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Live-Cell Fluorescence Microscopy of Directed Cell Migration on Partially Etched Electroactive SAM Gold Surfaces

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The ability of a cell to adhere, polarize, and migrate is influenced by the complex and dynamic extracellular matrix. The role of the extracellular matrixin directed cell migration has been under intense investigation and is important for a number of fundamental biological processes, including tissue repair, immune response, embryogenesis, and cancer metastasis.[1] Before cells can migrate they must first polarize in response to external cues from the local microenvironment and establish spatial, temporal and functional asymmetry internally.[2] Cell polarization involves the complex interplay of various signaling pathways that induce reorganization of the cytoskeleton and key organelles to initiate directed migration.[3] The development of molecularly well defined model substrates that combine sophisticated surface chemistry, microfabrication technology, molecular biology, and live-cell fluorescence microscopy imaging would allow for further investigation into these complex cellular processes.

We believe that the most flexible model surfaces for studying biointerfacial science are based on self-assembled monolayers (SAMs) of alkanethiolates on gold.[4] These surfaces have four principle advantages over other surfaces for studying biospecific cell behavior: they are 1) synthetically amenable to a wide range of terminal groups to generate many different tailored surfaces,[5] 2) inert to unspecific protein adsorption,[6] 3) redox active monolayers,[7] and 4) compatible with several surface spectroscopy techniques used for the characterization of interfacial interactions and reactions.[8] However, until now, two major limitations have hindered the study of cell biology on SAMs. Due to gold's fluorescent quenching, SAMs of alkanethiolates on gold are incompatible with the live-cell high resolution fluorescence microscopy needed to visualize dynamic features within cells.[9] Additionally, the directional path of cellular migration must be determined ex post facto because a ligand pattern cannot be independently visualized during migration.

To study complex cell behavior, especially directed cell polarity and migration, a molecularly well-defined surface that can be patterned with a chemoselective immobilization strategy would be of tremendous utility. Additionally, the surface should be compatible with live-cell high resolution microscopy and simultaneous visualization of the cell-path trajectory.

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Herein, we introduce three new, straightforward technologies that combine synergistically to provide complete spatial and visual control of directed cell polarity and migration. This strategy uses: 1) partially etched gold surfaces to determine the precise track or path of cell migration trajectory, 2) microfluidic lithography (μFL) to pattern gold surfaces rapidly with a variety of alkanethiols for biospecific cell interactions, and 3) live-cell high-resolution fluorescence microscopy on gold surfaces to visualize internal organelle dynamics during polarity and migration. To obtain ligand control over the surface and characterize the patterning, we utilize a chemoselective electroactive SAM immobilization methodology to pattern ligands on the partially etched gold surfaces.[10] We show live-cell directed migration of a mammalian cell line in which the nucleus and Golgi have been fluorescently labeled to determine the role of cell polarity on motility. The strategy is based on the use of microfluidic cassettes to partially etch the gold surface and then install a monolayer on the etched regions for subsequent immobilization of biospecific adhesive peptides. By using a slightly modified inverted microscopy set-up (see the Supporting Information),[11] routine live cell fluorescence microscopy is now possible on gold surfaces. This study is the first demonstration of live-cell fluorescence microscopy of directed cell migration on tailored gold surfaces.

The strategy to chemoselectively immobilize ligands, visualize the ligand patterns, and monitor directed cell migration through live-cell fluorescence microscopy is outlined in Figure 1. First, a polydimethylsiloxane (PDMS) microfluidic cassette was reversibly sealed to a bare gold surface to achieve spatial control. To partially etch and pattern the gold surfaces, a mild tri-iodide solution (18 mM KI, 4.3 mM I_2) was flowed into the microchannels and allowed to react for 10 s.[12] Without removing the PDMS stamp, the microchannels were subsequently flushed with water and then ethanol by flowing each into the channels for 10 s. To form a patterned SAM on the etched regions, we developed a new technology termed μFL. To perform μFL, an ethanolic alkanethiol solution containing either a terminal ligand or reactive head group was flowed into the channels and allowed to self-assemble on the partially etched regions. By controlling the concentration of alkanethiol solution and duration of SAM formation, mixed SAMs and even gradients can be patterned. It should be noted that the μFL strategy can be used to pattern a variety of alkanethiols rapidly onto flat or etched gold surfaces (Supporting Information).

