From combinatorial chemistry to chemical microarray
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Combinatorial chemistry was first applied to the generation of peptide arrays in 1984. Since then, the field of combinatorial chemistry has evolved rapidly into a new discipline. There is a great need for the development of methods to examine the proteome functionally at a global level. Using many of the techniques and instruments developed for DNA microarrays, chemical microarray methods have advanced significantly in the past three years. High-density chemical microarrays can now be synthesized in situ on glass slides or be printed through covalent linkage or non-specific adsorption to the surface of the solid-support with fully automatic arrays. Microfabrication methods enable one to generate arrays of microsensors at the end of optical fibers or arrays of microwells on a flat surface. In conjunction with the one-bead one-compound combinatorial library method, chemical microarrays have proven to be very useful in lead identification and optimization. High-throughput protein expression systems, robust high-density protein, peptide and small-molecule microarray systems, and automatic mass spectrometers are critical tools for the field of functional proteomics.

Introduction
Combinatorial chemistry began in 1984 when Geysen et al. [1] first reported the 96-well plate multi-pin system for peptide synthesis. Later, Frank [2] modified this approach by synthesizing and analyzing multiple peptides as spots on cellulose paper, the so-called 'SPOT libraries' method. In 1991, Fodor et al. [3] reported the use of the light-directed, spatially addressable parallel chemical synthesis method to generate peptide microarrays. These three methods all involve efficient synthesis of an array of peptides on solid-supports followed by analysis with standard immunodetection methods. In 1986, Geyesn et al. [4] introduced an iterative approach to analyze multi-pin peptide arrays. In 1991, Lam et al. [5] first described the ‘one-bead one-compound’ (OBOC) combinatorial peptide library method. An OBOC combinatorial bead-library can be considered as a huge spatially separable but non-addressable chemical microarray because only one peptide entity is displayed on each bead. About the same time, Houghten [6] reported the use of a tea bag technique to synthesize peptide mixtures, and the use of an iterative approach or positional scanning approach [7] to analyze these libraries. Since then, the field of combinatorial chemistry has taken off and has now become a major discipline.

Although the chemical microarray technology pioneered by the Affymax group [3] preceded the DNA microarray era [8,9], it was not until the past three years when chemical, peptide or protein microarrays resurfaced as a popular research approach in the field of proteomics. This delay is due in part to the lack of commercially available instruments for spotting high-density arrays until the past few years. Interestingly, the highly successful Affymetrix oligonucleotide chip initially designed for high-speed DNA and RNA sequencing [8,10], and later widely used in the DNA microarray field, is in fact generated with methods based on the original light-directed synthesis method described by the Affymax group (Affymetrix is a spin-off company of Affymax). About the same time, rather than relying on in situ oligonucleotide synthesis, Brown and co-workers [9,11,12] invented and popularized the DNA microarray technique by developing methods and more affordable instruments to spot cDNAs directly onto glass slides in a microarray format. The success of DNA microarray technologies in the analysis of thousands to tens of thousands of genes on one glass slide [12] revitalized the interest in chemical microarrays. The field of protein microarrays has been recently reviewed [13,14••]. In this minireview, we focus our attention on the development of chemical microarrays (small-molecule compound, peptides or proteins).

Like the gene chips, chemical microarrays are prepared by immobilizing a large number of chemical or biological molecules on the surface of a solid-support by one of three methods: first, in situ synthesis; secondly, chemical ligation through a covalent bond; or thirdly, non-covalent binding. Although small-molecule or peptide microarrays can be prepared by any of the above three approaches, the protein microarray is amenable only to the two latter methods.

Solid-support
Amongst the large number of solid-support materials described for the production of microarrays, silica or glass is most often used because of its great chemical resistance against solvents, its mechanical stability, its low intrinsic fluorescence properties, and because it can be readily functionalized. Other solid-supports include cellulose sheets [2], polymer-based membranes, or other materials. Glass coated with polymers [15] or dendrimers [16] have also been reported.
In situ synthesis

