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MAMMALIAN CELL CLONAL GROWTH AND SECRETION MEASUREMENTS USING GEL MICRODROPLETS AND FLOW CYTOMETRY.

by

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Table of Contents

Abstract 4
Introduction 5
Background 9
Protocol 19
Growth in GMDs 28
Flow cytometry measurements 33
Secretion 63
Screening effects of medium composition 81
Other technical problems 90
Summary 92
References 94

MAMMALIAN CELL CLONAL GROWTH AND SECRETION MEASUREMENTS USING GEL MICRODROPLETS AND FLOW CYTOMETRY.

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Abstract

Flow cytometry is a relatively new technology which can rapidly acquire optical measurements on thousands of individual cells. Hydrodynamic focusing is used to pass a suspension of fluorescence-labelled cells single file through a laser beam. Scattered and fluorescent light is collected, filtered, quantified and digitized, yielding measurements on individual cells at the rate of up to 10,000 cells per second. This thesis demonstrates that this capability for rapid measurement can be extended to monoclonal microcolonies of mammalian cells enclosed in microscopic (30-100μ) droplets of gelled agarose.

Bacteriology has long benefitted from the ability to rapidly separate and clone subpopulations of cells from a mixed sample. This is accomplished by streaking the mixed sample onto a solid surface in a petri dish so that individual cells are separated from their neighbors. The solid growth medium keeps clonal progeny and their secretions separate from other colonies. A parallel technology for mammalian cells uses microtiter wells (0.1-1.0 ml) and plate readers capable of scanning 96-well plates at about 1 well/second. The new methodology presented here immobilizes individual mammalian cells in gel microdroplets (GMDs, volume = 0.0000001 ml), cultures up to 1,000,000 GMDs in a T-75 flask (the size of two 96-well plates), and uses flow cytometry to measure the growth and secretory products of monoclonal microcolonies at up to 100 occupied GMDs/second.

GMDs are made by melting a 2.5% suspension of ultralow gelation temperature agarose in 0.5 ml of growth medium, cooling to 37 C, gently mixing in the cells, adding 10 ml of mineral oil and vortexing to produce a suspension of liquid microdroplets in the oil. The suspension is cooled for 20 minutes in ice water and the gelled microdroplets are transferred by centrifugation into growth medium for incubation. Microcolonies grow within the solid matrix of the gel. Fluorescent RNA and DNA dyes may be used to measure growth, thereby quantifying plating efficiency, growth rates, and even the variability in growth rates for any subpopulations within the sample. These measurements can be made as early as a single doubling time.

By coentrapping binding sites within the GMDs, cell secretions can be captured and labelled as in a solid phase immunoassay. This permits analysis and separation of those clones secreting desired products from a mixed population. Unlike encapsulation methods, which enclose microcolonies within an alginate-polylysine membrane, this coentrapment method permits the use of antibodies as labels. About 1000-4000 polystyrene beads (0.8μ) coated with goat anti-(mouse IgG) antibody were coentrapped in 53-88μ GMDs with various mixtures of nonproducing mouse mastocytoma cells (P815) and secreting mouse hybridoma cells (ATCC HB118). After 11 hours incubation (less than the 12-15 hr replication time) the GMDs were washed, stained with FITC-conjugated goat anti-(mouse IgG) antibody and washed again. GMDs containing secreting cells were thus labelled with green fluorescence and detectable using the flow cytometer. All labelling and washing steps are done on bulk suspensions of the GMDs, offering a considerable labor savings compared to 96-well plates. The screening rate of the flow cytometer is 100 times faster than comparable plate readers. Finally, the screening step can be performed after a few hours incubation, eliminating problems of overgrowth by nonsecreting cells.

Chapter 1

Introduction

Screening is an important procedure in many research problems in biology, medicine, and biotechnology. Ever since Koch demonstrated the ability to culture and separate individual colonies of bacteria, this ability has played a central role in bacteriology.[1] In contrast, the study of pathogens such as viruses, mycoplasma, and other obligate intracellular microorganisms has been hampered by the inability to rapidly culture and isolate the entity of interest. Selection (culturing a mixed population under conditions which favor only the desired organism) is a powerful isolation method in biology. Selection has many inherent advantages over screening (culturing a mixed population in a manner which allows individual clones to be isolated and individually tested). Selection is generally a better technique whenever it is applicable. However screening is often the only method available, especially when the criterion of interest is a cell function such as secretion or gene expression.[2]

Current technologies for screening are very tedious and time consuming. The slower growth rates of mammalian cells compared to bacteria aggravate the problem. Most bacteria have optimal doubling times of 20-60 minutes, which produces colonies visible to the naked eye within a single day. Yeast are slightly slower. Growth, detected as suspension turbidity, may be measured after a few hours. Plating yeast for subcloning requires 48-72 hrs. Mammalian cells double once or twice in a day, thereby

increasing the time needed to perform growth measurements and typically requiring up to 7-11 days for each subcloning step.

The biotechnology industry and medical research require methods for measuring the growth of mammalian cells. Both also need the ability to clone, analyze, and isolate mammalian cells. Biotechnology is keenly interested in screening cells based on their secretions, since secreted products are much easier to separate and purify than products that remain intracellular. Screening for secretors has importance in medical research as well, especially in the area of gene therapy.[2-6]

The goal of this thesis project was to develop and prove the feasibility of a new method for cloning and screening mammalian cells which could significantly decrease the labor, tedium and cost of current methods. Two recently developed technologies, flow cytometry and gel microdroplets (GMDs), were combined into a single method for mammalian cell culture such that the clonal growth and secretion of individual, monoclonal microcolonies can be rapidly and conveniently assayed. Flow cytometry is already used for screening and isolating individual mammalian cells based on surface proteins or internal products.[2,7] GMDs are a means for isolating individual clones and their progeny within a solid matrix.[8,9] The high throughput rates achievable by flow cytometry suggested that clonal growth and secretion measurements could be rapidly performed if the "containers" of individual microcolonies could be miniaturized to the point where flow cytometry could be utilized. This meant replacing the 0.1-1.0 ml microtiter well commonly used for screening with an easily manipulated 0.0000001 ml gel microdroplet in which individual cells can be immobilized, cloned, screened and isolated.

GMDs permit growth measurements on a large number of individual clones, allowing the identification of subpopulations with different growth rates. As long as the entity to be screened for (*i.e.* a particular secreted product) can also be trapped within

the same matrix and measured, GMDs plus flow cytometry offer a much faster and less tedious screening alternative to microtiter wells and soft agarose plates. The GMD methodology demonstrated in this thesis is especially useful because it can screen for a secreted product, not just the internal or membrane bound products. Since the same technologies used in flow cytometry for cell sorting should be extendable to GMDs, rapid isolation of interesting clones will be possible. In less than an hour 10^5 clones could be individually screened for growth or secretion and interesting clones isolated.

Proof of Feasibility

In order to demonstrate the feasibility of this new methodology, four major technical points needed to be demonstrated. These points are the focus of this thesis.

(1) A methodology for putting mammalian cells inside of GMDs, with adequate yields and viability.

(2) Ability for those cells to live, grow, and function inside the GMDs in a normal manner.

(3) Capability of the flow cytometer to measure the cell growth inside the GMDs.

(4) Adequate capture of secretions within the GMDs to perform a solid phase immunoassay within the GMDs and to detect the results with the flow cytometer.

If these major capabilities could be demonstrated (as they are in this thesis), then a wide variety of applications would be anticipated for this new combined technology. It was therefore desirable from the start of this project to keep the development of the technology as general as possible. GMDs of agarose were selected because of the established history of mammalian cell culture in agarose.[10-13] The technology can easily adopt most of the techniques of fluorescent labelling already in common use in

immunology, biology and medical research. The many capabilities of the flow cytometer, including multiple fluorescence measurements, can also be utilized.[14]

The next few chapters establish and evaluate the performance of GMD based flow cytometry on these four major points, along with some technical details.

Chapter 2 briefly reviews how work on immobilization moved from proteins and active enzymes in the 1970's to various bacteria, protoplasts and finally animal cells. Adaptations of some of Mosbach's techniques plus some techniques developed in our lab during the early 1980's provide the basic technology for creating GMDs as miniature microtiter wells and petri dishes.[8-11]

While the methodology is simple and robust to the degree that it has worked in several people's hands, there are many fine points not published in the general literature which are important for producing satisfactory results. These are outlined in chapter 3.

Chapter 4 provides experimental results demonstrating growth inside the GMDs for two different cell lines. The growth rates are approximating those of cells in free suspension. Cell viability and plating efficiency is also demonstrated to be satisfactory.

Chapter 5 extends the work of chapter 4 to the flow cytometer. It assesses the precision, accuracy, repeatability, validity and capability of flow cytometer measurements of cell growth inside GMDs. It shows that cell growth inside GMDs can be measured at the fundamental limit of a single doubling time.

Chapter 6 demonstrates the ability to capture secreted products within the GMDs, perform qualitative immunoassays and detect the results with the flow cytometer.

Chapter 7 presents data showing how this GMD based flow cytometry can be used to test for growth differences in various medium compositions.

Chapter 8 examines some technical issues involved in sorting and recovering interesting clones from a mixed population.

Chapter 2

Background

Two methodologies are usually used for mammalian cell cloning. These methods are the baseline technology against which this new method of GMD based flow cytometry will compete.

The most common method for screening mammalian cells uses a microtiter plate, which consists of a plate of 96 quarter milliliter plastic wells into which individual cells can be placed and grown until such time as their properties can be measured. This process is very tedious and labor intensive. The past ten years has seen a boon in gadgets and machinery to simplify and speed up this task. Eight and twelve tip pipettors, washing gadgets, automated plate washers and plate readers are but a few of these devices. All are descendants of a basic concept that human labor can be directly replaced by robotic control.[15] While functional, this technology is inherently slow and limited by practical considerations, not the least of which is how many plates will fit into a single incubator.

The second screening method clones cells in soft agar or agarose.[10, 12, 13] This technique is analogous to plating bacteria. A petri dish is partly filled with culture medium in gelled agarose. Cells are mixed with more medium and agarose. This liquid suspension is poured into the petri dish and spread as a thin layer before it is allowed to gel. Only a few hundred cells can be plated as clones on a single dish, so many dishes

are required. Colonies form in agarose gel matrix in the petri dish in much the same manner as in GMDs. The petri dish is slow and cumbersome to wash due to long distances through which diffusive equilibration must occur. Therefore, secretions are usually assayed by blotting the surface of the gel onto filter paper, performing the assay on the paper, and identifying positive spots on the filter paper with the corresponding location in the petri dish.[16] Interesting colonies are manually plucked from the gel and subcultured. This blot assay technique has some advantages. Very sensitive radioimmunassays can be performed. The cells themselves are not exposed to the assay, so toxic reagents and harsh conditions such as elevated temperature can be used in the assay. Multiple different assays can be performed in parallel. However, the need to transfer to filter paper is inherently less sensitive since only a fraction of the colony's secretions are absorbed. Furthermore, the blotting and harvesting steps are very time consuming. These can be avoided if the assay can be performed directly on the petri dish, despite the long wash steps.

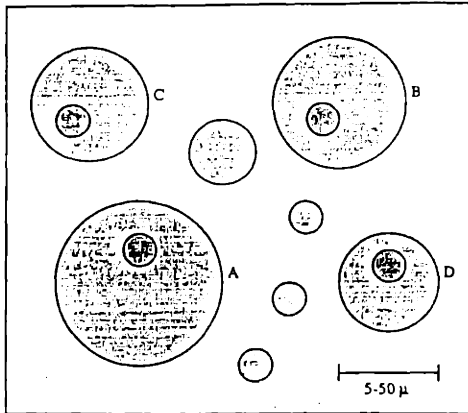
Recent advances in computing power and image recognition do offer the possibility of observing petri dishes under a robotically controlled fluorescence microscope system.[17] This method of scanning microfluorimetry continues to be developed and may become a competitor to GMD based flow cytometry. Both have theoretical advantages and disadvantages over the other. The merits of each system change as each develop. The GMD technology could also be adapted for use with scanning microfluorimetry. In general, flow cytometry seems to be faster while microfluorimetry can be more sensitive at very slow speeds. Microfluorimetry may retrace and reexamine interesting events, but is more susceptible to false events from nonspecific binding to the larger supporting matrix of the petri dish. This thesis is based on a preference for the already existing technology of flow cytometry.

History of the idea of GMDs

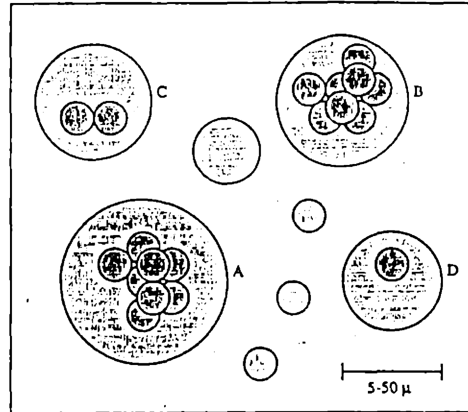
The initial concept of this project was to convert the microtiter well/petri dish into a miniaturized form which could be rapidly and easily manipulated in large numbers, with the intent of using a modified flow cytometer for analysis. Ideally, the form was to allow detection and analysis of growth at the fundamental limit of one replication. Its small size would also minimize the prospects for nonspecific binding to the matrix and improve the signal-to-noise ratio for detecting secreted products. Several ideas were considered.

One technology which had recently been developed was the microencapsulation of cells in an alginate-polylysine membrane.[18] While this method had great potential as a means of manufacturing products such as antibodies,[19,20] the very fact that the membrane is impermeable to antibodies is detrimental to screening since antibodies are extremely useful labels. Microcarriers, another possibility, would not work for growth measurements since cells can travel between carriers. It also would be unsatisfactory for secretion detection.

Therefore, attention was turned to a method of entrapment. The cells are entrapped in a solid matrix capable of isolating the clonal progeny as a single microcolony, yet permeable enough to allow nutrients and growth factors to diffuse in and out. (See schematic 1.) To enable the use of antibodies as a label, the matrix had to be permeable to large proteins. If secreted product was also one of the items of interest, separate binding sites could be immobilized in the gel matrix to capture the secretions. Hence the idea of entrapping cells in GMDs for growth measurements, and coentrapping coated beads as binding sites for their secreted products. The microcolony entrapped in the GMD may also permit screening based on internal or membrane constituents when the signal-to-noise ratio of single cell flow cytometry is not adequate.[7]



GMDs immediately after formation. At this inoculation density, most GMDs initially have 0 or 1 cell.



GMDs after incubation. The cells in A and B have replicated 3 times to form microcolonies of 8 cells. C contains a slower growing cell. The cell in D appears non-viable.

Mammalian cell growth within a solid matrix (agar) was first accomplished in 1964.[21] Various improvements on this technology have occurred, including use of methyl cellulose[22] and ultralow gelation temperature agarose.[12] The next major step was to obtain growth in entities smaller than the commonly used 35 mm petri dishes. Various methods for immobilizing enzymes and microorganisms on or within small (0.2-3.0 mm diameter) gel drops had been produced in the 1970's. Viability of the droplet entrapped animal cells was first demonstrated in 3000μ agarose beads formed by Teflon molds.[23] However, the ability for growth was originally seen only in the microencapsulated cells.[18] Finally, polyclonal growth was demonstrated in the solid matrix of fibrin and in 80-200μ agarose droplets.[10,24] Our lab was also involved in attempts to immobilize enzymes and microbes during this time.[25] Partly through independent work in our laboratory and partly guided by these new developments, G.B. Williams, a postdoctoral researcher in the laboratory was developing similar techniques but for smaller droplets, of 10-80μ diameter, for use in examining single enzyme molecules. My early work extended these methods to entrapping mammalian cells in 30-100μ GMDs and adapted the technology to the flow cytometer.

Several compounds were considered or experimented with for the manufacture of microdroplets, including including fibrin, alginate, and gelatin. Agarose was chosen for several reasons. Culture in agarose gel in petri dishes is an established method for mammalian cell cloning. Cells can be recovered from the gel and grown again in normal culture. The physical properties of agarose are satisfactory. It is optically clear, strong enough for the gel microdroplets to be manipulated, available in forms with usable gelation temperatures, and permits the diffusion of large macromolecules. Agarose is an inert matrix used for electrophoresis of many organic compounds, so it will not interfere with most staining protocols. Finally, a large technology already exists whereby various compounds are attached to agarose. While this technology

usually uses crosslinked agarose, it is expanding to other forms.‡ Extensions of this technology to GMDs will quickly permit a large number of compounds to be used as binding sites for secretion measurements.

Uniform size gel microdroplets would be beneficial for many reasons, including simplification of some types of data analysis. In an ideal case, individual cells would be entrapped near the center of uniformly sized spherical droplets. Previous work had centered on a droplet generator, but clogging was a major problem. Rayleigh dispersion of a capillary jet produces droplets whose diameters are mostly in the range of 1-4 times the nozzle diameter.[27] To produce gel microdroplets of 30-100μ diameter, the required nozzle is so small that clogging will always be a major problem. The pressure drops and shear forces will also be detrimental to cell viability.

Droplet size uniformity is not a critical item. It is unimportant for many potential uses of this technology. Indeed, the protocol for making GMDs that has already been developed is a satisfactory method for most experimental applications. Droplet size can also be measured with the flow cytometer by using signal width (time of flight) or by making the gel itself fluorescent and using its fluorescence to measure the volume. This size measurement could then be used in future applications where it is important.

The protocol for making GMDs in chapter 3 is a variant of several approaches tried by others in our laboratory, which in turn include variants of many techniques in the literature, especially the work of Nilsson and Mosbach.[10,11] However, unlike their method of making 80-500μ GMDs, my aim was to produce a majority of the GMDs with diameters less than 100μ, preferably about 30-100μ. This size produces an order

‡ Pharmacia P-L Biochemicals, Inc. of Piscataway, N.J. manufactures many of these compounds, also available through Sigma Chemical. Many of the binding techniques are published.[26]

of magnitude more GMDs per milliliter, which is important for screening monoclonal microcolonies. This also allows the GMDs to be used in an Ortho Cytofluorograf flow cytometer with a minimum of modifications. I made several changes to the work of G.B. Williams, who worked out the first effective methods for making these small GMDs by vortexing. In his enzyme work, Dr. Williams used a 5% solution of low gelling temperature agarose (Sigma Type VII, approximately 37° C gelling temperature for a 5% solution). I was concerned about exposing mammalian cells to the 40-45° C initial temperatures of the molten gel. Furthermore, working with an agarose solution near its gelling temperature meant small changes in temperature introduced significant variations in viscosity. The resultant GMD production was unreliable and of variable quality and yield.

I therefore chose to use ultralow gelation temperature agarose (Sigma Type IX, 15° C gelling) and expose the cells briefly to 5-10° C during the gelling phase. In order to obtain a less viscous solution so that smaller droplets could be obtained, I tested and ultimately used 2.5% rather than 5% agarose. This required finding ranges of centrifugation and pipetting forces which were gentle enough not to break the GMDs. The 2.5% agarose GMDs are easily pelleted at 400 to 800*g* without breakage. The 2.5% agarose is also stiff enough to resist deformation during sieving with nylon mesh. This means that the size range of GMDs fractionated between two sieves is close to range of the mesh openings. GMDs made with lower concentrations of agarose deform easily, slipping through the mesh and complicating the sieving operation. Finally, in order to obtain a large yield of GMDs in the desired small size class, I added fetal bovine serum to the agarose solution to act as a surfactant. This combination of a lower concentration of agarose plus the effect of the surfactant allowed vortexing to reliably create sufficient quantities of small GMDs.

There are many fine points in the GMD making procedure which influence its quality. Some of these tricks are based on repeated observations, while others are based on a single instance where a particular detail of the protocol appeared to help. Everyone in our laboratory uses slightly different methods, based on small differences in research projects and individual experience with the GMD making process. A large number of variables affect the dispersion of the agarose solution into droplets of the desired size. Not the least of these are the viscosities of both the oil and the agarose, which are very temperature dependent. I therefore recommend following the instructions for my protocol closely.

I have taught my protocol to a new post-doc who is using GMDs as a method for measuring growth following electroporation. This demostrates that my protocol is repeatable in another's hands. That protocol is explained in chapter 3, along with various comments, observations, superstitions, and plausible suggestions for scaleup if that is desired.

Basics of Flow Cytometry

Flow cytometry is a relatively new technology designed to rapidly acquire optical measurements on thousands of individual cells.† Hydrodynamic focusing is used to pass a suspension of fluorescence-labelled cells single file through a laser beam. Scattered and fluorescent light is collected, filtered, quantified and digitized, yielding measurements on individual cells at the rate of up to 10,000 cells per second.

† For basic information on flow cytometry, see books by Shapiro[28,29] and Melamed et al.[30] Many applications of flow cytometry are published in the journal **Cytometry** by the Society for Analytical Cytology, first published in 1981.

In hydrodynamic focussing, an aqueous suspension of cells is injected as the sample stream into the centerline of a larger sheath stream in laminar flow. As this combined sheath and sample stream enters a conical nozzle, both streams narrow in laminar fashion. In this manner, the objects (cells or GMDs) contained in the sample are aligned in single file and passed with spatial precision through a focussed laser beam. In the Ortho Cytofluorograph IIs, the sample stream is ejected from tubing 200µ in diameter at a flow rate of less than 0.5 ml/min into a sheath of deionized water flowing at 10 ml/min. It then enters an optically square and clear quartz flow cell where the combined stream narrows to a 250µ square channel. This results in the sample stream being narrowed to a 10-50µ core stream at the center of the channel. Optical measurements are taken while the objects are still within this channel. Other brands of flow cytometers squirt the stream into air and take the measurements from the capillary jet just distal to the nozzle but before the jet has broken into droplets.

A variety of lasers and fluorescent dyes may be used with the flow cytometer, giving it great flexibility. The experiments presented in this thesis all used the 488 nm (blue) line of an argon-krypton laser operated at 35 mW light output. The laser beam is focussed into an ellipse which is several hundred microns wide but only 5 to 8 microns high in the direction of flow. Microscope lenses, dichromatic mirrors and various color filters are all optically aligned to collect and separate by wavelength the scattered and fluorescent light from objects as they pass through the laser beam. This collected light is focussed into fiber optics which convey it to photomultiplier tubes where it is quantified. Fluorescent light is collected at 90° to the incident laser and the flow stream. This orthogonal position provides the darkest possible background. Blue light scattered from the objects can be collected at either 90° or at low forward angles. The forward blue scatter was used for the experiments in this thesis. Green fluorescence from fluorescein was measured through an FITC bandpass filter with very sharp cutoffs

outside 515 to 530 nm. Red fluorescence from propidium iodide was measured through a 630 nm long pass filter.

