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Single molecule detection of proteins in microfluidic platform

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Title (English)

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Abstract

The development of novel method for measuring low concentrations of protein. Two antibody-linked single-stranded oligonucleotides are ligated specifically only when the target protein is present. A circle is formed by connecting the ends of the oligonucleotide to each other. A long molecule complementary to the circle is rolled of and hybridized with a large number of oligonucleotides connected to a fluorescent dye. These big molecules can be detected visually in a confocal microscope, counted with a pattern-recognition program and the initial concentration can be calculated.

Keywords

Micro fluidic platform, protein concentration, protein detection, proximity ligation, rolling circle amplification, single molecule detection

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Jonas Henriksson

Sammanfattning

Proteiner är en grupp molekyler som inte bara bygger upp muskler, senor och hår, men även är inblandade i många av kroppens inre funktioner. Detta innebär att om man till exempel är sjuk kan proteinnivåerna i olika delar av kroppen påverkas på olika sätt. På grund av att nivåerna i fråga många gånger är väldigt låga, är de med dagens metoder oftast omöjliga att mäta. Hade man emellertid kunnat mäta lägre koncentrationer, och se skillnader mellan dem, hade man kunnat skaffa sig mer information om hur dessa sjukdomar påverkar kroppen och i förlängningen även avgöra huruvida en person är sjuk eller inte.

Jag har jobbat med att utveckla en ny metod för proteinmätning som potentiellt ger möjligheten att mäta både lägre koncentrationer och med högre noggrannhet än vad som idag är möjligt.

Metoden går ut på att kopiera en DNA-molekyl som man via en igenkänningsmolekyl binder till proteinmolekylen. DNA är en molekyl som man länge kunnat kopiera i tusental, och jag har använt mig av ett sätt där jag låter av DNA-molekylen bilda en cirkel som sedan rullas varpå en lång kopia bildas – som i en tryckvals. Den långa kopian trasslas ihop, märks sedan in med en molekylspecifik färg och kan sedan studeras som en liten prick i ett mikroskop. Varje prick härrör från en enda proteinmolekyl, och varje färg kan representera ett proteinslag.

Examensarbete 20p inom
Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet januari 2007

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1. Background

1.1 Why measure protein concentrations?

”DNA makes RNA makes protein makes life”

The sequencing of the human euchromatin is finished to 99%[1]. Since the last publishing in 2001[2] today the international human gene consortium (IHGC) has apart from lowering the error rate and the amount of gaps in the sequence, also lowered the suggested amount of genes from 30,000 to between 20,000 and 25,000.

-How can about 3GB of information[1], no more that can be stored on a DVDR, and coding for no more than 25,000 genes, contain all the properties and differences that makes us human beings?

-Well, the answer is that we don’t know. Our newly won knowledge of the human genome only describes the most fundamental theory of life in a molecular point of view. The diversity of it is also dependent of RNA-level phenomena such as multiple start- and endpoints and alternative splicing, as well as protein level – posttranslational – modifications.

-Can’t we just measure levels of DNA, RNA and protein at the same time, in the same method?

-The answer is, for the moment, no.

The common way to detect DNA molecules today usually involves amplifying of the molecule[3]. This is in the nucleic acid case rather simple during to the complementary in the double stranded molecules. In the protein case however there is no complementary sequence, making the amplification process, and therefore the detection method, a bit trickier.
We need to develop better methods to measure protein concentrations.

The lack of good methods to detect low abundant proteins has resulted in limited information of how proteins affect the body. Our present information covers only those proteins we can detect without any amplification, and the only information known of what really happens derives from data achieved by interaction studies performed in far from realistic circumstances.[1, 4]

This lack of information is in itself probably the main reason for developing new tools to measure protein concentrations. By monitoring different protein's roles in the complex pattern of protein interactions and signal paths, we can be sure to achieve more information of these.

One also understands that in parallel to achieving this information one can detect abnormalities in protein concentrations. With more information about how proteins affect the body one can hence connect an abnormality in protein concentration to for example a specific disease and in the extension diagnose patients via more refined protein concentration measurement.

1.2 How are protein concentrations measured today?

Methods used to measure protein concentrations today could be divided into two groups, those who are dependent of molecules that specifically recognize and bind to the certain protein (affinity reagents), and those who are not.

There are of course pros and cons with both of the strategies but simplified one can say that detection molecule dependent assays are more selective[5, 6]. They pay their price however by requiring a priori information about the protein in order to construct detection molecules for the specific application. The binders used in detection molecule dependent methods may be of different kinds and play a crucial role for the properties of the method. In order to discuss these binders there are some concepts that needs a short explanation.

Affinity is the term used to describe a detection molecule's tendency to bind its protein, easiest measured by their dissociation constant (K_d), defined as the concentration of free detection molecule when the probability of finding a bound protein is 0.5. A lower K_d corresponds to a higher affinity.

Selectivity can be explained as a detection molecule's ability to bind to its protein without interference from other molecules in the solution.

Sensitivity is the detection molecules ability to bind to rare molecules in a sample, easiest quantified as the limit of detection, the lowest amount of protein resulting in a detection signal two standard deviations over the systems background.

Other crucial concepts are ease of preparation, stability and reproducibility. In general a good detection molecule has high affinity, high selectivity and high sensitivity to the protein concerned. I will present three different kinds of molecules used today, antibodies, nucleic acid aptamers and affinity ligands.

1.3 Detection molecules

Antibodies

Antibodies are the natural occurring detection molecules. With its variable and constant regions antibodies builds up a library of detection molecules that our body uses for immunological defense. They have a very high typical maximal affinity[5] in vivo that corresponds to a K_d of 10^{-11}M. Produced antibodies are of two different kinds. The traditional antibody is made by infusing the protein to a host animal, and retrieves its immunological reply, polyclonal antibodies – a mix of different binders that has affinity to different regions on the protein. Depending on the batch, polyclonal antibodies vary in affinity, selectivity and sensitivity. Monoclonal antibodies are made using hybridoma cells, which produces one clone of binders with the same affinity, selectivity and sensitivity which makes them – in contrary to polyclonal antibodies – reproducible. Another aspect is the price and accessibility. Polyclonals are cheaper and easier to produce. In general one can add that antibodies easily decompose during storage and freeze/thawing[5].

Nucleic acid aptamers

Nucleic acid aptamers are strands of DNA selected by randomly having affinity for different protein molecules due to their tertiary structures. They are typically strong binders[5], with K_d of about 10^{-9}M and are therefore used as a complement to traditionally antibody applications. Their main advantage is their stability, being nucleic acid chains and not denaturable proteins. They are also easy and cheap to produce and copy using standard DNA amplification technique.

Affinity ligands

Affinity ligands, also named peptide aptamers or scaffold proteins, are usually weak binders selected for their affinity to a protein in analogy to nucleic acid aptamers, but being peptide chains instead of nucleic acids. Due to their rather low affinity[5], K_d of typically 10^{-7}M they are mostly used in affinity chromatography related methods.

1.4 Methods used today

The most commonly used method in each group will be discussed.

