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High Content Analysis of Proteins and Protein Interactions by Proximity Ligation

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Abstract

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Fundamental to all biological processes is the interplay between biomolecules such as proteins and nucleic acids. Studies of interactions should therefore be more informative than mere detection of expressed proteins. Preferably, such studies should be performed in material that is as biologically and clinically relevant as possible, i.e. in primary cells and tissues. In addition, to be able to take into account the heterogeneity of such samples, the analyses should be performed *in situ* to retain information on the sub-cellular localization where the interactions occur, enabling determination of the activity status of individual cells and allowing discrimination between e.g. tumor cells and surrounding stroma. This requires assays with an utmost level of sensitivity and selectivity.

Taking these issues into consideration, the *in situ* proximity-ligation assay (*in situ* PLA) was developed, providing localized detection of proteins, protein-protein interactions and post-translational modifications in fixed cells and tissues. The high sensitivity and selectivity afforded by the assay's requirement for dual target recognition in combination with powerful signal amplification enables visualization of single protein molecules in intact single cells and tissue sections.

To further increase the usefulness and application of *in situ* PLA, the assay was adapted to high content analysis techniques such as flow cytometry and high content screening. The use of *in situ* PLA in flow cytometry offers the possibility for high-throughput analysis of cells in solution with the unique characteristics offered by the assay. For high content screening, it was demonstrated that *in situ* PLA can enable cell-based drug screening of compounds affecting post-translational modifications and protein-protein interactions in primary cells, offering superior abilities over current assays.

The methods presented in this thesis provide powerful new tools to study proteins in genetically unmodified cells and tissues, and should offer exciting new possibilities for molecular biology, diagnostics and drug discovery.

Keywords: in situ, proximity ligation, flow cytometry, high content screening, rolling circle amplification, in situ PLA, single-cell, single-molecule, protein interactions, drug screening, post-translational modifications

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To my family

"Nature composes some of her loveliest poems
for the microscope and the telescope"

Theodore Roszak

List of Papers

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

I Söderberg O.*, Gullberg M.*, Jarvius M.*, Ridderstråle K., **Leuchowius K.J.**, Jarvius J., Wester K., Hydbring P., Bahram F., Larsson L.G., Landegren U. (2006) *Direct observation of individual endogenous protein complexes* in situ *by proximity ligation.* Nature Methods, **3**(12): 995-1000. **equal contribution*

II Jarvius M.*, Paulsson J.*, Weibrecht I.*, **Leuchowius K.J.**, Andersson A.C., Wählby C., Gullberg M., Botling J., Sjöblom T., Markova B., Östman A., Landegren U., Söderberg O. (2007) In situ *detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method.* Mol Cell Proteomics, **6**(9):1500-9. **equal contribution*

III **Leuchowius K.J.**, Weibrecht I., Landegren U., Gedda L., Söderberg O. (2009) *Flow cytometric* in situ *proximity ligation analyses of protein interactions and post-translational modification of the epidermal growth factor receptor family*. Cytometry A, **75**(10):833-9.

IV **Leuchowius K.J.**, Jarvius M., Wickström M., Rickardson L., Landegren U., Larsson R., Söderberg O., Fryknäs M., Jarvius J. (2010) *High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation*. Mol Cell Proteomics, **9**(1):178-183.

Related Work by the Author

Peer Reviewed Research Articles

Berglund M., Thunberg U., Fridberg M., Wingren A.G., Gullbo J., **Leuchowius K.J.**, Amini RM., Lagercrantz S., Horvat A., Enblad G., Söderberg O. (2007) *Establishment of a cell line from a chemotherapy resistant diffuse large B-cell lymphoma.* Leuk Lymphoma, **48**(5):1038-41.

Boström H.*, **Leuchowius K.J.***, Hallböök H., Nordgren A., Thörn I., Thorselius M., Rosenquist R., Söderberg O., Sundström C. (2008) *U-2973, a novel B-cell line established from a patient with a mature B-cell leukemia displaying concurrent t(14;18) and MYC translocation to a non-IG gene partner.* Eur J Haematol, **81**(3):218-25. **these authors contributed equally*

Vermeer M.H., van Doorn R., Dijkman R., Mao X., Whittaker S., van Voorst Vader P.C., Gerritsen M.J., Geerts M.L., Gellrich S., Söderberg O., **Leuchowius K.J.**, Landegren U., Out-Luiting J.J., Knijnenburg J., Ijszenga M., Szuhai K., Willemze R., Tensen C.P. (2008) *Novel and highly recurrent chromosomal alterations in Sezary syndrome*. Cancer Res, **68**(8):2689-98.

Wensman H., Göransson H., **Leuchowius K.J.**, Strömberg S., Pontén F., Isaksson A., Rutteman G.R., Heldin N.E., Pejler G., Hellmén E. (2009) *Extensive expression of craniofacial related homeobox genes in canine mammary sarcomas*. Breast Cancer Res Treat, **118**(2):333-43.

Review Articles

Söderberg O., **Leuchowius K.J.**, Gullberg M., Jarvius M., Weibrecht I., Larsson L.G., Landegren U. *Characterizing proteins and their interactions in cells and tissues using the* in situ *proximity ligation assay*. Methods 2008;**45**(3):227-32.

Weibrecht I., **Leuchowius K.J.**, Clausson C.M., Conze T., Jarvius M., Howell W.M., Kamali-Moghaddam M., Söderberg O. *Proximity ligation assays: a recent addition to the proteomics toolbox.* Expert Rev Proteomics 2010, *in press*.

Book Chapters

Söderberg O., **Leuchowius K.J.**, Kamali-Moghaddam M., Jarvius M., Gustafsdottir S., Schallmeiner E., Gullberg M., Jarvius J., Landegren U. *Proximity ligation: a specific and versatile tool for the proteomic era*. In Genet Eng (N Y) 2007;**28**: 85-93.

Leuchowius K.J., Söderberg O., Kamali-Moghaddam M., Jarvius M., Weibrecht I., Pardali K., Landegren U. *Protein diagnostics by Proximity Ligation: Combining Multiple Recognition and DNA Amplification for Improved Protein Analyses*. In Molecular Diagnostics 2009, Patrinos G.P., Ansorge W.J. (eds). Elsevier/Academic Press, Burlington, CA, pp. 299-306.

Contents

Abbreviations

BiFC	bimolecular fluorescence complementation
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CD	cluster of differentiation
CFP, GFP, YFP	cyan, green and yellow fluorescent protein
Co-IP	co-immunoprecipitation
DARPin	designed ankyrin repeat protein
DNA	deoxyribonucleic acid
EGF(R)	epidermal growth factor (receptor)
FACS	fluorescence activated cell sorting
FRET	Förster resonance energy transfer
HCS	high content screening
HRP	horse radish peroxidase
IF	immunofluorescence
IgG	immunoglobulin G
IHC	immunohistochemistry
MS	mass spectrometry
MSR	minimum significant ratio
PDGF(R)	platelet-derived growth factor (receptor)
PLA	proximity ligation assay
PMT	photomultiplier tube
PTM	post-translational modification
RCA	rolling circle amplification
RCP	rolling circle product
RNA	ribonucleic acid
SNR	signal to noise ratio
TAP	tandem affinity purification
Y2H	yeast two hybrid

Introduction

The living cell is a truly remarkable entity. In many ways it resembles a large city; instead of streets and buildings it has structural proteins, instead of cars and trucks it has transporter proteins, and instead of factories it has ribosomes (using RNA as the blueprint for what proteins to manufacture), etc. To fully understand how the city works, it is imperative to know not only its components, but also how the components function and how they interact with each other. Otherwise, how could we possibly understand what for example automatic teller machines are for, or why people go to them to receive pieces of colored paper, then go around the city, carefully distributing these pieces of paper to different locations? Analogously, how can we ever hope to understand a disease such as cancer if we do not know the function of the relevant proteins, or how their altered behavior affects their interactions with other biomolecules?

The challenge of studying the function of the cell lies in its enormous complexity and range of proteins and other molecules. Just like in the city where some parts (say, red-nosed clowns) are much less common than other parts (lawyers), the number of copies of a certain protein in the cell can vary from just a few (e.g. receptors and transcription factors) up to hundreds of millions (structural proteins). To further complicate matters, proteins can exist in many different variants as a consequence of alternative splicing of gene transcripts, and because of post-translational modifications (PTMs) such as phosphorylation, glycosylation, ubiquitination, acetylation, etc. All of these different changes can influence not only the function of the proteins but also their choice of interaction partners, and are thus of great importance for an improved understanding of the cellular processes.

To address the challenges of studying proteins and protein interactions, several methods have been developed, each with their own advantages and disadvantages. These methods can loosely be divided into three broad categories: methods in which the proteins are modified by the introduction of a new protein domain to enable detection, methods in which proteins are studied indirectly by using reagents that bind to them, and methods in which the physical properties of the proteins are studied directly, without the need for modifications or binding reagents. In this introduction, I will try to give a brief overview of some of the commonly used methods from these categories as used for *in situ* analyses. But first I will try to explain why I believe proteins should be studied *in situ*, i.e. directly in cells and tissues.

Seeing is believing – analyses of proteins *in situ*

There is a wide range of methods for analyzing proteins *in vitro*, from classical methods such as ultracentrifugation and gel separation to the use of different kinds of biosensors. What many of these techniques have in common is that they provide ensemble measurements, i.e. measurements that are based on an average of a large number of molecules, usually from homogenized cells or tissues, or from purified proteins. This is required to obtain robust signals that can be distinguished from background noise, and is usually sufficient in many applications. There are also *in vitro* assays that can be used to study purified single molecules, for example by using atomic force microscopy [1], but these assays put very high demands on the sample preparation and the quality of the measurements to obtain reliable results.

The problem with averaged measurements lies in the heterogeneity of biological systems: not all cells are created equal. In the case of cancer, for example, there can be a large difference in the expression of certain proteins between malignant cells and surrounding stroma. With averaged measurements such important differences could be overlooked because they would be averaged out. Also, the expression profiles of rare cells could go unnoticed, since they would be drowned in the cacophony of protein expression from cells more common. There is the possibility of isolating cells of interest from surrounding tissues by e.g. laser capture microdissection [2], and then perform averaged measurements of the enriched cells, but this is very laborious. Any information on sub-cellular localization of proteins would also be lost in the process.

