Published in final edited form as:
Nat Rev Drug Discov. 2006 April ; 5(4): 310–320.

Multiplexed protein measurement: technologies and applications of protein and antibody arrays

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Abstract

The ability to measure the abundance of many proteins precisely and simultaneously in experimental samples is an important, recent advance for static and dynamic, as well as descriptive and predictive, biological research. The value of multiplexed protein measurement is being established in applications such as comprehensive proteomic surveys, studies of protein networks and pathways, validation of genomic discoveries and clinical biomarker development. As standards do not yet exist that bridge all of these applications, the current recommended best practice for validation of results is to approach study design in an iterative process and to integrate data from several measurement technologies. This review describes current and emerging multiplexed protein measurement technologies and their applications, and discusses the remaining challenges in this field.

Longitudinal and cross-sectional studies to measure the levels of individual proteins in cells, tissues, extracts and biological fluids have been a long-standing and crucial component of progress in life-science research and clinical practice. Such measurements have three key purposes. First, they are surrogates (or biological markers) for organ activity, disease processes or drug action — examples include serum cardiac troponin I measurement for diagnosis of myocardial infarction[1] and B-type natriuretic peptide measurement for diagnosis of congestive heart failure[2]. Second, they are proxies for activity when the measurement of organ, cell, pathway or protein activity is onerous — for example, using the titre of influenza-antigen-specific immunoglobulin rather than virus-neutralizing activity for the determination of immunity. Third, they are an end measure — for example, measuring the mass of protein in a timed urine sample when assessing glomerular function.

Longitudinal study

A study in which measurements are made over a time course within an individual or individuals.

Cross-sectional study

Competing interests statement
The author declares competing financial interests: see web version for details.

FURTHER INFORMATION
PRoteomics IDEntifications (PRIDE): http://www.ebi.ac.uk/pride
Expert Protein Analysis System: http://www.expasy.org
Plasma Proteome Database: http://www.plasmaproteomedatabase.org
Global Proteome Machine: http://www.thegpm.org
Human Protein Reference Database: http://www.hprd.org
PhysioNet: http://www.physionet.org
Access to this interactive links box is free online.

A study in which measurements are made at a single time point in many individuals.

Single nucleotide polymorphisms

A specific location in a DNA sequence at which different people can have a different DNA base. Differences in a single base could change the protein sequence, leading to disease, or have no known consequences.

The accepted 'gold standard' for single-protein measurement is immunoassay, which exploits the diversity and specificity of antigen binding by immunoglobulins. Commonly used affinity ligands are monoclonal immunoglobulins or their antigen-binding domains, or polyclonal antisera. These assays use either single antigen-specific antibodies (as in immunohistochemistry) or, more commonly, two antibodies (as in 'sandwich' or competitive immunoassays). A wide range of labelling and signal-enhancement strategies have been developed that allow ligand binding to be detected through association of the ligand with a 'read-out' antibody that has particular fluorescent, colorimetric, histochemical or radioactive properties, or through changes in density or mass. Assay formats exist to measure proteins in solutions (enzyme-linked immunosorbent assays (ELISA) and immunospot assays), on the surface of cells (flow cytometry), within cells (immunohistochemical and immunofluorescent microscopy) and in organs (*in vivo* imaging with labelled antibodies). The most widely used format is ELISA, which has a well-established typical specification for measurement of single proteins in a solution; a lower limit of reliable quantitation of $\sim$1 pg ml^{-1}; a dynamic range of 3 logs; coefficients of variation between replicate measurements of 5–20%; undetectable non-specific binding in the dynamic range; time to first-result of 1–2 h; and 96- or 384-well formats.

Recently, there has been increasing interest in the simultaneous measurement of many proteins in experimental samples. Predominantly, this interest has been prompted by the impact that highly multiplexed assays have had on genomics. During the past ten years, comprehensive surveys of genes, transcripts and single nucleotide polymorphisms have become commonplace for genetics and molecular biology discoveries. Furthermore, it is now recognized that single nucleic-acid measurements can lead to spurious conclusions in, for example, studies of transcriptional regulation or heritability of complex traits[3,4].

Multiplexed measurement is logical for biological discovery with proteins because they constitutively function within networks, pathways, complexes and families[5-7]. The activity of an individual protein is dependent not only on its abundance, but also on the effects of interacting, modifying, antagonistic and synergistic proteins. Cytokine biology provides an example of the complexity of protein networks and inadequacy of monoplex measurement. In living systems, effects on target-cell activities are a dynamic aggregate of multiple agonist and antagonist cytokines, associated modifier proteins, ligands, receptors and receptor antagonists. Measurement of the level of a single cytokine *in vivo* is therefore a poor surrogate for activity, and integration of results from multiplexed measurement of component cytokines in a network is much more likely to be descriptive of biological processes (see later for further discussion).

Box 1 Protein array substrates and attachment chemistries

Five types of protein arrays are in common use today:

Planar glass or silicon chips. These were the first surfaces to be used in protein arrays. Advantages include low cost, low lot-to-lot variability, low fluorescent background and extreme 'flatness' (important for accurate array scanning). However, glass or silicon protein chips require the onerous application of a surface chemistry for protein attachment; many

attachment chemistries have been described[6,58]. Given a practical limit of multiplexing of sandwich immunoassays of ~50, the parallel processing advantage of planar arrays is typically achieved by assaying many samples in partitioned sub-arrays on a single chip. The disadvantages of glass or silicon chips are the difficulties and lot-to-lot variability associated with attachment chemistry, and denaturation of most of the attached capture agent. Recent innovations have been silicon chips, either in a conventional array[132] or in an etched, 'compact disc' format[133].

