Applications of in situ proximity ligation assays for cancer research and diagnostics

LIZA LÖF
Abstract

In the field of cancer research and diagnostics it is crucial to have reliable methods for detecting molecules involved in the disease. New and better methods for diagnostics, prognostics and drug delivery therefore remain a permanent aim. In this thesis applications of the in situ proximity ligation assay (*in situ* PLA) were developed for diagnostics and research. Two new methods were developed, one more cost effective proximity assay without the use of enzymes and one method for loading pharmaceuticals in lipid rafts made from detergent resistant membranes (DRMs) to be used as a drug delivery platform.

In **Paper I** the aim was to develop a flow cytometric detection method of the fusion protein BCR-ABL that is the hallmark of chronic myeloid leukemia (CML). By using *in situ* PLA the malignant cells carrying the fusion protein could be detected in patients in a convenient workflow.

**Paper II** describes an application of multiplex *in situ* PLA, where extracellular vesicles (EVs) are detected and identified using flow cytometry. Up to five different antigens are targeted on the EVs, reflected in three different colors during detection in the flow cytometer. By using antibodies targeting proteins specific for prostasomes a population of prostasomes could be identified in human blood plasma.

In **Paper III** a new method is described for using lipid raft for drug delivery. In this method, lipid rafts, derived from prostasomes or erythrocytes, are loaded with pharmaceuticals. The vehicles were loaded with doxorubicin, added to cells and counted. Cells that received the vehicle with doxorubicin stopped proliferating and died, while controls that received the lipid raft vehicle without doxorubicin were not affected, suggesting that the vehicles are effectively loaded with the drug and that they are safe. This lipid raft vehicle could provide a safe drug delivery system.

**Paper IV** investigates the crosstalk between the two major signal pathways Hippo and Wnt, and how these are affected in gastric cancer. When looking at different colon cancer tumor stages, we found that the cellular localization of TAZ/β-catenin interactions were different. We also found that protein complexes involved in the crosstalk increased in sparsely growing cells compared to more densely growing cells. On the basis of these results the protein E-cadherin, involved in maintenance of the epithelial integrity, was investigated and was found to have a probable role in regulating the crosstalk between Hippo and Wnt.

A new method for localized protein detection is described in **paper V**. Here a proximity assay, based on the hybridization chain reaction (HCR), was developed. This assay, proxHCR, is more cost effective than *in situ* PLA because no enzymes are required. ProxHCR successfully detects protein interactions and can be used together with both fluorescence microscopy and flow cytometry.

**Keywords:** Cancer, Diagnostics, Research, Prognostics, Method development, Flow cytometry, Drug delivery

*Liza Löf, Department of Immunology, Genetics and Pathology, Molecular tools, Rudbecklaboratoriet, Uppsala University, SE-751 83 Uppsala, Sweden.*

© Liza Löf 2016

ISSN 1651-6206
ISBN 978-91-554-9638-8
urn:nbn:se:uu:diva-300191 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-300191)
Applications of *in situ* proximity ligation assays for cancer research and diagnostics

Liza Löf
“The difficult we do immediately. The impossible takes a little longer.” Author unknown
-My Mother taught me this

To All of My Family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III  Dubois, L.*, **Löf, L.*., Larsson, A., Hultenby, K., Waldenström, A., Kamali-Moghaddam, M., Ronquist, G., Ronquist, KG. Detergent resistant membranes (DRM) from erythrocyte ghost can vesiculate and form the basis for delivery of pharmaceuticals: A model drawn from experiences with prostasomal DRM. *Manuscript*

IV  Arngården, L., **Löf, L.**, Grannas, K., Raykova, D., Zieba, A., Grabek, A., Oelrich, J., Figueiredo, J., Dahlin, J., Kamali-Moghaddam, M., Seruca, R., Söderberg, O. Crosstalk between Wnt and Hippo signaling pathways changes upon colon cancer stage and is affected by cell density and loss of or mutated E-cadherin protein. *Manuscript*

*Shared first author

Reprints were made with permission from the respective publishers.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>13</td>
</tr>
<tr>
<td>Cancer</td>
<td>15</td>
</tr>
<tr>
<td>Proteins</td>
<td>17</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>17</td>
</tr>
<tr>
<td>Current methods</td>
<td>19</td>
</tr>
<tr>
<td>In situ PLA</td>
<td>21</td>
</tr>
<tr>
<td>Affinity binders</td>
<td>21</td>
</tr>
<tr>
<td>Modification of the affinity binders through conjugation</td>
<td>24</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>26</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>31</td>
</tr>
<tr>
<td>Extracellular vesicles</td>
<td>33</td>
</tr>
<tr>
<td>Present Investigations</td>
<td>34</td>
</tr>
<tr>
<td>Paper 1: Sensitive flow cytometric detection of BCR-ABL fusion protein positive cells in blood from patients with chronic myeloid leukemia</td>
<td>34</td>
</tr>
<tr>
<td>Introduction</td>
<td>34</td>
</tr>
<tr>
<td>Aim and procedure</td>
<td>34</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>35</td>
</tr>
<tr>
<td>Paper II: Detecting extracellular vesicles using a multicolor in situ proximity ligation assay with flow cytometric readout</td>
<td>36</td>
</tr>
<tr>
<td>Introduction</td>
<td>36</td>
</tr>
<tr>
<td>Aim and procedure</td>
<td>36</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>36</td>
</tr>
<tr>
<td>Paper III: Detergent resistant membranes (DRM) from erythrocyte ghost can vesiculate and form the basis for delivery of pharmaceuticals: A model drawn from experiences with prostasomal DRM</td>
<td>38</td>
</tr>
<tr>
<td>Introduction</td>
<td>38</td>
</tr>
<tr>
<td>Aim and procedure</td>
<td>38</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>39</td>
</tr>
</tbody>
</table>
Paper IV: Crosstalk between Wnt and Hippo signaling pathways changes upon colon cancer stage and is affected by cell density and loss of or mutated E-cadherin protein. .....................................................40
   Introduction .........................................................................................40
   Aim and procedure ..............................................................................40
   Results and discussion .........................................................................40
Paper V: Proximity-dependent initiation of hybridization chain reaction .....................................................................................................42
   Introduction .........................................................................................42
   Aim and procedure ..............................................................................42
   Results and discussion .........................................................................42
Conclusion and future perspectives ..............................................................43
Acknowledgements .......................................................................................45
References.....................................................................................................49
Abbreviations

AO  Acridin orange
AP  Active phase
ATP  Adenosine triphosphate
BME  Beta-mercaptoethanol
BP  Blast phase
BSA  Bovine serum albumin
CCyR  Complete cytogenetic response
CML  Chronic myeloid leukemia
Co-IP  Complex-immunoprecipitation
CP  Chronic phase
CSC  Cancer stem cell
DBCO  Dibenzocyclooctyne
DRM  Detergent resistant membrane
DTT  Dithiothreitol
EGF  Epidermal growth factor
ELISA  Enzyme-linked immunosorbent assay
EV  Extracellular vesicles
FACS  Fluorescence activated cell sorter
FISH  Fluorescence in situ hybridization
FITC  Fluorescein isothiocyanate
FBS  Fetal bovine serum
FSC  Forward scatter
GCP  Good clinical practice
GFP  Green fluorescent protein
HCR  Hybridization chain reaction
IMS  Imaging mass spectrometry
IF  Immunofluorescence
IHC  Immunohistochemistry
IgG  Immunoglobulin
iRCA  Immune rolling circle replication
INF-α  Interferon alfa
MRD  Minimal residual disease
MS  Mass spectrometry
NH₂  Primary amine
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-protein interactions</td>
</tr>
<tr>
<td>ProxHCR</td>
<td>Proximity hybridization chain reaction</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tubes</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
</tr>
<tr>
<td>RCA</td>
<td>Rolling circle amplification</td>
</tr>
<tr>
<td>RCR</td>
<td>Rolling circle replication</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH</td>
<td>Sulphhydryl</td>
</tr>
<tr>
<td>SMCC</td>
<td>Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Introduction

When I was thirteen years old I was interviewed for a book, and they asked me what I wanted to work with when I grew up. I replied that I wanted to be a detective in Los Angeles. I was always intrigued by solving mysteries and puzzles. But then I got interested in biology, and later, physiology and soon learned that working in the field of medicine is like being a detective. Today when a patient is seeking medical advice or needs to be diagnosed the doctor needs to take tests to see what this patient is suffering from. The doctor must solve that patient’s individual puzzle to make the correct diagnosis, and to start the appropriate treatment. In order to find the pieces of the puzzle in each individual the appropriate tests needs to be ordered, and for that we need sophisticated methods that give the correct answers. Developing these methods is the ultimate detective work. I have chosen the field of cancer. In cancer research, whether basic research, diagnostics, prognostics or drug delivery platforms, the need for new technologies and methods is always there. The work in this thesis has the focus on development of methods for cancer research.

