

Biophysics advanced internship - experiment L2B

DNA origami

Working group of Prof. Tim Liedl

Summer semester 2020

Preliminary remarks

The DNA origami technique uses a long, viral, single-stranded DNA molecule as a framework strand (the scaffold), which is brought into the desired shape by hundreds of short pieces of DNA (the staples). The aim of this internship is to gain insight into the DNA origami technique. For this purpose, each student will produce the scaffold DNA with the help of bacteria and bacteriophages, design a DNA origami structure on the computer, manufacture a predesigned structure in the laboratory and then analyze it with the help of electron microscopy. For the Corona-affected term, we will skip the scaffold production and proceed straight to DNA origami design, purification and analysis.

Preparation

Please read these instructions carefully to prepare for this experiment. The person in charge explains the individual steps involved in carrying out the experiment in more detail in the laboratory. In addition, you need to download, install and try out the **caDNAo** (<http://cadnano.org>; download the installer from https://drive.google.com/file/d/1LkIRJznDhtB_zmfuN_coNeR4I_9k1kok/view?usp=sharing) software on your computer, which is used for structural design of the DNA origami. The following two tutorial videos give a very understandable overview of how the geometry of DNA can be used to build three-dimensional structures:

- <https://youtu.be/cwj-4Wj6PMc>
- <https://youtu.be/EabqNaYAI7o>

The following publications should also be read for further preparation:

- Paul Rothemund: Folding DNA to Create Nanoscale Shapes and Patterns (Nature 2006)
- Shawn Douglas et al.: Self-assembly of DNA into nanoscale three-dimensional shapes (Nature 2009)
- Tim Liedl et al.: Self-assembly of three-dimensional prestressed tensegrity structures from DNA (Nature Nanotechnology 2010)

A good overview of the various promising uses of the DNA origami with a good introduction to the method is the following article:

- Thomas Tørring et al.: DNA origami: a quantum leap for self-assembly of complex structures (Chemical Society Reviews 2011)

If you have any questions about the experiment, you can of course contact the supervisor at any time in advance.

Experimental timeline (regular)

Day 1

Start: 9:00 a.m.

Duration: approx. 10 hours

- Oral query about the understanding of the subject
- Creation of the scaffold DNA
- Computer-aided design of a DNA origami structure

Day 2

Start: 9:00 a.m. - Duration: approx. 3 hours

- Gel electrophoresis of the prepared scaffold
- Staining of the folded samples
- TEM analysis of the stained samples

Experimental timeline (Corona)

Day 1

Start: 9:00 a.m.

Duration: approx. 10 hours

- Oral query about the understanding of the subject
- Folding of DNA origami
- Computer-aided design of a DNA origami structure
- Staining of the folded samples
- TEM analysis of the stained samples

1 Theoretical foundations

1.1 The DNA molecule

Deoxyribonucleic acid (from here on always referred to by the much more common English abbreviation **DNA**) is an essential component of every known living being as a store and carrier of genetic information from one generation to the next. DNA is a polymeric macromolecule and consists of repeating subunits, the nucleotides. Each nucleotide consists of the monosaccharide deoxyribose, one of the four nucleobases adenine (A), cytosine (C), guanine (G) and thymine (T) and a mono-, di- or triphosphate. The nucleobase is connected to the 1' C atom of deoxyribose and together they form a so-called nucleoside. The phosphate is bound to the 5' C atom of the sugar ring in the nucleoside and thus completes the nucleotide. Nucleotide molecules play a central role in the metabolism of all living organisms: they serve as energy carriers (e.g. adenosine triphosphate ATP), participate in signal cascades (e.g. cyclic adenosine monophosphate cAMP) and are part of important cofactors in enzymatic reactions (e.g. flavin mononucleotide FMN).