Since the height of the laser beam in the direction of flow is less than the object size (cells of 10-20μ and GMDs of up to 100μ), the measurement is made in slit-scan mode.[28] The resulting signal from the photomultiplier tubes must be integrated with time for each event as it occurs in order to quantify the total amount of fluorescence from that object. This is referred to as area mode, since the value output is proportional to the area under the signal curve, rather than its peak height or width. Integration is performed in analog mode and the final value digitized to 12 bits in a custom built data acquistion system.[†]

Flow cytometry is often combined with electrostatic sorting. Based on the values measured as the cell passes through the laser beam, a decision can be made to accept or reject that individual cell. As the stream is ejected into air it is vibrated by a piezoelectric at a frequency which causes the capillary jet to break up into uniform size droplets of water. The breakoff point in space and in time delay after passing through the laser is constant enough to permit electrical charging of each droplet at the moment it breaks away the capillary stream. The electric field between two capacitively charged plates can then deflect the flight of the desired charged droplets into a collection vessel. Alternatively, with the use of stepping motors, a 96 well plate can be moved mechanically to allow deposition of a single object in each well. Done under sterile conditions, this method allows the isolation, recovery and monoclonal culture of interesting cells from a mixed population.

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† Many thanks to Jon Bliss, who built the electronics and wrote the controller software necessary to acquire the data and transfer it to a computer for analysis.[31]

Chapter 3

Protocol

Mammalian cells and antibody coated beads were coentrapped in agarose GMDs by the steps listed below. The original work on this dispersion method for forming GMDs dates back to 1983,[25] but the current protocol was also influenced by the work of Nilsson and Mosbach.[10] These original protocols have been modified to produce GMDs with high yield in the range of 30 to 100μ in diameter. In volume terms, this produces over 10 times more GMDs per ml than the 80 to 200μ range of Nilsson and Mosbach, a significant advantage when the intent is to have single cell inoculations in order to study individual clones. Flow cytometry is also faster with these smaller particles, in the ideal case by a factor proportional to the diameter squared (maximal laminar flow velocity based on the Reynolds number is inversely related to channel diameter, and the minimum object spacing in the flow direction is directly proportional to object size). The method is designed for 15 ml test tubes, with suggestions for scale up if desired.

1) Combine 15-17 mg of agarose (Type IX, ultralow gelling temperature, Sigma) with 0.4 ml tissue culture medium containing 10% fetal bovine serum in a 15 ml polystyrene tissue culture tube (Falcon #3033).

Larger amounts could be made up, presterilized by autoclaving, and stored in liquid or solid form.[11] This is especially true if lower concentrations of agarose are used, since

the 4% agarose at this stage is difficult to pipet. However, the individual tube method is generally satisfactory, and has been performed many times without contamination problems, even though the powdered agarose is not sterile (see below).

Sigma's type IX agarose is recommended for several reasons. While many other ultralow temperature agarose preparations have been marketed recently, they are untested in my protocol. Small variations in the viscosity of either the oil or the agarose suspension may influence the size class of the GMDs produced.

Type IX agarose's gelation temperature is around 15° C, which is far below the 30° C of many low temperature agaroses. This simplifies handling. The tubes may be briefly exposed to room temperature without gelling. The cells are not exposed to 40-45° C as they must be with Sigma Type VII agarose. Finally, use of a low temperature agarose near its gelation temperature allows significant changes in viscosity with small temperature changes. Type IX seems to produce more consistent results with better yields of GMDs in the desired 30-100μ size class.

The chief problem with Type IX is a difficulty in adding liquid to the powder to form uniform suspensions. Type IX tends to form a more concentrated button of gel at the bottom of the tube. This may pose a major problem in scale up if larger samples are desired. The following are some suggestions for scale up that come partly from my own attempts and partly from literature.[11, 13]

Add the agarose by sprinkling it slowly into a continuously stirred bottle of phospahte buffered saline (PBS). Mix well and weigh bottle. Autoclave at 120° C for 30 minutes. Cool, reweigh, and add sterile water to correct weight. The use of PBS is suggested mainly because the heating process can cause precipitation of some amino acids and will degenerate others. As long as the GMDs will later be resuspended in growth medium, the composition of the medium used for GMD making is unimportant, with the probable exception of the fetal bovine serum (fbs). The serum appears to be

important as a surfactant for very small GMD formation and the oil to water transfer step. Use of serum provides larger yields of smaller, more uniform size GMDs. Extra serum could be added with the cells if not present when the agarose is dissolved.

The advantage of this bulk method for making up the agarose is the savings in time when large batches of GMDs are needed. Its use may be limited to agarose concentrations less than 4-5%. The more concentrated solutions are too viscous to pipet accurately. Using the tube protocol, the powdered agarose is not measured under sterile conditions, nor is it autoclaved. The next step in the process will heat the suspension enough to kill most microorganisms. No problems with contamination were ever noted in the short (1-3 day) experiments commonly performed for this thesis, nor on those few occasions where GMD growth was allowed to continue for up to 10 days. The tube method is often quicker for simple experiments.

The use of the Falcon #3033 round bottom polystyrene tube is important. Attempts to make GMDs in polypropylene tubes, glass tubes, or centrifuge tubes with conical bottoms all produced unsatisfactory results.

2) Heat for 4 minutes in an 85-90° C water bath to dissolve and melt the agarose.

This accomplishes two key purposes. First, it sterilizes the suspension for most common microorganisms. Second, this temperature helps disperse the agarose. When the powdered agarose is weighed into the tube and then the media added, the agarose tends to form a highly concentrated gel button at the bottom of the tube, rather than a uniform suspension. If still present, this button can be partly dispersed by mechanical stirring with the tip of a sterile rod or pipet. However, heating to this temperature range and vortexing for 5 seconds was often found to be sufficient. It most cases, the tubes were vortexed after 2 minutes in the hot water bath and then the loose caps were tightened to prevent further evaporation.

3) Cool for 4 minutes in a 37° C water bath.

Tubes may be kept at least half an hour at this temperature. Waiting time at this temperature also improves the uniformity of the agarose suspension.

4) Add and thoroughly mix by vortexing for 5 seconds 0.1 ml of a 2.0% suspension of 0.8μ antibody coated polystyrene beads as binding sites.

If no binding sites are desired, add 0.1 ml tissue culture medium with serum instead. This serum may be important, since part of the previous solution's serum may have been denatured by heating. Vortexing at this point seems to create fewer trapped air bubbles than mixing by pipet.

The beads used for these experiments are polystyrene assay particles from Pandex Laboratories (Mundelein, IL). The beads come coated with goat anti-(mouse IgG) antibody. To remove the sodium azide preservative, the beads are rinsed twice with sterile Hank's BSS, pelleting at 11000g x 5 min. They are resuspended by vortexing with a smaller quantity of serum free tissue culture medium to produce a bead suspension of 1.5-2.5%, which is 6 to 10 times greater concentration of beads than the purchased product. This increased bead concentration was needed for adequate capture of secretions.

These beads may be purchased from Pandex Laboratories with a large variety of coatings. The beads may also be purchased with activated surfaces that permit the user to coat the beads with almost any protein. It is this potential flexibility that prompted the use of these beads as binding sites in these experiments.

There are other potential types of binding sites. Cells, such as erythrocytes, might be coentrapped in the GMDs as binding sites. The technology for attaching various substrates directly to agarose is well established.[26] Agarose was chosen for GMDs because it was hoped that these other technologies would be easily adapted. Pharmacia

(Piscataway, NJ) sells a variety of products linked to agarose droplets for use in affinity columns. Most such droplets have been crosslinked and cannot be melted for use in making GMDs. However, non-crosslinked versions of agarose with covalently attached biochemicals have recently appeared on the market, as well as some very high molecular weight dextrans which could also be coentrapped in the gel matrix. These increasingly available products and technologies create great versatility for GMD based flow cytometry. The Sepharose 4B droplets (Pharmacia), sieved to the correct size, also provide useful test droplets for immunoassay protocol development and for flow cytometer adjustment and calibration.

5) Add 0.1 ml of tissue culture medium containing approximately $0.2\text{-}2.0 \times 10^7$ cells/ml, depending on how critical single cell occupancy (inoculation of the GMD with a single cell) is for that experiment.

If it is important to have mostly empty and singly occupied GMDs, aim for the lower range of this value. This inoculum produces an average of of 0.1 cells per 80μ GMD. Under these circumstances, Poisson statistics predict that 95% of occupied 80μ GMDs will be occupied by a single cell. On the other hand 90% of all 80μ GMDs will be empty. Smaller GMDs will be even more sparsely occupied. This slows down the rate of data collection by the flow cytometer and may also require extra work making extra tubes of GMDs. Use of an occupancy of 0.3 to even 1.0 cell per 80μ GMD produces faster results if they can be adequately interpreted. For screening purposes, it is possible to work at even higher cell densities, since purity can be achieved by a successive subculture. For experiments investigating growth, GMDs can be sieved to less than 60μ diameters in order to improve the fraction of occupied GMDs which are inoculated with a single cell.

6) Mix gently with the pipet or very gently with the vortexer (*i.e.* two 1 second bursts on the vortexer set at moderate.)

Mammalian cells are fragile and easily killed in this step. The need for viability vastly outweighs any disadvantage of nonuniformity in dispersing cells. To get true Poisson distributions the cells must be uniformly dispersed in the gel. However, the GMD size distribution is a much larger factor than Poisson statistics in determining the distribution of single and multiple occupancy, so thorough mixing is not critical nor encouraged.

7) Add 5 ml of autoclaved mineral oil (U.S.P. type, from Borden & Remington Corporation, Everett, MA) at 37° C and vortex maximally (Vortex-Genie from Fisher Scientific) for 15 seconds, thereby dispersing the aqueous phase into liquid microdroplets,

8) Then add an additional 5 ml of mineral oil at 37° C, and vortex maximally for 15 more seconds, creating more droplets and diluting them to reduce coalescence.

Many variations have been tried. The main reason for initially adding only half the oil is to increase the power of the first vortex in dispersing the agarose into the smallest possible liquid microdroplets (LMDs). Use of room temperature oil is possible but has a different viscosity than the 37° C oil recommended. The remaining oil is added to dilute the suspension and reduce coalescence of the LMDs before they are gelled. Adding ice cold oil at this point has been tried to help speed up the gelling, a problem at lower agarose concentrations (*i.e.* 0.5%). However, cold oil is harder to pipet and unnecessary in the standard protocol. Adding all 10 ml of oil at once produces nearly the same results as this two step addition. A ratio of 1 ml of agarose to 10 ml oil in a single tube has also been used with satisfactory results. The exact amount of oil is not

critical, so accurate pipetting is not required.

The oil should be sterilized by autoclaving. It will turn cloudy as the moisture introduced by the steam condenses, but this will clear in a few hours if the cap is loosened. Before autoclaving, the oil may be rinsed with water or PBS by use of a separatory funnel. This may remove potentially toxic contaminants. However, no specific problems were noted using unwashed oil. When attempting to grow cells in GMDs suspended in oil, the oil was allowed to equilibrate before use for several days in a 5% CO_2 37° C incubator. This equilibration is unnecessary if the GMDs are to be immediately transferred to growth medium.

9) Chill 20 minutes in an ice water bath, causing agarose gelation and GMD formation.

Gelation is a function of both temperature and time. Measurements indicate that 6° C is reached in the core of the tube after 5 minutes. Tubes were usually mixed by inversion every minute for the first 5 minutes to accelerate the gelation.

10) Pour the mineral oil-GMD suspension over 3 ml cell culture medium in a 15 ml polypropylene centrifuge tube.

Polypropylene appears important here. Transfer between phases is less efficient in polystyrene tubes.

11) Centrifuge at 400*g* x 5 min, then increase to 800*g* x 5min to transfer GMDs to the culture medium.

Attempts to transfer GMDs solely by rotation of the tubes on a rotator produced lower yields. The 2.5% Sigma Type IX agarose GMDs appear to tolerate 800*g* without any breakage.

12) Aspirate the mineral oil and the clumps of agarose and oil at the oil-water interface.

A small fraction of the agarose GMDs will clump at the oil-water interface. In many cases this layer is composed of GMDs which have entrapped small oil droplets, giving them a density between oil and water. It is best to discard these, since they will also float during any future centrifugations. However, if a large amount of the agarose is at the interface rather than in a pellet at the bottom, then the GMDs have stuck together in a clump encasing oil droplets. To remedy this, aspirate all the clear oil above this layer, recap the tube, gently agitate to break up the clumps, and pellet again for 4 min at 800*g*. The yield in the pellet will increase markedly. Residual oil can then be aspirated.

13) Rinse once with medium and pellet at 800*g* for 4 min.

This is useful for removing the oil which can cause trouble in future steps such as staining. Aspirate the residual oil before transferring the GMDs to a flask for incubation. Estimate the size of the GMD pellet to determine the correct dilution for incubation. Incubate GMDs in flasks or wells at cell densities similar to those used for normal cell culture.

This process yields GMDs suspended in the aqueous cell culture medium. The cell inoculum can be controlled. For growth experiments or high purity screening, about 10% of the GMDs will be occupied by cell(s), with 95% of those singly occupied (cloned). For low purity screening, initial occupation by multiple clones can be more frequent or even the norm. The coentrapped bead inoculation results in about 3800 beads per 80μ GMD.

For flow cytometry, the GMDs must be sieved to a size class usable by the flow cytometer. This sieving can occur immediately after GMD creation if aseptic

technique is used, or individual aliquots GMDs can be sieved after sampling and staining. With experience, 25-40% of the agarose by volume is converted to GMDs in the 44-88μ size class. This corresponds to a yield of 900,000 GMDs per test tube, not all of which may be occupied by a cell. An additional 10% of the agarose is converted to GMDs in the ≤ 44μ size class. While this is not as good as 100% conversion into uniform size GMDs (the ideal, from a data analysis standpoint), it is quite satisfactory for most applications. The product of several tubes can be pooled if necessary. For flow cytometry, aim for 10-50,000 occupied GMDs in each final sample to be analyzed. With some loss in accuracy, it is possible to divide samples into as few as 1000 occupied GMDs. If this is done, some spare samples should be created for use in determining and adjusting electronic gain settings on the flow cytometer.

This protocol, or very similar variants of it, have been used with 3 cell types: mouse hybridoma cells (PA2.6, ATTC HB118); mouse mastocytoma cells (p815, ATCC TIB64); and Chinese hamster ovary cells (CHO). Three types of culture media have been used: DMEM, RPMI 1640, and F12. The protocol has worked in the hands of three different people. Other variants of the protocol have been used by two other investigators working with yeast and bacteria rather than mammalian cells. This initial usage demonstrates adequate robustness in the protocol. It is therefore concluded that this protocol demonstrates the feasibility of using GMDs as miniaturized petri dishes and microtiter wells for the study of mammalian cell clonal growth and secretion.

Chapter 4

Growth in GMDs

Growth of the mammalian cells within the GMDs was the second major point that needed to be demonstrated to show feasibility of the flow cytometry based GMD technology. It is of course a misnomer to speak of normal growth since there is nothing "normal" about growing mouse cells outside of a mouse. However, *in vitro* cell culture is important for both biotechnology and medicine. The goal of this chapter therefore is to show that the growth and function of model cell lines inside agarose GMDs are similar to their growth and function when cultured in free suspension or in monolayer. The culture of mammalian cells in soft agarose is already an established technology.[12] Growth of mammalian cells in larger droplets of agarose is also established.[10] It therefore seems reasonable to believe that mammalian cells which can be cloned in soft agarose will also grow in these smaller GMDs providing they are not injured by the GMD formation protocol. Some key points shown in this thesis which support this belief are 1) the plating efficiency is satisfactory at 80-95%, 2) cell division occurs at rates approximating those of free suspension, and 3) normal cell functions such as secretion and metabolism are also occurring.

Measurements of growth were obtained in several ways, each showing some different aspects of growth. The first method provided visual confirmation of

viability, growth and cell division. Cells were stained with carboxyfluorescein diacetate (cFDA) and counterstained with propidium iodide (PI).[32] The cFDA is a nonfluorescent fluorescein molecule with two acetate groups attached via ester bonds. The nonpolar cFDA molecule can diffuse into cells through intact cell membranes. Inside cells with intact enzyme functions ("viable"), nonspecific enzymes cleave the ester bonds. This creates free fluorescein, which fluoresces green and does not rapidly diffuse back out of the cell. PI on the other hand is excluded by intact cell membranes but rapidly diffuses through holes in "dead" cell membranes and binds reversibly through intercalation to double-stranded RNA and DNA. Using this method, under fluorescent microscopy live cells appear green and dead cells appear red.

Methods Two cell lines were used for most tests. The first is p815, also known as ATCC TIB64, a mouse mastocytoma.[33] The second is PA2.6, also known as ATCC HB118, a mouse hybridoma cell line.[34] The standard culture medium was K medium, which consists of RPMI 1640 medium supplemented with 10 mM HEPES buffer, 10% fetal bovine serum, 300 mg/L L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were entrapped in GMDs by a standard protocol. Half of the sample was incubated with the GMDs still in the oil phase (aqueous GMDs surrounded by oil). The remaining GMDs were immediately transferred into K medium for incubation. Incubation was at 37° C, 5% CO_2. Aliquots were taken at various time points. In addition, after 42 hours incubation in oil, some GMDs were transferred from oil into K medium for further incubation. All aliquots of oil suspended GMDs were transferred to K medium before staining.

The stock solution of 10 mM cFDA dissolved in DMSO is stored frozen. It is diluted to 30 μM cFDA with PBS (Dulbecco's phosphate buffered saline without Ca^{+2} and Mg^{+2}, pH 7.3) Each sample of GMDs was pelleted (600*g* x 2 min) and resuspended in the 30 μM cFDA. This was incubated for 20 minutes at 37° C. The samples were then washed twice with PBS to remove extracellular cFDA and fluorescein, and finally resuspended in 10 μM PI in PBS. PI is an equilibrium dye and must be present during observation and measurement. Its affinity for double stranded nucleic acids is high enough that the dilute dye in solution does not give noticeable background fluorescence. The GMDs were observed on a haemocytometer under the blue or green excitation of a fluorescence microscope.

Observations The two cell lines produced similar results. When the GMDs were incubated while suspended in medium, cell growth was observed. The single cells entrapped in the gelled agarose matrix became microcolonies of 2 and then 4 distinct spherical cells. After longer incubations, *i.e.* at 42 hours, microcolonies of ≥ 16 cells appeared. In these large microcolonies the morphology of each cell, presumably squeezed by the displaced agarose, was altered to form a spherical microcolony. In early experiments, plating efficiency (based on green/red fluorescence and appearance of cell division) ranged from 20-80%. With experience and protocol refinements, the plating efficiency increased to 80-95%. The observed doubling times of 10-15 hrs were approximately those of the same cell line when grown in standard tissue culture.

Red and green stained cells were observed to be disjoint sets, correlated with replication. After 24 hours incubation, red (=dead) cells always occurred as unreplicated single cells. Microcolonies of 2 and 4 cells were all green. This

verified the red/green staining protocol for identification of viability. In larger microcolonies all the cells were green for the first 3 to 5 days. After this time, some large microcolonies would become mottled red and green, showing the presence of both live and dead cells as the cell density of the microcolony became too great and/or the medium became depleted of nutrients.

The cell lines were also tested for growth while the GMDs were suspended in the oil phase. This manner of incubation was motivated by future experiments in which transfer of water soluble compounds from one clonal microcolony to another is to be avoided. The oil phase could serve the same purpose as the plastic of the microtiter wells, separating one well from another. Unfortunately, when the GMDs were incubated while still in the oil phase, most entrapped cells did not replicate. A small increase in cell size was noted. A small fraction of the cells did undergo a single replication. But normal growth did not occur. The cells did retain their viability for ≥42 hours in the oil, as demonstrated by their green fluorescence upon exposure to cFDA. This viability was further demonstrated by transferring these oil incubated cells/GMDs to tissue culture medium and observing normal replication over the following 24 hrs. As Chapter 6 will discuss, hybridoma cells in oil-suspended GMDs do secrete antibodies. The reason for the lack of growth in oil suspension is not known, but it is easy to speculate either **a)** important nutrients or growth factors partition from the aqueous medium into oil phase or **b)** more likely, partition into the large oil-water interface surrounding the GMDs. Cytostatic toxicity of the oil was also not thoroughly investigated.

GMDs were produced where each cell type was coentrapped with a large number (100's) of 0.8μ polystyrene beads coated with goat anti-(mouse IgG) antibody (Pandex Laboratories, Mundelein, IL). These coentrapped beads were

used in subsequent experiments as binding sites for cell secretions. When incubated in oil suspension, cells in GMDs with beads were all stained red (indicating death) and showed no cell division. This is evidence of a toxic agent. It was later discovered that the manufacturer of the beads adds sodium azide (a potent cytocidal agent) as a preservative. For GMDs incubated in conventional growth medium, the presence of these 0.8μ beads had no demonstrable effect on viability, presumably due to dilution of the azide in the flask of tissue culture medium. Other experiments, wherein the beads were washed with PBS to remove the azide, showed that cell viability could be maintained inside GMDs with coentrapped beads, even while incubated in oil suspension. However cell division still did not occur as long as the GMDs remained suspended in oil.

These experiments visually confirmed the viability of the cells after the GMD forming process which entrapped them in the agarose matrix. The plating efficiency inside the GMDs was found to be satisfactory. The conversion of cFDA to fluorescein and its retention in the viable cells demonstrated that some metabolic function was still intact. The experiments confirmed growth and cell division inside GMDs except when incubated in oil suspension, in which case only maintenance of viability was observed. Chapter 5 will show that growth in GMDs is exponential at growth rates approximating those of standard tissue culture with the cells in free suspension. The preservation of secretory function for the hybridoma cell line will be shown in chapter 6. This secretory function was even maintained during oil suspension of the GMDs. Overall, these key features of cell activity, combined with fact that cloning in soft agarose is an established technology, demonstrate the feasibility of using agarose GMDs as miniaturized, individual microtiter wells and/or petri dishes.

Chapter 5

Flow Cytometry Measurements

Once it had been shown that mammalian cells could be entrapped inside gel microdroplets (GMDs) without adverse affects on growth or function, the next major step was to prove feasibility of using the flow cytometer to measure that growth inside the GMDs. There are several ways of using the flow cytometer to detect or measure growth. Each has its advantages and disadvantages.

Scatter The simplest method is to detect changes in the amount of light scattered by the microcolony as it grows. The flow cytometer uses the blue (488 nm) line of an argon-krypton laser to excite the fluorescent molecules. Light striking the GMD/cell scatters due to reflection and refraction. Scatter can be measured at 90° to the laser beam or at low forward angles. It is a "free measurement" since it is always available, does not require a fluorescent label, and does not interfere with any other fluorescence measurement. However, light scatter off the cells is not linear with cell number in the microcolony. Empty GMDs themselves also scatter some light. Still, forward blue scatter (FBS) provides a trigger and coincidence gating signal which is useful when measuring weak fluorescence events. The scatter signal is also monotonic with microcolony size, even though it is not linearly proportional. It may therefore be useful for distinguishing between single cells and microcolonies, thereby separating events

due to microcolonies from events due to single cells which have not replicated and are presumably dead.

