Biochips beyond DNA: technologies and applications

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Abstract. Technological advances in miniaturization have found a niche in biology and signal the beginning of a new revolution. Most of the attention and advances have been made with DNA chips yet a lot of progress is being made in the use of other biomolecules and cells. A variety of reviews have covered only different aspects and technologies but leading to the shared terminology of "biochips." This review provides a basic introduction and an in-depth survey of the different technologies and applications involving the use of non-DNA molecules such as proteins and cells. The review focuses on microarrays and microfluidics, but also describes some cellular systems (studies involving patterning and sensor chips) and nanotechnology. The principles of each technology including parameters involved in biochip design and operation are outlined. A discussion of the different biological and biomedical applications illustrates the significance of biochips in biotechnology.

Keywords: protein, chips, arrays, microfluidics, lab-on-a-chip, micro total analysis systems, immunoassays, diagnostics, electrophoresis, drug screening, drug discovery, proteomics, expression profiling, photolithography, soft lithography, patterning, tissue array, nanotechnology, nanolithography, cell assays.

Introduction

The completion of the sequence of the human genome commenced a new era in biology. For a long time, biology has been the poor cousin of the physical and chemical sciences due to the lack of quantitative rigor for characterizing biological phenomenon. This is mainly due to the inherent complexity of biological systems and the lack of tools to track these systems. Advances in technology and information sciences put humanity on the verge of conquering biological complexity.

An effective way to address biological complexity is to isolate the different components of the system and to understand how the different components interact. Historically, biology has been focused on studying one component at a time. The advent of Systems Biology has accelerated the shift to understanding multiple components at a time, which is more appropriate to characterize biological phenomenon. One area that will have immediate impact toward a better understanding of biology is through the use of chip technology to capture the different components in a miniaturized, well-defined, and quantifiable environment. The applications of chip technology in biology dawned during the genomics

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revolution. The power of DNA chips became imminent with its applications in profiling the expression of thousands of genes arrayed in a silicon chip. From then on the concept of chips have been extended to a variety of biomolecules such as proteins and including cells leading to the coining of the word ''biochips.''

Although many reviews have been written on biochips (see Table 1), the aim of this particular review is to provide the users or potential users of non-DNA biochips with an understanding of a wide enough breadth to realize the different options that exist and, if pursued further, evaluate the effectiveness,

Table 1. Literature references (books, review articles, or journal publications that review or provide an overview of the particular subject area).

Category	Subject area	References
Book	Microsystem technology	Manz and Becker, editors [563]
	μTAS	van den Berg *et al.*, editors [564]
General	Protein arrays	Wagner and Kim [59]
		Mitchell [79]
		Templin *et al.* [125]
		Stoll *et al.* [565]
		Wilson and Nock [9]
		Cahill [348]
		Ekins and Chu [5]
		Zhu and Snyder [496]
		Schweitzer and Kingsmore [389]
		Kodadek [3]
		Mirzabekov and Kolchinsky [150]
	Peptide arrays	Frank [513]
	Cell-based microarrays	Blagoev and Pandey [367]
	Tissue arrays	Kallioniemi *et al.* [464]
	Microfluidics	Freemantle [12]
		Mitchell [38]
		Reyes *et al.* [294]
		Auroux *et al.* [307]
	Microfluidics on PDMS	McDonald *et al.* [10]
Technologies	Microfabrication	Qin *et al.* [104]
	Patterning proteins	Blawas and Reichert [116]
		Bernard *et al.* [218]
		Kane *et al.* [51]
	Patterning cells	Kane *et al.* [51]
		Bhadriraju and Chen [566]
		Folch and Toner [238]
	Soft lithography	Whitesides *et al.* [213]
		Xia and Whitesides [216]
		Zhao *et al.* [217]
	Microfluidics microfabrication	Reyes *et al.* [294]
		Becker and Gärtner [61]
		Chovàn and Guttman [41]

(Continued.)

Table 1. Continued.

Category	Subject area	References
	Microfluidics on PDMS	McDonald *et al.* [10] Anderson *et al.* [251] Jo *et al.* [252]
Applications	Microfluidics (μTAS)	Auroux *et al.* [307] McDonald *et al.* [10]
	Proteomics	Figeys and Pinto [568]
	Capillary electrophoresis on chips	Dolník *et al.* [75] Effenhauser *et al.* [411] Regnier *et al.* [532] Kutter [73]
	Chromatography on chips	Regnier *et al.* [532] Kutter [73]
	Protein expression profiling	Jenkins and Pennington [344]
	Dielectric separations	Hughes [296]
	Clinical and functional target validation	Mousses *et al.* [569]
	Drug delivery	Santini *et al.* [518]
	Immunoassay and other ligand assays	Wan [529] Ekins and Chu [524]
	On-chip enzymatic assays	Wang [529]
	Medical diagnostics	Talary *et al.* [567]
	Patterned cells	Kane *et al.* [51] Singhvi *et al.* [187] Bhadriraju and Chen [566]

appropriateness, and ultimately the value of biochips (from those that may or will be commercially available to those that will be designed to suit particular needs). This review also provides a perspective for those interested in the biotechnological aspect of biochips: many technical issues are not trivial but that research leads to knowledge, discovery, and/or even achievement. Furthermore, a list of abbreviations and a glossary are provided as appendices to cope with the complex terminologies that have floated in the field.

Microfabrication of chips

The development of a biochip involves several considerations: what type of experiments needs to be performed on the chip, what support is needed, how to apply the protein on the chip, and how to immobilize the protein without losing its functionality. The type of experiment will determine the chip format. In constructing a chip, an important consideration is what material works best or is compatible for a given type or range of required experiments. The chip in its final state goes through a number of steps from applying the proper surfaces that are compatible with the biomolecules of interest, in this case, proteins or cells, to the

techniques used to attach these to the surfaces in a functional form, *i.e.*, keeping the proteins active or cells viable.

Biochip formats

Biochips have different configurations depending on the application. They include microfluidic chips, nanovials, nanoplates, three-dimensional pads, patterned arrays, and flat-surface spot arrays [1]. These can be classified mainly into two types: arrays and microfluidics. Arrays have sample or ligand molecules (*e.g.*, antibodies) at fixed locations on the chip while microfluidics involves the transport of material, samples, and/or reagents, on the chip. A third type of chip is a biosensor where a chip can be composed of several components including electronic circuitry, electrodes, sample vials, or channels. When sufficiently miniaturized, in principle, a sensor system can also be arrayed. Examples of the different chip formats are shown in Fig. 1.

Arrays

An array encompasses a number of variations including nanovials, nanoplates, patterned arrays, and flat-surface spot arrays. So long as proteins or their ligand molecules are delivered in a regular fashion or present in certain groupings on the chip, an array is created, regardless of whether the same sample or different samples of the same type are deposited onto the chip. One important factor is spot size. An array, specifically a microarray, will have spot sizes in micrometer dimensions. Whether or not a filter membrane containing spots of a few millimeters in diameter, for example, should be considered an array is debatable. An array might contain variants of a bioactive peptide or protein (domains, mutants, or splice variants), components of a protein pathway, or the entire proteome of an organism [2]. The protein of interest can be in solution as in nanovials or held in place by some gel, matrix, or capture agent. Arrayed molecules referred to in this review include protein antigens, ligand molecules or binders (*e.g.*, antibodies), cells, and tissues. In the case of proteins, the strategy is to have an array with only antigen molecules (antigen arrays), or its reverse, with only antibodies (antibody arrays). Other terms that have been used include protein function arrays (containing antigens) and protein detecting arrays (containing ligand molecules) [3].

Arrays have been a popular chip format in the biotechnology sector due to their adaptability to automation and high-throughput systems and because they are adapted from or a logical extension of the equivalent format containing DNA molecules. Although this may be a general notion, Roger Ekins (Department of Molecular Endocrinology, University College London Medical School) conceived, used, and patented ligand-binding assays in a microarray format in the 1980s [4,5]. Ekin's patent covers "all microarray-based ligand assays" (including immunoassays) and DNA/RNA analysis [6]. The key concept regarding microarrays according to Ekins is that when a spot is small enough,

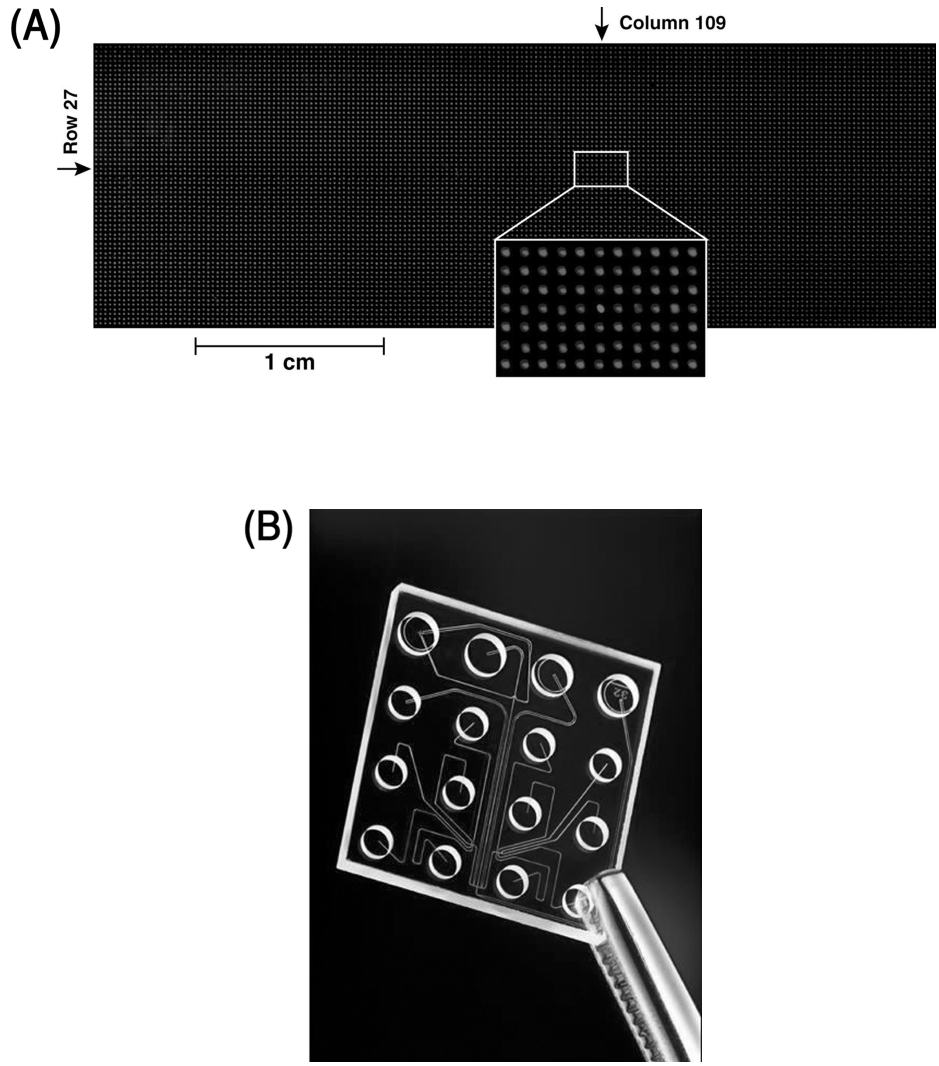
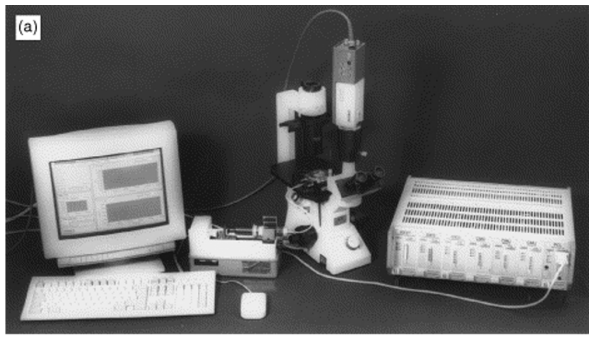
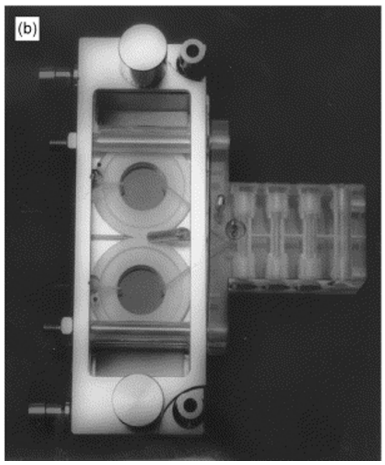


Fig. 1. (A) Microarray format. A glass-coated slide with 10,800 protein microspots. Array density is 1600 per cm^2 and spot size is 150–200 μm in diameter printed via split-pin arraying. Protein G was printed 10,799 times and FRB was printed once in row 27, column 109 as indicated (visible in fluorescence analysis, see [118]). Reprinted with permission from [118]. Copyright 2000 American Association for the Advancement of Science. (B) Microfluidics format. A microfluidic device for electrophoretic separations. Copyright Agilent Technologies. (C) Sensor chip experimental setup. (a) Photograph of an experimental setup. The culture/sensor unit (b) is placed on the microscope stage. (b) Photograph of the two-channel version of the culture/sensor unit. The sensor chips from the bottoms of the two culture chambers. These chambers are connected to a sensor module on the right side, equipped with microelectrodes for pH and oxygen-concentration [48]. Copyright 1998 Elsevier Science Ltd. (D) Illustration of the sensor chip in (C). This design of the silicon sensor chip includes IDES-structures, ISFETs, oxygen sensors, temperature sensors, and a transparent aperture. Because each type of sensor element is at least present in pairs, a chip allows redundant data acquisition of each parameter [48]. Copyright 1998 Elsevier Science Ltd.

(C)





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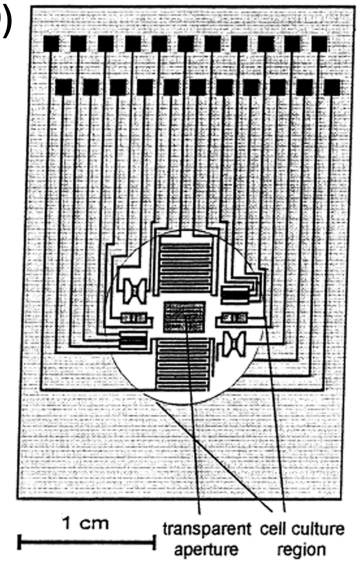

Fig. 1. Continued.

the signal obtained is independent of the sample volume and the amount of binder (*e.g.*, antibody) within this spot, and dependent only on the concentration of target (antigen) molecules in solution that the binders in this spot are exposed to. When operating within this region, termed ambient analyte assay limits, greater sensitivity and faster results can be obtained [4,7,125]. (See Fig. 2; see also Biochip applications, Clinical Applications, Diagnostics, Immunoassays.) (However, microarrays can also be analyzed on the basis of the total mass of the analyte bound to capture antibodies rather than on concentration [8].) Nevertheless, such insights led to the idea of the microarray and that with a drop of sample, thousands of different substances can be analyzed in parallel [4].

The advantages of arrays lie in the minimal sample consumption and high-throughput capability inherent in its configuration. Compared to a 96-well microtiter plate, a single array spot may contain a million times less sample, which means less than a picogram or femtomole of protein [9]. And yet, the amount of information that can be generated is tremendous. Even chips that are biochemical sensors involving SPR-BIA (surface plasmon resonance-biomolecular interaction analysis) are amenable to an array format for high-throughput

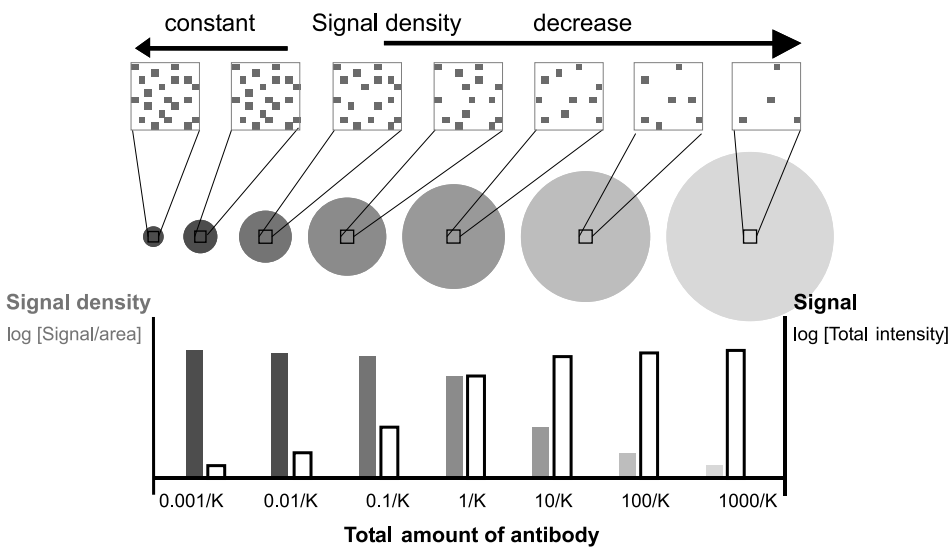

Fig. 2. Signal and signal density in microspots. Signal density (signal/area, relative intensities, log scale) and signal (total intensity, log scale) or captured targets in microspots are shown for different concentrations of capture molecules. The capture molecules are immobilized with the same surface density on all spots. The signal (total signal) increases with increasing amount of capture molecules at growing spot size. When most of the targets are captured from the solution the signal reaches its maximum. By contrast, signal density (signal/area) increases with decreasing amount of capture molecules (decreasing spot size), reaching a constant level when the capture molecule concentration is $<0.1/K$ (K is the association constant). Under these ambient analyte conditions, target concentration in solution is minimally altered by the amount of captured targets on the microspot. The figure was adapted from Ref. [328]. Reprinted with permission from Ref. [125]. Copyright 2002 Elsevier Science Ltd.

applications (for examples, see Table 2, Drug discovery and development, Proteomics and drug discovery tools) (see Biacore, HTS Biosystems, and Prolinx, Table 3; Nanoplex, Table 4).

One of the relevant and widespread applications of arrays containing proteins or cells is in immunodiagnostics. The other is expression profiling along with the elucidation of protein–protein interactions, which the proteomics field has fostered. Other applications are enumerated in Table 2 (see also Biochip Applications). Nevertheless, the technology is not without its challenges. Difficulties arise in surface effects that lead to protein adsorption and denaturation and in detector sensitivity. A lot of research and development has gone into patterning and immobilization, exploring and using various surface chemistries to arrive at appropriate surfaces to anchor either sample or ligand molecules (see Technologies, Array technologies). The importance of detector sensitivity is obvious from the minute amounts of proteins that may be arrayed. In addition, some research has focused on the issue of tailor-made molecular tags that address specificity in detecting particular proteins in sandwich assays and in multiplexed analyses.

Microfluidics

Microfluidics involves manipulating fluids (gases and liquids) in a closed channel system with cross-sectional dimensions in the range of 5–200 μm [10–13] and total chip area is 2–3 cm^2 [12,13]. A miniaturized gas chromatographic (GC) system on a silicon wafer, developed in the 1970s at Stanford University by S.C. Terry *et al*. is considered to be the first microfluidic device [14]. The technology, however, did not develop much further until molecular biology, particularly the area of genomics, made a major impact in academic, pharmaceutical, and biotechnological research. Because it was possible to carry out experiments such as separations and assays on a chip, a microfluidic chip was termed lab-on-a-chip. The type of experiments can range from biochemical or chemical synthetic reactions to analytical separations including electrophoresis. Extending this capability and incorporating different experimental steps so that a full analysis can be performed on a chip has led to the term μ-TAS (micrototal analysis system), a concept discussed in a 1990 paper by Andreas Manz (Center for Analytical Sciences, Department of Chemistry, Imperial College of Science, Technology and Medicine, London, UK) [15]. Such a chip may incorporate functions such as fluid transport, mixing, incubation, and even particle sorting [16]. Microfluidics for aqueous systems, which are relevant for biochemical and biological applications, came out of the research labs of Andreas Manz [7,17–20], D. Jed Harrison (Department of Chemistry, University of Alberta, Edmonton, Canada) [21–26], J. Michael Ramsey (Oak Ridge National Laboratory, Oak Ridge, Tenn.) [27–32], and Richard A. Mathies (Department of Chemistry, University of California, Berkeley, CA, USA) [33–36] with technologies adapted from the microelectronics industry (photolithography and wet etching) [10,37].

Table 2. Summary of applications (a list of biochip applications including those cited in this review).

Application area	Chip format	Description	Reference
Proteomics and Drug discovery tools			
Electrophoresis, CE	microfluidics	Electrophoresis, early demonstration	Manz *et al.* [15]
Electrophoresis, CE	microfluidics	Electrophoresis, high-speed separations	Jacobson *et al.* [30,402]
Electrophoresis, CE	microfluidics	Electrophoresis, commercial chip	Owen [11], Kuschel [473]
Electrophoresis, CE	microfluidics	Electrophoresis + protein digestion and peptide separation	Gao *et al.* [474]
Electrophoresis, CE	microfluidics	Electrophoresis + digestion, separation, and post-column labeling	Gottschlich *et al.* [570]
Electrophoresis, CE	microfluidics	Analyze kinase A assay	Cohen *et al.* [475]
Electrophoresis, CE	microfluidics	Immunoaffinity capillary electrophoresis	Dodge *et al.* [80], Ensing and Paulus [471], Phillips and Chmielinska [472]
Chromatography, EC	microfluidics	EC and ion-exchange chromatography; chips and capillaries have comparable resolution.	Ericson *et al.* [423]
Chromatography, EC	microfluidics	Open-channel EC	Jacobson *et al.* [403]
Chromatography, EC	microfluidics	Reverse-phase EC on peptides and amino acids; outperformed capillary separations in speed and efficiency	Throckmorton *et al.* [476]
Chromatography, EC	microfluidics	Micellar electrokinetic chromatography; cyclic channel system; theophylline analysis	von Heeren *et al.* [418]
Chromatography, EC	microfluidics	2D EC/CE, 1st dimension: open channel EC, 2nd dimension: CE	Gottschlich *et al.* [571]
Particle sorting	microfluidics	Trapping and separation of latex beads, TMV, below 1 μm	Green *et al.* [298]
Particle sorting	microfluidics	Dielectrophoresis of particles and cells	Fiedler *et al.* [299]
Particle sorting	microfluidics	Separation, multicomponent fractionation	Hughes [296]
Flow cytometry	microfluidics	Flow cytometry of *E. coli*	McClain *et al.* [477]
Mass spectrometry	sensor	Proteomics, chip-MS SPR-BIA applications	Nelson *et al.* [44]

(Continued.)

Table 2. Continued.

Application area	Chip format	Description	Reference
Mass spectrometry	sensor	MALDI/MS-BIA analysis	Sönksen *et al.* [45]
Mass spectrometry	array	SELDI-TOF MS	Merchant and Weinberger [54]
Mass spectrometry	array	SELDI protein chips	Weinberger *et al.* [55]
Mass spectrometry	array	SELDI profiling, cancer research	von Eggeling *et al.* [56]
Mass spectrometry	microfluidics	High-throughput CE/ESI-MS	Zhang *et al.* [480]
Mass spectrometry	microarray	Sample chip for ESI-MS	Prosser *et al.* [481], Schultz *et al.* [482]
Mass spectrometry	array	Multichannel device for ESI-MS	Liu *et al.* [483]
Expression profiling	array (antibody)	Proteomic profiling in cancer	Knezevic *et al.* [484]
Expression profiling	array (cDNA expression)	Protein expression and antibody screening	Lueking *et al.* [485], Büssow *et al.* [486]
Expression profiling	array (antigen)	multiplex protein profiling; immunoRCA on 75 cytokines, proteomic survey	Schweitzer *et al.* [388]
Expression profiling	array (antigen)	cytokine profiling chip, commercial	Wagner and Kim [59]
Expression profiling	array (antigen)	SELDI profiling of tumor tissue lysates	von Eggeling *et al.* [56]
Expression profiling	array(antibody)	60 antibodies against CD antigens on arrays; studied patterns from blood leukocytes of leukemic patients	Belov *et al.* [534]
Expression profiling	microarray (tissue)	Biomarker profiling of tumor samples	Kononen *et al.* [465], Nocito *et al.* [467]
Expression profiling	microarray (tissue)	Analyzed prognostic markers in 553 breast carcinomas	Torhorst *et al.* [466]
Expression profiling	microarray (tissue)	Verified sample quantity representative of whole tissue and use of archival tissue	Camp *et al.* [539]
Expression profiling	array (cell lysates)	Molecular profiling of tumor samples by layered expression scanning	Englert *et al.* [468]
Expression profiling ELISA	array (antibody)	Simultaneous detection of IgG as antigens (direct) and cytokines (sandwich) on PVDF-immobilized antibodies; ECL detection	Huang [490]

Expression profiling ELISA	microarray (antigen)	ELISA for autoimmune diagnostics, monitoring 18 autoantigens in human sera	Joos *et al.* [121]
Expression profiling ELISA	microarray	High-throughput ELISA with IgGs as antigens	Mendoza *et al.* [74]
Molecular screening	arrays (antigen, antibody)	Arrays on membranes, binding interaction detected with HRP-conjugated antibodies; visualization by ECL	Huang [572]
Molecular screening	array (cDNA expression)	Antibody specificity screening	Lueking *et al.* [485]
Molecular screening	array (cDNA expression)	Screening antibody–antigen interactions	Holt *et al.* [491]
Molecular screening	array (antibody)	High-throughput screening of antigen–antibody interactions; expression monitoring	De Wildt *et al.* [340]
Molecular screening	array (target protein)	Identification of protein targets of small molecules	MacBeath and Schreiber [118]
Molecular screening	microarray (yeast proteome)	High-throughput screening of biochemical activity (phosphoinositide-binding)	Zhu *et al.* [128]
Molecular screening	microarray	Immunoassay and enzymatic reactions	Arenkov *et al.* [137]
Molecular interactions	array	Screening protein interactions with p52, dsDNA, ssDNA, and simian virus 40 early pre-mRNA	Ge [514]
Molecular interactions	microarray	Tested 3 interactions: protein G with IgG, p50 with IκBα, and FRB with FKBP12	MacBeath and Schreiber [118]
Molecular interactions	microarray (yeast proteome)	Protein–protein interactions in yeast	Zhu *et al.* [128]
Drug screening and testing			
Drug screening	microarray	Determining compound selectivity for subtypes of adrenergic receptors	Fang *et al.* [153]
Drug screening	sensor	Multiparametric screening of the effects of cytochalasine B and D on the cytoskeleton of LS 174T cells.	Ehret *et al.* [47]
Drug delivery	microfluidics	Controlled release; silicon chip with electrochemical dissolution of chemicals	Santini *et al.* [517]

(Continued.)

Table 2. Continued.

Application area	Chip format	Description	Reference
Clinical applications			
Direct-binding immunoassay	array sensor (antibody)	Measurement of binding kinetics and the effect of spot size on the association rate	Sapsford *et al.* [573]
Direct-binding immunoassay	microfluidics	IgG analysis with a post-separation CE chip	Mangru and Harrison [405]
Direct-binding immunoassay	microfluidics	hCG in serum	Schneider *et al.* [71]
Direct-binding immunoassay	microfluidics	hCG in undiluted whole blood	Schneider *et al.* [72]
Direct-binding immunoassay	microfluidics	Analysis of serum cortisol with no extraction or sample preparation	Koutny *et al.* [454]
Immunoassay	microarray	Micromosaic immunoassay: patterning by microfluidics; rows of antigens perpendicular to sample solutions; antibody screening for immunodiagnostics	Bernard *et al.* [249]
Immunoassay	array (antigen)	AFM detection	Jones *et al.* [69]
Immunoassay	sensor	Multianalyte analysis with an integrated optical waveguide sensor	Plowman *et al.* [574]
Immunoassay	microarray	Immobilized IgG in polyacrylamide gel pad; FITC-labeled antigen	Arenkov *et al.* [137]
Immunoassay	microfluidics	Automated heterogeneous assay with PA/rIgG affinity model in less than 5 min	Dodge *et al.* [80]
Immunoassay	microfluidics	Micellar electrokinetic chromatography; cyclic channel system; theophylline analysis	von Heeren *et al.* [418]
Immunoassay	microfluidics	Simultaneous assays for ovalbumin and anti-estradiol	Cheng *et al.* [541]
Competitive immunoassay	sensor	Serum theophylline determination for point-of-care diagnostics	Schult *et al.* [575]
Competitive immunoassay	microfluidics	Distinguish between IgG subclasses; immunoaffinity CE	Dodge *et al.* [80]

Competitive immunoassay	microfluidics	Electrophoresis; analysis of monoclonal antibodies and theophylline	Chiem and Harrison [25,525]
Sandwich immunoassay	array (antibody)	High-throughput screening of multiple cytokine expression in sera; ELISA with ECL detection	Huang *et al.* [526]
Sandwich immunoassay	array (antibody)	High-throughput protein quantification; multiplexed ELISA; human cytokines	Moody *et al.* [527]
Sandwich immunoassay	microarray (antibody)	Multiplexed ELISA; option for analyte quantification in complex biological samples	Wiese *et al.* [528]
Sandwich immunoassay	microarray (antibody)	Mass-sensing, multianalyte analysis of 4 human IgG subclasses	Silzel *et al.* [8]
Sandwich immunoassay	microarray	Detecting hCG with FTTC-labeled secondary antibody	Arenkov *et al.* [137]
Sandwich immunoassay	array sensor	Simultaneous multianalyte fluoroimmunoassays on a planar waveguide; antibody lanes patterned perpendicular to sample lanes	Rowe *et al.* [119]
Sandwich immunoassay	array sensor (antibody)	Simultaneous multianalyte identification (bacterial, viral, and protein analytes)	Rowe *et al.* [576]
Sandwich assay	microarray	High-throughput ELISA with IgGs as antigens	Mendoza *et al.* [74]
Fluorescent-sandwich immunoassay	microarray	Multiplexed profiling of induced secretion of inflammatory cytokines with RCA detection	Schweitzer *et al.* [388]
Enzymatic assay	microfluidics	β-galactosidase (120 pg), using RBG (7.5 ng)	Hadd *et al.* [32]
Enzymatic assay	microarray	Assays on HRP, AP, and β-D-glucuronidase; accelerated by microelectrophoresis	Arenkov *et al.* [137]
Enzymatic assay	microfluidics	Fluorogenic enzyme assay on β-galactosidase after on-chip cell lysis and protein extraction	Schilling *et al.* [542]
Enzymatic assay	microarray (target proteins)	Phosphorylation assay	MacBeath and Schreiber [118]
Diagnostics	array (antibody)	Analyze blood leukocytes for diagnosing leukemia based on expessed CD antigens	Belov *et al.* [534]
Diagnostics	microarray (antigen)	Screening 18 diagnostic markers for systemic rheumatic disease; ELISA	Joos *et al.* [121]
Reverse immunoassay	microarray	ImmunoRCA on allergen-specific IgEs	Wiltshire *et al.* [535]

(Continued.)

Table 2. Continued.

Application area	Chip format	Description	Reference
Reverse immunoassay	microarray (antigen)	ImmunoRCA for femtomolar antigen detection	Schweitzer *et al.* [387]
Biomarkers	microarray	Detection/quantitation of specific proteins and antibodies in complex solutions	Haab *et al.* [375]
Biomarkers	See also Proteomics and Drug Discovery tools, Expression Profiling on tissue microarrays		
Disease monitoring	microarray (antigen)	Analysis of proteomic changes in cell lysates relevant to prostate cancer progression	Paweletz *et al.* [120]
Biological and medical research			
Biochemical assay	microfluidics	β-galactosidase (120 pg), using RBG (7.5 ng)	Hadd *et al.* [32]
Enzymatic assays	See Enzymatic assays under Clinical applications		
Sensor	microfluidics	Differences in electrical signals between 2 cell populations: normal and those exposed to an irritant or toxin	DeBusschere *et al.* [543]
Sensor	See also Drug screening and testing		
Cellular manipulation	microfluidics	Transported cells through a microchannel network	Li and Harrison [26]
Cell sorting	microfluidics	With cell trapping algorithm, PDMS chip with pneumatic control	Fu *et al.* [16]
Cell sorting	microfluidics	Dielectrophoretic sorting of particles and cells	Fiedler *et al.* [299]
Cell sorting	microfluidics	Fluorescence-activated cell sorter	Fu *et al.* [275]
Cell shape/behavior	patterning	Regulating apoptotic behavior (vs. growth) by restricting extent of spreading	Chen *et al.* [52]
Cell shape/behavior	patterning	Cell shape depends on surface characteristics	Kleinfeld *et al.* [114]

Cell shape/behavior	patterning	Neurite outgrowth dependence on bioactive laminin	Hammarback *et al.* [546]
Cell shape/behavior	patterning	Limiting cell extension controls growth and protein secretion	Singhvi *et al.* [187]
Cell shape/behavior	patterning	Cell spreading changes hepatocyte behavior from differentiation to proliferation	Mooney *et al.* [548]
(Cell behavior)	Patterning; microelectrode array	Development strategy for chip substrates to study chemical biology in cells	Yeo *et al.* [547]
Cell behavior	microfluidics	Cell fusion	Chiu [540]
Cell migration	Microfluidics (gradient formed)	Study of neutrophil chemotaxis in an interleukin-8 gradient	Joen *et al.* [240]
Toxicity	Microelectrode array	Brain slice cultures grown on chip to monitor electrophysiological signals	Kristensen *et al.* [70]

Table 3. Companies with biochip products and technologies.

Chip company (References)	Chip products and services (Instrumentation/Software)	Technology
Agilent Technologies Palo Alto, CA www.chem.agilent.com [38], [11], [152]	LabChip® (see Caliper) (2100 BioAnalyzer with Cell Assay Extension cartridge available)	Electrophoretic DNA, RNA, protein analysis; cell assays (fluorescently labeled annexin-V detection for apoptosis; transfection efficiency monitoring by GFP detection or antibody staining); microfluidics on glass quartz
Aurora Biosciences Corp. San Diego, CA http://www.aurorabio.com (now PanVera LLC, a subsidiary of Vertex Pharmaceuticals)	NanoWell™ assay plates (3456 wells)	UHTSS (ultra high-throughput screening system) with FRET based detection; cell-based assays; automated screening of 100,000 compounds and 2400 re-tests per day; automated plate replication for 96- and 384-well screening plates [57,58].
Biacore International AB Uppsala, Sweden http://www.biacore.com [79]; [146]	Sensor chips, 10 different SPR-based protein chips (gold-coated glass) Biacore 3000: automated SPR-based biosensor system	Technology involves (1) SPR which detects local concentration changes due to binding interactions at the surface, (2) the Sensor chips which provides for surface attachment of molecules of interest, and (3) a microfluidic system which delivers sample to the surface of the chip. Single spot format.
BioArray Solutions Warren, NJ http://www.bioarrays.com [522], [580]	(offers complete assay solutions and on-demand high volume production of custom chips with proprietary assay designs and software analysis, chips in disposable fluidic cartridges which hold patient samples)	LEAPS (Light-controlled Electrokinetic Assembly of Particles near Surfaces): optically programmable assembly of thousands of "bead" microparticles, computer-controlled illumination for biochemical analysis; sorting and light-directed transport of cells on chip Custom Bead Array Technology: high-volume production of customized bead arrays; monitoring molecular binding events; bead selection from encoded bead libraries, array assembly, and immobilization; decoding image with bead position and color tag provided; 4000 binding events recorded in one CCD camera snapshot.

		SMARTCHIP-Integrated Assay Solutions: bead array assay format with programmable fluidic sample/reagent delivery, temperature control, and image analysis Applications in protein profiling, quantitative assays, array cytometry, clinical diagnostics
Biolog, Inc. Hayward, CA http://www.biolog.com	Phenotype MicroArrays™(PM) Cell-based array that tests up to 2000 cellular properties simultaneously OmniLog™ instrument (reader/incubator that monitors cellular changes in 50 arrays simultaneously)	Integrated system of cellular assays, instrumentation, and software for HTS of cells; identify new drug targets by cell-to-cell comparison, also for lead characterization and optimization. Cell-based assays available in PM format now number 760. Simultaneous measurement of effects on one gene in a cell under different growth conditions [456,580].
Biosite Diagnostics, Inc. San Diego, CA http://www.biosite.com [456], [522]	Triage® protein biochips 1. Cardiac system: point-of-care blood test to diagnose AMI (screens CK-MB, myoglobin, and troponin 1 markers) 2. BNP test (point-of-care blood test to diagnose CHF by measuring BNP marker) 3. DOA panel differential diagnosis of drugs of abuse, DOA, covers eight drug classes) 4. Micro Parasite Panel (for diagnosis of 3 pathogenic parasites) 5. *C. difficile* Panel (to identify *Clostridium difficile*, an infectious organism causing severe diarrhea especially in hospital settings)	Triage® protein biochip immunoassays: microfluidics in plastic; 100 assays in 15 min; picomolar sensitivities from several drops of whole blood or plasma; uses antibodies with a near-infrared fluorescent label which is read by a portable battery-powered fluorometer [522]. Triage® Cardiac system: microfluidics in immunodiagnostics, only a few μl of blood needed [456]; 10 antibodies arrayed in 6 zones [522]

(Continued.)

