Cornell NanoScale Facility
Research Accomplishments
2003-2004
Abstract:
In increasing complexity, we have published in the year 2003 on: construction of nanoscale channels for biological applications, a new technique to separate biological objects in a deterministic manner, measurements of the elasticity of erythrocytes, and the collective behavior of bacteria undergoing quorum sensing in a microfabricated structure.

Summary:
The sacrificial polymer work [1] used a combination of nanoimprinting technologies and sacrificial polymers to create nanochannels for elongation of genomic length DNA molecules. In related work, we have been using the ebeam capabilities at CNF to do direct writing of nanochannels at the 30 nm level. These nanometer channels will be used to extend genomic length DNA molecules.

The erythrocyte work studied the heterogeneity and time-dependence of cell flexibility [2]. Red cells were aspirated into microchannels, data for many individual red cells was rapidly acquired, and the fundamental heterogeneity of cell membrane biophysics analyzed. Fluorescent labels were used to quantify red cell surface and cytosolic features of interest simultaneously with the measurement of area and volume for a given cell.

In a related area, we developed a technique to sort magnetically labeled cells using microfabricated magnetic structures combined with microfluidics. We label white blood cells with superparamagnetic nanobeads and run them over magnetized stripes. The stripes create a series of linear magnetic traps that alter the movement of magnetically beaded cells and displace the labeled beads from the main blood flow.

We have shown that the environmental topology of complex structures is used by E. coli to create traveling waves of high cell density [3, 4], a prelude to quorum sensing. This was first observed in mazes designed to mimic complex environments, then more clearly in a simpler geometry consisting of a large open area surrounding a 250 µm x 250 µm square with a narrow opening of 10 to 40 µm. Our results showed that under nutrient-deprived conditions bacteria search out each other in a collective manner and that the bacteria can dynamically confine themselves to highly enclosed spaces.

We developed a continuous-flow technique for separating and concentrating particles according to size, where dispersion is virtually eliminated [5]. The technique uses a microfabricated matrix designed to unequally bifurcate laminar flow streams. Spheres of 1 µm diameter were sorted in 40 seconds with a resolution of 1% in diameter. When used to concentrate biological samples, the technique potentially increases the detection limits of many devices, as the bacterial artificial chromosomes were enriched by two orders of magnitude.

References:
Separation and Analysis of Cell Contents

CNF Project # 398-91
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**Figure 1, top right:**
Bacteria seek each other out and collapse into 200 µm squares through 10 µm channels.

**Figure 2, below left:**
A titled array of posts can be used to separate 1 µm diameter beads with 10 nm precision, as high speeds.

**Figure 3, below right:**
Complex microfluidics for combining stream-line flow and particle separation.
**Abstract:**

Biophysicists and Biochemists spend much of their time studying the interactions between important biomolecules. An understanding of these interactions is critical to generating a clear and accurate picture of cellular function. The development of fluorescent markers has made spectroscopic, single molecule techniques extremely useful in this field. However, the range of concentrations that researchers can work at has limited this utility. We have been investigating Zero Mode Waveguides, simple optical nanostructures, as a tool for studying molecular interactions at micromolar and higher concentrations.

**Summary:**

Fluorescence Correlation Spectroscopy has been used for more than four decades as a technique for studying molecular interactions both *in vivo* and *in vitro* [1]. The technique reveals both the average number of molecules in the focal volume as well as the average time a fluorescent molecule spends in the focal volume. Like most single molecule techniques, the size of the focal volume dictates an upper limit for the concentrations that can be used. Traditional techniques are limited to the pico to nanomolar range. Some alternative techniques such as total internal reflection fluorescence correlation spectroscopy increased the available concentration range. However, none have produced the five order of magnitude reduction in focal volume necessary to study interactions with kinetic constants in the micromolar range.

Zero Mode Waveguides are structures small enough that when illuminated by a source, no guided modes are excited. Instead, a radially confined evanescent field is produced near the opening of the waveguide [2]. We fabricate these devices by depositing a thin metal film onto a fused silica cover slip. E-beam lithography is then used to pattern the waveguides, which are then dry etched. The resulting structure behaves very much like a cylindrical metallic waveguide. Diameters, ranging from less than 30 nm to 100 nm, ensure an extremely high cutoff frequency. The small evanescent field generated by excitation is further reduced by ohmic losses in the metal film. This produces a focal volume on the order of zeptoliters.

We have used Fluorescence Correlation Spectroscopy in Zero Mode Waveguides to study the behavior of Bacteriophage lambda’s repressor protein. The repressors form dimers, tetramers and octamers in solution [3]. The small observation volume generated by the Zero Mode Waveguides allows us to characterize the oligomerization at high concentrations. Working at total repressor concentrations of roughly 1 mM, we were able to determine the kinetic constant that characterizes the octamerization process. In addition, we were able to confirm the repressors biological activity by observing binding between repressor dimers and a DNA fragment containing the repressor binding site. The binding causes a significant change in the diffusion constant and hence the diffusion time of the molecules.

This demonstrates that two important families of experiments can be carried out using Zero Mode Waveguides: (1) Determination of kinetic constants for reactions that occur at high reagent concentration and (2) ligand binding assays. The devices also offer a number of other benefits. Fluorescence Correlation Spectroscopy in Zero Mode Waveguides requires little reagent and experiments can be carried out quickly. Determination of diffusion times and concentrations takes only a few minutes of data acquisition. Additionally, the small size scale of the waveguides makes them ideal for massive parallelization.

**References:**


Protein Binding Studies at High Concentration using Zero Mode Waveguides

**CNF Project # 551-95**

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- Zero Mode Waveguides allow the use of Fluorescence Correlation Spectroscopy at greater than 10 micromolar concentrations.

- Reaction kinetics can be determined by observing the relative number of reagents in the focal volume.

- Ligand binding can be detected by measurement of the diffusion constant.

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**Figure 1:** SEM of a Zero Mode Waveguide.

**Figure 2:** Fluorescence autocorrelation curve showing two distinct species in solution.

**Figure 3:** Fluorescence autocorrelation curves for (1) 21bp operator sequence tagged with Alexa488 and (2) the same fluorescent DNA fragment with repressor protein. The increase in diffusion times is clearly visible by observing the FWHM of the autocorrelation curves.
Abstract:
This project utilizes a combination of fluorescence microscopy and nanofabricated structures to perform single molecule studies. Single molecule studies allow the direct detection of molecular properties including size, diffusion characteristics, and binding processes [1]. The ability to detect single molecules also enables a tremendous reduction of sample volume, conserving rare and precious samples, and bypassing biological amplification processes such as PCR.

Introduction:
Signal to noise ratio is the limiting factor in many single molecule studies [2]. A number of techniques are employed to achieve the signal to noise ratio necessary for single molecule detection. Focal volume confinement is the principal method. By reducing the focal volume, the number of unwanted fluorophores and corresponding background noise in the detection volume is reduced. Raman scattering, another significant source of background noise, is also reduced.

Focal volume confinement is accomplished by two means. The first level of focal volume confinement is realized by the use of confocal optics, limiting both excitation of the sample and collection of emission to the focal plane of interest. Additionally, when operating in epi-illumination mode, a diffraction-limited laser spot size with lateral dimensions of less than 500 nm can be produced. The next level of focal volume confinement is the use of a nanofluidic channel, which physically constrains the sample to an axial depth of 500 nm, and in one lateral dimension to a width of 500 nm.

In addition to reducing the focal volume of the sample of interest, nanofluidic channels enable the rapid interrogation of every molecule in the sample. In many cases, other single molecule detection techniques rely on diffusion to bring the molecule of interest into the detection region. This process can be slow, and does not provide precise control over which molecules are detected and analyzed. In nanofluidic channels, sample speeds of several millimeters/second can be achieved, enabling truly rapid analysis.

The material properties of the nanofabricated structure are very important for achieving the maximum attainable signal to noise ratio. Autofluorescence is an intrinsic source of noise and must be minimized by careful selection of device material. Additionally, the device should be chemically inert so as not to interact with the sample in any way. Electrical properties must also be considered if electrokinetic drive is to be used to move the sample through the detection volume.

Summary:
Nanofluidic channels were fabricated for use in single molecule studies. A fusion bonding technique was chosen for its simplicity, high throughput and low cost. Devices were fabricated using standard photolithographic processes. Fused silica was selected for the substrate and cover wafers for its outstanding optical, chemical, thermal and electrical properties.

Preliminary results demonstrate a signal to noise ratio high enough for single fluorophore detection. A number of samples including fluorescent microspheres and Bacteriophage-λ digest have been used to characterize the performance of the system. A plethora of biological and nanoscale samples are being considered for analysis, including mRNA, DNA, various proteins, and more.

Future work includes the fabrication of smaller channels for increased signal to noise ratio, and the simultaneous detection of single molecules driven through a linear array of channels.

References:
Nanofluidic Channels for Single Molecule Studies

**CNF Project # 551-95**

Principal Investigator: Harold G. Craighead

- Increased signal to noise ratio through focal volume confinement.
- Nanofluidic channels enable rapid interrogation of every molecule.
- Fused silica fusion bonding for rapid fabrication, superior material properties.

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Figure 1: Reservoir and powder blasted inlet hole.

Figure 2: 10 µm transport channel array.

Figure 3: 500 nm detection channel array.
Abstract:
Conventional DNA analysis uses radiochemical or fluorescent methods to detect the binding (hybridization) of unknown DNA (target DNA) to known complementary DNA (c-DNA). Drawbacks include the need to attach fluorescent or radioactive labels, and the inability to follow time-varying concentrations of DNA.

The alternative is to make use of large negative charge of DNA in solution (about one charge per base pair) and detect the charge transfer during matching. E.g., a recent electro-chemical sensor constructed at MIT senses transfer by measuring the resistivity of a thin silicon beam containing a near surface p-n junction. During binding, depletion layer thickness increases, increasing the resistivity of the beam.

A more sensitive technique is to use a thin film transistor. The c-DNA is attached to the gate of TFT-FET transistor operated in the sub-threshold regime.

The two problems to be solved are the selective attachment of c-DNA and to quantitatively correlate of potential changes measured with selective binding as seen by fluorescence. To simplify these investigations, we separated sensing from amplification and currently fabricate simplified test structures. The inner Au electrode mimics the Au gate of our previous TFT sensors. The outer “reference” electrode is fabricated with either Au or Al.

Four series of experiments have been conducted to understand the origin of the potential that develops between the inner Au electrode and the outer Al reference electrode under various conditions. In all cases, a 2x SSPE/0.2% buffer was used. This is a near neutral, pH 7.4, solution of 3.0 M NaCl, 0.2 M NaH₂PO₄, 0.02 M EDTA. Potential was measured as function of time with an electrometer with an input impedance in excess of 10¹⁴ Ω.

