

Fabrication of Multifaceted, Micropatterned Surfaces and Image-Guided Patterning Using Laser Scanning Lithography

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Abstract

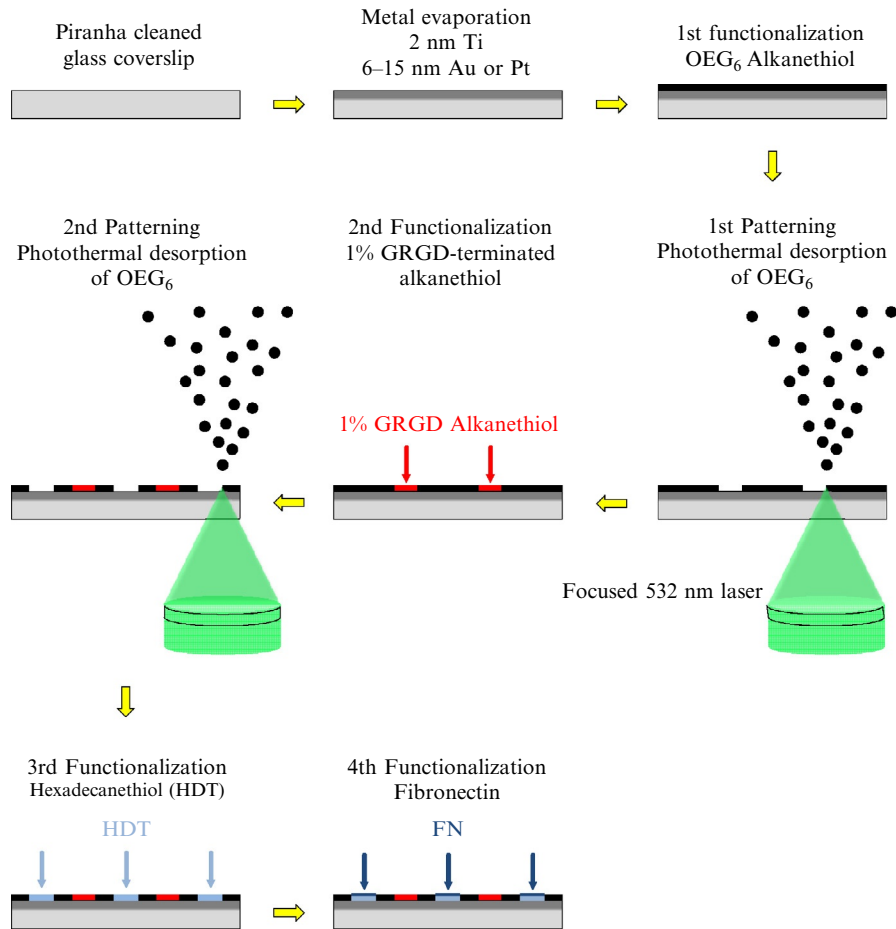
This protocol describes the implementation of laser scanning lithography (LSL) for the fabrication of multifaceted, patterned surfaces and for image-guided patterning. This photothermal-based patterning technique allows for selective removal of desired regions of an alkanethiol self-assembled monolayer on a metal film through raster scanning a focused 532 nm laser using a commercially available laser scanning confocal microscope. Unlike traditional photolithography methods, this technique does not require the use of a physical master and instead utilizes digital “virtual masks” that can be modified “on the fly” allowing for quick pattern modifications. The process to create multifaceted, micropatterned surfaces, surfaces that display pattern arrays of multiple biomolecules with each molecule confined to its own array, is described in detail. The generation of pattern configurations from user-chosen images, image-guided LSL is also described. This protocol outlines LSL in four basic sections. The first section details substrate preparation and includes cleaning of glass coverslips, metal deposition, and alkanethiol functionalization. The second section describes two ways to define pattern configurations, the first through manual input of pattern coordinates and dimensions using Zeiss AIM software and the second via image-guided pattern generation using a custom-written MATLAB script. The third section describes the details of the patterning procedure and postpatterning functionalization with an alkanethiol, protein, and both, and the fourth section covers cell seeding and culture. We end with a general discussion concerning the pitfalls of LSL and present potential improvements that can be made to the technique.

INTRODUCTION

Surface patterning strategies that allow for the immobilization of a biomolecule in a well-defined pattern array against a biologically inert background have provided a foundation for the creation of advanced biosensors (Senaratne, Andruzzi, & Ober, 2005) and a tool for investigating local environmental cues and mechanotransduction processes on cell behavior (Goffin et al., 2006; Kilian, Bugarija, Lahn, & Mrksich, 2010; McBeath, Pirone, Nelson, Bhadriraju, & Chen, 2004; Senaratne et al., 2005; Singhvi et al., 1994; Sniadecki, Desai, Ruiz, & Chen, 2006). A multitude of

patterning strategies exist and a number of reviews cover them in detail (Hook, Voelcker, & Thissen, 2009; Singh, Patil, Thombre, & Gade, 2013). Although patterning a single biomolecule is relatively straightforward using existing strategies such as microcontact printing, the ability to pattern multiple biomolecules in close proximity without cross-contamination is still challenging. A handful of techniques, some based on variations of microcontact printing (Bernard, Renault, Michel, Bosshard, & Delamarche, 2000; Chiu et al., 2000; Desai, Khan, Gopal, & Chen, 2011; Shen, Thomas, Dustin, & Kam, 2008; Tien, Nelson, & Chen, 2002) and others on photochemical reactions (Ryan et al., 2004; Yang, Frey, Oliver, & Chilkoti, 2000), have been implemented to pattern multiple proteins or cells, for alignment of multiple alkanethiol self-assembled monolayers (SAMs), or for uncaging biotin species for streptavidin-based immobilization but it is still difficult to pattern multiple biomolecules in close proximity with high resolution. Additionally, all of the aforementioned techniques rely on the use of a physical master to act as either a template for stamp creation or as a mask for photochemical reactions. The use of a physical master limits the ability to change pattern design quickly and requires costly, time-consuming fabrication of a new master. To alleviate this issue, a few maskless patterning strategies have been developed (Demers et al., 2002; Doyle, Wang, Matsumoto, & Yamada, 2009; Rhinow & Hampp, 2006; Shadnam, Kirkwood, Fedosejevs, & Amirfazli, 2004). A noteworthy example, micro photopatterning (μ PP), implements a focused two-photon laser to ablate patterns of a thin poly(vinyl) alcohol (PVA) film tethered from a silane functionalized glass surface (Doyle et al., 2009). μ PP has been implemented to pattern multiple proteins in close proximity with each protein confined to its own pattern (Doyle, 2009; Doyle et al., 2009). The process of ablating a thin PVA film by raster scanning a focused laser is similar to the technique described here although the substrate preparation and physical mechanism of patterning are extremely different (Doyle, 2009).

