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“MICRO- AND NANOSCALE ENGINEERING OF CELL SIGNALING”

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Abstract

It is increasingly recognized that cell signaling, as a chemical process, must be considered at the local, micrometer scale. Micro- and nano-fabrication techniques provide access to these dimensions, with the potential to capture and manipulate the spatial complexity of intracellular signaling in experimental models. This review focuses on recent advances in adapting surface engineering for use with biomolecular systems that interface with cell signaling, particularly with respect to surfaces that interact with multiple receptor systems on individual cells. The utility of this conceptual and experimental approach is demonstrated in the context of epithelial cells and T lymphocytes, two systems for which the convergence and balance of multiple signaling pathways has dramatic impacts on the ability of these cells to provide their physiological function.

Keywords

Micropatterning; Epithelial; T lymphocyte; Immune synapse

INTRODUCTION

Cells possess the remarkable ability to recognize and respond to the microscale organization of molecular signals in their extracellular environment. As the basic structural unit of life, proper coordination of these inputs is essential for formation and subsequent function of tissues, organs, and, ultimately, entire organisms. This is seen in the ability of epithelial cells to form integrated monolayers, driven by the coordinated effects of multiple extracellular cues on a range of cellular functions. Similarly, formation of functional neural circuits relies on interpretation of complex combinations of signaling molecules by growing neurons. The stem cell niche is a more recently described example of microscale signaling, in which local

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interactions direct stem and progenitor cell senescence, proliferation, and differentiation on a cell-by-cell basis. At the subcellular level, the organization of cell-matrix interactions is known to drive an expansive array of functions, including spreading, polarization, and even cell fate. Most recently, juxtacrine signaling, in which cells communicate with each other through membrane receptor-ligand pairs formed within areas of direct intercellular contact, has emerged as a powerful example of how microscale organization can drive cell function.

A common theme in across these systems is that while the signaling pathways involved are reasonably understood at the biomolecular level, understanding of the spatial aspects of these networks remains incomplete. Unveiling the mechanisms through which cells interpret the microscale details of their environment is consequently a vital area of contemporary research. Spatial modulation of intracellular signaling is undoubtedly an important aspect of these mechanisms. The theoretical foundations for this are well established for large distance scales, in phenomena such as chemotaxis in which cells sense the chemical gradient of soluble factors produced by a cellular- or tissue-level source. Turing (1) further developed this reaction-diffusion concept through a mathematical framework that showed that interactions between two compounds (morphogens) could give rise to highly complex, often dynamic patterns of chemical concentration that are not possible through a single cue acting alone. Notably, in both scenarios the spatial extent over which a reaction can be modulated is related to the balance between diffusion of the chemical species and regulation of activity. For soluble molecules, these distances are on the order of tens of micrometers, placing stringent requirements on the spatial kinetics of cell signaling with regards to responding to microscale organization. However, emerging concepts in biophysics, such as membrane microdomains and intracellular compartmentalization, suggest mechanisms through which these molecules may exhibit dramatically reduced mobility and consequently regulation of chemical kinetics over smaller distance scales. Understanding of these concepts has advanced dramatically in recent years, driven in large part by improvements in microscopy, such as development of fluorescent protein tags, superresolution imaging, and improved time resolution. The field of spatially resolved cell signaling is thus enjoying a period of renewed advances in which analysis techniques provide the means to examine molecular processes at the spatial scales appropriate for cells. The ability to define and control the spatial organization of signaling molecules involved in these pathways is an important counterpart to this analysis. This review focuses first on contemporary advances in micro- and nano-scale fabrication techniques that have propelled this field, and then on two physiological systems that demonstrate the possibilities of this approach.

FOUNDATIONS AND TOOLS

Origins of Spatially Resolved Cell Signaling

The microscale dimensions of cells impose stringent criteria on intracellular signaling reactions. Fundamentally, the pathways that define migration, polarization, and other spatially selective processes must themselves be modulated over these same distances. The concept that spatial modulation of the concentration of a chemical species conveys information is well established in chemotaxis and morphogenesis, where dispersion of bioactive molecules from a source into a large space sets up gradients that can be recognized

by cells. However, these gradients are of multi-cellular distances, and are limited in how rapidly they can change with position; in the absence of reactive interaction with other molecules, the concentration of a freely-diffusing compound can only decrease as fast as $1/r^2$, where r is distance from the origin. Moreover, the limited volume of a cell makes dispersion alone not a useful model for spatially resolved signaling at subcellular scales. Instead, intracellular signaling typically couples activation of a signaling molecule, through conformational change or reversible, covalent modification (*e.g.*, phosphorylation or palmitoylation), with one or multiple mechanisms for deactivation; initiation of downstream process occurs during the transient period of activation, the details of which are dictated by the configuration of component involved in the signaling event. In the case of intracellular signaling in response to an extracellular ligand, activation of a cell surface receptor initiates an intermediate signaling molecule which diffuses from the site of interaction. This process serves many functions, including amplification of the receptor signal and communication of this event to other parts of the cell. The spatial extent to which this event is communicated (or, conversely, restricted) is determined by the mobility of intermediate signaling molecule, balanced against the concentration, location, and rate of action of deactivating molecules. In a key analysis, Brown and Kholodenko (2) examined the steady-state activity profile of a soluble, cytosolic signaling molecule that is activated uniformly across the surface of a spherical cell and deactivated by a second agent distributed throughout the cytoplasm. Using physiologically realistic values for the activity and mobility of these molecules, Brown and Kholodenko demonstrated that specific combinations can establish gradients that decrease over distances of tens of to a few micrometers. This mechanism thus offers spatial resolution that is subcellular, potentially describing a wide range of functions such as establishment of cell polarity. However, many cellular processes, such as lamellipodial ruffling, are defined at smaller dimensions, and require correspondingly finer resolution in chemical signaling. This can be achieved through the involvement of signaling molecules with lower mobility, for which an increasingly wide range of mechanisms have been proposed. For example, association of a protein to the cell membrane dramatically reduces mobility. Lipids and other freely diffusing molecules in these membranes exhibit diffusion coefficients on the order of $1 \mu\text{m}^2/\text{sec}$, which is 1 – 2 orders of magnitude lower than that of cytosolic proteins. In addition, association of proteins with structures, including purported lipid raft microdomains in the membrane or the underlying cytoskeletal network, provide mechanisms for further reducing the long-range diffusion coefficient by several more orders of magnitude. Strategically, it is becoming clear that membrane-associated signaling proteins are involved in many signaling pathways of interest to cellular engineering, providing a provocative and emerging area of contemporary research. Finally, clustering of receptors and intracellular signaling proteins is a common event in signaling. Such clusters were identified first in the context of the immune synapse, but later in the context of fibroblasts (3, 4). These clusters are actively transported in the cell interface, and the dynamics of these systems is a topic of contemporary research.

