UTRECHT UNIVERSITY

THESIS

Guanine Quadruplex Samples for a Centrifugal Force Microscope

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Abstract

This thesis describes the optimalization of a newly designed sample for a Centrifugal Force Microscope. It starts of with a theoretical background about G-Quadruplexes and follows up with a step by step process for making the G-Quadruplex DNA involved and the sample itself. The alterations are described fully, complete with their reasons. Measurements regarding the tethering of beads are presented and discussed. It ends with a prognosis of the future and its possibilities.

Acknowledgements

I'd firstly like to thank Gerhard Blab for supervising me and helping me throughout the process. I'd further like to thank Dave van den Heuvel for helping me with the microscope and in the lab. I'd also like to thank the whole Biophysics group of the Debye Institute for helping me along the way and coming up with valuable advise and support. I'd like to thank you all for this opportunity.

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Introduction

Nearly every person on earth once started with only 46 strands of DNA. A large part of these humans leaves it because of damage done to it. Playing such a leading role throughout our lives it is imperative that we learn more about this complex molecule.

Research on DNA is often done with classic single molecule methods, however those methods are often very time-consuming. Using the Centrifugal Force Microscope it is possible to perform tests on hundreds of samples of telomeric material at the same time. Thus making it possible to get vast sets of data on Guanine-Quadruplexes, a structure arising there which can prematurely end life cycles of cancerous cells. Using this set-up one could test drugs targeting this Quadruplex on potency in a fast and inexpensive way. Developing it further in terms of precision and consistency are therefore a must.

This report will form a theoretic basis for these experiments as well as delve deeper into optimizations done with a new sample design. This new design incorporates both a new design and some new possibilities for the way force is exerted on the G-Quadruplex DNA as well as showing the problems that arise with them.

Chapter 2

Theory on G-Quadruplexes

2.1 History of the G-Quadruplex

The DNA of any human being, and even any other organism, is only composed of four different nucleotides. These are Adenine, Thymine, Cytosine and Guanine. In 1910 it was discovered by Ivard Bang[1] that a Guanine related substance had interesting additional properties. He observed that Guanylic acid in concentrated solutions formed a gel.

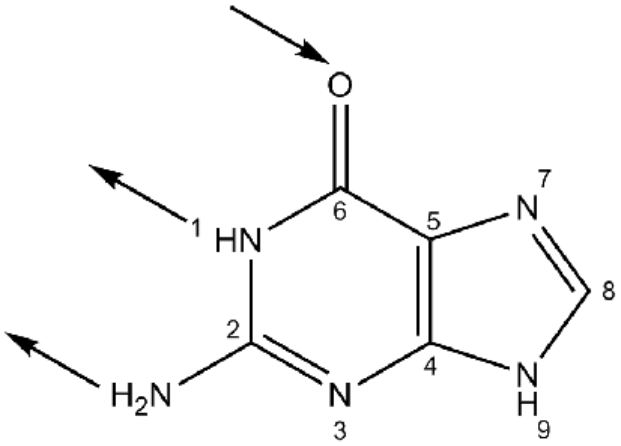

Figure 2.1: A schematic model of the Guanine molecule, with arrows, illustrating hydrogen bonds reacting to a Cytosine partner, pointing in the electronegative direction.

This discovery then was largely forgotten until more than 50 years later when its value suddenly came to light. In 1962 Marie Lipsett[2] was working on making Guanine Polymers in the presence of potassium chloride, but thought she failed when her 5-GMP (Guanine-mono-phosphate) formed a viscous solution that looked like DNA. She notified her coworker David Davies, who was thinking of Bangs paper, of her findings. After performing fiber diffraction he found that four Guanines in a plane could be the cause of this. It was named a G-tetrad and its structure is the basis for all later theories about complex Guanine structures (G-Quadruplexes).

In the following decade it was confirmed that it was not the pH of a solution but the presence of a Sodium ion that made it possible for the G-tetrad to stabilize[3]. Experiments from 1975-1978 further showed that some other monovalent cations could also produce similar results[4], specifically: Rb^+ and K^+, which both preferred different confirmations from Na^+.

In the early 1990s after years of dwindling interest for the subject it was shown that G-Quadruplexes had a biological relevance. And thus widespread research on this exciting structure flared up once again. Several studies have been done since on the self assembly of 5'-GMP with very promising results[5].

Sudden global interest, however, developed after the discovery of G-Quadruplexes *in vivo*. In 2009 H. J. Lipps and D. Rhodes[6] were the first to show the formation and a certain stability of these complexes in a one celled organism. They focused on the telomeric region; the single stranded ends of chromosomes, which play a vital role in protecting the genome from damage during DNA-replication. Telomeres are also heavily involved with the life cycles of cancerous cells. So when it became undeniably clear that these structures arise also in human DNA[7] researching it was no longer relevant only for biophysicists but also for the medical world.

2.2 Structures and Conformations

If one wishes to understand the G-Quadruplex it is imperative that one first understands its basic building blocks: Guanine. It is a derivative of a purine and has the ability to make 3 hydrogen bonds with Cytosine in helical DNA, as described by Figure 2.1. Standard helically shaped DNA is also called B-DNA. This figure doesnt show the DNA backbone but it is located at place 9 instead of the single Hydrogen. Now 4 Guanines can sometimes align themselves in a square plane using Hoogsteen hydrogen bonding. This shape is called a G-tetrad and can be seen in Figure 2.2.

A stack of these planes is then called a G-Quadruplex and it is as such a quite 3-dimensional structure. However calling it just a stack of planes is selling the G-Quadruplex short. Saying that it's the defining quality in the collection of possible G-Quadruplexes is much more accurate. Now in further

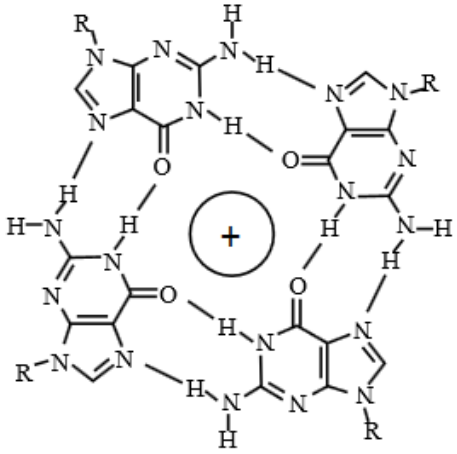

Figure 2.2: A schematic G-tetrad complete with stabilizing cation.

describing the structure it is essential to elaborate on four of its qualities:

- Anti/Syn Configuration
- Inter- or Intramolecular
- (Anti-) Parallel strands
- Grooves

The configuration depends on the 3-dimensional position of the nucleotide in relationship to its backbone. This angle is called the glycosidic bond angle (GBA) and in is limited to only two shapes in the DNA, namely the *syn* and the *anti* shape[8], that are visible in Figure 2.3.

As obvious as it may seem a G-Quadruplex is not necessarily limited to only one molecule. Multiple loose strands can work together to create the structure and they are labeled according to this: Di-, Tri and Tetramers. G-Quadruplexes created in this way are called intermolecular, because they exist in between different strands, be it partially or entirely. Of course there is also a single molecule equivalent called a Monomer. Here a G-Quadruplex is Intramolecular, for it is "inside of the molecule".

A G-Quadruplex is called parallel if all four of the strands can be followed in the same direction. Reversely it is called anti-parallel if at least one of the strands goes the opposite way. A parallel configuration will always insure an *anti* shape for all the Guanosines[9]. An anti-parallel configuration can contain both *syn* and *anti* shapes.

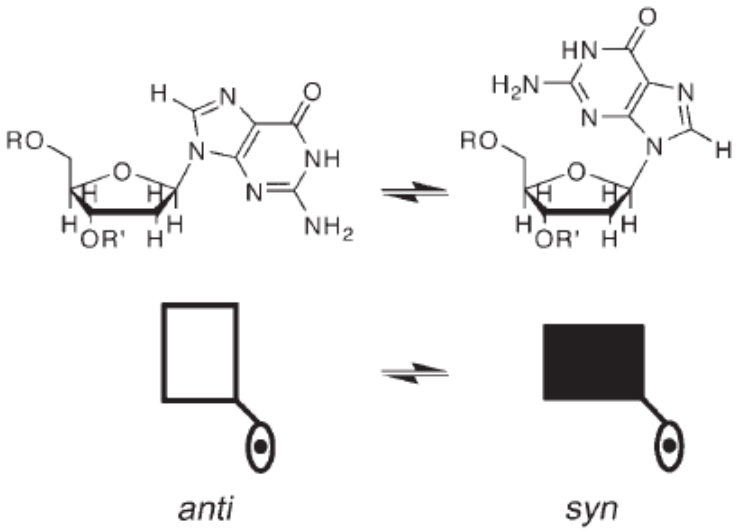

Figure 2.3: The difference in spatial conformation between *anti* and *syn*[8].

