Chapter 2

Protocols for Self-Assembly and Imaging of DNA Nanostructures

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Abstract

Programed molecular structures allow us to research and make use of physical, chemical, and biological effects at the nanoscale. They are an example of the "bottom-up" approach to nanotechnology, with structures forming through self-assembly. DNA is a particularly useful molecule for this purpose, and some of its advantages include parallel (as opposed to serial) assembly, naturally occurring "tools," such as enzymes and proteins for making modifications and attachments, and structural dependence on base sequence. This allows us to develop one, two, and three dimensional structures that are interesting for their fundamental physical and chemical behavior, and for potential applications such as biosensors, medical diagnostics, molecular electronics, and efficient light-harvesting systems. We describe five techniques that allow one to assemble and image such structures: concentration measurement by ultraviolet absorption, titration gel electrophoresis, thermal annealing, fluorescence microscopy, and atomic force microscopy in fluids.

Key words: DNA, Self-assembly, Atomic force microscopy, Fluorescence microscopy, Nanostructures

1. Introduction

Many of the properties that make DNA useful for genetic information transfer also make it useful for self-assembly of nanostructures. Researchers from physics, chemistry, biology, and computer science use DNA self-assembly to examine the fundamental theories and optimal conditions of self-assembly (1), cooperative effects and emergence; thermodynamics and mechanics of polymers (2–4); and biochemical algorithm execution, logic modules and circuits, error correction techniques, and computational demonstrations (5, 6).

DNA as a molecule has several advantages when compared with other molecules. It is simple enough to be relatively well

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understood (compared with proteins), complex enough to build technically advanced structures (compared with many natural and artificial polymers); it can be chemically synthesized (and nowadays ordered from companies over the internet); and it is stable, reliable, and predictable enough to be confidently handled by researchers with little in the way of chemistry background.

A major challenge in the field to date has been coping with exact stoichiometry requirements needed for high numbers of and/or physically large perfectly assembled structures. Typically, several different short (~5–100 bases) strands bind to each other, and if they are not at the correct absolute and relative concentrations then significant defects occur in the final assembled structures.

Three ideas have been introduced in recent years to overcome this problem. Structures have been assembled that require only one carefully designed sequence which takes advantage of sequence symmetry principles (7). Examples of this include single-sequence DNA nanotubes shown in Figs. 3 and 4. Two other techniques: error avoidance protocols and DNA "origami" that have been also introduced are left for another discussion (8–10).

To face the challenges of stoichiometry, one measures the absolute or relative concentrations of DNA very precisely. This is done in one of the two ways. The absorption of DNA at a light wavelength of 260 nm is dependent on its concentration, base sequence length and structure. If the DNA has a known base sequence and length, and does not have any structure (secondary structure), then its concentration can be related to its absorption (11).

If the DNA does have some structure, then the correct concentration ratio with its complementary strands can be chosen by mixing it at different ratios ("titrating"), and analyzing these using titration gel electrophoresis (12).

Having determined the concentrations of the DNA strands, they can then be mixed in appropriate buffer conditions and slowly annealed over several days from ~90 to 20°C to assemble the desired structures.

To visualize the structures several options may be used (see Note 1). Fluorescent molecules that bind to DNA may be added and the structures viewed with a fluorescence microscope, which is relatively quick and easy. Significantly more challenging is to use an atomic force microscope and fluid cell, visualizing the structures using a scanning probe. This provides much higher resolution.

Several examples of structures that are relatively stable and/or simple to assemble have been developed by Mao and colleagues. These include single sequence lattices and nanotubes and structures consisting of three sequences, including lattices and polyhedra (7, 13–15).

2. Materials

The following lists of materials and equipment are suggested, along with recommended suppliers. There are often many other good suppliers for these, the following are suggestions only, in particular with regards to equipment. All water used should be 18 MΩ and of pH 7–8.

2.1. Absolute Concentration by Ultraviolet Absorption Measurements

1. UV spectrometer (see Notes 2 and 3) (we use a V-630Bio, Jasco, Japan).
2. Cuvettes: 2 (Hellma, Germany).
3. Ultrapure water: 18 MΩ, pH 7–8.
4. Pipettes: 100, 2.5 μL (Eppendorf, Germany).
5. Centrifuge tubes: 0.5 mL (Eppendorf, Germany).
6. Clean compressed air/nitrogen and/or lens cleaning tissue.
7. DNA (Integrated DNA Technologies, USA).

2.2. Relative Concentration by Polyacrylamide Gel Electrophoresis

1. Gel electrophoresis system (such as the PerfectBlue Dual Gel System, Peqlab Biotechnologie, Germany).
2. Electrophoresis Power supply (such as the EPS301, GE Healthcare, USA).
3. Circulating cooling water at 4°C (see Note 4).
4. Detergent (such as 1104-1, Alconox, USA).
5. Ethanol in squirt dispenser.
6. Acrylamide–bisacrylamide: (Rotiphorese Gel 40, Roth, Germany). Warning: Acryilamide is a neurotoxin and carcinogen and should be handled with care in a fume cupboard.
7. 10× TAE Buffer: 400 mM Tris–acetate, 10 mM EDTA, pH 8.3.
8. Ultrapure water: 18 MΩ, pH 7–8.
9. $MgCl_2$: 1 M (Sigma-Aldrich, Germany).
10. TEMED (Tetramethylethylenediamine, Sigma-Aldrich, Germany).
11. APS: (Ammonium persulfate, Sigma-Aldrich, Germany) prepare fresh solutions weekly at 10% w/v in water.
12. Glass beakers: 2, 150 mL (Duran Group, Germany).
13. Pipettes: 100, 2.5 μL (Eppendorf, Germany).
14. Vacuum chamber and pump (such as model 2478257, Duran Group, Germany, or model MVP 015-4, Pfeiffer Vacuum, USA).
15. Aspirating pipettes: 25 mL (BD Falcon, USA).

