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Micropatterned ligand arrays to study spatial regulation in Fc receptor signaling

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Summary

Fc receptor signaling plays a fundamental role in the adaptive immune response. A plethora of Fc receptors (e.g. Fc gamma, Fc-alpha and Fc-epsilon) are expressed on different immune cells, including natural killer cells, macrophages, mast cells and neutrophils. Receptor clustering and activation by multivalent ligands or opsonized particles induces a signaling cascade that leads to targeted secretion of chemical mediators (i.e. histamine, cytokines and chemokines) and phagocytosis, among other responses. Spatial targeting and compartmentalization are common mechanisms of regulation in Fc receptor signaling. However, the tools for studying these dynamic interactions have been limited. To overcome these limitations in our model system, microfabricated surfaces containing spatially defined ligands are used to cluster and activate IgE receptors (FcεRI), involved in allergic responses by mast cells. Micron-scale control of cell activation allows investigation of spatially regulated mechanisms for intracellular signaling with fluorescence microscopy. This approach in conjunction with biochemical techniques has proven to be valuable for investigating immune receptor signaling.

1. Introduction

Cellular receptors for immunoglobulins (Fc-receptors) are widely expressed on the surface of highly specialized cells that form part of the adaptive immune system (1, 2). These cells are sensitized by binding of antibodies produced in response to foreign molecules. Receptor activation elicits different cellular responses that depend mainly on the cell and receptor type but also on the presence of other co-stimulatory signals (3). Such responses include: release of preformed mediators, de-novo protein synthesis, phospholipid metabolism, changes in cell morphology, endocytosis and phagocytosis. One important class of Fc receptors is the receptor for IgE (FcεRI), which is responsible for the release of chemical mediators that causes inflammatory and allergic reactions (4), and is found primarily on mast cells and basophils.

Cross-linking of IgE-FcεRI by multivalent antigen (ligand) triggers intracellular signaling events, leading to multiple cellular responses. In the earliest signaling events, antigen-induced clustering of IgE-FcεRI causes stable association with ordered lipid domains, cytoskeletal redistribution and Lyn kinase phosphorylation of the FcεRI β and γ subunits (5). The latter initiates a series of signaling events that induces Ca²⁺ mobilization and other downstream signaling steps leading to degranulation and secretion of preformed mediators (6).

Elucidation of signaling pathways involved in receptor signaling are crucial for the development of therapeutics and prevention of immune diseases. Current understanding of FcεRI signaling has been mostly dependent upon biochemical and genetic characterization

of protein interactions. Spatial orchestration of these signaling events is a critical aspect, but studies have been limited by these common approaches. We have recently established the use of microfabricated surfaces containing spatially defined ligands to investigate spatial regulation of cell signaling (7, 8).

A recent review discussing the applications of micro- and nanofabrication in receptor signaling and cell biology has been published elsewhere (9). Herein, we present a detailed description of an approach we developed to investigate spatial regulation in IgE-FcεRI signaling on RBL-2H3 cells by microfabricated surfaces using the polymer lift-off method and 2,4-dinitrophenyl (DNP) ligands. It should be noted that even though the procedure described here is specific for the IgE receptor system, the same principles are applicable to other receptor systems and can be adapted by selecting the appropriate ligands and surface functionalization schemes.

2. Materials

2.1. Microfabrication

1. Parylene C dimer (Speciality Coating System, Indianapolis, IN)
2. Parylene Deposition System PDS 2010 LABCOTER (Speciality Coating System, Indianapolis, IN)
3. Computer assisted design (CAD) software.
4. Cleaning solution (piranha etch) composed of a freshly prepared mixture of concentrated sulfuric acid and 30% (v/v) hydrogen peroxide solution (3:1 v/v) (see **Note 1**).
5. Silicon wafers containing a ~ 50–100 nm oxide layer or glass wafers (optical quality for microscopy, see **Note 2**).
6. Positive photoresist (see **Note 3**).
7. Resist spinner and hotplates.
8. Chrome coated mask.
9. Developing solutions for dissolving exposed photoresist. The choice of developing solution depends on the photoresist used.
10. Optical pattern generator for mask design (e.g. DWL66 Heidelberg Mask Writer)
11. g-line (435nm) or i-line (365nm) stepper
12. Reactive Ion Etcher (e.g. Oxford PlasmaLab 80+)

