

Cell growth and division

a practical approach

**Edited by
Renato Baserga**



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 **OIRL PRESS**
at
OXFORD UNIVERSITY PRESS
Oxford New York Tokyo

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
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

ABBREVIATIONS

xiv

1. MEASURING PARAMETERS OF GROWTH

1

R. Baserga

Introduction

1

Growth Parameters

1

Counting the number of cells in cultures

2

Indirect measurements of cell number

3

DNA amount

3

RNA amount

4

Protein amount

4

Mitoses

4

To clean coverslips for tissue cultures

5

Cells arrested in mitosis by nocodazole

6

DNA Synthesis

6

Autoradiography

6

Determination of Parameters of Growth by Autoradiography

with [^3H]Thymidine

11

Growth fraction

12

Quiescent cells and stationary cell populations

13

Phases of the cell cycle

13

Short method for growth fraction and cell cycle phases

14

Flow of cells through the cell cycle

15

Quiescence of Cells

15

References

16

2. PRIMARY AND MULTIPASSAGE CULTURE OF MOUSE EMBRYO CELLS IN SERUM-CONTAINING AND SERUM-FREE MEDIA

17

D. Loo, C. Rawson, T. Ernst, S. Shirahata and D. Barnes

Introduction

17

Primary and Precrisis Culture

17

Primary culture of late stage mouse embryos

17

Primary culture of earlier stage mouse embryos and rat embryos

25

Passaging and cloning mouse embryo cells

26

Crisis and Immortalization

27

Extended culture in serum-containing media

27

Extended culture in serum-free medium

30

Plasmid Transfection and Selection of Transformed SFME Cells

in Serum-free medium

32

ix

	Serum-free transfection procedures: calcium and strontium phosphate precipitates	32
	Selection of oncogene-transformed serum-free derived cells	33
xix	Conclusion	34
	Acknowledgements	34
i	References	34
	MEASURING PARAMETERS OF GROWTH	
	R. Baserga	
	Introduction	
3	EFFECTS OF ONCOGENE EXPRESSION ON CELLULAR GROWTH FACTOR REQUIREMENTS: DEFINED MEDIA FOR THE CULTURE OF C3H 10T1/2 AND NIH3T3 CELL LINES	37
	D. Greenwood, A. Srinivasan, S. McGoogan and J. M. Pipas	
4	Introduction	37
4	General Methods	38
2	Laboratory facilities and equipment	38
6	Required solutions	38
6	Cell lines	41
6	Plasmids	41
	General Procedures	42
11	Notes	42
12	General protocols for culturing in defined media	43
13	Defined Media Protocols for NIH3T3 and C3H 10T1/2 Cells	44
13	General notes on NIH3T3 in defined media	44
14	General notes on C3H 10T1/2 in defined media	45
12	Miscellaneous Procedures	45
12	General notes on transfection	46
16	General notes on focus assay	47
	Picking colonies	48
	Staining dishes with crystal violet or Giemsa stain	49
17	EXPERIMENTS	50
17	Assays of response to growth factors	50
	Selection of transformed cell lines in defined media	51
	Modifications of a media formulation for a variant cell line	52
17	Example Results	53
17	7-day assay of growth of NIH3T3 in the presence of pairs of growth factors	53
22	Titration of response to FGF by NIH3T3	54
26	Changes in growth factor requirements of C3H 10T1/2 cells after transformation by SV40	55
27	Selection of cells transformed by tumour DNA using defined media	55
30	Comparison of growth in defined media of P86 and NIH3T3	58
	Acknowledgements	59
32	References	59