16. Pipette Filler (VWR International, USA).
17. Bulldog clips.
18. Centrifuge tubes: 0.5 mL (Eppendorf, Germany).
19. 20 mL Syringe.
20. Needle (G 14 0.60 × 30 mm).
21. Gel loading buffer: 4 g sucrose, 25 mg bromophenol blue, 25 mg xylene cyanol, 25 mg Orange G (Sigma-Aldrich, Germany), H_2O to 10 mL. Store in small aliquots at 4°C.
22. DNA ladder (Low Molecular Weight, such as N3233, New England Biolabs, USA).
23. SYBR Gold (Invitrogen, USA). This is toxic and should be handled carefully according to the manufacturer's instructions.
24. Stiff plastic/card sheet larger than the gel plates.
25. Aluminum foil.
26. Staining tray: opaque plastic box with lid slightly larger than the size of the gel.
27. Gel documentation system (Molecular Imager Gel Doc XR, Bio-Rad, USA).
28. DNA strands (Integrated DNA Technologies, USA).

2.3. Thermal Annealing of DNA Nanostructures

1. 10× TAE buffer: 400 mM Tris–acetate, 10 mM EDTA, pH 8.3.
2. $MgCl_2$: 1 M (Sigma-Aldrich, Germany) (see Notes 5 and 6).
3. Water: 18 MΩ, pH 7–8.
4. Membrane filter: 0.02 μm (Anotop 25 Plus, Whatman, England).
5. Beaker: 2–4 L (Duran Group, Germany).
6. Styrofoam box to fit beaker (see Note 7).
7. Boiling water to fill beaker (see Note 8).
8. Screw-top microtubes: 0.5 mL (VWR International, USA).
9. Zip-lock bag.
10. Metal weights (nuts and bolts).
11. Glass thermometer 0–100°C.
12. Pipettes: 100, 2.5 μL (Eppendorf, Germany).
13. DNA strands (Integrated DNA Technologies, USA).

2.4. Fluorescence Microscopy of DNA Nanostructures

1. Fluorescence microscope (see Note 9) (Olympus IX71, Olympus, Japan).
2. 10× TAE buffer: 400 mM Tris–acetate, 10 mM EDTA, pH 8.3.
3. Water: 18 MΩ, pH 7–8.

4. YOYO-1 (Invitrogen, USA). Warning: This is toxic and should be handled carefully according to the manufacturer's instructions.
5. Microscope slides or cover slips, thickness 0 (Menzel, Germany).
6. Fingernail varnish.
7. Wavelength filter (U-MWIB2, Olympus, Japan).
8. Light source (X-Cite Series 120, EXFO Photonic Solutions, Canada).
9. Ascorbic acid (see Note 10).
10. Pipettes: 100, 2.5 μL.
11. DNA product.

2.5. Atomic Force Microscopy in Fluid of DNA Nanostructures

1. Atomic force microscope (we use a Multimode V, Veeco Instruments, USA): operated in intermittent contact (tapping) mode.
2. Fluid cell (Veeco Probes, USA).
3. Mica (50, Ted Pella, USA).
4. Metal puck (Ted Pella, USA).
5. Cantilevers (model DNP-S10, Veeco Probes, USA).
6. 10× TAE buffer: 400 mM Tris–acetate, 10 mM EDTA, pH 8.3.
7. Ultrapure water: 18 MΩ, pH 7–8.
8. Membrane filter: 0.02 μm (Anotop 25 Plus, Whatman, England).
9. Optical microscope.
10. Tweezers (such as model 5599, Ted Pella, USA).
11. Pipettes: 100, 2.5 μL (Eppendorf, Germany).
12. DNA product.

3. Methods

3.1. Absolute Concentration by Ultraviolet Absorption Measurements

1. Single-stranded DNA can have significant secondary structure (where bases in the same strand bind to each other). This alters the extinction coefficient and leads to incorrect concentration determination. With current models and technology, there is no way around this (apart from using strands that are designed not to have secondary structure) and the best way to circumvent this is to use titration gel electrophoresis. However, titration gel electrophoresis requires much more time and effort, and thus is usually only conducted when it is found the

lattice is not forming as desired from the concentrations determined by UV absorption measurements.

