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Improvements and Applications of *in situ* Proximity Ligation Assays

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Abstract

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The cells building up the human body is in constant communication with each other. This communication is done through large complex networks of signaling pathways for inter- and intracellular signal transduction. The signaling activity regulates many important processes, for example cell death, proliferation and differentiation. Information within the signaling networks is communicated over the cell membrane, through the cytoplasm and entering the nucleus by protein activities such as protein-protein interactions (PPIs) and post translation modifications (PTMs). The cells adapts to their own environment, responding to multiple stimuli from their surroundings. This in combination with memory of previous responses, difference in cell cycles stages and sometimes altered genetic background generates heterogeneous cell populations in which every cell is slightly different from its neighbor. This calls for methods to study the activity of endogenous proteins in individual cells within a population.

In situ proximity ligation assay (*in situ* PLA) was originally developed to visualize interaction between endogenous proteins in fixed cells and tissue and can also be applied to detect PTMs. This thesis describe the application of *in situ* PLA to study PPIs in signaling pathways and the work to further develop and improve techniques for proximity dependent detection.

In paper I *in situ* PLA is used to study cross talk between the Hippo and the TGFβ signaling pathways. The study shows the complex formation by the transcription co-factors of the Hippo pathway, Yap and Taz, and the main effectors of the TGFβ pathway Smad2/3. Furthermore the density dependent localization of the interaction is described.

Paper II presents a new version of the *in situ* PLA probes for simultaneous detection of multiple complexes. Visualization of various complexes involving EGFR, Her2 and Her3 is presented as a proof of concept.

The efficiency of *in situ* PLA is limited by several factors, one being the design of PLA probes and oligonucleotide systems. Even upon proximal binding of the probes there is a risk of formation of non-circular ligation products, which cannot be amplified and detected. In Paper III two new PLA probes are presented aiming to reduce the formation of non-circular ligation product and hence increase the detection efficiency of *in situ* PLA.

Paper IV presents a new method for detection of protein complexes and phosphorylation; proxHCR. ProxHCR combines signal amplification by enzyme free hybridization chain reaction (HCR) with the requirement of proximal binding of two affinity probes. As a proof of principle the method is used to detect multiple complexes and protein phosphorylation in fixed cells and tissue.

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To my family and friends

List of Papers

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

I **Grannas, K**.*, Arngården, L.*, Lönn, P., Mazurkiewicz, M., Blokzijl, A., Zieba, A.**, Söderberg, O.** Crosstalk between Hippo and TGFβ. Subcellular localization of YAP/TAZ complexes. *Journal of molecular biology, in press*

II Leuchowius, KJ.*, Clausson, CM.*, **Grannas, K**., Erbilgin, Y., Botling, J., Zieba, A., Landegren, U., Söderberg, O. (2013) Parallel visualization of multiple protein complexes in individual cells in tumor tissue. *Molecular & Cellular Proteomics*, 12(6):1563-71

III **Grannas, K**.*, Klaesson, A.*, Koos, B., Nong R., Arngården, L., Söderberg, O.,** Landegren, U.** Increasing efficiency of *in situ* proximity ligation assay by enzymatic conversion dependent proximity probe design. *Manuscript*

IV Koos, B., Cane, G., **Grannas, K.**, Löf, L., Arngården, L., Heldin, J., Clausson, CM., Klaesson, A., Hirvonen, MK., Souza de Oliviera, FM., Talibov, V., Pham, N., Auer, M., Danielson, H., Haybaeck, J., Kamali-Moghaddam, M., Söderberg, O. Proximity dependent initiation of hybridization chain reaction. *Nature communications, in press*

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Related Work by the author

Original Articles

I Christerson, L., Blomqvist, M., **Grannas, K.**, Thollesson, M., Laroucau, K., Waldenström, J., Eliasson, I., Olsen, B., Herrmann, B. (2010) A novel Chlamydiaceae-like bacterium found in faecal specimens from sea birds from the Bering Sea. *Environmental Microbiology Reports*, 2(4):605-610

II Figueiredo, J., Söderberg, O., Simões-Correia, J., **Grannas, K.**, Suriano, G., Seruca, R. (2013) The importance of E-cadherin binding partners to evaluate the pathogenicity of E-cadherin missense mutations associated to HDGC. *European Journal of Human Genetics*, 21(3):301-309

Reviews

I Zieba, A., **Grannas, K.**, Söderberg, O., Gullberg, M., Nilsson, M., Landegren, U. (2012) Molecular tools for companion diagnostics. *New Biotechnology*, 29(6):634-640

II Gremel, G., **Grannas, K.**, Sutton, LA., Pontén, F., Zieba, A. (2013) *In situ* Protein Detection for Companion Diagnostics. *Frontiers in Oncology*, 3:271

Book Chapters

I Koos, B., Andersson, L., Clausson, CM., **Grannas, K.**, Klaesson, A., Cane, G. Söderberg, O. (2014) Analysis of protein interactions in situ by proximity ligation assays. *Current Topics in Microbiology and Immunology*, Springer Berlin Heidelberg, 377:111-126

Contents

Abbreviations

BiFC	Bimolecular fluorescence complementation
BRET	Bioluminescence resonance energy transfer
CTC	Circulating tumor cell
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EndoIV	Endonuclease IV
FFPE	Formalin fixed paraffin embedded
FLIM	Fluorescence lifetime imaging microscopy
FPALM	Fluorescence photoactivation localization microscopy
FRET	Förster resonance energy transfer
HCR	Hybridization chain reaction
HRP	Horse radish peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
In situ PLA	*In situ* proximity ligation assay
K_D	Dissociation constant
LOD	Limit of detection
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
PALM	Photo-activated localization microscopy
PCA	Protein-fragment complementation assay
PDGF(BB)	Platelet-derived growth factor B-chain
PLA	Proximity ligation assay
PTM	Post translation modification
RCA	Rolling circle amplification
RNA	Ribonucleic acid
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
SPR	Surface plasmon resonance
TGFβ	Transforming growth factor beta
UNG	Uracil-DNA glycosylase

Introduction

Life started on earth billions of years ago. How and in what form we can probably never know for sure but that life form has developed into a huge range of organisms, from simple single-celled bacteria to large complex organisms with their individual cells collaborating to make up organs and systems. The cell theory was established in the late 1830's by Matthias Schleiden and Theodore Schwann, when they stated that cells are the building blocks of all plant and animal tissue[1]. The human being is far from the largest organism on earth, compare us to the blue whale that can reach a weight of 200 tons. Still our bodies consist of about 40 trillion cells[2], building up all our organs and tissue, interacting to make us one single organism. All cells in a human body contain an identical, or almost identical, copy of our genomic DNA. It is the regulation of which part of the DNA is expressed and transcribed to messenger RNA, and in the next step which proteins translated, that decides the functional status of the cell. These proteins are responsible for almost all cellular functions such as cell structure, movement and communication[3].

The proteins largely determine the characteristics of a cell and their presence can be used as a marker of the cell status. But mere knowledge of the presence or absence of a protein in a cell usually contributes insufficient information. Knowing the amount and cellular localization will give a hint of the protein function but knowing if the protein is alone or takes part in complexes with other proteins further improves our knowledge. During my PhD studies I have been working with development and use of *in situ* proximity ligation assays (*in situ* PLA) for visualizing and analyzing proteins, protein-protein interactions and post translational modifications (PTMs) where they naturally occur, i.e. *in situ.*

Signaling networks - Communication and coordination

All the cells in an organism are engaged in a complex communication with each other to share information and to coordinate functions and activities. The cells act within their environment by enacting their differentiated gene programs, responding to stimuli from their surroundings and communicating with other cells. One approach to communication between cells is the release

of ligands that can bind to receptors of other cells to start a series of events; a signaling pathway. The signaling is initiated by the binding of a ligand to a receptor, creating a conformational change that causes recruitment of other proteins to the receptor. This is the starting point of a signal transduction, converting the signal to a cellular response[3]. Through the signaling pathway information is transferred by PTMs[4], protein-protein interactions [5] and translocation of proteins. This results in either activity change of existing proteins or in a change in DNA transcription, for example by protein binding to DNA[6]. Signaling pathways regulate important functions such as proliferation, differentiation, migration and apoptosis[7-10]. Describing this signaling as independent pathways is really a too simplified explanation. The signaling pathways interact with each other and should rather be seen as complex signaling networks[11].

