Chapter 3
Antibody Microarrays for Cell-Based Assays: The Use of Micro-Arrayed Antibodies for Exploring Cell Surface Diversity or Whole Cell Functionality

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Abstract Since the early 1980s, microarrays have gained increasing interest due to their tremendous field of applications. A large repertoire of biomolecules has been micro-arrayed for the specific and parallelized detection of targets contained in a single biological sample. Most individual binding events target soluble compounds although pioneering microarray developments have been undertaken with whole cell binding on micro-arrayed glass slides. In this review, we wish to focus on micro-arrayed antibodies and their use for cell-based analysis. We will illustrate the wide range of applications that can be explored by site-specifically immobilizing eukaryotic or prokaryotic cells on a surface.

Abbreviations

AML	Acute myeloid leukemia
CCM	Culture-capture-measure
CD	Clusters of differentiation
CFU	Colony forming units
CTC	Circulating tumor cells
DNA	Deoxynucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-assisted cell sorting
GNP	Gold nanoparticles
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus

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ICS	Intra-cellular cytokine staining
IFNγ	Interferon gamma
IL	Interleukin
IS	Immunological synapses
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
LOD	Limit of detection
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
RLS	Resonant light scattering
SECM	Scanning electrochemical microscopy
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SPR	Surface plasmon resonance
STEC	Shiga toxin-producing *E. coli*
TCR	T cell receptor
TNFα	Tumor necrosis factor

3.1 Introduction

Micro- and nanotechnologies have been acquiring increasing interest for more than two decades. This general enthusiasm is motivated by several reasons; the most important is undoubtedly due to the scale of these new generations of devices. By decreasing the size of experimental setups, one can reasonably expect the decrease in volume of samples required to run an analysis on a micro-system. This feature might be critical in biological or biomedical applications as, in most of them, access to large sample volumes might be challenging. Another important advantage in using micro- and nano-systems in biological studies is the decrease in time usually required for running multistep processes. This aspect is illustrated by recent advances in Laboratories-On-A-Chip (Lab-On-Chip), where integrated functions allow, for instance, the efficient analysis and sorting (i.e., physical separation) of rare circulating tumor cells (CTCs) carried in the blood stream [1]. Last but not least, another major benefit in using miniaturized devices is the possibility to run parallelized assays on a single support. This multiplexed analysis might be carried out according to two aspects: one is based on a series of samples that can be handled and analyzed in parallel, thanks to dedicated microfluidic architectures [2], while the other is focused on one single sample interrogated at several points throughout a single analysis. A typical example of this latter point is illustrated by microarrays where series of probes (DNA [3], proteins [4], peptides [5], carbohydrates [6], etc.) are immobilized on predetermined locations of a surface. Upon incubation with a sample, individual responses of each micro-arrayed probe are simultaneously

analyzed and compared to control responses, thus producing a significant amount of information through a single assay.

So far, the most abundant applications of microarrays are issued from DNA microarrays dedicated to the probing of transcriptomes, genomes, and searches for single nucleotide polymorphisms (SNP). Pioneer work on DNA microarrays was led at Stanford University by Pat Brown's group which first published the use of DNA microarrays for gene expression level assessments [7]. Although tremendous interest arose from DNA microarray-based technologies, these miniaturized systems functionalized by biomolecular probes are not the oldest format of parallelized arrays. Indeed, besides Fodor's group which is often cited as the first example of micro-arrayed peptides [8], Ekin's lab proposed in the late 1980s the simultaneous arraying of antibodies for immunoassay applications [9, 10]. This original work was based on a "multi-analyte microspot" assay where series of antibodies were simultaneously immobilized on a solid support and then incubated with biological samples. Using such formats, both noncompetitive and competitive assays were led by combining secondary radio-labeled antibodies and labeled antigens, respectively [9, 11]. Data produced by these protein microarrays gave both relative occupancy and quantitative data on surface immobilized antibodies. Since then, protein microarrays met an increasing enthusiasm, giving rise to two major classes of protein microarrays: protein function arrays made of immobilized libraries of proteins for assessment of their function; and protein detection arrays which consist of immobilizing proteins with defined binding properties used for probing targets contained in a sample [12]. Antibody microarrays definitely fall into this latter class and are systematically used for antigen binding, followed by detection.

Soluble antigen detection using antibody microarrays [13] or microarrays designed for cell lysate and tissue extracts [14] will not be described here as this chapter is focused on cell-based assays led on antibody microarrays. By carefully looking at the literature, this latter category appears as the pioneer demonstration of the microarray concept ever described and illustrated in Chang's work [15], published in 1983, describing immobilization of anti-Lyt 2.1 and anti-Lyt 2.2 antibodies (1, 0.5, and 0.25 mm spots in diameter) on a solid support. Several hundreds of features (up to 400) were micro-arrayed on 1 cm^2 and the whole microarray was incubated with cell lines expressing either one or the other antigen on their cell membrane. After washing, AKR thymocytes expressing the Lyt 2.1 antigen and BALB/c mouse thymocytes expressing the Lyt 2.2 antigen specifically bound to the anti-Lyt 2.1 and anti-Lyt 2.2 spots, respectively. Individual cells bound to each feature were counted and the measured counts of cells were consistent with the spot area (more captured cells on larger spots), thus validating the surface antigen exploration using a solid support. This work appears to be the very first example of micro-arrayed biomolecules, and interestingly was dedicated to whole-cell analysis. More recently, several examples of cell microarrays have been published [16, 17], driven by an important demand for new devices specific to cell analysis [18]. Blood cells are of special interest due to the ease of collection by blood tests [19], although protein microarrays have also been described for phenotyping adhering cells (mesenchymal stem cells) [20] or for probing cell adhesion properties of hepatocytes [21]. Most blood

cells show a weak ability for spontaneous binding onto a surface, which means that specific cell capture and analysis on a microarray implies specific cell surface interactions with functionalized spots. These cell surface recognition events are mostly ensured by recognition of clusters of differentiation (CDs) by specific antibodies as shown in the following examples. This strategy has been used for functionalizing patterns [22] or microstructures [23] with one type of antibody to specifically capture individual cells on defined locations. In both examples, the whole microarray is then turned into a matrix of identical cells enabling either easy readout for subsequent bioassays [22] or even sorting by dissociating pieces of the solid support bearing immobilized cells [23]. This chapter will focus on recent developments on protein microarrays grafted by a series of different antibodies.

3.2 Antibody Microarrays for Cell Typing

3.2.1 Phenotyping Blood Cells

CDs are surface motifs identified as specific to a cell type, sub-cell type, differentiation stage, or physiological process, etc. By immobilizing antibodies targeting a specific CD, one may expect to obtain some cell arrangement on two dimensions depending on their surface antigen. This strategy has been successfully used to distinguish undifferentiated from differentiated fetal neural cells [24]. In this work, authors showed that the number of differentiated neural cells was higher on anti-CD56 and anti-O4, lower on anti-CD57 and similar on anti-CD44, CD81, and anti-CD90 spots, by comparison to similar experiments done with undifferentiated cells. These data were confirmed by counter-staining of the captured cells by labeling of intracellular markers. Multiplexed information is then accessible by combining microarray binding position to cross-labeling specific to another antigen. This explains why this approach is sometimes referred as “solid-phase cytometry” [24].

