Combining microscience and neurobiology

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There is a wide range of literature on soft lithography, organic surface science (especially self-assembled monolayers of organic thiols adsorbed on gold) and microfluidics. These areas have developed in the fields of physical and surface chemistry, materials science and condensed matter physics, but they offer broad new capabilities in the development of relevant micro- and nanosystems to users in biology in general, and in cell biology in particular. The ability to integrate these techniques for fabricating materials and for controlling the chemistry of surfaces with electrical and electrochemical measurements should be especially relevant in neurobiology. The major impediment to the development of a field of 'microfabrication and measurement' in neuroscience is the absence of effective collaborative interactions between the communities of fabricators and neurobiologists.

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Introduction

The interface between the living and the non-living is an important one for cell biology. Much of what we know about mammalian cells comes from studying them in culture, so the techniques of anchorage dependent cell culture are highly developed, and used in virtually all laboratories involved in this subject. Cell culture is convenient, but the cells are unquestionably in a different environment — to an extent that is currently difficult to define — from the one that they experience *in vivo*. There is, at present, no substitute for cell culture for detailed, reductionist studies in cell biology. It is, therefore, particularly important to understand, define and control the interface(s) between the cell and the solid material that supports this cell. This interface is not a bystander in the biology: it is a vital part of the system, and one that has a strong influence on the behavior of the cells. It is crucial to controlling the attachment, spreading, differentiation and death of cells, and it provides an important window that can be used for observing cells [1–4].

The relationship between 'microstructures' and 'cell biology' is a surprisingly natural one. The mammalian cell is an entity with typical dimensions of approximately tens of microns (neurons, with their elongated axons and dendrites, are exceptional in their extension). To study and manipulate cells, one needs tools for observing, patterning, assaying and stimulating that have a range of dimensions [5]: it would be useful to have structures both larger (e.g., microchannels that surround the cell and control the flow of medium over it) and smaller (such as subcellular probes) than cells. The ability to constrain cells growing in culture into ordered patterns proves especially useful in a variety of studies: for example, examination of the cytoskeleton [6,7] and cell motility [8–11] have been extensively studied using patterned cells. The ability to assemble regular networks of neurons should be particularly useful in neurobiology, but is just beginning to develop as an area of technology [12–14,15•,16–18]. This paper provides an overview and guide to the relevant literature of soft lithography, organic surface science (with an emphasis on self-assembled monolayers of organic thiols adsorbed on gold), and microfluidics.

The soft lithography tool box

Soft lithography is a collective name given to a set of techniques that make microstructures by printing, molding and embossing.

History

Soft lithography developed as an alternative to photolithography and electron-beam lithography (see glossary), as a method of making networks of electrical connections for use in microelectronics [19,20]. The development of photolithography and related techniques has continued beyond what seemed, ten years ago, to be its intrinsic physical limits. It is now possible to foresee commercial electronic devices — operating with familiar principles of physics — with wires that are less than 20 nm in width. These techniques (photolithography and electron-beam lithography) have become almost unbelievably sophisticated; they are also very specialized, expensive, difficult to use and are unfamiliar and inaccessible to biologists.

It seems unlikely that soft lithography can compete with these other methods on the basis of feature size alone

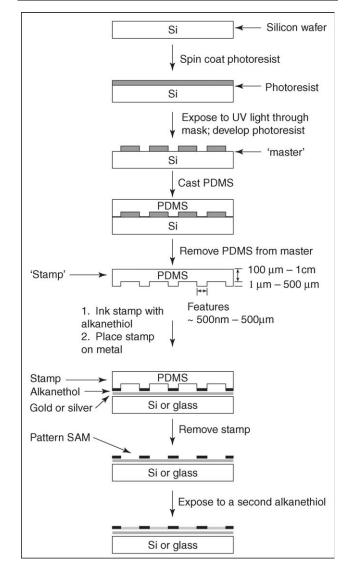CONEUR 312

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(although it is possible to perform astonishing feats of replication using soft lithography: for example, replica molding can reproduce features that are less than 1 nm in size [21]). Soft lithography continues, however, to retain characteristics that are very important for applications in cell biology: these include simplicity, low cost, rapid prototyping, compatibility with cells and ease of use.

