

REVIEW

Antibody-based Proteomics: From bench to bedside

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Over the past 75 years, antibodies have gone from being recognized as disease biomarkers to being used as very powerful therapeutic tools. This evolution has been accelerated by the identification of mAb and the extensive use of immunological tools both at fundamental and clinical levels. In this review, we evaluate how antibodies can be used to assess the proteome of cells or tissues and their relevance for clinical applications. These antibody-based proteomics approaches also require analytical and technological pipelines as well as specific enabling tools which are described. Our first objective was to establish how large-scale datasets (provided by high-throughput studies such as proteomics and transcriptomics) can be integrated with literature searches and clinical data to identify potentially relevant markers against which antibodies should be raised. Then based on an extensive literature review and our experience, we compare the methodologies developed to produce specific antibodies either *in vivo* or *in vitro*. This is followed by the description of the validation tools currently available and it also includes the use of antibody-based approaches in the establishment of molecular signatures utilized at the bench and soon available for bedside use.

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1 Introduction

For the last 75 years, antibodies have been used in everyday life sciences research and in clinical settings as specific diagnostic tools and/or more recently in a battery of different therapeutic strategies. This has been rendered possible through a series of technological and methodological advances beginning at the end of the 1940's with the introduction of Freund's adjuvant [1], a powerful activator of the host immune system. Shortly thereafter, this was followed by the development of experimental procedures depending on the

specific protein antigen-binding nature of antibodies such as hemagglutination and immunoprecipitations [2, 3], immunofluorescence and immunomaging [4], ELISA and related immunoassorbant methods [5, 6], Western blotting [7], and protein arrays [8]. Recent years have seen the discovery and market introduction of other protein-binding molecules (aptamers, phage libraries, single chain Fv, *etc.*) claiming to be specific, quicker to generate and to circumvent some of the limitations associated with "conventional" antibody production. In this review, we will at first describe the basis on which "target antigens" – worth raising antibodies to (*e.g.*, biomarkers) – are selected (Fig. 1.1). Then as illustrated in Fig. 1.2, *in vivo* versus *in vitro* methodologies for the production of antibodies are presented. Finally, the application of these antibodies in the clinic is reviewed either at the level of detection (diagnostics) or as therapeutic tools (Fig. 1.3). This

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Abbreviation: scFv, single chain variable fragment

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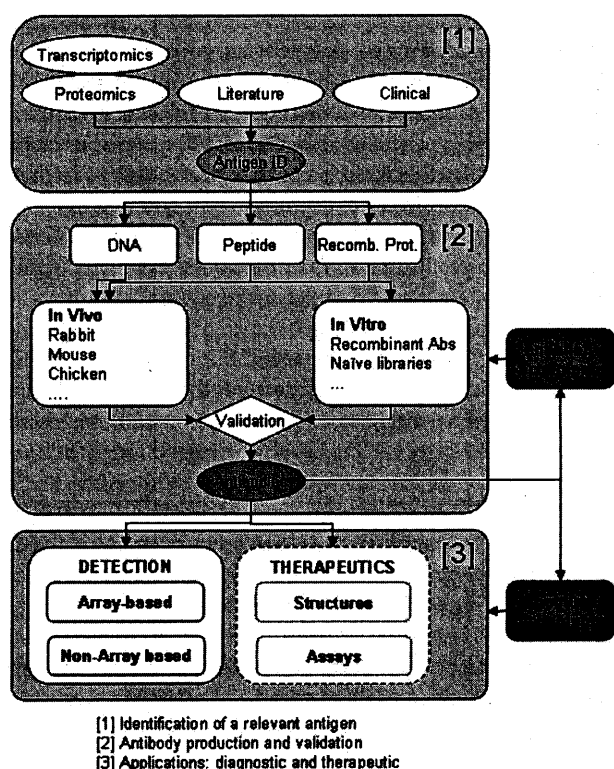


Figure 1. Schematic representation of experimental antibody-based analytical pipeline. The first step of our pipeline resides in (1) antigen identification, selection, and design, and this is followed by the second phase of (2) antibody production *in vivo* or *in vitro*. Finally, once antibodies are produced and characterized they can be used for (3) detection/diagnostics/prognostics and possibly for therapeutics.

review has for objective to cover a large spectrum of applications of antibody-based proteomics approaches at both fundamental and clinical levels. Although we do not develop in depth each aspect of the approaches described, our intention is to provide a global overview of both strategies and experimental designs involved in this process.

2 Antigen identification and selection

In the course of the last 50 years, clinical diagnostic tests have successfully made use of over 100 blood proteins as indicators of various pathologies. To complement this list, a battery of additional markers have been investigated as evidenced in part by the doubling in the number of studies in the last decade which aimed at identifying novel biological markers for disease. The vast majority of those studies have pursued various genomics approaches, using DNA- or mRNA-based methodologies, or proteomics technologies, identifying novel proteins by MS and/or protein arrays. DNA arrays and other molecular biology tools have been key to the identification of dangerous pathogens clearly laying the

