites, of antigens, hormone receptors, ion channels, and so on. Since the extracellular matrix is connected directly to the cytoskeleton via transmembrane molecules,

changes in the conformation of one or more matrix components can directly influence cell shape *(77–79)*. The soluble signals that have a more rapid turnover, on the order of seconds to minutes, activate signal transduction processes that induce a specific physiological process such as growth or expression of tissue-specific genes The effect of a soluble factor is entirely dependent, both qualitatively and quantitatively, on the matrix chemistry associated with the cell

2.5. Changes in Matrix Chemistry with Respect to Lineage Position

The lateral matrix chemistry, found between homotypic cells, consists of CAMs and proteoglycans, but no collagens, fibronectins, or laminins *(55–70)* The basal matrix chemistry, found between closely associated heterotypic cell types (e.g , epithelia and mesenchymal cells), consists primarily of CAMs, proteoglycans, basal adhesion molecules (fibronectins or laminins), and collagens The collagen scaffoldings in the basal matrix chemistry of two adjacent cell types are cross-linked, thereby physically connecting the two cell layers.

The chemistry of the basal and lateral matrices change in predictable ways within a maturational lineage *(57,58,68,70)*. All known determined stem cells are associated with a fetal lateral and basal matrix chemistry consisting of fetal isoforms of CAMs and proteoglycans, fetal isoforms of laminin, type IV collagen, and often high levels of hyaluronates. Commitment and subsequent maturation of the cells are associated with a shift, in gradient fashion, in the lateral and basal matrix chemistry. The shift includes a decline in the amount of basal matrix components, a decline or loss in the expression of hyaluronates, a conversion to more and more stable forms of collagens, and a shift to adult isoforms of basal adhesion proteins, of CAMs, and of proteoglycans.

Although all tissues studied have similar basal and lateral matrix chemistries within the stem cell compartment, there are three major variations known in basal and/or lateral matrix chemistry occurring with maturation (summarized in Table 4)·

1. Preservation of expression of all major classes of matrix components but with gradients in isoforms within those classes. Some lineages, exemplified in gut and liver, are associated with a preservation in the expression of all classes of matrix components, but a shift in which isoforms are expressed The liver progenitor cells and intestinal stem cells are associated with a classic embryonic basal lamina consisting of laminin, type IV collagen, fetal CAMs, and specific forms of syndecan, a heparan sulfate proteoglycan Maturation is associated with a gradual shift to increasingly more stable forms of fibrillar collagens, fibronectin, and a heparin-like heparan sulfate proteoglycans, located at highest concentrations in association with the most mature cells (e.g , around the parenchyma near the central vein in the liver) In the past, the liver was considered an "epithelioid

Table 4
Summary of Major Changes in Matrix Chemistry During Development[a]

Known classes of mature matrix		
Gastrointestinal tissue[b]	Epidermal and neuronal tissues	Hemopoietic cells[c]
Retention of all major classes of matrix components for basal and lateral matrix but conversion to mature isoforms	Loss of basal matrix components (collagens, basal adhesion proteins, and integrins) with the committment to terminal differentiation; lateral matrix components convert to mature forms	Lack of collagens; loss of cell binding domains in adhesion proteins in basal states of the mature cells but with possible expression in activated states of those cells; conversion to mature isoforms of other classes of matrix components

[a]Stem cell compartment—embryonic matrix. type IV collagen, fetal laminins, fetal CAMs, fetal proteoglycans (tend to be forms of heparan sulfate protcoglycans), hyaluronates Mature cells—mature matrices various fibrillar collagens, fibronectins, mature CAMs, mature proteoglycans (tend to be higher proportion of dermatan sulfate proteoglycans and heparin proteoglycans)

[b]Also many tissues that can assume a quiescent state (e g , liver, prostate, lung)

[c]Mostly relevant to lymphocytes The literature is still scanty on maturational effects in hemopoietic tissues on extracellular matrix chemistry Therefore, it is difficult to discern patterns that may be true for all classes of hemopoietic cells

organ" and not a true epithelium, because most of the mature liver cells were associated with a form of extracellular matrix in the Space of Disse that is distinct morphologically from basement membranes. Now it is realized that the liver and the gut are both true epithelia with gradients of matrix chemistry paralleling and coordinate with maturing lineages of cells

2 Loss in expression of basal matrix components: The skin and nervous system undergo maturational processes in which there is a loss in the expression of basal matrix components, but retention in expression of lateral matrix components. The lateral matrix components undergo the maturational shifts in chemistry known for all lineages.

3. Loss in expression of collagens and shift to regulated expression of adhesion proteins containing cell-binding domains. Hemopoiesis is associated with loss of collagen expression and with loss of expression of exons encoding the cell binding domains in adhesion proteins in those cells that become free floating Expression of isoforms of the adhesion proteins still containing those cell binding domains is retained by immunocytes, but utilized in a highly regulated way for the many complex cellular interactions involved in immunological responses. Other hemopoietic cell types, especially the monocytic–macrophage lineage, retain the capacity to produce collagens; their matrix chemistry maturational patterns are too poorly characterized to permit generalizations.

Table 5
Common Requirements for Growth by all Maturational Stages of Epithelia and for Tumors

Substratum: type IV collagen, laminin.
Basal medium: any nutrient rich medium.
Calcium concentration. typically below 0.5 m*M*.
Other nutrients: putrescine, folinic acid, hypoxanthine, nicotinamide
Trace elements. varies with the tissue and consist of the co-factor requirements for enzymes typically in the tissue.
Glycosaminoglycan/proteoglycan: tissue-specific species of heparan sulfate proteoglycan, or if it is not available, the heparan sulfate chains added to the basal medium

2.6. Ex Vivo Maintenance of Cells at Distinct Maturational Stages

For optimal maintenance of cells ex vivo, one must define the maturational stage of the cells, the soluble signals, and the matrix signals. By defining all of these, one can reproducibly and with considerable accuracy know how the cells will behave. In the first edition of this review *(80)*, detailed methodologies for the defined conditions for various cell types were provided. There are summary overviews of the principles that guide the development of these protocols given. In particular, there is an update on some of the generic rules for defined ex vivo conditions for cells (Tables 5–8).

2.6.1. Hormonally Defined Media (HDM) Containing Rigorously Defined Nutrients, Hormones, and Growth Factors

Any cell type can be maintained ex vivo, if it is provided with appropriate substratum of extracellular matrix and with a serum-free HDM containing purified hormones, growth factors, trace elements, and nutritional factors *(81,82)*. The exact composition of HDM is tissue-specific and, very rarely, is species-specific. Thus, for example, an HDM developed for optimal growth of rat lung tissue will be virtually identical to that for growth of human lung tissue. For the few tissues for which cells at distinct maturational stages have been isolated in bulk (hemopoietic cells, liver), the exact composition of HDM contains subsets of factors that are identical for all maturational stages and others that are distinct *(17,57)*. Complementing this is the realization that the exact composition of HDM is distinct for cells in an optimal growth versus optimal differentiation state *(57)*.

Classical cell-culture conditions, tissue-culture plastic and serum supplemented medium (SSM), produce cell cultures with minimal differentiated functions caused by inhibition of transcription of tissue-specific mRNAs and

Table 6
Growth Requirements that are Maturationally Stage-Specific

Variables or conditions	Stem cells	Committed progenitors	Mature cells
Lipids	LDL/HDL + FFA + PL[a]	LDL/HDL + FFA + PL[a]	Linoleic acid will suffice, although the ideal is as for progenitors
Feeder layers	Strict dependence on embryonic, tissue-specific stromal feeders and on feeders of support cells	Strict dependence on stromal feeders (tolerant of stromal feeders from diverse sources).	No dependence on feeders.
Mitogens	Local signaling dominant (e.g., IGFII, bFGF, transferrin)	Local signaling dominant (e.g., IGFII, bFGF, transferrin).	Systemic signaling dominant (e.g., insulin, T3, EGF)
Effects of malignant transformation (Tumors)	Poorly differentiated tumors have identical requirements to those of stem cells except for a loss of dependence on age- and tissue-specific mesenchymal feeders and a reduced dependence on other feeders	Well-differentiated carcinomas have requirements that are identical to that of committed progenitors except for loss in the requirement for all feeders	Not relevant.

[a]FFA a mixture of free fatty acids bound to crystalline pure bovine serum albumin and that include linoleic acid, palmitic acid, oleic acid, and stearic acid, PL phospholipids that include phosphatidylcholine, lysophosphatitlylcholine, phosphatidylethianolamine, and sphingomyelin LDL/HDL, low and high density lipoproteins

destabilization (i.e., short-half-lives) of those mRNAs *(14,17,57,81,82)*. Common gene mRNAs (e.g., actin, tubulin) are stabilized by these conditions. Moreover, normal cells, especially epithelial cells, survive for only a few days when cells are plated on tissue-culture plastic substrata and in a serum supplemented medium. Tumor cells and stroma are better able than normal epithelial cells to survive the deleterious effects of serum, a factor in their preferential selection (e.g., stromal cell overgrowth) in classical cell cultures. Serum-free HDM and tissue-culture plastic result in cell cultures that have far more stable tissue-specific messenger RNAs, hence greatly enhanced differentiated function. However, the cells still cannot synthesize their tissue-specific mRNAs at normal rates *(81,82)*. Thus, the cells maintained on tissue-culture plastic and in

Table 7
Differentiation Conditions: Common Requirements for All Maturational Stages and for Tumors

Substratum: flexible, porous substratum coated with fibrillar collagens (e g , type III), fibronectin.[a]
Basal medium. any nutrient rich medium
Lipids LDL, HDL, FFA, and PL [b]
Calcium above 0.5 m*M*
Hormones: glucocorticoids.
Glycosaminoglycan/proteoglycan: tissue-specific forms of heparin proteoglycans or, if not available, unbleached heparin saccharides

[a]The cells, at all maturational stages, must be able to polarize such that their basal surface is specialized for uptake of nutrients and for signaling *(80,81)*

[b]FFA a mixture of free fatty acids bound to crystalline pure bovine serum albumin that include linoleic acid, palmitic acid, oleic acid, and stearic acid, PL phospholipids that include phosphatidylcholine, lysophosphatitlylcholine, phosphatidyletlianolamine, and sphingomyelin LDL/HDL, low and high density lipoproteins

Table 8
Requirements for Differentiation In Vitro that Vary with the Maturational Stage or that Vary with Respect to Distinct Fates

Requirements for polarity
Stem cells: essential for survival of the cells as well as for function
Committed progenitors optimal but not essential for survival, essential for function
Mature parenchymal cells optimal but not essential for survival, growth, or for posttranscriptionally regulated tissue-specific functions; remains essential for full transcriptional regulatory mechanisms
Tumors. optimal but not essential for survival, growth, or some functions
Requirements for feeders
Stem cells. age- and tissue-specific stromal feeders.
Committed progenitors dependent on stromal feeders but can utilize ones that derive from diverse sources
Mature cells. stromal feeders are optimal but not essential for any known physiological state
Tumors: optimal but not essential
Known soluble signals dictating fates
Membrane-permeant cAMP derivatives, TPA, phorbol esters, dimethyl-sulfoxide (DMSO), tissue-specific heparin saccharides
Signals dictating optimal gene expression
Heparin proteoglycan or its heparin chains are required in combination with specific sets of soluble signals for full transcriptional regulation of tissue-specific genes by the indicated soluble signals Each gene requires a precise mix of soluble signals for optimal expression and regulation

HDM regulate entirely or almost entirely under posttranscriptional regulatory mechanisms. Survival of both normal and tumor cells maintained as primary cultures on tissue-culture plastic persist for only a few days, a week at most.

2.6.2 Extracellular Matrix Substrata

Matrix substrata are essential for cell survival and for stabilizing cells in phenotypic configurations that facilitate signal transduction mechanisms activated by soluble signals. The extracellular matrix components can be provided ex vivo either by soluble signals that induce the synthesis of the appropriate matrix components and/or by presenting the cells with prepared substrata of tissue extracts enriched in extracellular matrix or purified matrix components. The purified matrix components, many of them commercially available, produce highly reproducible biological responses in cells particularly when they are used in combination with HDM The availability of some classes of matrix components (e.g , proteoglycans) in rigorously prepared forms, remains quite limited. No individual purified matrix component will enable cells to survive and function for more than a week or two. Rather, long-term stability of survival and physiological responses has been afforded only by extracts enriched in extracellular matrix and, to a lesser extent, mixtures of matrix components. Implicit in these findings is that the matrix chemistry is complex and multifactorial in its effects. Matrix extracts, such as "matrigel" *(17,63,82)*, biomatrix *(17,82)*, and amniotic membrane extracts *(82)*, have induced cells into far more complicated physiological states than any known purified matrix component and are the only conditions known that have permitted differentiated cells to survive ex vivo almost indefinitely. A major function of the matrix is to induce three-dimensional states in cells, essential for achieving full normalcy in cellular phenotype and for normal transcription rates of tissue-specific genes *(78,79)*. The future, therefore, will be in identifying precise mixtures of purified matrix components that confer stable survival and physiological responses.

2.7. Comparison of Normal and Tumor Cells

Neoplastic cells have phenotypes that are quite similar to those of stem cells and committed progenitors *(84–91)*. Therefore, it is logical that tumor cells have ex vivo maintenance requirements that overlap extensively with that of normal progenitor cells, their normal counterparts (Zvibal et al., submitted). The fallacy in many of the prior assumptions about tumors has been in the comparison of them with normal, mature cells. For example, the requirements for ex vivo maintenance of hepatoblastomas are almost identical to that of hepatoblasts, whereas well-differentiated hepatomas or cholangiomas have requirements close to that of the committed hepatic or biliary progenitors, respectively. The phenotypic expression of tumors and of cultures of them also

parallels that in their normal counterparts. Thus, as Van Potter and Pierce *(83–85)* have long argued, tumors are maturationally arrested progenitors, and the expression of fetal genes is simply part of the normal phenotype of the progenitor cells *(80,83)*. Proof of Van Potter and Pierce's hypothesis has been supported by more recent studies comparing normal hepatic progenitor cells versus hepatic tumor cells (Zvibel et al., submitted). For example, matrix/hormonal synergies have been found to regulate fetal (early) genes such as insulin-like growth factor-II (IGF-II) and transforming growth factor-beta (TGFβ), both transcriptionally and posttranscriptionally in tumors *(73)* and in their normal counterparts—hepatic progenitors *(21)*; identical hormone/matrix synergies regulate adult-onset genes in mature parenchymal cells *(17,81)*. If the tumor cells express any adult-onset genes, they are regulated, invariably, by posttranscriptional mechanisms entirely or with muted transcriptional regulatory mechanisms in combination with the posttranscriptional mechanisms.

3. Methods and Protocols

Many of the methods and protocols are as reported in the original edition of this chapter *(80)*. Updates include the modifications to acknowledge ex vivo conditions that must be tailored to maturational stages of cells. Several tables (Tables 5–8) provide summaries of the rules governing variations on the major protocols.

3.1. Isolation of Lineage Position-Specific Cells

The behavior of cells ex vivo depends, in part, on the microenvironment offered to them, and also on the maturational stage of the cells. Identical microenvironments will elicit qualitatively distinct growth responses and expression of tissue-specific genes depending on whether the cells are progenitors, intermediates in the maturational lineage, or terminally differentiated cells. Although this concept is considered a given by investigators in the hemopoietic field, it has only relatively recently been considered by investigators working with cells from other tissues (e.g., ref. *13*).

Numerous specific protocols used for isolation of purified cell populations enriched for specific maturational stages have been reported for various tissues. However, there are some highly successful approaches used by hemopoietic biologists and that are applicable broadly to all tissues (*see* Chapters 16–24). These are the ones described in general (and in brief) *below*. Investigators are encouraged to review the literature relevant to the cell of interest to them to learn of cell parameters possibly unique to their cells and that might offer approaches for purification.

As cells progress through maturational stages, they change in characteristic ways defined rigorously for various tissues by developmental biologists and/or

by those studying apoptosis or senescence, programmed cell death, or necrosis. Among the most common changes that occur with respect to maturation are:

1. Increase in cell size: Stem cells and early progenitors are typically small with cell diameters ranging from 7–15 μm, whereas many fully mature cells can range from 20–50 μm or even larger.
2. Increase in cell "granularity". Granularity is a crude measure of the number of cytoplasmic particles such as mitochondria, ribosomes, and other organelles and is detected by the "side scatter" feature in flow cytometry.
3 Increase in autofluorescence Older cells have more lipofuscins and other factors that fluoresce
4. Changes in antigenic profile· Each cell type has a distinct antigenic profile for each of the maturational stage Investigators can use this fact to develop flow cytometric sorting strategies that with cell sorting result in purification (or enrichment) of these cells The most ideal antigenic profiles for purification of maturationally staged cells are those of surface markers enabling one to purify viable cells
5. Lineage position-dependent changes in tissue-specific gene expression. As stated in more detail in Section 2 3 , every tissue lineage has genes that are characteristically expressed at early, intermediate, or late stages of the lineage.
6. Chemistry of extracellular matrix produced by the cells A review of this is given in Section 2 5 This fact enables investigators to purify cells by matrix molecules (or by receptors for them) that are dominant at particular maturational stages.

To purify particular cells at specific maturational stages will necessitate protocols employing multiple parameters, since there are no known single parameters that uniquely identify cells at a single maturational stage. Also, it is important to note that one must use such purification approaches whether one starts with embryonic, neonatal, or adult tissues. The full maturational lineage exists at all developmental stages, but the proportions of cells that are early, intermediate, or late in the lineage change with developmental age. Embryonic tissues consist of cells predominantly early in the lineage; with age the lineage is truncated increasingly towards cells late in the lineage. The following is an example of a straightforward approach that can be used for multiple tissue types to isolate particular maturational stages of cells. It is to be considered as representative and not as the only approach to achieving the goal of maturationally defined cell populations.

1 Dissociate tissues by protocols minimizing the loss of surface markers (therefore, avoid harsh enzymes)
2. Identify antigenic surface markers of cells to be eliminated. The antibodies that recognize these surface markers must not crossreact with antigens on the cell(s) of interest). Critical control studies are to verify that the antibodies do not crossreact remembering that most antibodies have been screened for crossreactivity

with one but not all maturational stages, therefore, an antibody that does not crossreact, for example, with mature cells may, nevertheless, crossreact with progenitor cells

3 Use the antibodies that are unique and do not crossreact to the cells of interest to eliminate those cells via panning protocols or, for rigorous purification, by flow cytometry

4 After eliminating the cells that are not of interest, use antibodies (or parameters) that identify cells of interest (and ideally of cells at specific maturational stage) Select by panning or by flow cytometry

5 If the purification scheme eliminates many, or most, of the cell types not of interest, one can use measures of "forward scatter" (defining cell size) and/or "side scatter" (defining cell granularity) on flow cytometers to select cells that are broadly defined at early, intermediate, or late maturational stages This works only secondarily to the purification of cells to as few cell types as possible within the samples. Cell size and cell granularity do not, in general, define cell types but rather only maturational stages within a cell type

3.2. Defining the Ex Vivo Requirements for Specific Cell Types

The detailed protocols for defining nutritional, hormonal, extracellular matrix and other requirements for cells are as published previously *(80)* and are not given here. Rather, only summaries are provided.

An approach to defining the soluble signals from cell–cell interactions has been to replace the serum supplement to media with known and purified hormones and growth factors *(80)*. This enables one to produce serum-free HDM Recipes for HDM for other cell types are widely available in the literature. Use of HDM results in selection of the cell type of interest from primary cultures containing multiple cell types. Also, distinct HDM are needed for optimal growth vs differentiation of cells and for optimal expression of specific genes (*see* Tables 6–8 for summaries of generic conditions).

The requirements for tumors or tumor cell lines are identical to, or overlap extensively with that of their normal counterparts, the stem cells (the blastomas and anaplastic cell lines) or committed progenitors (the well-differentiated tumors and cell lines) (*92*; Zvibel et al., submitted), supporting the hypotheses of Van Potter *(84)* and of Sell and Pierce *(85)*. Therefore, the development of an HDM for a cell type can begin by defining the requirements for a cell line that can be used in clonal growth efficiency (CGE) assays (percent of cell colonies that grow at low seeding densities) to assess the effects of candidate factors.

To start the process, one should establish on tissue-culture plastic and in a serum supplemented medium (SSM) the minimal cell densities at which one can detect viable colonies (typically 100–1000 cells/60-mm dish) when developing HDM for cell growth or the minimal cell densities for cell survival (typically 10,000–100,000/60 mm) when developing HDM for optimal cell

differentiation. Use the lowest cell density at which one can routinely obtain colonies (in defining growth conditions) or survival (for defining differentiation conditions) in SSM and on tissue-culture plastic Then establish a negative control at the same density in serum-free medium (SFM):

1 Negative control cells on tissue-culture plastic and in SFM. Minimal survival or growth.
2 Positive control· cells at low densities on tissue-culture plastic and in SSM Maximal survival (differentiation) and or maximal number of colonies (growth)

Substrata of extracellular matrix are essential for the survival of cells and the specific chemistry of the matrix will determine whether the cells are in a state permissive for growth or differentiation. Development of HDM is most easily and readily achieved by first assessing the effect of extracellular matrix on the ability of the cells to undergo clonal growth (for optimizing growth) or on the extent of survival (for optimizing differentiation). As a guide to defining these variables, use those conditions listed in Tables 5 and 7 and found to be the common generic conditions required for growth or differentiation. Therefore, by assessing these generic conditions, one can achieve a new set of base conditions: Most cells will attach and survive on appropriate matrix substrata for 1 wk or more, even if there are no hormonal or growth factor additives in the medium. Therefore, one can use this condition as a base condition in which to test the effects of soluble signals, one by one. Ideally, use purified matrix components (purified collagens, adhesion proteins, etc.) and not matrix extracts (e.g., matrigel, biomatrix) for this process, since it will reduce enormously the number of undefined variables in the microenvironment. Using the matrix substrata, add the cells at the cell densities just defined and establish new base conditions:

3 Negative control using matrix: Number of colonies (for defining and HUM for growth) or number of cells surviving on matrix (for defining an HDM for differentiation) when cells are on an optimal matrix substrata and are in SFM
4. Positive control using matrix. number of colonies (in development of HDM for growth) or number of cells surviving on matrix (in development of HDM for differentiation) when cells are on an optimal matrix and in SSM.

Once these four control conditions are defined, one can then assess soluble factors one by one for their influence on colony growth or on expression of a specific gene. Replicate plates of cells are plated on the appropriate generic matrix substratum optimizing growth (or that optimizing differentiation) and in a plating medium containing the base medium plus additives that will permit attachment to the matrix (e.g., if enzymes were used to prepare single cell suspensions, then the plating medium must contain appropriate inhibitors of those enzymes or even low levels, 1–2% of serum to inactivate them). After a few

hours under the plating conditions, the cultures are rinsed and given the test conditions: SFM plus the test factor(s). In all experiments, replicates under each of the four controls should be included.

The purified hormones and growth factors to be assayed for efficacy for growth or differentiation are prepared as stocks at concentrations that are 1000X of the expected final concentration. The initial concentrations to be tested should be based on any information available from in vivo or in vitro studies on the relevance of the factor to the cell type of interest. Plan to do medium changes that take into account the lability of the factor(s) being tested. In optimizing growth conditions, study further any factor(s) that increases the number of colonies (or increases the cell survival). In surveying for soluble factors relevant to differentiation, one must plate the cells at high cell densities.

4. The Future

The prospects are bright that in the near future, the rapidly developing fields of stem cell biology and lineage mechanisms, matrix biology and chemistry, signal transduction, and tissue engineering will provide information that enables investigators to understand the fundamental principles and molecular mechanisms governing the formation and maintenance of tissues and, on the practical side, enables investigators to maintain any tissue ex vivo in a physiological state approximating that in vivo.

Our recognition that all tissues are organized or "formatted" as progenitors and lineage systems provides an explanation for many past mysteries or paradoxes in basic and clinical research. Several examples are noted:

1. The well-known difficulties by everyone to grow ex vivo any adult, quiescent tissue is owing, in part, to the fact that most of the cells plated in culture are mature cells or cells late in the lineage and with limited growth potential, whatever growth is experienced by the progenitors in those cultures is obscured by sheer mass of more mature cells that rapidly go into growth arrest. In addition, the ex vivo requirements for growth of the progenitors are distinct from those of the mature cells. Therefore, one must purify the progenitors and establish them ex vivo using the defined conditions unique to them Only progenitors from a tissue should be clonable and able to demonstrate extensive proliferative potential
2. The heterogeneity of gene expression within a tissue and the variability in responsiveness of cells to pharmacological and physiological agents is the result of mechanisms governing lineage position-dependent gene expression in combination with the microenvironment. Only by isolating cell populations that are from specific lineage positions can one have cells that uniformly express specific genes It is likely but, in most cases, not yet shown that molecular mechanisms governing gene expression (transcription factors, processing, mRNA metabolism, protein turnover) incorporate lineage position-dependent facets

3 Regenerative processes in all adult tissues should derive from a combination of committed progenitors and from more mature cells; however, steady replacement of the committed progenitors, and, therefore, the ultimate source of regeneration of tissues, will derive from determined stem cells, if they exist in an adult tissue.
4. Aging of an individual is a phenomenon keyed to the collective properties of stem cells in all tissues. The absolute regenerative potential of a tissue over a lifetime is dependent on the number of progenitors in that tissue and their rate of utilization By contrast, apoptosis incorporates molecular mechanisms associated with the maturation of a lineage, whether in an embryo or an elderly person and is secondary to stem cell properties Therefore, most past studies on "aging" were actually studies of apoptosis
5 Gene therapy, to date, has succeeded only using ex vivo modification of hemopoietic progenitors *(93)* Other approaches, particularly use of "targeted injectable vectors," ones injected systemically that naturally target the relevant tissues, have failed either by failure to be taken up uniquely by the relevant targets and/or by transient and very muted expression caused primarily by immunological rejection mechanisms *(93)* Although the huge number of ongoing studies in this area may eventually offer successful methods for "targeted injectable gene therapy," it is more likely that the most successful approaches will be "ex vivo gene therapy" in which investigators will isolate relevant determined stem cells or committed progenitors, expand, transfect and select transfected cells ex vivo, and then reintroduce the transfected cells in vivo
6 Tumor biology is a subset of stem cell biology. That is, tumor cells are stem cells or committed progenitors with mutations that alter their ability to complete the maturational lineage process and their interdependence with support cells Most of the phenotype of tumors is identical to that of their normal counterparts, thus, most so-called tumor markers are actually "early genes" expressed by and regulated identically in normal and transformed progenitors Only by comparing tumor cells that can be mapped to particular stages of the early lineage to their normal progenitor counterparts at the same stages can investigators identify the true distinctions between the normal and the transformed progenitors. The effectiveness of cancer therapies should be dependent on whether the toxic effects of a particular therapy targets the stage of the lineage containing the malignantly transformed cells. Some of the therapies fail because they target and kill more mature cells in the lineage, leaving a transformed progenitor at an earlier stage of the lineage to grow rapidly in a form of regenerative response to compensate for the cellular "vacuum" left by the cancer treatment. In the future, when we know markers defining lineage stages for many tissues, and when we have identified which lineage stages are affected by which cancer therapies, a patient's tumor can be staged for its position in the lineage enabling clinicians to use the optimal cancer therapies that kill cells at that stage of the lineage. Alternatively, if we learn enough of the molecular mechanisms of commitment and how the lineage is driven, we should be able to develop therapies to differentiate tumors, ending their aberrant growth patterns.

In addition, the fields of artificial organs, plastic surgery, drug testing, vaccine production, cell therapy, gene therapy, bioreactors containing mammalian cells to be utilized for production of specific products, and many others will be radically redirected and advanced by some of the understandings now emerging from stem cell and lineage biology, signal transduction and matrix biology and chemistry It is an exciting time and one that will benefit patients and industry as well as research

Acknowledgments

Funding for research derived from an American Cancer Society grant (BE-92C), from an NIH grant (DK44266), a grant from The Council for Tobacco Research (1897), the North Carolina Biotechnology Center, a Cancer Center grant (P30-CA13330), a Liver Center Grant (AM17702), and a CGIBD Center Grant (AM532361).

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5

Scale-Up of Suspension and Anchorage-Dependent Animal Cells

Bryan Griffiths and Denis Looby

1. Introduction

In this chapter, scale-up is described in a laboratory context (10–20 L), but the principles and techniques employed have been successfully adapted so that cells are now grown industrially in unit volumes of up to 10,000 L for vaccine, interferon, and monoclonal antibody production. The need to scale-up cell cultures has been expanded from the historical requirement for vaccine manufacture to include not only interferon and antibodies, but many important medical products such as tissue plasminogen activator, erythropoitin, and a range of hormones and blood factors. The low productivity of animal cells, resulting from their slow growth rate and low expression of product, plus the complexity of the growth conditions and media, led to attempts to use recombinant bacteria to express mammalian cell and virus proteins. However, this has proven unsuitable for many products, mainly because of incomplete expression and contamination with bacterial toxins, and more importance is now being put on expression of recombinant proteins from mammalian cells. This has allowed the use of faster growing and less fastidious cell lines, such as CHO, and amplification of product expression by multiple copies of the gene.

2. Principles of Scale-Up

Animals cells are grown in two completely separate systems. For those cells that grow individually in suspension, the range of fermentation equipment developed for bacteria can be readily modified. This is a great advantage, since these culture vessels are economic in terms of space, the environment is homogeneous and can be critically controlled, and scale-up is relatively straightforward. Many cell types, however, will only grow when attached to a substrate or, in some cases, will only produce significant levels of a product when grown

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

in this mode. Scale-up of substrate attached cells is far more difficult to achieve and has given rise to a wide range of alternative culture systems.

Two approaches to scale-up can be taken. The first is volumetric—a simple increase in volume while retaining the same cell density or process intensity. The second method is to increase the cell density/unit vol 10–100-fold by means of medium perfusion techniques. Cell densities of over 10^8/mL can be achieved in a variety of systems, but they are difficult to volumetrically scale-up because of the effects of nutrient and waste metabolite concentration gradients, however, the development of cultured systems based on the immobilization of cells in porous carriers has overcome this limitation. Compromise is possible with large-scale (100–500 L) cultures operating at just 10–20 times above the conventional cell densities ($1–3 \times 10^7$/mL) by means of special perfusion devices, such as the spin filter.

The environmental factors that can most readily be controlled are pH (and redox) and oxygen. The limiting factor in scale-up, particularly in cell density, is usually oxygen. Surface aeration used in small cultures soon becomes inadequate, since the volume (and therefore depth) of medium increases. Bubbling of air/oxygen mixtures into cultures, with turbulent stirring/agitation, is the most efficient means of oxygenation. Unfortunately, cells are fragile, compared to bacteria and only slow stirring and bubbling rates can be used, which are often inadequate for maintaining a sufficient oxygen supply. To overcome this problem, most cultures rely on several oxygenation methods, and many ingeneous methods have been developed for this purpose *(1)*.

Two further points should be taken into account during scale-up. The first of these is the increased risk of contamination and the proportionally higher costs of culture failure. The second is a question of logistics in the preparation of medium and particularly cell inocula. It is a small matter to harvest 10^8 cells and inoculate them in a good physiological state into a new culture. An inoculum of 10^{10} cells takes a long time to prepare, cells can be left for long periods in damaging conditions, and media can lose its temperature and pH can change while these handling procedures are carried out. The objective is to keep both the process and the culture system as simple as possible, having everything well prepared and ready, and not to be over-ambitious with regard to scale. This will ensure that cultures are initiated with cells in good physiological condition and reduce the risks of microbial contamination

3. Methods

3.1. Suspension Culture

3.1.1. Culture Vessels (Fig. 1)

The simplest means of growing cells in agitated suspension is the spinner culture vessel. The culture pot has a magnetic bar, usually placed a few milli-

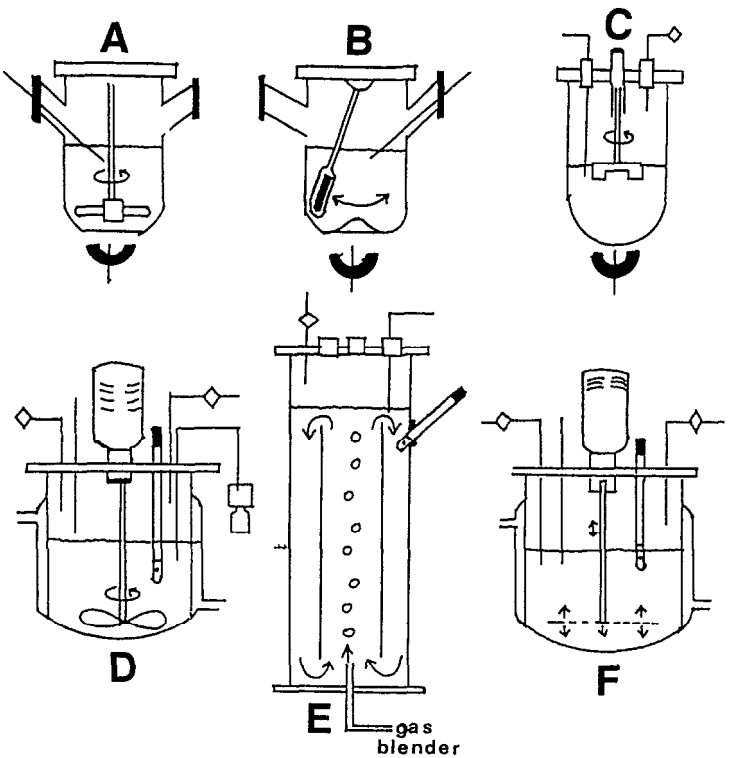

Fig. 1. Range of culture apparatus for suspension cells **(A)** magnetic bar spinner culture, **(B)** Techne MCS stirrer, **(C)** surface stirrer (Techne BR-05/06), **(D)** small scale fermenter with marine impeller, **(E)** airlift fermenter, **(F)** vibro-fermenter (Chemap)

meters from the bottom of the vessel, and is placed on a magnetic stirrer. As long as the bar is able to rotate freely and the stirrer is of sufficient quality to maintain constant stirring speeds, and not overheat, this methodology works extremely well for growing most cells up to densities of 1–2 × 10^6/mL These glass spinner vessels are available from a wide range of suppliers (e g , Bellco, Wheaton) in sizes from 0.2–20 L *(2)*. A modification of this principal for shear-sensitive cells is the spinner vessel using surface agitation as exemplified by the BR-06 Bioreactor (Techne). Spinner vessels are only satisfactory up to a certain size—between 2 and 10 L depending on the cell line and its required use. Above 10 L, glass vessels become inconvenient to handle and the progression to *in situ* stainless steel vessels should be considered. The other reason for change is that, with scale-up, the need to control the culture environment and carry out specialized manipulations (e.g., perfusion, media changes, and so on)

increases. For this level of sophistication, a fermentation system needs to be used. The main differences are that stirring is by a direct-drive mechanism with a motor, and the vessel has a complex top that allows the inclusion of a range of sensors, probes, feed supply lines, and sampling devices for contamination-free monitoring and control. Fermenter kits (laboratory scale) are available in the range from 1–40 L and cost in the region of $4500–37,500 (£3,000–25,000) (with control of stirring speed, temperature, pH, and oxygen).

Culture vessels for animal cells should have the following features·

1. Curved or domed bottom to increase mixing efficiency at the low stirring speeds that are used (100–350 rpm)
2. Water jacket temperature control to avoid the use of immersion heaters that give localized high temperatures Electrically operated silicone pads are also suitable at volumes up to 5 L—the only disadvantage is the reduction in visibility into the vessel
3. Absence of baffles and other sharp protrusions that cause turbulence The interior is finished to a high grade of smoothness to minimize mechanical damage and for cleanliness.
4 An aspect ratio (height to diameter) of 2 1 maximum, and preferably no more than 1.5.1.
5 Suitable impeller to achieve nondamaging bulk flow patterns (e g , modified marine or pitched blade impellers) with top drive, so that there are no combinations of moving parts that would grind up the cells

Some animal cells, including many hybridoma lines, are sensitive to the mechanical effects of stirring. For such cells, there are two alternative means of mixing besides stirring.

1 Vibromixer This is a nonrotary device using a plate that vibrates in the vertical plane a distance of 0 1–3 mm. Conical perforations in the plate affect the mixing (Vibro-fermenter, Chemap)
2 Airlift Medium is circulated in a low velocity bulkflow pattern by being lifted up a central draft tube by rising air bubbles, and recirculated downward in the outer ring formed by the draft tube. This system forms a near ideal mixing pattern and allows near-linear scale-up to at least 1000 L. Unfortunately, the apparatus, with a 12 1 aspect ratio, is very high and a 30-L fermenter needs 3-m ceiling height. Airlift fermenters are available commercially either as complete systems (e.g., Incelltech) or as disposable 570 mL unit (Cellift, Fisher Scientific).

3.1.2. Culture Procedure for Suspension Cells

1 Inoculum should be prepared from a growing suspension of cells (i.e., in mid-to-late logarithmic phase). Stationary phase cells are either slow to start in a fresh culture or do not grow.
2 Prewarm to 37°C, and equilibrate the pH of the culture medium with the CO_2/air gas mix, before inoculating the cells.

3 Inoculate cells at over 1×10^5/mL. Recommended level is $2–3 \times 10^5$/mL for many cell (hybridoma) lines
4 Stir the culture within the range 100–300 rpm This speed depends on the individual type of culture reactor. Stir at a speed sufficient to keep the cells in homogeneous suspension Do not use speeds that allow cells to settle out at the bottom of the culture
5. Monitor cell growth at least daily by taking a small sample, either through a special sampling device or removing the vessel to a laminar flow cabinet and using a pipet, and carry out a viable cell count (Trypan blue stain and a hemocytometer, *see* Chapter 1)
6 pH. If the culture is closed (i.e., all ports stoppered with no filters), then the pH will fall. You should remove the culture to a cabinet and gas the head space with air If the culture is very acid, sterile sodium bicarbonate (5 5%) can be added or, when the cells have settled out, remove 50% of the medium and replace with fresh (prewarmed) medium Return culture to stirrer. It is preferable to have inlet and outlet filters, so that there is continuous head-space gassing, initially (i.e., first 24 h) with 5% CO_2 in air, followed by air only. Suitable filters are nonwettable with a 0 22-μm rating.
7 After 3–4 d, the saturation density of $1–2 \times 10^6$ cells/mL should be attained Most suspension cells will then die at a rapid rate unless harvested or maintained with medium changes

3.1.3. Special Procedures

1 Airlift. Follow the protocol in Section 3 1 2 except, instead of stirring, a gas flow rate of approximately 5–20 cc air/L/min is used for mixing
2. Increase cell density by perfusion To perfuse a culture (i e., the continuous or semicontinuous addition of fresh medium and withdrawal of an equal volume of spent medium) means that methods of separating the cells from the medium are needed There are a number of ways to do this, i.e., spin filters, membranes (e g , hollow fibers), and porous carriers

3.1 3.1 Spin Filter

The problem with most filtration techniques is that the filter rapidly becomes blocked with cells. A spin filter, so called because it is attached to the stirrer shaft, reduces the problem of blockage, because as it spins it produces a boundary effect on its surface that reduces cell contact. Also, they normally have a large surface area and, thus, have a low flow rate at any one point. A porosity of about 6–10 μm is needed, and stainless steel mesh can be used. This is thin enough to be cut to form a cone in which the join can be double-folded and machine-pressed (Fig. 2). This allows a perfusion rate in the order of 1–2 vol/d. This device will allow cell densities of over 10^7 cells/mL to be achieved *(3)*.

3.1.3.2. Hollow-Fiber Cartridges

Hollow-fiber units are available at both ultrafiltration and micro-filtration membranes. For the purposes of withdrawing medium and returning the cells

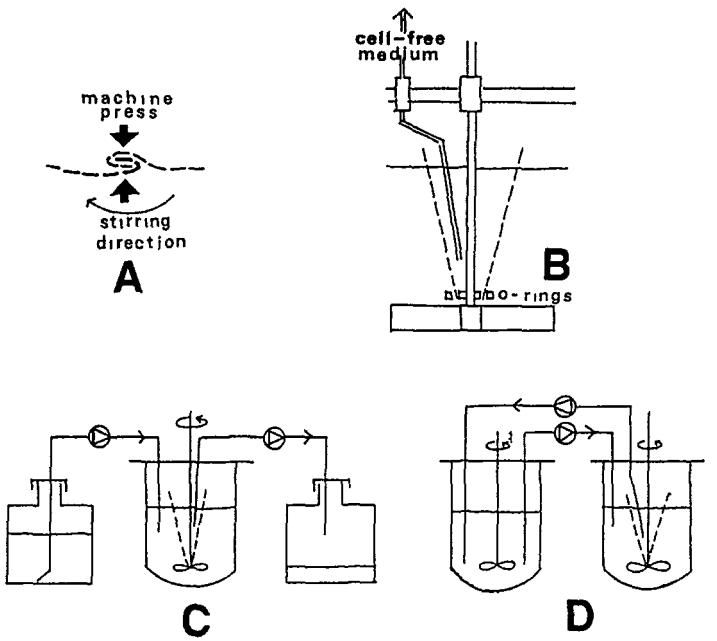

Fig. 2. Spin filters· **(A)** construction using folded interleaving edges, **(B)** diagrammatic representation of a spin filter; **(C)** open perfusion systems, **(D)** perfusion system with media recycle

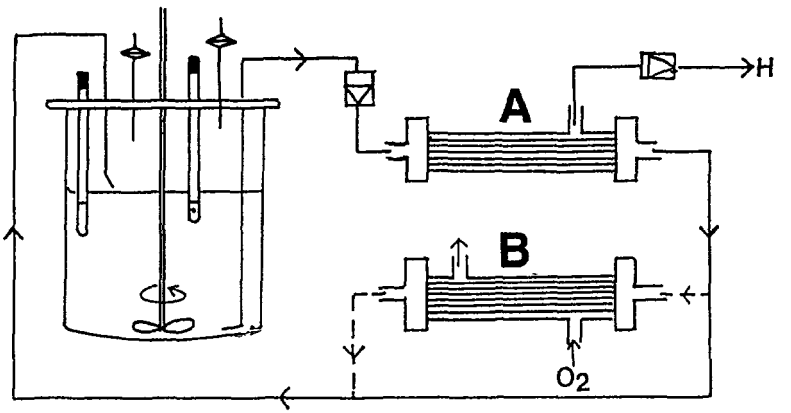

Fig. 3. Hollow-fiber cartridges in perfusion loop for **(A)** removing spent medium (H) and returning cells to the culture and **(B)** oxygenating the medium

to the culture in a loop outside the vessel filtration 0.22 μm is sufficient. The scheme is shown diagrammatically in Fig. 3. Many fiber cartridges are not steam sterilizable, but polysulfone and teflon fibers are. The quantity of medium withdrawn must be balanced by adding fresh medium from a reservoir. Using flow rates of up to 1 vol/h, cell densities in the region of $2–5 \times 10^7$/mL can be achieved, but oxygen is a limiting factor and an additional filtration cartridge should be put in the external loop as an oxygenator.

3.1.4. Notes–Suspension Culture

1 Perfusion Media becomes limiting because of the depletion of nutrients (glucose and glutamine), build-up of waste metabolites (lactate and ammonium), and insufficient oxygen More efficient media usage (cells produced/maintained per unit media consumed) can be achieved in spin filter systems with media recycle through an external reservoir because of improved oxygenation (i e , increased mixing and sparging rates can be used in the cell free environment of the reservoir)
2 Media: Serum is a very high cost addition to culture media, but alternatives such as specialized serum-free media that include a range of growth factors usually work out as expensive, or more so Although cultures may have to be initiated in a complex media, as cell density increases, so cells become less dependent on serum and growth factors Thus, at densities above $5 \times 10^6–10^7$/mL, serum concentration can be drastically reduced (to 2%) or even excluded If serum-free medium is used, cells are more susceptible to damage by stirring and sparging, but this can be offset to a certain extent by adding pluronic F68 (polyglycol) at 0 1% (*See* ref. *4* for other means of cutting the costs of media)
3 Contamination/sterility· The larger the scale, the more expensive a culture failure becomes Carry out stringent quality-control procedures by testing growth media several days before it is to be used for bacterial contamination. Do not take shortcuts on the support equipment, but use specialized tubing connectors and sampling devices supplied by fermenter equipment companies Also, do not overuse the air filter (6–10 sterilization cycles maximum), and do not allow them to get wet (either during autoclaving or with media or condensation)
4 Suspension culture Many cells either attach to the surfaces of the vessel or form unwanted clumps. Media for suspension culture should have a reduced calcium and magnesium-ion concentration (special formulations are commercially available) because of the role of these ions in cell attachment Attachment to the vessel can be discouraged with a pretreatment of a proprietary silicone solution (e g., Repelcote, Hopkins and Williams).

3.2. Anchorage-Dependent Culture

3.2.1. Materials

Anchorage-dependent culture systems are far more difficult to scale-up than suspension cultures because of the additional requirement of providing the extra surface area in an economical (in terms of space) way and still maintain

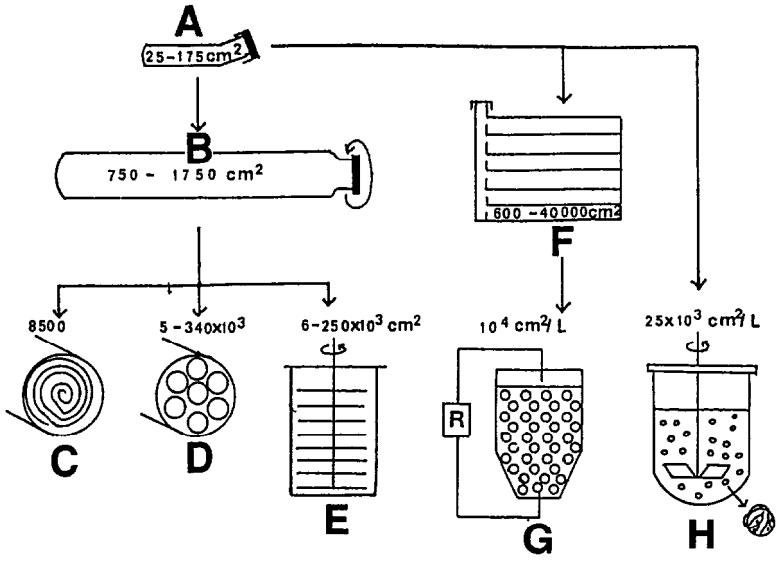

Fig. 4. Scale-up of anchorage-dependent cultures (a) flask, (b) roller bottle, (c) plastic spiral (Sterlin), (d) glass tubing (Belco-Corbeil); (e) stack plates; (f) multi-tray units (NUNC); (g) immobilized bed (glass spheres); (h) microcarrier culture The figures define the surface area for each culture vessel

homogeneity throughout the system. For this reason, suspension culture, in which a 1-L stirred vessel is conceptually similar to a 1000-L vessel, is always the preferred culture method. The first step in scale-up (Fig. 4) usually involves the change from stationary flasks (available in sizes up to 200 cm^2) to roller bottles (sizes up to 1750 cm^2) The larger size of roller bottle will yield in the range of 2–5 × 10^8 cells, and therefore, for most purposes, a multiplicity of rollers has to be used. The next step in scale-up is to use roller bottles that have an increased surface area resulting from the inclusion of glass tubing (Belco-Corbeil, Chemap Gyrogen) or plastic spiral films (Sterlin). By this means, the surface area within a roller bottle can be increased to 8500 cm^2 (spiral film) and 15,000 cm^2 (glass tubing). An alternative to investing in specialized, and costly, roller culture equipment is to use plastic multi-tray units (Nunc). Each tray has a surface area of 600 cm^2 and units of 6, 10, and 40 (24,000 cm^2) plates can be obtained. Two systems that allow a huge unit scale-up of substrate attached cells are immobilized beds (e.g., constructed of glass spheres) and microcarrier culture (cells growing on 200-μm spheres that are stirred in suspension culture apparatus). Microcarriers can provide 5000 to 50,000 cm^2/L and are currently being used in commercial production systems at the 1000-L

scale (a total of 15×10^6 cm^2 surface area, which has the potential of supporting 15×10^{11} cells).

3.2 1.1 Roller Culture

Reutilizable glass or disposable plastic roller cultures are used. The most commonly used sizes are 750–850 cm^2 and 1500–1700 cm^2. Complete modular systems holding up to 48 large bottles can be purchased either free-standing, for use in hot rooms, or within an incubator cabinet.

3.2 1 1.1. Procedure The following procedure is based on a 1500 cm^2 (24 × 12 cm) roller bottle.

1 Add 200–300 mL of medium
2 Add 1 5 × 10^7 cells (observe previously listed advice on preparation of inocula and medium).
3 Revolve the culture at 15 rph
4. Cell growth can be observed under an inverted microscope with a long-distance objective
5 After 3–5 d, the cell sheet will be confluent yielding from 1 5 × 10^5 (human diploid) to 5 × 10^5 (heteroploid cells, e.g., HeLa) cells/cm^2
6 Pour off the medium, wash the cell sheet with prewarmed phosphate buffered saline, and add 20 mL trypsin (0 25%). Place culture back on roller, and allow to revolve for 10–20 min The cells will detach and can be harvested, diluted in fresh medium and serum, and passaged on.

This outline protocol can be considerably modified. An advantage of this method is that the medium volume:surface area ratio can be altered easily. Thus, after a growth phase, and when a product is to be harvested, the medium volume can be reduced to 100 mL in order to obtain higher product concentration.

3.2.1.2. Glass Bead Immobilized Beds *(3,5)*

This type of culture system apparatus is easily fabricated in the laboratory (Fig. 5) A suitable cylindrical glass container is packed with borosilicate glass spheres (minimum diameter 3 mm, optimum diameter 4 or 5 mm). Medium is perfused by means of a peristaltic pump from a reservoir (which ideally is a stirred tank reactor), which can be monitored and controlled for pH, oxygen, and so on. The productive capabilities of the system are give in Table 1.

3.2.1.2.1. Procedure

1. Prepare growth medium and add to reservoir (2 mL/cm^2 culture surface area)
2. Equilibriate the system for temperature and pH, and circulate the media through the packed bed (allow sufficient time for the solid glass spheres to reach 37°C).
3 Inoculate cells (1–2 × 10^4/cm^2) into a volume of medium equal to the void volume of the bed (250 mL/kg 5 mm spheres)

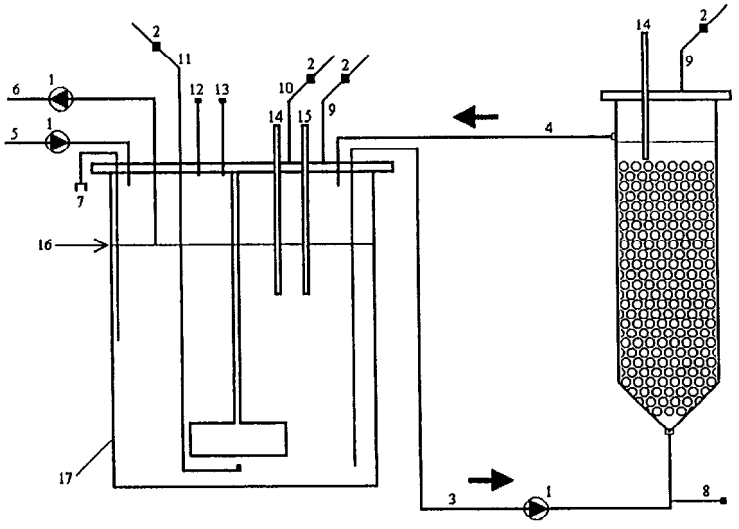

Fig. 5. Schematic diagram of the fixed bed culture system (1) peristaltic pump, (2) air filter, (3) media recycle out, (4) media recycle return, (5) media feed, (6) harvest line, (7) samples port, (8) inoculum line, (9) off gas, (10) head space air/CO_2, (11) air/O_2 sparge, (12) addition lines, (13) DO probe, (15) pH probe, (16) medium level in reservoir, (17) reservoir vessel

4 Allow the cells to attach (3–8 h depending on cell type), but the culture can safely be left overnight (16 h) at this stage
5. Start medium recycle, initially at a rate of 0.1 linear cm/mm, but as the culture progresses, this rate is increased to a maximum of 5 cm/min
6 Cell growth can only be monitored by indirect measurement, and the glucose utilization rate is the most convenient An alternative is oxygen utilization rate Growth yields should be determined for the particular cell line and nutrient, so that an approximation of cell numbers can be made (e.g., glucose utilization is usually within the range of 2–5 × 10^8 cells produced/g glucose)
7 When the culture is estimated to be confluent (after 5–7 d), drain the medium, wash the bed with phosphate-buffered saline wash, and add trypsin/versene to harvest the cells. The efficiency of cell detachment can be increased by intermittently draining and pumping back the trypsin solution The bed acts as a depth filter, and to recover a high percentage of the cells, the bed should be washed through several times with medium after the trypsin has been drained off (Note 5 mm beads allow a better drainage and cell recovery than 3 mm)
8 Wash out the culture bed immediately with a detergent, so that cell debris does not become fixed onto the glass beads

This culture method is basically very simple, and the apparatus is cheap and reutilizable. It has a large potential scale-up and has been proven at the 100 L

Table 1
Physical Characteristics of Glass Sphere Beds

Bead diameter	3 mm	5 mm
Surface area (cm^2)		
Total	7400	4600
Available (70%)	5200	3200
Void medium vol (cc)	295	250
Total vol (cc)	675	625
Cell count (x 105/cm^2)	0.78	2.50
(x 108/kg)	4 0	8 0

Table 2
Commercially Available Microcarriers

Name	Manufacturer	Type	cm^2/g[a]
Acrobead	Galil	Derivitized	5000
Bioglas	Solo Hill Eng.	Glass/latex	350
Bioplas	Solo Hill Eng	Polystyrene	350
Biosilon	Nunc	Polystyrene	255
Cytodex 1,2	Pharmacia	Dextran	6000
Cytodex 3	Pharmacia	Collagen	4600
Cytosphere	Lux	Polystyrene	250
Dormacell	Pfeifer & Langen	Dextran	7000
Gelibead	Hazelton Lab.	Gelatin	3800
Mica	Muller-Lierheim	Polyacrylamide	[b]
Micarcel G	Reactifs IBF	Polyacrylamide	5000
Microdex	Dextran Prod	Dextran	250
Superbeads	Flow Labs	Dextran	6000
Ventregel	Ventrex Lab	Gelatin	4000
Ventreglas	Ventrex Lab	Glass/polystyrene	300

[a]A guideline only as different types vary two- to fivefold in cell yield/cm^2
[b]Porous matrix beads

vol scale (30 L bed of 3 mm beads) *(5)*. It is more suitable for harvesting a secreted cell product over a long period of time, rather than acting as a source of cells resulting from the difficulties of removing cells from the bed.

3.2.1.3 Microcarrier Culture

The advantage of this methodology is that the cells, when growing on small carriers, can be treated as a suspension culture with all the advantages of large unit scale-up, homogeneity, and easily controlled environmental conditions. The range of microcarriers commercially available is extensive (Table 2) *(2,6)*,

and at least one type is suitable for all cell types, however demanding. The decision of which one to use is influenced by whether a dried powder or already-prepared sterile solution is preferred, the cost/cm^2, whether a special derivitized surface is needed for a particular cell, or whether one wishes to harvest cells by dissolving the carrier (gelatin, collagen) and thus producing a higher quality cell suspension. Experience has shown that it is worthwhile to evaluate several types in small-scale cultures for each particular cell line, since significant differences in cell yield, cell-specific productivity, and longevity of culture (before cell detachment) are seen

3 2 1.3.1 Culture Apparatus Modifications of suspension culture vessels are used. Spinner flasks with a magnetic bar are unsuitable, but versions are available with large paddle-type impellers (Bellco) or specially modified stirring actions (e.g., Techne MCS) Stirring rates are much slower (20–70 rpm) than for suspension cells, and thus more efficient mixing at low speeds is required. Scale-up in laboratory fermenters can also use the large-bladed paddles, but there are several modifications of the marine impeller available that are very efficient (e.g., SGI ascenseur)

3.2 1.3.2. Procedure Microcarrier culture is not a difficult technique, but it does require more critical attention to experimental detail than most methods and the use of the correct culture vessels. The following procedure is based on using Cytodex 3 (Pharmacia) or Dormacell 2.3 (Pfeifer & Langen) at 3 g/L. Prepare the microcarriers according to manufacturer's recommendations.

1 It is essential that the medium with microcarriers be prewarmed and stabilized before inoculating the cells Cell attachment to moving spheres requires conditions to be just right It is even more important with this method than with previously described ones to initiate the culture with growing (logarithmic cells) and not stationary-phase cells, and cells that have been rapidly prepared and are in good physiological condition (i e , have not been standing in trypsin for extended periods)
2 Inoculate at 2×10^4/cm^2 into 30–50% of the final volume Stir at the minimum speed to maintain homogeneity (20–30 rpm) for 4–8 h.
3. When the cells have attached (expect 70–90% plating efficiency), the volume can be increased to the full working volume. (Stirring speed may also have to be increased to give complete mixing)
4. A great advantage of the microcarrier system is that samples can be readily removed and microscopically examined. Unstained preparations will show whether or not cells have attached, spread out, and then begun to grow Cell counts can be made by the standard nuclei-counting procedure, which releases the stained nuclei from the attached cells
5. As the culture progresses, the stirring rate can be increased to prevent cell-to-cell attachment, bridging microcarriers and causing clumps to form A maximum of 75 rpm should be achieved.

6 In nonenvironmental controlled cultures, the media will become acidic after 3–4 d and a partial (50–70%) media change should be carried out Stop stirring, allow beads to settle (10 min), decant spent medium, add fresh (prewarmed) medium, and start stirring, gradually increasing the rate
7. Cells can be harvested when confluent by allowing the carriers to settle out, giving a serum-free wash, allowing the carriers to settle out again, decanting off as much of the free fluid as possible, adding trypsin, and restarting stirring, but at slightly faster speed (75–125 rpm) After 20 min, allow the beads to settle out for 2 min Cells can either be removed by decanting, or the mixture can be filtered through a coarse sintered glass filter that allows passage of the cells, not the microcarriers. If gelatin or collagen carriers are being used, then cells can be released by treatment with trypsin/EDTA (which solubilizes gelatin) or collagenase.

3.2.2. Scaling-Up

This can be achieved by increasing the culture volume, and increasing the microcarrier concentration from the suggested 3–15 g/L If higher concentrations are used, then it is imperative to have a perfused system with full environmental control. The easiest means of perfusing is the spin filter (as described for suspension cells), but a much larger pore size can be used (60–100 μm). This allows much faster perfusion rates to be attained (1–2 vol/h) Perfusion from a reservoir that is adequately gassed is an efficient means of oxygenating the culture. Spin filter systems are commercially available (Appeillon, New Brunswick).

3.3. Porous Carrier Culture

Porous carriers can be used for the immobilization of both anchorage-dependent and suspension cells to high densities (0 5–2.0 × 10^8 cells/mL carrier). They can be used in stirred tanks *(6,7)*, fixed beds *(8–10)* and fluidised beds *(8–12)*. Porous carrier systems like hollowfiber systems are high-density continuous perfusion processes, but unlike hollow-fibers they can be scaled-up volumetrically (>100-L bed volumes) The properties of several commercially available porous carriers are given in Table 3.

3.3.1. Fixed Bed Porous Glass Sphere Culture System

This is the same design as the fixed bed system described in Section 3.2.1.2., except that the solid glass spheres are replaced with Siran porous glass spheres (Table 4). This can lead to an increase in unit cell density of up to 50-fold. The system is very suitable for secreted cell products and can be operated as a continuous perfusion process for many months.

1 Set up the system as in Fig 5.
2. Add 10 L of cell culture media to the reservior
3. Equilibrate the system for temperature, pH, and dissolved oxygen (DO)

Table 3
Commercially Available Porous Carriers[a]

Name	Material	Diameter, μm	Pore diameter, μm	Void volume, %
Siran[b] (Schott Glaswerke)	Glass	400–5000	60–300	60
Cultispher[c] (Hyclone)	Gelatin	170–270	10–20	50
Microsphere[b] (Verax)	Collagen	500–600	20–40	75
Cytocell[c] (Pharmacia)	Cellulose	180–210	30	95
Cellsnow[c] (Kirin Ltd)	Cellulose	800–1000	>100	—
IAM carrier[b]	Polyethylene	1200–1500	50–300	—
ImmobaSil[2] (Ashby Scientific)	Silicone rubber	1000	100	40

[a]These include carriers with a high specific gravity suitable for fixed and fluidized beds, and almost neutrally buoyant carriers for use in stirred tank reactors In this chapter, the fixed bed porous glass sphere reactor and the Verax fluidized bed reactor are described

[b]High-specific gravity

[c]Low-specific gravity

Table 4
Characteristics of Siran Porous Glass Spheres

Material	Borosilicate glass
Average diameter	3–5 mm
Pore size	60–300 μm
Pore volume	60% Open
Total surface area	75 m^2/L^a
Biocompatible	Yes
Steam sterilizable/autoclavable	Yes
Reusable	Yes

[a]Surface area based on fixed bed volume

4 Add the inoculum (700 mL of cell suspension containing 12×10^9 cells) to the fixed bed (1 L) of dry porous glass spheres.
5 For suspension cells start media recycle immediately at a linear flow velocity of 2 cpm increasing to 20 cpm with cell growth

6. For anchorage-dependent cells leave the bed stationary for 3–6 h to allow the cells to attach before starting media recycle as in step 5
7. Take a sample from the reservoir daily and carry out the following analysis
   a. Free cell count
   b. Glucose concentration
   c. Product concentration
8. Start media feed and harvest when the glucose concentration drops below 1.5 mg/mL (i.e., for a feed glucose concentration of 4 mg/mL)
9. Adjust the media feed rate to give a glucose concentration in the reservior of 1.5–2 mg/mL (typically 10 L/day in steady-state culture).

3.3.2 Verax Fluidised Bed Culture System

The Verax fluidized bed culture systems range from the System One with a 16 mL fluidized bed capable of producing about 1 L of harvest per day to the System 2000 which has a 24 L fluidized bed capable of producing 1000 L of harvest per day. The process is based on the immobilization of cells (anchorage-dependent and suspension) in porous collagen carriers. The carriers are weighted (specific gravity 1.6) so that they can be used in fluidized beds with a high recycle flow rate (typically 70 linear cpm). The microspheres have a pore size of 20–40 μm, and pore volume of 85%, allowing the immobilisation of cells to high density (1–4 × 10^8 cells/mL). The culture system is based on a fluidised bed bioreactor containing the carriers, through which the culture fluid flows upward at a velocity sufficient to suspend the microspheres in the form of a slurry (i.e., approx 70% bed expansion). For oxygenation the medium is recycled through a membrane oxygenator. The system is run continuously for long culture periods (typically 100 d) (Fig. 6).

This procedure is based on the Verax System One:

1. Set up and calibrate the system as per the manufacturer's instructions
2. Set recycle flow rate to 90 mL/min.
3. Concentrate 5 × 10^7 viable cells into approx 2.1 in a 30-mL syringe with 25-gage needle.
4. Swab the septum 70% with alcohol.
5. Insert the needle through the septum and inject half the inoculum, wait 30 s, and inject the remaining inoculum
6. Take a 3-mL sample from the reactor using a syringe with 25-gage needle daily
7. Carry out the following analysis
   a. Free cell count
   b. Glucose concentration.
   c. Lactate concentration
   d. Product concentration.
8. Start media feed and harvest when the glucose concentration drops below 1.5 mg/mL (i.e., for a feed glucose concentration of 4 mg/mL)

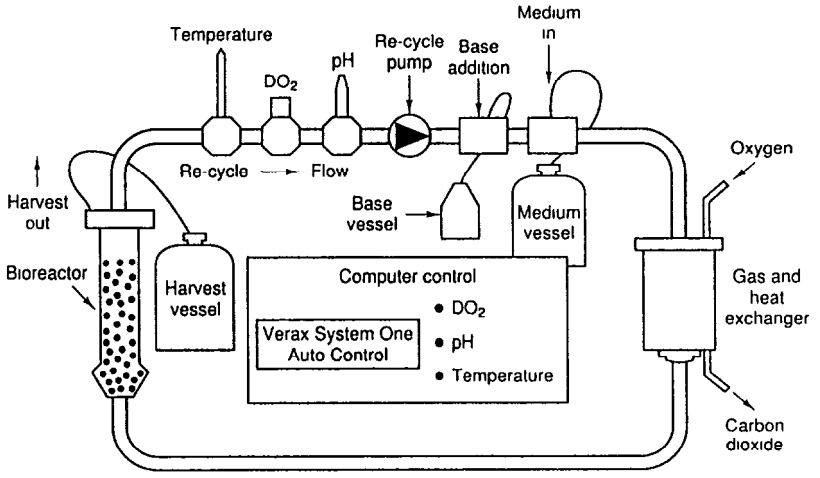

Fig. 6. Schematic diagram of Verax fluidized bed culture system

9 Adjust the media feed rate to give a glucose concentration in the reservoir of 1 5–2 mg/mL (typically 1 L/day in steady-state culture)

4. Conclusion

The stirred tank bioreactor is the most widely accepted culture system for both cells in free suspension and attached to solid microcarriers. It allows volumetric scale up to 10,000 L for batch suspension culture (1–5 × 10^6/mL), 1000 liters for batch microcarrier cultures (1–5 × 10^6/mL), and density scale up (to 1–2 × 10^7/mL) in perfused spin filter cultures. However there are disadvantages with solid microcarrier-based systems that make them unsuitable for some cell lines. In particular, the effects of sparging and microcarrier collisions on the cells can lead to inefficient cell attachment, poor cell growth, and stripping of cells from confluent microcarriers (particularly in long term perfusion culture systems). These limitations are overcome by using porous instead of solid microcarriers because the cells in the porous carriers are protected from the effects of sparging and carrier collisions, and can withstand much higher sparge rates and stirrer speeds than cells attached to solid microcarriers. Porous carrier culture systems have been proven by many workers at the Laboratory scale and by Verax at an industrial scale. Although the superiority of porous carrier systems has been demonstrated, they are not yet widely used in industry.

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6

Hollow-Fiber Cell Culture

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1. Introduction

Hollow-fiber mammalian cell culture systems were first conceived *(1)* to mimic the in vivo cell environment. In tissues, cells exist immobilized at high density, and are perfused via capillaries having semipermeable walls. Fluid (blood) circulating within the capillaries brings oxygen and nutrients and removes CO_2 and other waste products. This description applies equally to hollow-fiber culture systems but with culture medium in the place of blood, and with capillaries made from ultrafiltration or microfiltration membranes.

Hollow-fiber systems have been found to have a number of advantages over other culture systems. These include:

1. High product concentrations: Where a cell secretes a product of higher molecular weight than the cut-off of the fiber membrane, it accumulates in the cell-containing compartment (normally the extracapillary space). As the vast majority of the medium circulates in the intracapillary space, the product is not diluted in this, as would be the case in a homogeneous system (e.g , a stirred tank)
2. A higher ratio of product to culture medium derived contaminants: This greatly facilitates the purification of the secreted product.
3. Reduced requirements for high molecular weight supplements· If the cells require supplements of higher molecular weight than the cut-off of the fiber, then they only need to be supplied to the small proportion of medium in the extracapillary space Often, these supplements can be reduced or omitted entirely once the culture is well established, as the cells themselves may secrete factors sufficient to maintain their own viability
4. A low shear environment The main medium flow is separated from the cells by the capillary membrane Oxygenation also takes place in the intracapillary circuit and/or uses a silicone membrane or similar gas exchange system, and thus the cells are not subjected to potentially damaging contact with bubbles

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

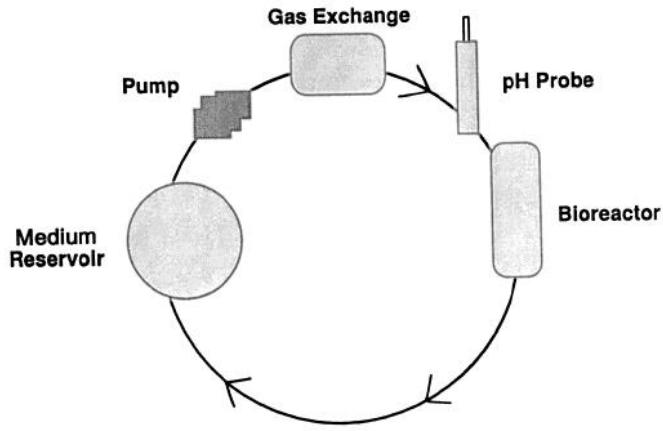

Fig. 1. Schematic diagram of a basic hollow-fiber culture system.

5. Convenience: Compared to a stirred tank capable of producing an equivalent amount of material, a hollow-fiber system requires only a supply of CO_2 and electricity (i.e., no O_2, N_2, compressed air, steam, and drain). It is also a much smaller, bench-top unit.
6. Cost: Again, compared to a stirred tank of similar productivity, hollow-fiber systems are much cheaper *(2)*.

For these reasons, hollow-fiber systems have been widely used for the production of monoclonal antibodies and other high molecular weight secreted products, and this is the type of application that is be described here. These systems have also found favor for many other purposes, such as the extracorporeal expansion of tumour-infiltrating lymphocytes *(3,4)*, and, in particular, as the basis for bioartificial organs such as liver *(5–7)*, but such applications are beyond the scope of this chapter.

1.1. Principles

A basic hollow-fiber system is shown diagrammatically in Fig. 1. Oxygenated medium at the appropriate pH for the cells is circulated through the thousands of capillaries within the hollow-fiber cartridge before being returned to the reservoir and recirculated. In the simplest systems, oxygenation and pH control is achieved by having a gas exchange surface (usually silicone tubing) in a CO_2 incubator. More complex systems have a self-contained gas exchange unit with the mixture of CO_2 and air passing through it controlled by feedback from a sterile pH probe situated in the medium flow. The cells are situated on the outside of the capillaries, in the extracapillary space (ECS). In such a system, nutrient and waste product exchange is largely by diffusion, supplemented

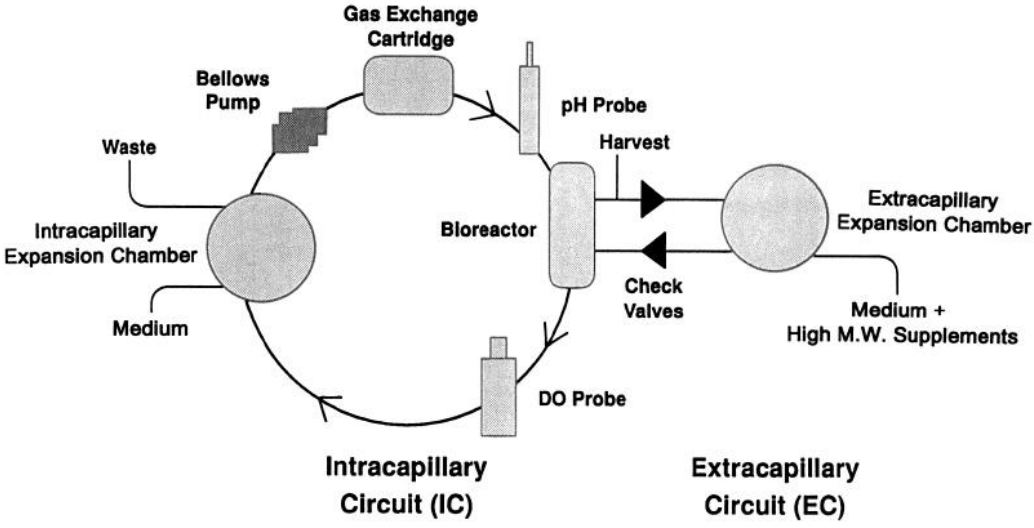

Fig. 2. Schematic diagram of an AcuSyst hollow-fiber culture system.

by "Starling" flow *(8)*—flow out of the capillaries into the ECS at the upstream end of the fibers, and back into the capillaries at the downstream end, due to the pressure drop along the length of the fibers. This can result in the formation of large axial and radial gradients of both nutrients and waste products within the cartridge, leading to nonuniform (and thus suboptimal) colonization of the ECS. This effect can be partially overcome by the periodic reversal of the direction of medium flow in the capillaries *(9)*, but the system which will be used as the basis for the rest of this chapter, the Cellex (Minneapolis, MN) AcuSyst-Jr and related Maximizer 500 and 1000, overcomes these problems by inducing mass flow of medium across the fiber walls. This is achieved by the application of a pressure differential, to first push medium from the capillaries into the ECS and then, by reversing the pressure differential, back again into the capillaries ("cycling"). This system is shown diagrammatically in Fig. 2. The cycling (as well as waste outflow) is controlled by ultrasonic detectors in the base of the extracapillary (EC) and, in some machines, intracapillary (IC) expansion chambers.

Although what follows applies to the specific system mentioned, and is based on the use of hollow-fibers with a 10,000 Da cutoff, the methods have been couched as far as possible in general terms, and should be applicable, with greater or lesser modification, to most hollow-fiber systems.

2. Materials

2.1. Cell Line Characterization

1 Culture media and supplements
2 75-cm^2 Tissue-culture flasks
3 Carbon dioxide/air mixture(s)

2.2. Flowpath Preparation

1. Autoclavable pH and dissolved oxygen (DO) probes and cases
2 DO probe membranes and electrolyte
3 Phosphate-buffered saline.
4 Autoclave (must be big enough to accept the pH and DO probes standing vertically)
5. DO and pH probe leads
6 Temperature probe
7. Gas lines.
8 Endotronics multiport white caps with air vent filters (0 2 μm)
9. Sterile muslin gauze swabs
10. 0 2% Hibitane in 70% ethanol solution
11. Sterile injection site septa.
12. Sterile container with 3 l of sterile water, another with 10 l of basal medium, a third with medium supplemented with any high molecular weight substances required by the cells, and a fourth for waste. Bottles can be connected to the flowpath using item 8, other containers (e.g., sterile bags) should be equipped with female luer connectors

2.3. Inoculation

1 A healthy culture of a sufficient number of appropriate cells in mid-log growth phase.
2 80 mL of Culture medium
3 60- and 10-mL Syringes

2.4. Growth Phase

1. Glucose and lactate assays.
2. Cell-line characterization/set point determination data (*see* Section 3.1.).

2.5. Monitoring and Maintenance

1. pH Meter with standards.
2. DO Meter with standards.
3. Assays for glucose and lactate (and ammonia and glutamine if required), and product.
4. Sterility assays
5. Syringes for sucking cell debris from the flowpath.

2.6. Removal of Cells

1 Gate clamps
2 250 mL of basal (or supplemented) medium in a sterile bottle, fitted with tubing and adaptor to connect into the EC circuit between the lower nonreturn valve and the hollow-fiber cartridge

3. 250 mL (or larger) sterile bottle fitted with tubing and adaptor to fit EC bioreactor out/inoculation port
4. Peristaltic pump, e.g , Watson-Marlow 500 series

3. Methods

3.1. Cell Line Characterization/Set-Point Determination

Before attempting to culture any cell-line in a hollow-fiber system, it is essential to characterize the cells' behavior in culture. In particular, it is important to ascertain the optimum pH for growth of the cell line, since the sooner after inoculation the cells multiply to the point where they have fully colonized all the available space within the ECS ("pack out" the ECS), the sooner high levels of product can be harvested. The cells' tolerance to lactate, and requirements for glucose are also useful to know, as these will help in making decisions on the replacement rate of the basal medium (that circulated in the IC circuit). It is also useful to have some idea of the lowest pH at which the cells retain viability, since the range between this and the optimal growth pH will constitute the pH range within which the system can be run. Data on productivity of the substance of interest at different pHs is also of use. Sadly, there is no simple way of quickly getting all of this information. However, a growth study in T-flasks is a good starting point; armed with the data from this, optimization experiments can then be performed in the hollow-fiber system itself (*see* Note 1).

1 Decide on suitable culture media and supplement types to test, and adapt the cells to these media by multiple passage in tissue-culture flasks.
2 Pre-gas three 75-cm^2 tissue-culture flasks per test medium with carbon dioxide/air mixture(s) according to media requirements.
3. Prepare inocula by centrifuging mid log-phase cell suspensions and resuspending the pellets in the minimum volume of chosen media. Use cells at maximum possible viability
4. Inoculate two 75-cm^2 flasks with between 1×10^5 and 2×10^5 viable cells/mL (or a lower concentration if possible) for each medium. The final culture volume should be 50 mL. The remaining flasks are blank controls with 50 mL of medium only.
5 Incubate the flasks at 37°C
6. Aseptically remove a 3-mL sample from each flask 2 h after inoculation Record this as time zero. The sample volume may be adjusted according to assay requirements (*see* step 8)
7. Sample each flask daily for the following 10 d
8. Measure pH and cell viability immediately after sampling Also measure glucose, lactate, ammonia, glutamine, and product concentration. Control flasks are assayed for pH, ammonia, and glutamine only

9. Display the data graphically. If the cultures have not reached a stationary/death phase by d 10, continue sampling every other day until d 14 If a stationary/death phase has still not been reached, repeat the procedure with double the inoculum cell density (*see* step 4)

10 From the graph identify
   a The pH at which maximum cell growth rate occurs. This is used to determine the "growth" phase pH
   b. The pHs at which maximum product secretion with minimum growth rate occur This is used to decide on the "production" phase pH.
   c The glucose, lactate, and glutamine concentrations at and around maximum growth and maximum product secretion rates These act as a guide in determining the glucose, lactate, and glutamine "growth" and "production" setpoints to aim for, and identify limits during the culture
   d. The ammonia production profile This indicates ammonia concentration limits during culture

3.2. Flowpath Preparation

Different machines have flowpaths with different degrees of re-usability However, in the AcuSyst systems, the pH and DO probes are the only major components of the flowpath that can be re-used; the rest comes as a unit that has been presterilized with ethylene oxide. Preparation involves sterilizing the probes and connecting them to the rest of the flowpath, installing the flowpath in the incubator/control module, pressure testing the assembly, and flushing the system (to remove toxic ethylene oxide residue, and glycerine from the hollow-fibers) and filling with medium.

In this method, the details relate specifically to the AcuSyst-Jr., and will need adapting for use with any other system.

1 Re-membrane DO probe according to manufacturer's instructions Check operation. A 1% solution of Na_2SO_3 is useful in order to check a probe's ability to give a zero reading, and the speed with which it will do so
2. Assemble pH and DO probes in their respective cases (these cases enable the probes to be plumbed into the flowpath such that medium in the IC circuit will flow past them continuously).
3 Place a small amount of phosphate-buffered saline in each probe case Bag both probes in autoclave bags
4. With the probes in an upright position (use a suitable rack or basket) autoclave the probes using a liquid cycle at 121°C for at least 15 min
5. Remove the pre-sterilized cultureware from its box. Spray and swab down the covering with 70% ethanol in water, and allow the package to dry under HEPA filtered laminar-flow air in a clean safety cabinet Check package for damage or punctures.
6. Open and discard the packaging and remove the cultureware Untape and discard padding.

7 Immediately check and tighten all connections The flowpath is a closed system with all possible routes into the system protected by filters, covers or plugs Ensure all these are in place
8. Close all clamps, unkink all tubes Check all lines are labeled correctly.
9 Aseptically connect the pH and DO probe assemblies following the instructions supplied with the flowpath To facilitate aseptic manipulation of the connections they may be wrapped for a minimum of three min in autoclaved muslin gauze swabs soaked in 0.2% Hibitane in 70% ethanol (*see* Note 2).
10. Aseptically attach sterile injection site septa to the sample ports.
11. Remove the flowpath from the safety cabinet and gently slide it into the incubator chamber. Load the circulation pump into its holder, and secure
12. Plug the leads from the DO, pH and temperature probes, and IC and EC ultrasonic detectors into the appropriate sockets Position the temperature probe in its holder in the flowpath.
13 Attach the gas lines to their correct ports, and their corresponding positions in the flowpath.
14. Assemble the peristaltic pump heads and load all except the outflow pump tube segments.
15. Test the integrity of the flowpath following the steps detailed on the liquid crystal display (LCD) unit
16. Reverse-load the outflow tubing into its pump head, aseptically connect 3 L of sterile water to this line and flush the system with it, again following the instructions on the LCD unit. Swap the vessel which had the water for one containing sterile basal medium, and flush 3 L of this through the system.
17. Reload the outflow tubing, this time in its correct orientation, and connect to a waste vessel Connect basal medium to the medium pump (which supplies the IC circuit) and medium containing any high molecular weight substances to the F3 pump (which supplies the E C. circuit). If required, pump a suitable volume of this supplemented medium into the ECS to condition the outside of the fibers prior to inoculating with cells.
18 Set the medium pump at 25 mL/h and set the machine running (without cells) for 2 d. Check for the presence of any residual cytotoxic compounds in the flowpath by then taking a sample of the medium from both the IC and EC circuits, and check that cells plated out at low density will grow in this medium. If not, continue to run the machine, with the medium pump set at 25 mL/h for several more days and try again Once growth in both IC and EC samples is satisfactory, the flowpath is ready for inoculation

3.3. Inoculation

Cells can grow to very high densities (1–2 × 10^8/mL) in hollow-fiber systems, but must also be introduced in quite large numbers to ensure growth within the system In our experience with antibody producing cells, the number that should be inoculated to ensure growth is at least 2 × 10^6/mL of EC space in the hollow-fiber cartridge, and it may be advantageous to add larger

numbers, up to 8×10^6/mL of EC space. (These figures are equivalent to 2×10^8 and 8×10^8 cells/m^2 of fiber surface area, respectively.) The number will, however, vary with the cell line. Fast-growing rodent cells may thrive from a smaller inoculum, whereas anchorage-dependent and genetically engineered cells may need to be used at the upper end of this range.

1 Culture the cells so that they are growing at maximum rate and with maximum viability (*see* Note 3). It is often best to feed the culture with fresh medium 1–3 d before inoculation
2. Concentrate the cells by centrifuging under conditions that will maintain maximum viability (other strategies, such as settling out, are also possible). Resuspend the pellets in a total of 60 mL of medium, which may be either fresh or from the culture supernatant, depending on the preference of the cell-line in use
3 Transfer these cells to the 60 mL syringe Add 10 mL of medium to the smaller syringe
4. Connect the cell-containing syringe to the inoculation port (which connects with the ECS of the hollow-fiber cartridge) and gently inject the cells Swap syringes and flush the line through with the 10 mL of medium In the AcuSyst, this step should be performed following the prompts on the LCD screen

3.4. "Growth" Phase

After inoculation there follows a period when the cells are actively multiplying to pack out the ECS of the hollow-fiber cartridge (the growth phase). The conditions used at this time are generally those for optimal growth as ascertained in Section 3.1. In machines equipped with it, cycling is not started immediately, in order to give the cells the opportunity of colonizing the fibers undisturbed by mass flow of medium. Similarly, the addition of supplemented medium to, and harvesting of product from, the ECS is delayed to allow the cells to condition the medium and grow to higher densities before medium removal starts. This has to be balanced against the possible build-up of any high molecular weight toxic compounds that a cell-line might produce.

1. Set pH to the optimum for growth as previously ascertained (Section 3.1), set basal medium feed to 25 mL/h, and program in appropriate delays for cycling and for supplemented medium and harvest pumps (suggested delays· 12 h to 7 d for cycling, 3–14 d for supplemented medium/harvest, depending on cell line). In the AcuSyst, this step should be done prior to inoculation, as it will automatically switch into growth phase process control once inoculation is complete.
2. Assay glucose and lactate levels in the medium in the IC circuit every 24–48 h (*see* Note 4)
3. When glucose and lactate assays indicate that glucose levels are starting to become limiting, or more commonly when lactate levels reach 50% to 75% of the maximum acceptable level defined previously, increase the medium pump rate Increasing it from 25–50 mL/h will be adequate for most cell lines at this stage.

4. Repeat steps 2 and 3, increasing the medium pump rate as required until the conditions in step 6 are fulfilled
5 When addition of supplemented medium to the EC compartment and harvesting of product start, make sure the two pump rates are adjusted to provide the cells with the required concentration of supplements The best way to do this is to supply the supplements at the required concentration and harvest product at the same rate
6 When the medium pump rate reaches its maximum (or the maximum rate at which you are prepared to supply medium to the machine, whichever is lower), or the glucose and lactate (and product) levels reach a plateau, the growth phase is at an end

3.5. "Production" Phase

Where the media supply rate has been the deciding factor, you are now no longer able to cope with the metabolic demands of the cells at this pH and it is necessary to reduce the pH with reference to the data obtained in Section 3.1, the aim being to reduce the growth and consequent metabolic activity of the cells while retaining product secretion. The hollow-fiber cartridge may still not be completely packed out with cells at this stage If, however, the glucose, lactate, and product levels have reached a plateau, then the cartridge has become completely packed out with cells without cellular metabolism placing inordinate demands on the medium supply. This usually only happens with slow-growing cell lines. Thus, although the growth phase has ended in terms of the cells multiplying freely to fill the cartridge, the culture may continue at this same pH, or may be switched to a lower pH if it is advantageous in terms of product secretion rate.

The characteristic of the production phase is that the cells are held under more or less constant conditions (usually with lower cell growth rate) than in the growth phase (*see* Note 5). Thus the frequency of monitoring assays can be reduced. However, a new requirement now arises because, as the hollow-fiber cartridge is now filled with cells, excess cells and cellular debris are deposited in the bottom of the EC expansion chamber. If left to accumulate, this material will eventually affect the fluid flow and the ultrasonic detectors in the chamber, which can lead to all medium being removed from the cells in the hollow-fiber cartridge. Thus regular removal of this material is essential.

Assuming no contamination, the length of the culture is determined by one of two factors. The culture may lose productivity over a period of months, usually the result of increasing quantities of dead cells and cell debris in the hollow-fiber cartridge. Alternatively one or other part of the flowpath may become blocked, again due to the accumulation of dead cells and debris. This is usually first noticed in the AcuSyst when it is unable to maintain its cycling time. In our experience, runs of 4–6 mo are normal.

3.6. Monitoring and Maintenance

1. Inspect system daily for leaks, irregular cycling, adequate liquid levels in media feeds, harvest and waste containers, and generally for normal functioning Keep a log of every action taken. Do not forget to check the CO_2 supply—running out can kill the culture (*see* Note 6)
2. Calibrate the pH and DO probes prior to inoculation of the cells and then at least once a week. Recalibrate both probes immediately after a power interruption or microprocessor communication failure
3. During the growth phase assay glucose and lactate (and, if required, ammonia and glutamine) levels every 24–48 h. Once in production phase, these measurements may be made less frequently, but not less than once a week, and additionally if a process control parameter (e g , pH, media feed rate) is changed
4 Measure product concentration regularly, i e , on every batch of product, plus samples from the EC circuit if required
5 Both IC and EC samples should be checked at least once a month for sterility
6. Check for the accumulation of debris in the EC chamber This should be removed at regular intervals that will depend on the cell line Too much debris will lead either to blocking of the EC circuit nonreturn valves, or malfunction of the ultrasonic detectors

3.7. Removal of Cells from the Hollow-Fiber Cartridge at the End of a Run

Eventually a run will be terminated, either for one of the reasons mentioned in Section 3.5., or because enough product has been made. At this time it may be appropriate to remove a sample of the cultured cells from the hollow-fiber cartridge, possibly for testing immediately, but more often to enable samples to be frozen down for future reference, i.e., for making a post-production cell bank (*see* Note 7). The method that follows has proven satisfactory for human lymphoblastoid cells, yielding (after 4 mo of culture) post-production cell banks indistinguishable by both single- and multilocus DNA fingerprinting from the cells used for inoculation. Other approaches to extracting the cells can be taken, and in particular it may be necessary to use enzyme treatment to remove cells which are normally anchorage-dependent.

1. Abort the process (this stops all pumps, cycling, etc).
2 Clamp off IC circuit tubing into and out of the hollow-fiber cartridge
3. Clamp EC circuit tubing, upstream of lower nonreturn valve, and between the harvest removal line and the upper nonreturn valve
4. Using aseptic technique, pull EC circuit tubing off the downstream end of the lower nonreturn valve, and connect to the bottle containing 250 mL of medium
5 Connect empty bottle to EC bioreactor out/inoculate port.
6. Check that all clamps are open along the path that the medium will take from the bottle through the hollow-fiber cartridge to the receiving bottle

7 Load part of the tubing between the medium bottle and the flowpath into the peristaltic pump.
8 Pump the medium rapidly through the hollow-fiber cartridge (but not so fast as to blow off the tubing connectors). This will flush a significant proportion (but not all) of the cells from the cartridge, along with dead cells and debris
9. Separate the viable cells from the dead cells and debris, either by culturing them or by using a physical separation technique
10 If a post-production cell bank is to be made, culture the cells to obtain maximum viability and freeze according to standard procedures.

4. Notes

1. Although the technique described is easy to carry out, extrapolating the data from such a "closed" system, where cells experience constantly changing pH, nutrient, and waste levels, to the more "open" hollow-fiber system where most parameters are held constant by the supply of fresh nutrients/buffering agents and the constant removal of waste products, is difficult In practice, the growth pH is usually easy to ascertain, whereas the production pH is usually a compromise between productivity and excessive metabolic demands of the cells Using the data gained from culture in flasks the growth pH is usually accurate, but the pH which appears to be optimum for the production phase frequently proves to be too low when tried in a hollow-fiber system. Err, therefore, on the side of caution, or you could kill your cells by using too low a pH The question of whether, in fact, a pH change is required between growth and production can only be answered by experimentation using a hollow-fiber system.
2. This, and all the subsequent steps in running a hollow-fiber culture system, requires aseptic technique of the highest calibre A large number of aseptic procedures are involved, particularly if one is to run the system for 4–6 mo, and each carries with it a risk of contaminating the culture Given good technique, it is possible to run a system in an ordinary laboratory, without the use of antibiotics, for 6 mo, as we have done on numerous occasions
3. The condition of the cells used for inoculation is of paramount importance to the subsequent success and productivity of the culture. Do whatever is necessary to get cells of the highest possible viability, that are growing as fast as possible and are in mid-log phase Be careful not to damage the cells when concentrating them prior to inoculation Similarly do not inject them into the hollow-fiber system too fast, as they may be damaged by high shear forces when passing through narrow-bore tubing.
4. Keep a close eye on the glucose and lactate levels at this stage, as these can change quite quickly and if adjustments are not made, for example to the medium flow rate, may kill the culture. We always measure the glucose and lactate levels every day for at least the first 10 d, and longer if necessary My advice is to do the same, and arrange that someone who is competent to make decisions regarding pump rates and so on, be available to take measurements over the weekend(s)

5. Barring contamination, the production phase should be the vast majority of the culture period It is less demanding on labor and assays than the growth phase, and weekend working should not be necessary on a routine basis
6. The key here with monitoring and maintenance is just to keep a careful eye on everything, both the culture itself and the hardware containing it—and never relax the quality of your aseptic technique! If microbial contamination should occur it is usually obvious, characterized either by a rapid drop in dissolved oxygen levels in the medium, or a disruption of the normal relationship between glucose utilization and lactate production However, cryptic contaminations can occur, and hence the recommendation to perform sterility tests on a regular basis
7. It is useful to remove cells from the hollow-fiber cartridge at the end of a run and make a post-production cell bank at the end of every run, as material is then always available for reference if any queries arise regarding a culture at a later date. Cells can also be extracted from the system during the course of a culture, and cell banks made if required, by removing medium from the harvest line prior to it reaching the peristaltic pump head. The medium will again contain a mixture of live cells, dead cells and cell debris It is usually necessary to remove a significant volume (e.g , 50 mL) in order to get sufficient cells for most purposes, such as culture to make a cell bank, as in our experience the sort of viable cell densities are well below 10^5/mL. This will, however, vary with the stage of the culture and the cell line.

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7

Separation and Maintenance of Primary T- and B-Lymphocytes

Derek Kinchington and Tony Ng

1. Introduction

Two distinct populations of lymphocytes have been identified: T-lymphocytes, which are thymus dependent, and B-cells, which were first observed in the Bursa Fabricus of birds. Mammals do not have an equivalent structure, and there are varying opinions as to the similarity of these cells between species. In humans, current theories are that B-lymphocytes differentiate in the fetal liver and in the bone marrow of adults. Human T- and B-cells are most easily obtained either from peripheral blood or from biopsy of lymphoid tissues (lymph nodes, spleen, Peyer's patches from gut, tonsils, and adenoids)

To establish T- and B-lymphocytes from clinical material requires three steps: separation from blood or other tissues, enrichment for either B- or T-cells, and finally the maintenance of the primary cultures It may be necessary to carry out several enrichment steps to get >90% purity. These methods are described in turn.

2. Materials

1. 1 μg/mL Acridine orange.
2. 2-Aminotrylisotriouronum bromide hydrobromide (AET) treatment of sheep red blood cells (SRBG)· Dissolve 402 mg AET in 10 mL distilled water and adjust the pH to 9.0 with 1*M* NaOH. Do not overshoot Mix one packed volume washed SRBG in 4 vol AET, incubate at 37°C for 25 min, and mix regularly Wash five times in saline, and then resuspend in RPMI and HEPES buffer. This will keep for 1 wk at 4°C.
3. B-cell growth factor (BCGF).
4. *Clostridium perfringens* neuraminidase
5. Epstein Barr Virus (EBV) suspension, 5×10^4 transforming units (TFU)

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

6 2 μg/mL Ethidium bromide
7 F(ab')$_2$ fragment of goat antihuman IgM (μ-chain specific)
8. Ficoll-paque
9 RPMI growth medium contains 5, 10, or 15% fetal calf serum (FCS) (*see* Note 1), 2 m*M* L-glutamine, 100 μg/mL penicillin, and 100 mg/mL streptomycin
10 Growth medium containing 10% FCS, and 2-mercaptoethanol (2×10^{-5}*M*).
11. Hanks' balanced salt solution, pH 4.0 (*see* Appendix).
12 *Helix pomatia* Lectin-Sepharose 6 MB.
13. Heparin
14. IgG: antihuman.
15 IgG. pan-T or pan-B.
16. Immunomagnetic beads (Dynabeads and DETAGHaBEADS Dynal, Wirral, UK)
17 Interleukin 2,10–40 U/mL final concentration (*see* Note 2)
18. Nylonwool: sterile
19 Pharmacia column K9/15 fitted with an 80-μm mesh net
20. Phosphate-buffered saline (Dulbecco A) (PBSA)
21 PBSA containing 0.2% human serum albumin and 0 02% sodium azide
22. Phytohemagglutinin (PHA) (1 mg/mL stock)
23. Propidium iodide, diluted to 50 μg/mL in PBS (pH 7 4) containing 0 1% Triton X-100, 0 1 m*M* disodium EDTA and RNase at 50 μg/mL
24 Protein A-Sepharose 6 MB (Pharmacia).
25. 0.1% Trypan blue.

3. Methods

3.1. Separation of Lymphocytes from Blood Using Ficoll

Commercial products such as Ficoll-paque mixtures are available to separate human mononuclear cell populations contaminated with RBG and granulocytes.

1. Collect blood into tubes containing 10 units/mL preservative free heparin
2 Dilute blood 1·2 in serum-free medium and layer it carefully onto the Ficoll-paque using a Pasteur pipet to produce a clean interface between the two layers To obtain the maximum yield, it is advisable to keep the proportion of blood to Ficoll in a ratio of 1 3. If small volumes of blood are being separated, an 11-mL centrifuge tube is used with 3 mL blood layered onto 7 mL Ficoll-paque
3. Centrifuge at 800*g* for 25 min at room temperature.
4 Collect the white opaque mononuclear fraction from the interface between the diluent and the Ficoll-paque, and add at least 5 vol of serum-free medium. Centrifuge at 400*g* for 10 min Repeat for two more times to remove the Ficoll-paque, which can be toxic to cultured cells
5. Count the lymphocytes using Trypan blue exclusion (*see* Chapter 1) and culture in RPMI containing 10% FCS (Fig. 1). A small volume of medium containing cells is mixed with an equal volume of 0.2% trypan blue dissolved in PBS Cells are counted using a hemocytometer or disposable counting chambers (Kova, Hycor Biomedical Inc , Irving, CA). Alternatively cells can be resuspended in an

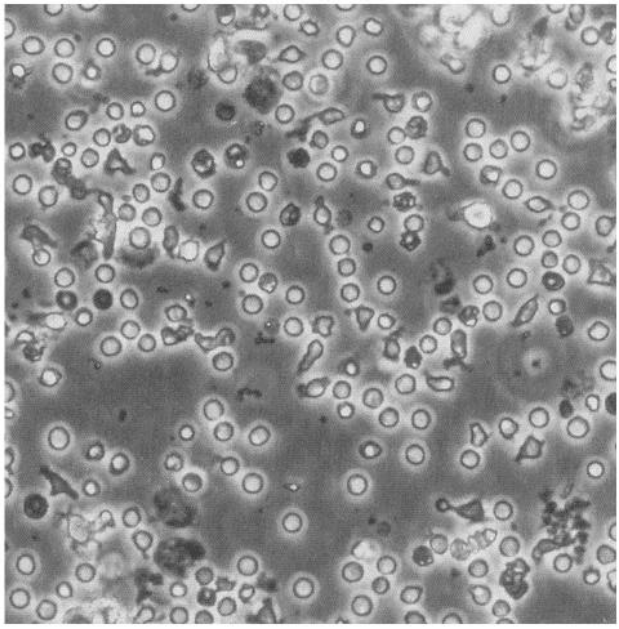

Fig. 1. Phase contrast photomicrograph of human lymphocytes prepared using a Ficoll-paque gradient. The preparation also contains some cell debris and a small number of monocytes (magnification: 440×).

equal volume of PBS containing 1 μg/mL acridine orange and 2 μg/mL ethidium bromide and viewed under a microscope equipped with a halogen quartz light source and appropriate filter. Live cells are viewed as green, dead cells are orange and any red blood cells in the preparation are dark.

3.2. Separation of Lymphocyte Suspension from Biopsied Material

1. Collect biopsy material in a sterile manner. Wash off any contaminating blood using sterile PBS. If the material is grossly contaminated, antibiotics can be added at this point.
2. Remove any surface fat, and then chop the tissue into small pieces about 1 mm diameter. Two sterile scalpels are used for this operation, and make sure that the tissue is cut and not torn.
3. To prepare a cell suspension, two methods can be used:
   a. Place the tissue into sterile stomacher bags with a small volume of medium. Agitate for 10 s and then collect the suspension through a sterile gauze filter.
   b. Press the soft tissue with a pipet tip against a sterile sieve, and collect the resultant cell suspension.

4 If the cell suspension is grossly contaminated with blood, this can be separated from the lymphocytes by density centrifugation using Ficoll-paque as described in Section 3.1.
5 Count the lymphocytes using trypan blue exclusion and culture in RPMI containing 10% FCS

3.3. T- and B-Cell Enrichment Methods Using Nonimmunological Substrates

Having obtained a separate mononuclear cell fraction, it is necessary to distinguish and separate the T- and B-lymphocyte populations. Techniques based on different adherence properties are used.

3.3.1 Rosetting

T-lymphocytes spontaneously form rosettes when added to sheep erythrocytes, that are then separated from B-cells by centrifugation.

1. Count viable lymphocytes using trypan blue exclusion, and adjust to 5×10^6 lymphocytes/mL.
2. Adjust freshly prepared AET-treated sheep erythrocytes (*see* Section 2.) to a 2.5% (v/v) suspension in PBS
3 Mix lymphocyte suspension, FCS, and sheep erythrocytes in a 1·1.1 ratio, and centrifuge at 200*g* for 5 min at room temperature
4 Incubate for 1 h at 4°C
5 Resuspend this cell mixture by gently tapping the tube, mix 50 µL of trypan blue with 50 µL of the cell suspension and pipet sample into a 0 2-mm deep hemocytometer
6 Count 200 lymphocytes and determine the percentage of rosettes, i e , cells with three or more attached erythrocytes.
7 Layer the rosetted suspension onto Ficoll-paque (3 7 mL, respectively, *see* Section 3.1.) and centrifuge at 800*g* for 10 min.
8 Harvest the nonrosetted B-cells at the interface Rosetted T-cells will be spun down to the base of the tube These may be obtained by removing and washing the rosettes, the erythrocytes are lysed with sterile distilled water or high salt

3.3.2. Lymphocyte Fractionation Using Helix pomatia Lectin-Sepharose 6 MB

T-cells possess a receptor for *Helix pomatia*, and this property is used to bind T-lymphocytes to a Sepharose column *(1)*.

1 Treat human peripheral lymphocytes (12 5 × 10^6 cells/mL) with neuraminidase, 5 µg/mL in Tris-buffered Hanks' solution pH 7 4 for 45 min at 37°C *(2)*
2. Wash the lymphocytes free of neuraminidase with PBS containing 0.2% human serum albumin and 0 02% sodium azide
3 Pipet 10^8 cells in 1 5 mL of the PBS buffer onto a 3-mL column of *Helix pomatia* lectin-Sepharose 6 MB equilibrated with the same PBS buffer, and leave for 15 min at room temperature

4. Add 80 mL of the PBS buffer to the column at a flow rate of 6–10 mL/min; this elutes unbound cells (enriched for B-cells)
5. Add 80 mL of PBS buffer containing 0.1 mg/mL *N*-acetyl-α-D-galactosamine; this releases a population of weakly bound cells (mixture of T- and B-cells).
6. Finally, add 80 mL of buffer containing 1.0 mg/mL *N*-acetyl-α-D-galactosamine, which elutes the enriched T-lymphocyte fraction Total cell recovery is about 70%.

3.4. Nylon-Wool Columns

At 37°C in the presence of serum, B-cells will bind preferentially to a nylon-wool column. Most of the T-cells and "null cells" remain unbound and pass through the column This method is rapid and is suitable for the separation of large numbers of cells *(3)*.

1. Pack 600 mg of sterile nylon wool into a 20-mL plastic syringe barrel (a 100-mL syringe can be used for large-scale preparation), and wash with growth medium containing 5% FCS
2. Seal the column and incubate at 37°C for 1 h
3. Rinse the column with 5 mL of warm growth medium containing 5% FCS
4. Add 2 mL of the cell suspension dropwise to the top of the column. Then, add 1 mL of the warm growth medium, and ensure that there are no air bubbles in the column. Use the 20 mL syringe for about 10^8 cells and the 100-mL syringe for more than 5×10^8 cells
5. Seal the column and incubate at 37°C for 1 h
6. Wash the column with 25 mL of warm growth medium Collect the eluent which will consist predominantly of T-lymphocytes and null cells The lymphocytes are concentrated by centrifugation (200*g* for 10 min at 4°C) and cell viability may be determined by trypan blue exclusion. Some of the B-cells adhering to the column may be recovered by mechanical elution
7. Wash the column with 100 mL of warm growth medium and discard this volume
8. Seal the syringe tip with a needle and rubber bung For safety it is possible to buy a small plastic two or three way valve connector to fit the syringe tip Add 2 mL of warm growth medium and squeeze the nylon wool with blunt stainless steel forceps.
9. Remove the needle and bung and wash with 10 mL of warm growth medium and retain Replace the syringe piston and expel the remaining growth medium
10. Concentrate these cells by centrifugation (200*g* for 10 min at 4°C). The number of viable lymphocytes is assessed by trypan blue exclusion These cells will be mostly B-lymphocytes but contaminated by some T-lymphocytes and "null" cells.

3.5. T- and B-Cell Enrichment Using Specific Immunological Binding Methods

Lymphoid cells are separated from each other by exploiting differences in the molecules expressed on their surfaces, for example; immunoglobulin molecules, histocompatibility or blood group antigens and cell surface receptors.

Immunoabsorbents consisting of a specific antibody coupled to some form of matrix either macrobeads (column) or plastic are used.

3.5.1. Enrichment Using Antihuman IgG coated Petri Dishes

1 Pour 4 mL of 1% antihuman IgG in to a sterile Petri dish and leave for 1 h at 4°C
2. Rinse the coated Petri dish twice with PBS
3 Add 10^8 cells (maximum) in 4 mL of growth medium containing 5% FCS Incubate for 1 h at 4°C Gently swirl the dish several times during the incubation but keep it level
4. Remove the medium containing the nonadhering T-cells using a sterile Pasteur pipet Gently rinse the dish with growth medium containing 5% FCS and discard
5 Add 4 mL fresh medium and vigorously aspirate to obtain adherent B-cells. Count the different lymphocytes suspensions using either trypan blue exclusion or the ethidium bromide/acridine orange stain and prepare them for culture

3.5.2. Separation Using a Protein A-Sepharose 6 MB Column

Protein A binds to the Fc portion of IgG and, when coupled to Sepharose 6 MB, will be adsorbed by cells that have been coated with a specific IgG antibody. The immobilized cells are then released by adding excess soluble IgG, that displaces the antibody-coated cells from the Protein A-Sepharose 6 MB column *(4)*.

1 Take 10^8 cells/mL from a separated lymphocyte culture (*see* Sections 2.1. and 2 2.) and treat either with pan-B or pan-T antibody (20 µg/10^7 cells/mL) at 4°C for 30 min.
2 Wash the cells three times with RPMI containing 10% FCS Spin down and resuspend in 0 25 mL of the same medium
3 Pipet the treated cells into a small plastic column (0 5 cm in diameter) containing 1 5 mL Protein A-Sepharose 6 MB, close the column, and incubate at room temperature for 20 min
4. Add 20 mL medium at a flow rate of about 2.5 mL/min to remove the unbound cells
5 Add 2 mL of dog IgG (20 mg/mL), close the column, and incubate at 37°C for 60 min.
6. Elute the cells by adding 3 mL of dog IgG (20 mg/mL) followed by 15 mL of buffer, both at 37°C. Approximately 60% of the bound cells are recovered

3.5.3. Positive or Negative Selection of Lymphocyte Subsets by Immunomagnetic Separation

T-lymphocyte subsets ($CD4^+$ T-helper and $CD8^+$ T-suppressor cells) and B-lymphocytes ($CD19^+$) can be positively or negatively selected from whole blood or peripheral blood mononuclear cells (with >90% purity) using commercially available magnetic beads that are precoated with monoclonal anti-

bodies (MAbs) against specific surface antigens. For instance, $CD4^+$ T-lymphocytes can be isolated using magnetic beads coated with an anti-CD4 antibody (MAb 66.1), e.g., Dynabeads M-450 CD4 (Dynal) and subsequently detached from the magnetic beads using DETACHaBEAD (Dynal). Separation from mononuclear cell suspension rather than whole blood is preferred since a much larger beads to target cell ratio is required for the latter. Target lymphocytes obtained by detachment should be thoroughly washed and rested for at least 2 h in humidified CO_2/air (1:19) at 37°C before used for in vitro functional assays.

1 Estimate the number of target lymphocytes in the mononuclear cell suspension. Add the required number of beads (beads: target cell = 3 1) to cell suspension in a test tube that is kept on an apparatus that provides gentle tilting and rotation (e g., Spiramix Mixer, Griffiths & Nielsen, Billingshurst, Sussex, UK) for 30 min at 4°C.
2. Place the test tube in a Dynal magnetic particle concentrator (MPC) for 2–3 min. The immunomagnetically selected, e.g., $CD4^+$ cells will be attracted to the wall of the test tube. Aspirate and discard the supernatant (or use as CD4-depleted cell suspension)
3. Resuspend the selected cells in RPMI growth medium. Repeat steps 2 and 3 five times and then resuspend in 100–200 µL of RPMI growth medium.
4 Add 10 µL (per 100 µL cell suspension) of DETACHaBEAD (Dynal) to the test tube that is then kept rotating on Roller SRT1 at room temperature for 1 h.
5. Following incubation, add 500 µL of RPMI growth medium and vortex for 30 s before placing the test tube back in Dynal MPC.
6. After 2–3 min, aspirate and keep the supernatant while the detached beads are retained on the wall of the tube by the magnet.
7 Repeat steps 5–6 three times and combine the supernatants that contain the cells of interest
8. Wash the detached cells three times to remove DETACHaBEAD

3.6. Establishment of the Activation Status of Freshly Prepared Lymphocytes Using DNA Staining Dyes and Flow Cytometry

To establish the activation status of freshly prepared lymphocytes, a number of DNA staining dyes such as propidium iodide (PI) can be employed, in conjunction with RNase (to remove RNA which also binds PI). Cells which have been fixed with ice-cold ethanol take up PI, giving rise to various DNA fluorescence patterns *(5)* according to their activation status. Figure 2 demonstrates the DNA fluorescence profile/ cell cycle histogram of PI-stained H9 T cells, examined using a FACScan. Cells in the G_0/G_1 region are in the resting phase whereas those in the A_0 region are nonviable/apoptotic.

1. Spin down the cell suspension (0.5×10^6 cells/sample) and wash once with 2 mL of PBS. Resuspend in 400 µL of PBS and add 400 µL of ethanol dropwise at 4°C.

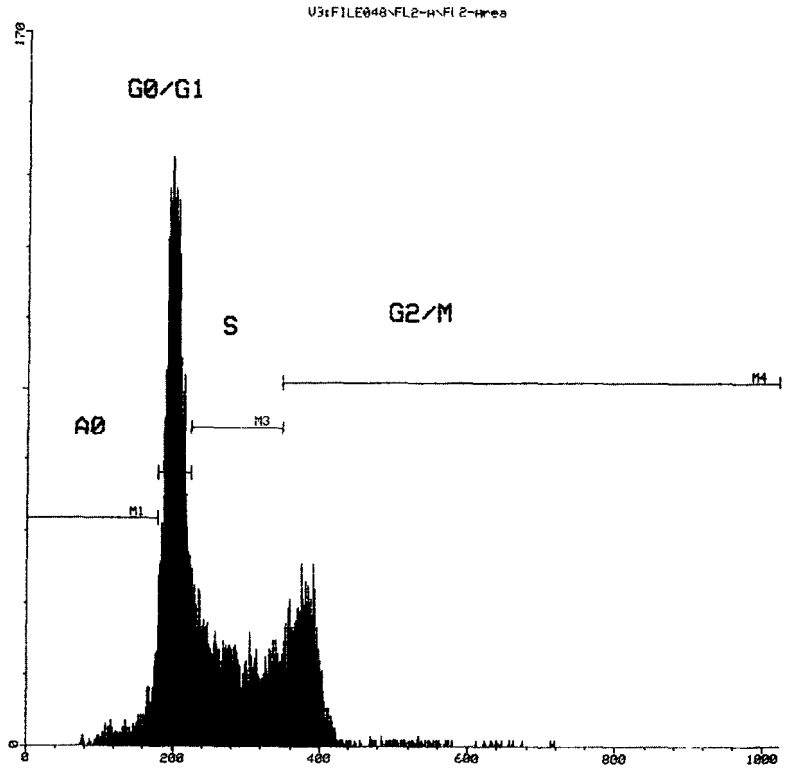

Fig. 2. Cell cycle histogram of H9 T-cells stained with propidium iodide The *y*- and *x*-axis represent cell count and DNA fluorescence, respectively

2. After overnight incubation at 4°C, wash once with 2 mL of PBS and resuspend in 300 µL of PI (final concentration 50 µg/mL) diluted in PBS (pH 7.4) containing 0 1% Triton X-100, 0.1 m*M* disodium EDTA, and 50 µg/mL RNase. Keep the sample in the dark at room temperature and analyze within 1 h using a FACScan *(5)*

3.7. Establishment of Primary Lymphocyte Cultures

Primary lymphocytes can be maintained by mitogens, e.g., plant lectins PHA, Concanavalin A (Con A), and allogenic transplantation antigens and feeder layers; or by the addition of specific growth factors to culture media, e.g., IL2 (*see* Notes 2 and 3).