My thesis is based on five papers and they extend over a wide range of cancer research and diagnostics. Paper I describes the development of a new method for research, diagnostics, and prognostics in chronic myeloid leukemia, with applications both in the research lab and in clinical settings. Paper II describes the development of a method for detecting extracellular vesicles. Extracellular vesicles play an important role in cell-to-cell communication and cancer metastasis, and they may represent important biomarkers. This method can bring new insights into how extracellular vesicles are involved in cancer metastasis. Both the molecular methods in paper I and II are developed for flow cytometry. Paper III concerns new ways to deliver drugs to cells. Here we have developed a new, biological drug delivery system built from cell membranes, which can be loaded with drugs of interest and delivered to the target cell. In paper IV we apply the molecular detection techniques in focus in this thesis, and we investigate signaling processes of relevance for cancer research. How do cellular pathways interact, and how are they altered when mutations are introduced? It is also important to strive towards less expensive methods, and one way of doing this is to develop procedures with no need for expensive enzymes. Accordingly, we developed in paper V a method, proxHCR, as a cost-effective technique for detection of protein interactions and post-translational modifications, utilizing HCR for
signal amplification. The amplified, fluorescent signal of proxHCR can be detected using either microscopy or flow cytometry.
I was involved in all aspects of the research for and preparation of papers I and II, in all the cellular work and writing of paper III, cellular work and the microscopy for paper IV, and the flow cytometric work for paper V.
In summary, the focus of this thesis is on methods for cancer research and diagnostics. It is important to have good, specific, robust, dependable and cost-effective methods. We need to be able to trust our results, in order to unravel the cancer puzzle.
Cancer:

In 1971, the United States President Richard Nixon signed the National Cancer Act into law. This declared a national crusade to reduce the incidence, morbidity, and mortality of cancer by strongly supporting cancer research. During the ensuing two and half decades there were considerable changes in this field of research, influencing everything from cancer research, prevention to diagnosis and treatment. Therapy changed from a strong reliance on irradiation and radical surgery to the more successful and today standardized, multimodal therapy with a combination of surgical resection, radiation and chemotherapy with increasingly targeted drugs. The crusade paid off, in the decade of 1990 there was an overall decrease in cancer mortality for the first time.

Today we know many of the factors that lead to cancer; a number of different agents are able to cause cancer by modification of the cellular DNA, including hereditary factors, chemicals, viruses, bacteria, radiation, air pollution and life style factors. We also have more knowledge about the cancer genesis today, something that has been a matter of speculation over the course of history. The first time a cancer cell was observed by microscopy was in 1838 and it would take until the late 1960s before the term oncogene was first recognized after the discovery that retroviruses can introduce normal genes into healthy cells where they act as oncogenes by causing cancer. Actually, the theory of oncogenes had been presented earlier by the Danish physicist Niels Henrik Arley. He presented his idea around 1950, but the theory was rejected as nonsense. Proto-oncogenes and tumor suppressor genes are two basic classes of genes normally present in our cells that when they acquire mutations may cause cancer. These two classes of genes work as an orchestra to regulate the proliferation of normal cells. The proto-oncogenes encode proteins that are normally important in embryogenesis, and the normal maintenance of human organs and tissues. These are proteins that stimulate cell division, or inhibit cell differentiation and apoptosis. The tumor suppressor genes codes for proteins that prevent cells to proliferate, or that stimulates apoptosis when needed. These proteins can also be important in the DNA repair process. There is an axiom in cancer research that cancers in adults typically evolve over decades, that there is a multistep process with an accumulation of mutations in several genes over time. Childhood tumors usually originate from DNA mutations that occurred early in life or before birth. There are differences of opinion how progression of the cancer starts. According to the clonal evolution hypothesis tumor progression is...
driven by selection of the fittest tumor cell that emerges as cancer\textsuperscript{9}. One other model is the cancer stem cell (CSC) hypothesis, were only the stem cell acquire mutations and drive the development of cancer\textsuperscript{10}. There are a number of different types of mutations that can cause these cells to become cancerous and many carcinomas displays mutations in a number of proto-oncogenes and tumor suppressor genes. The genetic aberrations can be categorized into four different groups. i) Minor sequence changes involving limited deletions, insertions or base substitutions involving a few nucleotides, which cannot be detected via cytogenetic analysis. ii) Alterations of chromosome numbers, called aneuploidy that involves gains or losses of entire or large parts of chromosomes. These changes can be detected via cytogenetic analysis and are recorded in most cancer types. iii) Chromosome translocations are fusions within or between different chromosomes that can be detected cytogenetically. These new chromosomes can contain a fusion between two genes thus giving rise to gene products that displays tumorigenic properties. iv) The fourth group is gene amplifications creating multiple copies of a gene, for instance growth promoting genes. These changes can often be visualized both cytogenetically and at a molecular level\textsuperscript{11}. Point mutations, insertions and deletions lead to increased transcription or hyperactive gene products. Gene amplification events or chromosomal translocations leading to extra chromosomal copies or a relocation of one piece of one chromosome to another can lead to a higher expression of the gene product\textsuperscript{12}. These events, may lead to cancer, but the molecular alterations can also serves as targets for diagnosis and prognosis, and in some cases also for drugs to treat the disease. Even if mutational events that can be used to make a diagnosis and prognosis are relatively rare today, more and better methods become available to find increasing numbers of them. There are several examples of gene aberrations that are used today as diagnostic and prognostic markers. Mutated KRAS genes involving a single amino acid replacement can represent an activating mutation, such that the resulting protein cannot downregulate signaling of the vascular endothelial growth factor VEGF) and epidermal growth factor (EGF) signaling pathways, which leads to excessive proliferation of cells. Such mutations are found in many carcinomas, like pancreatic cancer, colorectal cancer and adenocarcinoma of the lung were they are associated with a poor prognosis and aggressive disease\textsuperscript{13-15}. Another example of markers are the chromosomal translocations, where one of the most well-known chromosome rearrangement is the Philadelphia (Ph) chromosome where the c-abl gene on chromosome 9 is joined to the bcr gene on chromosome 22, giving rise to the fusion product bcr-abl - the hallmark of chronic myeloid leukemia (CML)\textsuperscript{16}. 
Proteins

The blueprint of our body is to be found in the genomic DNA. DNA is transcribed into mRNA, which in turn is translated into proteins. During recent decades research has taken great leaps towards a better understanding of the molecular biology of the human body. The great achievement of sequencing the human genome\textsuperscript{17} has given us many of the clues that we need. The next step was to look at the proteins in the body. The human genome contains approximately 22,000 protein-coding genes\textsuperscript{18}, giving rise to perhaps 500,000-5,000,000 different proteins, when taking into account, differential transcription start sites, splice variation, processing, and post-translation modifications such as glycosylations and phosphorylations\textsuperscript{19,20}. Proteins vary enormously in structure, function and stability, and they are considered to be the final products of many genes. They are the functional and an informative output of the cell, and can tell us about the biology of the cell. Many genes do not give rise to any proteins and there is a poor correlation between mRNA and the corresponding translated protein. This notion is now accepted since some RNA transcripts do not give rise to any proteins - they are non-coding. Alternative splicing of mRNA that encodes several protein products and the post-translational modification that cannot be predicted from nucleic acid sequences and the fact that some proteins are unstable and will be degraded are other reasons for the poor correlation between the mRNA and the corresponding protein\textsuperscript{21}. The study of proteins - proteomics - does not only look at individual proteins, it also takes on higher-order complexes by examining interactions among proteins as well as the modifications of the proteins and pathways they are involved in.