1.4.1 Detection molecule independent methods

Two dimensional gel electrophoresis (2DGE)

2DGE separates proteins on a polyacrylamide gel in two directions. Horizontally separation is based on the proteins isoelectric point (pI) i.e. at which pH their net charge is zero. Vertically separation is made by size just as in normal SDS-PAGE (Figure 1). When proteins are separated, one can quantify the different proteins using e.g. mass spectrometry. This way of quantification is time-consuming and the instruments used are expensive and requires skilled personnel[7]. This makes the method suited for analytical investigations rather than a "screening approach" or for diagnostic purposes. The main advantage is as mentioned earlier it's independent of detection molecules. Because the contribution from random diffusion in the gel, the proteins tend to "blur out" on the gel, which leads to poor separation of proteins with similar properties. The main advantage is that the method can apart from concentration measurement also be used for characterizing new proteins[8] and study their post transcriptional modification patterns.

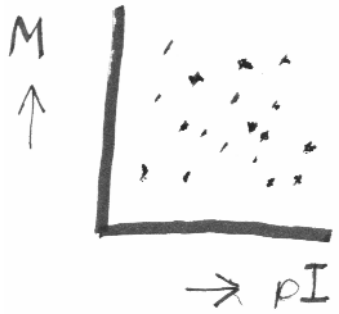


Figure 1. In 2DGE proteins (black dots) are separated on a gel based on their molecular weight and isoelectric point

1.4.2 Detection molecule dependent assays

Enzyme-linked immunosorbent assay (ELISA)

ELISA is the most common strategy of determining protein concentrations today[5, 6, 7, 9]. The format includes detection molecules, usually antibodies, in a dual layer assay (Figure 2). In the original assay the protein in the sample is first bound nonspecifically to plastic wells, and after washing the primary detection molecule is added. After another round of washing the secondary detection molecule that binds to the primary detection molecule is added. A third round of washing then is required before the intensity can be measured from a third molecule that changes its fluoroforic properties when cleaved by an enzyme connected to the secondary detection molecule. The good thing with the method is that the dual recognition events increase selectivity as well as the limit of detection[5, 6]. Nowadays ELISA is often performed as a sandwich assay with radiolabeled antibodies and fuorofores as readout molecules.

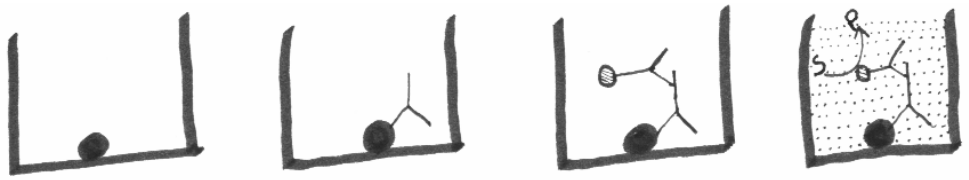


Figure 2. In ELISA concentration is measured via dual detection molecules (Y-shaped) binding to immobilized protein (big black dot). One of the detection molecules is linked to an enzyme (white dot) that catalyzes (S→P) a detectable coloring of the surrounding liquid (small black spots).

1.5 What needs to be done now?

In order to be able to measure low abundant protein concentrations simultaneously from a small volume of sample, one has to develop a new multi compatible method with better sensitivity and selectivity.
The method needs to give a reliable result even when small volumes of sample is used, if today's methods uses all the blood of a mouse to measure the level of interesting proteins not much work can be done on that mouse later. Another wish is that the method can be used to measure several different proteins in parallel, and it certainly needs to be quick, inexpensive and easy to use.

1.6 Nucleic acid ligation based methods

In order to fulfill these wishes there have been some research on methods where the discrimination by ligation together with the ease of amplifying a DNA signal might do the trick.

1.6.1 Discrimination by ligation

DNA ligases have the property of efficiently repairing a break in one of the strands in double stranded DNA[10] (Figure 3). Its ability to serially join two single stranded DNA molecules is however strongly limited. This is due to the slow reaction rate that requires the fixed position supplied by the hybridization. As a result of this the ligase´s ability to join a mismatched nucleotide is very low whereas their ability to ligate matching nucleotides are very high[11].

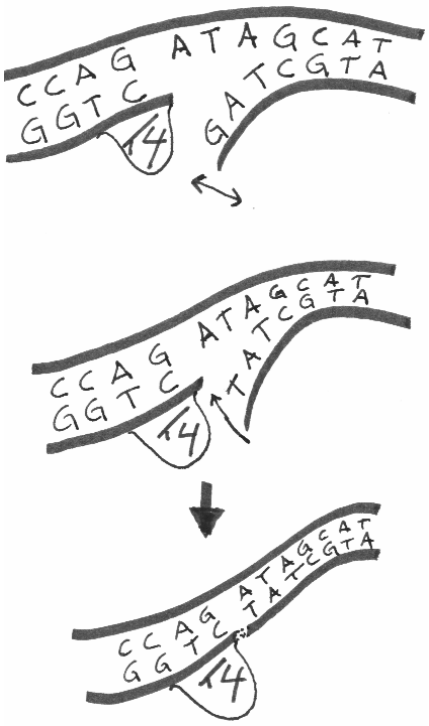


Figure 3. The ligase (T4) highly prefers to ligate the matched sequence (lower two) wich gives rise to the discrimination by ligation methods.

1.6.1.1 Padlock probes

Padlock probes use discrimination by ligation described earlier to detect specific DNA sequences. The probe is simply a single stranded DNA molecule about 100 nt long designed to form a circle when hybridizing to its target molecule. In this state the outer ends are forced by the hybridization to come end to end to each other. If the molecule shows a perfect match at the ends a ligase can join them together (Figure 4). The ligated strand can be amplified and quantitatively examined using the PCR technique. Today padlock probes are used to analyze sequence variants in specific places both in DNA and RNA[12, 13].

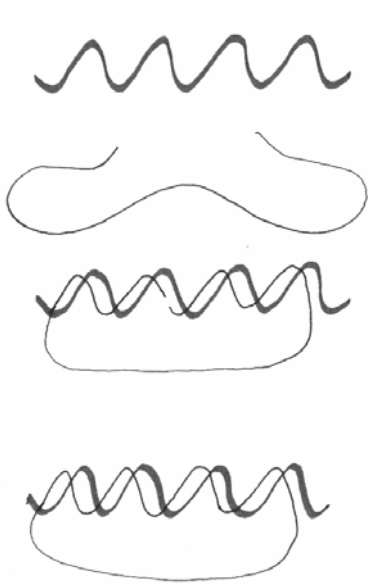


Figure 4. A Padlock probe (thin strand) can be hybridized to DNA (thick strand) and ligated (lower picture) forming a" padlock" around the DNA.

1.6.1.2 Proximity ligation

Proximity ligation is a method developed from padlock probes used to quantify proteins[5, 6].

This is how it all works: Two different detection molecules e.g. antibodies or aptamers are pre-conjugated individually to approximately 50 bases long strands of DNA, containing protein independent PCR-primer hybridization sites. In addition one of them contains a sequence tag that is unique to the specific protein. When the two detection molecules bind the same protein molecule, they are close enough to each other to be joined with a ligase. (Figure 5) As mentioned before the ligase can only join double stranded molecules, this is solved with a short complementary strand of DNA – henceforth referred to as "splint". By diluting the sample before ligation one can minimize the risk of two DNA-strands being in proximity by chance. Having built this "proximity complex" one easily gets a read-out by amplifying the ligated strand using a real time PCR and some sort of detection probe. Please note that only the tag sequence is designed specifically for each different protein. Several different proteins could therefore be measured simultaneously using the same splint and primer-strands.