From a quantitative point of view, absolute weighing of different cell populations is delicate as the cell capture is highly dependent on several factors such as the antibodies ability to bind to its antigen or the abundance of the antigen on the cell membrane. Nevertheless, semiquantitative data has been reported [24–26]. Some authors even showed how real-time monitoring of cell binding on an antibody microarray could yield information on diffusion coefficients for membrane antigens diffusing around the cells [26]. The measured values were consistent with diffusion coefficients measured by photo-bleaching experiments. Within one blood cell family, subsets of cells were also distinguished by micro-patterning anti-CD4 and anti-CD8 antibodies on a surface [25, 27]. Ratios of these T-lymphocyte cells were also confirmed by control flow cytometry experiments. The coupling of such microarrays with dedicated microfluidic systems showed remarkable results obtained by flowing fleshly drawn peripheral blood mononuclear cells (PBMCs) [27], which might be a useful tool for blood analysis in General Practitioner offices. Other screens were also done for exploring a larger repertoire of surface antigens. Ellmark et al. [28] arrayed

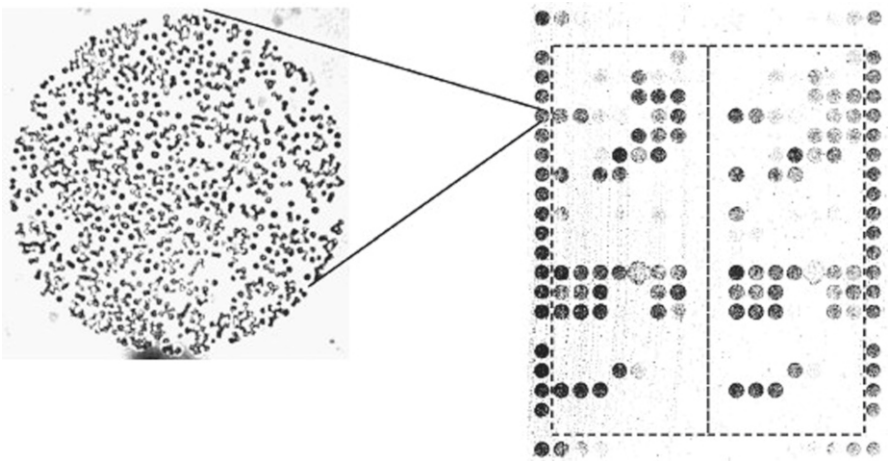

Fig. 3.1 Dark-field microscopy of a B cell sample analyzed on an antibody microarray. Anti-CD44 spots applied down the left and right sides of the duplicate arrays provide array alignment for defined antibody addresses. Anti-CD44 titrations are shown at the top and bottom of the array. The magnified image shows individual cells captured on a dot. Reprinted from *Cancer Letters,* 265, Ellmark P, Hogerkorp CM, Ek S, Belov L, Berglund M, Rosenquist R, Christopherson RI, and Borrebaeck CA, Phenotypic protein profiling of different B cell sub-populations using antibody CD-microarrays, 98–106, Copyright (2008), with permission from Elsevier

82 different antibodies on a chip (DotScan™ antibody microarray) and incubated T cells collected from human tonsils. After incubation and washing, the slides were scanned and images were acquired with an 8-bit camera (Fig. 3.1). Greyness levels for each spot were processed according to an ANOVA test and four clusters were identified and associated to B-lymphocyte subpopulations. These phenotypic profiles allowed the characterization of naïve B-lymphocytes, Germinal Center founder cells, Germinal Center cells, and memory B cells. mRNA expression levels of the targeted CD were also analyzed on genechips. DNA microarray data were similarly processed by running an ANOVA test, and in concordance with results obtained on antibody microarrays, 4 clusters corresponding to the same B cell subpopulations were distinguished. Among the 82 antibodies screened in this assay, 13 were clearly identified as key antigens for sorting the B cell subfamilies by antibody microarray analysis, 23 were identified as key antigens for sorting the B cells by gene expression analysis, and only 8 arose from both analyses. This feature thus strengthens the interest of using antibody microarrays for getting better insights in phenotypic analysis.

3.2.2 Typing Bacteria

CDs are often used as a target of choice for eukaryotic cell binding. But other surface antigens might also be selected in the search for contaminating bacteria, for instance. Due to its large social impact on public health and cost for society

(U.S. Department of Agriculture [29]), foodborne pathogens constitute a major issue in the development of fast, reliable, affordable, and easy-to-operate biosensors. Here, we will focus on the development of antibody microarrays for immunoassays developed to that end.

Since control assays are done on a routine basis, food-specific assays should allow highly parallelized analysis. This has been done, for instance, by microarraying antibodies in 96-multiwell microtiter plates [30]. After bacterial enrichment and incubation in the plates, *E. coli* and *Salmonella typhimurium* bacteria were successfully detected with 10^7 and 10^8 colony forming units (CFU)/mL. After labeling of the captured cells with a fluorophore-conjugated secondary antibody, immunoassays were automatically analyzed with a microplate reader. Distinguishing different bacterial strains or species is important, but sometimes is not sufficient: one of the most important examples is the characterization of *E. coli* strains, as some of them are harmless while other strains—Shiga Toxin-producing *E. coli* (STEC)—are the most researched foodborne pathogens. Among the STEC, six serogroups are frequently encountered and named "the big 6" [31, 32]. One must add the O157:H7 *E. coli* serogroup, which is also regularly involved in foodborne diseases. Recently Gehring et al. designed a high-throughput microarray capable of distinguishing these serotypes [32]. This antibody microarray was capable of specifically capturing each serotype—O26, O45, O103, O111, O121, O145, O157—with minimum cross-reactivity between each strain. The microarray readout was done by fluorescent scanning of the plates after bacteria capture and intracellular labeling by a nucleic acid targeting reporter molecule. However, bacterial serotyping on protein microarrays is not limited to STEC foodborne pathogens and can be extended to clinical applications. *Streptococcus pneumoniae* is responsible for thousands of deaths worldwide each year. One of the most important factors of pathogenicity of this bacterial strain is due to the composition of the capsule covering the bacterial surface. This surrounding layer is made of polysaccharides whose composition fires a specific immune response by the infected host. So far, 91 different *S. pneumoniae* serotypes have been identified [33]. Clustering their capsular antigenic determinants identifies these serogroups. In order to facilitate the easy determination of *S. pneumoniae* serotypes, Marimon et al. designed a protein microarray (Fig. 3.2) containing antibodies targeting 83 of the 91 serotypes described [34]. 226 mucoid isolates were analyzed (106 invasive isolates and a subset of 120 randomly selected noninvasive isolates) and data were compared with the results obtained by PCR analysis. Among this set of clinical samples, 37 different serotypes were distinguished among the 83 different serotypes targeted by the micro-arrayed antibodies and no statistically relevant differences were observed by comparison with standard methods used for *S. pneumoniae* serotyping.