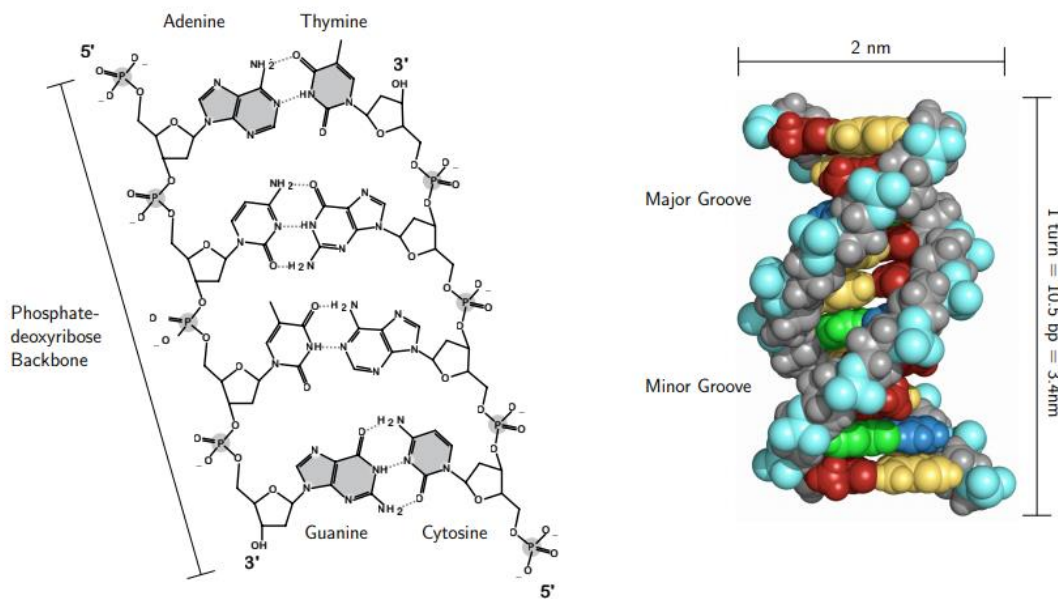


Figure 1: Chemical structure of the DNA double helix (left) and a ball-and-stick model of the B-form DNA (right)

Each of the four possible monophosphate nucleotides can be connected to the 3' C atom of the sugar ring of another nucleotide via the phosphate. This means that long polymers with any composition of nucleotides and thus any sequence can arise. These molecules are then called polynucleotides or single-stranded DNA (ssDNA). As a consequence of the asymmetrical phosphodiester bond between two neighboring sugar rings, the polynucleotide can now be assigned a direction. One end of the ssDNA ends with a phosphate group at the 5' C atom (5' end) and the opposite end with a hydroxyl group at the 3' C atom (3' end) of the deoxyribose (see Figure 1). This directional information plays an important role in the enzymatic amplification of the DNA and thus each DNA strand has a reading direction (DNA sequences are usually specified from the 5'-end to the 3'-end).

Hybridization

The four bases can form base pairs (bp) via hydrogen bonds. A and T together form two, G and C together form three hydrogen bonds. In aqueous solutions, the four bases also form hydrogen bonds

with the water molecules. Since the aromatic rings of the nucleotides are positioned almost perpendicular to the length of the DNA strand, the π -orbitals of neighboring rings overlap. The carbon rings align with each other and water molecules are displaced from the space between the bases. This process is known as base stacking and the sum of these stacking interactions stabilizes the DNA double helix. Two ssDNA molecules with an opposite complementary sequence can thus join together. This process is called hybridization, and the two antiparallel strands together form a double helix. The right-handed B-form DNA is present under physiological conditions. One full turn of the double helix corresponds to 10.5 bases and extends over approximately 3.4 nm. The diameter of a double helix is 2 nm and the vertical distance between two adjacent bases is 0.34 nm. Other shapes are e.g. the A-form and the Z-form DNA, which, however, do not play a role in understanding this practical experiment.

The hybridization of two single strands to form a double-stranded molecule can be reversed by supplying heat. This process is known as melting. Every dsDNA molecule has a characteristic melting temperature, which depends on the sequence and especially the length of the strand: long molecules have a higher melting temperature than short molecules.

Optical properties

While each nucleobase has a different absorption maxima, we consider a general DNA molecule as having an absorption maximum of UV light at a wavelength of 260 nm. The total absorption of a DNA molecule depends on the sum of the absorption of the individual nucleotides and the interactions between neighboring bases. Because of these interactions, a ssDNA molecule absorbs less light than the pure sum of the individual bases and a dsDNA molecule absorbs less light than the sum of the two individual strands it contains. The difference in the absorption of ssDNA and dsDNA is used, among other things, for the experimental determination of the melting temperature of DNA molecules with the help of UV photometry. The determination of the concentration of DNA in solutions is also carried out with the help of UV photometry: For this purpose, the absorption is measured at 260 nm and the concentration is calculated with the help of Lambert-Beer's law (more on this later).

1.2 DNA as a building material in nanotechnology

DNA has emerged as a promising material in nanobiotechnology recently. The following properties make it an ideal building material: DNA is stable and easy to handle in aqueous solutions and a variety of buffers. It is commercially available for a relatively low price and sequences up to 100 bases in length can be chemically synthesized easily. DNA is biocompatible, modular and has a programmable sequence.

