J. Biomater. Sci. Polymer Edn, Vol. 19, No. 6, pp. 725–753 (2008)

Also available online - www.brill.nl/jbs

Review

Diagnostic devices as biomaterials: a review of nucleic acid and protein microarray surface performance issues

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Received 9 May 2007; accepted 14 July 2007

Abstract—This review of current DNA and protein microarray diagnostic and bio-analytical technologies focuses on the different surface chemistries used in these miniaturized surface-capture formats. Description of current strategies in bio-immobilization and coupling to create multiplexed affinity bioassays in micrometer-sized printed spots, problems with current formats and review of some detection methods are included. Recommendations for improving long-standing challenges in DNA- and protein-based arrays are forwarded. The biomaterials community can contribute relevant expertise to these formidable bio-interfacial problems that represent significant barriers to clinical implementation of microarray assays.

Key words: Microarray; antibody; DNA; surface chemistry; immobilization; assay; diagnostics; assays; detection; protein array; analysis.

INTRODUCTION TO DNA AND PROTEIN MICROARRAYS

The microarray is a two-dimensional multiplexed surface-capture bioassay format comprising tens to thousands of microspots of immobilized capture agents (probes) with binding activity against libraries of soluble analyte molecules (targets). Capture spots of nucleic acids (e.g., DNA, aptamers), proteins (e.g., antibodies) or

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other affinity capture agents (e.g., lectins, metal chelates, inclusion complexes) are surface-immobilized in pre-determined spatial patterns or 'mapped' arrangements onto various solid substrates [1]. Capture of analyte to each spot from unknown solutions is monitored with several different possible detection technologies (optical, electrochemical, vibrational/mechanical). Computer processing of the microarray signal 'map' set allows rapid, simultaneous determination of multiple analyte presence in samples containing possibly thousands of analytes. The microarray concept — as a miniaturized, multiplex spotted format — was initiated by the ambient analyte model of Ekins and colleagues [2–6]. This largely theoretical notion has asserted that 'microspot' assays relying on immobilization of capture agents in a few square micrometer areas should be capable of detecting target analytes in solution with much higher analytical sensitivity than conventional macroscopic immunoassays. This assay performance advantage through capture area miniaturization has recently received some experimental validation [7]. Boosted by the completion of whole-genome sequencing projects and convenient reliable micro-printing access, DNA microarray technology rapidly became the first proving ground for Ekins' hypothesis [8, 9]. DNA and other nucleic-acid-based microarrays have made the most significant advancements to date in facilitating gene expression and genotyping studies over the past decade. However, two other motivations have prompted more recent development of protein-based microarray assays. First, genetic profiling alone does not provide sufficient information to decipher complex cellular signaling pathways and communication networks. Lack of correlations between cellular gene up-regulation, resultant mRNA transcriptional products and translated protein abundance begs for a more direct method to screen specific protein products. Second, accurate profiles of differentially expressed proteins by cells, tissue and indeed, whole organisms, required a convenient multiplexed format beyond the ELISA assay format for better defining biomarkers for both normal homeostasis, as well as distinguishing a wide range of diseases and responses to therapeutics. As a requirement of increasing demand for these capabilities, protein-based microarrays (including arrays of synthetic or recombinant peptides and proteins, single chain, synthetic, monoclonal or polyclonal antibodies and lectins) have exhibited rapid growth and diverse forms over the past few years to become important research tools for identification, quantification and functional analysis of proteins in basic and applied proteome research [10–13].

DNA MICROARRAYS

The DNA microarray exploits classic Watson–Crick and Hoogsten nucleotide base-pairing interactions to pair target DNA in solution with complementary probes immobilized on the array surface. While traditional nucleic acid analytical methods are limited to "one gene at a time", DNA microarray technology enables parallel processing of thousands of gene species concurrently by reducing individual assay features to sub-millimeter scales (50–200 μm) and putting them all on a single assay

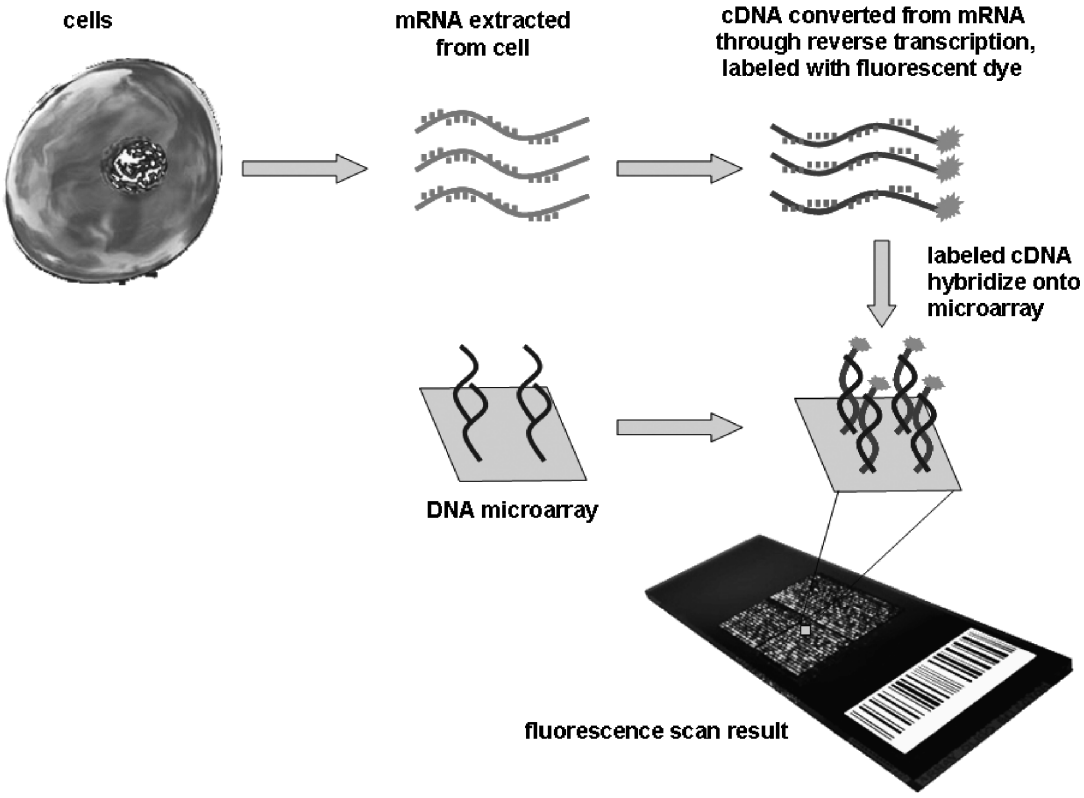

Figure 1. DNA microarray assay format: sample messenger RNA (mRNA) is extracted from a target cell or tissue, purified and converted into complementary DNA (cDNA), using reverse transcriptase polymerase chain reaction (RT-PCR) that provides fluorescence labeling. Fluorescently labeled sample cDNAs are then exposed and hybridized to a DNA microarray.

piece. This miniature assay format (Fig. 1) greatly reduces sample consumption, assay time and provides high-throughput assay technology for genomic studies profiling up to thousands of DNA targets in a single experiment.

To build a printed DNA microarray, synthetic or amplified libraries of DNA fragments representing a complete set (e.g., mouse or human), or a sub-set (e.g., human inflammatory markers) of genes are microscopically spotted, often with a robotic ink-jet style or contact pin-type printer [14], to predetermined spatial locations on a solid substrate, usually a low-fluorescence glass microscope slide. Robotic spotters (either contact pin printers or non-contacting inkjet piezo-droplet ejectors) pull aliquots of DNA probe aqueous solution from pre-formulated individual wells on 384-well or 96-well 'source' plates, depositing spot volumes of less than 1 nl onto these supports. These spots usually dry under an air environment within a few seconds onto the slide, forming roughly circular dried DNA probe domains with diameters of 100–250 μm, spaced 100–400 μm on center. With successful development of highly accurate robotic spotters, tens of thousands of such DNA probe spots, each representing a unique nucleotide sequence, are routinely immobilized on a single slide in a few hours, allowing printing of pieces of virtually every gene present in an organismal genome. As described below, this printing process requires some sort of substrate-reactive immobilization chemistry, embodied in a coating or crosslinking process to keep printed spots immobilized [15]. In many custom-made DNA microarrays, nucleic acid probes are usually

obtained in a library from oligo-DNA vendors and printed onto solid substrate. Probes bind to surfaces either through direct attachment, or adsorbed as insoluble aggregates. Recent work has shown that printed spot variability upon drying is not understood and perhaps a significant source of array signal variation [16].