Further analysis of these systems requires robust experimental models that can capture the spatial configurations of signaling molecules. Traditional methods of investigating cell interaction with a ligand that is associated with the extracellular environment is to coat this molecule onto a solid support; by providing micro- and nano-scale control over the

localization of these molecules, contemporary fabrication techniques are emerging as a powerful part of these experimental systems.

Surface Bioengineering at micro- and nano-meter scales

Micro- and nano-scale fabrication is rapidly growing as a technique in cellular engineering. The basic methodologies were originally developed for integrated circuit production, which focuses on patterning metals and other materials. Various steps consequently involve processes or chemicals that while effective on microelectronics materials, are not compatible with proteins and other biomolecules. The last two decades have seen the adaptation of these techniques away from these harsh processing conditions, now providing near-arbitrary control over the layout of proteins and other biomolecules molecules on a substrate surface. A key study in this process by Kleinfeld and colleagues (5) used photolithography to create complementary regions of hydrophilic and hydrophobic molecules on a material surface, which were effective in directing the growth of cultured neurons and demonstrated the promise of microfabrication in cellular engineering. Subsequent developments, and particularly the “soft lithography” approaches including microcontact printing, brought surface patterning out of the cleanroom and into traditional cellular and molecular biology laboratories. The successes of these techniques in understanding cellular function, along with biotechnological applications such as DNA/RNA microarrays, have driven the development of biology-specific equipment, methods, and facilities. While this development continues on multiple fronts, two themes have particular promise of advancing the study of micro- and nano-scale signaling. The first is the expansion of these techniques to include a wider range of biomolecules on the patterned surface, allowing the exploration of new signaling pathways. The second is the development of methods for presenting multiple, independently defined patterns of biomolecules on a single surfaces, particularly with subcellular resolution. The following sections describe key advances in these directions, and will be followed by contemporary examples of how such capabilities can advance understanding of cell function and provide new tools for the clinical manipulation of cells.

Optics-based micropatterning—Photolithography continues to be the workhorse of microscale fabrication. In this method, a working surface is coated with a light-sensitive resist layer, which is then exposed to a desired pattern of light. The resist is then developed to selectively remove either the exposed or unexposed regions, thus selectively masking off regions of the surface from a subsequent processing step such as metal deposition or etching. These steps, as well as removal of the remaining photoresist used to mask the surface, often involve high vacuum, organic solvents, or reactive plasmas, which are not generally compatible with biomolecules. Kleinfeld *et al.* partially addressed this problem by patterning hydrophilic and hydrophobic organosilanes, which are less sensitive to environment than proteins and can survive photolithography development, but this approach relied on selective adsorption of proteins to each region to control cell attachment. Soft lithography approaches, described in the following section, have dramatically changed the way biomolecules can be patterned, but are not suited for all applications. Emerging light-sensitive materials and chemistries provide an intriguing set of tools to produce sophisticated micropatterned surfaces. In particular, Doh and Irvine (6, 7) developed an approach using a novel, aqueous-compatible photoresist which after processing presented a

patterned layer of biotin moieties, to which proteins can be captured (Fig. 1A). This approach avoids processing conditions that are incompatible with protein such as organic solvents or dehydration, and offers spatial resolution down to 1 μm . Moreover, the ability to sequentially expose different features into the resist provides a clear pathway to patterning of multiple proteins on the same surface (8) (Fig. 1B). In a complementary approach, photolithography was combined with oxygen-based reactive ion etch to pattern parylene on silicon surfaces (9, 10), which then were used to delineate arrays of supported lipid membrane patches tethered with immunoglobulin (IgE) for studying receptor mediated signaling on spatially confined membrane domains in mast cells (11-13). Instead of exposing a photoresist layer, light can be used to directly control the modification and even polymerization of hydrogels and other biomaterial scaffolds. In this direction, Hahn *et al.* (14) used photoinitiated cross-linking to pattern bioactive RGDS peptides onto photoactive poly(ethylene glycol)(PEG)-based hydrogel substrates. Another unique example is shown by Mandal *et al.* (15), in which a thermoresponsive polymer was micropatterned with photolithography. Cells were allowed to attach to areas of the polymer patterns at physiological temperature, but forced to detach from the surface when the temperature was below 32°C, the transition temperature for the polymer.

Another emerging direction is maskless photo-patterning, which has allowed control over the distribution of biomolecules in both 2- and 3-dimensional contexts at micrometer resolutions. In laser scanning lithography (LSL)(16, 17), a laser beam is focused onto substrate material at the focal plane, producing a diffraction-limited spot. This point is raster-scanned across the sample to achieve desired spatial pattern, which totally eliminates the need for physical photomasks in photolithography. The West group (17) and Sia group (18, 19) have used scanning laser on standard confocal microscope to induce crosslinking of photocurable hydrogels to achieve selective cell adhesion areas and rigidities (Fig. 1C). Alternatively, PEG (20) and polyvinyl alcohol (21) layers, which both repel cell adhesion, were ablated with laser beam to allow controlled coating of extracellular matrix protein coating on defined areas for cell shape and migration studies. These techniques provide rapid iteration of pattern geometry on a sample-by-sample basis, using chemistries that are compatible with biomolecules and cells.