Biomass The next simplest method for growth measurement is to directly stain the cells with a fluorescent dye whose quantity per cell is relatively constant. Many such dyes exist, staining cellular constituents such as protein, nucleic acids, or membrane components. The flow cytometry literature is replete with various methods, most of which could be adapted for GMDs.[29,35] Some stains can be used with live cells while others are designed for fixed cells. Fixed cell methods are easier for data analysis of time course experiments since the growth is stopped, allowing all the samples to be processed together. As a demonstration experiment, cells were fixed with methanol and stained with propidium iodide (PI). PI stains double-stranded DNA and RNA with red fluorescence. DNA and RNA content per cell is relatively constant, so this method provides a measurement related to "biomass" or the number of cells present.

DNA Measurement The most accurate method in flow cytometry for measuring cell growth is measurement of DNA. Mammalian cells in G0/G1 (resting) phase of the cell cycle kinetics have one diploid complement of DNA which is essentially identical from cell to cell. Flow cytometry can measure this DNA with a coefficient of variance of about 3%. Cells in G2/M (mitotic) phase have twice as much DNA, while the small fraction of S (synthesis) phase cells have intermediate values. Some dye systems used with flow cytometry can accurately measure DNA of live cells without harming them. Notable are the Hoechst dyes, such as Hoechst 33258 and 33342.[36] Most of these dyes are excited in the ultraviolet range. Since the flow cytometer used for these experiments was not equipped with a UV laser, an alternate method was used. The cells were

fixed, an enzyme was used to digest and remove the RNA, and the remaining DNA was stained with propidium iodide (PI).[37]

In assessing the use of the flow cytometer for GMD measurements, the key factors to be established are the precision, accuracy, repeatability, validity and capability of the measurements. Each will be illustrated in the experiments of this chapter. Precision is assessed using GMDs purposely occupied by multiple cells. The source of errors related to precision is confirmed by the uniform variance in log mode. Accuracy is evaluated by comparing free cells and cells in GMDs, and by comparing the measured values from microcolonies to integer multiples of values from single cells. Repeatability is assessed in the analysis of the many samples from a study of growth as a function of glutamine concentration. Validity is confirmed by showing exponential growth, comparing growth in free culture and growth in GMDs, and by comparing direct observation under the microscope of 2 and 4 cell microcolonies with flow cytometry data. The system's capability is thus demonstrated in many ways. It can measure the growth characteristics of several thousands clones in a few minutes of flow cytometry, detecting growth in each clone at the fundamental limit of a single replication. It is capable of counting cells in GMDs with sufficient resolution to determine plating efficiency. Together, these factors prove the feasibility and utility of this new methodology of GMD based flow cytometry.

Methods

Mammalian cells (either p815 or PA2.6) in exponential growth phase were entrapped in gel microdroplets according to the standard protocol, either with or without coentrapped beads. They were diluted in K medium to achieve an inoculum of about 100,000 cells/ml and incubated in 5 to 10 ml of culture medium in T-25 or T-75 flasks at 37°C, with high humidity and 5% CO_2. Aliquots were taken at various time points including time zero.

The cells were fixed with methanol by the following protocol: A 1.0 or 1.5 ml aliquot of GMDs was pelleted and rinsed twice in PBS (phosphate buffered saline) to remove the protein in the medium which would flocculate in methanol. Rinse steps involve suspending the GMD pellet in 1.0 or 1.5 ml of PBS, mixing by inversion, centrifuging for 2 minutes at 600*g*, and aspirating the supernatant. The pellet was resuspended in 0.5 ml of PBS to which was added dropwise 0.5 ml of methanol, thereby fixing the cells. These tubes of fixed cells inside GMDs could be stored in the refrigerator for several days or even weeks without degradation.

At time zero, an aliquot of the free cell suspension used to inoculate the GMDs was also fixed to serve as a standard. Some of the free cell suspension was used to directly inoculate a new flask of culture medium which was sampled at the end of the experiment as a second control to confirm steady state properties for the cells grown in free suspension.

After all samples had been collected and fixed in methanol for at least 4 hours, they were prepared for flow cytometry. This involved resuspending in PBS and sieving through nylon mesh filters to remove GMDs too large for the flow cytometer. For growth experiments GMDs were sieved through a mesh with

nominal 62 or 88μ openings. The 1 ml samples containing about 5-50,000 GMDs were sieved through a 0.7 cm^2 mesh and rinsed with 10 ml of PBS to assure adequate yields of small GMDs passing through the mesh. These small GMDs were pelleted and resuspended in 1 ml of PBS with 10 μM PI. The PI freely enters the methanol fixed cells and stains the double-stranded DNA and RNA with red fluorescence (RF). This provides an RF signal which is proportional to the number of cells in the microcolony. Free cells and single cells in GMDs are stained in as little as five minutes. Microcolonies in GMDs were given at least 45 minutes to equilibrate. This extra time was shown to be necessary by successive measurements on aliquots from the same sample. The extra time is needed to stain cells at the interior of the microcolony which are surrounded by other cells that absorb the dye as it diffuses by. These tables illustrate the validity of this staining protocol, using flow cytometry measurements on free cells.

PI dilution from 1 mM stock	median red fluorescence	coefficient of variance
1 / 1024	192	0.70
1 / 256	588	0.51
1 / 64	1088	0.46
1 / 16	1392	0.43
1 / 4	1604	0.41

minutes after addition of PI	median red fluorescence
1	460
2	640
3	697
4	720
5	748
47	712

From these tables, a 1/100 or 1/50 dilution (10 or 20μM) is seen to be adequate to stain most of the binding sites without excessive nonspecific binding. The time constant for staining free cells is seen to be about 1 minute.

In some experiments the fixed cells/GMDs were treated with RNAse before the staining step. This removed the RNA so only DNA was left to be stained by the PI. This decreases the signal strength by 30-50%, but dramatically decreases cell to cell variance in staining, which improves the precision and resolution of the cell count inside each GMD. Cell-to-cell variance in DNA content, or more exactly the staining of DNA content, is less than the 2 to 3% measurement variance of the flow cytometer.

RNA digestion is performed after sieving the GMDs by rinsing the fixed cells/GMDs with PBS and incubating 50 μL of GMDs in 0.5 mL of PBS containing 10 Kunitz Units per ml of RNase (Type Xa, Sigma) for 30 min at 37° C. For staining, the GMDs can be pelleted and resuspended in PBS with PI. Alternatively, the correct amount of concentrated PI can be added directly to the mixture without washing, since the digested RNA does not interfere with staining.

Addition of concentrated PI is simpler and faster by one centrifugation step, but pelleting and resuspending the GMDs in a common solution of PI in PBS reduces variations in PI concentration from tube to tube due to decanting and pipetting variability. GMDs occupied by single cells produced the same RF histograms as free cells, confirming that RNase worked even on cells inside the GMDs. The accuracy of the DNA measurements on microcolonies confirms that this RNase protocol also works on microcolonies.

The samples were run through the flow cytometer. Forward blue scatter (FBS) and red fluorescence (RF) were measured on several thousand occupied GMDs from each sample. Noise events were reduced by coincidence gating during collection and further computer processing. Histograms were computed in a linear mode and the logarithmic transform computed. (Note- simply plotting histograms on a log axis does NOT achieve the desired goal.[38]) For RF, measurement errors of the flow cytometer and the biological variation in cellular RNA and DNA content are both well approximated as percentage errors, *i.e.* the errors are relative to the size of the measurement rather than being absolute. When transformed into the log domain, the variance thus becomes more nearly constant over the entire range. This is important in theory because most statistical tests assume constant variance. It is important practically because when the data are presented graphically, the log histogram has peaks which are visually the same width and peak heights which are more representative of the number of counts in the peak.

The histograms can also be integrated to give an average RF value per occupied GMD. This average can be calibrated by the average RF for free cells and plotted as a function of incubation time. This average value corresponds to the typical result obtained through methods such as turbidity measurements to

estimate cell concentrations. Those methods lack information available through GMD based flow cytometry about growth variations within the cell population.

The use of the median rather that the mean average is often warranted. Flow cytometry data frequently contains undesired events. Noise events and coincidence events are frequent and occur at variable rates. A small fraction of the GMDs may contain multiple initial cells which will bias the computed average. They may also result in some events with offscale measurements. All of these events distort the measurement of the properties of a single microcolony. Since these undesired events tend to have values at high or low extremes, their influence on the mean average is amplified. The median on the other hand is relatively insensitive to these events. The median therefore tends to be more repeatable and a better estimate of the true population average.

Observations

Fluorescence microscopy and microphotography were used to monitor the result obtained using the flow cytometer. Photo #1 can be compared with the schematic on page 12, showing the cells inside the GMDs. Photo #2 is the same as photo #1 but taken under green excitation light to visualize the red fluorescence of the PI stain. Photo #3 shows how microcolonies form after incubation. The edges of the 4 to 8 cells within each microcolonies can barely be outlined in the photograph because an entire microcolony is not within the depth of field of the microscope. As a result, the photo is blurred. Individual cells are identifiable within the microcolonies when visualized under the microscope, since small adjustments to the focus allow the observer to examine each cell independently.

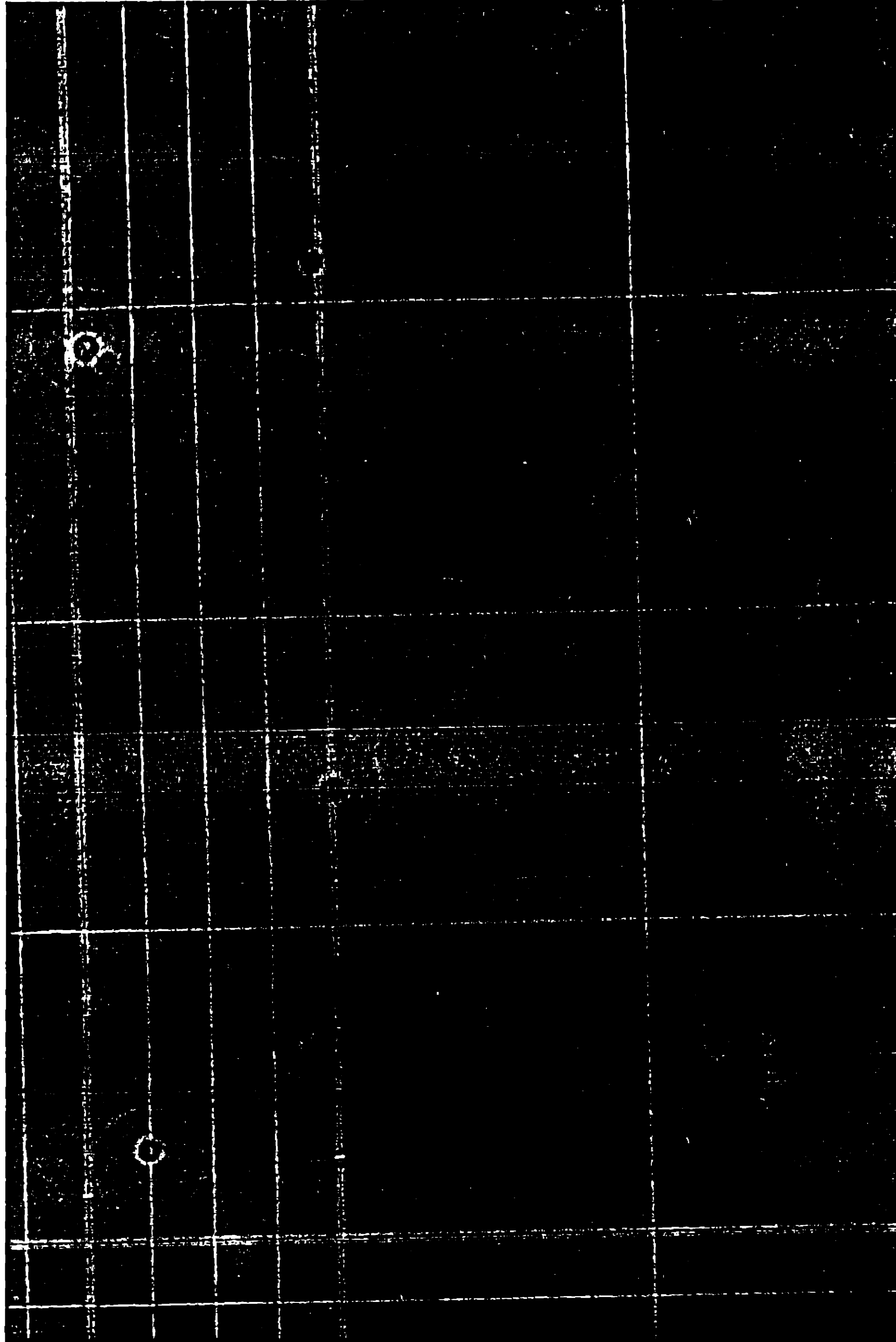


Photo 1. PA2.6 cells inside GMDs, fixed immediately after inoculation and stained with propidium iodide. The large circles are GMDs, while the cells are the small black dots inside.

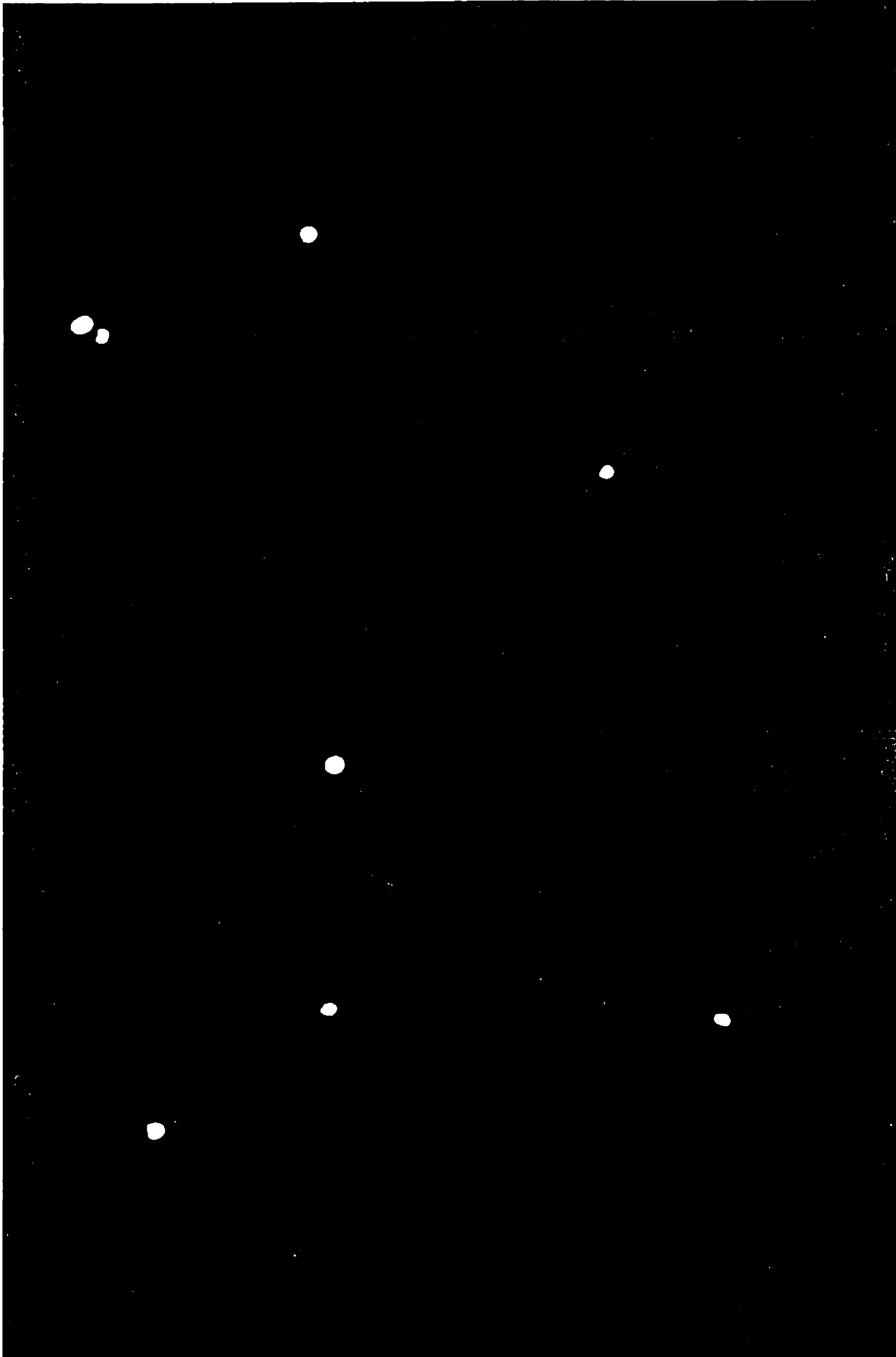


Photo 2. Same frame as photo #1, but under green excitation to reveal only the red fluorescence of the individual cells.

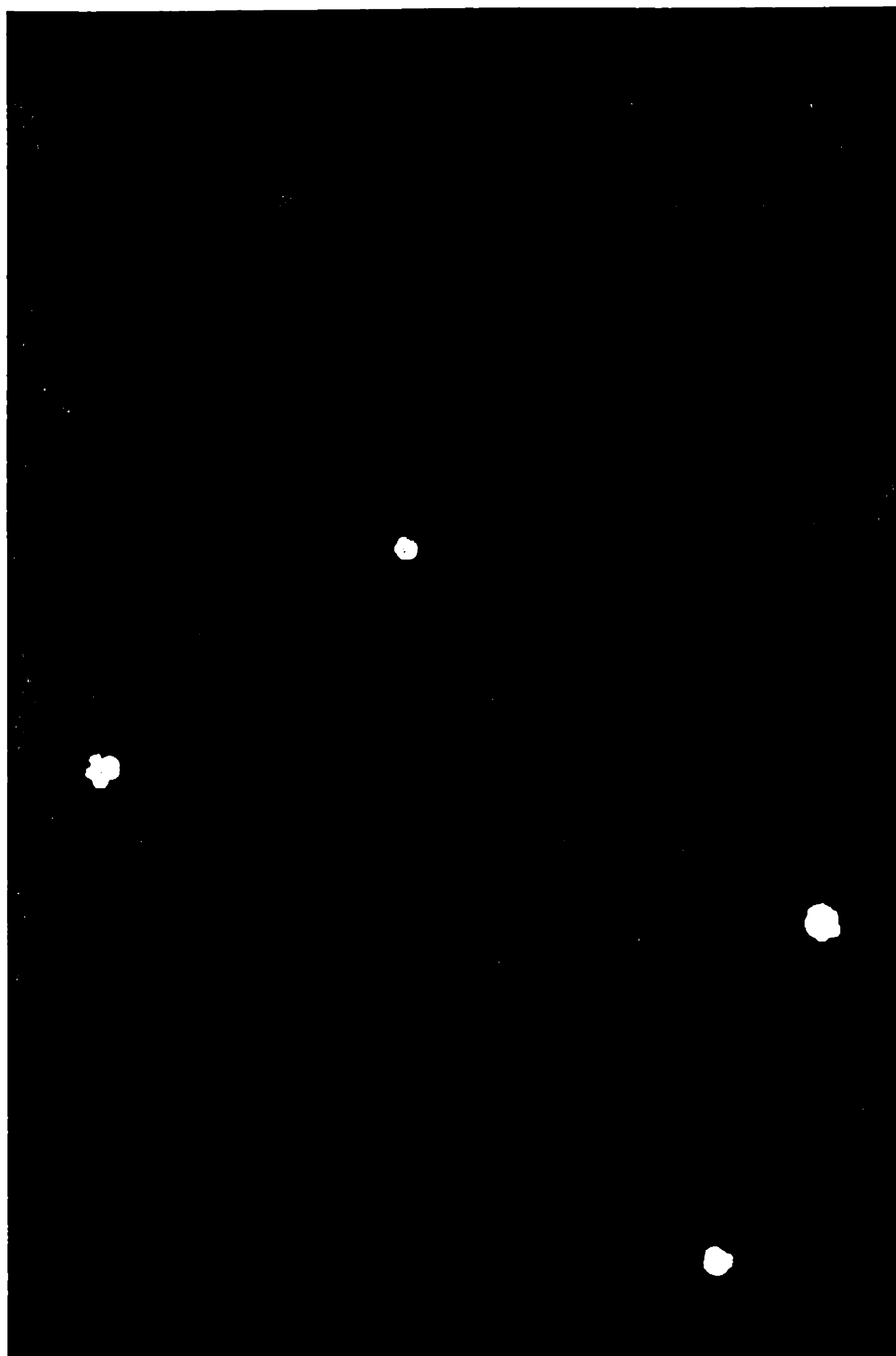


Photo 3. Microcolonies inside GMDs, red fluorescence. The GMDs are from the same batch as photos #1 and #2, but were allowed to incubate for 40 hours prior to fixation. The GMDs now contain microcolonies of 8 cells.

Precision and Accuracy Figure 1 shows a typical histogram of the red fluorescence (RF) signal from free cells. Note the difference caused by treating with RNase. While smaller, the value of the DNA only signal has markedly less cell to cell variation. The precision of the measurement can be estimated by computing the coefficient of variance (c.v.) for the G1 peak. Given problems due to noise events, coefficient of variance in flow cytometry is calculated by the relationship

$$C.V. = 0.425 \text{ (Peak Width)} / \text{(Peak Channel)}$$

where peak width is measured at one half the height of the peak channel.[35] The factor 0.425 arises from assuming a normal distribution.

Figure 2 shows a histogram of the DNA related RF signal from free cells and from cells immediately after entrapment in GMDs. Note that the measurement of cells inside GMDs is approximately as precise as the measurement of free cells. The two curves nearly overlap, showing the accuracy of the measurement in GMDs. The inaccuracy in the mode average is usually less than 7%. In the majority of cases, the GMD measurement is the lower value because the cell in the large GMD tends to be further away from the centerline of the flow cytometer optical alignment than free cell suspensions. Figure 2 is a minority case in this regard.

The histogram in figure 3, plotted on a logarithmic scale, is for the same cells as in the previous figure, but the cells have not had the RNA digested away. The RF signal is therefore related to the "biomass," or more exactly to the double stranded RNA and DNA content of the cell or microcolony. Again, accuracy is demonstrated by the agreement between free cells and cells in GMDs.