3.7.1. Maintenance of T-Lymphocytes Using IL2

1. Dilute the enriched T-cell suspension (with growth medium containing 10% FCS) to give a density of 5×10^5–10^6 cells/mL. Depending on the volume, place either

200 µL aliquots in a microtiter plate, 1 mL/well in a 24-well plate, or 3–7 mL in a 25-cm^2 flask

2. Add PHA to give a final concentration of 2 µg/mL Incubate at 37°C in a humidified 5% CO_2/95% air incubator, and observe cell cultures daily for cell growth and evidence of cell death
3. After 3 d, count the cells, using trypan blue exclusion, harvest cells, and wash (centrifuge at 1000 rpm for 5 min)
4. Gently resuspend the pellet in fresh growth medium (RPMI plus 15% FCS) supplemented with IL2 (10–20 U/mL, *see* Note 3) at a cell concentration of 5×10^5–10^6 cells/mL, and culture but do not add PHA
5. Continue to adjust the cell concentration every 3 d, adding fresh medium (plus IL2) each time After 2 wk, the individual T-cells may be cloned and separated for functional tests

3.8. Maintenance of B-Lymphocytes Using EBV to Transform Cells

1. Take 2×10^6 of enriched B-lymphocytes and pellet (200g for 10 min at room temperature)
2. Resuspend the cells in 1 mL of EBV suspension (5×10^4 TFU), and incubate at 37°C for 1 h in a humidified 5% CO_2/95% air incubator
3. Wash three times with PBSA to remove unadsorbed virus, and resuspend in growth medium containing 10% FCS
4. Culture in 24-well plates at a cell concentration of 10^6 cells/mL of growth medium at 37°C in a humidified 5%CO_2/95% air incubator Check for proliferation after 7 d
5. Remove medium and cells from wells, spin down, and gently resuspend transformed cells in fresh medium Continue to culture in 24-well plates. These cells can be grown in limiting dilution for B-cell characterization with monoclonal or polyclonal antibodies or functional assays

3.9. Maintenance of B-Cells Using B-Cell Growth Factor

Recently, B-cells from peripheral blood from normal donors grown in the presence of BCGF and anti-IgM has led to the establishment of a long-term cultured human B-cell line *(6)*

1 Pipet out 1 mL enriched B-cells (10^6 cells/mL) into 24-well plates with growth medium (plus 10% FCS) containing 20 µg/mL F(ab')2 fragment and 10% BCGF. Culture cells at 37°C in humidified 5% CO_2/95% air incubator
2 Feed cells every 3–4 d by replacement of 50% of culture-well contents with an equal volume of fresh medium containing 10% BCGF and 20 µg/mL anti-IgM Check for cell growth and viability using Trypan blue exclusion until cell culture is established.

4. Notes

1 FCS may vary from batch to batch set up established T- or B-cell lines in culture and count cells on a daily basis to check which serum gives maximal cell growth; or [^{3}H] thymidine may be used to measure cell division rates

2 IL2 (T-cell growth factor) also shows variation and ideally should be assayed on established cell lines: increased growth is monitored by increased ^{3}H-thymidine incorporation
3 Optimal growth conditions should be established with T-cell lines before embarking on experiments using the valuable material just prepared, i e., check that the sterile 1X medium will maintain T-cell lines.

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8

Human Pilosebaceous Culture

Terence Kealey, Michael Philpott, and Robert Guy

1. Introduction

The pilosebaceous unit is named after *pilus*, the Latin for hair, and *sebum*, the Latin for grease, since it is made of up two components, a hair follicle and a sebaceous gland (Fig. 1). Its culture is important to the study of its diseases, including alopecia and acne; but its culture is also important to the study of skin epithelial stem cells because the epithelial cells of the outer root sheaths of the hair follicle are pluri-potential in their phenotype, and can re-epithelialize the denuded epidermis to heal wounds *(1)*.

The pilosebaceous unit is characteristic of all mammals, and many of the techniques described here may be applied to units isolated from species other than the human, but it should be noted that the sebocytes of different animals respond differently to retinoids and other pharmacological agents *(2)*.

The pilosebaceous unit is sufficiently small to allow organ maintenance, which encourages the optimal differentiation of cells in culture, since they grow on their native basement membrane, adjacent to their native cell neighbors. Some of the different cell types of the unit can also be cultured as cell lines. Central to the successful culture of the human pilosebaceous unit is its isolation, and this is described.

There are at least four types of human pilosebaceous unit:

1 Terminal pilosebaceous units (which contain large hairs and large sebaceous glands and which are found on the scalp),
2. Vellus pilosebaceous units (which contain small hairs and small sebaceous glands, and which cover most of the body),
3 Sebaceous pilosebaceous units (which contain small hairs and large sebaceous glands, and which develop on the face and upper trunk at puberty—they are the units responsible for acne), and

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

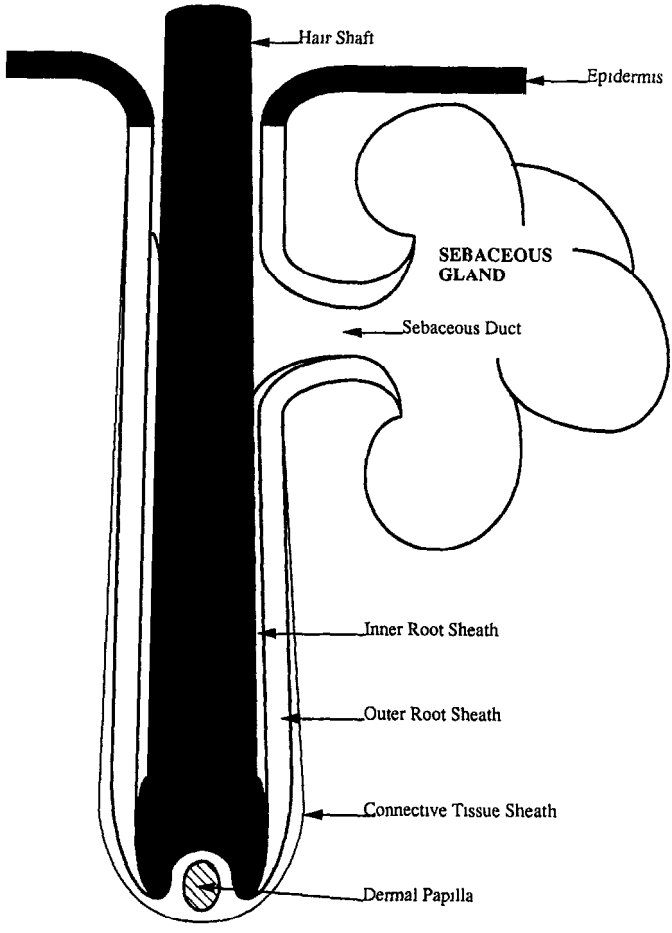

Fig. 1. Diagram illustrating the major regions of the pilosebaceous unit

4. Apo-pilosebaceous units (which are terminal axillary units into which apocrine sweat gland ducts empty)

Moreover, the hair grows cyclically, with an active growth stage, anagen (from the Greek *ana*, *genein*, to produce), followed by a regressive, catagen, stage (from the Greek *kata*, down, and *genein*, to produce) which gives rise to a resting, telogen, follicle (from the Greek *telos*, end, and *genein*, to produce). Unless otherwise stated, the methods described here apply to the human terminal pilosebaceous unit isolated in anagen.

There are a number of different cell types, both mesenchymal and epithelial, within the pilosebaceous unit.

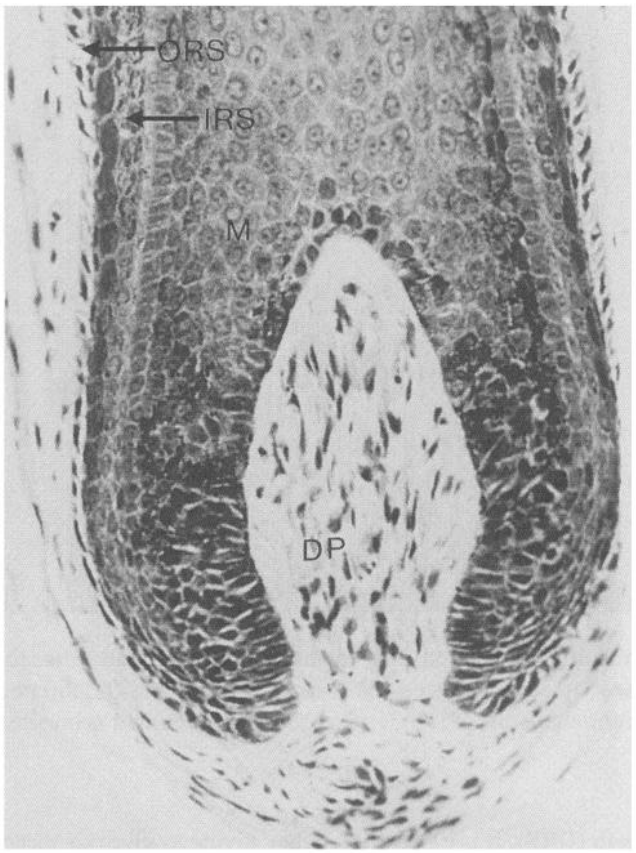

Fig. 2. Histological section of the bulb region of isolated human hair follicle showing the dermal papilla (DP) matrix cells (M), inner root sheath (IRS), and outer root sheath (ORS).

1. Mesenchymal: The unit is closely ensheathed by concentric layers of fibroblasts that are phenotypically different from interfollicular fibroblasts. This phenotypic difference is most demonstrated by the fibroblasts of the dermal papilla (Fig. 2). The dermal papilla invaginates the base of the hair shaft and it is believed to direct, to a degree, the cell division and differentiation of the epithelial cells of the unit.

   Dermal papilla fibroblasts, when grown in primary culture, do not spread as a monolayer, but grow in clumps, as if mimicking dermal papilla morphology. Moreover, dermal papilla fibroblasts grow more slowly, and demonstrate a larger size, than do interfollicular fibroblasts on primary culture *(3)*.
2. Epithelial: There are a number of different epithelial cell types in the pilosebaceous unit. The epithelial stem cells are found within the upper part of the outer

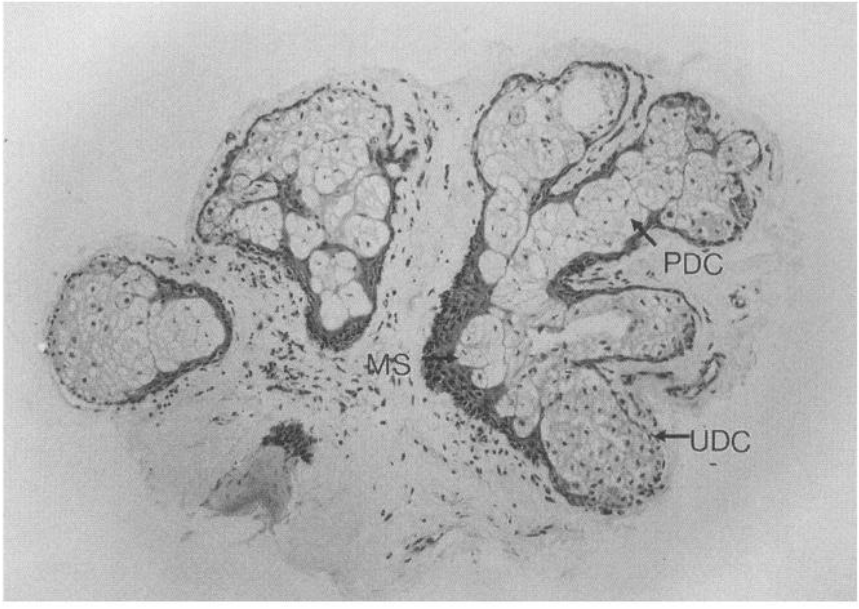

Fig. 3. A histological section of a freshly isolated human sebaceous gland. The three distinct sebocyte cell types can be seen: peripheral undifferentiated cells (UDC), partially differentiated cells (PDC), and mature differentiated sebocytes (MS).

rooth sheath (ORS). It is believed that they divide to give rise directly, within the pilosebaceous unit, to the peripheral layers of the ORS, to the matrix cells, and to the basal sebocytes as three distinct populations of transient amplifying cells *(4)*. As noted, they may also give rise to epidermal cells *(1)*.

The sebaceous gland is made up of one cell type, the sebocyte, that engages in holocrine secretion (i.e., it dies as it releases its secretory product of lipid) (Fig. 3). The transit time of the sebocyte is about 3 wk *(5)*. It arises peripherally by the division of basal sebocyte transient amplifying cells, and as it matures it moves centrally and fills with lipid droplets. These droplets eventually rupture the cell, killing it, to release their lipid that is pushed out on to the skin surface, via the sebaceous duct, and the pilosebaceous infundibulum, by the growth and expansion of new sebocytes.

The matrix cells, which divide every 24 h, are among the most rapidly dividing cells in the body. They are the cells that terminally differentiate to give rise to the hair shaft. They are found at the base of the hair follicle, surrounding the fibroblasts of the dermal papilla, from which they are separated by a basement

membrane The more peripheral matrix cells give rise to the inner root sheath (IRS) and possibly to the inner part of the outer root sheath *(6)*.

The successful organ maintenance of the hair follicle, sebaceous gland, and entire pilosebaceous unit have all been reported, as has the primary culture of ORS cells, matrix cells and sebocytes. The primary culture of IRS cells would seem to be impossible as the IRS is terminally differentiated *(7)*. The successful culture of stem cells has not been reported.

2. Materials

Media and supplements may be purchased from any company supplying tissue culture products and must be of tissue-culture grade. For some products, however, we indicate which company we have found optimal.

2.1. Medium for Hair Follicle Organ Maintenance

1 Williams E· We buy this as liquid medium (1X). In our experience, medium made up from powder only sustains suboptimal growth
2. L-Glutamine Bought as liquid medium at 200 m*M* (100X). Stored frozen in aliquots of 10 mL One milliliter is added to 100 mL of Williams E immediately prior to use
3. Hydrocortisone· Vials of 1 mg powder are bought, to which 1 mL of 100% ethanol is added, followed by 19 mL of Williams E Kept frozen in 1 mL aliquots, 20 µL is added to 100 mL of Williams E immediately prior to use
4. Insulin· Vials of 100 mg powder are bought, to which 10 mL of sterile pure water is added, followed by 100 µL sterile glacial acetic acid Stored frozen in 100 µL aliquots, 100 µL added to 100 mL of Williams E medium immediately prior to use.
5 Penicillin and streptomycin These are bought as a single solution containing both antibiotics at 100X concentration Kept frozen, aliquots are thawed and 1 mL added to 100 mL of Williams E prior to use

2.2. Medium for Infundibular Organ Maintenance

Keratinocyte serum-free medium (SFM) (Gibco, Gaithersburg, MD) is supplied as a liquid basal medium (1X) which is supplemented with:

1 50 µg/mL Bovine pituitary extract (supplied with basal medium) stored frozen
2 100 U/mL Penicillin and 100 µg/mL streptomycin (Section 2 1 , item 5) Add at 1/100 mL medium
3 2 5 µg/mL Amphotericin B, supplied as a solution (100X) Store frozen and add at 1/100 mL medium
4 $CaCl_2$ $10H_2O$ to a final Ca^{2+} concentration of 2 m*M* Dissolve 2 79 g ($CaCl_2 \cdot 10H_2O$) in 100 mL keratinocyte serum-free basal medium and add 1/100 mL of medium Store at 4°C.

2.3. Medium for Sebaceous Gland Organ Maintenance

Phenol red-free Williams E medium is supplied as a liquid medium (1X) and is supplemented with:

1. 2 m*M* L-Glutamine, supplied as a solution (100X) Store frozen and add 1/100 mL medium
2. 100 U/mL Penicillin and 100 µg/mL streptomycin, as in Section 2.1 , item 5 Add 1/100 mL medium
3. 2 5 µg/mL Amphotericin B Add 1/100 mL medium
4. 10 µg/mL Bovine insulin, supplied as 100 mg powder Reconstitute and store as in Section 2 1 , item 4 Add 1/100 mL medium
5. 10 ug/mL Transferrin, supplied as 10 mg powder Reconstitute with 10 mL Williams E and store frozen in 1-mL aliquots Add 1/100 mL medium
6. 10 ng/mL Hydrocortisone, supplied as 1 mg powder Reconstitute with 500 µL ethanol, followed with 9.5 mL Williams E Store frozen in 10-µL aliquots and add 10 µL/100 mL medium
7. 10 ng/mL Sodium selenite, supplied as 1 mg powder Reconstitute with 10 mL Williams E and store frozen in 10-µL aliquots Add 10 µL/100 mL medium.
8. 3 n*M*-Triiodothyronine, supplied as 1 mg powder Reconstitute with 1 mL 1*M* NaOH, swirl until dissolved, and add 49 mL Williams E Store frozen in 10-µL aliquots and add 10 µL/100 mL medium
9. 1% (v/v) Trace elements mix (Gibco), supplied as crystals Add 10 mL distilled deionised water and store frozen Add 1/100 mL medium
10. 10 µg/mL Bovine pituitary extract, supplied as 5 mg powder Reconstitute with 1 mL Williams E and add 200 µL to 100 mL medium

2.4. Medium for Primary Explant Culture of Outer Root Sheath Keratinocytes

Keratinocyte SFM (Gibco) is supplied as a liquid basal medium (1X) which is supplemented with:

1. 50 µg/mL Bovine pituitary extract (supplied with basal medium) stored frozen.
2. 100 U/mL Penicillin and 100 mg/mL streptomycin Section 2.1 , item 5) Add at 1/100 mL medium
3. 2.5 µg/mL Amphotericin B. Add 1/100 mL medium.
4. 5 ng/mL Epidermal growth factor (EGF) (supplied with basal medium) stored frozen

3. Methods

3.1. Isolation of Human Hair Follicles

We usually isolate human hair follicles from full thickness skin taken from women undergoing facelift surgery, but this method can also be used for the isolation of hair follicles from beard, axillary, and pubic skin. Before obtaining

skin, ethical committee permission must be obtained, as must the consent of the patient. These are not usually difficult to obtain, since the removal of a sliver of skin from the incision neither affects wound healing nor produces a worse scar. It is essential, when isolating human hair follicles, that the subcutaneous fat should not be removed from the skin because it is important to the successful isolation *(8,9)*.

1 The skin is cut into thin strips (approx 10 mm × 5 mm) which are placed in isolation medium, Earle's balanced salt solution (EBSS):PBS^+ (with Ca^{2+} and Mg^{2+}) (1.1), until required for isolating hair follicles
2. Hair follicles are isolated by taking a strip of skin and, under a dissecting microscope, cutting very carefully through the skin at the dermosubcutaneous fat interface using a sharp scalpel blade (Swann-Morton No 12). This cuts the hair follicles just beneath the opening of the sebaceous gland, resulting in the lower portion of the hair follicle, including the bulb, being left in the subcutaneous fat.
3 The hair follicle bulbs are then easily isolated from the subcutaneous fat by placing the fat, cut side upwards, under the dissecting microscope Using watchmakers forceps (No. 5), carefully grasping the ORS of the cut end of the hair follicle, the hair follicle can be gently removed from the fat. Isolated hair follicles are placed in EBSS:PBS^+ (1 1) prior to being used for experiments Using this method we routinely isolate several hundred hair follicles within 2 h from a piece of skin 2 × 1 cm The yield of follicles from an individual piece of skin is variable, depending on the thickness of the skin and underlying fat, the size of the follicles and the depth at which they penetrate into the fat (*see* Note 1)
4. Isolated hair follicles are initially placed in a 15 mm Petri dish containing isolation medium, before being placed into tissue-culture medium (*see* Note 2)

3.2. Organ Culture of Isolated Human Hair Follicles in Serum-Free Defined Medium

1 Isolated human hair follicles are maintained in individual wells of 24-well multiwell plates containing 500 µL of Williams E medium supplemented with 2 m*M* L-glutamine, 10 µg mL insulin, 10 ng/mL hydrocortisone, 100 U/mL penicillin, 100 µg mL streptomycin at 37°C in an atmosphere of 5% CO_2/95% air *(10)*. Usually, hair follicles will sink to the bottom of the multiwell, though follicles will occasionally float at the air liquid interface This can then cause problems when trying to measure growth as the follicles usually lie at an angle; follicles found floating at the air liquid interface can be encouraged to sink by gentle prodding with a syringe needle.
2 Length measurements are made on isolated hair follicles using a Nikon Diaphot inverted microscope with an eye piece measuring graticule (2.5 mm)
3. Medium is usually changed every 3–4 d, although we have found that hair follicles will grow normally for up to 10 d without a medium change (*see* Note 3).
4. Follicles will grow in vitro for up to 10 d at 0.3 mm a day, the normal in vivo rate, and they will retain their *in situ* morphology over that time (Fig. 4) On exposure

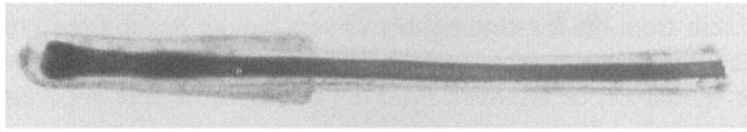

Fig. 4. An isolated human hair follicle maintained for 5 d in culture showing the increase in hair fiber production over this time.

to the appropriate growth factors, they will respond in vitro as they do in vivo *(11)*.

3.3. Primary Explant Culture of ORS Keratinocytes

1. We culture ORS keratinocytes directly onto tissue culture plastic *(9)*. Hair follicles are isolated either by dissection or plucking and placed under a dissecting microscope where, with a scalpel blade, the hair follicle bulb is removed and the remainder of the hair follicle cut into a number of small pieces (approx 1 mm in length).
2. Pieces of follicle (5–10) are placed into 25 mL flasks and covered with a thin layer of culture medium consisting of keratinocyte SFM (Gibco) supplemented with 5 ng/mL EGF, 50 µg/mL bovine pituitary extract, and incubated at 37°C in an atmosphere of 5% CO_2/95% air.
3. After 24 h, most of the hair follicle pieces will have attached to the tissue culture plastic, and 5 mL of fresh medium can be added (*see* Note 4).

3.4. Primary Culture of Dermal Papilla Fibroblasts

Human dermal papilla fibroblasts are cultured following the method described by Messenger *(3)*.

1. Hair follicles are isolated from human skin and placed in isolation medium as described in Section 3.1.
2. Using a scalpel blade, the lower hair follicle bulb, containing the dermal papilla, is removed by transecting the hair follicle through the keratogenous zone.
3. The lower follicle bulb is placed in fresh isolation medium containing 1% BSA, penicillin (100 U/mL), and streptomycin (100 µg/mL). Addition of BSA to the isolation medium reduces adhesion of the isolated dermal papilla and facilitates easy transfer for subsequent culture *(12)*.
4. Dermal papillae are isolated from the hair follicle bulbs as follows. First, using either fine dissecting needles (Watkins and Doncaster, Maidstone, Kent, UK), or 25-gage Microlance syringe needles attached to 1 mL plastic syringes, the hair follicle matrix cells are removed. This is achieved by holding the follicle bulb to the bottom of the Petri dish with watchmakers forceps, and using a needle to

remove the matrix cells from the cut end of the follicle bulb These cells are easily removed; frequently applying pressure to the base of the follicle bulb will result in the matrix being ejected from the cut end of the follicle

5 The remaining hair follicle bulb contains the connective tissue sheath, and the dermal papilla, which sits centrally within the follicle bulb The connective tissue sheath is then cut open with syringe needles, and the dermal papilla isolated by careful dissection This procedure is difficult but can be easily mastered with practice, and up to 20 dermal papillae can be isolated within 2 h

6 Once isolated, up to five dermal papillae are placed in individual wells of 24-well multiwell plates and covered with 100 µL of culture medium, which consists of either minimal essential medium (MEM) or Williams E medium supplemented with 2 m*M* L-glutamine, 20% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 µg/mL)

7 Cultures are maintained at 37°C in an atmosphere of 5% CO_2/95% air Within 48 h, most dermal papillae will have attached to the tissue-culture plastic, the old medium can be removed by aspiration, and 500 µL of fresh medium added.

8 Medium is usually changed every 3 d Initial growth is usually slow, but within 7 d explants from the papilla can usually be seen under phase contrast microscopy Primary explants usually aggregate, and so rarely achieve confluence, and we usually passage cells after 4 wk.

3.5. Germinative Epithelium Cell Cultures

The human hair follicle germinative epithelial cells, also known as matrix cells, can be cultured using the method recently published by Reynolds and Jahoda *(13)* for the culture of germinative epithelium from rat vibrissae follicles Their culture requires a dermal papilla fibroblast feeder layer. The feeder layer is prepared as described in Section 3.4. *(3,12)*. Because dermal papilla fibroblasts grow slowly and rarely achieve confluence, it is not necessary to arrest their growth prior to overlaying them with germinative epithelial cells.

1 Hair follicles are isolated from human skin using the method described in Section 3 1 Under a dissecting microscope, the lower hair follicle bulb containing the dermal papilla is removed using a scalpel blade (Swann Morton no 15).

2. Using fine dissecting needles (Watkins and Doncaster, Maidstone, Kent), or 25-gage Microlance syringe needles, the hair follicle matrix and germinative epithelium are removed from the hair follicle bulb, leaving behind the dermal papilla and connective tissue sheath

3 Isolated germinative epithelial tissue is then placed in either 24-well multiwell plates or 35-mm Petri dishes on feeder layers of human dermal papilla fibroblasts, and covered with culture medium consisting of MEM containing 20% FCS, 2 m*M* L-glutamine, 10 µg/mL epidermal growth factor, 50 µg/mL bovine pituitary extract

4. Cultures are maintained at 37°C in an atmosphere of 5% CO_2/95% air. Under these conditions, cells explant out from the germinative epithelium, forming colo-

nies of small rounded cells The human germinative epithelial cells, like those of the rat, appear much smaller than ORS keratinocytes in vitro.

3.6. Isolation and Maintenance of Human Sebaceous Glands by Shearing

To isolate human skin glands we use sagittal, frontal, midline chest skin (5 × 60 mm) from patients undergoing cardiac surgery

1 It is important that the incision is made with a scalpel, since diathermy may damage the glands The sliver of skin should then be placed in a suitable buffer, such as PBS^+ or EBSS If the media are bicarbonate buffered, the containers should be airtight Bicarbonate buffered media are preferred for transport from the operating theatre (room) because they seem to promote the better retention of glandular lipogenesis, possibly caused by the bicarbonate dependence of lipogenesis
2 In the laboratory, subcutaneous fat is trimmed from the skin and discarded.
3. For shearing *(14)*, the skin is washed four times in sterile EBSS to elute the bactericidal solution that surgeons apply to the skin, and then cut into small pieces (<5 mm) in length and sheared in 10 mL of PBS^+, by the repeated action of very sharp scissors, until a porridge-like consistency is obtained (bicarbonate-buffered medium cannot be used during shearing as it will be exposed to the air for some time) (*see* Notes 5 and 6)
4 Samples of this suspension are diluted in a Petri dish with PBS^+ to facilitate microscopy, and the skin glands identified under a binocular microscope
5 Glands are isolated using watchmakers forceps (No 5) taking great care not to damage them.
6 Isolated glands are placed in fresh EBSS until required for experiments
7. We routinely maintain our glands (*see* Note 7) overnight at 37°C on sterile nitrocellulose or millipore filters, pore size 0 45 μm, floating on 5 mL of phenol red-free Williams E medium supplemented with 2 m*M* L-glutamine, 10 μg/mL insulin, 10 μg/mL transferrin, 10 ng/mL hydrocortisone, 10 ng/mL sodium selenite, 3 n*M* triiodothyronine, trace elements mix, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 5 μg/mL Fungizone, in an atmosphere of 5% CO_2/95% air
8 Glands maintained for longer periods are also cultured on nitrocellulose filters in the same medium, which is changed every other day (*see* Note 8)
9 We have found that glands maintained in this way retain their *in situ* morphology over seven d. In addition to this, both rates of DNA synthesis and lipogenesis are retained over this period, as are the appropriate responses to steroid hormones *(15,16)*

3.7. The Primary Culture of Sebocytes from Isolated Sebaceous Glands

Primary explants of sebocytes can be established from sebaceous glands *(17)*.

1 Five glands are plated out on 3T3 feeder layers (2×10^4 cells/cm^2) in 25 cm^2 flasks and covered with culture medium consisting of DMEM containing 4 5 g/L

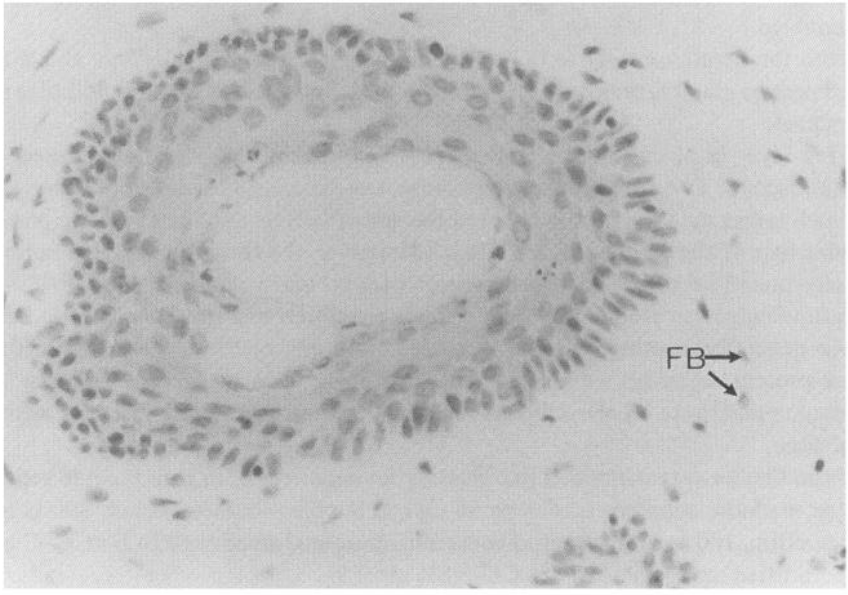

Fig. 5. A histological section of a freshly isolated human pilosebaceous infundibulum. The duct has a stratified squamous appearance with regions of columnar cells in the periphery. Toward the lumen of the duct the cells become more rounded and less intensely stained. A number of fibroblasts (FB) can be seen dispersed around the infundibulum.

glucose and Ham's F12 medium (3:1) supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL EGF, 0.4 µg/mL hydrocortisone, 10^{-9}*M* cholera toxin, and 3.4 m*M* L-glutamine.
2. Cultures are maintained at 37°C in an atmosphere of 95% air/5% CO_2 and the medium is changed every 3 d.
3. The feeder layer is renewed every other week by selectively removing the 3T3 cells with 0.02% EDTA.
4. These sebocytes may be subcultured. They are detached with 0.25% trypsin and 0.02% EDTA in PBS at 37°C for 15 min. Trypsin is inhibited by the addition of culture medium.
5. Sebocytes are replated at a density of 4×10^3 cells/cm^2 on a 3T3 feeder layer. By the third passage, however, rates of sebocyte proliferation fall to about 25% of the rate seen at the first passage.

3.8. Isolation and Culture of the Human Sebaceous Pilosebaceous Infundibulum (Sebaceous Duct)

This is the structure involved in acne (Fig. 5). It can be isolated from non-hair-bearing facial skin taken from women undergoing facelift surgery *(18)*.

1 Using a keratotome, the top 0.1 mm of the skin containing the epidermis is removed
2. With the keratotome set to 0.2 mm, the upper portion of the dermis above the sebaceous gland is removed. It is in this layer that the sebaceous infundibulae are located.
3 This layer is placed in sterile PBS and transilluminated under the dissecting microscope The sebaceous infundibulae can be easily identified, as they are much larger than the infundibulae of the vellus follicle and they lack the prominent hair of the terminal hair follicle. Moreover, the infundibulae also contain large quantities of sebum which appears dark on transillumination
4 Infundibulae are isolated by gentle microdissection and placed in sterile PBS We generally isolate 20–30 infundibulae from any one individual where they are present, although we are only able to identify them in 50% of subjects We assume that these are the subjects with high sebum secretion rates and a history of acne
5 Infundibulae are maintained free floating, in a multiwell, in keratinocyte serum-free medium, supplemented with 50 µg/mL bovine pituitary extract, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 µg/mL amphotericin B at 37°C in a humidified atmosphere of 5% CO_2/95% air *(19)*
6. Culture medium (*see* Note 9) is changed every other day
7. To prevent infundibulae from adhering to the surface of the multiwell, it is gently agitated every day
8. When infundibulae are organ maintained thus, they will cornify centrally to mimic the development of a stratum corneum, but otherwise they will retain their *in situ* pattern and rate of cell division In response to the appropriate cytokines, they will mimic the various in vivo expressions of acne in vitro *(19)*

3.9. Primary Explant Culture of Infundibular Keratinocytes

This is performed as described for the primary explant culture of ORS keratinocytes (*see* Section 3.3.).

4. Notes

1. We usually use skin within several hours of removal from the patient, although we have found that follicles isolated from skin left overnight in a refrigerator at 4°C will still grow in vitro
2. It is important that hair follicles are left in isolation medium for the shortest time possible (<2 h) and that, when hair follicles are being handled, only the upper ORS (the cut end) is held in the tweezers, and not the rounded hair follicle bulb This is important, because the follicle bulb contains the highly proliferative matrix cells that give rise to the keratinized hair fiber, and these cells are very sensitive to trauma, and damage to them will prevent normal hair growth taking place in vitro As soon as hair follicles have been isolated and placed in a Petri dish, damaged follicles can be identified under a dissecting microscope and discarded; healthy follicles can be transferred for maintenance

3 Great care should be taken when changing medium because it is very easy to discard hair follicles with the old medium Two methods of changing medium are recommended, to place fresh medium into new multiwell plates and transfer follicles from their old medium by gently grasping the ORS with watchmakers forceps, or to aspirate medium very carefully using a 25-mL plastic syringe and 21 gage needle, taking great care not to suck up the follicle as well
4 Under these conditions, we find that ORS keratinocytes readily explant onto tissue-culture plastic. We have found that it is unnecessary to use trypsin or collagenase to promote cell disaggregation, presumably because the basal, dividing, cells of the ORS are located around the periphery of the isolated hair follicle However, a short period of enzyme digestion (we use 20 min in 1mg/mL collagenase in PBS at 37°C) in some cases aids attatchment of the pieces of ORS by promoting the adhesion of the surrounding collagen to the plastic
5. Sometimes glands are not completely isolated by shearing, but remain attatched to lumps of collagen. They can be easily teased away from the collagen with watchmakers forceps, taking care to pull at the collagen rather than grasp the glands
6 It is important to use medium at room temperature Ice-cooled medium seems to impair glandular viability, possibly for the same reason that adipocytes are killed at 4°C, namely that the intracellular fat or sebum solidifies and so ruptures the cell
7 Glandular lipogenesis in vitro is much greater after the overnight maintenance of the glands at 37°C in bicarbonate-buffered medium than in freshly isolated glands *(15)* and this is most likely owing either to the bicarbonate dependence of lipogenesis or the temperature sensitivity of the glands.
8. We have found that phenol red-free medium is superior to medium containing phenol red We attribute this to the estrogen-like actions of phenol red *(16)* since estrogens are known to inhibit lipogenesis in sebaceous glands. Apart from its role as an indicator, phenol red also protects medium from damage by light. For this reason phenol red-free Williams E medium must be handled in the dark, and we achieve this by wrapping the bottles in two layers of aluminium foil Although it is obviously impossible to carry out tissue culture in complete darkness, it is important to expose the medium to as little light as possible by turning out the lights in the tissue-culture room and flow hood. The Petri dishes are also covered with foil Despite these precautions we have found that a bottle of medium will only keep for 7 d and must be replaced after this time.
9 Keratinocyte serum-free medium contains Ca^{2+} at a concentration of 0.09 m*M* We supplement this with $CaCl_2 \cdot 10H_2O$ to give a final Ca^{2+} concentration of 2 m*M* since we have found that low calcium concentrations disrupt infundibular morphology on maintenance. We attribute this to desmosomal disassembly, a phenomenon already described for keratinocytes cultured in low calcium-containing media *(20)*

Acknowledgements

We thank Martin Green, Barbara Langlois, Deborah Sanders, Darren Thompson, and Gillian Westgate for all their help, and we thank Unilever PLC for financial support

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9

Keratinocyte Culture

Yvonne Barlow and Richard J. Pye

1. Introduction

The in vitro growth of keratinocytes has proved to be an important tool in the study of the normal biology and disease processes involving the skin, e.g , the influence of extrinsic regulators of growth and differentiation, effects of pharmacological agents, dermo-epidermal interactions, tissue antigenicity, and models of carcinogenesis

Fibroblasts adapt well to culture conditions. However, keratinocyte culture has been hampered by the inadequacy of media, which had hitherto been optimized for fibroblasts, and the overgrowth of cultured keratinocytes by more vigorous stromal elements. These difficulties were initially overcome by the use of lethally treated 3T3 feeder layers, which supported keratinocyte growth and inhibited overgrowth by fibroblasts *(1)*. This technique, with or without minor variations, is still widely used today It led to the supposition however, that undefined elements from the dermal components were essential to keratinocyte growth. Later, Eisinger et al. *(2)* demonstrated that by altering seeding density, pH, and incubating conditions, keratinocytes could be grown in the absence of specialized substrates, conditioned medium, or media supplements. These procedures still required large seeding densities, and passage either on plastic or 3T3 feeder cells was limited. More recently, the development of the MCDB series of media *(3,4)* has permitted serum-free, clonal growth of keratinocytes with as few as 400 cells/cm^2 of substrate. The optimization of nutrients, particularly the reduction in calcium concentration and the omission of serum, favors proliferation rather than differentiation, extending the life of the culture and permitting serial propagation. This model is particularly useful because of the absence of undefined supplements in the medium.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

The design or selection of the tissue-culture system to be used does, however, depend on the question to be explored. Indeed, central to our understanding of epidermal–stromal interactions has been the development of the dermal equivalent model *(5,6)* as a useful working alternative to organ culture of skin. In this way, collagen lattices, seeded with living fibroblasts, are placed on rafts. Keratinocytes are then either incorporated into the lattice as an explant that grows over the surface, or seeded as a single-cell suspension on top of the lattice. These simplified skin models are cultured at an air–liquid interface that promotes differentiation of keratinocytes. In this chapter, the techniques of keratinocyte culture on tissue-culture plastics, extracellular matrices, 3T3 feeder layers, and in the dermal equivalent system are described.

2. Materials

Media and supplements may be purchased from any company supplying tissue-culture products and must be of tissue-culture reagent grade.

1 Keratinocyte growth medium Keratinocyte growth medium BulletKit® (Clonetics) is supplied as a basal medium (unsupplemented MCDB 153) to which the following aliquots (also supplied) are added: 0.1 ng/mL human recombinant epidermal growth factor, 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 0.15 m*M* calcium, 2 mL bovine pituitary extract, 50 µg/mL gentamicin, and 50 ng/mL amphotericin-B. The complete medium is stored at 4°C and has a shelf-life of 10 wk

2. Alternative Keratinocyte medium This is a mixture of Dulbecco-Vogt's modification of Eagle's minimum essential medium and Ham's F12 (3/1 [v/v])—3DMEM:F12

   a. Basal medium—3DMEM:F12: 10 03 g DMEM powdered medium, 4.77 g HEPES, 0 80 g Sodium bicarbonate, and 2 65 g Ham's F12 powdered medium.

      Constituents are made up to 1 L with deionized, double distilled water, brought to pH 7 2–7.4 with 5*M* sodium hydroxide and filter-sterilized Sterility checks should be made Alternatively, three parts single strength liquid DMEM containing 20 m*M* HEPES buffer are mixed with one part Ham's F12 liquid medium and sterile sodium bicarbonate (0 8 g/L) and L-glutamine (2 m*M*) added before use Glutamine is unstable at 4°C and should be kept frozen Its half-life in medium stored at 4°C is approx 3 wk, and medium stored for long periods should be supplemented with fresh glutamine

   b Supplements to 3DMEM F12 keratinocyte medium. Stock solutions

      i Epidermal growth factor (EGF). Dissolve 0 1 mg EGF from mouse submaxillary glands in 10 mL of distilled water (10 µg/mL). Filter-sterilize and store frozen in 1-mL aliquots at –20°C

      ii. Insulin: Dissolve 100 mg of bovine insulin in 20 mL of 0.005*N* HCl (5 mg/mL) Filter-sterilize and store in 1-mL aliquots at –20°C.

      iii Hydrocortisone: Dissolve 5 mg in 1 mL of 95% ethanol (5 mg/mL) and

Table 1
Keratinocyte Seeding Density on Plastic, Extracellular Matrix, and 3T3 Feeder Cells in Vitro

Substrate	Keratinocyte seeding density/80 cm^2 flasks
Tissue-culture plastic	$5–10 \times 10^6$
Extracellular matrix	$3–5 \times 10^6$
3T3 Feeder cells	$1–2 \times 10^6$

store this concentrated stock at 4°C Add 0 4 mL of concentrated stock to 9.6 mL of serum-free medium, filter-sterilize, and store in 1-mL aliquots at –20°C.

iv Cholera toxin: Dissolve 1 0 mg of cholera toxin in 1.18 mL of distilled water ($10^{-5}M$) Store this concentrate at 4°C Add 0 1 mL of concentrate to 9 9 mL of medium containing 10% serum Filter-sterilize into 1-mL aliquots and store at –20°C

v. Transferrin/Triiodo-l-thyronine (T3): Dissolve 100 mg of human transferrin in 12 mL of phosphate-buffered saline Dissolve 13 6 mg of T3 in the minimum volume of 0 2*N* sodium hydroxide, and make up to 100 mL in sterile distilled water ($2 \times 10^{-4}M$). Add 0 2 mL of the T3 concentrate to 12 mL of transferrin, and make the total volume up to 20 mL with distilled water Filter-sterilize into 1-mL aliquots, and store at –20°C

vi Adenine Dissolve 243 mg in the minimum volume of 1*N* HCl, and make up to 100 mL with sterile distilled water ($1\ 8 \times 10^{-2}M$). Filter-sterilize and store in 10-mL aliquots at –20°C

vii Serum. Heat-inactivated fetal calf serum (FCS, 10%) is used to culture epithelial cells All batches of serum must be tested for their ability to support keratinocyte growth at clonal densities (see Chapter 1) (Table 1)

3. Feeder cell medium.
   a. Basal medium DMEM powdered medium: 13 37 g *N*-2 hydroxyethyl piperazine N-2 ethane, 4.77 g sulfonic acid (HEPES), and 0.80 g Sodium bicarbonate. Constituents are made up to 1 L with deionized, double-distilled water, brought to pH 7 2–7.4 with 5*M* sodium hydroxide and filter-sterilized Sterile single-strength, liquid DMEM containing 20 m*M* HEPES can be purchased ready for use Sterile sodium bicarbonate (0.8 g/L) and L-glutamine (2 m*M*) are added separately
   b. Serum· Heat-inactivated newborn calf serum (10%) is added to DMEM before use
4 Antibiotics.

Antibiotic	10X solution	1X solution
Penicillin	1000 U/mL	100 U/mL
Streptomycin	1000 µg/mL	100 µg/mL
Fungizone	2 5 µg/mL	0 25 µg/mL

5. Dispase (Protease type IX from *Bacillus polymyxa*). Dissolve 250 mg dispase in 100 mL serum-free DMEM Filter-sterilize and store in 10-mL aliquots at –20°C
6. Trypsin
   a. Routine subculture of cells: Trypsin (Difco, 1 250) 0 25% solution containing 0.02% ethylene-diamine-tetra-acetic acid (EDTA) is made up in HEPES-buffered calcium and magnesium-free Hanks' balanced salt solution
   b. Keratinocyte suspensions Trypsin (1 300) 0 25% containing penicillin 200 U/mL, streptomycin 100 µg/mL, and 0.5 g/L sodium bicarbonate in calcium and magnesium-free Hanks' balanced salt solution are stored in 5-mL aliquots at 20°C Before use, each 5-mL aliquot is made up to 20 mL with calcium and magnesium-free Hanks' balanced salt solution
7. Trypan blue solution for cell viability counts Dissolve 400 mg Trypan blue, 810 mg sodium chloride and 60 mg potassium dihydrogen orthophosphate. Adjust the pH to 7 2–7 3 with 1*M* sodium hydroxide, and make up to 100 mL with distilled water
8. Dermal equivalents:
   a. Medium for preparation of collagen rafts: 9.78 g Eagle's minimum essential medium powder, 5 0 g HEPES, 2 0 g sodium bicarbonate, and 535 mL distilled water
   b. Collagen Collagen can be purchased from a number of commercial sources Pure Type I calf skin collagen (1 mg/mL) is dialyzed against several changes of distilled water until the pH is no lower than pH 4 5 Dialyzed collagen is stored in 5-mL aliquots at –20°C until required
   c. Reconstitution of dermal equivaltents 2.3 mL Eagle's MEM, 1 5 mL collagen, 0 25 mL sodium hydroxide (0 1*N*), 0 45 mL fetal calf serum, and 0.5 mL fibroblast cell suspension (8×10^5 cells/mL)

3. Methods

All procedures using human tissue are carried out in a Class II laminar flow hood using aseptic technique.

3.1. Preparation of Keratinocytes

1. Human skin may be obtained from surgical specimens removed at circumcision, mastectomy, apronetomy, or during cosmetic surgery Not all skin grows equally well in culture. In our experience, skin from ears, hands, and feet are less successful than skin from breast, abdomen, or preputial skin. Growth rate is also affected by donor's health and age Keratinocytes from children and young healthy adults undergo more population doublings The skin sample should be placed in a dry sterile container and stored at 4°C until collection can be arranged. For maximum viability, skin should be processed as soon as possible after excision However, where necessary, skin can be stored in this way for several days with a small loss of viability. If prolonged storage is contemplated, a small quantity of phosphate buffered saline containing penicillin and streptomycin should be added, to prevent drying of the sample

2. Fatty tissue should be removed from abdomen and breast samples, otherwise tissues should be directly immersed in 10 x strength antibiotic solution for 30 min. After several washes in serum-free medium, tissues are left to soak in single-strength antibiotic solution for a further 30 min (*see* Note 1)
3 The skin is transferred to a sterile 90-mm Petri dish with the sample placed dermal side down. Using sterile scissors and forceps, the skin is raised in the forceps, and small pieces of skin (0.5 × 0.5 cm) are cut from the surface leaving behind as much of the connective tissue as possible
4 The pieces of tissues are placed in Dispase solution at 4°C overnight with approx 4–5 cm^2 of tissue/mL of Dispase For convenience, tissues may be left in Dispase solution for 48 h without significant loss of viability.
5 After 24 h, the tissue incubated in Dispase solution is transferred to a sterile 90-mm Petri dish Using sterile forceps, the epidermis is peeled very easily from the dermis. If the epidermis does not peel easily, the tissue can be incubated for several hours at 37°C, or placed in fresh Dispase and incubated for a further 24 h at 4°C The pieces of peeled epidermis should be kept moist in calcium and magnesium-free Hanks' balanced salt solution during this procedure
6 The pieces of epidermis are transferred to a sterile universal, containing 10 mL of prewarmed trypsin solution and incubated at 37°C for 30–45 min.
7 After this time, the contents of the universal container are shaken once or twice to loosen the trypsinized cells, which are released into suspension The solution is allowed to settle briefly, after which the pieces of epidermis float to the surface while cellular debris falls to the bottom of the universal.
8 The keratinocyte suspension is carefully pipeted into a centrifuge tube. To each 5 mL of keratinocyte cell suspension, 5 mL of DMEM containing 20% serum is added and the suspension centrifuged at 200*g* for 5 min
9. The supernatants are aspirated and 1 mL of fresh medium containing 10% serum added to each cell pellet. Using a plugged sterile Pasteur pipet, the cell pellets are very gently resuspended to form a single-cell suspension
10. More fresh, prewarmed trypsin solution is added to the pieces of epidermis, and the process is repeated Three trypsinizations may be performed to maximize cell yield. The cells harvested in this way are pooled and counted.
11. An equal volume of cell suspension and Trypan blue solution are mixed and left at room temperature for 5 min. Using the improved Nebauer hemocytometer, both chambers are filled by touching the edge of the coverslip with the pipet and allowing the suspension to be drawn up by capillary action. Avoid flooding the chamber.
12 Each hemocytometer chamber is divided into nine large squares delineated by triple white lines The center square is further subdivided into 25 squares, which are again subdivided into 16 squares Viable cells (i. e., those not stained blue) are counted in the two central squares of each chamber and the mean value for one large square calculated. The number of cells in one large square is multiplied by the dilution factor for Trypan blue, i.e., x 2 = *n* cells. Multiply the number of cells *n* by 10^4 = cells/mL The number of cells/mL multiplied by the number of mL of cell suspension = total number of cells harvested

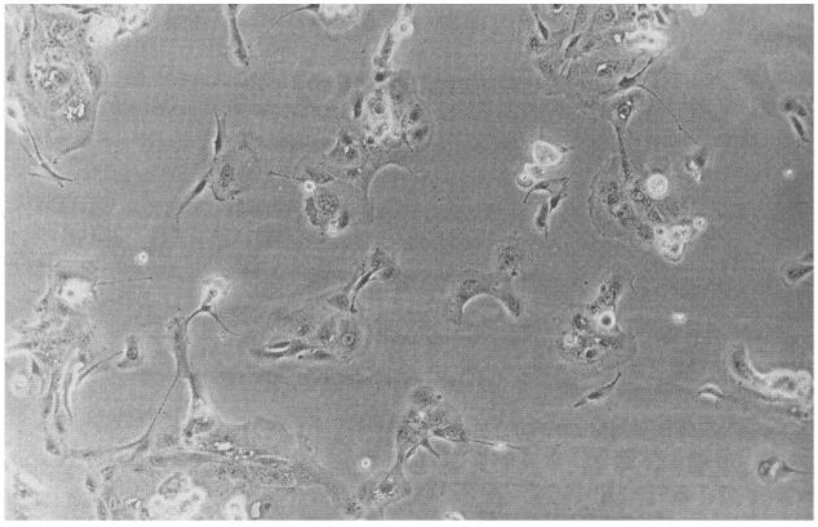

Fig. 1. Human foreskin keratinocyte culture 3 d after plating (200×).

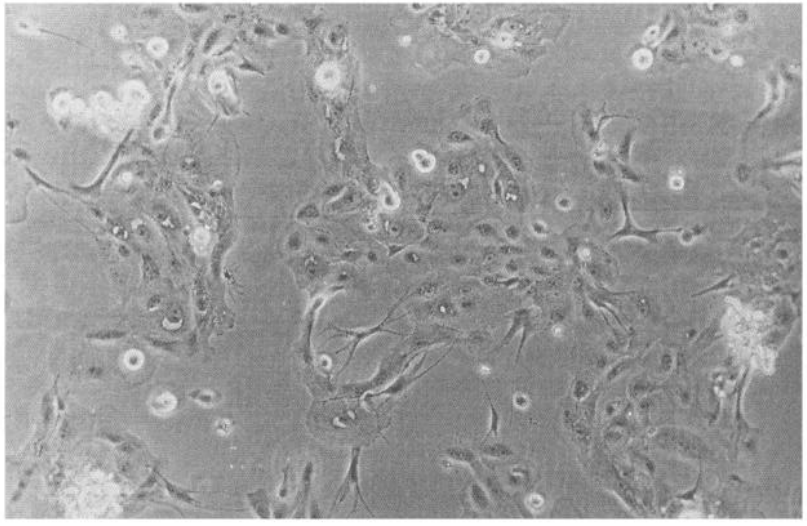

Fig. 2. Human foreskin keratinocyte culture 7 d after plating (200×).

3.1.1. Seeding of Keratinocytes

Keratinocytes may be seeded into tissue-culture flasks, onto 3T3 feeder layers, or onto an extracelluar matrix.

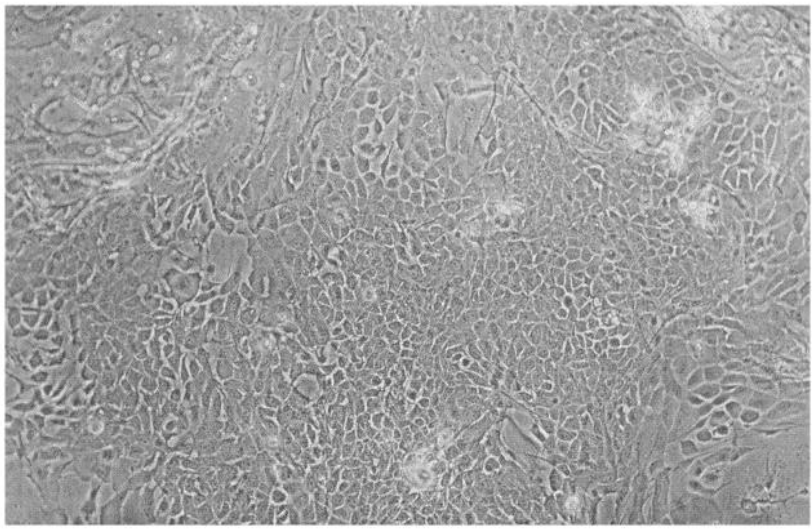

Fig. 3. Human foreskin keratinocyte culture 12 d after plating. Keratinocytes assume typical pavement-like structure as cultures approach confluence (200×).

3.1.1.1. Tissue-Culture Flasks

1. The suspension of cells is diluted in an appropriate volume and seeded into plastic tissue-culture flasks at a density of 6.25×10^4 to 1.25×10^5 cells/cm^2.
2. After the cells have attached (1–2 d), cultures should be refed with complete keratinocyte medium every 2–3 d. Cultures should be fed with keratinocyte medium without EGF when first seeded into flasks. When cells approach confluence, they may require to be fed every day. Cultures are incubated at 37°C.
3. Cells may appear flattened a few days after plating (Fig. 1), but as the culture becomes denser, cells assume the typical pavement-like appearance of epithelium (Figs. 2 and 3). Cultures should approach confluence within 10–15 d and should be subcultured (*see* Section 3.2.) before they reach confluence. Cultures held at confluence differentiate, and blistering may occur.

3.1.1.2. 3T3 Feeder Layers

1. 3T3 Swiss albino transformed mouse embryo fibroblasts may be obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology, Porton Down, Salisbury (ref. no. CCL-92). 3T3 cells are grown in DMEM plus 10% newborn calf serum. Cultures are routinely passaged two or three time/wk.
2. Prior to seeding 3T3 cells with keratinocytes, cells are passaged and grown to 50–70% confluence (Fig. 4). The 3T3 cells are then lethally irradiated with 6000 rads using any appropriate source or treated with mitomycin C (*see* Note 2). Cul-

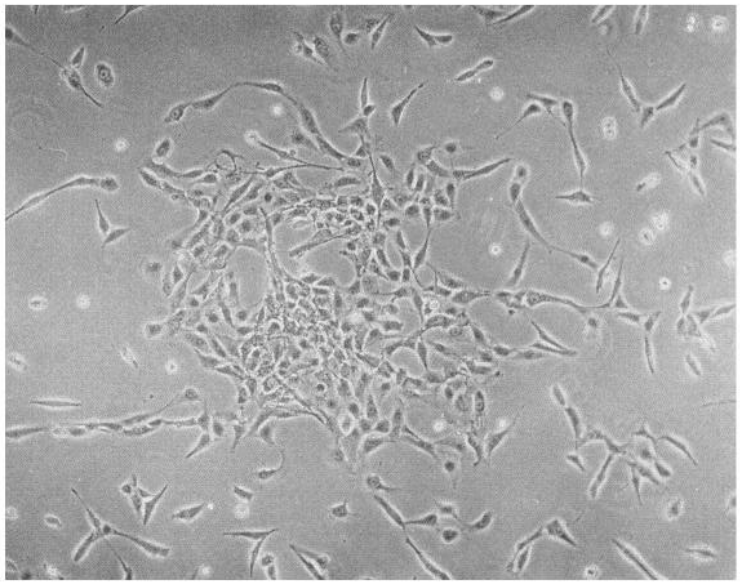

Fig. 4. Swiss 3T3 cells 2 d after seeding at 1.5×10^6 cells/80 cm^2 flask (100×).

tures 50–70% confluent are incubated with medium containing mitomycin C at 5–10 µg/mL (*see* Note 2) for 1 h at 37°C.

3. The cells are then thoroughly washed with serum-free medium before keratinocytes are seeded onto the 3T3 cells.
4. Keratinocytes are seeded at a concentration of 2.5×10^4 cells/cm^2, fed with keratinocyte medium without EGF, and incubated at 37°C.
5. After cells have attached, cultures should be fed with complete keratinocyte medium. Clones of epithelial cells may not be apparent among the 3T3 feeder cells for several days, but with increasing incubation, small islands of 20–50 cells should be easily visible (Fig. 5). The cultures should approach confluence in 10–14 d and must be subcultured before cells reach confluence. Keratinocytes may be subcultured at seeding densities as low as $1.25–2.5 \times 10^4$ cells/cm^2 onto new lethally treated 3T3 cells.

3.1.1.3. Extracellular Matrix (ECM)

1. ECM made in this laboratory from 3T3 cells or human foreskin fibroblasts is less successful in promoting growth of keratinocytes than commercially prepared bovine corneal endothelial cell matrix *(7)*. Once confluent, these cultured cells are lysed with 0.5% Triton-X-100 in 0.0125*M* ammonium hydroxide for 5–10 min and washed in balanced salt solution before use (Fig. 6).

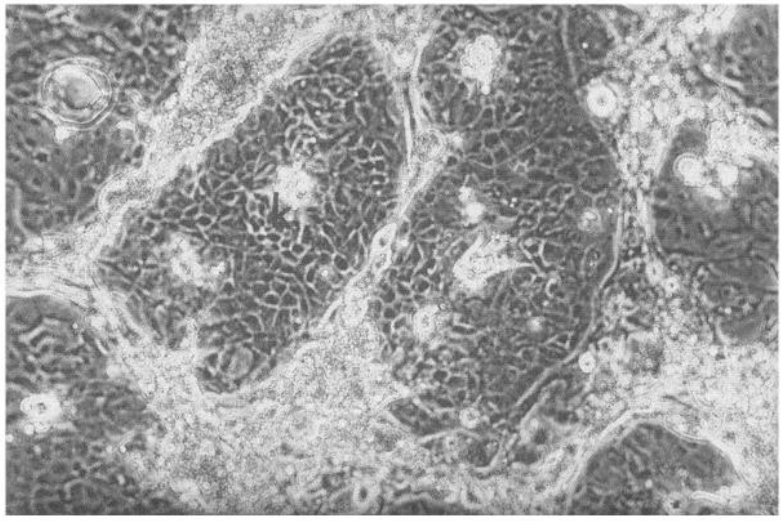

Fig. 5. Colonies of keratinocytes (K) growing on lethally treated 3T3 feeder cells, 7 d after seeding at 2×10^6 keratinocytes/80 cm^2 flask (200×).

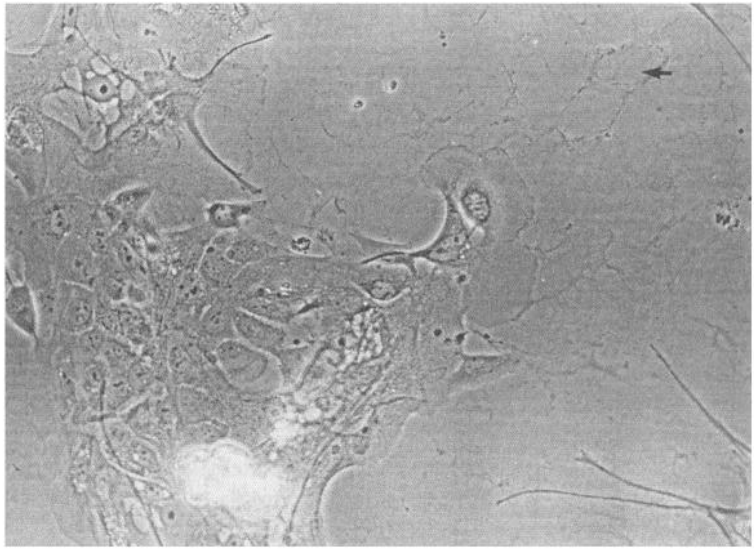

Fig. 6. Keratinocytes from 60-yr-old female after third passage on extracellular matrix from bovine corneal cells. The ghosts of the corneal cells can clearly be seen.

2 Keratinocytes seeded onto bovine cornea ECM have up to two- to threefold greater plating efficiency than on tissue-culture plastic, and confluence may be achieved in up to 2 wk with seeding densities of 3.5–6.0 × 10^4 cells/cm^2 in 80-cm^2 flasks
3 The cultures are fed with keratinocyte medium without EGF for the first day, after which cultures are refed as required with complete keratinocyte medium Cells should be subcultured before confluence is reached.

3.2. Subculture

1 Nearly confluent flasks of keratinocytes are washed twice in calcium and magnesium-free Hanks' balanced salt solution
2. To each 80-cm^2 flask, 5 mL of trypsin–EDTA solution is added, and the flasks incubated for 5–10 min at 37°C
3. The cells are dispersed into solution by tapping the flasks
4 Trypsinization is stopped by the addition of 0 5 mL of 20% FCS to each flask
5 The cell suspensions are centrifuged at 200*g* for 5 min and the supernatants aspirated
6 The cell pellet is gently resuspended in 1 mL of MCBD 153 medium and the cells counted in a hemocytometer The cells are diluted to an appropriate volume for subculturing onto various substrates (Table 1)
7 Cells are fed with keratinocyte medium, but without EGF EGF is added to the cultures at the first feeding *(7)* Proliferation rate is dependent on culture conditions, age, health of donor, and the site of donor skin Table 1 is a guide to seeding densities used in this laboratory on different substrates.

3.3. Dermal Equivalents

Keratinocytes attach to the surface of the dermal equivalent, divide, and grow to cover the collagen lattice. Differentiation into a multilayered epidermis occurs, and keratinization is much more complete in these cultures incubated at an air–liquid interface (Fig 7). Dermal equivalents are made in this laboratory in 60-mm diameter Petri dishes, but can be scaled up or down to the required size.

1 Sufficient collagen, medium, serum, and human skin fibroblasts (as described in the Materials section) are mixed and poured into 60-mm diameter Petri dishes
2 The dishes are incubated in humidified boxes at 37°C, and within a few hours, the collagen gels solidify.
3 At this stage, a skin plug approx 2–3 mm in diameter can be inserted into the gel, which, on contraction, will hold the skin plug tightly in place
4. Keratinocytes migrate from the explanted tissue across the surface of the collagen/fibroblast matrix The gels will normally contract within 2–3 d (Fig 8), but the degree and speed of contraction of the gel depend partially on the seeding density of the fibroblasts

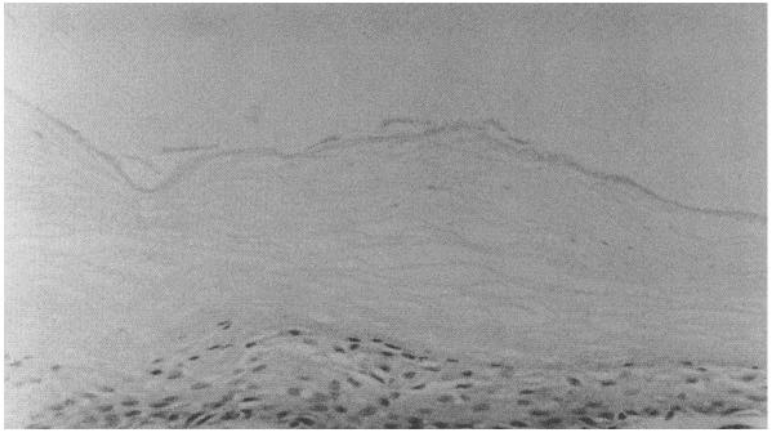

Fig. 7. Keratinocytes cultured on dermal equivalent (100×).

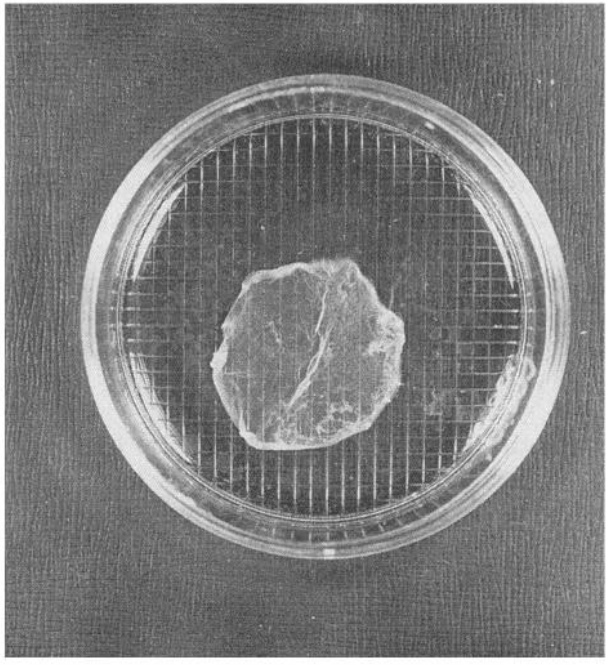

Fig. 8. Contracted collagen–fibroblast matrix 3 d after seeding. Original diameter of the gel was 60 mm (100×).

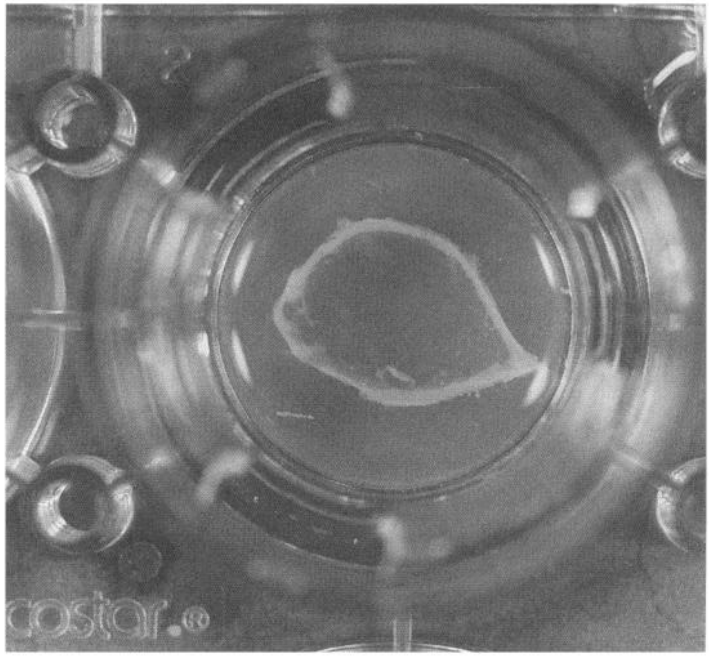

Fig. 9. Collagen–fibroblast matrix raised to air–liquid interface on stainless-steel grid (200×).

5. The contracted rafts are raised onto transwell plates (Fig. 9). Rafts without epithelial plugs may be seeded with keratinocytes at this stage (3×10^5 cells/mL). This procedure can be standardized using stainless-steel O rings.
6. The cultures are then fed with enough keratinocyte medium to reach just below the level of the raft. The medium is drawn up by capillary action.
7. Cultures are refed every 2–3 d.

4. Notes

1. Several other antibiotics have been tested in this laboratory for use with keratinocyte cultures. Vancomycin was found to be very effective when tested against 10 methicillin-resistant strains of Staphyloccocus and has very low toxicity to cultured keratinocytes. Washing in medium containing 1 mg/mL vancomycin for 30 min is recommended for infected cultures. After several washes in serum-free medium, cultures can be incubated with 100–200 μg/mL of vancomycin. Vancomycin is only recommended in special circumstances and should not be used routinely.
2. Mitomycin C, which disrupts microtubule formation in cells and prevents completion of cell division, is often used as an alternative to lethal irradiation.

Potency and toxicity of different batches of mitomycin C varies, and each batch should be tested In this laboratory, mitomycin C has been found to be effective at concentration of 5–10 μg/mL. If none of the available flasks of 3T3 cells are at a suitable stage, a fully confluent culture of 3T3 cells can be lethally treated, and subcultured into a flask freshly seeded with keratinocytes the same day or the following day with beneficial results

Acknowledgments

Our thanks to Debbie Coakley for excellent technical assistance. Our grateful thanks to J. K. Wright for assistance with photography, to T. E. Cawston, Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge for the collagen used in the dermal equivalent, and to R. Guy, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

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10

Tissue Culture of Skeletal Muscle

Terry A. Partridge

1. Introduction

In its simple forms, no special difficulty attaches to the tissue culture of skeletal muscle. Indeed, it is one of the easiest tissues to culture in large amounts because the starting material, skeletal muscle, is plentiful and readily obtainable from a wide variety of species, including humans. Moreover, the stem cells from which muscle develops in tissue culture seem to be very resilient; they will survive anoxic conditions within muscle at temperatures between 4 and 37°C, for up to a few days, and when obtained in suspension, are easy to freeze (with 10% dimethyl sulfoxide [DMSO] as a cryoprotectant) and store in liquid nitrogen.

Mature muscle fibers are sensitive to physical damage and trauma, making it difficult to maintain them in good functional condition out of the body for more that a few hours It has therefore been common practice to grow new muscle fibers afresh from their mononucleate precursor cells (mpc). These latter cells may readily be obtained in large numbers by mincing and enzymatic disaggregation of growing or regenerating muscle and in smaller, but still usable numbers from mature normal muscle. It is of biological interest that the thymus is also a source of such cells *(1)* and that apparently nonmyogenic mesenchymal cells may be converted to myogenesis *(2,3)*. More recently, methods have been developed for obtaining and maintaining isolated viable fibers from commonly used rodent muscles. These preparations provide fully mature muscle fibers which remain viable for several days. In addition the satellite cells which emigrate from these fibers can be harvested to produce pure myogenic cultures.

Here, the disaggregation method we use to grow skeletal muscle from neonatal mouse muscle and a recently described technique for growing high purity

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

myogenic cultures from isolated muscle fibers are described in detail *(4)*. The disaggregation method gives satisfactory results with fetal human (15 wk) and neonatal rabbit muscle and is useful where large amounts of source muscle are available and where muscle with undamaged fibers, which is necessary for the isolated fiber technique, is difficult to obtain. A simple method we have used for growing avian muscle in culture is also described, and the use of some mouse cell lines is commented on. In addition, the derivation of conditionally immortal myogenic clones from mice transgenic for an inducible construct for controlled expression of the SV40 large T immortalizing gene is described. Complications, problems and refinements are indicated in the Notes section.

2. Materials

1 All glassware is washed in 1.5% RBS 25 detergent (Chemical Concentrates, Sittingbourne, Kent, UK), rinsed ten times in tap water and twice in double-glass distilled water, and then air-dried in a warm oven

2 Muscle cells are usually grown in vented polystyrene tissue culture Petri dishes, 35 or 60 mm diameter. Where we need to make permanent microscopic preparations, immunostained preparations, or microscopic observations at high resolution on the cultures, we grow the cells on a sterile coverslip, placed in the Petri dish prior to addition of the cell suspension and removed when it is required. We routinely coat tissue-culture surfaces with sterile rat-tail collagen in distilled water and allow it to air dry This is not strictly necessary for the mixed cell cultures described here

3 For filtration of the single-cell suspension, three layers of 45-µm pore size nylon cloth are placed in sterile Swinnex filter holders.

4 Phosphate-buffered saline (PBS): 8 0 g NaCl, 0 2 g KCl, 1 12 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1 L distilled water, adjusted to pH 7 2 with NaOH The solution is dispensed in 100-mL aliquots and sterilized by autoclaving

5. Antibiotic solution: 100 mL sterile PBS, 10 mL Fungizone (amphotericin B, 250 µg/mL), and 2 mL penicillin/streptomycin (P 5000 IU/mL, S 5000 µg/mL) Can be stored at –20°C

6 HEPES-buffered calcium and magnesium-free Hanks' balanced salt solution (BSS), pH 7 4 (Ca^{2+}- and Mg^{2+}-free BSS) Purchased as a 10X solution and diluted 1/10 with double-glass distilled water This solution is buffered by the addition of 1*M* HEPES solution to give a final concentration of 25 m*M* HEPES, sterilized by filtration through a 0.45-µm Millipore filter and stored at –20°C

7 Trypsin stock solution: Trypsin 0 25% porcine trypsin (Sigma) stored at –20°C in 20-mL aliquots

8. Pangestin stock solution. Pangestin (1.75, Difco, East Molesy, Surrey, UK) mixed in a 0.5% ratio (w/v) in Ca^{2+}- and Mg^{2+}-free BSS for 2 h at 37°C, centrifuged at 2600*g* for 15 min at 4°C, and filtered through a Whatman No. 1 filter paper. The pH is adjusted to 7 4 with 1*N* NaOH The solution is sterilized by filtration through a 0 45-µm Millipore filter and stored in 20-mL aliquots at –20°C

9 Enzyme solution On the day prior to use, 60 mL of trypsin stock solution, 20 mL pangestin stock solution, and 20 mL Ca^{2+}- and Mg^{2+}-free BSS are mixed under aseptic conditions The solution is dispensed in 5-mL aliquots in sterile universal bottles, stored at 4°C overnight, and brought to room temperature immediately before use

10. Inhibition medium 100 mL Hanks' BSS + 20 mL fetal calf serum (FCS) and 2 mL penicillin/streptomycin (P 5000 IU/mL, S 5000 µg/mL). Store at 4°C until use.

11 Culture media· Basic medium constituents Eagle's minimum essential medium (EMEM), Dulbecco's minimum essential medium (DMEM), FCS, horse serum (HS), chick embryo extract (CEE), penicillin and streptomycin stock solutions, and Fungizone solution are all obtainable from a number of commercial sources (*see* Note 1).

We make our own CEE by a standard method *(4)* Fertile chicken eggs, 10–12 d incubation, are held blunt end uppermost, swabbed with 70% ethanol, and given a sharp tap, delivered horizontally, with a sterile scalpel handle at about the lower edge of the air space (this can be seen by holding the egg against a bright light). The cap of the shell and adherent shell membranes are then carefully lifted off with sterile coarse forceps One prong of a second pair of sterile coarse forceps is plunged through the embryonic membranes and beneath the neck of the embryonic chick, which can then be lifted carefully from the egg and transferred to a sterile beaker When all the embryos have been removed, they are washed free of all traces of blood and yolk with several changes of BSS These embryos are then crudely homogenized by removing the plunger from a 20-mL syringe, loading the embryos, a few at a time, into the barrel, carefully reinserting the plunger, and pushing it home to express the contents, via the needle fitting, into sterile plastic universal containers To the mashed embryo in each container an equal quantity of Hanks' BSS is added, and the two are mixed thoroughly by stirring with a sterile glass rod After 30 min incubation at room temperature, the containers are centrifuged at 2000*g* for 20 min and the supernatant CEE is stored in small aliquots at –20°C. On thawing, the CEE is recentrifuged before use

12 Media for mouse primary cultures. 100 mL DMEM, 10 mL FCS, 2 mL CEE

13 Media for single fiber culture·

a Plating medium DMEM + 10% HS, 0.5% CEE,

b. Proliferation medium· DMEM + 10% HS, 20% fetal bovine serum, 1% CEE.

c Differentiation media.

i DMEM + 10% HS, 2% FCS, 0.5% CEE. This is replaces the growth medium for up to 1 wk.

ii DMEM + 2% FCS, 0 5% CEE. This replaces the previous medium for the final few days of differentiation.

14. Cell lines and their growth media

a. G8-1. For growth 100 mL DMEM, 10 mL FCS; for differentiation 100 mL DMEM, 2% HS.

b. C2. For growth 100 mL DMEM, 20 mL FCS; for differentiation· 100 mL DMEM, 2% H S

c MM14: 50 mL DMEM, 50 mL Ham's F10, 15 mL HS, 3 mL CEE
d Human primary cultures. 100 mL DMEM, 20 mL FCS.
e. Avian cells Following the methods described by Konigsberg *(5)*, we have used "high growth" media consisting of: For chick, 79 mL EMEM (with Earle's salts), 15 mL HS, 5 mL CEE, 1 mL Penicillin/Streptomycin, 0 25 mL Fungizone For Quail, 74 mL EMEM (with Earle's salts), 15 mL HS, 10 mL CEE, 1 mL Penicillin/Streptomycin, 0 25 mL Fungizone. Primary cultures, given one change of "high growth" medium on the day after they were set up, and left in this medium, initially proliferate and subsequently, as they exhaust the nutrients, differentiate to form large numbers of myotubes However, Konigsberg *(4)* recommended reducing the concentration of CEE to produce a "low growth" medium that, on addition to the culture, limits proliferation and encourages differentiation

15 Culture substrata
a Collagen· This is made by a standard method *(5)* Rat tails are sterilized by steeping them in 95% ethanol for a few hours Each tail is then broken into short segments, starting at the tip To do this, the tail is grasped crossways at the distal end with two pairs of sterile Spencer-Wells forceps, held side by side, the tip is snapped off by a sharp rotation of the more distal of the two forceps, and carefully drawn away to pull out the long white tendons that insert into the tip of the tail These are then cut off with a pair of sterile scissors and allowed to fall into a beaker containing sterile 1% acetic acid in distilled water (150 mL/tail) This process is repeated, working progressively toward the base of the tail When all of the accessible tendon has been removed, the beaker is covered to maintain sterility and kept for a few days at 4°C, after which the solution is centrifuged for 1 h at 2500*g* The supernatant is then dialyzed, in autoclave-sterilized dialysis tubing, against daily changes of distilled water until it begins to become viscous This solution is removed, after sterilizing the outside of the dialysis tube with a wash of 70% ethanol, and can be stored for several months in aliquots under sterile conditions at 4°C Should the solution become too gelatinous, its viscosity can be reduced by addition of a little sterile dilute acetic acid A drop of collagen solution is placed on the culture surface (glass or plastic), spread thinly with a glass spreader (made by using a fine flame to seal the tip of a Pasteur pipet and to bend the terminal cm or so at right angles to the main stem), and left to air-dry in a sterile flow hood Such collagen coated surfaces can be stored dry for several weeks
b. Gelatin· A 0 01% solution of gelatin (Porcine skin, Type I) in distilled water is distributed into 1–2-mL aliquots and sterilized by autoclaving. A drop of the solution is placed on the culture surface, spread, and air-dried as for collagen
c Tissue-culture substrata for single fibers
i. In general, myogenic cultures grow better on plastic than on glass substrata. Primary cells obtained from by dissociation of minced muscle

fragments are routinely grown in vented polystyrene tissue culture Petri dishes, 35 or 60 mm in diameter, or for large numbers of cells, in Falcon culture flasks The single fiber cultures develop most satisfactorily in 24-well Falcon Primaria plates

ii. For the mixed cell cultures obtained from disaggregated muscles, it is sufficient to coat the plastic with type I rat-tail collagen or gelatin or the commercial collagen preparation Vitrogen 100 However, we find that, in general, myogenic cultures differentiate better on a matrix containing laminin, such as Matrigel, which we use as a 10% solution in DMEM (approx 1 mg protein/mL). This is applied to the wells, ice cold to delay gelation, and the excess drained before incubation at room temperature for 90 min An isolated muscle fiber placed on this surface adheres firmly within a few minutes but can be removed, intact, with a fine pipet when sufficient cells have emigrated from it

3. Methods

3.1. Tissue Culture of Cells from Neonatal Mouse Muscle

1 Newborn mice, (1–2 d, *see* Note 2) are killed by decapitation, the tail and paws are cut off, and the body washed in 70% ethanol, then soaked in antibiotic solution By slitting the skin along the length of the spine, it can be peeled off in one operation by means of two pairs of coarse forceps. one pair is used to grasp the mouse by the exposed end of the spine at the neck and the second pair to pull the free edge of the skin at the sternum back toward the tail

2. The skinned carcass is then placed in a sterile Petri dish where as much as possible of the skeletal muscle is removed from the limbs and trunk, with the aid of watchmakers forceps and microscissors, and placed in a second sterile Petri dish where it is kept damp with a drop of BSS Care must be taken not to include fragments of bone or of the brown fat that is present in large amounts in these young animals, especially between the shoulder blades. It is also important, particularly if the animal has suckled, not to puncture the gut Depending on the age and size of the mice and the skill of the operator, approx 0 5 g of muscle can be obtained in this way from 3–5 mice in about 2 h: this is the optimal amount to be taken through the remainder of the procedure by one person

3. The muscle is minced into small fragments with a pair of sharp, curved fine scissors (if well dampened with saline, the fragments tend to be kept together by surface tension) and divided into 10 equal portions, each of which is placed in 5 mL of enzyme solution in a sterile plastic universal bottle

4. These are placed in a 37°C water bath and shaken at 2 Hz for 10 min. Each aliquot is gently sucked into and expelled from a Pasteur pipet continuously for one min to disperse the cells. Remaining muscle fragments are allowed to settle, and the supernatant containing the released cells is decanted into 5 mL of inhibition medium

5 Fresh enzyme solution (5 mL) is then added to the remaining muscle fragments in each universal bottle and the disaggregation procedure repeated

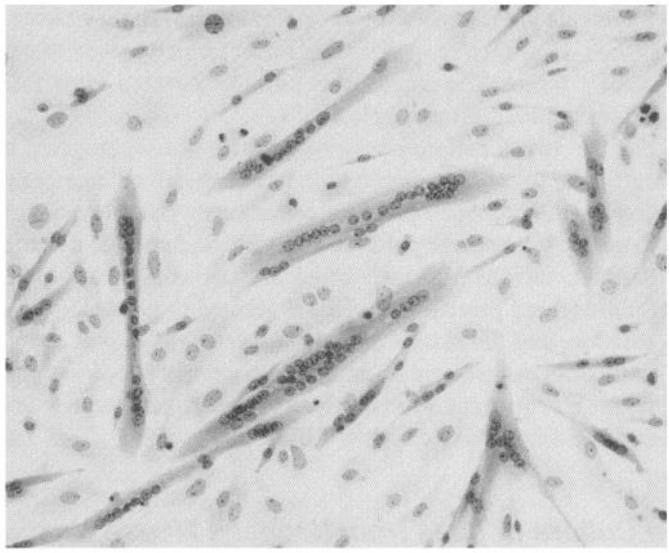

Fig. 1. Seven day primary culture of cells obtained by enzymic disaggregation of neonatal mouse muscle, stained with H&E, 100×. Note small multinucleated myotubes and the numerous unfused cells. Of the latter, some of the small spindle-shaped cells are probably myoblasts.

6. The inhibition medium containing all the released cells from both incubations is poured into a large, sterile plastic syringe fitted with the Millepore filter holder containing the 45-μm nylon cloth, and slowly expressed, thus removing any remaining clumps of cells and muscle fragments to give a suspension consisting almost entirely of separated mononucleate cells.
7. This is centrifuged at 350*g* for 10 min at 4°C, and the resultant cell pellets are resuspended in a known volume of growth medium.
8. A 100-μL sample of this suspension is added to 50 μL of a 0.5% solution of Trypan Blue in PBS, and a count of viable cells (i.e., those that exclude the dye) is made in a hemocytometer.
9. For culture in 35-mm plastic Petri dishes, cells are suspended in culture medium at concentrations of between 10^4 to 2×10^5 cells/mL, and plated out at 2 mL of cell suspension/dish. It is common to coat dishes with collagen or gelatin. This is important when cloning myogenic cells, but with cultures prepared as in the previous steps, we can detect no advantage in doing so.
10. The Petri dishes are placed in a 37°C incubator in an atmosphere of 10% CO_2 and examined daily under an inverted microscope fitted with long-working distance phase contrast objectives. By 5–7 d, numerous myotubes will have begun to form (Fig. 1, *see* Note 3). If longer term cultures are required, the medium must be replaced by one containing less FCS and no CEE, so as to discourage overgrowth of the culture by nonmyogenic cells.

3.2. A New Method for Preparation of Pure Myogenic Cells from Rodent Muscle

A major problem in tissue culture of rodent muscle is the high level of contamination of the cultures by nonmyogenic cells. A second common difficulty with conventional tissue-dissociation methods is that useful numbers of myogenic cells can be directly derived only from muscles of young animals; beyond the first week after birth the yield of myogenic cells from a 10-mg muscle, such as the mouse extensor digitorum longus, falls to single figures and the nonmyogenic contamination becomes yet more prominent. Recently, we have developed a means of overcoming both of these problems. It consists of isolating single muscle fibers from all of the surrounding connective tissue and using these to initiate the cultures. The isolated muscle fiber preparation has been used for some years to study satellite cell proliferation within the muscle fiber basement membrane, but has been practiced only on small muscles such as the flexor digitorum brevis. We have adapted the separation method to permit isolation of large living muscle fibers, 1 cm or more in length, from the major limb muscles of the mouse and rat. The method is described in detail in ref. *6*, but the main points are given here:

1 The donor animal is killed by cervical dislocation and the muscles to be used are removed immediately. First, the skin of the lower leg is shaved and sterilized with 70% ethanol An incision is made and the skin is deflected to expose the musculature of the lower hind limb The chosen muscle is dissected carefully, being handled only by the tendons The best muscle is the extensor digitorum longus which can be removed intact with both tendons, but we also use the soleus muscle and the tibialis anterior The latter muscle arises proximally from the crest of the tibia and must be removed from the latter together with the periosteum in order to preserve the muscle fibers undamaged.
2 The intact muscle is washed in PBS and incubated for 1 5–2 h in 8 mL of freshly prepared filter-sterilized 0.2% type I collagenase in DMEM in a 5-cm Sterilin plastic Petri dish
3 After incubation, the muscle is removed to fresh 5-cm Petri dish containing 8 mL DMEM. To prevent sticking of muscle fibers, this and all further transfers of the muscle are performed with a wide-bore flame-polished Pasteur pipet, prerinsed with 10% HS in DMEM The Petri dishes are prerinsed with undiluted HS to prevent adhesion of the accumulated single fibers prior to plating.
4 The muscle is gently sucked in and out of the wide-bore pipet, while observed under a dissecting microscope, to release fibers from its surface Once 20–30 fibers have been released, the mass of muscle is removed to a second similar Petri dish and the procedure of sucking in and out of the pipet is repeated As the mass of muscle diminishes, successively narrower pipets are used
5 The procedure is continued until sufficient single muscle fibers have been released Dishes containing separated undamaged fibers plus damaged hyper-

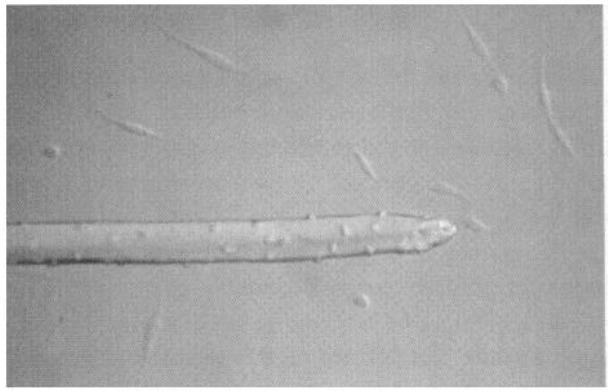

Fig. 2. Phase contrast image of part of single fiber isolated from a mouse extensor digitorum longus muscle and plated out in a Matrigel coated well of a tissue-culture plate. After incubation for 3 d a number of satellite cells have emigrated from the muscle fiber and have undergone a number of divisions.

contracted fibers and cellular and extracellular debris are kept in a humid incubator at 37°C and 5% CO_2 until sufficient dishes have been prepared.

6. The intact, nonhypercontracted fibers are picked individually out of the debris with a Pasteur pipet and each is placed individually in a single well, precoated with 10% Matrigel in DMEM, of a 24-well Falcon Primaria tissue-culture plate.
7. Within 3 min, the fiber has become attached to the Matrigel coating and it is possible to slowly add 0.5 mL of "plating medium," taking care not to disturb the fiber. The culture is then returned to the incubator for 3 d.
8. Cultures are observed daily, but must not be kept out of the incubator for more than 15 min for the first few days. Satellite cells begin to emigrate from the fiber within 24 h, increasing progressively in number, up to 300 cell/fiber, over the next 2 d (Fig. 2).
9. At 3 d the original fiber may be removed (although this is not necessary) and the plating medium replaced with the "proliferation medium." When the cultures become dense (Fig. 3), they may be trypsinized and subcultured or allowed to differentiate. The latter is accelerated if the "proliferation medium" is replaced progressively by the two low serum "differentiation media."

The above technique produces cultures most of which consist entirely of myogenic cells (Fig. 4). These cultures can be passaged once or twice to produce between 3×10^4 and 1.5×10^5 cells/murine muscle fiber. The yield from rat muscle fibers is proportionally greater. To achieve such pure cultures, it is important only to plate nonhypercontracted fibers which can be seen to be clear of adherent wisps of connective tissue.

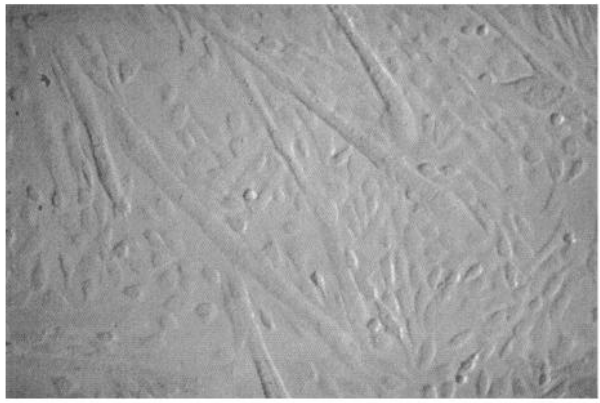

Fig. 3. Phase contrast image of cells grown from a culture of the type initiated in Fig. 2 after removal of the original fiber and incubation for a further 7 d in growth medium. Note the formation of small myotubes in this dense culture.

In terms of yield of myogenic cells per weight of muscle, this method is 10^3 to 10^4-fold more efficient than standard methods of dissociating cells from minced muscle. Moreover, it can be applied to muscles of all ages of animal. Its main drawback is that it is quite labor intensive if very large numbers of myogenic cells are required.

3.3. Tissue Culture of Avian Muscle

Chick and quail embryonic muscles are among the easiest to prepare and culture. No sterilization of the carcass is required. At 9 d for the quail and 11 d for the chick, the muscles can be disaggregated enzymically as described for the mouse, but are also sufficiently soft that the mpc can be released by mechanical disruption of the tissue. Further advantages of the avian muscle culture systems, especially quail, are that a greater proportion of the cells differentiate into myotubes and that there is less experiment to experiment variation than with mouse muscle. For both chick and quail muscle, we have found the following method, based on that described by Caplan *(7)* and by Konigsberg *(8)* to be a very quick and satisfactory means of obtaining large numbers of mpc.

1. Eggs of the correct incubation age are swabbed with 70% ethanol and opened at the blunt end. The method normally recommended for doing this is to cut around the end of the egg with sterile scissors, but this generates egg shell dust, which may fall onto the embryo. We find it more satisfactory to use the method described for the preparation of CEE (*see* Section 2., item 11): breaching the shell with a blow of a sterilized scalpel handle, lifting off the cap of the egg shell,

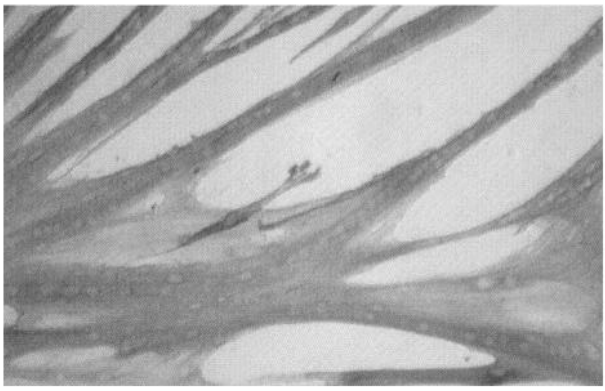

Fig. 4. Well-developed myotubes in a subculture of cells from a primary culture initiated from a single muscle fiber, fixed and immunostained for neonatal muscle myosin. Such myotubes are formed when dense cultures, as shown in Fig. 3, are kept in differentiation medium for 4–5 d.

still adherent to the underlying membrane, with sterile coarse forceps, then using a second pair of forceps to lift out the embryo by the neck, without squeezing it, and transfer it to a sterile Petri dish.

2. The skin overlying the breast muscle is dissected away, and the muscle is carefully removed and placed in a drop of sterile PBS in a second Petri dish.
3. The pooled muscle is finely minced with curved scissors (as for the mouse), and the fragments are suspended in sterile growth medium; approx 0.5 mL/quail breast, 1 mL/chick breast.
4. This suspension is distributed in 2-mL aliquots into large (20–25 mL) conical centrifuge tubes, and each tube is mixed for 30 s on a laboratory vortex mixer at high speed.
5. After allowing the large fragments to settle, the supernatant is drawn off. The remaining fragments can be subjected to a second agitation in growth medium.
6. The combined supernatants are filtered through three layers of 20-μm pore nitex mesh mounted in a Millepore filter holder and samples counted for viable cells as with the mouse cell preparations.
7. Depending on the desired timing and degree of myoblast fusion, the cells can then be seeded out in growth medium at 10^4–10^6 cells/35-mm Petri dish. Cell debris and nonviable cells initially contaminate cultures prepared in this way, but they can be removed by giving the cultures a brief wash and change of medium on the following day.

3.4. Cell Lines

It is possible to trypsinize primary cultures of muscle cells, using standard tissue-culture methods (*see* Chapter 1), and to propagate them for several gen-

erations. With an increasing number of passages, there is a tendency to lose the myogenic phenotype This loss of myogenicity can be offset by seeding the cells at low densities, such that clones develop from individual cells, and subcloning from the nonfused cells present in myogenic clones *(9)*. Stable continuous myogenic lines sometimes appear after several such serial passages. Continuous lines are available from the rat (L6, L8) and mouse (MM14, G8-1, C2), but not from human or avian origins. These lines provide the most experimentally reliable material, in most of them, the majority of cells fuse into myotubes in the right conditions and they present little difficulty in tissue culture. Of the lines of which we have experience, the C2 line *(10)* is the most satisfactory, being almost compulsively myogenic. It should be kept in mind, however, that these cells are to some extent neoplastic (all lines we have tested form tumors as well as muscle when implanted into nude mice) and should not be accepted uncritically as models of normal myogenesis (*see* Note 3).

4. Notes

1 Culture media· Sera and commercially available CEE are extremely variable and must be batch tested As a general rule, all procedures, especially those concerning biologically derived components, must be rigidly standardized.

2 Age of muscle· In general, the older the starting muscle, the longer the disaggregation period required, and the lower the yield of mpc We obtain 10^7–10^8 total cells/g from neonatal muscle; 1–5 × 10^5 cells/g from muscle of 6–7 wk-old mice This problem can be partially overcome by causing the muscle to start to regenerate in vivo prior to taking it for extraction of mpc this has been done by ligaturing the vascular supply of the muscle, by injecting myotoxic agents such as marcain or notexin, or as we have sometimes done with good results, removing, mincing, and reimplanting the muscle as an autograft This latter procedure, applied to 7-wk mouse muscle, 3–5 d after grafting yields some 10^8 cells/g About half of these cells are macrophages, but these can be removed by virtue of their rapid adherence to glass, and the yield of mpc is still much improved

3 Conditionally immortal myogenic cells: With rodent cells, extensive culturing runs into two related problems First, there is a limited number of cell doublings before there is a slowing and eventual cessation of proliferation. a proliferative "crisis" Second, the occasional cells which pass through this crisis are immortalized and frequently develop abnormalities of cell behavior, in particular they tend to become neoplastic *(11,12)* To avoid these problems, attempts have been made to immortalize rodent myogenic cells by transfection with the thermolabile mutant of the SV40 large T transforming antigen, tsA58 *(13)* A more convenient method of obtaining cells which can be regulated in tissue culture by means of this system, is to use, as a source of muscle tissue, a mouse which is transgenic for a construct containing this gene in a form whose expression can be regulated. In the so-called immortomouse, designated H2-TS6 *(14)*, the transgene consists of a promoter from the H2k^b Class I major histocompatibility com-

plex locus regulating expression of the tsA58 gene. This promoter is inducible in many cells by exposure to murine interferon-γ and causes expression of the tsA58 protein which is functional, maintaining cells in proliferation, only at temperatures below those of the normal body, e g., 33°C Myoblasts (as well as many other cell types) derived from animals homozygous or heterozygous for this transgene can be maintained in permanent proliferation under "permissive conditions" –33°C in the presence of 20 U/mL interferon-γ. On return to normal culture conditions, i e , 37–39°C in the absence of interferon-γ, the cells cease proliferation and synchronously differentiate into skeletal muscle We have derived a number of clones from this mouse itself and from mice in which the transgene has been crossed onto the mdx dystrophic background, and have maintained these as clones for several years Provided that the cultures are kept at low density, the cells retain a normal muscle phenotype over at least 40–60 passages (eightfold splits) and form normal muscle fibers when injected into degenerating muscles of dystrophic immunodeficient hosts *(15)* Because all cells from this animal contain the same transgene in the same genomic site, it is possible to rederive further completely equivalent clones at will

These cells have proved useful in experiments where there is a call for generation of large numbers of myogenic cells from small starting numbers or for clonal expansion of a single cell. By using muscle from this animal in the single fiber culture method described in Section 3 2., we have found it possible to produce highly myogenic clones of 10^6 or more cells from a selected satellite cell over a period of a few weeks

4 Enhancement of differentiation In vivo, motor innervation and the physiological workload greatly influence the state of differentiation of skeletal muscle fibers Addition of extracts of peripheral nerve has been reported to produce better differentiated and longer lived cultures than media lacking such extracts *(16)* By far the most effective in vitro model of skeletal muscle, however, is the nerve muscle coculture system developed by Peterson and Crain *(17)* In such cultures, slices of fetal mouse spinal chord are placed in culture together with fragments of skeletal muscle Outgrowths from the spinal chord of nonneural cells, when they contact the muscle fragment, stimulate the proliferation and early differentiation of myogenic cells within it. Subsequently, axonal outgrowths of nerve cells make synaptic contacts with the developing muscle fibers, causing them to twitch and to express many of the phenotypic characteristics of normal skeletal muscle The chief disadvantages of this method of culture are that some experience and a meticulous technique are required to set up and maintain the cultures, and that the amount of well-differentiated muscle tissue formed within them is too small to be of use for many biochemical techniques

5. Problems: Two major problems of tissue culture in general seem to be exemplified in extreme forms by skeletal muscle in tissue culture
   a. It is difficult to set up tissue cultures in which a high proportion of cells become differentiated and form muscle fibers The principal reason for this is the difficulty of ridding cultures of cells with no myogenic potential

However, even with cloned myogenic cells, not all, and sometimes only a minor proportion, develop into muscle cells

b The degree of differentiation attained by muscle fibers grown in tissue culture falls far short of what is found in vivo The innervated cocultures described in Note 4 are the most effective attempt so far to remedy this.

6. Two practical difficulties we have encountered are
   a Poor reproducibility Even with standardized techniques and batched media components, variation between individual primary culture preparations is often too great to permit comparisons to be made between them. It is safest, therefore, to study the effects of variables within particular preparations. Cultures of muscle of the Japanese quail seem less susceptible to this problem
   b Long-term cultures· As the contractile myofibrils assemble within developing muscle fibers, these cells respond to minor disturbances, such as illumination under a microscope, change of medium, or addition of more cells, by contracting violently This may cause irreversible detachment of the entire monolayer of cells from their substratum

In general, it is best to use the simplest culture system for skeletal muscle that will critically test the hypothesis under consideration If it is necessary to use one of the more complicated methods, it can save much time and effort to acquire experience of it in a laboratory where it is used routinely, because written accounts of a technique do not record all of the subliminal practices that are frequently crucial to its success

Acknowledgments

I thank Jennifer Morgan, Diana Watt, and David Rosenblatt for their advice and criticism. The work on mouse muscle culture is supported by the Muscular Dystrophy Group of Great Britain and Northern Ireland.

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11

Isolation of Rat Liver Hepatocytes

David S. Neufeld

1. Introduction

The liver is composed of hepatocytes, endothelial, Kupffer, fat-storing, and pit cells. This chapter describes a technique for isolating hepatocytes, which represent 60–65% of the cells of the liver and about 80% of the volume of the organ (since they are larger than the other cells) *(1–3)*. Two years after Howard et al. *(4)* discovered that collagenase was a very important tool in the separation of liver cells, Berry and Friend *(5)* introduced the technique of collagenase perfusion of the liver.

Collagenase perfusion has two conflicting requirements. On one hand, the cells must be exposed to very low concentrations of Ca^{2+} to allow cleavage of hepatic desmosomes On the other hand, collagenase activity requires the presence of Ca^{2+}. To resolve this conflict, two different procedures have been employed. These have been termed the "one-step" and "two-step" procedures *(6)*. The one-step procedure takes advantage of the fact that the requirement of Ca^{2+} for collagenase activity is substantially less than that required for disruption of the desmosome The two-step procedure utilizes a pre-perfusion step with Ca^{2+}-free medium for at least 10 min, during which the desmosomes are irreversibly cleaved, followed by the addition of Ca^{2+} in physiological or supra-physiological concentrations to the perfusate. Both methods are widely used successfully in various laboratories. The two-step method is described in detail in this chapter.

2. Materials

2.1. Stock Buffers:

1 10X Leffert's buffer 100 m*M* HEPES, 30 m*M* KCl, 1 3*M* NaCl, 10 m*M* NaH_2PO_4, 100 m*M* D-glucose (all solutions should be made with highly purified

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
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H_2O). This solution is sterile-filtered (through a 0.22-μm filter using a pressure vessel coupled to a filtration apparatus) Store at 4°C

2 10X EGTA solution (5m*M*)· 1.9 g EGTA are added to 100 mL 10X Leffert's buffer and 800 mL H_2O 10*N* NaOH are added until the solution clears (EGTA does not readily dissolve in H_2O) and the pH is adjusted to a normal pH Finally, the volume is adjusted to 1 liter. This solution is sterile-filtered Store at 4°C

3 100X $CaCl_2$ Solution: A 2 79% solution of $CaCl_2$ is made by adding 27 9 g $CaCl_2$ to 1 L H_2O. This solution is sterile-filtered Store at room temperature

2.2. Buffers for Day of Perfusion

1 1X Leffert's buffer. 900 mL H_2O are added to 100 mL of 10X Leffert's buffer The pH is adjusted to 7.4 (7 35–7 45). Sterile-filter 100 mL of 1X Leffert's buffer and store in a water bath at 37°C

2. EGTA solution· 30 mL 10X EGTA solution are added to 270 mL 1X Leffert's buffer The pH is adjusted to approx 7 4 and the solution sterile-filtered and stored at 37°C (*see* Note 1)

3. $CaCl_2$ solution (wash buffer) 6 mL 2.79% $CaCl_2$ solution are added to 600 mL 1X Leffert's buffer The pH is adjusted to 7 4 and half the solution is sterile-filtered and stored on ice.

4 The remaining 300 mL of the $CaCl_2$ solution are mixed with 100 mg collagenase (*see* Notes 2–4) The pH is adjusted to 7 4 and the solution sterile-filtered and stored at 37°C

2.3. Other Materials

1. A rat (any rat 200–250 g will do) However, you may want to change the concentration of collagenase if the size is substantially different
2. Peristaltic pump capable of delivering 4–60 mL/min.
3 Silastic tubing (Dow-Corning [Midland, MI] Medical Grade tubing, 0 078 in ID × 0.125 OD) as well as other tubing (such as tygon or latex)
4 Oxygen tank
5 Bubble trap (an IV set is used in our laboratory)
6 Instruments and surgical supplies (scissors [large and small], forceps [large and small full curve], cannula, hemostats, silk suture, sterile cotton tipped applicators, gauze sponges, ethanol).
7. Sodium pentobarbital
8 80-mesh micron filter (Tetko Inc., Briarcliff Manor, NY)
9. Hepatocyte culture medium· RPMI plus 10% FCS, 10^{-7}*M* dexamethasone 10 μg/mL insulin, 5 μg/mL transferrin.

3. Methods

The various components of the perfusion system are assembled as shown in Fig. 1: A reservoir (which can be a sterile bottle) is connected to the peristaltic pump by a short piece of tubing which is then connected to the water bath.

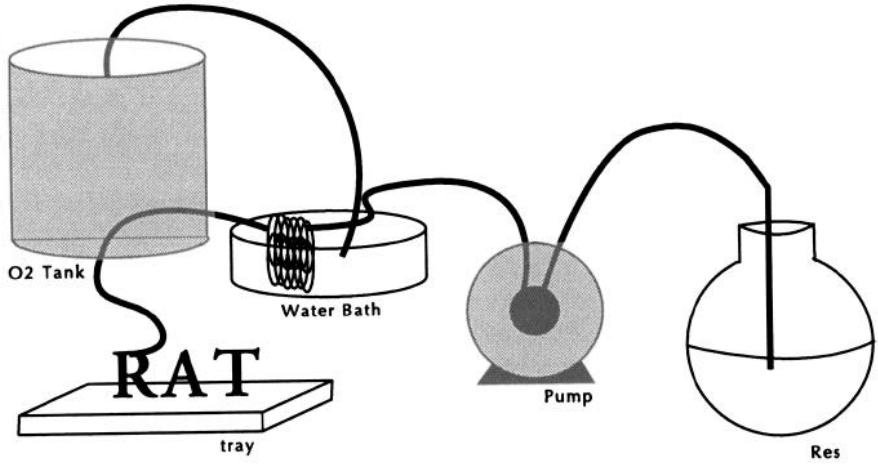

Fig. 1. Components of the perfusion system.

Approximately 40 ft of silastic tubing are run through the water bath. This, in turn, is connected to the bubble trap, that is then connected to the cannula and, finally, to the animal.

1. Allow 500 mL H_2O to run through the tubing (*see* Note 5).
2. Anesthetize the rat with an intraperitoneal injection of sodium pentobarbital (0.2 mL/200 g rat).
3. Begin bubbling O_2 into the water bath.
4. Tape the sedated rat to the tray, abdomen upwards, and swab the abdomen with 70% ethanol.
5. Start the EGTA solution running through the line.
6. Grasp the abdominal wall with a pair of forceps and open the abdominal cavity from the pubis to the xiphoid process along the midline.
7. Commencing at the midpoint of the vertical incision, make a horizontal cut on each flank from the center line around toward the back.
8. Wet a cotton tipped applicator with EGTA solution, and move the intestine to your right side, thus exposing the liver.
9. Gently push the main lobes of the liver upwards (toward the animal's head), uncovering the portal vein and the inferior vena cava.
10. With the aid of a blunt edged hemostat, draw silk suture under the vena cava, above the branch to the right kidney, and tie a loose half-knot around the vein, ensuring it is not obstructed (I fold the lowest lobe over the ends of the ligature and deflect the ends to my left).

11 With the aid of a full curved forceps with fine-serrated points, put a suture at the back of the portal vein (farthest from the liver); then put a second one in closer to the liver Once again, tie sutures loosely but do not tighten the knot
12 To cannulate the portal vein (*see* Note 6), fix the vessel by grasping a small portion of its uppermost wall with very fine forceps, and puncture it with the trocar about 1 5 cm from the point where the vein branches to enter the liver lobes
13 The trocar should be held almost parallel to the portal vein during the insertion, to avoid piercing the opposite wall
14 You may want to maneuver the tip of the cannula to lie approx 0.5 cm proximal to the branch point. This should be executed with the needle pulled back
15. Immediately remove the trocar and make a very tight double knot with the ligatures around the portal vein (*see* Note 7)
16 Connect the tubing to the cannula and start the flow of the perfusate
17 Cut the vena cava well below the kidney The liver should blanch immediately to a beige color (*see* Note 8)
18. Cut into the thoracic cavity and cut the vena cava Tie the suture tightly around the vena cava above the kidney, and put a piece of gauze into the thoracic cavity to draw out the liquid that accumulates in the cavity Remove the blood that has accumulated in the chest cavity
19 Allow the EGTA solution to continue to run through the liver for a total of 4 min
20 Stop the pump, change the buffer to 1X Leffert's buffer, and allow that to run for 2 min (*see* Note 9)
21 Turn off the pump. Add the digestion buffer and allow this to run until the liver is broken apart.
22 Turn off the pump and remove any accumulated liquid from the chest cavity
23 Using a new set of forceps and scissors, cut out the lobes of the liver (discard lobes that are not well digested and that have not blanched to a uniform beige color) and place the lobes in a sterile culture dish.
24 In a sterile hood, add the ice-cold $CaCl_2$ solution to the digested lobes of the liver Using two scalpels, cut apart the lobes This should release many cells and the liquid should become cloudy
25 Filter the cells through an 80-μm mesh filter stretched over a funnel (*see* Note 10)
26 Pour the cells into 50-mL centrifuge tubes and spin the cells at 50*g* for 1–4 min
27. Remove and discard supernatant and resuspend the cells with wash buffer (*see* Note 11). Repeat this procedure until you have washed the cells three times
28 Resuspend the cells for a final time in 10–20 mL of $CaCl_2$ solution
29 To 80 μL Hank's balanced salt solution (HBSS), add 10 μL cells and 10 μL 0.04% trypan blue and determine the percent viability under the microscope (*see* Note 12)
30. Plate the cells at a concentration of 6–8 × 10^6 cells/100-mm dish in hepatocyte medium. The media should be changed after 4 h, however, one can wait to change the media till the next morning. For best results, the media should be changed daily (Fig. 2).

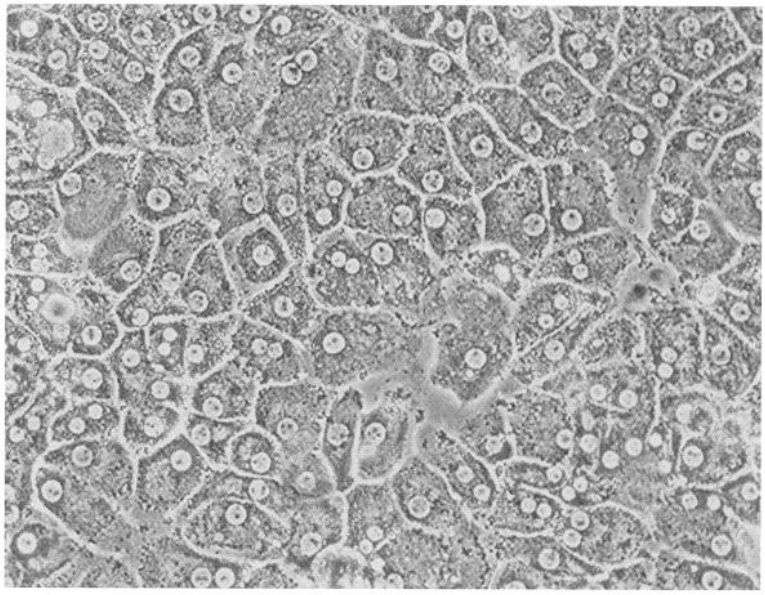

Fig. 2. Adult rat hepatocytes cultured for 24 h, magnification 64×.

