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Making Visible the Proximity Between Proteins

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Abstract

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Genomic DNA is the template of life - the entity which is characterized by a self-sustaining anatomical development, regulated signaling processes, the ability to reproduce and to respond to stimuli. Through what is classically known as the central dogma, the genome is transcribed into mRNA, which in turn is translated into proteins. The proteins take part in most, if not all, cellular processes, and it is by unraveling these processes that we can begin to understand life and disease-causing mechanisms.

In vitro and *in vivo* assays are two levels at which protein communication may be studied, and which permit manipulation and control over the proteins under investigation. But in order to retrieve a representation of the processes as close to reality as possible, *in situ* analysis may instead be applied as a complement to the other two levels of study. *In situ* PLA offers the ability to survey protein activity in tissue samples and primary cell lines, at a single cell level, detecting single targets in their natural unperturbed environment.

In this thesis new developments of the *in situ* PLA are described, along with a new technique offering *in situ* enzyme-free detection of proximity between biomolecules.

The dynamic range of *in situ* PLA has now been increased by several orders of magnitude to cover analogous ranges of protein expression; the output signals have been modified to offer a greater signal-to-noise ratio and to limit false-positive-rates while also extending the dynamic range further; simultaneous detection of multiple protein complexes is now possible; proximity-HCR is presented as a robust and inexpensive enzyme-free assay for protein complex detection.

The thesis also covers descriptions on how the techniques may be simultaneously applied, also together with other techniques, for the multiple data-point acquisition required by the emerging realm of systems biology. A future perspective is presented for how much more information may be simultaneously acquired from tissue samples to describe biomolecular interactions in a new manner. This will allow new types of biomarkers and drugs to be discovered, and a new holistic understanding of life.

Keywords: Proximity ligation assay, In situ PLA, rolling circle amplification, protein interaction, protein-protein interaction, in situ, single cell, single molecule, protein complex, antibody, cancer, tissue section, microscopy, image analysis, system biology, multiplex, dynamic range, methods development, systems biology

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“Chaos is the key to creativity.”
John A. McCarthy

To my family and friends

List of Papers

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

I **Clausson, C.M.**, Allalou, A., Weibrecht, I., Mahmoudi, S., Farnebo, M., Landegren, U., Wählby, C., Söderberg, O. (2011) Increasing the dynamic range of in situ PLA. *Nature Methods*, 8(11):892–893

II **Clausson, C.M.***, Arngården, L.*, Ishaq, O., Krzywkowski, T., Koos, B., Brismar, H., Wählby, C., Nilsson, M., Söderberg, O. Compaction of rolling circle amplification products increases signal strength and integrity. *Manuscript*

III Leuchowius, K.J.*, **Clausson, C.M.***, Figueiredo, J., Erbilgin, Y., Botling, J., Landegren, U., Seruca, R., Söderberg, O. (2013) Parallel visualization of multiple protein complexes in individual cells in tumor tissue. *Molecular & Cellular Proteomics*, 12(6):1563-71

IV Koos, B., Cane, G., **Clausson, C.M.**, Grannas, K., Arngården, L., Klaesson, A., Söderberg, O. Proximity dependent initiation of hybridization chain reaction. *Manuscript*

* Equal contribution

Related work by the author

Review articles

i. Weibrecht, I., Leuchowius, K.J., **Clausson, C.M.**, Conze, T., Jarvius, M., Howell, W. M., Kamali-Moghaddam, M., Söderberg, O. (2010) Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert review of proteomics*, 7(3):401-409

ii. **Clausson, C.M.**, Grundberg, I., Weibrecht, I., Nilsson, M., Söderberg, O. (2012) Methods for analysis of the cancer microenvironment and their potential for disease prediction, monitoring and personalized treatments *The EPMA journal*, 3(1):1-9

Book chapters

i. Koos, B., Andersson, L., **Clausson, C.M.**, Grannas, K., Klaesson, A., Cane, G., Söderberg, O. Analysis of Protein Interactions in situ by Proximity Ligation Assays. In: *Current Topics in Microbiology and Immunology*. Springer Berlin Heidelberg, 2013;334: 1-16

Patents

i. Clausson, C. M., Gullberg, M., Söderberg, O., Weibrecht, I. Dynamic Range Methods (2013). Publication numbers: US20130288249 A1; EP2627781 A1; WO2012049316 A1. Application numbers: US 13/879,038; EP20110773711; PCT/EP2011/068039.

Contents

Abbreviations

AP-MS	Affinity purification-mass spectrometry
BiFC	Bimolecular fluorescence complementation assay
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine monophosphate
Co-IP	Co-immunoprecipitation
CyTOF	Time of flight cytometry
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
FACS	Fluorescently activated cell sorting
FlAsH	Fluorescein arsenical helix binder
FRET	Förster resonance energy transfer
HCR	Hybridization chain reaction
HER2	Human epidermal growth factor receptor 2
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
LAMP	Loop-mediated isothermal amplification
MAPK	Mitogen-activated protein kinase
MELC	Multi-epitope-ligand cartography
mRNA	Messenger-ribonucleic acid
PCA	Protein fragment complementation assay
PCR	Polymerization chain reaction
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLA	Proximity ligation assay
PRINS	Primed *in situ* labeling
qPCR	Quantitative polymerization chain reaction
RCA	Rolling circle amplification
ReAsH	Red arsenical helix binder
RNA	Ribonucleic acid
SH2	SRC Homology domain 2
SH3	SRC Homology domain 3
SKBR3	Sloan-Kettering human breast cancer cell line
SPADE	Spanning-tree progression analysis of density-normalized events
ssDNA	Single-stranded deoxynucleic acid
TEV	Tobacco-etch virus

TMP	Trimethopim
Y2H	Yeast-two-hybrid

Introduction

I will begin by identifying the setting in which my biological investigation presented herein takes place.

Humans have an unending compulsion to ask why. It is something that sets us apart from our closest evolutionary relative, the chimpanzee. When confronted with problems of reasonable difficulty, chimpanzees tend not to find gratification in the problem solving itself while humans do[1], even when there is no direct survival benefit from solving the problem. Among all the questions humans ask, the ultimate may be "what is life?" This question can be confronted from many different viewpoints, which in turn can be grouped into either the humanities or the natural sciences. Both categories find their base in philosophy, but a main feature of natural sciences, which sets it apart from the humanities, is the empirical approach to answering questions. The empirical procedure works by observing a phenomenon and experimenting with its constituents in order to gather evidence to describe what the phenomenon depends upon. This restrains natural sciences from going beyond factual investigations when answering questions, but has proven very powerful for the human understanding of the mechanisms of nature.

Biology is one of the classical branches of natural science, and it is under this category that my work comes in. Biology is delimited to study living organisms, which are defined as entities that are self-sustaining by regulated signaling processes and have reproduction, anatomical development and respond to stimuli. This is what natural sciences define as life, and it will be the definition of life in this thesis. Life is very complex and can be studied at different levels. There are evolutionary biologists who may wander out into the wild and study populations of animals, investigate their behavior and environment in order to understand evolution. On a different level another type of biologist may be concerned with what genes underlie a certain type of ability that a species has developed in order to fill an ecological niche. Such a biologist may be said to be a geneticist. On a deeper level, we will find people concerned with how genes exert their functions in cells by being transcribed and translated into proteins, which exert their functions through interactions with other proteins. This is the level of biochemistry, and it is the specific subcategory of biology under which my work is done.

Life ultimately depends on the interactions between biomolecules in the cells and the sub-cellular compartments. The interactions manifest themselves on higher levels in tissue functions, organ functions and organismal functions. It is by understanding the molecular interactions, or the micro-ecology of cells, that we also hope to understand life. To this end I have taken part in developing tools for investigation.

Natural sciences have since long had a profound preference for the reductionist approach, where the system under study is regarded as nothing more than the sum of its parts. The expectation is that by understanding the function of each part, the system will also be understood. On the other side of the scale resides holism, where a system is considered as a whole and where the parts of the system are understood in relation to the function of the whole system.

From my perspective systems biology - the interdisciplinary science of biology and physics - should become increasingly important. With a holistic view of systems within a cell, or a system that a group of cells make up in a tissue, terminology and concepts from physics can be applied in biology. New types of questions will be posed, making possible new approaches to the understanding of how biological mechanisms work. The main hurdle to overcome for enabling deeper analyses of the micro-ecology in both healthy and diseased tissues is to generate multiple data points in single cells while also preserving the information of tissue architecture, as I will explain further.

The four papers in this thesis describe new tools offering certain properties, which might be advantageous in the realm of systems biology, where high-content data is needed. I will first describe the basic principles of the *in situ* proximity ligation assay, around which most of my work revolves. I will discuss other methods related to the area of protein interaction detection, their validity and finally I will discuss important factors for the application of the methods in systems biology.

Single-cell technology

Tolstoy started a novel with the words "happy families are all alike; every unhappy family is unhappy in its own way"[2]. This perspective reflects how we humans focus on identifying problems, which is the first step to solving them. Happy and unhappy tissues may be regarded in the same manner. There are always variations among cells within tissues, happy or unhappy, but we are often more interested in understanding and solving the problematic ones. The view of cancer tissues has become more complex in recent years as interrogation techniques have advanced. The idea of cancer has previously been that it is arising from a single progenitor cell dysfunctional in a specific way through mutations, leading to unregulated cell division that will result in a tumor. However, the closer we look the more complex the tumors will appear. The cancer cells evolve and are subjected to natural selection through interplay with its microenvironment. As is the case with other ecosystems, also the tissue microenvironment changes and responds to the populations in its niche and to external interference. For example, one cell population of the same cancer clone may be eradicated by external interference, e.g. chemotherapy, only to leave room for another cancer clone to thrive[3]. To solve the problem, we need to understand the communication within cells and between neighboring cells, and how it may be controlled. To do this we need methods capable of single-cell analysis.

In order to retrieve the best picture possible of the molecular interaction networks taking place, clinical tissue specimens are required. The techniques then applied for analysis can either leave the tissue intact to perform the study *in situ*, or they may extract the cells of interest and perform experiments *in vitro*. Two main disadvantages of *in vitro* analysis of clinical tissue specimens is the yet unavailable single-cell resolution of data, and that the spatial information is lost.

The method most used for protein detection *in situ* is immunohistochemistry (IHC)[4], which is a modification of immunofluorescence (IF)[5]. These techniques allow for a semi-quantitative protein level detection in individual cells of tissue sections, and not single-molecule detection. Although they have been used in routine diagnostics for decades, they struggle with unspecificity and in setting up new assays validation by other techniques, such as mRNA sequencing, is required. These techniques are discussed in more detail later.

Single-cell measurements of proteins in cells located in suspension, e.g. blood cells, can be performed by fluorescently activated cell sorting (FACS), image flow cytometry[6] or mass cytometry[7]. FACS and image flow cytometry both target proteins via IF, with which follows unspecificity problems as just

mentioned. The image flow cytometry technique offers the advantage of capturing an image of each cell, which permits image analysis and co-localization studies of proteins. Although the throughput of thousands of cells per second is impressive, the alternative of microscopy scanning of cells fixated onto glass slides also offers data sets of thousands of cells and at much greater image resolution. The multiplex detection of protein targets has reached more than 30 via mass cytometry, where the limiting spectral properties of applying fluorophore-labeled affinity reagents is circumvented by instead using lanthanide isotopes for labeling[7]. However, protein interaction detection is still lacking here, and so is single-molecule detection.

Assays providing spatial relationships between protein expression level and status, protein interaction, surface markers and relayed intra- and intercellular signals, will facilitate improved tissue profiling that can be used to model the micro-ecology *in vivo*. In essence, this will shed light upon the complex communication between and within cells taking place in a microenvironment.

