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Probing Biology with Small Molecule Microarrays (SMM)

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Abstract In the continuous drive to increase screening throughput and reduce sample requirement, microarray-based technologies have risen to the occasion. In the past 7 years, a number of new methodologies have been developed for preparing small molecule microarrays from combinatorial and natural product libraries with the goal of identifying new interactions or enzymatic activities. Recent advances and applications of small molecule microarrays are reviewed.

Keywords Activity profiling · Combinatorial libraries · Diagnostic · Screening · Small molecule microarray (SMM)

Abbreviations

Ab	Antibody
AGT	Alkyl guanine transferase
Boc	*tert*-Butoxycarbonyl
Cy3, Cy5	Cyanine-3, cyanine-5
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocarbamate
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GST	Glutathione-*S*-transferase
MALDI	Matrix-assisted laser desorption ionization
NHS	*N*-Hydroxysuccinimide
NVOC	Nitroveratryloxycarbonyl
PNA	Peptide nucleic acid
SMM	Small molecule microarray
SPR	Surface plasmon resonance

1 Introduction

Astute observation of unanticipated results has often contributed to scientific breakthroughs. As famously said by Louis Pasteur, "chance favors the prepared mind". To explore biology, screening for small molecules that perturb processes has been a fruitful approach by providing the means to dissect the role of individual actors in complex biological networks. With the advent of automation, much effort has been devoted to increase our capability for discovery-driven research and to investigate the millions of interactions that make up a biological organism. Combinatorial chemistry has enabled small teams of chemists, as well as academic laboratories, to prepare large compound libraries. As for screening, the 96-well plate has gradually been substituted by the 386- or 1544-well plate, reducing the sample requirement from 50 μL/well to 5 μL/well. This means that 10 000 compounds now require a total volume of 50 mL using 1544-well plates (5 μL/well). Using microarrays, 10 000 compounds can be screened in less than 100 μL without sophisticated automation. More importantly, ligands can be identified for

proteins obtained from crude cell extract without further purification or labeling, thus enabling discoveries of unanticipated interactions. This level of miniaturization is not only important for screening but also to measure multiple enzymatic activities in parallel from complex proteomic mixtures. While the first small molecule microarrays (SMM) were reported in the early 1990s using photolithography for their preparation, it was the success of DNA microarrays that inspired the widespread exploration of SMM as a tool to probe biological events. This chapter is divided into two main sections: (i) the preparation of SMM with a discussion of microarray surfaces, in situ microarray synthesis or immobilization of existing libraries, and (ii) the screening of SMM with a discussion of ligand discovery, enzymatic activity measurement, and carbohydrate recognition as well as non-immobilized SMM for multicomponent screening and cellular assays.

2
SMM Preparation

Several techniques have been reported for preparing microarrays (including photolithography, contact printing, and inkjet), yielding arrays with densities ranging from 1000 to 500 000 features per square centimeter [1, 2]. While photolithography is used on an industrial scale (Affymetrix) to pro-

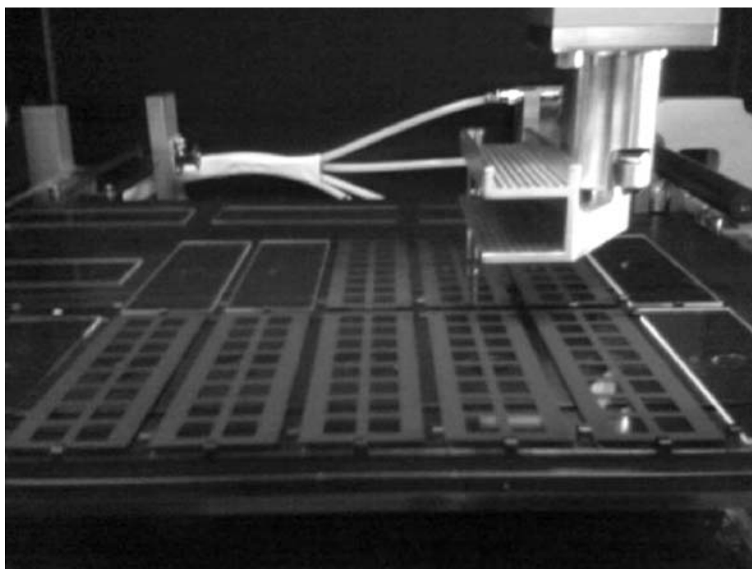

Fig. 1 Contact printing robot: expanded view of the needles with capillaries for solution pick up and delivery

duce oligonucleotide microarrays with feature size from 14 to 50 μm, most SMM have been prepared using a contact printing robot. In its simplest format, the robot picks up a solution from a microtiter plate by capillary action and delivers nanodroplets of solution (ca. 1 nL) on the array simply by getting into contact with the surface in a similar fashion as does an ink pen (Fig. 1). Depending on the conditions, features ranging from 100 to 300 μm are obtained by this method. The first such robot was assembled according to a protocol published by the Brown laboratory in the mid 1990s [3]. By the end of the 1990s, such robots had become commercially available; current versions have the ability to print 10–50 microarrays containing 10 000 features in a matter of hours.

2.1 Microarray Surface

Most contact printing is carried out on standard 25 mm × 75 mm glass microscope slides, which can be functionalized but are otherwise chemically inert and have low intrinsic fluorescence. As shown in Fig. 2, the silanol glass surface can be treated with (3-aminopropyl)triethoxysilane, which reacts covalently with the glass via hydrolysis/condensation resulting in aminosilanized slides (careful preparation is crucial to ensure an even surface) [4]. The amino groups can be subsequently coupled with a bifunctional linker (e.g., Fmoc-8-amino-3,6-dioxaoctanoic acid [5], poly(ethylene glycol) diglicydyl ether [6], or 1,8-diamine-3,6-dioxaoctane via carbonate formation [7]) and termi-

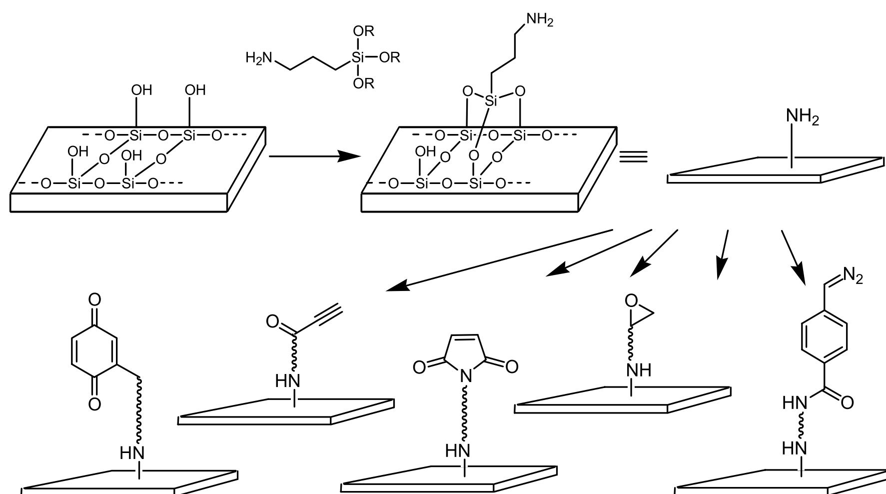

Fig. 2 Functionalization of glass slides. The silanol surface is reacted with aminopropylsilane, which is further reacted with a bifunctional linker and adequate spacers to obtain the desired functionalized surface

nated with a functional group for covalent attachment of small molecules (maleimide, diazobenzylidene, *N*-hydroxysuccinimide, epoxide, alkyne, isocyanate, etc.). While it has been demonstrated that a small molecule–protein interaction is possible with small molecules attached directly to the glass surface [8], it has generally been noted that higher sensitivity and better signal-to-noise ratios are obtained if a PEG spacer is used [7, 9, 10]. To improve loading and homogeneity of the glass surfaces, aminosilylation followed by coupling with dendritic molecules (PAMAM) and subsequent crosslinking was explored. Optimized procedures led to a chemically activated polymer film with a tenfold increase in loading (150 fmol/mm^2 vs. 5 fmol/mm^2 for the aminosilane slides) and high resistance [11]. The polarity of the surface formed was modulated by changing crosslinking reagents, which allowed modifications of the spot size and sharpness. Alternatively, uncoated glass slides were functionalized with methacryloxypropyl-trimethoxysilane and incubated with tetraethylenepentamine, thereby introducing multiple reactive groups per attachment point to the surface. This surface was further functionalized with acryloyl chloride, epichlorohydrin, or 1,4-butanediol diglicydyl ether to obtain acrylic, epoxy hydrophobic, and epoxy hydrophilic surfaces, respectively [12]. Aside from glass, arrays were also prepared on a gold surface, which can be used directly for surface plasmon resonance (SPR) or mass spectroscopies. Generally, a self-assembled alkanethiolate monolayer was partially functionalized with a handle for subsequent derivatization such as a hydroquinone [13], or carboxylic [14] or NHS groups [15].