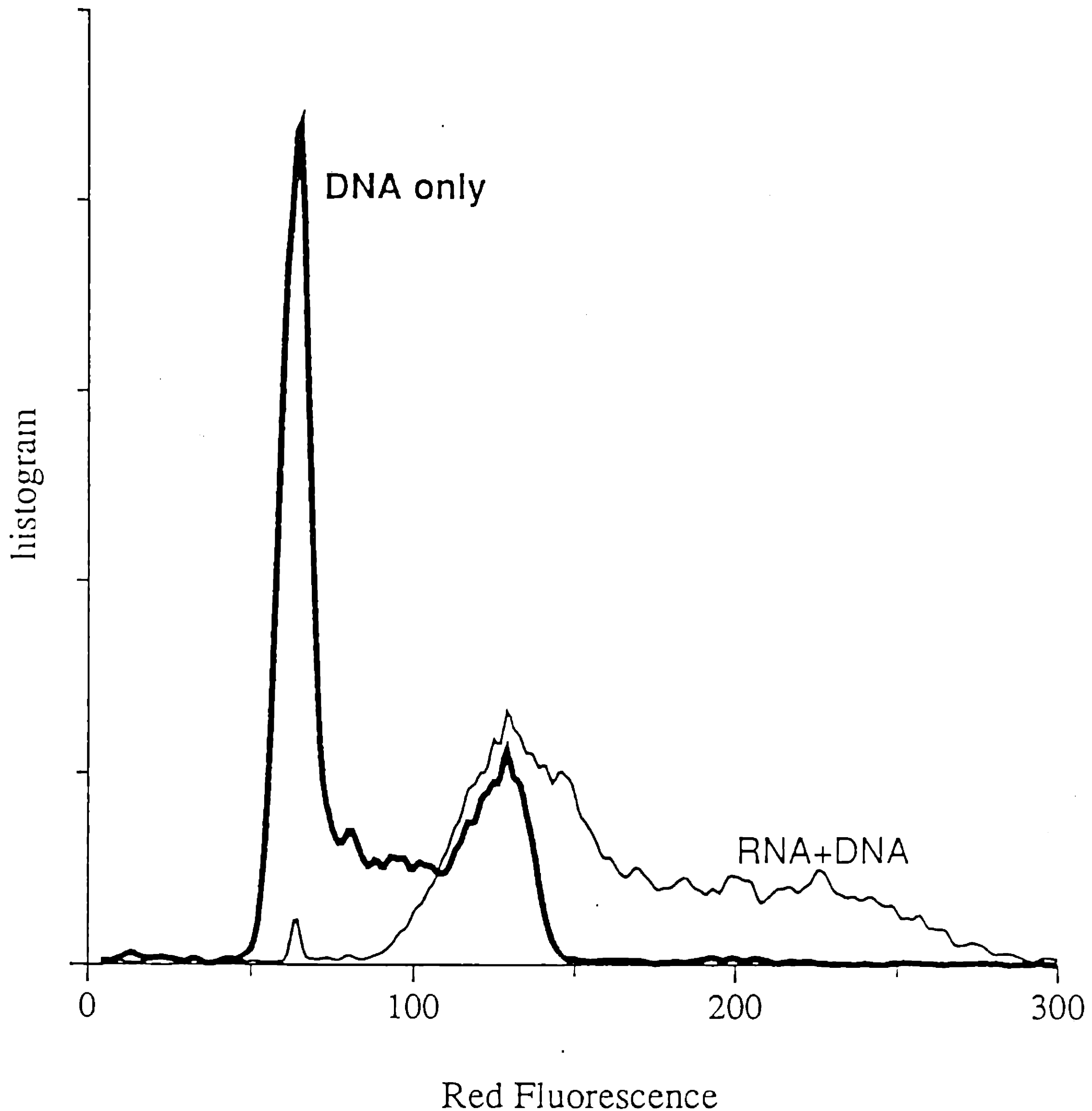


Figure 1. Comparison of measuring DNA and RNA+DNA using propidium iodide (PI), which stains double stranded RNA and DNA with red fluorescence. P815 cells were fixed with methanol. One of the two samples was incubated with RNAse to digest away the double stranded RNA, leaving only the DNA to be stained by PI. Both samples were stained with PI and the red fluorescence of 5000 cells was measured with the flow cytometer, yielding these histograms. Note that the DNA signal is about half as strong as the RNA+DNA signal, but has less variance.

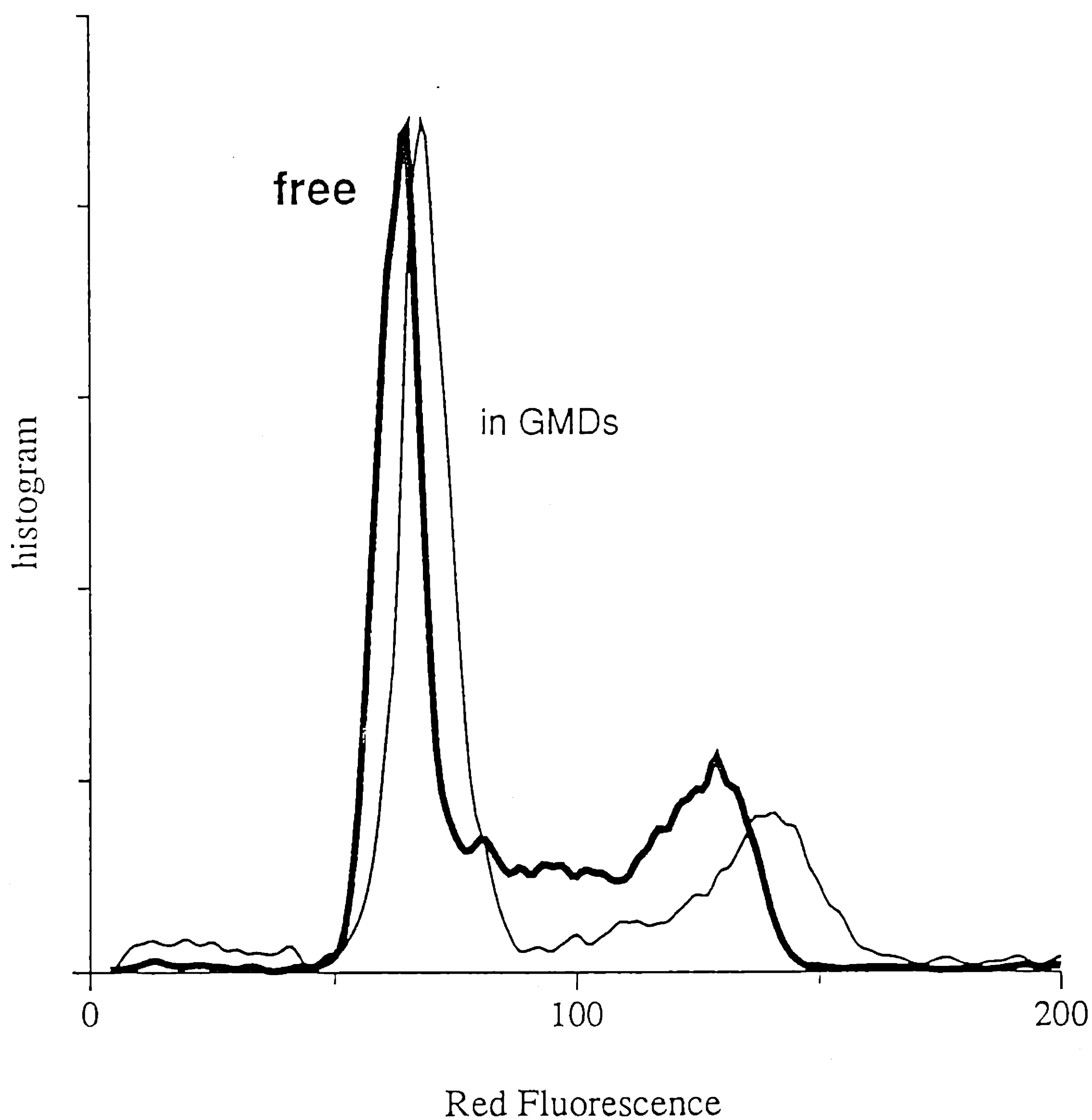


Figure 2. Comparison of the DNA signal from free cells and cells in GMDs. P815 cells were immobilized in GMDs. Free cells and cells in GMDs were fixed with methanol, treated with RNAse, and stained with PI. The flow cytometer gives essentially the same red fluorescence histogram for free cells as for cells in GMDs.

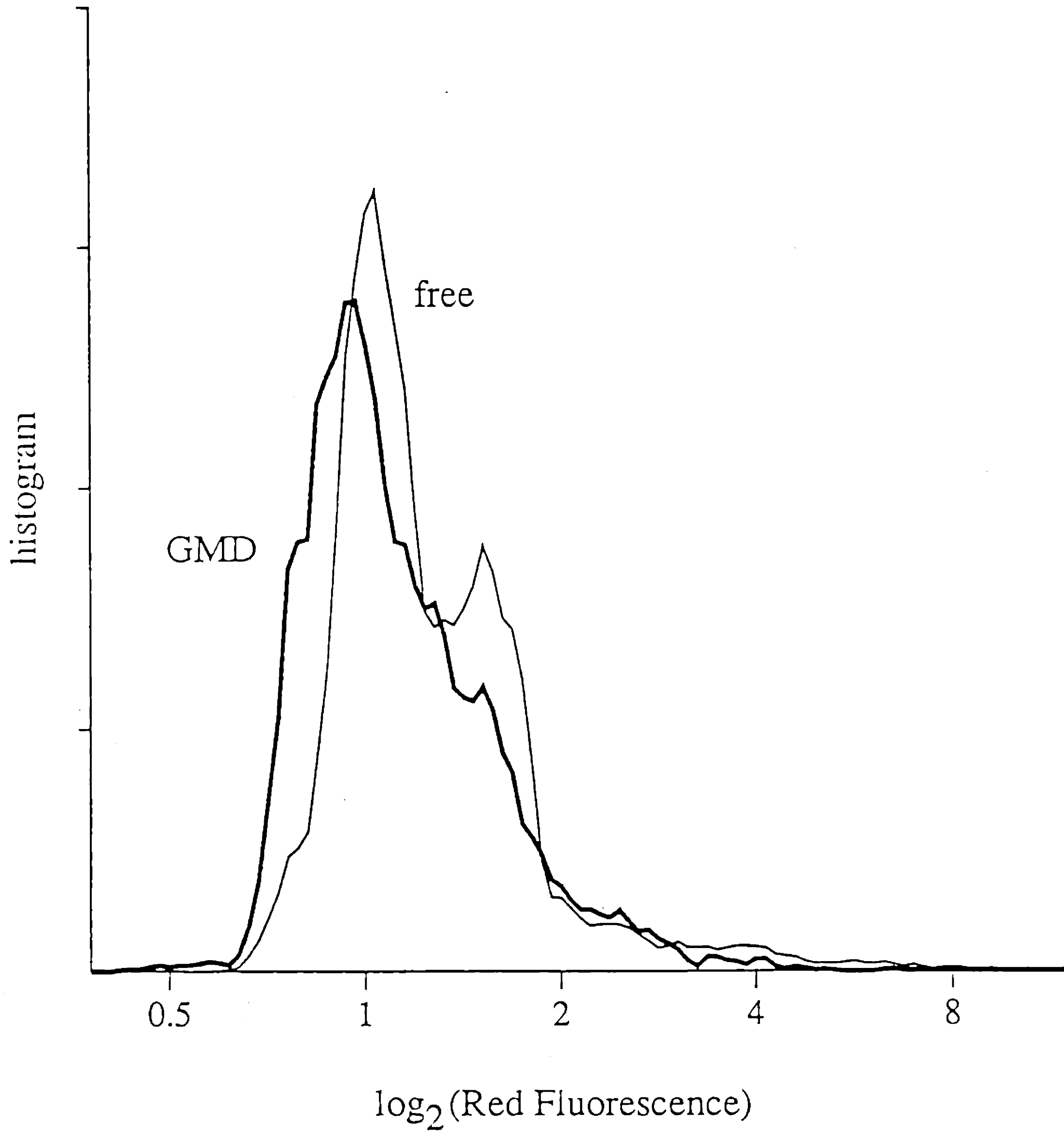


Figure 3. Comparison of the RNA+DNA signal in free cells and in GMDs. Similar to figure 2, but the RNA has not been digested away. The abscissa is also converted to a logarithmic scale. Again, note the agreement of the red fluorescence histograms of free cells and cells in GMDs, demonstrating the accuracy and validity of flow cytometry of GMDs.

The inoculated cell density can be increased so that the occupancy of GMDs by cells is increased to the point where many GMDs are multiply occupied. Figure 4 shows the histogram for such a case. The inoculum produced an average of about 1.4 cells/GMD. Unoccupied GMDs do not fluoresce and therefore were not measured. Note the ability to resolve RF histogram peaks for up to 5 cells per GMD. A simple model for predicting cell occupancy would be to assume a uniform size GMD and apply Poisson statistics.[9]

$$P(\mathbf{n},\lambda) = \lambda^{\mathbf{n}} e^{-\lambda} / \mathbf{n}!$$

$P(\mathbf{n},\lambda)$ is the probability of a GMD containing exactly **n** cells, given an average occupancy of λ. Since GMDs are not uniform in size, this formula gives only a coarse approximation. In figure 4b, the RF histogram was obtained after the GMDs were incubated to permit cell replication. The large peak at 4 represents mostly GMDs initially occupied by a single cell which has grown to form a microcolony of 4 cells. In figure 5, the data from figures 4 and 4b is transformed into the log domain and presented as overlaid curves on a logarithmic axis. As stated before, this is the more statistically correct method, since the variance in the data tends to be proportional to the measured value. Again, note the resolution achieved is more than adequate to count the number of cells per GMD. The uniformity of the peak widths in log domain confirms the hypothesized nature of the measurement errors as relative errors, and documents the precision of the flow cytometer over its entire range. The coefficient of variance for the peaks in figure 5 range from 8 to 13%. Linearity is good, with a slight nonlinearity detected because the peaks do not exactly line up with the logarithmic scale integer values. This nonlinearity is estimated to be within 10% of the predicted value over the full measurement range, and may be due to imprecise tuning of the analog-to-

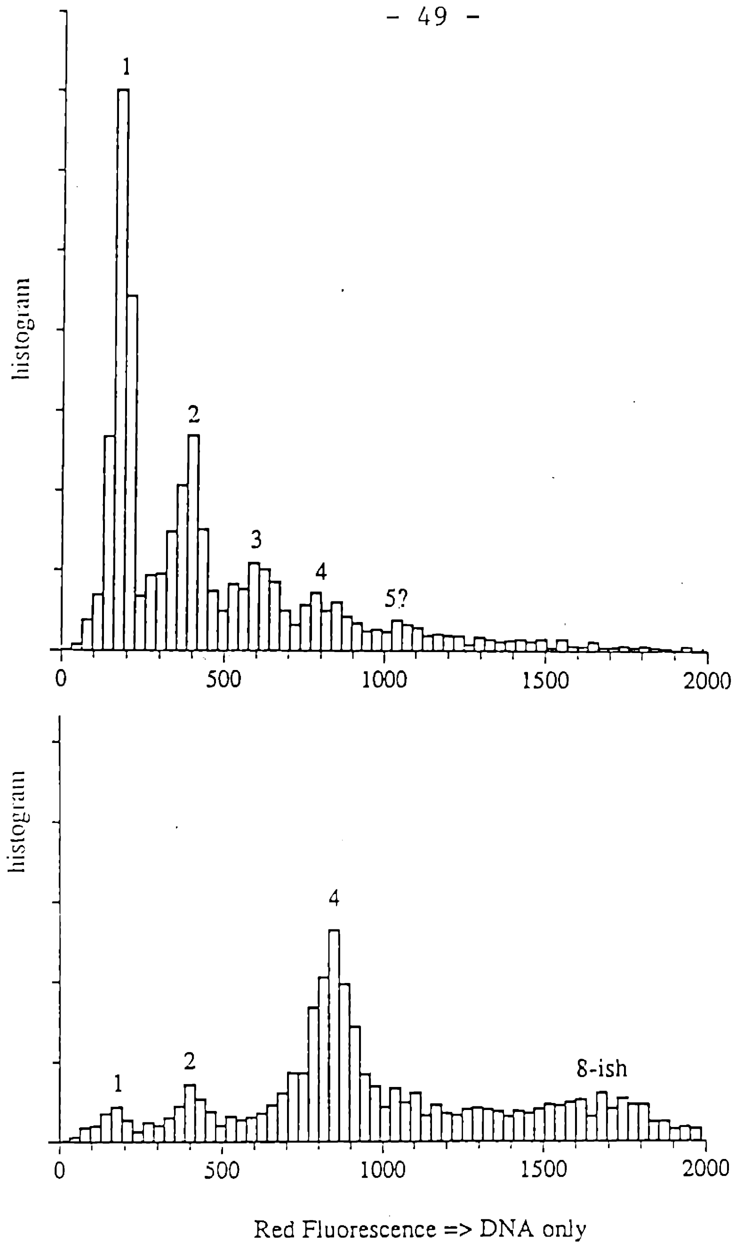


Figure 4. PA2.6 cells immobilized in GMDs at an inoculation density such that many GMDs were initially occupied by more than one cell. In 4a, the cells in the GMDs were immediately fixed with methanol, treated with RNAse, and the remaining DNA stained with PI. The red fluorescence (RF) was measured on 5000 occupied GMDs, yielding this linear histogram. The RF is measured with arbitrary units, but peaks in the histogram appear at values which are multiples of the RF of single cells. These peaks correspond to GMDs inoculated with 1, 2, 3, 4, 5, or more cells. Most GMDs are singly occupied.

In 4b, the cells were incubated for 24 hours before fixation. This allowed DNA replication to occur. The largest peak in the histogram corresponds to microcolonies of 4 cells cloned from a single cell inoculated into a GMD. Microcolonies of 2 and 8 are also distinguishable.

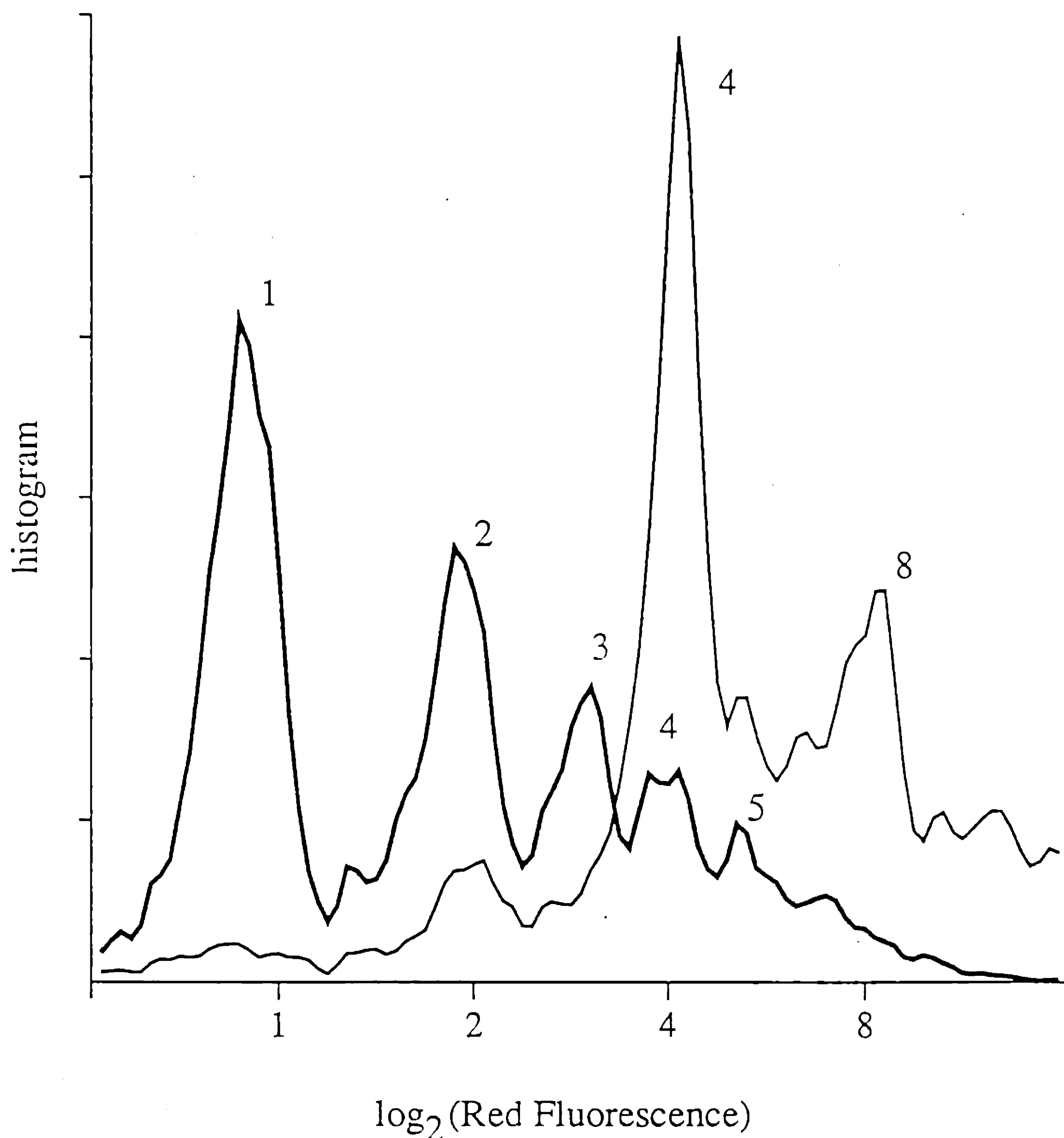


Figure 5. The data from figures 4a and 4b is transformed into histograms of the logarithm of red fluorescence, scaled to the RF of a single cell. The two datasets are plotted as overlaid curves rather that bar histograms, with the thicker line corresponding to the data from figure 4a. Note the improved resolution of the peaks, especially at RF=8, and the increased uniformity of the peak widths in the log domain. Note also the alignment of the peak corresponding to a microcolony of 4 cells and the shorter peak corresponding to a GMD inoculated with 4 individual cells. Both situations give the same RF value, demonstrating the accuracy of the flow cytometer with GMDs.

digital flow cytometer electronics, which were under development at the same time as the linearity experiments.

As mentioned above, one sample in figure 5 was incubated for 24 hours (about 2 doubling times) to allow cell growth before measurement. Microscopic observation confirmed the formation of microcolonies of 2, 4 and 8 cells. Note the peaks at 2, 4 and 8 on the log histogram. These peaks are of the same width as the original inoculum, demonstrating the precision of the measurement. The peaks lie very close to the values of 2, 4, and 8 computed from the data on the original inoculum, confirming the accuracy of the measurements and the overall methodology.

Repeatability The next figure (#6) is an overlay of many histograms from an experiment which will be described in more detail in chapter 7. Five flasks were inoculated with a pooled sample of GMDs containing p815 cells. All of the flasks contained K medium except that the concentration of L-glutamine was varied. One of the flasks contained no L-glutamine (except a trace carried in with the GMDs) while the other four flasks had 60, 240, 600 and 1200 mg/L. The normal value is 600 mg/L. After 40 hrs of incubation, two replicate samples were taken from each flask. Each sample was fixed and stained by standard protocols outlined above. Based on median RF measurements, the four flasks with glutamine supplement showed no significant differences in growth. Growth in the flask without glutamine was stunted. The RF (RNA + DNA) histograms from these ten samples are overlaid in figure 6. Note that the curves for the two samples from the glutamine free flask are clearly different from the eight samples obtained from the other four flasks.

