Retinal Implant Project

CNF Project Number: 657-97
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Abstract:
The purpose of the Retinal Implant Project is to restore useful vision to patients who are blind with degenerative retinal diseases. The primary illnesses we hope to treat are retinitis pigmentosa (a primary cause of inherited blindness) and age-related macular degeneration (the leading cause of blindness in the developed world). Both these diseases cause the eventual destruction of the photoreceptor cells – rods and cones – in the retina, leaving intact the ganglion cells that transmit electrical impulses (and hence visual information) to the brain. The ganglion cells may be stimulated, however, with biphasic current pulses from a microfabricated electrode array. Blind surgical volunteers have consistently described visual percepts that resulted from such stimuli, and this has led our team to develop a wireless, implantable retinal prosthesis.

Summary of Research:
The implanted portion of our device consists of power and data secondary receiving coils, and in a sealed Ti can a small number of discrete components, and a custom designed integrated circuit (IC) that consists of circuitry for clock and data recovery, current drivers for electrodes in a stimulating electrode array, and a programmable function generator capable of stimulating with a wide range of pulse widths and amplitudes. The current outputs drive high-charge capacity sputtered iridium oxide film (SIROF) stimulating electrodes, which in turn give rise to the visual percepts mentioned above.

CNF-fabricated components of this system have included various proof-of-concept test structures and tools used in the research effort and an integrated combination flexible circuit and stimulating electrode array. Si wafers serve as carriers for these freestanding films during processing. The electrode leads are fabricated inside of ‘sandwiches’ of polyimide and amorphous silicon carbide (SiC), while the SIROF electrodes are reactively sputter-deposited.

Assembly of the intraocular components of the prosthesis is accomplished by flip chip stud bumping of the IC and solder attachment of discrete components onto an internal flexible circuit board, which is hermetically sealed into an ultraminiature Ti can. The RF coils are soldered and glued to the integrated external flex-array, which is in turn thermosonically bonded to the hermetic feedthrough of the Ti can. Finally, the thermosonic bonds are protected and insulated with an overmold. An external patient interface unit will consist of a video camera for capturing images, a digital signal processor, and a radio frequency (RF) transmitter and coil to relay power and data to the implanted device.

Scientific challenges still remain in realizing a chronically implantable retinal prosthesis. While our first generation device was primarily encapsulated in polymers for short term proof-of-concept implant studies, our second generation system focused on a system that would last many years in vivo. Our latest efforts focused on developing a device with 256+ stimulation channels, which is still small enough and of a configuration to be easily implanted in the ocular orbit and continue to function for many years in vivo. Thus, a major effort has been the development of a technological platform to build a robust, hermetically packaged, high-density subretinal visual prosthesis with a lifetime of > 10 years in biological saline that is scalable to hundreds of I/O channels.

Recent efforts have focused on optimizing the configuration, fabrication, assembly and surgical techniques in order to ready the project for pre-clinical trials. Toward this end, test devices for various purposes — including assembly, surgical fit, life-time testing, etc. — have been fabricated at the CNF.

References:
Microfluidic and Nanofluidic Devices for Epigenetic Analysis

CNF Project Number: 762-99

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Abstract:

We are developing microfluidic and nanofluidic devices for analysis of epigenetic modifications on single cells and single chromatin fibers. One aspect of our research is focused on microfluidic devices for the extraction, purification, labeling, and stretching of human chromosomal DNA or chromatin from single cells and small cell populations. This device offers the capability of trapping and chemically processing the genomic contents of multiple single cells in parallel within microfabricated pillar arrays, while immobilizing the chromosomal material. Another, makes use of submicron channels and nanoslits to analyze individual native chromatin fibers. Our submicron-sized fluidics allow optical evaluation of the epigenetic state (DNA methylation or histone tail modifications) of short individual chromatin fibers by single-molecule fluorescence analysis of bound probes. This method, known as Single-Chromatin Analysis at the Nanoscale (SCAN), allows us to determine the abundance of specific epigenetic states in native chromatin samples. Our nanoslit devices represent a platform for electrokinetically stretching longer native chromatin fibers with the purpose of better understanding the localization of different epigenetic modifications. These devices represent a step forward in better understanding epigenetic states with single-cell and single-molecule resolution.

Summary of Research:

Our microfluidic single-cell processing devices are simple, valveless PDMS devices capable of efficient isolation of DNA from individual cells by trapping and stretching long strands of human genomic DNA in a two-dimensional array of micropillars. Briefly, single cells are trapped within individual capture cups under flow while excess cells flow out to waste. Upon lysis, the flow of DNA/chromatin through an array of obstacles results in entanglement and immobilization. Once immobilized, the chromosomal DNA/chromatin can be processed in multiple ways.

We have fluorescently labeled different epigenetic modifications such as methylated DNA or histone modifications, allowing us to quantify the abundance of specific histone modifications on cancer cell lines. Specific DNA hybridization with fluorescent-labeled probes has also been successful. Isothermal amplification methods provide a unique application of these devices, since the template DNA is preserved within the channels after the reaction, while amplified material can be recovered off chip. In summary, our current microfluidic single cell processors represent a simple, inexpensive platform for studying the genomic contents of individual cells to assess cell-to-cell heterogeneity.

Single-molecule techniques could enable the analysis of sample-limited material such as chromatin from single cells. Using submicron-sized fluidics developed at the CNF, biomolecules including chromatin, DNA, and related probes are electrophoretically driven through the submicron channels ($W = 500$ nm, $H = 250$ nm), which limits the optical detection volume to approximately 100 aL and enables single-molecule detection.

By performing single-molecule fluorescence analysis on the probes bound to trinucleosome chromatin fibers, we have been able to quantify multiple epigenetic marks on a genome-wide scale. Further, we have developed methods for molecular identification based on single-molecule mobility measurements. Using a 600 µm long channel for better resolution, we have been able to identify different biomolecules, all identically labeled with the same fluorophore, based on their transit times through the channel. Additionally, we have observed binding induced mobility shifts which have been used for epigenetic mark detection and for assaying chromatin quality.
We use nanoslits as a tool to stretch and image fluorescently-labeled chromatin by tethering it to an antibody-coated microsphere and anchoring it at the entrance of the nanoslit by an electrophoretic force on the DNA. Single chromatin fibers were controllably stretched into the nanoslits by applying a constant electric field. The field-extension of tethered chromatin in the nano-confinement slits was measured and described using modified worm-like chain field-extension models. Our current nanoslit chromatin linearization method is a simple platform for studying chromatin dynamics and determining the localization of epigenetic marks to assess human epigenomic mapping.

Figure 1:
A. Single-cell DNA immobilization channel showing (a) a trapped cell and (b) fluorescent labeled DNA immobilized in a micropillar array.

B. (a) Submicron fluidic channel (L = 50 µm, W = 500 nm, H = 250 nm) used for SCAN. The white dots (not to scale) signify typical laser locations for fluorescence generation and detection. Laser separation is 25 µm. (b) Submicron fluidic channel (L = 600 µm, W = 500 nm, H = 250 nm) used for single molecule mobility analysis.

C. (a) Schematic of chromatin stretching nanoslit. The height of the slit is ~ 100 nm. (b) Native chromatin fiber extracted from HeLa cells expressing GFP on histone H2B. DNA is stained with DAPI. Histone modification sites are labeled with anti-H3K79me2 antibody. Scale bar is 3 µm. (c) Voltage-extension curves for HeLa chromatin fibers. The stretch (black circle) and release curves (gray circle) do not coincide and the process displays hysteresis.

References:
Fabrication and Demonstration of a Microplate-Based Enrichment Device Used for the Selection of Aptamers

CNF Project Number: 762-99
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Abstract:
Here, we describe the fabrication, and usage of a versatile microcolumn chromatography device that can be directly coupled to a standard 96-well microplate. This device, called a Microplate-based Enrichment Device Used for the Selection of Aptamers (MEDUSA), was designed for the purpose of performing aptamer selections to a variety of targets simultaneously, as well as characterization and optimization of aptamer selections, and also has the capability of being used for other chromatography-based applications. MEDUSA is fabricated using a CO₂ laser at 10.6 µm (Universal Laser Systems, VersaLaser) at the Cornell NanoScale Facility, which is a very rapid fabrication method, allowing MEDUSA to be customizable. This device was designed to be programmable enabling both serial and parallel flow configurations through the 96 microcolumns. The functionality and versatility of MEDUSA was demonstrated through binding characterization experiments using known ribonucleic acid (RNA) aptamers and their targets, as well as a multiplex novel aptamer selection.

Summary of Research:
Aptamers are nucleic acid ligands that bind with high affinity and specificity to their target compound. These ligands are discovered via a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) [1,2]. This is an iterative process of binding, partitioning, and amplification of nucleic acid sequences that bind to the target, starting from a very large, diverse library of random sequences. Aptamers are useful tools that have applications in biotechnology, diagnostics, and therapeutics [3].

We have developed a device designed for high-throughput SELEX for novel aptamer discovery, and characterization of the SELEX process to improve its efficiency, specifically to generate aptamers that bind tightly and specifically in less time and using fewer reagents. Our device, a Microplate-based Enrichment Device Used for the Selection of Aptamers (MEDUSA), is a layered device consisting of 96 microcolumns in the same configuration as a standard 96-well microplate, making it compatible with these standard microplates and potentially automatable using existing microplate-based liquid handling systems. MEDUSA is also programmable in that flow through the microcolumns can be in series or parallel, thereby increasing its versatility.

MEDUSA consists of layers of different materials of varying thicknesses, and these materials are cut using a CO₂ laser at 10.6 µm (Universal Laser Systems, VersaLaser) at the Cornell NanoScale Facility, and the laser parameters, including intensity, speed, and density of laser pulses, are adjusted based on the type and thickness of the material. Schematics of MEDUSA showing all of the layers for the fully parallel and a combination of serial and parallel configurations are shown in Figure 1.
Layer “1” is 1/2” thick poly(methyl methacrylate) (PMMA), and contains 96 microcolumns capable of holding 10 µL of target-immobilized affinity resin that are organized with identical spacing to a 96-well microplate. The layers on either side of the microcolumn layer, “5”, are 1/16” adhesive silicone gaskets that provide a liquid-tight seal across all 96 microcolumns, and contain 96 2 mm holes that hold polyethylene frits above and below each microcolumn to retain the target-bound resin within the microcolumns. In a serial configuration, a set of non-adhesive 1/32” gaskets (“6”) are used to connect adjacent microcolumns via small channels. The next layers are 1/4” PMMA capping layers (“2”) with 1/16” PMMA washers (“3”), which assist in the precise alignment of the nanoports (“4”) to the capping layers, as well as distribute the forces from assembly of the device.

