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Multiplex immunoanalysis

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SUMMARY

Multiplex analysis enables a simultaneous determination of multiple targets in one sample. This approach has been largely adopted in genomics and progressively expands to various domains of bioanalytics. In protein analysis, immunoassays play a fundamental role and their multiplexing and miniaturization is of great applicability to both basic and applied research. Furthermore, these high-throughput methodologies have a considerable potential in the field of laboratory diagnostics. The following text describes planar and bead-based arrays, two main strategies of immunoassay multiplexing. Principles, detection methods and strengths of each are shortly discussed. Finally, we mention several challenges linked with the integration of these methods to diagnostics.

Key words: immunoassay, multiplex analysis.

SOUHRN

Vostrý M.: Multiplexní imunoanalýza

Multiplexní analýza umožňuje současné stanovení více analytů v tomtéž vzorku. Tyto postupy, původně rozšířené především v genomice, nyní významně pronikají i do dalších oblastí bioanalytiky. V analýze proteinů hrají zásadní roli imunochemické metody a jejich multiplexace a miniaturizace je široce využitelná jak v základním, tak v aplikovaném výzkumu. Také možnosti uplatnění těchto vysokokapacitních metod v laboratorní diagnostice jsou značné. Text popisuje dva hlavní přístupy k multiplexní imunoanalýze – provedení v planárním a suspenzním uspořádání. Krátce diskutuje principy, detekční metody a přednosti obou těchto strategií. V závěru jsou zmíněna některá úskalí spojená s integrací multiplexní analýzy do diagnostiky.

Klíčová slova: imunoanalýza, multiplexní analýza.

Introduction

Immunoassays allow for sensitive and specific detection of various target structures in complex biological samples. Since their introduction in early 1960s (RIA) or 1970s (ELISA) [1], immunochemical methods have become an indispensable analytical tool in wide range of applications including clinical diagnostics. ELISA is invariably considered the gold standard for single protein measurement. Annual sales for immunoassay reagents and supplies are $7 billions worldwide [2]. Although the first steps towards immunoassay miniaturization were done already in the 1960s [3], it was only recently that technology (e.g. microfluidics) and informatics enabled a real high-throughput immunoanalysis. In 1989, Ekins described microarray technology principles in the ambient analyte theory and envisaged the immense potential for research and laboratory diagnostics [4]. Today, the increasing awareness of the multifactorial nature of various diseases and pathological states (e.g. cancer, sepsis) really calls for simultaneous, time-saving and cost-effective measurement of multiple analytes. This multi-marker strategy could be then translated into more robust diagnostic algorithms, better-fitting prognostic models and more effective population screening. Of note, multiplex immunoanalysis is perfectly suited for diagnostics of immune system disorders (autoimmune diseases, allergies). Widely used in basic research, the multiplex methodologies are slowly penetrating the *in vitro* diagnostics market and the time of their significant implementation is probably about to come.

Currently, two main streams of multiplex immunoanalysis exist – planar microarrays (protein chips) and bead-based microarrays (suspension arrays). These approaches vary greatly in many aspects, but primarily in the way of the individual analyte identification (position vs. bead characteristics).

Planar microarrays

Microarrays are highly miniaturized and parallelized assay systems in which the capture molecules are immobilized in microspots to a solid support. In a protein chip, in general, spots (< 300 µm) are arrayed with a density of < 2000/cm^2. Current nanotechnology enables the fabrication of highly dense protein nanoarrays (< 10^6 spots/mm^2) suitable for the whole proteome profiling [5]. Arraying technology, widely established during the genomic era, has thus expanded far beyond DNA chips.

Actually, proteins are the executive power of the cell. Their levels do not always correlate with appropriate mRNA levels and they often exist in various functional states. Thus, protein chips (analytical or functional) supplement the genomic data and provide further insight in the complex biological processes. However, several difficulties related to the nature of proteins had to be overcome before a successful construction of protein microarrays. In contrast to nucleic acids, no simple principle of capture agents design is available. The binding of proteins depends on complex tertiary

structure and is highly affected by the post-translational modifications. The immobilization to the solid support (as well as direct labeling) may alter the antigenic properties. Therefore, each capture molecule must be individually screened for affinity, selectivity and cross-reactivity. Glass slides (used in DNA arrays) have low binding capacity for proteins and multiple surface treatment options have been adopted to enhance it (hydrogel, aminosilane, dendrimers, aldehyde activation etc.). Specific binding systems may also be used to fix the capture molecule (streptavidin – biotin, His-tag/nickel chelate). Membranes (e.g. nitrocellulose, poly-lysine) guarantee a high binding capacity as well.

The most common capture reagent is an antibody. Monoclonal antibodies provide standard, specific binding and unlimited supply but their development is costly and time-consuming. *In vitro* display techniques (phage display, ribosome display) enable an efficient screening of binding properties and creation of large recombinant antibody libraries. Non-protein binders (e.g. aptamers) and protein scaffolds (e.g. affibodies) are novel and promising classes of binding molecules [6].