Cell signaling

Starting from the end of my thesis, but the most important part of the protein studies: How does the tumor cell differ functionally from a normal cell? We need to access the cell’s dynamics, investigate signaling pathways and look closer to see what changes characterize the cell. The signaling is complex, there is cross-talk between signaling pathways and there are abundant positive and negative feedback loops\textsuperscript{22}. The complexity of alterations in cancer is multimodal and will reflect on more than one signaling pathway, it will alter the growing tumor’s proliferation and apoptosis. Insights in key networks
involved in tumorigenesis will give us clues to the cancer initiation and progression, including metastasis. Two of the signaling pathways I have looked more closely at in this thesis are the Hippo and Wnt signaling pathways. Hippo signaling is controlling organ growth by regulating apoptosis and proliferation in various cell types. Hippo signaling is regulated by cell density. The Hippo effector proteins YAP/TAZ are targeted by degradation in cells that are growing at high density. When cells are sparse YAP/TAP move into the nucleus leading to that transcription of target genes. Wnt signaling is regulated in three manners: the WNT/\(\beta\)-catenin pathway, the canonical pathway WNT/\(\beta\)-catenin, which is the most studied pathway, and the non canonical pathway. When WNT is in an off state, there is no receptor signaling from the cell membrane and the pathway is tightly regulated. The regulation is enacted in the cytoplasm by a \(\beta\)-catenin destruction complex, a complex that needs to be orchestrated to perfection. Axin is the scaffold for all other proteins in the destruction complex and it makes the complex ready for ubiquitinylation when TRCP is phosphorylated and the complex can be targeted for degradation. When WNT binds its receptor Frizzled this leads to a cascade of signaling where the effector proteins are no more bound to the complex of destruction. The signal is no longer inhibited; \(\beta\)-catenin is free to move into the nucleus directing target genes to be transcribed.
Current methods

The proteomics era arrived with a strong desire for methods that can be applied in functional studies of proteins in order to generate therapeutic agents, diagnostic methods and prognostic markers. There are a number of sophisticated methods out there that we can use to look deeper into the protein interactions and find potential therapeutic agents.

There are different attributes of proteins that catch our interest. We want to look at the structure, the profiling of their expression levels in relevant samples, and the signaling pathways. We are also interested in functional properties of proteins through analyses of properties like phosphorylation state and activity profile.

I will just mention mass spectrometry (MS) since this is one of the most accurate and reliable methods for identifying proteins in a complex sample, provided sufficient amounts are available for analysis. However, I will not discuss the technology further since it is not relevant for the affinity-based methods presented in this thesis. MS is a sophisticated, relatively complicated and expensive method, allowing proteins to be identified by recording the amino acid sequences of peptides after enzymatic digestion using trypsin.

Unlike MS, the methods considered in this thesis are all affinity based, and face problems that are associated with affinity-based reactions. I will start with presenting the different methods and later discuss challenges and potential solutions.

Western blotting is a method where proteins separated by electrophoresis are transferred to a membrane for antibody-based detection, as a means to visualize proteins in a cell or tissue lysate. This method does not give any indication about the position of the protein in the sample. It is a widely used technique and fairly easy and inexpensive to use. Once the sample is lysed and the protein is in solution it is added to a native sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated according to its size, after which the proteins are transferred to a membrane, which is then blocked for unspecific binding of antibodies. The proteins are then visualized using first a primary antibody and then a secondary antibody. The secondary antibody is coupled either to a fluorophore or an enzyme that can...
be reacted with its substrate to create a signal that is recorded on the membrane, allowing the size of the protein to be estimated and the protein identified\textsuperscript{28}.

Western blot can be used in combination with co-immunoprecipitation (CoIP), a method that is widely used to look at protein interactions in a sample. The preparation steps are the same as for westerns blot, where the sample is lysed and then analyzed. By antibody “fishing” or “pull-down”, using antibody-conjugated agarose beads proteins of interest are captured from a cell lysate and after gel separation another antibody can be used in a western blot to detect other proteins in interaction with the captured ones\textsuperscript{29}.

Immunofluorescence (IF) uses antibodies conjugated with a fluorophore dye for imaging protein distribution among cells or in tissues. Two classes of techniques are available, primary or secondary detection. In the former case a single, primary antibody labeled with a fluorophore is used for detection and visualization. The secondary uses a primary antibody against the protein target and then a secondary, labeled antibody directed against the primary antibody raised in a distinct species than the primary. The success of the method depends on the antibody used and the level of expression of the targeted proteins.

Immunohistochemistry (IHC) is commonly used in both research and clinical settings where the labeling is done by enzymes that generate a colored precipitate at the site of binding by enzyme-conjugated antibodies in a tissue section\textsuperscript{30}.

The enzyme-linked immunosorbent assay (ELISA)\textsuperscript{31} is the most commonly used immunoassay in the clinic and the sandwich ELISA depends on binding by pairs of antibodies. The dual recognition reduced risk for cross reactivity. A solid support is coated with capture antibodies that serve to immobilize the proteins of interest, followed by washes. Then an enzyme-conjugated detection antibody is used to generate a signal. When the ELISA method introduced the solid support it also introduced a successful removal of excess reagents due to the allowance of washing. The dual recognition together with the washing step gives a low background signal, however since the methods are only as good as our antibodies there will still be some signal from antibodies binding un-specifically.

To ensure optimal detection of antigens by the method used the protocol needs to be optimized for each antigen and each antibody. This is often very time consuming but well worth the time. Firstly we would like to enrich the protein of interest and this is usually done by a capturing step followed by washing to remove any irrelevant proteins from the samples. This step is usually followed by some kind of blocking to avoid any unspecific binding of the probe. The probes are the affinity reagents that are used to label the
target of interest. This step is followed by detection; nowadays the detection step can be amplified and then the signal is recorded. In order to enhance specificity one could use dual binders for detection.

**In situ PLA**

The method I use in my research is the *in situ* PLA that was first reported in 2006\(^3\). Fredriksson et al. first described the PLA in 2002 for specific and sensitive measurement of proteins in solution\(^3\), and Söderberg et al. adapted the method for visualization and measurement of proteins, their interactions and posttranslational modifications (PTMs) in cells and tissue sections\(^3\). In 2009, Leuchowius used this method in combination with flow cytometry readout to detect extracellular proteins and protein interactions\(^4\). Development of the method to detect multiple protein complexes came 2013\(^3\). The method uses two affinity binders recognizing target proteins. The targets could be two different epitopes on one protein, two interacting proteins, or one protein and its PTM such as a phosphorylation or glycosylation. The affinity binders are coupled to DNA oligonucleotides. Once the two binders have recognized their targets, the two DNA oligonucleotides are brought in close proximity to facilitate circularization of another pair of DNA oligonucleotides via enzymatic ligation. This newly formed DNA circle then serves as a template for a localized DNA amplification through rolling circle amplification (RCA). Ninety minutes of amplification will generate approximately 1,500 complements of the circular, 100 nucleotide long template DNA strand. To each of these copies a fluorophore-coupled DNA oligonucleotide can be hybridized such that detection event that creates a circle will generate a signal that can be recorded in a flow cytometer or a fluorescence microscope\(^3,4\).

**Affinity binders**

The key components for the best immune based methods are the affinity binders – those are the antibodies. In order to find the best antibody for the analysis, one can choose either a polyclonal or a monoclonal antibody. There are some key differences between the two and it is a good idea to consider which that will do the job for you.

I cannot stress enough that our methods are no better than the antibodies we choose. And choosing between a polyclonal and a monoclonal antibody is one of the big decisions. There are great differences in the two kinds of antibody reagents. The production of polyclonal antibodies is less expensive. They are also faster to produce and the skills required to make them are less
than for monoclonal antibodies. The price you pay for your polyclonal antibody comes with the price of specificity; the polyclonal antibody is less specific against its target, since it will recognize a number of epitopes on any one antigen. Polyclonal antibodies have proven to be clinically effective. However, the production will generate batch to batch variability which will limit their use in the clinic\textsuperscript{36}. The production of a polyclonal antibody is typically done by inoculation of a mouse, rabbit or goat etc that will induce the B-cells to produce large amount of IgG immunoglobulins recognizing the antigen from the target of interest. The animal will generate antibodies that recognizes multiple epitopes on an antigen and the serum will contain multiple IgG antibodies from a heterogenous mixture with different affinities.