2.2 In Situ Synthesis

2.2.1 Light-Directed Synthesis

The first microarrays reported in the literature were prepared by synthesizing the small molecule directly on the array surface in a combinatorial fashion using monomers blocked with a photolabile protecting group (nitroveratryloxycarbonyl, NVOC) and photolithography masks to achieve selective deprotection at a given coordinates (Fig. 3). This light-directed in situ synthesis was demonstrated to be effective for the preparation of oligopeptide and oligonucleotide microarrays [16] and was later used for the preparation of unnatural oligomers (carbamates) [17]. Larger oligonucleotide arrays were subsequently reported using a new photolabile protecting group – α-methyl-6-nitropiperonyloxycarbonyl (MeNPoc) – allowing the synthesis to be carried out on features of 5–10 μm (10^6 sequences/cm^2) [18]. The spatial resolution achieved using that method is close to the physical limitations of diffraction for high contrast. Using an effect of the non-linear response of semiconducting photoresistant films to light, thus lowering the contrast requirements,

Fig. 3 Light-directed synthesis. An area of the array is selectively irradiated with UV light using photolithographic masks to trigger the deprotection of photolabile groups such as Nvoc (**a**) or the generation of an acid for deprotection of acid labile groups (**b**)

it is possible to further improve the density. The polymeric film is used to construct a pattern, which masks selected regions of the substrate from exposure to standard chemical reagents during synthesis, whereas the unprotected areas are exposed to coupling of new monomers. A microarray of 8 μm feature size was prepared with this method, raising the possibility of further optimization [19]. Alternatively, as the cost of preparing photolithographic masks is considerable, a maskless method using a digital micromirror device (digital light processor, DLP) was developed [20]. Using the DLP technique and a single photolabile reagent (MeNPoc-protected glycolic acid) a peptoid array was synthesized [21]. Shortly after, a strategy for the synthesis of spatially addressable arrays of cyclic peptides was also reported [22]. These experiments expanded the scope of light-directed synthesis of microarrays beyond specialized environments. DLP has also been applied to the preparation of oligonucleotide and peptide microarrays using building blocks with standard acid-labile protecting groups (DMT and Boc, respectively). Instead of directly removing the protecting group, light is used to generate an acid with spatial resolution resulting in deprotection of the standard Boc and DMT groups (Fig. 3b) [23–25]. Although the microarray density is lower than in the masking methods (ca. 3000 features/cm^2), the technique is more flexible than classical mask photolithography.

2.2.2 SPOT-Synthesis

In the late 1980s, Frank and coworkers developed a method for parallel solid phase synthesis where the molecules were linked to a membrane rather than to conventional polystyrene beads [26, 27]. Libraries were prepared by adding the different reagents at each of the spots on the membrane. This SPOT-synthesis method can be carried out on cellulose and polypropylene sheets (loading: 100–10 000 nM/cm^2 or 10–100 nM/cm^2, respectively) and requires no specialized infrastructure to obtain libraries. The mean spot size

is 6 mm, which makes these arrays several orders of magnitude less dense than microarrays. However, the spots are sufficiently large that they can be excised and the molecules recovered from the membrane after cleavage [28]. For preparation of small molecule arrays, the cellulose has to be activated (epibromohydrin or tosyl chloride) to introduce a flexible diamino spacer allowing for further functionalization. In the case of polypropylene membranes, photoinduced coupling with acrylic acid or methyl acrylate prior to the spacer introduction proved to be effective. Such surfaces can be further modified with linker such as Rink or Wang linkers. Macroarrays of 1,3,5-triazines [29], cyclic peptidomimetics [30], 1,3,5-hydantoins [31], natural product fragments (from sorangicin and epothilone) [32], chalcones [33], and α-acyl amino amides [34] have been reported, testifying to the flexibility of this method. The synthesized libraries can be screened against the biological targets directly on the support (e.g., protein binding, antibody binding, or metal binding assays) or reformatted in a microtiter plate or microarray after cleavage.

2.3 Chemoselective Immobilization

As an alternative to in situ synthesis, molecules can be immobilized to the surface after their synthesis. To this end, it is critical to have a method that is chemoselective and operates under mild conditions to avoid degradation of the molecule or to preclude it from interacting with a protein. In the case of combinatorial libraries, the design of the synthesis can include a specific functionality, which can be leveraged to ligate the small molecule to an appropriately functionalized surface with controlled orientation. Existing methodologies are listed below (Fig. 4).

2.3.1 Immobilization of Thiols on a Maleimide Surface

The first SMM microarray prepared by contact printing using a chemoselective immobilization was reported by Schreiber and coworkers in 1999 [35]. Amine-functionalized slides were treated with a bifunctional linker (NHS/maleimide) to obtain a maleimide-functionalized surface (Fig. 4a). Three molecules (biotin, digoxigenin, and a pipecolyl α-ketonamide – an FKPB ligand) conjugated to a cysteine residue were spotted and the array was then probed simultaneously with FITC-conjugated streptavidin, Cy3-conjugated DI-22 (an anti-digoxigenin Ab), and Cy5-conjugated FKPB. After a brief washing, the three differently labeled proteins were detected at the respective wavelength of their fluorophore only in the locations corresponding to their cognate ligands, thereby demonstrating that SMM could be used to probe small molecule–protein interaction and be multiplexed.

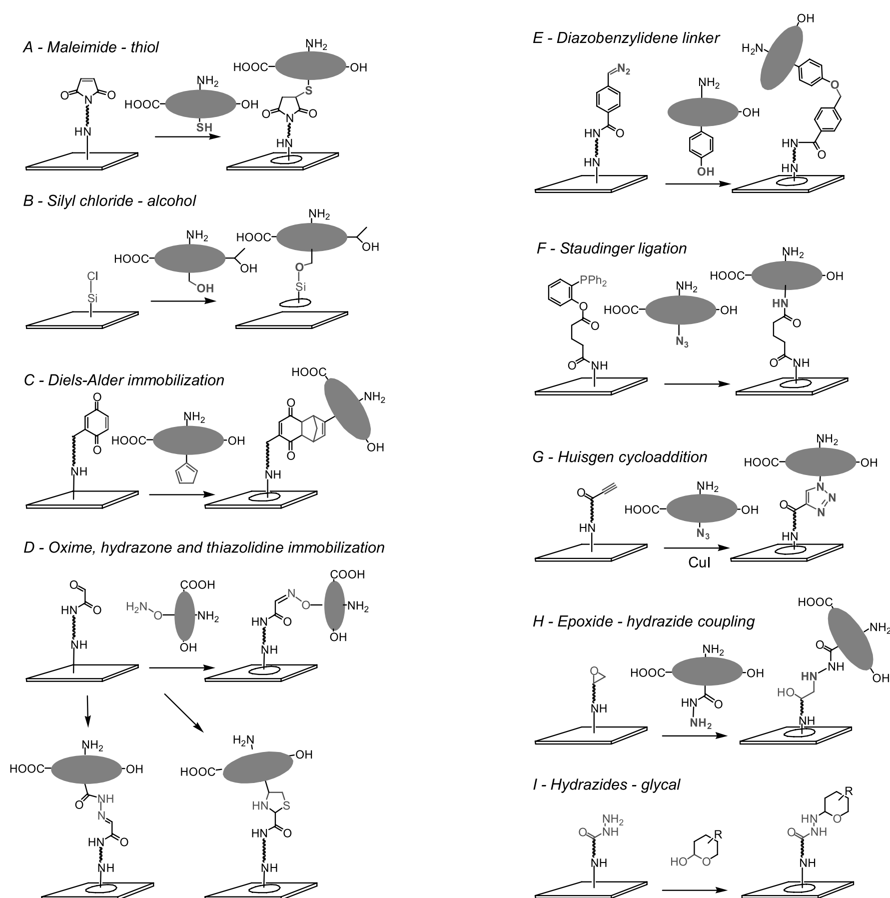

Fig. 4 Chemoselective ligation methods: **a** maleimide linker, **b** silyl linker, **c** Diels–Alder ligation, **d** glyoxylyl linker, **e** diazobenzylidene linker, **f** Staudinger ligation, **g** Huisgen [3 + 2] cycloaddition, **h** hydrazide linker, **i** glycal immobilization

2.3.2
Immobilization of Alcohol on a Silyl Chloride Surface

While most SMM have some form of a linker that distances the small molecule from the surface so as to minimize steric interactions between the protein and the surface, direct immobilization on the glass surface has also been reported to be effective. Treatment of the glass surface with $SOCl_2$ in THF using catalytic amounts of DMF transformed silanols on the surface into chlorosilanes [8]. This chlorosilane was found to react readily with primary alcohols but rather slowly with secondary alcohols or phenols (Fig. 4b). To validate the detection of small molecule–protein interactions, the interactions of three known ligand–protein pairs (α-ketoamide, digoxigenin, and biotin)

were investigated and led to the observation that, despite the lack of linker, binding was detected. An important motivation for the development of this immobilization strategy was that it is compatible with libraries synthesized by the same group using a silyl linker. The viability of this immobilization method beyond the proof of concept has been validated with the discovery of new ligands from large libraries (> 1000 microarrayed compounds) [8, 36]. Furthermore, it could also be used to immobilize natural products, which often bear a primary hydroxyl group.

