New applications of the *in situ* proximity ligation assay, and their positions amongst contemporary protein detection methods

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Table of Contents
Summary .................................................................................................................................................... 3
Abbreviations ............................................................................................................................................. 4
Introduction ................................................................................................................................................ 5
From genome to interactome ................................................................................................................... 5
The *in situ* proximity ligation assay ......................................................................................................... 5
Multiplexed *in situ* proximity ligation assay ........................................................................................... 6
Adapting chromogenic *in situ* hybridisation to the *in situ* proximity ligation assay ............................ 7
The scientific context surrounding proximity ligation assay ................................................................... 7
Aims ........................................................................................................................................................... 8
Results ........................................................................................................................................................ 9
Testing two anti-HER2 antibodies on breast cancer tissue ................................................................. 9
Testing two anti-CK17 antibodies on normal tissue ............................................................................... 10
Investigating functionality of splint lengths and inverted arms in the *in situ* proximity ligation assay. 10
Analysing feasible false positives in immunohistochemistry with *in situ* proximity ligation assay...... 11
Multiplexed *in situ* proximity ligation assay on breast cancer tissue ................................................. 13
Examination of antibody concentration through varying backpiece concentrations ....................... 14
Discussion ................................................................................................................................................ 15
Strengths of the emerging *in situ* proximity ligation assay ............................................................... 15
Potential weaknesses affecting the *in situ* proximity ligation assay .................................................... 16
Materials and methods.............................................................................................................................. 18
Acknowledgements .................................................................................................................................. 22
References ................................................................................................................................................ 23
Appendix – Contemporary protein detection methods ......................................................................... 25
Fluorescent protein tags ......................................................................................................................... 25
Protein fragment complementation assay ............................................................................................... 25
Resonance energy transfer systems ....................................................................................................... 27
Bioorthogonal chemical reporters ......................................................................................................... 27
Yeast two-hybrid and its related methods ............................................................................................... 28
Affinity purification - mass spectrometry ............................................................................................... 31
Immunohistochemistry .......................................................................................................................... 33
Summary

In the post-genomic era, many methods have emerged to study proteins, their functions and localisations. One of them is the proximity ligation assay, the big advantage of which is the capability to be applied on patient tissue specimens for detection of protein interactions. This degree project was aimed at testing new applications of the in situ proximity ligation assay. The general approach of the method is to probe for a protein in tissue specimens with two oligonucleotide coupled protein specific antibodies. If the antibodies become situated next to each other, two purposefully designed DNA strands complementary to the mentioned oligonucleotides become hybridised to them. Ligation can occur and by incubation with φ29 DNA-polymerase, a long DNA strand emerges to which fluorescently labelled detection probes can hybridise. The protein complex is then visible as a sharp dot in a fluorescence microscope.

In this project, this procedure was modified somewhat to produce different readouts. The oligonucleotides coupled to the antibodies were changed in one setup together with the complementary DNA strands, making a multiplexed assay possible. This was tested by probing for human epidermal growth factor receptor 2 and epidermal growth factor receptor homo- and heterodimers in breast cancer tissue, with positive readouts. This report is first in another demonstration, where haptens (small intermediate molecules) were conjugated to the primary antibodies instead of oligonucleotides, which were instead placed on standardised secondary antibodies. The assay was used probing for villin and myotonic dystrophy protein kinase, also yielding positive results after being adapted to brightfield analysis through chromogenic in situ hybridisation. The importance of the proximity between antibodies in the experiments also was explored through length variations of the intermediate DNA strands. Such experiments confirmed the hypothesis of spatial relationships between the involved oligonucleotides impacting signal abundance. An approach to protein level detection was tried out, through a multiplex assay with intentional variations in the ability to detect proteins, with a positive outcome. The different experiments together enhance diversification of the proximity ligation assay, showing strengths in new areas. It now lends itself to convenient use by histologists through the chromogenic in situ hybridisation and is easily applied to any set of antibodies by hapten conjugation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AP-MS</td>
<td>Affinity purification - mass spectrometry</td>
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<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CISH</td>
<td>Chromogenic <em>in situ</em> hybridisation</td>
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<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
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<td>DMPK</td>
<td>Myotonic dystrophy protein kinase</td>
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<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>HER</td>
<td>Human epidermal growth factor</td>
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<td>HPA</td>
<td>Human protein atlas</td>
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<td>HPR</td>
<td>Human proteome resource project</td>
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<td>HT</td>
<td>High throughput</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>PCA</td>
<td>Protein fragment complementation assay</td>
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<td>PLA</td>
<td>Proximity ligation assay</td>
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<tr>
<td>PrEST</td>
<td>Protein expressed sequence tag</td>
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<td>RCA</td>
<td>Rolling circle amplification</td>
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<td>RCP</td>
<td>Rolling circle product</td>
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<td>RET</td>
<td>Resonance energy transfer</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TBS</td>
<td>Tris-base saline</td>
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<tr>
<td>TBST</td>
<td>Tris-base saline 0.05% Tween-20</td>
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<td>TMA</td>
<td>Tissue microarray</td>
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<td>TxRed</td>
<td>Texas Red</td>
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<td>Y2H</td>
<td>Yeast-two-hybrid</td>
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Introduction

From genome to interactome
After the mapping of the human genome, the first draft released in June 2000, possibly bigger problems arose. The sequences of more than 20 000 genes had become known, but for the most part their functions were still unidentified. Previously, methods for DNA studies had been somewhat of a centrepiece of biological research. A novel paradigm was at hand, focusing on the products constructed from DNA templates – proteins.

The Human Proteome Resource Project (HPR) is currently in the midst of mapping the whole human proteome. This is done by producing antibodies targeted against protein expressed sequence tags (PrESTs), in a high throughput (HT) manner, which are then used in immunohistochemistry (IHC) on many different tissue samples. The output is high resolution images of the 3,3'-diaminobenzidine (DAB) stainings published in the Human Protein Atlas (HPA). In IHC, DAB is turned into a dark brown precipitate through catalysis by horseradish peroxidase (HRP), which in turn is coupled to an antibody probing a protein of interest. Up until now, several thousands of proteins have been mapped in normal tissue types, cancer types and cell lines. Of course, this information is useless if the functions of the proteins are unknown. To obtain knowledge of large numbers of proteins, the interactome concept has been brought into brighter light. By establishing what proteins interact with each other throughout a complete organism, important biomolecular pathways will emanate. Were this to be in done in a HT procedure in the human being, the purposefulness of the resulting mapped pathways cannot be exaggerated. It will be of special interest in drug development contexts.

To study protein interactions at higher resolution and in greater detail, however, other methods have to be employed. A range of such methods is available, all with their own advantages addressing different experimental circumstances.

The in situ proximity ligation assay
The proximity ligation assay (PLA) method was initially invented by Fredriksson et al. in 2002, when DNA molecules were used as aptamers in vitro. Two years later, in 2004, Gullberg et al. made modifications to the method by using antibodies in the place of the aptamers. This made the method more convenient to use and showed the possibility of detecting all proteins to which antibodies can be produced. However, an in situ adaption was first presented by Ola Söderberg et al. in 2006 showing great potential in several different areas of biological research. It was demonstrated that individual proteins could be visualised in subcellular contexts. The method, also sometimes termed P-LISA (Proximity Ligation in situ Assay), has already enjoyed being part of several different scientific advancements. In one of them, in situ PLA played a key role in demonstrating the position of the protein aurora B kinase during anaphase in cell mitosis (Fuller et al. 2008). In the experiment, oligonucleotides were conjugated to anti-tubulin and anti-xenopus aurora B kinase antibodies. The method described is the standard in situ PLA method, also conveyed in figure 1. When aurora B kinase and tubulin were in very close proximity to each other, two other DNA-strands (of which the short one is called splint, and the longer one backpiece) could hybridise to the antibody conjugated oligonucleotides. The DNA-circle that had formed could then be ligated, after which a φ29 DNA polymerase was introduced. Since the template was circular, a long strand of DNA was formed in this rolling circle amplification. The product created is denominated rolling circle product (RCP). To this, fluorescently labelled complementary oligonucleotides were hybridised. The localisation of the protein was then evident in an
epifluorescence microscope, and it could be determined that aurora B kinase played a decisive role during anaphase while interacting with tubulin in the midzone of the cell.

Figure 1 – Schematic representation of in situ PLA. A complex of two proteins (yellow and blue) is probed with one oligonucleotide coupled antibody for each protein. To the oligonucleotides, two pieces of single stranded DNA (the short one is named splint and the long one is termed backpiece) can hybridise. After ligation of them, a DNA polymerase (green) uses the circle as a template, producing a long strand of ssDNA to which fluorescently labelled detection probes are able to hybridise. The analysis is then continued by fluorescence microscopy.