Instead of focusing on intracellular pathological pathways, larger pathological intercellular pathways may be discerned in the tissue microenvironment. New multi-target drugs may be developed which obstruct particular interaction patterns between cells in a microenvironment, which are unique or altered in disease. By controlling the microenvironment, hopefully the size of the malignant niches and cancer cell mobility can be suppressed. Drugs may function by disengaging cellular functions non-essential to individual normal and abnormal cells, but essential to a pathological interplay between abnormal cells.

Single-cell analysis is also important as embedded assay controls, to determine biological and technical variation in normal tissue samples or cell lines for model building in systems biology. An obstacle concerning tissue specimens is that the measurement of data-points per cell may be precluded by the fact that whole cells are seldom available in regular tissue slices. Tissue specimens are commonly sliced at four micrometer sections, while cell nuclei themselves have diameters on the order of tens of micrometers. To manage this situation, adaptation of sample preparation, or of the wet-lab assay or of data processing, may be required. Samples may be sliced thicker, or measurements of a grid of area units may replace per-cell measurements, or the quantification of a house-keeping protein may be used for data-point normalization.

Basics of the *in situ* proximity ligation assay (PLA)

The *in situ* PLA[8] is a modification of the original PLA version[9], for localized detection *in situ*. The original PLA version employs DNA aptamers for target protein binding in solution. When the target protein forms a dimer upon interaction and the aptamers have bound a monomer each, the aptamers come into proximity of one another. Each aptamer has a tail of ssDNA, and when two aptamers are close to one another a connector oligonucleotide brings together each aptamer tail via hybridization. The tails are then ligated together, and the successful ligation is read-out via qPCR. In a later report, exchanging the DNA aptamers as binders for DNA-conjugated IgG in solution phase PLA resulted in a femtomolar sensitivity in the detection of cytokines[10]. The use of IgG rather than DNA aptamers is beneficial also because it provides a huge repertoire of high quality affinity reagents, developed for methods such as IHC, which can be readily implemented in PLA. The *in situ* version of PLA works upon the same basic principles of target binding and proximity-dependent ligation, but the method of read-out is different. The qPCR read-out is exchanged with rolling circle amplification (RCA), since an anchored signal is required for localized detection of the targets.

In situ PLA[8] is used to determine close proximity between two proteins in fixed cells or tissue sections on a microscopy slide (**figure 1**). In order to do this, the first step is to locate the proteins of interest in the tissue sample with an affinity reagent that provides a handle for further steps. In *in situ* PLA this reagent consists of IgG coupled to a short single-stranded DNA oligonucleotide (together becoming a proximity probe; sometimes called PLA probe). When two of these proximity probes are localized close to one another, i.e. when they have bound adjacent epitopes, their oligonucleotide arms are available to DNA manipulation tools. Two circularization oligonucleotides are added to the reaction, and if the two arms are in proximity and hybridization occurs successfully, this facilitates the formation of a continuous single stranded DNA circle upon ligation by T4 DNA ligase. Signal generation is the next step in the method, where the circle is used as a template for rolling circle amplification and the oligonucleotide of the proximity probe as a primer. The amplification creates a large bundle of single-stranded DNA that will be connected to the targeted antigen via the proximity probe. Subsequently, hundreds of short fluorescently labeled DNA oligonucleotides are hybridized to this bundle. The high local concentration of the fluorophores is then visible as a bright fluorescent dot of approximately 1 μm in a regular fluorescence microscope. *In situ* PLA has since been used for the detection of several different proximity events[11], such as post-translational modifications[12-14], protein-DNA interactions[15,16], protein-RNA interactions[17], for detection of fusion tags[18,19] and combined with padlock probes for simultaneous measurement of proteins and mRNA[20,21].

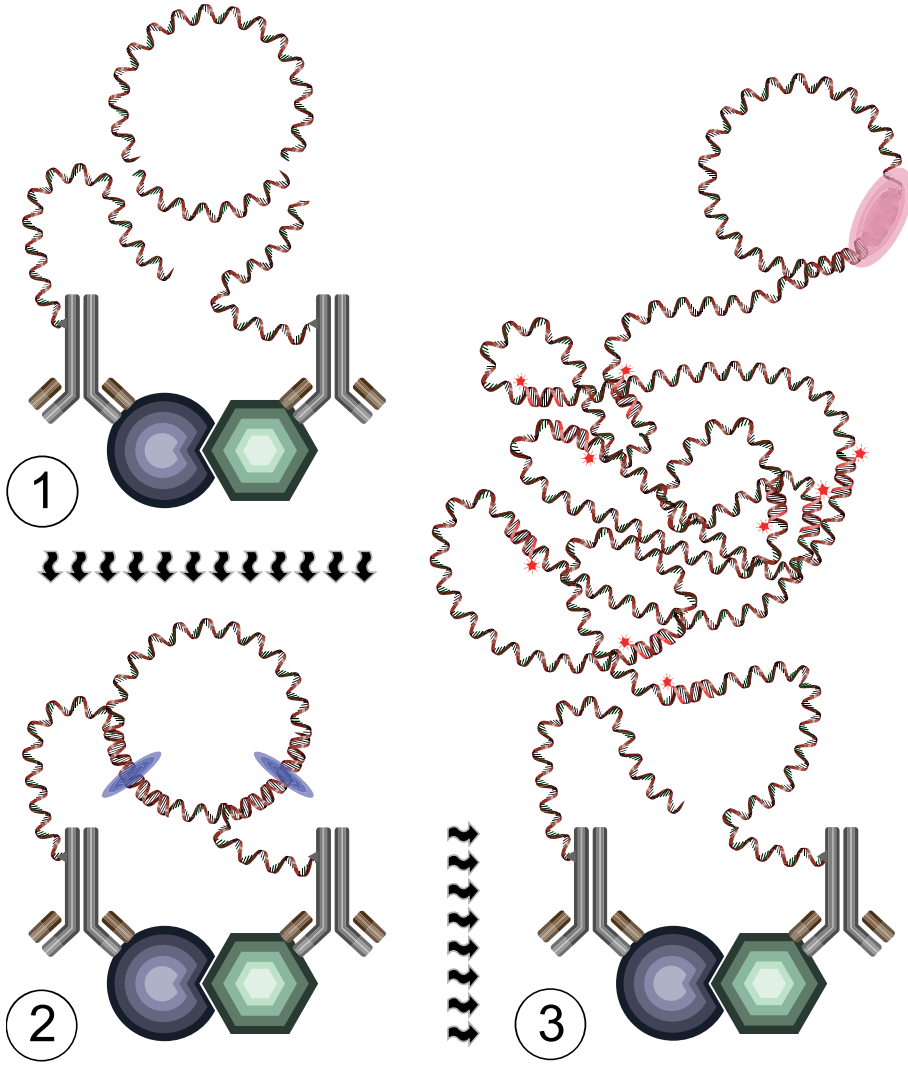

Figure 1 - Principle of *in situ* PLA.
A protein complex is targeted with PLA proximity probes, and a short and a long circularization oligonucleotide are thereafter hybridized to the oligonucleotide arms of the proximity probes (1). After the successful proximity-dependent hybridization, the circularization oligonucleotides are ligated into a continuous DNA circle (2) for the following rolling circle amplification. The RCA product becomes a concatemer of copies complementary to the DNA circle, and each repeat contains a detection sequence to which a fluorophore-labeled detection oligonucleotide may hybridize (3). The RCA product collapses onto itself, and the high local concentration of fluorophores becomes visible as a bright dot of ~1 μm in an epifluorescence microscope.

Other techniques for protein interaction detection

The IHC[4] and IF[5] techniques brought up earlier are single-recognition immuno-assays. In IF antibodies are labeled with fluorophores and in IHC with enzymes, that generate a colored precipitate at the site of antibody binding. IHC has successfully been used both in research and in clinical settings to determine protein levels in individual cells within a tissue section. However, the selectivity of an assay is fully dependent on the antibody used. It should be emphasized that all antibodies bind multiple proteins, but with different affinity. False positive signals are therefore common due to the fact that only a single binder is required for signal generation. Up to 19% false positive signal generation has been reported in cell lines, by comparison to RNA sequencing[22], and the rate is expected to be higher in more complex samples such as tissue.

The IF technique has been applied in an early manner to investigate the co-localization of two proteins, via double-staining[23,24]. The resolution is far too low for the technique to be able to infer a physical interaction between proteins, but despite this it is still used regularly in some settings where the interactions have previously been validated by other means[25,26]. With super-resolution techniques it may however be possible to detect close proximities between single proteins in clinical tissue samples, with a resolution down to 20-40 nm in 3D, without signal amplification[27,28]. However, super resolution techniques are currently hampered by their limited multiplexing capabilities, as well as the high cost of the instruments and demanding sample preparation.

Förster resonance energy transfer (FRET) is a widely adopted technique that has had a major impact for protein complex detection *in vivo*, but which can also detect protein complexes *in situ* in tissue. The basic principle of FRET is to use the energy transfer between two compatible protein fluorophores to report on proximity. The emission spectra of the first fluorescent protein (donor), needs to overlap with the excitation spectra of the second fluorescent protein (acceptor). The donor and the acceptor are fused to one protein each, and if the two proteins are in close proximity the energy transfer can occur. FRET has developed throughout the years mostly with new designs of fluorescent proteins[29], new fusion proteins and three-plex FRET[30]. More has happened with the instrumentation used to investigate FRET[29]. Super resolution microscopy has made possible single-molecule FRET[31] and trajectory mapping[32]. Tracking of small molecules such as sugar, Ca^{2+}, cAMP and PKA activity is a big advantage of FRET[33,34]. A weakness is the complex analysis methods involved and the advanced expertise required[35,36]. The spectral overlap requirement of fluorescent proteins in FRET makes it difficult to investigate several protein interactions simultaneously. Two interac-

tions have been studied at the same time, via the use of two pairs of FRET donors and acceptors[37,38]. FRET multiplexing has reached the ability to measure three events by identifying the spectral fingerprint of each fluorophore later used as reference in post-image acquisition unmixing of the fingerprints. This multiplex adaptation holds a long-term promise to detect up to 10 or more simultaneous proximity events through the development of optimized fluorophores[34].

Bioluminescence resonance energy transfer (BRET) is a relative of FRET, where the autofluorescence of the surrounding tissue is avoided by obviating the need for an external excitation light source. The light source is instead the light-emitting conversion of a substrate by luciferase, which has taken the place of the donor fluorescence protein in FRET[39]. BRET has evolved in conjunction with photo-switchable fluorophores, making it possible to determine the ratio between non-interacting proteins and interacting proteins[40]. A similar technique is used with positron emission tomography (PET), with the acceptor fluorophore being excited by β-particles[41].

In general, BRET and FRET have evolved deeper into the area of *in vivo* studies, where transfection of fusion proteins is needed. For instance, BRET-FRET techniques have evolved to study membrane proteins, which are difficult to study via co-immunoprecipitation, and also to increase the order of complexity possible[42]. New species of fluorescence molecules have made their way into FRET and BRET techniques, with lower spectral overlap, better photostability, intensity, possibility to conjugate to live cells inserted into live mice[43-45], and the possibility of quantum dots to be both acceptor and donor of light in FRET-FRET techniques[46]. Also, a new type of quantum dot working in the infra-red range has increased the sensitivity of FRET, which otherwise is unsuitable for the detection of scarce events using other parts of the spectra which yield higher autofluorescence of tissues and cells[47]. The FRET limitation of around 10 nm of proximity renders the quantum yield of FRET, and thereby also the mentioned techniques, highly sensitive to the precise locations of the target epitopes and in what direction the fluorophores end up. However, the limitations on biochemical flexibility of FRET may instead be turned to an advantage, e.g. for *in vivo* measurement of minute conformational changes of a protein. While BRET and FRET techniques provide sensitive *in vivo* assays with a future promise of a higher level of multiplexing, their application to clinical tissue is not straightforward. As transfection is not possible, affinity reagents need to be used targeting the biomolecules of interest[48,49]. Unfortunately, the autofluorescence of clinical tissue samples is often too high to be overcome by regular FRET based on flurophore-labeled antibodies, and require more complicated solutions such as time-resolved FRET[50]. A more straight-forward way to measure protein complexes in tissue is offered by *in situ* PLA. Albeit, meas-

uring kinetics and conformational changes in living cells is instead something *in situ* PLA cannot do.