ground work for their current and continued use as routine diagnostics (for review see ref. [9]). DNA/RNA microarrays have also been used to identify differential mRNA expression between a number of normal and disease conditions. Their use, though informative, has limitations in reliability since the analysis is restricted to changes in mRNA levels which do not necessarily reflect changes in protein expression. For example, mRNA levels and protein expression of *S. cerevisiae*, cultured in rich or minimal medium, weakly correlated (correlation factor of 0.45 for 678 loci) with each other, and indeed a negative correlation was reported for the expression of selected genes associated with stress response [10]. Similar results were found in mammalian cells where mRNA levels (up or down) correlate at most 40% with the variation in protein expression in two hematopoietic cell lines at distinct stages of myeloid differentiation as well as in the livers of mice treated for different periods of time with three different peroxisome proliferator-activated receptor (PPAR) agonists [11]. Moreover for diagnostics, noninvasive procedures based on the collection of biofluids for biochemical analysis or for various imaging methods, making the link between the expression of an mRNA, which is intracellular, and the expression of the protein it codes for extracellularly in blood, urine, or other fluids is not so straightforward and indeed requires substantial investment in comprehensive (and costly) validation efforts.

Proteomics-based approaches using MS or protein arrays on the other hand allow for the direct analysis of biological fluids not only for the presence of various proteins but also for specific protein modifications and metabolites. And as for transcriptomics, proteomics approaches have technical issues of their own. While protein arrays (described in more detail below) measure the differential expression of known proteins, protein modifications or metabolites, MS, which can identify novel protein targets is limited in its ability to quantify protein expression changes. To address the latter, several methods have been adopted to measure differential expression such as isotope-coded affinity tags (ICAT) [12] which rely on the selective conjugation of cysteine thiol groups in proteins, followed by enzymatic digestion and quantitative analysis of peptide conjugates by MS, *in vacuo* isotope-coded alkylation technique (IVICAT) [13], a methylation reaction adding a permanent positive charge at the N-terminus of peptides, MS peak height [14] or even the more controversial peptide counting methods [15].

Known biomarkers such as cytokines, growth factors, and intracellular proteins, thought to arise in blood plasma by tissue leakage, abnormal cell secretion, alternative cleavage, or unusual modifications, are typically present in minute ng/mL quantities in blood, essentially at the very limit of detection of mass spectrometers. Thus in highly complex samples such as plasma, high abundance proteins which take over the sequencing capacity of the mass spectrometer are best removed prior to injection to obtain any meaningful data. Indeed in efforts to “dig deeper” and improve detection of low abundance markers, various affinity columns have

been developed, and are available commercially, to deplete blood plasma of Igs, albumin and other high abundant proteins of low interest; fractionation procedures have been implemented to proteomics pipelines to decomplexify cellular/tissue samples toward the identification of proteins for use in diagnostics analyses and/or immunotherapeutics purposes. Multiple studies have demonstrated the feasibility of these techniques for the identification, both of serum biomarkers identification in early detection of breast, ovarian and prostate cancer [16–18], and molecular markers of tumor invasion identified from invasive bladder cancer tissue biopsies [19].

3 Antibody production

When producing antibodies various factors warrant mindful consideration with regards to the system of choice, being either *in vivo* or *in vitro*. The latter will be discussed in a later section (see Section 3.2). *In vivo* approaches necessitate selection of the best adjuvant, the animal host, the antigen of interest, the monoclonal or polyclonal nature of the antibody desired as well as for clinical purposes, a definition of optimal strategies to produce large volumes of reproducible, specific antibody.

3.1 Antibody production *in vivo*

3.1.1 Animal hosts

Since Freund's pioneer work more than 100 adjuvant preparations have been described [20]. Adjuvants effectively increase the host immune response through one or more of the following modes of action: "depot effect", antigen presentation and targeting, humoral system activation, and cytotoxic lymphocyte induction. Rabbits, rats, or mice are the most frequently used hosts in the generation of antibodies for experimental and clinical research. Although horses have been used for rabies antiserum production as an essential method for treating people exposed to rabies [21, 22], larger mammals (*i.e.*, goat, sheep, and donkey) have also been used, most frequently for the generation of secondary/complementary antibodies requiring significantly higher production volumes owing to their widespread use as routine experimental tools for bioresearch. More exotic systems are available – and recently increasingly in higher demand – to work with antigens harboring characteristics that prevent or limit the production of antibodies in conventional mammalian hosts.

We make use of chicken hosts to generate high quality antibodies. One of the many advantages of using hens is the phylogenetic distance with mammals that allows for the production of antibodies against highly conserved sequences among mammals (*i.e.*, extracellular domains of G protein-coupled receptors, transporters, *etc.*). This approach allows the production of antibodies with enhanced recognition-,

binding-, and affinity-related properties above that observed in antibodies raised in conventional mammalian hosts. Immunization of hens represents a sensible alternative to generate polyclonal antibodies, because chicken housing is inexpensive, egg collection is nonstressful to hens, isolation and affinity purification of antibodies (IgY) is fast (~2 months), consisting of a simple two-step process involving delipidation followed by immunoaffinity isolation. This is in contrast to production in mammalian hosts in which antiserum must be harvested either by bleeding the animals or for larger volumes by cardiac puncture under terminal anesthesia; both of which must be carried out by trained staff and typically cause significant anxiety to the animals. While representing a practical source of antibodies, chicken eggs are a clean, noninvasive means by which large volumes of antibody can be repeatedly harvested. Notably, this method is in agreement with the European Union Directive 86/609/EEC for the protection of animals used for experimental use requesting methods reducing the suffering of animals.

