Nanobiotechnology and Cell Biology: Micro- and Nanofabricated Surfaces to Investigate Receptor-Mediated Signaling

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IgE receptor, patterned surface, lithography, supported membranes, immune cell, cell adhesion

Abstract

Advances in microfabrication and nanofabrication are opening new opportunities to investigate complicated questions of cell biology in ways not before possible. In particular, the spatial regulation of cellular processes can be examined by engineering the chemical and physical environment to which the cell responds. Lithographic methods and selective chemical modification schemes can provide biocompatible surfaces that control cellular interactions on the micron and submicron scales on which cells are organized. Combined with fluorescence microscopy and other approaches of cell biology, a widely expanded toolbox is becoming available. This review illustrates the potential of these integrated engineering tools, with an emphasis on patterned surfaces, for investigating fundamental mechanisms of receptor-mediated signaling in cells. We highlight progress made with immune cells and in particular with the IgE receptor system, which has been valuable for developing technology to gain new information about spatial regulation in signaling events.

Contents

CELL BIOLOGY AND THE OPORTUNITIES OF NANOBIOTECHNOLOGY

Nanobiotechnology: micro- and nanofabricated materials, structures, and devices used to examine and engage with biological systems on subcellular and molecular levels

GFP: green fluorescent protein

The subject of cell biology covers the hierarchical organizations of diverse biomolecular constituents that enable cells to maintain homeostasis in a particular biological environment and to respond appropriately to external signals. This review describes a nanobiotechnology approach to understanding the complex operation of cells and focuses on receptor-mediated cellular signaling. As an example we highlight the receptor (FcεRI) for immunoglobulin E (IgE) on mast cells that operates in the allergic immune response. Multivalent ligand (e.g., an inhaled allergen) binding to and clustering cell surface IgE receptors initiates a transmembrane signal, stimulating intracellular pathways that lead to a number of cellular responses including gene transcription and secretion of chemical mediators. The secreted molecules typically interact with other cells in the surrounding tissues resulting in systemic changes, and ultimately the person who encountered the allergen sneezes or suffers other, sometimes life-threatening, symptoms of allergies and asthma. This example illustrates the remarkable efficiency with which biology integrates response mechanisms over multiple length scales: The nanoscale encounter of allergen and receptor initiates a response that cascades through collective interactions over micrometers in the cell and increasing length scales beyond, ultimately to the macroscale system of the human being.

In recent years, many laboratories have investigated signal transduction pathways that are stimulated in a large variety of physiologically relevant cell types, and by incorporating genetic, biochemical, and physical approaches, key enzymes as well as regulatory structures and mechanisms have been defined. However, the necessary spatial arrangements and regulation are difficult to approach with many current methods. Although advances in genetically encoded fluorescence tags, such as green fluorescent protein (GFP), and confocal fluorescence microscopy allow distributions and some dynamics of particular components to be monitored, spatial resolution is limited by the diffraction of light (roughly half the wavelength, typically >250 nm). Usually more limiting are cellular autofluorescence and morphological heterogeneities that confound interpretations of fluorescence images. Other challenges include heterogeneities in the local environment and in properties of the stimuli that tend to increase experimental variables. This review describes developments of micro- and nanofabrication techniques to provide spatial and chemical control at the level that the cell operates. Combined with other approaches, these fabrication methods offer a powerfully expanded set of tools for addressing the complexities of cell biology.

Micro- and nanofabrication tools emerged in large part from the realms of electrical engineering and materials science. Photolithography and other methods developed for reducing the size of electronic devices now reach easily into submicron length scales, the same regimes within which biomolecules are organized in cells. Advances in materials science include biocompatible polymers and modified surfaces developed in part for effective engineering of tissues and physiological implants. Together with fabrication methods, materials can be designed with chemical features that resemble spatial cues sensed by cells or, alternatively, can interfere selectively with those cues. Many of these advances have occurred under the broad umbrella of nanotechnology—or nanobiotechnology if they relate directly to biological systems. Strict definitions of nanotechnology usually focus on materials properties and manipulations corresponding to length scales of 1–100 nm. This would apply to, for example, structures of individual proteins or brightly fluorescent quantum dots that can be used to monitor individual proteins on cell surfaces. The realm of nanobiotechnology appropriately extends beyond 100 nm and upward to the micron scale, so to consider biomolecular structures operating collectively within a living cell, the cellular structures are built from biomolecules and provide the relevant context. Thus, the convergence of nanobiotechnology (that includes both fabrication and chemical modification on cellular and biomolecular length scales) with the more standard biochemical and biophysical tools provides exciting new opportunities for cellular investigations.

Herein we describe current developments that draw from the toolbox of nanobiotechnology, focusing particularly on patterned surfaces that are already proving valuable in investigations of receptor-mediated cellular signaling. After briefly describing basic schemes for surface engineering and chemical modification, we describe early applications to address mechanistic questions in cell biology. Then, as a set of connected examples, we focus on our collaborative efforts to develop these approaches for gaining new information about spatial regulation of IgE receptor signaling in mast cells, and briefly describe new efforts in other immune cell systems. We assess current limitations and suggest future directions.

Photoresist: photosensitive material used for engineering surfaces in photolithography

MICRO- AND NANOFABRICATION FOR SURFACE PATTERNING

Modern fabrication technology is based primarily on the principles of lithography: top-down techniques to pattern features on a surface starting from bulk material. Micro- and nanofabrication (reviewed in Reference 83) were advanced particularly by the semiconductor and integrated circuits industry to produce electronic components with designed features of increasingly small size. Photolithography is most amenable to patterning surfaces for spatially controlled interactions with cells that can be visualized and analyzed. Useful feature dimensions range from a few hundred nanometers (as limited by the wavelength of light) to several or tens of micrometers (the size of cells). In addition to a large engineering literature, a useful primer on photolithography is available (10), and detailed descriptions for particular biological applications are referenced in sections below.

The basic photolithographic method generates patterns on a surface (typically a silicon wafer) coated with a photoresist by selectively irradiating the surface with light through a predesigned mask (**Figure 1*a***). The mask contains the features of desired shapes and sizes in an opaque material (usually chromium) on a transparent background. UV light shining through the transparent

