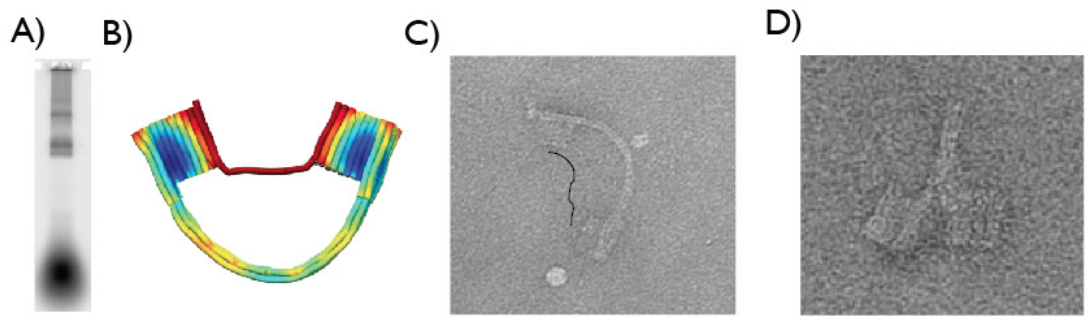

Figure 7.9: Effects of shortening the tightrope. A) Agarose gel showing successful folding of tightrope structures with shortened linear gBlock strands. B) CanDo [347] analysis showing the expected bent structure backbone resulting from mechanical strain. C) TEM image confirming the bent configuration; the tightrope is visible and an offset black line is drawn in to highlight its position. D) Another configuration observed in TEM, probably representing mechanical buckling of the structure backbone under strain.

scaffolded DNA origami techniques[166,108], whereby the m13mp18 genome is folded via 175 short staple strands. The tightrope strand begins as a 300 to 500-mer dsDNA "gBlock" available commercially from Integrated DNA Technologies. This gene block is then amplified by PCR with a phosphorothioate forward primer and a phosphate reverse primer, and the amplicons are subsequently exposed to lambda-exonuclease. The phosphate-primed strands are digested by the exonuclease, while the phophorothioate-modified strands remain intact, and can then be integrated with the support to form the ssDNA tightrope.

7.4 NM2CM: STRATEGIES FOR INTEGRATING TOP-DOWN AND BOTTOM-UP NANOTECHNOLOGY, TO CONSTRUCT FULLY PROGRAMMABLE BIO-CHIPS

No integrated architecture has yet been proposed which fully specifies the steps necessary to produce structures with a) overall sizes on the scale of today's computer chips (centimeters), b) addressable features on the 10 nm scale, and c) the ability to attach a wide range of discrete components at customizable locations. We have performed initial theoretical and experimental investigations into a scheme for nanometer-to-centimeter fabrication integration via top-down organization of DNA nanorods using

DNA hybridization interactions. In particular, George Church and I – leveraging previous work by the Church lab and collaborators on lithographic fabrication of periodic, chemically uniform nano-arrays for sequencing applications, as well as of aperiodic DNA microarrays – defined a deterministic strategy for ordering molecular components across millimeter or centimeter length scales while (we expect) maintaining few-nanometer precision in the placement of individual components. We proposed a solution that combines two levels of photo-lithography with DNA nano-structure patterning.

7.4.1 de Bruijn rod bridging

Although conventional lithographic techniques can produce patterns at high spatial resolution, they provide no means to specifically interface these patterns with diverse molecular-scale components. In contrast, the self-assembly of information-bearing bio-polymers exploits the specificity of molecular recognition to generate combinatorial numbers of specific binding interactions. Here we outline – based on a proposal by George George, which was created in response to discussions with the author and others – a method which specifies the construction of an array of uniquely addressable bio-molecular lattice points (UALPs) on a surface, with spacing on the order of 500 nm. Such an array could be used to provide docking and interconnection sites for inorganic components templated on DNA nanostructures. Such an array could also serve as a surface-based seed layer for growth into the third dimension via templated self-assembly of subsequent layers. Several capabilities in nanotechnology can be leveraged to create fully addressable nano-arrays.