Although IgYs are not recognized by protein A or G, their applicability is comparable to that of mammalian Igs – an increasing supply of "secondary" tools (untagged or tag-labeled antichick antibodies) are commercially available. In our experience generating custom chicken antibodies to purified proteins, recombinant proteins or to peptides, the signal is very strong and specific for concentrations ≤ 0.1 – $2 \mu\text{g/mL}$ of total IgY, or even for 1:2000 dilutions of straight yolk. Furthermore, the IgY has a number of intrinsic biochemical advantages: the fact that IgYs do not activate mammalian complement and show no interaction with mammalian Fc receptors, which could mediate inflammatory responses in the gastrointestinal (GI) tract, makes IgY antibodies very attractive immunotherapeutics for peroral (oral) administration of specific antibodies against GI pathogens in both humans and animals as well as for antibody-based diagnostics. Other clinical IgY applications, such as inhibition of xenograft rejection, are also being developed for pig-to-human organ transplants [23]. Considering the benefits of IgY methodologies in polyclonal antibody production – including the massive quantities (100 mg total IgY *per* egg; 2–10 mg of specific IgY *per* egg) that can be produced – and the universal application of these antibodies in research and medicine, it is expected that in the near future IgY technologies will play an increasingly greater role in research diagnostics and immunotherapy.

3.1.2 Source of antigen for immunization

The easiest method to produce polypeptide antigens for immunization involves the cloning and expression of the chimerical polypeptide of interest with GST, maltose-binding protein (MBP), or another purification system. Following expression of the protein antigen, protease-specific cleavage site(s) are used in the final purification step to generate the highly purified desired antigen. Bacterial expression, being the most widely used expression system, however is limited

in that no PTMs are added to synthesized proteins, and transmembrane proteins can only be expressed without their hydrophobic membrane-spanning domains. Proper folding of proteins can also be affected (by the latter bacterial system shortcomings) effectively limiting the possibility to generate antibodies recognizing the protein in its native conformation. Mammalian or insect cell expression systems can be used as alternatives to resolve some of the issues associated with bacterial systems.

In cases where antibodies against peptides or modified peptides are required, coupling to a carrier is necessary to induce a humoral (antibody) response. That being said, the use of peptides as antigens should nevertheless be used cautiously since they have lower probabilities of being antigenic and antigen determinant prediction softwares have limited capabilities in the identification of antigenic peptides; 50–60% correct predictions when hydrophobicity and accessibility are used and up to 70% when prediction for turns in protein structure are taken into consideration [24]. Alternatively, and more successfully, animals can be immunized using the whole protein and domain-specific antibodies can be purified through affinity purification using the polypeptide sequence of interest. Using similar approaches, specific antibodies can be screened using peptides of interest during the generation/selection of a mAb.

Antibodies can also be generated by DNA immunization. This approach has the advantage of bypassing antigen (protein or peptide) expression and purification and thus allows for fast-tracking antibody production. Recently, limitations in the clinical use of this approach has been put forward due to poor antibody production and more importantly its inability to work well in primates and humans (for review see ref. [25]).

A polyclonal humoral response is comprised of antibodies derived from various clonal populations having varying specificities (for different epitopes on the same molecule), affinities, and classes. Polyclonal antiserum can be obtained in a relatively short time frame (2–4 months) in contrast to standard mAb production procedures that can be quite labor intensive and require 4–8 months. Polyclonal antibodies (pAb) show different affinities for different epitopes and thus may demonstrate overall excellent binding achieved by adherence to a number of different sites on a complex immunogen or antigen. MAb on the other hand will recognize a specific antigen with constant biochemical properties. Moreover through simple cloning and molecular engineering modifications of the Ig sequence, antibodies can be custom designed for specific uses (e.g., humanization to prevent/limit immunoreactivity in the course of immunotherapy). Chickens are commonly misbelieved to be limited to producing only polyclonal antibodies, however, hybridomas expressing monoclonal IgYs against human prion proteins previously have been generated by fusion with a myeloid cell line [26]. The instability of hybridomas and the low levels of secretion require cloning of the IgY mRNA and the use of other expression systems for efficient production.

Production systems of mAb by *in vitro* culture of hybridomas can consist of either one of two established culture systems: the first is made up of a single compartment, and consequently allows only low-density cell culture; the second system is made up of two compartments that allow high-cell density growth. For production of mAb on a small scale (<0.1 g), simple low-density cell systems such as tissue culture flasks and gas permeable bags can be easily employed. For production of larger quantities, in the 0.1–0.5 g range, high-density cell culture systems are often required. All production systems mentioned above are dependent on intrinsic characteristics of hybridomas (medium, growth conditions, etc.) and require highly controlled environments [27, 28].

3.2 Antibody production *in vitro*

In vitro, antibody production methods can be used to identify binding partners as alternatives to antibodies for low antigenicity peptides. The first recombinant antibody fragment reported to be expressed in the yeast *P. pastoris* was a rabbit single chain variable fragment (scFv) selected from a bacterial phage display library [29]. scFvs contain heavy and light chain variable regions connected by a small, flexible peptide; these fragments can be modified – disulfide-stabilized scFv's and bivalent scFvs – to enhance their stability or avidity, or to add further specificities or effector functions [30]. Furthermore, owing to their reduced size (27 000–30 000 M_r) scFvs can penetrate tissues and are cleared from the blood more rapidly than whole antibodies rendering them well suited for many *in vivo* diagnostic and therapeutic applications.