Instead, *in situ* methods can provide *localized* detection, allowing measurements to be performed directly in cells and tissues, revealing any heterogeneity among the cells. This can help to identify important differences between different cell types, and enable studies of individual cells in the context of their natural microenvironment. The challenges of *in situ* analyses are the difficulties of obtaining robust and sensitive measurements that are also selective, i.e. can distinguish between the intended target molecule and the surrounding myriad of other molecules. Studies *in situ* should be able to provide more biologically *relevant* data than other *in vitro* studies do, since cells and tissues can be preserved morphologically, ensuring that proteins and other biomolecules remain in a cellular environment that resembles *in vivo* conditions more closely.

Of course, to obtain the most biologically relevant data, studies should ideally be performed *in vivo*, i.e. in living cells or intact animals or humans. However, *in vivo* analyses may not be feasible for diagnostic investigations, and they can be extremely technically challenging and sometimes raise serious ethical issues, limiting their application in practice. *In situ* analyses provide a good middle-ground between the relative simplicity of *in vitro* assays and the biological relevance of *in vivo* analyses.

Fuse them, then use them – genetically encoded detection moieties

Proteins can be studied by incorporating a detectable tag, i.e. by fusing the gene encoding the protein of interest with a sequence encoding a detectable protein domain and a suitable promoter into a DNA vector. This construct can then be introduced into cells, causing the fusion protein to be expressed. The tag to be incorporated depends on the type of application. For example, by joining the protein of interest with a fluorescent protein tag such as the green fluorescent protein (GFP), cloned from the jellyfish *Aequorea victoria*, the sub-cellular localization of the protein can be studied in transformed cells and tissues, both living and fixed (reviewed in [3]).

It is also possible to genetically include a much smaller, non-fluorescent tag in the protein, and then detect the protein by adding small fluorescent molecules that covalently bind the introduced tag [4]. The red and green fluorescent dyes FlAsH and ReAsH are examples of such molecules, targeting a 12-residue peptide sequence. Other examples include the 20 kDa SNAP and CLIP tags, based on mutated versions of the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase, that react specifically with benzylguanine (BG) and benzylchloropyrimidine (CP) to create a covalent bond between the tag and the small molecular substrate [5]. By attaching a fluorescent label to the substrate, the location and dynamics of the tagged protein can be visualized.

Although these techniques can yield valuable information on single proteins, more elaborate techniques are needed in order to study how proteins interact with each other or with other macromolecules inside cells. One common extension of the use of fluorescent protein tags to label proteins is the use of the Förster resonance energy transfer (FRET) effect [6] to measure protein-protein interactions. To achieve this, two different fluorescent proteins, such as the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), can be used to tag two potentially interacting proteins (Figure 1a) [7-9]. If the two fluorescent protein tags are brought into close proximity, typically within ten nanometers since the energy transfer is inversely proportional to the sixth power of the distance, and have the correct orientation, the absorbed energy of the light-excited “donor” CFP can be transferred radiation-less to the “acceptor” YFP. The “acceptor” fluorescent protein, excited by the transferred energy, then emits light at the longer wavelength typical of YFP. When this shift in emission is observed, one can surmise that the two proteins interact, are in the same complex, or at least are very close to each other. If the protein modified by the addition of the YFP tag is not expressed, or if the two fluorescent tags are not close enough, light will only be emitted by CFP and not by YFP. FRET-based detection can also be applied to study interactions between proteins and nucleic acids [10-11].

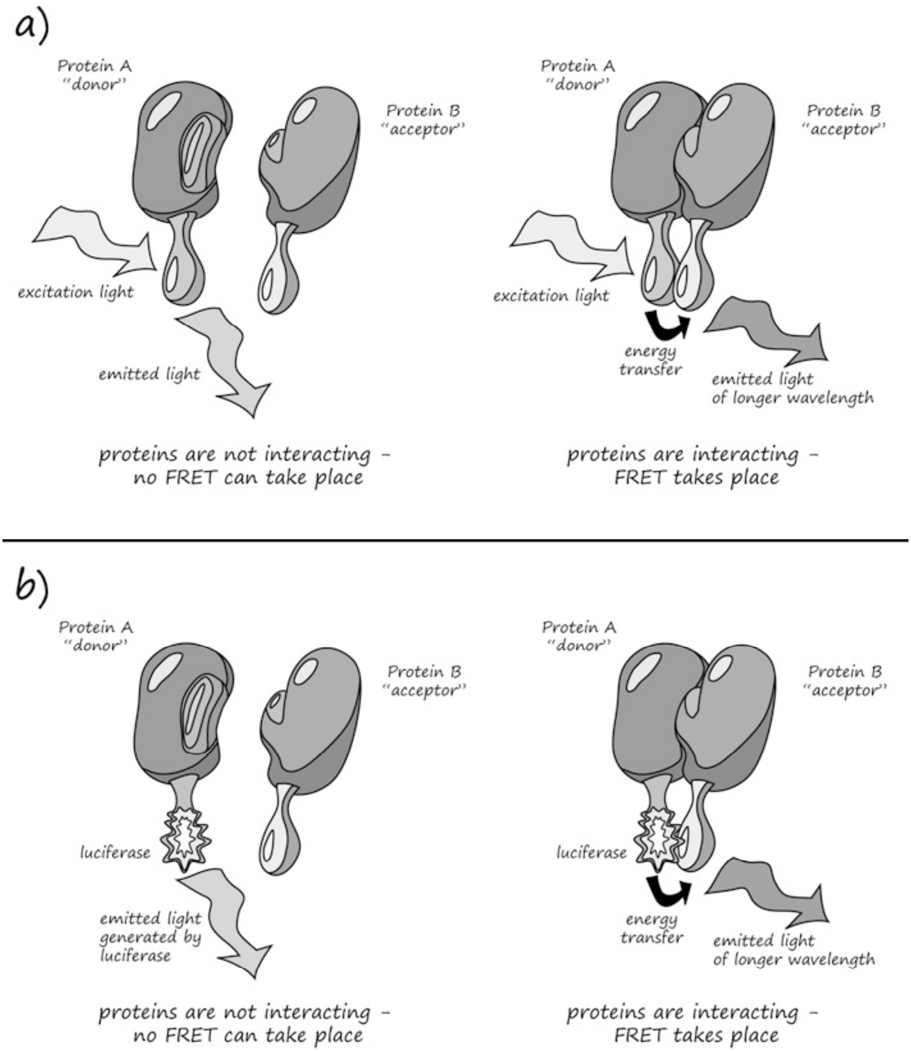

Figure 1. FRET-based detection. FRET can be used to detect protein-protein interactions by tagging potentially interacting proteins with fluorescent proteins such as CFP and YFP (**a**), or by tagging one protein with luciferase and the other protein with a fluorescent protein (**b**).

Bioluminescence resonance energy transfer (BRET; Figure 1b) is a variant of fluorescent protein FRET in which the fluorescent tag of the donor has been replaced by luciferase [12-13]. The light-producing luciferase is used as the energy donor for the energy transfer to the fluorescent protein, and thus no external light source is required for the FRET reaction. This reduces the problem with background autofluorescence and undesired direct excitation of the acceptor by external light.

Bimolecular fluorescence complementation (BiFC; Figure 2a) also uses a fluorescent tag to visualize protein interactions, but instead of detecting FRET between two different fluorescent proteins, only one fluorescent protein is used. The fluorescent protein is split into two parts with the non-fluorescent N-terminal and C-terminal parts fused genetically to two different proteins [14-15]. If these two proteins interact, then the two fragments of the fluorescent protein can be brought together and reassemble into a functional fluorescent protein. For the fluorescent protein to become fully functional it needs a certain time for maturation, typically one hour; therefore, rapid dynamics cannot be studied using this technique. Instead of using fluorescent proteins, fragments of a split enzyme such as horse radish peroxidase (HRP) can be used to catalyze a colorimetric reaction that can be read out by other means than fluorescence-based detection. BiFC can also be combined with FRET to enable studies of ternary protein complexes [16].

Another widely used method for elucidating protein interactions with fusion proteins is the yeast two hybrid (Y2H) assay, in which the DNA binding domain of a transcription factor is fused to one protein and a transcription activating domain is fused to another protein, restoring the function of the transcription factor only if the two proteins interact (Figure 2b). Thus, if the two proteins interact, the expression of a reporter gene is induced. Although Y2H assays have been proven useful to screen massive numbers of protein interactions, they tend to give a high number of both false positive and false negative identifications [17]. They also do not provide any information about the subcellular localization or the dynamics of the protein interactions.

An advantage of using tags to study proteins in cells is that the labeling of the proteins is inherently very specific, since only the expressed fusion proteins contain the introduced tag. An even greater advantage is that live cells can be studied with many of these techniques, since the fluorescent protein-tagged fusion proteins are detectable by fluorescence microscopy without the need to fix and permeabilize the cells (reviewed in [7]), reducing the risk for artifacts introduced by fixation [18]. This also means that the dynamics of the protein interactions can be studied in real time in living cells, without having to resort to the use of time series of cells fixed at different time points, subject to the relatively slow maturation of the fluorescent proteins upon translation, as mentioned earlier [19].

On the other hand, a limitation of methods involving fusion proteins is that artificial DNA-constructs must be introduced and expressed in the cells, which means that clinical material such as cancer tissues cannot be studied with these methods. In addition, fusion proteins are generally not expressed at the endogenous level, and the properties of the fusion proteins may differ from those of the endogenous proteins. This can lead to changes in the protein's subcellular localization and its pattern of interactions, so controls are needed to verify that the modified protein faithfully represents the properties of the endogenous protein.

