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REVIEW

The method developer's guide to oligonucleotide design

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ABSTRACT

Introduction: Development of new methods is essential to make great leaps in science, opening up new avenues for research, but the process behind method development is seldom described.

Areas covered: Over the last twenty years we have been developing several new methods, such as in situ PLA, proxHCR, and MolBoolean, using oligonucleotide-conjugated antibodies to visualize protein-protein interactions. Herein, we describe the rationale behind the oligonucleotide systems of these methods. The main objective of this paper is to provide researchers with a description on how we thought when we designed those methods. We also describe in detail how the methods work and how one should interpret results.

Expert opinion: Understanding how the methods work is important in selecting an appropriate method for your experiments. We also hope that this paper may be an inspiration for young researchers to enter the field of method development. Seeing a problem is a motivation to develop a solution.

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KEYWORDS
Method development; oligonucleotide design; microscopy; Protein-protein interactions; antibodies; proximity ligation

1. Introduction

Life is a dynamic process of protein-protein interactions, so to understand life in health and disease methods are required to monitor not only levels of proteins but also take in to account which proteins they interact with. This is especially important, as proteins will change their functions with changing interaction partners. The mere presence of a protein will not provide information on their functional states (i.e. active or inactive), nor about which function it performs at the moment. With the development of methods for genomics and proteomics, we are aware of the architecture of the genome and the presence of the proteins. Although there are still a lot of unknowns, we have a rough understanding of how genetic aberrations results in diseases and how these are propagated into the proteome. As individual cells are controlled by epigenetic regulation of the genome and external signals from their microenvironment, as well as, signaling network topology inside the cell. Analysis should preferably be performed at a single cell level and if possible, also retain information on its surroundings. To allow for studies of cellular communication and signal transduction, we have during the last two decades been developing methods to visualize levels of protein interactions in individual cells *in situ*. The common theme of these methods is that they target two proteins of interest, determine if these proteins are in close proximity and then generate a signal that will allow visualization of interaction-events by microscopy. Our goal was to examine endogenous protein-protein interactions in cells and tissue sections. To achieve this, we relied on antibodies to target the proteins of interest. To determine if the pair of antibodies bound to the target proteins were within in close proximity, we used short DNA oligonucleotides conjugated to the antibodies (proximity probes) to set an upper threshold on the distance between antibodies. The distance between two adjacent nucleotides in a double-stranded DNA molecule is 0.34 nm, so the distance the attached oligonucleotides can span is dependent on how many nucleotides they contain. We designed the methods to take advantage of the predictable hybridization between single-stranded DNA molecules. A proximal binding of two proximity probes would then enable hybridization between the attached oligonucleotides or template hybridization with subsequently added DNA oligonucleotides. The hybridized DNA oligonucleotides could then be modified, be joined by ligation or cut by DNA modifying enzymes, to create a DNA molecule that would be a reporter of a proximity event. The final step of the methods is then to utilize the DNA molecule, formed by the proximal binding of a pair of proximity probes, to generate a strong fluorescent signal that easily could be detected by standard microscopy. To achieve this, we utilized two techniques: (1) rolling circle amplification (RCA), involving circular DNA molecules formed through proximity probe-templated ligation, and (2) hybridization chain reaction (HCR) for methods where the proximal binding of proximity probes reveals an initiator sequence.

In this paper we will in depth describe the design of these methods, their advantages and limitations, and considerations for their use and interpretation of data.

2. Design of the *in situ* proximity ligation assay

In order to visualize protein interactions in fixed cells and tissue sections, we developed the *in situ* Proximity Ligation

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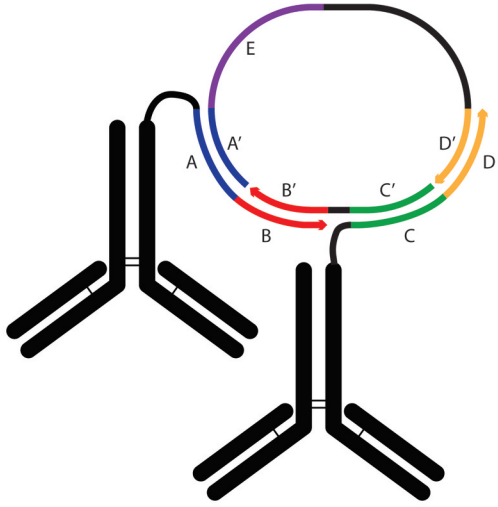
Article highlights

- Description of how to develop new molecular biology methods
- A guide on the workflow for oligonucleotide design, for molecular biology methods
- Considerations for the selection of a method and interpretation of data

Assay (*in situ* PLA), originally called Proximity-Ligation *in situ* assay (p-LISA) [1]. The method was inspired by the proximity-dependent DNA ligation assay (PLA) [2]. In PLA the oligonucleotide portion of the proximity probes can be ligated together when a pair of proximity probes binds the same protein. The ligation is templated by hybridization to a separate linear DNA oligonucleotide. As each of the proximity probes are equipped with a primer site, only the ligated proximity probes, containing both forward and reverse primer sites, can then be amplified by PCR. In order to visualize the location of protein-interactions inside a cell, we had to use another way to generate a detectable signal as PCR products would diffuse away. Instead we opted for RCA [3] to provide a localized amplification of a circular DNA template. RCA products are physically linked to the proximity probe and therefore stay in place even after washing. We thus had to develop a method where proximity probes rather than being ligated together, would instead act as a template for the ligation of two subsequently added circularization oligonucleotides into a circular DNA molecule. Only if the two proximity probes were in close proximity, would it be possible to ligate both sides of the circularization oligonucleotides and form a circular DNA molecule. The formation of DNA circles, thus report on proximal binding of the proximity probes. To visualize the DNA circles, these are amplified using RCA primed by the oligonucleotides of the proximity probes. The RCA product formed will hence be an extension of the oligonucleotides of the proximity probes consisting of multiple repeats of the reverse complement of the DNA circle, one after the other. An hour amplification with Phi29 DNA polymerase will result in several hundreds of repeats per molecule. The single-stranded RCA product will form a bundle of DNA, that is bound to the targeted protein by the antibody part of the proximity probes. The sequence of this RCA product is a concatemer of repeats, so each molecule will bind hundreds of detection oligonucleotides. The detection oligonucleotides are the reverse complement of a repeated element of the RCA product. By using fluorophore-labeled detection oligonucleotides, each RCA product will be stained with several hundred fluorophores. The stained RCA product can easily be detected by microscopy, as bright dots of around 1 µm. These *in situ* PLA products can then be enumerated using image analysis software, such as CellProfiler [4] or ImageJ software, providing quantification of the number of proximity events per cell.

When designing a new method, we start with paper and pencil and draw the design. For you reading this review, it might be easier to follow the text if you look at the figures to identify the different designs described in the text. For *in situ* PLA, we will need five different oligonucleotides (Figure 1(a)). The two Proximity probe oligonucleotides need to contain elements that are reverse complementary to the Circularization oligonucleotides, positioned so that they bring the 3´end of one together with the 5´end of the other. In Figure 1 we call these elements A, B, C and D. In addition, we include a spacer region at the 5´end to provide some flexibility, and a chemical moiety (in the example in Figure 1, we used an aldehyde modification for conjugation with SANH) that can be used for conjugation to the antibodies. Circularization oligonucleotide 1 will need to contain a element reverse complementary to element A in its 5´end, this we call A´, and a element reverse complementary to element D in its 3´end, called D´. Between these we add a element that we will use as a target for the Detection oligonucleotide, we call this E, and a spacer region to allow for some flexibility. Circularization oligonucleotide 2 is designed to contain a element reverse complementary to element C in its 5´end, called C´, and one reverse complementary to element B in its 3´end, called B´. Between these we add a short spacer region. Both Circularization oligonucleotides are equipped with a 5´-Phosphate to allow them to be ligated together. Once they have been ligated into a circular molecule it would be the template for an RCA, primed by either of the Proximity probe oligonucleotides. As the detection oligonucleotide will hybridize to the RCA product, which is a concatemer of the reverse complementary sequence of the ligated Circularization oligonucleotides, it will target E´ in the RCA product. Hence, the sequence of the Detection oligonucleotide will be of the same sequence as element E, but equipped with a fluorophore. The next task is to design the different elements. The good thing with DNA is that it is predictable, the strength of hybridization can be predicted by computer software. We nowadays mainly use NuPack software [5] for designing and evaluating oligonucleotides. We use a library of random nucleotides and through an iterative process we select elements that hybridize well to each other at the temperature of interest, for *in situ* PLA 20–37 degrees Celsius, with as little cross-hybridization as possible. With NuPack, the complex formed can be visualized, which makes it easier to optimize the design. For the *in situ* PLA design we made elements A-D of 11 nucleotides long, to get stabilization when both circularization oligonucleotides hybridize to a proximity probe oligonucleotide, and element E of 20 nucleotides. Next, the spacer regions in the Circularization oligonucleotides are added, to provide some more flexibility. As there are optimal sizes of the circular DNA templates for RCA, the ligated product of the Circularization oligonucleotides should be around (0.5+N) x10.5 nucleotides [6]. For Circularization oligonucleotide 1 we added a 23-nucleotide long spacer region to get a total length of 65 nucleotides, and for Circularization oligonucleotide 2 we added 3 nucleotides to get a total length of 25 nucleotides. The distance threshold for *in situ* PLA will be determined by the length of the Proximity probe oligonucleotides. To add some distance we incorporated a spacer region of 5 nucleotides in each oligos, a stretch of polyA:s. This design gives a total maximal distance between antibodies of 35 nucleotides, which will be around 14 nm. If one would require a more stringent assay, one could reduce the spacer region. For extreme distance threshold, the chemical moiety used for conjugation can be place at the 3´end of Proximity probe oligonucleotide 1, reducing distance

