Microarrays in infection and immunity

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Over the past decade, microarrays have revolutionized the scientific world as dramatically as the internet has changed everyday life. From the initial applications of DNA microarrays to uncover gene expression patterns that are diagnostic and prognostic of cancer, understanding the interplay between immune responses and disease has been a prime application of this technology. More recent efforts have moved beyond genetic analysis to functional analysis of the molecules involved, including identification of immunodominant antigens and peptides as well as the role of post-translational glycosylation. Here, we focus on recent applications of microarray technology in understanding the detailed chemical biology of immune responses to disease in an effort to guide development of vaccines and other protective therapies.

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Introduction

Remarkably, live rotavirus vaccines, which were introduced in 2004, were developed using essentially the same empirical methods for attenuation as the Sabin polio vaccine nearly 50 years earlier: loss of virulence through multiple passages in non-human cells. The vaccine seems to be safe and effective and is desperately needed to prevent the 600 000 deaths annually that are attributed to rotavirus [1]. But its history underscores that, despite the profound role of vaccines in reducing human and animal morbidity and mortality, the field has relied on technological advances in other areas to spur its own development. In particular, the dual advances of genomic sequencing and microarray design have resulted in a renaissance of research in immunity and infectious diseases.

The applications of microarrays span from the bench to the bedside, providing tools that require less effort, expense and sample than other technologies and which are also highly multiplexed, building on established pattern-recognition techniques and statistics [2]. In this review, we do not discuss technical aspects common to all arrays (e.g. statistical analysis and immobilization chemistry) because these are extensively reviewed elsewhere. Instead, we limit ourselves to novel applications in infection and immunity using four variants of array technology: DNA, antigen, peptide–MHC complex (pMHC) and carbohydrate (Figure 1). Collectively, these technologies are already advancing our understanding of the interplay between immunity and disease, providing a rational basis for the design of vaccines and agents that interfere with disease progression (Table 1).

Genome arrays for disease surveillance, diagnosis and characterization

Since the days of Pasteur, pathogen identification has been accomplished using a combination of culture and serological techniques, all of which are labor-intensive, require highly trained personnel and incur a delay of hours to days before a conclusion can be reached. Furthermore, many organisms are refractory to culture, whereas serotyping is limited by the availability of specific antisera — a situation especially problematic when emerging or evolving pathogens are considered. Because of the availability of numerous genome sequences and the need to contain rising healthcare costs, DNA microarrays that can simultaneously probe clinical and environmental samples for the presence of conserved viral and bacterial sequences, specific virulence factors and antimicrobial resistance genes, and can even identify point mutations, present a novel alternative (Figure 2) [3,4].

This concept is elegantly illustrated by the pan-viral DNA microarray (Virochip), which comprises highly conserved 70-mer oligonucleotides from every partially and fully sequenced viral genome in GenBank (as of June 2004). The third generation chip includes 22 000 oligonucleotides, representing ~277 000 sequences [5,6]. Patient samples are collected by nasal lavage, the ribonucleic acids are purified, reverse-transcribed, amplified by random-primer PCR, and finally incubated with the array under stringent binding conditions. Using such an array, deRisi and colleagues were able to correctly identify a range of viruses from the RNA of infected tissue culture cells and human samples [5]. More impressively, the array was subsequently used to identify, isolate and even sequence ~1000 bases of a virus now known as the severe acute respiratory syndrome coronavirus (SARS-CoV) [7–9]. Originally designed as an experimental

Figure 1
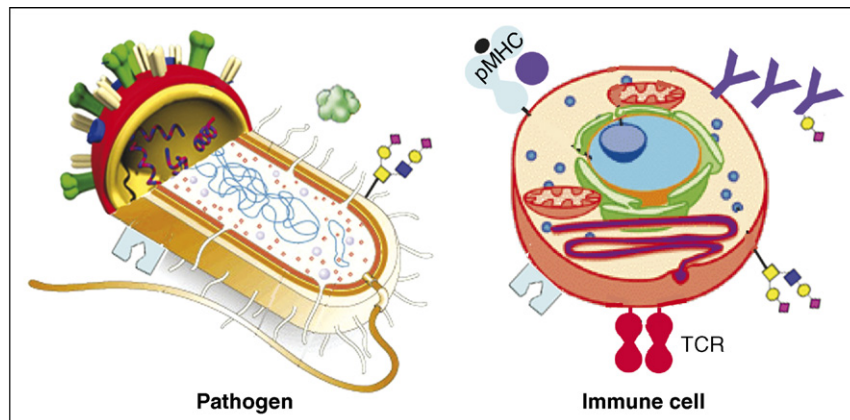
Opportunities for arrays in infection and immunity. Aspects of a pathogen that are now accessible to array analysis include the genetic material (transmissible elements and gene variants) and the binding specificity and temporal expression of carbohydrates and lectins involved in, for instance, host cell attachment. The antigenicity of surface exposed and secreted molecules can also be assessed at the genome-wide scale to aid in vaccine and diagnostics development. From the host perspective, arrays can reveal immune cell responses in terms of transcriptional responses, antibody-binding specificity, T cell–pMHC reactivity and the functional consequences of T cell activation.