Table 3. Continued.

Chip Company (References)	Chip products and services (Instrumentation/Software)	Technology
Caliper Technologies Mountain View, CA http://www.calipertech.com [38]	LabChip® system (for electrophoretic separation; for vacuum-driven cell assays; products and services from benchtop to HTS systems; see also Agilent) Caliper®250 HTS system (uses LabChip, performs fluorogenic and mobility shift assays currently, other cell-based assays are under development) Library Card™ device (reagent array for HTS of chemical compound libraries)	Microfluidics lab-on-a-chip technology: quartz glass chips with photolithographically etched channels sealed by a second glass layer; fluid motion induced by electrokinetic pressure; demonstrated screening of 750,000 compounds in a drug library against a protein target, consuming 750 ng of protein (equivalent to 1 million compounds per μg of target) [38] Partnered with Agilent on LabChips
Ciphergen Biosystems, Inc. Fremont, CA http://www.ciphergen.com [146], [79]	ProteinChip® Biomarker System (tools for biomarker discovery from identifying potential markers to resolving multivariate patterns in 16-spot ProteinChip Latex Arrays, including the ProteinChip Reader and ProteinChip and Biomarker Patterns™ Software) ProteinChip® Products (8 spots/chip; 6 different surfaces including affinity capture applications; ProteinChip Reader) Biomarker Discovery Center® Services (R and D facilities to help Ciphergen customers solve complex, biological research problems	SELDI ProteinChip technology for rapid separation, detection, analysis of proteins; protein capture chips for MS analysis Aluminum chip with 8 spots; 12 side by side is the size of a 96-well microtiter plate
Combimatrix Corp. Mukilteo, WA www.combimatrix.com [260]	(microelectrode array [260])	*CMBX biological array processor system*: semiconductor array chip coated with a *3D porous reaction layer* in which DNA, RNA, peptides or small molecules can be synthesized or immobilized; *virtual flask technology* using chemistry to build virtual walls around each electrode beneath each microspot to avoid chemical contamination, each virtual flask has a diameter about that of human hair [260]; *proprietary software* to control each electrode at each microspot

Fluidigm Corp. South San Francisco, CA http://www.fluidigm.com (formerly Mycometrix) [11]; [38]; [67]	Topaz™ Protein crystallization Starter Kit (chips with integral valves that meter and mix precise amounts of sample and reagents, to crystallize proteins from nanogram amounts)	MSL microfluidics™: using proprietary MSL (Multi-layer Soft Lithography) process to fabricate PDMS microfluidic chips with microscale pumps and valves, including a cell sorter and a rotary pump that accelerates reaction kinetics on a chip; enables precise fluid manipulation; chips have up to 600 valves and 12 control lines
Gyros AB Uppsala, Sweden http://www.gyros.com [11]	Gyrolab MALDI SP 1 (CD microlabs) (Gyrolab Workstation, Cutter and Adaptor for Bruker MALDI)	CDs that use centrifugal forces to move samples through microstructures (nanoliter scale versions to perform specific tasks, *e.g.*, volume definition, chromatography columns and chambers for enzymatic reactions and cell growth). Microstructures have the appropriate surface chemistries. Monitoring results by LIF Concentrates, desalts, mixes with matrix and crystallizes protein samples for MALDI-MS; processes 96 samples in parallel per CD [11]
HTS Biosystems, Inc. Hopkinton, MA www.htsbiosystems.com [79]	FLEX CHIP™: Kinetic Analysis System: BMT chip (for kinetic analysis of up to 400 binding events, 400 test sites/cm^2) FLEX CHIP™ Reader (for data collection) CHEMI FLEX™ System (microarray biochip; chemiluminescence-based detection for quantitative multiplexed assays PHASE FLEX™ System (disposable HTS plates for cell-based assays)	Proteomatrix™ technology portfolio: 1. FLEX CHIP™ Kinetic Analysis System based on *grating-coupled SPR (GCSPR)*, parallel detection of binding events in microarray format without relying on a reporter molecule. Protein interactions; gold-coated plastic optical grating 2. CHEMI FLEX™ technology: *chemiluminescence detection* of quantitative multiplexed assays in microarray format with integrated fluidics and reagent reservoirs for expression proteomics, target screening 3. PHASE FLEX™ *phase fluorescence* system: Lifetime fluorescence system using standard dyes (GFP, FITC); fluorescence lifetime, characteristic of fluorescence emission and the excited state lifetime (or half-life), has advantages over intensity and ratiometric measurements (independent of the measurement platform, immune to photobleaching and fluorophore concentration, allows multilabel detection)

(Continued.)

Table 3. Continued.

Chip Company (References)	Chip products and services (Instrumentation/Software)	Technology
Large Scale Biology Corp. Vacaville, CA http://www.lsbc.com [38]	(Plastic-based antibody arrays; protein chips developed for in-house clinical diagnostics [38])	ProGEx™ proteomics technology for the separation, analysis, identification, and quantitation of proteins in biological samples Patented methods for recombinant animal viral nucleic acids to extend proteomics development capabilities in protein chips; study human proteins in animal cells Bioinformatics: eFLOW, a custom computing cluster used for high-throughput processing and analysis of genomic and proteomic sequence data and for computing applications involving protein chips and discovery of protein markers
LumiCyte, Inc. Fremont, CA http://www.lumicyte.com [79]	Uses Protein BioChips as a service for clinical diagnostics; selling SELDI-based services and information to the life science industry BioPhore Knowledgebase™	Seldiography (patented, proprietary SELDI process): detection and quantitative mapping of protein biomarkers from biological fluids; protein capture chips for MS analysis
Micronics, Inc. Redmond, WA http://www.micronics.net Ref: Chip description in Freemantle pp. 32–33 []	Provides lab card development and microplumbing solutions: 1. Liquid/Liquid Extraction System (LLES)(using H-Filter® technology) 2. Chemical Analysis System (using T-sensor® technology) 3. μFluidic™ Evaluation Kit (for testing different channel configurations, sold by Pierce Chemical Co.) 4. Access™ Lab Card (for quantitative analtye detection, *e.g.* in immunoassays, in H-Filter® or T-sensor® format)	Diffusion-based detection, filtration methods, and microcytometry Patented technology platforms: 1. H-Filter® technology: diffusion-based separation in aqueous laminar flow streams, preparation of complex samples, *e.g.* whole blood, without centrifugation or solvent extraction 2. T-sensor® technology: multiple input flows are simultaneously analyzed, differentiating large from small particles by diffusion 3. Microcytometry™: intergrated chemical diffuser and cytometer channel, allows uniform controlled lysing of blood cells prior to cytometry 4. ORCA Microfluidics™: integrates sample preparation with point-of-care analysis, integrates H-Filter® and T-sensor® platforms in a disposable card

Nanostream, Inc. Pasadena, CA http://www.nanostream.-com [38]	Customized microfluidic solutions	Snap-n-Flow™: rapid prototyping modular approach to fabricating microfluidic components (*e.g.*, 3 configurations for splitters, valves, filters, and mixers) Customized microfluidic solutions (*e.g.*, a device for high-throughput separations; a multiplexed assay chip that performs serial dilutions and dose–response curves are generated; multiplexed cellular assays; optimization of cellular growth conditions)
Packard Bioscience Co. Meriden, CT http://www.packardbio-science.com [146], [152]	Hydrogel™ coated slides (array substrates) SpotArray™ 72 (for high-throughput contact printing, 1536 spots onto 72 slides in 1 h) SpotArray Enterprise (PiezoTip™ piezoelectric dispensing, 325-pL drops, 108 array slides per run, 20,000 spots per slide) ScanArra (Way scanners)	3-D Hydrogel technology to maintain protein activity in a porous polyacrylamide gel pad; proteins are immobilized via a coupling agent that covalently links protein amine groups to the gel. Piezo Tipnology (non-contact printing technology based on piezoelectric dispensing)
Prolinx, Inc. Bothell, WA http://www.prolinx.com [79], [456]	Versalinx Chemical Affinity Tools (for assay development) Versalinx Protein Microarray Technology (array substrate with universal high-capacity binding surface that retains protein activity and minimizes background)	Versalinx™ chemical affinity technology: synthetic chemical affinity technology involving the P(D)BA-SHA (phenyl(di)-boronic acid-salicylhydoxamic acid) pair, complex formation enables solution-phase bioconjugation for immobilization, purification, and analysis with low NSB. Versalinx Protein Microarray Technology: developed for protein immobilization on array surfaces, based on the reversible complexation of P(D)BA-SHA; P(D)BA-conjugated proteins (and peptides) attach to SHA moieties displayed on chip surface; results in uniform spot morphology, high signal intensities and does not require blocking to prevent NSB. Acapella™ Molecular Interaction Analysis System: instrumentation for label-free molecular interaction analysis based on SPR

(Continued.)

Table 3. Continued

Chip Company (References)	Chip products and services (Instrumentation/Software)	Technology
Sense Proteomic Ltd. Babraham, Cambridge, UK www.senseprotomic.com	Expressarray™ p53 (for cancer research, 200 spots in 2.5 cm × 2.5 cm chip) Sense Kv subunit protein array (for cardiovascular research, 96-well plate) Sense p450 protein array Sense human liver proteome array (both for drug metabolism and toxicology, available for compound screening services or for technology establishment in-house)	Functional protein arrays based on specific disease tissues COVET™: high-throughput expression of proteins with a proprietary tag that serves both for affinity capture purification and a folding marker Protein Function Arrays: purification and printing of COVET proteins in arrays, preserving biological activity for protein activity profiling Scinticell™: cell-based scintillation proximity assay, scintillant molecules inserted into the cell membrane and experiments are carried out with radio-ligands; to study receptor–ligand interactions in cells
SomaLogic, Inc. Boulder, CO http://www.somalogic.com [79]	Services: Research Proteomics: target identification, surrogate marker development Clinical Research: patient stratification and response monitoring in clinical trials; product development Commercial Diagnostics: screening, diagnosis, and patient management	Photoaptamers: as capture agents; 10^{15} library size PhotoSELEX: in vitro, combinatorial chemistry technique used to identify photoaptamers to specific target proteins, photochemically links them; evolutionary process that results in highly specific photoaptamers; automated process with current capacity of 200 new photoaptamers/week for development of high-density aptamer arrays
Umedik, Inc. Toronto, Ontario, Canada http://www.umedik.com [383]	DIA/PRO™ BioChip (separates contaminating particles in a drop of fluid; contains assay(s) and pertinent biochemical analysis components; disposable plastic) DIA/PRO Electronic Reader (identifies and analyzes specific tests with bar coding)	ICEflo™ technology: on-chip particle separation, eliminating centrifugation. Works well with blood, saliva, urine, and feces samples. Applications in biomarker detection, diagnosis, food safety and testing, environmental testing

Zeptosens AG Witterswil, Switzerland http://www.zeptosens.com [565]	ZeptoMARK (protein microarray for proteomics) ZeptoREADER ZeptoVIEW software SensiChip (for low abundance gene expression analysis; planar waveguide substrate with 6 microarrays, each enclosed in a low-volume microfluidic structure) SensiChip Reader	PLL-PEG surface chemistry for immobilizing proteins on microarrays, minimizing NSB; cDNAs, 70mer oligonucleotides, or antibodies as recognition elements or capture probes; fluorescent planar waveguide detection by evanescent field excitation to minimize background and achieve detection limits in the zeptomole range; bioassays are multiplexed nucleic acid hybridization, immunoaffinity, and membrane receptor based Zepto^{TM} READER: capture molecules in a microarray are immobilized on a thin film (100–200 nm Ta_2O_5, a high-refractive index material) planar waveguide deposited on a transparent support [565]
Zyomyx, Inc. Hayward, CA http://www.zyomyx.com [59]	Protein Profiling Biochip^{TM} System (for simultaneous multianalyte analysis from biological mixtures, *e.g.* serum, cell lysates; system includes fluorescent reader and assay components)	Possesses technologies in microsystem design, fluid mechanics, and non-contact printing (10 nL reservoirs and zero dead volume [59]); surface chemistry for protein immobilization; detection physics for optical parallel detection (also electronic biosensors and MS); and biochemistry for protein expression, purification, and characterization

This table is of companies with non-DNA biochip products and technologies. Although they may have DNA chip products and technologies, these are not mentioned. A number of chip companies have been excluded because either they have no products out yet or there is no clear description of the technology. Information was obtained from the companies' respective websites, if no additional reference citations are indicated.

Microfluidics systems have several technical advantages:

1. Minimal/economical use of sample and reagents (nanoliter amounts) and limited evaporation in closed channels. This low reagent use minimizes costs and, with toxic reagents, minimizes or even totally eliminates, disposal and environmental concerns [11,38].
2. High performance due to rapid mixing; reactions/processes are more accurate and faster, hence more efficient, when compared with normal systems (discussed below). This is an important factor in high-throughput applications [11,38].
3. Smaller size, which means lower power consumption and more lab space, possibly even portability [11].
4. Integration of mechanical, electronic, and optical capabilities, which not only leads to more reliable and reproducible results [11], but also enables μ-TAS [38].
5. Relatively inexpensive fabrication techniques, which translates to multiplexing and to mass production [11].

A summary of the advantages of microfluidics with respect to specific properties such as heat and mass transfer are summarized in Sanders and Manz [13]. In terms of applications, the advantages in biological and medical research stem from compatibility with biological systems, *e.g.*, microfluidic channels and *in vivo* capillary systems have comparable sizes and fluid flow rates ($\sim$ 10 μm, 0.1 cm/s) [39], and similar elasticities allow its use in devices for physiological research and diagnostics [10].

In microfluidic devices, fluid flow through the channels is characterized by what is referred to in separation science as a low Reynolds number [12,40–42]. This means the flow is laminar or turbulence-free, resulting in mixing that is controlled solely by diffusion (see also Glossary). When a system is 10-fold smaller, diffusion-controlled mass transport is 100 times faster. Hence, chemical separations, *e.g.*, electrophoresis, can also be performed 100 times faster. The same type of relationship also holds with heat transport [12,13,41]. Because of more efficient power dissipation, higher voltage gradients can be used [13]. Synthetic reactions also result in higher yields and kinetic parameters are easily extracted from the data [41]. Reactions and separations done on microfluidic chips are hence described as high performance.

Fluids are pumped through the channels by a number of techniques. The most common is electrokinetic or electroosmotic flow (EOF) where an applied electric field induces the bulk fluid in the channel to move [22,23]. (Other means of fluid transport are discussed under Technologies, Microfluidic technologies, Fluid Flow.) Hence a microfluidic chip includes other elements, which may comprise pumps, valves, mixing and reaction chambers, reagent and waste reservoirs, and detection cells [11].

Microfluidic systems are commonly used for electrophoretic separations, but other separation methods including electrochromatography and ion-exchange chromatography have also been done (see Table 2). Other applications include immunoassays, flow cytometry, sample injection of protein samples into mass

spectrometers, cell manipulation and sorting, including chemical gradient formation. Thus, the four major application areas are: miniaturized analytical systems, biomedical devices, chemistry and biochemistry tools, and systems for fundamental research [10]. A wide range of applications indicates a variety of measurements that can be performed (viscosity, pH, kinetics, molecular diffusion, and binding coefficients) and different sample types and reagents that have been used (whole blood, cellular suspensions, protein solutions, and various buffers) [11].

An interesting, recent development is the Lab CD, a compact disc system that does sample preparation by using centrifugal force to "spin out" the sample from the center of the disc outward through the channels (see Gyro in Table 3) [38].

Although microfluidics chips are gaining wide usage, it is not without problems and technical challenges. Because of the small dimensions and the higher surface to volume ratio, surface effects should be controlled or compensated for, if not prevented with proper surface treatment during chip fabrication or just prior to use. Protein adsorption has been a major issue but there are a variety of chemistries and patterning techniques to use on chip surfaces to render them adherent or nonadherent to proteins and cells (see Technologies, Array technologies). A second issue is detector sensitivity. Optical detection systems are path-length dependent. Hence, laser-induced fluorescence detection, an inherently sensitive method, has been used (see also other detection systems under Principles of Design and Operation, Microfluidic detection systems) [11].

A number of techniques address other issues including integrating required components on the chip (pumps, valves, reservoirs), pumping fluids efficiently, monitoring their flow, and avoiding bubble formation as well as evaporation in the microchannels (see Technologies, Microfluidic technologies). Nevertheless, research in microfluidics includes the development of novel microfabrication methods, the integration of novel components that increase chip complexity, and the fundamentals of fluid flow in narrow channels [43]. Specifically, the development of μTAS systems is focused on micro- and nanofluidic control, and the development of microreactors and high-throughput screening systems [41].

Sensors and other chips

Chips that involve miniaturized experiments or sample spots and wells that are not within the specifications described earlier for microarrays or microfluidic systems are described here in this section. With further miniaturization or addition of certain procedures or components, they would fit the categories described above. Nevertheless, they have been used by the biotechnology community in various relevant applications.

Several sensor chips perform miniaturized experiments; one is the biochemical sensor involving the principle of SPR-BIA [44–46]. Another is a sensor that can monitor cell signaling and metabolism where cells are cultured and their behavior recorded without any transport [47–49]. The whole field of patterning cells to

study their function also falls in this area [40,50–53] (see also Biochip Applications, Biological and Medical research).

ProteinChip Arrays (see Ciphergen in Table 3) are chips used for surface-enhanced laser desorption and ionization (SELDI) applications. Although formatted on a chip with 8 or 16 spots, they are not, by definition, microarrays. Each spot, which is about 2 mm in diameter, binds several nanomoles of protein [54]. They have been used especially in protein capture experiments and biomarker profiling with interesting and useful results [54–56] (see Table 2).

Nanowells in multiwell plate format have samples that are not immobilized. They have been used in high-throughput cell-based arrays [57,58] (see Aurora Biosciences, Table 3) and the analysis of yeast kinases [136].

Materials

Protein chips consist of a substrate (see Glossary) with or without a surface coating. Substrates can be made from glass, quartz, polymers, or even silicon. The coating or the appropriate surface chemistry that is applied to the chip surface (in arrays) or the channel walls (in microfluidic systems) is one of the most important considerations because the key issue is to preserve protein activity [10]. Other criteria for choosing the appropriate material include chemical inertness, nonspecific binding properties, uniform density of the molecular surface with accessible end groups for protein binding [59], and compatibility with other components of the system, often the detector. The characteristics and properties of chip materials are outlined in Table 5.

Glass and oxidized silicon are very different materials yet share some similarities (see Table 5). They are the immediate choices for mechanical and electronic devices but their use in chips for biological or even chemical applications is not as straightforward. An exception for glass is that it has been used successfully for DNA separation and sequencing [33–36,60]. Their surfaces can be coated, however, with monomolecular thin films (*e.g.*, SAMs) or functionalized polymers with appropriate protein-binding end groups [59]. One advantage in microfluidics is that the etching process used to fabricate the channels also cleans the surface [10].

Plastic has been used and come in a wide range of size and thickness. The attraction is that it is considerably cheaper than glass and can be disposable, convenient for single-use applications [38].

Chips fabricated from polymers have also been popular. There are three types of polymers classified according to their degree of pliability or softness, which is a function of the extent of molecular cross-linking within the material. These are thermoplastic, elastomeric, and duroplastic polymers [61]. To date, only the first two have been used for microfluidic devices. Duroplastics are more cross-linked, harder, and more brittle. As an important fabrication parameter, the glass transition temperature, T_g, of the polymer determines the temperatures at which it can be molded; some guidelines and precautions are given by Becker and

Gärtner [61]. Polymers can have different mechanical and optical properties and resistance to various types of chemicals and reagents (organics, acids, and alkalines). In particular, a distinct mechanical property of elastomers is that its Young's modulus can be varied over two orders of magnitude by controlling the amount of cross-linking between polymer chains [62]. Biodegradable polymers are also available. A list of polymers for microfabrication, along with their physical and chemical properties, is provided in Becker and Gärtner [61]. For drug screening in particular but also for other analytical applications, suitable polymers are PDMS, PMMA, PC, and Teflon [41].

Advantages specific to polymers are the following:

1. Some polymers can seal effectively against silicon and glass, allowing hybrid devices to be designed and fabricated [62]. For example, microfluidic channels were constructed of SU-8, composite-quartz SU-8, and silicon to be able to perform UV/VIS spectroscopy [63].
2. Fabrication conditions are not as stringent as with silicon [62,64], hence is favorable with academic researchers (see also Table 5).

The additional advantages below benefit commercial applications:

3. Little capital equipment is needed to set up a fabrication facility [62].
4. They are relatively inexpensive. For example, the elastomer PDMS is 50 times cheaper than an equivalent volume of silicon [62]. On the one hand, they can be easily mass-produced so that high-volume production of disposable units is possible [61,62]. On the other hand, it is affordable to produce small quantities [62].
5. The rapid turnaround time for fabrication allows easier design modification and iteration [65].
6. A low barrier of entry should speed up innovation and benefit both research and development in industry [62].

A large part of the literature on polymeric material for protein chips is focused on PDMS. It has several properties that make it well suited for patterning proteins and cells [66] as well as for microfluidic systems [10] (see Table 5). It is durable and has been used about 100 times over several months with "no noticeable degradation in its performance" [51]. It has a hydrophobic surface and adsorbs lipophilic compounds but the elastomer can be modified or its surface treated after manufacture to render it hydrophilic [10,67]. Its surface can be modified by plasma treatment (see Glossary) and subsequent formation of monomolecular films on the resulting oxidized surface [10,51,68]. The fact that it has poor thermal conductivity and is highly permeable to gases is a double-edged sword. For applications like electrophoresis, low thermal conductivity means a build-up in temperature due to resistive heating and results in peak broadening. However, for an incubator system, low thermal conductivity is desirable to maintain elevated temperatures. Gas permeability is not desirable when evaporation or oxidation can be a problem but is desirable for cell culture, for example, and in instances where liquid is to be exchanged [10]. Lastly, in microfluidic systems, several PDMS layers containing channels,

Table 4. Companies with biochip-related products and technologies.

Chip-related Company (References)	Product/Services (Instrumentation/Software)	Technology
Advion BioSciences, Inc. Ithaca, NY http://www.advion.com	ESI Chip™ (100-nozzle chip for nanoelectrospray infusion of samples, automated) NanoMate™ (automated nanoelectrospray system compatible with several MS systems)	Electrospray nozzles on a chip: a sample aliquot (1–25 μl) is transferred from a standard 96-well microtiter plate into a disposable conductive tip; the filled tip is aligned and sealed against a chip nozzle; spraying is initiated when head pressure and voltage are applied to the tip
Akceli, Inc. Cambridge, MA http://www.akceli.com [79]	(Seeks partnerships to apply reverse transfection technology in drug discovery)	Reverse transfection technology: enhances drug discovery and development (target identification and lead optimization, HTS); improves basic understanding of cellular pathways; enables high-throughput cell- and protein-based assays. Transfected cell microarrays: human cells are added to a slide with 10,000 or more different gene sequences in a hydrogel matrix. The cells are analyzed to study how the newly expressed proteins affect cellular networks and pathways
Affinity sensors Cambridge, UK http://www.affinity-sensors.co.uk [44]	IAsys (optical biosensor for direct analysis of unfiltered lysates, whole cells; measures kinetic parameters; choice of pre-derivatized surfaces)	
Beecher Instruments, Inc. Sun Prairie, WI http://www.beecherinstruments.com [1]	ATA-27 automated arrayer (0.6–3 mm punch size; punch speed: 120-180 per h) MTA-1 manual arrayer (0.6 min punch size; 30–70 per h)	Tissue microarray instruments generate multiple specimen slides that contain hundreds of individual tissues

BioCrystal, Ltd. Westerville, OH http://www.biocrystal.com	BioPixels™ (novel nanocrystalline fluorescent markers for tagging biomolecules) PhyloM™ (family of proprietary molecules with very specific binding properties for targeting, imaging, detection, etc.)	BioPixels are functionalized to enhance their stability and allow for coupling to biomolecules, provides high-resolution imaging due to their small size (<20 nm), resists photobleaching, results in high fluorescence intensity due to high quantum efficiency
Biomedical Photometrics, Inc. Waterloo, Ontario, Canada http://www.confocal.com [383]	Designs and manufactures laser scanning products to accommodate many types of biochips, materials, and even live specimen MACROscope® (for tissue imaging and materials analysis scanning) DNAscope™ (for microarray and biochip scanning) MACROview™ analysis software (for image display and analysis)	Confocal laser imaging technology for biological and medical diagnostic applications MACROscope® technology: enables design of high optical resolution and wide field-of-view simultaneously for fewer scanning steps
GeSiM mbH (Gesellschaft für Silizium-Mikrosysteme mbH) Grosserkmannsdorf, Germany http://www.gesim.de	Micro Pipettes (piezodispensers that deliver 30 pl up to 2 nl drops) Nano-Plotter NP 1.2™ (modular micropipeting system, 384 samples in 45 min) microfluidic components and services (silicon/glass micromachining)	Microfluidic Manifold Systems: integrating fluidic structures (capillaries and reaction chambers) in a single chip; micromachining and rapid prototyping; customized lab-on-a-chip solutions
Phylos, Inc. Lexington, MA http://www.phylos.com [79], [146]	TRINECTIN™ binding proteins as capture molecules for protein microarrays (seeking collaborations where partners will develop and commercialize protein arrays)	PROfusion™ technology: establishing a system to produce high quality binding proteins from TRINECTIN™ scaffolds. TRINECTIN™ binding proteins (8.5 kDa) are based on an IgG domain scaffold of fibronectin; these proteins are covalently linked to an mRNA tag; attachment to the chip is via the mRNA tag for consistent orientation; also being developed as biotherapeutics (*E. coli* expression; subnanomolar affinities)

(Continued.)

Table 4. Continued.

Chip-related Company (References)	Product/Services (Instrumentation/Software)	Technology
Nanoplex Technologies, Inc. Mountain View, CA http://www.nanoplextech.com	(seeking partnerships to exploit technologies)	Nanobarcodes™ Particles: self-encoded submicron metal stripes for multiplexed biological applications Nanoparticulate Raman Tags: SERS-based quantitation tags (a SERS band is 1/50 the width of a fluorescence band), for multiplexed analyte quantitation Self-assembled Metal Surfaces: nanoscale architecture by surface assembly from spherical colloidal gold nanoparticles in a planar arrangement on, *e.g.*, glass Colloidal Gold Amplified SPR: instrumentation and methods for colloidal gold-amplified imaging SPR for high-throughput microarray applications with SPR sensitivity
Quantum Dot Corp. Hayward, CA http://www.gdot.com	Qbead™ microspheres (polymer latex beads with quantum dot bar coding for multiplexed assays) Qcell™ encoded cells (as above, using cells instead of beads)	Qdot™ bar codes: as bar codes in multiplexed assays. Quantum dot nanocrystals have 1) a semiconductor core whose composition and size determines the emission wavelength, 2) an inorganic coating to amplify optical properties, insulate the core, and provide chemical stability, and 3) an outer organic coating for chemical coupling to biomolecules

TeleChem International, Inc. Sunnyvale, CA http://www.arrayit.com Boguslavsky [146]	ArrayIt™ Products: Microarray substrates (SuperAmine, SuperAldehyde, SuperEpoxy), Micro Spotting Pins, Printheads; SpotBot® personal microarrayer	Special protein microspotting pins and nickel-plated substrates to immobilize histidine-tagged proteins

This table is a list of biochip-related companies. Although not a comprehensive list, these are companies that support chip technology, *e.g.* with detection systems or equipment and instrumentation. Companies that sell arrayers, in general, have been excluded unless they provide other chip-related products (see TeleChem). Companies like Affibody, Cambridge Antibody Technology, Dyax, and Protein Sciences have been excluded because although they may collaborate with chip companies, they do not produce chip-related products as their main line of business. Information was obtained from the companies' respective websites, if no additional reference citations are indicated.

Table 5. Chip materials.

Substrate	Advantages	Disadvantages
Silicon also silicon dioxide (SiO_2) and silicon nitride (Si_3N_4) [70]	Surface negative charge supports EOF (oxidized Si) [10] Can be coated with monomolecular thin films or functionalized polymers displaying protein binding moieties [59] Can be coated onto glass substrate (140 nm silicon nitride waveguide layer deposited onto a BK-7 glass substrate) [71] Biocompatible [70][1] Compatible with a variety of microfabrication techniques already available [12,70]	Relatively expensive [10] Not UV/VIS transparent; cannot use optical detection [10] Sealing processes require a cleanroom, high voltages and temperatures [10] Etching is expensive, time-consuming [10] Quite stiff; cannot make moving parts; surface chemistries require high temperatures [62] A semiconductor, cannot apply high voltages [12]
Quartz glass	Good electrical insulator hence suitable for electrophoresis [12] Transparent; UV transparent for absorption and fluorescence detection [10,12] Surface negative charge supports EOF [10] (see also very flat glass)	Amorphous, relatively difficult to etch vertically [10] Sealing processes require a cleanroom, high voltages and temperatures [10] Etching is expensive, time-consuming [10] Quite stiff, cannot make moving parts; Surface chemistries require high temperatures [62]
Very flat glass (Corning (GLW))	Chemically inert [74] analyses in organic solvents or low molecular weight organic materials [12] Has low background fluorescence, gives higher signal to noise ratio [152] Can be coated with monomolecular thin films or functionalized polymers displaying protein binding moieties [59] Compatible with a variety of microfabrication techniques already available [12]	(see quartz glass)

Polylysine-coated glass slide	Readily available [59] Direct protein immobilization [125] Positively charged [125] [2]	Inconsistent spot morphologies due to non-specific, non-covalent bonds formed between surface and protein, resulting in poor reproducibility [59]
Nitrocellulose	Readily available [59] Direct protein immobilization [125]	Inconsistent spot morphologies due to non-specific, non-covalent bonds formed between surface and protein, resulting in poor reproducibility [59] Hydrophobic [125] [2]
PVDF	Readily available [59] Direct protein immobilization [125]	Inconsistent spot morphologies due to non-specific, non-covalent bonds formed between surface and protein, resulting in poor reproducibility [59]
Plastic	Less expensive, more rugged, easy to work with, transparent [12]	Hydrophobic [59] [2]
Organic polymers (general)	Inexpensive [62,67] Thermal sealing or use of adhesives possible [10] Good for biological applications; can be mass produced inexpensively [12]	Should control surface chemistry [10] Incompatible with organic solvents or low molecular weight organics [10]
PDMS (an elastomer)	Inexpensive [67], 50 times cheaper than silicon on a per volume basis [62] Optically transparent to 280 nm [10], to 300 nm [51] Compatible with aqueous media and some alcohols [51] Non-toxic to cells, can be used for cell culture [10,51]; high permeability to gases [10][3] Cures at low temperatures, can be molded with delicate features, deforms and seals onto itself reversibly [10]; contacts nonplanar surfaces conformally [51]; material strength can be controlled by extent of crosslinking between polymer chains; can form a tight seal against silicon or glass so can construct hybrid devices; optical structures can be fabricated (optical gratings and waveguides) as well as difficult geometries (coils and basket weave structures) [62]	Some geometries collapse because of elasticity [10] Poor thermal conductivity ($\sim$0.2 Wm/K) [10][3] Most organics are soluble in the bulk polymer and swell the polymer [51] (see also Organic polymers)

(Continued.)

Table 5. Continued.

Substrate	Advantages	Disadvantages
	Excellent material for EOF and pressure pumping; its surface can be charged; elastomeric so easy to incorporate pumps and valves [10] Less stringent fabrication conditions than silicon; fabrication with non-lithographic techniques, i.e. soft lithography [62]; application of rapid prototyping and replica molding; replicates reproducibly [10], durable with repeated use [51]	
PDMS (Sylgard 184 by Dow Coming)	High transparency above 230 nm with little autofluorescence [61,105]; stiffer than RTV 615 (see below); chemically inert to acids and bases [16]	(see Organic polymers)
PDMS (RTV 615 by General Electric)	Less stiff compared to Sylgard 184 (see above) [16]	(see Organic polymers)
COC	High chemical stability; optically transparent [61,90]	(see Organic polymers)
Polystyrene	(see Organic polymers)	Hydrophobic [9,125][2]
Bacterial S-layers	Immobilization matrix for biomolecules [78]; immobilization matrices for binding of monolayers of functional molecules (*e.g.* enzymes, antibodies, and immunogens) in a geometrically well-defined way [76]; exhibit identical physicochemical properties on each molecular unit, down to the sub-nanometer scale; a broad spectrum of very precise chemical modifications can be applied [77]; can be coated or recrystallized on silicon wafers [78]	Not characterized enough

[1]Biocompatible means that the material does not induce toxic reactions in tissues that the material is placed intimately in contact with. In particular, silicon, silicon dioxide, silicon nitride, and platinum were used [70].
[2]Hydophobicity and hydrophilicity of chip surfaces, as an advantage or disadvantage, ultimately depend on the particular proteins that are immobilized on the chip (in arrays). They also affect surface wettability in microfluidic systems. In many cases, the substrates are surface-treated or coated to provide a compatible system with a given application, and hence the inherent surface characteristics may not be a major consideration.
[3]This property can also be an advantage or disadvantage depending on a particular application.

valves, and reservoirs, can be aligned and sealed for the fabrication of three-dimensional devices [10].

For protein arrays, glass and silicon have been widely used. Some, however, prefer the use of polymers, because they are nonreactive and can be coated over other sturdier surfaces such as glass. Their fabrication also is relatively simpler. Silicon, silicon dioxide, silicon nitride, platinum, and gold are also suitable [69] and, in particular, considered biocompatible to tissues, *i.e.*, they do not induce toxic reactions in tissue material that they are in contact with [51,70]. Although compatible with a variety of microfabrication techniques, silicon is a semiconductor, which means high voltages cannot be applied. This is taken advantage of on sensing chips where the entire chip is coated with a 500-nm thick silicon dioxide except the sensing regions, where a thickness of 40 nm was used [71,72].

For microfluidic systems, glass or quartz is inert to the chemicals (*e.g.*, low molecular weight organics) and reagents used in the various processes that are carried out on chip [73–75]. Furthermore, transparency is necessary for detection systems involving absorbance and fluorescence. Glass slides and quartz glass continue to be used both academically and commercially. Quartz glass is used for commercially available chips for protein separation (see Caliper in Table 3). More recently, polymeric materials have also been used in chips that perform capillary electrophoresis [75]. Silicone rubber (PDMS) has properties that make it suitable for microfluidics, *e.g.*, devices with moving parts such as pumps and valves are best made of soft materials [62].

In the area of nanotechnology, researchers have been studying the crystalline surface structures on archaea and bacteria. These surface layers, termed S-layers, are one of the most abundant cellular proteins on such organisms. They are composed of protein subunits that form self-assembled crystalline lattices of monomolecular thickness [76,77]. They have center spacings of less than 25 nm and therefore may find biomimetic and nanotechnological applications [77]. In particular, S-layers have the appropriate structures to be used as an immobilization matrix [78]. Although not in the mainstream, they have potential use in protein microchips.

Technologies

It would seem that, especially for arrays, the technology with which to fabricate protein chips would already be in place, given the wide usage of DNA microarrays and that commercially available DNA arrayers and contact printers could be adapted for use with proteins. This assumption, however, cannot be made. The making of a protein chip is more complicated; it involves more steps and more scientific know-how [79]. Proof of this is the many commercial entities, especially biotechnology companies, which have development projects on protein chips (see Table 3), not to mention the amount of literature covering this topic (see Table 1). Although some technologies are proprietary and therefore inaccessible, a number

of techniques have been published with success and will be discussed. The main issues in miniaturization are the more pronounced surface effects and detection sensitivity. When volumes are down to the picoliter, even nanoliter level, the surface to volume ratio increases dramatically [9]; proteins will denature especially due to dehydration and will adsorb on chip substrates such as glass, plastics, steal, etc. [9]. Counteracting these surface effects with various chemistries are an essential part of chip fabrication. The modes of detection are discussed in the subsequent section on Principles of Design and Operation.

The technologies mentioned below are based on fabrication technologies used for glass, silicon, and polymers. They have been adapted from existing technologies used in other industries such as in the electronics and manufacturing sectors. Polymer technologies possess the flexibility with which different surface chemistries can be used depending on the application. For microfluidics, clearly, they have been used because of the various microstructures required on chip.