**Multiplexed in situ proximity ligation assay**

The possibility of further development of PLA from the basics just stated is evident. For instance, complexes of more than two proteins can be detected through addition of a third oligonucleotide conjugated antibody. This oligonucleotide then needs to hybridise to the circularising DNA strands for them to be ligated and then serve as a template for RCA. Also, as in an experiment in this project (figure 6), the antibody arm (i.e. the oligonucleotide localised on the antibody probing the yellow protein in figure 1) can be inverted for demanding an even closer proximity between the two antibodies before ligation of splint and backpiece can occur. It is inverted by the means of conjugating its 3’ end to the antibody instead of its 5’ end. In this particular case, its 3’ end also contains three uracils (L10317, table 2), making it possible to detach from the antibody with uracil DNA glycosylase and formamidopyrimidine DNA glycosylase.

Figure 2 – Schematic representation of multiplexed in situ PLA. The reactions forming RCA products (to the right) take place in the same sample and at the same time. By allowing the detection sequences (red and green) of the circular DNA strands to depend on one of the antibodies (as the arm of one of the antibodies probing the dimer is complementary to the detection sequence), in turn dependent on the proteins in the complex, the colours of the resulting RCA products will specify the constituents of the original complexes.
The multiplexed PLA is somewhat more complicated - another adaption of the original PLA idea. The word multiplex is an abridgement of the words multiple and complex, implying the possibility of distinguishing several protein complexes in the same sample. PLA can be multiplexed in several different ways. For instance, multiplexed PLA was first used in vitro, probing for protein cancer biomarkers and amplifying the signal in quantitative PCR (Fredriksson et al. 2007). The concept in this project is to let the part of the circular strand hybridising to the detection probes rely on one of the two antibodies in the system (figure 2). The antibodies in turn probe a dimer, where one of the included proteins can vary in the same sample or tissue. This way, several different interactions of a certain protein with other proteins can be monitored in the same experiment.

Adapting chromogenic in situ hybridisation to the in situ proximity ligation assay
A possible limitation of the in situ proximity ligation assay is the presumed need of an epifluorescence microscope in order to deduce results. However, other detection methods are possible. Returning to the RCA product, the oligonucleotides hybridising to this long single DNA strand have the possibility of being labelled with a chosen molecule or compound. This molecule can be selected so that there are antibodies directed towards it. Such an antibody then can be coupled to an enzyme, for instance alkaline phosphatase, which will be able to dephosphorylate a specific substrate creating a precipitate with certain colour (figure 3). This is the way the chromogenic in situ hybridisation (CISH) operates. This way, the RCA products can be localised indirectly in the tissue sample. The surrounding tissue can then be visualised through hematoxylin staining, a commonly used method giving blue-purple colour to cell nuclei and other parts of the tissue able to oxidise the hematoxylin. These procedures result in more easily handled samples, as they can be stored at room temperature and will not bleach for decades. Another positive aspect is that histologists are more familiar with this appearance of the samples, in the end making PLA more easily accessible to this professional society.

Figure 3 – Schematic representation of CISH. The antibody, linked to alkaline phosphatase (represented by a pair of scissors), is targeted towards the fluorophore TxRed residing on the detection oligo hybridised to an RCA product. Analysis can be done by brightfield microscopy.

The scientific context surrounding proximity ligation assay
Quite many protein detection methods have emerged during the last decades, each with its advantages and disadvantages. The technique of choice depends largely on the desired output from the experiment, on the input material and the method experience of the laboratory in question. To compare in situ PLA with other valuable techniques in the protein detection area, a short description of a number of them follows in appendix A.
Aims
The aim of this degree project was not to deduce any new knowledge regarding a certain disease or biological phenomenon, but rather to further evaluate the capabilities of new applications of PLA, specifically in situ. The possibility of detecting multiple protein complexes in the same sample was explored, as was the use of hapten conjugated primary antibodies. Moreover, the ability of applying in situ PLA to protein detection with antibodies from HPR was of interest, as well as the technicality of protein quantification.
Results

**Testing two anti-HER2 antibodies on breast cancer tissue**
Four primary antibodies targeted against human epidermal growth factor 2 (HER2) were employed in sets of two, using *in situ* PLA and CISH. The antibodies of special interest were HPA001060 and HPA001338, rabbit antibodies from HPR (unpublished) targeted to the same epitope but produced in one rabbit each. This was to test the hypothesis that *in situ* PLA and CISH could be applied to antibodies produced by HPR. Both of these rabbit antibodies were used together with either goat or mouse derived anti-HER2 antibodies targeting different epitopes (figure 4). The signal amount in experiments with primary antibodies is easily distinguished from the controls. The results were compared with another 3,3'-

![Figure 4: Visualisation in red of HER2 protein in breast cancer tissue with *in situ* PLA and CISH. Two rabbit derived antibodies were evaluated. Since they are both targeted towards the same epitope of the HER2 protein, they were used together with commercial anti-HER2 antibodies from goat and mouse targeted towards different epitopes. Panels A and B represent rabbit derived HPA001060 together with anti-HER2 from goat and mouse respectively. Secondary antibodies were then anti-rabbit and anti-goat or anti-rabbit and anti-mouse. Controls were without primary antibodies, but with secondary antibodies anti-mouse and anti-rabbit (C) or anti-goat and anti-rabbit (F). Primary antibodies employed together with HPA001338 (from rabbit) were from goat (D) and mouse (E). Results were compared with a DAB staining (showing brown staining at the localisation of the antibody) from HPA of HER2 probing antibody HPA001383 (G). Primary antibodies in the PLA experiments were incubated with the tissue sections at RT for 40 minutes. Tissue slices are neighbouring and scalebars represent 50 μm.**

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9
diaminobenzidine (DAB) staining from HPR with HPA001383 also targeting HER2 (figure 4 G), giving a similar staining pattern. The in situ PLA was then concluded to be functional. This means that in situ PLA now easily lends itself to the common practice of histologists.

**Testing two anti-CK17 antibodies on normal tissue**

*In situ* PLA and CISH were used on normal tissue, involving two primary antibodies targeted towards cytokeratin 17 (CK17) from HPR (figure 5). Their numbers in the proteinatlas are CAB000029 and HPA000453. Secondary antibodies were anti-mouse IgG and anti-rabbit IgG. By comparison to the DAB stainings (the regular immunohistochemistry [IHC] experiments) of the individual antibodies, *in situ* PLA could be said to serve its purpose well, as the staining patterns were similar.

![Figure 5: Visualisation in red of CK17 protein in prostate tissue with *in situ* PLA and CISH. The control was without primary antibodies but with secondary antibodies anti-mouse and anti-rabbit (A). In *in situ* PLA, primary antibodies were CAB000029 from mouse and HPA00453 from rabbit (B). DAB staining with HPA000453 (C) and CAD000029 (D) were collected from HPA, with brown staining at localisation of antibodies. Primary antibodies in the PLA experiments were incubated with the tissue sections at RT for 40 minutes. The tissue sections in A and B are close to neighbouring with one slice in between them. Scalebars represent 50 μm.]

**Investigating functionality of splint lengths and inverted arms in the *in situ* proximity ligation assay**

In this experiment, dimers of HER2 were probed for on frozen SKBR3 cells (a commonly used human breast carcinoma cell line). By varying the length of the splint (elongated with a varying number of non-interacting adenosines) and the kind of arm (regular versus inverted), the proximity between the proteins in the complexes was subject of examination (figure 6). The exposure time for each channel was kept the same between all the photos, and the digital enhancement procedure of images was identical. The photos are thus comparable, and the following conclusions can be drawn: The antibodies with inverted arms can be used with confidence in the place of antibodies with regular arms, as they gave a good amount of signal (figure 6 A and F). The polymerase was not impaired by the inverted arm, since detaching it in the RCA reaction (through degradation of the uracils in the 3’ end of its sequence close to the antibody) did not produce more rolling circle products (RCPs) (figure 6 A and E). There was a great variation of HER2-EGFR signal amounts (figure 6 A, B, C, D).
Figure 6: Fluorescent images showing the impact on signal amount of three different complexes by varying splint lengths and inverted arms in multiplexed in situ PLA on frozen SKBR3 cells. HER2-HER3 dimers red; HER2-EGFR green; HER2-HER2 blue. White spheres are nuclei. No secondary antibodies were utilised. Two antibodies coupled to different arm oligonucleotides were tested – one with a regular arm (F) and one with the arm inverted (L10317). The splints had a differing number of adenosines inserted in the middle – 0A (L8410) (A,E,F), 5A (L10249) (B), 10A (L10250) (C) and 20A (L10251) (D). For (E), the inverted arm was detached in the RCA reaction. (F), positive control with a regular arm and a 0A splint (L8410). Exposure times between photos and image handling were the same. Scalebar represents 10 μm.

