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Review Article

Proximity assays for sensitive quantification of proteins

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ABSTRACT

Proximity assays are immunohistochemical tools that utilise two or more DNA-tagged aptamers or antibodies binding in close proximity to the same protein or protein complex. Amplification by PCR or isothermal methods and hybridisation of a labelled probe to its DNA target generates a signal that enables sensitive and robust detection of proteins, protein modifications or protein–protein interactions. Assays can be carried out in homogeneous or solid phase formats and *in situ* assays can visualise single protein molecules or complexes with high spatial accuracy. These properties highlight the potential of proximity assays in research, diagnostic, pharmacological and many other applications that require sensitive, specific and accurate assessments of protein expression.

Contents

1. Introduction

The remarkable advances made over the last fifty years or so in all areas of the life sciences, medicine, diagnostics, forensics and biotechnology are inconceivable without the contributions from two key technologies: the polymerase chain reaction (PCR) for the detection of nucleic acids and antibody-based methods for the detection of proteins.

- PCR is typified by its exquisite sensitivity and simplicity of use, for example the ease with which specific primers can be synthesised and modified. These properties have led to the widespread use of PCR and its complement, reverse transcription (RT)-PCR, for the analysis of mutations, SNPs and DNA methylation, the analysis of gene expression, as well as a pervasive presence in diagnostic assays aimed at identifying pathogens [1]. The introduction of real-time quantitative PCR (qPCR) [2–4], which uses fluorescence to detect PCR amplicons provided a simple and reproducible method for the detection of nucleic acids and, crucially, affords the very large dynamic range required for accurate quantification of mRNA.
- Antibodies are characterised by their diversity, specificity and ability to bind to target epitopes in complex biological samples such as serum and whole cell lysates. They are used in a wide range of immunoassays, *e.g.* the enzyme-linked immunosorbent assay (ELISA) [5], which measure signals emanating from the affinity interactions of antibodies with their target molecules. Antibodies are also an essential component of flow cytometry, which allows the analysis of the expression of cell surface and intracellular molecules, characterisation and definition of different cell types in heterogeneous cell populations, assessment of the purity of isolated subpopulations, and analysis of cell size and

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volume. This has enabled the detailed study of cellular protein expression, location, modification and interaction [6], the discovery of protein biomarkers in serum and plasma for diagnostic applications such as early detection and monitoring of disease [7] and the rapid and specific detection of pathogen-specific proteins [8] together with the emergence of antibody-derived drug-conjugate molecules as promising next generation therapeutics [9].

The ever-increasing availability of new antibodies continues to expand the potential of the immunohistochemical repertoire. At the same time, there has been a continuous stream of improvements and novel developments of nucleic-acid detection methods, including the emergence of isothermal amplification methods such as rolling circle amplification (RCA) [10]. The combination of these technologies, leading first to the development of immuno-PCR (iPCR) and, more recently proximity ligation (PLA) and extension (PEA) assays, couples the detection specificity of the antibody with the amplification power of PCR or RCA. This arsenal is beginning to provide researchers with a powerful tool for the detection and quantification of cellular, pathogen and GMO-specific proteins as well as diagnostic biomarkers [11]. This emergence of proximity assays into the main stream of proteomic research is reflected in the number of papers citing the technology, which have increased fourfold between 2010 and 2014 from 41 to 156, with 55 papers already published in 2015.