Although most antibody microarrays used for bacteria typing or serotyping involve surface scanning for fluorescent signal analysis, other approaches have been described for microarray readout. For instance, surface-bound bacteria have been labeled by gold nanoparticles (GNP) functionalized by lectins [35]. By constructing a sandwich assay involving on one side an antibody/bacterial antigen recognition and a lectin/bacterial sugar binding on the other side, a high selectivity was obtained

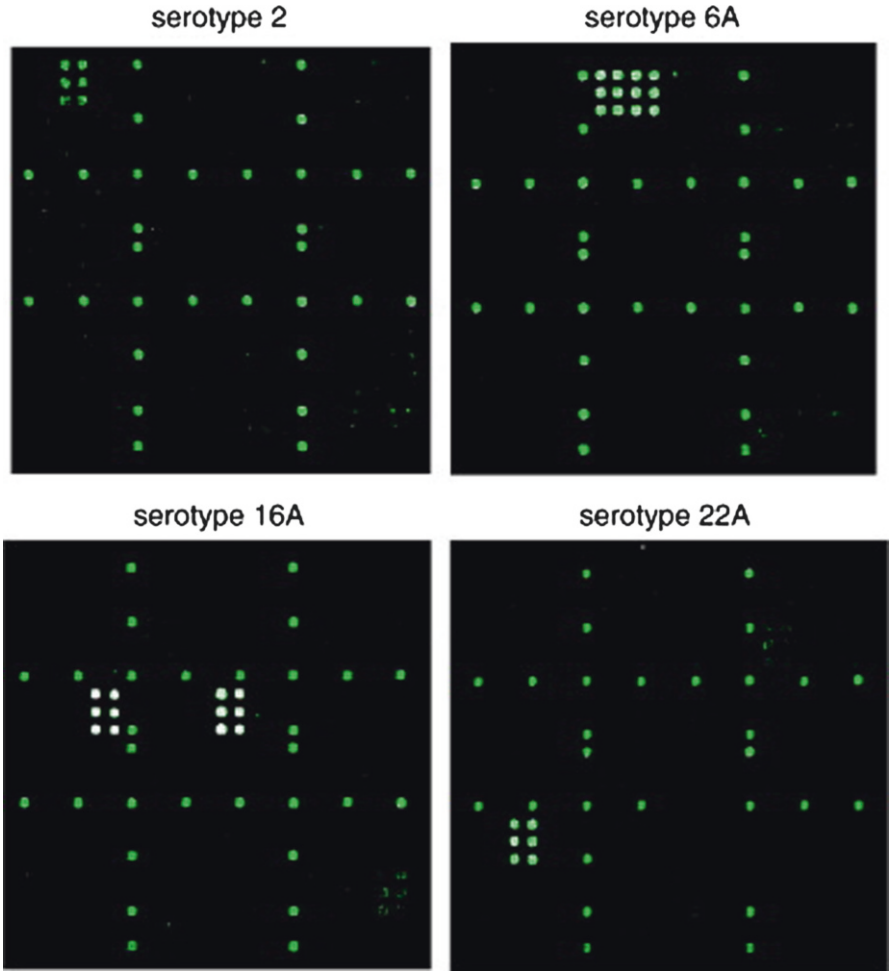

Fig. 3.2 *Streptococcus pneumonia* typing on antibody microarrays. After binding of fluorophore-labeled bacteria on microarray-wells, fluorescent signal is detected wherever the printed antibodies (specific to serotypes 2, 6A, 16A, and 22A) interact with the capsular antigens carried by the isolate being tested. The reference grid, made up of spots containing the fluorophore streptavidin-Cy3, allows easy identification of the antisera. Reprinted from *J Microbiol Methods* 80, Marimon JM, Monasterio A, Ercibengoa M, Pascual J, Prieto I, Simon L, and Perez-Trallero E, Antibody microarray typing, a novel technique for Streptococcus pneumoniae serotyping. 274–80, Copyright (2010), with permission from Elsevier

for *E. coli* and *Bacillus subtilis* strain detection. The labeling of bacteria with GNP followed by incubation with silver nitrate and hydroquinone initiates the electro-less silver deposition on the GNP and enables the identification of positive features by resonant light scattering (RLS). Such amplification processes gave significant results, as the limit of detection (LOD) was as low as 10^5 bacteria/mL. Although most antibody microarrays are analyzed after targeted labeling, there is a growing interest for

label-free technologies, particularly on surface plasmon resonance (SPR) detection [26, 36–38]. Since samples are analyzed by the time they are deposited onto the microarray, real-time analysis of cell capture can be recorded. In the specific case of bacterial detection on microarray red by SPR, the usual LOD cannot be below the 10^4–10^5 CFU/mL range. Although such levels might be observed in naturally contaminated samples, international standards request the detection of contaminating bacteria present at concentrations as low as only a few bacteria per milliliter. The difference of several orders of magnitude between the desired concentration for detection in food and the LOD observed in standard bacterial assays requires one to first run an amplification step to enrich the sample with bacteria. This phase usually lasts for one night to a few days and thus significantly impacts the overall assay duration. Recently, Bouguelia et al. proposed an original approach based on a culture-capture-measure (CCM) of living bacteria on antibody microarrays [39]. The method is based on the incubation of raw sample—diluted to 1/10th in liquid culture medium according to standard methods—on a microarray and incubation at optimal temperature in an SPR imager. By monitoring in real-time the bacterial growth on the protein microarrays, remarkable LOD were reached with the detection of only a few Salmonella in less than 15 h. Interestingly, data analysis allowed the access to initial contamination levels, regardless of the strain detected in the sample. Due to the significant improvement in assay duration and easy operation, such label-free methodologies appear as promising approaches for living cell detection.

Besides food safety and clinical applications, the detection and characterization of bacteria in the environment might also be of significant interest. To that end, antibody microarrays have also been shown to be interesting tools, as they may appear as complementary to genetic-based assays (mostly PCR-based detection) [40, 41]. Series of soil samples (sediments, rock, granite) and water-based samples (ice, hydrothermal spring, mud) worldwide (Yellowstone National Park, Argentina desert, South African mine, Iceland, Antarctica, Atacama Desert, etc.) have been processed on antibody microarrays for detecting environmental bacteria. Using a 200-antibody microarray, Rivas et al. managed to map microbial biomarkers specific to the geological environment [41]. *Vibrio* spp., *Pseudomonas* spp., and *Actinobacteria* spp. were successfully identified and confirmed by control sample cloning and sequencing of the bacterial 16S rRNA gene. Although assays are undertaken on lysed bacteria—which supposedly improves the LOD—the bacterial concentration required for specific detection is still quite high (around 10^5 CFU/mL) by comparison to concentrations that might be encountered in their native environment. This limit thus implies the necessity of either a pre-concentration step or an enrichment step before running the microarray-based immunoassay. Nevertheless, the antibody microarray approach turns out to be an interesting tool for environmental applications, mainly due to the optimized storage protocols, and to the robustness of these microarrays, making them ideal candidates for extreme conditions such as planetary exploration [42, 43]. Dedicated instruments containing a sample preparation unit (for extraction and ultra-sonication) and a sample analysis unit (for fluorescent detection) were developed and optimized to detect bacteria and spores at levels of 10^3 particles/mL.