Analysing feasible false positives in immunohistochemistry with in situ proximity ligation assay

The two proteins villin and Myotonic dystrophy protein kinase (DMPK) were probed with two antibodies each in an in situ PLA experiment made available to brightfield analysis owing to subsequent CISH. The four antibodies were provided by HPR, and were precisely the same as the ones applied in the DAB stainings on the HPA (HPA006884 and HPA006885 for villin; HPA007164 and HPA008905 for DMPK). The two antibodies in each pair were conjugated to FITC and biotin respectively, and used in the in situ PLA, proceeding with CISH (figure 7 and figure 8). Image B of each figure shows successful PLA, due to co-localisation of antibodies (C and D in each figure, www.proteinatlas.org). With a higher certainty than with IHC, it was then possible to conclude that villin was localised in kidney. Image F in each figure shows unsuccessful in situ PLA, which correlates with the fact that only one of the two antibodies showed signal in the HPR DAB stainings (G and H in each figure, www.proteinatlas.org).
**Figure 7:** *In situ* PLA and CISH of two hapten conjugated antibodies targeted towards one epitope each of villin. The top row is kidney tissue, while the bottom row is placenta tissue. *In situ* PLA controls were without primary antibodies (A and E). *In situ* PLA was done with haptenised primary antibodies HPA006884 (conjugated to FITC) and HPA006885 (conjugated to biotin) (B and F). C and G, DAB stainings from the HPA of anti-villin HPA006884 (www.proteinatlas.org), show brown signal where anti-villin antibodies were bound. D and H, DAB stainings from the HPA of anti-villin HPA006885 (www.proteinatlas.org). In the *in situ* PLA reaction, an oligonucleotide coupled antibody was targeted towards the FITC conjugated HPA006884 while another oligonucleotide coupled antibody was targeted towards the biotin conjugated HPA006885. Tissue sections in B and F are four slices apart from A and E. Scalebar represents 50 μm.

**Figure 8:** *In situ* PLA and CISH of two hapten conjugated antibodies targeted towards one epitope each of DMPK. The top row is skeletal muscle tissue, while the bottom row is colon tissue. *In situ* PLA controls had no primary antibodies (A and E). *In situ* PLA was done with haptenised primary antibodies HPA008905 (conjugated to FITC) and HPA007164 (conjugated to biotin) (B and F). D and H, DAB stainings of anti-DMPK antibodies from the HPA of HPA007164 (C and G, www.proteinatlas.org) and of anti-DMPK HPA008905 (D and H, www.proteinatlas.org). In the *in situ* PLA reaction, an oligonucleotide coupled anti-FITC antibody was targeted towards the FITC conjugated HPA008905 while an oligonucleotide coupled streptavidine molecule was targeted towards the biotin conjugated HPA007164. Tissue sections in B and F are two slices apart from A and E. Scalebar represents 50 μm.
Multiplexed in situ proximity ligation assay on breast cancer tissue

A total of four frozen breast cancer tissue samples from two unique patients were used when analysing two sets of three dimers (HER2 with HER2, HER3 or EGFR and EGFR with HER2, HER3 or EGFR). The resulting images revealed in figure 9 made a successful experiment apparent. Dissimilar expression patterns could be seen in different cell populations (figure 9 left column) and also between individual cells (figure 9 right column), as resulting PLA signals were not evenly spread. Over all, EGFR and HER2 dimers showed greater abundance than dimers with HER3. Future experiments should correlate this with patient diagnosis.

A.a

A.b

B.a

B.b

Figure 9: Fluorescent images of multiplexed in situ PLA, probing for HER2 and EGFR dimers in breast cancer tissue. The two proteins form hetero- and homodimers with HER2 (blue), HER3 (red) and EGFR (green). Frozen breast cancer tissue from two patients (A and B) was used. Lower case letters denote how either HER2 (a) or EGFR (b) were allowed to dimerise with the three other proteins HER2, HER3 and EGFR. Scalebars represent 50 μm and 10 μm respectively.
Examination of antibody concentration through varying backpiece concentrations

Primary anti-HER2 antibodies were serially diluted from 10-fold to 10000-fold (1:10, 1:30, 1:100, 1:300, 1:1000, 1:3000, 1:10000) and incubated with SKBR3 cells. Three different backpieces were used, in one concentration each. The backpieces all shared a detection sequence for FITC labelled detection oligonucleotides, and also had one additional unique detection sequence each (targeted towards Pacific Blue, Alexa 555 or Cy5 labelled detection oligonucleotides). The purpose was to produce a dynamic range of the number of RCPs for determining concentrations of antigen binding antibodies. The expectation was to be able to distinguish (and count) RCPs using the low concentration backpiece at high concentrations of primary antibody. Conversely, the high concentration backpiece would be useful when discerning RCPs at low concentrations of primary antibody. Using only one backpiece and detection oligonucleotide would render the range non-dynamic and restrict it to a large extent. Here, eight samples were used, but the principle will be the same with only one sample. Photos of the cells were taken through a fluorescence microscope at the four different wavelengths (emitted by FITC, Pacific Blue, Alexa 555 and Cy5) for each primary antibody dilution (figure 10), and RCPs were counted. One dilution (1:1000) had to be skipped due to poor image acquisition. At higher antibody concentrations, light saturation occurred at high backpiece concentrations, which was expected. The lower backpiece concentration with Cy5 detection then seemed more reliable, and could possibly be used for quantification of antigen binding antibodies. Close to equal amount of Alexa 555 and FITC labelled RCPs were observed. This was partly due to the inherent high intensity level of the Alexa 555 dye, creating sharper RCPs than FITC. More autofluorescence is also a problem while using FITC. Concluding, the usage of three different backpieces with unique detection sequences, in one specific concentration each, appeared highly useful as an antibody quantification method. With knowledge of the number of RCPs per antigen in the tissue, the antigens (here HER2) should be quantifiable as well.

Figure 10: RCPs obtained with varying primary antibody and detection probe concentrations after multiplexed in situ PLA. HER2 proteins were detected in SKBR3 cells through seven different dilutions of anti-HER2 antibodies. Data from one dilution (1:1000) was discarded due to poor image acquisition. The three backpieces (L10394, L10395, L10396) complementary to the oligonucleotides conjugated to the HER2 probing antibodies had one common (for FITC [L9375]) and one unique detection sequence (for Alexa 555 [L10398], Pacific Blue [L10397] or Cy5 [L8672]). The backpieces were of three different dilutions (1:1, 1:5 and 1:25), accounting for the variations in RCP counts between the three different fluorescent dyes. During image acquisition, similar cell populations were photographed in the different antibody dilutions using identical camera settings. Intensity measurements were done in Visiopharm Integrator System, during which nuclear areas were excluded.
Discussion

Strengths of the emerging in situ proximity ligation assay

In situ PLA can be applied to clinical samples for detection of protein interactions, which is the major advantage of the technique since this cannot be accomplished using any other method. Appendix A provides explanations on other methods related to PLA (fluorescent protein tags; protein complementation assay [PCA]; resonance energy transfer systems [RET]; bioorthogonal chemical reporters; yeast-two-hybrid [Y2H] and its related methods; affinity purification – mass spectrometry [AP-MS]; immunohistochemistry [IHC]).

Amplification of signal is another very important attribute offered by in situ PLA. The ability of detecting single molecules (as shown in the brightfield CK17 and HER2 experiments) is not present in any of the techniques related to PLA. For that, in vitro techniques will have to be employed, such as the enzyme linked immunosorbent assay (ELISA). Single complexes can also be detected, composed of several proteins, with the possibility of discrimination on account of properties such as proximity or phosphorylation. Fluorescence analysis is most common for in situ PLA results, but can be replaced by regular brightfield analysis through chromogenic in situ hybridisation (CISH), adapting the in situ PLA technique to the common practice of histologists. The conjugation of haptons to primary antibodies and subsequent use of standardised secondary antibodies was shown for the first time by the experiments with villin and DMPK. Any interactions between molecules to which antibodies are available can now be discovered with in situ PLA. This also means that primary antibodies very well can be from the same species, which is not possible with secondary antibodies targeting species specific IgG. For the Human Proteome Resource project (HPR), this is a very interesting novelty, as the high specificity of PLA now has become easily applicable to the large quantity of antibodies in their library, derived almost solely from rabbit (Agaton et al. 2003). The mapping of the human proteome is executed by HPR through immunohistochemistry (IHC) (Uhlen et al. 2005), thus using only one antibody per protein, which sometimes results in false positives (as shown in the haptons experiment, figure 7 and figure 8). Through use of in situ PLA, specificity can be heightened to a large extent and false positives reduced. Arguably, the difference in the 3,3’-diaminobenzidine (DAB) signal in the mentioned experiment could also be explained as one of the antibodies yielding a false negative, as the proteins might have been expressed in different splice variants throughout the body and the part corresponding to the protein expressed sequence tag (PrEST) used to derive one of the antibodies had been spliced out. Reverse transcriptase PCR on the mRNA sequences of the proteins in question could settle the matter.

In multiplexed in situ PLA the number of complexes that can be analysed in the same sample is limited only by the availability of suitable fluorophores. This is another big advantage of in situ PLA, particularly since it does not require genetic modification of the host cell like several other techniques do. It is rather rapid and has the potential of being automated. The assay exploring HER2 and EGFR homo- and heterodimers in patient specimens is of diagnostic interest, as the HER proteins are thought to be important in breast cancer proliferation. There is one commercially available diagnostic kit quantifying HER2 proteins and HER2 homodimers in breast cancer tissue, the HERmark™ assay (Monogram Biosciences Inc., San Francisco, California, US). Unfortunately it requires the shipping of samples to a central laboratory. The patient samples analysed here clearly showed differences in expression patterns, which should be quantifiable through rolling circle product (RCP) counting. The multiplex setup where splint lengths were varied also showed the impact of small changes in proximity on detection, which is something the mentioned HERmark™ assay takes little notice of (Dua et al. 2007). Furthermore, the experiment of the protein
concentration determination presents the possibility of quantifying protein levels in precious samples by taking advantage of a simple backpiece dilution series in one sample (demonstrated here in a series of eight samples), circumventing the need for antibody titration on several samples. The dynamic range given by the approach is of interest, since up to only about 200 rolling circle products (RCPs) per cell can be counted in image analysis programs with good reliability. However, more experiments need to be done before any absolute conclusions can be drawn in this matter.