The nanoports provide a standard fluidic interface with the device, and are bonded to the capping layers, which contain small holes to allow solutions to flow in and out of the microcolumns. This device can be reconfigured between serial and parallel during an experiment without disturbing the samples contained within the microcolumns. Furthermore, by fabricating MEDUSA with the VersaLaser, a new device can be made in approximately one hour, which enables the production of customized devices for each new experiment. Even smaller versions that are easier to handle can be made if 96 microcolumns are not needed.

Images of both a 96x and a 48x MEDUSA in their fully assembled form in both configurations are shown in Figure 2.

Using MEDUSA to perform high-throughput characterizations of aptamers and aptamer selection conditions has been demonstrated. In this experiment, green fluorescent protein (GFP), human heat shock factor 1 (hHSF1), and negative elongation factor E (NELF-E), as well as their respective aptamers, were used as model systems. Here, 96 tests were carried out simultaneously to investigate the effects of immobilized target concentration on aptamer binding using a range of target concentrations, as well as the partitioning efficiency and binding specificity of aptamers against both their specific target proteins as well as non-specific targets by performing selections to these targets in series.

Additionally, the 48x version of MEDUSA was used to perform aptamer selections to 19 different targets simultaneously. This greatly reduced the time and reagent consumption, which ultimately reduced the cost significantly. Aptamer selections are not always successful in discovering an aptamer that binds with high affinity and specificity, so being able to perform many selections concurrently is a great advantage.

These two experiments have ultimately demonstrated MEDUSA as an extremely useful tool for high-throughput selections, characterizations, and optimizations.

References:
Scalable Sensor Array Platform for Analysis of Quantal Transmitter Release Events

CNF Project Number: 848-00
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Abstract:

Neurontransmitters are released in a quantal event by fusion with membranes. We develop and fabricate a complementary metal oxide semiconductor (CMOS) sensor array capable of parallel electrochemical detection of vesicle release events from chromaffin cells. To enable amperometry measurement, polarizable platinum electrodes are deposited on the Al/Cu metal contact on the CMOS chip by conformal e-beam evaporation. An SU-8 insulation layer is also applied to protect the surface structure of the chip and avoid incomplete coverage of the metal contact by shifting the position of the electrodes as well as form deep wells to trap cells.

Summary of Research:

Neurontransmitters are released into the extracellular space in a process known as exocytosis [1]. The amperometry measurement provides precise details about the released transmitters in a single quantal event. However, amperometric spikes vary from cell to cell even under the same condition [2]. Therefore, a large number of measurements for vesicle release events must be performed to achieve a change in the mean value. Here, we present the CMOS IC sensor array capable of parallel amperometry measurement of vesicle release events and the post-fabrication to enable its functionality.

We have developed a 10.10 CMOS IC sensor array for high throughput amperometry measurement with high signal to noise ratio (Figure 1) [3]. The IC sensor is fabricated at MOSIS by On Semiconductor C5F/N. Polarizable electrode materials such as gold or platinum are not offered in this process. Instead, Al/Cu metal contact are deposited to serve as interconnection of the chip. However, amperometry measurement requires polarizable electrodes for oxidation reactions to occur. Hence, it is necessary to have a post-fabrication process in the CNF clean room to deposit platinum electrodes directly onto the Al/Cu metal contacts for amperometry measurement. To have a complete coverage over the desired working region, conformal electron beam evaporation is performed by rotating shelf to deposit 10 nm of titanium and 200 nm of platinum [3].

Figure 1: CMOS IC sensor array. (a) Chip surface with working electrodes and other surface elements. (b) and (c) are microscopic images of single working electrode. The focal plane of (b) is the CMOS potentiostat circuit underneath the surface, while the focal plane of (c) is at the surface of the electrode. The dimension of each electrode is 15 µm x 15 µm. (d) Schematic of electrode topology [3].
To avoid possible defect such as incomplete coverage of the Pt electrode, a shift electrode strategy is performed to redefine the position and shape of the working electrodes (Figure 2) [4]. The shifted electrodes also enable cell trapping by SU-8 deep wells. The patterned poly(L-lysine) in register with the electrodes will promote cell adhesion, while poly(ethylene glycol) is applied in between wells will resist cell adhesion [5]. Pt electrodes are deposited over the Al/Cu contact, but instead of just covering the contact window, they are extended to cover some part of the overglass. 10 µm SU-8 2007 thick layer is fabricated on the surface of the CMOS chip.

Deep wells with 16 µm in diameter are opened by general lithography at the redefined electrode position. The round shape rather than the original square shape of the electrode opening is beneficial for cell trapping (Figure 3).

Microcontact printing of poly(L-lysine) and poly(ethylene glycol) will be performed for promotion and resistance of cell adhesion.

References:
Silicon Nitride Cantilevers for Muscle Myofibril Force Measurements

CNF Project Number: 1255-04
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Figure 1: Myofibril attached to a glass needle for stretch-shortening and nano-levers for force measurement. Example of a myofibril with eight sarcomeres in-series.

Abstract:
Custom fabricated silicon nitride cantilevers (Figure 1) were used to measure force in passively lengthened skeletal muscle myofibrils from patients with cerebral palsy (CP) and compared to samples from non-spastic patients. Passive forces in the CP patients were lower compared to non-spastic samples, when the sarcomere lengths were matched. However, the sarcomere lengths in CP patients are much longer than that found in non-spastic muscles. When forces between CP and non-CP are compared with physiologically relevant sarcomere lengths, the passive forces are higher in CP.

Summary of Research:
To measure muscle forces in the nano-Newton range, silicon nitride cantilever pairs were manufactured and used. Cerebral palsy (CP) is one of a group of motor disorders resulting from a brain lesion to the developing brain. Morphological and structural changes in CP muscle are well documented but the origin of the higher passive forces in CP muscle compared to normal is not. Elevated passive force is seen in the in vivo joint [1] and in fibre bundles [2] while at the single fibre level it has been reported as higher [3] or with no difference [2]. Elevated passive forces are attributed to increased collagen and laminin content, and while speculation about the passive force contributions of titin have been made, these speculations have never been tested. Myofibril work could assess titin’s passive force characteristics in CP muscle since structural proteins seen at higher levels (e.g., collagen, dystrophin, laminin) are absent in this preparation and the force observed is attributed solely to titin [4].

Titin is giant non-linear molecular spring present within the sarcomere which has two regions, an inextensible region which is bound to the thick filament and an extensible region spanning the I-band region composed of spring-like elements arranged in series.

Purpose:
To determine whether the forces at matched mean sarcomere lengths (SL) differ between CP and non-CP muscle.

Methods:
Single myofibrils were generated from biopsy samples obtained from children already scheduled for surgery: CP (n=9, mean age 102.6±47.2 months) and non-CP (n=2, mean age 37.5±22.5 months). CP participants GMFCS
scores ranged from III to V. The non-CP participants had hip dysplasia but not CP. CP samples were from the gracilis (G) and adductor longus muscles (AL), non-CP were from AL only.

Myofibrils were chemically and mechanically isolated as described in our previous work [5]. Single myofibrils were attached to nanofabricated silicon-nitride cantilevers (stiffness 68 pN/nm) [6] for force measurement at one end of the myofibril (resolution < 0.5 nN), and at the other end, a glass pipette needle attached to a piezo-motor for controlling specimen length (Figure 1). All testing was done in a relaxing solution which contained ATP but no calcium, pH = 7 [5].

Single myofibrils were obtained (CP-G: n=40, CP-AL: n=46, non-CP-AL: n=8) and myofibrils were placed in a relaxing solution at a mean SL of 2.0 µm. They were then lengthened passively to a mean SL of 2.4, 2.8, 3.2, 3.6 and 4.0 µm (and if possible, > 4) and held for two minutes at each length prior to measuring force. After the two minute hold at a target length, the specimen was shortened to a mean SL of 1.8 µm for 10 minutes before proceeding to the next (longer) target length. Forces were converted to stress by dividing the force by the area of the myofibril measured at initial SL.

Repeated measures ANOVA with significance at 0.05 were used.

Conclusions:

- Forces during passive lengthening in adductor longus CP single myofibrils are lower than non-CP samples (for matched sarcomere lengths) as seen in Figure 2 and this suggests CP tissues contains a larger titin isoform, which offers a more compliant sarcomere to reduce the risk of damage to the muscle.

- This is the first time that passive force behaviour has been investigated in CP myofibrils, offering insight into whether the high passive forces seen in C at the whole muscle level are attributed to mechanism within or outside of the sarcomere.

- Adductor longus-CP and Gracilis-CP myofibrils do not have different stiffness at matched sarcomere lengths (Figure 3), suggesting that the lower passive force in cerebral palsy myofibrils is a distributed phenomenon within the body.

- The differences between CP and non-CP are more pronounced as the muscle is elongated and since non-CP sarcomere length is normal (2.4 µm) while CP muscle sarcomere length is typically very long (3.5 µm), the differences in passive force at physiological length between groups is significantly different, with CP myofibrils sustaining higher passive forces compared to non-CP.

References:


Graphene-Channel Field Effect Transistors for Biosensing Applications

**CNF Project Numbers:** 1520-07, 762-99

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**Abstract:**

We demonstrate two different methods for fabricating large area graphene-channel field effect transistors for biosensing applications. The first method uses a soak-and-peel delamination of graphene using DI-water and the second method uses a combination of dry transfer and modified-RCA cleaning. Devices exhibit clear Dirac peaks close to zero volts. The suitability of these devices for detecting the binding of DNA and poly-L-lysine was investigated. Devices show a large shift (~15V) in Dirac voltage with exposure to DNA and poly-L-lysine.

**Summary of Research:**

Graphene channel transistors are promising for biosensing applications due to their remarkable electrical properties. Use of graphene as a biosensor has been previously demonstrated in variety of ways [1]. Most devices use exfoliated graphene. Although exfoliation yields the highest quality graphene, this process is not scalable. CVD grown graphene is the method for scaling to arbitrarily large device dimensions. Scaling the device size improves signal-to-noise ratio in biosensors. Though methods are well established for growing graphene in large length scale by CVD most reported devices so far have been limited to few micrometer lengths. Here, we demonstrate two simple, scalable CVD graphene transfer method for field effect transistors.