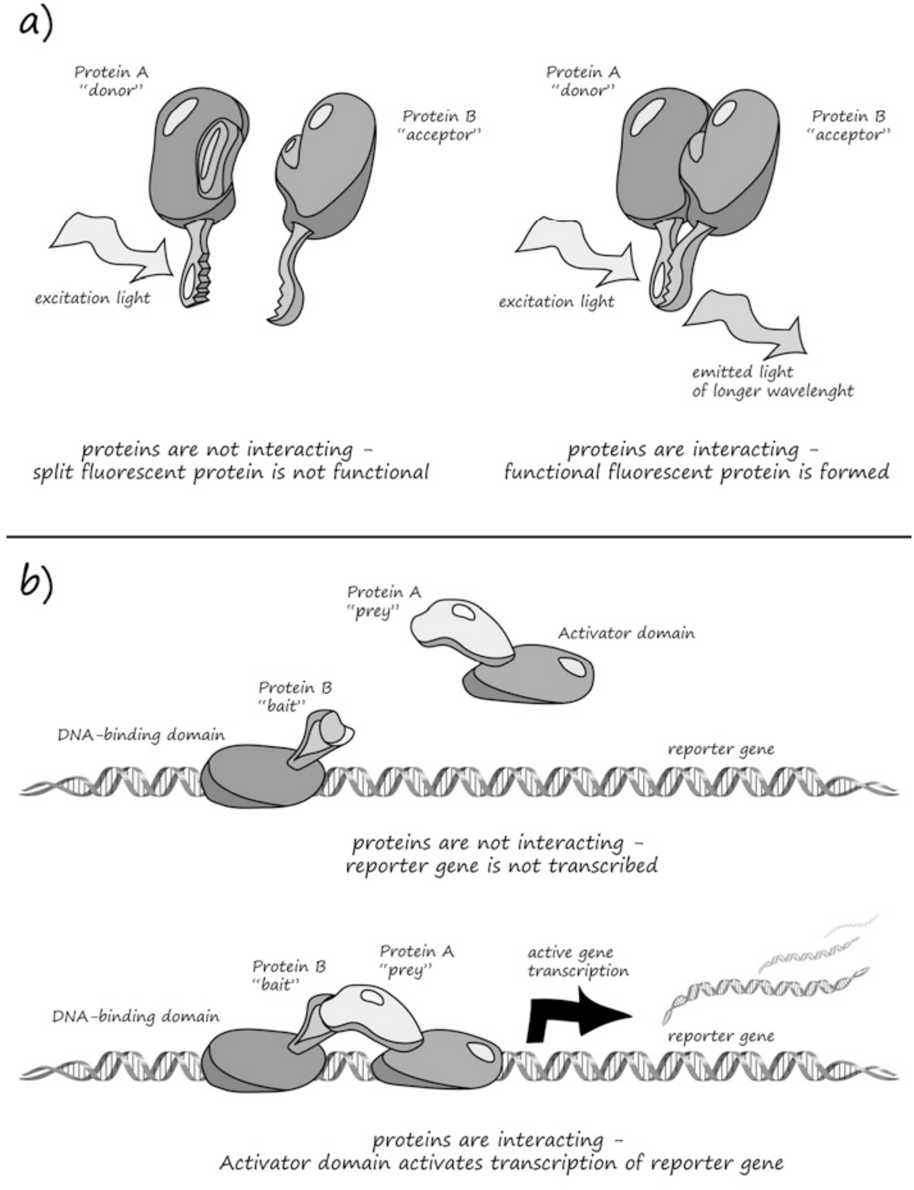

Figure 2. (**a**) Interactions between proteins can be measured through the use of split fluorescent proteins that can form functional fluorescent protein upon interaction of the tagged "donor" and "acceptor" proteins. (**b**) Another common method for measuring protein-protein interactions is with the yeast two hybrid assay, where transcription of a reporter gene can be activated as a result of the interaction between the "bait" and the "prey" proteins.

Look but don't touch – label-free detection

This category of protein detection methods makes use of sensitive instruments such as mass spectrometers and different types of biosensors to study proteins and other biomolecules without the need for fluorescent labels or other molecular tags. A benefit of using label-free detection methods is that the risk of possible disturbances of the biological system by an introduced label can be avoided. Unfortunately, label-free detection methods are plagued by problems of selectivity, especially when complex biological materials, such as whole cell extracts or sera, are analyzed. Without any selection or amplification it is difficult to study low abundant proteins when the difference in concentration between the most abundant proteins and the protein of interest can vary by many orders of magnitude. To overcome this problem of complexity, mass spectrometry is almost always combined with a preceding complexity reduction step in which interesting proteins are isolated by for instance 2D gel electrophoresis or liquid chromatography. In addition, affinity reagents such as antibodies can be used to fish out proteins and protein complexes of interest from the molecular soup before analysis [20].

When studying interactions between proteins, mass spectrometry can be used in conjunction with for instance tandem affinity purification (TAP)-tag purification [21], in which a dual peptide tag is fused to the protein of interest. This dual tag is then used in a two-step purification scheme after which the proteins that are attached to the modified protein can be analyzed by mass spectrometry. Obviously, since the TAP-tag method requires two protein purification steps, it is not applicable to study weak or transient interactions unless the proteins have been cross-linked.

The benefits of mass spectrometry includes the possibility to study biomolecules for which no binders are available, and that it can be used to study the proteome in a more unbiased way, since all the proteins in the sample can be detected, not just the ones that the researcher chooses to look for. In addition, it is also a powerful method for studying post-translational modifications (reviewed in [22]).

Recent advances in mass spectrometry include instrumentation and procedures for localized detection using so-called imaging mass spectrometry, but so far the resolution remains greatly inferior to methods based upon optical microscopy, and intact proteins are difficult to identify [23].

There are many other kinds of label-free detection systems, many of which are based on optical measurements, for example using surface plasmon resonance imaging [24] or stimulated Raman scattered microscopy [25]. There are also assays that measure electrical impedance changes [26]. Although these assays can be useful for measuring the cellular distribution of small molecules or studying cellular morphological changes upon stimulation or drug treatment, they cannot be used to study proteins selectively or perform studies of protein-protein interactions, at least not *in situ*.

Finding by binding – affinity reagent-based detection

Proteins can be detected through the use of different kinds of affinity reagents (binders) that bind the proteins of interest. The most commonly used binders are antibodies, either monoclonal produced in hybridoma cell lines, or polyclonal produced in animals such as rabbits and goats. There are several subclasses of antibodies, e.g. IgM and IgG, with IgG as the one predominantly used. The basic structure of IgG consists of two large heavy chains and two smaller light chains. Both the heavy and the light chains can be divided into two parts: a constant region and a variable region. The constant region of the heavy chain determines the antibody's isotype, while the variable regions of both chains mediate the binding specificity of the antibody. In the host animal, recombinations of the immunoglobulin genes in the antibody-producing cells generate a vast repertoire of unique binders that are then selected on the basis of their ability to bind their antigens. The affinity of the selected clones is further enhanced by gene conversion or somatic hypermutation.

For certain proteins it is not possible to raise antibodies, e.g. when the protein is too toxic to the host animal. In these cases other approaches such as single-chain fragments, DNA- or RNA-aptamers [27-29], so-called scaffold proteins such as Affibodies [30] or designed ankyrin repeat proteins (DARPins) [31] can be used instead. These types of binders are selected and perhaps also affinity-maturated *in vitro* by procedures such as SELEX for aptamers [27, 29] or phage, ribosome or RNA display for proteins, in analogy with the selection processes employed by the immune system when antibodies are produced *in vivo*. These artificial binders can have properties that make them more suitable for certain applications than antibodies, such as being smaller in size or easier and cheaper to manufacture. For detection of post-translational modifications of proteins, other binders can be useful, such as lectins that can be employed to detect protein glycosylation.

While not a method for studying protein interactions *in situ*, co-immunoprecipitation (co-IP) is worth mentioning since it is considered the golden standard technique for studying protein interactions in cell lysates. In co-IP a binder is used to fish out a protein of interest from cell extracts (Figure 3a) [32]. If the protein interacts with other proteins, they may also be fished out from the solution and subsequently identified by protein identification techniques such as gel electrophoresis, western blot, or mass spectrometry [33].

While binders are normally not detectable *per se*, they can be modified to enable detection. In immunofluorescence-based staining of cells, fluorophores are covalently coupled to the binders, either directly to the primary antibodies or, as is more commonly done, to secondary antibodies binding specifically to the constant region of the primary antibodies (Figure 3b). The reason for the latter approach is that it gives a stronger signal since several secondary antibodies can bind each primary antibody and thus increase the local concentration of fluorophores around the primary binder. It is also a benefit that the same fluorophore-coupled secondary antibody can be used to detect many different primary antibodies, as long as they are raised in the same species. Instead of fluorophores, enzymes such as HRP can be coupled to the binders to enable colorimetric readout by converting a substrate into a colored product, as is done for immunohistochemistry (IHC; Figure 3c).

By using a pair of antibodies, each labeled with different fluorophores such as Cy3 and Cy5, protein-protein interactions can be studied *in situ* by utilizing FRET effects between the fluorophores [34]. When in close proximity, the excited fluorophore on the first antibody can transfer its energy to the fluorophore on the second antibody, which can then emit the energy as light of a longer wavelength. This shift in emission wavelength can then be detected and the amount of interaction estimated, although the calculations can be quite complicated.

Immuno-RCA is a method in which a DNA oligonucleotide is attached to the binder (Figure 4) [35-37]. When the binder has bound its protein, a circular DNA strand is added. Using the oligonucleotide on the binder as a primer, the circular DNA molecule is replicated via rolling circle amplification (RCA) [38-39] into a long, single-stranded piece of DNA consisting of concatenated copies of the DNA circle. The long strand of DNA, still attached to the binder, then folds into a micrometer-sized ball of DNA. By hybridizing oligonucleotides labeled with fluorophores to the repeated sequences of these so-called rolling circle products (RCPs), the balls of DNA can be visualized by fluorescence microscopy and the location of the binding event can be deduced.

The problem with immuno-RCA is shared with other methods involving reagents binding the target protein: the problem of cross-reactivity [40]. Binders do not recognize the whole target protein, but merely accessible epitopes consisting of just a few amino acids of the protein, and can therefore frequently bind to other proteins that have similar epitopes. The selectivity of the detection reagent may be improved by increasing the selectivity of the binder itself, for instance using *in vitro* affinity maturation and selection of antibodies [41]. Alternatively, assays can be used where two or more binders are required to bind the same target protein simultaneously to elicit a detectable signal, thereby reducing the possibility that the wrong protein is recognized. The latter approach has been used with great success in so-called sandwich immunoassays, in which one immobilized antibody captures the protein of interest on a solid-phase support and another, labeled, antibody is used to detect the protein [42-43]. Such assays can reach pM detection levels. The approach is also used in the proximity ligation assay, presented in the next section.