4. Notes

1. The EGTA solution should be filtered through a different filter than the other solutions, that can be filtered in the following sequence: H_2O, 1X Leffert's buffer, $CaCl_2$ solution, and finally collagenase.
2. The powdered collagenase should be stored, at all times desiccated at –20°C. Once the bottle is opened, it should be aliquoted in the appropriate quantities for the best results.
3. The quality of the collagenase is very important. It will be the main ingredient in determining the viability of the isolated hepatocytes. Therefore, a 100–200 mg sample of a particular lot should be acquired from a company and tested (by performing an entire perfusion) to determine if a viability of 90% or greater can be attained with this collagenase. Companies that produce collagenase include Boehringer Mannheim Biochemicals (BMB; Indianapolis, IN), Worthington Biochemical Corporation (Freehold, NJ), Sigma (St. Louis, MO), Atlanta Biologicals (Norcross, GA), and Life Technologies (Gaithersburg, MD. Once a high quality lot of collagenase is found, a large quantity of that specific lot should be purchased (enough to last at least 1 yr), because the testing process is very tedious.
4. One hundred eight milligrams BSA Pentex Fraction V (from Miles Laboratory) may be added to the 100 mg collagenase. This will decrease the activity of trypsin

(which is deleterious to the hepatocytes, however, is necessary in small amounts) but will have no effect on the collagenase

5. During this time, adjust the speed of the pump to 16 mL/min
6. Use an 18-gage catheter except for very small rats or for mice. In those cases, a 22-gage catheter should be used. A 16-gage catheter may be used for large (350 g or larger) rats
7. Blood should begin flowing through the cannula immediately on removal of the trocar, so that there are no air bubbles introduced into the liver (which would seriously impair the perfusion). At the same time, check the line before connecting it to the cannula to make sure there are no air bubbles in the line.
8. Tape down the tubing coming into the cannula to insure the cannula does not slip out of the portal vein
9. The desmosome is a part of the junctional complex which must be cleaved in order to achieve a good preparation of isolated hepatocytes. The removal of Ca^{2+} results in an irreversible cleavage of the desmosomes. Therefore, the first two solutions are Ca^{2+} free and, in fact, the first solution contains EGTA, which is a Ca^{2+} binding agent. However, desmosomal separation is not instantaneous, and a too-rapid restoration of Ca^{2+}, will allow the desmosomes to reform with a consequent severely reduced yield of isolated cells. To ensure good yields of hepatocytes, Ca^{2+} must be removed for at least 10 min. However, it should be noted that collagenase needs Ca^{2+} to function and the hepatocytes can tolerate at most a 40 min period in the absence of Ca^{2+}. For this reason, Ca^{2+} must be reintroduced to the liver.
10. If a small amount of buffer is used, the mesh will quickly become clogged with cells. Therefore a large amount of buffer (at least 100–150 mL) should be used with a wing-tipped rubber policeman to flush the cells through the mesh.
11. Do not put the tip of the Pasteur pipet near the pellet when removing the supernatant. Since the cells are spun down at such a low speed, the pellet is very loose and can easily be suctioned up
12. Live cells (with intact membranes) exclude trypan blue, while dead cells take up trypan blue. The dead cells (blue) are more easily visualized without the use of the phase condenser. The viability should be >90%.

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12

Primary Kidney Cells

Mary Taub

1. Introduction

Hormonally defined serum-free media have been developed for growth and functional studies with kidney epithelial cell cultures. Not only can several established kidney tubule epithelial cell lines (MDCK and LLC-PK_1) be grown in a serum-free environment *(1,2)*, but in addition primary cultures of kidney tubule epithelial cells can also be grown serum-free *(1–4)*. Investigations with primary kidney cell cultures are particularly advantageous for several reasons. First of all, kidney cells can be grown in vitro from the animal of choice. Thus the results of tissue-culture studies can be more closely correlated with animal studies. Second, new tissue-culture systems can be developed that more closely resemble the kidney cells in vivo than presently available established kidney cell lines

This chapter describes the use of serum-free medium for the growth of primary rabbit kidney proximal tubule cell cultures which express renal proximal tubule functions. When following this procedure, rabbit kidney proximal tubules are first purified from the renal cortex by a modification *(4–6)* of the method of Brendel and Meezan *(7,8)* The purified renal proximal tubules are then placed into tissue-culture dishes containing serum-free medium supplemented with three growth supplements: insulin, transferrin, and hydrocortisone Within the first day of culture, the tubules attach to the culture dish. Subsequently, epithelial cells grow out from the tubule explants. After 1 wk, confluent monolayers are obtained that express a number of renal proximal tubule functions (Table 1). The cultures exhibit multicellular domes or hemicysts, indicative of their capacity for transepithelial solute transport (Fig. 1). These monolayer cultures can be used for a large number of purposes, ranging from infection with viruses, and subsequent viral production, to transfec-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
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Table 1
Properties of Primary Rabbit Kidney Proximal Tubule Cell Cultures

Morphology *(4,9,10)*
Domes
Form polarized monolayers
Adjacent cells form tight junctions
Brush border (although not as elaborated as in vivo)
Transport properties
Sodium/glucose cotransport system *(4,9)*
Sodium/phosphate cotransport system *(6)*
P-aminohippurate transport system *(11)*
Amiloride-sensitive sodium transport *(12)*
Hormone responses
Parathyroid hormone sensitive cyclic AMP production *(4)*
Enzymes, metabolic properties
Leucine aminopeptidase *(4)*
Alkaline phosphatase *(4)*
Gamma glutamyl transpeptidase *(4)*
Glutathione *(18,19)*
Glutathione S-transferase *(16,18)*
Angiotensin converting enzyme *(10)*
Phosphoenolpyruvate carboxykinase *(13)*
Aerobic metabolism *(9)*
Hexose monophosphate shunt *(9)*
Gluconeogenesis *(17)*
Growth properties
Cell growth in serum-free medium *(4)*
Growth in response to insulin, transferrin and hydrocortisone *(4,13)*
Growth in response to laminin, collagen and fibronectin *(14)*
Growth in Glucose Free Medium *(17)*
Can undergo two passages

tion with plasmid DNAs containing oncogenes for subsequent cell immortalization. The confluent monolayers are amenable to biochemical studies when cultured on plastic, as well as electrophysiologic studies when cultured on permeable supports. Now that many of the differentiated functions of the renal proximal tubule cells have been defined, and appropriate genes have been cloned, the primary cultures are amenable for molecular biology studies concerning the control of the expression of differentiated function.

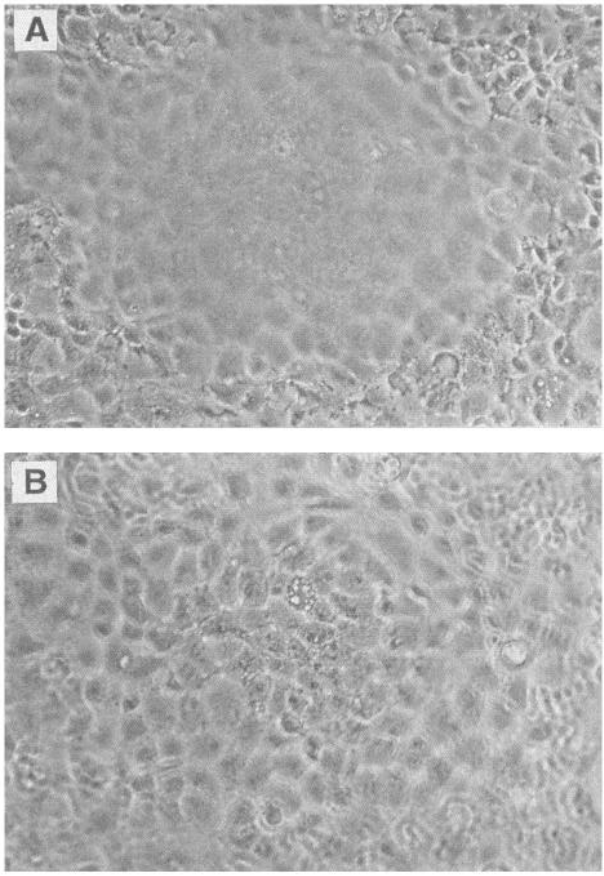

Fig. 1. Dome formation by rabbit kidney proximal tubule cell cultures. A photomicrograph was taken of a confluent monolayer under an inverted microscope at 100× magnification. **(A)** The cells in the monolayer; **(B)** the cells in the dome.

2. Materials

1. Water and glassware: Water to be used for the preparation of sterile medium and other sterile reagents is purified with a Milli-Q reagent grade water system as previously described *(4,5)*. The feed water for the Milli-Q reagent grade water system is obtained from a Millipore Reverse Osmosis System. A separate set of glassware, bottles, and stir bars is utilized for the preparation of medium, and other tissue-culture solutions.
2. Culture Medium: The basal medium is a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/L D-glucose and L-glutamine, and without

either sodium pyruvate or sodium bicarbonate), and Ham's F12 Medium, that is further supplemented with 15 m*M* HEPES buffer (pH 7 4) and 20 m*M* sodium bicarbonate (DME/F12) If penicillin is to be added, a dosage of 0 92 × 10^5 IU/L is to be employed My laboratory has successfully cultured primary proximal tubule cells in medium that is completely antibiotic free

The medium is sterilized using a disposable Millistak filter unit with a 0 22-μm pore size A Millipore peristaltic pump is utilized to pump the medium from a reservoir (a 20 L carboy), through the Millistak filter, into sterile medium bottles Individual bottles of medium are stored frozen at –20°C, until ready for use Medium bottles which are under immediate use are kept at 4°C for up to 2 wk

The basal medium (DME/F12) is supplemented with insulin, transferrin and hydrocortisone on the day of use. Sterile stock solutions of bovine insulin, human transferrin and hydrocortisone are prepared for this purpose. Bovine insulin (Sigma [#I5500], St Louis, MO) is solubilized in 0 01*N* HCl at a concentration of 5 mg/mL, sterilized by passage through a 0.22-μ filter, and distributed into sterile 12 × 75-mm polystyrene tubes using 1-mL aliquots Insulin is kept at 4°C and is stable for up to 1 yr Human apo-transferrin (Iron poor > 97% pure; Sigma # T2252) is prepared at a concentration of 5 mg/mL in water, filter sterilized using a sterile 0 22-μ filter unit, and aliquoted into individual polystyrene tubes Individual aliquots are kept at –20°C Aliquots of transferrin may be frozen and thawed up to four times Hydrocortisone (Sigma H-4001) is solubilized in 100% ethanol at 10^{-3}*M*, aliquoted into polypropylene tubes, and stored at 4°C for 3–4 mo Sterile 0.25% trypsin/1 m*M* (ethylenedinitrilo)tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (Life Technologies Corporation, Bethesda, MD) is used for the trypsinization of renal proximal tubule cell cultures

3. Iron oxide solution During the perfusion procedure, a 0 5% iron oxide solution (w/v) is used. The iron oxide solution is prepared as described by Cook and Pickering *(14)* To summarize, 2 6-g sodium hydroxide and 20-g potassium nitrate are dissolved in 100 mL of oxygen saturated water Ferrous sulfate (9 g) is dissolved in 100-mL aliquots of oxygen saturated water These two 100 mL solutions are mixed, and the mixture is boiled for 20 min. The resulting black precipitate of magnetic iron oxide is washed 5–10 times with water. In each wash, the precipitate is resuspended in water, and is then attracted to the bottom of the wash flack using a strong magnet. The wash water is then decanted away After the final wash, the iron oxide is resuspended in one liter of 0 9% NaCl, distributed into 250-mL bottles, and autoclaved for future use Immediately prior to use, a portion of the iron oxide solution is diluted three- to fourfold in PBS
4 Collagenase and Soybean Trypsin Inhibitor· Collagenase class 4 (162 U/mg) is obtained from Worthington (Freehold, NJ, Cat. # 4188), and soybean trypsin inhibitor from Life Technologies (Grand Island, NY). A collagenase solution is prepared in DME/F12 medium at 10 mg/mL, and filter sterilized. The collagenase solution is only utilized on the day of preparation Because of lot-to-lot differences, each particular lot of collagenase should be tested to evaluate whether the particular lot does indeed promote the outgrowth of cells from proximal

tubules, or have deleterious effects A stock solution of 10 mg/mL soybean trypsin inhibitor in PBS is prepared, filter sterilized, and stored in frozen aliquots

5 Rabbit kidneys· Kidneys with the renal vein and artery intact are obtained from male New Zealand white rabbits (2–2 5 kg) that are sacrificed in a closed container using 100% CO_2 The renal vein and artery are separated, using a forceps, while in the animal, and kept completely intact with the kidney, while the ureter is removed Each kidney is transferred into a sterile 50-mL polypropylene tube in ice The left kidney has the larger artery and vein of the two kidneys

6 Implements for primary cell culture Immediately prior to primary cell culture, implements are sterilized in an autoclave, including a sterile curved nose scissors, 2–3 forceps, 2–3 100-mm diameter glass Petri dishes, heavy black suture thread, a Kelly hemostat (5½" straight end), a 20-gage metal needle (blunt ended with a file, so as not to slit the renal artery), a 50-mL glass syringe, a 15-mL Dounce homogenizer (with a Type A, loose pestle), a metal spatula, a 2" magnetic stir bar, and a 1000-mL beaker Heavy black suture thread and a Kelly hemostat are used to suture the renal artery over a 20-gage needle The needle is attached to a 50-mL glass syringe containing perfusate After perfusion, the kidney is sliced in a Petri dish, using the scissors and forceps. The Dounce homogenizer is used to disrupt the renal cortical slices, to obtain nephron segments

The preparation of renal proximal tubules for tissue-culture entails the use of Nylon nitrex screening fabric both 253 and 85 μm (TETCO, Depew, NY), as well as a sterilized, 1000-mL beaker, a metal spatula and a magnetic stir bar The nylon mesh is held in place in 4" diameter embroidery hoops

3. Methods

1 Add 5 μg/mL insulin, 5 μg/mL transferrin, and $5 \times 10^{-8} M$ hydrocortisone to the culture medium (DME/F12) on the day of preparation of rabbit kidney proximal tubule cells for tissue-culture

2 Implements to be used for primary renal proximal tubule cell culture described in Section 2 6. are sterilized in an autoclave. Nylon mesh in embroidery hoops are sterilized in a large beaker containing 95% ethanol.

3 A sterile collagenase solution is prepared on the day of culturing, at a concentration of 10 mg/mL in DME/F12 medium

4. A rabbit is killed in a container filled with 100% CO_2 The kidneys are removed (with renal artery and vein intact) using sterile scissors, and each kidney is placed in a sterile 50 mL conical tube with ice cold DME/F12 medium containing 192 IU/mL penicillin and 200 μg/mL streptomycin The kidneys are kept ice cold throughout the procedure

5 Each kidney is perfused as follows The kidney is placed in a 100-mm diameter glass Petri dish, and washed with ice cold DME/F12 containing penicillin and streptomycin A sterile blunt ended needle (18-gage) is inserted into the renal artery, and sutured The needle may also be kept in place using a hemostat The kidney is then perfused, first with ice cold PBS (to remove blood), and then with PBS containing iron oxide (the kidney becomes grey black in color)

6 The renal capsule is removed using sterile forceps, and the kidney immediately transferred into another sterile 100-mm glass Petri dish containing ice cold DME/F12 At this point great care must be taken with regard to sterility Slices of the renal cortex are removed from the kidney using a sterile, curve nosed scissors Care should be taken not to remove the medulla at this step, as subsequent processing with medullary slices as well, results in the presence of a large quantity of debris in the final cell cultures (which may be prohibitive to cell growth) The medulla may be identified from the cortex, as only the cortex becomes grey black in color following a successful perfusion of the kidney with iron oxide

7 The slices of the renal cortex are transferred into a sterile 15-mL Dounce homogenizer containing DME/F12 medium. The tissue is disrupted with 4–5 strokes of a loose pestle (type A) After tissue disruption, the homogenizer can be covered with the lid of a sterile tissue-culture dish, to maintain sterility while setting up the sieves for the next purification step.

8 The nephron segments are separated using the nylon mesh sieves. Towards these ends, an 85-μm sieve is placed directly over a sterile 1000-mL beaker Then the wider 253-μm sieve is placed over the 85-μm sieve The sieves are washed with DME/F12 containing 192 IU/mL penicillin and 200 μg/mL streptomycin to remove the ethanol from the sieves while maintaining sterility The suspension of disrupted tissue in the Dounce homogenizer (which contains tubule segments and glomeruli) is poured over the top sieve. Subsequently, 700–800 mL of DME/F12 medium with penicillin and streptomycin is poured over the sieves, to facilitate the passage of tubules and glomeruli through the sieves Undisrupted material remains on the top of the first, 253-μm, sieve Proximal tubules and glomeruli collect on the top of the second, 85-mm sieve, due to their large diameter Narrower tubule segments and debris pass into the beaker The tubules and glomeruli are removed from the top of the 85-μm sieve using a sterile metal spatula (preferably with a rounded end), and are transferred into a sterile 50-mL plastic conical tube containing 40-mL DME/F12 with penicillin and streptomycin

9 In order to remove the glomeruli in the tubule suspension, a sterile magnetic stirbar is placed in the tube. Glomeruli (covered with iron oxide) adhere to the stir-bar The stir-bar is carefully removed (using a sterile hemostat). This process is repeated.

10 First soybean trypsin inhibitor, and then collagenase are added to the tubule suspension. The final concentration of both reagents is 0 05 mg/mL). The tubules are incubated with the collagenase solution for two minutes at 23°C (Empirically, we have observed that the use of collagenase is required if the outgrowth of epithelial cells from the tubules is to occur in vitro) In order to stop the collagenase treatment, the tube containing the tubules is placed in a desktop centrifuge, and spun for 5 min at 500 rpm (21*g*) The pellet containing tubules is resuspended in DME/F12 and again washed by centrifugation The medium used for the final resuspension of the tubules is the growth medium

11. After centrifugation, the tubules are suspended in DME/F12 medium supplemented with 5 μg/mL insulin, 5 μg/mL transferrin, and $5 \times 10^{-8} M$ hydrocortisone

The medium may be further supplemented with 192 IU/mL penicillin, but we have found that streptomycin is deleterious to our cultures (*see* Note 1) Generally, we use 100 mL of medium to suspend the renal proximal tubules, but in some cases we have used up to 200 mL of medium to suspend the proximal tubules obtained from a single rabbit kidney

12 The tubule suspension is then inoculated into 35-mm diameter tissue-culture dishes (at 2 mL/dish) Care should be taken to keep the tubules in suspension during this process, because they are denser than isolated cells in suspension, and may rapidly settle, even while pipeting To maintain uniformity, the tubule suspension can be placed in a sterile bottle with a sterile stir bar, and continuously stirred using a stirring motor

13. The culture dishes are placed in a 5% CO_2/95% air humidified environment at 37°C.

14. The culture medium is changed the day after plating the tubules (to remove debris and unattached nephron segments) The medium is changed routinely every two d thereafter. Confluent monolayers can be obtained after 6–7 d in culture (*see* Note 2)

15 Primary rabbit kidney proximal tubule cells on plastic dishes can be subcultured by trypsin treatment Confluent first passage cultures can be obtained if care is taken to minimize the trypsinization period. In some cases proximal tubule monolayers can even be obtained following a second passage into plastic dishes. In order to obtain first passage cells, the culture medium is removed by aspiration, and the cells are washed with PBS. A solution of EDTA-trypsin is then added. The majority of the EDTA/trypsin solution is immediately removed, so that only a film of trypsin covers the cells, providing a gentler trypsinization The cells are transferred to a 37°C incubator for a short time (as short as 1 min), and examined under an inverted microscope at 100× magnification, to determine whether they are rounded up (and detached from the dish). Trypsinization can even be conducted at room temperature, as the cells are loosely attached to the bottom of the culture dish. Trypsin action is stopped by the addition of an equimolar concentration of soybean trypsin inhibitor in PBS DME/F12 medium is added,the cells are removed from the dish into a 12-mL plastic centrifuge tube, and spun at 500 rpm (21*g*) The cells are resuspended in DME/F12 containing the three growth supplements (insulin, transferrin and hydrocortisone), inoculated into plastic Petri dishes

4. Notes

1. The medium used for tissue-culture studies should not be supplemented with streptomycin which is a nephrotoxin, impedes the initial attachment of nephron segments to the culture dish, and the initial outgrowth of cells from the nephron segments

2 If primary rabbit kidney proximal tubule cell cultures do not grow to confluence, a number of problems may have occurred First, enough tubules must be added to the culture dishes in order to obtain confluent monolayers (0 5 mg protein/mL

tubule suspension) The tubules may easily be lost if they are not carefully harvested at the end of the sieving procedure Second, cell cultures obtained from kidneys of young adults (as opposed to older animals) are the most successful A third point of concern is the tissue-culture medium The purity of the water is critical in defined medium studies Loss of purity caused by contamination from a dirty pH probe (for example) may result in medium that does not support cell growth Our laboratory determines pH using samples of the medium rather than placing the probe in the medium to be used for tissue-culture studies In addition, a set of glassware is used in medium preparation that is specifically designated for that purpose. Another point of caution with regards to medium is the hormone supplements. Improper preparation or storage of the growth supplements may be deleterious to cell growth The growth stimulatory effect of insulin may be lost if the stock solution is frozen Furthermore the medium supplements should be added to the medium immediately prior to use for tissue-culture, as these supplements are not necessarily stable in the tissue-culture medium Fourth, care should be taken that the incubator be maintained at a constant temperature of 37°C, and in a constant 5% CO_2/95% air environment Animal cell growth in the absence of serum is more sensitive to shifts in temperature, and to changes in the medium pH than in the presence of serum The addition of HEPES buffer to the medium alleviates this latter problem to some extent Finally, the primary rabbit kidney proximal tubule cells are less adherent to plastic dishes than many other cell types. Thus the cells may detach during their manipulation for cell growth studies or for transport studies (for example) The problem of adhesion may be alleviated by growing the cultures on tissue-culture dishes coated with laminin, or with laminin and type IV collagen *(15)*. Renal proximal tubule cell cultures on plastic cell culture dishes can readily be passaged once Although additional subculturings can be obtained, confluence is obtained with significant difficulty However proximal tubule cell cultures grow more rapidly, achieve a higher saturation density, and a higher passage number in laminin coated dishes

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13

Human Thyroid Epithelial Cells

D. W. Williams and David Wynford-Thomas

1. Introduction

The thyroid gland contains two populations of epithelial cells, of quite different embryological origin and function. Only the major component—the follicular cells—is considered here. The minor C cell population, which forms only a few percent, can be ignored for the purposes of primary culture.

The follicular cells are organized in the intact gland into discrete functional units or follicles that consist of spheres lined by a single layer of epithelium and filled with the secretory product of the lining cells—thyroglobulin. The follicles are embedded in a vascular stroma.

As with other epithelial tissues, almost all published methods for obtaining primary cultures have employed some form of proteolytic digestion combined with mechanical disaggregation. Earlier workers used trypsin in protocols similar to those used for embryo fibroblast preparation *(1–3)*. These yield mainly single-cell suspensions for which it is difficult to obtain epithelial cells free of fibroblast contamination *(4)*. A major improvement can be achieved by the use of collagenase (and/or dispase), which unlike trypsin does not digest the intercellular junctional complexes that hold together the apical poles of the follicular cells. As a result, provided that digestion and disaggregation are not excessive, the majority of the follicular cells can be released in the form of follicles rather than single cells *(5)*, which can be separated from the single-cell fibroblast component by differential sedimentation *(6)* or filtration *(7)*. In addition, it has been shown that, as expected, avoidance of trypsin improves the viability of the resulting cultures and their sensitivity to hormones that act through cell-surface receptors, notably thyroid-stimulating hormone (TSH) *(8)*. Finally, this method of preparation allows for culture of follicular cells as intact follicles in suspension as well as in monolayer. The follicular cell is

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

highly polarized both ultrastructurally and functionally (for example with respect to hormone and growth factor receptor distribution), and it has been shown that in monolayer its polarity is reversed, the apical rather than the basal surface being in contact with the environment *(9)*. This can result, particularly in confluent monolayers, in a loss or diminution of many responses to extracellular agents, for example, the stimulation of adenylate cyclase and iodide uptake by TSH *(10)*, and of proliferation by epidermal growth factor *(11)* Culture in suspension as intact follicles avoids such artifacts, since the normal polarity is maintained Indeed, the thyroid offers a rare opportunity to culture a glandular epithelium without disrupting its in vivo organizational unit, a condition that can never be entirely met with branched ductular-acinar structures such as breast or pancreas

All digestion protocols require that the tissue be first minced into small enough pieces to permit rapid diffusion of enzyme to all areas. Various strategies have been subsequently employed for digestion and disruption Most simply, the tissue fragments can be incubated for several hours at 37°C and then disrupted in one step, for example by pipeting, followed by harvesting of the released cells *(12)*. This inevitably results, however, in some cells being exposed to enzyme for too long and hence to an excessive proportion of single cells and poor viability. It is preferable to fractionate the procedure by harvesting at multiple intervals during the digestion (gentle mechanical disruption being applied throughout), so that cells (in the form of follicles) are removed from the enzyme as soon as possible after their release. One variation of this approach that we have found *(13)* to give reproducibly high yields of human follicles with minimal fibroblast contamination (<0 1%) is detailed here. Similar methods have been applied to cultures of dog *(5,14)*, sheep *(15)*, pig *(16)*, rat *(6)*, and human *(17)* thyroid, although not all including a fractionated harvesting protocol.

2. Materials

1. Hanks' balanced salt solution (HBSS). This is a simple inorganic salt solution buffered for use in atmospheric CO_2 We use the modified, calcium- and magnesium-free formulation supplied by Flow Laboratories
2. 200 U/mg Collagenase type A (Boehringer Mannheim): Store powder at 4°C
3 Dispase (Boehringer Mannheim)· Neutral protease from Bacillus polymyza, Grade II Store powder at 4°C
4. Enzyme mixture. Dissolve 40 mg of collagenase plus 60 mg of dispase in 60 mL of HBSS Filter through 0 45-μm nitrocellulose filter to sterilize. Prepare fresh each time; keep on ice until use
5 Nylon mesh· 200 μm Nominal pore size Sterilize by autoclaving
6. RPMI 1640 medium· The cells have also been grown successfully in Dulbecco's modified Eagle's medium (DMEM). We routinely add penicillin (50 U/mL) and streptomycin (50 μg/mL)

7 Fetal calf serum (FCS)
8 Newborn bovine serum (NBS)
9. Acridine orange and ethidium bromide Prepare solution containing 0 1 μg/mL of each in HBSS Store in dark bottle at 4°C These are mutagenic and gloves should be worn when handling them
10 Agar high gel strength· Make 100 mL of a 2% solution in double-distilled water Boil in a bottle with loosened lid to dissolve and sterilize Store at 4°C. Reboil before each use
11. Trypsin/EDTA solution· 0 05% (w/v) trypsin plus 0 02% (w/v) EDTA in Dulbecco's calcium- and magnesium-free phosphate-buffered saline Dilute from 10X stock (stored at –20°C) Store working solution at 4°C (up to 1 mo)
12 Dimethyl sulfoxide (DMSO) Tissue-culture tested product, supplied sterile Store at room temperature in dark Store freezing mix-ture (DMSO NBS/1 4) at 4°C in dark

3. Method

The following procedure is summarized in Fig. 1.

1 Human thyroid tissue is best obtained as freshly excised surgical material—most of our samples come from thyroid lobectomies performed for removal of "cold" nodules (*see* Note 1), which usually provide at least 1 g of histologically normal gland. The tissue sample is transported to the tissue-culture laboratory in HBSS on ice (speed at this stage is not particularly vital, since we have found no deleterious effect from storage at 4°C for up to 1 h)
2 Prior to enzyme digestion, the tissue is first minced with sterile scalpel blades as finely as possible (to around 2-mm cubes) The pieces are washed four times in 15 mL of HBSS at 4°C in a universal container to remove as much blood as possible (Each wash consists of manual shaking followed by sedimentation under gravity—there is no need to centrifuge)
3 The HBSS is removed and replaced by 10 mL of enzyme mixture consisting of collagenase (130 U/mL) and dispase (1 mg/mL) in HBSS The container is incubated in a static water bath at 37°C with intermittent gentle agitation by manual shaking for 20 s every 15 min.
4. After the first hour of incubation, the first "fraction" is harvested (*see* Note 2) The enzyme mixture (containing suspended follicles and single cells) is removed (leaving the undigested tissue fragments at the bottom of the universal container—*see* Note 3), and transferred to a sterile 15-mL centrifuge tube. FCS is added to a final concentration of 0 5% to neutralize proteases, and the tube placed on ice. Fresh enzyme mixture (prewarmed to 37°C) is added to the undigested tissue fragments and the incubation continued
5 After a further 30 min, the next fraction is harvested exactly as in step 4 The process is repeated until no more tissue remains (usually after 3 h)
6. While digestion is continuing, the fractions already collected are centrifuged at 200*g* for 2 min in a swinging bucket rotor to recover the cells and follicles The

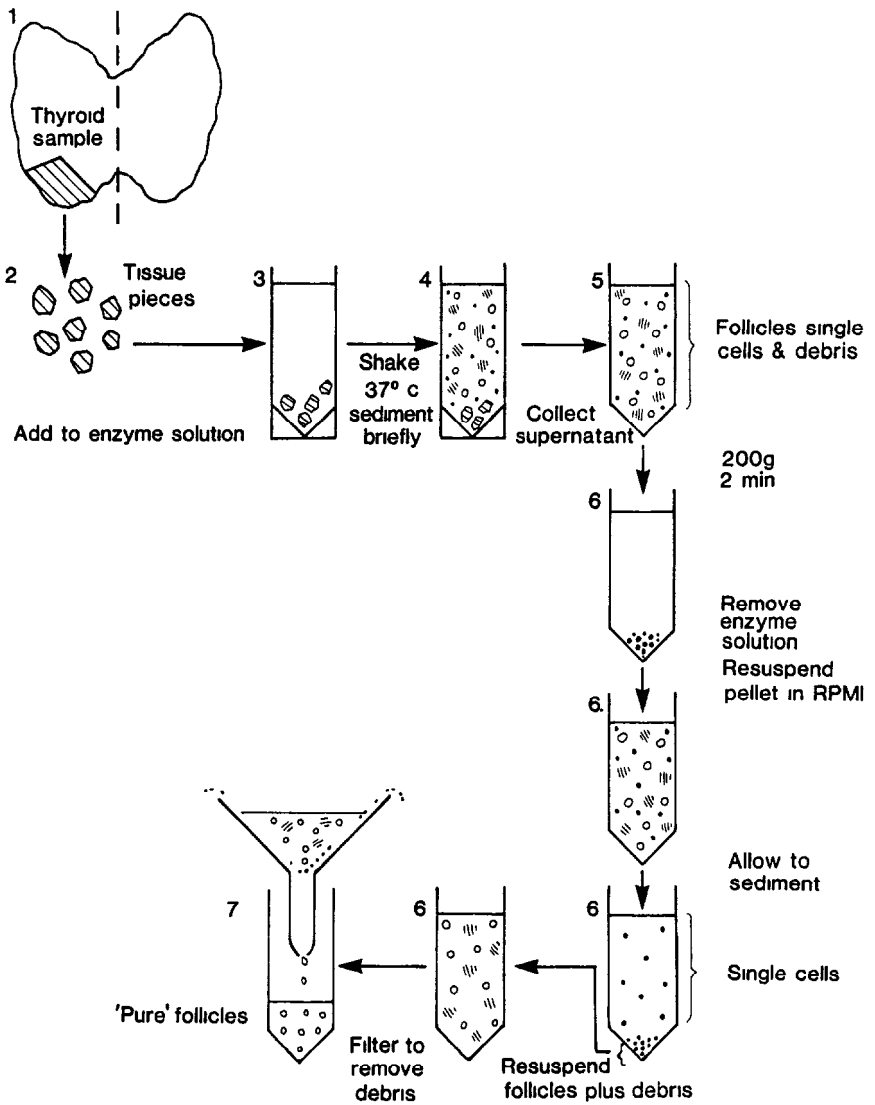

Fig. 1. Protocol for preparation of human thyroid follicles

supernatant (enzyme mixture plus FCS) is removed and the pelleted cells thoroughly resuspended by pipeting in 10 mL RPMI medium at 4°C They are then allowed to resediment under gravity for 0.5–1 h (*see* Note 4). The supernatant (containing single cells) is then discarded and the sedimented follicles from all fractions pooled in fresh RPMI.

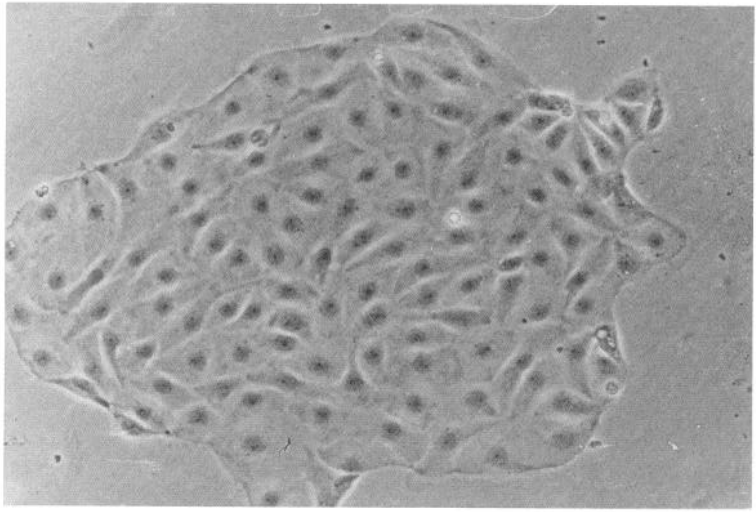

Fig. 2. Phase contrast of human thyroid follicular cells in monolayer culture, showing an island of cells derived from a single follicle 3 d after attachment (350×).

7. After the final fraction has been processed, the pooled follicles are filtered through a 200-μm nylon mesh to remove large debris such as partially digested tissue fragments. Finally, the follicles are washed twice by centrifugation (200*g*, 2 min) and resuspended in 1–4 mL of RPMI.
8. To obtain a crude cell count and viability index, 15 μL of follicle suspension is mixed with 15 μL of acridine orange/ethidium bromide solution and viewed in a hemocytometer under phase contrast (for cell/follicle counting) and UV light for assessment of viability (*see* Note 5).
9. Prior to plating out or freezing (see Note 6), the follicles are first incubated overnight (in a standard humidified 5% CO_2 atmosphere at 37°C) at a density of around $10^5/cm^2$ in RPMI (still serum-free) on Petri dishes coated with 2% agar (*see* Note 7) to prevent cell attachment. This maneuver allows most of the follicles that will have been ruptured during extraction to reform and leads to death of most of the small proportion of single cells (particularly fibroblasts) that may still be present.
10. Monolayer culture (Fig. 2): Follicles are plated on standard tissue-culture grade plastic Petri dishes in RPMI medium supplemented with 10% FCS. Plating efficiency is poor at low density; densities are best kept above $10^4/cm^2$. Initial doubling times average 2–3 d. At confluence, the cultures can be passaged (split ratio up to 1 in 8) by standard trypsinization schedules using trypsin/EDTA solution (*see* Section 2.). As expected for a primary culture, senescence occurs after a finite number of divisions—in 10% FCS between 2 and 4. The cells flatten, mitoses are no longer observed, and after several weeks, the culture eventually dies. Escape from senescence has never been observed in these human cultures.

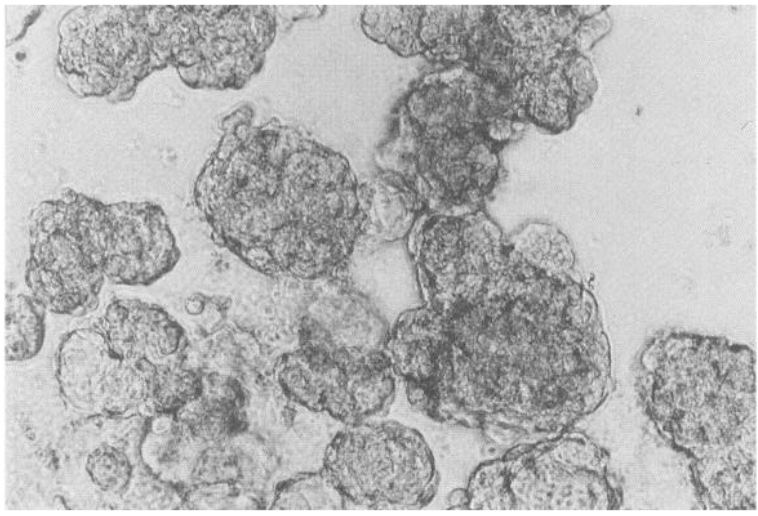

Fig. 3. Phase contrast of thyroid follicles in suspension culture (350×).

The cells retain the tissue-specific control of growth by TSH, which can be demonstrated by stimulation of [^{3}H]-thymidine labeling index in serum-free medium by TSH, in the presence of insulin-like growth factor-1 (IGF-1) *(18)*.

11. Suspension (follicle) culture (Figs. 3 and 4): Cells (follicles) are plated at 5×10^4–5×10^5/mL in RPMI supplemented with the desired serum concentraton. The cells will survive even in serum-free medium for many days (~95% viability after 1 wk). In 10% FCS, proliferation is very limited in comparison to monolayer cultures, [^{3}H]-thymidine uptake falling rapidly during the first few days. This is probably partly the result of a simple physical restriction on cell spreading in the follicle, but there is evidence also that the loss of proliferative capacity correlates with the development of follicular inversion, which occurs over the first week of culture (*see* Note 8). In the absence of serum, inversion is delayed, and clear proliferative responses to addition of pure growth factors (e.g., TSH plus IGF-1) can be observed for at least 1 wk. This culture system was the first to demonstrate the growth-stimulatory effect of TSH on human follicular cells in vitro *(13)*.

4. Notes

1. Cold nodules are most often a benign follicular adenoma; the surrounding unaffected tissue is usually clearly distinguishable macroscopically from the tumor, but the normality of the samples used for tissue culture is checked retrospectively by histological examination of tissue sections. Laryngectomies provide an alternative source of surgical samples. Some workers have successfully employed postmortem material, which is of course available in much larger quantity *(19)*.

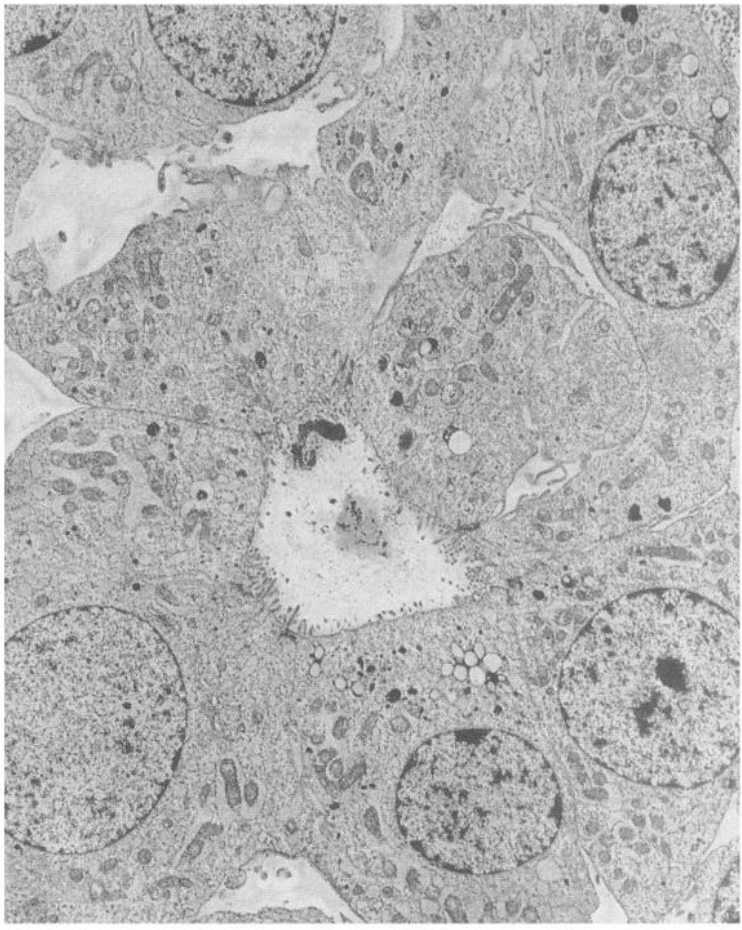

Fig. 4. Electron micrograph of follicles in suspension. The follicles were collected by centrifugation, fixed in 1% glutaraldehyde, and embedded in LR White resin. Sections were stained with uranyl acetate/lead citrate (8000×).

2. Very little release of cells occurs during the first hour of enzymic digestion. Most follicles are recovered in the next four successive 30-min incubations. The whole procedure usually lasts 3–4 h.
3. The undigested tissue pieces sink rapidly compared to the released follicles, so that on standing after a brief shake the fragments will all have settled in less than 1 min, leaving the follicles still in suspension.
4. At step 6, the tube is allowed to stand for a much longer period than in Note 3 (longer than 30 min). In this time, the follicles will sediment even under unit

gravity in a medium of sp gr 1, permitting adequate separation from the single cells (including fibroblasts), which remain in suspension. This is made possible by the large size of human follicles, with rat follicles, for example, a Percoll separation medium is needed *(6)*

5 Viable cells exclude the ethidium bromide and are stained green by the acridine orange. Dead cells take up the ethidium bromide and consequently show an orange fluorescence The quantitation of cell number is subject to observer error, because of the difficulty of distinguishing individual cells in some follicles This has not proven to be a problem provided absolute figures are not important and that all observations for a given experiment are made by the same operator If higher accuracy is needed, a follicle sample can be treated with trypsin to produce a single-cell preparation

6 Freezing: The follicles are first incubated for 24 h in suspension to allow reformation of closed follicles, since this has been found to improve subsequent viability after thawing. The follicles are harvested from the agar-coated dish by washing with RPMI medium into a 15-mL centrifuge tube, and then centrifuged at 200*g* for 2 min The pellet is resuspended in ice-cold RPMI containing 10% NBS at a maximum cell concentration of 10^7/mL An equal vol of a 1·4 (v/v) mixture of DMSO and NBS is added, and the cells thoroughly dispersed by gentle pipeting. The suspension is aliquoted into freezing ampules and allowed to cool slowly to –70°C inside a tightly closed polystyrene box The next day the ampules are transferred to liquid nitrogen for prolonged storage We have routinely obtained viability of >80% after 3 yr in store.

7 The agar is prepared by boiling a 2% solution in double-distilled water The hot solution is dispensed rapidly to avoid cooling, using a disposable plastic pipet. An excess is applied (around 5 mL for a 9-cm Petri dish) and allowed to cool for 1 min Most of the agar is then removed, leaving a thin coating.

8. Follicle inversion is a well-recognized process in serum containing media *(20)*. The mechanism is unclear, but the result is an inside-out structure in which the polarity of the cell is reversed, the microvillous border coming to lie on the outside in contact with the medium, accompanied by corresponding changes in position of the Golgi apparatus, nucleus, and intercellular junctions. Perhaps as a result of inappropriate fluid and electrolyte transfer, the lumen of the follicle becomes greatly distended, and the lining epithelium very attenuated Such cells show a marked alteration in both functional and proliferative responses *(13,21)*

Acknowledgments

We are grateful to the Cancer Research Campaign and to the Welsh Scheme for Development of Health and Social Research for grant support

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14

Derivation and Maintenance of Embryonic Stem Cell Cultures

Elizabeth J. Robertson

1. Introduction

The ability to derive permanent tissue-culture lines *(1)* of pluripotential stem cell lines (ES cells) from mouse embryos has provided a valuable model system for fundamental research into the cellular differentiation processes occuring in the normal embryo Perhaps the most attractive feature of embryonic stem cell lines is that they can be manipulated to differentiate into a diversity of cell types either directly in the tissue-culture dish or within the context of the normal developing embryo following their return to the embryonic environment.

ES cells can be maintained in the undifferentiated state for periods of several months without loss of their developmental capacity. When ES cells are reintroduced into host carrier embryos by the technique of blastocyst injection *(2)*, they resume a normal regulated pattern of proliferation and differentiation to form chimeric conceptuses. ES cells are unrestricted in their pattern of functional differentiation contributing extensively both to the somatic and germ cell lineages of adult chimeras *(3)* ES cells, in conjunction with protocols for targeted gene disruption, have made it possible to introduce mutations into specific genetic loci in mouse. The discovery that mammalian cells can undergo a process of homologous recombination (site-specific recombination between exogenously introduced, cloned DNA, and the chromosomal cognate gene) has facilitated the generation of mice carrying a range of targeted genetic alterations, from large deletions to single nucleotide changes *(4,5)*.

ES cell lines are generally freely available, and can normally be obtained on request from any laboratory routinely performing gene targeting experiments. However, since numerous protocols exist for the routine maintenance of different ES cell lines, it is advisable to ask for details of specific cell-culture condi-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
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tions used in the donating laboratory. This chapter presents methods for the derivation of new ES cell lines from blastocyst stage mouse embryos and the routine culture of cell lines generated using this procedure. Detailed protocols for the production of chimeric mice, stable transfection of ES cells, and the construction of gene targeting vectors can be found elsewhere *(5,6)*.

2. Materials

1. Complete growth medium. Dulbecco's modified Eagle's medium (DMEM) (high glucose formulation) plus 10% (v/v) fetal calf serum, 10% (v/v newborn calf serum, *see* Note 1), $5 \times 10^{-5}M$ 2-mercaptoethanol, antibiotics (50 U/mL penicillin, 50 µg/mL streptomycin), and 1% nonessential amino acids
2. Trypsin-EDTA; 0 25% (w/v) powdered porcine trypsin in 0 04% (w/v) EDTA in phosphate-buffered saline (PBS) (pH 7 6)
3. PBS (calcium and magnesium free)· 0 17*M* NaCl, 3 4 m*M* KCl, 4 m*M* Na_2HPO_4, 2 4 m*M* KH_2PO_4
4. Mitomycin C medium. DMEM supplemented with 10% (v/v) newborn calf serum and 10 µg/mL mitomycin C (prepare freshly at each use)
5. Lightweight paraffin oil

3. Methods

3.1. Isolation of ES Cell Lines (see Note 2)

3.1.1. Preparation of Embryonic Outgrowths

1. Prepare feeder layers in a 24-well tissue-culture plate Seed $5–7 \times 10^4$ mitomycin C-treated STO fibroblasts in 1 mL complete growth medium/well Feeder layers should be prepared 1 d prior to collecting embryos
2. Set up timed matings using the mouse strain of choice The blastocyst stage of embryonic development is reached 3 5 d after fertilization of the oocyte Inspect females daily for copulation plugs (day of plug is d 0) On d 3, kill the females by cervical dislocation, swab the abdomen with liberal quantities of 70% ethanol, and dissect out individual uterine horns (cut below the uterotubal junction and above the cervix). Remove the horns into a sterile Petri dish Flush the uterine lumen by introducing a 26-gage needle attached to a 1-mL syringe filled with complete growth medium (*see* Notes 3 and 4)
3. Remove the dish to a dissecting binocular microscope and locate the blastocysts. Using a finely drawn out Pasteur pipet attached to a mouth-controlled tube, collect the blastocysts. Transfer the embryos into a small drop of complete growth medium (overlay the drop with lightweight paraffin oil to prevent evaporation)
4. Transfer the embryos individually into the feeder wells taking care to place the embryo in the center of the well (*see* Note 5) Return the plate to the incubator and culture undisturbed for 4 d After this time, inspect the embryos The blastocysts will have attached by the outgrowth of the trophectoderm cells The inner cell mass (ICM) of the embryo will have proliferated to form a small discrete mass of cells (Fig 1)

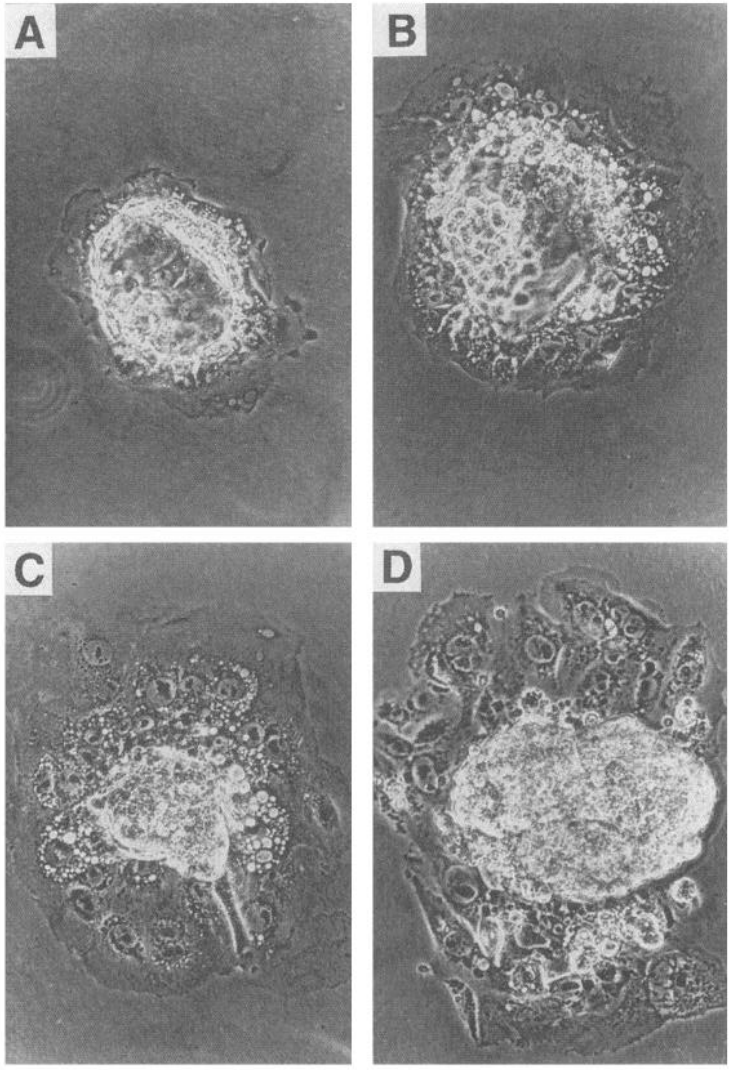

Fig. 1. Attachment and growth of a single blastocyst on a gelatinized tissue-culture surface. **(A)** 1.5-d culture, blastocyst attaching by trophoblast cell outgrowth; **(B)** 2.5-d culture, ICM component is revealed by the continued spreading of the trophoblast cells; **(C)** 4-d culture, cells of the ICM proliferating rapidly; **(D)** 5-d culture, ICM has formed a mass of cells.

3.1.2. Disaggregation of ICM Clumps

1. Prepare fresh feeder layers in a 24-well plate (as in Section 3.1.1.).
2. Aspirate the medium from the wells containing the embryos and add 1 mL of PBS.

3 Prepare a tissue-culture dish containing an array of small drops of trypsin-EDTA solution (approx 100 µL) Overlay the drops with lightweight paraffin oil
4. Place the 24-well plate under a dissecting microscope, and using a finely drawn out Pasteur pipet, carefully dislodge the ICM clumps from the trophectoderm cells. Transfer each ICM into a separate trypsin drop Return the dish to the incubator and leave for 5 min
5 Pull out a very fine diameter capillary in a bunsen flame from a Pasteur pipet. Attach the pipet to a mouth-controlled tube. Using gentle suction, suck a small quantity of complete growth medium into the pipet.
6. Return the dish of trypsin drops to the binocular microscope. Dissociate each ICM as follows. Blow a small quantity of medium from the pipet into the trypsin drop (to neutralize the trypsin action) Using mouth control, draw the ICM clump in and out of the end of the pipet (the end of the pipet should be approximately a quarter the diameter of the ICM clump) Fragment the ICM into several small cellular clumps, and transfer the fragments into a fresh feeder well Repeat for all embryos, and return the 24-well plate to the incubator.

3.1.3. Identification and Expansion of ES Cell Colonies

1 Incubate the ICM-derived fragments for 4 d. During this period, the fragments will attach and proliferate to form small primary colonies of cells
2 Inspect each well under high-power phase contrast (e.g., 200x). With reference to Fig. 2, classify the individual primary colonies according to morphology. A variety of differentiated phenotypes will be noted Mark potential ES cell colonies by circling the position of the colony on the underside of the well with a fine-tipped, indelible marking pen Return the plate to the incubator. Inspect the marked colonies daily for the following 3 d Eliminate colonies that differentiate to form large flat cells (*see* Fig 3). Genuine colonies of ES cells will increase in size in the absence of overt differentiation (*see* Fig 4 and Note 6).
3 Using the following procedure, subculture colonies of stem cells approx 6–7 d after the primary disaggregation event. To avoid the possibility of cross-contamination with nonstem cells, each putative ES cell colony should be dissociated individually
4. Prepare the feeder layers in 1-cm wells. Aspirate the well containing primary colonies and add 1 mL of PBS
5 Using a drawn out Pasteur pipet dislodge the marked colony, transfer it to a small drop of trypsin-EDTA, and incubate for 2–3 min in the incubator
6. Using a finely drawn out Pasteur pipet dissociate the colony into a single-cell suspension This should be achieved easily, since stem cells are more readily dissociated than the original ICM-derived clump.
7 Transfer the cell suspension to a 1-cm feeder well containing 1 mL of complete growth medium and incubate
8 Inspect the wells after 2 d for the presence of stem cells These are readily identifiable as having a very characteristic morphology (*see* Fig 5).
9. After 2 d of further culture, passage the wells containing ES cell colonies. Wash the well with 1 mL of PBS and trypsinize the cells by the addition of 100 µL

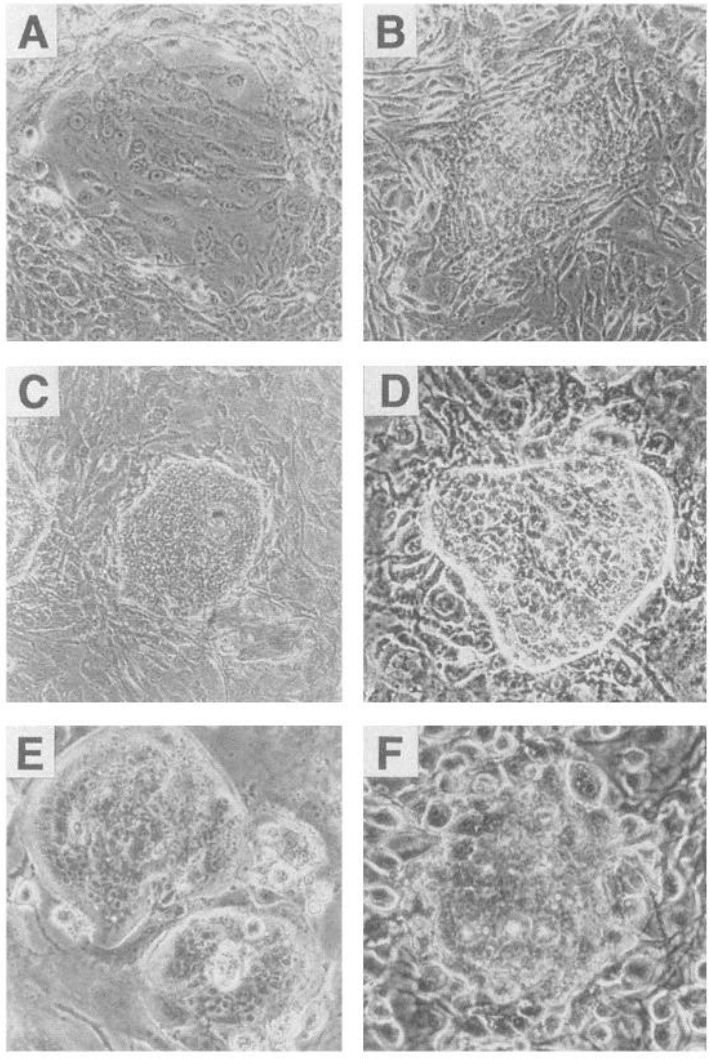

Fig. 2. Morphologies of primary colonies that result from the disaggregated ICM. **(A)** Patch of "spread" trophoblast cells; **(B)** fibroblast-like cells; **(C)** epithelioid-like cells; **(D)** epithelioid cells, single colony of cells at high power; **(E)** appearance at high power of trophoblast-like cells before cell flattening has occurred; **(F)** colony of stem cells.

trypsin-EDTA solution. Allow a 3–4 min incubation in trypsin. Then using a Pasteur pipet and bulb, add 100 µL complete growth medium to each well. Vigorously disperse the cells to ensure that a single-cell suspension is obtained.

10. Seed the single-cell suspension from each well into a 3-cm feeder plate containing 2 mL of complete growth medium.

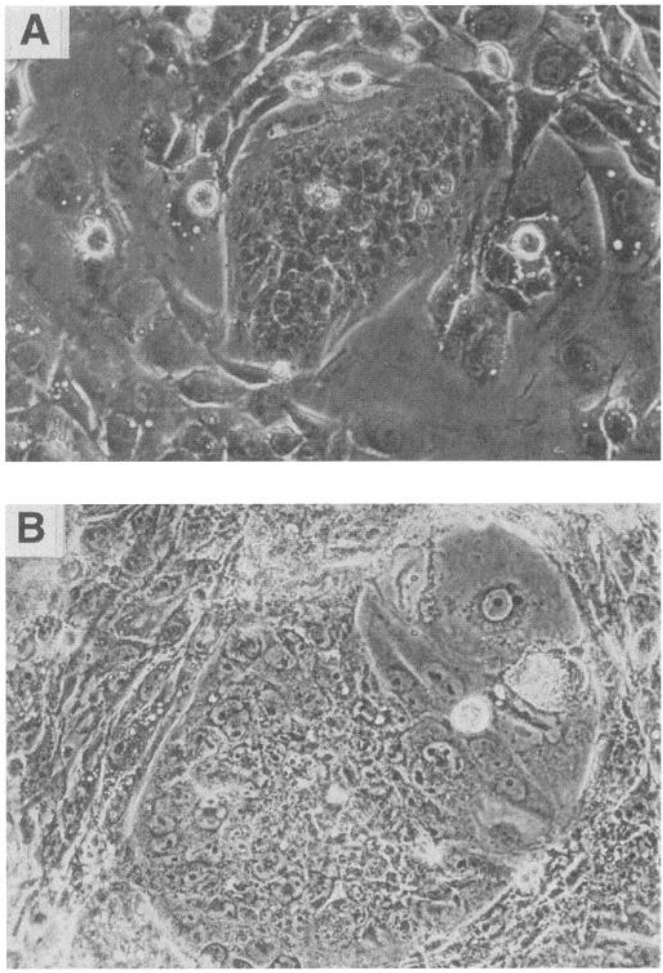

Fig. 3. Progressive alteration to the morphology of a putative stem cell colony over a 3-d culture period. **(A)** When first located 2 d after disaggregation of the ICM, the constituent cells superficially resemble stem cells. **(B)** Following a further 3 d culture, the colony has spread and flattened to form a patch of giant trophoblast-like cells.

11. Refeed the plates daily. After 3 d, sufficient stem cells should be present to necessitate subculture. Stem cells can also be frozen down at this stage (*see* Note 7).

3.2. Routine Culture of ES Cell Lines

It is recommended that ES cells be cultured exclusively on feeder layers. However, for some experimental procedures, it is desireable to omit feeder cells. This can be achieved by culturing the cells on gelatinized plates in complete growth

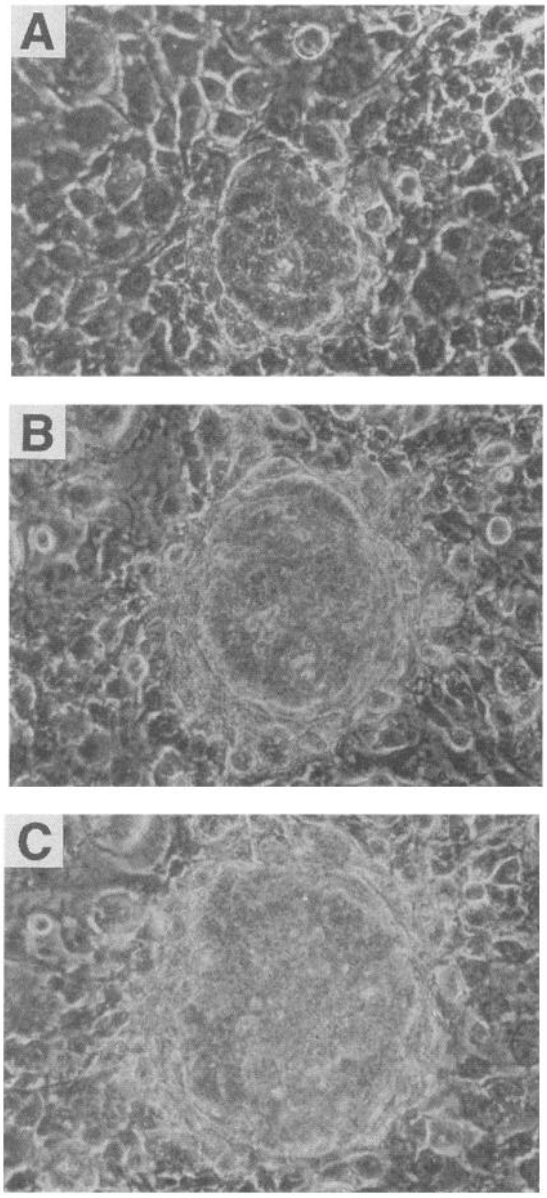

Fig. 4. Progressive growth of a colony of stem cells. **(A)** d 3; **(B)** d 4; **(C)** d 5. Note that the colony increases in size in the absence of overt differentiation. The cells pile up and maintain a discrete colony on top of the feeder cells. The constituent cells adhere tightly to one another making it difficult to see individual cells. This growth pattern is characteristic of stem cells, although the colonies may appear slightly flatter and less regularly shaped than the colony illustrated here.

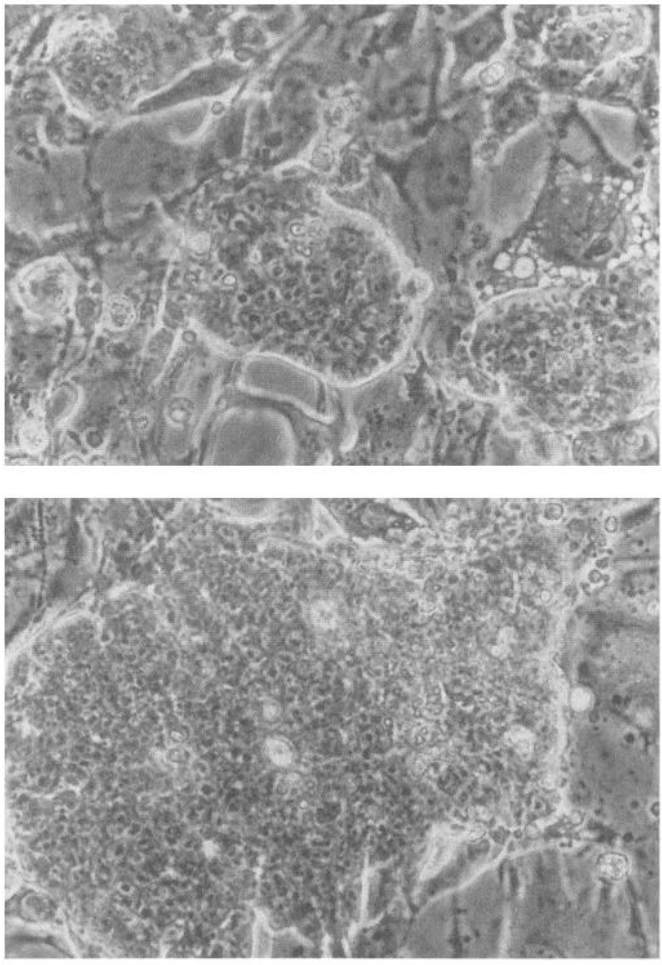

Fig. 5. Examples of typical areas of cells found within a subconfluent culture of established stem cells to illustrate the cellular morphology exhibited by pluripotential cells. The culture was seeded initially as single cells. These divide and the sister cells stay together to form small nests. With time the nests increase in size and merge to form a confluent layer.

medium supplemented with 100 U/mL ESGRO (Gibco-BRL, Gaithersburg, MD). ESGRO, recombinant soluble leukemia inhibitory factor (LIF), is sufficient to replace the LIF activity normally provided by the STO feeder cells *(7)*.

Stem cells grow rapidly and divide approximately every 15–18 h. The ES cell line should be maintained at relatively high densities to ensure maximal growth rate in order to minimize spontaneous differentiation.

1. Cultures of ES cells should be inspected and refed daily The cultures should be passaged when the stem cells have reached an approx 80% confluence.
2 Refeed the cultures 2–3 h prior to subculture to maximize the cell viability
3 Aspirate the growth medium and wash with 2–3 mL of PBS Add 1 mL of trypsin-EDTA solution, and return the plates to the incubator for 4–5 min.
4. Gently rock the plates to dislodge the cells Add 1 mL of complete growth medium, and using a Pasteur pipet and bulb, dissociate the cells to a single-cell suspension
5. Centrifuge the cell suspension at 1000 rpm for 5 min Aspirate the medium and resuspend the cells in 5 mL of complete growth medium Determine the cell density, and replate the cells into fresh feeder plates at a density of 10^6 cells/ 6-cm plate
6. Refeed and inspect the plates daily An 80% confluent dish of cells will be obtained after 3–4 d of culture, and at this time, a 6-cm plate will typically yield 2×10^7 cells

4. Notes

1 Both fetal serum and newborn calf serum are used as medium supplements For the successful growth of most tissue-culture cells, serum quality is critical, and this is especially true for blastocysts and embryonic cells. The use of unsuitable serum is undoubtedly one of the reasons for a failure to isolate stem cells from embryos All sera should be tested for their ability to support the growth of pluripotential stem cells serum testing is laborious, and the best strategy is to simultaneously request the maximum number of available samples from all the commercial suppliers The samples can then be tested in parallel and a bulk order placed for the most suitable sera. Serum can be stored for periods of up to 2 yr at –20°C The most sensitive test of the ability of a given serum to support the growth of stem cells is by a comparison of the plating efficiency of single cells from either an established embryo-derived stem cell line or, alternatively, feeder-dependent EC cell line (e g , PSA-4) The stem cells are coplated with feeder STO cells Control samples of fetal newborn calf sera are included in the test Only sera that are comparable to, or better than, the control sera should be bought. The technique is to set up duplicate plates containing a suspension of mitomycin C-treated STO fibroblast cells and a small number of stem cells in serum-free medium The plates are placed in sets with each set being allocated a specific serum batch. Serum is added to a final concentration of 10% To a single plate in each set, serum is added to a final concentration of 30% At this concentration, any toxic component will readily be detected The plates are incubated for 7–10 d, and the stem cell colonies stained and counted. A good quality serum gives a plating efficiency of 20% or higher Toxicity effects will be evidenced by a lower plating efficiency and/or the formation of smaller colonies at the 30% concentration Serum toxicity may be the result in part of high levels of complement. Heat treating serum (56°C for 30 min) to inactivate complement may dramatically decrease toxicity

2 Karyotype analysis procedures should be used to ascertain the chromosome complement and sex chromosome constitution of a cell line as soon as possible after it has been established The majority of ES cell lines derived, to date, have a normal euploid chromosome complement when established cultures are analyzed However, it is inevitable that cell populations will drift away from the normal genotype and subsets of aneuploid cells will be selected over a period of continuous culture For this reason, it is important to routinely check the chromosome complement of stem cells in culture Simple chromosome counts are used to determine the model number, and estimate the range and variability of the population Ideally, this should be complemented by G-banding analysis, which enables the exact chromosomal constitution to be determined

3 The starting material of choice for the isolation of stem cells is the blastocyst stage embryo Since the time course of mouse embryogenesis is well documented, and the time of fertilization can be readily determined, the recommended method for obtaining the maximum number of fully viable embryos is to leave the embryos to develop within the reproductive tract until they have reached the appropriate blastocyst stage Blastocysts are amenable to culture in standard tissue-culture media and do not require special requirements This is not the case for embryos at preblastocyst stages that have to be grown in a simpler serum-free medium. The mouse strain from which a cell line is to be made is another important consideration. The majority of inbred strains will, for example, yield relatively few embryos (normally between five and eight) If approaching this technique for the first time, it is recommended that more fecund outbred stocks of mice be used to generate embryos Outbred mouse stocks are readily available from commercial breeders, and matings provide large numbers of embryos on which to practice the various techniques before turning to the particular strain, or mutants, of interest The rate of recovery of stem cell lines is rarely higher than 30%, and may be lower than 10% Therefore, a large percentage of the embryos are wasted. If the mouse strain of interest is available in restricted numbers, or if the desired genotype is carried by only a fraction of the embryos from a given mating, attempts to isolate a stem cell line can be frustrating

4 Blastocysts that have undergone a period of implantational delay prior to flushing may be more suitable material from which to recover stem cells, the success rate can be significantly improved by the use of delayed blastocysts Implantational delay is brought about by altering the hormonal status of the pregnant female The source of estrogen production is removed by surgical ovariectomy 2 5 d postcoitum Lowering the level of estrogen, together with the postoperative administration of a synthetic progesterone, prevents the embryos from implanting. The embryos develop normally to the blastocyst stage, but since the tissues of the uterine wall have been artificially rendered nonreceptive, the embryos arrest and remain free floating within the uterine lumen. In the delayed state, the embryos remain viable for up to 10 d For the purposes of isolating stem cells, a 4–5-d delay period is normally used. Embryos are flushed from the uterine horns and treated exactly as described for normal blastocysts

5 There are a variety of technical considerations to be made regarding the best strategy for the culture of blastocysts. These are as follows:
   a Embryos collected on a single day can be kept together and cultured as one group, or split up into individual embryo cultures For most purposes, it is recommended that embryos be cultured singly This has the advantage of avoiding the necessity of recloning the stem cell cultures obtained, since a cell line derived from a single embryo is effectively a clonal population in terms of genotype and sex chromosome complement An additional advantage is that the monitoring and recording of embryos on an individual basis provides information about the overall viability of cultures and the efficiency of recovering stem cells The major disadvantage to this approach is that it takes considerably more time and materials to maintain large numbers of individual cultures.
   b. Blastocysts can either be grown in drop cultures under a layer of liquid paraffin oil or in larger tissue-culture dishes. The advantage of drop cultures is that embryos are easily located for observation because they are physically confined to a small area In my experience, however, embryos grown in drop cultures for the necessary 5–6 d fare less well. This is probably because of depletion of an essential growth requirement in the medium, or more likely results from the gradual accumulation into the medium of traces of toxic residues from the overlying oil The most satisfactory method is to use small tissue-culture wells (10 mm) that hold about 1 mL of medium
   c. Embryos may either be cultured directly on tissue-culture plastic or on feeder layers The use of feeder layers during initial culture appears to maximize the viability of blastocyst cultures The stage at which an individual ICM-derived component is selected for disaggregation is fairly critical Figure 1 shows examples of the morphologies of blastocyst outgrowths Under the conditions of culture described in Section 3 1 1 , embryos will normally attain a suitable morphology following a 5-d culture period. A degree of variability in the size of the ICM-derived cell clump between embryos of the same batch is a common observation As a result, it may be necessary to disaggregate ICM clumps over a 2-d period according to the morphology of individual cultures It is important to note that there is by no means an absolute correlation between the phenotype of the outgrowth and the successful isolation of a stem cell line; stem cells can be obtained from both vigorously growing ICMs and from small outgrowths. In my experience, however, ICM clumps in which there is extensive endoderm formation, or a relatively rapid progression to an overtly multilayered egg cylinder-like structure, tend to have a reduced chance of retaining pluripotential cells
6 It is important to be able to accurately identify colonies of pluripotential stem cells Positive identification is difficult unless one is familiar with the appropriate cellular morphology Pluripotential cells are typically small, have a large nucleus and minimal cytoplasm, and the nuclei contain one or more prominent dark nucleoli structures. The cells pack tightly together in small nests in which it

is difficult to discern the individual component cells Individual cells are most easily seen at the edges of the colony A helpful exercise, prior to attempting to isolate stem cells from embryos, is to plate out single cells from a feeder-dependent embryonal carcinoma cell line onto a feeder plate (e g , 5×10^2 cells on a 10-cm plate) Carefully observe the growth of the resulting colonies over a period of 5–8 d.

7. It is recommended that embryo-derived stem cell lines should be grown exclusively on feeder layers. This, together with careful culture in high-quality medium, acts to prolong the embryonic phenotype of the cell line specifically in terms of maintaining a high differentiation ability and euploid chromosome complement. As with all permanently established cell lines, long-term tissue-culture will, however, select for abnormal cells within the population For this reason, it is advised that many samples be frozen as soon as possible after founding a specific cell line Cultures can be replaced as necessary or recloned to establish euploid cultures. Recloning can be achieved by picking single cells into feeder wells. The resulting colonies of cells are re-expanded by the method described. For experimental procedures in which it is desirable to minimize the number of contaminating fibroblasts from the feeder layer, it is possible to passage the stem cells once or twice on gelatinized plates. If cultures are kept under these conditions for longer periods of time, extensive cell death occurs

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15

Culture of Cells From Human Tumors of the Nervous System on an Extracellular Matrix Derived from Bovine Corneal Endothelial Cells

Manfred Westphal, Alf Giese, Hildegard Meissner, and Dorothea Zirkel

1. Introduction

Cell culture has become an integral part of the daily routine of most biological laboratories. Many genetic tests, chemo-resistance testing for chemotherapy, and the study of deregulated cell proliferation in cancer, cannot be performed without cultured cells. In endocrinology and developmental biology, elaborate cell culture techniques allow the investigation of regulatory processes and cellular interactions in in vitro systems, in which the experimental conditions can be defined as in no other experimental paradigm.

With many tissues, either human or animal, normal or neoplastic, the initial problems are cell attachment and cell survival. Particularly, primary cultures derived from tumor specimens pose a problem to many laboratories One solution has been the modification of regular commercial tissue culture dish surfaces that are usually the patented secrets of the manufacturers. Among the other approaches to improve the adhesiveness of culture materials, are coatings with attachment enhancers such as polyamino acids, in particular poly-lysine *(1)*, fibronectin *(2)*, laminin *(3)*, or collagen *(4)*. The use of natural, complex substrates was based on the observation, that endothelial cells are capable of producing basement membranes in vitro. During the study of corneal endothelium and the differences between species in their capacity for regeneration of the cornea, bovine corneal endothelial basement membrane found broad application as a cell culture substrate for many different endothelial and epithelial cell types. This bovine corneal extracellular matrix (bECM) was then found to be useful in the cell culture of a wide range of different normal and neoplastic cell

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

types *(5,6)*. For example, bECM as well as other ECMs were used in the study of the cell biology of tumor cells derived from mammary carcinomas *(7)*, urological tumors *(8)*, adrenal adenoma *(9)*, different kinds of pituitary adenomas *(10,11)*, as well as central nervous system (CNS) tumors *(12)* that are the topic of this chapter.

Biologically produced matrices have the potential to account for a variety of biological effects, as one has to reckon, that they are part of a self-made cellular environment that is deposited in a kind of "autocrine/paracrine" fashion. One major effect is the facilitation of cell attachment by providing an anchor for cell surface adhesion molecules. The molecules inherent to most matrices like fibronectin, laminin, and collagen bind to the cells via cell surface receptors, many of them members of the integrin family *(13)*. By this way of interaction, a geometrical order can be imposed on the protein complexes in the membrane that in turn can lead to the activation of second messenger events similar to those mediated by soluble growth factors or hormones via their respective receptors. In addition it has been shown that ECMs, because of their high content of heparan-sulfate, retain large amounts of heparin binding growth factors *(14)* including the acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF) and the heregulin family of trophic and growth factors.

Because of these biologically active constituents, it has to be born in mind that any ECM is not an inert attachment substrate and that it may interfere with the cellular biology of normal as well as neoplastic cells *(15,16)* affecting cell differentiation and responsiveness. This problem becomes even more complicated as matrices from different sources may affect cells in a variety of ways *(17)*. In the neuro-oncological context it should be mentioned, that an ECM derived from human arachnoid cells has been shown to induce differentiation in one designated glioma cell line *(18)*. Apart from the endothelial cells from cornea or large vessels, many other cells, including those derived from neoplastic tissues show a capacity to produce matrices. This chapter focuses first on the production and applications of bECM in experimental neuro-oncology, and with the matrices produced by glioma cell lines and their usefullness in the study of cell migration and differentiation.

2. Materials

2.1. ECM from bECM

2.1 1. Bovine Eyes

Following the instructions from protocols published earlier and elsewhere *(12,20)*, fresh bovine eyes have to be obtained from the local slaughter house (*see* Note 1).

2.1.2. Isolation of the Cornea (see Note 2)

1. Instruments (sterile). No. 1 cannulae, angled microscissors, tight gripping forceps, and a grooved director or disposable plastic spatula.
2. Phosphate-buffered saline (PBS)
3. Dulbecco's modification of Eagle's medium (DMEM) with high glucose content supplemented with 10% fetal calf serum, 1 m*M* glutamine, 2m*M* pyruvate, 25 µg/mL gentamycin, and 2 5 µg/mL fungizone

2.1.3. ECM Production

1. Dextran (mol wt 40,000).
2. Basic bovine FGF *(see* Section 3.4 *)*.
3. 20 m*M* Sterile ammonium hydroxide solution.
4. Sterile PBS containing gentamycin

2.2. FGF Isolation (22)

1. Bovine brain (4 kg) (*see* Note 3)
2. Heavy duty Waring blendor
3. 0 15*M* Ammonium sulfate pH 5 6
4. 6*N* HCl.
5. Ammonium sulfate
6. Formic acid.
7. CM-Sephadex C-50
8. 0.1*M* sodium phosphate pH 6 0
9. 0.1*M* sodium phosphate pH 6 0, 0 15*M* NaCl
10. 0.1*M* sodium phosphate pH 6 0, 0 6*M* NaCl

2.3. Tumor Cell Isolation (see Note 4)

1. Hank's balanced salt solution, Ca^{2+}- and Mg^{2+}-free
2. Enzyme coctail. pronase (0.05%), collagenase (0.03%)
3. 0.01% DNAse in Hank's, filtered sterile

2.4. Immunostaining

1. Round 12-mm diameter glass cover slides
2. Antibodies.
3. Citifluor anti-fading reagent (City-University London)
4. Nail-polish

3. Methods

3.1. Preparation of bECM

1. Preparation of the cornea· Only eyes which are uninjured, show no signs of infection, inflammation, or ulceration are used. As shown in Fig 1, they are held with the left hand, rinsed thoroughly with alcohol from a spray bottle and then held down firmly on a paper towel that is also soaked in alcohol The eye is punctured

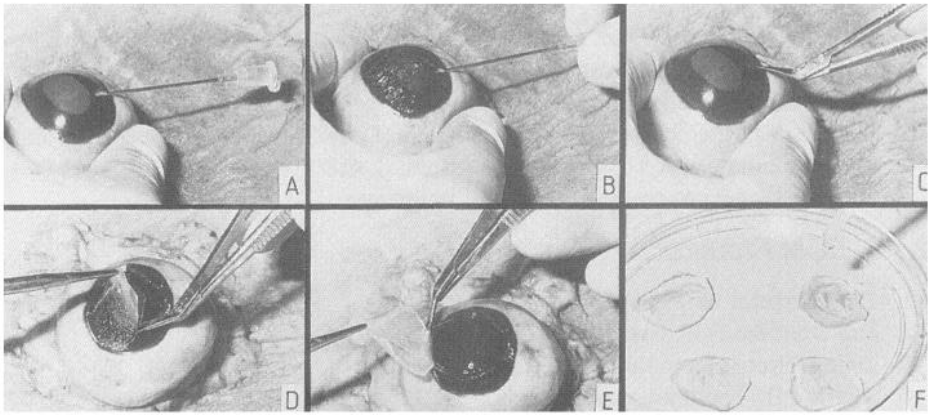

Fig. 1. Different steps in the production of ECM. **(A)** The eye is punctured with a 10-gage needle to drain the anterior eye chamber and relieve the pressure so that the eye wrinkles as seen in **(B)**. **(C)** Starting from the puncture hole, the cornea is excised, being lifted up halfway along the way **(D)** and then cut off after almost complete reflection **(E)**. Four cornea are put face-down onto a Petri dish and are covered with PBS **(F)**.

tangentially with a cannula so the fluid from the anterior eye chamber can drain allowing the cornea to shrink after the pressure is taken off the eyeball. Starting from the puncturehole made with the needle, the cornea is carefully excised with sharp scissors taking great caution not to contaminate it with other tissues from within the eyeball (Fig. 1A–E, *see* Note 2).

2. Preparation of the endothelium: Once the cornea is removed it is put with its external surface down onto a Petri dish where it will readily stick. It is then rinsed with a few drops of PBS at room temperature (Fig. 1F). Four corneae are placed into one Petri dish. Then the exposed inner surface is gently scraped once with a groved director or a disposable plastic spatula.Without the exertion of any pressure this maneuver will yield whole sheets of endothelial cells (Fig. 2). The groved director is then dipped into a tissue-culture dish containing the complete cell culture medium. Use one dish per eye. Process 10 eyes in one session. Because the cells will not all be removed with the groved director, it is useful to take one 1-mL pipet and "scavenge" the liquid left behind the four corneae and pool it into a fifth dish.
3. Primary culture and expansion of stocks: The dishes are then transferred to the incubator and left undisturbed for 5 d. At that point the first colonies of cells growing in a cobble stone pattern will have formed (Fig. 2). These may be few and far between but once colonies have formed after several days, FGF can be added and proliferation will take off. After the initial culture dishes are about 50% covered with colonies, these are detached with trypsin that may take up to

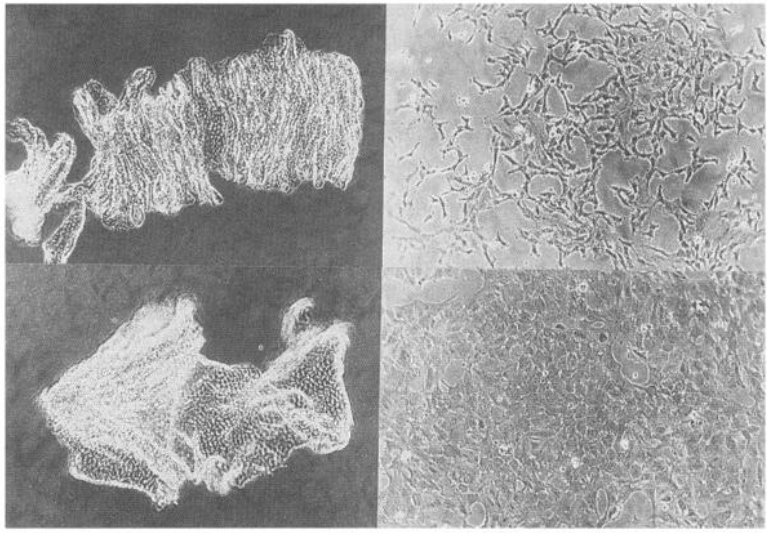

Fig. 2. Appearance of ECM. Two typical sheets of corneal endothelium as it comes off and is seen in the culture dish are shown on the left. Two different confluence stages of endothelial cells are shown on the right.

20 min because the primary cells attach firmly to their support (via an extracellular matrix). The detached and dispersed cells of 10 culture dishes are expanded onto 25 new dishes which have been precoated with gelatin.

4. Gelatin coating: 0.2% Gelatin is dissolved in PBS and autoclaved. It is advisable to filter-sterilize this solution because it may still contain granular matter that disturbs the cell culture and the homogeneity of the deposited matrix. The cell-culture dishes (10-cm diameter) are covered with 5 mL of the PBS-gelatin and left in the refrigerator overnight. Prior to use, the excess PBS-gelatin is removed. This gelatin coating facilitates the next passage (production step) because the cells are much easier to detach.
5. After the corneal endothelial cells have been expanded on the gelatin-coated dishes, they are best passaged at the point when they are just subconfluent because then there are fewer intercellular adhesions resulting in a very homogeneous solution of single cells. The cells from eight to ten 10-cm dishes are combined and added to a 550-mL bottle of complete medium that also contains 5% dextran (mol wt 40,000, dissolved by heating in a small volume of medium without additives and sterile filtered after cooling). Finally, crude basic bFGF is added (*see* Note 3). This cell suspension is plated onto any kind of culture dish which is to be covered with ECM. For example, 6 mL are added to a T25 flask, 25 mL to a T75, and 1 mL/well to 24-well multiwell tissue-culture dishes. At this density and in the presence of bFGF the cells should reach confluence within two to three

days After confluence the cells are left for one more week to ten days and then they are lysed. The biological properties of matrices produced over a short or long period are essentially the same (*see* Note 4). In any kind of multiwell plates, the cell number needs to be relatively higher than in larger dishes because it should be made certain that the cells become confluent without the necessity of a tedious second addition of bFGF to hundreds of wells

6 The cells are lysed by removing the culture medium and replacing it with distilled water containing 20 m*M* ammonium hydroxide. The cells burst rapidly by osmotic shock and within 3 min the gelatinous cellular debris can be removed and rinsed off with PBS (*see* Note 5). The ECM is left behind and should be washed at least once with PBS Finally the ECM-coated dishes can be stored at 4°C while being covered with PBS containing 25 µg/mL gentamycin At that time the matrix can be seen with a phase contrast microscope (*see* Note 6).

3.2. ECM From Human Brain Tumor Cell Lines (tECM)

1 Producer cells· Cells from a large, well-characterized panel of human glioblastoma cell lines *(21)*, most of which are positive for cell-surface fibronectin, have been assayed for ECM production They all produce different quantities of ECM that in a few cases can be seen in the phase contrast microscope More often the ECM is only detectable by immunofluorescence with the appropriate antibodies against fibronectin, laminin, tenascin, or other known ECM components (*see* Section 3 3) These immunostainings also show that the cells not only produce different amounts of ECM, but also vary in the composition of the ECMs Cells growing in a distinct geometrical arrangement may deposit their matrix in an orderly fashion and impose such order on cells seeded thereon *(19)*

2 For cell culture, the same conditions are used as in step 1 except that Earle's modified MEM is used as a basal medium.

3 The same procedure is used for isolation of tECM as for bECM.

3.3. Analysis of ECM by Immunostaining and Quantification of ECM Protein

3.3.1. Immunostaining of ECM

1 Producer cells are grown on a glass cover slide which is placed at the bottom of a multiwell culture dish The cells are lysed by the standard procedure, therafter the slide is thoroughly rinsed in PBS and set atop the small pedicles of a staining tray which can be covered to function as a wet chamber.

2 Primary antibody is added, left for 30 min at room temperature and then rinsed off

3 The second, fluorescent antibody is added and left for another 30 min and again rinsed off

4 The cover slips are then put face-down onto a heavier microscope slide with 20 µL of Citifluor antifading reagent between them. This "wet-chamber" is sealed off with nail polish These slides can be stored for many months at 4°C or viewed immediately with a fluorescent microscope

3.3.2. Protein Determination

Several papers have published the solubilization of ECM and measurement of protein *(22)*. In our hands, the solubilization of the protein with sodium hydroxide works better than any cocktail of detergents.

1 The cells from which a protein determination is desired are seeded onto 6-well multiwell dishes. We have arbitrarily decided to leave the cells for 8 d after reaching confluence
2 The cells are lysed with distilled water and the debris is rinsed off
3 600 µL of 0 5*M* NaOH are left for 3 h at 60°C on the ECM
4. The protein content of the lysate is determined with a commercially available BCA-ProteinAssay (Pierce, Rockford, IL) NaOH or bovine serum albumin dissolved in NaOH are used as reference point or as standard, respectively.

3.4. Isolation of FGF

The addition of FGF to the dispersed stock cells before plating is of great importance for the production of ECM This speeds up the rate at which the cultures become confluent and also influences the quality of the matrix, i.e., thickness and homogeneity. The isolation of basic FGF (bFGF) has been described by Gospodarowicz *(23)* but it is not necessary for the production of ECM to purify bFGF to homogeneity.

1 Eight frozen brains (*see* Note 7) are homogenized in 8 L of 0.15*M* ammonium-sulfate pH 5.6 at 4°C
2 The homogenate is adjusted to pH 4 5 with 6*N* HCl and stirred for at least 1 h at 4°C
3 The homogenate is then centrifuged at 20,000*g* and the burgundy-colored supernatants are collected **CAUTION:** If the color has turned brown because the pH has dropped too low during adjustment even for only a short moment, the proceedure should be stopped at this point and started anew because bFGF is very intolerant to acidic treatment
4 Ammonium sulfate is now added to the stirring extract to give a concentration of 200 g/L and the turbid suspension is centrifuged at 20,000*g* after having been stirred for 60 min
5. Again the supernatants are collected and again ammonium sulfate (250 g/L) is added to give a final concentration of 450 g/L After this centrifugation the supernatants are discarded and the pellets from this second "cut" are collected and pooled
6 The collected pellets are dissolved in a small volume of water (approx 250 mL), adjusted to pH 6.0 with formic acid, and dialyzed against water. (Use dialysis tubing with molecular weight cut-off between 10,000 and 12,000)
7. The dialyzate is loaded onto a CM Sephadex C50 column (3.5 × 20 cm) that has been pre-equilibrated with 0 1*M* sodium phosphate pH 6 0 The ionic strength of

the dialyzate should be equal or less than that of the running buffer Thorough washing of the CM-Sephadex before packing allows for a fast flow rate of 200–400 mL/h

8 The material retained on the column is eluted with a stepwise increase of NaCl concentration in the sodium phosphate buffer, first to 0.15*M* and then to 0.6*M*

9 The material eluting at the high salt concentration is collected. It is less than 0 1% pure but this is completely sufficient for use in the production of ECM It can be stored frozen as well as lyophylized until reconstitution The concentration at which the extract has to be used should be tested in a bioassay on endothelial cells. It is variable between extractions.

3.5. Isolation and Culture of Brain Tumor Cells

Brain tumors may arise from a broad range of different cells. The most common glial tumors are astrocytomas and oligodendrogliomas. Within one histological subtype the different entities are distinguished by their biological aggressivesness that is to be reflected by grades given according to various grading systems of which the WHO-grading seems the most widely used *(24)*. In general, it is possible from the imaging studies taken preoperatively to have a fair estimate whether the tumor is well differentiated (low WHO grade) or anaplastic (high WHO grade) This helps to decide which method should be taken for the initial dispersion of the tissue specimen

1 Representative specimens from a lesion are obtained at surgery (*see* Note 8) The selected tissue fragment or different fragments from different parts of the tumor are placed immediately into 15 mL of sterile Hank's BBS without calcium and magnesium (CMF) (Fig 3, *see* Note 9)

2 Enzyme digestion (*see* Notes 9 and 10) The tissue is cleaned of coagulated blood and minced with angled scissors The fragments are transferred into a centrifuge tube containing 10 mL of a cocktail of pronase (0 05%), collagenase (0 03%) and DNAse (0 01%) in Hank's BBS After 20-min incubation at 37°C and gentle shaking of the incubation mixture, the fragments are mechanically disrupted by repeated trituration The undispersed fibrous fragments should be allowed to settle and the turbid supernatant transferred to a centrifuge tube

3 Mechanical dispersion (*see* Note 10) After mincing, the fragments are agitated by repeated trituration (*see* Note 11) until a turbid solution is obtained Allow large pieces or undispersed fibrous fragments to settle and remove the supernatant into a centrifuge tube Sometimes an elaborate vascular network can be maintained at the end of the dispersion

4 The cells and small cell aggregates obtained from either of the dissociative proceedures are centrifuged at 80*g* for 10 min and thereafter redispersed in cell culture medium containing 10% FCS or in serum-free medium In the case of enzymatical dispersion great care should be taken to carefully remove all the enzyme, because otherwise the leftover enzymes, particularly the collagenase will digest the ECM To be on the safe side, the cells can be washed once in medium

Fig. 3. The arrow points toward the area in which a yellow discoloration of the tissue-culture medium around the tumor specimen can be seen after the tube has been standing for 30 min.

5. Before aliquoting the cell suspension to the culture dishes designated to be used in the subsequent experiments, a drop of the suspension should be microscopically examined to rule out the possibility, that either because of the nature of the selected tissue or as a consequence of the isolation procedure, it is composed of cellular debris. (A trypan-blue exclusion assay is optional at this point but with growing experience, the experimentor will be able to do without.)
6. The suspension is aliquoted onto ECM coated dishes and left to attach for at least 6 h but preferably overnight.
7. Cells are maintained at 37°C in a humidified atmosphere supplemented with 5–8% CO_2 depending on the medium used or the tissues cultured. We keep meningeomas in DMEM (DME-H21) with 10% fetal calf serum, 2 m*M* glutamine, 1 m*M* pyruvate, 25 μg/mL gentamycin, and 2.5 μg/mL amphotericin B. Gliomas are maintained in MEM-Earle with the same supplements. Splitting and passaging of the cells is done with a 0.05% trypsin/0.04% EDTA solution in PBS/CMF (STV).
8. Careful inspection of the cells on the next day is mandatory (*see* Notes 12–23). In extreme cases the cultures are confluent and the debris from red blood cells, unattached fragments and lymphocytes needs to be rigorously shaken up and rinsed off. In any case it is advisable to change the medium as early as possible (*see* Note 12). Even small fragments will have attached at this point (Fig. 4).

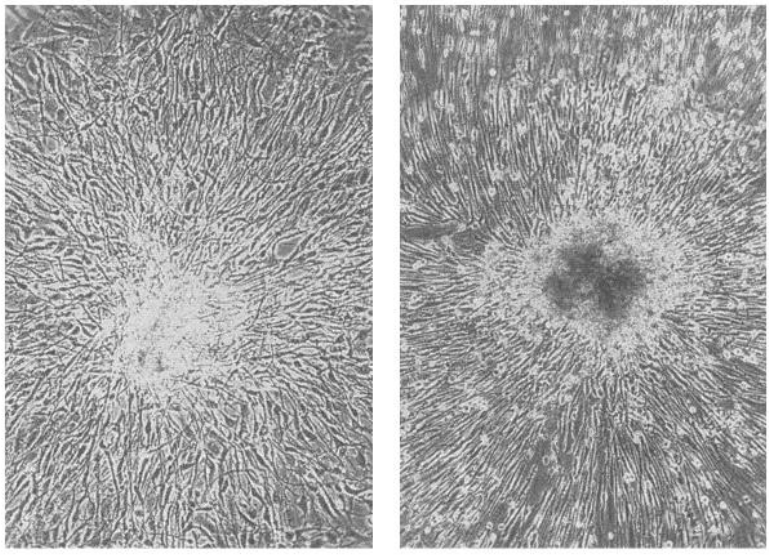

Fig. 4. Typical radial outgrowth of glial tumor cells from aggregates found in the primary cultures of a pilocytic astrocytoma (WHO grade 1, left) and an anaplastic astrocytoma (WHO grade 3, right).

4. Notes

4.1. Methods

1. The cornea is a very tough but slippery tissue and the forceps to lift the cornea after it is excised halfway should have a firm grip. Also, the scissors should be sharp (Fig. 1A–F).
2. Eyes can be conveniently transported in a plastic bag and do not need any special prerequisites or cooling for transportation. If they are obtained by slaughter house workers at inconveniently early hours they should be kept in a refrigerator until the experimentor will come to pick them up later. Although corneal endothelial cells are rather sturdy, it is advisable to use the eyes within 6–8 h after the animals have been killed (*see* Note 23).
3. The addition of FGF is not quantified in this methodology because it will depend on the respective batch how much needs to be added. The protein peak eluting from the CM-Sephadex C-50 contains both acidic and basic FGF. The shape of the peak will depend on the geometry of the column used and the exact running conditions. Also the final FGF concentration will depend on the width of the peak and when it is decided to stop the collection. It is best to take an aliquot and test various dilutions to find the most effective concentration.
4. It is possible that the quality and the composition on the matrix deposited by cells in vivo as well as in vitro depends on the growth phase and the density of the cells

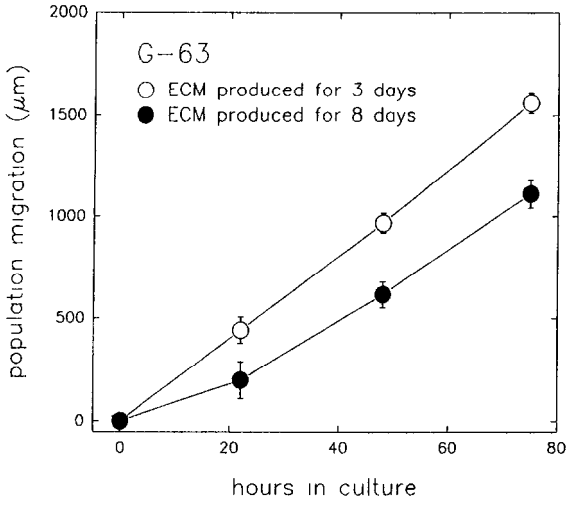

Fig. 5. Cell migration of a glioma cell line (NCE-G63) that was evaluated in a standardized migration assay on two different autologous matrices which were obtained from cells just at confluence (○) or 5 d past confluence (●)

(*see* Note 24) For example, confluent cultures may deposit signals into the matrix that result in a nonpermissive environment for proliferation and migration to the cells to be cultured Proof for this theory can be obtained by using autologous tECM in a migration assay in which the tECM is either prepared from cultures just grown to confluence or from cells grown several days past confluence In this paradigm, it was found that cell migration was enhanced on ECM derived from actively growing cells (Fig. 5). The producer cells for any ECM should be regularly tested for mycoplasma as otherwise contaminations may be spread. It is best to use the PCR-based kits. Although mycoplasma is unlikely to survive the hypo-osmotic lysis and the ammonia, it is safer to dispose of infected producer cells

5. The higher the passage numbers get for the endothelial cells, the more "resistant" they become towards osmotic lysis It is therefore important to scrupulously inspect random dishes after lysis to hunt for leftover cell islands. We have observed the phenomenon of survival of endothelial cells after presumed lysis and storage at 4°C for several days. In questionable cultures, a chromosome analysis should be helpful as bovine cells have a very distinct karyotype (Fig 6)
6 bECM will have a different appearance from batch to batch It may look homogeneous and bubbly, but it may also look more like a web or show pronounced crests and ridges, depending on how long the corneal endothelial cells had been propagated in culture, what the initial density was and how long it took for the cells to become confluent *(19)*. The matrix itself can be stored for up to two years as is our experience, maybe even longer. Likewise the bovine corneal endothelial

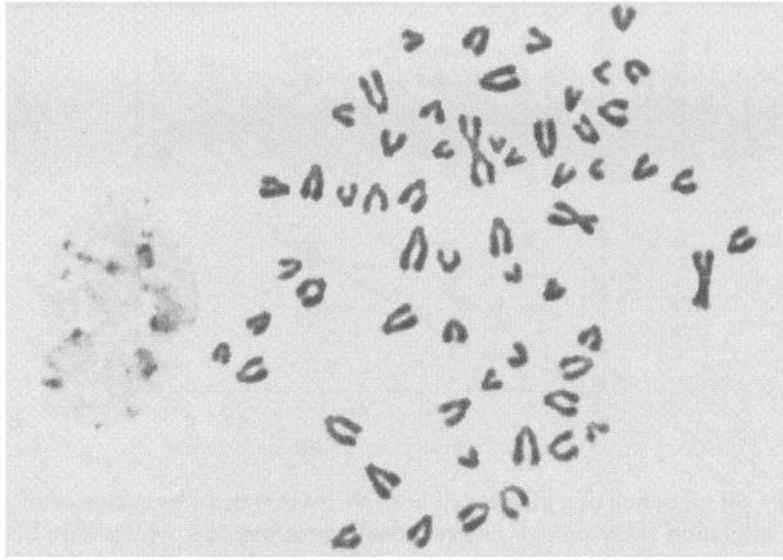

Fig. 6. Simple metaphase spread of bovine endothelial cells stained with Orcein. The normal chromosome set contains 60 chromosomes, which are mostly acrocentric and thus characteristically different from normal human chromosomes.

cells can be frozen in log-growth phase in a mixture of 4.5 parts medium 4.5 parts fetal calf serum and 1 part dimethyl sulfoxide. They have been stored in liquid nitrogen or at –80°C for up to 8 yr without losing their biological properties.

7. After the brains are obtained from the slaughter, they should be frozen rapidly. If they are kept at –20°C they should be put to 4°C the evening before the homogenization to gradually attain a waxy consistency. This will take longer when they are kept at –80°C. It is necessary that the brains go through one freezing process at least once before they are used because that facilitates the homogenization owing to denaturing of the tissue. Any attempt to use fresh tissue will turn out to be difficult and messy at the stage of homogenization and will fail ultimately at the point of the first centrifugation because no firm pellet will be obtained.
8. It is advisable to obtain a preliminary histological diagnosis by frozen section. Viable tissue can only be selected on the basis of surgical experience. In the ideal situation, the neurosurgeon is the principle investigator and decides what is the best specimen for the experiment. If this is not the case, a close colaboration between surgeon and scientist should be established with sufficient feedback to improve the sampling technique. In any case, frozen sections from the immediate vicinity of the removed specimen can be helpful.
9. In this solution the material can be maintained for at least 4 h at room temperature. The viability of the tissue is indicated by a yellow discoloration of the phe-

nol red indicator in the CMF around the tissue fragment at the bottom of the tube (Fig. 3) If the specimen was taken from an anaplastic lesion and there is no discoloration, one has to suspect that the material was mostly necrotic Otherwise the pH should change due to the metabolic activity which can even be seen in tumors of low cellularity such as benign neuromas Usually 1 cm^3 is plenty of material and any temptation to use more should be withstood When the tissue is derived from a primary, anaplastic glial tumor, the tissue is usually very soft and almost liquefied and can be easily dispersed mechanically If the specimen was obtained from a differentiated tumor with a lot of fibrous components such as a fibrillary astrocytoma or a tough meningioma or a tumor after irradiation, an enzymatic dipersion may be a valuable option

10. For gliomas and meningeomas alike the decision has to be made as to whether the tissue is to be digested with enzymes or whether a mechanical dispersion will suffice Enzymatic dispersion has the advantage that a more homogeneous suspension is obtained which can already be utilized in proliferation studies in which it will be advantageous to use aliquots which are comparable in cell number Purely mechanical dispersion has the advantage that only the loosely connected tumor cells will be obtained either as single cells or small aggregates The blood vessels are sometimes left behind as intact whole networks, thus eliminating the major source of contamination with other cell types. Even from small fragments mainly tumor cells will attach and migrate away from the small original spheroids (Fig 4)
11 If the decision is made to use mechanical dispersion by trituration with a tissue culture pipet, these should have smooth tips Some manufacturers leave their cut pipetips unrefined, resulting in very rough mouths which will tear up the cells The decision has to be made, however, on an individual basis depending on the softness of the tissue and the experimental goal. Medulloblastomas, pituitary adenomas, pineocytomas and many of the malignant primary gliomas are usually very soft and will readily disperse by repeated trituration through a 5 mL tissue-culture pipet alone Acoustic neuromas and other schwannomas as well as meningiomas and are often very tough and fibrous so that enzymes help to disrupt the tumor tissue
12. After the overnight attachment period, attention should be paid to the ECM which may be intact or can be completely lysed. Such slow lysis of the ECM has been noted in several cases of malignant gliomas in an experience of many hundred cases Such lysisis owing to release of proteolytic enzymes after cell death or due to the many proteases which are known to be produced by glioma cells and tumor cells in general for the process of invasion *(25)*

4.2. Applications of ECM

13. Meningeomas are lesions of the coverings of the brain which are regularly homogeneous, well vascularized, and of rather tough consistency. They occur in a variety of histological subtypes and despite many efforts, there is no reliable grading system. These tumors usually attach very well to cell culture substrates

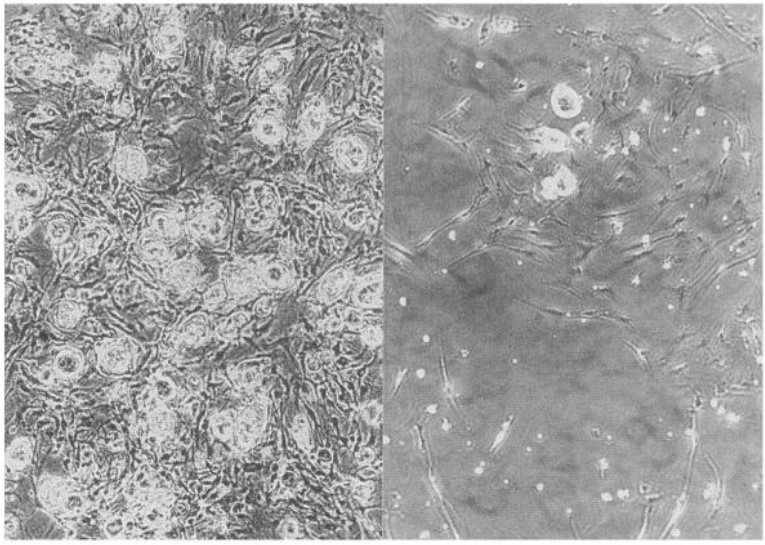

Fig. 7. Phase-contrast photomicrographs comparing the primary cultures from a meningioma grade WHO 2 on plastic (left) and on ECM (right). In this rare case the initial cultures on ECM look much better that on plastic.

even without bECM. This is most likely due to a rapid deposition of large amounts of a matrix rich in fibronectin and collagen by theses cells that are both very effective autologous adhesion factors but appear to be motogens for other cells types (Giese et al., manuscript in preparation). There are sporadic cases that do not attach to plastic but to ECM (Fig. 7). This seems to be a feature of atypical meningiomas, like those in patients with von Recklinghausen's disease, malignant meningiomas, or such secondary to cranial irradiation. In general, we have observed very little difference in the cellular morphology between the coated and uncoated dishes in the primary culture of meningeomas. The cultures on ECM reach confluency at an earlier time point and can be expanded more rapidly which may be advantageous for some applications such as karyotyping, reducing the concerns about additional tissue-culture artifacts. Second, bECM allows the rapid reduction of serum concentrations and helps with serum free conditions and therefore facilitates experiments evaluating growth factor requirements for tumor cells.

14. Gliomas have to be divided into differentiated, low-grade and anaplastic, and high-grade lesions. In addition primary tumors are to be distinguished from recurrent lesions that arise after irradiation or other treatments. The anaplastic lesions, are often very heterogeneous with a potential to generate numerous different cell lines from one tumor *(26)*. This heterogeneity is the prime basis for the desperate therapeutic situation in the management of these tumors. The understanding of this heterogeneity and the different growth control mechanisms

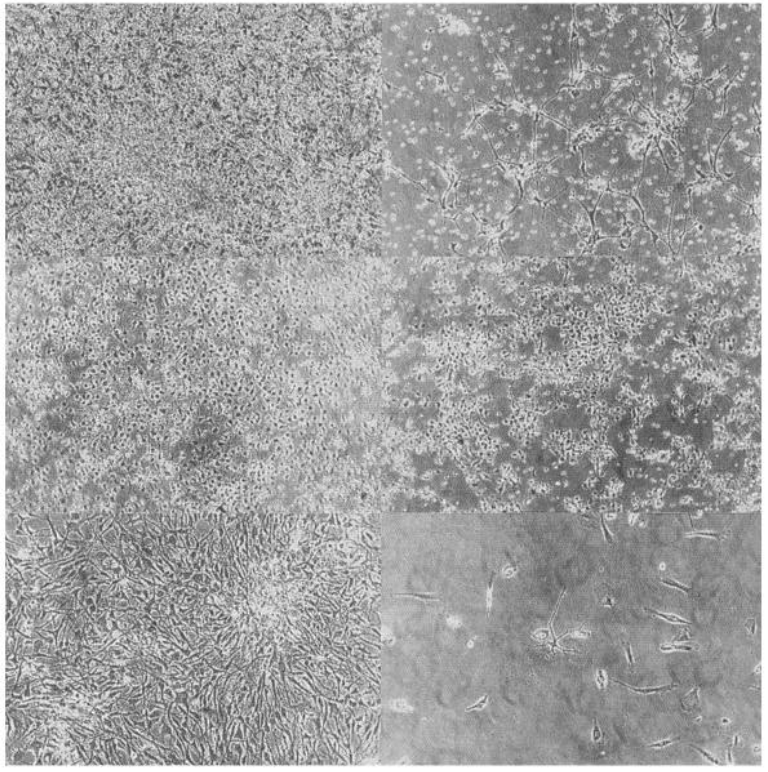

Fig. 8. Three different primary cultures which have been derived from different gliomas and were plated on ECM (left) are compared with the cultures on plastic (right). The first specimen was obtained from a fibrillary astrocytoma (WHO grade 2, top). The second specimen is derived from a differentiated oligodendroglioma (WHO grade 2) and the final specimen was taken from a glioblastoma (WHO grade 4).

simultaneously present in one glioma will be pivotal to the development of effective therapies. The use of ECM as an attachment substrate helps to maintain the heterogeneity of these tumors in early cultures.

15. Primary cultures: In contrast to meningeomas, the behavior of primary glioma cultures on plastic and bECM is difficult to predict. In general, the initial attachment allows a speculation about the biological aggressiveness of the tumor because the well-differentiated gliomas show only poor attachment to plastic and adhere well to ECM, both in medium containing serum and serum-free cultures to which conditioned serum-free medium from an established glioma cell line has been added. Also cultures from anaplastic tumors adhere better to ECM than to untreated culture dishes (Fig. 8). This phenomeneon is the result of the expres-

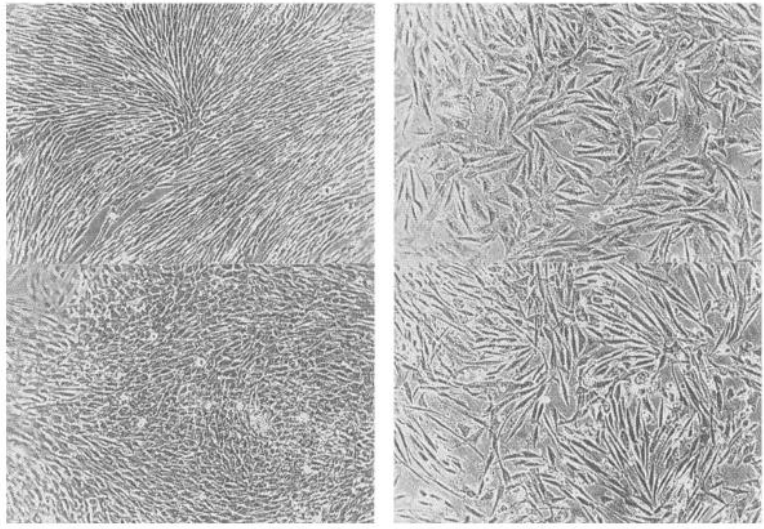

Fig. 9. Phase-contrast photomicrographs from two cell lines stablished from the same initial specimen obtained from a glioblastoma. The cell line depicted at the left (NCE G195) is shown in passage 26 in 10% FCS (top left) and in passage 11 on ECM maintained in serum-free medium (bottom left). The cell line shown on the right (NCE G168) is shown in passage 22 in serum-containing medium on plastic and in passage 11 in continuous serum-free culture on ECM (top and bottom, respectively). NCE-G195 shows a very different morphology in both conditions whereas the differences for NCE-G168 are rather gradual.

sion of integrins on the surface of malignant glioma cells which interact with many of the components contained in ECM *(27,28)*.