2. Turn on spectrometer; allow lamp and system to stabilize for 1–2 h.
3. Appropriate DNA sequences can be dissolved in water to a concentration of 100 μM, this can be determined from the information sheet accompanying the sequences. These should be briefly heated to 60°C and well vortexed to ensure complete mixing.
4. Calculate a molar extinction coefficient for each DNA sequence using the nearest-neighbor model (16–18) – for example using Scitools on the internet from Integrated DNA Technologies (see Note 11).
5. Rinse the cuvette under flowing water, shake water out by hand *hard*, repeat several times. Dry the outer surface with compressed air/nitrogen and lens cleaning tissue.
6. Load the cuvettes with 100 μL water, set parameters (depending on the model of spectrometer, these exact options may not be possible, but there should be similar possibilities):
   (a) Wavelength scan: 350–220 nm
   (b) Scan rate: 400 nm/min
   (c) Bandpass: 1 nm
   (d) Response (integration) time: medium
7. Measure baseline, set baseline subtraction.
8. Add 2 μL of DNA to the measurement cuvette without removing it from the spectrometer, stir with pipette tip for 10–20 s.
9. Measure absorbance, ensure that the absorbance lies between 0.1 and 1 or add or dilute DNA until this is the case. Also ensure that absorbance between 320 and 350 nm is extremely close to zero or apply an offset if it is not, read-off absorbance at 260 nm (note this may not be the peak maximum), (see Notes 12 and 13) see Fig. 1 for an example.
10. Calculate the concentration of DNA using the Beer-Lambert law:

$$\text{Absorbance} = \text{path length} \times \text{extinction coefficient} \times \text{concentration} \quad (1)$$

$$\text{Concentration} = \frac{\text{absorbance}}{\text{path length} \times \text{extinction coefficient}} \quad (2)$$

For example, with an absorbance of 0.5 and an extinction coefficient of 100,000 L/mol·cm and a cuvette of width 1 cm:

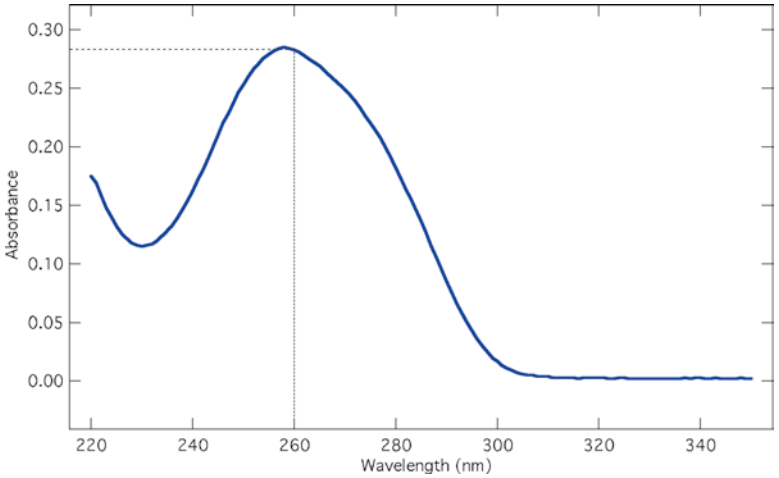

Fig. 1. A representative ultraviolet absorbance curve of a single-stranded DNA sequence. Note that the maximum is not exactly at a wavelength of 260 nm (this is dependent on the sequence); however, the absorbance is measured at 260 nm because this is the value that the extinction coefficient is normally calculated at. Note also that a baseline is measured directly before adding DNA, thus there is no vertical offset necessary, and this can be seen by the 0 values at higher wavelengths (320–350 nm).

$$\text{Concentration} = \frac{0.5}{1\text{cm} \times 100{,}000(\text{L/mol cm})} = 5\ \mu\text{M} \quad (3)$$

11. Calculate the amount of water needed to be added achieve a concentration of 1 μM (the DNA nanostructures are generally assembled at a DNA concentration of 1 μM or less). Continuing the example:

$$\text{Concentration}_{\text{initial}} \times \text{volume}_{\text{initial}} = \text{concentration}_{\text{final}} \times \text{volume}_{\text{final}} \quad (4)$$

$$\text{Volume}_{\text{final}} = \frac{\text{concentration}_{\text{initial}} \times \text{volume}_{\text{initial}}}{\text{concentration}_{\text{final}}} \quad (5)$$

$$\text{Volume}_{\text{final}} = \frac{5\ \mu\text{M} \times 102\ \mu\text{L}}{1\ \mu\text{M}} = 510\ \mu\text{L} \quad (6)$$

$$\text{Volume}_{\text{needed}} = 510 - 102\ \mu\text{L} = 408\ \mu\text{L}. \quad (7)$$

Add this amount and mix with pipette tip.

12. Transfer solution to a centrifuge tube (loss of small amounts here is not critical, if well mixed, the concentration will not change).
13. Repeat steps from three onward for all DNA strands.