a



Proximity probe oligonucleotide 1	Chem linker A B	Chem linker-AAAAAAAAAAGACGCTAATAGTTAAGACGCTT
Proximity probe oligonucleotide 2	Chem linker C D	Chem linker-AAAAAAAAAATATGACAGAACTAGACACTCTT
Circularization oligonucleotide 1	P- A′ E D′	Phosphate-CTATTAGCGTCCAGTGAATGCGAGTCCGTCT AAGAGAGTAGTACAGCAGCCGTCAAGAGTGTCTA
Circularization oligonucleotide 2	P- C′ B′	Phosphate-GTTCTGTCATATTTAAGCGTCTTAA
Detection oligonucleotide	Fluorophore E	Alexa 555-CAGTGAATGCGAGTCCGTCT

b

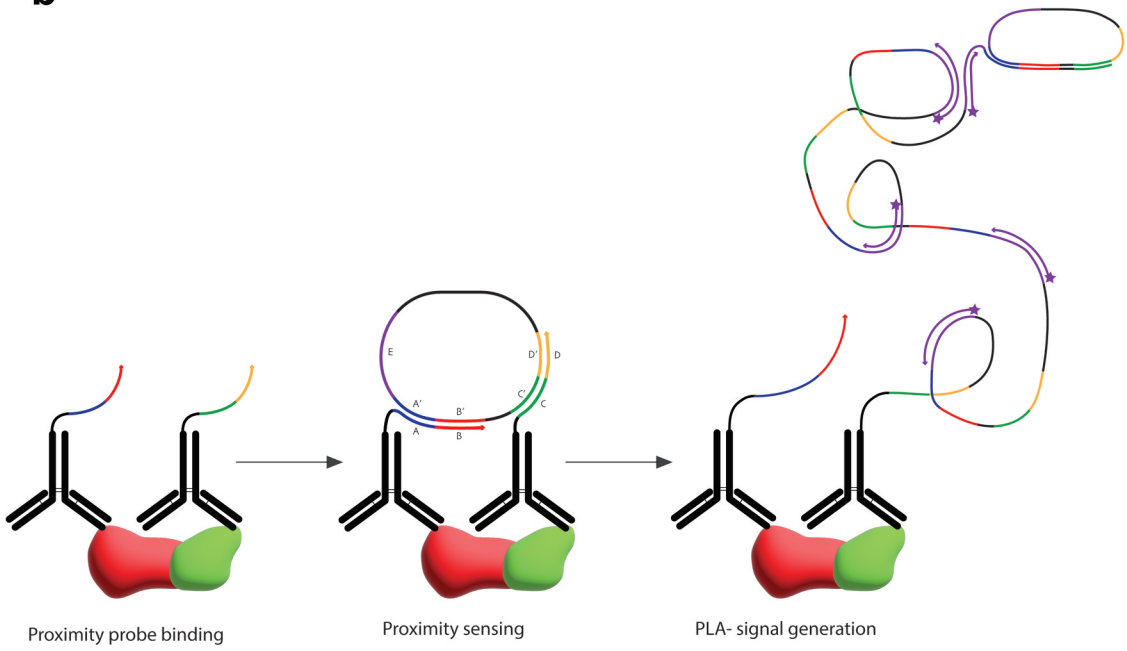


Figure 1. (a) A schematic presentation of the oligonucleotide design for *in situ* PLA. (A)The sequences of the different oligonucleotides are presented in the table below, color coded to identify the different elements. Proximity probe oligonucleotides are presented as conjugated to antibodies. The sequence of proximity probe oligonucleotide 1 was originally designed with three mismatched 2´-O-methyl-RNA at its 3´end to ensure that RCA could only be performed from proximity probe oligonucleotide 2. This is however not needed, and we have since then removed them as it does not affect efficiency of *in situ* PLA. Addition of a three mismatched 2´-O-methyl-RNA at the 3´end of the detection oligonucleotides can be used if one wants to combine the RCA step with detection, to prevent Phi29 DNA polymerase to prime when the detection oligonucleotides hybridize to the RCA products. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. (b) A schematic presentation of the *in situ* PLA reaction. The proximity probes bind their targets. Proximity sensing is achieved by hybridization of circularization oligonucleotides and their ligation into a circular DNA molecule (reporter of proximity events). PLA signal amplification is obtained by rolling circle amplification of the DNA circle, templated by one of the proximity probes, and subsequent detection of the RCA product with fluorophore-labeled detection oligonucleotides.

threshold to almost zero. The distance between targeted epitopes in the *in situ* PLA method will also be dependent on the size of the antibodies. Alternative affinity reagent may be used to reduce the distance threshold, such as recombinant antibody fragments or designed ankyrin repeat proteins (DARPins) [7]. Most applications using *in situ* PLA are done with species-specific secondary proximity probes [8]. The advantage with such an approach is that one does not need to conjugate every primary antibody, but can use the same proximity probes, targeting, e.g. mouse and rabbit antibodies. The distance threshold will of course be a bit larger as four antibodies will be used.

An *in situ* PLA reaction consists of three types of steps (Figure 1(b)). The first is the targeting of the proteins of interest, i.e. binding of the proximity probes. The second step is the proximity sensing, i.e. if the proximity probes have bound close enough to template the ligation of circularization oligonucleotides into a circular DNA molecule that acts as a reporter of proximity events. The third step is to generate an amplified signal, i.e. hybridization of fluorophore-labeled detection oligonucleotides to the RCA product of the DNA circle. To perform an *in situ* PLA reaction, cells must be fixed. If monitoring intracellular proteins, permeabilization of the cells is also necessary for antibodies and other reagents to enter the cell and recognize their targets. The slide is blocked to reduce nonspecific binding of antibodies and proximity probes, followed by incubation of a pair of primary antibodies. After washes, the slide is incubated with a pair of secondary proximity probes. The slides are washed to remove excess of proximity probes and circularization oligonucleotides are added, which will hybridize to the proximity probes and then ligated together by T4-DNA ligase. Following washes, the ligated circularization oligonucleotides are amplified by Phi29 DNA polymerase and the detection oligonucleotides hybridized to the formed RCA products. RCA can be combined with the detection step, if the detection oligonucleotides are modified to prevent priming. This can be achieved by adding a few mismatched 2´-O-methyl-RNA nucleotides at the 3´end of the detection oligonucleotide. If the detection oligonucleotides were not blocked, they would prime the DNA synthesis and result in a double-stranded product to which the detection oligonucleotides wouldn´t hybridize to. *In situ* PLA may also be combined with other methods, such as padlock probes, to in parallel monitor protein-interactions, or post-translational modifications of proteins, and mRNA expression [9].

Over the years we have developed several variants of *in situ* PLA to solve various limitations, or for different unique applications. We will herein describe the design of some of these.

3. Increasing the dynamic range

As described above, the threshold for detecting a proximity event is a few nanometers, which is far below the resolution of a conventional microscope. However, the RCA product is a much larger molecule. A one-hour RCA will generate an RCA product of around 1 µm in diameter, stained with several hundred fluorophores. The advantage is that it creates strongly stained object, with a defined morphology that makes them easily quantifiable by image analysis software. The disadvantage is that the resolution of the objects monitored will be worse and that there will be a limitation in how many RCA products that can be recorded in an individual cell. As the cells become filled with RCA products, they will merge, making it impossible to count individual RCA products. One solution to the problem was to decrease the size of the RCA products, to be able to fit in more in each individual cell. Decreasing the time for RCA would generate shorter RCA products. However, they would then be less fluorescent as fewer detection oligonucleotides would hybridize to each individual RCA product. To reduce the size of the RCA products, without compromising their fluorescence, we opted to compact the RCA products through hybridization with an additional oligonucleotide (Figure 2(a)). The RCA product of *in situ* PLA is a concatemer of repeats reverse complementary to the circularization oligonucleotides, to which the detection oligonucleotides hybridize. The RCA product, hence, consists mainly of single-stranded DNA. We designed the compaction oligonucleotide [10] to be a dimer of a sequence reverse complementary to the region flanking the hybridization site of the detection oligonucleotides in the RCA product (i.e. the spacer region of circularization oligonucleotide 1), separated by a short spacer region. The compaction oligonucleotide will hybridize to two distal repeats of the RCA product and keep them close together. The compaction oligonucleotide should not prime DNA synthesis. Therefore it has its 3´ end blocked with a few mismatched 2´-O-methyl-RNA nucleotides to avoid priming. Adding the compaction oligonucleotides during RCA reduces the diameter of the RCA products to approximately 200 nm. As the same amount of detection oligonucleotides will bind to the compacted RCA product, there will be a higher local concentration of the fluorophores and the compacted RCA products will appear brighter.

Another approach to increase the dynamic range is to use a combination of alternative circularization oligonucleotide 1, differing only in the spacer element which we replaced with elements for a second detection oligonucleotide [11] (Figure 2(b)). We made three versions of the circularization oligonucleotide 1, containing three different sequences for the second detection element (Element F, G and H), and corresponding detection oligonucleotides labeled with different fluorophores. If the three versions of circularization oligonucleotide 1 are used at different concentrations, the RCA products stained with detection oligonucleotide F, G or H will reflect the ratio between the different circularization oligonucleotide 1. By setting the ratio of circularization oligonucleotide F:G:H to 1:10:100, one can simply enumerate the signal with the optimal number of RCA products, i.e. highest amount of non-coalescing RCA products. If the amount of RCA products reporting with detection oligonucleotide H is too high, i.e. the RCA products coalesce, one can simply count the RCA products reporting with detection oligonucleotide G and multiply those by a factor of ten or multiply the number of RCA products stained with detection oligonucleotide F with hundred.