Soft lithography and micro-contact printing—In the illumination-based techniques described above, each working surface must be individually processed. Mask-based lithography of features of subcellular dimension typically requires clean room facilities, and the resources to develop and maintain these procedures made microfabrication initially impractical for cellular- and molecular-based researchers. The field of soft lithography, in which elastomer casts off a topological master are used to pattern the material of interest (10, 22) was developed in large part to address this need. Perhaps the best-known example of this type of approach is microcontact printing (μCP) (23), in which the elastomer cast is coated with the material to be patterned and placed in contact with a working surface, essentially stamping the compound onto a substrate. This was originally used to pattern alkanethiols onto gold-coated surfaces (23, 24), but ultimately was adapted for patterning a wide range of molecules, including silanes, proteins, and supported lipid bilayers (25-28). In a landmark paper, Chen *et al.* (24) used μCP to control the area of contact between

individual cells and a substrate, and demonstrated that increased cell spreading correlated to a switch from cell apoptosis to growth. Following this initial demonstration, μ CP has been used to demonstrate that a wide range of cellular functions, including differentiation, can be controlled to cell spreading and the microscale details of the cell-substrate interface (29).

The additive nature of μ CP is particularly well suited for creating multicomponent patterns of biomolecules. By combining multiple rounds of μ CP on a single surface, and using molecular affinity approaches to preserve the activity of sensitive proteins, we have demonstrated the use of multicomponent surfaces in understanding a range of cellular physiologies, including directed outgrowth of cultured neurons (Fig. 2A,B) (30) and, as will be described in the following sections, spatial cross-talk in T cell costimulation (31). The Chen group also introduced a modified μ CP method in which stamps are used to combined and selectively remove patterns of proteins from a single surface (32); this approach allowed co-patterning of multiple ECM proteins to study subcellular integrin interactions by human endothelial cells (33) (Fig. 2C).

Dip-pen and fountain-pen nanolithography—Spatial patterning can also be achieved through scanning probe techniques, where proteins and other molecules are delivered directly from a tip to specific regions of the substrate surface. For subcellular patterning, this is typically achieved with the tip of an atomic force microscope (AFM), which can perform either additive (34) or subtractive (35) actions. Two examples of this approach that have been fully developed into commercially-available systems are dip-pen nanolithography (DPN) (36-38) and fountain-pen nanolithography (FPN) (39-41). DPN uses AFM tips that are ‘ink’-coated from source reservoirs to pattern surfaces with molecules or proteins, and can achieve spatial resolution down to sub-50nm (38). DPN is more flexible than other techniques like μ CP in its ability to produce multi-component patterns, while the serial writing process limits its throughput. FPN uses modified AFM cantilevers that contain an ink reservoir and a connecting microchannel (39-41). Although still serial in process and the spatial resolution slightly lower (at 100-nm) (42), the advantage of FPN is that with the reservoir containing enough “ink” material, the technique is able to draw micrometer-size dots and lines in large areas.

Nanopatterning—While microelectronics has continued to push towards smaller scale fabrication, leading to lower power, lower cost, and faster devices, there is a substantial delay before these capabilities become available in the academic setting. Currently, large-scale photolithography in the academic setting is limited to dimensions of hundreds of nanometers, while processors for commodity-level electronics are being fabricated with features on the order of tens of nanometers. The ability to pattern biomolecules at these smaller scales would open up investigations into entirely different aspects of intracellular signaling and cellular-/molecular-engineering.

Electron-beam (e-beam) lithography is perhaps the most widely used approach to accessing these scales (43-45). It has been used for direct patterning of self-assembled monolayer (SAM) for spatial presentation of IgE to mast cells (44), and supported lipid bilayer for studying receptor dynamics in T cell immunological synapse (45-48).

Nanoscale patterning has also been achieved using a micellar lithography approach. In this method, a surface is coated with a dense layer of micelles composed of a block co-polymer shell surrounding a metal core. The polymer is subsequently removed, leaving behind an array of metal particles, the spacing of which is determined by the size of the polymer shell. The metal particles can then serve as anchors for proteins and other biomolecules, and has been effective in identifying critical dimensions of integrin-based sensing (49-53). Notably, this self-assembly approach offers high-throughput patterning, but does not directly offer the ability to create arbitrary patterns. Such capability is possible through the use of electron beam lithography (54). This nanopatterning technique has also been recently combined with supported lipid bilayer to present to cells a hybrid interface containing both mobile and immobile signaling components (55). It has also been used in combination with DPN to produce single-molecule arrays (56).

MICRO-/NANO-ENGINEERING OF SIGNALING IN CELLULAR SYSTEMS

Epithelial cells

An important concept in physiology is that larger organisms are divided into functional compartments. The barriers that separate these regions must often simultaneously resist bulk transport of compounds yet allow selective exchange of specific molecules between these spaces. Since diffusion is often the major driving force for this transport, these barriers must be thin while at the same time show a high degree of connectivity and integrity. This functionality is often provided by a single layer of cells forming a sheet or tubular structure, such as the intestinal epithelia of the gut or the closely related endothelium that separates blood from interstitial space. In the plane of such tissues, cells form sheets via strong intercellular junctions including tight junctions, adherens junctions, desmosomes, and gap junctions, which are maintained through homophilic binding of cadherins on the apposed cell membranes (Fig. 3A). Perpendicular to this plane, cells are polarized along the apical-basal axis with distinctive distributions of membrane proteins, cytoskeleton, and organelles that enable selective and/or directed transport across the layer. Knowledge of how cells balance cell-cell and cell-matrix interactions is essential for understanding tissue development, maintenance, and potential pathology, but the spatial architecture experienced by these cells is difficult to capture in traditional experimental systems. We focus here on advances in understanding of epithelial cell function that are made possible through surface micropatterning.

