Microengineering the Environment of Mammalian Cells in Culture

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Abstract

Assays based on observations of the biological responses of individual cells to their environment have the potential to make enormous contributions to cell biology and biomedicine. To carry out well-defined experiments using cells, both the environments in which the cells live and the cells themselves must be well defined. Cell-based assays are now plagued by inconsistencies and irreproducibility, and a primary challenge in the development of informative assays is to understand the fundamental bases for these inconsistencies and to limit them. It now seems that multiple factors may contribute to the variability in the response of individual cells to stimuli; some of these factors may be extrinsic to the cells, some intrinsic. New techniques based on microengineering—especially using soft lithography to pattern surfaces at the molecular level and to fabricate microfluidic systems—have provided new capabilities to address the extrinsic factors. This review discusses recent advances in materials science that provide well-defined physical environments that can be used to study cells, both individually and in groups, in attached culture. It also reviews the challenges that must be addressed in order to make cell-based assays reproducible.

Keywords: *biosensors, cell-based assays, diagnostics, microfabrication, microfluidics, PDMS, poly(dimethylsiloxane), soft lithography.*

Introduction

Cells are the fundamental living units of organisms: the response of an organism to disease, injury, or therapy is the response of its cells. To be able to model life and predict the response of organisms to therapeutic or pathological stimuli ultimately requires the ability to model the behavior of the cells (individually) and tissues (as collections of cells) to these stimuli. Thus, characterizing the full range of cellular behaviors, mapping these behaviors to normal function and to disease, and reducing these behaviors to molecular processes are three of the primary goals of biomedical research.

The study of the molecular aspects of cells, especially understanding the genome and how the information it encodes is converted into proteins, has exploded in the last 40 years. Understanding how the phenotype (the actualized cellular characteristics, processes, and behaviors, such as shape, size, growth rate, migratory behavior, and response to stimuli) arises from its genotype (molecular composition) is, however, a much more complex subject and is only just beginning to be explored. Both approaches—the "top-down" study of cells, beginning with phenotypic cellular behaviors, and the "bottom-up" study of nucleic acids, proteins, and networks of reactions—must ultimately combine if we are to fully understand the cell.

Even without this full understanding, however, the ability to modulate the phenotype of cells is potentially enormously useful. These behaviors are the result of the operation of all the processes in living cells and thus provide integrated, if very complex, information about the cellular response to stimuli (including the passage of time). As it is not necessary to understand everything about how a molecule interacts with the human organism in order to develop a useful drug, it is also not necessary to understand the molecular basis of all cellular behaviors to be able to use cells in assays, as sensors, and in engineered tissues.

These uses of cells do, however, require that their responses to stimuli be reproducible, especially if they are not entirely characterized. In science, it is understood that the reproducibility of an experiment is the *sine qua non* for transforming observational science into reliable engineering. There is no such understanding among cell biologists. One may well ask how it is possible to do science without the reproducibility of experiments, but cells are, in fact, very complicated systems and far from completely understood; the fact that metallurgy places much greater emphasis on reproducibility than cell biology stems from the fact that experiments in metallurgy usually *are* reproducible; those in cell biology often are not, and it is very difficult to understand all the reasons that might contribute the answer to the question, why not?

Characterizing cellular behavior requires, at minimum, two capabilities: (1) detailed control over the features of the environment that influence the behavior of the cell; and (2) access to cells that are themselves sufficiently well defined so that they respond in reproducible ways to environmental stimuli. Both of these subjects—the environment of the cell and the cell itself—are increasingly important research topics as their centrality to bioscience and biotechnology becomes more obvious.

Organisms are, of course, composed of cells. They are equally composed of tissues—functional assemblies of cells (usually of several types) and structural elements. In these tissues, cells are highly organized in their physical and chemical interactions with their neighbors and with other aspects of their environment. The architecture of the tissue—the spatial organization of cells and surrounding scaffolding—defines the local cues that may be experienced by an embedded cell. Such cues might include gradients (of concentrations) in soluble and immobilized factors (e.g., cell adhesion molecules and extracellular matrix proteins); networks of molecularly mediated adhesive and signaling interactions arrayed on adjacent cells and nonliving scaffolds; or static and time-dependent forces acting on the cells. The complex structure of tissue—from

molecular to organismic and from static to time-dependent—determines the world in which the cell lives. Tissue structure is critical to tissue function, and tissue function reflects collective behaviors of cells.[1,2]

Although the spatial presentation of environmental signals at cellular and subcellular length scales is essential in determining many cellular behaviors, understanding the interactions of cells with their environment has been much less studied than the internal, molecular workings of the cell. The origin of this disparity lies in the tools that are available: molecular biology and biochemistry have produced a flood of extraordinarily useful tools for exploring the molecular aspects of the cell;[3] there are fewer tools to define and manipulate its more macroscopic, external environment. Most of our current understanding of the interaction between the environment and behavior of cells is based on the presentation of soluble and insoluble environmental cues to large populations of a single type (ostensibly) of cell cultured as monolayer sheets on the surface of a "substrate" (typically, a lightly surface-oxidized polystyrene Petri dish). These limited conditions for presenting stimuli to cells have limited our understanding of how cells actually sense and respond to their environment. New tools are now becoming available that make it possible to (1) tailor many aspects of the surface on which attached cells rest at the molecular level; (2) determine the shape and location of individual cells; (3) pattern certain aspects of the liquid culture medium at subcellular scales; and (4) release cells from certain environmental constraints at a specified time during the experiment to study the time-dependence of their behaviors (for example, subsequent motion across the substrate).