3.2. Relative Concentration by Polyacrylamide Gel Electrophoresis

1. Clean electrophoresis plates thoroughly with detergent and rinse thoroughly with water, wipe with ethanol then wipe dry.
2. Place plates with spacers together and set in the electrophoresis unit, with the gap for the comb upward and inward.
3. Squirt ethanol in between the plates until approximately 1/4 full and leave for several minutes to ensure there are no leaks.
4. Mix Acrylamide–bisacrylamide, TAE buffer, $MgCl_2$ solution and water in the following ratio per 10 mL of resulting solution (see Note 14) (Table 1).
5. Place in vacuum chamber for 5 min to remove air from the solution (this speeds up polymerization).
6. Check electrophoresis plates to see that there are no leaks, pour out ethanol. If there are leaks, pull the plates apart, put them back together again and recheck.
7. Remove solution from vacuum chamber. Divide solution gently into two (for two gels) without mixing in unnecessary air.
8. Prepare pipettes and tips for the APS and TEMED solutions.
9. Under the fume-hood, working without pause, add APS solution at 50 μL per 10 mL of solution to the first flask, and swirl gently to mix. Add TEMED (closing TEMED lid immediately) at 10 μL per 10 mL of solution to the second flask, and swirl gently to mix.
10. Immediately pipette (slowly to avoid bubbles) solution between the first set of gel electrophoresis plates, making sure no air bubbles get trapped. Fill until the level reaches the bottom of the gap for the comb.
11. Insert the comb, ensure that it traps no air bubbles, if this is the case take it out and reinsert it. It is useful to have the top of the comb slightly (~1 mm) above the upper edge of the glass plates (see Note 15). Use bulldog clips to hold the comb securely into position (otherwise, the expanding polymerizing gel displaces it).
12. Repeat steps 9–11 for the second solution.
13. Optimally, wait 90 min for the gel to polymerize (shorter times and the gel does not have polymerized completely with even and static pore sizes, longer than a couple of hours and the gel swells and dries) (see Note 16).

Table 1
Pipetting instructions for 10 mL of a 20% TAE/Mg^{2+} polyacrylamide gel

Polyacrylamide gel (%)	Acrylamide–bisacrylamide (37.5:1) (mL)	10 × TAE (mL)	1 M $MgCl_2$ (μL)	H_2O (mL)
20	5.00	1	120	3.88

14. Fill buffer between the gels and into the reservoirs of the electrophoresis unit.
15. Remove one of the combs and immediately flush the wells completely with buffer using the syringe and needle to remove unpolymerized acrylamide (see Note 17).
16. Repeat for second comb.
17. Set out nine 0.5 mL centrifuge tubes in a holder.
18. Calculate – from the concentrations (in μM) determined by UV absorption – the volume of the first strand needed for 100 ng (shorter strands run relatively faster and spread relatively wider, thus may need to be relatively more concentrated when run with longer strands). Use the formula:

$$\text{Volume}(\mu\text{L}) = \frac{100 \times 10^{-9}\,\text{g}}{\text{molecular mass}(\text{g/mol}) \times \text{concentration}(\text{mol}/\mu\text{L})} \quad (8)$$

Add this volume of the first strand to each tube.

19. Calculate the quantity in moles of the first DNA strand that this volume holds using:

$$\text{Quantity} = \text{concentration} \times \text{volume} \quad (9)$$

20. Calculate the volume needed of the second strand for each these (suggested) factors of the first DNA strand: 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2.

$$\text{Volume} = \frac{\text{quantity}}{\text{concentration}} \times \text{factor} \quad (10)$$

21. Add each volume to one of the tubes (there is one tube left).
22. Calculate the amount of 1 M $MgCl_2$ solution needed to be added to each tube to give a concentration of 12.5 mM. Add this amount to each respective tube.
23. Close the tubes, label them, and vortex briefly.
24. Using a Polymerase Chain Reaction (PCR) machine, heat tubes to 90°C (lid temperature 92°C) for 1 min and cool evenly in small steps to room temperature over 1 h. This should ensure hybridization of the strands.
25. Add 0.4 μL of low molecular weight DNA ladder to the remaining tube. Pipette 3 μL of loading buffer into each tube and vortex.
26. Prepare running buffer and salt (1 L or more depending on the size of the electrophoresis unit): 20 mL 50× TAE buffer, 12.5 mL 1 M $MgCl_2$, and water to fill to 1 L. Refrigerate until at 4°C. The buffer stock should be the same as that used for the gel and at the same concentration (1X).
27. Flush wells again with buffer using the syringe and needle, immediately before loading wells with DNA.

28. Load the first well with prepared DNA ladder solution and the rest of the wells with the DNA strand solutions in order.
29. Connect the electrophoresis unit to the circulating cooling water. This ensures that the gels remain cool while running and do not thermally denature the hybridized DNA samples (see Note 4).
30. Connect the electrophoresis unit to the power supply and run at a constant voltage of 10 V per cm gel length until the yellow loading dye runs to the bottom of the gel (typically 1–3 h).
31. During this time, the staining solution can be prepared. An opaque plastic container with a flat bottom just larger than the gel is needed, and this is filled with buffer to a depth that would be the same as the thickness of the gel. SYBR Gold is added at a ratio of 1 μL per 10 mL, this is covered with an opaque lid or aluminum foil and allowed to mix on a rotator at a small angle to the horizontal at the lowest speed (<1 Hz).
32. Prepare a sheet of aluminum foil about three times the size of the gel flat on the table.
33. When the gel is finished, turn off the power supply, and remove the gel from the unit. Use a thin blade or plastic scraper to carefully remove the top gel plate. A few droplets of water between the plate and the gel can help. Spread the aluminum foil over the top of the gel, then place a flat stiff piece of plastic over the aluminum foil. Use this to support the gel "sandwich," as it is flipped up so that the bottom gel plate is now on top. This plate is also removed, two opposite sides of the foil are trimmed to the gel width, the remaining two sides are used to lift and support the gel, and the whole lot is placed in the staining container. The lid is placed on and the gel is left to stain on the rotator for 30–60 min.
34. The rotator is then stopped, the staining solution is removed using the 25 mL pipette and disposed of as toxic waste.
35. The gel is lifted out using the aluminum foil support and onto the UV light box. The gel can then be slid off the aluminum foil onto the glass using a few drops of water as lubricant if necessary.
36. The aluminum foil is stored safely for next time or disposed of as toxic waste.
37. The gel is examined/photographed using the gel documentation system and an appropriate wavelength filter for SYBR Gold making sure the focal distance of the camera is set to reach the gel and not to the inner UV bulbs (see Fig. 2 for an example).
38. The correct ratio of DNA strands is chosen by comparing the bands to see the one-to-one binding ratio. This ratio can be used with the excess (not used in the gel analysis) DNA to self-assemble the desired structure.

