REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	22.Apr.02	MAJOR REPORT

4. TITLE AND SUBTITLE
NANOMETER SCALE ANTIBODY PATTERNING FOR DIRECTED CELL IMMOBILIZATION AND STIMULATION

5. FUNDING NUMBERS

6. AUTHOR(S)
CAPT ORTH REID N

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CORNELL UNIVERSITY

8. PERFORMING ORGANIZATION REPORT NUMBER
CI02-58

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
THE DEPARTMENT OF THE AIR FORCE
AFIT/CIA, BLDG 125
2950 P STREET
WPAFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited distribution
In Accordance With AFI 35-205/AFIT Sup 1

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

20020523 157

14. SUBJECT TERMS	15. NUMBER OF PAGES
	5
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

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B. TITLE OF DOCUMENT: Nanometer-Scale Antibody Patterning for Directed Eosinophil Cell Immobilization and Stimulation

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Nanometer-Scale Antibody Patterning for Directed Cell Immobilization and Stimulation

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Antibodies have been patterned at nanoscale resolution for the precise immobilization and stimulation of immunological cells. We demonstrate that an antigen, bovine serum albumin (BSA), can be patterned on silicon using a photolithographically patterned polymer lift-off technique. The nanoscale pattern is realized as the polymer is mechanically peeled away in one contiguous piece in solution. Anti-BSA antibodies bound specifically to the BSA create a pattern of antibody F_c segments that provide the stimulus for eosinophils immobilization and degranulation. These patterns, ranging from sub 600 nm to 67 μm, provide a spectrum of stimuli for the 10-14 μm eosinophil cells. This method provides a new technique for capturing cells from solution, analyzing cellular biochemical cascades events such as degranulation, and studying cellular morphological changes in response to a finite, nanoscale antigenic stimulus.

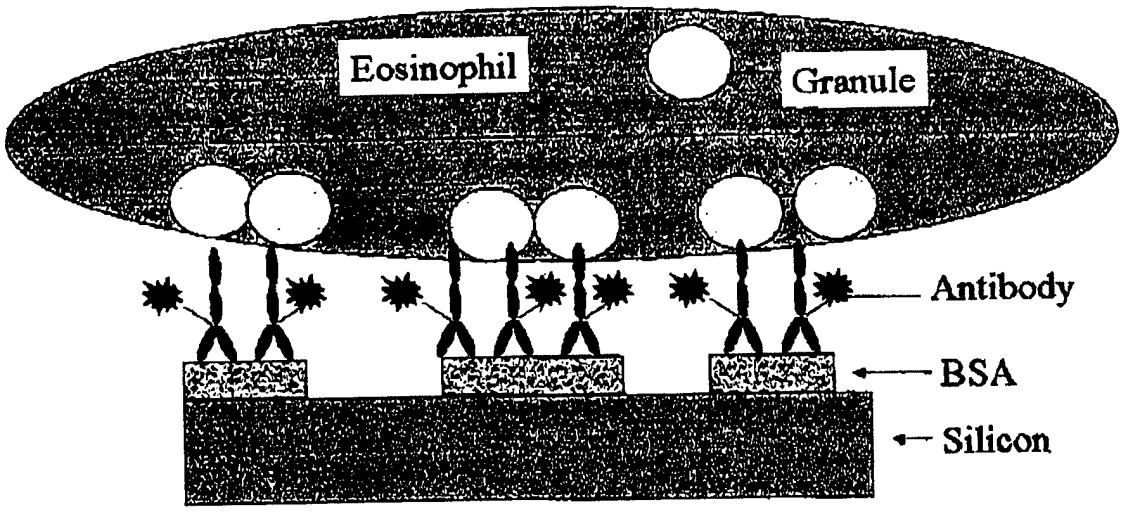

Figure 1. Schematic of eosinophil cell binding to F_c tail of Alexa 594 conjugated anti-BSA antibody on silicon substrate.