3.3 Characterizing Diseases Using Antibody Microarrays

Many pathologies trigger protein expression disorders. This is particularly the case with cancers, since they underlie several genetic disorders. By targeting specific cell surface markers, antibody microarrays turned out to be powerful tools for studying whole cells drawn from patients. From a practical point of view, fluorescence-assisted cell sorting (FACS) remains the technique of choice for probing the expression of cell surface markers. But, due to the limitation of excitation and emission spectra of fluorophores used in routine in flow cytometry, the multiplexed analysis of CD expression is often limited to 3–5 targets per cell sample. On the other hand, thanks to the high-throughput arraying of antibodies on surfaces, protein microarray developments are more often limited by the availability of a large repertoire of CD-specific antibodies than by surface density saturation.

The antibody microarray approach for disease or pathological disorder monitoring has been developed for more than a decade by Belov et al. on leukocytes from healthy or sick patients [44]. Early studies have been carried out on a series of 60 antibodies micro-arrayed on nitrocellulose membranes incubated with leukocytes. The fluorescent labeling of captured cells with either anti-CD3 FITC or anti-CD19 PE antibodies allowed the identification of T and B lymphocytes, respectively. Authors demonstrated that several stages of leukemia disease could be diagnosed by comparing signal patterns with responses from normal pre-sorted leukocytes. The results were confirmed by analysis on cDNA microarrays and flow cytometry analysis. Although protein microarrays appear to be a powerful tool for whole-cell analysis, one should indicate that this technology is not intended to replace flow cytometry, particularly when several questions have to be addressed at the individual cell level; as cells are captured on features functionalized by a single probe, the direct capture of cells only informs the presence of one CD on its surface. Sandwich assays—with the limitation of emission/excitation spectra—with fluorescently labeled antibodies might be done but cannot be extended to a large number of targets as signal deconvolution requires large computing resources. Similar experiments were also led on larger antibody collections (82 micro-arrayed antibodies) and allowed the identification of normal B cell populations and malignant B cell populations [28]: after cell binding, the specific response of each feature allowed a principle component analysis that clearly identified germinal center founder cell populations and normal naïve B cells. More recently, this group published results obtained on antibody microarrays with higher number of probes (147 antibodies) still targeting CD antigens. This new generation of microarrays (named DotScan™ microarrays) allowed the identification of expression profiles specific to discrete leukemia subtypes [45]. The thorough analysis of the collected data outlined several differences between normal leukocytes and acute myeloid leukemia (AML) with AMC cells expressing the myeloid antigens (CD13, CD15, and CD33), the stem cell precursor markers (CD43 and CD117) and a range of other antigens (e.g., CD4, which is usually highly specific to T lymphocytes). Once again, the authors outline the need to run counter labeling with appropriate antibodies to strengthen the data issued from one subpopulation.

Leukocyte populations can also be interrogated for non-cancer disorders; recently, an 82-antibody microarray has been designed and tested for the diagnosis of systemic lupus erythematosus (SLE) [46]. SLE is an immune inflammatory disease characterized by abnormal autoantibody production. This disorder is often difficult to characterize as the diagnosis is based on clinical symptoms that may take years to appear. Soluble biomarkers have been identified (serum complement C3 and C4 and anti-dsDNA antibody) but they are not sufficient to reliably characterize this disease. Using a specific set of immobilized antibodies, PBMCs from both healthy donors and SLE-diagnosed patients, these were incubated on protein microarrays. Interestingly, the data analysis not only allowed one to distinguish these two patient populations, but it also allowed the separation of patients with SLE from patients with other autoimmune disorders.

As explained in the introduction of this chapter, most cellular assays done on antibody microarrays are done with circulating cells. However, adherent cells have also been processed on protein microarrays for disease diagnosis [47, 48]. Kaufman et al. prepared a microarray specific for metastatic melanoma and processed clinical lymph node samples [47]. Such samples contain a large variety of cell types including cancer cells, leukocytes, and also lymph node stromal cells and endothelial cells. To increase the cell subpopulation specificity, samples were treated with anti-CD45 antibody-coated magnetic beads that allowed melanoma cells and leukocytes to be enriched and profiled separately. Other adherent cells have been studied on protein microarrays for determining disease-specific expression profiles. Liu-spotted antibodies (119 CD-specific antibodies) on polystyrene Petri dishes and screened several prostate cancer cell lines [49]. Although some heterogeneity was observed in expression profiles, abnormal profiles specific to each cancer cell line were identified.

Besides diagnosis applications, there has been a step forward in the monitoring of disease stages, such as in the case of HIV patients [50]. CD4- and CD8-expressing lymphocytes are commonly used as markers for HIV infection evolution in patients. To that end, absolute and relative counting of these T-lymphocyte populations is regularly followed. Belov's group studied purified CD4- and CD8-expressing sets of cells upon incubation on antibody microarrays. Three pools of blood samples were chosen: one from long-term non-progressor patients naturally controlling viremia; one from HIV+ patients under highly active antiretroviral therapy (HAART) with low viral load (<50 copies/mL); and the last from HIV patients under HAART with viremia. Experiments were led on microarrays functionalized with 135 antibodies targeting surface antigens. Pairwise analysis of the results allowed the identification of an antigen set containing 17 antigens, 5 antigens of which have never been described so far as involved in HIV disease progression. One year later, this group published other results of interest on the application of such microarrays for HIV-infected patient follow-up [51]. In this manuscript, there were two pools of HIV patients with HAART: one group of patients achieving sustainable response by maintaining below detectable plasma viremia, and the other responding intermittently to the treatment. Among the 135 antibodies used on this device, the expression of seven markers was common to the combined groups, whereas the expression

levels of ten markers were different for these pools. Interestingly, expression levels of CD9, CD11a, CD27, CD28, and CD11c, CD44, CD49d, CD49e, and CD54 were directly linked to CD4 and CD8 cell counts, respectively.

3.4 Functional Antibody Microarrays: Application to Cell Secretion Detection

3.4.1 Analyzing Cellular Populations on a Protein Microarray

As described earlier in this chapter, protein microarrays are divided into two major classes: analytical and functional devices [52]. The interest in developing functional microarrays is partly due to the benefits of assay miniaturization; indeed, by decreasing sample volumes, the surface-to-volume ratio increases dramatically. This specific feature thus motivated surface protein immobilization processes to go one step further into cellular biological insights [53]. By targeting circulating blood cells, biologists and physicians hope to get more details on physiological events occurring at the cellular level. Since flowing cells have poor physical interference with other cells, one of the most important physiological phenomena is the production and secretion of soluble products that react with other cells at a later time, and in this manner channel information through the body. The library of secreted products—sometimes referred to as a secretome—includes mainly hormones and cytokines. Hormones remain an important target of secreted products in many diagnosis assays. Their main characteristics reside in the fact that hormones are secreted by a specific cell type and are diffused through the body before reaching one unique target cell type. In the case of immunological responses, which are also of primary interest [54–56], the problem at hand shows a much higher degree of complexity, as the same cytokine might be secreted by several cell types and may target different cell types. The large repertoire of both cytokines and biological events fired by these soluble factors explains their importance for clinical diagnosis [54]. ELISAs are very popular assays because they allow the sensitive and qualitative detection of cytokines in vitro, although no information on secreting cells is produced. In vitro culture of immune cells and measurements of their reactivity toward extracellular agents (antigenic stimulation for instance) at the cellular level is also of important interest [57, 58]. This is motivated by the evidence that in vitro measurements of cytokine levels might be directly correlated to in vivo lymphocyte activity [59].