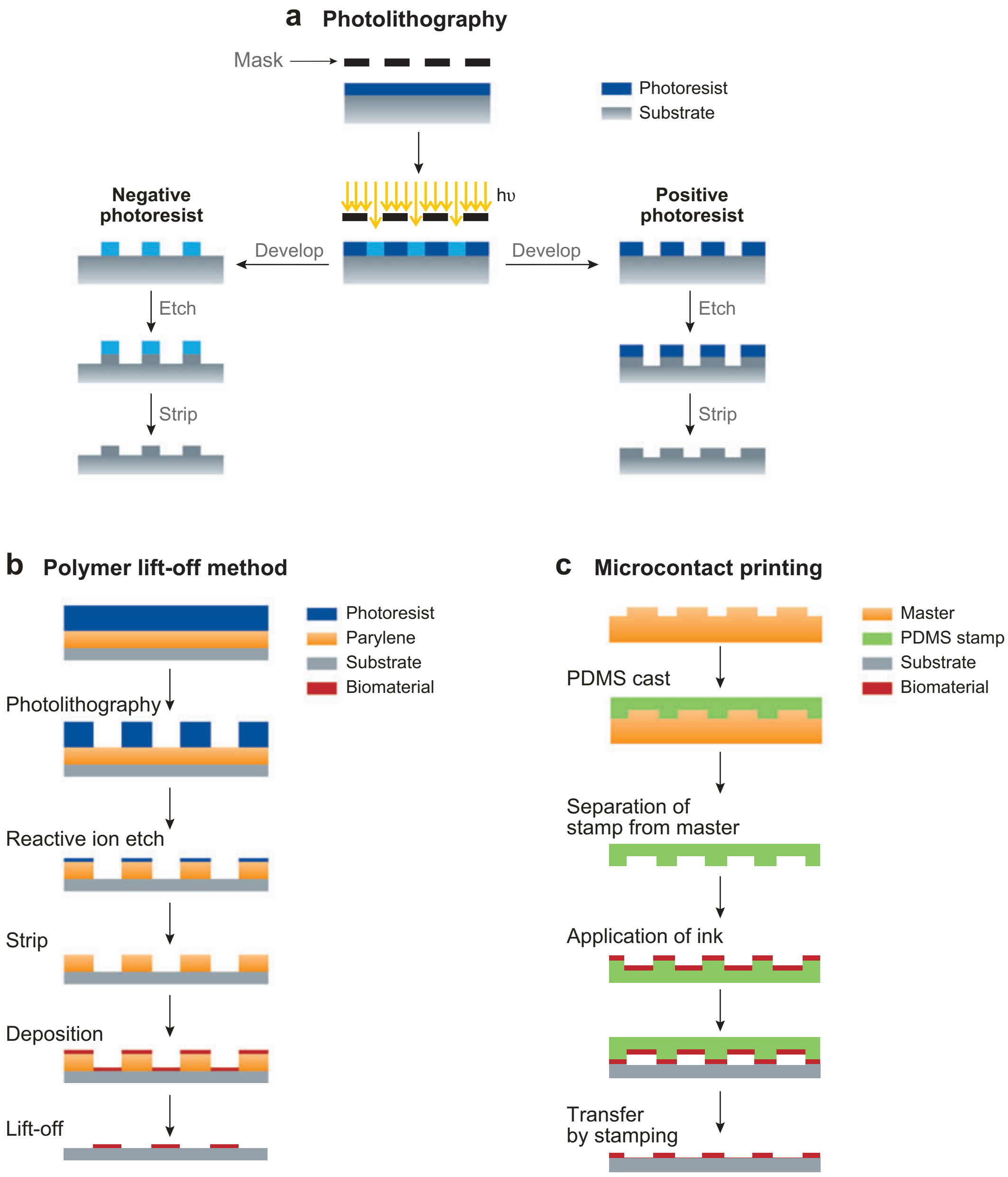

Figure 1

Schematic diagram of methods used in microfabrication. (*a*) Conventional photolithography. (*b*) Polymer lift-off. (*c*) Microcontact printing.

regions of the mask changes the chemical composition of the photoresist, making it soluble (positive) or insoluble (negative) to particular solvents. After exposure, unprotected sites can be either chemically or dry etched, and subsequent processing steps yield surfaces that have two (or more) patterned regions for distinctive chemical modification or construction of topographical features. Depending on the biological application, the fabricated surface regions that provide spatial control need to be selectively modified with materials that are biocompatible and provide chemical control.

The challenge of transforming microfabricated surfaces for effective interfacing with cells has been addressed by adapting the fabrication and the chemical modification schemes. Below we discuss two fabrication approaches that are particularly useful for investigating cell interactions: microcontact printing (μCP) and polymer lift-off. Other techniques are also being developed for biological or medical applications, such as patterning proteins or DNA for microarrays. Electron beam (E-beam) lithography, which can pattern features with resolution as small as a few nanometers, is a serial method that requires no mask: The pattern is created by controlling the beam as it scans across a resist-coated substrate. This technique is more difficult and expensive, and it requires a significant amount of time to pattern features over an area of centimeter dimensions. Other new technologies for patterning biological materials with high resolution include nanocontact printing (60), nanoimprint lithography (23, 32) and scanning probe lithography (1, 41, 65, 84). Although patterning on the nanometers length scale will ultimately be valuable for addressing biomolecular questions in cell biology, application of these approaches is currently limited by optical resolution and other experimental uncertainties.

Microcontact Printing

Soft lithography, developed in the 1990s (reviewed in Reference 86), opened up opportunities for culturing or probing cells with biologically friendly surfaces that can be engineered with chemical and topographical features (14). The term refers to a set of fabrication techniques that involve molding, imprinting, or embossing with a template made of polymers, such as the elastomer polydimethylsiloxane (PDMS). A particular example is μCP, which is readily applied for patterning flat surfaces (47, 66). A master, typically prepared by conventional photolithography, is used to create an elastomeric stamp with the desired features (**Figure 1*c***). The stamp is coated with the desired biomaterial (ink) and then pressed against a surface to deposit the material. The versatility of the method can be increased by sequential stamping with different materials to yield multiple regions that are chemically distinctive. Elastomeric templates are easy to use and inexpensive to produce with commercially available templates, thus eliminating the user's need for clean room facilities and expertise. Some drawbacks of μCP include PDMS pattern distortion, nonuniformity over the stamped area, and the potential for surface contamination with excess material on the stamp.

μCP: microcontact printing

Polymer Lift-Off

Another versatile approach to biocompatible patterning is photolithography followed by polymer lift-off (38) (**Figure 1*b***). For example, a thin layer (~1 μm) of parylene C is deposited over a silica or glass surface before the addition of the photoresist layer. This substrate is patterned with photolithography to produce the desired features and then subjected to a controlled reactive ion etch that removes exposed regions of parylene down to the silicon dioxide or glass surface. At this stage the sample is transferred to a biologically friendly environment and the biomaterial is added in aqueous solution to coat the entire surface before mechanically peeling off the polymer. The revealed pattern is cleanly presented over a bare substrate that can be subsequently modified with a chemically distinctive material.

Surface Interfacing with Biomaterials

Surface modification provides chemical control of cell surface interactions, and minimizing nonspecific interactions can be

Ligand/hapten: a molecule that specifically binds to or forms a complex with a target protein to mediate a biological response

SAM: self-assembled monolayer

as critical as selectively engaging receptors or other components in the plasma membrane. Patterned and functionalized surfaces are widely used for biosensor applications, such as antigen-based antibody detection (74, 82). However, cells offer more challenges for surface modification, both in terms of the stringency for biocompatibility (cell viability and function) and the capacity for nonspecific interactions (including binding to surfaces via cellular secretions). Some materials commonly used in cell studies are described below. For patterning, these materials can be, for example, stamped onto a prepared surface with μCP (**Figure 1*c***) or coated onto a photolithographed polymer prior to polymer lift-off (**Figure 1*b***).

Proteins and small-molecule ligands. Proteins are among the most widely used biomaterials to coat and modify surfaces, and early characterization of protein-surface interactions was carried out in the context of tissue engineering, as part of assessing cellular responses to nonnatural devices (45, 63, 75). Simple adsorption sometimes is sufficient: Particularly with hydrophobic surfaces, proteins adsorb and partially denature, usually causing an essentially irreversible association (77). However, this results in a highly heterogeneous arrangement with various protein domains exposed nonuniformly across the surface (17), and any functional activity may be impaired or lost. Methods to orient intact proteins and improve function have been developed, and these typically involve an initially immobilized layer that forms complexes with the desired protein added secondarily. Examples include protein A, which binds IgG antibodies (8), nickel chelators such as Ni-(nitriloacetic acid) that bind proteins containing histidine tags (1), and commercial products that bind other genetically encoded peptides. Proteins also provide a means to pattern small-molecule ligands (e.g., antigenic groups) that bind to specific receptors on cells by conjugating these molecules to proteins prior to deposition on the surface.

Lipids. Supported lipid bilayers (reviewed in Reference 7) form on glass or silica surfaces by spontaneous fusion of vesicles containing fluid-phase lipids (16, 44). These planar bilayers have proven useful for microscopic examination of cell surface or cell-cell interactions (28, 43), and they can be stably patterned with micrometer dimensions by μCP (36, 48) or polymer lift-off (61) methods. Lipids are versatile materials; the composition can be varied and the headgroups can be selectively conjugated with small molecules such as fluorophores, small-molecule ligands, or other functionalities (e.g., biotin that can bind to avidin in sandwich arrangements). Similarly, proteins can be presented in an oriented and mobile manner in patterned bilayers if they are appropriately lipidated [e.g., by conjugation to glycosylphosphatidylinositol, GPI (12)].