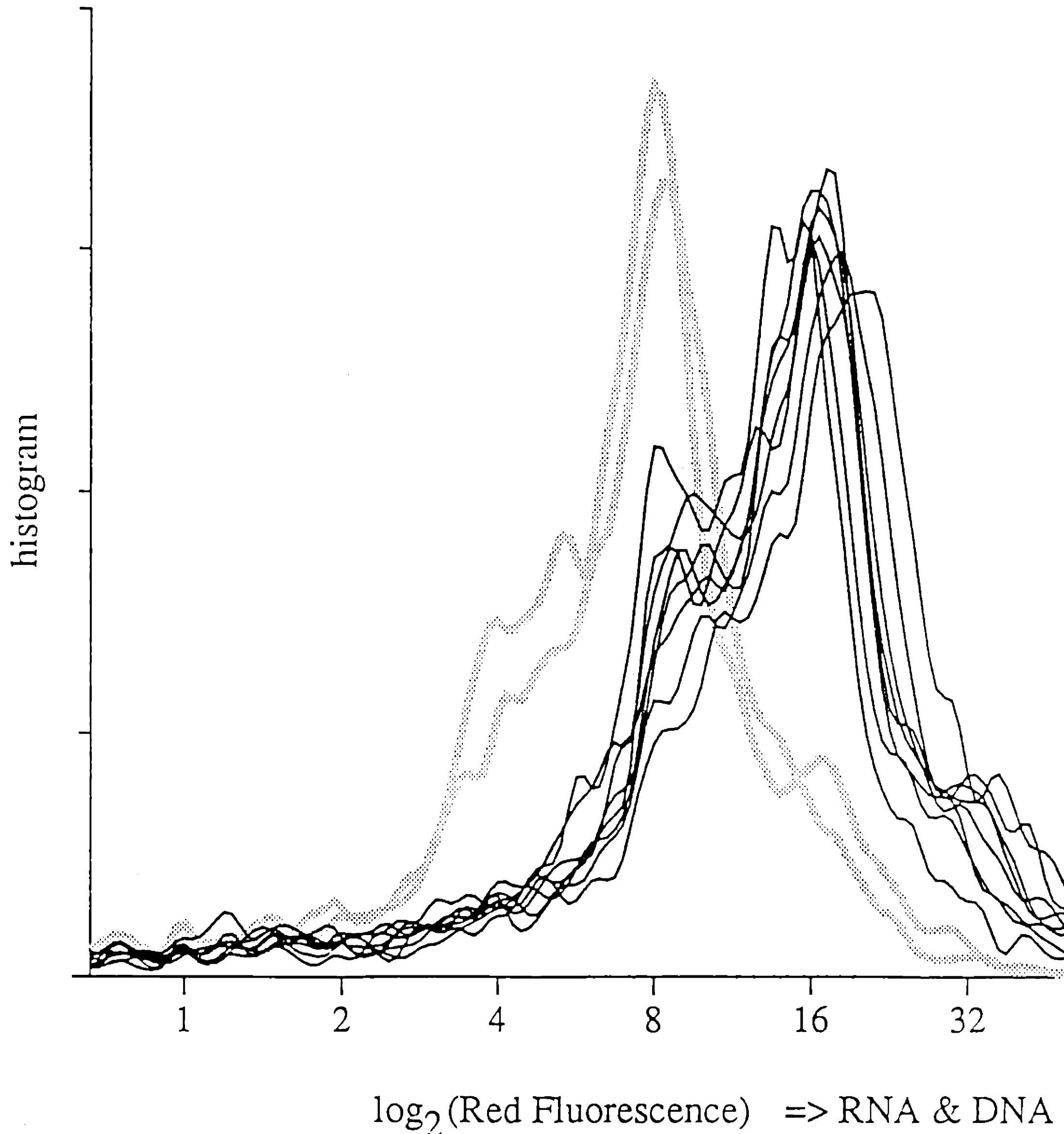


Figure 6. P815 cells were immobilized in GMDs and incubated in 5 flasks with varying amounts of L-glutamine. Two replicate samples from each flask were obtained after 40 hours incubations. The cells were fixed with methanol, stained with PI, and the RF from 5000 occupied GMDs was measured with the flow cytometer. These RF values were scaled by the RF from single cells and plotted as histograms in the log domain. Immobilized cells in the flask without glutamine (thick gray lines) stopped growing after 3 replications, forming microcolonies of 8 cells. In the other 4 flasks (a total of 8 samples drawn with overlaid, thin black lines) the typical result was 4 replications to form 16 cell microcolonies. Note the sample-to-sample variability compared to the ability to distinguish a 25% reduction in growth rate in the flask without glutamine.

This allows an estimate of the amount of sample-to-sample variability introduced in the steps of incubating, sampling, sieving, washing, staining, and flow cytometry. The coefficient of variance for the means of the eight similar datasets is 14%. This sample-to-sample variance is much larger than would be estimated by the standard error of the mean (SEM) of any individual data set, which averaged 1.3% for these eight datasets. This is often true in flow cytometry. Since flow cytometry involves a large number of data points from a single sample, the SEM of any data set is an estimate only of the variability introduced by the flow cytometry, and not the variability introduced by all the other steps listed above. The sample-to-sample variability is the correct estimate of repeatability.

Another interpretation and explanation is more intuitive and more valuable for day to day work. It also gives insight into the more complex (and unsolved) statistical problem of defining significant differences between population distributions rather than just differences between population means. There are 28 possible combinations of pairs from the eight histograms. The probability of a combination corresponding to the two most dissimilar curves is therefore about 4%. The range of variance of these eight curves is therefore a visual approximation of the 95% confidence limits for the distribution of RF in these samples. This therefore gives an intuitive feel for the repeatability of these measurements. Visually, it is obvious that the eight curves are significantly different than the histograms of the two samples taken from the flask without glutamine.

Validity and Capability The validity of the measurements can be strongly supported by reference to the fact that the cloning of mammalian cells in petri dishes containing soft agarose is an established technique. There is no reason to suspect culture of mammalian cells in small droplets of agarose will introduce any strange behavior. The validity will be further supported here by confirming that the morphology, growth and function of cells entrapped in GMDs corresponds to those in standard culture. Normal morphology and a functional enzyme system was confirmed by fluorescence microscopy using the cFDA/PI methodology discussed in chapter 4. The rest of this chapter will show that growth rates in GMDs are typical of free culture and that the plating efficiency is satisfactory. Later chapters will also show that the response of growth to changes in medium composition is normal, and that cell function, *i.e.* secretion, is normal.

Growth of mammalian cells within gel microdroplets was documented and quantified by sampling a flask of GMDs over time. Samples were taken as a function of time, fixed, stained and measured as noted in the methods section above. One experiment was done using the simpler method of staining both the RNA and DNA, without an RNase step. Figure 7 shows the histograms obtained at various time points. The shift to higher values is obvious. An average RF for each sample was computed at plotted with respect to time in figure 8. This shows that the amount of double-stranded RNA and DNA in each microcolony (mean RF per occupied GMD) increased in exponential fashion as would be expected. This was true of both cell types p815 and PA2.6, the mouse mastocytoma and hybridoma respectively. The aberrantly low data point at 40 hours for the p815 cell line is easily explained by reexamining figure 7 and noting that many events for the 40 hour curve are offscale to the high side. In computing the average, these events are counted only as a full scale value, causing the underestimate.

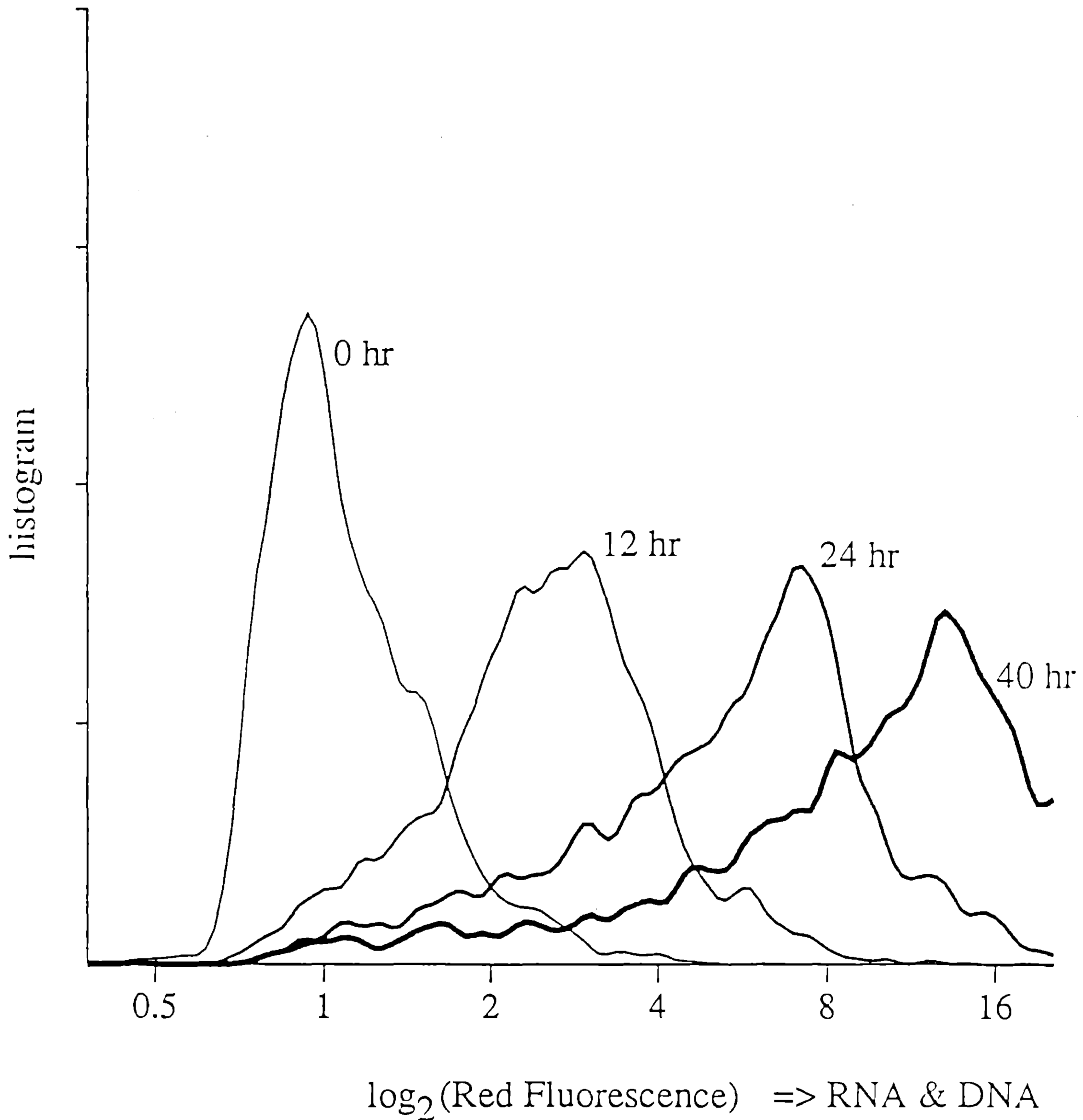


Figure 7. P815 cells were immobilized in GMDs and incubated at 37 °C, 5% CO_2. Samples were taken at 0, 12, 24, and 40 hours of incubation. The cells were fixed with methanol, stained with PI, and the RF from 5000 occupied GMDs was measured with the flow cytometer. These RF values were scaled by the RF from single cells and plotted as histograms in the log domain. The increase with time of the RF signal indicates cell growth.

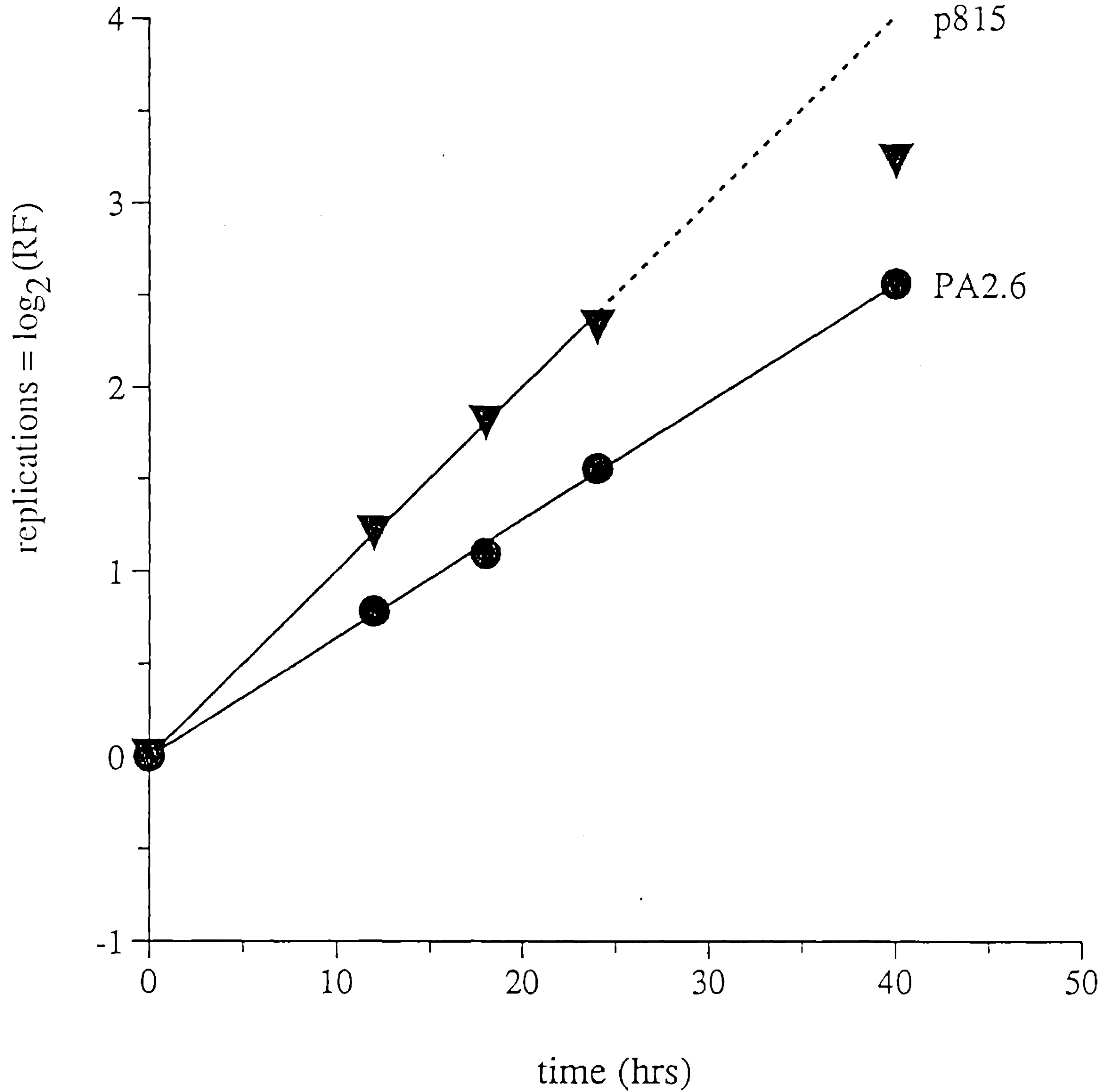


Figure 8. P815 and PA2.6 cells were immobilized in GMDs and incubated at 37 °C, 5% CO_2. Samples were taken at 0, 12, 24, and 40 hours of incubation. The cells were fixed with methanol, stained with PI, and the RF from 5000 occupied GMDs was measured with the flow cytometer. An average RF per occupied GMD was computed, scaled to the RF at time 0 hr, and plotted on semilog axes against incubation time. The ordinate therefore corresponds to DNA replications. Note the exponential growth without lag time. Note also the accuracy of the measurements except for p815 at 40 hr (see text).

Use of medians rather than means would have diminished this error. A similar experiment with coentrapped beads showed no difference in growth rate with or without the polystyrene beads, and no detrimental influence of the beads on the RF measurements.

In a repeat experiment, samples were taken at even earlier time points to demonstrate detection of growth at the fundamental limit of a single cell division. Figure 9 shows that detection, as the peak at 1 cell/occupied GMD decreases and the peak at 2 increases.

Growth of mammalian cells within gel microdroplets is better quantified by using RNase to remove RNA and then staining the remaining DNA. The increase with time of RF due to DNA replication is calibrated with respect to the RF measurements of individual cell DNA obtained from a control sample of free cells. This yields a relative value which corresponds closely to the number of cells in the GMD. The DNA measurement is valid for determining cell number because fluorescence microscopy confirms that cell membrane division took place at the same rate as DNA replication.

The increased precision of DNA measurements allows several important facts to be demonstrated. The shift in time of the histograms in figure 10 toward increasing values of RF shows the growth of the cells in the GMDs. The precision of the RF measurement is constant over time and magnitude. The peaks at the relative RF values of 2, 4, 8, and 16 cells seem to dominate. This shows that early progeny of a single clone tend to divide in synchrony. The alignment of the peaks with integral values also documents the accuracy of the measurements. The low fraction of occupied GMDs containing a single cell after 40 hrs of incubation attests to the high plating efficiency of the cells after entrapment in GMDs. Over 90% of the inoculum underwent at least 1 replication. The median

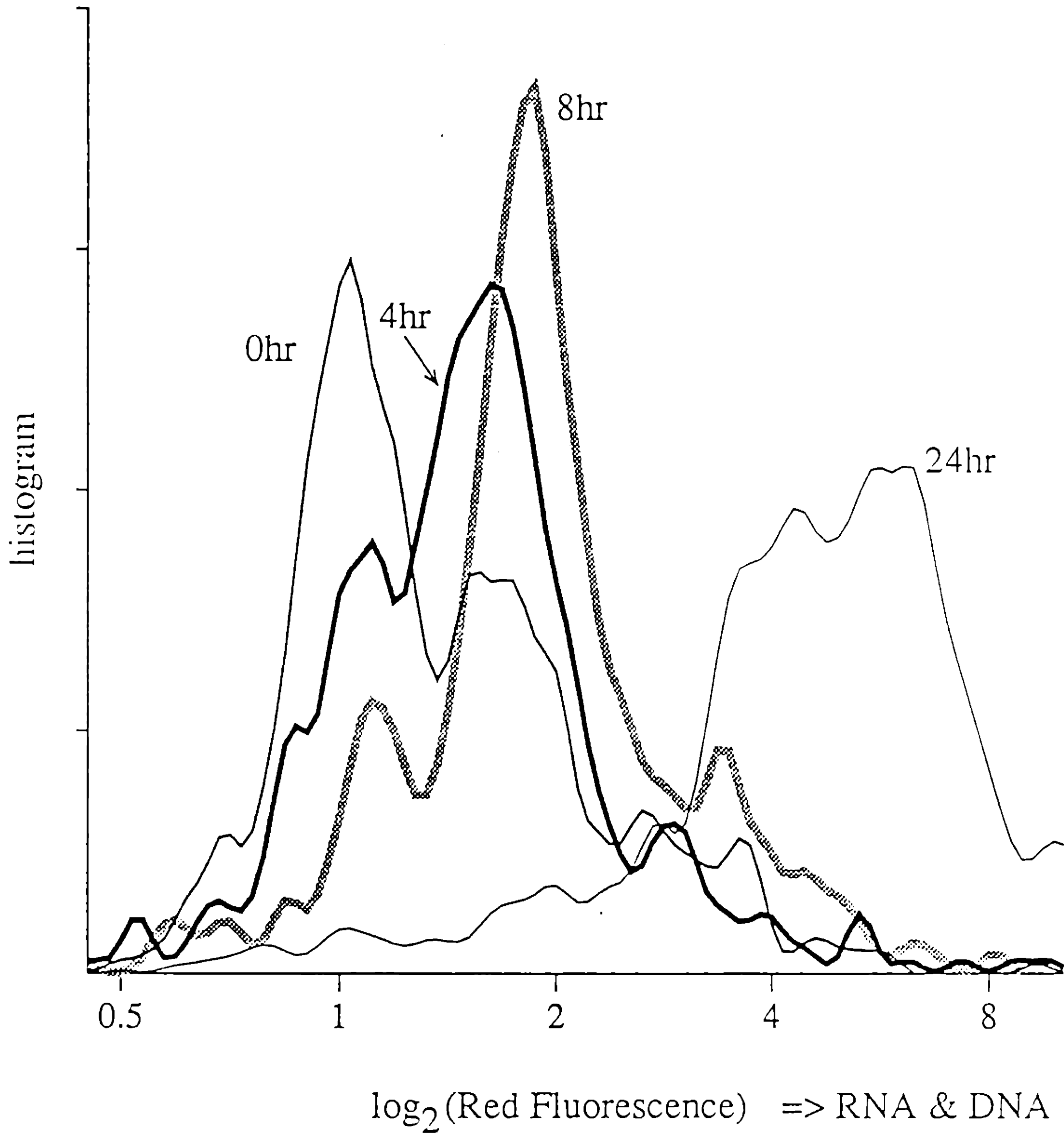


Figure 9. PA2.6 cells were immobilized in GMDs, incubated, sampled, fixed, and stained at early time points. Note the ability to detect growth (a shift in the RF histogram) as early as 4 to 8 hours, at the fundamental limit of one cell division.