The three general methods for \textit{in situ} synthesis of chemical microarrays are SPOT-synthesis \cite{2}, light-directed parallel synthesis with photolithographic masking methods \cite{3}, and the recently developed maskless light-directed synthesis using a digital micromirror array \cite{17**,18**,19}. Spot-synthesis uses the standard solid-phase peptide synthesis method, but the density of the spots obtained with this approach is low (e.g. 25 spots per cm\(^2\)). Fully automatic instruments such as the Auto-Spot Robot ASP222 (ABIMED, Langenfeld, Germany) for SPOT-synthesis are commercially available. Applications of peptide arrays made by SPOT-technology have been reviewed recently \cite{20}. The latter two methods of light-directed \textit{in situ} synthesis use advanced instruments and very high-density microarrays can be obtained. The photolithographic masking approach first reported by Fodor \textit{et al.} in 1991 \cite{3} used a photolabile protecting group (e.g. NVOC) for the building blocks and each peptide element measures 50 $\times$ 50 $\mu$m. In 1996, McGall \textit{et al.} \cite{10} further improved the technique by using photolithographic masking method to pattern a polymeric photoresist film on the glass surface. The patterned photosensitive film acts as a physical barrier to mask selected regions of the substrate from exposure to standard chemical reagents used in oligonucleotide synthesis. This newer approach has potential for generating microarrays with much higher densities.

In 1999, Singh-Gasson \textit{et al.} \cite{17**} reported the development of a maskless array synthesizer to generate \textit{in situ} oligonucleotide microarrays containing more than 76 000 features each measuring 16 $\mu$m\(^2\). This array synthesizer utilizes a digital micromirror device, which consists of a 600 $\times$ 800 array of micromirrors to form virtual masks, and theoretically can produce 480 000 pixels of synthetic oligomers in a 10 $\times$ 14 mm area. (R,S)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethyl chloroformate was used as the photolabile protecting group in the oligonucleotide synthesis. About the same time, Gao and associates \cite{18**} described a similar maskless microarray setup to synthesize oligonucleotide and peptide \cite{19} microarrays. Instead of using specialized building blocks with photolabile protecting groups, Gao used common building blocks with acid-labile protection groups that are commercially available. Spatially addressable deprotection was accomplished by the use of light to generate acid, which in turn removed the protecting group, allowing the growth of the oligomers \textit{in situ}. Although recent advances in \textit{in situ} synthesis are truly impressive, this approach is inherently inefficient as each compound spot has to be synthesized from its building blocks. In contrast, the high-throughput spotting of finished products by an automatic arrayer onto the surface is far more efficient because each compound needs to be synthesized only once, and multiple replicates can be produced simply by spotting.

Spotting methods

Instrumentation

Several automatic arrayers are commercially available. Spotting is based on direct-contact or non-contact deposition of the liquid sample. The pin-ring technique (e.g. GSM 417 arrayer, Affymetrix, Santa Clara, California) and the stamp micro-contact technique (e.g. SpotBot Personal Microarrayer, TeleChem International, Inc., Sunnyvale, California) allow disposition of spots of fairly reproducible size. The non-contact piezoelectric technique enables fast dispensing of monodisperse droplets. Moerman \textit{et al.} \cite{21} recently reported on the use of electrospraying in a stable cone-jet mode to generate a highly reproducible spot of biological material of 130–350 $\mu$m diameter, and as small as 50 pl.

Chemical ligation

For small molecules or short peptides, immobilization often requires covalent linkage of the compounds onto the solid-support. Schreiber’s group \cite{22} used Michael addition to link thiol-containing compounds to maleimide-derivatized glass slides to form a microarray of small molecules. In a subsequent report \cite{23**}, they described the covalent attachment of alcohol-containing small-molecule compounds onto chlorinated glass slides. We applied the highly selective chemo-selective ligation reaction to covalently attach peptides or small molecules onto the glyoxylyl-derivatized glass slide by oxime bond or thiazolidine ring formation \cite{24**}.

For protein microarrays, immobilization is accomplished by either covalent linkage or non-covalent adsorption. Schreiber’s group \cite{25} described the use of aldehyde-derivatized glass slide to print protein microarrays. The aldehyde groups on the glass surface react with primary amines on the protein to form Schiff’s base linkages. Bovine serum albumin (BSA) was used to block the remaining glass surface or other non-specific binding sites. Zhu \textit{et al.} \cite{26**,27} reported the development of a protein chip that consists of microwells fabricated with polydimethylsiloxane (PDMS). The microwell array has a density of 140 wells in an area of 1.8 $\times$ 2.8 cm. The wells are then activated by GPTPS, a crosslinking reagent, prior to protein immobilization. Lin \textit{et al.} \cite{28} recently reported the development of a microstamp-well system to print high-resolution protein microarrays on an aminopropyltrimethoxysilane surface that had been activated with bis-sultosuccinimidyl suberate. Each protein spot measures about 350 $\times$ 350 $\mu$m with a gap of 100 $\mu$m between each spot.