Lastly one should consider the distances between the Phosphodiester backbones of the G-Quadruplex. These are called the ”Groove distances” and are mostly determined by whether the strands are (anti-)parallel and which shape the Guanines take. These can basically be split up in three different categories[8]. If two adjacent Guanosines have the same GBA it is called a ”medium groove”. If they have opposite GBAs it can be either a ”narrow groove” or a ”wide groove”. Logically the ”narrow groove” is the form in which there is the least distance between the backbones.

All these things are needed to properly describe a certain topology. However not all topologies are realistically possible. For instance, if the connecting loops are too long the structure becomes unstable. The same applies for a topology with loops that can cross paths. This can happen with Diagonal loops, which connect non-adjacent strands. The opposite is called Lateral loops. Groove-widths also play a major role in the possibilities, as not all widths are allowed in all places and certain Grooves can favor certain loops. They can do this either clockwise or anti-clockwise.

2.3 Cation

The first sightings and successive successful sightings of the structure have been done mostly in the presence of a monovalent cation and this is not a coincidence. It can be seen in the chemical structure that a G-tetrad has a

strong negative electrostatic potential due to the O6 oxygen of the Guanine. This can be stabilized using a localized positive charge: an ion. Historically mostly Potassium and Sodium ions have been used due to their easy accessibility and biological relevance, however research has been done using Rubidium, Thallium[10] and Lithium[11]. Sodium can be found in a range of geometries ranging anywhere from in between tetrads to in the center of a tetrad. Potassium on the other hand is limited to only an equidistant position between tetrad planes, and forms the eight oxygen atoms in a symmetric tetragonal bipyramidal configuration[9]. This leads to a difference in preferred topologies for the G-Quadruplex when using either. And this should be remembered when planning experiments.

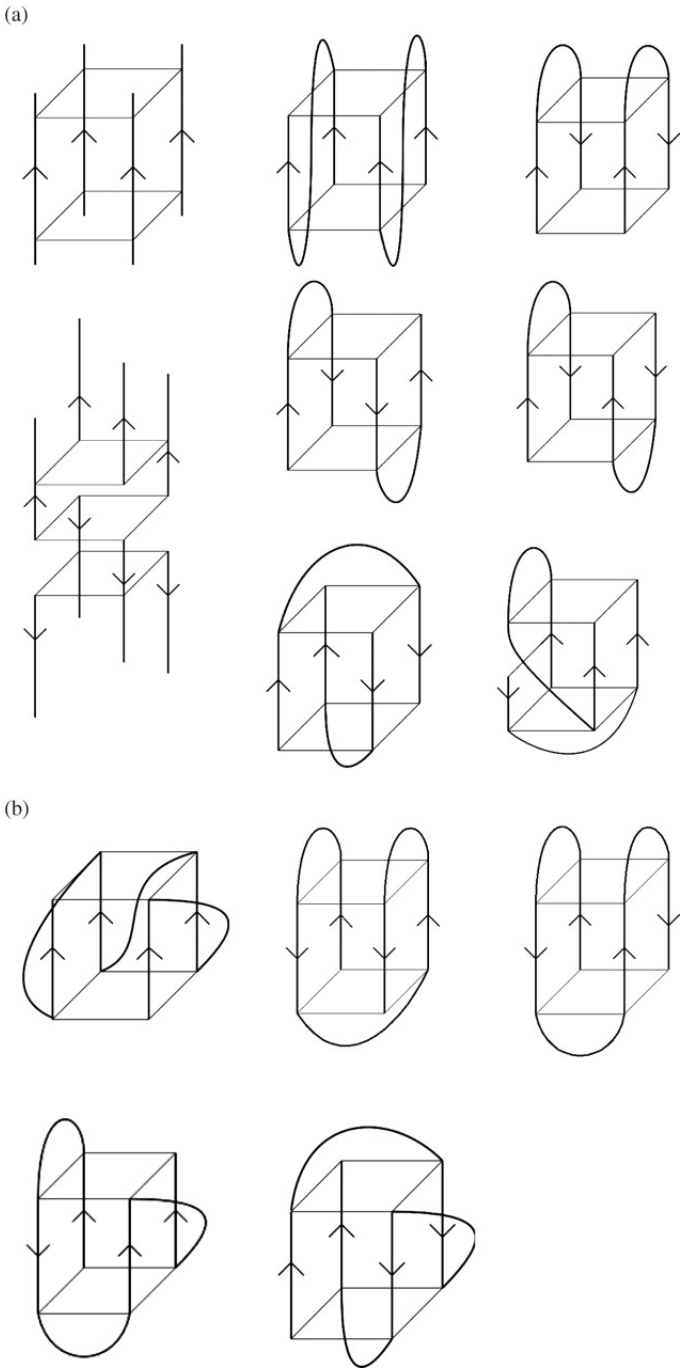

Figure 2.4: Different possible G-Quadruplex configurations with a) non-monomeric and hybrid configurations and b) monomeric (anti-) parallel configurations. The arrow shows the direction of the DNA.

Chapter 3

Research methods

During the research I worked on optimizing a new sample design for the Centrifugal Force Microscope. This chapter will give an overview of the process that are used, what they are and how they work, without going into the specifics of exactly what I encountered during my experiments. It consists of two parts: The first describing the production of the DNA that is used during my research. The second consists of using the DNA while actually building the sample.

3.1 Production of G-Quadruplex DNA

The DNA that we buy can't be directly used, it needs to be altered in such a way that it is usable. This is done by adding so called "handles" on the chosen G-Quadruplex DNA. An outline can be seen below.

3.1.1 Polymerase Chain Reaction

The start of the process consists of making high quantities of the DNA that contains the handles using Polymerase Chain Reaction (PCR). PCR is a method for enzymatic synthesis of specific DNA sequences[12]. It requires a template DNA, two *primers*, Deoxynucleotide Triphosphates (dNTPs), Polymerase and preferably a buffer.

So step one is finding the template DNA that contains the required sequence. In our case this was the pUC18 plasmid. A solution of all the required substances is heated until it undergoes denaturation and falls apart in single strands.

It is then somewhat cooled down again so that a site-specific *primer* can latch on. A primer is a strand of nucleic acid that serves as a starting point for DNA replication. This is necessary because it allows the Polymerase enzyme to start adding nucleotides to a complementing string, as it can only continue on an already formed piece of double stranded DNA. The primers we are using contain biotin or digoxigenin so that this is always included in final result.

The solution is then once again heated and the DNA splits up. When it cools the primers attach themselves somewhere on the new strands and thus form the first strands of proper length.

As this process is repeated a couple of times the number of molecules which have the right length grow exponentially whereas the too long ones only grow linearly. After a number of rounds this leads to the solution consisting of mainly the required molecule. A simplification of the process can be seen in Figure 4.1.

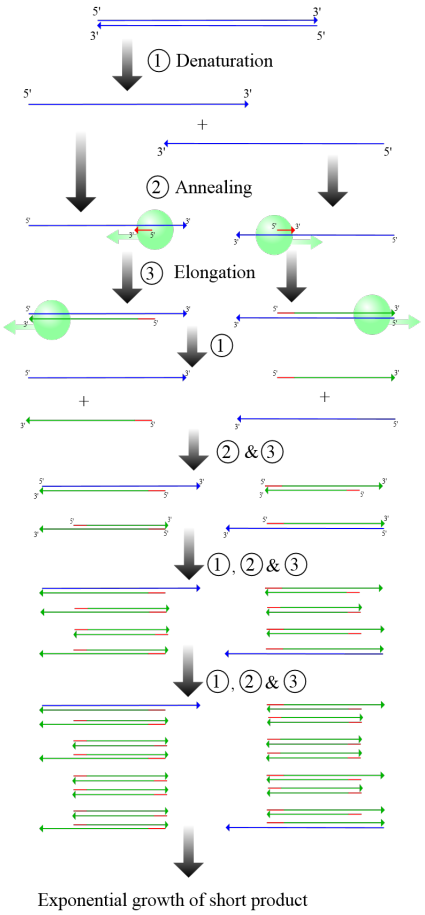

Figure 3.1: Example of a PCR-process.