Photolithography

Photolithography is used to prepare the surface of a silicon or glass chip, similar to the etching of semiconductor microchips [37,79,80]. The first step involves silanization, which deactivates the surface, before a photoresist is applied or spun on and prebaked. After UV exposure, developing and postbaking, the unmasked regions are etched and then the mask is stripped off. The etched areas reveal structures that are to be specifically surface treated for protein binding (in arrays) [81,82] or that define the required channels (in microfluidics) [37,75,80]. Photolithography on gold has been described [69]. X-ray lithography on PMMA has been used for the fabrication of microfluidic devices [83]; the channels formed for microelectrophoresis were approximately 20 μm wide (depending on the optical mask used) and 50 μm deep (depending on X-ray exposure time). The literature on photolithography can be referred to for more details [84–88]. (See Fig. 3 for an illustration of the photolithographic process applied to microfluidics.)

Photolithography is a well-established technology in the fabrication of microelectronic devices. Although it can result in features smaller than 1 μm, many chip applications today do not require features to be this small, especially in cell biology [51]. For academicians, disadvantages include the high cost of photolithographic equipment, the need to access cleanrooms, the relative inflexibility in the type of chemical functionalities that can be introduced onto surfaces, and the limitation to planar substrates [51]. Photolithography and wet etching have disadvantages for commercialization as well:

1. Cost of the substrate material: Compared to polymers, many types of glass (*e.g.*, borofloat such as Corning Pyrex, borosilicate such as Schott B270, and photostructurable glass such as Schott Foturan) are at least 2–10 times more expensive and is a factor in mass production [61]. The situation is worse then for silicon, which is more expensive than glass. It is 50 times more expensive than PDMS [62].

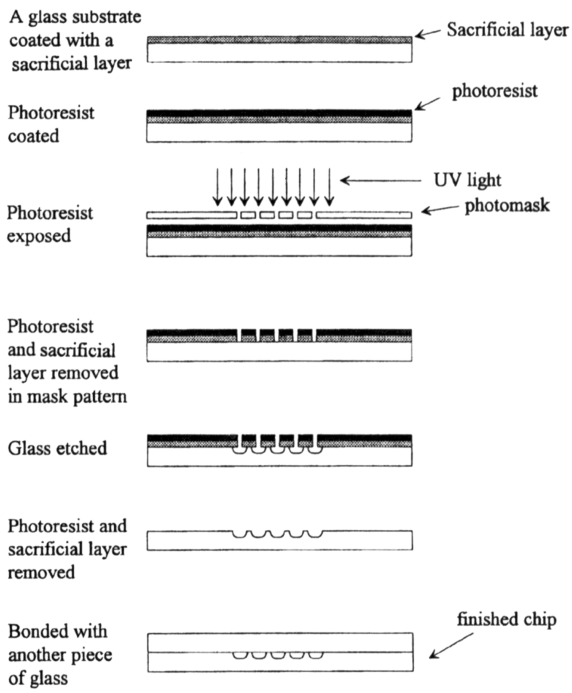

Fig. 3. Photolithographic process for making chips. Reprinted with permission from Ref. [75]. Copyright 2000 Wiley-VCH Verlag GmbH & Co. KGaA.

2. Difficult to automate: With many steps in the photolithographic process, as these steps should be done sequentially, there is the increased fabrication time and the higher risk for errors. Since a number of reagents and chemicals (some even harmful) are required, there are the substantial costs and attention required in both their use and their proper disposal.
3. Design limitations: The etching process on glass or quartz allows only the fabrication of structures with shallow, semicircular cross sections. But there are applications that require different features, *e.g.*, angled walls and different channel heights. These can be done on silicon by dry etching [89] but then the substrate cost becomes prohibitive.
4. Surface chemistry: With silicon and glass substrates, surface silicon bonds to biomolecules. Although silanization helps, this is an added step to an already long process [61,80].

Polymer fabrication methods

Polymers are an attractive alternative for chip fabrication. Once a master is made, mass replication is possible. Nevertheless, single devices can also be fabricated with a number of techniques. Polymer fabrication methods can be categorized into two: replication techniques, amenable to mass production,

and single-device techniques, which are ideal for producing prototypes. These technologies have been used, in particular, for microfluidics, although arrays have been fabricated with these technologies. The most popular replication methods, hot embossing and injection molding, have used PMMA and PC polymers. COC is also promising for molecular biotechnology applications [90]. A table of polymer physical properties can be found in Becker and Gärtner [61].

Master fabrication

The master can be fabricated by a number of techniques depending on the precision required and the channel dimensions. Larger structures (> 100 μm) can be made from computer numerical control (CNC) machining, as done on stainless steel [75]. The techniques below produce features < 100 μm in size.

Micromachining. Laser micromachining techniques (sawing, cutting, milling, turning) for miniaturized applications can produce structures down to tens of micrometers in size [41,61]. Advantages include a wide range of materials that can be machined, the short times required, and no additional steps of mask fabrication and lithography. A major drawback is that only simple structures can be made, *i.e*., straight walls; deep holes and structures with high aspect ratios cannot be fabricated well [61]. Micromachining techniques to produce 3D structures are discussed in Unger *et al*. [292].

Silicon micromachining. Silicon micromachining can be done by wet etching of silicon (the simplest technique) or dry etching, which includes the technique ASE (advanced silicon etch) [89]. Wet etching results in a trapezoidal channel whereas dry etching can result in deep vertical walls. The depth, however, depends on the etch rate: the faster the rate, the rougher the surface. Typical depths are 10–40 μm; with ASE, this is increased to 200 μm [61] (see also Glossary).

Electroplating. Electroplating is a commonly used method for the fabrication of masters. This means the master produced is made out of nickel or an alloy like NiCo or NiFe. The process starts with photolithography up to the development of the photoresist. The resist-free regions are then electroplated to form a metal structure shim, which is further processed. Typical structures have heights of 10–40 μm. Heights of up to 1 mm can be achieved with the use of thick resists such as EPON SU-8 [61].

A variation of electroplating is the LIGA technique. A combination of lithography, electroplating and molding, very small channels with high aspect ratios can be made [75]. In the lithographic step, thick PMMA layers are ablated by either synchrotron radiation [91] or, in the case of laser-LIGA, pulsed UV irradiation [92]. With the electroplating step, the resulting nickel surface is quite smooth but there are some drawbacks, including a slow growth rate (10–100 μm/h) that has a radial dependency leading to an uneven thickness [61].

Replication technologies

Polymer replication technologies are used in the commercialization of microfluidics systems because of the relatively low manufacturing cost [93]. The difficult and expensive step is the fabrication of the master or mold tool. Once this is made, it can be replicated into the desired polymer substrate by a number of techniques with a short turnaround time [61]. Beyond proper process control to minimize surface roughness, the main disadvantage is that undercuts or overhanging structures cannot be fabricated. Restrictions to replication that set constraints on how the master can be fabricated are summarized by Becker and Gärtner [61].

Hot embossing. A widely used replication method for the fabrication of microfluidic systems is hot embossing [94–100]. The embossing tool and the polymer substrate are heated separately (helps extend the lifetime of the mold tool) in a vacuum chamber to just above the substrate's T_g; this removes water vapor and prevents the formation of bubbles. Embossing is carried out at 0.5–2 kN/cm^2 for several minutes until the mold–substrate sandwich cools to just below T_g. Turnaround times are short, *e.g.*, 5–7 min for PMMA. Furthermore, high structural resolution can be achieved [61,75,101]. (See Fig. 4 for a diagram of a hot-embossing machine.)

Injection molding. Adapted from macroworld polymer manufacturing, raw, granular polymer material is put into a cylinder with a heated screw (see Fig. 5). The cavity containing the polymer is heated to a temperature close to the melting point of the polymer. As the polymer melts, it flows into the mold cavity, which is subsequently cooled to allow the removal of the replica [61,93,102]. This process, termed variotherm, is necessary for microfabrication but is distinctly different from the macro process where the molten polymer is injected with 60–100 MPa (600–1000 bar) pressure into the evacuated mold cavity. Typical turnaround times are 1–3 min. The process requires optimization for proper replication; shrinkage may occur due to the large temperature drop from injection to ejection [61]. The fabrication of microfluidic devices using this technique has been described [93,103].

Casting. Because of material flexibility and low cost in producing planar channel structures [104], the casting of silicone rubber (PDMS) elastomers has been widely used by academic researchers. The elastomer is poured on top of the mold and cured or hardened at room temperature and atmospheric pressure [105], although heating can accelerate curing [75]. It is the simplest of the three replication techniques but requires long (minutes to hours) contact with the mold [75]. A miniaturized separation device fabricated using this technique was patented by Ekström *et al*. [106] in 1990. The elastomer is sandwiched between two glass plates to provide mechanical support and seal the channels [61].

An overview of these replication methods is given by Becker and Gärtner [61]. Other polymer fabrication techniques that have not yet been used for microfluidics are also cited.

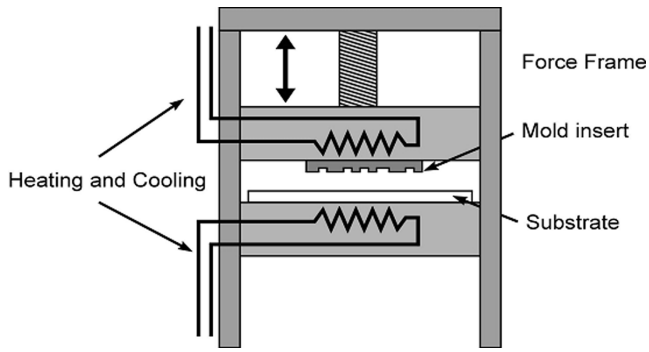

Fig. 4. Diagram of a hot embossing machine. Reprinted with permission from Ref. [61]. Copyright 2000 Wiley-VCH Verlag GmbH & Co. KGaA.

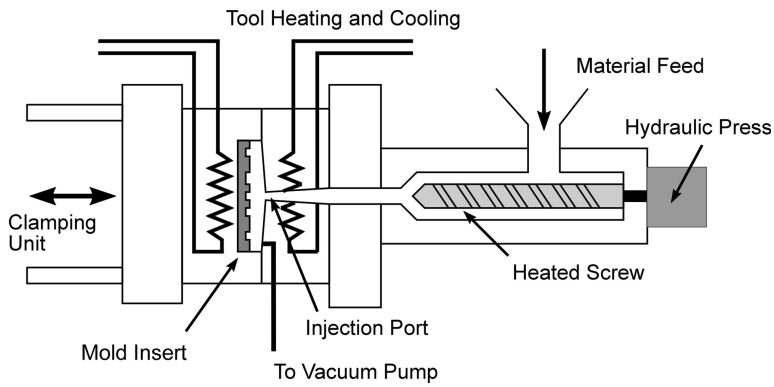

Fig. 5. Schematic diagram of an injection molding machine. Reprinted with permission from Ref. [61]. Copyright 2000 Wiley-VCH Verlag GmbH & Co. KgaA.

Single-device fabrication

Laser ablation. Laser ablation with an excimer laser has been widely used for the fabrication of microfluidic devices [107,108]. Various geometries can be patterned with the use of a mask. The structures formed have little thermal damage, straight vertical walls, and well-defined depth [75,109,110]. Electroosmotic flow in laser-ablated channels has been demonstrated [108]. Accurate patterning of parallel arrays has also been done [82]. Polymers that have been used with this technique include PMMA, PS, PC, PET (or Melinex [75]), cellulose acetate, nitrocellulose, polyimide, poly(tetrafluoroethylene) (Teflon), and photoresists [82,107,108,111–113].

Photoresist technology. The use of photoresists is a well-established technology, similar to photolithography, and can be adapted for use with arrays or

microfluidics. The process involves spin casting a photoresist layer over a substrate, masking, and exposing to UV irradiation. Unmasked photoresist decomposes under UV exposure and this defines regions for patterning (in arrays). Additional layers of photoresist and other treatments are required to form channels and to seal them (in microfluidics). One of the early applications of photoresist technology was controlling cellular growth within amino-silanized regions [114].

In fabricating arrays, silanes are used for attaching proteins to silica (glass or quartz) or metal surfaces [115]. They do not interfere with the optical properties of quartz or the electrical properties of metal substrates. They form covalent bonds with proteins and can withstand the harsh solvents used to dissolve photoresists. Once the unmasked regions have been defined after UV exposure, the silane (usually amino-terminated for protein linkage) is bound to these exposed regions and residual photoresist is sonicated away with acetone. The chip is then incubated in a hydrophobic, ''background'' silane to define the nonadherent regions. The result is then a mixed silane monolayer with protein adherent and nonadherent regions [116]. The proper choice of background silane can minimize nonspecific protein binding. However, nonspecific binding can occur with incomplete surface coverage or gaps in the silane monolayer. An additional disadvantage is that residual photoresist as well as solvent can denature proteins [116].

In fabricating microfluidic systems the use of thick resists such as SU-8 results in channel heights of over 200 μm (normal photoresists usually have 0.5–3 μm thickness) [61]. Starting with a base layer of SU-8, which is exposed to UV light and postbaked, a second SU-8 layer is spin-coated over the first layer and then processed by one of the following three general processes:

1. Fill: A sacrificial layer of another polymer (*e.g.*, Araldite) fills the gaps left on the second SU-8 layer after UV exposure. A third SU-8 layer is spun on top, exposed, and baked. The sacrificial layer is dissolved, thus defining closed microchannels.
2. Mask: The second SU-8 is not developed but instead a metal layer is laid on this as a mask. After the third SU-8 layer is spun on top and exposed to UV light, developing dissolves the resist directly underneath the metal, thus forming the channel. This process, however, is slow and would take several hours to produce a channel length of 1 cm [61,117].
3. Lamination: This is similar to the fill process up until the channels are to be closed. In this case, a layer of dry SU-8 film is laminated on top to close the channel. Advantages are the short processing times, no dissolution steps, and the channels are completely sealed [61].

(See Fig. 6 for an illustration of these three processes done with SU-8.)

Other polymer techniques (*e.g.*, layering) specific to a particular format are discussed in their respective sections below. Others involving electrochemical or ultrasonic techniques are found in the literature [41,104].

Apart from the polymer replication technologies and photolithographically related methods described above, a set of microfabrication techniques has made

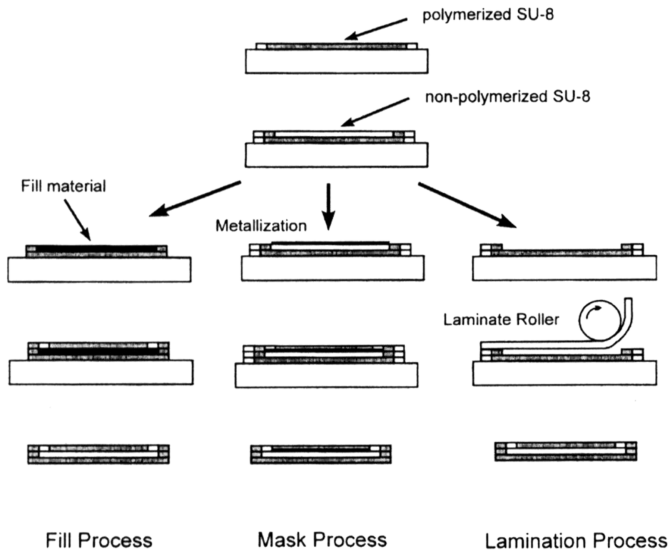

Fig. 6. Fabrication methods for microchannels using optical lithography with SU-8. Reprinted with permission from Ref. [61]. Copyright 2000 Wiley-VCH Verlag GmbH & Co. KGaA.

an impact in chip microfabrication. With much of the research on patterning proteins and cells done on "soft" PDMS elastomer, the term soft lithography came into use, stemming from research done in George M. Whitesides' laboratory (Department of Chemistry and Chemical Biology, Harvard University) (see under Array technologies, B. Patterning, Soft lithography, and Microfluidic Technologies, A. Fabrication techniques, Soft lithographic methods for microfluidics).

Array technologies

The crux in producing a protein array is immobilizing proteins that remain functional. Despite all the technologies discussed above, arrays are still being produced on the simplest substrate, glass slides [71,72,118–121]. The key of course is application of the appropriate chemistry to treat or coat the surface for protein immobilization. A number of protein immobilization techniques are discussed below. An important part of protein immobilization is patterning. Although the use of both terms is at times interchangeable, patterning specifically pertains to immobilization at specific locations in 2D or 3D space [116]. Several patterning techniques are discussed below. The application of proteins or protein ligands on the chip is implied with immobilization or patterning. However, this should not always be assumed. At times, immobilization or patterning only refers to the preparation of the chip surface and how the proteins are to be bound but does not include how the proteins or ligands are applied or put on the chip. Hence, protein-printing methods are

discussed, some of which are patterning techniques themselves. Finally, an issue with protein immobilization is proper orientation of protein molecules, specifically antibodies, so that binding sites are effectively directed toward the bulk solution and away from the chip surface. Some of these orientation techniques are summarized at the end of this section.

Protein immobilization

The efficiency of protein immobilization depends on the chip surface, the method of applying the protein on the chip and the immobilization method itself. At optimum conditions, the resulting density may be as high as 10^5 protein molecules in a 50-μm diameter spot [59]. Immobilization can be achieved by one of three methods: physical (noncovalent) adsorption, the use of high-affinity ligands, and covalent bond formation [59,116]. These methods may require that either the chip surface or the protein undergo some modification or treatment.

Noncovalent attachment. Physical adsorption is characterized by weak, noncovalent attractive forces such as ionic, hydrophobic, van der Waals, and hydrogen bonding [9,59,122]. Because of these nonspecific interactions, results lack reproducibility as evidenced by irregular spot morphologies. Furthermore, the immobilized protein can be denatured and sterically occluded [9,123,124]. This is the case when arrays are prepared from adhesive substrates, *e.g.*, those that are hydrophobic such as nitrocellulose, PVDF, and PS, or positively charged such as polylysine (coated glass slides) and aminosilane (*e.g.*, on glass or silicon) [9,59,125]. This type of immobilization is attractive because of its simplicity and is generally used in ELISAs [9].

High-affinity ligands. The use of high-affinity ligands result in strong noncovalent specific interactions between proteins and surface-bound ligands. The protein of interest then becomes attached to the surface only at a specific contact point, leaving it functional. Examples include biotinylated proteins on streptavidin-coated surfaces [126,127], His-tagged proteins on Ni^{2+}-chelated surfaces [9,125,128], and the use of lectins, protein G and protein A [129–132]. Another technique called DNA-directed immobilization (DDI) uses a microarray of DNA–streptavidin conjugates as an immobilization matrix. Biotinylated proteins can then bind in a highly parallel yet chemically mild process [133] (see Fig. 7).

Covalent attachment. In covalent attachment the chip surface is usually treated with an appropriate silane. The most common is an amine-reactive surface (brought about by reaction with an aminosilane reagent) that randomly conjugates to lysine residues on proteins [118,136,137]. An aldehyde-reactive surface reacts with oxidized glycoproteins [137] and with primary amines on proteins [9,118], forming a Schiff's base linkage [118]. Thiol-terminated silane surfaces

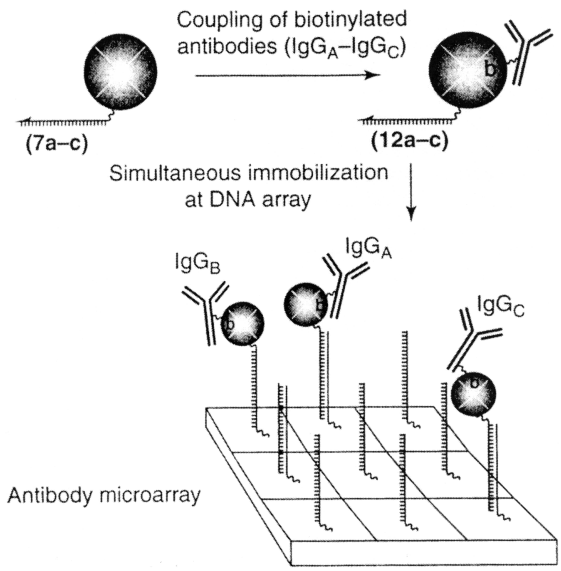

Fig. 7. Schematic drawing of the "DNA-directed immobilization" (DDI). A set of covalent DNA–streptavidin (STV) conjugates (7a–c) are coupled with biotinylated antibodies to generate tagged conjugates (12a–c). A microarray of capture oligonucleotides is used as the immobilization matrix. Note that owing to the specificity of Watson–Crick base pairing, many different compounds can be site-specifically immobilized simultaneously in a single step. Adapted from Ref. [581]. Reprinted with permission from Ref. [133]. Copyright 2002 Elsevier Science Ltd.

have also been used [138–141] and also in combination with heterobifunctional crosslinkers [142]. Another technique involves a mixed monolayer of benzoquinone and oligo(ethylene glycol) groups. Benzoquinone provides the selectivity via ligand immobilization through a Diels–Alder cyclopentadiene conjugation while the ethylene glycol "repel" proteins and reduce nonspecific adsorption [143–145]. Industrialized approaches to protein immobilization have also used proprietary surface chemistries [146,147] (see Zyomyx in Table 3). Covalent immobilization chemistries have been shown to preserve protein functionality for up to 2 years [134,135].

Another set of chemistries involving covalent attachment is the use of photochemistry and heterobifunctional crosslinkers (see Patterning, Photochemical techniques).

A method that combines covalent and strong noncovalent attachment is to derivatize the chip surface with poly(ethylene glycol) (PEG) [148,149]. PEG is effective for nonadherent regions of the chip surface. To render specific areas of the chip as protein-adherent or protein-binding, these regions are derivatized with biotin. Streptavidin binds to these surface biotin molecules and because it is tetrameric, it also binds biotinylated proteins to the chip surface. This method has been used to produce high-density protein microarrays [126,127].

Polyacrylamide gel pads. An immobilization technique that increases spot density as well as preserves biological activity is the use of gels. With the use of gel pads, the advantages are that evaporation is minimized and more proteins are immobilized because of a "third dimension" [150]. In fact, the immobilization capacity is said to be 100 times greater for proteins as well as oligonucleotides. After the polyacrylamide gel micromatrix is made and loaded with microliter amounts of protein solution, it is chemically activated. Chemical activation involves reducing the enamine bond formed between the aldehyde group on the glutaraldehyde-activated gel and the amino groups of the protein [151]. This step immobilizes the protein in the gel. In one account, microelectrophoresis accelerated diffusion in the gel [137]. Commercially available HydroGel-coated glass slides are made from "a modified polyacrylamide to optimize protein immobilization and spot morphology" [146] (see Packard Bioscience in Table 3). Immobilized proteins on these slides have shown retention of their activity in enzymatic and binding assays. Glass substrates are currently used although other materials may be used [152]. A reverse process applies proteins to the chip via a gel matrix (see Protein printing; Stamping).

Immobilization of membrane proteins. An interesting and important development is the immobilization of membrane proteins. A majority of the current drug targets are membrane proteins. Hence, they should be addressed by biotechnological tools such as protein chips in multiplexed compound screening but also in more fundamental studies involving cell–cell communication. Chip surfaces, modified with γ-aminopropylsilane (GAPS), bind the membrane proteins via their associated lipids. This technique was tested on GPCRs (G-protein coupled receptors), which showed high specificity with no apparent artifacts. Incidentally, gold as a substrate exhibited less nonspecific binding than ultraflat glass [153].

Patterning

Patterning is immobilizing proteins or cells in specific locations [116]. Protein patterning in dimensions of up to 100 μm is important for biochemical applications from binding assays to combinatorial screening [10,51] while patterning of cells is important for fundamental studies in cell biology [15,18,19] and for their use in cell-based sensors [17,39,65,154] and tissue engineering [42,51]. Patterning methods include: photoresist technology (discussed above under Polymer fabrication methods, Single-device fabrication), photochemical techniques, and techniques that involve the use of self-assembled monolayers (SAMs) [116], including soft lithography.

Photochemical techniques. Photochemistry involves the use of labile chemical species that are activated, in this case, with UV irradiation [155–157]. There are three commonly used chemistries for photopatterning: arylazide chemistry, diazirine chemistry, and nitrobenzyl caging chemistry.

Arylazide photochemistry involves a reactive nitrene that can insert into C–H bonds [157]. One application used a photoactive biotin (nitroarylazide attached to the biotin tail) which binds to adsorbed avidin on the chip surface. Incubation in protein solution and UV exposure allows the nitrene formed to insert into the protein [158]. Another type of photochemistry involving diazirine has been used to immobilize proteins in various applications [155]. Upon absorbing 350 nm irradiation, diazirines form highly reactive carbenes, which insert into covalent bonds. The main disadvantage with arylazide and diazirine chemistries, including benzophenone, is that the protein has to be present during the UV exposure. This may inactivate the protein and with the short half-life of the nitrene, in the case of arylazide chemistry, protein conjugation is not optimal and results in low yields [159]. Arylazide ring substitution to bring the required UV activation to longer wavelengths is a possible solution [160].

Nitrobenzyl chemistry is also referred to as caging chemistry; labile groups are attached to a molecule to prevent its activity. Two caging groups that are used are NVOC (nitroveratryloxycarbonyl) and MeNPOC (α-methyl-6-nitropiperonyloxycarbonyl) [161–166]. For a multianalyte sensor, antibodies were patterned onto waveguide surfaces where caged biotin (MeNPOC-biotin) was immobilized by conjugation to BSA, which was then adsorbed onto the glass substrate [165,167]. Upon UV exposure, the caging group breaks down into a ketone and carbon dioxide, harmless by-products, and the caged molecule, *i.e.*, biotin, retains its activity [156]. The sensing elements were therefore formed from repeating a sequence of UV exposures in selected areas and incubation in streptavidin and then biotinylated antibody. Advantages with nitrobenzyl chemistry are the harmless by-products from the break up of the caging molecules, as mentioned, and that protein binding occurs after the UV irradiation. This provides some flexibility and specificity in the immobilization step. In the above example, different types of biotinylated antibodies can be applied to the chip [132].

In general these photopatterning chemistries described above can be used repeatedly on the same surface with different proteins or protein ligands. However, a concern is proper control of the background surface. Blocking with BSA is an option [168]. But a more important issue is UV light: the chip must be kept under subdued lighting until the photochemistry is to be done and proper care should be taken during photolithographic masking so that the derivatized surface is not disturbed (damaged or smeared) and UV scatter is avoided [116]. Other references on photoimmobilization include papers by Hensakul *et al.* [169], Morgan *et al.* [170], and Delamarche *et al.* [171].

Apart from the chemistries described, photopatterning can be performed with heterobifunctional crosslinkers [138,172–175]. Several are commercially available. Many have aryl azides as the photoactivatable group but benzophenone (BP) reagents have also been used because of higher crosslinking efficiencies [172,175–177]. They can be handled at ambient light and activated at longer wavelengths [172,178–180]. These rigid molecules have also been made more

flexible with hydrophilic spacers, *e.g.*, the BP-oligo(ethylene glycol)-maleimides (BP-EG_n-MI), for better accessibility of immobilized proteins or ligands to their binding partners in bulk solution. In photopatterning antibodies, BP-EG_5-MI tested well on polystyrene. The BP group is attached to the PS surface and the MI end is available for protein binding via amino groups. Maximum sensitivity is achieved when the preparation is carried out stepwise on the chip rather than premixed [172]. Another heterobifunctional crosslinker is *N*-succinimidyl-4-maleimidobutyrate [116,138]. Immobilization with heterobifunctional crosslinkers can be done on glass, silica, silicon, or platinum substrates [116,138,181].

Self-assembled monolayers (SAMs). On silica or metal surfaces, alkylsilanes or alkane thiols assemble into organized layers [182,276]. One of the earlier works involved patterning with *n*-octadecyltrimethoxysilane (OTMS) to produce hydrophobic SAMs on SiO_2 [81]. The background regions were treated with UV irradiation and the use of a lithographic mask to strip off OTMS while the protein-binding regions were prepared by adsorption of BSA onto OTMS and layering biotin and streptavidin on top of this. With later developments came the use of mixed monolayers that defined the pattern on the chip. One end of the molecule binds to the chip surface and the other has an end group that can be varied to define adherent or protein-binding regions (hydrophilic) and nonadherent or background regions (hydrophobic). Examples of patterning mixed SAMs with UV irradiation are given in Blawas and Reichert [116].

The advantages of using substrates coated with SAMs are the variety of surface chemistries that can be achieved via the different end groups that can be attached [59] and the uniform surface characteristics that allows higher sensitivity and reproducibility, especially when compared to physical adsorption. Protein denaturation has therefore been minimized. The use of SAMs has tremendously improved the microfabrication of protein arrays. Binding protein molecules to alkanethiol SAMs by C–C bond formation using photoactivatable benzophenone has been demonstrated [183]. SAMs have been applied in different areas: polypeptide synthesis [166], microelectrode formation [184], cell adhesion [185–188], and protein adhesion [81,141,186,189–194].

Protein adsorption onto uncharged SAMs is dependent on protein size and the wettability of the surface. The general trend is that the less wettable the surface, the greater the adsorption. Exceptions, however, have been noted with both smaller and larger proteins [195]. SAMs that are protein-adherent, *e.g.*, are those presenting Ni^{2+} chelates for binding with His-tagged proteins from cell extracts [196], those presenting hexadecanethiol [116,197,198], those patterned with EDA ((*N*-(2-aminoethyl)(2-aminopropyl) trimethoxysilane on silica and platinum surfaces [188,199], and those ending with methyl groups [189]. Bovine capillary endothelial cells, *e.g.*, attached to the fibronectin-coated methyl end groups (as opposed to ethylene-glycol end groups) and remained attached for at least 5 days [200]. SAMs that resist protein adsorption, and hence are useful as background SAMs, are those made from alkanethiols

ω-substituted with oligomeric ethylene glycol [148,201], with tripropylene sulfoxide [202] or with sulfonate groups [203]. With UV irradiation, either strategy for patterning on SAMs is possible: start with an adherent surface (*e.g.*, EDA) and render specific regions as nonadherent (UV exposure and subsequent binding to 13F (tridecafluoro-1,1,2,2-tetrahydrooctyl-1-dimethylchlorosilane) to give a hydrophobic surface) [188,199,204] or start with a nonadherent surface (*e.g.*, chemically bonded OTS) and render specific regions adherent (UV exposure and subsequent binding of silanol groups to EDA) [81,205].

Currently, SAMs of alkanethiols are the most common and are usually formed on gold. (Silver films can be used except in the case of live cells because Ag^+, which is released upon exposure to air or oxidants, is cytotoxic [51].) The procedure involves laying a titanium thin film (1–5 nm in thickness) on the substrate to promote the adhesion of gold, gold (10–200 nm in thickness) is then applied [206]. The gold film is subsequently exposed to alkanethiol vapor or solution [207,208], whereupon the sulfur atoms are coordinated to the gold surface and the close-packed alkyl chains are characteristically tilted 30° from the surface [51,203]. SAMs can be used over several days, *e.g.*, in cell culture but they should be protected from temperatures above 70°C, UV light, and oxygen or ozone [51].

Patterning on SAMs can be done via a variety of techniques: UV microlithography [189], scanning probe lithography using AFM [209,210], microwriting [189], micromachining [104,189], stamping [189,211], microcontact printing [197,200], laser ablation [198], and photochemistry [171,211]. Some of these methods (stamping and microcontact printing) are discussed subsequently. Microwriting, micromachining, and stamping may have the advantage that UV exposure of the surface is not required. However, multiple protein patterning may not be possible because the protein solution is incubated in a single step whereas photochemical methods can pattern multiple proteins on a single surface [116]. But for high-throughput applications, these techniques are not suitable because of repeated processing. Protein printing methods by inkjet techniques or multiple-pin arrayers are more suitable (see Protein printing).

Soft lithography. Soft lithographic methods have also been used on SAMs of alkanethiols on gold. Soft lithography can pattern biomolecules and cells as well as form microfluidic channels and both surface chemistry and the cellular environment can be controlled [51,213]. They are performed on elastomeric stamps with raised patterns (bas-relief) produced via replica molding. PDMS elastomer has been used extensively where the master is fabricated from photolithography [51]. (See Fig. 8 for an illustration of the fabrication of a PDMS stamp.) The following general protocols depend on feature sizes generated in the master. For features ≥ 20 μm, the photolithographic mask can be flexible transparencies made from high-resolution laser printing at 3387 dots/in. [64,154] with patterns drawn from Freehand or AutoCAD software. This is the

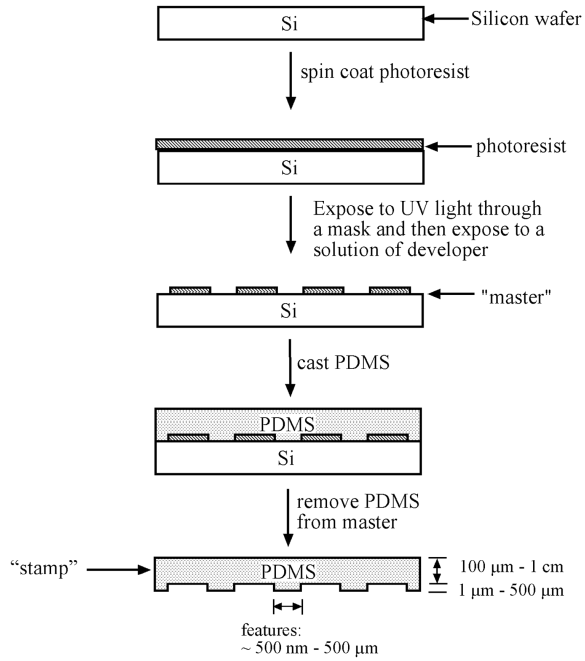

Fig. 8. Schematic illustration of the procedure used to fabricate a PDMS stamp from a master having relief structures in photoresist on its surface. Reprinted with permission from Ref. [51]. Copyright 1999 Elsevier Science Ltd.

basis for the term "rapid prototyping" – costs little and is done in hours. For features >10 μm, microfiche patterns are used as photomasks [214]. Finally, for small features (2–20 μm), commercial laser writing is used for fabricating the PDMS stamp [215]. Minimizing the use of specialized equipment, Deng *et al.* [64] have described a fabrication procedure producing features as small as 15 μm starting with CAD files printed at a resolution of 600 dots/in. Access to a cleanroom, however, is still necessary.

Soft lithographic patterning covers four techniques: microcontact printing (μCP) of alkanethiol SAMs on gold; patterning using microfluidic channels (or micromolding in capillaries, MIMIC); laminar flow printing [51,216–218] and patterning with 3D microfluidic systems [53].

1. Microcontact printing (μCP): Microcontact printing involves the application of material (proteins, thiols, etc.) from an elastomeric stamp to specific regions on a substrate [51,200,219] (see Fig. 9 on μCP using soft lithography). The stamp is made of silicone rubber, PDMS, fabricated by replica molding on a micropatterned silicon wafer. As an elastomer, PDMS can provide conformal contact between stamp and substrate, ensuring a homogeneous transfer of "ink" to the substrate [197,219]. The "ink" is a thiol-terminated alkane [197] or, in a recent report, PTMP [219]. It provides the highest resolution

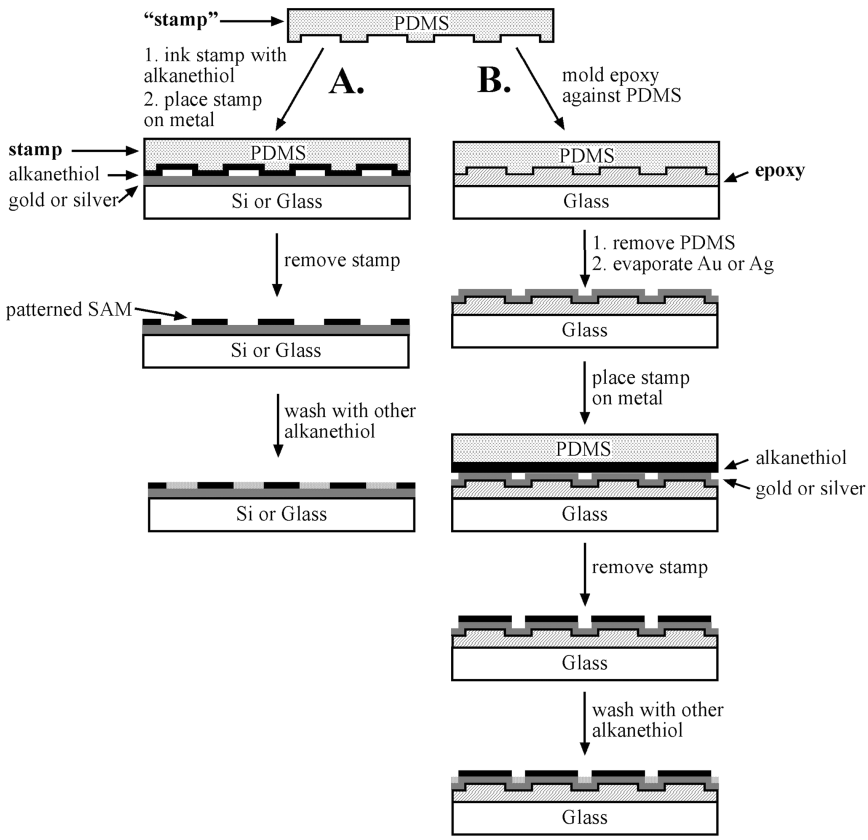

Fig. 9. Microcontact printing (μCP) and fabrication of contoured substrates using soft lithography. (A) A stamp is inked with an alkanethiol and placed on a gold surface; the pattern on the stamp is transferred to the gold by the formation of an SAM on the regions that contacted the substrate. The bare areas of the gold are exposed to a different alkanethiol to generate a surface patterned with an SAM that presents different chemical functionalities in different regions. (B) The PDMS stamp can also be used as a master to mold harder polymers and generate contoured surfaces. After evaporation of a layer of gold, these surfaces can be functionalized by μCP of one alkanethiol with a flat stamp. The grooves of the substrate can then be exposed to an alkanethiol presenting a different functional group to produce a contoured surface with patterned chemical reactivity. Reprinted with permission from Ref. [51]. Copyright 1999 Elsevier Science Ltd.

among the soft lithographic methods with features smaller than 1 μm. When patterning on SAMs, controlled adsorption has been observed [51]. It also allows control of surface chemistry at the molecular level [51] to promote certain properties such as wettability [220–222], protein repellency [187,189], crystal growth [223–225], and adhesion [226,227]. The most widespread application of μCP is in the formation of patterned SAMs by the printing of alkanethiols onto Au [200,228], Ag [200,229,230], Cu [231], Pd [232], or of silanes onto Si/SiO_2 [233–236].