The selectivity of the Proximity ligation method is very high due to its requirements for two recognition events together with the discrimination by ligation[14, 15]. By amplifying the signal with PCR the method also gets high sensitivity. To detect low concentrations of a protein only a small volume of detection molecules are needed. Even though the dynamic range of PCR is theoretically about 10^8 [24] the PCR readout gets limited by background ligations earlier.

Figure 5. In proximity ligation two detection molecules (Y-shaped) bind to a protein molecule (peanut-shaped). The detection molecules has DNA extensions that can be ligated by a splint (short upper ladder), and amplified using PCR.

1.6.1.3 Rolling circle amplification (RCA)

Amplification of circular DNA molecules doesn't necessary need the PCR reaction[13, 16]. By using a highly processive enzyme such as φ-29 a polymer of repeated complements can be rolled off in a way similar to an old newspaper press. The method is called rolling circle amplification (Figure 6).

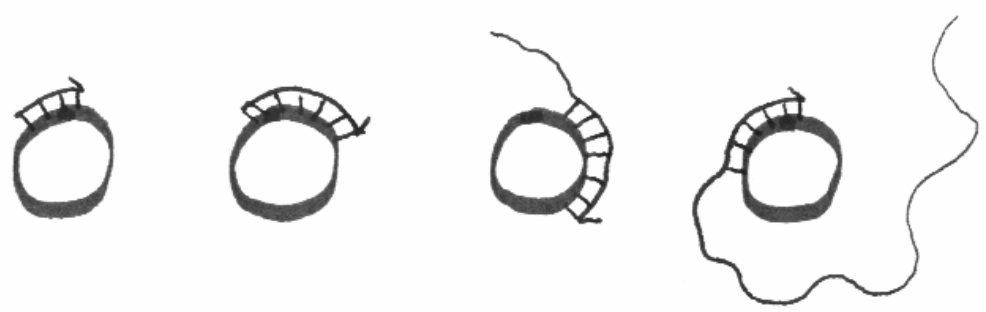


Figure 6. Rolling circle amplification. A primer sequence i hybridized to a circularized DNA strand. As the polymerase duplicates the steric force around the circle makes the duplicate displace from it, allowing amplification of the same segment over and over. The" spun of" strand then consits of a polymer of repeated complements to the initial circle.

1.6.1.4 Circle to circle amplification (C2CA)

C2CA is a method to increase the number of circles based on rolling circle replication[17]. After several revolutions of rolling circle amplification, short complementary DNA strands are added in excess. These molecules play three rolls in the process.

Firstly they hybridize to the rolling product to create double stranded regions that can be cut using restriction enzyme resulting in monomers. Due to their excess, intact complementary strands will replace the cleaved ones and act as splints allowing the monomers to form circles. Finally they can act as primers to an additional rolling circle replication reaction. These steps are then repeated for a desired number of cycles (Figure 7). The advantage of C2CA is the exponential amplification rate over a number of cycles[17].

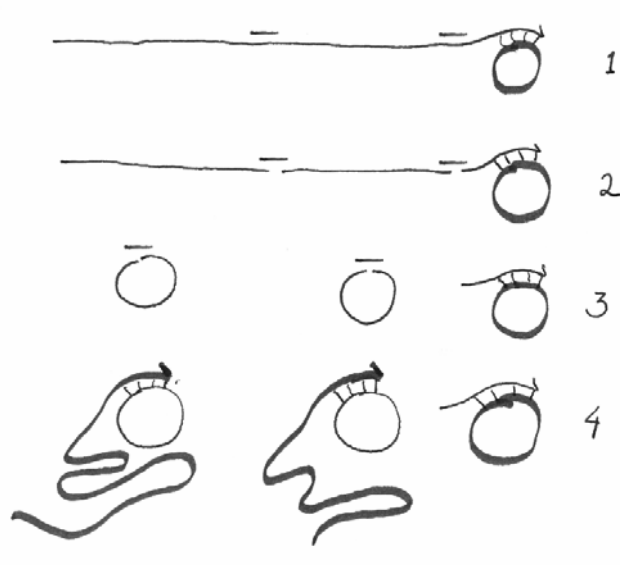


Figure 7 C2CA. A polymer of complements (thin strand) made with RCA (1) is cut into monomers (2) and recirculaed (3). The inverse circles are then rolled (4) to produce a polymer of the same polarity as the original circle (thick strand).

1.6.1.5 The Blob

Blobbing is alternative readout method for padlock probes.

Instead of a PCR-readout, one can make a large concatemeric DNA molecule using circular probes and RCA. This molecule can thereafter be fluorescence labeled using fluorofore-coupled DNA strands complementary to it forming a shining skein of DNA[18] (Figure 8). A set of probes designed to detect different DNA sequences can be rolled off in the same vial. By making them hybridize to oligonucleotides labeled with different fluorofores, one can investigate several sequence variants simultaneously. If the proximity probes are circularized, the blob readout can be used not only to distinguish DNA sequence variants but also to measure protein concentrations.

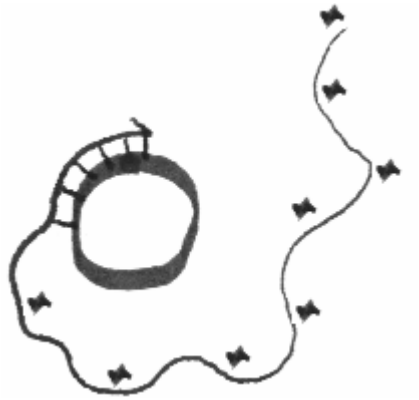


Figure 8. The Blob is a fluorofore linked molecule rolled off a DNA circle. The black stars represents fluorofor linked complementary DNA strands.

The main advantages of the blob readout are firstly a better accuracy compared to PCR because the amplification is linear in each step. Secondly the degree of amplification is only about 30 cycles in PCR[3]. In the blob readout one is not limited by anything else than the scanning time[18, 25]. This is due to optically defined sample volume and counting of discreet objects. Thirdly the possibilities of multiplexing the reaction is very limited in a PCR assay, whereas using a blob readout and a well compiled set of fluorofores[19] one can detect a number of colors in the same vial, without having as a problem that higher concentrations of another fuorofor results in unreliable values. This is a result of the fact that in a PCR assay one can only measure the total amount of fluorescence in one color. In the blob assay one measures a number of individual colored spots that can be detected and sorted out from the background. If blobing was made to measure protein concentrations under ideal circumstances, there would be one blob visible per protein in the sample, and by making it shine in a distinct color, it could be sorted out among thousands of other-colored.

1.7 Confocal microscopy and microfluidics

The shining skeins of DNA – the blobs – can be detected and quantified visually through a confocal microscope[20]. The microscope sends out a laser beam that exitates the fluorofore, witch sends out emission light with a higher wavelength. The main thing about the confocal microscope is however that the emitted light can be collected only from one conjugate plane simply by focusing using lenses and a pin hole. This property makes confocal microscopy ideal for counting blobs because background light from the bottom and top of the vial can thereby be avoided.