3.1.2 Clean up

The products have to be cleaned up after each step, to reduce contaminations that might lead to complications further along the line. Also it yields a more concentrated product, which is often desirable. During our process we used a *Wizard SV Gel and PCR Clean up System*[13], which consists of a set of tubes, a column with a membrane, Membrane Binding Solution and Membrane Wash Solution. The process uses the principle that the silica membrane will reversibly bind the product. It has a recovery rate of approximately 87% for lengths of between 100 and 500 bp (base pairs) [13].

There are two approaches given for the first step of the cleanup, depending on whether the product is in an agarose gel or not. If it is, it should be melted at 60 °C. Then 10 µL Membrane Binding Solution should be added for every 10 mg of gel. If it is just a regular solution, then the quantity of Membrane

Binding Solution should be equal to the amount of solution (1:1 in terms of volume). Either of these is then incubated at room temperature for 1 minute and poured into the column. The column is placed in a collection tube and centrifuged for 1 minute. Centrifugation always happened at 16,000g. We did two batches at once so that they could balance each other out in the centrifuge.

The flowthrough is discarded and the column is filled with 700 µL of Membrane Wash Solution (with Ethanol already added). This is centrifuged for 1 minute and the flowthrough is discarded. Then 500 µL of Membrane Wash Solution is added, with a centrifuge time of 5 minutes. Again the flowthrough is discarded. The membrane is then dried by centrifuging it for 1 minute.

Lastly nuclease-free water is used to free the DNA from the membrane. In this step the column is put inside a clean collection tube together with 50 µL of nuclease-free water. This is left to incubate for 1 minute and is then centrifuged for 1 minute. The resulting flowthrough contains the product.

3.1.3 Digestion

The next phase consists of taking DNA and 'cutting it into the actual handles. This done by using two restriction enzymes called HindIII and Eco24I (also called BanII). As can be seen in Figure 3.2 they dont cut straight but take out a piece in the middle of the DNA whilst also forming the attachment points (also called "sticky ends") for the G-Quadruplex DNA. The resulting handles have a length of respectively 207 and 201 bp, where both also have 4 single nucleotides. The removed piece has a total length of 41 bp and 8 singles.

```
  1  GTGAAATACC GCACAGATGC GTAAGGAGAA AATACCGCAT CAGGCGCCAT TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC
     CACTTTATGG CGTGTCTACG CATTCCTCTT TTATGGCGTA GTCCGCGGTA AGCGGTAAGT CCGACGCGTT GACAACCCTT CCCGCTAGCC ACGCCCGGAG
101  TTCGCTATTA CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT TCCCAGTCAC GACGTTGTAA AACGACGGCC
     AAGCGATAAT GCGGTCGACC GCTTTCCCCC TACACGACGT TCCGCTAATT CAACCCATTG CGGTCCCAAA AGGGTCAGTG CTGCAACATT TTGCTGCCGG
                HindIII                                                                  BanII
201  AGTGCCAAGC·TTGCATGCCT GCAGGTCGAC TCTAGAGGAT CCCCGGGTAC CGAGCTCGAA TTCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT
     TCACGGTTCG AACGTACGGA CGTCCAGCTG AGATCTCCTA GGGGCCCATG GCTCGAGCTT AAGCATTAGT ACCAGTATCG ACAAAGGACA CACTTTAACA
301  TATCCGCTCA CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT CACATTAATT GCGTTGCGCT
     ATAGGCGAGT GTTAAGGTGT GTTGTATGCT CGGCCTTCGT ATTTCACATT TCGGACCCCA CGGATTACTC ACTCGATTGA GTGTAATTAA CGCAACGCGA
401  CACTGCCCGC TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA ATCGGCC
     GTGACGGGCG AAAGGTCAGC CCTTTGGACA GCACGGTCGA CGTAATTACT TAGCCGG
```

Figure 3.2: The PCR product, with the cutting sites and their respective restriction enzymes clearly labelled.

3.1.4 G-Quadruplex DNA

It is important to pick the right experimental DNA; different sequences could have different preferences in their eventual configuration, and finding results for a sequence that might not translate to the region that you are actually interested in could be a waste of time. As such it was decided that we would use

hTel	AGCTCTCTAGA$(TTAGGG)_{12}$AGATCTCAGCT
ILPR	AGCTCTCTAGA$(ACAGGGGTGTGGGG)_2$AGATCTCAGCT

Table 3.1: Both DNA types.

DNA that is also found in the human genome. The two selected regions are the human Telomere(hTel) and the Insuline Linke Polymorphic Region (ILPR), which have the following sequences.

There is a relevant difference between the two in the sense that hTel can form three G-Quadruplexes with three planes, whereas ILPR can only form one G-Quadruplex, but with four planes. The difference between the folded and unfolded DNA can be calculated with the following values:

- Double stranded DNA has a length of 0.34 nm/bp
- Single stranded DNA has a length of 1 nm/bp
- Folded G-Quadruplex DNA has a length equal to that of double stranded DNA

So theoretically, unfolded hTel DNA (with handles) is 225 nm and entirely folded it is 164 nm, with a total difference of 61 nm. ILPR has an unfolded length of 181 nm and a folded one of 164 nm with a total length difference of 17 nm. In reality however this result will often vary as there are many more parameters to consider. For instance the length between the start and the end of the G-Quadruplex might be the lateral or the diagonal distance. Also when there is no external force the dimensions are often highly dependent on Brownian motion.Lastly, it is clear that the DNA might not be maximally folded, reducing the total length difference.

3.1.5 Ligation

After choosing the wanted experimental DNA, in our case hTel and ILPR, the next step should be the ligation. Here a ligation enzyme catalyzes the attachment of the sticky ends of two kinds of handles to a Quadruplex molecule.

3.1.6 Electrophoresis

This is a step that could be done at any time; however we only did it in the last steps. Gel ectrophoresis is a process that uses charge of macromolecules to sort parts according to their size. It does so by putting an electric field over a gel. The gel is porous. Now the DNA is combined with a loading dye, which serves a twofold purpose: Firstly it makes the moving DNA visible and secondly it

causes the DNA to sink in the gel as it is heavier than the buffer. The gel is also stained with Ethidium Bromide which fluoresces strongly when absorbed within a DNA molecule. An electrical field is then put on the gel pulling the negatively charged backbone of the DNA to the positive side. As stated the gel is porous and will much more easily allow small molecules to pass through than larger ones.

In this way a division is created between the different lengths of DNA parts in the solution. As it consists of mostly well defined lengths this can be made to be quite an exact stepwise division.

3.2 Centrifugal Force Microscope Sample

The making of a sample for the Centrifugal Force Microscope can be divided in two parts. First a glass container is made to which the material that is to be researched can attach itself or where it can reside in. This container is called a Sample Assembly. The second part involves actually building the experimental material inside the Sample Assembly in such a way that it can be researched.

There was a working method for making samples; however it was heavily reliant on capillary action and prone to contamination. There was also a poor control of possible air bubbles within the sample. This caused the process to be inconsistent, which is undesirable during repeated experiments. This led to the design of a new Sample Assembly, which during this project has been tested and modified.

3.2.1 Sample Assembly building

The Sample Assembly is a fairly simple construction. It consists of a thin piece of glass called a coverslip, which the microscope looks through, a thick piece of glass as a basis called a coverslide and a piece of parafilm to keep them apart. The piece of parafilm has a channel cut from it and this is the where the actual sample is contained. Figure 3.3 shows the exact dimensions of the Sample Assembly.

Close to each edge of the channel there is a tube. This is the key development in the design, as it allows almost direct manipulation of the inside of the channel. For this purpose two holes are drilled into the coverslide using a diamond drill with a 1mm head. As the drilling happens under water the coverslide should be washed with ethanol and demineralized water. The coverslides can then be stored in demineralized water for later use or be used right then. If they are to be used right then they should be blown dry using nitrogen.

Two outer and two inner tubes should then be cut. Here the inner tubes are placed in the holes and the outer tubes are placed about halfway over the

smaller tubes. This is then glued together using a fast drying acrylic glue.

While this is drying, a heating element is turned on to give it sufficient time to warm up. Then the parafilm is cut in the proper shape using a pre-made template. A properly cut parafilm should have smooth edges along its channel.

After drying the small side of the tube that protrudes from the coverslide should be cut off using a razor. The parafilm can then be placed on the conver-slide in such a way that it does not cover the openings of the tubes inside the channel. It should be rubbed on so that it sticks to the coverslide.