Cross-talk between cell-cell adhesion and cell-ECM interaction—While cadherins and integrins are involved in connecting a cell with its environment and share some similarities in downstream signaling events, they utilize distinct signaling pathways that influence a variety of cellular-level functions, including establishment of cell polarity (57). Homotypic E-cadherin interaction is required for the formation of junctional complexes that provide a diffusional barrier between the apical and basolateral membrane domains of epithelial cells, as well as the localization of exocyst complex (57, 58) and sites of basolateral vesicle delivery (59). However, this cell-cell adhesion alone is insufficient to generate full epithelial apical-basolateral polarity (57). Integrin binding to ECM provides a host of complementary functions; for example, adhesion to laminin-coated surfaces can

induce selective β -casein secretion from the apical surface of mammary epithelial cells (60). Importantly, these actions are not simply additive, as several studies have identified crosstalk between cadherin and integrin signaling, including those of inhibitory nature. For example, integrin-activated focal adhesion kinase (FAK) can phosphorylate β -catenin and thus induce degradation of E-cadherin cell-cell adhesion complex (61). A key study by Wang *et al.* (62) demonstrated that engagement of integrins by fibronectin-coated beads applied to the apical side of endothelial cell disrupted VE-cadherin-containing adherens junctions between endothelial cells within the monolayer. A complementary study combining micropatterning of ECM with magnetic-bead based (where beads were coated with E-cadherin) force measurement by Al-Kilani *et al.* (63) allowed quantitative insight into how the cadherin mediated adhesion is modulated by both the shape and extension of cell-ECM adhesion. These examples highlight the concept that such crosstalk may be localized, and studies into this epithelial cell function need to be considered using spatially resolved experimental systems.

In this direction, several studies have taken advantage of the ability to pattern multiple proteins on a single surface to investigate interactions between cadherin and integrin signaling in epithelial cells. Hunt and Nelson (64) used a μ CP approach to create complementary patterns of an E-cadherin/Fc fusion protein juxtaposed with an ECM protein (collagen VI) on planar glass substrates (Fig. 3B); E-cadherin interactions were re-oriented to the same plane as integrin-ECM adhesion to facilitate microscopy that is not possible in the natural cell-cell interface, while maintaining spatial separation of these signals. With this system, Hunt and Nelson demonstrated in MDCK cells highly targeted delivery of vesicles containing a basolateral membrane protein to sites of E-cadherin, supporting a role of E-cadherin in basolateral vesicle delivery. This system was subsequently used by Borghi *et al.* (65) to study lamellipodial activity and cell migration on alternating stripes of E-cadherin and collagen IV. Focusing still on MDCK cells, E-cadherin adhesion reduced lamellipodial activities on both proteins patterns, which mimics the decrease of lamellipodia activity in contacting and non-contacting membranes during intercellular adhesion between epithelial cells (66) (Fig. 3C). Meanwhile cell migration was not affected by the combination of adhesion cues, and traction force was found mainly on collagen-coated stripes, and oriented to the direction of collagen patterns, but the migration was biased parallel to the stripes, suggesting a role of E-cadherin in orienting cell migration and redirecting cytoskeletal force (Fig. 3D-E). These studies together demonstrate both local and cell-wide coordination of cell function by the convergence of integrin and cadherin signaling.

A surprising aspect of integrin-cadherin crosstalk was demonstrated by Tsai and Kam (67) who applied microcontact printing techniques to create complementary patterns consisting of microscale fibronectin (FN) dots surrounded and separated by E-cadherin/Fc on surfaces of different rigidities. On rigid (1 MPa Young's modulus) polydimethylsiloxane (PDMS) surfaces, engagement of FN inhibited formation of E-cadherin-based contacts in MCF-7 breast carcinoma cells. The lack of E-cadherin contacts on regions between FN features indicates that this inhibition is a global, cell-level effect, an observation that would not be possible on uniformly coated surfaces. Application of the inhibitor PIP2 allowed MCF-7 cells to establish both focal contact and E-cadherin-based contacts on these surfaces,

demonstrating a new role of Src-family kinases in spatial signaling and crosstalk. Intriguingly, MDCK cells did not show this inhibitory crosstalk, suggesting important differences between epithelial cell models. Lastly, by patterning FN and E-cadherin on elastomer surfaces, Tsai and Kam demonstrated that this inhibitory crosstalk correlated with substrate stiffness; as the Young's modulus of this system decreased the ability of MCF-7 cells to establish concurrent connections with FN and E-cadherin. Since it has been previously shown that epithelial cell invasiveness correlates with the overall elasticity of its cytoskeleton (68), their results implicate a role of the crosstalk between cadherin- and integrin-mediated adhesion in metastatic disease progression.

Mitotic spindle orientation and cell division in polarized epithelium—Proper cell division in the polarized epithelium is critical for the maintenance of epithelial integrity and tissue functions. For correct positioning of daughter cells and inheritance of localized molecules (69), the position and orientation of the mitotic spindle are coupled to cell polarity, which decides the axis of cell division and the future position and fate of the daughter cells. The local and global cortical cues such as cell polarity, shape (70) and the adhesive microenvironment (71, 72) have both been implicated in the determination of mitotic spindle orientation and position. Micropatterning of ECM molecules has provided an elegant solution to independently evaluate the contribution of these cues that are difficult to delineate *in vivo*. Thery *et al.* (73) designed a series of micropattern shapes of fibronectin (FN) to manipulate single HeLa epithelial cell adhesion. It was shown that, while spindle orientation is not completely driven by cell shape, the spatial distribution of the ECM affects the location of cortactin and ezrin, which dictates the polymerization of membrane-associated filamentous actin and in turn guides spindle orientation in mitosis. Interestingly, in a subsequent study, they further demonstrated that even a slight modification of ECM shape was sufficient to cause a transition from symmetrical to asymmetrical orientation of the spindle, leading to unequal cell division (74), suggesting that cell shape controls spindle orientation and epithelial cell division through mechanotransduction in the actin and microtubule cytoskeleton based on localized distribution and density of cortical cues.