Flow cytometric microbead assays. 'Suspension' arrays involve attachment of the capture agent for protein assays to microspheres, and their enumeration by flow cytometry[129,134]. Microspheres (and assays) can be multiplexed by using beads that differ in size or fluorescence intensity (with a fluorescent dye non-overlapping in emission spectrum to that used for the specific protein assay). Attractive aspects of suspension arrays are fluid-phase kinetics (which are faster than the solid-phase kinetics of planar arrays), custom multiplexing and greater precision (due to measurements of hundreds of beads for each analyte). Disadvantages of multiplexing of sandwich immunoassays are similar to those associated with bead and chip assays. The major limitation of cytometric bead assays is sample throughput. Microbead assays are available from Becton, Dickinson and Company (Cytometric Bead Array) and the partners of Luminex Corp. (xMAP).

Arrays in plastic microwells. Enzyme-linked immunosorbent assays (ELISAs) in 96- and 384-well plastic plates have dominated monoplex protein measurement for many years. Genometrix pioneered the printing of arrays in 96-well plate format[135]. Multiplexed, microplate immunoassays are available in a 384-well format that features up to 16 assays per well from Pierce Biotechnology Inc. (SearchLight). Sample consumption is greater than on chips and sensitivity is lower.

Nitrocellulose on chips. Nitrocellulose binds proteins in a non-covalent, irreversible manner. Modified nitrocellulose membranes have been used for many years for the detection of specific proteins that have been resolved by gel electrophoresis and western blotting. Attachment of nitrocellulose membranes to glass slides combines the advantages of microarrays with the protein binding capacity, long-term stability of printed proteins and assay methods of traditional blots. Nitrocellulose arrays are available from Whatman PLC (FAST Slides).

Three-dimensional gels. Coating glass slides with a porous, hydrophilic, three-dimensional surface chemistry comprising a crosslinked polymer containing amine-reactive groups was initially described by Khrapko *et al*[136]. The advantages of three-dimensional gels are reaction kinetics that are more similar to fluid-phase than solid-phase kinetics (due to holding attached biomolecules, such as capture antibodies, away from the surface of the slide), low fluorescent background and reduced protein denaturation[56,59]. These benefits translate into approximately tenfold higher sensitivity than planar glass. The disadvantages of three-dimensional gel microarrays are cost and novelty (and as such have an unproven track record). Several types of three-dimensional microarray are available, for example, CodeLink from GE HealthCare Co. or HydroGel from PerkinElmer Inc.

The same caveat applies to the measurement of single proteins as surrogates (or biological markers) that are predictive of organ activity, disease process or drug action. In diagnostic parlance, the lack of concordance between individual protein abundance and clinical status or outcome can be referred to as a false-positive or false-negative result. The usefulness of diagnostic tests can be measured by positive predictive value (PPV) and negative predictive value (NPV). A logical advance in biomarker-based and activity-based protein measurement is surrogate protein selection predicated by a comprehensive understanding of protein networks, pathways and dynamics.

Until recently, specific, sensitive multiplexed protein measurements have not been generally undertaken because of technical challenges (BOX 1). These challenges include the diverse physico-chemical properties of proteins, their lability and the interferences introduced as a by-product of multiplexed measurement reagents. Solution-phase immunoassays, for example, have proven difficult to multiplex for these reasons. The diversity in dynamic range of protein concentrations in living systems is also challenging. For example, detectable proteins in human serum range in abundance from grams to tenths of picograms per ml. Furthermore, serum protein abundance can change by as much as 10,000-fold on stimulation. This is particularly true of many of the proteins that are most frequently measured, such as acute-phase reactant pentraxins or chemokines. A practical impediment to multiplexed protein measurement has been the expectation of a scientific community reared with the specifications of ELISAs that has been reluctant to deploy ratiometric or semi-quantitative multiplexing technologies. Nevertheless, reports of discoveries made using such technologies are starting to drive adoption of these approaches. This article briefly reviews technologies for multiplexed protein measurement, emerging standards, applications and remaining challenges.

Multiplexed protein measurement

Although the specific needs of biologists for multiplexed protein measurement are heterogeneous, they can be grouped into four main categories that follow a logical research and development succession: surveys of changes in protein abundance; modelling networks, pathways, and physiological and disease states; biomarker validation; and clinical diagnostics (TABLE 1). The optimal technology for multiplexed protein measurement varies among the four main types of application, together with the associated degree of multiplexing, desired specification and type of experimental design. The two principal types of technologies in use today in these applications are mass spectrometry and protein arrays. Mass spectrometry is typically used for comprehensive proteomic surveys and has previously been reviewed in this journal[8], and so will be covered only briefly in this article, which focuses primarily on protein arrays.

Cytokine

Cytokines comprise several hundred small, soluble proteins that are powerful mediators of target cell activities such as migration, activation, phagocytosis, proliferation and apoptosis. Cytokines are secreted by producer cells into extracellular fluids, and act on target cells through binding to specific cell-surface receptors.

Positive predictive value (PPV). The probability that the patient has the disease when restricted to those who test positive.

Negative predictive value (NPV). An assessment of reliability of a negative test. The probability that the patient does not have the disease when restricted to those who test negative.

Proteomic surveys by mass spectrometry

The typical purpose of comprehensive proteomic survey experiments is to be initial exploratory tools for hypothesis generation. Usually, this involves the identification of a set of proteins with different levels of expression between two states. The technology specifications for comprehensive surveys of protein levels are: applicability to many sample types, including

tissue lysates; ability to measure simultaneously most (thousands) of the proteins/peptides in a small sample volume in up to 100 samples; and moderate price.