Recombinant antibody libraries have been constructed by cloning antibody heavy- or light-chain variable genes directly from animal or human lymphocytes and then expressing them as an scFv or as an antigen-binding fragment (Fab) using various display technologies [31–33]. Recombinant antibody technology, an alternative to traditional immunization of animals, facilitates the isolation of target-specific high affinity mAb, requiring no immunization, simply by virtue of high-throughput screening techniques. Phage display, ribosome display, and microbial cell display platform technologies have been extensively used to construct large scFv or Fab libraries [34–36].

In phage display libraries, an antibody fragment is expressed on the surface of a phage particle, its encoding gene is fused in-frame to one of the phage coat proteins and cloned in a vector that can be packaged as a phage particle (for review see ref. [37]). Different display systems can lead to monovalent or to multivalent display, which is convenient for selecting antibodies of higher affinity, achieved by using direct fusion or a disulfide-bridged link to a minor coat protein, pIII, and by using phagemids into which antibody libraries are easier to clone than phage vectors [38].

In ribosome display, the link between antibody and encoding mRNA is made by the ribosome; at the end of translation the mRNA is made to stop without releasing the

polypeptide and the ternary complex as a whole is used for the selection [39]. In mRNA display, there is a covalent bond between antibody and mRNA established *via* puromycin as an adaptor molecule [40]. These display methods are carried out entirely *in vitro*, thereby eliminating the need for cell transformation. The other advantage is that it is very amenable to mutagenesis to provide additional diversity between generations, without the need to transform the cloned library into *E. coli*.

Finally, in yeast cell display libraries, antibodies are displayed on the yeast *S. cerevisiae* cell surface *via* fusion to the α -agglutinin yeast adhesion receptor, which is located on the yeast cell wall [34]. Although the display level on the cell is irregular, with on average about 3×10^4 fusions *per cell per* scFv molecule, cell sorting “sorts” through this variability. By staining the cells with both fluorescently labeled antigen and/or anti-epitope tag reagents, the yeast cells can be sorted according to the level of antigen binding and antibody expression on the cell surface. This approach has been used to build a nonimmune human scFv library with over 10^9 clones, which is efficiently selected by magnetic bead capture and then sorted by flow cytometry to yield single nanomolar-affinity antibodies [34].

Despite the recent availability and market introduction of these protein-binding molecules shown to be specific and quicker to generate, these also on occasion have been demonstrated to be difficult to work with and to be of relatively low affinity (10^{-6} – 10^{-8} M compared to 10^{-9} – 10^{-11} M for conventional antibodies) requiring additional efforts of genetically fusing binding molecules to multimerization domains (e.g., fusing Jun to produce dimeric Fv fragments) [41]. Random or targeted mutation approaches by error-prone or window PCR, DNA shuffling, for example, have also been used successfully to increase the effective avidity of binders [42–45]. Indeed a benefit (results)-to-cost analysis should be evaluated before selecting conventional antibodies or protein-binding technologies.

Nucleic acid aptamers have also been developed as an alternative to conventional antibodies. These DNA- or RNA-based molecules have the recognition properties of antibodies and can be *in vitro* selected through rounds of binding/amplification cycles called systematic evolution of ligands by exponential enrichment or SELEX. Aptamers have the advantage of being chemically synthesized so they can easily be modified to improve their properties, and are fairly small compared to peptide-derived binders (for review see ref. [46]).

Clinical applications of recombinant antibodies often require large quantities of highly purified molecules, sometimes for multiple treatments. The development of very efficient expression systems is thus essential for the full exploitation of recombinant antibody technologies. Indeed, production of recombinant protein in the milk of transgenic dairy animals has been pursued for which several successfully expressed antibodies have been harvested from the milk of mice, rat, and goat transgenics [47].

Previous work suggests that plants will be a simple and economic bioreactor for the large-scale production of industrial and pharmaceutical recombinant protein [48, 49]. Genetically modified transgenic plants have several advantages as sources of protein over human or animal tissues, transfected animal cell lines, transgenic animals, or recombinant microbes, including: (i) the production of material on an agricultural scale at low cost; (ii) correct assembly of multimeric antibodies (unlike bacteria which lack the capacity to generate disulfide bonds); and (iii) increased safety, since plants do not serve as hosts for human pathogens such as HIV, prions, hepatitis viruses, etc. [50].

4 Antibody-based high-throughput analyses and their clinical applications

Several current and prospective applications of antibody-derived array-based or nonarray-based methodologies in the context of clinically relevant studies have been defined. In the past few years, it has become evident that an increasing number of clinical studies will see their needs to augment significantly for (i) biomarker discovery as well as for (ii) molecular signature identification [51–53]. Technological progress brought about by the improvement in DNA microarray technologies combined with improved detection methods and statistical analyses have led to the development of so-called antibody arrays to allow the characterization of protein expression levels or their PTMs. In addition, depending of the approach selected, the possibility of establishing specific profiles relevant to given diseases has also been proposed [51–54].

In the present section, the parameters which can be analyzed using antibody arrays including not only the evaluation of protein expression levels but also the analysis of their PTMs as well as the characterization of specific protein complexes will be defined, then the technologies and the approaches available, including their limitations (for instance real arrays *versus* bead-based assays), will be discussed. Finally, the impact these approaches can have on clinical research in the specific context of biomarker discovery *versus* molecular signature establishment will be illustrated.

