# Cell growth and division

## a practical approach

Edited by
Renato Baserga

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# Preface

Cell growth means different things to different people. For many investigators, cell growth is just a question of growth factors, that is of the environment that surrounds the cells, in culture or *in vivo*. For others, cell growth is a problem of gene expression, that is of the genes and gene products that interact with and respond to the growth factors in the environment. For all of us, though, whether we purify growth factors or clone genes, the success of our work depends upon the assays we use; which takes me back (many years, unfortunately) to when I was a graduate student, and one of my professors told me that my experiments would only be as good as the assays used.

Cells constitute the basis for any assay of cell growth and (with few notable exceptions) the cells used are cells in culture. And there lies the rub, as cells in culture are fickle: *'qual piuma al vento'*, as Verdi would say. Thrown, somewhat brutally, into a hostile environment, cells in culture respond with a number of tricks to ensure their survival. Some (like human diploid fibroblasts) maintain a rigorous growth control but offer a stubborn resistance to transformation. At the opposite end of the spectrum, HeLa cells have jettisoned all growth controls and can be reduced to a state of no growth only by the drastic expedient of removing all proteins, a stage that closely resembles death. In between lies all kinds of cell lines, each of them with different growth requirements, different stabilities and different ranges of behaviour. Hence each cell line requires a different assay, and it would be foolish to expect that blood lymphocytes (the best $G_0$ cells on our planet) should behave in the same manner as HeLa cells. This book attempts to define these different assays in selected animal cell lines. I have tried to include some of the cell lines most frequently used as well as those that are less popular, concentrating on those that show growth regulation. The book should be useful to cell biologists, but particularly to molecular biologists who are interested in growth factors, growth-regulated genes and transformation.

I would like to thank all the contributors to this book, who actually sent their chapters *almost* within the deadline, and the staff of IRL Press, who have displayed an interest in the proceedings which is almost unique in publishers of scientific books.

Renato Baserga

## Abbreviations

| | |
|---|---|
| ALL | acute lymphoblastic leukaemia |
| ANLL | acute non-lymphoblastic leukaemia |
| ATCC | American Tissue Culture Collection |
| BCGF | B-cell growth factor |
| BSA | bovine serum albumin |
| CEE | chick embryo extract |
| CK | creatine kinase |
| CPDL | cumulative population doubling level |
| CSA | colony-stimulating activity |
| CSF | colony-stimulating factor |
| DEX | dexamethasone |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulphoxide |
| EBV | Epstein–Barr virus |
| EGF | epidermal growth factor |
| FCS | foetal calf serum |
| FGF | fibroblast growth factor |
| FITC | fluorescein isothiocyanate |
| GCT | giant cell tumour |
| GM | granulocyte–macrophage |
| HDL | high density lipoprotein |
| HS | horse serum |
| HTLV | human T-cell leukaemia virus |
| IL | interleukin |
| INS | insulin |
| LCM | lymphocyte-conditioned medium |
| $\beta$-ME | $\beta$-mercaptoethanol |
| MEM | minimum essential medium |
| MLR | mixed lymphocyte reaction |
| MSA | multiplication stimulating activity |
| PAI | plasminogen activator inhibitor |
| PBS | phosphate-buffered saline |
| PDGF | platelet-derived growth factor |
| PHA | phytohaemagglutinin |
| PPLO | pleuropneumonia-like organism |
| PPP | platelet-poor plasma |
| SFME | serum-free mouse embryo |
| STI | soybean trypsin inhibitor |
| TCA | trichloroacetic acid |
| THR | thrombin |
| TPA | tetradecanoylphorbol-13-acetate |
| TRS | transferrin |

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## 3. EFFECTS OF ONCOGENE EXPRESSION ON CELLULAR GROWTH FACTOR REQUIREMENTS: DEFINED MEDIA FOR THE CULTURE OF C3H 10T1/2 AND NIH3T3 CELL LINES 37

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CHAPTER 1

# Measuring parameters of growth

RENATO BASERGA

## 1. INTRODUCTION

This chapter deals with the various parameters of growth and how they can be measured.

