Chapter 12

Controlled Confinement of DNA at the Nanoscale: Nanofabrication and Surface Bio-Functionalization

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Abstract

Nanopatterned arrays of biomolecules are a powerful tool to address fundamental issues in many areas of biology. DNA nanoarrays, in particular, are of interest in the study of DNA–protein interactions and for biodiagnostic investigations. In this context, achieving a highly specific nanoscale assembly of oligonucleotides at surfaces is critical. In this chapter, we describe a method to control the immobilization of DNA on nanopatterned surfaces; the nanofabrication and the bio-functionalization involved in the process will be discussed.

Key words: DNA, Self-assembly, Nanoscale, Nanotechnology, Fluorescence microscopy

1. Introduction

The ability to control the placement of biomolecules on surfaces with nanometer resolution is of great interest to the study of biological events at the single molecule level, and provides a platform for the development of biosensing devices with unparalleled sensitivity (1–9).

In particular, the confinement of DNA on surfaces has gathered a great deal of interest, as it can be employed for bioanalytical (genomic) studies and can be used to drive the self-organization of biological (e.g., proteins) and inorganic (e.g., nanoparticles) moieties with nanometer resolution (10–17).

Various chemical strategies have been employed to immobilize DNA on surfaces, ranging from electrostatic interactions, formation of (thiolated) self-assembled monolayers (SAMs), direct

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covalent attachment, and through biotin–streptavidin interaction (18–24). Different fabrication methods have been used to control such immobilization in order to generate micro- and nanoscale DNA features: contact printing (25–27), AFM-based methods (28), and nanopipette deposition (29) are among the most notable examples of the different approaches pursued.

In this chapter, we present a strategy merging top-down nanofabrication techniques with bottom-up self-assembly, to control the confinement of DNA molecules on substrates with nanometer resolution.

We will first describe the fabrication procedure employed to nanopattern glass substrates with Au/Pd nanodots. This consists of a direct electron-beam (e-beam) writing step that creates nanometer-scale voids in a resist polymer spun on the glass substrate; a subsequent metal evaporation fills the voids with Au/Pd and produces an array of sub-50-nm metal dots in the desired geometry (defined by the e-beam writing).

The Au/Pd nanodots so produced can be used as functional regions on the substrates for the controlled confinement of DNA. We will here describe a biotin–streptavidn-based functionalization methodology to immobilize oligonucleotide chains on the so-prepared nanopatterned surface (21, 22, 24, 30–32). The metal nanodots allow for the formation of SAMs of thiolated alkanes presenting biotin end groups, which can be used for the subsequent immobilization of streptavidin. Employing such a strategy, we immobilize double-stranded DNA (dsDNA) on every fabricated (and properly functionalized) nanodot. This functionalization procedure allows for the formation of non-sterically hindered DNA nanodomains at surfaces; a homogenous surface packing density can be envisioned, an advantage over thiolated DNA SAMs (20).

We verify the validity of our approach by EPI-fluorescence microscopy imaging.

2. Materials

2.1. Metal on Glass Nanopattern Fabrication

In our laboratory, we have employed the following instrumentation: a CEE Brewer 100 resist spinner (Brewer Science), an electron-beam writing system (FEI, with Nabity NPGS stage writing control), and an electron beam evaporator (Semicore).

1. Glass coverslips, 22 mm × 22 mm, No. 1.5 (Corning).
2. Acetone, HPLC grade.
3. Ethanol, 200 proof.
4. Sulfuric acid (H_2SO_4), concentrated.

5. Hydrogen peroxide (H_2O_2), 30% concentration. Store at 4°C.
6. 7× Cleaning solution (MDBio).
7. Polymethylmethacrylate (PMMA) photoresist: 495K and 25K molecular weight (MW), A3 concentration for 25K, A2 concentration for 495K (Microchem Corp.); store in the dark.
8. Aquasave conductive coating (Mitsubishi).
9. Isopropanol (IPA), HPLC grade, 99.8%.
10. Tweezers, round tip, stainless steel (SPI supplies).
11. Gold/palladium 60/40%, pellets (Plasmaterials).
12. Titanium (Plasmaterials).

2.2. Functionalization of Nanopatterned Surfaces

A Harrick PDC-32G plasma cleaner was used in our laboratory.