4. Increasing the complexity

To understand complex cellular events, it can be helpful to investigate multiple protein interactions in parallel. One option would be to design orthogonal *in situ* PLA systems that can be used in combination. One option is to design orthogonal *in situ* PLA systems for combined use. Alternatively, the oligonucleotide design of *in situ* PLA can be modified by incorporating unique

tag sequences in the proximity probe oligonucleotides, serving as barcodes for the generated RCA products [12]. If the detection elements F, G or H are incorporated between element C and D in the proximity probe oligonucleotide 2, these can be conjugated to different antibodies. Such proximity probes can be used to monitor proximity toward a common proximity probe 1 (Figure 2(c)). The proximity probes 2F, 2 G and 2 H will hybridize to their cognate tag oligonucleotide F´, G´ or H´. The circularization oligonucleotides will hybridize to the proximity probes 1 and 2F, 2 G or 2 H. For the pairs of proximity probes bound in close proximity the circular ligation product would hence contain either tag F´, G´ or H´. The different RCA product will be stained with corresponding detection oligonucleotide, providing the ability to monitor alternative protein interactions in parallel. To increase complexity further, one could introduce tag elements in both proximity probe 1 and 2, which would generate RCA product stained by pairs of detection oligonucleotides, identifying the proteins targeted in each recorded proximity event.

All assays shown so far aim at detecting proximity between two proteins, but there are occasions when one would like to visualize tertiary protein complexes. We therefore designed one system that requires three proximity probes to be bound in close proximity to allow the ligation of a circular reporter DNA molecule [1]. In this system we add a third proximity probe, in between the two standard ones. We thus had to replace circularization oligonucleotide 2 with two new ones, i.e. circularization oligonucleotide 3 and 4 (Figure 2(d)). The proximity probe oligonucleotide was designed with a short spacer region at the 5´end and two elements for hybridization to circularization oligonucleotides (Element J and K). This gives us a system where circularization oligonucleotide 1 hybridizes to proximity probes 1 and 2, via element A´ and D´. Circularization oligonucleotide 3 will hybridize to proximity probe 1 and 3, via element B´ and J´, and circularization oligonucleotide 4 will hybridize to proximity probes 2 and 3, via elements C´ and K´.

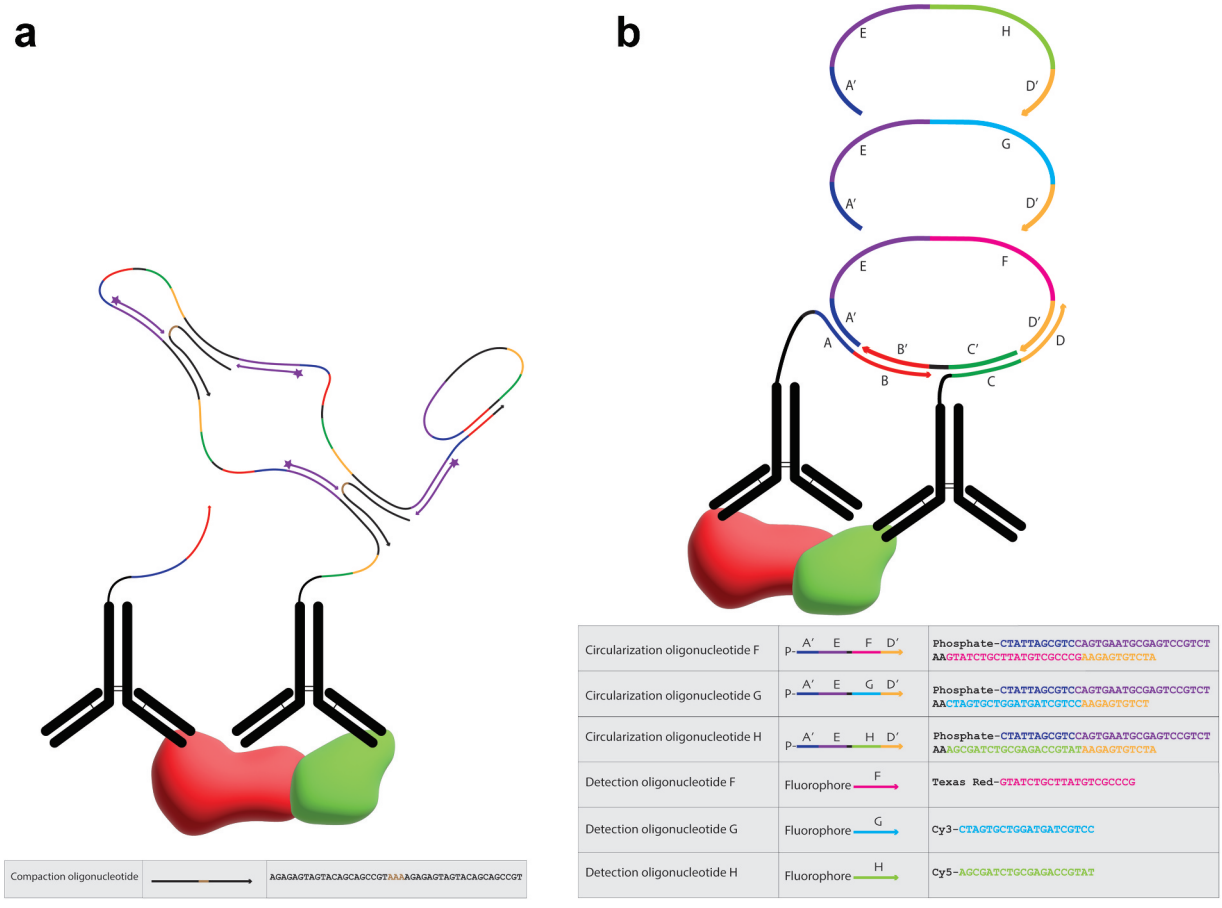
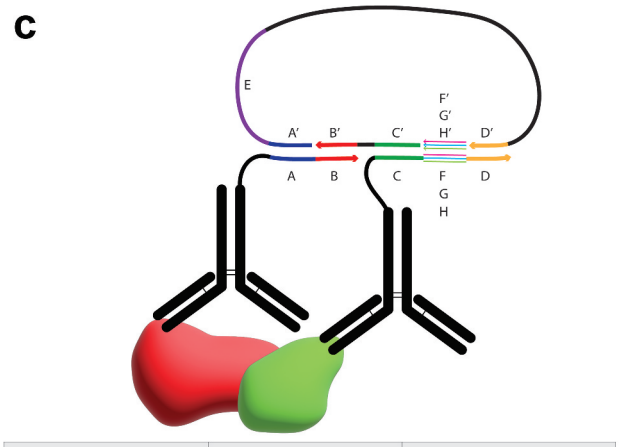
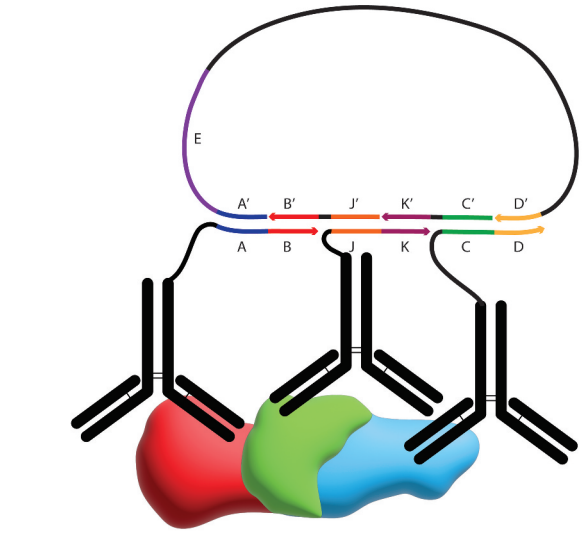


Figure 2. (a) A schematic presentation of the oligonucleotide design for variants of *in situ* PLA. (A) By just adding a compaction oligonucleotide, the RCA product of a regular *in situ* PLA will be condensed into a much smaller object. All other oligonucleotides are the same as in Figure 1. Addition of a three mismatched 2´-O-methyl-RNA at the 3´ends of the detection oligonucleotides and the compaction oligonucleotide is required to perform RCA combined with detection. (b) The circularization oligonucleotide 1 of the regular *in situ* PLA was replaced with three variants, having different detection elements (F, G or H), which can be targeted with the cognate detection oligonucleotide. All other oligonucleotides are the same as in Figure 1. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. (c) A tag sequence (F, G or H) was incorporated into proximity probe oligonucleotide 2 to allow multiplexing. The sequence of proximity probe oligonucleotide 1 and the circularization oligonucleotides are the same as the regular *in situ* PLA (Figure 1), with the addition of the tag oligonucleotides F´, G´ and H´. The DNA circles formed in the proximity sensing will contain the tag sequence corresponding to the specific proximity probe oligonucleotide 2. (d) To detect tertiary proximity events, we utilized the regular *in situ* PLA design, with addition of a third proximity probe oligonucleotide. Circularization oligonucleotide 2 was exchanged with two new ones, i.e. circularization oligonucleotide 3 and 4. All other oligonucleotides are the same as in Figure 1. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´.



Proximity probe oligonucleotide F	Chem linker C F D	Chem linker-AAAAATATGACAGAAC CGGGCGACATAAGCAGATACTAGACACTCTT
Proximity probe oligonucleotide G	Chem linker C G D	Chem linker-AAAAATATGACAGAAC GGACGATCATCCAGCACTAGTAGACACTCTT
Proximity probe oligonucleotide H	Chem linker C H D	Chem linker-AAAAATATGACAGAAC ATACGGTCTCGCAGATCGCTTAGACACTCTT
Tag oligonucleotide F′	p- F′	Phosphate-GTATCTGCTTATGTCGCCCG
Tag oligonucleotide G′	p- G′	Phosphate-CTAGTGCTGGATGATCGTCC
Tag oligonucleotide H′	p- H′	Phosphate-AGCGATCTGCGAGACCGTAT
Detection oligonucleotide F′	Fluorophore F′	Alexa488-TATCTGCTTATGTCGCCCG
Detection oligonucleotide G′	Fluorophore G′	Cy5-CTAGTGCTGGATGATCGTCC
Detection oligonucleotide H′	Fluorophore H′	Alexa555-AGCGATCTGCGAGACCGTAT



Proximity probe oligonucleotide 1	Chem linker A B	Chem linker-AAAAAAAAAAGACGCTAATAGTTAAGACGCTT
Proximity probe oligonucleotide 2	Chem linker C D	Chem linker-AAAAAAAAAATATGACAGAACTAGACACTCTT
Proximity probe oligonucleotide 3	Chem linker J K	Chem linker-AAAAAATGGCCGACTCACGAATTAGAT
Circularization oligonucleotide 3	p- J′ B′	Phosphate-GAGTCGGCCATAGTATCCTTTAAGCGTCTTAA
Circularization oligonucleotide 4	p- C′ K′	Phosphate-GTTCTGTCATATTTATCTAATTCGT

Figure 2. (Continued).