Once the monolayer was installed onto the partially etched regions, the microfluidic PDMS cassette was removed from the gold surface. The etched and patterned gold surface was then immersed in a tetra(ethylene glycol) undecane thiol ($EG_4C_{11}SH$) solution to backfill the unetched and unreacted regions of the gold surface. The ethylene(glycol) group prevents unspecific

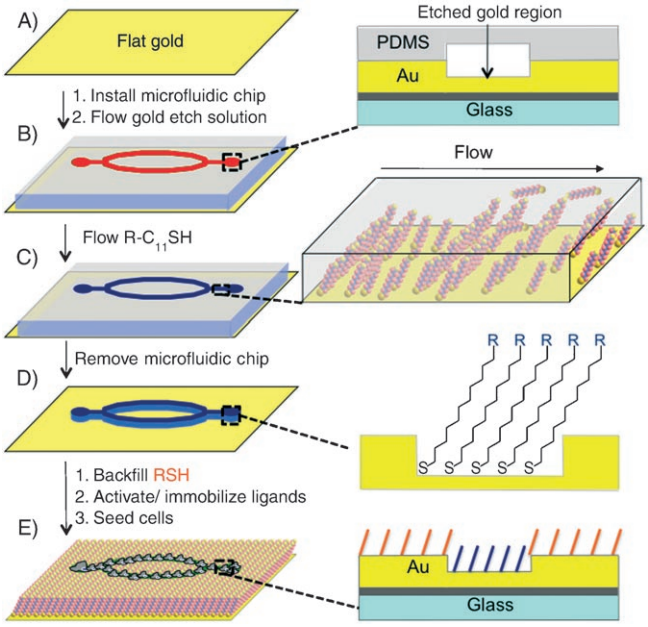

Figure 1. Schematic representation of the generation of patterned, partially-etched gold surfaces for chemoselective ligand immobilization and directed cell migration. A) A PDMS microfluidic cassette was reversibly sealed to a flat bare gold surface. B) A dilute tri-iodide etch solution was flowed into the microfluidic cassette for 10 s to create a recessed, patterned gold surface. C) Without removing the PDMS cassette, a rinsing solution of absolute ethanol (20 μL) and then water was flowed through the channel followed by the addition of 1 mM hydroquinone terminated alkanethiol in EtOH for 60 s. This generated a partial SAM on the partially etched gold regions. D) Removal of the cassette from the gold surface exposed the partially etched SAM-patterned regions and the bare gold regions. E) The entire substrate was then immersed in 1 mM $EG_4C_{11}SH$ solution for 8 h to create an inert SAM background; the unetched regions were resistant to protein and cell adhesion. Electrochemical oxidation of the hydroquinone to the quinone followed by chemoselective coupling with RGD–oxyamine peptides resulted in a molecularly defined surface, tailored for directed cell-migration studies. Cells only adhered to the ligand-presenting regions. Directional cell migration could be predicted and tracked by visualizing the cells and path trajectory with standard live-cell fluorescence microscopy.

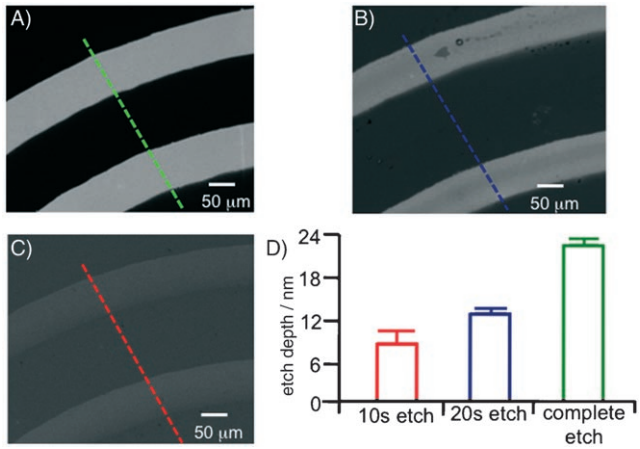

Figure 2. Controlling the amount of gold etch and therefore gold thickness by varying the duration of the flow of tri-iodide solution in the microfluidic channels. Gold thickness was determined by linescanning. A) A 24 nm gold surface on a glass slide was completely etched, and showed a patterned gold and glass substrate; etch depth: 24 nm. B) Partially etched surface obtained after 20 s flow with tri-iodide solution; etch depth: 13 nm. C) Surface etched after 10 s flow with tri-iodide solution; etch depth: 8 nm. D) Bar graph of etch results.