With thorough knowledge of the epitopes and antibodies involved in the PLA, the method could be used as a molecular ruler much like fluorescence resonance energy transfer (FRET) (Ciruela 2008). The localisation of the molecules detected by in situ PLA can also be determined to subcellular compartments (indicated in the splint length experiment and the breast cancer experiments), which is not generally possible using any of the techniques reviewed in appendix A. Furthermore, these techniques require modifications of the proteins, as in the usage of fluorescent protein tags, bioorthogonal chemical reporters, in PCA, in AP-MS, in Y2H and in RET. This can have adverse effects on protein folding, localisation and function. Additionally, their detection of complexes built up by more than two proteins is exceptionally complicated when at all possible (Shyu et al. 2008). Often, the proteins of interest also need to be overexpressed, further endorsing an unnatural environment. In situ PLA, on the other hand, does not suffer from this. Proteins have been produced in their genuine contexts, and the state in which they are in is reliable due to instant tissue fixation. The concept of choosing the right Y2H related method depending on whether the protein is cytosolic, from a mammal, naturally residing in the nucleus or in the membrane then has vanished. The false positives inherent in those techniques are solved by simple background measurements in the in situ PLA. Dynamics of proteins or their direct response to drug treatment or global variations in cell condition (i.e. during differentiation or mitosis) are possible to study with in situ PLA through examination before and after stimuli. However, methods like fluorescent protein tagging, FRET and certain PCAs might be more convenient in such matters granting that gene modification and vector transformation need to be carried out. The number of reporters offered by PCA can be hard to exceed by any other method, in situ PLA included. However, in situ PLA is not limited to fluorescence, as has been demonstrated in this project as well (figure 4, 5, 7, and 8). Antibodies targeting the RCA product, or the detection oligonucleotides, can be fused to reporter proteins. One example is horseradish peroxidase (HRP), another is alkaline phosphatase. Concerns brought up through use of fluorescence or inexperience thereof, are thereby escapable by the user. Moreover, the commercially available kits often further enhance the efficiency and convenience of methods, which is also the case for in situ PLA (www.olink.com). Using such a kit, an experiment typically takes an afternoon.

**Potential weaknesses affecting the in situ proximity ligation assay**

The biggest disadvantage of PLA is that it cannot be used in vivo. Several other protein detection techniques offer this benefit, such as bioorthogonal chemical reporters, fluorescent protein tagging, PCA and RET. Protein dynamics and kinetics in living cells are naturally of high interest, for which bioorthogonal chemical reporters, FRET and certain PCA setups might be best suited to study.

The rate at which AP-MS can detect interactions of proteins cannot be reached by in situ PLA, although theoretically it can have high throughput, in accordance with such a process utilised by HPR (Uhlén et al. 2005). The challenges endured by AP-MS, however, sometimes affect the credibility of various identified interactions. An important aspect of AP-MS is that
it detects novel protein interactions, whereas in situ PLA can be applied only when known interactions occur. Should an in situ PLA experiment require fluorescent analysis, a few issues arise not shared by some PCA setups, AP-MS, IHC and Y2H (and its related methods). Photobleaching, background, choosing appropriate filters with little bleed through between them, and autofluorescence, are problems requiring skills and experience to handle well. Unfortunately, FRET struggles with even bigger difficulties in this area, although it has been possible to turn some of the obstacles into advantages instead (Pfleger & Eidne 2006). The virtues of fluorescence overshadow the mentioned issues, however. The in situ PLA RCPs are easily distinguished, and by capturing images at different wavelengths more detailed analysis is possible.

In situ PLA inherently demands many different steps to be carried out flawlessly. Alas, this results in a large number of false negatives. My estimation is that one protein interaction in more than a hundred will be represented by an RCP in the end. This number naturally depends on factors such as antibody incubation times and temperatures, the quality and concentration of antibody, structural states of epitopes and the quality of reagents. As mentioned in appendix A, other related methods instead require a large number of positive signals for detection. GFP molecules, for instance, needed to be present in the range of hundreds to thousands of copies to be detected (Tsien 1998). False positives are also possible in in situ PLA, as an antibody targeted to an epitope absolutely unique to one protein is hard to produce. It is thought that the epitope can also be mimicked in its 3D-structure by other proteins, even though the primary structure is not the same (Burritt et al. 1998).

When proceeding with experiments of in situ PLA yielding RCPs, the reliability of the RCPs could be questioned. There could be a risk of the proteins in the complex being in very close proximity to each other, but without interacting. Experiments have been done to study this, when previous PLA signal indicating Myc and Max interactions were obstructed by 12-O-tetradecanoyl-phorbol-acetate and interferon-γ, resulting in a significant decline in RCP count (Söderberg et al. 2006). This shows how a small molecule merely destabilising an interaction is enough to vanquish formation of RCPs. Furthermore, the experiment presented with different splint lengths demonstrates the importance of small differences in proximity.
**Materials and methods**

**Preparation of tissue slides**
Three types of tissue slides were used: Formalin fixed paraffin embedded (FFPE) tissue microarrays (TMAs) from HPR (IH82, regular tissue; A56, cancerous tissue), frozen Zn-fixed SKBR3 cells (Olink Bioscience, Uppsala, Sweden) and frozen breast cancer sample slides from two patients provided by Akademiska Sjukhuset (Uppsala, Sweden). The FFPE slides were deparaffinised through incubation in the following solutions: xylene 2 x 10 min, 99.9% (percentages are given in v/v) EtOH 2 x 2 min, 95% EtOH 2 x 2 min, 80% EtOH 2 min, 70% EtOH 2 min, H2O 5 min and PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH2PO4, 1.5 mM KHPO4, pH 7.4) 2 min. Antigens were retrieved in 1 x citrate pH 6 retrieval solution (Dako, Glostrup, Denmark), at 120°C for 5 min and then 80°C for 20 min and approximately 40 min between those temperatures and room temperature (RT) (skipped when prepared slides from HPR were available). The breast cancer slides from Akademiska Sjukhuset were fixated by being kept in 2% paraformaldehyde for 15 min at RT, then in PBS for 2 min at RT and lastly in 70% EtOH for 15 min at RT. After then washing them in TBST (100 mM Tris-HCl, 1.5 M NaCl, 0.05% Tween-20, pH 7.7), in a cuvette, 2 x 5 minutes an ImmEdge™ pen (Vector Laboraties, Burlingame, USA) was used to create a hydrophobic border around the tissue samples. Afterwards, the samples were blocked when incubated at RT for 20 minutes in a humidity chamber with TBST containing 0.5 mg/ml bovine serum albumin (BSA), 2 μg/ml Salmon sperm DNA and 2 mM cysteine and lastly washed 2 x 3 minutes in TBST.

**Conjugation of antibodies to haptens**
Antibodies (table 1) were provided by HPR and were conjugated to biotin or fluorescein isothiocyanate using the LightningLink™ kit (Innova Biosciences, Cambridge, UK).

**Application of antibodies**
Primary antibodies were diluted in TBST with 0.5 mg/ml BSA, 2 μg/ml salmon sperm DNA and 2 mM cysteine, with which samples were incubated at 4°C ON in a humidity chamber. Afterwards, the slides were washed in TBST, in a cuvette, 2 x 5 minutes. Secondary antibodies (table 1) were diluted in TBST with 0.5 mg/ml BSA, and incubated with the samples for 60 min at RT in a humidity chamber, after which the samples were washed 2 x 5 minutes in TBST.

**Hybridisation and ligation of DNA strands**
For non-multiplexed experiments, hybridisation and ligation reactions of DNA strands forming the rolling circle were carried out using the Duolink® Detection Kit 613 (Art. No 90103) (Olink Bioscience, Uppsala, Sweden) in the following manner. The first solution contained 20% (percentages are given in v/v) hybridisation mixture and 80% ddH2O. Incubation was done in a humidity chamber at 37°C for 15 minutes, followed by washing 1 x 1 minute in TBST. After this, a ligation solution containing 20% ligation mixture, 0.025 U/μl T4 DNA ligase, and remaining part ddH2O, was applied to the samples. Samples were incubated at 37°C for 15 minutes, also in a humidity chamber. In multiplexed experiments, hybridisation and ligation steps were merged. The diluent was ddH2O with 0.05% Tween-20. The solution incubated with the samples at 37°C for 30 minutes in a humidity chamber contained ddH2O with 0.8 x dithiothreitol-free T4 DNA ligase buffer, 0.8 mM ATP, 0.2 M NaCl, 0.165 mg/ml BSA (New England BioLabs Inc., Ipswich, United Kingdom), 0.02 U/μl T4 DNA ligase (Fermentas Inc., Glen Burnie, Maryland, USA), 0.1 μM S3 backpiece L8552 (table 2), 0.1 μM splint L8410, 0.1 μM tag 1851 L8334, 0.1 μM tag 1852 L8331 and 0.1 μM tag 1854 L8328. Afterwards samples were washed 2 x 2 minutes in TBST.
Disengagement of inverted antibody oligonucleotide arm
To disengage the inverted oligonucleotide arm (L10317 [table 2]), 0.05 U/µl uracil DNA glycosylase and 0.2 U/µl formamidopyrimidine DNA glycosylase were introduced in the rolling circle amplification step (see below).