Thus far at least three different parameters can be directly evaluated using antibody array- or nonarray-based approaches. First, and probably the most obvious/logical parameter to be detected using antibody arrays is the expression level of either a given protein in a series of samples or the expression level of a large number of proteins in a single biological sample (see Figs. 2–4). Independently of the proteomic platform used, antibody-based protein profiling remains one of the principal methodologies for the application-oriented translation of proteomics in the context of clinical studies. The major advantages of antibody-based approaches are (i) the possibility of multiplexing, (ii) good to very good reproducibility; (iii) sensitivity, often being able to

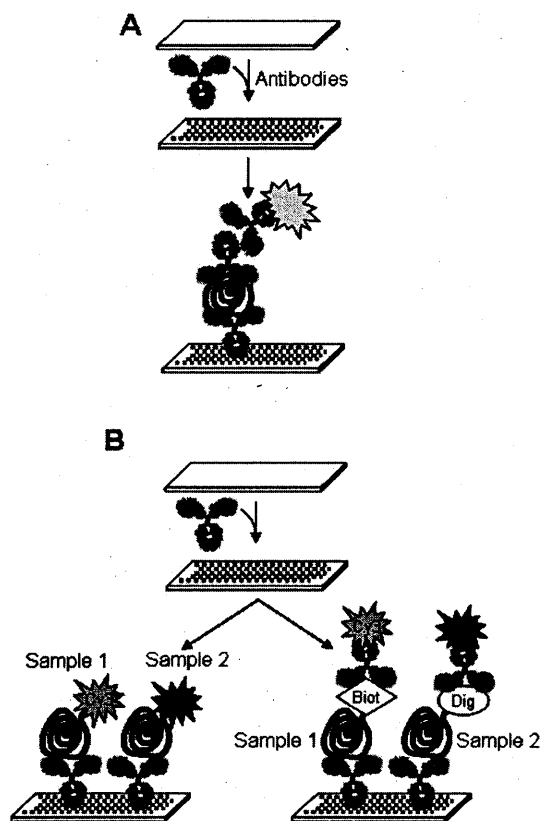


Figure 2. Array-based analyses: targeted approaches. Schematic representations of the possible targeted approaches using array-based methodologies: (A) Sandwich assay and indirect antibody labeling, (B) antibody capture and direct labeling by Cy3/Cy5 (left panel) or by biotin/digoxigenin followed by secondary immunogenic reaction using antibiotin and antidigoxigenin antibodies coupled to Cy3/Cy5 respectively (right panel).

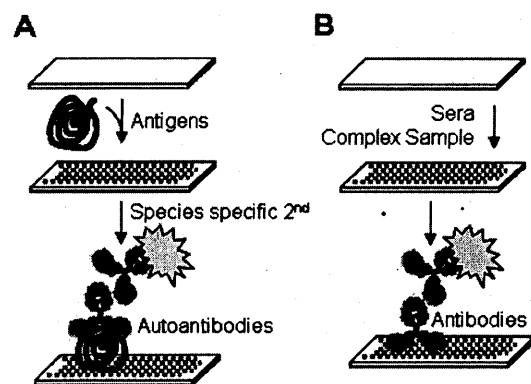


Figure 3. Array-based analyses: untargeted approaches. Schematic representations for two potential configurations of array-based untargeted approaches: (A) specific antigens are spotted on the array to identify and characterize antibody specificity. (B) When the antibody is characterized, it can be used as a tool to scan a large number of complex samples (*i.e.*, serum) for the presence of the antigen. The visualization is carried out through the use of a secondary antibody coupled to a fluorophore/enzyme.

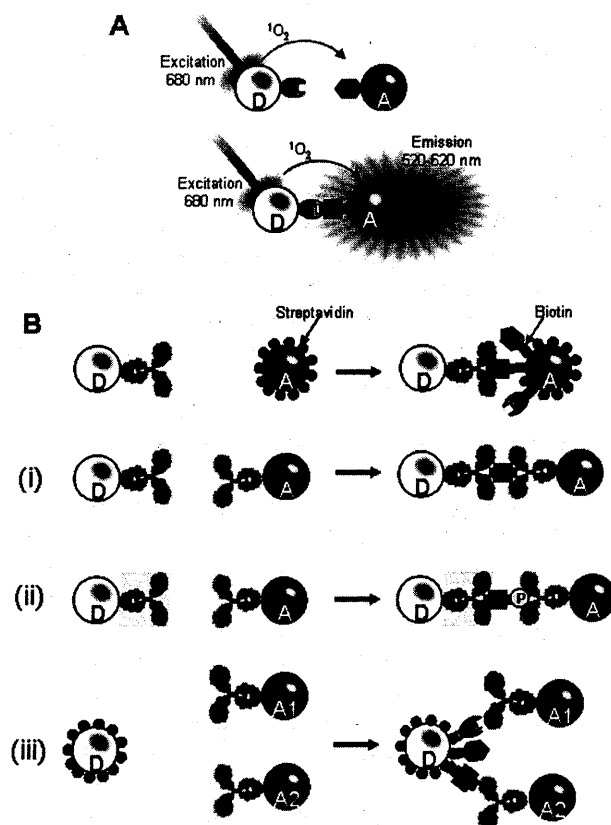


Figure 4. AlphaScreen principles and potential applications. (A) Principles of AlphaScreen (D, donor beads; A, acceptor beads). (B) Examples of various experimental settings allowing for the detection of one antigen in a complex sample following its biotinylation: (i) Sandwich detection of a given antigen by two different antibodies raised against the same protein; (ii) Sandwich detection of a PTM on a given antigen (here phosphorylation); (iii) finally, detection of multiple antigens present in a complex sample following its biotinylation. This type of approach requires the existence of acceptor beads emitting at various wavelengths.

detect proteins at lower concentrations [55, 56] than other proteomic methodologies (*e.g.*, 2-D PAGE followed by MS); and (iv) specificity, effectively lowering the effect of the dynamic range of protein concentrations in complex samples [55, 56]. To increase the throughput of these antibody-based analyses, several approaches have been developed either using array or nonarray methodologies.