The first method uses a soak-and-peel delamination of graphene using DI-water [2,3]. The second method uses a combination of dry transfer by PDMS and modified-RCA-cleaning [4,5]. In both cases, PDMS was used for graphene transfer and the graphene was transferred to prepatterned source-drain electrodes on Si/SiO₂ wafer, which avoids contact between lithographic resists and graphene. In the soak-and-peel scheme, water penetrates between hydrophobic Cu and hydrophilic graphene and separates them. The separated graphene is transferred on to the patterned electrodes. In the dry transfer scheme, after the Cu etching using wet chemicals, a modified RCA cleaning is carried out to clean the graphene-metal interface. Then graphene is transferred on to the patterned substrates. Using the second method graphene channel FETs with channel lengths as large as one millimeter was fabricated. Transistors transfer characteristics (source-drain conductance versus back gate voltage) exhibit a clear Dirac peak close to zero back gate voltage (Figures 1 and 2).

The devices fabricated using the soak-and peel-scheme were used for detecting the binding of double stranded DNA and poly-L-lysine [2]. The Dirac peak shifts by 17 V after exposure to ~ 580 pM of poly-L-lysine and by 14 V upon exposure to 300 pM of DNA (Figure 3). The polarity of the response changes to positive direction with poly-L-lysine and negative direction with DNA and a detection limit of 8 pM for 48.5 kbp DNA and 11 pM for poly-L-lysine are obtained.

**References:**


Figure 1: Optical micrograph of graphene transferred on to a pre-patterned source-drain electrode and current-gate voltage characteristic measurement of a bare GraFET. Inset shows the measured channel resistance as a function of back gate bias.

Figure 2: Total resistance between source and drain electrodes in a graphene FET array as a function of back gate bias. Dirac voltage occurs at a gate bias of 3 ± 0.5 V across the array.

Figure 3: Transistor curve for a device measured after exposure to a 300 pM solution of λ DNA. The Dirac point is close to zero after dipping in TE buffer, and shifts to negative voltages after exposure with DNA.
Abstract:

Integrated circuits (ICs) with 3D time-resolved imaging capabilities can image microorganisms and other biological samples given proper packaging. A portable, flat, easily manufactured package will enable users to place biological samples on slides directly above the imaging chip. We have developed a packaging procedure for our group’s imager IC chip using laser cutting, photolithography, epoxies, and metal deposition. This year in the Cornell NanoScale Science & Technology Facility (CNF), we have finalized this packaging procedure after experimentation with a flip-chip method, and we have aligned and adhered the chip to a holder wafer.

Summary of Research:

A. Flip-Chip Experimentation. We first attempted a flip-chip strategy incorporating a patterned fused silica wafer as a combination biological sample slide and connection between the imager chip and a printed circuit board (PCB). Flip-chip machines such as the Finetech FINEPLACER Lambda use vacuum chucks and micro-positioners to align bond-pads on a chip to another device. Researchers can pattern solder or gold ball bumps onto bond-pads and heat to cure after performing the flip-chip process. We unsuccessfully attempted flip-chip using anisotropically conductive adhesive (Creative Materials Anisotropic Conductive Thermoplastic Adhesive 111-05) to electrically and mechanically bond the imager chip to the patterned fused silica wafer [1].

After designing a pattern for a photomask to create metal connection lines between the chip’s bond-pads and a PCB, we patterned the mask using the Heidelberg Mask Writer DWL2000. We spun LOR10A and SPR220 photoresist (PR) onto fused silica wafers, exposed them with the mask, and developed them. We then evaporated titanium (adhesion layer), copper, and gold onto the wafers, and then performed lift-off and diced the wafers. Our preliminary tests bonding the patterned wafer pieces to the chip using 111-05 did not result in electronic connections.

B. Silicon Packaging for Best Fit. After moving on to a flat package concept, we sought to minimize the inevitable gap between the imager chip and its holder wafer. We designed a pattern for the mask with a gradient of squares (minimum size 5 mm², the size of the imager chip), which at the end of the process became cavities to find the best fit for the chip. We used the Oxford 100 plasma enhanced chemical vapor deposition system (PECVD) to deposit 4 µm of SiO₂ (oxide) on the top of the Si wafer and 1 µm on the bottom. The thick oxide on top serves to protect non-cavity areas of the wafer from the Si etch, and the thinner oxide on the bottom serves as a Si etch-stop.

We then spun SPR220-4.5 PR on the wafer, exposed with the cavity gradient mask, and developed. Then, the Oxford 82 was used to etch through all of the oxide in the square cavity areas. We used a CHF₃ etch process (etches oxide at ~ 35 nm/minute) until only 100 nm remained on the cavity areas, and then we switched to a CF₄ etch process (etches oxide at approximately 31 nm/min) because unlike CHF₃, CF₄ does not deposit unwanted polymer after etching all the way through the oxide. We subsequently used the Unaxis 770 Deep Si Etcher to etch through Si to form the square cavities in the Si wafer. The tool etches Si at around 2 µm/min and 1 µm oxide for every 150 µm Si.
Finally, we removed the 1 µm etch-stop layer of oxide by submerging the wafer in pure HF. Unfortunately, even the largest cavities (5080 µm²) were too small for the imager chips (Figure 1).

C. Imager Chip Alignment and Adhesion to Holder Wafer. After discussion with CNF staff, we decided to explore a process using one purposefully over-sized cavity in the center of a Si wafer because we figured a larger gap could be easier to fill with adhesive. We used three wafers in this process: a Si holder wafer with a square 6.5 mm² cavity, a fused silica handle wafer to help align the top of the handle wafer with the top of the chip, and a fused silica carrier wafer to push the adhesive into the gap between the holder and the chip (Figure 2).

We used the Lal group's LPKF ProtoLaser U tool to cut a 6.5 mm² hole in a Si wafer (holder wafer). Then, we spun SPR220-3 on a fused silica wafer (handle wafer), placed the holder wafer and the chip top-down onto the handle wafer, and baked the package to bind the pieces together. Outside of the CNF, we prepared Varian Torr Seal adhesive and applied it to the fused silica carrier wafer, then pressed the adhesive side onto the backside of the package (backside of the chip and holder wafer) [2]. After curing at room temperature for 24 hours, we soaked the package overnight to remove the handle wafer and PR. The top surfaces of the holder and the chip were sufficiently co-planar for our needs: the top of the chip is 8.7 µm above the holder wafer, with the Torr Seal protruding 10.2 µm above the holder wafer surface and 1.5 µm above the chip surface.

References:
Patterned Surfaces for Studying EGF and IgE Receptor Signaling and the Formation of the Lysosomal Synapse

CNF Project Number: 1726-08
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Abstract:
We demonstrate the use of lithographic processes in combination with chemical modification to prepare micron and submicron sized arrays of patterned biomaterial. We have explored the application of these patterns to characterizing epidermal growth factor receptor (EGFR) signaling in fibroblast cells [1] and to uptake mechanisms of aggregated low-density lipoproteins (agLDL) by macrophage cells [2]. Current work aims to investigate IgE-receptor (FcεRI) mediated mast cell responses and to study lysosomal synapse formation with fibrillar β-amyloid plaques formed in the brains of patients suffering from Alzheimer's disease.

Summary of Research:
Bio-patterned surfaces are a powerful tool for exploring a wide range of cellular activities. The surfaces described in this work have enabled further understanding of EGFR- and FcεRI-regulated signaling processes as well as the mechanisms underlying microglia and macrophage mediated β-amyloid degradation.

Through standard photolithographic techniques and a polymer lift-off method, receptor ligands or fibrillar β-amyloid may be patterned on selected substrates. Glass or silicon wafers, initially coated with a parylene-C polymer film and photoresist, are patterned using the ASML PAS 5500/300C DUV stepper. An oxygen-based etch then transfers the features in photoresist to the parylene layer, creating multiple arrays of individually patterned features (0.25 µm to 2 µm in size) at a range of periodicities (2 µm to 20 µm separation). Subsequent chemical modification on the exposed surface and the final peeling away of the parylene layer establishes immobilized protein or fluid supported lipid bilayers in spatially defined patterns.

This technique has become significant in revealing the formation of multi-protein signaling complexes in response to patterned ligand. In addition to the EGFR and actin cytoskeleton (Figure 1), we have observed and quantified the recruitment of various EGFR signaling partners, including Ras, MEK, and phosphorylated ERK [1]. Surface tethered EGF has also proven useful in exploring evidence on the cross-talk between integrin receptors and EGFRs. NIH-3T3 cells immunolabeled for β1 integrin exhibit preferential clustering of this integrin to patterned EGF features located at the cell periphery (Figure 2), an observation indicative of the specialized structural and regulatory functions of this protein within the larger EGFR signaling complex.

A similar pattern of recruitment for β1 integrin is observed on mast cells responding to surfaces with the IgE receptor ligand. Integrin receptors clustering with the IgE receptor is a novel result and offers new information on where mechanical signaling may be tightly coupled with biochemical signaling in these cells. Overall, our results emphasize the important roles that receptor clustering and organization play in regulating key signal transduction events. Ongoing work aims to further characterize the spatiotemporal characteristics of receptor signaling, associated plasma membrane phosphoinositides, and the actin cytoskeleton in response to patterned ligand.

We have also extended this patterning technique to study macrophage cell interactions with agLDL. To degrade agLDL, macrophages generate an extracellular hydrolytic acidic compartment, termed the lysosomal synapse [2]. J774 macrophages incubated on agLDL-patterned arrays present regions of low pH at patterned features. Since agLDL is immobilized, our results demonstrate these acidic, lysosomal synapse domains are extracellular. A medically important function of macrophages includes their interaction with agLDL in the walls of blood vessels. This interaction results in massive cholesterol uptake and macrophage transformation to foam cells [4]. An understanding of agLDL uptake, one that doesn't require receptor-mediated endocytosis, may help explain the role of foam cell formation in vivo and could thus be useful in preventing atherosclerosis.
Further, we are examining the interactions between fibrillar β-amyloid peptides and microglia or macrophages. Patterned surfaces, coated with fibrillar β-amyloid (Figure 3), enable localized secretory events and changes in pH to be studied with enhanced spatial control. Preliminary results indicate that J774 macrophages — with their lysosomes previously labeled with FITC-dextran — adhere to β-amyloid-coated surfaces and secrete their lysosomal contents in response to contact sites (Figure 4). This likely indicates a process involving extracellular digestion. A better understanding of microglia and macrophage mediated β-amyloid degradation processes may contribute to new insight on the pathomechanisms operating in Alzheimer’s disease.

