UCRL-TR-228478

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SINGLE-CELL LEVEL INVESTIGATION OF CYTOSKELETAL/CELLULAR RESPONSE TO EXTERNAL STIMULI

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February 26, 2007

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Auspices Statement

This work was performed under the auspices of the U. S. Department of Energy (DOE) by the University of California, Lawrence Livermore National Laboratory (LLNL) under Contract No. W-7405-Eng-48. The project (04-ERI-015) was funded by the Laboratory Directed Research and Development Program at LLNL.

FY06 LDRD Final Report

Single-Cell Level Investigation of Cytoskeletal/Cellular Response to External Stimuli

LDRD Project Tracking Code: 04-ERI-015

Amy Hiddessen, Principal Investigator
Jane Bearinger, Co-Investigator

Abstract

A detailed understanding of the molecular mechanisms by which chemical signals control cell behavior is needed if the complex biological processes of embryogenesis, development, health and disease are to be completely understood. Yet, if we are to fully understand the molecular mechanisms controlling cell behavior, measurements at the single cell level are needed to supplement information gained from population level studies. One of the major challenges to accomplishing studies at the single cell level has been a lack of physical tools to complement the powerful molecular biological assays which have provided much of what we currently know about cell behavior. The goal of this exploratory project is the development of an experimental platform that facilitates integrated observation, tracking and analysis of the responses of many individual cells to controlled environmental factors (*e.g.* extracellular signals). Toward this goal, we developed chemically-patterned microarrays of both adherent and suspension mammalian cell types. A novel chemical patterning methodology, based on photocatalytic lithography, was developed to construct biomolecule and cell arrays that facilitate analysis of biological function. Our patterning techniques rely on inexpensive stamp materials and visible light, and do not necessitate mass transport or specified substrates. Patterned silicon and glass substrates are modified such that there is a non-biofouling polymer matrix surrounding the adhesive regions that target biomolecules and cells. Fluorescence and reflectance microscopy reveal successful patterning of proteins and single to small clusters of mammalian cells. In vitro assays conducted upon cells on the patterned arrays demonstrate the viability of cells interfacing with this synthetic system. Hence, we have successfully established a versatile cell measurement platform which can be used to characterize the molecular regulators of cellular behavior in a variety of important biological processes. The achievements realized in this project have enabled presentations and publication within the international scientific community, new collaborations with researchers at the University of California, and successful competition for three additional, separate research grants on studies of stem cell fate commitment and pathogen-host cell interactions.

Background and Motivation

The many individual cells of a multicellular organism are typically exposed to hundreds of different signals from their unique microenvironments, and these signals ultimately control the growth, division, differentiation and all other functions promoting the life or death of the cell. Extracellular signal molecules (e.g., proteins) can be soluble, attached to the surface of an opposing cell or presented by the extracellular matrix. In general, a cell's response to signals not only depends on the types and combination of signal molecules in its environment, but also on the specific set of receptors and intracellular signal transduction machinery the cell possesses. If we are to completely understand molecular mechanisms controlling cell behavior, measurements at the single cell level are needed to supplement information gained from population level studies. One of the major challenges to accomplishing studies at the single cell level has been a lack of physical tools to complement the powerful molecular biological assays which have provided much of what we currently know about cell behavior. **The objective of this project is to develop a versatile experimental platform that facilitates measurement of the responses of single cells to controlled environmental factors to better understand signal transduction in a wide variety of biological processes.** While characterization of molecular regulators of cellular behavior is important to all biological processes involved in health and disease, this project was particularly motivated by the potential impact of enabling single cell studies in the areas of pathogenesis and stem cell fate commitment; hence, these subjects are the focus of this section.