Activity in the cell signaling networks is directly linked to the cell functions and status. Aberrant activity in a signaling pathway disrupts the control system and normal functions of a cell, and can cause diseases such as cancer [12]. Studying the cell signaling pathways and their interactions gives us information of how to characterize healthy and unhealthy cells and what differentiate them from each other[11,13]. Cancer cells often carry the properties of uncontrolled growth and genetic instability [14] leading to heterogeneity within tumors [15]. As the cancer cells divide they will compete with both other cancer cells and healthy cells about space and nutrition[16]. Metastatic cancer can be spread by cancer cells detaching from the main tumor by epithelial-mesenchymal transition (EMT). The detached cancer cells can enter the blood stream in the form of circulating tumor cells (CTC) and invade new tissue to form daughter tumors after mesenchymal-epithelial transition (MET)[17,18]. The combination of response to the external environment and the high mutation rate in many cancers further increases the diversity within and between tumors [19].

The standard *in vitro* approach of studying cell activity often involves studying the average of proteins, or protein-protein interactions, of a cell population. Besides loosing information about the cell heterogeneity this also omits spatial information. Studying cell signaling calls for methods to detect endogenous proteins as well as study their activity with single cell resolution to retain information about the heterogeneity of cell populations.

Methods for studying proteins *in situ*

Proteomics can be divided into expression proteomics; studying the abundance and location of proteins and functional proteomics focusing on protein activities such as interactions, PTMs and signal transduction[20]. A signaling

pathway can consist of a series of protein interactions and PTMs, transmitting the information from the cell membrane, through the cytoplasm and to the nucleus[21,22]. As signaling is a consequence of both the genetic program and the cell's interaction with its environment, detecting these events *in situ* will provide information on cellular communications and response to micro-environmental cues.

There are several methods available to detect the important protein-protein interactions and PTMs. This work revolves around one of these methods, *in situ* PLA. To give a perspective of available methods I will start by introducing a few other methods for detection of protein activity and interactions.

There are many methods available for detection of protein interactions *in vivo,* such as yeast-two-hybrid systems[23] and its mammalian equivalent[24], or *in vitro*; electrophoresis mass spectrometry (MS) in combination with gel electrophoresis[25], Surface plasmon resonance (SPR)[26,27], immunoprecipitation and TAG systems[28]. Even though these methods have proven very useful, they generally lack the ability to observe cell-cell variations. Hence, to be able to reveal heterogeneity and cellular communication analysis needs to be performed *in situ* on endogenous proteins, which calls for additional methods. This work will focus on methods primarily used *in situ.*

Immunohistochemistry (IHC) (figure 1a) and immunofluorescence (IF) (figure 1b) are two commonly used methods for detection of endogenous proteins *in situ*[29,30], both methods are used in many clinical applications[31,32]. The methods utilize antibodies, carrying marker molecules, to bind to the target protein. The marker molecule, that can be an enzyme or a fluorophore, is then visualized by wide-field or confocal microscopy. By using two different reporter molecules the co-localization between two proteins can be measured and indicate an interaction but these approaches are limited by light microscopy resolution or that of the signal generating system used (i.e. colored precipitates formed by attached enzymes). Enzymatic limitations and the difficulty to resolve colors in bright field imaging limit opportunities for multiplexing IHC, making IF an attractive alternative to study co-localization. The sensitivity of IF depends on the strength of the specific fluorescent signals compared to the autofluorescence background from the sample, a problem of greater concern when studying weakly expressed proteins. Both IF and IHC have the possibility to utilize secondary affinity binders; probes targeting the primary affinity binder. Using secondary binders the amount of reporter molecule per target molecule can be increased. IHC and IF have no proximity condition for signal generation, thereby these methods are highly dependent on the ability to visualize and analyze the localization with high resolution.

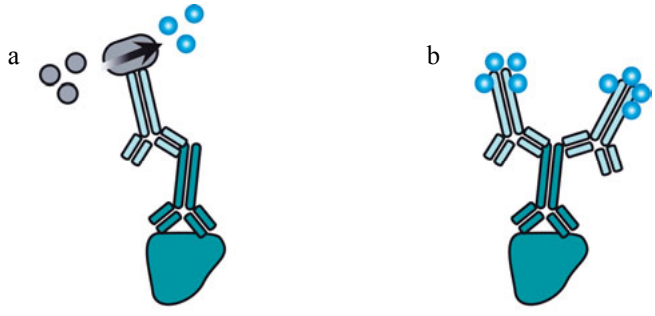

Figure 1. Protein detection using IHC and IF.

(a) IHC; secondary antibody carrying horseradish peroxidase (HRP) binds to the primary antibody. The HRP converts substrate to colored product to be visualized by bright field microscopy. **(b)** IF; fluorescently labeled secondary antibodies bind to the primary antibody. Visualization can be done by fluorescence microscopy.

Optical imaging by fluorescence microscopy is used in research and clinics all over the world. For a long time the resolution; the shortest distance between two imaged objects before they seems to amalgamate[33], was limited by the diffraction barrier. This limiting the resolution to around half of the wavelength of the light used to image the sample[34]. Developments in microscopy during recent years, such as stimulated emission depletion (STED) microscopy [35], photo-activated localization microscopy (PALM) [36], fluorescence photoactivation localization microscopy (FPALM) and stochastic optical reconstruction microscopy (STORM)[37] have moved the detection limits beyond the diffraction barrier down to around 20 nm[34,38,39]. In 2014 Erik Betzig, Stefan Hell and William Moerner were rewarded with a joint Nobel Prize in chemistry for their work developing super-resolved fluorescence microscopy. Even with the great developments in microscopy, identifying co-localization by fluorescence intensity in individual pixels will often end up with a poor resolution when considering the size ranges in a cell and between proteins. Furthermore the equipment for super-resolution imaging is expensive and requires training to use thus is not commonly available. Methods with a proximity condition for the generation of a detectable signal does not remove the need for high resolution imaging but enables detection of molecular proximity, rather than optical proximity identified by co-localization of pixels.

Förster resonance energy transfer (FRET) can be utilized to measure intra- and intermolecular distances between 2 and 10 nm. FRET is a phenomenon of distant dependent energy transfer between two fluorophores (in some applications called fluorescence resonance energy transfer), one donor and on acceptor fluorophore. The basic principle of FRET (figures 2a and 2b) is that the donor fluorophore absorbs a photon and thereby becomes temporari-

ly excited to a higher electron state. The excess energy can be transferred from the donor to the acceptor, without emission of the photon, by a dipole-dipole energy transfer if the following criteria are fulfilled; (i) an acceptor fluorophore is between 2 and 10 nm from the donor, (ii) the emission spectrum of the donor overlaps with the absorbance spectra of the acceptor and (iii) the donor and acceptor are sufficiently aligned to enable the donor to induce an acceptor dipole. The transfer efficiency is directly correlated to the distance between donor and acceptor and can be used as a distance measurement. There are several parameters to detect and measure the transfer efficiency; change in donor lifetime by fluorescence lifetime imaging microscopy (FLIM), quenching of donor fluorescence intensity, increase of the acceptor fluorescence intensity or a change in the fluorescence polarization (the orientation of the fluorophores)[40,41]. The change in fluorescence polarization, as well as changed distance can also be used to detect conformational changes of proteins[42,43]. By fusing the acceptor and donor with the target protein pairs in a complex, FRET can be used to study the dynamics of an interaction *in vivo* as well as *in situ*[44,45] If the acceptor and donor fluorophores are linked to antibodies targeting the proteins of interests then it is possible to detect interactions between endogenous proteins *in situ*[46,47]. There are also FRET methods available to study single proteins as well as three party complexes[48]. It is important not to confuse the distance between the donor and acceptor labeled proteins with the distance between the actual target proteins. This is especially important when using acceptor/donor-coupled antibodies since there is some spatial flexibility in the donor/acceptor – antibody construct.