2.3.3
Immobilization Through Formation of Oximes, Hydrazones, and Thiazolidines

Hydroxylamines or hydrazines are known to react selectively with aldehydes and even faster with glyoxylyl groups. Glyoxylyl-functionalized slides were obtained by derivatizing aminosilanized glass slides with serine which, after deprotection, was oxidized with $NaIO_4$ to obtain the glyoxyl functionality [9]. Alternatively, direct coupling of protected glyoxalic acid to aminosilanized glass slides and its subsequent deprotection also afforded glyoxylyl-functionalized slides. This surface was shown to react selectively with peptides labeled with hydroxylamine or a terminal cysteine residue, which yielded a thiazolidine ring (Fig. 4c). The authors noted that improved results were obtained in the spotting when the surface was prepared with a mixture containing protected glyoxalic acid and stearic acid to increase the hydrophobicity of the surface. The immobilization chemistry was validated using Cy3-labeled streptavidin to detect immobilized biotin. The possibility of using this array for functional assays was also demonstrated by treating an immobilized kinase substrate (octapeptide) with Src kinase in the presence of $[\gamma\text{-}^{33}P]$-ATP followed by autoradiography imaging of the phosphorylated peptide. It was further shown that arrayed peptides could be used for cell adhesion assay by means of a known ligand/cell surface receptor pair. For the purpose of cell adhesion screens, non-specific binding could be eliminated by derivatizing the glass surface with $PEG_{5000}NHNH_2$ after spotting of the small molecules.

Conversely, peptide microarrays prepared from glyoxylyl-labeled peptides and a semicarbazone-derivatized surface were also reported [37]. Glass slides covered by a semicarbazide sol-gel layer were found to reduce non-specific protein absorption and improve signal-to-noise ratio for antibody detection. Using three immobilized peptide epitopes from hepatitis C and Epstein-Barr virus, the concentration of specific antibodies in patients' sera could be measured. The microarray format was compared to traditional ELISA using 130 samples of sera from HCV-infected individuals, resulting in superior sensitivity and selectivity for the microarray format (there were 13 false positives by ELISA). This ligation method was also found to be effective for antibody immobilization. Periodate oxidation of the antibodies' carbohydrates yielded aldehydes that were conjugated to the semicarbazone.

2.3.4
Immobilization via Diels–Alder Cycloaddition

The orthogonality of the Diels–Alder reaction and its compatibility with most functional groups defined it as a suitable reaction for chemoselective immobilization (Fig. 4d) [13]. A self-assembled monolayer of alkanethiolates on a gold surface terminated with hydroquinone was activated after a mild reduction that converted the hydroquinone into benzoquinone, an excellent dienophile. Three known peptide substrates to kinases (*c-Src*, PKA, and c-Abl) were labeled with cyclopentadiene and then arrayed. Using [γ-^{33}P] ATP, it was shown that only the matched substrate was phosphorylated with Src kinase. It has also been demonstrated that a Diels–Alder reaction can be used to immobilize proteins as well [38]. In this case, a maleimide surface was used as the dienophile and spotted with hexadienoic acid-labeled proteins obtained by ligation reaction. As thiol groups from cysteine residues also reacted with the maleimide, the cysteine residues were protected prior to spotting using Ellman's reagent.

2.3.5
Immobilization to a Diazobenzylidene Surface

To broaden the range of functional groups compatible with an immobilization reaction, a diazobenzylidene-functionalized surface (Fig. 4e) was developed as this functional group is known to selectively react with heteroatoms bearing acidic protons (such as phenols, carboxylic acids, or sulfonamides) [39]. An aminosilane surface was derivatized with the toluenesulfonylhydrazone of 4-carboxybenzaldehyde, which was converted to the desired diazobenzylidene functionality following base-induced elimination. The functional group compatibility of this immobilization method was evaluated with a series of FKPB12 and biotin derivatives, demonstrating that the diazobenzylidene functionality reacts only with heteroatoms having a proton with a $pK_a < 11$. The effectiveness of this strategy for ligand discovery was confirmed with the discovery of new calmodulin ligands from a library of > 6000 immobilized phenols.

2.3.6
Immobilization via Staudinger Ligation

The mildness and orthogonality of the Staudinger ligation with respect to most functional groups [40, 41] makes this reaction ideal for immobilization of small molecules on an array with controlled orientation (Fig. 4f). To this end, an amine-functionalized slide with PAMAM dendrimer was derivatized with glutaric anhydride to introduce terminal COOH groups, which were subsequently esterified with 2-(diphenylphosphanyl)phenol.

Small molecules labeled with an azide were conveniently obtained directly from solid phase cleavage by using a Kenner-type linker followed by cleavage using 6-azidohexylamine. The immobilization chemistry was validated with a series of biotinylated peptides and a biphenyl-antibiotic, as well as manose, which were recognized selectively either by Cy5-labeled anti-biotin Ab or fluorescently labeled concanavalin A, which binds to manose. It was shown that the array could be washed and reused with nearly the same sensitivity of detection, which attests to the stability of the crosslinked dendritic surface. Concurrently, an alternative Staudinger ligation with a thioester was also used to immobilize peptides [42]. The authors prepared a 15-mer peptide which binds to RNase S and showed that the immobilized RNase S had nearly full activity. More recently, the Staudinger ligation was applied to site-selective covalent immobilization of proteins [43]. The azide-modified C-terminus of a protein was prepared by expressed protein ligation (EPL) in vitro. This chemoselective immobilization was validated with the microarraying of Ras, which was detected using a Cy5-labeled anti-Ras Ab.

2.3.7 Immobilization via Huisgen Cycloaddition

The Cu-catalyzed [3 + 2] Huisgen cycloaddition of azides [44] to alkynes has proven itself to be a very mild and reliable reaction, proceeding in aqueous environments and orthogonal to most other functional groups, thus being highly suitable for conjugation of highly functionalized molecules (Fig. 4g). This reaction was exploited for the preparation of carbohydrate arrays [45–48]. Commercially available amine-coated and NHS-functionalized glass slides were coupled with a linker terminated with an electron-poor alkyne and having an internal disulfide bond. The latter can be reductively cleaved from the support to analyze the degree and quality of carbohydrate immobilization. The reliability and efficiency of this method were not only demonstrated with several lectin-binding assays but also by its use to map epitopes of therapeutically important antibodies (vide infra).

2.3.8 Immobilization Using Hydrazides

Epoxides react faster with hydrazides than other nucleophilic functionalities such as hydroxyl, amine, carboxylic, and even thiol, which should allow for selective ligation of small molecules having hydrazide functionality (Fig. 4h). The epoxide surface was prepared from amine-coated slides immersed in a solution of poly(ethylene glycol) diglicydyl ether. Careful analysis of functional group compatibility using carbohydrate ligands showed that the immobilization reaction was selective for hydrazides in the presence of thiols at pH > 5 [10]. Carbohydrate arrays were also prepared from unlabeled saccha-

rides using a hydrazide surface (Fig. 4i). The unfunctionalized carbohydrates containing a hemiacetal at the reducing end were immobilized and probed with lectins. Notably, it was found that a hydrazide surface was superior to a hydroxylamine surface due to the predominance of the acyclic product oxime generated in the latter reaction, whereas the hydrazide coupling yielded mostly the β-anomeric cyclic product. Using this technique, glycan microarrays were prepared and their ability to detect pathogens was demonstrated [49].

2.3.9
Self-Sorting Supramolecular Immobilization

Instead of screening biological samples directly on the surface, as in the case of covalent attachment of small molecules or proteins to the glass slide, an alternative method has been developed where the small molecules are covalently tethered to a peptide nucleic acid (PNA) tag such that libraries can be screened in solution prior to self-assembly by sequence-specific hybridization to an oligonucleotide microarray [50, 51]. Aside from minimizing potential problems associated with the display of ligands or substrates on a surface (non-specific interactions with the surface, high local concentration), it also allows separation of ligands that are bound to a protein from unbound ones prior to hybridization and as such offers a detection method that is not possible with covalently immobilized compounds (vide infra) [52]. A second asset of the PNA tag is that it can be used to encode libraries prepared by mix-and-split combinatorial synthesis by using a unique PNA codon for every building block in the library [53]. Upon cleavage from the solid phase, the library is obtained as a mixture in solution; however, it sorts itself into an addressable microarray upon hybridization (Fig. 5). This PNA-encoded strategy has been validated with the discovery of inhibitors from libraries of > 1000 compounds and the profiles of substrate specificity and enzymatic activity from complex proteomic mixtures [54–58].