Non-covalent binding

In standard ELISA or dot-blot assays, most proteins are immobilized on the solid surface such as polystyrene plate or polymer membranes by simple adsorption. A similar approach has been applied for protein microarray assays. Martin \textit{et al.} \cite{29} described the generation of a hydrogel ‘stamper’ to generate microarrays (50–80 $\mu$m diameter spot) through non-covalent adsorption of the protein onto an aminosilane surface. Huang \textit{et al.} \cite{30,31} reported the use of hand spotting (0.25 pl) to apply 504 spots of a number of capturing antibodies onto the surface of Hybond ECL membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in an area of 6 $\times$ 8 cm. Ge \cite{32} described the use of a Bio-Rad 96-well dot block apparatus to generate a protein array on a 12 $\times$ 8 cm nitrocellulose membrane.
Arenkov et al. [33] described the preparation and application of a three-dimensional gel pad protein microchip that is comprised of proteins immobilized within polyacrylamide. The dimension of each gel pad ranges from $10 \times 10 \times 5 \mu m$ to $100 \times 100 \times 20 \mu m$. Knezevic et al. [34] used a GMS 417 arrayer to array a large collection of commercially available antibodies onto a thin film of nitrocellulose matrix bonded to a glass slide (FAST slides). Each protein spot measured 600 $\mu m$ in diameter.

In addition to direct adsorption onto the solid surface, the molecule to be microarrayed could also be biotinylated first and then printed onto a surface that has been precoated with a monolayer of streptavidin. We have successfully used NeutrAvidin (Pierce Chemicals) to coat polystyrene microscope slides, and blocked with BSA prior to spotting biotinylated peptides onto the slide using a GMS 417 arrayer (K Lam et al., unpublished data). The commercially available XNA on Gold™ microarray slide (NanoArc Corp) is precoated with streptavidin and the slide surface is patterned with a hydrophobic polytetrafluoroethylene to form 50 $\mu m$-deep wells. However, the array density of this microarray slide is relatively low (384 wells per slide).

Other types of chemical microarray

Microarray at optical fiber tip

In an effort to develop a highly miniaturized multianalyte sensor array, in 1994, Walt and colleagues [35] developed a four-sensor dye array at the distal surface of optical fibers. Three years later, the same group reported the use of a photodeposition patterning system to generate a polymer microarray that comprised thousands of individual elements photodeposited as hemispheres at the distal end of an optical imaging fiber, with each element corresponding to an individual core of the imaging bundle [36].

Multiplex analysis with quantum-dot-tagged microbeads

Han et al. [37••] recently reported on the development of polymeric microbeads with multicolor optical coding achieved by embedding different-sized hydrophobic quantum dots into these microbeads. Different known peptides, proteins or nucleic acids can be linked or adsorbed on small samples of each coded bead. Their interaction with fluorescent target molecules can be analyzed rapidly with flow cytometry. With DNA hybridization studies, these investigators demonstrated that the coding and target signals can be read at single-bead level simultaneously. A similar technology using traditional fluorophores has been commercialized by Lumines (Austin, Texas).