4.1 Array-based technologies

Protein arrays comprised of immobilized proteins are an emerging biochip format. The first steps of this technology development were carried out at the end of the 1990s and the beginning of the 2000s. Indeed, the first reports described the use of high-density filters or array-like structures to screen for antibodies [57, 58]. Similarly array-based technologies were used to either evaluate antibody–antigen interac-

tions with a high-throughput or characterize specific functional properties [59, 60]. Thus far protein biochips have been used for a spectrum of applications as broad as protein–antibody and autoantibody profiling, for the study of protein–ligand interactions, where the ligand can be either proteins, peptides, DNA or RNA, and for the determination of enzymatic activity, and substrate specificity of classes of enzymes [61]. In the context of antibody-based approaches, two major methodologies have been developed as “targeted” or “untargeted” [62–64]. The theoretical opposition between both themes is conditioned by the type of material deposited on the array (either antibodies or studied samples) and the type of the sample analyzed.

4.1.1 Antibody arrays: Targeted approaches

The two main types of formats are “sandwich” and “label-based” assays (Fig. 2; [61]). These approaches are complementary and have respective pros and cons. In the sandwich assay in which immobilized antibodies capture unlabeled proteins, the captured proteins are detected by another antibody (Fig. 2A). The signals for detection may be generated by a variety of methods (see Table 1). Sandwich assays can have higher specificity than label-based assays since two antibodies target each analyte instead of just one. Detection sensitivity also can be higher due to reduced background [61]. The assays are noncompetitive since only one sample may be incubated on each array, thus a “standard curve” of known concentrations of analytes is required to achieve accurate calibration of concentrations. In the label-based assay, the targeted proteins are labeled with a tag allowing detection after capture by an immobilized antibody. Two options for detection are available: (i) “direct labeling” where proteins are labeled with a fluorophore such as Cy3 or Cy5 and (ii) “indirect detection” where proteins are labeled with a tag such as biotin or digoxigenin and subsequently detected by a labeled antibody [61]. In addition, label-based assays allow the coinubation on the arrays of two different samples, each labeled with a different tag, thus for instance allowing the coinubation of a reference sample with a test sample to normalize for variation between spots in capture antibody concentration. In addition, this assay is competitive as the analytes in the test and reference solutions compete for binding to the antibodies [65]. Nevertheless, label-based methods also have drawbacks relative to sandwich assays. The use of just one antibody *per* target means that specificity can be lower and, as all proteins are labeled, backgrounds and detection limits can be higher. The approach is probably best used as a practical method to screen binding to many antibodies targeting a wide variety of proteins. Sandwich assays are more difficult to develop in a multiplexed setting relative to label-based assays. Indeed, matched pairs of antibodies and purified antigens may not be available for each target, and the possibility of crossreactivity between detection antibodies increases with additional analytes. Consequently, multiplexed sandwich assays are considered to have a prac-

Table 1. Detection methodologies for sandwich antibody-based arrays

Method	Description	Reference
RCA	Rolling circle DNA amplification	[82, 83]
TC-RCA	Two-color RCA	[84, 85]
RLS	Resonance light scattering	[86, 87]
ECL	ECL	[88, 89]
TSA	Tyramide signal amplification	[90, 91]
SA	Streptavidin	[92, 93]

tical size limitation of 30–50 different targets [61]. Arrays using label-based detection, on the other hand, are only limited by the availability of antibodies and the available space on a substrate.

4.1.2 Antibody arrays: Untargeted approaches

In contrast to the targeted strategies, this approach uses the characteristics of solid supports/arrays to deposit a large number of antigens either purified or in a complex mixture such as serum (see Fig. 3). Interesting developments have been proposed at an early stage to find application in the medical domain [66] but also more recently to be of interest in the bridging of genomics and proteomics [67, 68]. Under those conditions, each array will not be probed with a large number of antibodies but rather it will allow the multiparallel analysis of a large number of analytes. In particular, two major configurations can be developed with at first the spotting of specific antigens on the array (and again several supports are available ranging from NC to specific polymers). These antigens can be as varied as recombinant proteins, synthetic peptides, DNA, or RNA. The most appropriate objective of this type of assay may be either the identification of the antigen for a given uncharacterized antibody or the identification of an autoantibody pattern present in a given serum (Fig. 3A). In addition, these properties may allow us to consider the first option as an enabling technology to better characterize immunologic tools (either from *in vivo* or *in vitro* sources; Fig. 1). Under those circumstances, the number of antibodies tested is only dependent of the number of replicas made from the initial array. The other configuration which can be applied to this experimental strategy is the direct spotting on the array of a large number of biological samples (serum, cell, or tissue extracts). In this context, quantification would again require, as for the targeted sandwich approaches described in Fig. 2A, a standard curve of known concentrations of the antigen against which the antibody is raised, to achieve accurate calibration of concentrations.