1. Toluene anhydrous, 99.8%.
2. Ethanol anhydrous, >99.5% (200 proof).
3. Acetic acid, glacial ACS reagent grade.
4. Ethanol, ACS reagent grade.
5. Acetone, ACS reagent grade.
6. Gibco™ Dulbecco's phosphate buffer saline (PBS) 1× (no magnesium, no calcium, 2.7 mM potassium chloride, 0.14 M sodium chloride, 1.5 mM potassium phosphate, and 8 mM sodium phosphate, pH 7.4), Invitrogen; store at room temperature (RT).
7. ThermoScientific BupH™ PBS (TPBS) (0.1 M sodium phosphate and 0.15 M sodium chloride, pH 7.2); store at RT.
8. Deionized (DI) Millipore water (resistivity of 18 MΩcm).
9. HS–$(CH_2)_{11}$–$(C_2H_6O_2)_3$–OH and HS–$(CH_2)_{11}$–$(C_2H_6O_2)_3$–biotin (ProChimia); store at –20°C.
10. Polyethylene glycol (PEG)-silane, 5,000 MW (mPEG5000) (LaysanBio); store at –20°C.
11. Glass syringes, metal needles for the anhydrous solvents (Popper).
12. Teflon mini-rack (Invitrogen).
13. 6-Well plates (Falcon).
14. Parafilm.
15. Streptavidin (Invitrogen); store at –20°C.
16. Chicken egg albumin (Sigma); store at 4°C.
17. Oligonucleotides (IDT): one 20-mer with a biotin functional group at the 5′ position (5′-/52-Bio/GTC ACT TCA GCT GAG ACG CA-3′) and the complementary strand with a Cy3 fluorophore at the 5′-end (5′-/5Cy3/TGC GTC TCA GCT GAA GTG AC-3′); store at 4°C wrapped in aluminum foil.

2.3. Microscopy

In our laboratory, epifluorescence microscopy was performed on an inverted microscope, Olympus IX81 (Olympus) equipped with a Cascade II, 512×512 pixel CCD camera (Photometrics).

1. Cloning ring (Sigma/Aldrich).
2. High vacuum grease (Dow Corning).

3. Methods

Throughout the steps of the method described here, a great deal of attention should be focused on the cleanliness of both the laboratory working environment and the materials and tools employed.

Because of the nanometer scale of the features fabricated (and functionalized), we recommend carrying out the fabrication steps (Subheading 3.1) in an ultraclean environment, such as a clean room. Furthermore, in order to obtain a successful bio-functionalization (Subheading 3.2) of the fabricated nanopatterns, it is important to carry out the procedure rapidly with as short a time lapse as possible between steps. Moreover, all glassware and tweezers used must be dry, preferably stored in an oven at approximately 70°C, and cooled in air prior to use.

It should be noted that in order to verify the validity of our approach (i.e., the controlled DNA functionalization of nanopatterned surfaces), we have used a biotinylated and Cy3-labeled dsDNA. We have hybridized in solution, prior to the attachment to the surface, a biotinylated oligonucleotide (20-mer chain) and its complementary oligonucleotide labeled with a Cy3 fluorophore. (In Subheading 4, we report the alternative procedure to immobilize biotinylated single-stranded DNA).

3.1. Metal on Glass Nanopattern Fabrication

Cleaning is absolutely critical to the success of the procedure. Any contamination, even nanoscopic, not removed prior to patterning will result in defects in the surface passivation (see Note 1).

3.1.1. Preparation and Cleaning

1. Prepare piranha solution ($^1/_3$ volume of H_2O_2 plus $^2/_3$ of H_2SO_4).
2. Starting with a new glass coverslip, sonicate in ethanol for 2 min.
3. Blow dry with a stream of inert gas (Ar or N_2) (see Note 2).
4. Dilute 7× detergent 1:4 with deionized (DI) water and bring to boiling temperature on a hot plate at 200°C (goes from cloudy to clear).
5. Immerse the slide for 2 min in boiling solution and rinse with DI water for 10 min.
6. Immerse in piranha solution and let soak for 5 min.

7. Take the slide out of the piranha solution and rinse it for 10 min with DI water.
8. Rinse with ethanol.
9. Blow dry with a stream of inert gas (Ar or N_2) (see Note 3).

   It is now possible to proceed to resist deposition.

3.1.2. Resist Deposition

Again, cleanliness is paramount here. All work should be performed in a clean room, class 10,000 or better. A bilayer of higher MW resist is spun on top of a lower molecular weight resist to aid with the subsequent metal deposition and liftoff (discussed later). The high MW top layer has a slightly different dose curve and will develop with a narrower opening, creating an overhang, which ensures proper liftoff (see Note 4).