The protein fragment complementation assay (PCA) monitors protein interaction *in vivo* via the reconstitution of a reporter protein. In a dimeric protein interaction, each protein is a designed fusion protein with one of the two monomers of the split reporter. In the original version the reporter protein is β-galactosidase, transforming a substrate into a fluorescent signal upon reconstitution[51]. The bimolecular fluorescence complementation assay (BiFC) is a version of PCA, with the split reporter being a fluorescent protein. A disadvantage has previously been the high background caused by a too high affinity between the split reporter monomers, driving the interaction. Recent constructs have been proposed yielding less background[52]. A protease from the tobacco-etch-virus (TEV) has also been used as a reporter in a PCA. Upon interaction between two proteins, the protease can cleave off a transcription factor to enter the nucleus and start the transcription of a reporter protein[53]. However, already twelve years earlier the split-ubiquitin assay was published[54]. It is related to the split-TEV assay in that it includes protease activity. Upon reconstitution of the ubiquitin, a reporter protein is cleaved off from the C-terminus by endogenous ubiquitin-specific proteases[55]. Naturally, PCA cannot be used for analysis of endogenously expressed proteins as vectors carrying the genes for the fusion proteins need to be transfected into the host cell. Although PCA enables high throughput screening, and is as such a very useful tool for discovery of putatively interacting proteins, other methods will be needed for detecting protein interactions in clinical material.

There also exist the techniques of chemical protein tags for the detection of proteins[56] and interactions *in vivo*. The protein of interest can have its primary structure altered with an insert of a peptide which is chemically inert to naturally occurring compounds, but highly reactive with synthetic compounds. The peptide can be of varying types and sizes depending on the chemical tag, which may be FlAsH/ReAsH, the SNAP/CLIP tag, the TMP-tag, the HaloTag or the beta-lactamase tag. Instead of using energy transfer as in FRET, conversion of a compound into fluorescence as in BRET or reconstitution of a reporter protein as in PCA, these chemical protein tags can be used to report on close proximity between two tag-modified proteins by direct covalent attachment of a reporter molecule to one of the partners, facilitated by the other. The reporter molecule may be biotin or a reactive compound, which can act as a handle for further modification or purification, or a fluorescent molecule[57].

The HerMark™ or VeraTag™ assay is an approach to quantifying interactions in clinical tissue specimens, also fluorescently[58]. The sample tissue sections are placed on a microscopy slide before a close proximity between proteins is quantified. This is done via the cleaving off of a fluorescent molecule from one antibody via a photochemical reaction from a second antibody in the immediate vicinity. The read-out is the sum fluorescence intensity from the fluorophore in a specialized instrument. The technology does not allow subcellular localization of the interaction in the tissue, but has proven more sensitive than immunohistochemical methods such as the HercepTest, which is clinically used to stratify breast cancer patients[59]. The technique has limited abilities to multiplex, but has proven useful in pre-clinical settings to stratify patient cohorts with respect to their individual breast cancer types and treatment options[60,61].

All until now mentioned assays for protein detection label their targets. The assays either bring with them inherent false positives, from cross-reactive affinity reagents, or uncertainty towards to what extent genetic alteration of e.g. fusion proteins will perturb the true function of the native protein. A label-free *in situ* assay is therefore attractive in proteomics, and there exist versions of mass-spectrometry which enable this[62], capable of single-cell resolution down to 7 μm[63]. The principle is to first eject ionized molecules from the cell or tissue surface, by either a laser-, an ion- or an electron beam. The ions are transported to a detector, and quantitative data on the identified molecular species, collected at each ionization point in the section, may be translated to a visual depiction of the concentrations in the sample.

Apart from being label-free, this technique stands out with its unbiased approach, large dynamic range, its sensitivity and the degree of multiplexing that mass spectrometry brings with it. It shows great potential in diagnostic settings, with reliable application to clinical samples for identification of several types of proteins[64-66]. Two obvious drawbacks of this approach of *in situ* protein detection are the lack of means to detect protein interaction and single-molecules. Two other limitations are the relatively imprecise target quantification and the sample destruction, obviating further analysis.

The technique offers great potential from a systems biology point of view. It can perform tissue profiling based on the identification of hundreds, or thousands, of proteins and their abundance, at a single-cell resolution. However, activity status of intra- and intercellular communication pathways via protein interaction is not possible to map so far, due to the lack of methods for analysis of protein interactions.

Demands on methods

Interactions between biomolecules can take place in several ways. Stable interactions are typically characteristic of complex forming proteins while transient interactions relay signals within pathways. Hemoglobin is a workhorse in the human body and a very stable protein complex that does not dissociate. Other stable complexes may dissociate when they are not needed, such as the important RNA polymerase machinery. However, the majority of protein interactions are expected to be transient for events to occur at sufficient rates within cells[67].

The nature of interactions depends on the sequence, structure, posttranslational modification and the present allosteric state of the protein. Hydrogen bonds, salt bridges and van der Waal forces act as the chemical bonds between the interaction interfaces, which can be of different types. There are a few sequence motifs common to the interaction surfaces of several proteins, which are particularly suited for their purpose and may vary slightly in sequence resulting in altered affinities. Two of them are the classical SH2 and SH3 domains. They are often involved in signaling pathways and can be found in hundreds of different proteins[68].

Our understanding of the nature and significance of various types of protein interaction is likely not yet fully mature. It has recently been understood that a protein can exert its function in a cell by constant weak and non-specific interactions with many other proteins, abrogated by a post-translational modification such as phosphorylation[69].

All methods have their strengths and weaknesses. Thus, using multiple methods to validate the results is important, and to use methods appropriate for answering a specific question. As described above, signals produced by *in situ* PLA report on close proximities between epitopes (depending on the design, up to tens of nanometers). Consequently, successful *in situ* PLA cannot be used as proof of a physical interaction between the targeted proteins. However, this is true also for the other techniques. Co-immunoprecipitation is said to be the gold standard for protein interaction detection. However, experimental conditions, particularly the buffer conditions, which the interacting proteins are subjected to, are not the same as in their natural local cellular environment[70]. This may cause both false negatives and false positives. Also, when preparing cell lysates proteins are brought together which would otherwise be isolated from one another in cellular compartments.

Recent developments of co-immunoprecipitation read-outs has made possible quantifiable single-molecule detection and kinetics studies of interacting proteins from cell lysates[71,72] from as little as 10 cells[71] and on the order of tens of milliseconds[72]. However, the kinetics may not resemble the endogenous kinetics, as they will be affected by buffer conditions and concentrations.

Although *in situ* PLA can only suggest an interaction between proteins, it does so regardless of the affinity of the interaction, as the target proteins are fixated in the sample cells or tissue. Controls for interaction validation can be constructed in the form of obstructing the interaction with a drug if available[8], using transfection with a competing peptide[73], by introducing primary sequence changes at the interaction site[74], or by comparison to a knock-out cell line[8], or to an unstimulated sample or to tissue sections where it is known the proteins are expressed but do not interact.

In the identification of a problem within a method lies an opportunity for further development. I have within my doctoral education focused on a few limitations of *in situ* PLA.

(1) For a precise quantification of *in situ* PLA signals, the tools for image analysis require discrete signals of fluorescence decidedly stronger than the surrounding background autofluorescence. In order to meet this demand, RCA is applied to localize a great number of fluorophores to each target molecule. But as the RCA products have a large diameter of around 1 μm, the dynamic range over which targets may be quantified, is hampered. I have addressed this issue by two different approaches (Papers I & II), the second approach simultaneously limiting the production of false positive signals (Paper II).

(2) While *in situ* PLA is applied in more than one new publication every day, only very few conduct *in situ* PLA experiments in a holistic approach due to the restricted level of multiplexing. For the "next generation tissue profiling", high-content analysis of multiple proteins, protein interactions and post-translational modifications will need to be performed together with genotyping of mRNA. A first step towards this is development of a multiplexed *in situ* PLA system (Paper III).

(3) The dependency of enzymes for ligation and amplification makes *in situ* PLA costly and limits its usability in e.g. point-of-care devices. Hence, a different read-out of proximal binding without enzymatic steps would be very beneficial (Paper IV).

The dynamic range of *in situ* PLA

Assays have an upper and a lower limit of concentration within which it is possible to accurately measure analytes, and this is called the dynamic range. The lower limit is either two or three standard deviations above the background, depending on the accuracy required. The saturation limit is reached when an increased amount of target no longer gives a proportionately increased amount of signal.

For *in situ* PLA, the unit of measurement is usually signals per cell. Background measurements are often acquired as technical controls in assays precluding one of the primary antibodies in tissue experiments, but can also be biological controls by comparing the live experiment with cells or tissue known not to possess the protein interaction in question. A control may also be a cell line where the proteins of interest are mutated at the site of interaction, or it may be the disruption of interaction by a drug or small molecule, or it may be stimulation experiments.

Regardless of how the control is designed, the limit of detection will be two or three standard deviations above the background number of signals per cell. The saturation limit is reached when signals start to coalesce, because they become too crowded, and the saturation limit will be proportionate to the size of the cell.

For *in situ* PLA the dynamic range spans approximately from a few signals to several hundred signals per cell, or two orders of magnitude, depending on the cell size and the specificity of the antibodies applied. This may be considered low by comparison to the dynamic range of e.g. qPCR, which is theoretically nine orders of magnitude. The low dynamic range of *in situ* PLA can be attributed to the large size of the signals, typically around 1 μm in diameter. The problem arises when neighboring cells in a tissue or cell slide have largely diverging expression rates of a protein. For example, this range may be six orders of magnitude for HER2 in SKBR3 cells[75]. Also, in immuno assays a low dynamic range results in the need to titrate the antibodies, which is an obstacle to the use of precious clinical material or in high-throughput assays. However, the large signal size is otherwise an advantage of *in situ* PLA, as signals are easily distinguished over autofluorescence already at low magnifications (10 x or 20 x) and this also facilitates reliable signal quantification by image analysis software.

The dynamic range of *in situ* PLA may be slightly increased if the intensity per cell were to be measured instead of signals per cell, and thereby disregarding the possibility of signal localization. Instead we took another approach and created a new type of oligonucleotide hybridization step, with competing circularization oligonucleotides (**figure 2**). Three types of circu-

larization oligonucleotides are applied targeting the same proximity probe couple, but different in their respective detection oligonucleotide sequence. As they have the same target, they will compete for binding this target, and by adding them at a set concentration ratio (e.g. 1:10:100) they will give rise to an analogous ratio of different signals after the RCA.