Nucleic acid probes can also be synthesized directly, base by base, from the solid substrate. To date, the most popular microarray technology, Affymetrix's GeneChip® microarrays, comprises 25-mer probes synthesized directly on the solid support using a sequential, light-directed photolithography process [17, 18]. Surface chemistry exploited for this strategy uses a proprietary hydroxy-terminated organosilane layer on fused silica optimized for their particular synthetic approach [17, 18]. This is coupled with repeated phosphoramidite photo-deprotection cycles involving sequential surface exposure to alternating cycles of UV light then photo-protected DNA nucleotides in selective spatial locations using complex photomasks, creating DNA oligomer libraries *in situ* on surfaces. Resulting spot sizes are smaller (e.g., 6 μm diameter) and generally more uniform than oligomer spotting technologies.

In the typical DNA microarray assay format (see Fig. 1), messenger RNA (mRNA extracted from a cell culture, food, or tissue sample) is purified and converted into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction (RT-PCR) that often includes incorporation of fluorescently tagged nucleotides to produce cDNA amplicons with fluorescence labels. Once labeled, resulting cDNA target samples must be purified extensively and then are hybridized to a DNA microarray using high ionic strength solutions for hours. Each microarray spot contains approx. 10^{12} copies of one printed single-strand DNA probe sequence strongly bound to the surface, allowing fluorescently labeled cDNA targets to hybridize specifically to select complementary spots on the array. After stringency rinsing of the hybridized array and drying, signal is detected by exposing the dry microarray to an optical (e.g., fluorescence) scanner or microscope: hybridized spots are identifiable as fluorescent array spots, while spots with no target hybridized are not fluorescently visible. Microarray fluorescence data do not provide a directly quantitative measurement of gene presence for each gene represented in the microarray. Assay signal is generally judged to be significant when different than two log orders of spot intensity increase or decrease (i.e., $^{2}\log S$) over some baseline control, usually a fiduciary gene marker set or 'normal' expression sample [19]. Importantly none of the signals translate to specific quantities of genes or markers. Assay detection limits vary, depending on substrate, probe length, target length, detection milieu and spotting method, with femtomolar DNA target detection frequently reported from saline solutions [20].

At the end of 2004, the US Food and Drug Administration (FDA) cleared the AmpliChip™ Cytochrome P450 Genotyping test (Roche Molecular Systems) for use with the Affymetrix GeneChip Microarray Instrumentation System (Affymetrix). By analyzing expression of a key specific gene (cytochrome P4502D6) within the cytochrome P450 family that plays an important role in metabolism of commonly

prescribed drugs [21], this assay helps predict patient metabolic responses to certain drugs. This information assists physicians in prescribing proper drug dosing in patients at risk for drug toxicity. In 2007, the DNA microarray-based breast cancer prognosis test, MammaPrint® (Agendia, The Netherlands), was cleared by the FDA. MammaPrint®, produced by Agilent's inkjet printing array platform, is part of an *in vitro* diagnostic laboratory service that profiles the expression activity of 70 breast-tumor-associated genes in a surgically-removed suspect breast tumor biopsy, yielding correlative information about the likelihood of tumor recurrence [22]. The assay is only approved as an adjunct to traditional tumor oncology and histological profiling. Its clinical utility is in assisting selection of chemotherapy options to minimize recurrence.

Despite some success and rapid development of numerous DNA microarray technologies, numerous challenges remain in understanding the biological and clinical significance and metrics of, the assay signal, screened data and various practical issues pertaining to reproducibility, quality control and correlations among different microarray methods and platforms [23–25]. To address these challenges, the FDA has collected researchers from the government, industry and academia together to form the MicroArray Quality Control (MAQC) consortium, to identify and assess critical factors affecting array data quality and to optimize and standardize microarray procedures [23, 24, 26–33]. The FDA's active participation and the coordinated efforts of the MAQC project seek to validate microarray platforms in producing high-quality, reliable data useful in drug development, disease diagnosis and regulatory decision-making. Already the MAQC project has provided a solid foundation for 'best microarray practices', including the use of reference material, data assembly and formats [23]. Additionally, the standardization and validation of DNA microarray quality control procedures in the MAQC project should serve as a foundation for future development of the more complicated protein microarray.

PROTEIN MICROARRAYS

Protein microarrays can refer to either the protein-based affinity capture agent printed into the array, or to the proteinaceous analyte intended to be captured on-array, or both. Comprehensive analysis of target proteins is required to obtain a complete picture of normal *versus* disease processes in the body. Protein arrays offer new opportunities for full spectrum assay of multiple protein analytes, analogous to what DNA microarrays have already provided for genomic analytes in a massively parallel, miniaturized and automated routine. Representing a radical revolution from conventional "one protein at a time" methods such as ELISA, column chromatography and spectroscopic studies, protein microarrays promise exciting new capabilities for protein expression profiling, disease diagnosis and drug discovery [11, 34–36]. In particular, the concept of getting disease diagnosis one step closer than DNA genetic profiling, to direct proteinaceous agents in

pathogenesis, is clinically attractive, since, significantly, gene expression does not always correlate with translated protein product abundance.

The basic assay concept, processing steps and microarray fabrication robotic systems used for DNA microarrays can all be directly translated to protein microarray technology. However, as the fundamental difference between proteins and DNAs lies in their chemistry and the associated higher order structural adaptability of DNA, less robust protein microarrays remain in an early development stage. Proteins immobilized on array surfaces behave differently than immobilized nucleic acids. Thus, protein microarrays pose several other significant analytical challenges beyond those already recognized for DNA microarrays. First, the sheer number of targets in the human proteome (estimated 4×10^4 to 1×10^6) is several orders of magnitude more abundant than genes ($(2–2.5) \times 10^4$) [37]. Unlike DNA with its relatively simple primary structures and electrostatic properties, proteins have diverse chemical, structural and biological properties, producing widely different protein–protein, protein–antibody affinities and immobilized protein–microarraying surface interactions. The most common protein capture reagent printed into microarrays is the immunoglobulin G (IgG) antibody, generated synthetically or immunogenically against thousands of targets to date. However, even with nanomolar ligand affinities typical of commercial monoclonal antibodies, analyte capture fidelity, sensitivity and shelf-life issues plague capture agents for protein arrays. High quality sources of specific antibodies or suitable protein binding ligands remain the critical limiting factor for bioassay performance and a critical starting point for protein microarray improvements. Second, protein analyte concentrations of interest for clinical detection range widely from nanomolar (nM) to femtomolar (fM) to even attomolar (aM) and zeptomolar (zM) for the most to least abundant proteins. In relevant bioassay milieu, however, low abundance target analytes (aM to zM ranges) exist within complex biological milieu (e.g., serum) in a huge excess of non-target proteins, producing problematic antibody cross-reactions and unacceptable signal-to-background ratios [1]. Hence, improved surface chemistry and immobilization methods and highly specific capture agent selection are crucial to enhance difficult-to-detect analyte capture. Third, no direct amplification methods for protein analytes exist analogous to polymerase chain reaction (PCR) for DNA, making protein microarray detection sensitivity at fM or even lower ranges very difficult. Fourth, unpredictable levels of arrayed antibody cross-reactivity to identical analyte targets complicate reliability and signal in diagnostic tests. High-density multiplex protein microarrays require validated capture and sandwich antibodies with improved specificity to reduce current analyte or antibody–antibody cross-reactivity [38], an important unsolved problem whose significance is drastically under-estimated. Fifth, maintaining structural conformation and bioactivity of desiccated, printed/immobilized capture proteins on microarraying surfaces remains a major issue. Unlike single-strand DNA oligomers that remain stable under dry conditions and retain capture activity after long periods in storage, proteins are more susceptible to desiccation-induced damage [39–43]. For all these reasons, protein microarrays are much more

challenging than DNA microarrays and have not achieved assay performance comparable to DNA counterparts. Currently, the protein microarray market is at the same point that the DNA array market was five years ago. Despite having adequate miniaturization and speed to meet the demands of clinical diagnostic applications, protein microarrays have not yet overcome the scientific, clinical performance and economic hurdles to enter the clinical diagnostics market. Limited by accessible proteins/antibodies with low cross-reactivity, many research focused on low-density (3–50 analytes) protein microarrays. Given the well-recognized problems with proteins at interfaces in biotechnology and biomaterials [44, 45], many protein interfacial issues will be difficult to solve, stimulating development of alternative affinity reagents for protein diagnostic targets.