Genetic engineers have been using dsDNA molecules with complementary protruding single-stranded ends (so-called sticky ends) to produce new linear DNA constructs since the 1970s. In the early 1980s, the chemist Nadrian Seeman from New York University postulated that it was possible to create periodic three-dimensional networks from DNA. At the beginning of the 1990s, his group presented the first DNA-based supramolecular structure: a 3D cube made up of six single-stranded DNA loops (see Figure 2a).

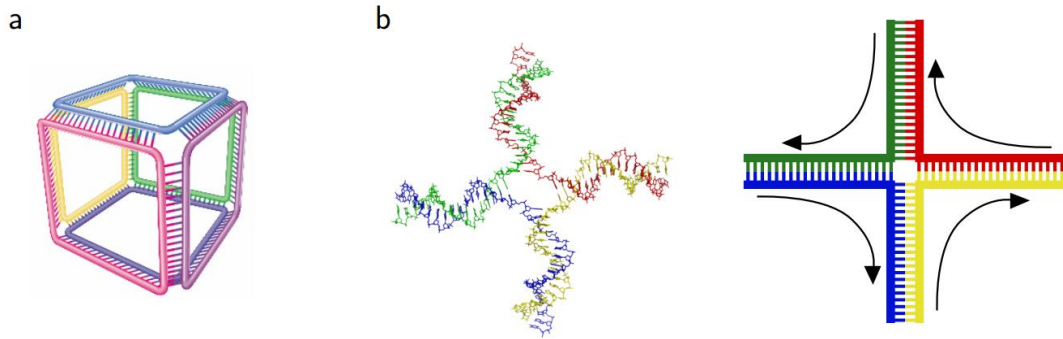


Figure 2: (a) Ned Seeman's DNA cube, consisting of 6 single-stranded DNA loops. (b) Holliday Junction: on the left is the molecular structure showing the single strands pass from one double helix to the other, none of the bases remains unpaired; on the right the same structure is shown again schematically.

A central motif in structural DNA nanotechnology is the so-called Holliday Junction (named after the Australian scientist Robin Holliday). Holliday junctions are covalent phosphate bonds between two DNA double strands. In organisms, these crossings play an important role in the recombination of homologous sequences during cell division. Here the Holliday Junction can slide freely between two connected double helices. An immobilized junction, on the other hand, can be achieved through asymmetry of the sequences. Such junctions can be used to create a spatially fixed connection between two double helices.

1.3 DNA origami

The DNA origami technique, invented by Paul Rothemund in 2006, uses a long, single-stranded viral DNA molecule (called a scaffold) to create desired patterns and shapes on the nanometer scale. The scaffold is folded into the desired shape by hundreds of short oligonucleotides (the so-called staples) (a schematic representation of this process is shown in Figure 3). Rothemund used a 7249 base long modified version of the genome of the M13mp18 bacteriophage as a scaffold (more information on this virus in 1.4). The sequence of this scaffold is known and it can either be purchased commercially or produced relatively easily in the laboratory with the help of *E. coli* bacteria. In contrast to the Japanese art of paper folding, the DNA origami technique described here is based on molecular self-assembly. The scaffold is mixed with the staples and divalent cations (usually Mg^{2+}) in a buffer solution.

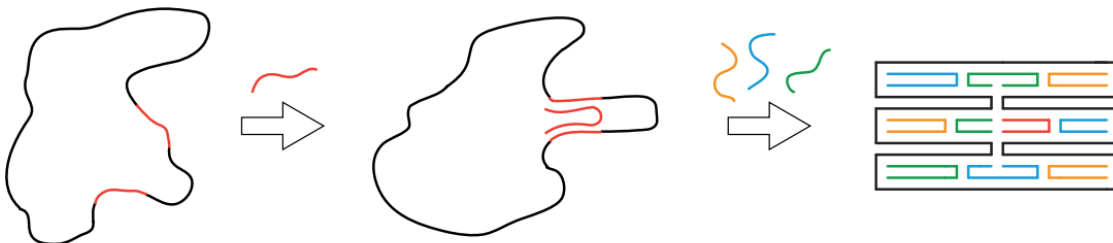


Figure 3: The long, single-stranded scaffold (left) is folded at a specific position by the short staple, which has two complementary points on the scaffold (middle). When all of the staples are added, the scaffold is folded into the previously defined shape (left). The adjacent anti-parallel double helices are held in the desired shape by staple double crossovers in the form of immobilized Holliday junctions.