research tool, the Virochip also seems to be capable of viral diagnosis in a clinical setting [6,10].

Although the success of the Virochip is impressive, significant hurdles remain for broad application of this technology, primarily in terms of sample amplification and probe design [11,12]. Other arrays under development operate at three levels of detail, each of which can be used in surveillance, diagnostic or vaccine development programs to answer the following questions:

Which organisms are present?
Arrays at this level include the Virochip and other microarrays for more specialized detection of viruses of the central nervous system [13] respiratory pathogens [14], and for bacterial strain identification [15••,16,17]. Importantly, these chips have demonstrated the ability to detect bacteria in the viable but non-culturable state and are amenable to automation [18].

Which genes are present?
Clinically, the presence of genes or mutations that confer antibiotic resistance influences treatment options

[19,20•,21], whereas transmissible virulence factors and serotypes can be used for strain identification and to indicate the pathogenic potential of the organism [15••,22]. Similarly, genes that are differentially present in pathogenic versus commensal or live attenuated strains can guide vaccine development [23].

Which gene variants are present?
Surveillance and molecular epidemiological programs are developing focused arrays to track antigenic drift, anticipate dominant serotypes and monitor the genomes of live attenuated vaccine strains [24•,25,26]. For instance, the low-density FluChip can distinguish all influenza A hemagglutinin and neuraminidase subtypes, and tiled resequencing arrays can detect single nucleotide polymorphisms within these genes [24•,27•,28,29].

The complement to specific pathogen hybridization is interrogation of host transcriptional responses [30,31], primarily using peripheral blood mononuclear cells. The premise is that all coronaviruses will induce similar host responses, and these responses will be distinguishable from those induced by, for instance, bacterial sepsis.

Table 1

Recent applications of arrays in infection and immunity

Array format	Biomarker discovery	Diagnostic profiles	Vaccine candidates	Surveillance/ epidemiology	Number of unique elements[a]
DNA	–	[7,15••,19,22,49]	–	[15••,20•,24•,27•]	55–22 000
Antigen/ORFeome	[51,55,57•]	[52,54,56•,58••,124]	[50,125]	–	22–232
pMHC	[65•,66]	–	[67••]	–	7
Glycan	–	[79,91•,97,126]	[80•,81•,90,93,127]	[95••]	9–200
Lectin	–	[105]	–	–	9–21

[a] Refers to the number of unique elements (e.g. DNA oligonucleotides) individually produced and immobilized on the array.