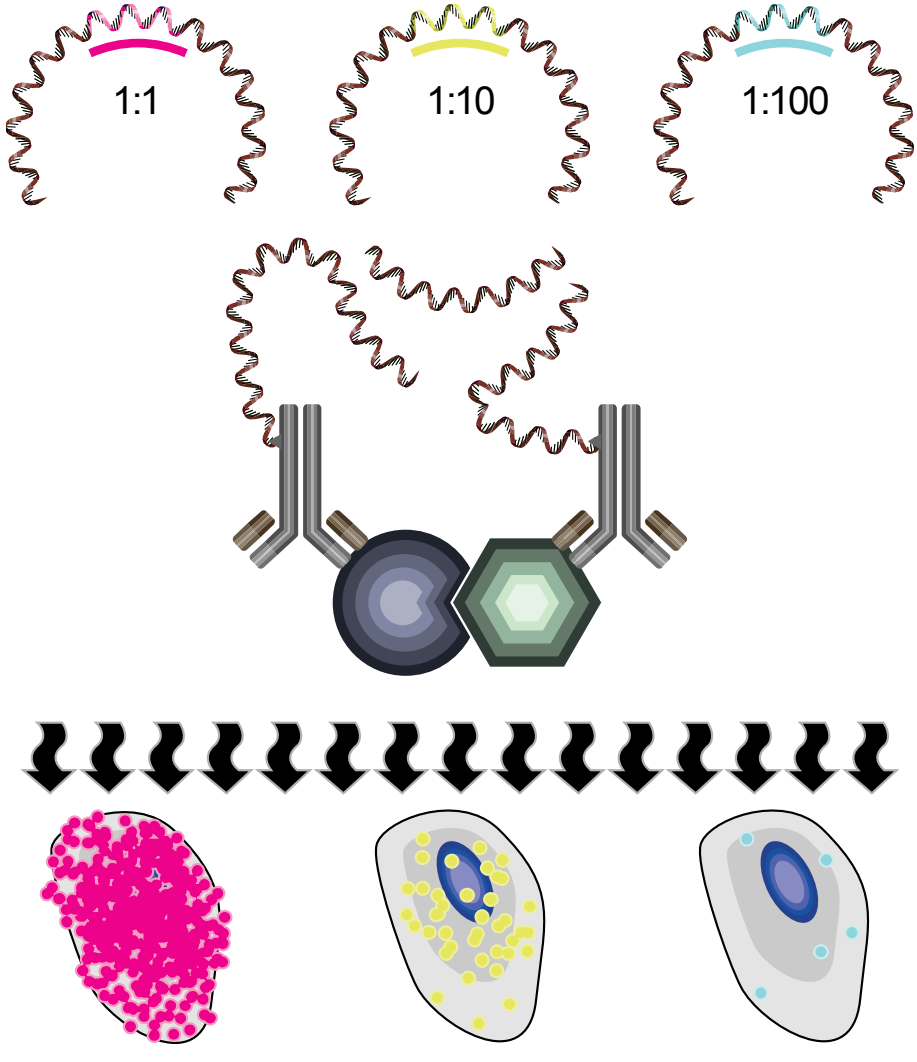

Figure 2 - An approach to extend the dynamic range of *in situ* PLA.
Multiple circularization oligonucleotides are applied, at a set concentration ratio, in the hybridization step of *in situ* PLA. They can hybridize to the same antibody-conjugated oligonucleotides, for which they will compete, but differ in their detection sequences. After RCA, signals of different colors will appear at a ratio analogous to that of the input concentrations.

The different RCA products are then detected by probing them with detection oligonucleotides of different fluorescent colors. Three different signal concentrations are then available in three separate fluorescence channels.

With this procedure there is theoretically no limit to the dynamic range of *in situ* PLA, and we demonstrated an increase by four orders of magnitude to cover the large concentration range of HER2 in SKBR3 cells and in breast cancer tissue sections overexpressing HER2. However, with this procedure the signals representing a single target occupy three fluorescence channels. This means the technique cannot be combined with multiplexed *in situ* PLA, where multiple targets occupy one fluorescence channel each, and in practice only four fluorescence channels can be used in the same assay. This calls for another approach to extending the dynamic range.

Compaction of the RCA product

An extended dynamic range would also be the result from smaller signals, or RCA products, as more signals per cell would be distinguishable. But making them smaller should not impede the detectability of the signals. For instance, performing the RCA for a shorter amount of time will decrease the size of the resulting ssDNA bundles, but will likewise decrease their intensity as there will be fewer copies of the detection sequence per RCA product to which detection oligonucleotides hybridize.

The detectability of the RCA products depends on the ratio, or the contrast, between the intensity of the local autofluorescence and the signal. The original *in situ* PLA signals generally exhibit a high contrast towards their local environment, but some formalin fixed and paraffin embedded (FFPE) tissues with exceptionally high autofluorescence, such as brain, fatty or connective tissue, still pose signal detection problems. While we expected the RCA products to collapse onto themselves into dense ssDNA bundles via unspecific hydrogen bonding of the bases and the inherent polarity of ssDNA, we have observed that the signals sometimes are stretched out and are not uniform. Digital enumeration of the signals through image analysis software would benefit from even more distinct and uniform signals.

As an improvement, we introduced a new type of short oligonucleotide to the RCA reaction itself, in essence pulling the RCA products together immediately during their generation to make them more condensed. The procedure is taking advantage of a previously unused spacer sequence of the RCA product (**figure 3**) in the original *in situ* PLA. To get into more details, the compaction oligonucleotide is 48 nucleotides long. Out of these, 42 nucleotides are two copies of the same 21 nucleotide sequence, which are spaced apart with 3 nucleotides. The 3’ end of the compaction oligonucleotide has 3

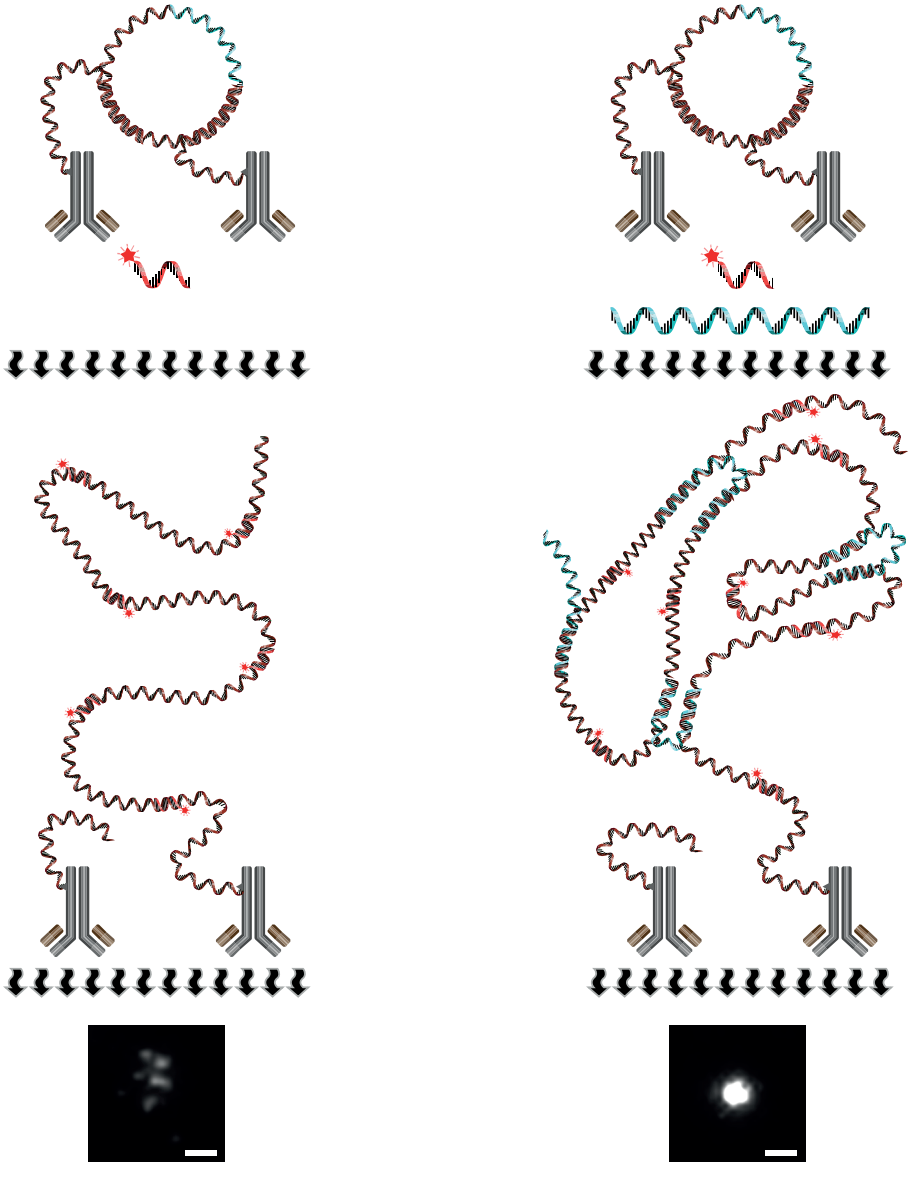

Figure 3 - Principle for compaction of RCA products in *in situ* PLA.
In regular *in situ* PLA (left panel) RCA products are left to collapse randomly onto themselves. The relatively high concentration of hybridized fluorophore-labeled detection oligonucleotides makes the ssDNA products easily detectable in an epifluorescence microscope (bottom left). With a cross-hybridizing compaction oligonucleotide in the RCA reaction (right panel), both adjacent and distal parts of the RCA product are bridged together, effectively condensing the RCA product. It will thereby occupy a smaller volume - resulting in a higher local concentration of the fluorophore-labeled detection oligonucleotides. Subsequently, the signal intensity increases and the signal shape becomes more distinctly round. Scalebars represent 1 µm.

residues of 2'O methyl-RNA to prevent priming by the phi29 DNA polymerase. The compaction oligonucleotide will thereby be able to hybridize to two repeats of the RCA product, bringing either close or distal parts together. This reduces the diameter of the RCA products from 1-2 μm to 0.2-0.5 μm. The condensation through cross-hybridization by compaction oligonucleotides results in a more distinct shape of the RCA products and also a higher local concentration of fluorophores, which increases the intensity of the RCA products.

There is an optimal concentration ratio between RCA products and compaction oligonucleotides. A too low concentration will simply lead to less condensed RCA products. But the concentration can be too high as well. A too high concentration of compaction oligonucleotides means that distant parts of the RCA products will not be as efficiently pulled together, as fewer hybridization sites for the compaction oligonucleotide are left unoccupied during the RCA. However, we have noticed that there is a concentration window of a few orders of magnitude within which the signals appear similarly condensed by image analysis software, and this is the main purpose.

Another approach to make RCA products more condense is to introduce strong secondary structures in them. Complete Genomics does this in the RCA products they refer to as DNA nanoballs - with parts of their adaptors being complementary[76]. While we have not observed the compaction efficiency reached through this approach, we assume that the cross-hybridization within the DNA nanoball would lower the compaction efficiency, as the hybridization target becomes immediately available emanating from the DNA polymerase and cannot hybridize to distal parts of the RCA product.

Compacted RCA products have a significant maximum intensity increase over regular RCA products and stand out much more from their local environment, with a 50% increase of signal-to-noise ratio in FFPE brain tissue. Furthermore, while regular RCA products may split up during RCA, so that one RCA product may be reported as several, the compaction oligonucleotide prevents this, creating a more accurate signal-to-target ratio.

Methods other than *in situ* PLA that apply RCA for signal generation will benefit from the same technique, e.g. padlock probe assays for genomic DNA or mRNA detection, or for *in situ* sequencing of mRNA transcripts[77]. The mentioned benefits of the compaction oligonucleotide are maybe particularly suitable for high-content screening. Another benefit of the more distinct appearance of the RCA products brought about by the compaction oligonucleotide is the possibility to use the same fluorescence channel for a staining of a different character, e.g. immunofluorescence. This would give high-content screening one more data-point per fluorescence channel, e.g.

for measuring the protein expression level. An increased accuracy of the signal-to-target ratio is important of course, and the extended dynamic range allows for greater biological variation. The increased ratio between signal intensity and autofluorescence intensity, and the homogenous shape of the signals facilitate accurate image analysis.