5. Increasing the efficiency

The proportion of potential protein interactions that can be recorded by *in situ* PLA is dependent on the affinity and specificity of the antibodies, as well as the following hybridization and enzymatic reactions. For all antibody-based methods, it is important to be aware of that what we observe is the presence of antibodies which will not always reflect the targeted proteins. All antibodies crossreact with other proteins, to what extent is determined by concentrations and affinity. The quality of the antibody will hence affect the outcome of *in situ* PLA. We have on several occasions failed to develop *in situ* PLA assays against certain proteins. One example for this is ERß that we later could show was dependent on antibody specificity [13]. As stated above, *in situ* PLA requires a pair of proximity probes to bind in close proximity to allow the formation of a circular DNA molecule. So if 30% of epitopes are bound by a proximity probe it will mean that 9% (30% x 30%) of interacting proteins will be bound by pairs of proximity probes. Requiring more proximity probes, as discussed in the example above with three proximity probes, will thus reduce efficiency. Another problem is availability of epitopes, some will be unreachable if they are shielded by protein interactions or antibodies bound to adjacent proteins. Additionally, the epitopes may be altered due to conformational changes of the protein, reflecting e.g. activity states, or due to fixation. The effects will be that *in situ* PLA will only report a fraction of all protein interactions occurring in a cell.

In addition to the antibody part of *in situ* PLA, the method is dependent on how well the oligonucleotides hybridize and the enzymatic reactions. These reactions are rather efficient as DNA hybridization can, depending on the sequence, reach near 100% and enzymatic reactions of ligation (95%-100%) [14] and polymerization are in a similar range. But although proximal binding of a pair of proximity probes is an absolute requirement for the ligation of circularization oligonucleotides into a circular DNA molecule it also produces undesirable linear ligation products. More than two circularization oligonucleotides may hybridize to a pair of proximity probe, or, if the proximity probes have more than one proximity probe oligonucleotide, the circularization oligonucleotides may hybridize to different proximity probe oligonucleotides (Figure 3(a)). To overcome this limitation, we designed an oligonucleotide system where circularization oligonucleotide is connected to proximity probe oligonucleotide 2, with deoxyUridine (dU) incorporated between the segments (Figure 3(b)) [15]. Utilizing the elements from *in situ* PLA design, the new proximity probe oligonucleotide will contain a chemical linker at the 5´end, followed by a spacer region, and elements C-D-U-A´-E-D´-C´-B´. This will ensure that every proximity probe oligonucleotide 2 carries its own circularization oligonucleotide. To activate the proximity sensing, the dU nucleotides are destroyed using a combination of Uracil-DNA Glycosylase (UDG) and Endonuclease IV (Endo IV). This will unfold the proximity probe 2, i.e. release the 5´end of the circularization oligonucleotide and allow it to hybridize to proximity probe 1. The proximity probe thus needs to be designed so that it cannot hybridize to circularization oligonucleotide segment of proximity probe 1 prior to the

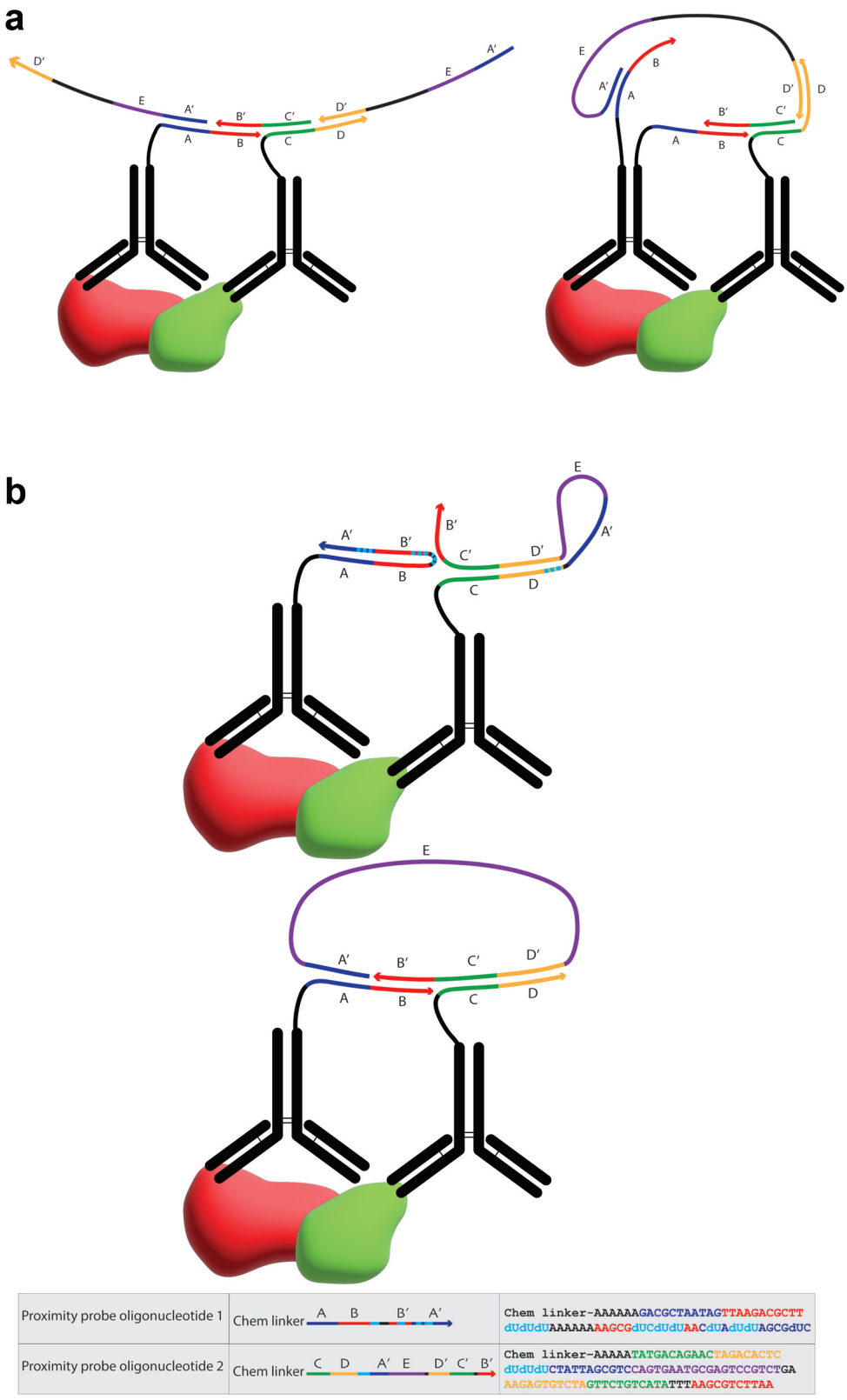


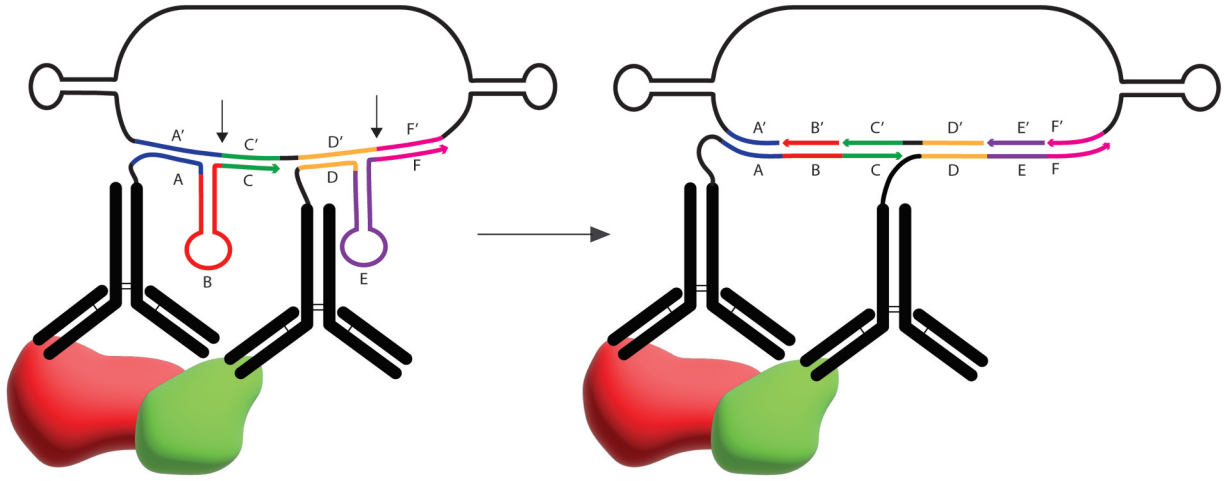
Figure 3. (a) A schematic presentation of the oligonucleotide design to increase the efficiency of *in situ* PLA. (A) A schematic presentation of possible linear ligation products in the proximity sensing step of *in situ* PLA. (b) a schematic presentation of the unfold oligonucleotide design, where the circularization oligonucleotides are incorporated into proximity probe oligonucleotide 2. dU represent deoxyUridine (dU) in the sequence. Proximity probe oligonucleotide 1 was designed as a hairpin to block interactions to proximity probe oligonucleotide 2. Upper panel shows proximity probe binding and lower panel shows proximity sensing after dU residues have been removed by uracil-DNA glycosylase and endonuclease IV treatment. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. The detection oligonucleotide is the same as the regular *in situ* PLA (Figure 1).

proximity sensing step. We have previously used DNA hairpins, where one side contains dU nucleotides, for this purpose [16]. Hence the proximity probe oligonucleotide 1 was designed with a chemical linker at the 5´end, followed by a spacer region, and elements A-B-loop-B´-A´ with several dU nucleotides in the loop region and elements B´ and A´. As such, when UDG and Endo IV are added, the dU nucleotides are destroyed leaving the A-B elements that will function as a ligation template for the circularization oligonucleotide. As for original *in situ* PLA, the method starts with the binding of the proximity probe. Proximity sensing is activated by addition of Uracil-DNA Glycosylase and Endonuclease IV, that cuts the proximity probes, liberating the circularization oligonucleotide of proximity probe 2 and fragmenting the blocking part of proximity probe 1. The circularization oligonucleotide will stay hybridized to proximity probe 2, due to the complementary elements C-D and C´-D´, and its 5´ and 3´end will hybridize to proximity probe 1 via elements A´ and B´ if the proximity probes are in close proximity. Ligation will produce a circular DNA molecule, that will be the template for the subsequent RCA primed by the proximity probes. The difference between this Unfold design and the original *in situ* PLA is that it requires only one ligation event, instead of two, and that as the circularization oligonucleotide already is bound to proximity probe 2 in a 1:1 ratio. This will result in more RCA products compared to *in situ* PLA, most likely due to less formation of linear ligation products.