The stamp (or mold, or microchannel)

The central object of soft lithography is a slab or membrane of poly(dimethylsiloxane) (PDMS), or occasionally some other polymer with similar properties. This block has, embossed on its surface, bas-relief features that typically have lateral dimensions of 1–1000 microns, and a height of a few hundred nanometers to tens or hundreds of microns. It is used in a wide variety of ways: as a stamp, to print patterns of molecules on substrates [22–24], or to print patterns of cells [6,25]; as a mold, to generate three-dimensional topography in a substrate [19]; or as one part of a larger structure that becomes a network of microchannels [26,27].

The processes that are used to make the stamp have been described in detail (Figure 1) [19,20,27]. Typically, they start with a bas-relief master (see glossary) prepared by photolithography using straightforward techniques. The patterns are drawn using a computer aided design (CAD)

Figure 1

A schematic representation of the procedure used to fabricate a PDMS stamp from a master that has relief structures in photoresist on its surface, and the process of microcontact printing using the PDMS stamp. The stamp is inked with an alkanethiol and brought into contact with a gold surface; the pattern on the stamp is transferred to the gold surface by the formation of a SAM. The bare areas of gold are exposed to a different alkanethiol to produce a surface patterned with a SAM that presents different chemical functionalities in the different regions. This figure was adapted from Kane et al. [33].

tool, and printed on a sheet of transparency that functions as the mask (see glossary); this pattern is transferred into a photoresist (see glossary) using photolithography; when the resist is developed, it gives the appropriate bas-relief structure. Liquid PDMS prepolymer (a commercial and inexpensive material) is poured over the bas-relief structure, cured and peeled away. This patterned polymer structure can be used directly as a stamp for contact printing molecules or cells [6,20], or as a mold for replica molding the pattern into another material (including,

polymer, gel or metal); when used to make a microfluidic system (see glossary), it is usually lightly oxidized on the surface by exposure to an oxidizing plasma before it is sealed against a flat surface. The entire process requires less than a day to go from the design on a computer to the microfabricated mold or microfluidic device [26,28]. The fabrication of copies of the elastomeric molds, using an existing template, requires only an hour or two. ('Foundaries' that provide stamps and other tools of soft lithography as a service to users who want to use the techniques, but do not want to learn how to make the stamps, are being set up at several universities, notably Harvard and the California Institute of Technology).
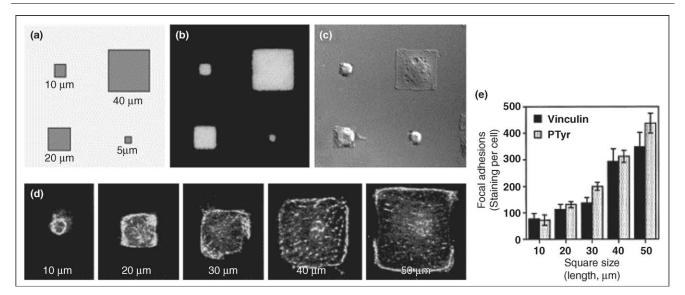
PDMS — the most commonly used material in soft lithography — has several very attractive characteristics from the vantage of cell biology: it is soft, transparent, permeable to gases, impermeable to water, biocompatible and has a low electrical conductivity. The characteristics of PDMS have been summarized in several reviews [29–31].

