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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 both DNA-protein interactions as well as 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.

Keywords

DNA; self-assembly; nanoscale; nanotechnology; fluorescence microscopy

1. Introduction

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

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

Various chemical strategies have been employed to immobilize DNA at surfaces, ranging from electrostatic interactions, formation of (thiolated) self-assembled monolayers (SAMs), direct 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 nano-scale 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-50nm 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. In this context, we will here describe a biotin-streptavidin 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 will discuss and demonstrate the ability to immobilize double stranded DNA (dsDNA) on each and every fabricated (and properly functionalized) nanodot. This functionalization procedure allows for the formation of non-sterically hindered DNA nanodomains at surfaces; an homogenous surface packing density can be envisioned, an advantage over thiolated DNA SAMs (20). We will verify the validity of our approach by EPI-fluorescence microscopy imaging.

2. Materials

2.1 Metal on Glass NanoPattern Fabrication

1. Glass coverslips, 22mm × 22mm, No. 1.5 (Corning).
2. Sulfuric Acid (H₂SO₄), 17.8M (EMD Chemicals).
3. Hydrogen Peroxide (H₂O₂), 30% conc. Store in refrigerator at 4°C (Fisher Scientific).
4. 7x Cleaning Solution (MDBio).
5. Polymethylmethacrylate Photoresist (PMMA)- 495K, and 25K molecular weight (MW), A2 concentration, (Microchem Corp.); store in dark.
6. CEE Brewer 100 Resist Spinner (Brewer Science).
7. Electron Beam Writing System (FEI, with Nabity NPGS stage writing control).
8. Electron Beam Evaporator (Semicore).
9. Aquasave Conductive Coating (Mitsubishi).
10. Acetone, HPLC Grade (Pharmco-Aaper).
11. Ethanol, 200 proof (Pharmco-Aaper).
12. Isopropanol (IPA), HPLC Grade, 99.8% (Pharmco-Aaper).
13. Tweezers, round tip, stainless steel (SPI Supplies).
14. Gold/Palladium 60%/40%, Pellets (Plasmaterials).
15. Titanium (Plasmaterials).

2.2 Functionalization of nanopatterned surfaces

1. Toluene anhydrous 99.8% (Sigma).
2. Ethanol anhydrous >99.5% (200 Proof) (Sigma).
3. Acetic acid, glacial ACS reagent grade (Pharmco-Aaper).
4. Gibco™ Dulbecco's Phosphate Buffer Saline 1X (PBS) (no magnesium, no calcium, 2.7 mM Potassium Chloride, 0.14 M Sodium Chloride, 1.5 mM Potassium Phosphate, 8mM Sodium Phosphate, pH= 7.4), (Invitrogen); store at Room Temperature (RT).
5. ThermoScientific BupH™ Phosphate Buffered Saline (0.1 M Sodium Phosphate, 0.15 M Sodium Chloride, pH=7.2) (TPBS); store at RT.

6. Ethanol, ACS reagent grade (Pharmco-Aaper).
7. Acetone, ACS reagent grade (Pharmco-Aaper).
8. Deionized (DI) Millipore water (resistivity of 18 MΩ*cm).
9. HS-(CH₂)₁₁-(C₂H₆O₂)₃-OH and HS-(CH₂)₁₁-(C₂H₆O₂)₃-Biotin, (ProChimia); store at -20°C.
10. PolyEthyleneGlycol (PEG) Silane, 5000 MW, (mPEG5000) (LaysanBio); store at -20°C.
11. Glass syringes, metal needles for the anhydrous solvents (Popper).
12. Harrick PDC-32G Plasma Cleaner;
13. Teflon mini-rack, (Invitrogen).
14. Six well plates (Falcon).
15. Parafilm (Pechiney Plastic Packing).
16. Streptavidin (Invitrogen); store at -20°C.
17. Chicken egg Albumin, (Sigma); store at 4°C.
18. 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 foiled at 4°C

2.3 Microscopy

1. Inverted Microscope, Olympus IX81 (Olympus).
2. CCD Camera, Cascade II, 512×512 pixels (Photometrics).
3. Cloning Ring (Sigma/Aldrich).
4. 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 as well as the materials and tools employed.