1. Preheat a hot plate to 180°C and place cleaned samples on the hot plate for 1 min to force off any adsorbed water molecules.
2. Let cool for 10 s before placing the sample on a resist spinner chuck (see Note 5).
3. Spin lower MW resist (25K) first at 4,000 rpm for 45 s, use a ramp rate of 1,000 rpm/s (see Note 6).
4. Bake for 5 min at 180°C on a hot plate.
5. Let cool for 10 s before placing the sample on the resist spinner chuck.
6. Spin higher molecular weight resist (495K) as top layer at 4,000 rpm for 45 s, use a ramp rate of 1,000 rpm/s.
7. Bake for 5 min at 180°C.
8. Let cool for 10 s before placing on the resist spinner chuck.
9. Spin on the Aquasave conductive discharge layer at 1,000 rpm for 45 s at a ramp rate of 300 rpm/s. It is important to make sure to dab any droplets of Aquasave at the edges (particularly corners) so that the sample is completely dry before being placed in an electron-beam writer.

   It is now possible to proceed to the e-beam writing step.

3.1.3. Electron-Beam Pattern Writing

An electron-beam writing system is an SEM that controls beam shuttering and position to generate patterns from CAD files. Process testing will be necessary to determine optimal doses for generating the desired features (see Note 7). We use a pattern which is 50 μm by 50 μm, and consists of 1 μm register squares spaced every 10 μm, with sub-50-nm dots filling every 2 μm between them. This ensures that each individual dot is optically resolvable and discrete once functionalized and imaged with a fluorescence microscopy (see Note 8).

1. Load samples in the e-beam writer, making sure that a good top contact is made with the mounting clips.
2. Pump down chamber. When pressure has reached or is below the specified value (in our case, 5×10^{-6} Torr), turn the beam on; use an acceleration voltage of 30 kV.
3. Check the beam currents of the spot sizes to be written within the Faraday cup. It is necessary to check beam currents each time in the Faraday cup, as currents drift over time.
4. Update the run files with the most recent beam currents to ensure that the doses are consistent.
5. Check gun tilt and stigmation, and adjust these until both are minimized (see Notes 9–11).
6. Move to the sample. Four-point focus (see Note 11) must be taken to correct for sample tilt. No sample is perfectly level, resulting in loss of focus over the writing area. Focus on the surface of the sample at four points around the region to be written, registering the points with the control software. A plane is then fit to these four points, and the focal depth interpolated and adjusted as the pattern is written (see Notes 10 and 11).
7. With all the steps taken to ensure proper focus and minimal stigmation over the area of the pattern, move to a position inside the four-point focus region.
8. Engage automated stage and beam control, and execute pattern writing in software.
9. After writing, it is necessary to remove the discharge layer, Aquasave. Rinse with DI water until all Aquasave is visibly gone.
10. Then blow dry with a stream of inert gas (Ar or N_2).

    The samples are now ready for development.

3.1.4. PMMA Development

A cold ultrasonic development process is used to achieve nanometer-scale features. The cold development sharpens resist contrast, resulting in the smallest possible features from the exposed regions.

1. Place rinsed and dry sample in a solution of 1:3 H_2O:IPA at 4°C. Sonicate for 1 min in a water bath sonicator at 4°C (see Notes 12 and 13).
2. Quickly remove the sample and immerse in 100% IPA at room temperature to halt development (see Note 14).
3. Blow dry with a stream of inert gas (Ar or N_2).
4. Examine pattern: large alignment marks should be visible; check further in an optical microscope at magnifications of 25–100×. The micron-sized features should be visible.

   It is now possible to proceed to the metal deposition step.

3.1.5. Metal Deposition

In our laboratory, metal deposition is performed in a Semicore electron beam evaporator. A focused beam of electrons is used to heat a target material held in a crucible. As opposed to a thermal evaporator, the heating is highly localized to the surface and allows for precise control of the thickness (at the Ångström level). The sample is held above the target some distance away, allowing for a highly directional and uniform material flux onto the surface.

1. Place the developed samples on the sample holder in the evaporator.
2. Pump the system to less than the suggested threshold pressure and begin deposition procedure.
3. Evaporate a 1-nm adhesion layer of titanium. This is necessary for the adhesion of the Au/Pd to the glass.
4. Next, deposit 3 nm of Au/Pd on top of the titanium.
5. Follow proper power-down and venting procedures, and remove the sample.

The metalized sample now consists of a layer of metal sitting atop the unexposed PMMA, with openings in the resist where the sample was exposed to electrons during the e-beam writing session (in these holes, the Ti adhesion layer and Au/Pd are deposited on the glass surface): see Fig. 1.