40	GROWTH OF HUMAN LEUKAEMIA CELLS <i>IN VITRO</i>	61
30	B. J. Lange	Assessing proliferative potential
40		Acknowledgements
40	Introduction	References
	Human Leukaemic Colony-Formation <i>In Vitro</i>	62
	Obtaining human leukaemic cells	62
	Preparation of leukaemic cells for culture	63
20	INTERLEUKIN-3-DRIVEN CLONAL GROWTH OF HUMAN LEUKAEMIA CELLS	67
	Procedure for colony-formation in CML	M. B. Przytowski 74
	Procedure for colony-formation in ALL	74
20	Procedure for Growing Leukaemic Cells in Suspension Culture	76
20	Short-term suspension culture	Preparation of Culture Medium 76
20	Long-term suspension culture	Preparation of stock solutions 77
20	Established cell lines	Culture medium 77
20	Virally transformed leukaemic cell lines	Obtaining a single cell suspension 78
20	Limitations of Systems for Growing Human Leukaemia Cells <i>In Vitro</i>	78
20	References	Preparing a single cell suspension 79
20		Deriving Cloned T-lymphocytes
20		Mixed lymphocyte culture
20		Isolating cloned T-lymphocytes
50	METHODS FOR CLONAL GROWTH AND SERIAL CULTIVATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTES AND MESOTHELIAL CELLS	81
100	J. G. Rheinwald	Characterization of cloned T-cells
100		Determination of DNA Content and Antigen Expression
100	Introduction	IL-2-driven T-cell proliferation 81
100	Basic Strategies and Considerations	Acknowledgements 82
100	Culture Media, Supplements and Solutions	References 83
	Growth factors and hormones	83
	Epidermal Keratinocyte Culture	84
	Culture medium	84
100	The 3T3 fibroblast feeder layer	MUSCLE CELL CULTURES 84
	Initiating cultures from skin	J. R. Florini, D. A. Ewton, E. Ferris and B. J. Lange 85
	Purifying keratinocyte cultures of contaminating fibroblasts	86
100	Growth of colonies and retention of clonogenic cell fraction	Introduction 87
100	Subcultivation	Properties of muscle cell cultures 88
100	Identifying keratinocytes in culture	Comparisons of cell lines and primary cultures 88
100	An alternative culture system for human epidermal cells	Conditions for culture 88
100	Mesothelial Cell Culture	Cell lines 89
114	Culture medium for human mesothelial cells	Primary muscle cells 89
118	Initiating cultures from peritoneal or pleural fluids	Modified or specialized media 89
118	Subcultivation	Serum-free media 90
118	Preparing 'differentiated' mesothelial cell populations	Use of hormones 90
118	Identifying mesothelial cells	Minimizing consumption of fetal bovine serum 91
100	General Methods	Acknowledgements 92
100	Staining cultures	References 92

Cryopreservation	92
Assessing proliferative potential	93
Acknowledgements	94
References	94
6. INTERLEUKIN-2-DRIVEN CLONED T-CELL PROLIFERATION	95
M. B. Prystowsky	
Introduction	95
Preparation of Culture Medium	96
Preparation of stock solutions	96
Culture medium	96
Obtaining a Single Cell Suspension of Murine Splenocytes	97
Harvesting spleens	97
Preparing a single cell suspension	97
Deriving Cloned T-lymphocytes	98
Mixed lymphocyte culture	98
Isolating cloned T-lymphocytes	98
Maintenance and expansion of cloned T-cells	99
General principles	99
Characterization of cloned T-cells	100
Determination of DNA Content and Antigen Expression	100
IL2-driven T-cell Proliferation	102
Acknowledgements	102
References	102
7. MUSCLE CELL CULTURES	105
J. R. Florini, D. Z. Ewton, E. Ferris and B. Nadal-Ginard	
Introduction	105
Properties of muscle cell cultures	105
Comparisons of cell lines and primary myoblast cultures	105
Conditions for Proliferation and Differentiation of Muscle Cell Cultures	106
Cell lines	106
Primary muscle cells	114
Modified or Specialized Media for the Growth of Muscle Cells	118
Serum-free media	118
Use of hormones and growth factors	118
Minimizing consumption of foetal bovine serum	119
Acknowledgements	120
References	120

8. CELL CULTURE OF HUMAN DIPLOID FIBROBLASTS IN SERUM-CONTAINING MEDIUM AND SERUM-FREE CHEMICALLY DEFINED MEDIUM	121
V. J. Cristofalo and P. D. Phillips	
Introduction	121
Propagation of Cells in Serum-supplemented Medium	122
Materials	122
Trypsinization and harvesting of the cells	122
Propagation of Cells in Serum-free Medium	126
Materials	126
Trypsinizing and harvesting the cells	128
A classification of growth factors for WI-38 cells	130
Conclusions	130
References	130
9. CELL SYNCHRONIZATION	133
G. S. Stein and J. L. Stein	
Introduction	133
Synchronization of Continuously Dividing Cells	133
Double thymidine block	133
Mitotic selective detachment	135
Monitoring Cell Synchrony	136
Rate of DNA synthesis in suspension cultures	136
Rate of DNA synthesis in monolayer cultures	137
References	137
10. GROWTH AND MAINTENANCE OF BALB/c-3T3 CELLS	139
W. Wharton and M. J. Smyth	
Introduction	139
Basic Tissue Culture Techniques	139
Solutions used in the culture of BALB/c-3T3 cells	139
Growth of BALB/c-3T3 cells	141
Permanent storage of cells	143
Mycoplasma	144
Spontaneous transformation	145
Specialized Uses of BALB/c 3T3 Cells	147
Mitogenesis	147
Quantitation of transformation	148
Tumorigenicity	151
Acknowledgements	152
References	153
APPENDIX	155
INDEX	157

