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.

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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
β-ME	β-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

Contents

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.

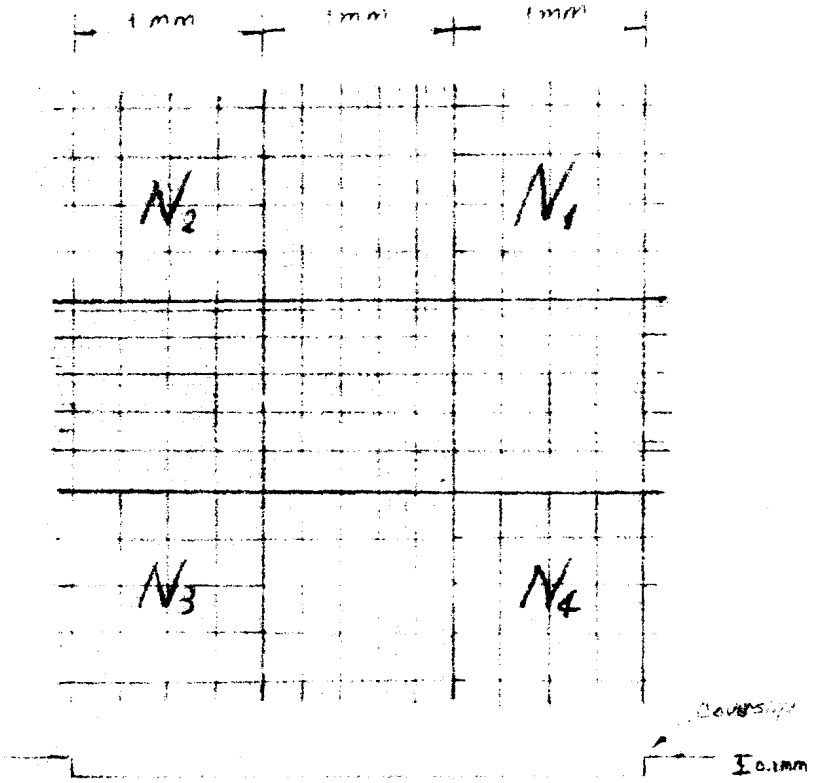

Figure 1. Diagram of haemocytometer's chambers. The grid is what one sees under the microscope. Below the grid is a cross-section of the haemocytometer, indicating that the space between the grid and the coverslip is 0.1 mm. Each *N* square thus has a volume of 0.1 mm^3. Multiplying the average number 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, 6×10^{-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 μg) the number of cells per μg of tissue. A rough (very rough) estimate is that 1 μg of tissue contains 5×10^8 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