A tissue can grow by: (i) increasing the number of cells; (ii) increasing the size of the cells; or (iii) increasing the amount of intercellular substance. Since the intercellular substance of a tissue is usually a secreted product of the cell, for example collagen, it can be considered, so to speak, as an extracellular extension of the cytoplasm. We can therefore consider an increase in intercellular substance as a variation of an increase in cell size and thereby reduce tissue growth to two mechanisms, growth in size and growth in the number of cells. This is true regardless of whether we are dealing with normal or abnormal growth. However, although both mechanisms may be operative, increase in cell number is by far the most important component in either normal or abnormal growth. Cells in culture can also grow either by increasing their size or by increasing their number.

There are also static and dynamic ways of measuring growth and cell division. For instance, counting the number of cells in a Petri dish tells us how much that cell population has grown. It does not tell us whether or not the cells are still proliferating. Other methodologies (autoradiography with [$^3$H]thymidine, flow cytophotometry, etc.) are necessary if we wish to examine cell proliferation and its pertubations in more detail.

In the following sections I will give a few simple techniques for measuring cell growth, and I will try to stress their interpretation and their limitations.

## 2. GROWTH PARAMETERS

From the foregoing, it is clear that there are several ways of measuring parameters of growth. The question is: which parameter of growth does one wish to measure? Take, for instance, a typical experiment in which one wishes to determine the effect that a growth factor has on a population of cells in culture. It is often stated in seminars and papers that a certain growth factor is mitogenic, but the only evidence we are shown to document its mitogenicity is a labelling index (with [$^3$H]thymidine) or, even worse, incorporation of radioactive thymidine into acid-soluble material. Mitogenic means that it induces mitosis: that is that cells divide and increase in number. Incorporation of [$^3$H]thymidine measures DNA synthesis, not cell division. The two processes often go together, but they can also

be separated (for a review, see ref. 1). If we wish to determine the effect of growth factors (or of any environmental change) on cell proliferation, that is their ability to stimulate or inhibit cell division, the best method is very simple, and it is to count the number of cells before and after treatment, possibly at 24-h intervals.

## 2.1 Counting the number of cells in cultures

(i) Prepare the dishes in which the cells have been grown (this example is for 100-mm dishes).

(ii) Prepare a trypsin solution, either 0.25 or 0.1% (see note i below) in Hanks' balanced salt solution (containing no $Ca^{2+}$ or $Mg^{2+}$).

(iii) Pour Hanks' solution (no $Ca^{2+}$ or $Mg^{2+}$) into 50-ml tubes.

(iv) Remove the medium from the dishes and set aside.

(v) Wash with 10 ml of Hanks'; remove.

(vi) Add 10 ml of trypsin solution and leave for 30 sec–1.5 min (note i).

(vii) Remove the trypsin solution and let stand at room temperature for 2–3 min (note i).

(viii) Add the growth medium (which includes 5% calf serum), 10 ml. At this point the cells will detach from the surface. In these days of very expensive serum we use the conditioned medium obtained from step (iv) to inhibit trypsin instead of fresh growth medium.

(ix) Mix well using a sterile pipette, drawing the cell suspension up and down the pipette 5–10 times.

(x) Count in a haemocytometer (*Figure 1*), by depositing a few drops of cell suspension under the coverslip. Use the four corners to count cells, divide by 4 and multiply by $10^4$ to obtain cells/ml.

For instance, if you count 140 cells in the four corners:

$$140/4 = 35;\ 35 \times 10^4 = 3.5 \times 10^5 \text{ cells/ml}$$

Notes

(i) Trypsin strength varies from one batch to another (regardless of what the manufacturer says) and, in addition, sensitivity to trypsin is different in different cell lines. Therefore, it is impossible to give a single optimal trypsin concentration. One has to go by trial and error, and the same comments apply to steps (vi) and (vii). The goal is to obtain a suspension of single cells with as little as possible cellular debris.