Figure 2
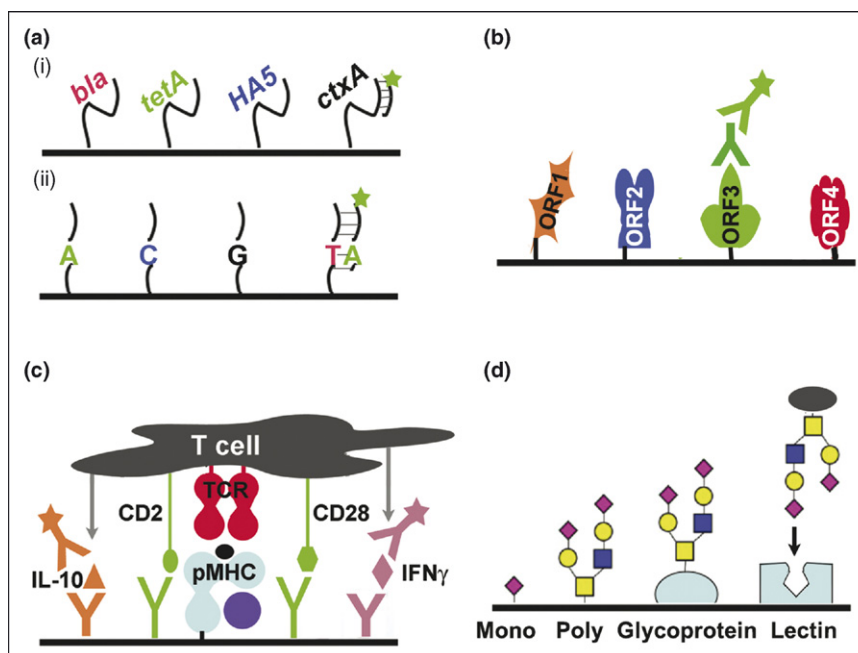
Arrays used to study infection and immunity. **(a)** DNA arrays. (i) Immobilized DNA sequences can be chosen to represent highly conserved regions from a broad range of organisms, as in the pan Virochip, and used for disease diagnosis. Alternatively, the DNA chosen can be more specialized, detecting clinically relevant genes (e.g. antibiotic resistance and export pump genes, key antigens for serotyping and virulence factors capable of horizontal transmission). Sample nucleic acids are purified, fluorescently labelled and allowed to specifically hybridize with immobilized complementary DNA. (ii) Use of resequencing arrays provides single base pair information, which is useful when tracking genetic drift and single nucleotide polymorphisms. **(b)** Antigen arrays to monitor humoral immunity. Open reading frames (or peptides) from a genome are expressed recombinantly, purified and immobilized in an array. Serum from an infected, recovered or vaccinated individual is incubated with the array; bound antibodies are detected by subsequent incubation with a fluorescently labeled secondary antibody. These arrays can be used to rapidly characterize the protective immunome of an organism, to identify novel vaccine candidates and to compare vaccine-induced humoral responses with those resulting from natural infection. **(c)** pMHC arrays to monitor cellular immunity. A variety of soluble peptide–MHC (pMHC) complexes are immobilized with co-stimulatory antibodies to form an array of artificial antigen-presenting cells. Fluorescently labeled T cell populations can be incubated with the array to quantify the fraction specific for a given pMHC. Additionally, antibodies can be co-deposited with the pMHC to capture locally secreted cytokines in a sandwich assay (illustrated here for interleukin 10 and interferon–γ). The nature of the cytokines released reveals the responses of the T cells to stimulation with a particular pMHC. Using secondary antibodies with different fluorescent labels, the assay can be multiplexed to detect multiple cytokines simultaneously. **(d)** Glycoarrays and lectin arrays. Monosaccharides, oligosaccharides and polysaccharides of varying sequence and structure, in addition to glycoproteins and lectins, are immobilized in an array. Fluorescently labeled proteins, viruses or bacterial cells are applied to the array to assess their carbohydrate-binding specificity; for example, the species specificity of bird and human influenza variants can be attributed to the glycan-binding propensitites of the respective hemagglutinin variants. In the case of lectin arrays, these arrays can rapidly assess the nature of the glycans attached to the protein or cell surface.

Thus, transcriptional profiling could be used to diagnose disease completely independent of any knowledge of the pathogen or even after the pathogen has been cleared from the system. Despite preliminary successes [32–34], this approach has met with several obstacles in terms of individual heterogeneity and strong stereotyped inflammatory responses mediated by the nuclear factor NFκ-B, which obscure pathogen-specific responses [35–37].

Antigen arrays to monitor humoral immunity

Once the genes harbored by an organism have been identified, the next step is to probe the host immune responses to the genes products. Which protein antigens are recognized? And which confer lasting immunity? Antibody recognition of a set of antigens can sensitively

diagnose disease [38] and an immunodominant antigen can be a candidate target for passive or active vaccination [39]. Antigen arrays are a natural fit for biomarker discovery and complement recent advances in vaccine development.

One advance, termed reverse vaccinology, capitalizes on the available genome sequence of a pathogen that is refractory to traditional vaccine development strategies [40–43]. *In silico* techniques identify conserved open reading frames (ORFs) predicted to encode surface exposed or secreted proteins; hundreds of these are cloned in *Escherichia coli*, expressed and purified in parallel and used to immunize mice [44]. Proteins that either protect mice from subsequent challenge or induce sera

that are protective in *in vitro* assays are pursued. First applied to serotype B *Neisseria meningococcus*, researchers ultimately queried a metagenomic database using sequence data from eight strains [44,45]. Five antigens were identified; none of these was broadly protective alone but together they induced bactericidal antibodies in mice against 66 of 85 meningococcal strains [45].