protein adsorption and cell attachment to the surface.[6] Depending on the nature of the alkanethiol used in the etch region, an immobilization reaction or an activation/immobilization strategy can be employed to chemoselectively install a variety of ligands in the etched regions.[13] We used an electroactive hydroquinone alkanethiol that can be electrochemically reversibly oxidized to the quinone before subsequent reaction with oxyamine containing ligands to generate an interfacial oxime conjugate.[10] For cell biology studies, cells can be seeded onto the substrate for biospecific attachment to the etched regions where the ligand pattern is localized. This methodology allows for ligand pattern visualization on biospecific-tailored surfaces that are compatible with live cell high resolution fluorescence microscopy for directed cell polarity and migration studies.

To characterize the thickness of the gold etch patterns generated by the flow of oxidants through the microfluidic channel, we etched several gold substrates under a range of conditions (Figure 2). We first evaporated titanium (6 nm) and then gold (24 nm) onto a thin glass coverslip and determined the precise thickness using a quartz crystal microbalance. Three sets of etching conditions were performed by using the microfluidic cassette on a bare gold surface. The first gold surface was completely etched away to the underlying titanium layer by using concentrated tri-iodide solution (5 M KI, 1 M I_2). The etch depth was 24 nm, as determined by a quartz crystal microbalance during gold deposition. The second was etched for 10 s by using the dilute etch solution (18 mM KI, 4.3 mM I_2), and the third surface was etched for 20 s with the same solution. To determine the amount of gold etched, phase contrast microscopy images were taken of the three substrates. B using image processing software, linescans of these substrates were used to measure the transmittance of light through the substrates, which was correlated with fully etched surfaces to calculate the amount of gold etched. For the 20 s etch, 13 nm of gold was removed and for the 10 s etch, 8 nm was removed. As controls, surfaces with each of these thicknesses were independently generated by e-beam evaporation, (characterized by a quartz crystal microbalance) and determined to have the same transmittance as the microfluidic/chemical partially etched gold surfaces. These results demonstrate that gold can be selectively etched and therefore patterned by using microfludic chips, and that the etch depth thickness can be quantified with simple image processing software.

In order to characterize the quality of the SAMs on the patterned, partially etched regions by μFL, a hydroquinone–tetra(ethylene glycol) undecane thiol ($H_2QEG_4C_{11}SH$) was assembled on the surface of partially etched regions of gold (1 mM, 30 s) and backfilled with the inert tetra(ethylene)glycol alkanethiol ($EG_4C_{11}SH$; 1 mM, 10 min). The hydroquinone group is electroactive and can be reversibly oxidized to the quinone, which can subsequently react chemoselectively with oxyamine tethered ligands to generate an interfacial oxime conjugate (Fig-

ure 3). Cyclic voltammograms (CV) were taken of the partially etched surface, and these showed two distinct redox peaks at 570 and 290 mV, which is characteristic of the hydroquinone–quinone redox couple. To demonstrate the chemoselectivity of