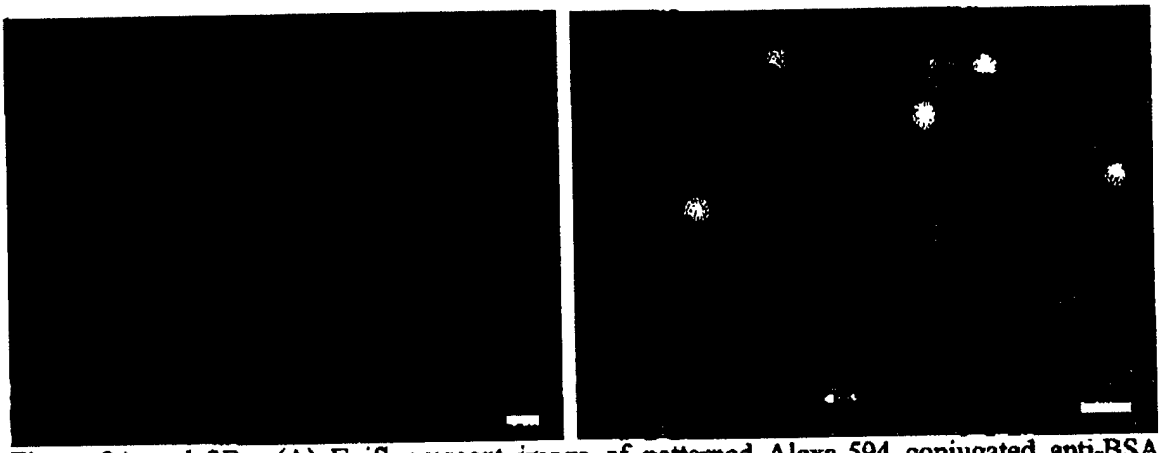

Figure 2A and 2B. (A) Epifluorescent image of patterned Alexa-594 conjugated anti-BSA antibodies specifically bound to patterned BSA on silicon substrate. Sacle bar is 70 μm and the largest square is 67 μm. (B) Eosinophil cells (green) immobilized to the patterned antibody matrix. Scale bar is 19 μm and the period between dots is 9.6 μm.

NANOMETER-SCALE ANTIBODY PATTERNING FOR DIRECTED CELL IMMOBILIZATION AND STIMULATION

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Abstract - **Antibodies (Ab) are patterned at nanoscale precision for the precise immobilization and stimulation of immune cells. We demonstrate that the antigen bovine serum albumin (BSA) can be patterned on silicon using a photolithographically patterned polymer lift-off technique. The nanoscale pattern is realized as the polymer is mechanically peeled away in one contiguous piece in aqueous solution. Anti-BSA Ab are bound specifically to BSA to create a pattern of oriented Ab that provides a surface for eosinophil immobilization and degranulation. The patterns ranged from 0.36 μm^2 to 4,489 μm^2, appropriate dimensions for the 10-14 μm diameter eosinophil cells. This method provides a new technique for immobilizing cells onto nano- and micrometer scale patterns for analyzing cellular biochemical cascade events such as degranulation and studying cellular morphological changes in response to defined nanoscale antigenic stimulus.**

Keywords - Immunology, nanofabrication, biomaterials, surface modification.

I. INTRODUCTION

Eosinophilic granulocytes (eosinophils) play an important role in the host immune defense against parasite invasion [1]. These exocytotic cells synthesize cytotoxic proteins and store these proteins in secretory granules, where they are ready to be released onto the surface of a parasite [2]. Eosinophils contribute to the pathophysiology of bronchial asthma [3] with activation of allergen-specific IgG1 and IgG3 induced through the Fc portion of the immunoglobulin molecules ($Fc_\gamma RII$) [4]. Eosinophil cell Fc and complement receptor numbers vary dramatically between healthy patients and patients with eosinophilia [5]. Eosinophils are stimulated and degranulate in response to zymosan (yeast cell walls) [6], IgG- coated Sephadex beads [7], IgA coated beads [8], and lipid mediators leukotriene B_4 (LTB_4) and platelet activating factor (PAF) [9]. Whole cell patch-clamp technique has been used to study the discrete electrical impedance changes during compound exocytosis and cumulative fusion [10]. Cellular adhesion through the B_2 integrin is an important step in eosinophil activation and accumulation as demonstrated by eosinophil binding to IgG coated sepharose beads and by binding inhibition with membrane antibodies against CD18 and CD11b--the B_2 integrin ligands [11].