Protein expression level may vary widely (~10^6 fold). Thus, the ideal detection method should offer an adequate sensitivity and the largest detection dynamic range possible. Chemiluminescence is a cost-effective solution providing a very high sensitivity. As for the glass slides, fluorescence is most commonly used. The fluorophore (frequently Cy3, 5 or Alexa dyes) can be directly labeled onto target proteins or coupled to a second detection antibody (sandwich format). Sandwich assays achieve higher sensitivity and specificity and are the method of choice for low abundance proteins. On the other hand, this format is not favorable for large-scale multiplexing (> 50 targets) due to an increased risk of cross-reactivity. Signal amplification methods such as tyramide amplification or rolling circle amplification are available for an additional sensitivity increase. Finally, label-free detection methods, surface plasmon resonance imaging (SPRI) for example, can be used in the microarray setting.

Protein chips are applied for the simultaneous detection of multiple targets, but also for their functional analysis. Post-translational modifications, protein-protein or protein-DNA interactions can be tracked. This has a substantial importance for drug discovery. In reverse-phase arrays, multiple samples are arrayed onto the chip and then analyzed for a single target structure. Several diagnostic arrays are commercially available [3]. Usually, (auto)antibodies, tumor, cardiac or infectious disease markers are the targets of interest. Many companies produce protein or peptide microarrays for research purposes (www.biochipnet.de).

Bead-based arrays

In the bead-based arrays (suspension or liquid arrays), capture molecules are immobilized to a microsphere and captured analytes are detected mostly using the flow cytometry principle. Utilization of microspheres as the solid support is not new. The application potential of differently sized beads coated with antigens has already been described about thirty years ago [7]. Later in early 1980s, microparticles were used for human IgG quantification [8]. As the number of discrete particle sizes distinguishable by a flow cytometer is limited, in the 1990s, different approaches to multiplexing have emerged (optical, graphical, electronic encoding) for different platforms (flow cytometry, fluorescence microscopy) [7, 9]. The Luminex® xMAP® (Multi Analyte Profiling) system is a well-known and popular bead-based flow cytometric platform dedicated to multiplex analysis. In its basic setting, it uses 5.6 µm polystyrene particles incorporating two fluorophores in 100 different ratios. This is the theoretical limit of multiplexing. The technology offers a robust and very flexible way to multiple analytes determination. Many domains of application exist since various capture molecules can be fixed to the particle surface (proteins, peptides or nucleic acids). Thus, immuno-, enzyme-, receptor-ligand or DNA/RNA assay may be performed [10].

The immunoassay protocol is usually similar to a classic sandwich ELISA protocol. Filter bottom microplate and a vacuum washing system are required. However, the use of magnetic beads enables the automation of washing steps as well as construction of fully automated analyzers. During the read-out, microspheres are aspirated, transported to the cytometer (fluorometer more precisely) and subjected to excitation by two different laser beams. The first laser (red) excites the internal fluorophores and allows decoding the spectral address and hence the analyte measured. The second laser (green) excites the fluorophore bound to the reporter molecule, phycoerythrin, and enables quantification. Laterally diffracted light is measured to detect and exclude bead doublets. Usually, 100 beads of each type are analyzed before skipping to the next well. This is in contrast to only a few spot replicates in planar arrays. In general, particles provide a large analytical surface (~10^6 capture molecules per bead for Luminex) and „near-solution" kinetics. However, at least for immunoassays, bead-based arrays are only favorable to a lower number of targets simultaneously analyzed.

Luminex is an open technology and many companies are licensed to produce proprietary analyzers and reagent sets (www.luminexcorp.com). Huge amounts of different analyte combinations are available. Bio-Rad has developed BioPlex™ 2200 system, a fully automated analyzer handling magnetic particles. The ANA Screen panel for this instrument is coupled with medical decision support software relying on multi-analyte pattern recognition. Luminex has recently added a third internal fluorophore and the new FlexMAP 3D™ system offers a higher degree of multiplexing (500 analytes theoretically) and a higher throughput.

Many studies have compared the analytical characteristics of Luminex assays with conventional ELISAs, especially in the field of cytokines determination. In spite of mostly high correlations, poor concurrence

of quantitative results is often reported. Nevertheless, when comparing identical reagents (antibodies, diluents etc.), similar results are achieved [11].

Another flow cytometric multiplex platform comes from BD™ Biosciences. Their Cytometric Bead Array (CBA) relies on different intensities of a single internal fluorophore. Favorably, it can be performed on a standard flow cytometer. Yet, the company offers also a highly flexible machine – FACSArrayTM Bioanalyzer. Prefabricated kits or individual beads (Flex Sets) may be purchased.

Conclusion

The modern and rapidly evolving technologies of protein chips or bead arrays have already shown their capabilities in research laboratories. Their potential for clinical diagnostics is evident. They could provide a cost-effective solution to the omnipresent demand for productivity increase. The aspect of minimum sample volume requirements is also important. Several challenges however have to be addressed before their wider implementation. The validity and a clear contribution to therapeutics have to be proved before being FDA-approved or CE-marked. Questions of standardization, quality control system and health insurance companies' reimbursement persist. The enormous amount of data resulting from laboratory "globalization" must be properly treated in the sense of statistical evaluation, clinical interpretation and regulatory aspects. If proved beneficial and efficient in the clinical setting, these novel analytical tools will substantially change the current system of laboratory testing.

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