UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 674

Visualizing Interacting Biomolecules *In Situ*

IRENE WEIBRECHT

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2011

ISSN 1651-6206
ISBN 978-91-554-8078-3
urn:nbn:se:uu:diva-151579

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Wednesday, June 1, 2011 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract
Weibrecht, I. 2011. Visualizing Interacting Biomolecules *In Situ*. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 674. 55 pp. Uppsala. ISBN 978-91-554-8078-3.

Intra- and intercellular information is communicated by posttranslational modifications (PTMs) and protein-protein interactions, transducing information over cell membranes and to the nucleus. A cells capability to respond to stimuli by several highly complex and dynamic signaling networks provides the basis for rapid responses and is fundamental for the cellular collaborations required in a multicellular organism. Having received diverse stimuli, being positioned at various stages of the cell cycle or, for the case of cancer, containing altered genetic background, each cell in a population is slightly different from its neighbor. However, bulk analyses of interactions will only reveal an average, but not the true variation within a population. Thus studies of interacting endogenous biomolecules *in situ* are essential to acquire a comprehensive view of cellular functions and communication.

In situ proximity ligation assay (*in situ* PLA) was developed to investigate individual endogenous protein-protein interactions in fixed cells and tissues and was later applied for detection for PTMs. Progression of signals in a pathway can branch out in different directions and induce expression of different target genes. Hence simultaneous measurement of protein activity and gene expression provides a tool to determine the balance and progression of these signaling events. To obtain this *in situ* PLA was combined with padlock probes, providing an assay that can interrogate both PTMs and mRNA expression at a single cell level. Thereby different nodes of the signaling pathway as well as drug effects on different types of molecules could be investigated simultaneously.

In addition to regulation of gene expression, protein-DNA interactions present a mechanism to manage accessibility of the genomic DNA in an inheritable manner, providing the basis for lineage commitment, via e.g. histone PTMs. To enable analyses of protein-DNA interactions *in situ* we developed a method that utilizes the proximity dependence of PLA and the sequence selectivity of padlock probes.

This thesis presents new methods providing researchers with a set of tools to address cellular functions and communication in complex microenvironments, to improve disease diagnostics and to contribute to hopefully finding cures.

Keywords: proximity ligation, in situ PLA, padlock probe, rolling circle amplification, flow cytometry, in situ, single cell, single molecule, protein interaction, protein-DNA interaction, posttranslational modification

Irene Weibrecht, Department of Immunology, Genetics and Pathology, Molecular tools, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-554-8078-3
urn:nbn:se:uu:diva-151579 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-151579)

To my beloved family

”It is very easy to answer many of these fundamental biological questions;
you just look at the thing!”

Richard P. Feynman

List of Papers

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

I Jarvius M[*], Paulsson J[*], **Weibrecht I**[*], Leuchowius KJ, Andersson AC, 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

II Leuchowius KJ, **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

III **Weibrecht I**[*], Grundberg I[*], Nilsson M, Söderberg O (manuscript) *Simultaneous visualization of both signaling cascade activity and end-point gene expression in single cells.* Accepted for publication in PLoS ONE

IV **Weibrecht I**, Gavrilovic M, Lindbom L, Landegren U, Wählby C, Söderberg O (manuscript) *Visualizing individual sequence-specific protein-DNA interactions* in situ. Submitted

[*] *equal contribution*

Reprints were made with permission from the respective publishers.

Related Work by the Author

Peer Reviewed Research Articles

Mahmoudi S, Henriksson S, **Weibrecht I**, Smith S, Söderberg O, Strömblad S, Wiman KG, Farnebo M (2010) *WRAP53 is essential for Cajal body formation and for targeting the survival of motor neuron complex to Cajal bodies*. PLoS Biol. 8(11): e1000521

Weibrecht I, Böhmer SA, Dagnell M, Kappert K, Östman A, Böhmer FD (2007) *Oxidation sensitivity of the catalytic cysteine of the protein-tyrosine phosphatases SHP-1 and SHP-2*. Free Radical Biol Med 43(1): 100-10

Review Articles

Leuchowius KJ, **Weibrecht I**, Söderberg O (2011) In situ *proximity ligation assay for microscopy and flow cytometry.* Curr Protoc Cytom 9.36.1-16

Weibrecht I, Leuchowius KJ, Clausson CM, Conze T, Jarvius M, Howell WM, Kamali-Moghaddam M, Söderberg O (2010) *Proximity ligation assays: a recent addition to the proteomics toolbox*. Expert Rev Proteomics 7(3): 401-9

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

Book Chapter

Grundberg I[*], **Weibrecht I**[*], Landegren U (2010) *Amplified single molecule detection.* Handbook of Nanophysics. Principles and methods, CRC Press, Taylor & Francis Group, ISBN: 978-1-4200-7540-3, pp 40-1-11
[*]*equal contribution*

Contents

Abbreviations

ACTB	β-actin mRNA
BiFC	Bimolecular fluorescence complementation
BJhTert	Immortalized human foreskin fibroblast cell line
bp	Base pair
BRET	Bioluminescence resonance energy transfer
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DUSP6	Dual specificity phosphatase 6 mRNA
EGF(R)	Epidermal growth factor (receptor)
EndoIV	Endonuclease IV
ERK	Extracellular signal-regulated kinase
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FLIM	Fluorescence life time imaging
FRET	Förster (Fluorescence) resonance energy transfer
GFP	Green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
H3K4 / 9	Histone H3 lysine 4 / 9
Hek293	Human embryonic kidney 293 cell line
HER2-4	Human epidermal growth factor receptor 2-4
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
In situ PLA	*In situ* proximity ligation assay
ISH	*In situ* hybridization
Kb	Kilobase
LNA	Locked nucleic acid
m	Meter
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MCF-7	Human breast adenocarcinoma cell line
MEK	Mitogen-activated protein kinase kinase
miRNA	MicroRNA
MKP	Mitogen-activated protein kinase phosphatase

mRNA	Messenger RNA
NIH3T3	Mouse embryonic fibroblast cell line
nm	Nanometer
OLA	Oligonucleotide ligation assay
PAEα/β	Porcine aortic endothelial cell line expressing PDGFRα/β
PCA	Protein fragment complementation assay
PCR	Polymerase chain reaction
PDGF-BB / -DD	Platelet-derived growth factor-BB / -DD
PDGFRα/β	Platelet-derived growth factor receptor alpha / beta
PDI	Protein-DNA interaction
pEGFR	Phosphorylated EGFR
PI3K	Phosphoinositide 3-kinase
PLA	Proximity ligation assay
PPI	Protein-protein interaction
PRINS	Primed *in situ* labeling
PTM	Posttranslational modification
pY	Phosphorylated tyrosine
qPCR	Quantitative PCR
RCA	Rolling circle amplification
RCP	Rolling circle product
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SH2	Src homology 2
SINE	Short interspersed nuclear element
SKOV-3	Human ovary adenocarcinoma cell line
SNP	Single nucleotide polymorphism
SOS	Son of Sevenless
TNFα	Tumor necrosis factor alpha
TSA	Tyramide signal amplification
U343	Human neuronal glioblastoma cell line
UNG	Uracil-DNA glycosylase
VEGFR	Vascular endothelial growth factor receptor

Introduction – take a closer look

The human body consists of about 10^{13} cells[1], all containing the same ~3 x 10^9 bp of genomic DNA[2], a ~2 m long molecule[3] packed into the nucleus. What information cells use from their genomic DNA, e.g. which genes and miRNA are expressed, will differ substantially between cell types and the expressed molecules span concentration ranges from only a couple of molecules up to millions of them[4]. Complexity is further increased by taking into account all intercellular communication that is required to sustain a multicellular organism. All cells in a given population, even within clonal expansions from each other – e.g. in immortalized cell cultures – are slightly different because of position in cell cycle or access to stimuli from surrounding cells. Even the molecules present in each cell vary: proteins with different modifications, expressed mRNAs or accessibility of certain DNA-sequences for transcription factors. When looking at this impressive complexity, it seems amazing that an organism like the human body functions properly most of the time, ensured by a concert of interacting molecules, transporting signals within one cell and from one to another, directing cells to e.g. division, differentiation or apoptosis. How cells cope with this seemingly overwhelming amount of – sometimes contradictory – information that they receive in every second of their lives can yet only be partly appreciated. But not until we understand how processes in a cell and body interweave and synergize, will we be able to efficiently treat diseases that occur when this teamwork is perturbed.

Despite this knowledge, investigations of signaling networks and regulating events have for a long time been recorded as averages for large numbers of cells or molecules, often investigated *in vitro*. Unfortunately, it is quite likely that such average analyses hide intercellular variations, and analysis of individual cells will thus retrieve more complex, but also more reliable information (Figure 1)[5]. In particular, as soon as we investigate not cell cultures with clonal expansions from one precursor cell but mixtures of cells, patient samples or tissue sections, averaging also means ignoring the heterogeneity of different cell types within a tissue and what effects the composition of this tissue has on each individual cell. *In situ* studies and studies focusing on single cells have the advantage that these can be correlated to the surrounding tissues. Thereby additional local, morphological and, when molecules are investigated *in vivo*, in the living cell or organism, maybe even spatiotemporal information can be retrieved.

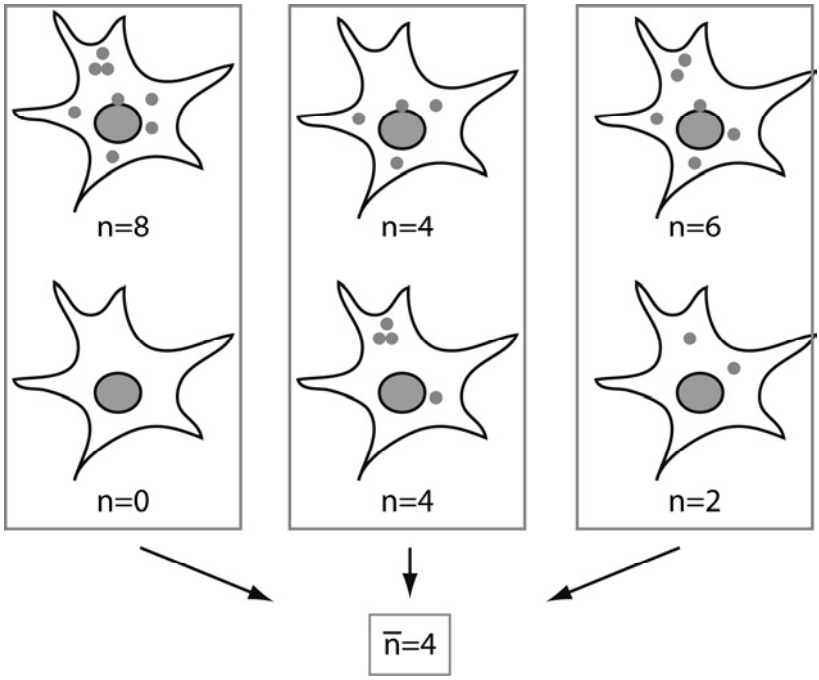

Figure 1. Difference between single cell- and average detection methods.

All three cell populations have an average of 4 molecules per cell, which would be the number detected by methods investigating the average of a sample. Studies with single cell resolution would however reveal different degrees of cell-to-cell variation in the left and right population and a truly homogenous distribution of signals in the middle panel.

Heterogeneity is not only found between individual cells but also between properties of individual molecules in these cells. In order to gain information about distribution of these molecules in time and space, their interaction partners and modifications, as well as about any changes upon different stimuli, molecules have to be studied individually. Even if not all molecules in a volume – for instance a cell – can be analyzed, single molecule analysis will result in more than one (average) data point for the whole sample and thereby strengthen the data[6-8].

Of course, not all questions need to, can or should be answered by single molecule / single cell methods. Very important findings were made with averaging methods and could only have been found in that way. Still, in order to comprehensively understand molecules, cells, tissues and organisms, in health and disease, we have to move closer, look deeper and in greater detail on what is happening to each molecule, in every cell in its natural environment.

Methods to study protein-protein interactions *in situ*

Cell signaling is achieved by a series of connected events were protein-protein interactions (PPIs) will add posttranslational modifications (PTMs) to interacting proteins, causing conformational changes and activating the modified protein for a subsequent PPI with another partner. This cascade of PTMs and PPIs can transmit and amplify signals through the different compartments of the cell, from the cellular borders to the nucleus and specific genetic loci, and it is regulated by feedback loops and crosstalk between pathways to ensure a controlled response to stimuli. Several methods have been developed to study such interactions *in vitro* either for screening purposes – e.g. 2D gel electrophoresis with mass spectrometry readout[9], yeast-two-hybrid systems[10-11] or protein microarrays[12-13] – or to investigate certain interactions in greater detail by methods like co-immunoprecipitation[14], protein fragment complementation assay (PCA)[15] and the proximity ligation assay (PLA)[16]. These methods have helped to unravel important information, however, with the exception of PLA they all provide averages for populations of molecules or cells. As pointed out above, *in situ* analyses are required in order to retrieve information on a single cell level.

To study proteins in intact cells and tissues, methods to recognize and label the proteins of interest are required, using either genetic modifications to directly attach a reporter molecule to the targeted protein, or affinity reagents (e.g. antibodies) to label the targeted protein with a reporter molecule.

Fluorescence immunostaining (IF) is an example of the latter, applying wide-field or confocal microscopy for readout. Interacting proteins are detected *in situ* by two different primary antibodies. Either these antibodies are directly labeled or they are in turn detected by fluorescence-labeled secondary binders. If the proteins are co-localized, the fluorophores also become co-localized in the cell (Figure 2). When the images derived from the individual fluorescence channels are superimposed, the area of co-localization can be calculated by comparing fluorescence intensities in each pixel of the image. Investigating PPI through confocal microscopy has the benefit that spatial information about the PPI can be obtained at microscopic resolution just below micrometers. In order to prevent detection of artifacts, the aim is to visualize interactions of endogenously expressed proteins, rather than overexpressed proteins, genetically modified with a fluorescence tag. However, in some cases, low abundance of proteins can make detection of few complexes challenging[17].