The technology of choice for proteomic surveys today is 'shotgun' mass spectrometry. Many technology variations exist that differ in specificity, sensitivity, dynamic range, ease of use and cost. However, all are based on conversion of intact proteins in a sample into peptide fragments, followed by their volatilization, measurement of their mass-to-charge ratio (*m*/*z* ratio) and intensity, and then off-line protein identification by comparison with a database of peptides and their masses. Mass spectrometry has an open architecture, and is therefore not constrained by hypotheses regarding which groups of proteins are differentially expressed. Experiments typically involve the comparison of results from two, small (~10–50), matched groups of samples, with identification of many intensity peaks that represent peptide expression differences. Database analysis translates these *m*/*z* ratio intensity differences into high-likelihood protein-abundance differences. Experiments are typically repeated, with protein abundance differences 'discovered' in a first study, and the differences 'validated' in a subsequent study. Mass spectrometry is ideal for rapid, cost-effective, initial proteomic surveys of experimental systems. However, it has limitations. First, analytical sensitivity for multiplexed protein measurement is much lower than ELISA, and limits the usefulness of the technology with small or 'rare' proteins, such as cytokines in blood samples. Sensitivity can, however, be enhanced by sample preparation to remove high-abundance proteins[9,10] (for example, albumin and immunoglobulins in plasma samples). Second, extensive sample preparation is frequently necessary, especially for intracellular or membrane-associated proteins, which can introduce systematic bias. Furthermore, many proteins are labile, and degradation during sample preparation or storage greatly impairs protein identification. Third, automated protein identification by database lookup is frequently plagued by false positives (due to imprecision in *m*/*z* ratio measurement, non-specific peptide cleavage, or incomplete or inaccurate peptide database entries) and false negatives (due to orphan peptide fragments from small proteins). Bioinformatic algorithms are typically used to select expression differences in proteins with high likelihood identities. The development of empirical databases of expressed peptides in specific tissues, using methods such as multi-dimensional protein identification technology[11-13], promises to assist in the correct identification of differentially expressed proteins. Similarly, use of statistical approaches, such as False Discovery Rate or Significance Analysis of Microarrays[14-18], that incorporate appropriate adjustments for multiple observations will assist in the identification of true protein abundance differences. Fourth, mass spectrometry is semi-quantitative and best used for identification of gross differences in expression (≥fivefold). Specialized techniques do exist to make results more quantitative, but these require the use of radiolabelling steps beyond the scope of many experiments. Fifth, day-to-day, run-to-run, operator-to-operator and machine-to-machine variation continue to constrain use of mass spectrometry to experiments in which all the samples are run in a single batch. However, coefficients of variation are improving, and efforts are underway to 'normalize' or standardize spectra, thereby enabling benchmarking[19]. A recent advance — multiplexed reaction monitoring — provides significant improvement in sensitivity and coefficients of variation, and has the potential to extend the applicability of mass spectrometry from biomarker discovery to multiplexed protein measurement[20].

For these reasons, multiplexed protein measurement by mass spectrometry is currently best used for hypothesis-generating, rather than hypothesis-testing, experiments. In experimental systems for which there is little knowledge of relevant protein networks or pathways, mass spectrometry can rapidly provide lists of candidate proteins and pathways[7]. An example of this is the discovery of serum protein biomarker candidates that are predictive of drug efficacy. Rigorous attention to experimental technique and bioinformatic analysis is crucial for success[21,22].

Arrays for multiplexed protein measurement

Conventional immunoassay platforms have limited multiplexing capacity and high sample volume requirement. Since the original description by Ekins[23,24] and early advances by Ward[25,26], Schreiber[27], Snyder[28] and Brown[29], high-throughput multiplex immunoassays that measure hundreds of proteins in complex biological matrices have become significant tools for quantitative proteomics studies, diagnostic discovery and biomarker-assisted drug development[5,6,30-39].

Three broad categories of antibody microarray experimental formats have been described (FIGS 1 and 2): first, direct labelling, single-capture antibody experiments[29,40,41]; second, dual antibody (capture and read-out antibody), sandwich immunoassays[25,26,42-46]; and third, antigen- or peptide-capture arrays, with single read-out antibodies[47-51]. In the direct labelling method, all proteins in a sample are tagged, thereby providing a means for detecting bound proteins following incubation on an antibody microarray (FIG. 1a). In the sandwich immunoassay format, proteins captured on an antibody micro-array are detected using a cocktail of labelled detection antibodies, with each antibody being matched to one of the spotted antibodies (FIG. 1b). In the antigen-capture arrays, the antigens are printed on the array and ligands in an experimental sample are detected on the basis of binding a labelled read-out antibody (FIG. 2). In each case, the spatial location of the specific capture reagent on the microarray defines the identity of analyte measured at that location: advantages and disadvantages of these formats are compared in TABLE 2.

Various microarray substrates have been described, including nylon membranes, plastic microwells, planar glass slides, gel-based arrays and beads in suspension arrays[42,45,52-59]. Numerous studies have been carried out to optimize antibody attachment to the microarray substrate[60-62]. In addition, various signal generation and signal enhancement strategies have been used in antibody arrays, including colorimetry, radioactivity, fluorescence, chemiluminescence, quantum dots, other nanoparticles, enzyme-linked assays, resonance light scattering, tyramide signal amplification, rolling circle amplification and eTag protein proximity assays[6,25,63-65]. Although most arrays use recombinant or monoclonal antibodies, alternative high-affinity ligands have recently been used in microarrays[66-70].