(ii) The amounts can be appropriately scaled down if one uses smaller Petri dishes. The amounts of growth medium we use *to grow* cells (not to trypsinize them) are 20, 8 and 3 ml respectively, for 100-, 60- and 35-mm dishes. In short-term experiments (24–48 h), the amounts of growth medium can be reduced to half the indicated volumes, resulting in considerable savings.

(iii) Counting the number of cells in a solid tissue is somewhat more complicated. The difficulty here is to obtain a satisfactory suspension of single cells. Perhaps, for solid tissues, DNA amount (see Section 2.3) is the best available method.

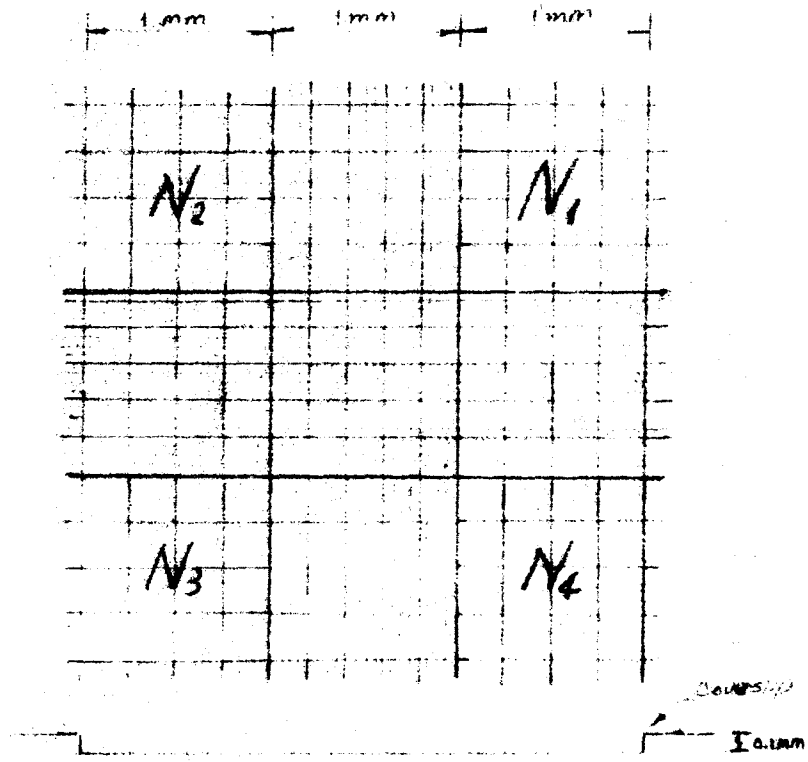


**Figure 1.** Diagram of haemocytometer's chambers. The grid is what one sees under the microscope. Below the grid is a cross-section of the haemocytometer, indicating that the space between the grid and the coverslip is 0.1 mm. Each *N* square thus has a volume of 0.1 $mm^3$. Multiplying the average number of cells per *N* square by $10^4$ gives the number of cells per ml. Further corrections are necessary if the cell suspension has been diluted before counting.

## 2.2 Indirect measurements of cell number

The number of cells per dish is logically the best parameter to use to indicate whether or not a population of cells is growing. There are, however, alternatives. For instance, one can measure the amount of DNA per dish, or the amount of RNA, or the amount of proteins, or count the number of mitoses.

## 2.3 DNA amount

The amount of DNA per dish can be determined, for instance, at 24-h intervals after plating. It is also a very simple procedure; indeed, for someone trained in biochemistry, it is easier than counting the number of cells. Since the amount of DNA per cell is *usually* constant (in mammalian cells, $6 \times 10^{-12}$ g/diploid cell in $G_1$), the amount of DNA per dish is an indirect measure of the number of cells. The $G_1$ amount of DNA in somatic cells is generally referred to as the $2n$ amount.

Cells in S-phase or in $G_2$ have increased amounts of DNA with respect to $G_1$ cells, but, if a population is truly growing, the increase in total amount of DNA per dish will go way beyond the error caused by the individual variations due to the distribution of cells throughout the cell cycle. DNA amount is probably the method of choice for solid tissues. Using the amount of DNA per cell given above, one can calculate (from the amount of DNA per $\mu$g) the number of cells per $\mu$g of tissue. A rough (very rough) estimate is that 1 $\mu$g of tissue contains $5 \times 10^8$ cells, but this estimate will vary greatly with the type of tissue.