2. Immuno-PCR

The original iPCR, which was first described in 1992 [12], involved amplification of a biotinylated, linear plasmid DNA linked to antigen/monoclonal antibody complexes immobilised on microtiterplate wells through a streptavidin-protein A chimera (Fig. 1). This modification significantly enhanced the sensitivity of an equivalent ELISA, permitting the detection of as few as several hundred targets by means of ethidium bromide-stained agarose gel electrophoresis. Additional changes created a more universal iPCR by substituting the fusion protein with commercially available biotinylated secondary antibodies, thus circumventing the variability and lack of specificity associated with the use of protein A [13]. Although assay throughput and sensitivity was increased further when readout by gel electrophoresis was replaced with fluorogenic PCR-ELISA [14], iPCR still required time-intensive and laborious post-PCR analysis. This was addressed by using qPCR to detect antigen/antibody complexes, which simplified iPCR by reducing the number of handling steps and, crucially, increased the dynamic range of the assay [15,16]. Eventually, the most advantageous assay format was identified as consisting of a sandwich assemblage: a capture antibody is adsorbed directly to the surface of a PCR plate well, sample and detection antibody, which is coupled to a DNA-label, are premixed and transferred to the PCR plate [17]. At the time, the marker DNA was covalently coupled to the antibody, but since the covalent conjugation of oligonucleotides to antibodies can be difficult and time consuming, this has now been largely replaced by a combination of biotinylated antibodies and streptavidin-linked oligonucleotides. Today, iPCR in its various manifestations has become a robust method that provides the specificity and sensitivity required *e.g.* for assessing the success of novel drug design [18] or measuring the pharmacokinetics [19] and toxicokinetics [20] of drug metabolism. It has also been used for the detection of protein biomarkers of cancers [21–25] and viral infectious agents [26,27]. Chimera Biotec (http://www.chimera-biotec.com) is the best-known provider of iPCR-based assays and assay development services with numerous applications targeting many kinds of macromolecular analyte.

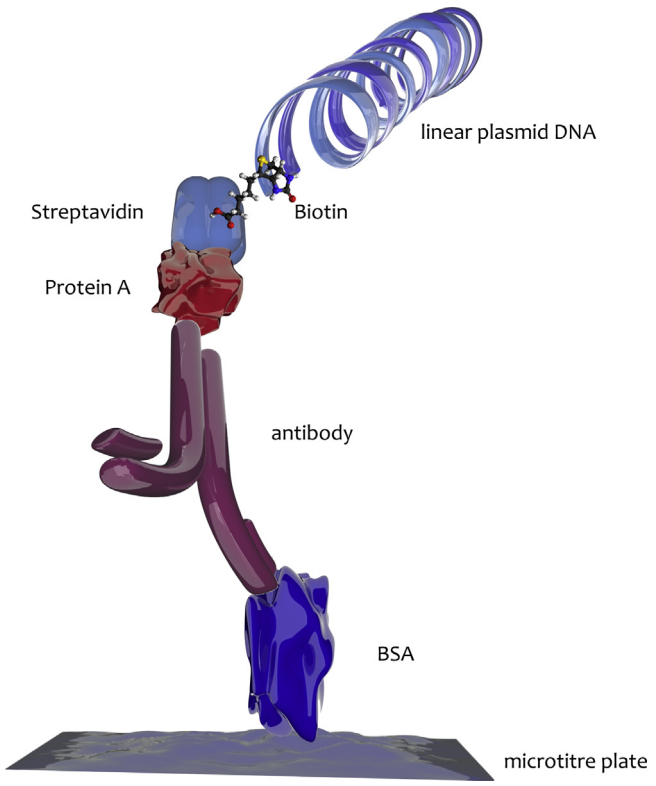

Fig. 1. The original iPCR made use of a recombinant streptavidin-protein A chimera with bispecific affinity for DNA and antibodies to link linear plasmid DNA to an antibody specific for bovine serum albumin (BSA), which was immobilised on the surface of microtitre wells. Binding of the antibody to BSA resulted in a specific antigen–antibody–DNA conjugate that was detected by agarose gel electrophoresis after PCR amplification with plasmid-specific primers.

3. Proximity ligation assays

Arguably the main drawbacks of iPCR are its non-homogeneous nature, which requires extensive washing steps to ensure minimal background signal. Proximity assays address this issue and the first of these, PLA, was first demonstrated in 2002 [28]. At first, PLA made use of two DNA aptamers [29], which bind their targets with affinities and specificities that are comparable to those of monoclonal antibodies [30] and can be designed so that they only require a single epitope on a protein surface [31]. However, difficulties with aptamer design and the availability of a vast pool of commercial antibodies has resulted in antibody-based PLAs becoming the most popular way of implementing this assay [32]. Today, the most common method uses two antibodies, with the requirement for a dual binding event making a false positive result less likely and thus reducing background noise.