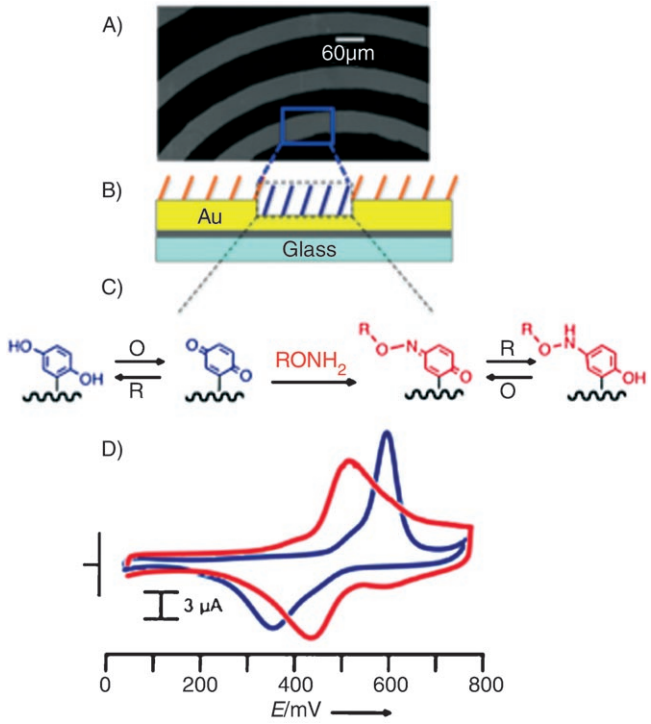

Figure 3. Electrochemical characterization of a chemoselective ligand immobilization strategy on a patterned partially etched gold surface. A) Bright-field micrograph of a partially etched surface. B) Side-view representation of a partially etched patterned SAM. In the etched regions, a hydroquinone alkanethiol SAM was patterned by using microfluidic lithography. C) The hydroquinone was activated by electrochemical oxidation to the quinone, and subsequently treated chemoselectively with oxyamine tethered ligands to generate a stable interfacial oxime linkage. D) Cyclic voltammetry of the surface showed diagnostic redox signals for the hydroquinone–quinone and oxime product. Integrating the waves provided a quantitative measure of the amount of oxime product on the partially etched regions. Intersection of crosshairs represents zero current.

the reaction, an oxyamine acetic acid was immobilized to the surface (250 mM, 2 h). The two redox peaks shifted to 483 mV for the oxidation and 342 mV for the reduction peak; this corresponds to the oxime product. The resulting redox peak shift and peak integration allows for a diagnostic and quantitative evaluation of reaction yield and therefore density of immobilized ligand.[10]

To verify the quality of the SAM and amount of electroactive molecules on the partially etched region, we used cyclic voltammetry within the corresponding patterned etch area.[10,14] For example, the total area etched with the microfluidic cassette and therefore total area of installed electroactive hydroquinone or oxime ligand conjugate was 0.375 cm^2 and the gold surface was 2 cm^2. The peak area for the hydroquinone was calculated to be 4.05 μC with the equation $Q = nFA\Gamma$ (where Q represents total charge, n is moles of electrons (2), F is Faraday's constant, Γ is molecules per surface area). From the peak area data, our 0.375 cm^2 surfaces should theoretically contain a total of 3.75×10^{13} electroactive molecules if a full monolayer is present. We found experimentally that 3.38×10^{13} molecules of hydroquinone were on the etched surface, which closely approximates the theoretical calculation. This demonstrates that an electroactive SAM can be installed rapidly by μFL, and the density of the molecules/ligands on the patterned surface can be precisely determined.

To observe directed cell migration on the partially etched surface, an RGD oxyamine peptide, which is the minimum adhesion peptide found in the extracellular matrix protein fibronectin,[15] was synthesized and chemoselectively immobilized to the etched and patterned quinone–alkanethiol surfaces. For cell biology studies, a mixed SAM containing $H_2QEG_4C_{11}SH$ (10%) and $EG_4C_{11}SH$ (90%) was flowed through the microchannels (1 mM total, 1 min), the microfluidic cassette was then removed, and the remaining bare gold regions were backfilled with tetra(ethylene)glycol alkanethiol (1 mM) for 8 h. The surface was activated with 750 mV for 10 s to oxidize the hydroquinone to the reactive quinone to which RGD–oxyamine was immobilized (1 mM, 2 h). A stably transfected fluorescent fibroblast cell line (Rat2) containing GFP-labeled Golgi and mCherry-labeled nuclei was then seeded onto the surface (Figure 4).