Besides whole-cell recognition and phenotype characterization, antibody microarrays have also been used in functional cell-based assays and more specifically, for the monitoring of cytokine release in the extracellular medium [60]. To that end, Kasai et al. developed glass slides functionalized with anti-IL1b antibody and deposited islets of cells entrapped in collagen gel droplets [61]. This approach permits the deposition of several cell types on a single microarray and allows interrogation of them for the production of one cytokine. The plotting of a calibration curve obtained

by incubating known amounts of cytokine and scanning electrochemical microscopy (SECM) analysis allowed the quantitative assessment of cytokine production at the droplet level. However, cell-specific capture and cytokine detection might also be done on the same antibody microarray [27, 62]. Revzins' group proposed biochips functionalized with series of spots containing antilymphocytes antibodies (CD4 and CD8) and spotted alongside antibodies specific to IFNγ and IL-2 cytokines [27]. After T lymphocyte capture on the spots and incubation, cytokines spontaneously diffused and were immobilized on cytokine-specific surrounding spots. Both CD4+ and CD8+ subpopulations were independently studied for each cytokine revealed in ELISA-like sandwich labeling. The same approach was also validated on hepatocytes (adherent cells), processed in a similar manner and interrogated for α1-antitrypsin production [62]. In this latter example, hepatocytes were immobilized using a protein from the extracellular matrix (collagen) rather than with an antibody targeting cell surface antigens. In an attempt to describe original antibody-based multiplexed platforms, some authors also engineered DNA-conjugated antibodies for microarray applications [36, 63]. This strategy was successfully applied to the simultaneous capture of cells and detection of secreted products [63] and showed remarkable LOD (10 fM for IL-2) due to a nanoparticle amplification effect.

Functional assays dedicated to immune responses after antigenic stimulation are important for control of vaccination efficiency, and also for epitope screening or identification of multiple epitope-specific T cell populations. An elegant way to sort these cellular subpopulations is based on the immobilization on a surface of peptides bound to class I or class II MHC (major histocompatibility complex) molecules rather than antibodies targeting CD [64]. Such an approach showed significant results, thus validating the use of such protein arrays for the identification of rare T lymphocytes from vaccinated mice. More recently, the same group increased the complexity of such microarrays by combining cytokine-specific antibodies to peptide-MHC [65]. Blood drawn from patients was processed on a biochip, captured cells were stimulated upon pMHC binding, and T cell responses were monitored (secretion of IFNγ, TNFα, Granzyme B, IP-10, IL-2, IL6, and IL-1b). Samples from patients suffering from melanoma and who received vaccination showed statistically different profiles than those who did not receive vaccination. One interesting conclusion of this work is the correlation between high levels of IFNγ and TNFα and the arrest of tumor development. Such tools thus provide an easy-to-process device for the fast assessment of vaccination efficiency.

A common feature for the example of functional protein microarrays presented here is the requirement of a labeling step for cytokine production read-out. Although fluorescent labeling or nanoparticle conjugation often permits an improvement to the LOD, the end-point analysis impedes access to kinetic parameters. Due to the very low amounts of cytokines secreted by few hundreds of cells immobilized on a surface, there are only a few examples of label-free detection of cytokines on protein microarrays [38, 66]. SPR turns out to be a suitable approach for monitoring cytokine capture on antibody microarrays; either when the analysis is done downstream of the cell-specific capture [66] or when cell and cytokine capture are completed on a single biochip [38].

3.4.2 *On the Use of Microtechnologies for Designing New Functional Microarrays*

Since the last two decades, device miniaturization for biomedical applications benefited from the developments in micro- and nanotechnologies, in particular for surface functionalization. For instance, micro-contact printing using polymeric molds enabled the deposition of proteins on features at the micrometer level [67]. Interestingly, by patterning a surface at length scales smaller than individual cells, new insights became accessible. This can be illustrated by the work of Shen et al. who patterned surfaces with different proteins and followed the secreting profiles as a function of stimulating protein localization (Fig. 3.3). During immune cascades involving T cells, it is known that cell–cell communication occurs mainly through small intercellular contacts (>100 μm^2) termed immunological synapses (IS). These synapses involve protein complexes, which will interact in a given order to eventually activate the T cell. Molecular studies identified the involvement of several complexes, specifically the TCR and CD28 complexes. Cellular co-stimulation, often initiated by complex binding, seems to be more important when CD28 complexes are segregated and surrounding the IS. This hypothesis was tested by arraying central features with anti-CD3 antibodies (binding and activating the TCR complex), and microarraying smaller features—functionalized with anti-CD28 antibodies—in an intermingled matrix. Monitoring IL-2 production at the individual cell level assessed stimulation efficiency. Data analysis confirmed that presenting anti-CD28 antibodies in the cell periphery of anti-CD3 spots enhances the IL-2 secretion by CD4+ T lymphocytes. These conclusions were strengthened by control experiments led on similar arrays where each feature contained a mixture of both antibodies, with the result being that they did not trigger a significant secreting amplification effect.

3.4.3 *Analyzing Individual Cellular Responses on an Antibody Microarray*

Another significant advance in cell-based assays due to recent improvements in microtechnology is the access to individual cell responses. On the contrary to single-cell assays where one unique object is observed [68], individual-cell assays enable the monitoring of physiological events occurring at the single-cell level from a large number of individual cells. The access to individual responses measured for each member of a cell population is important from a biological point of view, as significant variations have been observed even through genetically similar cells [69]. Surface patterning techniques permitted the creation of large series of microchambers where individual cells can be isolated and cultured for a few hours [70, 71]. Han et al. designed and fabricated parallelized micro-cuvettes by microengraving and incubating individual immune cells on this array. After covering the array with a cover glass slide functionalized with four different antibodies