The major applications for efficient primary culture, are in vitro chemoresistance testing in experimental oncology, cytogenetics and the analysis of differentiation events by immunocytology. In all instances, the major advantage of the ECM is the complexity of the culture composition which will be obtained. Thus, cellular responses influenced by the interactions of several components will be more adequately reflected in such cultures.

16. Established cell lines: The value of ECM in established cell lines is limited to two complexes: serum-free culture and analysis of migration. Research into cellular differentiation related to neurodevelopmental aspects has as its goal to fully define the experimental conditions and that excludes the use of serum in the culture medium. Such serum-free cultures require an adhesive substrate during each passage because the trypsinized cells will not readily attach without serum, stay afloat and die. It has to be conceded though, that the use of ECM as an attachment substrate is also not really defined, although somewhat less complex than serum. We were able to permanently establish cells in serum-free conditions that

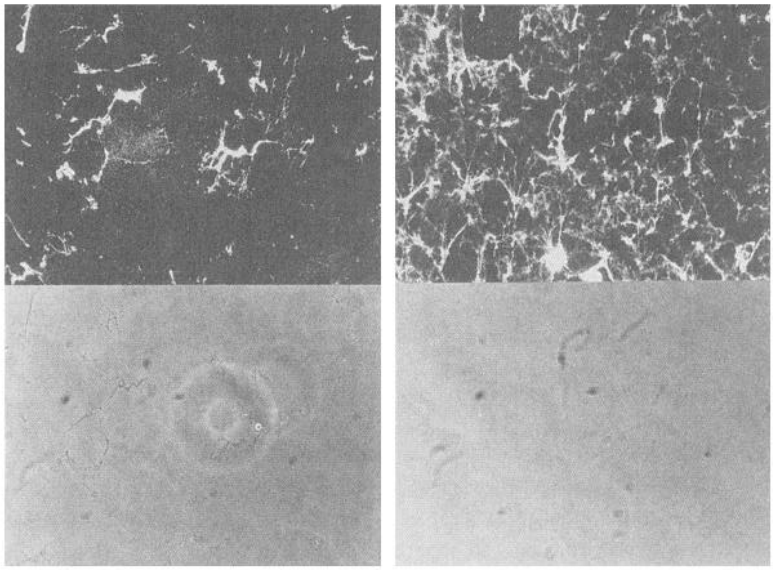

Fig. 10. Immunostaining of glass cover slides covered with bECM (right) and tECM from NCE-G 44, glioblastoma (left). The corresponding phase contrast photomicrographs (top) show only barely visible fine fibrous structures. Below, the bECM is stained with an antiserum against tenascin, the tECM is stained with antifibronectin

resulted in morphologies different from the cultures maintained in serum-containing medium (Fig. 9). The cells maintained in serum-free medium will have reached at most half the passage numbers of the parallel line maintained in the presence of serum. This readily illustrates, that the ECM in itself has a selective effect.

17. Application of tECM: The biological effects of ECM constitute an interesting field of research. In the context of gliomas, the anaylsis of cell migration in its causal relationship with the ECM is of profound clinical importance, as most glioma patients will eventually die from an unresectable recurrence of their lesion that has arisen far away from the original site due to the migratory activity of the tumor cells. Most normal cells as well as tumor cells in culture produce an ECM although it may be invisible to the naked eye and has to be visualized by means of immunofluorescence (Fig. 10). The influence of single, purified ECM components such as fibronectin or tenascin has already been shown to differentially affect cell migration *(27,28)*. This migration is somewhat slower on tumor produced ECMs (Fig. 11) but tECM is still a supportive substrate. Comparisons between tECM and purified components can only be made within certain limits as the immunostainings of tECM already indicate, that there is a complex three dimensional arrangement of ECM deposits with highly uneven distribution. The

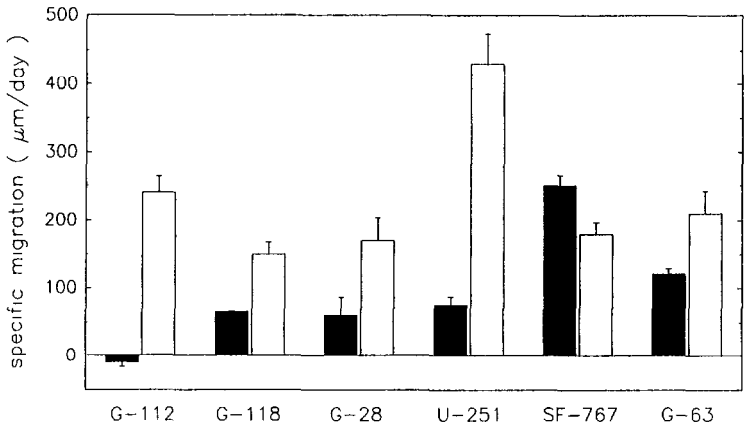

Fig. 11. Migration of six different established human glioblastoma cell lines as tested in a standardized migration assay The maximal migratory response to any of four different purified substrates (□) is compared to cell migration on autologous tECM in quadruplicate cultures. ■, migration on autologous tumor-derived ECM, □, migration on purified ECM proteins

role of such naturally produced EMCs may play a role in developmental biology and neurotransplatation, where they may have a role in the migration of precursor cells and astrocytes in the CNS in regional differentiation of cells to be grafted for therapeutic purposes *(29)*

18. Pituitary adenomas: Usually, these tumors are soft and can be dispersed mechanically. Unfortunately, the cells from these inevitably benign lesions (with very few exceptions) show almost no proliferation and therefore are prone to fibroblast overgrowth after 3–6 wk in culture. Adenomas are usually easily dispersed without the need for enzymes and the cells attach within a few hours Experiments studying the pharmacological regulation of hormone secretion can be started the next day after unattached cells, blood and debris are washed off The test substances can be added and the medium can be completely removed to be assayed without the cells coming off because they are firmly attached *Note the exception.* ACTH-secreting adenomas from patients with Cushing's disease and long-standing cortisol excess give rise to cells that remain round, attach much slower and sometimes need two days to flatten. The time it takes for these cells to attach is correlated to the degree of cortisol excess in vivo before surgery *(10)* In addition to the much improved attachment over plastic, the most striking effects of bECM are the rapid and obvious changes in cellular morphology after exposure to hypothalamic releasing factors, such as GRF for HGH-secreting cells and, CRF or vasopressin for ACTH-secreting cells *(10,11)* The facilitation of cell attachment is seen with all types of pituitary adenomas, indifferent whether they are hormonally active or inactive (Fig. 12).

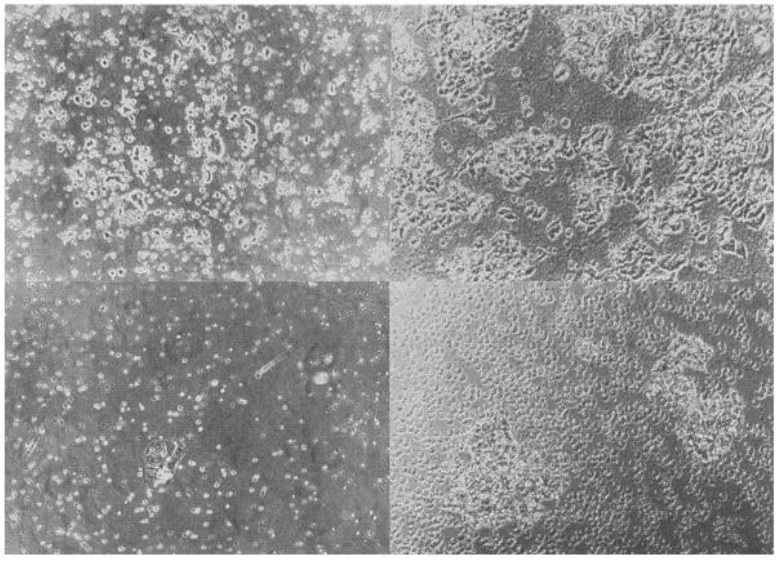

Fig. 12. Cell cultures from two different human pituitary adenoma specimens are compared on ECM and plastic, showing that after 24 h many single cells have attached on ECM whereas only very few cells stay attached on plastic (left). This phenomenon allows experimentation with hormonally active cells one day after explantation with ECM whereas without experimentation will not be possible for several days and even then, there will be less cells.

19. Acoustic neuromas as well as other benign schwannomas are difficult to culture even on ECM. If they are soft and cellular, they also attach well to untreated culture dishes. The same holds true for malignant schwannomas to which ECM does not appear to offer any advantage.
20. Pineal tumors are derived from a very heterogeneous group of tissue sources. Those that belong to the the glial cell lineage behave like the gliomas. Other derivatives such as germinomas or pineocytomas or pineoblastomas attachment well to bECM in comparison to plastic. In general these tumors behave much like pituitary adenomas, as without any specific additives, they do not appear to proliferate.
21. Medulloblastomas: This particular type of tumor is very difficult to maintain in culture, and bECM has not provided a major breakthrough. In addition to providing an attachment substrate, the addition of specific additives or conditioned media seems to be the crucial point in successful culture of this type of early glial tumor (numerous personal communications).
22. Neurocytoma: Neurocytomas are rare neuroglial precursor tumors located at the foramen of Monroi in the lateral ventricles. They are very interesting from the

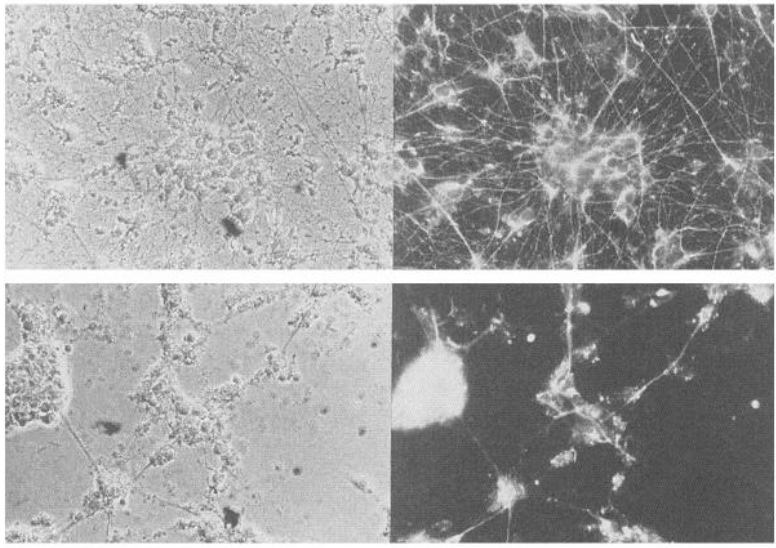

Fig. 13. In this particular case of a human neurocytoma culture, the cell differentiation was studied in relation to incubation conditions and substrates. The cells on plastic (below) show a much weaker attachment and no expression of their morphological characteristics, although the expression of GFAP as seen in the immunostainings which correspond to the phase contrast photomicrographs.

developmental aspect and because of the necessity to study the immunocytochemical markers, they cannot be studied without an attachment substrate *(30,31)*. In comparison with cells which were maintained on LabTec chamber slides which are said to allow optimal culture of cells designated to be immunostained, the attachment after 5 d is much superior on ECM and in comparison to the unshaped cells on plastic, the cells have a very distinct morphology (Fig. 13).

4.3. Final Comments

23. Corneal endothelial cells can be frozen and stored in liquid nitrogen like any other cell type. They have been cultured for many in vitro passages and continue to produce matrix. It has to be noted, however, that the bECM gets thinner and less homogeneous with increasing age of the corneal endothelial cells, probably because the cells take longer to reach confluency. ECM can be stored at 4°C for up to 2 yr. It can also be dried from distilled water to avoid the formation of salt crystals.
24. Composition of ECMs: ECMs are different in their chemical composition, depending on the tissue they are derived from. This relates to the matrix proteins as well as to growth factors which have been shown to be sequestered into the

ECM by the producer cells. The presence of all heparin binding growth factors has to be postulated and has to be reckoned with when the regulation of cell proliferation is to be investigated *(14,15,32)*. The ECM serves as a kind of slow release reservoir for these factors that can act as intrinsic mitogens Also binding studies on ECM may become difficult to interpret for that reason as it has recently been shown, that a basal lamina proteoglycan, perlecan, can promote growth factor binding *(33)*

Acknowledgments

The authors are grateful to D. Gospodarowicz in whose laboratory the introduction to ECM biology and the methodological training was obtained. We express our thanks to the departmental operating room staff for their collaboration and appreciate the photographical work of S. Freist. The karyotype analyses were performed by M. Haensel. Throughout the times the work in the Laboratory for Brain Tumor Biology was alternately supported by the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe, and the Heinrich-Bauer Stiftung für Hirntumorforschung. The continuous support of the department chairman, Hans-Dietrich Herrmann is vital to the ongoing work in the laboratory.

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16

Culturing Primitive Hemopoietic Cells

Long-Term Mouse Marrow Cultures and the Establishment of Factor-Dependent (FDCP-Mix) Hemopoietic Cell Lines

Elaine Spooncer and T. Michael Dexter

1. Introduction

The maintenance of normal primitive hemopoietic cell types in culture for long periods can be achieved either by culture of hemopoietic cells in association with bone marrow-derived stromal cells, as in long-term cultures, or by establishing factor-dependent (FDCP-Mix) primitive hemopoietic cell lines. The long-term culture technique and a reproducible method for establishing FDCP-Mix cell lines are described here *(1,2)*.

In the stromal cell-dependent long-term cultures, a wide range of hemopoietic cell types are maintained for several months, including the stem cells (as recognized by the CFU-S assay *(3)* and the ability of the cells to rescue potentially lethally irradiated mice), mature cell types of the myeloid lineage, and all the committed progenitor cell types. Cloned factor-dependent cell lines can be derived from long-term cultures and the method described here produces cells that maintain a homogeneous primitive nature under certain culture conditions and yet can be induced to mature in response to normal regulatory influences. Clearly, long-term in vitro growth of primitive hemopoietic cells is valuable for studying the maintenance of homeostasis in the hemopoietic system in which both stromal cells and hemopoietic growth factors (e.g., interleukin 3 [IL-3]) are thought to play important roles. These cultures are also useful for investigating the biochemistry of the response of hemopoietic cells to biological regulators, the molecular biology of the nature of stem cells and for testing the response of hemopoietic cells to drugs, chemicals, and other insults.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

2. Materials

1. Fischer's medium supplemented with 1.32 g/L sodium bicarbonate, 500 μ/mL penicillin, 50 μg/mL streptomycin, and 7 mL/L of 200 m*M* L-glutamine. Store at 4°C and use within 2 wk after the addition of the L-glutamine.
2. Horse serum each batch must be pretested for its quality to support long-term bone marrow cultures. We have used batches from Northumbria Biologicals (Northumberland, UK) and ICN Pharmaceuticals (Thames, Oxfordshire, UK) Store at –20°C until use and then keep the aliquot in use at 4°C for up to 10 d
3. Hydrocortisone sodium succinate Make a stock solution in Fischer's medium and sterilize through an 0 22-μm filter For use dilute to $10^{-4}M$ and store at –20°C
4. Complete growth medium made up in 100 mL aliquots containing 20–25% (v/v) horse serum (depending on batch quality) and 1% (v/v) $10^{-4}M$ hydrocortisone stock (final concentration $10^{-6}M$) in Fischer's medium
5. Donor mice, 8 wk or older

2.1. Additional Materials for Establishing Clonal, FDCP-Mix Cell Lines

1. NIH-3T3 producer cell line for the 2-1 recombinant src virus grown as a Moloney murine leukemia virus pseudotype *(4)*, *src* (MoMuLV) producer cells The *src* (MoMuLV) has been categorized in this institute to be used under the good microbiological practice Code of Practice by the local Advisory Committee on Genetic Manipulation
2. Dulbecco's modified of Eagle's medium supplemented with 10% (v/v) newborn calf serum (DMEM 10% NBCS). culture medium for *src* (MoMuLV) producer cells
3. Radiation source to irradiate producer cell cultures at a dose to kill all producer cells (for example, 20 Gy delivered at 84 Gy/min by linear accelerator)
4. Source of IL-3· filtered, conditioned medium (CM) from Wehi-3b-leukemic cell line established at the Walter and Eliza Hall Institute, cultures chemically purified IL-3 from Wehi-3b CM, or recombinant IL-3
5. Semi-solid media for plating cells in the granulocyte–macrophage colony-forming cell type assay, e g., Noble Agar final concentration 0.3% or methyl cellulose. For more details about working with semi-solid media (*see* Chapters 20 and 21)

3. Methods

3.1. Long-Term Mouse Bone Marrow Cultures (see Note 1)

1. Assemble the tissue-culture equipment and reagents in a microbiological safety cabinet. Prepare the complete growth medium, 10 mL/long-term culture, in 100-mL aliquots.
2. Kill donor mice by cervical dislocation. One femur (approx 1 5 –2 × 10^7 nucleated cells) is used for each long-term culture. Remove both femora from each donor, removing as much muscle tissue as possible and cutting first below the knee joint and then at the hip joint. Place the bones in a Petri dish containing a

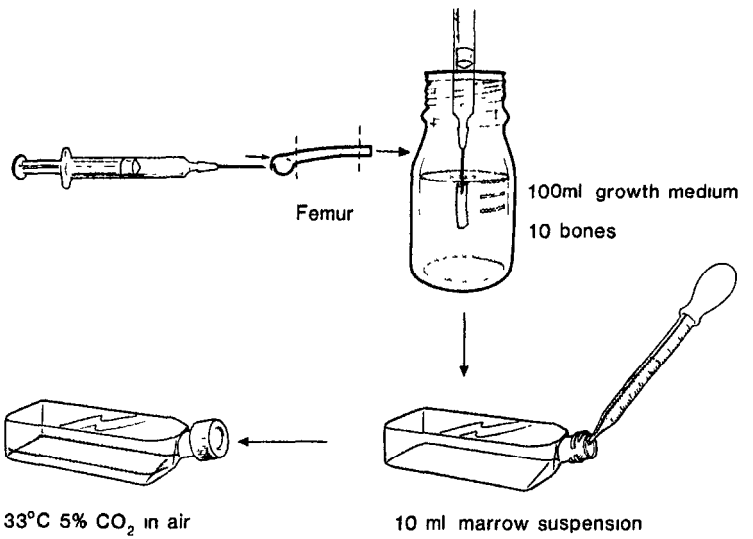

Fig. 1. Preparation of long-term bone marrow culture

small volume of Fischer's medium on ice. If mice are in short supply, then tibiae can also be removed. Use two tibiae to make one long-term culture The time between killing donor mice and flushing the cells into growth medium should not exceed 15–20 min Therefore, the speed at which you can work dictates how many donor mice to kill at once A competent operator should be able to work in batches of five mice (i.e., 10 cultures).

3 Take the femora to the microbiological safety cabinet Using sterile gauze swabs, clean off any remaining muscle tissue from each bone. Carefully cut off the ends of the femora and avoid splintering the bone (Fig. 1). Insert the tip of a 21-gage needle and 1-mL syringe into the knee end of the bone For an average sized mouse (approx 25 g) the needle tip should fit firmly into the bone cavity If the donor mice are very large or small, a slightly larger or smaller needle may be required to give a good fit. Hold the syringe and needle so the bone is just below the level of the medium and flush medium through the bone 5–10 times until the bone shaft is empty of cells and looks white Repeat with nine more bones into the same 100-mL aliquot of medium (*see* Note 2). It is not necessary to make a single cell suspension, but do ensure that whole marrow plugs are broken up into fragments. Dispense 10-mL aliquots of the marrow cell suspension into 10 tissue-culture flasks Connect a plugged sterile pipet to the 5% CO_2 in air supply line and turn on the gas at a low pressure. Gas the cultures by inserting the tip of the pipet into the air space of the flask for a few seconds, cap the flask firmly, and place the cultures horizontally in a 33°C incubator (preferably nonhumidified).

4. The cultures are routinely fed at weekly intervals by removing half the growth medium (5 mL) and replacing it with fresh medium. Gently agitate the flasks to

suspend nonadherent cells uniformly in the growth medium and carefully remove 5 mL, taking care not to disturb the adherent cells with the tip of the pipet. Tip the flask sideways and add 5 mL fresh medium down the side of the flask so it does not flow directly onto the adherent cells. Gas the flask gently and replace the top The feeding routine can be altered if required and the cultures will tolerate a complete media change weekly or more frequent feeding. Cells harvested during feeding comprise CFU-S, progenitor cells, and mature cells

5 During the first 1–3 wk of culture, a highly organized adherent layer develops on the base of the flask containing stromal cells of bone marrow origin The establishment of the adherent layer is essential to the subsequent onset of hemopoietic activity The development of the adherent layer can be observed using inverted phase-contrast or bright field microscopy The major cell types in this cellular environment are macrophages, fat cells, and the large, well spread "blanket cells" that can only be easily observed in the electron microscope. Within the adherent layer, foci of hemopoietic cells develop and grow (Fig 2A) These are known as "cobblestone" areas and from these foci hemopoietic cells are released into the growth medium Hemopoietic cells retained within the adherent layer are the most primitive population in the culture They include a high concentration of CFU-S and these CFU-S have higher self-renewal capacity than that of the CFU-S released into the growth medium The cells released into the growth medium include the bulk of the mature granulocytes, GM-CFC and CFU-S, with lower self-renewal capacity (as measured by serial transplantation) Between 3–4 wk after establishing a long-term culture, it will have achieved a relatively steady-state of hemopoietic cell production This level of hemopoietic activity should persist for at least another 8 wk and often longer. Signs that the hemopoietic activity is declining are a predominance of macrophages in the nonadherent cell population and a decline of hemopoietic cell foci in the adherent layer. Table 1 describes some of the characteristics of active long-term cultures and of nonhemopoietic long-term cultures

3.2. Establishing Cloned FDCP-Mix Cell Lines

1. Establish long-term bone marrow cultures. When active hemopoiesis is established (after 4–6 wk) infect the cultures with the *src* (MoMuLV) as described in steps 3–6 *(5)*
2. The NIH-3T3 *src* (MoMuLV) producer cells are cultured in DMEM containing 10% NBCS at 37°C in 5% CO_2 in air. Subculture twice weekly at a dilution of approx 1 3 using 0 25% trypsin to detach the cells, according to standard procedure for the culture of adherent cell lines
3 The day before infecting long-term cultures with *src* (MoMuLV), feed the long-term cultures by removing 5 mL of growth medium and replacing with 5 mL fresh medium.
4 Prepare NIH-3T3 *src* (MoMuLV) cultures so they will be subconfluent on the day of infection The day before infection remove all the growth medium and replace with half the usual volume of fresh growth medium.

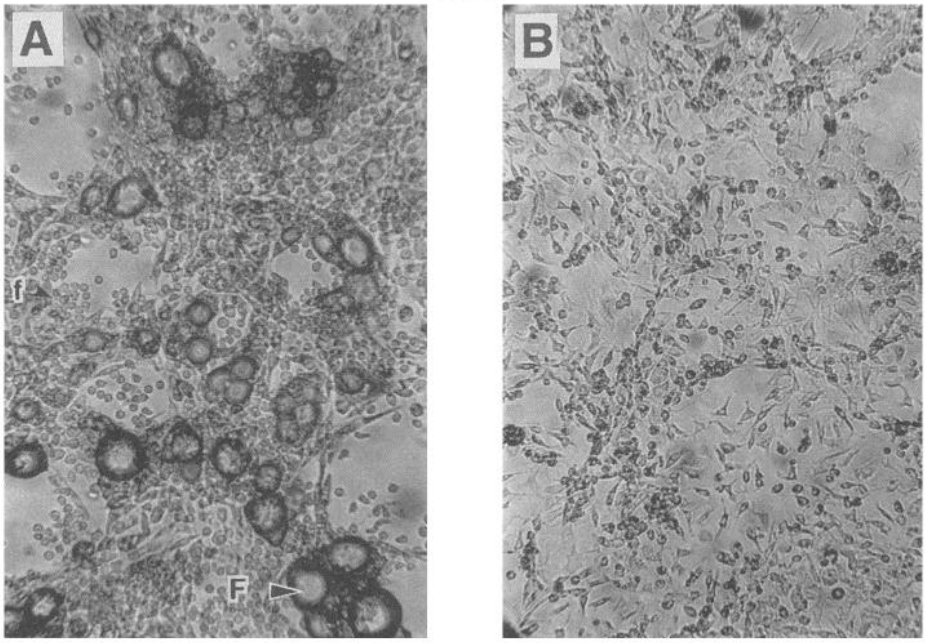

Fig. 2. (A) Inverted phase-contrast micrograph of hemopoietic long-term culture: "f" arrows, a small focus of hemopoietic cells; "F" arrows an area of fat cells. Note the heterogeneous arrangement of the adherent cell layer and the association of the hemopoietic cells (small round cells) with the fat cells and underlying stromal cells. **(B)** Inverted phase-contrast micrograph of long-term culture 15 wk after infection with *src* (MoMuLV). There is an absence of fat cells and extremely reduced numbers of hemopoietic cells. The highly organized structure of the adherent layer is lost and the predominant cell type is a macrophage, many of which have abnormal morphology.

5. On the day of infection irradiate the producer cell cultures with a dose of irradiation (for example, 20 Gy delivered at 84 Gy/min) sufficient to kill all the cells. Harvest the growth medium from the cultures and centrifuge at 1000–1500*g* on a bench-top centrifuge for 5 min. Remove all growth medium from the long-term cultures and add 2–4 mL of the supernatant growth medium from the irradiated producer cells. This should contain 2×10^4 to 2×10^5 focus-forming units of sarcoma virus when titered on Rat-1 fibroblasts *(6)*. The *src* (MoMuLV) is labile so the supernatant growth medium should be added to the long-term cultures within 10 min of harvesting the medium from the producer cells. Add 2–4 mL DMEM 10% NBCS to control long-term cultures.
6. At 4–6 h later, make the volume of media in the long-term cultures to 10 mL with complete long-term culture growth medium.

Table 1
Characteristics of Hemopoietic Long-Term Cultures[a]

Total cell production	Progenitor cell type	Progenitor cell production (nonadherent cells)			Differential morphology of nonadherent cells				
		Per 10^5 cells	Per culture/wk		Culture	Blast cells	granulocytes	Early granulocytes	Late Macrophages
Nonadherent cells/culture/wk $3–10 \times 10^6$ ($<10^6$)	CFU-S	15–30	450–3000	(<200)					
Adherent cells/culture $3–6 \times 10^6$	GM-CFC	150–300	4500–30,000	(<2000)	Active culture	10–20	10–20	55–75	<5
					Nonhemopoietic culture	<5	<5	20–40	50–70

[a]This table shows some examples for cell production in active long-term cultures The data shown are approximate ranges to illustrate the extent of hemopoietic activity that should be attained in long-term cultures Data in parentheses describe the characteristics of nonhemopoietic cultures that may be observed when cultures are very old or where culture conditions are not adequate to promote hemopoietic activity.

7 Feed the long-term cultures weekly by removing 5 mL and replacing 5 mL fresh medium Assess the cultures weekly for signs of viral transformation *(5)*, which is indicated by the following events
   a. A fall in the nonadherent cell count
   b A rise in the concentration of CFU-S and GM-CFC
   c. An increase in the self-renewal capacity of CFU-S (measured by serial transplantation in vivo) and GM-CFC (measured by replating in semi-solid media)
   d. Changes in the adherent cell morphology, including decrease in the number of hemopoietic foci and cobblestone areas, decrease in fat cells, loss of the cellular organization, and appearance of "transformed" stromal cells (Fig 2B)

   The changes in hemopoietic activity may first be observed 5–10 wk after infecting the cultures with *src* (MoMuLV). At this stage, factor-dependent cell lines may be derived from the cultures

8. Two methods have been used to establish FDCP–Mix cell lines from *src* (MoMuLV) infected long-term cultures.
   a Method A:
      i. Harvest nonadherent cells from src (MoMuLV) infected cultures and plate them in semi-solid medium with Fischer's medium and horse serum (10% [v/v]) Supplement the medium with IL-3 at a concentration that will promote optimal development of GM-CFC colonies from normal bone marrow in semi-solid media The number of cells plated should be such that when colonies have developed on the plate they are well spaced and individual clones can easily be harvested
      ii. Incubate the plates at 37°C in 5% CO_2 in air (humidified) for 7–10 d At this stage inspect the plates using, for example, an Olympus zoom stereomicroscope, and pick out individual colonies from the semi-solid medium with a sterile Pasteur pipet
      iii Disperse single colonies in a 2 mL vol of Fischer's medium, horse serum 10% (v/v), and IL-3 (FHS/IL-3) in 24-well cluster plates Inspect the individual clones daily using an inverted microscope Until the cells begin to proliferate, feed the cultures every 3–4 d by gently removing 1.5 mL FHS/IL-3 and replacing it with fresh medium The cells lie at the bottom of the well and should not be disturbed during a medium change
      iv. When the cells begin to proliferate, monitor the cell concentration and gradually expand the culture volume, maintaining the cell count at between 10^5/mL and 5×10^5/mL Not every clone will survive, but in a successful experiment (*see* Note 3) >80% of the colonies will establish a cell line
      v. When the clones are established and growing rapidly, freeze some in liquid nitrogen (using standard procedures, *see* Chapter 1) and determine the cell line characteristics
   b. Method B
      i. Harvest nonadherent cells from the long-term cultures. Wash the cells and suspend them in FHS/IL-3 at approx 2×10^5 cells/mL

11 Observe the cultures for growth of cells and when the cells are growing well, clone cell lines by plating in semi-solid medium with FHS/IL-3 exactly as described in Method A

9 The cloned cell lines die in the absence of IL-3 In liquid culture in FHS/IL-3 they have a doubling time of 15–24 h The log phase of growth occurs between about 8×10^4 to 8×10^5 cells/mL The plating efficiency of the cells in semi-solid medium with FHS/IL-3 is between 5–25% The plating efficiency tends to increase as the cell lines age. Cells may not continue to grow if they are diluted below 5×10^4 cells/mL in liquid medium and they begin to die when the cell density is above 10^6 cells/mL. Maintain the cell lines in active growth by subculturing to approx 6×10^4 cells/mL twice weekly If the cells are subcultured only once a week and are therefore in the plateau phase of growth for long periods, the plating efficiency (CFC content) of the cell population declines and the incidence of macrophages increases. Culturing cells in fetal calf serum causes a decline in the self-renewing cells and leads to extinction of the cell lines

10 The FDCP-Mix cell lines have the following characteristics *(2)*, many of which are in common with hemopoietic stem cells (*see* Note 3)

a. Survive and self-renew in response to IL-3 (some cell lines may also respond to granulocyte–macrophage colony-stimulating factor)
b Survive and undergo myeloid differentiation in response to an inductive hemopoietic environment in vitro (e g , irradiated long-term culture adherent layer) in the absence of added IL-3
c The cells can be induced to differentiate into mature macrophages, neutrophils, and erythrocytes in the presence of fetal calf serum and erythropoietin in semi-solid medium (the CFC-mix assay). Occasional megakaryocytes, eosinophils, and mast cells are also seen
d The cells are not infected with the *src* (MoMuLV) However, they do produce the ecotropic MoMuLV (helper virus)
e. The cells are nonleukemic.
f Early isolates of the cell lines (within 3 mo of establishment) can form spleen colonies in potentially lethally irradiated mice. The ability to grow in vivo is lost as the cell lines age The early isolates also establish long-term hemopoiesis in the presence of an inductive hemopoietic environment (i e , self-renewal of the cells as well as differentiation is maintained for many months).

11 Figure 3 shows the morphology of FDCP-Mix cell lines grown in liquid culture with FHS/IL-3. The majority of the cells have a primitive appearance and contain many basophilic granules The occasional mature macrophage may be observed (<3%).

4. Notes

4.1. Long-Term Bone Marrow Cultures

Establishing long-term cultures is technically straightforward, but achieving good growth in the cultures can be very difficult for many reasons (some of which are not defined!) Possible problems are listed

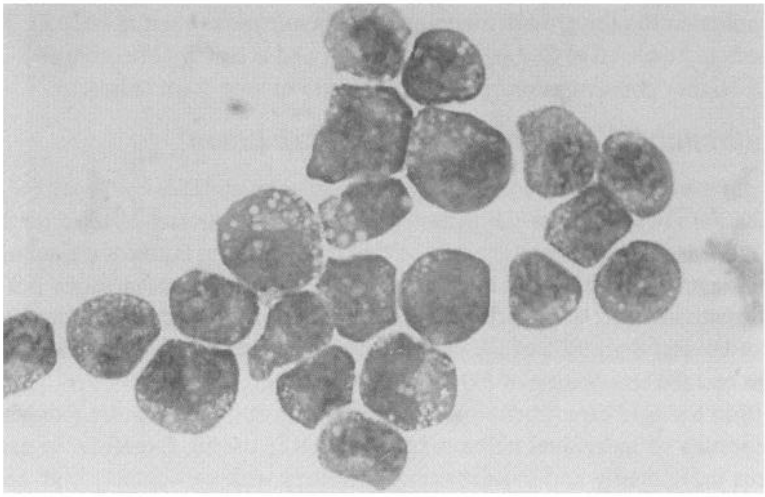

Fig. 3. Typical morphology of FDCP-Mix cells grown in liquid culture with FHS/IL-3.

1. The most likely problem is that the batch of horse serum is suboptimal. The best solution to this is to obtain a sample of horse serum from a laboratory that has established the technique and is willing to provide about 100 mL of their serum. Compare samples against the "good" batch by establishing cultures and measuring cell production, CFU-S, and GM-CFC for at least 6 wk.
2. Some strains of mice do not generate hemopoietically active cultures for a long duration. For example, C3H, CBA mice, and C57B1/6 mice *(7)*.
3. Growing cultures with loose caps in a humidified gassing incubator leads to a high level of fungal contamination because of the accumulation of spore-carrying moisture around the neck of the flask. These incubators are best for short-term cultures. If contamination of cultures is a problem, then the cultures can be established individually by flushing a single femur into 10 mL of growth medium in the flask, instead of by pooling 10 femurs into 100 mL of medium.
4. If the adherent layer is not well established within the first 2–3 wk of culture, hemopoietic activity will not occur. These cultures may sometimes be rescued by giving another inoculum of 1–2 × 10^7 fresh bone marrow cells at 3–4 wk.
5. Rough handling and frequent disturbance of cultures can inhibit the development of the adherent layer or even cause detachment of adherent cells. Handle the cultures gently and resist the temptation to inspect them on the microscope frequently during the first 3 wk.

4.2. Modifications to the Standard Culture Method

6. The technique can be scaled up if large numbers of cells are required e.g., flush three femora into 30 mL growth medium for culture in a T75 flask (75 cm^2 surface area).

7. Terminal erythroid differentiation in long-term cultures can be achieved by supplementing the growth medium with anemic mouse serum (AMS). The AMS needs to be tested to find an effective batch and is usually effective at 1–2% (v/v) *(8)*. Higher concentrations of AMS are toxic to long-term cultures.

4.3. Establishing Cloned, FDCP-Mix Cell Lines

8. In our experience the transformation of long-term cultures by the src (MoMuLV) does not always follow the pattern described in Section 3 2 7. We do not know the reasons for this, but they may be that the long-term cultures are suboptimal at the stage of infection or that the producer cell supernatant does not provide adequate infectious virus. In 12 separate long-term culture infections performed over the last 4 yr, 50% of the experiments have led to hematological transformation and the emergence of FDCP-Mix cell lines
9. Within a single experiment infecting long-term cultures with src (MoMuLV) the responses of individual cultures may vary. It is useful, therefore, to assess cultures individually and to select single cultures with particularly high concentrations of CFU-S and CFC from which to derive FDCP-Mix cell lines
10 When establishing FDCP-Mix cell lines by plating nonadherent cells from src (MoMuLV)-infected cultures in semi-solid medium we have, in some experiments, serially replated the colonies in semi-solid medium two to three times before selecting clones for expansion in liquid culture This procedure may enhance the concentration of CFC with high self-renewal capacity, which can establish FDCP-Mix cell lines, compared with the selection of clones from primary semi-solid media cultures

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Collagen Gel Culture of the Human Hematopoietic Progenitors CFU-GM, CFU-E, and BFU-E

Annie Allegraud, Irène Dobo, and Vincent Praloran

1. Introduction

The first evidence of clonal growth and differentiation of hematopoietic cells was obtained by Bradley and Metcalf in 1966 *(1)* by using an agar semi solid culture system. These progenitor cells, initially called "Colony Forming Units in Culture" (CFU-C) developed into colonies after several days of culture in the presence of different types of conditioned media containing "hematopoietic growth factors." They are found in bone marrow, blood, fetal liver, and spleen of most mammalian species.

The development of other semi solid culture systems, and the progressive discovery of specific hematopoietic growth factors showed that the population of CFU-C was heterogeneous, containing progenitors giving rise, after variable times of culture, to colonies of mature cells constituted by one or different myeloid lineages. The demonstration of a hierarchical distribution of progenitors, the purification of numerous growth factors regulating their proliferation and differentiation, and the results of other in vitro and in vivo experiments *(2)* led to a schematic three compartments model of hematopoiesis with:

1. A stem cell compartment, constituted by cells with extensive self-renewal and commitment potential
2. A progenitor cell compartment, heterogeneous and hierarchically distributed from multipotential to unipotential progenitors The differentiation of cells in this compartment is associated with a progressive loss of proliferative potential and is dependent on the presence of specific growth factors.
3. A compartment of maturing and mature cells, composed of morphologically identifiable cells restricted to one lineage, endowed with very limited proliferative potential, quickly acquiring the phenotypic and functional properties of circulating mature cells.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

Recently, it has been shown that pluripotent stem cells and part of the progenitors population can be phenotypically identified by the presence at their cell surface of a specific antigen classified as CD 34, which is recognized by specific monoclonal antibodies

The collagen gel technique described in this chapter, developed by Lanotte et al. *(3)* for the culture of mouse progenitors, was subsequently used for the culture of human colony forming units granulo-macrophagic (CFU-GM), colony forming units erythroid (CFU-E), burst forming units erythroid (BFU-E), and colony forming units granulocytic-erythroid-megacaryocytic-macrophagic (CFU-GEMM) progenitors *(3–6)*. These progenitors can be grown either from mononuclear cells or CD 34^+ cells with the addition of appropriate growth factors. It allows the direct counting of colonies in the culture dishes, as well as their in situ staining or immunocytochemical analysis after harvesting and treatment of the gels on glass slides. It is useful for:

1 Determining the frequencies of these types of progenitors in bone marrow or peripheral blood harvested for autografting procedures,
2. Screening and measuring the in vitro and in vivo effects of various substances (pharmacological agents or cytokines) on the development of these progenitor cells,
3 Identifying precisely the type, size, and maturation of the colonies developed by their morphological aspect after staining and/or cytochemistry; and
4. Analyzing membrane or cytoplasmic antigens with specific antibodies

2. Materials

1. Collagen: Human (placental extracts) or rat (tail) type I collagen are perfectly suitable. Bovine collagen (derma) must be avoided because it gives generally poor results due to its extraction procedure. Extraction from rat tails has been published in details *(3)*. It is not described here, because it is relatively time consuming, not easy, and now not useful since some companies (Institut J Boy, Reims, France; Imedex, Chaponost, France; Hemeris, Sassenage, France) provide rat or human "native aqueous collagen" in a ready-to-use form which gives good results. Some other commercial type I collagens contain rather high concentrations of acetic acid They must be extensively dialyzed under sterile conditions against good quality double-distilled water to eliminate this acetic acid The solution is adjusted to 3 mg/mL with culture quality sterile double-distilled water and stored at –20°C or at 4°C. Hemeris recently commercialized a ready-to-use Collagen culture kit
2. Culture medium· We use Iscove Modified Dulbecco Medium (IMDM)
   a 1X IMDM· liquid medium without supplements and with L-glutamine is stored at –20°C until use When stored at 4°C, the medium is kept for <15 d
   b 2X IMDM: one vial of powder (ready for the preparation of 1 L) is dissolved in 400 mL of sterile culture quality double-distilled water at room tempera-

ture The pH is adjusted to 7.5 with sodium bicarbonate (7 5% [w/v]) and the total volume is completed to 500 mL The medium is then filtered and stored in 100-mL aliquots at –20°C until use. When stored at 4°C, it is kept in sterile conditions for <15 d

3. Fetal calf serum (FCS) (*see* Note 1): Complemented FCS is inactivated by treatment for 30 min at 56°C, centrifuged (4°C, 30 min, 2000*g*), aliquoted in 50-mL sterile tubes and stored at –20°C until use
4. Albumin (*see* Note 2) 50 g of lyophylized fraction V (Gibco-BRL) is added very slowly to 90 mL of sterile double-distilled water in a sterile beaker, and left at 4°C with slow magnetic stirring overnight. Ten grams of deionizing resin (AG 501-X8, Biorad) is added to the beaker for 1 h with slow stirring; the beads are pelleted by a 10 min centrifugation at 4°C, the BSA solution harvested and the procedure repeated a second time. The solution (50% [w/v]) is adjusted to 37% with sterile 10X phosphate-buffered saline (PBS), then diluted with sterile 1X IMDM to a final concentration of 10% (w/v) This solution is filtered through a 0 45-μm filter, frozen at –20°C overnight, defrosted at 37°C, and centrifuged at 2000*g* for 30 min to eliminate agregates. This stock solution is distributed in small vials (1–5 mL) and frozen at –20°C until use The pH of one defrosted vial is adjusted to pH 7 5 with 7 5% sodium bicarbonate. Add the same quantity of sterile bicarbonate to all the other vials before their use in culture
5. 5637 conditioned medium (5637 CM): This adherent bladder carcinoma cell line spontaneously release several cytokines in its culture medium during its proliferation *(7)* Cells are seeded at 1×10^4/mL in IMDM plus 10% FCS and cultured for 7 d in 25 or 75 cm^2 flasks at 37°C, 5% CO_2. The supernatant is then recovered, centrifuged for 10 min at 2000*g*, filtered on a 0 22-μm filter, and stored at –20°C until use Optimal final concentrations in the culture media are usually 5–10%
6. Recombinant cytokines· They are generally purified and can be used at known concentrations, with perfectly controled combinations. All cytokines cited in Table 2 can be purchased from several companies All dilutions will be done in 1X IMDM with 2% bovine serum albumin (BSA) and stored according to the instructions provided by the company.
7. Heparinized vials. Bone marrow or blood are collected in a tube or syringe containing approximately 10 IU/mL of preservative free heparin
8. Ficoll (d = 1 077 g/mL). Several companies provide this ready to use reagent. Store in sterile conditions in dark at 4°C.
9. $CD34^+$ cells separation We use an indirect immunomagnetic two-step procedure according to the detailed information provided by Miltenyi (Bergisch, Gladback, Germany)
10. Antioxidants are mandatory for an optimal growth of colonies 2β mercaptoethanol or α thioglycerol ($1.88 \times 10^{-2}M$) are prepared extemporaneously in 1X IMDM medium and discarded after use
11. A standard water jacket incubator with an automatic regulation of CO_2 and a large water surface to maintain fully saturated humidity is necessary

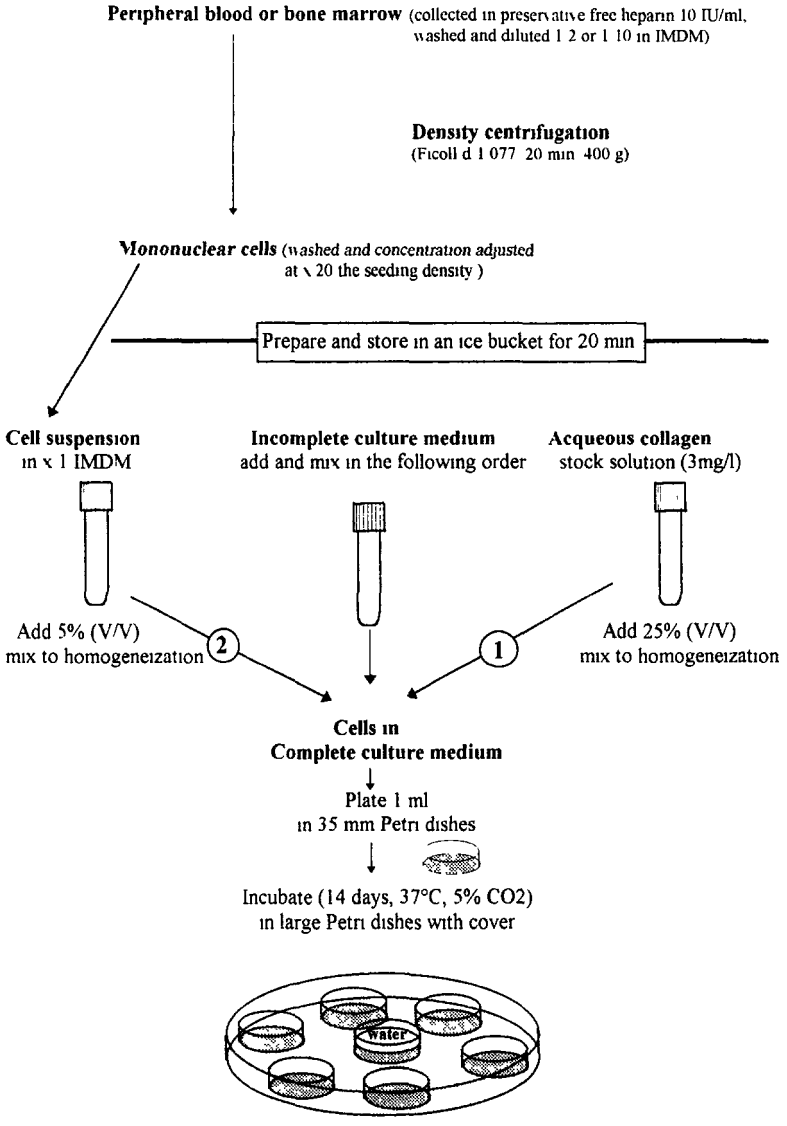

Fig. 1. Scheme for preparing and seeding hematopoietic progenitors in collagen gel matrix.

3. Methods

The procedure is schematically represented in Fig. 1.

3.1. Cell Harvesting and Separation of Mononuclear Cells

1. Blood is harvested in sterile vacum heparinized tubes, centrifuged once at 200g for 10 min to maximally eliminate the platelet rich plasma. The pellet (WBC +

RBC) is resuspended in a double volume of 1X IMDM Six milliliters of cell suspension are carefully layered on 4 mL of Ficoll in a 15-mL sterile round bottomed tube and centrifuged (400*g*, 18°C, 20 min). Mononuclear cells are aspirated from the interface and washed twice with 10 mL of 1X IMDM (400*g*, 10 min) in a 15-mL conical tube The pellet is resuspended in 1–5 mL of IMDM—2% FCS and a cell count performed The cell suspension can then either be seeded in culture or submitted to further enrichment of progenitor cells as described in Section 3 2.

2. One to four milliliters of bone marrow are aspirated from the posterior iliac crest or sternum with a 10 mL disposable plastic syringe This is immediately injected in an heparinized vial and agitated to avoid clotting.The cell suspension is diluted 1.10 with IMDM and processed as described for blood cells.

3.2. Positive Selection of CD34⁺ Cells (see Note 3)

The MiniMacs device (Miltenyi) gives us a final purification of 85–95% of $CD34^+$ cells. For obtaining of good results, it is mandatory to follow precisely the detailed manufacturers instructions.

3.3. Preparation of Collagen Culture Medium

Each experimental condition must be performed in triplicate or even more times depending on the expected colony frequency and of the statistical analysis required (*see* Note 4). Table 1 gives the percentage of each component in the preparation of the final collagen culture medium.

3.4. Serum and Growth Factor Requirements

1. The optimal development of CFU-GM, CFU-E, and BFU-E depends on several parameters· the FCS batch, the FCS concentrations (10–25%), the combinations and concentrations of cytokines used (Table 2), and the degree of purity of cells seeded (mononuclear cells or $CD34^+$ cells)
2. According to the type of progenitors grown and the type of cells cultured (mononuclear cells or $CD34^+$), selected batches of 5637 CM or various combinations of purified recombinant growth factors should be used Because of the presence of large numbers of accessory cells (able to produce cytokines), combination of numerous growth factors are generally not mandatory for an optimal development of colonies. We use recombinant cytokines for experiments needing a strict control of culture conditions (Table 2, Note 5)
3. CFU-GM: in most cases, d 14 colonies are counted. FCS at 10% is generally optimal; 5637 CM (5–10%) allows their growth with results equivalent to combinations of IL3 + GM-CSF + G-CSF or GM-CSF + G-CSF in our hands. If one wants to develop more primitive progenitors giving rise to colonies after 21 or 28 d of culture, addition of earlier cytokines such as Stem Cell Factor (SCF) and/or IL1β or IL6 often improves their growth.

Table 1
Percentages of the Various Components Used for Preparing the Collagen Gel

	2X IMDM	FCS	BSA	Thioglycerol	Growth factors	1X IMDM	Collagen	Cell suspension in IMDM
Percentage of the final volume	25%	10–25%	10%	0 4%	10%	0–15%	25%	5%

Table 2
Type and Concentrations of Cytokines Used for Cultures Needing Strictly Controlled Conditions

	SCF	IL1β	IL6	IL3	GM-CSF	G-GSF	Epo
Range of concentrations used in cultures (ng/mL) except Epo (U/mL)	10–100	1–10	10	5–40	10–20	10	0 5–3

a. CFU-E: Small hemoglobinized colonies (16–100 cells) are observed at d 7 with only the addition of Epo (1 U/ml), in a culture medium containing 10% FCS
b. BFU-E: Present in bone marrow and blood, they generate large hemoglobinized colonies (100 to >5000 cells) after 14 d of culture. FCS concentrations of 20–25 % is generally optimized for their growth (number and size of colonies) In our hands, they grow similarly when stimulated by 5637 CM plus Epo or by combinations of purified cytokines such as IL3 + Epo.

4. Blood or bone marrow mononuclear cells can generate d 14 CFU-GM and BFU-E. Although present at high frequency in bone marrow, d 7 CFU-E are absent from blood

3.5. Collagen Plating Procedure

1. Because of the presence of a high percentage of 2X IMDM in the incomplete culture medium, the collagen solution must be added before the cells. The collagen stock solution and the incomplete culture medium are distributed into individual tubes corresponding to each individual culture condition and these are stored at 4°C for at least 20 min before use. The collagen stock solution is aspirated with a pipet, added to the tube of incomplete culture medium, and pipeted up and down quickly until homogenization is achieved The cell suspension is then added and homogenized by pipeting up and down The mixture is quickly distributed to and swirled to cover 35-mm Petri dishes. These are placed in the incubator Cells are seeded respectively at 2–5 × 10^4 or –5 × 10^5 cells/mL for bone marrow or blood mononuclear cells, and at 5 × 10^2 to 1 × 10^3 for purified $CD34^+$ cells
2. The cultures are incubated at 37°C, 5% CO_2, fully saturated humidity (*see* Note 6) The CO_2 concentration (around 5%) must be adjusted in order to have a pH of 7.2–7 4 in the culture medium.

3.6. Colony Counting and Identification

1. Direct counting is performed under an inverted microscope. In most cases the cellular origin of colonies can be identified by an experienced observer by cell morphology. Erythroid colonies are generally made of one to multiple hemoglobinized (orange-red color) compact cells clusters. CFU-E contain 1 or 2 such clusters of less than 100 cells, mature at d 7. BFU-E contain 3–16 subunits of variable size and level of hemoglobinization (100–5000 cells) after 14 d of culture. Day 14 CFU-GM derived granulomacrophagic colonies appear as white and refringent, with a compact center and dispersed individual cells at the periphery. Granulocytes are smaller than macrophages. Colonies comprise from 100 to several thousands of cells.
2 To identify cells within the colony, the entire gel can be fixed and stained with May Grunwald Giemsa (MGG), allowing counting of colonies and identification

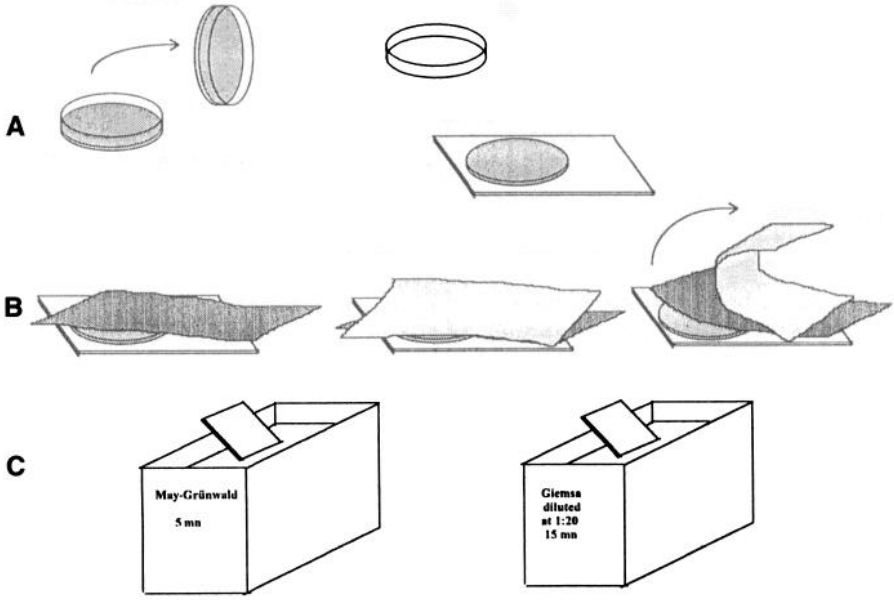

Fig. 2. Schematic representation of the harvesting, drying, and staining of collagen gels. **(A)** Gel are detached at the periphery of the culture dish with a Pasteur pipet and slipped out of the culutre dish onto a glass slide (40 × 70 mm). **(B)** The culture medium is blotted with monofilament nylon HC sifting fabric (37-μm sifting, Polylabo, France) in contact with the gel and a piece (40 × 60 mm) of Whatman 3 mm then placed on top of it for 5 min. The blotting paper and the sifting fabric are removed from the still humid gel by carefully pealing them apart. The gel layer is left to dry for several minutes to hours. **(C)** The collagen layer with colonies inside can then be stained with MGG.

of their cellular composition. For this purpose, the gels are harvested on glass slides (40 × 75 mm) and dried as represented in Fig. 2 before being submitted to the three steps of MGG staining. Other techniques of cytochemistry or immunocytochemistry can also be easily performed either on gels dried and then fixed, or on gels fixed in the dish and secondarily recovered. These techniques are not described here because they are out of the scope of this chapter. Some of them are described in detail elsewhere *(4–6)*. Most of the techniques described in the literature for cell smears or histological preparations are directly usable with collagen gels.

3. To recover cells, the collagen gel is subjected to collagenase digestion (collagenase Sigma type 1; 0.2 mg/mL) in serum free medium supplemented with 0.1% BSA for 30 min at 37°C. It is then possible to pick up colonies individually.

4. Notes

1. FCS is crucial for obtaining optimal results in colony growth. As some sera have inhibitory effects in these culture systems, several batches must be tested to select and order a sufficient amount of the best batch
2. Some people use human albumin (4% [w/v]) industrially prepared for therapeutic use However, in our experience, it gives lower numbers of colonies
3. Other purification procedures based on affinity columns or immunomagnetic beads are also available from other companies such as Cell Pro (immunoaffinity column) Baxter or Dynal (immunomagnetic beads) Several of them are used for therapeutic purposes to enrich the $CD34^+$ bone marrow or blood population "ex vivo" before autografting.
4. Prepare a quantity of medium larger (at least for one more dish) than required for the number of dishes due to the loss of medium in tubes and pipettes during the cell seeding
5. The optimal development of $CD34^+$ cells generally require higher FCS concentrations (25% for us) and combinations of 3–7 growth factors (Table 2) This cell population contains d 14 CFU-GM and BFU-E as well as earlier progenitors giving rise to large and often mixed colonies after longer times of culture (21–28 d) Some of them have a self-renewal potential In our experience, 5637 CM used in combination with IL3 (10 ng/mL) and SCF (10 ng/mL) gives good results for the growth of d 14 CFU-GM Addition of Epo (3 U/mL) to this combination allows the growth of hemoglobinized d 14 BFU-E. Combinations of purified cytokines (SCF + IL1 + IL3 + Epo) each at 10 ng/mL and 3 U/mL (Epo) allows the development of d 14 granulomonocytic and erythroid colonies together Mixed colonies derived from CFU-GEMM are generally observed with lower frequency
6. Six 35-mm Petri dishes are grouped in a large covered glass Petri dish (10-cm diameter) around an open 35-mm Petri dish filled with water This reduces drying, pH, and temperature variations during the daily openings of the incubator

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18

Long-Term B-Lymphoid Cultures from Murine Bone Marrow Establishment and Cloning by Using Stromal Cell Line AC 6.21

Cheryl A. Whitlock and Christa E. Muller-Sieburg

1. Introduction

Nearly all hematopoietic cells in mammals derive from precursors that undergo much or all of their development in the bone marrow. In vitro models for many lineages are available and represent modifications of the original bone marrow culture system designed by Dexter and Lajtha *(1)*. In this chapter, we describe a second bone marrow culture system, first reported in 1982 *(2)*, that provides an in vitro environment selectively supporting long-term proliferation and differentiation of early B-lymphocyte lineage cells. This method can be used to obtain heterogeneous populations of immature precursors of the B-cell lineage greatly enriched from other hematopoietic cell types. Clonal populations can also be obtained by extension of this method to limiting dilution culture.

A recent major advance in the technology of B-lineage cell culture has been the isolation of stromal cell lines that support B-lymphopoiesis from the primitive precursors through the pre-B-cell stage. These lines can substitute for the mixed stromal cell layer that provides the supportive microenvironment in the primary bone marrow cultures. We describe one such cell line, AC 6.21, and its use for expansion of established heterogeneous populations of B-lineage cells and for limiting dilution culture to obtain clonal lines or determine the B-cell precursor frequency of selected populations.

The technique of long-term bone marrow culture for the study of B-lymphopoiesis is still in its infancy, but much progress has been made since its inception in 1982. We now know something about the characteristics of the stem cell precursors that establish the continuous B-lineage cell lines and will

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

hopefully someday learn more about how these precursors are committed to the B-lineage by the culture environment. Many investigators are also isolating stromal cell lines that are able to support B-lymphopoiesis and, through their efforts, the factors and cellular interactions that are important in supporting B-cell ontogeny will be defined in the future

2. Materials

1 Culture medium RPMI 1640 medium, 5% (v/v) selected batch of fetal calf serum (*see* Note 1), $5 \times 10^{-5}M$ 2-mercaptoethanol, 2 m*M* glutamine, 1 m*M* sodium pyruvate, 100 U/mL penicillin, 50 μg/mL streptomycin. Antibiotics are recommended for initiating cultures, but may be omitted after 2 wk Antifungal agents are toxic to the stromal layer and should not be used β-mercaptoethanol is essential for viability and growth of the stromal cell line AC 6 21

2 Mice Balb/c mice provide the most consistent results, but continuous lymphoid cultures have been established using other mouse strains. Mice *must* be between 2½ and 3½ wk of age at the time of marrow harvesting. Balb congenic strains such as Balb B, Balb K, and Bab-14, are useful for preparing feeder layers (*see* Note 3)

3 Enzyme. Dispase-Collagenase is purchased in 100-mg vials from Boehringer-Mannheim (Indianapolis, IN) One vial is dissolved in 20 mL serum-free medium to prepare a 10X stock, then filtered through a 0 45-μm filter before aliquoting and storing at –35 to –70°C Thawed and unused portions retain significant activity when refrozen or stored for a few days at 4°C

4 Reagents and equipment for immunofluorescent staining and cell sorting Iscove's medium provides better viability of cells during these procedures. Propidium iodine is used in the final step of the staining procedure in order to detect and eliminate dead cells from the sorting Stocks are prepared in phosphate-buffered saline (PBS) at 1 mg/mL and stored at –20°C. Once stained, the cell suspension is filtered through a nylon screen with mesh 3-60/45 (Tetco Inc , Elmsford, NY) in order to remove cell clumps Sorting requires a fluorescence-activated cell sorter (FACS™) equipped with two lasers, one tuned to 488 nm to excite fluorescein isothiocyanate (FITC), and the other to 590 nm to excite Texas red

5. Antibodies· Antibodies used for enrichment of B-lymphocyte progenitors are listed in Table 1 with their corresponding references We have found that good quality antibody preparations are critical for successful purification of Thy-1^{lo} $T^-B^-G^-M^-$cells (*see* Section 3.2.4 and Note 4). We use antibodies derived from serum-free hybridoma culture supernatents that have been ~100-fold concentrated by ammonium sulfate precipitation (50% [w/v]) These antibodies can be labeled with biotin, FITC, or Texas red as described *(10)* and are always titrated prior to the FACS sorts to determine the optimal concentration for staining All antibodies are diluted in Iscove's medium with 3–5% serum and filter-sterilized through a 0 2-μm disk filter directly before use (Prewashing the filter with serum-containing medium will reduce loss of antibody by nonspecific binding to the filter)

Table 1
Antibodies and Their Specificities

Antibody	Specificity	Cells in bone marrow, %	Refs
RA3-6B2	B220 (pre-B- and B-cells)	25–30	*3*
M1/70	Mac-1 (macrophages)	23–28	*4*
RA3-8C5	Granulocytes (=Gran-1)	28–33	*5*
30H12	Thy-1 2	3–5	*6*
53-2 1	Thy-1 2	3–5	*6*
19XE5	Thy-1 1	3–5	*7*
31–11	Thy-1 (nonallelic)	3–5	*8*
GK1.5	L3T4, on T_H cells	1–3	*9*
5 3-6 72	Lyt-2, on T_K cells	2–3	*6*

3. Methods

3.1. Lymphoid Long-Term Bone Marrow Cultures (Whitlock-Witte Cultures)

The following technique is remarkable for its simplicity and reproducibility. The most critical ingredient for success is the batch of fetal calf serum used. Selection of a suitable serum batch is described below in Note 1. A recommendation for those initiating cultures for the first time or screening serum batches is to use Balb/c mice because marrow from these mice has proven to be best for rapidly and reproducibly establishing B-lineage cultures.

3.1 1. Harvesting Marrow and Initiating Cultures

1 Sterilize the surgical area of the mouse widely with ethanol
2 Open the skin overlying the femur anteriorly with sterile scissors from abdomen to well below the knee
3. Trim the thigh muscles away, then remove the bone by cutting just below the hip and knee joints
4 Transfer the bones to a Petri dish containing medium or balanced salt solution with 5% serum and keep on ice until all the bones are removed The knee joint is preserved until just before marrow harvesting because of the tendency of the marrow plug to be partially extruded when it is cut
5. Scrape the interior of the marrow cavity with a 25-gage needle while flushing out the marrow plug with 3–5 mL of medium or balanced salt solution containing 5% serum. All clumps of cells and bony spicules should be saved and cultured. (A goal of marrow harvesting is to obtain as many stromal cells as possible)
6 Pipet the cells and medium up and down to break up as many clumps as possible, then centrifuge the entire cell suspension at 300*g* for 10 min
7 Suspend the cell pellet in culture medium at 10^6 cells/mL

Table 2
Culture Volumes for Standard Tissue-Culture Vessels

Vessel type	Surface area, cm^2	Volumes for initiation mL[a]
24-Well plates[b]	2	1.0
6-Well plates	10	2.5
60-mm dishes	20	5.0
100-mm dishes	57	14.0
T25 flasks	25	7 0
T75 flasks	75	20 0

[a]The volume given is cell suspension needed to produce a similar density of cells per square centimeter in each vessel type For initiation of high-density cultures that will produce B-lineage cells, a cell suspension of 10^6 cells/mL is used For preparation of low-cell-density mixed stroma, 3×10^5 cells/mL is used

[b]For 24-well plates (and smaller vessels, if attempted) a higher density of cells per surface area must be used in order to seed enough cells to prevent well-to-well variability in the stromal layers

8. Dispense the cell suspension at approx $2\ 5 \times 10^5$ cells/cm^2 of the culture vessel (an exception is in vessels less than 10 cm^2, such as in 24- and 96-well plates) The volumes used for most conventional culture vessels are shown in Table 2
9 Incubate cultures at 37°C, in 7–8% CO_2, and in a well-humidified incubator. Cover multiwell plates in plastic wrap to decrease air circulation around them. Tissue-culture plates are best handled in stacks on trays, and individual trays can be wrapped with foil. Wrapping cultures serves to decrease evaporation and concentration of medium, as well as aid in containing and preventing spread of airborne contaminants, such as mold spores

3.1.2. Maintenance of Cultures

Once initiated, long-term lymphoid bone marrow cultures need only to be fed with fresh medium until the time nonadherent lymphocytes are harvested for analysis. The timetable for feeding long-term cultures is provided in Table 3. For 60- and 100-mm tissue culture plates, dehydration is a problem, and these cultures may require feeding twice a week. Flasks and multiwell plates have less of a dehydration problem and can be fed only once a week.

1 Initiate cultures as described in Section 3 1 1
2. For biweekly feedings, add an amount of fresh medium equivalent to ½ of the volume of culture medium used to initiate the culture without removal of any of the spent medium. (For once weekly feedings, this step is omitted.)
3. At the subsequent feeding, aspirate approx 80% of the spent medium (with care not to remove the nonadherent cells), then add a volume of fresh medium equiva-

Table 3
Schedule of Feeding for Culture Maintenance

Day	
0	Initiation of cultures
[3 or 4][a]	Feed with 1/2 volume [optional][b]
7	Removal of 80% spent medium; feed with full volume
[10 or 11]	Repeat of d 3 or 4
14	Repeat of d 7

[a]Tissue-culture dishes have a greater tendency for the medium to evaporate, therefore, they should be fed twice weekly to prevent concentration of the medium and poor culture growth

[b]Details of feeding steps are given in Section 3 1 2

lent to the amount used to initiate the culture (For once weekly feedings, cultures are aspirated at each feeding and fed with a volume of fresh medium equivalent to the amount used to initiate the culture.)

3.1.3. Collection of Cultured Lymphocytes for Seeding Secondary Cultures or for Experiments

The whole process of establishment of B-lymphopoiesis (*see* Note 2) can take 3–6 wk, depending on the serum batch. Nonadherent cell numbers steadily increase, after a brief decline, and reach a "maximum" that is characteristic for each individual culture and ranges roughly between 10^5 and 10^6/mL. When patches of small- to medium-sized lymphocytes predominate in the cultures (Fig. 1), they can be harvested for assay without sacrificing the primary culture.

1. Gently pipet the surface of the stromal layer to dislodge the lymphoid cells that are loosely adherent to a subpopulation of stromal cells.
2. Once dislodged, remove 90% of the medium and suspended nonadherent cells.
3. Add fresh medium to the primary culture vessel, and the remaining lymphoid cells will continue to divide until they reach their characteristic "maximum" density in 4–7 d
4. Transfer the harvested lymphoid cells to a new tissue-culture vessel and incubate at 37°C for 2 h to allow any dislodged stromal cells to adhere. (It is best to leave the harvested lymphocytes in their conditioned medium during this incubation. The adherence step is not required for routine expansion of lymphoid cultures.)
5. Harvest the lymphocytes a second time by gently pipeting the medium or swirling the culture vessel.

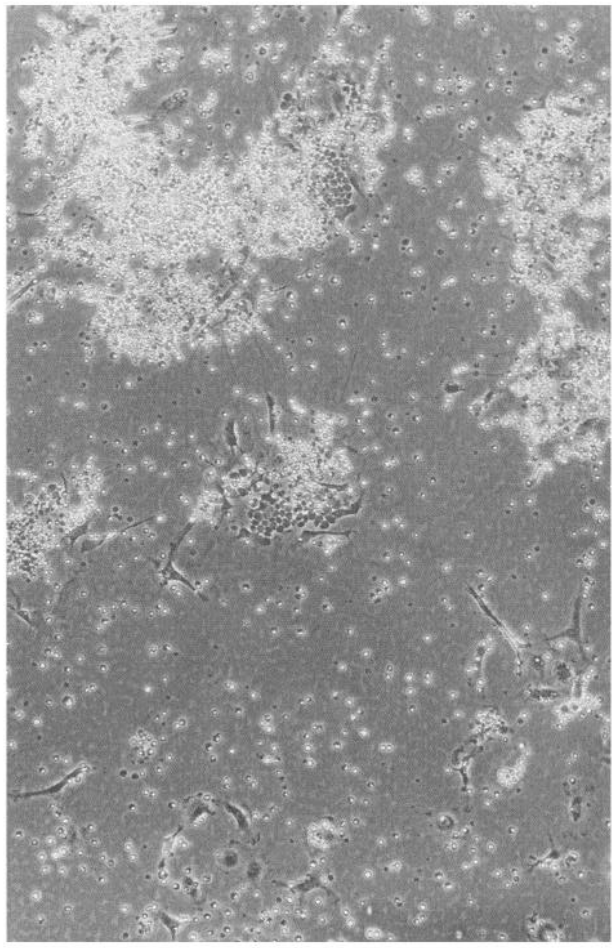

Fig. 1. Established long-term lymphoid bone marrow culture initiated at 10^6 cells/mL (high cell density). Patches of small- to medium-sized lymphocytes predominate and are closely associated with underlying stromal cells. Scattered nonadherent cells not associated with stromal cells have poor viability. Often lymphocytes are seen beneath the stromal cells (center/bottom), a process termed pseudoimperipolesis.

6. Repeat the adherence steps if a large number of stromal cells appear to be present.
7. Lymphocytes harvested from the primary cultures can now be used for experiments or subdivided onto established mixed stromal layers or stromal cell lines (*see below*) in order to expand the number of cells that can be obtained. The number of lymphocytes that is optimal to seed onto low-cell-density, mixed stroma has never been determined, but we know that primary cultures can be subdivided at least 10- to 20-fold.

3.1.4. Preparation of Mixed Stromal Cell Feeder Layers

Mixed stromal cell layers devoid of lymphoid cells can be prepared by initiating bone marrow cultures at a lower cell density than used to initiate cultures that produce B-lineage cells. Low-cell-density, mixed stroma proliferate to cover 50–75% of the culture vessel surface in about 3–4 wk. At this point, nonadherent lymphocytes harvested from high density cultures, as described in Section 3.1.3., can be seeded onto them.

1 Harvest bone marrow from young mice of the appropriate mouse strain or congenic type that will allow differentiation of the seeded lymphocytes from those that might derive from the feeder layer (*see* Note 3)
2 Suspend cells in culture medium at 3×10^5 cells/mL
3 Initiate cultures with the same volumes as indicated for high density cultures (Table 2)
4 Maintain cultures, as indicated, for high density cultures in Table 3 and Section 3 1.2.
5 After 3–4 wk of culture, check cultures by phase microscopy for degree of confluence (should be at least 50% confluent), types of stromal cells present (should be a variety of stromal cell types), and the presence of foci of nonadherent lymphoid cells (Fig. 1, discard cultures with lymphoid colonies since these will contaminate the seeded cells).
6 Continue to regularly feed the feeder layers until they are ready to be used, and they should be functional for 2–3 mo

3.1.5. Preparation of Stromal Cell Line (AC 6.21) Feeder Layers

Stromal cell lines that can substitute for the mixed stroma are currently becoming available. One of several lines we have prepared, AC 6.21 *(11)*, is especially good at supporting B-lymphopoiesis from a very primitive precursor through the cytoplasmic μ-positive stage (*see* Note 3).

1 Obtain confluent or nearly confluent stroma cell layers.
2 Aspirate medium.
3. Wash stroma three times with serum-free RPMI-1640
4 Add a volume of Collagenase-Dispase (0 5 mg/mL in serum-free medium) sufficient to cover the bottom of the vessel (approx ⅓ to ½ of the volume used to initiate the culture, Table 2)
5 Incubate at 37°C
6 Wait 15 min.
7 Check by phase microscopy for cell detachment Typically, AC 6 21 does not completely detach by this treatment until pipeted. If enzymatic digestion is complete, the membrane processes will be retracted to thin branching fibers that give a "spider web" appearance to the culture.
8. If necessary, mix gently and wait 10–15 min more

Table 4
Approximate Numbers of AC 6.21 Needed for Confluency in Various Culture Vessels

Vessel	No for confluency	Maximum density[a]
96-Well	4×10^3	—
24-Well	4×10^4	—
6-Well	2×10^5	4×10^5
60 mm dish	4×10^5	8×10^5
100 mm dish	$1\ 1 \times 10^6$	2×10^6
T25 flask	5×10^5	1×10^6
T75	$1\ 5 \times 10^6$	3×10^6

[a]AC 6.21 has an extensive membrane that can cover a large area of the culture dish Cultures that appear confluent can increase in cell number by reduction of the surface area occupied by each cell, therefore, the number of cells needed to achieve confluency and the maximum number of cells that can be harvested from a dense culture are slightly different This column is provided as an easy reference for determining how many flasks need to be prepared for large-scale experiments

9 Check by phase microscopy again for cell detachment.
10. Once enzymatic digestion is complete, harvest the cells by vigorously pipeting the vessel surface with a Pasteur pipet. (If most of the cells are still loosely adherent to the vessel before pipeting, then the Collagenase-Dispase solution can be removed by aspiration and the stromal cells harvested directly into a desired amount of fresh culture medium containing serum This allows elimination of step 12 and simplifies routine passage of the cells)
11. Check by phase microscopy for efficient removal
12 Pellet cells at 150*g* for 7 min if harvested into the enzyme solution
13 Once in culture medium, count viable cells.
14 For preparation of feeder layers that are to be used within 1 or 2 d, dispense the harvested stromal cells into the new cultures at a density that is about ¼ to ½ the density needed for preparing confluent stromal cell layers (Table 4).
15. For routine passage of the stromal cell line, it should be carried at subconfluency to prevent selection of variants that are not contact-inhibited. Our routine is to passage the cell line once per week and, at each passage, set up flasks that are 1:20 and 1:50 splits of a culture that is just nearing confluency This is approx 1×10^4 and 4×10^3 cells/mL, respectively

3.2. Cloning of B-Lineage Cells (Limiting Dilution Culture)

Precursors capable of long-term proliferation in lymphoid bone marrow cultures can be cloned by limiting dilution culture on mixed stromal cell layers or, preferably, on an established stromal cell line, such as AC 6.21. The source of

lymphocytes to be cloned can be either nonadherent cells from established Whitlock-Witte cultures or fresh bone marrow (*see* Note 4).

3.2.1. Preparation of Mixed Bone Marrow Stroma for Limiting Dilution

1 Harvest marrow from the appropriate congenic mouse strain (*see* Note 3) and prepare as for larger bone marrow cultures
2 Suspend cells in culture medium at 1 5 × 10^6 cells/mL
3 Initiate 96-well cultures with 3 × 10^5 cells/well in a vol of 0.2 mL
4 Wrap stacks of plates in plastic wrap to decrease dehydration.
5 Incubate at 37°C for 2–3 d
6. Pipet each well to suspend nonadherent cells This is best accomplished by using a 12-space multiwell pipetor set at 0 15 mL.
7 Remove all the medium and add 0 2 mL of fresh medium to each well
8 At weekly intervals, for a total of 3–4 wk, repeat steps 6 and 7 with the goal of depleting the stroma of any nonadherent lymphoid precursors prior to cloning
9. At 1–2 d prior to cloning, remove the medium.
10 Add 0 1 mL fresh medium containing 10% fetal calf serum to each well.
11 During this 2-d period, screen the cloning plates for wells that contain patches of nonadherent cells that may be mistaken for clones later on and eliminate these wells from the limiting dilution experiment (that is, simply ignore these wells when selecting and counting clones)

3.2.2. Preparation of Stromal Cell Line Feeders for Limiting Dilution

1 Harvest AC 6.21 stromal cells as described in Section 3 1 5 One T75 flask will be adequate for four or five 96-well plates The approximate numbers of stromal cells that can be obtained from confluent vessels of different sizes are provided in Table 4
2 Suspend the harvested stromal cells in culture medium with 10% fetal calf serum at 2 to 4 × 10^4 cells/mL
3. Aliquot 0 1 mL/well (75–100% confluency)
4. Use for limiting dilution experiments within 1–2 d of initiation.

3.2.3. Cloning of Established Lymphoid Cell Lines

1 Harvest and adhere lymphoid cells from established bone marrow cultures as described in Section 3 1.3.
2. Pellet and resuspend the lymphocytes in fresh medium with 10% serum at 3 × 10^3 cells/mL.
3. Make five threefold dilutions in the same medium to prepare suspensions of 1000, 333, 111, 33, and 11 cells/mL in quantities sufficient to plate 0 1 mL/well for the desired number of plates. (Often <10% of those colonies growing after 3 wk of cloning will continue to grow after expansion [*see* Note 4], therefore, multiple plates at the lower cell numbers need to be set up in order to obtain enough colonies that have a reasonable chance of being clonal and continued growth)

4. By using a 12-space multiwell pipetor, add 0.1 mL of each cell suspension to each of the appropriate wells.
5. Stack the plates, wrap in plastic wrap, and culture at 37°C.
6. Weekly, carefully remove 0.1–0 15 mL of spent medium by using the multiwell pipetor so not to disturb the cells at the bottom of the wells
7 Add an equivalent amount of fresh medium to each well
8 After 3 wk, screen wells by phase microscopy to detect wells with lymphoid colonies (The variety of colony types found on limiting dilution culture are described below in Note 5.)
9. Prepare a plot of the log frequency of negative wells vs the number of cells added to the wells to check for linearity at the lower cell densities, particularly in the range from which clones are to be selected (Typically, there is negative interaction at high cell densities and deviation from linearity)
10 To expand the clones, prepare stromal cell layers in 24-well plates and also in 6-well plates, 60-mm dishes, or T25 flasks for further expansion For mixed stroma, this process takes 3–4 wk, as described in Sections 3 1 4 and 3.2 1 , and should be started in all sized vessels around the time of initiating the limiting dilution cultures Feeders of stromal cell line AC 6 21 can be prepared 1–2 d prior to use.
11. Check each mixed stromal feeder layer carefully for lymphoid cell growth before use
12. Change the medium to medium containing 10% serum 1–2 d prior to using the mixed stromal layers for expansion of clones.
13 Vigorously pipet the limiting dilution well with the clone to be harvested to disrupt the stromal layer and release any lymphoid cells intimately associated with it
14. Transfer the entire contents of the pipeted well to one well of a 24-well plate containing an established feeder layer
15. Feed the expanded clones weekly by aspirating 80% of the medium and replacing it with fresh medium Care should be taken not to crosscontaminate wells, and the plates should be kept covered in plastic wrap to prevent dehydration.
16 Any well showing patches of 50 or more nonadherent cells, indicative of continued proliferation of the transferred cells, can be expanded further

3.2.4 Cloning of Selected B-Cell Progenitors from Fresh Bone Marrow

A subpopulation of fresh bone marrow cells, designated Thy-1^{lo} $T^-B^-G^-M^-$ (*see* Note 4) is enriched in progenitors capable of producing long-term B-lymphoid cultures. Our method for isolating these cells for limiting dilution is outlined:

1. Harvest fresh bone marrow as described in Section 3.1 1 for the initiation of long-term lymphoid cultures, except use Iscove's medium containing 5% fetal calf serum instead of RPMI-1640. (In Iscove's medium, bone marrow cells maintain good viability over a longer period of time)

2 Dilute appropriate antibodies (Table 1) in Iscove's medium and sterile filter through a 0 2-μm disk directly before use.
3. Pellet cells in a conical tube at 300*g* for 10 min
4 Suspend cells in rat anti-Thy-1 antibody (For this and all subsequent steps, 10 μL of antibody or avidin is used for each 10^6 cells.)
5 Incubate on ice for 15 min
6 Wash (i e., dilute the antibody/cell suspension with 2 mL Iscove's medium containing serum, then underlay with 50–100% fetal calf serum. Pellet the cells through the serum, then carefully aspirate the liquid from the tube being careful not to disturb the cell pellet, but remove as much serum and antibody as possible)
7. Suspend the cell pellet in FITC-goat antirat Ig and incubate 15 min
8 Wash as in step 6
9 Suspend the cell pellet in normal rat serum (diluted 1 4 in Iscove's medium) and incubate for 3–5 min
10. Add a cocktail of biotinylated antibodies. rat anti-B220, rat anti-Gran-1, rat anti-Mac-1, rat anti-Lyt-2, and rat anti-L2T4 Incubate 15 min on ice
11. Wash as in step 6
12 Suspend the cell pellet in Texas red-Avidin and incubate 15 min
13 Wash as in step 6
14. Suspend the cell pellet in Dulbecco's modified PBS containing 20 μg/mL propidium iodine
15 Filter the cell suspension through a nylon screen to remove cell clumps before sorting
16 The cell population of interest is that which stains weakly with anti-Thy-1 and not at all with the antibodies specific for B-cells, macrophages, mature T-cells, and granulocytes Generally, we obtain 1 to 2 × 10^4 Thy-1^{lo} $T^-B^-G^-M^-$ cells in a 2 h sort.
17 Collect sorted cells into ice cold Iscove's medium with 5–10% serum
18 Count the sorted cells
19 Suspend the cells in culture medium with 10% fetal calf serum (now back to RPMI-1640 medium) at 300 cells/mL Make threefold dilutions in quantities sufficient to plate 0.1 mL in each well of the number of desired limiting dilution plates (If bulk cultured at this point, suspend at 10^3–10^4 cells/mL)
20. Aliquot 0 1 mL of each cell suspension on established feeder layers in 96-well plates (*see* Section 3 2 3)
21 Feed weekly as described in Section 3 2 3 for limiting dilution cloning of established lymphoid cultures.
22. Screen wells after 3 wk of culture for lymphoid colonies, as described in Section 3.2.3
23. Select and expand clones as described in Section 3.2.3

4. Notes

1. Selection of a batch of fetal calf serum that efficiently supports B-lymphocyte proliferation in this culture system is the key to the success of all the above procedures Screenings we have done suggest that the highly defined serums do not

work well in this system Of those remaining, approx 20% will work. Two basic criteria are useful for comparing serum lots. One is its ability to permit good establishment (50–75% confluency at 3 wk of culture) of low-cell density, mixed stromal cell layers using Balb/c bone marrow. The second criterion is outgrowth of lymphoid colonies in high cell density cultures In a 60-mm dish, high-cell density (10^6 cells/mL in 5 mL) culture at 3 wk, there should be a minimum of five discreet patches of lymphoid cells containing more than 500 cells each Serum lots that have proven to work well for this culture system have been stable for up to 4 yr when stored at –35°C

2 Recognition of whether a culture is establishing adequately is important so that time is not wasted waiting for B-lineage cultures to establish when the potential of successful establishment is low The ability to assess how well a culture is doing comes only with experience, but the following gives a verbal description of how the cultures should look at different times after initiation, which may aid in assessment. The first phase is outgrowth of the stromal layer and death of the majority of the nonadherent cells. This progresses rapidly during the first 7–10 d of culture. Both lymphoid and myeloid precursors survive this initial phase to proliferate and form discrete foci of nonadherent cells Lymphoid colonies tend to be intimately associated with the stromal cells that underlie them and are composed of cells that vary in size from small to medium-sized lymphocytes (Fig. 1). Myeloid colonies are more uniform in cell size and do not conform to the shape of the stromal cells beneath them. Myeloid cells also are larger than even the largest lymphoid cells

The second phase is a crisis phase in which nonadherent cell numbers decrease and, with some serum batches, essentially disappear This occurs and lasts a variable amount of time after culture initiation, but a return of nonadherent cells should be seen after 3–4 wk of culture Only lymphoid cell proliferation survives this second phase. With good batches of serum, the crisis phase is short, and often the first phase overlaps with the third phase, characterized by steady increases of nonadherent cell numbers to the maximum cell density characteristic of the individual culture (1 to 10×10^5/mL).

The last or fourth phase is characteristic of Balb/c bone marrow cultures, but we have not determined whether it occurs in cultures initiated with other mouse strains. If the heterogeneity of immunoglobulin gene arrangements of the nonadherent cells is followed during phase three, it is found that the culture gradually become pauciclonal after 4–6 mo of culture The beginning of the fourth phase is heralded by a gradual increase in the maximum number of nonadherent cells in the culture and coincides with the cultures becoming more and more pauciclonal. The cultures progress to a stage when the nonadherent cells must be subdivided in order to maintain viability, and eventually a clonal, tumorigenic, stromal layer-independent cell line emerges. Because of the tendency of older cultures to be pauciclonal and less "normal" in their growth patterns, those interested in studying normal B-cell development should limit their experiments to cultures that are between 3- and 12-wk-old.

3. Feeder layers: Mixed stroma vs stromal cell lines and the use of congenic mouse strains Proliferation of B-lymphoid cells in this culture system is dependent on adherent stromal cells. In the primary bone marrow cultures, as described in Note 2, a mixture of adherent cells establishes what we call a "mixed stromal cell feeder layer." From one established culture, we isolated a number of stromal cell lines that can substitute for the mixed stroma. One such line, AC 6.21, is especially good at supporting proliferation

   Use of the stromal cell line, AC 6.21, for limiting dilution culture has many advantages First, a large number of 96-well plates can be prepared in a relatively short amount of time with a minimum of effort. Second, the microenvironment within each well is more consistent than with mixed stroma, and third, there is never any problem with contamination of the clones with stroma-derived lymphocytes. Irradiation of the stromal cells is not needed since this stromal cell line is contact inhibited and will remain viable for several weeks without passage if fresh medium is given weekly. This latter characteristic makes it highly useful for cloning and limiting dilution analysis of B-lineage precursors.

   A disadvantage of this stromal cell line over mixed stroma is that dead cell debris accumulates on the AC 6.21 The failure of this debris to accumulate on the mixed stroma probably arises from the presence of macrophages that phagocytize the debris rather than a lower turnover of the lymphoid cells.

   We have used the AC 6 21 stromal cell line for most of our limiting dilution studies and know that it can support proliferation and differentiation of primitive progenitors (Thy-2^{lo}T$^-$ B$^-$G$^-$M$^-$) to the pre-B-cell stage of differentiation with synthesis of cytoplasmic immunoglobulin *(11)* Differentiation to surface Ig-bearing cells does not proceed as well on the stromal cell line as it appears on mixed stromal cell feeders

   AC 6 21 is grown in the same medium used for long-term lymphoid bone marrow cultures β-mercaptoethanol is essential for viability of AC 6 21, and media should always contain 5 to 10 × $10^{-5}M$ fresh 2-mercaptoethanol.

   Stromal cell lines such as AC 6.21 have a tendency to select for variants that will overgrow the confluent cell layer if it is carried routinely in a confluent state. Therefore, cultures for passage should be no more than around 80% confluent at the time of subculture. It is also advisable to have a large number of vials of an early passage frozen for future use in the event the stromal cell line does start to overgrow.

   If mixed stromal cell layers are used as feeders for limiting dilution culture or routine passage or expansion of bulk cultures or clones, it should be kept in mind that the feeder layer may give rise to lymphoid cells that may contaminate the passaged cells. A simple method for being able to monitor feeder layer contamination is to use congenic mouse strains to prepare the feeders. Balb/c congenics at the histocompatibility locus (e g., Balb.B and Balb.K) and the immunoglobulin locus (e.g., Bab-14) work equally well in this culture system. No problem has arisen in our experiments when we have seeded B-lymphoid cultures onto feeders with a different H-2-type. Histocompatibility markers can be easily moni-

tored by surface immunofluorescence. Balb/c and Bab-14 cells can be differentiated by restriction-length fragment polymorphisms in the heavy chain constant region genes *(12)*.

4. Limiting dilution culture theories and practice: Limiting dilution culture simply means to culture a cell population at progressively lower densities until the cells of interest are at a frequency of <1/well The statistical theory used at the basis for this technique is beyond the scope of this manuscript, but in practice two points should be kept in mind. One is that if proliferation of the cell of interest is dependent only on the microenvironment provided by the culture conditions (in this case the medium and stromal cells) and if there is no negative or positive interaction between the cell of interest and other cell types in the mixed population cultured at limiting dilution, then a plot of the log of the frequency of negative wells (those not containing the cell of interest) versus the number of cells per well cultured will be linear. The second point to remember is that at a plating frequency of one cell of interest per well, approximately two-thirds of the wells will contain that cell. Since one-third of the wells do not contain the cell of interest, then a significant number of wells will contain two or more This latter point must be kept in mind when selecting the plates from which to choose clones.

   Approximately 1 in 1000 fresh bone marrow cells, when seeded at limiting dilution on an established bone marrow stromal layer, have the capacity to proliferate up to 4 wk As detailed, this frequency has been derived from limiting dilution analysis using AC 6 21

   Although the vast majority of nonadherent cells found in a well-established long-term lymphoid bone marrow culture are pre-B- and B-cells, bone marrow derived pre-B-cells (purified by flow cytometry as cells that express B220) do not have the capacity to initiate long-term lymphoid cultures We have found this capacity exclusively in a small population of bone marrow, characterized by expression of low levels of the cell surface antigen Thy-1. These cells lack B220, Mac-1, Gran-1, L3T4, and Lyt-2 antigens, and are thus termed Thy-$1^{lo}T^-B^-G^-M^-$ cells One in 15 Thy-$1^{lo}T^-B^-G^-M^-$ cells, which comprise 0 1–0.2% of total bone marrow, give rise to a lymphoid culture (Table 5). Thy-$1^{lo}T^-B^-G^-M^-$ cells are also highly enriched for pluripotent hematopoietic stem cells *(13)* This indicates that long-term lymphoid bone marrow cultures provide an in vitro system that allows the study of B-lineage differentiation from stem cells through mature B-cells. When established cultures are used for limiting dilution culture (3–8 wk post initiation), approximately 1 in 10 will proliferate to form a colony of 50 to several thousand cells after 3 wk. Only 10% or fewer of these will continue to proliferate after expansion Therefore, when planning a limiting dilution experiment for obtaining clones for future experiments, a large number of plates at the dilutions that should have approximately 0 3 clonogenic cells/well should be initiated to obtain a large number of clones for expansion Which clones will have long-term proliferative potential should be obvious after 2 wk of culture in the 24-well plate

5. Selecting lymphoid clones from the variety of colonies seen in limiting dilution cultures: The most striking finding on limiting dilution culture of bone marrow

Table 5
Frequency of B-Cell Precursors in Sorted Bone Marrow Populations

Population	Bone marrow, %	1/Frequency[c]
Total bone marrow	100	1200
B220+	30	>4000
$B^-M^-G^-$[a]	16	150
Thy-1^+[b]	5	70
Thy-$1^{lo}T^-B^-G^-M^-$	0 1–0 2	15 ± 5

[a]$B^-M^-G^-$. cells depleted of B220, Gran-1, and Mac-1 expressing cells
[b]Thy-1^+ contains both T lymphocytes and Thy-$1^{lo}T^-B^-G^-M^-$ cells
[c]Frequencies of wells containing lymphoid colonies were determined at 2 wk

cells on AC 6 21 is the complexity of the colony types observed The colonies not only differ greatly in cell number, but also in cell morphology. The most prominent colony that will be observed is one that consists of large cells, approximately three times the size of a lymphocyte and are round and nonadherent in the 1st wk in culture (Fig. 2A,B). With time, the cells in these colonies become granular and vacuolated, and they adhere to the stromal cells beneath them (Fig 2C) About 1 in 35–50 fresh bone marrow cells will form such a colony, therefore, these colonies present a problem in limiting dilution culture of lymphoid cells in whole bone marrow Large numbers of them in a well will compete with growth of lymphoid colonies Since lymphoid cell precursor frequency in whole bone marrow is 1 in 300–1000, then every well containing a lymphocyte precursor also potentially contains six or more colonies of these large, granular cells Fortunately, the large, granular cells do not have long-term growth capacity and, therefore, will be easily depleted with passage of the lymphoid clones

Lymphoid colonies can be recognized as those that consist of very small cells that vary in size and tend to adhere to the stromal cells (Fig. 2A). All colonies with this gross appearance can be shown to contain lymphocytes by Wright's-Geimsa staining and immunofluorescent staining of B220, a B-lineage specific surface marker.

A third group of colonies consists of cells that are more uniform in appearance and intermediate in size when compared to lymphocytes and large, granular cells (not pictured). Wright's-Geimsa staining of these cells most often shows them to contain predominantly primitive cell types with loose chromatin and basophilic cytoplasm. In some cases, these primitive cells are mixed with some lymphocytes or polymorphonuclear leukocytes Attempts to expand such colonies have failed thus far.

If nonadherent cells from primary lymphoid long-term bone marrow cultures are used as the source of cells for cloning, then the distribution of colonies

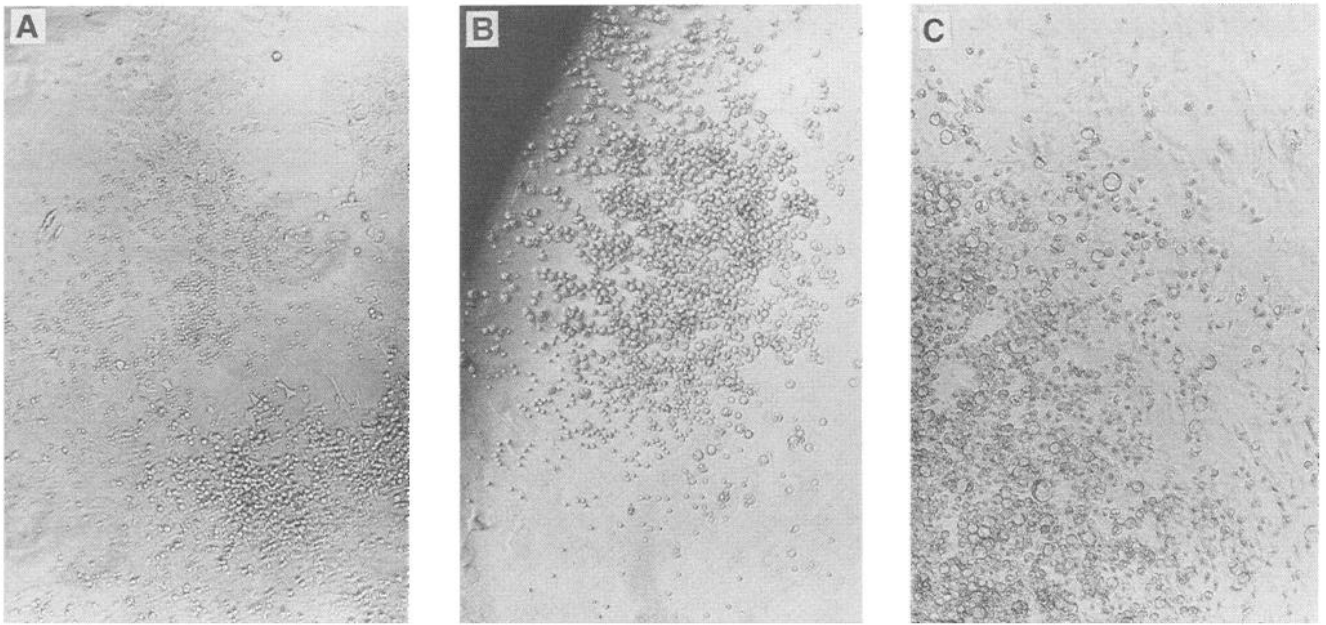

Fig. 2. Colony types in limiting dilution culture of fresh bone marrow cells on AC6.21. **(A)** A colony of small lymphocytes is shown adjacent to a young colony of "large-granular cells." The latter cells are at a stage in which most are still nonadherent, but many are beginning to increase their granularity. **(B)** A higher power view of the large-granular cell colony from (A). **(C)** Pictured is a high power view of a "large-granular cell" colony that is more mature than shown in (B). All of the cells are now highly granular and adherent. Many are swollen by a large intracytoplasmic vacuole that displaces the nucleus to one side (signet ring formation). Magnifications: (A) 25×, (B) 50×, (C) 50×.

obtained is more uniform. If young cultures are used (3-wk-old or less), then there is still a significant frequency of large, granular cell colonies that are obtained, but the frequency is about 10-fold less than for fresh bone marrow. In addition, the frequency of clonable B-lineage cells is 10-fold higher; therefore, it is simpler to obtain a large number of pure B-lineage clones by using established B-lineage cultures. However, our experience has shown that clones obtained from a single experiment can have the same immunoglobulin heavy chain gene rearrangements and, thus, are siblings

There are other cell types that undergo limited proliferation on AC 6.21 to give colonies of less than 50 cells Once such cell type is oblong in shape and forms a colony where the cells characteristically migrate away from each other (not pictured) We have not been able to obtain enough of these cells for Wright's-Geimsa stain or other methods of characterization

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19

Human Long-Term Bone Marrow Culture

Brian R. Clark, Catriona Jamieson, and Armand Keating

1. Introduction

The bone marrow is the primary site of hematopoiesis in adults. Accurate in vitro models of hematopoietic regulation and function should reflect the various hormonal and environmental regulators that act on developing hematopoietic cells in the marrow. Some of the regulatory elements acting in vivo are maintained when bone marrow cells are cultured in vitro under suitable culture conditions. Modifications of techniques initially described by Dexter (*1*; *see* Chapter 16) for culture of murine bone marrow led to the establishment of an in vitro system that supports human hematopoietic cells *(2,3)*. Functional hematopoiesis in these cultures was dependent on the formation, over 3–4 wk, of an adherent layer of cells derived from structural elements of bone marrow. This layer, termed "stromal" layer because of its likely derivation from the supportive elements in the marrow, has been extensively characterised *(4)*. Stromal cells have been shown to exert positive and negative regulatory effects on hematopoietic cells through cell-cell interaction and by the secretion of cytokines *(5,6)*.

Using variations on the basic long-term marrow culture (LTMC) technique described in this chapter, investigators can examine aspects of hematopoiesis in normal and disease states *(7)*. For example, long-term culture techniques can be applied to quantify primitive hematopoietic cells *(8)*, assess damage to stroma after chemotherapy, and examine physical interactions of hematopoietic cells with stroma *(9)*. Long-term cultures are also employed as a means of purging leukemic cells in vitro prior to autologous bone marrow transplantation for myeloid leukemias *(7)*.

Long-term cultures favor the generation of mature granulocytes but poorly support erythrocyte formation. However, the precursor cells of both granulo-

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cytes and erythrocytes (which are assayed by the ability to form colonies in semi-solid media) are present and can be maintained over several weeks. These cells, termed granulocyte/macrophage colony-forming cells (CFU-GM) and erythroid colony-forming cells (BFU-E and CFU-E), can be maintained for approx 14 wk and 8 wk, respectively. Although this system has limitations, it is a useful approximation to hematopoiesis in vivo (*see* Note 1).

2. Materials

1. Fetal bovine serum (*see* Note 2)
2. Horse serum (*see* Note 2)
3. Ficoll-Paque density gradient (density 1 077 g/mL) (Pharmacia, Uppsala, Sweden)
4. McCoy's 5A tissue culture medium (Gibco BRL, Grand Island, NY; cat no 21500)
5. Sodium bicarbonate (7.5% [w/v]) (Gibco, cat no. 25080)
6. 100m*M* Sodium pyruvate (Gibco, cat no 11360)
7. Liquid media supplements (with catalog numbers from Gibco). vitamins (11120), essential amino acids (21135), nonessential amino acids (11140), L-glutamine (25030) or equivalent (e g., GlutaMax) (Gibco)
8. Antibiotic-antimycotic solution (each mL has 10,000U penicillin, 10,000 U streptomycin, 25 µg amphotericin) (Gibco, cat. no 15245).
9. Hydrocortisone 1mg/mL in dimethyl sulfoxide (DMSO) (final hydrocortisone concentration in complete media of approx 1×10^{-6}*M*) (Sigma, St Louis, MO, cat. no. 40888).
10. White cell diluting fluid (3% acetic acid in water with a trace of methylene blue)

3. Methods

The procedure for LTMC generation is outlined schematically in Fig. 1 (*see* Notes 1 and 2).

3.1. Long-Term Culture Medium

1. Make up a medium supplement mix stock as follows:

Ingredient	Volume, mL
Vitamins	160
Bicarbonate	160
Pyruvate	160
Essential amino acids	120
Nonessential amino acids	65
Glutamine	160
Antibiotic solution	160
Hydrocortisone	0 5

Store supplement mix in 50-mL aliquots at –20°C Avoid repeated freeze-thawing

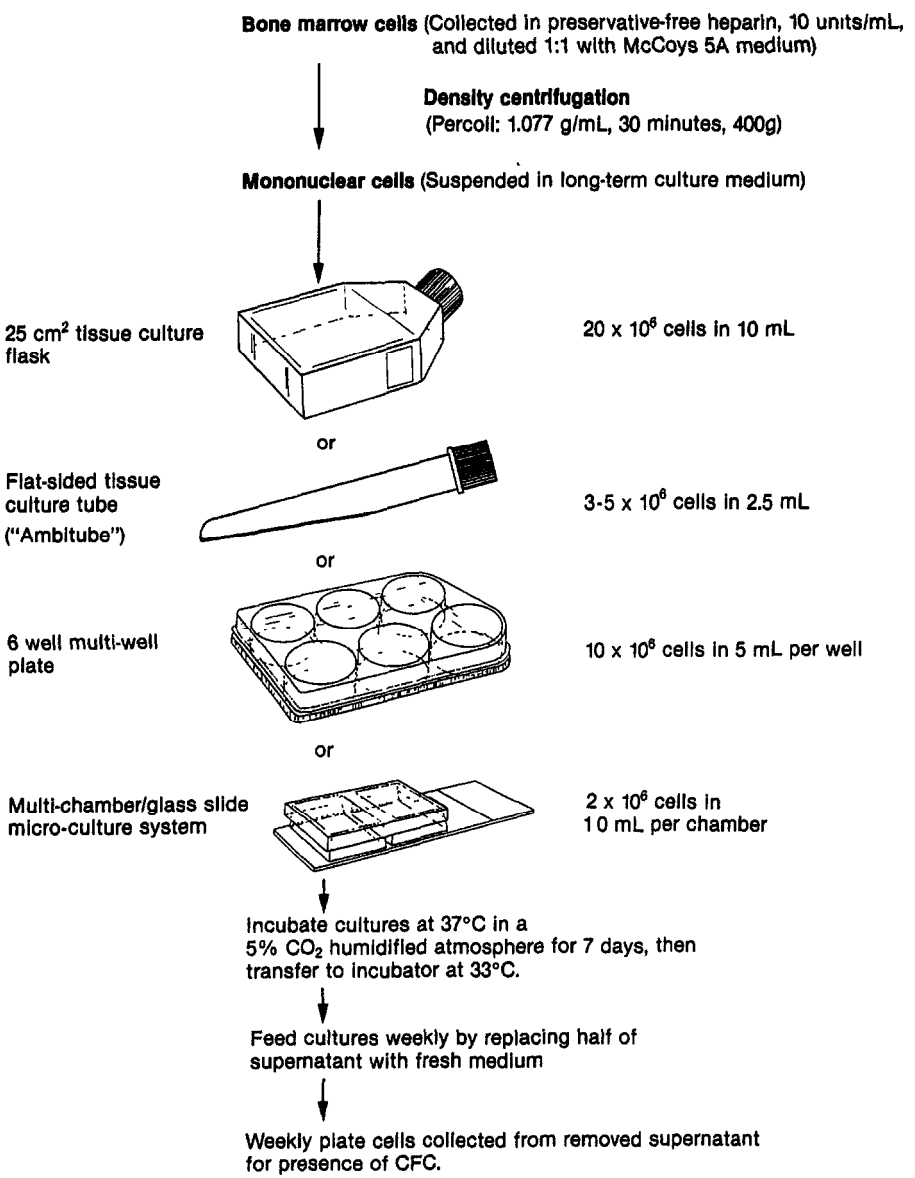

Fig. 1. Scheme for generating human LTMC

2. McCoys medium is prepared to 1X strength from liquid or powdered stocks according to manufacturer's instructions. Where appropriate, L-glutamine or sodium bicarbonate should be added. Long-term culture medium is made using (v/v): 12.5% Fetal bovine serum, 12.5% horse serum, 5% medium supplement mix, and 70% McCoys medium.

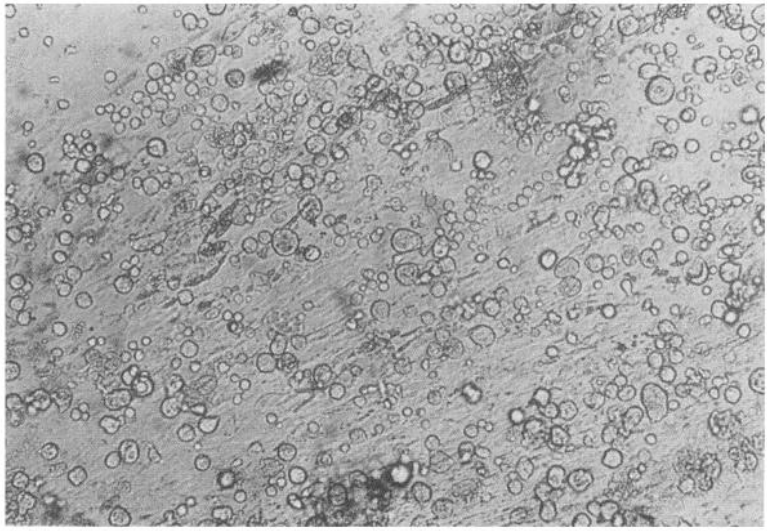

Fig. 2. Light micrograph of an adherent layer from a hematopoietically active human LTMC (x100).

4. The complete mixture may be passed through a 0.2-μm bottle-top filter to ensure sterility (optional). Store at 4°C for no more than 2–3 wk. Topping up with L-glutamine and antibiotics can be done, but may lead to increased variability.

3.2. Isolation of Marrow Cells

1. Marrow samples may be obtained from diagnostic aspirates, residual bone marrow contained in transplant bags and lines, or directly from bone fragments (typically ribs, sternum, or femoral head) (*see* Note 3). Aspirates (3–5 mL) should be drawn into a syringe containing 500 U preservative-free heparin (*see* Note 4).
2. Dilute marrow 1:1 with McCoy's medium.
3. Layer the diluted cells over Ficoll (*see* Note 5) and centrifuge at 400*g* for 30 min. Ensure the centrifuge brake is off to preserve the separation during deceleration. Collect the interface mononuclear cells and wash twice by centrifugation in McCoy's medium. Resuspend the cells in a small volume, count (*see* Note 6), and dilute to the required concentration in long-term culture medium (Fig. 1).

3.3. Generation of Long-Term Culture

1. Place the cells into desired tissue-culture vessel (*see* Note 7) (Fig. 2).
2. Incubate cultures at 37°C in a humidified atmosphere of 5%/$C0_2$ in air (*see* Note 8).
3. Feed the cultures after 7 d by replacing half of the supernatant with fresh, prewarmed medium. For optimal hematopoiesis, maintain the cultures at 33°C thereafter (*see* Note 9).
4. Continue feeding the cultures weekly in the same manner (*see* Notes 9 and 10).

3.4. Monitoring Outcome

Overall assessment of a successful long-term culture (*see* Notes 11 and 12) is made based on the confluence of the stroma (full confluence is expected after 3–4 wk), the presence of active regions of hematopoiesis (which appear as phase-dark groups of round cells, termed "cobblestone regions"), the persistence of hematopoietic progenitor cells (CFU-GM and BFU-E) and production of mature cells. The formation of cells with large lipid filled vacuoles (adipocytes) in the stroma is also an indicator of good hematopoietic conditions. However, the development of many lipid-filled cells throughout the layer may be a sign of culture senescence.

Hematopoietic activity is most readily quantified when media is removed at weekly feedings. The numbers of nucleated cells and colony-forming cells in the supernatant can be assessed by counting or plating cells in colony-forming assays (*see* Chapters 17, 20, and 21). In the later stages of culture, as hematopoietic activity declines, cells in the removed medium may require concentration by centrifugation to ensure accurate counts (remember to note the volume of the medium spun down to allow the calculation of cells or colonies per mL of culture medium!)

4. Notes

1. This method for generating LTMC is based on our modification of the Gartner and Kaplan technique *(2)* It is strongly recommended that any personnel handling human material are vaccinated against hepatitis B The stock numbers given herein for reagents supplied by Gibco do not include the suffix denoting the amount and packaging of the material Consult your catalog or sales representative for more details
2. The most crucial components in LTMC are the sera. These must be tested for activity in LTMC or colony-forming assays to determine their suitability There are extreme variations in the performance of sera in LTMC and time spent selecting a good batch from a number of suppliers is worth the investment Most suppliers will send 2 or 3 samples for you to test Set up LTMCs from the same marrow sample in media with different batches of horse serum (without fetal bovine serum) and observe stromal formation and hematopoietic activity. Batches of fetal bovine serum can be screened in colony assays (described in Chapter 20) for optimal colony formation, a good indicator of efficacy in LTMC Screened long-term culture media components are available from at least one supplier (Stem Cell Technologies, Vancouver, Canada).
3. Fragments of bones containing red marrow (e.g., rib fragments, femoral head) can be obtained from friendly thoracic and general surgeons after obtaining the appropriate consents. They should be placed directly into McCoys' medium with antibiotics and taken to the laboratory. The bones can be dissected with sterile bone cutters and the marrow scraped and flushed out into medium using a

16-gage needle and syringe. Because the marrow is being prepared into medium, the dilution step prior to Ficoll (Section 3.2., step 2) can be omitted. Marrow obtained from these samples may contain fat globules which can be discarded with the supernatant after pelleting the cells by centrifugation. Marrow aspirates can be shipped to the lab at ambient temperature via same day or overnight courier. Preservation of the sample may be enhanced by the addition of an equal volume of McCoy's/20% fetal bovine serum Observe all relevant shipping codes and protective measures.

4. Even in experienced hands, large marrow aspirations tend to have substantial contamination with blood. Taking smaller aspirates of 2–3-mL may actually give more bone marrow! Blood contamination leads to a lower concentration of marrow cells which can result in the seeding of too few marrow progenitor cells into a culture. This problem is most acute in samples obtained from patients with hypoplastic marrows Correction can be made for blood contamination by determining the haematocrit of the sample *(10)*. A standardized inoculum is essential for comparing the behaviour of samples from normal and disease states. Lyophilized preservative-free heparin can be obtained from Gibco. Resuspend in phosphate-buffered saline and filter prior to use.
5. An alternative to Ficoll separation is methylcellulose sedimentation of erythrocytes. Methylcellulose solution (typically 2.2% stock, *see* Chapter 20) added to a final concentration of 0 1% is thoroughly mixed by inverting the tube and allowing it to sit undisturbed in the tissue culture hood for 20–30 min. A large proportion of the erythrocytes will sediment. The supernatant (containing erythrocyte depleted leukocytes) can be removed and given one wash in McCoy's medium, counted, and used to establish LTMC. Two rounds of methylcellulose sedimentation may be required to reduce excessive red cell contamination and allow stroma to develop.
6. White cell diluting fluid (WCDF) lyses the red cells and acts as a diluent for cell counting. For example, 20 µL of a sample can be added to 180 µL WCDF, mixed by trituration and a portion placed onto a haemocytometer. The final count is then adjusted by x10 multiplication for the 1:10 dilution made in WCDF Viability can be assessed using the trypan blue dye exclusion assay (*see* Chapter 1)
7. We routinely use tissue culture flasks with areas 25–150 cm^2 for LTMCs and expansion of stromal cultures (*see* Note 10). LTMCs may also be established by seeding the following volumes of cells (resuspended at 2×10^6/mL) in the following tissue culture vessels:

96-well flat bottomed tissue culture well	100 µL
Nunclon multichamber slide	400 µL–1 mL
24 well plate	2.0–2 5 mL
6 well plate	5 mL
25 cm^2 flask	10 mL

Extrapolate values for larger flasks

8. Flasks can be gassed in the tissue-culture hood with a supply of sterile 5% CO_2/air, sealed and placed at 37°C in a standard incubator. In gassed incubators, we

routinely close lids after allowing flasks to equilibrate overnight to reduce the risk of contamination

9 Avoid disturbing the flask during the first 2–3 wk (e.g., to "check its progress") and take care when feeding cultures. Carefully add fresh medium down the side of the flask opposite to that supporting the stroma and ensure the pipet does not come into contact with the stroma.