6. Visualizing proximity and abundance

For analysis of protein-protein interactions it is important to determine the amount of the participating proteins, quantifying both free and interacting proteins (i.e. abundance and proximity). With the different versions of *in situ* PLA that we have developed, there are tools to investigate if proximity probes bind in proximity, reporting the presence of potential protein complexes. However, the method does not give information on the abundance of the interacting proteins and to what extent they interact. It is possible to combine *in situ* PLA, monitoring proximity between proteins, with immunofluorescence to monitor abundance of the participating proteins. With immunofluorescence one does not get the signal amplifications that one gets with *in situ* PLA, so the data would be comparison of fluorescence intensities for immunofluorescence and numbers of RCA products for *in situ* PLA. Another option would be to combine *in situ* PLA with immuno-RCA [3], allowing a similar readout for both interactions and protein levels, as both methods will generate RCA products. This will require that the antibodies will have to be conjugated with different oligonucleotide designs to generate either an immuno-RCA reaction or an *in situ* PLA. If the antibodies are conjugated with individual oligonucleotides, i.e. designed for immune-RCA or the *in situ* PLA, the probes will compete for binding of the proteins. Alternatively, if the antibodies are conjugated with both types of oligonucleotides, the resulting assay may be sensitive to the degree of conjugation of one or both oligonucleotides to the antibodies.

Instead of trying to combine *in situ* PLA with an orthogonal method, we designed a method to produce RCA products where each individual RCA product could be interrogated on whether they were derived from a proximity event or not. The method was named MolBoolean, using the Boolean operators at a molecular level, and is based on the incorporation of tag oligonucleotides into a preformed DNA circle [17]. Free proteins will only allow incorporation of one tag oligonucleotide, while proximity will facilitate incorporation of two tag oligonucleotides. The incorporated tags would be targeted with fluorophore-labeled detection oligonucleotides, generating single-colored RCA products for free proteins and dual-colored RCA products for proteins in proximity. Proximity sensing in the MolBoolean method is done by hybridization of the DNA circle to a proximity probe oligonucleotide. The double-stranded region where the DNA circle binds the proximity probe provides a restriction site for a Nickase (which only will cut one strand, i.e. only cut the DNA circle) and thereby open up for incorporation of a tag oligonucleotide, specific for each proximity probe oligonucleotide. For non-interacting proteins, the DNA circle will only bind one proximity probe oligonucleotide, and hence will a single tag oligonucleotide be incorporated into the DNA circle. For proteins in proximity, the DNA circle hybridized to a pair of proximity probe oligonucleotides, and two tags will be incorporated into the DNA circle. The proximity probe oligonucleotides in Molboolean will hence need to contain elements to provide hybridization to a DNA circle, and a tag element to identify to which antibody it has been conjugated to. The DNA circle needs to be designed so that the binding to a proximity probe oligonucleotide will enable cleavage of the DNA circle and hybridization of a tag sequence, followed by re-ligation into a DNA circle (Figure 4). Proximity probe 1 was designed with a chemical linker at the 5´end, followed by a spacer region, elements A-B-C. Elements A and C was used for hybridization to the DNA circle and element B was the tag sequence. When binding to the DNA circle, element B will loop out, bringing element A and C close together. To be able to cut the circle at a define position, to enable incorporation of tag oligonucleotide B´ into the DNA circle, we used the Nickase Nt.BsmAI that cuts one strand in a duplex at the site GTCTCN*N. As we wanted to cut the circle and not the proximity probe oligonucleotide a reverse complementary sequence of the of the non-palindromic restriction site was introduced in the 5´end of element C. As the Nickase requires a double-stranded template, we designed the proximity probe oligonucleotide so that the last three nucleotides of element A are the same as the last three nucleotides of the 21 nucleotides long element B. The 5´end of element A´ of the circle may thus hybridize to the 3´end of element A or B. To make it more favorable to use the 3´end of element B, we made the nucleotide 15 to 18 of element B reverse complementary to the last 3 nucleotides of element A and the first nucleotide on element B. This will form a short stem region of the proximity probe oligonucleotide, once it hybridizes to the DNA circle. Once the DNA circle is cut by the Nickase, element A will remain hybridized to A´ and element C to C´, with element B being single-stranded in between. The Tag oligonucleotide B´ is then added, and it will hybridize to element B. Proximity probe 2 was designed by the same approach, but with elements D and F for hybridization and element E was the tag sequence, i.e. a chemical linker at the 5´end,



Proximity probe oligonucleotide 1	Chem linker A B C	Chem linker-AAAAAAAAACTCCTGAGGTAGGTAGTTGAGCAG CATCCGCACTTATAGCTGCAGTGAGACGAGTGGACAC
Proximity probe oligonucleotide 2	Chem linker D E F	Chem linker-AAAAAAAAATGAGGTGGAGCATCAGACG GTAATTAACCCGCCCCGTACGAGAGACGTAAGTAGCGTGGCA
DNA circle	P- F′ D′ C′ A′	Phosphate-TTTATCTATATCTGCCACGCTACTTACGTCTCT* CGTCTGATGCTCCACCTCATATATAAATTGTGTCCACTCGTCTCA* CTGCTCAACTACCTACCTCAGGAGAAACCTTTACTT
DNA circle	-P	Phosphate-CGAGGTGCTTTTAGCACCTCGAAGTAAAGCTATCC ACTGTCACCAACTACTAGATAAACGTCACACTTTTCGTGTGACG
Tag oligonucleotide 1	P- B′	Phosphate-CTGCAGCTATAAGTGCGGATG
Tag oligonucleotide 2	P- E′	Phosphate-CGTACGGGGCGGGTTAATTAC
Detection oligonucleotide B	Fluorophore B′	Texas Red-CTGCAGCTATAAGTGCGGATG
Detection oligonucleotide E	Fluorophore E′	Atto647-CGTACGGGGCGGGTTAATTAC
Nickase Nt.BsmAI		GTCTCN*N

Figure 4. A schematic presentation of the oligonucleotide design for MolBoolean. The sequences of the different oligonucleotides are presented in the table below, color coded to identify the different elements. Proximity probe oligonucleotides are presented as conjugated to antibodies. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. The DNA circle is ordered in two pieces that contains complementary elements (the black hairpins of the DNA circle), so that spontaneously hybridize to each other and facilitates ligation into a complete circle. The recognition sequence for the nickase Nt.BsmAI is shown in the table and where it cuts is indicated by (*) in the DNA circle sequence. Once the DNA circle is cut by Nt.BsmAI (position indicated by black arrows) the tag oligonucleotides can hybridize to the proximity probe oligonucleotides. This allows the oligonucleotides to be re-ligated into a DNA circle, that would be the template for RCA.

followed by a spacer region and elements D-E-F. The DNA circle was designed to contain element F´ and D´ with a restriction site for Nt.BsmAI in between, a short spacer region followed by element C´ and A´, also here with Nt. BsmAI site in between. Elements F´ and A´ were connected with a long spacer region. As we couldn´t purchase circular DNA, we designed it so we could do the circularization at the lab. We therefore divided the DNA circle into two fragments, that we could ligate to form a DNA circle. The first DNA circle fragment was designed to hairpin structure at both the 5´and 3´ends, with flanking region reverse complementary to the ends of the second DNA circle fragment. The DNA circle fragments will hybridize to each other, once combined, and be joined into a DNA circle by addition of T4-DNA ligase.