Each of these formats and procedures has distinct advantages and disadvantages, relating broadly to sensitivity, specificity, dynamic range, multiplexing capability, precision, throughput and ease of use. In general, multiplexed microarray immunoassays are ambient analyte assays[71], involving picolitre antibody ‘spots’ and microlitre sample volumes. Besides low requirements for reagent and sample, miniaturization of reactions confers the benefits of reduced reaction times (as a consequence of short diffusion distances) and improved signal-to-noise ratios[72].

Despite the heterogeneity of formats and procedures available for multiplexed protein measurement, the ‘gold standard’ for specification remains the ELISA, albeit with additional specifications for a multiplexed assay environment. Unique considerations in assembling multiplexed immunoassays include the elimination of assay cross-reactivity, configuration of multianalyte sensitivities, achievement of dynamic ranges appropriate for biological relevance when carried out in diverse matrices and biological states, and optimization of reagent manufacturing and chip production to achieve acceptable reproducibility. Specifications and standards for antibody microarrays are being developed[73-75]. Specifically, requirements have been described for comprehensive validation programmes to identify and minimize antibody cross-reaction under highly multiplexed conditions, controls to normalize sample replicate measurements, and quality control testing of reagents, antibody microarrays, sensitivity, dynamic range and platform precision. A key remaining challenge for many analytes is the

availability of high-affinity reagents with specificity in multiplexed assays, because almost all monoclonal antibody pairs were selected for performance in a monoplex assay format. Another challenge, in common with other high-throughput technologies, is the development of statistical approaches to integrate data from replicated assays and to reveal the significance of biomarker findings.

Arrays for modelling networks and pathways

Modelling gene or protein networks or pathways in disease states is a recent field of endeavour. Proteins usually function in interdependent networks. The goal of model development is to understand inter-relationships among the members of a protein network or family in a disease state. Precise models enable prediction of the biological responses to a perturbation, and detection of novel associations among members[76].

Model development is a mathematical exercise. Two types of mathematical approach are being implemented in static and dynamic biological systems. The standard approach in biology has been reductionist analysis — division of the system into component variables and 'solving' a differential equation for each with empirical data[77]. Although biological systems are generally complex and nonlinear, much of our current biological knowledge has been derived from this type of deterministic, reduction-ist analysis[78]. Furthermore, disease states are frequently associated with linear dynamics[79] (or, more accurately, with the breakdown of multi-scale fractal complexity). For these reasons, reductionist methods are likely to remain useful for the foreseeable future for quantitative prediction of responses to perturbation of networks. Notable recent examples of progress are the development of dynamic models of yeast responses to hyperosmolar shock[80] and treatment of chronic myeloid leukaemia with imatinib[81]. The second, newer approach to proteomic modelling is finite-state modelling, which uses the changes in gene[3,4] or protein expression[76] that follow perturbation of a particular node to identify the topology of complex networks. This approach, although relatively recently introduced to systems biology[76,82], has proven useful in other nonlinear systems and enables detection of novel associations among elements[76] or assessment of network robustness.

Current biological experimentation, particularly for multiplexed protein measurement, mandates that these modelling approaches must have the flexibility to use noisy, incomplete data. When modelling networks, pathways and disease states, the technological specifications should include the following: assays for many of the major elements in that network or pathway (typically 25–200 proteins); measurement of those within the biological dynamic range with reasonable precision (coefficients of variation of ≤15%); and the ability to measure many samples (≥500) without confounding run-to-run imprecision. The technologies of choice for this application are immunofluorescent bead or planar arrays. Experiments typically involve following the time course or dose response in multiple individuals or following multiple treatments or interventions. As with ELISAs, fluorescent intensities are converted back to mass units using standard curves, and several replicate observations are made for each protein and sample to calculate standard errors of values[73].

One field of research that is being dramatically affected by this use of protein arrays is the characterization of humoral immunity in common allergies[34,47,83-87], autoimmune disorders[31,32,49,88-92], cancers[37,93,94] and infectious diseases[33,38,95-99]. Comprehensive characterization of changes in humoral immunity is starting to become possible through the development of protein chips containing hundreds or thousands of potential allergens, autoantigens or epitopes arrayed as microscopic spots on planar glass slides (FIG. 2). Arrays are incubated with serum, then washed, 'developed' by incubation with a fluorescently tagged anti-immunoglobulin (for example, anti-IgE for allergy detection) or anti-immunoglobulin subtype, and quantified in a fluorescence scanner. Antigen arrays are simple

to develop and calibrate. Hundreds of antibody specificities can be screened simultaneously by class, subclass and titre.

Clinical uses of such protein arrays include disease classification on the basis of reactive or autoreactive epitopes, monitoring disease progression by measuring the change in epitope dynamics, immunoglobulin class or sub-class switching, and monitoring disease activity by analysis of the antibody titre. In limited comparisons with traditional 'monoplex' *in vitro* diagnostics, multiplexed antigen arrays have shown similar or improved diagnostic sensitivity and specificity[83,85]. Although studies so far have been limited to descriptions of states or differences between states, antigen arrays have the power to enable development of quantitative mathematical models of the dynamics of humoral immune systems in health and disease[100,101]. Furthermore, the development of high-throughput peptide expression systems has the potential to greatly expand the repertoire of such studies by allowing a broad array of epitopes to be surveyed simultaneously for humoral immune responses.

A related, highly innovative development is the construction of microarrays of substrates for protein activities or protein modifications[28,36,50,51,102-110]. Rather than measuring multiplexed protein abundance changes between states or in disease processes, such arrays measure changes in both specific protein activity and in global patterns of activity.