Self-assembled monolayers. These hydrocarbon-based structures, which anchor to the substrate and interact closely along their chains to form a carpet-like presentation, are widely used in fabrication technology (reviewed in References 57 and 72). They are highly versatile and can control the surface physicochemical properties as well as present selected groups. Typically self-assembled monolayers (SAMs) are formed by coupling long-chain alkanethiols to a gold surface or alkylsilanes to surfaces such as silica and glass. SAMs containing oligomers of polyethylene glycol (PEG) in terminal regions have been valuable in many biological applications because this hydrophilic material resists nonspecific protein adsorption and cell adhesion (30). SAMs can also be terminated with a variety of functional groups for conjugation to a wide range of molecules and proteins (8, 56, 73, 82). A related self-assembly scheme with additional versatility is a polymer brush, typically assemblies of block copolymers in which the functional block is exposed. These can be patterned on surfaces with surface-initiated polymerization, where the initiator is patterned followed by addition of monomeric units and in situ polymerization (72).

EARLY APPLICATION OF PATTERNED SURFACES: FUNCTIONAL CONSEQUENCES OF CONTROLLED CELL ADHESION

Responsive interactions of cells with surfaces are fundamental to physiology, and if disregulated they can lead to pathological conditions such as tumor growth. In physiological tissues as well as in plastic culture dishes, adhesion of cells to substrates is primarily mediated by integrins in the plasma membrane binding to extracellular matrix (ECM) proteins. These cell surface interactions play a critical role in cell cycle regulation, leading to proliferation or death (5, 6), and they also modulate receptor-mediated responses to other signals. Surface patterning with μCP made it possible to examine systematically the importance of the spatial dimensions over which these chemical interactions occur. A pioneering study on endothelial cells in the late 1990s (13) utilized SAM-coated surfaces, with islands of ECM proteins (e.g., fibronectin), on a hydrophilic background that resisted nonspecific binding. Cell spreading was thus controlled by cell surface integrins binding ECM, and in this manner, cell size and shape were restricted to the patterned features. The authors could also vary the extent of cell spreading while keeping the direct contact area constant by adjusting the pattern feature sizes and the spaces between them. They correlated the imposed cell shape and spreading with cell cycle regulation, and found that the cellular footprint plays a significant role in switching between cell growth, proliferation, and apoptosis.

These and related studies also pointed to the importance of the actin cytoskeleton in mediating these cellular responses through cytoskeletal anchorage to the substrate via integrins in focal adhesion complexes (39). In the last several years a large number of studies have taken advantage of patterned surfaces to study spatial control of cell surface interactions and address specific questions of morphology and adhesion (25, 50, 79, 80), dendritic branching (15), migration (42), and mechanotransduction (how cells convert mechanical signals into chemical responses) (4, 19, 37, 52, 64, 78). As a recent, quantitative example, mechanical interactions were measured between cells and microfabricated surfaces containing needle-like posts made of elastomeric PDMS. Cells attached to and deflected multiple fibronectin-coated posts, and the degree of deflections could be interpreted in terms of the local subcellular distribution of traction forces (4, 78).

SPATIAL REGULATION IN RECEPTOR-MEDIATED CELL SIGNALING

Cells respond to chemical messages (e.g., antigens, growth factors, and cytokines) in the environment by means of specific cell surface receptors. Binding of these molecular stimuli to receptors stimulates transmembrane and intracellular signaling events that result in a global cellular response. In initial events, supramolecular complexes assemble dynamically at the plasma membrane as the signaling begins to cascade, bringing together the interactive proteins. Spatial and temporal targeting of signaling components in the membrane on micron and submicron scales is critical for overall efficiency and regulation of the cellular response. If the environment is engineered, then the spatially regulated response of the cell to defined stimuli can be investigated by monitoring redistributions of selectively labeled components or alterations in signaling pathways. Patterned surface approaches lend themselves readily to signaling that involves specialized regions or interactions such as membrane domains formed by clustered receptors or synapses formed at a cell-cell interface. These are features of immune cell signaling, and studies on IgE receptors and T cell receptors (TCRs) are

Integrins: family of transmembrane receptor proteins involved in adhesion of the cell to the ECM and to other cells

ECM: extracellular matrix

Focal adhesion: dynamic assembly of protein complexes formed on cells anchored to the ECM as a consequence of integrin activation, and through which the cytoskeleton of a cell connects to the outside environment

TCR: T cell receptor

Lipid rafts: dynamic domains in cell membranes, enriched in lipids with ordered acyl chains and cholesterol, that facilitate membrane compartmentalization involved in processes such as receptor-mediated signaling

DNP: 2,4-dinitrophenyl

making rapid progress with spatial and chemical control to gain new information about cellular mechanisms.

IgE Receptor Signaling

IgE receptors on RBL (rat basophilic leukemia) mast cells are a useful model for understanding signaling in hematopoietic cells and the role of the plasma membrane in this process. FcεRI, the high-affinity receptor for IgE, is, like the TCR, a member of the multisubunit immunoreceptor family that mediates cell activation in response to foreign antigens. IgE binds via its Fc segment to its surface receptors on mast cells or basophils, and the variable domains in the two Fab segments provide specific antigen recognition. As depicted in **Figure 2**, cross-linking of IgE-FcεRI by specific antigens (multivalent ligands) initiates the transmembrane signal (reviewed in Reference 26). Initial steps involve stable association of the clustered receptors with ordered regions of the plasma membrane (commonly called lipid rafts) where they encounter active tyrosine kinases (Lyn, of the Src family) that are anchored by acyl chains to the membrane inner leaflet. Tyrosine phosphorylation of FcεRI subunits by Lyn results in recruitment and phosphorylation of cytoplasmic Syk tyrosine kinase (of the Syk/Zap-70 family), which in turn phosphorylates phospholipase C (PLC) along with several other substrates. This leads to downstream signaling steps, such as Ca^{2+} mobilization, and results in release of secretory vesicles (degranulation) and other cellular responses.

Spatial regulation arises early in the signaling process at two stages: (*a*) ligand-dependent cross-linking of the IgE-FcεRI complexes required for cell activation and (*b*) selective targeting of signaling proteins to the region of the activated receptors. Our collaborative investigations of these questions utilize monoclonal IgE that is specific for the hapten 2,4-dinitrophenyl (DNP). DNP-conjugated ligands can be tested directly for binding to IgE-FcεRI on RBL cells, and the cell activation consequences of cross-linking these receptors can be assessed by monitoring various signaling steps including degranulation.

Structurally defined ligands to examine structural constraints in IgE-FcεRI-mediated signaling pathways. We have investigated the first stage of spatial regulation with a bottom-up approach of nanobiotechnology: synthesizing multivalent ligands with defined architectural features that control the manner in which the IgE-FcεRI come together when cross-linked to activate the cells. In one study we synthesized flexible spacers of poly(ethylene) glycol in the range of 10 nm and found that these effectively cross-link the two binding sites of IgE intramolecularly. Correspondingly, these serve as potent inhibitors of intermolecular IgE cross-linking and cell activation and provide a potential lead for allergy therapeutics (3).