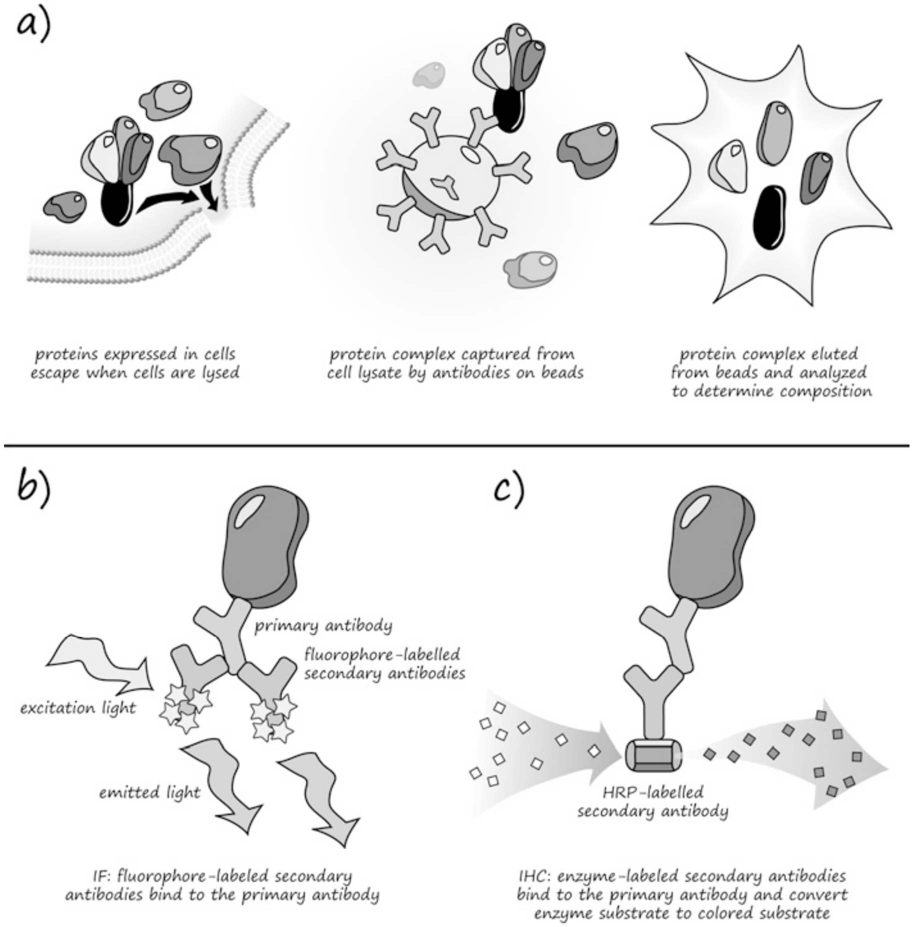

Figure 3. (**a**) Protein-protein interactions can be investigated through the use of immunoprecipitation, whereby antibodies are used to fish out proteins and their interaction partners from a complex mixture such as cell lysates, before the captured proteins are identified by e.g. mass spectrometry. (**b**) With immunofluorescence staining, proteins are detected through the use of binders that are fluorophore-labeled directly, or detected via fluorophore-labeled secondary antibodies. Alternatively, enzymes such as HRP can be used instead of fluorophores, converting a substrate to a colored product that can be visualized with bright-field microscopy (**c**).

The advantages of using binders to study macromolecules include the possibility to create binders against virtually anything, including proteins, nucleic acids, PTMs, carbohydrates, etc., which makes the methods very versatile. Another big advantage is that clinical material can be studied with these techniques, including the vast amounts of material being collected in biobanks worldwide, since the studied material need not be modified genetically.

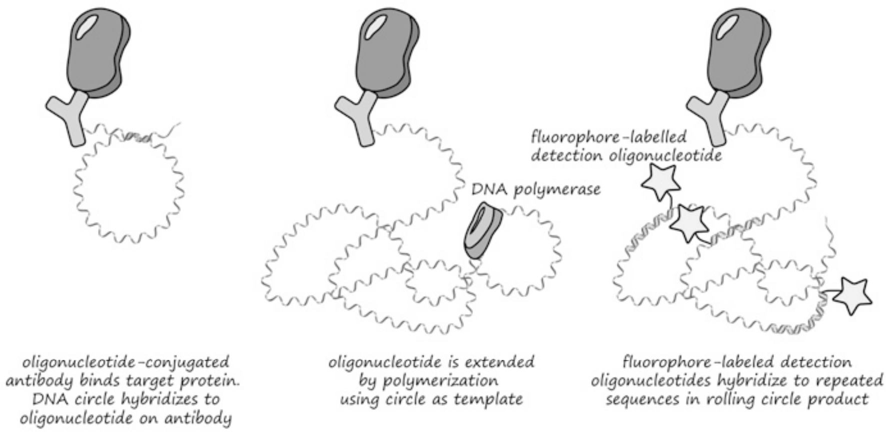

Figure 4. Immuno-RCA. Proteins can be detected through the use of oligonucleotide-labeled antibodies directed against the protein of interest. Upon binding, a DNA circle is added and used as a template for rolling circle amplification, extending the oligonucleotide attached to the antibody. The single-stranded rolling circle product can subsequently be detected by hybridizing fluorophore-labeled detection oligonucleotides to the repeated sequences of the rolling circle product.

Finally, a sense of closeness – *in situ* PLA

The *in situ* proximity ligation assay (*in situ* PLA) can be seen as a more advanced form of immuno-RCA that uses the principle of dual detection for greatly enhanced selectivity, as offered by the proximity ligation assay [44-45]. It is also, incidentally, the subject of this thesis. Just like in immuno-RCA, binders are modified by attaching oligonucleotides. However, instead of having only one binder, two or more binders are used for the recognition of the target protein or protein complex (Figure 5a). After removing antibodies that have failed to bind their targets, two linear oligonucleotides are added that can hybridize to the oligonucleotides on the binders and be joined into a circle by a DNA ligase (Figure 5b). This circle can then be amplified by RCA, using one of the antibody-bound oligonucleotides as a primer (Figure 5c), and the whole complex visualized using fluorophore-labeled oligonucleotides that can hybridize to the resulting RCP (Figure 5d). The requirement for dual recognition by the binders greatly improves the selectivity of the method as compared to immuno-RCA. The two binders need not bind the same molecule, but can, for instance, target two different proteins in a complex. Such an approach was used to study the heterodimerization between c-Myc and its binding partner Max, and it was shown that the method could be used to specifically visualize the heterodimer in fixed cells and tissues [46].

In situ PLA has also been used to study post-translational modifications by using one antibody directed against the modification, e.g. a phosphorylated tyrosine residue of a ligand-stimulated platelet-derived growth factor receptor beta (PDGFRβ), and one antibody towards the receptor itself [47]. This can improve the selectivity of the detection, especially with phospho-specific antibodies, since they often exhibit a high propensity for cross-reactivity towards other phosphorylated proteins. It was also shown that *in situ* PLA can be generalized by introducing the concept of secondary detection: a pair of primary antibodies raised in two different species can be used to detect the target proteins, while oligonucleotide-conjugated antibodies are used to bind the primary antibodies species-specifically. This means that the same oligonucleotide-conjugated secondary antibodies can be used for the detection of many different proteins if the appropriate primary antibodies are used, eliminating the need for constructing antibody-oligonucleotide conjugates for every primary antibody, saving time and effort for the researcher doing the experiments.

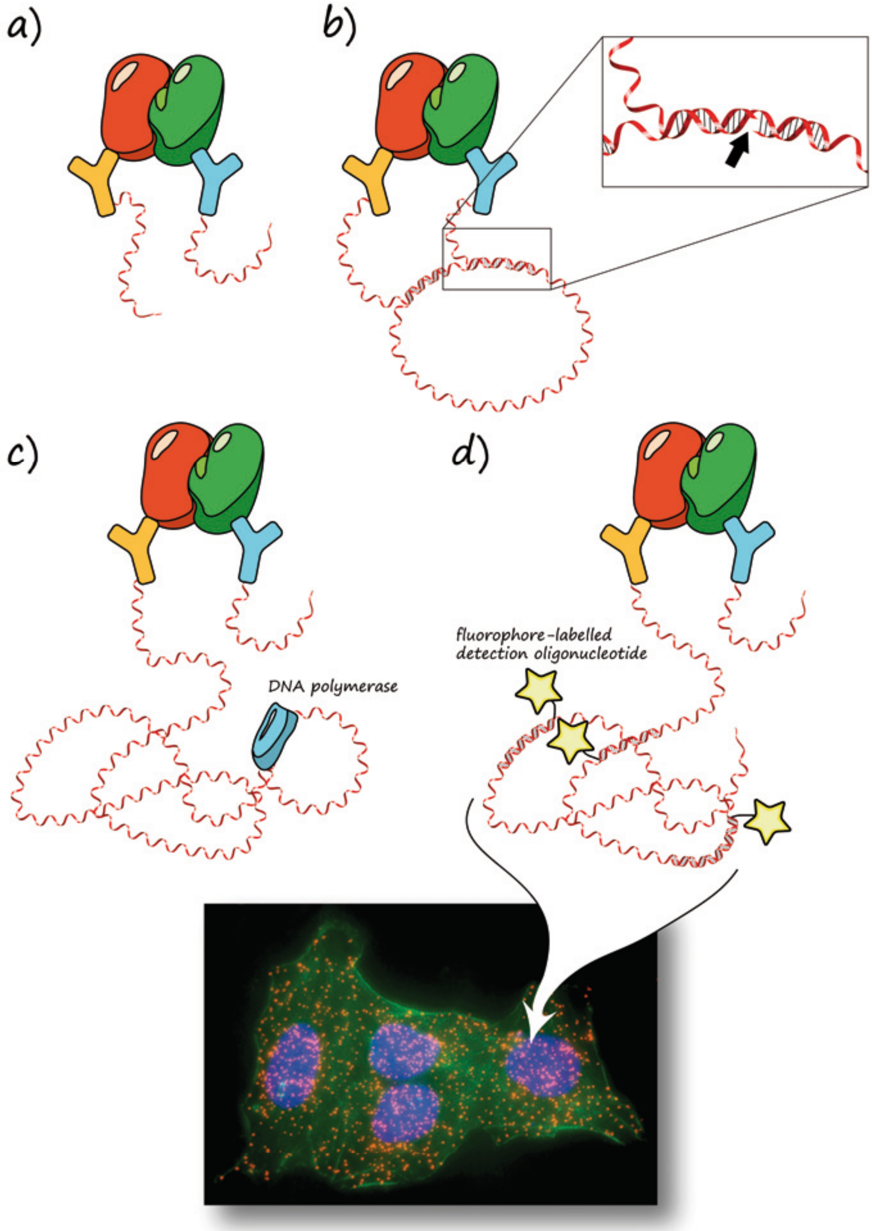

Figure 5. The principles of *in situ* PLA. (**a**) Proteins are detected by oligonucleotide-labeled antibodies. (**b**) If the proteins are in close proximity, the oligonucleotides on the antibodies can be used as a template for the formation of a DNA circle consisting of two linear oligonucleotides, joined through enzymatic ligation (indicated by an arrow). (**c**) The newly formed DNA circle can be amplified using rolling circle amplification, forming a long, single stranded concatemer of repeated copies of the circle. (**d**) By hybridizing fluorophore-labeled detection oligonucleotides to the rolling circle products, they can easily be visualized with fluorescence microscopy.