As noted above, a second area in which protein arrays are enabling new understanding of networks, pathways and disease states is in cytokine biology[43,45,111-114]. Multiplexed, immunoassay arrays allow quantitative, comprehensive measurement of cytokine families and networks. Cytokine arrays require less multiplexing than antigen arrays, but are technically more demanding, having the following requirements: they must be sandwich immunoassays (with antibodies capable of detecting two epitopes); pg ml^{-1} sensitivity; and a dynamic range of 3 logs (without detectable cross reactivity at the lower quantitation limits). Formats available for multiplexed cytokine measurement include bead arrays, planar glass arrays and nitrocellulose membrane arrays. Such arrays enable the multiplexed measurement of time course and dose response of cytokine production by specific leukocyte lineages following treatment with various lectins or ionophores or in disease states. These studies are essential for the validation of findings from gene expression or shotgun mass spectrometry studies, and are crucial for the development of mathematical models of cytokine networks. Such models permit prediction of responses to various stimuli and downstream changes in bioactivities. One such study examined cytokine network responses of dendritic cells to *in vitro* treatment with defensins[114]. RNAse, which was included in the study as a control treatment, was, unexpectedly, found to elicit dose-dependent secretion of pro-inflammatory cytokines. Subsequent studies confirmed that RNAse A superfamily proteins activate dendritic cells in a manner similar to that of tumour-necrosis factor — a hitherto unknown property.

21 CFR compliant

In compliance with Title 21 of the Code of Federal Regulations. This is the principal law enforced by the US FDA.

Protein arrays for biomarker development

A biomarker is a measurable 'biological marker' that correlates with a specific outcome or state: this subject has been reviewed recently in this journal[115] and is only briefly discussed here. As applied to drug development, the biomarker hypothesis states that changes in levels of blood or tissue proteins, individually or multiplexed, are highly and specifically characteristic of disease states and therapeutic outcomes. The basis of this hypothesis is that

biological systems are adaptive and that challenges to homeostasis effect characteristic topological perturbations of protein networks.

Biomarker development occurs in a four-step process of discovery, followed by replication in independent cohorts, validation of diagnostic sensitivity and specificity, and, finally, translation into a clinical diagnostic test or surrogate endpoint in a clinical study (FIG. 3). Candidate biomarkers are triaged at each stage of development. A biomarker can be a single protein (such as prostate-specific antigen), a panel of proteins or a combination of one or more proteins and other clinical measures, together with an algorithm that integrates individual values. In cases in which individual biomarkers lack adequate PPV or NPV, combinations of 3–10 protein profiles can be effective, providing a panel and algorithm with high PPV and NPV.

Given the magnitude of the current monoplex immunodiagnostic market (US$12 billion annually), interest is burgeoning in protein biomarker development for: first, early diagnosis, differential diagnosis, disease staging and determination of disease prognosis; second, patient selection and as surrogate endpoints during clinical trials of investigational drugs; and third, companion diagnostics for guidance in drug prescribing.

Although mass spectrometry is an excellent technology for the identification of biomarker candidates, it is currently inadequate for the later stages of biomarker development, or use in 21 CFR-compliant clinical trials or regulated *in vitro* diagnostics[116]. The technology specifications for multiplexed protein measurement for biomarker development in clinical studies are onerous: multiplexed assays must be available for most biomarker candidate proteins (typically 5–25 analytes) and be of sufficient sensitivity to measure analytes within the biological dynamic range; multiplexed assays must have similar precision to an ELISA (coefficients of variation of ≤10%); assays should have high-throughput capability; and have sample volume requirements and good laboratory practice (GLP) or good manufacturing practice (GMP) development times that concur with ‘go/no-go’ decision timelines. The technologies of choice for clinical studies are fluorescent bead or chip immunoassay arrays or ELISAs. Experiments typically involve analysis of samples from multiple time points or dose intensities in all patients in a clinical study, and comparison of biomarker value means or medians in groups of patients that differ in primary or secondary endpoints.

Most protein biomarker publications so far have involved the early diagnosis, differential diagnosis, disease staging or determination of disease prognosis[37,41,117-122]. Serum and plasma are the most widely examined specimen types until now, although interesting studies in saliva, tears, breath condensates, cerebrospinal fluid and tissue lysates have also been reported[123-125]. Studies have either used mass spectrometry for protein biomarker discovery, followed by array-based replication, or arrays both for biomarker discovery and replication[123]. No studies have been published that have taken biomarkers through the entire development process, and it is anticipated that many of the candidate biomarkers identified so far will lack sufficient predictive value for diagnostic development.

In particular, biomarkers are of great current interest in clinical trials of investigational drugs, given their potential ability to reduce trial duration and cohort size, or detect drug efficacy with greater sensitivity. Although only a few publications have substantiated this premise, most pharmaceutical companies are actively exploring the use of biomarkers in clinical trials. Two specific types of biomarkers that are highly prized are ‘bridging biomarkers’ that are useful both in preclinical and clinical development, and ‘surrogate markers’ that are sufficiently validated to substitute for a primary or secondary clinical endpoint or outcome in a clinical trial. In this author's experience, multiplexed protein measurement in Phase I and II clinical trials for several therapeutic areas can yield useful, objective measures of drug mechanism-of-

action, effect and efficacy. However, there has yet to be an example of a drug for which approval was expedited by the use of multiplexed protein measurement. Furthermore, claims for the use of multiplexed protein measurement in drug safety in clinical studies are, in this author's opinion, premature.