First, “bare” sensors were tested, with neither c-DNA attached to the inner Au electrode or matching t-DNA added to the buffer solution. Second sensors with single stranded c-DNA were attached to the Au electrodes. (The c DNA used was 5’TGTACCGTACCTGGTCGGAGTGCGA TCTTC 3’ with a molecular weight of 9205). No DNA was added to the buffer solution. Third, c-DNA was attached to the inner Au electrode and matching t-DNA was added to the buffer solution. Fourth, c-DNA was attached to the inner Au electrode and non-matching t-DNA was added to the buffer.

When neither c-DNA nor t-DNA was added to the buffer, no significant voltage change was observed with time. On the other hand, when c-DNA matched the t-DNA, output voltage decreased ~ 0.3 V after a time period compatible with hybridization taking place between c-DNA and matching t-DNA. The decrease is consistent, but much more experimenting will be needed to put this finding on a firm footing.

Summary:
Simplified test structures were fabricated to investigate the feasibility to construct a TFT-FET sensor to follow the hybridization between c-DNA and matching t-DNA. Initial results are encouraging, but cross correlation with fluorescent labeling experiments will be required to validate these preliminary findings.

References:
Thin Film Transistor Sensor for DNA

CNF Project # 564-95

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- Poly-silicon thin film transistor (TFT-FET) DNA sensor.
- Potential change during DNA hybridization.

Figure 1, top right:
Structure of TFT sensor (a) Side view of sensor (b) Top view of sensor.

Figure 2, below right:
Change in potential between inner electrode, covered with c-DNA, and outer Al electrode after matching t-DNA was added to buffer solution.
Introduction:

Multivalent binding of ligands on the surfaces of mast cells can lead to the cross linking of two molecules of IgE and lead to the clustering of high affinity IgE receptors (FceRI) on a cell surface. Aggregation of the high affinity IgE receptors then initiates a signaling cascade that terminates with the secretion of mediators of an allergic exocytosis reaction termed degranulation [1]. The desire to provide new insights into these processes and understand the spatially controlled signaling events initiated by ligand mediated clustering of receptors has produced an interest in using ligand-patterned surfaces [2, 3]. These would provide us with control over size, distribution and location of receptor clusters and enable us to systematically observe the spatially controlled events. We utilize self-assembled monolayers as molecular templates to engage and cluster IgE-receptors on RBL mast cells with the target spatial resolution [4, 5]. These types of “bioactive templates” were fabricated using electron beam lithography, and consist of gold arrays on silicon with patterns in the micron to submicron scale [6]. Gold arrays served as the molecular tethering sites, covalently binding self-assembled monolayers of alkanethiols.

The ligand, 2, 4-dinitrophenyl(DNP)-caproate interacts specifically with anti-DNP IgE bound to the high affinity cell surface receptors, FceRI of RBL mast cells. Present results indicate these arrays are a powerful tool for visualization and systematic characterization of submicron scale co-redistribution of membrane and cytosolic components involved in receptor-mediated cellular signaling.

Summary:

We utilize self-assembled monolayers as molecular templates to engage and cluster IgE-receptors on RBL mast cells with sub-micron scale spatial resolution. Bioactive templates were fabricated using electron beam lithography, and these consisted of gold arrays on silicon with patterns in the micron to submicron scale. Gold arrays served as the molecular tethering sites, covalently binding self-assembled monolayers of alkanethiols. The free end of the monolayers were functionalized with 2, 4-dinitrophenyl(DNP)-caproate based ligands which interact specifically with anti-DNP IgE bound to the high affinity cell surface receptors, FceRI of RBL mast cells. These arrays provide a powerful tool for visualization and systematic characterization of submicron scale co-redistribution of membrane and cytosolic components involved in receptor-mediated cellular signaling.

References:

Figure 1: SEMs of (a) 1 µm squares gold posts on silicon with pitch of 2 µm, (b) 200 nm squares with 400 nm pitch. Electron beam lithography was used for fabrication of these arrays using the lift-off approach.

Figure 2: IgE-Receptors are crosslinked by DNP-ligand SAMs on a patterned surface. Patterned SAMs provide a spatially localized stimulus to the cells and IgE-receptors on the cell membrane crosslink only on these regions. (a-c) Confocal images of RBL mast cells stimulated by DNP-ligand SAMs. Substrates imaged under confocal microscopy (468/568 nm laser lines) with 100X objective.
Abstract:

The goal of our research program is the restoration of useful vision to patients who are blind with degenerative retinal diseases. Acute human surgical trials performed by our group and others have demonstrated that some vision may be restored through electrical stimulation of surviving ganglion cells using a microfabricated electrode array. Volunteers reported visual percepts that corresponded to patterns of electrical stimulation in some cases, but more complex images could not be identified. This is likely due to the need for the nervous system to adapt over time to this new form of input. In order to ascertain the true potential of a visual prosthesis for the large numbers of patients who suffer from retinal degenerations, it will be necessary to simultaneously address a number of major interdisciplinary technical challenges to produce a successful, chronically implantable device—one that can safely operate in the saline environment of the eye over a period of years.

Summary:

A paradigm shift in our surgical and design methodology has resulted in a wireless implant, 99% of which is attached in the sterile environment of the back of the eye in such a way that it is invisible following implantation. Only the ultrathin and flexible electrode array passes through the wall of the eye, where it is positioned under the retina. This design places the power receiving circuit elements outside the eye’s wall, thus enabling a wide range of encapsulation options. We have now developed a prototype ab externo prosthesis.

This system is a wirelessly driven programmable low power retinal microstimulator built on a flexible parylene substrate with overall dimensions of 12 x 24 mm. It consists of secondary coils for receiving power and data signals from outside the body, circuitry for converting the RF carrier to a DC power supply, a parylene stimulating array containing iridium oxide micro-electrodes, and a test IC for evaluating the hermeticity of the encapsulating materials. Mock-up versions of this device have been successfully implanted, and we expect to evaluate the first fully implemented microstimulator in animal surgical trials in Fall, 2004. Meanwhile, an ongoing biocompatibility study has demonstrated the post-surgical recovery of healthy retina tissue after the subretinal introduction of coated electrode array materials, and significant reductions in the power requirements of the stimulator chip have been achieved by means of energy recovery from previously charged electrodes. In short, we are developing a new type of miniaturized neural prosthesis that embodies several simultaneous advances in bioengineering technology. It is necessary to perform extensive simulation and in vitro testing of the completed microstimulators prior to implantation, and we have assembled the transmitting apparatus, test equipment, and trained personnel to perform these tasks.

We are fortunate to be receiving ongoing in-kind support from MOSIS, Inc. for the fabrication of these circuits that form the heart of our prosthesis.

All of the above efforts are supported by and complementary to the work of our surgical and retinal physiology teams. In the near future, we will refine our ab externo surgical technique for implanting our device in animal surgical trials in a cooperative effort with our CNF-based engineering group. We look forward to the opportunity to solve the interdependent biomedical engineering and surgical problems outlined above in the years to come, and ultimately to the restoration of useful vision to blind patients.

References:

Full-text versions of recent articles published by our group may be found online at our Web site, http://www.bostonretinalimplant.org.
Retinal Implant Project

CNF Project # 657-97

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- **Goal:** Restoration of useful vision to patients blind due to retinal degenerations.

- **Method:** Wirelessly powered flexible implant stimulates remaining healthy nerve cells with visual information from an external camera.

- **Uses** iridium oxide microelectrode array fabricated on parylene substrate.

Figure 1, top right:
Mock-up version of ab externo retinal prosthesis.

Figure 2, below right:
Artist’s rendering of implant’s placement in eye orbit.
Microfabrication of Mixers for use in Macromolecular Folding Experiments

CNF Project # 692-98

Principal Investigator: Lois Pollack

Abstract:
This project continues to focus on the development of small volume, low background sample cells for use with x-ray scattering applications. Silicon nitride windows on the sample cell, and at other locations along the x-ray beamline, dramatically decrease the scattering background. Lower-background microfabricated mixers have been employed to measure global conformational changes that occur during macromolecular folding. We are also developing sample cells for use with static x-ray scattering measurements [1] as well as reduced sample volume mixers for use with confocal microscopy.

Summary:
To avoid significant background scattering from air, many x-ray experiments are performed in vacuum. However, liquid samples must be contained, therefore sample cells must be sealed with windows that pass x-rays with minimal attenuation. For practical reasons, it is difficult to construct refillable sample chambers that are vacuum compatible, so it often becomes necessary to break the vacuum. In this case, windows are inserted along the path of the x-ray beam in locations that minimize the air gaps. X-rays, in a typical scattering experiment, must pass through four or five windows; each adds to the background scatter. This high background can be problematic. If the scattering from molecules in small sample volumes, e.g. from within microfabricated flow cells [2], is weak, it is easily dominated by background scattering from the windows. We have been employing silicon nitride membranes as x-ray windows for both our sample cells and at other locations along the beam’s path. This year, using microfabricated mixers sealed with silicon nitride windows, we continued our experiments to probe the earliest events in RNA folding. We are now focusing on the role of the positively charged ions that are required to fold negatively-charged RNA. We have determined that the first phase of folding corresponds to an electrostatic collapse or relaxation [3]. This observation highlights the importance of the diffuse cloud of small ions that surrounds nucleic acids and has led to a second series of experiments probing the equilibrium distribution of cations around negatively charged DNA. The goal of these experiments is to determine the spatial distribution of ions as a function of increasing ion charge. The ions’ x-ray signature is quite small [4], therefore this experiment benefits greatly from the use of the CNF-fabricated silicon nitride windows along the beamline.

References:
Microfabrication of Mixers for use in Macromolecular Folding Experiments

CNF Project # 692-98

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Figure 1: Cartoon of an RNA folding experiment carried out within microfabricated mixers. Folding is initiated by the rapid addition of small Mg$^{2+}$ ions to unfolded RNA. The RNA folds as it flows down the channel; the x-ray beam is moved relative to the device to probe the conformation of the RNA at any position along the outlet channel, corresponding to different times after the initiation of folding. Schematic conformations (see Russell et al., PNAS 99 (2002)) of the RNA are shown at different locations along the outlet channel. Molecule a. is unfolded; molecules b. and c. are captured during folding. This mixer is sealed on both flat surfaces with small, silicon nitride windows.

Figure 2: Photograph of a silicon nitride membrane employed as a window during our run at the Advanced Photon Source. The square window is visible at the center of the square piece of silicon. These windows are fabricated at the CNF and are placed before and after the sample cell. The smaller windows used to seal the sample cell (caption, Figure 1) have dimensions on the order of tens of microns and are not shown in this picture.
Biomaterials offer enormous promise for the repair of lost tissue function. The physical structure of a biomaterial is now known to be a key factor that determines cellular responses and hence the range of biomedical applications suitable for a material. Both micrometer and nanometer scale features of a material significantly influence cell behaviors—such as morphology, migration, adhesion, proliferation and differentiation in vitro and in vivo.