2.2. RBL-2H3 cell culture

1. Minimum Essential Medium (MEM; Gibco, Invitrogen) supplemented with 20% (v/v) Fetal Bovine Serum (Atlanta Biologicals, GA) and 50µg/mL Gentamicin (Gibco, Invitrogen).
2. Trypsin Solution (Gibco, Invitrogen).

2.3. Supported lipid bilayer preparation

1. 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (16:0–18:1 PC) (POPC; Avanti Polar Lipids, Al).

2. 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[6-[(2,4-dinitrophenyl)amino]caproyl] (DNP-Cap-PE; Avanti Polar Lipids, Al).
3. 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Lissamine Rhodamine PE; Avanti Polar Lipids, Al). Stock solutions of lipids are prepared in chloroform at concentration ranging from 1–25 mg/mL. Store stock solutions in glass vials dessicated at –20°C.
4. Argon or nitrogen gas (Airgas Inc.).
5. Phosphate Buffered Saline (PBS; 137mM NaCl, 2.7 mM KCl and 10 mM sodium phosphate, pH 7.4).
6. Acrodisc 25mm syringe filter, 0.2µm HT Tuffryn membrane (Pall Co., NY).
7. Plasma cleaner PDC-32G (115V) (Harrick Plasma, NY) connected to a vacuum pump with a minimum pumping speed of 1.4 m³/hr and a maximum ultimate total pressure of 200 mTorr.
8. Parafilm M Laboratory Wrapping Film (Fisher Scientific).

2.4. Ligand carrier immobilization

1. (3-mercaptopropyl)trimethoxysilane (Sigma-Aldrich), stored under argon in a dessicator at 4°C.
2. 4-Maleimidobutyric acid N-succinimidyl ester, GMBS (Sigma-Aldrich). Prepare a 1M stock solution in Dimethylformamide (DMF) and store at –20°C in small aliquots (stable for weeks in DMF)
3. Toluene (Mallinckrodt Chemicals, NJ)
4. 2,4-dinitrophenylated bovine serum albumin, DNP-BSA (Molecular Probes, Invitrogen), conjugated to a fluorophore (e.g. Alexa 568 or Cy3).
5. Phosphate buffer (0.1M sodium phosphate and 0.15M NaCl, pH 7.3).

2.5. Transfection

1. Gene Pulser Xcell electroporator system (Bio-Rad Laboratories)
2. Electroporation buffer (137 mM NaCl, 2.7 mM KCl, 1 MgCl₂, 5.6 mM glucose and 20 mM Hepes (pH 7.4))
3. DNA construct encoding for the green fluorescent protein (GFP)-fusion protein of interest.

2.6. Cell activation

1. Monoclonal anti-2,4-dinitrophenyl IgE (Sigma-Aldrich).
2. Buffered Saline Solution, BSS (135 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose and 20 mM Hepes (pH 7.4)), containing 1 mg/ml BSA
3. 35 × 10 mm tissue culture dish.
4. Micro-patterned substrate.

2.7. Fixation and imaging

1. Formaldehyde 3.7%, freshly prepared in PBS from a 37% stock.

2. Bovine serum albumin (BSA)
3. Glass Bottom Culture Dishes, No. 1.5 (MatTek Co., MA)

3. Methods

3.1. Microfabrication

Due to the highly technical nature of the microfabrication process and the variations in instrumentation between different facilities, specific details are not described. Photolithography instrumentation is expensive and not easily accessible outside dedicated clean room facilities; moreover instrument models vary greatly between facilities, and some procedures will need to be adapted and/or optimized depending on the available instrumentation.