Arrays for panel-based clinical diagnostics

Multiplexed protein measurement is not new to clinical chemistry. However, until now, multichannel analysers (both discrete and continuous flow) have generally used large amounts of sample and reagents, carried out multiplexing by serial performance of single assays, and have not incorporated immunoassay or other ligand-binding assay techniques for low-concentration analytes. Furthermore, physicians have integrated the results of several single protein analyte measurements for the evaluation of organ function in hepatic, renal, myocardial, endocrine, immunological or infectious diseases. One example of a multiprotein diagnostic panel in common use is a combination of elevated levels of cardiac isoenzymes of protein markers, such as creatinine phosphokinase, troponin T, aldolase and lactose dehydrogenase, in serum in the diagnosis of acute myocardial infarction.

Two significant, recent advances in immuno-diagnostics are the development of miniaturized, multiplexed assays and panel algorithms that translate multiple analyte concentrations into a specific diagnostic or prognostic index[23,72,117]. Advantages of miniaturized, multiplexed assays are reduced time to first result, reduced cost of goods and smaller sample volume requirements (which is particularly important for paediatric assays). Early publications have engendered considerable excitement about biomarker-panel-based diagnostics or prognostics, especially for early diagnosis of cancer. It must be emphasized, however, that replication in independent cohorts, analytical validation by transfer to a qualified platform and biological validation by incrimination of a network or pathway in a particular disease process are essential for biomarker panels to have analytical and statistical rigour. With few exceptions, biomarker-based diagnostic candidates have not yet been examined for adequate PPV, NPV or area-under-the-curve of receiver–operator-characteristics[126]. Serum or plasma protein biomarker panels in development include those for differentiation of acute thrombotic and haemorrhagic stroke[117], and as objective outcome measures in sepsis and community acquired pneumonia.

Development of fully validated biomarkers into a clinical diagnostic test involves several steps (FIG. 3). The first phase involves multiplexed immunoassay development, in which optimized, multiplexed assays are developed for validated biomarkers and transferred to an immunodiagnostic platform. This stage is process delimited and guided by strict specifications and quality controls. The result is a panel of 2–10 dual-antibody sandwich, direct or indirect, immunoassays with documented specifications regarding multiplexed lower limit of reliable quantitation, precision, interferences, component stability, dynamic range, ease-of-use and turn-around time. The next stage is final *in vitro* diagnostic development, which is driven by regulatory, manufacturing and marketing considerations. Few established platforms exist for multiplexed *in vitro* diagnostics, and these capture the analytes of interest on chromatographic devices[127,128], on multiple labelled particles[129-131] or a single surface[132].

Conclusions

Multiplexed protein measurement is a rapidly advancing field (see TABLE 3 for primary, published manuscripts illustrating examples of uses of multiplexed protein measurement technologies) that has the broadest potential of any existing ‘-omics’ technology to transform drug discovery and development in the next 10 years. During this period, multiplexed protein abundance, activation state and activity measurement will become broadly applied for target validation and, with adaptation, will be applied to miniaturized, multiplexed high-throughput screens. In particular, quantitative, multiplexed protein abundance measurements will become

obligatory for the validation of the biological significance of results of functional genomics studies. Guided by FDA prompting, tort concerns and pipeline needs, biomarker data from multiplexed protein measurement technologies will become increasingly the norm, both in Investigational New Drug (IND) approval submissions and Phase IV (post-marketing) studies. Inexorably, we are entering an era of personalized medicine, in which inclusion criteria for clinical studies will be based increasingly on a patient's genetic or proteomic profile, surrogate endpoints will become more prevalent and approved indications will more routinely include biomarker-based diagnostics. In tandem, multiplexed protein measurement for biomarker-based diagnostic and prognostic testing will become the largest growth segment of the immunodiagnostics industry. Finally, multiplexed protein measurement has the capacity to identify surrogates that will be integrated into clinical indices, treatment algorithms, and, ultimately, into dynamic disease models that permit real-time, data-driven patient management.

Acknowledgements

A Deo lumen, ab amicis auxiliam (to God for illumination, to friends for help). This work was partially supported by a National Institutes of Health grant. Thanks to J. Huntley, D. Gessler and the referees for critically reviewing this manuscript.