Lastly, Fink *et al.* (75) combined micropatterns, direct laser-ablation of intracellular actin retraction fibers, and stretchable cell culture substrates to investigate the role of external forces and cytoskeletal mechanotransduction on dividing epithelial cells. On micropatterns, spindle orientation rotated strongly only when the retraction fibers facing mitotic spindle poles were ablated, confirming the importance of the local geometrical and mechanical signals in guiding the morphogenetic process.

Epithelial-mesenchymal transition—The loss of apical-basolateral polarity in epithelia has been regarded as a precondition and a hallmark of epithelial cancer (76). Metastatic cancers are typically characterized by loss or inactivation of E-cadherin or catenins and gain of mesenchymal N-cadherin expression, which promotes transformation of epithelial cells into migratory phenotype termed epithelial-mesenchymal transition (EMT) (77), during which the apical-basolateral polarity in epithelial cells is replaced by antero-posterior polarity. The mechanisms involving EMT process has largely known to be induced by cytokines, growth factors, and matrix metalloproteinases (MMPs) (78, 79). By culturing

single mouse mammary epithelial cells on precisely defined micropatterned areas, Nelson *et al.* (29) discovered that the cells' EMT response to MMP-3 was blocked by limiting cell spreading, which was not seen with TGF- β -induced EMT, revealing a previously unknown shape-dependent mechanism that controls EMT process. More recently, Gomez *et al.* (80) further demonstrated in TGF- β -induced EMT in a micropatterned (multicellular) culture that gradients of mechanical stress define the spatial locations where EMT occurs, and establishing a causal relation of tissue geometry and endogenous mechanical stress with EMT.

Together these studies illustrate the growing application of microengineered surfaces in understanding cell function. While this field began with a focus on cell spreading as initiated by ECM proteins in particular, this work with cadherin-based systems suggests the universality of the concept that spatial organization influences cell signaling. The next section continues on this theme, illustrating the use of contemporary surface patterning technologies in understanding cell signaling.

T lymphocytes and the immune synapse

Cellular and molecular immunology is a rapidly emerging focus for many areas of biomedical engineering. In particular, the ability to engineer surfaces at the micro- and nano-scale has provided new insights into the spatial dynamics of signaling in T cells, key mediators of the adaptive immune response. The archetypal activation event (Fig. 4A) begins with binding of the T Cell Receptor (TCR) complex with an appropriate peptide ligand, loaded into a Major Histocompatibility Complex (MHC) on the surface of an Antigen Presenting Cell (APC). This interaction provides the specificity of the adaptive immune response. However, functional activation of the T cell requires engagement of additional membrane receptors. Two of these have been of particular interest in the context of spatially resolved signaling (Fig. 4B). The first is the integrin receptor Lymphocyte Function-associated Antigen-1 (LFA-1), which provides attachment between cells when bound to its ligand, Intracellular Cell Adhesion Molecule – 1 (ICAM-1) and lowers the level of TCR signaling required to activate T cells. The second is CD28, an immunoglobulin superfamily receptor, that when bound to either CD80 or CD86 provides a costimulatory signal that augments TCR signaling and is required for full activation of many classes of T cells. While both LFA-1 and CD28 modulate and amplify TCR signaling, they involve fundamentally different intracellular pathways. These three systems together form a core set of T cell signaling mechanisms that have been explored using patterned surfaces.

Overlaid on this molecular-level understanding of T cell – APC communication, it is becoming clear that the micro- and nano-scale organization of signaling complexes in the cell-cell interface modulates cell signaling. Engagement of receptor-ligand pairs between T cells and APCs occurs within a small ($\sim 70 \mu\text{m}^2$) area of cell-cell contact, an interface termed the immune synapse (IS). This structure coordinates signaling at the cellular level by directing secretion of cytokines to the interface between these cells. In addition, receptor-ligand complexes in the IS exhibit spatiotemporal dynamics at finer, subcellular scales. Initial microscopy-based experiments in the late 1990's revealed the archetypal structure of a mature synapse, consisting of an accumulation of TCR-pMHC complexes in the middle of

this interface, a region termed the central Supramolecular Activation Cluster (cSMAC), surrounded by LFA-1-ICAM-1 in an area termed the peripheral Supramolecular Activation Cluster (pSMAC) (3, 81). Subsequent investigations revealed variations of this basic pattern in different cell-cell interfaces, depending on the specific type of T cell and APC that are interacting suggesting that microscale organization is part of the language of T cell – APC communication. The IS is also dynamic (3); upon initial formation, microclusters of TCR-pMHC form in the periphery of the IS, surrounding LFA-1-ICAM-1, and this microscale structure inverts over the initial minutes of interaction (Fig. 4C). CD28 microclusters exhibit a complementary spatial dynamic, forming first in the periphery concurrent with TCR complexes, but later dissociating from TCR at the cSMAC-pSMAC interface. Current models identifying these microclusters as the fundamental unit of signaling, outlining a role of spatial organization at increasingly finer scales in IS function. It is also recognized that many of the signaling molecules downstream of TCR/CD28/LFA-1 signaling involve molecules that associate with different molecules of the cell membrane, a structure that itself is increasingly recognized to be spatially complex at submicrometer scales. Finally, the engagement of multiple signaling pathways in the platform of the immune synapse illustrates the biological complexity of cell-cell interfaces.