Protein microarrays have many diverse formats for protein target analyte capture (Fig. 2). Recombinant proteins, antibodies, short peptides, aptamers and unique oligonucleotides can all be screened and used as protein-specific capture agents printed onto substrate surfaces in different applications [46]. Microarrays fabricated with printed recombinant proteins have been used in protein–protein and protein–drug interaction studies [47–50]. Antibody microarrays provide an excellent format for protein expression profiling [51, 52]. Both formats have produced impressive results for detection of pathogenic bacteria and viruses, cancer, autoimmune disorders, food allergies and other diseases [53–57]. Additionally, other affinity reagents including short peptides, synthetic oligonucleotides and aptamers are increasingly used in various assays, including diagnostics, epitope analysis and protein folding studies [1, 11, 35, 57–59].

While many different protein microarrays are reported, most assay processing steps beyond reagent selection are similar to DNA microarrays, including sample preparation and handling (probe and target), microarray fabrication, surface capture affinity assay (antibody–antigen binding, protein–protein interaction, protein–drug interaction, etc.), signal detection and data analysis.

MICROARRAY SURFACE CHEMISTRY AND COUPLING SCHEMES

Various strategies are used for microarray construction on a wide range of substrates. As summarized above, DNA microarrays can be manufactured either by synthesis of oligonucleotides *in situ* on chip, or by microarray printing using robotics or other delivery techniques of pre-synthesized oligonucleotides and cDNAs from solutions. By contrast, most protein microarrays can only be printed onto substrates because the capture elements required for analyte binding specificity exceed the size of peptides readily synthesized *in situ* [60], another important distinction from nucleotide arrays. Capture biomolecules modified with tags to effectively anchor onto chemically reactive microarray or sensor substrates are becoming more popular. Specially designed surface chemistries for tag binding improve surface coupling efficiency and minimize non-specific binding from undesired cross-reactive groups intrinsic to many DNA/protein combinations with surfaces [61].

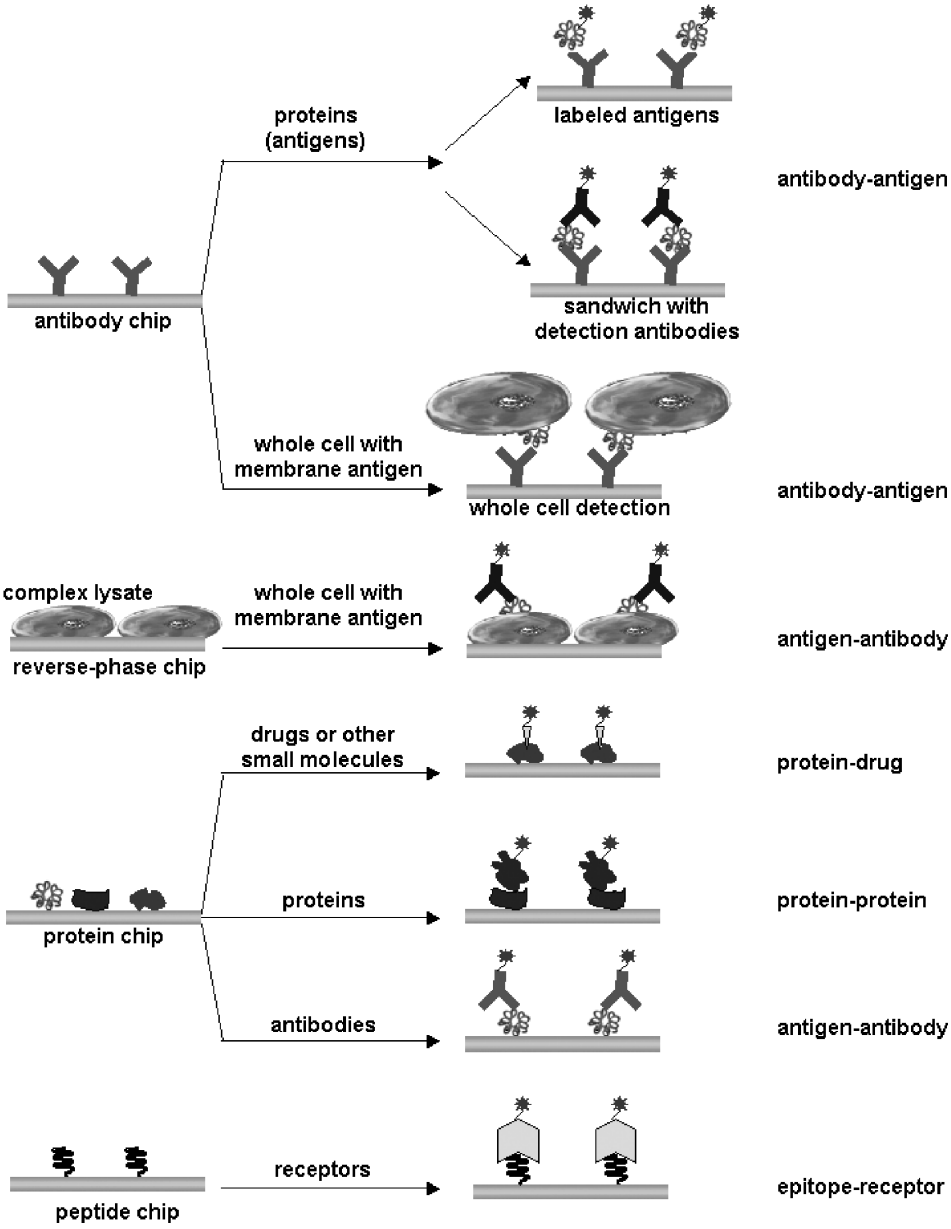

Figure 2. Different formats for protein microarray applications. Antigen and antibody microarrays have been used primarily in diagnostics, while protein microarrays have various potential in assaying a wide range of biochemical activities including protein–protein and protein–drug interactions [152]. Peptide microarray can be used in epitope analysis. Cell suspensions can also be incubated on microarrays of antibodies targeting cell surface antigens. Reverse-phase chips are fabricated by spotting complex cell lysates on substrate surfaces, then the presence of particular proteins in the lysates are quantified by incubation of antibodies targeting those proteins.

Nevertheless, many approaches for tailoring microarray surface properties to improve immobilized bioactivity of unmodified biomolecules exist [15]. Direct surface attachment avoids adverse effects of further capture molecule modifications (e.g., altering inherent biomolecular structure).

Microarray substrates

Microarrays are constructed on various substrates including glass, gold, silicon oxide and nitrocellulose membranes. Essential practical support features are

quite straightforward: inexpensive solid supports or membranes must reliably immobilize printed capture agents and retain their specific analyte capture activity in various assay and detection formats over time. Arrayed surfaces must also be amenable to sensitive detection methods and instrumentation. Importantly, the array surface community appears to be divided currently along lines drawn by key patents dictating freedom to operate in the commercial domain: (1) direct affinity reagent printing and dessication to an intrinsically adhesive solid support (i.e., nitrocellulose), (2) reactive organo-thin film modification (i.e., alkylsilanes, functional thiols and polymer monolayers) as a binding matrix coated onto a solid support and (3) thicker, three-dimensional reactive films (i.e., immobilized hydrogels, sol gels) coated over solid supports.

Much of the current microarray processing and fabrication equipment, including robot microarray printers and microarray fluorescence scanners, is built around the standard $1'' \times 3''$ low-fluorescent glass slide. Additionally, extensively studied reactive organosilane coatings on glass and other hydroxylated solid surfaces [62–64] pair well with glass slides in microarray applications. Chemi- and physi-sorbed functionalized alkylsilane films readily form from solutions onto glass and native oxides of silicon, titanium and many other metal substrates surfaces. These complex polysiloxane layers form spontaneously *in situ via* silane hydrolysis, achieving networks of inter-silane Si–O–Si bonded polymers as shown in Fig. 3, as well as silane–surface covalent and non-covalent associations. Silane layer control and reproducibility remain problematic for commercial use. The silane layer permits direct modification of glass surfaces for immobilization and binding of printed affinity surface capture agents. Surface reactivity to printed proteins and nucleic acids can be conveniently and inexpensively achieved using commercial alkylsilanes that bear terminal reactive groups, including epoxide, amine, carboxylate, vinyl, acrylate and alkylhalide functionalities.