Host-Cell Cytoskeletal Response to Chemical Signals of Pathogenic Origin

The cytoskeleton establishes cell shape, mechanical integrity and spatial organization in the cytoplasm, and facilitates vital cell functions, including migration [1, 2], division [3], adhesion [4] and intracellular positioning and transport of vesicles and organelles [5]. Cytoskeletal structure, and hence, cell morphology, is primarily governed by actin filaments, intermediate filaments and microtubules. Hundreds of different accessory proteins regulate the dynamic behavior of the filaments and microtubules, and thus, allow cells to respond to changing conditions through transduction of extracellular and intracellular signals. [6] Several cell types respond to chemical signals through a cytoskeleton-associated behavior known as polarization. Cell polarization, a prerequisite step to many other cell functions, is defined as the establishment of structural and molecular asymmetry in a cell (i.e., asymmetry in cell shape, protein distributions and functions) [7, 8] Polarity depends critically on stimulus-induced reorganization of the actin and microtubule cytoskeleton. For example, white blood cells called neutrophils hunt bacterial invaders by receptor-mediated sensing of a concentration gradient of N-formylated peptides (chemoattractants) released from the bacteria. Neutrophil binding of these peptides stimulates the growth of actin-rich protrusions in the direction of increasing chemoattractant concentration, thereby establishing the cell polarization required for cell migration. [9]

The importance of understanding cytoskeletal restructuring in response to chemical signals is not restricted to the immune system; cytoskeleton-mediated cell behaviors are also important in pathogenesis. Bacterial and viral pathogens have developed tools to hijack and exploit the host cell cytoskeleton to enter, move and replicate within host cells, as well as spread to new cells, in order to achieve successful infection. [10-14] For example, many pathogenic bacteria can promote their own internalization through surface proteins that mimic natural host cell ligands. The initial interactions between bacterial ligands and their host cell receptors mediate entry by triggering a cascade of cell signaling events, involving receptor clustering, recruitment of adapter and effector proteins, and activation and restructuring of cytoskeletal components, that culminates in the internalization of the bacterium.[15] In comparison to the processes of replication and spreading,

much less is known about host cell cytoskeletal response to pathogen docking and entry. Recent studies have identified several of the cellular factors involved in the various steps of the internalization process. However, a spatio-temporal description of the signal-driven response of the host cell cytoskeleton during the initial stages of pathogen contact and engulfment remains incomplete.

A comprehensive, fundamental understanding of pathogen infection and the host response is required for the development of new strategies for detection and therapeutic intervention. To date, investigations of cytoskeletal dynamics have been hampered because conventional fixation protocols for immunofluorescence studies insufficiently preserved the cytoskeletal structure.[16] This research aims to establish a new experimental platform, based on live-cell cytoskeletal labeling and bioengineered cell arrays, to acquire a more comprehensive description of cytoskeletal dynamics during initial host cell interactions with bacterial pathogens.

Stem Cell Fate Decisions in Response to Chemical Signals

Stem cells have the capacity to self renew indefinitely and to be induced to differentiate into cells with specialized functions. A major goal in stem cell biology and in stem-cell engineering is to both identify and determine the relative roles of intrinsic and extrinsic signals in controlling stem cell fate, and to determine if intrinsic events place a limit on the ability to control the fates of cells with external factors in order to use them for regenerative medicine.[17] Hematopoiesis, a hierarchical process in which hematopoietic stem cells (hsc) proliferate or differentiate into numerous highly specialized blood cells, is an exquisite example of the molecular complexity of signaling-dependent cell behavior. The ability of hematopoetic stem cells (hsc) to self renew or to commit to the cascading pathway of differentiation into highly specialized types of blood cells is well-documented (for a review of hsc biology see [18]). However, the molecular regulators underlying hsc fate decisions are poorly understood. [19, 20]