This mixture is then heated to a temperature at which all DNA molecules become single-stranded, i.e. melted, and slowly cooled to room temperature over a period of hours to days. As it cools, the staples bind to their complementary locations on the scaffold and an array of antiparallel double helices is formed. The rate of cooling is kept low to prevent the structures from being trapped in local minima in the energy landscape. In a folded structure, the position of each individual base is known. Chemical modification of the bases, such as the attachment of fluorescent molecules or biotin, now allow each individual base to be addressed within the structure with a theoretical resolution of 0.34 nm (the distance between two neighboring bases in the double helix). This property together with the high parallelism of the method (around 100 billion objects are created simultaneously by self-assembly in a 50 μL drop of sample solution) ensure the astonishing application possibilities of this technique. Figure 4 shows one of the two-dimensional structures that Rothemund presented in his publication.

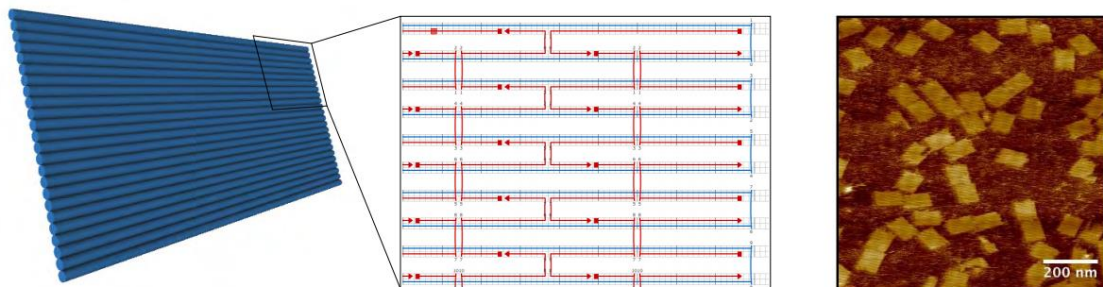


Figure 4: Representation of a 2D DNA origami rectangle. It measures 70 x 100 nm and is 2 nm high (corresponding to the diameter of the double helix). Left: schematic representation of the design: it consists of 24 anti-parallel double helices, shown as cylinders. Middle: Excerpt from the design scheme. It shows the path of the scaffold through the structure (in blue), which is held in the desired shape by the staples (in red) through Holliday Junctions between neighboring helices. The 5'-end of the staple is marked by rectangles, the 3'-end by triangles. Right: AFM image of folded objects.

In 2009, William Shih's group at Harvard showed that the DNA origami method can also be extended to three dimensions. Figure 5 shows the concept using the example of a bundle of six double helices (6-Helix Bundle): the double helices are arranged in a hexagonal honeycomb lattice with transitions to adjacent helices. The B-shape geometry of the DNA with 10.5 base pairs per revolution allows transitions with 120° angles to one another.

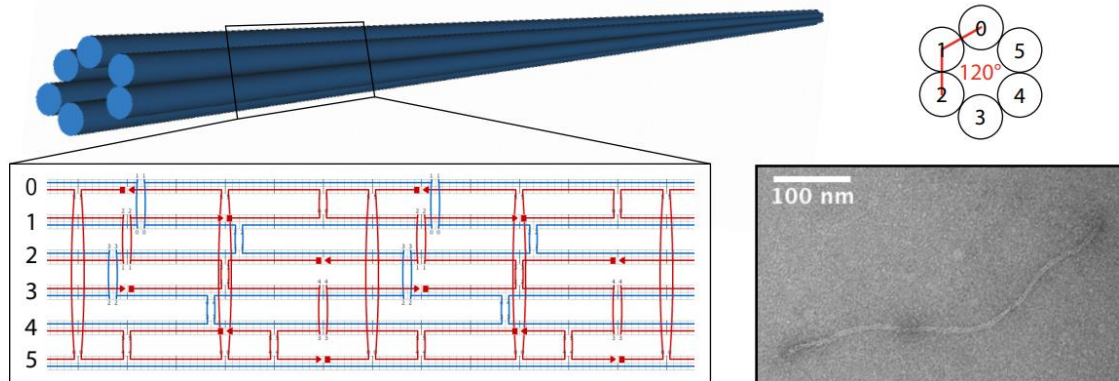


Figure 5: The Six Helix Bundle (6HB) can be represented in a simplified manner by six adjacent helices that have been folded into a hexagon. In this arrangement, each helix has 2 adjacent neighbors and transitions to these are possible at angles of 120° (or more accurately, 240° from the opposite direction). The excerpt from the design scheme shows a rolled out 6HB with the scaffold in blue and the staples in red. Transitions of the scaffold and the staple are only possible between helices which are adjacent to each other in the hexagon. The electron microscope image at the bottom right shows a 428 nm long 6HB with a diameter of ~ 6 nm.