Rolling circle amplification, detection and mounting
For non-multiplexed experiments, rolling circle amplification and detection steps were carried out using the Duolink® Detection Kit 613 (Art. No 90103) (Olink Bioscience, Uppsala, Sweden) in the following manner. The RCA mixture created was ddH₂O, 20% (percentages are given in v/v) amplification stock and 0.125 U/µl φ29 DNA polymerase. With this, samples were incubated at 37°C for 60 minutes in a humidity chamber. Afterwards, they were washed 2 x 2 minutes in TBST. The detection solution consisted of 0.015 mM S3 Texas Red (Olink Bioscience, Uppsala, Sweden) in ddH₂O, incubated at 37°C for 60 minutes in a humidity chamber. Instead of using the Duolink® Detection Kit 613 with its instructions and ready made solutions in multiplexed experiments, these were recreated manually in the following manner. The RCA solution incubated with the samples at 37°C for 60 minutes in a humidity chamber was ddH₂O with 0.5 mg/ml BSA, 10% φ29 DNA polymerase buffer (Fermentas Inc.), 0.24 mM dNTP and 0.24 U/µl φ29 DNA polymerase (Fermentas Inc.). Subsequently, the slides were washed 2 x 2 minutes in TBST. The detection solution contained the following: ddH₂O, 1.6 x saline-sodium citrate (3 M NaCl, 300 mM trisodium citrate and regulated to pH 7.0 by HCl), 0.171 mg/ml BSA, 5.13 ng/µl poly-A (Biomers.net, Ulm, Germany), 0.85 x Hoechst 33342 (Invitrogen, Carlsbad, California, US), 0.02 µM S3 1852 Cy3 L10068 (table 2), 0.02 µM S3 1854 Cy5 L8672, and 0.02 µM S3 1851 TxRed L8878. The slides were washed 2 x 2 minutes with TBST, and lastly also 2 x 1 minute with TBS before cover slips were mounted on the slides with Vectashield H-1000 (Vector Laboratories, Burlingame, USA).

Chromogenic in situ hybridisation, counterstaining and mounting
The DuoCISH kit (Dako, Glostrup, Denmark) was applied for the chromogenic in situ hybridisation (CISH) as follows. All steps took place at RT, and incubations were done in a humidity chamber. Samples were blocked by 100 µl of peroxidase block each, incubating for 5 minutes. The slides were then washed 2 x 3 minutes in TBST, and exposed to 100 µl of DuoCISH antibody mix for 30 minutes. They were washed anew 2 x 3 minutes in TBST after which a solution of 1% (percentages are given in v/v) red chromogen in red chromogen buffer was added to each sample, incubated for 10 minutes. Slides were washed in TBST 2 x 3 minutes before the counterstaining procedure was commenced. Samples were rinsed once in ddH₂O, and then transferred to Mayer’s hematoxylin (Histolab Products AB, Gothenburg, Sweden), in which they were incubated for 30 seconds. They were then incubated in 70% EtOH for 2 minutes and then in xylene for 5 minutes. After drying, a droplet of Aqua Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden) was applied on top of the tissue samples, followed by a cover slip.

Image acquisition
Fluorescence photos were taken using an Axioplan 2 (Carl Zeiss, Light Microscopy, Göttingen, Germany) while AxioVision (Zeiss) was used for image manipulation. In experiments demanding comparison in between images, exposure times for each wavelength were kept constant, as well as brightness, contrast and saturation levels. For brightfield analysis in relevant experiments, the MIRAX MIDI (Carl Zeiss MicroImaging GmbH, Jena, Germany) scanner microscope was used.
Table 1- Antibodies used in this project.

<table>
<thead>
<tr>
<th>ID number</th>
<th>Concentration</th>
<th>Target antigen</th>
<th>PLA type</th>
<th>Dilution in experiment</th>
<th>Experiment</th>
<th>Supplier</th>
<th>Host animal</th>
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<td>HPA006884</td>
<td>0.300 g/l</td>
<td>villin-1</td>
<td>Primary ab; FITC conjugated</td>
<td>1:100</td>
<td>1</td>
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<td>Rabbit</td>
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<td>HPA006885</td>
<td>0.275 g/l</td>
<td>villin-2</td>
<td>Primary ab; Biotin conjugated</td>
<td>1:100</td>
<td>1</td>
<td>Human Proteome Resource Project</td>
<td>Rabbit</td>
</tr>
<tr>
<td>HPA008905</td>
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<td>DMPK-1</td>
<td>Primary ab; FITC conjugated</td>
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<td>1</td>
<td>Human Proteome Resource Project</td>
<td>Rabbit</td>
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<td>Primary ab; Biotin conjugated</td>
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<td>1</td>
<td>Human Proteome Resource Project</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Prototype</td>
<td>80 μg/ml</td>
<td>FITC</td>
<td>Secondary ab; S3 arm</td>
<td>1:35</td>
<td>1</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Mouse</td>
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<tr>
<td>Prototype</td>
<td>85 μg/ml</td>
<td>Biotin</td>
<td>Secondary ab; S3 primer</td>
<td>1:35</td>
<td>1</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Mouse</td>
</tr>
<tr>
<td>90301</td>
<td>1:1</td>
<td>Mouse IgG</td>
<td>Secondary ab; S3 arm</td>
<td>1:2.5; 1:5</td>
<td>6; 3</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Donkey</td>
</tr>
<tr>
<td>90301</td>
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<td>Mouse IgG</td>
<td>Secondary ab; S3 primer</td>
<td>1:2.5; 1:5</td>
<td>6; 2</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Donkey</td>
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<td>Not available</td>
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<td>HER2</td>
<td>Primary ab</td>
<td>1:100; 1:100</td>
<td>6</td>
<td>Provided by Olink Bioscience</td>
<td>Mouse</td>
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<td>HER2</td>
<td>Primary ab; S3 Inv T L10317</td>
<td>1:200; 1:100</td>
<td>5;4</td>
<td>Roche Holdings Ltd., Basel, Switzerland</td>
<td>Mouse (humanised)</td>
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<td>EGFR</td>
<td>Primary ab; S3 Inv T L10317</td>
<td>1:100</td>
<td>5</td>
<td>Roche Holdings Ltd., Basel, Switzerland</td>
<td>Mouse (humanised)</td>
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<td>HER2</td>
<td>Primary ab; S3 Primer 1854 L10099</td>
<td>1:100; 1:100</td>
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<td>AM00055PU-N</td>
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<td>HER3</td>
<td>Primary ab; S3 Primer 1852 L10100</td>
<td>1:100; 1:100</td>
<td>5;4</td>
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<td>Mouse</td>
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<td>EGFR</td>
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<td>5;4</td>
<td>Roche Holdings Ltd., Basel, Switzerland</td>
<td>Mouse (humanised)</td>
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<td>90202</td>
<td>1:1</td>
<td>Rabbit IgG</td>
<td>Secondary ab; S3 primer</td>
<td>1:5</td>
<td>3</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Donkey</td>
</tr>
<tr>
<td>90302</td>
<td>1:1</td>
<td>Goat IgG</td>
<td>Secondary ab; S3 arm</td>
<td>1:5</td>
<td>3</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Donkey</td>
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<td>3</td>
<td>Human Proteome Resource Project</td>
<td>Mouse</td>
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<td>HPA001338</td>
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<td>HER2</td>
<td>Primary ab</td>
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<td>3</td>
<td>Human Proteome Resource Project</td>
<td>Rabbit</td>
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<tr>
<td>sc-31153</td>
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<td>Not available</td>
<td>0.1 g/l</td>
<td>HER2</td>
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<td>1:10</td>
<td>3</td>
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<td>Mouse</td>
</tr>
<tr>
<td>90202</td>
<td>1:1</td>
<td>Rabbit IgG</td>
<td>Secondary ab; S3 arm</td>
<td>1:5</td>
<td>2</td>
<td>Olink Bioscience, Uppsala, Sweden</td>
<td>Donkey</td>
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<td>M7046;</td>
<td>1:1</td>
<td>CK17</td>
<td>Primary ab; RNA arm</td>
<td>1:100</td>
<td>4</td>
<td>Roche Holdings Ltd., Basel, Switzerland</td>
<td>Mouse (humanised)</td>
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<td>Primary ab</td>
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<td>2</td>
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<td>Rabbit</td>
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<td>Primary ab; S3 arm L8697</td>
<td>1:100</td>
<td>4</td>
<td>Roche Holdings Ltd., Basel, Switzerland</td>
<td>Mouse (humanised)</td>
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</table>

Regarding antibodies used in more than one experiment, the order in columns “Experiment” and “Dilution in experiment” correspond. Humanised antibodies have had the Fc region replaced with a human type. Regarding experiment numbering, number 1 points to the hapten experiment, number 2 to the CK17 assay and number 3 to the regular in situ PLA experiment with HER2. Number 4 is assigned to the experiment with splint lengths, number 5 to the multiplexed experiment on breast cancer tissue and lastly number 6 to the protein quantification trial via RCP counts.
### Table 2 - Oligonucleotides used in this project.