Gold is another popular substrate for DNA and protein immobilization and microarray fabrication. The epitaxial gold–thiolate bond (RS–Au) is reasonably robust, with a dissociation energy of approx. 40 kcal/mol [65, 66], providing a convenient and reliable capability to modify gold surfaces from dilute solutions with various thiolated molecules, including proteins, aptamers and DNA. Self-assembled monolayers (SAMs) spontaneously formed by organosulfur compounds on gold have been extensively studied and well-characterized for decades [66–69]. Various terminal chemical functional groups yield surface properties relevant to immobilization and microarray fabrication. Gold surfaces can be easily fabricated by evaporation, sputtering, particle nucleation, or electroless redox chemistry and remain relatively stable (i.e., non-oxidized) under normal microarray fabrication conditions. Despite these advantages and abundant fundamental academic studies [70–75], widespread commercial use of gold-based microarrays is limited by cost and adlayer (e.g., RS–Au) thermal stability (i.e., unsuitable for high-temperature wash conditions), as well as by gold-fluorescence quenching, inappropriate for common fluorescence detection on many microarray formats. However, gold's

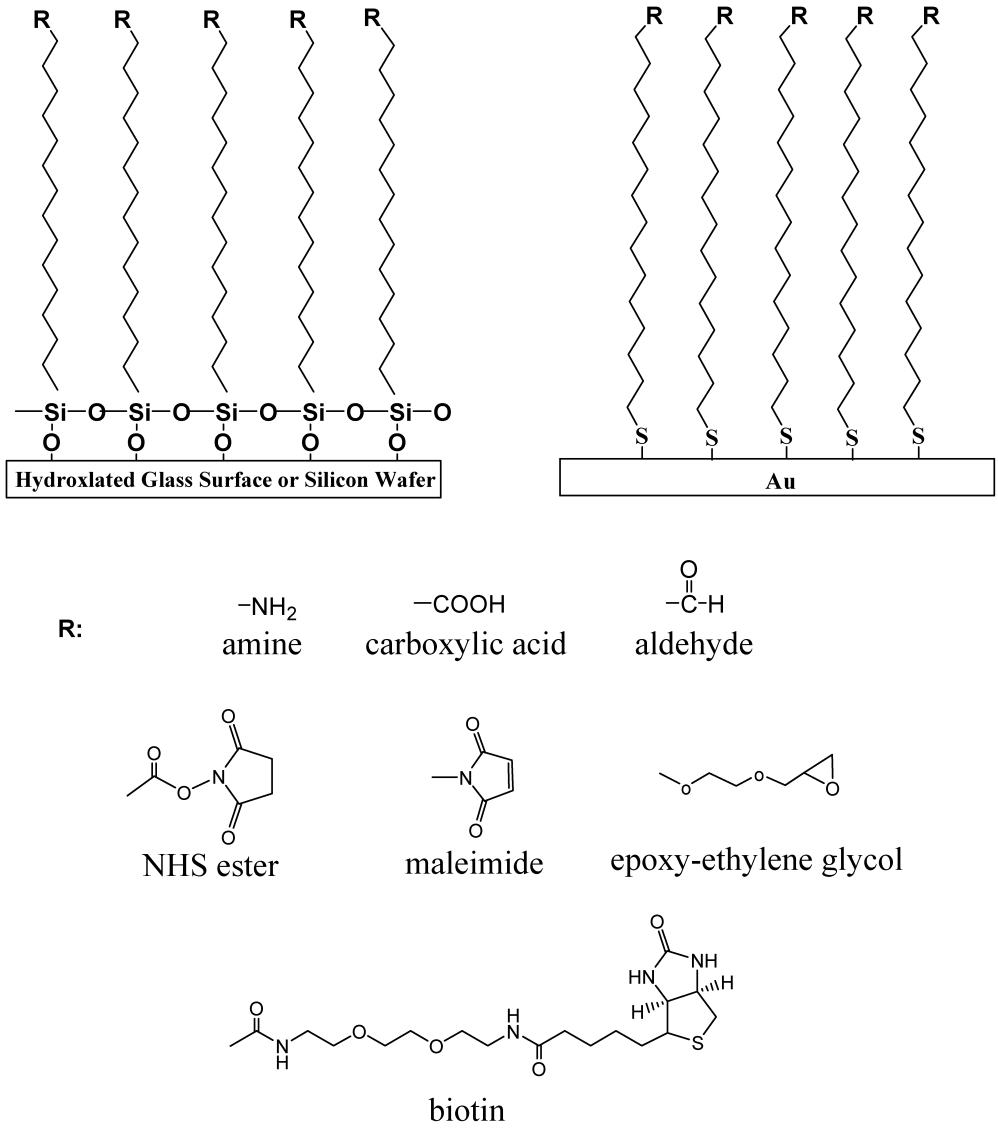

Figure 3. Schematic representation of polysiloxane adlayers formed from alkylsilanzation from solutions onto glass or silicon oxide wafers and alkanethiol (SAM) monolayers on gold surfaces. While alkylthiolate–gold adlayers are generally better controlled, alkysilanes are well-known to form multi-layer networks on these surfaces in addition to more idealized monolayers shown (left). Various terminal functional groups can be introduced to these substrate films for DNA and protein microarray immobilization.

intrinsic metallic properties in thin, evaporated films on solids permit use of label-free technologies such as surface plasmon resonance (SPR) for surface capture detection [76–79].

Three-dimensional surfaces, including porous bulk phases (e.g., zeolites) and porous membranes, are also applied in microarray technologies, especially in protein microarrays and some commercial formats. Theoretically, three-dimensional formats provide larger surface areas and higher biomolecule probe loading capacities and, hence, higher signal capture potential. Polymer membranes such as aminated nylon [80] and nitrocellulose [81] have been widely employed in microbiology laboratories as protein and DNA blotting supports [82, 83]. Gel-based immobilization studies combined with mass spectrometry are routine in proteomics investigations [61, 84]. Arrays fabricated on thin hydrogels on gold substrates have

dominated real-time protein interaction studies for the past decade because widespread application of SPR and SPR imaging (SPRI) technology [85–89]. Polymer films and hydrogels coated on glass are extensively used for conventional DNA and protein microarray formats [15]. Distinct in their three-dimensional film matrix properties over classic organic monolayers, coating thickness exceeds the dimensions of immobilized molecules. Many commercial available microarray formats based on three-dimensional reactive polymer films and hydrogels (e.g., GE's Codelink™ [70], Accelr8's Optarray™ [90], Whatman *Fast* protein microarray slides, Fisher Scientific) are often used for spot-printed array immobilization [15].

Surface coupling chemistry for array bio-immobilization

Various chemical functional groups for coupling DNA probes and affinity capture proteins onto glass, silicon and gold through self-assembled monolayer of organosulfur and silane compounds are shown in Fig. 3, with paired complementary surface attachment chemistries also listed in Table 1. Importantly, array-printed molecules dry rapidly on substrates under most spotting technologies and therefore are not simply immobilized by covalent forces. Covalent coupling is often presumed but is not the exclusive or even primary mode of affinity reagent–surface binding interactions. Significantly, printing of proteins produces substantial noncovalent forces upon microspot dessication [91], eliminating the need for covalency but compromising affinity capture bioactivity.

