

Use of the Fibroblast Model in the Study of Cellular Senescence

Vincent J. Cristofalo, Craig Volker, and Robert G. Allen

1. Introduction

In this chapter, we present standard procedures for the culture of human cells that exhibit a finite proliferative capacity (replicative life-span). The use of a cell culture model has the advantage of providing a controlled environment to study a wide variety of cellular phenomena. It also has the inherent limitation of isolating cells from the regulatory elements that might be provided by other types of cells *in vivo*. Nevertheless, cell culture models have been crucial to our current understanding of mechanisms of growth, differentiation, development, and neoplasia and numerous other disease states. In this chapter we present procedures for human fibroblast culture including serum-free cultivation of cells, which is necessary when the cellular environment must be fully defined. In addition, we present procedures for the determination of replicative life-span, saturation density, and assessment of replicative capacity from labeled thymidine incorporation in fibroblasts. The methods described here have been well tested and provide highly reproducible results (1,2).

1.1. Cellular Senescence

Phenotypically and karyotypically normal human cells exhibit a limited capacity to proliferate in culture (3,4). This finite proliferative potential of normal cells in culture is thought to result from multiple changes (5) and has frequently been used as one model of human aging. Although most replicative life-span data are derived from fibroblasts, other types of cells such as glial cells (6), keratinocytes (7), vascular smooth muscle cells (8), lens cells (9), endothelial cells (10), lymphocytes (11), liver (12), and melanocytes (13) are also known to exhibit a limited replicative life-span in culture. Both environ-

mental and genetic factors appear to influence the proliferative life-span of fibroblasts from normal individuals (5,14,15). Not all of the determinants of proliferative capacity are known; however, a variety of changes are associated with the decline of proliferative capacity including changes in gene expression, telomere shortening, and signal transduction. These are all thought to be important factors that influence replicative life-span (15–20).

1.1.1. *Telomere-Shortening*

Loss of telomeric repeats is tightly linked to the cessation of mitotic activity associated with cellular senescence (16,17,21,22). The telomeres of human chromosomes are composed of several kilobases of simple repeats (TTAGGG)_n. Telomeres protect chromosomes from degradation, rearrangements, end-to-end fusions, and chromosome loss (23). During replication DNA polymerases synthesize DNA in a 5' to 3' direction; they also require an RNA primer for initiation. The terminal RNA primer required for DNA replication cannot be replaced with DNA, which results in a loss of telomeric sequences with each mitotic cycle (21,23). Cells expressing T antigen are postulated to exhibit an increase in their proliferative life-span because they are able to continue proliferating beyond the usual limit imposed by telomere length (24). Immortalized and transformed cells exhibit telomerase activity that compensates for telomere loss by adding repetitive units to the telomeres of chromosomes after mitosis (23,25–27). Cultures derived from individuals with Hutchinson–Gilford syndrome (28) often exhibit decreased proliferative potential, albeit results with these cell lines are variable (29). Fibroblast cultures established from individuals with Hutchinson–Gilford progeria syndrome that exhibit a lower proliferative capacity than cells from normal individuals also exhibit shorter telomeres; however, the rate of telomere shortening per cell division appears to be similar in progeria fibroblasts and normal cells (16). It has recently been demonstrated that proliferative senescence can be delayed and possibly eliminated by transfection of normal cells with telomerase to prevent telomere loss (30). It is also interesting to note that other repetitive DNA sequences become shorter during proliferative senescence (31,32).

1.1.2. *Mitogenic Responses and Signal Transduction*

As a result of senescence-associated changes, cells assume a flattened morphology and ultimately cease to proliferate in the presence of serum (5). Numerous factors may contribute to the senescent phenotype; however, the principal characteristic of cellular senescence in culture is the inability of the cells to replicate DNA. Paradoxically, the machinery for DNA replication appears to remain intact, as indicated by the fact that infection with SV-40 initiates a round of semiconservative DNA replication in senescent cells (33).

Nevertheless, senescent cells fail to express the proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase δ , apparently as a result of a post-transcriptional block (34). Furthermore, senescent fibroblasts fail to complement a temperature-sensitive DNA polymerase α mutant (35,36). This may contribute to the failure of senescent cells to progress through the cell cycle because it is known that a direct relationship exists between the concentration of DNA polymerase α and the rate of entry into S phase (37). It has also been observed that replication-dependent histones are also repressed in senescent cells and that a variant histone is uniquely expressed (18).

It might also be noted that the senescence-dependent cessation of growth is not identical to G_0 growth arrest that occurs in early passage cells that exhibit contact inhibited growth or that are serum starved. Several lines of evidence suggest that senescent cells are blocked in a phase of the cell cycle with many characteristics of late G_1 . For example, thymidine kinase is cell cycle regulated; it appears at the G_1 /S boundary. Thymidine kinase activity is similar in cultures of proliferating young and senescent WI-38 cells (38,39). It should also be noted that thymidine triphosphate synthesis, which normally occurs in late G_1 , is not impaired in senescent cells (39). Furthermore, the nuclear fluorescence pattern of senescent cells stained with quinacrine dihydrochloride is also typical of cells blocked in late G_1 or at the G_1 /S boundary (33,40). In addition, Rittling et al. (41) demonstrated that 11 genes expressed between early G_1 and the G_1 /S boundary are mitogen inducible in both young and senescent cells. On the other hand, growth-regulated genes such as *cdc2*, *cycA*, and *cycB*, which are expressed in G_1 , are repressed in senescent cells (42). These observations suggest the possibility that senescent cells are irreversibly arrested in a unique state different from the normal cell cycle stages.

As cells approach the end of their proliferative potential in culture they become increasingly refractory to mitogenic signals (15,43,44). The signal transduction pathways that convey these mitogenic signals play significant roles in the regulation of cell proliferation and adaptive responses; hence, decline in the activity of elements in these pathways may contribute significantly to the senescent phenotype. For example, there is a senescence-associated loss in the capacity of cells to activate protein kinase C (45) or to increase interleukin-6 (IL-6) mRNA abundance (46) following stimulation with phorbol esters. Furthermore, transcriptional activation of *c-fos* following stimulation of cultures with serum is also diminished in senescent cells (18,47). Other genes such as *Id1* and *Id2*, which encode negative regulators of basic helix-loop-helix transcription factors, fail to respond to mitogens in senescent cells (48).