Many universities have access to clean room facilities and technical staff who train in the fabrication process. We suggest initial collaborations with groups with experience in microfabrication. The fabrication procedure described here is straightforward and can be easily followed by a person with basic knowledge in the field. Alternatively, some companies or facilities may be able to process custom orders.

The fabrication of the patterned substrate involves a series of steps that are summarized in Figure 1. Once the patterned parylene wafers are prepared, they can be stored for several months (in a dessicator) for further use. Immobilization of the biomaterial is then performed in the laboratory.

1. Design pattern with desired feature size and shape in a computer assisted design (CAD) software. The pattern size and spacing should be enough to include several features per biological cell and large enough (>500nm) for efficient visualization using fluorescence microscopy.
2. Design chrome mask. The mask is fabricated by transferring the CAD pattern using an optical pattern generator on a chrome (~80 nm layer) evaporated glass plate (5" by 5") coated with photoresist. After light exposure and photoresist development, the chrome is removed from areas unprotected by photoresist by using acid etch. Once prepared, the same mask can be used indefinitely for the photolithographic preparation of the patterned wafers and can be stored for years without significant lost of quality.
3. Clean wafer with piranha solution.
4. Coat the wafers with a thin layer (~1 μ m) of Parylene C polymer using the Parylene Coating System. The amount of parylene dimer loaded into the system and other instrument settings will have to be optimized to achieve the desired layer thickness.
5. Spin-coat wafers with photoresist to get a uniform layer of ~1.6–2.0 μ m and place wafers on a hot plate for ~0.5–2 min (soft-bake) to evaporate any excess solvent and promote adhesion of the resist to the wafer. Soft-bake conditions (baking time and temperature) will depend on the choice of photoresist.
6. Expose photoresist coated wafer to electromagnetic radiation through the mask (prepared previously as depicted in steps #1–2) by using a 5X g-line (435nm) or 10x i-line (365nm) stepper. The optimal focus and exposure time should be determined experimentally.
7. Place exposed wafers on a hot plate for ~0.5–2 min (post-bake) to anneal the surface. Post-bake conditions will depend on the choice of photoresist.

8. Dissolve the exposed photoresist (develop) using the appropriate solvent for the photoresist and characterize the photoresist film thickness. Some facilities have automatic instrumentation for wafer development such as the Hamatech-Steag wafer processors.
9. Etch wafer using reactive ion etcher (RIE) with oxygen as the reactive specie (see **Note 4**).
10. Dissolve remaining photoresist with acetone while spinning the wafer using a spin-coater.
11. Store wafer for future use.
12. Before ligand immobilization on the surface, cut silicon wafers into 8×8 mm pieces. These small pieces are used for cell experiments (see **Note 5**). When working with glass wafer, cut wafer into 22×22 mm pieces by scratching the surface on the side opposite to the parylene layer using a diamond scribing tool with the help of a rubber ruler. Carefully detach coverslip from a glass bottom culture dish (No. 1.5) and replace with patterned parylene glass piece by using an adhesive, such as Sylgard 182 silicone elastomer (Dow Corning), with the parylene layer facing the interior of the dish.

3.2. Ligand immobilization using supported membranes

Two different methods for immobilizing 2,4-dinitrophenyl (DNP) ligand to the patterned substrates are presented here. The first method requires supported lipid bilayers for ligand presentation while the other alternative method involves covalent coupling of a ligand carrier to the surface (see Figure 2). Both immobilization schemes are equivalent in their ability to induce receptor clustering. However, we find that supported lipid bilayers provide some significant advantages. Supported lipid bilayers tend to be inert and more resistive to nonspecific protein absorption, in contrast to the SiO_2 surface which is more prone to protein absorption and cell adhesion (10, 11). Our experimental system uses IgE antibodies specific to DNP groups. Cells are sensitized with anti-DNP IgE antibodies where they form tight complexes (high affinity, low dissociation) with FcεRI receptor on the cell surface. IgE-FcεRI clustering and activation by the immobilized ligands recruits a series of signaling proteins that, if labeled, can be visualized with fluorescence microscopy (Figure 3). For other experimental systems, the choice of ligand and immobilization scheme may need to be adapted (see ref. (9)). For example, biotin-streptavidin interaction can be used to tether biotinylated proteins on a surface modified with biotin groups attached to streptavidin (12).