1) Construction of periodic spot arrays: Interference photo-lithography[308] allows rapid and low-cost fabrication of periodic patterns over large areas. The resulting chips may consist of an array of positively charged amino-silane spots on silicon or glass surfaces. Changing the pitch is merely a matter of changing the incidence angle. As a rule of thumb, it is easy to obtain a 3× ratio between pitch and spot diameter but difficult to obtain a 10× ratio: thus 300 nm spot diameter at 1000 nm pitch is feasible, as is 100–150 nm spot diameter at 400 nm pitch, but 200 nm spot diameter at 2000 nm pitch

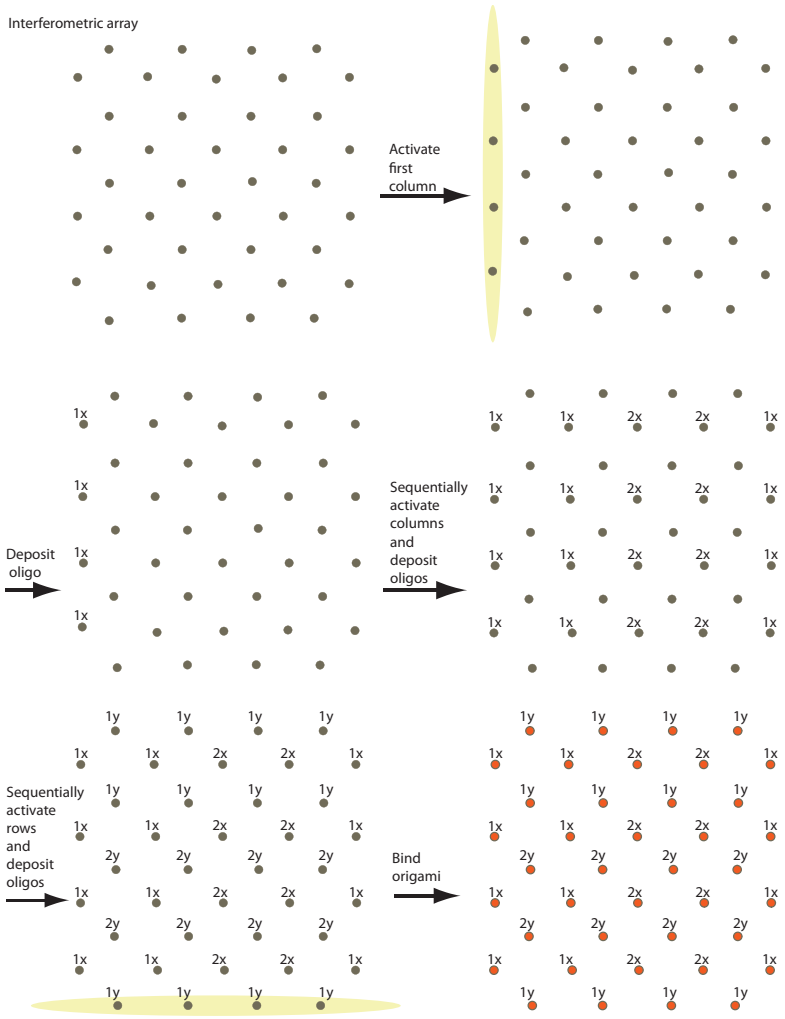

Figure 7.10: Construction of DNA spots in a de Bruijn pattern. A diffraction-limited UV line focus, produced by a cylindrical lens, is scanned through a series of discrete positions, aligned to a nano-grid fabricated using interference lithography. The line foci sequentially activate lines of nano-grid spots along the x or y axes via nitro-benzyl chemistry, followed by deposition of the corresponding oligo type. Each axis corresponds to a De Bruijn sequence of the different spot types, with sub-sequence length $s = 2$. Thus, each pair of consecutive DNA spots along an axis uniquely identifies the location along the axis. The alphabet size n is the number of DNA spot types along each axis. Shown here is the De Bruin sequence with $n = 2$ and $s = 2 : 1122(1)$. Note that, for a pattern with 10^8 UALPs, $n = 100$ and $2 * 