Combinatorial chemistry and chemical microarray

The microarray technology has emerged as a powerful technique for providing insights into molecular interactions, biochemical catalysis, drug development, and the field of proteomics. As mentioned earlier, the first peptide library or peptide array was described by Gysen et al. in 1984 [1]. In a way, the chemical microarray is nothing more than a library of immobilized compounds or proteins that are spatially addressable, highly miniaturized, and therefore requiring only a minimal amount of analytes for analysis. A decade ago, we reported the synthesis and screening of an OBOC combinatorial library [5]. In this method, using a split-mix synthesis approach [5,6,38], millions of peptide-beads were generated such that each bead displays only one chemical entity. Because each compound-bead is spatially separable, the OBOC combinatorial library can be viewed as a huge microarray that is not addressable. This array is highly miniaturized as each compound-bead measures only 50–80 $\mu m$ in diameter. The OBOC combinatorial approach is, in fact, the ultimate high-throughput synthesis and screening method available, as millions of compounds can be synthesized in a few days and screened concurrently. Using various screening techniques, we can identify compound-beads with a specific biological, chemical, or physical property, and isolate them for structural determination. Over the past 10 years, we [39] have successfully used this technology to identify ligands for various protein targets or small-molecule dyes, as well as peptide substrates for various protein kinases. In addition, using intact cell lines as a probe, we were able to identify ligands that bind to cell surface receptors [40]. We have also described a non-addressable chemical microarray system [41] in which the compound-beads were immobilized in a thin layer of soft agar together with cancer cells. After the linker was cleaved, compounds from each bead were released and diffused outward. A zone of growth inhibition around the positive bead was detected by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the culture plate. Schreiber's group [42] reported on the use of PDMS to generate plates of thousands of 1 mm diameter microwells into which a mixture of yeast and compound beads were distributed, such that the majority of the wells had 1–2 beads. After photolytic cleavage, the compounds were released and the biological assay proceeded in each well concurrently.

Recently, we reported the development of a new chemical microarray to complement the OBOC combinatorial library method [24••]. Ligands identified from the OBOC combinatorial libraries are resynthesized and immobilized on a glass plate or plastic plate in a microarray format and in multiple replicate sets. These ligand chips are then analyzed with multiple probes under multiple different conditions.

Schreiber's group has successfully used the OBOC combinatorial libraries with a cleavable linker to generate an array of stock solutions (5–10 mM in 5–10 µL) of single small-molecule compounds and printed these compound stock solutions on glass slides in a microarray format [43]. Small-molecule compounds that bind to human FKBP12, histone deacetylase-1, calmodulin, and a variety of glutathione-S-transferase-fusion proteins derived from yeast were identified with this approach. Schultz and his colleagues [44••] recently reported the use of split-mix synthesis method to generate compound libraries that contain a peptidomolecular (PNA) coding tag. The compounds are then cleaved off the solid-support, mixed
with a library of protein or crude cell extract, sized with a size-exclusion column, and the protein fraction (> 10 Kd) hybridized to an oligonucleotide microarray (Gen Flex tag array). This elegant approach enables them to spatially separate and decode the compounds that interact with the target proteins, based on the addressable oligonucleotide microarray.

Methods of analysis and applications
Most of the detection methods in chemical microarrays use standard immuno-detection techniques such as enzyme-linked colorimetric, fluorescent or luminescence methods. Binding assays are the most common, although functional assays such as protein kinase and protease activity profiling are also feasible. We have developed several on-bead screening assays for the OBOC combinatorial library method [39]. Essentially any of those on-bead screening assays can be applied to the chemical microarray method. For instance, immobilized peptides or protein substrates can be phosphorylated by protein kinases in the presence of [γ-32P] ATP, and the phosphorylated peptides or proteins can be detected by autoradiography or phosphor image analysis [24••,45]. Meldal et al. [46,47] developed a fluorescent-quenching approach to identify peptide substrate for proteases. This assay system can easily be applied to chemical microarrays as well. Other less-common assays such as time-resolved fluorometry [48], AC impedance, and electrochemical active labels may also be used to analyze a chemical microarray (see also Update). We used OBOC combinatorial library methods to identify peptide ligands that bind to intact cells [40] and have applied similar cell-adhesion assays to determine cell binding profiles of specific cancer cell line or cells from primary cultures to an array of immobilized ligands [24••]. Such profiling, in principle, will help to guide peptide-targeted therapies of cancer [49]. Furthermore, the cell-binding assay, when used in conjunction with appropriate fluorescent-labeled antibodies and confocal microscopy, will enable one to detect cell signalling or morphological changes of cells at the spots where cell attachment occurs.

Korbel et al. [50] recently reported the use of microarray technique to monitor chemical reactions by determining the enantiomeric excess of thousands of samples. Several investigators have successfully used protein microarrays to analyze different protein functions. Zhu et al. [27] used a microarray of 5800 yeast proteins to study their interactions with known proteins and phospholipids. Many new calmodulin- and phospholipid-interacting proteins were identified. Using similar technique, protein substrate profiles of 119 yeast protein kinases were evaluated [26••]. Huang et al. [31] used their antibody array to detect a large number of different cytokines in conditioned media and patient sera. Knezevic et al. [34] used an antibody microarray to analyze protein expression in tissue derived from squamous cell carcinoma of the oral cavity. Sreekumar et al. [51••] applied protein microarrays to discover novel radiation-regulated proteins. Zlauddin and Sabatini reported on the expression of defined cDNAs in microarrays of live cells [52••]. They first prepared a microarray of plasmid DNAs on a glass slide and then allowed a lawn of target cells to grow on the glass slide, resulting in a microarray of discrete spots of transfected cells of interest.