While useful and precise in many cases, FRET detection assays still struggle with issues some of which were addressed by the development of bioluminescence resonance energy transfer (BRET). BRET, like FRET, is based on distance dependent energy transfer between two molecules. In BRET the donor is bioluminescent luciferase and the acceptor is a fluorophore (figures 2c and 2d). Upon oxidation of its substrate luciferase emits luminescence light that will excite the acceptor. This causes a measurable increase of fluorescence intensity of the acceptor fluorophore.

In contrast to FRET the energy transfer between acceptor and donor in BRET is independent of their orientation due to the spherical distribution of luminescence emitted by the luciferase. Since BRET, in contrast to FRET, does not require optical excitation, the risk of photo bleaching and problems with background fluorescence are minimized[49,50]. BRET on the other hand depends on local access to substrate. Originally BRET showed less sensitivity compared to FRET, resulting in problems with single cell resolution and intracellular resolution. However, further development of BRET has improved sensitivity and enabled successful detection of interactions on subcel-

lular level[51-53]. Both FRET and BRET have problems with detection of low abundant proteins. The two methods are also mostly often used in applications where the acceptor and donor protein domains are fused to the target proteins, which might alter the protein properties and which precludes studies of endogenous proteins[54].

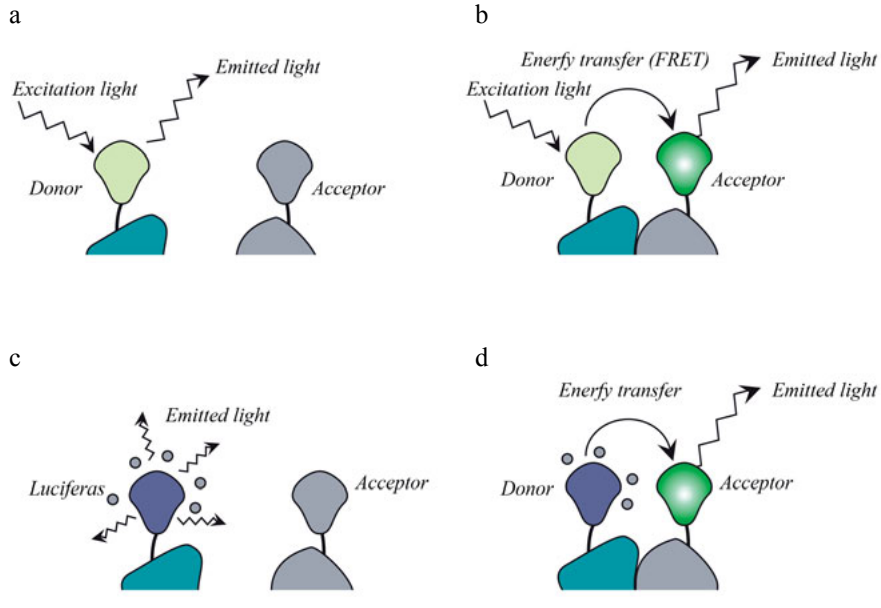

Figure 2. Detection of protein interactions using Förster resonance energy transfer (FRET) and Bioluminescence resonance energy transfer (BRET).

FRET **(a)** Two interaction partners not yet in interaction. One fused with a donor fluorophore and the other with an acceptor fluorophore. **(b)** Interaction between the two interaction partners brings the donor and the acceptor into 2-10 nm distances from each other, enabling energy transfer from the donor to acceptor. Excitation of the acceptor causes emission of light. BRET **(c)** Two interaction partners that are not in complex, one fused with luciferase and the other with an acceptor fluorophore. The luciferase converts substrate, which generates emitted light. **(d)** Interaction between the two proteins brings the luciferase close enough to excite the acceptor causing emission of light of a longer wavelength.

FRET and BRET both generate a detectable signal only if two molecules are in proximity of each other. This is however not always the same thing as being members of the same complex or in direct contact. The protein-fragment complementation assay (PCA) is a method where two, individually inactive, fragments of a reporter molecule are fused to two interaction partners of interest. Upon interaction the fragments are brought in proximity and can form an active reporter molecule. There are several versions of PCA,

many based on the formation of functional enzymes, and other on the formation of fluorescence reporter molecules, also called bimolecular fluorescence complementation (BiFC)[55,56]. By using multiple pairs of split fluorescence reporters BiFC can be multiplexed. BiFC has been shown to offer greater sensitivity than FRET and BRET, and it is suitable for detection *in vivo*. BiFC does not depend on the orientation of the interaction partners as FRET and does not require delivery of a substrate as in BRET, but the technique cannot be used to measure the distance, although the proximity requirements can be varied by the length of the linkers[57-59]. The methods have also successfully been combined in BRET-BiFC and FRET-BiFC[60,61].

PCA and BiFC are limited to ectopically expressed proteins since the reporter fragments must be fused to the target proteins. VeraTag is a technology (also used in the HerTag assay) for detection of endogenous proteins and protein-protein interaction in formalin fixed paraffin embedded (FFPE) tissue, and thereby suitable for analysis of patient samples. Even though the method is not detecting localized events *in situ*, the method is of clinical use and therefore well worth mentioning. VeraTag utilizes pairs of antibodies as affinity probes for the interacting proteins. One antibody is attached to a fluorescence reporter molecule and the other antibody to a biotin molecule. After binding of the two antibodies to the sample, streptavidin conjugated to sensitizer methylene blue is added, binding to the biotin labeled antibody. The sample is illuminated which releases reactive single state oxygen from the photosensitizer. The short lived oxygen will affect only its close surrounding, enabling a proximity dependent cleavage of the marker fluorophore from the antibody. The detached fluorophore can be collected and analyzed by capillary electrophoresis[62-64]. The VeraTag technology can only provide information at the bulk level and fails to offer spatial information about the target proteins.

Proximity ligation assay – visualizing proximity

The *in situ* proximity ligation assay is a method for visualizing proximity between two or three interacting molecules[65] *in situ*. The method is a further development of the proximity ligation assay (PLA), originally developed to detect proteins and protein interactions *in vitro*. In the original publication PLA utilized pairs of DNA-aptamers as affinity probes to detect homodimers of platelet-derived growth factor B-chain (PDGF-BB) or thrombin. The DNA-aptamers were extended with additional DNA sequences. Upon binding within proximity the two additional DNA sequences could hybridize to a common connector oligonucleotide, allowing the ends to be covalently joined by enzymatic ligation. The ligation product was then quantified by real time PCR[66].