If one would let the sample flow through microfluidic channels instead of just putting it on a microscope slide, one can ensure that a single blob is only counted once just by having fast enough flow through the channel. By doing this however, only a fraction of the sample is analyzed which could lead to a higher detection limit if the confocal microscope is not fast enough. The use of a microfluidic platform for the blob readout keeps the reagent volumes and analysis time down for the assay[20].

2. Introduction

The aim of this work was to create DNA circles from a protein recognition event, and subsequently perform rolling circle amplification to create blobs that can be quantifiably measured in a microfluidic system connected to a confocal microscope. The work was divided into three parts. In the first one I verified the standard proximity ligation with real time PCR readout, as a protein concentration measurement method[14]. In the second part parameters for the blob readout was optimized with circles ligated without protein present, and in the third one an attempt was made to get all the parts together from recognition complex to blob.

2.1 Part one – Real time PCR

To verify proximity ligation as a concentration measurement tool[14] known concentrations of the protein PDGF was measured using real time PCR amplification with SYBR green (see 3.1).

2.2 Part two – Optimizing the blob readout system

As mentioned in the background, the blob is a shining skein of fluorofor hybridized DNA, which was the result of a single protein detection event. In this part there was no protein recognition event. Instead circles of DNA were used as templates. The circles were made by ligating oligonucleotides using excess of splints, which later acted as primers for the blobbing. The

efficiency of the blob readout depends on how well the blobs were scanned in a confocal microscope and counted with an in-house made matlab based picture analyzing program. The aim of part two was to optimize the rolling and hybridization of fluorofore connected oligos to achieve the maximal selectiveness for the readout method. Optimization in confocal microscope settings has already been done with other assays.

2.3 Part three – From recognition complex to blob

In this part the two previous parts were put together, in an attempt to measure the recognition complex concentrations with the blob readout system. In order to achieve enough circles, a circle to circle amplification step had to be included. Since this is a complex task, a PAGE assay was run with fractions from each step in order to monitor the progress in terms of cutting and circularization of the recognition molecule, and forming the blob molecule.

3. Theory

3.1 Part one – Real time PCR

SYBR-green I:

SYBR-green I is a small molecule that increases its emission-intensity with a factor 50 when bound to a double stranded DNA-molecule[21]. When SYBR-green I is added to a standard PCR mix it can be used to detect concentrations of DNA. As the cycles go on the PCR mix increases its fluorescence as the amount of double stranded DNA is amplified.

C_t-value:

As PCR is an exponential amplification method the read-out as function of number of PCR cycles changes exponentially in a certain interval. The fractional cycle when the fluorescence exceeds a particular intensity is called the threshold cycle (C_t) and is reversely proportional to the log of the starting concentration[22] (Figure 9).

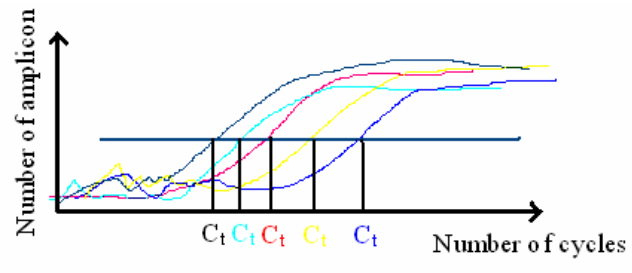


Figure 9. The C_t value is at which cycle a pre-determined threshold value of amplicons (horizontal line) is reached. The increase of amplicons is measured by an increase in fluorescence.

3.2 Part two – Optimizing the blob readout system

The fluorofore labeled single-stranded DNA molecules can be easily detected as shining dots in a confocal microscope.

To optimize the numbers of rolling products detected as blobs a series of optimization experiments were made. The main theory is that a rolling circle product has to emit sufficient light to be considered a blob in the evaluation program. The emitted light comes from detection probes – short oligonucleotides coupled to a fluorofor that hybridize to the single stranded rolling product. If the product is not long enough it will not be detected because the total amount of emitted light will not be high enough to distinguish it from the background. Even if the molecule is long enough, the hybridization has to be good enough to make the concentration of fluorofores sufficient to detect it as a blob. Remember – in this readout the total concentration of fluorofores is the same regardless of the protein concentration. What is measured is spatial accumulations of fluorofores due

to its hybridization to rolling products. The total amount of fluorofores is limiting for the maximum protein concentrations that can be measured with this technique. If the amount is too high, however, then the background concentration will limit the minimum concentration measurable.
A summary to this discussion is that the more blobs that are detected from a certain protein concentration, the better – as far as there is significance in the higher protein concentrations.

3.2.1 Rolling time

The polymerase needed to make a rolling product from the circles has to be a highly prossessive with a strand displacement effect. Earlier studies have shown that φ-29 polymerase is a good choice. Since the product yield is proportional to time under non substrate-limited conditions[23] I wanted to study whether the rolling time of the circles had an effect on the amount of blobs that were detectable. A longer time should be more effective due to longer rolling products (bigger blobs) that are more effectively detected in the confocal microscope. Too big blobs however could result in a depletion of the detection probes, and paler blobs, less easy to detect.

3.2.2 Hybridization conditions

The assay was set up to test the parameters disentangling temperature, disentangling time, hybridization temperature and hybridization time.

The theory prior to my investigation was that a rolling product, when diluted in PBS, collapses to a ball of single stranded DNA. When hybridizing fluorofor-attracted oligonucleotides to the product, one first needs to disentangle the ball by melting unwanted random hybridizations, making a more accessible structure. The time and temperature for the disentangling is varied. Too low disentangling temperature would probably result in a less accessible structure, and lower hybridization ratio, every thing else held constant. If too high temperature could have an effect to the hybridization is yet to find out. After decoiling, the time and temperature of the hybridization was also varied. A higher temperature would result in fewer oligos hybridized to the blob and a lower temperature could enable the different oligos to hybridize unspecifically. The time needed to perform maximal hybridization is only a convenience factor.

3.2.3 BSA concentration

When having optimized the times and temperatures, I wanted to see if the addition of BSA could further improve hybridization. Here the theory is that rolling products, polymerase and detection probes both sticks to the walls of the vials, reducing their bulk concentration. BSA is a protein that simply sticks to the walls instead of our reagents. Making the bulk concentration of rolling products and probes relatively higher could result in more blobs being detected.

3.3 Part three – From recognition complex to blob

In order to investigate cutting and circularization of recognition molecules as well as forming of the blob molecule an assay was set up where cutting, circularization and rolling of recognition molecules were monitored using PAGE.