Micron-scale patterning of biomolecules is an active area of research as the microelectronics technology merges with biology [12]. Patterning biomaterials on the micro- and nanometer scales allow a more focused method for cell stimulation than bath application of the biomaterial. Several different methods have been used to micropatterned biomaterials and chemicals on solid substrates for cell interrogation. Photopatterning has been used to spatially distribute biomolecules, such as enzymes, antibodies, and nucleic acids, for the development of biochips on silicon, glass, and plastic substrates [12]. A printing technique called microcontact printing (μCP) process uses a poly(dimethyl siloxane) (PDMS) elastomeric stamp to pattern a wide array of and biomaterials [14],[15],[16]. Microfabrication techniques were used to micropattern bovine serum albumin and horseradish peroxidase [17]. μCP has been used to pattern cell adhesion proteins to immobilize and direct neuronal and astroglial cell growth on glass substrates [18],[19]. Deep plasma etching and photoplastics were used to create PDMS stamps to pattern different cell suspensions to specific locations of a tissue culture substrate [19].

Many different cell types have been exposed to patterned biomaterials on solid substrates. *E.coli* O157:H7 cells were captured from a solution using PDMS stamped anti- *E.coli* O157:H7 for a diffraction grating biosensor [20]. Neuronal and glia cells were also patterned using light-assisted functionalised photoresists [22]. A silicon micromachined flow-through chamber has been designed for the 'entrapment' of chick embryo spinal cord neurons as a model system for biological neural networks [23]. Differentiated B104 neuroblastoma cells were micropatterned on four substrates to determine the preferred support substrate [24].

This paper presents a method for patterning a surface with antibodies that serve as a stimulus for eosinophil immobilization, activation and degranulation. A patterned BSA:anti-BSA IgG complex serves as a model stimulus for eosinophils. Vapor deposited Parylene, di-para-xylylene, is conformally deposited over the silicon substrate and used as a pinhole-free barrier between the solution and the substrate. Conventional photolithography and reactive ion etching (RIE) are used to pattern the polymer [25]. After the BSA incubation, the samples are incubated with Alexa-594-conjugated anti-BSA IgG solution. The immunospecificity of the antigen allows Alexa-594-conjugated anti-BSA IgG molecules to bind specifically to the patterned substrate. The samples are subsequently immersed in buffer solution and the Parylene layer is mechanically removed. Micro- and nanometer square patterns are formed using a polymer lift-off method [26] (Fig. 1). Eosinophils are pipetted onto

the patterned surface (Fig. 2). Time series epifluorescence microscopy occurs immediately after cell addition to detect real time cell immobilization, morphological changes, and degranulation.

II. Methodology

1) *Silicon Wafer Preparation and Parylene Deposition*: 3-inch <1-0-0> N/phos type wafers (Silicon Qwest, Int'l, Santa Clara, CA) are cleaned in base and acid baths to remove surface contaminants. The wafers are baked at 1100°C for 50 minutes and annealed for 10 minutes in a wet oxide process to grow a 500-nm thermal oxide layer in the silicon substrate. Fig. 1 details the fabrication steps used for the parylene lift-off technique. A pinhole-free conformal layer of Parylene is deposited onto 3-inch silicon wafers using the PDS-2010 Labcoater 2 Parylene deposition system (Specialty Coating Systems, Indianapolis, IN). The polymer thickness is dependent upon the amount of evaporated polymer. 1.5 g of Parylene C dimer is used to deposit a 1-μm thick Parylene film on five 3-inch silicon wafers.

2) *Photolithography*: 1.5 μm of OCG_OiR 897-12i photoresist (Shipley, Marlboro. MA) is applied to the Parylene-coated silicon wafers. The samples are pre-baked for 1 minute at 90°C and exposed using standard photolithographic techniques with a 10X stepper (Fig. 1a). After development, the exposed portions of the Parylene film are postbaked for 90 seconds at 115°C and are subjected to an oxygen-based RIE step using theP1 asma Therm 72 with an RF power density at 0.255 W/cm^2 (Fig. 1b). After etching, the samples are dipped into a beaker of acetone to remove residual photoresist, rinsed with isopropyl alcohol, and washed in deionized water. The samples are then dried with a nitrogen gas stream.

3) *Silanization of Silicon*: 3-aminopropaltriethoxysilane (3-APTS, Sigma-Aldrich, Milwaukee, WI) solution is prepared in a 50-mL amber bottle using 0.5-mL of 3-APTS and 24.0 mL of acetone in a nitrogen purged glovebox to create a 2% silane solution. The silanization step began by cleaning 1 cm^2 silicon chips in a Harrick Plasma Cleaner/Sterilizer PDC 3-G for 1 minute. The chips are removed and placed in 100°C Milli-Q filtered water for 30 minutes. The silicon chips are nitrogen dried then quickly inserted into the bottled silane solution and incubated in a closed container for 30 minutes. The chips are removed, washed in acetone for 5 minutes, immersed in isopropyl alcohol and deionized water, and baked on a hotplate at 70°C.