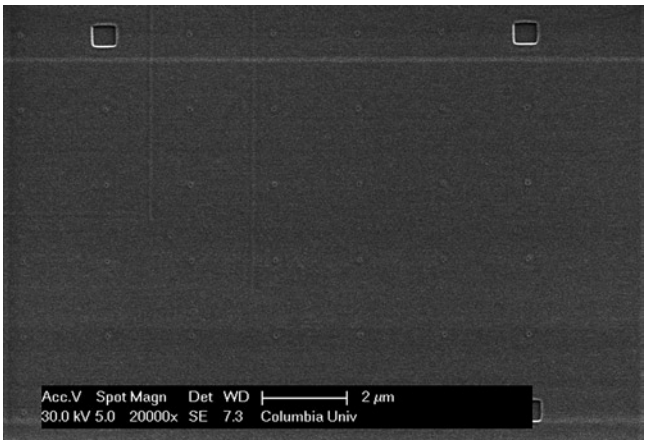

Fig. 1. Scanning electron microscope (JEOL JSM-5600 LV) image of nanopattern holes prior to liftoff of the PMMA resist layer. The pattern has been written in the electron-beam writer, developed, and the metal layers deposited; however, the unpatterned glass surface is still covered with the PMMA bilayer, with metal on top. The large 1 μm square registers are visible, with sub-50-nm holes in a square lattice with a 2 μm unit cell spacing in between.

3.1.6. Liftoff

Liftoff is the process to remove the remaining, unexposed resist that is now covered with metal. Recall that a slight overhang was created due to the use of a bilayer. Therefore, metals deposited inside the features on the glass substrate are not connected to the bulk of the metal sitting on top of the resist. Solvent is used to dissolve the resist, removing the metal while leaving behind only the metal deposited in the holes (directly on the glass) as defined by beam writing. The metal will appear to float off of the surface, hence the term liftoff.

1. To remove the resist, place the metal-coated sample in spectroscopic grade acetone (see Note 15).
2. Seal the container with parafilm tightly to avoid evaporation, and let sit overnight to remove the resist and metal layer on top (see Note 16).
3. When the sample is visibly clean of the metal layer, transfer it to a fresh beaker of acetone.
4. Remove the sample from the acetone, and rinse first with acetone from a squirt bottle, and then with ethanol from a squirt bottle (see Note 17).
5. Finally, blow dry with a stream of inert gas (Ar or N_2).

The Au/Pd nanopatterns on glass are now ready for surface functionalization. Figure 2 displays an atomic force microscopy (AFM) image and profile of the sub-50-nm dots fabricated on a glass coverslip.

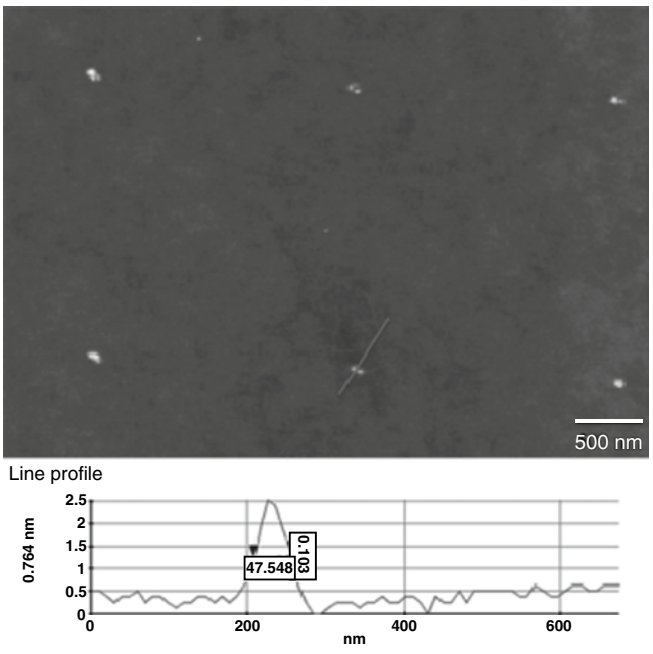

Fig. 2. Atomic force microscopy image of the Au/Pd nanodots array on glass. Imaging was performed with a XE-100, advanced scanning probe microscope (PSIA). The image has been analyzed with the XEI software, version 1.6.