There is evidence for both stochastic and non-random regulation of hsc fate [19]; however, the molecular mechanisms that generate hsc diversity are poorly understood. It is generally accepted that extrinsic and intrinsic signals guide hsc fate toward differentiation, self-renewal, apoptosis and migration. [20] Extrinsic signals include myriad combinations of growth factors, cell-microenvironment interactions, and cell-cell interactions. Intrinsic events are most likely regulated by coordinated expression of multiple genes. Recent gene array data comparing expression profiles of subsets of progenitor/hsc subpopulations suggest that hsc are transcriptionally active and that differentiation occurs by reducing the repertoire of expressed genes, while decreasing hsc related genes and increasing lineage specific genes. [21] While these data provide intriguing clues about molecular differences between stem and differentiating populations, the gene expression data are based on population averages, and thus, do not clearly address effects of the heterogeneous status of individual cells within the isolated population or the complex environment of multiple cell interactions. Since fate decisions are made by the individual cell, an experimental platform that enables investigation of fate commitment of individual hsc under controlled environmental conditions is needed.

A more complete understanding of the roles of external and internal signals in determining hsc fate has important implications in stem cell engineering, developmental biology, and in understanding aberrant signaling in disease (e.g., leukemia). Motivated further by discussions and collaboration with stem cell experts at UC Merced, this research proposes to develop a new experimental capability for monitoring stem cell proliferation and/or differentiation in response to controlled levels of chemical factors, such as growth factors. This work will contribute to goals within the stem cell biology community to map molecular pathways which control stem cell proliferation and differentiation, and to determine the relative roles of extrinsic and intrinsic influences on the fate decisions of stem cells.

Technical Approach and Results

The main research activities in this project focused on the bioengineering of an experimental cell measurement platform based on chemical patterning approaches. However, additional biological tools, including live cell labeling and genetic manipulation procedures, were also (fully or partially) developed to facilitate the goal of applying the engineered platform to the study of molecular signaling within cellular systems. The experimental activities and results are described further below.

- **Live cell labeling of cytoskeletal tubulin and actin with green fluorescent protein probes**

The current method of choice for visualizing the cytoskeletal molecular constituents in living cells is green fluorescent protein (GFP) and its derivatives. (see Yoon et al., 2002) GFP is well suited to the study of dynamic cell processes because it eliminates the need to fix cells prior to observation, and can be observed in living cells directly using fluorescence microscopy. The actin and microtubule cytoskeletons of living human fibroblasts (BJ1) and epithelial cells (HeLa) have been successfully labeled and visualized using yellow fluorescent protein-actin (YFP-actin) and green fluorescent protein-tubulin (GFP-tubulin). Briefly, the experiments consisted of establishing and maintaining cell lines for cytoskeletal labeling, transforming competent cells to produce working quantities of fluorescent protein vectors, including purification and gel electrophoresis analysis of the pure vectors, optimizing the chemically-aided transfection of cells and maintaining stable transfected (labeled) cell lines, and performing preliminary fluorescence microscopy examinations of the labeled cytoskeletons of transfected cells. (Fig. 1) By establishing these stably transfected cell lines, we have the cellular tools needed to examine cytoskeletal responses to varied extracellular stimuli.

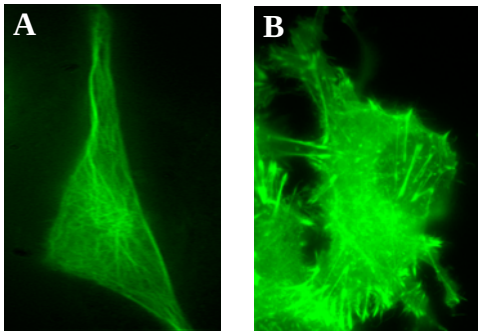

Figure 1. Fluorescence micrographs of single, *living* BJ1 or HeLa cells labeled via chemically-aided transfection of subcellular localization vectors. Representative micrographs for (A) individual, living fibroblast (BJ1) cell labeled via transfection of purified GFP-tubulin or (B) individual, living epithelial (HeLa) cell labeled via transfection of purified YFP-actin vector.