Multiplexing – the way forward

One main feature of *in situ* PLA is the incorporation of DNA assisted read-out of protein activity status, which makes available the highly versatile nucleic acids manipulation toolbox with ligases, restriction enzymes and polymerases among other DNA-interacting proteins, as well as several types of synthetic and natural nucleotides. *In situ* PLA is thereby a technique readily amendable to the needs of a scientist, where only the imagination is a limiting factor[78]. A natural way forward from the original *in situ* PLA has been to create a multiplex version, moving the detection sequence in the circular template from its back part to its proximity probe oligonucleotide parts. By this design a unique identifier, brought about by each different protein targeting proximity probe, will be incorporated into the circularized ligation products. Consequently the sequence of the ligated circle will be dependent on which proteins take part in the targeted complex. By giving the detection oligonucleotides different fluorophores, the color of an RCA product will reveal the interaction partner in an assay and several protein complexes can be viewed in parallel (**figure 4**).

As a model system we chose three members of the protein family of human epidermal growth factor receptors, namely EGFR, HER2 and HER3. The different homologs are caused by gene duplication and have arisen relatively late in evolutionary terms and HER4, the fourth and last member of the family, can in turn be differentially spliced into four isoforms of different function[79,80].

This is a well-characterized group of receptor proteins, with implications in several diseases. Approximately 15-20% of breast cancer tumors overexpress HER2 due to genomic gene amplification[81,82]. The EGFR family proteins can homo- and heterodimerise in ten different combinations[83], and the downstream signaling pathway affected is dependent upon the combination and what ligand has bound the receptor. The HER2 protein does not have a known ligand, but can dimerise with itself or any member of the family. The pathways affected include major ones such as the MAPK and the PI3K pathway[81], and several fundamental cellular processes end up being affected, such as survival, death, cell growth, proliferation and differentiation[84].

The combinatorial fashion of dimerization between the family members, the availability of high quality affinity reagents, cell line controls and the clinical importance made these proteins ideal choices for a proof-of-concept of the multiplex *in situ* PLA design. Using both a panel of cell lines and clinical tissue specimens we could confirm that the design enables parallel visualization of alternative protein complexes. In the version of multiplex *in situ* PLA presented here, three types of protein duplexes were investigated in parallel, with one of the two partners in the duplexes being the same. The oligonucleotide system employed here does not allow for much higher levels of multiplexing, as the limit is reached regarding the number of fluorophores which can be spectrally distinguished by microscopy, without elaborate optimization or spectral unmixing techniques.

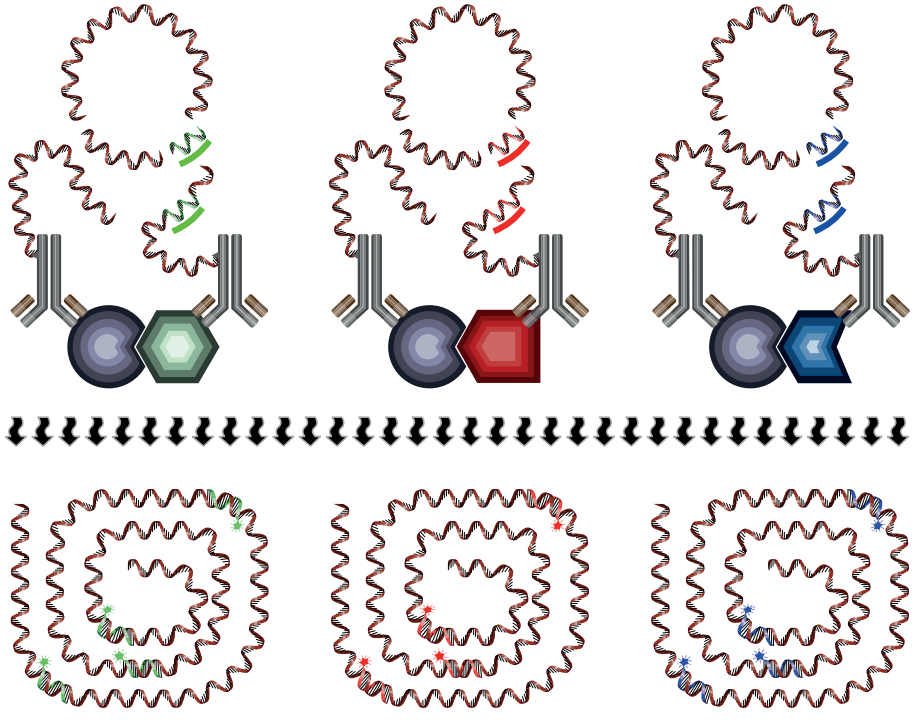

Figure 4 - Principle of multiplex *in situ* PLA.
Each of the PLA probes is unique in the sequence of its DNA-arm. To this sequence a complementary tag is hybridized in the formation of the circular RCA template (top panel). Then ligation of the template into a continuous circle takes place (not shown), and after RCA (not shown) the detection sequence has been repeated thousands of times in the RCA product (bottom panel). The detection oligonucleotides hybridized to the RCA products are labelled with different types of fluorophore. The color of each RCA product, or *in situ* PLA signal, reports on the proteins complicit in each detected complex.

To achieve a higher level of multiplexing, reiterative cycles of melting and re-hybridization of detection oligonucleotides can be used, as previously demonstrated for RCA products deposited on microscopy slides[85]. Hence, in each cycle a new set of interactions will be recorded. The number of recorded PLA signals will be increased linearly with the number of cycles.

After multiple steps of reiterative melting and re-hybridization of the detection oligonucleotides, the integrity of the RCA products becomes a concern. The heating during the melting step will cause them to float out, which in turn causes a decrease in the local concentration of fluorophores. This will in turn have complications on the robustness of the image analysis. While the compaction oligonucleotide described in this thesis has its greatest impact when implemented in the RCA reaction it has an effect, however limited, also when applied after RCA. The integrity of the RCA products might be better kept if instead of performing de-hybridization of the detection oligonucleotides via heat, they are rendered non-fluorescent by photo-bleaching or by chemicals, as in multi-epitope-ligand cartography (MELC). This is a recently developed technique with which it is possible to probe for more than a hundred targets in a serial fashion in a tissue section[86], through inactivation of the fluorophores in between each image capture. The resulting map of hundreds of layers of staining patterns identifies the co-localization of proteins, but cannot be said to detect interactions. Instead the technique yields a toponome map of the hierarchical organization of proteins within the tissue. The technique is of low-throughput as each assay takes days, but the amount of acquired information is extraordinary for being an immuno-assay.

A compelling approach to reach higher multiplex levels is to instead apply *in situ* sequencing techniques of RCA products, as has been done for mRNA transcripts reported upon via padlock probes[77]. Just by reading the initial four bases in the target specific detection sequence of the RCA products 4^4, or 256, different targets may be read out simultaneously in a sample section. The *in situ* sequencing readout of the identification of the RCA products is a relatively elaborate technique. It is based on sequencing by ligation, and requires reiterative steps of cleavage, hybridization, ligation and imaging for each interrogated base. Lastly, a comparatively advanced image analysis procedure is required, but the amount of information gained is striking.

The future multiplexing for interactomes and systems biology

In order to understand the complex interaction networks that govern cells within tissues, it is in practice a prerequisite to be able to measure the protein activity statuses of signaling pathways at a subcellular level, or larger complexes such as scaffold proteins[87]. The ability to localize multiple protein complexes and their activity statuses, and produce spatial information in fixed cells and tissue sections is a great advantage of the presented multiplex version of *in situ* PLA. It will reveal the spatial relationships and quantitative ratios between various protein complexes and activity statuses in individual cells. The protein complex content of individual cells will reveal their individual roles in tissue sections among neighboring cells, and it will be possible to reveal a hierarchical maturation scheme among the cells - similar to that of the SPADE trees built from individual cell type identification by the CyTOF in mass cytometry[7]. It paves the way for a systems understanding of biological variation, and the complex interaction not only between proteins inside cells, but between cells among cells in a tissue sample.

With techniques in place for antibody conjugation and validation, DNA assisted read-out and image analysis, the venue of subcellular interactome investigation in tissue samples and fixated cell lines using *in situ* PLA lays open for both a reductionist approach to understand isolated complex formations and the holistic approach to build models and shed light on larger systems of complex formation.

Non-targeted approaches will be essential to define possible targets for *in situ* PLA. During the last twenty years a few main techniques have brought promise to map complete networks of protein interaction, the interactomes, within organisms. These are the protein fragment complementation assay (PCA) and affinity purification with mass spectrometry (AP-MS). Interactome maps by yeast-two-hybrid (Y2H) assays, a type of PCA based upon complementation of one DNA binding and one transcription activating domain, can have largely diverging results, with only a few percent overlap of the detected interactions. The reliability of the output data from Y2H assays have since long been questioned, and has led to the view that high-throughput data is of lower quality. However, by careful experimental strategies including thorough controls and quality assurances, Y2H assays have become more reliable over the years[88,89].

The techniques just mentioned are either *in vivo* or *in vitro* assays, and they share the disadvantage of bringing the proteins of interest either into unnatural environments or to non-physiological concentrations, or both. While the idea of *in vivo* assays is to investigate the proteins in natural environments,

in living cells, the proteins are often heavily modified fusion proteins and overexpressed. AP-MS can leave the proteins unmodified, but instead brings them into the unnatural environments of purification columns. *In situ* assays aim to study the protein interactions unmodified in their local natural environment. Tissue samples are fixated, and the resulting stains are snapshots of the molecular events inside cells. However, *in situ* assays that rely on affinity reagents are biased in the way that they will only detect interactions of proteins that affinity reagents have been raised against. In view of this, *in situ* PLA is situated downstream of *in vivo* and *in vitro* assays that are used to fish for probable interaction partners.

High-content data is important for systems biology, both for the production of robust models with the use of rigorous assay-embedded controls and for finding novel quantitative relationships between detected targets. To this end, multiple techniques are preferably used concurrently. The research group I am part of has developed techniques covering the complete chain of the central dogma. Genomic alterations can be detected[90], as well as protein-DNA interactions[16], spliceotypes[91] and mRNA transcripts[20,92]. Proteins can be detected with high specificity through dual-recognition *in situ* PLA[14] and their modifications and interactions with multiple partners as well. An RCA read-out of these multiple methods in a single tissue specimen provides efficient detection over auto-fluorescence with the help of the proposed compaction oligonucleotide through image-analysis. Using a serial approach, a tissue section can be analyzed using the RCA-based methods for characterization of nucleic acids and protein activity, followed by IF/IHC for phenotypic evaluation and afterwards stained with e.g. hematoxylin-eosin for a histological evaluation. Retrieving data from all these levels will require sophisticated image analysis, and will end up characterizing the ecology of the micro-environment. This next generation tissue profiling should be a valuable tool both for research and diagnostics. The image output will no longer give us immediate answers, as for single-plex assays, but will require extensive further interpretation. The famous physicist Richard Feynman, who once said "It is very easy to answer many of these fundamental biological questions: you just look at the thing!" might have over-simplified the matter. The expertise of the physicist colleagues of Feynman, advanced image analysis, spatial statistics and mathematical descriptions will be required to make sense of the images.

Enzyme-free signal amplification

There are many ways to amplify DNA, but only a few which can tether the amplified product to a starting location. We have already mentioned the RCA as the signal amplification technique in *in situ* PLA. Other alternatives include the loop-mediated isothermal amplification (LAMP) technique[93,94] and the primed *in situ* labeling (PRINS)[95,96]. The downside of all of these methods is their dependence on enzymes.

The hybridization chain reaction technique has recently been developed, allowing enzyme-free DNA signal amplification[97]. The technique builds upon two fluorescently labeled short oligonucleotides, with high proportion of complementarity, both within and between themselves. Each will have a hairpin structure that has a high enough melting temperature to keep the closed conformation unless they are invaded by an initiator oligonucleotide. Once a hairpin has opened up, it will invade the next, generating a hybridization chain reaction that creates a long nicked and fluorescently labeled double-stranded DNA product.