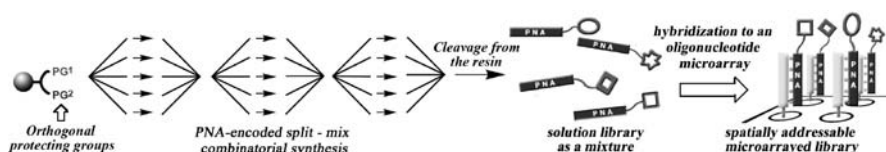

Fig. 5 Immobilization through self-sorting of PNA-encoded libraries. Starting from a bifunctional linker with orthogonal protecting groups, a library is prepared by split and mix combinatorial syntheses where a specific PNA codon is used to encode every building block. The libraries are then cleaved from the solid phase to obtain a mixture in solution, which is converted to a SMM by hybridization to an oligonucleotide microarray [51]

2.3.10
Physisorption Using Lipophilic Tags or Fluorous Tags

Rather than using a covalent reaction to immobilize the small molecule to a surface, a small molecule tagged with a tail having special physical properties could adhere to a surface by physiosorption. This was demonstrated for the preparation of carbohydrate arrays in microtiter plates where carbohydrate labeled at the anomeric position with saturated carbon chains between 13 and 15 carbons were retained on a hydrophobic surface and were resistant to aqueous washing. The viability of this immobilization was demonstrated with the detection of several carbohydrate–lectin interactions [59]. Conversely, it was also shown that such lipophilic tails could be introduced via Huisgen cycloaddition [60].

The unique properties of fluorinated alkanes (immiscible with water or organic solvent) have prompted the development of perfluorocarbon chains linked to small molecules as a handle for purification [61]. Recently, this principle has been applied to the preparation of carbohydrate microarrays [62]. Four sugar derivatives with a prefluorooctane tag linked to the anomeric position were prepared and spotted by a standard DNA arraying robot onto a commercially available glass microscope slide coated with a Teflon/epoxy mixture, or onto a surface prepared by the reaction of a fluoroalkylsilane with uncoated glass slides. The array was then successfully screened against respective fluorescently labeled lectins and the result was reproducible after rinsing with water, buffers, and detergent (Tween-20).

2.4
Non-specific Immobilization

All the specific immobilization methods listed above require a certain functional group to be present in the immobilized compounds. These methods are desirable for combinatorial libraries where the required functionality may be introduced but lend themselves poorly to immobilize natural products or known drugs that do not have a single common functionality that can be targeted. Two methods have been reported to address immobilization of natural products or libraries lacking a common functionality: photocrosslinking, and reaction with an isocyanate surface (Fig. 6).

2.4.1
Photocrosslinking

Amine-coated glass slides were derivatized with a linker terminated with the known photoaffinity reagent trifluoromethyldiazirinbenzoyl (Fig. 6), which upon UV irradiation releases a nitrogen molecule and is converted into a highly reactive carbene that reacts indiscriminately with small molecules.

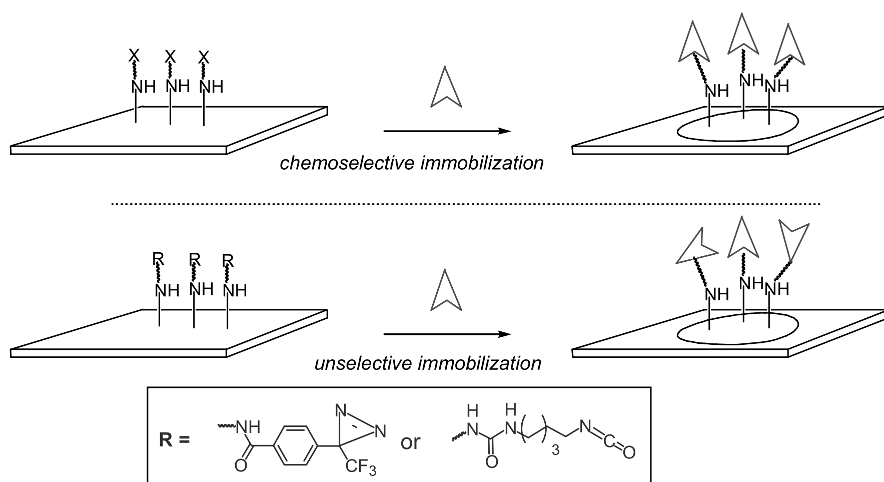

Fig. 6 Non-specific immobilization methods with covalent bond formation. Chemoselective immobilization will yield a SMM with a defined orientation (*top*) whereas unselective immobilization will allow different orientations of the small molecule on the array (*bottom*)

The lack of selectivity in the photocrosslinking was seen as advantageous because it means that small molecules will be presented into different orientations, which reduces the chance that the attachment point to the surface interferes with binding to its target. The method was validated with the immobilization of a variety of natural products such as the steroidal glycosides digoxin, digitoxin and digoxigenin, FK506, rapamycin and cyclosporine A. All the immobilized products were successfully recognized in a specific manner by their protein targets or antibodies [7].

2.4.2
Immobilization to an Isocyanate Surface

Aminosilanized glass slides were coupled with an ω-aminoacid linker functionalized with α,ω-diisocyanatohexane yielding an isocyanate-functionalized surface (Fig. 6). Isocyanates react with many nucleophilic functional groups and should also produce attachment of product in multiple orientations. It was demonstrated that alcohols, phenols, carboxylic acids, amines, and anilines all reacted with comparable efficiency on this surface. A "diversity microarray" containing nearly 10 000 bioactive small molecules, natural products, and compounds originated from several diversity-oriented syntheses were prepared and detected with antibodies against compounds of interest. It was also shown that by using an appropriate PEG spacer to avoid non-specific protein interactions, a crude proteomic mixture could be used directly in the screening without prior purification [5].

2.5 Other Microarray Preparation

2.5.1 Dip-Pen Nanolithography

Protein nanoarrays were prepared using a scanning probe microscopy (SPM)-based lithography technique by deposition of lines and dots of 16-mercapto-hexadecanoic acid (MHA) [14] or 16-mercaptoundecanoyl-NHS [15] on a gold thin-film substrate. The spot size ranged from 100 to 600 nm, which was significantly lower than the standard 5–20 μm features achievable by photolithography or the 100–300 μm achievable by contact printing. The surrounding area was passivated with 11-mercaptoundecyl-tri(ethylene glycol). Proteins were then immobilized by simple adsorption (MHA) or reaction with the NHS. In a proof of principle, it was shown that protein–protein interaction could be detected by atomic force microscopy or fluorescence.

2.5.2 Microarrays with Non-immobilized Small Molecules

While screening immobilized compounds in a microarray has already enabled the discovery of important inhibitors, the fact that the small molecules are covalently attached to the surface limits the types of screens that can be used, most notably in cell-based assays. Two approaches have been developed to address these limitations: (i) microarrays of nanodroplets, which have been used for multicomponent enzymatic assays, and (ii) microarrays of small molecules embedded in a biodegradable polymer for cell-based screens. For nanodroplet microarrays, it was found that small molecules could be deposited in a microarray format as glycerol nanodroplets (1.6 nL) by standard contact printing at a density of 400 spots/cm (similar density to contact printing) and that reagents or enzymes could be metered into each droplet using aerosol deposition without any cross-contamination amongst nanodroplets (Fig. 7). Because of water's fast evaporation in these small volumes, multiple additions by aerosol are possible. The utility of this method was demonstrated by screening a library of 352 compounds against thrombin, chymotrypsin, and three caspases [63]. More recently, this method has been used to identify inhibitors of SARS. A potential limitation of this approach is that the assays need to be carried out in a high concentration of glycerol to reduce the evaporation rate, but it was shown that at least several therapeutically important enzymes (proteases and kinases) are functional under these conditions.

To extend the utility of SMM towards cell-based screens, small molecules impregnated in a biodegradable polymer solution were microarrayed by contact printing on standard glass slides. Cells were cultured on top of the

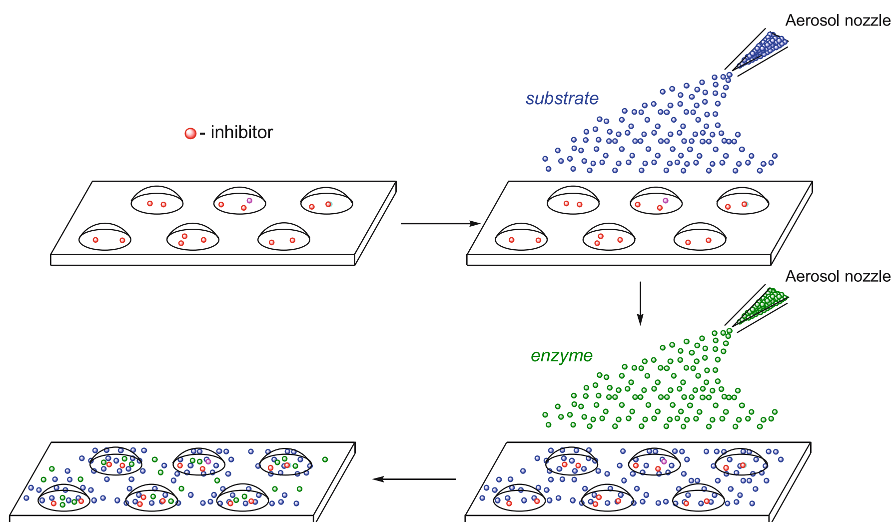

Fig. 7 Microarray of nanodroplets as individual reaction vessels. Small molecules are arrayed in a glycerol solution and the subsequent reagents are added as an aerosol

array allowing each compound to slowly diffuse out of the polymer and affect neighboring cells. After screening several polymers, polylactide/glycolide copolymer (PLGA) was identified as the best candidate [64]. As a proof of principle, it was shown that cytotoxic compounds indeed killed cells in the proximity of the compound spot but not beyond. It was also shown that this format was compatible with other phenotypes than cell death, using rapamycin for the inhibition of ribosomal phosphorylation. The readout was achieved by staining the cells with a specific anti-phosphoribosome Ab after they were fixed and permeabilized. This method was then used to evaluate the synergistic effect of 70 small molecule inhibitors in combination with seven different siRNA. These results demonstrated that high content cell-based assays can be performed in a highly miniaturized microarray format that does not require sophisticated automation. Using the standard microarray density with a 200 μm spot, less than 100 cells are necessary to screen each compound.