In this protocol we describe the implementation and details of laser scanning lithography (LSL) (Slater, Miller, Yu, & West, 2011), a photothermal desorption patterning strategy, for the fabrication of multifaceted, patterned surfaces and for generating image-guided patterns. In general, a focused 532 nm laser is raster scanned across the surface of a metal film supporting an alkanethiol SAM (Slater et al., 2011). The metal film absorbs the light energy, which at the correct wavelength induces the formation of localized surface plasmons that decay as heat thereby inducing thermal desorption of adjacent alkanethiols through disulfide bond formation once a temperature of ~ 212 °C is reached (Shadnam et al., 2004). We have harnessed this photothermal desorption mechanism to selectively remove patterns of an alkanethiol SAM. Upon removal, bare metal-patterned regions are exposed and functionalized with a second alkanethiol or protein. Successive rounds of alkanethiol removal followed by backfilling are implemented to pattern multiple biomolecules in close proximity with each molecule confined to its own pattern array (Figs. 11.1 and 11.8). Additionally, we outline the details of image-guided LSL which uses a custom-written MATLAB script to convert a user-chosen image into a digital “virtual mask” that can be used to guide the laser position during patterning.

**FIGURE 11.1**

Schematic outlining the fabrication of multifaceted, patterned surfaces with laser scanning lithography (LSL). A clean glass coverslip is coated with 2 nm of Ti and 6–15 nm of Au or Pt. The metalized coverslip is functionalized with an alkanethiol self-assembled monolayer (SAM); we utilized an OEG_6 -terminated alkanethiol to create a passive, biologically inert, and protein repulsive background. Desired patterns of the OEG_6 SAM are photothermally desorbed from the surface with a focused 532 nm laser. The bare metal patterns are functionalized with a 2nd alkanethiol; we utilized a solution consisting of 1% GRGD-terminated alkanethiol in OEG_3 . A 2nd round of photothermal patterning occurs followed by functionalization with a 3rd alkanethiol; we utilized HDT to render the 2nd pattern array hydrophobic. Fibronectin is adsorbed to the hydrophobic, HDT functionalized patterns.

This technique provides a means to fabricate complex pattern configurations containing intricate geometries with a wide range of shapes and sizes that would be difficult to pattern with existing techniques such as microcontact printing.

11.1 SUBSTRATE PREPARATION: PIRANHA CLEANING, METAL DEPOSITION, AND ALKANETHIOL FUNCTIONALIZATION

This section outlines preparation of the substrates prior to patterning and includes detailed protocols for piranha cleaning, thin metal film deposition via electron-beam evaporation, and alkanethiol functionalization (Fig. 11.1).

11.1.1 Materials

- 35 mm diameter, # 1, optical borosilicate circular glass coverslips (Fisher Scientific, Pittsburgh, PA. Custom order).
- Sulfuric acid (H_2SO_4 , Fisher Scientific, Pittsburgh, PA).
- 200 proof ethanol (VWR, West Chester, PA).
- Squeeze bottle filled with 200 proof ethanol.
- 500 ml glass beaker.
- 1 ml micropipette.
- 30% hydrogen peroxide (H_2O_2 , Fisher Scientific, Pittsburgh, PA).
- Millipore water (MPH_2O) with a resistance $>18 \text{ M}\Omega\text{-cm}$ (Millipore Super-Q water system, Billerica, MA).
- Glass stir rod.
- Ultrahigh purity nitrogen tank equipped with inline filters and Pasteur pipette or plastic pipette tip (Matheson TriGas, Basking Ridge, NJ).
- Pyrex crystallizing dish: 190×100 , No. 3140.
- 4 l glass beaker.
- 60×15 mm plastic Petri dishes (VWR, West Chester, PA).
- Parafilm.
- Scalpel.
- Ruler.
- 7 cc molybdenum crucibles (Kamis Inc., Mahopac Falls, NY).
- $1/8'' \times 1/8''$, 99.999% pure Titanium (Ti) shot (Kamis Inc., Mahopac Falls, NY).
- $1/8'' \times 1/8''$, 99.999% pure Platinum (Pt) or Gold (Au) shot (Kamis Inc., Mahopac Falls, NY).
- $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OH}$ (OEG₆, ProChimia, Sopot, Poland. Cat # TH005-02).
- Coverslip tweezers.

11.1.2 Equipment

- Electron-beam evaporator (Sharon Vacuum, Brockton, MA).
- Custom-built chamber for alkanethiol functionalization.

- 2—58 × 76 mm glass slides.
- Polydimethylsiloxane (PDMS). Sylgard[®] 184 (Dow Corning Corporation, Midland, MI).
- 1 l bottle filled with water.
- Chemical fume hood.
- Custom-machined Al sample holder for e-beam evaporation.

11.1.3 Method

11.1.3.1 Piranha cleaning of glass coverslips

Caution: Piranha solution is extremely corrosive, reactive, and potentially explosive. Perform the Steps 1–7 in a chemical fume hood with the appropriate protective wear. Contact your chemical safety officer for additional information.

1. Carefully pour 525 ml of H₂SO₄ into the crystallizing dish.
2. Slowly add 175 ml of H₂O₂ to the H₂SO₄ in the crystallizing dish.
3. Gently stir the solution with a glass stir rod.
4. Carefully add ~30 coverslips to the piranha solution.
Make sure that the coverslips do not float and keep them separated from each other using the glass stir rod.
5. Carefully stir the solution with the glass stir rod every 20 min.
6. After 1 h, quench the piranha solution by slowly adding MPH₂O to the crystallizing dish.
Do not fill the crystallizing dish completely; this could lead to spillage in the next step.
7. Pour off the quenched piranha solution into a 4 l glass beaker while assuring that the coverslips stay in the crystallizing dish.
Let the piranha cool to room temperature. Dispose of the quenched piranha solution properly (contact your chemical safety officer for proper disposal).
8. Add a copious amount of MPH₂O to the crystallizing dish with the coverslips.
9. Pour off the MPH₂O.
Make sure not to discard the coverslips when pouring off the MPH₂O.
10. Repeat Steps 8 and 9 three times.
11. Flow MPH₂O into the crystallizing dish for 10 min.
Keep the flow rate low enough not to crack or break the coverslips.
12. Pour off the MPH₂O and repeat the 10 min wash at least two more times.
The pH of the MPH₂O in the crystallizing dish can be measured to assure that all of the acid is removed.
13. Dry the coverslips with N₂.
Use low pressure (≤5 psi) to avoid cracking/breaking the coverslips.
14. Store the clean, dry coverslips in plastic Petri dishes wrapped with Parafilm or other container that prevents dust and atmospheric contaminants from adsorbing to the clean glass.

The piranha-cleaned glass coverslips are now ready for metal deposition (Fig. 11.1). We use the coverslips immediately since the clean, hydrophilic surface promotes better metal adhesion and uniform coating. Alternatively, the coverslips can be stored in a sealed container and used later if desired. If stored before use, it is best to expose them to oxygen plasma immediately before metal deposition.

11.1.3.2 Metal film deposition via electron beam evaporation

1. Secure the glass coverslips to the sample holder.

We machined an aluminum plate to act as a custom sample holder (Fig. 11.2). Alternatively, the coverslips can be secured to an existing sample holder using nonoutgassing metal tape or metal binder clips. Contact your clean room manager to decide which method is best for your evaporator.

2. Evacuate the deposition chamber until a pressure of at least 1×10^{-6} Torr is reached.
3. Deposit 2 nm of Ti at a rate of 1 Å/s.
4. Deposit 6–15 nm of Au at a rate of 1 Å/s or Pt at a rate of 0.1 Å/s.



FIGURE 11.2

Custom-machined coverslip holder for metal deposition. An aluminum slab was machined to deposit metal films onto a large number of coverslips simultaneously using an electron-beam evaporator. The coverslips fit snug into the holes and rest on the 2 mm wide lip.

