DNA Microscopy

Establishing an immuno protocol

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Abstract

As we continue to explore and understand the world, the demands on our methods and techniques evolve too. DNA microscopy is a developing method that offers an alternative to conventional light microscopy. Instead of relying on light and a convex lens, it utilizes nature's building blocks, DNA, to create spatial images and describe different proteins near one another using sequencing. Björn Högberg's lab has previously shown the method to work in an artificial system and the project is now ready to start exploring one of the options for a cellular setup.

One possibility is to create a multi-omic system that utilizes antibodies to deliver target strands to specific cells, mimicking the original artificial setup. This requires the conjugation of designed DNA strands, targets, to the antibody. An appropriate protocol was developed and established in this thesis. The four cell lines used were A549, HeLa, MCF-7, and MDA-MB-231. In situ immunocytochemistry (ICC) was used to visually confirm the relative specificity of the four cell types to the nine different antibodies. Both SiteClick and oYo-Link were trialed for the conjugation of targets, using both ProFIRE and Amicon to purify the conjugates. The result showed that all four cell lines had general specificity to antibodies CD44 and BASP1, but only HeLa and A549 to SLC38A1. A549 was the only cell line that showed specificity for ALDH3A1 and AKR1B10. For the conjugation protocol SiteClick in combination with ProFIRE and Amicon gave the best result for conjugate concentration and purity.

The results of the thesis provide the beginning of a library with appropriate cell lines and antibodies to use in a cellular setup. It also establishes a protocol for the conjugation of target strands to antibodies. To fully realize a cellular version of the artificial system more study needs to be performed on appropriate cell lines and antibodies for a more complex setup. To further evaluate the conjugates' effect on the DNA reactions the reaction requires sequencing and re-construction.

Keywords: Microscopy, Immunocytochemistry, Conjugation, Spatial Transcriptomics.

Acknowledgements

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Anna Andersson, Gothenburg, May 2024

CONTENTS

1 Introduction **1**
- 1.1 Background . . . 1
- 1.2 Aim . . . 1
- 1.3 Limitations . . . 2

2 Theory **3**
- 2.1 DNA Microscopy . . . 3
  - 2.1.1 Background . . . 3
  - 2.1.2 General Setup . . . 4
  - 2.1.3 Cellular Transition . . . 8
- 2.2 In Situ Immunocytochemistry . . . 9
  - 2.2.1 Antibody Selection . . . 10
- 2.3 Conjugation . . . 11
  - 2.3.1 SiteClick . . . 11
  - 2.3.2 oYo-Link . . . 12
  - 2.3.3 Purification Methods . . . 12

3 Methods **13**
- 3.1 Cell Protocols . . . 13
- 3.2 Gel Protocols . . . 14
- 3.3 In Situ Immunocytochemistry . . . 16
- 3.4 Conjugation . . . 18
- 3.5 DNA Microscopy . . . 18

4 Results **21**
- 4.1 Antibody Specificity . . . 21
- 4.2 Conjugation . . . 24
- 4.3 DNA Microscopy . . . 27

5 Conclusion **29**

CONTENTS

1
Introduction

1.1 Background

As our knowledge of spatial biology advances, so do the requirements for our methods and tools. Since the first cell was discovered using light microscopy, the accuracy and capability of our methods have greatly advanced. However, conventional microscopy shows the existence of different elements, rather than their genetic expression or genotype[2]. On the opposite side, the use of modern sequencing of transcriptomics can describe the whole genetic content of a sample but fails to capture the spatial placements of the elements in their original position. DNA microscopy is a technique that aims to bridge this gap between techniques by showing both what and where different proteins and components are in a sample. By using sequencing, DNA microscopy could become an easy and customizable option for tissue and cell mapping[10].

Development of DNA Microscopy has been ongoing at Björn Högberg's Lab at Karolinska Institutet for the last few years, with proof of concept shown in an artificial setup[4]. The project is now at a stage where it is time to apply the method to a cellular version of the previous artificial design. One possible protocol would be to use conjugated antibodies to apply the existing procedure on a cellular setup. This thesis entails the creation of a library with appropriate cell lines and antibodies, a conjugation protocol, and confirmation that antibodies do not hinder the DNA microscopy reaction.

1.2 Aim

The project aims to create a library that can be used to transition the current artificial setup to a cellular one, produce an appropriate protocol for conjugation, and confirm its feasibility in DNA microscopy. This is done by performing in situ immunocytochemistry (ICC) with different cell lines and antibodies, comparing different conjugation methods, and running DNA microscopy with conjugated antibodies.

1.3 Limitations

For the ICC there will only be a visual comparison between the antibodies' affinity since the project at this stage doesn't require exact targeting of protein, but rather a simple setup that can show proof of concept. Only four different cell lines are trialed since the previous artificial system consisted of four different target sites. The conjugation protocol is only accessed based on one antibody since the protocol is non-dependent on which antibody is used. The thesis ends with a DNA microscopy reaction that includes the conjugated antibody to ensure that it does not interfere with the main reactions.

2
Theory

2.1 DNA Microscopy

2.1.1 Background

DNA microscopy is a measurement method that shows both the presence and location of the cellular transcriptome using sequencing. This is done by using synthesized DNA strands, called *seed strands*, which carry a unique DNA sequence called a *barcode* for identification. When allowed to react with other DNA strands in a cross-linking gel, the end concatemers contain a unique combination of barcodes that can be used to reconstruct spatial information about the gel content. These seed strands go through three main steps in a cross-linking gel: pre-activation of immobilized seed strands, activation of pre-activated monomers, and amplification of monomers into concatemers. After PCR, the gel is dissolved and the PCR product sequenced. Using computational analysis, the reconstruction does two things. Firstly it reconstructs the shape and content of the sample, i.e. creating a spatial image of the sample at the time of fixation. The second is to investigate the relative placement of different proteins to one another, organelles, or similar to find a potential correlation to cell behavior.

2.1.2 General Setup

DNA microscopy uses 5 different types of single-stranded DNA, shown in figure 2.1.

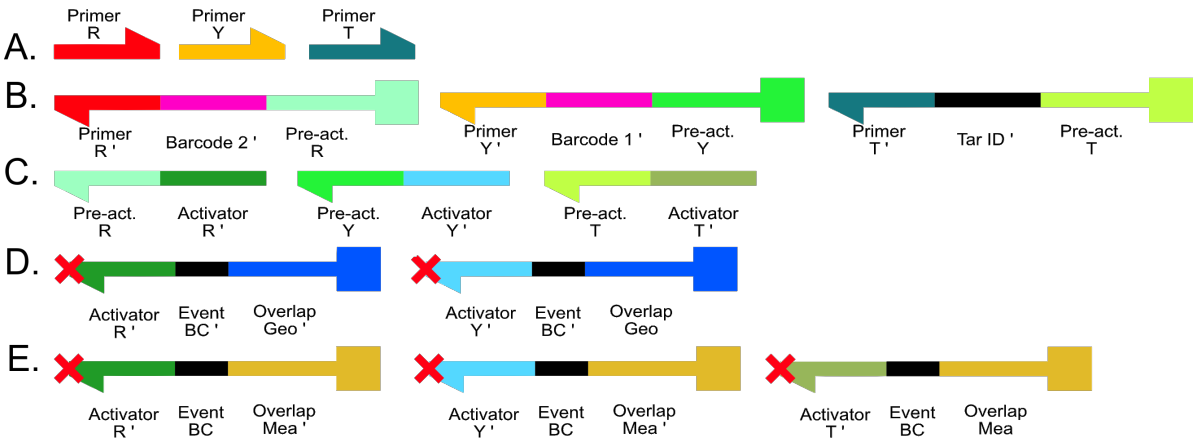


Figure 2.1: A. Primers, B. 2 Seed strands(most left) & target strand (most right), C. Exponential primer, D. Geometry activation strands, and E. Measurement activation strands.

Each color in figure 2.1 represents a different part of the strands, with complementary sequences bearing the same color. Barcodes and *event barcodes* are randomized sequences of determined lengths, and patterns, that enable the computational analysis to differentiate between the millions of seed strands used. This is the basis that enables the creation of unique concatemers. The squares on the 5' terminal on the seed strands, and both types of activation strands, represent an acrylate molecule that immobilizes the DNA strand in the gel throughout the reaction. The pre-activation and activation sequences act as activation steps for the molecules in the reaction and the two different overlap sequences determine the type of concatemer created. The red crosses on the 3' terminal end of the activation strands represent a polymerase blocker consisting of an inverted 3' stop.

Before PCR the targets are mixed with a cross-linking acrylate gel which contains all the components in figure 2.1. This body of gel dictates the 3D space that the DNA microscopy reaction will describe. The gel is then put through a PCR program yielding the following products over 35 cycles.