PLA probes are assembled either through noncovalent attachment of biotinylated oligonucleotides to streptavidin and subsequent interaction of that complex with biotinylated antibodies [33] or, more commonly today, through generation of oligonucleotides covalently attached to streptavidin at either their 5′- or 3′-ends, allowing them to interact directly with biotinylated antibodies. PLA can use either monoclonal or polyclonal antibodies, as well as a combination of the two. There are two alternative approaches for detecting the antibody/antigen interaction: one uses direct primary antibody conjugation (Fig. 2A), the other indirect scheme uses a secondary antibody linked to DNA for detection (Fig. 2B). It is also possible to conjugate oligonucleotides directly to Fab fragments, which improves the dimensional detection limit of PLA [34]. At its simplest, a single biotinylated monoclonal antibody can be divided into two groups for conjugation with a 5′- or 3′-oligonucleotide,

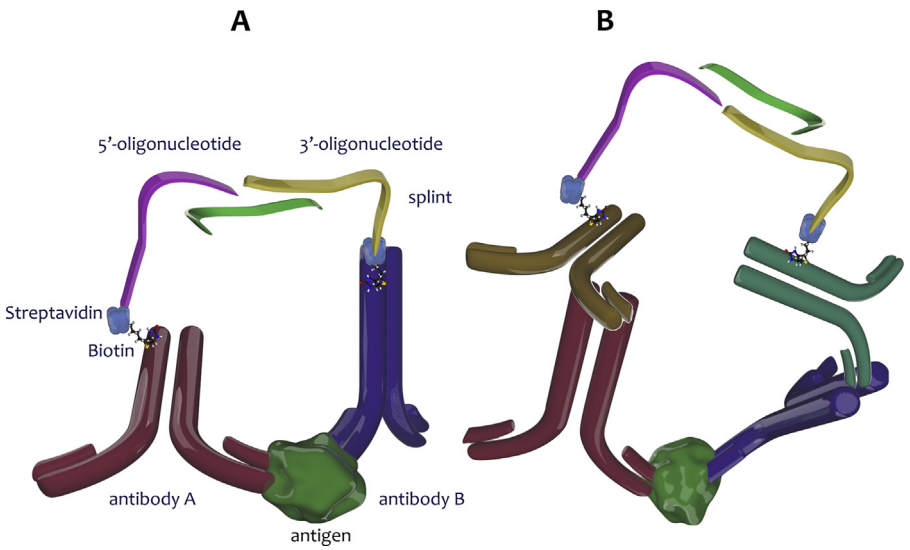

Fig. 2. Direct and indirect PLA. (A) Biotinylated antibodies bind pairwise to adjacent epitopes on target proteins. This brings the two streptavidin-oligonucleotide tails, one coupled through its 5′-end, the other through its 3′-end, into close proximity. The connector oligonucleotide (splint) hybridises to both oligonucleotides, resulting in adjacent free 3′-OH and 5′-phosphate moieties. The gap is ligated and the resulting continuous DNA strand can be amplified and detected. (B) The indirect form of PLA follows the same principle, except that unmodified primary antibodies are detected with secondary antibodies that are conjugated to the DNA strands.