T cells respond to the microscale organization of cell-cell interfaces—

Capturing the microscale organization of the IS is a clear and compelling application of microengineered surfaces. The approach that emerged in this direction is to replace the APC with a surface containing microscale patterns of proteins that bind to and activate specific receptors on the T cell surface (Fig. 4D); the layout of these proteins dictates the organization of signaling complexes in artificial synapses formed between the cells and substrates. These proteins include constructs based on the extracellular domains of the receptors' natural ligands (*i.e.*, pMHC, ICAM-1, or CD80) or antibodies that upon binding activate their target. This approach provides key advantages that are not available in other experimental systems. In particular, the use of surface microfabrication techniques allows creation of a wide range of geometries with minimal ancillary changes to the experimental system. A key study into immune synapse structure by Tseng *et al.* (82), carried out using cell-cell interfaces, suggested that segregation of CD28 and TCR by micrometer-scale distances enhances costimulation. Converting the native segregated pattern of CD28 and TCR into a colocalized counterpart was accomplished by truncating the cytosolic domain of CD80. While effective in altering TCR-CD28 colocalization and restricted to proteins in the APC, the mechanism by which this approach changed organization is not well understood, and may have had additional effects on cell signaling as the protein was modified. Such challenges are common in traditional molecular biology approaches, but are circumvented by the use of micropatterning. Micropatterned surfaces also allow the study of patterns that may be difficult to obtain in cell-cell systems, which while of potentially limited physiological relevance, may reveal new aspects of cellular signaling.

This approach was first demonstrated by Doh and Irvine (83). That study used a new photoresist system (Fig. 1A) developed in the Irvine group (84) to create arrays of features containing an activating antibody to the ϵ -chain of CD3 (anti-CD3), part of the TCR complex which upon this binding can initiate downstream signaling independent of antigen

recognition. The regions separating and surrounding these features were modified with a protein consisting of the extracellular domain of ICAM-1 fused with the Fc region of human IgG providing activation of LFA-1. T cells on these arrays halt migration and initiate calcium fluxes upon interaction with the anti-CD3 features, recapitulating the events following contact with an APC. Moreover, localization of TCR and LFA-1 on these surfaces followed the pattern geometry (Fig. 4E). Doh and Irvine demonstrated with these surfaces that secretion of IL-2 and IFN- γ , two high-level measures of functional activation of these CD4⁺ T cells, was differentially sensitive to whether the anti-CD3 pattern was a single central feature or an annular ring (Fig. 4E). Subsequently, Shen *et al.* (31) combined multiple rounds of microcontact printing to create surfaces containing either colocalized or segregated patterns of anti-CD3 and anti-CD28 (providing activation of the CD28 receptor) surrounded and separated by ICAM-1 (Fig. 4F). This system was designed to test the hypothesis of Tseng *et al.*, namely that segregation of CD3 and CD28 signaling enhances T cell activation. As shown in Fig. 4F, a segregated pattern consisting of a central feature of anti-CD3 surrounded by a constellation of anti-CD28 does promote higher levels of IL-2 secretion from mouse CD4⁺ T cells than a single, colocalized feature (Fig. 4F), which is consistent with this hypothesis. However, reversing the positions of anti-CD3 and anti-CD28 reduced IL-2 secretion, while placing colocalized CD3 and CD28 in the periphery of the cell-substrate interface promoted the highest levels of IL-2 secretion (Fig. 4F). This more extensive set of patterns, which were straightforward to produce by microfabrication but would be difficult to recreate in the cell-cell interface, clarified that it is the position of CD28 signaling within the interface, rather than the position relative to CD3, that influences cell activation.

Together, these studies provided the first direct demonstrations that T cells are sensitive to the micro- and nano-scale organization of signaling molecules with the immune synapse. Conversely, a practical application of this technique would be to control T cell activation using in a cell culture system. In the clinical setting, it may thus be possible to guide the expansion of T cells in the context of immunotherapy, recapitulating the control afforded by APCs but in a more reproducible and reliable synthetic system (85).

Microscale dynamics of T cell signaling—Beyond capturing the structure of the immune synapse, micro- and nano-scale surface engineering is a powerful approach for understanding intracellular signaling. While many of the pathways downstream of TCR, CD28, LFA-1, and other membrane receptors have been largely delineated at the molecular level, knowledge of how multiple mechanisms intersect and converge to promote a wide range of possible T cell responses remains incomplete. A major practical barrier in these studies is that the activity of the intermediate signaling molecules is tightly regulated. Many intermediates, and the molecules that regulate them, are associated with the cell membrane or other intracellular structures, thus providing mechanisms for spatial regulation of signaling but pose challenges in the analysis of such systems. Particularly in the immune synapse, the ability to spatially control the presentation of ligands at subcellular scales through surface engineering provides an important experimental counterpart to microscopy-based analysis. An early demonstration of this approach used immunoprecipitated aggregates of the anti-CD3 antibody OKT3 to locally stimulate TCR activity in Jurkat cells