Since LSL is a photothermal process the thermal properties of the supporting metal film influence the pattern resolution. Important aspects to consider when choosing the metal support layer (Au or Pt) and film thickness are outlined in [Section 11.5](#).

5. After depositing the metal films let the samples cool in the chamber for 30 min.
6. Remove the samples from the deposition chamber.
7. Blow the metalized surfaces with N₂ to remove any surface contaminants.
8. Store the metalized coverslips in plastic Petri dishes wrapped with Parafilm or other container that prevents dust and atmospheric contaminants from adsorbing to the surface.

The metalized coverslips are now ready for alkanethiol functionalization ([Fig. 11.1](#)). Au films undergo Ostwald ripening ([Voorhees, 1985](#)) and Pt films oxidize in ambient conditions ([Petrovykh et al., 2006](#)) and therefore best functionalized immediately. We typically begin the alkanethiol functionalization within 1 day of the metal deposition.

11.1.3.3 Alkanethiol functionalization of metalized coverslips

1. Carefully mark a cross hair pattern in the middle of the metalized coverslip using a ruler and scalpel by very gently dragging the tip of the scalpel across the surface of the metal.

This will provide a reference point and a feature for focusing on the surface for patterning.

2. Blow the metal surface with N₂ to remove any loose metal.
3. Prepare a 4 mM solution of an OEG₆-terminated alkanethiol in 200 proof ethanol. A volume of ~350 μl will be needed for each 35 mm diameter coverslip. In our studies we wanted a passive, protein repulsive, and biologically inert background so we implemented an OEG₆-terminated alkanethiol as the first SAM. An alkanethiol with a different terminal functionality can be used if desired.
4. Insert the metalized coverslip into the functionalization chamber with the metal side up ([Fig. 11.3A](#)).

The functionalization chamber was constructed from two 58 × 76 mm glass slides, a PDMS spacer that acts as a tight seal, and a water filled bottle to supply pressure ([Fig. 11.3B](#)). The PDMS was cured at 60 °C for 2 h in a 60 mm diameter plastic Petri dish. After curing, the plastic Petri dish was carefully broken and the PDMS removed. A hole in the PDMS large enough to house a 35 mm diameter coverslip was created using a biopsy punch, scalpel, or razor blade.

5. Slowly add ~350 μl of the OEG₆ solution to the metalized side of the coverslip using a micropipette.
6. Add the top glass slide to complete the functionalization chamber ([Fig. 11.3A](#)).
7. Add a 1 l bottle filled with water to the top glass slide to act as a weight sealing the chamber tightly ([Fig. 11.3B](#)).

The PDMS will conform to both glass supports acting as a tight seal.

8. Leave the OEG₆ solution overnight, ~16 h, at room temperature.

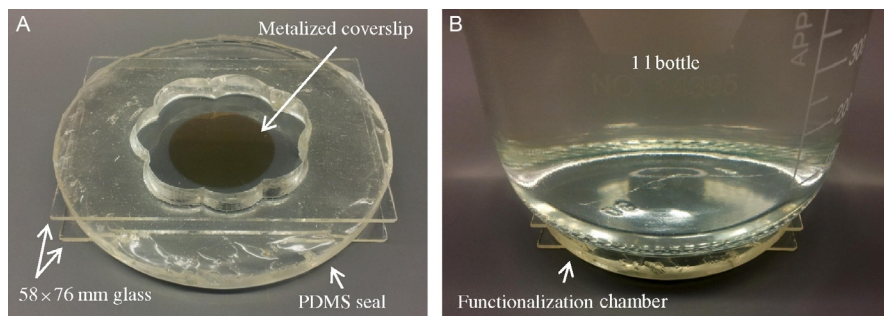


FIGURE 11.3

Functionalization chamber. (A) A functionalization chamber was constructed from two 58×76 mm glass slides and a PDMS ring. (B) A 1 l water filled glass bottle was added to the top of the chamber after assembly to apply pressure to the PDMS ring in order to form a tight seal to prevent solution evaporation during alkanethiol functionalization.

9. Disassemble the functionalization chamber and remove the coverslip.
10. Rinse the coverslip with a stream of 200 proof ethanol for 1 min using a squeeze bottle.
11. Wash the coverslip in a 500 ml beaker filled with 200 proof ethanol for 1 min by gently moving the coverslip back and forth while completely submersed in the ethanol.
Move the coverslip slowly and gently to prevent cracking or breaking.
12. Rinse the coverslip with a stream of 200 proof ethanol for 1 min using a squeeze bottle.
13. Dry the coverslip with a stream of low pressure (≤ 5 psi) N_2 .

The alkanethiol functionalized coverslip is now ready for patterning (Fig. 11.1) and can be stored in a N_2 filled container at $4^\circ C$ protected from light for up to 1 month but we typically pattern the samples within a week to prevent oxidation of the alkanethiol SAM.

11.2 GENERATION OF VIRTUAL MASKS

This section describes two methods for defining pattern configurations. The first method details how to enter the pattern coordinates and dimensions manually using Zeiss AIM software and is recommended for simple pattern geometries. The second method describes how to generate pattern configurations from images using a custom-written MATLAB script. The algorithm exports the image-based pattern coordinates and dimensions as a file recognized by Zeiss AIM software and is recommended when creating patterns with complex geometries.

11.2.1 Equipment

- Zeiss AIM software (Carl Zeiss, Munich, Germany).
- MATLAB (MathWorks, Natick, MA).
- ImageJ (NIH, Bethesda, MD) or other image analysis software.

11.2.2 Method

11.2.2.1 Pattern generation using Zeiss AIM software

1. Acquire an image with each of the objectives ($20\times$ NA0.8 air, $63\times$ NA1.4 oil, etc.) using the same pixel density (512×512 or 1024×1024 , etc.) and laser source (532 nm) that will be used for patterning.
2. Click the “info” button (Fig. 11.4A.1) in the “Image” window (Fig. 11.4A) to determine the pixel size ($\mu\text{m}/\text{pixel}$) for each image acquired in Step 1.