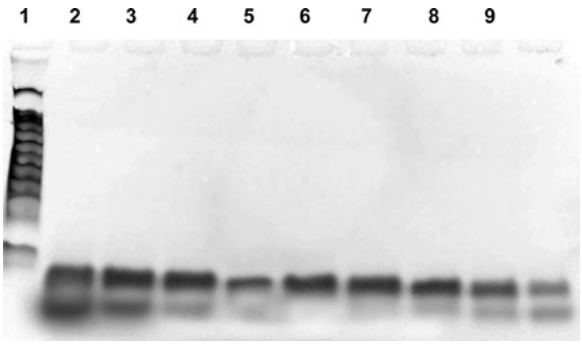

Fig. 2. A native PAGE gel electrophoresis titration analysis of the concentration of two complementary DNA strands. Each strand is 8 bases long, the gel is 20% and was run at 10 V/cm for 3 h. Lane 1: low molecular weight DNA ladder (766–25 bp); Lanes 2–9: relative concentration increments from factors of 2 to 0.25 as listed in the protocol. The upper band represents the hybridized DNA, the lower band excess single-stranded DNA: Lane 6 has the correct ratio of the two strands in this case, as there is no excess single-stranded DNA.

3.3. Thermal Annealing of DNA Nanostructures

1. The DNA strands are mixed at the correct concentrations and thermally annealed in appropriate buffer conditions (see Note 18).
2. All stock buffer and salt solutions should be filtered with a 0.02 μm membrane filter before use, this is quite critical.
3. The final volume and concentration of required DNA product is decided. Typically, volumes between 20 and 2,000 μL are produced, at concentrations between 50 and 1,000 nM. As an example, 100 μL of 5-stranded lattice at 500 nM total concentration is chosen.
4. The concentration needed of each strand is calculated, this depends on the stoichiometry of the strands in the final structure.

$$\text{Concentration of strand} = \frac{\text{stoichiometry of strand}}{\text{total stoichiometry}} \times \text{total concentration} \quad (11)$$

5. For a strand mixed at a ratio of 1 with a total of five strands, this is

$$\text{Concentration of strand} = \frac{1}{5} \times 500\ \text{nM} = 100\ \text{nM} \quad (12)$$

6. The volume needed from each DNA strand solution is calculated using Eq. 4. For example, if each strand solution has been diluted to 1,000 nM, then for 100 μL final volume:

$$1{,}000\,\text{nM} \times \text{volume}_{\text{initial}}\,\mu\text{L} = 100\ \text{nM} \times 100\ \mu\text{L} \quad (13)$$

$$\text{Volume}_{\text{initial}} = \frac{100\ \text{nM} \times 100\ \mu\text{L}}{1{,}000\ \text{nM}} = 10\ \mu\text{L} \quad (14)$$

7. The calculated volume of each strand is pipetted into a screw-top microcentrifuge tube.
8. The volume of 50× TAE buffer needed for a 1× solution of 100 µL is calculated using the Eq. 4. For example:

$$50 \times \text{volume}_{\text{initial}}\ \mu\text{L} = 1 \times 100\ \mu\text{L}. \quad (15)$$

$$\text{Volume}_{\text{initial}} = \frac{1 \times 100\ \mu\text{L}}{50} = 2\ \mu\text{L} \quad (16)$$

   This volume is pipetted into the screw-top microcentrifuge tube.
9. The volume of 1,000 mM (1 M) $MgCl_2$ solution needed for a 12.5 mM solution of 100 µL is

$$1{,}000\ \text{mM} \times \text{volume}_{\text{initial}}\ \mu\text{L} = 12.5\ \text{mM} \times 100\ \mu\text{L}. \quad (17)$$

$$\text{Volume}_{\text{initial}} = \frac{12.5\ \text{mM} \times 100\ \mu\text{L}}{1{,}000\ \text{mM}}\mu\text{L} = 1.25\,\mu\text{L} \quad (18)$$

   This is pipetted into the screw-top microcentrifuge tube.
10. Enough water is added (here 46.75 µL) to make up the total required volume (here 100 µL).
11. The lid is screwed *tightly* onto the tube, and it is briefly (10 s each) centrifuged, vortexed, then centrifuged again.
12. The tube is placed into the zip-lock plastic bag with enough weights (nuts and bolts) to make sure it sinks, the bag is rolled up and secured with a rubber band, and a few small holes are made in it to allow the air to escape.
13. Enough (tap) water is boiled to fill the large (2–4 L) beaker. The bag with weights and microcentrifuge tube is placed in the bottom of the beaker, along with the thermometer. The beaker is filled with water just below boiling point.
14. The beaker is placed inside the Styrofoam box which is closed, and this is placed in a safe place and left for 48 h or until the water has cooled to room temperature (~20°C).
15. The microcentrifuge tube is then taken out and dried, to ensure that no water droplets on the outside enter upon opening the lid of the microcentrifuge tube.