The development of tools to build structured environments for cells is making it possible to study how cues presented with cellular and subcellular spatial granularity affect cell behavior and function. Results from experiments carried out in these engineered environments establish clearly that cells have a remarkable ability to sense and respond to their environment.[4,5] They also demonstrate that the "cell"—as often used in bioengineering and some areas of cell biology—is ill defined as an experimental object and so heterogeneous and irreproducible in its response to stimuli that the development of better-defined systems for studying cells must be a central infrastructural objective for cell biology and bioengineering.

The understanding that cells in culture are heterogeneous, but heterogeneous in ways that we do not understand and cannot easily characterize, emphasizes the importance of reductionist studies of single cells. It also suggests important limitations of these studies: a single cell can never speak for a tissue, any more than an individual can speak for a crowd.

New Tools to Control the Environment of Cells in Culture

The last 10 years have seen the rapid development of methods of presenting spatially and chemically well-defined, soluble, adhesive, and mechanical cues to cells; the spatial resolution of these techniques extends easily to the 10 μm scale and, with increasing difficulty, to sub-100-nm scales.[6,7] The majority of these techniques have arisen from two new capabilities: (1) the ability to pattern surfaces with biologically active (or inert) molecules and (2) the ability to build microfluidic systems with dimensions relevant to cell biology and that take advantage of the characteristic fluid physics of liquids flowing in microchannels.

The tools used to pattern surfaces use, and are related to but quite distinct from, the photolithographic tools used in the microelectronics industry.[6,8,9] Photolithography is one of the most successful of all technologies within its domain, but it is not well suited to the materials needed in cell biology, nor is it well suited to manipulating organic molecules at the interfaces with a sophistication equivalent to that which is possible and demanded in molecular biology and biochemistry. The emphasis in microelectronics is on defect elimination, small feature sizes, extreme accuracy in complex patterns, and long device lifetimes; the needs in cell biology are experimental simplicity, one-time use of devices, low cost, the ability to produce relatively simple patterns, and compatibility with cells and biomolecules. Feature sizes for microfabricated devices used in cell biology are usually relaxed, as compared with traditional microelectronic devices (1–100 μm features cover the needed range of sizes), and defects are not important.

The patterning of substrates and the fabrication of microfluidic systems rely on a common technology—soft lithography.[6,8,10] Many aspects of this technology have been described in reviews; from the vantage of the discussions here, their most important aspects are that they are relatively straightforward to use experimentally, and they provide molecular-level control of surfaces using the often structurally complex, delicate organic molecules required in cell biology. The extreme experimental care needed to manufacture microelectronic devices is not required to make patterned (printed and molded) microsystems for use in cell biology and bioengineering.

Adhesive cues can now be easily and reproducibly presented to cells in specific geometries by patterning the adhesive extracellular matrix proteins onto cell culture substrates.[11–13] Soluble factors can be delivered to cells as gradients[4,14] or in concentrations that vary with time, using microfluidic systems (Figure 1).These patterning techniques have been used to demonstrate the importance of the spatial presentation of soluble and adhesive cues to basic cell functions such as migration, proliferation, differentiation, and apoptosis (cellular "suicide").[5,11,15,16]

Studies of Cell Function and Behavior Using Well-Defined, Patterned Environments

Here, we sketch representative experiences with these new tools for fabricating micropatterned environments to use in cell biology and biotechnology. These techniques are bringing a new level of reproducibility to certain types of experiments in cell biology and to enable certain studies—especially studies involving adhesion of cells to patterned substrates and movement of cells on substrates—with levels of precision that have not been possible in the past. With this capability has come both the ability to do new kinds of experiments that explore the relationship between the cell and its environment and the suggestion that cell populations, as they are currently studied, are more heterogeneous in their response than has been fully appreciated in the past. This realization provides important challenges for the future: high-resolution studies of phenotypic behaviors of cells is probably going to require better-defined environments (to which one currently can see a clear experimental pathway) and better-defined cells (to which the paths now appear more arduous).

It has long been known that cells are able to sense spatially encoded soluble and adhesive cues.[3,17,18] Immune cells, for example, can migrate to sites of infection and inflammation by moving through gradients in the concentration of soluble cytokines, in processes broadly called chemotaxis.[19,20] In cell culture, chemotaxis can be observed by placing the tip of a micropipette filled with a chemotactic agent in a culture of responsive cells; cells soon migrate and accumulate at the pipette tip.[20,21] Similarly, adhesion to an extracellular matrix also carries spatial information. Adhesion of cells to a flat surface causes them to polarize numerous subcellular organelles with respect to the surface plane. Adherent cells also sense and migrate toward a higher density of extracellular matrix ligands.[22–24] Unfortunately, in many of the experimental accounts of this type of study, the cues used to stimulate the cells were spatially

ill defined; this poor definition made the characterization of the cell response ambiguous. For example, for cells migrating in chemotactic gradients, it has been difficult to define whether cells respond to absolute concentrations of soluble factors, gradients in concentration across the cell surface, or some time-averaged sampling of the environment as cells move irregularly across the surface.