Self-assembled monolayers

Self-assembled monolayers (SAMs) are structures that have transformed surface science from a field focused on single crystals of metals to one concerned primarily with organic surfaces. The catalyst for this transformation was SAMs, which made it possible to prepare, routinely and easily, organic surfaces — SAMs of alkanethiols (see

glossary) supported on thin gold films — in which the composition of the surface was precisely specified, and in which there was a high degree of order in the structure. SAMs are based on spontaneous chemisorption and self-organization of functionalized, long-chain organic thiols onto the surfaces of appropriate substrates. The particular advantage of SAMs from the vantage of cell biology is that the extensive body of methodologies that has been developed for their preparation makes it straightforward to attach ligands relevant to biology on surfaces, the other properties of which — especially their tendency to adsorb proteins non-specifically — are well-controlled. The field of SAMs has been reviewed extensively [20,24], as has that of SAMs specifically relevant to cell biology [6,32,33]. There are four major components to this field. First, patterning; PDMS stamps wetted with an alkanethiol are very effective at patterning gold and palladium films; this technique is referred to as 'microcontact printing' and is discussed in more detail below. Several methods are available that can be used to present ligands that promote cell attachment on the surface of SAMs that are otherwise inert [25,34,35]. These systems give very precise control over the nature of interactions between cells and surfaces (Figure 2). Second, inert surfaces; there are now several functional groups which, when presented at the surface of a SAM, effectively prevent the adsorption of proteins from the growth medium onto the surface [25,36]. Thus, non-specific adsorption of proteins does not occur. Because cells attach to surfaces by specific interactions

Figure 2

The control of the shape of cells using microcontact printing to pattern adhesive islands of fibronectin and the distribution of focal adhesions in shaped cells. **(a)** A diagram of square, adhesive islands with sides ranging from 10 to 50 mm in length; the islands were micropatterned onto gold substrates using microcontact printing. **(b)** An immunofluorescence micrograph showing that the distribution of adsorbed fibronectin is limited to the square islands. **(c)** A differential interference contrast micrograph of bovine capillary endothelial (BCE) cells cultured on square islands of different sizes coated with fibronectin. **(d)** Fluorescent confocal micrographs of individual cells labeled for vinculin that were cultured on square islands of different sizes (the lengths of the sides are indicated). **(e)** Quantification of total vinculin and total phosphotyrosine labeling per cell, for cells cultured on square islands of different sizes. More than 30 cells per condition were averaged; error bars indicate the standard error of the mean. This figure was reproduced from Figure 1 by Chen et al. [7].

with ligands (either on adsorbed proteins or those presented specifically), these so-called 'inert' surfaces — surfaces that do not adsorb proteins — make it possible to prepare substrates to which cells do not attach with submicron resolution. (In the best systems — SAMs terminated in oligo(ethylene glycol) groups, adsorbed on surfaces of palladium — attachment and spreading of representative cell types is prevented for approximately a month [37].) Third, analysis; the thin gold films used as supports for SAMs are exactly those required for surface analysis by surface plasmon resonance (SPR) spectroscopy, and by (less useful, in this context) ellipsometry (see glossary) [38,39]. These surfaces are also compatible with analyses carried out by infrared spectroscopy and mass spectrometry [40]. Fourth, biocompatibility; SAMs on films of gold and palladium seem to be non-toxic to cells (silver, by comparison, is cytotoxic) [37].

Molecular control of interfaces
The general principles of soft lithography
Soft lithography forms patterns and structures with dimensions usually between 1–1000 microns using 'soft' materials (organic polymers, SAMs, gels, or other structures based on organic matter.) The word 'soft' in this context is a little misleading. It is a term taken from physics, and has come to mean most types of organic matter, not just matter that would meet the intuitive criteria for soft (elastomers, gels). Soft lithography refers both to the pattern-forming tool (e.g. a PDMS stamp) and to the material being patterned (a SAM, an organic polymer). There is sometimes confusion between related techniques for forming patterned polymers, depending on whether they use stamps that are soft or hard (the latter, for example, fabricated in metal or silicon). These distinctions are not relevant to our discussions; both types of stamps have their uses.

Microcontact printing
Microcontact printing provides a very convenient method of patterning surfaces into regions having different molecular surface chemistries, and thus, different interactions with cells [20]. The techniques used for this type of patterning are readily accessible to a cell- or neurobiology- laboratory, and many of the relevant organic thiols used for this technique are now commercially available.