We have produced biomaterials with complex structures in which features of different length scales can be hierarchically organized. To accomplish this objective, we mimicked natural material formation processes such as bone, tooth and shells. The key to ordered structures in biological systems typically involves hierarchical construction, with separate steps for first building the foundation and then transcription of a finer-scale pattern. In our hierarchical fabrication method (Figure 1), micrometer scale architecture was created by microfabrication technology; nano-structured hydroxyapatite (HAP) minerals were then synthesized onto the preset structure using natural mineralization approaches. It should be noted that nano-structured HAP is regarded as the major component of bone minerals and the formation of such material may have direct application in bone implantation and tissue engineering.

Summary:

We first patterned parallel ridges/channels (R/C) (Figure 2a) as a framework to guide the spatial organization of nanostructured HAP. This pattern was selected because it was expected to have immediate visible effect on cell morphology and orientation, enabling determination of its biological impact. Other patterns, such as pillars, can be easily generated depending on particular applications (Figure 2b). Silicon was chosen as a starting material because it is the most widely used material in microfabrication; however, the design principle defined in Figure 1 can be applied to other materials of biological relevance with minor technical modification.

After chemical modification, micropatterned materials were immersed in supersaturated calcium phosphate solutions for mineral deposition for up to 3 days. The resulting mineralized structures were examined by scanning electron microscopy (SEM) at various times after initiation of mineralization. SEM images indicated that nucleation and crystal growth appeared on the patterned substrate within 8 hours (Figure 3a). The initial precipitates were round and distinct. After 24 hrs, a nearly continuous layer of minerals was formed on the patterned surface (Figure 3b) and the mineral morphology changed to thin and plate-like. As the reaction continued past 24 hours, the appearance of minerals remained the same while the patterned ridges became evidently wider (Figure 3c). Similar results were obtained for the mineral formation on pillared structures (Figure 3d). Close examination of the mineral revealed nanometer-scale texture with thin plates of less than 100 nm on the patterned surface (Figure 3e). The mean size of the minerals was 56±32 nm as determined by AFM. The size did not change to any significant degree with reaction time up to 3 days (Figure 3f).
Biomaterials with Hierarchically Defined Micro- and Nano-Scale Structure

CNF Project # 757-99

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Figure 1: Schematics of the steps for producing micro- and nano-structured materials. a) A clean silicon wafer; b) Micrometer scale structures were created using photolithography and reactive ion etching techniques, top view; c) Surface modification, side view. Please note that features were not drawn to scale. Acidic moieties (-COO-) were generated on the surface through silanization and succinylation; d) A layer of nano-structured calcium phosphate was formed on the patterned surface in supersaturated calcium phosphate solution.

Figure 2: SEM images of micro-scale structures. a) Parallel ridges/channels patterned on silicon substrate. The width and height of the ridges are 4 µm and spacing is 10 (m. b) Regular arrays of pillars (4x4x4 µm) patterned on silicon substrate. Scale bars represent 4 µm.

Figure 3: SEM images of micro- and nano-scale structures. a) After 8 hrs in supersaturated calcium phosphate solution, discrete and round initial crystallites were formed on the patterned substrate. b) After 24 hrs, the crystallites became thin-plate like and started to form a continuous layer on the patterned substrate. The width of the ridges was 5.1 µm. c) After 48 hrs, the morphology of crystals remained the same while the mineral layer became more uniform and continuous. The width was increased further to 5.7 µm. d) Nanostructured HAP was organized into pillared structural form. e) High magnification image of minerals shows a clear thin-plate structure with the plate thickness at ~100 nm. Scale bars on SEM images represent 4 µm. f) AFM images of minerals, the average grain size was determined at 56±32 nm, in agreement with the SEM study.
Abstract:
In this study, we fabricated microfluidic channels on glass wafers for electrophoresis of protein mixtures. The fabrication involved mainly dry etching and thermal bonding of the glass wafer. A plug of protein mixture was injected into the separation channel using an electrokinetic valve at the cross of the channels. The different protein bands were detected at the end of the separation channel using laser-induced fluorescence. The electrophoresis data showed very high reproducibility with the standard deviation in the residence time of the peaks smaller than 0.5%. We also demonstrated a novel mechanism that was able to separate surfactant-denatured proteins without gels. The separation is believed to be based on the difference in the amino acid sequence instead of the molecular size. Further work is underway to use similar devices to separate real biological samples such as cell lysates.

Summary:
Polyelectrolytes such as DNA and denatured proteins normally have free solution mobilities independent of the molecular size unless their sizes are very small. Therefore, separation without a sieving matrix is not possible. Here, we report a novel mechanism for separation of denatured proteins in free solution.

In our experiment, a mixture of sodium alkyl sulfates with different carbon chain lengths (C_{12}, C_{14}, and C_{16}) was used for protein denaturation. Four model proteins (α-Lactalbumin, Ovalbumin, Conalbumin and β-Galactosidase) had significantly different electrophoretic mobilities in free solution under the above conditions, which led to complete separation.

We also found that the presence of a binary mixture of sodium dodecyl sulfate (SDS, C_{12}) and sodium tetradecyl sulfate (STS, C_{14}) could lead to separation to a similar degree. The effective mobilities of the four proteins were estimated to range from -1.7 to -3.4 x 10^{-4} \text{cm}^2\text{v}^{-1}\text{s}^{-1}. No separation was observed when SDS or STS alone was used. We attribute the difference in the electrophoretic mobility among various denatured proteins to the heterogeneous distribution of SDS and STS on the protein surface, which is dependent on the amino acid sequence. The characteristic binding of different surfactants along the polypeptide chain determines surface charge density of the protein-detergent complex, and therefore determines the unique free solution mobility for each protein.

We demonstrated the separation in a microfluidic chip and the same protocol can be implemented in conventional capillary tubes. Without the need for sieving matrices or complex instrumentation, this separation technique offers shorter analysis time and higher sensitivity. More importantly, because the mechanism is orthogonal to the existing separation modes, this method can potentially be applied in multidimensional separation systems, complementary to gel electrophoresis and isoelectric focusing.
Separation of Denatured Proteins in Free Solution on a Microfluidic Chip

CNF Project # 762-99

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• Microfluidic channels fabricated based on glass wafers.

• Plugs of protein mixture injected at the cross using an electrokinetic valve.

• Surfactant-denatured proteins separated in free solution.

Figure 1: The layout of the microfluidic channel.

Figure 2: The injection of mixture plugs into the separation channel using a gated electrokinetic valve.

Figure 3: Proteins separated by mixed surfactant coating. The peaks are from proteins: 1. β-Galactosidase (116 kDa); 2. Conalbumin (80 kDa); 3. α-Lactalbumin (14 kDa); 4. Ovalbumin (45 kDa).
Abstract:

We have developed methods for patterning receptor-populated lipid bilayers and multilayer protein domains at the micrometer scale. The receptors contained in the lipid bilayers patterned onto silicon or glass substrates are functional and have been used to bind and detect toxin fragments. Protein domains have been patterned onto both gold electrodes and silicon substrates and used to selectively bind targeted antibodies. To pattern these domains we have made use of a polymer lift-off technique, which involves etching a parylene-C coating to expose specific areas of a silicon substrate [1]. Biomolecules are then incubated with the substrate and bound onto it with appropriate chemistry. Polymer lift-off following biomolecule attachment reveals patterns on the exposed areas of the substrate. We have achieved patterning with optimized fluorescence intensity, reducing non-specific binding with the aid of the polymer lift-off and an underlying hydrophilic layer as a secondary blocking agent.

Summary:

Biosensors that rely on the binding specificity of a biomolecule to its corresponding lipid or protein receptor have an important application in field-testing of samples to detect infectious agents or clinical markers. The reduction of the size of the biosensor, as well as the sample volume, and the capability of integration of receptors for multiple toxin detection in a single chip still remains a challenge. We present an approach to create micrometer-scale lipid bilayer domains that mimic cellular membranes targeted by two bacterial toxins and micrometer scale multilayer protein that selectively bind targeted antibodies.

To pattern the biomolecule domains, we made use of a novel micro-patterning technique which involves the etching of a polymer coating to expose specific areas of a silicon, glass or gold substrate [1]. The substrate was then incubated with lipid vesicles (for lipid bilayer domains) or proteins (for protein domains). Lipid vesicles fused onto the silicon or glass surface and formed lipid bilayers as described by Orth et al. [2]. Polymer lift-off, following lipid deposition, revealed lipid patterns on the exposed areas of the silicon chip. The lipid domains obtained ranged in size from 0.5-76 µm with square and rectangular shapes. In this fashion we have created lipid domains populated with gangliosides G_{M1} and G_{T1b}. These domains were capable of binding cholera and tetanus toxin fragments respectively.

Protein patterning was optimized on both silicon surfaces and gold electrodes with covalent thiol-gold linkage. Bath-applied recombinant protein G was used as the covalently linked layer. Polymer lift-off following covalent linkage of protein G revealed protein patterns with similar sizes as those obtained for lipid domains. The protein G layer was used to specifically bind the F_\text{ab} regions of an applied immunoglobulin, orienting the F_\text{c} regions of this primary antibody properly for optimized capture efficiency of a secondary antibody.

Lipid bilayer domains populated with gangliosides G_{M1} and G_{T1b} were used to bind cholera toxin subunit B (CTB) and tetanus toxin fragment C (TTC) respectively. Concentrations of 20 pM and 100 pM for TTC and CTB were detected optically. This suggests the use of this patterning technique for lipid bilayer domains in biosensor applications for the detection of specifically bound toxins. Furthermore, the ganglioside populated lipid domains remained functional and in distinct patterns even after a year of storage at 4°C.

Fluorescently labeled antibodies were used to demonstrate preferential binding to the protein G patterned areas. A green fluorescently labeled primary antibody was bound to the patterned protein G layer and used subsequently to capture a specific secondary antibody (labeled with red fluorescence). Polymer lift-off combined with hydrophilic passivation significantly reduced nonspecific binding to non-patterned areas. We propose that patterned antibodies on silicon can also be used in biosensing applications based on immune recognition of analytes.

References:


Lipid and Protein Patterning for Biological Applications

CNF Project # 762-99

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- Patterning of lipids is performed with a polymer lift-off technique.
- Bilayers populated with gangliosides are patterned in features with sizes from 0.5-76 μm.
- Patterned bilayers are incubated with fluorescently labeled toxin fragments.
- Lipid patterning and selective toxin binding can be used for biosensor applications.
- Protein domain patterning is performed through a polymer lift-off technique.
- Protein G is covalently linked to the exposed areas of the gold substrate via thiol chemistry.
- Polymer lift-off reveals micrometer sized patterns of covalently linked protein G.
- Fluorescently labeled primary antibody is applied and bound via Fc region to the protein G layer. Non specific binding is prevented with the underlying hydrophilic passivation layer.
- Fluorescently labeled secondary antibody is captured from solution via specific interaction with primary antibody.
- Protein multilayered domains can be used in combination with gold electrodes for sensing or capturing of target analytes.