Because of the nanometer scale of the features fabricated (and functionalized) we recommend carrying out the fabrication steps (section 3.1) in an ultra clean environment, such as a cleanroom. Furthermore, in order to obtain a successful bio-fucntionalization (section 3.2) of the fabricated nanopatterns, it is important to rapidly carry out the procedure with as small a time lapse as possible between steps. Moreover, all glassware and tweezers used must be dry, preferably stored in an oven at *ca* 70 °C and cooled in air prior 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-labelled, dsDNA. We have hybridized in solution, prior to the attachment to the surface, a biotinylated oligonucleotide (20-mer chain) and its complementary oligomer labeled with a Cy3 fluorophore. (In the notes section we will discuss how, and why, the same DNA immobilization procedure can be alternatively employed to bind a biotinylated single stranded DNA).

3.1 Metal on Glass NanoPattern Fabrication

3.1.1 Preparation and Cleaning—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).

1. Prepare Piranha solution, one part H₂O₂ to three parts H₂SO₄.
2. Starting with a new glass coverslip, sonicate in Ethanol for 2 minutes.
3. Blow dry with a stream of inert gas (Ar or N₂) (see Note 2).
4. Dilute 7x detergent 1:3 with deionized (DI) water, bring to boiling temperature on a hot plate at 200°C (goes from cloudy to clear).
5. Immerse slide for 2 minutes in boiling solution, rinse with DI water for 10 minutes.
6. Immerse in Piranha (1:3 H₂O₂: H₂SO₄), let soak for 5 minutes.
7. Take the slide out of the Piranha solution and rinse it for 10 minutes with DI water.
8. Rinse with Ethanol.
9. Blow dry with a stream of inert gas (Ar or N₂) (see Note 3).

It is now possible to proceed to resist deposition (see next section).

3.1.2 Resist Deposition—Again, cleanliness is paramount here. All work should be performed in a cleanroom, class 10,000 or better. A bi-layer 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 Hot plate to 180°C, place cleaned samples on hot plate for 1 minute to force off any adsorbed water molecules.
2. Let cool 10 seconds before placing sample on resist spinner chuck (see Note 5).
3. Spin lower MW resist first at 4000 RPM for 45 seconds, use a ramp rate of 1000 rpm/sec (see Note 6).
4. Bake for 5 minutes at 180°C on a hot plate.
5. Let cool 10 seconds before placing the sample on the resist spinner chuck.
6. Spin higher molecular weight resist as top layer at 4000 RPM for 45 seconds, use a ramp rate of 1000 rpm/sec
7. Bake for 5 minutes at 180°C.
8. Let cool 10 seconds before placing on resist spinner chuck.

¹Make 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 N₂).

²When handling samples, especially washing and drying with tweezers, always make sure to rinse and blow dry towards the tweezers, this prevents the transfer of any contaminants from gloves and tweezers onto the samples

³Clean the coverslips immediately prior to the resist deposition; if they are allowed to sit in air they will accumulate dust.

⁴Time is of the essence; the quicker one works while spinning resist layers the less likely the surface is to be contaminated.

⁵Make 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.

⁶Observe 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 try again. Properly spun resist should uniformly coat the surface to the edges, looking smooth and glassy.

9. Spin on the Aquasave conductive discharge layer at 1000 RPM for 45 seconds with a ramp rate of 300 RPM/sec. It is important to make sure to dab any droplets of Aquasave at edges (particularly corners) so 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, as described in the next section.

3.1.3 Electron Beam Pattern Writing—An electron beam writing system is a SEM, which additionally 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 between them every 2 μm . This ensures each individual dot is optically resolvable and discrete once functionalized and imaged with a fluorescence microscopy (see Note 8).

1. Load samples in the machine, making sure that 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 kilovolts (kV).
3. Check the beam currents of the spot sizes to be written with in 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 doses are consistent.
5. Check gun tilt and stigmation, adjust these until both are minimized (see Notes 9, 10 and 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

⁷Larger features (micron sized) are written at spot size 5, while the nano-features are written with spot size 1.

⁸The 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.