Although signal transduction efficiency declines with replicative age, the members of affected pathways are seldom influenced uniformly by senescence. For example, both the number of receptors (per unit cell surface area) and

receptor affinities for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor-one (IGF-one) remain constant throughout the proliferative life of fetal lung WI-38 fibroblasts (49–51); however, senescent WI-38 cells produce neither the mRNA nor the protein for IGF-I (52). Similarly, young and senescent WI-38 fibroblasts have similar baseline levels of intracellular Ca^{2+} and exhibit similar changes in cytosolic Ca^{2+} fluxes following growth factor stimulation (53); however, the expression of calmodulin protein is uncoupled from the cell cycle and exists in variable amounts in senescent WI-38 cells (53). The calmodulin-associated phosphodiesterase activity also appears to be diminished in late-passage cells (Cristofalo et al., unpublished results). At least some of the changes in signal transduction associated with senescence may also stem from alterations in the cellular redox environment, because the rate of oxidant generation increases during senescence (54) and some steps in various signal transduction pathways are highly sensitive to changes in redox balance. The protein abundances of protein kinase A (PKA) and various isoforms of protein kinase C (PKC) are unchanged or slightly increased by senescence (20,55); however, PKC translocation from the cytoplasm to the plasma membrane is impaired in senescent fibroblasts (45,56).

Changes in signal transduction efficiency associated with senescence are not necessarily the result of any decrease or loss of components of signaling pathways. Experiments performed in various types of immortal and normal cells reveal that increases in signal transduction components can also impede signaling pathways. This is most clearly seen in the case of the extracellular signal-regulated kinase (ERK) pathway where the correct sequence and duration of activation and inactivation of ERKs at the G_1/S boundary (57–59) is required for entry into S phase. Indeed, constitutive ERK activation has an inhibitory effect on cell cycle progression, both in NIH 3T3 fibroblasts (58) and in *Xenopus* oocytes (60). Furthermore, overexpression of oncogenic *ras* in human fibroblasts leads to a senescent-like state rather than to an immortal phenotype (61). Thus, increases as well as decreases in individual components of pathways may contribute to senescence-associated changes in signal transduction. Taken together, senescence-associated changes in mitogenic signaling pathways occur for a variety of reasons that may include any imbalances in or dysregulation of controlling pathways. Interestingly, these effects are largely confined to proliferation and noncritical functions because, if maintained, subpopulations of cells can survive indefinitely in a senescent state.

1.2. Relevance to Aging

Before beginning our discussion of methods for the propagation of human fibroblasts and determination of replicative life-span, we digress briefly to discuss interpretation of this type of data. We shall also consider the relationship

between changes observed during senescence in vitro and aging in vivo. Finally, we will examine a second hypothesis that suggests that senescence in vitro recapitulates at least some aspects of developmental changes associated with differentiation.

The finite replicative life-span for normal cells in culture is thought to result from multiple environmental and genetic mechanisms (5) and has frequently been used as a model of human aging. Historically the use of replicative life-span of cell cultures as a model for aging has been accepted because (1) fibroblast replicative life-span in vitro has been reported to correlate directly with species maximum life-span potential (62), and most importantly (2) cultures of normal human cells have been reported to exhibit a negative correlation between proliferative life-span and the age of the donor from whom the culture was established (8,16,63–68). Other types of evidence also appear to support the strength of the model. For example, the colony-forming capacity of individual cells has also been reported to decline as a function of donor age (69,70). Various disease states of cell donors have been found to significantly influence the proliferative life-spans of cells in culture. For example, cell strains established from diabetic (68,71) and Werner's patients exhibit diminished proliferative potential (19,28,65,72,73). Cultures derived from individuals with Hutchinson–Gilford syndrome (28) and Down's syndrome (28,74) may also exhibit decreased proliferative potential, albeit results with these cell lines are more variable (29). Collectively, these observations have been interpreted to suggest that the proliferative life-span of cells in culture reflects the physiological age as well as any pathological state of the donor from which the cells were originally obtained.

It must be noted that interpretation of replicative life-span data is often difficult owing to large individual variations and relatively low correlations. For example, one large study (75) determined replicative life-span in more than 100 cell lines, yet obtained a correlation coefficient of only -0.33 . Hence, it is difficult to assess whether the reported negative correlations between donor age and replicative life-span indicate any compromise of physiology or proliferative homeostasis in vivo (75,76). A major factor that has influenced the results of most studies is the health status of donors when tissue biopsies were taken to establish the cell cultures (68,75). Most studies include cell lines established from donors who were not screened thoroughly for disease, as well as cell lines derived from cadavers to determine the effects of donor age on proliferative potential. Variations in the biopsy site have also been a factor that probably influenced the results of many studies (68,75).

Studies of rodent skin fibroblasts appear to support the existence of a small, but significant, inverse correlation between donor age and replicative life-span (67,77,78). Furthermore, it has also been observed that treatment of hamster

skin fibroblasts with growth promoters can extend the proliferative life of cultures established from young donors but has negligible effects on cultures established from older donors (79). Aside from inherent species differences and the effects of inbreeding that may influence these results, it is also apparent that rodent skin is better protected from some types of environmental injury such as light exposure. However, even in rodents, the relationship between donor age and proliferative potential is not entirely clear. For example, an examination of hamster skin fibroblast cultures established from the same donors at different ages reveals no age-associated changes in proliferative potential in animals older than 12 mo (78).

To address these issues, we recently examined the proliferative potential of 124 human fibroblast cell lines from the Baltimore Longitudinal Study of Aging (BLSA) (80). All of these cell lines were established from donors described as healthy, at the time the biopsy was taken, using the criteria of the BLSA. This study revealed no significant change in proliferative potential of cell lines with donor age, nor did we observe a significant difference between fetal and postnatally derived cultures (80). Goldstein et al. (68) also reported that no relationship between proliferative life-span and donor age could be found in healthy donors but did observe a relationship in diabetic donors. In addition, we performed a longitudinal study by determining the replicative life-span of cell lines established from individuals sampled sequentially at different ages. As in the case of the cross-sectional analysis, no relationship between donor age and replicative potential was found in this longitudinal study. Indeed, cell lines established from individuals at older ages frequently exhibited a slightly greater proliferative potential than the cell lines established from the same individuals at younger ages (80).

1.2.1. Relationship of In Vitro and In Vivo Models

One of the underlying assumptions of in vitro aging models is that the changes observed during proliferative senescence bear at least some homology to those observed during aging in vivo. In fact, both similar (concordant) and dissimilar (discordant) changes have been observed between aging-associated changes observed in vivo and in vitro; these are summarized in **Table 1**.