First step: Activation amplification

For the first cycles, the immobilized seed strands will anneal to a primer which creates a copy without the acrylate tail, meaning it can diffuse away from the original seed strand. If the mobile seed strand is then annealed to an exponential primer, a pre-activation sequence will be transcribed onto the seed strand, creating pre-activated monomers. In short, the first cycle creates mobile copies of the original seed strands while exponential primers add a pre-activation sequence, resulting in pre-activated monomers shown in figure 2.2.

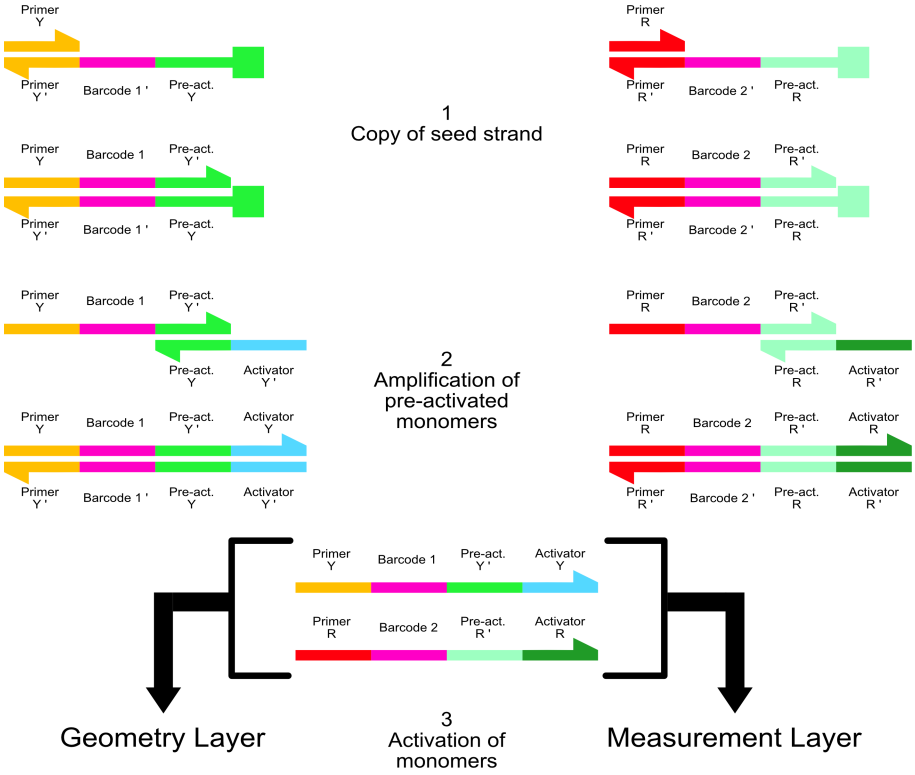
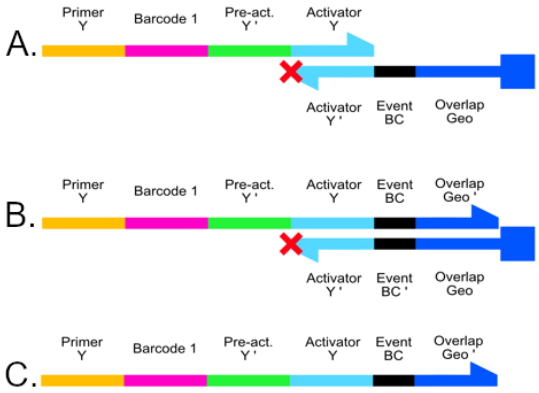


Figure 2.2: 1. Primers and immobilized seed strands produce mobile copies of seed strands. 2. Exponential primers and mobile seed strands produce pre-activated monomers. 3. The pre-activated monomers diffuse outward and interact with activation strands.

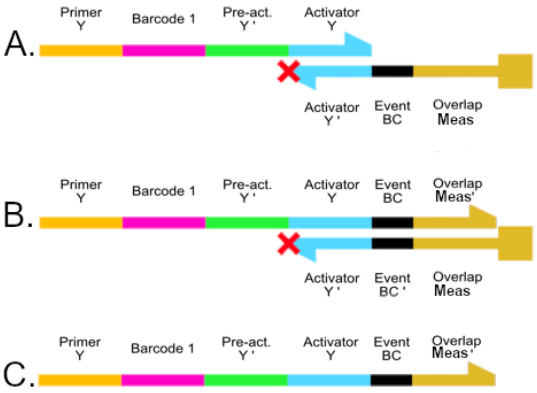
At the end of the activation amplification step, there should be a growing sphere surrounding the immobilized seed- and target strands, containing both mobile seed- and target strands respectively, and pre-activated monomers. The pre-activated seed strand monomers will then continue into either the geometry- or measurement layer. The former will create the reference nodes the reconstruction uses to model the space encompassed by the gel. Instead, the seed strands in the measurement layer will produce concatemers that measure the activity of the chosen gene. Note that while figure 2.1 only shows one target strand, there could technically be as many different ones as there are genes you are investigating.

Second step: Activation of pre-activated monomers

For the second cycle, the pre-activated monomers have an equal chance of annealing to either a geometry or measurement activation strand. It is possible to alter the odds of the pre-activated monomers interacting with either a geometry- or measurement strand, by changing their original concentration. With the activation strands being immobilized due to the acrylate molecule, the pre-activated monomers need to diffuse outwards to interact with one. Both the geometry- and measurement activation strand reactions are illustrated in figure 2.3.



(a) Second cycle reaction for the geometry layer.

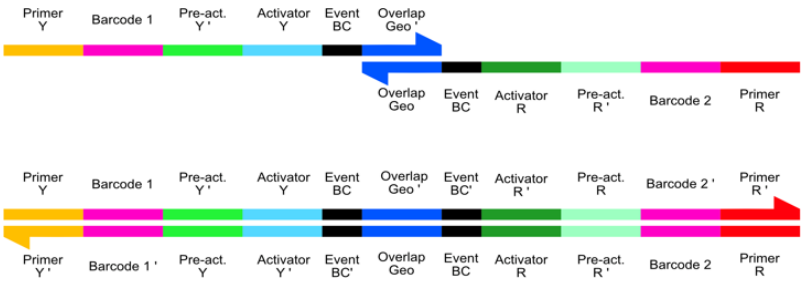


(b) Second cycle reaction for the measurement layer.

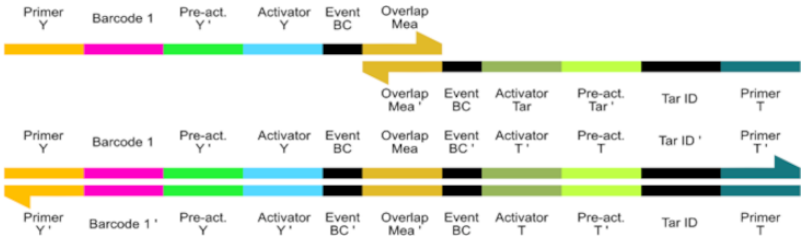
Figure 2.3: A. The pre-activated monomers anneal to an activation strand. B. Due to the polymerase blocker on the activation strands 3' terminal, illustrated with a red cross, only one complementary strand is extended. C. A complete, activated, monomer.

Third step: Synthesis of concatemers

During the final cycle both the geometry and measurement monomers create the final concatemers. The geometry layer creates concatemers as the complementary 3' ends anneal and elongate. This means that the geometry concatemers can only be produced from seed strands that are amplified from activated monomers originating from a red and yellow seed strand. This is to prevent self-interaction for the different nodes. For the measurement monomer, the overlap sequences are identical, meaning that they can not anneal to one another. Instead, the measurement monomer anneals to the 3' terminal of activated target monomers which have amplified parallel to the seed strands. Figure 2.4 shows the two reactions that produce the two layers.



(a) Geometry monomers from red- and yellow seed strands respectively anneal and create concatemers for the geometry layer.



(b) The measurement monomers anneal with the complementary target monomers and create concatemers for the measurement layer.

Figure 2.4: The complementary activated monomers anneal and create concatemers with unique barcode combinations.

The resulting concatemers now contain an unique combination of barcodes that enables the reconstruction of the original sample. Barcodes 1 and 2 show which seed strand the concatemer was originally amplified from, with a higher number of copies indicating how close they were. The event barcodes enable the reconstruction to angle the nodes to one another by comparing how close the immobilized activation strands seem to have been. Note that due to the red and yellow seed strands being non-complementary to one another, a node can only anneal to another node of a different color, stopping self-interaction which creates cleaner data.

2.1.3 Cellular Transition

The original, artificial system, placed four drops of four different target strands sequentially on a slide. Due to the order and placement of the droplets, this creates a reference to the result from the reconstruction program, making it easier to confirm if it worked. In figure 2.5 the light blue color represents the cross-linking gel while each circle is a different target sequence.