In the experiments no protein was present. In order to make recognition molecules a 1:2:3 relationship between 3´ arm, splint and 5´ arm was used. Excess of 5´ arms will force almost all of the splints to hybridize to them, and excess of 5´ arm – splint complexes will force almost all of the 3´ arms to hybridize to them. Hereby almost all of the 3´ arms will be in a recognition molecule state at a given time. By radioactively labeled 3´ends of both splints and 3´ arms it is possible to detect the hybridization and cutting of the recognition molecule as the bands wary in molecular weight when the labeled molecule was hybridized, ligated, cut, and replicated. When a DNA molecule is circularized it can either travel slower or faster than an uncircularized molecule of the same length. In this study any variation in "apparent molecular weight" is considered as a circularization of the molecule provided no other operations are made. According to similar experiments performed by my supervisor however, circles of this size (roughly 100 bases long) travel slower than uncricularized products.

4. Materials and methods

4.1 Part one – Real time PCR

Two different detection molecules pre-conjugated with antibodies specific for PGBF-β were provided by S. Gústafsdóttir, Department of Genetics & Pathology, Uppsala University.

1 µl of sample (different concentrations of PGBF-β in PBS / 0.1% BSA) was added to 4 µl solution containing 20 pM of each antibody-conjugated detection molecule in a buffer containing PBS, 1% BSA, 16 µg/ml polyA and 1 mM free biotin . The mix was then incubated in 37˚C for one hour to allow the protein-antibody connection.

The 5 µl of solution was added to 45 µl of PCR-mix containing 0.4 µM splint, 80 µM ATP (NEB), 0.4 U T4 Ligase (Fermentas), 1×SYBR® Green (Molecular Probes), 0.1 µM forward primer, 0.1 µM reverse primer, 0.2 mM dNTP (Fermentas), 1.5 U platinum® *Taq* DNA polymerase (Invitrogen) in 1×PCR-buffer (Invitrogen) containing MgCl (Invitrogen) The fluorescence for the different concentrations of protein was monitored using a ABI prism 7700 real time PCR instrument and plotted in Figure 10.

Sequences used in proximity ligation experiment:

Arm1: Antibody-ttttttttatgtggtctatgtcgtcgttcgctagtagttcctgggctgcac

Arm2: tcgaggcgtagaattccccccgatgcgcgctgttcttactcatttttt-Antibody

Splint: aaatacgcctcgagtgcagcccattt

Forward primer: atgtggtctatgtcgtcgttcg

Reverse primer: tgagtaagaacagcgcgcat

4.2 Part two – Optimizing the blob readout system

The oligonucleotides were circularized by adding oligo, ligation splint and T4 ligase in a 1×φ-29 buffer (NEB) to a final concentration of 200 nM oligo, 600 nM splint and 1 U T4 ligase (Fermentas). This was used as a stock solution of circles and regarded as 200 nM circularized oligos. This is of course not true since the circulization is only about 50% effective (see figure 18). The circles were rolled in 50 µl solution containing 1×φ-29 buffer (NEB), 1 mM dNTP (Fermentas) and sterile filtered water. Then the samples were diluted one time in a detection mix containing 10 nM detection probes in 1×PBS buffer.

Sequences used in the optimization assays.

Oligo #3 tcggcgctgaccaga-ttccgagatgtaccgctatcgtttccacgc-ctagtgctggatgatcgtcc-ttctcgaccgttagcagtt-ccgtggcaactcacc

Oligo #5 gggattataaagaactgttgc-ccgagatgtaccgcccacgc-gttgatgtcatgtgtcgcac-ttctcgaccgttagcagtt-ttcttctgggctaattacagc

Splint #3 tctggtcagcgccga-ggtgagttgccacgg

Splint #5 gttctttataatccc-gctgtaattagccca

Detection probe #3 Bodipy-ccacgc-ctagtgctggatgatcgtcc

Detection probe #5 Cy5-ccacgc-gttgatgtcatgtgtcgcac

The blobs were detected in a Zeiss LSM 510 Meta confocal microscope. The settings of the confocal were as follows:

Objective:	40× Plan neofluar NA1.3 oil immersion
Excitation wavelength Cy5:	633 nm
Excitation wavelength Bodipy:	488 nm
Emission bandwidth Cy5:	>650 nm
Emission bandwidth Bodipy:	505-530 nm
Pinhole:	300 (optical slice <5 μm)
Scanning mode:	Linear, both directions. Voxel time 1.6 μs.
Scanning time:	approx. 30 s (30 000 lines)

The sample flowed in a 200 μm wide and 40 μm deep microchannel of PDMS sealed with a regular objective glass and was examined in a microscope. The flow rate was 3 μl/min.

4.2.1 Rolling time

For this experiment I used 20 pM circles and hybridized for 20 minutes in 37°C, without BSA.

4.2.2 Hybridization conditions

I used 20 pM circles in this assay, and the rolling time was set to 30 minutes (37°C). No BSA was added in the hybridization.

4.2.3 BSA concentration

I used 20 pM circles, hybridized for 20 minutes in 37°C, and the rolling conditions were 30°C for 30 minutes.

4.3 Part three – From recognition complex to blob

4.3.1 Making blobs

The following protocol was used for making blobs from protein recognition complexes:
Firstly the ligation molecules were cut using 0.1 μM short complementary DNA-strands and 10 U RSA1 (NEB) incubated in 37°C for one hour, followed by a enzyme denaturizing step in 65°C for 10 minutes.
Then the detection molecules were circularized by adding 1 mM ATP (NEB) and 1 U T4 ligase (Fermentas). 37°C, 15 minutes.
In the next step 0.2 μM dNTP (Fermentas) and 30 ng φ-29 polymerase (NEB) was added to replicate the circles to a first degree rolling product for an hour in 37°C, followed by a 10 minutes denaturation of the enzyme in 65°C.
Due to the small amount of circles the amount of rolling products is not sufficient for making blobs. Instead two Circle-to-circle-amplification rounds were performed with cutting, recirculization and rolling with 0.3 μM short complementary DNA-strands and 10 U RSA1 (NEB), 0.6 mM ATP (NEB) and 1 U T4 ligase (Fermentas), 0.1 μM dNTP (Fermentas) and 90 ng

φ-29 polymerase (NEB) respectively. The same conditions as in the first round was used.

The rolling products obtained were then hybridized using the optimized protocol obtained from part two:

70°C in 5 minutes for disentangling, 37°C in 20 minutes for hybridization and no BSA added.

The blobs were then examined in the confocal microscope as in part two.

Sequences used in making blobs experiment:

3´arm: ttttttccgagatgtaccgctatcgttttgggctagttaccgt

5´arm: gtgagtatgaggaagttctagtgctggatgtcgtccttccgagatgtaccgctatcgtccttcc

Splint: cttcctcatactcacacggtaactagccca

1°, 3°scDNA: acgatagcggtacatctcgg

2° scDNA: ccgagatgtaccgctatcgt

4.3.2 Tracking the process with PAGE

In the experiment two recognition molecules (single stranded DNA) was to connect together aided by a splint without any protein or antibodies being present. To accomplish this 20 pM recognition molecules with a 3´ complex binding end were incubated together with 40 pM splint and 60 pM recognition molecules with 5´ complex binding end. This 1:2:3 relationship makes every splint bind to a 5´arm, and every 3´arm bind to a 5´-splint complex. This relation thereby forces every 3´arm to form a recognition complex. To keep track of the molecules in the gel, the 3´arms and splints had previously been labeled with ^{32}P γ ATP at the 5´end.