Micropatterning of cells has been done with μCP on SAMs of alkanethiolates. The appropriate extracellular matrix is important to be able to pattern any adhesion-dependent cell line. In other patterning techniques, defining adherent and nonadherent regions require the adsorption, *e.g.*, of albumin, which after several days starts to degrade and leak into the nonadherent regions. With μCP on SAMs, an advantage is that no leakage is observed either from albumin degradation or extracellular matrix. It should be noted, however, that the μCP process does affect cell shape and size [51]. Pattern resolution is typically 1–2 μm with island separations of 20–40 μm. Hence, this is one application where the resolution obtained with photolithography is not necessary. Patterning of cells is important fundamental studies in cell biology and the development of biosensors requiring precise cell control and positioning on the chip. Furthermore, because cells can be accurately arrayed, automated systems can be developed for their incorporation into drug screening with large compound libraries [52].

2. Patterning using microfluidic channels: Patterning with microfluidic channels relies on restricting fluid flow to selected regions on the chip surface. The technique, micromolding in capillaries (MIMIC), developed by Kim *et al.* [237], involves allowing solutions to flow into channels formed by a PDMS mold in conformal contact with the substrate (see Fig. 10). PDMS seals reversibly and thus can be used to pattern proteins and cells by covalent or noncovalent attachment, or dissolution [10,238–240]. This technique allows the fabrication of 3D structures.

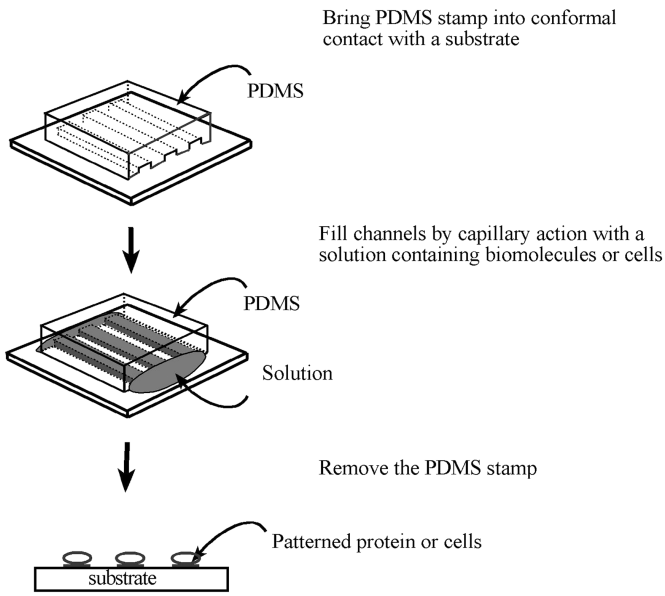

Fig. 10. Illustration of the procedure used to pattern proteins and cells using microfluidic channels. Reprinted with permission from Ref. [51]. Copyright 1999 Elsevier Science Ltd.

MIMIC has been used on several substrates such as glassy carbon or ceramics [241,242], sol-gel materials [243], inorganic salts [244], polymer beads [245], and colloidal particles [244]. It has also been used to pattern proteins using sol-gel microstructures on silicon [246]. MIMIC has been used to pattern immunoglobulins with submicron resolution on glass, gold, and PS for IgG detection in immunoassays [247,248]. In one application, capture agents were immobilized in stripes and samples were delivered in parallel channels perpendicular to the rows of capture agents. Binding reactions result in fluorescent signals [249]. Microfluidic channels were also used to pattern an immunosensor array to simultaneously detect clinical analytes [119]. To generate patterns of biotinylated ligand on a biodegradable polymer, Patel *et al.* [250] allowed a solution of avidin in elastomeric channels to flow over the biodegradable polymer PLA-PEG-biotin (biotin attached to the terminal PEG group of a polylactide-PEG copolymer). The avidin molecules bound to surface biotin moieties can then bind to biotinylated proteins or ligands in solution. Several cell patterning studies using MIMIC include the use of PLA-PEG-biotin to study the adhesion and spreading of bovine aortic endothelial cells and PC12 nerve cells [250] and the selective adherence of cells on various biomedical polymers and other substrates [238,239].

3. Laminar flow patterning: This technique was developed by Takayama *et al.* and takes advantage of microfluidic laminar flow [40]. With laminar flow conditions, several different fluids can flow next to each other in a channel without mixing except at their interfaces where diffusion occurs. This provides a means to selectively pattern cells (or portions of cells) and culture medium in a highly controlled manner [10,40,51]. *Escherichia coli* and chick erythrocytes were patterned side by side via a hydrophilic PDMS channel mold reversibly sealed onto a silanized silicon chip; other fluids (nutrients, fluorescent labels, enzymes, drugs) were delivered to these immobilized cells by the same technique [40] (see Fig. 11). The technique is simple and allows multicomponent patterning in relatively few steps as well as patterning over delicate surfaces like mammalian cells [51]. It also should enable applications such as array screening of cells, cell-based sensors, and the use of microfluidic systems in metabolic and toxicological studies [40].

4. Patterning using 3D microfluidic systems: The three-dimensional microfluidic system in PDMS is actually made from a 3D MIMIC technique [251] and is related to techniques for the 3D fabrication of microchannels [252]. It can be used to pattern multiple types of proteins and cells in a complex, discontinuous pattern on planar surfaces [53] (see Fig. 12).

Protein printing

Protein printing, spotting, or deposition encompasses a set of techniques that apply protein or protein ligand solutions to the chip. These techniques can be classified as either a contact or a noncontact printing method. Contact methods include several of the patterning techniques mentioned including μCP and the

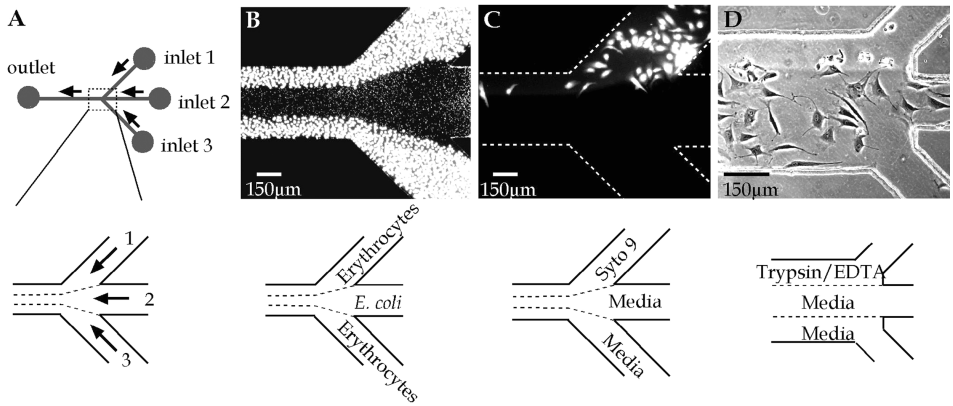

Fig. 11. Schematic representation of a laminar flow patterning experiment. (A) Top view of the capillary network. A poly(dimethylsiloxane) (PDMS) membrane containing micron-sized channels molded in its surface was placed on the flat surface of a Petri dish to form a network of capillaries. Micrographs were obtained for the area of the capillary system at which the inlet channels converge into a single main channel. (B) Two different cell types patterned next to each other. Chick erythrocytes and *Escherichia coli* were deposited selectively in their designated lanes by patterned flow of cell suspensions. Adherent cells were visualized with a fluorescent nucleic acid stain (Syto 9). (C) Pattern of selectively stained BCE cells. A suspension of BCE cells was introduced into capillary network (pretreated with fibronectin) and allowed to attach and spread. After removing nonadherent cells by washing with media, Syto 9 and media were allowed to flow from the designated inlets. (D) Patterned detachment of BCE cells by treatment with trypsin/EDTA. Cells were allowed to adhere and spread in a fibronectin-treated capillary network, and nonadherent cells removed by washing. Trypsin/EDTA and media were allowed to flow from the designated inlets. Pictures B and C are fluorescence micrographs taken from the top through PDMS. Picture D is a phase contrast image observed by an inverted microscope looking through the polystyrene Petri dish. White dotted lines identify channels not visible with fluorescence microscopy. Reprinted with permission from Ref. [51]. Copyright 1999 Elsevier Science Ltd.

other soft lithographic methods where a microfluidic system is used. Several more are discussed below. Noncontact printing comprises several techniques of which the inkjet is the most popular.

Contact printing methods.

1. Multi-pin arrayers: Photolithographic methods have been the most common method employed in the fabrication of microarrays. Instruments used to print DNA arrays have been used with proteins. These arrayers are robotic systems that perform high-precision contact printing [118,121]. Contact printing arrayers with needle-like dispensing tips deliver sub-nanoliter to nanoliter sample volumes directly on the chip surface [125]. (This should be distinguished from μCP which uses a stamp.) In one account, with nanoliters of sample solution delivered, spot diameters of 150–200 μm are typical (1600 spots/cm^2) [118]. A disadvantage is that with long printing times and small volumes of solution, the proteins dry out. One solution is to humidify the spotting chamber [118,253] and/or to add glycerol to the protein solutions [118]. A potential problem is that the chip

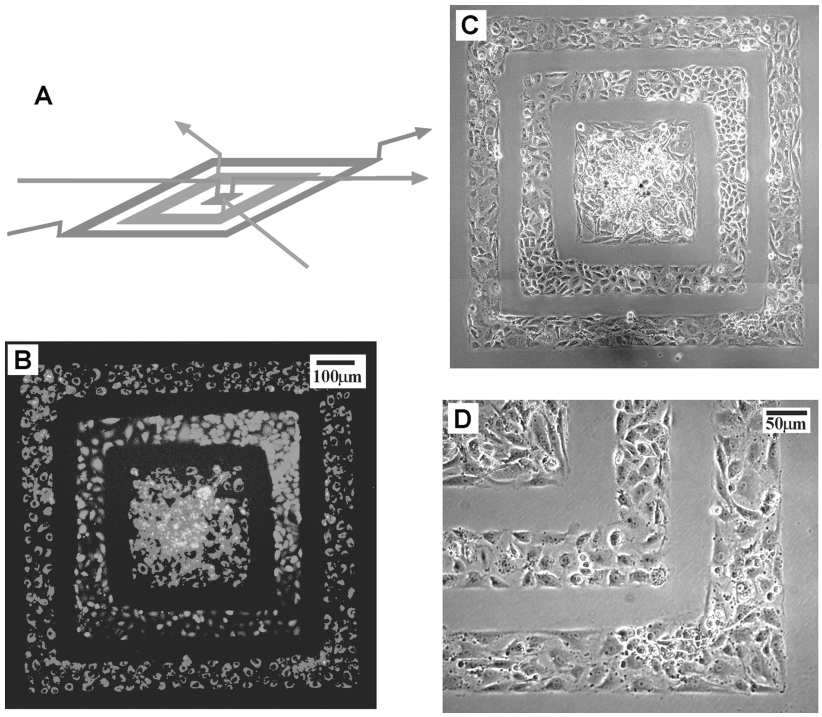

Fig. 12. Patterning by using 3D microfluidic systems. Fluorescence (B) and phase contrast (C and D) pictures of two cell types, ECVs and BCEs, deposited on a tissue culture dish in a concentric square pattern by using the 3D stamp depicted in A. These cells were labeled fluorescently before being deposited. Before patterning cells, the PDMS stamp first was autoclaved for 20 min, then the channel walls were coated with BSA (30 mg/ml in pH 7.4 phosphate buffer for 1 h) for preventing cell attachment to the PDMS stamp. Suspensions of cells ($\sim$ 5 million cells per ml) were introduced into the three sets of channels and were allowed to sediment and attach to the surface of the tissue culture dish. These cells were cultured with the stamp in place for $\sim$ 24 h to grow and spread into a confluent layer. The pictures were taken immediately after the PDMS stamp was removed; these cells were immersed under media and were alive. An expanded view of the lower right corner of C is shown in D. Reprinted with permission from Ref. [53]. Copyright 2000 National Academy of Sciences, USA.

surface may be damaged mechanically, which results in irregular spot shapes and other defects. This inhomogeneity that may result from contact pin printing may affect signal reproducibility [59].

2. Scanning probe lithography: Scanning probe lithography is nanopatterning of SAMs using atomic force microscopy [209,210,254]. This is a form of contact printing because of local interactions between the chip surface and the tip of the AFM [254].

3. Microcontact printing (μCP): Because an elastomeric stamp is used, it is possible to use different types of "inks." Many studies involving μCP has been done on SAMs on gold [51]. A procedure is detailed in Kumar *et al.* [184]. Most of the literature describing this printing method was published in the 1990s

[184,197,216–218,228,255–257]. A novel approach is positive microcontact printing with the use of PTMP inked onto PDMS [219]. Since not all patterns on a stamp are stable or transfer properly, the use of protrusions (in $+\mu$CP) rather than recessions (in $-\mu$CP) on a stamp provides more stability. The use of PTMP also improves the wettability of the gold surface. The use of μCP to deliver hydrogel spots onto surfaces has been demonstrated [258].

4. Stamping: Similar to putting a rubber stamp onto paper, a hydrogel stamper makes brief contact with an aminosilylated surface and deposits a protein film [212]. The stamper is a micromolded, elastic hydrogel loaded with the protein solution. The stamp prints a protein spot 10–80 μm in diameter as a submonolayer as observed from AFM (atomic force microscopy) studies and ^{125}I labeling. Martin *et al*. [212] did a 4-element pattern in 2 min and complete patterning was done in 25 min. The stamping procedure can be configured for multiplex array fabrication, where stamping can be done by several stampers working in parallel. The protein loaded into the hydrogel is immobilized within the gel matrix via an amine-coupling agent. This is an advantage for the production of arrays since an immobilization step is not necessary.

Noncontact printing methods. Noncontact printing comprises methods that use capillaries or inkjet technology to deposit nanoliter to picoliter drops onto the chip without touching the surface [125]. Although mechanical damage is minimized, the inside walls of the spotters should be made biocompatible to avoid protein inactivation [59].

1. Inkjet printing: Of these ''ink'' techniques, inkjet technology is the most established and has been largely used for DNA arrays on glass slides [11,152] as well as ligand arrays [4,5]. Protein solutions are aspirated from a reservoir and less than 1 nl is spotted onto the chip to yield spot diameters of about 80 μm on polystyrene [4]. Some applications of inkjet printing in protein microdeposition include work by Roda *et al*. [259] and Silzel *et al*. [8].

2. Piezoelectric printing: This is a variation of inkjet printing where, as opposed to back-filling, samples are aspirated from the front. The glass capillary dispensing tip has a piezo-electric collar that compresses the sample upon applying a voltage and dispenses 325-pl drops without surface contact [146,260]. An advantage with front-filling is less sample is required and there is minimal sample heating so that proteins remain active [152]. Piezoelectric printing has been used with in the production of hydrogel-coated slides [152] (see Packard Bioscience in Table 3).

3. Dip-pen nanolithography (DPN): This technique by Chad Mirkin (Department of Chemistry, Northwestern University, Evanston, Illinois) is a high-resolution patterning technique that can be used to fabricate protein arrays with spot diameters of approximately 100 nm [261–264] (see also Glossary). Molecules are transported from the AFM tip to the solid substrate via capillary action [264]. (See also Nanotechnology, Arrays: nano-lithography, Dip-pen nanolithography).

4. Electrospray deposition (*ESD*) is done on slightly conductive surfaces [265] through a dielectric grid ''mask''. The electrospray process stops as the distance between tip and chip substrate is increased about 100 μm. Hence, the printing process can be automated using a computer-controlled x-y-z stage [265,266]. Spot sizes can be as small as 2–6 μm in diameter but are usually ≥ 50 μm under laboratory conditions [265].

Protein orientation

For optimum performance and increased sensitivity, ligand molecules should have their binding sites directed away from the chip surface. This is especially important for immunodiagnostics. Immobilization of whole antibodies [267–269] and antibody fragments should be done with the proper orientation. The simplest way to achieve this is by the use of amino- or carboxy-terminal tags with recombinant proteins, *e.g.*, with His-tags [128] or via an amino-terminal serine or threonine residue [270]. Depending on the application, it may be sufficient to orient whole antibodies by binding to immobilized protein A or G [271]. However, this is not very stable. Several methods have been developed specifically to orient antibodies and antibody fragments [272].

One method is by oxidizing and biotinylating the conserved glycosylation site on the Fc region of the antibody [9]. Another method involves obtaining the dimeric Fab′ fragments by cleaving with pepsin, reducing the disulfide bond that holds these fragments together in the hinge region, and conjugating the reduced disulfide to a thiol-reactive surface or biotin-maleimide. The resulting antibody or Fab′ fragments have their antigen-binding sites directed away from the chip surface [9].

Heterobifunctional crosslinkers have been used as well to immobilize antibodies. Densities of 74–220 ng/cm^2 were achieved on glass cover slips [138]. (Assuming a 10 kDa protein in a 100 μm × 100 μm spot, the spot would contain low to subfemtomole amounts, 0.74–2.20 fmol, or picogram amounts.) Carbohydrate-reactive crosslinkers bind to the carbohydrate moiety in the Fc region of the antibody. At least eight different crosslinkers were tested and no dependence or correlation could be found with length, reactive group present, or rigidity of the linkers. Compared with previous studies with thiolsilanes, a distinct difference is that these carbohydrate-reactive linkers showed higher activites. The immobilization procedure, however, is more complex and tends to cause some denaturation.

Apart from ligand molecules, the protein molecules themselves can be oriented in a more random manner to enable different sides of the molecule to effectively interact with the components in bulk solution and obtain maximum detector sensitivity. In using an aldehyde silane reagent on glass slides, the aldehydes react with primary amines on the protein. Since a typical protein will have several surface lysines and have also the amino-terminal group free, the binding reactions with the aldehydes on the chip surface lead to bound protein molecules on the chip with varying orientations [118].

Microfluidic technologies

Fabrication techniques

Early microfluidic devices were used in DNA analysis [273,274] and cell sorting [275]. They were made from elastomers and, to this day, although glass and quartz are used even in commercially available microfluidics chips (see Table 3), the fabrication of polymer microfluidic devices is a growing field because of tremendous savings in time and cost compared to fabricating devices out of silicon and glass [10]. Currently, technological efforts are focused on the integration of more capabilities on chip, *i.e.*, sample preparation and/or detection systems, high-volume production (millions of chips per year), and further developments in micro- and nanofabrication as well as in surface modifications [61]. In the fabrication of microfluidic devices, important considerations are the development time from design to working prototypes, the design and layout of the chip considering channel size and geometry, and the integration of the required components (*e.g.*, for injection, separation, and/or detection) [10].

With polymers, microfluidic channels are formed by some of the technologies already discussed under arrays and several more to be discussed below. Two general categories are, as mentioned earlier, the replication technologies and the single-device technologies [61]. Replication technologies include molding, embossing, and casting (discussed above) as well as the use of SAMs and soft lithographic techniques (discussed below) [10,61]. As basic principles in patterning with soft lithography, rapid prototyping and replica molding are also used for microfluidic systems [10,65,154,216]. The single-device technologies include laser ablation and photoresist technology (discussed above), as well as stereolithography and layering techniques (discussed below). Techniques for fabricating and patterning 3D structures are discussed in Reyes *et al*. [294] and in Blawas and Reichert [116].

Soft lithographic methods for microfluidics.

1. Rapid prototyping: Rapid prototyping [65,154] combines high-resolution commercial printing, photolithography, and soft lithography to produce a master at a fraction of the time and cost that it takes to produce one by photolithography using a chrome mask (see also Glossary). Instead of a chrome mask, a high-resolution transparency is used as a photomask for the fabrication of the master by contact photolithography. The resulting master is a positive relief (protrusions) of SU-8 photoresist on a silicon wafer. For extended lifetimes, the master can be made of a single piece of hard polymer (structural polyurethane or epoxy) [10].

The major difference in resolution, >20 μm features against ~ 500 nm for a chrome mask, can be improved with the use of image setters with >3386 dpi resolution. If features <20 μm are required, a chrome mask should be used instead. The channels formed have cross-sectional diameters in the 50–100-μm range. The resulting rough edges on two walls do not affect the performance

of a 50×50 μm channel, when compared to separations done with fused silica capillaries [10].

2. *Replica molding (casting)*: This method involves fabricating channels from bulk polymer. The technique involves using the positive master and creating a negative PDMS replica by casting the prepolymer. After an hour of curing, the PDMS replica is cooled and peeled off the master. Replica molding can be used with any master fabrication method [10].

Since the PDMS replica would have three walls after casting, a fourth wall then seals the channel. This fourth wall is a flat material that can be PDMS or some other material. Sealing can be done reversibly by conformal contact or irreversibly by sealing onto certain substrates upon exposure to air plasma [277] (see also Glossary). Reversible sealing is due to van der Waals interaction between PDMS and the material of the fourth wall. Although it is watertight, the seal will not hold greater than 5 psi pressure in the channels. Irreversible sealing is due to the covalent bonds formed upon exposure to the plasma. For example, when PDMS is irreversibly sealed against glass, Si–O–Si bonds are formed with the loss of water. The seal can withstand 30–50 psi. Duffy *et al.* [65] list the different materials that will work or not work with this plasma sealing technique.

Plasma oxidation serves another purpose. PDMS is hydrophobic, difficult to wet, and to easily nucleate bubbles. Plasma oxidation transforms the PDMS surface to a hydrophilic one with exposed silanol groups. Although the adsorption of hydrophobic and negatively charged proteins is minimized, some still do adsorb [65]. An advantage is that these silanol groups support a cathodic EOF upon interacting with ionized or ionizable species in neutral or basic solutions. Hence, it is useful for capillary zone electrophoresis (CZE). This oxidized surface, however, is stable for only about 30 min and reverts back to its hydrophobic state [10]. Nevertheless, the oxidized PDMS surface can be modified by adsorption of charged and neutral polymers [65] or covalent attachment of trichlorosilanes [277,278].

Single-device techniques for microfluidics.

1. *Stereolithography*: Stereolithography is a method that requires no masks or steps aside from the laser time required to ''draw'' the structures on the chip (see also Glossary). The technique starts with the liquid polymer and as the focused laser hits the polymer surface, the polymer at the focal point cures and solidifies. The fabrication of a microfluidic device using this technique has been described [279]. The features of the chip are drawn on CAD software and this controls the movement of the laser and the chip container. Although the technique fits the label of rapid prototyping, a chip can take several hours to fabricate, depending on how many features need to be defined on the chip [61]. Stereolithography can be used to fabricate 3D structures.

2. *Layering*: The technique has been described by Carlos H. Mastrangelo and his group (Department of Electrical Engineering and Computer Science,

University of Michigan, Ann Arbor MI, USA) [280–282]. Thin layers of different polymers define the chip including a sacrificial layer in between that is etched away to define the channel [61] (see Fig. 13). Parylene can be used to define the channel walls and cover. Metal can be deposited onto the parylene by sputtering or evaporation to define electrode structures. The etchant, usually acetone, enters the chip via holes and carry with it dissolved material. The etching process can last for an extended period of time because the transport of etchant is done only by diffusion [61]. A similar technique uses multilayer structures on flexible rubber chips and is referred to as multilayer soft lithography. The interesting aspect of this proprietary technology is that miniature pumps and valves are built on the chip [11,38,67] (see Fluidigm in Table 3).

Manufacturing technologies. Besides forming microchannels, a set of methods is employed to seal them and put in other structures such as through holes and electrodes. Bonding techniques such as lamination, gluing, applying heat

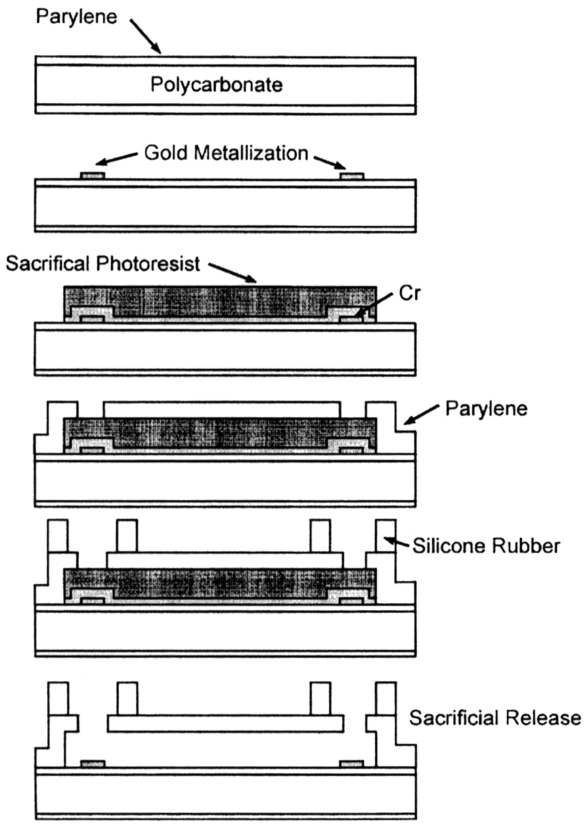

Fig. 13. Diagram of the fabrication process for layered structures. Reprinted with permission from Ref. [61]. Copyright 2000 Wiley-VCH Verlag GmbH & Co. KGaA.

and pressure, and welding, and other manufacturing steps including dicing and electrode fabrication are discussed in Becker and Gärtner [61].

Microfluidic functions: fluid flow and control
There are several methods of actuating flow based on principles such as electric field, pressure, and surface tension. A number of these techniques are discussed below. Apart from fluid transport, another important aspect in operating microfluidic systems is flow control. Various techniques exist from microvalves and the use of hydrogels and hydrodynamics to valveless flow switching and virtual walls. The integration of other components on the chip, especially optical components for detection, and interfacing to other instrumentation systems are discussed in the next section, Principles of Design and Operation.

Fluid flow. Fluid flow in microchannels can be controlled via different principles. Some give only localized control, while others influence the entire fluidic system in the device. In a complex microfluidic system, the active control and switching of fluid flow is crucial. In other words, it should be possible to change flow rates at different stages of the experiment and at different points within the chip. This can be achieved with actuation principles that directly operate on fluids, such as electroosmotic force (EOF) and electrocapillary pressure, as opposed to local electrostatic generation of pressure or the use of centrifugal force [38]. However, more active control can be achieved if the fluids on the chip can be properly controlled by a system of pumps and valves as it is done in the macro world. These methods are discussed or cited below. For both EOF and pressure pumping, the use of PDMS as a chip material has distinct advantages. Its surface can be charged and, when sealed irreversibly, can withstand high pressure. It is also easy to incorporate membrane pumps and check valves because the material is elastomeric [10]. A number of actuation principles involving the induction of surface tension differences will also be discussed.
1. *Electroosmotic flow*: The early development of microfluidic chips in the 1990s can be attributed to the poor performance of microfabricated pumps. The early microfluidic devices used electrokinetic or EOF control. To this day, many microfluidic devices transport fluids in this manner [12]. A fluid transport method used for both soft and hard materials [283], electroosmotic pumping was first demonstrated by Harrison *et al*. [23]. Molecules pick up a charge from the channel walls and are driven through the channel via an applied voltage. The applied fields are high (over 2 kV/cm) [18]. Microfabricated electrodes made of platinum, chromium, or gold wires are located on the channel walls, *e.g*., at the interface of the polymeric substrate and the glass cover plate [38,284]. Gases generated electrolytically at the electrodes are removed by permeation through polymeric substrates. Furthermore, the flat flow profile, as opposed to a parabolic one in pressure systems, results in less band broadening. Therefore, besides simplicity in design and ease of operation, separation efficiencies are also

improved [73]. Variations to EOF have also been described. Electroosmotic hydraulic pumping has been demonstrated where fluid flow relies on the applied voltage and not the field strength. It is also independent of the entire channel length that is electroosmotically pumped [284]. Opposite flows can be generated with flowFET, EOF with field-effect flow control, analogous to a field-effect transistor (FET) [285]. This illustrates the potential of flow switching in microfluidic systems using EOF.

The use of EOF in contrast to pressure-driven flow has several advantages: experimental simplicity, maintaining fluid flow without mechanical parts (pumps or valves), and minimal back pressure. The presence of an applied field already assists applications involving electrophoretic separation [41]. But it can also be a disadvantage in some cases. Electric fields are an important means of molecular separations; however, they pose some difficulties as a method for manipulating fluids. Pumping heterogeneous solutions causes "electrophoretic demixing" [283]. Changing solution conditions such as buffer composition or salt concentration requires some readjustment of the voltage applied. Thus, depending on the experiment, difficulties may arise from having to reset voltages to adjust for changes in ion concentration, unequal pressures, and evaporation [16]. Voltage control is adequate for a simple one-junction system but in more complicated systems with several switching junctions, voltage adjustments to compensate for different pressures and resistances throughout the device will be difficult [62].

2. *Manetohydrodynamics (MHD)*: A pumping method developed by Ingrid Fritsch (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR, USA), magnetohydrodynamics relies on electric and magnetic fields to "push" fluids down the channel. Sample solutions are spiked with redox species such as ferric cyanide or nitrobenzene. When the fields are applied orthogonally to the direction of flow, the redox species move down the microchannel and drag along the rest of the fluid, effecting a flow. Typical flow velocities are up to a few millimeters a second, comparable to EOF. Voltage required is about one volt and the magnetic fields are "moderate." Nonaqueous solutions (*e.g.*, dichloromethane and ethylbromide) can be used which is useful for on-chip organic synthesis [38]. Lemoff and Lee [286] performed AC magnetohydrodynamic pumping where solution is driven along a circular silicon channel.

3. *Electrocapillary pressure (ECP)*: A technique developed by Menno W.J. Prins (Philips Research Eindhoven, Eindhoven, Netherlands), ECP is a means of moving fluids through a channel by controlling the position of a fluid–fluid interface (see Fig. 14). Electrocapillary pressure refers to the voltage-dependent pressure on the meniscus of a fluid in a microchannel. The position of this meniscus is controlled by a localized force that is generated by the voltage on the electrode that is embedded in the channel walls, giving the method a high spatial resolution in terms of actuation [287]. Fluid transport is sustained by a proper sequence of voltages. It should be distinguished from electrowetting because it is

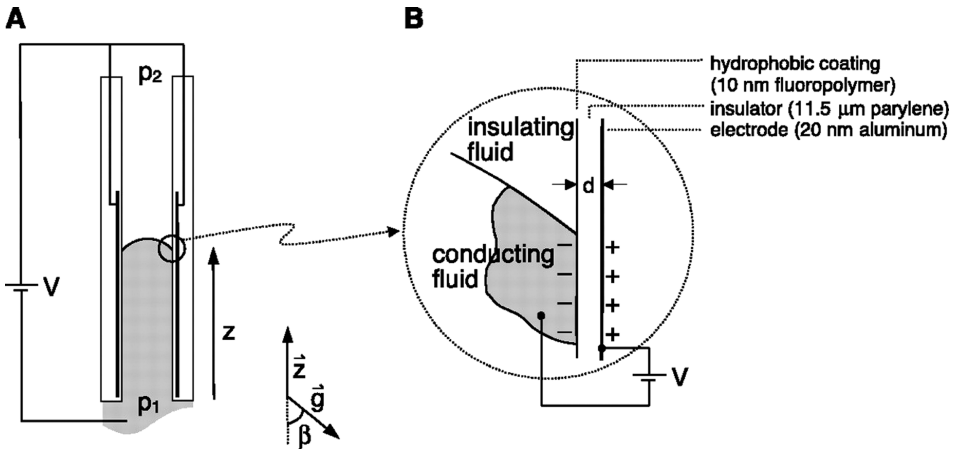

Fig. 14. Electrocapillary pressure. (A) Sketch of fluid in a microchannel. The fluid at the bottom (shown in gray) is electrically conducting (an aqueous salt solution). The top contains an insulating fluid (either air or a nonpolar oil). A voltage V is applied to the electrode; the aqueous solution is at ground potential. The pressures at the bottom and top openings of the microchannel are expressed as p_1 and p_2, respectively. The angle between the gravitational force and the long axis of the microchannel is given by β. (B) Close-up of the meniscus edge. Inside the microchannel wall, a 20-nm-thick aluminum electrode is present, separated from the aqueous solution by an insulating layer of thickness d. The insulating layer consists of an 11.5-μm thickness of parylene (deposited by chemical vapor polymerization of di-*p*-xylylene) with a 10-nm fluoropolymer coating (AF1600 of DuPont, deposited from solution). Because of the applied electric field, opposite charge densities are induced at the fluid/solid interface and in the subsurface electrode. The electrostatic attraction between the aqueous solution and the electrode enhances the coverage of the wall by the aqueous solution. Reprinted with permission from Ref. [287]. Copyright 2001 American Association for the Advancement of Science.

independent of the contact angle with the applied voltage [287]. It should also be distinguished from electrocapillarity in the classical mercury/electrode system where all fluids are electrically conducting [287]. ECP is electrostatic in nature with very low charge densities (10^{-4} C/m^2) and displacement currents; electrochemical reactions and electrolytic bubbles are absent. Consequently, power consumption is very low. This pumping method does not rely on mechanical valves or pumps and is suitable for microfluidics applications that require "repeated low-power operation." But as with electrokinetic flow, the surface chemistry in the channels has to be well controlled. The smaller the dimension of the channels, the greater is the influence of, and problems associated with, surface effects [38]. Because of precise control, flow switching is possible as well as guiding fluid flow through a 3D-channel network [287]. Fluid velocities can be several centimeters per second, almost 2 orders of magnitude higher than other methods for electrofluidic actuation [285,288].

4. Thermocapillary pumping (TCP): Given a nanoliter- or picoliter-sized drop, TCP involves heating one end of this drop to create a surface tension difference with the opposite end of the same drop. This surface tension difference causes a

capillary pressure difference and induces motion. Typical TCP velocities run up to 20 mm/min (approximately 300 μm/s). References on this technique are Kataoka *et al*. [289] and Burns and Troian. [290].
5. *Electrochemistry*: Fluids can be actively controlled electrochemically with the use of redox-active surfactants. Surfactant species are generated at one electrode and consumed at the other. Electrochemical control of the position of these species then controls fluid motion [288].
6. *Pneumatic actuation*: Valves fabricated from elastomers can have precise fluid control over a wide range of conditions [62,292]. They can attain complete valve sealing, have fast response times, need low actuation forces, and have a small footprint. The first devices occupied an area of 100 μm × 100 μm and now the valves' working area has been reduced to 20 μm × 20 μm. A peristaltic pump results from having three consecutive valves in a row. Flow rates are up to a few nanoliters per second, compatible with that which is required for assays, and dead volumes of both valves and pumps are negligible [62]. Mechanical pumping can transport and mix liquid samples [291,292]. Pressure-driven flow will allow the use of more types of solutions in microfluidic devices since it is less sensitive to solution properties than EOF [67].
7. *Light-driven fluid motion*: Asymmetrical irradiation of a substrate surface with "photochromic azobenzene units" causes a gradient in surface free energy due to azobenzene photoisomerization. The direction and velocity of fluid motion is determined from the direction and steepness of the light intensity gradient [293].