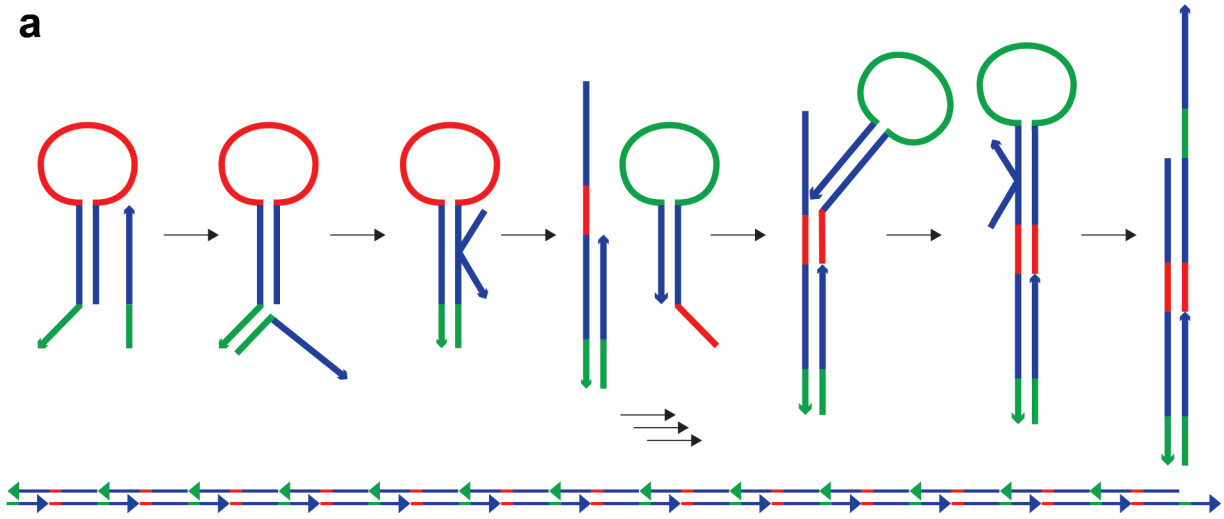
The Molboolean method is performed by letting the proximity probes bind to their targets. In case of secondary proximity probes that means binding to the respective primary antibodies. After washes, the DNA circle is allowed to hybridize to the proximity probes and the Nickase is added. As the Nickase requires a double-stranded DNA restriction site, it will cut the DNA circle only if it hybridizes to proximity probe 1 and/or proximity probe 2. Hence, it will cut bound DNA circle at one or two positions. The tag oligonucleotides (B´ and E´) will hybridize to the proximity probes, in between the

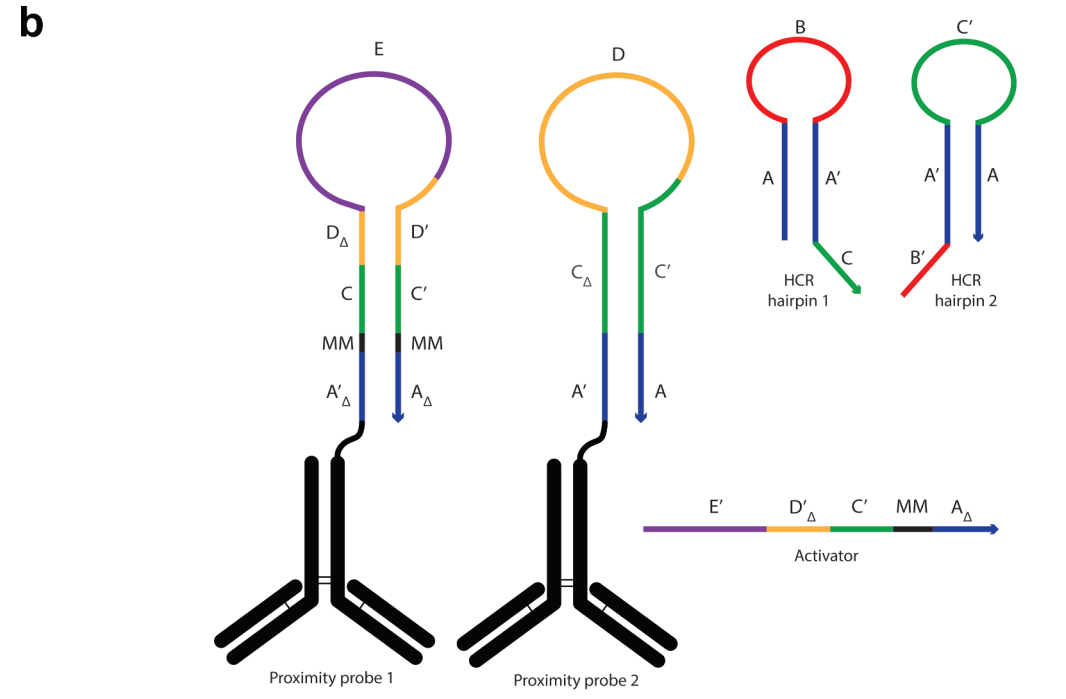
elements used to hold the DNA circle (Figure 4). The oligonucleotides will be aligned and the nicks can then be sealed by addition of a DNA ligase, re-creating the DNA circle with addition of tag B´ and/or E´. Information on which proximity probes the DNA circle has hybridized will thus be incorporated into the sequence of the DNA circle. Proximity sensing is provided by incorporation of both tags into the DNA circle. RCA is then performed, using the re-created DNA circle as a template, and the RCA products will subsequently be labeled with detection oligonucleotides (B´ and/or E´). As the detection oligonucleotides are labeled with different fluorophores, each individual RCA product will be stained by either one fluorophore (detection oligonucleotide B´ or E´) or a combination of both. The dual stained RCA products will report on proximity events, while the single stained for non-proximity events.

7. Enzyme-free detection of proximity

Both *in situ* PLA and Molboolean rely on RCA to generate a fluorescent signal to indicate a proximity event. There are several advantages with RCA such as each individual molecule containing hundreds of fluorophores, making single molecules easily detectable. Furthermore, RCA generates defined objects that can be enumerated. On the other hand, one disadvantage is that the large RCA products distort the resolution of the subcellular location of the proximity event (an uncompacted RCA product has a diameter of around 1 μm). In addition, the methods also use enzymes, which are the reagents that are most expensive in those techniques and put demand on storage at very low temperatures. We therefore set out to develop a method that does not rely on enzymes for fluorescent amplification of DNA. A beautiful oligonucleotide design to produce a long double stranded DNA molecule was developed by the Pierce lab almost two decades ago, called hybridization chain reaction (HCR) [18]. The method is based on the use of DNA hairpins that upon addition of an initiator oligonucleotide will hybridize to each other and form a nicked double-stranded DNA molecule (Figure 5(a)). The two essential hairpins for HCR amplification are designed with a stem region, a loop, and a toehold. HCR hairpin 1 elements are A-B-A´-C, and HCR hairpin 2 elements are B´-A´-C´-A. The initiator oligonucleotide is composed of elements C´-A, or B-A. The single-stranded toehold of HCR hairpin 1, element C, hybridizes to element C´ of the initiator oligonucleotide. Once bound, the element A of the initiator oligonucleotide competes with element A of HCR hairpin 1 for hybridization to element A´ of HCR hairpin 1. When the initiator oligonucleotide is fully hybridized, element B of the now opened HCR hairpin 1 will hybridize to element B´ of HCR hairpin 2 and the HCR hairpin 1 can invade HCR hairpin 2 through competition for hybridization to element A´ of the HCR hairpin 2. This will expose elements C´-A (which is the same sequence as the initiator oligonucleotide) and the chain reaction of hybridization events will continue. More and more double-stranded DNA is hence formed, and the reaction is driven by the reduction in Gibbs free energy this causes as double-stranded DNA is thermodynamical favored over single-stranded DNA. The design of the HCR hairpins requires that they are metastable. This means that they are kinetically hindered and therefore do not hybridize to each other in the absence of an initiator oligonucleotide. There is a delicate weighing involved considering the lengths of the different elements. The stem needs to be long enough to provide a stable hybridization between elements A and A´, ensuring metastability. On the other hand, by having a longer stem the kinetics of the invasion will be slower. The toehold and loop should be short so that they don´t form stable complexes in the absence of an initiator sequence. On the other hand they should be long enough to enable hybridization once the HCR hairpins are opened up [19].

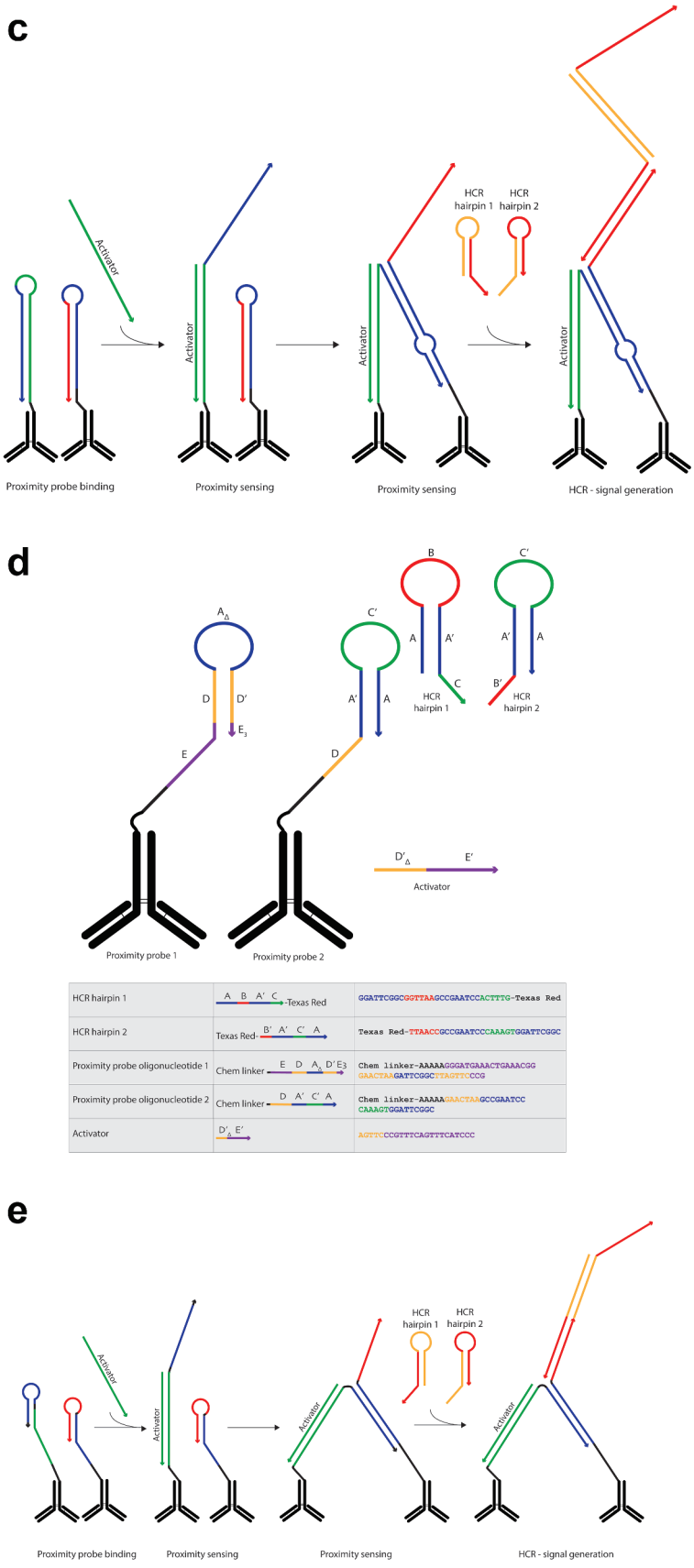
We developed a method based on Hybridization Chain Reaction (HCR) for visualizing protein interactions. This method involves a proximity sensing step to expose the initiator sequence and trigger an HCR reaction, obtained through the proximity-dependent initiation of HCR (ProxHCR) [20]. For the design of the oligonucleotide system, the initiator sequence (elements C´-A) needed to be incorporated into proximity probe oligonucleotide 2. This sequence should only be exposed through an interaction with proximity probe oligonucleotide 1. The proximity probe oligonucleotides have to be designed to prevent them from hybridizing to each other, allowing them to independently bind to their protein targets. Proximity probe oligonucleotide 1 had to be designed so that proximity sensing could be activated, exposing an element that could hybridize to proximity probe oligonucleotide 2. Using these requirements, we designed the proximity probes sequences as hairpins with the initiating sequence hidden in the proximity probe 2 oligonucleotide stem. This would only be opened and exposed via interaction with an invading sequence hidden in the proximity probe 1 oligonucleotide stem. This, in turn, is only exposed once a single-stranded activator oligonucleotide is added. To be more detailed proximity probe oligonucleotide 2 has the elements C´-A at its 3´end. Elements A´-C are therefore included in the 5´end to keep the initiator sequence hidden in a stem region. An element D was included as a loop, serving as the binding site for proximity probe oligonucleotide 1 after activation. As we wanted the proximity probe oligonucleotide 1 to hybridize to elements A ´-C-D of proximity probe oligonucleotide 2, the 3´end will be composed of elements D´-C´-A. Element D would be the toehold for the strand invasion. The 5´end would hence be composed of elements A´-C-D with an element E as loop in between. To activate proximity sensing, the activation oligonucleotide was designed to consist of elements E´- D´-C´-A. The oligonucleotide system next needed to be modified (Figure 5(b)) to avoid that proximity probe oligonucleotide 1 could initiate an HCR and to avoid that the activator oligonucleotide could activate proximity probe oligonucleotide 2. We did this by deleting parts of elements and by introducing mismatches. The sequence of proximity probe oligonucleotide 2 starting with a chemical linker at the 5 ´end was then: a spacer element, element A´, element C_{Δ} (where the 3´end was truncated), element D, element C´ and element A. The loop of proximity probe oligonucleotide 2 consisted of element D and the 5´ end of element C´. The sequence of proximity probe oligonucleotide 1 starting with