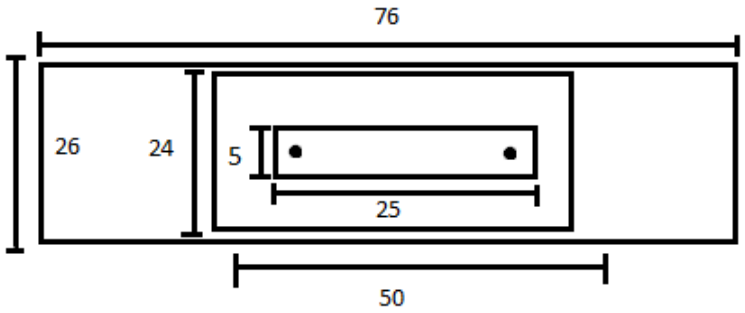

Figure 3.3: Schematic image of the Sample Assembly, showing its outline and dimensions in mm.

A coverslip etched with hydrofluoric acid (HF) should then be taken from a pre-made stock, dried off with nitrogen and put on the parafilm so that it sticks a little. Cleaning this way removes the upper layer of the glass, including possible pollution of the surface, which might induce non-specific binding. It also etches the glass making it easier for the anti-digoxigenin used in a later step to latch onto it. Handling HF, however, is highly dangerous and should only be performed by someone with the proper training. Symptoms of contact sometimes only show one or two days later. As HF is strongly corrosive it can cause burns which go untreated for a relatively long time before being noticed. HF can also react with the calcium in the body leading to serious health complications.

The last step consists of melting the parafilm and finishing the assembly. It can then be filled with demineralized water, while making sure there are no air bubbles left.

3.2.2 Sample preparation

The CFM is a machine that can both exert force on a test sample and measure it. The mechanism for the application of this force is partially inherent: the microscope can spin. However the build of the sample is also critical for it to work. Within the sample there should be an attachment between one part of the DNA and the coverslip, and another one between the other side of the DNA and some sort of mass. Granted that this mass has a higher buoyant mass than the medium, this will allow the setup to put a variable strain on the experimental DNA.

The sample is made by letting anti-digoxigenin bind non-covalently to the coverslip. While this is happening a batch of a variant of PTC, which also contains a blocking agent. After the appropriate time has passed this is then used to flush the chamber. The PTC is then left to settle at the coverslip reducing the amount of further non-covalent binding later along the line.

A combination of an excess of microspheres (spheres of the order of magnitude of 1 µm) covered in streptavidin are added to a solution of the finalized DNA. This is solution is then gently rotated for a while allowing the two components to bind but keeping it from settling at the bottom.

The chamber is again flushed to remove any blocking agent that is not attached to the coverslip, but is somewhat settled at the bottom of the chamber. After which the DNA-Sphere solution is added. The unoccupied side of the DNA can then bind to the anti-digoxigenin; creating the coverslip-molecule-sphere configuration that is required for CFM testing.

The injection tubes are then cut of and the holes are sealed off with acrylic glue. The sample is then ready for testing. A complete guide for all the steps can be find in the appendices.

Chapter 4

Experiments

The experiments we did consisted mainly of trying to follow the standard procedure. As it turned out however often I had to deviate from this or consider entire alternatives if I wanted to optimize the end product. Described below are the specifics of the process I followed.

4.1 Production of G-Quadruplex DNA

The process of creating the DNA didn't require a lot of alteration, as it was a well established method. As such I focused on following it instead of improving it. At the end however this did not turn out to be sufficient and a thorough evaluation of the process had to be made.

4.1.1 PCR

I started normally with the PCR process by mixing the following.

Substance	Quantity
G4HandleAntiS primer (contains biotin)	30 µL
G4HandleSense primer (contains digoxigenin)	30 µL
Dreamtaq DNA Polymerase	3 µL
Fermentas pUC18	< 1 µL
dNTP's 10 mmol L^{-1}	6 µL
Purified water	231 µL
Total	SI300

Table 4.1: PCR mixture.

4.1.2 Clean up

This was then properly sealed off with parafilm, to prevent evaporation and the mixture is placed in a PCR machine, which changes temperature on predetermined intervals. The PCR cyclus then starts.

1. 98 °C for 30 seconds: The DNA strings are pulled apart
2. 55 °C for 45 seconds: The Primers attach
3. 72 °C for 30 seconds: Polymerase attaches the dNTPs

These steps were then repeated 34 times and afterwards left at 72 °C for 10 minutes so that the single strands could form into the required final product.

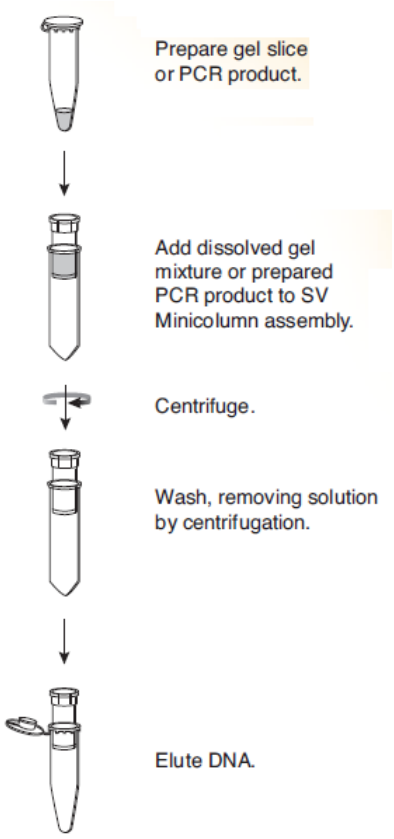

Figure 4.1: A graphic representation of the Clean up process[13].

I then proceeded to clean up the PCR product, following the procedure as described in Research Methods, for a normal solution. After testing it with Nanodrop Spectrophotometer, which uses very small amounts of the solution to determine DNA concentrations, it was found that our product contained about 65 ng μL^{-1} DNA with about a 5% uncertainty. The DNA at this point is 457 bp, and assuming a base pair weighs about 615.5 Da, this means that the solution is 0.234 µmol L^{-1}.

4.1.3 Digestion

We decided to use around 600 ng of DNA, which was 10 µL of the cleaned up PCR solution.

We mixed the substances described in Table 4.2 and left this mixture rest for roughly an hour. We then proceeded to clean it up with the method described above. Afterwards the Nanodrop machine was used to determine the amount of DNA in the solution: 10 ng μL^{-1}.

Substance	Quantity
PCR product	10 µL
10x Buffer Tango	5 µL
HINDIII	1 µL
BANII	1 µL
Purified water	33 µL
Total	50 µL

Table 4.2: Digestion mixture.

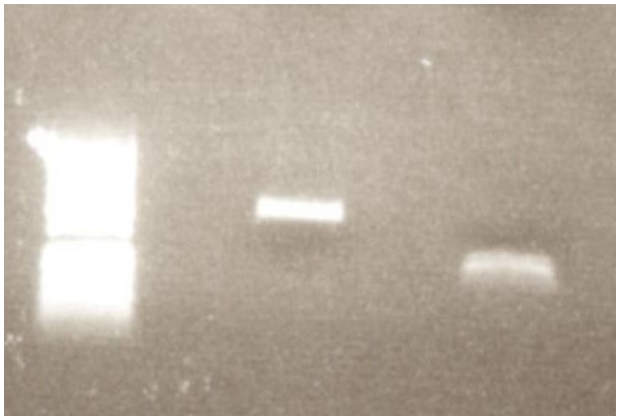

Figure 4.2: Gel showing Fastruler LR DNA ladder, PCR product (at around 460 bp) and Digestion product (somewhere around 200-250 bp), as the ladder is very unseparated there are no reference values given in terms of base pairs.

4.1.4 Ligation

We let a combination of 2 µL of demineralized water, 5 µL of 10x Ligation buffer, 15 µL of handle solution and 6 µL of G-Quadruplex solution sit overnight at 16 °C.

4.1.5 Electrophoresis

In our experiments we used a 1% agarose gel. We added 1 µL of Loading dye to 4 µL of the digest product and to both ligation products. For reference 5 µL of Thermo Sicentific Fastruler Low range DNA ladder was used. All these were put into separate wells and for 30 minutes a voltage of 120 V was placed over the gel. The resulting densities of lengths can be seen in figure 4.3.