After incubating 3´arms, 5´arms and splints together with T4 ligase in 37° for 30 minutes and 70°C in 5 minutes, the molecules were cut in the back end with RsaI and short complementary DNA strands. (RsaI only cuts double stranded DNA) This was done in 1×φ-29 buffer for 15 minutes. In the third step 1 U T4 ligase was added to circularize the molecules.

The experiments were run on a 6% acryl amide gel (with 7 M Urea, 5%APS, 1%TEMED) with 0.6×TEB running buffer. The films were developed in a Phosphoimager.

Sequences used in gel experiment:

3´arm: ^{32}P γ ATP-ttttttccgagatgtaccgctatcgttttgggctagttaccgt

5´arm: gtgagtatgaggaagttctagtgctggatgtcgtccttccgagatgtaccgctatcgtccttcc

Splint: ^{32}P γ ATP-cttcctcatactcacacggtaactagccca

scDNA: acgatagcggtacatctcgg

5. Results

5.1. Part one – Real time PCR

Seven concentrations of PDGF in the pM range were measured with the standard proximity ligation method. The SYBR-green detected in the real time PCR machine is only visible when bound to enough amount of doublestranded DNA. The amplification of DNA is only possible when PDGF is present.

As the C_t value is inversely proportional to the log of the amount of DNA, this correlation is also valid for the amount of PDGF. For more theory regarding proximity ligation the earlier chapters of this work are recommended.

The correlation log [PDGF] / C_t seems to be inversely proportional for concentrations between 0.1 to 100 pM (figure 10). Hence the Ct value from the real time PCR reaction is a way of measuring PDGF concentration in a solution if a titration of known concentration is prepared in the same way.

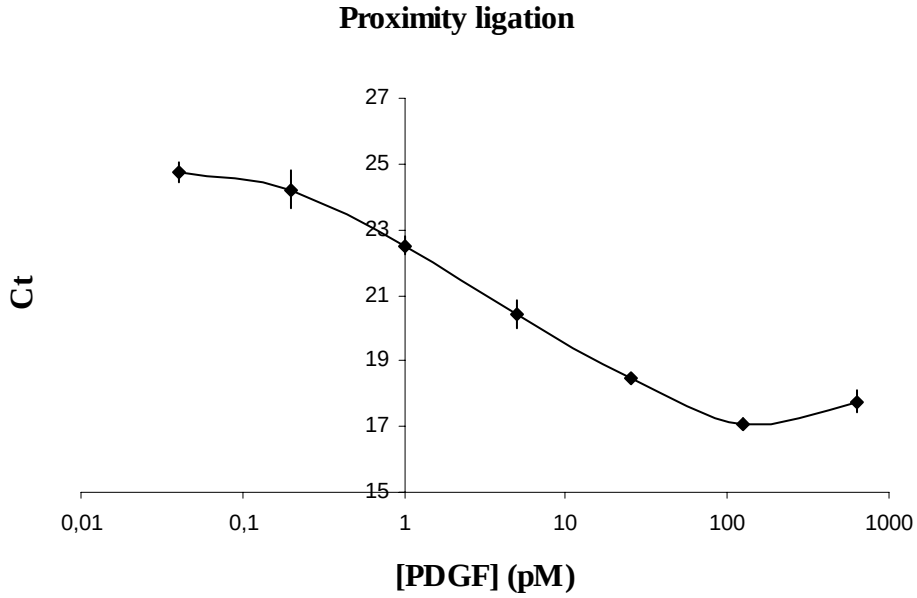


Fig 10. Proximity ligation. Concentrations of PDGF plotted against C_t value. The deviation of the duplicates is shown as vertical bars. The slope is linear between 0.1 to 100p

5.2 Part two – Optimizing the blob readout system

5.2.1 Rolling time

In this experiment ligated circles of DNA were replicated for different amounts of time with φ-29 polymerase. The readout was made by counting blobs in a confocal microscope followed by enumeration using an in-house developed matlab picture recognition program. It seems that the longer you let the reaction proceed the larger amount of blobs are detected. (Figure 11).

N.b. a doubling of the time does not give a doubling in the amount of blobs detected.

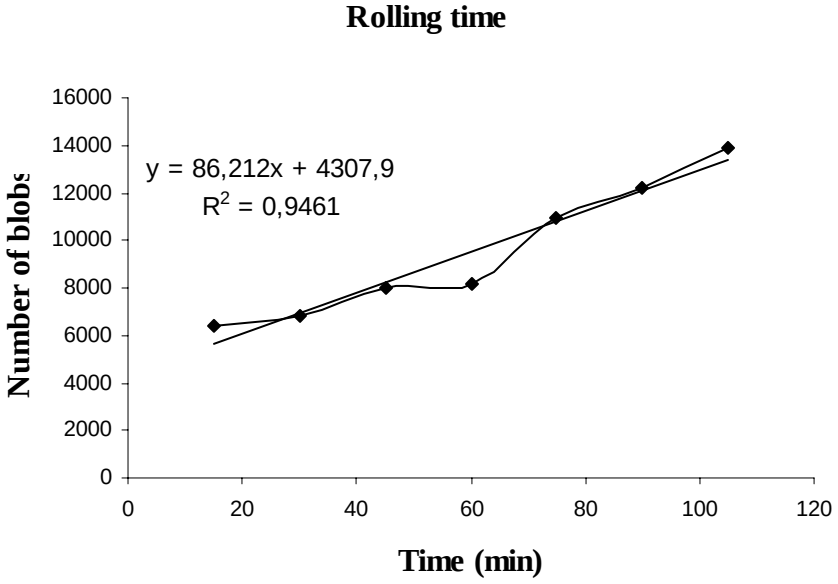


Figure 11. How rolling time influences the number of blobs in a blobbing assay with confocal microscopy readout. The numbers of blobs seems to be proportional to the rolling time with the rate-constant 86 min^{-1}.

5.2.2 Hybridization conditions

In these experiments ligated oligos were replicated using φ-29 polymerase and one parameter a time was altered. The readout was made by counting blobs with a confocal microscopy using a matlab-based picture analysis program.

5.2.2.1 Disentangling time

The time parameter of the disentangling step in the hybridization reaction of the blobbing assay was investigated. In the time span investigated the disentangling time seems to be of minor importance. (Figure 12.)

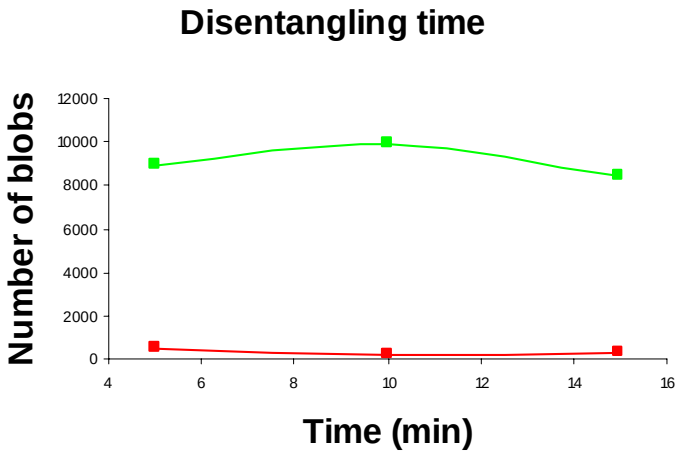


Figure 12. The green (upper) and red (lower) curves represent two different circles with different detection probes. A shorter disentangling time does not seem to affect the result negatively in the time-span examined

5.2.2.2 Disentangling temperature

The temperature for the disentangling step in the hybridization reaction of the blobbing assay was investigated. In the selected temperature span, the number of blobs seems to reach a maximum at 70°C. (Figure 13.)