References:


Progress Towards Nanophotonic Trapping for Precise Manipulation of Biomolecular Arrays on Si$_3$N$_4$ Waveguides

CNF Project Number: 1738-08
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Abstract:
Optical trapping is a powerful manipulation and measurement technique widely employed in the biological and materials sciences. Our lab has developed and implemented an on chip device based on Si waveguides, coined a nanophotonic standing-wave array trap (nSWAT), that allows for controlled and precise manipulation of trapped nano/micro particles [1]. Here, we report our ongoing investigation of Si$_3$N$_4$-waveguide based nSWAT devices. The Si$_3$N$_4$ nSWAT works at 1064 nm laser input, and has improved biocompatibility.

Summary of Research:
Optical trapping is a powerful manipulation and measurement technique widely employed in the biological and materials sciences. Miniaturizing bulky and expensive optical trapping instruments onto optofluidic platforms holds promise for high throughput lab-on-chip applications. In 2014, using the CNF, Wang Lab researchers (Soltani, et al.) developed and implemented such a device, named the nanophotonic standing-wave array trap (nSWAT), that allows for controlled and precise manipulation of trapped particles [1]. Using photonic interference functionalities, an array of stable, three-dimensional on-chip optical traps is formed by the evanescent field at the anti-nodes of a standing-wave in a nanophotonic waveguide. By employing the thermo-optic effect via integrated electric microheaters, the traps can be repositioned at high speed (~ 30 kHz) with nanometer precision. "Dumbbells" were formed with deoxyribonucleic acid (DNA) tethered between two polystyrene (PS) beads, and these could be precisely manipulated and stretched within the nSWAT. Figure 1 (adapted from Ref. [1]) shows an array of DNA dumbbells stably trapped on, and precisely manipulated along, two parallel Si waveguides in an nSWAT device.

The first generation nSWATs are Si waveguide based devices that work at 1550 nm for low optical losses. Although water absorption at 1550 nm is already quite low, a blue shift of the working wavelength to 1064 nm would reduce the water absorption ~ 100 fold [2], indicating an ideal compatibility with biological species. To explore this compatibility, we began to investigate Si$_3$N$_4$ waveguides, which at 1064 nm, have the aforementioned decreased optical loss and also have considerable smaller nonlinear effects, as compared to Si, which is vital for high power applications. Microparticle trapping on Si$_3$N$_4$ was first demonstrated in 2005 [3], however burnt Si$_3$N$_4$...
waveguides at high power have been an unsolved issue [4]. We have used finite element method (FEM) based full wave electromagnetic simulations in COMSOL to solve for the optimal Si₃N₄ waveguide dimensions for maximized trapping force and minimized optical loss.

Using CNF equipment, we have been working on the following goals: (1) fabricate Si₃N₄ waveguides using DUV lithography, instead of e-beam, to achieve low loss waveguides that can potentially tolerate high laser power; (2) create fabrication recipes for protection of microheaters in the presence of biological buffers; (3) optimize Si₃N₄ surfaces for nanoparticle manipulation. These improvements should significantly reduce the nSWAT production time and cost per device without compromising the quality of these devices. Figure 2 displays an SEM image of a fabricated Si₃N₄ waveguide with a highly smooth top surface and side walls.

In conclusion, we are working on the fabrication of low loss Si₃N₄ waveguides on thermal oxide coated Si wafers in order to create the next generation nSWATs.

References:


BIO

The Electrochemical Sensor’s Platform Fabrication for Antibody Detection Using the Antibody Catalyzed Water Oxidation Pathway

CNF Project Number: 1757-09
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Abstract:
Biosensors, which are analytical devices that convert biological responses to electrochemical signals, serve as cheap and effective surveillance systems and are currently being developed to optimize the efficiency of detection of antibodies by increasing both diagnostic speed and detection limits. Our study focuses on the responsive nature of polymer brushes and the ability to pattern them using photolithographic methods in order to create a unique platform designed for antibody detection. This new biosensor takes advantage of the Antibody Catalyzed Water Oxidation Pathway (ACWOP), which is the inherent capacity of all antibodies to catalyze the production of hydrogen peroxide (H₂O₂) from water in the presence of singlet oxygen. This report focuses on the fabrication of two types of platforms for the device — native silicon substrate and gold-plated quartz crystal.

Summary of Research:
Infectious diseases as well as the risk of pandemics represent a longstanding global problem. Antibodies are generated naturally as part of the adaptive immune response and are early markers for many ailments. Because of their capacity to recognize a monumental range of antigenic groups with great specificity, antibody detection provides a powerful means by which to monitor the spread of particular diseases. The general platform of our biosensor consists of patterned poly(oligoethylene glycol methacrylate) (POEGMA) polymer brushes surrounded by a photosensitizer which can be excited with UV light to generate singlet oxygen from ambient oxygen as shown in Figure 1. Two separate platforms were fabricated using photolithography. Native silicon chips were employed as the main substrate for the production of the device, whereas gold-plated quartz crystal microbalances (QCMs) were utilized for antibody quantification purposes (Figure 2).

The fabrication of the native silicon platform comprises spin coating a bi-layer of a lift-off resist (LOR-5A) and a positive-tone photoresist (SPR-220.3.0) to get the desired undercut profile, isolating the final pattern, and preventing “fencing” issues (lift-off residuals). Contact lithography (ABM contact aligner) was used to expose a bi-layer of resist followed by the development of the patterns in a base solution. Subsequently, 10 nm of titanium (adhesive...
layer) and 90 nm of gold layers were deposited on the silicon substrate using electron gun evaporation system (SC4500 odd-hour evaporator). Finally, lift-off process was performed in order to remove the resist and obtain patterns of gold lines (Figure 3). One of the major challenges associated with the lift-off process was the reattachment of gold particles onto gold patterns. This was addressed by soaking the silicon wafer face-down in a Remover 1165 solution for four hours and then cleaning it in an ultrasonic bath for two hours. Lastly, the silicon wafer was cleaned multiple times with deionized water and dried with a nitrogen gun.

For a gold-plated quartz crystal microbalance, the fabrication of this platform included spin coating a layer of SPR220-3.0 photoresist, exposing with the ABM contact aligner, and developing in a base solution to get the desired gold patterns (Figure 4). To allow for the maximum yield of both the polymer brushes and photosensitizer on the same surface, it is crucial to immobilize a thiol initiator on the exposed surface before removing photoresist. Our final goal is to transform these types of platforms into microfluidic device that will minimize nonspecific adsorption of biomolecules and enable delivery of H$_2$O$_2$ in a separate part of the device for enhanced measurements.

References:
Biomechanics of Bacteria

CNF Project Number: 1970-10
Principal Investigator: Christopher J. Hernandez
Users: Jason D. Guss, Melanie Roberts

Abstract:
The mechanical properties of the bacterial cell envelope influence cell growth, cell division and subcellular localization of membrane proteins. Here we demonstrate the ability to apply mechanical loads to live bacteria, the first step toward determination of mechanical properties of bacterial components in vivo. Additionally, we show that devices based on the same concept have the ability to separate bacterial species/strains from one another based on the cell mechanical phenotype.

Summary of Research:
In bacteria, the ability to resist mechanical forces is necessary for survival and growth, allowing cells to withstand osmotic pressures while maintaining cell shape, cell growth and division. Hence, the mechanical properties of bacteria and bacterial structural components influence species competition and resistance to toxins and antibiotics. Our work involves the use of micro/nano fabricated devices as tools for mechanical testing of live bacteria. Within our devices individual bacteria are flowed into tapered channels and trapped at points within the channels based on whole cell stiffness in which less stiff cells are able to travel further in to the channels (Figure 1). Key advantages of this microfluidic platform for profiling the biomechanical properties of bacteria include: minimal sample preparation, no chemical immobilization or labeling, and the ability to analyze hundreds of cells at once.

In our first series of experiments we manufactured devices on silica glass wafers using deep UV photolithography to achieve nano-scale features (250 nm smallest dimension).

A device design in which cells from a population are submitted to up to 12 different applied pressures was used to establish the biomechanical profile of two model organisms, Escherichia coli (E. coli) and Bacillus subtilis (B. subtilis) (Figure 2). Our results demonstrated differences in stiffness between E. coli and B. subtilis (Figure 3) and suggest that a device with a shorter channel length would allow transport of E. coli but not B. subtilis, potentially allowing for separation of bacteria based on the biomechanical properties.

In additional experiments, we have applied super-resolution microscopy to bacteria trapped within the devices, enabling more detailed mechanical analyses of the cells.

References:
2014-2015 Research Accomplishments

Figure 2: (A) A schematic of the biomechanical profiling chip with five individual devices is shown. (B) A single biomechanical profiling device. Each device consists of twelve sets of trap channels. The pressure across each set of trap channels is greatest closest to the inflow (bottom of the figure) and decreases linearly up to the top bypass channel (top of the figure). (B1–B3) Micrographs of single channel set. Each channel set contains five tapered channels. Bacteria trapped in the traps appear dark using phase contrast microscopy (white arrow heads). Note bacteria travel further into the channels when the pressure across the trap is greater (compare B1 to B3). (C) A hydraulic circuit model representing the segments of bypass channels and sets of trap channels is shown.

Figure 3: (A) The position of bacteria occupying trap channels at twelve different pressure levels (where level 1 is lowest and level 12 is greatest) in a single experiment are shown. Horizontal lines indicate averages at each pressure level. E. coli travel further into the traps than B. subtilis overall (p < 0.0001, ANCOVA) as well as at each individual pressure level (p < 0.0001, t tests). Data shown are representative of three different experiments. (B) Differences in bacteria stiffness between species can be detected in a mixed culture. E. coli expressing GFP (green, indicated by horizontal arrows) traveled further into the trap channels than B. subtilis (indicated by tilted arrows). The results suggest that, if trap channel length were reduced, it would be possible to design traps to allow transit of E. coli while preventing transit of B. subtilis, enabling separation of bacteria based on mechanical phenotype.
Unidirectional Migration of Cancer Cells During Cancer Metastasis Recapitulated by Micromolding Collagen

CNF Project Number: 2040-01
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Abstract:
During cancer metastasis, cancer cells often migrate in a unidirectional way through the extracellular matrix. We created an *in vitro* micromolded collagen microtrack system to recapitulate this unique mode of migration using a silicon master produced by photolithographic techniques. We investigated the reproducibility and fidelity of collagen microtrack molding as a function of microtrack size (width ranging from 2 to 20 µm). Our results showed that fidelity of collagen microtracks decreases with decreasing track width due to the high aspect ratio.

Introduction:
One of the leading causes of cancer-related fatalities is metastasis, where cancerous cells escape from the primary tumor and migrate to spread throughout the body. Cancer cells use various migration techniques during metastasis to escape from the primary tumor. It has been established that metastatic cancer cells often show unidirectional migration by forming microtracks within the extracellular matrix *in vivo* using proteolytic enzymes, primarily matrix metalloproteinases (MMPs).