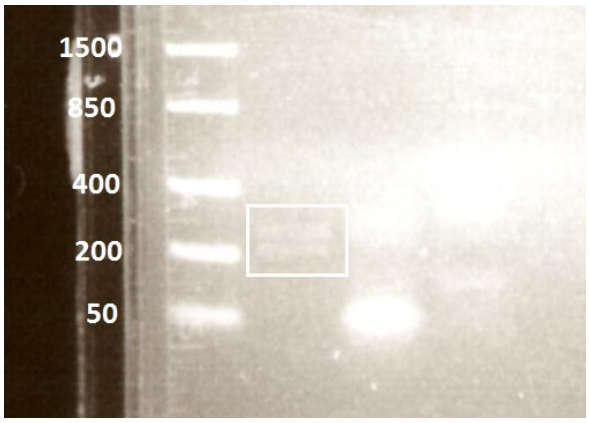

Figure 4.3: Gel showing Fastruler LR , Digestion product and both the ILPR and hTEL ligation products. The values presented are in base pair lengths. The white box outlines two distinct layers in the digestion product.

4.1.6 Complications

Using the gel ectrophoresis method it became clear that the ligation had not been successful. The bands could be expected at somewhat above the 400 bp band, but were seen closer to 250 bp. Earlier made gels had seemingly shown solely the expected band in the digestion step. However on closer inspection of the new gel, which once again had a Digestion ladder, there were actually two bands quite close to each other, as can be seen in the white box in Figure 4.3. As both optimal handles are almost identical in length this was hinting towards a problem with one of the restriction enzymes. The difference in length is then caused by the middle part that is usually cut out but now still attached to one of the handles. As it turned out the HindIII we were using had expired seven years earlier.

Upon checking for further errors it was also found that we were using a $100\,\mu mol\,L^{-1}$ stock of the G-Quadruplex DNA instead of a $100\,nmol\,L^{-1}$ one. It was however concluded that it was unlikely that this contributed to the unsuccessful ligation. According to the producers of the ligation enzyme keeping it overnight should also not be a problem.

As such we decided to retry with the following adaptations:

- New HindIII was used.
- Only half the amount of restriction enzymes previously described was used.
- In the ligation step 8 µL of $100\,nmol\,L^{-1}$ G-Quadruplex DNA solution was used.

After completing this cycle the products were once again put on a gel. As can be seen in Figure 4.4 the steps were now all a success, with the ligation bands on the expected length of somewhat above 400 bp. TheFastRuler LR ladder was then used to estimate the amount of DNA in the finished product. Every step of the ladder contains approximately 20 ng of DNA, and its light intensity corresponds to that. As all the DNA from the 5 mL of ligation product is in one band, which has a light intensity very similar to that of the ladder light bands, it is probable that the the ligation is a 4 ng μL^{-1} solution.

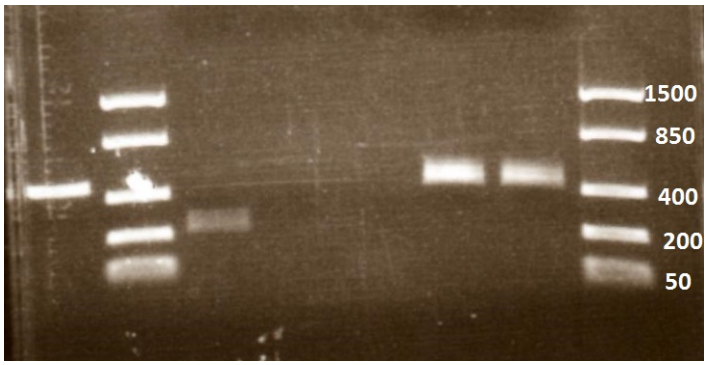

Figure 4.4: Gel showing (from left to right) PCR product, Fastruler LR, new Digestion product and both the new hTEL and ILP ligation products, followed by another Fastruler LR. The lengths are given in base pairs.

4.2 CFM Sample Preparation

Before I started with the research there was already a way present that made it possible to create acceptable samples. It was, however, time-consuming and unreliable. In the hope of creating a more streamlined process a change was made in the way the sample chamber is filled. Going from a capillary force versus an injection method. Although seemingly a minor change it resulted in a lot of complications that I have tried to tackle in the course of the project.

There was also the issue of the broken Centrifugal Force Microscope, which didnt allow me to actually test the samples. Instead I relied on Gerhard Blabs experience on the subject to find the key points I used to verify whether my sample could be used for testing. I also used a regular optical microscope to look at my sample at different magnifications in a way that would be similar to a zero force measurement in the CFM.

4.2.1 Beads

The first change I made was to change the material of the beads I used. In the former experiments silica beads were used as they are relatively inexpensive and damage proof. More importantly, they have very good density, allowing the eventual researcher to work with a range a forces that is interesting for a G-Quadruplex. Gerhard advised me to look at polymer beads, as it is easier to work with in terms of the blocking agent used. Its density also allows me to make better zero force measurements. As such I also had to change the procedure using the new blocking agent. As I applied my results to the silica beads might be problematic and also because the new blocking agent is preferable, I searched for alternative materials.

One of the things I found were Dynabeads MyOne[14]: superparamagnetic polymer microspheres. These beads contain 25% ferrites, which are normally used to magnetize the beads, but also serve to heighten the density to RE-FREF which is very similar to that of the silica beads. As such they might be used while maintaining most of the protocol in terms of the blocking agent. However it should be kept in mind that it could give rise to problems as the magnetizable beads might complicate relations between the molecule and the ions.

For the experiments that I did during my project this was already a step too far. The beads that were readily available were 0.53 µm diameter Polystyrene beads, with a density of $1.06\,\mathrm{g\,mL^{-1}}$. Using this information I could calculate how much it had to be diluted for the optimum DNA/beads ratio. I looked up the water/bead ration and as it turned out 1% of the weight consisted of beads. Firstly I calculated the weight of a single bead:

$$1.06\,\mathrm{g\,cm^{-3}} * \pi/6 * (0.53\,\mu\mathrm{m}/10^4) = 8.3 * 10^{-14}\mathrm{g} \tag{4.1}$$

Which meant that there are approximately $1.21 * 10^{13}$ per dried gram of beads. And as such $1.2 * 10^{11}$ beads per mL of bead solution. Which was about a factor 10 more than the silica bead solution. I also wanted to make changes in the DNA/Bead ratio, as before it had been a 1/12 ratio. The Poisson distribution shows that between a 1/10 and a 1/50 is good. I decided to get close to the upper limit and go for 1/50. I diluted the DNA with a factor 10,000.

$$DNA/\mu\mathrm{L} = 8.526 * 10^9 * 10^{-4} = 8.526 * 10^5\,\mu\mathrm{L}^{-1} \tag{4.2}$$

$$PolymerBeads/\mu\mathrm{L} = 1.211 * 10^8 \tag{4.3}$$

$$1.211 * 10^8 = 8.526 * 10^5 * 50 * X \tag{4.4}$$

Here X is the is the ration of DNA to Bead solution. As it turned out it was about 0.284, I opted for a composition of 1 µL Bead solution to 3 µL DNA solution, with a ratio of 1/47.3

Substance	Grams
Tris	2.41
KCl	9.69
$MgCl_2 + 6H_2O$	0.81
Heparin	0.08
EDTA	0.037
BSA	0.02

Table 4.3: The contents of regular PTC.

I did a similar equation for the silica beads and found that 4 µL of Bead solution to 1 µL of DNA, with a ratio of 1/44 would work well.

4.2.2 PTC Buffer

Using new beads also meant I had to find a new composition of PTC. With silica beads there was a problem with using α casein. This was because of the fact that ,not only is the coverslip is made of glass; so are the beads. This led to the beads sticking even more to the glass and was resolved by using a higher concentration of Bovine Serum Albumin (BSA) in the PTC.

A liter of PTC typically contains, next to a liter of purified water, the following substances.

Gerhard was concerned about possible ferrite leakage from the Dynabeads. I found that EDTA has the possibility to sequester certain metal ions, one of which is iron. So the use of PTC should keep this problem from happening. Here Fe^{2+} has a dissociation constant of $4.76 * 10^{15}$[15] and Fe^{3+} of $1.00 * 10^{-25}$, which is considerably less stable, but should still suffice. It, however, shouldnt sequester Potassium and Sodium ions.

With the addition of $10\,mg\,mL^{-1}$ PTC α casein it is called PTC1 and the newer formula using $10\,mg\,mL^{-1}$ PTC BSA was called PTC2. In my research I was surprised that there was $MgCl_2$ in the original PTC composition as this adds extra charged ions to the mixture, which could make the G-Quadruplex fold in uncontrolled ways. So I opted to remove it, and name the result PTC0. Any reference to PTC1 and PTC2 in the rest of my research and in part REFREF refers to the mixture using PTC0 as a base, not normal PTC. Ofcourse the EDTA also sequesters the Magnesium where Mg^{2+} has a Kd of $2.04 * 10^{-9}$. Then again I saw no need to add the Magnesium if it served no purpose and needlessly burden the EDTA. Thats why PTC0 seemed like the best choice.