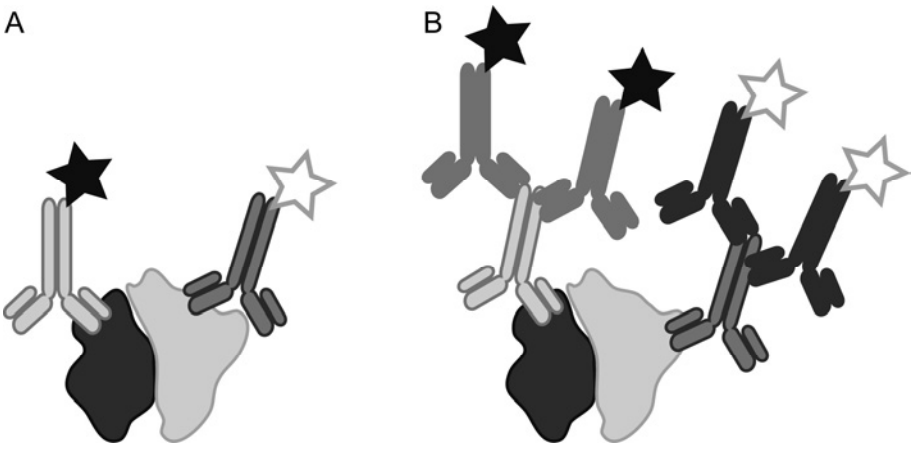

Figure 2. Immunofluorescence (IF).

(A) Interacting proteins are detected by directly fluorescence-labeled antibodies or (B) by primary antibodies derived from different species, which are in turn visualized by fluorescence-labeled secondary antibodies.

The sensitivity of immunostaining is highly dependent on whether the signal generated by binding of the affinity reagents, i.e. fluorescence-labeled primary or secondary antibodies, can be distinguished from background autofluorescence of the cells. Therefore, application of secondary fluorescence-labeled antibodies instead of directly labeled primary antibodies has the advantage of signal amplification: Every primary antibody can be bound by several secondary antibodies (Figure 2B). This leads to an increase of fluorophore concentration at the site where the primary antibody has bound and hence a stronger signal.

To increase the spatial resolution of PPI detection, methods based on resonance energy transfer can be used, reducing the distance required between two proteins to record them as interacting molecules. Förster resonance energy transfer (also: Fluorescence resonance energy transfer (FRET)) exploits a nonradiative energy transfer between two adjacent fluorophores with compatible excitation and emission spectra. Since the emission spectrum of a fluorophore is of a longer wavelength span than its excitation spectrum, a donor molecule can emit energy that will excite a second fluorophore (acceptor) (Figure 3). This is however only possible if the excitation spectrum is overlapping with the donors' emission spectrum and if the donors' emission dipole and the acceptors' excitation dipole are not perpendicular orientated towards each other. Furthermore, nonradiative energy transfer between donor and acceptor can only occur in a range of 2-10 nm. Beyond this distance, the energy has decayed too much to excite the acceptor molecule[18].

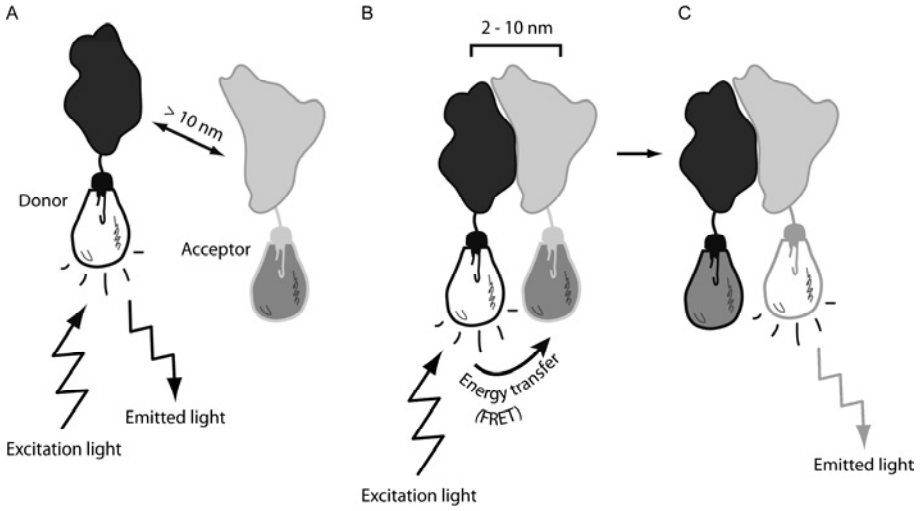

Figure 3. Förster resonance energy transfer (FRET).

(A) Two potentially interacting molecules are fused to two different fluorophores with overlapping excitation- and emission spectra. When the proteins are not interacting i.e. their distance from each other is larger than 10 nm, the donor is excited and emits light at a longer wave length. (B) Upon interaction of the molecules, the fluorophores are brought into FRET distance. If now the donor fluorophore is excited, an energy transfer to the acceptor fluorophore occurs, leading to (C) excitation of the acceptor which in turn starts to emit light.

Donor and acceptor molecules – fused to the proteins of interest – are transfected into cells to be studied. When the target molecules are interacting and thereby brought within FRET distance, energy transfer from the excited donor to the acceptor occurs, leading to immediate excitation of the acceptor. This energy transfer is detectable by different means, either by detecting the increase of acceptor fluorescence intensity, changes in donor lifetime (fluorescence lifetime imaging (FLIM)) or donor intensity upon addition or photobleaching of the acceptor. Alternatively changes of the fluorophore orientation towards each other can be detected[18]. By using FRET, the dynamics of proximity between two proteins can be detected *in situ* and *in vivo*[19]. Another possibility is to detect endogenous proteins with a pair of antibodies labeled with the donor and acceptor molecules, respectively[20]. Furthermore, protocols are available for single molecule detection *in situ*[21], as well as for detection of three interacting proteins[22].

Bioluminescent resonance energy transfer (BRET) is closely related to FRET, but employs a bioluminescent luciferase instead of the donor fluorophore. Under oxidative conditions, the luciferase converts a substrate, leading to the emission of light. If the acceptor fluorescent molecule is in close proximity, then Förster resonance energy transfer will transport the energy to the acceptor molecule and the fluorophore will be excited (Figure

4)[23]. BRET is able to circumvent some of the problems encountered with FRET e.g. the risk for simultaneous excitation of both, donor and acceptor fluorophores, due to overlapping excitation spectra. Furthermore, BRET reactions do not require a certain orientation of the interacting proteins towards each other since the energy that the luciferase sets free is spherically distributed. The assay is not impaired by autofluorescence and does not damage light sensitive tissue. BRET has recently been applied for detection of PPI in living mice[24] and at subcellular localization[25].

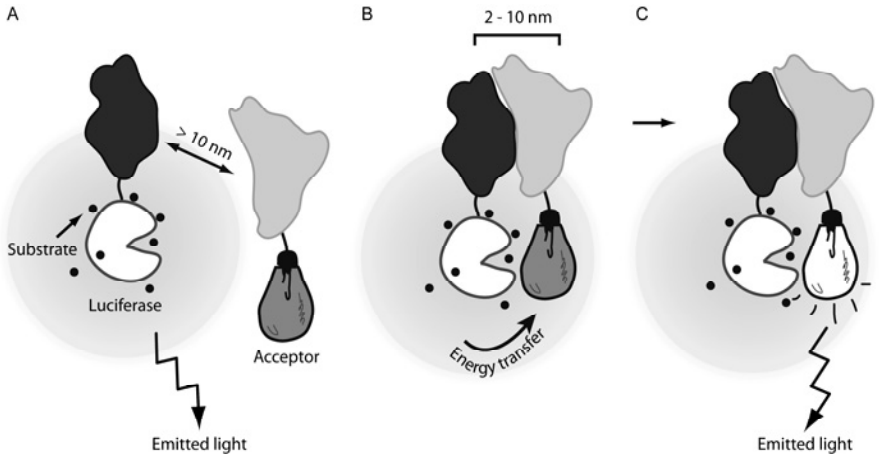

Figure 4. Bioluminescent resonance energy transfer (BRET).

(A) One of the interacting proteins is fused to a luciferase, the other one to a compatible fluorophore. The luciferase converts a substrate leading to the emission of light as long as the proteins of interest are spatially separated by > 10 nm. (B) When the proteins interact, the energy releasing enzyme and the fluorophore are brought in proximity to each other and hence the energy is transferred to the fluorophore, (C) excites it and light is emitted.

However, both FRET and BRET, by fusing the fluorophores / luciferase to the proteins of interest, alter the proteins and their function may be compromised. Hence careful controls are needed to prevent false-positive and -negative signals. Expression levels have to be sufficiently high, otherwise the energy transfer cannot be detected but not so high that the physiological function of the proteins is impaired. For FRET, precautions have also to be taken for the design of the fusion construct because the orientation of donor and acceptor towards each other plays an important role for FRET efficiency. Unfortunately, FRET is prone to photobleaching and difficult to use in samples with high autofluorescence, limiting its usefulness for studies in tissue sections. Furthermore, it may be challenging to find fluorophores with excitation and emission spectra suited for higher multiplexed FRET assays to simultaneously detect multiple interactions.

However, BRET also as drawbacks in that it requires a substrate which has to be delivered into the cell. Furthermore, the substrate is not locally anchored, and it is therefore difficult to create a localized signal[23].

PCA is another approach that can be used to detect PPIs, based upon reassembly of a split reporter molecule to obtain a functional reporter molecule. The approach was originally described using ubiquitin as the reporter molecule[15]. Bimolecular fluorescence complementation (BiFC) is a special case of PCA, based on the assembly of a fluorescent protein from two non-fluorescent domains, each domain fused to one protein of a complex. Upon complex formation of these proteins the fluorescent protein is reconstituted from both domains, hence fluorescence from non-interacting molecules is avoided (Figure 5)[26]. This property renders BiFC more sensitive than F/BRET[26-27]. When the two proteins to be investigated interact in a living cell, the location of the association can be visualized *in situ* and even weak and transient interactions can be detected as the interactions are preserved by the assembled fluorophores. Interaction partners do not need to be positioned in a certain orientation towards each other as it is the case with FRET, and the distance range for BiFC depends on the length of the linkers. An advantage of BiFC over other forms of PCA is that it does not depend on exogenous substrates[28]. BiFC can be utilized to study PPIs in living cells[29] and has been used for interaction studies in several organisms, e.g. mammalian cells, plants[30] and yeast[31]. Multiplexed detection is enabled by using several pairs of split fluorescent molecules[32].

The fact that BiFC suffers from a delay of one hour or more between complex formation of the proteins and reconstitution of the fluorescent molecule is however a drawback. Moreover, dissociation detection is difficult due to the stability of the reconstituted protein domains. These two properties severely limit opportunities to use the method for studies on dynamic events[26], and the stable complexes may interfere with cellular physiology.

Since it might be challenging to multiplex each method in itself, detection of higher order complex formation is accomplished through combinations of the methods described above: BiFC and FRET[33], BiFC and BRET[34] or FRET and BRET[35].

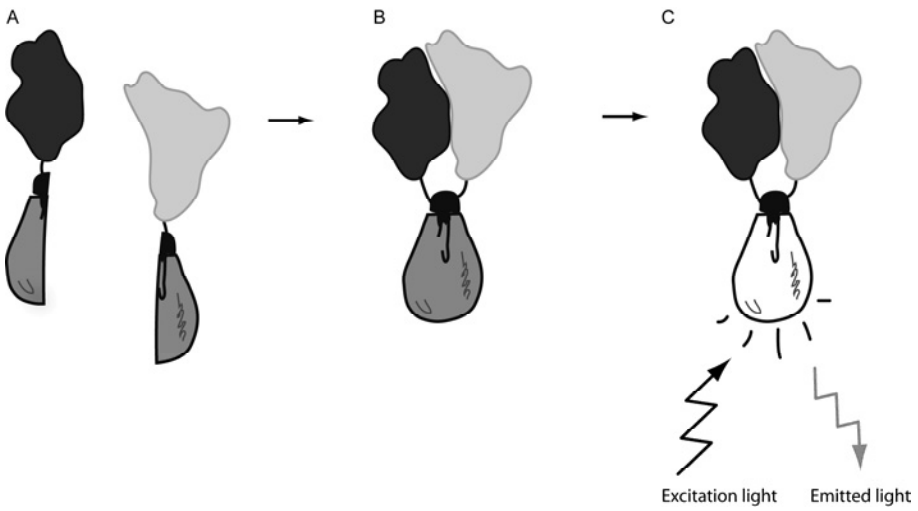

Figure 5. Bimolecular fluorescence energy transfer (BiFC).

(A) A fluorescent molecule is split into two halves that are fused to the proteins of interest. (B) Upon interaction of the target proteins the halves are brought in proximity to each other, and can reconstitute to form a fluorescent molecule (bulb), (C) which can subsequently be excited to emit light.

The methods discussed so far for *in situ* detection of PPI share the limitations that they often require the studied proteins to be fused to domains and transfected into cells, potentially leading to unphysiological overexpression. The fluorescence signal generated may be difficult to detect in cells and tissue sections with high autofluorescence. To overcome these limitations, namely to detect endogenous PPIs in fixed cell lines and tissues, the *in situ* proximity ligation assay (*in situ* PLA) was developed (Figure 6)[36]. This method is based on the proximity ligation assay (PLA)[16], where a double recognition of the protein of interest through oligonucleotide-carrying antibodies (PLA-probes)[37] or aptamers[16] is required to produce an amplifiable reporter molecule. Upon proximal binding of the PLA-probes, the oligonucleotides can be joined by ligation and amplified by a PCR reaction.