High-throughput analyses of cells

Go with the flow – flow cytometry

Parallel to the development of optical microscopy systems, the development of flow cytometry has followed. Originally based on techniques used for blood cell counting, flow cytometry has developed greatly since its inception in the 1960s, with many enhancements added such as fluorescence detection and the possibility to sort detected cells. One of the first flow cytometers, the Rapid Cell Spectrophotometer, was built by Louis Kamentsky, and was later used in the development of the Fluorescence Activated Cell Sorter (FACS) by Leonard and Leonore Herzenberg with colleagues [48]. Since then, better flow cytometers have been developed by introducing more and better excitation sources, improved electronics and data analysis capabilities, etc., but the principle remains the same.

The basis for flow cytometry is the optical analysis of cells or other particles suspended in liquid. The cells in the liquid are focused hydrodynamically into a thin stream, and travel in single file through one or several laser beams that excite the fluorescently labeled cells. The emitted light can be collected by one or several detectors, and the results processed in real-time. In addition to detecting fluorescent light emitted from the cells, the scattering of the laser beam by passing cells is usually also measured as it provides a measurement of physical properties of the cells such as size and morphology. The cells are commonly labeled with fluorophore-labeled antibodies to enable measurements of protein expression, but can also be labeled by introducing fluorescent protein fusion tags such as GFP. In addition, FRET between fluorophore-labeled antibodies or fluorescent protein tags can be detected and used to measure protein-protein interactions [34, 49-50].

Flow cytometry allows quantification of cellular nucleic acid content through the use of intercalating dyes such as DAPI or Hoechst. Since the DNA-intercalating dyes can be used to measure the amount of cellular DNA, they can also be used to obtain cell cycle information as the amount of DNA depends on the phase of the cell cycle. It also facilitates detection of apoptotic or dead cells, and gain or loss of genetic material (aneuploidy) in cancer cells. Other parameters such as cellular pH or calcium flux can be measured with specific probes, e.g. pH- or calcium-sensitive fluorescent dyes.

With fluorescence microscopy, light-induced bleaching of the fluorophores can be a big problem, limiting the use of powerful lasers for the illumination of cells. With flow cytometry, fluorophore bleaching is not a problem since each cell is only analyzed once and for a very short time, enabling the use of powerful lasers. In combination with very sensitive detectors such as photomultiplier tubes (PMTs), this makes flow cytometry a very sensitive method for analyzing total fluorescence in single cells. In addition, the measured fluorescence response can be used to physically sort cells automatically into different fractions. As mentioned before, this technique is known as FACS, and can for instance be used to separate different subpopulations of a heterogeneous mixture of cells for subsequent analyses.

Since flow cytometry uses cells in solution and is well suited for analyses of heterogeneous cell populations due to its throughput, it is not surprising that it is commonly used for analyzing blood samples. By employing labeled antibodies that bind to different surface proteins known as cluster of differentiation (CD) antigens, leucocytes can be separated into different subpopulations, e.g. CD4 positive cells, indicating T helper cells, often measured in HIV diagnostics. Further delineation of subpopulations can be attained by including several antibodies directed at different CDs simultaneously, then identifying cells types based on the pattern of expressed CDs.

Flow cytometry has also been employed for drug discovery (reviewed in [51]), for instance to perform simultaneous measurements of phosphorylation levels of a number of proteins involved in signaling pathways under the influence of inhibitors [52-53]. Another interesting approach is to label cells in different samples with fluorophores in a combinatorial fashion, so that each cell from a sample carries a kind of fluorescent barcode identifying which sample it came from. By combining three different fluorophores at different ratios, all the different samples in a 96-well plate can be labeled uniquely [54]. The samples can then be mixed and treated with a drug, stained for markers that could measure the cellular response induced by the drug, and then be analyzed by flow cytometry. From the measured data, the sample identity of the responding cells can be determined.

So-called imaging flow cytometry has been around for many years [55], and is a technology that combines flow cytometry with microscopy to enable cellular analyses with the benefits of both technologies, i.e. analysis throughput and imaging. For many years it seems to have mostly combined the disadvantages of both techniques rather than the advantages, but recently the instrumentation has matured enough to provide imaging data comparable in quality to what can be obtained by microscopy, at a rate of up to one thousand cells per second [56-57].

Content is king – high content screening

High content screening[1], or HCS, was invented and first presented by Cellomics Ltd. in the mid-1990s with the ArrayScan system, and has since then gained much interest from both industry and academia. Inspired by the advances in flow cytometry and automation, the pioneers of HCS sought to automate fluorescence microscopy and combine it with powerful image analysis software to enable high-throughput analysis and quantification of cellular and subcellular features. Since the process was fully automated, cells could be analyzed faster and with much less labor, resulting in more reliable statistics and less risk for bias in the evaluation of the results. This enabled researchers to use cells in discovery-driven research, for instance cell-based drug screening [58], whereas previously only hypothesis-driven research was feasible.

Instrumentation for HCS includes an automated digital microscope, either based on laser-scanning confocal or epi-fluorescence imaging, software that performs image analysis and quantification, and robotics to handle plate loading, etc. The different optical setups each have their advantages and disadvantages: with confocal imaging only a thin optical section is imaged, resulting in less stray light from objects out of focus and better image contrast with thick specimens, but in some cases a thicker optical section, as can be provided by epi-fluorescence imaging, is desirable.

With HCS, cells can be analyzed using ordinary molecular analysis tools, i.e. immunofluorescence staining, fluorescent protein-tagging, RNA interference studies, etc., but instead of performing the reactions on coverslips or microscopy slides the reactions are performed in microtiter plates with 96, 384 or 1,536 wells per plate. Reactions in microtiter plates are easy to automate with standard robotics that can handle everything from seeding cells, incubating the plate at certain temperatures and performing washes between different reaction steps, to loading the plates in the HCS instrument for analysis.

Although both flow cytometry and HCS are techniques that are similar in many ways, HCS differs from flow cytometry in that it is more adapted to analysis of adherent cells, whereas flow cytometry is more commonly used for the analysis of suspension cells, although adherent cells can be used if they are detached from the surface they grow on. And while flow cytometry has a much higher throughput in the number of cells analyzed per second, HCS can gain more information per cell, e.g. cellular morphology and subcellular localization of proteins, and perform analysis of subcellular structures such as fibers, neurite outgrowths and spots. On the other hand, flow cytometry is currently more suitable for multiplexed detection of spectrally

[1] Although screening by e.g. flow cytometry could also be considered high content screening, throughout this text I will use the word exclusively to denote *imaging*-based high content screening.

distinct fluorophores, for instance attached to antibodies binding target proteins, since the response from up to 18 fluorophores can be measured simultaneously [53, 59]. There are ways of adapting fluorescence microscopy to even higher degrees of spectral multiplexing, for example using multispectral imaging in combination with spectral unmixing of the acquired image data [60], but so far it has not been used for HCS, in all likelihood due to low imaging throughput and a lack of *in situ* assays with such requirements.

Putting a number on it – characterization of screening assays

When setting up high-throughput screening assays there are a number of metrics that can be used to assess the assay's suitability for screening applications. The most well-known is the Z' factor, described by Zhang *et al* [61]. The Z' factor is calculated according to equation (1), where n is the number of replicates, m_t is the mean of the maximum controls, m_b the mean of the minimum controls, and s_t and s_b the standard deviations of the maximum and minimum controls, respectively. The Z' factor grows asymptotically towards 1 when the dynamic range, i.e. the difference between the positive and negative samples, increases and when the standard deviations of the samples decrease.

$$Z'factor = 1 - \frac{3 \cdot (s_t + s_b)/\sqrt{n}}{|m_t - m_b|} \qquad (1)$$

An assay with a Z' factor value between 0.5 and 1 is considered an excellent assay that can be used for screening applications, while values between 0 and 0.5 indicate a poor assay, which might suffice in some cases. Values below zero indicate an assay where positive and negative samples cannot be distinguished from each other because of low dynamic range or large variability of the measurements. The large emphasis on the variability of the measured sample, i.e. that an assay must have at least 12 standard deviations of difference between the positive and negative sample means for it to be considered excellent, is needed because a high-throughput screen typically involves measuring a very large number of samples. Related to the Z’ factor is the signal window [62], calculated according to equation (2). The recommendation for the signal window is that it should be above 2, but values over 1 are also acceptable, while values below 1 are considered unacceptable.

$$SW = \frac{m_t - m_b - 3(s_t + s_b)/\sqrt{n}}{s_t/\sqrt{n}} \qquad (2)$$

Signal to noise ratio (SNR) is another measurement, derived from the field of electrical engineering. It compares the signal, i.e. difference between the means of the positive and the negative samples, to the noise, i.e. variability of the samples. SNR is calculated according to equation (3). The signal to noise ratio should not be confused with signal to background, which is calculated as the ratio between the mean of the positive and negative samples. The higher SNR the more reliably the maximum and minimum controls or samples can be separated.

$$SNR = \frac{|m_b - m_t|}{\sqrt{s_b^2 + s_t^2}} \tag{3}$$

The Z' factor, signal window and SNR are used to characterize the suitability of a screening assay in regards to the rate of false positives and negatives when measuring a large number of samples. However, they do not give any estimation of the precision or accuracy of potency estimates from potency assays, i.e. assays to measure dose responses. In these cases the minimum significant ratio (MSR) can be used instead [63]. The MSR measures the smallest potency ratio between any pair of compounds that is statistically significant, and can be used to estimate inter- or intra-assay reproducibility. It can also be used to verify that an assay is producing equivalent results when transferred from one laboratory to another. MSR is defined as equation (4) where n is the number of replicates per sample, and SD the standard deviation of the difference between the logarithms of two potency estimates. A MSR below 3 is usually desirable.

$$MSR = 10^{2\sqrt{2*SD/\sqrt{n}}} \tag{4}$$

Present investigations

The current investigations describe the development of the *in situ* proximity ligation assay (*in situ* PLA) as a way to study proteins, protein-protein interactions and post-translational modifications in fixed cells and tissues in a highly selective and sensitive manner. The first paper describes the original development of the technique, and the following papers describe further enhancements and applications of the original method.

