High density single molecule surface patterning with colloidal epitaxy

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Simple and inexpensive methods for dense surface patterning of single molecules will help realize the massive potential throughput of molecular arrays in biology and nanoscience. To surpass the resolvable density limit imposed by random deposition, the authors have developed a method that uses colloids to pattern single molecules at a fixed length scale. They demonstrate the ability to pattern fluorescently labeled DNA such that ~38% of the available diffraction-limited regions contain exactly one molecule. This density is slightly less than the theoretical limit suggested by Monte Carlo simulations but surpasses the random deposition limit by more than threefold.

With the application of spectroscopy to studies that require the interrogation of many molecules in parallel, there is growing interest in developing methods for strategically patterning different molecules on surfaces at small fixed length scales. A number of approaches have been developed to control the deposition of DNA molecules on surfaces, including molecular combing by capillary flow,1 casting solutions on a surface prepatterned with polydimethylsiloxane (PDMS),2 spin stretching,3 and using a PDMS stamp inked with DNA for printing on mica.4 Ordered DNA arrays have been generated by drop projection,5 microcontact printing,6 deposition on surfaces prepatterned by e-beam lithography,7 and dippen nanolithography.8 These approaches typically either yield high densities over small areas or low densities over large areas and are not well suited for single molecule deposition. Nanopatterning of DNA has been demonstrated on large surfaces of anodic porous alumina9 and through the use of micron-sized beads to deposit nanoscale DNA spots,10 but neither of these approaches has yet been demonstrated for single molecule deposition. Optical traps have been used to deposit single colloidal particles in a designed pattern,11 but this approach has not yet scaled to deposit millions of features in a dense ordered array.

Optical resolution is traditionally determined by the Rayleigh criterion with the diffraction limited resolution defined by \( d_R = 0.61 \lambda / NA \), where \( \lambda \) is the wavelength of collected photons and NA is the numerical aperture of the system. The theoretical density limit for single molecule surface patterning is the circle packing density limit: ~91% surface coverage and molecular spacing defined by \( d_R \). There have been efforts to surpass the resolution limit using structured illumination patterns,12 photoswitchable fluorophores,13 and centroid localization methods14 designed to resolve multiple single molecules within a diffraction limited region (DLR). While these techniques could conceivably be used to increase the maximum number of resolvable molecules while using a random deposition strategy, they may be difficult to scale up for large single molecule arrays.

Here, we demonstrate an approach to single molecule surface patterning that uses colloidal epitaxy to deposit individual double stranded DNA molecules with a minimum pitch of \( d_R \). Briefly, silica colloids with diameter \( d_R \) are functionalized with fluorescently labeled DNA; the colloids are self-assembled to form a monolayer on a coverslip using the DNA as a tether to the surface, and the colloids are subsequently removed to leave behind an ordered pattern of single DNA molecules. If the length of the tether between the colloid and the surface is small in comparison to the colloid diameter, this deposition strategy will theoretically allow every deposited single molecule to be spaced by at least a colloid diameter and be resolvable. An illustration outlining the colloidal epitaxy process is shown in Fig. 1.

We performed Monte Carlo simulations to determine the highest theoretical density achievable with a variety of patterning strategies, including colloidal epitaxy, random deposition, random deposition with image processing, and surface prepatterning methods. For the random deposition of molecules on a surface, we assigned \( N \) molecules a random position on a continuous \( XY \) grid containing \( d \) DLRs. If \( \theta = N / d \) is taken as the average number of deposited molecules per DLR and \( \theta \) is the fraction of DLRs containing exactly one molecule, we found that \( \theta \) reaches a maximum of

![FIG. 1. Illustration of single molecule colloidal epitaxy (not to scale).](image)

Aminated silica beads are coupled to thiolated double-stranded DNA decorated with Cy3 and biotin. (2) The DNA-bead conjugates are tethered to the surface through a biotin-neutravidin binding interaction. (3) Unoccupied neutravidin sites are filled with free biotin and the surface is incubated with a type II restriction enzyme to cleave the double-stranded DNA. (4) The beads are washed from the surface leaving behind an array of single molecules with a minimum pitch approximately equal to the colloid diameter.
To find the conditions with the highest fraction of DLRs containing \( m \) molecules, we set \( \theta = \frac{\theta_m}{\theta + K_D} \exp(-sD_0 D a (\theta_m + K_D) \tau) \).