10. Stromal cells from long-term cultures may be expanded by trypsinization of the phosphate-buffered saline-washed monolayer and seeding into fresh culture vessels The seeding of cells into new vessels should not exceed 1:4, but growth may be increased by the addition of basic fibroblast growth factor (βFGF, 10ng/mL), allowing weekly passaging of 1:5–1:10 Cultures should be passaged just prior to reaching confluence The repeated passaging of stroma results in the elimination of hematopoietic foci and generates a uniform cell population with a flat angulated appearance that lack hematopoietic cell antigens.

11 Rarely, endogenous EBV-transformed B-lymphocytes may emerge in cultures: very large numbers of nonadherent cells, frequently in clumps, appear on the base of the vessel and give a "milky" appearance to the culture. Microscopic and flow-cytometric analysis demonstrate $CD19^+$ cells. Cultures, or sets of cultures, behaving in this manner should be discarded without opening

12. Even between normal marrow samples, there can be some variability in the ability to establish LTMC Leukemic bone marrow, in particular, can fail to form stroma *(11)*

Acknowledgments

We are grateful to the contribution of Paul Toor to the first version of this chapter published in 1990, and helpful discussions with colleagues including Xing-Hua Wang, Kathryn M. Matthews, Rakesh Nayar, and members of the Department of Experimental Haematology, Christie Hospital, Manchester, UK.

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*Readers are particularly directed to refs. *2* and *7*, which cover a range of topics relating to long-term culture

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20

In Vitro Clonal Culture of Human Hematopoietic Progenitor Cells

Brian R. Clark, Catriona Jamieson, and Armand Keating

1. Introduction

The human bone marrow is one of the most dynamic tissues in the adult body, generating over 2×10^{11} new blood cells everyday. The production of distinct blood cell lineages from a common pool of hematopoietic stem cells is tightly regulated. Substantial progress in the study of hematopoietic cell ontogeny and marrow biology under normal and disease conditions has resulted from the development of culture methods to detect clonogenic hematopoietic progenitor cells *(1)*.

Clonogenic assays define individual hematopoietic progenitors cells by the phenotype of the daughter cells produced during the formation of a colony in semi-solid culture medium. The formation of colonies is influenced by cytokines including the hematopoietic growth factors and colony stimulating factors. Colony-forming cells (CFC) can be classified by the production of macrophages, granulocytes, erythrocytes, or megakaryocytes in the colony. This gives rise to the nomenclature describing colony forming units (CFU) with a suffix denoting the lineage characteristics of the CFC—e.g., CFU-GM *(2)*, granulocyte/macrophage; -Meg, megakaryocyte *(3)*; -GEMM, granulocyte, erythrocyte, macrophage, megakaryocyte *(4)*. There are two clearly distinguishable erythroid CFC: CFU-E and BFU-E *(5)*. The latter is the "burst forming unit-erythroid," an erythroid progenitor which gives a cluster of discrete subcolonies under typical assay conditions and is thought to be more primitive than CFU-E.

In this chapter, we describe a method that conveniently allows detection and enumeration of hematopoietic progenitor cells capable of colony formation.

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2. Materials

1. Erythropoietin (Stem Cell Technologies, Vancouver, Canada).
2. Ficoll-Paque (Density 1.077 g/mL) (Pharmacia, Uppsala, Sweden)
3. Iscove's modified Dulbecco's medium (IMDM) supplemented according to manufacturer's instructions (e.g., with bicarbonate and L-glutamine)
4. Human plasma (*see* Note 1).
5. Methylcellulose stem cell solution (2.2% [w/v]). (Follow manufacturer's instructions about supplementation, e g , with L-glutamine)
6. Leucocyte conditioned medium or recombinant growth factors (*see* Note 2)
7. Fetal bovine serum (FBS) (*see* Note 3).
8. $5 \times 10^{-3} M$ 2-Mercaptoethanol stock solution.
9. Preservative-free heparin (Gibco BRL, Grand Island, NY, cat. no 15077).

3. Methods

The procedure is outlined diagrammatically in Fig. 1 (*see* Note 4)

3.1. Isolation of Cell Populations

Best results are obtained if the marrow or blood specimen is used within 2–3 h after collection. If this is not possible, the specimen (with preservative-free heparin to prevent coagulation) may be left overnight at room temperature for use the next morning.

1. Dilute sample 1:1 with IMDM (*see* Note 5).
2. Isolate the mononuclear enriched fraction by density centrifugation over Ficoll (*see* Chapter 20). Ensure brake is off during deceleration to preserve interface. Collect the interface cells and wash twice in IMDM.
3. Resuspend in IMDM and count on a hemocytometer (*see* Chapter 1) Adjust cell concentration prior to plating (*see* Note 6)

3.2. Preparation of Colony Assay Mix

1. For each (1 mL) assay prepare the following. 10 µL 2-mercaptoethanol stock (final $5 \times 10^{-5} M$), 300 µL human plasma (final 30% [v/v]), 50 µL leukocyte conditioned media (final 5% [v/v]), erythropoietin (final 2 U/mL), IMDM varies to volume for a total of 500 µL
2. Aliqot 500 µL of this assay mix into a 5 mL tube. Add 100 µL of the cell population to be assayed (*see* Note 6).
3. Using a 1-mL syringe (without a needle) add 0.4 mL of 2 2% (w/v) methylcellulose stock to each assay tube. Cap tube and vortex mixture. This gives the desired final methylcellulose concentration of 0.9% (w/v) Add a 16-gage needle to a 1-mL syringe, remove the mix and plate in the culture vessel (*see* Note 7).
4. Incubate in a humidified atmsophere of 5%CO_2/air for 14 d (*see* Note 8).

3.3. Scoring and Interpretation of Results

Colonies can be monitored with an inverted microscope from d 10 onward without affecting growth. When colony formation is judged optimal, the dishes

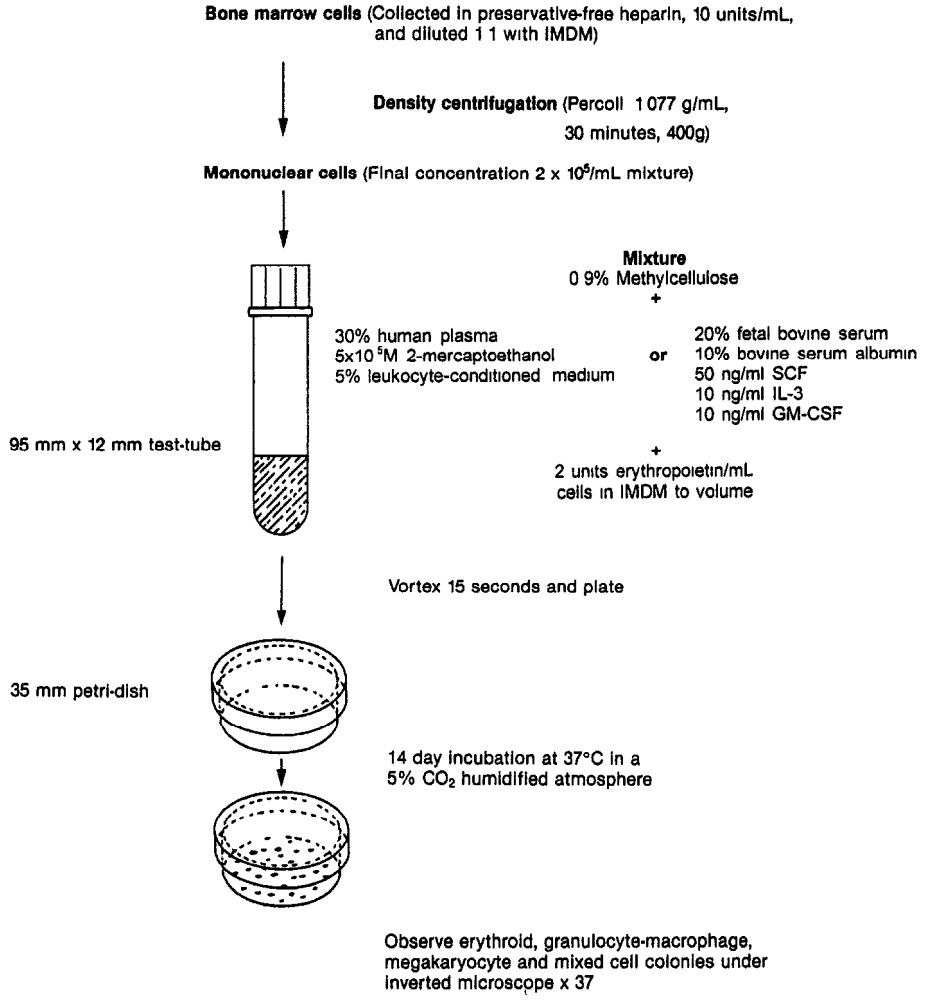

Fig. 1. Scheme for CFC assay

are removed and colonies assessed and enumerated under 37× magnification. Figure 2 shows a mixed cell colony (CFU-GEMM), whereas Fig. 3 contains hemoglobinized erythroid cells (BFU-E) and a loose macrophage colony (CFU- M).

A useful tip when enumerating colonies is to scratch 8–10 parallel lines on the lid of a Petri dish. This lid can be left on the microscope and the base of each dish to be assessed can be fitted into it, allowing an ordered approach to scoring. Similarly, the underside of wells in a 24-well plate can be scratched with 3 or 4 parallel lines using a sharp pair of scissors using the edge of a table to provide a straight edge (this can be done by reaching under the plate and not

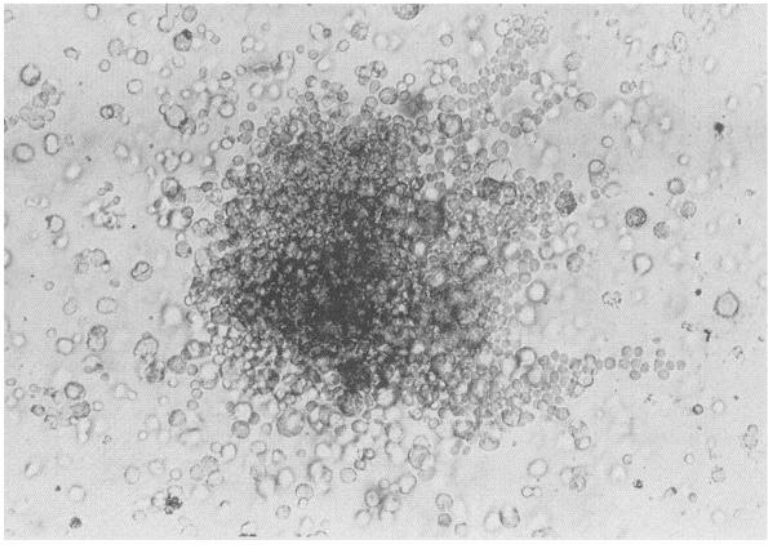

Fig. 2. Light micrograph (×125) of mixed cell colony (CFU-GEMM). The colony contains erythroid cells, granulocytes, megakaryocytes, and macrophages.

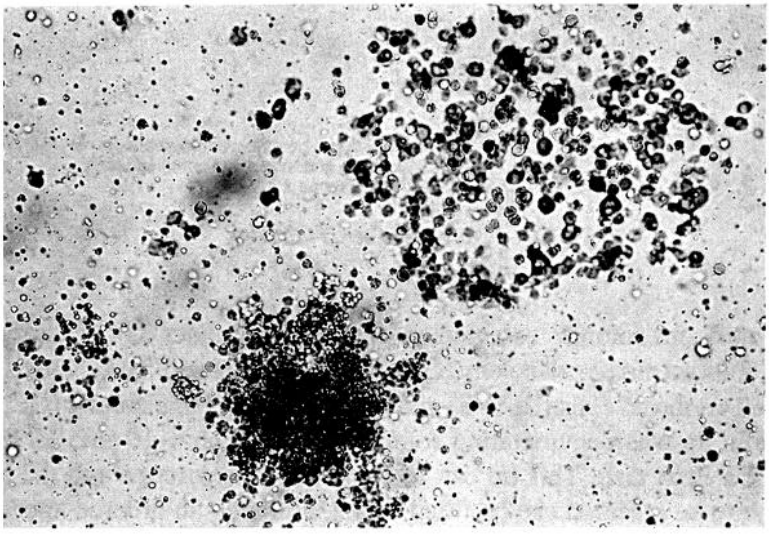

Fig. 3. Light micrograph (×80) of erythroid colony (BFU-E, lower left) and a macrophage colony (CFU-M, note dispersed monocytes-macrophages, upper right).

by inverting the plate[1]) A video and manual describing colony assays are also available from reagent suppliers (Stem Cell Technologies, Vancouver, Canada).

Most workers do not directly compare absolute CFU numbers from results in different laboratories—every laboratory has slightly different conditions. However, colony counts (in optimized assays) may be considered accurate based on the presence of granulocytic and erythroid colonies (BFU-E and CFU-E) in approx 1:2 ratio in normal samples (*see* Note 6).

4. Notes

1. Human AB^+ plasma is theoretically the best type, although donors may not always be available Do not use expired blood bank plasma Human plasma can be obtained by collecting 50 mL of blood in preservative-free heparin from consenting adults. Centrifuge in order to remove the cells and platelets, and remove the supernatant (plasma) Aliquot and freeze at –70°C for up to 6 mo Prior to use, thaw out plasma and centrifuge at top speed in a microfuge for 10 min to pellet cryoprecipitate. Use supernatant in assay. As with all biological material, plasma will have some variability from person to person For consistent assays, a stock may be prepared by pooling plasma from several donors Take appropriate protective measures when handling plasma
2. You can make phytohemaglutinin leucocyte-conditioned medium (PHA-LCM) easily Separate peripheral blood lymphocytes using Ficoll density gradient centrifugation (*see* Section 3 1), wash the cells twice and resuspend at 1×10^6/mL in IMDM 10% FBS Add 1% (v/v) of PHA, and incubate for 7 d at 37°C Remove the cells and debris by centrifugation, collect the supernatant, filter sterilize, and store aliquots at –70°C Do not repeatedly freeze-thaw Test optimal levels of PHA-LCM in colony assays (expected range 2–10% [v/v]) If colony levels are not optimal at 5–10% PHA-LCM, you should make another batch Alternatively, recombinant human hematopoietic growth factors are an excellent source of defined colony stimulation *(6)*. A typical core mixture is 50 ng/mL stem cell factor (SCF, kit-ligand, mast cell growth factor) 10 ng/mL IL-3 and 10 ng/mL GM-CSF IL-6 (10 ng/mL) may also be added These may be obtained from one of several biological suppliers
3. The use of FBS instead of human plasma is an option To avoid biohazard or collection problems associated with human plasma use a mixture of fetal bovine serum (20% [v/v] in mix) and bovine serum albumin 10 mg/mL stock (Boeringher-Mannheim) (10% [v/v] in mix). Omit the mercaptoethanol. This may be the method of choice when megakaryocyte growth is not required. FBS for colony assays should be selected after extensive screening of different batches from several suppliers for activity upon the same sample. Keep the good batch for your colony assays (or long-term cultures; Chapters 16 and 19). You should re-titrate the PHA-LCM for this system. If your work involves the plating of antibody-labeled progenitor cells, it is a worthwhile precaution to heat inactivate serum at 56°C for 25 min to destroy complement This should not affect colony

formation. Serum should always be stored at –20°C until use Aliquot to avoid repeated freeze-thawing.

4 Colony assays using agar as the gelling agent are now rarely performed since methylcellulose has a number of advantages, including ease of handling and the ability to readily allow the removal of individual colonies for analysis.

5. Typical samples are bone marrow aspirates, peripheral blood, or cord blood. Samples should be harvested into containers or syringes containing preservative-free heparin. If you are setting up the assay, cord blood may be a convenient source of tissue with abundant hematopoietic cells Provide tubes containing preservative-free heparin, rather than obtaining samples in standard heparin

6. Changing the cell concentration ensures you do not have too many or too few colonies in assays of different types of samples For example, the incidence of CFU in blood is about 10-fold lower than in bone marrow (incidence/nucleated cell) If any sample is suspected to contain fewer or greater CFU than normal, it is wise to plate a range of cell concentrations Good mid-range cell stock concentrations are 10^6/mL for bone marrow, 5×10^6/mL for blood, and around $1–2 \times 10^6$/mL for cord blood. Thus, the addition of 100 µL of these cell stocks to each 1-mL assay would mean plating 10^5 marrow cells, 5×10^5 blood cells, and $1–2 \times 10^5$ cord blood cells Expect a total of 100–200 colonies from a 1 mL mix of an average marrow However, in order to obtain strictly clonal cultures (colonies that are reliably derived from a single cell), reduce the number of cells plated to obtain approximately 50–100 colonies. When setting up the assay, plate a wide range of cell concentrations. You can use the assay with confidence if there is a linear relationship between the number of cells plated and the number of colonies obtained. Results are typically reported as CFU/10^5 nucleated cells plated.

7 Dispense 1 mL of mix into a 1 × 35-mm Petri dish or 4 wells of a 24-well plate. Ensure an even spread of the mixture by gently rocking the dish by hand

8 Inadequate humidity will lead to drying of the methylcellulose and poor colony formation. This can be prevented by not plating in the edge wells of a 24-well plate and adding sterile water instead or by arranging 35-mm Petri dishes within a larger dish and adding an open dish with sterile water at the centre of the large dish to provide humidity

Acknowledgments

We acknowledge the contributions of Paul Toor and Xing-Hua Wang to the development of these techniques in our laboratory.

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21

High Proliferative Potential Colony-Forming Cells

Ivan Bertoncello and Anthony B. Kriegler

1. Introduction

High proliferative potential colony-forming cells (HPP-CFC) are among the most primitive hematopoietic cells yet identified in vitro They share many of the properties of transplantable primitive hematopoietic stem cells (PHSC) with long-term hematopoietic reconstituting ability in vivo *(1)*, and the HPP-CFC assay is routinely used in many laboratories as a surrogate, short-term, in vitro assay for the murine PHSC compartment.

HPP-CFC were first identified in mouse bone marrow by Bradley and Hodgson *(2)* by their ability to preferentially survive treatment with high doses of 5-fluorouracil (5FU), and to form large colonies in agar cultures >0 5 mm in diameter containing an average of 5×10^4 cells. These HPP-CFC grew in response to a mixture of pregnant mouse uterus extract (a source of CSF-1) together with a second crude source of growth factors such as medium conditioned by human placenta or spleen, or the 5637 cell line *(2,3)* It was soon realized, however, that the marrow contained at least two populations of HPP-CFC with differing growth factor requirements, and potentiality *(3)*. With the advent of recombinant cytokines, and the development of sophisticated cell separative strategies able to resolve closely related subpopulations of primitive hematopoietic cells on the basis of their cell surface phenotype, cell cycling characteristics, or the uptake of the fluorochromes, rhodamine 123 (Rh), or Hoechst 33342 (Ho) *(1,4–6)*, it has been possible to define the HPP-CFC compartment more precisely.

The most primitive of these two populations (HPP-CFC-1) *(2,7)* is:

1. Responsive to the factor combination, CSF-1 plus IL-3 plus IL-1 *(8)*,
2. Largely noncycling as it preferentially survives treatment with high doses of 5FU and is concentrated in the Sca-1$^+$, Rh-dull, or Ho-dull fractions after sorting *(4–6,9)*,

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

3 Multipotential *(3)*,
4 Detectable at an incidence of less than 1 in 400 cells *(2)*, and,
5. Able to proliferate and differentiate to form a second more mature population designated HPP-CFC-2 *(3,7)*

The more mature HPP-CFC-2 population is:

1. Responsive to the combination, CSF-1 plus IL-3 *(3,7)*,
2 Largely in cycle as it is preferentially killed by high doses of 5FU and is enriched in the Rh-bright or Ho-bright fractions after sorting *(4–6)*, and
3 Gives rise to single factor-responsive, lineage restricted GM-CFC *(3,7)*

Single factors, including CSF-1, GM-CSF, and IL-3 also stimulate a HPP-CFC subpopulation that is more mature than HPP-CFC-2 *(2,7)*, and closely related to lineage restricted hematopoietic progenitor cells of low proliferative potential.

More recently we have identified HPP-CFC which require four growth factors. These have been designated pre-HPP-CFC-1, and appear to be the most primitive hematopoietic progenitor cells detectable in agar culture *(7)*. However, HPP-CFC subpopulations requiring more than three growth factors cannot be resolved using current cell separative strategies.

It is evident that a great deal of plasticity exists in the growth factor requirements of HPP-CFC. For example, IL-6, stem cell factor (SCF) and GM-CSF can also be included in a variety of three and two growth factor combinations to stimulate HPP-CFC-1 and -2, respectively *(7,9)*. Other workers *(10,11)* have found that the six factor combination of CSF-1, G-CSF, GM-CSF, IL-1, IL-3, SCF, or these six factors plus basic fibroblast growth factor (bFGF) was optimal for the growth of these very early HPP-CFC. Using these combinations of growth factors, HPP-CFC can be grown in serum-free medium *(10)* to form colonies containing macrophages, granulocytes and megakaryocytes *(11)*. Other growth factors such as IL-4, IL-11 *(12)*, and Flt3/Flk2 ligand *(13)* have also been implicated in the stimulation of early HPPCFC populations.

The agar culture assay will detect pre-HPP-CFC-1, HPP-CFC-1, HPP-CFC-2 plus the single factor-responsive HPP-CFC in the six factor system, the last three populations in the presence of three factors (e.g., CSF-1, IL-3, and IL-1), the last two with two factors (e.g., CSF-1 and IL-3) and the last population with a single factor. As a general rule, synergism between growth factors is an absolute requirement for stimulation of HPP-CFC: the more primitive the HPP-CFC subpopulation, the greater the number of growth factors required for optimal growth.

Transforming growth factor beta (TGF-β) inhibits both HPP-CFC-1 and HPP-CFC-2 *(14,15)*, but MIP-1α has no effect *(15)*. Since these experiments

were carried out using Rh-dull, Ho-dull, Lin^-, $Sca\text{-}1^+$ cells, it can be concluded that these inhibitors probably have the same effects on pre-HPP-CFC-1, which are also present in these cell fractions.

2. Materials

1. Agar gels: Stocks of 1% and 0.66% Difco Bacto-agar (*see* Note 1) are prepared in batches sufficient for use over 1 mo. Weigh out the solid agar into a sterile conical screw-topped flask containing a sterile magnetic stirrer bar. Add the requisite amount of sterile distilled water. Stir and heat until the agar is melted and just commences to boil, and then cool at room temperature. Repeat this procedure, and then store agar at room temperature until required. It is important to make sure the caps are loose during melting and boiling to prevent shattering of the flasks.
2. Culture medium: Double strength medium is made from powdered stock. We use the alpha-modification of Eagle's MEM, but any basic medium that can be made to double strength may be used. Alpha MEM medium has consistently given better results than most other media. Since we use large quantities of medium, we routinely prepare a more concentrated stock with vitamin supplementation. This concentrated stock is stored frozen, and used to prepare double-strength medium each day as follows:
   a. Concentrated stock. Dissolve powdered alpha medium without nucleosides sufficient for 10 L of medium in approx 1500 mL sterile distilled water by stirring for 4–6 h. Add 100 mL Eagle's MEM vitamins (100X) and 10 mL phenol red (1% aqueous). Determine the osmolality of the concentrated medium and dilute to 1300 mosmol (*see* Note 2), and gas with CO_2. Sterile filter (0.22-µm filters) and store frozen at –20°C.
   b. Double strength medium for agar cloning: 32 mL Concentrated stock, 2 mL sterile glutamine (200 m*M*), 40 mL sterile serum (*see* Note 3), 8 mL sterile sodium bicarbonate (5.6%), 8000 U gentamycin (*see* Note 4), and 18 mL sterile distilled water. This formula is for 100 mL of double-strength medium ready for use (*see* Note 5).
3. Balanced salt solution (BSS):
   a. 1 5*M* HEPES buffer. Dissolve 35.75 g HEPES (acid salt) and 0.2 mL phenol red (1% [w/v]) in approx 60 mL distilled water and titrate to pH 7.2 with NaOH. Make up to 100 mL and filter through a 0.22-µm filter and store at 2–8°C.
   b. Concentrated BSS stock: This is prepared as a 10X concentrated stock solution containing 80 g/L NaCl, 4 g/L KCl, 10 g/L glucose, 20 mL/L of a 1% (w/v) solution of phenol red, and 2×10^5 U/L gentamycin. The concentrated stock is filtered through a 0.22-µm filter and stored at 2–8°C.
   c. Isotonic BSS: Add 10 mL HEPES buffer (1.5*M*) and 5 mL $NaHCO_3$ (5.6% [w/v]) to 100 mL of 10X concentrated BSS stock. Distilled water is added to bring the volume to 1 L, and the solution is filtered through a 0.22-µm filter and stored in aliquots of 100 mL at 2–8°C.

5 Growth factors are prepared at suitable concentrations (*see* Section 3.2) in single strength medium
6. Incubator boxes (*see* Note 6) Since the dishes should be incubated at 7% oxygen gas phase, we routinely use 5 L capacity polystyrene plastic boxes with rigid, tight fitting lids into which perforated aluminium trays are placed. The tray is 2 cm above the bottom of the box Two diagonally opposite holes (18-gage needle size) are drilled in the lids of the boxes for gassing. Boxes are washed and dried, and aluminium trays are autoclaved, between incubations
7. Gas mixture Plastic boxes are gassed with a gas mixture of 5% 0_2, 10% CO_2, and 85% N_2, which is ordered from regular suppliers (*see* Note 7)

3. Methods

3.1. Harvesting of Bone Marrow Cells

Groups of at least five mice are used to provide femurs. The femoral shafts are stripped of all tissue and are flushed from one end and then the other with chilled BSS containing 2% newborn calf serum using a 1-mL syringe fitted with a 23-gage needle. Marrow cells are collected at a concentration of one femur equivalent per milliliter (approx 2.0×10^7 cells/mL) and kept on ice until used.

3.2. Plating Procedure (Double Layer)

Bone marrow cells can be cloned in nutrient agar using either a double, or a single layer system. We routinely use a double-layer culture system with growth factors included in a 1 mL underlay, and cells plated in a 0.5 mL overlay, since we find that colony formation is better in this system than in the single layer system.

1 Sterile growth factors to give the desired final concentrations are added directly to 35-mm Petri dishes (*see* Note 8). Typically we find the following concentrations of recombinant growth factors to be optimal for HPP-CFC growth in the 1 5 mL double layer culture system 1×10^3 U/dish rhCSF-1, 4000 U/dish rhIL-1α, 25 U/dish rmIL-3, 100 ng/dish rrSCF, 100 ng/dish rhIL-6, 20 ng/dish rmGM-CSF, 100 ng/dish rhG-CSF. However, all growth factor preparations should be tested for the concentrations necessary to achieve optimal colony formation
2. Melt the 1% agar in a conical flask to just boiling and put in a 37°C bath to cool
3 Calculate the amount of double-strength medium for the number of dishes to be plated allowing for additional dishes to ensure that sufficient medium is available Decant this volume in a conical screw topped flask and warm in the 37°C bath
4 When the agar is cooled to 37°C (test it by agitating it in the flask and feeling the temperature), add an equal volume of agar to the double-strength medium and mix thoroughly (either by pipet or with a sterile magnetic stirrer bar previously placed in the flask) and dispense 1-mL aliquots to each of the dishes, shaking the dishes from side to side to ensure complete coverage of the dish and mixing with

growth factors (*see* Note 9). This basal layer should gel within a few minutes at room temperature (20°C)

5 For the overlay (containing the bone marrow cells), melt and just boil the 0.66% agar and place in the bath to cool (*see* Note 10). Add the required number of bone marrow cells to prewarmed medium (double-strength) and immediately add an equal volume of agar After mixing thoroughly, dispense 0 5 mL aliquots to the dishes—no shaking is required After a few minutes the upper layer should gel and the dishes can be incubated (see Note 11) For normal bone marrow we routinely use 2 5 × 10^3 cells/dish; for FU_{2d} bone marrow, 2 × 10^4 cells/dish; and for FU_{8d} bone marrow, 1 × 10^3/dish For fractionated enriched bone marrow populations, the cell numbers are reduced appropriately (*see* Note 12).

6 Fifty milliliters of sterile distilled water are placed under the aluminium tray of the plastic box to provide humidity during incubation. Stack the dishes in the box. Each box can take up to 150 dishes

7 The box lids are then taped on with three layers of tape which is left taut and wound around the lid-bottom box junction. No wrinkles should be evident and the taping should be finished by smoothing it down over the junction

8 The boxes are then gassed at the rate of 2.5 L/min for 30 min to give a final O_2 concentration of 7% The gassing holes are sealed with three layers of tape, and the box is placed in the 37°C room or incubator (*see* Note 7)

9. Colonies are scored at 14 d of incubation (*see* Notes 13–17) using a dissecting microscope at 20X magnification with a calibrated grid in one eyepiece to measure colony diameters. Normally the colonies originating from HPP-CFC are clearly visible without magnification but are checked to observe that they are tightly packed with cells (*see* Note 14)

4. Notes

1. Various agarose preparations may be used instead of agar, provided that they gel adequately at room temperature. However, with myeloid cells cloning efficiencies, and colony growth, in agarose are lower than in agar. Likewise, sterilization of agar by autoclaving should be avoided as it reduces colony formation *(16)*

2 It is important to measure the osmolality of media and sera to be used in order to ensure reproducibility and uniformity of culture conditions On testing a range of media, we have found a final medium osmolality of 280–300 mosmol to be optimal for mouse bone marrow colony formation

3. We routinely use medium supplemented with 20% serum. Batches of sera must be tested to choose a pool suitable for the next 6 mo to 1 yr work. We often find batches of newborn calf serum, to be better than fetal calf serum for clonal agar culture. Recent batches of Hyclone iron-loaded newborn calf serum have consistently given good results However, it has recently been shown that murine HPP-CFC will grow efficiently in serum-free conditions using cocktails of up to seven recombinant cytokines *(11)*.

4. Gentamycin has been used routinely for several years to replace penicillin and streptomycin because of its spectrum and stability.

5 The bicarbonate concentration we use gives strong buffering with 10% CO_2 in the gas phase
6. Although incubators are available which allow the experimenter to regulate the concentration of O_2, CO_2, and N_2 in the gas phase, individually gassed plastic boxes have several advantages
   a Each experiment can remain undisturbed over the incubation period.
   b Contamination during incubation is not a problem.
   c. They are cheap and, if a 37°C room, or nonhumidified incubator is available the number of experimenters who can do clonogenic experiments is greatly increased for little change in cost
   d They can be used to test numerous gas concentrations for various cell and culture types.
7 The gassing of the incubation boxes is calculated to pass 75 L of gas through a box with a volume of 5 L A brief passage of gas will not suffice to achieve the desired optimal O_2 and CO_2 concentrations
8. The total volume of growth factor preparations added to the underlay should preferably be less than 0.15 mL, and no more than 0.3 mL/dish (10–20% of the total volume of the double layer system) in order to avoid the gels losing their firmness
9. Continuous pipeters can be used for plating large numbers of dishes (e.g , above 100 dishes)
10. For the single layer system the 0.66% agar is melted and placed in the 37°C bath The volume of double-strength medium is placed in another flask and warmed, the requisite numbers of cells are added, and the equal volume of agar added immediately mixed and plated.
11 The most common failure of cultures arises from overheating pipets during routine flaming, or using agar that is too hot On the other hand, care must be exercised to ensure that the agar-medium mixture does not gel before dispensing into the dishes.
12. It has been observed that colony formation with fractionated enriched populations of bone marrow is usually better than with unfractionated marrow *(17)*.
13 It is essential to ensure that low cell densities are used if optimal colony growth is to be achieved When the incidence of cells present at low frequency in the population is being measured, the number of replicate dishes must be increased rather than the cell density per dish. In practice, 20–50 colonies/dish is an optimum to achieve, and especially with an enriched population, dishes should be set up at different cell densities The use of low cell densities will prevent
   a. Secondary effects occurring as a result of stimulation of accessory cell production of growth factors and/or inhibitors; and
   b The inhibition of colony formation or colony growth that can occur when too many colonies develop in the dish.
14 Colonies from different bone marrow populations may develop at different rates For example, using FU_2 bone marrow cells, clones start developing at 6 d of incubation and grow rapidly to form large colonies over the next 4–8 d. On the

other hand, colonies from FU_{8d} bone marrow develop more rapidly to form large colonies by 8–10 d of incubation

15 HPP-CFC have been detected in all mouse strains tested

16 Since colony formation takes place over a lengthy incubation period (10–14 d), the HPP-CFC may develop by sequential action of the growth factors initially placed in the cultures on cells generated within the colonies during colony development Also, combinations of other growth factors than those discussed here may detect cells with high proliferative potential with their actions being either additive or synergistic

17. At the present time the size of the colony, and more particularly the number of cells generated per single HPP-CFC, are the criteria for detection of these cell types. The development of large colonies in the primary cultures at 14 d does not exhaust their total proliferative potential since these colonies can be sampled, dispersed into single cells, and replated to give further colony formation, although the secondary colonies are smaller and have more restricted growth factor requirements. For example, large colonies developed from $FU_{2d}BM$ cells with CSF-1 plus IL-3 plus 5637 conditioned medium can be replated with CSF-1 alone to ultimately yield 27×10^6 cells/initial HPP-CFC

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22

Production of Human and Murine Eosinophils In Vitro and Assay for Eosinophil Differentiation Factors

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1. Introduction

Bone marrow cells from a number of animal species have been used extensively in liquid and semisolid cultures to study hemopoiesis and to produce functional mature cells and factor-dependent cell lines (for review *see* ref. *1*). Neutrophils and macrophages are produced without added growth factors from murine long-term bone marrow cultures *(2)*, whereas lymphoid cells *(3,4)* and megakaryocytes (reviewed in ref *5*) can be induced under certain conditions.

When bone marrow cells from mice or humans are established in tissue culture in the presence of eosinophil differentiation factor (EDF), mature functional eosinophils are produced and liberated into the nonadherent cell population *(6,7)*. This factor was originally proposed as interleukin-4 (IL-4) *(8)*, but is now accepted as interleukin-5 (IL-5). Other names for IL-5 are eosinophil colony stimulating factor (CSF-Eo), B-cell growth factor type II (BCGFII), and T-cell replacing factor (TRF).

Eosinophils are produced for only a relatively short time when marrow is cultured in the presence of IL-5. This suggests that IL-5 stimulates the differentiation of eosinophils from existing progenitor cells present in the marrow with no recruitment of eosinophil progenitor cells from stem cells. When the animal has a pronounced eosinophilia, subsequent culture of the marrow results in enhanced eosinophil production compared to cultures established from normal marrow. This is interpreted as an increase in eosinophil progenitors in the animal. We use the Helminth parasite *Mesocestoides corti* Hoeppli

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

1925 to effect such an increase in eosinophil progenitor numbers in the mouse. This parasite has been used extensively to study host/parasite interactions and causes a well-documented eosinophilia *(9)*, with peak numbers of eosinophil progenitors appearing in the bone marrow 10 d after infection *(10)*.

The assessment of eosinophil numbers from cultures can be done in one of two ways: total cell counts (obtained from an electronic particle counter, e.g., Coulter counter, or by using a hemocytometer) and the percentage of eosinophils (obtained from a differential count on Giemsa-stained smear or cytocentrifuge preparation) are used to calculate the number of eosinophils, or by assaying for eosinophil peroxidase, which can be related to eosinophil numbers *(11)*.

A microplate modification of the culture system *(12)* provides a method for assaying sources of IL-5. This assay is based on assessment of eosinophil numbers by quantifying eosinophil peroxidase in microplate bone marrow cultures that have been incubated with IL-5 samples. This assay also detects the eosinophil differentiation activity of granulocyte macrophage colony stimulating factor (GM-CSF) and IL-3. The B-cell activity of mouse IL-5 can be measured using the B-cell lymphoma (BCL1) cell line (8) though this assay also detects IL-4 (B-cell-stimulatory factor [BSF1]).

2. Materials

1. Parasite: The second stage larvae (tetrathyridia) of the Cyclophyllidean Cestode *M corti* are maintained by intraperitoneal passage in mice, where it reproduces itself vegetatively The larvae can be stored in Dulbecco's phosphate-buffered saline (PBS) "A" at 4°C for several weeks, and for longer periods if fetal calf serum (FCS) is added *(13)* To our knowledge there have been no recorded cases of human infections with *M corti*, although there have been several cases of infection by other species of *Mesocestoides (14,15)* Parasites for reinfecting mice are harvested from mice that have been infected for several weeks. The only noticeable visible effect of the infection is an increasing abdominal distension It may be necessary to occasionally passage the parasite into another strain of mouse (e.g., CBA) because it seems to lose the ability to stimulate high eosinophilia after several months passage through the same strain
2. Mice: We routinely use Balb/c nimr mice, 6–8 wk-old, that are maintained under specified pathogen-free (SPF) conditions and are allowed free access to food and water Any infections the mice contract before the marrow is harvested for culture may result in changes in the cell numbers and types seen in the marrow cultures.
3. Media: The basic medium used is RPMI 1640 purchased in powder form and reconstituted as recommended by the manufacturer. We use two basic forms of RPMI as indicated:

	HEPES Medium	Culture Medium
HEPES buffer	20 m*M*	10 m*M*
Sodium bicarbonate	None	24 m*M*
Glutamine	2 m*M*	2 m*M*
Sodium pyruvate	None	1 m*M*
Penicillin	100 U/mL	100 U/mL
Streptomycin	100 µg/mL	100 µg/mL
Monothioglycerol (Sigma)	None	75 µ*M*

The above media are stable at 4°C for several months. Glutamine is unstable, so fresh glutamine is added from a frozen (–20°C) 200 m*M* stock solution and the medium used within 2 wk. Glutamine is a general requirement in tissue-culture medium and has been found to be necessary for hemopoietic cell differentiation *(16)*

The media are further supplemented and used as follows.

a Bench medium HEPES medium is supplemented with newborn calf serum to 5% (v/v). This is used for cell and tissue collections and preparation, and is stored at 4°C It has the advantage over bicarbonate-buffered medium in that it does not change its pH while outside a CO_2 environment

b. Bone marrow culture medium Culture medium is supplemented with $10^{-6}M$ hydrocortisone and 15% FCS. The pH of this medium is maintained by culturing in an atmosphere of 5% CO_2 in air

Media additives:

a. Hydrocortisone. A $10^{-2}M$ (48 45 mg/mL) stock solution of hydrocortisone sodium succinate in PBS is filter sterilized (0 22-µm pore) and stored in 50–100 µL aliquots at –20°C. 10 µL stock solution is added to 100 mL bone marrow culture medium Any remaining stock hydrocortisone solution is not refrozen, but discarded

b FCS· This has to be selected for optimal eosinophil growth, since some batches result in negligible eosinophil production in cultures Whether this is caused by inhibitors or lack of growth factors is not known It is preferable to test the FCS in both the EDF assay and in long-term cultures

c Gentamicin· A stock solution of 5 mg/mL gentamicin sulphate in PBS is filter sterilized, stored at –20°C, and diluted 1.100 into medium for use Gentamicin is occasionally required when assaying column fractions for IL-5

4. Sources of IL-5 Native murine IL-5 can be obtained from mitogen- or antigen-stimulated spleen cells *(11)*, T-cell clones *(17)*, or the EL4 lymphoma cell line *(18)* However, these sources also contain other lymphokines The T-cell hybrid NIMP-TH1 produces IL-5 in the apparent absence of other known lymphokines *(19)* and has provided the most useful source of IL-5 until recombinant material from transfected monkey COS cells became available *(20)*. The cross-species reactivity of murine IL-5 *(21)* was employed on studies with human cells until recombinant human IL-5 became available *(22)*. IL-5 has also been detected in the serum of parasitized animals *(8)*, and recent work has demonstrated the presence of a factor stimulating human eosinophil differentiation in serum from

patients undergoing eosinophilia *(23)* Recombinant mouse and human IL-5 are now available from a number of commercial sources

IL-3 and GM-CSF have some eosinophil differentiation activity *(6)* so limited number of eosinophils can be produced in cultures using WEHI-3-conditioned medium or commercially available GM-CSF When sources of IL-5 such as crude spleen conditioned medium that contain other lymphokines as contaminants are used, large numbers of neutrophils and/or macrophages are produced so that eosinophils represent only a small percentage of the nonadherent cells. Methods detailing production of the above sources of IL-5 are given in ref. *24*

5 Eosinophil cultures A Class II Microbiological safety cabinet is required for human cultures, and all waste materials should be autoclaved Murine cultures are established under standard tissue-culture conditions. A gassed 37°C incubator is required, which has to be humidified for agar, cluster, and microplate cultures

a. Agar cultures We use Difco Bacto-Agar that is preselected for colony growth. A 5% (w/v) stock solution is prepared by suspending the agar in distilled water and placing in a boiling water bath for 5 min The agar is stored at room temperature We do not autoclave the agar since this seems to introduce some toxicity into the system Leukocyte migration plates (Sterilin, Teddington) are used in place of Petri dishes as this allows the whole agar culture to be easily recovered and stained for assessing colony number and type The small culture volume (400 μL) results in savings in materials and reagents A square 100-m*M* Petri dish is used as the container for the leukocyte migration plate

b. Long-term cultures: These cultures are established in either flasks or Cluster plates We use 25- or 80-cm^2 flasks from which large numbers of eosinophils can be produced for functional studies Most experimental work is done using 24-well Cluster plates. A cytocentrifuge is useful to prepare slides for staining and differential counts, the morphology being clearer than on smears

c Microplate cultures. The cultures are established in 96-well round (U) bottomed microtiter plates. The flat bottomed 96-well microtiter plates are not satisfactory for this assay system

6 Eosinophil peroxidase assay

a. Peroxidase buffer. 0 05*M* Tris-HCl, pH 8 0 Filtered (0 45-μm pore) and stored at room temperature

b. o-Phenylenediamine (OPD). a stock solution of 10 mg/mL in distilled water is stored in 1-mL aliquots at –70°C, where it is stable for several months

c. 30% (w/v) Hydrogen peroxide stored at 4°C

d. Triton X-100. a 10% (v/v) stock solution in water is stored at 4°C

e. 4*M* Sulphuric acid.

f. Complete substrate solution: to 48.5 mL peroxidase buffer add 1 mL OPD stock solution, 0 5 mL Triton X-100 and 6 μL hydrogen peroxide OPD is light sensitive and so this solution should not be prepared until it is required An automatic microplate reader with a 490-nm filter is recommended for reading the plates

7 Giemsa Stain Buffer; Sorensens buffer concentrate pH 6.8, diluted to 3 3 m*M* in distilled water. Giemsa (10%) stock solution is diluted 1·5 with buffer for use
8. Congo Red Stain Dissolve 5 g Congo Red in 50 mL distilled water then add 50 mL ethanol. The solution is stable and can be re-used. Different batches of Congo Red seem to vary in their ability to stain eosinophils.
9. Toluidine Blue Stain Add 1 g Toluidine Blue to 100 mL methanol Acidify by adding 5 mL 2*M* HCl The solution is stable and can be reused.
10 Luxol-Fast-Blue Stain. Add urea to 70% ethanol until saturated (approx 250 g/L), then filter through a Whatman No. 1 filter paper. Dissolve 1 g Luxol-Fast-Blue in 100 mL of the urea-saturated 70% ethanol The solution is stable and can be re-used
11 Harris' Hematoxylin Stain: Dissolve 1 g hematoxylin in 50 mL ethanol, and 100 g aluminum ammonium sulphate (or aluminium potassium sulphate) in 1 L distilled water (with gentle heating). Add the hematoxylin solution to the salt solution and bring to the boil rapidly CAREFULLY add 2.5 g mercuric oxide (a violent reaction may occur if added too rapidly) and allow to cool Filter Add 4 mL glacial acetic acid to each 100 mL of stain and store at room temperature. The stain can be reused extensively. Alternatively, obtain from a commercial source

3. Methods

3.1. Parasite Passage and Harvest

With the parasite in a plastic Universal tube in PBS:

1 Aspirate 100–200 µL of PBS into a 1-mL syringe and expel the air bubble, allow the parasites to settle in the Universal at unit gravity, then insert the end of the syringe into the parasite pellet and fill the syringe
2 Invert the syringe and allow the parasites to settle Expel excess PBS and refill with more parasites if necessary.
3 Fit an 0.8 × 40 mm needle, expel air, and excess PBS, then inject 100 µL parasite ip into each mouse
4. To harvest the parasite, kill the mouse by cervical dislocation, then cut the abdominal skin and expose peritoneal wall (*see* Section 2 , item 1).
5. Using a 5- or 10-mL syringe with a 0 8 × 40-mm needle, inject 5–10 mL of PBS into the peritoneum Withdraw the parasite/PBS into the syringe
6. Expel parasite/PBS into a sterile plastic universal bottle
7. Wash the parasites several times by filling the bottle with PBS, allow the parasites to settle, and pour off the supernatant.
8 Use the parasites to infect more mice (*see* Section 1) and store the remainder at 4°C.

3.2. Marrow Collection and Cell Preparation

3.2.1. Mouse Marrow (see Note 1)

1. Fill a suitable container with 70% ethanol and immerse a pair of scissors and forceps Use a tissue to dry the instruments before use and replace them into the alcohol between each procedure
2 Kill mice by cervical dislocation

3 Fill a 5-mL syringe with bench medium and fit a 0 4 × 12-mm needle We use the syringe type that is packed in an outer polypropylene case, the case being used as a sterile "home" during the procedures.
4 Wet the animal's fur with 70% ethanol and place the animal on its back. Pull up the abdominal skin with forceps and make a cut across the abdomen with the scissors held vertically. Pinch the skin on each side of the cut between thumb and forefinger, and enlarge the incision by pulling the skin toward the head and tail The incision should enlarge around the animal and the skin finally break at the animals back Continue to pull the skin back toward and over the tail and legs until the muscles of the upper and lower legs are exposed. Complete the skinning by grasping the tibia/fibula with forceps and the skin with the other hand and pulling the leg from the skin until the foot is clear and the whole leg completely skinned.
5 Remove the legs from the animal by a combined movement that both dislocates the leg from the pelvis and cuts the muscles, ideally leaving the leg complete with femoral head
6 Holding the femur with forceps, cut across the femur with the scissors to remove the head
7. Flush out the marrow by holding the femur with forceps (foot held upwards) and inserting the needle as far as possible up into the femur, being careful not to put the needle through the knee joint With the needle in the femur, bend the needle to an angle of about 45° to prevent the cells running down the outside of the needle and onto the syringe Position the leg above the collection tube and when the medium is injected into the femur the cells will be flushed out and drip into the tube The cells often congregate as a lump at the end of the bone, so withdraw the needle and touch the cut end of the femur onto the tube and dislodge the cells using the syringe needle and flush them into the tube Pool the marrow from several mice into one tube
8 Centrifuge the cells (300g for 8 min) and remove the supernatant
9 Resuspend the cells in 5 mL of medium and break up any large cell clumps by repeated aspiration into a 10 mL pipet If the cells are not to be established in culture immediately, then resuspend them in bench medium, otherwise use bone marrow culture medium
10 Aspirate the cells into a 10-mL syringe via a 0 8 × 40-mm needle and expel them back into a tube through a 0 4 × 12-mm needle Count the cells and adjust to the required density.
11. A differential count can be done to assess the degree of marrow eosinophilia (*see* Note 2).

3.2.2. Human Marrow

Marrow can be obtained from aspiration of the iliac crest or the sternum (during cardiothoracic surgery) or from ribs removed during thoracotomy. Use of marrow samples for research purposes requires the informed consent of the patient and the approval of the local Hospital Ethics Committee.

1 Resuspend marrow in bench medium to 1–2 × 10^7 cells/mL and layer 10 mL cells onto 10 mL Ficoll-Paque in Universal tubes.
2 Centrifuge at 600*g* for 35 min at room temperature. Transfer the interface cells (mononuclear cells) to a new tube and add bench medium
3. Centrifuge (600*g* for 10 min) and resuspend pellet in bench medium
4. Centrifuge (500*g* for 10 min) and resuspend pellet in bone marrow culture medium at 10^6 cells/mL. Approximately 0 5–1 5 × 10^7 mononuclear cells are obtained from 1 mL of normal marrow aspirate

3.3. Eosinophil Cultures

3.3.1. Murine Microplate Cultures (see Note 3)

1. Adjust the marrow cell concentration to 1 × 10^6 cells/mL in bone marrow medium and aliquot 10 µL of sample (or sample dilutions if titrating) into duplicate microplate wells.
2. Add 100-µL cells/well (including several wells without IL-5 to use as control cultures) and incubate in a humidified atmosphere at 37°C and 5% CO_2 in air
3. After 5 d assay the cultures for eosinophils by either total cell counts and differential counts or by peroxidase (*see* Section 3 5.).

3.3.2. Human Microplate Cultures

1 Set up cultures as for mouse microplate cultures (Section 3 3 1., steps 1 and 2 above)
2 Every 7–10 d carefully aspirate 50 µL of medium and replace with fresh medium containing sample
3 After 21–28 d in culture, determine total cell numbers (Coulter counter or hemocytometer, *see* Chapter 1) and perform differential cell counts on a cytocentrifuge or smear preparation, or perform a peroxidase assay.

3.3.3. Agar Cultures (see Note 4)

1 Melt the agar in a boiling water bath and hold at 45°C
2 Adjust the bone marrow cells to 2 × 10^5 cells/mL (or dilutions of cells if required) in bone marrow medium and hold in a 37°C water bath
3 Aliquot 40 µL of IL-5 or other growth factors or their dilutions into each well of a leukocyte migration plate.
4 Pipet 1 vol of agar into 15 vol of the cell suspension, mix well, and immediately aliquot 400 µL/well
5 Place the plate into the upturned lid of a 100-mm square Petri dish containing a moist piece of tissue or filter paper and cover with the square Petri dish base
6 Allow the agar to solidify, putting the plates for a short time at 4°C if the laboratory temperature is high Place in a humidified incubator at 37°C in 5% CO_2 in air.
7. Fix and stain mouse cultures after 5–7 d, and human cultures after 14–21 d (*see* Section 3.4.).

3.3.4. Long-Term Cultures (see Note 5)

1 Adjust the bone marrow cells to 1 5 × 10^6 cells/mL If large numbers of eosinophils are required within 1 wk use marrow from parasitized animals, 9–20 d postinfection
2 Aliquot the cells into flasks or Cluster plates, 10 mL of cells for a 25 cm^2 flask, 30 mL for an 80 cm^2 flask, or 1-mL/well for 24-well cluster plates
3 Add IL-5 at a predetermined optimal dilution and place in a 37°C incubator in 5% CO_2 in air The Cluster plates will require a humidified incubator.
4 Every 6–7 d, gently tap the cultures to dislodge the nonadherent cells and remove all the supernatant and nonadherent cells Add fresh medium containing IL-5 and place back into the incubator
5 Determine the total cell number of nonadherent cells and prepare a smear or cytocentrifuge preparation.

3.4. Fixing and Staining the Agar Cultures

We find it necessary to stain the cultures to accurately count eosinophil colonies since assessing the cultures by morphology alone under the inverted microscope is misleading as we have found both "tight" and "very loose" colonies of eosinophils. Mouse cultures are stained using Congo Red and counterstained with either Harris hematoxylin or Toluidine Blue. We have had little success using Luxol-Fast-Blue for staining mouse agar cultures. Human cultures can be stained with any of these stains including Luxol-Fast-Blue. Colonies are defined as clusters containing more than 40 cells.

3.4.1. Preparation of Agar Disks

1. Set the leukocyte migration plate at an angle of about 30° in a retort stand With a slide held horizontally and resting against the rim of a well, direct the agar disk onto the slide by flushing with a stream of PBS from a wash bottle It is possible to mount three agar discs on one standard 26 × 76-mm microscope slide
2 Cover the agar disk(s) with a piece of dry Whatman No 1 filter paper and put onto a warm plate to dry Do not have the warm plate too hot
3 Remove the filter paper just before it is completely dry and allow the agar disc to dry completely Fix in fresh methanol for 15 min before staining (*see* Section 3.4 1.)

3.4.2. Eosinophil Stains

1 For Harris hematoxylin and Congo Red, place the fixed slide in acidified Harris hematoxylin for 5 min. Wash under gently running tapwater until blued. Place in Congo Red and stain for 15 min. Wash briefly under gently running tapwater and dry. Nuclei stain blue, eosinophil granules red.
2 For Congo Red and Toluidine Blue, stain the fixed slide for 10 min in Congo Red. Wash in 50% ethanol for 5 min Stain in Toluidine Blue for 5 min. Rinse in water and dry. Eosinophil granules stain reddish-brown, mast cell granules dark blue Neutrophils and macrophages should not have any cytoplasmic staining

3 For Luxol-Fast-Blue, stain the fixed slide for 1.5 h in Luxol-Fast-Blue Wash under gently running tapwater for 3 min and dry Stain with Harris hematoxylin for 2 min (30 s for cytocentrifuge preps) Wash under gently running tapwater for 3 min. Nuclei stain blue, eosinophils have green granules.

3.5. Eosinophil Peroxidase (EPO) Assay (see Note 5)

1 Aspirate most of the medium from the bone marrow cultures, taking care not to suck out the nonadherent cells of the pellet The medium does not interfere with the assay, but the wells become overfilled if it is not removed
2. Add 100 µL of substrate solution to each well and leave at room temperature for 30 min It is not necessary to incubate in the dark for this short period
3. Stop the reaction by the addition of 50 µL of 4*M* sulphuric acid, and determine the absorbance at 490 nm Once the acid has been added the color is stable for several hours.

3.6. Fixing and Staining Cytocentrifuge Preparations

1 Ensure the cytocentrifuge preparation (or smear) is dry then fix in methanol for 5 min
2 Stain the fixed slide in Giemsa for 2 min and wash under tapwater
3 Blot dry and examine with oil immersion, a green Kodak Wratten filter No 11 may assist in identifying eosinophils
4 Nuclei are reddish-purple, eosinophils have red to orange granules Basophilic granules are blue The eosinophil stains in Section 3 4 1. can be used instead of Giemsa for staining cytocentrifuge preparations and smears

4. Notes

1 When taking bone marrow we work in the open laboratory, using alcohol dried instruments There are very few problems with contamination at this stage The animal's fur is wetted with alcohol to prevent possible contamination by the fur being flicked
2 The importance of using parasitized mouse marrow for eosinophil production is illustrated in Fig. 1 It shows the production of eosinophils after 7 d incubation using marrow harvested from mice on different days after infection Uninfected mice usually produce relatively few eosinophils We generally use marrow from mice infected for 9–20 d (*see* Note 6)
3 The short-term microplate culture system is used to assay IL-5 samples, to find the optimal amount of IL-5 to produce eosinophils in the longer term cultures, and to test FCS batches In assaying for IL-5 in column fractions, it is advisable to include gentamicin in the bone marrow culture medium Use marrow from mice infected for between 9 and 20 d for establishing the cultures Cells of the neutrophil lineage constitute 30–60% of total normal human marrow cells and, in the absence of a suitable growth factor, such cells do not survive in the microplate cultures for longer than 21 d. Since the EPO assay detects human myeloperoxidase (even in the presence of the enzyme's inhibitors such as

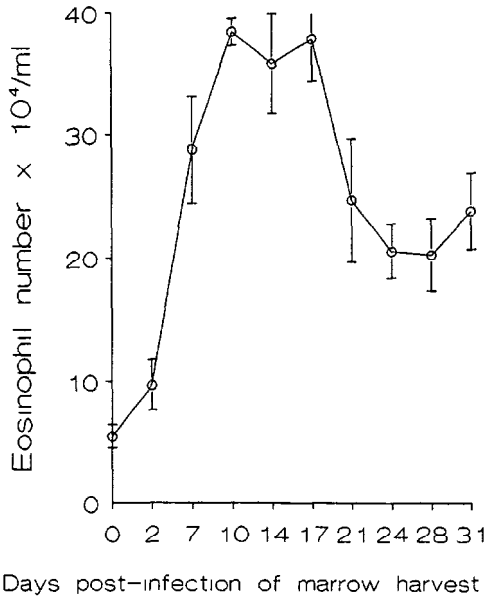

Fig. 1. Number of eosinophils in supernatant from murine bone marrow cultures after 7-d incubation. Marrow was harvested from mice on different days postinfection with *M. corti*. All cultures were established in the presence of IL-5 from a T-cell clone-conditioned medium Eosinophil numbers represent the mean ± 1 SD of three replicate cultures

KCN), the assay is only suitable for use in the human microplate culture system after 21-d culture with samples that are known not to contain neutrophil growth factors. Figure 2 shows the production of human eosinophils (and total cells) from microplate bone marrow cultures stimulated with NIMP-TH1-conditioned medium Samples that are cytotoxic and kill the marrow inoculum may appear positive in the peroxidase assay, since eosinophil peroxidase has not been degraded during the culture period. For this reason it is prudent to examine the cultures before assaying for EPO, and wells that have no visible cell pellet noted

4. Agar cultures· Some workers use slides precoated with 0.3% agar to mount their agar disks on. This is to ensure that the agar sticks to the slide and is not pulled off when the filter paper is removed. Figure 3 shows the time course of eosinophil production of cultures established in the presence of NIMP-TH1 conditioned medium. The marrow was harvested from mice infected for 14 d with *M corti* with a marrow eosinophilia of 45%. When IL-5 was added from d 0 of culture, most of the eosinophils were produced in the first 2–3 wk when over 80% of the nonadherent cells were eosinophils (85.7 ± 5.1% at 7 d and 83.7 ± 1 2% at 14 d.

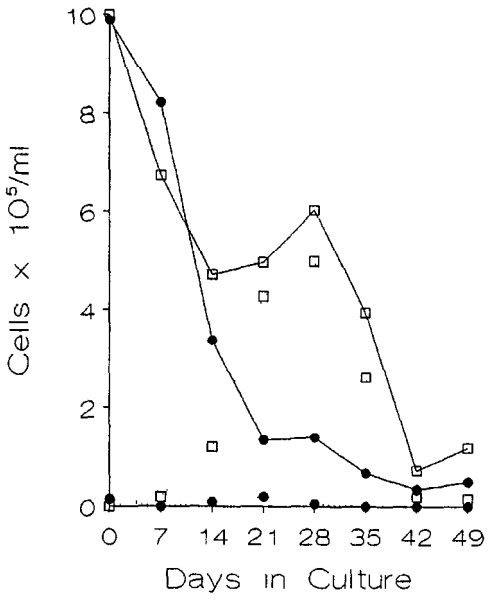

Fig. 2. Production of total cells (——) and eosinophils (•••••) from microwell cultures of normal human bone marrow established in the presence (□) and absence (●) of murine EDF (NIMP-TH1-conditioned medium)

Without IL-5, the eosinophils present in the marrow samples disappear at the end of 7 d, and addition of IL-5 for the first time at this point ensures that subsequent eosinophils are produced in vitro from the precursors and are not surviving eosinophils from the inoculum. In cultures that have the addition of IL-5 delayed for 7 d, it takes another 14 d before peak eosinophil numbers are produced, when the eosinophils may represent up to 90% of nonadherent cells Fewer eosinophils are produced if the addition of IL-5 to the cultures is delayed for 14 d, when at peak production time the eosinophils only represent 27% of supernatant cells

Recombinant mouse IL-5 (rmIL-5) is active in stimulating the production of eosinophils in murine marrow cultures. Table 1 shows the effect of different concentrations of rmil5 and NIMP-TH1-conditioned medium on eosinophil production after 7-d culture. It must be noted that eosinophil production is very variable between experiments, as well as having large variations between "replicate" cultures This variation between replicates tends to increase as the cultures get older, particularly in Cluster plate cultures. This may represent cloning of the very young committed eosinophil progenitors that are present in the marrow at a low frequency A total lack of eosinophil production in the presence of a known IL-5 sample may be due to an unsatisfactory batch of FCS, or a fault in the medium or its preparation

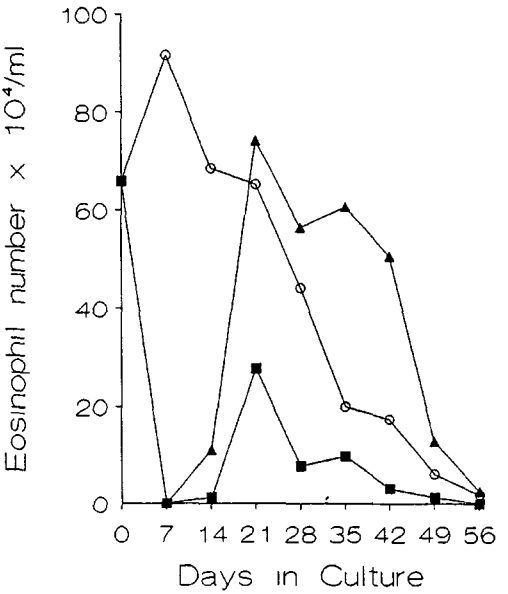

Fig. 3. Production of eosinophils from bone marrow cultures established from *M corti*-infected mice. Cultures were incubated continuously in the presence of NIMP-TH1 conditioned medium from d 0 of culture (○), d 7 of culture (▲), and day 14 of culture (■). Eosinophil numbers represent the mean + 1 SD of three replicate cultures

5. Use of donor horse serum (DHS) in long-term cultures: If a good batch of FCS has been obtained, we do not find it necessary to include DHS in the bone marrow culture medium. Table 2 shows the cumulative numbers of eosinophils produced over 35 d in culture together with the percent of nonadherent cells that are eosinophils It can be seen from Table 2 that the presence of DHS suppresses eosinophil numbers, though the percentage of nonadherent cells that are eosinophils is increased because of reductions in the other cell types. Hydrocortisone has a short-term suppressive effect in cultures without DHS, but is beneficial for the longer term cultures.
6 Other sources of eosinophil progenitors. Spleen and fetal liver are focal points of hemopoiesis We have no experience with fetal liver, but spleen cells have been used in agar cultures and in the IL-5 assay. Uninfected mouse spleen cells produced no eosinophil colonies when incubated in agar in the presence of EL4-conditioned medium, but spleen cells from mice infected for 12 d do produce eosinophil colonies. Cells from the same spleen gave a positive assay result when used in the IL-5 assay, but had to be partially purified and the EPO level above background (i e., unstimulated cultures), was not as great as marrow cells IL-5 transgeric mice can also serve as a source of progenitors *(25)*

Table 1
Number of Eosinophils Produced from Mouse Bone Marrow After 7 d Incubation in the Presence of IL-5

	Source of IL-5			
EDF	NIMP-TH1		rmIL-5	
dilution	Number[a]	%	Number[a]	%
1 100	63 36 ± 6 99	52.2 ± 5 1	nd	nd
1 200	n d	n d	20.52 ± 1.89	25 9 ± 3 0
1·300	22 00 ± 0 40	28.0 ± 2 6	n d.	n d
1.400	n d	n d.	18 49 ± 0.54	21 4 ± 4 8
1·1000	3 95 ± 0 30	7 0 ± 1 1	22 60 ± 1 56	34 3 ± 1 8
1:3000	0.76 ± 0 17	1 9 ± 0.1	10 14 ± 0.65	14.3 ± 0.1
None	0 11 ± 0 15	0 2 ± 0 3		

[a]Number given as eosinophils $\times 10^4$/mL Both number and percent given as mean ±1 standard deviation of 4 replicate cultures.

Table 2
The Production of Eosinophils in the Presence and Absence of Hydrocortisone and Donor Horse Serum

Medium additives[a]			Days in culture				
DHS	Hc		7	14	21	28	35
+	+	[b]	35 90	41 94	42 3	42.33	42 34
		[c]	58 ± 9	43 ± 6	8 ± 4	0 5 ± 1	0 2 ± 0 4
+	–	[b]	27 10	37.50	38 61	38 63	38.64
		[c]	41 ± 10	33 ± 4	7 ± 4	0 3 ± 0 5	0 2 ± 0 1
–	+	[b]	46.50	54 00	54 84	54 96	55 02
		[c]	63 ± 5	38 ± 12	11 ± 5	2 ± 3	1 ± 1
–	–	[b]	52 50	53 19	54.26	54.30	54.30
		[c]	64 ± 7	32 ± 18	8 ± 3	1 ± 1	0 ± 0

[a](+) indicates medium supplemented with 5% donor horse serum (DHS) or hydrocortisone (Hc), (–) indicates absence of DHS from the medium

[b]Cumulative total of eosinophils $\times 10^4$/mL

[c]Eosinophils as percent of total nonadherent cells (mean ± 1 standard deviation of six duplicate cultures) Bone marrow from parasitized mice was incubated in the presence of T-cell clone conditioned medium

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Flow Sorting for Isolating CFU-E

Suzanne M. Watt and John M. Davis

1. Introduction

Erythroid progenitor cells have been classified into three groups of increasing maturity: the primitive burst forming unit (p-BFU-E), the mature burst forming unit (m-BFU-E), and the erythropoietin responsive colony forming unit (CFU-E). This classification is based on their time of maturation in vitro, their proliferative capacity, and their responsiveness to growth factors *(1)* The CFU-E can be distinguished from the more primitive erythroid progenitors by their ability to proliferate and mature in response to a single growth factor, erythropoietin. In clonal assays in vitro, the CFU-E form single or double clusters characteristically containing 8–64 mature or maturing erythroid cells 2 d after cultures have been initiated with mouse bone marrow or fetal liver *(2,3)*.

The availability of enriched populations of CFU-E is important to our understanding of the function and mode of action of growth factor receptors in normal cells; the influence and regulation of molecules, genes, and viruses that are specific to the erythroid lineage; and as a baseline for understanding errors in gene regulation or function that govern the development of leukemic or preleukemic states. One approach to purifying CFU-E has relied on the use of fluorescently tagged monoclonal antibodies as probes to cell surface molecules together with flow cytometry *(4,5)*. Although probes that specifically identify CFU-E are not available, phenotypic analysis has revealed that CFU-E can be segregated from more primitive erythroid precursors, from morphologically recognizable erythroid cells, and from mature myelomonocytic cells and their progenitors with a series of rat monoclonal antibodies *(4–6)*. Hemopoietic tissues vary in their content of different types of hemopoietic progenitor cells and of maturing cells of particular lineages *(6)*. Since mouse fetal liver is a major site of erythropoiesis and contains high numbers of CFU-E, the strategy for

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

isolating CFU-E described here relies on the fractionation of low density fetal liver cells on the basis of their forward light scatter characteristics and differential reactivity with two rat monoclonal antibodies, YBM 42.2.2 and YBM 10.14.9 using flow cytometry *(4–6)*. The anti-T200 antibody YBM 42.2.2 does not react with the CFU-E or more mature erythroid cells *(4)* but reacts with all leukocytes, thus allowing segregation of CFU-E from both myelomonocytic and lymphoid cells and from all other hemopoietic precursors. The YBM 10.14.9 antibody is then used to separate CFU-E from more mature erythroid cells *(5)*. This approach yields cell populations containing at least 60% CFU-E, whereas 80% of the cells have the morphology of early erythroid blast cells and do not stain with benzidine, which identifies hemoglobin containing cells (*5–7*, *see* Note 1).

2. Materials

2.1. Reagents for Media Preparation

1. Powdered Iscove's Modified Dulbecco's Medium (IMDM) containing (per liter) 3 024 g $NaHCO_3$, 60 mg penicillin, 100 mg streptomycin, $5 \times 10^{-5}M$ 2-mercaptoethanol. After preparation, do not adjust pH Store at 4°C Light sensitive. This should be prepared at 1X and 2X strength.
2. IMDM without bicarbonate but with all other additions. Adjust to pH 7 3 with 5M NaOH or pH 5 1 with HCl.
3. Powdered modified Eagle's minimal essential medium with Earle's salts, but without phenol red, containing 20 g/L bovine serum albumin (BSA) and sodium azide (0.02%).
4. Sodium bicarbonate ($NaHCO_3$)
5. Sodium benzylpenicillin
6. Streptomycin sulphate (745 U/mg).
7. 2-Mercaptoethanol
8. 1*M* HEPES buffer pH, 7.3 (commercially available)
9. 10X PBS: 0.2*M* sodium phosphate buffer with 1 48*M* sodium chloride, pH 7 3
10. 4% (w/v) Sodium azide

2.2. In Vitro Culture Reagents

1. Methylcellulose (65 HG, 4000 mPa, Fluka, AG, Buchs, Switzerland) prescreened to support CFU-E growth as described in Note 2.
2. BSA· Store at 4°C
3. Lipids.
   a. Cholesterol (5-cholesterol-3-β-ol)
   b. Oleic acid (*cis*-9-octadecanoic acid)
   c. L-α-phosphatidyl choline dipalmitoyl
4. Human transferrin.
5. Erythropoietin (Epo): Epo step 1 (1000 U/mg) or pure Epo (80,000 U/mg) obtained from Terry Fox Laboratory Media Preparation Service (Vancouver,

Canada) or commercial recombinant Epo Store in 200-µL aliquots at –70°C in 0 1% BSA or prescreened fetal calf serum (FCS). Do not refreeze after thawing.
6. Commercial Ficoll-Hypaque (density 1.077 g/cc) Light sensitive. Store at room temperature.
7. FCS: Prescreened for supporting CFU-E growth. Store in 10-mL aliquots at –20°C.

2.3. Animals

Day 12–13 pregnant CBAf/CaH mice 8–12 wk of age. Day 0 of pregnancy is taken as the day of appearance of the vaginal plug.

2.4. Hybridomas

1 YBM 42 2 2 (Rat IgG2a antibody): This does not bind protein A at neutral pH.
2. YBM 10.14.9 (Rat IgG2c antibody): This antibody binds protein A at neutral pH.

2.5. Antibody Purification

1 Commercial rabbit anti-rat Ig
2. Commerical protein A-Sepharose CL-4B
3. 0.1*M* Phosphate buffer, pH 8 0.
4. 0.1*M* Glycine-HCl buffer, pH 3 0
5. 2*M* Trizma base in water
6 Phosphate-buffered saline (PBS): 0 02*M* phosphate buffer with 0 148*M* sodium chloride, pH 7 3.
7 Saturated ammonium sulfate solution, pH 6 8
8. 0 1*M* Borate buffer, pH 8 2
9. 0.2*M* Triethanolamine, pH 8.2
10. 20 m*M* Dimethylpimelimidate dihydrochloride in 0 2*M* triethanol-amine, pH 8.2

2.6. Antibody Labeling

1. Commercial fluorescein isothiocyanate (FITC) coupled protein A Store at 4°C
2. FITC (Isomer 1) Store dessicated at 4°C.

2.7. Stains

1. Commercial May-Grunwald Stain prefiltered through a Whatman 1MM filter paper
2 Commercial Giemsa R66 improved stain.
3. Benzidine stock solution: 0.2% (w/v) benzidine hydrochloride in 0.5*M* acetic acid This can be stored in the dark at 4°C for 3–4 wk. **Caution,** benzidine is a carcinogen.
4. 30% (w/w) H_2O_2 solution.

2.8. In Vitro Culture Reagents

2.8.1. 2% Methylcellulose

1. Add 20 g methylcellulose to 500 g sterile glass freshly double-distilled deionized boiling water

2 Boil for 2 min with great care to avoid excess foaming Control the level of heating
3 Add sterile double-distilled deionized water to 520 g to correct for water loss resulting from evaporation
4 Cool to approx 37°C
5 Add 500 mL of double-strength IMDM Keep covered with foil
6 Cool on ice with mixing for 2–3 h
7 Stir on a magnetic stirrer at 4°C overnight to allow the methylcellulose solution to clear
8 Store in 50-mL aliquots protected from light at –20°C for up to 4 wk

2.8.2. Deionized and Delipidated BSA

1 Dissolve 400 mg of Dextran T40 in 400 mL of glass double-distilled deionized water
2 Add 4 g of activated charcoal (Norit A or SX-1) and leave at room temperature for 30 min with occasional mixing
3 Add 20 g BSA to the surface of the dextran-coated charcoal solution and leave for 2–3 h at 4°C to dissolve without mixing
4 Titrate to pH 3 0 with concentrated hydrochloric acid (HCl) to inhibit heat-induced polymerization of the albumin
5 Incubate for 30 min at 56°C in a shaking water bath
6 Centrifuge at 40,000*g* for 20 min and Millipore filter the supernatant
7 Adjust the pH to 5 5 with 2*M* NaOH
8 Deionize the BSA solution overnight at 4°C over 40 cm^3 of Amberlite MB-1 mixed in exchange resin
9. Concentrate the solution to 150 mL on an Amicon UM10 membrane at 4°C.
10 Adjust the pH to 7 0 with 2*M* hydrochloric acid
11. Millipore filter and store in 10-mL aliquots indefinitely at –20°C or 4°C

2.8.3 Lipids

1 Dissolve 4 mg L-α-phosphatidyl choline dipalmitoyl, 3 9 mg cholesterol, and 2 8 mg oleic acid in a few drops of chloroform at room temperature or ethanol at 50°C in a 25-mL glass beaker Completely evaporate the solvent under a stream of nitrogen, leaving the film of lipid on the bottom of the beaker
2 Add 10 mL of bicarbonate-free IMDM pH 5 1 containing 1% of the deionized and delipidated BSA.
3 Immerse the beaker containing the lipids in ice and sonicate under air for 10 min at maximum energy just below the foaming point so that the lipids form small micelles
4 Millipore filter through 1.2- and then 0 45-μm filters Store indefinitely at 4°C

2.8.4 Other Reagents

1. $5 \times 10^{-2}M$ 2-mercaptoethanol in double-glass distilled deionized water. Millipore filter Prepare fresh stocks before use

2. Ferric chloride stock ($FeCl_3$). Since ferric chloride is hygroscopic, weigh out a large piece of $FeCl_3$ $6H_2O$ and immediately dissolve in $10^{-3}M$ hydrochloric acid (HCl). Dilute to a $7.9 \times 10^{-3}M$ stock solution in $10^{-3}M$ HCl. Store at –20°C.
3. Transferrin: Dissolve 360 mg of human transferrin in 4 mL of bicarbonate-free IMDM pH 7.4 and 1.15 mL of $7.9 \times 10^{-3}M$ $FeCl_3$ in $10^{-3}M$ HCl. Millipore filter and store indefinitely at 4°C.

3. Methods

3.1. Antibody Preparation

1. Collect the supernatant from the hybridoma cell lines grown in IMDM with 1% FCS, 24 h after cells reach confluencey.
2. Precipitate the immunoglobulin (Ig) by adding an equal volume of saturated ammonium sulfate solution (pH 6.8). Mix for 1 h at 4°C.
3. Centrifuge at 10,000*g* for 10 min at 4°C.
4. Resuspend the pellet to one-tenth the original volume in PBS and dialyze against $0.1M$ phosphate buffer pH 8.0 at 4°C.
5. Estimate the protein concentration by measuring the absorbance at 280 nm.

3.2. Antibody Purification

1. Mix purified rabbit anti-rat Ig with protein A-Sepharose to a final concentration of 11 mg Ig/mL of Sepharose beads in $0.1M$ borate buffer pH 8.2 for 30 min at room temperature.
2. Wash the beads in excess borate buffer and then in $0.2M$ triethanolamine pH 8.2.
3. Resuspend the Sepharose in 20 vol of 20 mM dimethyl pimelimidate dihydrochloride freshly made in $0.2M$ triethanolamine, pH 8.2. Mix for 45 min at room temperature. This will covalently crosslink the Ig to the protein A and prevent it from leaching from the column.
4. Spin the beads at 500*g* for 1 min and resuspend in an equal volume of 20 mM ethanolamine, pH 8.2, for 5 min at room temperature.
5. Wash the beads three times in $0.1M$ borate buffer pH 8.2.
6. At the same time, equilibrate protein A-Sepharose with $0.1M$ borate buffer, pH 8.2. YBM 10.14.9 will bind to protein A-Sepharose at neutral pH, whereas YBM 42.2.2 will not.
7. Apply the concentrated YBM 10.14.9 sample to a protein A-Sepharose column and the YBM 42.2.2 to the rabbit antirat Ig-protein A Sepharose column; 10–20 mg of Ig can be applied/mL of beads.
8. Elute the bound Ig with $0.1M$ glycine-HCl buffer, pH 3.0, and neutralize the eluted material immediately with Trizma base.
9. Dialyze the antibodies against PBS and store in small aliquots with 0.1% BSA.
10. If the purified antibodies are to be coupled to FITC, dialyze against $0.1M$ bicarbonate buffer, pH 9.3, instead of the PBS, and couple with FITC immediately. Do not store antibodies for an extended period of time in the bicarbonate buffer.

3.3. Fluorescein Labeling of Antibodies

1. Dialyze the antibodies against 0 1*M* bicarbonate buffer, pH 9 3, for 2–5 h at 4°C.
2. Dissolve the FITC at 1 mg/mL in dimethyl sulfoxide (DMSO). Add 25 μg of FITC/mg of purified YBM 42.2.2 Ig for 2 h at room temperature with constant rotation to give a fluorescein to protein ratio of approx 3·1
3. Separate the FITC-conjugated Ig from the free FITC by passing through a 5-mL Sephadex G-25 column equilibrated with PBS Collect the Ig fraction and store in small aliquots containing 1% BSA and 0.02% NaN_3 at –20°C

3.4. Cell Preparation and Labeling

1 Dissect the livers from d 12–13 mouse fetuses using cataract knives and place in chilled bicarbonate-free IMDM containing 10% FCS
2 Prepare a single-cell suspension by gently syringing the fetal livers through 19-, 21-, and 25-gage needles sequentially attached to a 2-mL syringe.
3. Place the cells in a 10-mL centrifuge tube and allow cell clumps to settle at 4°C for 5 min. Pass the supernatant through sterile nylon gauze to remove smaller clumps
4. Centrifuge 5 mL of cells (10^7 cells/mL) over 4 mL of Ficoll-Hypaque (density 1 077 g/mL) at 600*g* for 30 min at room temperature.
5 Collect the low density cells from the Ficoll-Hypaque interface and wash three times in Eagle's-HEPES medium with BSA and sodium azide Approximately 10^5 cells are recovered/fetal liver processed
6. Add normal mouse serum, heat inactivated at 56°C for 30 min to the cell suspension at a final concentration of 10% to block Fc receptors. Incubate the cells for 20 min at room temperature, and wash the cells in Eagle's-HEPES medium containing BSA and sodium azide.
7. Centrifuge the antibodies in a Beckman airfuge at 26 lb/in^2 for 10 min at room temperature to remove aggregrates and further minimize Fc binding
8 Label cells (10^7/mL) with saturating levels of FITC-tagged YBM 42 2 2 (approx 400 μg/mL) at 4°C for 30 min Include propidium iodide (50 μg/mL) during the incubation
9 Wash and resuspend the cells to 2 × 10^6 cells/mL in Eagle's-HEPES medium containing BSA and sodium azide at 4°C

3.5. Cell Sorting (see Note 1)

1. Separate the labeled cells on a fluorescence-activated cell sorter by the two parameters of forward light scatter and fluorescein fluorescence.
2 For a FACS-II cell sorter, cellular excitation is achieved with an argon ion laser at an output power of 0 3 W and an emission wavelength of 488 nm Set the light scatter gain at approx 4. For fluorescein fluorescence, set the photomultiplier voltage at 650 V with an amplifier gain of 8–16. These voltages and gains will vary with the instrument used. The fluorescein fluorescence is detected by placing a 530-nm long pass interference filter and a 530-nm long pass filter in front of the appropriate photomultiplier tube.

3 Sterilize the tubing and nozzle by passing ethanol through the cell sorter for 30 min. Wash out the ethanol with sterile distilled water, and pass sterile distilled water through the tubing and nozzle for at least 1 h, following this with a 0 9% saline wash for 30 min prior to sorting.
4 Run the cells at 4°C through the cell sorter, collecting cells with intermediate to high forward light scatter characteristics that are negative for YBM 42.2.2 labeling when compared to control cells labeled with an irrelevant FITC-tagged rat monoclonal antibody of the same subclass (*see* Fig. 1A,B)
5 Collect the sorted cells in bicarbonate-free IMDM, pH 7 3, containing 20–50% FCS at 4°C in earthed siliconized glass tubes. Note CFU-E will die if the collection medium becomes alkaline.
6 Spin the sorted cells at 200*g* for 15 min at 4°C
7. Resuspend the cells to 2×10^6 cells/mL in Eagle's-HEPES medium with BSA and sodium azide
8 Label with FITC-tagged YBM 10 14.9 or with unlabeled YBM 10 14.9 (using 400 μg/10^7 cells) for 30 min at 4°C.
9. Wash the cells twice with Eagle's-HEPES medium with BSA and sodium azide Resuspend to 2×10^6 cells/mL in the same medium.
10 When using the unlabeled YBM 10 14.9, a two-stage indirect labeling procedure is necessary For this, incubate the cells with FITC-protein A (20 μg/mL final concentration) for 30 min at 4°C prior to washing, and resuspension in Eagle's-HEPES medium with BSA and sodium azide Include propidium iodide (50 μg/mL) in the final incubation step. Dead cells labeled with propidium iodide give a very high fluorescence signal in the fluorescein channel and can be gated out when only two parameters are available for sorting.
11 Sort the labeled cells selecting the YBM 10.14.9 positive cells (avoiding the very highly propidium iodide labeled nonviable cells) using conditions described for the first sort and shown in Fig. 1C.
12 Centrifuge the sorted cells at 200*g* for 10 min at 4°C. Resuspend in bicarbonate-free IMDM containing 10% FCS at 105 cells/mL. Keep at 4°C until cultured.

3.6. CFU-E Culture (see Note 2)

1. Thaw the 2% methylcellulose stock at room temperature.
2 Set up triplicate cultures in 35-mm plastic Petri dishes in a final vol of 1 mL containing. 0 8% (w/v) methylcellulose, 10% (w/v) FCS, 1% (w/v) deionized and delipidated BSA, 0.3 mg transferrin saturated with $FeCl_3$, 5×10^{-5}*M* 2-mercaptoethanol, 0 05 U erythropoietin, 8 μg L-α-phosphatidylcholine dipalmitoyl, 7.8 μg cholesterol, 5.6 μg oleic acid, and 10^2–10^3 sorted fetal liver cells or 10^4 unsorted fetal liver cells all in single-strength IMDM, pH 7.3.
3. Incubate the cultures at 37°C in a humidified incubator gassed with 5% CO_2 in air for 2 d.
4. Score single- or double-cell clusters containing 8–64 mature or maturing erythroid cells using an inverted microscope with a 100-fold magnification (*see* Note 3)

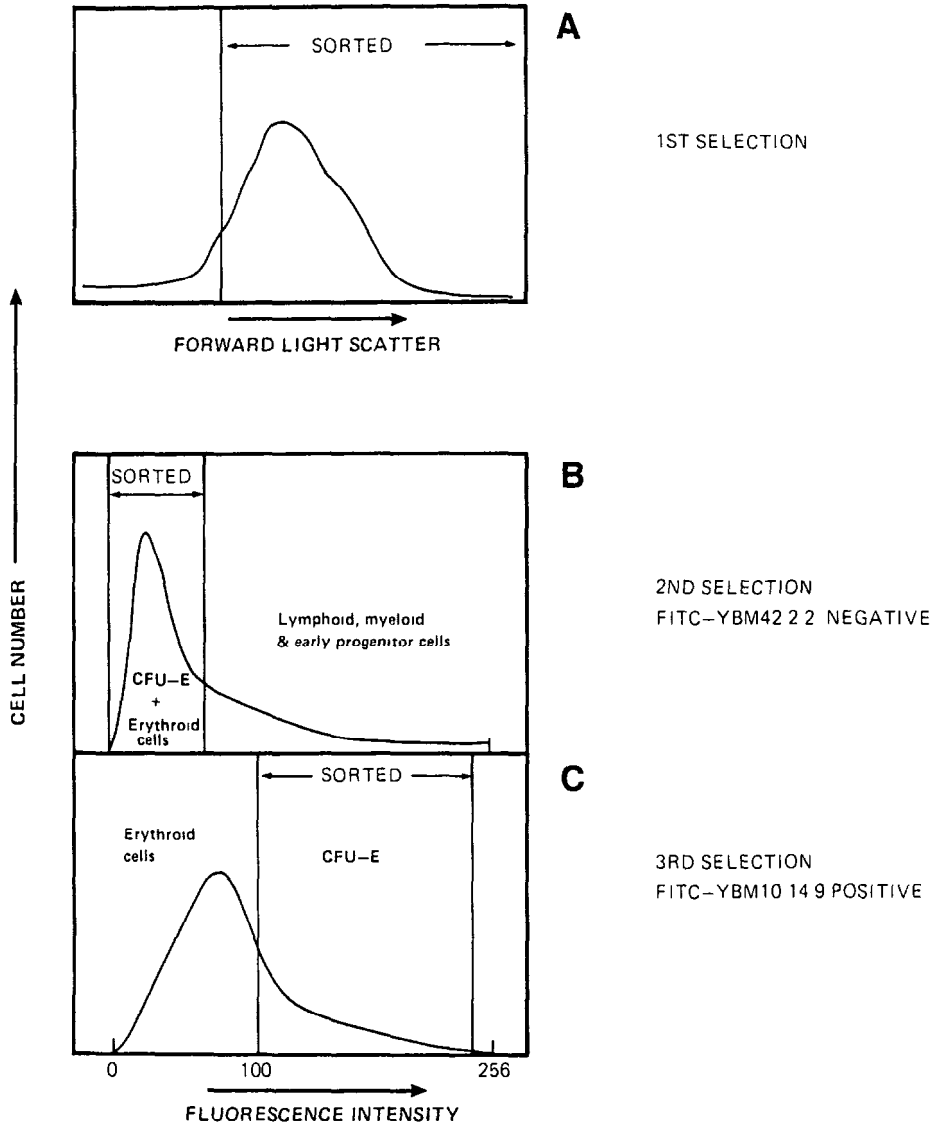

Fig. 1. Typical histograms showing the regions selected for isolating CFU-E The fetal liver cells are first selected on the basis of their intermediate to high forward light scatter characteristics **(A)** and the YBM 42.2 2 negative cells **(B)** are sorted. The isolated cells are labeled with FITC-tagged YBM 10 14.9, and the positive cells **(C)** are collected. Care is taken to exclude the highly positive cells that are stained with propidium iodide and represent the nonviable cell fraction

3.7. Morphology

1 Cytocentrifuge sorted cells and air dry
2 Fix the cells with methanol for 10 min at room temperature
3 Incubate in May-Grunwald stain for 20 min at room temperature and then in 3% Giemsa in tap water for 20 min at room temperature
4. Wash the slides in tap water Air dry and mount the coverslips with DPX

3.8. Benzidine Staining

1. Suspend the cells in tissue-culture medium (without NaN_3) to a concentration of 1 5 × 10^6 cells/mL
2 Pipet 150 µL of the cell suspension into the well of a 96-well-flat bottom microtiter plate.
3. Prepare the staining solution, add 10 µL of 30% H_2O_2 to 1 mL of benzidine stock solution. Mix and use within 30 min
4. Add 50 µL of staining solution to cells, and mix quickly by pipeting up and down several times
5 Wait 5 min. At this time, cells containing at least 10% hemoglobin will have stained a dark blue Estimation of the number of hemoglobin containing cells is best achieved by photographing the cells at this stage, since both the color of the stain and the number of stained cells will change with time
6 The early erythroid blast cells represent the most immature and largest erythropoietic precursors recognizable, containing a prominent nucleolus, basophilic cytoplasm, and loose chromatin pattern, and are negative for benzidine staining More mature erythroid cells containing hemoglobin will stain with benzidine

4. Notes

1 The method describes the isolation of CFU-E using sequential sorting with two monoclonal antibodies, YBM 42 2 2 and YBM 10 14 9. Relatively high recoveries of CFU-E (40%) can be achieved in this way. These studies could be done equally well using a single multiparameter sort with anti-isotype reagents labeled with fluorescein and phycoerythrin or directly conjugated reagents Substantial enrichment for CFU-E from both normal fetal liver and bone marrow can also be achieved with a set of monoclonal antibodies listed in ref *7* Indeed, more efficient purification may be obtained for CFU-E by combining three probes, such as YBM 10 14 9, YBM 42 2 2, and YW 13.1 1, since all these antibodies exhibit different patterns of reactivity with normal hemopoietic cells. Studies using simultaneous two- and three-color sorting with a variety of antibodies to human erythroid precursors show the potential benefit of such approaches to cell fractionation *(8)*. Other procedures that allow substantial enrichment for CFU-E include multiparameter sorting using fluorescein-conjugated pokeweed mitogen and rhodamine-conjugated anti-neutrophil/monocyte antibodies *(9)*. In addition, Nijhof and Wierenga *(10)* obtained sufficient numbers of highly purified CFU-E for biochemical analysis in a relatively short time by using density separation and elutriation of spleen cells from thiamphenol-treated mice

2 The isolated cells are analyzed for CFU-E by their growth in methylcellulose *(3)* Details of methylcellulose preparation are also given in ref. *11*. Fetal calf serum can be omitted from the cultures, since the BSA, lipid, transferrin, and erythropoietin additives have been designed to allow CFU-E growth in serum-free conditions *(3)*. The approximate concentrations of each additive are described, but it is essential to test and titrate each additive in order to obtain the best conditions for CFU-E growth.
3. Details of antibody purification are given in ref. *12* The benzidine staining technique described here was adapted from ref. *13* Single cells from the purified CFU-E can also be sorted directly into 150 µL of the methylcellulose-supplemented culture medium in a microtiter tray. The colonies are allowed to develop for 2 d at 37°C, and the maturing erythroid cells can be stained after cellulase digestion of the methylcellulose *(14)*. For this, 75 µL of FCS containing 1.08 mg/mL cellulase (1943 cellulase U/g) is added to each well, and the cultures are incubated overnight at 37°C. The following day, the contents of each well are transferred as drops to a glass microscope slide and allowed to air dry. The cells are fixed and stained with May-Grunwald/Giemsa stain Alternatively, the cells may be stained *in situ* with benzidine with or without digestion of the methylcellulose with cellulase.

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Murine Bone Marrow-Derived Macrophages

E. Richard Stanley

1. Introduction

The molecular phagocytic lineage comprises, in order of increasing maturity, the committed macrophage precursor cell, the monoblast, promonocyte, monocyte, and the macrophage. Methods for the preparation and culture of bone marrow-derived macrophages, developed by Stanley and colleagues (*1,2*; Tushinski, R. J., personal communication), provide large numbers of mononuclear phagocytes that are capable of extensive cell proliferation. Since their proliferation can be stimulated by colony-stimulating factor-1 (CSF-1), granulocyte macrophage colony stimulating factor (GM-CSF), or interleukin-3 (IL-3), they represent an important primary cell source for studies of the actions and interactions of these three growth factors. The principles underlying the method are:

1. To generate and expand primitive mononuclear phagocyte precursor cells by culturing bone marrow cells in a combination of partially purified CSF-1 and IL-3 for a period of 3 d,
2. To remove contaminating red cells, fibroblasts, and mature macrophages and disrupt aggregates of proliferating cells by proteolytic digestion of the nonadherent cells at d 1 and 3 of culture; and
3. To obtain a population of mononuclear phagocytes that is relatively homogeneous with respect to their state of differentiation by recovering only those cells (i.e., monoblasts, promonocytes) that acquire the capacity to adhere to tissue-culture plastic during d 4–5 of culture

About 95% of the bone marrow-derived macrophages so obtained possess the CSF-1 receptor, 93–98% proliferate in response to CSF-1, and 90% of cells die on removal of CSF-1 from the serum-containing medium *(1,3,4)*. This latter observation reflects the absence in these cultures of contaminating, fibro-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc., Totowa, NJ

blast-like, CSF-1-producing cells and enables this population to be used not only to study the effects of growth factors on macrophage proliferation, but also on their survival.

2. Materials

1 Dulbecco's modified Eagle's medium (DMEM)
2. Pronase solution: 0.02% (w/v) Pronase (B grade, Calbiochem), 1.5 m*M* EDTA in phosphate-buffered saline (PBS)
3 Complete medium: DMEM supplemented with 15% (v/v) fetal calf serum (FCS), 0.292 mg/mL glutamine, 0.02 mg/mL asparagine, 0.5 μ*M* 2-mercaptoethanol, 0.2 g/L penicillin, 0.2 g/L streptomycin
4. CSF-1: Stage 1 L cell CSF-1 prepared as in ref. *5* or purified human recombinant CSF-1
5 IL-3: Purified recombinant murine IL-3 or medium conditioned by serum-free cultures of the myelomonocytic leukemia cell line WEHI-3 *(6)*
6 Zwittergent stock solution: 1% (w/v) Zwittergent 3–14 (Calbiochem). Stored at 4°C.
7 0.005% Zwittergent: Zwittergent stock solution diluted 1 in 200 with PBS. Stored at 4°C.

3. Method

The method is divided into:

1. Isolation of bone marrow cells,
2. Generation of mononuclear phagocyte precursor cells,
3 Differentiation of precursor cells to adherent mononuclear phagocytes,
4 Culture of bone marrow-derived macrophages *(1,2)*, and
5. Determination of macrophage concentration *(3)*

The last section is included since adherent cultured macrophages cannot be easily detached by trypsinization and alternative methods for quantitating cell number are required.

3.1. Isolation of Bone Marrow Cells (see also Chapters 16 and 21)

1. Remove the tibias and femurs from C3H/HeJ mice by cutting the proximal end of the femur and the distal end of the tibia, leaving the other ends intact.
2. Insert a 23-gage needle into the intact ends and flush bone marrow out through the cut ends with ice-cold DMEM.
3 Dispense marrow plug by three passes through a 22-gage needle and centrifuge (1200*g*, 5 min, 4°C).
4 Resuspend the pellet in ice-cold DMEM and determine the nucleated white cell concentration by counting an aliquot of the resuspended cells in 2% acetic acid.

3.2. Generation of Mononuclear Phagocyte Precursor Cells

1 Adjust the cell density to 10^6 cells/mL in complete medium containing 500 U/mL (0 22 n*M* CSF-1) of stage 1 CSF-1 or 6 ng/mL (0.44 n*M*) pure human recombinant CSF-1, and 1 n*M* IL-3 (or 10% WEHI-3 conditioned medium)
2. Seed cells into tissue culture flasks at a density of 2 9 × 10^5 cells/cm^2 and incubate (37°C, 10% CO_2 in air) for 24 h
3. Collect nonadherent cells and discard adherent cells (fibroblast-like cells, mature mononuclear phagocytes, and other cells adhering to the flask)
4. Centrifuge the nonadherent cells (800*g*, 10 min, 4°C), resuspend in 1 mL of Pronase solution/10^7 cells and incubate for 15 min at 37°C
5 Stop Pronase digestion by the addition of horse serum (0 2/10 mL Pronase solution) and layer the suspension onto 15 mL of ice-cold horse serum
6. Incubate on ice for 15 min to allow clumped erythrocytes and debris to settle through the horse serum
7 Remove the cell suspension from the horse serum by Pasteur pipet, overlay onto another 15 mL of ice-cold horse serum, centrifuge (1200*g*, 10 min, 4°C), disperse the pellet in DMEM as described in step 2, and seed into the original number of flasks
8. Incubate the flasks at 37°C for 2 d, collect the nonadherent cells, and discard the adherent cells. Pronase treat the nonadherent cells as described in steps 4–7 and resuspend the pellet in complete medium containing 0 22 n*M* stage 1 CSF-1 or 0 44 n*M* pure human recombinant CSF-1.
9 At this stage, if desired, adjust the cell suspension to 10% (v/v) with respect to dimethyl sulfoxide and store at −196°C for later use

3.3. Differentiation of Precursor Cells to Adherent Mononuclear Phagocytes

1. Adjust concentration of the pronase-treated nonadherent cells to 10^5 cells/mL
2 Seed into tissue-culture dishes at a density of 1.9 × 10^4 cells/cm^2 in complete medium containing 0 22 n*M* stage 1 CSF-1 or 0.44 n*M* pure human recombinant CSF-1 and incubate for 2 d at 37°C in 10% CO_2 in air.
3. Remove the nonadherent cells with two washes of sterile PBS.

3.4. Culture of Bone Marrow-Derived Macrophages

1. Maintain log-phase growth by culturing in 4.4 n*M* CSF-1 in complete medium. At high cell densities it may be necessary to periodically change the medium (*see* ref. *1* for formula predicting the CSF-1 consumption by bone marrow-derived macrophages). (Note that 1 unit of CSF-1 is equivalent to 0.44 n*M* or 12 pg of pure human or mouse CSF-1.)
2. Log-phase cells may be rendered quiescent by washing once with PBS, replacing the growth medium with complete medium (without CSF-1) and incubating for 16 h
3 Remove adherent macrophages for subculture by scraping with a sterile cell scraper.

3.5. Determination of Macrophage Concentration

1. Wash the cell monolayer with ice-cold PBS
2. Pipet 0.005% Zwittergent onto the cells
3. Incubate for 5 min at 20°C.
4. Examine with a microscope to ensure cell detachment and remove by pipet using several up and down pipetings.
5. Dilute appropriately and count by electronic cell counter or hemocytometer

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25

Routine Testing of Cell Cultures and Their Products for Mycoplasma Contamination

Alison Stacey and Alan Doyle

1. Introduction

Regular and routine testing of cell cultures for the presence of all types of adventitious agents should be a requirement in any tissue-culture facility. Although bacterial and fungal contaminations can frequently be detected by macroscopic examination, the detection of mycoplasma and virus contamination, which are not readily discernable to the naked eye, necessitate the introduction of specific test regimes. This obviously will incur some additional costs to the running of the facility but the need for quality controlled cell cultures in both research and commercial situations cannot be underestimated.

Microbial contamination may exert numerous effects on cells in culture, some of which may eventually result in cell death. Mycoplasmas alone have been shown to alter the growth rate of cells in culture *(1)*, induce chromosomal abberations *(2)*, influence amino acid *(3)*, and nucleic acid *(4)* metabolism, and cause membrane abberations *(5)*.

The sources of potential contamination should be borne in mind. Although most tissue-culture reagents are screened for the presence of mycoplasma, cases of *Mycoplasma hyorhinis* in trypsin have been documented. The most common sources of infection are from human operators (*M orale* and *M fermentans*) or other cell lines possibly brought in from other laboratories (*M hyorhinis*) The frequency of mycoplasma infection in some tissue-culture laboratories may be as high as 90–100%, and mycoplasmas within individual cell cultures may reach titers of between 10^6–10^8 colony forming units (CFU) per milliliter, which is approx 100–1000 times the cell density.

A range of assays are available for the detection of mycoplasma, including DNA staining, culture, DNA probes, and more recently polymerase chain reac-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

tion (PCR). Each test has its merits as well as disadvantages and therefore it is recommended that, where possible, two test systems be used in parallel.

2. Materials

2.1. Mycoplasma Detection by DNA Staining

1 Carnoy's fixative 75 mL methanol, 25 mL acetic acid (glacial). For each sample to be tested prepare 4 mL of fixative. Note Care must be taken when disposing of used fixative.
2 100 mL Hoechst stain stock solution. Add 10 mg Bisbenzimide Hoechst 33258 to 100 mL of distilled water. Allow to dissolve and filter sterilize using a 0.2-μm filter unit. Wrap the container in aluminium foil and store in the dark at 4°C. Note The toxic properties of Hoechst 33258 are unknown, therefore, gloves should be worn at all times when handling the powder or solutions.
3. 50 mL Hoechst stain working solution· Add 50 μL of stock solution to 50 mL of distilled water. PREPARE IMMEDIATELY BEFORE USE
4 Mountant· 0 1*M* citric acid (22 2 mL), 0 2*M* disodium phosphate (27 8 mL). Autoclave and then mix with 50 mL glycerol. Adjust to pH 5 5 Filter sterilize and store at 4°C

2.2. Mycoplasma Detection By Culture

1 Agar preparation
   a. Agar media: Dissolve 2.8 g of mycoplasma agar base in 80 mL distilled water, and autoclave at 15 lb/in^2 for 15 min Prepare fresh as necessary
   b Yeast extract: Dissolve 7 g of yeast extract in 100 mL distilled water, and autoclave at 15 lb/in^2 for 15 min Using aseptic technique dispense into 10 mL aliquots and store at 4°C.
   c. Pig serum: Using aseptic technique dispense into 10 mL aliquots and heat inactivate by incubation of serum at 56°C for 45min Store at 4°C.

   Allow the autoclaved agar media to cool to 50°C and mix with 10 mL of heat-inactivated pig serum and 10 mL yeast extract (both prewarmed to 50°C) Dispense 8 mL/5-cm diameter Petri dish. Seal in plastic bags and store at 4°C. Agar plates must be used within 10 d of preparation
2. Broth preparation
   a Broth media· Dissolve 2 g of mycoplasma broth base in 70 mL distilled water, and autoclave at 15 lb/in^2 for 15 min.
   b. Yeast extract· Dissolve 7 g of yeast extract in 100 mL distilled water, and autoclave at 15 lb/in^2 for 15 min. Dispense into 10 mL aliquots and store at 4°C
   c Horse serum: Dispense into 20 mL aliquots Store at –30°C Do NOT heat inactivate.

   Allow the autoclaved agar media to cool to 50°C and mix with 20 mL of horse serum and 10 mL yeast extract (both prewarmed to 50°C). Dispense 1.8 mL/glass vial and store at 4°C Prepared broth may be stored without deterioration for several weeks.

2.3. Positive Control Organisms

Both of the tests described in this chapter should be run in parallel with positive controls. In general three species are used: *M. orale* and *M. hyorhinis* for DNA staining and *M. orale* and *M. pneumoniae* for the culture method. In all cases the positive control organisms are inoculated at 100 CFU/mL.

1 Mycoplasma broth and agar (*see* Section 2 2)
2 Preparation of stocks of positive control organisms.
   a Thaw existing stocks or reconstitute lyophilized stocks and inoculate 100 µL into 10 broths Place at 36 ± 1°C for 5–7 d. Observe daily for changes in broth color.
   b Once a distinct colour change has been observed, transfer the entire contents of the broths into 1 mL cryotubes, assign a batch number, and snap freeze in vapour phase of liquid nitrogen.

2.4. Enumeration of Positive Control Stocks

1 Thaw an ampule of the batch to be enumerated, serially dilute in 10-fold dilutions
2 Inoculate 3 × 10 µL aliquots of each dilution onto agar plates that have been air dried for 30 min prior to use. Plates should be labeled with the organism name, the batch number, and the dilution.
3. Plates should then be incubated anerobically for 2–7 d prior to counting. The time required for colonies to appear is dependant on species. As a rough guide 2–3 d are required for *M hyorhinis*, 4–5 d for *M. orale*, and 5–7 d for *M pneumoniae*.
4. Using an inverted microscope count the colonies of the dilution where between 10 and 100 are observed
5. Calculate the number of CFU in the cyropreserved stock using the equation: No of CFU/mL = mean no of colonies counted × 100/dilution factor.

3. Methods

Prior to testing, cell cultures should undergo at least two passages in antibiotic-free medium, since infection may be masked by the presence of antibiotics. Equally, cryopreserved stocks should also undergo two passages in antibiotic-free medium because cryoprotectants may also hide infection. All cultures should be incubated for 3 d prior to testing.

3.1. Mycoplasma Detection by DNA Staining

1 Using a routine method of subculture, harvest adherent cells with trypsin or EDTA and resuspend in the original cell culture medium at a cell concentration of approx 5×10^5 cells/mL
2. Test suspension cell lines direct from culture at about 5×10^5 cells/mL An accurate cell count may not be necessary but sufficient cells should be used so that a semiconfluent spread of cells is obtained by the time of observation (1–3 d incubation)

3 Add 2–3 mL of cell suspension to each of two tissue-culture dishes containing glass coverslips Coverslips should be sterilized by autoclaving prior to placing in the Petri dishes.

For control dishes, plates inoculated with 100 CFU of two species of mycoplasma should be included as positive controls (*see* Section 2 3) Additionally 1 pair of dishes should be left un-inoculated as a negative control

4 Incubate at 36°C ± 1°C in a humidified 5% CO_2/95% air atmosphere for 12–24 h
5 Remove one dish and incubate the remaining dish for a further 48 h.
6 Before fixing, examine the cells for the presence of bacterial or fungal contamination
7 Fix the cells by adding 2 mL of Carnoy's fixative dropwise at the edge of the dish to avoid disturbing the cells Leave at room temperature for 3 min.
8 Decant the fixative and tissue-culture medium to a waste bottle and add 2 mL of fixative to the dish Leave for 3 min
9. Decant the fixative to waste.
10 Invert the lid of the dish and, using forceps, rest the coverslip against the lid for 30 min to air dry.
11 Wearing gloves return the coverslip to the dish and add 2 mL Hoechst stain (working solution) Shield the coverslip from direct light and leave at room temperature for 5 min
12. Decant stain to a waste bottle.
13 Add 1 drop of mountant to a labeled slide and place the coverslip cell side down onto the appropriate slide
14. Examine the slide at 100x magnification with oil immersion under UV epifluorescence Cell nuclei will fluoresce In mycoplasma negative cultures the nuclei will be seen against a dark background In mycoplasma positive cultures the cell nuclei will be seen amongst fluorescing thread-like or coccal structures (Fig 1).

In an alternate system, cells of the test culture can be inoculated onto coverslips pre-inoculated with an indicator cell line such as the Vero African Green Monkey cell line. The Vero cells should be inoculated at a cell concentration of 1×10^4 cells/mL and left for 4–24 h prior to addition of the test sample.

The major advantage of this system, which overcomes the additional time required to set up, is the increased sensitivity achieved by the increased surface area of cytoplasm in Vero cells which aids in revealing the mycoplasma. This system also enables the mycoplasma screening of serum and other reagents that can be inoculated directly onto the indicator cell line.

3.2. Microbiological Culture for the Detection of Mycoplasma

1. Using a routine method of subculture, harvest adherent cells with trypsin or EDTA and resuspend in the original cell culture medium at a concentration of approx 5×10^5 cells/mL
2. Test suspension cell lines direct from a culture at about 5×10^5 cells/mL.

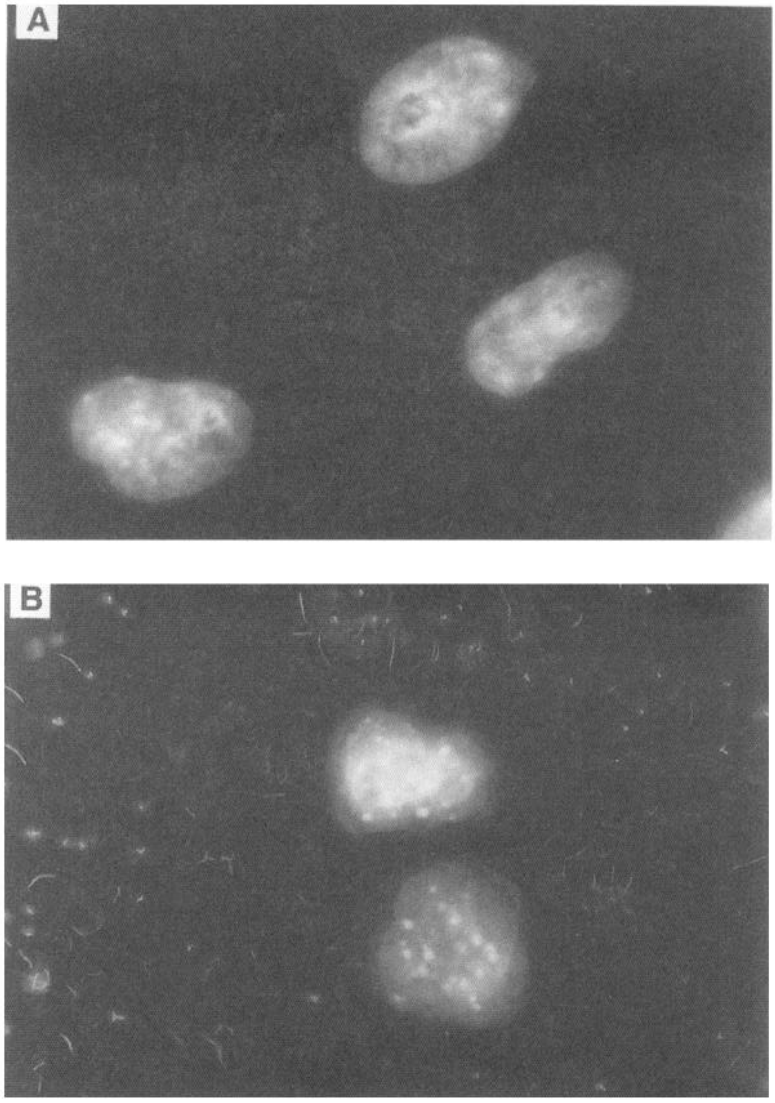

Fig. 1. (A) Noninfected cell culture; **(B)** mycoplasma-infected cell cultures.

3. Inoculate an agar plate with 0.1 mL of the test cell suspension, and incubate anaerobically at 36°C ± 1°C for 21 d.
4. Inoculate a broth with 0.2 mL of the test cell suspension and incubate aerobically at 36°C ± 1°C. At approx 7 and 14 d postinoculation, subculture 0.1 mL of the inoculated broth cultures onto fresh agar plates and incubate as in step 3. Plates

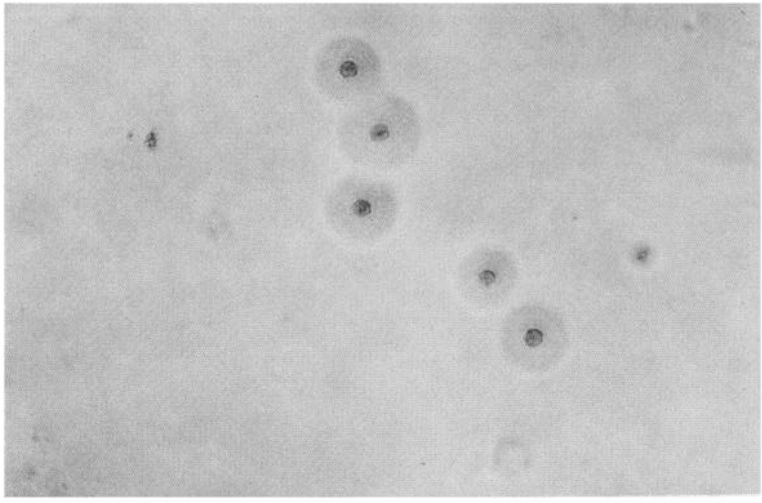

Fig. 2. Mycoplasma colonies, grown from an infected cell culture, showing a typical "fried egg" appearance.

inoculated with 100 CFU of two species of mycoplasma should be included as positive controls (*see* Section 2.3.). Additionally one plate should be left un-inoculated as a negative control.

