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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.

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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.

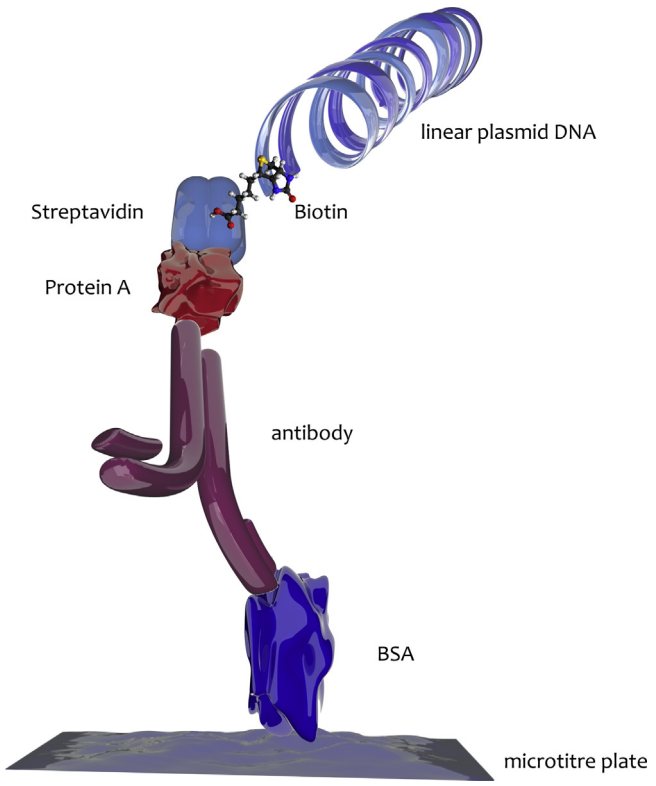

Fig. 1. The original iPCR made use of a recombinant streptavidin-protein A chimera with bispecific affinity for DNA and antibodies to link linear plasmid DNA to an antibody specific for bovine serum albumin (BSA), which was immobilised on the surface of microtitre wells. Binding of the antibody to BSA 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,

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