So is the monoclonal antibody always the best choice then? Well, to make it easy one can say that a monoclonal is everything that a polyclonal is not. The production is more expensive and more difficult to produce, but will generate a never-ending source of antibodies. They are produced by hybridomas that take a long time to make but will however produce a very specific antibody recognizing only one epitope of the protein of interest. This antibody will create less staining background since it is only detecting one target epitope and the likelihood of cross reaction is less than for polyclonal antibodies. The optimization of the protocol is kept to a minimum, since it only has to be optimized once. The antibody is usually made from hybridoma cells originating from mice. Spleen cells from mouse immunized with a specific antigen are fused to a cell line. This will create a hybridoma that is immortalized and capable of producing only one type of antibody. When the immunogen is a peptide the target epitope will be smaller and may have a homology to other epitopes that will increase the risk of cross reactivity. The risk of cross reactivity makes it necessary to check the sequence to avoid homology and overlapping\textsuperscript{37}.

An antibody has a size of approximately 150 kDa and consists of two light chains of about 25 kDa each and two heavy chains of approximately 50 kDa each. There are κ or γ light chains and α, β, ε, γ, μ heavy chains. The type of the heavy chains will define the isotype (IgA, IgD, IgG, IgE, IgM) of the antibody. To exemplify the structure one often uses IgG that comprise of two identical light and heavy chains that are linked together via disulphide bonds (Figure 1). The N-terminal (variable region) of the heavy and light chains is the antigen recognition site, where the antibody binds its target or epitope. The constant region on the heavy chains, the Fc-region, of the antibody, has affinity for Fc receptors on cells, which can cause nonspecific background of antibodies binding cells via their Fc regions\textsuperscript{38}.
Figure 1. Immunoglobulin structure showing the heavy (light blue) and light chains (dark blue) peptide chains with the antigen binding site as well as the Fc- and the F(ab’)2-fragment of the antibody. Residues available for covalent modification, the primary amines (NH2) and disulphide bonds are also displayed. At the top of the antibody the variable regions are located and the lower part is the constant region.
Another class of affinity binders is the single chain-binding proteins. These consist of the antibody variable amino acid sequence of the light and the heavy chain and are about 26 kDa. The single chain binders are recombinant polypeptides representing antibody variable sequences transcribed and expressed as proteins in *Escherichia coli* bacteria. These binders have the same affinity and specificity as antibodies. They can produce a lower background, since they lack the Fc-portion of full length antibodies that is recognized by Fc-receptors on some cells\textsuperscript{39}.

Affibodies are another class of affinity binders. They are small, only 6.5 kDa, 58 amino acids, generally cysteine free, and can be produced with affinity to any target protein. They are derived as mutant variants of domain B of the staphylococcal protein A, which is an immunoglobulin binding protein. Since they are small and lack the structural parts of an antibody they have advantages such as higher stability and less unspecific binding\textsuperscript{40}.

Another type of small affinity binders is the DARPin(s (designed Ankyrin repeat proteins). They are, similar to affibodies, single domain proteins, only 14 kDa, and can also be made to bind any target protein with a high affinity. DARPin(s derives from natural ankyrin repeat proteins, a very abundant binding protein in the human body\textsuperscript{41}.

Aptamers are yet another class of affinity binders. They are single-stranded DNA or RNA molecules that have been selected to bind different targets, peptides, amino acids and proteins. The aptamers bind the functional domains of the target protein, which make them very interesting in the field of diagnostics and therapy\textsuperscript{42}. Aptamers shows great sensitivity, and PLA using aptamers can detect proteins down to zeptomolar concentrations\textsuperscript{33}.

**Modification of the affinity binders through conjugation**

The affinity binders need sometimes to be functionalized prior to use in an assay. They can be conjugated with different moieties such as enzymes, fluorophores, DNA oligonucleotides, or chelated metal ions in order to be visualized or quantified. There are a number of labeling methods available and I have used two of them in my projects, namely copper free click chemistry conjugation\textsuperscript{43,44} and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)-conjugation\textsuperscript{45}. These are the ones I will be discussing here. Modifications of proteins are most often done in one of two ways. by forming bonds with either the primary amines (NH\textsubscript{2}), or with sulfhydryls (SH), also called thiols.

The thiols, are naturally occurring in the side-chain of the amino acids cysteine in proteins, they need to be reduced to free sulfhydryl groups (-SH) before reacting with the thiol-reactive compounds. In the antibody the cysteine sulfhydryl groups are coupled by disulphide bonds (Figure 1). There are a number of chemical agents that are sulfhydryl-reactive; acryloyls, haloace-
tyls, maleimides, and 5-thio-2-nitrobenzoic acid (TNB)-thiols to name a few. The SMCC-conjugation utilizes maleimide, which reacts with the SH-group at a pH between 6.5 and 7.5 forming a non-reversible thioether linkage. The disulphide bonds in the antibody are reduced by dithiothreitol (DTT) or beta-mercaptoethanol (BME) to make the sulfhydryl groups available for the maleimide, after which any remaining DTT or BME is removed by buffer exchange since they contain thiols and will compete for reaction sites. After the conjugation remaining maleimides in excess can be quenched by addition of compounds with free thiols.

The other main conjugation method is using chemicals that react with the primary amines on the antibody. The primary amines are positively charged at pH 7.4, and they are therefore often located on the outside of the protein’s tertiary structure and therefore accessible to the conjugation chemicals. Among the many agents that can be coupled to primary amines are NHS esters, acyl azides, isocyanates, aldehydes and carbonates. In the click chemistry conjugation an NHS ester is used for formation of amide bonds through crosslinking. The NHS esters allow the introduction of dibenzocyclooctyne (DBCO) groups in the proteins, and these groups can then be reacted with azides, placed on the labelling agent.
The analytical method I have chosen for many of my projects is flow cytometry. *In situ* proximity ligation assays (PLA) and flow cytometric applications are a perfect fit for each other. Leuchowius et al. were the first to use *in situ* PLA for protein detection in flow cytometry\(^{34}\). Since each signal from *in situ* PLA is amplified using rolling circle amplification (RCA) the flow cytometer can record abundant signals representing the target proteins. This is very useful when the targets are limited in numbers.

Flow cytometry was first invented in the 1960s\(^{47}\) as an instrument used to, correctly, analyze blood cells\(^{48}\), and the first fluorescence activated cell sorter (FACS) came just a few years later\(^{49}\). FACS made it possible to analyze the function and properties of cells isolated from the blood and organs of the body. What really made FACS take the step into the clinical setting was the discovery and development of the hybridomas and the monoclonal antibodies as well as the development of an increased range of fluorophores\(^{37,50,51}\).