4.2.3 Acrylic Glue

In the old process transparent nail polish was used to seal of the sample after completing it. Evidently this was not the optimal way, as it took long to dry and was likely to contain unwanted substances. As such I searched for an alternative that could also be used to keep the tubes in place. In the end it became clear that two-component glues were the way to go. As they dried quickly, had no need of moisture to do so, could stick to a multitude of surfaces and were also transparent. This posed a mild health concern as this glue is a known carcinogenic and damages the respiratory organs. As such I worked with it in a fume hood as much as I could. The specific glue we eventually went for was Kombi Turbo by Bison[16].

4.2.4 Tubes

When I started working the only available tubes were PTFE (Teflon) tubes. As described earlier I used an inner and an outer tube, corresponding to the small tube that fit into the holes in the coverslide and the tube placed over this one, in which I could inject. The inner tube had an inner diameter (ID) of 0.4 mm and an outer diameter (OD) of 0.9 mm. The outer tube had an ID of 0.8 mm and an OD of 1.6 mm. I also had tubing available with an ID of 1 mm and an OD of 1.6 mm which I used later on.

The first combination produced unsteady results. The inner tube had space left and as acrylic glue only poorly sticks to PTFE this sometimes resulted in the entire tubes being pulled out. The outer tube came with its own set of problems in terms of injection difficulty. If the tip of the pipette wasnt held exactly right on the opening of the tube everything would drip away without even entering the chamber. Not only would this lead to an uncertain amount of the desired fluid in the chamber, but the act of trying to get it right was prone to introducing air bubbles into the chamber.

I tried several things to solve this problem. Practice with always getting it just right turned out to be impractical in the timeframe of my research. I also used a small pipette point as a funnel, which I attached in several ways, including: plugged in, gluing and with melted parafilm. Although this solved the problem of accurate aim, it would produce air bubbles in different ways and led to less control and precision in the injection.

Eventually a new tube arrived which worked very well with the third tube mentioned. It had an ID of 0.5 mm and an OD of 1 mm. I also started making a small incision in the opening of the outer tube, so that it could form its own makeshift funnel, if need be. This combination already produced more consistent results. However shifting of the tubes relative to each other sometimes also introduced both bubbles and spillage. I tried all the methods of attachment that I also used for the funnel, but to no real avail.

The final improvement consisted of using silicon tubing. I only used it for the outer tube and it had an ID of 1 mm and an OD of 3 mm. This tube had more friction, causing it to shift less, and was quite flexible, allowing me to gently push out any air bubbles. This in contrast with how I earlier had to either suck them out or blow them out from the other side.

I also did some calculations on the best lengths for the tubes. As several of my samples showed way less beads than expected, as can be seen in Figure 4.8, I wondered whether the solutions even totally reached the chamber. The tubes I were using were quite long, because it allowed me to spot air bubbles before they actually entered the chamber. I figured it would be optimal to have the chamber filled with the excess fluid filling both tubes equally, but the length should minimally fill one tube and the chamber.

Using the dimension seen in Figure 3.3, combined with the thickness of the coverslide I calculated that the volume of the chamber is 12.5 µL. As the minimal amount of fluids that I wanted to use was 30 µL, I s every step in the sample making process that involved smaller quantities up. This left me with a minimum of 17.5 µL to divide over both tubes. I calculated the volume per length for the inner tube with an ID of 0.5 mm and the outer tube with an ID of 1 mm. These were respectively 2 and 7.9 µL cm^{-1}, which led me to the following equations.

If I want to divide what's rest of the minimal fluid over the two tubes, and I want the outer tube to be either 0.5 or 1 cm, the outcomes are the maximal inner tube lengths.

$$(17.5/2 - 1 * 7.9)/2 = 0.4\,\text{cm} \tag{4.5}$$

$$(17.5/2 - 0.5 * 7.9)/2 = 2.4\,\text{cm} \tag{4.6}$$

So either one of these two combinations would be optimal. However I don't necessarily need an optimal configuration, but do want my outer tube to be as long as possible, as it allows me to easily extract air bubbles. Therefore I also made equations to find the minimum.

$$(17.5 - 1 * 7.9)/2 = 4.8\,\text{cm} \tag{4.7}$$

$$(17.5 - 0.5 * 7.9)/2 = 6.8\,\text{cm} \tag{4.8}$$

I then considered that I also wanted decently sized inner tubes. As this keeps them from slipping of from the outer tubes, which also creates bubbles. As such I settled for an inner tube length of 1 cm and an outer tube length of 2.5 cm.

4.2.5 Tools

In the process of making samples I often resorted to using improvised methods for very common things like holding up the sample assembly. So with the cooperation of Gerhard and his 3D printer, I created a couple of new tools for my successor. Using them is fairly straightforward.

The first tool is used to outline the locations of the holes on the coverslide, allowing for a more streamlined process in drilling them. Before, this consisted of using the parafilm template to mark the location two holes in a more arbitrary way somewhere on the coverslide.

The second tool was an improved parafilm template, as the one we had was made for the old process of samplemaking which used a smaller coverslip. The new one has the correct size, so that the parafilm can be cut with just the template instead of also contaminating a clean coverslip.

The third tool was a simple coverslide holder. It keeps the sample elevated, making the glue step easier, as the tubes can be put all the way through, and the outer side of the coverslip clean. Figure 4.5 shows from top to bottom: the hole outline holder, the improved parafilm template and the general purpose holder.

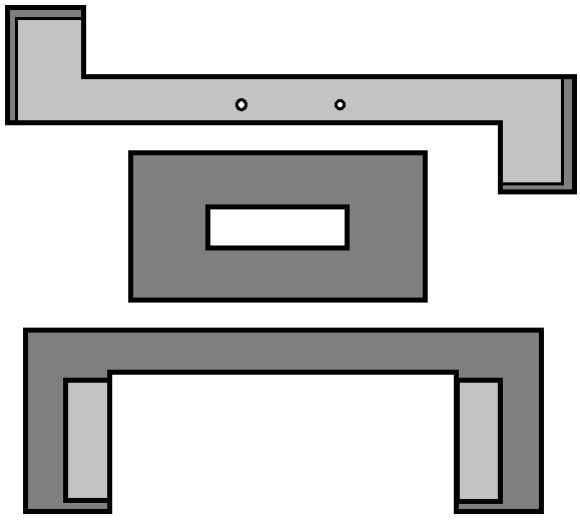

Figure 4.5: A schematic image of the developed tools. The dark grey is as much thicker than the light grey as the thickness of the coverslide.

4.2.6 Parafilm cutting

Cutting the parafilm correctly is deceptively hard. Edges that are not cleanly cut or angles that are not right can lead to even more serious malformations when the parafilm is melted. This can result in air bubbles that are hard or impossible to get out and fluid flows that do not reach the entire surface. I have tried several ways: with the parafilm both towards the template and not, cutting directly or outlining the shape and cutting it then. I also contemplated the idea of having a sort of metal cookie cutter shape, but making it wouldve been more effort than it was worth.

The method that got me the best results was as follows: The template is placed on the parafilm side and the long sides of the chamber, and the outline are cut. Then the template is removed and the parafilm flipped over. The long sides are then cut a few millimeters longer and the short sides are cut on the ends. Overcutting a little isnt a problem as it will melt back together in the steps to come, cutting not enough, however, is, as it can lead to raveled edges.

In the future it might be a good idea to have a template, that is made of a more solid material. Now it would sometimes happen that I would accidentally cut into the template, making for less clean edges and eventually a ruined template.

4.2.7 Tips and Tricks

During my research I found that there were a great deal of little tricks that greatly improved both my speed in working and the overall final product. As it is the full intention of this thesis that my work will be continued in the future I decided to write them down here and save the next person a lot of time.

Drilling

Always drill under water, and change the water regularly as it will get filled with glass quickly. Move Drill through the glass at a constant speed. If you start breaking an usual amount of glass, change the drillhead; it is probably not sharp anymore.

Drying

When drying, hold the nozzle so it wont pop off. Instead of trying to blow off all the water, blow it to one end and let the drops be absorbed by a clean tissue.

Storage

I always drilled enough coverslides for a week. I would then clean them with purified water and ethanol and store the ones that I wasnt going to use in a 50 mL tube filled with purified water. I also tried ethanol, but was told that, unlike water, it attracts biological material.