- **Methods for genetic manipulation of model progenitor stem cells investigated**

We explored the use of gene silencing approaches in preparation for application of the engineered cell platform to studies of stem cell fate. This ability to genetically manipulate cell

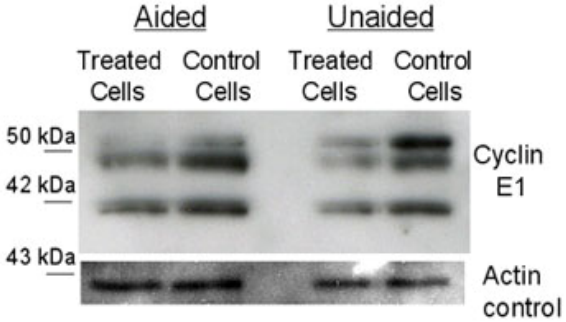

Figure 2. Results of Western blot for suspension cells treated with gene silencing molecules via chemically-aided transfection or unaided transfection. First demonstration of partial gene silencing (reduced expression) in treated versus untreated control cells for (A) chemically aided or (B) unaided transfection of NB4 suspension cells treated with silencing molecules targeting Cyclin E1gene. Protein levels measured at 42 hrs post transfection. Actin protein expression used as experimental control.

gene expression can be a useful way to isolate molecular pathways and the molecules which regulate them. In consultation with Dr. Larry Dugan (LLNL), we designed silencing molecules for a cell cycle gene known as Cyclin E1 using the web-based Dharmacon siDesign Center resource. We decided to take on the additional challenge of developing a method for suspension cells, for which silencing protocols are few as compared to adherent cell types. In fact, our results are the first demonstration of gene silencing in a particular type of non-adherent, adult human leukemia cells (a model progenitor stem cell line) known as NB4 cells. These cells were treated with a short, interfering RNA:DNA molecule targeted to Cyclin E1 using chemically-aided or unaided transfection. The effect of the silencing molecules was assessed using Western blotting, a technique which allows comparison of protein expression levels within cell populations, *e.g.* post-transfection. Our preliminary results are shown in Fig. 2.

- **Novel chemical patterning method applied to fabricate regioselective microarrays**

In collaboration with Dr. Jane Bearinger (LLNL), chemical micropatterning techniques have been developed to construct biomolecular [23] and cell microarrays that will facilitate integrated observation and characterization of cell behavior in response to chemical factors. In this chemistry-based method, invented by Dr. Bearinger, red light is used to chemically activate material on a PDMS polymer stamp in contact with an oxidizable thin film, e.g., an unsaturated silane film, on a silicon or glass substrate. This leads to selective removal of the thin film where contact is made. (Fig. 3) Next, a non-fouling polymer layer consisting of interpenetrating

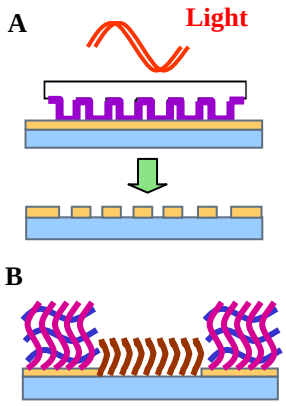

Figure 3. Schematic representation of the chemically-based patterning method used to fabricate cell microarrays. (**A**) Red **light** chemically activates **photocatalyst molecules** on a PDMS stamp positioned in contact with an oxidizable **thin film** on a **silicon** or **glass substrate**. Localized oxidation selectively removes the thin film where contact was made with the stamp. Next, (**B**) a non-fouling IPN (**AAm** and **PEG**) is covalently bonded to the regions of the patterned substrate where **thin film** remains. Bare **substrate** regions are back-filled with bioadhesive chemistry (e.g., **aminopropylsilane**) to complete the chemically coded microarray.

polyacrylamide (AAm) and polyethylene glycol (PEG) polymers (interpenetrating polymer network, or IPN) [22] is covalently bonded to regions of the patterned silicon where the thin film remains. The bare Si or glass regions of the pattern are backfilled with adhesive chemistry, e.g., aminopropylsilane, completing the array of adhesive islands surrounded by a non-adhesive polymer matrix.