Paper I: Direct observation of individual endogenous protein complexes *in situ* by proximity ligation

Aim of the study

The aim of the work in Paper I was to establish a method to visualize interacting proteins or other macromolecules in fixed cells or tissues. Earlier methods for studying these kinds of interactions have limitations that we wished to address. These limitations include problems with low sensitivity, low selectivity, and the difficulty of studying protein interactions in clinical material.

The *in situ* proximity ligation assay

The *in situ* proximity ligation assay uses a pair of proximity probes as a means to detect the target proteins. The proximity probes consist of a binding part, such as an antibody, to which an oligonucleotide has been attached. The oligonucleotide is coupled to the antibody by a heterobifunctional linker that couples primary amines on the antibody to a sulfhydryl group on the oligonucleotide. The proximity probes are allowed to bind to their target proteins in the cells or tissues after a blocking step, in which a blocking compound such as bovine serum albumin (BSA) or normal serum is used to block non-specific binding sites in the cells. This step is included to minimize the amount of non-specific binding of the antibodies, which can otherwise lead to false positive signals. Unbound proximity probes are washed away.

If the two proximity probes are bound to their targets sufficiently close to each other, their attached oligonucleotides can be used together as templates for the ligation of two linear oligonucleotides into one circular strand of DNA. By contrast, if the two proximity probes are not in close proximity, e.g. if the two target proteins do not interact, then no DNA circles can be created. Any DNA circles that form can be used as a template for the extension of one of the oligonucleotides of the proximity probes by continuous rolling circle amplification (RCA), whereas the linear pieces of DNA can only be rendered double stranded before the reaction ends.

Through a one-hour RCA reaction, a single stranded DNA molecule is created that consists of approximately one thousand concatenated complementary copies of the DNA circle. This DNA strand is tethered to one of the antibodies and thus anchored to the site of its formation. Because of intra- and intermolecular interactions, the single stranded DNA collapses into a ball of DNA, referred to as a rolling circle product (RCP), with a diameter of approximately one micrometer. Finally, by using fluorophore-labeled oligonucleotides that hybridize to the repeated sequences of the RCPs, they can be detected by fluorescence microscopy.

Summary and results

The proximity ligation method, which was first developed to detect proteins in solution [44-45], was adapted for *in situ* applications by combining proximity-dependent ligation, using the antibody-oligonucleotide conjugates as templates, with signal amplification via rolling circle amplification (RCA) [38] for localized visualization of the detection event. The resulting method, originally named P-LISA (proximity-ligation *in situ* assay, or now *in situ* PLA), was used to study interactions between the oncoprotein c-Myc and its binding partner Max in a number of different cell lines, and in fresh-frozen tissues.

Several controls were used to validate the method, including a *myc* knock-out cell line, attempts to visualize interaction between non-interacting proteins, and the use of small molecular dimerization-inhibitors or IFN-γ to perturb c-Myc/Max interactions. In addition, the generality of the methods was shown by visualizing interactions between the HER2 protein with the epidermal growth factor receptor (EGFR) in addition to the c-Myc/Max interactions.

By slightly modifying the method, we could show that it was sensitive enough to visualize single endogenous protein complexes in the highly complex mixture of macromolecules that make up the cell. This was done by using two different versions of one of the linear oligonucleotides that make up the DNA circle as a result of a successful detection event. The RCPs from the two different circles were detected using two different detection oligonucleotides. Statistically, half of the RCPs should therefore be of one color, while the other half should be of the other color. If the bright spots detected in the cells emanated from several detection events, the spots would be dual colored since they would contain RCPs from both colors; however, since they in fact were *not* dual colored, we concluded that the spots were single RCPs, generated from separate detection events.

The method was also further extended so that it could be used to detect interactions between three different interacting proteins in one complex, through the use of three different proximity probes simultaneously. This was used to visualize the interaction between c-Myc, Max and RNA polymerase II.

Paper II: *In situ* detection of phosphorylated platelet-derived growth factor receptor β using a generalized proximity ligation method

Aim of the study

In Paper II, our aim was to make the *in situ* proximity ligation assay more general. This was done by using primary antibodies to bind the target proteins or PTM of interest in combination with species-specific secondary antibodies conjugated to oligonucleotide arms, to detect the primary antibodies in the same manner as described in Paper I. The new method was used to study the phosphorylation of the platelet-derived growth factor receptor β (PDGFRβ) in cells stimulated by PDGF-BB, and in tissues. With current methods, such studies were complicated by the low degree of selectivity of existing binders, posing a need for more selective methods.

Method

The method presented in Paper II was a further development of the method presented in Paper I. Instead of using proximity probes to bind the proteins of interest directly, we used one unconjugated mouse monoclonal antibody raised against the phosphorylated form of the PDGFRβ, and one unconjugated rabbit polyclonal antibody raised against the PDGFR itself. These primary antibodies bound to their target, and were detected by proximity probes directed against the immunoglobulins from the different species, with one proximity probe binding the mouse monoclonal antibody and the other one binding the rabbit polyclonal antibody. The rest of the detection, including the ligation of two linear oligonucleotides into a DNA circle with the proximity probes' arms as templates, the RCA of the circle, and the hybridization of detection oligonucleotides was performed as previously described.

Results

Tyrosine phosphorylated PDGFRβ was visualized in human embryonic kidney cells and pig aortic endothelial cells, both overexpressing the PDGFRβ, but not in the same cells transfected with the alpha-form of the receptor, demonstrating the selectivity of the method. Measurements of the level of phosphorylation of the receptor by *in situ* PLA as a result of increasing concentrations of ligand were compared to results obtained by immunoblotting, showing similar results. To show that the method was sensitive enough for the detection of endogenous levels of protein, detection of phosphorylated PDGFRβ was also demonstrated in immortalized human foreskin fibroblasts (BJ hTERT), with or without stimulation with PDGF-BB. In addition, phosphorylated PDGFRβ was visualized in fresh-frozen human scar tissue undergoing wound healing.

Discussion

When studying post-translational modifications of proteins *in situ*, current methods such as immunohistochemistry and immunofluorescence present problems with respect to sensitivity and selectivity, especially when tissue sections are studied. With the requirement for dual recognition of the target protein or post-translational modification, and the powerful signal amplification of RCA, *in situ* PLA was shown to alleviate these problems. In this case, a generalized *in situ* PLA, using species-specific proximity probes in combination with primary antibodies derived from two different species, was used to visualize and quantify the phosphorylation of the PDGFRβ.

The benefit of using this indirect approach instead of the method presented in Paper I, in which the proximity probes were used directly to detect the target proteins, is that the same proximity probes can be used to detect many different proteins or post-translational modifications without the need for conjugating new antibodies. This saves time and effort, since the conjugation process can sometimes present problems with certain antibodies. In addition, batch-to-batch variation between different conjugates can be reduced as large quantities of secondary antibodies can be conjugated at the same time.

Paper III: Flow cytometric *in situ* proximity ligation analyses of protein interactions and post-translational modification of the epidermal growth factor receptor family

Aim of the study

Even though *in situ* PLA is a sensitive and selective method for detecting and quantifying protein interactions or post-translational modifications, the readout format where fluorescence microscopy is used can be limiting when studying heterogeneous samples. In such studies, large amounts of cells often need to be evaluated to obtain statistically reliable results. This can be both quite labor-intensive and time-consuming with ordinary fluorescence microscopy. Flow cytometry, on the other hand, is a very efficient way of analyzing large numbers of cells and is commonly used for analysis of heterogeneous cell populations such as leucocytes, where thousands of cells can be analyzed in a few seconds. Thus, our aim of the study was to combine the sensitivity and selectivity of *in situ* PLA with the efficiency of analysis of flow cytometry.

Methods

In situ PLA, using directly conjugated primary antibodies, was used to detect homomeric and heteromeric interactions between EGFR and HER2 in three different cancer cell lines: SK-OV3, U343 and MCF-7. To measure the relative amounts of EGFR and HER2 in the cell lines, flow cytometric immunofluorescence staining was performed with fluorophore-labeled antibodies binding the receptors. The proximity probes used in the *in situ* PLA reactions were created by attaching oligonucleotides to antibodies through a hydrazine-aldehyde coupling reaction, in contrast to the maleimide-thiol method used in Paper I. In addition, the conjugates were purified by HPLC to remove unreacted oligonucleotides. Two types of oligonucleotides were used for each antibody-conjugate, so that homomeric and heteromeric interactions between EGFR and HER2 could be studied with different combinations of proximity probes in separate reactions. Monoclonal antibodies had to be used to ensure that no more than one antibody could bind each receptor monomer. Cells to be analyzed were gently detached and either treated with EGF or left untreated as controls before being stained with the proximity probes in the *in situ* PLA reactions. The stained cells were then analyzed by flow cytometry, recording the fluorescence intensity of tens of thousands of cells in each staining reaction.

In addition to detecting dimerization among EGFR and HER2, phosphorylation of EGFR was also detected by using unconjugated primary antibodies directed against phosphorylated EGFR in combination with *in situ* PLA with immunoglobulin-specific proximity probes, similar to what was described in Paper II. The results from *in situ* PLA were compared to results obtained with ordinary immunofluorescence staining with the same primary antibodies, used one at a time.

Results

Using *in situ* PLA in combination with flow cytometric readout, the amount of dimerization between EGFR and HER2 was monitored in separate reactions in SK-OV3, U343 and MCF-7 cells. The expression of the receptors was determined in these cell lines by IF so that the amount of monomers could be compared to the amount of homomeric and heteromeric interactions. In U343, having a high expression of EGFR and a low expression of HER2, homomeric EGFR-EGFR interactions were abundant while the amounts of EGFR-HER2 and HER2-HER2 interactions were much lower. In contrast, although SK-OV3 has the same level of EGFR expression as U343, the amount of EGFR-EGFR interactions was low. On the other hand, SK-OV3 has a very high expression of HER2, and since HER2 is the preferred interaction partner of the epidermal growth factor receptor family it is not surprising that both the amounts of HER2-HER2 and the HER2-EGFR interactions were high. In MCF-7 cells, having a low expression of EGFR and a moderate expression of HER2, the amount of detected interaction between EGFR and HER2 was negligible, possibly due to HER2-HER3 interactions.