(86). Single particle tracking revealed that the diffusion coefficient of the Src family kinase Lck doubled as a function of position over distances of micrometers from the site of stimulation, suggesting that TCR activation modulates the local synapse architecture. The report by Doh and Irvine (7) describing micropatterned anti-CD3/ICAM-1 surfaces expanded this methodology by allowing the definition of more complicated geometries. With these surfaces, they demonstrated strong spatial correlation of PKC θ , a novel member of the PKC family of protein kinases that provides critical roles in T lymphocyte function, with a range of different anti-CD3 patterns (Fig. 4G) suggesting a localized interaction between this surface receptor and downstream signaling molecule; such associations are difficult to determine on surfaces that are uniformly coated with a stimulatory protein. However, the study by Shen *et al.* (31) found a weaker correlation between PKC θ and TCR signaling. It is noted that these two studies focused on different mouse CD4+ T cell preparations, which may be responsible for the contrasting degrees of TCR / PKC θ colocalization; ongoing studies seek to understand these subtle distinctions, which would not be possible without micropatterned surfaces. Finally, surfaces presenting multiple, independently defined patterns of proteins promises a new approaches to exploring intracellular signaling. Specifically, it may be possible to spatially dissect a signaling network by creating independent patterns of ligands that bind to and localize membrane receptors; the ability of mouse cells in the Shen *et al.* report to respond to segregated patterns demonstrates the feasibility of this approach. Surface micro- and nano-engineering has been particularly successful in advancing another experimental model that has been important in the context of the immune synapse, the substrate supported lipid bilayer (87-90). The basic supported bilayer system consists of a single, continuous lipid membrane in close apposition to an appropriate substrate, most commonly glass or other silicon oxide-based material (Fig. 5A). A thin (nanometers thick) layer of interfacial water separates the lipid bilayer from substrate while maintaining association between these structures. Importantly, this system maintains the 2-dimensional fluidity of natural membranes in a controllable, synthetic system. Tethering of proteins to this membrane imparts this fluidity to the proteins, capturing the natural environment that proteins experience when presented on the surface of a living cell. For T cells, tethering of pMHC-, ICAM-1-, and CD80/86-derived constructs to the bilayer has been used extensively as a model of the APC, and was essential for the early observation of synapse maturation, including formation and inversion of the “bullseye” pattern of TCR and LFA-1 (3). However, it is increasingly recognized that the cell plasma membrane exhibits structure over scales from tens of nanometers to several micrometers in extent, modulating the mobility of membrane-associated proteins. To capture this important architecture, a number of methods were developed for patterning the basic supported lipid bilayer system (26, 91-93). As a general principle, micro- and nano-scale fabrication can be used to define barriers that interrupt the supported lipid bilayer, blocking and directing the mobility of membrane biomolecules (Fig. 5B). Based on the contemporary fabrication techniques available at various times, these barriers were initially of micrometer-scale dimension, but by migration from photolithographic patterning to electron-beam-based lithography, have been reduced to nanoscale dimensions (94), which are well suited for the scales of the immune synapse. These patterned bilayers have provided new insight into coupling between the actin cytoskeleton and membrane receptors, most notably the TCR. Such interactions were suggested by the initial descriptions of the immune synapse by

Grakoui *et al.*, in that inhibition of actin cytoskeleton dynamics with cytochalasin D reduced TCR-pMHC motion along the supported lipid bilayer. Given recent reports that TCR signaling can be initiated through mechanical forces (95, 96) and, more recently, that activation of CD4+ T cells is sensitive to the mechanical rigidity of an underlying substrate (97, 98), understanding the details of TCR-cytoskeleton interaction is of increasing interest. The initial study into T cell response to micro-patterned supported lipid bilayers (45) used metal grids to break up a bilayer presenting pMHC and ICAM-1, restricting mobility at a subcellular level (Fig. 5C). Within each region of corralled lipid bilayer, pMHC migrated towards the center of the cell, while ICAM-1 was excluded to regions towards the cell periphery, suggesting local coupling between the cytoskeleton and TCR. Moreover, restricting inward motion of TCR-pMHC complexes in this manner enhanced T cell activation, further suggesting the importance of mechanical forces and mechanical response of the pMHC. Later, DeMond *et al.* (46) demonstrated that TCR microclusters could move along small barriers to lipid diffusion, even when this direction was not parallel to the direction of actin flow, suggesting a frictional slip coupling between TCR and the actin cytoskeleton (Fig. 5D). Most recently, Yu *et al.* (99) demonstrated that trapping of TCR-pMHC motion with a lipid diffusion barrier slowed down actin retrograde flow, further supporting a substantial, but incomplete, interaction between TCR and the actin cytoskeleton.

Division of a membrane into an array of separated corrals also revealed new aspect of T cell sensitivity to low numbers of pMHC complexes. The inspiration for these recent experiments is that engagement of T cells with soluble MHC class II dimers is sufficient to activate T cells, even when one of the halves of the dimer is a very weak self-pMHC (100). The same investigators demonstrated synergy of agonist pMHC and self-pMHC in supported planar bilayers in the presence of ICAM-1 and CD80. This is significant because in the bilayer setting the distance between the agonist pMHC and self-pMHC is not fixed and greatly increases the entropic penalty to form a dimer. It is interesting to consider that conserved *cis* interactions may contribute to oligomerization as recently described for adhesion systems (101, 102). Working with only ICAM-1 and pMHC in bilayers, both Varma *et al.* and Ma *et al.* inferred that single agonist pMHC are sufficient to trigger formation of signaling microclusters (103, 104). A caveat with both of these studies is that single molecule triggering is inferred from assumptions of random MHC-peptide distribution rather than directly observed positions, which would be required to form a definitive conclusion. Furthermore, recent studies using micropatterning argue that 2-3 agonist pMHCs are needed to form a signaling unit for Ca^{2+} mobilization (105). This experiment built on the earlier result by Mossman *et al.* (45) in which the bilayer was partitioned into small corrals. In Manz *et al.*, the size of each compartment was reduced in size to 0.5 μm ; they found that the barriers decreased signaling at low pMHC density, suggesting that effective signaling required that most corrals contain 2-3 pMHC (105). One interpretation of this finding is that a single TCR microcluster requires the formation of a TCR dimer and that this requires at least 2 pMHC in a 0.2 μm^2 area. When a few corrals contained only one pMHC, Ca^{2+} signaling could not be triggered suggesting that single pMHC are generally incapable of triggering this mode of signaling. This argues against the suggestion in the Varma *et al.* and Ma *et al.* studies that a single ligand can trigger signaling

microclusters. However, there is another possible interpretation related to the need to segregate from ICAM-1. In experiments where ICAM-1 and pMHC are freely mobile, they can readily rearrange to accommodate segregated LFA-1 and TCR based microclusters (Fig. I2C, top). When barriers are placed in the bilayer, they prevent long-range diffusion of both pMHC and ICAM-1, which may hinder segregation. Under the conditions of these experiments there are ~40 ICAM-1 per corral, which would outnumber pMHC by at least 10-fold. Given that ICAM-1 is also restricted to move within the 0.5 μm corrals, the requirement for pMHC in each corral may be related to the number of pMHC needed to locally compete with and even compress ICAM-1 molecules in the same corral to enable microcluster formation. It would be interesting to repeat these experiments with CD48 as the principal adhesion molecule, the CD2-CD48 interaction is predicted to cooperate directly in TCR-pMHC interaction (106).