Although the results presented in **Table 1** clearly demonstrate that some similarities do exist between aging in vivo and replicative senescence, it remains unclear whether these changes arise through a common mechanism or via distinct pathways. As seen in **Table 1**, senescence in tissue culture and aging in the intact organism are not homologous. Others have noted that progressive morphological changes begin to develop in diploid cell cultures shortly after they are established regardless of the donor age; no cells are found in vivo

Table 1
Aging in Cell Culture Replicative Senescence vs Donor Age

Concordant features		Discordant features	
Collagenase	(↑) ^a (81,82)	c- <i>fos</i> induction	(↓) (20,83,84)
Stromelysin	(↑) (85)	<i>EPC-1</i> mRNA	(↓) (86,87)
PAI-1	(↑) (88,89)	<i>H-twist</i> mRNA	(↓) (90; unpublished)
IGF-BP3	(↓) (91)	<i>G-6-PDH</i> mRNA	(=) ^b (54,92)
TIMP-1	(↓) (85)	Fibronectin	(↑) (93)
HSP 70	(↓) (94,95)	<i>ND-4</i> mRNA	(↑) (96,97)
Response to Ca ²⁺	(↓) (98,99)	<i>p21</i> mRNA	(↑) (100,101)
		<i>MnSOD</i> mRNA	(↑?) (102,103)
		β-Galactosidase	(↑) (Cristofalo, unpublished)
		Chemiluminescence	(↑) (54,96)
		H ₂ O ₂ Generation	(↑) (54,96)
		<i>Collagen a(1)I</i> mRNA	(↓) (100,104,105)
		Proliferative capacity	(↓) (80)
		Saturation density	(↓) (80)

^aArrow indicates direction of change in replicative senescence.

^bIndicates no change.

G-6-PDH, glucose-6-phosphate dehydrogenase; HSP 70, heat shock protein 70; IGF-BP3, insulin-like growth factor binding protein-3; PAI-1, plasmogen activator inhibitor-1; SOD, superoxide dismutase; TIMP-1, tissue inhibitor of metalloproteinase-1.

at any age that exhibit the morphological phenotype of cells, in vitro, at the end of their replicative life-span (106).

Rubin (76) suggests that the limited replicative life-span in vitro may be an artifact that reflects the failure of diploid cells to adapt to the trauma of dissociation and the radically foreign environment of cell culture. However, that hypothesis ignores factors such as telomere shortening that appear to influence proliferative life and that are not dependent on the culture environment. Presently, it is possible to state that the loss of proliferative potential in vitro does not directly reflect changes in replicative capacity that occur in vivo during aging and that changes in gene expression associated with replicative senescence are not completely homologous with changes observed during aging in vivo.

1.2.2. Relationship Between Senescence and Development

One view of the limited proliferative capacity of cells in culture is that it stems from the effects of the culture environment on the state of differentiation of the cells (107–113). Although the state of differentiation may change in

Table 2**Comparison of Replicative Senescence of Fetal Cells In Vitro with Differences Between Fetal and Adult Cells?**

Concordant		Discordant	
ND-4 mRNA	(↑) (96,97)	<i>c-fos</i> induction	(=) ^b (84)
MnSOD activity	(↑) (103,114)	<i>EPC-1</i> mRNA	(↑) (86,87,97)
Catalase activity	(↑) (92)	<i>Cu/Zn SOD</i> mRNA	(↑) (102) ^c
IL-1 α	(↑) (103,115)	<i>MnSOD</i> mRNA	(↑) (102,114) ^c
IL-1 β	(↑) (103,115)	<i>Cu/Zn SOD</i> activity	(↑) (102) ^c
Response to PDGF-BB	(↑) (116)	<i>COX-1</i> mRNA	(↑) (96) ^c
		<i>SD</i> ^d mRNA	(↑) (96) ^c
		<i>COX</i> activity	(↑) (96) ^c
		<i>ND</i> ^d activity	(↑) (96) ^c
		<i>SD</i> activity	(↑) (96) ^c
		<i>G-6-PDH</i> mRNA	(↑) (92)
		PDGF requirement	(↑) (117)
		<i>Collagen $\alpha(1)I$</i> mRNA	(Ø) (100,118)
		β -Actin	(Ø) (100; unpublished)

^aArrow indicates direction of difference between proliferatively young fetal and adult cells.

^bIndicates no change.

^cBased on observations of changes during proliferative senescence, made in this laboratory that will be presented elsewhere (54).

^dND=NADH dehydrogenase; SD=succinate dehydrogenase.

cells that senesce in vitro, there is, in fact, no evidence that the changes in gene expression observed in fetal cells as they senesce in vitro, are tantamount to differentiation, in vivo. While some analogous changes can be found they are greatly outnumbered by the discordant differences that characterize these two distinct phenomena. Hence, a comparison of senescence-associated changes and differences that exist between fetal and postnatal cells reveals little similarity (Table 2).

At least some analogous similarities exist between senescence in fetal fibroblasts and developmental changes that occur in vivo. For example, it has been observed that addition of PDGF-BB stimulated an increased mRNA abundance of the transcript encoding the PDGF-A chain in fetal and newborns; however, the response was greatly decreased in adult cells. Senescence in vitro of newborn fibroblasts appears to result in the acquisition of the adult phenotype (116). In contrast, there are a number of differences reported between fetal- and adult-derived cell lines related to growth factor requirements for proliferation and migration (117,119–121) that remain disparate even as these cultures become

senescent. For example, Wharton (*119*) has shown that fetal dermal fibroblasts will proliferate in plasma or serum while adult dermal fibroblasts require serum. It is also noteworthy that the expression of some genes, such as *SOD-2*, increases during proliferative senescence but only in some types of fibroblasts (*114*); in other types of fibroblasts no change is observed (*54,114*). It might be expected that cells placed in culture will be deprived of those signals that direct the normal sequence of developmental pathways and that differentiation, if it occurs, is to an aberrant state. Alternatively, fetal cell lines may arise from different precursor cells than do adult fibroblasts and thus merely differentiate to a different fibroblast type.