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Name</th>
<th>Purpose</th>
<th>Experiment</th>
<th>Concentration used (μM)</th>
<th>Supplier</th>
<th>5’ Modification</th>
<th>Sequence (5’ to 3’)</th>
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<td>L9309</td>
<td>S3 backpiece</td>
<td>Ligation</td>
<td>4</td>
<td>0.1</td>
<td>Eurogentec, Oslo, Norway</td>
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<td>CTATTAGTGCGTCCAGTGAATGCGAGTCCGTCTAAGAGTAGTACAGCAGCCGTCAAGAGTGTCTA.</td>
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<td>L8552</td>
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<td>5</td>
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<td>TriLink BioTechnologies, San Diego, California, USA</td>
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<td>L10394</td>
<td>S3 1851 Backp</td>
<td>Ligation</td>
<td>6</td>
<td>0.76</td>
<td>Integrated DNA Technology, Coralville, Iowa, USA</td>
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Oligo IDs and names are generally applicable in our research group only, and aldehyde labeled oligos were conjugated to antibodies with SANH (succinimidyl 4-hydrazinonicotinate acetone hydrazone). Regarding experiment numbering, number 4 is assigned to the experiment with splint lengths, number 5 to the multiplexed experiment on breast cancer tissue and lastly number 6 to the protein quantification trial via RCP counts. Referring to oligonucleotides used in more than one experiment, the order in columns “Experiment” and “Concentration used (μM)” correspond.
Acknowledgements
First and foremost, my gratitude is directed towards my supervisor Ola Söderberg, for making this project possible, offering valuable expertise and support. Karl-Johan Leuchowius is thanked for discussions and his service. I thank Tim Conze for the same reasons. The tips offered by Irene Weibrecht were also well appreciated. Lastly, I would like to thank the Ulf Landegren group at the Institution of Genetics and Pathology for generating such a good working atmosphere. Also, the offered PhD position flatters me.
References


Appendix – Contemporary protein detection methods

Fluorescent protein tags
*Caenorhabditis elegans* is a 1 mm long nematode which has acquired great fame among the general public by being involved in research leading to Nobel prizes: in 2002 for organ development and programmed cell death, in 2006 for RNA interference studies and in 2008 for experiments with the green fluorescent protein (GFP). The GFP originates from *Aequorea victoria*, a jellyfish found off the west coast of North America. In *A. victoria*, the Ca\(^{2+}\) triggered protein aequorin emits blue light, which excites GFP causing it to emit green light. The ecological advantage of such a system is unknown and considerably more effort has been devoted to this single protein than to the host organism. The scientific virtue of the protein cannot be hyperbolised, and has been extensively used for protein detection and localisation in vivo. This has been done through fusing the GFP gene to the gene encoding the interesting protein, which will be visible after correct folding in a fluorescent microscope (figure 11). Since only one GFP is tagged to each protein of interest, quite a large number of molecules are needed to overcome autofluorescence in the tissue. An estimated 300 to 3000 copies of correctly folded GFP are required to overcome autofluorescence locally and present a distinguishable green dot. If the protein of interest is present throughout the whole cell, autofluorescence becomes even harder to defeat. Moreover, the fact that the GFP is physically linked to the protein of interest might cause problems in itself. The protein in question can have problems folding correctly, natural localisation might be impeded and function of the protein of interest can possibly fail. A positive aspect, however, is that cells do not need to be fixed but can be studied live in media. The dynamics of the protein in the cell can also be studied over a time range. Additionally, GFP can be engineered with mutations, so that its fluorescence becomes more sensitive to environmental factors such as pH or more prone to phosphorylation state. Also, through mutation experiments, other variants of GFP fluorescing in other colours have been produced. Furthermore, GFP is sometimes used in Förster resonance energy transfer (FRET) analysis and protein fragment complementation assay (PCA) setups, which are covered in later sections. (Tsien 1998, Sekar & Periasamy 2003)

![Figure 11 – Schematic representation of protein tagging by GFP. The GFP is genetically linked to the protein of interest, and will be possible to detect through fluorescence microscopy.](image)

Protein fragment complementation assay
The nature of the signal produced when detecting interactions of proteins can be quite diverse in PCA. This method, like the use of fluorescent protein tags, requires coupling of protein sequences to the proteins of interest. At the same time, it also resembles the split tobacco etch virus (split-TEV) assay or the split ubiquitin assay (explained further down) in that it requires the reconstitution of a protein. In PCA, the idea is to dissect strategically a chosen protein able to produce a specific signal into two parts. The DNA sequence corresponding to each part is then inserted in-frame with the DNA sequence of one of the two interacting proteins of interest. When the proteins of interest interact, the two protein fragments will refold and reconstitute a functional protein (figure 12). This is why a rational dissection is required,
rather than to simply dock two subunits of the same protein. Demanding refolding denotes a very precise spatial relationship between the two fragments, and close to eradicates false positives. The number of positive signals observed is thereby proportional to the number of protein complexes. This situation has been exploited when studying ligand activation of the dimeric erythropoietin receptor, which has cytosolic domains 73 Å apart when unstimulated. A split PCA reporter (dihydrofolate reductase) was fused to these domains and able to reconstitute during receptor stimulation as the domains in question then reside only 8 Å from each other.

Figure 12 – Schematic representation of PCA. A reporter protein (purple) is strategically dissected into two halves, each of which is genetically linked to one of the two proteins of interest (yellow and blue). If interaction between them occurs, the cleaved protein will be able to reconstitute and produce a detectable product (orange).

Regarding the readout, one alternative is to produce fluorescent signals, by allowing the protein fragments to reconstitute GFP or versions of it. This technique has been well used and is often referred to as bimolecular fluorescence complementation (BiFC). However, a list of other reporter proteins is available, comprising three different luciferases (from firefly, *Gaussia princeps* and *Renilla reniformis*), monomeric red fluorescent protein (from *Discosoma coral*), adenylate cyclase CyaA, murine dihydrofolate reductase, glycaminide ribonucleotide transformylase and aminoglycoside kinase to name a few. They all contribute to increasing the areas of use for PCA, as the readouts differ in between assays, and thereby also their applicability to various experiments. (Ciruela 2008).

After prey and bait proteins interact, and when refolding of the PCA reporter has been completed, the reporter protein is active and the complex is ordinarily irreversibly trapped. Dissociation of the complex is generally not possible, which can impact its natural localisation. On the positive note, however, this actuality can be useful when studying rare or volatile complexes. Unfortunately, kinetics of the interactions among the proteins normally are not possible to study through PCA, since the majority of reporter proteins need a time frame of minutes to fold. These conditions have been true for some time, but certain reporter proteins have been developed that both fold under a minute and can be disassembled.

One of the more promising areas of usage for PCA is drug discovery. By introducing what is called PCA sentinels into a known pathway of importance to specific clinical diagnoses, that pathway can be monitored. The PCA sentinels are nothing more but regular PCA modifications to sets of proteins throughout a pathway, functioning as indicators for what parts of the pathway interact. When such a setup exists, the cell line in question can be subjected to diverse drugs. The sentinels will then reveal what part of the pathway is disrupted and by what drug. This can be of high-throughput, and naturally care must be taken not to interrupt the pathway completely by the sentinels themselves. (Remy & Michnick 1999, Remy & Micknick 2006, Michnick *et al.* 2007)
Resonance energy transfer systems

There are two resonance energy transfer (RET) systems, namely bioluminescence-RET (BRET) and Förster-RET (FRET), also known as fluorescence-RET. The phenomenon upon which FRET depends is the ability of one fluorescent compound to excite another within a certain distance and at a specific orientation. The BRET technique likewise relies on energy transfer, but with the energy originating from substrate oxidation by luciferase (figure 13).

In order to study the interaction of a protein pair using FRET, they need to be fused to one other protein each. These are fluorescent proteins of differing fluorescing capabilities, and are referred to as donor and acceptor. During the detection of an interacting complex, the donor is excited in a specific wavelength. The wavelength thus emitted by the donor in turn will excite the acceptor, which successively will radiate at a third specific wavelength unveiling an interacting complex. As the energy transfer occurring is highly dependent on the distance and orientation of donor and acceptor, FRET can also be used as a “molecular ruler” through calculation of light intensity.

![Figure 13 – Schematic representations of FRET (left) and BRET (right). Acceptor (YFP) and donors (CFP and luciferase) are genetically linked to the proteins of interest. When interaction occurs in FRET, CFP will be close enough to transfer energy for YFP to fluoresce after first being excited itself by a different wavelength. This wavelength exciting CFP is in BRET replaced by luciferin (red), which produces light when oxidised by luciferase. If YFP is in proximity, it will be excited by this specific wavelength.](image)

In BRET, one of the two fluorescent proteins in FRET is replaced by luciferase. Luciferase oxidises luciferin, and energy transfer from the emitting substrate to the acceptor can occur if the acceptor is within range.