A possible source of error is polyploidy, that is an increase in the amount of DNA per cell from $2n$ to $4n$ or even $8n$, in which case one could have an increase in the amount of DNA per dish without a concomitant increase in the number of cells. This is not a frequent occurrence, but it can happen, for instance, in certain pathological conditions, in response to certain drugs or when a large proportion of cells is blocked in $G_2$ (reviewed in ref. 1).

There are several methods for determining the amount of DNA in a culture dish or in a tissue. I prefer the classic method of Burton (2).

### 2.4 RNA amount

For a given cell type, the amount of RNA ought to be constant. As with DNA, $G_2$ cells will have roughly twice the amount of RNA as $G_1$ cells. As a measure of cell number, however, RNA amount is less accurate than DNA amount because, in several conditions, cells can grow in size and increase their RNA amount without cell division (3). Indeed, RNA amount is a good indicator of cell size, rather than cell number. Most of the cellular RNA that is measured by bulk chemical methods or individual cell histochemical methods is ribosomal RNA (rRNA ~85% of total cellular RNA). Since rRNA forms a part of the ribosome, on which protein synthesis is carried out, it seems logical that RNA amounts ought to be a reasonable indicator of cell size, a hypothesis that has been empirically confirmed. Classic methods for the determination of RNA amounts can be found elsewhere. If one wishes to determine the amount of RNA in individual cells, one needs expensive equipment that, in addition, require technical expertise to operate. I prefer computerized microspectrophotometry (4) to flow cytofluorimetry, but both are complex and unless one is a devoted cell biologist who loves to look at single cells, my advice is that when these instruments are needed one should seek collaboration. Flow cytometry has already been discussed in a previous book from this series (5).

### 2.5 Protein amount

The same comments apply here as to RNA amount. The amount of cellular protein is a reasonable indicator of cell size, but a poor indicator of cell number.

## 3. MITOSES

Surely, if one is looking at the mitogenic effect of a substance, the number of mitoses ought to be the best indicator of such an effect. However, mitoses are

fleeting; in most cells they last only 45 min and, unless one looks at precisely the right moment, one may miss them. Furthermore, the duration of mitosis can increase in certain cells, especially in transformed cells (6). Everything else being equal, if the duration of mitosis in cell line A is twice that of cell line B, the number of mitoses in A will also be twice that of B, although the two cell lines may grow at the same rates. In tissues, the number of mitoses per 1000 cells (the mitotic index) is a reasonable measure of the *proliferating activity* of a cell population, but not of its growth. For instance, in the crypts of the lining epithelium of the small intestine, there are many mitoses. Fortunately for us, the small intestine in the adult individual does not grow, because, for every new cell produced in the crypts, one dies at the tips of the villi. So, in any given cell population, one must distinguish between cell division and increase in cell number.

There are also technical problems. To begin with, if we wish to determine the mitotic index of cells in culture a 22-$mm^2$ coverslip will have to be placed into the culture dish (see below for the preparation of coverslips). Mitoses can then be counted directly on the coverslips after fixation and staining (see below). Staining and counting of mitoses directly on plastic surfaces is not advisable. However, suppose that a wave of mitoses occurse 25–27 h after stimulation of a quiescent cell population with growth factors. One may miss it, unless samples are taken practically every hour. More economic in terms of time and money is to add a drug that will arrrest cells in mitosis. One can then count the percentage of cells that *accumulate* in mitosis over a certain period of time. The three main drugs for this purpose are colcemid, colchicine and nocodazole.

Colcemid and colchicine are very similar but the latter is more toxic. For mitotic arrest, the optimal concentration of colcemid is 0.16 $\mu$g/ml for human cells or 0.04–0.08 $\mu$g/ml for rodent cells. I like to leave the drug in for 4 h, then fix the coverslips. By dividing the time period into 4-h blocks (for instance, 16–20 h; 20–24 h; 24–28 h) after serum-stimulation, once should be able to get a pretty good idea of the mitotic activity of a cell population. If cells are left in colcemid (and especially colchicine) for more than 4 h, cell damage occurs with loss of mitotic figures.