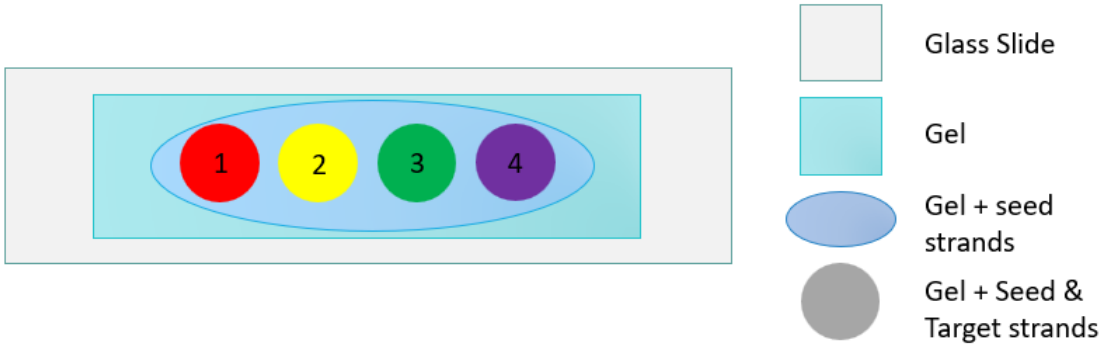


Figure 2.5: The artificial setup contains target strands 1, 2, 3, and 4. These contain all DNA microscopy components, while the surrounding gel lacks target strands. This is all covered in a gel without any DNA microscopy components.

For the cellular, multi-omic setup, instead of having four drops of targets in a gel, the four different target strands were attached to four types of cells using antibody conjugation. These cells were placed in four respective corners inside a gel, mimicking the four-point reference system in the artificial setup. Figure 2.6 illustrates the placement of the cells within the gel where the different colors of the antibodies represent the four different targets that were conjugated to them.

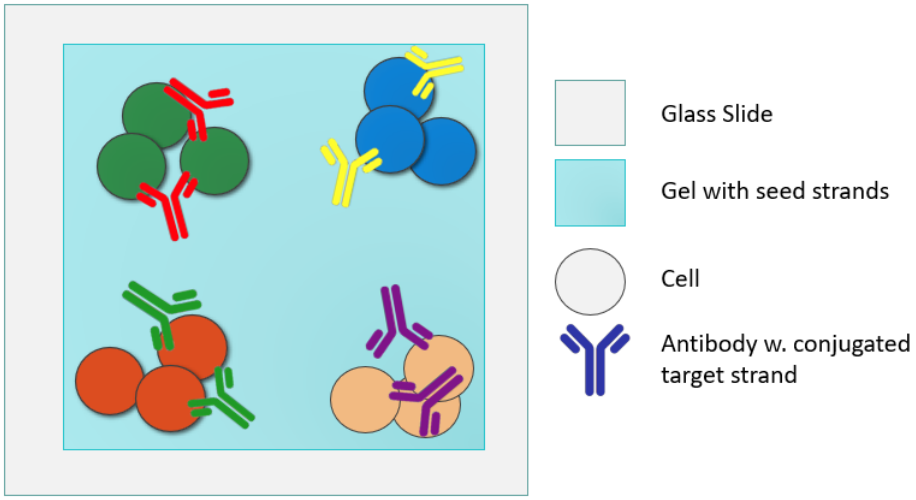


Figure 2.6: Illustration of the multi-omic system using target strand conjugated antibodies attached to four different cell types.

2.2 In Situ Immunocytochemistry

As previously mentioned the target strands in the cellular setup of DNA microscopy utilize antibodies. To choose which antibodies are suitable for the experiment the visual method immunocytochemistry (ICC) was used. For this thesis, ICC refers specifically to indirect immunofluorescence (IF) which uses secondary antibodies with attached fluorophores to indicate specificity for different membrane-bound proteins. The general protocol includes fixation of the cell, blocking buffer, primary antibody incubation, and secondary antibody incubation. Figure 2.7 illustrates how the antibodies attach to the protein of choice for indirect IF.

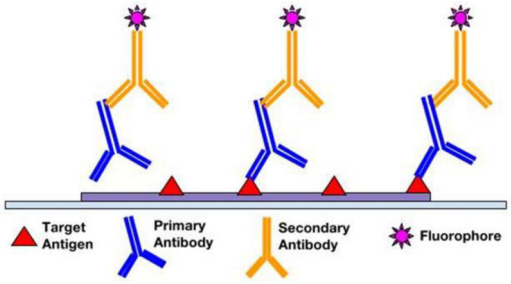


Figure 2.7: Illustration of indirect immunofluorescence with the target protein representing a membrane-bound protein.

The result is not a quantitative measurement of how well the antibodies bind to each cell type but offers a sufficiently accurate differentiation in specificity between the different cell types.

Cell lines

The four different cell lines were picked from a pre-existing stock in the lab. For protocol simplicity, only attached and established cell lines were chosen. The four cell lines all come from different types of cancer so there would be a difference in gene expression. The four cell lines were A549 (lung cancer), HeLa (cervical cancer), MCF-7 (estrogen-positive breast cancer), and MDA-MB-231 (triple-negative breast cancer).

2.2.1 Antibody Selection

Due to the nature of the experiment, only membrane-bound proteins were considered when choosing primary antibodies since it removed the need for permeabilization of the cells. When choosing which antibodies to investigate both a literature study and The Human Protein Atlas were used, the latter showing the relative abundance of each protein for the different cell lines. The goal was to find both a general antibody with a similar affinity to all four cell lines, as well as a specific one respectively. In table 2.1 the antibodies with an asterisk were chosen based on the literature study, not on the normal transcripts per million (nTPM) value from The Human Protein Atlas version 23.0[1].

Table 2.1: Each column presents the registered (nTPM) of each protein for the respective cell lines on the Human protein atlas, .

Antibody Name	A-549 (nTPM)	HeLa (nTPM)	MCF-7 (nTPM)	MDA-MB-231 (nTPM)	Literature Reference	The Human Protein Atlas
AKR1B10	3229,7	1,3	2,1	3,0		[12]
ALDH3A1	2070,1	74,6	3,8	3,0		[13]
BASP1	297,8	1259,6	247,4	126,8		[14]
∗CD44	247,0	166,1	29,2	722,6	[9]	[15]
∗ESR1	0,2	0,2	78,9	0,0	[22]	[17]
∗EpCAM	6,2	2,6	335,0	18,8	[3][7]	[16]
∗MUC1	3,9	70,4	77,1	30,4	[5][11][21]	[18]
SLC38A5	0,4	327,9	0,2	3,2		[19]
SPINK13	0,4	276,5	0,0	0,1		[20]

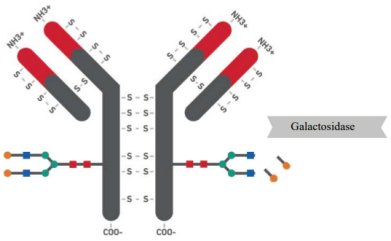
All primary antibodies, besides CD44, were paired with the secondary antibody Goat anti-rabbit, while CD44 was paired with Goat anti-rat. Both secondary antibodies had the Alexa Fluor 488 fluorescence molecule.

2.3 Conjugation

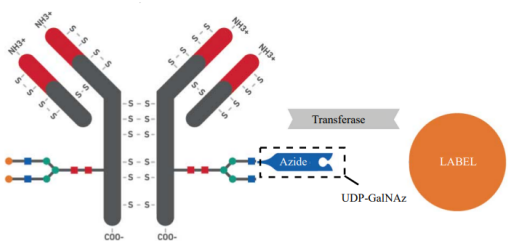
Antibody conjugation is when a drug, enzyme, or other moiety is chemically linked to a primary antibody[6]. There are several ways to do this with the vast majority including a transformation of the FC-chains on the antibody.

2.3.1 SiteClick

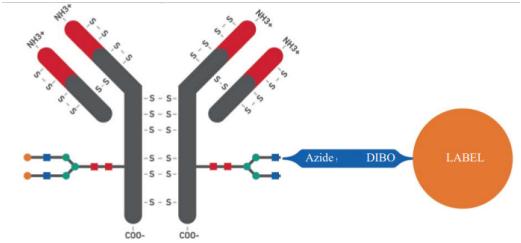
SiteClick is a conjugation method that modifies the carbohydrate chains on the FC region of the antibody. It then attaches an azide molecule which the DIBO-modified target strand clicks into, as illustrated in figure 2.8.



(a) β-Galactosidase modifies the carbohydrate chains on the antibodies FC-domain.



(b) The azide attaches to the modified carbohydrates.



(c) The DIBO-labeled target strand clicks into the azide attachment.