HCR hairpin 1	Fluorophore- A B A′ C	Fluorophore-ACAGACGACTCCCACATTCTCCAG GTGGGAGTCGTCTGTAACATGAAGTA
HCR hairpin 2	B′ A′ C′ A -Fluorophore	CTGGAGAATGTGGGAGTCGTCTGTTACTTCATGTT ACAGACGACTCCCAC-Fluorophore
Proximity probe oligonucleotide 1	Chem linker A′Δ C DΔ E D′C′ AΔ (MM, MM)	Chem linker-AAAAATCGTCTGTGGCATGAAG GCCCGCTGTATTCAGTGAATGCGAGTCA GACGAATACAGCGGGCCTTCATGCCACAGACGA
Proximity probe oligonucleotide 2	Chem linker A′ CΔ D C′ A	Chem linker-AAAAAGTGGGAGTCGTCTGT AACATGAAGGCCCGCTGTATTCGTCT TACTTCATGTTACAGACGACTCCCAC
Activator	E′ D′Δ C′ AΔ (MM)	GACTCGCATTCACTGAATACAGCGGGC CTTCATGCCACAGACGA

Figure 5. (a) A schematic presentation of the oligonucleotide design for proxHCR. (A) A schematic presentation of HCR. An initiator oligonucleotide (green and blue) will bind and invade an HCR hairpin. As it opens up it will reveal the initiator sequence (red and blue) to bind and invade an HCR hairpin of the other polarity. As it opens up it will reveal the first initiator sequence (green and blue). The HCR reaction will continue and generate a long nicked double-stranded DNA molecule. (b) A schematic presentation of the oligonucleotide design for proxHCR, where the activator oligonucleotide targets the loop region. The sequences of the different oligonucleotides for proxHCR are presented in the table below, color coded to identify the different elements. Proximity probe oligonucleotides are presented as conjugated to antibodies. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. (c) A schematic presentation of the proxHCR reaction, using the oligonucleotide above. The different elements are not presented in the figure, instead focusing on how the different oligonucleotides bind to each other. (d) A schematic presentation of the oligonucleotide design for proxHCR, where the activator oligonucleotide targets an external toehold. The sequences of the different oligonucleotides for proxHCR are presented in the table below, color coded to identify the different elements. Proximity probe oligonucleotides are presented as conjugated to antibodies. The 3´end of the oligonucleotides are indicated as arrowheads, the sequences are written 5´ to 3´. (e) A schematic presentation of the proxHCR reaction, using the oligonucleotide above. The different elements are not presented in the figure, instead focusing on how the different oligonucleotides bind to each other.



HCR hairpin 1	A B A′ C -Texas Red	GGATTCGGCGGTTAAGCCGAATCCACTTTG-Texas Red
HCR hairpin 2	Texas Red- B′ A′ C′ A	Texas Red-TTAACCGCCGAATCCCAAAGTGGATTCGGC
Proximity probe oligonucleotide 1	Chem linker- E D A_Δ $D'E_3$	Chem linker-AAAAAGGGATGAAACTGAAACGG GAACTAAGATTCGGCTTAGTTCCCG
Proximity probe oligonucleotide 2	Chem linker- D A′ C′ A	Chem linker-AAAAAGAACTAAGCCGAATCC CAAAGTGGATTCGGC
Activator	D'_Δ E′	AGTTCCGTTTCAGTTTCATCCC

Figure 5. (Continued).

a chemical linker at the 5′end was then: a spacer element, element A'_Δ (truncated at the 5′end), element C_{MM} (containing mismatches: the first two nucleotides were replaced with GG), element D_Δ (truncated at the 3′end),

element E, element D′, element C'_{MM} (containing mismatches: the last two nucleotides were replaced with CC) and element A_{Δ} (truncated at the 3′end). The loop of proximity probe oligonucleotide 1 consisted of element E and the 5′ end of element D′. The activation oligonucleotide was modified to bind proximity probe oligonucleotide 1 and was hence: E′- D'_{Δ} - C'_{MM} - A_{Δ}.

As for the previous methods, proxHCR also contains steps of targeting proteins of interest, proximity sensing and signal amplification (Figure 5(c)). The proximity probe oligonucleotides are conjugated to antibodies, and these proximity probes are used to stain fixed cells or tissue sections. Excess of proximity probes are removed by washes and then the proximity sensing is induced by addition of the activation oligonucleotide. This will bind the loop of proximity probe 1 and invade the stem, which will expose the 3′end of the stem of proximity probe 1. If the pair of proximity probes have bound in close proximity, the exposed 3′end will hybridize to the loop of proximity probe 2 and invade the stem. This will expose the 3′end of the stem of proximity probe 2, which contains the initiator sequence, and it will bind HCR hairpin 1 and start the HCR. As the HCR hairpins are fluorophore-labeled it will generate a fluorophore-labeled nicked double-stranded DNA molecule as an extension of the interacted pair of proximity probes.

To increase efficiency of proxHCR we designed an oligonucleotide system [21], were also the proximity probe oligonucleotides had an external toehold rather than within the loop. For this design we aimed to keep the oligonucleotides as short as possible (Figure 5(d)). Proximity probe oligonucleotide 2 was designed with a chemical linker at the 5′end, followed by a spacer region, an external toehold element D, element A′, element C′ and element A. For proximity probe oligonucleotide 1, the chemical linker and spacer region was followed be an external toehold element E (extending three nucleotides into the stem), element D, element A_{Δ} (truncated one nucleotide at the 5′end, the first and last nucleotide now being part of the stem region), element D′ and E'_3 (the three nucleotides reverse complementary to the 3′end of element E). The activation oligonucleotide then was designed to be composed of element D'_{Δ} (the last five nucleotides) and element E′. The assay is performed as above, with the exception that invasion is done via an external toehold (Figure 5(e)).

8. Considerations

All the methods described above consist of three steps: targeting of the proteins, proximity sensing and signal amplification. By scrutinizing how the steps work, one can identify the factors to consider in analyzing the data.

The first step is targeting the protein of interest, which we do by using antibodies. It is important to have in mind that all antibodies crossreact with other protein than the intended. To what degree, depends on affinity and concentration. The paratope of an antibody consists of only a few amino acids [22] and the epitope of the antigen may be shared in parts with other proteins. In addition, the remaining amino acids of the antibody may also interact additional proteins. As for all antibody-based methods, the readout reports amount of bound antibodies and NOT the amount of the targeted protein. However, the data is interpreted as showing the presence of the protein. This is an important distinction that we always need to bear in mind. For all antibody-based method it is crucial to validate the antibodies specificity [13]. For the methods presented herein, the antibodies are modified, by the attachment of oligonucleotides, i.e. proximity probes. For most cases we have used bifunctional linker, that utilizes NHS esters to bind the antibodies. The oligonucleotides will hence be conjugated to different parts of the antibodies, which may interfere with antigen binding. There are several approaches to conjugate antibodies to defined positions, i.e. site-specific conjugation, that can be used to get a controlled conjugation distal from the paratope.