- **Process developed for constructing biomolecule and cell microarrays**

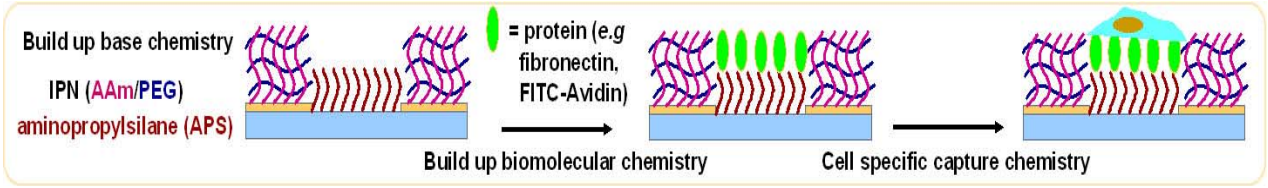

Figure 4. General scheme of process developed during this project to construct biomolecule and cell arrays.

• Fabrication of chemically-patterned protein microarrays

After back-filling with aminopropylsilane (APS), patterned silicon and glass substrates were incubated with a solution of fluorescein-labeled Neutravidin protein. (Fig. 5)

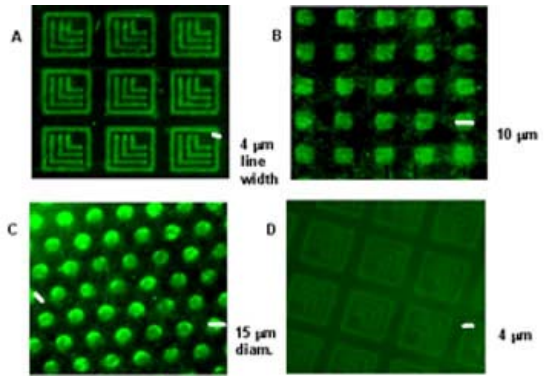

Figure 5. Fluorescence micrographs of chemically patterned biomolecular microarrays. Stamps bearing various features were used to pattern silane films on (A,B) silicon wafers or (C,D) glass coverslips. After back-filling with APS, the substrates were incubated with a solution of fluorescein-labeled Neutravidin. Fluorescence micrographs show protein selectively adsorbs to APS regions, but is repelled by the surrounding non-fouling polymer matrix (20× magnification). Similar results were obtained using antibody solutions.

• Fabrication of chemically-patterned single-cell and multiple cell-cluster microarrays, demonstrated using adherent mammalian cells

Unlabeled and GFP-tubulin (green fluorescent protein tubulin)-labeled HeLa cells (human epithelial cells) were loaded onto patterned silicon and glass substrates (back-filled with APS). Cells were incubated with the substrates for approximately 8-12 hours and then imaged using reflectance, phase contrast, or fluorescence microscopy. (Fig. 6)