In summary, the untargeted approaches allow for the analyses of a large number of different samples and the principal limitation comes from the capacity to replicate the array. In addition, a sensitivity issue may be raised since, in

contrast to sandwich arrays, the antigen will not be enriched by antibody capture on the array.

4.2 Nonarray-based approaches

Nonarray-based technologies represent a significant alternative to array-based methods as they make use of homogeneous solutions in contrast to array-based methodologies thus expanding their versatility, their sensitivity as well as their potential throughput. Examples of three nonarray-based technologies (TR-FRET (LANCE™, HTRF®), Luminex, and AlphaScreen™) will be briefly described with more emphasis placed on the AlphaScreen technology.

4.2.1 TR-FRET-based technologies

Time-resolved fluorometry is commercialized at least by two companies under the copyrighted denominations LANCE (Perkin-Elmer) and HTRF (Cisbio). This technology exploits the unique fluorescence properties of lanthanide chelates which display (i) long fluorescence decay after excitation (up to a millisecond) and (ii) high signal-to-background ratio. Homogeneous assays (which do not require washing steps) particularly benefit from time-resolved fluorometry because the sample constituents present during detection cause very high background fluorescence when conventional fluorochromes are used. TR-FRET is based on the proximity of a donor label (europium chelate/cryptate) and an acceptor label (Allophycocyanin, APC, XL665) which are brought to close proximity by a specific-binding reaction. The excited energy of the Eu-chelate/cryptate is transferred by a non-radiative resonance energy transfer mechanism to an acceptor within a short distance. Fluorescent lanthanide chelates with long excited state lifetimes are used to avoid interference caused by short-lived emission from acceptor molecules excited directly rather than by energy transfer. TR-FRET has been widely used, for example, in kinase, nuclear receptor, and protein-binding assays [69–75]. However, very few assays have in fact been applied to clinical settings [76].

4.2.2 The Luminex technology

The Luminex technology is built on a combination of flow cytometry, microspheres, lasers, signal processing, and traditional chemistry. This technology can be configured to perform a wide variety of bioassays quickly, cost-effectively, and accurately. First, Luminex color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. This technology allows therefore multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely. A significant number of

products are already available commercially and a significant number of clinically oriented research projects have been carried out using this technology [77–80].

4.2.3 AlphaScreen

AlphaScreen relies on the use of “donor” and “acceptor” beads that are coated with a layer of hydrogel providing functional groups for bioconjugation. When a biological interaction between molecules brings the beads into proximity, a cascade of chemical reactions is initiated to produce a greatly amplified signal [73, 81]. Upon laser excitation, a photosensitizer in the donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse and react with a chemiluminescer in the acceptor bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light with a wavelength ranging from 520 to 620 nm.

Basically, as described in Fig. 4A, illumination at long wavelengths ensures that few biological or assay substances will interfere. A high concentration of photosensitizer in each donor bead generates up to 60 000 singlet oxygen molecules *per* second. This results in a very high signal amplification that contributes to detection sensitivity to the attomole (10^{-18}) level. Singlet oxygen has a short lifetime in an aqueous solution ($\sim 4 \mu\text{s}$) that allows diffusion of no more than 200 nm, consequently, only beads within that close proximity will emit light. This distance is approximately ten times longer than what energy transfers allow in TR-FRET experimental setups, thus providing a selection advantage for the study of larger complexes (including protein complexes) and find application in sandwich-based approaches usually carried out using ELISA (AlphaLISA™ methodology, see Fig. 4B) [82–84]. The acceptor beads contain a thioxene derivative that reacts with the singlet oxygen molecule to generate a chemiluminescence at 370 nm. This energy is immediately transferred to fluorophores within the same bead, shifting the emission wavelength to 520–620 nm. A short half-life decay reaction allows detection in a time-resolved mode. Several applications of AlphaScreen at the bench have led to significant enhancement of biochemical assays such as measures of enzymatic activities like GTPase or protein ubiquitination [83, 85–87]. Thus far, AlphaScreen-based assays have mostly been used in industrial settings for high-throughput screening.

A significant advantage of this technology resides in its enhanced flexibility (as illustrated in Fig. 4B), its very low background and finally its high-throughput capacity. Experimental configurations similar to the targeted or untargeted array-based methodologies can be designed without much variation in the assay, thus allowing a direct comparison between both approaches. In addition, as the spectrum of fluorophores contained in the donor beads will expand, the possibility of testing multiple analytes in a single well will considerably enhance the throughput of the technology to reach potentially ultrahigh-throughput capacity.

4.3 Applications of array- and nonarray-based methodologies: From bench to bedside

Thus far immunophenotyping has notably been used in the diagnosis of leukemia. It involves the labeling of white blood cells with antibodies directed against surface proteins on their membrane. By choosing appropriate antibodies, the origin of leukemic cells can be accurately determined. The labeled cells are processed in a flow cytometer. The whole procedure can be performed on cells from the blood, bone marrow or spinal fluid in a matter of a few hours. This concept can certainly be extended using the technologies mentioned above to characterize at specific antigen expression levels/modification in a disease state and consequently not only represent a good characterization tool at the bench but may also potentially constitute diagnostic/prognostic detection methods at the bedside in the near future.