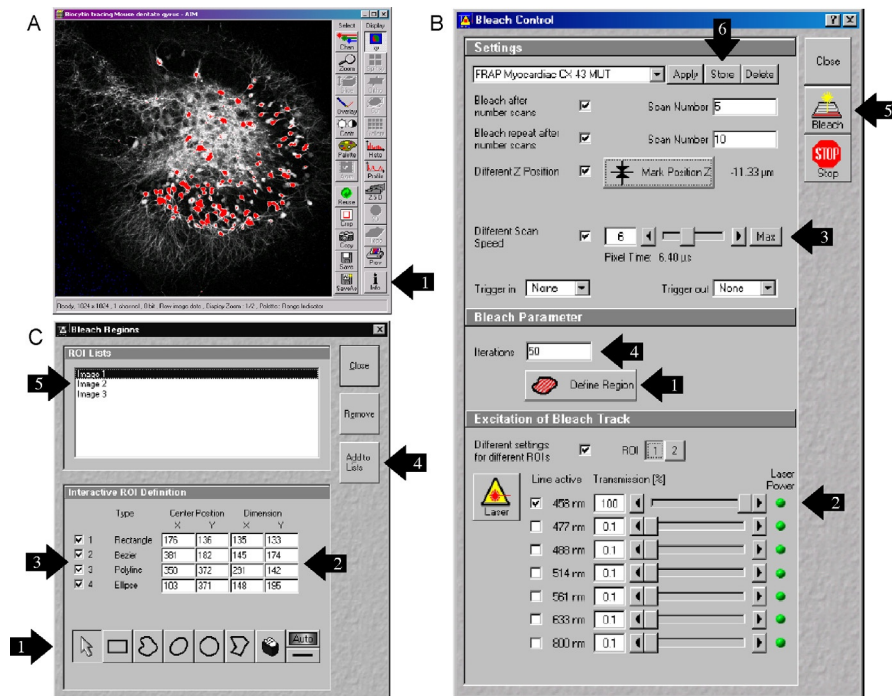


FIGURE 11.4

Zeiss AIM software windows. (A) The “Image” window used to determine the image properties (pixel calibration, viewfield size) for defining pattern dimensions. (B) The “Bleach Control” window used to define and store the patterning settings and to perform test patterns. (C) The “Bleach Regions” window used to define and store regions of interest (ROIs) and pattern configurations.

Courtesy of ZEISS Microscopy.

3. Determine the geometry (location, size, spacing, orientation) of the desired pattern or pattern array and locate the center point of each pattern and its horizontal and vertical dimensions.

You will need to define all coordinates and dimensions in units of pixels. Use the calibration from Step 2 to convert the pattern dimensions to pixels.

4. Open the “Bleach Control” window (Fig. 11.4B).
5. Click the “Define Region” button (Fig. 11.4B.1) in the “Bleach Parameter” section of the “Bleach Control” window (Fig. 11.4B).
6. Using one of the various shapes (rectangle, circle, ellipse, etc.) (Fig. 11.4C.1) available in the “Interactive ROI Definition” section of the “Bleach Regions” window (Fig. 11.4C) to draw the desired pattern in the “Image” window (Fig. 11.4A).

Make sure that the correct image from Step 1 is loaded in the “Image” window (Fig. 11.4A). Higher resolution patterns require a higher NA objective whereas lower resolution patterns can be created with a lower NA objective.

7. Adjust the center point and spatial dimensions of the pattern (Fig. 11.4C.2) in the “Interactive ROI Definition” section of the “Bleach Regions” window (Fig. 11.4C).

It is important to note that the laser scans in the horizontal direction. It is therefore more efficient during patterning to orient the long axis of any elongated patterns in the horizontal direction.

8. Repeat Step 7 for all of the desired patterns in the viewfield.

Each individual pattern will be displayed as a different region of interest (ROI) (Fig. 11.4C.3) in the “Interactive ROI Definition” section of the “Bleach Regions” window (Fig. 11.4C).

9. Once all of the patterns/ROIs are drawn in the proper geometry use the “Add to Lists” button (Fig. 11.4C.4) in the “Bleach Regions” window (Fig. 11.4C) to save the pattern configuration.

11.2.2.2 Image-guided pattern generation using MATLAB

1. Obtain an image that you want to define your pattern configuration (Fig. 11.5A).
2. Convert it to a binary image with the desired pattern features as white (pixel intensity of 256 for an 8 bit image) and the background black (pixel intensity of 0) (Fig. 11.5B).

We typically use the “auto threshold” or “auto local threshold” algorithm in ImageJ to binarize our images. Each image will have to be carefully processed to retain the desired features.

3. Scale, resize, and/or crop the image to match the window size (512×512 , 1024×1024 , etc.) and pixel calibration ($\mu\text{m}/\text{pixel}$) determined in Step 2 of the previous section (Fig. 11.5B).

We typically use ImageJ for these operations.

4. Save the image as a .tif file.
5. Using the custom-written MATLAB scripts convert the .tif file to a .rls file.

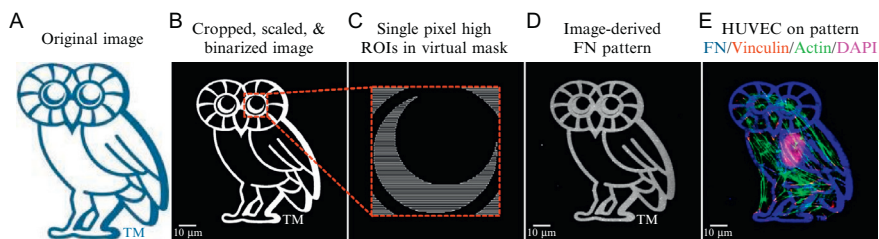


FIGURE 11.5

Image-guided LSL. (A) An image is chosen to define the pattern configuration. (B) The original image is cropped, scaled, and binarized. (C) The binarized image is processed with a MATLAB script to generate a virtual mask that approximates the image features as a mosaic of one pixel high quadrilaterals. (D) The virtual mask guides the laser position and shutter during photothermal patterning. The bare metal patterns are backfilled with a biomolecule, fibronectin in this example, to create an image-derived biomolecular pattern. (E) Cells (HUVECs) were seeded on the patterned surface, cultured for 24 h, then fixed and fluorescently immunolabeled for fibronectin (blue), vinculin (red), actin (green), and the nucleus (magenta). SB = 10 μm.

The complete lines of code for the MATLAB script and the proper procedure for using it are described in detail in the Supporting Information of reference (Culver et al., 2012).

6. Place the .rls file generated by the MATLAB script in the C:\AIMROIS folder on the computer that controls the microscope.

The desired pattern configuration is now saved in the AIM software and can be loaded using the “Bleach Regions” (Fig. 11.4C) or “MultiTime” window (Fig. 11.6A). Additionally, the pattern configuration may be easily modified by simply deleting existing ROIs or adding new ROIs and resaving the configuration. Simple patterns can be drawn by hand and saved in a few minutes while more extensive pattern arrays can take hours. Alternatively, the MATLAB script converts images to the file format used to define the ROI content in Zeiss Aim software. This allows a user to define complex pattern arrays or intricate pattern shapes using any software that allows for binary file output as a .tif file. Additionally, it provides a means to convert any binary image into a pattern configuration composed of single pixel high quadrilateral ROIs (Fig. 11.5C). Due to the ease of use of the MATLAB algorithm almost all pattern configurations are now derived from binary images created in basic image analysis software such as ImageJ.