Figure 1: Patterns of fluorescently labeled toxins bound to lipid domains populated with ganglioside receptors. (a) Alexa 594 conjugated CTB bound to lipids containing GM1; (b) Alexa 488 conjugated TetC bound to lipids containing GT1b. Both toxins at a concentration of 100 nM.

Figure 2: Patterns of covalently linked unlabeled protein G (20 μm squares) with successive attachment of Alexa 488-labeled goat anti mouse IgG and Alexa 594-labeled mouse anti human IgG.
Abstract:
Nanofluidic channels are fabricated using a non-lithographic method. Using electrospinning, we deposited HDPC (Heat Depolymerizable Poly-Carbonate) fiber (several hundred nanometer diameter on average) on various substrates. We then used this HDPC fiber as the sacrificial layer of nano-scale channels. We also developed a size controlling mechanism for the fibers. These channels can be used as a DNA separation device.

Summary:
Recently, microfluidic and submicron-size channels have been a topic of focus because of their biologically relevant size [1]. Sorting DNA molecules, and synthesizing and controlling chemicals in extremely small quantities are the examples of applications of nanofluidic channels [2]. In this research, we used a non-lithographic method to fabricate nanofluidic channels. Conventional methods of fabricating sub-micron channels with lateral dimension of less than 200 nm typically use lithographic methods. However, our method enables us to fabricate without these difficult and expensive techniques.

HDPC is a good material for the sacrificial layer of nanofluidic channels. Using electrospinning, we were able to deposit fibers on the various substrates. Electrospinning is a novel method to fabricate small fibers with diameter of less than 1 micron. The target substrates used in this experiment consisted of silicon, thin film silicon dioxide on silicon, and glass substrates. The substrate was mounted on a counterelectrode located approximately 1.5 cm from the tip. Twenty microliters of a 25 wt% solution of HDPC (Mw~100,000) in chlorobenzene was dispensed onto the tip and voltage was applied between the tip and the counterelectrode. At a critical voltage around 5 kV, a Taylor cone formed and a polymer jet was extracted from the source. As the jet traveled toward the counterelectrode, the solvent would have evaporated and deposited a cylindrical fiber on the substrate [3]. The diameter of these fibers was measured to be around 100–1,000 nm by atomic force microscope right after electrospinning. After depositing HDPC fibers, SOG (spin on glass) was spun with a spinner. We could produce the thickest capping layer around 1 micron. Transparency of SOG strongly suggests that these channels be used for the application of biological experiments. In order to provide fluid inlet and outlet ports, both sides of the fiber and capping layers have been etched in the Plasma Therm 72. The layer of photoresist protected the main channel area. Since the HDPC fibers are evaporated into non-toxic vapor above 250°C, HDPC was removed from the channels by heating it up 450°C for 48 hours on a hot plate. Finally, we attached PDMS gaskets onto the fluid inlet and outlet ports for fluid reservoir.

We also developed a method to control the size of the channels. A plasma etch was performed in a plasma cleaner using an oxygen plasma to etch the fibers deposited on the substrate. This plasma provides isotropic etching which preserves the original elliptical fiber shape after the etch process. With a low power plasma, we could get a linear relation between time and the amount of etched fiber. Therefore, we could control the size of the sacrificial layer so that we could also control the size of the channels.

To confirm open channels, a fluorescent dye solution of 100 mg rhodamine B in 5 ml methanol and 5 ml H₂O was pipetted into an inlet port and made to fill the structure by capillary force.

References:
Nanofluidic Channels

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• HDPC fiber was deposited on the various substrates by electrospinning.
• This fiber acts as the backbone of nanoscale channel.
• Transparent SOG layer was spun on the substrate.
• The control of the channel size is possible by using a plasma etcher.
• Fluorescent dye solution was used to confirm open channels.

Figure 1: AFM depth profile before and after oxygen plasma etch. 5 minute etch with low power. (a) Before etch the width and height of the channel is 976.56 nm and 590.20 nm respectively and, (b) after etch, 566.41 nm and 299.62 nm respectively. In total, 50% of fiber diameter is removed.

Figure 2: SEMs showing cross section of nanofluidic channel. SOG layer deposited over the fiber formed the channel. The shape of the channel is elliptical. The length of the major and minor axis are 522.7 nm and 98.44 nm respectively.

Figure 3: Fluid (rhodamine B) filling the channels. Left channel is already filled with fluid and the other is being filled. This process took approximately 5 minutes.
Abstract:
Neurons, the signal generating cells of the mammalian nervous system, attach and grow processes in response to a number of chemical and physical surface cues. Some of these physical cues are on the micrometer scale. We have generated surfaces with arrays of pillars of varied widths and spacings to study how these cells extend their processes and to determine how they can be guided. Cells were grown on surfaces fabricated from silicon wafers as well as those micromolded into polystyrene using the silicon structures as masters. The latter allows production of a large number of surfaces inexpensively. Rat hippocampal neurons were grown on the surfaces and process outgrowth, both axonal and dendritic, was quantitated as a function of pillar size and spacing.

Summary:
In the brain, neurons grow in distinct patterns, forming complex electrical circuits. To accomplish this, they extend processes, axons for sending signals and dendrites for receiving them, to contact other neurons. This biological phenomena is extremely complicated, making model systems composed of isolated cultured neurons a powerful method to understand neuronal function and network formation. The growing processes of one neuron are guided to their target neuron(s) by a number of biochemical and physical cues. Some of the physical cues are thought to be textural and some of these are on the micrometer scale. Thus, we have fabricated regularly textured surfaces and grown neurons on them to better understand the outgrowth of these fundamental neuronal processes.

Pillars 1 micrometer tall with widths of 1.5 or 2.0 µm with spacings, interpillar distances, of 1.5 to 4.5 µm were fabricated in silicon using standard processing, including reactive ion etching [1]. The surfaces were composed of arrays of pillars and smooth surfaces arranged such that each neuron grew completely on a smooth surface or on pillars that all had the same width and spacing. Polystyrene replicas were micromolded from silicon masters using a soft (PDMS) intermediate mold [2]. All surfaces were treated with polylysine that is required for neuron attachment. Neurons were grown on the surfaces and fluorescently labeled for specific proteins by immunocytochemistry for observation in the light microscope or were critical point dried and observed in the scanning electron microscope.

Processes, both axons and dendrites, projected from the cell bodies and grew randomly on smooth surfaces. Neurons extended their processes in patterns extremely faithful to the pillar geometry when the pillars were spaced close to each other but less faithfully as the spacing increased. Process growth was nearly random when the spacing was 4.5 µm. We have quantitated this process outgrowth using custom software developed in collaboration with Prof. Roysam of the Rensselaer Polytechnic Institute and CenSIS an NSF Engineering Research Center.

References:
Neurons attached and grew on pillars, extending axons and dendrites along the pillars’ base or on their tops. Figure 1.

The pillar size and spacing strongly influenced neuronal outgrowth. When the pillars were close together, 1.5 µm in Figure 2, processes followed the geometry, but when they were further apart, 4.5 µm in Figure 3, their grow was nearly random. The pillars in both figures were 2.0 µm wide and 1.0 µm tall.
Abstract:

The techniques of selective filtration and equilibrium dialysis are often used to analyze complex mixtures of cellular products and smaller molecules in solution. For example, solutions such as blood typically contain complex mixtures of biomolecules in the size range of 1-10 nm, which must be separated and purified prior to testing. To perform genetic testing on a blood sample, DNA (2 nm diameter, linear) must be separated from hemoglobin (5.5 nm diameter, globular). Attempts at a size-based separation face the problem that even e-beam lithography cannot reliably generate features smaller than 20 nm laterally. However, metal films thinner than 10 nm can easily be deposited, capped with an SiO₂ film, and later wet-etched away as a sacrificial layer. The resulting channels, as well as the entry and exit holes, may measure many microns laterally. Yet by fully offsetting the entry hole (through-etched in the substrate) from the exit hole (etched through the oxide cap), filtrate solutions are forced along a ‘double-L’ path with the middle leg thin enough to exclude by size alone molecules larger than the sacrificial film thickness.

In addition, a dissimilar metal may be deposited atop the oxide cap to act as an electrical gate, either sorting or detecting filtrate molecules according to charge. While a few other groups have developed membrane systems which incorporate some of these features [1, 2], we believe that our device may ultimately prove more robust, require fewer steps to fabricate, and yet competitively meet the concurrent requirements of nanometer-scale channel sizes and integrability with Si electrical devices.

Summary:

We have successfully fabricated prototypes of a singly-gated device with a channel height of 25 nm. Much of the initial fabrication was done by students in the Research Experience for Undergraduates (REU) program [3]. Experiments are underway by another REU student to test device throughput and durability in a direct flow geometry. In these experiments, a device array approximately 1 cm x 1 cm is clamped between two chambers of PDMS. A dye-containing solution is then pumped through the device with a syringe pump, and the outflow monitored via UV/vis spectrometry for passage of the dye molecules. The current student will also incorporate two electrical gates per channel, creating an electrical gradient along the channel to promote charge—as well as size—based separation. Finally, we plan to attempt separations of realistic test solutions (e.g., DNA and hemoglobin) with thinner channel constrictions.

References:

Nanofiltration Through Microfabricated Sieves of Novel Geometry

**Goal:** Fabricate lithographically patternable, integrated, porous film devices for molecular filtration.

**Figure 3, below right:** SEM cross-section of single device. Left hole through-etched in wafer. Right hole 8 µm diameter. Embedded electrical gate visible (bright, center) over etched sacrificial channel (dark black horizontal line).

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**Figure 1:** Completed device, packaged in PDMS.

**Figure 2:** Optical microscope image (reflected light, from top) of array of devices before etch of 25 nm Al sacrificial layer.
Abstract:
Bioanalytical microsystems for the detection of viable pathogenic organisms are being developed. Pathogenic organisms such as B. anthracis, C. parvum, Dengue virus and E. coli are identified via specific mRNA molecules or their genomic RNA (in the case of the virus) with detection limits between 5 and 40 cells/mL. The microsystems consist of modules in which bioanalytical operations are being performed. These modules are for example a laser-induced cell lysis system, an RNA purification system, a nucleic acid sequence-base amplification module and a biosensor component for the detection and quantification of the RNA molecules.

In addition, research is being performed on the design of mixers, microheaters and electrochemical transducers. In the last year, the main work focused on the development of a robust, sensitive, portable and reliable biosensor module. A biosensor based on fluorescence detection using a fluorescence microscope and one based on electrochemical detection using an interdigitated ultramicroelectrode array (IDUA) as transducer were designed and tested [1]. They were based on an earlier prototype developed in our lab [2] and were made in polydimethylsiloxane (PDMS) and packaged in a polymethyl methacrylate housing that allowed easy connection to external syringe pumps. Extremely low detection limits of 0.1 and 1 fmol of RNA, respectively, were obtained. The electrochemical biosensor can be operated by a micropotentiostat with microprocess controller and is thus a core module of the portable bioanalytical microsystem.