To investigate signaling mechanisms stimulated by cross-linked IgE-FcεRI, we prepared bivalent ligands with rigid spacers by conjugating DNP to 5′ ends of double-stranded DNA (dsDNA) which has a persistence length of ~50 nm (81). These ligands stimulated low-level degranulation and also revealed a length dependence, such that bivalent ligands with rigid spacers of 4–5 nm were more effective than longer ligands of the same structure (62). Because trimeric and larger cross-linking of IgE receptors causes significantly higher levels of cellular responses (24), we took advantage of branched dsDNA constructions (59, 71), in particular a series of Y-shaped trivalent ligands with rigid arms of different lengths (76) (**Figure 2**). We found that the trivalent Y-DNP_3 ligands trigger robust signaling responses and that stimulated phosphorylation of Lyn and other steps leading to degranulation depend on ligand length. However, another pathway leading to Ca^{2+} release from intracellular stores does not show this length dependence. This spatial differentiation provides direct support for transphosphorylation as a key mechanistic step in signaling, leading to degranulation, and also

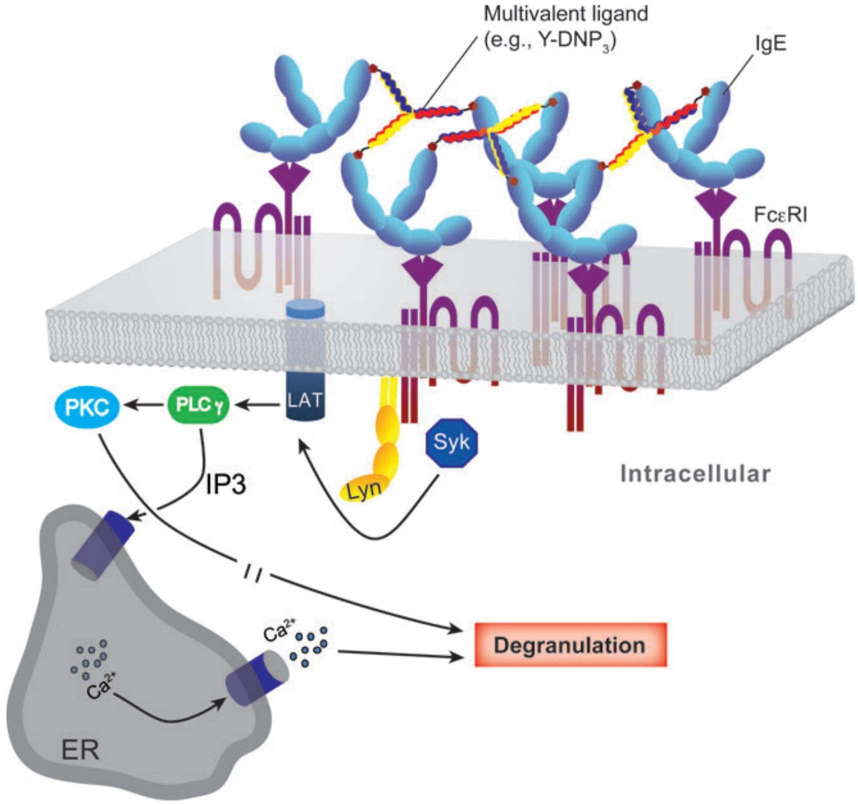

Figure 2

Basic scheme for IgE-receptor (IgE-FcεRI)-mediated signaling in mast cells. Intracellular signaling is initiated after FcεRI are clustered by cross-linking of bound IgE by multivalent ligand. This clustering drives association with lipid rafts, where active Lyn kinase phosphorylates the immune tyrosine activation motifs of the FcεRI β and γ subunits. Lyn binding directly to phosphorylated FcεRI-β amplifies this response, and Syk tyrosine kinase is recruited to phosphorylated FcεRI-γ where it is activated by Lyn. Phosphorylation by active Syk causes activation of other downstream signaling molecules such as LAT (linker for activation of T cells) and phospholipase C (PLCγ). Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by PLCγ produces inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, which are responsible for the release of Ca^{2+} from endoplasmic reticulum (ER) stores via IP3 receptors (leading to influx of extracellular Ca^{2+}) and activation of protein kinase C (PKC), respectively. These and other pathways are responsible for mobilization of intracellular Ca^{2+} and consequent degranulation.

reveals branching of pathways in signaling events.

Patterned ligands to examine membrane-compartmentalized signaling. Spatial targeting of signaling components at the plasma membrane has been a subject of considerable interest and accumulating evidence points to the participation of membrane domains, including lipid rafts (2, 40, 91). IgE-FcεRI were among the first complexes shown to involve lipid rafts in the signaling mechanism, using a number of correlative approaches that related physical properties of these membrane complexes with their biological consequences (reviewed in Reference 33). However, direct methods to investigate spatial rearrangements in the membrane and targeting of components were initially limited by typical heterogeneity of the

CTB: cholera toxin B

cells and stimuli, as well as by the diffraction limit of light. For example, we originally observed with fluorescence microscopy that Lyn coredistributes with antigen-cross-linked receptors in large patches that form after cold incubation for several hours (35). By fabricating surfaces that are patterned with receptor-specific ligands, we examined labeled cellular components coredistributing with the receptor clusters in the same pattern, in real time at 37°C and more physiological conditions (61, 89).

Patterned-supported lipid bilayers prepared by polymer lift-off for these studies contained, as desired, fluorescent lipid analogs for visualization as well as phospholipids modified with DNP for specific binding of anti-DNP IgE on RBL cells (61). **Figure 3*a*** shows a schematic of cells incubated with patterned bilayers containing specific ligand. Clustering of IgE-FcεRI on the cell surface is visualized with fluorescently labeled IgE as the cells settle on the substrate (**Figure 3*b***), and, as expected, cells are activated as observed by their ruffling response and secretory activity. Coredistribution within the cell of fluorescent membrane probes or GFP-labeled intracellular components can be monitored simultaneously. Intracellular enzyme activities, in particular tyrosine phosphorylation, can also be monitored with the use of labeled antibodies and cells that are fixed at specified time points. We found that intracellular tyrosine kinase activity is visualized (with antiphosphotyrosine antibodies) within the same few minutes that the cells settle on the patterned substrate and IgE-FcεRI clusters on the cell surface, whereas detectable accumulation of the Lyn-GFP kinase occurs only after about 15 min (**Figure 3*b***). A GFP construct containing only the palmitate/myristate (PM) acylated region of Lyn that anchors it to the membrane also coredistributes with clustered IgE-FcεRI but at somewhat longer times (~45 min). Under these conditions, the inner leaflet lipid PIP_2 bound to a specific GFP construct (PH domain of PLCδ) does not coredistribute with the clustered IgE-FcεRI, nor do outer leaflet lipid raft markers, including fluorescent lipid $DiIC_{16}$ and fluorescently labeled cholera toxin B (CTB) bound to ganglioside GM1 (89).

The flat surfaces with patterned lipid bilayers lend themselves to other optical measurements, including fluorescence photobleaching recovery. We used this approach to evaluate the dynamic nature of the proteins concentrating in the regions of patterned ligands and clustered IgE-FcεRI. Whereas IgE is immobilized by binding to its ligands, **Figure 3*c,d*** shows that Lyn-GFP and PM-GFP repopulate the bleached regions, indicating that diffusive exchange occurs for these membrane-anchored proteins while the environment that causes their preferential localization is maintained. Lyn-GFP exhibits a mobile fraction that is significantly smaller than that for PM-EGFP. This difference suggests that the SH2 and SH3 protein domains in the Lyn construct (but not in the PM construct) are involved in protein-protein interactions in the region of the clustered IgE-FcεRI, as would be expected for this kinase that phosphorylates FcεRI and other substrates during early stages of signaling.