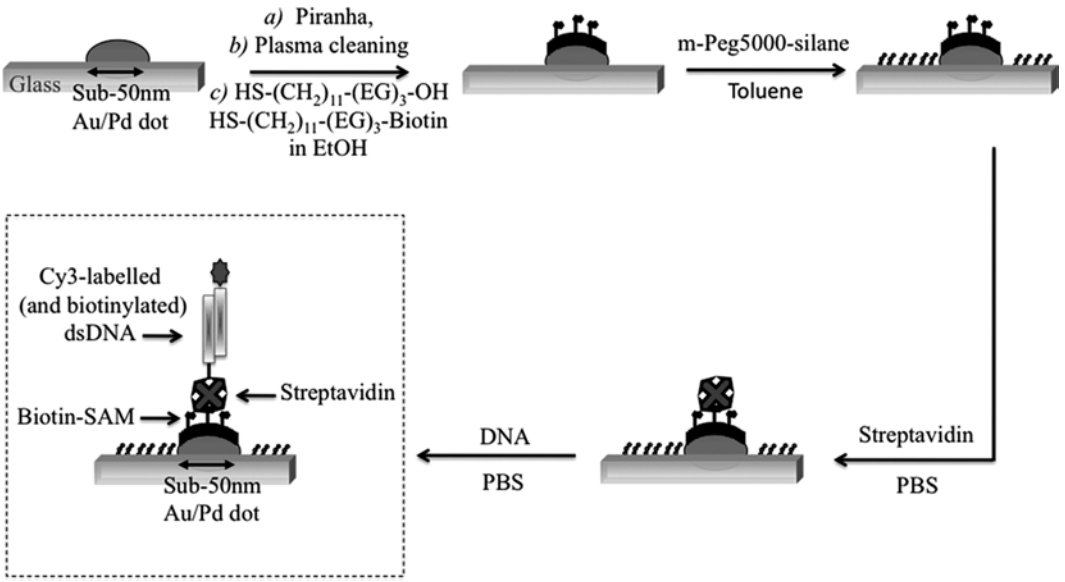

Fig. 3. Scheme of the main steps of the bio-functionalization procedure.

3.2. Functionalization of Nanopatterned Surfaces

Figure 3 schematically displays the main steps of the bio-functionalization procedure.

Starting with a nanopatterned surface, i.e., Au/Pd nanodots on a glass slide, carry out the following steps:

1. Prepare fresh piranha solution (see *above*).
2. Clean the nanopatterned surface slide by immersion for 3 min in 1 h 30 min-aged piranha solution (if piranha is used as freshly prepared, it will eat away the metal). The immersion can be done using a Teflon rack to hold the sample/s in the container.
3. Take the sample out of the piranha solution with clean tweezers and rinse in DI water for at least 5 min.
4. Rinse the sample with ethanol and blow dry with a gentle stream of inert gas (Ar or N_2).
5. Place the dry sample in a plasma cleaner for 5 min at 18 W.
6. Prepare a 1 mM anhydrous ethanol solution of HS–$(CH_2)_{11}$–$(C_2H_6O_2)_3$–OH and HS–$(CH_2)_{11}$–$(C_2H_6O_2)_3$–biotin: mix at 3:1 ratio (see Note 18).
7. Pull the sample out of the plasma cleaner and immediately incubate (see Note 19) it in the freshly prepared EG/biotin–thiol solution (1.5 mL per 6-well plate is sufficient to incubate the glass slide of the dimension used here). Seal the 6-well plate container with parafilm, cover with Al foil, and incubate on a shaker overnight (12–18 h). In this way, a SAM exhibiting functional biotin end groups will be formed on the metal nanodots; such biotin groups can then be used to immobilize streptavidin on the dots.

8. Prepare a fresh solution of 2 mg of mPEG 5000-silane in 25 mL of anhydrous toluene and add 30 μL of acetic acid (as a catalyst) (see Notes 20 and 21).
9. Rinse the sample in ethanol and blow dry with a gentle stream of inert gas (Ar or N_2).
10. Place the dry sample in the PEG solution. Incubate in a glass container, sealed with parafilm and covered with Al foil for at least 24 h (up to 48 h). This step is fundamental to assure proper passivation of the glass surface surrounding the dots: the formation of a PEG layer prevents (or at least minimizes) any nonspecific protein and DNA adsorption from taking place.
11. When PEGylation is done, rinse the glass slide with acetone and then ethanol, and blow dry with Ar or N_2.
12. Prepare a solution of PBS with 10 μg/mL of streptavidin and 1 mg/mL of albumin in PBS (see Note 22). Place the dry slide in 2 mL of such solution, seal the container with Parafilm, cover with Al foil, and incubate on the shaker at RT for 2 h (see Note 23).
13. Rinse the sample thoroughly with PBS (do not let the sample dry). Then, move the sample to a fresh well filled with PBS and incubate it for at least 30 min, while storing it foiled, on a shaker (see Note 24).
14. Meanwhile, prepare a solution of biotinylated DNA (see Note 25). For biotinylated dsDNA, prepare a solution of two complementary strands of 2 μM in TPBS at RT, one biotinylated at the 5′ end and its complement labeled with a fluorophore (Cy3 in our case) at the 5′ end. Heat the solution gradually (steps of approximately 2°C per min) to a temperature of 65°C; leave the solution at this temperature for 15 min. Then ramp the temperature up to 75°C and leave for 1 h 30 min. Then proceed in the reverse order, ramping down the temperature, let cool at 65°C for 15 min and then take gradually to RT. When the solution has reached RT, it can be employed as described in the next step.
15. Incubate the (wet) coverslip from step 13, in at least 1.5 mL of the 2 μM biotinylated dsDNA solution prepared as described above (see Note 25); cover the container with Al foil during the incubation and seal with Parafilm. The minimum incubation time recommended is 3 h (see Note 26).
16. Take the sample out of the previous solution and rinse it with PBS. Then move the sample to a fresh well and incubate it for 30 min in PBS, while storing it foiled, on a shaker.