The principle of flow cytometry is the ability to measure the fluorescence and optical characteristics of the cell, when the cell is passed through a light source in a fluid stream (Figure 2). The light source used to excite a fluorescent tag is most often a laser beam. The sample is injected into the flow chamber and the laser beam will hit the cell. When the sample is hit by the laser the electrons on the fluorophores in the sample absorb light at a specific wave length to become energized and when they fall back to their lower orbitals light is emitted. The photons are converted by photomultiplier tubes (PMT) to an electrical impulse and recorded as numerical signals. The computer system can visualize the signals in a histogram or in a dot plot, displaying the intensity of fluorescence of each cell. This distinguishes weakly fluorescent from strongly fluorescent cells. There is diffraction of light around the cell, producing a pattern of light that is detected. This is the scattered light that is somewhat proportional to the size and granularity to the cell. There are two types of scattered light detected, namely forward scatter (FSC) and side scatter (SSC), which are affected by the characteristics of the cells (Figure 3). Light is scattered in all directions and in the forward direction it is collected within a range of 0.5-10°. The FSC is proportional to the size of the cell and is used for immunophenotyping. SSC, on the other hand, is detected with an approximately 90° angle and is affected by the cells inner complexity and shape. Different cell populations can be separated based on the SSC-signal that reflects the different characteristics of the cell, the cell membrane, granularity and nucleus. When fluorescent probes are used, they
are also excited by the laser light and emit a longer wavelength that can be detected. The fluorescent probes are used to detect certain molecules on the cells that can be used to further characterize the cell. While in 1995 only five parameters could be measured, the instruments are today equipped with optical systems to distinguish more than 20 parameters. The fact that the flow cytometers are multiparametric is what makes them so useful. They can be used to measure several components on each cell, making it possible to identify subpopulations in a heterogeneous cell population. The most common way of analyzing the data obtained from flow cytometry is to do a percentage analysis of the positive from the negative cells, to separate the subpopulations from each other. There is a need for advanced computer programs since we are now analyzing more parameters of the cells. The fluorescent probes that are used to label the cells are commonly used to visualize cell surface receptors, organelles inside the cell, DNA content, whether the cells are apoptotic and to measure the enzymatic activities of the cells. When staining the cells, it is important to think about, not only the antibody, but also which fluorochrome that is to be used. It is important to think about the separation between emission and excitation of the light of the fluorophore and the intensity of fluorescence. When applying optimal conditions for different fluorophores it is possible to do a multiparametric analysis of the cells. Another important aspect of the flow cytometer and the multiparameter analysis is the possibility to sort cells, which allows separation and collection of subpopulations of cells. This requires another type of flow cytometer, a FACS. A sorter lets the cells flow through a series of droplets and the cells are isolated from these small drops. When the parameters in the instrument are correctly set the cell is collected by a change in an electrical charge that is applied to the droplet. It is the sorting criteria that determine the polarity of the charge, the positivity or negativity, and the cell will move to the right or the left. When examining a cell in a flow cytometer the optimal size of the cell is between 1-30 µm in diameter. Smaller cells can be missed because of the sensitivity of the instrument and larger can block the system. Small particles, such as virus, bacteria, DNA content can be detected with less precision or with more advanced machines that have an increased specificity. Cells from solid tissues can also be analyzed if they are first separated from each other and the cells are dispersed. To continue with the immunophenotyping of the cells there are a number of protocols available. In the United States the National Committee for Clinical Laboratory Standards (NCCLS) and in Europe the European Working Group for Clinical Cell Analysis have set quality controls and standardizations, which helps to reduce inter laboratory variations in the clinical setting. There are a number of protocols for a number of different types of analyses, such as viability, DNA content, platelet activation and cytotoxicity. It is advantageous, and of course sometime necessary to use live cells and analyze them as soon as possible, suspended in physiological medium, like PBS or tissue media like RPMI-1640 with addi-
tional proteins like bovine serum albumin (BSA) or fetal bovine serum (FBS). Live cells have less unspecific binding of antibodies than fixed or dead cells. Even if it is sometimes problematic it can be advantageous to fix cells prior to analysis. If the cells cannot be processed in enough time the cells can be fixed, but it is important to keep in mind that there are problems associated with fixation. It can increase unspecific binding of the antibodies and also lead to decreased binding of the antibody and can also cause high autofluorescence in the cells. Fixation can lead to epitope masking by changing the structure of the proteins and the fixatives can cause cells to aggregate and stick to the inside of plastic tubes, so there can be a loss of rare cells. To preserve the ultrastructural integrity of the antigens it is important to test different fixatives. For flow cytometry formaldehyde, alcohols or acetones are used, where formaldehyde is a crosslinking agent and alcohols or acetones are precipitating fixatives. Alcohols will permeabilize cells and the nucleic acids can leak out of the cells, thus it is not suitable for DNA analyses. Formaldehyde fixatives will form hydroxymethyl groups and cross-link peptides by reacting with free amino side chains. Formaldehyde is a cross-linking fixative and it is a good fixative for lipids that stabilizes the membrane and the permeability of the membrane will increase. Formaldehyde fixation after immunolabeling serves to cross-link the antibody and the covalently bound fluorophore to the cells, which will preserve the cells and the fluorescent signal\textsuperscript{52}. Krutzik et al. discuss formaldehyde and methanol fixation for flow cytometry\textsuperscript{58}.

For analyzing intracellular components of the cells, either cytoplasmic or nuclear, there is a need to permeabilize the cells. Formaldehyde fixation will lead to some permeabilization of the cells, but additional agents are usually needed. When internal antigens are to be labeled with antibodies permeabilization must be performed before or during the antibody labeling, otherwise the antibody cannot enter the cell. Once the cells are fixated and permeabilized, if the protocol requires, it is time for the labeling of the antigens of interest.

The binding of an antibody to its epitope is a non-covalent binding and the interactions are reversible. Different staining targets require different times to obtain the best staining results. To optimize the protocol one needs to titrate the antibodies and once all parameters are to satisfaction it is time to analyze the sample in the flow cytometer to detect the signal from the fluorescent probe. The fluorophores can be divided into different groups; for example, organic dyes like fluorescein isothiocyanate (FITC) is one of the most commonly used dyes in flow cytometry\textsuperscript{59}. Other examples of fluorophores are the biological fluorophores and quantum dots. A popular biological, organic dye is green fluorescent protein (GFP) which was originally isolated from jellyfish\textsuperscript{60}. The quantum dots are inorganic nanocrystals made from cadmium selenide and zinc sulfide. They have been successfully used in polychromatic analysis\textsuperscript{61}. 
Figure 2. Basic principles of a flow cytometer. The sample runs through the instrument in a fluid and is passed through a light source, often a laser and the resulting scattered light is collected.
Figure 3. Side scatter (SSC) can be used to characterize the cells granularity and forward scatter (FSC) can be used to estimate the cell size.
Chronic myeloid leukemia (CML) is a cancer of the blood. Of all cases of leukemia in adults CML accounts for approximately 15%, with an annual incidence of 1-2 cases per 100,000 worldwide. 95% of the patients that are diagnosed with CML carry the translocation t(9:22)(q34; q11.2) that gives rise to the fusion gene, and translates into the fusion protein BCR-ABL, which is a hallmark of the disease.

In 1960 a minute chromosome was discovered that was later named the Philadelphia chromosome, which was demonstrated in malignant cells of patients with CML. In 1973 it was shown that it is a translocation between chromosome 9 and 22 that gives rise to the Ph chromosome, and the resulting p210 fusion protein BCR-ABL was described in 1986. CML originates in a bone marrow myeloid pluripotent cell that carries the Ph chromosome, and will lead to a chronic phase (CP) of the disease with proliferation of myeloid cells in the blood, bone marrow, and spleen. If the disease is left untreated the next phase is the accelerated phase (AP) or the blast phase (BP), where an accumulation of genetic instability and chromosomal aberrations cause the cells to go into blast crisis where they are unable to differentiate. The diagnosis is most often made in the CP phase, usually after an unexplained leukocytosis, often without symptoms. The patients that do display symptoms exhibit fatigue, weight loss and some pains. These symptoms are usually due to anemia and/or enlargement of the spleen. Today, the disease is diagnosed using a number of molecular methods. Fluorescence in situ hybridization (FISH) is used to demonstrate the presence of the fused gene BCR-ABL, cytogenetics is used to identify the presence of the Ph chromosome, and reverse transcriptase-polymerase chain reaction (RT-PCR) measures the presence of the mRNA transcript of BCR-ABL. RT-PCR can be used qualitatively, to reveal the presence of the transcript, or quantitatively to measure the amount of remaining transcripts after treatment.

After the discovery that BCR-ABL is a constitutively active tyrosine kinase that drives the disease, the treatment changed from nonspecific agents such as interferon alfa (INF-α), hydroxyurea and busulfan, to highly specific tyrosine kinase inhibitors (TKIs). Development of these small molecules, which block the proliferation of the malignant cells by interfering with the BCR-ABL protein and its substrate adenosine triphosphate (ATP), drastically increased the 10-year overall survival from 20 to 80-90%. Once treatment is started the molecular response is measured. Complete cytoge-
Hematologic response (CCyR) is reached when no Ph+ cells can be detected by cytogenetic or FISH analysis. RT-PCR is used to investigate minimal residual disease (MRD), and once the level is down to below 0.1% (international standard - IS) CCyR is considered as having been reached. If the RT-PCR results are negative for the BCR-ABL transcript, the patient is in molecular remission.\textsuperscript{70}
Extracellular vesicles

Our body consists of some $10^{14}$ cells. These cells consist of a lipid cell membrane, a cytoplasm with organelles, a nuclear membrane and a nucleus. Cells from different organs and tissues have widely different characteristics, as a consequence of expressing different sets of genes. All cells communicate with their surroundings; they can interact with neighboring cells or with cells that are further away. Besides through cytokine signaling and endocrine secretion, communication may also occur through EVs. EVs are membrane secreted vesicles that appear to be involved in cell-cell communication and transportation, however many different functions have been suggested, and their true functions are yet to be proven.