⁹It is important to correct for beam tilt and stigmation. If the beam is tilted the projection of the feature will be an oval rather than a 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 up 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 exhibits itself when imaging as 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 degrees with respect to the edge. Adjusting the stigmation will correct for the aberration, resulting in better focus and thus smaller features.

¹⁰A 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. Sprinkling these particles at the corners of the slide and using them to define the four point focus as well is a commonly used technique. The edge is actually a few hundred nm above the surface however, so the focus is not entirely accurate. This method is best for features down to ~35nm, below that, the “spot” method should be used (see below).

¹¹“Spot” method: This requires using a 15nm 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 manually expose a spot on the surface, and then subsequently optimize focus and stigmation on the spot until it is sharp. This must be done iteratively, the resulting spot coming 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.

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 focus and stigmatism over the area of the pattern move to a position inside the four-point focus region.
8. Engage automated stage and beam control, 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 N₂).

Samples are now ready for development.

3.1.4 PMMA Development—A cold ultrasonic development process is used to achieve nm 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 3:1 H₂O:IPA at 4°C. Sonicate for 1 minute in water bath sonicator at 4°C (see Notes 12 and 13).
2. Quickly remove 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 N₂).
4. Examine pattern: large alignment marks should be visible; check further in an optical microscope at magnifications of 25–100x. The micron-sized features should be visible.

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

3.1.5 Metal Deposition—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 angstrom 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 1nm adhesion layer of Titanium, this is necessary for the adhesion of the Au/Pd to the glass.
4. Next deposit 3nm 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

¹²We recommend keeping the sonicator, and water/IPA development solution in a refrigerator prior to and during development. If that isn't possible be sure bath and H₂O/IPA are chilled to 4°C before proceeding.

¹³Development solutions can be reused, but should be changed periodically (every 10 uses) and stored at 4°C.

¹⁴The 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 one minute, transfer the sample directly into the IPA at room temperature.

writing session (in these holes, the Ti adhesion layer and Au/Pd are deposited on the glass surface): see figure 1.

3.1.6 Liftoff—Liftoff is the process to remove the remaining, unexposed resist now covered with metal. Recall a slight overhang was created due to the use of a bi-layer. Therefore metal 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 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 spectroscopy 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 spray bottle, then with ethanol from a spray bottle (see Note 17).
5. Finally blow dry with a stream of inert gas (Ar or N₂).

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.

3.2 Functionalization of nanopatterned surfaces

Figure 3 schematically displays the main steps of the biofunctionalization procedure.

Starting with a nano-patterned surface, i.e. Au/Pd nanodots on a glass slide:

1. Prepare a piranha solution: $\frac{1}{3}$ H₂O₂, $\frac{2}{3}$ H₂SO₄.
2. Clean the nanopatterned surface slide by immersion for 3 minutes in 1h30 minutes 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 N₂).
5. Put the dry sample in an UV-ozone cleaner for 5 min at 18 W.
6. Prepare a 1 mM anhydrous-ethanol solution of HS-(CH₂)₁₁-(C₂H₄O₂)₃-OH and HS-(CH₂)₁₁-(C₂H₄O₂)₃-Biotin: mix at ratio 3:1 (see Note 18).

¹⁵It is important to prevent any re-deposition of the lifted off metal.

¹⁶When 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.

¹⁷Never allow the samples to go dry in the acetone or upon removal during the rinsing steps.

¹⁸Always 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, then Ethanol. Blow-dry the needles before storage in oven.

7. Pull the sample out of the plasma cleaner and immediately incubate (see Note 19) it in the EG/Biotin-thiol solution freshly prepared (1.5mL per six well plate is sufficient to incubate the glass slide of the dimension used here). Seal the six-well plate container with parafilm, cover with Al foil and incubate on a shaker overnight (12/18 hours). 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 mPEG5000-silane in 25 ml of anhydrous toluene and add 30 μ L 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 N₂).
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 hours (up to 48 hours). This step is fundamental to assure a proper passivation of the glass surface surrounding the dots: the formation of a PEG layer prevents (or at least minimizes) any non-specific protein and DNA adsorption from taking place.
11. When PEGylation is done, rinse the glass slide with acetone then ethanol, and blow dry with Ar or N₂.
12. Prepare a solution of PBS with 10 μ g/mL of streptavidin and 1 mg/mL Albumin in PBS (see Note 22). Place the dry slide in 2 mL of such solution, seal with Parafilm the container, cover with Al foil and incubate on the shaker at RT for two hours (see Note 23).
13. Rinse the sample thoroughly with PBS (do not let the sample dry). Move then the sample to a fresh well filled with PBS and incubate it for 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 2mM in TPBS at RT of two complementary strands, one biotinylated at the 5' end and its complement labeled with a fluorophore (Cy3 in our case) at the 5' end. Heat up the solution gradually (steps of *ca* 2°C per