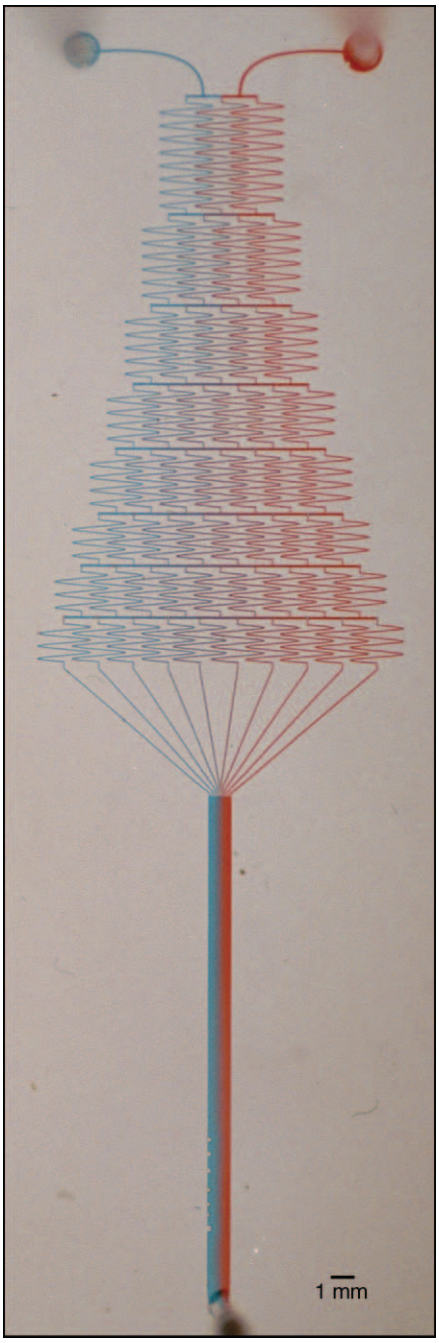

Figure 1. Photograph showing a microfluidic device used for generating gradients of soluble species. The operation of the device is illustrated with blue and red dyes. Solutions in channels split, mix by diffusion, split again, and ultimately combine into a single wide channel (bottom); the stream in this channel forms a gradient perpendicular to the direction of flow.

Here, soft lithography has made it possible to define, with high spatial resolution, environmental cues—both those immobilized on surfaces and those dissolved in the medium—that influence cell behavior. In so doing, they make it possible to study the ways in which cells examine and respond to their surroundings. For example, by using parallel laminar flows in a microfluidic channel, we and others have been able to expose cells to simple linear gradients to study chemotaxis (Figure 1).[5,25] Microcontact printing or stencil printing with elastomeric membranes can also be used to generate well-defined, micrometer-scale patterns of proteins on surfaces.[26,27]

These approaches have demonstrated that changes in cell shape that result as cells attach, spread, and flatten on an adhesive surface can regulate cell growth and death (Figures 2a and 2b).[11,15] Earlier studies had shown that increasing the density of extracellular matrix ligands on a substrate could change the shape and behavior of cells, but it left unresolved whether the changes in cell shape were causally linked to the changes in cell behavior.[28] Culturing cells on progressively larger islands coated with extracellular matrix enabled cells to spread to greater extents without the need for varying the density of immobilized ligands. This more direct approach to modulate cell shape was shown to be the critical factor in triggering informative phenotypic behaviors in cells.[11,15]

In some cases, the capability to modulate a particular environmental cue has provided the means to discover previously unknown cellular responses. For example, soft lithography made it possible, for the first time, to pattern surfaces into adhesive and non-adhesive regions and to define the shapes of these regions and, hence, the shapes of cells that adhered to and spread on them (Figures 2a and 3). This new capability has enabled studies of cells growing into shapes defined by printing to be regular geometrical figures (e.g., squares, triangles, and polygons) and demonstrated that both the extent of cell spreading and flattening and the geometric shape of the adhesive area could regulate cell behavior.[29,30] Cells conforming to equilateral triangles or squares polarize their migrating leading edges (i.e., their lamellipodia) toward one of the corners of the polygon rather than along a straight edge (see the bright areas on the corners in Figure 3a).[30] How cells detect such geometric features, however, remains to be discovered.[30,31]