Summary:
The biosensor modules of the bioanalytical microsystem are being fabricated in polydimethyl siloxane (PDMS) using soft-lithography technology. Two channels, 100 µm wide and 50 µm deep, are made in PDMS, covered with a glass slide and packaged in a polymethyl methacrylate housing. In the case of the electrochemical biosensor, IDUAs are fabricated on the glass slide and are thus put in direct contact with the microchannels. Syringe pumps are connected to the channels through tubing and the sample solutions are actively pumped through the channels at flow rates between 0.5 and 20 microliter/min. A mixture containing magnetic beads coated with DNA oligonucleotides (capture probes), liposomes entrapping either dye or electrochemical marker molecules and tagged with a second DNA oligonucleotide (reporter probe), sample RNA and a hybridization buffer is pumped through the main microchannel. The magnetic beads are caught on a magnet capturing a complex of hybridized RNA, liposomes and magnetic beads. Optically, the amount of RNA in this zone is then quantified using a fluorescence microscope. In the case of the electrochemical detection, the liposomes are lysed via a detergent that is pumped through the second channel and the released electrochemical markers are quantified via oxidation and reduction reactions on the IDUA. The same lysis can also be used for optical detection in order to increase the fluorescence signal by approximately an order of magnitude. The overall assay can be accomplished in only 10 minutes and extremely low detection limits of 0.1 and 1 fmol, respectively, were obtained.

References:
Development of Microfluidic Biosensor Devices Based on Liposome Amplification Strategies

CNF Project # 802-99

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- Microbiosensor, core module of a bioanalytical microsystem for pathogen detection:
- Optical and electrochemical microbiosensors fabricated using softlithography.
- Detection limits of 0.1 and 1 fmol of RNA, respectively.
- Detection of Dengue virus, B. anthracis, C. parvum, E. coli.
- Signal amplification using liposomes entrapping optical and electrochemical marker molecules.

Figure 1: Microchannel layout (A) and final microbiosensor device (B)

Figure 2: Signal enhancement through detergent-caused liposome lysis. (A) intact liposomes, (B) lysed liposomes.
Abstract:
We are presently investigating various surface chemistry approaches for the
development of highly sensitive and specific substrate platforms for use in
integrated surface plasmon resonance (SPR)-based biosensor devices. We
report here a multi-step route for the immobilization of DNA and proteins on
chemically modified gold substrates using fourth-generation NH2-terminated
poly(amideamine) (PAMAM) dendrimers [1] supported by an underlying
amino undecanethiol (AUT) self-assembled monolayer (SAM).

Using this procedure, bioactive ultrathin organic films were prepared via
layer-by-layer self-assembly methods and systematically characterized by
fluorescence microscopy, variable angle spectroscopic ellipsometry (VASE),
atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and
attenuated total internal reflection Fourier transform infrared spectroscopy
(ATR-FTIR). The thickness of the AUT SAM base layer on the gold substrates
was determined to be 1.3 nm from ellipsometry. Fluorescence microscopy and
AFM measurements, in combination with analyses of the XPS/ATR-FTIR
spectra, confirmed the presence of the dendrimer/biopolymer molecules on the
multilayer sensor surfaces. Model proteins, including streptavidin and rabbit
immunoglobulin (IgG) proteins, were covalently attached to the dendrimer
layer using linear cross-linking reagents. Through surface plasmon resonance
(SPR) measurements, we found that sensor surfaces containing a dendrimer
layer displayed an increased protein immobilization capacity, compared to AUT
SAM sensor surfaces without dendrimer molecules [2].

Other SPR studies also revealed that the dendrimer-based surfaces are useful
for the sensitive and specific detection of DNA-DNA interactions.
Significantly, the multi-component films displayed a high level of stability
during repeated regeneration and hybridization cycles, and the kinetics of the
DNA-DNA hybridization process did not appear to be influenced by surface
mass transport-limiting effects.

Summary:
Various surface chemistry approaches were investigated for the development
of novel types of surface plasmon resonance (SPR) biosensor matrices. One
promising approach involves the fabrication of a dendrimer-based multilayer on
a 50 nm-thick gold film uniformly coated with an amino-functionalized
alkanethiol self-assembled monolayer. These surfaces were characterized using
various surface analytical techniques such as AFM, ATR-FTIR, XPS etc. The
successful immobilization of proteins and DNA to the 2-D dendrimer surfaces
was achieved by using an intermediate cross-linker layer, and different types of
biomolecular interactions were studied by SPR. These surfaces were found to
be efficient in enhancing the SPR signal. The developed surfaces will be
modified further to improve the specific binding; also, new chemistries based
on mixed SAMs will be explored.

References:
Molecular-level control of size, shape, surface chemistry, topology, and flexibility
from atoms to macroscopic matter. Angewandte Chemie, International Edition in

Functionalized Self-Assembled Monolayers as a Surface Plasmon Resonance (SPR)
Development of Bioactive Surface Chemistries for use with Nanoscale Biosensing Systems

Development of a novel biosensor matrix for an integrated surface plamson resonance (SPR) device.

A multi-step route for the immobilization of proteins and DNA on chemically modified gold substrates using fourth-generation NH₂-terminated poly(amidoamine) (PAMAM) dendrimers.

The dendrimer-based surfaces were found to be efficient in enhancing the SPR signal response.

Figure 1:
Scheme of immobilization of DNA molecules on gold using fourth-generation NH2-terminated poly(amidoamine) (PAMAM) dendrimers as a mediating linking layer.
Abstract:
Many cell types including neurons, chromaffin cells of the adrenal gland and mast cells, release transmitter molecules, stored in ‘vesicles,’ by a process called exocytosis. In this process, an intracellular vesicle loaded with molecules to be secreted, fuses with the cell membrane, forming an initial opening called a fusion pore, and emits its contents into the extracellular space. The process of fusion pore formation and expansion is not well understood. Our goal is to develop microchip devices that detect exocytosis of oxidizable molecules from single vesicles electrochemically, to better understand the process of exocytosis and the role of drugs in the modulation of loading and release of single vesicles.

Summary:
Release of the catecholamines dopamine, adrenaline, and noradrenaline from single vesicles can be detected amperometrically as a brief spike of oxidation current using carbon fiber electrodes [1]. We microfabricated platinum electrochemical detector (ECD) arrays on microscope cover-glasses to obtain spatial information about the release sites [2]. The ECD arrays successfully detected adrenaline release events of single vesicles from chromaffin cells. Release positions were determined based on the relative fraction of molecules that is detected by each of the four electrodes of the array [2] and verified by simultaneous fluorescence microscopy.

We investigated the time course of the signals at the individual electrodes in detail. It was found that even for the electrodes that were far away from the release site, the time course of the signals was much slower than expected for the diffusion coefficient of adrenaline in aqueous solution. We conclude that adrenaline binds reversibly to the cell surface leading to an apparent diffusion coefficient near the cell surface that is about an order of magnitude lower than the aqueous diffusion coefficient.

By combining the ECDs with total internal reflection fluorescence (TIRF) imaging, we found that many of the release events did not occur as expected. In TIRF imaging, fluorescence is excited only in a shallow layer (~150 nm) at the glass-water or glass-cell interface. It is usually thought that release occurs from vesicles that are docked or tethered at the plasma membrane. Docked or tethered vesicles are clearly visible preceding their exocytosis under TIRF excitation. About half of the observed events were of this type. However, release often also occurred at sites where no vesicle was seen prior to release [3]. We hypothesize that vesicles rapidly approach the plasma membrane followed by exocytosis without involving a sustained docking period.

Various drugs affect release from single vesicles (also called quantal release). For example, the drug L-Dopa, used to treat Parkinson’s disease increases quantal size, while drugs such as reserpine and amphetamines decrease quantal size. The changes in quantal size are associated with changes in vesicle size [4]. We began to develop amperometric electrode arrays with integrated electronics that can perform high throughput measurements of quantal size and the effect of drugs on the spatio-temporal properties of single vesicle exocytosis.

References:
Microfabricated Devices to Study Single Vesicle Release

CNF Project # 848-00

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Sunitha Bandla

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- Spatio-temporal localization of neuraltransmitter release.
- Measurement of chemical signals from one or more cells using electrochemical detection.
- Utilizes nanofabricated electrode arrays.

Figure 1: Four Electrochemical Detector Arrays (ECDs) are fabricated on a microscope coverglass. Each ECD site consists of the exposed tips of four platinum wires.

Figure 2: Current traces measured by the electrodes of an ECD during a single release event.

Figure 3: Prototype four pixel “electrochemical camera” array, and N x M array schematic. Pixels are approximately 20 x 25 µm.
A Microfabricated PCR-Based Biosensor

Abstract:
A portable, fully-automated, PCR-based detection system has been developed for the rapid detection of bacterial pathogens. Microfabricated DNA purification and real-time PCR microchips were fabricated and tested for their ability to purify and detect DNA sequences from the Gram positive bacteria Bacillus globigii and Listeria monocytogenes. The microfabricated detection chips contained 10 µm² by 50 µm tall pillars that were etched into the silicon substrate and coated with PECVD silicon dioxide for DNA purification. Using these silica-coated microstructures, bacterial DNA could be bound, washed and eluted for subsequent real-time PCR. These microstructures were included in an integrated detection microchip containing two regions, one for DNA purification and one for real-time PCR. The real-time PCR chamber consisted of a molded poly(dimethyl siloxane) (PDMS) structure that was bonded directly to the silicon chip. Due to its placement above the silicon chip, the chamber could be laterally illuminated for fluorescence excitation. In turn, the fluorescence emission wavelength could be monitored from the top of the chip, providing a means of performing real-time PCR. Using an automated detection platform with integrated microprocessor, pumps, valves, thermocycler and fluorescence detection modules, microchips were used to purify and detect bacterial DNA by real-time PCR using SYBR Green fluorescent dye. Between $10^7$ and $10^4$ L. monocytogenes cells could be detected using this system with an average turnaround time of less than 1 hour. The fully automated detection platform is completely portable, making the system ideal for the detection of bacterial pathogens in the field or other point-of-care environments.

Summary:
There is a need for rapid and accurate methods to detect pathogenic bacteria, viruses and other disease-causing agents. Devices designed to meet this need incorporate multiple laboratory processes in a semi-automated, miniaturized format [1, 2]. Sample preparation for these devices is often carried out off-line which complicates the entire process. We sought to develop a microchip-based detection system capable of purifying DNA from complex samples and performing real-time PCR to detect bacterial pathogens. As a result, a portable, fully-automated, PCR-based detection system has been developed for the rapid detection of bacterial pathogens. The detection platform with integrated microprocessor, pumps, valves, thermocycler and fluorescence detection modules was used to detect bacteria by real-time PCR amplification.