Nocodazole (7) offers the advantage that it can be used for longer periods of time, 16–24 h. We use it at concentrations of 0.04–0.2 $\mu$g/ml, and mitoses, clearly identifiable, continue to accumulate. Depending on the cell line, and up to 12–16 h, nocodazole arrest is reversible (so is colcemid-arrest but only up to 4 h).

### 3.1 To clean coverslips for tissue cultures

(i) Pour chromic sulphuric acid (enough to cover the coverslips) into a large Petri dish.
(ii) One by one, place each coverslip in the acid. Leave to soak for 30–45 min.
(iii) Remove the acid and place the coverslips in a beaker. Let water run over them for 1–2 days.
(iv) After 1–2 days rinsing, rinse again with deionized water.
(v) Then take three large Petri dishes. Fill the first with methanol; the other two with 80% ethanol. Place all the coverslips in methanol; then individually

rinse each coverslip — first in one dish of ethanol, then in the other. Lay them out to dry on paper towels or wipe with gauze pads. Then autoclave. All handling after the chromic sulphuric acid is done with tweezers.

(vi) Coverslips are placed in tissue culture and, at the desired times, they are removed, washed three times in buffer and then fixed in methanol at −20°C for 15 min. The coverslips are then mounted (cells up!) on a regular glass slide for convenient handling, using ordinary nail polish.

### 3.2 **Cells arrested in mitosis by nocodazole**

(i) Coat the coverslips (four per 100-mm Petri dish) with poly-L-lysine (Sigma 3000 mol. wt) at 1 mg/ml dissolved in Hanks' (calcium, magnesium free solution). Leave for 24 h.

(ii) Remove the polylysine solution and allow the coverslips to dry.

(iii) Plate $5 \times 10^5$ cells per 100-mm dish in normal growth medium, each dish containing two polylysine-coated coverslips.

(iv) After 18 h remove the medium and add fresh growth medium plus 0.1–0.2 $\mu$g/ml of nocodazole dissolved in dimethylsulphoxide (DMSO).

(v) Leave for the desired period of time and then fix using the method given above and stain with Giemsa/Sorenson's buffer.

## 4. DNA SYNTHESIS

It is often desirable to measure DNA synthesis instead of cell proliferation. This is especially true if one wishes to study $G_1$ events leading to the replication of DNA or if one wishes to know the fraction of proliferating cells in a given cell population. Measurements of DNA synthesis are also much more impressive than counting cell number. For instance, a mitogenic stimulus may double the number of cells in a Petri dish in 24 h. In the same time, the fraction of cells labelled by [$^3$H]thymidine will go from 0.1 to 90%, virtually eliminating the need of statistical analysis.

The method of choice here is high-resolution autoradiography with [$^3$H]thymidine. I will first outline the technique and then discuss its advantages and disadvantages.

### 4.1 **Autoradiography** (modified from Baserga and Malamud, 8)

Coverslips for autoradiography are prepared as outlined in Section 3.1. Cells are grown on coverslips and fixed as described in Section 3.1.

#### 4.1.1 *Preparing tissue sections for autoradiography*

(i) Mounting the section on precleaned slides rubbed just prior to use with fresh egg albumin (egg white). (Avoid commercial albumin since it contains phenol, a reducing agent which causes a high background.)

(ii) Cut tissue sections of 3–10 (usually 5) $\mu$m.

(iii) Deparaffinize slides; set up 11 staining dishes:
(a–d) xylene — 5 min each.
(e) 50% xylene and 50% absolute ethanol — 5 min.
(f–j) 100% alcohol — 3–5 min, then 95, 70, 50, 35%, always for 3–5 min.
(k) distilled water — until slides are ready to be dipped. Do not allow to stand in water longer than 0.5–1 h.