(3)

Assuming that \( K_D \) is small for a biotin-streptavidin binding interaction, Eq. (3) simplifies to

\[ \theta(\tau) = 1 - \exp(-sD_0 \theta_m \tau). \]

(4)

The \( k_{on} \) rate constant for streptavidin binding to biotinylated bovine serum albumin has been reported to be \( 1.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}. \) Since this constant is directly proportional to the diffusion coefficients of the reacting species, we expect that biotin coupled to a 300 nm bead will reduce the rate constant by two orders of magnitude to \( 10^{-2} \text{M}^{-1}\text{s}^{-1}. \) Using experimental values, one can then estimate that the time constant for deposition in Eq. (4) is \( 3 \times 10^4 \text{s}^{-1}. \) This is in reasonable agreement with our experimental observations of 38% coverage after \( 40 \text{ h} \) of binding.

Double stranded DNA was coupled to 300 and 640 nm amminated silica colloids (corpuscular Inc.) as described elsewhere, \(^{16} \) with stoichiometries experimentally determined such that the average number of DNA molecules per colloid was near unity [Fig. 1(a)]. The DNA sequence was designed to contain a Cy3 fluorophore, a BsaI restriction site, a 5’-biotin, and a 3’-thiol (Integrated DNA Technologies, sequences 5’-Bio-CGC TCT ATC CTC CCT CCA TTC CAA CCA GAC GCC ACC CTC AGT CAT TTG TA-SH—3’ and 5’-TAC AAA TGA CTG AGG GT—3’, restriction site in bold). Imaging Cy3 with a 1.45 NA objective gives \( d_R = 240 \text{ nm} \); thus 300 nm colloids were chosen to ensure slightly more than adequate spacing. Glass coverslips (Precision Glass & Optics, D-263T cut glass, 0.15 mm, \( 2 \times 1.1 \text{ in}^2 \) 40/20 surface quality) were RCA cleaned, coated with a polyelectrolyte multilayer, and functionalized with biotin-PEO-amine (Pierce), as previously described. \(^{17} \)

Surfaces were then washed with 1 mL of 10 mM Tris, 50 mM NaCl, pH 7.5 buffer, and incubated for 45 minutes with 1 mg/mL neutravidin (Pierce) in 0.01% sodium azide, 20 mM Tris, 100 mM NaCl, and pH 8 buffer. DNA-colloid constructs were resuspended in 100 \( \mu \text{L} \) of 1% BSA, 1X phosphate buffered saline (PBS), pH 7.4, and allowed to bind to the neutravidin surface for 20 h at 4 °C [Fig. 1(b)]. The surfaces were then washed with 1X PBS and the deposition process was repeated with a fresh batch of constructs. After the first deposition period of 20 h, the fraction of beads containing DNA in solution decreased slightly (\( \sim 5\% \)) due to deposition. Repeating the process serves two purposes: first, it allows us to verify and quantify the deposition; second, it helps to improve the deposition rate and surface density for the second incubation. After the final wash, unoccupied neutravidin sites on the surface were filled by incubation with 50 mM biotin for 30 min. The colloids were then removed with the addition of ten units of BsaI in 1× buffer No. 4 (New England Biolabs) and 1% BSA for 2 h at 37 °C [Fig. 1(d)], followed by washing the surface extensively with de-ionized H\(_2\)O [Fig. 1(d)]. The surface was imaged on a Nikon TE2000-S microscope in total internal reflection fluorescence mode with a Hamamatsu ORCA-ER charged coupled device. A Cy3 filter set (HQ535/50, Q565LP, HQ610/75, Chroma) and a Nikon Plan Apo total internal reflection fluorescence (TIRF) \( 60 \times 1.45 \text{ NA} \) objective with a low-fluorescence immersion oil was used (\( n = 1.515 \)).
Figure 2 shows brightfield, scanning electron microscopy (SEM), and TIRF images of colloidal epitaxy using 300 nm silica colloids pre- and postenzymatic cleavage. Restriction enzyme treatment and washing was >90% successful at removing the colloids. Control experiments with nonbiotinylated or nonthiolated DNA showed little nonspecific binding and DNA without Cy3 showed no sign of fluorescence. Images were processed using a custom script in MATLAB and single molecules were identified based on their fluorescence. Images were processed using a custom script in MATLAB and single molecules were identified based on their fluorescence.

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