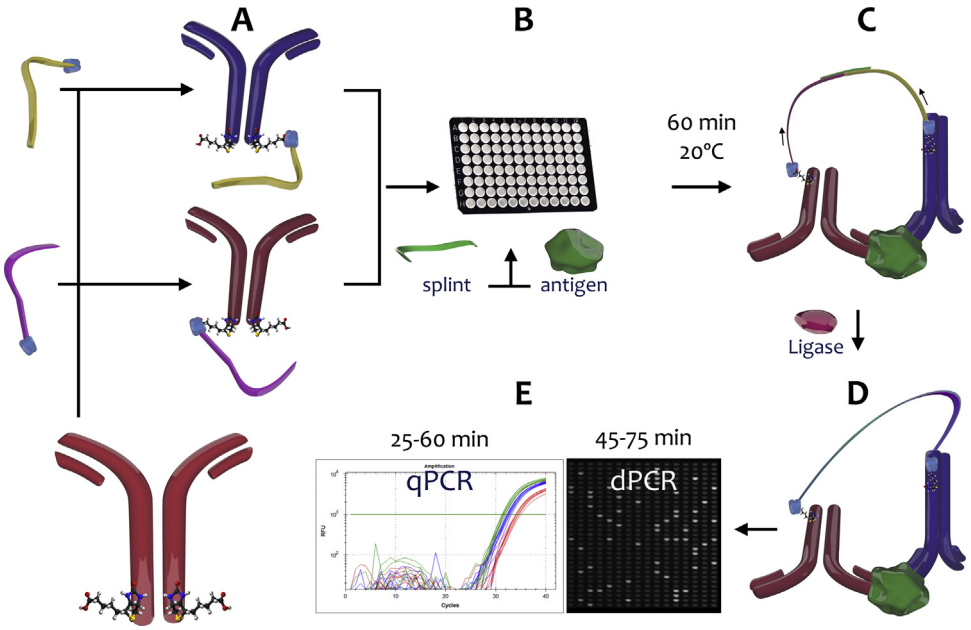

Fig. 3. PLA workflow. (A) Oligonucleotides synthesised with a streptavidin molecule at their 5′- or 3′-ends are combined with biotinylated antibodies to form proximity probes. (B) Probes, sample and splint are combined and both probes bind simultaneously to their epitopes on the target antigen, if present, and the 5′- and 3′-oligonucleotides are joined by the splint. (C) DNA ligase connects the gap and thus joins the two oligonucleotides. (D) This generates a full length DNA amplicon that can be amplified and detected by several methods. (E) Detection by qPCR or dPCR is shown.

respectively (Fig. 3A). This design is applicable to antigen targets assembled in multimeric formats such as protein homodimers and repeated motifs expressed on the surfaces of virions. The subsequent workflow is both simple and rapid and following a 60 min annealing of antibodies, antigen and splint in a microtitre plate (Fig. 3B), a brief 10 min ligation (Fig. 3C) generates amplifiable DNA templates (Fig. 3D) that can be detected using a number of different readout formats. Fig. 3E shows results obtained using a standard qPCR thermal cycler (CFX Connect, Biorad, Hercules, CA, USA) or a digital PCR (Constellation, Formulatrix, Bedford, MA, USA) setup. In addition, flow cytometry [35], loop-mediated isothermal amplification [36] or DNA sequencing [37,38] have also been employed. The indirect form of PLA follows the same principle, except that unmodified primary antibodies are detected with secondary antibodies that are conjugated to the DNA strands.

The intra-assay coefficients of variation (CVs) with qPCR as readout range from less than 10% to greater than 30% [39]. This has been improved by the development of a digital PLA (dPLA) based on amplified single molecule detection, which shows significant improvements in precision and detection sensitivity over qPCR readout [40]. The dPLA workflow is the same as that for standard PLA up to and including the ligation step. Instead of PCR amplification, two oligonucleotides complementary to either end of the single stranded oligonucleotides connecting the two antibodies are added, generating two restriction sites. Following digestion, released DNA strands are circularised *via* a second DNA ligation

reaction and the circularised reporter DNA molecules are amplified, in this case by RCA. Amplification products form random coils, which after are detected by hybridisation with fluorescence dye labelled probes and counted using a dedicated microfluidic detection instrument.

PLAs can be carried out in solution as a homogeneous assay, which has the advantage of minimising operator intervention, obviating the requirement for washes and hence facilitating maximum speed. Like immuno-PCR, PLA can also be configured in a solid phase format using an immobilised capture antibody, with two proximity probes detecting captured target molecules [41], an approach that may be more suitable for detecting protein directly from biofluids such as blood or faeces (Fig. 4A). Configuring the assay through the binding of three independent affinity reagents to the same target molecule can further enhance specificity of signal generation [42]. In this triple antibody specific proximity ligation assay, the third proximity ligation probe replaces the connector oligonucleotide as a ligation template. Short blocking oligonucleotides prevent ligation in the absence of the target molecule and considerably minimise background noise.