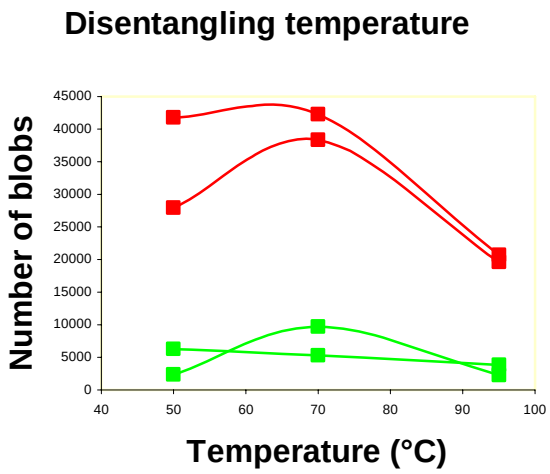


Figure 13. The green (upper) and red (lower) curves represent two different circles with different detection probes. Double series of experiments are presented in the figure. The disentangling temperature seems to have a maximum at 70°C even though one of the green curves does not have a maximum in detected blobs at this temperature.

5.2.2.3 Hybridization time

To achieve a high number of detected blobs in the assay the time parameter for the hybridization of fluorofores was investigated in a blob readout experiment. (Figure 14.)

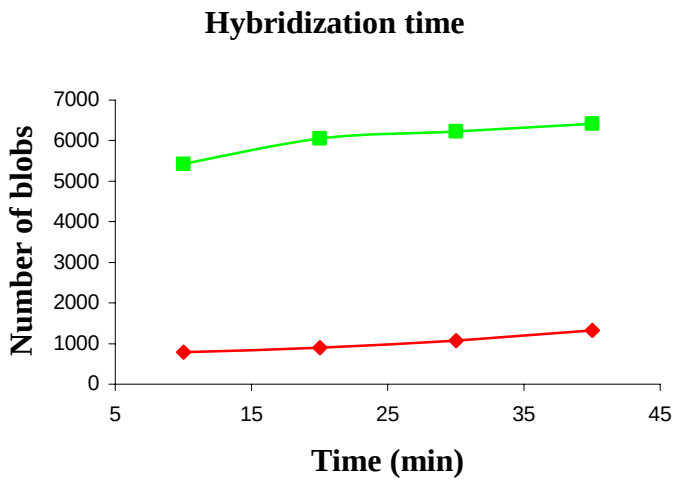


Figure 14. A longer hybridization time seems to be advantageous. Since the curve in the replication time experiment is steeper this relation is of minor difference in the end. The green (upper) and red (lower) curves represent two different circles with different detection probes.

5.2.2.4 Hybridization temperature

For the same reason the temperature parameter for the hybridization of fluorofores was investigated in a blob readout experiment. (Figure 15.)

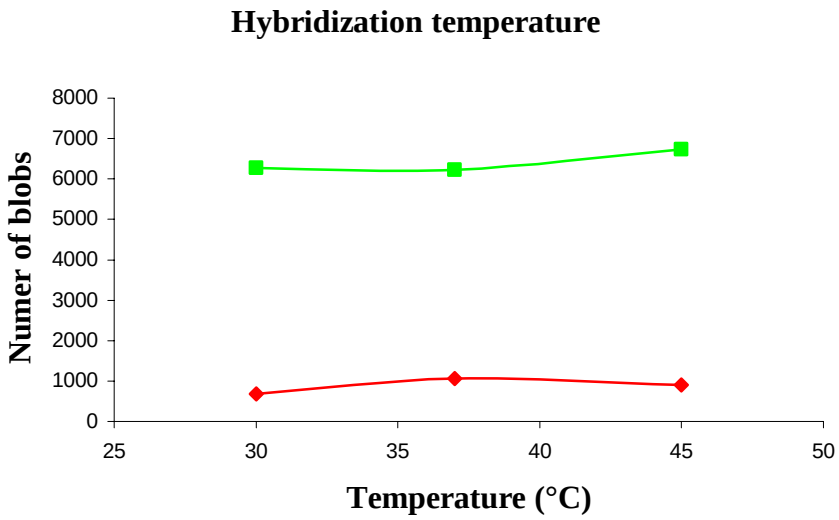


Figure 15. The hybridization temperature does not seem to affect the result substantially. The green (upper) and red (lower) curves represent two different circles with different detection probes.

5.2.3 BSA concentration

To study the effect of an addition of BSA to the hybridization mix different concentration of BSA were added and blobs were counted as in the experiments above. The result is showed in Figure 16. In the vials containing high concentrations of BSA more aggregates of blobs were detected. To illustrate this, a picture taken in the confocal microscope is added (Figure 17.)

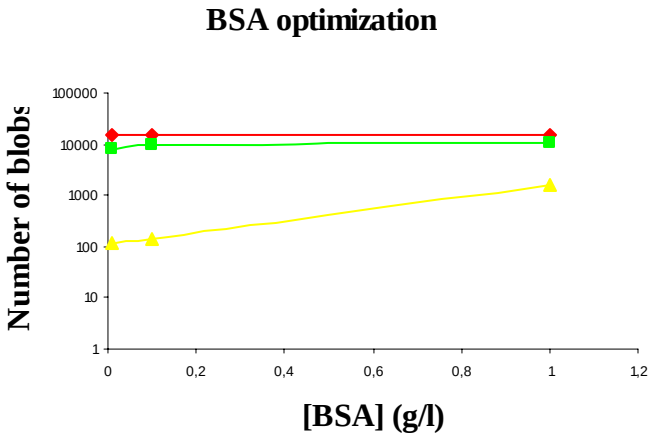


Figure 16. The green and red curves (two upper) seems to be unchanged by adding BSA of concentrations up to 1 g/l. The yellow (lower) curve represents aggregates of blobs. They seem to increase with higher BSA concentration.