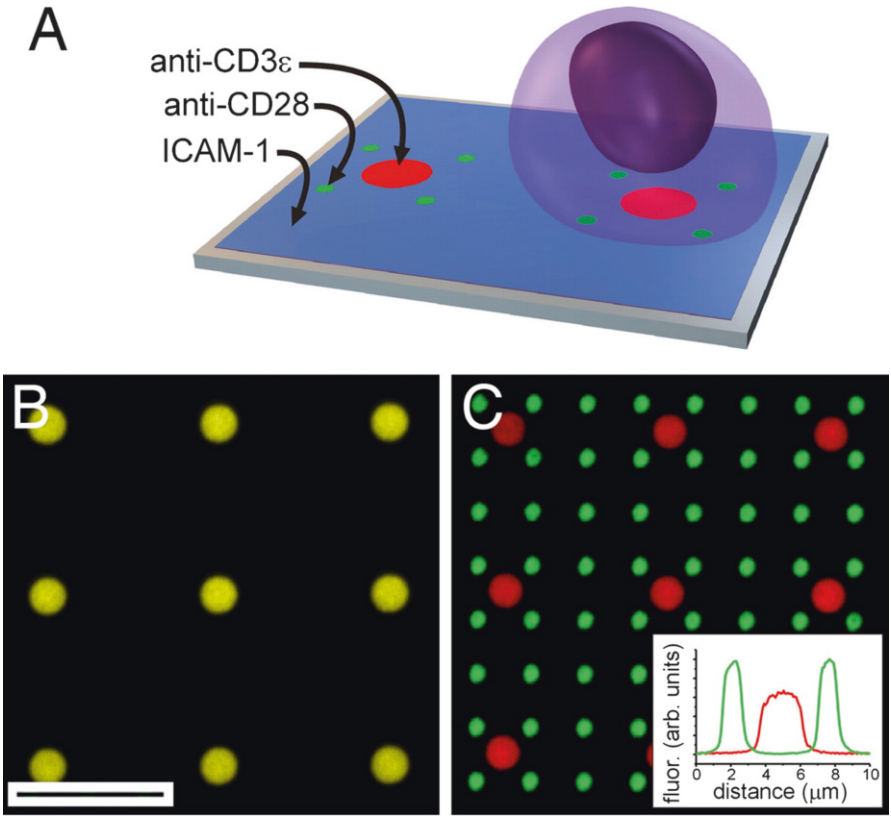

Fig. 3.3 Multiplexed detection of T cell interaction with micro-patterned, co-stimulatory arrays. (**a**) $CD4^+$ cells were presented with micro-arrayed antibodies that capture the microscale organization of ligands associated with T cell co-stimulation. Colocalized patterns were created by mixing anti-CD3 and anti-CD28 antibodies (*yellow*) in a single step (**b**), while segregated patterns were defined by sequential patterning of anti-CD3 (*red*) and anti-CD28 (*green*) on a single surface (**c**). (*Inset*) Fluorescence profile across a segregated site. ICAM-1 was coated onto the remainder of these surfaces but is omitted here for clarity (scale bar: 10 μm). Reprinted from Shen K, Thomas VK, Dustin ML, and Kam LC (2008) Micropatterning of costimulatory ligands enhances CD4+ T cell function. *Proc Natl Acad Sci U S A* 105, 7791–6 with permission from PNAS

(anti-IL-6, anti-IL-17, anti-IFNγ, anti-IL-2, and anti-TNFα), secretion levels were determined by molecular sandwich-like construction with fluorescently labeled antibodies and secreting rates per cell were calculated for each cytokine. Microfluidics also significantly impacted individual cell-based assays by enabling the channeling and isolation of individual cells [72] (Situma Biomol 2006). On an experimental point of view, the usage of polydimethylsiloxane (PDMS) molds paved the way to a large panel of applications focused on cell analysis. This is illustrated either by static assays where microstructures (Fig. 3.4) were easily processed by PDMS molding [71] or on dynamic assays where microstructures are used for injecting, separating, and studying cells on antibody microarrays [73, 74]. In their former work, Heaths'

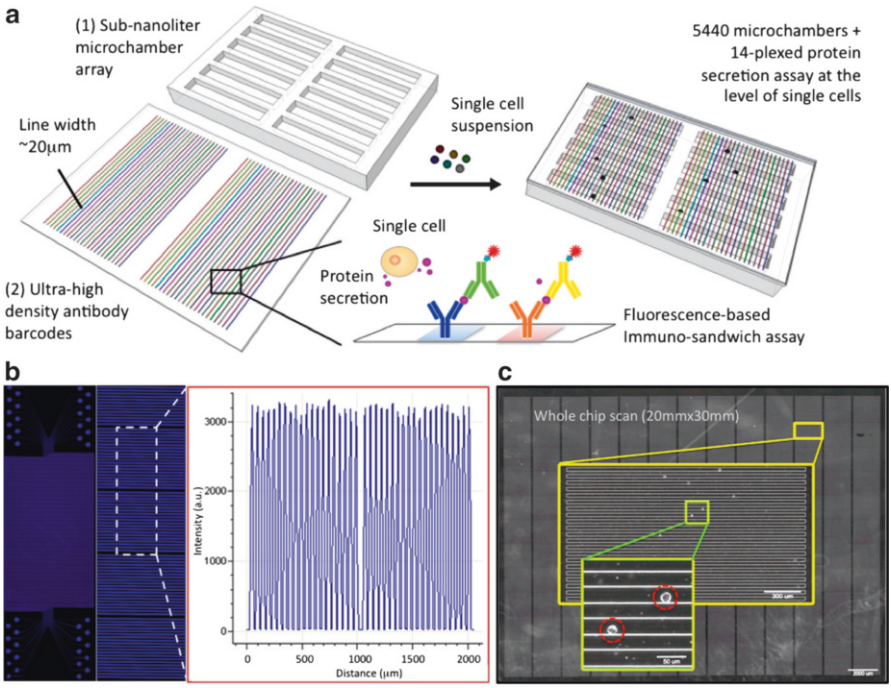

Fig. 3.4 A high-throughput multiplexed single cell secretomic assay on antibody microarray. (**a**) Schematic illustration showing integration of a high-density antibody barcode array chip and a subnanoliter micro-chamber array chip for a protein secretion assay at the single cell level. (**b**) Scanned fluorescence image showing high uniformity of protein loading across the entire barcode microarray. (**c**) Photograph stitched from a large number of individual pictures collected by an automated, motorized phase contrast microscope. Scale bar 2 mm. The first enlarged image shows a column of micro-chamber array (scale bar 300 μm). The second enlarged image shows individual cells loaded in micro-chambers (scale bar 50 μm). Reprinted with permission from Lu Y, et al. *Analytical Chemistry,* (2013), 85(4):2548–2556. Copyright (2013) American Chemical Society

group fabricated a microfluidic platform designed for multiplexed analysis of individual cells isolated in more than 1,000 nL-volume micro-chambers [73]. This device is composed of 80 microchannels actuated by valves, enabling the isolation of more than 10^4 cells in individual reaction chambers. Each chamber is functionalized by 12 stripes of proteins specific to secreted products. Human macrophages were chemically activated for cytokine production and production of IFNγ, TNFα, IL-2, IL-1a, IL-1b, IL-6, IL-10, and IL-12 was subsequently measured. Control experiments were led by intra-cellular cytokine staining (ICS) of cytokines and flow cytometry. This analysis emphasized the discrepancy of secreting and non-secreting cells, although macrophages were differentiated from the THP-1 human cell line. A similar experiment was also undertaken with PBMC cells, enriched for CD3 and CD8 markers. The authors observed a high level of functional heterogeneity within this subpopulation sorted with these two markers. The simultaneous assessment of secreting properties for the set of twelve proteins confirmed the polyfunctional

property of these cells, since an average of more than five different secreted proteins were detected. Such a complex network of secretion profiles fuels the need for new tools suitable for the multiplexed analysis of individual cells.