The sample is now ready: i.e., Au/Pd nanodot is properly and specifically functionalized with the oligonucleotide of interest.

In the next section, we will describe how we characterized the functionalized surface, verifying by fluorescence microscopy the presence of Cy3-labeled dsDNA immobilized on sub-50-nm Au/Pd nanodots on glass.

3.3. Fluorescence Microscopy

3.3.1. Sample Preparation

Inverted microscopes are designed to accept coverslips mounted on standard glass slides. We have devised a simple mounting scheme with our coverslips for fluorescence microscopy (see Fig. 4). It is an open design, which allows for additional processes to be carried out in situ on the microscope. Microfluidics can and have been used: here, we present a setup offering most of the functionality without the complexity of microfluidics.

1. Remove the bio-functionalized sample from PBS rinse solution and place face up on a clean surface.
2. Apply vacuum grease to the edge of a cloning ring and place it on the coverslip, with the pattern centered as well as can be done by hand. The grease creates a watertight seal.
3. Fill the ring with buffer solution (PBS). At this point, check for leaks around the ring. If any are detected, a slight twist of the ring is often adequate to seal the leak; if not, additional grease applied to the region of the leak will solve the problem.
4. Suspend the slide across the cutout section of the aluminum holder of the microscope. We use an aluminum plate cut to fit

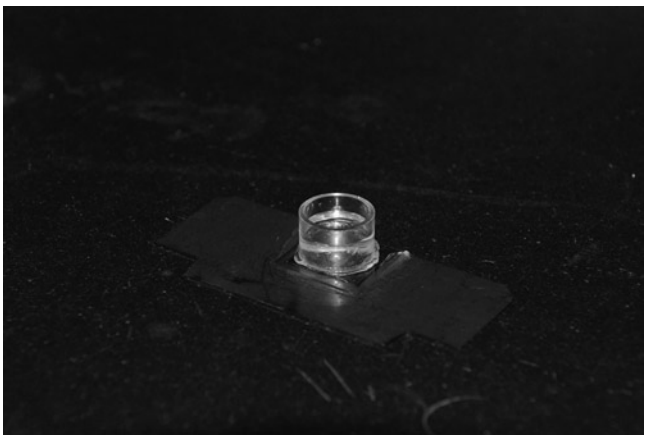

Fig. 4. Photograph (Nikon D80) of the fully functionalized nanopatterned slide prepared for fluorescence microscopy. The coverslip sits atop an aluminum carrier, machined to fit the microscope mounting hardware, and is suspended above a cutout section allowing clearance for the optics. It is held in place by a thin bead of grease along each edge. A cloning ring adhered with a thin bead of grease encircles the patterned region and contains the necessary buffer solution.

the microscope mount, with a gap cut into it to allow for the objective.

5. Use vacuum grease to seal the coverslip to the aluminum holder along the edges. Proceed to the microscope for imaging.

3.3.2. Microscopy

An inverted fluorescence microscope capable of epifluorescence microscopy is used to image the samples. Oil immersion lenses with 60× and 100× magnification are best used for imaging of the nanopatterns. The camera we use is a photometrics Cascade II; it is cooled to −70°C and has on-chip amplification for low noise, high-sensitivity imaging.

1. Make sure the optics are aligned.
2. Find the pattern in differential image contrast (DIC) mode.
3. Switch from DIC to the Cy3 (excitation 550 nm/emission 568 nm) fluorescence channel. Use live imaging with <100-ms exposure to adjust the focus again as there is a slight difference in focal plane with the different optics.
4. Proceed to record desired data, static images, or time sequences (see Note 27).