1. Prepare a solution containing 89 mol% POPC, 10 mol% DNP-cap-PE and 1 mol% lissamine rhodamine PE by mixing stock solutions in chloroform (see **Note 6**). The amount of lipids used should amount to a total lipid concentration of 1mM after reconstitution in buffer (see step 3). Use a glass vial to mix the lipids.
2. Evaporate solvent with a stream of argon or nitrogen gas to create thin lipid film in a glass vial. Lipid films may be stored under argon for weeks at -20°C in a dessicator.
3. Hydrate film by adding ~2mL of PBS for 5min. The temperature of the buffer should be above the gel-liquid crystal transition temperature of the major lipid and should be maintained during the hydration period. The effects of buffer pH and ionic strength on supported lipid bilayer formation and stability are addressed in reference (13).
4. Vortex for 30s to create large multilamellar vesicles (LMV).

5. Sonicate the samples with a probe sonicator with suspension at $\sim 10^{\circ}\text{C}$ higher than the transition temperature of major lipids until the suspension becomes clear corresponding to unilamellar vesicles (between 5–10 min). Use water bath to control temperature and prevent overheating.
6. Filter the liposome suspension through a $0.2\ \mu\text{m}$ filter (Acrodisc) to remove particles deposited during sonication.
7. Plasma clean substrate (parylene patterned surface) for $\sim 10\text{s}$ to make surface hydrophilic.
8. Cut a piece of Parafilm M and place the patterned parylene substrate on the film, add lipid suspension, and incubate for $\sim 10\text{min}$. The hydrophobicity of the parafilm will cause the solution to remain on the substrate.
9. Fill four $100 \times 50\ \text{mm}$ crystallizing dishes with distilled de-ionized water.
10. Rinse the substrate vigorously in water iteratively in each of the crystallizing dishes. Use a small ($35 \times 10\ \text{mm}$ or smaller) dish to transfer the substrate from each crystallizing dish to the other to avoid drying the surface and hence destroying the bilayer. After the second rinse, mechanically peel of the parylene layer with tweezers. This will yield supported lipid bilayers patterned on the substrate. Rinse once more with water (see **Note 7**).
11. Place the patterned substrate on the $35 \times 10\text{mm}$ culture dish and replace water with BSS. Keep the substrate hydrated at all times.
12. Experiment with cells should ideally be done same day as lipid preparation (see **Note 8**).

3.3. Covalent ligand (attached to carrier protein) immobilization using silane chemistry (alternative method)

1. Rinse patterned parylene substrate gently with acetone and dry with nitrogen gas in a glass dish.
2. Prepare a 2% (v/v) solution of (3-mercaptopropyl)trimethoxysilane (MPTS) in toluene. Aminosilanes and their fumes are irritants and highly corrosive. The reactions must be done carefully in a chemical hood.
3. Incubate patterned parylene substrate with the MPTS solution for 20–30 minutes in the hood at room temperature in a glass dish to functionalize the surface with thiol groups. Rinse surface with acetone and dry using nitrogen or argon gas. When working with glass wafers, this reaction should be carried out before using adherents to attach the glass piece to the plastic dish (as described in step 3.1.13)
4. Rinse the surface with absolute ethanol and add the 2mM of the GMBS cross-linker solution in absolute ethanol for 1 hour (see **Note 9**).
5. Rinse substrate with phosphate buffer.
6. Immediately incubate patterned parylene substrate with 25–100 $\mu\text{g/ml}$ of protein (DNP-BSA) in phosphate buffer for 2 hours (up to overnight).
7. Rinse with phosphate buffer to remove unbound protein.
8. Mechanically peel-off parylene under buffer to reveal the patterned protein and rinse the surface with buffer.
9. Block surface with PBS containing 10mg/mL BSA for 2 hours (up to overnight) to minimize nonspecific binding.