3.4. Fluorescence Microscopy of DNA Nanostructures

1. If the chosen DNA structure has dimensions on the order of several micrometers or greater (for example, a large two-dimensional lattice), then it may be viewed with a fluorescence microscope if it is "dyed" using an intercalating

fluorescent molecule, such as YOYO-1 (Invitrogen). This binds between base pairs of double-stranded DNA, optimally at a ratio of 1 dye molecule per 5 base pairs (see Note 19).

2. Thus, if the total number of bases of the 5 strands is 100, then the number of base pairs is 50. The YOYO-1 stock solution is 1,000,000 nM (1 mM) and typically one dyes a DNA structure solution of 10 μL. The volume of YOYO-1 needed is

$$1,000,000\,\text{nM} \times \text{volume}_{\text{initial}}\,\mu\text{L} = \frac{50\,\text{base pairs}}{5} \times 500\,\text{nM} \times 10\,\mu\text{L} \quad (19)$$

$$\text{Volume}_{\text{initial}} = \frac{(50\,\text{base pairs}/5) \times 500\,\text{nM} \times 10\,\mu\text{L}}{1,000,000\,\text{nM}}\,\mu\text{L} = 0.05\mu\text{L} \quad (20)$$

3. This volume is not realistic to pipette, so the YOYO-1 is diluted in 1× TAE buffer, for example (100× dilution): 0.5 μL YOYO-1 stock solution, 2 μL 50× TAE buffer, 97.5 μL water in a plastic (YOYO-1 binds to glass containers) microcentrifuge tube.
4. With 100× dilution, 5 μL of this is pipetted into a microcentrifuge tube.
5. Using a cut-off tip 10 μL of DNA structure solution is added (see Note 20).
6. Ascorbic acid is used to minimize photobleaching of the fluorescent molecules. It is prepared at 100 mM in a volume of, for example, 10 mL. With a molecular mass of 176.12 g/mol this is:

$$\text{Mass} = \text{molecular mass} \times \text{concentration} \times \text{volume} \quad (21)$$

$$\text{Mass} = 176.12(\text{g/mol}) \times 100 \times 10^{-3}(\text{mol/L}) \times 10 \times 10^{-3}\,\text{L} = 176\,\text{mg} \quad (22)$$

This mass is dissolved in 10 mL of water and stored in a light proof jar. New solutions should be made every week.

7. This is added to a final concentration of 10 mM, so with 5 μL of YOYO-1 solution and 10 μL of DNA solution, one adds approximately 1.5 μL.
8. The fluorescence microscope is prepared, the light source is switched on, the correct filter is loaded, and an appropriate objective (40× air) is chosen.
9. For a very quick look, 1 μL of dyed-DNA solutions can be pipetted using a cut-off tip onto a Number 0 cover slip and placed on the microscope. There are large amounts of background fluorescence, but normally the structures themselves can also be seen.

10. For a better image, this process is repeated but the droplet is covered with a second cover slip and the edges of the cover slip are sealed with fingernail varnish. There is much less fluorescence background using this technique.

3.5. Atomic Force Microscopy on Fluid of DNA Nanostructures

1. The DNA structures bind in solution to a mica surface given the correct conditions, and the topography of the structure can then be measured/"visualized" using an atomic force microscope. The precise details of this protocol vary greatly depending on the model of atomic force microscope used.
2. The microscope and control computer are switched on, and the software loaded.
3. It is generally much easier to set the correct engage height of the cantilever above the mica surface in air, as the surface of the mica is difficult to see with an optical microscope when submerged in buffer.
4. The cantilever holder is set to a distance far enough from the surface to ensure that upon loading the cantilever the tip of the cantilever does not contact the surface.
5. Using a small optical microscope and tweezers, the tip is loaded correctly in the fluid cell.
6. The mica is loaded into the microscope (initially without any sample).
7. The fluid cell/holder is loaded into the microscope.
8. With the aid of the optical microscope that comes with the atomic force microscope, the laser spot is aligned onto the very end and center of the cantilever. This is important!
9. If there is a reflecting mirror, its angle is adjusted so the laser shines close to the center of the photodiode window.
10. The photodiode position is adjusted so that the laser is reflected directly at its center.
11. The steps 8–11 are repeated to fine-tune the system to ensure that the detected signal is high (with the laser reflecting very close to the end of the tip) and the deflection signal (relating to the laser reflecting onto the center of the photodiode) is minimal.
12. The surface of the mica is brought into focus of the optical microscope. The surface can be difficult to observe (being semitransparent), it can help to move around looking for cracks on the surface. The cantilever is brought to a level just before it comes into perfect focus, indicating that it is very close to, but not in contact with the surface (see Note 21). As a guide, as the cantilever moves closer to the focal height, a double image of the cantilever is seen, and this merges into one at the focal height.