However, to allow localized detection of the protein complexes *in situ*, another method for DNA amplification is required, i.e. rolling circle amplification (RCA). For *in situ* PLA the oligonucleotides on the PLA-probes instead act as a template for hybridization and ligation of two subsequently added connector oligonucleotides, which can form a circular DNA molecule. Once ligated, this circular molecule can be amplified by RCA, primed from the oligonucleotide attached to the PLA-probe. After the first round of amplification, phi29 DNA polymerase – a polymerase with a strong strand displacement activity – displaces the newly created strand

leading to the production of single-stranded concatemeric complements of the original DNA circle[38-39]. As the RCA-priming molecule stays attached to the detected protein complex via the antibody, the amplified DNA-concatemer will remain located at the site where the protein complex was detected. Such immobilized RCA-products (RCPs) are visualized by hybridization of fluorescence-labeled detection oligonucleotides binding to a hybridization sequence encoded in the DNA-circle. Since the amplified product consists of hundreds of copies, hundreds of detection oligonucleotides will hybridize to the same molecule. The resulting bright signals, approximately one μm in size, are easily distinguishable from potential background fluorescence in an epifluorescence microscope. Each detected interaction event gives rise to one distinct RCP and it is therefore straightforward to enumerate detected complexes. *In situ* PLA provides an assay for detection of endogenous proteins and PPIs in cells and tissue sections, with a single-molecule resolution and the possibility for relative quantification of the signal. Further developments of the method include the development of a brightfield variant[40], application of secondary PLA-probes for detection of PTMs (paper I), flow cytometry as readout platform (paper II) and for detection of three proteins forming one complex[36].

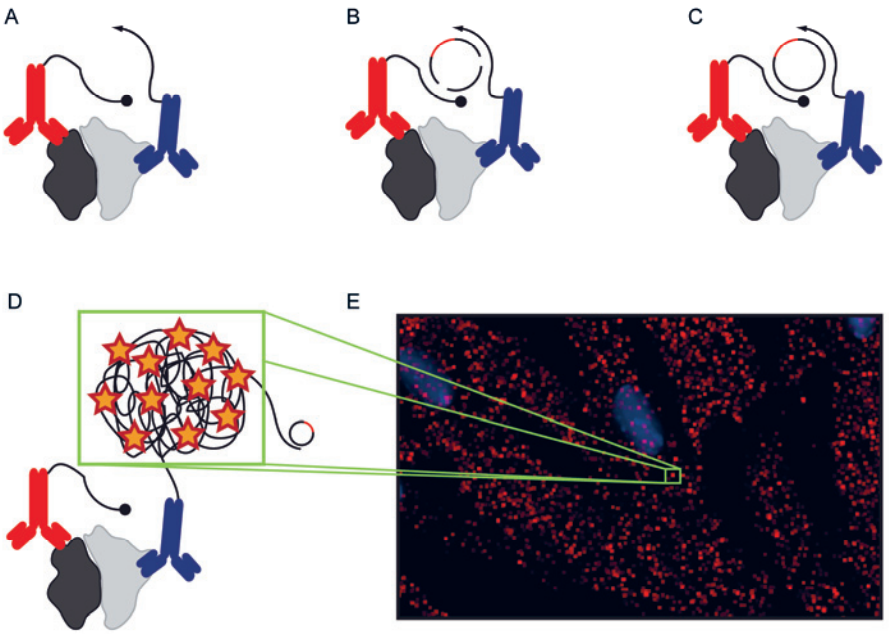

Figure 6. In situ proximity ligation assay (*in situ* PLA).

(A) Two PLA-probes (red, blue), consisting of an antibody and an oligonucleotide each, can bind to the protein of interest. (B) Upon complex formation, the PLA-probes are brought in proximity. Now two connector oligonucleotides can hybridize and (C) be ligated forming a circular molecule. (D) This DNA circle can now be amplified as a concatemeric copy of the circle by RCA and be detected with fluorescence-labeled detection oligonucleotides (stars). (E) Each resulting RCP and with that each detected interaction or modification (red dots, see green rectangle) is visualized as single bright spots in a fluorescence microscope. Here phosphorylated PDGFRβ in BJhTert cells detected by *in situ* PLA with secondary PLA-probes is depicted.

Methods to study protein-DNA interactions *in situ*

Although all cells in the body contain the same genomic DNA, the accessibility of this information for the cell is closely regulated by proteins, e.g. the DNA is tightly wrapped around histones organizing the DNA in nucleosomes. It is also proteins that direct transcription of the genes for expression (transcription factors and RNA polymerases) and copy DNA prior to cell division (DNA polymerases and ligases). For all such and many more events, proteins are interacting with nucleic acids. Therefore, I think that it is as important to study protein-DNA interactions (PDI) and histone modifications, how access of DNA is regulated and what impact that might have on a cell's fate *in situ*, as it is important to study protein and mRNA

expression, PPIs and protein modifications. However, today there is no method available to study individual PDIs in single eukaryotic cells and it can therefore only be hypothesized that there is an equally large variation between individual cells within a seemingly homogenous population as it has been seen for proteins and mRNA-molecules[41-43].

There are several methods that can extract information about proteins binding to DNA-sequences, one of the most popular ones being chromatin immunoprecipitation (ChIP)[44-45]. ChIP extracts DNA-sequences interacting with a protein by applying an antibody against the protein of interest to fragmented nuclear extract. By doing so, the DNA fragments bound to the protein will be retrieved together with the protein. After reversal crosslinking, DNA bound by the protein can be determined by PCR, microarrays (ChIP-on-chip) or sequencing (ChIP-seq)[46-47]. With ChIP, new interactions can be found but they are averaged over hundred[48] to millions of cells and no spatial information can be retrieved, rendering the method unsuited to study cell-to-cell variations.

PLA has also been applied for detection of PDIs. The binding of recombinant transcription factors to synthetic oligonucleotides spiked into nuclear extracts has been investigated. By using a panel of synthetic oligonucleotide sequences containing potential binding sites for the transcription factor, a consensus sequence for the transcription factor recognition site could be determined. As described above, one PLA-probe was used to detect the protein. Instead of the second PLA-probe, a part of the target DNA-sequence was allowed to participate in the ligation reaction. Upon binding of the protein to the right DNA-sequence, the oligonucleotide on the PLA-probe could be ligated to the bound DNA with the help of a connector oligonucleotide. Detection of interactions of the amount of proteins corresponding to 1-10 cells was analyzed using qPCR-readout[49].

Nevertheless, none of the methods described so far can be used to retrieve spatial information from individual cells – in which cells do these interactions or epigenetic modifications occur and how often and where in the tissue? In order to answer questions related to variation in the population, *in situ* methods are required. The major obstacle for such analyses is to find a compromise between making the DNA-sequence accessible and at the same time keeping proteins bound to the DNA. Furthermore, detection of a specific DNA-sequence can be challenging, especially if this sequence is short. For detection of PDIs *in situ* FRET based assays have been used. In one study the proteins to be analyzed were fused to green fluorescent protein (GFP), serving as donor fluorophore, and transfected into mammalian cells. A fluorescent dye, intercalating with genomic DNA, was used as acceptor (Figure 7). Upon close proximity between the investigated protein (e.g. the glucocorticoid receptor and heterochromatin proteins 1α and 1β) and the genomic DNA, energy is transferred from the excited GFP fused protein to the DNA intercalating dye and changes in donor lifetime can be measured by

FRET-FLIM[50]. Even though this method allows investigation of differences between individual cells, no information about the DNA-sequence can be retrieved.

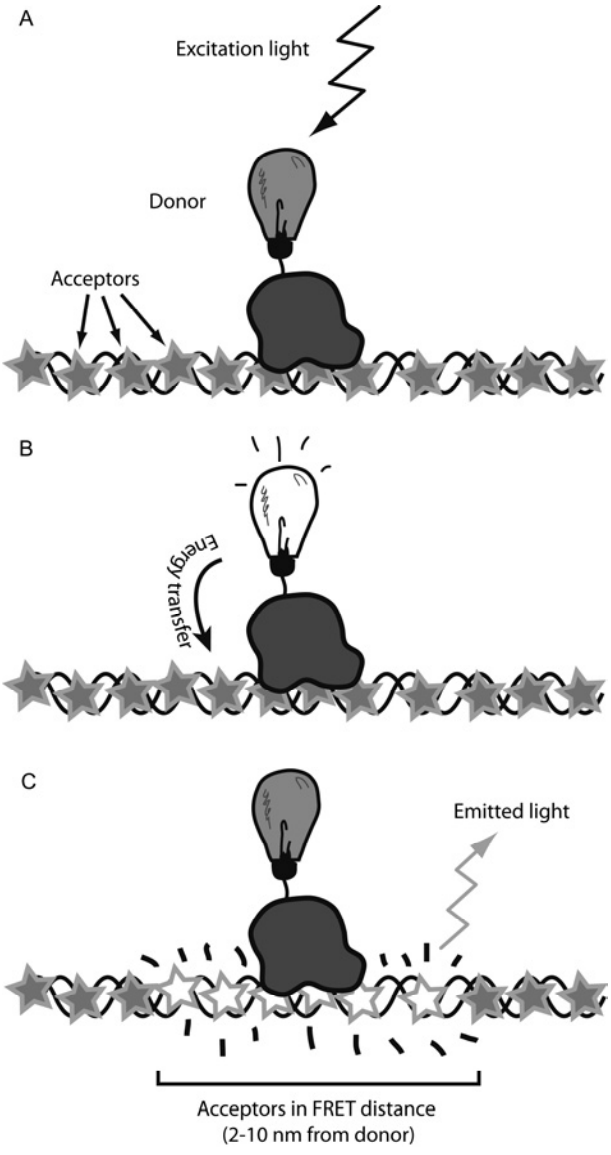

Figure 7. FRET-FLIM.

(A) Genomic DNA is labeled with a fluorescent dye suitable as FRET acceptor (dark grey stars) and DNA-binding proteins are labeled with the corresponding FRET donor. (B) The fluorophore on the protein can be excited and upon binding of the protein to the DNA FRET occurs between fluorescent molecules within FRET distance. (C) This leads to excitation of the acceptors which in turn start to emit light.

To detect a specific DNA-sequence and its co-localization with a particular protein, IF and fluorescence *in situ* hybridization were combined to immuno DNA fluorescence *in situ* hybridization (immuno DNA FISH) and immuno RNA FISH [51-53]. First, antibodies against the protein of interest are applied, followed by secondary fluorescence-labeled antibodies. The cells are then postfixated before the genomic DNA is denatured and the target sequence detected by hybridization of hapten labeled detection oligonucleotides.

These haptens can in turn be detected by IF utilizing antibodies against the label (Figure 8). By superimposing the different fluorescent channels, co-localization of the nucleic acid sequence and the protein can be detected and visualized *in situ*. Immuno DNA FISH has e.g. been applied for co-localization studies of histone methylations in chromosomal subdomains and at differentially expressed gene loci in human cells[54]. Although immuno DNA / RNA FISH presents a valuable tool for investigation of PDIs *in situ*, it is still limited in resolution since the target nucleic acid sequence is recognized by hybridization and has to be quite large (see below). Furthermore, spatial resolution is restricted to the resolution of the microscope used and proteins can only be detected if they are so abundant that IF can distinguish them from background.

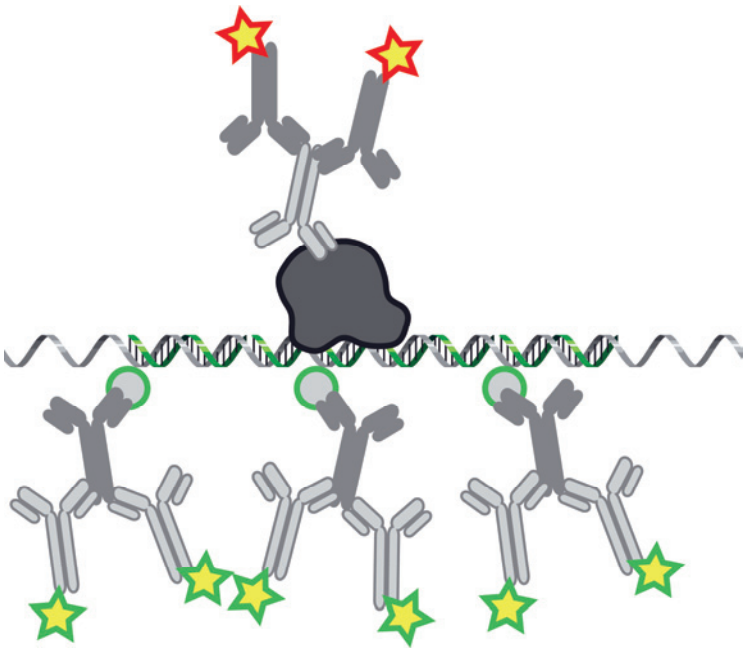

Figure 8. Immuno DNA / RNA FISH.

Upon fixation, the protein of interest is detected by IF and the secondary antibodies are labeled with one color (red stars). Subsequently, the DNA-sequence is detected with several labeled probes (green sequence), which in turn are detected by IF (green stars).

Methods to study nucleic acids *in situ*

To my knowledge there is today no method available for *in situ* PDI detection with a sequence resolution better than several kbs to Mbs. However, methods for detection of plain nucleic acid sequences that can discriminate between shorter sequences exist:

In *in situ* hybridization (ISH) the target DNA-sequence is detected by hybridization, as described for immuno DNA FISH, with radioactively labeled probes[55-56], but this detection is limited in resolution and by the probe instability. Therefore ISH was further developed, utilizing other means to label the detection probe e.g. enzymatic reactions and fluorescence-labeled probes (FISH), testing different probe lengths and extent of probe labeling. Using either directly fluorescence-labeled or hapten-labeled probes amplified by secondary detection, signals from hybridized sequences could be visualized: several Mbs large DNA-sequences in metaphase spreads and down to ~50 kb in interphase chromatin[57]. On the other end of the scale, larger sequences up to whole chromosomes – even in 24-plex – and individual chromosome arms in 48-plex could be simultaneously and differentially labeled[58-60].