From structural design to the finished object

The design of DNA origami structures has been extremely simplified through the development of a CAD software package called *caDNano*. Basically, the structural design process can be divided into four steps. In the first step, a geometric model is defined that roughly reproduces the desired structure. This geometric model is then filled with a scaffold path in which anti-parallel double helices are routed through the entire structure using crossovers at permitted positions. In the next step, staples complementary to the scaffold are created automatically. These staples must then be divided into segments between 18 and 49 bases in length (the aim is to achieve an average staple length of 32 to 49 bases). In the last design step, the scaffold path is populated with the sequence of the intended scaffold and thus the staple sequences are determined. Depending on the design and the length of the scaffold, between 200 and 300 different staples per design must be synthesized.

These synthesized staples are then mixed with the scaffold in a ratio of 10:1 in a buffer solution with MgCl_2 as a source of divalent cations. The sample is then heated to 80°C and slowly cooled to room temperature over a period of one hour up to several days, depending on the geometry of the structure. This process is known as annealing. The concentration of MgCl_2 and the duration of the annealing vary from design to design and must be experimentally determined individually for each geometry.

1.4 Production of the scaffold strand

In order to be more flexible in the design of DNA origami structures, it is desirable to have DNA scaffolds of different lengths on hand. For this purpose, PCR-amplified fragments of bacteriophage λ -DNA were incorporated into the M13mp18 RF plasmid (see <https://international.neb.com/products/n4018-m13mp18-rf-i-dna#Product%20Information> for more information on the M13 Vector).

Currently we have versions of the original 7249 bases long M13mp18 vector of different lengths in our lab: 7308, 7560, 8064 and 8634 base long scaffolds. During the praktikum, each student will make one of these scaffolds himself.

We produce the scaffold with the help of the bacteria *E. coli*. We infect a bacterial liquid culture with the virus, the bacteria multiply and reproduce the virus into the surrounding liquid medium. *E. coli* cells produce the virus particles, which we then separate from the medium and isolate the genomic DNA from the viruses: we further purify this DNA and then use it as a scaffold. Below is some information about the M13 bacteriophage and its cycle of replication.

Bacteriophage M13

The bacteriophage M13 (Figure 6) is a filamentous virus that infects *E. coli* bacteria. The native form of the virus is approximately 900 nm long and 6.5 nm wide. The DNA of the virus is stored within a protein envelope and the length of the genome determines the length of the virus particle. A phage particle for the 8634 base long scaffold is thus longer than a phage particle for the 7249 base long scaffold.

The M13 phage attacks *E. coli* by attaching to the receptors at the end of so-called F-pilus on the bacteria. This F-Pilus is used in the world of bacteria for horizontal gene transfer, i.e. for the exchange of genetic information in the form of plasmids between two cells of the same generation. It is therefore important to use a suitable *E. coli* strain for scaffold making. The virus particle docks with the help of a surface protein at one end to the F-pilus and the infectious single-stranded virus DNA is introduced into the host cell.