Previously, we used state-of-art microfabrication technologies to recapitulate the unidirectional migration of cancer cells *in vitro* using micromolded collagen microtracks. To micromold the collagen, we produced a silicon master (using a photolithographic technique) that contains parallel patterns shaped like tracks (15 µm (W) × 25 µm (H) × 1100 µm (L)), which span the size of microtracks created by cells (~ 15 µm in width). And we have successfully shown that we can use micromolding to recreate the *in vivo* tracks *in vitro*.

Here, we investigated the reproducibility and fidelity of collagen track micromolding as a function of microtrack size. We created a silicon master with track-like patterns that range from 2 µm to 20 µm in width. Overall, this system provides a platform to investigate the molecular mechanism by which cancer cells migrate in microtracks.

Materials and Methods:
Silicon Mold Fabrication. A silicon master (100 mm in diameter) was created with track-like patterns using a photolithographic technique in the Cornell NanoScale Facility. Patterns were pre-designed (width: 2, 5, 10, 20 µm, height: 25 µm, length: 1100 µm) using Layout Editor (L-Edit, a computer aided design (CAD) software) and printed on a photomask using the Heidelberg Mask Writer (DWL 2000). The silicon wafer was then pre-baked; spin-coated with SU-8 (epoxy based negative photoresist) and post baked twice. The ABM contact aligner was used to transfer the patterns from photomask to SU-8 coated silicon wafer by exposing to UV light. This silicon master wafer was then pre-baked; spin-coated with SU-8 (epoxy based negative photoresist) and post baked twice. The ABM contact aligner was used to transfer the patterns from photomask to SU-8 coated silicon wafer by exposing to UV light. This silicon master wafer was used to cast polydimethylsiloxane (PDMS) stamps that allowed molding collagen into microtracks.

Collagen Microtrack Fabrication. PDMS stamps were used to fabricate collagen microtracks. Stamps were inverted on a drop of neutralized Rat Tail Type I collagen and allowed to polymerize for 90 minutes at 37°C temperature. After polymerization, stamps were removed and grooved collagen wells were capped with a collagen lid to enclose the microtrack.

Patterned Collagen Microtrack Analysis. Confocal reflectance microscopy was used to image the microtracks, and collagen pattern fidelity was characterized by measuring dimensions. Zen 2010 software was used to visualize the tracks in xy, yz and zx plane.
2014-2015 Research Accomplishments

Results:

In vivo microtracks found in the extracellular matrix (ECM) of a tumor microenvironment were re-created in vitro by micromolding collagen using the silicon master (Figure 1). We found that fidelity of collagen microtracks decreased with decreasing track width. Collagen microtracks with 10 and 20 µm width were successfully reproduced (Figures 2 and 3 respectively).

We were unable to recreate microtracks with 2 and 5 µm widths due to high aspect ratio (width: height (µm) = 2:25 (µm) and 5:25 (µm) respectively). The PDMS stamps, prepared directly from casting on the silicon master, mainly failed to capture the structure of the silicon mold for the decreased microtrack widths (2 and 5 µm).

Conclusion:

We have created an easily reproducible platform in vitro to study the unidirectional mode of migration during cancer metastasis. These microtracks seeded with highly metastatic cancer cells will allow us to compare the molecular mechanisms of cancer cells during unidirectional migration in cancer metastasis as a function of microtrack size.

References:

Micropatterns and Microfabricated Devices to Assess Cardiac Function in iPSC-Derived Cardiac Cells

CNF Project Number: 2065-11
Principal Investigator: Jan Lammerding
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Primary Sources of Research Funding: National Institutes of Health award R01 HL082792; Department of Defense Breast Cancer Idea Award BC102152; National Science Foundation CAREER award CBET-1254846; Pilot Project Award by the Cornell Center on the Microenvironment & Metastasis through Award Number U54CA143876 from the National Cancer Institute; * 2014 NNIN REU Program @ CNF
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Abstract:

Cardiac disease is the leading cause of death in the USA. Mutations in the nuclear envelope proteins lamins A/C play an important role in dilated cardiomyopathy: up to 10% of inherited cases are caused by lamin mutations. However, it remains unclear how mutations in a nuclear envelope protein expressed in every type of tissue can primarily affect the heart. In this project, we are using microfabricated substrates that enable quantitative analysis of the effects of lamin A/C mutations on the organization and function of sarcomeres, the force generating structural units in muscle cells. We will use cardiac muscle cells derived from induced pluripotent stem cells (iPSCs) to assess the effect of specific lamin mutations on cardiac structure and function. The iPSCs were generated from the skin cells of patients affected by a cardiac laminopathy and healthy relatives. We have already conducted proof-of-principle studies with the microfabricated substrates and are currently in the process of applying the experimental approach to a broader panel of cells. Ultimately, the experimental platform will provide a powerful tool to not only study the molecular mechanism underlying cardiac defects caused by lamin mutations but also to screen pharmaceutical reagents that can be used for therapy.

Summary of Research:

Mutations in nuclear envelope proteins cause at least 10 different human diseases that include dilated cardiomyopathy, Emery-Dreifuss muscular dystrophy, and Hutchinson-Gilford progeria syndrome [1]. Mutations in the gene encoding lamins A/C (LMNA) alone are responsible for up to 10% of all cases of familial dilated cardiomyopathy [2]. Intriguingly, the mechanisms by which mutations in these ubiquitously expressed nuclear envelope proteins give rise to diseases with often a high degree of tissue-specificity remain elusive. Studies into the cardiac-specific effects of lamin mutations have been hampered by the limited availability of human cardiac cells for in vitro studies; while animal models of the disease have provided many useful insights, they do not fully recapitulate many important aspects of the human disease. To address the cardiac-specific aspects of this disease, we are developing microfabricated substrates to study cardiac-specific function in cardiac cells differentiated from induced pluripotent stem cells (iPSCs) derived from patients with lamin A/C mutations and healthy relatives. iPSCs derived from skin fibroblasts of laminopathy patients offer the exciting possibility to study the effect of genetic mutations in human cells differentiated into the cell types of interest. Lamins, as the main component of the nuclear lamina, play an important role in providing structural support to the nucleus and in mechanically connecting the nucleus to the cytoskeleton [3]. This intracellular mechanical feedback is essential for cytoskeletal organization. Efficient functioning of cardiac tissue requires a highly ordered assembly of parallel sarcomeres in cardiac cells, enabling maximum contractile force. Studies with cells and tissues derived from lamin A/C-deficient mice have revealed impaired sarcomere organization and disturbed cytoskeletal organization.
Motivated by these findings, we hypothesize that lamin mutations affect the organization of sarcomeres in cardiac cells, impeding the ability of heart cells/tissue to contract the heart efficiently. To test this hypothesis, we are developing microfabricated substrates that enable quantitative evaluation of sarcomere structure and contractile force generation in cardiac myocytes. We plan to use these substrates with cardiac laminopathy patient-derived and healthy cardiac myocytes.

To study cytoskeletal organization in cardiac cells, we have developed two types of surfaces engineered to induce cell alignment, which promotes sarcomeric organization and alignment in healthy cells. The first approach utilizes high aspect ratio rectangular polydimethylsiloxane (PDMS) pillars (1:7) that can be used as a stamp for microcontact printing of cell-adhesive proteins (e.g. fibronectin, laminin). The second approach comprises surfaces with microgrooves (2-4 µm wide, 3-4 µm deep) from which replicas can be made in PDMS or in other polymer surfaces by hot embossing. Tests using healthy cardiac cells have confirmed that the microgrooves induce sarcomere organization and alignment (Figure 1.)

To study the forces exerted by the cardiac cells, we have microfabricated molds to produce thin, flexible microposts with precisely defined bending rigidity. When cells are grown on these substrates, the deflection of the posts is used as a measure of the force exerted by the cell on each pillar (Figure 2.) Tests are currently underway to improve the adhesion of cells to the tops of the pillars only. Once the experimental conditions have been optimized, we will grow cardiac myocytes from patients and healthy controls on these substrates and compare cell/sarcomere alignment and force generation between mutant and healthy cells. Pharmaceutical and genetic manipulation of structural and cell signaling pathways can then be used to identify molecular mechanisms and potential therapeutic targets for the laminopathies.

References:


The Cooperative Role of Topography and Interstitial Flows on Breast Tumor Cell Invasion

CNF Project Number: 2068-11

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Abstract:
Malignant tumors often feature an elevated fluid pressure due to abnormal vascular vessels, and thus an increased interstitial flow rate out of the tumor. Collagen alignment outward from the tumor margin is another critical topography feature in the tumor microenvironment [1]. A key step of cancer metastasis is tumor cell migration through the interstitial space under the influence of elevated interstitial flows and aligned collagen [2]. We developed a microfluidic device with topography features to mimic the alignment of collagen and is capable of applying interstitial flows in three dimensional collagen matrix to investigate the biophysical roles in the tumor microenvironment on the morphology and motility of human breast tumor cells.

Summary of Research:

Device Design.
The goal of designing the topography microfluidics device was 1) to engineer micro-sized topography to mimic in vivo aligned collagen fiber and study the topography guidance on tumor cell invasion, and 2) to study the synergistic role of interstitial fluid flows and micro-sized topography on guiding tumor cell migration in a 3D collagen matrix environment, in which tumor cells reside on the PDMS topography side and were fully covered by collagen matrix.

A two-step etching method was used to fabricate the topography device, first the depth of the main channel and second the depth of the ridges. The basic design of the microfluidics is shown in Figure 1. A main channel with height of 120 µm was divided into five sub-channels by walls. Interstitial flows were applied through it. Four sub-channels were fabricated with ridge features (20 µm height), and the center sub-channel was a control channel with no topography.

Topography Guided Tumor Cell Migration.
Cells migrating along ridges can clearly be seen to be guided by the ridge topography, as shown in Figure 2 and the trajectory plots in Figure 3 A and B. Cells were observed to migrate along the sides of the ridges, as well as the top of ridges. Cells on ridges may migrate in one direction, then switch directions on the ridge, or leave the ridge. The cells that migrated along ridges also had significantly higher speed and persistence lengths than cells without ridge guidance (Figure 3 C and D). These high persistence lengths are due to the migration of cells along the ridges, highlighting the importance of surface microtopography in guiding persistent cell migration.

The Cooperative Role of Interstitial Flows and Topography.
When interstitial flows were applied, we observed similar trend as no flow, in which the cell migration speed and persistence were enhanced on topography compared to in control case. We also observed no significant directional cell migration along or against the flow direction in the presence of flow.