In situ PLA was developed to visualize proximity between endogenous proteins using pairs of antibodies as affinity probes (figure 3). Each antibody is conjugated to an oligonucleotide creating a PLA probe. When a pair of these probes binds in proximity to each other, they can template the hybridization of two additional oligonucleotides to form a circular DNA molecule. After ligation by T4 ligase the circular DNA molecule can be amplified by phi29 DNA polymerase, in a process called rolling circle amplification (RCA), using one of the probes as a primer. The phi29 polymerase continuously displace the newly produced strand from the template DNA circle to continue the RCA, thereby producing a long single stranded DNA molecule consisting of repeats complementary to the DNA circle. One hour of phi29 polymerase-driven RCA generates a single DNA molecule, consisting of approximately 1000 monomers of complements to the 100 bases nucleotide DNA circle. The RCA product remains attached to the protein complex via one of the PLA probes. The DNA circle includes a detection sequence to allow the RCA products to be visualized by the hybridization of hundreds of fluorophore labeled oligonucleotides, detection oligonucleotides, complementary to the repeated detection sequence in the RCA product. Each such product is seen as a bright dot of about 1 μm size by fluorescence microscopy[65,67]. The great amount of fluorophores per RCA product decreases problems with background fluorescence from the sample compared to IF and FRET/BRET. Every RCA product serves as a localized marker for proximal binding of the two *in situ* PLA probes.

By introducing secondary *in situ* PLA probes; oligonucleotide-coupled species-specific pairs of antibodies can be used as general *in situ* PLA probes in multiple applications where primary antibodies of different species are used[68]. A drawback of using secondary *in situ* PLA probes is the size of the primary antibody-secondary *in situ* PLA probe complex, increasing the distance criteria for detection. Besides antibodies, recombinant affinity binders, such as DARpins, can be utilized in *in situ* PLA[69].

Immunohistochemical detection methods have a widespread use and are not affected by autofluorescence from the sample. By attaching HRP to a detection oligonucleotide the RCA product can be enzymatically visualized by a protocol for bright-field microscopy *in situ* PLA[70]. Besides protein-protein interaction *in situ* PLA has been used for detection of protein-RNA interaction[71], protein-DNA[72] interactions and PTMs[73].

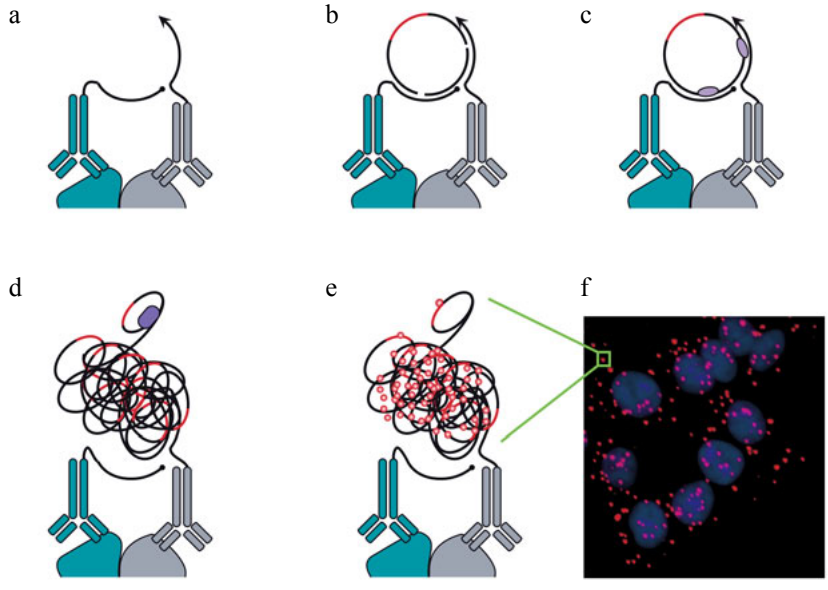

Figure 3. Principle of *in situ* PLA

(a) Two PLA probes; oligonucleotide equipped antibodies, binds to the target proteins. **(b)** Upon proximal binding the PLA probes acts as template for hybridization of two subsequently added oligonucleotides. **(c)** The ends of the hybridized oligonucleotides are ligated by T4 ligase to form a circle. **(d)** The DNA circle is amplified by Phi29 driven RCA. **(e)** The RCP product is hybridized by fluorescently labeled oligonucleotides. **(f)** Visualization of cell nuclei (blue) and RCA product (red) by epifluorescence microscope. In this example detection of Mek-Erk interaction in Hct116 cells.

Studying cell signaling pathways using *in situ* proximity ligation assay

In situ PLA can be used to study signaling pathways in their natural environment, revealing heterogeneity among cell populations and within tissue samples[74]. Important information about the activity status of different members of the signaling pathway ca be obtained by monitoring and quantifying interactions and PTMs involved in signal transduction[75].

The hippo signaling pathway is one of the control systems for cell growth and organ size, deregulation of the pathway is associated with tissue overgrowth and tumorigenesis. The pathway was first discovered in *Drosophila melanogaster* with many components strongly conserved in humans[76]. At active signaling the two co-transcription factors of the Hippo signaling pathway; Yap and Taz, are translocated to the nucleus where they can promote transcription. The Hippo signaling pathway is partly regulated by cell-

to-cell contract and in dense cell cultures Yap and Taz become phosphorylated and bind to the 14-3-3 protein binding site. As a consequence Yap and Taz are accumulated in the cytoplasm and degraded by ubiquitin-mediated proteolysis[77,78]. The controlling components of the Hippo signaling pathway is relatively well understood but the up- and down-stream regulatory systems are not fully known[78,79]. As a part of the widespread cell signaling networks the Hippo pathway is believed to interact with components of other important pathways such as the transforming growth factor beta (TGFβ) signaling pathway. Yap and Taz have been shown to form complexes with and influence the nuclear shuttling of Smad2 and Smad3, effectors of the TGFβ pathway[80,81]. In the TGFβ signaling pathway Smad2 and Smad3 are phosphorylated upon interaction between TGFβ receptor I and II. Phosphorylated Smad2 and Smad3 forms a complex with Smad4 and enters the nucleus where they together with other transcription factors influence the transcription[8,82].

Using *in situ* PLA the activity of signaling pathways can be studied in cell cultures where both dense and sparse areas of cells are represented[83]. In **paper I (Crosstalk between Hippo and TGFβ - Subcellular localization of YAP/TAZ complexes)** we show density dependent formation of Yap-Smad2/3 and Taz-Smad2/3 complexes and visualize the subcellular localization of these complexes.

Signaling networks consists of proteins with important regulatory functions and many interaction partners, so called nodes or hubs, as well as proteins engaged in only a few interactions[84]. The different interaction options for theses nodes induce different signaling outcomes and alterations in the nodes has been shown to have a great effect on signaling and development of diseases[85-88]. Mapping protein nodes and their interaction partners as well as determining the balance between the different interaction events provides a more detailed picture of cellular functions. In **paper II (Parallel visualization of multiple protein complexes in individual cells in tumor tissue)** we present a multiplex *in situ* PLA method for simultaneous detection of multiple protein complexes *in situ*.

Characteristics of a successful detection method

As all other detection methods *in situ* PLA has its advantages and drawbacks. The list of characteristics of a successful method for detection of molecular events will change depending on the application and the user. But some characteristics are more general and to start with the obvious; a detection method need to be sensitive and specific. Enough sensitive to detect the amount of molecules in a sample, and specific to detect the analytes intended with a minimum of false positives.

The limit of detection (LOD) is the lowest number of analytes that can be detected in a robust and reproducible fashion. LOD is often defined as the number of molecules that give rise to a detection signal either 2 or 3 standard deviations above the background[89,90]. The sensitivity and LOD depends on; the background signal, signal variation among replicates and detection efficiency. Detection with affinity reagents *in situ* has many causes contributing to the background; unspecific binding of the affinity reagents, unspecific binding of the detection reagents and background autofluorescence from the sample are a few examples. Interactions detected by *in situ* PLA are visualized by the binding of detection oligonucleotides to RCA products, creating high local concentrations of fluorescence that are relatively easy to distinguish from background fluorescence and non-specifically bound detection oligonucleotides. *In situ* PLA has the advantage that detection by two, or sometimes three, independent affinity binders is required to produce a detection signal. This serve to reduce risks of false positive signals due to unselective affinity binders. The use of commercial antibodies makes the method versatile and easy for users to apply towards their specific research question. That said, one must always take care to find the best binders available for the application as well as do proper validation. An antibody can cross react with a related epitope on an irrelevant molecule, or bind to a closely related similar, homologous protein. Furthermore *in situ* PLA detects proximity not interaction, this is something the method has in common with other methods such as FRET, BRET, and IF. Even though the distance criteria is small enough to indicate interaction, there is always the possibility that the proteins are just very close to each other.