Do those microengineering technologies that have already been developed and

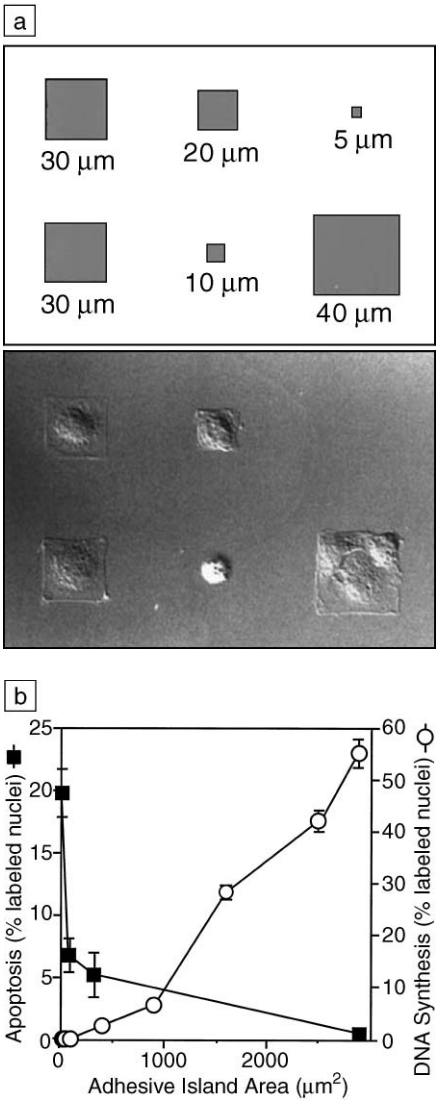

Figure 2. The influence of the footprint of a cell on its choice between growth and apoptosis. (a) (top) Schematic diagram showing the pattern: square islands of self-assembled monolayers (SAMs) to which proteins stick, surrounded by a different SAM to which proteins do not adsorb; (bottom) Nomarski microscopic image of the shapes of bovine adrenal capillary endothelial cells confined to the patterns. Scale labels indicate lengths of the sides of the squares. (b) Changes in cell shape as cells attach, spread, and flatten on an adhesive surface can regulate cell growth and death: apoptotic (cell death) index versus DNA synthesis (cell growth) index after 24 h, plotted as a function of the area of the spread cell.

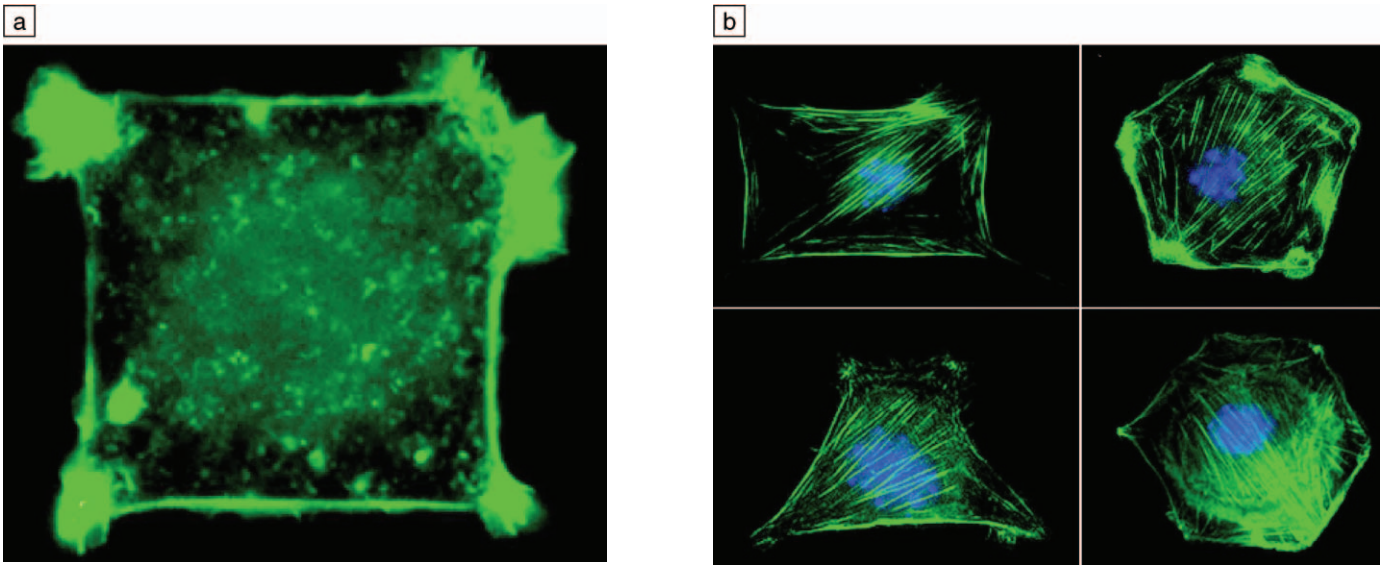

Figure 3. (a) A cell spread on a square 30 μm × 30 μm island; the cell was stimulated with platelet-derived growth factor (PDGF) and stained for F-actin with fluorophore-labeled phalloidin. (b) Cells confined to various shapes (all with areas of 900 μm^2) were stimulated with PDGF and stained with phalloidin and 4′-6-diamidino-2-phenylindole to visualize F-actin (green) and nuclei (blue). Images obtained by fluorescence microscopy.

demonstrated in biomedical research represent the majority of work that must be done to enable hypotheses in cell biology to be tested in reliable experimental systems? Hardly. We know only a few of the environmental signals that influence cells and are unable to investigate most of the ones that we have identified. For example, cell shape, cell structure, cell–substrate adhesion, and cell mechanics are believed to be linked. Cells attached to soft substrates form fewer adhesions than do cells on stiff substrates.[32] Cells also generate contractile tension; this tension generates mechanical stresses at the cell–substrate boundary, and these stresses in turn seem to influence the strength of adhesions. Because adhesions and mechanical stress are linked, it has been difficult to examine the influence of adhesion, cell shape, and cellular mechanics independently: the tools necessary to separate them have not been available.