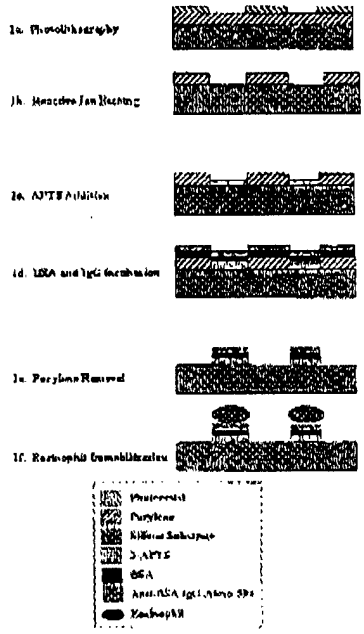


Fig. 1. Process flow schematic of the fabrication steps. (a) Patterning of 1.5 μm of OCG_OiR 897-12i photoresist using optical lithography. (b) Reactive ion etching of 1.0 μm layer of Parylene C dimer and subsequent removal of the top photoresist layer. (c) Application of 3-aminopropyltriethoxysilane layer to a plasma cleaned silicon substrate. (d) Application of 100 μg/ml BSA and 50 μg/ml anti-BSA IgG. (e) Mechanical parylene removal with tweezers, resulting in a surface with patterned Alexa 594-BSA-anti-BSA complex. (f) Immobilization of eosinophils to the patterns Fc(IgG) surface.

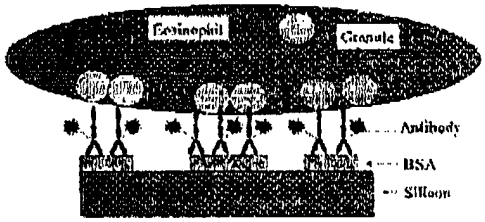


Fig. 2. Schematic of eosinophil cell binding to F_c tail of Alexa 594 conjugated anti-BSA antibody on silicon substrate.

4) *BSA and Anti-BSA IgG Preparation*: Bovine Serum Albumin (BSA) (Sigma-Aldrich, Milwaukee, WI) is reconstituted to 10 mg/ml and used to create 100, 10, and 1 μg/ml dilutions. A 30 μl drop of 2 mM BSA solution is placed on the Parylene-patterned substrate for 60 minutes, as illustrated in Fig. 1c. Polyclonal, mouse anti-BSA IgG molecules (Sigma-Aldrich, Milwaukee, WI) and stained with NHS-Alexa 594 dye (Molecular Probes, Eugene, OR). The IgG stock solution is diluted to 100 μg/ml, 7.4 pH in phosphate buffered saline (PBS). Samples are incubated in

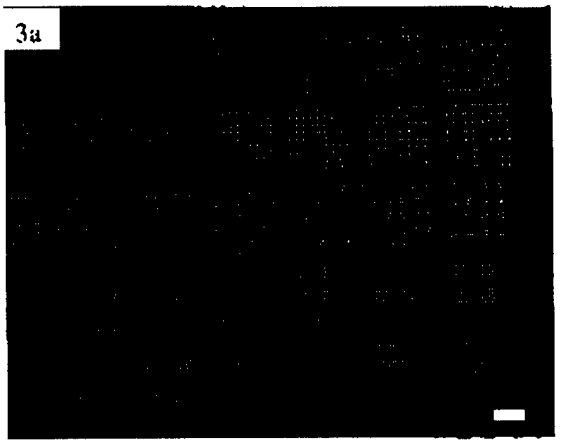
35-mm plastic Petri dishes (Fisher Chemicals, Pittsburgh, PA). Alexa 594-conjugated anti-BSA IgG is applied onto the pattern and incubated for 30 minutes. After the incubation, the sample remains immersed in aqueous solution while being transferred to a second Milli-Q water beaker in a 35-mm Petri dish. The Parylene is removed mechanically by peeling it off the substrate with tweezers in solution. The polymer film is shed easily in one contiguous piece from the substrate. The resulting sample contains patterned antigen-Ab complexes as illustrated in Fig. 1d.