Figure 2.8: Overview of the SiteClick reaction for conjugation of the target strands to the antibody.

2.3.2 oYo-Link

While SiteClick chemically alters the antibody, oYo-link uses UV light, a conjugation type that is referred to as Light-Activated Site-Specific conjugation (LASIC). This method used a specific kind of photo-crosslinker which forms a covalent bond within the FC-binding site. One of the main differences between oYo-Link and SiteClick is that oYo-Link produces a maximum of two conjugate targets to each respective heavy chain, while SiteClick can hypothetically attach several.

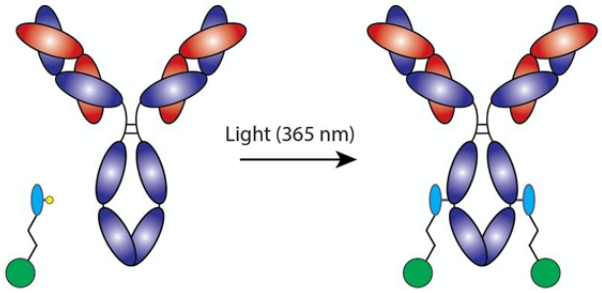


Figure 2.9: oYo-Link reaction using UV light to covalently attach the modified target strands.

2.3.3 Purification Methods

After conjugation, the conjugated antibodies must be purified from unconjugated antibodies and unbound target strands of DNA to ensure that the reconstruction of the sequenced data doesn't contain incorrect information. This is commonly done using Amicon, which is a type of column that mechanically filters out any impurities of a certain size. As a fail safe, one of the samples was also run through proFIRE prior to Amicon. proFIRE works similarly to a HPLC which filters out components based on their charge.

3
Methods

3.1 Cell Protocols

All of the following cell protocols were performed in a biosafety level 2 lab (BSL-2) according to safety regulations. Growth media always refers to 10% FBS, 1% Pen-Strep in Gibco DMEM media heated to 37°C.

3.1.1 Thawing and freezing of cells

All cell lines were thawed from pre-existing stocks in the lab.

Thawing of frozen stock

The following protocol was used to thaw all 4 cell lines which had been stored at -120°C.

1. Thawed the frozen tube in a 37°C water bath.
2. Resuspended the cells in 4 mL growth media.
3. Centrifuged at 300 RFC for 4 min.
4. Removed supernatant and resuspended in 4 mL growth.
5. Added all of the cell suspension and 4 mL growth media to a T25 flask.
6. Incubated at 37°in an incubation chamber.

Preparation of frozen stock

The following protocol was used to prepare stock from all 4 cell lines. Previous to the protocol the cell culture was split into a T75 flask instead of T25 for a greater volume of cells before freezing.

1. Removed growth media from the flask using an aspirator.
2. Washed with 8 mL of D-PBS.
3. Added 8 mL of dissociation media and incubated for 5 minutes.
4. Pipetted the dissociated cells into a 15 mL plastic tube and centrifuged at 300 RFC for 4 minutes.
5. Removed supernatant using an aspirator and resuspended in 2 mL 10% DMSO in FBS.
6. Added 1 mL of resuspended cells into freeze tubes and placed in room temperature cooling container.

7. Placed the cooling container at -80°C overnight before moving the cell tubes to -120°C storage.

3.1.2 Cell Splitting

Cell splitting was performed to extract cells for experiments or when the cell culture had reached 80-100% confluency.

1. Removed old growth media using an aspirator.
2. Added 5 mL D-PBS and slightly swirled it in the flask.
3. Removed the D-PBS using the aspirator and added 5 mL dissociation liquid.
4. Incubated for 5 minutes or the cells had detached.
5. Pipetted the dissociated cells into a 15 mL plastic tube and centrifuged at 300 RFC for 4 minutes.
6. Removed supernatant using an aspirator and resuspended the pellet in 4 mL growth media.
7. Added 6 mL growth media to a new T25 flask.
8. Added between 5-50% of the resuspended cells to the T25 flask depending on the time before the next experiment.

3.2 Gel Protocols

The following protocols describe the gels used throughout different portions of the experimental period. All gels were imaged with a Bio-Rad ChemiDoc MP©and processed with Imagej software©.

3.2.1 Urea PAGE gel

Urea PAGE gels were used to inspect free-floating DNA after conjugation protocols and control expected reactions in DNA microscopy.

1. Set up gel equipment with 1 mm glass.
2. Measured 8 mL of 19:1 UREA 8% TBE and added 8 µL TEMED inside fume hood.
3. Added 80 µL APS outside of fume hood and quickly vortexed before pouring into the glass set up.
4. Added either an 8 or 15-well comb depending on the number of samples.
    - *Note. The gel mixture sets quickly and the above steps need to be followed through in quick succession.*
5. Left to set for 15 minutes.
6. Moved the gel into a container and filled it with 1X TBE buffer.
7. Removed comb.
8. Ran the gel in the buffer at 300 V for 20 minutes to ensure consistent temperature.
9. Prepared the samples and ladders accordingly before running at 95°C for 5 minutes:
    - For the 20/100 oligo ladder and samples, 3 µL of formamide and 1 µL of the sample were added to PCR tubes.

- For the Ultra Low range Ladder (ULR) 6 µL formamide and 0.4 µL URL were added to a PCR tube.

10. Used a syringe to wash the wells in the gels after the first run.
11. 3.9 µL of each ladder and samples were pipetted into individual wells and ran at 300 V for 30 minutes.
12. Prepared the 0.1% SYBR™ Gold Nucleic Acid Gel Stain liquid by adding 1.4 µL SyBR gold to 14 mL 1X TBE buffer.
    "Note. The stain should not be prepared more than 2 minutes ahead of use to ensure efficiency."
13. The gel was removed from the glass and incubated with the staining liquid at room temperature in a plastic container for 5 minutes.
14. The gel was then imaged before being discarded.

3.2.2 Silver staining gel

Silver staining was used as a complement to urea PAGE gel for the conjugated antibodies to see where the protein had collected throughout the gel. The Sigma-Aldrich ProteoSilver™ Plus Silver Stain Kit with the accompanying protocol was used on urea PAGE gels for the silver staining.

3.2.3 SDS PAGE gel

SDS gels were used to check protein composition, specifically to control the correct conjugation of the antibodies. All SDS gels used were Mini-PROTEAN TGX Stain-Free Precast Gel from Bio-Rad and imaged twice, once for protein and once with SYBR™ Gold Nucleic Acid Gel Stain to observe potential DNA.

1. Prepared 0.1% SDS TRIS-glycine buffer by adding 10 mL SDS and 100 mL 10X TRIS-glycine to the measuring cylinder. It was then filled up to 1 L with dH2O.
2. Prepared samples by adding 3 µL of sample to 9 µL of Loading buffer.
3. Ran the samples at 95°C for 5 minutes.
4. Removed gel from packaging and added to the container.
5. Filled the container with the buffer and removed the comb.
6. Added 11 µL of the samples to each respective well.
7. Added 3 µL of an unstained ladder and all blue ladders respectively to individual wells.
8. Ran the gel at 120 V for 1 hour and 30 minutes or until the all-blue ladder has migrated through all 10 bands.
9. 14 mL of the buffer was removed from the container before the rest was poured out.
10. The gel was imaged before incubation for 5 min in 0.1% SYBR™ Gold Nucleic Acid Gel Stain before being imaged again.

3.3 In Situ Immunocytochemistry

In situ Immunocytochemistry (ICC) was used to confirm and differentiate different antibodies' specificity to the four different cell types. Before the ICC protocol, glass slides were treated with poly-L-lysine, as described below, for increased cell adhesion.

3.3.1 Poly-L-lysine

The following protocol was performed in a sterile environment in a BSL-2 lab.

1. Double-frosted micro slides were wiped with 70% ethanol and placed in individual Petri dishes.
2. 1 mL of poly-L-lysine was pipetted onto the clear portion of the slide, coating it evenly.
3. The slide was left to incubate without the petri dish lid for 15 minutes.
4. Remaining poly-L-lysine was removed by pipette and aspirator.
5. Petri dish lid was added and the slides were stored in a 37°C incubator.

3.3.2 Preparation of cells

The following protocol is identical to protocol the cell splitting protocol 3.1.2 until step 7.

1. After the cells were resuspended 20 µL of the cell suspension was added to 20µl of Trypan Blue stain in a new tube. The mixture was pipetted up and down to ensure even suspension.
2. 10 µL of the Trypan cell mixture was added to each side of a Countess™ Cell Counting Chamber Slide.
3. Based on the number of live cells the cell suspension was diluted to 250.000 cells/mL with growth medium into an Eppendorf tube.
4. Used the undiluted cell suspension to continue the cell splitting protocol.

3.3.3 ICC Protocol

The first half of the protocol was performed at a BSL-2 lab but later moved to a BSL-1 lab after the cells were fixated. All slides were imaged using a BIC microscope.