Microfabrication of fluidic Microsystems
Microfluidic systems are formed when embossed stamps are sealed to flat surfaces. These systems are readily prepared, and are very useful in the study of cells; they make it possible to study the behavior of individual cells in defined microenvironments. The two components of these systems — the embossed stamp and flat surface — can be joined reversibly (so that the components can be separated, and the interiors of the channels are accessible for other types of analyses), or permanently (through covalent bonds).

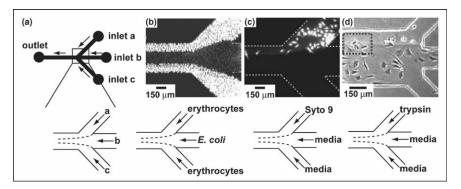## Other types of soft lithography potentially relevant to cell biology
There is a wide range of variants of soft lithography, many of which are relevant to biology. First, printing proteins and cells; the stamps used in microcontact printing are typically fabricated in PDMS, but a wide range of other soft materials can also be used as stamps. Gels are particularly interesting in these applications, and surface-patterned agar stamps are effective in patterning proteins and both bacterial and mammalian cells [41–43]. The latter application is interesting, in the sense that the 'ink' of the stamp is self-renewing. If the agar contains nutrients, cells will grow on its surface, and after an initial inoculation, in principle, it should be possible to use these stamps to print patterns for long periods of time without subsequent re-inking. Second, electrochemistry on surfaces; the SAMs that are so useful in providing biospecificity to the interactions at surfaces can, in some cases, be released from a gold surface by applying a brief, reducing electrochemical potential [44,45]. We illustrate uses of this technique below; here we only point out that this approach makes it possible to modify the properties of surfaces used in experiments in cell biology in real time, during the experiment. Third, irreversibly sealed membranes as a method of patterning cells; a straightforward method of patterning cells is to bring a patterned membrane into contact with an appropriate surface, grow cells in the holes in the membrane, and then release the cells from their artificial constraints by peeling off the membrane [46]. This type of experiment is especially useful in the study of cell motility: when the membrane is removed the cells are released, and they migrate across the surface. Fourth, mass spectrometry (MS) on molecules adsorbed at surfaces; MS has become one of the most information-rich methodologies in biochemistry and molecular biology, and is revolutionizing the study of proteins. SAMs on gold are good substrates for matrix-assisted laser desorption ionization MS (MALDI) [40].

Laminar flow
An important characteristic of applications of microfabricated structures for the transport of fluids (channels with widths and heights in the order of tens to hundreds of microns) in cell biology is laminar flow [27,47]. In small channels, the flow of liquids with low viscosity (on the order of the viscosity of water) is laminar: that is, two streams of fluid introduced into a channel flow parallel to one another, without turbulence; the fluids only mix by diffusion at their point of contact (the interface). This ability to generate parallel flows of liquids makes it possible to pattern the deposition of cells in a channel [48] (Figure 3), to expose different populations of — or individual — cells in a channel to reagents, drugs, dyes, or temperature gradients [49•,50], and even to 'paint' different parts of the same cell with different molecules [50,51]. This technique also makes it