Gluing

Always try to work in a fume hood and wear latex gloves while gluing, as acrylic glue is hazardous. Using a tiny flashlight can be useful to help you see whether youre applying the glue correctly. A white pipette tip is a perfect tool to place glue around the tube; work only with drops at a time.

Heating

Heating should be done in an almost horizontal fashion on the edge of the heating element. Make sure that it melts evenly, using a tool to push down upon it can be useful for that. Do make sure not to melt it too long as it will distort the sides of the chamber.

Flow speed

Pipetting too fast can destroy the carefully crafted composition within your chamber. The chamber only contains a small volume, so pipetting 74 µL in a second can cause strong currents within. Using 10 seconds for every 30 µL is a safer standard that I mostly used. It might however still be too much.

4.3 Measurements

As stated, I used an optical microscope to look at my samples. I used 40x and 60x magnifications. The former mostly to search for beads that I could use and the second to look at them in detail. Instead of relying purely on what I could see from the microscope, I used the digitally translated images mostly, which allowed me to enlarge the image digitally. In this way I could look more carefully at regions of interest. In this way I could see changes in the image even if the difference was only a few pixels through the live camera feed. Before getting down to the actual results I have to clarify a few terms. Within the sample there are three basic states: so called 'stuck, 'loose and 'tethered beads.

A stuck bead is non-covalently bound to either the coverslip or coverslide. It will not move at all, because the binding energy is higher than forces generated by Brownian motion. Finding them is easy as they compose the first and last layer that can be seen when going through different depths of the sample. I often found the highest bead density in these areas, although there should optimally be none, and they can be used as guidelines as to where the other beads should be found between. A loose bead is not bound to anything. It may have picked up a DNA molecule, but that too is not bound to the anti-digoxigenin on the coverslip. Because its movement is decided purely by Brownian motion and drift they can be seen to do a variety of things. It can make random walks that look like the bead is DNA is stuck to the coverslip, it can be almost still, it can move almost linearly. The last is mostly caused by internal currents within the sealed off chamber, which are called 'drift, although they can happen by chance. An important thing here is that they can be found anywhere between the stuck layers.

A tethered bead is a bead that was successfully put in the required conformation of coverslip-DNA-bead. Differentiating between a tethered and a loose bead can be quite difficult: a bead should move, but only ever so slightly. So using the Pythagorean theorem with half the diameter of the sphere added to the DNA length as the hypotenuse and once again half the diameter as one of the legs. Twice this distance yielded the maximal distance that the bead can move on the x,y-plane, which is the one that can be seen: 0.82 µm. So if a bead moves more than this it is obviously not tethered. Also, if a bead is more than a DNA length away from the stuck beads, it is impossible that it's tethered.

When I went deeper into the research for what I expected to see I also found a piece by Nelson, Zurla and Durlap[17]. They conducted experiments to relate the length of a DNA tether to the mean excursion of the bead. In their research they used polymer beads with a density highly similar to the beads I used and a diameter of 0.48 µm[17], which is less than a 5% difference from my diameter of 0.53 µm. Because of this high similarity I could use their finding to predict the expected movement in my measurements.Using a graph I determined that the likely movements would range between 155 and 185 nm, capturing most folding possibilities for hTel DNA. Ultimately their data could also have been used to measure the difference in tether lengths and spot possible Quadruplex formations, however due to the nature of my samples I wasn't quite at that point yet.

It should be kept in mind that the pixels are 16x16 µm divided by the magnification. The magnifications that I used most are 40x and 60x giving pixel sizes of 400x400nm and 267x267 nm. If one keeps in mind that the movement of a tethered bead is usually around one pixel and that it can only be found fairly close to the stuck beads, combined with loose beads that look like tethered beads, one can imagine finding them to be quite a tedious task.

A typical image of what I would see can be seen in Figure 4.6. As can be seen it seems to consists of different wave-pattern like circles of different sizes

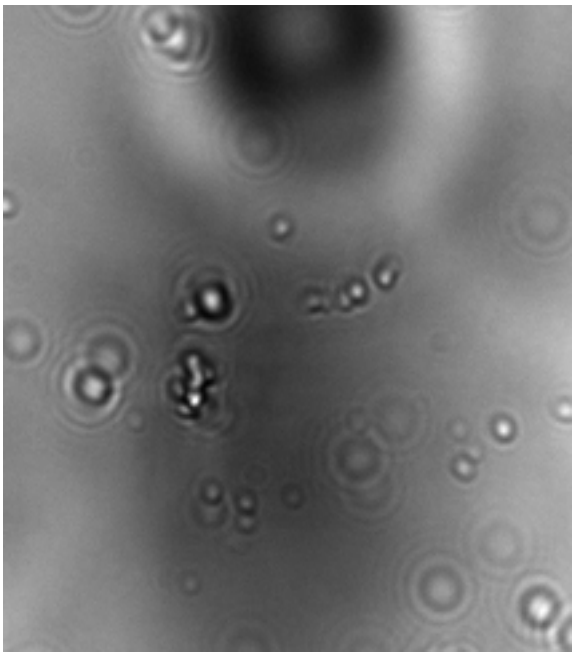

Figure 4.6: An image of the Sample 60x enlarged.

and intensities and a big blob like pattern in the background. The latter is some form of dirt present in the microscope that I could not remove without risking damaging it. The former however are the microspheres. The wave patterns are created by self-interference of the light used to look at them, and depending on whether the beads are below or above the focus they can have a black or a white center, with their size proportional to the distance from the focus.

4.3.1 beads

When first starting I found two mayor problems that were prevalent throughout my research:

- There don't seem to be enough beads
- There are clusters

Here, clusters are defined as non-covalently bound beads that form conglomerations ranging from 2 to upwards of 10 beads. I decided to tackle this problem first as it seemed like something that could be solved by changing or adding only a single step in the creation and storage process. I consulted

the website of the beads' producer. They advised the following in ascending severity[18]:

1. Pipetting roughly
2. Sonication
3. Vortexing

I tried all of them and compared them under a microscope to an untreated sample of beads. Sadly, pipetting roughly did not seem to produce the necessary force to take apart the beads and seemed almost identical to the untreated sample. Vortexing had the unpleasant side effect of pushing most of the beads to the bottom of the tube, packed together so tightly that they stay in that layer. As such sonication seemed to be the way to go.

Sonication consists of placing the tube containing the bead solution in a water bath. The tube is then bombarded by ultrasonic sound waves and this energy then breaks the non-covalent binding. I was somewhat wary of using sonication as the energies involved can also end up damaging the beads. This is especially unwanted in the actual CFM tests as the forces need to be highly precise and rely on the exact bead mass. The producer mentioned that the heating up of a sample is a characteristic symptom of beads getting damaged, as they then have too much thermal energy. As such I decided to permanently add a 30 second sonication step to my sample making process.

Upon trying this I did notice a slight increase in temperature and when I looked under the microscope I saw some beads that seemed asymmetrical, which were probably beads, which lost fragments. An example of a broken bead can be clearly seen in Figure 4.7 I also, sadly, still saw clusters, but significantly less than before. Over the course of my research I have tried many sonication time lengths: 30 seconds, a minute and even 10 minutes. I found that sonication almost always seemed to have a positive influence on the number of clusters in about the same amount. The number of damaged beads however seemed to stay roughly the same too, growing only slowly as time increased.

Figure 4.7: An broken bead next to a normal one for comparison.

4.3.2 Lack of beads

The purpose of my research was to produce samples with high quantities of tethered beads and low amounts of loose or stuck beads. However, as stated

before, there were not as much beads as I expected and it was very time-consuming to follow beads long enough to say with some certainty that it was tethered. A persisting problem has been the low amount of beads in the sample: a 15x magnification can be seen in Figure 4.8, showing only sporadic beads when the expected amount of beads should cover a great part of the surface.