A high detection efficiency for a detection method implies a low LOD and often also a wide dynamic range. 100% detection efficiency would mean that every single target gives rise to a detectable signal. This is not the case for *in situ* PLA, or any other *in situ* detection method. There are many factors limiting the detection efficiency of *in situ* PLA; the dissociation constant (K_D) of the affinity binders, the efficiency of the enzymatic steps, the composition and complexity of the sample as well as the design of the oligonucleotide system. All these factors can also be affected by the experimental conditions such as buffers, temperatures and time of reaction. One particular factor that may limit the detection efficiency of *in situ* PLA is that not every interaction will give rise to a circular DNA ligation product, even when the two interaction partners are successfully bound by PLA probes. This can be due to inefficient enzymatic reactions but it might also be caused by the design of the oligonucleotide system used for the PLA probes. Furthermore the formation of a circular ligation product can be limited by assembly of several independent oligonucleotides prior to ligation. In **paper III (Increasing efficiency of *in situ* proximity ligation assay by enzymatic conversion dependent proximity probes**) we present and evaluate the efficiency of three

different oligonucleotide systems by their ability to form a circular ligation product as well as detect PTMs *in situ.* The oligonucleotide systems evaluated are; *in situ* PLA[65], ReLig *in situ* PLA and Unfold *in situ* PLA.

ReLig *in situ* PLA

ReLig *in situ* PLA is a further development of the previous presented *in situ* PLA detection of protein-DNA interaction [72]. The system is designed to limit the difference in concentration and ratio between the *in situ* PLA probe and the circularization oligonucleotides. In ReLig *in situ* PLA one of the PLA probes to carry the circularization oligonucleotide while the other PLA probe is necessary for priming the RCA reactions, creating a one-to-one ratio between PLA probe and circularization oligonucleotide.

The ReLig *in situ* PLA probes are equipped with linear oligonucleotides (figure 4). One of the PLA probes carries a padlock-like oligonucleotide that includes two hairpin structures. The carrier PLA probe is blocked from priming the RCA reaction by three 2'-O-methyl bases at the 3'-end. The two hairpin structures contain deoxyribonucleic uracil and can thereby be opened for hybridization to the second PLA probe by enzymatic digestion. The uracil is released by Uracil-DNA Glycosylase (UNG) and the apurinic/apyrimidinic sites are removed by Endonuclease IV (EndoIV). The now open oligonucleotide can hybridize to the second PLA probe, and together with an additional tag oligonucleotide, filling in a short gap between the two ends of the circularization oligonucleotide, form a circle that can be ligated and amplified by RCA. Both the circularization and the tag oligonucleotides contain detection sequences. This enables dual colored RCA products and also ensures that the RCA product detected is generated from hybridization with the second PLA probe. Probe specific tag oligonucleotides can also be used for multiplexing of the method.

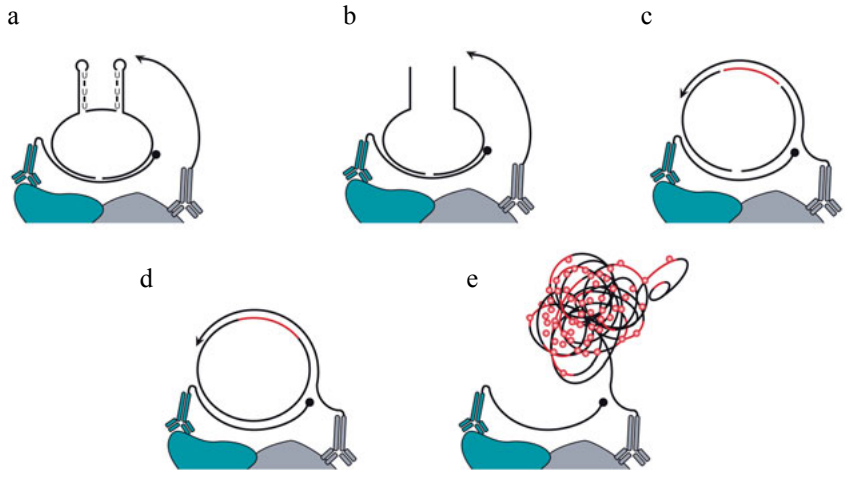

Figure 4. The principle of ReLig *in situ* PLA.

(a) The PLA probes, one probe carrying a circular oligonucleotide, binds to two interacting proteins. **(b)** The circle is opened by enzymatic digestion and now available for hybridization to the other probe. **(c)** The open circle and a tag oligonucleotide hybridize to the second PLA probe. **(d)** The circle is closed by ligation. **(e)** The DNA circle is amplified by RCA and the RCA product is visualized by hybridization of detection oligonucleotides.

UnFold *in situ* PLA

The UnFold *in situ* PLA design includes two PLA probes, consisting of antibodies conjugated to self-hybridizing oligonucleotides. One of the probes includes the circularization oligonucleotide sequence (figure 5). This probe consist of a hairpin structure with a large loop, at the end of the loop three deoxyribonucleic uracil residues are situated. The second probe consists of a hairpin structure with multiple deoxyribonucleic uracil residues in the stem of the hairpin shape. The deoxyribonucleic uracil residues can be enzymatically digested by UNG and EndoIV. Upon enzymatic digestion the probe containing the circularization oligonucleotide sequence is cleaved in two parts; one part remains attached to the antibody and hybridized to the other part containing the circularization sequence. The other probe oligonucleotide will after digestion remain as a single stranded sequence and can now template circularization of the DNA strand of the first PLA probe when bound in proximity. Finally the circle is enzymatically closed and the circular DNA molecule can be amplified by RCA.

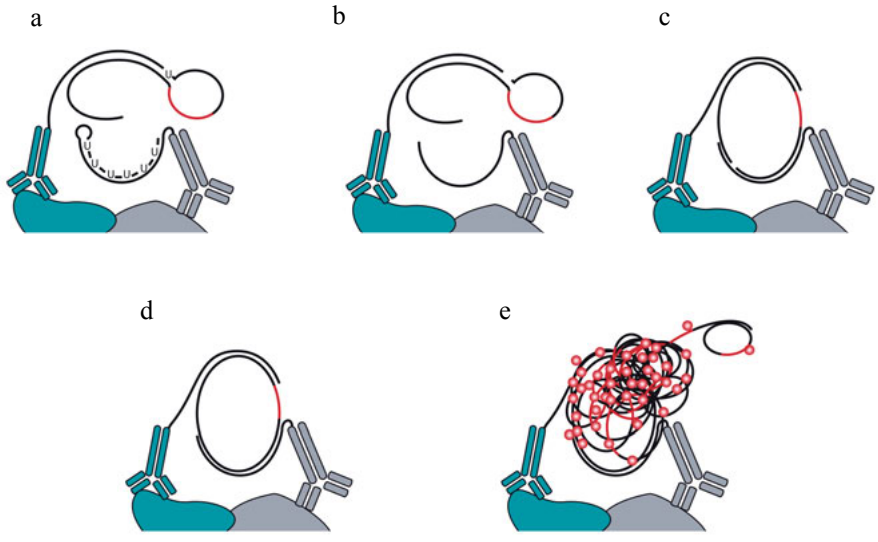

Figure 5. The principle of UnFold *in situ* PLA.