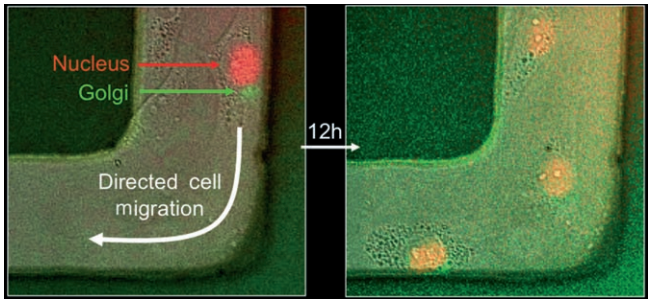

Figure 4. Multiwavelength time-lapse live-cell fluorescence microscopy of transfected Rat2 fibroblast cells during directed migration on partially etched electroactive RGD-presenting SAM gold surfaces. The partially etched regions appear lighter in the micrographs. A) Initial position of cells on the etched region of the gold; red: nucleus, green: Golgi. B) An image taken 12 h after cell migration in the etched regions of the gold.

To measure the role of cell polarity in directed cell migration, which is a fundamental question in cell motility, the relative positional vector of the concentrated Golgi and nucleus can be determined as the cell polarizes and migrates.[15] We were interested in studying the relative orientations of the Golgi and nucleus when the cell changes direction and migrates around corners. It is unclear how the nucleus to Golgi vector is oriented during directed cell migration through a turn. A number of different experimental observations and theories have been proposed: 1) The vector always points in the direction of migration; 2) the vector is random and does not consistently point in the direction of migration; 3) the Golgi remains concentrated, but moves to the rear of the nucleus during a turn; it then becomes diffuse, reorients itself to the front of the

nucleus in the direction of migration, and reconcentrates after the turn is completed.[3,15–17] With the biospecifically etched, patterned surfaces, the nucleus to Golgi vector can be measured as cells predictably change their direction of migration. Upon cell seeding, the cells adhered only to the partially etched regions of gold because the RGD ligands were localized in these areas only. These regions appear lighter than the thicker gold regions, and clearly show the etch pattern, ligand pattern, and cell-path trajectory. Over a period of 12 h, cell migration was observed on the partially etched surface. The cells stayed confined to the etched regions, and the Golgi was concentrated toward the leading edge of migration. Our results suggest that the polarity vector (nucleus to Golgi) reorients after completion of a directional turn around corners (see movie in the Supplementary Information for an example of directed migration around corners). No cells adhered to control surfaces that were not treated with RGD–oxyamine or with a scrambled RDG–oxyamine peptide.

In conclusion, we have developed a methodology to visualize ligand patterns of SAMs of alkanethioalates on gold. PDMS microfluidic cassettes provided spatial control over gold etching and μFL, which is the methodology used to pattern ligands of alkanethiolates in the same etched regions on the gold surface without the need for a separate pattern registration step. Microfabrication methods to generate 3D microfluidic cassettes will allow for the etching and/or patterning of alkanethiolates in isolated regions on to generate complex but addressable geometries and patterns on the surface. To characterize the surfaces and immobilize ligands chemoselectively, a $H_2QEG_4C_{11}SH$ SAM was formed in the etched channels by using a μFL strategy. The electroactive alkanethiol was used to determine the amount of ligand immobilized on the electroactive area patterned on the substrate. Furthermore, these surfaces were used to determine the directed polarity and migration of a fluorescent cell line. Finally, live cell fluorescence microscopy was used to observe both the Golgi and nucleus, in order to monitor directed cell polarity and migration. Gradient peptide surfaces and peptide nanoarrays are being investigated to determine the effect of the underlying surface on cell polarity and migration.[8,15,18] The combined ability to pattern partially etched gold surfaces, install and pattern a variety of alkanethiolates and be compatible with live-cell fluorescence microscopy provides a synergistic methodology that enables the observation and study of a variety of cell adhesion, migration, and growth dynamics on molecularly tailored gold surfaces.[18]

Experimental Section

Synthesis of alkanethiols: The undecane thiols terminated with tetra(ethylene glycol) and hydroquinone–tetra(ethylene glycol) were synthesized as reported previously.[10]

Solid-phase peptide synthesis: Peptide synthesis of RGD–oxyamine was performed as previously reported.[10]

Electrochemistry: All electrochemical measurements were made by using a Bioanalytical Systems Epsilon potentiostat. An Ag/AgCl electrode served as the reference, the gold monolayer acted as the working electrode, and a Pt wire served as the counter electrode. The electrolyte was $HClO_4$ (1 M) and the scan rate was 100 $mV s^{-1}$. All measurements were made in a standard electrochemical cell.