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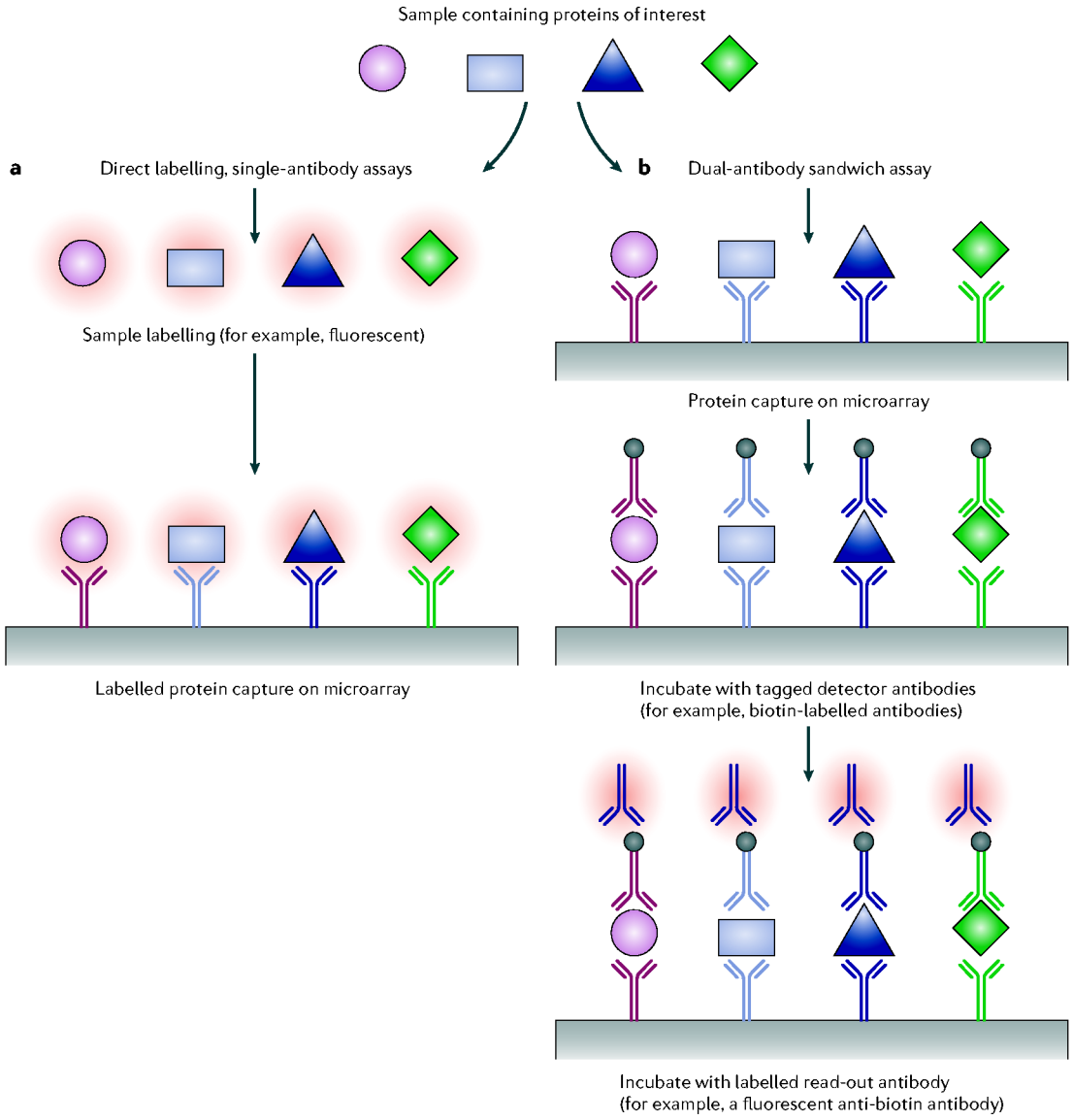

Figure 1.

Schematic representation of the two antibody microarray experimental formats. a | Direct labelling, single-capture antibody experiments. All proteins in a sample are labelled (red haloes), thereby providing a means for detecting bound proteins following incubation on an antibody microarray. **b** | Dual-antibody (capture and read-out antibody) sandwich immunoassays. Proteins captured on an antibody microarray are detected by a cocktail of tagged detection antibodies, which are matched to the spotted antibodies. The detector antibody tag is then measured by binding of a labelled (red halo) read-out antibody.

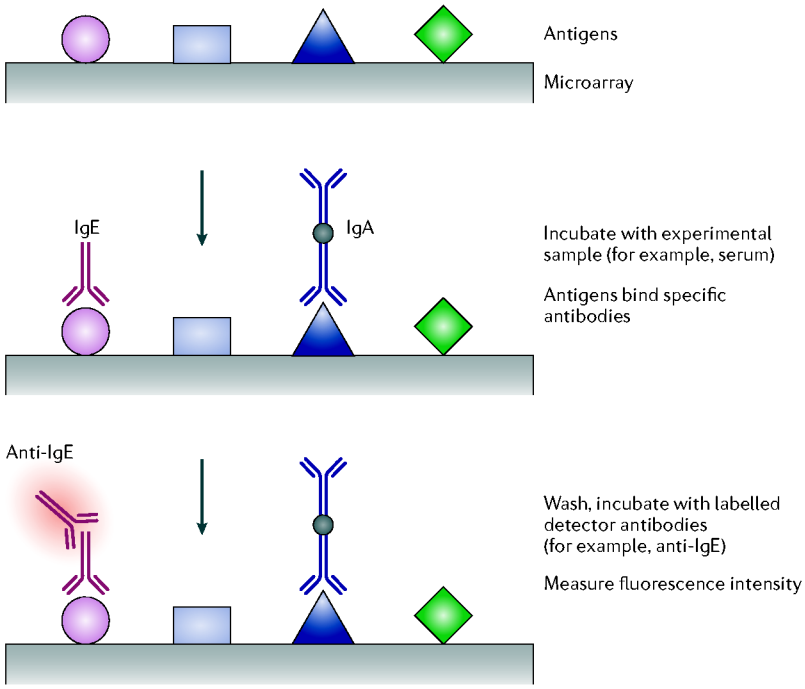

Figure 2.

Schematic representation of antigen or peptide capture arrays. Antigens are printed on the array, which is then incubated with an experimental sample containing antibodies. Binding of the antibodies to the antigens can then be detected by binding a secondary, anti-immunoglobulin (a read-out antibody) that has been fluorescently labelled (red halo). Ig, immunoglobulin.