11.3 SURFACE PATTERNING WITH LSL: GENERAL METHOD AND FABRICATION OF MULTIFACETED SURFACES

The following section describes how to setup and perform patterning using a Zeiss 5LIVE DuoScan laser scanning confocal microscope equipped with Zeiss AIM software. New Zeiss software, ZEN, is now available and includes many of the same

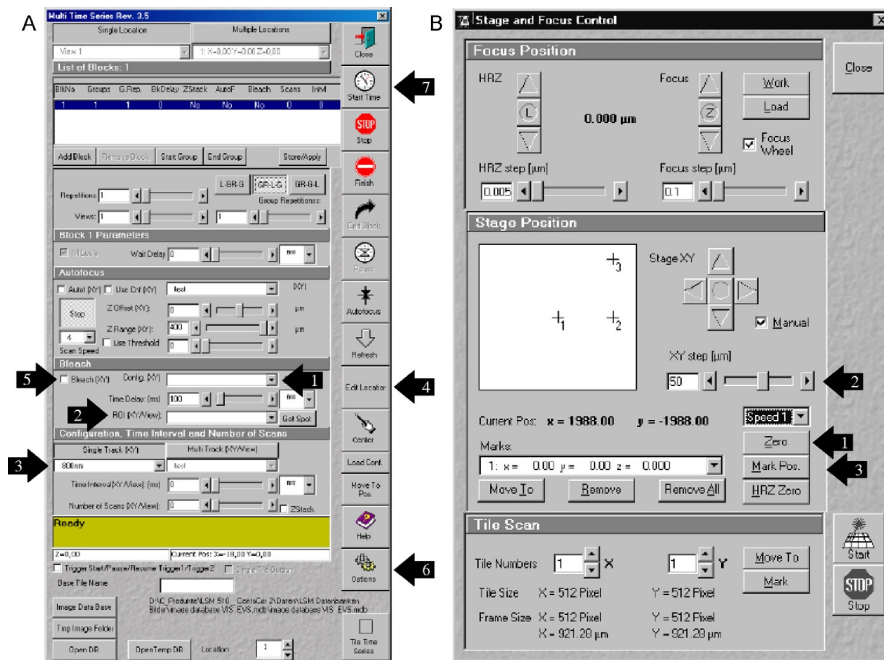


FIGURE 11.6

Zeiss AIM software windows. (A) The “MultiTime” window used to automate the patterning process. (B) The “Stage and Focus Control” window used to locate and mark the desired viewfields where patterning will occur.

Courtesy of ZEISS Microscopy.

functions described in the following section but there may be differences in the location, name, and operation of specific functions. Additionally, patterning can be performed on other models of Zeiss laser scanning confocal microscopes and potentially on non-Zeiss microscopes assuming that the software provides proper control over the scanning parameters as outlined here. The following protocol assumes that you are an experienced user of both confocal microscopes and Zeiss AIM software and does not describe details concerning basic operation of a confocal microscope but does include the details of setting up the software correctly for patterning. Contact an experienced user or a Zeiss representative for basic operational procedure or with any questions concerning software details.

11.3.1 Materials

- Ultrahigh purity nitrogen tank equipped with inline filters (Matheson TriGas, Basking Ridge, NJ).

- Ultrahigh purity nitrogen tank equipped with inline filters and Pasteur pipette or plastic pipette tip (Matheson TriGas, Basking Ridge, NJ).
- Alkanethiol functionalized, metalized, 35 mm diameter, # 1, optical borosilicate circular glass coverslips.
- 200 proof ethanol (VWR, West Chester, PA).
- 70% ethanol.
- Squeeze bottle filled with 200 proof ethanol.
- 500 ml glass beaker.
- HS(CH₂)₁₁(OCH₂CH₂)₃OH (OEG₃, ProChimia, Sopot, Poland. Cat # TH 002-02).
- HS(CH₂)₁₁(OCH₂CH₂)₆GRGD (GRGD, ProChimia, Sopot, Poland. Cat # TH 024-002).
- 1 ml micropipette.
- Human plasma fibronectin (FN, Sigma Aldrich, St. Louis, MO. Cat # F2006-2MG).
- 1-Hexadecanethiol, >95% (HDT, Sigma Aldrich, St. Louis, MO. Cat # 52270-100ML).
- Sterile 60 × 15 mm plastic Petri dishes (VWR, West Chester, PA).
- Sterile PBS.
- Sterile MPH₂O.

11.3.2 Equipment

- Zeiss 5LIVE DuoScan laser scanning confocal microscope equipped with an environmental sample holder, motorized stage, Zeiss AIM software, 532 nm laser source, and the appropriate objectives (20 × NA0.8 air, 63 × NA1.4 oil, etc.) (Carl Zeiss, Munich, Germany).
- Custom-built chamber for alkanethiol functionalization.
 - 2—58 × 76 mm glass slides.
 - PDMS. Sylgard[®] 184 (Dow Corning Corporation, Midland, MI).
 - 1 l bottle filled with water.
- Sterile tissue culture hood.
- Incubator set to 37 °C and 5% CO₂.
- Water bath set to 37 °C.

11.3.3 Method

11.3.3.1 General method

1. Place an alkanethiol functionalized coverslip onto the appropriate sample holder.

We used an environmental sample holder designed to accommodate a 35 mm diameter coverslip that was fit with a tight sealing top that allowed for gas flow into the chamber. These stages are typically used to heat and deliver CO₂ for time-lapse imaging.

2. Connect the gas line that feeds into the top of the chamber to a filtered ultrahigh purity N₂ source.
3. Add the top of the chamber to the base creating a sealed environment.
4. Flow N₂ into the chamber at a pressure of ~3 psi.

Use a high enough pressure to provide a steady stream of N₂ but not so high that the coverslip cracks.

5. Adjust the stage position so that the crosshair on the metal is centered above the objective.

When creating micron-sized features (~1 μm or larger) over large areas we used a 20× NA0.8 air objective. When creating submicron-sized features (~450 nm) we used a 63× NA1.4 oil immersion objective.

6. Use the eyepieces to locate and focus on the crosshair in differential interference contrast (DIC) mode using white light illumination.
7. Find and focus on the crosshair in DIC mode using the 532 nm source in transmitted light mode.

It is important to use the 532 nm laser at a very low power setting (0.1–0.3% transmission) and fastest scan speed. The 532 nm laser can induce local heating of the metal film and desorption of the alkanethiol. To prevent this from occurring during focusing and alignment deliver the least amount of energy to the sample possible using very low laser power, fast scan speeds, and by focusing quickly. The gain and brightness/contrast will have to be adjusted to achieve an acceptable signal. The potential of using alternative wavelengths not adsorbed by the metal film for focusing purposes is outlined in [Section 11.5](#).

8. After focusing on the crosshair move to a new viewfield and refocus.

The metal film will typically have some structure or small defects that can be used to refocus in the new viewfield.

9. Save the settings used to focus on the sample in the “Scan Control” window.
10. Open the “Bleach Control” window ([Fig. 11.4B](#)).
11. Click on the “Define Region” button ([Fig. 11.4B.1](#)) in the “Bleach Parameter” section of the “Bleach Control” window ([Fig. 11.4B](#)).
12. Locate the saved pattern configuration in the “ROI Lists” section ([Fig. 11.4C.5](#)) of the “Bleach Regions” window ([Fig. 11.4C](#)).
13. Click on the desired pattern configuration in the list ([Fig. 11.4C.5](#)) to load it.

Be patient, complex pattern configurations containing hundreds or more individual ROIs ([Fig. 11.5B](#) and [C](#)) can take minutes to load. Patterns created manually and saved and those developed using the MATLAB algorithm that were pasted into the C:\AIMROIS folder will appear in the “ROI Lists” ([Fig. 11.4C.5](#)).

14. Once the pattern configuration has loaded, define the pattern settings (active laser source and % transmission ([Fig. 11.4B.2](#)), scan speed/pixel dwell time ([Fig. 11.4B.3](#)), number of iterations ([Fig. 11.4B.4](#))) in the “Bleach Control” window ([Fig. 11.4B](#)).
15. Run a series of test patterns using the “Bleach” button ([Fig. 11.4B.5](#)) in the “Bleach Control” window ([Fig. 11.4B](#)) to determine the optimal settings.