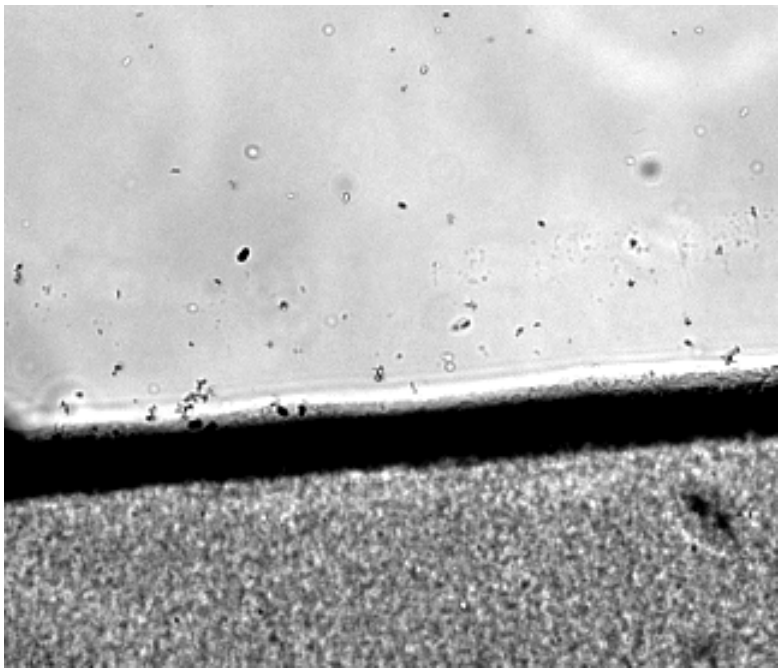

Figure 4.8: 15x Image of sample with visible parafilm border.

Because the former process did seem to have a lot more beads, and the most important difference is the terms of getting the solutions into the chamber (injection vs. capillary) this is the most likely suspect. I concluded that a very possible reason could be the forces induced by pipetting. The composition is fragile and with 75 µL going through the only 12.5 µL chamber quickly this can be a huge displacement. Doing this carefull however did not seem to make the improvement that I hoped it would, this makes me question whether this is really the problem, and, if it is, whether it is possible to pipette slowly enough to cope with this problem.

Another possible cause could be that sonicating the beads destroys the streptavidin on their surface. This would be a possible reason why there seemed to be even less beads after I started sonicating them. Sadly, I only thought of this when there wasn't enough time left to test this.

4.3.3 Tethered beads

I also tried to find evidence that there were actually tethered beads present in my sample. I did this by taking the TIFF files that I got from the microscope and analyzing them with FIJI[19]. I set a threshold value in terms of luminosity that only incorporates the bead and use the Tracking functionality to create an image showing the path. If the path is a lot more than 400 µm it is unlikely that the bead is tethered. An example can be seen in Figure 4.9. This image is 60x magnified and the bead is clearly loose as it moves substantially more than the two pixel movement expected.

Figure 4.9: 60x Magnified, ImageJ analyzed image of a loose bead.Every pixel is 267x267 nm.

I also manually checked whether the Tracking function was capable, and as it turned out it worked perfectly. Having gained trust in this method I started analyzing my data and finally got trustworthy results. In Figure 4.10 a bead can be seen, which I overlaid with its path, which is visible in white, in the center of the bead. This image is also done with 60x magnification, and as can be seen the bead only moves 4 pixels. This corresponds roughly with about a 600 nm movement, which is well within the possible range, making it very likely that this bead is indeed tethered.

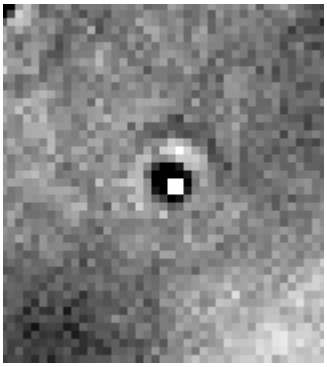

Figure 4.10: 60X Magnified image, with the path outlined in white. Every pixel is 267x267 nm.

Chapter 5

Future Research

There are a lot of possibilities in the future to continue my research, as it was far from complete when I stopped. I will look at it within three timeframes, all with their own respective magnitude.

5.1 Sample testing

The sample contains by far not as many beads as expected, but the exact reason for this is unclear. It could be that the DNA does not attach to the coverslip, or that the beads do not attach to the DNA. Maybe they all attach, but are flushed away in a later stage. Before the problem can be solved it has to be known what the problem is. As such, future tests can be done to find where it goes wrong. I have a few propositions for these tests.

5.1.1 Labeled anti-digoxigenin

It might firstly be very useful to check whether the non covalent bind between Antidoxigenin and the coverslip can be maintained during the harsh process of pipetting new substances and flushing. This could be done using Fluorescently labeled anti-dig. The sample making process should just be followed normally, only exchanging the DNA-bead mixture for the anti-dig. If this does not lead to a clear fluorescent layer after flushing, this is obviously a problem.

5.1.2 Quantum dots

Dave van den Heuvel works with Quantum dots: nanomaterials of semiconductive materials. He informed me that Quantum dots coated with strepta-

vidin could be an excellent way of testing the attachment of DNA to the coverslip, as they can be made very visible, but can be quite small. This would mean they would only put minimal forces on the DNA, allowing to really only look at the coverslip-DNA interaction.

5.1.3 YOYO-1

YOYO-1 is a green fluorescent dye that has the ability to enter and stay within DNA. Bound YOYO-1 produces a light intensity 3200 times larger than unbound YOYO-1[20]. It has the added benefit of being able to bind to DNA once every 3 base pairs, further heightening the signal produced. It could be used to test the same thing as Quantum dots, but could also be used to test the binding between beads and DNA, and its survivability during the sample making process, or even if there are problems with the streptavidin.

5.2 Centrifgual Force Microscope

Once the sample can be made successfully the original scope of my research could be resumed. It is than possible to search for the unfolding of the G-Quadruplex DNA. Solving the problems with the non-collimated light source and getting to the right precision to actually detect this change could pose a challenge to the person who will do the follow-up research. There is also plenty of room for improvement in the software section, which could be looked at: streamlining the interface and optimizing mass measurements.

5.3 Practical use

There are many possible ways to apply a working CFM to research. Perhaps even other types of molecules can be tested using it, and if not then there are always a lot of different DNA shapes that can be tested. I want to however focus on something a little less obvious.

5.3.1 Telomestatin

If all this is possible, and the method of measuring is well established it can be used for more practical ends. One of the possibilities that I found is using the CFM for testing the anti-cancer drug Telomestatin and similar substances. They fight cancer in the following way: Cancerous cells divide very rapidly, an act that would normally deplete the telomeres, which triggers apoptosis. These malignant cells, however, mangage to, through mutation, activate the

enzyme Telomerase, which elongates the telomeres again. Thus allowing the cells to keep going through mitosis. Research has shown Telomerase activity scales inversely with G-Quadruplex folding energy in the telomeres, which is also what was used in this research. As such new materials are developed that increase this energy[21].

One of the most tested is Telomestatin, but research on it, and other drugs, are mostly down slowly through single molecule methods. Massive experiments using the CFM could prove to be a great tool in testing new and even more effective drugs quickly on potency in terms of G-Quadruplex folding energy.

Chapter 6

Conclusion

In conclusion I can say that the research on this fascinating subject is not done by far. Although I may have found a few tethers, the CFM should have tethered beads aplenty to measure. I have, however, successfully managed to create the DNA used in this research. This success coupled with my detailed steps should mean that my successor should be able to do this as well.

I also managed to optimize many ways in which the sample for the new design can be made. I terms of the tools I developed and the optimalization in tubes, glue and beads. I provided many tricks and detailed reasoning, that will get that person started and allowing him or her to easily follow the logic in why I did what I did.

Lastly I provided many possible research goals that can be used in the future, thinking in different timescales. Allowing this thesis to be useful not only for my direct successor, but also for sequential researchers.

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Appendices

Appendix A

Sample preparation overview

Time	Antidig	DNA&Beads	Finish	Antidig Comments	DNA& Beads Comments	Finish Comments
0:00	Anti body			Channel: 7.5µl antidigoxigenin + 22.5 µl pure H20 **20 min attachment time**		
0:04		PTC		2ml/week PTC1 (Polystyrene beads): 1.425 ml PTC0 + 75 µl Casein stock PTC2 for (Silica beads): 1.97 ml PTC0 + 30 µl BSA stock		
0:23	Rinse			2 x 75 µl PTC(1 or 2) **30 min attachment time**		
0:42		Bead solution			In microtube: For Polystyrene: 1 µl bead solution + 26 µl PTC1 For Silica : 4 µl bead solution+ 25 µl PTC2	
0:44		Sonicate			20 seconds	
0:46		DNA			Add: For Polystyrene: 3 µl DNA For Silica: 1 µl DNA	
0:48		Rotate			Rotate the mixture **10 min attachment time**	
0:55	Rinse			2 x 75 µl PTC(1 or 2)		
0:59		DNA solution				Add 30 µl DNA/bead solution to the channel **5 min attachment time**
1:07			Rinse			2 x 75 µl PTC(1 or 2)
1:10			Cut			Use scalpel
1:11			Seal			Use acrylic glue

Figure A.1: An overview of the sample creation, given that there is a Sample assembly present.