Also, detection of single mRNA molecules by multiple fluorescence-labeled probes was accomplished. Single mRNA molecules were either detected by few, multiply labeled, or by several, singly labeled oligonucleotides[43,61-62]. Incorporation of the RNA analogous locked nucleic acid (LNA) into the probe was utilized to increase hybridization affinity. Hence higher hybridization temperatures could be applied and with that higher sequence discrimination accomplished. Utilizing LNA-containing probes, miRNAs could be detected *in situ* applying signal amplification schemes like tyramide signal amplification (TSA) or enzyme-labeled fluorescence. With the latter approach even individual miRNA molecules could be visualized[63-64]. LNA as well as peptide nucleic acid – DNA analogs which bind stronger to DNA due to their uncharged peptide backbone – containing probes form more stable complexes with the target DNA and enhance thereby detection sensitivity in FISH[65]. Furthermore, FISH has been applied for detection of DNA and mRNA-sequences *in vivo*[66-67].

Primed *in situ* labeling (PRINS)[68] is mostly applied for detection of repeated sequences and utilizes a specific oligonucleotide to hybridize to the target sequence in denatured chromosomes. Upon hybridization, the primer can be elongated by a DNA polymerase, incorporating hapten labeled nucleotides. These nucleotides can be detected by fluorescence-labeled avidin molecules or anti-hapten antibodies allowing visualization of the target sequence *in situ*. Even though single nucleotide polymorphism (SNP)-discrimination can be accomplished when positioned at the 3' end of the primer, detection of individual genes is difficult, probably since the created signal is not strong enough and not reaching high enough detection

efficiency – impaired by gaps in the target DNA, which can serve as undesired primers. However, single gene detection has been accomplished combining PRINS with signal amplification by TSA[69-70].

In situ PCR and *in situ* reverse transcriptase PCR amplify detected DNA or cDNA-sequences exponentially, thereby increasing their availability for FISH detection, which leads to a decrease in the limit of detection. Detection is utilized either directly by fluorescence labeling the PCR product or indirectly utilizing haptens. Since the signal is amplified, shorter sequences (150-350 bp) can be visualized and *in situ* PCR was successfully applied to single copy genes and low copy RNAs. Although the resulting PCR-products are fixed to the cells, they are not anchored at the site where they were generated. PCR-products can therefore diffuse away, causing false positive signals while false negative signals occur due to inefficient *in situ* PCR reactions[71-72].

Another approach to amplify the detected target sequence is by the branched DNA technique[73]. The target DNA-sequence is detected by several oligonucleotides hybridizing only at the 3' end to the target sequence. The free 5' end can in turn be bound by a preamplifier sequence consisting of tandem repeats, which are bound by several amplifier oligonucleotides. This structure can be detected by alkaline phosphatase labeled probes directed against the preamplifier creating a comb-like structure which strongly amplifies the localized signal.

Padlock probes – a technique capable of distinguishing single molecule sequence variants *in situ* – is a further development of the oligonucleotide ligation assay (OLA)[74]. In OLA, two oligonucleotides that hybridize adjacent to each other are ligated by T4-DNA ligase if they form a perfect match with the target sequence. For padlock probes these two hybridization oligonucleotides are linked together by a spacer region. Hybridization to its target sequence brings the 3' and 5' ends together, facing each other[75-76]. Only a perfect match in the junction enables ligation, which creates a circular DNA molecule that subsequently can be detected. For *in situ* detection of single molecules however detection by hapten- and fluorescence-labeled probes was not sensitive enough. Therefore, padlock probes were combined with efficient and locally anchored signal amplification by RCA, primed from enzymatically cleaved target DNA[77]. The RCP can then be detected by hybridization of fluorescence-labeled detection oligonucleotides (Figure 9). Padlock probes have been applied for detection of mitochondrial DNA in fixed cells *in situ*[77] and genomic DNA in comet assays[78] as well as recently for detection of mRNA *in situ*[79]. For this application the mRNA molecules are first converted to target cDNA through reverse transcription using specific LNA-containing primers. mRNA is then degraded with RNase H to provide a template for padlock hybridization, except for the part where the primer is bound, since the LNA bases protect the mRNA from degradation. This ensures that the cDNA stays attached to

the place where the mRNA was located and provides a cDNA template for the padlock probes, as ligation of DNA probes hybridized to RNA is less efficient. Padlock probes have been applied for SNP discrimination *in situ* and multiplexed mRNA detection, enabled by the linker sequence including the detection sequence, which can be freely chosen[79].

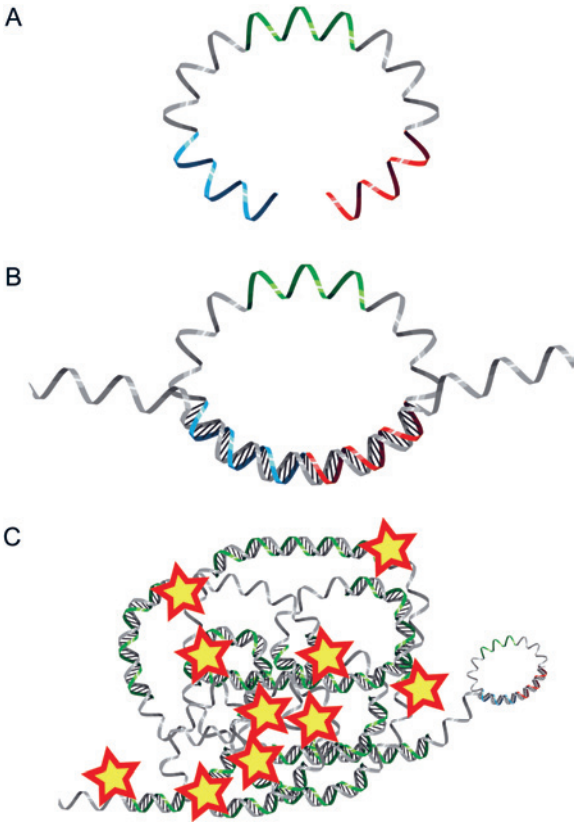

Figure 9. Padlock probe. (A) A padlock probe is a single stranded DNA molecule with the 3' and 5' ends (red, blue) complementary to the target strand. A segment of the noncomplementary part of the probe is utilized as detection site (green). (B) Upon hybridization of the probe to its target sequence, the 3' and 5' ends of the probe are brought together and can be ligated to a circular DNA-molecule. (C) Now an RCA can be primed from the target molecule and the resulting product can be detected by a fluorescence-labeled detection oligonucleotide (green sequence with red stars).

Model systems

PDGFRβ and *DUSP6*

Platelet-derived growth factor receptor beta (PDGFRβ) belongs to a family of receptor protein-tyrosine kinases (RTKs), characterized by an extracellular, ligand binding domain composed of five immunoglobulin-like loops, a transmembrane domain, a juxtamembrane domain, two tyrosine kinase domains separated by a kinase insert domain and a C-terminal domain. Stimulation with platelet-derived growth factor-BB (PDGF-BB)[80] or -DD[81] leads to dimerization of two receptor molecules, conformational changes and autophosphorylation at multiple tyrosine residues in the intracellular domains of the receptors. Thereby binding sites for SH2-domain containing downstream signaling mediators are created e.g. the RAS-RAF-MEK-ERK-pathway mediating an increase in cell survival, proliferation and motility[82-83]. Upon sustained signal activation the receptor is downregulated by internalization of the ligand-receptor complex. Dysregulated PDGFRβ signaling is found in malignancies like dermatofibrosarcoma protuberance or glioblastoma multiforme[82] and the receptor is therefore a potential target for treatment[84]. However, detecting the PDGFRβ specifically is challenging, since the PDGFRβ shares ~80% sequence homology in the kinase domain with the alpha isoform of the PDGFR[85] and both isoforms are equally well stimulated by PDGF-BB[86]. This has rendered it difficult to distinguish between PDGFRβ and PDGFRα, especially in their activated forms, using methods like IF and immunohistochemistry (IHC).

Dual-specificity phosphatase 6 (*DUSP6*, alternatively called *MKP-3*) is an ERK-specific cytosolic member of the MAP kinase phosphatases (MKP), exhibiting phosphatase activity specific against threonine and tyrosine residues in the activation loop of MAPK[87]. ERK is activated by RTK signaling (including PDGFRβ[88]) among others via GRB2 and SOS and the RAS-RAF-MEK-pathway. Upon activation, phosphorylated ERK translocates into the nucleus and activates transcription of target genes. One of these targets is *DUSP6*, which in turn becomes expressed, dephosphorylates ERK and therefore forms a feed-back-loop for ERK activation.

EGFR family

The epidermal growth factor receptor (EGFR) belongs to the family of receptor tyrosine kinases; consisting of four members: EGFR and HER2-4. All members comprise an extracellular binding domain, a lipophilic transmembrane segment, and an intracellular tyrosine kinase domain, which is inactive in HER3. Through stimulation with different ligands, the EGFR family members can form homo- and heterodimers. Binding of six different ligands to EGFR has been described, among them EGF and TNF-α, while no ligands have yet been reported for HER2. Upon stimulation, EGFR is phosphorylated at tyrosine residues in the intracellular domain, leading to recruitment of effector proteins and signal propagation in the cell, via RAS-RAF-MEK- and PI3K- pathways[89]. Whether the receptor dimerizes upon stimulation[90] or exists as preformed dimers/complexes that undergo a conformational change upon ligand binding, leading to phosphorylation of the intracellular domains[91], is however still a controversy. EGFR and HER2 are both overexpressed in many human cancers – breast, ovarian and stomach cancer to name a few – and are therefore possible targets for cancer therapy. Monoclonal antibodies against HER2 – Trastuzumab – and EGFR – Cetuximab – are currently applied in the clinic, i.e. Trastuzumab was approved for treatment of HER2 overexpressing metastatic breast cancer and Cetuximab for treatment of colorectal cancer as well as head and neck cancer.

Chromatin structure and Alu-repeats

In its smallest entity, chromatin is organized in nucleosomes, each consisting of an octamer histone core of two halves built from histone H2A, H2B, H3 and H4. Around this core, genomic DNA is wound in two turns consisting of 147 bp. Where the DNA enters and leaves the histone core, it is retained by one of two histone molecules, either H1 or H5, which compact the DNA further into a fiber structure (Figure 10). Whether a certain DNA-sequence is accessible for transcription is regulated by epigenetic signals acting either in trans – through feedback loops of transcription factors back on the gene itself and RNA-directed positioning of epigenetic signals – or cis – modifications, which are stably associated with the DNA-sequence and inherited with it[92]. Such cis-acting modifications are e.g. methylation of DNA in CpG islands and histone modifications such as methylation, acetylation, ubiquitylation and phosphorylation to name a few. Certain histone modifications are associated with specific effects on the transcriptional activity. For example, H3K9 methylation is associated with transcriptional inactivity, while H3K4 methylation is associated with active transcription.

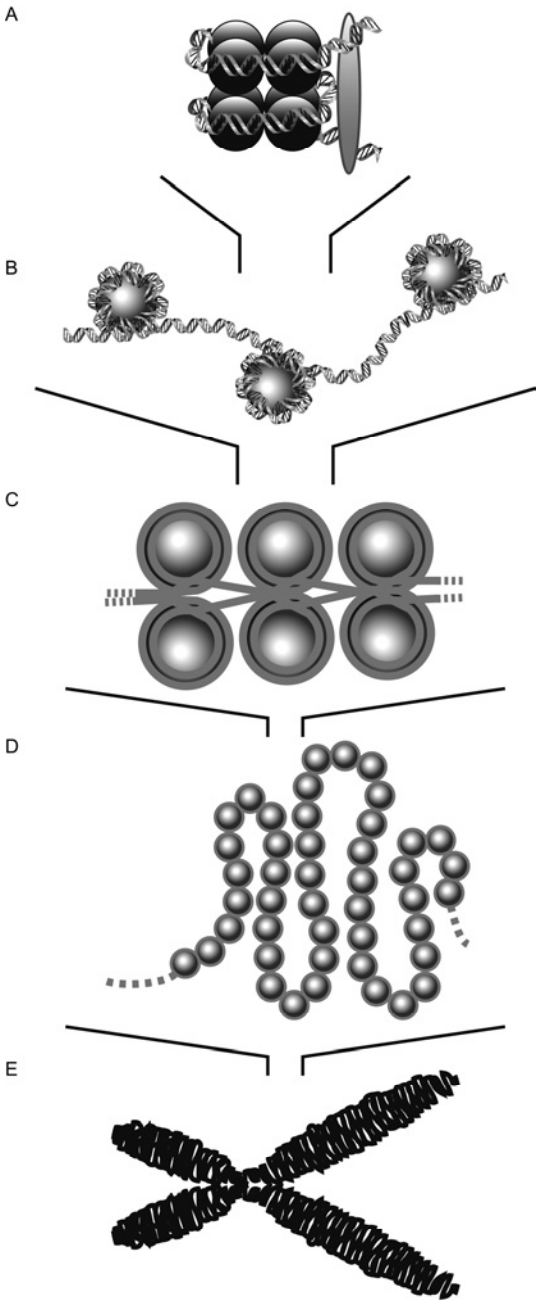

Figure 10. Chromatin structure.

(A) Genomic DNA is wrapped around a histone core consisting of 8 histones (two H2A, H2B, H3 and H4 respectively, dark grey circles). Histone H1or H5 is utilized as linker histone (light grey). (B) The nucleosomes are then organized like "beads-on-a-string" and (C) condensed into a ~30 nm fiber. (D) This fiber is in turn abridged into active chromatin, which (E) can be further condensed to a chromosome.