The appropriate variables to adjust are % transmission, scan speed/pixel dwell time, and number of iterations. The general idea is to pattern as quickly as possible: high power, high scan speed, and low number of iterations but to not provide so much energy that the metal film is damaged (Fig. 11.7). Below are typical settings used based on the supporting metal film and objective:

- 10 nm Au film using a 20 × NA0.8 air objective:
532 nm laser, 100% transmission (~4.1 mW), 1.61 μs pixel dwell time, and 125–500 iterations.
- 10 nm Au film using a 63 × NA1.4 oil immersion objective:
532 nm laser, 100% transmission (~3.6 mW), 1.61 μs pixel dwell time, and 125–500 iterations.
- 8 nm Pt film using a 63 × NA1.4 oil immersion objective:
532 nm laser, 30–40% transmission (~1.4 mW), 1.27 μs pixel dwell time, 2–5 iterations.

16. Image the test patterns in transmitted DIC mode to determine the best settings. Patterns that appear blackish or darker in color indicate damage to the supporting metal film (Fig. 11.7A and B). Whitish or lighter colored patterns indicate regions where the alkanethiol was removed without damaging the metal film (Fig. 11.7A and C).

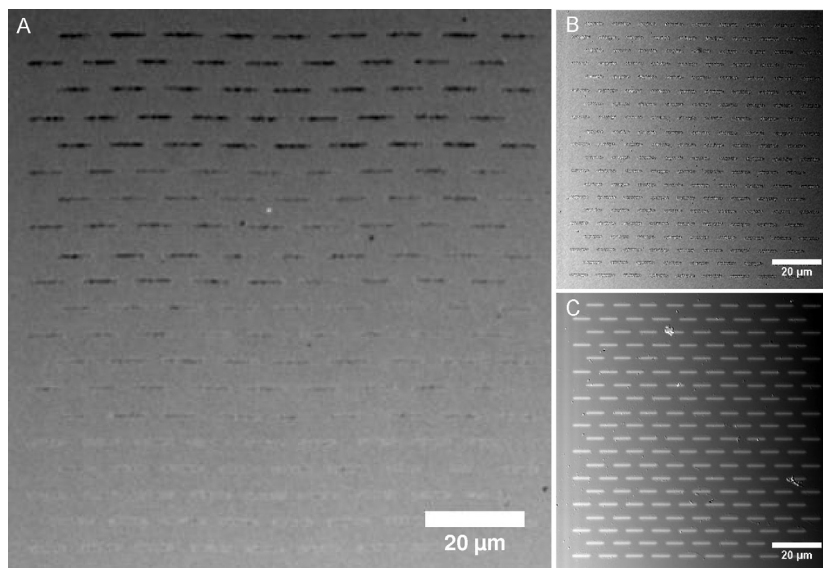


FIGURE 11.7

DIC images of pattern arrays to determine the proper patterning settings. (A) A DIC image of a series of $1 \times 8 \mu\text{m}$ test patterns fabricated with a 20 × NA0.8 objective with decreasing energy from top to bottom. The darker colored or black patterns in the DIC images (top part of (A) and all of (B)) indicate where too much energy was delivered and subsequent damage to the metal film. The lighter colored or whitish patterns (bottom of (A) and all of (C)) indicate alkanethiol desorption without damage to the metal film using the proper settings. SB = 20 μm.

17. Store the best patterning setting (laser power, dwell time/scan speed, and number of iterations) using the “Store” button (Fig. 11.4B.6) in the “Settings” section of the “Bleach Control” window (Fig. 11.4B).
18. Open the “MultiTime” macro (Fig. 11.6A).
19. Load the stored bleach/patterning settings using the “Config.” drop down menu (Fig. 11.6A.1) in the “Bleach” section of the “MultiTime” window (Fig. 11.6A).
20. Load the saved ROI configuration that contains the features for patterning using the “ROI” drop down menu (Fig. 11.6A.2) in the “Bleach” section of the “MultiTime” window (Fig. 11.6A).
21. Load the saved imaging configuration using the drop down menu (Fig. 11.6A.3) in the “Configuration, Time Interval, and Number of Scans” section of the “MultiTime” window (Fig. 11.6A).

This step only has to be completed if imaging of the patterns is desired.

22. Focus on the sample in the first viewfield.
23. In the “Stage and Focus Control” window (Fig. 11.6B) zero the stage position using the “Zero” button (Fig. 11.6B.1) in the “Stage Position” section.
24. Set the xy translation using the “XY step” slider (Fig. 11.6B.2) to the desired step size.

We often used one viewfield or larger for the step size depending on the pattern type and purpose. The viewfield size can be determined by clicking the “info” button (Fig. 11.4A.1) in the “Image” window (Fig. 11.4A). The geometry of the pattern array must be known to determine the appropriate step size between viewfields, especially for continuous pattern arrays. We optimized our pattern geometry so that the stage translated exactly one viewfield to create a continuous array.

25. Using the “Stage and Focus Control” window (Fig. 11.6B) move to the desired patterning locations, refocus on the surface, and click the “Mark Pos.” button (Fig. 11.6B.3) in the “Stage Position” section.
26. Once all of the desired positions have been marked, load the positions into “MultiTime” (Fig. 11.6A) using the “Edit Location” button (Fig. 11.6A.4).

At this point the bleaching parameters and locations for patterning have all been loaded into “MultiTime” and the system is ready for patterning.

27. Make sure that the “Bleach” box (Fig. 11.6A.5) is checked in the “Bleach” section of the “MultiTime” window (Fig. 11.6A).

If desired you can image each viewfield for patterning either before, after, or both. Alternatively, you can setup the system not to acquire any images. These adjustments can be made using the “Options” button (Fig. 11.6A.6) in the “MultiTime” window (Fig. 11.6A). We often elected to not acquire any images in order to reduce the patterning time.

28. Click the “Start Time” button (Fig. 11.6A.7) in the “MultiTime” window (Fig. 11.6A).

At this point the desired pattern configuration will be scanned at each location using the loaded bleach settings. It takes time to master the software and some practice is required to get the “MultiTime” settings correct. Contact an experienced confocal user or a Zeiss representative if help is needed. Depending on the number of ROIs in the pattern configuration, scan speed/pixel dwell time, number of iterations,

and number of viewfields to be patterned the time to execute the “MultiTime” patterning can be as quick as seconds or many hours.

29. After the “MultiTime” program has finished remove the sample from the stage.
30. Blow the patterned surface with low pressure (≤ 5 psi) N_2 .
31. Rinse the coverslip with a stream of 200 proof ethanol for ~ 1 min using a squeeze bottle.
32. Rinse the coverslip in a beaker filled with 200 proof ethanol by gently moving the coverslip back and forth while completely submersed in the ethanol.
33. Rinse the coverslip with a stream of 200 proof ethanol for ~ 1 min using a squeeze bottle.
34. Dry the sample with low pressure (≤ 5 psi) N_2 .

The bare, metal patterns are now ready for functionalization with either a 2nd alkanethiol (Fig. 11.1) or with a protein (Fig. 11.5D). The functionalization should be performed immediately to prevent the OEG₆ alkanethiol from migrating into the patterned regions. The following section describes the functionalization procedure for the fabrication of multifaceted, micropatterned surfaces (Fig. 11.8). If a single functionalization is desired then perform it now without a 2nd patterning session.