Approaches to these sorts of complex problems—problems that span a hierarchy of dimensions, from the molecular to the cellular—are now beginning to emerge. For example, the ability of soft lithography to form beds of soft, elastomeric microneedles by molding has made it possible simultaneously and independently to measure cellular stresses and to provide a well-defined adhesive substrate (Figure 4).[31] We have used this combination to begin exploring the relationships between cell shape, contractile stress, and cell–substrate adhesion.[31] This prototype study demonstrates the potential for microfabricated structures to provide informative new approaches to the study of the mechanics of cells.

An Emerging Picture of Cellular Mechanics

Together, these and other studies of the influence of cell–substrate adhesion, cell shape, and cell mechanics, and the influence of soluble factors on cells, combine to suggest several hypotheses.

First, all of these environmental signals seem to be linked: that is, these cues are nonorthogonal.[2] This observation raises the question of how to design and execute a well-defined study of cellular responses. For example, at the simplest level, if the shape of a cell affects its response to a growth factor, then a complete understanding of the influence of the growth factor on cellular behavior must include integrated studies of the shape of the cell: practically, the influence of the growth factor must be determined by using it to stimulate cells having many, well-defined shapes. We do not currently know what a complete set of cellular shapes would be. We are beginning to understand how to control the shape of cells attached to planar (two-dimensional) substrates, but have only a few tools to generate defined three-dimensional scaffolds for cell growth; we have only weak control over the nature of contacts between cells in cell culture and especially in cell cultures that define cellular shapes.

Second, cells interact dynamically with their environment: they both respond to and influence that environment. Most tools for micron-scale investigation of cells give only limited information about dynamics, although this situation is now beginning to change. Mrksich[33,34] (see article in this issue) and we[35] have introduced techniques that enable cells to be patterned in shape and location using printed self-assembled monolayers (SAMs) and then released from the constraints imposed by this patterning using a short (and apparently non-damaging) pulse of electrical current (Figure 5). This procedure offers the examination of a range of cellular behaviors that are reflected in motility. It provides, however, only the crudest glimpse into the time domain. Providing better—and ideally, interactive—control of the environment of cells in culture will be useful in extending the range of biologically relevant processes that can be studied using cultured cells.

Third, using cells in well-defined environments for experiments increases the signal and reduces the noise and variability in the experimental system. Processes that were difficult to study can become tractable with the use of microengineered substrates. For example, cell-to-cell communication between neighbors has long been suspected to be an important regulator of cell behavior. Cell-to-cell contact, and presumably communication, increases when cells are seeded at increasing surface density. However, because cells are randomly distributed on the surface, any one cell may contact none, one, or even six