OUTLOOK

Cells are complex and intricate constructs, with the control systems that coordinate their function being embodied in biomolecular reactions, rather than electrical or mechanical signals. Understanding how these systems regulate cellular processes is a key contemporary challenge and opportunity for Biomedical Engineering. This field is at a strategic juncture as many of the underlying tools have been developed, but come from a wide range of disciplines. Modern advances in microscopy provide an astounding level of spatial and temporal detail of signaling proteins in the complex environment of the cell. The ability to numerically model the dynamics of signaling systems has also advanced dramatically, allowing the inclusion of realistic cell shapes and the interaction of multiple pathways. As reviewed in here, adaptation of micro- and nano-scale fabrication techniques to be compatible with biological molecules offers control over the layout of cell signaling that is essential for bridging these other tools into a complete understanding of spatially resolved signaling. Clearly, uniting these technologies into a complete model of micro- and nano-scale cellular systems is an interdisciplinary endeavor. Assembling the expertise that can best leverage each underlying field, yet ensuring a shared vision of the capabilities and limitations of the complementary areas of research, is a major contemporary challenge to the next generation of scientists and engineers.

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Figure 1. Optics-based micropatterning

(A) Photolithographic processing of a water-soluble, biotinylated copolymer photoresist. The full procedure illustrated in the right-hand column demonstrates a pattern-and-backfill process for two different streptavidin (SAv) molecules. Adapted from reference (6), Copyright 2004 National Academy of Sciences, USA. (B) A three-component SAv patterned surface created by sequential lithography. Adapted with permission from reference (8). Copyright 2010 American Chemical Society. (C) Directed-exposure fabrication of a multicomponent hydrogel. Adapted from (18) with permission of The Royal Society of Chemistry.

Figure 2. Contact-based micropatterning of multicomponent surfaces

(A) Multiple rounds of microcontact printing can be aligned and combined on a single surface. This surface illustrates nodes of poly-L-lysine (PLL, red) connected by hexagonal grids of Protein A (blue). (B) Directed outgrowth of hippocampal neurons on a surface patterned with hexagonal grids of Ncad-Fc (blue), square grids of L1-Fc (dash lines), and nodes of PLL (at the intersections of hexagonal grids, outlined by green circles). Neurons were stained for β -tubulin (green) and MAP2 (red). (C) A stamp-off process provides an alternative method for creating multicomponent surfaces that are more than simply two complementary patterns. The stamp is then placed in conformal contact with the stamp-off template to remove protein on the stamp from areas in contact. The stamp can be subsequently coated with a second protein and/or undergo additional stamp-off steps, or placed in contact with final cell culture substrate to generate multi-component protein patterns (represented by the three component surface on the right). Adapted from reference (33), with permission of The Royal Society of Chemistry.

Figure 3. Capturing spatial separation of E-Cadherin and integrin signaling

(A) The integrity of epithelial sheets relies on balancing of cell-cell and cell-matrix interactions. Micropatterning of surfaces captures the natural separation of these signals on an experimentally convenient surface. Two variations on this normal architecture will be discussed, epithelial-mesenchymal transition (EMT) and spindle orientation. (B) Micropatterning process of a dual substrate display (DSD) surface. Adapted with permission from reference (64). (C) E-cadherin-GFP-expressing MDCK cells on collagen IV : E-cadherin/Fc micropatterned surface. (Red: CollagenIV; black: E-cadherinFc; scale bar: 10 μm). (D) Local traction stress magnitude and orientation (black vectors, arbitrary units) exerted by a single MDCK cell (delineated by the green line) on collagenIV-printed (inside dash lines) polyacrylamide gel sheet. (E) Top: Lamellipodia activities L_C and L_E on collagenIV (red) and E-cadherinFc/Fc (or PEG) stripes (black), respectively, as a function of E-cadherinFc percentage on non-collagenIV stripes. Bottom: Migration rate (average cell velocity at 10 min intervals) on patterned surfaces as a function of E-

cadherinFc percentage on non-collagenIV stripes and on plain EcadFc surfaces. (C)-(E) are adapted from reference (65), Copyright 2010 National Academy of Sciences, USA. (F) Integrin-cadherin crosstalk as a function of rigidity. On rigid FN/E-cadherinFc-patterned PDMS surfaces, MCF-7 cells establish only FAs (red paxillin staining); on soft micropatterned surfaces they are able to concurrently form FAs and CAs (green E-cadherin staining). Adapted, with permission, from reference (67).

Figure 5. Patterned supported lipid bilayer in studies of T cell signaling

(A) The supported lipid bilayer has emerged as a powerful tool for capturing the fluidity of cell membranes in juxtacrine signaling. In these studies, the APC is replaced with a supported bilayer presenting ligands to TCR, LFA-1, and CD28. (B)

Defining barriers to diffusion into supported lipid bilayers allows the capture of the natural complexity of the cell outer membrane. The blue squares represent material (photoresist, protein, or metal) barriers to lipid diffusion. (C) Control over the morphology of interfaces between T cells and supported lipid bilayers using grids of diffusion barriers. In the bottom panel, metallic barriers are indicated by the square, blue-green grid. Adapted from reference (45), with permission from AAAS. (D) Non-contiguous barriers allow deeper study into the dynamics of the immune synapse. This image illustrates the positions and trajectories of TCR signaling clusters (grey-white signal) formed by T cells interacting with a supported lipid bilayer that has been patterned with barriers indicated by the green lines. Adapted with permission from reference (46).