3.4. RBL-2H3 cell culture

1. Grow adherent RBL-2H3 cells in media to pre-confluence in 25 cm² flask in a 5% CO₂ incubator.
2. Harvest cells using trypsin solution to detach cells from the flask and resuspend in media or BSS at 0.5×10^6 cells/mL.

3.5. Cell transfection

1. Harvest the cells and resuspend at 1×10^7 cells per ml in electroporation buffer.
2. Mix ~5–10 µg of plasmid DNA with 500 µL cells suspension.
3. Electroporate cells by using an exponential pulse of 280 V and 950 µF in a 4-mm cuvette by using a Gene Pulser Xcell electroporator system.
4. Recover electroporated cells (avoid floating dead cell debris formed during electroporation), plate cells in medium at ~60% confluency and incubate at 37°C for 24 h (see **Note 10**).

3.6. Cell activation with patterned ligand

1. Sensitize cells by adding IgE to a final concentration of ~0.5 µg/mL and incubate for 1 hour keeping cells in suspension at 37°C (overnight incubation of attached cells with IgE is also possible). Remove excess IgE by washing and resuspension in BSS containing 1 mg/mL BSA and resuspend cells at a concentration of $\sim 0.5 \times 10^6$ cells/mL.
2. Place patterned substrate on 35 × 10 mm tissue culture dish and add ~ 1mL of cell suspension (see **Note 11**).
3. Incubate cells with the patterned substrate for 15–45 min at 37°C. It will take about 5 minutes for the cells to settle and begin attaching to the surface. For live cell imaging, skip steps 5–6 and immediately image cell using fluorescence microscope.
4. After the appropriate incubation time, rinse the cells with BSS to remove cells that are still in suspension.
5. Fix cells by adding 3.7% formaldehyde (in PBS) and incubate for 10 min. Remove fixative and quench reaction by adding PBS containing 10mg/mL BSA and incubate for 5min.
6. Rinse cells with PBS. Fixed cells can be stored at 4°C in PBS with 0.01% (w/v) NaN₃, to prevent microbial growth, for weeks.
7. Image cells using fluorescence microscope. If using inverted fluorescence microscope and patterned silicon substrate, turn substrate upside down inside a glass bottom dish filled with PBS buffer to visualize fluorescence features (see **Note 12**).

4. Notes

1. Piranha solution is extremely corrosive and reactive, and handling and disposal should be done with extreme caution (always use a hood and protective equipment). Some facilities may have access to automated wafer cleaning instrumentation such as the Hamatech Wafer Processor that are useful for reducing exposure to dangerous chemicals.