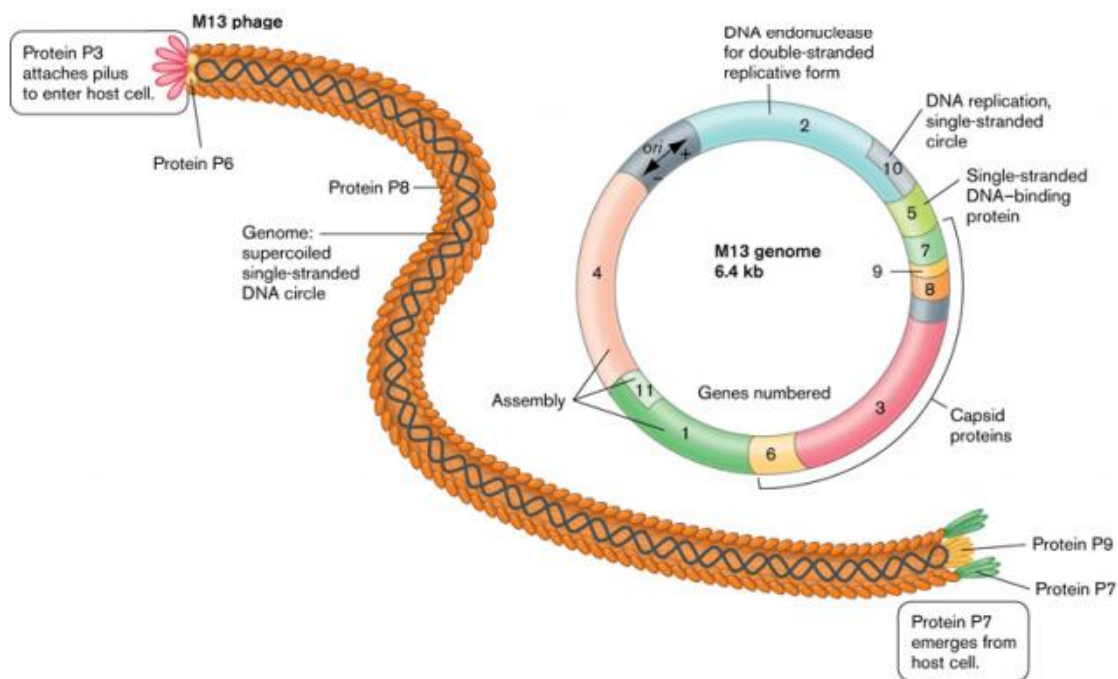


Figure 6: Schematic representation of M13 bacteriophage.

Replication cycle

As soon as the infectious DNA ((+) strand) is in the host cell, the replication cycle begins (Figure 7). A complementary, (-) strand is synthesised by the bacterial enzymes and the DNA becomes the double-stranded, replicative form (RF DNA). The (-) strand serves as the template for transcription and mRNA transcribes it into phage proteins. The amplification of the offspring (+) strand takes place by means of a so-called rolling circle amplification. The newly created (+) strands are packed into the protein envelope and the following virus generation is released into the surrounding medium. The host cell is not killed in this process and continues to grow, but at about half the normal rate. Per each host cell a few hundred new phages are produced. After a few hours of incubation of the infected culture there are enough new phage particles in the growth medium for further processing. These phages can then be separated from the E. coli cells by centrifugation and harvested from the growth medium with polyethylene glycol (PEG) precipitation. The protein coats need to be denatured to release the circular genomic DNA which is subsequently purified. The DNA can then be used as a scaffold of DNA origami.

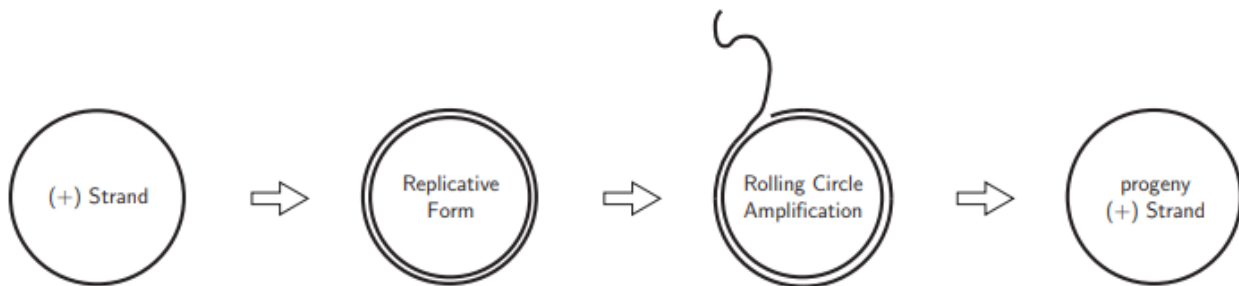


Figure 7: Schematic representation of the replication cycle inside the host cell.

1.5 Transmission electron microscopy

In transmission electron microscopy, the transmission electron microscope (TEM) irradiates a thin sample with an electron beam. The electrons which pass through the sample (i. e. they are not scattered or absorbed by the sample) are visualised on a fluorescent screen or converted into photons using a scintillator and detected on a CCD or CMOS camera. Because of the small de Broglie wavelength of the electrons one can reach much higher resolution with a TEM than with optical microscopes. For biological samples, an accelerating voltage between 80 kV and 120 kV is usually used to generate the electron beam. The samples are immobilized on a so-called TEM grid. This consists of a metallic grid, with the gaps consisting of a thin, electron-permeable film. In this experiment we use copper grids with a carbon/Formvar film. In Figure 8 you can find a schematic representation of the beam path in a TEM and an image of a TEM grid.