Table 1.
Methods to bio-immobilize DNA and proteins onto microarray platforms [61]

Functional chemistry on protein/peptide or DNA/oligonucleotide	Available surface functional groups	Type of surface binding
Polyphosphate backbone	Amine, poly(L-lysine)	Electrostatic
–COOH (carboxylic acid): Asp, Glu		Electrostatic
		Covalent amide (after carboxy activation)
$-NH_2$ (amino): Lys, Gln, Arg	Carboxylic acid, active ester, epoxide, aldehyde, isothiocyanate	Covalent amide
–SH (thiol): Cys	Maleimide, disulfide, vinyl sulfone	Covalent thiol ether
	Gold	Gold–thiolate bond
–OH (Ser, Thr)	Epoxide	Covalent ether
Synthetic 6 × His-tag	Metal complex	Coordination complex
Biotin	Streptavidin	Affinity complex
Various	Azidophenyl	Covalent bond (photochemical radical insertion)

Cationic surfaces. Many amine-terminated organosilane-coated slides provide cationic surfaces for electrostatically binding anionic DNA probes during microarray printing, the basis for several popular commercial silane-based glass microarray substrates. A post-print UV or thermal cross-linking step can be used to achieve covalent linkage between printed DNA molecules and these surfaces *via* the thymidine nucleotide's known photochemical reactivity with nucleophiles [92]. Protein microarrays have also been generated on aminosilane cationic surfaces through non-covalent binding [93]. Poly(L-lysine) (PLL)-modified slides also provide cationic immobilization of DNA probes and proteins. PLL, a basic, synthetic, positively charged poly(amino acid) polyelectrolyte, adsorbs from aqueous solution to negatively charged glass surfaces with sufficient residual cationic charge to bind DNA and proteins through electrostatic interactions. Such microarrays have relatively low stability, especially for protein microarrays and lack requisite non-specific binding properties (noise shedding) during assay [15]. Negatively-charged analytes and many other assay reagents abundant in physiological milieu also adsorb onto these cationic surfaces.

Amine-reactive surfaces. Surface covalent attachment of DNA and proteins improves attachment reliability and stability and generally lower assay background compared to cationic surfaces. Many microarray substrates are designed to be amine-reactive for covalent immobilization of many synthetic printable oligonucleotide probes, native peptides and proteins containing endogenous or exogenously added nucleophilic primary amine groups, respectively. Well-known amine-reactive immobilization chemistries (Table 1) include epoxides, aldehydes (Schiffs base formation), isothiocyanate, isocyanate and reactive esters including *p*-nitro- and perfluoro-phenyl and N-hydroxysuccinimide (NHS) reactive esters [94]. Epoxide reactivity leaves residual secondary hydroxyl groups; aldehyde coupling with amines requires an additional reduction step for stability; isothiocyanate is relatively unstable in ambient or aqueous conditions and is best stored desiccated at low temperature; reactive esters (e.g., NHS groups) exhibit high intrinsic reactivity to nucleophiles and are available in many bioconjugation formats for surfaces, but also suffer from competitive hydrolysis under ambient conditions [94]. Although the NHS ester hydrolysis side-reaction is usually slow below pH 9, humidity causes significant loss of binding reactivity for oligonucleotides after two months [95, 166]. A one-step reaction to regenerate NHS-reactive chemistry *in situ* on microarray surfaces has been reported to perform equally to freshly prepared microarray slides in print-immobilization of oligonucleotides [95]. Amine coupling is less suitable with acidic ligands (pI $<$ 3.5), ligands where an amine is in the active site and some cases of molecules possessing many amine groups [96].

Thiol-reactive surfaces. Thiol groups are either endogenous to many biomolecules (e.g., cysteine) or relatively easy to introduce synthetically. Thiol-reactive surface chemistry is more robust than amine reactive chemistry, so coupling conditions

are less critical [96]. DNA oligonucleotides and proteins bearing thiol groups are covalently immobilized onto many types of thiol-reactive surfaces. In proteins, thiol groups are present in isolated cysteine residues, but are also generated by selectively reducing exposed cystine disulfides with appropriate reagents (e.g., dithiothreitol, DTT, 2-mercaptoethanol (β-mercaptoethanol) or tris-(2-carboxyethyl)phosphine, TECP). Surface chemistries for covalent thiol coupling include maleimide [97] and other activated vinyl groups, pyridyl disulfide and vinyl sulfone chemistries. Reaction of any of these functional groups with thiols usually proceeds rapidly at or below room temperature in the physiological pH range (pH 6.5–8.0) to yield chemically stable thioethers. Thiolated molecules also immobilize directly *via* metal thiolates onto gold, silver, platinum, copper, zinc and other coinage metal substrates. Oligonucleotides immobilized on gold provide a simple two-dimensional model for studying DNA surface density, probe orientation and hybridization efficiency of immobilized DNA oligonucleotides [71–74, 98–100], as well as ready nanoparticle conjugates for other complex assay formats [101].

Photochemical coupling. Due to convenience, direct processing and speed, photochemistry has been widely used in biomolecule patterning on substrate surfaces [102–106]. Photochemistry can facilitate both surface chemistry patterning on and biomolecule reactions with surfaces. In the former case, a surface pattern can be created using spatially selective or masked photochemistry to produce reactive functional groups only at specific surface sites for binding proteins, DNA and other biomolecules. In the latter case, photo-actively derivatized biomolecules can be deposited uniformly onto surfaces bearing homogeneous films of reactive functional groups, followed by exposure to light of appropriate wavelength, through a mask if a pattern desired. Only biomolecules exposed to light should covalently attach to surfaces; unexposed biomolecules can (in theory) be washed off the surface. They can also photo-oxidize, non-specifically immobilize and degrade if the process is too aggressive.

Azidophenyl functionalization is a prominent example of photochemistry used to immobilize DNA and proteins through photochemical coupling. Excitation wavelengths for the photoreaction can be adjusted using additional substituent groups on the azidophenyl ring [107, 108]. Upon irradiation, these compounds generate a nitrene radical that readily inserts non-specifically into various chemical bonds (C–H, O–H, N–H, N–O, etc.) (Fig. 4). This compound has been widely employed

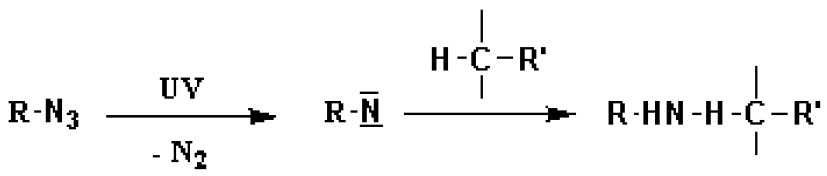

Figure 4. Schematic representation of the insertion reaction into C–H bonds of the nitrene radical (R–N), formed upon irradiation of azidophenyl-functionalized compound (R–N_3). R′ can be protein, DNA, organic surfaces and other organic molecules.

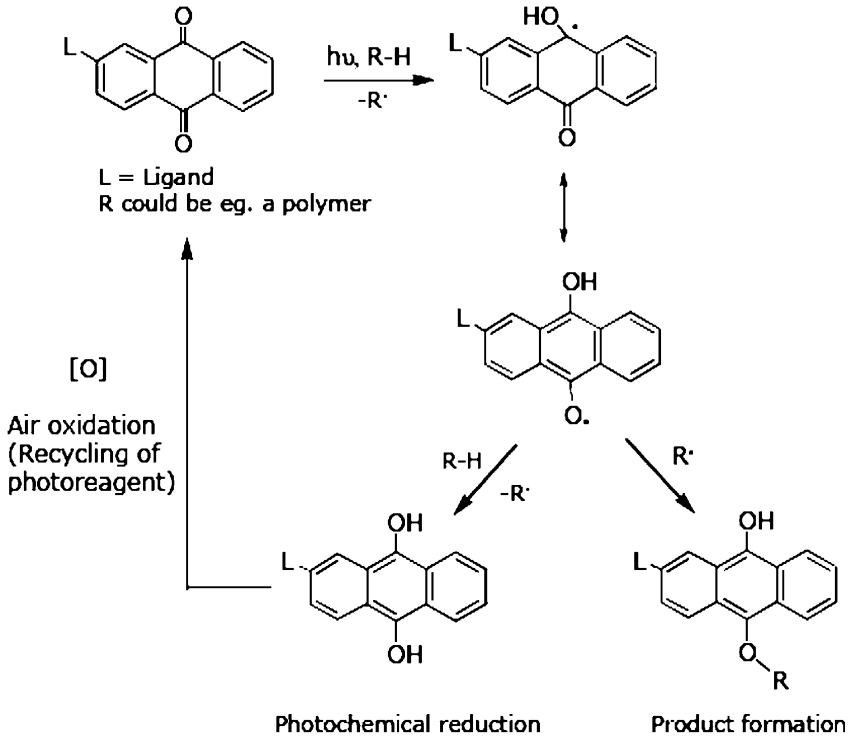

Figure 5. Exiqon's patented anthraquinone (AQ) photochemical attachment method [109].

in reactions with organic surfaces and conjugations with proteins and DNA [104]. Another photochemical coupling method uses an anthraquinone linker (AQ) developed by Exiqon (Vedbaek, Denmark) permitting radical coupling to a wide variety of organic substrates upon irradiation (e.g., polystyrene, polypropylene, PMMA) and conjugation with biomolecules, ligands and small reactive groups (carboxylic acids or amines) [109]. AQ photoreagents can even be recycled *via* photochemical reduction and air oxidation (Fig. 5).