Day 1

The following steps were done in a BSL-2 lab.

1. Attached two ibidi™ 25 micro-Inserts 4 Well stickers to a poly-L-lysine treated slide while in the petri dish.
2. Pipetted 10 µL of the diluted cell suspensions into each respective well. This was done for both stickers on each slide.
3. Added 1 mL of D-PBS beside each slide in the petri dish to decrease evaporation.
4. Incubated overnight at 37°C in an incubation chamber for 18-24 hours.

Day 2

1. Prepared 4% PFA by adding 1 mL of 16% PFA to 3 mL of D-PBS.
   Note. All pipette tips that had contact with the 16% PFA and the original ampule were discarded and enclosed in a plastic tube.
2. Removed growth media from the wells and surrounding D-PBS.
3. Added 10 µL of 4% PFA to each well and incubated for 15 minutes.
4. Removed the PFA with an aspirator and added 200 µL D-PBS to the whole sticker. Incubated for 5 minutes.
5. Removed D-PBS with aspirator and repeated step 4 two more times.
6. After adding D-PBS for a third time the petri dish lid was put on and slides were moved to a BSL-1 lab.
7. Pipetted and discarded the D-PBS.
8. Removed the slide from the petri dish and replaced the sticker with an incubation well, careful not to damage the cells.
9. Made 5% BSA in PBS blocking buffer and added 100 µL to each well, ensuring all 4 cell squares are covered.
10. Incubated for 3h.
    Check every 1 hour to ensure all squares are still covered. If there has been too much evaporation add more blocking buffer.
11. After incubation each antibody was diluted to the recommended ratio in PBS.
12. The blocking buffer was removed and 100 µL of the diluted antibody was added.
13. Two stickers were added to cover the well and stop evaporation.
14. Incubated the slides at 4 °C overnight.

Day 3

1. Removed the stickers and primary antibody.
2. Washed each well with 100 µL D-PBS three times.
3. Diluted each secondary antibody according to the manufacturers' recommendation in PBS.
4. Added 100 µL of secondary antibody to respective primary antibody chamber.
5. Incubated for 1 hour in the dark at room temperature.
6. Removed secondary antibody and washed each well with 100 µL D-PBS three times.
7. Removed both wells from the slide and Embedded with a DAPI-containing embedding medium.
8. Left the slides to set at room temperature for 24 hours and were then placed in storage at 4 °C.

3.4 Conjugation

3.4.1 SiteClick

The Invitrogen SiteClick™ Antibody Labeling Kits with accompanying protocol were used. Optional step 1 was performed due to low original antibody concentration, but optional step 6 was skipped for other purification methods as described further down. The conjugation was performed with antibody CD44 and measurement target strand 3.

3.4.2 oYo-Link

The oYo-Link® DBCO kit with accompanying protocol was followed. Antibody CD44 was used in combination with a DBCO-modified target 1.

3.4.3 Purification

To concentrate the conjugated antibodies and remove unattached targets that did not conjugate to the antibody, two different purification pipelines were investigated.

3.4.3.1 proFIRE

For purification of a SiteClick sample proFIRE was used to separate the desired conjugate from other impurities in the sample. This was done using the proFIRE machine with accompanying components. 120 µL was purified into 12 different samples.

3.4.3.2 Amicon

Amicon® Pro Purification System was used to purify and concentrate samples from SiteClick and SiteClick after proFIRE. The accompanying protocol was followed for both the SiteClick only and SiteClick with proFIRE samples. For the SiteClick with proFIRE samples, the first 3 samples were combined due to low conjugate concentration.

3.5 DNA Microscopy

The following protocol was originally based on the paper [8] but has since been modified, e.g. to include the original method for the geometry layer.

3.5.1 Enzyme mix

The PCR-enabling enzyme was KAPA HiFi HotStart plus dNTPs and was right before PCR was performed.

1. Removed enzyme from the freezer and pipetted 5 µL into 10 µl of DNTP before returning the enzyme to the freezer.
2. Added 15 µL of MQ and pipetted up and down to mix.

3.5.2 Seed mix

The seed mix always contained both B&Y and R&G strands and was diluted in dH2O before experimentation.

3.5.3 Overhang extension mix

Depending on which kind of sample was under investigation, different overhang extension mixes were used. For if looking at the geometry reaction only primers, exponential primers, and geometry activator strands relevant to the geometry reaction were added. If a component was skipped it was replaced with MQ.

Type	Name	Concentration (µM)	Amount (µL)
Primer	IGBA_DM5_RED_5N	6000	3
Primer	IGBA_DM5_YEL_5N	6000	3
Primer	IGBA_D5_Tar_P_5N	6000	3
Overhang extension	IGBA_D5_ExMoPG1	600	0.3
Overhang extension	IGBA_D5_ExMoPB1	600	0.3
Overhang extension	IGBA_D5_ExGAP1	600	0.3
Overhang extension	IGBA_D5_eLocT15_GR	8000	4
Overhang extension	IGBA_D5_eLocT15_BY	8000	4
Activator strands	IGBA_D5_eTGR_8N	8000	4
Activator strands	IGBA_D5_eTBY_8N	8000	4
Activator strands	IGBA_D5_eTTar2_8N	8000	4
Dilution	MQ		20.1
Total			50

3.5.4 Gelling components

The following proportions were used for a two-part gelling system with acrydite and PEG to create a 12.5% PEG/Acrylate gel. The 4-arm-PEG-ACRYD-10K and HS-PEG-SH-1500 were equilibrated at room temperature for 20 minutes before the protocol started. Specifications for the components are below the protocol.

Master mix 1 (MM1)

Note that the total volume for the protocol below yields 286 µL of MM1.

1. Weighed 55 mg of 4-arm-PEG-ACRYD-10K into an Eppendorf tube.
2. Added 114.5 µL of 5X Taq buffer and 172 µL of MQ to the Eppendorf tube and vortexed until homogeneous.

Master mix 2 - MM2

Note that the total volume for the protocol below yields 257 µL of MM2.

1. Weighed 30 mg of HS-PEG-SH-1500 into an eppendorf tube.
2. Added 26 µL of Recombinant Albumin, 165 µL of glycerol (50%), and 67 µL of MQ to the Eppendorf tube and vortexed until homogeneous.

3.5.5 Dissociation liquid

Dissociation liquid was used to dissolve the cross-linking bonds in the gel to prepare it for denaturing, either for control on gel or sequencing.

1. Made the dissolution premix by weighing 2.44 g of 0.167 M EDTA in a 1.5 mL Eppendorf tube before adding 38.3 mL of 0.767 mM KOH and 11.6 mL of MQ.
2. Added 120 µL of the dissolution pre-mix to a new tube with 1 µL 1M DTT and 72 µL MQ.
3. Pipetted mixture to mix.

3.5.6 DNA Microscopy Protocol

1. Added the following to a 50 µL PCR tube:
   - 3 µL Enzyme mix
   - 1 µL Seed mix
   - 1 µL Overhang extension mix
   - 5 µL MM2
2. Lastly 10 µL MM1 was added and the mixture was pipetted up and down quickly 15 times to mix. This was done in rounds of 4 tubes that were quickly centrifuged before the next round started.
3. This ran on the PCR cycle described below.
4. After PCR an equal volume of dissociation liquid to the total volume in the PCR tube was added and left at 4°C overnight.

3.5.7 PCR Program

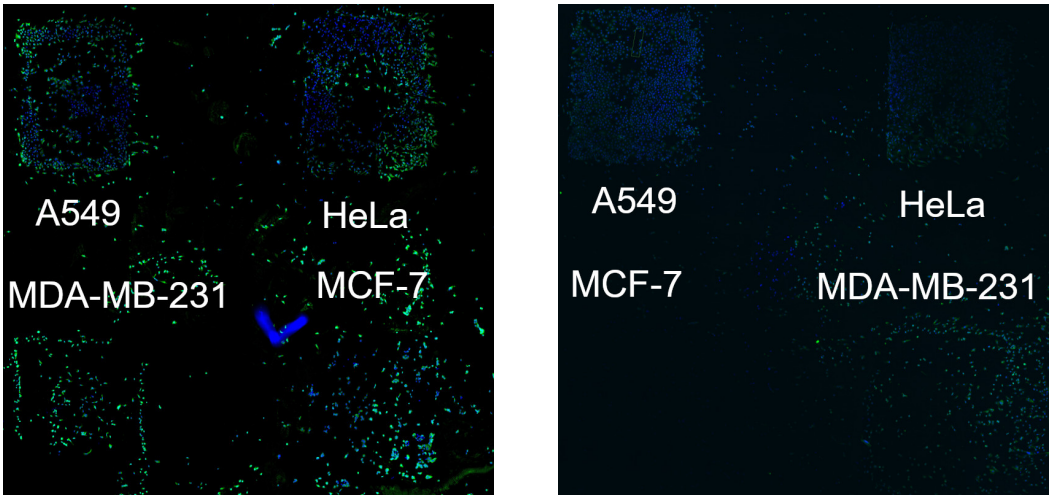
1. Incubate at 22C for 01:00:00
2. Incubate at 95C for 00:03:30
3. Incubate at 98C for 00:00:10
4. Incubate at 67C for 00:00:30
5. Incubate at 72C for 00:00:30
   - Cycle step 3-5 9 more times
6. Incubate at 98C for 00:00:10
7. Incubate at 50C for 00:00:30
8. Incubate at 72C for 00:00:30
   - Cycle step 6-8 1 more time
9. Incubate at 98C for 00:00:10
10. Incubate at 67C for 00:00:30
11. Incubate at 72C for 00:00:30
    - Cycle step 9-11 22 more times
12. Incubate at 72C for 00:02:00
13. Incubate at 4C