Fluid control. A number of systems for flow control exist. A review by Reyes *et al*. [294] provides extensive literature coverage on this topic. Below, some techniques developed to control fluids, with or without the use of valves, are described.
1. *Pneumatic actuation*: The same principle discussed above in Fluid Flow is basically a fluid control device. A valve is created by introducing gas pressure (air or nitrogen) into a control line, which in turn deflects a thin membrane downward to seal off a channel. This downward sealing motion is made possible by having a two-layer system; all microchannels wherein sample flows through are in the bottom layer and the control lines for pneumatic actuation are in the top layer [16].
2. *Passive microvalves*: Fluid control or control of the flow sequence is achieved by a network of passive microvalves (or restrictions). The valves can be modified according to the channel geometry and the sequence of fluid delivery is programmable [295].
3. *Valveless switches*: One type of a valveless switch is the principle of dielectrophoresis. Dielectrophoresis is particle motion caused by polarization effects in nonuniform electric fields, *i.e*., AC rather than DC fields as in the case of electrophoresis [12,296–298]. In sorting mammalian cells, dielectrophoretic field cages and switches can trap and direct particles, respectively [299]. Another

method is pressure switching [300]. Both techniques were used as a valveless switch to separate cells and other particles in microchannels [16].

4. Hydrodynamic flow control: Chien and Parce [301] designed a chip with hydrodynamic flow control. An algorithm and a strategy for calculating pressures were developed to control flow from multiple reservoirs via a multiport system on the chip. Its application in a study of enzyme kinetics was straightforward and reproducible and used only pressure-driven flow. The response was linear with a dynamic range of over 2 orders of magnitude. The key was an appropriate network structure to apply the proper pressure control [41].

5. Self-regulating flow switch with hydrogels: Depending on the pH of the solution, hydrogels can either swell or shrink. The response time for this speeds up tremendously in micrometer dimensions to less than 10 s [302]. Stable hydrogel jackets were designed around prefabricated posts that provide support as well as volume, allowing a short diffusion path to speed up both sensing and actuating functions. This behavior is taken advantage of as self-regulating shut-off valves to stop fluid flow in particular microfluidic channels, without any external controls or circuitry. Response times, however, were much slower than mechanical valves [62]. Changing the hydrogel composition can modify the pH behavior. Furthermore, hydrogels have other characteristics besides pH sensitivity that may enable other functional components [302].

6. Virtual walls with surface-directed flow: Fluid flow can be directed by patterned surfaces or polymerized coatings on a chip. At low pump pressures, fluid flow is confined to adherent surfaces and will avoid the hydrophobic nonadherent regions. Hence, it is possible to set a flow path without valves or channel walls and without the need of electrical power [38,291]. The chip surfaces or channel walls are treated with SAMs either by multistream laminar flow (laminar flow patterning) or photolithography. Multistream laminar flow required preformed channels where the depth used was ~ 180 μm. Photolithography was performed with 2-nitrobenzyl photochemistry to have hydrophilic carboxylate groups in the exposed regions [303]. SAMs can be used to modify the surface wettability [291,304–306] and can be formed on substrates such as silica in minutes. Contact angle measurements showed complete coverage of a glass substrate with SAMs of trichlorosilanes (OTS and HFTS) in less than 2 min [291]. One function that surface-directed flow can perform is the concentration of samples on chip [291].

With a wide choice of materials and technologies, hybrid devices in microfluidics may likely be developed [38]. Different materials have different desirable properties, *e.g.*, glass for optical windows and plastics for channels. Different technologies have different advantages in achieving a given end product with respect to fabrication time and features that can be built on the chip: microstructures with replica molding and 3D structures with stereolithography. For fluid manipulation and control, there are numerous techniques, some of which have been developed probably only within the last 3 years. It will be some time before the value of some of these technologies can be properly assessed. Furthermore although certain techniques may be robust enough, they are

proprietary technologies of commercial entities. And with their products still under development, it will be a while before one can pass judgment on winning combinations. Nevertheless, the enormous interest and research in the field will only benefit innovation and progress. The potential applications of microfluidic devices are outlined in McDonald *et al*. [10] and a two-part review on μTAS from Manz's group provides more than 600 references on technologies and applications [294,307].

Nanotechnology

Progress in the area of biochips has extended to nanotechnology. Both array and microfluidics chips are benefiting from technologies that can write nanometer features. Although producing these nanometer features may be more time-consuming and expensive, this may apply only for fabricating the master or mold tool and, hence, should not deter their commercial or industrial use. The resolution of replication technologies is determined by the size of the features on the mold or master [62]; replica molding or casting has been used to replicate nanometer features. Mold replication would allow mass production of the device, which would offset the cost of fabricating the master. Nanoscale molecular tags, *i.e*., nanobarcodes and nanocrystals, are discussed under Principles of Design and Operation.

Arrays: nanolithography

Lithographic techniques that write nanometer features can collectively be called nanolithography. Several techniques used in fabricating nanoarrays are mentioned below. Zhao *et al*. [217] discusses soft lithographic methods for nanofabrication. Nanopatterning, along with three-dimensional patterning, has been a growing interest since the late 1990s [116].

Electron beam lithography (EBL). Although it is a time-consuming and expensive process, electron beam lithography can be used to make nanometer features on a master [62]. Certain chemicals change their properties when hit with an electron beam. With computer control of the beam position, structures can be written in the range of up to about a hundred nanometers [308,309].

Dip-pen nanolithography (DPN). Using DPN, arrays were fabricated with 100–350-nm features. Optimization should reduce this closer to 10 nm, the limit of conventional DPN [262–264]. Molecules transported via capillary action from the AFM tip to the substrate are chemisorbed [264] (see Fig. 15). An important feature is that there is almost no detectable background due to nonspecific binding (NSB) and the chemisorbed proteins remain biologically active [261]. An array was fabricated by patterning 16 mercaptohexa-decanoic acid (MHA) on a gold thin-film in the form of dots or grids. Protein non-adherent regions were treated with 11 mercaptoundecyl-tri(ethylene glycol) [261]. AFM recognised binding interactions when it detected a doubling in height indicating

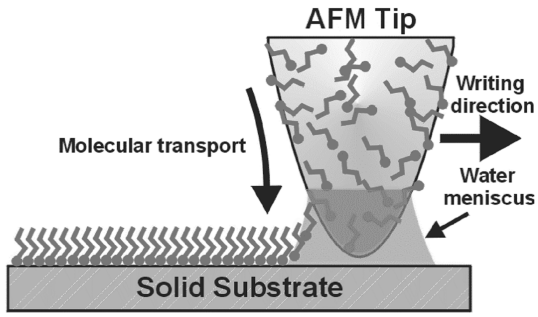

Fig. 15. Schematic representation of DPN. A water meniscus forms between the AFM tip coated with ODT and the Au substrate. The size of the meniscus, which is controlled by relative humidity, affects the ODT transport rate, the effective tip-substrate contact area, and DPN resolution. Reprinted with permission from Ref. [264]. Copyright 1999 American Association for the Advancement of Science.

the presence of 2 proteins. It is interesting that under these nanoscale conditions, whatever the orientation of the IgG relative to the chip surface was, it reacted with the anti-IgG in the protein solution. (See also Array Technologies, Protein printing, noncontact printing methods.)

Scanning probe lithography. Based on atomic force microscopy, scanning probe lithography is an alternative to photolithography in terms of fabricating structures less than 0.1 μm (100 nm). SAMs have been nanopatterned by scanning probe lithography [209,210,254] (see also Array Technologies, Protein Printing, Contact printing methods).

Finely focused ion beam lithography (FFIB). FFIB is a short wavelength lithographic technique using 30-keV indium ions to write small features (defect pits) on a gallium arsenide surface having 60-nm diameters with 185-nm spacing between them [310]. Adsorption of human serum albumin was limited to the inside portion of these pits, as seen on TM-SFM (tapping mode scanning force microscopy) images. Hence, the spatially controlled adsorption of proteins was achieved on a nanometer scale. Since no such adsorption was observed with silica, the choice of material is important. It is speculated that even single molecules can be adsorbed. In well-ordered arrays where well-defined lines or defects are created, the scanning probe tip can easily go or return to specific locations on the chip to within a few nanometers [310]. Potential applications therefore are the study of specific protein adsorption behavior as well as protein–protein interaction studies on single molecules [310].

Nano-imprint lithography (NIL). This embossing technique developed by Stephen Y. Chou and his group (Department of Electrical Engineering, Princeton University) can write nanometer features [62,311,312]. Relief patterns in a thermoplastic resist layer can be created by simply compressing it after it has been thermally softened [313]. However, problems occur in trying to get a clean release of the resist from the mold and the resulting imprinted structures become

distorted. With room-temperature NIL (RT-NIL), compression leads to the free-volume contraction and plastic deformation of the polymer [314]. Amorphous polymeric material has vacancies called free volume such that a polymeric film with a thickness of 100 nm can be compressed to a depth of 10–30 nm solely from compacting or collapsing the free volume. Beyond free volume compaction, further imprinting can be done through plastic deformation (see Glossary).

S-layer proteins. Discussed under Materials, these cellular protein layers have a repeating pattern of nanometer dimensions. The S-layer proteins of *Thermoproteus tenax*, for example, have hexagonal channels that are 6 nm in diameter [315]. In one application, the S-layer was used as a template to create metal masks [316].

Microfluidics: integration of optical elements and nanostructures

Integrating optical elements. Optical signals resulting from biochemical assays or other chip experiments need to be captured and recorded. The best solution would be to have light going in and out of the microfluidic devices and to have the necessary optics near the flow channels. Optical elements such as lenses and beam splitters can be fabricated within glass or elastomers [62]. Fine structures such as narrow line gratings and pillar arrays have been made with elastomers by embossing and replica molding [62,311,317]. Diffractive lenses can be defined on PMMA resist with subwavelength features by using electron beam lithography, rather than photolithography, followed by replication in quartz or silicon by using the PMMA mask in an ion etching process [62,318]. Silicon molds can be even etched at a faster rate with XeF_2 and results in high-resolution features as small as 50 nm [62,319]. Some replication guidelines are given by Quake and Scherer [62].

Nanostructures. Some of the same technologies used for arrays such as electron beam lithography (EBL) can be applied to microfluidics to create nanostructures. EBL and highly anisotropic pattern transfer have been used to construct nanostructures with lateral dimensions as small as a few nanometers [62,320]. With this high-resolution lithography and anisotropic ion etching, nanopillars that were 6 nm in width and lines 10 nm in width were fabricated [62,321]. The limit in developing nanodevices is in constructing molecular nanostructures using single molecules as templates [62]. Examples include single-molecule nanobridges in silicon and, out of single DNA molecules, nanowires less than 10 nm thick and nanoresonators with predicted gigahertz frequencies [62,322–324]. It would be interesting to see where all this will play a role in the biological and medical fields.

Principles of design and operation

This section on principles of design and operation provides the logic behind the tools, the rationale behind the choice of which materials and methods

make sense in any one particular chip application as well as discusses the other aspects involved in operating a biochip, *e.g.*, the detection system. This section is divided into a discussion on arrays and microfluidics. A separate section is dedicated to cell and tissue biochips because there are inherently different issues to be addressed as well as a different set of required conditions for operation.

Arrays: capture agents, molecular tags, detection

Generally, a key element in microarray is the occurrence of a binding event, *e.g.*, antigen–antibody interaction. As such, there are many situations involving all ligand-binding events where a microarray format is applicable [125]. How many samples constitute a microarray? This number can vary greatly from less than 1000/cm^2 [324] to a range of 1600–1800 protein spot cm^2 [118,128]. Another example is a dipeptide array with over 250,000 elements/cm^2 [166]. But in comparison, an array of cDNA sequences can be over 400,000/cm^2 [325]. In the final analysis, the relevant issues still are preserving protein activity and detector sensitivity. The protein should be active in order to take part in a binding interaction. And when this interaction occurs, it should be detectable.

Protein concentrations should be within detection range. For proteins to be immobilized on arrays MacBeath and Schreiber [118] observed saturated fluorescence signals above 1 mg/ml and kept the concentration at 100 μg/ml. Hence, for high-throughput applications, a microliter of sample may be enough to spot a whole array. Proteins in free solution, however, need to be at much lower concentrations. Sample concentration at the lower limits of detection is a more critical issue. Specific binding of Cy5-FKBP12 (Human immunophilin) could be detected at 150 pg/ml ($\sim$12.5 pM) [118]. Ekins *et al.* [125,326–329] quantified proteins such as thyroid-stimulating hormone (TSH) and Hepatitis B surface antigen (HbsAG) down to femtomolar levels or the equivalent of 10^6 molecules/ml. For high-throughput applications, a microliter of sample may be enough to spot a whole array [118]. But for detection systems, although there may be inherent sensitivity, background interference should be minimized (see Array detection systems, Nonspecific binding).

Assuming one already has a large library or repertoire of binders to target proteins in the sample and the goal is to simultaneously identify and quantify different types of molecules and interreactions, the choice in strategy is between immobilization via a capture agent or molecular tagging [330]. Immobilization involves keeping one set of molecules bound at specific sites on an array and the other free in solution to react with it. Although techniques to orient protein molecules exist (see Microfabrication of chips, Technologies, Array Technologies, Protein orientation), there is still the possibility and limitation that an interaction site on a bound molecule may be blocked and inaccessible. Molecular tagging, on the other hand, allows molecules to be unconfined. However, methods of identification and detection of the tag and the interacting

partner become more complicated. Both strategies are discussed in the following sections.

Capture agents

Capture agents need to be stably positioned on the chip. They must also be able to bind their target proteins present in nanomolar or even picomolar solutions [8,59,331]. Capture molecules include antibodies, antibody mimics, and aptamers [79,125,146,331].

Antibodies. Antibodies are the most prominent type of capture molecules. However, because monoclonal antibody production is labor-intensive, the development and use of alternative methods become imperative [125]. Antibody arrays have since been made [332–339], some using an scFv antibody library [335,340–347] isolated by phage display [332,333]. Monoclonal antibodies are known to have high binding specificity and be stable enough to last several weeks [348]. Nevertheless, recombinant scFv are relatively stable. Immobilization of the antibody on the chip surface can be done via reductive amination, direct avidin–biotin attachment, via epoxide linkers or by immobilization to the GOPS (3-glycidoxypropyldimethyl ethoxysilane) film [72,136,349]. The last method, which involves direct, covalent coupling, minimizes losses and is straightforward when compared with avidin–biotin coupling [72].

Antibody mimics. A disadvantage with antibodies is that they tend to denature with heat or pH changes. Another limitation is that sandwich assays, which require a second antibody, may give unreliable results when the antibodies bind to nontarget proteins. Alternative capture molecules are other protein scaffolds or antibody mimics [125,146,331]. They are more stable than antibodies, are smaller, and can be synthesized chemically. A 100-amino-acid domain of human fibronectin has been used as a pseudo-antibody. The variants are called trinectins and a proprietary mRNA display is performed on a library size of 10^{13} [350]. Another scaffold, an affibody, uses a 58-amino acid domain of a staphylococcus surface protein where the binding surface is 13 amino acids with IgG molecules [351]. The library size is claimed to be as large as 10^{16} in theory whereas phage display can only accommodate about 10^{11} members [334,335]. Affibodies, from heterologous expression of microorganisms [125,352,353], are labeled with 2 fluorophores such that when they bind their target protein, the labeled segments move away from each other. This results in a change in fluorescence thereby signaling a binding event [354]. This phenomenon has been termed fluorescence resonance energy transfer (FRET) [57,354,355] (see also Array detection systems).

Aptamers. Aptamers are single-stranded nucleic acid structural units that have been used as capture molecules [125,334,356–359]. There are also peptide aptamers [125,361]. A key advantage with their use is that they are synthesized *in vitro* and hence can be chemically modified efficiently [356]. Single strands

of DNA can mimic antibodies as they can assume three-dimensional shapes. Selection is done via SELEX (systematic evolution of ligands by exponential enrichment) [356,360] (Brody *et al.*; Brody & Gold 2000) or mRNA display [125,362]. It should be noted that RNA aptamers are susceptible to nucleases in complex protein mixtures [344,363].

A photoaptamer system has been developed where thymidine has been replaced with bromodioxyuracil in a 10^{15} DNA library [360]. In this system, aptamers are covalently attached to the chip and target proteins in the sample bind to their respective aptamers on the chip. Upon exposure to UV light, the captured target proteins are cross-linked via the bromodioxyuracil. After washing unbound proteins, detection can be done via a general protein stain. This is a particular advantage since aptamers are not protein molecules; hence, the readout can be reliable and rapid. In contrast to DNA microarrays, fluorescent labeling will not work in this case because different proteins take up the label to different extents [331,360,364].

Templin *et al.*[125] presents a table of capture molecules including their source, the techniques used to produce them, and corresponding references. Other capture molecules not discussed here include synthetic receptor ligands generated by combinatorial chemistry [365,366], enzyme substrates [118,136,137], and the potential use of molecularly imprinted polymers (MIPs) for affinity capture [344].

Molecular tags

Radioactive or fluorescent labels are most commonly used in detecting binding interactions [80,344,367] (see also Array detection systems). But with fluorescence measurements, specificity can be a problem. Organic dye molecules (*e.g.*, fluorescein, rhodamine, and cyanine dyes) and light scattering particles such as gold or silver have broad spectra making it impossible to generate unique tags for different types of molecules within a useful wavelength range [330]. Alternatives include semiconductor crystals called quantum dots and metallic bar-coded rods called nanobarcodes.

Quantum dots. Quantum dots are nanocrystals of semiconductors (*e.g.*, calcium selenide) that have much narrower spectral bandwidths [368–371]. They emit light at a number of precise wavelengths and the exact color depends on dot size. One advantage is stability; the dots can withstand hours of repeated cycles of excitation and fluorescence, compared to conventional dyes that last only minutes. The other advantage is activation of many colored dots using a single laser; several single wavelength activations of conventional dyes would imply the use of multiple lasers, where the cumulative energy would damage biological material [368,370–372]. They are an important innovation in cellular imaging and biosensing. Their applications are in screening drug leads and in providing a rapid readout for proteins present in a cell or tissue. These dots have been linked to antibodies to label target proteins in human cancer cells [370]. Currently under study are the extension of their excitation wavelength range beyond the visible

(trying indium arsenide), the mechanism by which cells take up the dots, and their surface chemistry (increase or change their binding specificity; make them stable, resistant to oxidation and high salt concentrations) [368] (see also BioCrystal and Quantum Dot in Table 4).

Nanobarcodes. Nanobarcodes are metallic rods 0.5 μm in diameter and about 10 μm long with electrochemically deposited thin metal stripes. Rods have been made with as many as 13 distinguishable stripes using seven different metals (Pt, Pd, Ni, Co, Ag, Cu, Au) in 10 nm to μm segments. With two metals, 4160 unique barcodes are possible; with three, the number increases to 8.0×10^5 distinct stripes [373]. Since derivatization chemistry for metal surfaces is well developed, the rods are attached to a variety of sample molecules via a number of techniques [373]. The barcodes are read by reflectance microscopy and the analytes are detected by fluorescence or mass spectrometry [373]. Hence, theoretically, many types of bioanalytical assays can be performed in a multiplex fashion. There are, however, technical challenges to be overcome: the making of these nanobarcodes and the attachment to analyte molecules can be automated but thus far is not a multiplexed or highly parallel process. In addition, the readout of a system with multiple barcodes has not yet been demonstrated. To be able to read thousands of different bar-coded samples is a complex image acquisition and pattern recognition problem. After an assay, the rods are placed on a flat surface at a low enough density and should overlap only to the extent that the coding information is not obscured. Decoding requires imaging resolution approaching the wavelength of visible light [330,373]. In a sandwich immunoassay, Nicewarner-Pena *et al*. [373] showed that barcoded human IgG and rabbit IgG were analyzed simultaneously with secondary antibodies given specific fluorescence labels. The reflectivity image was enough to distinguish the two different IgG molecules so the experiment could have been performed even with only one fluorophore (see Nanoplex in Table 4).

DNA labels. Co-tagging beads with short pieces of DNA is another labeling strategy termed "encoded synthetic libraries." Since DNA sequences in large numbers can be made quite easily, this method does not have the limitations associated with spectral coding [374]. A similar approach is being used commercially [330].

Array detection systems

In array systems, the detection of binding interactions or of specific molecules themselves depends highly on their abundance and on the binding affinities [375]. Since the range of concentrations of different proteins in a biological sample can be several orders of magnitude, dynamic range is an important factor in the choice of a detection system. The other factor to consider is quantitation. Firstly, the amount of deposited protein in a microsport may often be only femtograms, thus extremely sensitive detection will be required to detect them and their interactions [59]. Secondly, for the quantitation itself, some important issues are

the chip surface itself that has to be structurally well-defined and inert to nonspecific protein adsorption and the method of protein immobilization that should be generally applicable to most polypeptides but with adequate selectivity [143]. Signal intensities can be increased with proper site-specific orientation of molecules on the chip, especially with antibodies (see also Array Technologies, Protein orientation). This should give a higher binding probability than with conventional ELISA [59]. Nevertheless, signal intensities from binding interactions should be relatively quite high because the reactions are confined to a small area where there is a high local concentration. Spot size and spot density are responsible for the optimal signal level in miniaturized systems [125]. In a two-color labeling procedure, Haab *et al.* [375] detected different concentrations of captured antigens in the picomolar range. A few nanoliters of a picomolar solution correspond to femtogram amounts on the chip. In a peptide example, high local densities reduced nonspecific binding and permitted the detection of low-affinity interactions [376].

Nonspecific binding. Nonspecific binding (NSB) effects should be addressed especially in complex biological samples such as serum or whole blood [72]. Approaches to reduce NBS effects involve the following: signal amplification, sample dilution, surface-blocking treatments, and signal referencing. All except signal amplification involve reducing background noise. Sample dilution is not an option. Signal referencing aims to cancel out NSB signal either by direct optical interferometric referencing or indirect electronic background substraction, which may lead to signal degradation due to the loss of phase information [72]. In practice, there are several ways to reduce nonspecific binding. Jones *et al.* [69] used a monolayer of octadecanethiol (ODT); its hydrophobicity minimizes the NSB of protein material to the grid regions. Many have reduced nonspecific binding by introducing a monolayer of bovine serum albumin (BSA) on unspotted surfaces of the array chip [81,116,118]. In order not to obscure peptides and small proteins, BSA-NHS (BSA-N-hydroxysuccinimide) can be used instead [118], detailed protocols are given in Science Online (http://www.sciencemag.org/feature/data/1053284.shl). Mendoza *et al.* [74] blocked nonspecific binding sites by preincubating with Blocker Casein in PBS. In peptide arrays, the SPOT synthesis method, which involves the derivatization of Fmoc-β-alanine groups by hydroxyl groups on cellulose, reduces nonspecific interactions [377,378]. Several have resorted to a monolayer presenting poly(ethylene glycol) (PEG) groups in the background regions of the chip to make them nonadherent to proteins [51,71,72,116,143]. Background signals are nearly zero due to the absence of nonspecific adsorption [143]. In experiments that involve binding with scFv antibodies, detection sensitivity can be improved via scFv multimerization [379,380] and signals can be amplified with the use of biotin tyramine [381,382].

Detection with labels. In line with the foregoing sections on capture agents and molecular tags, detection systems should address the fact that the molecules have

labels or are label-free. For systems involving molecular tags, the most widely used detection method is the measurement of fluorescence. The analytes can be fluorescently labeled directly or indirectly via labeled secondary antibodies in a sandwich format [59]. Alternatively, the incorporation of fluorescently labeled nucleotides during replication of template DNA provides increased signal intensity in a technique called immunorolling circle amplification. Other options for label detection methods include luminescence, chemiluminescence, and radioactivity [59,125].

Fluorescence detection is done using charge-coupled device (CCD) cameras or laser scanners with confocal detection optics [125]. Confocal laser imaging with full quantitation is available commercially [383] (see Umedik and BioMedical Photometrics in Tables 3 and 4, respectively). Software solutions that do image analysis and classify results are also available [1]. A variation in fluorescence detection is fluorescence resonance energy transfer (FRET) with affibody capture molecules that are engineered with two tags, a donor and an acceptor fluorophore. When the acceptor fluorophore is in close proximity, as when binding occurs, the fluorescence emission of the donor disappears as it transfers energy to the acceptor and the resulting emission is at a longer wavelength. The appearance/disappearance of fluorescence emission at the two different wavelengths can then be monitored [57,354,355,384]. An advantage of this technique is that because the readings from two wavelengths are ratioed, signal artifacts, noise, and random errors are minimized. Such a detection scheme is useful for cell-based or biochemical assays and was the basis for a commercial high-throughput screening system [57] (see Aurora Bioscience in Table 3). With fluorescent labels an improvement in sensitivity was observed with waveguide technology [119,385] and an even greater improvement with planar waveguide technology [385,386] (see Fig. 16).

Sandwich assays, such as enzyme-linked immunosorbent assays (ELISAs) are an alternative to the detection of molecular labels. Since different samples can take up molecular labels to different extents, this variability is not a factor in sandwich assays where no labels are required [9]. Joos *et al.* [121] had a microarray ELISA for immune diagnostics and detected 40 fg of a protein standard. Because two different reagents are needed to recognize the protein, the specificity of the method is higher [9]. The use of ELISAs for microarrays, however, is met with several criticisms [9]. One is that the method may not be robust enough because each protein spot on the chip requires two binders. It is very difficult to make different ELISAs work under the same conditions on one chip [79]. Mendoza *et al.* [74] performed high-throughput ELISA. The chip had a 96-well format on optically flat glass plate with a black Teflon mask; each well had 144 elements made up of four identical 6×6 arrays, each 6×6 array having eight antigens at least in duplicate along with markers and blanks. Each antigen's monoclonal antibody was biotinylated while detection was done with alkaline phosphatase-streptavidin conjugate and ELF® (Fluorescent alkaline phosphatase substrate). In such a multianalyte array analysis, it is important to carefully

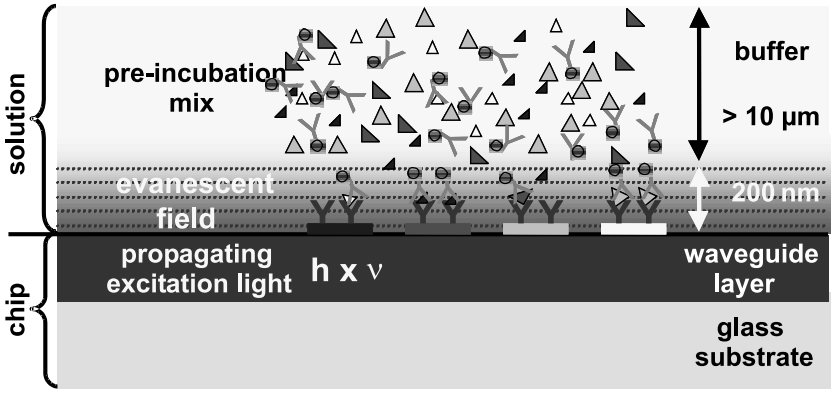

Fig. 16. Planar waveguide technology. Capture antibodies are immobilized on the planar waveguide in a microarray format. A preincubated cocktail mix of sample and fluorescently labeled detection antibodies is added onto the planar waveguide microarray. The formed sandwich immuno-complex can be detected without washing away unbound fluorescently labeled detection antibodies. Laser light of the desired wavelength is coupled into planar waveguide and generates an evanescent field, which extends only a few hundred nanometers into the solution. Therefore, only the surface-bound fluorophores are selectively excited. Individual signals correlating to the amount of captured analytes on each microspot are monitored by means of imaging detectors, such as CCD cameras. Reprinted with permission from Ref. [385]. Copyright 2001 Elsevier Science Ltd.

screen each monoclonal antibody clone against all antigens in the array to avoid cross-reactivities.

An interesting detection method is immunorolling circle amplification (immunoRCA). In this method, an oligonucleotide is coupled to a binder and upon binding and washing, a circular template DNA is added. In the presence of DNA polymerase and fluorescently labeled nucleotides, many copies of the template DNA are produced if the binder is present and the signal is amplified from the incorporation of the labeled nucleotides. Because of signal amplification, this technique has high sensitivity and a very wide dynamic range making it suitable for single molecule detection. Antigens at femtomolar levels can be detected where the antibodies are each labeled with a unique oligonucleotide primer. Multiplexed immunoassays can therefore be carried out and multiple antigens can be quantitated simultaneously [385,387–389].

Label-free detection. When capture molecules are used and the sample molecules are label-free, detection is based on the physicochemical changes that occur upon binding of the target protein to the capture molecule. Label-free detection schemes overcome the limitations of a sandwich assay in terms of cross-reactivity and the need for reagents [59]. Examples of label-free detection include surface plasmon resonance (SPR), mass spectrometry, and atomic force microscopy [59,125].

SPR involves surface refractive index changes due to changes in mass upon protein binding; an optical sensor measures the change in the angle of incident

light [72]. Some commercial systems are available [1,146,344] (see Biacore, HTS Biosystems, and Prolinx in Table 3).

Mass spectrometry (MS) can be used to identify sample molecules and covalently bound interacting partners, such as in the photoaptamer system. An advantage is that the measurement does not require that the protein is still active or in its folded state. Surface-enhanced laser desorption/ionization (SELDI) chips with different treated surfaces enable the capture of specific protein molecules and analyzes them in a time-of-flight (TOF) MS and, more recently, in a quadrupole-TOF instrument to achieve higher resolution for more precise mass measurements [1]. It has been used to analyze differential protein expression and profiling to identify disease marker proteins [55]. Proper identification requires enriching the sample first by affinity separation and then identifying via Edman sequencing, western blot, or MS partial sequencing or peptide fingerprinting [56]. SPR-biomolecular interaction analysis (SPR-BIA) has been done in combination with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS instrument [44,45]. In addition, MS can be used for quality control of proteins [390]. A drawback, however, is that MS is not quantitative [344].

Atomic force microscopy (AFM) provides topographic imaging of the chip surface [2,69,261,391]. In particular, it has sufficient sensitivity to detect a height increase upon antibody–antigen binding through cantilever deflections. It takes 1–5 min to scan an area more than 100 μm^2 [69]. This may be a problem for high-throughput applications. However, there are current efforts to adapt this to a high-throughput format [392].

Other detection systems include the use of piezoelectric devices in quartz crystal microbalances which measure changes in refractive index upon binding (has low sensitivity for smaller proteins below 10 kDa) [344,393–395]; the use of ion channels with subpicomolar sensitivity (is relatively complex) [396,397]; ellipsometry, an optical technique [59]; and electrochemistry applied to enzymatic systems [125,344,398–401].

A detection scheme involving both imaging and quantitation was in a kinase chip. A high-resolution phosphorimager was used to quantify phosphoryl transfer [136].

Microfluidics: channel dimensions, separations, integration, simulation, detection

Channel dimensions

Design parameters for microfluidic systems include the channel size and geometry [10]. Channel dimensions have to be carefully considered: length should be short enough for a minimum transport distance to mixing points or reaction chambers but long enough to prevent hydrostatic induced flow [80]. Furthermore the depth should be enough to allow a detectable volume while avoiding the effects of hydrostatic pressure. Supply channels for reagent and sample should be separate to avoid cross-contamination and facilitate

automation. The layout of the channels, the reagent reservoir, and the points where mixing will occur have to be optimized (see Simulation). For capillary electrophoresis, channel depths are typically 15–40 μm and widths are 60–200 μm [75,402–405]. Typical chip sizes are 2–3 cm^2 [12] and are made of silicon, glass, quartz, plastic, or polymer a few millimeters thick. The chips are also covered with a glass plate, the fourth wall of the channels. In a microfluidic system for heterogeneous immunoassays, the chip dimensions were 6.7×2.8 cm, the channel dimensions were 50 μm × 20 μm, and the reaction chamber was 165 pl [80]. In achieving the separation of two dye compounds in under a millisecond, Jacobson *et al*. [30] had a separation length of 200 μm. But channel lengths for separation also go up to a few mm [405]. Using multilayer soft lithography and elastomeric material, it is possible to build active systems with valves and pumps. Furthermore, the resulting devices are reduced in size by more than two orders of magnitude compared with devices using silicon [292].

Separations

In electrophoretic separations, the length determines the separation efficiency and resolution. Two means of increasing the channel length have problems with peak broadening: a meandering path that avoids sharp bends [406] and a square channel configuration for synchronous cyclic capillary electrophoresis (SCCE) [407,408]. Effenhauser *et al*. [409] had optimized parameters including short injection plugs, a high electric field strength, and a short effective separation length to obtain rapid separation in seconds with extremely high efficiencies. There is a limit to how much field strength can be applied in order to prevent excessive Joule heating [406], arcing between electrodes, and to avoid dielectric breakdown of the chip substrate itself [73]. One solution is having wide channels that narrow down over a short distance (the potential drop being limited to the narrow section of the channel) (see Fig. 17). The separation length was 200 μm and the field strength was 53 kV/cm. Separation for two dye compounds was achieved in less than 1 ms [30]. Another solution is increasing the field strength by having a microelectrode array a few millimeters apart inside a channel. By synchronizing the applied voltage with the moving sample bands in the channel, high field strengths are obtained at relatively low voltages and thereby achieving faster separations [410]. There were, however, some practical problems with compatibility between buffer composition and electrode materials, not to mention electrolytic effects [73].

CE chips have been made from the micromachining of glass substrates [30,37,405]. A CE chip using a glass/quartz substrate is commercially available (see Agilent and Caliper in Table 3). The use of cross-channels and double T injectors allowed the electrophoretic analysis of well-defined sample quantities on microchips [411]. Depending on the mode of operation (injection or separation), the electric fields are applied across a channel but to a lesser extent, at the orthogonal channel or reservoirs to prevent sample bleeding,

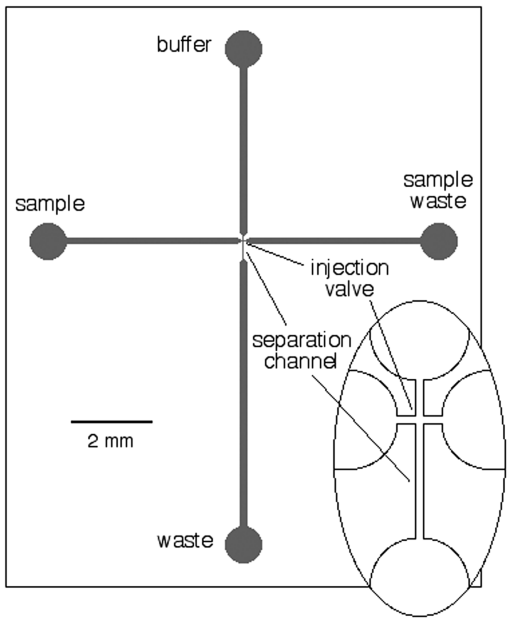

Fig. 17. Schematic of microchip used for high-speed electrophoretic separations. (Inset) Enlargement of the injection valve and separation channel. Reprinted with permission from Ref. [30]. Copyright 1998 American Chemical Society.

a procedure termed ''pinching'' [30,80,412]. (See Fig. 17 for the reservoir configuration.)

The first miniaturized system for capillary electrophoresis (CE) on PDMS was developed by Effenhauser *et al*. [10,105]. Their device sealed reversibly and hence could be re-used after cleaning. Without any surface modification, the channel walls were uncharged and hydrophobic, thus supporting only a weak EOF. A later development was a CE device also on PDMS but irreversibly sealed by plasma oxidation [413,414]. Since this process also modifies the surface, separation of amino acids and negatively charged proteins were achieved. But for positively charged proteins, the channels were coated with Polybrene®, a polymer containing quaternary amines, to make the charge ladder (successive acylation of lysines on a protein) positively charged. This reduced adsorption and reversed the EOF toward the anode. In several instances, the addition of zwitterions reduced adsorption of proteins and their derivatives [415].

Electrophoretic separations on chip is far advanced compared to on-chip chromatography. Difficulties arise from the use of stationary phases that in the macroworld has relied on pressure pumping. Because of the incompatibility of packed microchannels and EOF, most stationary phases that have been implemented in microfluidics use micellar phases [416–419] or are coated on

channels for an ''open column'' configuration [29,403,420,421]. Both methods proved to be cumbersome. New types of stationary phases are being developed and are compatible with chromatography using EOF, hence termed electrochromatography [73]. The criteria are that they pack or fill a column, they have compound selectivity, and they sustain EOF [73]. The type of stationary phase that fit this description are those that involve *in situ* polymerization. Usually, the polymerization is thermally induced as with polyacrylamide or methacrylates, styrene-divinylbenzene polymers, and those based on silicates, the sol-gel approaches [73]. But in one case, the polymerization was photo-induced and this is interesting in terms of being able to pattern microchannel manifolds [73,422]. With polymer-filled microchannels, the same resolution and van Deemter plots were obtained from separations done in fused silica capillaries and in microchips, of both low molecular weight (alkyl phenones, antidepressants) and high molecular weight analytes (proteins) [423]. In a different application, reagent-carrying beads were packed in a microchip for on-chip solid phase extraction; packing was done by applying both pressure and electrokinetic flow [424].

Integration

A key concept in integration and of μTAS is being able to put together different techniques and technologies so that the whole is more than the sum of its parts [73]. A high level of integration would accommodate different components that are required in a device from sample preparation to detection. The design of the chip must then consider how these components will be fabricated and how should the actual experiment be carried out (where to position these components and how will the sample be transported to and from different components). In general, this is more feasible when the components have as few moving parts as possible to avoid complicated chip fabrication and risk of breakdown during operation [10].