1.2.3. Limitations and Strengths of the System

Although the loss of proliferative potential in vitro may not directly reflect changes in replicative capacity that occur in vivo during aging, cell cultures remain a powerful tool for a variety of aging-related studies. These include studies of heritable damage to cell populations that simulate the effects of aging in vivo (*76*), a variety of chemical and molecular manipulations used to induce a senescence phenotype, the effects of stress (*61,76,122–125*), and as a system to study abnormal growth or quiescence (*5*). The model may also help to further elucidate the effects of diseases that alter proliferative life-span (*19,28,65,68,71–73,126*). Loss of capacity for senescence is a necessary step for immortalization and transformation to a malignant phenotype. The model may also prove useful in studies of the relationship between differentiation and replicative aging (*117,119–121*).

2. Materials

The serum-supplemented and serum-free, growth factor-supplemented formulations presented each give optimal growth of human diploid fibroblast-like cells. We also present methods for growth of cells in a defined medium using a defined growth factor cocktail (*2,127*). All reagents and materials for cell culture must be sterile, and all manipulations should be performed in a laminar flow hood. Serial propagation is generally performed in serum-supplemented media, yet serum is a complex fluid with numerous known and unknown bioactive components. For many studies, it is often desirable if not crucial to use a serum-free growth medium of defined composition.

2.1. Serum-Supplemented Medium

Suppliers and more detailed information on the items required for the preparation of serum-supplemented media are listed in **Table 3**.

1. Incomplete Eagle's modified minimum essential medium: Cells are grown in Eagle's modified minimum essential medium (MEM). Although the medium can

Table 3
Components of Standard Growth Medium

Component	Amount/L	Supplier	Cat. no.
Auto-Pow™, autoclavable powder Eagle MEM with Earle’s salts without glutamine and without sodium bicarbonate	1 pkg	ICN	11-100-22
100× Basal medium Eagle vitamins	10 mL	ICN	16-004-49
200 mM L-Glutamine	10 mL	Sigma	G3126
Sodium bicarbonate (7.5% solution)	26 mL	Sigma	S5761
FBS ^a	100 mL	Various	

^aFBS is from a variety of suppliers and tested on a lot-by-lot basis. See **Note 1**.

be purchased in liquid form, it is considerably less expensive to prepare the medium from a commercially available mix. In our laboratory incomplete MEM is prepared by dissolving Auto-Pow™ powder (9.4 g) and 100× basal medium Eagle vitamins (10 mL) in 854 mL of deionized, distilled water. After the incomplete medium has been mixed and dissolved, it should be divided into two equal portions (432 mL each) and placed in 1-L bottles (*see Note 2*). The caps are screwed on loosely, autoclave tape is applied, and the bottles are autoclaved for 15 min at 121°C (*see Note 3*). As soon as the sterilization cycle is finished, the pressure is quickly released and the bottles are quickly removed from the autoclave. The bottles are allowed to cool to room temperature in a laminar flow hood. When the bottles have cooled, the caps are tightened. Incomplete medium is stored at 4°C in the dark.

2. 100× Basal medium Eagle vitamins: Filter-sterilized 100× basal medium Eagle vitamins are purchased in 100-mL bottles and stored at –20°C. When first thawed, using sterile procedures, the vitamin solution is divided into 10-mL portions and stored in sterile 15-mL centrifuge tubes at –20°C until use.
3. L-Glutamine (200 mM): L-Glutamine (14.6 g) is dissolved in 500 mL of deionized, distilled water without heating. This solution is then sterilized in a laminar flow hood using a 0.2 µm pore size bottletop filter. Aliquots (50 mL) are added to sterile 100-mL bottles that are then capped and stored at –20°C until use. When thawed for use, the glutamine solution is divided into 5-mL portions and stored at –20°C in sterile 15-mL centrifuge tubes until use.
4. Sodium bicarbonate (7.5% w/v): Sodium bicarbonate (37.5 g) is dissolved in 500 mL of deionized, distilled water. This solution is then filter sterilized using a 0.2-µm pore size bottletop filter. The sterile solution is stored at 4°C.
5. Fetal bovine serum (FBS): Prior to purchase, various lots of fetal bovine serum (FBS) are tested for 3 consecutive weeks to determine their effects on the rate of cell proliferation and saturation density. The serum lot that gives the best growth response is chosen, and quantities that will last about 1 yr are purchased. The serum is stored at –20°C until use. Once thawed, serum is stored at 4°C for subsequent use; it should not be refrozen.

Table 4
Components of Serum-Free Growth Medium

Component	Amount	Supplier	Cat. no.
MCDB-104, a modified basal medium with L-glutamine, without CaCl ₂ , without Na ₂ HPO ₄ , without NaHCO ₃ , and without HEPES, and with sodium pantothenate substituted for calcium pantothenate	1 pkg/L	Gibco-BRL	82-5006EA
Sodium phosphate, dibasic	0.426 g/L	Sigma	S5136
Sodium chloride	1.754 g/L	Sigma	S5886
Calcium chloride dihydrate	1 mM	Sigma	C7902
Sodium bicarbonate	1.176 g/L	Sigma	S5761
HEPES ^a	11.9 g/L	Sigma	H9136
1 M Sodium hydroxide ^a	25 mL/L	Sigma	S2770
EGF), human recombinant	25 ng/mL	Gibco-BRL	13247-010
IGF-I, human recombinant	100 ng/mL	Gibco-BRL	13245-014
Insulin	5 µg/mL	Sigma	I6634
Ferrous sulfate heptahydrate	5 µM	Sigma	F8633
1 M Hydrochloric acid	Trace	Sigma	H9892
Dexamethasone	55 ng/mL	Sigma	D4902
95% Ethanol (not denatured)	Trace	Pharmco	111000- 190CSGL

^aNot used in growth medium. See **Note 1**.

- Standard serum-supplemented growth medium (complete medium with 10% v/v FBS): To prepare the standard serum-supplemented growth medium (complete medium with 10% v/v FBS), add 13 mL of filter-sterilized 7.5% (w/v) sodium bicarbonate to 432 mL of sterile, incomplete Eagle's MEM. The sodium bicarbonate must be added first because low pH can affect glutamine and serum components. After addition of the sodium bicarbonate add 50 mL of sterile FBS. Just before use the medium is prewarmed to 37°C in a warm water bath, then transferred to a laminar flow hood where 5 mL of a 200 mM solution of filter-sterilized L-glutamine is added. Complete medium is generally prepared fresh for each use. If this medium must be stored for periods exceeding 1 wk, additional filter-sterilized L-glutamine (1 mL/100 mL of complete medium) is added just before use.