4

Results

4.1 Antibody Specificity

The four cell lines' specificity to each antibody was based on the comparative strength of the fluorescence of the secondary antibody. For all the images, blue fluorescence is nuclei staining from the embedding media and is non-selective. The green is due to the modified second antibody. All images below, besides one, show all 4 cell lines and were processed using the auto mode on ImageJ.



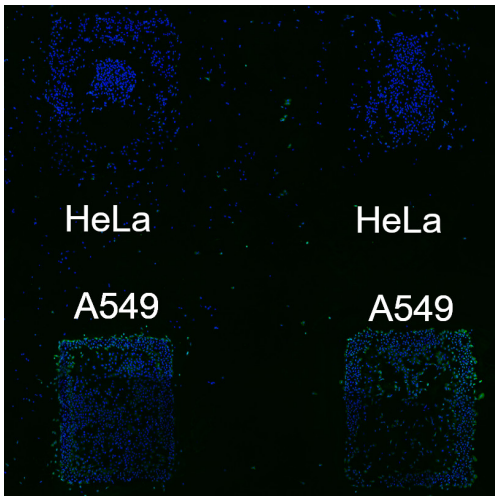
(a) Antibody BASP1 with secondary antibody goat anti-rabbit.

(b) Primary antibody CD44 with secondary antibody goat anti-rat.

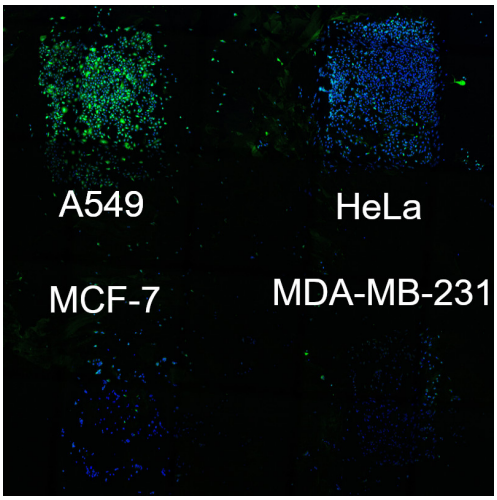
Figure 4.1: Antibody BASP1 and CD44 for all four cell lines.

Antibody BASP1 and CD44 in figure 4.1 show similar fluorescence across all four cell lines, indicating a general specificity. The result corresponds well to the nTPM values in table 2.1 and shows promise to be used as a general antibody. There was quite low cell adhesion for BASP1 which makes it difficult to separate the four different cell lines, but due to the overall fluorescence, it can be considered a general antibody. For the image of antibody CD44, the cell line MCF-7 is almost invisible. This could be due to low cell adhesion, and it was washed away during preparation, or that the microscope did not capture the fluorescence properly. Both cell lines show promise and should be trialed more for higher cell adhesion, with antibody BASP1 showing the most promise.

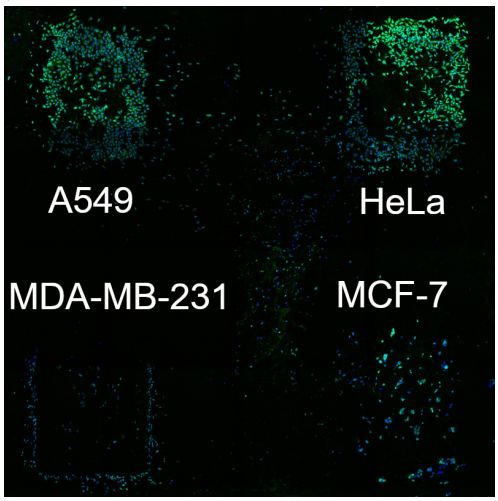
The following three antibodies, AKR1B10, ALDH3A1, and SLC38A5 all showed specificity for cell line A549, the latter also to cell line HeLa as seen in figure 4.2.



(a) AKR1B10



(b) ALDH3A1

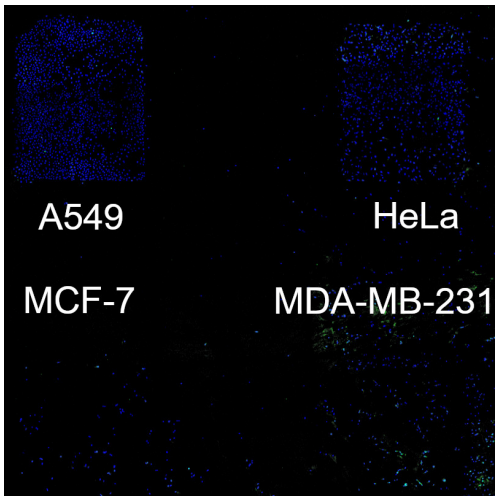


(c) SLC38A5

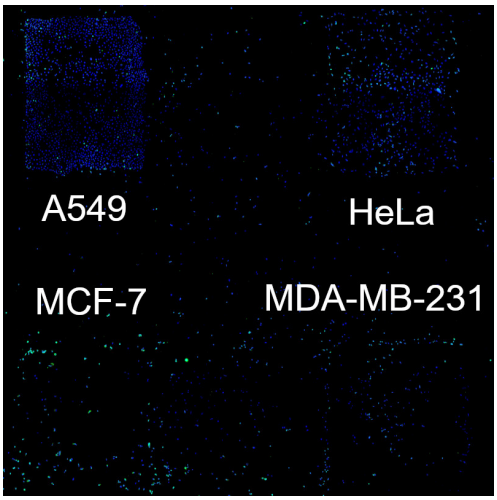
Figure 4.2: The three antibodies AKR1B10, ALDH3A1 and SLC38A5 showed specificity to cell line A548, with SLC38A5 also giving a strong signal for HeLa.

Note in the images above that for figure 4.2 a), there are only two of the four cell lines on the slide. The antibody was tested on all four cell lines but similarly to BASP1 there was low attachment, and exact specificity could not be confirmed. Due to this, it was tested with only A549 and HeLa, where A549 showed the most promise. Both for antibodies AKR1B10 and ALDH3A1 the images correspond with the expected results based on the transcription expression described in table 2.1. Antibody SLC38A5 appears to bind similarly to both A549 and HeLa, which only aligns with the nTPM for HeLa.

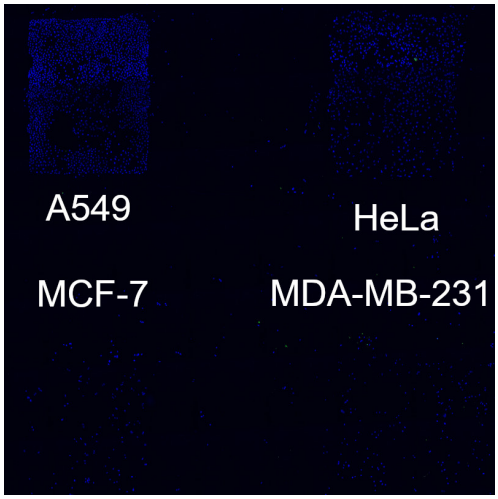
The four antibodies EpCAM, Estrogen, MUC1, and SPINK13 showed low or unclear signals, as seen in figure 4.3.



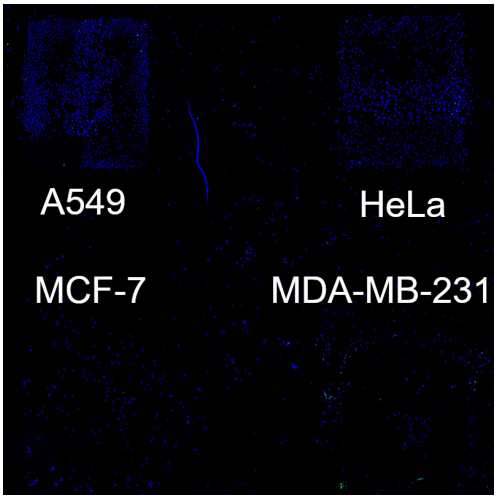
(a) EpCAM



(b) Estrogen



(c) MUC1



(d) SPINK13

Figure 4.3: The four antibodies EpCAM, Estrogen, MUC1 and SPINK13 show weak signal for all four cell lines.