A key element of reverse vaccinology is analysis of immune sera reactivity and it is here that protein antigen arrays are beginning to play a role [46]. Instead of immunizing mice directly, proteins corresponding to the entire ORFeome (or fractionated lysate [47]) are spotted onto a glass slide. Naïve, convalesent or immune sera are applied to the array, followed by a fluorescent secondary antibody (e.g. goat anti-mouse IgG). After washing, the array is scanned and fluorescence intensity is used to indicate the presence of antibodies that recognize the antigen immobilized in that spot (Figure 2). In this way, antigenic proteins can be rapidly recognized, the response of an individual to vaccination or infection monitored, and infections past and present diagnosed. Therapeutic monoclonal antibodies, destined for passive vaccination, can also be documented for cross-reactivity on a broad scale [48].

Such arrays are being developed for multiple diseases, including tuberculosis [49], *Yersinia pestis* [50], *Neisseria meningitidis* [51], leprosy [52] and HIV [53], and also for autoimmune diseases [38,54] and tumor-associated antigens [55]. Early successes include a vaccinia viral array consisting of 185 proteins that were probed using sera from naïve and immunized mice, non-human primates and humans [56•]. Interestingly, the three species did not recognize the same subsets of viral proteins. The array was later used to identify the H3L envelope protein as the immunodominant antigen in the live viral vaccine [57•], perhaps paving the way for a less traumatic subunit vaccine. Similarly, a diagnostic array representing the entire ORFeome of SARS-CoV and portions of five additional coronaviruses was developed and tested using serum from 400 Canadian and 206 Chinese patients [58••]. The array was shown to be at least as sensitive as and more specific than enzyme-linked immunosorbent assay (ELISA) tests for diagnosing SARS, requiring minimal sample processing compared with genome chips.

pMHC arrays to monitor cellular immunity

Cellular responses have always been harder to study than antibody responses: antigen binding, as opposed to the high-affinity binding reaction between two soluble molecules, involves a low affinity tri-molecular interaction that comprises two membrane-bound molecules and a post-translationally processed peptide. From the standpoint of vaccine development or targeted therapies, is it important to identify not only the amino acid sequence corresponding to a key peptide epitope but also the functional T cell responses that result from recognition.

Given these constraints, it is hard to imagine a screening technology that does not involve a cellular readout. Phage and cDNA display technologies, widely used to study antibody–antigen interactions, have been difficult to apply to analysis of T cell receptor (TCR)—pMHC interactions [59–61]. Non-genetic approaches involve incubating synthetic peptides with antigen-presenting cells and T cells, with stimulatory peptides identified by interleukin 2 release [62]. Computational prediction methods, especially for class I MHC, are also improving but still require experimental validation [63]. For epitopes that have been identified, enzyme-linked immunosorbent spot (ELIspot) and flow cytometry assays using tetramerized pMHC have found wise-spread use to monitor the spatial and temporal presence of cognate T cells [64].

The opportunities for arrays in analysis of cellular immunity are threefold: (i) to quantify the fraction of T cells in a population reacting with a given pMHC; (ii) to identify crucial peptide epitopes from candidate sequences; and (iii) to assess T cell responses resulting from recognition of these peptides. Recent reports have devised strategies to achieve these goals by using pMHC arrays [65•,66,67••,68]. In their current form, each feature on the array contains immobilized pMHC molecules, co-stimulatory antibodies and cytokine capture antibodies. Thus, a fluorescent T cell recognizing a particular pMHC will bind to the spot, be activated and secrete cytokines locally. These cytokines are captured and subsequently detected using fluorescent antibodies (Figure 2). Not only can the number of T cells bound to a spot be counted, replicating flow cytometry assays, but cellular responses can also be elucidated based on the cytokines released. Variations in design reveal the detailed consequences of binding to a single pMHC (using multicolor secondary antibodies to quantify levels of a series of cytokines) or to a peptide sequence (using arrays of peptide variants to identify agonist and antagonist peptides).