Another proximity-mediated detection system, *in situ* PLA, can detect and visualise target proteins and protein complexes expressed by fixed cells and on tissue slide sections [43,44]. *In situ* PLA uses RCA to amplify ligation products, which are generated through the ligation of two connector oligonucleotides to the two oligonucleotides conjugated to the antibodies. This results in the formation of a circular, single stranded DNA molecule, with one of the antibody-conjugated DNA molecules serving as a primer for the RCA. Following the addition of a DNA polymerase, a long DNA product is formed that remains covalently attached to one of the PLA probes. Consequently, a concatameric repeat of the same sequence aggregates in submicron spots, generating discrete, localised signals after hybridisation of fluorescence-labelled oligonucleotide probes complementary to the RCA product. Signals can be detected and digitally counted using a standard fluorescence microscope [45–49]. The reaction is so efficient that care must be taken when using RCA in a quantitative manner due to the possibility of non-linear saturation of the RCA signal [50].

Multiplex PLAs have been developed, where the ligation of numerous PLA probes linked to different combinations of 5′- and 3′-oligonucleotides results in unique sequences that serve as primer sites for target-specific amplification and quantification by qPCR [51]. This has been extended so that four 24-plex panels profiling 74 putative biomarkers can be analysed with high sensitivity, yet using very low levels of sample [52]. Kits for carrying out homogeneous PLAs are commercially available from Life Technologies, Carlsbad, CA, USA, those for *in situ* PLA from Olink Bioscience, Uppsala, Sweden or Sigma–Aldrich, St. Louis, Mo, USA.

4. Proximity extension assays

PEA is an alternative to PLA and was developed because proximity probes joined by a DNA ligase suffer from recovery loss in complex biological fluids [53]. The main difference between the two is that in a PEA the ligation event is replaced by a DNA polymerisation step. In its original design, one of the PEA probes consisted of a double stranded-oligonucleotide attached to the antibody at its 3′-end, with a nine nucleotide or so 3′-overhang at its 5′-end. This overhang was complementary to the 3′-end of the oligonucleotide bound to the other antibody partner (Fig. 4B). Following incubation of the proximity probes with a sample containing antigen recognised by the probes, the overhanging 3′-end could hybridise to the 5′-oligonucleotide and, following the addition of a DNA polymerase, the free 3′-OH was extended in the 5′–3′ direction towards the attachment site of the 5′-oligonucleotide. This generated a full-length amplicon and hybridisation site for the upstream primer and thus allows for the amplification and detection of the target antigen by PCR. This arrangement has now been replaced by a modification, where each of the two single-stranded oligonucleotides contain a complementary site for pair-wise annealing with the other oligonucleotide, allowing extension by a DNA polymerase [54]. This obviates the requirement for a double-stranded oligonucleotide with a 3′-overhang (Fig. 4C). Furthermore, judicious choice of DNA polymerase allows minimisation of background noise and so improves the sensitivity of the assay [54]. PEA is amenable to multiplexing [54,55] and features the same advantages of PLA, including very low sample consumption, high sensitivity and specificity detection in a homogeneous reaction [7]. For maximum sensitivity, PEA requires the use of DNA polymerases with intact 3′–5′ exonuclease as they reduce background noise by degrading non-proximal DNA strands. Kits for carrying out PEAs are commercially available from Olink Bioscience, Uppsala, Sweden.