2.6 Commercially Available Functionalized Surfaces

Slides for microarrays have become commercially available with a variety of functionalized surfaces for oligonucleotide, protein, and small molecule immobilization. Aminopropylsilane with different surface properties are available from Asper, Corning, and Schott; amine-reacting NHS slides are available from GE Healthcare and Schott; epoxide-functionalized slides are available from Corning and Schott; aldehyde-functionalized surface for im-

mobilization through reductive amination are available from Schott; and isothiocyanate-functionalized surface are available from Asper. Industrialized and standardized preparation conditions ensure high and reproducible quality of the surfaces, which are often superior to those prepared from the underivatized slides directly before microarray spotting.

3 SMM Screening

As seen in the previous section, a number of methods have been developed for displaying small molecules in a microarray format. This section describes various methods for SMM screening [65]. For screening of protein–ligand interactions, the simplest method is to obtain a fluorescently labeled protein; however, such labeling procedure can be cumbersome and may potentially inactivate the protein. Alternatively, specific antibodies can be used if available or the protein can be genetically tagged with a His-6 tag, GST tag, with fluorescent proteins such as GFP [66], or with AGT, which specifically reacts with 6-*O*-alkylguanine-fluorophore conjugates (Fig. 8) [67]. Fluorescently tagged proteins have also been obtained from crude lysates by labeling the C-terminus using a fluorophore–puromycin conjugate [68].

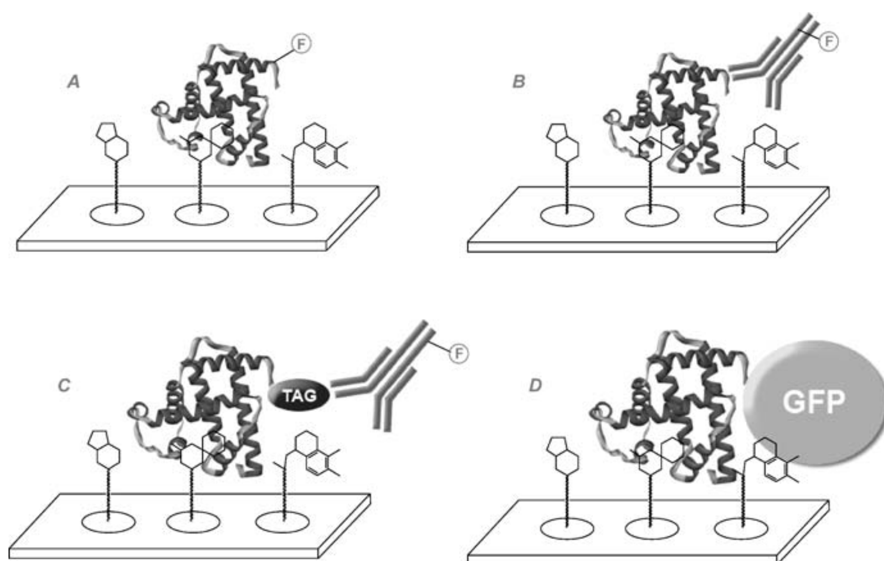

Fig. 8 Detection of a small molecule ligand interaction. **a** The protein of interest is itself labeled with a fluorophore F. **b** A specific labeled antibody is used for detection. **c** The protein of interest is expressed with a tag such as His-6 or GST and a labeled anti-tag Ab is used. **d** The protein of interest is expressed fused to green fluorescent protein (GFP)

3.1 Screening Ligand–Protein Interactions

Most methods that have been reported in the literature to immobilize small molecules on microarrays validate the immobilization strategy using well-known interactions such as biotin-streptavidin, digoxigenin-Ab or a known carbohydrate–lectin interaction. Beyond these proofs of principle, the first report of a new small molecule–ligand interaction was reported by Schreiber and coworkers with the discovery of a ligand for Ure2p [36], a central repressor of genes involved in yeast nitrogen metabolism. While Ure2p has been widely studied, there was no known small molecule inhibitor of this protein. Screening a library of 3780 small molecules microarrayed on the silyl chloride slides with fluorescently labeled Ure2p, the first ligand for this protein was discovered and named uretupamine (Fig. 9). Interestingly, uretupamine was found to inhibit only a subset of Ure2p's functions and as such provided the means to deconvolute Ure2p's different roles. It should be noted that the screen only required 4 μg of protein! Whole-genome transcription profiling and *URE2* gene depletion experiments showed that this inhibitor modulated the glucose-sensitive genes controlled by Ure2p. This discovery not only shed new light on the multiple function of Ure2p but also demonstrated the power of SMM in providing rapid screens for the small molecule probes necessary to dissect complex biological networks. Using the same approach, but an extended microarray containing 12 396 small molecules from several different

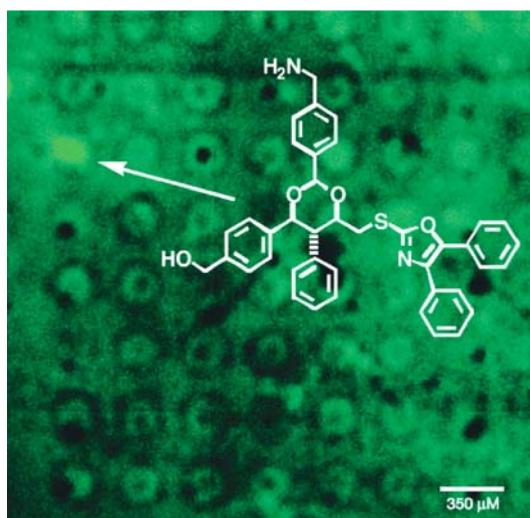

Fig. 9 Expanded view of the region of the 3780 compound SMM showing the signal from Cy5-labeled Ure2p interaction with uretupamine (reprinted with permission from reference [36])

combinatorial libraries, Hap3p, a subunit of a yeast transcription factor complex involved in yeast aerobic respiration, was screened. For this purpose, Hap3p was expressed with a GST tag and visualized with a Cy5-labeled Ab against GST. Two hits were identified from the microarray and one was reconfirmed to be a good Hap3p ligand by SPR (K_d 5 μM). Interestingly, the other hit was a GST ligand. Importantly, the identified molecule was found to function in vivo using a reporter assay [69]. In a third example from the same group, a microarray containing 6336 phenols immobilized using the diazobenzylidene was screened with Cy5-labeled calmodulin. Sixteen hits were identified of which 13 were reconfirmed using SPR (K_d 0.12 μM for the best compound) [39]. Together these results clearly show the power of SMM screen to discover new ligands for proteins of interest.

The first peptide microarrays were used to map the epitope of antibodies [16]. More recently, researchers have used an array of peptides to find an inhibitor of angiotensin II, one of the strongest vasopressors that regulates the cardiovascular system and blood pressure. An array of 8mer peptides spanning the sequence of the endogenous receptor of angiotensin II was prepared by SPOT-synthesis. Screening of this macroarray containing 352 unique peptides with fluorescently labeled angiotensin II afforded four concurring hits [70]. It was further shown that the best hit inhibited the contractile response of angiotensin II in a phenotypic assay.