10^4$ separate oligo deposition steps are required. Assuming that one activation and deposition step occurs every 5 seconds, the entire process (up to rod deposition) takes one day. For comparison, if each spot had to be individually activated and deposited at 5 seconds per step to ensure unique addressability in 2D, the process would take 5 seconds $* 10^8 = 15$ years. The use of a de Bruijn spot pattern and cylindrical lens allows us to circumvent this problem, as would the use of DMD-driven parallel oligo synthesis or deposition.

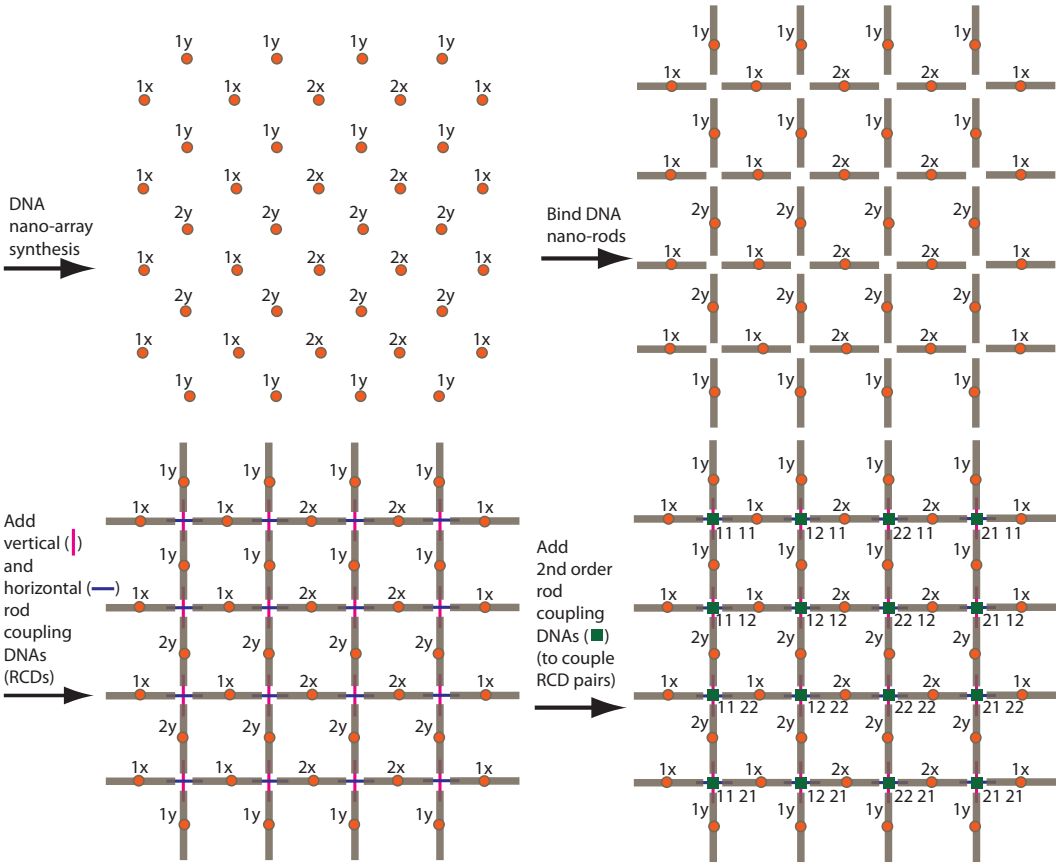

Figure 7.11: Conversion of the de Bruijn DNA origami pattern to a set of uniquely addressable bio-molecular lattice points (UALPs). Rigid DNA nanostructure rods bind to individual spots on the surface via binding sites on the DNA origami adaptors (orange). Contact points between rods bound to adjacent spots define unique x or y coordinates. Cooperative hybridization to markers (rod coupling DNAs) indexing these x and y coordinates allows unique addressing of 2D positions. Rods have directionality to define ordered pairs. i_x and i_y rod types are distinct to prevent mixing of the two coordinates. The total number of UALPs along an axis is n^2, where n is the number of distinct spot types per axis. The total number of UALPs in 2D is n^4. In the example shown: there are $n = 2$ distinct spot types, $n^2 = 4$ unique positions along each axis, and $n^4 = 16$ UALPs in 2D.

may not be feasible.