4.3.1 Measuring protein expression levels

One of the best illustrations of the application of the above-mentioned methodologies is the measure of protein expression levels. In this context, the use of label-based arrays was made to identify potential biomarkers for prostate cancer and cystic fibrosis. Serum proteins were labeled with Cy3 and Cy5 and profiled on antibody arrays [88]. Similarly, this strategy has also been used to look at protein expression in human tissue samples following laser capture microdissection [89]. In another interesting study using resected tumor tissue [90], proteins isolated from 30 different hepatocellular carcinoma tumors were compared to those from 15 normal liver specimens using Cy3 and Cy5 labeling on microarrays containing approximately 80 different antibodies. In total, 32 of the proteins exhibited differential expression between the tumor and normal groups. Moreover, a pancreatic cancer analysis was carried out by profiling the binding of samples from 142 patients to approximately 90 antibodies. This revealed multiple proteins associated with both malignant and benign pancreatic disease, some of which were previously unknown to be associated with pancreatic cancer [91]. Finally, antibody array approaches were also specifically designed to identify serum markers of bladder cancer [92] or lung cancer [93]. Thus far, nonarray-based methods have not yet been used in similar experimental setups. However, Luminex platforms offer a multiplex analysis of several of proteins which may be already in preclinical development. More recently, the coupling of array-based antibody technologies with microfluidics has led to the development of a point-of-care diagnostic and demonstrated its applicability to rapid, reliable measurement of proteinaceous disease biomarkers in biological fluids [94].

4.3.2 Measuring PTMs

Only recently has the identification of PTMs been recognized as potential biomarkers. Indeed, the biological roles of pro-

teins are determined not just by their abundance, but also by modifications occurring post-translationally such as phosphorylation, glycosylation, proteolysis, acetylation, methylation, nitrosylation, oxidoreduction, or lipidation. A novel application of antibody arrays has been to detect certain PTMs on multiple proteins which could be of interest at the clinical/diagnostic level [95]. One strategy employs a modified sandwich assay in which the detection antibody targets a particular modification instead of the core protein. Schematic representations of "heterogeneous" sandwich assays are shown in Fig. 2 for the measurement of phosphorylation and glycosylation states. In this experimental setting, proteins are captured by immobilized antibodies and are probed with antibodies targeting either the proteins themselves or phosphotyrosine [96]. By looking at the ratio of phosphotyrosine to total protein, the level of activation of the proteins can be measured. A related approach to measure the protein modifications using antibody arrays consists in immunoprecipitating whole-cell extracts using antibodies against phosphotyrosine, ubiquitin, or acetyl lysine, and the precipitates are fluorescently labeled and applied to antibody microarrays [97]. The level of binding to each antibody reflects the level of modification of each target protein.

For glycosylation profiles, the use of lectins, which are plant and animal proteins with natural carbohydrate affinity, can be of major interest. Each lectin type has specificity for a particular glycan structure, so the glycan structure measured on the captured proteins can be specified by the lectin used for detection. To measure both protein levels and glycan levels in the same experiment, the proteins can be tagged to allow detection with a different color fluorophore than that used to detect the glycan. This method is currently being used to profile the variation in glycan levels on serum proteins in cancer patients [98]. Such studies might reveal novel cancer markers or could identify glycan alterations involved in cancer progression.

4.4 Future perspectives

A major limitation for most antigen array analyses is that the antigens used are limited to proteins and other biomolecules that are known to represent candidate targets, and for which synthetic or purified preparations are available. Consequently, any initiative aiming to improve our understanding/knowledge of binder pairs will dramatically facilitate the conception of new arrays to evaluate protein expression profiles, PTMs, *etc.*

Thus far, at least two major initiatives have been launched to facilitate the characterization of antibodies and enhance their characterization rates. The first, named ProteomeBinders, is a new European consortium proposing to establish a comprehensive infrastructure resource of binding molecules for detection of the human proteome, together with tools for their use and applications in studying proteome function and organization (www.proteomebinders.org) [99]. The second, named The Human Protein

Atlas, displays expression and localization of proteins in a large variety of normal human tissues and cancer cells. The data are publicly available and presented as high-resolution immunohistochemical images of stained tissues and cell lines. Available proteins can be found through searches for specific genes or by browsing individual chromosomes (<http://www.proteinatlas.org>) [100].

In addition, provided the enhanced discovery rate of binders and the known *in vivo* efficiency of intrabodies for altering protein trafficking and/or processing or signaling pathways [101–103] or transbodies [104], we can also anticipate that these engineered proteins will find in the near future a tremendous field of applications both at the bench [105, 106] and at the bedside [107, 108].

In conclusion, in the course of this review we have described several strategies to maximize antigen selection and design, we also have listed the methods to produce antibodies *in vivo* using more traditional methodologies or *in vitro*, using approaches based on recombinant DNA. In the last section, we have described how these antibodies can be used at the bench or at the bedside to improve diagnostics or prognostics for patients suffering from various complex diseases. It has become obvious that the development of such immunologic tools can also lead to successful therapeutic strategies [109–111]. This is particularly well illustrated with the highly publicized example of herceptin (<http://www.herceptin.com>), which is an antibody specifically targeting the EGF receptor and commonly used to treat HER2 positive breast cancer patients [65, 109–114], thus demonstrating the increasing interest of the use of antibodies as accepted and successful therapeutics.

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