References:
Figure 1: Schematic of microfluidic device to study the effect of topography and interstitial flows on cell migration.

Figure 2: Micrograph of breast tumor cells migrate along the topography in 3D collagen matrix.

Figure 3: Topography guidance on tumor cell motility. Migration trajectories in control (A) and along topography (B). Cell migration speed (C) and persistence (D) in control and topography channel.
Abstract:

Successful mammalian reproduction requires that sperm migrate through a long and convoluted female reproductive tract before reaching oocytes. For many years, fertility studies have focused on biochemical and physiological requirements of sperm. In this project, we have shown that the biophysical environment of the female reproductive tract critically guides sperm migration, while at the same time preventing the invasion of sexually transmitted pathogens. Using a microfluidic model, we demonstrate that a gentle fluid flow and microgrooves, typically found in the female reproductive tract, synergistically facilitate bull sperm migration toward the site of fertilization. In contrast, a flagellated sexually transmitted bovine pathogen, *Tritrichomonas foetus* (*T. foetus*), which closely resembles human analogue *Trichomonas vaginalis*, is swept downstream under the same conditions. We attribute the differential ability of sperm and *T. foetus* to swim against flow to the distinct motility types of sperm and *T. foetus*: specifically, sperm swim using a posterior flagellum and are near-surface swimmers, whereas *T. foetus* swims primarily via three anterior flagella and demonstrates much lower attraction to surfaces. This work highlights the importance of biophysical cues within the female reproductive tract in the reproductive process and provides insight into coevolution of males and females to promote fertilization while suppressing infection. Furthermore, the results provide previously unidentified directions for the development of *in vitro* fertilization devices and contraceptives.

Summary of Research:

Device Design. We designed a two-layer device to mimic the *in vivo* structures, with a layer of microgrooves connected to a main lumen, as shown in Figure 1. The fabrication of this master mold uses a combination of PECVD oxide and photore sist to mask the etching to achieve the two-level etchings. FOTS treatment was done before using the wafer for PDMS soft lithography. To ensure the surface properties are the same within our microfluidic channels, we bonded the PDMS devices onto PDMS coated glass slides, using standard oxygen plasma treatment method [1].

Emergence of the Upstream Swimming. Sperm were seen to reorient and swim against the flow as the flow rate exceeded a critical value. In the absence of the flow or when the shear rate was below the critical value, sperm swam in all directions, and each sperm followed a clockwise (CW, viewed from above) circling trajectory. When the shear rate exceeded the critical value, sperm swam predominantly upstream in nearly straight lines. We show that there exists a critical shear γc. Below γc, no steady state solution exists. The CW circling bias dominates, the sperm swim clockwise following right-hand circles, and stay in bound states. Above γc, there exist two steady states (or fixed points). Using the acquired images, we made precise measurements of sperm head orientation θ as a function of flow rate near the transition point, and found that it follows saddle-node bifurcation as described by our theoretical model (Figure 2) [2].
Microgrooves and fluid flows cooperatively provide preferential migration pathways for sperm over *T. foetus*.

When subject to both a gentle flow and microgrooves, sperm quickly gained access to the microgrooves and swam upstream efficiently, whereas *T. foetus* did not enter the microgrooves and were swept downstream by the flow (Figure 3). Figure 4 shows that almost all (99%) *T. foetus* but only 2% of sperm, were swept away close to the grooved surfaces in the presence of a flow of 3 µL/min. In the case of smooth surfaces, 32% of sperm were swept away using the same flow rate of 3 µL/min. If we examine the distribution of instantaneous velocity parallel to the flow direction (x component), sperm were overwhelmingly found to move against the flow, and *T. foetus* was found to move with the flow.

The different responses of sperm and *T. foetus* demonstrate that fluid flows and microgrooves on channel surfaces cooperatively facilitate sperm upstream swimming, whereas microgrooves have no impact on *T. foetus* migration, and fluid flows sweep *T. foetus* downstream [3].

**Acknowledgements:**

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**References:**

Fabrication of Silicon Photonic Structures for High-Sensitivity Biosensing

CNF Project Number: 2133-12
Principal Investigator: Benjamin L. Miller
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Abstract:
Photonic crystals (PhC) have attracted exceptional interest as label-free optical biosensors because of their high sensitivity, small scale, and compatibility with fabrication methods and materials drawn from the microelectronics industry. In that context, we have been pursuing the design, fabrication, and use of nanocavity-coupled w1 PhC waveguide biosensors for label-free, high sensitivity optical detection of viruses. Fabricated on a silicon-on-insulator (SOI) substrate using electron-beam lithography and reactive ion etching, the PhC nanocavity provides optical detection based on resonant mode shifts in response to ambient refractive index changes produced by infiltration of target biomaterial. During the past year, we have focused on the design and fabrication of new types of two-dimensional PhCs. These structures are anticipated to be useful for capture of single virus particles via size selection and antibody-mediated capture. We also propose that infiltration of material into different regions of the sensor will allow for “self-referenced” discrimination of specific vs. nonspecific binding interactions.

Summary of Research:
Interest in label-free optical biosensing is driven by an ever-increasing need for simple, rapid, and sensitive methods for detecting biological molecules of interest in a diagnostic or research context, as well as by fundamental aspects of materials science. Over the past several years, we, and others [1] have pursued 1-and 2-D photonic crystal structures produced in silicon as optical biosensors. Two-dimensional photonic crystals (2D PhC) are particularly interesting given their exceptionally small size (microns), high sensitivity (detection of single virus-sized latex particles has been demonstrated) and compatibility with standard silicon wafer processing protocols.

The sensor design we have studied consists of a 2D PhC silicon slab structure with a triangular lattice of air holes. A linear defect is created in the PhC by removing a single array of central air holes, resulting in a w1 waveguide that allows modes to be guided through the crystal within its photonic band gap (PBG) [2]. Light propagation in the waveguide is confined by the PBG in the plane of periodicity and by index guiding in the direction perpendicular to the silicon slab [3]. By introducing a spatial point-defect formed by modifying the radius of a single air hole next to the W1 PhC waveguide, light is transmitted through the PhC waveguide at all wavelengths within the PBG except at the resonant wavelength of the confined cavity mode. At this wavelength, a dip is observed in the output transmission spectrum. Capture of a biological target within this defect or in the surrounding holes causes a change in the position of the resonant wavelength. To develop sensors able to specifically detect human pathogens such as viruses, our efforts this year have particularly focused on the design of new structures with defect “holes” size-matched to the virus target. These devices should be able to capture viruses using size selection in addition to antibody-mediated recognition.

Figure 1: Schematic of a two-dimensional photonic crystal (PhC) designed for size-selective capture of virus particles.
One of the significant challenges for label-free sensors is that of differentiating specific binding (capture of the target molecule, virus particle, or other analyte of interest) from nonspecific binding (everything else in the sample interacting with the sensor). Typically, a reference sensor is employed as an external control to correct for nonspecific signals. Over the past year, we have also used simulations of PhC behavior to propose that these devices can act as “self-referencing” systems able to directly discriminate specific from nonspecific binding [4]. Simulations suggest that material infiltrating into the defect hole of the device shown in Figure 1 produces a strong red shift in the defect resonance but not the band edge of the optical spectrum, while material infiltrating elsewhere (as would be expected for nonspecifically bound material) produces a shift in the band edge. We anticipate testing this concept experimentally in the near future.

References:


Fixed Path Length Silicon Sample Holders Improve CryoSAXS Measurements from Sub-Microliter Volumes

CNF Project Numbers: 2157-12, 2158-12
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Abstract:
CryoSAXS is a new method that gives structural information on biological macromolecules in frozen solutions [1]. However, changes in sample size that occur during freezing complicate data collection and analysis. Here we present microfabricated silicon sample holders for cryoSAXS with a fixed path length and low volume sample [2]. These sample holders make cryoSAXS a more accessible technique capable of probing a wide range of biological molecules.

Summary of Research:
Small angle x-ray scattering (SAXS) gives structural information about biological molecules in solution. However, large (~30µL) sample volumes are needed to mitigate radiation damage, limiting the use of SAXS in studying rare or expensive molecules. By cryocooling SAXS samples, radiation damage and required sample volumes are reduced by orders of magnitude [1], but challenges in creating identically-sized frozen samples complicate data acquisition and analysis. We microfabricated fixed path length sample holders that simplify cryoSAXS data collection and make the technique more routine.

Silicon has many characteristics that make it an ideal material for these sample holders. It is rigid, which prevents a change in the x-ray path length when the sample freezes, and it has low x-ray absorption. Additionally, the availability of high aspect ratio fabrication techniques allows the production of x-ray windows which are thin enough to have high x-ray transmission, but tall enough for the beam to pass through without clipping the edges and generating extraneous scatter. A simple parallelogram design, shown in Figure 1, was etched into a <110> silicon wafer with potassium hydroxide (KOH). The resulting sample holders are open-topped parallelepipeds with thin vertical x-ray windows, as shown in Figure 2. The high aspect ratio x-ray windows are 30 µm thick but over 600 µm tall, and have high x-ray transmission while still accommodating the entire beam. The relatively small thermal mass of the silicon sample holders allows rapid freezing of a drop of sample in a cold gas stream. Additionally, the required sample volume is less than 0.8 µL, which is less than 3% of the volume needed for a standard SAXS experiment.

One challenge in the successful fabrication of these sample holders is obtaining extremely smooth surfaces on the x-ray windows. X-ray scattering off of rough interfaces can be large enough to obscure the relatively weak scattering from biological molecules, and therefore

![Figure 1: Sample holder etch mask, patterned in low stress SiN onto a <110> Si wafer. Tiling the unit horizontally across the wafer avoids the need for corner compensation during the KOH etch.](image1)

![Figure 2: (Left) Top-down composite image of a silicon sample holder. Features not defined in the etch mask result from the anisotropic nature of the KOH etch. (Right) Cartoon sample holder rotated 80° from the photo. The walls have been made semi-transparent to show that the internal features do not encroach on the x-ray beam, shown as a cylinder.](image2)
smooth x-ray windows are essential. Etch parameters were optimized to give a very smooth finish: an etch at 60°C in 45% w/w KOH solution inside an ultrasonic tank resulted in an RMS roughness is 1.27 nm over 100 µm² as determined from AFM analysis.