For all four antibodies, the blue nuclei stain shows that there was a successful attachment for the four cell lines, but there is low to no green fluorescence. This indicates that the antibodies should not be further used in experiments, rather other antibodies should be trialed.

4.2 Conjugation

To compare the results of the different conjugation protocols, the samples were put on three different gels. Urea PAGE gel was used to check for DNA contamination, silver staining for protein movement throughout the urea PAGE gel, and finally, an SDS gel to compare the conjugate to the heavy and light chain of the antibody.

UREA Page

The 12 fraction samples from the proFIRE purification were put on urea PAGE gel to confirm the proFIRE profile. Figure 4.4 shows that all samples contained DNA, but only samples 6-12 had free-floating DNA.

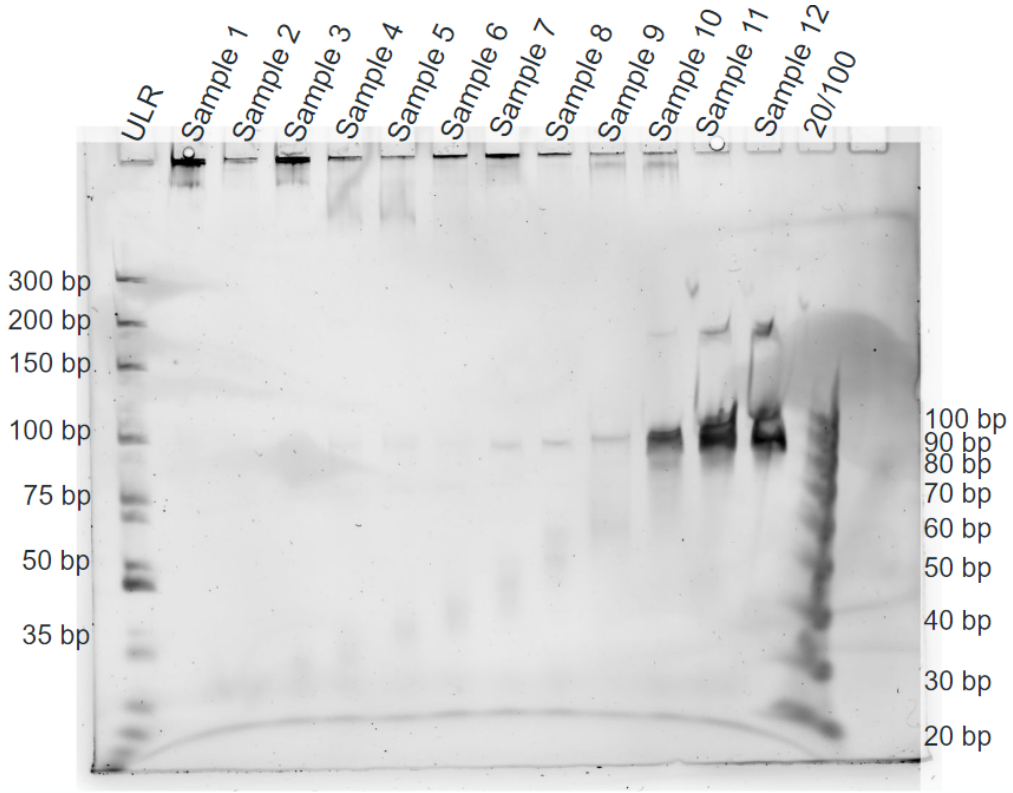


Figure 4.4: Urea PAGE gel of proFIRE samples.

Silver staining of the same gel was performed to see if there was also protein present, seen in figure 4.5. It shows that there was protein in all samples, but for samples 1-4, there seems to be protein that didn't enter the gel. Samples 9-12 show protein confirmation further down the gel.

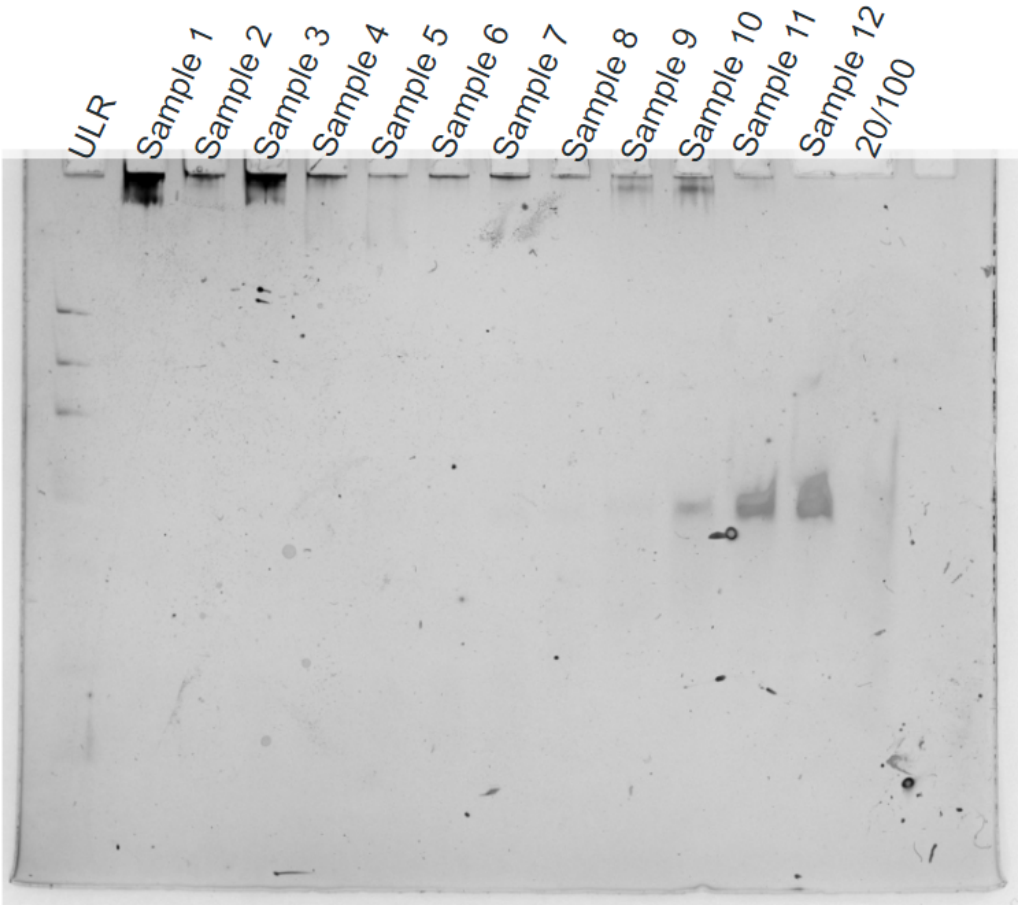


Figure 4.5: Silver stained Urea PAGE gel of the proFIRE samples.

SDS gels

To check the conjugation of the target to antibodies, a protein SDS gel was used to assess all three conjugation protocols. Both SiteClick protocols were purified using Amicon, and the CD44 samples were unconjugated antibodies. The samples were run both with and without fluorescently-labeled complementary DNA to further see if there was DNA present in the samples, which due to being cy5-labeled is blue in figure 4.6.