Miniaturization of sample preparation has several benefits such as reduced sample requirements, faster analysis times, and minimized exposure to contaminants [425]. There are a few examples where sample preparation has been integrated on chip. Sample derivatization and enrichment, with or without separation has been done [27,412,426–429], so with incorporation of a dialysis sampling unit [430] and interfacing to an FIA unit [431]. Though not often applicable, postcolumn derivatization has also been done on-chip after separation [432].

The incorporation of electrodes has always been part of microfluidic microfabrication [61,287]. The integration of electrodes for electrochemical detection has also been done [73]. A more critical aspect is the integration of optical components such as light sources, waveguides, optical filters, refractive elements, diffraction grating, and even light-sensitive diodes (see also Nanotechnology) [24,433–437]. Especially for highly sensitive fluorescence detection, the integration of micro-optical elements or, at least, the proper interfacing to optical detection systems is essential.

Interfacing to detection systems may not be straightforward mainly because different detection schemes have different requirements. Mass spectrometry (MS) is one detection system where there has been quite a lot of effort put into interfacing with chip devices. The impetus has been both from chromatographers and mass spectrometrists because of the advantages and capabilities in hyphenating the technique. Most of the interfacing has been done with electrospray ionization. (See Fig. 18 for two types of chip–MS interfaces.) In general, better and consistent electrospraying is achieved if capillary tubing is used after the separation channel [73,425,438,439] (see Fig. 19). Oleschuk and Harrison [425] have a review on microfluidic devices interfaced to mass spectrometry including a section on sample preparation. Reyes *et al*. [294] has a section in their review on interfacing chips to mass spectrometers. Although there have been attempts to miniaturize a mass spectrometer, doing so does not enhance sensitivity or improve the speed of analysis. It would be more convenient to improve on interfacing rather than reconstructing a new system [73,425].

Simulation

Simulation is an efficient way to design microfluidic devices from CAD software and to virtually test fluid and mass transport in both two- and three-dimensional

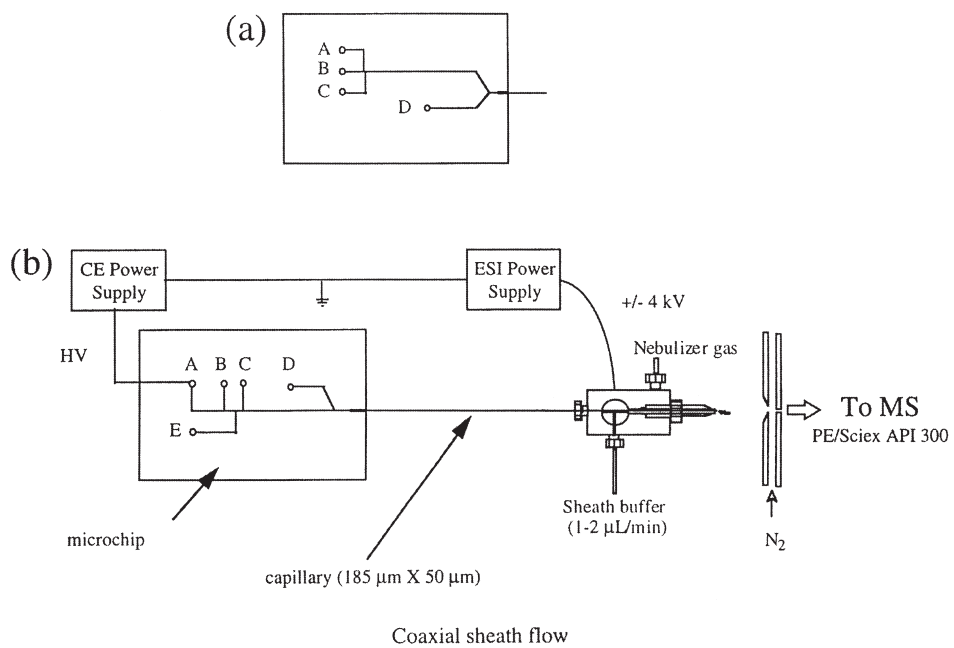

Fig. 18. Schematic drawings of two different chip-mass spectrometer interfaces: a disposable nanoelectrospray emitter (a) and a sheath flow electrospray interface (b). Reprinted with permission from Ref. [438]. Copyright 1999 American Chemical Society.

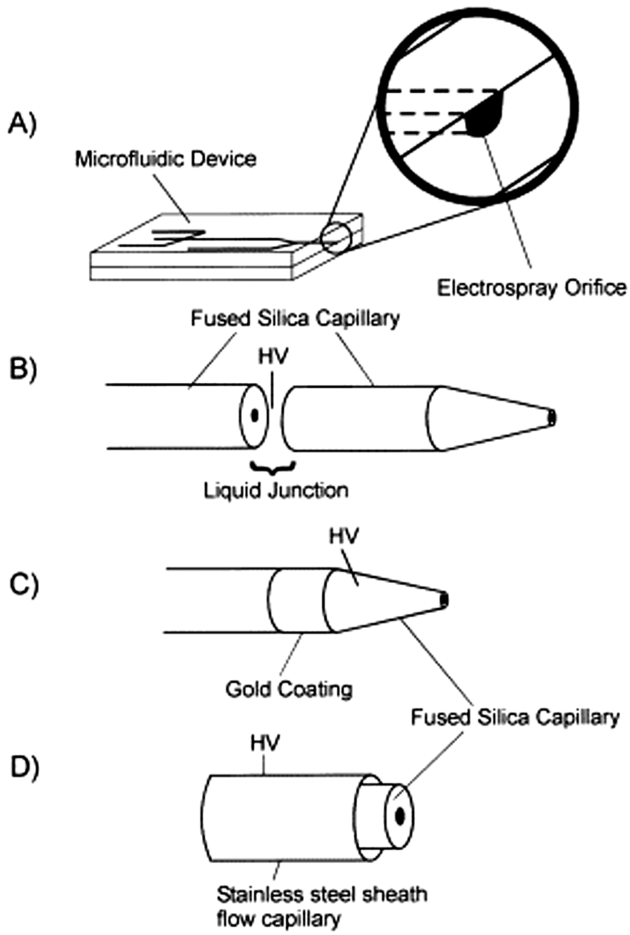

Fig. 19. Schematic diagram of different electrospray interfaces that have been developed for chip-ESI-MS: (A) spraying directly from an exposed channel at the edge of a chip; (B) liquid junction capillary interface; (C) gold-coated capillary interface; and (D) coaxial sheath flow configuration. HV denotes points to which the electrospray voltage is applied. Reprinted with permission from Ref. [425]. Copyright 2000 Elsevier Science Ltd.

flow systems. Computational fluid dynamics simulation can provide the information required from fluid mixing and transport to biomolecule separation and cell sorting [440]. Some specific methods include:

1. Galerkin fine-element method: a general, steady-state approach to simulate 2D and 3D fluid flow, thermal fields, and chemical concentrations in microfabricated devices [441].
2. FlumeCAD (Coventor, Inc.): a fine-element method for the general design and simulation of electrophoretic, electroosmotic, and pressure systems. It can be used to analyze electrokinetic injections [442] and to predict the electrophoretic motion in microchannels [443].

3. Multicomponent continuous media mechanics: describes electrokinetic motion in microchannel networks [444].

Ermakov *et al*. [445] developed a comprehensive computer program to simulate mass transport in any 2D channel structure, which considers electroosmosis and electrophoresis as well as diffusion. Besides fluid flow, their simulation of microfluidic systems also assisted in optimizing operational parameters for gated or pinched injection (cross-channel type) [446,447], where each technique has its own set of parameters and operational sequence. It has been demonstrated that T-structures are suited for sample mixing and cross channels, for electrokinetic focusing [448].

A microfluidic separation chamber for cell sorting was modeled on a three-step process [41]: (1) electrode geometry was optimized with the Ansoft Maxwell 2D-field simulator modeling the different electric fields in the device, (2) effective cell separation from various mixtures was modeled with MATLAB software based on dielectric properties [449], and (3) flow and thermal conditions were predicted using lattice-Boltzmann simulation [450]. Additional information regarding surface adsorption properties and surface interfacial forces can also be entered.

Microfluidic detection systems

For microfluidic systems, laser-induced fluorescence (LIF) in a confocal system is highly sensitive and is the most popular for microchip applications involving separation [41,451]. As channel dimensions are reduced, so too are detection volumes. The ultimate limit is single molecule detection, which can be done with LIF [73,105,452,453]. A detection cell with improved sensitivity can detect 6 μM fluorescein [24,75]. However, the use of LIF in a multichannel protein microchip remains a challenge [75]. Chemiluminescence detection limits, *e.g*., 7–35 nM HRP-F1 (fluorescein conjugate of horseradish peroxidase) [405], are 50–100 times better than on-chip absorption detection [24]. These limits, however, are higher than the mid-picomolar detection limits for on-chip LIF [25,454].

An alternative to detecting fluorescence is detecting changes in index of refraction. Two components manipulate the light beams: liquid-core waveguides and diffraction gratings [10]. Advantages of the method include universality (*i.e*., analytes do not need labeling), concentration-dependence and optical simplicity (*i.e*., less expensive than fluorescence) [433]. However, disadvantages include its temperature-dependence and poor detection limits [436].

Interfacing microfluidic devices to a mass spectrometer can be viewed in two ways: the MS as the detector or the chip as the sample introduction method. Depending on the type of instrument chosen, Mass Spectrometry gives high resolution, is sensitive enough for trace analysis, and can provide structural information and identify analytes. Since efficient means for interfacing the chips to electrospray ionization sources are in place (see Integration), the developments have been in increasing the sample handling capabilities of the chips [425].

Other detection schemes include electrochemical detection, Raman spectroscopy, and holographic refractive index detection [75].

Cell and tissue biochips

Cells on chips

Some applications involving cells on chips are not conveniently categorized into established formats. Patterned cells on a chip to study cellular behavior is a typical example. One that is clearly in an array format, that of single live cells, is being developed (see below). Several examples integrate different functions on the chip, and as it is necessary to transport material (sample and reagents), they fall under microfluidics. In some cases, cells on chips are used to sense microenvironmental changes, and hence are sensor chips. The different examples cited below exemplify a wide range of applications.

Cell patterning has been done by a number of researchers to study various cellular responses including the influence of cell shape and size on cell differentiation and function (see also Biochip Applications, Biological and medical research). Folch *et al*. [238,239] used deep (20–30 μm) channels on PDMS to transport material more slowly (for comparison, see Delamarche *et al*. [247,248]). As a base layer, collagen or fibronectin was patterned over different substrates before applying cells on them. A second cell type was applied to regions on the chip that did not require this protein base layer for adhesion [238]. Microfluidic channels were also used to pattern the cells directly onto the substrate such that two different cell types were patterned simultaneously [239]. Other examples of cell patterning have been described. Growth of bacterial cultures on a PDMS device has also been demonstrated [41].

The use of live cells and maintaining their viability is the crux of cell biochips. One type of cell array, single live cell arrays, is being developed by David Walt (Department of Chemistry, Tufts University) using optical imaging fiber (OIF) technology at a density of 10 million wells per cm^2. MH 3T3 mouse fibroblast cells are used in this sensor. With one cell per well, cell positions are encoded and physiological and genetic responses are simultaneously recorded (*e.g*., pH and O_2 measurements, promotor responses via fluorescence produced by the lacZ and phoA reporter genes, and protein–protein interactions using the yeast two-hybrid system) [455,456]. Assuming the cells remain viable and enough cells are monitored for statistical significance, the use of such a system for screening the physiological effects of a combinatorial drug library can be speculated [456].

With live cell microarrays, signal transduction pathways, protein–protein interactions, expression cloning, and cell localization for encoded proteins can be determined or performed. A current limitation is the availability of cDNAs for such experiments. One method that uses live cell microarrays for cDNA expression is the living protein chip for expression monitoring where proteins

were expressed in human embryonic kidney (HEK) cells to examine their effects on the cells themselves, *e.g.*, the induction of apoptosis [457]. The HEK293 cells are added to DNA–lipid complexes (reverse transfection) arrayed on a glass slide. These cultured cells can be made to express excess amounts of a protein whose cDNA has been introduced into these cells; the cDNAs need only to be spotted on to the array and the cells take them up. The microspots are 120–150 μm in diameter consisting of a cluster of 30–80 fluorescent cells. With these living arrays, the proteins do not need purification because their concentrations are so high relative to the HEK cell's endogenous proteins. The proteins expressed are 10–15% of the transfected plasmid [458]. These cells can even express membrane proteins like GPCRs. The expressed proteins can then be used for functional studies, *e.g.*, its interaction with small molecules. After the protein of interest is expressed within the cells, the array is removed from the culture and the cells are fixed. The cells are then visualized or subjected to other detection schemes [367,455].

In live cell microarrays, visualization is done via fluorescently labeled antibodies or by direct fluorescence microscopy with GFP-tagged proteins. Other methods of detection for cell microarrays include western blots and radiolabeled ligands [367].

The transport, cultivation, and manipulation of living cells have been performed on microfluidic devices [26,459]. One interesting device is a cell sorter. This integrated cell sorter incorporated several coordinated functions on a microfluidic device that included sorting, sample dispensing, flushing, recovery, and absorption of fluid perturbations [16]. Sorting required the use of peristaltic pumps, dampers, and switch valves. An actuated valve on this sorter can have an active volume as low as 1 pl while the volume allotted for optical detection is about 100 fl [16]. This pressure-driven device has a T-cross for sorting [16,275,292] as it was in a previous electrokinetic sorter [275]. Electrokinetic flow, however, presented some difficulties so this improved sorter with microvalves and micropumps comes even closer to μTAS. Using multilayer soft lithography for fabrication, this device has a top layer where all the valves and pumps are located and a bottom layer that has all the fluidic channels. For sorting, the channels start with a width of 30 μm which tapers down to 20 μm and finally to 6 μm at the sorting T. By detecting fluorescent signals, certain valves are opened or closed so that the cells are pushed into one or the other branches of the T-junction [292]. The literature presents more details on the valve dimensions, the use of dampers, and their locations [16]. The fabrication of switching valves and peristaltic pumps for sample dispensing and switching [292] and rotary pumps for mixing and incubation [460,461] have been described previously.

Another cell sorter was used to separate two types of white blood cells, T-lymphocytes and granulocytes. This device was also fabricated in PDMS, since glass and silicon dioxide proved to be too adhesive to effectively carry out the experiments with these cells. This chip had a network of small channels

with different lengths mimicking capillaries *in vivo* where cells are subjected to size restrictions. As predicted, the cells' passage through the microchannel network depended on their size and nuclear morphology [462].

For the separation of particles and cells in microfluidic systems, besides the components and methods described above which concentrated mainly on fluid flow and control, *i.e.*, pumps and valves, some additional components have been cited in the literature. For incubation purposes, spatially fixed temperature zones are situated along various sections of the microchannel [20]. For dispensing reagents to use in enzymatic reactions and dilution, multiple ports and plugs can be incorporated [26,463].

Another type of biochip involving live cells was a sensor chip used in multiparametric cellular monitoring for drug screening [47] (see also Biochip Applications, Drug Screening). Functional studies usually comprise measuring the effects of drugs on intracellular Ca^{+} concentration, pH, and membrane potential in cells using fluorescent dyes. Hence, different microelectronic sensors were used: pH-ISFET transducers and pH glass electrodes to measure extracellular acidification, a Clark-type oxygen electrode to measure cellular oxygen uptake, interdigitized electrode structures (IDES) to detect changes in cell adhesion and morphology, and temperature sensors. Furthermore, maintaining a physiological environment involved thermostated chambers and a fluid exchange system. Two test versions were made; one on glass and the other on silicon. Details on their operation were presented [47].

Tissue microarrays

Tissue microarrays (TMAs) were developed for molecular pathological studies on a genomic scale [464,537]. Molecular alterations in thousands of tissue specimens can be analyzed in parallel. They can be complementary to DNA arrays because they would help in validating and understanding the physiological relevance of the gene changes taken from DNA array data. However, TMAs can also be used to test the toxicity of drug leads by evaluating the drug target distribution in normal tissue, hence, accelerating the drug discovery process in being able to predict clinical failure [456].

Tissue microarrays (TMAs) have been applied in the high-throughput molecular profiling of tumor specimens [465,537]. A robot was designed to punch out cylinders (0.6 mm wide, 3–4 mm high) from 1000 individual tumor biopsies embedded in paraffin and to array them in a 45×20 mm paraffin block. The robot is now commercially available (see Beecher Instruments, Table 4). The tumor sections are analyzed in parallel by immunohistochemistry and *in situ* hybridization [1,465]. An important advantage is that archival tissue (paraffin blocks dated 20–40 years that are fixed in 4% buffered formalin) can be used since it may be difficult to gather fresh samples in a high-throughput setting [464]. Most of the profiling done has been in cancer; the TMA studies done on various cancers including tissue types, sample size, methods used, molecular

targets, and the corresponding references are outlined in Kallioniemi *et al*. [464]. The types of studies performed include the frequency of molecular alterations in large tumors (as part of a genomic scale study in molecular pathology), tumor progression, identification of prognostic markers, and validation of novel genes as diagnostic and therapeutic targets [464,466]. The information on the cellular origin of the molecular targets is also inherently available. TMAs are meant to be research tools to study population-related trends and not to diagnose individual cases. Different types of TMAs are available for cancer research such as multi-tumor arrays (containing different tumors), tumor progression arrays (tumors at different stages), and prognostic arrays (tumors with clinical endpoints) [467].

Molecular profiling of tumor samples can also be done using the technique referred to as layered expression scanning [468]. Cells or tissue samples are made to pass through a layered array of capture membranes (nitrocellulose or agarose gel layers) where each layer is linked to a specific antibody or cDNA sequence. Target proteins are then confined to specific layers so that a molecular profile for each cell type is generated.

Biochip applications

Drug discovery and drug development

Proteomics and drug discovery tools

The term proteomics can mean any of several different strategies aimed at unraveling the human proteome. The proteomics field can be categorized so as to provide a means of better understanding the complex field which leads to several approaches at solving the problem, each complementary to the others [469,470]. Profiling and cataloging proteins in normal and diseased tissue constitute expression proteomics, an area where protein arrays have been applied. Another important part of current proteomics research is in cell-map or interaction proteomics involving the investigation of molecular interactions and the determination of proteins that are involved in signaling pathways. All this is to be able to gain insight into biological processes, which is an end in and of its own. However, pharmaceutical research aims to identify or validate protein function in order to discover novel diagnostic and therapeutic drug targets, the realm of functional proteomics. To them, protein arrays help to identify disease-related proteins, or better still, determine their function. Last but not least, protein arrays can also help identify novel, therapeutic compounds. Hence, in achieving any or all of the above, biochips are becoming an indispensable tool in proteomics and drug discovery.

Separations. One of the first demonstrations of the usefulness of doing electrophoresis on microchips was in 1990 [15]. With cross-channels and double

T injector setups, well-defined sample quantities could be separated by on-chip electrophoresis [411]. An electrophoretic separation completed in less than a millisecond has been carried out [30]. Such a separation may be useful for ultrahigh throughput drug discovery, in monitoring millisecond timescale kinetics, or as a final cleanup or separation step, *e.g.*, in multidimensional separations. In a technique called immunoaffinity capillary electrophoresis, antibodies were immobilized directly onto fused-silica capillaries [80,471,472].

A commercially sold lab-on-a-chip can perform electrophoresis on 10 protein samples (cell lysates, column fractions, or purified proteins) in less than 30 min. The chip is put into a ''reader'' with software that is linked to allow the separations to be viewed in real time. After the analysis is done and data is stored, the chip can be disposed of without any toxic waste chemicals to worry about [11,473] (see also Agilent in Table 3). On a per chip basis, it is less costly than standard SDS-PAGE and has been used for recombinant protein expression analysis, optimization of protein purification procedures and for QA/QC of protein-based drugs [11].

An integrated microfluidic system that incorporates protein digestion and peptide separation for protein identification has also been developed [474]. The chip contains a trypsin membrane reactor for rapid protein digestion. The resulting peptides are concentrated and separated by capillary isotachophoresis (CITP)/capillary zone electrophoresis (CZE) prior to MS analysis. Cohen *et al.* [475] used on-chip electrophoretic separation to assess the result of an enzyme assay for kinase A. On-chip dilution of the peptide substrate was also performed to determine kinetic constants. A reverse-phase chromatographic separation of peptides and amino acids on a microchip ''outperformed capillary-based separations in both speed and efficiency''; six peptides were separated in 45 s with up to 600,000 plates/m [476]. For other examples of on-chip separations, see Table 2. The separation of cells is covered under Bioparticle manipulation and sorting (see below).

Bioparticle manipulation and sorting. Bioparticle separation and manipulation is based on the principle of dielectrophoresis. It has been used in biotechnological applications including the purification, fractionation, and concentration of cells and microorganisms. The size limit of the method has been shown to be below 1 μm with the trapping and separation of latex beads and tobacco mosaic virus (TMV) [298]. Strategies from a binary separation to multicomponent fractionation in microfluidic devices are discussed by Hughes [296]. As a means of separation, negative dielectrophoresis is implemented via electrode arrays in the microchannel and are driven with high-frequency AC. Laminar flow carries the particles past the electrodes while the AC strength determines particle trajectory. A particle sorter has been described to concentrate the sample down to a narrow stream, break up aggregates, trap and finally sort [299]. Flow velocities are at approximately 10 mm/s. When combined with sensitive optical detection, the technique may be applied to perform miniaturized flow cytometry [299].

Flow cytometry. Flow cytometry of *E. coli* has been carried out in a microfluidic device. Labeling efficiencies for the dyes and the antibody were greater than 94% [477]. Such an application is promising for drug discovery. (See also Bioparticle manipulation and sorting, above, and Biological and medical research, Cell manipulation and sorting.)

Mass spectrometry. Besides being a mode of detection, mass spectrometry in itself has been an indispensable tool in proteomics, especially in protein identification through partial sequencing and peptide fingerprinting as well as in quality control of reagents and in drug screening. At low array density, SELDI mass spectrometry and SPR-BIA, although they operate on very different principles, can monitor intermolecular interactions and can be linked to some means of identifying the interacting partners [44,45,54,55,344,478,479]. SELDI has also been used quite extensively for profiling and biomarker discovery. (See also Clinical Applications, Biomarkers.) Whole tissue extracts or microdissected material can be analyzed [56] (see also Table 2).

In a high-throughput CE/MS analysis, automated sampling from a 96-well microtiter plate held vertically in front of an electrospray mass spectrometer, was followed by CE separation through a microfabricated device fitted for electrokinetic and pressure-induced fluid flow [480]. Other high-throughput options include electrospray nozzles on a microchip [481,482] (See Advion, Table 4) as well as a multichannel device for high-throughput electrospray infusion equipped with individual electrospray exit ports via a microchannel/electrode system [483]. MS can also be used for screening applications as well as for quality control of small molecules, reagents, and proteins [390].

Expression profiling. Proteomic profiling involves measuring and recording the expression patterns of proteins in a given tissue, cell, or subcellular component. Comparing expression patterns of normal versus diseased states, provides some insight on possible disease-related proteins, as diagnostic markers or as drug targets. Confirmation of disease-relatedness, however, needs to be validated [470] (see also Identification of protein function). In the case of cancer, the aim of proteomic profiling is to understand how protein expression patterns influence the tissue microenvironment as well as the initiation and progression of tumor growth. Because most of the proteins identified in connection with oral cavity cancer, both in normal cells and tumor cells, are involved in signal transduction pathways, complex cellular signaling must play a key role in cancer progression [484]. Such a hypothesis may be answered with chip technology.

The combination of 2D-electrophoresis and mass spectrometry can study only a limited number of proteins. The use of protein profiling arrays can complement or replace 2D-gel electrophoresis and would have a significant impact on proteomics [344]. It can be a general resource for expression or interaction (cell map) proteomics, *i.e.*, high-throughput screening of gene expression and receptor–ligand interactions [1,485,486]. In a cytokine profiling chip, Fab antibody fragments are deposited on array posts and oriented for increased

detector sensitivity. Lower limits of detection achieved so far is 0.1 pM and the quantitation limit is 0.5 pM. Apart from performing multianalyte cytokine profiling, these chips can be used for the identification of possible drug targets or diagnostic markers using expression pattern data [59] (see Zyomyx, Table 3).

1. cDNA arrays. Initial screening of gene expression profiles via cDNA arrays is rapid but other methods are required to validate whether differences in hybridization signals actually correlate to physiologically relevant differences in gene expression [487]. From running DNA arrays to profiling proteins encoded by differentially expressed cDNA clones requires a high-throughput approach for parallel protein expression analysis. (See Fig. 20 on how cDNA libraries on chips are processed.) A large number of cDNA clones have to be expressed simultaneously with the appropriate vector system [1,128]. Gene expression data from DNA arrays and/or protein expression profiling data from 2D electrophoresis and MS can be correlated with data from protein chips [488,489]. DNA sequence information and protein expression levels can also be linked. Expressing cDNAs of interest can also be done via live cell microarrays [367,457] (see also Principles of Design and Operation, Cell and tissue biochips).

2. Enzyme-linked immunosorbent assay (ELISA). In principle, expression profiling can also be done using high-throughput enzyme-linked immunosorbent assay (ELISA) [74] (see also sandwich assays in Principles of Design and Operation; Arrays: capture agents, molecular tags, detection, Array detection systems; Detection with labels). The profiling of cytokine levels in biological samples with high specificity and sensitivity has been done by microarray

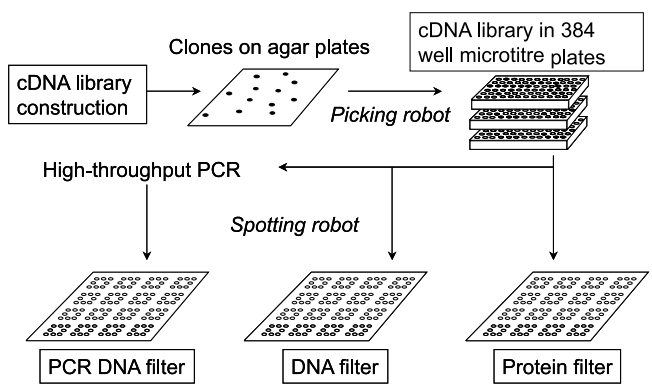

Fig. 20. High-density arrays of cDNA libraries. Using a picking/spotting robot, cDNA clones are picked from agar plates into 384-well microtiter plates, and identical patterns are spotted at high density onto nylon or polyvinylidene difluoride filter membranes that are subsequently processed for DNA hybridization (DNA filters) or protein detection (Protein filters), respectively. For microarrays, cDNA inserts are first amplified by high-throughput PCR, or proteins are expressed in microtiter plates before spotting. Reprinted with permission from Ref. [1]. Copyright 2000 Elsevier Science Ltd.

sandwich immunoassays [490]. To avoid cross-reactivity, the antibodies have to be screened against all antigens on the array. (See also Table 2.)

3. Tissue microarrays (TMAs). In cancer, molecular profiling of tumor samples with TMAs generated data that correlated with tumor progression and frequency of molecular alterations in tumors, as well as the identification of prognostic and diagnostic markers, and drug targets [464–468]. At the National Human Genome Research Institute, a multi-tumor array (~5000 specimens, and sections from 36 normal and 800 metastatic tissues) and a normal tissue array (76 tissue and 332 cell types) have been built [465]. Kallioniemi *et al.* [464] outlines the different studies that have been done with TMAs on a variety of cancers. With the technique called layered expression scanning, a type of tissue array, it is possible to produce a molecular profile for each cell type present in the tissue [468].

Molecular screening

1. Specificity screening. An offshoot of molecular profiling is antibody specificity screening against complex mixtures of proteins. Since antibodies may cross-react with unrelated proteins, protein arrays would help in determining antibody specificity. However, determination of cross-reactivity using protein chips is limited by the number and type of proteins arrayed on the chip. High-throughput specificity screening was done with 92 expression clones from the human fetal brain cDNA expression library, hEx1, using selected monoclonal antibodies and scFv fragments from phage display [485]. This was done as a quality control step to check for cross-reactivity as well as to detect common epitopes. This reverse antibody array would be particularly important for reagents used in immunohistochemistry and physiological studies on whole cells or tissues. Walter *et al.* [1] described the first use of a high-density protein filter. They screened the hEx1 cDNA library array with monoclonal antibodies against mouse GAPDH (glycerol aldehyde phosphate dehydrogenase), the heat shock protein 90-alpha (HSP90α), and tubulin-alpha. Holt *et al.* [491] screened protein array filters of hEx1 with antibody fragments to generate specific antibodies without the need to immunize or resort to display methods.

2. Screening for modification, expression, activity. Biochips can be used for the simultaneous determination of chemical modifications of many proteins on a given chip [59,128]. For example, molecular screening with antibody arrays can be used for detecting posttranslational modifications or smaller molecules, such as peptide hormones [492,493] and carbohydrates [494,495]. Furthermore, biochips can be used to monitor the expression of one or more proteins in a mixture such as a cell extract just as 2D gel electrophoresis has been widely used to profile protein expression and detect protein modifications [340].

Profiling biochemical activities in parallel for a large number of proteins would be a task that biochips should fulfill [59]. In a microarray format, the proteins should be properly immobilized on the chip surface and should maintain their

activity (not be denatured) [496]. Quantitating inhibitor–enzyme interactions [137] and screening a yeast proteomic array for phosphoinositide-binding activity [128] have been done. Similar studies that have analyzed protein biochemical activity in microwell plates [136,497] have the potential to be carried out in miniaturized chip or nanowell format.

3. Protein availability for screening. More often than not, the amount of protein isolated from 2D gels is not sufficient for screening, much less for monoclonal antibody production [344,498–500]. Even with advances in antibody production by phage display [501–506], significant amounts of protein are still required. De Wildt *et al.* [340] compared mass array screening with several rounds of phage display in screening antigen–antibody interactions. Some antibodies were selected with only 0.005% or 0.0005% recombinant protein in the lysate. Furthermore, parallel screening should be able to identify antibodies that recognize differentially expressed proteins [340]. Nevertheless, an important consideration is an adequate supply or source of proteins. With this comes the improvement of protein expression systems. A dual expression system in *E. coli* and *Pichia pastoris* has been used to produce soluble proteins in the native state [507]. Soluble expressed proteins find uses in antibody generation, crystallization, NMR studies, therapeutics, and *in vitro* interaction studies. A parallel, miniaturized expression of recombinant proteins in *E. coli* and in the baculovirus system has been developed [488,508].

An arrayed expression library can be a source of recombinant proteins [390]. The Resource Center of the German Human Genome Project (RZPD, http://rzpd.de) has been an important resource in this regard. For example, the human fetal brain cDNA expression library hEx1 (RZPD library no. 800) was used in experiments by Leuking *et al.* [1,485]. Although the availability of full-length human cDNAs is limited, there are on-going projects that address this [457,509–512].

4. SPOT synthesis peptide arrays. Using peptide arrays, the SPOT synthesis method can detect low-affinity antibody interactions or receptor contact sites [376]. This is due to reduced nonspecific binding and the increased binding affinity because of high local peptide density on the cellulose substrate [377,378]. Although not sufficiently miniaturized, SPOT synthesis involves straightforward and reliable experimental protocols, inexpensive equipment, a highly parallel process, and adaptability to different assays and screening methods. These peptide arrays prepared by the SPOT technique have been used in molecular recognition studies in molecular immunology and can be used in *in situ* screening of chemical libraries [513].

Molecular interactions and signaling pathways. Studies on molecular interactions include peptide–protein, protein–protein, and protein–drug interactions and can also extend to epitope mapping [348]. Early research in this area involved the use of a low-density universal protein array (UPA) system to screen the interactions

of p52 against 48 purified proteins spotted on nitrocellulose [514]. This system can also be used to study protein interactions with DNA, RNA, and other ligands as well as small chemical entities. Hence, it can be used to screen drug targets. MacBeath and Schreiber [118] studied the limit of their system (FKBP12-rapamycin-binding domain) and found specific binding was detected using concentrations as low as 150 pg/ml (approximately 12.5 pM). This implies being able to detect such interactions in cell lysates when using fluorescently labeled proteins. Their microarray also works in detecting transient signals, *e.g.*, enzyme–substrate interactions as with kinases. Zhu *et al.* [128] studied interacting proteins using a yeast proteome microarray. Since it was possible to observe almost the entire yeast proteome on one array, this strategy helps to map out pathways of protein–protein interactions, which is currently done by two-hybrid assays. One difference is that because much less material is needed on the chip (10–950 fg per protein) [9,128], the method is 10–100 times faster [79].

The study of multiprotein complexes is an extension of interaction studies. It is important for understanding cellular processes [348] and a basis for studying signal transduction. Signaling involves posttranslational modifications, *e.g.*, phosphorylations, which cannot be accessed by DNA methods. Since protein complexes are formed in stimulated cells, they cannot be studied in *in vitro* cell-free systems. Hence, live cell microarrays can be used to study signal tranduction. Signaling can be induced by a number of stimuli and assays are available in different formats. Activation is detected in microarrays expressing hundreds of cDNAs (*e.g.*, phosphorylation of a downstream kinase) or with antibodies specifically recognizing the relevant protein form [367]. A kinase chip specifically for evaluating the function of protein kinases in cell signaling is discussed below (see Identification of protein function). There have been studies done, albeit not chip applications, on protein interactions and epitope mapping with synthetic peptides [377,378,515]. Geysen *et al.* [515] used a peptide array to identify an immunogenic epitope preset on the VP1 coat protein of the foot-and-mouth-disease virus. Such studies can be done today more efficiently in microarrays.

Identification of protein function. The goal of molecular profiling is the identification of disease-related proteins that may be novel drug targets and prognostic and diagnostic markers. One possible strategy is to have organ and disease-specific protein arrays [348]. However, a disease-associated protein does not necessarily imply an appropriate drug target or biomarker. Molecular profiling catalogs the up- and down-regulation of proteins, the functional relevance however, for any one of these over- or under-expressed proteins should be determined by another means [470]. The probability of functional relevance increases if this protein's participation in a signaling cascade is established. The definitive criterion for function should then be the causative role of the protein in a given disease or physiological state.

Many types of functional studies can be carried out on protein arrays. Live cell arrays are appropriate for large-scale characterization of protein function [367]. Information on protein function can be derived from the analysis of biochemical activities [118,128,136,496,497,516]. Kinase chips are specifically used to determine function in the context of cell signaling, to evaluate substrate selectivity, to identify physiologically relevant enzyme–substrate pairs, and to discover novel inhibitors [128]. MacBeath and Schreiber [118] described their preliminary results in developing microarrays to study function in human proteins. Their major efforts involved immobilizing functionally active, folded proteins and detecting the interactions. They plan to assign function at a broader level, running miniaturized assays in parallel. Whether it is necessary to express and purify thousands of proteins or cellular systems or cell lysates sufficient to use to carry out functional arrays remains to be seen.

Identification of novel therapeutic compounds. The use of protein arrays to identify and validate novel therapeutics necessitates the development of assays. For example, identifying relevant enzyme–substrate pairs is a means to discovering new kinase inhibitors with the use of protein chips [349]. Some commercial entities intend to sell arrays containing whole protein families for use in drug lead optimization [260]. Before actual optimization studies begin, the viability of a compound as a drug lead is determined from its binding kinetics to a specific protein drug target. SPR-BIA technology can be used for this purpose. A commercial collaboration intends to carry out SPR-BIA technology in an array format for the next step, characterizing lead compounds via ADMET (Adsorption, Distribution, Metabolism, Excretion and Toxicity) assays [383].

Drug screening and testing

Protein chips can screen potential lead compounds at high throughput and can be used for toxicological testing as well. Arrays are well suited for this application when there is only a limited supply of cells or amount of drugs available for testing [47], especially in the high-throughput screening of lead compounds. Microfluidics has been used to screen 750,000 compounds from a drug library against a protein target, consuming only 750 ng of the protein (see also Caliper in Table 3). Although most protein arrays are based on soluble proteins, progress has been made in arraying of functional membrane proteins, which represent the majority of drug targets [153].

In experiments designed to detect protein–lipid interactions, proteins that were bound to low molecular weight compounds were identified. With specificity toward other proteins, lipids, and even small molecules, these functional protein arrays will enable the direct screening of entire proteomes for protein–drug interactions [79,128]. They can aid in determining the selectivity and specificity of drug leads in further downstream testing. For example, genetic variants of HIV reverse transcriptase and protease enzymes can be screened for efficacy against drug-resistant enzymes. In a clinical setting, pharmacoproteomic screening of

known population genetic variants of HMG-CoA reductase should help define patient types so that the most efficacious statin can be prescribed to a particular patient [59].