References:
### A Microfabricated PCR-Based Biosensor

**CNF Project # 884-00**

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USDA, FDA, Alliance for Nanomedical Technologies

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<table>
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<tr>
<td><strong>•</strong> An integrated DNA purification / PCR amplification device has been fabricated in a microfluidic format.</td>
<td><img src="image" alt="Figure 1: DNA purification microchip consisting of RIE-etched silicon and PECVD oxide. The pillars are approximately 10 µm square and 50 µm tall. (Provided by Dennis Kunkel.)" /></td>
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<td><strong>•</strong> The device is capable of purifying DNA from bacterial cells and performing PCR amplification via a miniaturized thermocycler.</td>
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<td><strong>•</strong> Real-time PCR detection is performed by lateral excitation of the PCR reaction chamber and monitoring of the emission wavelength from above the chip.</td>
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<td><strong>•</strong> This system has been used to accurately purify DNA from and detect between $10^4$ and $10^7$ <em>Listeria monocytogenes</em> cells.</td>
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**Figure 1:** DNA purification microchip consisting of RIE-etched silicon and PECVD oxide. The pillars are approximately 10 µm square and 50 µm tall. (Provided by Dennis Kunkel.)

**Figure 2:** An integrated DNA purification / real-time PCR microchip. The nucleic acid purification region is shown in (A) while the real-time PCR region is shown in (B). The fluid connections are 1) sample input, 2) waste outlet, 3) PCR reagent input, and 4) reaction outlet. The large white arrow denotes the position of the lateral path for fluorescent excitation for real-time PCR.

**Figure 3:** Detection of *Listeria monocytogenes* was performed by performing microchip DNA purification and subsequent real-time PCR with SYBR Green fluorescent dye. This chart shows the fluorescence as a function of PCR cycle number. An increase above a fluorescence threshold of 5 units was used to determine a positive detection result.
Abstract:
This project focuses on developing a micromachined device for fractioning whole blood using physical methods to enrich and/or isolate and then characterize rare cell types from peripheral circulation for non-invasive prenatal diagnosis and cancer applications. Our long-term goal is a stand-alone system capable of automated cell sorting and genetic analysis.

Our device has four segments of microfluidics channels. Each segment consists of a two dimensional array of columns. The gap width between the columns in the cross direction (perpendicular to the flow) narrows as cells traverse the device. The current design has channels spaced at 15 µm, 10 µm, 5 µm, and 2.5 µm intervals. The channel depth, constant over all segments of any single device, is either 5 or 2.5 µm. By applying different cell types to our device, we were able to show that we can separate cells based on their size and deformation characteristics. Neuroblastoma cells, averaging 10 µm in size, are retained in the 10 µm wide by 5 µm deep channels. Goose red blood cells, averaging 12 µm in size, can greatly deform and pass through channels as narrow as 5 µm wide by 2.5 µm deep; these are retained at the 2.5 µm wide by 2.5 µm deep channels. Red blood cells from human cord blood are retained in region of 2.5 µm wide by 5 µm deep channels. We are expanding these experiments to more fully characterize our device using a series of dyes to identify and track individual cell types.

Summary:
The original design had channels spaced 20 µm, 15 µm, 10 µm, and 5 µm. The channel depth, constant over all segments in a single device, was 5 µm. The current design has channels spaced at 15 µm, 10 µm, 5 µm, and 2.5 µm intervals. The channel depth is either 5 or 2.5 µm. The device has two reservoirs to create an inlet and outlet for fluids; each reservoir has two holes for easier fluid feed. The channels can be sealed using a glass wafer or a polystyrene top.

To make a contact mask with all of these channel segments going directly from a CAD file to the pattern generator would require over 90 hours of continuous equipment time. An alternative method was used to generate four masks for the different channel dimensions in conjunction with the 10X stepper to produce the final contact mask. The pattern was then transferred to silicon wafers using standard photolithography techniques and image reversal to achieve the negative structure. Wafers were dry-etched using deep reactive ion etching and plasma cleaned to remove remaining photoresist.

A polydimethylsiloxane (PDMS) mold for the device was made using the silicon wafer as the master and sealed. The device is assembled by plasma cleaning the PDMS bottom and the polystyrene (or glass) top followed by light pressure to contact seal.

We observed a difference in cell migration through the channels of the device: goose red blood cells stop at the entrance of a channel with only one cell per channel. Human red blood cells, whether fetal or adult tend to aggregate in groups and pause longer while exiting a channel. Occasionally, human red blood cells will stop in the open area between two successive rows of channels. The possibility that the cell died will be explored using vital dyes and cell surface marker stains to complete this study.
# Microfabricated Devices for Sparse Cell Isolation

*CNF Project # 905-00*

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### Figure 1, top right:
Current device, showing the two reservoirs, each with two connectors, and the channels area (between the reservoirs).

### Figure 2, below left:
Cultured breast cancer cells (MDA231), in medium only, retained at the third segment of the device.

### Figure 3, below right:
Adult blood spiked with cultured breast cancer cells (MDA231) migrating through the device, the cancer cells are retained at the third segment of the device while all healthy cells proceed to the output reservoir.
Abstract:
We have created a platform that provides a one-step membrane-based molecular separation, using conventional silicon processing and wet inversion to cast cellulose membranes directly onto the silicon nitride substrates. This process allows precise control of membrane thickness and pore size distribution. We have fabricated devices that were found to be very robust with molecular weight cutoffs of approximately 350 Da as measured by solute flux in a dialysis mode of operation. These devices were also found to be suitable for cell culture systems, and have been used as substrates to grow fibroblasts. A wide range of molecular separation and cell co-culture applications is being developed.

Summary:
Membrane separation may be an efficient method for size- and charge-based purification in the lab-on-a-chip environment. However, research incorporating membranes into these systems has been relatively lacking. Some researchers adhered small pieces of commercial membranes over microfluidic channels for separations [1, 2]. In these cases, the membrane pore sizes ranged from 0.1 μm with adsorbed protein as the separator, to as small as 8000 MWCO for dialysis. In other cases, membranes were used as cell culture substrates in lab-on-a-chip devices [3, 4]. Larger pore sizes were used to keep the cells in a specific location, but they are often so large that cells grow processes through the membrane.

By direct casting, we have eliminated the use of adhesives and have produced membranes that do not allow even the smallest cell process to grow through. In addition, they reject molecules as small as 355 Da. A silicon nitride layer is deposited on silicon wafers and a pattern of windows is opened using reactive ion etching. The windowed area is opened from the back-side using KOH and acts as a fret to support the cellulose polymer membrane that is spun across it. A solution of the polymer is spun across the silicon nitride surface using typical photore sist application methods. The membrane thickness is controlled by varying the speed and duration of the spinning process. Adhesion to the silicon nitride is very strong with no fluid paths around the membrane/fret, i.e. all flow is through the membrane and the windows in the silicon nitride film. The membrane has a thin skin on the top and bottom surfaces with a fine meshwork of structure and large voids. Tangential flow flux experiments can measure either the depletion of molecular species from the feed solution or the accumulation of molecules in the filtrate. The flux of molecules across the membrane is strongly dependent on molecular weight with a cut-off at 355 Da.

References:
Equilibrium Dialysis Systems for Selective Molecular Filtration

CNF Project # 937-01

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- Single step on-chip molecular separation of small molecules.
- Freeze fractured cellulose membrane showing top skin and internal structure (Figure 1; bar = 5 µm).
- Plot of molecular flux versus molecular weight. Strong dependence on molecular weight and cut-off at 355 Da (Figure 2).
Abstract:
The long term objective of this project is to develop microfabricated devices capable of performing automated cerebrospinal fluid (CSF) protein analysis for reliable antemortem diagnosis. Such devices would be useful in the clinical setting and be very useful in the identification of molecular markers for disease.

We have been using separately affinity and isoelectric focusing based separations of cerebrospinal fluid proteins. We previously demonstrated the use of microscale devices fabricated from borosilicate glass wafers to achieve affinity separations of protein, and we now fabricate devices out of polymeric materials for other separations and coupling to electrospray mass spectrometry. We have also developed techniques to probe protein-protein interactions using a combination of GMR materials and magnetic beads.

Summary:
It is important to use separations as a front end to mass spectrometry (MS) in the industrial proteomics community [1, 2]. In cerebrospinal fluid, there is an abundance of albumin (60-80% of the total protein content) which is usually not related to disease. Therefore, the possibility of using affinity-based separations for prefractionation (removal of albumin) of cerebrospinal fluid proteins using microfluidic devices has significant potential. We previously validated the use of Cibacron Blue 3G-A as the affinity ligand to remove albumin [3] in devices. To perform a second dimension of separation, we are testing devices made from cyclic olefin copolymeric materials for use in isoelectric focusing and electrospray mass spectrometry. These devices have channel widths of approximately 80 to 100 µm. Finally, we are adapting existing nanoelectrospray technology on board these devices in collaboration with the Craighead laboratory.

We are also developing the chemistry necessary to probe protein-protein (e.g., antibody-antigen) interactions using GMR sensors and magnetic beads. Thus far, we have demonstrated the ability to selectively probe transferrin-anti-transferrin, protein A-IgG and hrpW-antihrpW interactions. The last molecule, hrpW, is a useful indicator of type III protein secretion. These experimental observations are based on optical measurements of the binding of beads to derivatized silicon nitride surfaces.

References:
Multidimensional Microscale Separations of Cerebrospinal Fluid Proteins

CNF Project # 942-01

Principal Investigator: Kelvin H. Lee

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Figure 1: Isoelectric focusing of fluorescent-labelled carbonic anhydrase in microchannel.

Figure 2: Transferrin-coated bead based detection by anti-transferrin. Left: Anti-transferrin derivatized surface interacts with transferrin beads. Middle: Albumin derivatized surface does not interact significantly with transferrin beads. Right: IgG derivatized surface shows some nonspecific interactions with transferrin coated beads.
Abstract:
By using the parylene lift-off technique, we could control the location and size of receptor clusters by patterning micron-sized lipid bilayers containing liganded lipids, and thus enable direct visualization of structural reorganization of cellular components. Subsequent to concentration of mast cell IgE receptor, we observed co-localized tyrosine phosphorylation activity and structural rearrangement in plasma membrane inner leaflet. These data suggest such patterned surface can serve as a powerful tool to study cellular signaling events where localization is critical.

Summary:
The concept of membrane heterogeneity has been supported by accumulating data from biophysical and biochemical studies on cellular membranes and model membranes. The molecular basis of such membrane compartmentalization still remains largely controversial. Understanding the compartments can be facilitated by our ability to control the formation of such compartments and to decouple the different regulation mechanisms by additional biochemical manipulation.

In our research, spatially controlled stimuli on a 2D surface are utilized for systematic examination of localized signaling. Patterning supported lipid bilayers with liganded lipid on a silicon oxide surface using the parylene lift-off process [1] provides the mobility of ligands for the cellular receptors as well as the localization at the interface. Uniform arrays of haptenated lipids with feature sizes down to ~1 µm width are revealed after the polymer is peeled away in one contiguous piece under water, ready for controlled engagement of cellular receptors and monitoring of subsequent cellular responses.