The family of Alu repeats belongs to the family of short interspersed repeats (SINE) present in over 1 million times in the human genome[93], being one of the larger families of the SINE accounting for ~5% of human DNA[94]. The family is divided in several subfamilies with AluJ and AluS being the oldest and most abundant ones and AluY, which is still active as transposable element. Most of the Alu-repeats can be cleaved by AluI restriction

endonuclease. The primate sequence consists of ~300 bp, two imperfect repeated monomers, whereof one is well conserved between different Alu-family members and the other monomer being more variable. The rodent sequence contains only one monomer of ~130 bp. In 1995, a short 26 bp Alu consensus sequence was identified[95], which is also present in the left arm of the ~300 bp Alu consensus sequence presented by Deidinger *et al.*[96]. This short consensus sequence is present ~60,000 times in the human genome (perfect matches counted in NCBI, assembly march 2006). Alu repeats are not randomly distributed in the genome but rather seem to accumulate in gene rich regions[93]. Alu elements are mobile sequences, their genome interactions are suggested to be recombinatorial hotspots and involved through homologous recombination in most gene rearrangements[95]. Alu repeats can cause diseases by creating new insertions into coding sequences or promoters and by altering the level of gene expression, changing the reading frame and causing deletions and duplications by unequal cross over[94]. Furthermore, Alu repeats were found to be hypomethylated in colon cancer cell lines when compared to normal colon epithelial cells and the CpG island methylation level at one specific AluY repeat correlated with the amount of trimethylated histone H3K9 in these cell lines[97]. Hence analysis of the methylation status of repetitive elements at specific genomic loci could provide new insights into the mechanisms for cancer development.

Present investigations

The papers included in this thesis are all based on the development of novel methods for *in situ* detection of individual PPIs and protein modifications by *in situ* PLA, either alone (paper I and II) or together with simultaneous detection of individual mRNA-molecules by padlock probes (paper III). In paper IV, *in situ* PLA and padlock probes were combined as a novel method for detection of individual, sequence specific protein-DNA interactions.

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

Aim of the study

In situ PLA[36] was generalized by exploiting secondary, species-specific PLA-probes directed against primary antibodies derived from different species. These probes were applied for detection of individual phosphorylated PDGFRβ in transfected as well as endogenously expressing cells and tissue sections. Earlier, the phosphorylation status of the PDGFRβ has been investigated *in situ* using IHC or IF with phospho-specific antibodies against PDGFRβ[98-99]. Due to high sequence homology in the kinase domains[85], it has been difficult to distinguish phosphorylated PDGFRβ from the alpha-isoform. By combining the phospho-tyrosine-specific antibody with a receptor-specific antibody, requiring the binding of two antibodies to generate a signal with *in situ* PLA, we gained the selectivity that allowed us to distinguish both isoforms and a signal amplification to visualize individual receptors. An additional value by using secondary PLA-probes was that they could be conjugated in larger batches, and the need to conjugate every primary antibody was thus circumvented.

Methods

All cell types utilized in this paper – HEK293-PDGFRβ, PAE cells overexpressing either PDGFRα (PAEα) or PDGFRβ (PAEβ) and human fibroblasts (BJhTert) endogenously expressing the receptor – were starved

for 24 h prior to stimulation with PDGF-BB at indicated concentrations. After fixation and blocking, primary antibodies rabbit-anti-PDGFRβ and mouse-anti-PDGFRβ-pY751 were applied. Upon binding of the primary antibodies to the same target protein complex, secondary PLA-probes directed against mouse- or rabbit-IgG could bind to their target proteins thereby bringing the oligonucleotides in close proximity to each other. Subsequently, the assay was performed as described above for the original *in situ* PLA[36], and RCPs representing single phosphorylated PDGFRβ-molecules were quantified by image analysis. Detection of phosphorylated PDGFRβ in fresh frozen tissue sections was similar to detection in fixed cells except that higher concentrations of primary antibodies, PLA-probes and phi29-DNA polymerase were used.

For immunoblot analysis of phosphorylated PDGFRβ, the cells were starved and stimulated with different concentrations of PDGF-BB before they were lyzed and subjected to SDS-PAGE and immunoblotting. The membrane was probed with rabbit-anti-PDGFRβ antibody and subsequently stripped and reprobed with mouse-anti-pY751 antibody.

Results

Phosphorylation of PDGFRβ was first investigated in HEK293 cells stably over-expressing PDGFRβ by *in situ* PLA. In unstimulated cells, minimal amounts of signals were detectable. Upon stimulation with PDGF-BB, the amount of signals increased dramatically. As expected, technical controls confirmed, that presence of the phosphorylated receptor, primary antibodies as well as both PLA-probes were required for RCPs to arise.

Selectivity of *in situ* PLA was demonstrated using stably transfected PAE-cells expressing either the alpha- or beta-isoform of PDGFR. Both isoforms are stimulated by PDGF-BB to the same extent and closely related[85-86]. Using *in situ* PLA we detected PDGFR phosphorylation in the beta-isoform expressing cells, while only negligible amounts were found in the alpha-isoform expressing cells.

It was possible to detect phosphorylated PDGFRβ even in endogenously expressing human fibroblasts. When stimulating the cells with different amounts of PDGF-BB, *in situ* PLA revealed a similar dose-response curve as obtained with immunoblotting.

To test if the assay would be applicable for analyses in tissue sections, fresh frozen human scar tissue was analyzed for the presence of phosphorylated PDGFRβ. Receptor expressing stromal fibroblasts and pericytes are stimulated by PDGF-BB secreted during wound healing[100], so *in situ* PLA signals were found in the fibrotic dermal stroma beneath the epithelium and the stroma around venules, in accordance with literature.

Discussion

In situ PLA was shown to be a potent method also for detection of PTMs. Double recognition of the target protein for signal generation and the therewith accomplished selectivity, made it possible to discriminate between the phosphorylated PDGFRβ and -α. The potent amplification step integrated in the protocol enabled detection of single phosphorylated receptors. By utilizing species-specific secondary PLA-probes, the need to conjugate every primary antibody was overcome. Thus, secondary PLA-probes can be produced in large batches and be stored for a long time, ensuring reproducibility of the experiments. Any pair of primary antibodies can now be applied as long as the two antibodies are derived from two different species.

Since the RCPs stay attached to the site where the primary antibodies have bound, the results can hence be interpreted both with regard to the spatial distribution of the signals within the cells, as well as the variation within the cell population.

Paper II: 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

In paper II, *in situ* PLA was performed using flow cytometry as readout, which would enable analysis of thousands of individual cells in one run. In this study, homo- and heterodimers between EGFR and HER2 were determined and detection of the phosphorylation status of EGFR by *in situ* PLA was compared to IF.

Methods

For relative quantification of EGFR and HER2; SKOV-3, U343 and MCF-7 cells were stained with fluorescence conjugated primary antibodies and analyzed using a flow cytometer. The same cells were analyzed with *in situ* PLA for EGFR and HER2 interactions using PLA-probes made from Cetuximab and Trastuzumab. To visualize phosphorylated EGFR in EGF-stimulated U343 by *in situ* PLA, primary antibodies goat-phosphorylated-EGFR (pEGFR, pY1173) and rabbit-pEGFR (pY1045) together with secondary PLA-probes were applied. The same primary antibody (rabbit-pEGFR pY1045) was also used in IF and both samples were analyzed by flow cytometry.

For comparison, cytospins of the same EGF-stimulated cells were used to detect phosphorylated EGFR either by IF using the rabbit-anti-pEGFR antibody or by *in situ* PLA.

Results

Levels of homo- and heterodimers of EGFR and HER2 were investigated in three different cell lines: U343, SKOV-3 and MCF-7. First the protein levels were determined by IF-based flow cytometry: U343 and SKOV-3 expressed high levels of EGFR while MCF-7 expressed only low levels; for HER2, U343 expressed low, SKOV-3 high and MCF-7 intermediate levels of protein. Next, *in situ* PLA-based flow cytometry was applied to detect levels of EGFR-EGFR, EGFR-HER2 and HER2-HER2 dimers: U343 showed high levels of EGFR-EGFR homodimers and low levels of the hetero- and HER2 homodimers. In SKOV-3 cells low levels of EGFR-EGFR but high levels of the other two investigated dimers were detected. Finally, for MCF-7 none of the investigated dimers could be detected even though HER2 was expressed in these cells. However, MCF-7 also expresses HER3, which is a preferred interaction partner of HER2 and might bind most of the HER2 molecules.

In the next step, to detect changes in dimerization upon stimulation with EGF, the three cell lines were stimulated with varying amounts of EGF for different amounts of time and at different temperatures. Since no changes in dimerization were detected in any of these conditions, the phosphorylation status of the EGFR in U343 cells upon stimulation by EGF was instead determined by both *in situ* PLA and IF. When utilizing one of the primary anti-pEGFR antibodies for IF to detect pEGFR, the signal increased only slightly over background upon stimulation. With *in situ* PLA utilizing both pEGFR-antibodies, the ligand dependent increase in signal was dramatic. The same observation could be made when these experiments were performed on cytospins of the stimulated cells.

Discussion

In our work we were able to detect homo- and heterodimers of EGFR and HER2 by *in situ* PLA, and the results for U343 and SKOV-3 are in agreement with what is described in the literature, namely that the preferred interaction partner is HER2. In the MCF-7 cell line, we could not detect any of these dimers, even though HER2 is expressed in this cell line but most probably engaged with HER3. Therefore, expression levels alone do not provide enough insight into how the proteins interact with each other. We did not detect any changes in dimerization levels in any of the investigated dimers upon EGF-stimulation, but could detect changes in the amount of pEGFR by *in situ* PLA, confirming that the EGF-stimulation was successful. This phosphorylation was difficult to detect by IF, due to unspecific staining

of the primary antibody, again showing that dual recognition provides advantages over single recognition. The lack of response in dimerization, upon ligand stimulation, might be due to that the distance requirement of our *in situ* PLA (estimated to be below 30 nm) is not sufficient to detect the change in distance between the epitopes of the EGFR, although for other receptors such as VEGFR[101] receptor dimerization upon stimulation has been detected. Whether the members of the EGFR family exist as pre-formed clusters in the cell membrane that change conformation upon stimulation with the ligand, or whether they exists as inactive monomers, which associate with each other upon stimulation, is widely discussed in the literature[90-91].

In this paper we have successfully used *in situ* PLA together with flow cytometry, which increases the through-put compared to *in situ* assays by microscopy, allowing investigation of thousands of individual cells. We could also show that *in situ* PLA can be used for detection of PTMs even if each antibody alone, when used in IF, is not selective enough to distinguish its target molecule from the background.

Paper III: Simultaneous visualization of both signaling cascade activity and end-point gene expression in single cells.

Aim of the study

Two methods recently developed by my group were combined for detection of PTMs of receptors (*in situ* PLA) and individual mRNA molecules (padlock probes)[79]. *In situ* PLA was utilized for the detection of phosphorylated PDGFRβ together with padlock probes for detection of *DUSP6* mRNA, a downstream target of the PDGFRβ signaling. The aim of the study was to determine if the two protocols could be combined, since the ability to address several nodes of a pathway at the same time would be a valuable tool for e.g. drug screening.

Methods

BJhTert cells were starved for 48 hours in Modified Eagle Medium + 0.1% fetal calf serum. For stimulation, PDGF-BB was added at different time points and the cells were incubated at 37^{o}C. When inhibitors (Gleevec or 5-Iodotubercidin) were applied, these were added at a final concentration of 10 μM 1 h before stimulation with PDGF-BB was started. The drug concentration was kept constant throughout the stimulation. After fixation and permeabilization, mRNA detection by padlock probes was performed as

described in Larsson *et al.*[79]. Alternatively, PDGFRβ-phosphorylation was detected as described in paper II, with the exception that the pY751-antibody was exchanged for the pY100-antibody.

To accomplish simultaneous detection of phosphorylated PDGFRβ and *DUSP6* molecules, padlock probes were ligated to the target cDNA before the cells were blocked and primary antibodies and PLA-probes were applied. After hybridization and ligation of the circularization oligonucleotides for *in situ* PLA, RCA was performed for both methods together and detected by hybridization of differently labeled oligonucleotides. In all cases, the cell membranes were stained with FITC-labeled wheat germ agglutinin and the nuclei were labeled with DAPI. Images were taken with an epifluorescence microscope, three images per condition, enhanced in ImageJ[102] and analyzed in CellProfiler[103] on a single cell level.

Results

First the kinetics of PDGF-BB stimulation was determined for PDGFRβ phosphorylation and *DUSP6* expression levels. As expected, PDGFRβ phosphorylation increased steeply within the first 5-10 min of stimulation before the levels gradually decreased towards baseline levels, due to internalization of the receptor. Maximal *DUSP6* expression was detected after 3 hours of PDGF-BB stimulation, in the same range as described earlier, despite differences in the applied model system[88]. Then *in situ* PLA and padlock probe detection were combined in one protocol. To access what effect the combination of these two methods had, we investigated *ACTB* mRNA instead of *DUSP6* since *ACTB* is an abundantly expressed housekeeping gene and changes in detection efficiency would be more readily detectable. Even though the combining of both protocols decreased the detection efficiency by 50% compared to the individual detections, the levels of *ACTB* were overall unaffected by treatment with PDGF-BB, indicating that a higher number of *in situ* PLA signals does not inhibit detection by padlock probes.

Then, *in situ* PLA and padlock probes were combined to detect, which effects the treatment with either Gleevec or 5-Iodotubercidin had on the PDGFRβ pathway at the two nodes investigated. As expected, treatment with Gleevec prior to PDGF-BB abolished the increase in signals from both PDGFRβ phosphorylation and *DUSP6* expression. 5-Iodotubercidin on the other hand affected only signals from *DUSP6*, since 5-Iodotubercidin acts on the pathway downstream of PDGFRβ.