Increased demand for high-throughput cell-based formats to screen for drug leads will require engineered cell lines with diverse yet well-controlled characteristics for more critical applications. To identify and validate lead compounds, *in vitro* cellular screening is necessary before further animal testing. Furthermore, it may be helpful in cellular pharmacokinetics, *i.e.*, analyzing drug action from the onset to reversibility of effects [47]. There is also demand for using primary tissue arrays from patients as a better predictor of disease. Arrays of functional cells or tissues, ideally primary diseased tissue, can provide better understanding of disease physiology and may serve as surrogate for the disease eventually replacing the use of animal disease models. Considering the argument of pharmacogenomics, if drug response is influenced by genetic factors, disease-specific protein chips or primary tissue arrays from patients can be used to determine whether individuals will respond favorably or adversely to a given drug. Some may not even show a response due to a defect in the drug target. Such information is relevant and should be exploited for the development of new therapeutics, as well as the interpretation of clinical trial results and even treatment [348].

Cell biosensors. Multiparametric cell monitoring with the Cell-Monitoring-System CMS® [48,49], can detect relative changes in cellular behavior *in situ*. Different sensors on the chip monitored different parameters (see also Cells on chips). Cellular oxygen exchanges would indicate mitochondrial and photosynthetic activity, changes in cell adhesion or morphology would correspond to physiological responses, and extracellular acidification rates were monitored to determine metabolic activity. Fast pH changes (minutes) would correspond to cellular activation events such as receptor-mediated signaling; slow changes (hours or longer) meant cell growth or proliferation or cell death, respectively. The signals from the different sensors were correlated to give a proper interpretation of the cellular response to a particular drug. Cell-based assays would be simpler since metabolic activity does not play a role but nevertheless, there is the restriction that only adherently growing cells can be used [47]. After the successful testing of this sensor chip, an array format is a logical extension for high-throughput screening. The major hurdles would be in managing a more complex fluidic system and having appropriate data acquisition and processing [47].

Screening tissue arrays. Tissue microarrays can be used to predict the probable toxicity of lead compounds by evaluating the distribution of drug targets in normal tissue [465].

Monitoring drug metabolism and toxicity. Protein biochips may be utilized in monitoring drug metabolism and toxicity especially during preclinical

pharmacokinetic studies, to supplement *in vitro* and *in vivo* model systems. Furthermore, biochips used in drug metabolism and toxicology studies could be tools to optimally design clinical trials and exclude individuals with potentially deleterious drug-metabolizing enzyme (DME) profiles [59]. Xenobiotics or their metabolites can tightly bind to their corresponding DMEs and inhibit enzymatic activity. This is the major cause of adverse drug–drug interactions observed in patients on an incompatible drug regimen [59]. DMEs can be classified according to phases of metabolism:

1. Biotransformation: includes oxygenases, *e.g.*, cytochrome P450s and flavin monooxygenases (FMOs), that catalyze the hydroxylation and demethylation of hydrophobic xenobiotics.
2. Bioconjugation: includes transferases that take biotransformed intermediates as substrates and conjugate polar adducts, to make xenobiotics more hydrophilic enabling renal excretion.

Potential toxicity can be determined from information on xenobiotic–DME interaction. For example, xenobiotic toxicity involves a measurable increase in DME expression levels, particularly the P450s. Since many of the preclinical pharmacokinetic and pharmacodynamic analyses are performed in rats, protein biochips containing the human-equivalent P450s should lead to more medically relevant results [59] and may eventually circumvent the use of animals for toxicology studies.

Drug metabolism biochips would have two assay components: (1) DME expression profiling and (2) activity profiling of DMEs in the presence of selected xenobiotics. An ELISA-type analysis can be used for profiling the DME, which can be derived from cell or tissue extracts.

Protein microchips may therefore have a significant impact on the development of safer drugs through the comprehensive profiling of drugs or leads for effects on DMEs. However, challenges remain especially toward the expression of membrane-bound DMEs, which have complicated folding pathways due to the presence of the prosthetic heme group [59].

Drug delivery. Drug efficacy is affected by the means in which drugs are delivered in the human system. Silicon microchips enable drug release rates to be varied over time. The chips can store and release multiple chemicals on demand. In the future, integration of microprocessor control or biosensors may enable smart implants or tablets for controlled drug delivery [517,518] (see also Table 2).

Clinical applications

Immunoassays

To reiterate the principle in carrying out assays in microarray format, as one goes down with the amount of binder on the chip, the detected signal is a function of the concentration of target or analyte molecules in solution and is

independent of the solution or sample volume and the amount of binder in the microspot [4,326]. This is because the fraction of analyte captured reflects the solution concentration [121]. There is a limit of course to the amount of binder as statistics starts to be a problem if the number goes too low or as detection systems are challenged in sensitivity. Microarrays that operate in this realm, termed ambient analyte assay limits, yield faster results and obtain greater sensitivity due to optimal signal to noise ratios [4,121]. These conditions are represented in spot sizes less than 100 μm^2 containing 10^6–10^8 binders [121]. On the other hand, mass-sensing arrays can yield overall stronger signals because practically all the analyte molecules in solution are captured. However, incubation times can take 1–3 h. In this case, spots are about 200 μm in diameter with 10^{10} binders [8,121].

The most common use of microarrays is in immunoassays [9]. In particular, antibody-based immunoassays are the most common type of diagnostic assay because of antibody specificity; technologies that involve the use of these molecules develop at a rapid pace [519]. The assay is usually run in a multiplexed mode where the antibodies or other capture agents are immobilized and then exposed to a biological sample [9]. There are four immunoassay formats: direct-binding, sandwich (ELISA), competitive, and displacement [520]. Direct-binding and sandwich assays are the most common. There are some reports on the use of competitive assays and displacement assays, which are usually associated with high surface area/volume systems.

Direct-binding immunoassays. Immunoassays can be homogeneous or heterogeneous. Homogeneous assays refer to a binding reaction where both components are in the same phase, *i.e.*, in solution, while heterogeneous assays have one component in solution and the other fixed to a solid support, analogous to the use of capture agents in a microarray.

In homogeneous assays, it is not always easy to detect a binding event. A solution to this is to do sample separation on chip, *e.g.*, capillary electrophoresis. However, with such a format, only a limited number of assays are available [80]. Furthermore, a problem with precipitation is often observed when the antigens are large proteins and more so when the antibodies are polyclonal. This implies that the homogeneous format is more applicable for small antigens or haptens, such as drugs and some hormones [405].

In heterogeneous assays, one of the binding partners, usually the antibody, is attached to the chip surface. A key advantage of solid-phase immunoassays is that dilute solutions can be concentrated on the chip surface, making the binding event more easily detectable.

Mangru and Harrison [405] have performed an IgG immunoassay using chemiluminescence detection in a postseparation reactor capillary electrophoresis chip. In another case, an optical immunoassay microfluidic chip was developed to analyze human chronic gonadotrophin (hCG) in human serum [71] and in undiluted, whole blood [72]. Sensitivities were in the nanomolar

range: 0.1 ng/ml (human serum) and 0.5–5 ng/ml (whole blood), whereas the clinically relevant range is 0.3–1.5 ng/ml [72]. With rather high detection limits at 200–300 picograms, their conclusions were that better control of NSB effects is expected with improved surface blocking and this should in turn lower the detection limits. A microchip electrophoretic immunoassay analysis on serum cortisol was done in 30 s and within range of clinical interest (1–60 μg/dl); no extraction or sample preparation was done [454].

A fluorescence-based immunosensor was developed for simultaneous multi-analyte analysis [119,521]. Recognition elements on the chip capture sample analytes and the detection system involves a small diode laser, CCD camera, and image analysis software to correlate the positions of fluorescent signal and analyte identity. Although only spiked samples were tested, physiologically relevant concentrations of staphylococcal enterotoxin B (SEB) (a common cause of food poisoning), F1 antigen from *Yersinia pestis* (the etiologic agent of plague), and D-dimer (a marker for sepsis and thrombosis) were detected. Rapid detection of all three was performed by sandwich fluoroimmunoassays performed on a planar waveguide [119]. For other immunoassay applications, see also Table 2.

For point-of-care diagnostics, biochips are promising, especially since immunosensors for clinical diagnostics would require one-time-use, disposable chips [172] (Liu *et al.*, p. 760). In a microtiter well plate where molecules have to travel a distance in the order of millimeters, incubation times take several hours because it will take most analytes that long to diffuse throughout the entire well volume. Therefore, a much-reduced size is necessary and would be compatible with a chip format. Another complicating factor is the fact that automation equipment (*e.g.*, fluid handling/dispensing systems or robots) is bulky [80] (Dodge p. 3400–3401). The solution lies in microfluidics [62,522]. Most diagnostic assays have reagents bound to a solid support as in ELISA. When probing for multiple targets, the assay sensitivity becomes limited by the distribution of sample over a bigger area and by how much analyte can be pulled down from solution. Fluidic flow that transports material to different possible binding sites is a more efficient means of performing the assay and increasing detector signals, rather than passive diffusion. Complete recycling of sample can be achieved by active pumping, allowing optimal sensitivity [62]. Since assays are done in a flowing system, *i.e.*, nonequilibrium conditions, analysis times can be short, *e.g.*, less than 5 min [80]. This is a major factor in considering a microfluidic platform for automating heterogeneous immunoassays. In simulating this time advantage with biotin pixels and neutravidin-coated beads, passive diffusion for 4 h resulted in only a fraction of bound pixels whereas active pumping for 4 min resulted in greater than 80% of the beads binding to the pixels [62].

A low-cost diagnostic chip could be one based on dielectrophoresis technology. This technique is gentler on cells than DC techniques and can separate particles including various cells and bacteria [12]. It is capable of single

particle detection but at least 10 are preferred for better statistics. Organisms can also be tagged with antibodies coated on polystyrene beads. Current prototypes are on glass prepared by laser ablation and photolithography; commercial versions are expected to be on plastic [12].

Competitive assays. Competitive assays, by definition, involve measuring the labeled antibodies that are unbound [4,523]. (It is distinguished from non-competitive or direct-binding assays where only occupied binding sites are measured. Direct-binding, however, represents a better strategy because it generally gives higher sensitivity [4].) Microfluidic channel layouts and the operational sequence for a two-step competitive assay are illustrated in Dodge *et al*. [80]. Chromatography-type competitive assays in both single and sequential addition modes are possible [80]. The competitive immunoassay of the drug theophylline in human serum involved an immunoreactor with subsequent capillary electrophoretic separation on the chip and fluorescence detection [25,525].

Sandwich assays. Parallel detection of 25 cytokines was performed in a simple, low-density filter array in a sandwich format where detection limits were at physiological concentrations [526]. In contrast, sandwich ELISAs performed on high-density microarrays have also been done [74,527,528]. They can be used for detection of infectious agents or in cancer diagnostics [74,490]. Examples of other sandwich assays on microarrays include the simultaneous detection of multiple IgG subclasses [8], antigen–antibody reactions on a polyacrylamide gel substrate with immobilized capture molecules [137], and a fluorescence-based immunosensor (as mentioned above) [119] (see also Table 2).

Enzymatic assays. Apart from immunoassays, enzyme assays can also be used to screen for certain substrates in a clinical diagnostic setting. The benefits of performing enzymatic assays on microchips are the analytical power and minimal reagent use in microfluidic systems combined with the selectivity and amplification factors that come with biocatalysis [529]. Hence, for clinical testing of substrates, on-chip enzymatic assays have advantages over conventional systems in terms of speed and performance, not to mention sample volume since reagents and the enzymes themselves can be costly. At nanoliter volumes, these chips reduce their enzyme consumption by about four orders of magnitude over standard assays. Enzymes can convert 10^2–10^5 substrate molecules to product within 1 s and thus has been used in FIA [529,530] and CE. An enzymatic assay procedurally involves a derivatization reaction where a nondetectable species is transformed into a detectable one [529,533] and hence has steps including mixing, reactions, and separations. Fluid control was via electrokinetic pumping with voltages applied at the enzyme, sample, and buffer reservoirs. The application of this type of on-chip assay to complex samples such as biological fluids would benefit from additional sample processing functions on chip such

as cleanup and preconcentration [529]. Wang [529] summarized applications of on-chip enzymatic assays and provided references.

Diagnostics

Protein chips benefit clinical diagnostics and the diagnosis of complex ailments (*e.g.*, allergies, cancer, and autoimmunity) [1]. Diagnosis of leukemias and lymphomas is currently done using a combination of morphology, immunophenotype, cytochemistry, and karyotype. The different leukemias are differentiated by particular subsets of the 247 cluster of differentiation (CD) antigens on the plasma membrane. Belov *et al.* [534] constructed arrays with 60 different antibodies against CD antigens. These arrays can capture whole cells because leukocytes express subsets of CD antigens (dependent on leukemia type) on their plasma membranes. The binding pattern of leukocytes from leukemic blood samples on these arrays can be used for diagnosis, as well as drug discovery [9]. Conventional flow cytometry can analyze only up to three CDs in any one assay. Since results from this microarray method compare well with those from flow cytometry, faster and more extensive immunophenotyping should be now possible.

Autoimmune diagnostics profile the presence or absence as well as specificity of antibodies involved in autoimmune diseases [348]. The presence of certain serum antibodies then would enable the diagnosis of various autoimmune diseases. An example is the unambiguous diagnosis of systemic rheumatic disease based on simultaneous screening 18 autoantigens (diagnostic markers) by microarray ELISA [121]. Antibody titers were determined with high accuracy with less than 1 μl of patient serum [121].

It is also possible to diagnose allergies by using reverse immunoassays (*i.e.*, immobilized antigens or tissue extracts) in microarray format [390,535].

Biomarkers

The discovery of biomarkers, both for prognostics and diagnostics, is a very active research field. Protein expression profiling of cancer-specific biomarkers is a relatively recent development in diagnostics [59]. Sera and other body fluids, both in normal and diseased states, are profiled to identify novel biomarker candidates. Sometimes large antibody arrays are used [348]. Since screening single biomarkers cannot properly diagnose the disease and their clinical value is questionable (*e.g.*, in the case of CA125, an ovarian cancer protein biomarker) [59], protein microarrays are used to measure the abundance of specific proteins and characterize patterns of up to thousands of proteins. Haab *et al.* [375] tested the specificity and sensitivity of their assay with 115 antibody/antigen pairs in complex solutions using quantitative comparative fluorescence. Of all the arrayed samples, 50% of the antigens and 20% of the antibodies gave specific and accurate results on their binding partner at or below 0.34 and 1.6 μg/ml, respectively. This means the sensitivities are sufficient for many clinically relevant proteins in blood samples, even more so since antibodies used in clinical

diagnostics have much higher affinities than those used in research. For example, threshold prognostic concentration values of various breast cancer markers are as follows: c-erbB-2, 15 μg/ml; CEA, 5 μg/ml; and CA 15.3, 35 μg/ml [375,536], all within the limit detected in this study. Other concentration ranges for other clinically relevant proteins were cited [375]. Such an approach, combinatorial biomarker profiling, covers multiple cellular pathways thus increasing the probability of correct diagnosis, or even prognosis. It is promising in early cancer detection and in selecting therapeutic strategies for specific patient groups. In therapeutic strategies that fail, a compensatory mechanism may lead to the discovery of secondary drug targets. Wagner and Kim [59] listed differentially expressed proteins relevant in oral cavity and prostate cancers.

Prognostic and diagnostic markers can also be analyzed via tissue microarrays (TMA). Three TMA studies on breast cancer corroborate the estrogen and progesterone receptors and the HER-2 oncoprotein as biomarkers [464,466,538,539].

Disease monitoring

Organ and disease-specific protein arrays can be used in expression profiling to identify disease-related proteins. These arrays can also quantify specific sample proteins in terms of abundance, localization, and modification [146] as the disease progresses. On a large scale, arrays can provide the means to characterize the pattern for clinical or research purposes [375]. Hence, there is overlap with applications in diagnostics and drug discovery.

By studying protein regulation and expression, clinicians and researchers alike can predict predisposition to disease. Once the disease is manifest, data gathered can be used for monitoring disease progression, determining response to treatment, and providing overall prognosis [348]. It is also possible to screen for molecular markers and diagnostic and/or therapeutic targets in patient-matched tissue during disease progression [120].

Kononen *et al.* [465] used tissue arrays to do molecular profiling of tumor specimens obtained from 1000 tissue biopsies. This involved the simultaneous analysis of samples from many different patients at different stages of the disease. Englert *et al.* [468] also used tissue arrays in rapid molecular profiling of tumor samples (see also Principles of Design and Operation, Cell and tissue biochips, Tissue microarrays).

Paweletz *et al.* [120] used protein arrays to support a hypothetical model of prostate cancer progression. The researchers needed to gain insight into certain molecular events in how the disease progressed in individual patients. Whole protein lysates obtained from laser capture microdissection of histopathologically relevant cells were immobilized in reverse-phase arrays. They had a 20 mm × 30 mm slide with 1000 individual cellular lysates from 1 μl of lysate per spot. They expect that protein arrays will be used in the high-throughput analysis of the proteomic changes in tissue cells before and after treatment of the disease [120].

Biological and medical research

The use of biochips in basic biological and medical research has provided information that was heretofore inaccessible. Molecular profiles of cell types provided by tissue microarrays and the layered expression scanning technique [464,468] are relevant in understanding biological processes. In addition, many other types of experiments performed on microchips from assays to mimicking cellular migration in tissues and neuronal behavior *in vivo* give new insight into processes relevant in cellular biology and medicine.

Biochemical assays
Technology has led to advances in the understanding of genes and proteins particularly in how these biomolecules function in a cell. Comparing functional properties as well as screening for novel substrates can be performed simultaneously in an array format. Both biochemical [32,540] and enzymatic [118,125,137,542] assays on chips have been performed (see also Table 2).

Sensors
Cellular responses have been studied with the use of biosensor chips that incorporate several components to detect different types of response. The multiparametric microsensor chip is one example [47–49] (see Drug screening and testing). Another is a cell-based biosensor that detects a differential response in cell populations when exposed to irritants [10,543].

Cell manipulation and sorting
The ability to manipulate and separate cells facilitates the further study of cells and has practical applications. Microchip cell sorters have advantages over conventional FACS machines: reduced background fluorescence from precise control of the interrogation volume, increased sensitivity through the use of optics with high aperature numbers, and chip disposability [16,275,544]. Although the throughput is higher with a FACS machine, a chip format allows more flexibility in the types of experiments (including kinetic studies) that can be performed as well as the incorporation of other chip functions [16]. Electrokinetic manipulation of cells was done by Li and Harrison [26]. Flow cytometry on a microchip was used to sort and isolate fused cells and their viability in cell culture can be checked on chip as well [540,545]. (See also Principles of design and operation, Cell and tissue biochips, Cells on chips; and Biochip applications, Drug discovery and drug development, Proteomics and drug discovery tools, Bioparticle manipulation and separation.)

Cell shape and cellular behavior
A fundamental question in human biology that also has a disease-relevant aspect is the central role of cell shape in cell function. Cells micropatterned by SAMs

of alkanethiolates on gold were used to address these questions. As a matter of fact, the control of cell position is central to the developments in cellular biosensors and tissue engineering. It has been observed that nerve cells attach to and extend neurites only within patterned adhesive areas on chips [114,175,185,546]. The size and shape of these patterns affect cellular behavior [175,187]. By progressively reducing the micropatterned islands of extracellular matrix where endothelial cells were confined to, the transition from cell growth to apoptosis was observed. Therefore, apoptosis increases with "cell rounding" [52]. Furthermore, understanding the mechanism involved in apoptotic signaling implies the possible development of new anticancer therapies, *e.g.*, through the inhibition of angiogenesis (capillary formation) to block solid tumor growth. Cell growth and differentiation are responses to changes in ligand composition within the ECM [547–549], so is tumor metastasis [547,550]. Cell shape also seems to regulate differentiation [187,548]. Studies of cellular processes were performed using a dynamic substrate where immobilized ligands were electrochemically released [547]. Micropatterning technology is critical then for controlling cellular responses *in vitro* and for future work in tissue engineering where cell–cell and cell–ECM interactions can be studied [52]. Cell fusion has also been studied by microfluidics [540].

Cell migration and protein gradients

Generally, by immobilization techniques, cell migration can be controlled by manipulating substrate adhesiveness [175]. However, gradients of various molecules may be the determining factor in cellular migration within specific locations in a particular tissue. Hence, studies on cellular migration have benefited from biochips with gradients of biological molecules. However, gradient profile (the degree of steepness) may be the key in effecting this migratory behavior. Concentration differences are detected by receptors on the cell membrane; thus, only when these receptors detect a change will the cell elicit a response. For example, only when a sharp change is sensed do neutrophils exhibit chemotactic behavior in an interleukin-8 gradient [240]. Protein gradients in microfluidic systems can be used to study chemotaxis (directed cellular migration in chemoattractant gradients). Cell receptors bound to certain soluble molecules are potential targets in cancer metastasis, wound healing, and inflammation [240,551–553]. Other examples of gradients of physiological relevance include the morphogen gradient in Drosophila embryos [554,555] and angiogenic activity as a function of distance to the tumor site [556].

Microgradients have been made in two ways: by the use of photocrosslinkers [175] and by multistream laminar flow [540]. With the use of photolinkers, a laser rasters through the chip surface at progressively faster speeds where the amount of gradient molecules immobilized at a particular chip location is a function of the exposure time to the laser. Multistream laminar flow, on the other hand, involves protein and chemical gradients formed within microchannels [557–560]. Gradients can consist of ECM proteins, growth factors, and peptides [175].

Tissue engineering

Different types of basic biological research contribute to the understanding of tissue structures [52,187,546,561]. Certain cells may have to be situated at specific locations to achieve organized structures relevant to tissue formation [51]. Tissues formed *in vitro* can study how cells native to these tissues migrate. Cell migration enables the investigation of matrices and cellular paths that aid the formation of ''innervated, vascularized tissue'' [175]. The use of 3D microfluidic systems can study functionality in tissue architecture by addressing the molecular interactions between cells and cell types that are the bases in processes such as embryonic morphogenesis, formation of the blood–brain barrier, and tumor angiogenesis [53].

Toxicity

An example of arrays used in medical research was the study of electrophysiological parameters that are influenced by neurotoxicity in the brain. In the future, this research can help in understanding how electrophysiological activity is developed or organized in the brain. It can also lead to screening methods that detect toxic effects that lead to neurodenegeneration. Rat hippocampal and corticostriatal brain slice cultures were grown on perforated silicon in microelectrode arrays [70,562]. The neurons in culture developed normally and showed the susceptibility to the neurotoxin trimethyltin (TMT) and the excitotoxin NMDA (N-methyl-D-aspartate), as would neurons *in vivo*.

Conclusion

Recent advances in miniaturization, incorporation of complex components and adaptation of chips for non-DNA molecules signals a new era in biology. The shift towards studying biology as a complex interacting system (systems biology) requires the use of robust and reliable experimental systems that are amenable to analysis and quantitation of complex behavior. The use of biochips represents a platform to accelerate studies in systems biology and its applications in medicine and other fields are only limited by one's imagination. Further advances in this field and nanotechnology will lead to convergence of the physical, chemical, and biological sciences with great impact on biotechnology and improving quality of life.

The economic potential is enormous. Protein arrays were a $45 million business in 2000 and expected to be $500 million by 2006 [79] whereas microfluidics alone has been estimated to be a $1.5 billion business by 2005 [38]. The biochip business as a whole was already a $300 million business in 2000 and is growing annually at 40% [38].

However, for the nascent technologies to reach its full potential, several critical factors need to be addressed. Currently, the main limiting factor is the availability of appropriate reagents that can be incorporated on chips and not the technology for fabricating the chip itself. Stability of the capture reagents or

proteins needs to be addressed in order to preserve their functionality and to facilitate uniform coupling of these reagents on the chip for reliable detection. Continuous innovation is rapidly developing in search of alternative ligands to antibodies and proteins. Advances in detection technology will help in addressing specificity and increasing signal-to-noise ratio. Another critical factor will be to determine which specific capture agents should be included in a chip. Arraying thousands of proteins or capture reagents is meaningless if the results cannot be interpreted, thus it would be necessary to array the proper molecules. Regardless of these limitations, this field is still in its infancy yet making giant strides. It will be clear in time which technologies are more robust. Nevertheless, exciting developments in the technology and applications are expected in the coming years.

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Appendix 1: Abbreviations

AP	alkaline phosphatase
AFM	atomic force microscopy
AMI	acute myocardial infarction (heart attack)
ATP	adenosine triphosphate
BCE	bovine adrenal capillary endothelial (cells)
BNP	B-type natriuretic peptide (cardiac hormone)
CAD	computer-aided design
CCD	charge-coupled device
CE	capillary electrophoresis
CHF	congestive heart failure
CK-MB	creatinine kinase-MB
COC	cycloolefin copolymer

CNC	computer numerical control (machining)
CZE	capillary zone electrophoresis
DOA	drugs of abuse
DME	drug metabolizing enzymes
DPN	dip-pen nanolithography
EBL	electron beam lithography
ECL	enhanced chemiluminescence (detection)
ECM	extracellular matrix
ECP	electrocapillary pressure
ECV	human bladder cancer cells [53]
EDA	N-(2-aminoethyl)(2-aminopropyl) trimethoxysilane
ELISA	enzyme-linked immunosorbent assay (see Glossary)
EOF	electroosmotic flow
EPON SU-8	(see Glossary)
ESD	electrospray deposition
ESI	electrospray ionization
FFIB	finely focused ion beam (lithography)
FIA	flow injection analysis
FKBP12	human immunophilin
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GOPS	3-glycidoxypropyldimethyl ethoxysilane
GPCR	G-protein coupled receptor
HEK	Human embryonic kidney (cells)
HFTS	heptadeca-fluoro-1,1,2,2-tetrahydrodecyltrichlorosilane
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HTS	high throughput screening
IDES	interdigitized electrode structures
ISFET	Ion-sensitive field effect transistor
LIF	laser-induced fluorescence
LIGA	(see Glossary)
MALDI	matrix-assisted laser desorption ionization
MEMS	microelectromechanical systems (American usage)
MHA	16-mercaptohexadecanoic acid
MHD	magnetohydrodynamics
MIMIC	micromolding in capillaries
MIP	molecularly imprinted polymer
MS	mass spectrometer; mass spectrometry
NIL	nano-imprint lithography
NSB	nonspecific binding
ODT	1-octadecanethiol
OTCS	*n*-octadecyltrichlorosilane, more reactive than OTMS
OTMS	*n*-octadecyltrimethylsilane
OTS	N-octadecyltrichlorosilane

PBS	phosphate buffered saline
PC	polycarbonate
PDMS	poly(dimethylsiloxane)
PEEK	polyetheretherketone
PET	poly(ethylene terephthalate)
PLL-PEG	poly(L-lysine) grafted with poly(ethylene glycol), polycationic
PMMA	polymethylmethacrylate
PS	polystyrene
PTMP	pentaerythritol-tetrakis(3-mercaptopropionate)
PVDF	polyvinylidene fluoride
R_e	Reynolds number (see Glossary)
RCA	rolling circle amplification
RMS	root mean square
SAMs	self-assembled monolayers
SBIR	small business innovation research
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SELDI	surface-enhanced laser desorption ionization
SELEX	systematic evolution of ligands by exponential enrichment)
SERS	surface-enhanced Raman Spectroscopy
SPL	scanning probe lithography
SPR	surface plasmon resonance
SU-8	(see Glossary)
T_g	glass transition temperature (see Glossary)
TM-SFM	tapping mode scanning force microscopy
TMA	tissue microarray
TMV	tobacco mosaic virus
μCP	microcontact printing
μTAS	micro Total Analysis Systems

Appendix 2: Glossary

Bold-faced words indicate terms that are defined in this Glossary.

Actuator. (1) A peripheral device which translates electrical signals into mechanical actions; *e.g.*, a stepper motor which acts on an electrical signal received from a computer instructing it to turn its shaft a certain number of degrees or a certain number of rotations. [FDA, US Glossary of Computerized System and Software Development Terminology 1998, http://www.fda.gov/ora/inspect_ref/igs/gloss.html] (2) An actuator, the reverse of a sensor, is a device that converts an electrical signal to an action. Actuators are further divided into three categories: simple actuators that move valves or beams using one simple physical law, micromotors, more complex in the design and the possibilities, and microrobots which are the latest release in microtechnology. [National Textile Center, ''MEMS: An Overview'' 1998,

http://www2.ncsu.edu/unity/lockers/project/ntcprojects/projects/F98-S12/mems-overview.html, also in
http://www.genomicglossaries.com/content/labels_sig_and_detection_gloss.asp]

Atomic force microscopy (AFM). Scanning probe microscopy (SPM) involves methods of imaging surfaces with atomic or near-atomic resolution wherein a small tip is scanned across the surface of a sample in order to construct a 3D image of the surface. AFM is a type of SPM where, in particular, any type of surface can be probed by the molecular forces exerted by the surface against the tip. The sample must be less than 25 mm thick, up to 200 mm in diameter, and have an area of interest less than 5 μm total relief. Advantages of the technique include high lateral and vertical resolution, little or no sample preparation or alteration, digital data acquisition, manipulation, and storage of surface images [http://www.cea.com/tech.htm#afm1]. The newer proximal probe can be used in a number of modes of operation. Included are a contact mode, in which the tip touches the specimen surface and senses internuclear repulsive forces between nuclei in the tip and sample, and a noncontact mode that exploits electrostatic or van der Waals forces [http://www.sunyx.de/sunyx_engl/htm/tech/glossary.html]

Air plasma. See ***Plasma***

Anisotropic. Not exhibiting the same physical properties in all directions [http://www.mdacomposites.org/Glossary.htm]

Anisotropic etching. (1) Etching which involves different etch rates in different directions in the material [http://www.csa.com/hottopics/mems/gloss.html]. (2) Etching that results from reactive ion etching (RIE) which can etch surfaces normal to the direction of bombarding ions at a faster rate than surfaces parallel to the ion motion [adapted from http://www.icknowledge.com/glossary/r.html]. See also ***Reactive Ion Etch***

Aspect ratio. (1) Ratio of length to diameter [http://www.mdacomposites.org/Glossary.htm]. (2) The ratio of width-to-height or height-to-width in a feature formed by ablation or lithography [http://www.tamsci.com/library/library.html]

Bas-relief. Patterns or structures that are raised or protrudes from a flat background [Webster's New World Dictionary © 1990 Simon & Schuster, Inc.]

Capillary pressure. (1) Difference in pressure across the interface between two fluids [http://water.usgs.gov/pubs/wri/wri974097/text/glossary.html]. (2) The difference in the pressure for the nonwetting phase and the pressure for the wetting phase [http://www.ces.clemson.edu/ees/lee/napl/interfacial_tension.htm]. See website for a more in-depth explanation

Casting. A microfabrication method that can be used for replication. The elastomer precursor and its curing agent are poured over the mold. After curing, which may take several hours, the soft elastomer copy or replica is peeled off the mold. The formed microstructures can simply be placed against a planar surface, *e.g.*, a glass slide, a plastic sheet, or a printed circuit/ board containing electrodes to form closed channels (adapted from Ref. [61]).

Charge-coupled device (CCD) and CCD camera. A CCD is a charge-coupled device – a silicon chip whose surface is divided into light-sensitive pixels. When a photon (light particle) hits a pixel, it registers a tiny electric charge that can be counted. With large pixel arrays and high sensitivity, CCDs can create high-resolution images under a variety of light conditions. A CCD camera incorporates a CCD to take such pictures. [Xenogen website, Glossary, http://www.xenogen.com/glossary.html; also in http://www.genomicglossaries.com/content/imaging_glossary.asp and http://www.itl.arizona.edu/Library/glossary.htm (with animation on a CCD device).]

Chemisorption. (1) Adsorption involving very strong bonding forces [http://www.swbic.org/education/env-engr/tools/glossary.html#C]. (2) A process, related to adsorption, in which atoms or molecules of reacting substances are held to the surface atoms of a catalyst by electrostatic forces having about the same strength as chemical bonds. Chemisorption differs from adsorption chiefly in the strength of the bonding, which is much stronger in chemisorption [http://www.msu.edu/~svobodar/glossary.htm]. (3) The assimilation of gas, vapor, or dissolved matter by the surface of another substance resulting from electron transfer and a bond-forming chemical reaction between the surface and the gas, vapor, or dissolved matter
[http://www.lungusa.org/pub/cleaners/air_clean_app4.html]

Chemoattractant. A substance exhibiting chemical properties that attract biological cells during chemotaxis
[http://www.neuroprobe.com/global/glossary.html]

Chemotaxis. (1) Directed migration of cells in gradients of soluble molecules termed chemoattractants [240]. (2) Directional movement (migration) of biological cells or organisms in response to concentration gradients of chemicals, whereby the cells are attracted or repelled by substances exhibiting chemical properties
[http://www.neuroprobe.com/global/glossary.html]

Confocal laser imaging. See ***Confocal Scanning Laser Microscopy***

Confocal Scanning Laser Microscopy (CSLM). Also referred to as Laser Scanning Confocal Microscopy (LSCM), is used to obtain high-resolution images and 3-D reconstructions of a variety of biological specimens.

In CSLM, a laser light beam is expanded to make optimal use of the optics in the objective. Through an x–y deflection mechanism, this beam is turned into a scanning beam, focused to a small spot by an objective lens onto a fluorescent specimen. The mixture of reflected light and emitted fluorescent light is captured by the same objective and (after conversion into a static beam by the x–y scanner device) is focused onto a photodetector (photomultiplier) via a dichroic mirror (beam splitter). The reflected light is deviated by the dichroic mirror while the emitted fluorescent light passes through in the direction of the photomultiplier. A confocal aperture (pinhole) is placed in front of the photodetector, such that the fluorescent light (not the reflected light) from points on the specimen that are not within the focal plane (the so-called out-of-focus light) where the laser beam was focused will be largely obstructed by the pinhole. In this way, out-of-focus

information (both above and below the focal plane) is greatly reduced. This becomes especially important when dealing with thick specimens. The spot that is focused on the center of the pinhole is often referred to as the ''confocal spot.'' (A simple arrangement of a LSCM illustrating the confocal principle can be viewed in the website.)

A 2-D image of a small partial volume of the specimen centered on the focal plane (referred to as an optical section) is generated by performing a raster sweep of the specimen at that focal plane. As the laser scans across the specimen, the analog light signal, detected by the photomultiplier, is converted into a digital signal, contributing to a pixel-based image displayed on the attached computer monitor. The relative intensity of the fluorescent light, emitted from the spot that the laser hits, corresponds to the intensity of the resulting pixel in the image (typically 8-bit grayscale). The plane of focus (Z-plane) is selected by a computer-controlled fine-stepping motor, which moves the microscope stage up and down. Typical focus motors can adjust the focal plane in as little as 0.1 micron increments. A 3-D reconstruction of a specimen can be generated by stacking 2-D optical sections collected in series.

It should be noted that most laser scanning confocal microscopes consist of a confocal unit attached to a conventional fluorescence microscope. (The general setup of an entire LSCM system is illustrated in the website.) [Adapted from http://www.cs.ubc.ca/spider/ladic/intro.html.]

Compared to another form of laser scanning microscopy, multiphoton laser scanning microscopy (MPLSM), CLSM is less sensitive. MPLSM gives a sharper image and the photomultiplier is situated very close to the specimen without all the intervening optics and pinhole as in the case with CLSM [Bruce Jenks, Department of Cellular Animal Physiology, University of Nijmegen, Netherlands, http://www.sci.kun.nl/celanphy/Bruce%20web/scanning%20microscopy.htm, also in http://www.genomicglossaries.com/content/Microscopy.asp].