GFP-actin also coredistributes with the clustered IgE-FcεRI, pointing to participation of the actin cytoskeleton in observed coredistributions with the clustered receptors. Cytochalasin D inhibits actin polymerization, and incubation of cells with this reagent prior to the patterned bilayers prevented detectable GFP-actin, Lyn-GFP, and PM-GFP, but not clustering of IgE-FcεRI or the tyrosine phosphorylation activity that occurs at early times. These results are consistent with the progressive nature of targeted signaling and our current picture based on these and several other types of data (34): Initial phosphorylation of clustered IgE-FcεRI by Lyn in ordered membrane domains creates new binding sites for Lyn and assembly of other signaling components, and the actin cytoskeleton further stabilizes and regulates these protein-protein interactions. Overall, these studies demonstrated that patterned lipid bilayers provide new information and insights about spatial

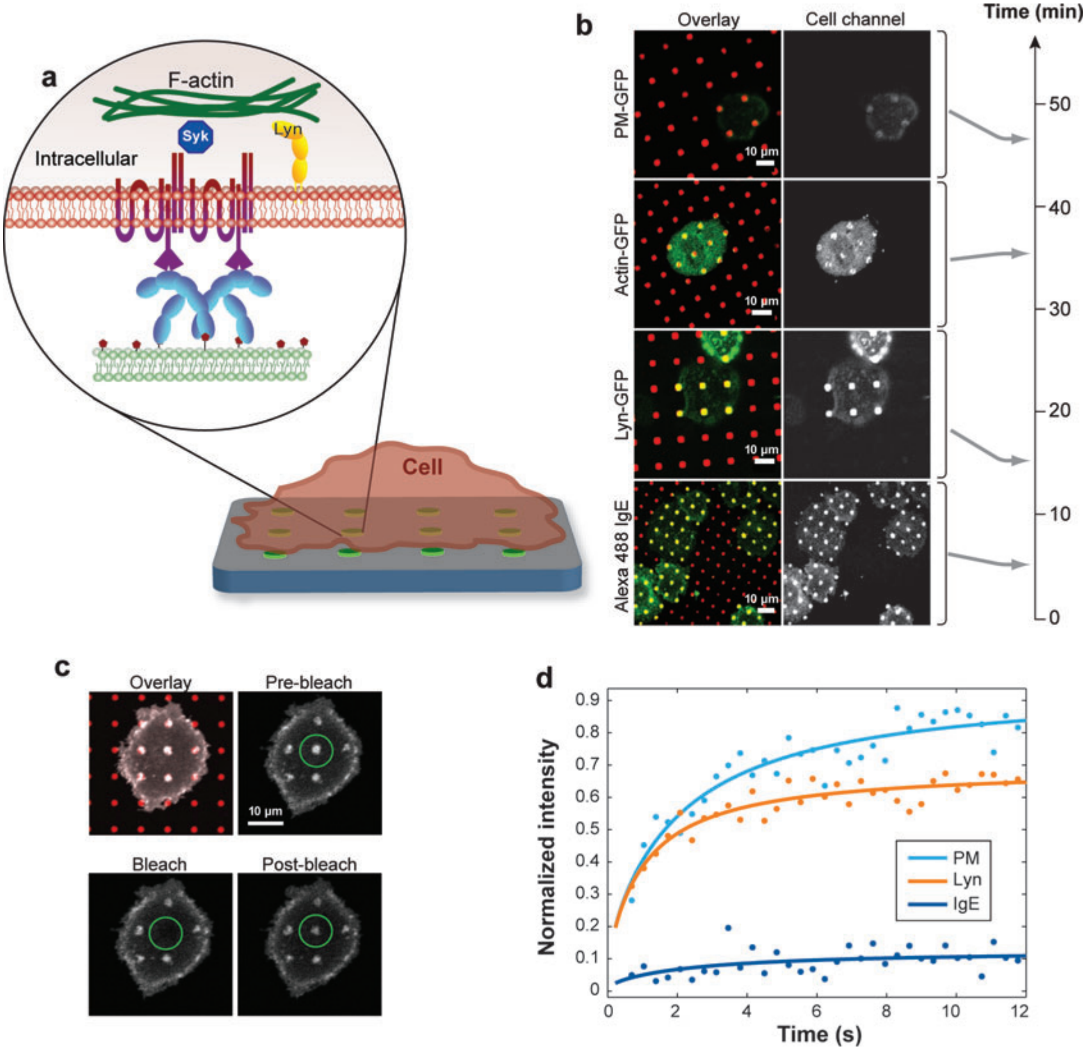

Figure 3

Patterned lipid bilayers to investigate spatial regulation of IgE-FcεRI-mediated signaling. Supported lipid bilayers, patterned by the parylene lift-off method contain a fluorophore (for visualization) and DNP conjugated to dipalmitoylphosphoethanolamine (for binding anti-DNP IgE). After incubation of RBL cells with these substrates, IgE-FcεRI on cell surface are clustered into the same pattern, and assembly of signaling components within the cell can be visualized with selective fluorescent labeling and microscopy. (*a*) Schematic of IgE-FcεRI clustering on cell surface by the patterned DNP ligands. (*b*) Confocal micrograph of fluorescently labeled cells interacting with the patterned surface. Colocalization of different GFP-labeled intracellular components (*green*) with the patterned bilayers (*red*) occurs at distinctive times; arrows correspond to the approximate time for the first appearance of clustering of the different probes. Concentrated tyrosine phosphorylation is observed by about 5 min, significantly before Lyn kinase accumulates detectably in the patterned regions (89). (*c*) Fluorescence photobleaching recovery in a cell membrane labeled with a GFP construct. In this representative example, cells were labeled with Lyn-GFP. A single spot was bleached in patterned regions where IgE-FcεRI were clustered, and recovery is monitored after the initial bleach. (*d*) Fluorescence photobleaching recovery curves show redistribution of Lyn-GFP and PM-GFP to be dynamic, and the latter has a higher mobile fraction than the former. Alexa 488–labeled IgE-FcεRI is immobilized by binding to the patterned ligand and shows no recovery after photobleaching. Adapted from References 87–89.

(micron scale) and temporal (minutes) aspects of receptor-mediated responses, including phosphorylation activity, selective targeting of signaling components, involvement of the cytoskeleton, and uncoupling of outer and inner leaflet components of lipid rafts. Moreover, the periodicity of the patterns enables reliable and quantitative evaluation of localized receptors and subsequent signaling events.

Patterned bilayers to examine cytoskeletal interactions with clustered IgE-FcεRI. The actin cytoskeleton and actin binding proteins participate in integrin-mediated adherence of cells to substrates via focal adhesion complexes. Consistent with studies on T cells showing interplay between integrin-cytoskeletal interactions and receptor-mediated signaling pathways, RBL mast cells show enhanced levels of stimulated degranulation when they are adherent to ECM (29), indicating cross talk between both pathways (46, 67). The picture is complicated by other potentially relevant structural interactions such as that between Lyn and the cytoskeletal adaptor protein paxillin, which was previously shown by co-precipitation studies with cell lysates (53). The use of patterned bilayers provides the means to spatially separate cytoskeletal interactions with integrins from those with clustered IgE-FcεRI, thus dissecting the contributions from each of these pathways.

Using patterned bilayers presenting DNP, we observed that, in addition to actin, several focal adhesion proteins such as vinculin and paxillin concentrate in the RBL cells under the clustered anti-DNP IgE-FcεRI. Under the same conditions, a GFP construct of α-5 integrin (the most prominent integrin in RBL cells) is excluded from the regions of the antigen-presenting patterned bilayers and preferentially associates with the silica surface between the patterned features. Thus, actin, vinculin, and paxillin associate with the clustered IgE-FcεRI independently of integrin association. Our recent biochemical studies in conjunction with the patterned bilayers provide evidence for paxillin participation in the regulation of signaling and suggest that this local recruitment is mediated by interactions with Lyn kinase (A.J. Torres, L. Vasudevan, D. Holowka & B. Baird, manuscript submitted).

Targeted trafficking of endosomes and secretory granules. Membrane trafficking among intracellular pools (e.g., Golgi, endoplasmic reticulum, endosomes) and the plasma membrane occurs continuously during the metabolic lifetime of most cells. Trafficking can be altered during the course of receptor-mediated cell stimulation, particularly in the case of mast cells and other cells where stimulation leads to degranulation of secretory vesicles. Our studies of RBL mast cells revealed that recycling endosomes significantly increase in net outward trafficking after IgE-FcεRI are clustered by antigen (58). The question arises: What spatial relationship do these stimulated release activities bear toward each other and the region of the initial stimulus? Polarized responses toward a contact interface are a common theme in intercellular signaling. For example, degranulation of a cytotoxic T cell in the synapse it forms with its target cell ensures specific killing. Similarly, mast cells release granules at the interface of a cell-size, antigen-coated bead (49). With the micron-size patterned ligands it is possible to differentiate a localized response on the scale of the distinctly clustered receptors from a polarized response across the entire cell substrate contact region.