Discussion

The results presented herein show that *in situ* PLA can be combined with padlock probes for detection of individual proteins and mRNA molecules *in situ*.

Since data analysis was done on a single cell level, cell-to-cell differences were readily accessible. Even though the cells were synchronized by starvation, the variation in response to PDGF-BB treatment was quite large at the investigated time points in particular for *DUSP6*, where some cells seemed to burst with expression while others were lacking behind. Alternatively, transient bursts of expression in between the time points of analysis were not detected. This heterogeneity would have gone unseen with an averaging method. Furthermore, as the molecules are detected *in situ*, not only information about the amount of detected signals per cell can be retrieved, but these data can even be put in context of the morphology and surrounding of the cell. The method presented here for simultaneous detection of individual PPIs, PTMs of proteins and mRNA molecules should be particularly interesting in drug screening applications for discovery of new drugs that affect a pathway at a certain point but leave other parts unaffected.

Paper IV: Visualizing individual, sequence-specific protein-DNA interactions *in situ*

Aim of the study

The aim of this paper was to develop a method for detection of protein-DNA interactions *in situ* that can discriminate between closely related genomic sequences. As a model system, histone H3's binding to a 26 bp-Alu repeat consensus sequence (5'CCT**G**TAATCCCAGCACT**TT**GGGAGGC 3')[95] was utilized. This Alu-repeat sequence is highly abundant in humans (~60,000 copies per genome) but not present in mice, although they carry an orthologous sequence present in ~6,000 copies (5'CCT**T**TAATCCCAGCACT**C**GGGAGGC 3'; differences to the human sequence are indicated in bold)[104].

Three different approaches for detection of protein-DNA interactions *in situ* were investigated. The most selective – "padlock probe based *in situ* PLA" – based protein recognition on *in situ* PLA with a single antibody and a single PLA-probe and detection of the genomic sequence on modified padlock probes.

Methods

Human (BJhTert) and mouse (NIH3T3) fibroblasts were seeded on glass slides and allowed to attach over night. Subsequent to fixation and permeabilization, the cells were treated with AluI and Lambda-exonuclease to cut the genomic DNA and make it partially single stranded. Now the cells were subjected to one of the following protocols:

Hybridization based *in situ* PLA

As DNA-detecting PLA-probe, an oligonucleotide complementary to the target sequences was utilized. This oligonucleotide hybridized to BJhTert and NIH3T3 cells before incubation with anti-histone H3 antibody and anti-rabbit PLA-probe. Subsequently, two circularization oligonucleotides were allowed to hybridize and ligate, and the resulting DNA-circle was amplified by RCA and detected by fluorescence-labeled detection oligonucleotides.

Genomic DNA-templated *in situ* PLA

The next approach was to use single stranded genomic Alu repeats as templates for ligation of the circular probes. Cells were first incubated with primary antibody and secondary PLA-probe as described for hybridization based *in situ* PLA. Subsequently, the two circularization oligonucleotides were hybridized directly to Alu-repeats at the genomic DNA and the secondary PLA-probe, followed by ligation using T4 DNA ligase. The resulting circular DNA molecule was amplified by RCA as described for hybridization based *in situ* PLA.

Padlock probe based *in situ* PLA

In the final approach, we used a padlock probe modified with two internal uracil-containing hairpin structures. The padlock probe was first hybridized and ligated to the genomic DNA, using Amp-ligase at 45^{o}C before primary antibody and secondary PLA-probe were added. Treatment with Uracil-DNA-glycosylase (UNG)/EndoIV was then performed to degrade the hairpin-structures of the padlock probe, allowing it to hybridize to the PLA-probe. Upon hybridization of an additional splint oligonucleotide to the PLA-probe, a circular DNA molecule could be created, amplified by RCA and detected by fluorescence-labeled detection oligonucleotides (Figure 11).

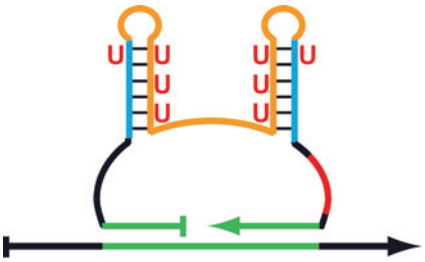

Figure 11. Hairpin-containing padlock probe.

The hairpin-containing padlock probe hybridizes to the target sequence via the target complementary sites of the probe (green) and is ligated. Subsequently, the hairpin structures (orange) can be resolved by removing the uracil bases. Now the parts of the probe complementary to the antibody-bound PLA-probe (blue) are liberated and can hybridize to the PLA-probe forming a circular DNA molecule.

Results

Hybridization based *in situ* PLA

To test if the selectivity of hybridization would be sufficient to discriminate between human and mouse sequences, binding of the Alu-specific probe alone was detected by a padlock probe hybridizing to the free 3' end of the PLA-probe. Even though the staining was more intense in human cells, there was still substantial staining found in mouse cells, indicating unspecific hybridization. The primary antibody was tested by IF and was found to give a similar staining in both human and mouse cells.

When using probes for *in situ* PLA to detect protein-DNA interactions, we observed ~160 interactions per human cell which was a 5-fold increase compared to the mouse cells, where ~30 interactions per cell were detected.

Genomic DNA-templated *in situ* PLA

Since detection of target DNA by hybridization was not specific enough, genomic DNA should be tested directly as PLA-probe. After incubation with primary antibody and secondary PLA-probe, the circularization oligonucleotides could hybridize to both the PLA-probe and genomic DNA, and then be ligated. An additional tag oligonucleotide was incorporated into the final circle to avoid unspecific ligation between two genomic sequences, i.e. increasing the requirement for a perfect match at the PLA-probe. This tag oligonucleotide served as a second detection site in the final RCP and thus only double colored RCPs were regarded as true signals. With this second design ~13 times more signals were detected in human compared to mouse cells.

Padlock probe based *in situ* PLA

To further improve the selectivity of the DNA detection we switched ligase to Amp-ligase that tolerates higher temperatures for ligation and thus is more specific than T4 DNA ligase. Since the antibodies utilized for detection of the protein part might not endure temperatures exceeding 37^{o}C, the DNA

had to be targeted prior to the protein. Circularization oligonucleotides were converted into a padlock probe to couple both sequences together and with that increase the likelihood that both would bind to the same target sequence. This necessitated however that the padlock probe contained sequences that were complementary to the oligonucleotide carried by the PLA-probe. Hence, the PLA-probe would be attracted to the site where the padlock probe had bound without the requirement to recognize a primary antibody in its proximity. To circumvent this problem, two hairpin structures were introduced in the padlock probe, shielding the PLA-probe complementary parts. Subsequent to ligation of the padlock probe to genomic DNA and binding of the primary antibody and the secondary PLA-probe, the hairpin structures in the padlock probe were opened. This was achieved by degradation of the uracil residues incorporated in the padlock probe, hence allowing the probe to hybridize to the PLA-probe. As the hairpin-containing padlock probe could be amplified by RCA like a regular padlock probe, it had to be ensured that the probe had been cleaved by UNG/EndoIV and religated. We therefore introduced a tag oligonucleotide into the final circular DNA (as described above) that served as a second detection site, confirming that the padlock probe had been cleaved and religated. Only double colored signals were regarded as true detected interactions whereof ~25 signals could be detected for interaction of Alu-repeats with histone H3 in human cells and almost no interactions were detected in mouse cells (median = 0). Hence, padlock probe based *in situ* PLA resulted in a ~500 times higher signal in humans than in mice, showing the highest selectivity of the methods tested herein.

Discussion

We have herein described the first method that enables a highly selective detection of PDIs *in situ*, potent to discriminate between highly homologous sequences. However, only a small fraction of all Alu repeats was detected, giving a detection sensitivity of ~0.13%. Thus we are still far from being able to detect transcription factors' interaction with single copy genes. To improve this method further and make it applicable for less abundant interactions, accessibility of genomic DNA to the probe needs to be improved without affecting the protein binding to DNA. To gain access to genomic DNA, the nucleus had to be permeabilized and the degree of permeabilization to gain access to the nucleus had to be balanced to the amount of protein that was lost due to permeabilization. With padlock probes, ~0.13% of all Alu-repeats could be detected, which suggests that a great part of the genomic DNA is not accessible to restriction digestion and exonuclease treatment or inaccessible for the probes.

Summary and future perspective

Analyses on a single cell level are essential to determine cell-to-cell variations in a population and assays where this can be performed in complex material, such as tissue sections, are needed for the methods to also be applicable in a diagnostic setting. The papers presented in this thesis all utilize *in situ* PLA in one way or another to investigate interacting biomolecules and their modifications, as a measurement of protein activity status. In paper I, we developed secondary PLA-probes to detect the phosphorylation of individual PDGFRβ molecules. These probes allowed us to apply primary antibodies, without the need for conjugation, as long as these antibodies were derived from different species. Individual *in situ* PLA signals could be quantified and with that provide a measurement of how many receptors were phosphorylated upon different treatments. To increase the throughput, we investigated in paper II if *in situ* PLA would be compatible with flow cytometer readout for detection of homo- and heterodimers of EGFR and HER2, as well as phosphorylation of EGFR. In addition, we could also show that *in situ* PLA was superior to IF for detection of phosphorylated EGFR due to its dual recognition. In paper III, we combined *in situ* PLA detection of PDGFRβ phosphorylation with padlock probe detection of *DUSP6* mRNA, both being induced by PDGF-BB stimulation. The ability to simultaneously measure activity status at different nodes in a signaling cascade provides a tool to study kinetics at a single cell level and can be utilized to dissect the molecular effect of drug candidates. In paper IV, we developed a method for detection of PDI *in situ* utilizing both *in situ* PLA and padlock probes, providing a highly selective method and paving the way for future developments of assays to determine protein interactions with single copy genes. The papers included in this thesis are a contribution to the molecular toolbox offering means to deepen our understanding of how cells are regulated through interactions between various biomolecules and of what variation that occurs within a seemingly homogenous cell population.

Of course, still too many questions remain unanswered, and are not possible to answer with current available methods or have not yet been asked. Therefore, new methods and improvements of existing methods are always required. To retrieve as much information from each investigated cell as possible, not only should the type of binders that can be used with *in situ* PLA be expanded, but also the number of molecules that can be investigated

simultaneously. Therefore a multiplex version of *in situ* PLA has been developed to determine the balance between alternative interaction partners and to follow signaling pathways (Leuchowius *et al.*, submitted). Furthermore, the dynamic range of *in situ* PLA's single molecule discrimination is restricted since it has to be possible to distinguish individual signals. To expand the dynamic range and dissolve signals that have converged, a novel oligonucleotide design has been tested that can increase the dynamic range by several orders of magnitude (Clausson *et al.* in preparation). Also, other designs that provide the ability to decrease or increase the distance requirement for the generation of *in situ* PLA signals have been developed (Leuchowius *et al.*, submitted). Utilization of screening and scanning devices as well as flow cytometers makes *in situ* PLA applicable even for high content analyses as demonstrated by Leuchowius *et al.*[105]. Now it is possible to screen new drugs not only for their effect on the expression of a protein but also for their impact on the function, and the analyses can be done either on cell lines or in patient cells to support the choice of therapy.

Methods to detect protein-DNA interactions for single copy genes interacting with rare proteins like transcription factors are a future goal. To achieve this goal with *in situ* PLA, the greatest current obstacle is that genomic DNA has to be made accessible to probes, while also the proteins have to stay in place. Therefore fixation and permeabilization need to be improved. Furthermore, all enzyme and binder based reactions need to be performed with high efficiency. Whether an interaction is detectable will also depend on the place in the nucleus where this interaction occurs, and how condensed the chromatin is at this place. Interactions between other types of biomolecules such as protein-RNA also require method development. As the efficiency of the DNA ligases for a DNA/RNA hybrid is very poor, our method for PDI detection described herein would require development of a ligase efficient in ligating a DNA molecule templated by RNA. Alternatively, if an RNA molecule can ligate on an RNA template, an RNA polymerase (or reverse transcriptase) would be capable to perform an RCA.

However, despite all efforts to improve the sensitivity of the target molecule detection – protein, nucleic acids and all combinations hereof – it will be challenging to detect 100% of endogenously expressed, untagged molecules present in a sample. What might be the greatest obstacles is the accessibility of the target molecules and the fact that all types of binders and enzymes have a limited affinity for their target but unfortunately also an affinity for other, irrelevant molecules.

But where does that leave us? As mentioned in the beginning, in addition to *in vitro* methods that provide important basic knowledge, methods that visualize endogenous molecules in cells, tissues and whole animals will contribute substantially to a deeper understanding of the processes of life. As

Richard P. Feynman said: “It is very easy to answer many of these fundamental biological questions; you just look at the thing!”. Being able to look at single molecules and how they interact in signaling cascades, in individual cells and potentially *in vivo*, is desirable so that we one day can understand how cells and whole organisms work. It is tempting to speculate that methods could be developed to detect all proteins belonging to one pathway and their interactions in multiplex, so that the signaling cascade could literally be “looked at” in individual cells. If further protein detection could be combined with multiplexed detection of RNA and DNA molecules *in situ*, then gene expression profiles and mutations as well as changes in signaling cascades could be studied in healthy and diseased cells. In that case questions like: Where in the cell and tissue are the interactions taking place? How often do certain interactions occur and how are they distributed in the cell, the population, the tissue and the organism? – all leading up to the ultimate question: Which molecular events cause or perpetuate a disease and how can it be cured? – could be answered. The methods presented in this thesis will hopefully be one step in the relay to answer these questions.

Acknowledgments

The work presented in this thesis was done at the Department of Immunology, Genetics and Pathology at Uppsala University. First of all, I would like to thank all organizations which have financially supported our work, in particular: Beijerstiftelsen, Knut and Alice Wallenberg stiftelse, the Swedish Foundation for Strategic Research, Vetenskapsrådet, Cancerfonden, the National Biobank Platform, the EU-FP6 projects ENLIGHT and MolTools, and the EU-FP7 project DiaTools. Without your support, this research would not have been possible.