Conformal contact. (1) The condition where a mold is in contact with a master or a substrate with a mold such that there are no gaps and all the features are represented in the other material in reverse. (2) To conform to the configuration of the object in contact with [adapted from the definition of *conformal coating* in http://developer.intel.ru/download/design/packtach/gloss.pdf]

Contact angle. (1) The angle of intersection of the interface between two fluids at a solid surface. It is measured from the solid surface through the water phase, or in an oil and gas test through the oil phase [http://www.glossary.oilfield.slb.com/Display.cfm?Term=contact%20angle]. (2) Depending on the surface tension of different materials, a well-defined angle is obtained for the contact of a liquid on a solid. The contact angle is defined as the angle between the tangent to solid–vapor interface and the line of the solid–liquid interface. The angle is chosen to be that containing the liquid. The angle can be zero if the liquid fully wets the solid surface; 180° for immiscible liquids; and 90° for meniscus maintaining fluids. The advancing and receding contact angles of a liquid on a surface are often different [http://www.sunyx.de/sunyx_engl/htm/

tech/glossary. html, with additional information from http://simscience.org/membranes/advanced/glossary/c.html]

Copolymer. Polymers that are formed from one or more monomers so that the molecule consists of chemically heterogeneous components [http://www.ch.kcl.ac.uk/kclchem/staff/arr/glossold.HTM#Colloid]

Cure. (1) A change in the physical properties of a material via chemical reaction or by reaction to temperature–time profile [http://www.tkb-4u.com/glossarylist/glossary_ac.php]. (2) To irreversibly change the properties of a thermosetting resin by chemical reaction, that is, condensation, ring closure, or addition. Cure may be accomplished by addition of curing (cross-linking) agents, with or without heat and pressure [http://www.mdacomposites.org/Glossary.htm]. (3) The irreversible process of polymerizing a thermosetting epoxy in a temperature-time profile [http://e-teknet.com/pcb_glossary.htm]

Curing agent. A catalytic or reactive agent that, when added to a resin, causes polymerization. Also called hardener [http://www.mdacomposites.org/Glossary.htm]

Dicing. The action of slicing a silicon wafer into its respective parts is called die. Due to the precision needed for such a feat, a special saw, similar to a circular saw only with a blade approximately 2 inches in diameter, is used for dicing silicon [adapted from http://www.itl.arizona.edu/Library/glossary.htm]

Dielectrophoresis. The induced motion of polarizable particles in nonuniform alternating electric fields. The effect is governed by the relative magnitudes of the dielectric properties of the medium and the particles [296,298]

Dip pen nanolithography (DPN). An AFM-based soft-lithographic technique where stable nanostructures are formed from the chemisorption of molecules that are transferred to the substrate surface. When T-substituted alkanethiols are patterned on a gold substrate, a monolayer is formed in which the thiol headgroups form relatively strong bonds to the gold and the alkane chains extend roughly perpendicular to surface. The thiol lattice formed is identical to that of a monolayer obtained via solution deposition of alkanethiols on gold. Creating nanostructures using DPN is a single-step process which does not require the use of resists. One of the most important attributes of DPN is that because the same device is used to image and write a pattern, patterns of multiple molecular inks can be formed on the same substrate in very high alignment [Chad Mirkin group, Department of Chemistry, Northwestern University, US "Surface Science and Dip Pen Nanolithography" 2001, adapted from http://www.chem.northwestern.edu/~mkngrp/dippen.html, also in http://www.genomicglossaries.com/content/miniaturization_glossary.asp].

Dry etch. See ***Etch***

Electrocapillarity. (1) The change in surface tension due to electric potential gradients in the presence of a surface charge [Ken Casson, Department of Chemical Engineering, University of Alabama,

http://www.eng.ua.edu/~checlass/Seminar/spring2001/0426.html]. (2) An actuation principle that uses surface tension gradient caused by electric potential difference [Jung hoon Lee and Chang-Jin "CJ" Kim, Mechanical and Aerospace Engineering Department, University of California, Los Angeles, CA, http://cjmems.seas.ucla.edu/papers/Jung_imece98.PDF]. (3) The dependence of the interfacial tension on the electrical state of the interphase [IUPAC Compendium of Chemical Terminology, 1986, 58, 446. 2nd ed. 1997, http://www.iupac.org/goldbook/E01941.pdf]

Electron beam lithography (EBL). A method of fabricating sub-micron and nanoscale features by exposing electrically sensitive surfaces to an electron beam. The method is similar to photolithography, but uses electrons rather than photons. Since the wavelength of an electron is far smaller than that of a photon, diffraction is not a limit to the resolution. While EBL is more expensive and less parallel than photolithography, its resolution is higher and it is frequently used to create photolithographic masks [http://www.allaboutmems.com/glossary.html#E]

Electroosmotic flow (EOF). Most surfaces have an electric charge and a double layer of counter ions forms at the walls of the microchannels. When an electric field is applied across the channel, the ions in the double layer move toward the electrode of opposite polarity. This movement is transferred by viscous forces into convective motion of the bulk fluid and a uniform velocity profile is created [11].

Enzyme-linked immunosorbent assay (ELISA). An immunoassay for detecting antigens involving the coupling of an enzyme (*e.g.*, alkaline phosphatase or horseradish peroxidase) to the detection reagent such as antibody that leads to a colorimetric readout upon reaction in the presence of a substrate. The direct ELISA assay format starts with an antigen where an antibody selectively binds to the antigen and a secondary antibody covalently linked to an enzyme recognizes the first antibody leading to a colorimetric readout upon addition of the appropriate substrate. The ELISA "sandwich assay", utilizes two antibodies that simultaneously bind the same antigen: one antibody is immobilized onto the surface, and the other one is fluorescently labeled or conjugated to an enzyme that can produce a fluorescent, luminescent or colored product when supplied with the appropriate substrate. The antigen is between two antibodies hence it has been termed a sandwich assay (sandwich assay definition adapted from Ref. [9])

EPON SU-8. See ***SU-8***

Etch. (1) A process using a chemical bath (wet etch) or a plasma (dry etch) that removes unwanted substances from the wafer surface [http://www.logicorp.net/glossary_terms.htm, http://www.forums.pctechguide.com/glossary/wordfind.php?wordInput = Etch]. (2) Removal of portions of a layer of conductive material from a usually insulating base through chemical or electrolytic means. In wet etching, the material is dissolved when immersed in a chemical solution. In dry etching, the material is sputtered or dissolved using reactive ions or a vapor phase etchant

[http://www.csa.com/hottopics/mems/gloss.html]. Hydrofluoric acid (HF) or potassium hydroxide (KOH) are used as etchants [61]

Evanescent field. In a waveguide, a time-varying field having an amplitude that decreases monotonically as a function of transverse radial distance from the waveguide, but without an accompanying phase shift
[http://www.atis.org/tg2k/_evanescent_field.html,
http://www.bandwidthmarket.com/resources/glossary/E6.html,
http://www.its.bldrdoc.gov/projects/devglossary/_evanescent_field.html]

Expression cloning. A molecular cloning approach in which expression of the gene to produce an identifiable gene-product is the basis of the selection of the desired clone. One such strategy uses antibodies to recognize epitopes expressed as part of a fusion protein in *E. coli*. Another variation involves the production of protein molecules by *in vitro* transcription of cloned cDNAs, followed by microinjection into *Xenopus* oocytes to allow expression of ion channels for patch-clamp methods [adapted from
http://www.pain.med.umn.edu/csn/e66/66-E.html].

Finite element. A discrete entity used to subdivide the geometry of a structure in order to create an idealized structure for finite element analysis. Each finite element is a simple shape (such as a rectangle or a triangle) for which the software has information to write the governing equations in the form of a stiffness matrix. The unknowns for each element are the displacements at the points where the elements are connected. The behavior of the structure is approximated by the aggregate behavior of all of the elements
[http://www.ces.clemson.edu/~lonny/courses/me818/glossary.html].

Finite element analysis. (1) An approximate method for calculating the behavior of a real structure. An idealized model structure is created by subdividing the geometry of a structure into elements which are connected at nodes. The method is based on the generalized method of weighted residuals using locally based approximations [http://www.ces.clemson.edu/~lonny/courses/me818/glossary.html]. (2) A computer-based analysis method which calculates the response of the model by solving a set of simultaneous equations that represent the behavior of the structure under external loading [http://www.cosmosm.com/support/glossary.htm]. (3) A technique for predicting the response of structures and materials to environmental factors such as forces, heat and vibration. The process starts with the creation of a geometric model. Then, the model is subdivided (meshed) into small pieces (elements) of simple shapes connected at specific node points. In this manner, the stress–strain relationships are more easily approximated. Finally, the material behavior and the boundary conditions are applied to each element [http://www.cosmosm.com/support/fea1.htm]

Glass transition temperature (T_g). (1) The temperature at which many polymers/high molecular weight substances begin to solidify. Cooling below this temperature results in a solid that is as hard as glass and brittle [61]. (2) The temperature at which a polymer changes from hard and brittle to soft and pliable

[http://www.psrc.usm.edu/macrog/glossary.htm,
http://www.epolybags.com/GLOSSARY.htm].

Hydrogel. (1) A water-based gel; a gel whose liquid constituent is water [http://www.solgel.com/educational/glossary.htm]. (2) The hydrogel polymer network is a cross-linked poly-(6-acryloyl-β-*O*-methyl-galactopyranoside, a highly hydrophilic polymer with ordered pendant-type sugar repeat units. When permitted to swell in protein solution, an initially dry polymer will reach an equilibrium solution content of about 99.1 wt% [212,577,578]

Injection molding. One of the widespread standard processes to fabricate polymer parts. The process starts with the raw polymer material in granular form. These granules are fed into the cylinder (Fig. 5), a heated screw, where the pellets start to melt. This melt is then transported forward toward the mole cavity. Typical temperatures in this region range from 200°C (for polymers such as PMMA and PS), over ~280°C (for PC), up to ~350°C for materials like PEEK. The molten material is then injected under a high pressure (typically 60–100 MPa = 60–1000 bar) into the evacuated cavity, which contains the mold insert/as the master structure. With microfabrication, therefore less material injected into the cavity and the surface-to-volume ratio increases, the cavity has to be heated closer to the melting point of the polymer material to allow the polymer to flow into the small structure of the mold insert. The cavity will then be cooled to allow the ejection of the microstructured part. This process, called variotherm, allows the fabrication of smaller structures than the equivalent cold-cavity process for macroscopic systems, but increases the cycle time due to the heating and cooling (adapted from Ref. [61])

Joule heating. The production of heat in a conductor as a result of the passage of an electric current through the conductor. The quantity of heat produced is given by Joule's law ($Q = I^2Rt$ where Q is the heat produced when an electric current I flows through a resistance R for a given time t.) [http://physics.miningco.com/library/dict/bldefjouleheating.htm, http://physics.about.com/library/dict/bldefjouleslaw.htm?terms = Joule%27s + law]

Laminar flow. Streamlined flow with no turbulence [http://www.allaboutmems.com/glossary.html#E]. See also ***Reynolds number***

Laser ablation. The photoablation process involves absorption of a short-wavelength pulse to break covalent bonds in long-chain polymer molecules with production of a shock wave that ejects decomposed polymer fragments [75,109]. A typical laser ablation setup consists of an excimer laser (ArF: 193 nm, 10–100 Hz; or KrF: 248 nm, several kHz), a mask or aperture, and an *xy*-table on which the substrate is mounted. The mask defines the ablated region, while moving the substrate on the *x–y* stage underneath the mask completes the pattern. Depending on the substrate material and on the energy available per laser pulse, typical ablation rates per laser pulse range between some hundred nanometers and 5 μm. The accuracy of the process in the *x–y* direction is largely determined by the energy distribution in the beam (which is normally constant to about 5% across the mask or aperture) and the quality of the *x–y* stage.

Typically, they are of the order of a few microns. Depth control, which ultimately also determines the wall roughness, is of the order of 0.1 μm. Due to the interaction of the laser light and the polymer material, however, certain surface modifications are induced in comparison to the untreated material [61,108]. See also ***Pulsed lasers***.

Layering. (1) Providing layers of various materials to complete the fabrication of components on the silicon surface [http://www.cabotcmp.com/glossary_semiconductor.htm]. (2) It is compatible with integrated circuit (IC) technologies and involves the growth of thin layers of polymers on planar substances and the use of sacrificial layers to create open volumes between these layers. A thin layer of parylene is deposited onto a substrate, either silicon or polycarbonate. Onto this layer, metal can be deposited using evaporation or sputtering methods to form electrode structures. This structure is then covered with a thin chromium layer and a layer of a sacrificial polymer. The thickness of this sacrificial polymer defines the channel height. The next step consists of depositing an additional layer of parylene on top of this structure to form the channel walls and cover. This represents a big advantage because the closing of the channel structure is naturally included in this fabrication method. Through-holes are defined in the parylene which allow fluid connections in the completed system as well as access of the etchant (usually acetone) for the subsequent sacrificial etch. Due to the comparatively small channel cross-section, this etch can take several hours as the dissolved material can only be transported by diffusion. Cast silicone structures on top of the parylene layer then define fluid reservoirs (see Fig. 13) [61,280–282]

LIGA. A German acronym for a microfabrication technique that combines lithography (Lithographie), electroplating (Galvanoformung, Galvanoplastie), and molding (Abformung) [41,61]. Originating from the Nuclear Research Center in Karlsruhe, Germany, the technique involves deep-etch X-ray lithography, electroplating, and injection molding. [http://ranier.hq.nasa.gov/Sensors_page/Terms.html]. It is suitable for making parts with depths significantly greater than lateral dimensions from metals, metal alloys, plastics or ceramics. Synchrotron radiation is used to create a pattern in an X-ray resist, usually PMMA. The use of synchrotron radiation allows lateral feature sizes that are as small as a few microns in dimension, and straight, smooth vertical sidewalls that can be as tall as several millimeters. Once the PMMA is exposed, it is chemically developed and used as a mold for electroforming metal or metal alloys [http://www.eetimes.com/reshaping/microstructures/OEG20020912S0029].

Lithography. The transfer of a pattern or image from one medium to another, as from a mask to a wafer. Photolithography involves the use of light to effect this pattern transfer. Microlithography refers to the process as applied to images with features in the micrometer range [http://www.cabotcmp.com/glossary_semiconductor.htm], also in [61]. See also ***Microlithography***, ***Photolithography***.

Magnetohydrodynamics. Better known in the astrophysics of plasmas and liquid metal pumping, MHD is the study of plasma motion and dynamics in the presence of a magnetic field [http://www.spacescience.org/ExploringSpace/

Glossary/1.html]. It involves three physical fields that are at right angles to each other: electric, magnetic, and flow. Application of an electric and magnetic field in small channels or microscopic reactor or sensor vessels containing aqueous or nonaqueous solution results in controlled flow or stirring [adapted from http://www.uark.edu/depts/cheminfo/uarkchem/facultystaff/faculty/fritsch/].

Mask. A device that acts as a barrier to the passage of a reagent (often light, see ***Photolithography***). A pattern of holes in the mask allows selective passage of reagent and results in a corresponding pattern of reagent deposition or photodeprotection on a surface placed behind the mask [IUPAC Combinatorial Chemistry, also in http://www.genomicglossaries.com/content/microarrays.asp]. See also ***Photomask***

Master. A microfabricated mold tool representing the negative (inverse) structure of the desired polymer structure [61]; a ''negative'' from which microdevices are replicated. The composition of the master used in production may depend on the production run. Masters made from metal or other hard materials may be used in manufacturing when the production run is large because of their durability; the expense of the master becomes negligible after many uses. For prototyping new devices and for limited run productions, however, metal or silicon molds are time-consuming and expensive to make, especially in a program of research and development where several iterations are necessary for the development of a final design. A master in SU-8 photoresist on a silicon wafer is durable and can be used indefinitely, although proper precaution should be taken by the user against breaking the fragile silicon wafer or when releasing the photoresist from the wafer. Replication of the master as one piece in a hard polymer, *e.g.*, structural polyurethane or epoxy, can further extend its lifetime [10]

Micromachining. (1) The application of integrated circuit fabrication processes to the manufacture of miniature machines, sensors and switches [http://www.ultratech.com/products/glossary.shtml]. (2) Machining (*i.e.*, performing various cutting or grinding operations on a piece of work) for the purpose of fabricating memsmechanical parts [http://www.csa.com/hottopics/mems/gloss.html]

Microcontact printing (μCP). (1) For patterning, a technique that uses the relief pattern on the surface of an elastomeric PDMS stamp to form patterns of SAMs on the surfaces of substrates. The stamp is 'inked' with a solution of an alkanethiol in ethanol, dried, and brought into conformal contact with the gold surface for 10–20 s. The alkanethiol is transferred to the gold substrate only in the regions where the PDMS stamp contacts the substrate. Subsequent exposure of the remaining bare gold substrate to a second alkanethiol generates a surface patterned into regions presenting different terminal groups (see Fig. 9) [51,228,579]. (2) A planar or rolling stamp is used to transfer molecules of the ''ink'' to the surface of the substrate by contact, only really suited to single layer fabrication [http://www.npl.co.uk/npl/cmmt/functional/func_process.html]

Molecularly imprinted polymers (MIPs). A new class of materials that have artificially created receptor structures. Since their discovery in 1972, MIPs have attracted considerable interest from scientists and engineers involved with the

development of chromatographic absorbents, membranes, sensors and enzyme and receptor mimics. [Piletsky S *et al*. Molecular imprinting: at the edge of the third millennium. Trends in Biotechnology 2001 Jan:19(1):9–12, Jan. 2001, also in http://www.genomicglossaries.com/content/biomaterials.asp].

Monolithic. (1) Formed from a single crystal, such as a monolithic silicon chip. (2) Produced in or on a monolithic chip, as a monolithic circuit. A monolithic integrated circuit (MIC) is an IC having elements formed in place on or within a semiconductor substrate, with at least one element being formed within the substrate [http://www.csa.com/hottopics/mems/gloss.html]

Microlithography. (1) The science and art of building a mask pattern on top of a substrate (*e.g*., quartz glass) and then transferring this pattern into another substrate. This process is repeated many times to form an integrated circuit, MEMS, etc. [http://www.tamsci.com/library/glossary.html]. (2) A manufacturing process for producing highly accurate, microscopic, two-dimensional patterns in a photosensitive resist material. These patterns are replicas of a master pattern on a durable photomask, typically made out of a thin patterned layer of chromium on a transparent glass plate [http://www.tamsci.com/library/glossary.html]. See also ***Lithography***.

Multilayer soft lithography. See ***Soft lithography***

Multilayer structures. See **Soft lithography**, Multilayer soft lithography

Nanopatterning. Creating patterns on the order of nanometers, the same size as a protein, by placing the proteins in specific locations [116]

Nanotechnology. (1) The application of science to developing new materials and processes by manipulating molecular and atomic particles [http://www.nanoelectronicsplanet.com/glossary/article]. (2) The science of building microscopic devices out of individual molecules or small numbers of molecules. The devices are measured in nanometers. A nanometer is one millionth of a millimeter, or about 10 carbon atoms long. The products of nanotechnology are likely to include tiny mechanical devices and computer circuitry far smaller than is possible with today's semiconductor technology [http://www.trnmag.com/Glossary/NglossaryN.html].

Phage. A collective term for viruses that infect bacterial hosts.

Phage display. A technique in which phage is engineered to display proteins on the virus particle's surface by fusing a recombinant peptide or protein (*e.g*., antibodies) with the virus capsid or coat protein genes. The phage may then be used to screen for specific ligands that bind to an immobilized antigen.

Photolithography. (1) A type of lithographic process used in the manufacturing of semiconductor devices, integrated circuits, and photomasks. It comprises applying a layer of material known as photoresist or simply resist, exposing the resist to UV light, developing the exposed resist [http://www.tamsci.com/library/glossary.html]. (2) Process by which selective masking generates light patterns which direct chemical transformations to certain areas of a photosensitive surface. Coupling of different building blocks to discrete sites may give rise to

spatially addressable arrays of compounds. [IUPAC Combinatorial Chemistry, also in http://www.genomicglossaries.com/content/microarrays.asp] See also Lithography, Photoresist

Photomask or Photolithographic mask. (1) A template used in photolithography that allows selective exposure of a photosensitive surface [http://www.allaboutmems.com/glossary.html#E] (2) A plate of material that is transparent at a certain wavelength (*e.g.*, SiO_2 at UV wavelengths) coated according to a certain pattern with material that does not transmit (mostly reflects) at the same wavelength (*e.g.*, chrome, aluminum, dielectric, etc.) [http://www.tamsci.com/library/glossary.html]

Photoresist. (1) A layer of material that will react when exposed to actinic energy, UV light (or X-ray, e-beam etc.). The resist layer exposed to UV light, in certain portions, undergoes a change in its solubility. It is then developed by washing it with a basic developer solution, thereby removing the nonirradiated (in a negative-tone resist) or irradiated (in a positive tone resist) portions [http://www.tamsci.com/library/glossary.html]. (2) A class of polymers used in microsystem technology and for microfluidic systems. Irradiation, with electrons, ions, X-rays, UV or visible light, leads to a photochemical reaction of the resist material, which is coated onto a carrier substrate (typically either silicon or another polymer). In the case of the so-called positive tone resists (or singly positive resists), the solubility of the irradiated areas increases; in negative tone resists, it decreases. The irradiated or nonirradiated areas, respectively, will be removed by a developer. For deep X-ray lithography, the standard resist material is PMMA which acts as a positive tone resist. Polyactide or copolymers of lactide and glycolide can be used as resist material for X-ray lithography. In the longer wavelength range, SU-8 is a resist material developed by IBM for UV-lithography which allows the fabrication of structures with heights of more than 1000 μm [61].

Piezoelectric. (1) A substance that becomes electrically charged by pressure [http://www.geology.wisc.edu/~jill/glossary.html, http://www.genomicglossaries.com/content/microarrays.asp]. (2) Having the ability to generate a voltage when mechanical force is applied, or to produce a mechanical force when a voltage is applied, as in a piezoelectric crystal [http://www.csa.com/hottopics/mems/gloss.html].

Plasma. A conductive assembly of charged particles, neutrals and fields that exhibit collective effects. Plasmas carry electrical currents and generate magnetic fields. Because plasmas are conductive, respond to electric and magnetic fields, and can be efficient sources of radiation, they can be used in innumerable applications when special sources of energy or radiation are required (adapted from http://www.plasma.org/basics.htm].

Plasma oxidation. (1) Plasma oxidation involves the growth rather than the deposition of high quality thin layers of silicon oxide, at room temperature. The technique employs O^- produced in an oxygen plasma [http://www.liv.ac.uk/EEE/research/sse/project4.htm]. (2) The process involved when a silicon

substrate is in contact with a plasma at floating potential during oxidation [http://researchwed.watson.ibm.com/journal/rd/431/hess.html]. (3) PDMS comprises repeating units of $–O–Si(CH_3)_2–$. Exposing a PDMS replica to air plasma introduces polar groups on the surface. One view is that the methyl groups ($Si–CH_3$) are replaced with silanol groups (Si–OH). These silanol groups then condense with appropriate groups (OH, COOH, ketone) on another surface when the two layers are brought into conformal contact. For PDMS and glass, this reaction yields Si–O–Si bonds after loss of water. These covalent bonds form the basis of a tight, irreversible seal: attempting to break the seal results in failure in the bulk PDMS. The seal withstands pressures of 30–50 psi. It is possible to seal PDMS irreversible to the surfaces of a number of materials: PDMS, glass, Si, SiO_2, quartz, silicon nitride, polyethylene, polystyrene, and glassy carbon. This method, however, does not work with all polymers, *e.g.*, Saran, polyimide, polycarbonate, and poly(methylmethacrylate) [10,65].

Plastic deformation. (1) Atoms in a material are permanently rearranged due to a stress-induced change. Removal of stress does not result in a restoration of the original shape [http://www.phy.davidson.edu/StuHome/BeKinneman/metal/glossary.htm]. (2) The permanent change in shape or size of a body without fracture, produced by a sustained stress beyond the elastic limit of the material [http://www.vishay.com/brands/measurements_group/guide/glossary/plas_-def.htm].

Polymers. Macromolecular substances with a relative molecular mass between 10 and 100 kDa with more than 1000 monomeric units. The polymerization process requires an initiator substance or a change in physical parameters (light, pressure, temperature). Due to the great length of the polymer chain, polymers are bulk materials. In most cases, polymers are amorphous but in some cases, microcrystalline, where the polymer chain is longer than the size of the crystallites. The chain length of the polymer molecules varies in bulk material; therefore, a polymeric material does not have a precisely defined melting temperature. Instead, a melt interval exists where the material becomes a highly viscous mass. The decomposition temperature is another characteristic point above which the thermal cracking of the material starts and the material ceases to function (adapted from Ref. [61]).

Prototyping. See ***Rapid prototyping***

Pulsed lasers. Lasers can be continuous wave (cw) or pulsed. Pulsed lasers with very short pulse duration (on the order of nanoseconds) are critical for achieving ablation since the laser energy has to be delivered in a very short amount of time. Hence, the laser energy will be confined to a very thin layer of material inducing intense photochemical or photothermal reaction, which results in ablation [http://www.tamsci.com/library/glossary.html].

Rapid prototyping. (1) Any of a variety of processes that avoids tooling time in producing prototypes or prototype parts and therefore allows (generally nonfunctioning) prototypes to be produced within hours or days rather than weeks. These prototypes are frequently used to quickly test the product's

technical feasibility or consumer interest [http://www.shapetomorrow.com/resources/r.html#rapid]. (2) The combination of high-resolution commercial printing, photolithography, and soft lithography, allowing microsystems to be designed and fabricated rapidly and inexpensively [10,65,154]. For microfluidic systems, it begins with creating a design for a device in a computer-aided design (CAD) program. A high-resolution commercial image setter then prints this design on a transparency. This transparency serves as the photomask in contact photolithography to produce a positive relief as a master; it is used for the replication (via casting) of PDMS devices [10].

Reactive ion etch (RIE). A technique whereby radio frequency radiation is coupled with a low-pressure gas to ionize the gas producing disassociation of the gas molecules into more reactive specie, and the substrate being etched is biased to induce ion bombardment. Compounds containing carbon (C) and halogens such as, fluorine (F), chlorine (Cl) or Bromine (Br) are typically used as gases. When the compounds dissociate in the plasma, both highly reactive halogen atoms or halogen compounds, and polymers that may deposit on the substrate blocking the highly reactive species are generated. Ions accelerated towards the substrate being etched by the applied or induced bias remove polymers on substrate surfaces oriented normal to the direction of ion motion, polymers coat substrate surfaces that are oriented parallel to the ion motion and block etching of those surfaces. Ion bombardment may also activate or accelerate chemical etching reactions. RIE therefore has the capability to etch surfaces normal to the direction of ion motion at a higher relative rate and surfaces parallel to the ion motion at a lower relative rate resulting in anisotropic etching. Typical RIE conditions are low pressure, low ionization levels and high ion energies relative to other dry etch techniques [http://www.icknowledge.com/glossary/r.htm].

Refractive index. (1) The ratio of the velocity of the radiation (*e.g.*, light) in vacuum to the velocity of radiation (*e.g.*, light) in the material [http://www.tamsci.com/library/glossary.html]. (2) The refractive index indicates the extent to which a light beam is deflected when passing from vacuum into a given substance [http://www.photometer.com/en/abc/abc_015.htm].

Registration. Proper positioning and exact alignment, term usually used in printing and image assembly [adapted from http://www.uniprint.com/terms/r.htm and http://www.ideaexchange.sappi.com/home.asp?pid = 89]. Example sentence from Chiu *et al.* [53]: ''Patterning of multiple ''colors'' . . . requires multiple steps of registration.''

Replica molding. The process involves the casting of prepolymer against a master and generating a negative replica of the master in PDMS with micron-scale relief patterns, *i.e.*, ridges on the master appear as valleys in the replica. The PDMS is cured in an oven at 60°C for 1 h, and the replica is then peeled from the master. Access holes for channels and reservoirs for buffer can be added in the replication step by appropriate placement of posts on the master or punched out of the cured layer by using a borer [10,51].

Reverse transfection. A microarray-based system for the functional analysis in mammalian cells of many genes in parallel. Mammalian cells are cultured on a glass slide printed in defined locations with solutions containing different DNAs. Cells growing on the printed areas take up the DNA, creating spots of localized transfection within a lawn of nontransfected cells. By printing sets of complementary DNAs (cDNAs) cloned in expression vectors, microarrays can be made whose features are groups of live cells that express a defined cDNA at each location. These ''transfected cell microarrays'' should be of broad utility for the high-throughput expression cloning of genes, particularly in areas such as signal transduction and drug discovery. For many applications, these arrays can serve as substitutes for protein microarrays, particularly for proteins that are difficult to purify, such as membrane proteins. [David Sabatini ''Reverse transfection'' Whitehead Institute, MIT, Cambridge, MA, USA, http://staffa.wi.mit.edu/sabatini_public/reverse_transfection/frame.htm, also in http://www.genomicglossaries.com/content/microarrays.asp].

Reynolds number (R_e). (1) A dimensionless parameter relating the ratio of inertial to viscous forces in a specific fluid flow configuration, and is a measure of the tendency to the liquid to develop turbulence [51]. 2. The nature of a flow of fluid is characterized by the Reynolds number, $R_e = \rho v l/\eta$, where ρ is the density of the fluid, v is the velocity, l characterizes the shape and dimensions of the channel (the cylinder diameter), and η is the viscosity of the fluid. Flows that have $R_e < 2000$ are laminar. When two or more streams with low R_e are combined into a single stream, the combined streams flow side by side without turbulent mixing; mixing occurs only by diffusion [10,41,440].

Self-assembled monolayer (SAM). (1) A layer of material that is grafted at a surface or interface formed from a reagent that forms a single uniform layer. (A grafted layer is chemically attached by bonds to an interface rather than adsorbed. This provides distinction between chemisorbed and physisorbed species.) Typical reagents will use the reaction of a thiol with gold or a trichlorosilane with SiOH groups. A wide variety of groups can be attached in this way to modify the stability of colloidal particles or to change surface properties [http://www.ch.kcl.ac.uk/kclchem/staff/arr/glossold.HTM#SAM]. (2) The resist used in the Harvard/NIST method is a self-assembled monolayer of organic molecules called alkanethiolates which is adsorbed on the surface of the gold. An alkanethiolate molecule can be thought of as a ball and chain in a weightless environment. The thiol ball of the molecule bonds strongly to the gold, while the hydrocarbon chain floats away from the surface. On gold and a number of other metallic and oxide surfaces, these and other appropriate compounds will self assemble into a single layer of tightly packed molecules a few nanometers thick, much thinner than a typical photoresist [http://physics.nist.gov/News/Releases/n95-28.html].

Silicone rubber. PDMS [116]

Silane (SiH_4). (1) A gas that readily decomposes into silicon and hydrogen, silane is often used to deposit silicon-containing compounds. It also reacts with

ammonia to form silicon nitride, or with oxygen to form silicon dioxide [http://www.appliedmaterials.com/products/glossary.html]. (2) A pyrophoric, toxic gas with a TLV (threshold limit value) of 5 ppm, and is combustible in the range of ~1–96%. Silane is widely used to deposit silicon-containing films in a variety of CVD (chemical vapor deposition) reactions [http://www.icknowledge.com/glossary/s.html].

Silanization. Silanization of items prevents compounds from adhering to the inside walls of the glassware. To silanize glass, the product is totally immersed in a silane solution for up to 30 min, followed by appropriate rinses chemically binding a nonreactive silicone layer to the glass surface, thus deactivating the surface and allowing for more accurate quantitative analysis [http://www.sun-intl.com/html/glossary.cfm].

Sintering. The process in which fine particles of a material become chemically bonded at a temperature that is sufficient for atomic diffusion [http://www.solgel.com/educational/glossary.htm].

Soft lithography. (1) A set of nonphotolithographic, microfabrication techniques for patterning surfaces used in biochemistry and biology. An elastomeric material with the patterns embedded as a bas-relief on the surface acts as the pattern transfer agent. These methods have the characteristic that routine access to a cleanroom is not necessary when producing most structures relevant to microfluidics (20–100 μm). They are also able to pattern transfer to curved materials. This method can be used to fabricate channels in bulk polymer [10,51]. (2) A technique where patterns are incorporated by curing the elastomer on a micromachined mould. Features that can be made include channels, diffraction gratings, and three-dimensional structures, such as coils and rings. These can be as small as 80 nm in diameter but typical channel widths are 50–100 μm [62,67].

Multilayer soft lithography is a micromachining technique that exploits the elasticity and the surface chemistry of silicone elastomers in order to create monolithic microvalves within microfabricated devices [292]. This technique is based on rapid prototyping and replica molding methods of soft lithography. A monolithic chip can be made of multiple layers of elastomeric channels [16]. Each layer is cast separately and bonded irreversibly to the next using the elastomer's chemical curing process, eradicating the problems of adhesion failure and thermal stress that occur when layering hard materials. This technique was developed by Stephen R. Quake and his group (Department of Applied Physics, California Institute of Technology, Pasadena, CA, USA) and is proprietary technology of Fluidigm Corp. (see Table 3) [67]

Sol-gel. Transition of a system from a liquid sol (colloidal suspension of minute solid particles in a liquid) to a viscous gel in which the suspended particles are organized in a loose, but definite three-dimensional arrangement. The thin film gel is dried (this process can be repeated several times to achieve the required film thickness) and finally sintered [http://www.npl.co.uk/npl/cmmt/functional/func_process.html].

Sol-gel coating. A coating produced by the sol–gel process of glassmaking, in which glass is formed at low temperatures from suitable compounds by chemical polymerization in a liquid phase; a gel is formed from which glass may be derived by the successive elimination of interstitial liquid and the collapse of the resulting solid residue by sintering [http://www.solgel.com/educational/glossary.htm].

Spin-coating. Solution is poured onto the substrate surface, which is then spun to expel excess fluid and create a uniform thickness [http://www.npl.co.uk/npl/cmmt/functional/func_process.html].

Scanning probe lithography (SPL). A high resolution patterning technique that uses a sharp tip in close proximity to a sample to pattern nanometer-scale features even below 30 nm with nanometer-scale alignment registration. It is a relatively simple, inexpensive, reliable method and can be used on various substrates. Some applications include nanometer-scale research, maskless semiconductor lithography, and photomask patterning. With better control of the tip, scanning speed has been increased to millimeters per second. Both noncontact and in-contact writing have been demonstrated as controlled writing of sub-100 nm lines over large steps on the substrate surface. (Adapted from: Soh HT, Guarini KW, Quate CF. Scanning Probe Lithography. (Microsystems, Volume 7) Kluwer Academic Publishers, 224 pp., July 2001, ISBN: 0-7923-7361-8; http://www.neutrino.co.jp/abi_mict/0-7923-7361-8.PDF]

SU-8. A photocurable, epoxy-based, negative photoresist that is used to make high aspect ratio features in electroplating, polymer casting and UV-LIGA. LIGA yields better results but due to cost considerations, it is well suited as a mold for electroplating because of its relatively high thermal stability ($T_g > 200°C$ for the cross-linked, *i.e.*, exposed, resist).

SU-8 is based on EPON SU-8 epoxy resin (from Shell Chemical) that has been originally developed, and patented by IBM (US Patent No. 4882245 (1989) and others). This photoresist typically is 200 μm but can be as thick as 2 mm and aspect ratio >20 have been demonstrated with standard contact lithography equipment. These astounding results are due to the low optical absorption in the UV range which only limits the thickness to 2 mm for the 365-nm wavelength where the photoresist is the most sensitive (*i.e.*, for this thickness 100% absorption occurs). Normal resists range from 0.5 to 3 μm in thickness. Microchannels with depths of some tens of micrometers can be fabricated. Although it is durable and can be used indefinitely, it not easy to process, has a large internal stress, and once developed, is difficult to remove. [Adapted from http://aveclafaux.freeservers.com/SU-8.html and http://www.allaboutmems.com/glossary.html#E, with additional information from McDonald *et al*. [10], Becker and Gärtner [61], and http://www.mems-exchange.org/catalog/UMich-B-0120/].

Substrate. (1) The material of which something is made and from which it derives its special qualities. In electronics, it is the physical material on which a MEMS circuit is fabricated; used primarily for mechanical support and insulating purposes, as with ceramic, plastic, and glass substrates;

semiconductor and ferrite substrates may also provide useful electrical functions [http://www.csa.com/hottopics/mems/gloss.html]. (2) A substance which an enzyme acts on.

Systems biology. (1) The simultaneous study of complex interactions of multiple levels of biological information including DNA, RNA, proteins and biochemicals. [http://www.paradigmgenetics.com/content/aboutus/glossary.asp] (2) A powerful approach to studying genes and proteins, made possible through technological advances. Unlike traditional biology that has examined single genes or proteins in isolation, systems biology simultaneously studies the complex interaction of many levels of biological information-genomic DNA, mRNA, proteins, functional proteins, informational pathways, and informational networks, to understand how they work together. [Institute for Systems Biology, Seattle WA, US ''What is systems biology?,''
http://www.systemsbiology.org/overview.html, also in
http://www.geocities.com/pribond/bioinfo/glossary/cell-biology.htm].

Teflon. Poly(tetrafluoroethylene), can be used for laser ablation [75].

Titer. (1) The term refers to antibody titer, which is a measure of the concentration of specific antibodies to selected microbes that are circulating in an individual's bloodstream [http://www.healingwithnutrition.com/cdisease/chronicfatigue/glossary.html]. (2) A titer is the amount of a substance found in certain tests [http://www.mtio.com/lupus/lfanp2.htm].

Waveguide. (1) Structure that guides electromagnetic waves along its length. An optical fiber is an optical waveguide [http://www.csp.com/html/tech_library/fiber_glossary/fbgl_t-z.htm]. (2) A material medium that confines and guides a propagating electromagnetic wave. In the optical regime, a waveguide used as a long transmission line consists of a solid dielectric filament (optical fiber), usually circular in cross section. In integrated optical circuits, an optical waveguide may consist of a thin dielectric film. [http://www.atis.org/tg2k/_waveguide.html, also in http://www.fiber-optics.info/glossary-wxyz.htm#Waveguide]

Wet etch. See ***Etch***

Young's modulus. A measure of the strength of a material expressed as the stress divided by the strain [http://www.youngsmodulus.com].

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