No differences in the interaction patterns between EGFR and HER2 could be detected upon stimulation with the receptor ligand EGF. However, phosphorylation of EGFR could be detected via *in situ* PLA by using a pair of primary antibodies directed against phosphorylated EGFR in combination with immunoglobulin-specific secondary proximity probes. In comparison, only a minuscule difference between EGF-stimulated cells and the control cells could be detected with IF using the same primary antibodies, probably due to unspecific binding of the primary antibody to unphosphorylated EGFR in the unstimulated control cells.

Discussion

For many years, the predominant view on EGF receptor signaling has been that upon binding of a ligand, monomeric EGF receptors in the plasma membrane associate to form receptor dimers. The receptor dimers subsequently autophosphorylate and relay the external signal internally through a range of different signaling pathways [64-67]. It therefore came as a surprise to us that upon EGF stimulation, no changes in the interactions between the receptors could be detected by *in situ* PLA, even though we could clearly detect the activation of the receptor. For a long time we believed our results erroneous, and we tried many variations of our assay in attempts to verify our findings. We modified our proximity probes to reduce the maximum distance allowed between the probes during signal generation, but still could see no difference between the stimulated and unstimulated cells. Replacing the antibodies with smaller binders, in this case Affibodies, was also to no avail. Finally, we discovered that the established view of the receptor signaling mechanism was beginning to be questioned by several groups that had obtained contradicting results, indicating that the EGF receptors can exist in preformed complexes in the cell membrane and that they undergo conformational changes upon ligand binding, leading to receptor activation [49, 68-70]. We therefore believe that the reason that we cannot detect any difference in homomeric or heteromeric dimerization between EGFR and HER2 upon stimulation with EGF is that we detect interactions within these clusters of receptors, and that the stimulation does not change the clusters sufficiently for us to be able to detect any difference between stimulated and unstimulated cells. The reason why other methods, such as co-IP, can detect changes in the amount of dimerized receptors upon stimulation could be due to stimulation-induced conformational changes of the receptors that help stabilize the interactions between the receptor monomers [71].

With the combination of *in situ* PLA and flow cytometry, we have set up a method to visualize protein-protein interactions and post-translational modifications in genetically unmodified cells such as cells from patients, where we can read out the results in thousands of cells per second. This enables studies of heterogeneous cell populations and rare cells detection in a way that would be impossible to match with slide-based microscopy. There is also the possibility for easy readout of multiplexed *in situ* PLA reactions, since flow cytometry can be used for the simultaneous analysis of cells stained with many fluorophore-labeled antibodies [53, 59]. The technique should therefore also be used to detect rolling circle products labeled with different fluorophores resulting from a multiplexed *in situ* PLA assay.

The benefits come at a price, however; the precise digital quantification of rolling circle products by microscopy is lost, since ordinary flow cytometers can only analyze the total fluorescence intensity of the cells, making the quantification more imprecise when low abundant proteins are studied. In addition, cells need to be in suspension during the *in situ* PLA reactions, which increases the assay time and the risk of losing cells during washing steps.

Paper IV: High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation

Aim of the study

Drug discovery is a costly process with many developed drugs failing clinical approval due to problems with efficacy and toxicity. Methods for drug screening by high-throughput screening have evolved tremendously in capacity in the last decades but have failed to improve on the clinical success rate. There is therefore a great need for methods that can give a better estimation on the *in vivo* effects and side effects of drugs early in the drug discovery process.

The use of *in situ* PLA for drug screening should enable more biologically relevant assessment of drug effects on protein-interactions and post-translational modifications with endogenously expressed proteins in genetically unmodified cells, or even primary tumor cells from patients, than what can be done in e.g. cell lines with overexpressed fusion-tagged proteins. In addition, as was shown in Paper III, *in situ* PLA can, at least for certain antibodies, provide superior results compared to immunofluorescence staining, a commonly used technique for cell-based drug screening.

We therefore wanted to test if *in situ* PLA could be used for drug screening in primary cells. As a model system we chose to screen for inhibitors of phosphorylation of the PDGF receptor β.

Methods

In situ PLA with secondary proximity probes directed against rabbit and mouse immunoglobulins was used in combination with a rabbit PDGF receptor β antibody and a mouse antibody specific for phosphorylated tyrosine residues to detect phosphorylated PDGF receptors in primary human fibroblasts. The assay presented in Paper II was adapted for high content screening by performing the reactions in flat bottom microtiter plates instead of on microscopy slides. This makes the assay much more suitable for automation, as required for large-scale drug screening. A Cellomics ArrayScan II instrument was used for automatic fluorescence imaging and image quantification. The image analysis software was set up to analyze cells by delineating the nuclei and cytoplasm boundaries with the help of fluorescent markers, and then quantifying the number of rolling circle products in each cell by spot counting. Around 1,000 cells were analyzed for each reaction condition.

To characterize the assay, the Z' factor was calculated based on data from sixteen samples of PDGF-BB-stimulated fibroblasts, where half of them had been treated with imatinib (Gleevec), a known inhibitor of PDGFR phosphorylation, and the rest had been left untreated as controls. The same procedure was also performed with immunofluorescence staining with the phospho-specific primary antibody used for the *in situ* PLA, or with an antibody specific for phosphorylated PDGFRβ, in combination with a fluorophore-labeled secondary antibody. Since it was not possible to quantify the results by spot counting, the total fluorescence intensity for each cell was measured instead, as is normal for these kinds of assays.

The assay was used to analyze the amount of phosphorylated PDGF receptor in stimulated human fibroblasts either treated with a kinase inhibitor or left untreated as controls. The Screen-Well Kinase Inhibitor library, consisting of 80 known kinase inhibitors to which we added six additional clinically used tyrosine kinase inhibitors, was used for the screening of inhibitors affecting the receptor activation. The compounds that elicited a response that was more than three standard deviations below the mean of the untreated negative controls were classified as hits and were further analyzed by measuring the dose responses with the same assay. To show that the assay could be used to measure protein-protein interactions, perturbations of interactions between PDGFRβ and PI3-kinase and between PDGFRβ and actin were also studied in cells either treated with imatinib (Gleevec) or left untreated.

Results

The *in situ* PLA assay, using a pan-specific antibody for phosphorylated tyrosines in combination with an antibody specific for PDGFRβ, achieved an excellent Z' factor of 0.71. In comparison, immunofluorescence staining, using the same primary antibody as for the *in situ* PLA in combination with a fluorophore-labeled secondary antibody, achieved a poor Z' factor of 0.25. However, since the pan-specific phospho-tyrosine antibody alone is not specific for the PDGF receptor, phosphorylation of other proteins is also detected. When the pan-specific phospho-tyrosine antibody was replaced with an antibody specific for phosphorylated PDGFRβ the Z' factor was only -3.1, rendering it unsuitable for screening purposes. Although it is not shown in the paper, the antibody specific for phosphorylated PDGFRβ can be substituted for the pan-specific phospho-tyrosine antibody for *in situ* PLA in combination with the PDGFRβ-specific antibody with similar results.

By screening through the library of kinase inhibitors with *in situ* PLA, 13 out of the 86 compounds were identified as hits. All hits except one were known PDGFR inhibitors; however, the unknown inhibitor was found not to have an effect during the dose response studies and was therefore considered a false positive. The clinically used PDGFR-specific tyrosine kinase inhibitors all had a strong impact on the level of receptor phosphorylation, in stark contrast to the EGFR-specific inhibitors that did not elicit such responses.

Interaction between PDGFRβ and PI3-kinase was shown to be significantly upregulated in stimulated cells compared to unstimulated cells. Upon treatment with imatinib, the amount of interactions was reduced significantly. This observation is in accordance with published data showing that the PDGF receptor needs to be phosphorylated for PI3-kinase to associate with it [72]. Interactions between PDGFRβ and actin were not significantly affected by stimulation or by inhibitor treatment although a small decrease was noted upon stimulation, probably caused by proteins binding to the phosphorylated receptor, thereby causing steric hindrance of the detection.

Discussion

In this paper we have shown that *in situ* PLA can be used for drug screening, with superior performance compared to immunofluorescence staining. We used our assay with a library of known kinase inhibitors and showed that we could identify specific hits. Furthermore, we showed that *in situ* PLA can be used for screening of inhibitors of protein-protein interactions, something which is difficult if not impossible to do in genetically unmodified primary cells with other techniques.

The use of primary cells for drug screening should be able to provide more biologically relevant data on the drug effects than what can be obtained from immortalized cell lines [52, 73], since the cell lines have often been cultured for a long time and in the process likely adapted to life on a plastic surface by genetic and epigenetic changes. Indeed, in many cases the cells might not even be the intended cell line, having been contaminated and overtaken by other, faster growing cell lines [74]! The problem with using primary cells for screening applications is that they can only be cultured for a certain number of passages before they reach cellular senescence and stop dividing [75]. There can also be difficulties adapting the cells to *in vitro* cultivation. It has been proposed that these problems could be solved by culturing stem cells, which in principle can be cultured indefinitely, and differentiate them into the cell type of interest when needed [76], although they will in all likelihood not fully resemble true primary cells.

The possibility to use primary cells for drug discovery means that also cells from patients can be used, enabling characterization of drug effects directly on clinical samples. This could be an important tool for assessing drug efficacy and toxicity in different patient populations before going into costly large clinical trials, and it could furnish a valuable tool for companion diagnostics, allowing the effects of a drug to be monitored in patients before selection of drug regimen and during the course of treatment.

Summary and future perspectives

The papers presented in this thesis introduce the *in situ* proximity ligation assay and the improvements and diversifications to the method that has followed since the first paper. In the first paper describing the *in situ* PLA reaction, assays were run on cultured cells and tissues with conjugated primary antibodies. A small attempt to show how *in situ* PLA can be used for drug discovery was performed with two inhibitors that inhibit the interaction between c-Myc and Max. However, since the experiments were performed on ordinary microscopy slides, and the fluorescence imaging and signal quantification was performed by hand, it hardly qualified as a high-throughput method. With Paper II the concept of using species-specific secondary oligonucleotide-conjugated antibodies for the detection of primary antibodies was introduced, circumventing the need for conjugating antibodies specific for the target proteins, and simplifying the adaptation of the assay to different targets. Automated image analysis and quantification of the microscope images could now also be performed by the freeware software BlobFinder [77], which was the result of a collaboration with the Center for Image Analysis in Uppsala.