2. Fabrication on glass wafer is desired when a transparent substrate is required. For example, total internal reflection fluorescence microscopy (TIRFM) requires a transparent substrate for propagation of an evanescent wave. Fabrication of glass wafers involves the same steps as silicon wafers but can be more difficult because of the fragility and thickness of optical glass wafers, which are much thinner (0.16–0.19 mm) than standard silicon wafers (>0.4 mm). For confocal and epifluorescence imaging, silicon wafers are sufficient. In some confocal systems scattered light from the silicon surface can be visualized as background noise. This background noise is small compared to the signal obtained from the labeled cells and can be improved by collecting fluorescence from wavelength far from the excitation wavelength.
3. Many different options are available for photoresist and the choice will mainly depend on the desired feature size and the available instrumentation (i.e. i-line or g-line stepper). We found that both SPR 220-3.0 (i-line or g-line) and SPR 955 cm-2.1 (i-line) (Rohm and Haas Electronic Materials LLC, MA) work well with our fabrication scheme.
4. The optimal time for oxygen etching should be optimized experimentally. Ideally the majority of the ~1.6–2µm photoresist layer should be consumed without exposing the parylene layer. This step is important because under etching will leave residual parylene on the patterns and over etching will consume the parylene layer. After etching the remaining thickness of the parylene features should be confirmed with a profilometer.
5. The lattice structure of the silicon wafers is responsible for its brittle nature. This property allows easy cutting of wafers by slightly scratching a corner with a diamond scribing tool and applying small pressure on both side of the scratch.
6. The ligands are chosen to be specific for the experimental setting. For other receptors the lipid composition may be changed, and other lipids with modified head groups may also be used for ligand presentation. For example, avitin-biotin interactions exploited by using biotinylated lipids. As an alternative to ligand presentation with supported lipid bilayers, an alternate method for covalently immobilizing ligands is presented in section 3.3.
7. Once the supported lipid bilayer has formed it is important to keep it hydrated. Exposure to air will destroy the supported lipid bilayer (13).
8. Although it may be possible to use the supported membranes days after preparation, it is recommended to use them within a few hours of preparation. Supported membranes tend to expand within hours of preparation in a pH and ionic strength dependent manner (13), and this will affect the quality of the patterned ligands.
9. After the MPTS covalently attaches to the silicon dioxide surface, its thiol group reacts specifically with the maleimide moiety on the GMBS cross-linker. This activates the surface with succinimide residues that can react with amine groups of an antibody or other protein to form stable amide bonds (14, 15).
10. Chemical transfection methods may also be use to introduce GFP encoding DNAs into the cells. We chose to describe electroporation for its simplicity.
11. Although supported lipid bilayers are usually a better choice for preventing non-specific interactions, one advantage of covalent immobilization of proteins is that the modified surfaces can be stored for weeks at 4°C without significant loss of functionality and short periods of drying do not affect the immobilized ligand.

12. In cases where the protein to be investigated is not available as GFP fusion protein immunofluorescence microscopy can be used to detect proteins. In such cases, fluorescent lipids cannot be used to visualize the patterned features because the detergent used to permeabilize cells (Triton X-100) partially dissolves the supported lipid bilayers. To overcome this technical limitation, fluorescently labeled anti-DNP IgE can be used to sensitize the cells and hence visualize the clustered receptors (see ref. (11) for example). Covalently immobilized protein carriers are not affected by permeabilizing reagents.

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Figure 1. Schematic diagram of patterned surface micro-fabrication and deposition of biological material (e.g. ligand).

Figure 2.

Micro-patterned ligand array after polymer lift-off. A) Array of immobilized fluorescent BSA visualized after partially peeling the parylene layer. Nonspecific bound material is removed with the polymer, leaving a surface with clearly defined features and low background fluorescence. B) Cartoon showing possible ligand immobilization schemes. The ligand of interest (e.g. DNP) can be incorporated into a supported lipid bilayer (top) or covalently attached to the surface via a protein carrier such as BSA (bottom).

Figure 3.

Cell activation with the patterned lipid bilayers containing DNP ligands. A) Cartoon representing RBL mast cells interacting with the patterned ligand array. FcεRI-IgE binding and clustering by the immobilized DNP molecules causes localized recruitment of signaling proteins such as Lyn kinase (zoomed box). B) Confocal images of RBL cells stimulated by ligand containing lipid patterns at 37°C for ~30 min. Distinctive local accumulation of Lyn kinase (upper) and actin (lower) shown in green can be visualized under the clustered FcεRI-IgE. Supported membranes (red) are labeled by adding 1 mol% Lissamine Rhodamine PE to the lipid mixture. Scale bar corresponds to 20 μm.