The sample holders were tested at station G1 at the Cornell High Energy Synchrotron Source. The smooth surfaces produce very small amounts of parasitic x-ray scattering, comparable to the scattering produced by sample holder materials used in standard SAXS experiments, as shown in Figure 3. Scattering profiles of glucose isomerase, a SAXS protein standard, taken in the silicon sample holders at 100 K agree well with scattering profiles of the same protein at room temperature in a conventional SAXS sample holder, as shown in Figure 4.

These silicon sample holders are a step towards making cryoSAXS data acquisition and analysis more routine and accessible. Eventually, they could enable high throughput experiments and increase opportunities for researchers without easy access to a synchrotron by allowing scientists to freeze their samples in their own labs and mail them to the beamline for analysis. Additionally, these low volume sample holders could allow SAXS experiments on molecules that are too difficult to express or too expensive to produce in the quantities required for conventional SAXS.

References:
Abstract:
We report a method for directly and quantitatively measuring the interactions between nanoparticles and surfaces by looking at the light that they scatter when interacting with a nanophotonic structure. This technique relies on measuring the statistical distributions in the light scattered by particles trapped in the evanescent field of a photonic crystal resonator. Particles experience optical gradient forces that attract them towards the surface as well as electrostatic forces in the screened electrical double layer that repel them from the surface, and undergo biased Brownian motion about the equilibrium of these forces, scattering more light when they are close to the surface and less when they are further away. This allows for measurements that are not limited by thermal noise. We measure interactions on the sub-kBT scale and forces on the single picoNewton scale.

Summary of Research:
Nanoparticles are quickly becoming an important component of many technologies, including medical therapeutics and diagnostic tests, cosmetics and sunscreens, as well as engineered suspensions for many applications including enhanced oil recovery. One major issue in the design of nanoparticles is ensuring their colloidal stability — if nanoparticles aggregate, adsorb onto surfaces, or flocculate out of the suspension, the products function may be compromised and in some cases it may cause harm. As a result, a variety of particle coating and solution-based stabilizing mechanisms have been introduced. However, these systems are often very complex and predicting the stability of an engineered suspension from first principles is usually not possible with the currently established theoretical frameworks. As a result, it is desirable to measure these interactions directly in order to gain insight into how the suspension behaves and to determine whether the stabilization procedures used were effective.

Measuring the potential energy surface of the interaction either between two nanoparticles or between a nanoparticle and a surface can help in answering these questions since this allows for knowledge of the energy barrier that must be overcome in order for particles to stick. Previous techniques for making these measurements include atomic force microscopy with the colloidal probe technique, and total internal reflection microscopy have limitations in dealing with particles smaller than around 1 µm in the true nanoparticle regime. We have developed a new technique called Nanophotonic Force Microscopy [1] capable of overcoming these limits and measuring sub-100 nm particles with interaction forces on the pN scale and energies on the sub-kBT scale. This technique relies on the enhanced optical gradient provided by a photonic crystal resonator [2].

Briefly our technique works by measuring the light scattered by particles as they undergo Brownian motion in an evanescent field. Due to the exponential dependence of the light with distance from the surface, the amount of light scattered by a particle can be related to its height. As many measurements of intensity are taken, the intensity distribution can be related to the probability distribution of finding the particle in a given state. Assuming that these states are related according to the Boltzmann statistical distribution, the relative probability of finding the particle in two of these states is related to the potential energy difference between the states. In this way, the potential energy well is mapped. This well contains contributions
from the optical gradient of our device as well as the particle-surface interaction we are interested in as it relates to stability. Fortunately, the form of the optical force is well understood and can be subtracted giving the surface contribution. The derivative of this gives the force.

Figures 1 and 2 show a measurement made using our device on silver nanoparticles with nominal diameters of 100 nm. Here we report the energy relative to equilibrium in units of kBT. As the figures show, we are successfully able to measure energies on the thermal scale and forces smaller than 1 pN on these true nanoparticles. We have also measured 100 nm polystyrene particles [1] and 50 nm gold nanoparticles, better than the current state of the art. Building on this work, this technology has now been licensed and developed into a commercial product with sufficient throughput to characterize 1000 particles per hour.

References:
Zero-Mode Waveguides on Thin Silicon Nitride Membranes for Efficient Single-Molecule Sequencing

CNF Project Number: 2214-13
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Abstract:

Single DNA molecules may be sequenced by imaging their replication by a polymerase in a zero-mode waveguide (ZMW). To increase the efficiency of this process, we fabricated ZMWs on a 35-nm-thick silicon nitride substrate, in which sub-10 nm nanopores may be milled. When biased, these pores pull molecules toward the bottom of the ZMW. We also coated the ZMWs with a thin layer of silicon dioxide using atomic layer deposition. This protects the aluminum from electrochemical reactions induced by voltage in the presence of a chloride solution.

Summary of Research:

Zero-mode waveguides (ZMWs) are wavelength-scale apertures in metal films, which may be used for single-molecule detection at micromolar concentration [1]. By immobilizing DNA-DNA polymerase complexes in ZMWs and imaging the incorporation of fluorescently-labeled nucleotide pentaphosphates, one may read the sequence of single DNAs [2]. These structures are traditionally fabricated on quartz substrates for imaging on a microscope. In 2014, we showed that by making ZMWs on 35-nm-thick silicon nitride membranes, and drilling a 3-5 nm pore at the ZMW base, the efficiency of molecular loading into these structures could be enhanced by orders of magnitude (see Figure 1 for a schematic) [3]. These structures were fabricated with e-beam lithography at the CNF.

We have continued to fabricate these devices for DNA sequencing experiments. Figure 2 shows a transmission electron microscope image of a newer version of the device. The ZMW aperture has been increased from 60 nm to 100 nm to facilitate more efficient capture of large DNA molecules. The entire surface has been passivated with 12-13 nm of atomic layer deposition silicon dioxide to protect it from electrochemistry with chloride buffer that may occur during voltage bias experiments. The bright spot in the ZMW center is a 4 nm nanopore (fabricated outside of CNF).

These structures may be used for capturing DNA-polymerase complexes. In Figure 3, we show a sample time trace from a nanopore-localized polymerase. Each fluorescent burst corresponds to a C or A nucleotide being incorporated into a new DNA strand.

References:

Figure 1: Schematic of an array of ZMWs on a thin silicon nitride membrane with nanopores at their bases. Upon voltage bias, these pores create an electric field, which pulls sample molecules into the ZMW detection volume.

Figure 2: Transmission electron micrograph of a ZMW with a nanopore at its center (bright spot). The 12-13 nm layer of ALD silicon dioxide is identified with arrows.

Figure 3: ZMW time trace of fluorescence as a DNA polymerase incorporates nucleotides into a new DNA strand. Each fluorescence burst corresponds to a C or A being added to the growing strand.
Fabrication of DNA Stabilizing Nanofluidic Channels for High-Resolution Optical Imaging of Transcription in vitro

CNF Project Number: 2230-13

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Abstract:

In this project we are developing a series of microfluidic devices optimized for studying DNA-protein interactions. The device design is similar to that constructed by Eric Greene at Columbia University in which thousands of DNA molecules are tethered to a lipid bilayer and placed under hydrodynamic flow to co-align DNA molecules perpendicular to nanofabricated barriers [1]. The devices will be used to quantify transcription factor binding affinities and kinetics at specific loci, using both synthetic DNA and chromatin preparations obtained from isolated nuclei.

Summary of Research:

Transcriptional activation is a primary mode of regulating gene expression. Genetic studies and genome-wide assays have provided valuable insights on transcription. However, the underlying mechanisms regulating transcription at the single-molecule level have yet to be thoroughly investigated. Much of our understanding of the complex process of transcription comes from chromatin immunoprecipitation (ChIP) methods in which a snapshot of the transcription state is taken by chemical cross-linking DNA, RNA and proteins, fragmenting the chromatin, and then pulling out protein-DNA complexes with antibodies to suspected transcription factors (TFs). Subsequent sequencing is used to identify the location of targeted TFs on the DNA at the time of cross-linking. Although this provides clear evidence of DNA-protein interactions in vivo, it is largely a binary approach and delivers only a crude estimate of the binding constants at play, with no information on the dynamics of the interaction. There is a critical need for new methods capable of providing quantitative kinetic information about TF binding so that accurate models of transcription may be established. The microfluidic device designed in this project aims to provide a platform for obtaining high-resolution transcription factor binding data at the single-molecule level.

To accomplish this goal, we have developed a method to prepare the required chrome-patterned surfaces using standard photolithography techniques. Clean glass wafers, coated with photoresist, are first patterned using the ASML PAS 5500/300C DUV stepper. Subsequent chrome evaporation and a final lift-off step yields multiple arrays of ~ 20 nm tall chrome patterns. These patterns exploit a saw-tooth design (Figure 1) to provide for effective DNA

Figure 1: Microfluidic device with SEMs of individual chrome barriers on a glass surface. The saw-tooth design is significant for trapping the DNA tethered to a lipid bilayer that is presented on either side of these chrome barriers. Left image: ~ 1 µm wide barrier. Right image: ~ 0.5 µm wide barrier.
capture at regularly spaced intervals; this ensures uniform presentation of DNA for imaging. Finally, the microfluidic device assembly makes use of double-sided tape to form a single channel between cut pieces of the patterned wafer and a microscope coverslip. Nanoports are attached over holes drilled into the glass surface to complete the device.

The surface surrounding the chrome barriers, inside this microfluidic device, is coated with a lipid bilayer. This lipid bilayer surface contains a biotinylated lipid that provides a binding site for streptavidin, to ultimately anchor biotinylated DNA to the surface. We are currently investigating the use of a biotinylated DNA binding protein, LacO, which binds the specific LacI site on DNA. In addition to effectively anchoring the DNA to the bilayer, this would circumvent any low efficiency in the biotinylation of DNA harvested in vitro. DNA bound to the bilayer surface is then able to diffuse in the direction of flow until it reaches a chrome barrier. Here, the free end of the DNA extends under the force of flow to create rows of “DNA curtains,” allowing for the observation of many co-aligned stretched DNA molecules within a single microscope field of view (Figure 2).

The interaction of proteins and DNA can be visualized within these devices using prism total internal reflection fluorescence microscopy (TIRFM), a high-resolution fluorescence microscopy technique [2]. Our goal is to adapt this research tool to study chromatin activity (Figure 3) and transcription activator-like effectors (TALEs) (Figure 4), primarily during the Heat Shock Response, at the single-molecule level [3]. These studies should greatly expand our understanding of gene regulation derived from current optical and biochemical analyses [4]. Applications for the “DNA curtains” approach extend far beyond these two specific applications, as many DNA-protein interactions are either poorly understood, or have not been previously investigated via optical methods.