The step of proximity sensing will determine if the proximity probes have bound sufficiently close to allow the oligonucleotides attached to reach from one proximity probe to the other. For *in situ* PLA can a circular ligation product only be created if the proximity probes have bound close enough. For MolBoolean can the incorporation of a dual tag only occur if the proximity probes have bound close enough. For proxHCR will the initiator sequence only be exposed if the proximity probes bind to each other. A positive proximity sensing shows proximal binding of proximity probes and NOT the presence of a protein-protein interaction. However, the data is interpreted as showing the presence of a protein-protein interaction. This is yet another crucial distinction that we need to bear in mind. *In situ* PLA commonly relies on the use of secondary proximity probes. In analogy with immunofluorescence, a control used is omitting the primary antibodies. For both methods will the amount of bound secondary antibodies, or proximity probes for *in situ* PLA, be used to set the background in the methods. For immunofluorescence, a threshold of fluorescence is set and all pixels (or voxels) above are counted as positive. However, there is always some amount of secondary fluorophore-labeled antibodies bound. Also, for *in situ* PLA there will be some amount of secondary proximity probes bound and some of these may bound in close proximity. The proximity sensing will accurately report the amount of proximity events, proximity probes bound with the distance threshold. The problem arises if the user assumes that the method reports protein-protein interactions, and classifies the signals as 'false positive' rather than background antibody binding. A true false positive signal would for *in situ* PLA would be formation of a circular DNA molecule, or RCA product, in the absence of one, or both, proximity probes. The probability that the circularization oligonucleotides will be ligated into a circular molecule, in the absence of a template is very low.

This leads us into the signal amplification. The advantage with RCA is that it creates highly fluorescent objects, that enables visualization of individual molecules. This provides an easy way of quantification; the numbers of RCA products can be counted. For proxHCR the fluorophore-labeled nicked double-stranded molecule will not form as clearly defined objects, so analysis will be based on fluorescence intensity of the cells as for immunofluorescence. The defined RCA products are rather large

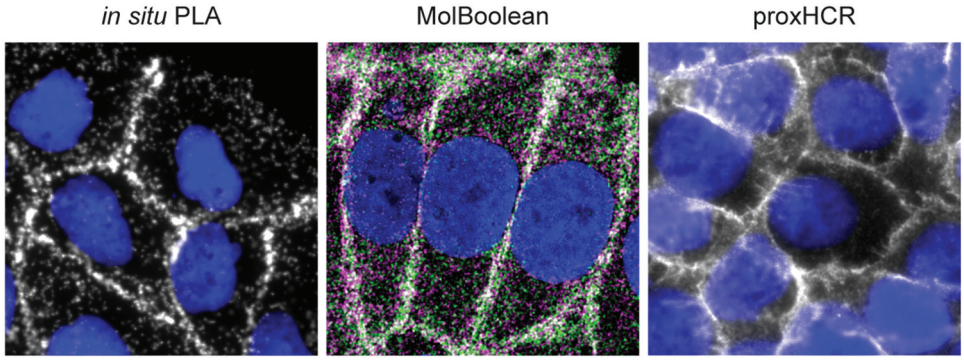


Figure 6. Example microscopy images. The microscopy images show E-Cadherin/β-catenin with *in situ* PLA, MolBoolean and proxHCR staining. Blue shows Hoechst staining of nuclei, white represent proximity events of E-Cadherin/ß-catenin. For MolBoolean, E-Cadherin is shown in magenta and ß-catenin in green. The MolBoolean image is taken by confocal microscopy and *in situ* PLA and proxHCR images with Epi-fluorescence microscopy. Hence, the images have different resolution.

objects, so one does not require advanced microscopy to record single molecules. However, as the RCA products are large the resolution of the protein-protein interaction will be poorer, it may be located somewhere within a 1 µm sphere. HCR-based approaches have the advantage that they provide a better resolution of structures within a cell. It is important to be aware of that both *in situ* PLA and proxHCR will produce a background staining, as the proximity probes bind non-intended targets. For fluorescence measurements one sets a threshold for what is considered background using a technical control of omitting e.g. primary antibodies, and records levels of fluorescence that exceeds the threshold and for RCA-based method one records the numbers of RCA products generated using similar technical controls, and determines the numbers exceeding this. Examples on how microscopy images of E-Cadherin/β-Catenin using *in situ* PLA, MolBoolean and proxHCR look like are shown in Figure 6.

The selectivity and affinity of the antibodies are crucial factors, and the antibodies need to be validated. Even if one will get a positive signal above background with well-validated antibodies, it shows that the proximity probes have bound in proximity. It provides an increased likelihood that the proteins are interacting or participating in a protein complex. In order to determine if two proteins can interact, different types of biochemical methods are required. The power of *in situ* PLA, MolBoolean and proxHCR is that they allow us to monitor possible protein interactions in the complex environment of a cell. The knowledge of potential protein interactions is important in the selection of antibodies to avoid targeting of elements used for protein interactions, as the epitopes will be shielded. Even when highly selective antibodies are used, biological controls, e.g. ligand-induced activation of protein interactions or transfecting cells with competing proteins, are important to validate that the assays are likely detecting protein-protein interactions.

9. Future perspectives

The aim with this review was to provide researchers with a guide of oligonucleotide design and method development, as this is a subject that seldom is addressed. The review also may provide scientists with a deeper understanding on how the methods work. All antibody-based methods report the amount of antibodies bound, and for *in situ* PLA, MolBoolean and proxHCR wether they have bound in proximity, they can never be used as an absolute proof that the protein is present or that proteins interact. But the likelihood that the data reflects that is something we can use to understand biology. There is of course no perfect method, there are always things to optimize. For proxHCR the quality of the oligonucleotides has a great impact on signal strength and leakage [23]. There is still a lot of optimizations and novel designs possible to increase efficiency further and to provide ability for multiplexing. Apart from the designs of our lab presented herein, there are other nice designs that we would like to make the readers aware of [24,25].

Although MolBoolean provides a richer set of data, including also information on non-proximity events, the method requires more sophisticated image analysis. The individual RCA products need to be classified as single or double-stained, which preferably should be done in 3D. An alternative approach, that we yet haven´t tested is to equip the two detection oligonucleotides with short overhangs. These will act as a split hybridization template, that will hybridize to the detection oligonucleotides. This third detection oligonucleotide will be labeled with a third fluorophore. The hybridization will be stabilized if it hybridizes to a pair of detection oligonucleotides, bringing the 5´end of one split hybridization template to the 3´end of its partner. To further stabilize the hybridization, the first two detection oligonucleotides could be ligated together. This will allow non-proximity events to be recorded in be two specific fluorophores and proximity events by a third fluorophore, in addition to the presence of the combination.

Understanding how a method works is important knowledge, when selecting a method to provide information on a scientific subject. All methods have their pros and cons. For analysis of dynamics in living cells, neither of the methods presented herein will work. Better options would be to use FRET-based assays [26] or protein fragmentation complementation assays, such as BiFC [27]. If further enhancement of the resolution is needed, expansion microscopy could be an option [28]. Here the RCA products would be connected to a hydrogel matrix polymerized onto the slide. After digestion of the cellular proteins, the gel can be expanded yielding

potentially a higher resolution. If no available method fits your requirement, try to develop a new one.

10. Conclusions

The herein described workflow for method development, especially oligonucleotide design may be a framework for other researchers to develop new methods. The in-depth description on how the different method work is essential for the understanding of the advantages and disadvantages a method has, which is important in selection of appropriate methods for any research purpose.

11. Expert opinion

Method development is a branch of science that is crucial to make new leaps in science. Novel methods provide researchers with new research tools, opening new avenues of what can be explored. Yet, the creative side of method development is rarely described in literature, how methods are conceived. In this work we describe the approach we have used, which we hope can be an inspiration to those entering the field of method development. All method development starts by the identification of an unmet need, for us the ability to visualize endogenous protein-protein interactions in fixed cells and tissue sections. To both be able to monitor where in a cell a protein-protein interaction occurs, and the amount of interactions. Next, we have to determine what functions are needed for the method and the components needed. Which for us was: targeting the proteins of interest, proximity sensing and signal generation. To do this, we used antibodies for targeting and oligonucleotides for proximity sensing and signal generation. The use of oligonucleotides has the advantage that hybridization is highly predictable, and it can be modeled using computer software. An additional benefit with oligonucleotides is the vast repertoire of modifying enzymes that can cut, ligate, degrade and copy DNA. Proximity sensing can hence be designed, based on oligonucleotide hybridization and subsequent alterations caused by the different enzymes. In the examples herein, we have used the restriction enzyme Nt.BsmAI (Nickase), T4-DNA ligase, Uracil-DNA Glycosylase, Endonuclease IV and Phi29 DNA polymerase. Then we needed to decide on how the oligonucleotides should be designed, and what enzymes would be needed, to perform the functions. When we design new molecular biology methods it is helpful to start by drawing the system and different elements. To visualize how we want the different oligonucleotide elements to hybridize to each other, where the different enzymes should act and how this will provide proximity sensing and signal generation is important to communicate your ideas to your group members, or colleagues. The design always needs some tweaking, so input from others is extremely valuable. Finally, the sequence of the oligonucleotides has to be determined. This can be done using computer software, such as the NuPack software [5], but when we designed most of the methods described herein such software wasn´t available. Instead, the oligonucleotides were designed manually, using a library of random 20-mers. We color code the sequence, as presented herein, to visualize the different elements, and evaluate using *in silico* predictions to determine that no unwanted hybridizations occur and that the intended ones are sufficiently stable at the temperature intended for the method. After this the oligonucleotides are ordered from a vendor and tested in the lab. Usually, it takes several rounds of re-designing to end up with a design that performs as intended, it is an iterative process of designing, testing and evaluating performance. The detailed description on how the different methods work will facilitate a deeper understanding of what conclusions can be drawn from experimental data and the advantages/disadvantages the different methods have.

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Declaration of interest

O Soderberg is inventor on several patents covering the methods described herein, variants of *in situ* PLA, UnFold, MolBoolean and proxHCR. BK is inventor of the patent for proxHCR.

Reviewer disclosure

One reviewer on this manuscript received an honorarium from Expert Review of Proteomics for their review work but have no other relevant financial relationships to disclose. The remaining reviewers have no other relevant financial relationships or otherwise to disclose.

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