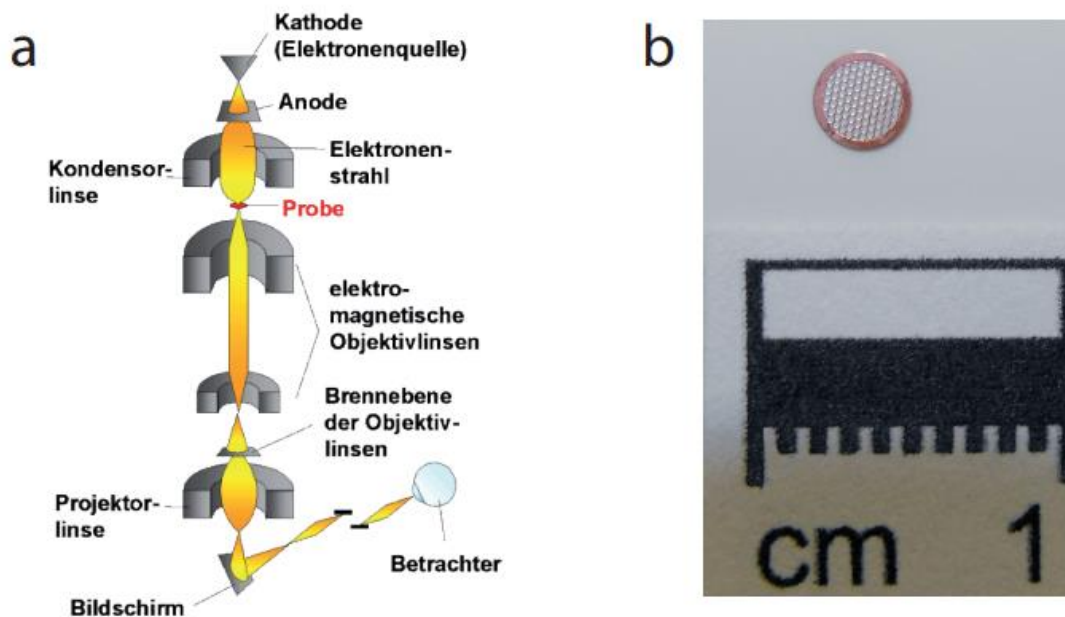


Figure 8: (a) Schematic representation of beam path in a TEM. (b) A copper TEM grid.

Most biological samples (as well as the DNA to be analysed in this experiment) have a very low contrast for electrons at these energies and therefore need to be stained before imaging. For this purpose, heavy atoms such as lead or uranium, which strongly scatter electrons, are used and thus increase the contrast. In this experiment we use uranyl formate solution to negatively stain the DNA origami sample.

2 Experimental plan

Day 1

Amplification of the bacteriophage

The phage is multiplied in an *E. coli* liquid culture. For this you need a suitable strain of *E. coli* bacteria, which can be infected by the phage (the cells must have an F-pilus). We use *E. coli* K91endA cells for this. Because these are stored in a freezer at -80°C , they become viable after thawing and begin to divide. You also need a suitable growth medium. The most commonly used medium for cultivating *E. coli* bacteria is LB medium (see https://en.wikipedia.org/wiki/Lysogeny_broth). To get an optimal phage yield we use a modified LB medium: the 2xYT medium. It contains 16 g tryptone, 10 g yeast extract and 5 g NaCl per liter.

A liquid starter culture is available at the beginning of the first day of the experiment. It is prepared the day before by placing deep-frozen cells in 50 ml LB medium and kept overnight at 37°C (corresponds to the optimal growth temperature for the cells) in a shaking incubator (ensures a high oxygen content in the liquid). The cells multiply exponentially and on the morning of the first day of the experiment there is an overnight culture with a high cell density. This is then put into 200 mL of 2xYT medium with 5 mM MgCl_2 by the supervisor and incubated again at 37°C .

The liquid culture should be incubated until it reaches an optical density of 0.5 at 650 nm:

- Determine cell growth every 15 minutes by measuring the OD_{650} until it reaches a value of 0.5 (this corresponds to a cell density of 4×10^8 cells/mL)
- Add the phage in a phage-to-cell ratio of 1
- Incubate for 3-5 hours at 37°C, 300 rpm, then remove from the shaking incubator
- Transfer to centrifuge tubes and centrifuge at 3000 rcf, 4°C for 30 min

This centrifugation step forms a solid pellet of cells at the bottom of the vessel, whereas the phages produced by the bacteria remain in the solution. The liquid will be used for further processing and the bacterial cells can be disposed of.