Figure 3

A schematic representation of an experiment in which laminar flows were used to pattern cells. **(a)** Top view of the microfluidic network. A PDMS slab containing micron-size channels embossed on its surface was pressed against the flat surface of a Petri dish to form a network of channels. Micrographs were obtained for the area of the capillary system at which the inlet channels converged into a single channel. **(b)** Two different cell types were patterned next to each other. Chick erythrocytes and *Escherichia coli* were deposited selectively in their designated lanes by patterning flows of each suspension of cells. Adherent cells were visualized with a fluorescent nucleic acid stain (Syto 9). **(c)** A pattern of selectively stained BCE cells. A suspension of BCE cells was introduced into the capillary network (pretreated with fibronectin) and allowed to attach and spread on the surface. The non-adherent cells were removed by washing with media, and then Syto 9 and media were introduced from the designated inlets. **(d)** Patterned detachment of BCE cells by treatment with trypsin and EDTA. Cells were allowed to adhere and spread in a fibronectin-treated microfluidic network; non-adherent cells were removed by washing. Trypsin, EDTA and media were flowed into the channel from the inlets indicated in the figure. Images b and c are fluorescence micrographs imaged from above (through the PDMS). Image d is a phase contrast micrograph acquired using an inverted microscope to image through a polystyrene Petri dish. White dotted lines indicate channels that were not visible with fluorescence microscopy. This figure was reproduced from Figure 10 by Kane *et al*. [33].

possible to pattern gradients of small molecules or proteins on surfaces, to produce gradients in solution, and to study the behavior of cells in these environments [52,53,54••].

Observation and readout

The most general methods of assaying the behaviors of cells in cell culture are those that use optical microscopy. Optical methods are directly compatible with cells grown on patterned substrates. PDMS is transparent to visible and UV wavelengths of light, and makes it possible to observe cells through slabs of PDMS [6]. Techniques other than optical have not, yet, been extensively developed for microfabricated systems, although the potential to use other methods — especially electrochemical measurements — certainly exists.

Electrical connections

The combination of soft lithography, to fabricate microstructures, and electrochemistry, to modify, stimulate and detect biochemical events, is clearly a promising one. Microcontact printing on metal (especially gold) surfaces is especially relevant, because the substrate in these experiments (the gold film) also functions as an electrode, and because microcontact printing can be used both to pattern SAMs on the surface and (in combination with procedures based on selective etching) to fabricate electrically distinct circuits and electrodes. The resolution of microcontact printing makes it possible to fabricate tens of distinct electrodes (or more) reliably in the area occupied by a single cell spread on a surface. Because elec-

trical measurements are centrally important in neurobiology, this combination is one with great promise. Its development is still at the stage of demonstrating proofs of principle; the important biological applications remain to be explored.

Electrochemistry in microfabricated structures relevant to cell biology has found two types of applications: in modification of the structure and composition of surfaces that are in contact with cells and in the measurement of cellular, electrochemical potentials.

Electrochemistry to control surface chemistry

There are two broadly useful applications of electrochemistry in modifying synthetic surfaces fabricated using soft lithography. The first takes advantage of the fact that application of a cathodic (reducing) pulse of current to an electrode supporting an alkanethiolate SAM releases the organic group as an alkanethiol, and generates a bare gold surface. If other molecules that are present in the solution adsorb on this surface, the electrochemical step has the effect of changing the interface from one presenting a SAM that, for example, resists the adsorption of proteins and cells, to one that enables cells to attach or spread [44,45]. A second class of experiments takes advantage of functional groups on the surface of the SAM that can be interconverted between reactive and unreactive forms by electrochemical redox reactions to trigger chemical reactions at surfaces [55,56]. These methods have been used to attach and release ligands that interact biospecifically with cells.

Electrical measurements of cellular potentials and electrochemical stimulation of cells

There have been several experiments demonstrating the co-fabrication of electrodes and microchannels using soft lithography [57,58]. The applications of these systems has largely been restricted to demonstrations of conventional electrochemistry, but the same principles can, in principle, be used to stimulate cells electrochemically, or to detect electrical responses of cells.

Experimental reproducibility and statistics

Cells are not well-defined objects. One cell can differ from another, even within a common population, by virtue of its position in the cell cycle, its passage number, its history of environmental stimuli, and many other factors [6,32]. These differences have been one factor that has made cell biology a technically difficult area, and one in which the standards for reproducibility of experimental results has always been more difficult than in other areas of science that involve non-living systems.