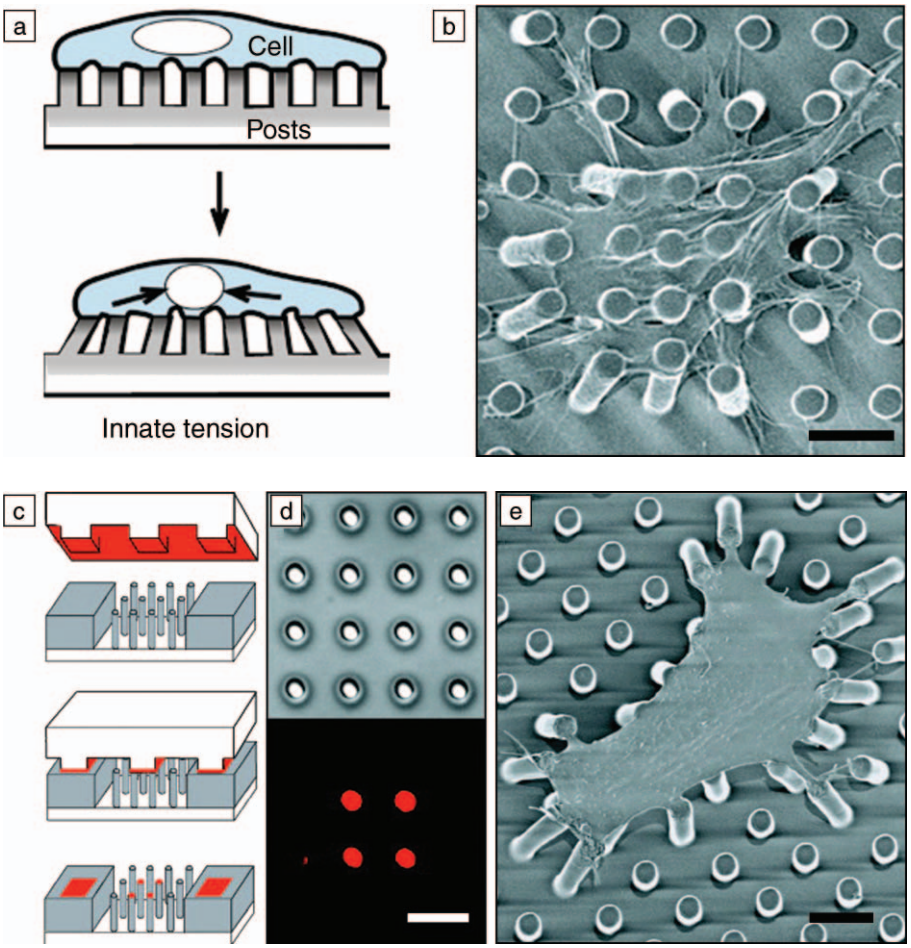

Figure 4. Elastomeric silicone microposts used to modulate the deformability of a substrate to cellular contractions. (a) Schematically, cells adhere to the tips of an array of closely spaced, vertically oriented elastomeric posts. When cells contact and probe the surface, the microposts deflect, depending on their mechanical stiffness, which can be easily manipulated by altering their dimensions. (b) Scanning electron micrograph showing a cell attached to the entire micropost substrate, including the shafts of the microposts and the underlying base. (c) Schematic illustration showing the process for microprinting adhesive proteins on the microposts. (d) Differential interference contrast (top) and immunofluorescence (bottom) micrographs of the same region of posts where a 2 × 2 array of posts has been printed with fibronectin. When adhesive proteins are selectively microprinted on the tips of the microposts (e), cells only attach on the tips. Scale bars indicate 10 μm.

neighbors. As a result, the effects of contact and number of contacts are often difficult to characterize. We have developed methods that restrict cells to forming pairs (Figure 6). This approach has allowed us to show that cell proliferation increases with a small number of contacts and then subsequently decreases.[36] Without these well-defined systems, this biphasic response was not previously seen. Exposing statistically significant numbers (hundreds to thousands) of cells to indistinguishable conditions is necessary to distinguish variability to response caused by variations in the environment from intrinsic variability in the cells themselves.

The Single-Cell Advantage (or Disadvantage)

Perhaps one of the most useful aspects of soft lithographic technologies that enable patterning of the shape and location of individual cells is the ability to study the behaviors of individual cells in parallel experiments; these sorts of experiments have the potential to bring a new level of precision and reproducibility to cell-based experiments. Conventional cellular assays use large numbers of cells (thousands to tens of millions) that are pooled into a single assay. These sorts of assays accept the fact that individual cells may be entirely distinct in their response to a stimulus and assume that a response averaged over a population of cells provides the most relevant measure of the stimulus–response characterization of cells in realistic biological environments. These experiments also assume that any variations in the local environment (e.g., the local density of cells on a surface) will average in the population response. In addition, they ignore the obvious differences among cells—their position in the cell cycle, their lineage, their history of epigenetic modification, and all the other characteristics that make individual cells individual.

Every field has its own justifications for using averaged behaviors, but whatever their virtues, it is almost always useful to be able to also look at individual behaviors. (Even in fields such as neuroscience and tissue engineering, where collective behaviors are the real focus of research, characterizing the behaviors of individual cells is important.) Microengineered surfaces and microfluidic channels now make it possible to provide indistinguishable—thus effectively identical—environments for multiple, individual cells and open the door to experiments that will deconvolve the variability in biological responses of cell populations from those that reflect differences between individual cells in the cell population.

In those instances in which statistically significant numbers of individual cells have been studied in parallel experiments, what have we learned about the robustness of cellular responses? The answer is, only enough to begin to phrase intelligent questions. It has been said that cells sense an analog world, but make binary decisions. That is, cells cannot and do not partially replicate, divide, differentiate, or die: they either do or they do not. They assess their environment and, based on that assessment, commit irreversibly to a process. (If responses to major choices were not binary, the opportunities for cellular dysfunction that could result, say, from having some components preparing for mitosis while others prepared for apoptosis would be very large).

Thus, when individual cells are allowed to spread to cover different "footprints," the cells that are most spread have the greatest tendency to divide.[28,37] Loosely interpreted, single cells must occupy an area above some threshold value before they can switch into the proliferative response. Experimental observations of populations of individual cells, uniformly patterned into indistinguishable shapes, show that different cells in these populations respond at different thresholds. That is, a sharp transition does not exist where, below a certain size of footprint, all cells do not divide, and above that size, all do; rather, within the population, an ever larger fraction of cells will