It is suggested that there are two classes of EVs that are secreted from the membrane and they can be separated by size. Exosomes are smaller, in the range of 30 – 200 nm in diameter, while microvesicles are larger, up to 2,000 nm depending on their origin. Their biogenesis also differs, where the microvesicles are derived from membrane budding, the exosomes are released through the endolysosomal pathway. In the late 1970s vesicles that were surrounded by a membrane were discovered in human prostatic fluids, and in the early 1980s exosomes were given their name, and since then several different functions of EVs have been suggested. EVs share similar characteristics in size, conserved protein content, and a lipid bilayer that can be visualized when imaging them with transmission electron microscope (TEM). Investigations have identified many proteins expressed in EVs. One group of proteins, tetraspanins i.e. CD9, CD63, CD81, CD82, are commonly used as EV markers.

Many cells release vesicles upon stress, such as in apoptosis and disease. It has been suggested that some types of cancer cause cells to shed an excess of vesicles. Since tumor EVs express similarities together with an array of tumor antigens that could reveal the identity of the originating tumor cell, they are of great interest in biomarker analysis. EVs could potentially be used as biomarkers for different types of cancer, and prostasomes in the plasma may prove a diagnostic tool for prostate cancer. Recently EVs have been identified as potential therapeutic vehicles. There are data that indicates that EVs could be used in both tissue regeneration in cardiac disease and as immune response modulators in cancer. Isolation and detection of EVs is done by, for example, sucrose gradient ultracentrifugation and flow cytometry of exosome coated beads.
Present Investigations

Paper 1: Sensitive Flow Cytometric Detection of BCR-ABL Fusion Protein Positive Cells in Blood from Patients with Chronic Myeloid Leukemia

Introduction
Analysis of neoplastic cells using flow cytometry has been the method of choice by hematologists for a long time. Leukemia is characterized by an abnormal proliferation and/or defective apoptosis of leukocytes. Leukemias are subdivided according to the nature of the proliferating cells. This is done using microscopy and flow cytometry. In many of the different forms of leukemia the diagnosis is made using flow cytometry after staining for different protein markers. One of the advantages of the flow cytometric methods is that the pathologist can inspect the blood cells to see if there are any other abnormalities that could for instance cause a poor response to treatment. It has not been possible to make the final diagnosis in chronic myeloid leukemia (CML), using flow cytometry, since more than one type of leukocytes are involved. The diagnosis is made by PCR or FISH analysis for patients with an excess of white blood cells.

Aim and Procedure
The aim of this project was to use in situ PLA with flow cytometric readout using in situ PLA to detect the fusion protein BCR-ABL in blood from CML patients. The assay was first established using K562 cells, a cell line positive for the BCR-ABL fusion protein, and a negative cell line, U937, for comparison. Once the protocol was developed, blood samples from CML patients were analyzed for the fusion protein, and the results were compared to the routine analysis through RT-PCR. The assay was also combined with immunofluorescence staining, so other markers on the surface of the CML cells could be identified.
Results and Discussion

The *in situ* PLA-based method described here proved highly sensitive and specific, opening up the possibility to stain for other surface markers at the same time. We can now simultaneously analyze surface markers on the cells when we stain for BCR-ABL fusion protein. This allows us to investigate which immune cells express the fusion protein and if we stain for CD34, a stem cell marker, we can investigate whether any cancer cells remaining after a patient has received treatment are stem cells. Also, by staining for surface markers, we can see if a patient that does not respond to one treatment has a different staining pattern compared to ones who do.

Measurement of BCR-ABL protein positive cells in CML patients correlate well to routine RT-PCR analysis, I don’t think our method will replace the established PCR method, but I do believe it can offer an important complement to the existing methods.
Paper II: Detecting individual extracellular vesicles using a multicolor in situ proximity ligation assay with flow cytometric readout

Introduction
Extracellular vesicles (EVs) are a promising next generation class of biomarkers. It is reported that some cancer cells shed more EVs than normal tissues. The EVs exhibit a protein profile similar to that of their originating tissue. Utilizing these two circumstances one could, in theory, use the EVs as biomarkers for different forms of cancer. EVs are often detected in bulk by flow cytometry after capture on beads, but the small size of the EVs represents a challenge for individual detection in conventional flow cytometry. The EVs are difficult to detect over the background noise when using forward scatter. This is because they are smaller than the wavelength of the laser light used. Their refractive index is very close to that of the fluids used in the system. Today, this problem is addressed by using beads and log side scatter to obtain a comparative plot with microvesicles and beads.

Aim and Procedure
We aimed to develop a multicolor in situ PLA method for flow cytometry readout, to detect extracellular vesicles. By using the multicolor version of in situ PLA with flow cytometric readout, we are able to label the EVs with both common and selective markers, and detect them through multicolor flow cytometry. Currently, in our multicolor in situ PLA we utilize four PLA probes, where some are directed against targets that are present on most EVs (the common markers), while other probes are directed against antigens that are only present on one population of EVs (selective markers) in the sample to be analyzed. Using a set of common markers the assay was developed to detect the total EVs in a sample using flow cytometry. When exchanging one of the probes to the selective marker the assay successfully identified one specific population of EVs.

Results and Discussion
We developed a multicolor in situ PLA-based method that we call ExoPLA for detecting EVs, with multi-recognition of up to five antigens present on their surfaces. Using flow cytometry readout we could clearly distinguish the true signals from background noise. The results for detection of a specific class of EVs – prostasomes - were also confirmed using fluorescence microscopy. Using ExoPLA with detection of selective markers together with probes against the common antigens present on the EVs, we can distinguish specific populations of vesicles within a mixed sample of EVs. Using this
method we also selectively identified prostasomes from other EVs in a complex matrix, 10% female plasma.
Paper III: Detergent resistant membranes (DRM) from erythrocyte ghost can vesiculate and form the basis for delivery of pharmaceuticals: A model drawn from experiences with prostasomal DRM

Introduction

Drug delivery systems need to be safe and deliver the drug to the correct “address.” Administration of pharmaceuticals may cause side effects and great efforts are being undertaken to find new drug delivery systems with minimal adverse effects. There are system tested for targeted drug delivery using synthetic vehicles and also vehicles from virus, but they are attacked by the patient’s immune system, which leads to a short half-life of the vehicle. Liposomal drug delivery systems are also used, but there are problems associated with loading the liposomal vehicle and there is sometimes leakage of pharmaceuticals from the vehicle. To circumvent these problems the membranes are usually made synthetically by reconstructing them, changing the bilayer from a liquid to a solid and adding cholesterol and sphingomyelin to the liposomes. The use of a biological vehicle from the patients’ own extracellular vehicles would solve the problem with the immune system. One of the tasks of EVs in the body is to deliver molecules between cells. These features make EVs good candidates for drug delivery vehicles.