All those reports required pre-purification or modification of the target protein with a label in order to visualize the signal on the array. Such labeling has limitations, including the need for additional steps in or prior to the assay in order to chemically or genetically encode the tag. Furthermore, proteins may need to be in a complex to be functional or properly folded. Perhaps the most important limitation of labeling is the inability to identify unanticipated (and thus unlabeled) proteins in crude cell lysates. Furthermore, it would be difficult for labeling techniques to be useful for diagnostic purposes because they are not applicable to complex mixture of proteins such as crude cell lysates. Nevertheless, they remain essential for screening SMM against target enzymes. In an alternative strategy, it was shown that PNA-encoded small molecules could be screened against crude cell lysates by using a size-exclusion separation to remove small molecules that do not interact with a macromolecule (Fig. 10) [52]. Since all the PNA tags also bear a fluorophore, hybridization of the selected compounds to a DNA microarray reveals the hits. Following speculations that acute respiratory allergy may be accentuated by residual proteolytic activity in allergens such as dust mite feces, a 4000 compound library targeting cysteine proteases was screened against crude fecal extracts, resulting in the discovery of a potent inhibitor of Derp1 [56]. Using a phenotypic assay, this inhibitors was used to correlate the function of Derp1 and T-cell replication, the phenotype of allergy. In a more targeted approach, it was also shown that the inhibitors bound to an enzyme could also be isolated using a gel-based separation. This approach was used to iden-

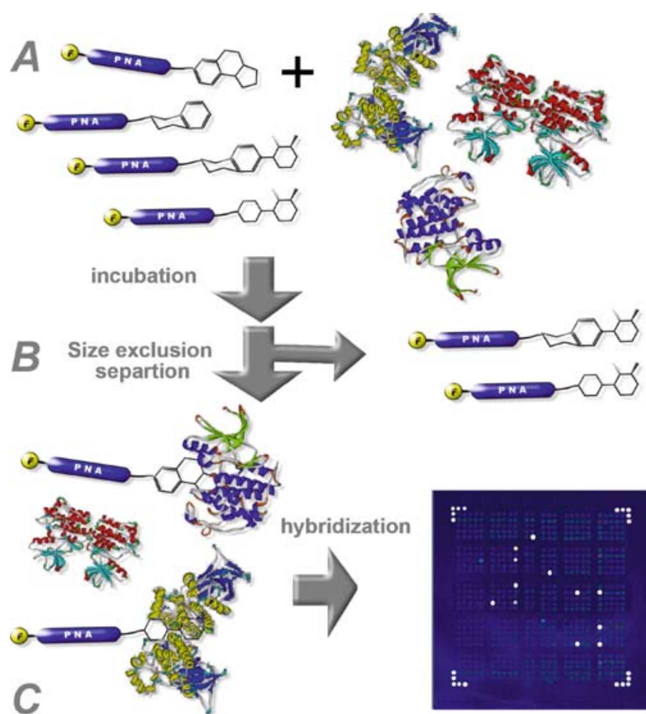

Fig. 10 Screening PNA-encoded libraries. **a** PNA-encoded library is incubated with unlabeled proteomic mixture. **b** The mixture is passed through a size exclusion filter and PNA-encoded molecules that are not bound to a protein are removed. **c** The hits are hybridized to the microarray for readout (all PNA are labeled with a fluorophore)

tify orthogonal inhibitors to the closely related cathepsins K and F. While several compounds inhibited both cathepsins, two compounds were found to be nearly ten times more potent for their respective targets [54]. Recently, it has been shown that an optimized microarray surface containing short PEG spacer could also be used to screen proteins directly from crude cell lysates using either genetically tagged proteins (GST) or specific antibodies towards untagged proteins. The ability to screen directly from crude lysates is significant as it saves substantial time and effort and may be more relevant as many proteins require a partner to remain active [5].

As protein rarely binds a single compound in a combinatorial library, the pattern of binding can be used as a specific fingerprint of a given protein [71]. To demonstrate that principle, a microarray of peptoids (7680) was probed with three proteins: maltose-binding protein, glutathione *S*-transferase, and ubiquitin. Each of these proteins gave a unique pattern for interaction. Knowing the fingerprint of a protein, such array could be used to detect specific proteins from proteomic mixtures. In a related effort to find a general ligand for IgG as an alternative method to protein A or G for antibody purifica-

tion, a SMM of 2688 triazines was screened, yielding a ligand with a K_d of 2 μM [72].

3.2
Profiling Substrate Specificity of an Enzyme or Enzymatic Activity from Complex Mixtures

A second important application of SMM has been to profile the substrate specificity of given enzymes or to measure the activity of enzymes from crude cell lysates. This has been demonstrated for kinases, proteases, and glycosidases.

3.2.1
Kinases

Phosphorylation of proteins by kinases provides an essential posttranslational mechanism to regulate the activity of targeted proteins. Kinases are involved in most signal transduction pathways and have been implicated in cell proliferation, differentiation, metabolism, and apoptosis. Information about cellular substrates of kinases and a method to measure kinase activity in relation to a particular cellular state are essential for dissection of kinase networks. Researchers in the kinase area recognized the power of peptide libraries toward this goal. It was demonstrated in 1995 that macroarrays on cellulose paper could be used to define optimal substrates for PKA and PKG [73] or to define peptide sequence with high affinity to cGPK [74]. As previously discussed, these macroarrays are two to three orders of magnitude larger than microarrays and require significantly more protein for screens.

More recently, a number of reports have appeared demonstrating that phosphorylation could be measured in a microarray format using a known kinase–substrate pair. Two different detection approaches have generally been used, the first makes use of $[\gamma\text{-}^{32/33}P]$-ATP to label the immobilized substrate with a radioactive phosphate, which can be detected by autoradiography, phosphor imager, or silver staining [9, 13, 75–79]. The second detection method relies on phosphospecific antibodies, which are fluorescently labeled [13, 51, 58, 76, 80]. While the fluorescent detection may be preferable as it avoids working with radioactive ATP, it has been shown that only monoclonal anti-phosphotyrosine antibodies showed reliable results [76]. Alternatively, fluorescently labeled phosphor-chelators have been used to detect the phosphorylated peptide in an array [81]. Preliminary results have also been reported for mass spectrometry detection and surface plasmon detection (vide infra).

In an impressive step from a proof of principle to a useful tool, Schutkowski and coworkers reported the preparation of peptide microarrays (13mers) containing 700 to 1300 kinase substrates identified bioinformatically from se-

quence analysis of the human genome [78]. Using [γ-^{33}P]-ATP, the authors showed that these arrays could be used to identify the preferred substrate of a given kinase, as exemplified by profiling two kinases – protein kinase A (PKA) (Fig. 11) and 3-phosphoinositide-dependent protein kinase (PDK1). The same strategy was used to identify the preferred substrates of CK2, a serine/threonine kinase [76]. Rather than using specific peptide sequences of predicted phosphorylation sites from database analysis, it was also shown that the preferred substrate of Abl could be inferred from the phosphorylation of a random array of 1433 peptides using a weight matrix-nearest neighbor algorithm [77]. In an other example, this technique was used to define the substrate specificity of Dbf2 a yeast kinase which, together with its binding partner Mob1, is an important component of the mitotic exit signaling network. The Dbf2–Mob1 complex was found to preferentially phosphorylate substrates that contain an RXXS motif and it was shown that proteins containing this motif were phosphorylated in vivo. However, the relatively low degree of sequence restriction suggested that Dbf2 achieves specificity by docking its substrates at a site that is distinct from the phosphorylation site [75]. Together, these studies clearly demonstrate that peptide microarrays represent a useful tool in identifying the preferred substrate of a kinase, which can be used to predict the cellular target of a kinase or for screening and diagnostic applications.

It was also demonstrated in a model system that the microarray format could be used to determine the K_i [13] and selectivity [82] of inhibitors. In

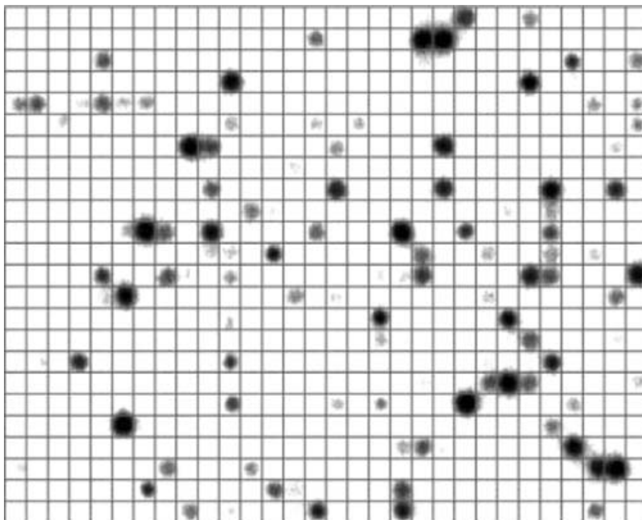

Fig. 11 Profiling substrate selectivity of PKA. A peptide microarray displaying 710 peptides derived from annotated human phosphorylation sites after incubation with PKA and ^{32}P-ATP and phosphoimaging (reprinted with permission from reference [78])

the later case, microfluidics was used to deliver separate kinase/inhibitor mixtures over a set of kinase substrates.

3.2.2 Proteases and Hydrolases

Proteases hydrolyse the amide bonds of proteins. While some proteases have a purely metabolic function, a number of proteases are involved in the post-translational regulation of protein activity and are essential for cellular function. Many pathways such as hormone activation, apoptosis, coagulation, or viral infection are dependent on the action of specific proteases. As for kinases, there is widespread interest in methods that define the preferred substrate of a protease and in being able to correlate their activity to the cellular state. Three detection methods have been developed based on irreversible inhibitors that selectively label active proteases [52], fluorogenic substrates [55, 83], and substrates flanked by two FRETing fluorophores [58].