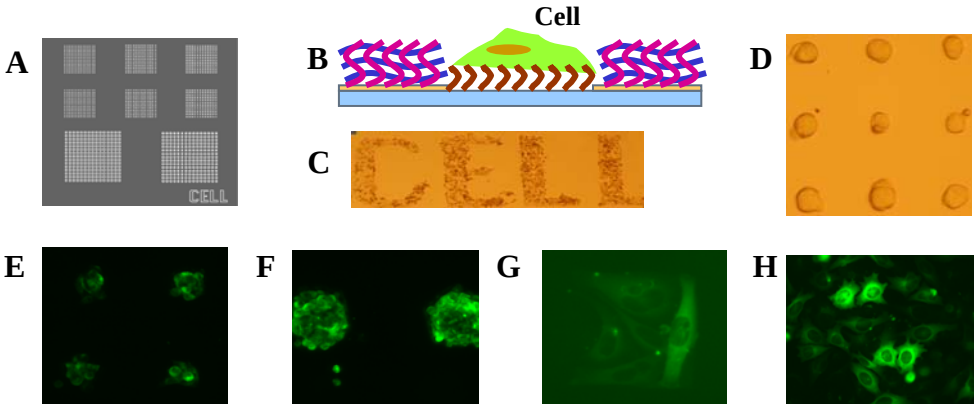

Figure 6. Fabrication of chemically-patterned single-cell and multiple cell-cluster microarrays demonstrated using adherent mammalian cells. A stamp master pattern (A) was designed with eight separated regions of circles or squares of increasing size (20, 30, 40 and 100 μm in diam. or width). Circles and squares within all regions are separated by 100 μm gaps. A stamp with this pattern was used to pattern silicon and glass substrates (Figure 1). After back-filling with APS, unlabeled or GFP-tubulin-labeled HeLa cells were loaded onto the microarrays. Cells preferentially adhered to APS regions, and were repelled by IPN regions (see B). Reflectance microscopy reveals patterning of (C) cell clusters (1.5×) and (D) a single cell array (20×) (30 μm circles). Fluorescence microscopy shows cell cluster arrays on (E) 40 μm circles, (F) 100 μm circles, and (G) one 100 μm square island (all at 20×). An

• Development of cell patterning chemistry to support non-adherent cell types, demonstrated using mammalian suspension cells

With the goal of creating a versatile cell patterning approach for making cell microarrays, we decided to develop additional cell capture chemistries to enable capture of non-adherent or suspension cell types. Additional motivation for pursuing methods for suspension cells was catalyzed by our collaborators at UC Merced (Pallavicini Lab), a group of stem cell biologists

interested in methods for patterning suspension, blood-forming stem cells for studies of cell fate commitment. For these experiments, following the creation of patterned protein islands surrounded by a non-fouling polymer matrix (as depicted in Fig. 3), a commercially available lipid-like/fatty-acid-NHS linker was chemically attached to the protein coating. Next, unlabeled suspension cells (NB4, adult human leukemia cells) were loaded these substrates, incubated for approximately 8-12 hours and then imaged using phase contrast microscopy. (Fig. 7)

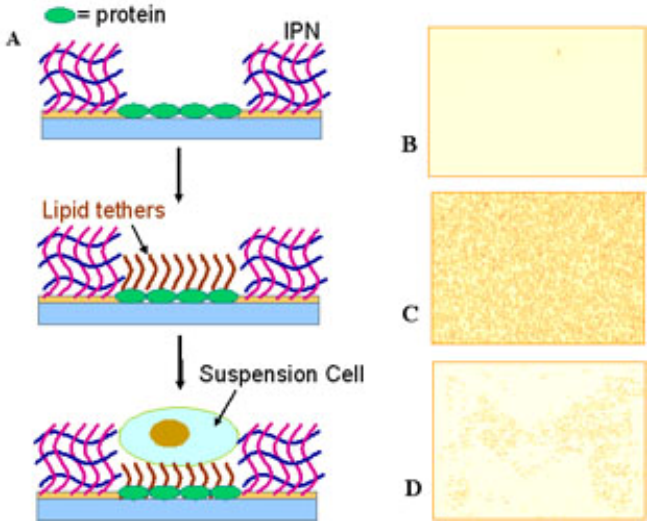

Figure 7. Development of chemistry to create patterned suspension cell substrates. Following the scheme depicted in (A), lipid-like, cell membrane tethers (unsaturated fatty acid-NHS linkers) are bound to protein-coated capture sites that ultimately attract suspension cells. Cell capture is mediated by insertion of synthetic lipid-like tethers into the living cell's lipid membrane. Phase contrast micrographs show that NB4 cells will not adhere to unpatterned glass substrates coated with protein only (B), while unpatterned substrates treated with both protein and the lipid-like tethers readily attract cells (C). Furthermore, a substrate patterned with an "M" demonstrates that NB4 cells selectively attach to regions treated with protein/lipid tethers, but are repelled by the surrounding non-fouling polymer matrix (D).