3.5 Conclusion

The development of surfaces functionalized with series of probes in the 1980s paved the way for a tremendous number of applications based on biomolecular recognition. The parallelized analysis of a large number of assays, conjugated to recent developments in microtechnologies, i.e., microfluidics, brought a large number of advantages such as reduction in regent cost, enhancement in assay speed, high potential for mass production of devices at low cost, the ability to integrate several processing steps into a single system, and easy operation by untrained technicians. Although early examples of microarrays were described with immobilized proteins for parallelized immune-recognition assays, the most rapid development in the biomedical field was observed with DNA microarrays. This feature might be partly explained by some limitations linked to protein (mostly antibody) handling, which are more expensive to produce than short DNA strands, more difficult to site-specifically immobilize on a surface, and more sensitive to environmental conditions (buffer, salt concentration, humidity, adjuvants, etc.) after deposition. However, proteins are involved in almost all biochemical processes that occur in a cell, most of which cannot be investigated by utilizing DNA microarrays. DNA biochips thus fail to shed light on events occurring downstream of protein expressions. Significant efforts have been made in engineering alternative probe molecules exhibiting specific binding properties and chemical stability, along with easy chemical derivatization. Among potential ligands, aptamers have had much focused attention and effort for more than two decades, but so far have failed in replacing proteins in biochemical assays used on a routine basis in analysis laboratories [75]. The unique binding properties and diversity of proteins explain the constant development of protein microarrays [76]. Among the proteins used as probes immobilized on surfaces, antibodies represent an abundant class of molecules deposited on microarrays. As demonstrated in the very first example of microarrays [15], the targeting of whole native cells is an important issue in several biomedical applications. More sophisticated antibody microarrays are arising, pushed forward by the developments led in parallel in micro- and nanotechnologies [72]. The recent developments remain focused on whole eukaryotic or prokaryotic cell identification, but may also allow access to physiological responses, as illustrated in cellular detection assays carried out on antibody biochips. However, cell-based miniaturized assays do not allow the investigation regarding physiological events at the individual cell level. An emblematic example is the search for rare cells—either stem cells or CTCs—that remain highly challenging to detect on microarrays because they are present at low levels in a biological sample and only a tiny amount of material is usually loaded on each run.

Nevertheless, antibody microarrays continue to raise interest for simple, robust, fast, and specific assays, as illustrated by recent examples of biochip fabrication for space missions and extraterrestrial life detection on Mars [43].

References

1. Jin C et al (2014) Technologies for label-free separation of circulating tumor cells: from historical foundations to recent developments. Lab Chip 14(1):32–44
2. Haeberle S, Zengerle R (2007) Microfluidic platforms for lab-on-a-chip applications. Lab Chip 7(9):1094–1110
3. Grant SF, Hakonarson H (2008) Microarray technology and applications in the arena of genome-wide association. Clin Chem 54(7):1116–1124
4. Zhu H, Qian J (2012) Applications of functional protein microarrays in basic and clinical research. Adv Genet 79:123–155
5. Foong YM, Fu J, Yao SQ, Uttamchandani M (2012) Current advances in peptide and small molecule microarray technologies. Curr Opin Chem Biol 16(1–2):234–242
6. Oyelaran O, Gildersleeve JC (2009) Glycan arrays: recent advances and future challenges. Curr Opin Chem Biol 13(4):406–413
7. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270(5235):467–470
8. Fodor SP et al (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251(4995):767–773
9. Ekins R, Chu F, Biggart E (1989) Development of microspot multi-analytite ratiometric immunoassay using dual fluorescent-labelled antibodies. Anal Chim Acta 227:73–96
10. Weller MG (2003) Classification of protein microarrays and related techniques. Anal Bioanal Chem 375(1):15–17
11. Ekins R, Chu F, Biggart E (1990) Fluorescence spectroscopy and its application to a new generation of high sensitivity, multi-microspot, multianalyte, immunoassay. Clin Chim Acta 194(1):91–114
12. Kodadek T (2001) Protein microarrays: prospects and problems. Chem Biol 8(2):105–115
13. Lynes MA (2005) Solid-phase immunoassays. Curr Protoc Toxicol Chapter 18:Unit18 17. Wiley
14. Dexlin-Mellby L et al (2011) Design of recombinant antibody microarrays for membrane protein profiling of cell lysates and tissue extracts. Proteomics 11(8):1550–1554
15. Chang TW (1983) Binding of cells to matrixes of distinct antibodies coated on solid surface. J Immunol Methods 65(1–2):217–223
16. Castel D, Pitaval A, Debily MA, Gidrol X (2006) Cell microarrays in drug discovery. Drug Discov Today 11(13–14):616–622
17. Chen DS, Davis MM (2006) Molecular and functional analysis using live cell microarrays. Curr Opin Chem Biol 10(1):28–34
18. El-Ali J, Sorger PK, Jensen KF (2006) Cells on chips. Nature 442(7101):403–411
19. Utz PJ (2005) Protein arrays for studying blood cells and their secreted products. Immunol Rev 204:264–282
20. Kato K, Toda M, Iwata H (2007) Antibody arrays for quantitative immunophenotyping. Biomaterials 28(6):1289–1297
21. Flaim CJ, Chien S, Bhatia SN (2005) An extracellular matrix microarray for probing cellular differentiation. Nat Methods 2(2):119–125
22. Kim H, Doh J, Irvine DJ, Cohen RE, Hammond PT (2004) Large area two-dimensional B cell arrays for sensing and cell-sorting applications. Biomacromolecules 5(3):822–827

23. Revzin A, Sekine K, Sin A, Tompkins RG, Toner M (2005) Development of a microfabricated cytometry platform for characterization and sorting of individual leukocytes. Lab Chip 5(1):30–37
24. Ko IK, Kato K, Iwata H (2005) Antibody microarray for correlating cell phenotype with surface marker. Biomaterials 26(6):687–696
25. Fujii Y, Anderson JM, Matsuda T (2008) Antibody-bound cell microarray for immunophenotyping: surface modification and lymphocyte subpopulations. J Biomed Mater Res B Appl Biomater 87(2):525–537
26. Suraniti E et al (2007) Real-time detection of lymphocytes binding on an antibody chip using SPR imaging. Lab Chip 7(9):1206–1208
27. Zhu H et al (2008) A microdevice for multiplexed detection of T-cell-secreted cytokines. Lab Chip 8(12):2197–2205
28. Ellmark P et al (2008) Phenotypic protein profiling of different B cell sub-populations using antibody CD-microarrays. Cancer Lett 265(1):98–106
29. Anonymous (2010) Response to questions posed by the food safety and inspection service regarding determination of the most appropriate technologies for the food safety and inspection service to adopt in performing routine and baseline microbiological analyses. J Food Prot 73(6):1160–1200
30. Gehring AG, Albin DM, Reed SA, Tu SI, Brewster JD (2008) An antibody microarray, in multiwell plate format, for multiplex screening of foodborne pathogenic bacteria and biomolecules. Anal Bioanal Chem 391(2):497–506
31. Gehring A et al (2013) A high-throughput antibody-based microarray typing platform. Sensors (Basel) 13(5):5737–5748
32. Hegde NV, Praul C, Gehring A, Fratamico P, Debroy C (2013) Rapid O serogroup identification of the six clinically relevant Shiga toxin-producing Escherichia coli by antibody microarray. J Microbiol Methods 93(3):273–276
33. Park IH et al (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J Clin Microbiol 45(4):1225–1233
34. Marimon JM et al (2010) Antibody microarray typing, a novel technique for Streptococcus pneumoniae serotyping. J Microbiol Methods 80(3):274–280
35. Gao J, Liu C, Liu D, Wang Z, Dong S (2010) Antibody microarray-based strategies for detection of bacteria by lectin-conjugated gold nanoparticle probes. Talanta 81(4–5): 1816–1820
36. Bombera R, Leroy L, Livache T, Roupioz Y (2012) DNA-directed capture of primary cells from a complex mixture and controlled orthogonal release monitored by SPR imaging. Biosens Bioelectron 33(1):10–16
37. Dudak FC, Boyaci IH (2009) Rapid and label-free bacteria detection by surface plasmon resonance (SPR) biosensors. Biotechnol J 4(7):1003–1011
38. Milgram S et al (2011) On chip real time monitoring of B-cells hybridoma secretion of immunoglobulin. Biosens Bioelectron 26(5):2728–2732
39. Bouguelia S et al (2013) On-chip microbial culture for the specific detection of very low levels of bacteria. Lab Chip 13(20):4024–4032
40. Parro V et al (2011) A microbial oasis in the hypersaline Atacama subsurface discovered by a life detector chip: implications for the search for life on Mars. Astrobiology 11(10):969–996
41. Rivas LA et al (2008) A 200-antibody microarray biochip for environmental monitoring: searching for universal microbial biomarkers through immunoprofiling. Anal Chem 80(21):7970–7979
42. de Diego-Castilla G et al (2011) Assessing antibody microarrays for space missions: effect of long-term storage, gamma radiation, and temperature shifts on printed and fluorescently labeled antibodies. Astrobiology 11(8):759–773
43. Parro V et al (2011) SOLID3: a multiplex antibody microarray-based optical sensor instrument for in situ life detection in planetary exploration. Astrobiology 11(1):15–28
44. Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI (2001) Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. Cancer Res 61(11):4483–4489