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 [^3H]thymidine, flow cytophotometry, etc.) are necessary if we wish to examine cell proliferation and its perturbations 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 [^3H]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 [^3H]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.

Measuring parameters of growth

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 j).
- (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.

There are also static and dynamic methods of counting cells. For instance, counting the number of cells in a Petri dish tells us how much that cell population has grown since the last time we counted it.

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.

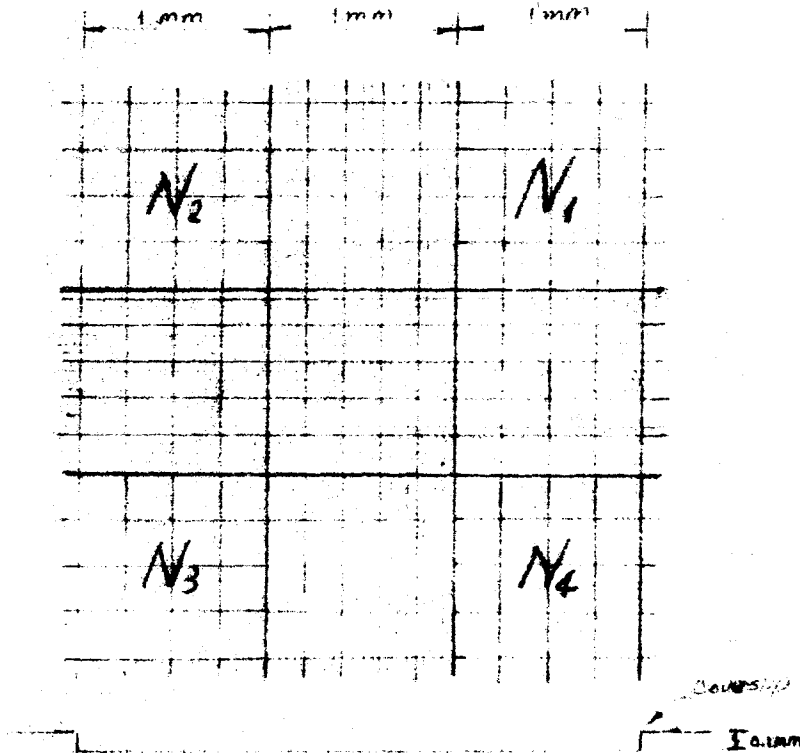


Figure 1. Diagram of haemocytometer's chambers. The grid is what one sees under the microscope. Below the grid is a cross-section of the haemocytometer, indicating that the space between the grid and the coverslip is 0.1 mm. Each N square thus has a volume of 0.1 mm^3 . Multiplying the average number 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, $6 \times 10^{-12} \text{ g/diploid cell in } G_1$), 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.

Measuring parameters of growth

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 cytometry, 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-mm² 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 occurs 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 arrest 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 $\mu\text{g/ml}$ for human cells or 0.04–0.08 $\mu\text{g/ml}$ 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, one 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 $\mu\text{g/ml}$, 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

Measuring parameters of growth

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 5×10^5 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 $\mu\text{g/ml}$ 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 [^3H]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 [^3H]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) μm .

- (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 $10\mu\text{Ci}$ of $[^3\text{H}]\text{Tdr}$ per mouse require about 10–12 days' exposure time.

Cell cultures: $0.02\mu\text{Ci/ml}$ of $[^3\text{H}]\text{thymidine}$ for 24 h labelling require 3–4 days' exposure. For short labelling pulses (30 min or so) use $0.5\mu\text{Ci/ml}$ and 3 days' exposure. If you are in a hurry, simply increase the concentration of $[^3\text{H}]\text{thymidine}$, 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 $595\mu\text{g}$ in 3.8 litres, or $156\mu\text{g}$ 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 ($97\mu\text{g}/500\text{ml}$) (Eastman Kodak Co., Rochester, NY — 1 lb package — $3800\text{ml H}_2\text{O}$ 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.