• **Manipulation of *viable* microarrayed cells demonstrated with an "on-chip" transfection**

Unlabeled HeLa cells were loaded on a patterned silicon substrate (back-filled with APS) at 24 hrs prior to aided transfection (Lipofectamine reagent, Invitrogen) of then arrayed cells with GFP-tubulin vectors. The cell array was rinsed to remove excess vectors and then imaged via fluorescence microscopy at 18, 24 and 96 hrs post-transfection. Apart from demonstrating the ability to manipulate cells after capture on the patterned array, an increase in the number of GFP labeled cells with time indicates cells remain viable on the array for at least 96 hrs. (Fig. 8)

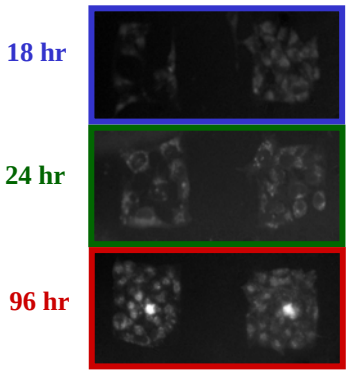

Figure 8. Demonstration of the ability to manipulate microarrayed cells. HeLa cells were transfected with GFP-tubulin vectors 24 hours after being loaded onto the array. Excess vector was rinsed away and the cell microarray was imaged via fluorescence microscopy at 18, 24 and 96 hrs post-transfection. The micrographs show the same two 100 μm square islands at all time points (20× magnification).

In summary, single cell and cell cluster microarrays have been developed using a novel chemical patterning method. The utility of this method has been demonstrated via selective patterning of proteins and cells, and by subsequent successful manipulation of arrayed live cells. A manuscript describing this work is currently being prepared. [24] *The ability to array cells and then manipulate them is an essential step toward developing the capability to investigate single-cell behavior in the context of well-characterized environments.*

Summary

We developed a new surface patterning methodology, based on photocatalytic lithography, to construct biomolecule and cell arrays that facilitate analysis of biological function. Our patterning techniques rely on inexpensive stamp materials and visible light, and do not necessitate mass transport or specified substrates. Patterned silicon and glass substrates are modified such that there is a non-biofouling polymer matrix surrounding the adhesive regions that target biomolecules and cells. Fluorescence and reflectance microscopy reveal successful patterning of proteins and single to small clusters of mammalian cells. In vitro assays conducted with the patterned cell arrays demonstrate the viability of cells interfacing with this synthetic system. We are currently pursuing applications of these cell microarrays to studies of stem cell fate commitment and pathogen-host cell interactions.

Exit Plan

In this project, we established a versatile cell measurement platform which can be used to characterize the molecular regulators of cellular behavior in a variety of important biological processes. The achievements realized in this project have enabled presentations (multiple talks (two invited) and posters) and publication (conference proceedings [23] and peer-reviewed journal article [24]) within the international scientific community, new collaborations with researchers at the University of California, and successful competition for three additional, separate research grants on studies of stem cell fate commitment and pathogen-host cell interactions.

Acknowledgements

We thank J. Hamilton, C. Stockton, L. Dugan, K. Wu, G. Stone, N. Shen, B. Stankovich, M. Colvin and M. Pallavicini for their contributions during the course of this project. NB4 suspension cells and the lipid-like/fatty-acid molecules used in this project were generously donated by our collaborator, Professor M. Pallavicini, at UC Merced. We gratefully acknowledge financial support from the LLNL LDRD program. We gratefully acknowledge partial financial support for J.B. from NIH R21 EB003991-01, and for A.H. from the DOE Campus-Laboratory Exchange program (with UC Merced collaboration).

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