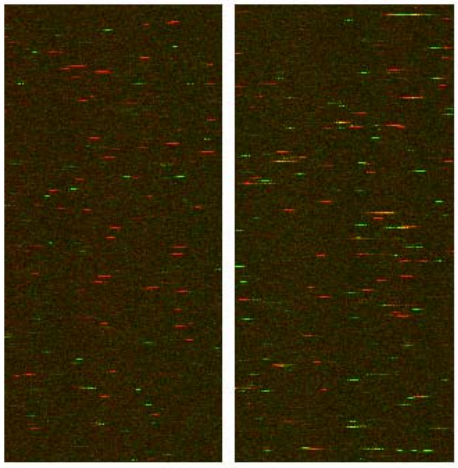
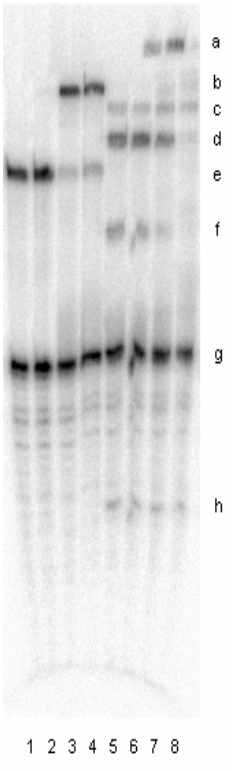


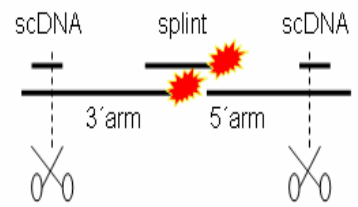
Figure 17. In the confocal microscope one can get a good estimation of the higher amount of aggregates (yellow) in 1 g/l BSA (right) compared to 0.01 g/l BSA (left)

5.3 Part three – From recognition complex to blob

5.3.1 Gel

To monitor the different steps in the experiment where blobs were to be counted from recognition molecules instead of preligated circles an assay was performed where the oligonucleotides used as recognition molecules and rolling template were detected using radioactive labelling in a PAGE set up. The gel is displayed in Figure 18 alongside an explaining schematic picture (flash indicates radio labelling) and a table showing the content of each vial. (Y indicates present regent)





Lane	3´Arm×	5Arm	Splint×	T4 lig	scDNA××	RsaI	T4 lig
1	Y	Y	Y	-	-	-	-
2	Y	-	Y	Y	-	-	-
3	Y	Y	Y	Y	-	Y	Y
4	Y	Y	Y	Y	-	Y	-
5	Y	Y	Y	Y	20 nM	Y	-
6	Y	Y	Y	Y	1 µM	Y	-
7	Y	Y	Y	Y	20 nM	Y	Y
8	y	Y	Y	Y	1 µM	Y	Y

Figure 18. X Left: PAGE gel. Bands in lanes 7 and 8 have been dislocated slightly to the left at a. a-h are used for describing bands in the discussion. Upper right: a scheme of the ligation and cutting of the recognition complex. Lower right: Table of contents in each lane. ×Labelled with ^{32}P γ ATP, ××short complementary DNA used to perform enzymatic cutting with RsaI.

5.3.2 Making blobs

There were no detectable blobs in the experiment.

6 Discussion

6.1 Part one – Real time PCR

In this experiment the standard proximity ligation method with real time PCR readout was used to verify proximity ligation as a concentration measurement tool. The method is suitable for measuring concentrations between 0.1 – 100 pM due to the linearity of figure 10. The reason for the levelling of at higher concentrations is due to the ratio of detection molecules per protein decreasing to fewer than 2. This makes ligation impossible. The method also has a lower detection limit for PDGF at 0.1 pM. This limitation is a result of the antibody's dissociation constant[6].

6.2 Part two – Optimizing the blob readout system

6.2.1 Replication time

An assay was set up where the replication time of the circles that later where to form blobs were altered and everything else was held constant. The graph displayed in the result (Figure 11.) shows as mentioned that the rolling time is proportional to the amount of blobs detected for the evaluated span of rolling times. The slope is not steep enough however, that doubling of the replication time would be suitable to lower detection limit for the blob readout. A better way would for example be to use C2CA on the rolling products before hybridizing fluorofores to create blobs of them. The fact

that there were several thousand of significant blobs detected even for the shortest rolling time in the assay therefore makes the conclusion simple – there is no need to amplify for more than 15 minutes. The effect that longer rolling time will result in a decrease of the number of blobs as a result of depletion of flouorophore coupled oligonucleotides is not shown in the graph. Further experiments with longer rolling times would therefore be of interest.

6.2.2 Hybridization conditions

Different experiments were set up where the time and temperature for two different steps of the reaction – disentangling and hybridization – where altered one at a time. The results are displayed in figures 12-15.

The time parameters do not seem to affect the result in my assay. This confirms that disentangling time and hybridization time could be set to the lowest values examined, five and ten minutes respectively. The hybridization temperature seems to be of little importance and can therefore be set to 37°C for convenience, whereas the disentangling temperature seems to have a maximum at 70°C. The reason for the dip in the graph at higher temperatures could be the result of unspecific hybridization taking place already in the disentangling step, or that they simply break when exposed to high temperatures. Why one of the green series in the disentangling temperature assay is not following the other in terms of having a maximum is considered as a coincidence.

6.2.3 BSA concentration

In the experiment different concentrations of BSA were added to the hybridization mix in an attempt to reduce the amount of blobs attaching to the vial by letting a layer of BSA function as a barrier.
The effect of BSA increasing the number of blobs is very small as one can see in Figure 16. This, combined with the effect of increasing aggregates with an increased BSA concentration, suggests that either BSA binds to the blobs, making the quantification a lot harder or that there are hyphae growing in the BSA interrupting the experiment. However, this experiment shows that the absence of BSA is preferred when making blobs. One explanation to why BSA induces aggregates could be that the BSA is already in aggregates once added to the mixture. Anyway one could try to eliminate the problem and see whether sterile-filtered BSA increases the amount of detectable blobs.

6.3 Part three – From recognition complex to blob

6.3.1 Gel

In order to follow all the cutting and ligation steps when making a rolling circle replication product of a protein recognition complex a PAGE assay was performed where the splint and one of the recognition arms where labeled with ^{32}P γ ATP. As one can see by the gel picture in Figure 18. all dark grey segments in the table are needed for forming the recognition

complex. Both light grey segments are also needed for cutting the outer ends of the recognition molecules. In lanes one and two band e represents radiolabeled 3´arms and band g represents the splint. In lanes three and four the recognition complex is formed but no end pieces are cut off. In lanes five and six the complex has been cut in one or both ends represented by the bands c and d respectively. Band f probably represents cut, unligated 3´arms. The appearance of band h is not fully understood. In lanes seven and eight a circle is formed represented by band a. For 1 M scDNA the circularization is fairly good, this is shown by the distinct a-band and small amount of residues in lane 8. This seems to compare well with the sketch presented in the upper right of Figure 18. The question is can one replicate the circles formed, and can they form blobs detectable in a confocal microscope?

6.3.2 Making blobs

To give an answer to the question above a setup was made where the circles were made from a protein detection molecule in the same way as in the PAGE experiment with the only difference that no radio labeling was made. The rolling product where both hybridized with fluorofore coupled oligonucleotides as well as replicated one and even two times with RCA to get enough blobs to detect them in the confocal microscope.
The fact that there were no detectable blobs when only rolled once was no surprise as the number of blobs formed was so low. Why no blobs were detected in the experiments when the molecules were rolled two or three

times could only be explained by some sort of error in the circle to circle amplification part of the experiment.

6.4 Conclusion

As a conclusion the proximity ligation and the blob-readout methods were both verified.
An optimization of the replication parameters resulted in these values:
Replication: 15 minutes 37°C
Disentangling: 5 minutes 70°C
Hybridization: 10 minutes 37°C
BSA: no BSA added
The protein recognition molecules could be cut and circularized. The circles could however not be detected in a blob readout.

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