13. The mica is then removed (this probably entails removing the fluid cell/holder also) and a fresh surface is prepared using (opaque) masking tape (see Note 22). This is best done by pressing firmly a strip of tape flat onto the mica on a table, lifting the far edge of the mica up so that it stands perpendicular to the table on its bottom edge, and peeling the tape slowly and evenly downward. A thin, complete, *shiny* layer of mica should have adhered to the tape. Quality of results may depend on the orientation of the tape relative to the mica, there is an optimal direction found by experimenting.
14. 5 μL of DNA structure solution is carefully pipetted onto the center of the mica using a cut-off pipette.
15. 1× TAE 12.5 mM $MgCl_2$ (filtered through a 0.02 μm membrane filter before use) buffer solution is added to the mica, and/or the surface of the fluid cell/holder and/or through a tube into the fluid cell, dependent on the system. Care should be taken that no air bubbles are trapped on the cantilever.
16. The mica is carefully reloaded into the microscope.
17. The buffer has a different refractive index so that the laser beam travels a slightly different path, steps 8–11 may need to be repeated with small changes to optimize the measured laser signal.
18. The cantilever is tuned (generally using a function in the software) to ~5% below its resonant vibration frequency (see Notes 23 and 24). The amplitudes used are much smaller than those in air, and should be adjusted to be above the level at which the tip sticks to the surface when imaging, but not so large that the sample is damaged by the tip's vibrations. This is best determined through trial and error.
19. The amplitude set point (ratio of the free amplitude of vibration to the amplitude while imaging) is generally set just below 1, for example 0.98 (or 98%). However, this can vary greatly dependent on the system.
20. The imaging parameters are then set. Initial scan sizes and speeds are set small (1 μM) and slow (0.5 Hz) to prevent damage to the tip as it first "contacts" the surface.
21. The most important two other parameters are the integral and proportional gains, these should be initially set extremely small (exact values are system dependent).
22. The number of measurements per scan line (pixels) can be set to 256.
23. The "engage surface" function of the microscope is actuated. Several errors may occur during this process (see Note 25).
24. When correctly engaged on the surface, the imaging parameters are optimized. It is generally helpful to first withdraw

the cantilever from the surface slightly (several hundred nanometers), retune the cantilever to the correct frequency and drive amplitude, before reengaging the surface.

25. There are generally at least two "views" in the software, an "image" view and an "oscilloscope" view of the trace and retrace of the current line scan profile. The amplitude set point, which is slowly increased until the tip just no longer contacts the surface, is the best seen in the oscilloscope mode when the trace and retrace scan profiles significantly depart vertically from each other. It is then decreased to just below the value when they come back vertically on top of each other for optimal imaging.
26. The integral gain is gradually increased so that the trace and retrace scan profiles correlate optimally with one another without excessive noise being introduced into the signals.
27. The proportional gain is then adjusted similarly.
28. The scan size and speed can then be increased and suitable DNA structures for imaging are found.
29. The desired scan size is set, and the scan speed is slowed to 1–3 Hz, and the number of measurements per line is increased to 512 or 1024. An image is then captured.
30. Care should be taken that no imaging artifacts like double tip images (coming from broken tips with two or more points) or material sticking to the tip occur, if so the cantilever should be changed and the whole process repeated.
31. When a new sample is required, the process can be simplified if care is taken. The tip is withdrawn approximately 100 μm from the surface. If the cantilever is not moved within its holder, a thin layer of mica is removed from the same mica sample, and the mica is returned afterward to the same position in the microscope, then the cantilever should be relatively close to the surface and should not need long for the engage procedure.
32. Examples of atomic force microscopy images taken using this method are shown in Figs. 3 and 4.

4. Notes

1. There are other options, such as Transmission Electron Microscopy, that are not discussed here.
2. There are now ultraviolet absorption spectrometer systems that are designed to quickly measure μL volumes in the μM range. These may not be accurate enough for the standards required here.

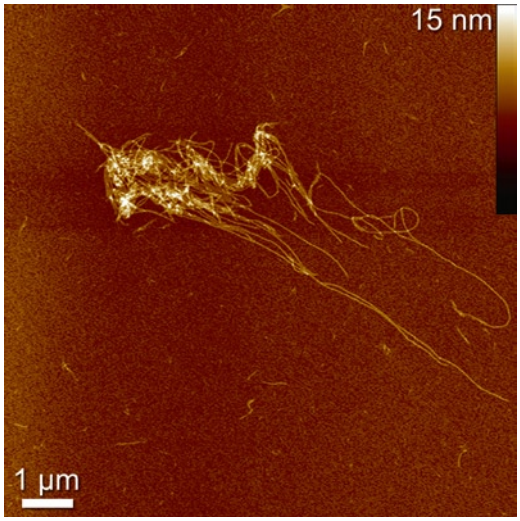

Fig. 3. An atomic force microscopy image of self-assembled DNA nanotubes that have clumped together. Excess DNA that did not form nanotubes can be seen as a background carpet. This height image was captured using "tapping mode" in buffer on mica. Scale bar 1 µm, height scale 15 nm.