In comparison to each other, it is easy to think BRET superior since the light source is from within the cell and easily can be turned on or off via substrate regulation. When using FRET, considerable attention needs to be directed towards the fluorescence complications. Autofluorescence of tissue is one problem, especially in pigmented tissue samples. Acceptor and donor proteins need to be carefully selected, with considerations of overlapping spectra. Photobleaching of the proteins is another issue. As these problems do not affect BRET, the big obstacle with BRET is the inability of subcellular protein localisation studying. The reason for this is the substrate for the luciferase, which cannot be distributed to merely one subcellular region. (Pfleger & Eidne 2006, Ciruela 2008)

Bioorthogonal chemical reporters

When studying biomolecules, it is of great importance to perturb the natural state of the interactions among them as little as possible. GFP tags, for instance, have been shown to occasionally disturb their host proteins beyond natural activity. To circumvent this problem, bioorthogonal chemical reporters such as fluorescein arsenical helix binder (FlAsH) have been developed. In their review, Perscher and Bertozzi defined bioorthogonal chemical reporters in 2005 as “non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes”. When discussing FlAsH, the non-native and non-perturbing chemical handle is made up of a small protein sequence (CCXXCC, where XX can be practically any two kinds of amino acid but preferably P and G) inserted in the protein to be studied (figure 14). The exogenously delivered probed is a biarsenical - a small molecule with two trivalent arsenic atoms able to
react with the cysteine thiols. The biarsenical likewise holds a fluorescent compound which fluoresces only faintly when unbound and much more strongly when bound to the protein sequence handle. There are also other bioorthogonal chemical reporters targeted to other biomolecules than proteins. (Prescher & Bertozzi 2005)

![Figure 14 – Schematic representation of how bioorthogonal chemical reporters function. The protein of interest is genetically modified, placing four cysteines in the right relation to each other for two arsenics in the biarsenical compound (linked to the green star) to bind. The fluorescein in the compound can then fluoresce by illumination of the right wavelength.](image)

Furthermore, there are several other versions of the described FlAsH system, making use of the same non-native protein motif but with dissimilar probes. An example is the red arsenical helix binder (ReAsH), where the fluorescent part of the probe is resorufin, emitting red light. The biarsenicals used are able to bind with great affinity and specificity to the thiols. To reduce background of binding to other endogenous thiols or lipoamide cofactors, small vicinal dithiols such as 2,3-dimercaptopropanol can be added, which at higher concentrations also can strip off the biarsenicals from the proteins if wanted. Development has also led to the knowledge of membrane impermeant biarsenicals, for limiting the probes to detect membrane proteins. Another positive remark is that the biarsenicals also can be modified for usage in affinity purification of the proteins they are bound to, and are then easily washed off competitively with 2,3-dimercaptopropanol. Furthermore, synthesis of proteins can be studied using these biarsenicals since they bind and start to fluoresce in a time span of seconds after protein synthesis, which is much less than the tens of minutes required for GFP to form the chromophore. On the negative note, however, the biarsenicals themselves and the other compounds used in the protocol such as the background reducing 2,3-dimercaptopropanol can infer toxicity to the cell. (Adams et al. 2002).

Yeast two-hybrid and its related methods

The yeast two-hybrid (Y2H) technique was first described by Stanley Fields and Ok-kyu Song in a letter to Nature in 1989. Originally, the idea was to use a gene vital for growing as a reporter gene if an interaction between the proteins in question occurs. A known protein of interest (called bait) is fused to a DNA binding domain (DBD), and the possibly interacting protein (called prey) is fused to an activation domain (AD). If bait and prey interact, the DBD and the AD will also interact causing transcription of the reporter gene (figure 15 A). Selection can then occur if the reporter gene is the often used E.coli lacZ producing β-galactosidase, and the yeast is grown on minimal medium with lactose. Several different variations of this procedure have been made available. For drug screening, one can use reversed yeast two-hybrid (figure 15 C), which means that the reporter gene instead is not activated if a compound added interferes with the protein interaction of interest. The yeast three-hybrid (figure 15 B), however, requires an intermediary molecule (such as a small molecule, a protein or several proteins) between bait and prey to transcribe the reporter gene. Another version is the membrane yeast two-hybrid technique, where interactions between membrane proteins are looked at.
Figure 15 – Schematic representations of Y2H (A), Y3H (B) and rY2H (C). In Y2H, protein interaction (yellow and blue) promotes DBD and AD interaction, so that β-galactosidase can be transcribed as a reporter gene. In Y3H, the procedure is the same, but with the additional third party molecule that is required (between the yellow and orange interacting proteins) for reporter transcription. In rY2H, the third party molecule instead obstructs the protein interaction so that no transcription occurs.

The bait is fused to the C-terminal domain of ubiquitin, and the prey is fused to the N-terminal domain of ubiquitin. If the two membrane proteins interact, native ubiquitin will be formed which in turn is recognised and split by an endogenous protease. A transcription factor, fused to the ubiquitin, can then enter the nucleus and activate the reporter gene expression. The cytosolic yeast two-hybrid functions likewise, with the only difference of examining the bait and prey in the cytosol and not bound to the membrane. As seen in figure 16, these two setups highly resemble the mating based split ubiquitin system (mb-SUS). The membrane can be of any cellular compartment (i.e. vacuole, plasma, mitochondria or nuclear membrane). The method is therefore suited for membrane proteins, and can be of high throughput. During high throughput experiments with this method, however, there can be a problem of unintended release of the transcription factor. This naturally produces a false positive signal, and can occur when the protein of interest contains a membrane protein like core and is therefore interpreted as a membrane protein, but does not have a real membrane protein domain. A false positive can also be due to unfortunate endogenous cleavage of the transcription factor, owing to the protein it is fused to. In both cases, the transcription factor could be able to enter the nucleus and start transcription. (Lalonde et al. 2008, Suter et al. 2008)

Figure 16 – Schematic representations of mb-SUS (A, B, C) and split-TEV (D). When ubiquitin (green protein in A, B and C) is reconstituted through successful protein interaction (between yellow and blue boxes), endogenous proteases can recognise it and release the transcription factor (grey box) into the nucleus (for transcription start of reporter) by cleavage. Bait and prey can both be membrane proteins (A) or prey can be cytosolic (B) or bait can be anchored to the membrane via a lipid molecule (C). In the split-TEV assay (D), the protease (pair of scissors) is reconstituted through interaction by bait and prey, and can cleave cytosolic or membrane bound recognition sequences (sandglass shaped) to release transcription factor into nucleus.

As Ito et al. explained in 2001, the Y2H can be of high throughput, which has permitted mapping of the interactome of *Saccharomyces cerevisiae*. An obvious disadvantage of the yeast two-hybrid system and some of its derivatives is that the interaction takes place in the yeast nucleus, which may not be the natural environment for the proteins. Another point worth mentioning is that false positives are likely, created by self activation of the reporter gene by individual bait and prey proteins. There are a few techniques that are somewhat related to the Y2H, in that they involve transcription of reporter genes. One of them is the split tobacco etch virus protease assay (split-TEV assay, figure 16 D). In this process the
protease is split into two parts, each coupled to either the bait or the prey. When reconstituted, it will be able to cleave specific protein sequences in certain other protein constructs, releasing transcription factors activating a reporter gene. Releasing luciferase is also a possibility in the assay. The reconstitution of protease naturally requires interaction between bait and prey, a circumstance resembling PCA. However, the difference is that PCA does not activate reporter genes, but produces a readout directly from the reconstituted protein. Split-TEV is generally used to examine constitutive or regulated protein-protein interactions and especially suited to study transmembrane proteins. The method has been used in both yeast and mammalian fibroblasts. (Wehr et al. 2006, Suter et al. 2008)

Another method taking advantage of the TEV protease is the Tango assay (figure 17 left), which is used to monitor receptor activation by ligand binding. It functions by having a transcription factor fused to a TEV cleavage site and also the receptor of interest. The TEV protease is fused to the signalling protein, which in turn becomes recruited by the receptor after activation. The transcription factor is subsequently cleaved off, and able to enter the nucleus to start transcription of a reporter gene. Three kinds of receptors have been studied. G-protein coupled receptors, receptor tyrosine kinases and steroid hormone receptors. An advantage of the Tango assay, apart from its sensitivity, is that it is insensitive to interference by endogenous proteins. (Barnea et al. 2008, Suter et al. 2008)