We have demonstrated the effectiveness of such surfaces for specific stimulation of mast cells as indicated by specific antibody binding and redistribution, morphological changes and degranulation in the previous research [2]. Here we show that these patterned surfaces provide a tool for visualizing localized intracellular signaling events, as well as providing new insight in the structural basis and functional relevance of membrane compartmentalization. Lyn kinase accumulates over the patterned DNP-haptens, pointing to the functional relevance of these spatially restricted membrane domains. Lipidated GFP constructs that associate with the cytosolic side of the plasma membrane also concentrate over the patterns, suggesting reorganization of this inner leaflet upon clustering of the transmembrane IgE-FcRI. These studies lay the groundwork for more general studies of cellular receptors and responses at interfaces.

References:
Figure 1, top right: Cartoon illustration of detailed molecular interaction at the interface between mast cell and patterned lipid bilayer.

Figure 2, below left: Lipid pattern indicated by fluorescent lipids.

Figure 3, below right: Same field of view as Figure 2. Visualization of redistribution of cell surface marker upon stimulation, in this case, fluorescently labeled antibody.
Abstract:
A biomicrochip for purifying DNA from bacterial pathogens was designed and fabricated in plastic. The fabricated plastic biomicrochips were tested for their ability to purify DNA from the Gram positive bacterium *Listeria monocytogenes*. Hot embossing and plastic casting were the two fabrication techniques used to create these microchips. Silicon molds fabricated by photolithography and dry etching were used for chip prototyping. Zeonor plastic (polycycloolefin resin) and epoxy microchips were fabricated using hot embossing and plastic casting, respectively. The purification channel contains an array of features that were 10 x 100 x 25 µm to increase the surface area for DNA binding and purification. Low temperature sputtering techniques were used to coat a layer of silicon dioxide onto the channel region, since DNA can bind to silicon dioxide in the high ionic strength environment [1, 2]. The channel was sealed with a poly (dimethylsiloxane) (PDMS) layer after oxygen plasma treatment. DNA was quantified with PicoGreen fluorescent dye and the quality of the material as a substrate for polymerase chain reaction (PCR) was tested using target specific primers. A minimum of 10⁶ *L. monocytogenes* cells could be detected. The chip efficiency is still being investigated using alternative buffers and microchip configuration. Furthermore, a real-time PCR chamber is being designed for integration with the DNA purification biomicrochip.

Summary:
This research is part of an overall effort to develop a portable, fully-automated, PCR-based system for the detection of pathogenic bacteria, viruses and other disease-causing agents. We sought to develop a detection system based on plastic that is capable of purifying DNA from complex samples and performing real-time PCR to detect bacterial pathogens. Thus far, plastic microchips have been fabricated by hot embossing and plastic casting.

Using these chips, we have been able to selectively purify DNA from bacterial cells and have utilized this DNA for PCR amplification.

References:
Plastic Biomicrochip for Nucleic Acid Purification

CNF Project # 1006-02

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- Plastic biomicrochip with silicon dioxide coating is a novel component for DNA purification.

- Plastic microchip is capable of purifying DNA from bacterial cells.

Figure 3, below: Top figure: Detection of Listeria monocytogenes DNA purified with the plastic microchip. Bottom Figure: Detection of L. monocytogenes by PCR amplification of the hlyA gene. (1a) Negative control (1b) Positive control (2) 10⁵ L. monocytogenes cells (3) 10⁶ L. monocytogenes cells (4) 10⁷ L. monocytogenes cells (5) 10⁴ L. monocytogenes cells. For lanes 2-5, “a” and “b” are the 1st and 2nd elution volumes of DNA, respectively.

Figure 1: DNA purification plastic microchip fabricated by hot embossing containing 10 µm x 100 µm features that are 50 µm tall.

Figure 2: A plastic microchip. The chip was covered by PDMS and attached to input and output tubing. The square in the center is the active region for DNA purification.
Abstract:
The internal transport of materials is essential for the survival of plants and animals. This process is usually accomplished by the convection of a fluid through vascular networks that deliver nutrients to and carries toxins away from living tissue. The networks enhance internal transport by providing low-resistance pathways that allow fluids to rapidly travel macroscopic distances; diffusive transport processes are too slow or ineffective over these distances. We are developing two applications that benefit from the introduction of vascular systems into synthetic materials: 1) a hydrogel-based vascular material for wound dressings, and 2) a leaf-like evaporative microfluidic pump. To assist us in the development of these applications, we use the tools of the CNF to fabricate two-dimensional, micrometer-scale vascular systems in synthetic soft materials.

Summary:
The Wound Dressing: In deep burns and wounds, very often the vascular network necessary for tissue repair and regeneration is compromised; this results in an impaired healing process, due to the fact that the required nutrients and reagents must travel from the sides of the wound towards the center. As well as lacking any capacity for delivery of reagents, conventional wound dressings have to be changed often in order to keep the exudates from accumulating in the wound bed, i.e. they also lack the capacity to remove reagents from the wound site on a continuous basis. The vascular wound dressing we are building will address both of these issues by using an embedded set of microfluidic networks to both deliver and extract fluids from a wound bed. The fabrication process involves molding PDMS or hydrogels onto pre-defined features (SU-8 resin on silicon) and then sealing this patterned sheet to a macroporous hydrogel sheet, which will act as the exchange interface with the wound bed. We are currently in the process of testing different types of hydrogels and measuring their diffusive permeabilities. We are also investigating the sealing chemistry for joining layers of hydrogel and PDMS together.

The Synthetic Leaf: The vascular system of the synthetic leaf is designed to transport water from a central inlet to a two-dimensional surface without sacrificing the mechanical strength of the leaf material. It consists of a reticulate network of four generations of channels arranged in concentric square grids, which imitates the arrangement and sizes of vessels observed in the leaves of plants. The channel network is defined in PDMS using SU-8 based soft lithography [1]. However, the smallest water conduits found in actual leaves (inner diameter ~10 nm) are too small and too numerous to be explicitly defined using lithography techniques. Instead, a hydrogel with nano-scale pores is placed on top of the PDMS substrate containing the channel network. The air-water interface in these pores is the heart of the pumping ability of the leaf. The capillary pressure generated by the menisci within the pores is large enough to not only wick water into the hydrogel but also to pull water against a hydrodynamic load (e.g. gravity in trees, or long, narrow microchannels in our experiments). At present, we are testing several types of hydrogels and designing experiments that will allow us to select the best candidate(s).

References:
# Defining Microfluidic Vascular Systems in Soft Materials

**CNF Project # 1119-03**

**Principal Investigator: Abraham D. Stroock**

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| Tobias D. Wheeler |
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- SU-8 based soft lithography used to define two-dimensional, micrometer-scale vascular systems in soft materials to enhance their mass transport properties.
- Applications under investigation include a vascular wound dressing and a leaf-mimicking evaporative microfluidic pump.
- Results: We have fabricated a hydrogel-based wound dressing with an internal vascular network and a synthetic leaf capable of generating hydrostatic pressures of up to ~1 atmosphere.

**Figure 1:** Soft lithography-based scheme for fabricating vascular wound dressings.

**Figure 2:** Sealed wound dressing device with dye filling its internal channel network. The channels are defined in a poly(hydroxyethylmethacrylate) hydrogel cast over a multi-level SU-8 master. The outermost channels are 6 cm long.

**Figure 3:** Pumping rate of a synthetic leaf prototype over time as the hydrodynamic resistance against which it must pump is cycled on and off. The observed pumping rate recovery is reminiscent of the recovery of the pumping rate of actual leaves under a changing load.
Abstract:
Microfluidic devices were fabricated to study the adhesion and biofilm formation by *Xylella fastidiosa*, a xylem-inhabiting bacterium that causes Pierce’s Disease in grapevines. These fluidic chambers were constructed, in part, from silicone elastomer and are 100 µm deep, 100 µm wide, and 14 cm long. A GFP strain of *X. fastidiosa* cultured on nutrient agar media and subsequently suspended in liquid media was used to fill one region of the fluidic chamber. Once *X. fastidiosa* cells attached to the chamber surfaces, non-attached bacteria were flushed out with sterile liquid media. The chamber was then infused with sterile media using a syringe pump, and growth of *X. fastidiosa* and colony development were assessed temporally using video imaging methods.

Summary:
Many of the *X. fastidiosa* cells actively migrated along the glass surface of the microfluidic chamber against flow velocities up to 20,000 micrometers per minute. Both the movement and velocity profiles of migrating *X. fastidiosa* cells were evident of twitching motility previously observed in *Neisseria gonorrhoeae* [1]. The twitching *X. fastidiosa* cells initiated movement by reversibly attaching to the substrate and then traversing end-on along the surface in intermittent jerks. The cells moved via one of the polar ends, with the other end freely wafting in the stream. They often paused briefly to complete division before continuing on their path. These twitching *X. fastidiosa* cells often approached a microcolony, aligned in parallel with a nearby cell, and forced their way between two adjacent cells. The maximum twitching velocity achieved by *X. fastidiosa* was about 2 micrometers per minute.

References:
Bacterial Colonization and Communication in a Microfabricated Fluidic Channel

**CNF Project # 1165-03**

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**Figure 1, top right:**
Top: Schematic views of microfluidic chamber. Access ports (1, 2) for introduction and removal of non-attached bacteria. Bottom: Setup of presently used flow chamber on inverted microscope stage.

**Figure 2, below left:**
Selected time-lapse video images showing the twitching and migration pattern of *Xylella fastidiosa* cell (black arrow). Time displayed as day:hour:minute.

**Figure 3, below right:**
Velocity profile of twitching *X. fastidiosa* cell.
Abstract:
We have been focused on the electrical signaling of cells in a multi-unit nerve bundle of the red swamp crayfish, Procambarus Clarkii, during regeneration from trauma. It has been shown that crayfish motorneurons regenerate by fusion of the severed axon with the surviving distal segment [4]. We are focused on correlations between electrical activity of neurons at points proximal and distal to a site of lesion and success or failure of regeneration. We have designed and fabricated a multi-unit cuff microelectrode array in polyimide for chronic implantation in living, regenerating animals. The polyimide array attaches to a nerve bundle and allows us to cause a neural trauma between groups of electrodes.

Summary:
We have designed and tested two geometries for cuff electrodes which utilize out of plane bending and therefore can be fabricated using standard two-dimensional photolithographic processes. Our initial prototype allowed us to obtain considerable insight into the manipulation of small polyimide devices post fabrication. Our second generation device bends out of plane to weave around a nerve bundle of interest in a living animal. The array is approximately 1 mm wide, 10 μm thick, and two groups of three recording sites (15 μm diameter) are separated by 40 μm in groups and approximately 150 μm between groups. The arrays are fabricated using a three mask photolithographic process in the photosensitive polyimide, Photoneece (Dow Corning). A backing layer is followed by a lift-off electrode pattern which is insulated with a second layer of polyimide to create our final devices.

We have also designed custom instrumentation to interface with these electrode arrays. Eight Channel flat flex cables interface (six recording electrodes, one stimulation electrode, and one reference electrode) to our electronics. Custom electronics modules include a 6 channel surface mount instrumentation amplifier, an automated electrode platination and impedance characterization unit, and an isolated and regulated DC power supply.