The first proof of concept that protease activity could be measured from crude cell lysates using microarray-based technologies was reported in 2002. Using a set of PNA-encoded irreversible inhibitors to label active proteases in complex mixtures, the authors showed that they could measure the difference in activity of caspase-3 between apoptotic and healthy cells [52]. Concurrently, it was shown that immobilized coumarin-based fluorogenic substrates (Fig. 12) could be used to define substrate specificity of proteases, as exemplified with thrombin. Importantly, it was shown that the relative K_{cat}/K_m was comparable for solution substrates and immobilized substrates [83]. It was later shown that PNA-encoding could also be used to prepare fluorogenic substrate libraries. Aside from defining the substrate specificity of a given protease, the method was shown to be robust enough to be used with more complex mixtures such as crude cell lysates or clinical blood samples. It was

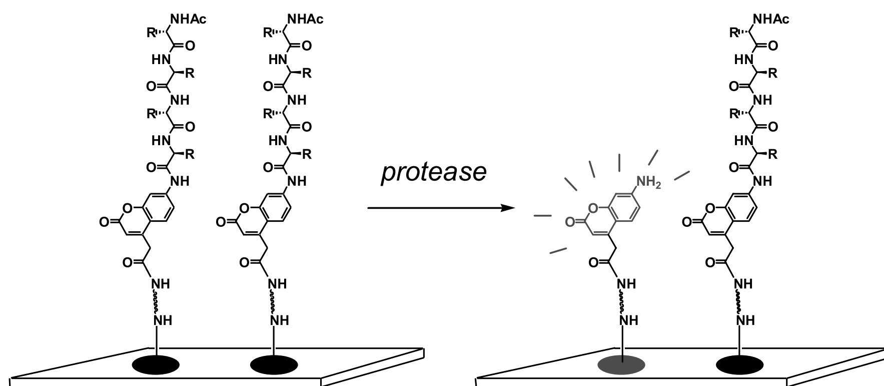

Fig. 12 Profiling substrate selectivity of protease using fluorogenic substrates

also sufficiently sensitive to measure differences in proteolytic activity between apoptotic and healthy cells as well as between sera from patient on anticoagulation therapy and healthy patients [55]. Conversely, it was also shown in a proof of principle that the activity of pepsin could be detected from a PNA-encoded library of substrates using dabcyl as an internal quencher and fluorescein [58]. More recently, PNA-encoded substrate containing fluorescent probes was used to perform β-secretase assay, a protease involved in Alzheimer's disease. In addition to the gain in miniaturization, the authors reported that this assay format was ten times more sensitive than assays in solution [57].

Alternatively, it was shown that fluorogenic substrates could be deposited as a glycerol/DMSO nanodroplet on a surface of a glass slide with the same density as covalent microarrays and that an enzyme of interest could be introduced by aerosol [84, 85].

3.2.3 Glycosidase

Shin and coworkers demonstrated that enzymatic glycosylations were possible on microarrays by converting GlcNAc to Sialyl-Lex using three separate glycosidase (β-1,4-galactosyltransferase, α-2,3-sialyl transferase and α-1,3-fucosyl transferase), followed by the detection of the product using a fluorescently labeled anti-Sialyl-LeX Ab [86]. An interesting application of this principle would be to use a microarray of substrates to profile the substrate specificity or activity of carbohydrate-processing enzymes. As a model study for this, a microarray containing GlcNAc and fucose was treated with β-1,4-galactosyltransferase and UDP-Gal. Revealing the product of the reaction with specific lectins showed that only GlcNAc was converted suggesting that this method should be more broadly applicable.

3.3 Carbohydrate Arrays

The glycobiology community was quick to recognize the potential of microarray technologies to screen glycan interactions. Microarrays are particularly well suited for this purpose as complex glycans are difficult to obtain in large quantities and the presentation of glycans on a surface with controlled density mimics the natural display of carbohydrates on the surface of proteins or phospholipid bilayers. This "cluster" effect may also be important to yield strong multivalent interactions, which have been shown to be important for affinity and selectivity in natural systems. Oligosaccharides have been immobilized using maleimide–thiol chemistry [86–88], Diels–Alder reaction [89], the reaction of p-aminophenyl glycosides with cyanuric chloride-coated slides [90], Huisgen cycloaddition [45, 46, 48, 59, 60], Staudinger lig-

ation [91], amine-NHS chemistry [92], and hydroxylamine-aldehyde [93]. Several reviews have already appeared highlighting the first proofs of principle in preparing and screening carbohydrate microarrays [10, 47, 94]. Notable applications of carbohydrate arrays have been to profile the specificity of therapeutically relevant antibodies against a panel of oligosaccharide. Wong and coworkers used a panel of epitopes to dissect the specificity of 2G12, a human antibody that neutralizes a broad range of HIV-1 strains and is known to recognize mannose-rich carbohydrate ($Man_9GlcNAc_2$) present on gp120. The profile revealed that the carbohydrate specificity of 2G12 is less restrictive than originally believed and that in a multivalent display, a single Manα1-2Man is sufficient for strong binding [46]. In a similar study, Wong and coworkers profiled the specificity of monoclonal antibodies to Globo H, a hexasaccharide antigen found on the surface of several cancer cell lines [48]. For this purpose they prepared a microarray with seven oligosaccharides (the Globo H hexamer and truncated forms) at 15 different concentrations. It was found that in a multivalent format, the truncated tetrasaccharide had similar activity to the Globo H hexasaccharide, suggesting that the more accessible tetrasaccharide should also be effective for vaccine preparation. Carbohydrate and glycoprotein microarrays have also proven useful to study carbohydrate–protein interactions beyond the known model systems. Seeberger and coworkers used an array of seven carbohydrates and five glycoproteins to define the interactions of gp120 and its glycans with five different binding proteins (CD4, CVN, Scytovirin, 2G12, and SC-SIGN) [88]. Aside from CD4–gp120, all other interactions required the glycan. However, the promiscuity of binding varied greatly amongst the four proteins, with Scytovirin being the most specific for the exact glycan structure present on gp120. More recently, Seeberger and colleagues used an array of six carbohydrates of varying length and sulfation pattern spotted at 12 different concentrations to probe interactions between these heparin-like glycans and fibroblast growth factors (FG1 and 2) [92]. This analysis led to the identification of a monosaccharide ligand bearing a sulfation pattern not found in nature that had similar affinity to tetra- and hexasaccharides. In another study on the sulfation pattern of chondroitin, a glycosamine glycan, Hsieh-Wilson and coworkers discovered a novel and specific interaction between chondroitin sulfate and TNF-α that can inhibit its activity [93]. In the most extensive study of lectin interactions, an array of 69 carbohydrates and glycoproteins were probed with 24 lectins at varying concentrations. This extensive study led to the identification of several unexpected lectin interactions and in a larger context clearly demonstrated the potential throughput of carbohydrate microarrays [95].

3.4
Solution-Based and Cell-Based Screening in a Microarray Format

Beyond proof of concept, nanodroplet technology was used to screen a library of 352 small molecules against three caspases leading to the identification of a selective inhibitor (high μM) [63]. This method was later used to screen 1000 pharmaceutically active compounds against human cathepsin L, a cysteine protease, based on the speculation that cathepsin L may be important in the activation of severe acute respiratory syndrome coronavirus (SARS). The screen led to the identification of a dipeptide aldehyde with an IC_{50} of 2.5 nM for cathepsin L. This compound was then shown to block SARS entry into host cells, thereby validating the role of cathepsin L in the pathology of SARS. This compound could potentially be used to develop a new therapy against the SARS virus [96].

The SMM developed for cell-based screens [64] using biodegradable polymer to imbed the small molecule was used to evaluate the synergistic effect of 70 small molecule inhibitors at three different concentrations in combination with seven different siRNA in two different cell lines. This small pilot experiment required the collection of 50 000 data points, which attests to the necessity for miniaturization. The screen led to the identification of four hits where a compound had reduced effect in the presence of an siRNA. These compounds were retested in conventional cytotoxic assays, which confirmed that macbecin II had reduced cytotoxicity when TSC2 was knocked down, suggesting that enhanced cellular growth resulting from decreased TSC2 activity can reduce a cell's sensitivity to DNA damage. Together, these results demonstrated that high content cell-based assays can be performed in a highly miniaturized microarray format, which greatly reduces the need for complex automation.

3.5
Beyond Fluorescent Readout

Fluorescence has thus far been the method of choice for detecting interactions in most microarray applications. As discussed earlier, detection of phosphorylated substrates has been successfully achieved using [γ-$^{33-32}$P] ATP for the phosphorylation followed by autoradiography as there are a number of limitations with fluorescent detection of phosphorylated residues [9, 13, 77, 78]. Alternatively, Mrksich and coworkers have also shown that mass spectrometry (MALDI) can be used to quantify phosphorylation of substrates microarrayed on gold surfaces [82] as well as small molecule–protein and protein–protein interactions [97]. Becker and coworkers have shown that MALDI is also suitable to detect protein–protein interactions for protein microarrays prepared by self-assembly of DNA-tagged proteins onto a DNA microarray [98]. Although the throughput of MALDI is much lower than fluorescence,

it provides a means to identify the protein and as such may lead to discovery of unanticipated interactions. Alternatively, surface plasmon resonance (SPR) has been used to detect phosphorylated peptides. As the difference between a peptide and the phosphopeptide is not sufficient to produce a change in SPR, an antiphosphotyrosine [13] and a novel zinc-based phosphochelator group coupled to biotin/streptavidin [99] were used to produce a shift in SPR. In a proof of concept, it was also shown that SPR could be used to detect interaction between small molecules immobilized via photocrosslinking (vide supra) and proteins or antibodies [100].