Photoaptamer techniques (e.g., see SomaLogic, USA) represent another versatile method for photochemical cross-linking of captured proteins to surfaces. Aptamers are synthetically manufactured, single-stranded DNA and RNA molecules selected to bind target molecules with extraordinary affinity and specificity through large contact areas between the selected aptamer and its target (e.g., protein). Photoaptamers recognize both the complex shape and charge distribution of their targets and the presence of specific amino acid residues at specific sites, imparting important specific binding [110]. SomaLogic's photoaptamers contain 5-bromodeoxyuridine (BrdU) in place of deoxythymidine. These halogenated bases provided photoreactive sites to cross-link bound target proteins. Aptamer arrays are printed, exposed to analyte targets and then irradiated with UV light, photo-cross-linking the aptameric BrdU to electron-rich amino-acid side-chains on the captured target protein.

Affinity complex binding. The biotin-streptavidin binding architecture has been widely used for immobilizing biomolecules to surfaces. Biotin binds to one of four streptavidin tetrameric binding sites with strong but reversible affinity

binding energy (K_d approx. fM, about 20 kcal/mol) [111, 112] in aqueous solutions. Two pairs of biotin binding sites lie on opposite faces of streptavidin, allowing crosslinking of biotinylated surfaces [113, 114]. Nucleic acids, proteins and even cells can all be biotinylated using a variety of reagents and functional groups and immobilized by passive incubation [115]. Many commercially available biotinylation kits allow facile modification with biotin; technology for stable non-covalent biomolecule surface binding is growing rapidly [116–119].

Another affinity label in protein chemistry is the 6 × His affinity tag [120–122] comprising approximately six histidine residues introduced artificially near the N- or C-terminus of a protein, generally using fusion protein technology. Nickel ion-chelate nitrolotriacetic acid (Ni-NTA) complexes introduced onto biochip surfaces are exploited for metal-ion coordinated immobilization of 6 × His-tagged proteins [61, 123–125]. One advantage of these metal chelate systems is their reusability. After capture assay experiments, immobilized proteins can be removed using a strong metal-complexing agent such as ethylenediaminetetraacetic acid (EDTA), or changing pH, or competitive elution using histidine. However, nickel ion chelate binding of His-tagged proteins is not very stable, susceptible to interference by commonly used salts and chemicals [126]. Several commercial suppliers provide Ni^{2+} ion-based affinity kits and substrate products for 6 × HIS biomolecular screening (e.g., Greiner and Qiagen).

Background surface passivation

Removing residual surface reactivity after DNA/protein immobilization on substrate surfaces is a crucial step in microarray fabrication. Excess reactive groups on substrate surfaces must be eliminated to avoid non-specific binding, but without influencing binding activity of surface-bound DNA/protein capture agents. Depending on specific surface chemistry and characteristics, various reactive small molecules are used to quench surface chemistry after affinity agent immobilization and prior to assay use. Common quenching agents for surface passivation include mercaptoethanol for thiol-reactive surfaces [91], ethanolamine for amine-reactive surfaces [95, 127]), surfactants (e.g., Tween 20 [128]), hydrophilic polymers (e.g., PEG [129]) and non-reactive proteins (e.g., albumin [130]). Importantly, passivation can directly influence microarray performance [128, 130], even producing totally different assay results using different passivation agents [128]. Additionally, passivation introduces yet another processing step and variable into the array assay. Future surface chemistry designs might seek to eliminate this step and associated assay reliability issues if it might reduce the background in other ways.

STRATEGIES FOR IMPROVING PROTEIN MICROARRAY IMMOBILIZATION

Microarray printing: non-equilibrium drying is different than solution immobilization

In typical microarray printing applications, robotic pins deliver 1–2 nl drops of printing solution onto microarraying slides. Under normal conditions on the print deck (room temperature, relative humidity 50–70%), such small volumes evaporate in a few seconds. Biomolecules in the printing solution never reach equilibrium with the reactive immobilization surface in this short period. The question really is what chemistry can occur between the printed solution and surface reactive groups before? Immobilization of DNA and proteins from these nanodrops onto substrate surfaces under non-equilibrium conditions has not been thoroughly investigated or clearly described. Nonetheless, this appears to be a natural mechanistic consequence of common microarray printing/fabrication formats. Our investigations of capture agent printing on two-dimensional maleimide-modified silicon nitride surfaces and commercial three-dimensional amine-reactive polymer microarraying slides revealed that covalent reactions were important for reliable DNA microarray immobilization, but not necessary for protein/antibody microarray immobilization [91, 127]. Differences between micro-printed DNA and protein immobilizations result from this fast evaporation, non-equilibrium microarray printing condition and the inherent differences between DNA and protein structural interactions with surfaces under drying conditions. Proteins contain substantially more higher-order structures than DNA and a wider diversity of chemistry (i.e., many more physical interactions possible from protein chemistry and protein-surface interactions). Diffusion coefficients for DNA 20-mers (approx. 5.7×10^{-7} cm^2/s [131]) are several orders of magnitude larger than that for immunoglobulin (IgG) antibodies (molecular mass approx. 150 kDa) [132]. Hence, in equal concentration, DNA diffuses to surfaces much more rapidly than most protein molecules, resulting in a higher probability to covalently react with surface reactive groups before printed drop desiccation. Additionally, compared to DNA (approx. 14 nm^2 for DNA 20-mer) [71], antibodies have a much larger surface footprint (approx. 100 nm^2) [133], producing substantially stronger physical adsorption and becoming almost irreversibly adsorbed after droplet evaporation. However, accurate reaction-diffusion models for microarray immobilization systems [7] in non-equilibrium drying conditions is difficult to establish as concentrations of salt and biomolecule change dramatically during the few seconds of evaporation.

Amounts of antibodies or proteins in printed nanoliter droplets for microarray spots (about 10^{-15} mol) greatly exceed that required for an immobilized antibody/protein monolayer. After complete drop evaporation in the few seconds following printing, stacked, aggregated multi-layers of antibody/protein form on each microarray spot with no control over deposited thickness, antibody immobilization modes, molecular orientation and intermolecular aggregation. This is important for the resulting antibody analyte capture performance and it is puzzling that so little

research has been directed at this issue. Dried antibodies aggregate extensively [134, 135] and adsorb onto surfaces in random orientations through multi-point contacts. Rapid spot drying onto array surfaces promotes irreversible surface adhesion of printed proteins [91], resulting from uncontrolled protein denaturation, aggregation and conformational changes in drying proteins on all surfaces. During microarray processing and blocking steps, loosely attached printed proteins are washed away from the surface — only strongly adsorbed antibodies remain [91]. In later surface capture assays, target analyte solution is usually incubated on-array from 30 min to 12 h. However, capture antibodies immobilized in microspots are only able to capture specific analytes from sample solutions if their binding domains are (1) exposed to the external milieu containing analyte and (2) remain structurally active to recognize and bind ligand. Additionally, physically attached printed capture antibodies or proteins in array spots can also desorb from microarray surfaces after extended immersion in incubation sample solutions [136]. These diverse uncontrolled parameters, poor reproducibility between different batches of printed microarrays and preparation methods, variance from spot to spot and difficulties to produce consistent results between different laboratories and different platforms of microarray systems plague the clinical capabilities of these arrays as quantitative, metric diagnostic platforms.

Site-specific protein microarray immobilization

One key focus in protein microarray technology is to enable protein immobilization in optimized analyte-recognizing orientations, especially for antibodies. Surfaces designed for attaching antibodies selectively through heavy chain Fc-domains allows Fab-domains (antigen binding fragments) to be oriented outward, away from the surface and more accessible to analyte capture from solutions.