¹⁹Incubate 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.

²⁰When preparing the PEG-silane solution make sure to work in a cold environment chamber (temperature below 5°C).

²¹A functionalization procedure incubating the sample in the PEG-silane solution first and then thiolating the gold works but gives rise to a less reliable passivation, probably due to physisorption of biotin-alkane chains on the PEG layer, that can then play an active role as functional spots for the attachment of streptavidin on surfaces.

²²The purpose of Albumin is to block non-specific binding between streptavidin and any passivation defects on the glass surface.

²³It is recommended to incubate in the streptavidin solution immediately after drying the sample.

²⁴It 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 non-specific adsorption.

²⁵Either single stranded (ssDNA) or dsDNA can be immobilized on the metal nanodots depending on the application: dsDNA nanodomains, for example, can be meaningful 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 section 3.2, incubate the sample in a ssDNA solution at a concentration of 2 mM in TPBS at RT for at least 3h (up to 12/18h, 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 then the sample in a 2mM solution of the complementary strand in TPBS adding 3mM NaCl. 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. 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 over-night (minimum incubation time 12h, up to 36h); do not shake the container during the incubation. After incubation rinse the sample in PBS and incubate in PBS for 1h: the rinsing is very important to get rid of residual NaCl salt physisorbed on the substrate.

minute) to a temperature of 65°C; leave the solution at temperature for 15 min. Then ramp the temperature up to 75°C and leave for 1h30 minutes. 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) cover slip from step 13, in at least 1.5 mL of the 2mM 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 3h (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 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-50nm Au/Pd nanodots on glass.

3.3 Fluorescence Microscopy

3.3.1 Sample Preparation—Inverted microscopes are designed to accept coverslips mounted to standard glass slides. We have devised a simple mounting scheme with our coverslips for fluorescence microscopy (see Figure 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 but would warrant a whole other methods chapter unto themselves. Here we present a setup offering most of the functionality without the complexity.

1. Remove biofunctionalized 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. We use an aluminum plate cut to fit 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.

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

²⁶The incubation with biotinylated dsDNA can go up to 12/18h (i.e. overnight).

1. Make sure optics are aligned.
2. Find the pattern in Differential Image Contrast mode (DIC).
3. Switch from DIC to the Cy3 (excitation 550nm/emission 568nm) fluorescence channel. Use live imaging with <100ms 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 EPI-fluorescence microscopy image of the Cy3 labeled dsDNA on the sub-50nm Au/Pd nanodot array.

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²⁷Imaging 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 100–300ms as this provides adequate signal to noise with most fluorophores we work with.

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Figure 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 micron square registers are visible, with sub 50nm holes in a square lattice with a 2 micron unit cell spacing in between.

Figure 2. Atomic Force Microscopy image of the Au/Pd nanodots array on glass. The inset shows a profile taken on a single sub-50nm Au/Pd dot. Imaging was performed with a XE-100, Advanced Scanning Probe Microscope (PSIA). The images have been analyzed with the XEI software, version 1.6.

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

Figure 4. Photograph (Nikon D90) of the fully functionalized nanopattern prepared as described above 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.

Figure 5. Epi-fluorescence image of the nanopatterned dots functionalized with a Cy3 labeled dsDNA: excitation 550nm, emission 568nm. Every fifth sub-50nm dot, a 1 μ m register dot is clearly visible, and brighter in the fluorescence image. Exposure time: 300ms. Image size 80 μ m $\times$ 80 μ m.