5. After 7, 14, and 21 d incubation the agar plates should be examined under 40× or 100× magnification using an inverted microscope for the presence/absence of mycoplasma colonies. Typically mycoplasma colonies will have a "fried egg" appearance (Fig. 2), but this may not be the case for all strains. However, it is of course necessary to distinguish mycoplasma colonies from "pseudocolonies" and cell aggregates. The use of Dienes stain that stains true mycoplasma colonies blue but leaves pseudocolonies and fungal and bacterial colonies unstained can be used. Additionally by using a sterile bacteriological loop cell aggregates can be disrupted, but mycoplasma colonies will leave a central core embedded in the agar.

3.3. Elimination of Contamination

In the event of cultures becoming infected with mycoplasma the best course of action is to discard the cultures, and following extensive decontamination of the tissue-culture cabinets and work surfaces, resuscitate "clean" cell stocks. However, in the case of irreplaceable stocks this may not be practicable and antibiotic therapy is required.

1. Culture cells in the presence of the chosen antibiotic(s) (*see* Table 1) for a period of 10–14 d during which time most cultures will be passaged approximately four times. Each passage should be performed at the highest dilution that the cell will tolerate.

Table 1
Antibiotics for Use in the Elimination of Mycoplasma Infections

Agent	Source	Effective concentration, mg/L
Ciprofloxacin	Bayer	20
MRA (mycoplasma removal agent)	ICN-Flow	0 5
Novobiocin	Sigma	44
BM Cyclin (two antibiotics)	Boehringer Mannheim	10 5

2 Test the culture for the presence of mycoplasma by Hoechst stain. If mycoplasma is still detectable it is unlikely that this antibiotic will be successful and an alternative should be tried on a fresh batch of cells.
3 If the Hoechst stain is negative then the cells should be cultured in antibiotic-free medium for a period required to conduct 10 passages. Testing should be conducted at every passage to monitor treatment success since mycoplasma may persist at low levels immediately after antibiotic treatment
4 If the culture is mycoplasma negative after 10 passages in antibiotic-free medium, the mycoplasma may be considered to be eradicated and a bank of mycoplasma-free cells should be prepared immediately

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Fluorescent *In Situ* Hybridization (FISH) for DNA Probes in the Interphase and Metaphase Stages of the Cell Cycle

Linda A. Cannizzaro and Guangping Shi

1. Introduction

Fluorescent *in situ* hybridization (FISH) is a sensitive and powerful method for mapping and positioning DNA sequences in mammalian and other genome systems *(1–3)*. DNA sequences ranging in size from <1 kb to several megabases can be localized to a specific chromosome site. The DNA is first labeled by nick translation with a non-radioactive immunofluorescent compound such as biotin-11-dUTP or digoxigenin-11-dUTP, then hybridized overnight to cell or chromosome preparations. The resulting signal can be detected under ultraviolet light with filters of wavelengths specific to the fluorescent compound.

FISH is now the method of choice for mapping DNA inserted into plasmid, phage, cosmids, P1, and yeast artificial chromosomes (YACs) *(4–6)*. In addition, Alu-PCR products isolated from megabase sized YACs, have been mapped by us and others, directly to a chromosome locus by FISH without background contamination from yeast DNA *(7)*.

The precision and speed of mapping DNA probes by FISH, make it possible to construct a physical map of genomic regions that have a critical role in genetic or malignant disorders. It becomes feasible to position a gene in relation to other genes so as to define the gene's orientation to a chromosome segment altered as a result of either a translocation or an inversion event *(1,8,9)*. This provides important information regarding the series of events that may ultimately transform or mutate the cell genotype. A gene placed in a new position as a result of a chromosome alteration, will most likely result in producing an altered form of the gene product *(10)*. This usually has a domino effect and can influence the transcription or function of neighboring genes.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

FISH technology also permits mapping, ordering, and positioning genetic loci in the nondividing or interphase stage of the cell cycle *(11–17)*. This aids molecular cytogenetic analysis in cases where the cells are not proliferating or are in a terminal differentiative stage. For instance, tissue samples obtained from patients undergoing chemo- or radiation therapy, usually contain only a few dividing cells as a result of the trauma induced by the therapeutic regimens. The patients condition and response to the therapy can still be evaluated in the interphase stage, especially for diseases such as chronic myelogenous leukemia (CML) where a specific chromosome alteration in the appearance of a Philadelphia chromosome causing fusion of the *bcr/abl* oncogenes, is diagnostic of the presence of disease *(8)*. Such probes are now commercially available, and are usually prelabeled ready for use for FISH analyses. A number of chromosome translocation-specific commercial probes have been developed to diagnose other forms of leukemia as well.

DNA probes can now be isolated from any chromosome region or band. This has been achieved by flow sorting specific human chromosomes and polymerase chain reaction (PCR) amplification of DNA from somatic cell hybrids retaining only one human chromosome or one segment of one chromosome. The end products are designated as chromosome "painting" probes which can detect signal over one specific chromosome or segment in either the metaphase or interphase stages.

Probes have also been designed that detect microdeletions, such as that specific for Williams syndrome on chromosome region 7q11.2. This deletion is not usually visible even with high resolution banded chromosomes, yet is readily detected with a 7q11-specific DNA probe. FISH technology has advanced cytogenetic analysis to a higher level of resolution never before achieved by routine chromosome analyses.

FISH is valuable for mapping and positioning multiple probes simultaneously along the same chromosome by labeling individual probes with different fluorescent compounds. Quantitative measurements of physical distance between two or more DNA probes is achieved with the help of a digital image analysis system equipped with a CCD camera and can be performed in the interphase or metaphase stage *(5,12,18,19)*.

A new technology, chromosome microdissection, has recently been developed to isolate DNA probes from any area of the genome irrespective of the structural elements which comprise the region. This has become a valuable method for identifying the chromosomal origin of marker chromosomes. The marker chromosome is first dissected out of the metaphase with a micromanipulator. The dissected unknown DNA is PCR amplified, then hybridized to normal metaphases by FISH in order to identify the origin of the marker chromosome (Cannizzaro et al., unpublished). This has become a powerful modifi-

cation of the FISH technology and has been undertaken in a number of prenatal and cancer diagnostic laboratories. FISH in combination with microdissection technology is valuable in cases where unusual and unidentifiable marker chromosomes are found in patients or fetuses with a high risk of a genetic disorder.

This chapter focuses on performing a nick translation procedure for labeling DNA probes with any immunofluorescent compound and a FISH procedure that can be readily modified for differing sizes of DNA probes inserted into various types of vector systems. The procedures can be applied to any FISH analysis irrespective of the cell type under investigation.

2. Materials

2.1. Chromosome/Cell Preparations

1. RPMI medium supplemented with 15% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD)
2. 200 m*M* Glutamine (100X) (Gibco).
3. 5000 U/mL, 5000mg/mL Penicillin/streptomysin (Gibco).
4. 10 ug/mL Colcemid (Gibco)
5. 0 075*M* KCl
6. 3·1 Methanol·glacial acetic acid

2.2. Probe Labeling

1. BioNick Labeling System (BRL, Gaithersburg, MD).
2. G-50 Sephadex Columns for biotinylated DNA purification (Boehringer Mannheim, Indianapolis, IN).
3. 2X SSC buffer: 300 m*M* NaCl, 30 m*M* sodium citrate, pH 7.0.
4. 2% Agarose gel.
5. 1kb DNA ladder (Gibco)
6. 1XTE buffer 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA

2.3. In Situ Hybridization

1. 100 µg/mL RNase (Sigma) in 2X SSC.
2. 70, 90, 100% Ethanol series
3. Hybridization mix· 50% formamide, 10% dextran sulfate, 2X SSC (0.3*M* NaCl; 0.03*M* trisodium citrate), 0 1% Tween-20.
4. Probe concentration. 20 ng/µL biotinylated DNA.
5. *Cot* 1 DNA concentration: 0 1–1 µg/µL human *Cot* 1 DNA (BRL Life Technologies). Stable at –20°C.
6. 3*M* NaOAc, pH 5 2
7. 70% Formamide, 2X SSC, pH 7.0. Store at 4°C
8. 50% Formamide, 2X SSC, pH 7.0. Store at 4°C.
9. 1X PN buffer 0 1*M* NaH_2PO_4 and 0 1*M* Na_2HPO_4 to pH 8 0, 0 1% Nonidet P40 (Sigma, St. Louis, MO).

2.4. Probe Detection

1. 1X PN buffer (*see* Section 2.3)
2. FITC avidin (Vector Laboratories, Burlingame, CA) Store at 4°C
3. Biotinylated anti-avidin (Vector Laboratories) Store at 4°C
4. Antifade: 1:1 glycerol Citifluor AF3 containing 3 75 μg/mL DAPI and 0.75 μg/mL propidium iodide Filter through 0.2-μm filter to remove excess particles Store in dark at –20°C
5. Normal goat serum
6. 1% Bovine serum albumin (BSA)
7. Borate buffer 50 m*M* Na_2SO_4, 2 5 m*M* $Na_2B_4O_7$, adjust pH to 9 2
8. Wright-Giemsa stain
9. Glass slides
10. 22-mm Square coverslips
11. Rubber cement
12. Humid chamber (square Petri dish with wet filter paper on bottom and containing pipets/toothpicks to place slides on)

3. Methods

3.1. Chromosome Preparations

1. Chromosome preparations for FISH analysis (*see* Note 1) can be used from any type of tissue, with modifications dependent on the growth characteristics of the cell type Cells grown in suspension such as peripheral blood lymphocytes, bone marrow, and lymphoblasts, require minimal pretreatment and incubation with colcemid, whereas, preparations from solid tissues, such as tumor biopsy specimens and fibroblast/epithelial cell lines, require longer incubation times in colcemid
2. Optimal growth and division of cells for suspension cultures is obtained if the cells are split and fed 24 h prior to harvesting In the case where cells are attached to the flask, optimal chromosome preparations are obtained after cells are trypsinized, split, and fed 48 h before harvesting for chromosomes

3.2. Cell Harvesting

1. Preincubate cells with colcemid at a final concentration of 0.02 μg/mL for 20 min to several hours depending on the cell type
2. At the end of the incubation, centrifuge cells at 1000 rpm for 10 min Discard supernatant, then add approx 5 mL of 0 75*M* KCl Leave at room temperature for 15 min, then add 1 mL of 3:1 methanol:glacial acetic acid fixative Recentrifuge at same speed for 10 min
3. Remove supernatant, then add 5–10 mL fixative solution Leave at room temperature for 20 min to 1 h Spin down in the same manner, wash with fixative at least one to two more times, then centrifuge Remove supernatant and place final cell suspension in a small volume of 3 1 fixative
4. Place several drops of cell suspension on cold wet slides Air dry or leave on warm plate at 56°C until dry

FISH can be performed on freshly prepared slides, or slides that are several months old, stored at 4°C We have had consistent success by storing slides overnight in 2X SSC at 37°C.

3.3. Probe Labeling

Commercially available DNA probes are prelabeled with either biotin or digoxigenin. Such probes are ready to use for FISH without further treatment. All other DNA probes must be labeled with either fluorescent compound before FISH is undertaken (*see* Note 2) The BioNick Labeling System (BRL Life Technologies) is used to label DNA probes with biotin and to generate optimal sized fragments for FISH hybridizations (500–1000 basepairs). For labeling with digoxigenin, the Nick Translation Kit (Boehringer Mannheim) with digoxigenin-11-dUTP is used.

1 Add 1 µg of the DNA probe to a tube that contains 5 µL of 10X dNTP along with 5 µL of 10X enzyme mix (DNA polymerase I/DNase I).
2 Mix the components together and spin in cytocentrifuge briefly (15,000*g* for 5 s).
3 Incubate at 16°C for 1 h
4 Just before adding stop buffer, take out 1/10 volume of the mixture and run on a 2% agarose gel to determine whether label is incorporated and whether the optimal sized fragments have been obtained. A 1 kb ladder size marker (BRL Life Technologies) is run alongside the labeled probe If the level of label incorporation is adequate and appropriate sized fragments are obtained, then 5 µL of stop buffer is added to the reaction tube to stop the enzyme activity
5 Unincorporated nucleotides are separated from the labeled DNA with a G-50 Sephadex column (Boehringer Mannheim) Follow the instructions for use provided with the spin column
6 The resulting biotin/digoxigenin-labeled DNA probe is stored at –20°C in TE buffer Immunofluorescently labeled DNA probes are stable and can be stored up to one year at this temperature

3.4. In Situ *Hybridization* (3,7)

1 Slides are pretreated with 10 µg/mL RNase. Forty-five microliters is added to each slide, then coverslipped. Slides are placed in a humid chamber for 1 h at 37°C
2. After RNase treatment, slides are washed in 2X SSC three times, for 2 min each, then dehydrated through an alcohol series (70, 90, 100%) for 2 min each. Air dry.
3 Chromosomal DNA is denatured at 70°C in 70% (v/v) formamide, 2X SSC for 2 min, then passed through a cold (chilled at –20°C) ethanol series. Air dry.
4 The probe DNA is prepared by adding a 20–50 1 ratio of *Cot* 1 DNA.probe DNA (usually about 50 ng probe is used per experiment). Add 1/10 volume of 3*M* NaOAc (pH 5 2) and 2 volumes of 100% ethanol. Put at –20°C for 30 min, centrifuge at 4°C for 10 min Wash pellet with 70% ethanol, and air dry pellet

5 Dissolve DNA in 10 µL hybridization mixture, vortex, then denature it at 75°C for 10 min, and preanneal it at 37°C for 30 min About 10 µL probe mixture is added to coverslip, then inverted onto slide Seal with rubber cement, and incubate overnight or up to 16 h in humid chamber at 37°C
6 The next day, remove coverslip and remnants of rubber cement from slides
7 Wash at 45°C in 50% formamide in 2X SSC (pH 7.0) three times for 5 min each, then in 2X SSC once for 5 min Place slides in 0 1*M* PN buffer containing 0 1% NP-40 (pH 8 0) at 45°C once for 5 min, then put in fresh 0 1*M* PN buffer at 4°C The slides can be stored this way for up to 1 wk, and it is important to never let the slides dry

3.5. Probe Detection (3)

1 Store slides in 0 1*M* PN buffer until ready for use
2 Incubate slides for 5 min with 40 µL 1% BSA for 5 min at room temperature Coverslip with parafilm.
3. Add 35 µL of 1 to 400 dilution FITC-Avidin in 1% BSA in 4X SSC and incubate at room temperature for 30–60 min, then wash in PN buffer at room temp three times
4. To amplify, incubate for 5 min at room temp with 1 to 10 dilution of normal goat serum, add 35 µL of 1 to 100 dilution of biotin-antiavidin in 4X SSC and incubate at room temp for 30–60 min, wash in PN buffer at room temperature three times
5. To detect, add 35 µL of a 1 to 400 dilution of FITC-avidin in 1% BSA in 4X SSC and incubate at room temperature for 30–60 min, wash with PN buffer at room temperature three times
6 If a second amplification is required (very little signal can be detected with little background), add 35 µL of 1 to 100 dilution of biotin-antiavidin in 4X SSC and incubate at room temperature for 30–60 min, wash in PN buffer at room temperature three times (*see* Note 3)
7 To detect, repeat step 5 (*see* Note 4)
8 For digoxigenin-labeled probe, after step 2, add 35 µL of rhodamine antidigoxigenin (Boehringer Mannheim) (5 µg/mL) in 1% BSA in 4X SSC and incubate at room temperature for 30–60 min Wash in 1X PN buffer at room temperature three times.
9. Slides are counterstained by adding 40 µL of propidium iodide at 1 µg/mL/slide and incubate at room temperature for 2 min; rinse 0 1*M* PN buffer
10. Slides are mounted in antifade solution Stored in the dark to minimize fading. Visualize on an appropriate fluorescence microscope (*see* Fig. 1) (*see* Notes 5 and 6)

3.6. Chromosome Identification (20)

1. Wash slides with distilled water and allow to dry (removes antifade solution and immunofluorescence).
2. Place slides in a Coplin jar containing borate buffer (pH 9.2) at 37°C for 5 min
3. Submerge the slides in a horizontal position, in a Petri dish containing a 3 1 mixture of borate buffer and Wright-Giemsa stain solution for 6 min at 37°C
4 Rinse with distilled water and air dry

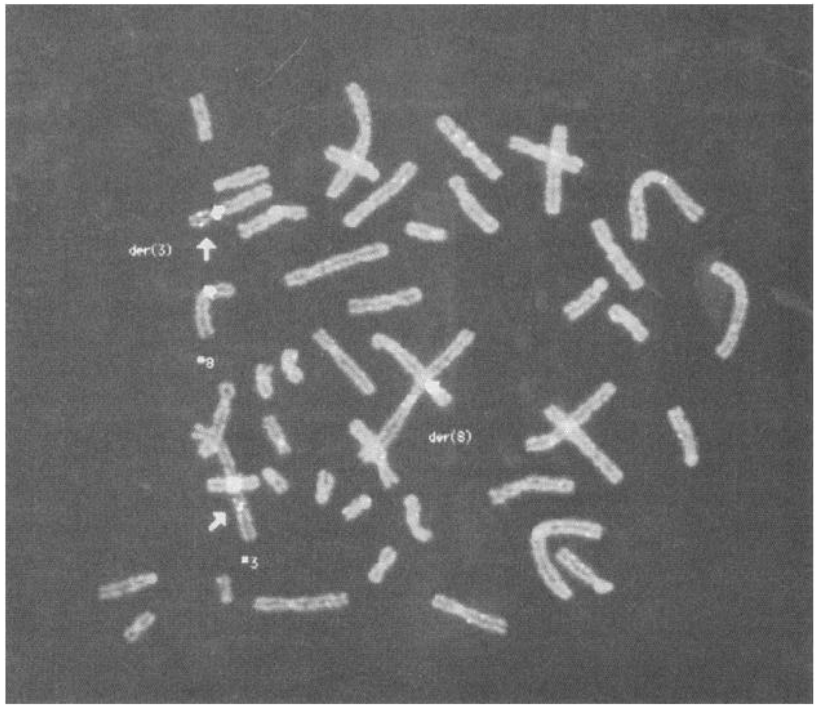

Fig. 1. FISH mapping of cosmid clone 11C12 (arrows) to a metaphase containing a t(3;8)(p14;q24) chromosome translocation. The cosmid maps to band 3p14 of chromosome 3. Normal and abnormal (der) chromosomes 3 and 8 are identified with an alpha satellite sequence which hybridizes to the centromere region and is specific for these 2 chromosomes.

4. Notes

1. A key to consistent success with the FISH technique is to make sure the chromosome or cell preparations are of the highest quality. If chromosome preparations contain a significant amount of cytoplasmic material, it will be difficult to resolve the signal, especially from a small-sized piece of DNA. Optimum quality preparations will decrease the likelihood of background problems or difficulties in resolving the fluorescent signal over a specific chromosome region. Wash cells several additional times in 3:1 fixative to improve cell resolution and remove excess cytoplasmic material.
2. Insufficient incorporation of label is a problem usually attributed to imprecise determination of the DNA concentration or indicates the DNA has not been properly purified. Run a gel with appropriate size standards before attempting nick translations to determine the DNA concentration. Otherwise, under-estimation or

over-estimation of the concentration will result in an inefficient labeling reaction Difficulties in nick translating the DNA are also encountered when there is an abundance of RNA still present in the DNA preparation Contamination of RNA must be eliminated by appropriate purification procedures before the DNA can be nick translated.

3 If a fluorescent signal is not detectable after the final amplification of fluorescent label, this can also be an indication that the DNA concentration may not have been correct. Lack of signal detection can also be attributed to an excess amount of suppressor or Cot DNA. Several experiments may be necessary to determine the appropriate ratio of Cot DNA to probe DNA Modifications may be necessary for different sized DNAs, due to varying amounts of repetitive and non-repetitive sequences in a particular sized DNA insert One can also reduce the stringency of the posthybridization washes in 50% formamide-2X SSC by either reducing the amount of time each slide is washed in the 50% formamide-2X SSC, or by reducing the number of washes.

4 Excess background signal is the most frequently encountered problem with FISH First, try reducing the concentration of DNA in the hybridization mixture One can also increase the stringency of the post-hybridization washes in 50% formamide-2X SSC by either adding several more washes or by increasing the amount of time in each wash. The stringency can also be increased by increasing the amount of formamide in the solution It is also important to use slides and coverslips cleaned with 70% ethanol Excess dirt should be avoided in order to diminish the amount of background signal.

5 When signal is detected, but it is very fuzzy and fades after a short time under the ultraviolet light, this can be attributed to the shelf-life of the antifade solution. This is easily solved by replenishing the antifade solution It has been our experience that this solution will function optimally for at least 3 mo Thereafter, it usually depends on where it is being stored It is best to make it fresh every 3 mo

6. Faint signals from small inserts are usually difficult to photograph This is compounded by detection with the triple band pass filter. Use of this filter diminishes the intensity of the signal, and in some cases, causes the fluorescence to quench much more rapidly This makes recording the signal impossible This problem can be overcome by scanning the preparation first with a double band pass filter, then switching to the triple pass filter just before taking a photograph In such instances, small signals can best be recorded with a very high speed/sensitive film, usually ASA 400 speed is adequate. We have successfully used ASA 400 for recording very faint signals. However, some laboratories have used ASA 1000 in such cases

Acknowledgments

This chapter was prepared while the authors research was supported by the American Cancer Society

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27

Cytogenetic Techniques for Human Leukemias

G. John Swansbury

1. Introduction

Since the previous edition of this book was published there have been two major developments that have greatly extended the application of cytogenetic techniques: The introduction of fluorescence *in situ* hybridization (FISH), which has had as dramatic an effect as the introduction of banding over 20 yr ago, and the increasing use of automated karyotyping systems both for the preparation of G-banded karyograms and for the manipulation of fluorescent images (see Chapter 26). Closely related advances in molecular genetics, including various forms of polymerase chain reaction such as comparative genome hybridization, mean that the cytogeneticist now has the means of resolving the most complex of translocations, with more techniques available than time to apply them all During the same period it has been confirmed that many of the acquired chromosome abnormalities seen in malignancy are intimately associated with specific stages of the neoplastic process, rather than being mere epiphenomena, and are also becoming ever more useful in differential diagnosis and prognosis. Consequently, clinical colleagues now expect that cytogenetic analysis of human leukemia will provide rapid, accurate, and specific results to help them in the choice of treatment and management of patients.

It follows that a chapter on cytogenetic techniques for studying leukemic tissue is appropriate in a series on molecular methods since these subjects are increasingly closely related and ever more complementary. Good, basic, conventional cytogenetic analysis has not been superseded by the many developments of molecular analysis: Although FISH and molecular studies can meet some of the limitations (detailed later) of cytogenetics, many analyses still require that there is at the very least some tentative indication of what abnor-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

malıty may be present. The effort and care spent on cytogenetıc analysıs as a first-line test are therefore possibly more ımportant than ever.

It is saıd that ıf there are many methods toward one end, then ıt usually follows that not only ıs none much better than the others but none ıs very good at all. Thıs still applies to cytogenetic studıes of leukemıc tıssues· There are probably as many subtly different methods as there are cytogeneticists and no one method has been ıdentıfied that gıves the consıstent, good qualıty that can be obtaıned wıth, for example, phytohemagglutinin (PHA)-stimulated normal lymphocytes. The observations that consistently applıed technique can give vastly dıfferent results ın cases processed sımultaneously and that populatıons of dıvısıons wıth different chromosome morphology can occur on the same slıde ındıcates that there ıs a dısease-derıved effect on marrow kınetıcs and chromosome morphology. Most ımprovements that mıght be obtained by the variatıon of techniques are slight and are swamped by this ıntrınsıc varıabılity. A survey of laboratory methods undertaken by the U.K. Associatıon of Clınıcal Cytogeneticists *(1)* revealed that almost every aspect of the processıng of marrows for cytogenetıc studıes was subject to substantıal varıatıon—and ıt can be assumed that every varıatıon worked for at least some cases.

Despıte all this, there has been some overall ımprovement ın success rates, abnormalıty rates and chromosome qualıty durıng the last decade, largely caused by·

1 Better qualıty samples beıng sent for analysıs,
2. The better selectıon of appropriate cultures for the dıagnosis, and
3 The more wıdespread use of the "ımmedıate" technıque, ın whıch the sample ıs not cultured at all but ıs collected straıght ınto hypotonıc solutıon

It stıll remains, however, that one of the most sıgnıfıcant factors ın maxımızıng success ıs the settıng up of multıple cultures to ıncrease the chance of catchıng abnormal dıvısıons at theır best.

The techniques for cytogenetic studıes ın leukemıa are simple and robust and can be learned ın a few hours; the analysıs of the chromosomes usually takes lıttle more than a few months of traınıng for most people; the ınterpretatıon of the results, however, requıres more experıence and a wıllingness to keep up-to-date wıth published work.

2. Materials

Most of the solutions should be kept in the dark at 4°C The dilutıons given here of most of the reagents are such that 0.1 mL may be conveniently added to a 10-mL culture.*

*NOTE· some of the chemıcals used—ındıcated by (*)—are known or potentıal carcınogens/poısons and should be handled wıth due care and attentıon

1. Containers: Sterile, capped, plastic, 10-mL centrifuge tubes. The caps should be tight-fitting to prevent leakage of fixative. For cultures lasting longer than 24 h, use 50-mL Universal tubes.
2. Pipets: plastic, disposable. Glass pipets should not be used because of the risk of needlestick injury.
3. Medium: For most cultures this laboratory routinely uses RPMI 1640, which was developed specifically for leukemic cells; many other media may be used successfully. To each 100 mL bottle add antibiotics (e.g., 1 mL penicillin + streptomycin) and 1 mL of preservative-free heparin. Medium used for longer cultures should have L-glutamine added (final concentration 0.15 mg/mL), this is an essential amino acid that is unstable and has a short life at room temperature.
4. Serum. Fetal calf serum is preferred but pooled human AB serum can be used. The proportion routinely added is 15 mL serum to 100 mL medium. For longer term cultures, each batch of serum should be tested to ensure that it supports cell growth.
5. Mitogens
   a. PHA stimulates T-lymphocytes to divide, acting via monocytes which produce interleukin 2.
   b. Pokeweed mitogen (PWM) indirectly stimulates both T- and B-lymphocytes.
   c. 12-*O*-tetradecanoyl-phorbol-13-acetate, also Phorbol 12-myristate 13-acetate (*) (TPA) is more specific for B-cells.

   PHA and PWM for cytogenetic use are obtained freeze-dried or lyophilized, ready for reconstitution to the appropriate concentration. TPA is obtained as a powder which is prepared by dissolving in 10% ethanol and then further diluting 1:19 with water to make a stock solution of 10 µg/mL in 0.5% ethanol. This should be stored in aliquots at –20°C.

   Other mitogens which can be used include Protein A and lipopolysaccharide (*) (LPS). Some laboratories use "cocktails" of several mitogens, but this increases the likelihood of stimulating normal cells into division.
6. Blocking agents
   a. FluorodeoxyUridine (FdUr) (*): Stock solution: 1 part FdUr (25 µg/mL) to 3 parts Uridine (1 mg/mL), giving final concentrations of 0.1 and 4.0 µ*M*. Small aliquots should be frozen, once thawed the effectiveness declines after a week.
   b. Excess thymidine (XT): Stock solution: 0.05 m*M* (30 mg/mL).
7. Releasing agents:
   a. Thymidine: 10 µ*M* stock solution. 0.05 g in 100 mL distilled water; filter sterilize (0.22-µm millipore filter). Store in aliquots at –20°C; the thawed solution keeps at 4°C for at least 1 mo (do not re-freeze).
   b. Deoxycytidine. Stock solution: 10 µ*M* (10 mg/44 mL).
8. Arresting agents: colcemid or colchicine. Colcemid is said to be less toxic than colchicine, and has a reversible effect. Both of these arresting agents act by preventing spindle formation and so the chromosomes remain dispersed in the cytoplasm. Colcemid (*) (Also called demecolchicine, from deacetylmethylcolchicine) Stock solution 1 µg/mL. Colchicine: (*) Stock solution: 20 µg/mL.

9 Ethidium bromide. (*) 10 μg/mL in saline (0 9% NaCl)
10. Hypotonic solution 0 075*M* potassium chloride (KCl, 5 59 g/L) Use at room temperature or at 37°C Note that the effectiveness does not derive from just the osmolarity The K^+ ions have a physiological action, so no advantage is obtained by diluting further With longer chromosomes, twisting or overlapping can be a problem and the use of 19 parts KCl to 1 part 0 8% sodium citrate is sometimes helpful.
11 Fixative· Absolute methanol, 3 parts, and glacial acetic acid, 1 part. This should be freshly prepared just before use although it may be kept for a few hours if chilled.
12 2X SSC· Salt solution used for banding NaCl (17 53 g) + sodium citrate (8 82 g) made up to 1 L of aqueous solution
13. 2.5% Trypsin: stored frozen in 1 mL aliquots Diluted 1:50 in buffer (Ca^{2+}- and Mg^{2+}-free, e.g , Hank's solution) when required
14 Phosphate-buffered saline· pH 6 8 for stains
15 Slides· Frosted-end variety are preferable for convenience of labeling The slides must be free of dust and grease Specially cleaned slides may be purchased, otherwise wash in detergent, rinse well in water, then in dilute HCl and alcohol These may be stored dry, protected from dust, or in acidified alcohol, or in a freezer
16. Stains. Giemsa (Gurr), Wright's and Leishman's stains are all suitable Leishman's stain is said to be better for the fuzzy chromosomes that occur in leukemias
17 Coverslips 22 × 50 mm, grade 0 preferred, but the thicker grade 1.5 may be acceptable
18. Mounting medium Gurr's neutral mounting medium is routinely used in this laboratory; in our experience it does not leach stain if it is not diluted with xylene. Other suitable mountants are XAM, DPX, Histamount, and so on. Mounting slides has the advantage of protecting delicate chromosome spreads from dust and scratches. However, if it is likely that a slide might need to be destained and processed for FISH analysis, then it should not be mounted.

3. Methods

As already stated, every cytogeneticist has his or her own variations on basically similar processes. Furthermore, it is often found that what worked well in one laboratory needed modification in another. The following is a summary of the general basic technique which will be found to be generally applicable anywhere; variations to suit particular conditions need to be derived locally.

In short, chromosomes are prepared from dividing cells (mitoses) since at metaphase, just before division, they shorten and become recognizable, discrete units. The cells may be already dividing in the tissue supplied or, in certain circumstances, may be stimulated into division. They are arrested and accumulated in metaphase or prometaphase by destroying the spindle, e.g., with colcemid. The cells are treated with a hypotonic solution to encourage

spreading of the chromosomes. They are then fixed, after which they can be stored indefinitely. Fixed cells are spread on slides and air-dried. They can be stained immediately, but are usually first treated to induce banding patterns on the chromosomes to assist in their identification.

3.1. Collection of Samples

Bone marrow is the preferred tissue for investigation of most hemopoietic disorders. Valuable results can sometimes be obtained from blood, spleen, and so on, especially if there is myelofibrosis, and a blood sample can be a valuable back-up if the supplied bone marrow sample is small or has clotted. Details relevant to culturing blood are given in Note 1; for the methods described here it is assumed that a sample of bone marrow is being studied.

Cytogenetic studies of bone marrow are expensive because they are highly labor-intensive, and so a lot of time can be wasted on inadequate samples. Ideally, a generous portion of the first spongy part of the biopsy should be sent: Later samples tend to be heavily contaminated with blood. Re-siting the needle, through the same puncture if necessary, gives better results than trying to get more material from the same site. If there is plenty of material, consider storing some in liquid nitrogen (*see* Note 2). Heparinized bone marrow samples can be transported with or without medium; however, use of medium will reduce the risk of the sample clotting or drying out. If harvesting can be started within about 25 min of aspiration, some of the sample should be processed by the immediate technique. The samples should be sent to the laboratory as quickly as possible without exposure to extremes of temperature, although divisions can sometimes be obtained even from samples a few days old, blood samples being more robust than bone marrow. In cases of acute lymphoblastic leukemia (ALL), however, or in any sample with a high white cell count, detection of a clone usually depends on the sample getting prompt attention. The samples should not be refridgerated: The cells may take an unpredictable time to start dividing again after exposure to cold conditions. It is very important that the marrow aspirate for cytogenetic study of cases of acute leukemia is taken before any cytotoxic therapy is given: Any clone usually disappears once treatment has started, even if the disease is still active.

3.2. Handling of samples

Careful sterile technique should be developed, though since most cultures are short-term, contamination is not usually a problem. All samples should be handled as carefully as if they might have hepatitis or HTLV/HIV (AIDS). The risk from aerosol formation from marrow or blood is low and can be minimized by the use of screw-capped tubes and by conducting all processing in a laminar flow cabinet. The greatest risk follows direct introduction of infected serum, e.g., through

a cut on the skin or by penetration with a needle or glass Pipet. Therefore, rubber gloves should be worn and plastic pipets or "quills" should be used (rather than needles or glass pipets) while processing unfixed tissue

3.3. Choice of Cultures in Hematology Cytogenetics

It has been shown that erythropoietic divisions predominate in the first few hours of culture with granulopoietic divisions appearing subsequently *(2,3)*. This corresponds to the observation that for erythroleukemia (acute myeloid leukemia [AML-M6]) short cultures are most likely to be successful and for other AMLs the sample should be cultured for at least 16 h (overnight). The immediate preparation or a same-day (direct) culture are often best for ALL and also for chronic myeloid leukemia (CML). Chronic lymphocytic leukemia (CLL) and ALL which have B- or T-cell phenotype need 3–5 d culture with appropriate mitogens, as well as having some unstimulated cultures.

In addition, because the cell cycle time is unpredictably affected by the disease, a variety of cultures should be set up in all cases to maximize the chances of getting divisions at a suitable stage. Occasionally very small sample size limits the number of possible cultures, so the following guide will help to choose the most appropriate one

3.3.1. Unstimulated Cultures

1. Immediate preparation without culture. Where local conditions allow prompt attention, part of the sample is put straight from the syringe into KCl with colcemid and heparin, some protocols also include some trypsin Twenty-five minutes later the tube is centrifuged and fixed according to the usual following procedure This is often the optimum culture for ALL and is also successful in other conditions Divisions from ALLs often have particularly poor morphology, especially when derived from a clone with high hyperdiploidy. More suggestions worth trying may be found in the detailed methodology published by Williams et al. *(4)* who have a consistently high success rate in their laboratory
2. Direct preparation. The sample is harvested the day it was taken Colcemid may be added immediately when setting up cultures or after a short incubation. Harvesting is usually begun about an hour after adding colcemid This is a good culture for ALL, CML, and AML-M6, it is not recommended for other AML types An occasionally useful variation is to add ethidium bromide *(5)* and leave the culture for 2–4 h This retards chromosome condensation
3. Overnight culture: Colcemid is added to the culture, which is then incubated overnight and harvested next morning This is the culture most likely to be successful in terms of numbers of divisions, although many of them will probably have short chromosomes· The longer the cells are exposed to colcemid, the more divisions will be collected—but the shorter the chromosomes will become In practice, however, there are often some with good-quality, long chromosomes

In this laboratory we have found that longer chromosomes may be obtained if the culture is placed in a fridge until about 4 PM and then incubated until harvesting at 9 AM next morning.

4 Twenty-four-hour culture The sample is incubated overnight and colcemid is added at some convenient time next day for 1 h or so before harvesting

5. Forty-eight-hour and 72-h cultures The sample is incubated for 2 or 3 d before harvesting These longer cultures may be useful if there is enough material to set up extra cultures, or if a weekend makes other cultures less convenient

6 Synchronization or blocking· The divisions are probably not truly synchronized, but there is an effect arising through a retardation of S-phase These methods were introduced to increase the number of divisions collected with a short exposure to colcemid, thus obtaining long, prometaphase chromosomes, in practice, the number of divisions is usually reduced but the chromosome morphology may be improved The cell cycle duration (and therefore release time) of malignant cells is more variable, and usually considerably longer, than that of normal tissues, so it is difficult to determine the optimum release duration. However, because of the good quality chromosomes sometimes obtained, blocked cultures are always worth doing if there is sufficient material

Commonly used synchronizing agents are Methotrexate (MTX) *(6)*, good for AML but possibly less so for ALL; FdUr *(7)*, which may bias towards normal cells, and XT *(8)*, which is probably good for all types but metaphases often do not spread so well This laboratory usually sets up both FdUr and XT cultures The agent is best added after a few hours of culture, e g , at the end of the afternoon, and the culture is incubated overnight. Next morning the block is released using thymidine for FdUr and deoxycytidine for XT.

Culture for 5 h after release of MTX block was defined as optimal for normal lymphocytes Gallo et al *(9)* showed that the time should be 9 5–11 5 h for myeloid and leukemic cells, Morris and Fitzgerald *(6)* confirmed that the time varies between patients and have shown that the cell cycle time is generally shorter in CML than AML Despite these studies, it seems that many laboratories routinely allow 6 h before adding colcemid for 10–15 min before harvesting.

3.3.2. Mitogen-Stimulated Cultures

1 Two- to three-day culture with PHA· PHA-stimulated culture of a small part of the bone marrow or blood sample should be used in all new cases to determine the patient's constitutional karyotype against which to compare any acquired abnormalities found. It is also an essential culture in all T-cell disorders

Note that allowance should be made for the response of diseased T-cells being reduced or retarded, so 72-h cultures are more likely to be successful than 48 h At the end of the culture time, the divisions are collected as described above In this laboratory the practice is to have two cultures, one harvested after overnight colcemid, the other after blocking

2. Three- to five-day cultures with B-cell mitogens· For B-cell lymphoid disorders, including lymphomas, it is essential to set up several of these using a selection of B-cell mitogens, again, blocking may be used

3.4. Setting Up Cultures

1. If the marrow aspirate arrives in medium known to be fresh and the patient is not on chemotherapy, then warmed serum and more medium can added directly It is better, however, to spin down the sample and resuspend it in fresh medium and serum
2. Use 5 or 10-mL cultures with a cell density of about 10^6/mL It is easy to over-inoculate cultures from patients with CML and sometimes ALL These often have high white cell counts. Adding too many cells almost always results in failure to obtain any divisions at all.
3. Add mitogens or blocking agents as appropriate, as described in Section 3 3 1
4. Cultures can be gassed with 4% carbon dioxide in air to help maintain the pH of the culture medium if it is bicarbonate buffered An increased partial pressure of carbon dioxide is not as important for cell growth as a decreased partial pressure of oxygen· 2 5% may be optimal for longer cultures
5. Stand the culture tubes in the incubator (at 37°C) at an angle, rather than upright, as this increases the surface area of the deposit and reduces local exhaustion of the medium
6. After the appropriate culture time, having released any blocked cultures, add colcemid (0 1 mL stock to 10 mL medium)

3.5. Harvesting

Samples must be in capped tubes during centrifugation and the centrifuge buckets should have secure lids to avoid aerosol dispersion.

1. Spin down the cells, remove the supernatant, and add hypotonic KCl solution, mixing thoroughly by tapping the base of the tube after the first few drops have been added Make up to 10 mL, and leave for 7–15 min
2. Add 4–6 drops of fixative, spin again, remove the supernatant, and tap the base of the tube to loosen the pellet. Add a drop or two of hypotonic if necessary to ensure dispersal of the cells, as they are useless if they are fixed in a lump Add 1–2 mL of fresh fixative while tapping the tube. The first few drops of fix are the most important and can have a big effect on chromosome quality Make up to 10 mL and invert the tube to ensure thorough mixing.

   Tip: *If the volume of red blood cells was large and inadequately dispersed in hypotonic, they may fix into a gelatinous mass, trapping the white cells, if this happens, resuspend in hypotonic for a few minutes and then re-fix*
3. Leave in the first fix for 15–20 min before spinning again and adding fresh fixative. After this, the sample can be stored at –20°C with further changes of fix when convenient If there was a lot of fat in the specimen, it may be removed by washing once with Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid)

   The fix should be changed at least three times (until the solution is completely colorless) before spreading. Stored at –20°C, the DNA will remain good for chromosome spreads or for molecular analysis for several years

3.6. Spreading

1. Change the fixative shortly before spreading, then spin again and add a few drops of fresh fixative to obtain a slightly cloudy suspension. Judging the correct dilution comes with experience· If the cells are not sufficiently dispersed, then the chromosomes will not spread properly, if the dilution is too great then time will be wasted in screening nearly blank slides. In this laboratory clean slides are kept in a freezer for a while prior to use, as the film of frost helps spreading. However, dry slides, or slides dipped into water may be used
2. A couple of drops onto each slide is adequate Spreading may be helped if the suspension is dropped from a foot or more above the slide but this is risky and should not be done if the sample is small and precious. Spreading may be improved by "huffing" on each slide immediately afterwards or by waving it in the hot moist air above a flame Adding a further drop of fresh fix to the spread is sometimes beneficial Another occasionally useful variation is to spread from 60% aqueous acetic acid, which usually gives fair spreading of chromosomes but possibly at the expense of reduced banding quality. There are several factors which affect spreading, and it has been shown that the air temperature should be about 25°C and humidity should be about 50–55%
3. If possible, check the first slides immediately under phase-contrast microscopy for cell density and chromosome spreading. If these are not optimal, try varying the spreading procedure
4. Place the slides on a hotplate or in an oven (at 60°C) for a few hours to dry. Some of the slides may be stained immediately but banding is not very effective until the slides have "aged" for a few days Some aging effect can be obtained more quickly by incubating the slides at 60°C overnight in an oven

3.7. Banding

There are several methods of producing bands on chromosomes (*see* Note 3). Band patterns are broadly grouped into G (Giemsa) bands and R (reverse) bands which are largely complementary *(10)*. Some methods specific for identifying certain limited chromosome regions exist but these have become largely redundant since the introduction of FISH (*see* Chapter 26).

This laboratory routinely uses G-banding produced by incubation in 2X SSC followed by treatment with trypsin (modified from ref. *11*). By this method the trypsin serves to enhance the bands produced by the 2X SSC, and may even be omitted. Trypsin may be used alone, but without the incubation in 2X SSC the banding tends to be unpredictable and it is difficult to control the amount of digestion needed. Chromosomes banded with 2X SSC may have a slightly hairy appearance; trypsin alone tends to produce rounded-off bands and thus an apparently clearer outline.

3.7.1. 2X SSC Banding Procedure

1 Heat up a Coplin jar containing 2X SSC to 60°C in a water bath Incubate the slides for 60–90 min for bone marrow, 1 h for blood.

2 Cool the slides and rinse in water or buffer (The slides may be dehydrated through alcohols and left to dry—overnight if wished—before continuing with trypsin treatment.)
3. Add 1 mL of trypsin to 50 mL of Ca^{2+}- and Mg^{2+}-free Hank's solution, the temperature should not be >10°C.
4 Transfer the slides to a jar containing Hank's solution Each slide is then immersed in the trypsin solution for a few seconds, washed in buffer or dipped in dilute serum solution to arrest the enzyme action. The time needed in trypsin is very variable and may need adjusting for each case. It is affected by variations in spreading technique, age of the slides, degree of contraction of the chromosomes, general chromosome morphology, and so on.

3.7.2. Trypsin Banding Procedure

This is as from step 4 in Section 3.7.1., but usually performed at room temperature. The duration of exposure needs careful attention especially if the slides have not been sufficiently aged. It can be helpful to watch a metaphase under 40× phase contrast to see when the chromatids start to swell. A variation is to dip the slide for 30 s into 25 vol hydrogen peroxide (1:3 in tap water) then rinse in tap water before proceeding to use trypsin.

3.8. Staining and Mounting

1 For Giemsa staining, dip the slides in buffer first, to reduce the risk of picking up oxidized Giemsa from the surface of the stain. Use freshly diluted stain in pH 6 8 buffer 1 part stain to 25 parts buffer for 5 min. (For Leishman's stain, the slides should first be air-dried and should be stained lying flat, use 1 part stain to 4 parts 6 8 buffer for 3–4 min For Wright's stain use 1 4 for 3–5 min)
2. Rinse the slides in buffer, blot gently and stand to dry· use of a fan will help to ensure that drying is rapid and uniform.
3. Mount the slides, if desired, using a small quantity of mounting medium Leave to dry overnight before examining the slide with an oil immersion objective, as oil and soft mountant tend to mix Do not leave the slides exposed to direct sunlight.

3.9. Chromosome Analysis (see Note 4)

Use a 10× objective for screening and an oil-immersion 100× objective for studying metaphases. Analysis directly down the microscope is possible with experience but at least one division from each case should be photographed and karyotyped as a record and as part of laboratory quality control, especially in apparently normal cases. Most laboratories which undertake any substantial volume of cytogenetic work now use an automated karyotyping system, which is invaluable for confidence in one's analysis, for checking, and for training.

When screening, it is important to bias toward cells with poor morphology where there is a mixed population (*see* Note 5). Selection of only good mitoses

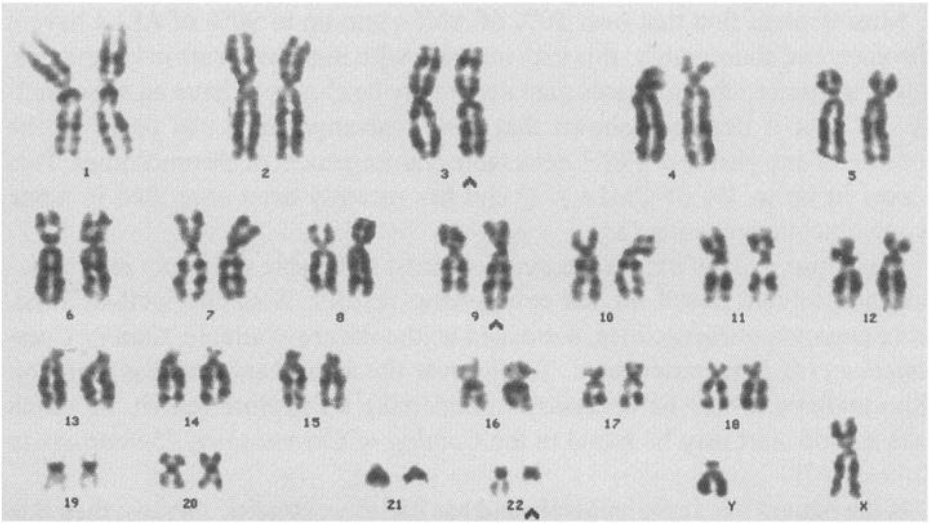

Fig. 1. G-banded karyotype, showing two abnormalities: The Philadelphia translocation, t(9;22)(q34;q11), most common in CML but also found in AML and ALL, which results in rearrangement of the ABL and BCR genes, and an inversion of part of the long arms of a #3 chromosome, inv(3)(q21q26). This abnormality is associated with abnormal megakaryocytes and platelet function. The breakpoint at 3q26 involves the EVI1 gene.

can lead to failure to detect the presence of an abnormal clone. If the quality of metaphases is poor then full analysis may not always be possible. However, even if the chromosomes can only be counted and/or grouped, useful information can sometimes be obtained. Be alert to the possibility of multiplier abnormalities; Fig. 1 shows a karyotype with an obvious, common abnormality and a rarer, subtle one that could easily be missed.

Where resources are available, at least 25 divisions should be fully analyzed unless a clone can be adequately defined with fewer. Chromosome abnormalities are described according to an International Nomenclature *(10)*. It has been calculated that if 29 cells are normal then one can be 95% confident that a clone involving 10% or more of the population is not present *(12)*. Even if a clone is detected after analyzing just a few cells, more may be analyzed in case of clonal variation and even the presence of multiple clones—a rare finding. A clone is defined as at least two cells with the same abnormal or gained chromosome or at least three cells with the same missing chromosome. Note that clonal loss must be intelligently differentiated from technical, random loss that can be at a high frequency.

Most centers find that over 50% of AMLs and up to 90% of ALLs have a chromosome abnormality; this may increase with improvements in techniques It was suggested that all cases may eventually be shown to have an abnormality *(13)* but it has been shown that gene rearrangements can occur in the absence of any visible or FISH-detectable rearrangement of chromosomes. This occurs in up to 3% of CMLs *(14)* and has recently been described in acute promyelocytic leukemia *(15)*.

Clinicians usually expect the cytogeneticist to be able to supply interpretation and advice based on the cytogenetic results. With the well-defined, more common abnormalities, published textbooks are available. *Cancer Cytogenetics (16)* is recommended. To discover the significance of less common abnormalities it may be necessary to undertake a literature search, in which case a good start may be found in the *Catalog of Chromosome Aberrations in Cancer (17)*

If the patient has acute leukemia and has had any cytotoxic therapy, then it is highly probable that any clone will become undetectable even if remission has not been achieved. Conversely, if a clone is found in a patient on treatment, then it may indicate resistant disease requiring more intensive therapy. Therefore, proper interpretation of results requires knowledge of the patient's treatment status. In CML the Philadelphia translocation (Ph) usually persists after treatment, though in some patients response to interferon is indicated by reduction or disappearance of the Ph-positive clone

It can be discouraging to work in leukemia cytogenetics when even experienced workers can find that up to one third of samples may fail to give a useful result or the chromosomes of abnormal divisions have refractory morphology. The work remains interesting, however, because of the high frequency and variety of chromosome abnormalities, their clinical importance, and their continued contribution to biological research.

4. Notes

For more notes on all aspects of cytogenetics than those given here, the second edition of a practical textbook by Rooney and Czepulkovski is recommended *(18)*

1 Blood cultures are used for the following purposes.
   a. T-lymphocytes that are cultured with PHA in the presence of monocytes for at least 48 h are transformed and start dividing. These cells can usually be used to establish the patient's constitutional karyotype against which any acquired abnormalities found can be compared Other tissues, e g., skin, may be used instead, but blood lymphocytes are usually the most convenient Note that if the patient has a T-cell disease, then some or many of the divisions may derive from a clone, also, diseases of stem cells or early pluripotent progenitor cells

(including CML) may also result in abnormal divisions being found in PHA-stimulated cultures Their presence does not mean that they are constitutional

b In the acute leukemias, cultures of blood leukocytes unstimulated by any mitogens may be useful if marrow is not available or has failed to produce a result Mitoses may be found in approx 10% of unstimulated cultures where there are blasts in the blood from the bone marrow or from extramedullary sites of hemopoiesis. If a chromosomally abnormal clone is found, these cells can be scored as bone marrow cells, though it must be borne in mind that some of these cells may have left the marrow weeks or months previously, so the population found may not represent an up-to-date picture of the state of the marrow If no clone is found then the result should be regarded as a failure, since normal divisions can occur due to causes unrelated to the disease Other malignancies, e g , multiple myeloma, may also release dividing cells into the blood, usually at a low frequency

c Cytogenetic studies of chronic lymphocytic leukemia (CLL) and B-cell disorders can be made on 3–5-d blood cultures stimulated with B-cell mitogens separately and in combination, as described earlier

2. Use of frozen material The custom in some hospitals and laboratories of cryopreserving viable samples of blood (and other tissues) in liquid nitrogen has been of great help to the development of molecular techniques and has permitted retrospective analysis of rare tumors. It is also possible to establish cultures for cytogenetic studies on frozen material if sufficient care is taken in the freezing and thawing processes A minimum procedure is given here. refinements are according to the facilities available

   Freezing. Suspend the cells in a mixture of 10% dimethyl sulphoxide (DMSO), 20% serum and 70% medium and put in 2 mL aliquots into the polythene tubes specifically produced for cryopreservation. Start freezing straight away. 1°/min for 30 min then 3°/min to the end. If a regulated system is not available, small quantities of cells can usually be safely cooled in the vapor phase at the top of a tank of liquid nitrogen

   Thawing· Warm up quickly (shake the vial in warm water) and wash with medium + serum to remove the toxic DMSO Culturing, and so on, is as described for fresh tissues, though some cultures may need an extra day before harvesting.

   Although some samples fail to recover, this Department has occasionally obtained chromosomes from frozen material which were of better morphology than those from a fresh portion of the same biopsy.

3. Fluorescence banding· Standard banding produced by fluorescent dyes is not recommended for routine cytogenetic work with leukemic tissues The interpretation requires considerably more experience and the rapid fading during exposure to UV light is a major drawback to the prolonged analysis of complex abnormalities down the microscope Photography gives a poor record because of the low contrast. In the past there were occasions when fluorescent dyes were helpful in identifying the Y chromosome and certain centromeres, but the application of FISH technology has superseded these uses

4 Applications and limitations of conventional cytogenetics, and the place of molecular analysis It is important to be aware of the applications and the limitations of conventional cytogenetics, and to know when molecular analyses may be more appropriate The usual clinical applications of the cytogenetics of acquired abnormalities in hematology are
   a To establish the presence of a malignant clone Detection of a karyotypically abnormal clone is almost always evidence for the presence of a malignancy, a rare exception being trisomy for chromosome 14 in reactive lymphocytes around renal tumors Demonstrating that there is a clone present is particularly helpful in distinguishing between reactive myelodysplasia and dysmyelopoietic syndrome, for example Note that the finding of only karyotypically normal cells does not mean that there is no malignant clone present
   b To clarify the diagnosis. Some abnormalities are closely associated with specific kinds of disease
   c To indicate prognosis, independently or by association with other factors Note that the presence of a clone does not automatically mean that the patient has a poor prognosis· Some abnormalities are associated with a better prognosis than a normal karyotype and some with worse
   d To monitor response to treatment Although conventional cytogenetic studies are not efficient for detecting low levels of a particular clone, and therefore should not be used routinely to monitor remission status, there have been occasions when this laboratory has found persistent disease in patients presumed to be in clinical remission with less than 5% blasts seen by cytology. Other uses include the determination of origin of hemopoietic tissue after allogeneic transplant, whether of bone marrow or of blood-derived stem cells, and the detection of response of CML to interferon, currently monitored by 6-mo scoring of the presence of the Philadelphia translocation Note that occasional clonal cells may be seen posttransplant without necessarily indicating relapse, their *persistent* presence or increasing incidence of abnormalities is of greater concern, although it is worth noting that the t(8;21)(q22;q22) translocation has been found at low levels in long-term remission *(19)* and without apparent significance
   e To indicate areas of particular interest for research.

The limitations are:

a. Only dividing cells can be assessed. If it is already known what specific abnormality is present and there are suitable probes available, then molecular analysis is complementary in being applicable to nondividing cells.
   b Studies are expensive because of the lack of automation in sample processing and the time needed to analyze each division, consequently only a few divisions can be analyzed It has been estimated that in an adult about 40 thousand million new cells are produced in the bone marrow every hour, so only a very small proportion is being examined. If available, molecular analysis has the advantage that several hundred cells can be screened efficiently.

c There is no useful result from some patients, e.g., if insufficient, unanalyzable, or only normal divisions are found Molecular analysis, by screening with a panel of probes, can be helpful.

d Sometimes the result is of obscure significance Rare or apparently unique abnormalities still occur and determining their clinical significance depends on a willingness to take the trouble to contribute to the literature. Molecular analyses are generally used to detect already-known abnormalities, so the substantial proportion of patients with unusual abnormalities that still need researching is an argument in favor of retaining full conventional cytogenetic analysis for all cases of leukemia at diagnosis.

Molecular analysis and FISH are particularly useful in identifying inv(16)(p13q22) and subtle translocations like t(15,17)(q22,q21) that can easily be missed if the quality of chromosome spreads is very poor

When DNA probes were developed for the Philadelphia chromosome translocation, t(9,22)(q34;q11) *(20)*, it was supposed that the labor-intensive cytogenetic screening for this abnormality (and similarly for other translocations) might become redundant However, important information would be lost if there was dependence solely on molecular detection: It is impracticable to screen cases for all the presently identifiable abnormalities, some translocation breakpoints have been found to be spread over very large distances, so the translocation could be missed if it was beyond the range of the probe being used, and the presence of further, nonspecific abnormalities can have a profound effect on the clinical significance As a general although not invariable rule, complex clones often imply a worse prognosis than simple clones

5. Distinguishing between significant abnormality and normal variation
   a. Polyploid mitoses (polyploid = numbers of chromosomes in multiples of 23 such as 69 and 92, not the usual 46) may be common, especially in actively regenerating marrow; particularly large accumulations of chromosomes usually derive from megakaryocytes These are usually normal but should not be ignored even if the analysis of so many chromosomes appears daunting In some cases their morphology is better and so abnormalities may be more apparent
   b. In some patients a high frequency of different chromosome abnormalities is found, including gaps, breaks, and rearrangements. This may indicate that the patient has an inherent chromosome fragility syndrome (which should be confirmed as it will have an effect on sensitivity to treatment) or has been previously exposed to occupational or therapeutic clastogens These include radiation and many cytostatic drugs but also more commonplace exposures such as smoking. Infection by viruses may sometimes be inferred when there are divisions found with vast numbers of fragments, but this picture may resemble that obtained in malignancy when there has been gene amplification resulting in the presence of paired "double minutes "
   c. The centromeres of chromosomes 1, 9, and 16 can have wide variation in size between individuals and can sometimes look abnormal. Checking a few divi-

sions from a PHA-stimulated culture should confirm that unusual appearances are part of the patient's constitution Similarly, a pericentric inversion of chromosome 9 occurs in up to 10% of the population without any major clinical effect These variations are sometimes useful after a bone marrow transplant in determining the origin of divisions when the host and donor are of the same sex

Acknowledgments

Methodology is a constant topic of conversation among cytogeneticists and I am indebted to the many, particularly fellow workers in this Department, Toon Min, Melissa Dainton and Lynne Hiorns, who have contributed to this chapter by sharing experience, theories and advice. I am also grateful to the Royal Marsden Hospital NHS Trust, under whose auspices this chapter was written

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28

Visualization of Cell Replication Using Antibody to Proliferating Cell Nuclear Antigen

James Hyde-Dunn and Gareth E. Jones

1. Introduction

The proliferating cell nuclear antigen (PCNA) is the 37-kDa molecular weight auxiliary protein of DNA polymerase δ *(1)*. PCNA is a very conserved and highly regulated protein necessary for cell cycle progression. PCNA is associated with cyclin A in both soluble and insoluble fractions, whereas it is associated with cyclin D1 only in the soluble fraction *(2)*. PCNA translocates from the cytoplasm to the nucleus at the beginning of S-phase, is required for entry into S-phase, and forms multiple high molecular weight complexes with cdk2 at late G1 or early S-phase *(2)*. This cell-cycle distribution of PCNA may be employed as a marker of DNA replication.

The soluble form of PCNA protein is sensitive to organic fixation and is not involved in replication whereas the insoluble fraction is associated with ongoing DNA synthesis *(3)*. Maximal levels of PCNA are found tightly bound to chromatin during the late G1 and S-phases. S-phase cells label with anti-PCNA monoclonal antibodies with much greater intensity than cells in the other phases of the cell cycle *(4)* When cells are fixed and probed with an antibody directed against PCNA, it is akin to taking a "snap shot" of the cell-cycle and visualizing the "window" of DNA synthesis, namely S-phase *(5)*. The Hoechst reagent will stain all nuclei, and can be used to give a value for the total nuclear population. By scoring the percentage of PCNA-labeled nuclei in a background of Hoechst-stained cells, it is possible to determine the proliferative activity of a population of cells.

Growth factors or cytokines are implicated in a wide variety of physiological and pathological processes such as embryogenesis, hemopoiesis, wound healing, and tumor progression. By employing the method outlined in this chap-

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

ter using serum as a mitogen, it is possible to assay the mitogenic capacity of specific cytokines on cells in vitro using nonradioactive detection techniques. The method is sufficiently sensitive to detect the effects of different doses of cytokine on cell populations. This indirect immunofluorescence protocol has been utilized to trace myogenesis of satellite cells in isolated cultured fibers and employed to analyse the role of growth factors in adult myogenesis *(6)*. An antibody against PCNA was used to distinguish proliferating cells from the rest of the myofiber nuclei and in so doing, study satellite cell proliferation.

2. Materials

1. Dulbecco's modified Eagle's medium (DMEM) (ICN Flow, Thames, Oxfordshire, UK) with bicarbonate. Keep at 4°C.
2. Heat-inactivated (HI) fetal calf serum (FCS) (Globepham) (*see* Note 1). Aliquot in sterile 20-mL tubes and keep at –20°C
3. Penicillin/streptomycin stock (ICN). 5,000 IU/mL, 5,000 µg/mL Aliquot in sterile 10-mL tubes and keep at –20°C
4. L-Glutamine stock (ICN). 200 m*M* (29 23 mg/mL) Aliquot in sterile 10-mL tubes and keep at –20°C
5. Complete DMEM. DMEM containing antibiotics (penicillin, 50 IU/mL, 50 µg/mL), L-glutamine (2 m*M*), and 10% HI FCS To make 200 mL, mix 176 mL of DMEM, 2 mL of stock penicillin/streptomycin, 2 mL of stock L-glutamine, and 20 mL of HI FCS Filter-sterilize (0 22-µm filter) and keep at 4°C.
6. Serum free DMEM. DMEM containing antibiotics (penicillin, 50 IU/mL; 50 µg/mL) and L-glutamine (2 m*M*). To make 50 mL, mix 49 mL of DMEM, 0.5 mL of stock penicillin/streptomycin, and 0 5 mL of L-glutamine Filter-sterilize (0.22-µm filter) and keep at 4°C
7. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS-A) (Oxoid, Basingstoke, Hampshire, UK)
8. PBS with Ca^{2+} and Mg^{2+} (PBS-ABC) (ICN). Sterile, keep at 4°C
9. Trypsin/EDTA (ICN) 0.05% trypsin, 0.02% EDTA in salt solution (SSS). Aliquot in sterile 10-mL tubes and keep at –20°C
10. 0 4% Trypan Blue (stock) (Sigma, St Louis, MO). 0.2% Trypan Blue (working solution) made up in PBS-ABC, keep at room temperature
11. 11-mm diameter No. 1½ thickness circular glass coverslips (BDH, Merk, Poole, Dorset, UK) (*see* Note 2)
12. Minimum essential Eagle's medium (modified), HEPES buffer (ICN). Keep at 4°C
13. PC10 (anti-PCNA) mouse monoclonal IgG_{2a} (Santa Cruz Biotechnology, Santa Cruz, CA, SC-56) 50-µL aliquots in sterile Eppendorff tubes Store at 4°C
14. Goat antimouse IgG TRITC-conjugated (Sigma). 50-µL aliquots in sterile microfuge tubes and keep at –20°C protected from the light
15. Hoescht stain (bisbenzamide) (Sigma): Make a stock solution of 10 µg/mL in PBS-A. Store at 4°C in the dark. Working solution: 1 µg/mL in PBS-A, make up fresh on day of use.

16 Bovine serum albumin (BSA) fraction V (BDH, Merk) Store at 4°C
17 Blocking buffer· PBS-A + 1% (w/v) BSA To make 100 mL, weigh out 1 g of BSA and complete to 100 mL with PBS-A Dissolve the BSA with gentle stirring and a low heat. Make fresh on day of use.
18. PC10 working solution: dilute stock monoclonal 1:10 in blocking buffer Make up fresh on day of use.
19. TRITC antimouse IgG working solution dilute stock 1·50 in blocking buffer and protect from light Make up fresh on day of use
20. Hoescht stain working solution: 1 μg/mL in PBS-A Keep in the dark at 4°C
21. Mounting medium Gelvatol containing 0 1% of the antifading agent *p*-phenylenediamine (*see* Note 3)
22 Glycerol (BDH, Merk) Analytical Grade: Store at 5°–30°C.
23 Mowiol 4-88 (Calbiochem, Nottingham, UK): Store at room temperature
24. Tris [hydroxymethyl] aminomethane, (Trizma BASE) (Sigma) Store at room temperature.
25. *p*-phenylenediamine (Sigma). Air and light sensitive. May darken on storage, store at room temperature under vacuum.

3. Methods

3.1. Coverslip Preparation

1. Use 11-mm diameter No. 1½ glass coverslips for best optical properties.
2 Sonicate coverslips in analytical grade acetone for 10 min.
3 Etch coverslips overnight in a 5 1 volume ratio of concentrated sulphuric acid/ nitric acid. Manipulate the coverslips with forceps and employ the precautionary procedures of wearing gloves, goggles, and a face mask while handling concentrated acid
4 Transfer the coverslips to a large beaker of distilled water and boil-wash five times using a fresh change of distilled water for each wash
5. Dry coverslips in an oven at 140°C for 2 h in order to sterilize
6. Transfer to a laminar flow hood in a sterile container

3.2. Preparation of Cells

1 Harvest cells (*see* Note 4) using trypsin/EDTA and seed in complete DMEM (*see* Note 5) at 2 × 10^4 cells/mL onto 11-mm diameter No 1½ glass coverslips in bacteriological grade Petri dishes.
2. Leave the cells to adhere and spread overnight in a 37°C humidified 5% CO_2 incubator.
3. Observe on an inverted microscope under phase-contrast optics to ensure cells are viable and well spread
4 Aspirate the medium and rinse the cultures twice for 30 s in an excess of prewarmed (37°C) DMEM to remove traces of serum.
5. Flood the coverslips with prewarmed (37°C) serum-free DMEM. Note the time and return to a 37°C humidified 5% CO_2 incubator overnight

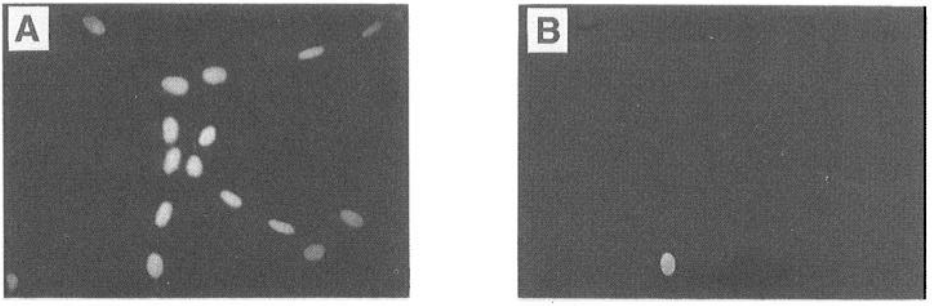

Fig. 1. (A) Rat-2 fibroblasts starved to mitotic quiescence. Hoescht stain. **(B)** Same field of view as (A) PCNA/TRITC.

6. Starve the cells in this media for 24 h in order that they may become mitotically quiescent (*see* Note 6). At this point a coverslip may be fixed and processed employing the immunocytochemistry methodology (*see* Section 3.3.) to determine the degree of mitotic quiescence (*see* Figs. 1A,B).
7. After 24 h of serum deprivation, cells may be stimulated to re-enter the cell cycle by aspirating the existing media and replacing it with prewarmed (37°C) complete DMEM, or by adding appropriate test dilutions of polypeptide growth factors in serum free media. Return to a 37°C humidified 5% CO_2 incubator and culture for a further 18 h, or less if desired.
8. After 18 h incubation observe again on an inverted microscope, then fix and label according to the immunocytochemistry methodology outlined in Section 3.3.

3.3. Immunocytochemistry

1. Remove coverslips from the culture medium and place them into individual 30-mm diameter bacteriological grade dishes containing prewarmed (37°C) DMEM. Rinse briefly in two washes with gentle agitation (*see* Note 7).
2. Prefix the cells for 1 min by adding an equal volume of prewarmed (37°C) acetone-methanol (1:1 volume ratio) to the remaining DMEM wash (*see* Note 8).
3. Aspirate and fix in a 1:1 volume ratio of acetone-methanol for 5 min at room temperature. Aspirate and air-dry. Check that cells are not detaching at this point using an inverted microscope.
4. Wash the coverslips three times for 10 min at room temperature with three changes of HEPES buffered MEM (*see* Note 9). Prepare required volumes of primary antibody dilution during these washes. Dilute stock monoclonal 1:10 in blocking buffer (PBS-A + 1% [w/v] BSA).
5. Place tissue paper saturated in distilled water within the lid of a small plastic box. Arrange the caps of cryovials or microfuge tubes evenly over this saturated surface, then with the aid of forceps carefully place the coverslips, cell side up, on top of the caps (*see* Note 10). Add 50 µL of PC10 mouse monoclonal primary

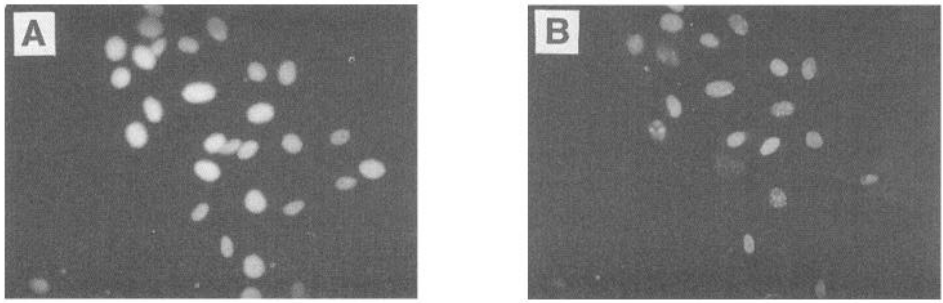

Fig. 2. (A) Rat-2 fibroblasts starved to mitotic quiescence then stimulated with complete media for 18 h. Hoescht stain. **(B)** Same field of view as (A) PCNA/TRITC.

antibody made up to 10 μg/mL to each coverslip. Secure the box on its lid and leave for 60 min at room temperature (*see* Note 11).

6. At the end of the incubation pick up the coverslips with forceps, drain the antibody off by touching the edge of a tissue paper and place it, cell side up, in a 30-mm diameter dish containing 2 mL of PBS-A + 1% BSA, (*see* Note 12). Rinse three times for 10 min in blocking buffer with gentle agitation. (During these washes prepare the required volume of secondary antibody dilution).
7. Place the coverslips back onto the cryovial caps, cell side up and incubate with the secondary antibody, goat antimouse IgG TRITC for 60 min at room temperature in the dark (cover incubation box in foil).
8. At the end of this incubation, handling the coverslips with forceps, drain the antibody off by touching the edge of a tissue paper, and place it cell side up in a 30-mm diameter dish containing 2 mL of blocking buffer. Rinse three times for 10 min in blocking buffer with gentle agitation in the foil-covered box.
9. Aspirate and rinse briefly for 10 min in HEPES-buffered MEM with gentle agitation. Again keep the coverslips in the foil-covered container.
10. Aspirate and flood the coverslips in an excess of Hoescht stain working solution. Incubate in the dark for 10 min at room temperature with gentle agitation.
11. At the end of the incubation aspirate and rinse the coverslips three times for 3 min in distilled water in the dark (*see* Note 13).
12. Manipulating the coverslips with forceps drain excess water by touching the edge of a tissue paper. Pipet 10 μL of Gelvatol antifade mountant centrally onto the cell surface and mount cell side down onto a clean glass slide.
13. Allow to polymerize overnight at 4°C in the dark.
14. Observe under a fluorescence microscope equipped with Hoechst and rhodamine filter sets for 450 ± 33 and 590 ± 18 nm emissions, respectively. Photograph using film of choice, following the manufacturer's instructions (*see* Note 14 and Figs. 1A,B and 2A,B). Store slides in the dark at –20°C. They are usable for up to 3 mo.

4. Notes

1 To heat inactivate FCS, incubate at 56°C for 2 h. The batch of serum should be pretested for its capacity to support your cell cultures and, once satisfied, use this same batch in all your subculture procedures for a particular experimental program. This will avoid problems associated with batch variation between seemingly identical serum.

2 Using small coverslips in the immunocytochemistry requires much lower volumes of antibody solutions (50 µL is sufficient to cover the surface of an 11-mm diameter coverslip), saving on antibody while providing scope for varying treatments and replicates

3. Preparation of gelvatol mountant·
   a. Place 6 g of analytical grade glycerol in a 50 mL disposable plastic centrifuge tube
   b. Add 2.4 g of Gelvatol (Mowiol 4-88) and stir thoroughly to mix the two
   c Add 6 mL of ultra pure water and stir the solution for 2 h at room temperature.
   d In the meantime, make up a 0 2*M* solution of Tris-buffer by dissolving 2.42 g of Tris-Base in 100 mL of ultrapure water and bring to pH 8.5 with HCL Add this to step c above and incubate the solution in a water bath at 50°–60°C for at least 15 min with occasional stirring to dissolve the Gelvatol
   e Add one grain (approx 2 5 mg) of the antiquench agent *p*-phenylenediamine/mL of the mixture and mix at 4°C for 30 min on a rotating wheel.
   f. Clarify the mixture by centrifugation at 5000*g* for 15 min and aliquot 1 mL into glass or plastic (airtight) bijoux bottles.
   g. Store at –70°C and protect from light. Warm the mixture to room temperature prior to mounting coverslips Aliquots warmed to room temperature have a shelf-life of 7–10 d.
   h. The mounting medium will set overnight. To observe immediately, secure the coverslip to the slide by painting nail polish on the edge of the coverslip.

4. Prior to plating, cell suspensions should be gently but thoroughly triturated to facilitate even distribution.

5. It is important to seed the cells down in complete media as the serum contains proteins such as fibronectin and vitronectin that form a matrix on which cells adhere and spread

6 Cultured Rat-2 fibroblasts cease to proliferate and arrest in the G_0 phase of the cell cycle when they are thoughly rinsed and maintained for 24 h in serum free media. Other cell types will have their own characteristic response to serum depletion.

7 It is important to remove coverslips from their culture medium and wash them prior to fixation in serum free-media, as acetone/methanol fix will precipitate such supplemented protein, leading to high nonspecific background staining.

8 Pre-fixing the cultures at physiological temperature reduces the risk of cellular detachment particularly among populations which have been starved to mitotic quiescence

9. These washes remove residual traces of fixative and neutralize the associated acidity. MEM with HEPES is used for such rinses as it maintains a physiological pH when eqilibrated against air
10 Handling the coverslips is a delicate procedure Should one fall from the forceps, pick it up, submerge it in a Petri dish of blocking buffer, and examine it on an inverted microscope to determine the cell orientation.
11. Prior to preparing antibody dilutions routinely centrifuge the aliquots of antibody to precipitate any insoluble colloid that may otherwise cause nonspecific background staining. We use a microfuge set at 13,000 rpm for 5 min
12 Never allow the cells to dry out at any stage
13. Washing cultures in distilled water removes the PBS-A which, if present when mounting, may result in salt crystal formation within the preparation
14 If the background fluorescence is high with Hoescht stain, reduce the concentration to 0 5 μg/mL If there is unacceptable background with TRITC, modify the blocking buffer by increasing the BSA concentration or replacing it with 2% serum

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Proliferation of Murine Myoblasts as Measured by Bromodeoxyuridine Incorporation

Bina Shah, James Hyde-Dunn, and Gareth E. Jones

1. Introduction

Growth factors or cytokines have been implicated in a wide variety of physiological and pathological processes. It has been shown that growth factors bind to specific receptors, and generate multiple signals that interact synergistically to initiate a proliferative response *(1)*. Synergistic effects are of crucial importance in assessing the growth-promoting activity of any agent in low serum or serum free, chemically defined conditions.

Measurements of DNA synthesis are often taken as representative of cell proliferation. Incorporation of ^{3}H-thymidine (^{3}H-TdR) or ^{3}H-deoxycytidine are the usual precursors measured in such assays. Exposure may be for short periods (½–1 h) for rate estimations or for 24 h or more to measure accumulated DNA synthesis where the basal rate is low, for example, in high-density culture. This incorporated tritium label is measured by autoradiography ^{3}H-TdR represents a particular hazard because of induced radiolytic damage to DNA through incubations longer than 24 h or at high specific activities. Another inconvenience is the requirement for the use of photographic emulsions and the prolonged development times *(2)*.

The incorporation of bromodeoxyuridine (BrdUrd) is a convenient nonradioactive method for assessing proliferative activity of growth promoting cytokines. Incubation in media containing BrdUrd results in incorporation of this analog in competition with thymidine during DNA replication (S phase). The BrdUrd can be detected through indirect immunocytochemical labeling using polyclonal or monoclonal antibodies raised against this molecule. By employing a horse radish peroxidase conjugated secondary antibody with high catalytic activity, an intrinsic amplification of the signal may be obtained.

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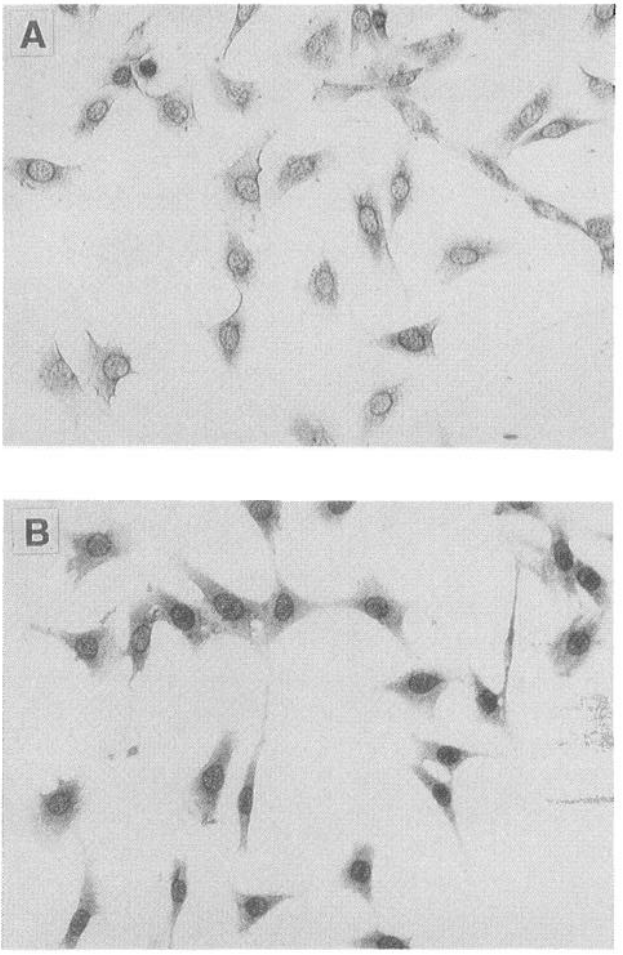

Fig. 1. (A) Low serum control; murine myoblasts starved for 48 h. **(B)** High serum control; murine myoblasts starved for 24 h then stimulated with 10% FCS.

In our studies, murine myoblasts are used but the protocol described here works well on other cells with very little modification. In order to obtain quiescent cultures, cells are plated in low serum media (Fig. 1a). After a suitable period these cells are exposed to high serum media (Fig. 1b), or a range of cytokine concentrations in low serum media, to stimulate re-entry into the cell cycle during the subsequent growth phase and then cell counts are performed to determine percentage of cells labeled.

All immunoassays rely on secondary reagents for detection. Certain controls are subsequently required to show that the assay is providing accurate

data, free from artefacts of the immunoassay procedures. Four types of controls are used: high serum control (maximum nuclei staining), low serum control (minimum nuclei staining), BrdUrd control, or no BrdUrd control added to a set. Finally primary antibody is not added to another set. In the latter two cases there can be no specific staining.

The introduction of multiwell plates revolutionized the approach to replicate sampling in tissue culture. They are economical to use and can be of good optical quality. The most popular, and the one we use, is the 96-well microtitration plate, each well having 32 mm^2 growth area and capacity for 0.1 or 0.2 mL medium and up to 10^5 cells.

2. Materials

All sterile unless otherwise stated

1. Phosphate buffered saline with Ca^{2+} and Mg^{2+} (PBS-ABC); store at 4°C
2. Phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS-A), store at 4°C.
3. Dulbecco's modified Eagle's medium (DMEM) with sodium bicarbonate Store at 4°C
4. Heat-inactivated fetal calf serum (HIFCS) (*see* Note 1); store at –20°C in 20-mL aliquots.
5. L-glutamine 200 m*M* stock, use at 2m*M*; store at 4°C
6. Penicillin/streptomycin· 5000 IU/mL/5000 µg/mL), store at 4°C.
7. High serum medium contains 88% DMEM, 1% penicillin/streptomycin, 1% L-glutamine, and 10% HIFCS Filter sterilize (0 22-µm filter) and store at 4°C
8. Low serum medium contains 97 9% DMEM, 1% penicillin/streptomycin, 1% L-glutamine, and 0 1% HIFCS Filter sterilize and store at 4°C
9. Trypsin EDTA 0 05% Trypsin and 0.02% EDTA in salt solution (ICN). Store at –20°C
10. Trypan Blue (Stock) 0 2% working solution made in up in PBS-ABC. Nonsterile, store at room temperature
11. Minimum essential medium (MEM), HEPES buffered, store at 4°C.
12. Polypeptide growth factors· Choice of experimenter Employed by our lab in this assay·
    a. Human recombinant platelet-derived growth factor BB, Homodimer (PDGF-BB) Stock concentration 20 µg/mL Working concentration between 0 and 60 ng/mL Prepared following instructions of suppliers. Dilute in low serum media
    b. Human recombinant colony-stimulating factor 1 (CSF-1) Working concentration between (0 and 3,000 U/mL) Dilute in low serum media
13. 2.5 m*M* (BrdUrd) Store at –20°C. Stable for 1–2 yr
14. 200 µ*M* Fluorodeoxyuridine (FldUrd) Store at –20°C Stable for 1–2 yr
15. Acid alcohol. 95% absolute ethanol and 5% glacial acetic acid. Store at –20°C.
16. 4*M* Hydrochloric acid Store at 4°C.
17. 50 m*M* Sodium chloride, 100 m*M* Tris-HCl, pH 8 4. Store at 4°C

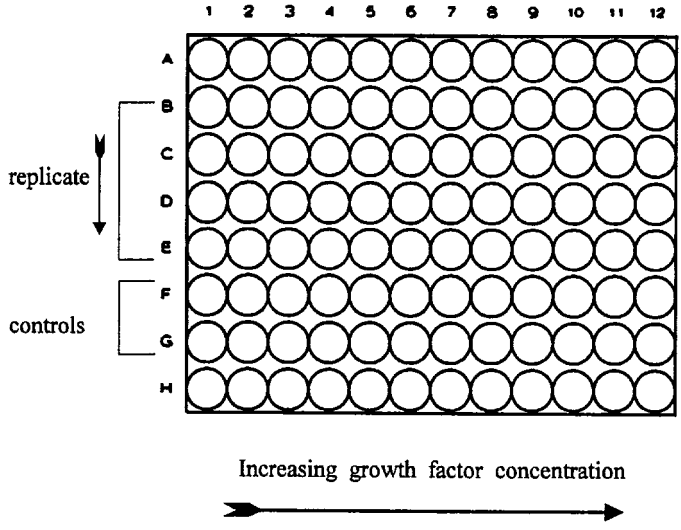

Fig. 2. Design for a 96-well plate

18. 50 m*M* Tris-HCl, pH 7 6 Store at 4°C
19 Bovine serum albumin (BSA), fraction V Store at 4°C Nonsterile
20 Blocking buffer. PBS-A + 1% (w/v) BSA Make fresh on day of use Nonsterile Keep at room temperature
21. Antibodies·
    a Rat anti-BrdUrd, diluted 1 10 in blocking buffer. Undiluted aliquots can be stored at –20°C for up to 6 mo
    b. Antirat IgG horse radish peroxidase conjugate, diluted 1 50 in blocking buffer Undiluted aliquots can be stored at –20°C for up to 6 mo
22 0 3% Nickel (II) chloride This stock stores at 4°C for 6 mo.
23. 3% Hydrogen peroxide (BDH) Make fresh on day of use.
24. Diamino Benzadine (DAB) metal stain. Make fresh immediately prior to use. Dissolve 6 mg DAB tetrachloride in 9 mL Trizma base buffer (50 m*M*, pH 7.4). Add 1 mL of nickel chloride stock. Add 100 µL of 3% hydrogen peroxide Filter before use
25 0.1% Gelatin in PBS-ABC Working dilution is 0 01%, store at 4°C
26 Tissue-culture grade sterile 96-multiwell plates.

3. Methods

3.1. Design for Multiwell Plates

The multiwell plates (*see* Fig. 2) should have four repeated wells for:

1. High serum media replacement (maximum stained nuclei, positive control)
2. Low serum media replacement (minimum stained nuclei, negative control).
3 Each concentration of cytokine

Other controls include

4. Control for incorporation of BrdUrd (i e , no BrdUrd added, for one well of each of the aforementioned experimental conditions)
5. Primary antibody control, no Rat anti-BrdUrd added, for one well of each of the aforementioned experimental conditions

3.2. Cell Culture

1 Harvest cells using standard dissociation methods and seed in low serum media DMEM media at 1×10^4 to 2×10^4 cells/mL. Add 100 µL of cell suspension per well (*see* Note 2)
2 Incubate for 24 h at 37°C in a 5% CO_2 humidified incubator In low serum conditions the cells become mitotically quiescent (*see* Note 3)
3. Dump (*see* Note 4), to discard the media. Rinse the plate twice with prewarmed PBS-ABC Dumping out the liquid each time.
4. Restimulate the cells by changing the medium to media containing a range of concentrations of cytokines in starvation media. Incubate for 6 h at 37°C 5% CO_2 humidified incubator
5 Now add 1·100 dilution (e g , 1 µL each/100 µL) of both BrdUrd and FldUrd (*see* Notes 5 and 6) Leave for 18 h at 37°C 5% CO_2
6 After a total of 24 h add fixative to all wells as shown in Section 3.3 , steps 1 and 2

3.3. Antibody Staining

All solutions are pre-warmed at room temperature unless otherwise stated.

1 Pre-fix Add 100 µL acid alcohol to wells (*see* Note 7) Leave at room temperature for 10 min
2 Dump. Add 200 µL of cold (–20°C) acid alcohol to wells and leave at –20°C for 20 min
3 Dump. Rinse three times for 10 min in MEM with HEPES (*see* Note 8), 200 µL/well, at room temperature on a shaker.
4 Dump. Add 200 µL 4*M* HCl (*see* Note 9) to wells. Leave at room temperature, on a shaker for 1 h
5. Dump. Rinse three times for 10 min in 50 m*M* NaCl, 100 m*M* Trizma, pH 8.4 (*see* Note 10), 200 µL/well, at room temperature, on a shaker
6. Dump Rinse three times for 10 min in MEM with HEPES, at room temperature, on a shaker
7. Dump. Add 50 µL/well Rat anti-BrdUrd. This is diluted 1·10 in blocking buffer *(3)*. Incubate for 1 h at room temperature (*see* Note 11).
8 Dump Rinse three times for 10 min in MEM with HEPES; at room temperature.
9. Tap out Add 50 µL/well antirat HRP. This is diluted 1:50 in blocking buffer (*see* Note 11) Incubate 1 h at room temperature, in the dark because this antibody conjugate is light sensitive.
10. Dump Rinse three times for 10 min in MEM with HEPES; 100 µL/well at room temperature.

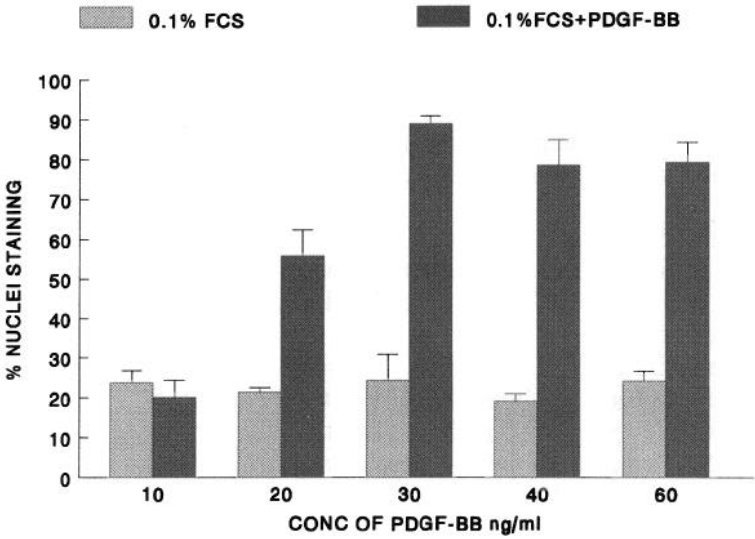

Fig. 3. Cell proliferation assay using PDGF-BB.

11. Dump. Add 100 µL/well DAB/metal (*see* Notes 12 and 13) reaction mix. Incubate for 5–15 min at room temperature, monitor the reaction on an inverted light microscope.
12. Look for brown/black stain in nuclei (*see* Note 14).
13. Rinse in excess distilled water to stop the reaction. Dump.
14. Air dry.
15. Using an inverted microscope, view under brightfield optics. Check the antibody control wells are negative.
16. Cell counts are performed to calculate the percentage of cell nuclei labeled in a field of total number of cells. Use a statistical package to analyse the data (*see* Fig. 3).

4. Notes

1. To heat inactivate FCS, incubate at 56°C for 2 h.
2. If cells do not adhere and spread onto the mutiwell plate, precoat the surface with 0.01% gelatin in PBS-ABC, as follows:
   a. 100 µL 0.01% gelatin in PBS-ABC is added to wells.
   b. Leave for 3–5 min at room temperature.
   c. Tap out and air dry for 5 min.
   d. Add cell suspension.
3. This protocol has been employed using murine myoblasts. An optimum incubation period will vary for different cells.
4. Dumping out: Invert the plate and hit it twice onto sterile or non sterile tissue paper. This will discard most of the liquid.

5 BrdUrd addition The unpredictable variable that effects the nuclei staining, is the variation in chromatin structure between different cell types Hence the concentration of BrdUrd should be optimized for a particular cell type by titration assay
6 FldUrd addition Uridine competes with thymine in the DNA FldUrd in this context contests the excess thymine, so that a lot more BrdUrd can be incorporated
7 Pre-fixing This is a gentle fixation for the cells, particularly necessary for a serum starved population of cells
8 The MEM with HEPES washes removes traces of fixative, acid, and antibodies. HEPES helps to maintain physiological pH and it is buffered against air
9 HCl disrupts the DNA, so that rat anti-BrdUrd antibody can locate the incorporated BrdUrd.
10 The NaCl in Trizma base neutralizes the HCL prior to antibody incubation
11. Working dilution of the antibodies are determined by titration assay
12. DAB is extremely toxic so use gloves and face mask If spilt remove with diluted chloros solution
13. The DAB substrate for horse radish peroxidase is made more sensitive by adding metal salts such as nickel chloride This is an enzymatic reaction, the enzymatic action of HRP results in an intrinsic amplification of the signal and therefore a dark brown/black staining.
14. Coomassie blue may be used as a counter-stain to visualise unstained cells. This stains for protein and is hence a total cytoplasmic stain.

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Analysis of Cell Proliferation Using the Bromodeoxyuridine/Hoechst-Ethidium Bromide Method

Michael G. Ormerod

1. Introduction

Measurement of a DNA histogram can be achieved by fixing or permeabilizing cells and staining them with a DNA-binding dye, such as propidium iodide (PI). The histogram will yield the percentage of cells in the G1, S, and G2/M phases of the cell cycle (for further discussion, *see* ref. *1*). Although some inferences about the movement of cells through the cycle may be drawn, the information gained is essentially static. For example, it is not known whether a cell with S phase DNA content is actually synthesizing DNA; also the presence of subpopulations with different cycle times cannot be detected

Dynamic information about cell cycle progression can be obtained by labeling cells with 5'-bromodeoxyuridine (BrdUrd) that is incorporated into DNA in place of thymidine. Detection of BrdUrd in the DNA allows the fraction of cells in S phase to be enumerated and, if samples are taken at different time intervals, also gives information about the cell cycle kinetics.

Three methods have been used. After the application of a pulse-label, monoclonal antibodies that react specifically with BrdUrd reveal those cells which are in S phase. Counter-staining with propidium iodide shows the cell cycle. If cell are harvested at times after the application of the pulse-label, the movement of labeled cells through the cell cycle can be followed. This method has been applied in vitro *(2)* and in vivo *(3,4)*. The other two methods exploit the observation of Latt that the fluorescence of *bis*-benzimidazoles (Hoechst 33342 and 33258) bound to DNA is quenched by BrdUrd *(5)*. One method, in which the cells are pulse-labeled and then stained with a combination of Hoechst 33258 and mithramycin, requires a dual laser flow cytometer and a complex

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

analysis in which fluorescence signals are subtracted in real time *(6)*. In the method described in this chapter, the cells are continuously labeled with BrdUrd, and permeabilized and stained with Hoechst 33258 and PI. The quenching of Hoechst/DNA fluorescence reveals whether the cells have incorporated BrdUrd; the PI/DNA fluorescence (unaffected by BrdUrd) gives the cell cycle phase. This method was originally applied to quiescent cells which had been stimulated into the cell cycle *(7)*. It can also be applied to asynchronous populations of cells *(8)*. Although the data analysis is more complex, a wealth of information can be derived. In particular, cell cycle specific effects of toxic treatments can be observed without resorting to artificial cell synchronization *(8–11)*.

It should be noted that, in all three methods, the cells have to be fixed or permeabilized to allow access of reagents to the DNA Analysis of viable cells is not possible. In addition, for the antibody method, the DNA of the cells has to be partially denatured to allow access of the antibody to the BrdUrd incorporated into the DNA.

In the method described here, BrdUrd is added and cells are harvested at fixed time intervals, typically, every 4 h for 36 h. Samples may be collected and the cells frozen prior to analysis For analysis, cells are suspended in a buffer containing Hoechst 33258 and a detergent, which releases the nuclei, and PI is then added. Hoechst 33258 is exited in the UV and the analysis requires a flow cytometer equipped with a source of UV light.

2. Materials

1 BrdUrd· Make up a stock solution of 10 m*M*. Store frozen
2 Hoechst 33258
3 PI Make up a stock solution of 100 µg/mL in distilled water Store in the dark at 4°C Stable for at least 6 mo
4. Ethidium bromide: Make up a stock solution of 100 µg/mL in distilled water Store in the dark at 4°C Stable for at least 6 mo
5. Staining solution The staining solution consists of (final concentration)· 100 m*M* Tris, pH 7.4, 154 m*M* NaCl, 1 m*M* $CaCl_2$, 0 5 m*M* $MgCl_2$, 0.1% (v/v) Nonidet-P40, 0.2% (w/v) bovine serum albumin, 1 2 µg/mL Hoechst 33258 Make up at 10X final strength and store in the dark at 4°C. The solution is stable for at least 6 mo. Batches of staining buffer can be prepared weekly from the 10X concentrated stock solution in distilled water.

3. Methods

3.1. Analysis of Synchronous Cells

1 Arrest cells in G0/G1 phase of the cell cycle (*see* Note 1)
2 Add a suitable growth factor or activating agent to the cell culture (*see* Note 2).
3 Add a suitable concentration of BrdUrd to the cell culture (*see* Notes 3–5).

4. If desired, at a suitable time after stimulation, treat the cells (radiation, drug, heat, and so on)
5. At fixed time intervals (6–24 h, depending on the type of cell), harvest an aliquot of cells (*see* Notes 6–8).
6. Centrifuge the cells and resuspend in 500 µL ice-cold staining buffer Briefly vortex mix. Stand on ice for 15 min.
7. Add 10 µL PI solution. Briefly vortex mix. Store on ice. (*see* Notes 9 and 10)
8. Analyze on the flow cytometer recording red (PI/DNA) and blue (Hoechst/DNA) fluorescences. Adjust the flow rate to about 500 particles/s If possible, perform a pulse shape analysis on the red fluorescence signal and gate to exclude any clumped nuclei (ref. *1* and Fig 1) Display a cytogram of red versus blue fluorescence (*see* Notes 11–14)

3.2. Analysis of Asynchronous Cells

1. If desired, treat the cells (radiation, drug, heat, and so on)
2. Immediately after the treatment, add a suitable concentration of BrdUrd to the cell culture (*see* Notes 3–5).
3. At fixed time intervals (3–8 h, depending on the cell cycle time), harvest an aliquot of cells (*see* Notes 4–6)
4. Continue as in steps 6–8

3.3. Data Analysis

Figure 1C shows a cytogram obtained from human peripheral blood lymphocytes 72 h after adding BrdUrd and phytohaemagglutin (PHA). Three cell cycles can be distinguished. The first cycle is labeled G1, S, G2. Note that, as the DNA content increases during S phase, the red fluorescence increases but the blue fluorescence decreases, because of the quenching of the Hoechst/DNA fluorescence by bromine. The cells which are in their second cycle are labeled G1', S', G2', and those in the third cycle, G1", and so on.

Figure 2 shows cytograms obtained from asynchronous cells (an untreated human embryonic fibroblast cell line–MRC5/34C). At time 0, G1, S, and G2/M phases of the cell cycle could be identified from both the red (PI) and blue (Hoechst) fluorescence. When the cells were incubated in 40 µ*M* BrdUrd, as the cells progressed through S phase, their red fluorescence increased but their blue fluorescence (which was partially quenched by BrdUrd) did not change. After 4 h in BrdUrd, there had been a small progression giving the S phase a slight bow shape on a plot of red versus blue fluorescence. At 8 h, the bow shape was more pronounced; also many of the cells originally in G2/M had divided and moved into G1 (unlabeled). At 16 h, all the cells in S phase at time 0 had reached G2 (labeled G2*) and a few had divided (G1*). Some of the cells which had been in G1 at time 0 h were now in S phase (Sf); some had progressed as far as G2/M (G2f). By 28 h, some cells that had begun the experiment in G2 had completed a cell cycle and returned to G1 again (labeled G1').

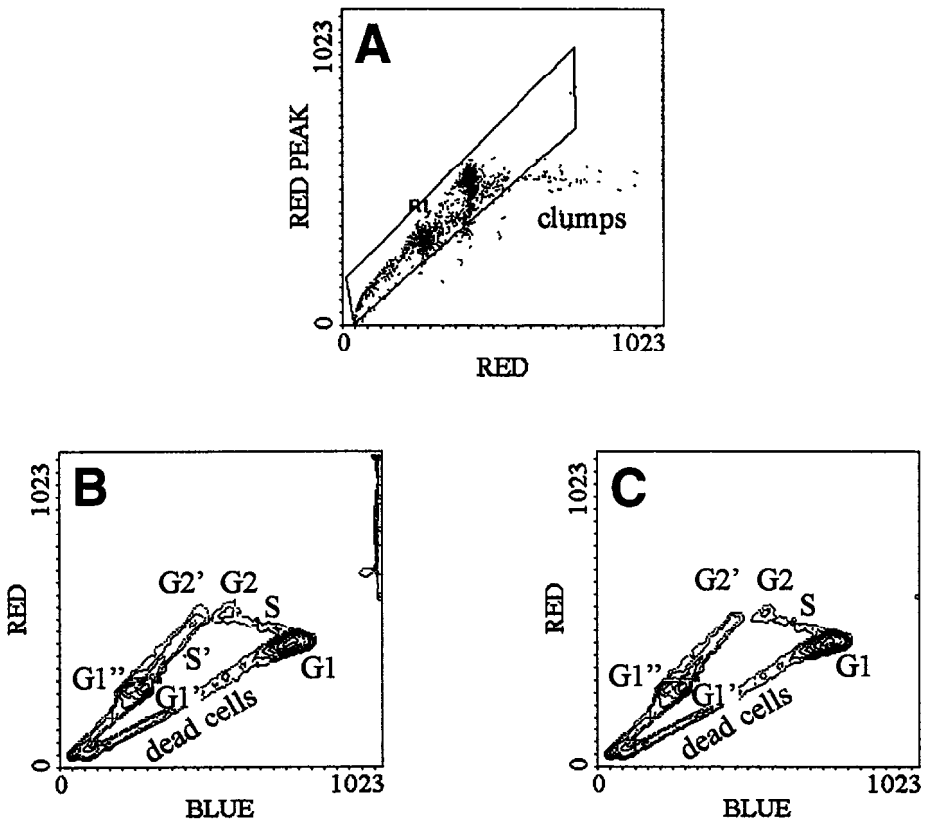

Fig. 1. Hoechst 33258-PI analysis of nuclei from human peripheral blood lymphocytes incubated for 72 h in 100 μ*M* BrdUrd, 100 μ*M* deoxycytidine after addition of PHA **(A)** PI/DNA fluorescence (red) showing a cytogram of the peak of the red fluorescence signal versus the integrated area A gate has been set to include single nuclei and to exclude clumps. The cytogram is displayed as a "dot plot" **(B,C)** Hoechst/DNA (blue) versus PI/DNA (red) fluorescence (cytograms displayed as contour plots). The cytogram in panel B shows ungated data; that in panel C shows the effect of gating on region, R1, in cytogram A For further discussion of the interpretation of the data, see text Cells prepared by Dr John Peacock and data recorded on a Coulter Elite ESP using a Spectra-Physics argon-ion laser tuned to produce 100 mW in the UV Red (>630 nm) and blue (460 nm) fluorescence were measured Data were acquired on an IBM-PC compatible computer and the figure was prepared using the WINMDI program supplied by Dr Joe Trotter, Salk Institute (La Jolla, CA)

Figures 3–5 illustrate typical effects of cell cycle perturbation. Figure 3 shows MRC5/34 cells which had been given 5 Gy γ radiation immediately prior to adding the BrdUrd. The cells suffered a G2 block. Cells that were

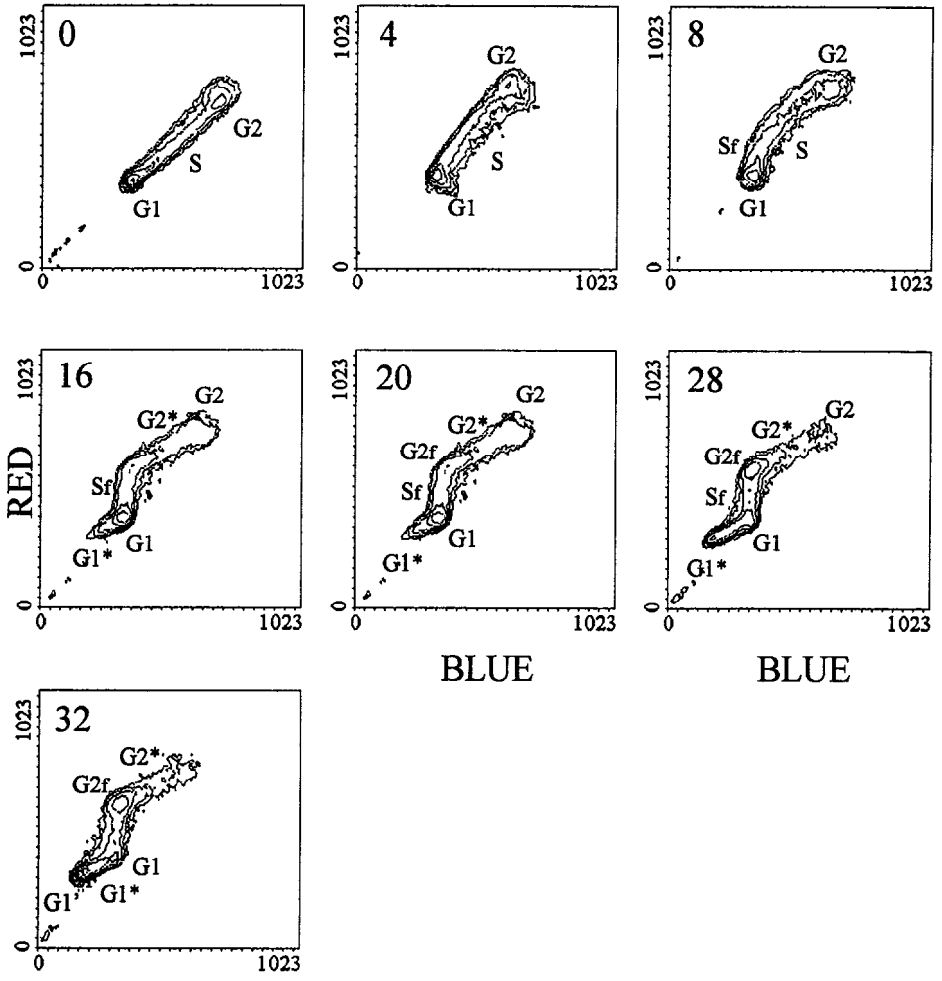

Fig. 2. Cytograms of PI-DNA (red) versus Hoechst 33258-DNA fluorescence of nuclei from MRC5/34 cells incubated in 40 μM BrdUrd and 40 μM deoxycytidine BrdUrd for the times shown on the panels. Cells prepared by Dr. David Gilligan and recorded by Mrs. Jenny Titley (Institute of Cancer Research, Sutton, UK) Other details as in Fig 1 For a description of the data see the main text

irradiated in all phases of the cell cycle have become arrested in G2. (Compare this figure to Fig. 2, 32 h time point.) Figure 4 shows W1L2 cells (human lymphoblastoid cell line) that had been incubated with cisplatin for 2 h before

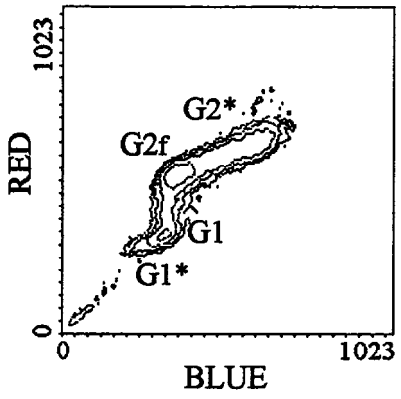

Fig. 3. A cytogram of PI-DNA (red) versus Hoechst 33258-DNA fluorescence of nuclei from MRC5/34 cells given 5 Gy γ radiation and then incubated in 40 μ*M* BrdUrd and 40 μ*M* deoxycytidine BrdUrd for 32 h Other details as in Fig 1 For a description of the data see the main text

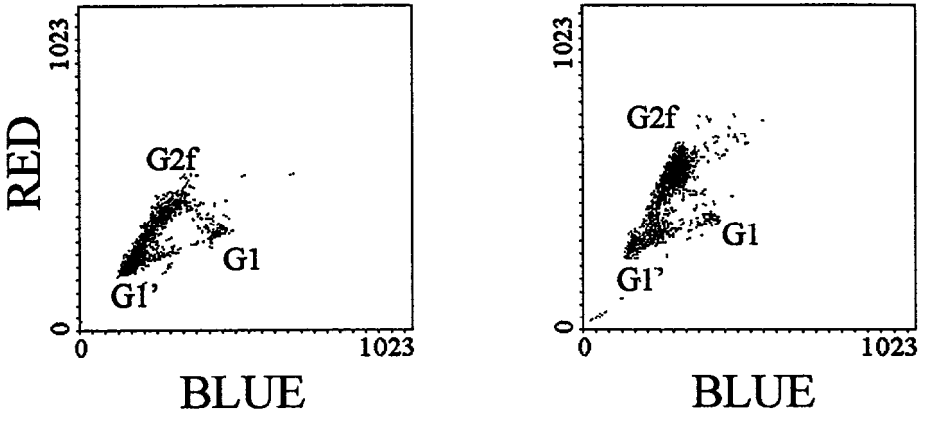

Fig. 4. Cytograms of PI-DNA (red) versus Hoechst 33258-DNA fluorescence of nuclei from W1L2 (human lymphoblastoid cell line) cells either untreated (left panel) or incubated for 2 h with 20 μ*M* cisplatin (right panel) and then incubated in 40 μ*M* BrdUrd for 24 h. Experiment run by the author Other details as in Fig 1

adding BrdUrd. Only cells treated with drug in G1 and early S phase are arrested in G2. The other cells have divided. Figure 5 shows a human medulloblastoma cell line (D283) that underwent a G1 block after γ radiation. Only a proportion of the cells in G1 became blocked in G1, some of the G1 cells progressed through the cycle and divided (in compartment G1'). Presumably these were cells irradiated in late G1 phase

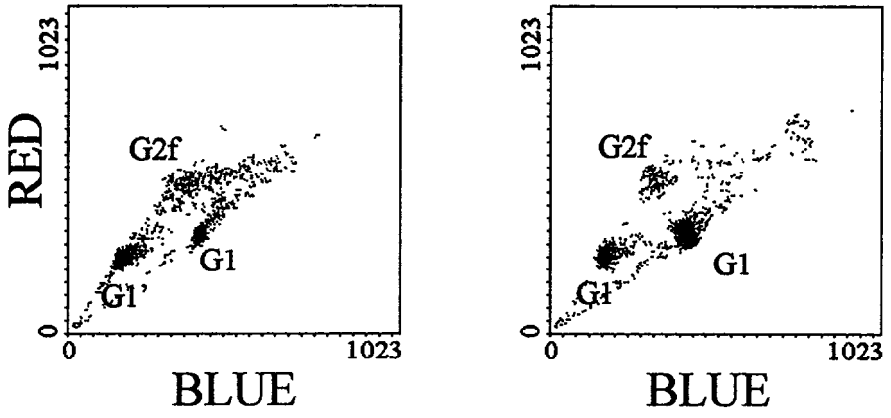

Fig. 5. Cytograms of PI-DNA (red) versus Hoechst 33258-DNA fluorescence of nuclei from a medulloblastoma cell line (D283) either untreated (left panel) or given 8 Gy γ radiation (right panel) and then incubated in 40 μ*M* BrdUrd and 40 μ*M* deoxycytidine BrdUrd 32 h. Cells prepared by Mrs Cyd Bush Other details as in Fig. 1.

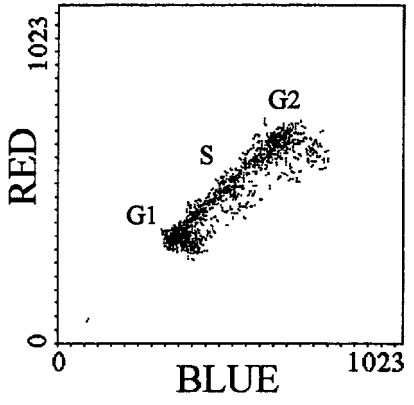

Fig. 6. A cytogram of PI-DNA (red) versus Hoechst 33258-DNA fluorescence of nuclei from MRC5/34C cells The cells had not lysed completely The nuclei and the partially lysed cells stain slightly differently creating a "shadow" in the cytogram. Experimental details as in Fig 1

4. Notes

1. Peripheral blood lymphocytes should be isolated by density gradient centrifugation Anchorage dependent cells are often rendered quiescent by reducing the concentration of serum.
2. PHA (typically, 200 μg/mL) is usually used to stimulate peripheral blood lymphocytes. Serum is added to adherent cell cultures rendered quiescent by incubation in a reduced concentration of serum

3. The correct concentration of BrdUrd must be determined by a preliminary experiment The concentration should be such that cells in G1 after one round of replication have about half the blue fluorescence of unlabeled cells in G1.
4 Concentrations of BrdUrd vary from 10–100 μ*M* If the concentration of BrdUrd is >20 μ*M*, the BrdUrd may become exhausted by 24 h The remedy is to either work at a lower cell density or to replenish the BrdUrd every 12 h
5 A trial experiment should be performed to check that the incorporation of BrdUrd into the DNA is not inhibiting the progression of the cells through the cycle After different times of incubation with BrdUrd, fix cells in 70% ethanol, centrifuge, and resuspend in phosphate-buffered saline containing 20 μg/mL PI and 0.1 mg/mL RNase Incubate at 37°C for 1 h and record the DNA histogram Addition of deoxycytidine (equimolar with the BrdUrd) can reduce any effect of BrdUrd
6 For suspension cultures, shake to resuspend cells and remove 3 mL For adherent cell cultures, use a separate flask for each time point Harvest the cells by a short incubation with trypsin
7. After harvesting, cells can be frozen and stored for later analysis
8 The detergent in the staining buffer releases nuclei At this stage, the samples are stable for several hours on ice
9 The cell concentration is important and should be between 5×10^5 and 2×10^6. If the concentration is too high, the nuclei will be under-stained and may give distorted cytograms
10 Either propidium iodide or ethidium bromide (EB) may be used as a counterstain for DNA.
11 When setting a gate on a plot of DNA-peak signal versus DNA-area, be careful to include material of DNA content less than that of cells in G1.
12 If it is not possible to perform a pulse shape analysis, clumps may usually be differentiated on a cytogram of forward light scatter versus red fluorescence
13 A PI/DNA complex is excited by UV It also absorbs blue light and will be excited by energy transfer from the Hoechst dye When the Hoechst fluorescence is quenched, there will be a consequent reduction in PI fluorescence. If a He-Cd laser is used as a source of UV (325 nm) (rather than an argon-ion laser, 360–390 nm), direct absorption will predominate and the secondary quenching will be reduced For this reason, it is preferable to use a He-Cd laser *(12)*
14. If the pattern produced from the sample at 0 h has a shadow (*see* Fig 6), the cells have not lysed properly Incubate the samples at 37°C for 5 min and re-run

Acknowledgments

I thank Jenny Titley, Cyd Bush, David Gilligan, and John Peacock, Institute of Cancer Research, Sutton, England, for supplying the data used to illustrate this chapter Their work was supported by the Cancer Research Campaign

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mRNA *In Situ* Hybridization to In Vitro Cultured Cells

Anton K. Raap, Frans M. van de Rijke, and Roeland W. Dirks

1. Introduction

In basic and applied biomedical research there is a considerable interest in having reliable RNA *in situ* hybridization techniques, since these allow the detection of RNA expression in a morphological context at the individual cell level. Conventional molecular biology techniques for RNA analysis such as Northern hybridizations, RNase protection assays, and reverse transcription-polymerase chain reactions (RT-PCR) demand the homogenization of the tissue or cell sample. In these cases the possibility of relating (changes in) RNA expression to histo- and cytomorphological information is lost to a very large extent.