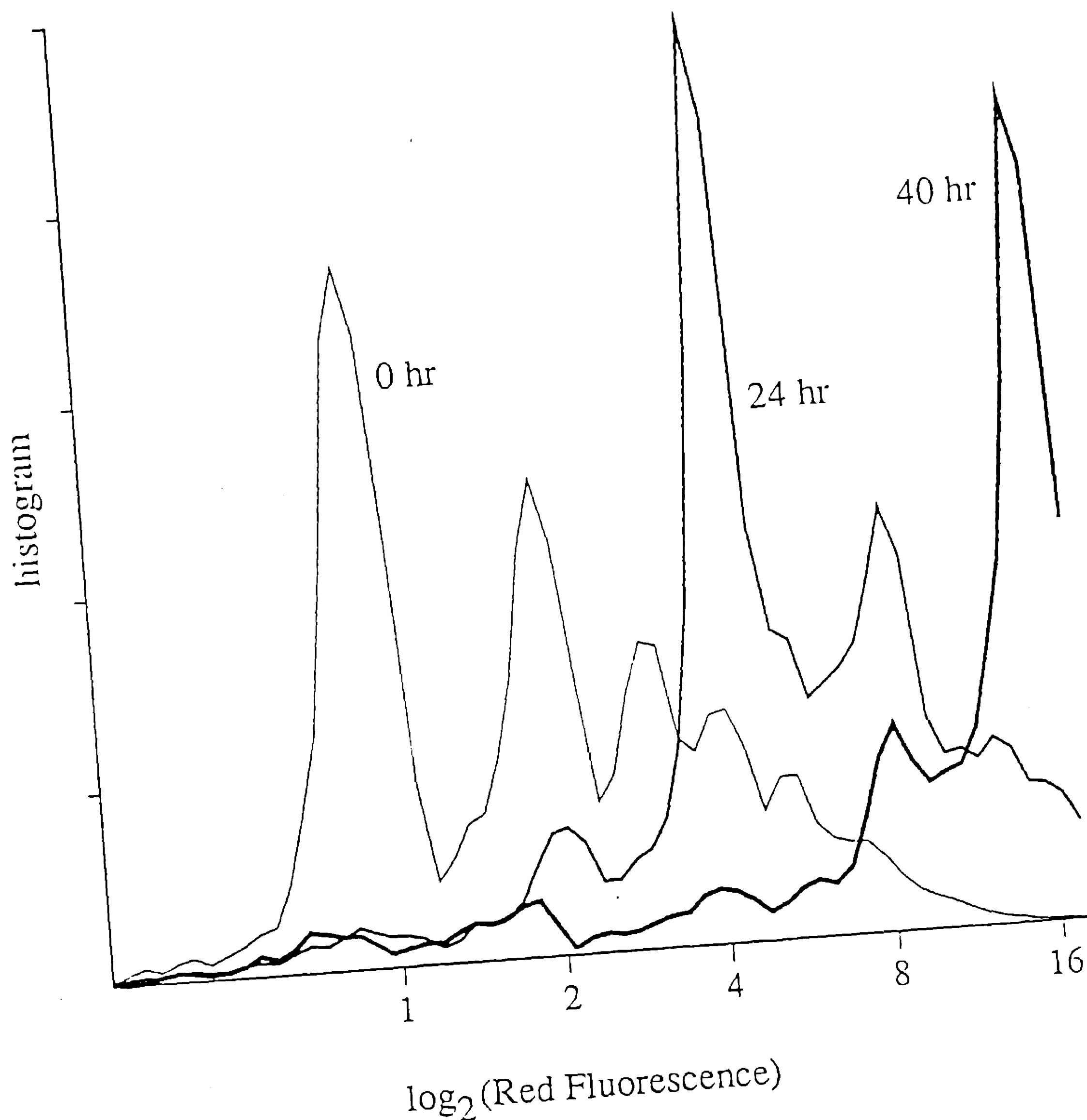


Figure 10. PA2.6 cells were immobilized in GMDs, incubated, sampled at various intervals, fixed with methanol, treated with RNAse, and their DNA stained with propidium iodide. The red fluorescence (RF) of 5000 occupied GMDs was obtained, calibrated to single cell RF, and the data transformed into histograms in the log domain. Note the ability to resolve peaks corresponding to 1, 2, 3, 4, and 5 cells in the inoculum, peaks corresponding 4 and 8 cell microcolonies at 24 hours, and peaks corresponding to 16 cell microcolonies at 40 hours. Note also the improved resolution achieved by using RNAse, as compared to figure 7.

value of RF in each sample was computed and plotted with respect to time. As shown in figure 11, growth is exponential. The growth rate of these PA2.6 cells was also measured in free suspension using actual cell counts on a haemocytometer. Growth in 2.5% agarose GMDs is slightly slower than free suspension. Nilsson et al. have also noted this slight loss in growth rate in larger agarose droplets, especially at higher concentrations of agarose.[11] However, in general the growth rates and characteristics appear normal. Given that soft agarose cloning is already an established technology, one can expect that growth in GMDs will be similar to, and as normal as, any other *in vitro* method of culturing mammalian cells.

These experiments also show another advantage of GMDs for growth measurements. The p815 cells tended to adhere to the flasks during culture in free suspension. Enough cells did remain nonadherent to permit the flasks could be subcultured ("split") without using trypsin to remove the adherent cells. However, this adherence complicates measuring growth rates in free suspension, since errors are introduced by cells that remain adherent to the flask even after treatment with tryspin. The GMDs are useful because they eliminate this problem, allowing growth rates to be easily determined even for this cell line.

At this time, truly anchorage dependent cells have not been grown in GMDs in this laboratory, nor has much effort been dedicated to that task since growth of such cells by soft agarose plating has also failed. However, most cell lines commonly cultured for biotechnology and medicine are not anchorage dependent.

These experiments clearly show that nonanchorage dependent cells can be inoculated into GMDs and cultured to become clonal microcolonies. The clones, entrapped within the solid matrix, undergo DNA replication and cell division in a manner identical to their counterparts grown in standard culture. Flow cytometry

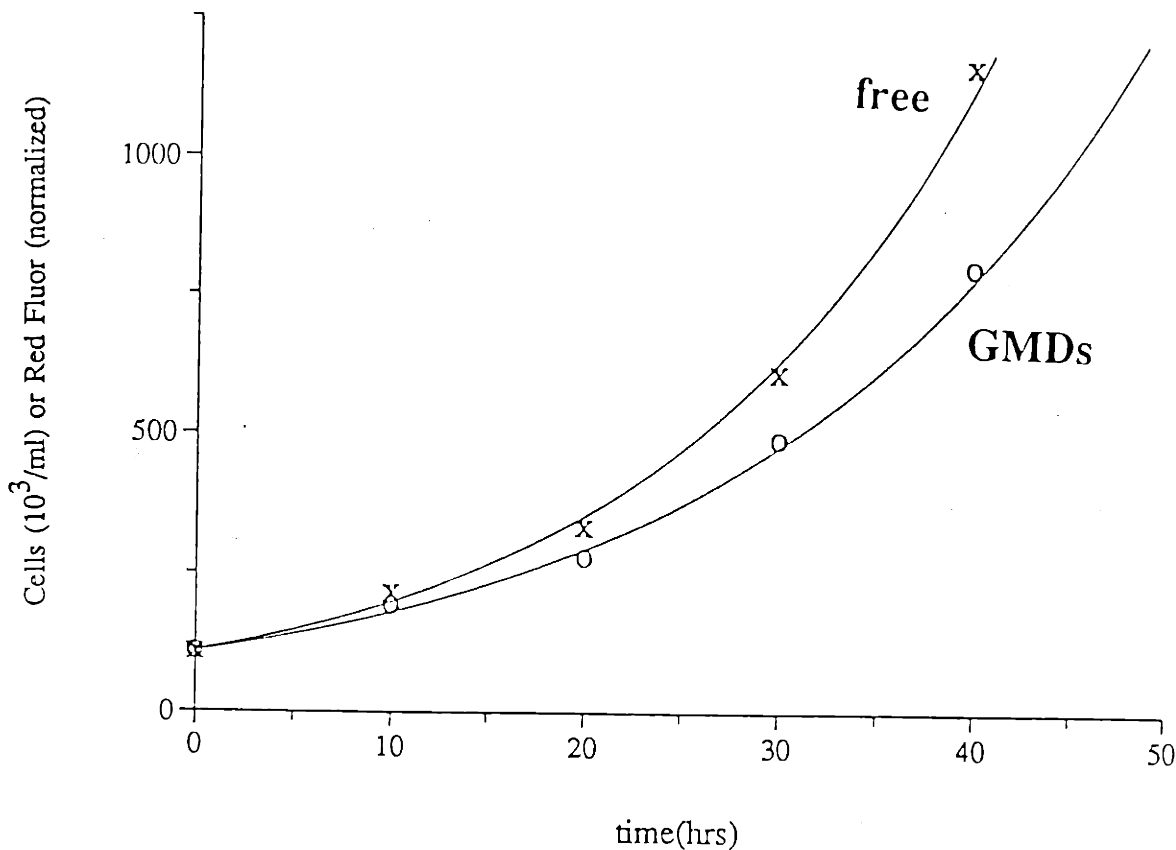


Figure 11. PA2.6 cells were grown in free suspension and immobilized in GMDs. Growth in free suspension was measured by haemocytometer cell counts. Growth in GMDs was measured by aliquoting samples at appropriate intervals, fixing the cells, digesting the RNA, staining the DNA with PI, and finding the median red fluorescence of occupied GMDs within the sample, compared to that at time zero. In both cases, growth is exponential, as shown by the best fit exponential curve. Growth in free suspension (-X-) is slightly faster than growth in GMDs (-O-).

is capable of measuring that growth with adequate precision, accuracy, and repeatability.

Chapter 6

Secretion

Secretion is an important function of mammalian cells in both biotechnology and medicine. Screening for subpopulations which are secreting or hypersecreting desired products is a frequently encountered problem in both fields. The 96-well microtiter plate is a common device used for this a task. This labor intensive and tedious technology can be replaced by GMDs and flow cytometry if the secretions can be trapped in the same GMD as the cell(s) producing them. Those secretions can then be labelled, measured by the flow cytometer, and the sorting capability of the flow cytometer used to isolate the cells of interest.

One of most common tools for screening is the immunoassay. The high specificity of antibodies make them very useful labels. The screening of hybridomas is also a very common problem in immunology. Therefore, to demonstrate the feasibility of using gel microdroplets and the flow cytometer for screening secreting cells, a model system was selected using both of these elements, hybridomas and antibody labels.

Methods

Using the standard protocol, GMDs were produced containing binding sites and a mixture of secreting and nonproducing cells. For simplicity, the binding sites chosen were 0.8μ polystyrene beads coated with goat anti-(mouse IgG) antibody (Pandex). The beads were washed with Hank's Basic Salt Solution (Gibco) to remove the sodium azide preservative and then concentrated by a factor of 6 to 10 fold so that adding 100 μL of the bead suspension during step 4 of the standard protocol incorporated about 3800 beads into an 80μ GMD. The protein binding capacity of this mixture is 75 μg/ml of agarose, or 20 pg/80μ GMD.

The cell lines chosen for this work were a mouse hybridoma (PA2.6, ATCC HB118) which secrets IgG and for comparison a mouse mastocytoma cell (p815, ATCC TIB64) which does not produce nor secrete antibody. The PA2.6 line produces IgG at a final concentration of 38 μg/ml of antibody when grown to medium exhaustion. This corresponds to a secretion rate on the order of 0.1 to 1.0 pg/cell/hr. This particular mouse IgG happens to be specific for human major histocompatability antigens, but this is irrelevant. The mouse IgG itself is an antigen for the polyclonal goat anti-(mouse IgG) antibody which coats the coentrapped beads and is used as the fluorescein-conjugated label. Mixtures of these two cell lines were created, so that the GMD inoculum could be all one cell type or a mixture which contained relatively few secretors in order to better simulate the common anticipated use of this technology.

The cell/bead/agarose mixture was dispersed in oil by the standard protocol used to form GMDs. GMDs were then either incubated while still in the oil phase or transferred by centrifugation to culture medium for incubation. The binding sites supplied in the GMDs capture only a fraction of any secretions for the cells

inside that GMD. Some secreted molecules (perhaps the majority) may diffuse past these binding sites and escape from the GMD. These molecules may then diffuse through the medium, enter and become bound within other GMDs, a phenomenon termed here as "crosstalk." Incubation in the oil phase was tried as a method for preventing crosstalk between the GMDs with secreting cells and other GMDs in suspension. For the GMDs incubated in medium, lower dilutions of GMDs were tried to reduce the crosstalk. Alternatively, some GMDs were incubated with a large number of the free Pandex beads (twice the number of beads entrapped within the GMDs) added to the culture medium to serve as external binding sites, scavenging the medium for escaped secreted molecules.

The GMDs were incubated for various amounts of time, including two experiments where a sample was obtained at 11 hours, slightly less than the doubling time of these cells. The GMDs were fractionated to a narrow size class using 10 cm^2 nylon sieves (nominal 44 and 88µ or 53 and 88µ openings). The sieves were rinsed with 50 ml PBS to assure adequate efficiency of the sieving. This also rinsed away any free secreted antibody dissolved in the suspension. The fractionation eliminated the free 0.8µ beads added to some suspensions to scavenge free secretions. The free beads were also removed during ensuing rinse steps because the low speed centrifugation used to pellet GMDs is far below that needed to pellet the beads.

The sieved GMDs were rinsed once more with 1 ml PBS. Centrifugation of GMDs was done at 600g for 2 minutes. An aliquot of 30 µL of pelleted GMDs was resuspended in 0.75 ml of PBS containing 4 µg of a fluorescein-conjugated polyclonal goat anti-(mouse IgG) antibody to act as the green fluorescent label (Organon Teknika, West Chester, PA). This produced a GMD suspension where the amount of fluorescent label added was about twice the maximal binding

capacity of the GMDs/beads for mouse IgG. Experiments using twice as much label gave identical results, confirming the adequacy of this choice.

As controls, GMDs with beads but without cells were produced and incubated in solutions spiked with commercially available mouse IgG (Organon Teknika). Different concentrations were used to estimate the linearity of the green fluorescence measurement. Linearity was poor and impossible to study due to the variability in coating on the beads, the polyclonal nature of both the bead coatings and the label, the variability in GMD diameters, and instrument limitations. Still, the degree of linearity was adequate to determine satisfactory concentrations for beads and stain.

The narrow size class of 53-88μ GMDs was run through the flow cytometer at up to 10,000 GMDs per minute. A 488 nm blue laser operated at 35 mW was used to excite the fluorescent label. Forward blue scatter (FBS) and green fluorescence (GF) measurements were collected on 10-40,000 events (GMDs) from each sample. Fluorescence microscopy verified visually and photographically the results obtained by the flow cytometer.

Data Presentation

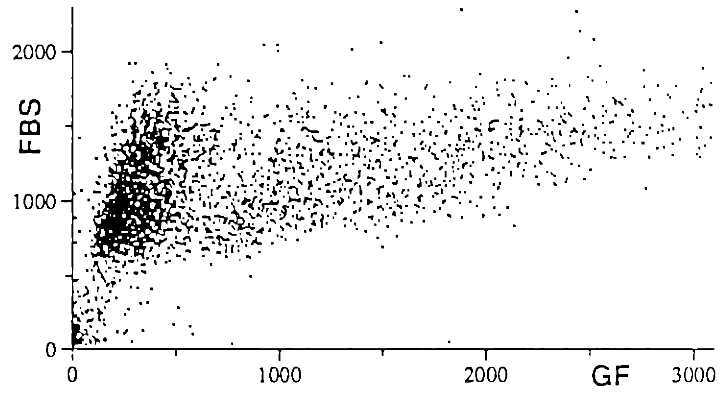
The forward blue scatter measurement is needed because the GMDs are not all of the same size. The forward blue scatter (FBS) gives a parameter roughly proportional to the size of the GMD. The high degree of linear correlation between the FBS signal and the green fluorescence (GF) signal suggests that FBS is proportional to volume. Even when sieved to the narrow size range of 53-88μ, the volume differential between the largest and smallest GMDs is still a factor of 5. The GF from nonspecific labelling of the large GMDs may therefore overlap

with the specific labelling of the small GMDs. Bivariate histograms of FBS and GF are needed for accurate interpretations.

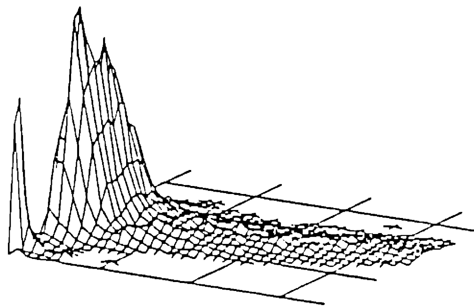
To understand the presentation of this data, a brief tutorial follows on the presentation of bivariate data, using as an example the dataset obtained from a case where secreting cells were entrapped in GMDs, incubated, and then the secretions stained. To reduce crosstalk, free beads in the medium absorbed some of the uncaptured antibody. However, some uncaptured antibody did diffuse into and label nearby GMDs. (This could be referred to as a nonspecific label, but is in reality a specific labelling resulting from crosstalk.)

When passed through the flow cytometer, two measurements are made on each GMD, a size measurement (FBS) and a label measurement (GF). Upon collecting 10,000 such measurements, the data is an array of size 2 x 10,000. One option would be to simply compute histograms for each variable independent of the other. This oversimplified presentation loses important information. An alternative presentation which preserves the covariance in the dataset is to make a scatter plot (known as a cytogram in the flow cytometry literature). The x-axis is the value of the GF measurement and the y-axis is the FBS measurement. A dot is placed on the graph at the coordinates corresponding to each data point. Figure 12A is the result of this plot. One dense cluster and one sparse cluster can be seen, representing the empty GMDs and those occupied by secreting cells. There is also a cluster of "noise" events near the origin. The cytogram is a useful presentation for a small number of data points (a few thousand), and is convenient if the graphics are performed real time on a storage oscilloscope attached to the flow cytometer. However, as larger numbers of data points are collected the center of a cluster saturates while the cluster's size continues to grow due to variance in the data. At this point, a bivariate histogram is more useful.

Scatter plot



Hidden line plot



Contour plot

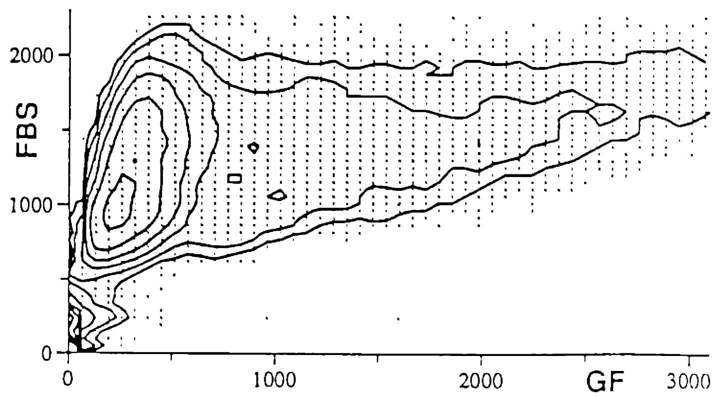


Figure 12. The same data is presented in 3 different forms. See text for explanation.

In bivariate histograms, the x- and y-axes are the values of the measurements while the z-axis represents the frequency of occurrence. There are two common representations of bivariate histograms. Conceptually, instead of putting a dot on the scatter plot, think of placing a grain of sand there instead. As more data is accumulated, the clusters of data will become mountains that correspond to the peaks in a one-dimensional histogram. To draw these mountains, the hidden line plot of figure 12B can be used. In other cases, it is better to use the contour plot of figure 12C, which is analogous to topographical maps used in surveying. To highlight features, the contour plot may be drawn with contours that are not evenly spaced. The hidden line plot is very useful when the peaks are distinct and arranged in a convenient manner for observation. When the peaks are less separable and have markedly different maximums the contour map is superior. The contour map is also easier to use when trying to draw boundaries around individual clusters for statistical analysis. The drawback of contour maps is that experience may be needed to accurately interpret them from visual cues.

One other option is to transform any of these plots onto logarithmic axes. The statistical basis for using log axes is less well grounded in this situation than for the earlier case of the histograms showing cell growth. Still, it is a useful technique when it clusters the data in patterns which the human eye can use to recognize various subpopulations of events within the data.

Results

Within as little as 11 hours (less than a doubling time), the GMDs containing secreting cells captured enough secretions to permit them to be labelled bright green. (See photos 4-6). This showed that cells in GMDs did secrete their products and that the GMD/bead method could capture adequate amounts of the secreted product to be useful as a screening tool. Crosstalk is apparent, but does not present an insoluble problem, as the quantitative results will show later. Given the secretion rate of the PA2.6 cell line, the production of a single cell in 11 hours would be on the order of 1 to 10 pg of IgG (10^7 to 10^8 molecules). This amount was detectable within the GMDs.

Figure 13 shows the results from flow cytometry of GMDs that were incubated in medium, with some samples from flasks containing the scavenging beads to reduce crosstalk. Based on control samples that lacked the green stain and/or the beads, the source of this background "noise" can be identified. Since the control sample without stain shows GF values lower than the other control, one source of noise is the slight amount of green stain which remains nonspecifically bound to all GMDs after washing, even the empty GMDs. The flasks containing only nonproducing cells have GMDs with a very dim fluorescence, giving GF values just slightly more GF that the no cell control. Fluorescence microscopy shows that the membranes of the nonproducing cells themselves are nonspecifically stained dim green by the labelled goat antibody, possibly on F_C receptors. This is therefore a second source of noise. The differences between contour plots of the two samples of nonproducers (with and without scavenging beads) is not significant. The difference in appearance is mostly due to the computer's attempt to draw contours on a very sharp cliff along

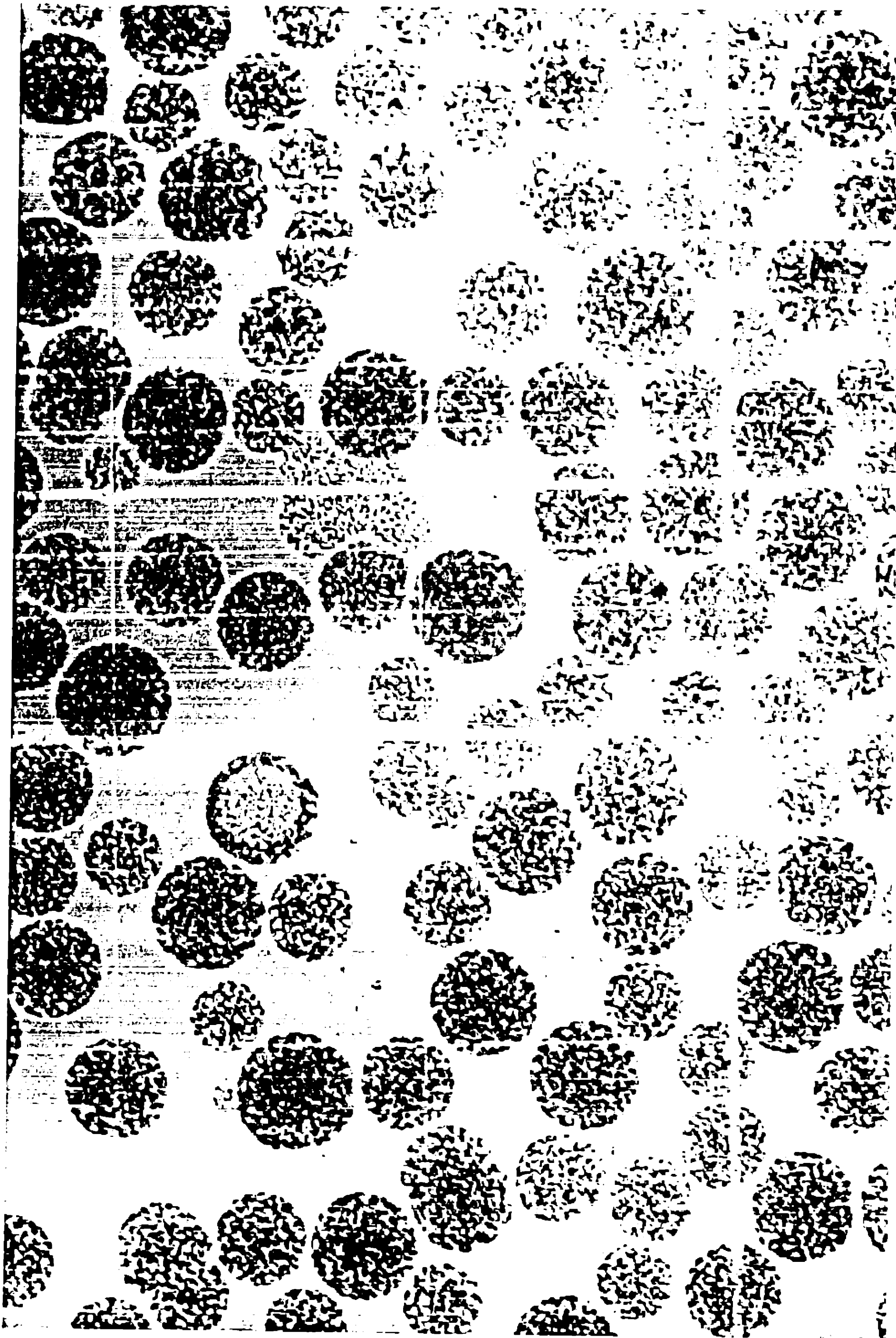


Photo 4. This microphotograph shows cells (15µ) immobilized in 30-100µ GMDs with coentrapped 0.8µ beads. The small squares are 50µ wide. The cells are a 90:10% mixture of P815 mastocytoma cells and PA2.6 hybridoma cells. The hybridoma cells secrete antibody that became bound to beads inside those GMDs containing the secreting cells. Other beads free in suspension captured most of the antibody which leaked out of the GMD. They were removed, and the GMDs were stained with an FITC-conjugated goat anti-(mouse IgG) antibody. The beads give the GMDs a gray, granular appearance under white light.