A particularly interesting capability that soft-lithographic procedures bring to cell biology is the ability to carry out experiments on indistinguishable systems (e.g. indistinguishable cells) in parallel; this capability makes it possible to average measurements over large numbers of individual cells or populations. For example, we have patterned a large number of bovine capillary endothelial cells into 'teardrop' shapes, released them electrochemically, and observed whether the direction of their motion correlated with the orientation of their shape [59••]. By performing measurements on a statistically significant number ($\sim$100) of individual ('indistinguishable') cells, we found that the shape of a cell does determine the direction of its motion — a result that would be virtually impossible to obtain through a smaller number of observations. We believe that these kinds of experiments have the potential to begin to generate data in cell biology that can be associated with reproducibility and variance in the way that data in the harder sciences (chemistry and physics) has always (ideally) been treated.

Nanofabrication using soft lithography

Many of the applications of soft lithography in biology have been in the fabrication of structures significantly larger than the cell. These structures — especially surfaces patterned into regions that are adhesive and non-adhesive to cells, and systems of microchannels that confine the growth of cells — usually have dimensions of tens to hundreds of microns. Other types of applications — especially those involving features significantly smaller than cells that cannot be easily replicated using soft lithography — will require new methods of fabrication.

This area is one in which development is now very active. 'Unconventional nanofabrication' — nanofabrication that does not involve e-beam or UV photolithography — produces a range of structures that are potentially useful in cell biology [60–62]. Some of these structures include nanorods (see glossary) grown electrochemically through pores in membranes or in phase-separated block copolymer films (dimensions of 5–100 nm; see glossary), thin, electrically connected wires embedded in polymers (smallest dimensions $\sim$20 nm) [63], and steps in substrates; these materials will be useful in studying the response of motile cells to changes in topography (down to 1 nm) [21]. To find application in biology, nanostructures must satisfy a different set of criteria than those required for micro- and nanoelectronics. The application of nanostructures in micro and nanoelectronics is concerned with the myriad details of a very demanding and highly developed technology requiring the generation of circuits of high complexity, with essentially no defects. The introduction of nanostructures into research in neurobiology will require (at least at the outset) development of techniques that are convenient and flexible chemically (especially with respect to surface chemistry).

Conclusions and future directions

Micro and nanofabrication and cell biology are just beginning to overlap. Soft lithography and related techniques are providing one bridge between these areas. These techniques make it possible to fabricate biologically relevant structures — with dimensions from a few nanometers to millimeters — easily (at least relative to conventional microfabrication), and with exquisite control over the composition and properties of the interfaces. These systems already offer useful control over electrical connectivity, and their integration with optical methods of detection is well advanced. The electrical methods have not been applied seriously to problems in neurobiology for two reasons: first, many of the relevant soft-lithographic techniques have been developed only in the past few years, and have not yet diffused across the border between 'fabrication' and 'biology'; second, the communities that are interested in these techniques have not yet formed effective, productive collaborations. The fabricators do not know what structures to make for the neurobiologists, and the neurobiologists do not know what structures (and what associated electrical and optical systems) might be available if asked for.

Neurobiology is a field that is developing very rapidly. The development of fields, however, is often limited by the availability of new tools. Genomics has grown rapidly because it has been able to take advantage of the remarkable toolkit of molecular biology to sequence and synthesize nucleic acids, and mass spectrometry to analyze and detect proteins; cell biology has relied on advanced optical microscopy, combined with structure-selective dyes, for much of its advance. There is an opportunity for neurobiology to develop, based on methods in organic surface chemistry, in microfabrication (especially using

soft lithography), in microfluidics, and in electrochemistry, a new set of tools for patterning neurons and supporting cells, the development of 2D 'tissues', and the electrical and optical stimulation of cells and of detection of cellular responses. These techniques will also be applicable to the fabrication of new types of electrodes and junctions, and to the design and fabrication of nanostructures that can act as 'reporters' for composition, movement, and organization within cells.

The technology is now available; what remains to be developed are effective collaborations that develop the relevant tools, demonstrate their use, and transfer them to the community of neurobiologists.

Acknowledgements

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