Several direct and indirect nonradioisotopic nucleic acid labeling formats are now available (for reviews, *see* refs. *1–3*). In conjunction with advanced molecular cloning, PCR, and automated DNA synthesis methods, they provide excellent tools for DNA and RNA *in situ* hybridization studies.

Improved labeling, immunocytochemical detection, and microscopic imaging procedures, in conjunction with optimization of chromatin preparation, have already resulted in reproducible DNA *in situ* hybridization protocols that permit sensitive and high resolution mapping of small unique DNA *(4–6)*, as well as large genomic clones by incorporating the principle of suppression hybridization *(7–9)* The ability of nonradioisotopic methods to discriminate, within one and the same preparation, multiple DNA sequences simultaneously has contributed significantly to these developments *(10–14)*.

Although significant progress has been made in the methodology of RNA *in situ* hybridization, its nonradioisotopic counterpart has not reached the level of sensitivity and reproducibility that has currently been achieved for DNA *in situ* hybridization protocols for molecular cytogenetic and clinical applications,

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

such as gene mapping and interphase diagnosis. Here we describe a protocol for fluorescence RNA *in situ* hybridization using double-stranded (ds) DNA probes to in vitro cultured, adherent cells such as HeLa. The protocol permits the detection of abundantly expressed housekeeping gene transcripts like human elongation factor (HEF) and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA with high signal-to-noise ratios. It is estimated that transcripts that have an abundancy that is about 10-fold less, should also be detectable.

2. Materials

1. Sterilized microscope object slides coated with gelatin/chrome alum or polylysine
2. Fixatives: 4% formaldehyde, 5% acetic acid in 0.9% NaCl, and 1% formaldehyde in PBS
3. 0.1% Pepsin in 0.01*M* HCl, freshly prepared from a 10% stock in H_2O
4. Biotin-, digoxigenin-, fluorescein-, or rhodamine-labeled cDNA probes
5. Formamide
6. 20X SSC: 3*M* NaCl, 0.3*M* Na-citrate, pH 7.0.
7. Dextran sulfate.
8. Herring sperm DNA
9. Yeast RNA.
10. Biotin- and digoxigenin detection reagents: avidin-FITC, and avidin-TRITC (Vector, Burlingame, CA), biotinylated goat antiavidin (Vector); sheep antidigoxigenin-FITC, and -TRITC (Fab fragments, Boehringer Mannheim, Mannheim, Germany); mouse monoclonal antidigoxigenin (Boehringer), digoxigeninylated-sheep antimouse-Ig (Boehringer)
11. 10X PBS: 80 g NaCl, 2 g KCl, 14.49 g Na_2HPO_4, 2.49 g KH_2PO_4/L
12. Immunology buffer (TNT). 0.1*M* Tris-HCl, pH 7.4, 0.15*M* NaCl, 0.05% Tween-20.
13. Blocking reagent (Boehringer)
14. 4',6-Diamidino-2-phenylindole (DAPI)
15. Vectashield embedding medium (Vector)
16. Modern epifluorescence microscope equipped with appropriate filter sets
17. Graded series of ethanol: 70, 90, and 100%
18. Hybridization buffer: 60% deionized formamide, 2X SSC, 50 m*M* sodium phosphate, 5% dextran sulfate, pH 7.4

3. Methods

3.1. Cell Culture and Fixation

1. Culture cells in the appropriate medium and CO_2 atmosphere. For convenience of subsequent handling, seed the cells in a Petri dish containing gelatin/chrome alum or poly-L-lysine coated glass object slides.
2. Rinse briefly with PBS
3. Fix with 4% formaldehyde, 5% acetic acid in 0.9% NaCl for 20 min at room temperature (*see* Note 1)
4. Wash in PBS for 5 min.

5 Dehydrate through a graded series of ethanol, 5 min each
6 Immerse slides in 100% xylene, or Histoclear for 10 min to remove lipids
7 Rehydrate in PBS for 5 min
8. Incubate in 0 1% pepsin in 0 01*M* HCl at 37°C for 10 min (*see* Note 2).
9. Rinse in PBS.
10. Postfix in 1% formaldehyde in PBS for 10 min.
11 Dehydrate as in step 5

3.2. Probe Preparation

Procedures for labeling DNA with haptenized (e.g., biotin or digoxigenin) or fluorochromized (e.g., fluorescein or rhodamine) labeled dUTPs are described in ref. *15* (*see also* Notes 3 and 4)

1 Purify the labeled probes by gel filtration
2 Add 50-fold excess of sheared herring sperm DNA and ethanol precipitate.
3 Dissolve the pellet in hybridization buffer at a stock probe concentration of 20 ng/μL Final probe concentrations for *in situ* hybridization are 5 ng/μL.

3.3. RNA In Situ Hybridization

1. Denature the probe at 80°C for 10 min.
2 Apply the denatured probe solution to the target area, e.g., 5 μL under a 18 × 18 mm^2 coverslip (Optionally, probe and target RNA can be denatured simultaneously at 80°C for 10 min.)
3 Hybridize overnight at 37°C in a humidified atmosphere (humidifier is 60% formamide, 2X SSC)
4 Wash twice for 10 min each in 60% formamide/2X SSC at 42°C, and twice for 10 min each in the same solution at room temperature
5 Rinse in TNT buffer

3.4. Immunocytochemical Detection and Embedding

1 Incubate for 15 min at 37°C in TNT buffer containing 0.5% blocking reagent.
2. Wash with TNT buffer three times for 5 min each.
3. For all antibody incubations, supplement the TNT buffer with 0 5% blocking reagent.
4. Incubate with antibodies at 37°C for 30–45 min.
5. Perform all washing steps with TNT buffer three times for 15 min each

In the following sections, we describe one-, two-, and three-step procedures for biotin- and digoxigenin-labeled probes. Recommended dilutions are indicated for the reconstitution volume suggested by the suppliers.

6. In the one-step procedure. For digoxigenin-labeled probes, incubate with 1 200 of sheep antidigoxigenin-FITC or -TRITC (Fab fragments). For biotin-labeled probes, incubate with 1·1000 dilution of avidin-FITC or -TRITC

7 In the two-step procedure for digoxigenin-labeled probes. Incubate with 1 200 dilution of mouse monoclonal antidigoxigenin, followed by 1 500 dilution of rabbit-antimouse-FITC or -TRITC
8 In the three-step procedure. For biotin-labeled probes, incubate with 1 1000 dilution of avidin-FITC or -TRITC, followed by 1 100 dilution of biotinylated goat-antiavidin, followed by 1·1000 dilution of avidin-FITC or TRITC For digoxigenin-labeled probes, incubate with 1 200 dilution of mouse monoclonal antidigoxigenin, followed by 1:200 dilution of digoxigeninylated sheep antimouse-Ig, followed by 1:200 dilution of sheep antidigoxigenin-FITC or -TRITC (Fab fragments)
9 After the last wash, dehydrate, air-dry, and embed in Vectashield containing 75 ng/μL DAPI. Slides hybridized with fluorochrome-labeled probes can be embedded directly after the posthybridization washings

See Notes 5–10 for discussions on procedural variations

4. Notes

We have previously described many variations in the specific steps of this general protocol (*see* refs *16–19*). Objective evaluation of the effects of these procedural variations on the final signal-to-noise ratios requires cytofluoro- or photometric analyses. Such studies are still scarce Hence, only qualitative statements can generally be made In the following notes, we describe our experience with a number of these variations in such qualitative terms.

1 Fixation Fixation by crosslinking with formaldehyde for limited time periods is traditionally used for mRNA *in situ* hybridization of cultured cells possibly because it provides, in conjunction with limited protease digestions, a workable balance between the retention of RNA and cell morphology, and accessibility of macromolecular reagents *(20,21)* In our studies, we found a beneficial effect of adding 5–20% acetic acid to the 4% formaldehyde The optimal concentration may vary for different cell lines *(19)*
2 Protease pretreatment. A positive effect of protease treatment is beyond doubt, although the optimal concentration and/or time of treatment should be optimized for each application *(16,22)* We prefer pepsin over proteinase K because it results in better preservation of cell morphology, and is less critical in terms of the intensity of the treatment than it is with proteinase K *(21)*
3. dsDNA or ssRNA probes. Although the protocol is written for standard nick-translated dsDNA probes, purified cDNA plasmid inserts, or PCR products thereof, we have used in vitro transcribed single-strand (ss) antisense RNA probes *(26)* and have obtained somewhat better results in terms of intensity of signal. However, critical titration of the ssRNA probe concentration, and high stringency hybridization conditions are recommended to prevent cross hybridization with rRNA. Also, probe lengths are critical and should not exceed 500 bp.
4. Direct and indirect methods. Although the protocol describes indirect detection procedures, we have obtained good results with rhodamine- or fluorescein-

labeled probes for such abundantly expressed transcripts as elongation factor mRNA *(19)* The three-step procedure yields the strongest signals

5. Heat treatment of the cells: This has been shown to be a useful step Since such a treatment may lead to DNA denaturation, in studies dealing with the spatial relationship of genomic DNA and nuclear RNA, discrimination between the gene and its primary transcript may become difficult *(19,25–27)* Lower temperatures of denaturation as recommended in the protocol have been proven to yield the same beneficial effect on RNA detection without obtaining genomic DNA signals (P. Lichter, personal communication).
6. Preventing exo- and endogenous RNase activity: In initial studies, we have investigated measures to prevent RNase activity For the PBS solutions used before the immunocytochemical detection steps, we have performed autoclaving in the presence or absence of diethyl pyrocarbonate (DEPC) For the targets under study, we saw no difference between the two treatments With the exception of the short PBS wash shortly before fixation and hybridization, the successive solutions used can in fact be considered as inactivating for RNases (e.g , ethanol for dehydration, xylene, pepsin in HCl acid, formamide-containing solutions). After hybridization the target is in a duplex and therefore protected from RNase action. However, situations may be envisaged in which RNases hydrolyze RNA sequences flanking the hybrid leading to dissociation of the *in situ* hybrid. However, comparison of incubations with and without vanadylribonucleoside complex present as an RNase inhibitor during immunocytochemical incubations showed no difference in signal intensities We therefore conclude that it is sufficient to work with sterile solution without DEPC or specific RNase inhibitors
7. Controls As a positive procedural control, we frequently use ribosomal RNA as a target *(19,28)* Very bright signals should be obtained, before proceeding with *in situ* hybridization for less abundant targets. To ascertain penetration of probes, *in situ* hybridization of nonexpressed probes (e.g., alphoid DNA in human cells) to denatured cells is recommended As specificity controls, several irrelevant probes should be brought through the same procedure. Trivial controls such as RNase treatment and omission of one or more of the key reagents in the immunological detection steps should also be performed

   The issue of specificity of RNA *in situ* hybridization should not be underestimated because supportive proof of specificity can not be obtained on the basis of molecular weight of the target, as is offered by Northern hybridizations Spurious *in situ* hybridization of probes to rRNA may be obvious from nucleolar localization of signals
8. Sensitivity. Figure 1A,B gives typical results of *in situ* hybridization of a HEF-mRNA to pepsin- and heat-pretreated HeLa cells, and of a control hybridization, respectively. The specific hybridization gives rise to numerous dot-like signals, whereas in the control the number of signals drop to a few per cell. On the basis of such results, we estimate that RNAs present in a 10-fold less copy number should be detectable by the method As an illustrative comparison, the HEF mRNA was detected as a strong band with standard Northern hybridization

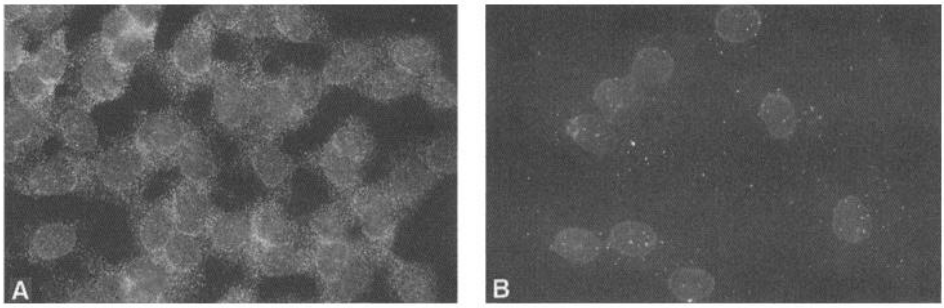

Fig. 1. Fluorescence *in situ* hybridization to human elongation factor mRNA in pepsin- and heat-pretreated HeLa cells. **(A)** Specific hybridization with digoxigenin-labeled 1.4 kb cDNA *(32)* using the three-step detection procedure and fluorescein as the reporter molecule. **(B)** Control hybridization with digoxigenin-labeled heterologous probe (pSS-DNA, *see* ref. *27*) and identical detection procedure. Nuclear counterstain was DAPI.

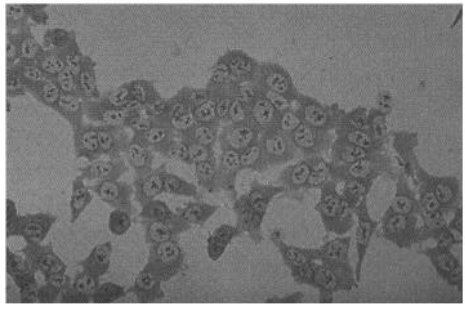

Fig. 2. *In situ* hybridization to rRNA in pepsin- and heat-pretreated HeLa cells. Hybridization was with a digoxigenin-labeled 2.1 kb 28S-rDNA fragment *(28)*. One-step-detection was with peroxidase-conjugated antidigoxigenin and the diaminebenzidine reaction.

using ^{32}P as the label, 10 μg of total cytoplasmic RNA as input material, and 2–6 h of autoradiographic exposure. Further research is necessary to more objectively determine the lower limits of the present protocol.

9. Brightfield detection: As an alternative to fluorescence detection, brightfield detection of enzyme labels instead of fluorescent labels can be used. For that purpose, the flurochrome-conjugated antibodies used in the indirect procedures are substituted by peroxidase or alkaline phosphatase conjugates. The enzyme labels are visualized, for example, using the diaminebenzidine reaction for peroxidase, or the nitroblue tetrazolium reaction/bromo-chloro indolyl phosphate reaction for alkaline phosphatase (*see* Fig. 2 for an example of rRNA detection using peroxidase as the final label). For other enzyme cytochemical reactions,

see ref *29* With these enzymes, bicolor detection of mRNAs can also be achieved However, for multicolor detection of different RNA species that reside in the same cell, fluorescence *in situ* hybridization is recommended *(17,18)*

10. Potential remedies for the autofluorescence problem A factor limiting sensitivity of fluorescence *in situ* hybridization is undoubtedly autofluorescence of the cells *(30)*. Such autofluorescence is composed of natural fluorescence of the cells as well as fixation-induced fluorescence. Glutaraldehyde, for example, is well known for its induction of high autofluorescence levels and is therefore not recommended for fluorescence *in situ* hybridization. Limited formaldehyde fixations give reasonably low levels of autofluorescence, but for small and low copy number targets it may still be too high to permit genuine *in situ* hybridization signals to reach the detection level A potential remedy for this fundamental problem may be the use of final reporter molecules that possess long luminescence decay times so that the generally short-lived autofluorescence can be gated out in time using time-resolved microscopy Encouraging results with such time-resolvable luminescent reporter molecules have recently been described *(31)*

Acknowledgments

The authors thank Mels van der Ploeg for valuable discussions, and Ton Maassen and Jan Bauman for the HEF and rDNA probes, respectively, and interest in our work. This work was supported in part by NWO-grant no. 534-060

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32

Transmission and Scanning Electron Microscope Preparation of Whole Cultured Cells

Josef Neumüller

1. Introduction

Despite the enthusiasm of the first investigations of cell ultrastructure, morphological studies have since lost some of their importance for biomedical research. The development of quantitative biochemical methods has been the cause of this reduced interest in morphology Biochemical reactions, however, take place in compartments of the cell and the extracellular matrix. This compartmentation, in an ultrastructural dimension, is the prerequisite for a systemic discharge of metabolic processes in temporal continuity. This compartmentation is provided by phospholipid biomembranes serving as support for sets of enzymes or receptors and as vessels for internalized or synthesized substances. Investigations of the biochemical processes in compartments are only possible if these compartments can be separated by ultracentrifugation or by ultrahistochemical methods Routine preparation for transmission electron microscopy (TEM) preserves only the morphology of cells and limits the possibilities for staining to electron-dense reaction products. Furthermore, the electron beam causes considerable contamination and damage to the object. In the past decade several attempts have been made to overcome these problems in order to obtain realistic morphological representations of cells and to perform ultrahistochemistry. First of all the development of cryomethods has made it possible to retain and preserve substances at their original site. However, the use of only "soft aldehyde fixatives"and the renunciation of crosslinking reagents such as OsO_4 causes a loss of contrast of the ultrastructural components The newer techniques of·

1. Cryotransfer from the ultramicrotome to the electron microscopy (EM) in a vitrified state;

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

2 Cryosubstitution that involves a gradual substitution of ice with organic solvents and finally with resins that polymerize at low temperatures,
3 Controlled freeze drying over a molecular sieve; and
4 The replica techniques taken from freeze-fractures can partially resolve the problems of conventional preparation methods

The problem of contrast can also be solved in some cases by electronic contrast enhancement and image processing.

The lack of information about absent ultrastructural details in sectioned cells can to some extent be compensated for by investigations using scanning electron microscopy (SEM) or high voltage electron microscopy (HVEM). This chapter deals with preparation schedules for several anchorage-dependent and suspension cell cultures as well as for cell cultures grown in gels

In the early days of EM, whole cultured cells on formvar-coated grids were investigated, but gave poor resolution under the normal 50-kV electron beam. After the development of ultramicrotomy these methods receded into the background. Metallurgists, however, were faced with the need to develop a HVEM allowing penetration of thin metal foils. Appropriate electron microscopes using a voltage of 1 MeV were constructed. Biologists, too, became interested in the features of these microscopes. Above all, the microtrabecular lattice was clarified by Porter and his colleagues. Only few biological investigations have been carried out because of the small number of HVEMs available in the world. New generations of high-resolution electron microscopes provided a compromise by working at a high voltage (HV) up to 400 kV. With this HV setting it is also possible to penetrate through cells that have been spread on grids *(1–5)*. This method is very powerful, particularly for investigation of cell motility, the cytoskeleton, and its interactions with the extracellular matrix (ECM) using immunogold particles and contrasting with silver stain.

2. Materials

1. Fixative 2% glutaraldehyde (GA) in 0 1*M* Na-cacodylate-HCl-buffer + 0.1*M* sucrose (pH 7 2), total osmolarity· 510 mOsM, vehicle osmolarity· 300 mOsM. GA is commercially available as a 25% aqueous solution (prepare fresh before each use); 1% OsO_4 in Na-cacodylate-HCl buffer (can be stored at 4°C for some months).
2 0.1*M* Na-cacodylate-HCl buffer as in ref. *1* without GA and sucrose as a washing solution (can be stored at 4°C for some months)
3 A gradual series of ethanol. 30, 50, 70, 80, 90, and 95% ethanol in double-distilled water; absolute ethanol that has been previously purified of any water and particles, using a molecular sieve that absorbs molecules with an effective diameter <0 3 nm (store at 4°C)
4. Intermediate fluids· Freon TF; gas flasks with Freon 13 or 116, or pure CO_2.

5 Reagents for embedding: Propylene oxide (1,2-epoxypropane); *N*-butylglycidyl ether; ultralow viscosity resin (ULVR)· 100 g ERL 4206 resin (vinylcyclohexene dioxide), 200 g HXSA (hexenylsuccinic anhydride), 25 g Araldite RD2 (DYO26) resin, and 25 g DMAE (dimethylaminoethanol) (store at 4°C)
6. Formvar resin, 0 2%, in water-free chloroform in order to prepare supporting film on EM grids (prepare fresh)
7 Materials spheroids, microcarriers, or coverslips for sticking cells, onto the specimen mount for SEM 10 mg poly-L-lysine in 100 mL double-distilled water; a double-sided adhesive tape, a silver-loaded epoxy adhesive.
8. Reagents for removal of resin from embedded cells in gels: 0.5 g crystalline NaOH in 50 mL absolute ethanol; a gradual series of 30, 60, 70, and 90% amyl acetate in absolute ethanol and pure amyl acetate (prepare fresh)
9 Chemicals for the freeze-fracture· A 5 m*M* aqueous solution of poly-L-lysine (mol wt 2000–4000), 0 5% boiled and filtered starch solution in double distilled water (both to be prepared fresh); Freon 22, 10% hydrofluoric acid (store at 4°C).
10 Materials for the preparation of microchambers: Beem capsules (size 00); nylon gauze (100-μm mesh width); dialysis tubes (10-mm diameter, separation at mol wt 50,000)
11. Small Beem capsules (size 3) for direct embedding of selected areas of monolayers.
12. Other materials. Round coverslips (10-mm diameter); glass fiber grids (0 5-mm mesh width) for handling of floating monolayers; gold grids G 200; copper grids with a hexagonal pattern (repeat distance 460 lines/in); appropriate flat embedding forms made of silicone rubber

3. Methods

3.1. Method 1: Simultaneous Preparation of Cell Cultures for SEM and TEM (6)

3.1.1. Fixation

1 Fix in 2% GA in 0 1*M* Na-cacodylate-HCl buffer plus 0 1*M* sucrose (pH 7.2), total osmolarity· 510 mOsM, vehicle osmolarity: 300 mOsM (*see* Note 1). Add prewarmed (37°C) fixative in equal volume to the culture medium for 5 min without moving the culture vessel (*see* Note 2).
2. Replace the fixative/medium mixture with pure fixative at room temperature for 1 h and gently agitate the culture vessel.
3 Change the fixative and fix for a further 24 h at 4°C.
4. Rinse three times with Na-cacodylate-HCl buffer at room temperature.
5 Carry out postfixation in 1% OsO_4 in 0.1*M* Na-cacodylate-HCl buffer (pH 7.2) at room temperature

3.1.2. Dehydration

Use gradual steps of ethanol as follows: twice for 5 min in 30% and in 50%; 10 min in 70, 80, 90, 95%, twice for 10 min in 100% (*see* Note 3).

3.1.3. Drying at the Critical Point: Preparation for SEM

1 Incubate with Freon TF as intermediate fluid (can be omitted if polystyrene vessels are used).
2 Transfer the sample to the specimen boat of the critical point drying apparatus (CPDA) In order to avoid artifacts the specimen has to be kept immersed in Freon TF or absolute ethanol (Fig 1)
3 Put the specimen boat into the CPDA cooled to 10–15°C Close CPDA and fill with CO_2 or Freon 13 or 116
4 Allow the fluid in the specimen boat to escape and change the intermediate fluid three times at 5-min intervals
5 Increase the temperature until the critical point is reached (it differs in respect to the intermediate medium used; Tc, critical temperature, Pc, critical point)

Carbon dioxide (CO_2)	Tc = 31 3°C
	Pc = 75 5 kg/cm^2
Freon 13 ($CClF_3$).	Tc = 28.9°C
	Pc = 39 5 kg/cm^2
Freon 116 (CF_3–CF_3)	Tc = 19 7°C
	Pc = 30 4 kg/cm^2

6. When the critical point is reached, the liquid interface becomes opalescent and disappears Allow the gas to escape slowly in order to avoid recondensation

3.1.4. Sputter Coating

1 Place the specimen onto a specimen mount using a conductive adhesive
2 Put the specimen mount into a sputter coater and cover it with a gold-palladium (80/20) layer of 1.5–2 nm (*see* Note 4)

3.1.5. Alternative Embedding: Preparation for TEM (continue after Section 3.1.2.)

1. Put the specimen into propylene oxide for 10 min (this step may be omitted if polystyrene vessels are used).
2. Change the ULVR three times at 4-h intervals or transfer the specimen directly from 100% ethanol to the resin in a flat embedding form The resin is miscible with absolute ethanol
3. Polymerize in an appropriate oven at 70°C for 12 h.

3.2. Method 2: Preparation of Cell Pellets, Spheroids, Cell Suspensions, and Cells Grown on Microcarriers for SEM

3.2.1. Cell Harvesting

1. Filter the cell pellets, spheroids, and microcarriers through a nylon gauze (100-µm mesh width) placed in a small glass funnel.
2. Rinse with 20 mL culture medium (without fetal calf serum [FCS]), prewarmed to 37°C.

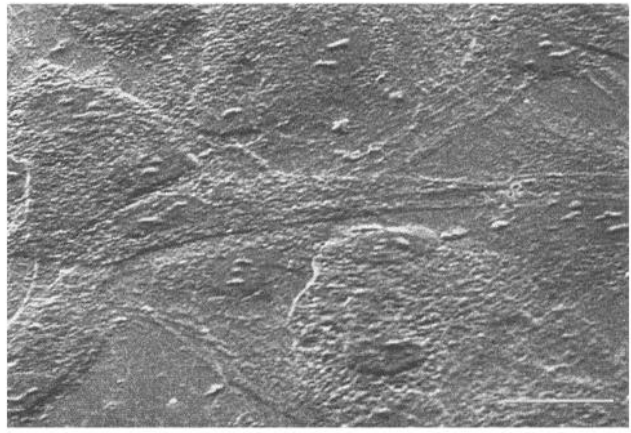


Fig. 1. Human skin fibroblasts grown on glass coverslips. Artifact by drying (bar = 10 μm).

3. Incubate a dialysis tube (separation at mol wt 50,000, 10-mm diameter) in phosphate-buffered saline (PBS) for 30 min before use. Knot one end of the tube, cut out the tip of the filter with the cell aggregates or microcarriers, and place it into the tube. Fill the tube with medium without FCS and knot the second end of the tube.
4. Alternatively, centrifuge cell suspensions at 800*g* for 10 min, resuspend in a small amount of culture medium without FCS, and fill directly into the dialysis tube.

3.2.2. Fixation, Dehydration, and Critical Point Drying

Perform according to Method 1. The cells, aggregates, or microcarriers remain in the dialysis tubes, which are placed into 20-mL tumbler vials, completely filled with the respective liquid and moved by means of a rotation tumbler. Avoid flushing too rapidly and changing the pressure in the CPDA too fast during outstreaming of gas in order to prevent bursting of the tubes.

3.2.3. Coating with Gold-Palladium

1. Cover the SEM specimen mounts with a double-coated adhesive tape (the tape should be somewhat smaller than the top surface area of the specimen mount). Open the dialysis tube and disperse the contents of the filter over the sticky surface of the tape.
2. Invert the specimen mounts and remove the excess of particles by generation of a soft air stream from an air puffer.
3. Connect the edge of the tape to the specimen mount with a droplet of conductive epoxy adhesive.

4 Put the samples in a desiccator which contains silica beads, evacuate and allow the glue of the tape to dry for at least 24 h.
5 Perform sputter-coating according to Method 1.

3.3. Method 3: Preparation of Cell Pellets, Spheroids, Cell Suspensions (Fig. 2A–C), and Cells Grown on Microcarriers (Fig. 3A–F) for TEM

1 Cell pellets from monolayers or centrifuged cell suspensions, spheroids, and sectionable microcarriers can alternatively be processed for TEM. Instead of the dialysis tubes, Beem capsules covered with a 100-µm-mesh-width nylon gauze are used. Cut the gauze to an appropriate size and clamp to the capsule with the bored capsule cap (Fig. 4, *see* p 386)
2. Fix and dehydrate according to Method 1 (the chambers made from the Beem capsules must be submersed completely in the respective fluids of the tumbler vessels). If polydextran beads are used, the incubation time should be extended two to three times to permit sufficient infiltration.
3 After dehydration in absolute ethanol submerse the capsule chambers in changes of ULVR four times for 4 h Each change of resin should take place in a separate tumbler vial moved by means of a rotation tumbler
4. Perform the infiltration with resin at 4°C After the last incubation bring the vials into a vertical position and clean the outsides with a filter paper wetted with propylene oxide
5. Allow the particles in the capsule to settle and perform polymerization at 70°C for 12 h.
6. Trim the blocks. This is quite easy because the particles are concentrated in the tip of the capsule
7. For sectioning, the use of a diamond knife is recommended, above all if microcarriers are embedded

3.4. Method 4: Preparation of Monolayers Grown on Coverslips or Plastic Petri Dishes for SEM

1. To grow cells use plastic Petri dishes with four ring-divisions fitted with 1-cm diameter coverslips previously marked with an asymmetric letter and sterilized (Fig. 5; *see* p. 386) or 6-cm diameter Petri dishes.
2. Fix and dehydrate cells after spreading and multiplication according to Method 1
3 Displace the coverslips carefully from the dish with a preparation needle and a tweezer with angled parallel edges and put into a special coverslip tray (Fig. 6; *see* p. 387) that fits the specimen boat of the CPDA Drying can be easily performed (Method 1) in this tray that can be made by anyone with do-it-yourself experience

If cells were plated on plastics, strips of the bottom of the dishes can be cut out with a hot scalpel blade. This has to be carried out very quickly to avoid desiccation of cells. The plastic strips can be marked as previously mentioned and processed in tumbler vials. They can be dried without the use of Freon TF, e.g., directly from absolute ethanol to CO_2

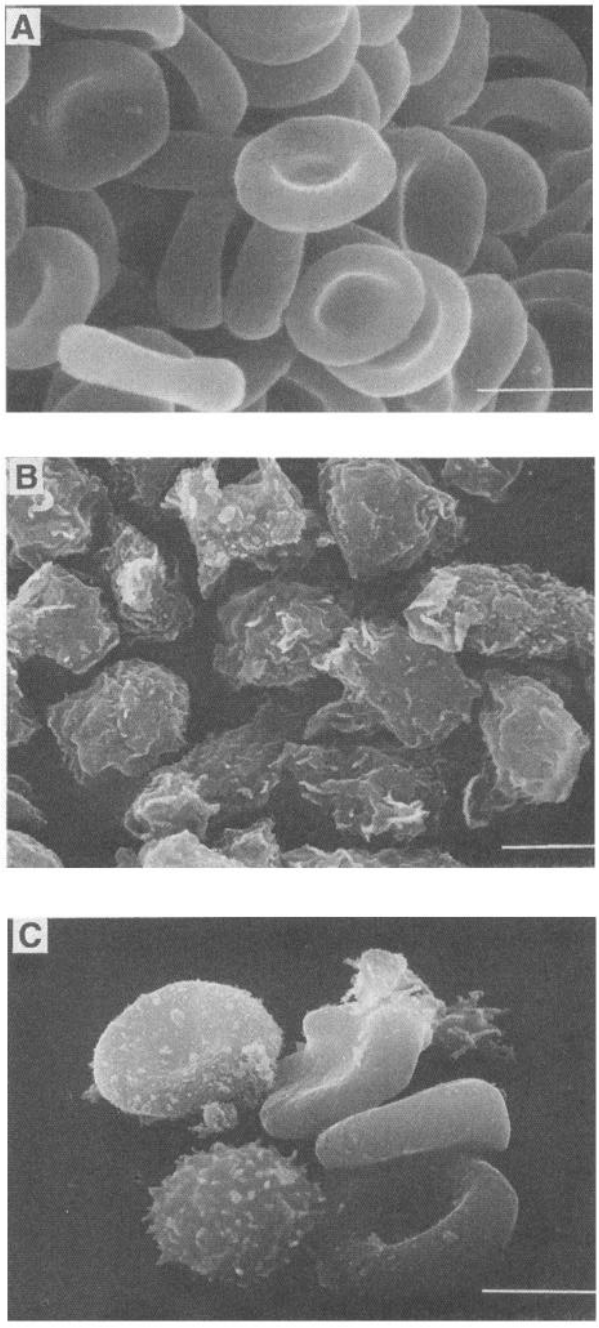
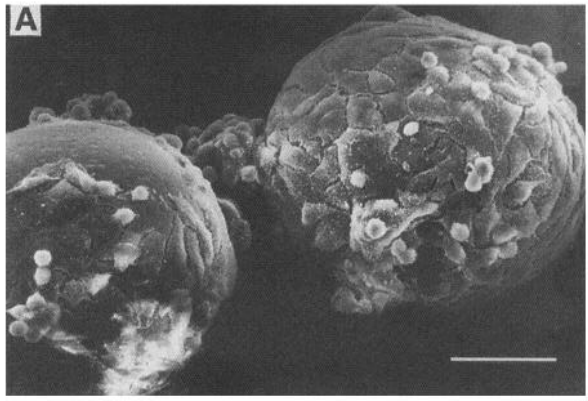
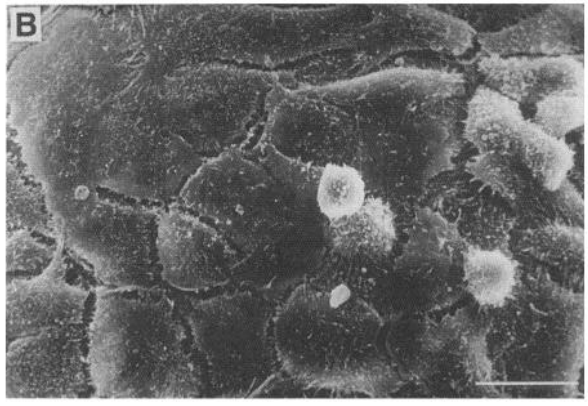


Fig. 2. (A) Attachment of human erythrocytes to an adhesive tape. Preparation by using dialysis tubes (bar = 5 μm). **(B)** The same preparation as in A, but of human granulocytes (bar = 5 μm). **(C)** The same preparation as in A, but of human buffy coat cells (bar = 4 μm).


A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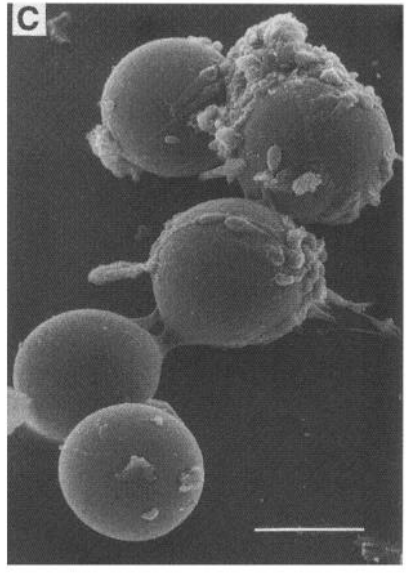
C

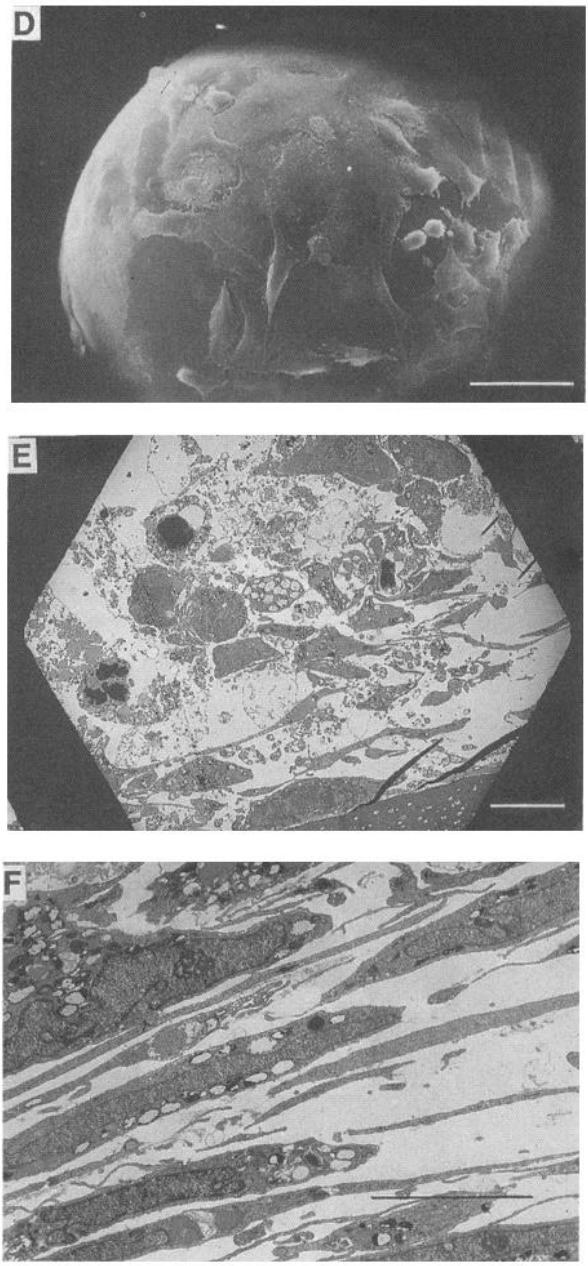

Fig. 3. *(shown on opposite page)* **(A)** HeLa cells grown to a high density on glass microcarriers (bar = 10 μm). **(B)** Cell contacts and surface projections of HeLa cells (detail of A; bar = 5 μm). **(C)** Human skin fibroblasts grown on microcarriers (Cytodex 3) (bar = 100 μm). **(D)** *(above)* HeLa cells grown on microcarriers (Biosilon) (bar = 30 μm). **(E)** TEM at low magnification of human tendon fibroblasts grown to a high density on Biosilon microcarriers which have not been stirred (* = microcarrier; bar = 1.5 μm). **(F)** As in E, but at higher magnification (bar = 5 μm).

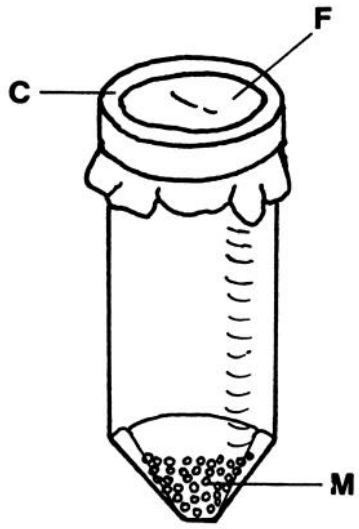

Fig. 4. Beem capsule with a filter (F) clamped to the perforated cap (C) containing microcarriers (M).

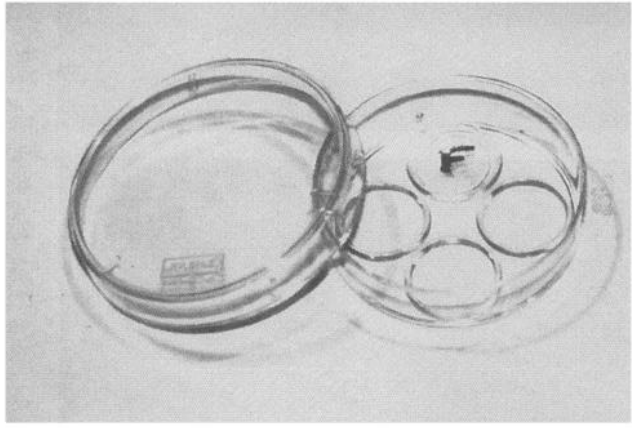

Fig. 5. Petri dish with ring-divisions containing marked coverslips (F).

4. Mount the coverslips or plastic pieces on to the specimen mounts with a conductive epoxy adhesive, place into a desiccator under vacuum for 24 h, and sputter coat as described under Methods 1 and 2.

3.5. Method 5: Simultaneous TEM Preparation of Cultured Cells

Process as in Method 4 until dehydration in 100% ethanol.

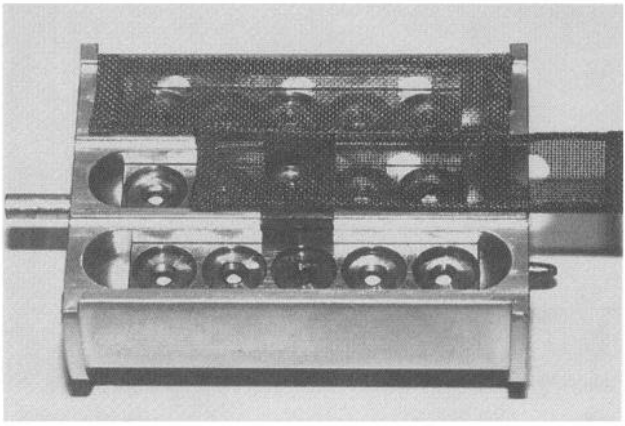

Fig. 6. Specimen boat of a CPDA with metal grid trays (T).

3.5.1. Cells Grown on Round Coverslips

1. Fill the rings containing the coverslips with a large drop of ULVR, which is changed three times at 10 min intervals at 4°C.
2. Fill a Beem capsule completely with ULVR, inverse rapidly, and place over the coverslip in a vertical position. The diameter of the Beem capsule should be <1 cm.
3. Polymerize in this position at 70°C for 12 h.
4. Break away the Beem capsule from the Petri dish. Split the coverslip from the resin block with a scalpel blade or by rapid cooling with CO_2 snow.
5. Carry out appropriate trimming after orientation under an inverted light microscope.

3.5.2. Cells Grown on Plastic Dishes (7)

1. Mark the edge of a selected area of cells inside the Petri dish with a thin preparation needle.
2. Replace the 100% ethanol in the dish by 2 mL ULVR.
3. Change it three times at 10-min intervals at 4°C.
4. Cover the marked area with an inverted and resin filled Beem capsule as mentioned in Section 3.5.1, step 2 and then process further as described there.
5. When the Beem capsule containing the resin block is split from the dish, the marks are visible as replicas on the flat surface at the aperture of the Beem capsule and serve as a guideline for trimming.
6. Orientate the block in the ultramicrotome at an exact 90° angle to the cutting axis. After one semi-thin section, perform ultrathin sections parallel to the monolayer plane.

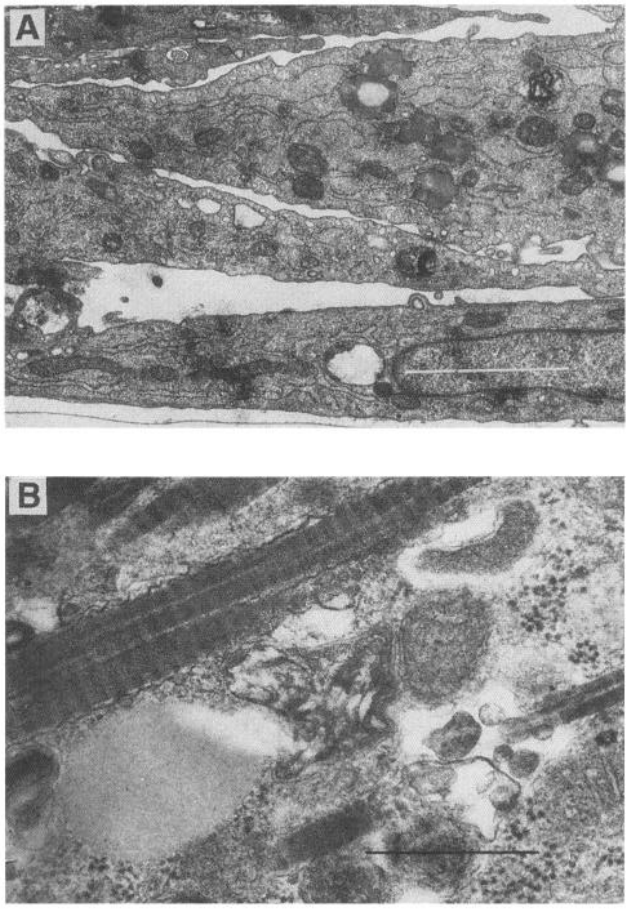

Fig. 7 (A) TEM preparation of "floating sheets" of human ligamentous fibroblasts. Note the electron-dense glycoprotein layer (▲▲) and the overlapping of these cells of a confluent monolayer (bar = 2 μm). **(B)** TEM preparation of "floating sheets" of human ligamentous fibroblasts. Note the production of collagen fibrils (↑) in deep recesses of the cell (bar = 0.5 μm).

3.6. Method 6: "Floating Sheet" Preparation of Monolayers for TEM (Fig. 7A,B) (8) *(see Note 5)*

1. Grow cells in 6-cm diameter plastic dishes.
2. Fix and dehydrate according to Method 1.
3. Add 5 mL propylene oxide or *N*-butylglycidyl ether to the dishes. Put a white porcelain plate under the dishes.
4. Move the dishes gently when the plastic appears rippled (after about 5 min). The monolayer floats up to the surface of the liquid.

5. Harvest the floating monolayer with a glass fiber grid (0.5-mm mesh width), invert the grid, and place it carefully in a flat embedding form filled with ULVR Press the grid slightly against the resin surface using a glass rod in order to separate the monolayer from the grid
6 Polymerize at 70°C for 12 h

3.7. Method 7: Whole Mount Preparation of Cultured Cells on Grids (9)

3.7.1. Preparation of Grids

1 Wash slides in distilled water and dry with Kleenex™ Remove remnant dust particles by using a gas jet duster Do not clean with soap, solvent, or acids because in this case the separation of films is very difficult.
2. Prepare a 0.2% solution of formvar resin in water-free chloroform. The resin must be dissolved completely in the solvent Use a magnetic stirrer.
3. Run a thin preparation needle along the edge of the slide Seize the coated surface and dip the slide slowly at an angle of 45° into a glass trough filled with water Under appropriate illumination the floating of the formvar film separating from the slide can be distinctly seen.
4 Transfer the films to gold grids using a grid filming device. The grids are held by a Teflon™ plate at the bottom of the trough. The surface of the liquid is slowly depressed and the films come in contact with the grids without any folds forming.
5. Remove excess water and dry the grid plate
6 Stabilize the filmed grids with a carbon layer using a vacuum coater.
7. Make the carbon surface hydrophilic by irradiation with a UV lamp for 30 min

3.7.2. Attachment of Cells to Grids

1. Cells are grown on the slides by incubation in small plastic dishes Avoid sliding together of grids Petri dishes with divisions (Fig 4) may be used

   Nonadherent cells can be attached to the carbon coated surface by dipping the grids into 10 mg/mL H_2O poly-L-lysine and washing three times in distilled water. Place the grids into the plastic dishes and allow the cells to settle and adhere to the coated surface
2 Transfer the grids for further processing into small Beem capsules that have been perforated with some holes at the conical part and on the sides in order to permit a good fluid exchange.

   As an example, HeLa cells grown on filmed grids are shown in SEM (Fig. 8A) and in TEM mode as whole cell mounts (Fig. 8B)

3.7.3. Fixation, Dehydration, Critical Point Drying, and Carbon Coating

All techniques are performed according to Method 3. The grids with the attached cells are coated a second time with carbon.

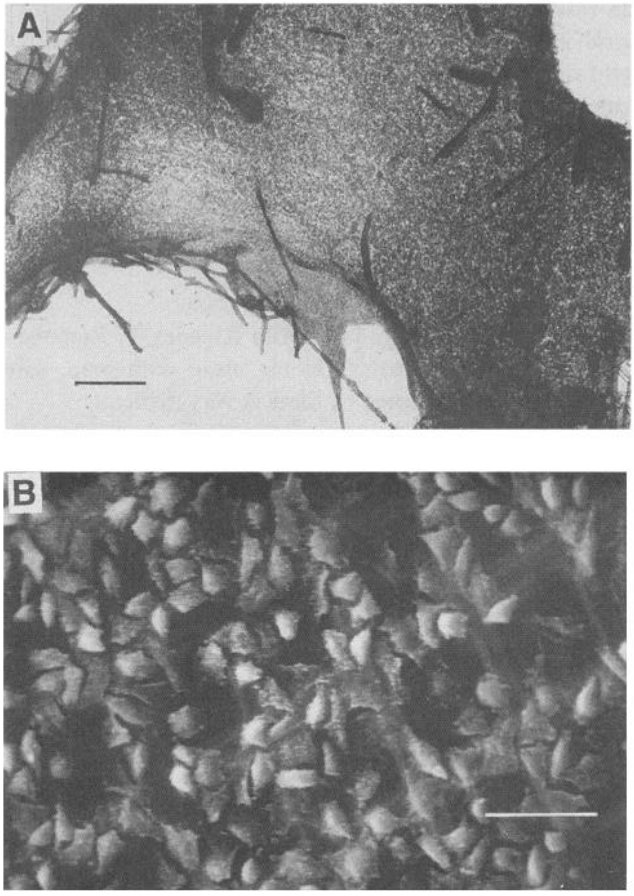

Fig. 8 (A) HeLa cells in TEM mode prepared by the whole cell mounting technique (bar = 1 μm). **(B)** HeLa cells prepared in SEM mode by the whole cell mounting technique (bar = 50 μm).

3.8. Method 8: Preparation of Cells Cultured on, or in, Gels for TEM and SEM (see Note 6)

1. Perform fixation *in situ* according to Method 1.
2. Extend the rinsing with Na-cacodylate-HCl buffer to twice the usual time.
3. Cut the gel into small cubes that then are transferred to tumbler vessels and dehydrate according to Method 1. If the cells are grown on the surface of the gels, dry the cubes in small gaskets in the CPDA.
4. Embed cells grown inside the gel in ULVR after dehydration in tumbler vials: 100% Ethanol three times for 1 h, propylene oxide for 1 h, 1 part ULVR + 3 parts propylene oxide for 1 h, 1 part ULVR + 1 part propylene oxide for 1 h, 3 parts

ULVR + 1 part propylene oxide for 1 h, ULVR alone two times for 3 h Prepare cells grown on the surface of the gels according to Method 1

5 Place the cubes in silicone embedding molds containing ULVR and polymerize at 70° C for 12 h
6. Trim the blocks and make sections for TEM
7 After the TEM observation, shorten the resin block to a flat disk on which the trimmed pyramid remains (compare with ref. *10*).
8 Incubate the disk in 0 5 g of crystalline NaOH in 50 mL of absolute ethanol for 30–60 min while the solution is agitated with a magnetic stirrer Avoid evaporation of the solvent.
9. Perform critical point drying when the removal of the resin is completed, using intermediate fluids as indicated in Method 1.
10 Incubate in absolute ethanol three times for 10 min, and change stepwise to amyl acetate (7 3, 4.6, 3 7, 1 9, and 100% amyl acetate) for 10 min at every step.
11 Dry in the CPDA with CO_2 for 30 min
12 Sputter with gold–palladium (*see* Method 1).

3.9. Method 9: Freeze Fracture of Monolayer (11) (see Note 7)

1 Attach the cells to a glass coverslip using an aqueous solution of 5 m*M* poly-L-lysine (mol wt 2,000–4,000)
2. Coat the slide with a thin film of a 0 5% boiled and filtered starch solution
3 Bring the coverslip side with the attached cells into contact with the filmed side of the slide without applying any pressure
4 Place the slide with the attached coverslip in a freezing container filled with Freon 22 that has been precooled with liquid nitrogen to –160°C
5 Separate the coverslip from the slide using a razor blade mounted on a polyethylene sheet
6 Place the broken parts in a freezing stage that is put in a vacuum unit on a liquid N_2 cooled shield N_2 is released and the system evacuated at 10^{-6} torr
7 Increase the temperature to –80°C using an internal heater inside the vacuum system
8. Remove the cooling shield and shadow the specimen with platinum at a 20° angle and with carbon at a 90° angle
9. Float off the replica in 10% hydrofluoric acid, rinse with distilled water and mount on grids according to Method 7 *(4)*

4. Notes

1 There is one basic principle that needs to be followed in order to get good preparation results. As mentioned, cultured cells are very sensitive to changes in pH, osmolarity, and variations in temperature These parameters have to be maintained during the aldehyde fixation steps at a level that is similar to that found in living cells. Aldehyde fixatives do not completely break down the semipermeability of the plasma membrane. Therefore the osmolarity should be at 510 mOsM (vehicle osmolarity 300 mOsM) *(6,12)* and it is recommended to check the osmo-

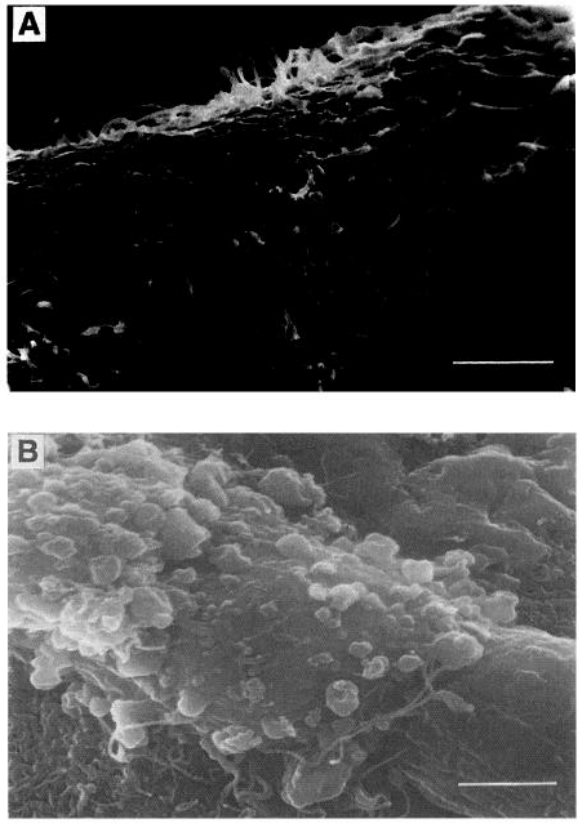

Fig. 9. (A) Spread human skin fibroblast. Cell fixed with GA and OsO_4. Note the well preserved microvilli (bar = 4 μm). **(B)** Human fibroblast grown on a microcarrier coated with gelatin (Cytodex 3) fixed only with GA. Note the bubble-like contracture of microvilli (bar = 4 μm).

larity with an osmometer. Even in cryomethods, in which cells are frozen with the addition of a cryoprotectant *(13)*, the osmolarity is of great importance.

2. During fixation, changes in temperature or vigorous shaking can completely alter the shape of the cells. Therefore one should add GA at 37°C to the complete medium for only a few minutes. This precaution stabilizes the plasma membrane and its projections. If the mixture of GA and medium remains in the culture vessels for more than a few minutes, protein aggregates from FCS cover the cells and cannot be removed. In a second step GA is added to an adequate fixation buffer (always at 37°C) that is allowed to cool down to room temperature after 1 h, the fixative is replaced again and the fixed cells are kept at 4°C for 12 h.

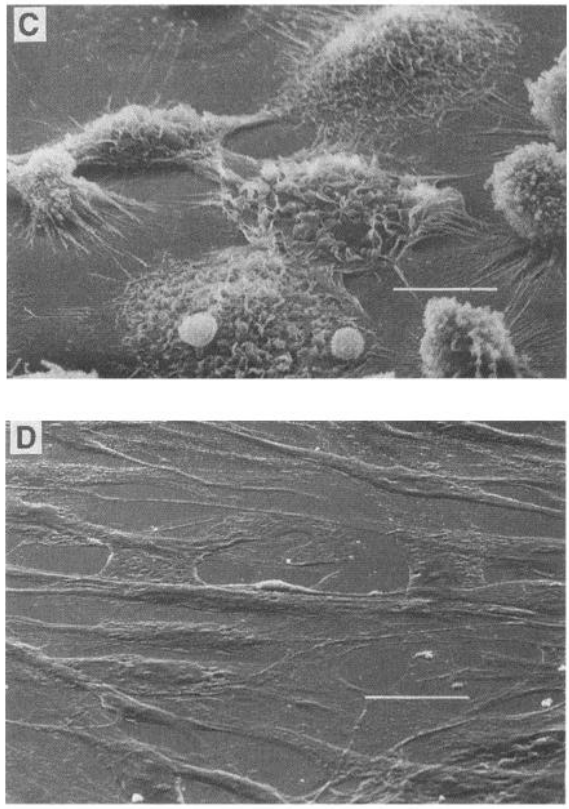

Fig. 9. *(continued)* **(C)** Human synovial fluid cells attached to glass coverslips. Fixation with cooled GA in cacodylate buffer (4°C). Note the retraction of the cytoplasm at the lobopodia. Only radial remnants of the cytoskeleton are visible (bar = 10 μm). **(D)** Good preservation of the cell shape of human skin fibroblasts (bar = 20 μm).

If one is interested in a good preservation of microvilli and other cell membrane projections, postfixation with OsO_4 is indispensable in order to avoid bubble-like swellings. OsO_4, on the other hand, increases the rigidity and fragility of the cell membrane. This leads to clefts and fragmentation. Therefore, the appropriate fixation and the right concentration of OsO_4 have to be found (Fig. 9A–D).

3. For dehydration in ethanol or acetone the cells do not need longer than a few minutes, but care has to be taken to make sure that the substratum is also well infiltrated and dehydrated, above all if microcarriers made of DEAE cellulose are used.

4 After critical point drying the specimen is placed onto a specimen mount using a double-sided adhesive tape or a drop of silver-loaded epoxy adhesive In order to avoid charge problems it is recommended that conductivity is provided between the specimen and the specimen mount by a droplet of a conductive adhesive Such adhesives are available and they dry in a few minutes The specimen mount with the specimen should be stored in a vacuum desiccator overnight before coating. A sputter coater is usually used for coating. In this apparatus the specimen is put into a vacuum jar in a holder that is at the same time the anode of a large diode The cathode is situated above the specimen and holds a gold target

The system is evacuated until <0 05 torr are reached Pure argon is let in several times in order to replace the remaining air When the vacuum of 0.05–0 07 torr is reached again, an HV of about 1 kV is set Because of the electron-push the argon atoms are ionized before they reach the gold target and cause an emission of gold from the cathode to the specimen surface at the anode This process causes a violet light in the jar. The particles collide and are thereby slowed down Thus the vacuum decreases and is kept at 0 1–0 2 torr by a minimal influx of argon through a needle valve The argon influx triggers the electric current that should be maintained at about 10 mA during sputtering. By these three parameters (voltage, electric current, and sputter time) the thickness of the gold layer can be gaged It should be about 0.15 nm in order to avoid "burying" fine surface detail under the metal coating. The size of the gold grains can be diminished by using gold-palladium targets *(12)*.

Mostly the observation in SEM is performed using secondary electrons In immunohistochemical methods with gold particles bound to receptors or antigens, the detector for back-scattered electrons is used, which gives good contrast between the gold particles and the rest of the cell surface *(14–22)* (Fig 10)

5 For the simultaneous processing of cell cultures for TEM, there are numerous possibilities Cells can be grown in a second Petri dish of the same size but without divisions, and prepared as a floating sheet *(8)* By this method cells are placed in several layers in which they maintain their contacts and are sitting on a thin glycoprotein film, which in cross-sections is visible as a distinct, electron-dense line (Fig 7A). As an example, with this method it was possible to show the formation of collagen fibrils in deep recesses of the cell surface of fibroblasts in confluent cell cultures (Fig 7B)

6. If cells are grown in semisolid gels no cubes can be cut. Therefore, embedding is performed *in situ* in the culture vessel Appropriate cubes are cut after polymerization Further processing is the same as the preparation of cells in gels

7 The main problem in cryomethods is the formation of ice crystals To overcome this, sucrose, dimethyl sulfoxide, or glycerol is added to the culture medium before freezing *(13,23)*. These substances, however, particularly alter the lipid and protein constitution of the cells Therefore freezing methods without cryoprotectants have been developed. Immediate immersion into liquid nitrogen is not useful because the contact of the warm surface bearing the monolayer causes the formation of bubbles. For this reason precooled polished copper plates

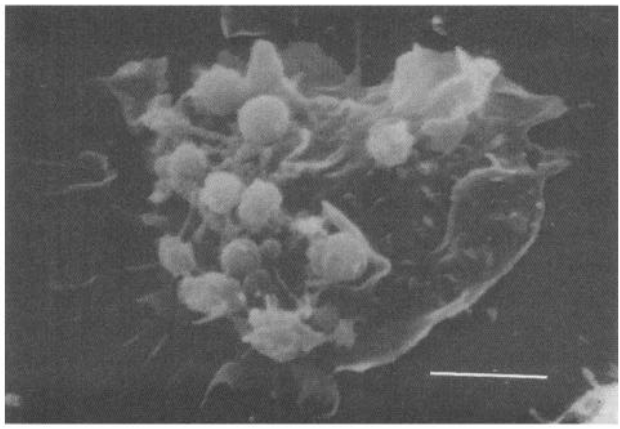

Fig. 10. Macrophage attached to a glass coverslip labeled with a monoclonal antibody against HLA DR (BMA 020) and in a second step with a goat-anti-mouse immunogold probe (▼) (bar = 2 μm).

are commonly used to which the monolayers are rapidly attached *(24,25)*. The procedure described in Method 9 has the advantage that the two complementary parts of the broken cells can be investigated separately. This method is very powerful in combination with immunohistochemical techniques using antibody or protein A-labeled gold particles.

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Adhesion Molecules on Isolated and Cultured Microvascular Endothelial Cells Demonstrated by Immunofluorescence and Immune Electron Microscopy

Josef Neumüller and Johannes Menzel

1. Introduction

In vitro systems of cultured endothelial cells (EC) represent appropriate tools for simulating interactions of cells involved in leucocyte homing or in tumor invasion. Such processes require the adhesion of cooperating cells via receptors classified as adhesion molecules (AM). EC equipped with AM are able to interact with other cells that bear counter-receptors (cell–cell communications) or with molecules of the extracellular matrix (ECM; cell–matrix communications). AM are routinely demonstrated in cell cultures or tissue sections by immunofluorescence or immunohistochemistry at the light- or electron-microscopical level.

In addition cell–cell and cell–matrix interactions can also be provided by carbohydrate moieties at the cell surface. Carbohydrates on the cell surface occur either as single or as composite chains, attached to the cell surface (e g., hyaluronan) via specific receptors *(1,2)*. The glycosylation of proteins takes place in the Golgi complex. In pathological situations sugars can also be bound to proteins by nonenzymatic glycation generating the advanced glycosylation end products (AGE) moieties *(3)*. AM or carbohydrates on the cell surface can be demonstrated in cell cultures or tissue sections by lectins or antibodies, labeled with fluorochromes, enzymes, or gold compounds.

1.1. Endothelium and Endothelial Cell Cultures

The endothelium of the human blood and lymphatic vascular system comprises an area of 1000 m^2 *(4)*. Since EC represent not only the antithrombogenic

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

inlay of the vascular bed but play an important role in the active or passive uptake of substances essential for the survival of tissues of surrounding organs as well as in the interaction with cells of the blood stream, they exhibit a significant morphological and functional heterogeneity. In the adult, resting EC form a highly heterogeneous cell population that varies not only in different organs but also in different vessel calibers within an organ. EC can either form a tight, continuous monolayer in organs where they perform important barrier functions (e.g., in the brain or the lungs), or they can form a discontinuous layer of cells with intracellular gaps or intracellular fenestrae *(5)*. Whereas the endothelium of large vessels is involved in the control of vasoconstriction and vasodilation, blood pressure, and other physiological parameters, the microvascular endothelium provides the blood–tissue exchange of oxygen and nutrients. Above all, in contrast to the endothelium of large vessels that of the microvasculature is able to promote neovascularization and interact with pericytes or smooth muscle cells in the vessel wall *(6,7)*. A special variant of EC with high-prismatic cells is represented in the high endothelial venules (HEV) of lymph nodes and Peyer's patches. The main function of the HEV consists in the recruitment and recirculation of lymphocytes *(8,9)*. The activation of EC during the course of inflammation can lead to a transition from flat to high-prismatic EC where adhesion and transmigration processes also take place as they do in HEV *(10,11)*

The expression of AM differs significantly depending on the origin of the endothelium. Even in endothelium from the same organic site it can change significantly either permanently or transitorily because of inflammatory processes *(10–26)*.

The tremendous increase in investigations concerning the modulation of EC function by cytokines and by the composition of the ECM has encouraged the development of EC cell culture techniques *(27,28)*. It was possible to obtain a scaling up of EC by isolation and culturing of human umbilical vein EC (HUVEC, *29*) or human arterial EC *(30)*. However, despite atherosclerotic lesions in the aorta, EC from large vessels are not involved in inflammatory processes in vivo. Such events occur within microvascular EC (MEC), predominantly in postcapillary venules. In this respect, the isolation and culture of MEC can be helpful in the introduction of in vitro models for the study of homing and transmigration of inflammatory cells. Such models allow investigations about the regulation of the cell surface expression of AM by cytokines *(18,20,24,26,30–37)* and about the interaction of EC with the ECM *(38–54)*. However, it is very important to mention that the isolation of MEC induces a significant change in the natural environment of the cells. Otherwise EC in vivo are also organized in monolayers covering the basement membrane of the vessels. Therefore the EC in in vitro models should be seeded on basement

membrane equivalents such as Matrigel® (a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma) collagen type IV, laminin, or fibronectin *(32,35,42,44,47,55)*. Nevertheless, EC can also be successfully cultivated on gelatin or poly-L-lysine-coated culture vessels. For immunofluorescence or immunohistochemical preparations, an inoculation of MEC onto poly-L-lysine coated LabTek chambers can be recommended There is a strong evidence that matrix proteins highly affect the growing behavior of EC. For instance, the cultivation of MEC in gels (e.g., Matrigel®) can result in the formation of a vessel-like cell assembly *(55)*.

MEC from human, bovine, sheep, rabbit, rat, and mouse have been cultivated from brain *(56–60)*, retina *(61–64)*, skin *(65)*, lung *(66–68)*, intestinum *(69)*, heart *(70–72)*, liver *(73)*, corpus luteum *(74,75)*, meniscus *(76)*, and synovium *(77)*. Since investigations are performed in the field of rheumatology in our laboratory, techniques appropriate for isolating, cultivating, and immunostaining synovial microvascular EC (SMEC) are described in this chapter. These techniques can however, be easily adapted for other MEC cultures (for reviews *see* refs. *78–81*)

1.2. Adhesion Molecules

AM are subdivided into six classes: integrins, molecules of the immunoglobulin superfamily, selectins, mucin-like AM, cadherins, and other AM *(13,15,16,21, 82)*. The family of integrin molecules (Table 1; for reviews *see* refs. *83–86*) is of ancient origin and has been highly conserved during evolution. Subfamilies of integrins are characterized by a common β subunit (β_1 = CD29, β_2 = CD18, β_3 = CD61 and β_4 = CD104) which is linked to the α subunit by noncovalent binding. So far at least eight different β and 14 α subunits are known. Each β subunit can be combined with any of the α subunits. Both subunits are characterized by three domains: a short intracellular domain, a short transmembrane domain, and a long extracellular domain. A ligand recognition motif for several, but not all, integrins provides the binding of peptides containing the amino acid sequence Arg-Gly-Asp (RGD). The α subunits possess three or four tandem repeats of a putative divalent cation binding motif and require Ca^{2+} and Mg^{2+} for ligand binding and conformation changes occurring after activation. Although the $\alpha_4\beta_7$ integrin is known to be a "mucosal homing receptor," the demonstration that it also recognizes VCAM-1 and FN (as does $\alpha_4\beta_1$) suggests that it might act at sites of inflammation. The second β_7 integrin $\alpha_E\beta_7$ is the first receptor shown to recognize E-cadherin *(87)*.

AM of the immunoglobulin superfamily (Table 2) are characterized by a number of repeats of immunoglobulin-related domains and, in many cases, by several membrane proximal repeats of a second type of protein module known as fibronectin type III repeat Such repeats are present not only in matrix mol-

Table 1
Integrins and Their Ligands

AM	CD nomenclature and synonyms	Distribution	Ligand	CD nomenclature and synonyms	Interacting with cells or matrix
Integrins					
$\alpha_1\beta_1$	CD49a/CD29 VLA-1	All EC	Collagen Laminin	—	ECM
$\alpha_2\beta_1$	CD49b/CD29 VLA-2	All EC	Collagen Laminin Fibronectin	—	ECM
$\alpha_3\beta_1$	CD49c/CD29 VLA-3	All EC	Collagen Laminin Fibronectin Epiligrin	—	ECM
$\alpha_4\beta_1$	CD49d/CD29 VLA-4	Activated EC	Fibronectin VCAM-1	— CD106	Lymphocytes
$\alpha_4\beta_7$			VCAM-1	CD106	Lymphocytes
$\alpha_5\beta_1$	CD49e/CD29 VLA-5	All EC	Fibronectin (CS-1) RGD-motivs	—	ECM
$\alpha_6\beta_1$	CD49f/CD29 VLA-6	All EC	Laminin	—	ECM (basement membrane)
$\alpha_7\beta_1$	CD49g/CD29 VLA-7	All EC	Laminin	—	ECM (basement membrane)
$\alpha_E\beta_7$	—	—	E-cahedrin	—	—

Table 2
Adhesion Molecules of the Immunoglobulin Superfamily

AM	CD nomenclature and synonyms	Distribution	Ligand	CD nomenclature and synonyms	Interacting with cells or matrix
ICAM-1	CD54	All EC	$\alpha_L\beta_2$	CD11a/CD18 LFA-1	Lymphocytes, monocytes, granulocytes
ICAM-1	CD54	All EC	$\alpha_M\beta_2$	CD11b/CD18 CR3	Macrophages
ICAM-1	CD54	All EC	$\alpha_X\beta_2$	CD11c/CD18 gp 150/95	Monocytes, NK-cells, dendritic cells
VCAM-1	CD106	Act EC	Fibronectin with CS-1	—	ECM
$\alpha_4\beta_1$	CD4d/CD29	Act EC	VCAM-1	CD106	Lymphocytes

Table 3
Selectins

AM	CD nomenclature and synonyms	Distribution	Ligand	CD nomenclature and synonyms	Interacting with cells or matrix
E-selectin	CD62 E ELAM-1 LECAM-1	Activated EC	Sialyl Lewis^X	CD15s	Lymphocytes
P-selectin	CD62 P PADGEM LECAM-3	Activated EC	Sialyl Lewis^X	CD15s	Lymphocytes, platelets

ecules but also in cytokine receptors. The binding to corresponding counter-receptors is independent of divalent cations *(82)*. EC express ICAM-1 with five immunoglobulin-like domains and ICAM-2 with two of such domains. Both of them bind to the integrin LFA-1 (CD11a, CD18) on lymphocytes *(13)*. In contrast to ICAM-2, ICAM-1 is strongly upregulated by the action of the cytokines IFN-γ, IL-1, and TNF-α. In this respect these ADM play an important role in the homing of lymphocytes at the site of the microvasculature of inflamed tissues *(15–21)*. Another AM, VCAM-1 (CD106) appears on cytokine- or lipoplysaccharide (LPS)-activated EC. Because of alternative splicing VCAM-1 may contain either six or seven immunoglobulin-like domains of the H type *(21)*. Like the ICAMs, VCAM-1 also contributes to the homing process by the interaction with the counter-receptor VLA-4 (CD49d/CD29), expressed on T-lymphocytes but binds additionally to fibronectin *(15,19,21)* These interactions are most likely responsible for the accumulation of $CD8^+$ T memory cells at sites of inflammation and important for the regulation of T cell immune responses *(21)*.

1 Selectins (Table 3, for reviews *see* refs *88–92*) are characterized by variable numbers (from 2–9) of complement regulatory repeats, an epithelial growth factor-like domain, and an amino-terminal lectin domain As is the case for lectins, their natural ligands are carbohydrates Their binding to selectins is provided by the *N*-terminal lectin-like domain Based on their cell-surface expression, selectins on EC, leucocytes, or platelets are termed E-, L-, or P-selectins respectively
2 Mucin-like adressins (Sialomucins, Table 4) Recently four members of this new family of AM have been cloned GlyCAM-1, MAdCAM-1, BSGL-1, and CD34 *(93–96)* These molecules are characterized by regions, rich in *O*-linked sugars MAdCAM-1 consists of two *N*-terminal domains with homology of the IgG superfamily members, ICAM-1 and VCAM-1, a mucin-like domain and an IgA-like domain Thus this ligand supports both L-selectin binding via its mucin-like domain and $\alpha_4\beta_7$ binding via its *N*-terminal immunoglobulin-like domains
3. Cadherins are predominantly involved in homotypic cell interactions *(97)*. All cell types that form solid tissues express some members of the cadherin molecule family. They are also thought to play an important role in tumor invasion These molecules are also present on EC *(98)*
4 Further AM on EC (Table 5) A vascular adhesion protein 1 (VAP-1) was recently described as a 90-kDa adhesion protein expressed in human synovium *(99)* In contrast to E- and P-selectin, VCAM-1, ICAM-1, and ICAM-2, VAP-1 is not found on unstimulated or LPS- or cytokine-activated HUVEC. An additional putative 70-kDa AM for lymphocytes (L-VAP-2) has been identified Its constitutive expression on HUVEC cannot be upregulated by cytokines *(100)* A CD44-like EC transmembrane glycoprotein (GP116) interacts with ECM molecules (e g , hyaluronan) and ankyrin Since it binds a wheat germ agglutinin binding site one can assume that it has an extracellular domain It is strongly suggested

Table 4
Mucin-Like Adressins

AM	CD nomenclature and synonyms	Distribution	Ligand	CD nomenclature and synonyms	Interacting with cells or matrix
—	CD34	EC	L-selectin	CD62L MEL-14	T-cells, neutrophils, eosinophils, monocytes
GlyCAM	—	HEV	L-selectin	CD62L MEL-14	T-cells, neutrophils, eosinophils, monocytes
MAdCAM-1	—	Venules of the mucosa	L-selectin	CD62L MEL-14	T-cells, neutrophils, eosinophils, monocytes
MAdCAM-1	—	Venules of the mucosa	$\alpha_4\beta_1$	CD49d/CD29 VLA-4	Lymphocytes, neutrophils,
BSGL-1	—	Venules of the mucosa	P-selectin E-selectin	CD62P CD62E	Lymphocytes, neutrophils

Table 5
Further Adhesion Molecules

AM	CD nomenclature and synonyms	Distribution	Ligand	Interacting with cells or matrix
VAP-1	Vascular adhesin molecule-1	EC	?	Lymphocytes
L-VAP-2	Vascular adhesin molecule-2	EC	?	Lymphocytes
GP116	CD44-like adhesion molecule	EC	Proteoglycans of the basement membrane	ECM

that phosphorylation of this molecule by protein kinase C is required for effective interaction with ankyrin during EC adhesion events *(101)*

1.3. Immunolabeling of AM on the Surface of EC

1 3.1. Immunofluorescence

The demonstration of AM by using fluorolabeled monoclonal antibodies (MAbs) provides significant advantages in comparison to other methods such as immunoenzyme histochemistry. If the cell cultures to be stained are kept at nearly 10°C (in order to avoid endocytosis of the fluorochrome-conjugated MAb), the immunolabeling can be performed when cells are still alive. Using different fluorochromes (fluorescein-isothiocyanate [FITC], phycoerythrin [PE], and tetramethyl-rhodamin-isothiocyanate [TRITC] are commonly applied) a multiple simultaneous staining can be performed in one step (direct immunofluorescence). For this method one needs a fluorescence microscope (for working with cell cultures an inverted microscope is recommended), equipped with appropriate excitation filters, dichroic mirrors, and barrier filters. The aforementioned fluorochromes exhibit the following physical characteristics in respect to their mean excitation wavelength (EXW) and their mean fluorescence emission wavelength (FEW):

1 FITC· EXW, 492 nm (blue light); FEW, 515 nm (green fluorescence).
2. PE EXW, 490 nm (blue light), FEW, 575 nm (orange fluorescence)
3. TRITC: EXW, 549 nm (green light); FEW, 574 nm (red fluorescence) *(102)*

The direct immunofluorescence on living cells makes sure that the conjugate binds only to the cell surface AM and is not allowed to enter the cytoplasm. In relation to the amount of antigens at the cell surface, the primary antibody should be applied in excess. The membrane fluorescence demarcates the cell and appears very faintly in the projection of a cell

If one is interested in the cytoplasmic pool of AM, the EC can be fixed briefly with acetone, precooled to –20°C, before immunolabeling. In contrast to immunohistochemistry that uses enzyme-conjugated MAb, there is no need for inactivation of endogenous enzymes. By this technique either the whole cytoplasm or the perinuclear region is stained. At the site of the nucleus the fluorescence appears weaker.

If the number of receptor molecules at the cell surface or the affinity of the respective MAb are accidentally low, the fluorescence signal can be insufficient (above all, when a photodocumentation is requested). In such cases an indirect immunofluorescence has to be carried out where the EC are incubated with an unlabeled primary antibody, washed with buffer, and incubated again with a xenogenic fluorolabeled second antibody, directed against the immunoglobulin species of the primary MAb. Usually the second antibody is poly-

clonal. Unfortunately the indirect fluorescence has several disadvantages: a multiple staining can only be obtained if the primary MAb differ in respect to their species- or at least their class-specificity and appropriate second antibodies can be found. Furthermore, the multiple wash procedures after the incubation of EC with antibodies can induce the loss of larger parts of the EC monolayers, above all, if the cells are in a confluent stage. This problem can be diminished by coating the growing support of the cultures with poly-L-lysine.

1.3.2. The Demonstration of AM by Using Electron Microscopy

The investigation of cell surface antigens by electron microscopy allows the determination of their exact localizaton and distribution AM are usually expressed more strongly at the apical than at the basal aspect of the cell membrane. They can be arranged in clusters or be spread uniformly. In addition, AM may be either present or not present along cell contacts. All these aspects can be revealed best by electron microscopy although the introduction of laser confocal microscopy has opened up new possibilities for their demonstration.

Living EC can be immunolabeled using peroxidase-conjugated antibodies *(59,103)* or with immunogold preparations *(103,104)* in direct or indirect procedures. As was mentioned in the explanation of the immunofluorescence technique, the cultures have to be kept at about 10°C to avoid phagocytosis of gold particles These particles are available at different sizes (a range of 10–20 nm particles is suitable for the immunolabeling of AM on EC).

2. Materials

2.1. Reagents for Cell Culture and Other Additives

1 EC growth medium (EGM; Promacell, Heidelberg, Germany) supplemented with 50 µg/mL endothelial cell growth factor (ECGF) heparin, 1 µg/L recombinant human fibroblast growth factor (FGF), 100 ng/L recombinant human epidermal growth factor (EGF), 1 mg/L hydrocortisone and 100 mg/L gentamicin + 2 5 mg/L amphotericin B

2 Paramagnetic beads·
   a. Dynabeads® M-450 (goat-antimouse-IgG labeled, Dynal, Oslo, Norway), additionally coated with CD31 (Immunotech, Marseille, France),
   b. Dynabeads® M-450 (tosyl-activated, Dynal), coated with ulex europaeus lectin-I (UEA-1, Vector Laboratories, Burlingame, CA);
   c Dynabeads® M-280 (streptavidin-coated), additionally labeled with biotinylated UEA-1 (Vector Laboratories).

3 Poly-L-lysine: Sterile stock solution of 100 µg/mL poly-L-lysine (hydrobromide, MW: 300,000)

4. Gelatin solution, 2%, type B, from bovine skin, endotoxin-free (Sigma-Aldrich, Vienna, Austria).

5 Collagenase solution EGM medium + 0.15% collagenase type XI.

6. 200 µg/mL Dil-Ac-LDL (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Biomedical Technologies, Stroughton, MA)
7. Phosphate-buffered saline (PBS) buffer solution, pH 7.2–7.4: 0.14*M* NaCl, 2.7 m*M* KCl, 0.9 m*M* KH_2HPO_4, 6.4 m*M* Na_2PO_4 (anhydrous).
8. Hank's solution, pH 7.3: 0.14*M* NaCl, 5.4 m*M* KCl, 0.4 m*M* $MgSO_4$, 1.3 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 0.3 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4, 5.1 m*M* glucose, adapted to pH 7.3 with $NaHCO_3$ solution
9. Hank's/bovine serum albumin (BSA) solution: Hank's solution + 2% BSA
10. Hank's/fetal calf serum (FCS) solution: Hank's solution + 5% FCS
11. EDTA-PBS I: EDTA (ethylene-dinitrilo-tetra-acetic acid disodium salt) buffer, pH 7.3: Basic salt solution (0.14*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, 1.06 m*M* glucose) complemented with 0.54 m*M* EDTA or 0.4 m*M* EDTA
12. EDTA-PBS II: Basic salt solution complemented with 0.2 m*M* EDTA

2.2. Reagents for Fixation, Embedding, and Electron Microscopy

1. Cacodylate buffer, pH 7.3: 0.1*M* Na-cacodylate, 0.15*M* saccharose.
2. Fixative for the immunofluorescence preparation protocols: Hank's solution, pH 7.3, +1% paraformaldehyde
3. Fixative for the electron microscopical preparation protocols (modified Karnovsky fixative), pH 7.3: Cacodylate buffer complemented with 0.1% glutaraldehyde and 1% paraformaldehyde
4. Postfixation reagent for the electron microscopical preparation protocols: Cacodylate buffer complemented with 2.5% glutaraldehyde
5. Tris HCl/sucrose buffer, pH 7.4: 0.05*M* Tris HCl, 0.22*M* saccharose
6. Tris-HCl/sucrose/BSA buffer, pH 7.4: 0.05*M* Tris-HCl/sucrose/buffer + 2% BSA.
7. Tris-HCl/glycine buffer, pH 7.5: 0.05*M* Tris, 0.1*M* glycine
8. DAB (diaminobenzidine-tetrahydrochloride)/Tris/sucrose buffer: Tris/sucrose buffer + 5.55 m*M* DAB
9. DAB/Tris/sucrose/H_2O_2 buffer: DAB/Tris/sucrose buffer + 14.9 m*M* H_2O_2
10. Immunogold reagents
    a. Auroprobe™, EM streptavidin, G15 (15 nm gold particles)
    b. Auroprobe™, EM GAM-IgG (goat-antimouse-IgG), G15 (15 nm gold particles), (both Amersham, Little Charfont, UK)
11. Fluorostab (Euro-Diagnostics, Apeldoorn, The Netherlands)
12. Spurr Low Viscosity Resin (Polysciences, Warrington, PA)

3. Methods

3.1. Isolation and Cultivation of SMEC (77,104)

1. Prepare gelatinated 50-mL polystyrene flasks. Add 0.7 mL sterile gelatin solution to the flasks, close flasks, and incubate them for 24 h at 4°C. After removal of the liquid phase the flasks are ready for use.
2. Collect synovial biopsies (appropriate size: 4–25 cm^2) in 25-mL sterile polystyrene tubes in complete EGM medium

3 Transfer the tissue to a plastic Petri dish (10-cm diameter) and dissect the vascularized synovium from the collagen rich tissue of the joint capsule in a sterile Petri dish by using scissors
4 Transfer the pieces to a further Petri dish of the same size which contains the cutting table of the tissue chopper
5 Adjust the McIlwain chopper to generate 25-µm tissue fragments. After finishing the first cutting series turn the cutting platform by 90° and start a second cutting series in order to obtain cubic fragments
6. Transfer the tissue fragments to a 25-mL polystyrene tube filled with 20 mL collagenase solution
7 Incubate the tubes at 37°C for 1 h, shaking them every 10 min to provide an appropriate infiltration of the tissue
8 Place a sterile filter holder with a 40 mesh stainless steel sieve over a Petri dish and filter the suspension with the digested tissue Squeeze the fragments that remain on top of the sieve by using a sterile rubber policeman (This procedure is important in order to press out the SMEC from the microvessels)
9 Wash the cells twice with Hank's/FCS by centrifugation of the tubes for 10 min at 350*g* Resuspend the cells with 10 mL EGM medium complete with all supplements and transfer to gelatinated culture flasks
10 After 1 h, gently shake the flasks and remove the nonadherent cells by changing the medium
11 Incubate the cells in a CO_2 incubator for about 1 wk and after reaching confluency change the culture medium every 3 d

3.1.1 Immunomagnetic Isolation of CD31$^+$ Cells (see Note 1)

1 Incubate paramagnetic beads coated with sheep-antimouse IgG, with MAb against CD31 (0 2 g/bead) for 30 min at 4°C (*see* Note 2)
2 Wash the beads three times with Hank's/FCS using the magnet particle concentrator (MPC)
3. Detach the cells of the primary culture containing SEC by incubation with 5 mL of EDTA/PBS-I prewarmed to 37°C for 5–10 min and incubate them with paramagnetic beads (*see* Note 1).
4. Wash the detached cells with EDTA/PBS-II for 5 min at 400*g*
5 Resuspend the cells in 5 mL EDTA/PBS-II, add the pre-estimated number of paramagnetic beads and incubate this mixture for 5 min at 4°C.
6 Remove the unrosetted cells by washing 3 times with EDTA/PBS-II using the MPC
7. Mix the rosetted cells with 5 mL of complete EGM medium and transfer this suspension to gelatin-coated culture flasks
8 Change the medium after 24 h and subsequently every 3 d The cultures remain stable for at least 3 passages in respect to their constitutive intracellular expression of von Willebrand factor (vWF) and their surface expression of fucosyl residues or AM-like CD31 and ICAM-1 and 2.

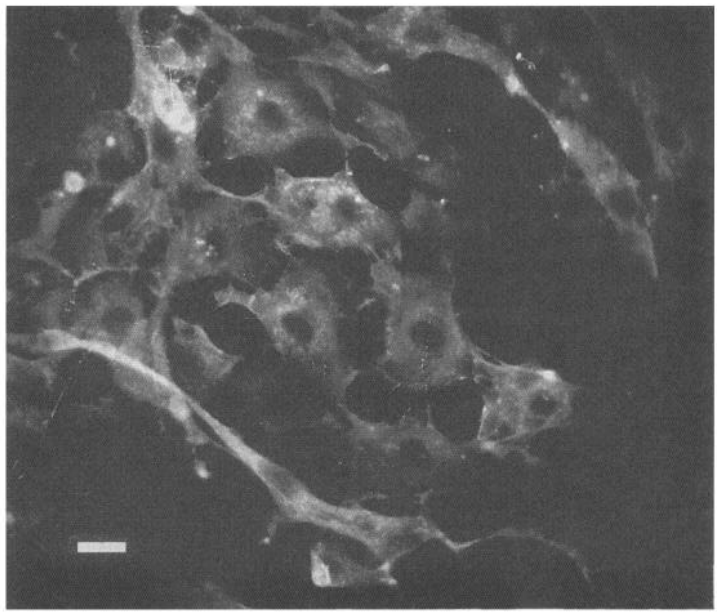

Fig. 1. Fluorescence microscopical characterization of SEC by their uptake of Dil-Ac-LDL.(EXW: 546 nm, FEW: 590 nm, bar = 30 μm).

3.2. Immunostaining Protocols

3.2.1. Ascertainment of the EC Phenotype

3.2.1.1. By Fluorescence Microscopy Using Dil-Ac-LDL

1. After reaching preconfluence add 1 mg/mL Dil-Ac-LDL (*see* Section 2.1., item 6) to the culture medium (Fig. 1) *(65,66,68,71,72,77,78,105,106)*.
2. Identify the EC after 24 h of incubation by using the fluorescence microscope (excitation: 546 nm; *see* Note 3). The EC are characterized by their distinct perinuclear red fluorescence (Dil-Ac-LDL is ingested via the scavenger receptor by EC and macrophages but not by fibroblasts).

3.2.1.2. By Demonstration of the vWF-Related Protein

Fix the cells with precooled acetone and use indirect immunofluorescence as described in the next section (Fig. 2) *(6,28,41,63,65,69,71,72,73,76–78,106)*.

3.2.2. Preparation of EC for Investigations Using Immunofluorescence (Figs. 2–4)

1. Coat eight compartment LabTek chambers with poly-L-lysine: Dispense about 150 μL of a sterile poly-L-lysine solution in each compartment of the chamber. After 10 min remove the liquid using a transfer pipet with a 200-μL tip and allow

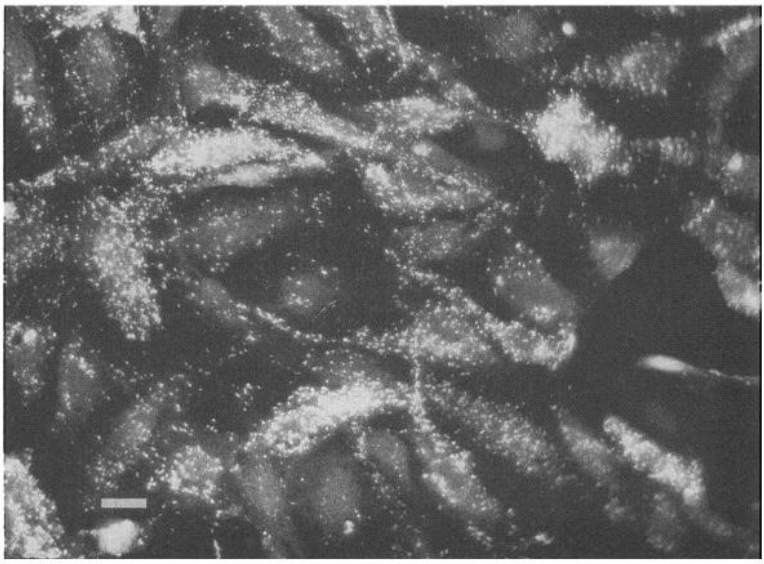

Fig. 2. Fluorescence microscopical identification of SEC by intracytoplasmatic demonstration of vWF using a FITC-conjugated polyclonal antibody (The Binding Site, Birmingham, UK) (EXW: 450-490 nm, FEW: 520 nm, bar = 20 μM).

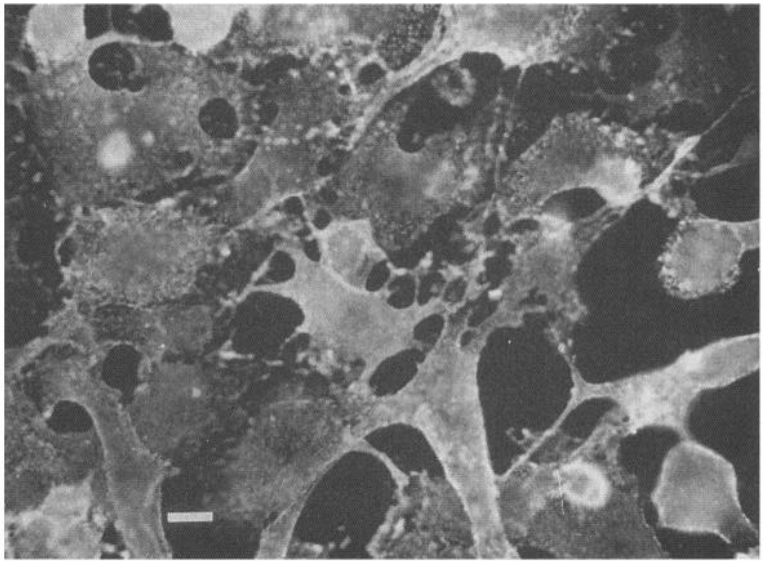

Fig. 3. Fluorescence microscopical demonstration of ICAM-1(CD54), present at the cell surface as well as intracellularly in LPS activated and acetone-fixed SEC using a FITC-conjugated MAb (clone: 84H10, Immunotech) (EXW: 450-490 nm, FEW: 520 nm, bar = 20 μM).

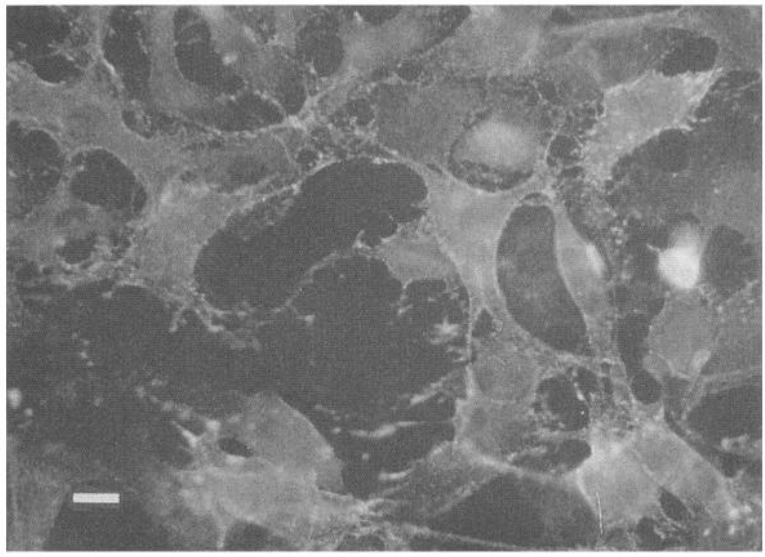

Fig. 4. Fluorescence microscopical demonstration of ICAM-1 (CD54), present only at the cell surface in LPS-activated, but unfixed SEC using a FITC-conjugated MAb (clone: 84H10, Immunotech), (EXW: 450-490 nm, FEW: 520 nm, bar = 20 μm).

the protein film to dry for 30 min. Wash the compartments briefly with distilled water and remove the liquid. The chambers are now ready for use.

2. Plate in every compartment 5×10^4 SEC/400 μL complete EGM medium. Usually the cells adhere within 1 h and form clusters. At this stage the cells are not connected to each other and do not express the typical cobblestone-like morphology.
3. After 2 d of incubation change the medium and wait for a further 2–3 d until confluence has been reached.
4. Activate the cells with thrombin, LPS, or with cytokines of interest, for an appropriate time (usually between 3 and 24 h, *see* Note 4).
5. Fix the cells with 1% paraformaldehyde (prewarmed at 37°C) for 30 min if the AM is to be demonstrated on the cell surface or with 70% acetone (precooled at –20°C) for 30 min if the AM is to be demonstrated intracellularly; or do not fix but cool the cells to 10°C if the AM containing aldehyde-sensitive epitopes are to be demonstrated on the cell surface. (Work very carefully avoiding agitation of cells as much as possible since under this condition the monolayers like to detach from the growing support.)
6. Replace the medium of every compartment with 400 μL of Hank's/BSA containing 0.2 mg/mL mouse immunoglobulin in order to block nonspecific binding sites.
7. Replace this solution with 200 μL of a 1:20 diluted (with Hank's BSA) unconjugated or fluorolabeled primary antibody against the AM of interest (0.2 mg/mL).

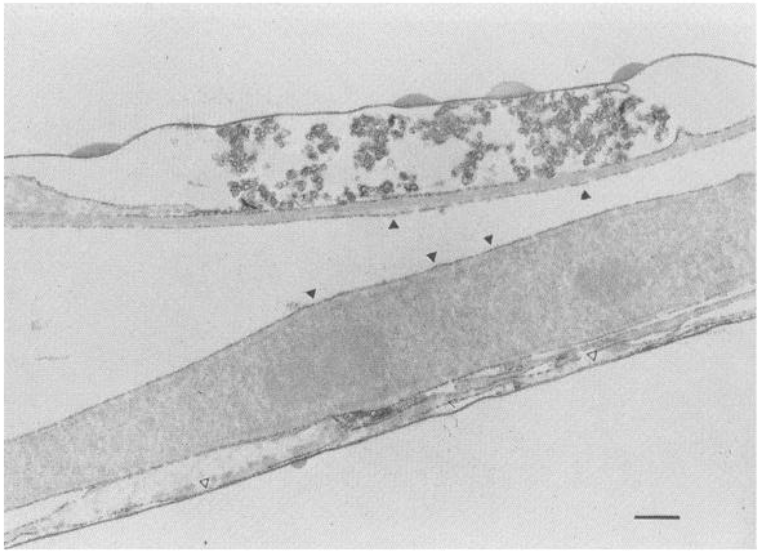

Fig. 5. Immune electron microscopical demonstration of fibronectin (FN) bound to the cell surface of SEC (arrowheads) using a primary monoclonal antibody against FN (clone FN15, Sigma, St. Louis, MO, USA) and a secondary peroxidase-conjugated polyclonal goat-antimouse antibody (Immunotech). The SMEC were not contrasted with uranyl aceatate or lead citrate (bar = 0.7).

8. After 30 min of incubation at room temperature in the dark, remove the primary antibody by carrying out three wash steps (5 min each) with Hank's/BSA.
9. If the primary antibody was used as a fluorolabeled conjugate (direct immunofluorescence method) perform the last wash step using Hank's without BSA. Remove the plastic part of the chambers and postfix with 70% ethanol. Change the ethanol after 30 min incubation at 4°C. Mount the slides in Fluorostab using a large coverslip and seal them with silicon grease.
10. For the indirect immunofluorescence method incubate the cells with 200 µL of 0.2 mg fluorolabeled antimouse immunoglobulin from rabbit or from goat, diluted 1:50 in Hank's/BSA (*see* Note 5).
11. After 30 min of incubation carry out the last washing, postfixation, and mounting procedures as described for direct immunofluorescence.
12. Keep the cells at 4°C until shortly before the observation of the immunostaining of EC under the fluorescence microscope but allow the slides to reach room temperature (approx 10 min).

3.2.3. Immune Electron Microscopy (Preembedding Methods)

3.2.3.1. Enzyme Immunolabeling Methods (Figs. 5–7)

1. Prepare poly-L-lysine-coated LabTek chambers with EC monolayer as described in Section 3.2.2.5., step 1.

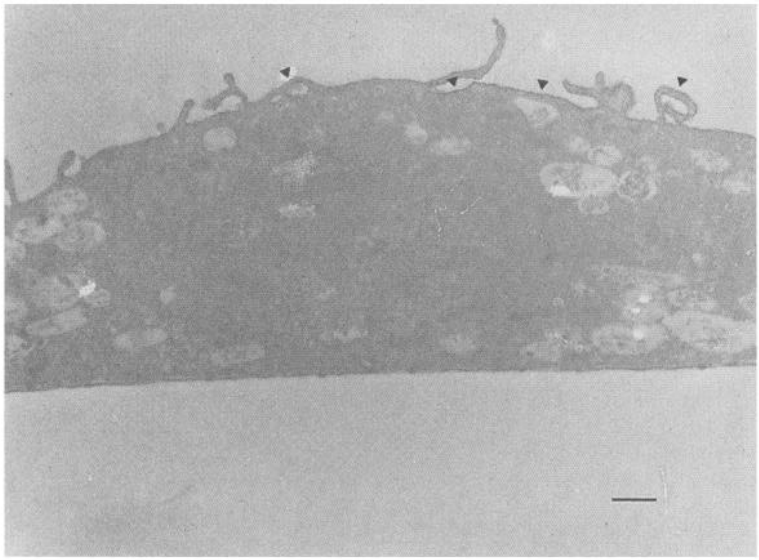

Fig. 6. Immune electron microscopical demonstration of VCAM-1 (CD106) bound to the cell surface of LPS-activated SEC (solid arrowheads) and to the growing support (empty arrowhaeads) using a primary MAb against this antigen (clone: 1G11) and a secondary peroxidase-conjugated polyclonal goat-antimouse antibody (both antibodies from Immunotech). The SMEC were not contrasted with uranyl aceatate or lead citrate (bar = 0.7 μm).

2. Remove the culture medium from each compartment and fix the EC with modified Karnowsky fixative (prewarmed to 37°C) for 10 min. Change the fixative (precooled to 4°C) and fix the cells for a further 30 min. Carry out the following preparation steps at 4°C.
3. Wash the EC twice with Tris-HCl/glycine buffer (each step 5 min) in order to quench aldehyde-induced nonspecific binding sites.
4. Wash the EC three times with 0.15*M* Tris-HCl/sucrose buffer (each step 5 min).
5. Block the endogenous peroxidase activity with 3% H_2O_2 in pH 7.4 Tris-HCl/sucrose buffer.
6. Wash the EC twice with Tris-HCl/sucrose/BSA buffer (each step 5 min).
7. Replace this solution with 200 μL of an 1:20 diluted (with 0.15*M* Tris-HCL/BSA) unconjugated or conjugated primary antibody against the AM of interest (2 mg/mL). Incubate for 30 min.
8. Wash the EC three times with Tris-HCl/sucrose/BSA buffer (each step 5 min).
9. Incubate the cells with 200 μL of an 1:50 diluted (with 0.15*M* Tris-HCL/BSA) peroxidase-labeled antimouse immunoglobulin from rabbit or goat for 30 min.
10. Remove the plastic part of the LabTek chamber from the slide and wash the EC three times with Tris-HCl/sucrose buffer (each step 5 min).

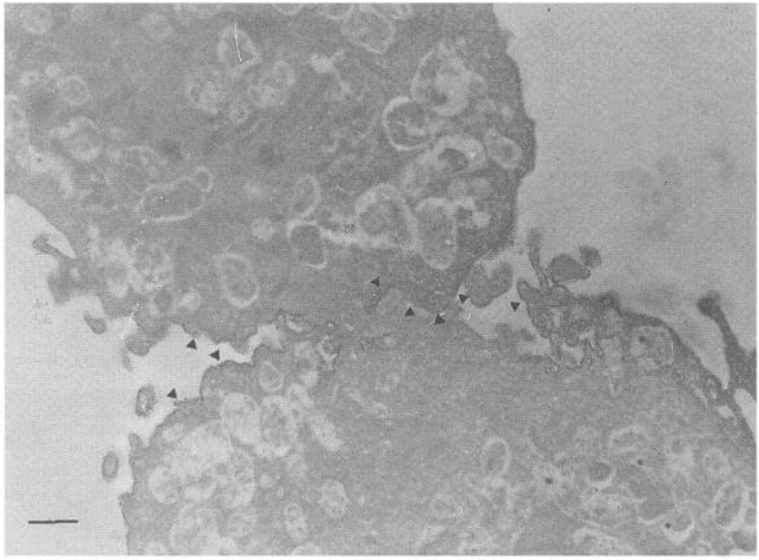

Fig. 7. Immune electron microscopical demonstration of CD31 bound to the cell surface of activated SEC using a primary MAb against this antigen (clone 5.6E) and a secondary peroxidase-conjugated polyclonal goat-antimouse antibody (both antibodies from Immunotech). The SMEC were not contrasted with uranyl aceatate or lead citrate. Note: the concentration of this ADM at the site of cell contacts (arrowheads; bar = 0.4 μm).

11. Preincubate the EC with DAB/Tris-HCl/sucrose for 5 min.
12. Incubate the EC with DAB/Tris-HCl/sucrose + H_2O_2 for 15 min.
13. Wash the EC three times with Tris-HCl/sucrose buffer (each step 5 min).
14. Postfix the EC in 1% OsO_4 in cacodylate buffer for 90 min. This step can be omitted if the staining of the AM appears very weak.
15. Wash the EC twice with cacodylate buffer (each step 10 min).
16. Incubate the slides in increasing ethanol concentrations (30, 50, 60, 70, 80, 90, and 95%), twice in absolute ethanol and finally in propylene oxide for 10 min each.
17. Cover the slides with the following mixture ratios of propylene oxide (PO) and Epon 812 (each step at least 3 h but no longer than 24 h at 4°C in a dessicator): 75% PO/25% Epon 812; 50% PO/50% Epon 812; 25% PO/75% Epon 812, followed by two incubation periods with undiluted Epon 812 at room temperature. For changing the resin dilutions dip the slides with one corner on a filter paper and cover them with the next higher dilution of resin.
18. Cover the EC of every compartment with inverted beem capsules completely filled with pure Epon 812 and press slightly on the top of the capsules to ensure that the aperture of the capsule is in contact with the upper surface of the slide.
19. Polymerize the resin for 24 h at 70°C.

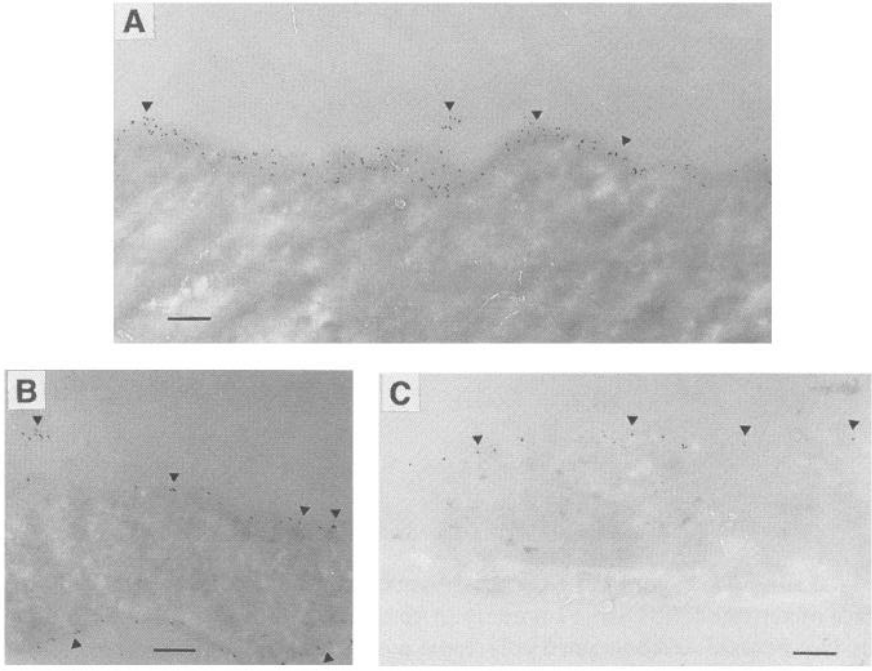

Fig. 8. (A–C) Immune electron microscopical demonstration of ELAM-1 (CD62E) expressed at the cell surface of activated SEC (arrowheads) using a primary MAb against this antigen (clone: 1.2B6, Immunotech) and a secondary immunogold-conjugated polyclonal goat-antimouse antibody (G15, Amersham, Little Chalfont, UK). Sections were obtained by planparallel cutting (**A** and **B**, or by orientation and reembedding, **C**).The SMEC were not fixed with OsO_4 and not contrasted with uranyl aceatate or lead citrate. Note the strong labeling of AM in oblique sections as in **A**. Clear crossections were obtained in cell projections **(B)** and in orientated and re-embedded monolayers **(C)** (bars: 0.4 μm in **A**, 0.7 μm in **B** and **C**).

20. Place the slides on a hot plate (about 80°C) and remove the resin blocks inside the inverted beem capsules by breaking away.
21. Trim the blocks and cut planeparallel sections from the flat surface of the blocks using an ultramicrotome equipped with a diamond knife (*see* Note 6).

3.2.3.2. Immunogold Method (Fig. 8)

1. Prepare LabTek chambers and plate the EC as described in Section 3.2.2, step 1.
2. Remove the culture medium from each compartment and fix the EC with modified Karnowsky fixative (prewarmed to 37°C) for 10 min. Change the fixative and fix the cells for a further 30 min (*see* Note 7).

3 Wash the EC three times with 0 15*M* Tris-HCl/sucrose buffer (each step 5 min)
4 Replace this solution with 0 2 mg/mL of a primary antibody against the AM of interest, diluted 1·20 in 200 µL of 0.15*M* Tris-HCl/sucrose/BSA buffer. Incubate for 30 min
5. Wash the EC three times with Tris-HCl/sucrose/BSA buffer (each step 5 min)
6 Incubate the cells with 200 µL of gold-labeled goat antimouse immunoglobulin, diluted 1 10 with 0.15*M* Tris-HCl/sucrose/BSA buffer for 1 h
7 Remove the plastic part of the LabTek chamber from the slide and wash the EC three times with Tris-HCl/sucrose buffer (each step 5 min).
8. If the cells remained unfixed during the immunolabeling, fix them with 2.5% glutaraldehyde in Tris-HCl/sucrose for 30 min. Change the fixative and fix the cells for a further 1 h Owing to the fixation, the cells adhere firmly to the slides
9 Wash the EC twice with cacodylate buffer (each step 10 min)
10 Postfix the EC in 1% OsO_4 in cacodylate buffer for 90 min.
11 Wash the EC twice with cacodylate buffer (each step 10 min).
12. Carry out the dehydration and embedding procedures as described in Section 3.2.3 1, steps 16–21

4. Notes

1. Usually the rheumatoid synovium contains a remarkable number of leucocytes that express the CD31 antigen. This leads to an unwanted and preferential isolation of these cells by the armed paramagnetic beads In this respect a coating of tosyl-activated beads with Ulex europaeus lectin (UEA-1) or an arming of streptavidin-coated beads with biotinylated UEA-1 can be recommended The coating of the beads as well as the isolation procedure can be performed in the same manner as described in Section 3 1.1 It is very important not to exceed the beads/SMEC ratio since SMEC like to phagocytose the attached beads. An overloading of EC with beads (that cannot be degraded by the cells) inhibits the cell attachment to the growing support and leads to cell death
2. The ratio of beads to SEC should be adjusted to approx 1 5 For this purpose 100 µL of the suspension of detached cells is mixed with 10 µL of paramagnetic beads and centrifuged at 400*g* for 1 min in a 1.5-mL Eppendorf tube The counting of rosetted cells (SEC) and of the cell number of total detached cells allows the calculation of the appropriate number of paramagnetic beads to be added to the detached cells
3 Light microscopy· An inverted microscope with a table that holds clamping devices for 50-mL polystyrene flasks as well as for slides. It should be equipped with a halogen lamp and a condensor for bright field and phase contrast, an epifluorescence system with an HBO lamp and a fluorescence filter pack for blue (480–490 nm wavelength) and green light excitation (546 nm wavelength), and suitable bandpass filters (BP, dichroic mirrors [DM] and long pass filters [LP] in the following configuration. BP450-490, DM510, and LP520) and (BP546, DM580, LP590), as well as a microscope camera for 35-mm films. The use of sensitive films (≥400 ASA) is recommended The objectives should be suitable

for phase contrast and fluorescence with a focus as long as possible and a numeric aperture as high as possible (recommended objective magnifications: 10×, 25×, 40×, and 63×)

4. After inoculum of the primary culture, SMEC can persist in the state of activation that was initiated in vivo because of the inflammatory processes. At this stage SEC express at their surface AM such as CD62E and CD106 Furthermore AM that are constitutively expressed can be upregulated (CD54, CD102, CD29/CD49d and CD29/CD49e) For the demonstration of the differential expression of this in vitro activation of AM α-thrombin, LPS, and several cytokines are useful The optimal concentrations per milliliter culture medium as well as the incubation times that give an activation response in SEC are indicated in the following If a combination of particular cytokines is used their concentrations may be reduced, but an individual dose-optimum curve should be established empirically

   EC activators

   a 1 IU/mL α-thrombin, 3 h of incubation,
   b. 1 ng/mL LPS, 24 h of incubation,
   c. 100 IU/mL (1 IU = 4.367 ng) recombinant human IFN-γ, 24 h of incubation,
   d 200 IU/mL (200 pg/mL) recombinant human interleukin-1α (IL-1α), 24 h of incubation;
   e 20 IU/mL (20 pg/mL) recombinant human interleukin-1β (IL-1β), 24 h of incubation,
   f 1000 IU/mL (10 ng/mL) recombinant human tumor necrosis factor-β (TNF-β), 24 h of incubation;
   g 500 IU/mL (10 ng/mL) recombinant human transforming growth factor-β (TGF-β), 24 h of incubation,
   h. 5 ng/mL recombinant human platelet-derived growth factor (PDGF), 24 h of incubation

5 This concentration provides an excess antibody concentration necessary for immunostaining of surface molecules For immunolabeling of the intracellular distribution of AM an appropriate concentration of the primary and—if necessary—of the second step antibody should be determined by titration

6. In order to avoid damage to the diamond knife during the planeparallel sectioning, the vertical plane of the block along the cutting axis may be adjusted by using a plastic block on which an eyelash has been mounted *(107)* Moving the block allows the adjustment of the same distance of the eyelash at all positions of the cutting plane The ultramicrotome is set to produce 0 5 μm semi-thin sections If a section is obtained that contains cut cells, the microtome is switched to produce 60-nm ultrathin sections. The method requires some practice and it is unfortunately not possible to make sure that all of the sections contain cross sectioned cells over the entire section area. Alternatively the blocks can be re-embedded in resin in such a way that the cutting plane is orientated at 90° to the growing plane of cells This results in good cross sections but the resolution is only one line of few cells

There is a further possibility to obtain more than one line of cells. the preparation of "floating sheets" *(108)*. By this method the EC are grown on 60 cm plastic dishes They are fixed and dehydrated as described above. After the incubation in 100% ethanol, 5 mL of propylene oxide or *N*-butylglycidyl ether is added to the dishes that are placed on top of a white porcelain plate. After 3–5 min the cell monolayer floats up to the surface of the liquid and can be harvested by means of a glass fiber grid (mesh width 0.5 mm) and transferred to flat embedding forms filled with resin. The grid is pressed slightly against the surface of the resin using a glass rod in order to separate the monolayer from the grid. If necessary the cells can be covered by a thin layer of resin before polymerization

7 Alternatively the cells can remain unfixed as described for the immunofluorescence method In this case take care not to detach the monolayer from the growing support.