References:
Polyimide Substrate Electrode Arrays for Chronic Implantation in Invertebrates

CNF Project # 1174-03

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- Designed and fabricated 6 channel polyimide microelectrode arrays for implantation.
- Custom metrology interfaces our micro-devices with the outside world.
- Impedance characterization hardware and software incorporates seamlessly into the system to monitor the dynamic bio-electronic interface.
- Initial implantations and recordings are underway.

Figure 1, top right:
The Electrode Array Tip. Two groups of three recording sites for longitudinal and lateral separation of signals. One reference electrode and one stimulation electrode.

Figure 2, below right:
Initial implantation into a tail segment of the crawdad. Array interconnect will mount on the animal’s carapace. Ventral Nerve Chord (VNC) and Nerve Three (N3) are labeled.
Flexible Microfluidic Probes for Drug Delivery to Neural Tissues

CNF Project # 1195-04

Principal Investigator: William L. Olbricht

Abstract:
Convection enhanced delivery (CED) of therapeutics has been proposed as an alternative to systemic drug delivery for the treatment for tumors, neurodegenerative diseases, and epilepsy [1]. CED uses pressure driven flows to distribute drugs into diseased tissues. Compared with diffusion mediated delivery methods, CED can achieve deeper drug penetration and nearly uniform drug concentrations within the affected tissue. Concentration profiles obtained using CED are relatively insensitive to molecular size or elimination mechanisms. Despite these potential advantages, results with animal models and clinical trials have shown it is difficult to control the fate of drugs infused using CED [2].

In the brain, the problem is exacerbated by variations in hydraulic permeability between white and gray matter. Relatively high permeability white matter tracts act as fluid sinks that can transport therapeutics into healthy tissues and into the cerebral spinal fluid, bypassing targeted tissue. Flexible polymer microfluidic devices that allow for real time monitoring of the infusion pressure were developed as a potential strategy for improving the performance of CED.

Summary:
Traditional CED involves inserting a needle or catheter into disease-afflicted tissue and pumping at a constant flow rate via an external pump. This method has been effective in proving that higher drug concentrations can be achieved by direct infusion than by systemic delivery. However, delivering therapeutics over several centimeters requires relatively high pressures that may result in irreversible tissue damage, uncontrolled drug distribution, and backflow up the needle. The deformation of the surrounding tissue creates high permeability fluid tracts that ultimately lead to loss of drug into the cerebral spinal fluid. Implantable microfluidic devices with multiple channels that allow for simultaneous drug delivery and monitoring of interstitial pressure may lead to enhanced therapeutic efficacy.

The development of acute and chronic microfluidic devices for implantation into neural tissues was achieved using both flexible and stiff substrates. Channels were formed using photoresist as a sacrificial layer and parylene as a structural layer. Channel dimensions of widths ranging from 10-50 µm and heights from 5-20 µm have been fabricated and tested using this method. The use of photoresist as a sacrificial layer allows for channels with relatively high aspect ratios, as compared to most thin films, which ultimately leads to low pressure drops (~1 psi) at moderate flow rates (~1 microliter/minute) over several centimeters of length. Channels have been fabricated on both silicon and parylene substrates. Silicon is used as a substrate for acute studies where implantations only last several hours. The rigidity of silicon allows for direct implantation into brain tissue without any coatings. Alternatively, parylene is used for chronic studies which last as long as six weeks. The flexibility and strength of parylene is ideally suited for long term implantation because it is conformal to the dynamic movements of neural tissues. Biocompatible and biodegradable coatings have been developed for monolithic parylene devices that provide the mechanical rigidity to penetrate tissue. Multiple channels with outlets along the implantable portion of the devices yield superior control of the spatial distribution of drugs compared to single channel systems. Furthermore, the hydraulic resistance to flow is monitored via the in-line pressure of each channel. The change in hydraulic resistance offers insight into the structural changes of tissues before, during, and after infusion, ultimately resulting in better drug delivery strategies.

References:
Flexible Microfluidic Probes for Drug Delivery to Neural Tissues

**CNF Project # 1195-04**

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• Flexible implantable fluidics for drug delivery.

• Microfluidic channels from parylene structural layers.

• In-line pressure measurement for feedback control.

• Superior drug distribution over conventional infusion methods.

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**Figure 1:** Monolithic parylene implantable microfluidic drug delivery system.

**Figure 2:** Inlet manifold for connection to external pump.

**Figure 3:** Cross section of 50 µm x 15 µm parylene channel.
Abstract:
Metal lift-off, SU-8 processing and gold electroplating have been used to create AC electrokinetic components for the isolation and manipulation of cells in lab-on-chip devices. These devices will be used to develop a quantitative system for evaluating the capacity of dielectrophoretic (DEP) based devices to isolate rare cells within heterogeneous cell populations. Different AC electrokinetic devices will be used to examine variations in the dielectric structure of specific cell types, providing an understanding of variation between cells of the same type, and cells of different types within the same population. The tools developed in this project will also be used to evaluate methods of altering the dielectric structure of targeted cells as a means of improving their separability.

Summary:
Dielectrophoresis (DEP) and electrorotation (ROT) are the motion of neutral bodies resulting from the interaction of the induced polarization of the body and a nonuniform or rotating electric field [1, 2]. In recent years, the use of such tools has grown significantly due to the suitability of thin-film microelectrode arrays for the production of highly non-uniform electric fields and the ease with which these devices can be integrated with conventional lab-on-chip (LOC) technologies.

The DEP force experienced by a particle is dependent upon the volume of the particle, the magnitude of the applied field gradient, and the relative polarizabilities of the particle and its surroundings. Polarization of the particle and its surroundings is dependent on the frequency of the applied electric field. Thus the magnitude and direction of the force experienced by a given particle is frequency dependent. Consequently cells or particles with different dielectric structures (i.e. different membrane capacitances) will experience forces of different magnitudes and even opposite directions in the appropriately chosen electric field. Therefore separation of cells with sufficiently different dielectric structures is feasible. However, a rigorous examination of exactly just how different two cell populations need to be before they are distinguishable in a DEP device has yet to be done.

To that end, a variety of tools for evaluating the dielectric structure, or more specifically, the DEP forces acting on cells of different types have been fabricated. Individual DEP Traps & ROT Spectrometers were constructed to perform preliminary experiments on model systems. DEP crossover measurements and ROT measurements were performed on polystyrene flow cytometer beads and SK-N-MC neuroblastoma cells. However, in order to get a more complete picture of the variation in the DEP forces generated on cells within the same population, it was determined that arrays, or ensembles, of individual DEP cell traps should be utilized to determine the holding forces on an ensemble of individual cells.

Arrays of planar quadrupole electrodes were formed using metal-lift off processing to pattern Au/Ti thin films on 100 millimeter diameter pyrex wafers. Electrodes were selectively insulated using a thin layer of SU-8 patterned by contact lithography. Fluidic networks were then fabricated by patterning between 50 and 100 µm of SU-8 around the array area. The wafers were then diced into individual chips and packaged in plexi-glass flow cells sealed with PDMS gaskets. Current work is focused on the modeling and measurement of trapping forces generated by these arrays. Work is also being done to determine how targeted modifications to cell surfaces affect these trapping forces.

References:
Dielectrophoresis (DEP) is an AC electrokinetic phenomena that can be used to isolate and manipulate cells based on differences in their dielectric structure.

- Single cell DEP traps have been fabricated within fluidic networks using conventional metal lift-off and SU-8 processing. (Figure 1, top right)
- DEP trapping of mammalian cells and polystyrene beads at electric field minima has been demonstrated. (Figure 2, below left)
- Arrays of DEP traps were fabricated to examine variations of holding forces within cell populations. (Figure 3, below right)
Development of Model Neural Prostheses
with Integrated Microfluidics for Drug Delivery

Abstract:
Two-sided Deep Reactive Ion Etching (DRIE) of silicon has been used to fabricate three dimensional model silicon neural probes employed in biocompatibility studies aimed at characterizing the cell and tissue reactive responses to neural probes chronically implanted in the brain. Surface micromachining techniques were integrated with existing DRIE processes to create model probes with integrated microfluidics to test the feasibility of using localized drug delivery to improve the long-term functional stability of devices implanted in the brain. Analytical and finite element modeling techniques have been combined with in vitro and in vivo experiments to develop an understanding of how these devices can alter the chemical make-up of tissue surrounding chronically implanted neural probes.

Summary:
Implantable electrode arrays capable of recording local field potentials from individual neurons within the cortex are an integral part of developing brain computer interface technologies. These technologies have the potential to replace communication and motor control signal pathways that have been damaged by disease or acute trauma. However, progress towards use of such devices in humans has been impeded by a general lack of functional device stability. This is attributed to the reactive cell and tissue responses to devices chronically implanted in the brain.

The chronic response to implanted devices is characterized by the formation of a dense cellular sheath that forms independently of the amount of damage caused during the initial device insertion. It has been proposed that local delivery of pharmacological agents within the wound site could be used to disrupt the formation of this sheath, thus improving the functional stability of implantable neural probes. Integration of microfluidic channels into neural probes is being explored as a method of providing dynamic control of the chemical environment surrounding chronically implanted devices.

Model neural probes without fluidic channels have been fabricated using a DRIE process that employs bosch etching [1] on both sides of a silicon substrate to form a three dimensional implant. Surface micromachining techniques similar to those previously described for the fabrication of vacuum sealed microlamps [2] and microfluidic needles [3] were integrated with the existing bulk subtractive techniques to create free standing, three dimensional silicon structures with integrated microfluidics [4].

In vitro characterization of device performance examined the real time release and diffusion of fluorescent molecules from device microchannels into agarose (1%w/v) gels intended to simulate brain tissue. Fluorescence intensity profiles were examined and compared with analytical predictions of transient concentration profiles for molecules of different molecular weights. In vivo studies were performed by inserting devices loaded with fluorescently labeled transferrin, a 74kD iron transport protein, into the brains of adult male rats. At various time points, brains were removed, fixed, sectioned and analyzed using confocal microscopy. Fluorescence intensity profiles were compared with the steady state analytical model for diffusion-mediated transport of molecules in tissue exhibiting first order elimination. Results from these studies, when compared with studies aimed at defining the extent of the reactive tissue volume, indicate that diffusion mediated transport of pharmacological agents from microfluidic channels integrated into the structure of a neural probe may be a viable approach to altering the reactive response to chronically implanted devices.

References:
Model silicon neural probes with integrated microfluidic channels were fabricated using deep reactive ion etching and surface micromachining techniques.

A 3.5 μm high channel extends the length of the device with four pairs of outlets spaced at 500 micrometer intervals along the shank. (Figure 1, top right)

Devices were characterized in vitro by recording and analyzing the release of fluorescent markers into agarose brain phantoms. (Figure 2, below left)

In vivo performance was examined at various time intervals using confocal microscopy to observe release into neural tissue. (Figure 3, below right)