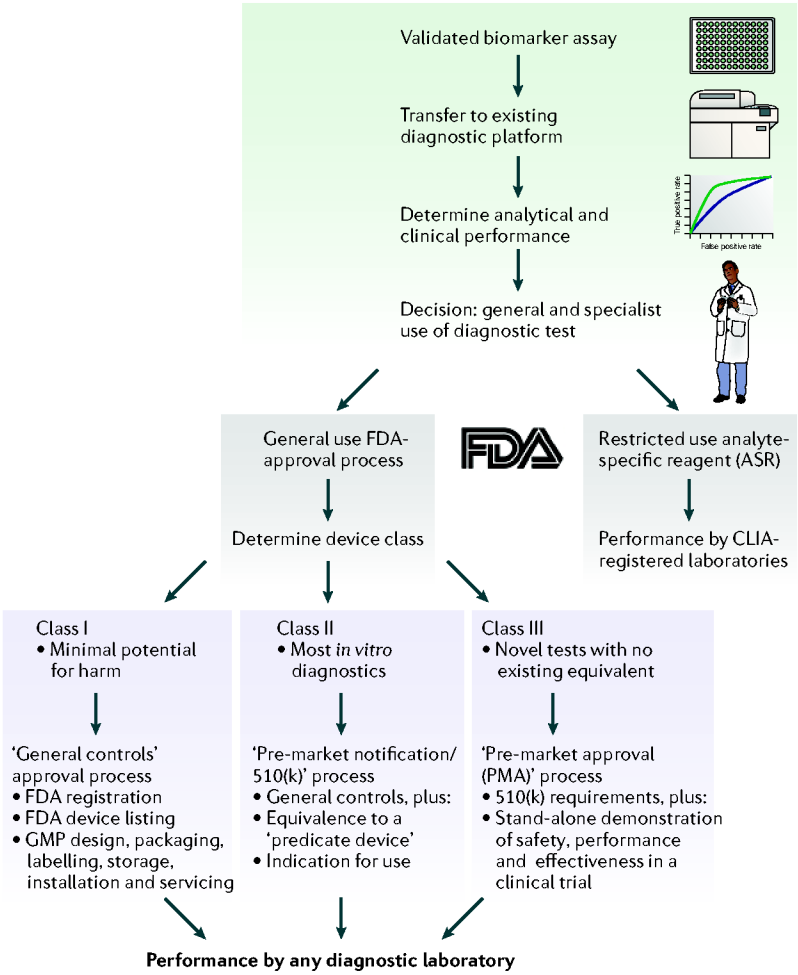

Figure 3.

Steps for the development of validated biomarkers into a clinical diagnostic test.

Optimized, multiplexed assays are developed for validated biomarkers and transferred to an immunodiagnostic platform. Then specifications are determined regarding analytical and clinical performance. Finally, *in vitro* diagnostic development is driven by regulatory, manufacturing and marketing considerations. CLIA, Clinical Laboratory Improvement Amendment (the US law to ensure quality laboratory testing); GMP, good manufacturing practice.

Table 1

Applications and technologies for multiplexed protein measurement

Application category	Technology	Approximate multiplexing	Technology characteristics	Desired specification	Experimental design	Refs
Surveys of most, large changes in protein abundance	'Shotgun' mass spectrometry	2,500	Universal, open architecture	Semi-quantitative; CV 30%	Iterative, cross-sectional; 50 samples	137
Modelling networks, pathways, physiological and disease states	Bead and chip protein arrays; indirect immunoassays	250	Multiple sets of canonical optimized assays	Quantitative; CV 10%	Longitudinal or dose-response; 500 samples	7
Biomarker validation; outcome surrogates in clinical trials	Bead and chip protein arrays; sandwich immunoassays	25	Flexible platform with rapid assay development	Quantitative; CV 5%	Cross-sectional or longitudinal; 500 samples	115
Panel-based clinical diagnostics	Multiplexed immunodiagnostic platform for rapid, point-of-care testing or automated central laboratory testing	5	Specific, highly optimized sets of assays	Quantitative; CV 3%; rapid format	Single sample comparison with reference range; >10,000 samples	127

CV, coefficients of variation.

Table 2

Pros and cons of array formats for multiplexed protein measurement

Format	Advantages	Disadvantages	Refs
Single antibody	Capacity to multiplex hundreds of analytes Require single capture antibodies	Currently semi-quantitative (ratiometric) Low specificity Low sensitivity	29
Dual antibody	High specificity High sensitivity Quantitative	Limited to ~50 analytes per array Requires matched antibody pairs	25,26
Antigen array	Capacity to multiplex hundreds of analytes Require single read-out antibody Capacity to measure certain activities	Generally limited to assays of antibodies, for example, autoantibody detection, infectious disease serodiagnosis Variability in specificity, sensitivity and quantitativeness	47,49

Table 3

Proof-of-concept applications of array-based multiplexed protein measurement

Application	Technology	Disease or biological process	Refs
Autoantibody profiling	Antigen arrays	Allergy	47,83,84
		Autoimmunity	31,32,49
		Cancer	138
Antibody response profiling	Antigen arrays	Immunization, exposure to infectious agents	33,139
		Diagnosis of parasite differential	140
		Determination of response to infection	141
Cytokine network profiling, enzyme substrate profiling	Antibody arrays, peptide arrays	Determination of antibody specificity	102
		Discovery of new biological activities of substances	28,114,103-109
Clinical biomarker development	Antibody arrays	Inflammatory bowel disease	119
		Sepsis	Personal communication
		Lung and ovarian cancer	121,122
		IND studies	142
Cytokine network profiling	Antibody arrays	Development of network/pathway models	5,45,114
Understanding disease process or mechanism of action	Antibody arrays	Severe combined immunodeficiency	Unpublished observations
		Cerebral palsy	118,143
		Systemic lupus erythematosus	Unpublished observations
		IND studies	142
Detecting adverse events	Antibody arrays	Immunization	Unpublished observations
Clinical diagnoses	Antibody arrays	Point-of-care stroke	117
		Diagnosis of myocardial infarction	126,128
		Screening of drugs of abuse	127
		Diagnosis of infection differential	

IND, Investigational New Drug application.