Mammalian protein-protein interaction trap (MAPPIT) is an additional method activating transcription of reporter genes (figure 17 centre). Instead of freeing transcription factors by cleaving protein sequences, this method does so by phosphorylation in a relatively complicated way. If bait and prey interacts, a ligand dependent cytokine receptor signalling pathway is restored. Signal transducer and activator of transcription 3 (STAT3), a transcription factor, can then start the transcription of a reporter gene. More in detail, the bait protein is fused with the intracellular part of the receptor to which the janus kinase (JAK) protein (able to phosphorylate STAT3) is also fused for the sake of proximity. At the same time, the prey protein is fused to another protein containing docking sites for STAT3. When the bait and prey proteins interact, JAK and STAT3 will be in proximity with each other and, upon phosphorylation by JAK, STAT3 is released and can enter the nucleus. An advantage of the whole concept is that proteins interact in the cytoplasm and not in the unnatural environment of the nucleus. Simply by regulating the presence of receptor ligand, induction of protein interaction is also possible. In a reversed version of MAPPIT (rMAPPIT), the reporter gene becomes activated due to interruption of the PPI (figure 17 right). The difference from

![Figure 17](image_url)

Figure 17 – Schematic representations of the Tango assay, MAPPIT and rMAPPIT. The tango assay (left) works by cleaving a protease recognition sequence (green sandglass) upon ligand (red) binding of the prey requiring receptor. The transcription factor is cleaved off in case of successful protein interaction and can start transcription of reporter gene in the nucleus. In MAPPIT (centre) and rMAPPIT (right), the transcription factor STAT3 requires phosphorylation by JAK to be released into the nucleus. In MAPPIT this will take place after ligand binding of receptor and interaction between bait and prey (yellow and blue, respectively). In rMAPPIT, STAT3 will be released into the nucleus as long as the bait and prey proteins are obstructed from interaction.
MAPPIT is then that the intracellular part of the receptor contains docking sites for both JAK and STAT3, so that JAK is possible to phosphorylate STAT3. This intracellular part is also fused to the bait protein, and at the same time the prey is fused to an inhibitory domain. If bait and prey interacts, the inhibitory domain will physically occupy the space where STAT3 needs to be located for JAK to phosphorylate it. Then it cannot translocate into the nucleus and start transcription of the reporter gene. rMAPPIT has been used for studies of drugs, able to disrupt the bait-prey interaction. (Eyckerman et al. 2005, Suter et al. 2008)

**Affinity purification - mass spectrometry**

The majority of the methods covered up to the present all concern the detection of one or a few protein complexes in cells or tissue. There is however a concern to determine the interactomes of different species, that is essentially to lay out maps of what proteins within a species interact. A high-throughput method is needed for this to become reality, and affinity purification – mass spectrometry (AP-MS) has proven advantages in such an area (figure 18 left). Y2H was used to establish the very first interactome, that of *S. cerevisiae*. The technique of AP-MS on the other hand is substantially different. The ORF of the proteins of interest are modified with a small in-frame tag on either the N or the C terminus. After the growth of sufficient study material, induction of promoters and cell lysis, this tag is captured by antibodies in an affinity purification step. When eluting, the tagged protein will be accompanied by interacting partners. After proteolysis, mass spectrometry and database matching, proteins previously present in the elution can be identified.

This method of course presents its own complications that need to be contemplated before practicing the experiment. One obfuscation involves the homogenisation of the sample before affinity purification. The protein of interest will become unnaturally exposed to numerous different proteins not present in its conventional subcellular environment. This circumstance produces background as unspecific binding occurs. However, extensive reduction of such background is possible through tandem affinity purification (TAP), where secondary affinity purification is carried out using a second purification handle inserted into the ORF. For the same reason of unspecific binding, the protein of interest needs to be expressed in close to physiological amounts, as overexpression leads to improper folding. Possible dealings with chaperones could then maneuver the protein to unsuitable cellular locations. Furthermore, chaperones are among the recurring contaminants in the affinity purifications in addition to naturally abundant proteins (i.e. actin). Another solution to lessen their impact on the reliability of results is to include certain proteins in the experiment, whose various interactions formerly have been deduced before. False negatives are another matter worth reflecting over. After an AP-MS experiment, interactions expected from previous results might fail to display. One explanation for this could be the growth condition, which often is quite uncompromising in an AP-MS trial. Continuing, the AP condition could be too tough for certain naturally occurring interactions. Furthermore, the tag inserted for the purpose of AP might have obstructed the interaction. The most serious problem causing false negatives, however, usually is the ratio between bait and prey. Unstable interactions can be outcompeted when the bait is overexpressed. Also worth mentioning is the phenomenon of multiple alternative complexes all sharing the same bait. During such occurrences, the complexes formed are exceptionally difficult to discern. Sometimes, the bait is rendered useless and another experimental strategy needs to be set up for deciphering the various proteins’ inherent relationships. Related to this issue is the circumstance where two or several proteins share the same binding site on a bait protein and therefore only can interact with that protein one at a time. In an AP-MS experiment, readouts from purification of such a bait protein will incorrectly show a multiple protein complex. (Ito et al. 2001, Gingras et al. 2007)
Variations of AP-MS have been developed with their own benefits, bypassing a few problems of the described regular AP-MS. If novel interactions are not sought after, known or foreseen protein interactions are quickly confirmed by the purposefully developed cross-and-capture system. Bait and prey proteins each have specific handles and have their ORFs in one *S. cerevisiae* strain each. The strains are crossed, lysed and bait proteins are isolated.

Figure 18 - Schematic representation of AP-MS (left) and QUICK (right). In AP-MS, the protein of interest (yellow) is genetically linked to an immunoprecipitation handle (green). The hosting culture is homogenised and put through immunoprecipitation (cylinder). After elution, MS (graph) is done, revealing interacting proteins through databank comparison. In QUICK, two strains are grown in one type of media each, with a certain carbon isotope (brown and grey), and one is subjected to RNAi of the protein of interest. Homogenisation is carried out and pooling is done followed by immunoprecipitation, and then tandem mass spectrometry. Out of the levels of each isotope corresponding to the different proteins in the sample, the interactions can be concluded.

Electrophoresis and western blot analysis then follows. The prey handle will be available for antibody targeting if the prey interacted with the bait. This method has been useful in studies of protein modifications.

Luminescence based mammalian interactome mapping (LUMIER) is an automated screening procedure for mammalian protein interactions. Such interactions pose a problem to the regular AP-MS technique, as protein amounts often are insufficient. LUMIER works by attachment of luciferase to prey proteins, and use of antibodies to capture bait proteins. When the immunoprecipitation is completed, luciferin is added to the sample, which will light up in the presence of prey protein.

Unspecific interactions are another reason to devise a variation of AP-MS. Quantitative immunoprecipitation combined with knockdown (QUICK) settles this matter (figure 18 right). The first step is to grow cultures in two different media containing a certain carbon isotope (\(^{12}\text{C}\) or \(^{14}\text{C}\)), and in one of them use RNAi to silence the bait protein. After sufficient growth, the two cultures are mixed and lysed together. Immunoprecipitation of the bait protein is the next step, after which tandem mass spectrometry follows to create a staple
diagram. The bait protein will be revealed as of one isotope (i.e. $^{12}\text{C}$) of it will here show a much higher concentration than the other (i.e. $^{14}\text{C}$). The two isotopes of a protein taking part in unspecific interactions during cell lysis will be of the same concentration. Lastly, the two isotopes of the prey proteins will be of different concentrations, since only one of them was grown together with the bait protein. (Suter et al. 2008)

The method of affinity purification – mass spectrometry has become rather well developed, and there is a vast bulk of information and knowledge surrounding it. In brief, there are more interesting techniques such as crosslinking of proteins and application of other purification strategies than affinity purification, i.e. size exclusion chromatography. Additionally, there is the whole concept of MS data handling. Unfortunately, this essay is unable to cover all of it. In spite of all the difficulties that the AP-MS approach poses, it is of extensive use. Complete interactomes of different species can now be unravelled with a reasonable amount of resources. Numerous complete metabolism pathways can be unveiled, being of obvious great utilisation in many areas, not to mention drug discovery.

**Immunohistochemistry**

Albert H. Coons *et al.* demonstrated in 1942 the immunohistochemistry technique the way it is in general aspects still used today. In the experiments, antibodies were collected from pneumococcus immunised rabbit and conjugated to fluorescein isothiocyanate (FITC). Pneumococcus infected mice were killed and harvested for their organs, which in turn were cut in thin slices, bathed in the antibody solution and lastly mounted on glass slides. In a fluorescence microscope, the antibody conjugates were visible primarily in liver and spleen. This procedure is sometimes referred to as immunofluorescence, while IHC is often associated instead with 3,3'-diaminobenzidine (DAB) staining. The difference is that the antibodies are then conjugated to Horseradish peroxidase (HRP), capable of cleaving DAB into a brown precipitate (figure 19). Neighbouring tissue can be counterstained with hematoxylin, for visualisation, and brown areas will represent antigens. Low sensitivity is a general problem with the method, since a significant number of antigen molecules often need to be present for a visible signal to be produced. However, this can be solved to a certain extent by coupling the antibodies to polymers of HRP. The setup is also restricted from detecting a number of antigens at the same time in the same tissue, as well as complexes of proteins (Ramos-Vara 2005).

**Figure 19 – Schematic representation of IHC.** A protein of interest (blue) is probed for with an antibody which in turn is probed for with a standardised secondary antibody coupled to HRP (symbolised by a pair of scissors). HRP is then able to cleave DAB into a precipitate (brown). Analysis is done by brightfield microscopy.