2) Decoration of periodic spot arrays with DNA nano-structures at one-to-one occupancy: Rolling circle nano-balls (rolonies, see Figure 7.12) have been shown to bind size-matched positively charged surface spots fabricated by methods such as interference lithography[170]. Rolonies sterically and electrostatically exclude one another on size-matched spots. Therefore the rolony concentration in solution can be held well above that needed to achieve a Poisson average spot occupancy of one, driving the system to stoichiometric spot occupancy. Rolonies have customizable size via the duration of the RCA reaction and each rolony may possess a unique 5' end with a hybridization-addressable sequence, e.g., defined by an overhang on the RCA primer.

Similarly, DNA origami bind to size and shape matched e-beam lithographic spots with one to one occupancy[345], and this property should extend to size-matched spots fabricated by interference photolithography or other low-cost methods for creating periodic arrays of nano-sized spots. Lambda-inside-m13 phage scaffolds have also been used to make lambda-sized origami. Furthermore, single-stranded tile (SST) structure fusion may be a viable approach to make larger SST structures[695,723]. All these facts suggest that 200 nm × 200 nm rectangles or circles should be achievable and possibly larger.

Nano-structures would be greatly superior to rolonies due to their precision and addressability. Note that in practice, attaching rolonies to DNA origami in a well-controlled fashion has seemed non-trivial due to non-specific aggregation.

3) Rigid DNA rods on the 1 um scale: 6-helix-bundle DNA origami rods span 450 nm per monomer and 900 nm per dimer, while the largest fully addressable 12-helix-bundle single-stranded tile rods currently span 1 um.

4) Custom aperiodic oligonucleotide arrays with micron-scale feature sizes: these can be readily generated using light-driven chemistry[112,613], thus placing distinct user-defined DNA sequences at defined locations on a surface. Digital micro-mirror devices (DMDs) and spatial light modulators (SLMs) can be used to activate chemical steps in parallel, or alternatively, 2D or 3D spot-scanning or line-scanning