5. Applications

The ultra-low sample requirement of homogeneous PLA and the ability of solid phase PLA to investigate larger sample volumes has made this technology a useful tool for a wide range of proteomic studies [56]. PCR-based assays only detect nucleic acids and cannot reliably provide information about the relationship between RNA and cellular protein levels, the extent of post-translational modifications or protein/protein interactions. This is important, since transcriptome and proteome are in a constant state of dynamic flux involving the transcription or degradation of RNAs and the translation, modification, interaction and turnover of proteins. This results in a complex, variable and sample-dependent [57] relationship between mRNA levels and protein expression, making extrapolation of changes in RNA levels to those of proteins difficult. Indeed, somewhere between 30 and 70% of the variability in protein levels can be explained by concordance to mRNA levels [58,59] and translation efficiency turns out to be the best predictor of protein levels [60]. This challenge is not confined to nuclear genes, as mitochondrial proteins can also show significant upregulation in the absence of any change in mRNA levels [61]. Furthermore, correlations are very much gene-dependent and are substantially stronger for genes involved in the maintenance of cellular processes and structural properties compared with regulatory genes [60].

PLA encourages an integrated approach for measuring relative changes in miRNA, mRNA and protein expression from the same starting sample and on a single analytical platform [62]. This makes it a useful method for the validation of potential biomarkers for clinical diagnostic needs, for example by simplifying the analysis of cellular protein/protein interactions [63,64], screening for inhibitors of such interactions and posttranslational modifications [65,66] or the interaction of cellular and viral proteins [67]. PLA is finding increased use for cancer biomarker profiling, where its multiplexing capabilities [51,68] allow the simultaneous analysis of tens of proteins at a time [69] and make possible the parallel visualisation of multiple protein complexes in tumour cells [70,71]. It provides an ultrasensitive assay for the detection of PSA [72] and has been used for the *in situ* quantification of EGFR receptor dimerisation and activation [63]. PLA has identified functional differences between different mutations, which may help with the development of mutation-specific targeted therapies [73]. Specifically, its ability to identify mutant EGFR dimer configurations that can evade blockade by anti-EGFR treatment may permit a more accurate patient selection for EGFR-targeted treatment in glioblastoma multiforme, the most common primary brain tumour [74]. In breast cancer, it has been used to demonstrate that elevated levels of HER2:HER2 and HER2:HER3 [75] as well as protein kinase

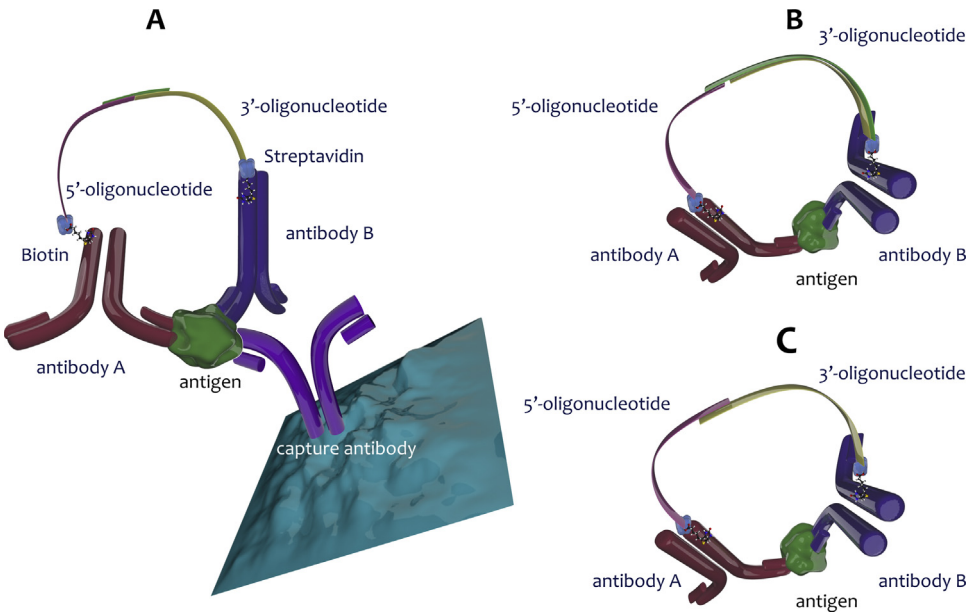

Fig. 4. A. Solid phase PLA. (A) Capture antibody immobilised in a microtitre well binds target antigen, with unbound particles and other sample components removed by washes. Proximity probes are then added to the well and a PLA is carried out. (B) First generation PEA. The 3′-oligonucleotide is double stranded with a 3′-overhang. In the presence of target antigen, the probe oligonucleotides can hybridise to each other, leading to the extension of one oligonucleotide into a DNA template that can be detected and quantified. (C) Second generation PEA. Single stranded oligonucleotides hybridise directly to each other, with one becoming extended by a DNA polymerase to generate an amplifiable DNA template.