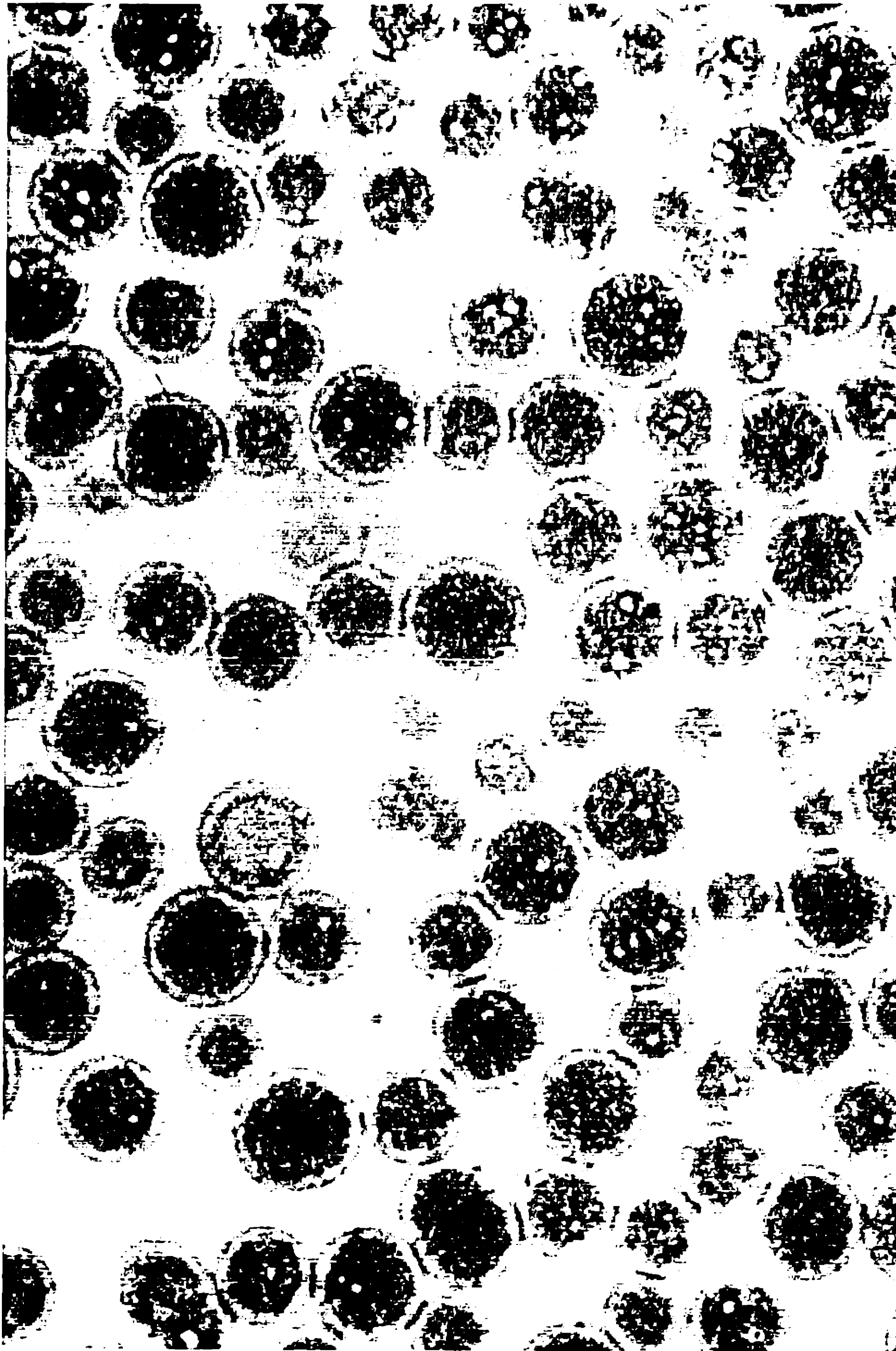


Photo 5 is the same as #4, but was taken out of focus, so that the cells now appear as clear circles inside the large GMDs.

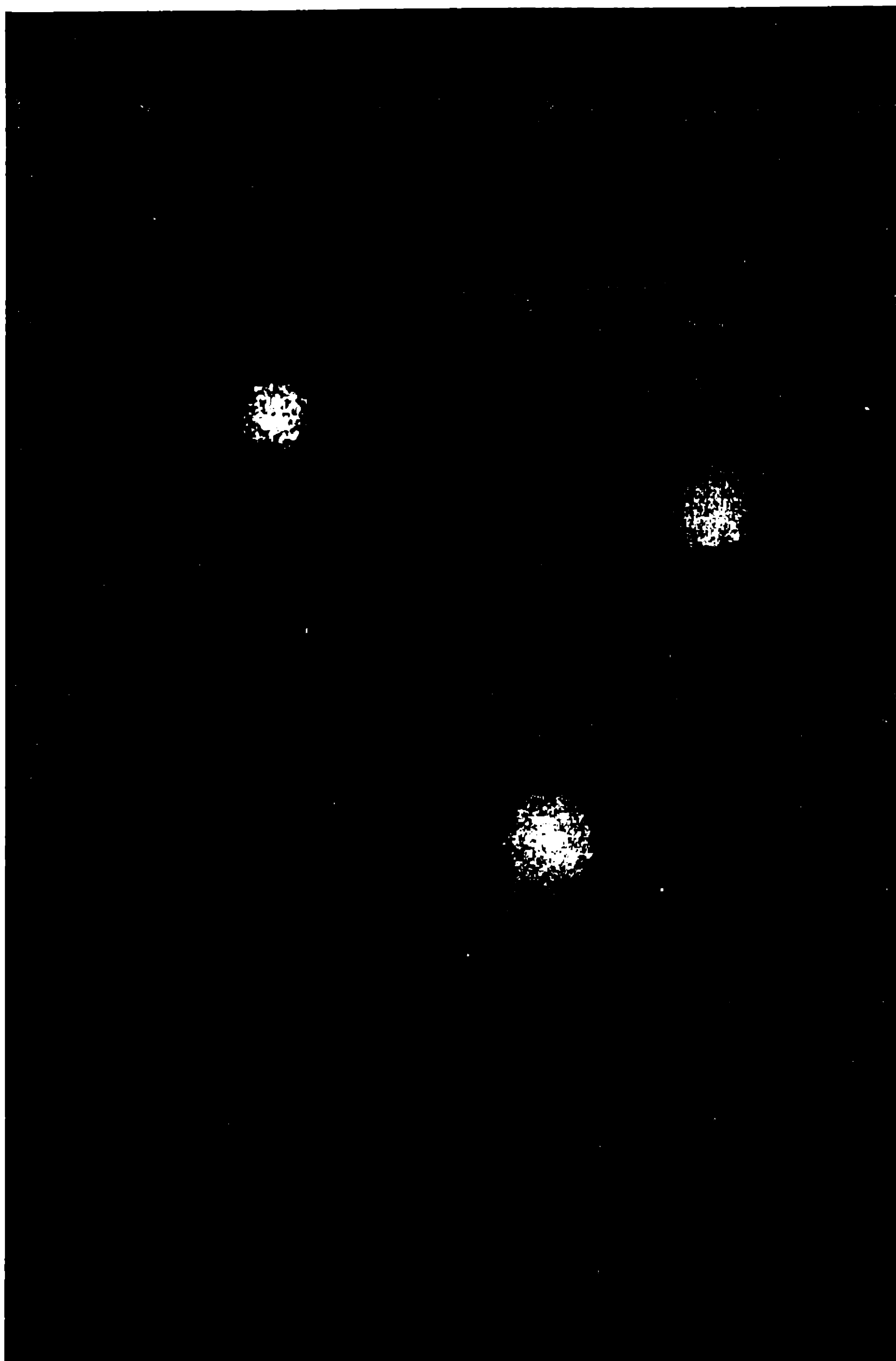


Photo 6 is the same frame as #4 and #5 but taken with a blue excitation only as lighting, thereby showing the green fluorescent label in 3 GMDs that contained cells which secreted mouse antibody.

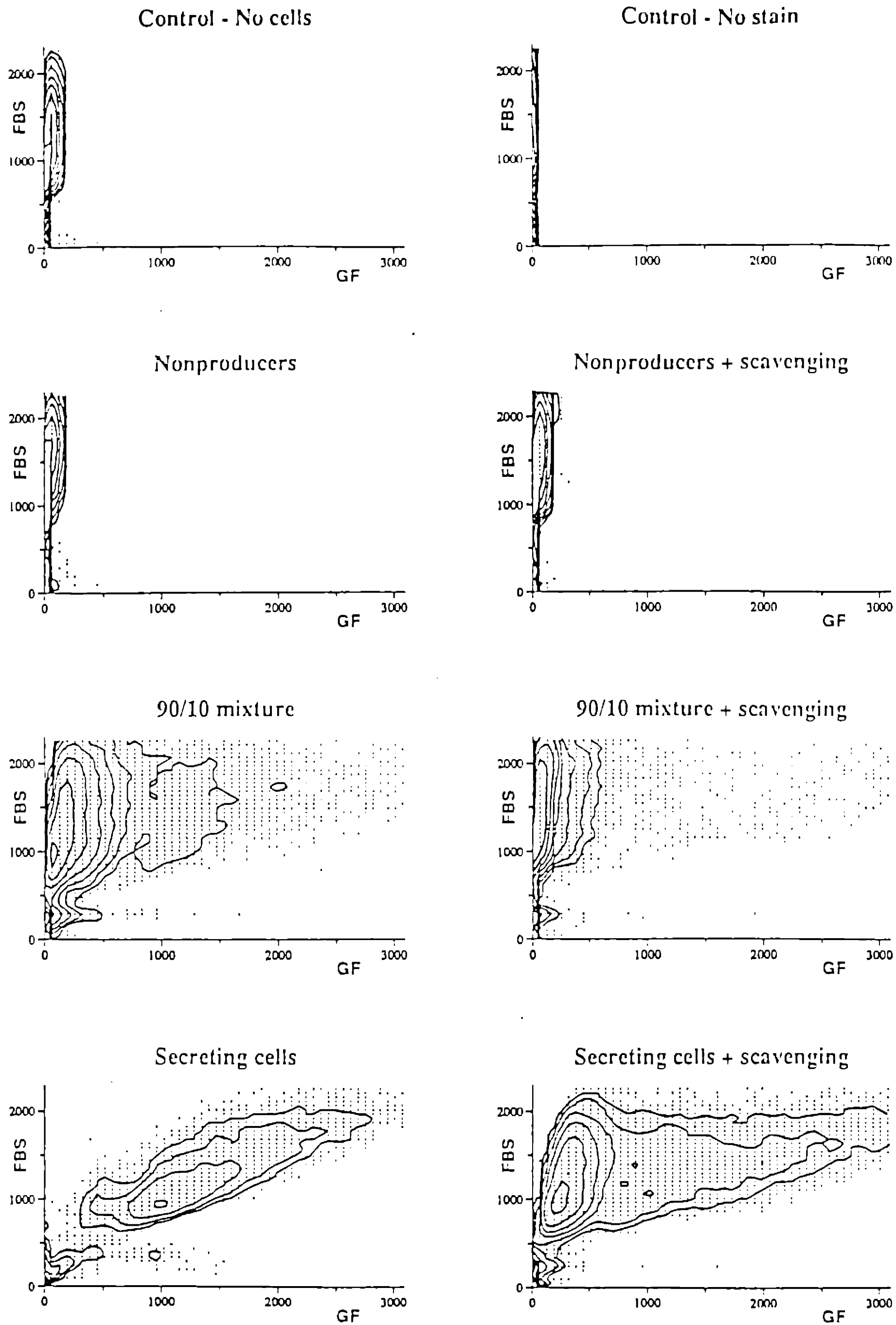


Figure 13. P815 cells (nonproducers) and PA2.6 cells (secretors) or mixtures of the two cell lines were immobilized in GMDs along with beads to act as binding sites for secreted antibody. In some cases the GMDs were incubated with a suspension of more beads that scavenged any antibody leaking out of the GMD in which it was produced. After 11 hours incubation, the GMDs were washed and captured antibody was labelled with green fluorescence using an immunoassay. Flow cytometry was used to measure the forward blue scatter (FBS) and green fluorescence (GF) from 20,000 GMDs. The data is plotted as contour plots, with contours at 5, 10, 20, 40, 80, 160, and 320.

the y-axis. A control sample of GMDs without beads, cells or stain (not presented here) shows a third source of noise. The beam splitting optics and optical band pass filters do permit a very small fraction of the scattered light from the blue laser into the GF photo multiplier tube. Much of this scattered light comes from the beads inside the GMDs. The amount of noise each of these three mechanisms contributes could not be accurately determined due to the resolution of the analog-to-digital converter at low signal values and the wide variance in GMD diameters. In any case, it is very small compared to the signal from GMDs containing secreting cells.

This background signal from each GMD determines the sensitivity of GMD based flow cytometry. Another practical measure of the sensitivity is the ability of GMD based flow cytometry to detect large differences between secretors and nonproducing cells in as little as 11 hours. This is excellent sensitivity for most applications. Many variables could be optimized to improve the sensitivity. These include the optical filters, the number of beads per GMD, wash steps, replacing beads with binding sites that do not scatter light, and preadsorbtion of the label onto blank GMDs to absorb some of the nonspecific binding molecules. Such improvements are not appropriate for the model system used here, and should only be considered when a particular application requires them.

When a small number of secreting cells are inoculated into the GMDs along with the larger number of nonproducing cells, the bivariate histogram (contour plot) in figure 13 shows that a small population of brighter green GMDs has appeared. These are so few in number that they are mostly represented by the small dots on the contour plot, rather than mountain contours. Furthermore, the larger group of events, consisting of the empty GMDs and GMDs occupied by only nonproducing cells, also shows increased GF values due to crosstalk of

antibody which has escaped from the originating GMD and become bound to another GMD. The addition of scavenging beads reduces this effect. In the case where the cell inoculum is entirely secreting cells, crosstalk is as large as the locally captured secretion and only one population of events is seen. Without the scavenging beads, the escaped antibody has nearly uniformly labelled all the GMDs in that sample. As a reminder, an 80μ GMD in this experiment could only hold 20 pg of IgG. A 50μ GMD, the smallest GMD in this fraction, could hold at most 5 pg, yet still produces a measurable signal. The addition of scavenging beads to reduce crosstalk in the 100% secreting cell sample again permits the GMDs to be grouped into a large peak at low GF values and a tail extending to higher values. These correspond to empty GMDs dimly labelled by crosstalk and the brighter GMDs containing secreting cells.

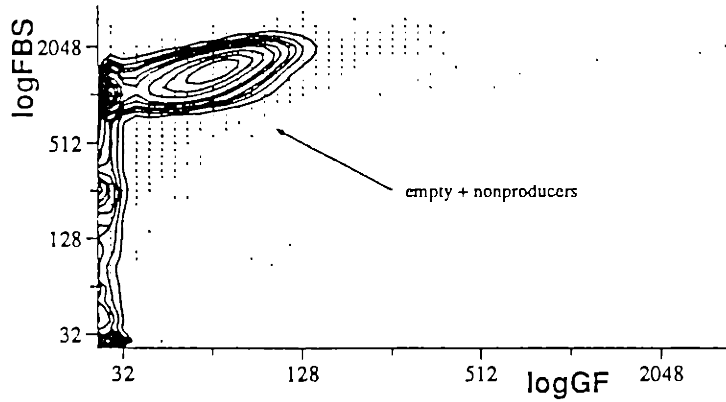
One attempt to reduce crosstalk is to incubate the GMDs in a more dilute suspension. This does not help because the affinity of the binding sites within GMDs is high enough to scavenge free secretions even at low concentrations.

Another attempt to reduce this crosstalk is to incubate the GMDs in an oil suspension. The oil is supposed to prevent the secreted antibody from diffusing over and contaminating other GMDs. Experiments also showed that this alone did not help. It is hypothesized that while the oil keeps the antibody within the originating GMD, the antibody is partitioned to the oil-water interface. The fraction of the secreted antibody captured by binding sites within the originating GMD is therefore not markedly increased. Later, during transfer steps from the oil phase to the aqueous phase for staining and flow cytometry, this partitioned antibody is released into the bulk aqueous mixture and evenly distributed to all GMDs in the suspension.

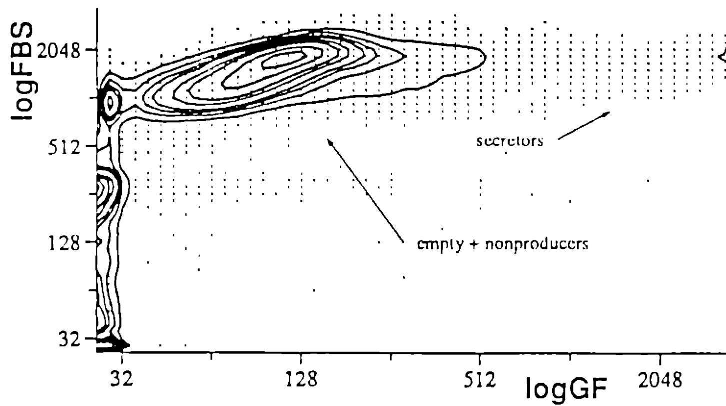
As alluded to before, the successful method for reducing crosstalk is to include free binding sites in the aqueous suspension which can scavenge the leakage. This can be accomplished by adding to the medium the same beads used for binding sites inside the GMDs. In the case of the experiments presented in this thesis, twice as many beads were free as were entrapped in the GMDs. These free beads are removed by sieving and by low speed centrifugation when pelleting the GMDs during rinsing steps. The results presented on the right side of figure 13 can be compared directly to their counterparts without the scavenging beads. Clearly, an improvement is seen in identifying those GMDs containing secreting cells. In the bottom right of the figure, it is easy to distinguish the unoccupied GMDs which are in the high peak and the occupied GMDs which have a brighter green fluorescence (GF). This improvement is easier to visualize in figure 14 where the data is presented on log-log axes. In figure 14C, two populations (mountain peaks) are easily identified, along with noise events near the origin. By use of the log axes, the long tail in figure 13 has become a distinguishable cluster. Analysis shows that the peak on the far right contains 33% of the nonnoise events. The estimated cell inoculation density was 34%, based on an average 80μ GMD. Fluorescence microscopy showed that 43 of 121 GMDs (36%) were occupied. This excellent agreement confirms that a subpopulation of brighter green GMDs exists which correspond to GMDs containing secreting cells.

The dim group of GMDs, which make up the left peak in figure 14C, are empty GMDs. They have been dimly stained due to crosstalk from the secreting cells in other GMDs. This is confirmed by comparing them to the upper graphs, which had 10% and 0% as many secreting cells. In these plots, the peak GF of the empty GMDs is at dimmer GF values. The mean brightness of the brighter group in figure 14C is 5 times that of the dim group. This separation between the

Nonproducers + scavenging



90/10 mix + scavenging



Secreting cells + scavenging

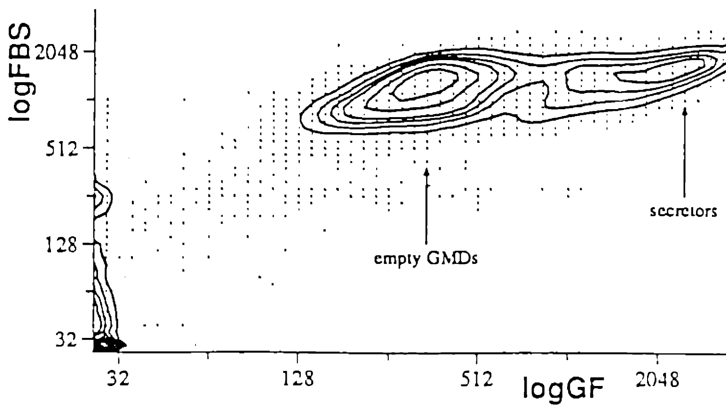


Figure 14. Three plots from figure 13 with the data converted to logarithmic domain to better highlight the different populations. Contours are at 25, 50, 75, 100, 150, 200, 300, 400 and 500.

subpopulations of GMDs with secreting cells and other GMDs is more than adequate for a flow cytometer with sorting capability to screen for and isolate the desired GMDs.

Conclusions

GMDs are therefore capable of capturing enough secretions to serve as miniature cloning and screening vessels. Crosstalk can be reduced or eliminated with large numbers of scavenging binding sites. Screening can occur in the flow cytometer at over 100 GMDs per second. The fastest plate readers for microtiter wells can read about 1 well per second. The GMD based flow cytometry method is therefore over 100 times faster during the analysis step. Other parts of the protocol are also faster, because 100,000 GMDs can be washed and stained in a single tube. Washing and staining an equal number of microtiter wells would be prohibitively time consuming. Because of the small volume of the GMD, it is expected that sensitivity and background noise will compare favorably to microtiter wells for most applications. Even without optimization, it was possible to detect as little as 1 - 10 pg of antibody secreted from a single cell. Finally, the electrostatic or fluidic sorting of flow cytometry is much faster than the manual retrieval from microtiter wells or petri dishes.

The occupancy of the GMDs can be controlled depending on the purity of the sort desired, as has been the case with microtiter wells. When seeking very rare events, the inoculation need not aim for monoclonal occupancy. Several cells can be inoculated into each GMD or microtiter well. Interesting GMDs or wells can be isolated and subcloned. With microtiter wells, this multiple occupancy method carries the risk that nonproducing cells will grow faster and overgrow the

interesting clones. GMDs eliminate this problem in two ways. First, secretion can be detected earlier, even before a single cell division has occurred, due to the much smaller volumes. Second, because GMDs are less cumbersome and faster to analyze, lower occupancies can be used.

These attributes of the GMD based flow cytometry technology give it many inherent advantages over the microtiter wells, soft agarose cloning, and scanning microfluorimetry. While it may not be the best method in every case, clearly its unique capabilities will be valuable for many applications.

Chapter 7

Screening effects of medium composition

The primary motivation for this thesis was the development of a GMD based flow cytometry technology which could analyze, screen, and ultimately sort and isolate clones based on their growth characteristics and secreted products. In this usage, it directly replaces the 96-well microtiter plates when the wells are filled with a common medium and inoculated with a mixed population of cells. As seen in previous chapters, GMDs have many inherent advantages over microtiter plates for this type of analysis and screening.