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34

Production of Heterologous Proteins Using the Baculovirus/Insect Expression System

Caroline M. Griffiths and Martin J. Page

1. Introduction

An important consideration for the expression of cloned genes in recombinant expression systems is the ability of the foreign host to produce the protein faithfully in a form that is similar or identical to that found in the cell type from which the gene was cloned. For eukaryotic proteins, this frequently involves many posttranslational modifications of the protein, such as glycosylation, phosphorylation, processing, and secretory events Additionally, very precise interactions are essential for the correct folding of the polypeptide to achieve the final tertiary structure. If the folding is incorrect, then the molecule will often be biologically inactive.

These considerations have led to the increasing use of recombinant eukaryotic expression systems to express cloned genes accurately. In particular, recombinant mammalian systems have been used extensively to achieve this aim, with considerable success. More recently an alternative higher eukaryotic expression system involving the use of a recombinant baculovirus and insect tissue-culture cells has demonstrated considerable advantages. These include the speed of obtaining expression of the recombinant product, the potential for high yields and large-scale production, and the option of expressing proteins that would otherwise be toxic at high levels in mammalian cells (e.g., c-*myc*, *see* ref. *1*). Examples of recombinant proteins produced using the insect expression system have so far (with some exceptions) shown them to be appropriately modified, processed, secreted, and correctly folded to give high yields of biologically active proteins, such as human interferon *(2,3)*, human Factor VIII *(4)*, tissue-plasminogen activator *(5)*, β-galactosidase *(6)*, c-*myc* *(7)*, interleukin-2 *(8)*, and influenza hemagglutinin *(9,10)*.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

The baculovirus expression system was conceived in the early 1980s *(2,3)*, and has developed into one of the most versatile eukaryotic expression systems to date. The insect baculovirus most widely used for expression of foreign genes in tissue-culture is the prototype baculovirus, *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV). Other baculoviruses have been used, but the cell lines required for such viruses are not so widely available as those used for AcNPV replication. However, if the foreign gene is to be expressed within insect larvae, as opposed to tissue-culture cells, the *Bombyx mori* NPV (BmNPV) is a better choice of vector. The double-stranded circular AcNPV genome is approx 128 kb in size, and replicated within the nuclei of lepidopteran species. The virus exhibits a biphasic life cycle both in the insect host and during tissue-culture conditions. The first phase (10–24 h postinfection) involves the fonmation of mature nucleocapsids that bud through the cellular membrane to form extracellular particles, known as budded virus (BV). These virus particles are infectious to neighbouring cells within the body of the host, or in tissue-culture, and serve to disseminate such an infection. The second phase (24–72 h postinfection) involves high-level expression of a few virus genes, in particular a 28 kDa protein known as polyhedrin. This protein is produced in such amounts that it can account for 50% of the total protein content of an infected cell, and is responsible for embedding the mature virus particles within the cell nuclei, generating very large viral occlusion bodies (*see* Fig. 1). These occluded viruses (OV) are released only after cell death and are essential for the lateral transmission of the virus, protecting the virus particles within the external environment

Several features of this biphasic life cycle make the insect baculovirus system amenable for genetic manipulation. First, the polyhedrin gene is unnecessary for the production of BV, which is infectious for insect cell cultures *(11)* Therefore the polyhedrin gene can be replaced with a foreign gene, so that it is suitably positioned under control of the powerful polyhedrin promoter. This was the basic design of the first baculovirus vectors developed, and is the most common vector type in use today. Second, the replacement of the polyhedrin gene with a foreign gene also gives a means of discerning recombinant viruses, since they will be unable to produce occlusion bodies in tissue-culture, whereas wild-type viruses still retain this capacity. Third, since the polyhedrin promoter is not activated until infectious virus particles are produced, the likelihood of any interference from a foreign gene expressed under control of this promoter on the production of virus is greatly minimized. A second late viral protein, p10, expressed to high levels during the occlusion phase, is also dispensible during infecions of tissue-culture cells, and for the development of the occlusion bodies. The promoter of this gene has also been used as an altennative to the polyhedrin promoter for high-level foreign gene expression in recombinant

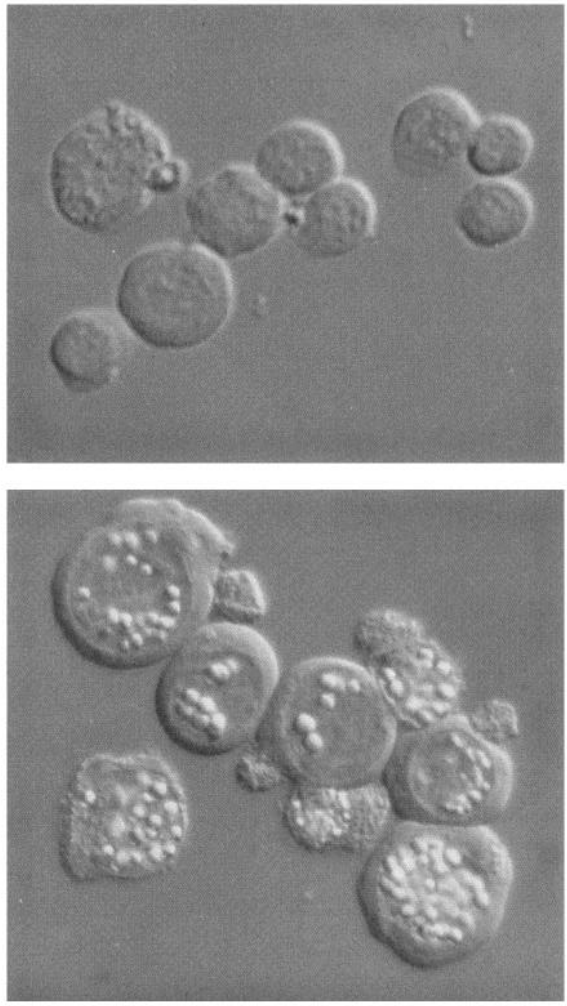

Fig. 1. The contrast between uninfected Sf insect cells (top) and cells 48h post-infection with wild-type AcNPV (bottom). The polyhedrin occlusion bodies are easily visible within the nuclei of the infected cells and give rise to occlusion-positive plaques that have a characteristic silver-gray sheen when examined against a light source.

baculoviruses. Other baculovirus promoters that are active at earlier times during the infectious cycle have also been used for foreign gene expression, but usually direct protein synthesis to lower levels than the polyhedrin or p10 promoters. For a review of baculovirus expression vectors, *see* refs. *12–14*, and references therein.

This chapter is aimed at introducing the technology to researchers who may have little or no previous experience of the insect baculovirus expression system. Throughout, it is assumed that some knowledge of basic recombinant DNA and tissue-culture techniques are known, although important aspects of each are emphasized.

2. Materials

1. Insect cell lines derived from the fall armyworm, *Spodoptera frugiperda*, are frequently used for expression studies with AcNPV Most widely used are the IPBL-*Sf*21 line and the clonal derivative *Sf*9. Both may be obtained from the American Type Culture Collection (Rockville, MD), or from any research group working in this area
2. Insect cell stocks are maintained at 18°C in Gibco LT TC100 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, as log-phase cultures in glass Techne biological stirrer flasks
3. Lipofectin
4. Low melting temperature ultrapure SeaPlaque agarose
5. Neutral Red.
6. X-gal in dimethylformamide
7. TE· 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA

3. Methods

To obtain successful expression of cloned genes using the insect expression system, it is necessary to clearly understand the principles underlying each experimental step For this reason, each of the steps are shown schematically in Fig. 2, and are described in detail.

3.1. Cloning of Foreign Genes into an AcNPV Transfer Vector

To obtain expression of recombinant proteins using this system, it is necessary to position the foreign gene under the control of a baculovirus promoter; usually the strong polyhedrin promoter. Owing to the very large size of the AcNPV genome (approx 128 kb), and a lack of suitable restriction sites at the desired position, this cannot be routinely achieved by direct cloning. Instead, the foreign gene is first subcloned into an AcNPV transfer vector. This consists of a cloned region of the AcNPV genome, usually the *Eco*RI-I fragment, within a simple plasmid background.

The AcNPV DNA region contains the polyhedrin gene, in which one or more unique sites have been engineered to facilitate cloning of the foreign gene adjacent to the upstream controlling promoter region. The rest of the AcNPV *Eco*RI-I fragment flanks the polyhedrin gene and allows recombination of the foreign sequences into homologous sequences at the polyhedrin gene region of the viral genome (*see* Section 3.2.).

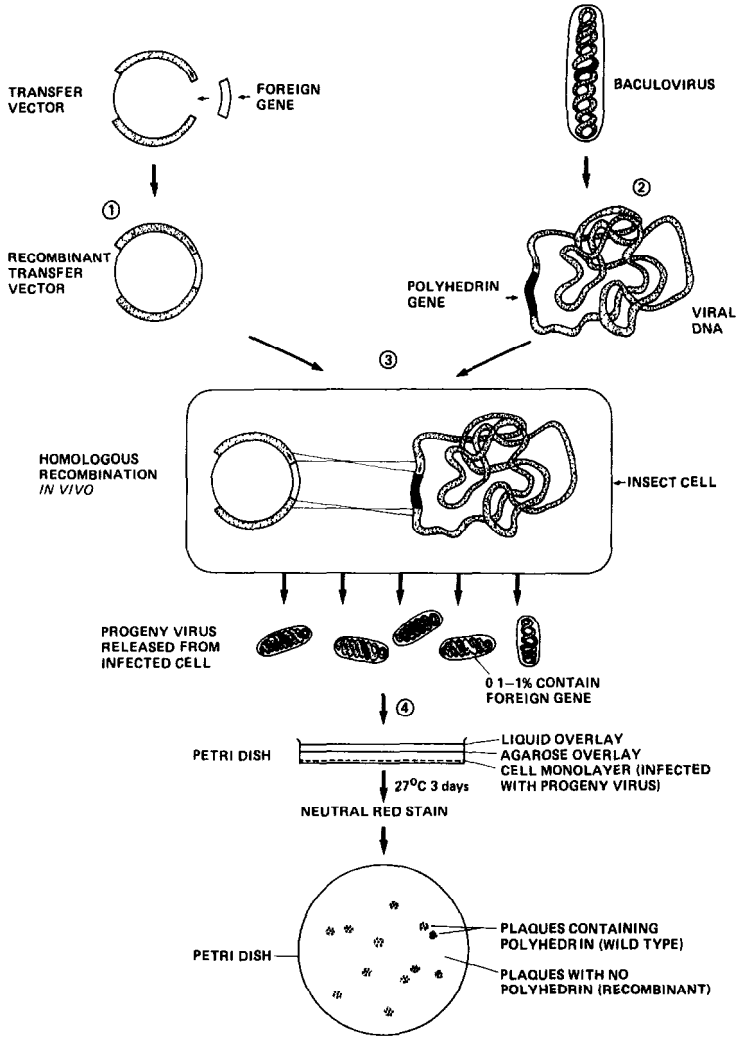

Fig. 2. Generation of a recombinant baculovirus for expression of a foreign gene. (1) Construction of the recombinant transfer vector (2) Extraction of the wild-type baculovirus DNA. (3) Cotransfection of recombinant transfer vector and baculovirus DNA. (4) Plaque assay for selection of recombinant baculoviruses Steps 1–4 are detailed in Sections 3 1.–3 4 , respectively.

Since the advent of baculovirus expression vectors, the range of plasmid transfer vectors has expanded remarkably, and the choice of vector for expression of foreign genes is important For a consideration of the range of vectors available, and their relative advantages or disadvantages for the expression of

various genes, the reader is referred to other reviews *(13,14)*. We recommend the use of polyhedrin promoter-based vectors such as pVL1392 and pVL1393 to achieve goods levels of expression of intracellular foreign proteins within recombinant baculovirus-infected cells. These two vectors are identical except for the orientation of their polylinker region and are available from Invitrogen (San Diego, CA) The following steps should be performed using standard cloning techniques *(15)* to insert the foreign gene into the transfer vector of choice The procedures described here are consistent with the use of pVL1392 or pVL1392 transfer vectors.

1 Digest the transfer vector with a suitable restriction enzyme for a unique cloning site positioned in the polylinker, immediately downstream of the polyhedrin promoter region.
2 Phosphatase the cut ends of the vector with alkaline phosphatase (calf intestinal or arctic shrimp) to reduce the high frequncy of self-ligation that would otherwise occur This stage is not required if the vector is digested with two enzymes in the polylinker which give noncompatible ends This procedure allows directional cloning of foreign DNA fragments
3 The foreign gene to be cloned must be prepared or manipulated such that it has homologous cloning ends for the digested/phosphatased transfer vector Ideally, both should have compatible "sticky" or overhanging ends, but if this is not possible, there are two options First, the ends could be altered by blunting and cloning suitable linkers onto the ends of the foreign gene to match the vector ends, or second, the ends of both transfer vector and foreign gene could be blunted, and directly ligated Ligate the foreign gene into the transfer vector using T4 DNA ligase
4. Transform competent *Escherichia coli* to ampicillin resistance (this gene is present within most transfer vectors) using an aliquot of the ligation mix
5 Prepare miniprep plasmid DNAs from picked colonies and identify by restriction enzyme analysis recombinants that have the foreign gene inserted in the correct orientation
6. Prepare full-scale DNA preparations from an identified positive clone and band through a cesium chloride density gradient
7 Perform a series of restriction enzyme digests to confirm that the recombinant transfer vector is correct. The foreign gene is now correctly inserted in the proper orientation immediately downstream of the polyhedrin promoter within a recombinant AcNPV transfer vector

3.2. Cotransfection of Viral DNA and Recombinant Transfer Vector into Insect Cells

The foreign gene contained within the AcNPV transfer vector must now be introduced precisely into the viral genome by homologous recombination within insect cells. Initially, wild-type viral DNA was used intact, but resulted in a very

high background of wild-type virus and only a small proportion (up to 0.1%) of recombinant viruses. The search for subsequent occlusion-negative recombinant virus plaques among an occlusion-positive wild-type virus background is difficult and time consuming. As with transfer vectors, there have been advances in the preparations of modified viral DNA used for recombinant virus production, and a short overview of the most commonly used viruses is pertinent.

The proportion of recombinant viruses obtained from a cotransfection can be increased by the use of linearized virus DNA. Linearized viral DNA genomes are not so efficient at initiating a replicative cycle as closed circular genomes, but a suitable transfer vector recircularizes the genome molecule during homologous recombination of the foreign gene into the viral genome. Therefore, recombinant viral genomes generated by recombination with linearized virus DNA are more efficient at producing infectious virus than the nonrecornbinant, linear genomes present. Homologous recombination of a polylinker containing a unique *Bsu*36I restriction site into the wild-type AcNPV genome in place of the polyhedrin gene has given rise to the occlusion-negative virus strain AcRP6-SC *(16)*. The genome of this virus can be linearized for use in cotransfections by digestion with this restriction enzyme. Linearization is also possible with recombinant viruses that contain the bacterial gene encoding β-galactosidase in place of the viral polyhedrin gene (e.g., AcRP23-lacZ or AcUVV1.lacZ, both available from Pharmingen), as the bacterial gene contains a single *Bsu*36I site. These viruses give blue plaque phenotypes in the presence of X-gal, but further recombinations that replace the β-galactosidase gene with other foreign genes results in colorless plaque phenotypes.

More recently, viruses have been engineered that can generate recombinant baculovirus expression vectors at frequencies approaching 100% *(17)*. *Bsu*36I restriction sites were inserted into the two viral genes flanking the polyhedrin gene, such that digestion of the genome at these sites with *Bsu*36I removes this region from the viral genome. The gene immediately downstream of the polyhedrin gene is essential for virus replication, so that although intact virus DNA is infectious, the digested genome is not. Transfer vectors containing the *Eco*RI-I fragment carry a copy of the missing sequences downstream of the site into which the foreign gene is to be inserted for expression. Hence, recombination between a transfer vector and the restricted viral DNA can restore the integrity of the essential gene, and allow replication of recombinant viruses to proceed. Several viruses of this type are commercially available from Pharmingen (BaculoGold) and Clontech (BacPAK5 and BacPAK6). The viral DNA preparations mentioned above can be used with any polyhedrin-based transfer vector, although they are often supplied with specifically designed transfer vectors. The exception to this is AcUW1.lacZ, which must be used with p10-based transfer vectors. We recommend the use of BaculoGold or BacPAK6

viral DNA preparations in cotransfections and procedures are described here assuming the use of BaculoGold virus DNA.

Cotransfection of the BaculoGold viral DNA and the smaller recombinant transfer vector into insect cells allows a proportion of the molecules to undergo double-reciprocal recombination at the homologous sequences which flank the viral polyhedrin gene region. The result is that the polyhedrin gene region within the viral genome is replaced by the foreign gene from the transfer vector.

1. Seed 2×10^6 insect cells into a 35-mm dish, and leave at 27°C for 1 h to attach
2. Into a sterile bijou, add 100 ng of BaculoGold viral DNA and 1–2 μg of transfer vector We recommend the use of wide-bore or cut-off pipet tips for all manipulations, to avoid shearing of the viral DNA Make the volume up to 50 μL with sterile distilled water and mix gently
3. Dilute Lipofectin reagent 1 in 10 with sterile distilled water, and add 50 μL to the DNA mixture. Mix gently and incubate at room temperature for 15 min.
4. Remove the medium from the monolayer of insect cells, and gently wash three times with TC100 medium which does not contain FCS. Incubate the cells in 1 5 mL of this serum-free medium for 10 min.
5. Add, dropwise, the Lipofectin-DNA mix to the cells, swirl gently to disperse and incubate for 4 h at 27°C
6. Add 1 mL of fresh medium (containing 10% FCS) to the well, and continue to incubate at 27°C for 2–3 d
7. At the end of this period, the culture medium will contain virus particles at a concentration of about 2×10^7 pfu/mL. Depending on the source of virus DNA used, anything from 0 1% (using uncut viral DNA) to over 90% (using BaculoGold viral DNA) will consist of recombinant viruses, which have to be identified and isolated by means of a plaque assay

3.3. Plaque Assay

Recombinant viruses present in the medium harvested following cotransfection can be identified and isolated by plaque assay. This method can also be used to determine the titer of an unknown virus stock solution. The principle of the method is to obtain well-isolated viral plaques in a confluent cell monolayer, which can be screened for recombinants or simply counted for determination of virus titer.

1. Prepare a series of 10-fold serial dilutions in culture medium of the virus stock, sufficient to cover each monolayer with 1 mL volume at each dilution. An appropriate range for culture supernatant harvested from cotransfections is 1 in 10–1 in 10,000, and for determination of virus titer, 1 in 10,000–1 in 1,000,000,000.
2. Seed 35-mm plastic Petri dishes with 2×10^6 insect cells, and incubate at 27°C for 1 h to allow the cells to attach. Seed enough for two plates/dilution for determination of virus titer

3 Remove most of the culture medium from the dishes, leaving a small amount to prevent the monolayer drying out. Gently drip 1 mL of the diluted virus onto the cells in the center of the dish. Incubate for 1 h at 27°C to allow adsorption of the virus.
4. Prepare a 1% agarose/culture medium overlay solution as follows. Autoclave an appropriate volume of 3% SeaPlaque agarose solution in distilled water (will need 0.5 mL/plate) and cool to 37°C. Warm twice this volume of culture medium to 37°C. Quickly mix the agarose and medium, and maintain at 37°C
5 Remove the inoculum from the Petri dishes using a sterile Pasteur pipet. Add 1.5 mL of the overlay mix to the side wall of each plate, allowing it to spread evenly over the monolayer Allow to set for 10–20 min at room temperature, and then overlay it with 1 5 mL of culture medium Incubate undisturbed in a humid environment at 27°C for 3–4 d Movement of the plates during incubation can cause smearing of the plaques.
6. After 3 d incubation, add 1 mL of a neutral red stock solution, diluted 1 in 10 in PBS, to the liquid overlay. Incubate for 1–2 h at 27°C. Tip off the liquid overlay containing stain, invert the plates, and leave for several hours or overnight at 4°C for the plaques to clear Plaques appear as circular regions of weak staining about 1–3 mm in diameter in a darker stained monolayer, and may show retarded cell growth.

3.4. Plaque Purification

Recombinant plaques may still contain some BaculoGold virus contamination because of diffusion from neighboring plaques. Thus, to isolate a pure viral stock, recombinants are purified by successive rounds of plaque purification.

1 Using a sterile Pasteur pipet or Gilson tip, pick a plug of agarose from directly over the potential recombinant plaque into 1 mL of culture medium. The plaque chosen should be well isolated within the cell monolayer, with no close neighbouring plaques. Vortex briefly, and leave at room temperature for 30 min or longer to allow the virus to diffuse from the plug. Keep an aliquot at 4°C in the dark for long-term storage
2. Carry out the plaque assay as in Section 3 3 , using the plaque suspension and 10-fold dilutions in TC100 down to 1 in 1000.
3. Stain and screen as described in the previous section, pick isolated plaques, and repeat.
4. Generally, two or three rounds of plaque purification are carried out to ensure elimination of BaculoGold virus, but the presence of the foreign gene can be confirmed by Southern Blotting or product analysis at an earlier stage if required

3.5. Analysis of Plaque-Purified Recombinant Viruses

It is good practice to verify that the recombinant virus isolated by plaque purification does indeed contain the inserted foreign gene, in the correct orientation, and without rearrangement or deletion. Similarly, it is essential that the

foreign gene is confirmed as expressing the desired protein under the direction of the baculovirus promoter. Both can be carried out from a single small-scale infection within a 35-mm dish as described. It should be noted that not all recombinant proteins will be expressed at levels where they are visible on the following protein gels, but the presence of the foreign gene can be confirmed by Southern blot analysis.

1. Seed a 35-mm dish with 2×10^6 insect cells as described in Section 3.2 , and infect with a small aliquot of the plaque purified virus derived from Section 3.4. Incubate at 27°C with a liquid overlay of 2 mL TC100 medium
2. After 2 d, remove the culture medium and clarify by centrifugation at 1500 rpm in a benchtop centrifuge for 5 min. Retain 1 5 mL in a sterile Eppendorf tube on ice for isolation of virus particles
3 Meanwhile, harvest the infected cell monolayer into 0 5 mL of sterile PBS and spin as for the culture medium. Remove the supernatant and lyse the cell pellet with 0 1 mL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Place in a boiling water bath for 5 min. The cell pellet can be stored frozen at –20°C
4 The clarified culture medium is spun at high speed (14,000 rpm) in a benchtop microfuge to pellet virus particles. The viral pellet is resuspended in 0.1 mL of 10 m*M* Tris HCl pH 7 6; 10 m*M* EDTA, 0 25% SDS and digested with 0 5 mg/mL proteinase K at 37°C for 1 h
5 Add 0 1 mL of phenol·chloroform (1 1) to the sample, and mix by gently inverting the tube several times Separate the phases by low-speed centrifugation, and discard the lower organic phase Repeat once more with phenol-chloroform, and once with chloroform alone
6 The viral DNA is precipitated with 0.1 vol of 3*M* sodium acetate and 2 vol of ethanol, at –20°C for at least 1 h, and pelleted by centrifugation Resuspend slowly in 15 µL of TE at 4°C overnight
7. Digest the viral DNA with restriction enzymes, and electrophorese the whole sample through an agarose gel Blot and probe as for standard procedures.
8. A proportion of the lysed cell pellet (10–20 µL) is analyzed by electrophoresis through a reducing polyacrylamilde gel (usually 10%), and visualized with Coomassic Blue staining It should be possible to distinguish the foreign protein in comparison to uninfected or BaculoGold virus-infected cells

3.6. Derivation of High-Titer Medium and Determination of pfu/mL

Once a recombinant plaque has been successfully identified, plaque-purified and confirmed, it is necessary to obtain culture medium containing high titers of the recombinant virus for further infections. This is achieved by a series of "scale-up" infections and the resulting high-titer culture medium is then titerd by plaque dilution assays to determine the pfu/mL of infectious recombinant virus.

1. The isolated pure recombinant AcNPV plaque is picked and transferred into a sterile Bijou (5-mL size) containing 1 mL of culture medium, and left overnight at room temperature to allow diffusion of the virus particles into the medium
2. The next day, 0.5×10^6 insect cells are seeded onto a 35-mm tissue-culture dish and left for 1 h at 27°C to attach. The medium is removed and replaced with 0.8 mL of the culture medium containing the virus plaque. The cells are left for 1 h at 27°C to allow infection to occur. The medium is removed and replaced with 2 mL fresh medium. The cells are left at 27°C for 4 d. The remaining 0 2 mL of culture medium containing the original plaque is stored in the dark at 4°C as a reserve, in case of contamination at this stage
3 After 4 d, the medium is removed and spun at 1500 rpm in a benchtop centrifuge for 5 min and the culture medium transferred to a labeled sterile tube. Of this, 1.8 mL is used to infect a T-25 flask seeded with 5×10^6 insect cells, as described above, and then replaced with 5 mL of culture medium, and left for a further 3 d at 27°C
4 The medium is removed and spun as described in step 3. Of this, 4.8 mL is used to infect a T-150 flask seeded with 30×10^6 insect cells, then replaced with 50 mL culture medium and left for a further 3 d at 27°C
5. The medium is removed and spun as described in step 3 At this stage, one has 50 mL of culture medium containing virus at a titer in the range of $1–4 \times 10^8$ pfu/mL. Of this, 1 mL is frozen at –80°C for long-term storage, the remainder is kept in the dark at 4°C and should retain its infectivity for at least 1 yr provided it is protected from the light
6. It is usual to determine the titer of the virus in the culture medium at this stage, and this is done by a dilution plaque assay. Assuming that the titer is about 10^8 pfu/mL, carry out the necessary dilutions to give 10–100 plaques/35-mm plate using a standard plaque assay as detailed in Section 3.3.

3.7. Isolation of AcNPV DNA for Characterization or Cotransfection

It may necessary to analyze the recombinant virus DNA further, or to use it as a source of virus DNA during other cotransfections. Additionally, it is economically desirable to prepare one's own viral DNA from the commercially available viruses such as BaculoGold or BacPAK6. We have therefore included a method for the extraction of viral DNA in larger quantities than described in Section 3.5.

1. Infect two T-150 flasks, each seeded with 30×10^6 cells at a multiplicity of infection of 2 pfu/cell as described *above*, and incubate at 27°C for 3–4 d
2 Harvest the culture medium and spin at 2000 rpm for 10 min to pellet the cells and debris. Discard the pellet.
3. Pellet the extracellular virus particles from the clarified supernatant at 100,000*g* in an SW40 swing-out rotor for 30 min. Wash the virus pellets in a total of 20 mL 0 1 × TE, and pellet again at 100,000*g*.

4. Resuspend the virus pellets in a total of 4 5 mL of 1 × TE, 0 2*M* NaCl, and incubate at 37°C for 2 h with 200 µg of proteinase K.
5. Add 0.5 mL of 10% Sarkosyl and continue to incubate at 37°C for a further 2 h
6 Gently extract the DNA twice with an equal volume of phenol·chloroform (1 1), and once with chloroform, as described previously. Use wide-bore tips to prevent shearing of the DNA
7. Precipitate the DNA with 10 mL of ethanol, and pellet at 8,000 rpm for 5 min Gently wash with 80% ethanol, and resuspend slowly in 500 µL of 10 m*M* TE at 4°C overnight The DNA will be viscous and fragile
8. The pellet will contain RNA and DNA at this stage Restriction enzyme digests can be performed on this material, but must include RNase A (preheated at 70°C for 10 min to inactivate any DNases present) Alternatively, the total nucleic acid preparation can be dissolved in TE buffer, incubated with RNase A (50 µg/mL) for 3 h at 37°C, then deproteinized again by adding NaCl to 0 15*M*, SDS to 1% and proteinase K to 20 µg/mL, and leaving for a further 2 h at 37°C The solution is then extracted with phenol:chloroform, and with chloroform as described *above*, and precipitated with ethanol The DNA contained after this step will be "purer" and more likely to digest completely with restriction enzymes The final DNA pellet is dissolved in sterile distilled water and quantified by UV absorbance at 260nm
9. If linearized (*Bsu*36I-digested) virus DNA is to be used for cotransfections, digest 1 µg of viral DNA with 5 U of restriction enzyme in a 50-µL reaction volume for 2 h at 37°C It is essential that the enzyme be heat-inactivated following digestion, by incubating at 70°C for 15 min. The DNA can be stored at 4°C, and is sufficient for 10 cotransfections (100 ng/transfection)

4. Notes

1 There are some unpublished observations that the various cell lines yield different levels of recombinant proteins, and it may be advantageous to test expression of a new gene in several cell lines. Insect cells grow optimally at 27–28°C, but we routinely maintain the cell lines in glass spinner vessels at a lower temperature (18°C) These slower growing cells seem to perform consistently better in plaque assays (carried out at the optimal temperature of 27°C).
2 Subculturing of the insect cell monolayer stocks is required at approx 1-wk intervals, while cells are still in log-phase growth (i.e., subconfluent). Viability counts should be done at this stage using trypan blue stain Cells are normally subcultured 1/10 for weekly maintenance Insect cells are considerably more fragile than most mammalian cells. For this reason, the cells should not be subjected to rapid pipeting or vortexing Otherwise, substantial cell death could occur.
3. Insect cells can be maintained in tissue-culture flasks, but we strongly recommend maintenance of cells as suspension cultures in glass spinner vessels However, cells that have just been thawed from liquid nitrogen storage should be scaled up in tissue-culture flasks for 3–4 passages before adapting to grow as suspension cultures. Cells grown in flasks are detached by sharply smack-

ing the flask several times or by scraping the cells off with a sterile rubber policeman Trypsin/versene is ineffective at removing insect cells and should not be used

4. Spinner cultures can be used for virus preparations or for large-scale production of recombinant protein and should be used at 27–28°C, which is the permissive temperature for virus replication. Standard glass spinner flasks are seeded at 5×10^4–1×10^6 cells/mL, stirred at 50–100 rpm Aeration is not required for volumes up to 500 mL but for larger scale cultures (*see* ref *18*).
5. As mentioned in Section 3.2., several recombinant viruses contain the bacterial β-galactosidase gene (BaculoGold, BacPAK6, AcRP23 lacZ, AcUW1 lacZ) and give blue plaques in the presence of X-gal When using these viruses as a source of viral DNA during cotransfections, the resulting new recombinant viruses containing foreign gene sequences in place of the β-galactosidase gene give colorless plaques This can be used as a screening mechanism during the plaque assay (*see* Section 3 3 , step 4) by including 120 g/mL of X-gal within the agarose/culture medium overlay
6. The factors affecting the levels of foreign gene expression within the baculovirus/insect cell system are still poorly understood Although polyhedrin can be expressed from the wild-type virus at levels approaching 1 mg/mL, the levels of foreign gene expression are usually in the range of 1–50 μg/mL The choice of transfer vector can influence the levels obtained For instance, transfer vectors that contain the polyhedrin gene ATG translational start followed by a unique cloning site shortly downstream of the *N* terminus result in foreign genes that are cloned in frame with the amino end of the polyhedrin gene The foreign gene is expressed as a polyhedrin/foreign gene fusion The levels of these proteins can often be considerably greater than obtained with mature proteins generated from transfer vectors that do not contain the polyhedrin ATG, but rely on the ATG of the foreign gene for translation initiation. For examples of both types of vector (*see* refs *12–14*)

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Transfection and Transformation of Human Thyroid Epithelial Cells

Nicholas Robert Lemoine and David Wynford-Thomas

1. Introduction

High-efficiency gene transfer into mammalian fibroblasts is a routine procedure that can be performed by a variety of methods *(1)*, but transfection of epithelial cells has been more difficult to achieve Each of the standard techniques has associated problems; special equipment is required for electroporation *(2)* and also for direct microinjection *(3)*. Protoplast fusion *(4)* is suitable only for cloned genes. Retroviral transduction of genes *(5)* is a method of high efficiency for animal cells, but again is applicable only to cloned genes of restricted size, and safety considerations are likely to restrict greatly the use of retroviral vectors suitable for human cells. Calcium phosphate coprecipitation has been used with success in several epithelial lines *(6–8)* and, more recently, strontium phosphate coprecipitation *(9)*. These techniques are very simple to perform, require no special equipment, and can be applied to genomic DNA transfection.

The human thyroid follicular cell appears to be a particularly suitable epithelial cell for gene transfection by coprecipitation, in which insoluble complexes of DNA with calcium or strontium phosphate are taken up by recipient cells, because phagocytosis can be specifically increased by thryoid-stimulating hormone (TSH). We have recently shown that this maneuver enhances the efficiency of gene transfer, with a fivefold increase in the frequency of cells transiently expressing SV40 large T-antigen as assessed by immunocytochemical assay 48 h after transfection *(10)*. In addition, these cells appear remarkably resistant to the toxic effects of calcium phosphate, with plating efficiencies of ≥95% even after 16 h exposure to coprecipitate.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

2. Materials

1. Plasmid DNA: Closed circular plasmid DNA (prepared by cleared lysate method with banding on a $CsCl_2$ gradient) *(11)* is dissolved in 1.0 m*M* Tris-HCl, 0.1 m*M* EDTA pH 8.0 at a concentration of 0.1 µg/µL.
2. Carrier DNA: High mol wt (*see* Note 1) carrier DNA (prepared by a method of caesium chloride banding) *(12)* is dissolved in 1.0 m*M* Tris-HCl, 0.1 m*M* EDTA pH 8.0 at a concentration of 0 5 µg/µL.
3. 25 m*M* HEPES. dilute 2.5 mL of sterile 1*M* HEPES to 100 mL with sterile water. Store at 4°C for up to 3 mo
4. 2X HEPES-buffered saline (2X HBS) dissolve 1 64 g NaCl plus 0.023 g Na_2HPO_4 $2H_2O$ in 90 mL of water Add 5 mL of 1*M* HEPES, then. for $CaCl_2$ coprecipitation, adjust pH to 7.1 with 0.5*M* NaOH; for $SrCl_2$ coprecipitation, adjust pH to 7.8 with 0 5*M* NaOH Adjust vol to 100 mL with water (*see* Note 2) Filter sterilize (*see* Note 3), and store at 4°C for up to 3 mo.
5. 2 5*M* $CaCl_2$: 10.8 g $CaCl_2$ $6H_2O$ in 15 mL of water Adjust vol to 20 mL with water Filter sterilize and store in aliquots at –20°C
6. 2*M* $SrCl_2$. 10.665 g $SrCl_2$ in 15 mL of water. Adjust vol to 20 mL with water. Filter sterilize and store in aliquots at –20°C
7. 1 0 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8 0. 100 µL 1.0*M* Tris-HCl pH 8.0 plus 20 µL 0 5*M* EDTA, pH 8.0 in 100 mL water Filter sterilize and store at 4°C
8. SF-12 medium: This must contain the appropriate concentration of sodium bicarbonate for the conditions of incubation for a pH of 7 4 at 37°C in 5% CO_2, add 2.9 mL of 7.5% $NaHCO_3$ solution/100 mL of medium (*see* Note 4)
9. RPMI medium: Prepare with sodium bicarbonate concentrations as in item 8
10. 11 8% (v/v) Glycerol: 11.8 mL Glycerol, diluted to 100 mL in Hanks' balanced salt solution (HBSS). Filter sterilize and store at 4°C.
11. TSH: Thytropar (Armour Pharmaceuticals, Tarrytown, NY) dissolve in HBSS at 1 U/mL as stock solution, filter sterilize and store in 100 µL aliquots at –20°C
12. 2*M* NH_4Cl: 1.06 g of NH_4Cl in 8 mL of water Adjust pH to 7.1 and adjust vol to 10 mL with water Filter sterilize and store at 4°C for up to 3 mo.

3. Methods

3.1. Thyroid Epithelial Cell Culture

1. Thaw human thyroid epithelial cells (*see* Chapter 13) from frozen stock, wash once with RPMI medium and plate out as a monolayer at 5×10^5 cells/60-mm dish in RPMI medium supplemented with 10% fetal calf serum (FCS)
2. Transfection is performed 4 d after plating (*see* Note 5). Six hours before transfection, replace the medium with 5 mL of warm SF-12 supplemented with 10% FCS (*see* Note 6).

3.2. Calcium Phosphate Coprecipitation

1. Prepare DNA for transfection by mixing x µL of plasmid DNA (containing 0.5–10 µg/DNA) solution with $(200 - x)$ µL of 25 m*M* HEPES in a sterile plastic

bijou If carrier DNA is used, then this is included to bring the final concentration of DNA to 10 μg/0 5 mL of coprecipitation suspension. Add 50 μL of $CaCl_2$ and swirl to mix.

2. Add this solution dropwise through a syringe and needle to 250 μL of 2X HBS in another sterile plastic bijou with continuous mixing by a stream of air bubbles via a plugged sterile pipet with plastic tip (*see* Note 7).
3. Leave the mixture to stand for 30 min, after which time the fully formed coprecipitate will have settled to the bottom of the container. Coprecipitates containing high mol wt carrier DNA have a coarser consistency than those containing plasmid DNA only
4. Five minutes before application of coprecipitate, add 50 μL of 1 U/mL TSH solution to the medium and swirl gently to mix (*see* Note 8)
5. Gently take up the suspension in a 1-mL Gilson pipet a few times, so that the coprecipitate is uniformly suspended through the solution
6. Add 100 μL of 2*M* NH_4Cl solution to the medium and swirl gently to mix (*see* Note 8).
7 Sprinkle the suspension over the medium of the epithelial monolayer
8. Incubate at 37°C for 16 h to allow uptake of the coprecipitate
9. Remove the medium containing coprecipitate and wash the cells twice with warm HBSS. Refeed with warm RPMI + 10% FCS.

3.3. Strontium Phosphate Coprecipitation

1. Prepare DNA for transfection by consecutively mixing x μL of plasmid DNA (containing 0.5–10 μg DNA), 30 μL of 2*M* $SrCl_2$, and $(220-x)$ μL of sterile water in a sterile plastic bijou See Section 3 1 , step 1 for details of carrier DNA.
2. Add this solution dropwise through a syringe and 23-gage needle to 250 μL of 2X HBS in another sterile plastic bijou with continuous mixing by a stream of air bubbles (via a plugged sterile pipet with plastic tip)
3 Leave the mixture to stand undisturbed at room temperature and observe the development of the coprecipitate After 5–10 min, the precipitate is seen as a fine dust and is then ready for application to the cells.
4. Gently take up the suspension in a Gilson pipet a few times, so that the coprecipitate is uniformly suspended throughout the solution.
5 Sprinkle the suspension over the medium of the epithelial monolayer in a 60-mm dish.
6. Incubate at 37°C for 90 min to allow adsorption of the coprecipitate.
7. Remove the medium containing coprecipitate, and wash the cells twice with warmed HBSS Apply 1 5 mL of 15% glycerol in HBSS and incubate at room temperature for 30 s, then wash three times with warm HBSS, and refeed with warm RPMI + 10% FCS.

3.4. Transient Expression Assay

The transient expression of genes that have been successfully transfected is maximal at 48–72 h after transfection in these cells. A convenient assay system

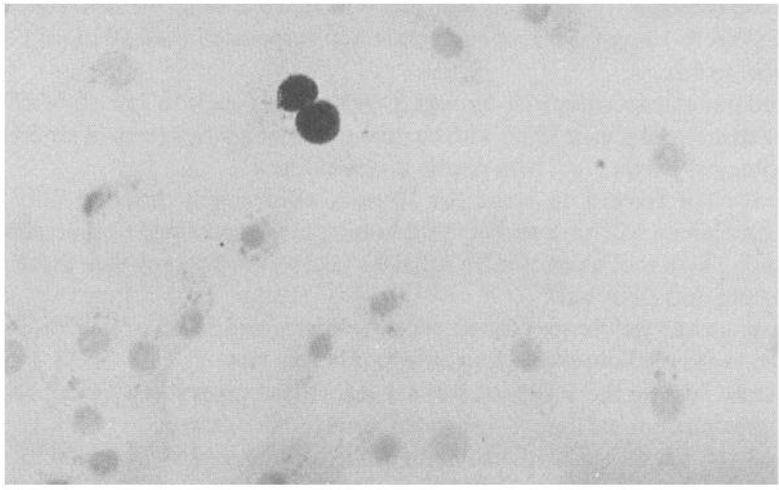

Fig. 1. Pair of recently divided thyroid epithelial cells showing stong nuclear immunoreactivity with anti-SV40 large T antibody pAB 419, 48 h after transfection with a plasmid containing the whole SV40 sequence. (Immunocytochemistry was performed on acetone-fixed monolayers using pAB 419 followed by rabbit antimouse immunoglobulin conjugated to horseradish peroxidase. Positivity is shown by deposition of the brown peroxidase-catalyzed polymer of diaminobenzidine.)

that allows calculation of transfection frequency is immunocytochemical demonstration of SV40 large T expression (Fig. 1) following the transfection of a plasmid such as pSVori$^-$ *(13)*.

3.5. Stable Expression Assay

The transfection of a dominant selective marker such as the neo gene that codes for resistance to geneticin in eukaryotic cells allows assay of stable transfection frequency. Primary human thyroid epithelial cells have poor cloning efficiency and require the use of a feeder layer (of geneticin-resistant fibroblasts) in order to develop viable clones from the low density required for geneticin selection.

Transfection of plasmids containing SV40 leads to the outgrowth of clones with extended life span that continue to proliferate after the untransformed cells undergo senescence (after an average of 3–6 doublings). These clones can be shown to express SV40 large T by the aforementioned immunocytochemical assay.

4. Notes

1. DNA prepared by methods that involve phenol/chloroform extraction of proteinase K digests for example will be of lower molecular weight, and this will reduce the efficiency of transfection.

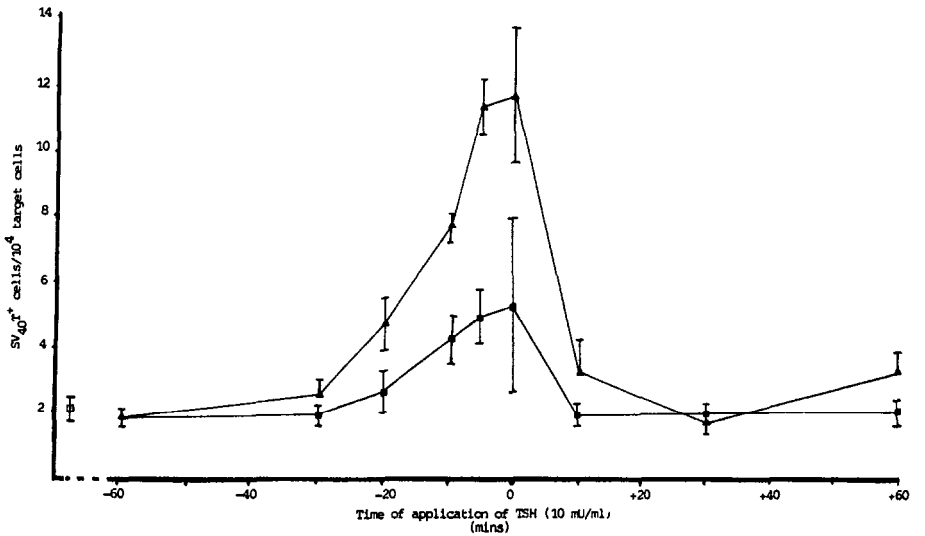

Fig. 2. Relationship between time of addition of TSH (10 mU/mL) to monolayer culture and efficiency of transfection (as assessed by proportion of cells showing transient expression of SV40 T antigen 48 h after transfection) □, no TSH (addition of NH_4Cl had no effect on this result) ■, TSH (10 mU/mL) added at indicated times ▲, TSH (10 mU/mL) added at indicated times plus NH_4Cl (20 m*M*) added at time 0 All points show mean +/– SE derived from 6 separate experiments

2. The effect of pH is critical, and it is recommended that pH of stock solutions should be checked at intervals after preparation, and if incorrect, new reagents should be prepared
3. 0 22-μm Filters should be flushed with sterile water before use This has been observed to reduce subsequent aggregation of the DNA-cation coprecipitates.
4. This medium has been found to be ideal for the culture of cells during transfection by coprecipitation. Some other media (including RPMI) are unsuitable because the product of divalent cation-phosphate concentrations is too high, causing excessive coprecipitation.
5. The growth rate of these cells (as determined by thymidine labeling) shows a peak on d 4 after stimulation with serum The highest efficiency of transfection is at this time *(10)*
6 Media and wash solutions should be prewarmed to enhance growth and transfectability of cells
7. Even mixing of the solutions is best achieved by a gentle stream of bubbles, and the pipet should have a plastic tip since the precipitate adheres to glassware.
8. The addition of TSH to stimulate phagocytosis significantly improves the efficiency of transfection by calcium phosphate coprecipitation when given within a critical time-window (*see* Fig. 2)· No effect is seen if it is given more than 20 min

before (or at any time after) addition of the coprecipitate Lysosomal function inhibitors, such as ammonium chloride, have no influence when used alone, but have a synergistic effect when used in concert with TSH in this system Interestingly, no effect of TSH has been demonstrable on the efficiency of strontium phosphate coprecipitation

Acknowledgments

We are grateful to the Cancer Research Campaign and to the Welsh Scheme for the development of Health and Social Research for grant support.

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Analyzing Chemotaxis Using the Dunn Direct-Viewing Chamber

Daniel Zicha, Graham Dunn, and Gareth Jones

1. Introduction

The Boyden chamber is the most widely used method for assaying chemotaxis in leucocytes *(1)* and is commonly used for cultured cells such as fibroblasts *(2)*. It is based on scoring cells that have migrated into or through a filter membrane toward a source of putative chemotactic factor. Although it is a sensitive method and very useful for the routine screening of potential chemoattractants, it can often give misleading positive results and has serious limitations for studying the mechanism of chemotaxis. The two most important shortcomings of the chamber are that the local concentration gradients of chemotactic factor, in and around the pores in the filter membrane, are variable and unknown and that the resulting cell behavior is unobservable and can only be deduced from the final distribution of the cell population. Zigmond and Hirsch *(3)* greatly improved the Boyden assay by devising a system of control experiments, now known as checkerboard analysis, in order to discount false-positive results owing to chemokinesis. Unequivocal confirmation of chemotaxis, however, still requires direct observation of the cells together with a knowledge of the direction and magnitude of the concentration gradient.

The currently available methods for directly observing chemotactic behavior on a plane substrate include the under coverslip assay *(3,4)*, the under agarose assay *(5)*, the Zigmond chamber *(6)*, and the Dunn chamber *(7)*. Of these methods, the concentration gradient approaches a linear steady state only in the Zigmond and Dunn chambers. Although similar in principle to the Zigmond chamber, the Dunn chamber was developed for studying long-term chemotaxis, particularly in slowly moving cultured cells such as fibroblasts, and the principal design criterion was therefore an improved stability of the gradient.

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

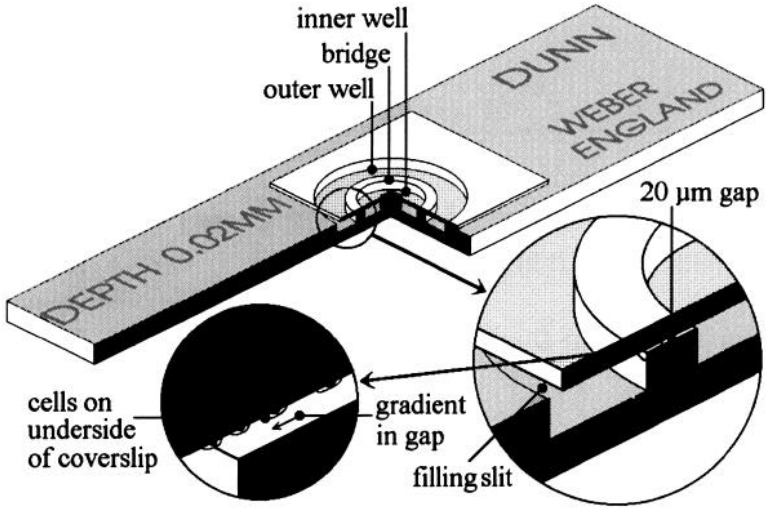

Fig. 1. Cutaway view of the Dunn chemotaxis chamber with the coverslip in the correct position to allow access to the outer well. The central pip shown in the inner well results from the manufacturing process and does not affect functionality.

Instabilities can arise in these two types of chamber from any of a number of causes, including thermal effects and mechanical creep. Even a slight change in the effective volumes of the two wells or in the height of the intervening diffusion gap can seriously disturb the gradient that forms in the gap. The improved stability of the Dunn chamber was attained chiefly by adopting a concentric layout for the two wells and the intervening bridge (Fig. 1) instead of the linear pattern devised by Zigmond. The concentric layout results in a blind inner well that, because it does not require sealing with wax, maintains a precisely constant volume. Provided that the culture medium in this well does not contain air bubbles, its incompressibility prevents any flow of medium between the wells that would otherwise destroy the diffusion gradient. A further improvement is that the Dunn chamber is made entirely from glass which permits the height of the diffusion gap to be predetermined by optical machining; this ensures that the gap maintains a constant height of 20 μm when the chamber is assembled correctly.

We have used the Dunn chamber to analyze the chemotactic responses of a variety of cell types. In this chapter we provide descriptions of two ways in which the apparatus can be used for the purposes of studying cell movement. The first describes a straightforward application requiring little detailed knowledge of mathematical principles that can generate data amenable to standard statistical analysis. The second example provides a more sophisticated

approach to the analysis of cell migration which lends itself particularly well to descriptions of the more subtle forms of cellular interactions.

2. Materials

1. Clean and sterile Dunn chamber: Now available commercially from Weber Scientific International Ltd (Teddington, Middlesex, UK)
2. Clean and sterile glass coverslips 18-mm square, Chance No. 2 or No. 3 (*see* Note 1)
3. Clean and sterile glass cloning cylinders having an internal diameter to match the outer diameter of the outer well of the Dunn chamber—usually around 12 mm
4. A syringe without needle containing silicon high vacuum grease This grease can be sterilized by autoclaving in a glass syringe but it is usually sufficient to transfer it from the manufacturer's tube into a sterile disposable syringe after wiping the mouth of the tube with a tissue soaked in 70% ethyl alcohol
5. Sterile syringe with fine needle (~25 gage) for refilling the outer well of the chamber
6. Hot wax sealing mixture, consisting of a 1:1:1 ratio of paraffin wax, beeswax, and Vaseline (petroleum jelly) mixed and used at around 60°C
7. Cell cultures· These are chosen by the investigator according to requirements of the experiment. Observe normal sterile techniques during routine subculture
8. Cell dissociation medium Normally trypsin/EDTA, but choose whatever is appropriate for the cell type.
9. Cell-culture medium suitable for maintaining the cells for the duration of the experiment in a sealed chamber without CO_2-gassing. Media based on Hank's salts will equilibrate with a bubble of air in a sealed chamber (*see* Note 2) but a better alternative may be to use an appropriate CO_2-independent medium
10. Cell-culture medium identical to the one in item 9 but containing the putative chemotactic factor at an appropriate concentration
11. Equipment for recording and analyzing cell behavior (*see* Section 3 2)

3. Methods

3.1. Assembling the Chamber

1. Seed the previously dissociated cells at low density onto the 18-mm square coverslip. The seeded area should be restricted to a circular region adjacent to one edge of the coverslip so that no cells come to lie outside the outer margin of the wells when the chamber is assembled. This can be attained by seeding the cells from a cloning cylinder attached to the coverslip using a minimum of the high vacuum grease (*see* Note 3)
2. Allow the cells time to settle and attach to the coverslip and then gently wash the coverslip several times with the cell-culture medium
3. Fill both the concentric wells of the chemotaxis chamber with the cell-culture medium In control experiments, both wells may be filled with medium containing the putative chemotactic factor at this stage.

4. Invert the coverslip onto the chemotaxis chamber, taking care to align the seeded area with the wells and to leave a narrow slit at one edge for draining and refilling the outer well (Fig. 1) There must be no air bubbles in the inner well at this stage (*see* Note 2).
5. Drain the outer well using absorbent tissue applied to the filling slit
6. Dry the coverslip and seat it firmly on the chamber by applying gentle pressure, through absorbent tissue, to the outer edges of the coverslip with the chamber supported on a perfectly flat and rigid surface (*see* Note 4).
7 Use the hot wax sealing mixture to seal the edges of the coverslip to the chamber but leave access to the filling slit.
8. Gently fill the outer well with medium containing the putative chemotactic factor using the syringe with fine needle Avoid any air bubbles unless performing a long-term experiment in Hank's-based medium (*see* Note 2)
9. Seal the slit with the hot wax sealing mixture

It is advisable to practice assembling the chamber using a dye to fill the outer well. Fluorescent dye experiments have confirmed that the gradient can be predicted reliably from diffusion theory *(7)*. According to theory, a protein with a molecular weight of the order of 10,000–20,000 will form a close approximation to a linear gradient within about 30 min of assembling the chamber and the half-life of the gradient (i.e., the time for its slope value to halve) will be about 30 h. A peptide of molecular weight 35,000–75,000 will form a linear gradient within 10 min and decay to half its initial value in 10 h. The times of gradient formation and decay are approximately proportional to the cube root of the molecular weight of the factor but a precise calculation requires knowing the diffusion coefficient *(7)*. When the gradient is initially established, the absolute concentration at the centre of the gradient will be close to half of the concentration in the outer well. It may therefore be appropriate to halve the concentration when filling both wells with factor in control experiments.

3.2. Recording the Cell Behavior

The annular bridge of the chemotaxis chamber is approx 1 mm wide and it is sometimes advantageous to include the full width of the bridge in the microscope recording field so that the direction of the gradient is always known and absolute concentrations can be estimated if required. Any small bubbles of air adjacent to the bridge (*see* Note 2) may locally distort the gradient and should be kept well away from the recording field. The exact method of time-lapse recording will depend on the video equipment, filming equipment or computer imaging equipment available. Using a CCD video camera, phase-contrast, or dark-field optics, and a frame-grabber, leukocyte behavior can be recorded digitally on computer disk and tracked automatically *(8)*. Cultured tissue cells may require more sophisticated optics, such as the DRIMAPS interference method,

and the recordings will usually need to be tracked manually or semi-automatically *(8)*. Whatever method is used, the net result of tracking a recording is a set of cell trajectories, each consisting of a sequence of (x,y) position coordinates obtained from a single cell.

3.3. The Trajectory Vector Plot Method of Analyzing Chemotaxis

A simple method for testing the direction of movement of cells tracked under the influence of a putative chemoattractant is provided in Webb et al. *(9)*.

1. Plot each cell trajectory in a set of recordings so that each cell has its starting position set to the zero point of a scatter plot. For ease of later presentation, arrange the scatter plot such that the source of putative chemoattractant (the outer well of the Dunn chamber) is at the top of the plot. Serially plot the subsequent sequence of position coordinates for each timed interval for every cell until the end of the recording time. Figure 2 demonstrates the results from the data obtained from the migration of a macrophage cell line in a linear gradient of the growth factor CSF-1, a potent chemoattractant for macrophages *(9)*.
2. Select the final position coordinates of each cell and use these data to calculate a simple mean; add this to your scatter diagram.
3. Test for bias in the final cell distribution. Use the Student's *t* test after first confirming that the data are normally distributed although we have always found this to be the case. In an isotropic environment, provided that there is no intercellular signaling, motile cells should be dispersed evenly into each of the quadrants of the scatter diagram and the statistical test will demonstrate no significant deviation from this prediction (*see* Note 5). Under the influence of a gradient of chemoattractant, as shown in Fig. 3, the cells will show a bias towards the source of the chemo-attractant. However, there should still be no significant bias in the direction of cell movement in the x-axis of the scatter diagram.

3.4. The Horizon Method of Analyzing Chemotaxis

A method we have developed for testing the directionality of a locomotory response *(10)* is first to reduce the trajectory data from each individual cell to a single angular direction. We call this the "horizon method" by analogy with methods for assessing the navigation abilities of released birds that record the direction in which each bird first disappears over the horizon. It has the advantages that well-established methods for the statistical analysis of directional data can be used and that stationary or sluggish cells which never reach the virtual "horizon" are automatically precluded from the analysis.

1. Convert the cell trajectories from a single experiment or group of similar experiments to a set of angular directions: $\theta_1, \theta_2, \ldots, \theta_n$. The direction θ_i is taken from the ith trajectory's starting position to the first position that is more than 30 μm from the starting position (*see* Note 6). Trajectories that never reach the 30 μm "horizon" are eliminated.

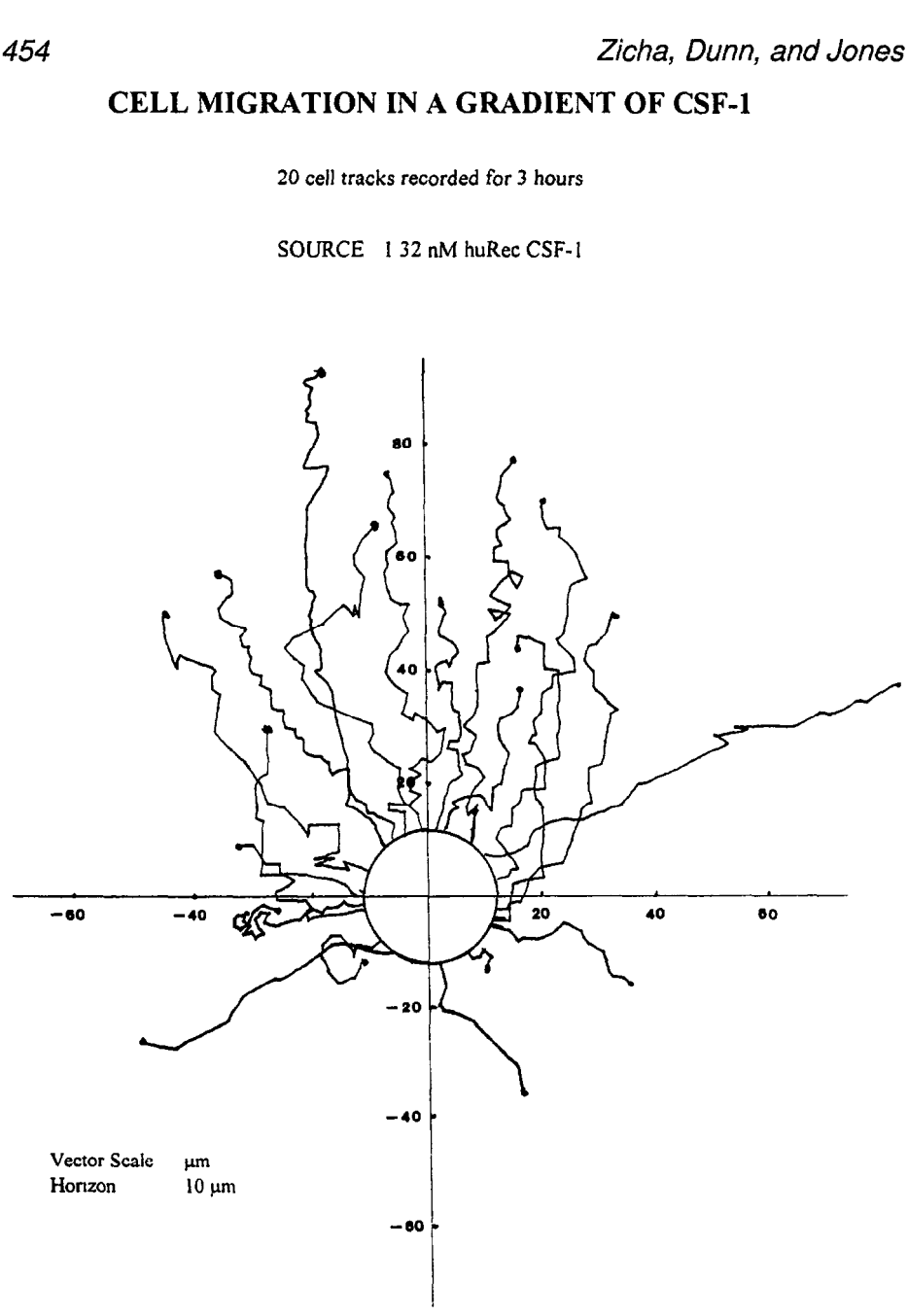

Fig. 2. Vector plot of the migration of 20 macrophages exposed to a gradient of human recombinant CSF-1 in the Dunn chamber. The outer well contained tissue-culture medium supplemented with 1.32 n*M* CSF-1 and timelapse recordings were taken at 2-min intervals for a total of 3 h Strong positive chemotaxis is evident and can be confirmed with standard statistical methods The source of CSF-1 is from the top of the figure.

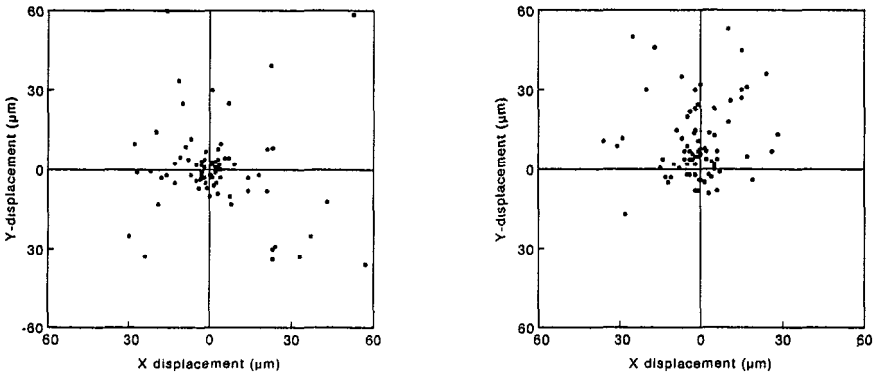

Fig. 3. Two plots in which only the final position of the cell is marked. A uniform concentration of CSF-1 stimulates cell migration, but there is no preference in directionality of cell movement In a gradient of CSF-1, the measured rates of cell movement are similar to those observed in isotropic conditions, but there is a strong statistical bias ($p < 0\ 01$) to migration up the gradient of CSF-1.

2. Calculate the mean direction, $\bar{D}$, from

$$\bar{S} = 1/n\Sigma\sin(\theta_i) \tag{1}$$

$$\bar{C} = 1/n\Sigma\cos(\theta_i) \tag{2}$$

$$\tan(\bar{D}) = \bar{S}/\bar{C} \tag{3}$$

3 Calculate the mean resultant length, $\bar{R}$:

$$\bar{R} = (\bar{C}^2 + \bar{S}^2)^{1/2} \tag{4}$$

4. Apply the Rayleigh test for unimodal clustering of directions by looking up the significance of the value for $\bar{R}$ in a published table *(11)*.
5. If the Rayleigh test indicates a significant unimodal clustering of directions, find the 99% confidence interval for the mean direction, $\bar{D}$, by using the values of n and $\bar{R}$ to find the deviation, δ, in a Batschelet Chart *(12)*

$$99\%\ \text{confidence interval} = \bar{D} \pm \delta \tag{5}$$

If the null hypothesis of a uniform distribution of directions is rejected by the Rayleigh test in step 4, then the cells are showing a significant directional response. In order to confirm that this response is a chemotaxis to the known gradient, however, it should also be demonstrated that the direction of the known gradient lies within the confidence interval for the mean cell direction. If the direction of the known gradient lies outside the confidence interval for the mean cell direction, a significant unimodal clustering of cell directions indicates that the cells are responding to some unknown factor which may be

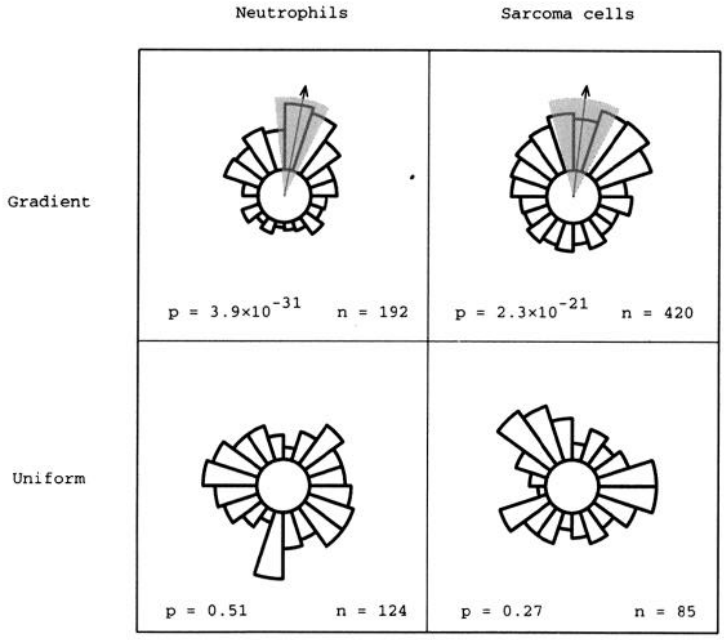

Fig. 4. Circular histograms demonstrating strong chemotactic responses of neutrophil leucocytes toward $10^{-8}M$ FMLP and rat sarcoma cells toward a combination of 80 ng/mL recombinant IGF II and 60 ng/mL recombinant PDGF BB. Arrows represent mean directions of significant directionality and grey segments mark their 99% confidence intervals. In both cases the confidence intervals include the direction of the gradient which was always vertically upwards with the source at the top.

an intercellular signal released by the cells *(10)*. Figure 4 illustrates the results of applying the Rayleigh test to neutrophil and sarcoma cell chemotaxis.

4. Notes

1. The coverslip should be as thick as the microscope optics permit in order to minimize its flexure.
2. If using a medium based on Hank's salts, a small bubble of air may be tolerated in the outer well. Any air bubble in the inner well will destroy the incompressibility of its contents and must be avoided.
3. Restricting the seeded area avoids cells being crushed and releasing unknown factors when the chamber is assembled.
4. Ideally, Newton's rings should appear at this stage as the medium beneath the edges of the coverslip is drained away. These indicate that a sufficiently close contact between coverslip and chamber has been achieved. They may not appear, however, in regions where high-vacuum grease was used to restrict the seeded area. This should not affect the stability of the gradient but it may result in the

diffusion gap being somewhat larger than 20 μm which may affect critical calculations of the half-life of the gradient.

5. This method of analysis will also identify any chemokinetic effect of a test molecule An increase in the rate of cell migration caused by the presence of appropriate stimulant can be tested for using standard statistical procedures
6. The starting position of each cell trajectory should ideally be taken as the cell's position at the start of recording. To avoid bias, only cells whose starting positions lie more than 30 μm from the nearest edge of the recording field should be analyzed If the cell distribution is approximately uniform, however, no bias should arise from including cells closer to the edge and even cells that migrate into the recording field from outside The horizon distance of 30 μm or approximately three cell diameters is fairly arbitrary and does not seem to be critical, but it should be chosen before analyzing crucial experiments

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Computer-Assisted Analysis of Single-Cell Behavior

Michael Cammer, Jeffrey Wyckoff, and Jeffrey E. Segall

1. Introduction

Recent advances in analysis of signal transduction mechanisms have demonstrated that changes in cell morphology are part of cellular responses to many important stimuli. In particular, growth factors and the signaling mechanisms stimulated by them can cause dramatic reorganization of the cytoskeleton and cell shape. These changes can affect cell motility, protein synthesis, adhesion, and, potentially, gene regulation. By measuring the kinetics and characteristics of changes in cell motility, cell morphology, and subcellular structures such as vesicles and the nucleus, the relationships of these changes to other biochemical events occurring inside the cell can be better understood.

The basis for analysis is time lapse microscopy—acquiring images of cells at a rate adequate for analyzing the response of interest, but not storing so much data that analysis becomes too time consuming. Parameters such as change in area, shape, motility on a planar surface, vesicle motility, nuclear morphology, and so on, can be assessed and quantitated by simple light microscopy. Developments in video and computer technology have resulted in the availability of relatively inexpensive components that can be added to microscope equipment already available in many laboratories *(1,2)*. In this chapter we describe what we consider to be one of the most inexpensive and user-friendly ways to allow a laboratory to perform computer-assisted analysis of cellular responses. The basic system is a video camera attachment to a light microscope that allows acquisition and analysis of images by a Macintosh running the program NIH *Image*. There are more sophisticated (and more expensive) systems available that can be obtained as the need arises. Some of these will be briefly described

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

in the Notes (*see* Note 8), but given the rapid rate at which systems are being developed, this can only be a start for a more extensive search.

2. Materials

2.1. Biology

Although the detailed descriptions here are for studying the rat mammary adenocarcinoma cell line MTLn3 *(3,4)*, appropriate modifications for other specific cell lines should be simple to design.

1. Growth medium. We use alpha-modified minimal essential medium (MEM) containing L-glutamine supplemented with 5% (v/v) fetal bovine serum and 1% stock penicillin/streptomycin antibiotics (*see* Appendix, p 478)
2. Experimental medium. Growth medium can be used, but since serum interferes with results in some experiments, one can use alpha-MEM with 0 35% bovine serum albumin (BSA) (osmotically equivalent to 5% serum) and 12 m*M* HEPES, pH 7 4
3. Stimulation dish For low to medium resolution with long working distance lenses, a 35 × 10-mm tissue culture dish or a 6-well flat-bottomed plate can be used For high resolution studies with oil immersion lenses, glass bottom microwell dishes (cat. no P35G-010-C, Mattek, Ashland, MA) or coverslips with incubation chambers are available
4. Extracellular matrix We typically coat the stimulation dish with 27 μg/mL of rat tail collagen I in Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium
5. Stimulus compound· 40 μ*M* transforming growth factor alpha (TGFα) or 50 μ*M* epidermal growth factor (EGF) is placed in 10-μL aliquots in sterile Eppendorf tubes and frozen at –70°C
6. Glass stimulus tubing For easy access to the Petri dish, a 100-μL glass pipet is bent in a 90° angle in the flame of a Bunsen burner and then sterilized by autoclaving
7. Silicon stimulus tubing We use silicon tubing to transport the stimulus compound to the Petri dish
8. Stimulus pump A peristaltic pump is used at maximum speed to assure addition of the stimulus within 30 s
9. Mineral oil To minimize evaporative cooling, the medium in the Petri dishes is covered with autoclaved mineral oil

2.2. Microscopy and Computer

1. Inverted microscope with planapochromat phase contrast optics, stable light source, camera port, and video camera A video recorder is useful for storage of the original data (*see* Notes 1–3).
2. Micrometer slide or other standard. We use 1 mm with 100 subdivisions. A micrometer slide is available from most lab suppliers or from most microscope suppliers

3 Macintosh computer with NIH *Image* software, video capture card for digitizing and sufficient memory and storage capacity for processing multiple images (*see* Notes 4–7).
4 Spreadsheet or plotting software, such as Microsoft Excel, for graphing and calculations (*see* Note 8).

3. Methods

3.1. Preparation of Cells for Experimentation

1. Petri dishes are coated with 27 μg/mL of collagen I in DPBS for 2 h
2 Cells grown to 60–80% confluency in a T75 flask are harvested by aspirating off the medium, rinsing with trypsin/EDTA, and incubating in 3 mL trypsin/EDTA for 3–5 min at 37°C. The trypsin reaction is stopped with 7 mL of growth medium.
3. After aspirating Dulbecco's PBS from the collagen-coated dishes, a final volume of 2 mL of growth medium containing 12 m*M* HEPES, pH 7 4 and cells at a density of 6×10^5 is added to the Petri dishes and allowed to incubate for 20–24 h.

3.2. Stimulation Protocol

1. On the day of the experiment, the growth medium is aspirated and 2 mL of experimental medium is added, then incubated for 3 h. Our cells can survive 9 h without serum, but other cell lines should be tested for special requirements
2 One hour before the experiment, warm the microscope chamber to 37°C and warm the tubing and oil
3 To minimize dilution errors, we use volumes of at least 5–10 μL For example, add 490 μL of experimental medium to 10 μL of EGF, or 390 μL of medium to 10 μL of TGFα for a final concentration of 1 μ*M* Add 250 μL of this 1 μ*M* stock to 4 75 mL of experimental medium (now 50 n*M*). Serially dilute 1 5 (1 mL into 4 mL medium) for concentrations of 10, 2, 0.4, and 0 08 n*M* Since these will be diluted twofold by adding 2 mL to the Petri dish during experimentation, the final experimental concentrations are 25, 5, 1, 0 2, and 0 04 n*M*, respectively A tube with 4 mL of experimental medium will also be needed as a control
4 A Petri dish is covered with a thin layer of mineral oil and moved to the microscope chamber and equilibrated for 30 min. (A new dish should be brought over from the incubator prior to the beginning of each experiment since the experimental time is approx 30 min each)
5. For viewing with a 10X lens, a field in which about 10 cells are visible, but not in contact with each other or any other cell, is chosen For higher magnification, the number of cells in the microscope field will be reduced
6 Once the field is chosen, record for 5 min prior to addition (*see* Section 3 3)
7 During these 5 min, prerinse tubing with 2 mL of experimental medium to prime tubing. Then pull up the remaining 2 mL for the stimulation
8 After the 5 min have passed, add the 2 mL of medium to the Petri dish by placing the tip of the bent micropipet just under the oil layer To prevent bubbles, stop

just after the last of the medium is added. The addition will take about 30 s and the media will be completely mixed in about 1 min

9 After addition, record for 15 min more or as long as appropriate (*see* Section 3 3)

10 Repeat steps 4–9 for each experimental condition.

3.3. Data Acquisition by NIH Image

1 Spatial calibration is necessary for proper measurements If all experiments will be performed with the same objective, a calibration frame needs to be collected only once. Collect an image of the micrometer slide in a horizontal orientation and one in the vertical Collection can be done by "Start Capture," "Stop Capture" in the Special menu and "Save" in the File menu Using the line measurement tool, check whether the pixels are true squares by measuring a given unit in the horizontal direction and the same unit in the vertical direction If they are equal, simply use the "Set Scale" command in the Analyze menu to choose the units and to input the known length in pixels If the number of pixels per given unit is different in the horizontal and vertical, an aspect ratio, the width divided by the height, also should be entered

One problem common to tube based video cameras is vigneting, the edges tend to appear stretched. If you are using a tube camera, collect additional images with the scale at each edge of the field If the field vignets, area measurements may not be possible and a different camera will need to be used.

2 For organization of data, utilize a separate folder for each stimulation. Prior to the experiment, create a folder for the day's experiment In this folder, create a folder for each experimental condition For instance, create a folder named "MTLn3 April 29, 1996." In this folder, create folders named " 10 n*M*," "2 n*M*," "0.4 n*M*," "0.08 n*M*," and "buffer"

3 If a video recorder is being used, begin recording. The video recording of the experiment is used as a backup to the NIH Image data.

4 Execute NIH Image. Go to the "Special" menu and select "Start Capturing." If the field is correct and in focus, draw a rectangular region of interest or go to the "Edit" menu and choose "Select All " In the "Stacks" menu, select "Make Movie " Input the number of frames and amount of time between frames (we use 20 frames at 60 s intervals) Pressing "OK" for the specified time interval begins the time lapse image collection.

5 After the experiment is complete, stop the VCR and in NIH Image go to "Save As" in the "File" menu. Save the file as a tiff or pics and in the appropriate folder

6 Repeat steps 3–5 for all conditions

3.4. Sample Area Measurements with NIH Image

Basic measurements of area, location change, or shape can be made within NIH Image. For example, Fig. 1 shows the area and speed of a cell at 36 different time points before, during, and after addition of EGF. Images of the cell at the 36 time points at 15 s intervals were stored on disk and measured by the following method:

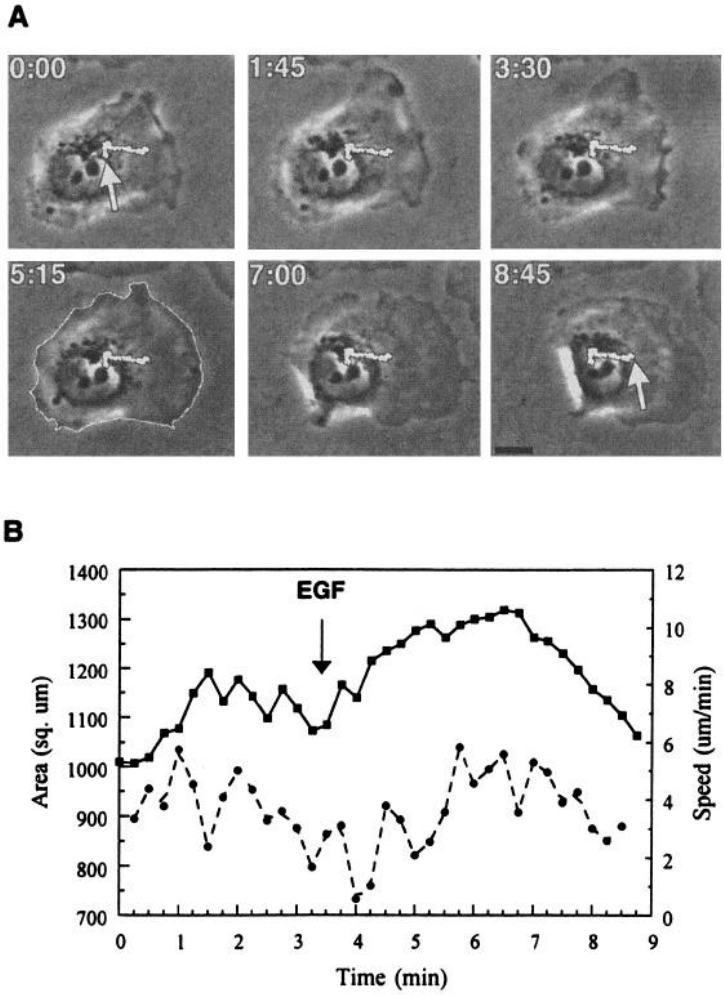

Fig. 1. Response of an MTLn3 cell to EGF. **(A)** Images of an MTLn3 cell at 1 min, 45-s intervals during an experiment. At 3.5 min the cell was stimulated with 5 n*M* EGF. The thick white line in the center of the cell tracks the path of the cell center for all 36 time-points. The arrow at 0:00 points to the initial cell center position and the arrow at 8:45 points to the terminal cell center position. The white line at the perimeter of the cell at 5:15 is an example of perimeter tracing. Scalebar is 10 μm. **(B)** Cell area (squares, solid line) and velocity (circles, hashed line) were calculated as described in the text. Soon after stimulation the cell stops briefly, then continues moving as it extends new lamellipods. The area increases by 10–20%, peaking 3–4 min after stimulation.

1. In the "Analyze" menu "Reset" was chosen to clear all past measurements.
2. In the "Options..." window under the "Analyze" menu bar "Area" and "X-Y Center" were checked. Note that X-Y center provides the x and y coordinates of

the center of the best fitting ellipse Another useful option is "Perimeter/Length" for calculating roundness

3. Using the freehand select region tool, the cell was traced in each consecutive time lapse image. The area was then measured by selecting "Measure" under the "Analyze" menu bar
4. "Show Results" under the "Analyze" menu bar was chosen to display the list of measurements
5. To save the results in a file, "Export" was chosen under the "File" menu bar "Measurements" was checked and a file name was given
6. This measurements file was opened into Microsoft Excel to plot the column of area measurements. Any spreadsheet or plotting program could have been used for the simple plot or for calculations such as plotting the change in area from one frame to the subsequent

To perform these steps and to calculate the velocity directly from the measured X-Y centers, we used the following macro (*see* Note 6 for more on macros):

```
{MACRO 'Measure and mark center movement [1]';
TO USE THIS MACRO:
1. Set the options for measurement in the Analyze --> Options menu.
   For instance: Area, Perimeter, X-Y Center, rUser1. This macro
   stores the distance between two consecutive cell centers in
   rUser1.
   Also click on a color to mark the distance between cell centers.
   If white is chosen, the complete path can be shown by performing
   a maximum pixels projection on the time lapse stack subsequent to
   measuring.
2. Reset the measurments by Analyze --> Reset.
3. Beginning with frame 1 of a stack of images, trace the cell. Type
   the number 1 or Special --> Measure and mark center movement. The
   macro automatically advances the stack to the next frame  Repeat
   until time lapse sequence is completed.
4. Show results in the Analyze menu. In the File menu Save As or
   Export as measurments.
*****This macro does not perform any error checking.*****
}
MACRO 'Measure and mark center movement [1]';
VAR
  x, y,                  {location of current cell center}
  oldx, oldy : integer; {location of previous cell center}

BEGIN
  InvertY(false);
  Measure;
  x := rX[rCount]; y := rY[rCount]; {center of traced cell}
  IF rCount > 1 THEN{if not the first timepoint, then measure the }
    BEGIN          {distance between this and last cell center    }
      MakeLineRoi(rUser1[rCount + 1], rUser2[rCount + 1], x, y);
      Measure;
      Fill;        {mark the distance traveled on the image       }
```

```
      rUserl[rCount - 1] := rLength[rCount];
      SetCounter(rCount - 1);
    END
  ELSE
    rUserl[rCount] := -1; {if first timepoint, no length stored}
  rUserl[rCount + 2] := x;
  rUser2[rCount + 2] := y;
  IF SliceNumber < nSlices THEN  {step to next timepoint}
    SelectSlice(SliceNumber + 1);
  KillRoi;
END; {'Measure and mark center movement [1]'}
```

3.5. Sample Vesicle Movement with NIH Image

A vesicle can be tracked by simply recording its location and measuring the distance between these locations. A simple macro could be written to record these locations. For instance, the measurement counter would be reset. Then, for each time lapse image in sequence, using the mouse the user would put the cursor over the vesicle and press a letter, for instance, "v." The macro would draw a straight line region of interest between the current point and the previous, measure this distance and put this result in a list The macro could also keep a running total of the distance between points The macros could also provide other information, such as angle.

3.6. Velocity

Velocity of vesicle or cell movement utilizes the *xy* coordinates of the centroid of the cell or vesicle as a function of time as described in Sections 3.4. and 3.5 By using a spreadsheet program these values can then be used to calculate velocity according to several different formulas. The simplest function to use is to calculate the distance between two points divided by the time interval between them: v = sqrt $((x_1 - x_2)^2 + (y_1 - y_2)^2)/(t_1 - t_2)$, where x_n, y_n, and t_n are the *xy* coordinates and *t* the time for a a particular centroid movement. A slightly more sophisticated calculation would average the speed between t_{n-1} and t_n with the speed between tn and t_{n+1} to determine the speed at t_n (which was done in Fig 1)

4. Notes

1. An inverted microscope with long working-distance optics is highly recommended for cell culture work. We typically use a Nikon Diaphot with a long working-distance phase contrast objectives (10X to 40X) and matching condenser. The predominance of imaging is done using phase contrast. One of our microscopes has a beam splitter that sends 50% of the light to the video camera port and 50% of the light to the occulars. This has the benefit of user ease in making microscope adjustments It has the detriment of lower light levels to the camera and, hence lower image quality Our other microscope has a 100% beam splitter which sends all of the light to the camera port or to the occulars

If cells need to be at a specific temperature or in a gaseous environement, an environmental chamber may be necessary. A stage heater may be adequate if stimulus solutions can be maintained at the proper temperature In a system with a chamber, solutions can be kept inside. Most microscope vendors can provide environmental chambers. Otherwise, resilient chambers can be fashioned from plexiglass or temporary ones from plastic sheeting and wooden dowels.

An alternative to an inverted microscope could be an upright microscope with water immersion optics that could be submerged in the cell culture medium.

2. A standard light source for video microscopy is a halogen bulb, but any lamp on a microscope will probably work Make sure the light source is stable. We have found that a high quality voltage regulator (e g., Type 1646 BK Precision) is more stable than the standard rheostat supplied by Nikon. Fluctuations may not be noticeable by eye, but they are especially problematic with a tube camera such as a Newvicon if quantitation of light intensity is desired.

   As for maximum performance with most black and white films, a green interference filter is recommended between the light source and the microscope condenser. A narrow band of wavelengths performs best with most microscope optics The green wavelength's position in the center of the visible spectrum helps protect the cells from ultraviolet light, which may be damaging, and from infrared light, which may be excessively heating. We also use UV and IR cut filters

   The system as described so far involves constant illumination of the sample, but some samples may act adversely to light To minimize this problem, a mechanical shutter may be introduced into the light path between the light source and the condenser lens. There are shutters that can be plugged into the computer serial port and simply commanded by macros. At each timepoint the shutter would be triggered to open immediately before collection of an image and would close immediately following collection

3. There is a wide range of black and white video cameras They run from high resolution and very expensive, including digital equipment which may replace video equipment in the future, to lower priced and more practical We use a Newvicon tube camera or a small CCD camera Companies that sell to scientists include Hamamatsu, Dage and Optronics among others, but now CCD cameras are available from a wide range of sources at reasonable prices For applications where neither high resolution nor linear light response is critical, just about any camera, including a color camera, is useable

   Although the video signal from the camera fed directly to the computer for digitizing is sufficient for the analysis outlined in this article, we send the video through other devices as well We loop the video signal through a monitor and through a video tape recorder before sending it to the computer digitizer.

   For convenience we send the video through a monitor so that we can see live, at video rates or "real time," the images that the camera is capturing. Security or surveillance monitors, such as the Panasonic TR-930B, are inexpensive and are useful for assessing focus or location of cells within the field, however, they cannot be used for assessing lighting conditions

We loop the video through a VCR so that we can video record each experiment independent of computer digitization. This archiving of raw data protects against computer problems during digitization and allows for subsequent digital resampling of timepoints at different intervals. (In the absence of a computer within proximity to a microscope, all experiments can be videotaped and digitized off tape later) Any VCR will work. We use SVHS (Panasonic AG-1970) because it is the highest quality currently available by a relatively affordable machine

4 Any recent Macintosh (such as the Centris, Quadra, PowerPC, some notebooks) can be used. The amount of RAM necessary depends on demand To view and analyze sequences image by image, 8 MB is sufficient To rapidly view full sequences of full window images, which are typically 640 × 480 pixels, more memory is needed Including the RAM needed by the system software, 24 MB is necessary for a 36 full window sequence. By using virtual memory, it is possible to extend RAM by using the hard disk as a working space, but this may be disturbingly slow If it is not necessary to animate sequences for viewing and if use of virtual memory is not preferred, long sequences can be collected, stored and analyzed from the hard disk, *see* Note 7

At the time of this writing, NIH *Image* supports four frame grabber cards. We have used three of them Our favorite is the Scion LG-3 (Scion Corporation, Frederick, MD) which we also use for other applications We also use the Data Translation QuickCapture DT2255 board (Data Translation, Inc , Marlboro, MA) The video card that is packaged with AV Quadras and Power Macintoshes can also be used for time lapse from macros where parameters such as shape, size and location are important, but it has poor resolution and cannot be used for densitometry because of an automatic gain function.

Because image files are large and a large number need to be analyzed, a data backup system is recommended There are a variety of solutions including magneto optical disks that provide random access workspace, tape drives for high quantity backup or access to a server. A current recommendation at the time of writing is the Zip Drive or the Olympus 230MB magneto-optical.

5. NIH *Image* is a comprehensive image processing and analysis software package that runs on the Macintosh (a version of *Image* for Windows95 is under development by the Scion Corporation). It is free (in the public domain), feature rich, highly flexible and user friendly. The software is continually updated and has a well subscribed bulletin board on the Internet where a wide range of expert users and developers communicate advice. The following summarizes some of the information provided in the manuals that can be downloaded with NIH *Image*
   a. If World Wide Web access is available, NIH *Image* can be found at the URL http://rsb.info nih gov/nih-image/.
   b. If complete Internet access is available, the best way to get NIH *Image* is by annonymous ftp Using Fetch or another ftp-like program, NIH *Image* software including documents, source code, macros and more can be retrieved from zippy.nimh nih gov (128 231 98 32) in the directory/pub/nih-image

c Fetch is one public domain software package for retrieving data over the network. In Fetch, open a connection to the host "zippy nimh.nih.gov" with the user id "anonymous," your email address as the password and "/pub/nih-image" as the directory In the customize menu under the topic "Formats," "BinHex 4 Format" and "MacBinary II Format" must be turned on

d If Internet access is limited to electronic mail, subscribe to the NIH *Image* mailing list by sending a message containing the line "subscribe nih-image <your name>" to listproc@soils.umn.edu Next obtain a list of the available NIH *Image* archive files by sending an "index nih-image" command to listproc@soils umn.edu. These files can then be retrieved by means of a "get nih-image filename" command. The files are Binhexed and broken into chunks less than 32K in size. The NIH mailing is maintained by the Soil Science Department at the University of Minnesota

e. On CompuServe, Library 9 (Graphics Tools) of the MACAPP forum contains the program and Library 6 of the MACDEV forum has the source code

f A recent version, but probably not as updated as available from zippy.nimh.nih.gov, is available by mail from National Technical Information Service (NTIS), 5285 Port Royal Road, Springfield, VA 22161, phone. 703-487-4650, fax· 703321-8547, order number PB95-50019SGEI ($100 check, VISA, or Mastercard).

6 Macros allow users to perform repetitive and multiple command sequences with a single keystroke or mouse click Macros are written in a text window within NIH *Image* The macro commands can be found at the end of the software documentation and are strung together within a pascal-like structure for interpretated execution when called

Many macros are available when retrieving the software The macro library includes extended manipulation of stacks, routines for processing data collected on other platforms and some image analysis, among many others

7. The "Make Movie.. " command for image collection requires sufficient computer memory to store the entire sequence If your computer has insufficient memory, there are three options for collecting and measuring longer sequences. One is to collect a small region of interest within the camera window. A second is to turn on virtual memory within the computer operating system and to increase the amount of memory available to NIH *Image* Another method is to save each image to disk as it is collected. The last method, with or without combination with the second method, allows for collection of images limited solely by free disk space

The following macro is an example of saving unlimited length sequences to disk. When executed, the macro prompts the user for the number of images to be collected, the time interval in seconds between images and a file name for the sequence of images. No error checking for valid values is performed.

To load macros from a file on disk, use the "Load Macros. " command in the "Special" menu. Macros are simple text files and can be typed in by opening a new text window within NIH *Image*. A single text file can contain many macros or procedures.

```
{ MACRO 'Collect unlimited images [c]';
TO USE THIS MACRO:
1. Use the Special --> Start Capturing command. When you see a field
   you want to collect an image of, run this macro.
2. Input the number of images you want to collect.
3. Input the time between each image in seconds  This time is nomi-
   nal  The time between each image is actually this wait time plus
   the time necessary for the videocard to perform a capture plus
   disk access time. The user needs to test times on his/her com-
   puter.
4. Input a file name. If the name does not include a complete path
   to valid folders, you will be prompted to check saving of the
   first image; all subsequent images will be saved unprompted.
5. The macro stops when all images are collected. To stop macro
   before this, use <open apple>. to cancel. All images up the the
   time of cancelation are saved.}

MACRO 'Collect unlimited images [c]';
VAR
  filename : string;  { name of image files }
  frames : integer;   { number of frames to be collected }
  time : integer;     { time in seconds }
  counter : integer,  { counts frame collection in FOR loop }
  year, month, day,
  hour, minute, second, dayofweek : integer; { used for timing }
BEGIN
  frames := GetNumber('How many images do you need? ', 0);
  time := GetNumber('Input time between images not including video card
and disk access time!, 0);
  filename .= GetString('Please name this sequence: ', 'untitled');

  SaveState;             { computer save old fonts, colors, etc. }
  SetFont('Helvetica'); { set font, colors for this routine }
  SetFontSize(10);
  SetText('Bold');
  SetBackgroundColor(255);
  SetForegroundColor(0);

  FOR counter := 1 TO frames DO { collect the time lapse images }
    BEGIN
      Capture; { collect the image }

      { stamp the time as text on each image }
      GetTime(year,month,day,hour,minute,second,dayofweek);
      MoveTo(8,8),
      Writeln(month, '/', day, '/', year);
      Writeln(hour, ':', minute, '.', second),

      SaveAs(filename, ' ', counter:3); { save to disk }

      Wait(time); { wait in seconds before next collection }
    END; { collection loop }

  RestoreState; { computer restores old fonts, colors, etc. }
END; { MACRO 'Collect unlimited images [c]' }
```

This macro performs a minimum of steps to collect the sequence Improvements might include allowing the user to collect a region of interest within the field instead of the whole field Another would be to change the "Capture" command to "AverageFrames" to improve image quality if either the experiment is of very slow moving objects or if the computer has a fast video card

8 The number of image analysis programs available for the Macintosh and PC is increasing rapidly Two programs that have a number of more sophisticated options for cell analysis are 2D-DIAS (Solltech, Oakdale, IA) and Image-1/Metamorph (Universal Imaging Corp., West Chester, PA). DIAS provides functions for automatically identifying cells through successive frames and measuring a number of additional parameters such as speed, shape, direction of movement, area flow in pseudopods, and so on.

9 Updates of the macros and biology protocol in Section 2 1 will be posted on the Analytical Imaging Facility web page accessible via the Albert Einstein College of Medicine webpage at http.//www aecom.yu edu/.

Acknowledgments

This work was funded in part by grants from the NIH and USAMRDC and performed in the Analytical Imaging Facility of the Albert Einstein College of Medicine Cancer Center supported by NIH grant P30 CA 13330.

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Appendices

Compiled by Jeffrey W. Pollard

Table 1
Balanced Salt Solutions[a]

Component	Earle's, g/L[b]	Hank's, g/L[b]	Gey's, g/L[b]	Puck's, g/L[b]	Dulbecco's PBS, g/L[c,d]
KCl	0 4	0 4	0 37	0 4	0 2
KH_2PO_4	—	0.06	0 03	0 15	0.2
$MgCl_2 \cdot 6H_2O$	—	0.1	0 21	—	0 1
$MgSO_4 \cdot 7H_2O$	0 2	0 1	0 07	0 154	0.1
$CaCl_2$	0 2	0 14	0 17	0 012	
NaCl	6 68	8	7	8	8
$NaHCO_3$	2 2	0 35	2 27	—	—
$NaH_2PO_4 \cdot H_2O$	0 14	—	—	—	—
$Na_2HPO_4 \cdot 7H_2O$	—	0 09	0 226	0 39[e]	2 16
Glucose	1	1	1	1	—
Phenol Red	0 01	0.01	—	0 005	—
References	*1*	*2*	*3*	*4*	*5*

[a]For detailed description of derivations, *see* ref *6*
[b]Sterilize by filtration
[c]Sterilize by autoclaving
[d]Dulbecco's PBS is often prepared without Mg^{2+} and Ca^{2+} for use in trypsinization
[e]$12H_2O$

From *Methods in Molecular Biology, Vol 75 Basic Cell Culture Protocols*
Edited by J W Pollard and J M Walker Humana Press Inc , Totowa, NJ

Table 2
Eagle's Minimum Essential Medium and Derivatives[a]

Component	Eagle's MEM, mg/L	Dulbecco's modification DMEM, mg/L	αMEM[b] mg/L	Iscove's modified DMEM, mg/L	Joklik's MEM, mg/L
Amino acids					
L-Alanine	—	—	25	25	—
L-Arginine HCl	126 4	84	126 4	84	105
L-Asparagine	—	—	50	25	—
L-Aspartic acid	—	—	30	30	—
L-Cysteine HCl H_2O	—	—	100	—	—
L-Cysteine	24	48	24	48	25
L-Glutamic acid			75	75	
L-Glutamine	292	584	292	584	294
Glycine	—	30	50	30	—
L-Histidine HCl · H_2O	42	42	42	42	42
L-Isoleucine	52 5	104 8	52 5	104 8	52
L-Leucine	52 5	104 8	52 5	104 8	52
L-Lysine HCl	73 06	146.2	73.1	146.2	72.5
L-Methionine	14 9	30	14 9	30	15
L-Phenylalanine	33 02	66	33.02	66	32
L-Proline	—	—	40	40	—
L-Serine	—	42	25	42	—
L-Threonine	47.64	95.2	47 64	95.2	48
L-Tryptophan	10 2	16	10 2	16	10
L-Tyrosine	36 22	72	36 22	—	37 8
L-Tyrosine disodium salt	—	—	—	104.2	—
L-Valine	46 9	93 6	46 9	93 6	46
Vitamins and lipids					
L-Ascorbic acid	—	—	50	—	—
Biotin	—	—	0 1	0 013	—
D-Ca pantothenate	1	4	1	4	1
Choline chloride	1	4	1	4	1
Folic acid	1	4	1	4	1
I-inositol	2	7 2	2	7 2	2
Nicotinamide	1	4	1	4	1
Pyridoxal HCl	1	4	1	4	1
Riboflavin	0 1	0 4	0 1	0 4	0 1
Thiamine HCl	1	4	1	4	1
Vitamin B12	—	—	1 36	0.013	—
Cholesterol	—	—	—	0 02	—

(continued)

Table 2 ***(continued)***

Component	Eagle's MEM, mg/L	Dulbecco's modification DMEM, mg/L	αMEM[b] mg/L	Iscove's modified DMEM, mg/L	Joklik's MEM, mg/L
$CaCl_2$	200	200	200	165	—
$Fe(NO_3)_3$ $9H_2O$	—	0 1	—	—	—
KCl	400	400	400	330	400
$MgSO_4 \cdot 7H_2O$	200	200	200	—	242 2
$MgSO_4$ (anhyd)	—	—	—	97 67	—
NaCl	6800	6400	6800	4505	6500
$NaHCO_3$	2200	3700	2000	3024	2000
NaH_2PO_4 $2H_2O$	158	141 3	158 3	125	1500
KNO_3	—	—	—	0.076	—
$Na_2SeO_3 \cdot 5H_2O$	—	—	—	0 0173	—
Others					
Adenosine	—	—	10	—	—
Cytidine	—	—	10	—	—
Deoxyadenosine	—	—	10	—	—
Deoxycytidine	—	—	10	—	—
Deoxyguanosine	—	—	10	—	—
Dihydrostreptomycin sulfate[c]	—	—	—	—	50
Bovine serum albumin	—	—	—	0 4	—
Glucose	1000	4500	1000	4500	2000
Guanosine	—	—	10	—	—
HEPES	—	—	—	5958	—
Lipoic acid	—	—	0 2	—	—
Penicillin G[c]	—	—	—	—	75000 IU
Sodium phenol red	17	15	10	15	10
Sodium pyruvate	—	110	110	110	110
Soybean lipid	—	—	—	0 1	—
Thymidine	—	—	10	—	—
Transferrin	—	—	—	0 001	—
Uridine	—	—	10	—	—
References	*7*	*8*	*9*	*10*	*11*

[a]For full lists of tissue-culture medium and references, *see* ref *11* It should be noted that medium formulations vary somewhat from company to company and list to list Confirm the exact composition of their media with the company that you purchase the media from

[b]αMEM is often supplied without ribo- and deoxyribo-nucleosides

[c]Antibiotics are often supplied with the medium or they can be added during preparation (*see also* Table 4)

Table 3
Other Useful Media[a]

Component	RPMI 1640, mg/L	HAM'S F12, mg/L	CMRL[b] 1066, mg/L	McCoy's 5A, mg/L	Medium 199, mg/L	Waymouth's MB752/1, mg/L
L-alanine	—	8 9	25	13 9	25	—
L-arginine free base	200	—	—	—	—	—
L-arginine HCl	—	211	70	42 1	70	75
L-asparagine	50	—	—	45	—	—
L-asparagine H_2O	—	15 01	—	—	—	—
L-aspartic acid	20	13 3	30	19.97	30	60
L-cysteine (free base)	—	—	—	31 5	—	61
L-cysteine · HCl H_2O	—	35 12	260	—	—	—
L-cysteine HCl	—	—	—	—	0 1	—
L-cystine	50	—	20	—	—	15
L-cysteine, disodium salt	—	—	—	—	23.66	—
L-glutamic acid	20	14 7	75	22 1	66 82	150
L-glutamine	300	146	100	219 2	100	350
Glycine	10	7 5	50	7 5	50	50
L-Histidine free base	15	—	—	—	—	128
L-Histidine HCl H_2O	—	20 96	20	20 96	21 88	—
Hydroxy-L-proline	20	—	10	—	10	50
L-Isoleucine	50	3 94	20	39 36	20	25
L-Leucine	50	13 1	60	39 36	60	50
L-Lysine · HCl	40	36 5	70	36.5	70	240
L-Methionine	15	4.48	15	14.9	15	50
L-Phenylalanine	15	4.96	25	16.5	25	50
L-Proline	20	34 5	40	17 3	40	50
L-Serine	30	10 5	25	26 3	25	—
L-Threonine	20	11 9	30	17 9	30	75
L-Tryptophan	5	2 04	10	3 1	10	40
L-Tyrosine	20	5 4	40	18 1	40	40

(continued)

Table 3 ***(continued)***

Component	RPMI 1640, mg/L	HAM'S F12, mg/L	CMRL[b] 1066, mg/L	McCoy's 5A, mg/L	Medium 199, mg/L	Waymouth's MB752/1, mg/L
L-Valine	20	11.7	25	17.6	25	65
Vitamins						
L-Ascorbic acid	—	—	50	0 5	0 05	17 50
Biotin	0 2	0.0073	0 01	0.2	0.01	0.02
Calciferol	—	—	—	—	0 01	—
D-Ca Pantothenate	0 25	0 48	0.01	0 2	0 01	1
Choline chloride	3	13.96	0 5	5	0 5	250
Folic acid	1	1.3	0 01	10	0 01	0.4
I-inositol	35	18	0.05	36	0 05	1
Menadione	—	—	—	—	0 01	—
Niacin	—	—	0 025	0 5	0 025	—
Nicotinamide	1	0 04	0 025	0 5	0 025	1
p-Aminobenzoic acid	1	—	0.05	1	0 05	—
Pyridoxal HCl	—	—	0 025	0 5	0 025	—
Pyridoxine · HCl	1	0 062	0 025	0 5	0 025	1
Riboflavin	0 2	0 038	0 01	0 2	0 01	1
Thiamine HCl	1	0 34	0.01	0 2	0.01	10
DL-α Tocopherol phosphate, disodium salt	—	—	—	—	0 01	—
Vitamin A acetate	—	—	—	—	0.115	—
Vitamin B12	0 005	1.36	—	2	—	0.2
Inorganic salts						
$CaCl_2$ · anhyd.	—	—	200	100	—	—
$CaCl_2$ $2H_2O$	—	44	—	—	264.9	120
$CaNO_3$ $4H_2O$	100	—	—	—	—	—
$CuSO_4$ $5H_2O$	—	0.0025	—	—	—	—
$Fe(NO_3)_3 \cdot 9H_2O$	—	—	—	—	0.72	—
$FeSO_4 \cdot 7H_2O$	—	0.834	—	—	—	—
KCl	400	223.6	400	400	400	150
KH_2PO_4	—	—	—	—	60	80
$MgCl_2 \cdot 6H_2O$	—	122	—	—	—	240

(continued)

Table 3 ***(continued)***

Component	RPMI 1640, mg/L	HAM'S F12, mg/L	CMRL[b] 1066, mg/L	McCoy's 5A, mg/L	Medium 199, mg/L	Waymouth's MB752/1, mg/L
$MgSO_4 \cdot 7H_2O$	100	—	200	200	200	200
NaCl	6000	7599	6799	6400	6800	6000
$NaHCO_3$	2200	1176	2200	2200	2200	2240
$NaH_2PO_4 \cdot H_2O$	—	—	140	580	158 3	—
$Na_2HPO_4 \cdot 7H_2O$	1512	268	—	—	—	566
$ZnSO_4 \cdot 7H_2O$	—	0.863	—	—	—	—
Other Components						
Adenine sulphate	—	—	—	—	10	—
5'-AMP	—	—	—	—	0 2	—
ATP, disodium salt	—	—	—	—	1	—
Bactopeptone	—	—	—	600	—	—
Cholesterol	—	—	0 2	—	0.2	—
2-deoxyribose	—	—	—	—	0 5	—
Glucose	2000	1802	1000	3000	1000	5000
Glutathione (reduced)	1	—	10	0 5	0 05	15
Guanine · HCl	—	—	—	—	0.3	—
Hypoxanthine	—	4 1	—	—	0.3	25
Linoleic acid	—	0 084	—	—	—	—
Lipoic acid	—	0.21	—	—	—	—
Phenol Red	5	1 2	20	10	15	10
Putrescine · 2HCl	—	0 161	—	—	—	—
Ribose	—	—	—	—	0 5	—
Sodium acetate · $3H_2O$	—	—	83	—	50	—
Sodium glucoronate	—	—	4.2	—	—	—
Sodium pyruvate	—	110	—	—	—	—
Triphosphopyridine nucleotide	—	—	1	—	—	—
Thymidine	—	0.73	10	—	—	—
Thymine	—	—	—	—	0.3	—
Tween-80	—	—	5	—	5	—

(continued)

Table 3 ***(continued)***

Component	RPMI 1640, mg/L	HAM'S F12, mg/L	CMRL[b] 1066, mg/L	McCoy's 5A, mg/L	Medium 199, mg/L	Waymouth's MB752/1, mg/L
Uracil	—	—	—	—	0 3	—
Uridine triphosphate $4H_2O$	—	—	1	—	—	—
Xanthine	—	—	—	—	0 3	—
CoCarboxylase	—	—	1	—	—	—
CoEnzyme A	—	—	2.5	—	—	—
Deoxyadenosine	—	—	10	—	—	—
Deoxycytidine HCl	—	—	10	—	—	—
Deoxyguanosine	—	—	10	—	—	—
Diphosphopyridine nucleotide $4H_2O$	—	—	7	—	—	—
Ethanol (for lipid component)	—	—	16	—	—	—
Flavin adenine *n* nucleotide	—	—	1	—	—	—
5-methyl-deoxycytidine	—	—	0 1	—	—	—
References	*12*	*13*	*11*	*14*	*15*	*16*

[a]For a full list of the medium, their modifications and references, *see* refs *11*, *13*, and *17*
[b]Can be made with Hanks' salts rather than Earle's salts

Table 4
Useful Antibiotics for Tissue Culture

Antibiotic	Spectrum of action	Recommended concentration μg/mL[a]	Approximate stability, d
Amphotericin B	Fungi and yeast	1	3
Ampicillin	Gram-positive and negative bacteria	100	3
Chloramphenicol	Gram-negative bacteria	5	5
Erythromycin	Gram-positive bacteria and mycoplasma	100	3
Gentamycin	Gram-positive and gram-negative bacteria and mycoplasma	50	5
Kanamycin	Gram-positive and negative bacteria	100	5
Nystatin	Fungi and yeast	50	3
Penicillin G	Gram-positive bacteria	100	3
Rifampicin	Gram-positive and gram-negative bacteria	50	3
Streptomycin	Gram-positive and gram-negative bacteria	100	3
Tetracycline	Gram-positive, gram-negative bacteria and mycoplasma	10	4

[a]The concentration given is sufficient to control a mild infection for the length of time stated at 37°C without undue toxicity to cells (*see* ref *18* for greater detail). Most media contain streptomycin and penicillin G The use of other antibiotics, particularly clinically relevant ones such as erythromycin, should not be encouraged unless absolutely necessary This is because media invariably goes into the drainage system and so increases the range of drug-resistant "wild" bacteria

Table 5
Insect Cell Medium

Grace's insect tissue-culture medium *(19)*	
Ingredient	mg/L
L-Isoleucine	50
L-Phenylalanine	150
L-Tryptophan	100
L-Leucine	75
L-Histidine · HCl H_2O	3700
L-Methionine	50
L-Valine	100
L-Arginine · HCl	700
L-Lysine · HCl	625
L-Threonine	175
L-Asparagine · H_2O	397.7
L-Proline	350
L-Glutamine	600
DL-Serine	1100
Glycine	650
L-Alanine	225
β-Alanine	200
L-Cystine disodium salt	22 69
L-Tyrosine disodium salt	62 15
L-Glutamic acid	600
L-Aspartic acid	350
D-Sucrose	26680
D-Fructose	400
D-Glucose	700
L-Malic acid	670
α-Ketoglutaric acid	370
D-Succinic acid	60
Fumaric acid	55
p-Aminobenzoic acid	0.02
Folic acid	0.02
Riboflavin	0.02
Biotin	0 01
Thiamine · HCl	0.02
D-Calcium pantothenate	0.02
Pyridoxine · HCl	0.02
Nicotinic acid	0.02
I-Inositol	0.02
Choline chloride	0.2
$NaH_2PO_4 \cdot 2H_2O$	1140

(continued)

Table 5 *(continued)*

Grace's insect tissue-culture medium *(19)*	
Ingredient	mg/L
$CaCl_2 \cdot 2H_2O$	1325
$MgCl_2 \cdot 2H_2O$	2280
$MgSO_4$ $7H_2O$	2780
KCl	2800
$NaHCO_3$	350

BML-TC/10[a] for insect cell culture and production of nuclear polyhedrosis virus *(20)*	
Ingredient	Values/L
Fetal calf serum	100 mL
Tryptose broth	2.6 g
KCl	2.87 g
NaH_2PO_4	1.14 g
$CaCl_2 \cdot 2H_2O$	1 32 g
$MgCl_2 \cdot 6H_2O$	2.28 g
$MgSO_4$ $7H_2O$	2.78 g
$NaHCO_3$	0.35 g
Glucose	1 g

[a]Plus Grace's vitamins and amino acids without β-alanine and D-serine as above

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