2.2. Serum-Free Medium

Suppliers and more detailed information on the items required for the preparation of serum-free media are listed in **Table 4**.

- Serum-free growth medium: This medium is prepared by dissolving a packet of powdered MCDB-104 medium (with L-glutamine, without CaCl₂, without

- Na_2HPO_4 , without NaHCO_3 , and without *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), with sodium pantothenate substituted for calcium pantothenate) in 700 mL of deionized, distilled water. The packet is also rinsed several times to dissolve any medium powder that may have adhered to it. The following additional components are then added in the order listed: 0.426 g of Na_2HPO_4 , 1.754 g of NaCl , 1.0 mL of a 1 *M* CaCl_2 solution, and 1.176 g of NaHCO_3 . For most studies HEPES is not used. The final volume is brought to 1 L with deionized, distilled water. Incomplete medium is sterilized by filtration through a 0.2- μm bottletop filter into sterile glass bottles. Using sterile procedures in a laminar flow hood, a 5% CO_2 /95% air mixture is passed through a sterile, cotton-filled CaCl_2 drying tube, through a sterile pipet, and bubbled into the medium (*see Note 4*). As the medium becomes saturated with the gas mixture, its color changes from pink to a salmon color. The final pH is 7.3–7.5. Incomplete medium is generally prepared fresh for each use, but it may be stored for up to 3 wk at 4°C. If unused complete medium is stored longer than 1 wk, additional L-glutamine (1 mL/100 mL of complete medium) should be added before use.
2. HEPES-buffered incomplete medium for stock solutions: The pH of carbon dioxide/bicarbonate-buffered MCDB-104 solutions rises during thawing, resulting in Ca_2PO_4 precipitate formation. Thus, growth factor and soybean trypsin inhibitor solutions that are stored frozen are prepared in HEPES-buffered solutions. To prepare 1 L of HEPES-buffered incomplete medium, mix medium as described previously except 11.9 g of HEPES free acid and 25.0 mL of 1 *M* NaOH are added instead of sodium bicarbonate. The pH of the medium is adjusted to 7.5 by titration with additional 1 *M* NaOH and the volume is brought to a final volume of 1 L with deionized, distilled water. The medium is sterilized by filtration through a 0.2- μm bottletop filter into sterile glass bottles. The HEPES-buffered incomplete medium may be stored at –20°C until needed.
 3. Concentrated growth factor stock solutions: For these procedures, use sterile plastic pipets and perform all manipulations in a laminar flow hood. Stock solutions of growth factors (100 \times) are prepared in HEPES-buffered incomplete medium at the following concentrations: EGF (2.5 $\mu\text{g}/\text{mL}$) and either IGF-I (10 $\mu\text{g}/\text{mL}$) or insulin (500 $\mu\text{g}/\text{mL}$) (*see Note 5*). All stock solutions are dispensed with sterile plastic pipets into sterile 1.0-mL cryogenic vials. The stock solutions may be stored at –20°C for short periods (up to 4 wk) or at –70°C for longer periods (3–4 mo). Dexamethasone (5 mg/mL) is prepared in 95% nondenatured ethanol. This solution is then diluted into HEPES-buffered incomplete medium to give a 100 \times stock solution (5.5 $\mu\text{g}/\text{mL}$). Stock dexamethasone is stored in sterile, siliconized test tubes. Ferrous sulfate is prepared fresh, just prior use. After preparation 5 μL of 1 *M* hydrochloric acid is added to each 10 mL of the ferrous sulfate 100 \times stock (0.5 mM). This solution is sterilized by filtration through a 0.2- μm filter.
 4. Complete serum-free growth medium: For 100 mL of complete serum-free growth medium, 1 mL of each of the 100 \times stock solutions are added to 96 mL of

Table 5
Components of Trypsinization Solution

Component	Final amount	Supplier	Cat. no.
Sodium chloride	6.8 g/L	Sigma	S5886
Potassium chloride	0.4g/L	Sigma	P5405
Sodium phosphate monohydrate, monobasic 0.14g/L	Sigma	S5655	
Glucose	1 g/L	Sigma	G6152
50× MEM amino acids, without glutamine	20 mL/L	Gibco-BRL	11130-051
100× basal medium Eagle vitamins	10 mL/L	ICN	16-004-49
0.5% Phenol red	10 mL/L	ICN	16-900-49
Sodium bicarbonate (7.5% solution)	5 mL/50 mL	Sigma	S5761
Trypsin, 2.5% in Hanks' balanced salt solution	5 mL/50 mL	Sigma	T4674
Soybean trypsin inhibitor, type I-S	1 mg/mL	Sigma	T6522

incomplete medium (MCDB-104). The resultant concentrations in the serum-free medium are: 25 ng/mL of EGF, 100 ng/mL of IGF-I, or 5 µg/mL of insulin (*see Note 5*); 55 ng/mL of dexamethasone; and 5 µM of ferrous sulfate.

5. Soybean trypsin inhibitor solution for serum-free propagation: Soybean trypsin inhibitor (100 mg) is added to 100 mL of HEPES-buffered incomplete medium. This solution is sterilized by filtration through a 0.2-µm bottletop filter into a sterile bottle. The sterile solution is then dispensed into sterile 15-mL centrifuge tubes in 7-mL portions and stored at -20°C. When needed, the solution is thawed and diluted 1:1 with bicarbonate-buffered incomplete medium.

2.3. Trypsinization

Suppliers and more detailed information on the items required for the preparation of trypsinization solution are listed in **Table 5**.