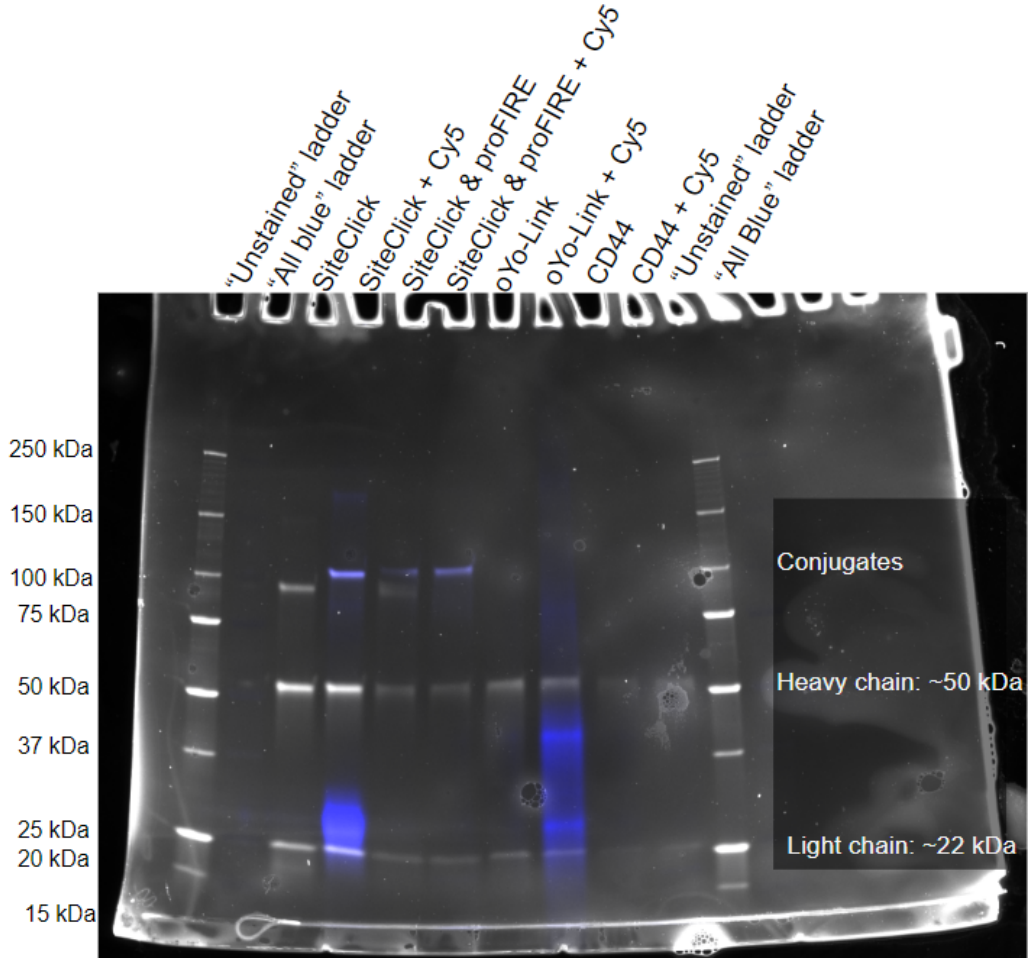


Figure 4.6: SDS PAGE gel of antibody conjugate samples, with cy5-labeled complementary DNA in blue.

There are clear bands for conjugates in both SiteClick samples at around 100 kDa, but none are present for the oYo-Link or CD44 sample. This indicates that SiteClick was successful at conjugating the target to the antibody, while oYo-Link was not. Furthermore, when looking at the blue staining there is a clear presence of target outside of the conjugate for both the SiteClick protocol that only used Amicon and oYo-Link. This gel indicates that the protocol using SiteClick purified with proFIRE and Amicon gives the cleanest sample of the conjugate.

4.3 DNA Microscopy

The DNA microscopy reaction was tested with the CD44 conjugate that was purified using both Amicon and proFIRE. The goal was to see if it had an effect on the reaction and was run on a urea PAGE gel, seen in figure 4.7.

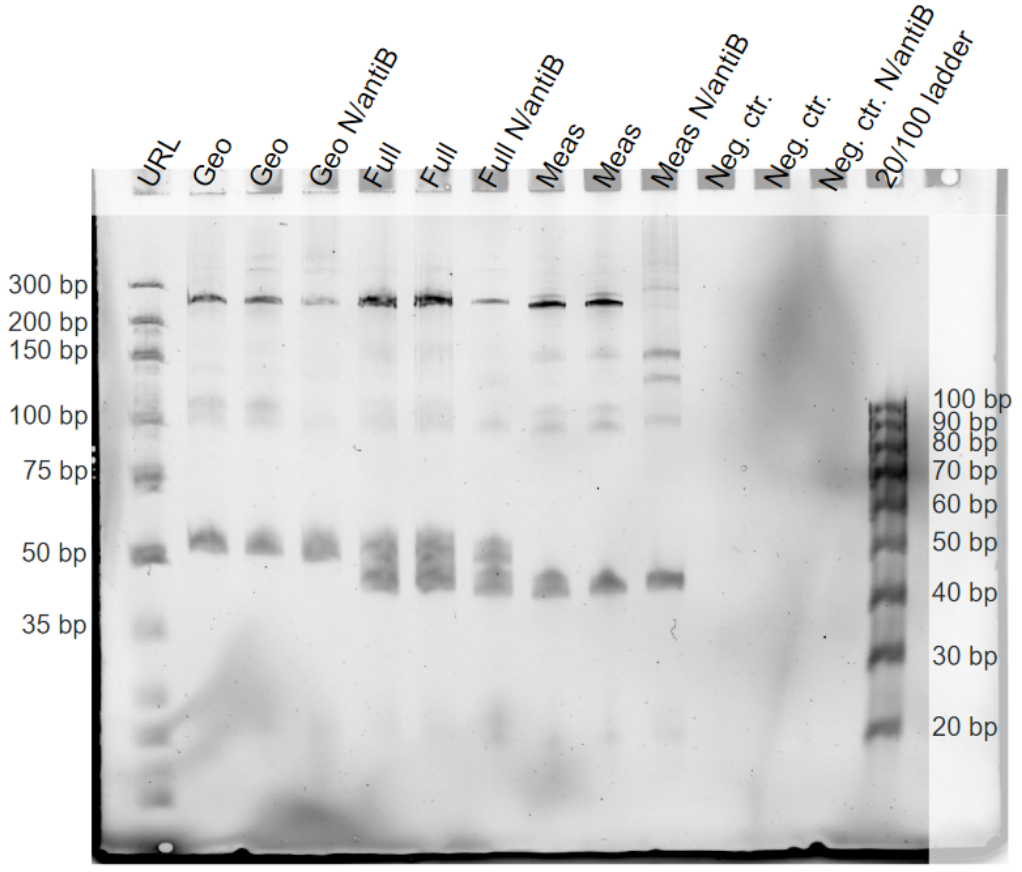


Figure 4.7: Urea PAGE gel of a DNA microscopy reaction containing conjugated antibodies. Geo means that the reaction only contained activation strands for the geometry layer, full that it contained both types of activation strands and meas that it only contained measurement activation strands. The negative control had no activation strands and for N/antiB there was no conjugated antibody added.

When looking at the geometry-only samples, you can see the bands for the activation strands at around 50 bp. The banding for the intermediate reactions between 70-200 bp, and final geometry concatemers at around 250 bp, look the same for the two duplicates with the antibody and the third without. This shows that the geometry layer can form despite the addition of an antibody. This is also true for both the full and measurement reactions with the antibody, while the measurement reaction lacks the clear concatemer band at around 250 bp. This indicates that a DNA microscopy reaction containing target-conjugated antibodies shows promise for future experiments.

5
Conclusion

The goal of the thesis was to lay a foundation for a cellular equivalent of the previous artificial set up to further develop DNA microscopy. There is proof of concept for the method but there are several options for how it might be applied in situ in the future. This thesis explores one of the alternatives which uses conjugated antibodies to deliver the measurement targets to different cell lines.

DNA microscopy uses seed strands, activator strands, and specifically designed DNA targets to reconstruct cellular spaces and quantitatively describe the vicinity of different proteins to one another. This was previously tested by placing target droplets sequentially in a gel that was then reconstructed for an expected visual. For this thesis, the targets were instead conjugated to antibodies which were then attached to different cell lines placed in a square pattern. There was no previous work on suitable antibodies, or appropriate conjugation protocol, which became the main goal of the thesis. A library of cell lines with appropriate antibodies was started. Antibodies CD44 and BASP1 showed promise as general antibodies while ALDH3A1 and AKR1B10 had high specificity toward cell line A549. Antibody SLC38A5 also showed specificity toward both A549 and HeLa compared to the other two. For a conjugation protocol, it was concluded that the use of SiteClick conjugation followed by proFIRE and Amicon purification yielded the best result. Furthermore, a DNA microscopy reaction that included a conjugated antibody showed low to no impact on the reaction.

One of the thesis's main problems was low cell adhesion after fixation throughout the ICC protocol. This made the analysis of the comparative specificity difficult since it was hard to identify the different cell types, making the results less reliable. The method of looking at comparative specificity instead of actual attachment could also skew the result due to a bias in the auto equalizer in ImageJ to amplify even low fluorescence. It could be beneficial to do an analysis that checks the fluorescence of the antibodies from a general reference point rather than within each four-cell square.

For the DNA microscopy analysis, there was no analysis further than seeing that the expected bands were on the gel. While it could be interesting to see the proportion of the different conjugation varieties and quantitatively compare the number of side reactions and concatemers that the two different protocols gave, there was no need for that information at this stage.

In conclusion, the thesis shows that antibody-delivered targets are a valid offer for a future cellular system and that the best way to conjugate and purify antibodies to remove free oligonucleotides requires several steps. The next step would be to incubate the 4 cell lines with the target conjugated CD44 antibodies, and see if the reconstruction can see the four different cell lines in each corner.

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