11.3.3.2 Fabrication of multifaceted patterned surfaces

This section describes the fabrication of multifaceted surfaces containing two independent pattern arrays of (1) a GRGD-terminated alkanethiol SAM and (2) human plasma fibronectin adsorbed to a HDT SAM with each biomolecule confined to its own pattern array (Fig. 11.8). The conditions were optimized to prevent FN adsorption to the GRGD patterns and to provide a hydrophobic surface for FN adsorption. The protocol can be modified to allow for multiple alkanethiols or for multiple proteins.

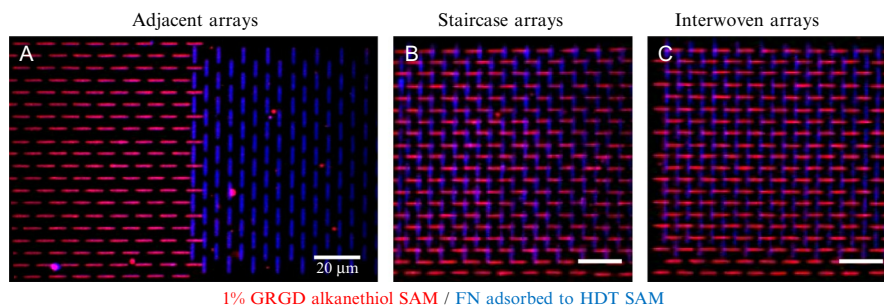


FIGURE 11.8

Example configurations of multifaceted, micropatterned surfaces. $1 \times 8 \mu\text{m}$ ellipses of a 1% GRGD-terminated alkanethiol in OEG₃ (red) patterned either (A) adjacent to, (B) in a staircase configuration, or (C) interwoven with ellipses of fibronectin adsorbed to a HDT SAM (blue). SB = $20 \mu\text{m}$.

1. Prepare a 1.98 mM solution of an OEG₃-terminated alkanethiol in 200 proof ethanol.
2. Prepare a 0.02 mM solution of a GRGD-terminated alkanethiol in 200 proof ethanol.
3. Prepare a 1% GRGD in OEG₃ solution by mixing equal volumes of the solutions from Steps 1 and 2.

A desired percentage of GRGD in OEG₃ can be prepared. We chose 1% GRGD in OEG₃ because it was a high enough concentration to induce adhesion formation yet low enough to prevent protein from adsorbing. We elected to use an OEG₃-terminated alkanethiol rather than OEG₆ to make the GRGD more accessible for integrin ligation (Houseman & Mrksich, 2001).

4. Insert the metalized coverslip into the functionalization chamber with the metal side up (Fig. 11.3A).
5. Slowly add ~350 μ l of the 1% GRGD solution using a micropipette.
6. Add the top glass slide to complete the functionalization chamber (Fig. 11.3A).
7. Add a 1 l bottle filled with water to the top glass slide to act as a weight sealing the chamber tightly (Fig. 11.3B).

The PDMS will conform to both glass supports acting as a tight seal.

8. Leave the alkanethiol solution for at least 1 h at room temperature.
9. Disassemble the functionalization chamber and remove the coverslip.
10. Rinse the coverslip with a stream of 200 proof ethanol for ~1 min using a squeeze bottle.
11. Rinse the coverslip in a beaker filled with 200 proof ethanol by gently moving the coverslip back and forth while completely submersed in the ethanol.
12. Rinse the coverslip with a stream of 200 proof ethanol for ~1 min using a squeeze bottle.
13. Dry the sample with N₂.
14. Place the sample onto the stage of the laser scanning microscope in the correct position for patterning.

If the 2nd pattern array needs to align to the 1st array, make sure that the sample is positioned correctly so that the 2nd array is in the correct position. The 1st array will be visible in DIC mode (Fig. 11.7C) and used to align the 2nd pattern configuration. Since the laser only scans horizontally, the sample may have to be turned 90° relative to its position during the 1st round of patterning to create interwoven arrays (Fig. 11.8C) when using elongated pattern shapes.

15. Repeat the patterning steps outlined in Section 11.3.3.1.

There is no need to retest the patterning conditions or to resave the settings once they have been optimized for this sample.

Once the sample has been patterned, rinsed, and dried the 3rd functionalization can take place. This can either be a 3rd alkanethiol, a protein, or both as outlined below.

16. Prepare a 2 mM solution of HDT in 200 proof ethanol.
17. Insert the metalized coverslip into the functionalization chamber with the metal side up (Fig. 11.3A).

18. Slowly add ~ 350 μl of the 2 mM HDT solution.
19. Add the top glass slide to complete the functionalization chamber (Fig. 11.3A).
20. Add a 1 l bottle filled with water to the top glass slide to act as a weight sealing the chamber tightly (Fig. 11.3B).
21. Leave the 2 mM HDT for 1 h at room temperature.

Since the HDT contains a smaller terminal group it will form a SAM faster. Additionally, since it is a shorter, smaller molecule if left too long it can displace the OEG- and GRGD-terminated alkanethiols.

22. Disassemble the functionalization chamber and remove the coverslip.
23. Rinse the coverslip with a stream of 200 proof ethanol for ~ 1 min using a squeeze bottle.
24. Rinse the coverslip in a beaker filled with 200 proof ethanol by gently moving the coverslip back and forth while completely submerged in the ethanol.
25. Rinse the coverslip with a stream of 200 proof ethanol for ~ 1 min using a squeeze bottle.
26. Dry the coverslip with N_2 .

The 2nd pattern array is hydrophobic after HDT functionalization and ready for protein adsorption. The wettability of the patterns can be tuned using alkanethiols presenting less hydrophobic or hydrophilic terminal groups. The following steps need to take place in a sterile tissue culture hood using proper sterile technique.

27. Immerse the coverslip into a sterile Petri dish filled with 70% ethanol for ~ 20 min.
28. Prepare a solution of FN in PBS at the desired concentration (10–25 $\mu\text{g}/\text{ml}$ is typical) and warm to 37°C in a water bath.

Follow the manufacturer's instructions for creating the FN solution.

29. Remove the coverslip from the 70% ethanol solution and allow it to air dry.
30. Rinse the sample thoroughly with sterile MPH_2O and allow it to air dry.
31. Place the coverslip metal side up in a sterile Petri dish.
32. Add ~ 500 μl of FN solution to the coverslip.
33. Place the Petri dish in a 37°C incubator for 30 min.
34. Remove the coverslip from the Petri dish and rinse it three times with 25 ml of sterile PBS.
35. Submerge the coverslip in a sterile Petri dish filled with PBS.

The multifaceted, patterned surface is ready for characterization or cell seeding. The protocol described the use of a peptide-conjugated alkanethiol SAM, a hydrophobic alkanethiol SAM, and a protein (Figs. 11.1 and 11.8). This protocol can be modified and optimized to allow for patterning of multiple peptides through the use of different peptide-conjugated alkanethiols or for multiple proteins through passive adsorption and proper blocking (Doyle, 2009; Doyle et al., 2009).

11.4 CELL DEPOSITION

This section describes the process of seeding human umbilical vein endothelial cells (HUVECs) (Fig. 11.5E) onto patterned surfaces.