The poly-His tag (*vide infra*) [123, 137] or biotin tag [138] can be engineered into either the C- or N-terminus of proteins: these tags generally do not adversely affect secretion, compartmentalization, or folding of fusion proteins within cells [123, 137]. Nitrolotriacetic acid- (NTA) [124, 136] or streptavidin-functionalized array surfaces [138] then can be used for the immobilization of oriented protein molecules bearing poly-His tags or biotin tags, respectively [125].

Protein A and protein G are known to bind the Fc region of most mammalian IgG subclones. Protein A is a cell-wall component produced by several strains of *Staphylococcus aureus* and protein G is a bacterial cell wall component isolated from group G *streptococci* [139] with different capabilities for binding particular species of IgG. Protein G also exhibits some non-specific binding characteristics. Both proteins are used for affinity purification of antibodies or for oriented immobilization of antibodies [140, 141]. Another genetically engineered Fc-binding fusion protein, known as protein A/G, is recombinantly expressed in *Bacillus sp*. Protein A/G is produced by gene fusion of the Fc-binding domains of protein A and protein G, possessing a more extended binding specificity than either protein A or protein G alone [142, 143]. Surfaces coated with protein A, protein G and pro-

tein A/G can all be used for site-specific IgG antibody immobilization [140, 141] with some improvement. As each protein A or protein G molecule has multiple binding sites for antibody IgG_c domains, reliable, high-density orientation of immobilized IgG on surfaces is likely. This leads to accessibility of antibody Fab-domains for protein analytes [144].

Commercialized surface chemistry called Mix&Go® (Bio-Layer) comprises polymeric coatings directly applied to microbeads or other substrates, combining two different proprietary approaches for capturing and orienting antibodies, respectively [145]. The first approach uses small molecule ligands screened and selected to reliably bind antibody Fc domains as an anchoring point. These small molecules create antibody-binding domains comprising two different small ligands mimicking binding domains from proteins A and G, respectively, and extending as two adjacent side-chains from a polymer backbone. Antibodies in solution are recognized and interact with this synthetic binding pocket through Fc domains. However, since this interaction is low affinity, a second approach adds another dimension to antibody binding stability on these surfaces. In this approach, metal complexes (created using combinatorial chemistry selection methods) are co-integrated into polymer chains, enhancing binding of Fc domains already in the synthesized binding 'pocket'. Combining these two approaches produces substantial improvements over either surface approach alone. In a multiplex flow cytometry assay for TNFα cytokine, a 70-fold signal enhancement was achieved compared to amide-coupled beads. Dramatic improvements were also demonstrated for over a dozen of other assays, including a membrane-based microarray immunoassay [145]. Additionally, structure-activity relationships for tailoring interactions between surfaces and other molecules might also be predicted for thousands of surface coatings using such a strategy to generate large libraries of novel polymer surfaces predicted to have superior characteristics for specific applications [145].

Printing additives to improve protein microarray performance

As mentioned above, many stresses influence immobilized antibody activity after microarray printing: printed proteins are subject to enormous local forces that readily dismantle native globular protein structure, reducing antibody bioactivity. Hydroxylated additives including trehalose, sucrose, glucose, poly(ethylene glycol) (PEG) and glycerol have all been commonly used as bulk protein cryo- and lyoprotectants, preserving bioactivity during bulk lyophilization processes [146, 147]. Many studies investigate effects of additives on antibody microarray performance. One study used direct-label assay with Cy3- or Cy5-labeled in antibody microarrays on home-made and commercially available slides [148]. Addition of trehalose into print buffer increased antibody capture activity on several home-made silane glass slides with cross-linkers. Surprisingly, different print buffers (carbonate buffer, pH 8.5; PBS buffer, pH 7.4; acetate buffer, pH 5.5; and citrate buffer, pH 4.5) had little effect [148]. In another study seeking to improve microarrayed protein stability on commercial aldehyde-coated glass slides [149], additives including

trehalose, sucrose, glycerol and PEG (200 Da) were added to PBS print buffer. PEG (200 Da, 30% w/v) greatly improved printed streptavidin stability and activity, whereas trehalose, sucrose and glycerol showed no improvement or even decreased activity compared to PBS buffer alone. In several other microarray contact printing studies, relatively high percent (20–50%) glycerol is added to protein microarray print buffers to inhibit nanoliter droplet evaporation [49, 150]. However, high concentrations of additives produces print problems including pin clogging during protein spotting, high spot background caused by spot smearing, especially in high viscosity solutions (e.g., with 20–50% glycerol additives). Additive adsorption on the interior walls of contact print pins increases the well-known 'carry-over effect' (e.g., residual print solution carried into the next print sample reservoir, even if these pins are repeatedly rinsed and dried between prints [151]). Analyte capture capabilities for printed, desiccated anti-human IL-1β, IL-4 and TNFα antibody microarrays on two commercial amine-reactive polymer slides were improved by addition of PVA (9000 Da, 0.05–0.5%) to the print buffer. PVA demonstrated the best performance among several hydroxylated additives in terms of spotted antibody distribution homogeneity, uniform microspot morphology, immobilized antibody bioactivity and spot-to-spot variance. PVA also maintained antibody capture activity reasonably well after 1-month storage under dry conditions at 4°C [127]. In addition, additives can end up heterogeneously distributed across printed microspots [16], yielding subsequent variability spot-spot in captured analyte uptake.

Although the printing/dried down immobilization format for protein and antibody microarrays does not control antibody orientation, surface density, or binding activity, the high-throughput capability, low sample volumes required and practicality for microarray manufacture and storage remain microarray attractions. Ideally, each capture antibody should reliably form a bound, bioactive monolayer in each microarray spot, retain full analyte selectivity and capture bioactivity without target cross-reactivity (an important, overlooked issue in itself). However, the influence of multiple factors including varying surface chemistry, printing conditions, different antibody sources and analyte affinities, assay conditions and print durabilities makes realization of universal 'one-size-fits-all' optimized printing and assay methods applicable to all capture antibodies on all arraying surfaces likely impossible [127].

MICROARRAY DETECTION TECHNOLOGIES

Nucleic acid and protein microarrays require reliable signal detection as their primary output. Microarray signals are detected using a variety of technologies including fluorescence labeling (including directly fluorescently labeled target [39, 152] and sandwich assay using secondary fluorescent detection agents [153]), radioactive labeling [41, 70, 72] and label-free methods including surface plasmon resonance [77, 154, 155], waveguide [51] and mass-based piezoelectric techniques [156]. Typically, analytical sensitivities in these detection methodologies extend

regularly to the ng/ml (SPR and piezoelectric)–pg/ml (fluorescence and radio labeling) range [78, 144, 157]. Importantly, many targeted analytes of clinical interest are present at 1000-fold less concentrations (fg/ml) and among non-analyte noise (i.e., in serum) at a million-fold higher concentration (mg/ml). To improve the sensitivity of these detection techniques, improvements in all other aspects of microarray fabrication, surface capture assay and biomolecular interactions are often considered (e.g., affinity of surface-capture interactions, use of labels with outstanding optical properties and microarray surfaces with intrinsically low non-specific binding/backgrounds) resulting in high signal-to-noise ratios. It is also possible to combine multiple techniques to provide overall information regarding the orientation of biomolecules, packing densities and also kinetic information not possible from a single detection technology [70].

While review of the various methods and innovations in microarray surface capture technologies is beyond the scope of this current review, the idea of integrating all the assay technical components into performance-improving system design strategies is important. All surface improvements have yielded incremental improvements in bioassay performance to date. Hence, it is likely that major breakthroughs in improving microarray detection limits in complex sampling environments will come from integrated system improvements. For example, reducing dimensionality of assay components may provide performance advantages (e.g., detection limits, reagent consumption, speed-to-answer and remote field deployment) for certain assay designs [7]. Microfluidics and nanoparticles have received substantial attention for these purposes and offer significant space for innovation through integration of new strategies and novelties surrounding assay miniaturization. Colorimetric properties of gold nanoparticles depend on particle size, providing highly selective detection technology for microarrays [158–162]. Detection principles, methods and various applications are described in a detailed review of nanostructures in biodiagnostics [101]. In a recent study, a 30 aM detection limit is reported for a nanoparticle-based bio-bar method for the detection of prostate-specific antigen (PSA) in goat serum [160]. Technical potential and opportunities to enact improvements with these approaches remain to be seen; many reported nano-strategies must be further developed and applied beyond initial proofs of concept to assays in relevant biological milieu.