Figure 5 displays an epifluorescence microscopy image of the Cy3-labeled dsDNA on the sub-50-nm Au/Pd nanodot array.

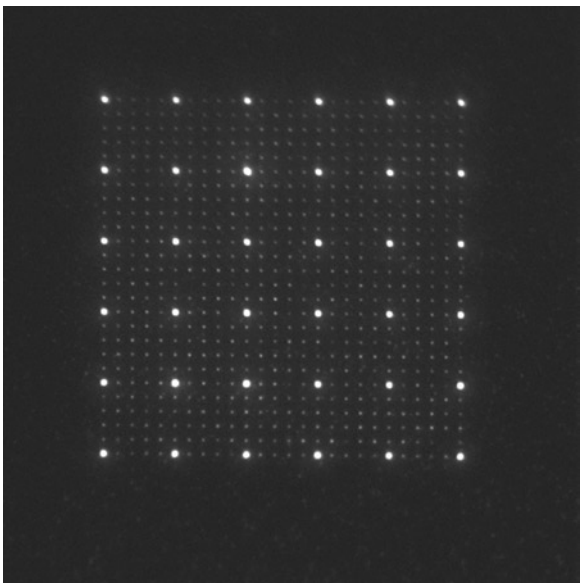

Fig. 5. Epifluorescence image of the *nanopatterned dots* functionalized with a Cy3-labeled dsDNA: excitation 550 nm, emission 568 nm. Every fifth sub-50-nm *dot*, a 1 μm *register dot* is clearly visible, and brighter in the fluorescence image. Exposure time: 300 ms. Image size 80 μm × 80 μm.

4. Notes

1. Make sure all glassware used is clean, i.e., washed with detergent, rinsed in DI water, rinsed with ethanol, and blown dry with a stream of inert gas (Ar or N_2).
2. When handling samples, especially washing and drying with tweezers, always make sure to rinse and blow dry toward the tweezers; this prevents the transfer of any contaminants from gloves and tweezers onto the samples.
3. Clean the coverslip immediately prior to the resist deposition; if it is allowed to sit in air, it will accumulate contaminants.
4. Time is of the essence; the quicker one works while spinning resist layers, the less likely the surface is to be contaminated.
5. Make sure the coverslip is cool before putting resist on, and never let resist sit for more than a few seconds before spinning. The solvent will evaporate slightly, especially at the edge, resulting in an uneven coating, which may or may not be obvious, but will affect the obtained features.
6. Observe the sample after each layer of resist is deposited. If there is streaking, this means the surface was not properly cleaned. Discard the sample and start again. Properly spun resist should uniformly coat the surface to the edges, looking smooth and glassy.
7. Larger features (micron sized) are written at spot size 5, while the nano-features are written with spot size 1.
8. The nanodots themselves are not visible in an optical microscope. The 1 μm registers are visible, and are intended to provide points of reference for the location of the nanodots, and to assist with focusing on the optical microscope. If you wish to characterize the nanodots, AFM is the best method.
9. It is important to correct for beam tilt and stigmation. If the beam is tilted, the projection of the feature will be oval rather than circle. This will affect feature shape and minimum achievable size, not to mention the fact that the focal plane of the beam will not be parallel with the plane of the sample. Stigmation results from the lens not being perfectly round, in fact no lens is. If you imagine the lens is an ellipse, and break the focus into orthogonal x–y coordinates aligned along the short and long axis of the lens, it becomes apparent that the lens will have different depth of focus for each axis. This shows when imaging has the ability to get one edge in perfect, crisp focus, while edges not parallel with the sharp edge will look out of focus, worsening as the angle of the two edges approaches 90° with respect to the properly focused edge.

Adjusting the stigmation will correct for the aberration, resulting in better focus and thus smaller features.