References:


Figure 2, top: Fluorescence image of YOYO1-stained DNA curtains. The left image shows DNA molecules after pausing buffer flow while the right image shows the same DNA now extended after returning buffer flow (arrow denotes direction of flow). Scale bar 15 µm.

Figure 3, middle: Fluorescence image of chromatin curtains obtained from Drosophila S2 cells. Scale bar 15 µm.

Figure 4, bottom: YOYO-1 labeled λ-DNA curtains with bound Alexa-647 labeled TAL1535. λ-DNA and TAL1535-Alexa-647 (arrowheads) were incubated together prior to injection into the microfluidic device. Scale bar 15 µm.
Microfabricated Devices for Cell Manipulation

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Abstract:
Cellular therapies to treat end-stage liver diseases require robust functional human hepatocytes for cell transplantation [1]. However, the paucity of donor liver tissue has limited these applications. Human pluripotent stem cells (hPSCs) represent a promising and potentially unlimited cell source to derive human hepatocytes. In this report we demonstrate enhanced function of human induced pluripotent stem cells (iPSCs)-derived hepatocytes (iPS-Hs) via 3D co-aggregation in PDMS microwells. The functions of iPS-Hs, albumin and urea secretion, were significantly enhanced via uniform 3D co-aggregation with stromal cells (SCs) in a PDMS microwell platform. Our results highlight the advantages of 3D microwell co-aggregation in derivation of functional iPS-Hs. This proof-of-concept study provides a robust approach to improve the function of iPS-Hs which holds great promise for drug screening, liver disease treatment, modeling, and personalized medicine.

Summary of Research:
Fabrication of PDMS Microwell. The photomask was prepared using DWL2000 mask writer (Heidelberg Instruments). The silicon wafer was spin-coated with SU-8 2150 photoresist (MicroChem) at 500 rpm for 40 sec and then 2500 rpm for 30 sec. The wafer was covered with the photomask and exposed by a UV photolithography machine (ABM Contact Aligner) for 32 sec. After being developed and post-baked, the SU-8 master wafer was fabricated. The SU-8 master wafer was then used to create PDMS (Sylgard 184, Dow Corning) mold. A mixture (10:1) of Sylgard 184 silicone elastomer components was casted onto the master wafer and cured at 60°C overnight to prepare a PDMS microwell. Figure 1 is a microscopic image of square micropattern on PDMS mold.

Formation of Cell Aggregates in PDMS Microwells. PDMS microwells were autoclaved, placed in 12-well plate, and coated with 1% (w/v) Pluronic® F127 solution before cell seeding to prevent cell attachment on PDMS surface and facilitate formation of cell aggregates. To form cell aggregates, cell suspensions of induced pluripotent stem cell-derived hepatocytes (iPS-Hs) alone (2.0 × 10⁶ cells) and iPS-Hs/stromal cells (SCs) mixture (iPS-Hs:SCs=2:1, total 2.0 × 10⁶ cells) was added to each well of 12-well plate with PDMS microwells inside. After four hours of static culture, the cells adhered on the interspace between microwells were removed by medium change. The cells fell into the microwells formed cell aggregates after overnight culture with gentle shaking. The cell aggregates were cultured in microwells for eight days.

Albumin and Urea Secretion. Albumin and urea secretion were analyzed by measuring the concentration of albumin and urea in culture medium. The medium was collected and replaced with fresh medium every two days. The collected medium was centrifuged at 1000 rpm for 5 min. The supernatant was stored at -20°C for analysis of albumin and urea secretion. Secreted albumin in the supernatant was quantified by an enzyme-linked immunosorbent assay (ELISA) kit using sheep anti-rat albumin antibodies and horseradish peroxidase detection. Urea concentration was assayed using a colorimetric endpoint assay using diacetylmonoxime with acid and heat.

References:
Figure 1: A microscopic image of iPS-Hs aggregates in PDMS microwells. Scale bar: 400 µm.

Figure 2: A microscopic image of iPS-Hs/SCs co-aggregates in PDMS microwells. Scale bar: 400 µm.

Figure 3, left: Albumin secretion of iPS-Hs during eight days of culture in PDMS microwells. Mean ± SD (n = 3). *p < 0.05, ***p < 0.001, NS: no significant difference.

Figure 4, right: Urea secretion of iPS-Hs during eight days of culture in PDMS microwells. Mean ± SD (n = 3). **p < 0.01, ***p < 0.001, NS: no significant difference.
Abstract:
The protein SNAP-25 plays a key role in transmitter release by fusion of neurosecretory vesicles. The temporal correlation between the conformational change of SNAP-25 and individual fusion events is investigated in SNAP-25 deficient mouse chromaffin cells overexpressing an improved SNARE Complex Reporter (mSCORE). The 4-electrode ECD chip was modified to support the direct culturing of chromaffin cells on top of electrodes. In the mSCORE expressing cells, we imaged the fluorescence resonance energy transfer (FRET) change of mSCORE localized to the plasma membrane by total internal reflection (TIR)-FRET imaging and simultaneously recorded individual fusion events as amperometric spikes with the microfabricated electrochemical detector (ECD) array.

Summary of Research:
In previous experiments with bovine chromaffin cells expressing a SCORE construct incorporating CFP and Venus as a FRET pair, a local rapid and transient FRET change was identified that occurred precisely where an individual vesicle released catecholamine [1]. Cyan fluorescent proteins (CFPs) are widely used as donor fluorophores in FRET experiment. It was reported that the CFP variant mCerulean-3 exhibits enhanced brightness and reduced reversible photoswitching [4]. In order to improve the signal-to-noise ratio of the FRET reporter, a modified probe named mSCORE was generated by replacing the FRET donor CFP in original SCORE [5] with mCerulean-3. Furthermore, to ensure that the mSCORE is actively supporting the release mechanism, chromaffin cells from SNAP-25 knock-out mice were used instead of bovine chromaffin cells to eliminate the contribution of wild-type SNAP-25 in the fusion mechanism.

As shown in Figure 1, the chromaffin cell expressing mSCORE was placed on the top of a 4-electrode ECD array and imaged in TIRF mode showing improved fluorescent intensity. In TIRF mode only the fluorescent molecules at the cell surface in contact with the coverslip are excited. In individual mSCORE-overexpressing chromaffin cells, we tracked conformational changes in SNAP25 by FRET imaging while exocytotic catecholamine release from single vesicles was simultaneously detected using an ECD array consisting of 4 Pt microelectrodes patterned photolithographically on a glass coverslip [2,3].
Initial experiments indicate that an mSCORE expressing SNAP-25 knock-out cell does show fusion events indicating that mSCORE is a functional SNAP-25 substitute.

In order to directly culture the mouse chromaffin cells on the top of ECD array, the individual ECD arrays patterned on the glass wafer were designed in a more compact way. The new design allows the glass chip to accommodate up to 14 4-electrode ECD arrays (Figure 2A), instead of only four in the previous design (Figure 2B). The compact arrangement of ECD arrays will increase the probability that a chromaffin cell will settle on top of the active region of an individual array when plated directly on the ECD coverslip. Direct culturing cells on the ECD chip will allow us to simultaneously perform electrophysiology stimulation and ECD recording, as well as to avoid the lifting of cells from culture dishes facilitating the experimental procedure and increasing the throughput accordingly.

References:


A Microfluidic Chip for Highly Parallel Single Cell Analysis

CNF Project Number: 2331-15
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Abstract:

We demonstrate a highly parallel workflow that allows robust and efficient single cell capture for downstream molecular biology analysis. A poly(dimethylsiloxane) (PDMS) microwell array is fabricated using well-established soft lithography techniques. Laser Capture Microdissection is subsequently used to transfer cells of interest into individual microwells with high accuracy. After cell capture, the PDMS chip is sealed with a semi-permeable membrane. Finally, a flow cell is attached onto the membrane-PDMS assembly to allow reagent delivery. We show preliminary results from the on-going development of the workflow.

Summary of Research:

PDMS microwell chips are fabricated using soft lithography techniques. Briefly, a silicon wafer is spin-coated with SU-8-2050 photoresist for 30 seconds at 500 RPM followed by one minute at 3000 RPM. A chromium mask with a 100 × 100 array of circles (diameter=100 µm, pitch=200 µm) is written using a Heidelberg Mask Writer DWL2000. The SU-8-coated wafer is then exposed to UV light through the chromium mask in an ABM Contact Aligner. After development in SU-8-developer, the wafer is rinsed with IPA, dried with nitrogen, and stored until PDMS molding. A Sylgard 184 (1:10) PDMS prepolymer mixture is poured over the silicon master wafer in a polystyrene Petri® dish and cured at 80°C for an hour.

A chemical gluing strategy is used to bond the PDMS chip to a polycarbonate (PC) membrane (GE Healthcare) following cell capture with Laser Capture Microdissection [1]. The PDMS chip and PC membrane are both plasma treated for 90 seconds before immersion in 1% aqueous 3-aminopropyltriethoxysilane (APTES) and glycidoxypropyltrimethoxysilane (GPTMS) at 80°C for 20 minutes, respectively. After silanization, the PDMS and PC membrane are briefly washed with DI water and dried with nitrogen.

The surface modified PDMS is then flipped and positioned over a Zeiss PEN-membrane slide containing cells of interest. The PDMS is elevated from the slide surface by 300-400 µm, as shown in Figure 1. A Zeiss PALM Microbeam microscope is used to cut and eject membranes into the microwells. The small distance between the PEN-membrane slide and the PDMS microwell chip ensures high accuracy of captured cell delivery. The cut membranes adhere to the PDMS via strong van der Waals interaction. After the collection of cells, the APTES-coated PDMS is irreversibly bonded to the GPTMS-coated PC membrane at room temperature for one hour. We then attach a SecureSeal hybridization chamber (Grace-Bio) onto the membrane-PDMS assembly, as shown in Figure 2. Reagents are introduced into the chamber and the microwells are dead-end filled. As a proof of principle demonstration, we collected 25 membrane samples into the microwells as shown in Figure 2.

To achieve highly parallel single cell analysis using our microfluidic device, we still need to improve a few aspects of the workflow. In particular, the longevity of the surface amine groups is crucial to allow sufficient time for sample collection as well as further surface modification with proteins such as bovine serum albumin [2].

References:

Figure 2: An assembled device. The PC membrane is bonded to PDMS via amine-epoxy chemistry. The flow chamber (red) on top of the membrane-PDMS assembly allows the introduction of liquid and application of pressure to dead-end fill the microwells.

Figure 3: An optical micrograph of 25 collected membrane cuts using a Zeiss PALM Microbeam Laser Capture Microdissection system.