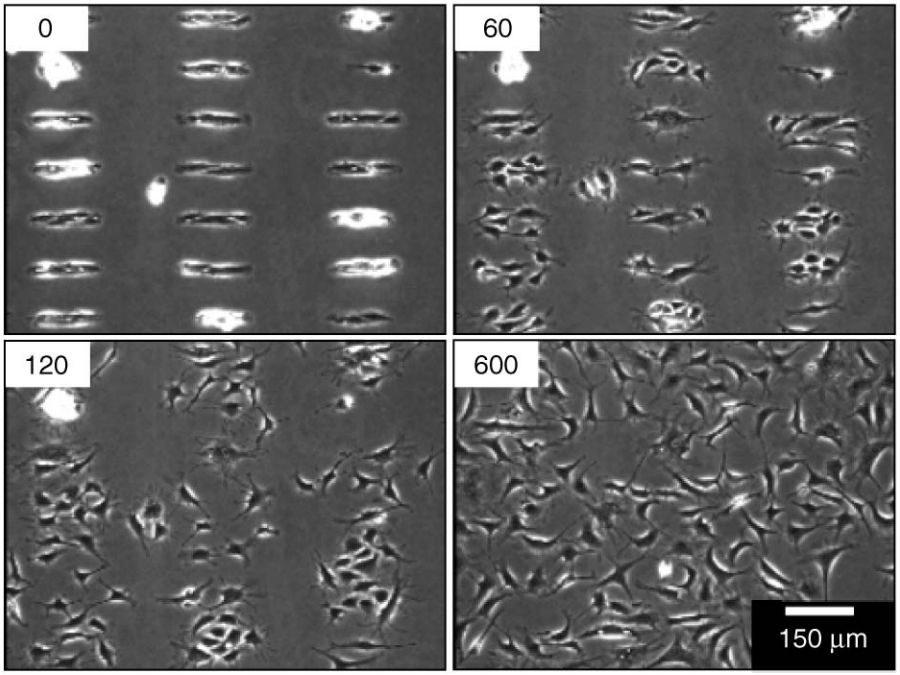

Figure 5. Bovine capillary endothelial cells were initially confined to rectangular patterns using methyl-terminated self-assembled monolayers (SAMs) surrounded by ethylene-glycol–terminated (EG-terminated) SAMs. Application of a cathodic voltage pulse (–1.2 V for 30 s) released the cells from the constraints of the microislands by desorbing the EG-terminated SAMs and enabling proteins to adsorb from the culture medium that allowed the attachment and movement of cells. The numbers indicate the time elapsed (in minutes) after the voltage pulse.

divide in response to incremental increases in footprint size. We do not understand the mechanistic basis of these observations. Do they reflect the fact that individual cells have different, preprogrammed thresholds required to trigger proliferation, or is there a stochastic aspect to the response of individual cells even within collections of cells that are otherwise identical (if "identical" cells actually exist)? If one were to examine the daughter cells of a cell that proliferated following an increase in its footprint, would the daughters predictably respond at the same threshold in footprint? In the case of a non-dividing response (e.g., the production of lamellipodia at the corners of polygons), only a certain fraction of superficially indistinguishable (in shape and lineage) cells again exhibit the behavior.[29] Are there responders and non-responders for every stimulus—a variability built into the population of cells—or do cells respond idiosyncratically at some times but not at others? Why?

These studies with ensembles of (apparently) indistinguishable isolated cells raise fundamental questions about the design of biological experiments. If individual cells are inherently different from one another—and different in ways that we cannot at present characterize—then what fraction of the ensemble must respond to constitute a statistically significant response? How many cells must be included in a study to produce a response that can be reproduced? How should statistics be treated in these experiments? What population of cells—prepared and characterized how—constitutes one cell type? These questions are uncomfortable ones for cell biologists, for whom thoughtful and rigorous statistical analysis is not a natural act.

To begin to explore these fundamental questions of experimental design—characterization of the starting sample of cells, understanding the significance of the experimental result—one experimental design (and unfortunately not one that is often practical) is to use the cell as its own control: that is, to repeatedly and non-invasively stimulate a single cell and characterize the range of its responses. This approach eliminates cell-to-cell variability by focusing on a single cell, but it must still deal with the ambiguities resulting from the history of that cell: its experience on multiple stimulations, its age, and its response to the rigors of being the object of investigation. Further, this approach is not applicable to the major events of the life and death of a cell: mitosis and apoptosis. Still, the availability of "well-defined environments"—defined shapes, on defined

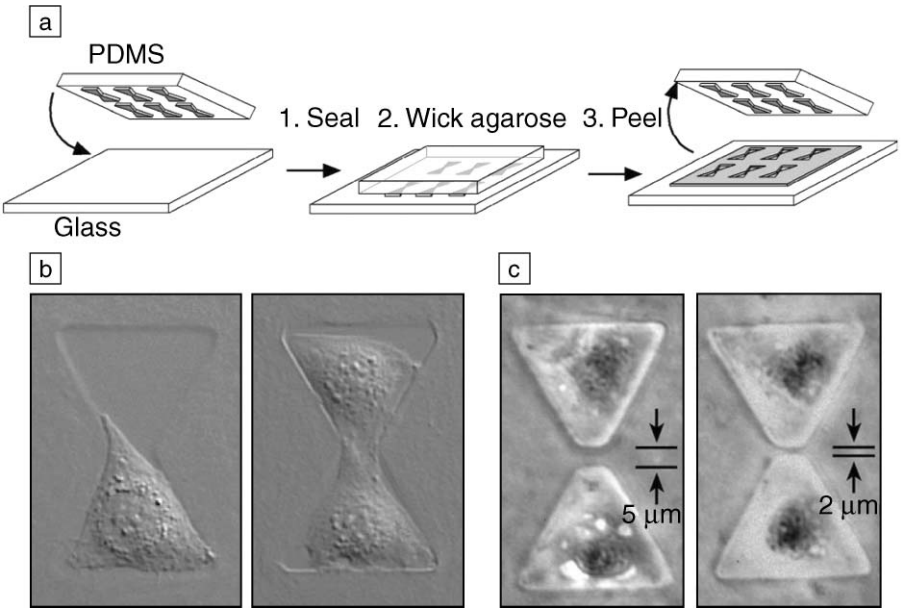

Figure 6. Method to induce cells to culture in pairs with control over the contact between them. (a) Agarose is wicked into channels formed by a poly(dimethylsiloxane) stamp sealed against a glass coverslip and allowed to gel before the stamp is peeled off. (b) When cells are seeded onto these substrates containing bowtie-shaped wells, cells attach and culture as either single cells or pairs. (c) The single cell-to-cell contact formed in these pairs can be blocked by fabricating substrates in which the agarose forms a thin wall, cutting the bowtie-shaped wells into separate, although closely spaced, wells.

substrates, in contact with defined culture media—constitutes the first step toward such experiments.