CONCLUSIONS: PERSPECTIVES AND RECOMMENDATIONS

Microarray technology is now fairly mature, facilitating high-throughput, parallel and semi-quantitative assessment of analyte abundance relevant to many useful applications. While DNA microarrays have been extensively used in gene expression and genotyping research work, they remain unapproved for clinical use in most formats. Reliability, consistency and absolute analyte quantitation issues remain to be solved, an issue substantiated and consistently aired within the research base reporting such data [163]. Little correlated overlap in gene expression results is reported

in several important dataset comparisons, including (1) on identical samples within major commercial array platforms [30, 163–165], (2) between PCR methods for gene expression and microarrays and (3) between laboratories on identical expression samples [30, 165]. This reputation has taken some time to infuse itself into the user base, undermining the confidence in these assays as a reliable means to document gene expression. From a clinical analytical perspective, this reduces to lack of array assay reliability, repeatability and consistency. From a practical perspective, this is manifested in comprehensive and uniform virtual invisibility of microarray assays in clinical patient treatment modalities. Despite the hype about these systems and the premature commercial expectations for their impact in medicine, few assays can respond to the NIH, FDA and EPA Critical Path initiatives that invoke reliable microarray performance in these health-care missions.

Very recently, a landmark coordinated study has provided the most comprehensive microarray results analyses to date, representing a mammoth collective effort (the MAQC project) comprising six FDA Centers, major commercial providers of microarray platforms and RNA samples, EPA, NIST, academic laboratories and other vested interests (137 scientists from 51 organizations). Significantly, the MAQC project intends to establish quality control metrics and limitations for "objectively assessing the performance achievable by various microarray platforms and evaluating the advantages and disadvantages of various data analysis methods" [30, 165]. Specifically, these studies now conclude that many earlier, substantial reports of lack of microarray correlation and reliability can generally be avoided when certain specific working protocols and standard methods are strictly followed. Importantly, intra-platform consistency across test sites and improved data repeatability and consistency in assessing gene differential expression was reported under these experimental conditions (coefficients of variation, CVs, improved from $>25\%$ to approx. 14%). The series of MAQC papers, published as an entire issue of Nature Biotechnology (Volume 21, No. 9, September 2006), is hailed as an important first step towards establishing a framework for standard use of microarrays in clinical and regulatory settings [30, 165]. Additionally, individual laboratories should now more easily identify and correct processing variables that account for problems and establish standard methods. These conclusions are extremely insightful in understanding the microarray analytical quandary, challenges for clinical adoption and the critical need to enforce standard procedures across their exploitation. However, this study is entirely process-oriented using commercial kits and focus only on assay results (signal generation); CVs are still unacceptable for bioanalytical reporting and no molecular mechanisms are understood to facilitate rational assay improvements or diagnostic metrics. Therefore, efforts to establish molecular insights into surface immobilization and assay capture processes that improve the understanding of assay variables and fabrication are important and needed (Table 2).

Characterization of microarray surfaces using the rigorous analytical protocols and routines common to the biomaterials field would benefit such a biomolecular-surface states understanding. Most methods to improve microarray surface as-

Table 2.
Performance benchmarks for assay capture surfaces in complex biological milieus

Reliable and consistent surface performance without surface defects or interfacial variability.
Substantial reductions in non-specific protein–surface interactions (NSB) to below 1% of a monolayer (<3 ng/cm^2) in solutions of cell or tissue lysates, serum, homogenized food or blood.
Elimination of 'blocking' steps using sacrificially adsorbed globular, surface-active proteins such as albumin, lactoglobulin, or casein, or adsorption of amphiphilic surface-active synthetic polymers (e.g., Pluronics™) to proactively mask surfaces against NSB from other sources.
Effective, direct surface immobilization of capture ligands to substrates to facilitate analyte capture, stringency washing, multiple-step processing under high-shear and extreme pH or solvent conditions.
Stabilization of immobilized affinity ligands in surface-capture environments to effectively preserve assay bioactivity under long residence times and lyophilized storage conditions.
Surface immobilization treatments and protocols compatible with substrates comprising metals, plastics, metal oxides and silicates with disposable economy.
High-density affinity capture ligand immobilization on surfaces with minimal lateral interaction, competitive interference in assay or steric hindrance issues.
Lot-to-lot and areal uniformity of affinity ligand immobilization and assay performance (signal:noise).
Direct assay from complex milieu (serum, cell lysate, food, environmental samples) without sample pre-purification or analyte amplification or enrichment steps that cost time and money.
Quantitative, direct correlation of assay analyte signal with sample analyte abundance.
Reduction in capture feature sizes, surface patterning fidelity, lateral feature spacing for miniaturized formats and integrated approaches to achieve sufficiently discriminating signal:noise performance.

say sensitivity and reliability are currently largely empirical because very little molecular-level information is reported or known about the chemical, physical or biological fate of DNA or its various known derivatives, or protein affinity agents immobilized on any of the diverse set of microarray surfaces. How immobilized single-strand DNA probe–surface disposition influences target DNA analyte capture (hybridization) efficiency, assay detection sensitivity, reliability of array data interpretation and assay quantification is correlated empirically. Surface analysis studies of dessicated immobilized probes and correlations to efficiencies of wet assay target capture will require some methods innovation. Only through these studies will sources of microarray assay variability be discovered and controlled and diagnostic meaning be developed.

A remarkable aspect of this timely and needed technical reporting of microarray assay problems is that these data, methods and analysis come a full decade after launch of the first microarray products and the subsequent decade-long frustrations of being unable to bring these tools to clinical applications. That such work and conclusions regarding the importance of data generation, process validation, interpretations and limitations come at this late point in microarray product and method maturity reflects the "cart before the horse" status of this technology in addressing important clinical use targets. Hence, while assay consistency under

certain strict operating conditions can now be asserted, the mechanism and meaning of how these microarray signals are generated in these assays is still a "black box". Furthermore, the ability to connect different assay signals from different array platforms (or various PCR methods) back to clinical analyte quantitative abundance in medically derived samples remains a pipedream to date.

Despite some commercial presence for research use, protein microarrays are still in early in the proof of concept stages. Substantial technological issues remain to bring these array formats to widespread practical use, including surface and materials performance problems, affinity reagent reliability and stability, limits of analyte cross-reactivity and detection sensitivity, quantitative capabilities and ease of use. For both microarray platforms, numerous challenges also plague understanding of the biological significance of the screened data (e.g., translating the quantitative features of the results). Intrinsic differences between protein and DNA structure, chemistry and interfacial behavior require careful distinctions between specific approaches to each format: to bring protein microarrays into routine use appears to face many more difficult challenges and problems than DNA microarrays.

As a last recapitulation, microarray printing is a non-equilibrium drying process, vastly different than solution phase bio-immobilization on surfaces where substantial history and experience exist. The challenges are distinct and the impact on the product profound. Microarray printing, involving nanoliter droplet depositions that dry within seconds on array surfaces, produces conditions for capture agents at surfaces that are not studied or understood. Experimental and performance challenges in this scenario — with their important implications for how microarrays respond to analyte capture, their performance reliability and sensitivity for clinical assays, must be addressed. Very little (i.e., no) surface data are available regarding this print deposition process and resulting DNA density, local electrostatic fields or charge densities and the impact on subsequent DNA target capture.

Therefore, our conclusion is that, under the current quest for clinical acceptance and general validation, both materials and methods for microarray analysis require substantial further elucidation, validation and quantitation. This is a remarkable parallel to the use of many biomaterials in other clinical applications where similar needs exist. Hence, diagnostic materials share many of the performance issues as clinical biomaterials. Clinical utility of this diagnostic information is in high demand but without the requisite understanding of the information yield limitations and meaning to clinical diagnosis and detection. These challenges are present at every stage of microarray technology from sample purification to microarray fabrication, analyte capture assay and signal detection. Hence, new strategies integrated across several disciplines that can design, apply and troubleshoot new assay reagents, materials, methods, detection techniques and data analyses might best be brought to bear on this field.

Acknowledgements

The authors are grateful for support from NIH grants EB00726, EB001473 and EB002027.

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