1. Ca²⁺/Mg²⁺-free medium: Cells tend to aggregate in media containing calcium; it is thus desirable to use a medium that is low in Ca²⁺ and Mg²⁺ for mixing trypsin solution. To prepare Ca²⁺/Mg²⁺-free medium, the following ingredients are added to 900 mL of deionized, distilled water with magnetic stirring: 6.8 g of NaCl, 0.4 g of KCl, 0.14 g of NaH₂PO₄ · H₂O, 1 g of glucose, 20 mL of 50× MEM amino acids without glutamine, 10 mL of 100× basal medium Eagle vitamins, and 10 mL of a 0.5% (w/v) solution of phenol red. The solution is then diluted to 1 L with deionized, distilled water and sterilized by filtration. The Ca²⁺/Mg²⁺-free medium is stored at 4°C until use.
2. Trypsin stock solution (2.5%): Filter-sterilized trypsin (2.5%) in Hanks' buffered salts solution is purchased in 100-mL bottles and stored at -20°C. Repeated

Table 6
Items for Thymidine Incorporation

Item	Supplier	Cat. no.
[³ H-methyl]-Thymidine, 2 Ci/mmol; 1 mCi/mL	Dupont NEN	NET-027A
Coverslip, No. 1, 22 mm × 22 mm	Thomas	6662-F55
Coverslip rack, ceramic	Thomas	8542-E30
Coverslip rack, glass	Fisher	08-812
Chloroform	Sigma	C5312
95% Ethanol, not denatured	Pharmco	111000190CSGL
95% Sulfuric acid	Sigma	S1526
70% Nitric acid	Sigma	25,811-3
Sodium hydroxide	Sigma	S5881
Petri dish, glass, 100 mm	Thomas	3483-K33
NTB-2 Emulsion	Eastman Kodak	165 4433
D-19 Developer	Eastman Kodak	146 4593
Acid fixer	Eastman Kodak	197 1746
Hematoxylin, Harris Modified	Fisher	SH30-500D
Permout	Fisher	SP15-100
Microscope slide, 3 in × 1 in	Thomas	6684-H61
Lab-Tek® Chamberslide™, two-chamber	Nalge Nunc	177380
Lab-Tek® Chamberslide™, four-chamber	Nalge Nunc	177437
Lab-Tek® Chamberslide™, eight-chamber	Nalge Nunc	177445
Sodium phosphate, dibasic	Sigma	S5136
Potassium phosphate, monobasic	Sigma	P5655
Methanol	Fisher	A408-1
Slide mailer, polypropylene	Thomas	6707-M27
Slide box, polypropylene	Thomas	6708-G08

freeze-thaw will very rapidly decrease activity. The bulk trypsin solution should be thawed only once, dispensed in 5-mL portions in sterile 15-mL centrifuge tubes and then stored at -20°C until use.

3. Trypsin solution (0.25%): Five milliliters of sterile sodium bicarbonate (7.5%) is added to 40 mL of ice-cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium. Subsequently, 5 mL of freshly thawed 2.5% trypsin stock is added to the solution. This solution should be prepared just before the cells are treated and should be kept on ice.

2.4. Thymidine Incorporation

Suppliers and more detailed information on the items required for measurement of thymidine incorporation are listed in **Table 6**.

1. [³H-methyl]-thymidine stock solution: Under sterile conditions, [³H-methyl]-thymidine (2 Ci/mmol, 1 mCi/mL) is diluted to a concentration of 5 $\mu\text{Ci/mL}$ in ster-

ile medium. This stock solution is aliquoted (5-mL portions) in a laminar flow hood using sterile procedures into sterile, 15-mL centrifuge tubes and stored at -20°C until use.

2. Phosphate-buffered saline (PBS) solution: dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , and 0.24 g of KH_2PO_4 in 900 mL of H_2O with magnetic stirring. The pH is adjusted to 7.4 with HCl, the volume adjusted to 1 L, and the solution is autoclaved for 20 min at 121°C .
3. Emulsion: Kodak NTB-2 emulsion is purchased in a lightproof container. The emulsion is stored at 4°C (see **Note 6**).
4. Developer and Fixer
 - a. Kodak D-19 developer is purchased in packets that make 1 gal when reconstituted. The entire packet is used at one time and the solution is stored in a brown bottle in the dark. The developer remains useable for 1–3 mo. When the developer turns yellow, it is discarded.
 - b. Acid fixer is made and stored in the same manner as the D-19 developer.

3. Methods

3.1. Cell Propagation in Serum-Supplemented Medium

Cells may be grown in a variety of culture vessels (see **Note 7**). Amounts described in the following procedure are for a T-75 flask. Proportional amounts are used for other size vessels; i.e., for a T-25 flask, one third of all of the amounts given is used. Trypsinization and seeding of flasks should be performed in a sterile environment (see **Note 8**).

To propagate adherent cells:

1. Prepare fresh trypsin solution (0.25%) and place it on ice; prepare fresh growth medium and warm it to 37°C .
2. Using sterile procedures in a laminar flow hood, remove spent growth medium from the culture vessel. For flasks and bottles, the medium should be removed by aspiration or decanting from the side opposite the cell growth surface. For cell culture plates and dishes, the medium should be removed by aspiration from the edge of the growth surface.
3. Gently wash the monolayers of adherent cells twice with 0.25% trypsin solution (4 mL).
4. Remove residual trypsin solution by aspiration from the side opposite the cell growth surface (flasks) or from the edge of the growth surface (plates, dishes, and slides) as appropriate.
5. Add enough trypsin solution (0.25%) to wet the entire cell sheet (2 mL/T-75).
6. The culture vessel should be tightly capped to maintain sterility and placed at 37°C .
7. The cells will assume a rounded morphology as they are released from the growth surface. Detachment of the cells should be monitored using a microscope. As a general rule, detachment will be complete within 15 min. The trypsinization process may be speeded up by gently tapping the sides of the flask. Care should be

taken to not splash cell suspension against the top and sides of the flask, because this will lead to errors in the determination of the number of cells in the flask.