Conclusions

The most elementary complete unit of biology is the cell: it is the simplest unit that is "alive." To understand the cell is to understand what it is to be alive. Among the many difficulties in the task of understanding the cell is the variability of cells. Even when a population of cells is monoclonal and shares an identical (or nearly identical) genome, individual cells are different. The potential contributions to these differences are beginning to be understood: the position in the cell cycle, a wide range of epigenetic phenomena (from gene silencing, through splice variation, to post-translational modification of functional proteins), and passage number (clearly marked in the structure of telomers, but certainly recorded in many less easily read parts of the cell) are a few. In general, these differences may reflect the fundamental truth that the history of experience for each cell, and the consequent changes that result from that history, are simply different. The individual and collective importance of these differences for the conduct of research in cell biology is not understood.

Cell biology has been a qualitative (or, at best, semi-quantitative), observational science. It has—understandably, in light of the complexity of the cell—lagged far behind genomics in moving toward a more quantitative basis, one in which defining the reproducibility and statistical significance of experiments and their interpretations becomes a routine part of interpreting the experiment. In bioanalysis, an area where the potential for applications that are immediately useful in the development of new pharmaceuticals has stimulated substantial effort to quantitate cell-based assays, the reputation for reproducibility of these assays is poor. There is an important opportunity to build the foundation for a "better-interpretable cell biology" by understanding the basis for the variability in cellular responses to stimuli (including the simple passage of time) and in improving the reproducibility of experiments involving cells—both individually and in ensembles—and the ability to design experiments that yield meaningful results.

One of the simplest parts of this problem is that of controlling the static physical environment of the cell: its location, its shape, the mechanical and chemical properties of the interface to which it is attached, and the composition of the medium in which it is immersed. The development of the tools to define these environmental factors is advancing rapidly: soft lithography, represented in microcontact printing (to control the surface) and micromolding (to control the topography of the surface and to fabricate microfluidic systems), and materials science to define the mechanical properties of the surface are important parts of this development. The toolset is not yet complete; it still is not possible to define the four important elements of the environment—the composition/properties of the support, the nature of the interface between the substrate and the cell, the shape and location of the cell, and the medium surrounding the cell—arbitrarily and independently, but the degree of control over these systems has increased enormously in the last decade.

Controlling the dynamic aspects of the environment of the cell is just beginning to be a focus of research. It is now possible to pattern the composition of the fluid medium surrounding the cell and to change that composition (within some range) with time constants of ~1 s; it is possible to illuminate or irradiate the cell; it is also possible to release the constraints that determine the shape of a patterned cell. These tools are making it possible to conduct experiments on ensembles of individual cells, in statistically significant numbers (hundreds to thousands), and in indistinguishable environments. These experiments will be the first step toward understanding the variation in response to a stimulus within a population of cells.

The next stages in the development of this technology have not really started. We know that cells attached to a culture plate are very different from cells in tissue; how can one mimic the complex, time-dependent, multidimensional (in both topography and in chemistry) world experienced by a cell in tissue? Moving in this direction will require integration of the mesoscopic, "cell-sized" tools developed by the bioengineer with the molecular approaches of the molecular biologist and geneticist.

Based on early experience, it appears that cells do not treat soluble ligands, surface-bound ligands, and substrate mechanics as orthogonal inputs to their function. Because the cellular control systems are interconnected, all factors in the environment must be simultaneously well defined. Biology needs, *inter alia*, (1) temporal control of cell–material interactions, (2) spatial control of chemistry and mechanics at the nanometer scale, (3) spatiotemporal control in three-dimensional settings, (4) presentation of complex (heterogeneous) surfaces, (5) understanding what properties of natural materials should be mimicked, and (6) ways of mimicking cell–cell and cell–tissue interactions.

Microengineered systems now enable the study of single cells in well-defined environments. An early lesson from these studies is that even in the best-defined environments now available, the response of cells to stimuli can be highly variable, and the reason for that variability is largely undefined. In particular, we do not understand how the relative contributions of cell-to-cell, cell-to-environment, and environment-to-environment variability contribute to this effect. Nor do we know whether cell-to-cell variability is inherent to the cells and deterministic, or idiosyncratic because of uncharacterized and idiosyncratic variability in response. Until we understand the fundamental basis for population variance, developing reliable assays based on cells will use population-averaged responses, and we will just have to live with the ambiguities that come with the difficulties of these systems.

Approaches to cell biology based on microengineering constitute necessary steps in understanding the processes used by individual cells as well as those used by ensembles of cells to form tissues that function in ways that individual cells do not. The materials science community will continue to play a critical role in generating the tools necessary to fuel this work in biomedical research.

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