5) *Eosinophil Cell Preparation and Immobilization:* Fresh blood is drawn from the jugular veins of horses, eosinophils are isolated, and are purified over discontinuous Percoll gradients as previously described [27]. Purified eosinophils are suspended in Medium 199 (Sigma-Aldrich, Milwaukee, WI) containing 4 mM glutamine, 4.2 mM NaHCO3 and penicillin/streptomycin (Sigma-Aldrich, Milwaukee, WI) (pH 7.2-7.3)| stored at room temperature and used within 24 hours. The cellsu spension is incubated for 45 minutes in LysoTracker Green (Molecular Probes, Eugene, OR). The samples are placed in the bottom indentation of 35mm petri dish (Mat Tek, Ashland, MA) containing 2 ml of PBS buffer. 50 µl of 1.9 x 10^6 cells/ml solution is pipetted directly onto the patterned silicon chips. Time series epifluorescence imaging begins immediately after cell application using an Olympus AX 70 upright microscope with a 60X water immersion objective and Omega Optical filter sets (Brattleboro, VT). Alexa 594 dye is observed with a 510-590-nm excitation/590-nm emission filter set and LysoTracker Green is observed with a 450-490-nm excitation/520-nm emission filter set. Images are captured using a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Cells are fixed with 3.7% paraformaldehyde (Sigma, Milwaukee, WI) for long-term storage.

III. Results

In this report, we describe the micro- and nanometer scale arrays of patterned Ab for cell stimulation, test the patterned surface antigenicity with functional antibodies, develop a method for immobilizing eosinophil cells to the pattern, analyze the cells' preferential binding to the patterned surface, and detect eosinophil degranulation events after cell incubation on the pattern.

Fig. 3a illustrates Alexa 594-conjugated anti-BSA IgG squares with sub 600-nm widths on the lower right to 67-µm squares on the upper left) on a silicon substrate using the Parylene lift-off technique. This matrix of patterns demonstrates the wide range of sizes attainable with this patterning technique. 500 nm resolution isapp roximately the lowest photopatterning threshold attainable using the 10X stepper. A statistical analysis showed that the average relative aerial density of fluorescently labeled antigen to the background is at least 150 times greater in the exposed regions than in the lift-off regions [28].



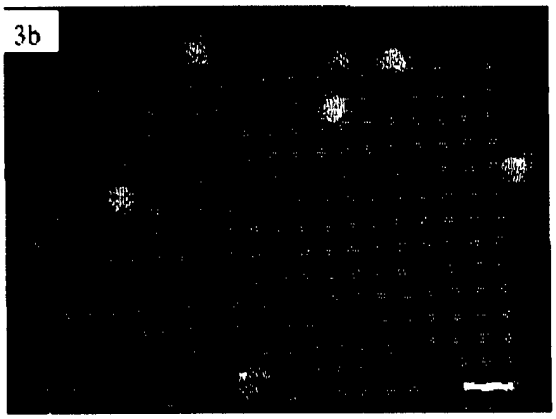


Fig. 3a and 3b. (a) Epifluorescent image of patterned Alexa-594 conjugated anti-BSA antibodies specifically bound to patterned BSA on silicon substrate. Scale bar is 70 µm and the largest square is 67 µm. (b) Eosinophil cells (green) immobilized to the patterned antibody matrix. Scale bar is 19 µm and the period between dots is 9.6 µm.

Fig. 3b illustrates the immobilization of horse eosinophils to the BSA-anti-BSA IgG complexes. Horse eosinophils were used since they have large granules that are easy to visualize during microscopy. This binding is similar to the experiments using immunoglobulin coated Sepharose beads detailed in the introduction. This method provides information that Sepharose beads do not offer. First, this method can be used to determine the spatial distribution and size of patterned Fc stimuli required for eosinophil activation and degranulation. Second, this technique allows eosinophil migration to be analyzed over the planar patterned surface.

Fig. 4a shows the initial state and Fig. 4b shows the same region after 5 minutes. These images demonstrate clear morphological changes are occurring on the substrate. Consequently, the circled regions in this image show possible fusion and/or degranulation events may be

occurring. Further experimentation with patch clamp amperotometry will be required to confirm that these changes are taking place.