8. When all of the cells have detached from the growth surface, as determined by inspection with a microscope, the flask is returned to the laminar flow hood. Complete medium with 10% v/v FBS is carefully pipetted down the growth surface of the vessel to neutralize the trypsin and to aid in pooling the cells. For a T-75 flask, 8 mL of complete medium is used. The final harvest volume is 10 mL.
9. Cell clumps should be dispersed by drawing the entire suspension into a 10-mL pipet and then allowing it to flow out gently against the wall of the vessel. The process is repeated at least three times. The procedure is then repeated with a 5-mL pipet. Until the procedure becomes routine, a sample is withdrawn and examined under the microscope to ensure that a suspension of single cells has been achieved. During this process, the cells should be kept on ice to inhibit cell aggregation and reattachment.
10. Using sterile procedures, remove an aliquot from the cell suspension, then dilute it into Isoton II in a counting vial. Typically, 0.5 mL of the cell suspension is diluted into 19.5 mL of Isoton II.
11. Count the sample with a Coulter Counter.
12. Calculate the number of cells in the harvest. Calculate the volumes of cell suspension and complete medium needed for new cell culture growth vessels. In most cases, cells are seeded at a density of 1×10^4 cells/cm² of cell growth surface, and the total volume of cell suspension plus complete medium added to the culture vessels is maintained at 0.53 mL/cm² of cell growth surface.
13. In the laminar flow hood, add the calculated amounts of complete medium to new culture vessels.
14. Dissolved CO₂ in equilibrium with HCO₃⁻ is the principal buffer system of the medium, although serum also has some buffering capacity. Because CO₂ is volatile, the gas phases in the flasks are adjusted to the proper pCO₂ to maintain the pH of the medium at 7.4. Using sterile procedures in a laminar flow hood, a 5% CO₂/95% air mixture is passed through a sterile, cotton-filled CaCl₂ drying tube, through a sterile pipet, and into the gas phase of the cell culture flask with the growth surface down. As the gas mixture is flushed over the medium surface, the color of the medium will change from a dark red toward a red-orange. The flask is flushed until the medium no longer changes color. At this point, the gas above the medium is 5% CO₂ and the pH of the medium is 7.4 (see **Note 4**). The flask is then tightly capped to prevent gas exchange with the outside environment. Cells grown in culture plates, dishes, and Lab-Tek® slides, which are not gas-tight, are not equilibrated with the gas mixture in this manner; instead they must be grown in incubators that provide a humidified, 5% CO₂ atmosphere.
15. The cell harvest is resuspended with 10-mL and 5-mL pipets, as before. Inoculate each culture vessel to a final density of 1×10^4 cells/cm² of growth surface.
16. Briefly flush the culture vessel a second time with the 5% CO₂/95% air mixture to replace the CO₂ lost when the vessel was opened. Cap the flask tightly and

incubate at 37°C. Periodically, examine the color of the medium to ensure that the seal is gas tight.

17. The cumulative population doubling level (cPDL) at each subcultivation is calculated directly from the cell count (*see Note 7*).

Example:

One week after seeding a T-75 flask with the standard inoculum of 7.5×10^5 cells at a cPDL of 37.2, the cells are harvested. One doubling would yield $2 \times 7.5 \times 10^5 = 1.5 \times 10^6$ cells; two doublings would result in $4 \times 7.5 \times 10^5 = 3.0 \times 10^6$ cells; three doublings would yield $8 \times 7.5 \times 10^5 = 6.0 \times 10^6$ cells, etc. Thus, the population doubling increase is calculated by the formula:

$$N_H/N_I = 2^X$$

or

$$[\log_{10}(N_H) - \log_{10}(N_I)] / \log_{10}(2) = X$$

where N_I = inoculum number, N_H = cell harvest number, and X = population doublings. The population doubling increase that is calculated is then added to the previous population doubling level to yield the cPDL. For example, if 9.1×10^6 cells were harvested, then the population doubling increase can be calculated from the expression:

$$9.1 \times 10^6 \text{ cells} = 2(X) \times 7.5 \times 10^5 \text{ cells}$$

$$X \log_{10} 2 = \log_{10}(9.1 \times 10^6) - \log_{10}(7.5 \times 10^5)$$

$$X = 3.6$$

The population doubling increase is added to the previous cPDL to give the new cPDL of the cell population. For this example, the new cPDL is $37.2 + 3.6 = 40.8$. The end of the replicative life-span was defined by failure of the population to double after 4 wk in culture with 3 wk of consecutive refeeding.

3.2. Cell Propagation in Serum-Free Medium

1. Because undefined mitogens and inhibitors present in serum complicate the interpretation of cell growth response results, soybean trypsin inhibition solution should be used to stop trypsin instead of complete medium with 10% v/v FBS to wash and collect the cells from the growth surfaces of flasks. Otherwise, cells are released from the surface of their culture vessel exactly as described previously for propagation of cells in serum-supplemented medium (**Subheading 3.1., steps 1–12**).
2. Wash the cells to remove residual mitogens and trypsin inhibitor, rather than using them directly to inoculate the culture flasks:
 - a. Under sterile conditions, the cells are pelleted by centrifugation at 75g for 5 min at 4°C.

- b. The centrifuge tubes are placed in ice, transferred to a laminar flow hood, the supernatant is removed, and the cells are resuspended in 10 mL of incomplete serum-free growth medium (**Subheading 2.1.**).
 - c. Under sterile conditions, the cells are again pelleted by centrifugation, and after removal of the supernatant, the cells are resuspended in 10 mL of complete serum-free growth medium (**Subheading 2.2.**).
3. Determine the cell number with the Coulter Counter as before, using an aliquot of the cell suspension (0.5 mL)
4. Cells are then seeded exactly as described in **Subheading 3.1., steps 13–17**, except that serum-free cell growth medium is used.

3.3. Replicative Life-Span

As noted previously, cells in culture exhibit a finite number of replications. At the end of their in vitro life-span substantial cell death occurs; however, a stable population emerges that can exist in a viable, though nondividing, state indefinitely (*128*). Furthermore, small subpopulations of cells may retain some growth capacity even after the vast majority of cells in a culture are no longer able to divide. As a practical matter, cultures of cells may be considered to have reached the end of their proliferative life-span when the cell number fails to double after 4 wk of maintenance in growth medium with weekly refeedings. The maximum proliferative capacity of the cells is determined as follows:

When cell cultures are near the end of their proliferative life-span, at least four identical sister flasks are prepared. One flask is harvested each week. If the number of cells harvested is at least double the number inoculated, cells are subcultivated as usual. One of the sister flasks may also need to be harvested to provide enough cells for subcultivation into four flasks. If the number of cells harvested is not at least double the number inoculated, all of the sister flasks are refed by replacement of the spent medium with fresh complete medium and equilibration with 5% CO₂/95% air mixture. This process is repeated three times. When cultures fail to double during this period, the culture may be considered to have reached the end of proliferative life or to be “phased out.”

3.4. Saturation Density

Cultures are grown until the cells are densely packed and no mitotic figures are apparent. This usually requires from 7 to 10 d after seeding for early passage cells, and more than 9 d for later passage cells. To estimate the saturation density, these confluent and quiescent cells are then harvested and counted as described previously.

3.5. Microscopic Estimate of Cell Density

It is often desirable to obtain an estimate of cell density without harvesting the cells. A stage micrometer is used to calibrate the eyepiece micrometer and

determine the diameter of the field of view for each objective and ocular lens used. The area of the field of view is calculated as $\text{Area} = \pi r^2$, where r is the radius of the field of view.