PEG precipitation of the virus particles

The phages are precipitated from the solution with the help of PEG-8000, NaCl and centrifugation. A solid pellet is formed again at the bottom, but this time it contains the precipitated phages and the supernatant can be discarded.

- Add 8 g PEG 8000 and 6 g NaCl to the supernatant of the previous step.
- Mix the solution with a magnetic stirrer for 10-15 min.
- Incubate on ice for 15-30 min
- Centrifuge at 10,000 rcf and 4°C for 20 min
- Discard the supernatant
- Remove the remaining liquid from the centrifuge tube with a pipette
- Resuspend phage pellet in 5 mL 10 mM Tris (pH 8.5)

Extraction of the viral single-stranded genomic DNA

Now we have purified phages in a buffer solution. Since the DNA is located within the protein envelope of the phage we now have to denature the envelope proteins.

Add to 5 mL of phage solution in Tris buffer:

- 5 mL PPB2 (0.2M NaOH, 1% SDS), mix gently by swirling, wait 3 min
- 3.75 mL PPB3 (3M KOAc pH 5.5 titrated with glacial acetic acid), mix gently by inverting the tube
- Incubate on ice for 10 min
- Centrifuge at 16,500 rcf and 4°C for 30 min

During centrifugation the denatured proteins will form a pellet and the DNA will stay dissolved in the supernatant: discard the pellet and collect the supernatant for the next steps.

Ethanol precipitation of the single-stranded genomic DNA

Now we want to purify the obtained DNA. This is done through ethanol precipitation. By adding 2 to 3 times the volume of the ethanol solution the polarity of the solution is reduced and thus the hydration shells, which shield the negative charge of the DNA, are broken open. The electrostatic interactions

between the negative-charged phosphate backbone of the DNA and positive ions in the solution (of added sodium acetate) are now so strong that the DNA precipitates. A pellet can now be formed again by centrifuging the sample.

- add twice the sample volume of chilled 100% ethanol
- add 1/10 of the sample volume of 3M sodium acetate
- incubate for 30 min on ice (or 2 min in liquid nitrogen)
- centrifuge at 16,500 rcf and 4°C for 30 min
- carefully remove the supernatant
- add 20 mL chilled 70% ethanol
- centrifuge at 16,500 rcf and 4°C for 10 min
- immediately remove the supernatant
- let the pellet dry
- resuspend the pellet in 1 mL 1xTE

Analysis of the obtained DNA

Now we have purified the obtained DNA and want to determine the concentration and purity. You can do this with a UV/Vis spectrophotometer, the Nanodrop. A 2 µL sample volume is sufficient for the measurement. In the nucleic acid mode, the Nanodrop measures absorption at several wavelengths in the UV spectrum from which the amount of DNA in the sample can be calculated. The software of the Nanodrop uses a standard absorption coefficient and outputs the concentration in ng/µL. By knowing the number of bases of the DNA molecule as well as the average molecular mass of the individual bases (= 330 g/mol) you can convert the mass concentration into molar concentration.

Day 2

Folding the DNA origami structures

As an example of a DNA origami structure you will fold a 6 helix bundle (6HB). The final concentrations in the folding solution should be 10 nM scaffold, 100 nM staples, 1xTE buffer and 14 mM MgCl₂. For a 20 µL sample pipette the following in a 200 µL PCR tube:

- 10 µL staples (200nM)
- 6 µL H₂O
- 2 µL 10xTE, 140mM MgCl₂
- 2 µL of the manufactured scaffold (100nM)

Place the sample in a thermal cycler, heat to 80°C and slowly cool to room temperature in one hour.

Staining of the folded samples

For even distribution of the sample on the TEM grid, the surface of the grid should be hydrophilic. You can achieve this by plasma treatment of the grids: expose the grids to argon plasma for 60 s at 240 V before applying the sample to the grid.

Nitrile gloves and a laboratory coat must be worn when staining the sample with uranyl formate!

- place 2 μL of sample on the grid, wait 1 minute
- pipette two 5 μL drops of 2% uranyl formate on parafilm
- wick off the sample liquid from the grid by gently touching a filter paper with the edge of the grid
- dip the grid in the first drop of uranyl formate and immediately remove the liquid with filter paper
- dip the grid in the second drop of uranyl formate and wait for 10 s
- again remove the liquid with the filter paper
- after 30 minutes of drying, the samples can be analyzed with the TEM

TEM analysis of the stained samples

Details of the TEM analysis of the samples are given at the device by the supervising person.