Then I would like to express my gratitude to my faculty opponent Prof. **Banafshe Larijani** and the examination board Prof. **Sophia Hober,** Assoc. Prof. **Johan Lennartsson** and Prof. **Chandrasekhar Kanduri** for taking the time to read my thesis and for coming to Uppsala to discuss it with me.

I would like to thank all people, near and far, who have been at my side during the last five years, in particular:

My supervisor **Ola Söderberg,** for introducing me to the wonderful world of *in situ* PLA and the magic we can do with it. Thank you for all your support during these years, for the open discussions we shared, for having a plan "B" when I needed it, for showing me that one sometimes just has to give it a try and for saving my life – from axe murderers on the plane and from drowning in the pool when "swimming" butterfly.

My co-supervisor **Ulf Landegren,** for bringing me into the group, for always having an open door and sharing your thoughts, no matter how busy you are, for creating such an open atmosphere at the lab, which makes this group a very special and inspiring place to do research in.

My collaborators on various projects: Thank you **Carolina Wählby,** for opening my eyes to image analysis and for being such a wonderful person; **Milan Gavrilovic**, for tireless explaining your algorithms and for counting my yellow blobs; **Amin Allalou,** for writing Blobfinder, it made my life a lot easier; **Dirk** "**Pacho" Pacholsky** and **Jan Grawé,** for keeping our microscopes up and running and for being at call, when they would not cooperate; **Nongnit Laytragoon-Lewin,** for sharing the cell lab with us;

Arne Östman, for your support on paper I and for always believing in oxPTP-*in situ* PLA, I still believe that it should be possible; **Janna Paulsson,** for the great work on paper I; **Markus Dagnell,** for your engagement in the oxPTP project and all **the rest of the Östman group,** for the great times I have had with you! Thank you **Johan Kreuger** and **Sébastien Le Jan** for our work on embryoid bodies. Seb, I really enjoyed our discussions during these looong hours at the microscope. Thanks to **Marianne Farnebo** and **Salah Mahmoudi** for the collaboration on Wrap53. A special thanks to **Frank** and **Annette Böhmer** for always asking the right questions, for inspiring me with science, music and books and for bringing *in situ* PLA to Jena.

Many thanks to the people from "my" **molecular tools / molecular medicine group** (current and former) for making our lab such a fantastic place to be at:

Ida, my second I^2 half, thank you for being there for me during all these years, for keeping the lab bench next to me, despite the RNases I worked with, for introducing me to the gym and the sensation that having pain can actually be fun, for teaching me Swedish and cross-country skiing, for your company at various conferences, in short – for your friendship! **Johan E,** for many evenings in your sofa discussing psychology and for your patience when Ida and I were busy doing girly things.

Thanks to my *in situ* PLA family: **Kalle,** my brother in *in situ* PLA ;-), for understanding my complaints about antibody-selectivity, for repeatedly listening to my explanations about complicated protocols for oxPTP and DNA-prot detection, for making Illustrator do what I want and for the great fun we had outside the lab singing "I can show you the world" and "swimming" butterfly; **Carl-Magnus,** for staying in the swimming team, when everyone else left and for enjoying non-black tea as much as I do; **Karin,** for joining our *in situ* PLA group and girly orders from the US; **Agata,** for enjoyable lab discussions and for trying to make people tell when they "borrow" your stuff; **Di,** for our microscope sessions philosophizing about *Drosophila* brains; **Gucci,** for introducing me to Chinese jazz music and many books worth reading; **Christina,** for finally realizing that *in situ* PLA is the right side of this lab, for testing cake recipes on us and for joining in on countless gym classes; **Tim,** for asking the right questions and for getting involved, for fixing my computer by just rolling over and for, together with **Lei** and **Louis,** all the wonderful dinners at your place; **Katerina,** for being such an amazing person and for bringing me down from "Åreskutan" in one piece, you are a great skiing teacher!; **Malin,** for taking me on as a student, for introducing me to *in situ* PLA and for valuable input on defense forms; **Georgia,** for bringing back some Greek attitude into your little *in situ* PLA group and for discussions about *in situ* PLA and life; **Andries,** for chats about transfection and about how to make a bacterium do

what I want; **Pier,** for discussions about protein-DNA interactions and your valuable help with the Bioanalyzer; **Caroline** and **Mikaela,** for just letting me stop by and have a (*in situ* PLA) chat;

A special thanks to **Lena**, first of all for your help on paper IV, without you, I would still stand in the lab and pipette! But thank you even more for your friendship and the great times I got to spend with your family (Tack, **Jesper** och **Anton** för mys- och filmkväller hemma hos er!); to **Sara,** for being my ”nucleus”-person, for introducing me to dancing Salsa and for all the fabulous times we spend together; to **Spyros,** for sharing the experience of the first Swedish winter, for the ”therapy” meetings at our office and for being 100% committed as actor in all spex movies; to **Mats** for your valuable input on padlocks, science in general and paper III in particular; to **Masood,** for the introduction to Jujitsu, for explaining how to cook rice properly and for your fabulous kind of humor; to **Lore,** for showing me how I would like to be when I grow up: interested, engaged and caring.

I would like to thank all current and former lab members for sharing science and parties at countless occasions:

Rachel, for your seemingly endless energy and gladness and for refreshing discussions; **Maria H,** for great lunch and fika company and excellent cake recipes; **Anja,** for evening discussions in German and for offering to go climbing with me (when this is over, I will join you!); **Elin FS,** for your help with counting sequences and for, together with Karin, starting in the swimming team; **Rongqin,** for long-distance relationship discussions at the microscope; **Marco,** for taking care of the microscopes and for doing ”whatever you want” for Spyros movie; **Johan V,** for joining the PhD-council and for visiting Jena; **Junhong,** for your company and great dumplings at the Xmas party; **Anne-Li,** for always being positive and engaged – in lab projects as well as spex movies, **Ling,** for patiently listening to my explanations about cell culture; **David,** for trying to built a “group memory”; **Jenny,** for your positive attitude and for knowing everything about blobs; **Mathias,** for engaging in zinc fingers and for many fun chats; **Sigrun,** for organizing the “come as you are not”-party together with Jenny; **Olle,** for valuable input at group meetings, your contagious laughter and for cocktail parties with “very interesting” cocktails; **Henrik,** for entertaining chats in the lab and for the sandwiches you prepared for us at 3:00 a.m. after a long night at BJ’s; **Magnus** and **Anna,** for your company at grill parties; **Yuki,** for delicious sushi nights and for importing culinary specialties from all over the world; **Chatarina,** for discussions about RGB and CYMK colors, **Jörg,** for introducing me to DNA-protein interactions; **Ida E,** for having such an irresistible laughter and **Lotte, Artor, Joakim, Camilla, Lei, Björn, Annika**, **Elin L, Anna E, Monica, Ellen, Jonas, Johan S**, **Sigrun, Heidi, Mia, Carolina R, Edith, Reza** and **Anna K** for

lively discussions and inspiring chats, for lunch and fika company and for making this group such a special place.

A huge thank you to the people in the group who are taking on the administration: **Elin E**, for taking care of all the orders and for being there when I needed a break from writing; **Carla,** for your help with antibody conjugation and both of you together with **Lena** for keeping the lab up and running and us organized; **Erik,** for answering all my questions about funding and for chairing this defense wearing a cerise colored shirt ;-); **Ulla,** for chats early in the morning when I was scuffling to the microscope and for patiently explaining in which order the registration forms should be signed; **Johan O,** for rescuing my external hard drive even though you had warned me that it was not secure; **Anders,** for having such a great kind of humor and for choir talks over lunch.

I would like to thank my students **Eric** and **Jane** for their work in and enthusiasm for the protein-DNA project and for believing in it as much as I did.

The **PhD student council,** for all meetings and discussions about things we were supposed to do and Rudbeck-life in general ;-).

People at **Olink: Kicki, Mats, Göran, Daniel, Erik, Gabriella, Andrea, Eva, Peter, Björn and the rest of the Olink team,** for their help with secondary *in situ* PLA, for countless "Duolink user" / Rudbeck people – Olink meetings and for providing us with kits and special wishes throughout the years.

I also would like to thanks some dear friends outside the lab: **Zandra,** for giving me a place to live when I first came to Sweden, for our TV-nights, delicious food and for always knowing how to do things; **Anna,** for singing with me (and we will give that concert!), for explaining the Swedish pension scheme and for our weekly therapy sessions in my sofa; **Toni,** for from time to time reminding me what we dreamt of when we were little and for finally visiting Uppsala; **Maria S**, for singing and playing the piano with me and for walking BACK from Hammarskog as well, even though we did not get cake; **Antje,** for our IKEA trip, for knowing who would win the Eurovision song contest 2008 and for joining me swimming, even though you were in Germany; "**die Mädels" – Annika, Berit, Bine, Susi and Janine,** for staying in touch and for the great reunion we had last year, let´s make it only the first of many; **Sina,** for the countless cookies you baked for me, for long discussions about science and life and for making me ski to Hammarskog.

”My” choir **Collegium cantorum**: Thank you for the music!!!, for listening to my German without an ”R” and for the amazing trip to Iceland last summer.

Ich möchte mich auch bei meiner Familie bedanken:

Bei meinen „Schwiegereltern” **Konni** und **Frank** sowie **Christian, Oma Käthe** und **Oma Renate** dafür, dass ihr mich so lieb in die Familie aufgenommen habt, für eure „Carepakete“ aus deutschen Landen und dass ihr auf Basti aufgepasst habt**.**

Danke auch an meine Großeltern: **Oma Rosi**, dafür dass du mich dazu gebracht hast, einmal darüber nachzudenken, was ich hier eigentlich mache, und für deine Bedenken, ich könnte deutsch verlernen; **Oma Inga** und **Opa Jochen,** für alle telefonischen Ferndiagnosen in den letzten Jahren und dass ihr euch Sorgen über meinen Heimweg durch die „dunklen Wälder” Uppsalas gemacht habt.

Opa, ich habe mir sehr gewünscht, dass du diese Arbeit noch lesen kannst, hätte sie dir gern erklärt und mit dir darüber diskutiert, doch es hat nicht sollen sein. Du fehlst mir!

Mama und **Papa,** danke, dass ihr immer für mich da seid, dass ihr euch jederzeit ins Auto nach Uppsala gesetzt hättet, um mich zu retten, danke für eure Liebe und dafür, dass ihr an mich glaubt! Vielen Dank auch an meine Geschwister: **Clausi** dafür, dass du meinen Computer soo viele Male per Ferndiagnose repariert hast, dass du zusammen mit Basti den Umzug nach Schweden geschmissen hast und für die interessanten Gespräche, die wir auf langen Autobahnfahrten und am Telefon gehabt haben. Danke auch an **Corinna** dafür**,** dass du auf Clausi aufpasst und ans Telefon gehst, wenn er mal wieder in der Firma verschollen ist. **Lieschen,** mein Wuschel, vielen Dank für all die tollen Bilder (vor allem für das auf dem Umschlag!), damit hast du mir immer eine große Freude gemacht.

Basti, mein Prinz, danke, dass du es trotz des großen Abstandes so lange mit mir ausgehalten hast. Danke, dass du immer für mich da bist, auch wenn das in letzter Zeit sicher nicht immer einfach war, für deinen Glauben an mich, dafür, dass alles wieder gut wird, wenn ich mich bei dir anlehne und vor allem für deine Liebe!

Was meinst du? Sollen wir es jetzt mal im selben Land, der selben Stadt, der selben Wohnung versuchen? Ich liebe dich! ♥

References

1. Savage, D.C. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* **31**, 107-133 (1977).
2. Finishing the euchromatic sequence of the human genome. *Nature* **431**, 931-945 (2004).
3. Misteli, T. Physiological importance of RNA and protein mobility in the cell nucleus. *Histochem Cell Biol* **129**, 5-11 (2008).
4. Pierige, F., Serafini, S., Rossi, L. & Magnani, M. Cell-based drug delivery. *Adv Drug Deliv Rev* **60**, 286-295 (2008).
5. Levsky, J.M. & Singer, R.H. Gene expression and the myth of the average cell. *Trends Cell Biol* **13**, 4-6 (2003).
6. Park, J.*, et al.* Single-molecule analysis reveals the kinetics and physiological relevance of MutL-ssDNA binding. *PLoS One* **5**, e15496 (2010).
7. Uyemura, T., Takagi, H., Yanagida, T. & Sako, Y. Single-molecule analysis of epidermal growth factor signaling that leads to ultrasensitive calcium response. *Biophys J* **88**, 3720-3730 (2005).
8. Greenleaf, W.J., Woodside, M.T. & Block, S.M. High-resolution, single-molecule measurements of biomolecular motion. *Annu Rev Biophys Biomol Struct* **36**, 171-190 (2007).
9. Monti, M., Orru, S., Pagnozzi, D. & Pucci, P. Interaction proteomics. *Biosci Rep* **25**, 45-56 (2005).
10. Fields, S. & Song, O. A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245-246 (1989).
11. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S.J. & Karin, M. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. *Mol Cell Biol* **17**, 3094-3102 (1997).
12. MacBeath, G. & Schreiber, S.L. Printing proteins as microarrays for high-throughput function determination. *Science* **289**, 1760-1763 (2000).
13. Zhu, H.*, et al.* Global analysis of protein activities using proteome chips. *Science* **293**, 2101-2105 (2001).
14. [Anon]. Identification of associated proteins by coimmunoprecipitation. *Nature Methods* **2**, 475-476 (2005).
15. Johnsson, N. & Varshavsky, A. Split ubiquitin as a sensor of protein interactions in vivo. *Proc Natl Acad Sci U S A* **91**, 10340-10344 (1994).
16. Fredriksson, S.*, et al.* Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol* **20**, 473-477 (2002).