Scan the sample to ensure that the cells are uniformly distributed. Then count at least 400 cells using random fields. Since the standard deviation of a Poisson distribution is the square root of the number, 400 cells are counted. The square root of 400 (**20**) is 5%, which is the limit of statistical reliability for most biological work. Record the number of cells and the number of fields counted. The cell density is then calculated as follows:

$$\text{cell density} = (\text{no. of cells counted}) / ([\text{no. of fields counted}] \cdot [\text{area per field}])$$

3.6. Thymidine Incorporation

3.6.1. Coverslips

1. Place coverslips in a clean, glass rack using forceps.
2. Lower the rack containing the coverslips into a solution of chloroform/95% ethanol (1:1) and allow to soak for 30 min.
3. Rinse the coverslips with deionized water.
4. Submerge the coverslips in a 95:5 solution of concentrated sulfuric acid (95%)/concentrated nitric acid (70%), previously prepared in a fume hood and allowed to cool to room temperature. Soak the coverslips in this solution for 30 min.
5. Rinse the coverslips thoroughly in deionized water.
6. The rack containing the coverslips should then be lowered into a solution 0.2 M NaOH and allowed to soak for 30 min.
7. Remove the coverslips from the NaOH solution and rinsed at least three times in deionized water.
8. Remove the coverslips from the rack and allow to air-dry on lint-free disposable wipes.
9. When completely dry, bake the coverslips for 3 h at 180°C for sterilization.

3.6.2. Cell Slides

1. In a laminar flow hood, under sterile conditions, cells are harvested and counted in the usual manner.
2. Cells are seeded at a density of 1×10^4 cells/cm² on Lab-Tek® slides or in cell culture dishes that contain coverslips (**Subheading 3.6.1.**). If using coverslips use sterile forceps to arrange them in the dish so that they do not overlap one another.
3. Immediately after seeding, the slides and dishes are placed in an incubator at 37°C in an atmosphere of 5% CO₂/95% air.
4. Twenty-four hours later, add the stock solution of [³H-methyl]-thymidine (specific activity 2 Ci/mmol; **Subheading 2.4.**) to the cultures to a final concentration of 0.1 μCi/mL.
5. After 30 h (**129**), the labeling medium is removed, and cells are immediately washed twice with PBS (**Subheading 2.4.**), fixed in 100% methanol for 15 min,

and air-dried. If cells are grown on coverslips, remove the coverslips from the dishes and place in a clean ceramic or glass rack using forceps prior to washing and fixing. If a Lab-Tek® slide is used, the plastic container and gasket must be removed prior to washing and fixing. These procedures should be done rapidly to limit damage to the cells. The cells must not be permitted to dry before they are fixed.

6. Mount coverslips with the cell surface up using mounting resin. Allow the resin to dry overnight.

3.6.3. Autoradiography

1. Remove the Kodak NTB-2 emulsion from storage at 4°C and place it in a warm room at 37°C. The emulsion will liquefy in 3–4 h. The emulsion may also be melted by placing it in a 40°C water bath in the dark for about 1–1.5 h. Do not shake the bottle because the resultant bubbles may cause irregularities in the final emulsion thickness.
2. In a dark room, the desired amount of emulsion is gently, but thoroughly mixed in a 1:1 ratio with deionized, distilled water.
3. Add 15–20 mL of the 1:1 emulsion/water solution to a container (a slide mailer works well for this) previously set up in a 40°C water bath in the dark.
4. Dip each slide individually into the slide mailer. One dip is sufficient to coat the slide.
5. Place each dipped slide in a standing (vertical) position in a wire test tube rack to drain off excess emulsion. The slides are allowed to dry for 30 min in the dark.
6. The dipped slides are placed into a slide box with a desiccant. The box is covered and sealed with black electrical tape. The box is placed inside a second light-tight container that also contains a desiccant and this is also sealed with electrical tape.
7. The container is placed at 4°C for 4 d.

Development of Cell Slides

1. Pour Kodak D-19 developer and acid fixer into large glass dishes.
2. Open the slide containers in a dark room (photo-safe light can be used), and remove the slides and place them in racks.
3. Place the slides in developer for 5 min.
4. Transfer the slides fixer for 5 min.
5. At this point, the room light may be turned on, if desired. Gently rinse the slides for 15 min in cold running water. The slides should next be lightly stained with Harris' modified hematoxylin stain to enhance nuclear visualization.

3.6.5. Staining Slides

1. Place the developed slides in staining dishes containing Harris' modified hematoxylin stain for 5–10 min. This amount of time is sufficient to produce light staining.
2. Drain slides in slide racks on paper towels.

3. Rinse the slides continuously with deionized, distilled water until the excess stain is removed and then drain them on paper towels.
4. Excess emulsion should be wiped from the back of slides while they are still damp
5. Air-dry the slides.

3.6.6. Counting Labeled Nuclei

1. For ease in identifying the limits of individual chambers under the microscope, if Lab-Tek® slides are used, the stain between the individual chambers can be removed with the end of a paper clip or push pin.
2. Silver grains over nuclei where [³H-*methyl*]-thymidine has been incorporated into the DNA will be readily visible at 400× magnification. Nuclei with five or more grains are considered labeled.
3. To determine the percentage of labeled nuclei, at least 400 cells are counted per coverslip or chamber using random fields. Typically, determinations are done in duplicate.

4. Notes

1. It is important that the highest quality deionized, distilled water is used to prepare growth medium and all other reagents used for cell culture.
2. It is important that the bottles not be filled to more than one half volume to prevent overflow during sterilization.
3. Prolonged heat destroys some medium components.
4. Cells in a culture environment require carbon dioxide for growth and survival, and we have found that well controlled CO₂/bicarbonate buffered media gives superior growth when compared with media containing synthetic buffers, such as HEPES.
5. Insulin and IGF-I both stimulate growth through the IGF-I receptor, although insulin has lower affinity for the IGF-I receptor and 50-fold higher concentrations are required to achieve comparable growth. Insulin is less expensive than IGF-I, and despite the reduced specificity, insulin is satisfactory for most experiments.
6. Emulsion should never be stored near high-energy sources of radioactivity.
7. Cell cultures are typically subcultivated weekly. Multiple identical sister flasks are prepared at subcultivation, as a hedge against potential contamination or other anomalies. Because a substantial fraction (25–60%) of the cells do not survive subcultivation (**130**), the number of cells does not generally increase above the seeded cell number until approx 24 h after subcultivation.
8. Cultures should routinely examined microscopically for contamination, and tested for mycoplasma at 5-wk intervals (**131**).

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