IV. DISCUSSION

This technique may be useful to determine if fusion events and degranulation events may be occurring on the patterned substrate using fluorescence imaging. The signal observed in unpatterned regions is comparable to the signal observed on a blank, new silicon wafer surface. Thus, the unpattterned regions show negligible binding. Uniformity of the final patterned biomaterial relies upon an optimized photoresist thickness of 1.5 μm and Parylene thickness 1.0 μm, photolithography precision, optimized RIE duration to prevent under- and overetching, and sufficient biomaterial incubation time. Application of 1 μm of Parylene and 1.5 μm of photoresist provided optimal conditions for polymer removal and resolution.

Anti-BSA Ab are used to confirm the specificity of the binding onto the patterned BSA and provide a surface rich in Fc fragments onto which eosinophils can bind and be stimulated. The patterned antigen did not spread from the confined pattern regions and were still isolated after a month of storage in aqueous solution.

The Parylene lift-off technique offers a rapid and precise way to create supported micro- and nanometer-scale patterns. These patterns can be used to capture other biomaterials from solution and integrated into biosensors, bioMEMS, and biological assay systems.

There are several advantages of this technique. The Parylene film removal can be performed at any step during the processing. Therefore, multiple reagents can be added to the initial patterned surface prior to Parylene removal. This would allow subsequent reagents to be added at high concentrations and maximal binding without concern for nonspecific binding on unpatterned surface areas. Parylene is biologically compatible polymer, provides a conformal coating with low permeability, and can be removed with a one-step mechanical lift-off. This technique permits the sample to remain submerged in solution so thatth e functional molecules are not denatured when dried. Parylene does not have a permanent bond with the substrate, thus allowing easy removal in one piece. The conformal film of Parylene is pinhole-free, so no unwanted patterning occurs in unexposed regions.

V. CONCLUSION

We have demonstrated a method for precise patterning antigen at the micro- and nanometer scale for cell stimulation. The antigenicity is confirmed by binding fluorescent antibodies onto the patterned surface. Eosinophil cells are effectively immobilized on the patterned surface. The eosinophils demonstrated preferential binding to the patterned surface. Time course imaging provides a means to study spatial-temporal changes associated with stimulation and degranulation.

This technique offers a new versatile tool to pattern antigen and other biomaterial onto solid substrates with feature sizes below 600 nm.

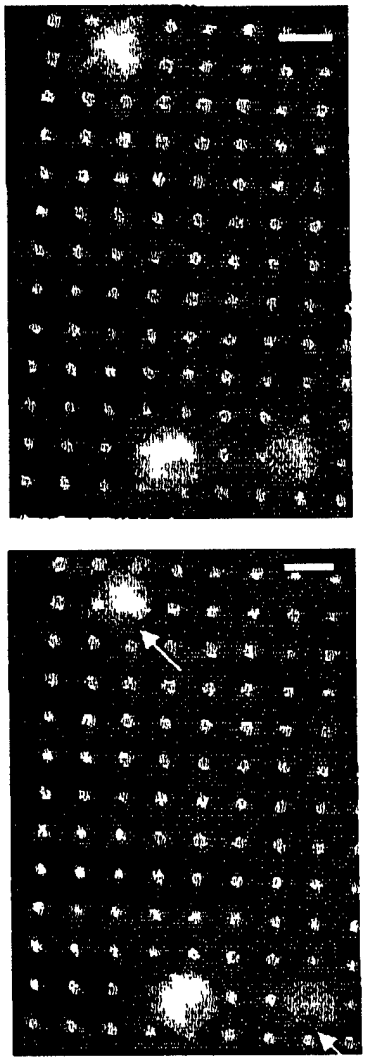


Fig. 4a and 4b. Epifluorescence images of eosinophils interacting with the patterned substrate. (a) Eosinophil interaction with the substrate 1 minute after application. (b) Eosinophil interaction with the substrate 6 minutes after application. Morphological changes can be observed in the cells with white circles. The cell with the top arrow appears to lose a green granule at its base. The cell with the lower arrow appears it may have degranulated from its center onto the pattern. Patch clampam, perotometry will be required to confirm that these changes are taking place Scale bar is 15 μm.

ACKNOWLEDGEMENT

We acknowledge the support of DARPA, NSF support through the Nanobiotechnology Center, and the resources of the Cornell Nanofabrication Facility. We would like to thank Dr. Ismail Hafez for his assistance preparing lipids and guidance on configuring a lipid extrusion system. We would also like to thank Mathieu Foquet for his designing the microscope set-up for this experiment and guidance with imaging. The views expressed in this article are those of the authors and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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