After the initial report on use of pMHC microarrays [66], Stone *et al*. used a modified approach to match T cell lines to activating viral epitopes [65•]. In the first clinical application of the technology, Chen *et al*. used the array to analyze patient T cell responses to a peptide vaccine against melanoma [67••]. Ten patients were immunized, and CD8+ T cell responses to seven pMHC and 26 secreted factors were measured using an array. Interestingly, the investigators were able to detect fractional T cell abundances as low as one cell in 10 000 (0.01%) and they identified a correlation between the functional profile and clinical outcome of the patients.

These early successes indicate that pMHC arrays might be used broadly in vaccine and therapeutic development [68,69]. For instance, they could identify tumor-associated antigens from a panel of candidates for

targeting by TCR therapeutics [70,71] or could identify viral peptides for inclusion in epitope vaccines. Conversely, the arrays could be used during de-immunization of therapeutic proteins. Although not yet demonstrated, the experiment could also be inverted, using soluble TCRs to capture cells that present specific pMHC for diagnosis or to assess vaccine-induced cellular immunity.

Carbohydrate arrays

Carbohydrates consist of sugar units, including monosaccharides, disaccharides, oligosaccharides and polysaccharides, that can be linked to proteins as linear or branched extensions with varying connectivity. These molecules can profoundly affect protein folding and solubility, pathogen infection and immune system responses [72] and can form the basis of several vaccines (e.g. *Haemophilus influenzae* type b) [73]. However, because of their intrinsic heterogeneity and non-template-driven biosynthesis, identifying and characterizing the linkage of sugar groups, for example by HPLC of enzymatically released carbohydrates, has been difficult. Recent and ongoing development of glycan arrays has been motivated largely by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/) and has been made possible by several technical breakthroughs, including advances in carbohydrate immobilization [74–78].

The first arrays consisted of a variety of sugars deposited on a slide and were used to profile the glycan-binding specificity of fluorescently-labeled anti-carbohydrate monoclonal antibodies, lectins and bacterial toxins [79,80•, 81•,82–88]. These might be useful for future development of multivalent toxin inhibitors [89] or peptide mimetics for immunization. Similar arrays have been used to demonstrate potential cross-reactivity between the immune response to an attenuated SARS vaccine and a self-carbohydrate [90]. Moving towards diagnostic glycoarrays, the GloboH hexsaccharide cancer marker and nine analogs were arrayed and used to test monoclonal antibodies and patient sera for GoboH-specific binding [91•].

A major application of these arrays has been to dissect the chemical biology of pathogen–host cell attachment. A rare and potently neutralizing antibody, 2G12, protects against viral challenge *in vivo* in animal models of HIV infection, by binding terminal Man α1-2Man residues on gp120 [92]. Carbohydrate arrays have been developed to characterize the affinity and structural specificity of 2G12 mannose recognition compared with other mannose-binding or gp120-binding proteins to develop a carbohydrate template for HIV vaccine design [81•,93,94]. With influenza A viruses, arrays have probed the basis of species specificity — a crucial aspect when evaluating serotypes for pandemic potential. The virus invades cells by hemagglutinin binding to cell surface sialic acid residues, which vary in structure based on the host species and anatomical location. Binding of hemagglutinin variants recovered from pandemic and circulating strains on a 260-member glycan array demonstrated differences in recognition of carbohydrate linkages (α2-3 or α2-6, characteristic of avian and human viruses, respectively), fucosylation and sulfation. Interestingly, a single amino acid change (Asp255Gly) in the pandemic 1918 H1 was found to switch specificity from exclusively α2-6 to mixed specificity, whereas Asp190Glu conferred complete reversion [95••,96].

A different approach is the use of these arrays to detect pathogens directly and indirectly. Bacterial glycoconjugates have been arrayed and interrogated with sera in an effort to determine an individual's prior exposure to the corresponding microbe [76]. Similarly, host carbohydrates and glycoproteins that are used as bacterial receptors have been arrayed, followed by specific capture of bacteria binding those receptors. This approach even allowed for microorganism recovery from arrayed spots for subsequent growth and antibiotic susceptibility testing [97,98].

Once lectin specificity has been determined (perhaps using comprehensive glycan arrays), the proteins can be employed to generate complementary lectin arrays. Here, the carbohydrate-binding proteins are immobilized and incubated with fluorescently labeled molecules or cells to assess the carbohydrate moieties [99–104]. Although this format is still in its early stages, one array that contains 21 commercially available lectins was used not only to discriminate between strains of laboratory and pathogenic *E. coli* bacteria based on whole-cell binding patterns, but also to track the temporal expression of different glycans during the growth cycle [105•]. A limitation is the number of well-characterized lectins and antibodies available, but mechanisms for creating lectin diversity exist in nature and could be applied to the engineering of specific lectin properties [106].