6:HER2 complexes [66] are significantly associated with differences in overall patient survival. Furthermore, specific interactions between Smad proteins and AP-1 transcription factors activate a programme of TGFβ-induced breast cancer cell invasion [76], with Akt activation identified as an important driver of progression [77]. Interestingly, measuring activated forms of Akt by *in situ* PLA does not correlate with phosphorylated Akt or the Akt isoforms as measured by IHC, suggesting that the isoform-specific PLA assays are providing additional clinically relevant information. PLA is showing similar promise for the molecular dissection of a number of other cancers, including lung [78,79], colorectal [80] and prostate [81] cancers. There are fewer publications describing the use of PEA, but this assay has been used to identify five plasma protein biomarkers associated with colorectal cancer, with three of them additionally found to be discriminators of early-stage cancer [55].

Pathogen detection is another application for proximity assays, suggested by the original proof of concept [41]. PLA has been used for bacterial [82,83] and viral [84,85] pathogen detection, identification of bacterial spores [86] as well as analyses of mechanisms underlying viral pathogenicity [87] and may have a role in the rapid identification of pathogens in environmental samples for biosecurity applications [88]. Proximity assays may be of particular interest for the development of assays targeting infectious fungal diseases, bloodstream and nosocomial infections, which are associated with high rates of morbidity and mortality. Early treatment is the foundation for successful disease management, yet its diagnosis is challenging due to the limitations of the methods currently used. Since pathogens are most effectively eliminated in the earliest stages of infection, there is an urgent need for early detection of infectious microbes. Current immunodiagnostic methods, *e.g.* ELISAs and lateral flow devices (LFD) lack the sensitivity for earliest possible diagnosis. PCR is more sensitive, but the detection of microbial DNA does not definitively prove the presence of a viable microorganism causing a given infection. Furthermore, there are contamination issues with PCR-based assays, not least because PCR reagents are produced in bacteria and fungi and may contain microbial DNA impurities. Proximity assays targeting proteins expressed only during active growth would provide functional information and allow earlier, clinically more relevant detection than ELISA or LFD but without the disadvantages associated with PCR-based detection [89].

6. Conclusions

Proximity assays expand the range of DNA amplification applications to include the sensitive, accurate and robust identification of proteins through the amplification of a surrogate DNA template after antibody binding [62]. This provides a variety of novel approaches for the direct detection of proteins, with the homogenous format of PLA allowing specific detection and quantification from unfractionated cell lysates and biofluids while *in situ* PLA provides a powerful means of localising proteins and interrogating protein/protein interactions. Today, most proximity assays make use of the extensive collection of commercially available antibodies, although advances in the selection and production of aptamers are likely to see their use increase in future [90,91]. This is going to increase the attractiveness of proximity assays for use as tools for next generation pathology surveillance [92], live cell imaging [93], unravelling the details of cellular migration characteristic of embryogenesis and cancer metastasis [94] as well as protein biomarker validation [95] essential for the implementation of personalised medicine [96]. Furthermore, there is an obvious application of proximity assays for the detection of pathogens where the detection of nucleic acids by itself does not indicate viability or ability to invade [89]. Finally, at this early stage it is also important to consider benchmarks for assay standardisation, reproducibility and transparency of reporting [11] analogous to the MIQE guidelines [97]. In conclusion, proximity assays provide an integrated approach to the measurement of changes in gene and protein expression, protein modification and interactions from the same starting sample and readouts on a single analytical platform.

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