Aim and Procedure

The aim of this project was to develop a new method to load EVs or erythrocytes with pharmaceuticals and use them for drug delivery. Prostasomes and red blood cells were used to test the method. Both prostasomes and erythrocytes were emptied and the membranes were washed, after which lipid rafts were isolated. Lipid rafts of both prostasomes and erythrocytes have the capacity to form vesicles. The vesicles formed from lipid rafts were loaded with pharmaceuticals. To test the system the vesicles were loaded with hemoglobin, acridin orange (AO), and doxorubicin. Vesicles from erythrocytes loaded with hemoglobin were tested by western blot to ensure that the loading was successful. Vesicles from prostasomes loaded with AO or doxorubicin were used to investigate the uptake of vesicles and the delivery of the drug on a prostate cancer cell line, PC3. The uptake of vesicles was tested by adding the AO loaded vesicles to the cells, and investigating the delivery of the drug. AO binds double stranded DNA and when bound it emits a green fluorescence. The uptake and delivery of doxorubicin was tested by a proliferation assay on the PC3 cells after adding the loaded vesicles to the cells and counting them after different times.
Results and Discussion
Lipid rafts from both erythrocytes and prostatesomes were able to form vesicles and were successfully loaded with pharmaceuticals. Using transmission electron microscope (TEM) the lipid rafts were visualized and they displayed a horse-shoe like form, suggesting an opening in the membrane. The loading of the vesiculated lipid rafts was confirmed using western blot, with strong bands representing hemoglobin in its both monomeric and dimeric forms. Using the prostate cancer cell line PC3, the uptake of the vesicles was tested in vitro. The green fluorescent light that is emitted by AO when it is intercalated into double stranded DNA could be detected in a fluorescent microscope demonstrating the uptake of the vesicles and delivery of AO to the cells. The proliferation assay on the PC3 cells also confirms delivery of doxorubicin to the cells as cell proliferation stopped completely. The empty control vesicles had no effects on the cells.

There is a great need for safe and direct drug delivery systems that are not attacked by the immune system. Using the patients’ own biological molecules to create such a system would circumvent the challenges of drug delivery systems that are currently in use. We produced a new type of delivery vehicle originating from prostatesomes and erythrocytes that was created during the separation step of lipid raft domains from their respective biological membranes. These lipid rafts vesiculated and loading was possible during the vesiculation step. Our data suggest that these vehicles can be loaded, taken up by cells and deliver their cargo to the cells without being cytotoxic to bystander cells.
Paper IV: Crosstalk between Wnt and Hippo signaling pathways changes upon colon cancer stage and is affected by cell density and loss of or mutated E-cadherin protein.

Introduction

There are a number of proteins and signaling pathways involved in gastric cancer. The Hippo signaling pathway regulates apoptosis and cell proliferation and thereby controls organ growth\textsuperscript{24}. The Wnt pathway is involved in stem cell self-renewal in the gastrointestinal tract\textsuperscript{99}. It has previously been demonstrated that cell density regulates the Hippo signaling pathway\textsuperscript{23}. The Wnt pathway, when in an OFF-state, is regulated by a destruction complex. This complex is composed of several proteins and bind β-catenin, one of the effector proteins of Wnt, targeting it for destruction\textsuperscript{25}. YAP/TAZ the effector proteins of the Hippo pathway has been shown to play an important role in the proteasomal degradation of β-catenin\textsuperscript{100,101}. E-cadherin is a protein that is found in cell junctions, and sometimes form complexes with β-catenin. These complexes can be affected in patients with gastric cancer that have a mutated E-cadherin protein. No previous work has reported whether mutated E-cadherin is affecting the crosstalk between Hippo and Wnt signaling.

Aim and Procedure

In this paper we wanted to investigate the crosstalk between the two signaling pathways, Wnt and Hippo. The cell lines were influenced in various ways to investigate the effects on protein localization and the protein interactions involved in the crosstalk between the two signaling pathways, analyzed using \textit{in situ} PLA. The different parameters investigated for their effect on signaling were cell density, Wnt treatment, different E-cadherin mutations and the complete absence of E-cadherin.

Results and Discussion

When looking at the cellular localization in different colon cancer tumor stages we found differences in TAZ/β-catenin interactions. In sparsely growing HEK293 cells there was a great increase in complex formation of the protein interactions involved in the crosstalk. Since cell density plays a role in the Hippo/Wnt crosstalk we further investigated this. We transfected CHO cells, cell that do not naturally express E-cadherin, with E-cadherin mutations and wild type E-cadherin. The mutations and cells without E-cadherin affected the part of the crosstalk that involves the formations of protein complexes. E-cadherin is important in the maintenance of epithelial integrity.
and the mutations, when found in hereditary diffuse gastric cancer, cause a more invasive cancer\textsuperscript{102}. Our results indicate that Hippo/Wnt crosstalk is regulated by E-cadherin dependent cell-cell adhesion.
Paper V: Proximity-dependent initiation of hybridization chain reaction

Introduction

Methods that utilize enzymes tend to be expensive, and they require low-temperature storage, and usually different temperatures during the assay. *In situ* PLA is an expensive method since it utilizes two different enzymes; T4 ligase and phi29 polymerase. T4 ligase is used to ligate the circularization oligonucleotides to create the circular template that is used for amplification. The phi29 polymerase is in charge of the amplification. A method that does not require enzymes could be less expensive but also more robust when it does not require an enzyme for amplification.

Aim and Procedure

The aim of paper V was to create a method that does not require any enzymes. To actualize this the hybridization chain reaction (HCR)\(^{103}\) was used to replace the enzyme-demanding RCA reaction. We combined HCR with a proximity assay. Four self-hybridizing oligonucleotides and one linear initiator oligonucleotide were designed. After optimization of the design of the oligonucleotides to avoid false positive reactions, two of the four self-hybridizing oligonucleotides were conjugated to antibodies and used as proximity probes against targets of interest. The other two self-hybridizing reagents are fluorophore-labelled oligonucleotides and bind the reporter DNA molecule built up from proximal pairs of proximity probes once the initiator binds and opens up the hairpin structure of the binding oligonucleotides. The signal was tested using both fluorescence microscopy and flow cytometry.

Results and Discussion

After designing the oligonucleotides mismatches were introduced during optimization to avoid false positives. The binding of the hairpin structured oligonucleotides to the proximity probes creates a starting point for the build-up of a long reporter molecule where oligonucleotide after oligonucleotide hybridize once the initiator is added and opens up the hairpin structure to expose the oligonucleotide sequence so they can hybridize. The reporter molecule carries several fluorophores, which creates a strong detectable signal that visible by both fluorescence microscopy and flow cytometry. The assay was evaluated and compared to *in situ* PLA and several protein interactions were successfully investigated. ProxHCR can be used to measure protein interactions and phosphorylations, and combined with flow cytometry. Since proxHCR does not require enzymes it will reduce the assay cost.
Conclusion and Future Perspectives

According to the Swedish cancer society the number of cancer patients will increase by 90 percent by the year 2040. 100,000 Swedish people will receive a cancer diagnosis in that same year. The cost for society will increase by 50 percent from present levels. There is a solution – and the researchers in the Swedish cancer society are all in agreement – more research and more money to research. There is not all bad news, however: with better methods to diagnose and treat the cancer the number of patients that live with their disease for ten years will also increase by 50 percent by year 2040.
I am happy that my research is in this field, developing new methods and making applications in the field of cancer research.

The method we developed in paper I can be used to simultaneously detect other types of proteins using IF. This opens up for more rigorous research on CML patients and personalized medicine. It gives us an opportunity to investigate each CML patient’s cell populations and see how they change over time with or without treatment. This could give a clue who will fail treatment and who will not. Moving forward with this method could be to combine PLA with mass cytometry\textsuperscript{104}, which could increase the analyzed parameters. Cytometry time-of-flight (CyTOF) does not use fluorophores, but metals, and are not limited by overlap between emission spectra by the fluorophores, and in the future potentially up to a 100 different labels can be detected.

The potential of the method in paper II is also interesting; it can detect and identify EVs in a complex matrix, like human blood plasma. With the knowledge that cancer cells may shed EVs\textsuperscript{84}, that cancer tumors can leak cancer cells into the blood and since cells express the protein pattern of their originating tissue we could, with this method, potentially, find EVs in the blood, which would indicate that the patient suffers from cancer.
EVs have potential not only as biomarkers, it can also be used for drug delivery. It has been previously shown that EVs can be loaded with small interfering RNA and delivered to cells\textsuperscript{89}. We present an additional method of loading vesicles and deliver pharmaceutical to cells. The use of EVs and their membranes reduces the immunological response of the patient, which thus reduces the side effects.
In situ PLA is the method that I have mostly worked with in my research presented in this thesis. So far this method is mostly used in research. With the methods I have presented in papers I and II we move some steps closer to the clinic. But there are still some problems associated with these methods in particular and affinity based methods in general.