In order to enable proximity dependent initiation of HCR for protein complex detection, we modified the design of the *in situ* PLA proximity probe. We exchanged the DNA arm on each PLA proximity probe with one of two species of hairpin oligonucleotides (proximity hairpins), to create a new type of proximity probes. When two such proximity probes are in close proximity, one hairpin can be induced to invade the other. This will liberate the initiator sequence from one of the HCR proximity probes, which in turn will start the HCR. A proximity HCR assay will retain the obligatory dependency of proximal binding from *in situ* PLA, without the dependency on enzymes for generation of signals (**figure 5**).

In a well performing HCR assay, the sequences and lengths of the hairpin species play a critical role. They should be stable enough not to unfold by themselves, but unstable enough to allow for invasion and efficient amplification. We carefully designed our hairpin species with special attention attributed to the free Gibbs energy of each species, and to the lengths of the stems and the loops. We also excluded sticky ends in the design, to avoid having the HCR perform a random walk process and instead force a fully dissociative way of hybridization, which is more reliable. Also, the buffer constituents play a major role in any HCR, with the critical factor being the ionic strength. The proximity HCR assay was tested both *in vitro*, using biotinylated proximity hairpins to target streptavidin-coated slides and beads, and *in situ*, using HCR probes to detect protein interactions in fixed cells.

The assay performed well in both settings and thus opens the door for further analysis to determine the virtues and drawbacks of proximity HCR in multiple applications.

Eliminating the enzyme dependence removes more than half of the reagent costs as compared to *in situ* PLA, and the assay becomes more robust. The target investigated is still determined by the affinity reagents, which means that posttranslational modification and protein complexes can still be probed for. Also, by directing the affinity reagents towards different epitopes on the same molecule a higher specificity is reached by comparison to single-epitope recognition such as IHC or IF. The assay may be multiplexed, by the parallel implementation of orthogonal species of HCR probes[98]. Through implementation of quenchers the HCR can be read out in real-time[99], and an exponential amplification can be employed[100].

The price and the robustness are two important factors for large-scale implementation of any assay. High-throughput experiments would be such a large-scale implementation, as well as a diagnostic setting. In poorer areas of the world robust and inexpensive diagnostics are beneficial, and a colorimetric read-out would be amenable to the technique for unaided visual assertion, e.g. for an on-site rapid diagnostic test.

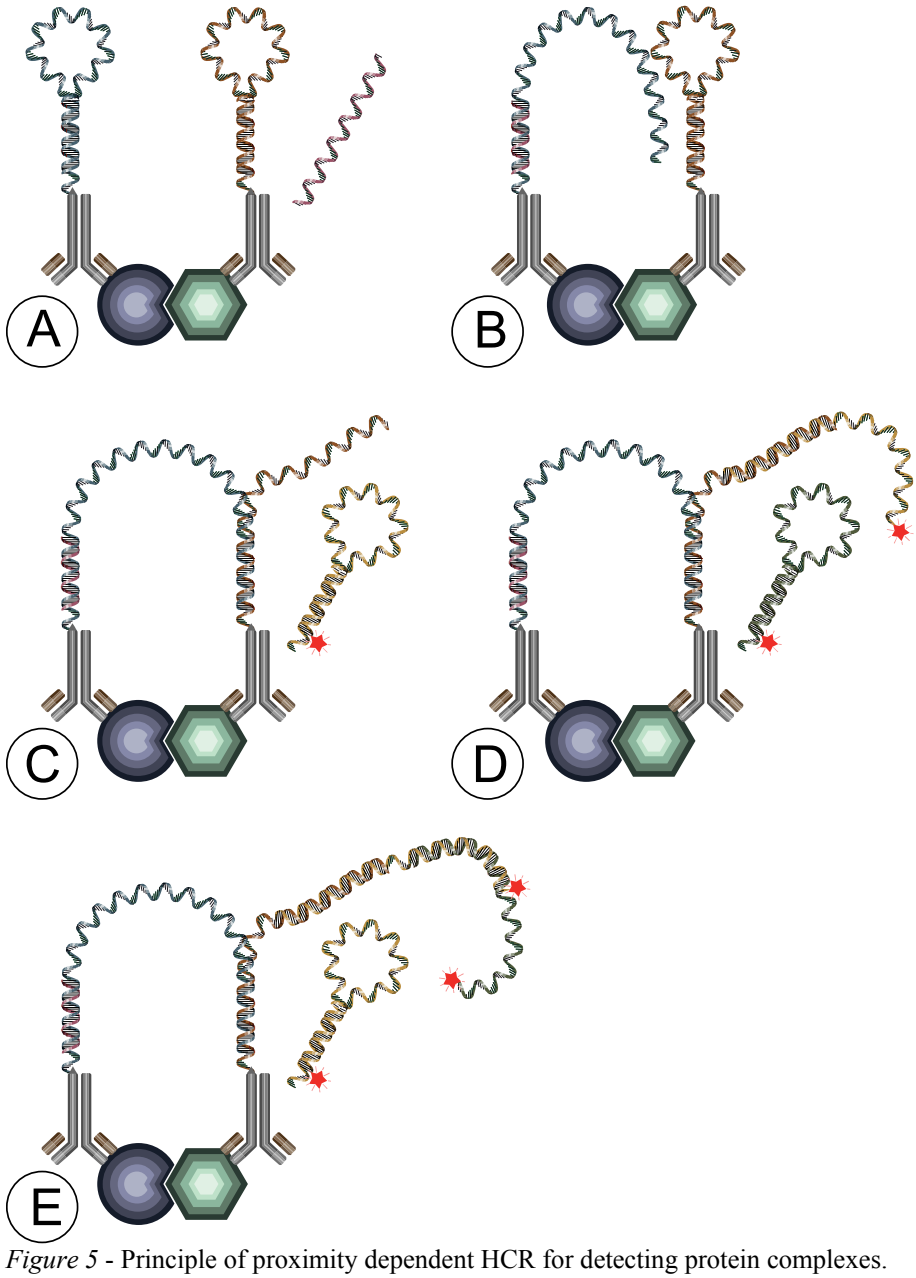

Figure 5 - Principle of proximity dependent HCR for detecting protein complexes. Two different DNA hairpin species are conjugated to one antibody each, forming proximity probes. The sequences of the hairpins have been carefully designed for stability (A). An initiating oligonucleotide invades and releases one of the hairpins (B), which can then invade and release the second hairpin (C). This second hairpin then functions as an initiator of the HCR by capturing and opening up one of two other hairpin structures, which are fluorescently labelled (D). The HCR between the two HCR hairpins is commenced (E), forming a nicked and fluorescently labelled double-stranded amplification product.

Present investigations

Paper I. Increasing the dynamic range of *in situ* PLA

Introduction

While *in situ* PLA offers bright and easily detectable signals, representing single protein complexes, the same signals infer a limitation on the method. They have large diameters of around 1 μm and a few hundred of them per cell are enough to cause saturation, limiting the dynamic range of the assay. Saturation is characterized by the signals coalescing, which causes them to become inseparable from one another. Quantification of the signals via digital enumeration by image analysis is no longer possible, although quantification by intensity measurement to a limited extent theoretically would be. As the signal amount is largely influenced by antibody concentration, apart from the endogenous protein expression, the issue is most often handled by performing antibody titration. This is unsuitable to precious tissue material, such as clinical samples, and consumes both time and money. Furthermore, the signal concentration might vary extensively among neighboring cells within a tissue sample along with their protein expression levels. This limits the analyzable tissue area, with some parts being saturated, and prohibits the comparison of protein expression levels between cells.

Aim of the study

The aim was to provide a technical improvement of *in situ* PLA, extending its dynamic range regardless of sample type or target.

Procedure

The aim was met through the addition of distinct circularization DNA oligonucleotides to the hybridization step of the *in situ* PLA protocol, at a predefined concentration ratio, to compete for binding to the PLA proximity probes. The oligonucleotides were identical in all parts but the detection sequence, which templates the sequences of the RCA products to which the detection DNA oligonucleotides hybridize. Each distinct detection oligonucleotide is coupled to a specific fluorophore, providing the RCA products with distinct colors during fluorescence analysis. Different types of RCA products will form at a ratio analogous to that of the input concentrations of the competing circularization oligonucleotides, so that the signal concentration can be chosen during analysis.

Two proofs-of-principle were given. One detected HER2 molecules on SKBR3 cells with varying concentrations of HER2-targeting antibody (from 0.0004 μg/ml to 4 μg/ml), to simulate differences in protein expression level among cells. Discrete, and thereby enumerable signals, were available for the whole concentration range.

The assay was then applied for detection of HER2 expression in breast cancer tissue. With ordinary *in situ* PLA, enumeration of signals would not be possible simultaneously in the cancer cells and normal stroma due to the limited dynamic range. However, by simply changing fluorescence filters during analysis, signal saturation was avoided in all cells and a pseudo-colored image was produced in the image analysis reflecting the number of signals in each cell.

Paper II. Compaction of rolling circle amplification products increases signal strength and integrity

Introduction

The signals generated from *in situ* PLA are RCA products of ssDNA. They tend to collapse into individual bundles of DNA that can be visualized by hybridization of fluorophore-labeled detection oligonucleotides. Hence, each RCA product will be labeled by hundreds of fluorophores. These often become intense enough to be easily identifiable over background autofluorescence already at low magnification, and digital enumeration is possible via image analysis.

However, there is a possibility of increasing the signal intensity further, while simultaneously making the signals smaller - providing an increased sensitivity and an extended dynamic range. In an effort to enhance the detection and quantification capabilities, we introduced a separate compaction oligonucleotide into the RCA reaction, effectively pulling the RCA products together during polymerization.

Aim of the study

Increasing signal intensity, extending the dynamic range and generating signals of greater integrity - facilitating more robust signal quantification.

Procedure

The compaction oligonucleotide comprises 48 nucleotides. Out of these, 42 were two copies of the same 21nt sequence spaced apart by 3 nucleotides. At the 3' end we inserted 3 residues of 2'O-methyl RNA to prevent priming and degradation by the phi29-polymerase.

The two-copy 21 nt sequence was designed to be complementary to a sequence replicated in the RCA products. This design permits cross-hybridization between both adjacent and distal parts within the RCA product.

By adding a compaction oligonucleotide to the RCA reaction, the signals exhibit a greater intensity, the signal-to-noise ratio is increased, the signals become smaller and more distinct, and tend not to split up into several smaller signals. This procedure is easily applicable to any technique employing RCA for signal generation.

Paper III. Parallel visualization of multiple protein complexes in individual cells in tumor tissue

Introduction

The original *in situ* PLA is able to detect only one type of protein complex, or protein modification, per sample. The detection sequence of the RCA product was dependent upon the standard circularization oligonucleotide, but could readily be made to depend upon the oligonucleotide arm of the PLA proximity probes. In this manner, RCA products would be distinguishable by their colors, and this would report upon the PLA-probes giving rise to each RCA product. In this way, multiple protein interactions and modifications would be detectable simultaneously in a tissue sample.

Aim of the study

The aim was to provide a means by which *in situ* PLA could provide simultaneous detection of several protein interactions.

Procedure

Antibodies were conjugated to oligonucleotides designed to harbor a tag sequence, which in turn was propagated into the RCA product to function as a detection sequence. In the two antibody conjugates facilitating the hybridization and ligation of the circularization and tag oligonucleotides in the assay, one contained no tag sequence but instead a generalized sequence. As such, both antibodies could not be varied simultaneously. One was kept constant while the tag-empowered conjugates varied in each assay in a combinatorial fashion, and the PLA-probe kept constant was varied between assays.

Proofs-of-concept were supplied in detections of known protein expression, complex formation and localization patterns of certain tissue types and cell lines. The detection specificity of selected protein complexes was assessed via cell line controls, with low native expressions of the protein complexes

under investigation, but transfected with vectors of various combinations of the proteins.