The other common use of microtiter wells is to screen various compounds, additives, or growth conditions with a standard inoculum of cells. The GMD technology does not have as great an advantage over microtiter wells in this case. Still, there are some potential applications of GMDs for this second class of problems. Inside GMDs, cell growth and secretion can be observed and individually quantified for a large number of clones. This provides the potential of identifying clones whose response to the item of interest is different from the response of the main population. For example, it could identify subpopulations and clones which will grow at normal rates despite the lack of a nutrient in the medium being tested. As a simple method of showing the potential of this new GMD based technology, some demonstration experiments were performed. The

experiments involved testing medium with reduced amounts of vital nutrients, namely serum and L-glutamine. Mammalian cells were incorporated into GMDs by the standard protocol, and aliquots were cultured in medium with different concentrations of these two supplements. Since the cell lines used are monoclonal, the presence of subpopulations was not expected nor were any convincingly found. The experiments do demonstrate the basic methodology and estimate the practicality of the experiments in terms of number of GMDs needed to get satisfactory results.

Methods In one experiment, the tissue culture medium was the standard K medium (RPMI 1640 supplemented with 10 mM HEPES buffer 300 mg/L L-glutamine, 50 u/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum), except that RPMI 1640 without L-glutamine was used and the glutamine supplement varied. Normally RPMI 1640 contains 300 mg/L of glutamine. K medium is supplemented with an extra 300 mg/L of L-glutamine from concentrated stocks of freshly reconstituted glutamine. In this experiment, the supplement of glutamine was varied to produce 5 flasks of growth media with concentrations of 0, 0.1, 0.4, 1.0 and 2.0 times the normal 600 mg/L value. Carryover of glutamine with the GMD inoculum raised the zero glutamine flask to a trace value, but that was calculated to be much less than 12 mg/L. L-glutamine, an amino acid, is an important source of energy and amino groups for many metabolic functions of mammalian cells, especially DNA synthesis. It was expected that the growth would be stunted in flasks with inadequate glutamine.

Mastocytoma cells (p815) were entrapped in GMDs by the standard protocol in four tubes. The tubes were pooled together and one half of the pool used for this experiment. The pooled sample was used to inoculate five T-75 flasks,

producing 5 ml of GMD suspension at each of desired glutamine concentrations. Time zero samples of GMDs and free cells were also set aside. Incubation of the flasks was carried out at 37° C, 5% CO_2. Samples of 1.2 ml were taken after 16, 24, and 40 hrs incubation. The 40 hr sample was obtained as 2 replicate samples. Immediately after sampling, the cells/GMDs were pelleted (600*g* x 2 minutes), rinsed once with PBS, and resuspended in 0.5 ml PBS. Then 0.5 ml of methanol was added to fix the cells. The fixed cells were stored refrigerated.

At a convenient time, the fixed samples were pelleted, rinsed once with 1 ml PBS, and sieved with 10 ml of PBS through a 0.7 cm^2 88μ nylon mesh, pelleted and resuspended in 20 μM PI in PBS. PI stains the cell's RNA and DNA with red fluorescence (RF). Flow cytometry was used to quantify the RF on 5000 occupied GMDs from each sample.

Results The results of this experiment were shown previously in figure 6. The flask with nominally zero glutamine showed significantly less growth after 40 hrs, with most cells in that flask undergoing 3 replications (to an 8 cell microcolony) rather than the 4 replications (to a 16 cell microcolony) seen in the other flasks. Glutamine levels of 60, 240, 600 and 1200 mg/L in the other 4 flasks yielded similar growth rates. This indicates that as little as 60 mg/ml glutamine is adequate to sustain growth, at least in the short term of 40 hrs incubation and 4 cell divisions.

This observed stunting of growth was a predictable result. The experiment was performed to demonstrate how GMDs can be used to investigate the effects of medium composition. Several points can be seen in this demonstration. On average, the 5000 occupied GMDs measured by flow cytometry required about 25% of each fixed sample. This varied from 10% to 100%, mostly due to

differences in yield while sieving of each sample. This variance was decreased in later experiments by using larger cross-sectional area sieves and larger rinses to get more consistent sieving. Still, even in this experiment 10 samples of 5000 occupied GMDs (most singly occupied) were generated from each test tube of GMDs produced by the standard protocol. On average, 40 such samples could have been generated. The 5000 occupied GMD sample was adequate to easily detect a difference of 25% in growth rate between the flasks. This implies that scale up of the protocol is unnecessary for many experiments.

The characteristic diffusion times for GMDs are on the order of seconds to minutes, so that the cells were exposed to culture conditions of the new growth medium almost immediately (relative to cell division time) upon transfer into the new flasks. Since growth in GMDs can be detected at the fundamental limit of a single division, data can be collected which shows any lag time between changing medium composition and changes in cell growth. Intermediate products of glutamine metabolism may permit growth to continue for some time after glutamine is removed from the medium. In this experiment, the GMD technology permits observing clonal growth rates at these early time points. If the longer term response to low levels of glutamine is of interest, larger GMDs could be used to study growth after 5 or 10 replications.

Methods In a parallel experiment, mouse hybridoma cells (PA2.6) were entrapped in GMDs by the standard protocol of chapter 3 and inoculated into the 5 flasks with varying concentrations of L-glutamine. At time points of 16, 24 and 40 hours, aliquots were taken and the cells fixed with 50% methanol as in the above experiment. Free cell and time zero samples of the GMDs were also fixed and sieved to below 88μ. The GMDs were then treated with 10 K.U./ml RNase

for 30 minutes at 37° C to digest the RNA. The remaining DNA was stained with 10 μM PI in PBS. Flow cytometry then measured the cell number in each occupied GMD.

Results Figure 15 shows the results of measuring forward blue scatter (FBS) and Red Fluorescence (RF) on several thousand GMDs after various incubation times. RF, measured in arbitrary units, can be calibrated to the value of a single cell. FBS measures light scatter from the microcolony in the GMD. The contour plots of FBS and RF show that FBS does not increase linearly with cell number. Still, the measurement of forward blue scatter (FBS) shows the microcolonies growing monotonically in time. The measurement is adequate to determine the difference between single cells and microcolonies, which can be useful in some experiments for eliminating from the dataset those events corresponding to cells which failed to replicate and are presumably nonviable. The FBS measurement is also useful for coincidence gating. The photo multiplier tubes used for RF measurement generate a large number of noise events. Noise may be from scattered laser light leaking through the red filter, from electronics, or from small but brightly fluorescent particles contaminating the samples. Many of these noise events can be eliminated, through either hardware trigger thresholds or computational thresholds that validate only those RF events coinciding with an FBS measurement indicative of a cell or microcolony.

Coincidence gating is especially important when measuring plating efficiency. The plating efficiency of the cell line in GMDs in the medium of interest can be determined by measuring which fraction of the inoculated cells have undergone at least 1 replication after an appropriate incubation. This is determined by setting a RF threshold between 1 and 2 cells and calculating the fraction of occupied

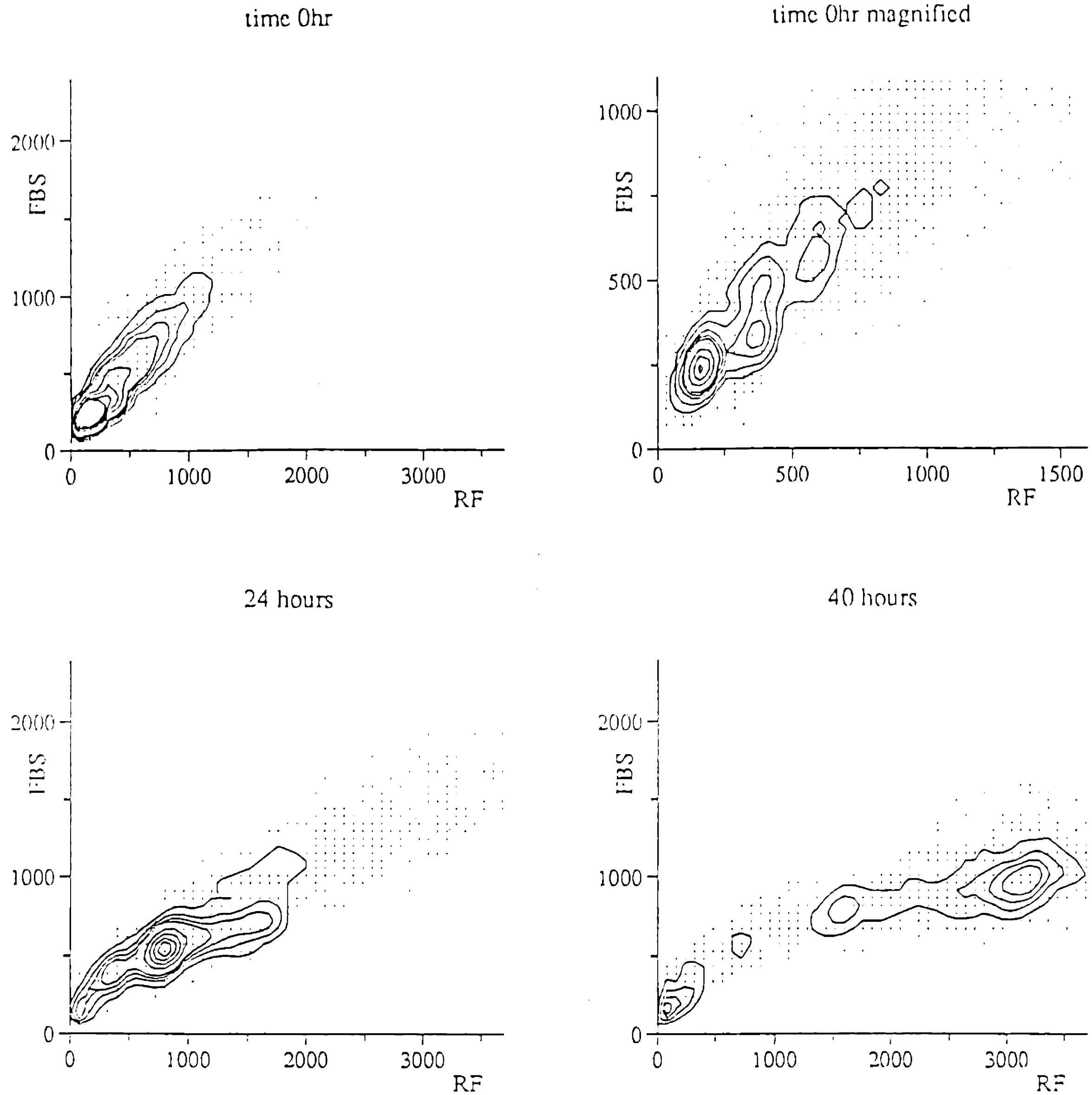


Figure 15. PA2.6 cells were immobilized in GMDs and aliquoted after various amounts of incubation. The red fluorescence (RF) and forward blue scatter (FBS) was measured for each occupied GMD. Contour plots show the increase in RF with time. A single cell produced about 200 units of RF in this experiment, as seen in the magnification of time zero. Note that multiply occupied GMDs give peaks at RF of 400 and 600 as well. At 24 hours, most occupied GMDs contain 4 cells. At 40 hours, 16 cell microcolonies produce about 3200 units of RF.

GMDs in which which growth has occurred, *i.e.* contain more than one cell. Given the possibility of multiple initial occupancy (2 cells inoculated within the same GMD, an alternative criteria of at least two replications (4 cells) can be used, with the incubation time extended appropriately. Electronic noise and particulate within the sample can generate a large number of flow cytometry events which do not correspond to cells, but have RF values below the growth criteria threshold. These events must be excluded if the measurement is to be accurate. Coincidence gating, by requiring that the RF and the FBS measurements both be consistent with measurements from cells in GMDs, permits exclusion of most of these unwanted noise events.

The upper right graph in figure 15 shows a detailed look at the RF and FBS measurements. Since this data is for time zero, no replication has occurred. The four large peaks on the contour plot correspond to GMDs containing 1, 2, 3, and 4 individual cells. The vast majority are contained in the singly occupied GMDs. Even more GMDs were unoccupied, but they are eliminated from the dataset by electrical gating due to their lack of RF. The contour plot also shows that for individual cells, FBS is linearly related to RF, with an offset due to scatter from the GMD itself. This demonstrates the effect of slit scan integration in flow cytometry of GMDs. If the cells are spatially separated, multiply occupied GMDs give scatter and fluorescence signals which integrate to multiples of single cell measurements. If the cells are packed into a microcolony, the FBS measurement increases asymptotically with the cell number as measured by RF. Hence, the plots at 24 and 40 hours show the correspondence between RF and FBS curving to the right.

The more important results of this experiment are shown in Figure 16. In this presentation of the data, the arbitrary RF value has been calibrated to the

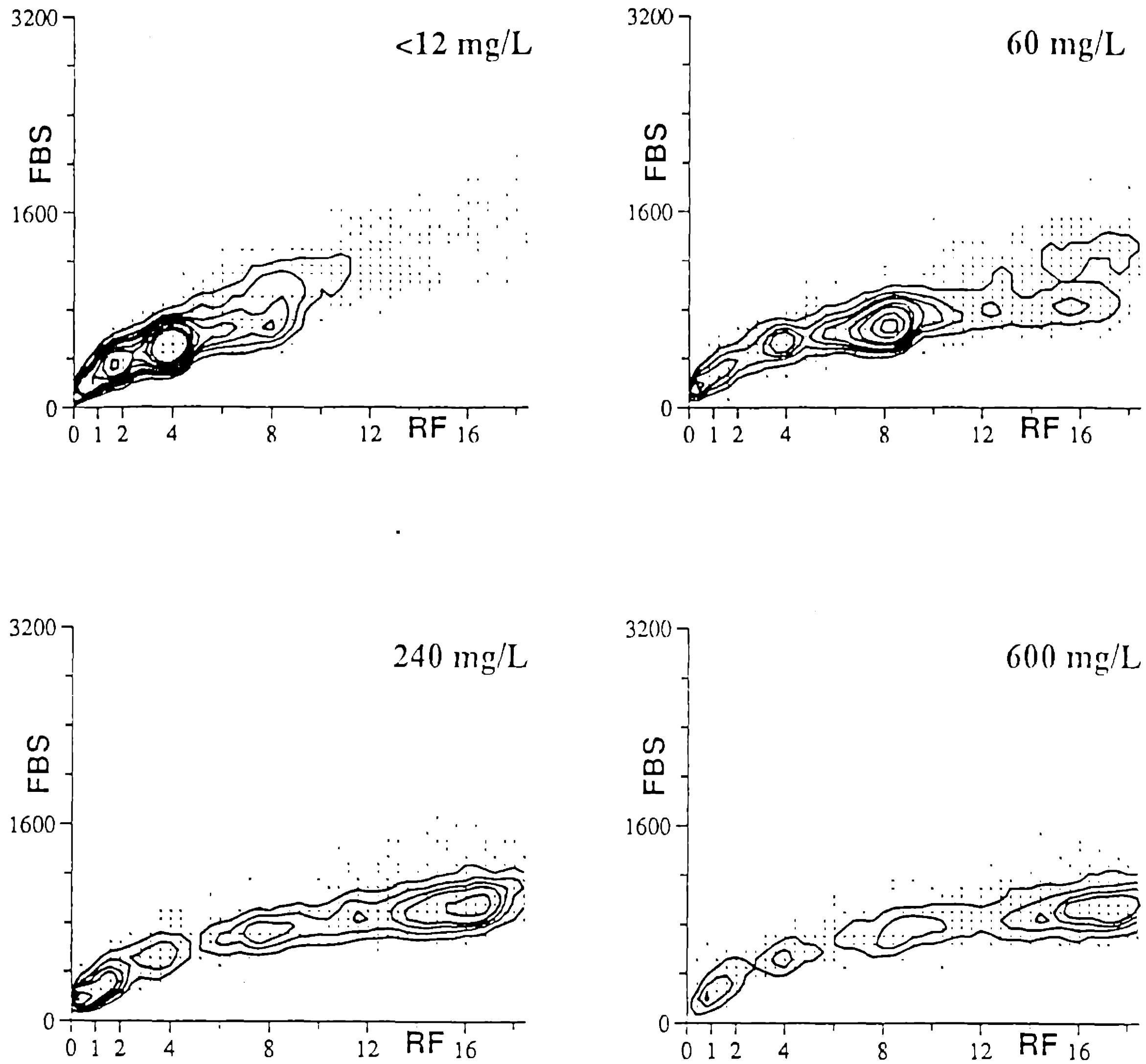


Figure 16. PA2.6 cells were grown in GMDs as in Figure 15. They were incubated in flasks with various concentrations of L-glutamine for 40 hours. FBS and RF (DNA) measurements were made on 5000 occupied GMDs. The RF axis is linear, but was scaled to the RF of a single cell. Note that the <12 mg/L flask stopped growing after 2 divisions (4 cell microcolonies). The 60 mg/L flask has its highest peak at 8 cell microcolonies, while the other flasks produced 16 cell microcolonies. Contours are at 4, 8, 12, 16, 24, 32, 48 and 64.

value of a single cell. The effect of reduced glutamine concentration on PA2.6 growth over 40 hours incubation is clearly shown. The zero (<12) glutamine flask shows stunting of growth. Most GMDs contain microcolonies of only 4 cells. In the 60 mg/L glutamine flask, 3 replications to form 8 cell microcolonies is the typical result. For 240, 600, and 1200 (not shown), the norm is 4 replications to form 16 cell microcolonies, although a subpopulation of the clones have only completed 3 replications. Analysis of data at 16 and 24 hours showed that the cell replication in all flasks occurred at essentially the same time. However, replication stopped after 2 and 3 replications in the two flasks with lower glutamine levels (<12 and 60 respectively), while the other flasks with adequate glutamine permitted replication to continue. This is consistent with a model suggesting that the exhaustion of some glutamine derived intermediate metabolite limits cell replication under these conditions.

Again, this result is not unexpected, given current knowledge of glutamine's effect on mammalian cell growth. The result is not the purpose of the experiment. Instead, the experiment demonstrates how GMDs could be used to replace microtiter wells when performing these experiments. Furthermore, because GMDs allow growth measurements on thousands of individual microcolonies, it will be possible to check for subpopulations which exhibit different growth characteristics. The simplest example of this, of course, are cells that do not grow at all. The plating efficiency measurement is such an example. In figure 16, it can be seen that even in the 600 mg/L flask, some cells do not replicate and have RF values of 1. These events represent less than 10% of all the events. The plating efficiency therefore was 90%. Note that GMD based flow cytometry can measure growth rates and plating efficiency simultaneously. This cannot be done in either microtiter wells or in petri dishes.

Chapter 8

Other technical problems

Sorting One of the great advantages of flow cytometry is the ability to perform cell sorting. The most common method of sorting is electrostatic. The capillary stream after interrogation by the laser is squirted into air while being vibrated by a piezoelectric transducer. Rayleigh's computations show that if a proper frequency is chosen, the capillary jet will break up into uniform size droplets with a spherical diameter about twice that of the capillary jet. Furthermore, each droplet will breakoff from the solid jet at the same point in space, or conversely with the same time delay from the laser interrogation point. The droplet stream may be charged just before breakoff, imparting a small amount of positive or negative charge to an individual droplet. The stream of droplets then pass through an electric field which deflects the desired droplet out of the mainstream of droplets and into a nearby collection vessel.

Many commercial flow cytometers are equipped with electrostatic sorting capability. These sorters are designed for sorting 10-20μ mammalian cells. They also work well for sorting smaller items, such as chromosomes. To sort larger items, such as plant protoplasts (40-60μ), the commercial flow cytometers must be modified. Sorting efficiency drops off rapidly as the ratio of particle size to capillary diameter increases. Solid objects whose diameter is greater than about

30% of the diameter of the capillary jet create too much flow disturbance and adversely affect the breakoff point.[39,40] The flow cytometer used for these experiments had a 100μ sorting nozzle, limiting efficient sorting to 30μ particles, too small for use with GMDs. However, jet ink printers use electrostatically controlled capillary jets with droplet sizes up to 1000μ. Therefore, the technology is developed and it is feasible to design such a system to sort 30-100μ GMDs.

Recovery Since cloning in soft agarose is an established technology in mammalian cell culture, no problems are forseen in recovering cells from the isolated GMDs. Indeed, the gel matrix may actually protect the cells from the mechanical forces involved in flow cytometry and sorting. Clones from soft agarose may be recovered by using a pipet tip to pluck from the petri dish a piece of agarose containing the clonal microcolony of interest. Established techniques for extracting the cells from the gel include enzyme degradation of the gel, mechanical breakage of the agarose by rapid pipetting or stirring rods, and simply allowing the cells to grow until they break out of the gel. To demonstrate the recovery from GMDs, experiments were conducted wherein several flasks of GMDs with cells were incubated for 7 days. In each case cells had escaped from the GMDs and gone on to reestablish themselves in free suspension in the growth medium.

Chapter 9

Summary

Overall, GMD based flow cytometry is clearly a feasible technology. All the major technical points have been demonstrated. GMD based flow cytometry appears to be a very flexible and useful alternative to microtiter wells for screening the growth and secretion characteristics of mammalian cells.

This thesis has outlined a protocol for entrapping the mammalian cells in small droplets of 2.5% agarose and culturing the cells in a normal manner. The simple protocol converts 25-40% of a sample into GMDs of a size class of 44-88μ which is useful for flow cytometers. The viability of the cells after this entrapment process is 80-95%, based on plating efficiency within the GMDs. Cell growth and division inside the GMDs appears normal. Cell functions, including enzyme activity, DNA replication, and secretion of protein products all appear normal inside the GMD.

The flow cytometer is capable of accurately measuring the number of cells inside GMDs with a precision of 8-13%. Accuracy is excellent (<7%), and linearity fair (departure <10%). The repeatability is more than adequate to test for relatively small changes (<25%) in growth rates in less than 24 hours. Unlike normal methods of measuring growth, the histograms computed from flow cytometry data allows simultaneous estimates of plating efficiency and permits

detection of subpopulations with different growth rates.

The inclusion of binding sites within the GMD allows the capture of some of the secreted products of the cells within that GMD. By tagging these products with a fluorescent label, mammalian cell clones can be screened in the flow cytometer at over 100 individual clones/second. Screening can occur as little as 11 hours after inoculation and can easily detect as little as 10 picograms of secreted antibody. This methodology is flexible enough to be adapted to a wide variety of binding sites, secreted products and labels.

Compared to microtiter wells, this is an increase of two orders of magnitude in rate of measurement. The simplified washing and staining provide significant savings in time, labor, and tedium. But an increase of this magnitude does more than just provide a cost savings. It opens up new possibilities and new applications that previously have been prohibitively difficult. This thesis has already demonstrated detection of growth at the fundamental limit of a single replication. The ability to screen several million GMDs in a day of flow cytometry is now feasible using this technology. Since each GMD could contain several clones if a coarse sort was desired, the potential exists for searching for events as rare as 1 clone in 10^7. This "needle in the haystack level" is within the range of the natural genetic mutation frequencies per generation for many cell lines. This is another important, fundamental limit which is being examined by medical research seeking to understand genetics and by biotechnology which is seeking to utilize mutant or deliberately mutated organisms.

Therefore, this thesis goes beyond demonstrating the mere feasibility of the new technology of GMD based flow cytometry It demonstrates the great potential that this technology might achieve, and justifies further development of the technology.

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