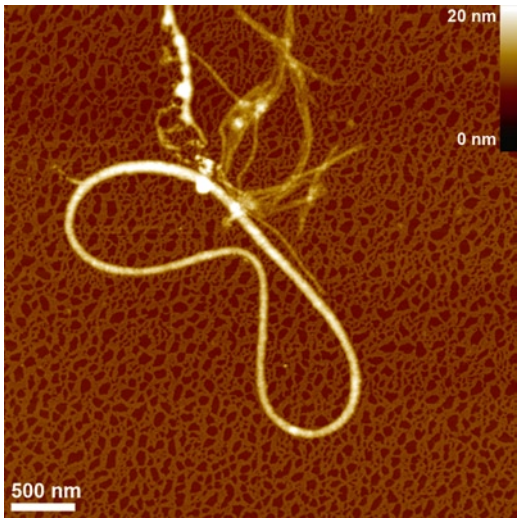

Fig. 4. An atomic force microscopy image of a self-assembled DNA nanotube that has connected both its ends (by chance). Thinner-tangled nanotubes can also be seen. This height image was captured using "tapping mode" in buffer on mica. Scale bar 500 nm, height scale 30 nm.

3. For DNA concentration measurements, temperature control (via a programmable water bath or Peltier element) of the sample while measuring absorption is not usually necessary; however, this is useful for making DNA melting measurements, often used to assist in analyzing these structures.
4. The gels should be "run" at 4°C, and this can also be achieved by placing the system in a cool room or refrigerator.

5. Salts are critical for these structures – they provide electrostatic shielding that allows the negative DNA strands to bind together. In standard DNA hybridization, salts with monovalent ions like Sodium Chloride are used. For the structures discussed here, salts with divalent ions such as $MgCl_2$ are used, and these allow the DNA to "fold" into the desired complex structures. 12.5 mM concentration is generally chosen, and this is high enough for binding and folding and allows the large DNA structures to bind to the mica surface in atomic force microscopy. Higher volumes may cause condensation of the DNA or unwanted significant binding of any excess single-stranded DNA to the mica surface.
6. Alternatively, a large thermos flask can be used instead of a beaker and polystyrene box.
7. Instead of annealing in hot water, a programmable PCR machine can be used with small temperature steps, ensuring that the lid is a few degrees warmer than the heating block.
8. This can be normal tap water.
9. A Total Internal Reflection Fluorescence (TIRF) microscope is advantageous to remove background fluorescent light from sources not in focus (at the surface), but imaging is certainly manageable without such a system.
10. This helps to prevent photobleaching of the fluorescent molecules.
11. The extinction coefficient is calculated using a nearest-neighbor model. One can, for example, make use of the online calculator "Scitools" provided by Integrated DNA Technologies at http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/
12. The absorption spectrum of DNA is sequence dependent, and thus the UV absorption peak of DNA may be found between approximately 260 ± 15 nm; however, the absorption should be measured at the wavelength that the extinction coefficient is calculated for, which is generally 260 nm.
13. Depending on the cuvette, it may be necessary to stir the sample with a pipette tip to remove an air bubbles and make a second measurement to ensure reproducibility.
14. This gel concentration is suitable for DNA strands up to 100 bases long, for longer strands smaller gel concentrations are needed.
15. The gels can be stored for several days in their glass plates if wrapped with tensioned rubber bands to keep the comb pressed securely into the wells (with the upper edge of the comb above the glass taking some of the tension) and kept in buffer. Metal clips oxidize in buffer and should not be used.

16. Gels may be stored for several days if wrapped securely with rubber bands – ensure that there is tension holding the comb correctly in place; otherwise, the wells fill with unpolymerized acrylamide – and stored in 1× TAE solution.
17. A small battery head lamp can help to make the gel wells more visible (Petzl Tikka).
18. We have also developed a technique that does not rely on thermal annealing but rather on the basis of dilution of DNA denaturing agents in the buffer (19).
19. One YOYO-1 molecule every five base pairs gives the best ratio of minimal structural deformation of the DNA helix to maximal fluorescence intensity, giving an optimal signal to noise ratio (20).
20. The DNA structures can be so large that the normal hole diameter of the pipette tip damages them as they pass through.
21. It is important to come into focus on the surface from a starting point far away from the surface; otherwise, one may focus on the reflection and not on the real surface.
22. One can see the thin peeled layers of mica with better contrast if the tape is opaque.
23. The feedback loop in the electronics of the microscope works optimally at values just below the resonance frequency of the cantilever.
24. In the 10 kHz range with small buffer volumes, there may be a resonance in the buffer itself which can be heard as a high-pitched tone. This is normal.
25. Several errors often occur while engaging, if these occur, the engage should be aborted. The amplitude may change significantly (more than 10%), thus the cantilever should be retuned with the correct amplitude. The deflection errors may increase significantly, particularly if the buffer was initially at a different temperature to the fluid cell/holder and/or mica, thus the errors should be brought to a minimum. Once corrected, the engage can be restarted.

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