45. Barber N et al (2009) Profiling CD antigens on leukaemias with an antibody microarray. FEBS Lett 583(11):1785–1791
46. Lin MW, Ho JW, Harrison LC, dos Remedios CG, Adelstein S (2013) An antibody-based leukocyte-capture microarray for the diagnosis of systemic lupus erythematosus. PLoS One 8(3):e58199
47. Kaufman KL et al (2010) An extended antibody microarray for surface profiling metastatic melanoma. J Immunol Methods 358(1–2):23–34
48. Rahman W et al (2012) Analysis of human liver disease using a cluster of differentiation (CD) antibody microarray. Liver Int 32(10):1527–1534
49. Liu AY (2000) Differential expression of cell surface molecules in prostate cancer cells. Cancer Res 60(13):3429–3434
50. Wu JQ et al (2007) Antibody microarray analysis of cell surface antigens on CD4+ and CD8+ T cells from HIV + individuals correlates with disease stages. Retrovirology 4:83
51. Wu JQ et al (2008) Longitudinal microarray analysis of cell surface antigens on peripheral blood mononuclear cells from HIV + individuals on highly active antiretroviral therapy. Retrovirology 5:24
52. Sutandy FX, Qian J, Chen CS, & Zhu H (2013) Overview of protein microarrays. Current protocols in protein science/editorial board, John E. Coligan … [et al.] Chapter 27: Unit 27 21. Wiley
53. Wilson DS, Nock S (2002) Functional protein microarrays. Curr Opin Chem Biol 6(1): 81–85
54. Bienvenu J, Monneret G, Fabien N, Revillard JP (2000) The clinical usefulness of the measurement of cytokines. Clin Chem Lab Med 38(4):267–285
55. Hernandez-Fuentes MP, Warrens AN, Lechler RI (2003) Immunologic monitoring. Immunol Rev 196:247–264
56. Schett G, Elewaut D, McInnes IB, Dayer JM, Neurath MF (2013) How cytokine networks fuel inflammation: toward a cytokine-based disease taxonomy. Nat Med 19(7):822–824
57. Bromage E, Stephens R, Hassoun L (2009) The third dimension of ELISPOTs: quantifying antibody secretion from individual plasma cells. J Immunol Methods 346(1–2):75–79
58. Cox JH, Ferrari G, Janetzki S (2006) Measurement of cytokine release at the single cell level using the ELISPOT assay. Methods 38(4):274–282
59. Brattig NW et al (2005) Relevance of ex vivo blood lymphocyte assay for in vivo lymphocyte function. Clin Exp Immunol 139(1):127–131
60. Gonzalez LC (2012) Protein microarrays, biosensors, and cell-based methods for secretome-wide extracellular protein-protein interaction mapping. Methods 57(4):448–458
61. Kasai S et al (2005) Real-time monitoring of reactive oxygen species production during differentiation of human monocytic cell lines (THP-1). Anal Chim Acta 549(1–2):14–19
62. Jones CN et al (2008) Multifunctional protein microarrays for cultivation of cells and immunodetection of secreted cellular products. Anal Chem 80(16):6351–6357
63. Bailey RC, Kwong GA, Radu CG, Witte ON, Heath JR (2007) DNA-encoded antibody libraries: a unified platform for multiplexed cell sorting and detection of genes and proteins. J Am Chem Soc 129(7):1959–1967
64. Soen Y, Chen DS, Kraft DL, Davis MM, Brown PO (2003) Detection and characterization of cellular immune responses using peptide-MHC microarrays. PLoS Biol 1(3):E65
65. Chen DS et al (2005) Marked differences in human melanoma antigen-specific T cell responsiveness after vaccination using a functional microarray. PLoS Med 2(10):e265
66. Stybayeva G, Kairova M, Ramanculov E, Simonian AL, Revzin A (2010) Detecting interferon-gamma release from human CD4 T-cells using surface plasmon resonance. Colloids Surf B Biointerfaces 80(2):251–255
67. Shen K, Thomas VK, Dustin ML, Kam LC (2008) Micropatterning of costimulatory ligands enhances CD4+ T cell function. Proc Natl Acad Sci U S A 105(22):7791–7796
68. Andersson H, van den Berg A (2004) Microtechnologies and nanotechnologies for single-cell analysis. Curr Opin Biotechnol 15(1):44–49

69. Niepel M, Spencer SL, Sorger PK (2009) Non-genetic cell-to-cell variability and the consequences for pharmacology. Curr Opin Chem Biol 13(5–6):556–561
70. Han Q, Bradshaw EM, Nilsson B, Hafler DA, Love JC (2010) Multidimensional analysis of the frequencies and rates of cytokine secretion from single cells by quantitative microengraving. Lab Chip 10(11):1391–1400
71. Lu Y et al (2013) High-throughput secretomic analysis of single cells to assess functional cellular heterogeneity. Anal Chem 85(4):2548–2556
72. Situma C, Hashimoto M, Soper SA (2006) Merging microfluidics with microarray-based bioassays. Biomol Eng 23(5):213–231
73. Ma C et al (2011) A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. Nat Med 17(6):738–743
74. Shi Q et al (2012) Single-cell proteomic chip for profiling intracellular signaling pathways in single tumor cells. Proc Natl Acad Sci U S A 109(2):419–424
75. Baird GS (2010) Where are all the aptamers? Am J Clin Pathol 134(4):529–531
76. Hall DA, Ptacek J, Snyder M (2007) Protein microarray technology. Mech Ageing Dev 128(1):161–167