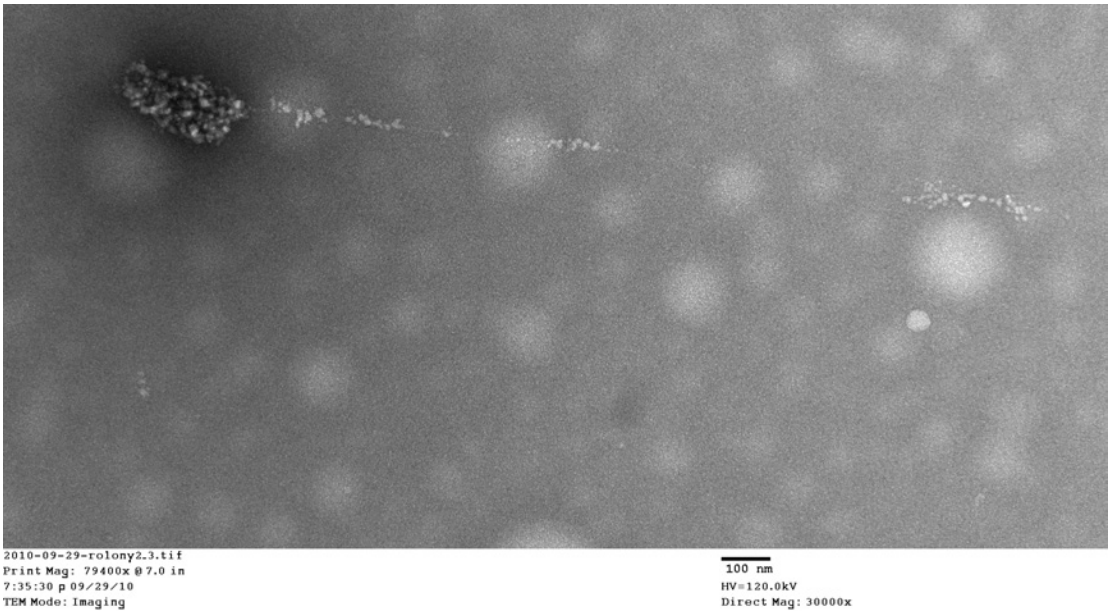

Figure 7.12: Negative stain TEM image of a single rolony on a glow-discharged carbon grid-suspended film.

optics can be used in a serial process. Any of these technologies can in principle generate diffraction-limited feature sizes on the order of 300 nm for UV light. In practice, oligonucleotide arrays have been limited to feature sizes of approximately 2 um, due to light scattering by the liquid medium in the synthesis flow cell. The feature size limit imposed by light scattering depends on the tolerance of the system with respect to optical crosstalk between spots: probably the micro-array manufacturers are being quite conservative about avoiding crosstalk at the expense of larger feature sizes. In the nm2cm application, we may be able to go well below 2 um features with the same optical setup if tolerances are high compared to those for oligonucleotide array synthesis. Furthermore, scattering can be suppressed by several methods, including drying the flow cell between chemical steps and using a dipping lens to limit the optical path length through the fluid. In principle there is no fundamental difference in theoretically attainable resolution between standard lithography and light-directed DNA synthesis/deposition.

Furthermore, the required resolutions of the optical systems involved depend on the parameters

of the components used in later stages of the fabrication process (see below). In the rod-bridging strategy described below, if interference lithography could pattern 200 nm spots at 2 um pitch, and if a 2 um origami rod was available, then DMD optics at the conservative tolerances used by micro-array manufacturers would already suffice for aperiodic light patterning, since DMD-directed DNA synthesis can already achieve $\sim$2 μm pitch in the micro-array field. Alternatively, construction of a periodic array with 200-300 nm spot size at 1 um pitch via interference lithography, followed by DMD-directed oligo synthesis at 1 um pitch would be sufficient, since 1 um rigid origami or SST rods are already available, as are 2D origami or SST assemblies with $\sim$ 200 nm diameter. In the below, we focus on a line-scanning scenario for illustration, assuming that light scattering can be minimized or tolerated, given the dimensions of the other components involved.

Combining capabilities 1 through 4 leads in principle to a strategy for construction of a uniquely addressable lattice in 2D. This strategy is shown in Figure 7.10 and Figure 7.11.

A. Use interference lithography to create a face-centered square lattice with uniform chemistry (i.e., no oligos yet).

B. Use nitrobenzyl photo-chemistry [247] to make these generic dots photo-activatable ("caged").

C. Use spatially patterned light, aligned to the underlying lattice generated in steps A and B, to selectively deposit or synthesize oligos within the interference lithographic spots. With sufficiently high-resolution digital micro-mirror device (DMD) or spatial light modulator (SLM) patterning, this in principle could result in a different oligonucleotide sequence being attached within each spot. Here, however, only $2n$ distinct oligonucleotide sequences ($1x$, ..., nx, $1y$, ..., ny) will be synthesized. Figure 7.10 shows an example where $n = 2$. Because the oligonucleotide sequences are to be arranged in non-crossing lines, it is possible to use focused lines of activation light from a cylindrical lens instead of DMD patterning, although DMD patterning would also be an excellent option. The line width could in theory be diffraction limited at $\frac{}{2*\mathrm{NA}} = 360$ nm for 360 nm UV light with NA $= 1/2$. In practice, the effects of light scattering must be taken into account to determine the effective line width

that is achievable (or the effective spot size, for DMD patterning).