We observed that stimulated outward trafficking of recycling endosomes marked with internalized CTB-GM1 is preferentially targeted toward the clustered receptors (88) (**Figure 4*a***). In contrast, fusion of individual secretory granules, marked with a genetically encoded probe (GFP-CD63), is targeted toward the cell substrate interface but spatially segregated from the clustered receptors and early-signaling complexes (**Figure 4*b***, left panel). Demonstrating spatial distinction required imaging the dynamic secretory events at the single-vesicle level

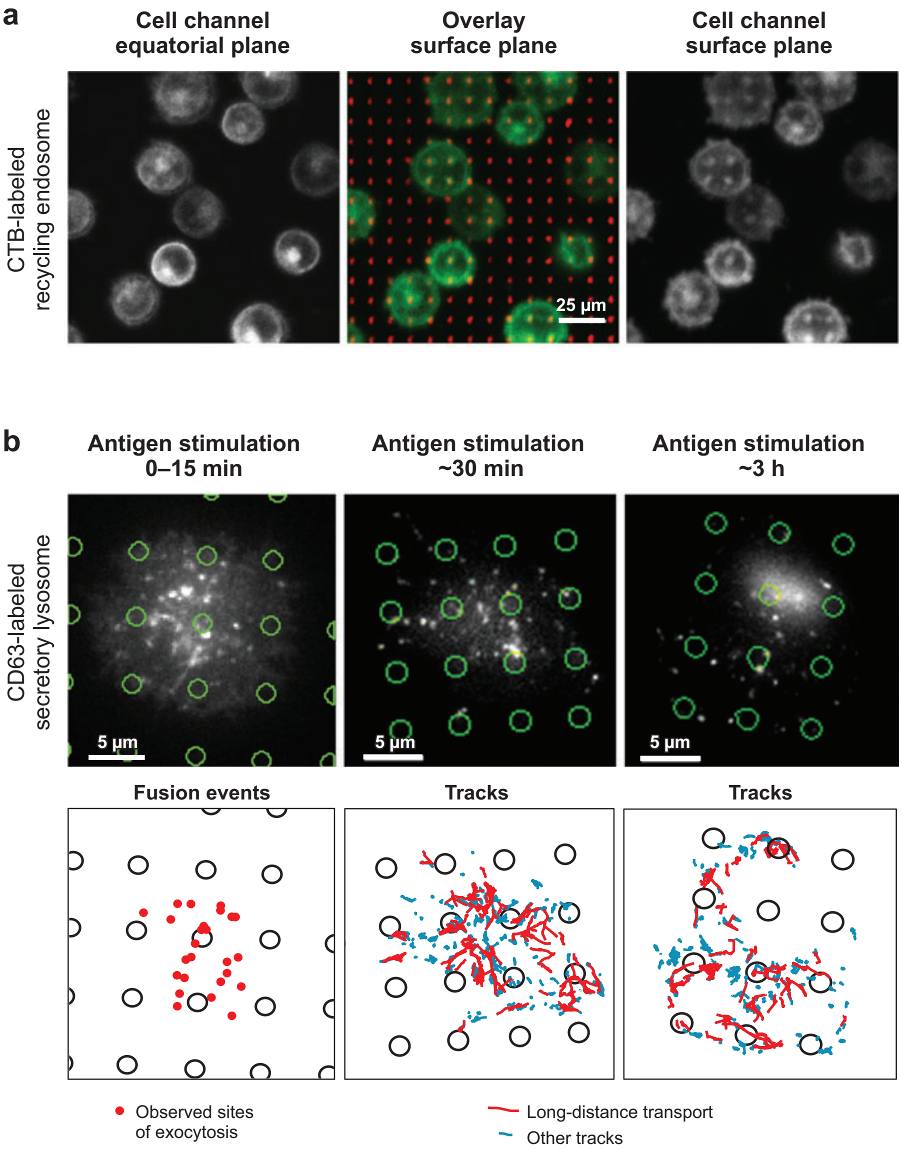

Figure 4

Differential trafficking of recycling endosomes and secretory vesicles in RBL cells stimulated by IgE-FcεRI clustered on patterned DNP ligands. (*a*) Recycling endosomes labeled by Alexa 488 CTB are targeted locally to the defined regions of clustered IgE-FcεRI. Confocal fluorescence micrographs show the intracellular pool of CTB when imaged at the equator and the enrichment of CTB at patterned features when imaged at the interface with substrate. (*b*) Secretory vesicles are labeled with CD63-GFP, and total internal reflection fluorescence microscopy (TIRFM) reveals that exocytosis occurs within 15 min, away from the defined regions of clustered IgE-FcεRI. During this early time period (<1 h) vesicle trafficking within the cell appears to be random. Prominent directed trafficking appears at later time points (>1 h), independent of the number of patterned features (that continue to cluster IgE-FcεRI) the cell is interacting with (87). All projections are based on tracks accumulated over a period of 2 min. Patterns are marked by green or black circles when overlaid with cell images or projected tracks. All image sequences were obtained by TIRFM at a frame rate of 5 per s. Reproduced from References 87 and 88, with permission from *The Company of Biologists* (for Reference 88).

APC: antigen-presenting cell

MHC: major histocompatibility complex

with total internal reflection fluorescence microscopy. We found that, although exocytosis of CD63-GFP-labeled secretory granules occurs within several minutes after stimulation on patterned surfaces at 37°C, only a small percentage of these granules undergo fusion. The majority of vesicles continue active trafficking that appear random without fusion (88) (**Figure 4*b***, middle panel). During later stages of activation, occurring on the timescale of hours, secretory granules acquire substantially more directed transport toward the receptors that remain clustered by the patterned haptens (**Figure 4*b***, right panel) (87). Secretory granules are involved in release of both prepackaged mediators (minutes after stimulation) and de novo synthesized cytokines, which occurs on the timescale of hours that corresponds to new protein synthesis (22, 27, 90). These different trafficking patterns may reflect distinct functional roles of mast cells in immediate hypersensitive reaction and in late-phase reactions.

T Cell Signaling

T cells, operating in the cellular immune response, are activated by physical association with antigen-presenting cells (APCs) and recognition of foreign peptides displayed in conjunction with the major histocompatibility complex (MHC) on the APC surface. TCR engagement of these complexes, together with integrin-mediated interactions, triggers the complex assembly of a microscale structure at the T cell–APC interface known as the immunological synapse (IS) (9, 68). Similar IS structures have also been identified between various effector and target cells (18), such as natural killer cells and target cells, and B cells and APCs (11). Supported lipid bilayers have been an important approach for investigating molecular redistributions that occur within the intact T cell membrane at the IS. In this experimental system the APC membrane is simulated by a supported lipid bilayer containing the relevant recognition proteins that are free to diffuse within the bilayer (28). By fabricating diffusion barriers before deposition of the bilayer, it is possible to constrain these dynamic redistributions, and the consequences for signaling can be observed. In one striking example (55), E-beam lithography was used to fabricate a grid of 100-nm-wide x 5-nm-high chromium strips with 1-μm spacing on a silica substrate. The supported lipid bilayer then prepared upon this grid contained mobile (GPI linked) forms of intercellular adhesion molecule 1 (ICAM-1) and MHC peptides, thereby representing APC. The chromium barriers confined lateral diffusion of both of these proteins within individual grid spaces. T cells interacted with this substrate primarily with TCR (specific for the MHC peptides) and the integrin LFA-1 (leukocyte function associated antigen-1, which is specific for ICAM-1), and the diffusion of these cognate membrane proteins was correspondingly restricted by the grid geometry. The TCR clusters formed within the grid space, with distinct orientation toward the center of the IS, suggesting a centralized, cytoskeletal based mechanism of synapse organization. New information about regulation of signaling within the IS was also revealed, in particular that the phosphorylation state of the TCR (visualized with antiphosphotyrosine antibodies) depended on their geometrical position. These results were consistent with a proposed mechanism indicating that spatial translocation of the TCR regulated their level of signaling activity.