Microfabrication: The microchips were fabricated by using soft lithography.[19] Patterns were fabricated by using masks drawn in Adobe Illustrator CS3 and photoplotted by Pageworks onto transparencies. The SU-850 (MicroChem, Newtown, USA) was patterned by using the manufacturer's directions, and a 100 μm channel depth was obtained with these masks. Slygard 184 (Dow Corning) was cast onto the mold by using a 1:10 ratio of curing agent to elastomer (*w/w*). The prepolymer was degassed for 15 min and then poured over the mold. The prepolymer was cured for 1 h at 75 °C. The PDMS was removed from the master and access holes were punched into the PDMS to allow fluid flow.

Preparation of monolayers: Gold substrates were prepared by electron beam deposition of titanium (6 nm) and gold (24 nm) on 24 mm × 100 mm glass microscope slides. The slides were cut into 1×2 cm^2 pieces and washed with absolute ethanol before use.

Chemical gold etch: A PDMS stamp was reversibly sealed to a gold substrate. To chemically etch the gold surface, a solution containing KI (18 mM) and I_2 (4.3 mM) was flowed into microchannels for various lengths of time to partially etch the gold layer. By controlling the duration of the etch conditions it was possible to control the amount of gold etched. Once the gold had been etched, flowing water and then ethanol (30 s each) were used to clean the microchannels.

Microfluidic lithography (μFL): For the patterned etched and electroactive surface characterization, a 1 mM solution of hydroquinone–tetra(ethylene glycol) undecane thiol in ethanol was flowed into the channels for 30 s to install a SAM on the etched regions. The microfluidic cassette was then removed and the remaining bare gold regions were backfilled with tetra(ethylene glycol) alkanethiol for 10 min. Cyclic voltammetry was used to determine the amount (density) of electroactive species on the etched regions. For cell biology studies, a mixed SAM that contained hydroquinone–tetra(ethylene glycol) alkanethiol (10%) and tetra(ethylene glycol) alkanethiol (90%) was flowed through the microchannels (1 mM total, 1 min). The microfluidic cassette was then removed, and the remaining bare gold regions were backfilled with tetra(ethylene glycol) alkanethiol (1 mM) for 8 h. The surface was electrochemically oxidized and the quinone surface was treated with RGD–oxyamine (1 mM, 2 h) to install the peptide on the surface for subsequent biospecific cell adhesion and migration studies.

Oxyamine coupling reaction: The surface was activated with the application of 750 mV for 10 s to oxidize the hydroquinone to the reactive quinone. For surface characterization, oxyamine acetic acid (250 mM) was added and incubated on the surface for 2 h. For cell biology surfaces the same activating conditions were used but RGD–oxyamine peptide was immobilized to the surface (1 mM, 2 h).

Cell culture: Histone-2B-mCherry and GFP-α-tubulin expressing Rat2 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (5%) and penicillin/streptomycin. Cells were removed from tissue culture flasks with trypsin/EDTA, added to serum-containing media, and centrifuged. Cells were resuspended in serum-free media and plated to the partially etched SAM-patterned substrates and incubated for 1.5 h. After a gentle rinse with PBS, cell-plated substrates were placed in culture media for 2 h prior to imaging.

Time-lapse microscopy: Microscopy images were obtained with a Nikon TE2000-E microscope. To enable fluorescence visualization, gold substrates were inverted onto the glass microscope chamber, which contained sandwiching-polystyrene beads (25 μm) that prevent contact between the cells and the chamber.[11] Fluorescent images were obtained at 10 min intervals over a period of 12 h. Data were analyzed with Metamorph imaging software.

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Keywords: cell migration · cells · electrochemistry · microfluidic lithography · self assembled monolayers (SAMs)

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