D. Build $2n$ different DNA nanostructure exclusionary objects (DEOs: orange disks in the figure), which bind to the corresponding DNA $2n$ spot types synthesized in step C. These are DNA origami, or other nano-structures, which can expose defined DNA adaptor sequences and which size-exclude one another on the spots from step C. Note that for a pattern with N UALPs, only $2 * n = 2 * N^{1/4}$ distinct DEOs must be synthesized.

E. Bind $2n$ types of rods to the corresponding adaptor sequences on the origami from step D, and at least $2 * n^2$ pairwise rod-coupling DNAs (RCDs: oligos or DNA nano-structures), which bind selectively to oriented rod-pairs via attachment sites on the ends of the rods. The rods will have a preferred orientation defining left-right, front-back and up-down axes for each rod. Each of the 4-way rod junctions will have two RCDs corresponding to the x and y axes. This results in a unique address for each of n^4 junctions, defined by the identity of the pair of RCDs at that site. Thus, each four-way rod junction comprises a UALP. The junction address can be queried by cooperative binding to its two RCDs, e.g., using cooperative hybridization probes. The cooperative hybridization probe can then be thought of as a *second-order RCD* specific to a given UALP. Note that for $n = 100$ spot types per axis, $2 * 100^2$ first-order RCDs must be synthesized. This can be achieved by amplification of oligo library synthesis (OLS) pools[375], which now routinely contain $5*10^4$ distinct user-specified sequences. In contrast, direct synthesis of $n^4 = 10^8$ second-order RCDs is a technical challenge. If not all UALPs must be uniquely addressed, redundancy can be encoded into the the first-order RCDs, allowing a much smaller number of second-order RCDs to be used. Alternatively, clever library synthesis methods may be employed, e.g., using sequential ligations, to produce $O(10^8)$ defined second-order RCDs.

F. Each axis (x and y) coordinate is set by adjacent DEO pairs. These can be thought of as a de Bruijn sequence with alphabet size n, subsequence length $s = 2$, full sequence length $n^s = n^2$. In general, this process leads to a grid of n^4 UALPs. *For* $n = 100$ *and grid spacing of 1 um, we have* $100^4 = 10^8$ *UALPs in a total grid size of 1* cm^2.

Findings from the nm2cm design exercise

Because of the inherent constraints on interference lithography (3× pitch to spot size ratio is easy while 10× is hard), if rigid 2 μm rods were used (for ease of a subsequent non-periodic photo-patterning step using a digital micro-mirror device or spatial light modulator), you would probably want 500 nm × 500 nm origami or SST spot-covering structures. This is still somewhat beyond the limits of DNA origami and SST technology, however. If a 1 μm pitch was used, then probably a 200-300 nm spot diameter would be sufficient, but the non-periodic light patterning would be somewhat more difficult.

7.4.2 Aperiodic polymer nucleation: a strand-efficient strategy for self-assembling long, rigid, fully-addressable nano-rods

We took initial steps to develop new ways to form rigid, multi-micron-long, fully-addressable nano-rods, as are required in the above deterministic nm2cm scheme.

Lambda origami

The phage lambda genome length is 48,490 bp, so a lamdba 6-helix origami bundle (6hb[165]) would have length (48,490)×(0.34 nm)/6 = 2747.76 nm. A dimer of lambda 6hbs would surpass 5 um length, which matches the "feature size" on an Affymetrix Human Tiling 1.0R Array Set. Similarly, a disk-shaped lambda origami would have > 200 nm diameter, matching the available interference lithography spot size. One technical issue is the the presence of single-stranded nicks in the commercially available lambda DNA, preventing its direct use as an origami scaffold. Various groups are pursuing in-house phage production or enzymatic repair of the commercial lambda DNA, in order to solve these problems. On the other hand, it is still unclear whether 3D folding of such a long scaffold will occur reliably. We therefore chose to take a different approach to constructing long nanorods, which we describe in the next subsection.