Another interesting study (20) used photolithography to create multicomponent protein surfaces containing restricted, micron-size regions of anti-CD3 (which binds and clusters TCR) surrounded by a field of ICAM-1 (which binds LFA-1), taking advantage of tightly associating biotin–streptavidin conjugates to anchor proteins to the substrate (**Figure 5*b***). In contrast to lipidated ligands that are mobile in supported bilayers, these APC ligands were immobilized to the defined regions on the substrate and thereby designed to constrain the geometry of the IS forming on

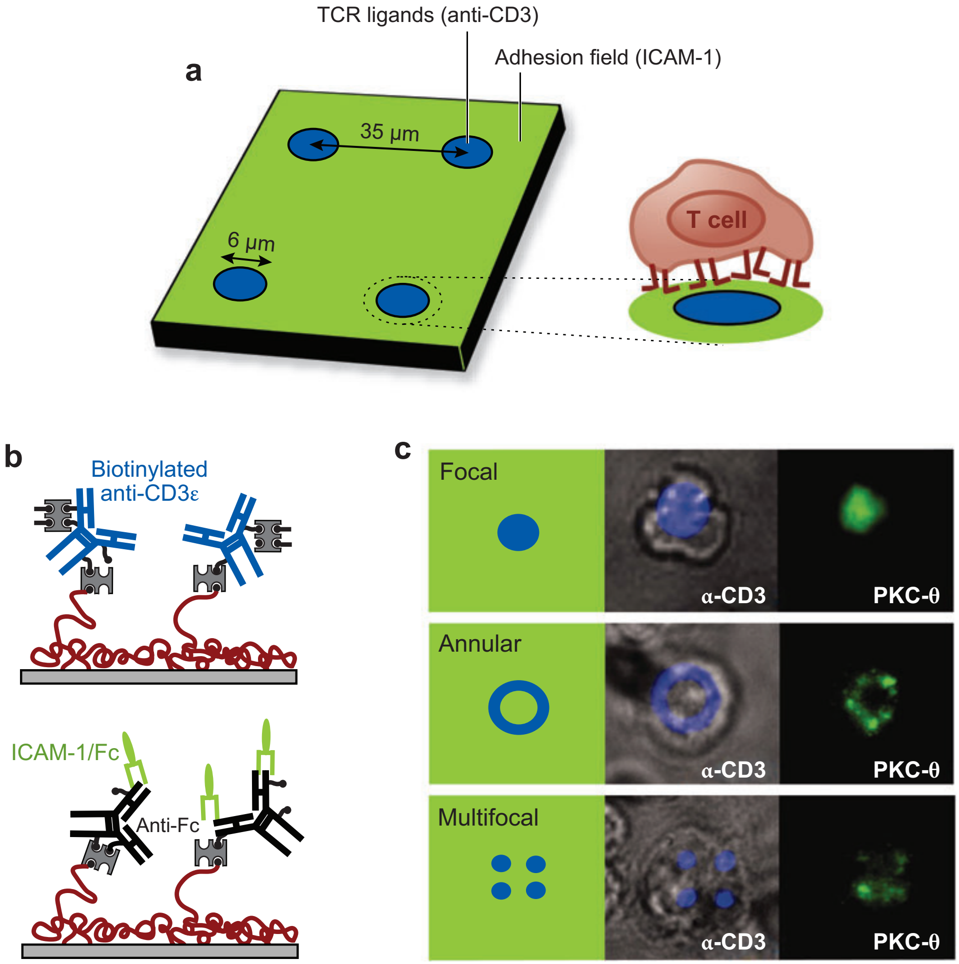

Figure 5

Patterned arrays used to investigate spatial constraints of immunological synapse, which forms between T cells and antigen-presenting cells (APCs). (*a*) Cartoon of a T cell interacting with the substrate differentially micropatterned with specific ligands. (*b*) Biotinylated proteins are immobilized by avidin-mediated sandwiching with biotin groups on a polymer resist. Biotinylated anti-CD3 binds to TCR, and biotinylated anti-Fc binds to Fc-ICAM-1 which binds to LFA-1. (*c*) Schematics of the anti-CD3/ICAM-1 substrate patterns with distinctive geometries used to activate the cells (*left panel*), and representative bright-field and immunofluorescence images at the T cell–substrate plane. Fixed cells showed different redistribution patterns of the signaling component PKC-θ (*green*) after incubation on substrates for 20 min. Adapted from Reference 20, copyright *Proc. Natl. Acad. Sci. USA* 2006.

T cells that could adhere to and be activated by these surfaces (**Figure 5*a***). Distinctive geometries were evaluated (**Figure 5*c***): focal (a central disc of anti-CD3 to resemble a normal IS), multi-focal, and annular. The authors observed that T cells stably interacted with and were activated by surfaces patterned with focal spots, whereas cells interacting with surfaces that prevented centralized clustering of TCR did not form the stable contacts that led to the same level of activation events. As exemplified by these two studies, spatially and chemically patterned surfaces are providing new insight into the mechanisms operating within the IS, particularly the functional relationship between the geometry of receptor-ligand interactions and T cell activation.

ZMW: zero mode waveguide

NANOAPERTURES TO EXAMINE PLASMA MEMBRANE DYNAMICS WITH SINGLE-MOLECULE RESOLUTION

As described in sections above, surfaces patterned with micron-size features have proven valuable in providing new information about spatial regulation of cell signaling that occurs proximal to the plasma membrane. However, visualization typically requires accumulation of many molecules in an area large enough to resolve. Ultimate elucidation of cellular homeostasis and stimulated responses requires investigations of molecular dynamics and interactions that occur on the length scale of ∼100 nm and may be obscured by visualization on the micron scale. E-beam lithography can be used to pattern on this scale, and a study on RBL cells was carried out with patterned SAMs on gold. The results demonstrated that anti-DNP IgE bound to FcεRI coredistributed on these patterned features when the SAMs were conjugated with DNP, and LAT accumulation and stimulated tyrosine kinase activity were visualized in the same regions (73). Although it was possible to pattern these features as small as 50 nm, visualization by fluorescence microscopy with live cells was not practical below 600 nm.

With other fabrication techniques, however, the diffraction limit of light can be overcome. Optical nanostructures, including zero mode waveguides (ZMWs), can be constructed to confine fluorescence excitation to subdiffraction volumes. These are fabricated as small holes, about 100 nm in diameter, in a thin metal film. Because of the subwavelength dimension, the ZMWs exhibit no propagating modes. Instead, a small evanescent field is generated with a typical decay length of 30 nm. Thus, illumination of the apertures confines the excitation volume to atto (10^{-18}) to zepto (10^{-24}) liters. The ZMWs were initially used to examine diffusion of fluorescent lipid probes on model membranes (70) and single-enzyme turnover of fluorescent substrates with micromolar concentrations (51, 69). Subsequent studies showed that the plasma membrane of live cells can be probed with high sensitivity with these optical nanostructures (21, 54, 85).

Characterization of RBL mast cell interactions with ZMW using a combination of fluorescence and electron microscopy showed that plasma membranes from live cells penetrate these nanostructures (**Figure 6**). Cellular exploration of the nanoapertures depends on actin polymerization but not on microtubules, suggesting that cells extend filopodia-like membranous extensions that elongate by the polymerization of actin filaments. The presence of actin filaments within ZMWs was confirmed by monitoring the fluorescence from genetically encoded GFP-tagged actin as it polymerized within the structures and entered the excited volume. This positioning of the plasma membrane into the apertures allows high-resolution examination of fluorescently labeled molecules. Fluorescence correlation spectroscopy (FCS) (reviewed in Reference 31) was used to monitor the diffusion of DiIC_{12} and DiIC_{16}, fluorescently labeled lipid analogs in plasma membranes, and these exhibited distinctive diffusive behaviors, suggestive of different membrane environments. These optical nanostructures offer the unique advantage of studying single-molecule dynamics in the plasma membrane, or within close proximity, at physiologically relevant molecular concentrations.