4.1.2 *Dipping technique*

(i) *Equipment.*
Slides to be dipped carrying a coverslip or tissue section complete with numbers applied with Indian ink at least 1 h before dipping, plus two trial slides
Slide boxes with bags of Drierite (Bakelite slide boxes, 25-slide capacity) Drierite, gauze sponges
NTB2 (Nuclear Track Emulsion) (Eastman Kodak Co., Rochester, NY)
Scotch Brand Pressure Sensitive Tape (Minnesota Mining & Manufacturing Co., St Paul, MN)
X-ray film envelopes, or aluminium foil
Red china marker
L-shaped galvanized tray
Timer
Coplin jar half-filled with distilled water at 40°C
Glass stirring rod
Scissors
Plastic bag
Log (batch no., slide no., date dipped, date developed, date stained, type emulsion, etc.)
Water bath at 40°C
Darkroom — use only Kodak Safe-Light 'Wratten' Series red lamp with 15-W bulb, with red filter, only when necessary.

(ii) *Procedure.*
(a) Melt the emulsion by placing it into constant temperature bath at 40°C for approximately 90 min.
(b) Hydrate the tissue slides or mounted coverslips in distilled water not more than 0.5 h before dipping (see above).
(c) If the emulsion is to be used undiluted, pour it into a beaker and keep in a water bath at 40°C.
(d) If the emulsion is to be used diluted: in complete darkness, except for a safe-light, fill the rest of the Coplin jar (see Equipment), with emulsion. (This 1 : 1 dilution may be discarded at the end of the experiment.)
(e) DO NOT LET ANY LIGHT FALL ON EMULSION (turn safe-light towards wall).
(f) Dip in two clean trial slides to test the consistency of emulsion.
(g) Dip the experimental slides back to back, for about 2 sec, vertically with

frosted ends up into emulsion. Separate the slides and place on an L-shaped tray, frosted ends forward and up. Be careful not to scrape the side of the emulsion container or touch the surface of slide; it may cause mechanical exposure.

(h) Slides should air-dry in 20–30 min, but may take longer in a small damp room. Test trial slides to see if they are dry.

(i) When the slides are completely dry place ten or less, in a bakelite box. Seal the closure edge with black tape. Wrap the box securely in light-tight film envelope or two layers of aluminium foil, then completely seal with black tape. Write the batch no. with a china marker on the tape. Place the boxes in a plastic bag, wrap it around them and fasten it closed.

(j) Place the batch in a refrigerator to allow for exposure.

4.1.3 *Exposure*

The exposure time of autoradiographs depends on the type and amount of isotope used. Mouse tissues treated with 10 $\mu$Ci of [$^3$H]Tdr per mouse require about 10–12 days' exposure time.

Cell cultures: 0.02 $\mu$Ci/ml of [$^3$H]thymidine for 24 h labelling require 3–4 days' exposure. For short labelling pulses (30 min or so) use 0.5 $\mu$Ci/ml and 3 days' exposure. If you are in a hurry, simply increase the concentration of [$^3$H]thymidine, but remember, long exposure of cells to high concentrations can cause radiation damage.

4.1.4 *Developing*

(i) *Equipment.*

Water bath at 18°C including two buckets of crushed ice and thermometer
Darkroom, using only a 15-W bulb with red filter
Six staining dishes placed in a water bath
Staining trays, timer
Distilled water
D-19 Developer, make up fresh every week, store in a brown bottle at room temperature, dilution 595 $\mu$g in 3.8 litres, or 156 $\mu$g in 1 litre. Always filter before use (Eastman Kodak Co., Rochester, NY)
F-10 Fixer, make every 3–4 weeks and store in a brown bottle at room temperature (97 $\mu$g/500 ml) (Eastman Kodak Co., Rochester, NY — 1 lb package — 3800 ml $H_2O$ at room temperature), filter and dilute 1:1 with distilled water before use.

(ii) *Procedure:* darken room.

(a) Fill the staining dishes with changes solutions (see below).

(b) Place the slides in racks.

(c) Slightly dirty the changes solutions by running an empty tray through them (it sounds magical, but it works better).

(d) Change solutions every ten slides.