As I have mentioned before, our methods are no better than the antibodies we use. First we have to find the right ones, and make sure they work for the task we want them for. Then we modify them by conjugating to oligonucleotides. This is a challenging treatment for antibodies and it might affect their affinity. There are a number of problems associated with conjugations. When we are using primary amines for our coupling to the protein we cannot control how many oligonucleotides are bound to the antibody. The oligonucleotides could also bind to the antigen-binding site and this would affect its affinity. This is also true for connecting fluorophores to antibodies\textsuperscript{105}. This could also affect their efficiency of binding target proteins. This can give a batch-to-batch difference in binding efficiency of conjugated antibodies, which demands much testing before the conjugated antibody may be applied in the clinic. The SH-groups could be better targets for conjugation than then NH$_2$- groups since they are not as numerous. One other solution is to choose other types of binders, which are not antibodies. For example recombinant binders that are smaller and many of them lack the structural components of an antibody. This could make them more stable during conjugation.

Protocols that requires fixation can also be a source of instability of the method. Protocol that uses formaldehyde as fixation needs to be optimized with care since formaldehyde denature protein, it could change the confirmation of the proteins or bind directly to the antigen binding site and change the efficacy of the antibody\textsuperscript{106}.

There is no method that has it all. We all wish for a method for detecting all targets down to single molecules, generating no false positives or negatives, and giving the same results each time. There is always something more to wish for. But we are making progress in advancing these methods; we are detecting diseases earlier, and are delivering drugs more safely, with fewer side effects. One day we will be able to lay down the entire puzzle for every patient.
Acknowledgements

My work presented in this thesis was performed at the Department of Immunology, Genetics and Pathology at Uppsala University, Uppsala, Sweden. Financial support was provided by the European project DiaTools.

I would like to start giving my gratitude towards my faculty opponent Prof. J. Paul Robinson, and my examination board Assoc. Prof. Taija Mäkinen, Prof. Sören Lehmann and Prof. Esbjörn Telemo. Thank you all for taking the time to read my thesis and coming to Uppsala to discuss my work.

I would like to extend my greatest gratitude towards my supervisor Masood Kamali-Moghaddam. It has truly been a pleasure being a part of your group the last couple of years. Thank you for letting me steer my own projects, and being there for me when I needed it. Thank you for listening to me, and discussing with me with you endless optimism. And most of all, thank you for believing in me!

Thanks also to my co-supervisor, Ulf Landegren. Thank you for letting me be a part of your amazing research group. You have a way of putting the best people together and creating a great work environment. Thank you for all the discussions over the years, and teaching me to listening and believing in myself.

Another person that has played a huge part in my work is Ola Söderberg, thank you for always having your door open, and letting me be a part of your group. Thank you for all the discussions about methods, cytometry and everything else.

Thank you to all amazing people, that without your help this work had not been possible; Ulla Strömberg, thank you for your always smiling face and that you always are positive when all I see is dark clouds in the sky. And thank you for all the time you have spent moving the projects forward. Joakim Dahlin, if your head had not popped up above the FACS that day I would not be where I am today. Thank you for all your help, and your patience with answering my questions and sharing sushi late nights by the flow cytometer.

Thank you, Joakim Klar for all your help and your time.
Thank you to all present and former members of the Kamali-Moghaddam’s group. Lotta, you are a true friend, always listening to me and giving me good advice and the best dinners. Felipé, thank for good company in Alpbach, that you always make me laugh and you are the best hugger. Jun-hong for being so helpful, Di for your dance moves, Sophie for being so kind and for all the good candies. Thank you Reddy for your efforts in the lab, Maria H for your patience and being a good roommate, and Spyros, Rachel, Gucci and Johan V for giving me such a good start in the lab. A special thanks to my students that I had the privilege to meet over the years; Olivia, Emma, Julia, Nick, Dahnu and Vasiliki thank you all for your hard work.

I have truly been lucky to be in a great lab, with amazing colleagues and friends. Linda you are such a friend, and travel companion. You are so helpful and funny. You are such an inspiration in all that you do. Karin G thanks for all the fun times and good food in Florida. Rasel, I really enjoy our work together and our talks about life. Thank you Elin for always keeping me going, with either chocolates or good advice. Johanna thanks for all your help and all your cookies. Christina, thank you for all the talks and drinks, I miss you! I also miss Marco, thank you for being you. Caroline, thank you for always helping me and answering my questions and being a good friend and listener. Joakim G for nice discussions, Erik for always being there when I need to talk, Marcus for your positive attitude and nice discussions, Andries for your endless energy and giving me so many new ideas, Lei for your patience in helping me understand sRCA, Tonge, you are one of a kind! Thank you for introducing wonderful foods and always being there for me when things were hard, David for all the discussions, Dorotheya for your smile, and the talks by the microscope, Johan H for always talking and making me smile, Peter I really enjoy our talks in the cell lab, and that you give me ideas what to eat for dinner, Hongxing for all the encouraging words, Johan B for teaching me about hybridizations and being such good company during lunch. Your lunch boxes beat it all! Thank you Björn for all the laughs and I really miss you bugging me. Just ask and I will lick your lab bench anytime! Johan O, you are amazing, without your skills I would not have a thesis, Carl-Magnus for Snow White, Pathu for all the interesting discussions, Radiosa you are so sweet and caring, Agata for your sense of fashion and all the talks about the good stuff, Camilla for being so nice and helpful, Gaelle for the nice evenings with wine and cheese, Axel you are the best lab bench neighbor I could ask for, and thanks for your PBS, TBST, EtOH… and that you put up with me. Lisa C, thank you for all the fun times by the FACS, even though nothing worked we had fun. Anna T, Jennifer, Simon, Pan thanks all for making the lab a fun place to work at. Ben thank you for helping me with R and for cheering me up.
Thank you Christina M for everything that you have helped me with over the years, your endless energy and that you always care. Tuulikki, thank you for always being helpful and for all the nice talks.

To the Stockholm crew, Mats thank you for all the nice discussions and laughs, Malte and Anja you were such good office mates. I miss you both, Elin L for the statistics, without you I would still sitting there crying, Ronquin, for the good teas, Lotte, for the help with presentations, Annika for good travel company in Paris, Thomas for remanding us what is important in life, Thomasz for helping out with the padlocks, Tagrid for always smiling.

The other Stockholm crew at KI that I have had the privilege to work with, Petter Brodin thank you for letting me spend time in your lab and for taking time to answer my questions. Kanth thank you for all the fun times by the CyTOF, I have really enjoyed every second. Axel thanks for all the help.

Thank you to the wonderful research nurses, Carina, Margareta and Emeilia. You are the best! Thank you Göran for all your help and for always being positive, Louise for all the great talks and fun time in the cell lab, Anders L for always answering your phone, Ingrid T for all your help with flow cytometry and getting me started in my projects, I could not have done it without your help, Richard R for always being positive and believing in the projects.

Thank you to all my friends and my family. I am grateful to have many friends and people who have helped me or influenced me over the years, thank you all! Mi you are the sweetest person I know, Nanna you are always happy and taught me what is important in life, Sofia the sushi nights need to be more frequent, Evelina wow, you are such a good friend and your baking skills will never be forgotten, I love you. Fredrik your jokes are the best and I am happy that you finally almost look your age, its only like 20 years left…. Elvira my wonderful friend, I love you, Gry there are not enough words in the dictionary for me to express how much you mean to me, you have helped me through so much, talking to you always make me feel better, and finally you are back for some more champagne! Per, Magnus and Toffe I miss you guys! Danne, you are an inspiration. Emelie and Kina you are always so supporting and such wonderful friends, Karin S I love our talks, and I miss them, Kicki Cool and Ejkej you two are the best, you always make me laugh and you have solutions to every problem, either if it is problems with neighbors or CAS numbers. Malin thank you for always being so sweet, Hanna you are a wonderful friend, thank you for convincing me that I could do this!
Mats S, thank you for everything that you taught me, Helene H thank you for believing in me. Maria Ferletta, you were my first supervisor, thank you for all that you taught me.


Dawn, Andy, David, Becky, Ali. I love you all so much, thank you all for loving me back!

Siv, Stefan, Maria tack för allt ni gör för oss.


Thank you all that helped me get through these last couple of months, visiting me at the hospital, sending flowers, buying slippers or just thinking about me. You made all the difference!
References

6. Mukherjee, S. The emperor of all maladies: a biography of cancer. (Scribner, 2010).


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)