One-third of approved biopharmaceuticals are glycoproteins, and the carbohydrate components of these have long been known to affect functions such as circulating half life, solubility and, for antibodies, complement activity and Fc-receptor binding specificity [107]. These effects have been primary forces in motivating the homogenization of carbohydrates on protein therapeutics expressed in recombinant hosts [108–110]. The recent discovery that naturally occurring antibody glycoforms vary in their ability to mediate inflammation and cytotoxicity (crucial for autoimmune and cancer treatments, respectively) [111•,112] has provided additional impetus to control post-translational modifications. Lectin arrays could aid in characterizing, optimizing and monitoring the quality of biologic therapies [99].

Emerging technologies

In addition to concerns common to all microarray applications, advances in several areas are likely to be directly applicable to studies in infection and immunity.

1. One approach is to streamline cloning, expression and purification of entire ORFeomes for antigen arrays. Such methods include those borrowed from structural genomics (e.g. tags for purification and immobilization [113]) and those that generate proteins directly from DNA (e.g. spotted viral or cell particles that present the protein of interest on their surface [55,114], *in vitro* transcription and translation of spotted transcriptionally active DNA to produce and directly capture expressed protein [56•,115], and spotted lentiviral arrays to directly transfect overlaid eukaryotic cells [116]).

2. A second important area of development is movement into the array format of technologies that have expanded capabilities compared with most current arrays, which measure binding under near-equilibrium conditions [117]. Newer formats can extract kinetic (e.g. the BIAcore flexchip), force (e.g. Bioforce Nanosciences) [118,119] or thermodynamic [120] parameters associated with the interaction, permitting rigorous quantitative comparisons and providing mechanistic insight.

3. A third approach involves high-throughput array-based analysis of additional post-translational modifications, such as phosphorylation [121] and lipidation [122,123].

Concluding remarks

Now that many of the technical hurdles have been addressed, microarrays with new and expanded capabilities (Figure 3) can monitor the genome of a pathogen with single nucleotide precision, identify antigens that stimulate both arms of the immune system, and even investigate the role of post-translational modifications. These new arrays are being used to probe the interactions between immunity and disease, and are already resulting in significant discoveries regarding the molecular mechanisms of disease, vaccine development and novel therapeutics. Consistent with the history of infectious diseases and technology, these newer technologies will lead to many more exciting discoveries.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Figure 3
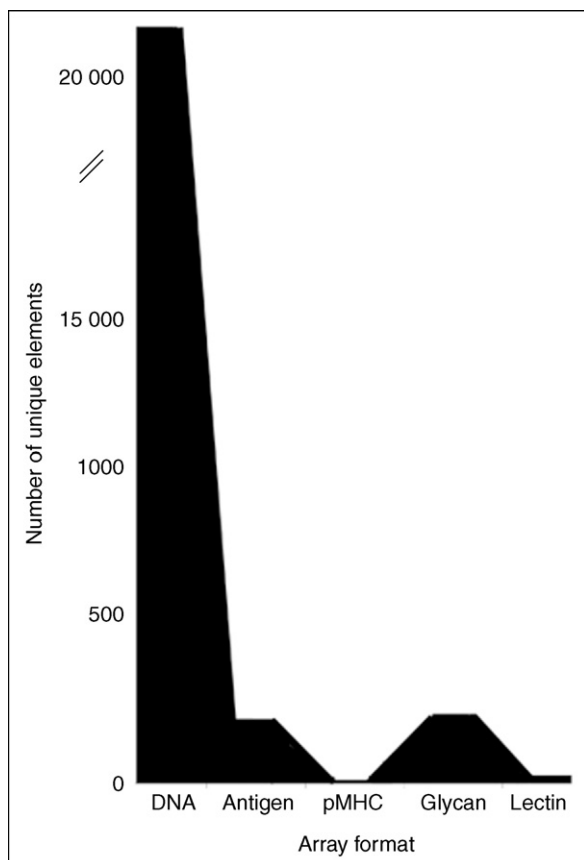
Complexity of arrays. Depending on the ease or difficulty of producing the material used for array elements, different array formats vary greatly in the number of unique molecules that are assessed. These range from as few as seven (for the newcomer, the pMHC array) to >22 000 (for DNA oligonucleotide arrays).

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