Both versions of the assay were used in Paper III, where conjugated primary antibodies were used for the detection of homomeric and heteromeric interactions between EGFR and HER2, and the secondary proximity probes were used for the detection of phosphorylated EGFR. In the first case, conjugated primary monoclonal antibodies had to be used for the detection reaction since we wanted to confine detection to receptor dimers without detecting receptor monomers. However, it has recently been shown by Clausson *et al.* (manuscript in preparation) that the problem can also be solved by conjugating one aliquot of the primary antibodies to one kind of hapten, e.g. biotin, and another one to a different hapten, e.g. fluorescein, then detecting the modified antibodies with secondary proximity probes specific for the two haptens.

By using flow cytometry as the readout method we were able to override the limitations imposed by manual fluorescence microscopy with regards to the number of cells that can be analyzed per reaction, enabling analysis of thousands of cells per second. However, this increase in speed meant that certain benefits of microscopy had to be sacrificed; information on the subcellular localization of the detected protein was lost since only the total fluorescence intensity for each cell can be measured by ordinary flow cytometry. In addition, this meant that the precise digital quantification of rolling circle products was not possible, making the analysis more vulnerable to interference from cellular background autofluorescence. With the advent of imaging flow cytometry, it is possible that these two drawbacks could be removed [56], but since we did not have access to such an instrument this was not tested. Another limitation of using flow cytometry as the readout method was the requirement that the cells should be kept in solution throughout staining and analysis. This limitation is less of a problem when it comes to analyzing non-adherent cells like leucocytes, but adherent cells need to be detached from the surface they grow on, which could potentially damage the cellular integrity and alter the state of activity, as well as introduce artifacts in the detection. It is also more difficult to automate the assay with the cells in solution since the washing steps require centrifugation of the cells.

To address the shortcomings of the flow cytometric readout of the *in situ* PLA reactions in Paper III, we instead used a high content analysis instrument in Paper IV. The instrument, which is basically a fast automated fluorescence microscope with sophisticated image analysis software, is not as fast as a flow cytometer but can provide more information per analyzed cell. The increase in speed and reduction in manual labor over manual microscopy provided by the HCA instrument was crucial in our attempt to prove that we could use *in situ* PLA for cell-based drug screening.

So where should we go from here? In my opinion, the most important enhancement of the assay would be the ability to perform multiplexed analyses, i.e. many simultaneous molecular measurements in the same sample. The basis of *in situ* PLA, whereby target molecules are detected through the use of a pair of target-specific affinity reagents to which pieces of DNA have been attached, should theoretically allow for the simultaneous detection of many protein-protein interactions in the same reaction, as long as the different detection events can be distinguished from each other. This has previously been done *in vitro* for proximity ligation-based measurements of proteins in serum, using quantitative PCR as a readout method [78]. However, this approach is not useful for localized detection. Instead, one possibility is to introduce a unique tag sequence in the DNA sequence of each proximity probe (Figure 6a), identifying each antibody. These tags could then be propagated into the rolling circle amplification products resulting from a successful detection event between two proximity probes that are binding interacting proteins. This should make it possible to deduce which combinations of proximity probes gave rise to signals, and thus which proteins interacted. The amplified tags in the RCPs could be detected with detection oligonucleotides designed to uniquely report the identity of the different tags by the use of different fluorophores for each tag (Figure 6b), similar to what has previously been done with RCPs on glass surfaces [79]. Each RCP would be stained with two types of detection oligonucleotides, revealing the identity of both proximity probes that gave rise to it. This detection scheme can then be used in a combinatorial fashion: by using three different proximity probes ("A", "B", & "C") targeting three pair-wise interacting proteins, three different types of RCPs incorporating different combinations of tags are possible ("A-B", "A-C" & "B-C"); four probes targeting four interacting proteins can give rise to six different RCPs, and so on.

To show the feasibility of the proposed setup, we have used it to detect interactions between EGFR family members in fresh-frozen human breast cancer tissue (Figure 6c), using a HER2-specific proximity probe in combination with EGFR, HER2 and HER3-specific probes. In this case, the setup is somewhat simplified, since the common proximity probe (HER2) has no tag sequence included, while the other three proximity probes (EGFR, HER2, HER3) all carry specific tag sequences. Thus, the interactions can simply be read out by hybridizing detection oligonucleotides that are specific for the three tag sequences to the RCPs.

A multiplexed assay would be able to save both time and effort, and, where applicable, precious clinical material. In addition, it would enable measurements of several concurrent events within the same cells, which could potentially provide entirely new opportunities to elucidate the intricate networks of protein interaction within cells [80]. This could prove crucial in the field of systems biology, where the interaction networks of the cells are mapped and described mathematically. Currently, to generate data for the mathematical models, large numbers of protein-protein interactions are measured one at a time by e.g. mass spectrometry [20]. But these measurements are of limited use when trying to model cellular interaction networks accurately since they only provide averaged measurements across large numbers of cells, completely disregarding cell-to-cell variations and the subcellular distributions of such protein interactions. In addition, the cells used in the experiments are generally modified genetically to express fusion-tagged target proteins in order to aid in the detection, and, as discussed, this may well perturb the true protein-protein interaction networks. Yet another consideration is that they often require too much material for the analyses to be performed on clinical material, confining the analyses to cultured cell lines that can differ greatly from cells found in living organisms, and thus preventing clinical application.

Expanding the knowledge of the cellular protein interaction networks is also vital for a better understanding of several types of disease, including cancer. Improved methods to study these interaction networks, especially in clinical material, should therefore be of great importance both for increasing the knowledge of the underlying disease mechanics, but also for finding new biomarkers to improve disease diagnostics. These types of biomarkers could be based on measuring interactions between proteins instead of measuring the up- or down-regulation of certain proteins [81].

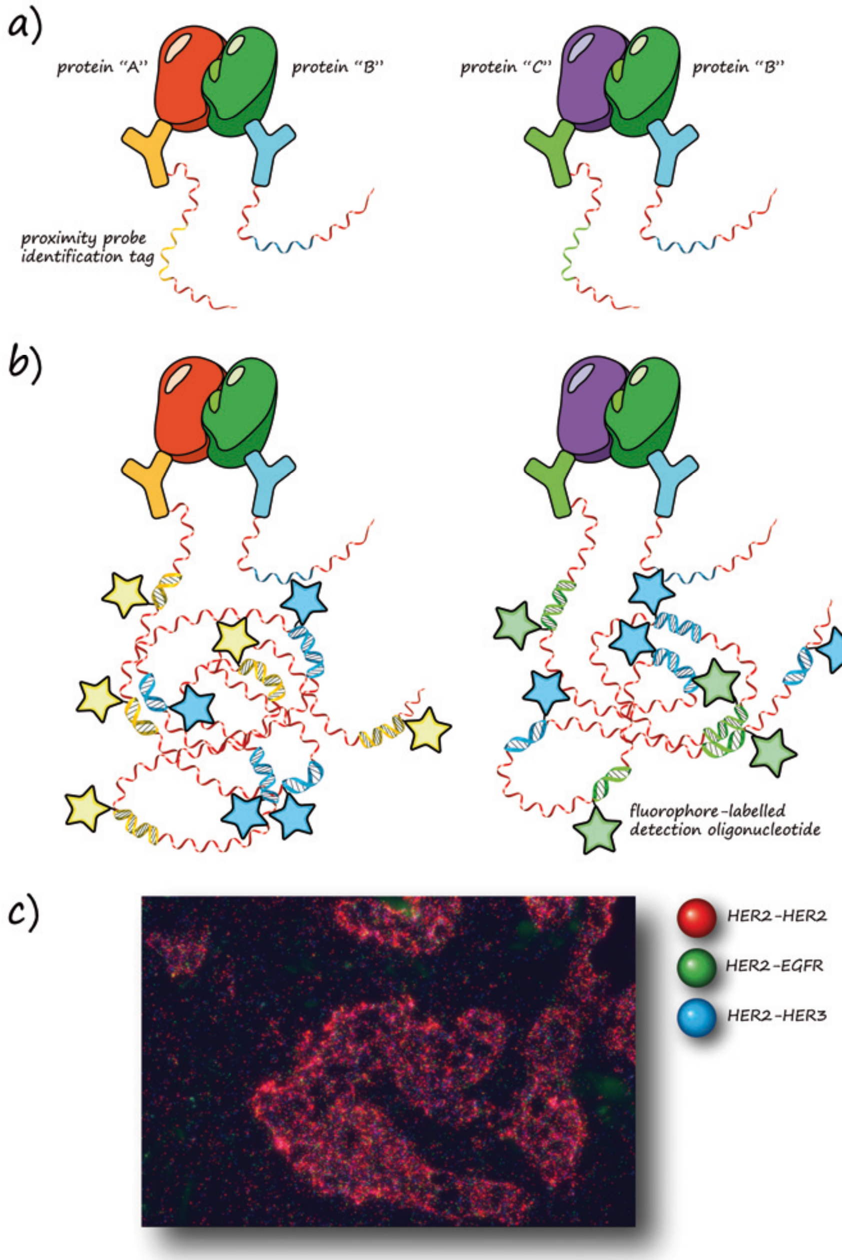

Figure 6. Multiplexed *in situ* PLA. (**a**) Proteins are detected using oligonucleotide-labeled antibodies. A unique identification sequence has been included for each type of antibody. (**b**) After the *in situ* PLA steps, the rolling circle products contain the tag sequences of the two antibodies that gave rise to the signal, indicating which proteins were interacting. The two identification tags can be detected through the use of specific detection oligonucleotides. (**c**) Multiplexed *in situ* PLA detection of interactions between HER2 and EGFR, HER2 and HER3 in breast cancer tissue.

Another context where multiplexed detection of protein-protein interactions could prove of decisive importance is in the field of network pharmacology, where drugs are designed to act on several drug targets simultaneously [82]. The rationale behind this is that the cellular interaction networks are quite robust because of their underlying structure [80], and to perturb these networks and also to avoid escape mutations in malignancy it may prove important to target several proteins simultaneously [80, 82-83]. Accordingly, methods are needed that can monitor the cellular interaction networks in order to assess the effects of these kinds of treatment protocols. Hopefully, this should become possible through simultaneous measurements of multiple protein-protein interactions by multiplexed *in situ* PLA.

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~THE END~

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