10. A faceted, crystalline particle such as Al_2O_3 is a good target for stigmation correction. When all edges appear clear, regardless of orientation, stigmation is minimized. A commonly used technique is to sprinkle these particles at the corners of the slide and use them to define the four-point focus as well. However, the edge is actually a few hundred nanometer above the surface, so the focus is not entirely accurate. This method is best for features down to ~35 nm; below that, the "spot" method should be used (see below).
11. "Spot" method: This requires using a 15-nm film of aluminum as the discharge layer, instead of Aquasave. Focus as best you can on the surface. Use the spot function in the scan menu to expose a spot on the surface manually, and then subsequently optimize focus and stigmation on the spot until it is sharp. This must be done iteratively; the resulting spot comes out smaller and sharper each time, enabling further refinement of the stigmation and focus. When the spot size is no longer getting smaller, focus is optimized. Always adjust focus first, before stigmation is adjusted. This is the best method for performing four-point focus, as it verifies that the focus is optimized for exposure of the smallest possible area. Spotting four corners in a box, immediately around the area to be written, ensures the best focus for writing. The 15-nm film of aluminum should be removed in NaOH prior to development.
12. We recommend keeping the sonicator and water/IPA development solution in a refrigerator prior to and during development. If that is not possible, be sure that the bath and H_2O/IPA are chilled to 4°C before proceeding.
13. Development solutions can be reused, but should be changed periodically (every ten uses) and stored at 4°C.
14. The development and quenching in room temperature IPA are time critical, so prepare everything in advance and move as quickly as possible. It is recommended to bring the IPA over to the cold sonication and after 1 min, transfer the sample directly into the IPA at room temperature.
15. It is important to prevent any re-deposition of the lifted off metal.
16. When placing the samples in the liftoff, keep them mostly vertical, but tilted slightly face down so the metal will fall away from the surface. Use one of the slotted Teflon racks as a holder.
17. Never allow the samples to go dry in the acetone or upon removal during the rinsing steps.

18. Always use separate dedicated syringes and needles for the anhydrous solvents employed. Rinse the needles and syringes after use, and store them in an oven at 70°C: the syringe and the needle used for toluene should be rinsed in acetone and then in ethanol. Blow dry the needles before storage in oven.
19. Incubate the samples in the biotin solution directly after plasma cleaning: allowing the sample to sit too long in air has a detrimental effect over proper SAM formation.
20. When preparing the PEG-silane solution, make sure to work in a cold environment chamber (temperature below 5°C).
21. A functionalization procedure incubating the sample in the PEG-silane solution first and then thiolating the gold works, but it gives rise to a less reliable passivation, probably due to physisorption of biotin–alkane chains on the PEG layer, which can then play an active role as functional spots for the attachment of streptavidin on surfaces.
22. The purpose of albumin is to block nonspecific binding between streptavidin and any passivation defects on the glass surface.
23. It is recommended to incubate the sample in the streptavidin solution immediately after drying.
24. It is possible to check the intermediate step by using a fluorescent-labeled streptavidin (Invitrogen): suggested fluorophores include Alexa 488 and Cy5. It is also possible to use avidin or neutravidin, instead of streptavidin, although avidin is not recommended due to a high degree of nonspecific adsorption.
25. Either single-stranded (ssDNA) or dsDNA can be immobilized on the metal nanodots depending on the application: dsDNA nanodomains, for example, can be useful to study protein–DNA interactions, while bound biotinylated ssDNA is available for in situ hybridization to its complementary sequence, a process of interest for bioanalytical applications. Furthermore, immobilized ssDNA can serve as an anchoring point on the surface to drive the self-assembly of biological and inorganic nano-objects properly functionalized with the complementary strand: the information encoded in the double helix can be a powerful tool for the fine control of such organization, noteworthy at the nanoscale. For ssDNA, at step 15 of Subheading 3.2, incubate the sample in a ssDNA solution at a concentration of 2 μM in TPBS at RT for at least 3 h (up to 12–18 h, i.e., overnight). In order to proceed with a RT in situ hybridization on the nanodots (fabricated as described above and functionalized with ssDNA), incubate the sample in a 2 μM solution of the complementary strand in TPBS adding 3 mM of NaCl and 125 mM of $MgCl_2$. The purpose of the high salt concentration is to screen any repulsive interactions

taking place between the phosphate groups of the complementary DNA strands, as they can prevent an effective hybridization from taking place at RT. The incubation should be done in at least 1.5 mL of solution and left in a container sealed with Parafilm and covered with Al foil overnight (minimum incubation time: 12 h, up to 36 h): do not shake the container during the incubation. After incubation, rinse the sample in PBS and incubate in PBS for 1 h: the rinsing is very important to get rid of residual salt physisorbed on the substrate.

26. The incubation with biotinylated dsDNA can go up to 12–18 h (i.e., overnight).
27. Imaging is always a balancing act between exposure time and amplification to achieve detection threshold. Longer exposure times integrate more light and result in better signal to noise, but result in loss of time resolution and lead to faster photobleaching. Increasing the gain amplification allows for lower exposures but increases the effect of noise, which can swamp out the fluorescence signal. We typically work at exposures of 100–300 ms as this provides adequate signal to noise with most fluorophores we work with.

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