11.4.1 Materials

- HUVECs (Lonza, Basel, Switzerland. Cat # C2519A).
- Endothelial basal media (EBM-2, Lonza, Basel, Switzerland).
- Endothelial growth media (EGM-2, Lonza, Basel, Switzerland. Cat # CC-3162).
- Sterile T-25 tissue culture flasks (VWR, West Chester, PA).
- Sterile PBS.
- 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) (Lonza, Basel, Switzerland).
- Sterile 60 × 15 mm plastic Petri dishes (VWR, West Chester, PA).

11.4.2 Equipment

- Incubator set to 37 °C and 5% CO₂.
- Sterile tissue culture hood.
- Centrifuge.
- Hemocytometer.
- Standard phase-contrast microscope for cell imaging.

11.4.3 Method

1. Culture HUVECs in FN-coated (10 µg/ml for 30 min) T-25 tissue culture flask in EGM-2 at 37 °C and 5% CO₂ until ~80% confluent.
2. Aspirate the media and rinse the flask three times with warm PBS.
3. Detach the cells using 3 ml of 0.25% trypsin and 1 mM EDTA in PBS at 37 °C for 3 min.
4. Collect the cells and centrifuge at 230 × *g* for 10 min.
5. Resuspend the cells in 1.5 ml of EBM-2 basal media
6. Measure the cell density using a hemocytometer.
7. Suspend the desired cell density, ~10,000 cells/ml, using 80,000 cells per sample in 8 ml of EBM-2 basal media.
8. Remove the patterned coverslip from the PBS and place in a fresh sterile Petri dish.
9. Slowly add the 8 ml of cells in EBM-2 to the Petri dish with the patterned coverslip.
10. Place the Petri dish in an incubator at 37 °C and 5% CO₂.
11. After 4 h, check the surface with a microscope to determine if the cells are attached and spreading.
12. Very gently rinse the surface with EBM-2 basal media to remove any loosely bound cells.
13. Exchange the EBM-2 for EGM-2.
14. Culture the cells for the desired time and perform time-lapse imaging or fixation and immunolabeling as desired.

For most applications we perform time-lapse image 4 h after switching to EGM-2 and fixation and fluorescent immunolabeling (Fig. 11.5E) 4–48 h.

11.5 DISCUSSION

11.5.1 Metal film influence on patterning resolution and alkanethiol SAM stability

Since LSL is a photothermal process the thermal properties of the supporting metal film influence the ability to generate and transfer heat to the Au–thiol bond for thermal scission and subsequent desorption and have a significant influence on the patterning resolution. In general, films with higher conductivity will lead to decreased resolution due to lateral spreading of the generated heat through the film and subsequent alkanethiol desorption outside of the focal diameter (Slater et al., 2011). To combat this issue one can pattern with finely tuned laser parameters that induce a temperature profile that reaches a local maximum of ~ 212 °C and therefore confine alkanethiol desorption only to the focal diameter. In theory, this also provides a means for subdiffraction limited patterning but in practice it is very difficult to implement. Fluctuations in laser power, local changes in film properties, or more influential, focus drift, make this a difficult endeavor to undertake over large surface areas although we have demonstrated the ability to pattern sub-Airy disk sized patterns over small regions (Slater et al., 2011). An alternative route is to change the thermal conductivity of the metal film by controlling its thickness. The in-plane thermal conductivity of Au films can decrease by a factor of approximately two when the film thickness decreases from 30 to 6 nm (Feng, Li, & Zhang, 2009). Accordingly, decreasing the film thickness allows for increased patterning resolution when using the same patterning parameters (Slater et al., 2011). Another method to decrease the thermal conductivity is to replace Au with Pt. Pt has an order of magnitude lower thermal conductivity than Au and therefore inherently provides increased patterning resolution.

While these approaches enhance patterning resolution there is a trade-off. Thicker (~ 15 nm) Au films are more uniform with fewer defects and therefore support stable alkanethiol SAMs for longer times. Cells can be confined to protein patterns surrounded by an OEG-terminated alkanethiol SAM for 10 days or more. As the Au film thickness is reduced, the morphology of the film is changed, is no longer as uniform, and the presence of small defects increases. While these thin films provide increased patterning resolution they do not support stable alkanethiol SAMs for long periods of time in our experience and we are often limited to 24–48 h for experiments. Similarly, while Pt inherently provides increased resolution its increased oxidation potential increases the oxidation rate of the thiol groups in alkanethiols and therefore the SAMs are not as stable for long periods of time (Petrovykh et al., 2006). Again we are limited to about 24–48 h for experiments. This trade-off between resolution and SAM stability must be taken into consideration when designing your experiments.

11.5.2 Focusing on the surface

In the protocol outlined here we described the use of a 532 nm laser source to focus on the surface when defining the viewfields for patterning. We cautioned that the 532 nm laser must be used at low power and a very fast scan speed for this procedure.

Since the metal film absorbs light at this wavelength local heating can be induced and therefore desorb some of the alkanethiol SAM. We did not find this to be an issue for thicker Au films since they need so much energy to induce heating but it can be an issue for thinner Au and Pt films. In these cases we were careful to focus quickly and to close the laser shutter when not in use. Alternatively, it would be much better to focus on the surface with a red-shifted laser source, 633 nm for example, where the metal films display increased scattering and lower absorption. For this to be a viable alternative one must assure that the focal planes of the two lasers match exactly. Due to how the lasers are coupled to the scan head on the 5LIVE DuoScan it was very difficult to maintain the focal planes of the 532 and 633 nm laser properly aligned and therefore we utilized the 532 nm source for both focusing and patterning. Other Zeiss models do not have this inherent problem and through proper maintenance the focal planes of multiple lasers are easily kept aligned. If using one of these other models we suggest utilizing a red-shifted source for focusing.

11.5.3 Biomolecule immobilization

In this protocol we outlined functionalization with both a peptide-conjugated alkanethiol and through passive adsorption of protein. Other procedures for biomolecule immobilization are also available although not discussed in detail. One could envision using only peptide-conjugated alkanethiols for each backfill functionalization step. This would provide better control over the orientation of the biomolecule, as defined by its conjugation procedure to the alkanethiol, and potentially allow for longer term storage. Additionally, this approach would allow for a final functionalization with an OEG₆ alkanethiol as the last step in the procedure to backfill any areas where unwanted desorption may have occurred during fabrication. One could also envision the use of alkanethiols with varying terminal functionalities to bind specific residues on biomolecules in order to sort multiple biomolecules after patterning. Alkanethiols with a variety of reactive terminal groups are commercially available (maleimide, NHS esters, biotin, azides, etc.) and would be beneficial for on chip sorting and conjugation. Alternatively, one could go the other route and use only passive adsorption of full proteins in each backfill step if proper blocking and rinsing measures were taken to avoid unwanted protein–protein interactions (Doyle, 2009; Doyle et al., 2009).

GENERAL CONCLUSIONS

We presented a detailed protocol for the fabrication of multifaceted, patterned surfaces and for image-guided patterning using LSL. The ability to pattern multiple biomolecules with subcellular resolution with each molecule confined to its own pattern array provides a mean to decouple the influences of matrix architecture and matrix composition on cell fate and should be useful for many studies. The ability to easily and quickly generate complex pattern configurations from user-chosen images, to

modify patterns “on the fly” with little to no lead time, to scale image-based patterns to various sizes, and to fabricate pattern arrays provides a high-throughput means to test microenvironmental influences on cell behavior.

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