(a) The UnFold *in situ* PLA probes. **(b)** Enzymatic digestion open the self-hybridized hairpin structure. **(c)** Upon proximity the UnFold *in situ* probes can hybridize to each other forming a circle. **(d)** After ligation the closed circle can be amplified by RCA and the RCA product can be visualized by hybridization of detection oligonucleotides.

ReLig *in situ* PLA and UnFold *in situ* PLA are designed aiming to reduce the fraction of non-circular ligation products. The UnFold *in situ* PLA design also reduces the number of ligation events needed to form a circle. The drawback is the addition of another enzyme dependent reaction, the digestion, which could possible reduce efficiency and increase cost.

Removing the enzyme dependency

A method that is complicated, expensive and that requires dedicated equipment will in most cases be of less use than an easy and cheap method that can be performed using standard equipment. The equipment needed to perform *in situ* PLA is mostly standard equipment found in most labs; possibility to heat to 37 °C and a bright-field or fluorescence microscope. But the reactions also depend on enzymes that, besides increasing the cost, have high demands on storage and control of the reaction temperature. Removing the enzyme dependency is a step toward a less expensive method with less demand on storage. The basic principles that makes *in situ* PLA unique is the proximal binding by pairs of affinity reagents providing localized detection with high specificity, and the amplification of detection signals to exceed background, enabling quantification of single detection events.

Recently an enzyme free amplification method based on sequential DNA hybridization called hybridization chain reaction (HCR) was described[91]. DNA is a versatile building block that is relatively easy to manipulate to use for different applications. By design of a DNA sequence a single stranded DNA molecule can self-hybridize or hybridize to other molecules into different secondary structures[92]. HCR is based on four meta-stable single-stranded DNA molecules that self-hybridize into hairpin structures; two HCR probes and two elongation oligonucleotides. Even though these molecules can hybridize to each other their strong self-hybridization prevents them from doing so. Exposure to an activator sequence that invades the stem of one of the HCR probes, renders part of the stem that now can invade the second HCR probe. The now opened second HCR probe will in turn invade one of the first elongation oligonucleotides, starting a chain reaction of these hybridization events that create a long double-stranded DNA molecule consisting of tiles of opened hairpins held together by base paring[91,93]. By using elongation oligonucleotides conjugated to fluorophores the hybridization product will contain a large amount of fluorophores. HCR has been utilized to detect cytokines by attaching the initiator to an antibody in a sandwich immunoassay format[94]. The method has also been shown to be possible to multiplex[95].

In **paper IV (Proximity dependent initiation of hybridization chain reaction**) we present a modified approach of *in situ* proximity probes that combines detection of proximity with the enzyme free signal amplification of HCR; proxHCR (figure 6). In order to introduce proximity dependence for detection of protein complexes and PTMs a pair of antibodies was equipped with two different oligonucleotides containing hairpin structures. In the presence of an activator oligonucleotide one of the hairpins opens up and can invade the other hairpin structure of the second proximity probe, provided that two proximity probes have bound in close proximity. This will lead to exposure of the initiator sequence that previously was hidden in the stem of the second proximity probe and will start a hybridization chain reaction. The reaction is driven by release of potential energy in the hairpin structure and will in presence of additional free fluorescently labeled DNA hairpins create hybridization products possible to detect by fluorescence. The reaction can continue until all available DNA hairpins are exhausted.

By avoiding enzyme-dependency the method results in a robust, cost efficient procedure. This is a step towards more portable methods adapted to point-of-care devices as well as for high throughput screening.

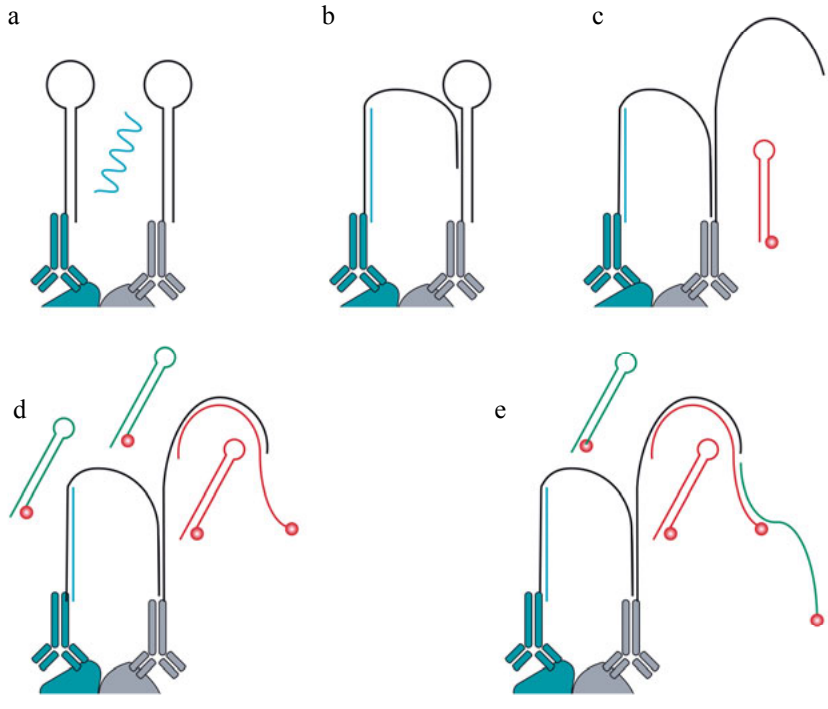

Figure 6. The principle of proxHCR.

(a) The proxHCR probes are brought in proximity by binding to two interacting proteins. **(b)** An activator oligonucleotide invades the oligonucleotide of one of the proxHCR probes, leaving parts of the invaded probe free to invade the second proxHCR probe. **(c)** The second proxHCR probe is invaded and now partly open to invade the hairpin shaped elongation oligonucleotide **(d)** The newly opened proxHCR probe invades a fluorescently labeled elongation oligonucleotide. **(e)** Elongation by invasion and hybridization of fluorescently labeled oligonucleotides.

Present investigations

Paper I: Crosstalk between Hippo and TGFβ - Subcellular localization of YAP/TAZ complexes

Introduction

The hippo signaling pathway is involved in functions such as growth control and proliferation, it is associated with the regulation of organ size and linked to tumorigenesis. The main effectors, Yap and Taz, and their roles in the regulation of the pathway have been described, but up- and downstream regulation remain only partially characterized[78,96]. The pathway is inactive in dense cell cultures, where cell-to-cell contacts cause phosphorylation and cytoplasmic retention of Yap and Taz[79,97]. Yap and Taz have been reported to be involved in the regulation of other important signaling pathways, one of them being the TGFβ signaling pathway by interaction and regulation of nuclear shuttling of the main effector proteins Smad2/3[80,81]. Smad2 and Smad3 are phosphorylated by the activated TGFβ ligand receptor complexes; the phosphorylation induces the formation of Smad2/3-Smad4 complexes that accumulate in the nucleus where they regulate transcription[8,98]. The aim of the project was to analyze the complex formation and complex localization of Smad2/3-Yap and Smad2/3-Taz complexes.

Procedure, findings and discussion

We investigated the effect of cell density and TGFβ treatment on the nuclear shuttling of Smad2/3/4, Yap and Taz in HaCaT and in Smad4 deficient HT29 cells. We showed that treatment with TGFβ causes a strong nuclear accumulation of Smad2 and Smad3 in sparse cell areas but less so in dense cell areas of HaCaT cells, while Smad4 display less density dependent nuclear shuttling. In Ht29 cells Smad2 show a similar pattern as in HaCaT cells while Smad3 shows less TGFβ induced nuclear accumulation. None of the cell lines had any change in total Smad expression upon TGFβ treatment. In HaCaT cells Yap and Taz accumulated in the nucleus in sparse growing cells, while being mainly located in the cytoplasm in dense cell areas. In HT29 cells Yap showed a nuclear accumulation in sparse growing cells but in dense growing cells Yap could be observed in both nuclei and in cyto-

plasms. Taz was absent in the nuclei of dense HT29 cells but was present in both nuclei and cytoplasms in sparse cells.