17. Berggard, T., Linse, S. & James, P. Methods for the detection and analysis of protein-protein interactions. *Proteomics* **7**, 2833-2842 (2007).
18. Vogel, S.S., Thaler, C. & Koushik, S.V. Fanciful FRET. *Sci STKE* **2006**, re2 (2006).
19. Sorkin, A., McClure, M., Huang, F. & Carter, R. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr Biol* **10**, 1395-1398 (2000).
20. Kenworthy, A.K. Imaging protein-protein interactions using fluorescence resonance energy transfer microscopy. *Methods* **24**, 289-296 (2001).
21. Sako, Y., Minoghchi, S. & Yanagida, T. Single-molecule imaging of EGFR signalling on the surface of living cells. *Nat Cell Biol* **2**, 168-172 (2000).
22. Galperin, E., Verkhusha, V.V. & Sorkin, A. Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nat Methods* **1**, 209-217 (2004).
23. Xu, Y., Piston, D.W. & Johnson, C.H. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc Natl Acad Sci U S A* **96**, 151-156 (1999).
24. De, A., Loening, A.M. & Gambhir, S.S. An improved bioluminescence resonance energy transfer strategy for imaging intracellular events in single cells and living subjects. *Cancer Res* **67**, 7175-7183 (2007).
25. Xu, X.*, et al.* Imaging protein interactions with bioluminescence resonance energy transfer (BRET) in plant and mammalian cells and tissues. *Proc Natl Acad Sci U S A* **104**, 10264-10269 (2007).
26. Hu, C.D., Chinenov, Y. & Kerppola, T.K. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell* **9**, 789-798 (2002).
27. Ciruela, F. Fluorescence-based methods in the study of protein-protein interactions in living cells. *Curr Opin Biotechnol* **19**, 338-343 (2008).
28. Kerppola, T.K. Visualization of molecular interactions by fluorescence complementation. *Nat Rev Mol Cell Biol* **7**, 449-456 (2006).
29. Kerppola, T.K. Complementary methods for studies of protein interactions in living cells. *Nat Methods* **3**, 969-971 (2006).
30. Stolpe, T.*, et al.* In planta analysis of protein-protein interactions related to light signaling by bimolecular fluorescence complementation. *Protoplasma* **226**, 137-146 (2005).
31. Sung, M.K. & Huh, W.K. Bimolecular fluorescence complementation analysis system for in vivo detection of protein-protein interaction in Saccharomyces cerevisiae. *Yeast* **24**, 767-775 (2007).

32. Hu, C.D. & Kerppola, T.K. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol* **21**, 539-545 (2003).
33. Shyu, Y.J., Suarez, C.D. & Hu, C.D. Visualization of AP-1 NF-kappaB ternary complexes in living cells by using a BiFC-based FRET. *Proc Natl Acad Sci U S A* **105**, 151-156 (2008).
34. Gandia, J.*, et al.* Detection of higher-order G protein-coupled receptor oligomers by a combined BRET-BiFC technique. *FEBS Lett* **582**, 2979-2984 (2008).
35. Carriba, P.*, et al.* Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat Methods* **5**, 727-733 (2008).
36. Soderberg, O.*, et al.* Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* **3**, 995-1000 (2006).
37. Gullberg, M.*, et al.* Cytokine detection by antibody-based proximity ligation. *Proc Natl Acad Sci U S A* **101**, 8420-8424 (2004).
38. Lizardi, P.M.*, et al.* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet* **19**, 225-232 (1998).
39. Baner, J., Nilsson, M., Mendel-Hartvig, M. & Landegren, U. Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res* **26**, 5073-5078 (1998).
40. Zieba, A.*, et al.* Bright-field microscopy visualization of proteins and protein complexes by in situ proximity ligation with peroxidase detection. *Clin Chem* **56**, 99-110 (2010).
41. Sigal, A.*, et al.* Variability and memory of protein levels in human cells. *Nature* **444**, 643-646 (2006).
42. Lahav, G.*, et al.* Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat Genet* **36**, 147-150 (2004).
43. Raj, A., Peskin, C.S., Tranchina, D., Vargas, D.Y. & Tyagi, S. Stochastic mRNA synthesis in mammalian cells. *PLoS Biol* **4**, e309 (2006).
44. Dedon, P.C., Soults, J.A., Allis, C.D. & Gorovsky, M.A. A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. *Anal Biochem* **197**, 83-90 (1991).
45. Ren, B.*, et al.* Genome-wide location and function of DNA binding proteins. *Science* **290**, 2306-2309 (2000).
46. Orlando, V. Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. *Trends Biochem Sci* **25**, 99-104 (2000).
47. Kim, T.M. & Park, P.J. Advances in analysis of transcriptional regulatory networks. *Wiley Interdiscip Rev Syst Biol Med* **3**, 21-35 (2011).
48. O'Neill, L.P., VerMilyea, M.D. & Turner, B.M. Epigenetic characterization of the early embryo with a chromatin

immunoprecipitation protocol applicable to small cell populations. *Nat Genet* **38**, 835-841 (2006).
49. Gustafsdottir, S.M.*, et al.* In vitro analysis of DNA-protein interactions by proximity ligation. *Proc Natl Acad Sci U S A* **104**, 3067-3072 (2007).
50. Cremazy, F.G.*, et al.* Imaging in situ protein-DNA interactions in the cell nucleus using FRET-FLIM. *Exp Cell Res* **309**, 390-396 (2005).
51. Zirbel, R.M., Mathieu, U.R., Kurz, A., Cremer, T. & Lichter, P. Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. *Chromosome Res* **1**, 93-106 (1993).
52. Brown, K. Visualizing nuclear proteins together with transcribed and inactive genes in structurally preserved cells. *Methods* **26**, 10-18 (2002).
53. Brown, K.E.*, et al.* Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**, 845-854 (1997).
54. Zinner, R., Teller, K., Versteeg, R., Cremer, T. & Cremer, M. Biochemistry meets nuclear architecture: multicolor immuno-FISH for co-localization analysis of chromosome segments and differentially expressed gene loci with various histone methylations. *Adv Enzyme Regul* **47**, 223-241 (2007).
55. Gall, J.G. & Pardue, M.L. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci U S A* **63**, 378-383 (1969).
56. Levsky, J.M. & Singer, R.H. Fluorescence in situ hybridization: past, present and future. *J Cell Sci* **116**, 2833-2838 (2003).
57. Speicher, M.R. & Carter, N.P. The new cytogenetics: blurring the boundaries with molecular biology. *Nat Rev Genet* **6**, 782-792 (2005).
58. Schrock, E.*, et al.* Multicolor spectral karyotyping of human chromosomes. *Science* **273**, 494-497 (1996).
59. Speicher, M.R., Gwyn Ballard, S. & Ward, D.C. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat Genet* **12**, 368-375 (1996).
60. Karhu, R.*, et al.* Chromosome arm-specific multicolor FISH. *Genes Chromosomes Cancer* **30**, 105-109 (2001).
61. Femino, A.M., Fay, F.S., Fogarty, K. & Singer, R.H. Visualization of single RNA transcripts in situ. *Science* **280**, 585-590 (1998).
62. Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A. & Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods* **5**, 877-879 (2008).
63. Silahtaroglu, A.N.*, et al.* Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. *Nat Protoc* **2**, 2520-2528 (2007).

64. Lu, J. & Tsourkas, A. Imaging individual microRNAs in single mammalian cells in situ. *Nucleic Acids Res* **37**, e100 (2009).
65. Volpi, E.V. & Bridger, J.M. FISH glossary: an overview of the fluorescence in situ hybridization technique. *Biotechniques* **45**, 385-386, 388, 390 passim (2008).
66. Dirks, R.W. & Tanke, H.J. Advances in fluorescent tracking of nucleic acids in living cells. *Biotechniques* **40**, 489-496 (2006).
67. Itzkovitz, S. & van Oudenaarden, A. Validating transcripts with probes and imaging technology. *Nat Methods* **8**, S12-19 (2011).
68. Koch, J.E., Kolvraa, S., Petersen, K.B., Gregersen, N. & Bolund, L. Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. *Chromosoma* **98**, 259-265 (1989).
69. Pellestor, F. Development and adaptation of the PRINS technology: an overview. *Methods Mol Biol* **334**, 211-220 (2006).
70. Kadandale, J.S., Tunca, Y. & Tharapel, A.T. Chromosomal localization of single copy genes SRY and SOX3 by primed in situ labeling (PRINS). *Microb Comp Genomics* **5**, 71-74 (2000).
71. Troyer, D.L., Xie, H. & Hu, J. Direct in situ single-copy (DISC)-PCR. *Methods Mol Biol* **71**, 71-76 (1997).
72. Bagasra, O. Protocols for the in situ PCR-amplification and detection of mRNA and DNA sequences. *Nat Protoc* **2**, 2782-2795 (2007).
73. Player, A.N., Shen, L.P., Kenny, D., Antao, V.P. & Kolberg, J.A. Single-copy gene detection using branched DNA (bDNA) in situ hybridization. *J Histochem Cytochem* **49**, 603-612 (2001).
74. Landegren, U., Kaiser, R., Sanders, J. & Hood, L. A ligase-mediated gene detection technique. *Science* **241**, 1077-1080 (1988).
75. Nilsson, M.*, et al.* Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science* **265**, 2085-2088 (1994).
76. Nilsson, M.*, et al.* Padlock probes reveal single-nucleotide differences, parent of origin and in situ distribution of centromeric sequences in human chromosomes 13 and 21. *Nat Genet* **16**, 252-255 (1997).
77. Larsson, C.*, et al.* In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. *Nat Methods* **1**, 227-232 (2004).
78. Shaposhnikov, S., Larsson, C., Henriksson, S., Collins, A. & Nilsson, M. Detection of Alu sequences and mtDNA in comets using padlock probes. *Mutagenesis* **21**, 243-247 (2006).
79. Larsson, C., Grundberg, I., Soderberg, O. & Nilsson, M. In situ detection and genotyping of individual mRNA molecules. *Nat Methods* **7**, 395-397 (2010).
80. Hart, C.E.*, et al.* Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* **240**, 1529-1531 (1988).
81. Bergsten, E.*, et al.* PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. *Nat Cell Biol* **3**, 512-516 (2001).

82. Ostman, A. PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev* **15**, 275-286 (2004).
83. Heldin, C.H., Ostman, A. & Ronnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* **1378**, F79-113 (1998).
84. Baselga, J. Targeting tyrosine kinases in cancer: the second wave. *Science* **312**, 1175-1178 (2006).
85. Matsui, T.*, et al.* Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* **243**, 800-804 (1989).
86. Matsui, T.*, et al.* Independent expression of human alpha or beta platelet-derived growth factor receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways. *Proc Natl Acad Sci U S A* **86**, 8314-8318 (1989).
87. Bermudez, O., Pages, G. & Gimond, C. The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. *Am J Physiol Cell Physiol* **299**, C189-202 (2010).
88. Jurek, A., Amagasaki, K., Gembarska, A., Heldin, C.H. & Lennartsson, J. Negative and positive regulation of MAPK phosphatase 3 controls platelet-derived growth factor-induced Erk activation. *J Biol Chem* **284**, 4626-4634 (2009).
89. Yarden, Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer* **37 Suppl 4**, S3-8 (2001).
90. Schlessinger, J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* **110**, 669-672 (2002).
91. Moriki, T., Maruyama, H. & Maruyama, I.N. Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. *J Mol Biol* **311**, 1011-1026 (2001).
92. Bonasio, R., Tu, S. & Reinberg, D. Molecular signals of epigenetic states. *Science* **330**, 612-616 (2010).
93. Lander, E.S.*, et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).
94. Deininger, P.L. & Batzer, M.A. Alu repeats and human disease. *Mol Genet Metab* **67**, 183-193 (1999).
95. Rudiger, N.S., Gregersen, N. & Kielland-Brandt, M.C. One short well conserved region of Alu-sequences is involved in human gene rearrangements and has homology with prokaryotic chi. *Nucleic Acids Res* **23**, 256-260 (1995).
96. Deininger, P.L., Jolly, D.J., Rubin, C.M., Friedmann, T. & Schmid, C.W. Base sequence studies of 300 nucleotide renatured repeated human DNA clones. *J Mol Biol* **151**, 17-33 (1981).
97. Rodriguez, J.*, et al.* Genome-wide tracking of unmethylated DNA Alu repeats in normal and cancer cells. *Nucleic Acids Res* **36**, 770-784 (2008).

98. Sorkin, A., Eriksson, A., Heldin, C.H., Westermark, B. & Claesson-Welsh, L. Pool of ligand-bound platelet-derived growth factor beta-receptors remain activated and tyrosine phosphorylated after internalization. *J Cell Physiol* **156**, 373-382 (1993).
99. Tidball, J.G. & Spencer, M.J. PDGF stimulation induces phosphorylation of talin and cytoskeletal reorganization in skeletal muscle. *J Cell Biol* **123**, 627-635 (1993).
100. Rajkumar, V.S.*, et al.* Platelet-derived growth factor-beta receptor activation is essential for fibroblast and pericyte recruitment during cutaneous wound healing. *Am J Pathol* **169**, 2254-2265 (2006).
101. Nilsson, I.*, et al.* VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. *EMBO J* **29**, 1377-1388 (2010).
102. Rasband, W.S. ImageJ. (1997-2005).
103. Carpenter, A.E.*, et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7**, R100 (2006).
104. Krayev, A.S.*, et al.* The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. *Nucleic Acids Res* **8**, 1201-1215 (1980).
105. Leuchowius, K.J.*, et al.* High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol Cell Proteomics* (2009).

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 674

Editor: The Dean of the Faculty of Medicine

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

Distribution: publications.uu.se
urn:nbn:se:uu:diva-151579

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2011