CONCLUSIONS AND PERSPECTIVES

Engineered surfaces that are spatially and chemically patterned on the micron scale are valuable for examining receptor-mediated signaling, including participation of the cytoskeleton and constrained targeting of

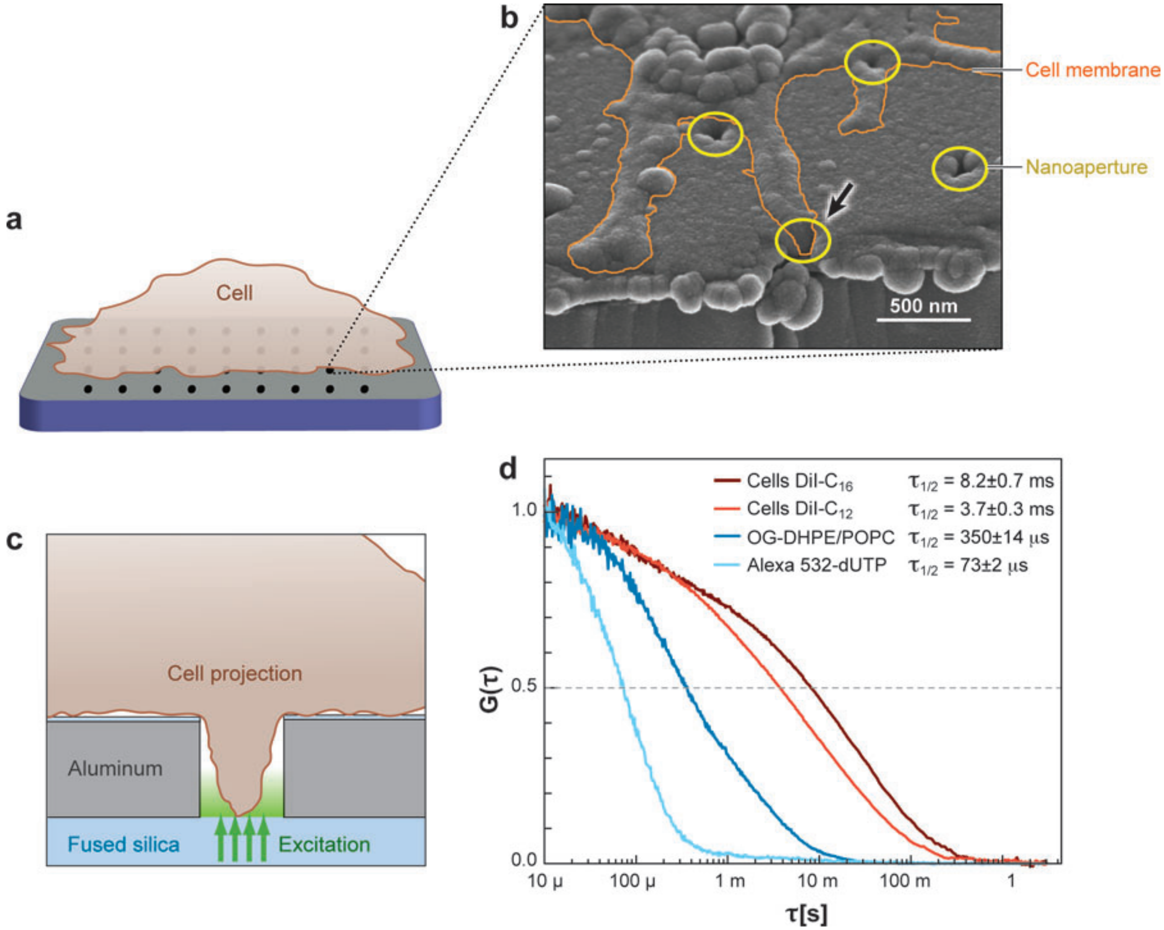

Figure 6

Invagination of RBL mast cells into arrays of zero mode waveguides (ZMWs) for observation of fluorescent membrane components. (*a*) Cartoon representing a cell interacting with the nanoapertures on the substrate. (*b*) Tilted cross-section of scanning electron micrograph showing cells exploring the nanoapertures after several hours of incubation at 37°C. The arrow points to a membrane protrusion entering the nanoaperture. (*c*) Schematic representation of membrane protrusion (not drawn to scale) illuminated at the bottom of the ZMW by an evanescent field. ZMWs have diameters of ∼100 nm and depths of ∼100 nm. (*d*) Normalized fluorescence correlation spectroscopy autocorrelation curves obtained with ZMW and compared for different systems: fluorescent lipid probes DiI-C_{16} and DiI-C_{12} in RBL cell plasma membrane, Oregon Green labeled DHPE in POPC model membranes, and Alexa 532-dUTP in solution. Residence times ($\tau_{1/2}$) within the excitation volume of the ZMW are estimated by the half-decay values of the curves. Adapted from Reference 54, with permission from *IOP Publishing*.

adhesion components, signaling pathways, and secretory vesicles. Together with current approaches of biochemistry and cell biology, these have already been successful in uncovering spatial regulation associated with immune cell signaling. Applications are not restricted to systems where receptor clustering is a necessary part of the activation mechanism. For example, micropatterned proteins or lipid-linked ligands could be designed to engage other receptors in discrete patterns to investigate stimulated recruitment and spatial organization of associated signaling components. The advantage of

Genetically encoded probes: engineered, tagged proteins that are expressed in living cells by transfection of corresponding DNA

the patterned ligand would be to increase the concentration of activated receptors into defined regions for visualization and analysis using fluorescence microscopy. Patterned surfaces have considerable flexibility, including design of the patterns as well as substrate composition and chemical modification to specify properties such as ligand specificity and two-dimensional mobility. Probes for visualizing cellular response mechanisms are also diverse, including genetically encoded probes (e.g., GFP conjugates) and probes incorporated into specific antibodies (for extracellular components or for permeabilized cells).

Most current studies with patterned surfaces are on the micron scale because this is easily accessible with relatively simple fabrication and chemical modification schemes, and also because this is the scale that is readily observable with fluorescence microscopy. However, this scale is large compared to the molecular scale, at which signaling initiates, and structural arrangements occurring on the micron scale may obscure the molecular interactions that bring them about. A promising step in the direction of reducing the length scale comprises the ZMWs and other optical nanoapertures that confine the observation volume to atto- or zeptoliters with spatial dimensions of ∼100 nm. FCS can be used to observe single-molecule dynamics, and fluorescence cross-correlation analysis can detect interactions of two components that are tagged with different fluorophores. The fabrication required for these nanostructures is considerably more demanding than that for micron-scale patterns, and it is also challenging to develop chemical modifications that restrict the stimulant to the same region of observation. However, with current capabilities it is reasonable to envision a device that confines both observation and stimulation to the length scale of molecular interactions, and also to probe temporal regulation with, for example, the use of photo-activatable ligands. An exciting near-term goal is visualizing individual molecular dynamics and associations as they occur in a living, responding cell. The ultimate importance of these technical accomplishments resides in cell biology problems that can be approached in these ways, which were not possible before. Essential to this success are increasing collaborations between the communities and the individuals who are advancing the fabrication and interfacing capabilities and those who will integrate necessary biological tools and knowledge.

SUMMARY POINTS

1. Engineered surfaces that are spatially and chemically patterned on the micron scale are valuable for investigating spatial regulation of cellular processes, including receptor-mediated signaling.
2. Studies with patterned surfaces and IgE receptors (FcεRI) in RBL mast cells have shown, for example, that signaling components target selectively to regions where receptors are clustered in a cytoskeleton-dependent manner, and that stimulated trafficking of secretory granules and recycling endosomes is differentially targeted.
3. Spatial resolution by microscopy is conventionally limited by light diffraction, but this can be overcome by fabrication schemes, such as ZMWs, that confine excitation regions to ∼100-nm dimensions; physiological concentrations of labeled components can be monitored at the single-molecule level.

4. An exciting goal within reach is observation of individual molecular dynamics and associations as they occur in a living, responding cell.
5. Continuing advances in nanobiotechnology to understand cell biology require close collaboration of life scientists, physical scientists, and engineers.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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