Paper IV. Proximity dependent initiation of hybridization chain reaction

Introduction

The *in situ* PLA is an enzyme-dependent technique for the detection of protein complexes. The T4 DNA ligase is used to ligate two circularization oligonucleotides together into a circular template for signal generation via RCA, during which the efficient and strand-displacing phi29 DNA polymerase is used. The enzymes infer demands on storage and are a major cost of the assay. With enzyme-free signal amplification, the assay may become less expensive and more robust. To this end, it may be possible to replace the original DNA-assisted reporter system of *in situ* PLA with the recently developed hybridization chain reaction (HCR). The HCR employs two types of short fluorescently labeled hairpin oligonucleotides, which invade and hybridize to each other in a chain reaction, to create a long nicked double-stranded DNA product, upon the activation by a third oligonucleotide.

Aim of the study

Design an oligonucleotide system capable of HCR, which is dependent upon activation by the proximity between two DNA-conjugated antibodies (proximity probes).

Procedure

Five types of oligonucleotides were needed, two of which were proximity-hairpins (78 and 67 nt long) which were to be conjugated to an antibody each (creating the proximity probes); an initiator oligonucleotide (44 nt), which was set to invade and open up one of the two proximity-hairpins; two amplification hairpins (both 50 nt long and conjugated to fluorophores) which should take part in the HCR signal amplification process. The oligonucleotides were carefully designed with respect to stability, in order to avoid initiator-independent HCR, which would be false-positive signal production. The functionality of the oligonucleotide system was determined by conjugation of the oligonucleotides to streptavidin beads, and incubation was performed at room temperature versus 37°C and with different types of buffers. Lastly, the oligonucleotide system was validated *in situ* on stimulated versus unstimulated DLD1 cells for HIF-1α/HIF-1β interactions.

Summary and future perspectives

My personal contributions to the projects have been project design, wet lab design and work, data acquisition, data analysis (including image analysis) and constructing major parts of some of the manuscripts. Each of the four papers makes possible new types of analyses in reductionist approaches. But more importantly, I think the new abilities together amount to unique possibilities to perform multiplex assays for high-content data acquisition of clinical tissue samples, in holistic approaches.

Comparing the tissue profiles between patients with the same diagnosis will give the possibility of identifying further stratification markers, and biomarkers (e.g. the interaction pattern being a biomarker, or the abundance of a specific cell type, or the spatial relations between cell types), which is useful for personalized medicine. It should be able to work in conjunction with standard diagnostics, e.g. HER2 amplification status, to stratify this subgroup even further in order to understand, for instance, why some patients respond well to therapies such as trastuzumab while others relapse.

The biological and technical variations will put higher demands on the lab design for the output of statistically significant high-content data. In the nowadays widely used single-plex *in situ* PLA, statistical methods are rather straightforward, measuring differences between a single experiment and a negative control. Trying to find correlations between targets detected in parallel in multiplex-assays requires more elaborate statistical techniques and power analysis. It is possible input from systems biologists will be required on how to best describe complex networks of interacting biomolecules.

Apart from data-analysis considerations, high-throughput wet-lab procedures need to be set up in order to cope with the larger number of reagents required. The best technique for DNA and antibody conjugation needs to be determined, as well as the DNA reporter system to be used. The high validity of these steps in a high-throughput tissue profiling setting is a prerequisite for later steps in the assay, and it might be found that the DNA reporter systems have varying efficiencies throughout the tissue sample.

The original *in situ* PLA technique is simple enough to be offered to researchers in ready-to-use kits. While *in situ* PLA advances, with parallel

read-out of multiple complexes, so does the expertise required. Unfortunately, this makes it difficult for other researchers to set up the technique in their labs. Also, it calls for close collaboration between technique-holders and clinicians, in order to pose relevant biological questions and interpret the output.

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References

1 Penn, D. C., Holyoak, K. J., Povinelli, D. J.
Darwin's mistake: explaining the discontinuity between human and nonhuman minds. *Behavioral and Brain Sciences.* **31:**109-178 (2008)

2 Tolstoy, L.
Anna Karenina. *The Russian Messenger.* (1877)

3 Clausson, C. M., Grundberg, I., Weibrecht, I., Nilsson, M. *et al.*
Methods for analysis of the cancer microenvironment and their potential for disease prediction, monitoring and personalized treatments. *The EPMA journal.* **3:**1-9 (2012)

4 Nakane, P. K., Pierce, G. B.
Enzyme-Labeled Antibodies for Light and Electron Microscopic Localization of Tissue Antigens. *Journal of Cell Biology.* **33:**307-318 (1967)

5 Coons, A. H., Creech, H. J., Jones, R. N., Berliner, E.
The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *Journal of Immunology.* **45:**159-170 (1942)

6 Adang, L. A., Parsons, C. H., Kedes, D. H.
Asynchronous progression through the lytic cascade and variations in intracellular viral loads revealed by high-throughput single-cell analysis of Kaposi's sarcoma-associated herpesvirus infection. *Journal of virology.* **80:**10073-10082 (2006)

7 Bendall, S. C., Simonds, E. F., Qiu, P., Amir el, A. D. *et al.*
Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science.* **332:**687-696 (2011)

8 Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K. *et al.*
Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nature methods.* **3:**995-1000 (2006)

9 Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C. *et al.*
Protein detection using proximity-dependent DNA ligation assays. *Nature biotechnology.* **20:**473-477 (2002)

10 Gullberg, M., Gústafsdóttir, S. M., Schallmeiner, E., Jarvius, J. *et al.*
Cytokine detection by antibody-based proximity ligation. *PNAS.* **101:**8420-8424 (2004)

11 Koos, B., Andersson, L., Clausson, C. M., Grannas, K. *et al.*
Analysis of Protein Interactions in situ by Proximity Ligation Assays. *Current Topics in Microbiology and Immunology.* (2013)

12 Conze, T., Carvalho, A. S., Landegren, U., Almeida, R. *et al.*
MUC2 mucin is a major carrier of the cancer-associated sialyl-Tn antigen in intestinal metaplasia and gastric carcinomas. *Glycobiology.* **20:**199-206 (2010)

13 Jarvius, M., Paulsson, J., Weibrecht, I., Leuchowius, K. J. *et al.*

In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. *Molecular & cellular proteomics.* **6:**1500-1509 (2007)

14 Leuchowius, K. J., Weibrecht, I., Landegren, U., Gedda, L. *et al.*
Flow cytometric in situ proximity ligation analyses of protein interactions and post-translational modification of the epidermal growth factor receptor family. *Cytometry A.* **75:**833-839 (2009)

15 Gomez, D., Shankman, L. S., Nguyen, A. T., Owens, G. K.
Detection of histone modifications at specific gene loci in single cells in histological sections. *Nature methods.* **10:**171-177 (2013)

16 Weibrecht, I., Gavrilovic, M., Lindbom, L., Landegren, U. *et al.*
Visualising individual sequence-specific protein-DNA interactions in situ. *Nature biotechnology.* **29:**589-598 (2012)

17 Jung, J., Lifland, A. W., Alonas, E. J., Zurla, C. *et al.*
Characterization of mRNA-cytoskeleton interactions in situ using FMTRIP and proximity ligation. *Plos One.* **8:**e74598 (2013)

18 Gajadhar, A., Guha, A.
A proximity ligation assay using transiently transfected, epitope-tagged proteins: application for in situ detection of dimerized receptor tyrosine kinases. *Biotechniques.* **48:**145-152 (2010)

19 Gu, G. J., Friedman, M., Jost, C., Johnsson, K. *et al.*
Protein tag-mediated conjugation of oligonucleotides to recombinant affinity binders for proximity ligation. *Nature biotechnology.* **30:**144-152 (2013)

20 Weibrecht, I., Grundberg, I., Nilsson, M., Soderberg, O.
Simultaneous visualization of both signaling cascade activity and end-point gene expression in single cells. *Plos One.* **6:**e20148 (2011)

21 Weibrecht, I., Lundin, E., Kiflemariam, S., Mignardi, M. *et al.*
In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay. *Nature protocols.* **8:**355-372 (2013)

22 Lundberg, E., Fagerberg, L., Klevebring, D., Matic, I. *et al.*
Defining the transcriptome and proteome in three functionally different human cell lines. *Molecular systems biology.* **6:**(2010)

23 Larson, B. A., Bern, H. A., Lin, R. J., Nishioka, R. S.
A double sequential immunofluorescence method demonstrating the co-localization of urotensins I and II in the caudal neurosecretory system of the teleost, Gillichthys mirabilis. *Cell and tissue research.* **247:**233-239 (1987)

24 Tramu, G., Pillez, A., Leonardelli, J.
An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. *The Journal of Histochemistry and Cytochemistry.* **26:**322-324 (1978)

25 Tanida, I., Ueno, T., Kominami, E.
LC3 and Autophagy. *Methods in molecular biology.* **445:**77-88 (2008)

26 Zhu, L., Yang, J., Zhang, J., Peng, B.
The presence of autophagy in human periapical lesions. *Journal of endodontics.* **39:**1379-1384 (2013)

27 Huang, B., Wang, W. Q., Bates, M., Zhuang, X. W.
Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science.* **319:**810-813 (2008)

28 Lew, M. D., Lee, S. F., Ptacin, J. L., Lee, M. K. *et al.*
Three-dimensional superresolution colocalization of intracellular protein superstructures and the cell surface in live Caulobacter crescentus. *PNAS.* **108:**E1102-1110 (2011)
29 Subach, F. V., Patterson, G. H., Renz, M., Lippincott-Schwartz, J. *et al.*
Bright monomeric photoactivatable red fluorescent protein for two-color super-resolution sptPALM of live cells. *Journal of the American Chemical Society.* **132:**6481-6491 (2010)
30 Galperin, E., Verkhusha, V. V., Sorkin, A.
Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nature methods.* **1:**209-217 (2004)
31 Roy, R., Hohng, S., Ha, T.
A practical guide to single-molecule FRET. *Nature methods.* **5:**507-516 (2008)
32 Manley, S., Gillette, J. M., Patterson, G. H., Shroff, H. *et al.*
High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nature methods.* **5:**155-157 (2008)
33 Miyawaki, A.
Development of Probes for Cellular Functions Using Fluorescent Proteins and Fluorescence Resonance Energy Transfer. *Annual review of biochemistry.* 357-373 (2011)
34 Woehler, A.
Simultaneous quantitative live cell imaging of multiple FRET-based biosensors. *Plos One.* **8:**e61096 (2013)
35 Nouar, R., Devred, F., Breuzard, G., Peyrot, V.
FRET and FRAP imaging: approaches to characterise tau and stathmin interactions with microtubules in cells. *Biology of the Cell.* **105:**149-161 (2013)
36 Padilla-Parra, S., Tramier, M.
FRET microscopy in the living cell: different approaches, strengths and weaknesses. *BioEssays : news and reviews in molecular, cellular and developmental biology.* **34:**369-376 (2012)
37 Ai, H. W., Hazelwood, K. L., Davidson, M. W., Campbell, R. E.
Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors. *Nature methods.* **5:**401-403 (2008)
38 Piljic, A., Schultz, C.
Simultaneous recording of multiple cellular events by FRET. *ACS chemical biology.* **3:**156-160 (2008)
39 Xu, Y., Piston, D. W., Johnson, C. H.
A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *PNAS.* **96:**151-156 (1999)
40 Zhang, L., Xu, F., Chen, Z., Zhu, X. *et al.*
Bioluminescence Assisted Switching and Fluorescence Imaging (BASFI). *The journal of physical chemistry letters.* **4:**3897-3902 (2013)
41 Thorek, D., Ogirala, A., Beattie, B. J., Grimm, J.
Quantitative imaging of disease signatures through radioactive decay signal conversion. *Nature Medicine.* **19:**1345-1350 (2013)
42 Carriba, P., Navarro, G., Ciruela, F., Ferre, S. *et al.*

Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nature methods.* **5:**727-733 (2008)
43 Alam, R., Zylstra, J., Fontaine, D. M., Branchini, B. R. *et al.*
Novel multistep BRET-FRET energy transfer using nanoconjugates of firefly proteins, quantum dots, and red fluorescent proteins. *Nanoscale.* **5:**5303-5306 (2013)
44 Petryayeva, E., Algar, W. R., Medintz, I. L.
Quantum dots in bioanalysis: a review of applications across various platforms for fluorescence spectroscopy and imaging. *Applied spectroscopy.* **67:**215-252 (2013)
45 So, M. K., Xu, C., Loening, A. M., Gambhir, S. S. *et al.*
Self-illuminating quantum dot conjugates for in vivo imaging. *Nature biotechnology.* **24:**339-343 (2006)
46 Algar, W. R., Wegner, D., Huston, A. L., Blanco-Canosa, J. B. *et al.*
Quantum dots as simultaneous acceptors and donors in time-gated Forster resonance energy transfer relays: characterization and biosensing. *Journal of the American Chemical Society.* **134:**1876-1891 (2012)
47 Xiong, L., Shuhendler, A. J., Rao, J.
Self-luminescing BRET-FRET near-infrared dots for in vivo lymph-node mapping and tumour imaging. *Nature Communications.* **3:**1193 (2012)
48 Grecco, H. E., Roda-Navarro, P., Girod, A., Hou, J. *et al.*
In situ analysis of tyrosine phosphorylation networks by FLIM on cell arrays. *Nature methods.* **7:**467-472 (2010)
49 Verveer, P. J., Wouters, F. S., Reynolds, A. R., Bastiaens, P. I.
Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. *Science.* **290:**1567-1570 (2000)
50 Saraheimo, S., Hepojoki, J., Nurmi, V., Lahtinen, A. *et al.*
Time-resolved FRET -based approach for antibody detection - a new serodiagnostic concept. *Plos One.* **8:**e62739 (2013)
51 Rossi, F., Charlton, C. A., Blau, H. M.
Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation. *PNAS.* **94:**8405-8410 (1997)
52 Cabantous, S., Nguyen, H. B., Pedelacq, J. D., Koraichi, F. *et al.*
A new protein-protein interaction sensor based on tripartite split-GFP association. *Scientific reports.* **3:**2854 (2013)
53 Wehr, M. C., Laage, R., Bolz, U., Fischer, T. M. *et al.*
Monitoring regulated protein-protein interactions using split TEV. *Nature methods.* **3:**985-993 (2006)
54 Johnsson, N., Varshavsky, A.
Split ubiquitin as a sensor of protein interactions in vivo. *PNAS.* **91:**10340-10344 (1994)
55 Dünkler, A., Müller, J., Johnsson, N. in *Gene Regulatory Networks* Vol. 786 *Methods in Molecular Biology* (eds Bart Deplancke & Nele Gheldof) Ch. 7, 115-130 (Humana Press, 2012).
56 Chen, Z., Cornish, V. W., Min, W.
Chemical tags: inspiration for advanced imaging techniques. *Current Opinion in Chemical Biology.* **17:**637-643 (2013)
57 Hinner, M. J., Johnsson, K.

How to obtain labeled proteins and what to do with them. *Current Opinion in Biotechnology.* **21:**766-776 (2010)

58 Huang, W., Reinholz, M., Weidler, J., Yolanda, L. *et al.*
Comparison of Central HER2 Testing With Quantitative Total HER2 Expression and HER2 Homodimer Measurements Using a Novel Proximity-Based Assay. *American Journal of Clinical Pathology.* **134:**303-311 (2010)

59 Larson, J. S., Goodman, L. J., Tan, Y., Defazio-Eli, L. *et al.*
Analytical Validation of a Highly Quantitative, Sensitive, Accurate, and Reproducible Assay (HERmark) for the Measurement of HER2 Total Protein and HER2 Homodimers in FFPE Breast Cancer Tumor Specimens. *Pathology research international.* **2010:**814176 (2010)

60 Han, S. W., Cha, Y., Paquet, A., Huang, W. *et al.*
Correlation of HER2, p95HER2 and HER3 expression and treatment outcome of lapatinib plus capecitabine in her2-positive metastatic breast cancer. *Plos One.* **7:**e39943 (2012)

61 Lipton, A., Goodman, L., Leitzel, K., Cook, J. *et al.*
HER3, p95HER2, and HER2 protein expression levels define multiple subtypes of HER2-positive metastatic breast cancer. *Breast cancer research and treatment.* **141:**43-53 (2013)

62 Caprioli, R. M., Farmer, T. B., Gile, J.
Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Analytical Chemistry.* **69:**4751-4760 (1997)

63 Schober, Y., Guenther, S., Spengler, B., Rompp, A.
Single cell matrix-assisted laser desorption/ionization mass spectrometry imaging. *Analytical Chemistry.* **84:**6293-6297 (2012)

64 Stoeckli, M., Chaurand, P., Hallahan, D. E., Caprioli, R. M.
Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nature Medicine.* **7:**493-496 (2001)

65 Norris, J. L., Caprioli, R. M.
Imaging mass spectrometry: A new tool for pathology in a molecular age. *Proteomics Clinical Applications.* **7:**733-738 (2013)

66 Norris, J. L., Caprioli, R. M.
Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. *Chemical reviews.* **113:**2309-2342 (2013)

67 Ozbabacan, S. E., Engin, H. B., Gursoy, A., Keskin, O.
Transient protein-protein interactions. *Protein Engineering, Design & Selection.* **24:**635-648 (2011)

68 Bhattacharyya, R. P., Remenyi, A., Yeh, B. J., Lim, W. A.
Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. *Annual review of biochemistry.* **75:**655-680 (2006)

69 Luh, L. M., Hänsel, R., Löhr, F., Kirchner, D. K. *et al.*
Molecular Crowding Drives Active Pin1 into Nonspecific Complexes with Endogenous Proteins Prior to Substrate Recognition. *Journal of the American Chemical Society.* **135:**13796-13803 (2013)

70 Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. *et al.*
Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *PNAS.* **95:**6803-6808 (1998)

71 Jain, A., Liu, R., Ramani, B., Arauz, E. *et al.*
Probing cellular protein complexes using single-molecule pull-down. *Nature.* **473:**484-488 (2011)
72 Lee, H.-W., Kyung, T., Yoo, J., Kim, T. *et al.*
Real-time single-molecule co-immunoprecipitation analyses reveal cancer-specific Ras signalling dynamics. *Nature Communication.* **4:**1505 (2013)
73 Baan, B., Pardali, E., ten Dijke, P., van Dam, H.
In situ proximity ligation detection of c-Jun/AP-1 dimers reveals increased levels of c-Jun/Fra1 complexes in aggressive breast cancer cell lines in vitro and in vivo. *Molecular & cellular proteomics.* **9:**1982-1990 (2010)
74 Figueiredo, J., Soderberg, O., Simoes-Correia, J., Grannas, K. *et al.*
The importance of E-cadherin binding partners to evaluate the pathogenicity of E-cadherin missense mutations associated to HDGC. *European Journal of Human Genetics.* **21:**301-309 (2013)
75 Shepard, H. M., Lewis, G. D., Sarup, J. C., Fendly, B. M. *et al.*
Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic. *Journal of clinical immunology.* **11:**117-127 (1991)
76 Drmanac, R., Sparks, A. B., Callow, M. J., Halpern, A. L. *et al.*
Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science.* **327:**78-81 (2010)
77 Ke, R., Mignardi, M., Pacureanu, A., Svedlund, J. *et al.*
In situ sequencing for RNA analysis in preserved tissue and cells. *Nature methods.* **10:**857-860 (2013)
78 Weibrecht, I., Leuchowius, K. J., Clausson, C. M., Conze, T. *et al.*
Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert review of proteomics.* **7:**401-409 (2010)
79 Arkhipov, A., Shan, Y., Kim, E. T., Dror, R. O. *et al.*
Her2 activation mechanism reflects evolutionary preservation of asymmetric ectodomain dimers in the human EGFR family. *eLife.* **2:**e00708 (2013)
80 Veikkolainen, V., Vaparanta, K., Halkilahti, K., Iljin, K. *et al.*
Function of ERBB4 is determined by alternative splicing. *Cell cycle.* **10:**2647-2657 (2011)
81 Kumler, I., Tuxen, M. K., Nielsen, D. L.
A systematic review of dual targeting in HER2-positive breast cancer. *Cancer treatment reviews.* **40:**259-270 (2014)
82 Perez, E. A., Cortés, J., Gonzalez-Angulo, A. M., Bartlett, J. M. S.
HER2 testing: Current status and future directions. *Cancer treatment reviews.* **40:**276-284 (2014)
83 Riese, D. J., Stern, D. F.
Specificity within the EGF family/ErbB receptor family signaling network. *BioEssays : news and reviews in molecular, cellular and developmental biology.* **20:**41-48 (1998)
84 Yarden, Y., Sliwkowski, M. X.
Untangling the ErbB signalling network. *Nature Reviews Molecular Cell Biology.* **2:**127-137 (2001)
85 Göransson, J., Wählby, C., Isaksson, M., Howell, W. M. *et al.*
A single molecule array for digital targeted molecular analyses. *Nucleic Acids Research.* **37:**e7 (2009)

86 Schubert, W., Bonnekoh, B., Pommer, A. J., Philipsen, L. *et al.* Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nature biotechnology.* **24:**1270-1278 (2006)
87 Good, M. C., Zalatan, J. G., Lim, W. A. Scaffold proteins: hubs for controlling the flow of cellular information. *Science.* **332:**680-686 (2011)
88 Braun, P. Interactome mapping for analysis of complex phenotypes: Insights from benchmarking binary interaction assays. *PROTEOMICS.* **12:**1499-1518 (2012)
89 Dreze, M., Monachello, D., Lurin, C., Cusick, M. E. *et al.* in *Methods in Enzymology* Vol. Volume 470 (eds Weissman Jonathan, Guthrie Christine, & R. Fink Gerald) 281-315 (Academic Press, 2010).
90 Nilsson, M., Krejci, K., Koch, J., Kwiatkowski, 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. *Nature Genetics.* **16:**252-255 (1997)
91 Conze, T., Goransson, J., Razzaghian, H. R., Ericsson, O. *et al.* Single molecule analysis of combinatorial splicing. *Nucleic Acids Research.* **38:**e163 (2010)
92 Larsson, C., Grundberg, I., Soderberg, O., Nilsson, M. In situ detection and genotyping of individual mRNA molecules. *Nature methods.* **7:**395-397 (2010)
93 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T. *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research.* **28:**e63 (2000)
94 Meng, Q., Wang, S., Zhang, L., Huang, X. *et al.* Development of an in situ loop-mediated isothermal amplification technique for chromosomal localization of DNA sequences. *Chinese Journal of Oceanology and Limnology.* **31:**128-133 (2013)
95 Harrer, T., Schwinger, E., Mennicke, K. A new technique for cyclic in situ amplification and a case report about amplification of a single copy gene sequence in human metaphase chromosomes through PCR-PRINS. *Hum Mutat.* **17:**131-140 (2001)
96 Koch, J. E., Kolvraa, S., Petersen, K. B., Gregersen, N. *et al.* Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. *Chromosoma.* **98:**259-265 (1989)
97 Dirks, R. M., Pierce, N. A. Triggered amplification by hybridization chain reaction. *PNAS.* **101:**15275-15278 (2004)
98 Choi, H. M., Chang, J. Y., Trinh le, A., Padilla, J. E. *et al.* Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nature biotechnology.* **28:**1208-1212 (2010)
99 Chemeris, D. A. Real-time hybridization chain reaction. *Doklady. Biochemistry and biophysics.* **419:**53-55 (2008)
100 Yin, P., Choi, H. M., Calvert, C. R., Pierce, N. A. Programming biomolecular self-assembly pathways. *Nature.* **451:**318-322 (2008)

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