Conclusions
There has been enormous progress in the development and application of chemical microarrays to high-throughput biological and chemical analysis. Thousands to tens of thousands of small molecules, peptides or proteins can now be immobilized in a small area on a solid-support in an addressable fashion. In the next two to three years, the quality of the chemical chips and instruments that produce them will further improve, and many will become commercially available. As the technology becomes more popular, many researchers will apply the technique to solving many different biological, chemical or physical problems. Newer and more sophisticated assay methods and instruments will be developed. Fully automatic mass spectrometers that enable one to instantly identify all the biological molecules or proteins that bind to each of the microarray spot will be developed. Combinatorial chemists will continue to develop small-molecule microarray techniques for efficient drug identification and optimization. Although the chemical microarray method is primarily a research tool at this time, it is expected that this approach will eventually develop into inexpensive and highly efficient medical diagnostics or environmental monitoring tools in the future.

Update
Recently, Fang et al. [53] reported on the fabrication of a membrane protein microarray. In this method, glass or gold-coated glass surface was first derivatized with γ-aminopropylsilane, and cell membrane preparations containing G-protein-coupled receptors were spotted with a quill-pin printer. Specific ligand binding to these membrane protein microarrays was observed.

Several groups have recently reported on the application of antibody microarrays for proteomic profiling of the cancer microenvironment [54], and immunophenotyping of leukemias [55]. Madoz-Gurpide et al. [56] used conventional chromatography or electrophoresis to fractionate total cell lysate. Each fraction was then printed on a microarray for subsequent analysis. Pawelczak et al. [57] described the so-called reverse-phase protein microarrays, in which serial dilution of total cell lysates derived from microdissected tissues are immobilized on nitrocellulose-coated glass slides. These cell lysates are then analyzed with different enzyme-linked antibodies against specific protein or phosphoprotein.

Park and Clark [58] described the development of a sol-gel encapsulated enzyme array for high-throughput screening of biocatalytic activity or enzyme inhibition. In this method, sol-gel microstructures containing enzymes were stabilized on glass, and a multi-well bilayer of polydimethylsiloxane
was used to support the enzyme array. Weng et al. [59] reported an addressable mRNA–protein fusion array that was formed by self-assembly of in vitro translation products, via hybridization, to surface-bound DNA capturing probes. Rakow and Suslick [60] described the development of a colorimetric sensor array that can detect volatile chemicals at a concentration below two parts per million. In this method, a microarray of metalloporphyrin dyes were immobilized on a flat surface, and a distinct color pattern emerged when the colorimetric sensor interacted with a specific volatile chemical. As an alternative to fluorescent or colorimetric detection methods, Morozov et al. [61] reported the use of a charge-coupled device to quantitatively detect isotope-labeled ligands bound to a protein microarray.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


This paper describes the development of maskless light-directed synthesis of a microarray, and introduces the concept of using photogenerated acids in the preparation of a chemical microarray.


This paper describes the covalent attachment of an alcohol-containing small-molecule library to chlorinated glass to form a microarray.


This paper describes the use of chemoselective chemistry to link compounds to glass slides to form a microarray. The three assays used in the analysis of this microarray involve protein binding, peptide substrate phosphorylation and cell adhesion.


This paper describes the development of a protein chip that consists of microwells fabricated with PDMs, and the application of this protein chip to analyse yeast protein kinase substrate profile.


This paper describes the development and use of polymeric microbeads for multiplexed optical coding of biomolecules. This method helps to sort and decode the ligands with bound target proteins.


This paper illustrates how a microarray of capturing antibodies can be used to profile cancer cell proteins.

52. Zlauddin A, Sabatini DM: Microarray of cells expressing defined cDNAs. Nature 2001, 411:107-110. This paper describes the use of a plasmid DNA microarray to transfect cells in situ to form a new cell microarray with newly expressed proteins encoded by the plasmid DNA.