The hippo signaling pathway has been associated with nuclear shuttling of Smads by interactions with Yap and Taz. We therefore continued by examining the formation of Smad2/3-Yap and Smad2/3-Taz complexes. We detected complex formation between Smad2/3 and Yap or Taz in HaCaT cells by immunoprecipitation while no complexes were found in HT29 cells. The localization of complexes was determined by using *in situ* PLA to visualize the increased formation of both Smad2/3-Yap and Smad2/3-Taz complexes upon stimulation with TGFβ in sparse HaCaT cells. The complexes were mainly located in the nuclei. In dense cell cultures less TGFβ induced complex formation was seen, and the complexes were predominantly localized in the cytoplasm. No complexes could be visualized in the HT29 cells using *in situ* PLA. To determine if the absence of complexes might be caused by Smad4 deficiency we treated HaCaT cells with siRNA targeting Smad4 and obtained a successful knockdown. This did not alter the expression or localization of any of the proteins analyzed other than Smad4. The Smad4 siRNA treatment did not decrease the number of Smad2/3-Yap complexes but the treatment resulted in more cytoplasmic complexes compared to no siRNA treatment. Also the siRNA control resulted in a reduced number of nuclear complexes. The reason for this difference in nuclear shuttling upon siRNA treatment is not known. The pattern of density dependent complex formation by TGFβ treatment was not affected by siRNA treatment.

Paper II: Parallel visualization of multiple protein complexes in individual cells in tumor tissue

Introduction

Many important cellular functions are regulated by cell signaling pathways in which the information is transferred between and throughout the cells by protein-protein interactions and protein phosphorylations. These pathways interact in large signaling networks, affecting each other. In the signaling networks there are hubs consisting of proteins with many interaction partners, while other proteins have only a few possible interactions. These hubs have important regulatory functions and aberrations in the hubs can have grave consequences[85,99]. The original *in situ* PLA technique[65] is described as a method for visualizing complexes involving two or three proteins. In this study we present a new version of the *in situ* PLA probe in which a protein specific sequence is inserted in the oligonucleotide of the *in situ* PLA probe. A probe specific tag must then be included to form a circular ligation prod-

uct upon hybridization of oligonucleotide to two *in situ* PLA probes bound in proximity to each other. The circle is amplified by RCA and the RCA product can be visualized by hybridization of detection oligonucleotides that uniquely recognize the tag sequence. This design enables simultaneous and localized visualization of the interaction partners of alternative protein-protein complexes.

As a proof of concept we visualize pairwise interactions between EGFR, HER2 and HER3, forming homo- and heterodimers, in breast cancer tissue. EGFR, HER2 and HER3 are all members of the epidermal growth factor family and can form homo- and heterodimers upon receptor activation. The total expression levels of EGFR and HER2 are used as prognostic and predictive biomarkers, but it has been shown that also the dimerization level has impact on drug effects[100,101].

Procedure, findings and discussion

The selectivity of the *in situ* PLA probes were verified by detection of EGFR, HER2 and HER3, one at a time, in transfected PAE cells, expressing different combinations of EGFR, HER2 and HER3. These results were congruent with detection of protein expression obtained by Western blot.

We continued with visualizing proximity between EGFR, HER2 and HER3 in fresh frozen human breast cancer tissue. The tissues had been characterized by immunohistochemistry measuring HER2 expression and been scored from 0+ (no visible stain) to 3+ (strong stain). Initially we used a general HER2-binding probe (with no tag sequence) in combination with specific (tag-containing) EGFR-, HER2- and HER3-binding probes. This way we could detect all three versions of HER2 complexes (HER2-HER2, HER2-EGFR and HER2-HER3). We saw higher levels of HER2 complexes in 3+ tissues compared to the 0+ tissues. We continued with replacing the general HER2-binding probe with general EGFR- and HER3 binding probes to visualize all pairwise complex alternatives. Complexes consisting of EGFR and HER3 were less abundant than the HER2 containing complexes in both 3+ and 0+ tissues, but the pattern of higher level of complexes in 3+ tissues compared to 0+ tissues remained.

This new *in situ* PLA probe design enables visualization of proximity of combinatorial proteins in fixed cells and tissue. The level of multiplexing by this design alone is limited by the number of wavelengths possible to distinguish by fluorescence microscopy but can be increased by serial hybridization of detection oligonucleotide[102]. Multiplexing allows several measurements on the same sample, which is valuable when working with precious

patient samples. Furthermore multiplex *in situ* PLA offers simultaneous visualization of several concurrent complexes with subcellular resolution.

Paper III: Increasing efficiency of in situ proximity ligation assay by enzymatic conversion dependent proximity probes

Introduction

The limit of detection of a method is coupled to the efficiency of the method. The efficiency of *in situ* PLA is limited by several factors such as enzyme activity, affinity reagents and oligonucleotide quality but also the design of the proximity probes and the oligonucleotide system. The formation of the correct circular DNA molecule depends on the assembly and correct hybridization of four different oligonucleotides and there is a risk that non-circular ligation products are formed besides the circular products required for detection.

In this study we present two oligonucleotide systems; ReLig *in situ* PLA and UnFold *in situ* PLA, designed to reduce the fraction of non-circular ligation products. Both the new systems are dependent on enzymatic conversion to form circular ligation products. The ReLig *in situ* PLA system carries the parts of the DNA circle by hybridization to one of the PLA probes. After enzymatic conversion the circle part can, together with a tag oligonucleotide, form a full circle by hybridization to the second PLA probe. The circle can be amplified by RCA. In the UnFold *in situ* PLA design, the circle parts are integrated in the sequence of one of the PLA probes. After enzyme treatment the circle parts are available for hybridization to the second PLA probe and can form a circular DNA molecule.

We evaluate the ability of the new *in situ* PLA systems to form circular ligation products in solution and to detect PTMs *in situ*. The systems are compared to the original *in situ* PLA design[65].

Procedure, findings and discussion

The original *in situ* PLA was shown to form both circular and linear ligation products by performing the ligation reaction coupled to beads. The ReLig and UnFold *in situ* PLA systems were designed aiming to limit the formation of non-circular ligation products. Their predicted secondary structures were analyzed using the Nupack nuclei acid system. To ensure that the additional

enzymatic step, the digestion by UNG and EndoIV, would not be limiting for the enzymatic conversion the efficiency of the different steps was evaluated and estimated to be almost 100% efficient. The efficiency in forming circular ligation products was evaluated and the original *in situ* PLA design displayed a larger proportion of non-circular ligation products compared to the other two designs, when the reaction was performed in solution.

All the designs successfully detected increase of phosphorylated epidermal growth factor receptor (EGFR) in A431 cell upon treatment with EGF. The UnFold *in situ* PLA system showed higher detection efficiency than the other two designs while The ReLig *in situ* PLA system showed less ability to detect the elevated phosphorylation level. The designs were also tested to detect elevated levels of phosphorylated Erk protein in Hct116 cells after stimulation with Phorbol-12-myristate-13-acetate (PMA). The UnFold *in situ* PLA system detected more phosphorylation events compared to the *in situ* PLA system, but the two designs showed similar ability to detect elevations of phosphorylated Erk. The ReLig *in situ* PLA system displayed inferior detection of elevated of phosphorylation.

In this study we present two enzymatic conversions dependent proximity probe designs; ReLig *in situ* PLA and UnFold *in situ* PLA, aiming to increase the detection efficiency of *in situ* PLA. The UnFold *in situ* PLA system show increased detection efficiency of phosphorylated proteins compared to the original *in situ* PLA design. Furthermore the design of the UnFold *in situ* PLA should enable easy multiplexing by adding a probe specific tag hybridization requirement and we hope to further develop this design to use for efficient multiplex detection of protein events in fixed cells and tissues.

Paper IV: Proximity dependent initiation of hybridization chain reaction

Introduction

The dependence on enzymatic reactions makes *in situ* PLA relatively expensive and with requirements for temperature regulation and storage. In this study we present proxHCR as a method for detecting protein complexes and PTMs, combining the proximity dependent dual binding requisite of *in situ* PLA[65] with the enzyme free hybridization chain reaction (HCR)[91] for signal amplification. By avoiding the requirements for enzymes the method is robust, inexpensive and possible to use with a minimum of equipment.

ProxHCR, like regular *in situ* PLA, utilizes a pair of antibodies with conjugated oligonucleotides as affinity binders. The oligonucleotides on the antibody pairs each have a hairpin structure. The DNA hairpin structures will remain stably self-hybridized until one of them is invaded by an activator oligonucleotide, opening up the hairpin structure. If the two antibodies bind in proximity to each other, for example to two interacting proteins in a complex, the free part of the opened affinity probe can invade the second probe. This will leave the second affinity probe with part of the sequence available for hybridization. In the presence of two additional fluorophore-conjugated hairpin shaped oligonucleotides this will start a hybridization chain reaction forming a fluorescently labeled amplification product that serve as a reporter molecule for the interaction.

Procedure, findings and discussion

We designed five species of oligonucleotides, four of them self-hybridizing and the fifth as a linear activator oligonucleotide. Two of the self-hybridizing oligonucleotides are conjugated to pairs of antibodies as parts of the proximity probes. The additional two hairpin shaped oligonucleotides are attached to fluorophores and acts as building blocks for the long hybridization product, that serves as a reporter molecule. These are added to the reaction upon removal of unbound proxHCR probes. A substantial part of the study was devoted to optimizing the oligonucleotide designs for proximity detection. For example mismatches were added to the proxHCR probe sequences to suppress generation of false positive signals.

The design of the interacting oligonucleotides was evaluated by surface plasmon resonance (SPR), showing efficient binding of the activator oligonucleotide and efficient hybridization of the two proxHCR probe oligonucleotides as well as the hybridization of the elongating oligonucleotides. The Opera high content screening system was used to determine the accumulation of fluorescence by elongation, showing a concentration dependent increase in fluorescence after 5 minutes of incubation. By epifluorescence microscope we observed no further increase of fluorescence after 30 minutes of HCR. Quantification of the signals is done based on fluorescence intensity, rather than counting individual amplification products.

ProxHCR as a means for detection of protein complexes and PTMs *in situ* was evaluated by comparison with established *in situ* PLA assays. We demonstrated successful detection of E-cadherin-β-catenin interactions in cells and in FFPE tissue sections. In preparations of cultured cells we also showed complexes of BCL2-BNP3, LC3-SQSTM1 and Mek-Erk, and we detected phosphorylation of PDGF-receptor β, Akt and Syk.

ProxHCR showed successful detection of both protein complexes and phosphorylations. In contrast to *in situ* PLA the method does not rely on enzymatic steps, which reduces its demands on storage and overall costs.

Summary and future perspectives

In this thesis I emphasize the importance to study not only protein expression but also the activity of proteins. It has been shown that the expression level of proteins alone, including proteins today used as clinical biomarkers, often do not serve as prognostic and predictive biomarkers [103,104], hence there is a need to study also the activity of proteins. Many well-established methods for evaluation of proteins, protein complexes and PTMs fail to observe the cell-to-cell heterogeneity in both cells and tissue. One important application when detecting molecular events is characterizing the molecular events in a population of cancer cells i.e. cancer tissue. Knowing that there is a great heterogeneity both between and within tumors[15], it is not enough to analyze the average of a cell population.

In paper I we present how *in situ* PLA can be used to visualize molecular events of crosstalk between the Hippo and the TGFβ signaling pathways. Complex formation is visualized with single cell resolution, showing differences depending on the microenvironment (cell-to-cell contact) within the same culture. Subcellular resolution facilitates analysis of how the molecular events act in the different compartments of the cell (cytoplasm vs. nuclei). The Hippo signaling pathway has been reported to be involved in the regulation of multiple important signaling pathways by interactions between Yap/Taz and the regulatory elements of other pathways.

The cell density regulation of the hippo signaling pathway has been suggested as a sensor also affecting the TGFβ signaling pathway. But recent papers report that basal–lateral restriction of the TGFβ receptors also limits Smad signaling, upon TGFβ stimulation, in high-density cell cultures[105,106]. This further emphasizes the need of monitoring the crosstalk between signaling pathways *in situ* to understand the impact of cell-to-cell contract. I believe there is a use for *in situ* PLA, together with other methods, in the continuous work to unravel the complex crosstalk between the Hippo signaling pathway and its regulation of interacting signaling systems.

In paper II we developed a method able to simultaneously visualize multiple molecular events in a sample. By introducing target specific tag sequences into *in situ* PLA probes we could simultaneously detect three different complexes in cells and tissue. Multiplexing detection methods are time and cost

efficient, and maybe most importantly reduce the amount of patient material used. Multiplexing beyond the three-plex system presented in this study would be beneficial but adds demands on the read-out since an epifluorescence microscope only can resolve a few emission spectra. This problem could be solved by sequential hybridization, with the drawback of increasing the hands-on-time needed and the risk of sample degradation. The problem will need some careful consideration but it would be well worth the benefit of a high-grade-plex system.

In paper III the efficiency of *in situ* PLA is discussed. In the original *in situ* PLA an assembly of two additional oligonucleotides besides the proximal binding of the PLA probes is required. Altogether four different DNA strands must hybridize correctly prior to successful ligation to form an amplifiable circle. We present two new oligonucleotide designs, both with the circularization oligonucleotide brought by one of the PLA probes. This aims to facilitate the formation of the correct circular ligation product and reduce the loss of efficiency due to non-amplifiable ligation products. We found that the UnFold *in situ* PLA system shows the potential of being an efficient alternative to the original *in situ* PLA system. By introducing a probe specific tag sequence in one of the arms there is a potential for multiplexing the UnFold *in situ* PLA system. This system should undergo further tests to ensure that the increase of efficiency is not outbalanced by the extra enzymatic digestion step added. I think it would be beneficial to further evaluating enzyme free means to make the oligonucleotide systems available for hybridization.

In situ PLA is a relatively easy method with no need for advanced equipment besides a light- or epifluorescence microscope and temperature regulated incubation. The enzymes used are a large contribution to the cost and the enzymes also need to be stored at specific temperatures. In paper IV we present an enzyme free detection method for proximity; proxHCR. As a proof of concept we show successful detection of protein complexes and protein phosphorylations *in situ*. We believe that the enzyme-free proxHCR method will provide advantages for inexpensive and robust high throughput detection of molecular events. The read-out is based on fluorescence intensity, which together with the low demands on equipment makes the method suitable for development into portable and point of care devises.

The perfect detection method can measure every single analyte, in all samples, regardless of complexity, without generating any false positive nor false negative and do so every time. Of course the method is also cost efficient, fast and easy to use as well as portable with no need of advanced equipment. Unfortunately this method does not exist. Nor should we hold our breath until the perfect method has been developed. When working with

development of methods we have to keep the purpose of the method in mind. Sometimes there is need for *in situ* detection with sub-cellular resolution, sometimes it is more important to use rapid and cost-effective analysis of an average cell population, and maybe the two can be combined. *In situ* PLA has been proven to be a useful tool for studying molecular events and it has potential to be further developed into more diverse and versatile applications.

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