4
Conclusion

Over the past 7 years a number of new technologies have been reported for the preparation of small molecule microarrays and there is now a broad repertoire of chemistries to immobilize small molecules with controlled or random orientation. Several groups have reported the preparation of SMM with libraries ranging from 1000 to over 10 000 compounds. The efficiency of SMM to screen and discover new ligands has been demonstrated with several landmark studies providing new ligands to explore important biological problems. The applicability of SMM to profile or to measure enzymatic activity from complex proteomic mixtures has also been demonstrated beyond simple proof of principle for kinases and proteases. In the carbohydrate field, the cluster effect of microarrays appears to be beneficial and carbohydrate arrays have also led to important new discoveries regarding therapeutically important antibodies, lectin specificity, and carbohydrate sulfation pattern. While the use of SMM is more challenging to implement than oligonucleotide microarrays, based on early success, there is little doubt that this format will become more widespread and will find applications in diagnostics.

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References

1. Heller MJ (2002) DNA microarray technology: devices, systems, and applications. Ann Rev Biomed Eng 4:129–153
2. Pirrung MC (2002) How to make a DNA chip. Angew Chem Int Ed 41:1276–1289
3. Brown P (2004) The MGuide. Version 2.0. http://www.cmgm.stanford.edu/pbrown/mguide/index.html, (last visited: 26 Jan 2007)

4. Conzone SD, Pantano CG (2004) Glass slides to DNA microarrays. Mater Today 7:20–26
5. Bradner JE et al. (2006) A robust small-molecule microarray platform for screening cell lysates. Chem Biol 13:493–504
6. Lee MR, Shin I (2005) Fabrication of chemical microarrays by efficient immobilization of hydrazide-linked substances on epoxide-coated glass surfaces. Angew Chem Int Ed 44:2881–2884
7. Kanoh N et al. (2003) Immobilization of natural products on glass slides by using a photoaffinity reaction and the detection of protein-small-molecule interactions. Angew Chem Int Ed 42:5584–5587
8. Hergenrother PJ, Depew KM, Schreiber SL (2000) Small-molecule microarrays: covalent attachment and screening of alcohol-containing small molecules on glass slides. J Am Chem Soc 122:7849–7850
9. Falsey JR, Renil M, Park S, Li S, Lam KS (2001) Peptide and small molecule microarray for high throughput cell adhesion and functional assays. Bioconjugate Chem 12:346–353
10. Shin I, Park S, Lee MR (2005) Carbohydrate microarrays: an advanced technology for functional studies of glycans. Chemistry 11:2894–2901
11. Benters R, Niemeyer CM, Wohrle D (2001) Dendrimer-activated solid supports for nucleic acid and protein microarrays. ChemBioChem 2:686–694
12. Hackler L Jr et al. (2003) Development of chemically modified glass surfaces for nucleic acid, protein and small molecule microarrays. Mol Divers 7:25–36
13. Houseman BT, Huh JH, Kron SJ, Mrksich M (2002) Peptide chips for the quantitative evaluation of protein kinase activity. Nat Biotechnol 20:270–274
14. Lee K-B, Park S-J, Mirkin CA, Smith JC, Mrksich M (2002) Protein nanoarrays generated by dip-pen nanolithography. Science 295:1702–1705
15. Lee SW et al. (2006) Biologically active protein nanoarrays generated using parallel dip-pen nanolithography. Adv Mater 18:1133–1136
16. Fodor SPA et al. (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251:767–773
17. Cho CY et al. (1993) An unnatural biopolymer. Science 261:1303–1305
18. Pease AC et al. (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci USA 91:5022–5026
19. McGall G et al. (1996) Light-directed synthesis of high-density oligonucleotide arrays using semiconductor photoresists. Proc Natl Acad Sci USA 93:13555–13560
20. Singh-Gasson S et al. (1999) Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. Nat Biotechnol 17:974–978
21. Li S et al. (2004) Photolithographic synthesis of peptoids. J Am Chem Soc 126:4088–4089
22. Li S, Marthandan N, Bowerman D, Garner HR, Kodadek T (2005) Photolithographic synthesis of cyclic peptide arrays using a differential deprotection strategy. Chem Comm pp 581–583
23. LeProust E et al. (2000) Digital light-directed synthesis. A microarray platform that permits rapid reaction optimization on a combinatorial basis. J Comb Chem 2:49–354
24. Pellois JP, Wang W, Gao X (2000) Peptide synthesis based on *t*-Boc chemistry and solution photogenerated acids. J Comb Chem 2:355–360
25. Pellois JP et al. (2002) Individually addressable parallel peptide synthesis on microchips. Nat Biotechnol 20:922–926

26. Frank R, Gueler S, Krause S, Lindenmaier W (1991) Facile and rapid spot-synthesis of large numbers of peptides on membrane sheets. Pept 1990, Proc 21st Eur Pept Symp, pp 151–152
27. Frank R (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron 48:9217–9232
28. Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports–principles and applications. J Immunol Methods 267:13–26
29. Scharn D, Wenschuh H, Reineke U, Schneider-Mergener J, Germeroth L (2000) Spatially addressed synthesis of amino- and amino-oxy-substituted 1,3,5-triazine arrays on polymeric membranes. J Comb Chem 2:361–369
30. Scharn D, Germeroth L, Schneider-Mergener J, Wenschuh H (2001) Sequential nucleophilic substitution on halogenated triazines, pyrimidines, and purines: a novel approach to cyclic peptidomimetics. J Org Chem 66:507–513
31. Heine N, Germeroth L, Schneider-Mergener J, Wenschuh H (2001) A modular approach to the SPOT-synthesis of 1,3,5-trisubstituted hydantoins on cellulose membranes. Tetrahedron Lett 42:227–230
32. Niggemann J, Michaelis K, Frank R, Zander N, Hoefle G (2002) Natural product-derived building blocks for combinatorial synthesis. Part 1. Fragmentation of natural products from myxobacteria. J Chem Soc. Perkin Trans 1, pp 2490–2503
33. Bowman Matthew D, Jeske Ryan C, Blackwell Helen E (2004) Microwave-accelerated SPOT-synthesis on cellulose supports. Org Lett 6:2019–2022
34. Lin Q, O'Neill JC, Blackwell HE (2005) Small molecule macroarray construction via Ugi four-component reactions. Org Lett 7:4455–4458
35. MacBeath G, Koehler AN, Schreiber SL (1999) Printing small molecules as microarrays and detecting protein–ligand interactions en masse. J Am Chem Soc 121:7967–7968
36. Kuruvilla FG, Shamji AF, Sternson SM, Hergenrother PJ, Schreiber SL (2002) Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays. Nature 416:653–657
37. Melnyk O et al. (2002) Peptide arrays for highly sensitive and specific antibody-binding fluorescence assays. Bioconjugate Chem 13:713–720
38. de Araujo AD et al. (2005) Diels–Alder ligation and surface immobilization of proteins. Angew Chem Int Ed 45:296–301
39. Barnes-Seeman D, Park SB, Koehler AN, Schreiber SL (2003) Expanding the functional group compatibility of small-molecule microarrays: discovery of novel calmodulin ligands. Angew Chem Int Ed 42:2376–2379
40. Nilsson BL, Kiessling LL, Raines RT (2000) Staudinger ligation: a peptide from a thioester and azide. Org Lett 2:1939–1941
41. Saxon E, Armstrong JI, Bertozzi CR (2000) A traceless Staudinger ligation for the chemoselective synthesis of amide bonds. Org Lett 2:2141–2143
42. Soellner MB, Dickson KA, Nilsson BL, Raines RT (2003) Site-specific protein immobilization by Staudinger ligation. J Am Chem Soc 125:11790–11791
43. Watzke A et al. (2006) Site-selective protein immobilization by staudinger ligation. Angew Chem Int Ed 45:1408–1412
44. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. Angew Chem Int Ed 41:2596–2599
45. Bryan MC et al. (2004) Covalent display of oligosaccharide arrays in microtiter plates. J Am Chem Soc 126:8640–8641

46. Calarese DA et al. (2005) Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc Natl Acad Sci USA 102:13372–13377
47. Feizi T, Fazio F, Chai W, Wong C-H (2003) Carbohydrate microarrays – a new set of technologies at the frontiers of glycomics. Curr Opin Struct Biol 13:637–645
48. Huang CY et al. (2006) Carbohydrate microarray for profiling the antibodies interacting with Globo H tumor antigen. Proc Natl Acad Sci USA 103:15–20
49. Lee MR, Shin I (2005) Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. Org Lett 7:4269–4272
50. Winssinger N, Harris JL, Backes BJ, Schultz PG (2001) From split-pool libraries to spatially addressable microarrays and its application to functional proteomic profiling. Angew Chem Int Ed 40:3152–3155
51. Harris JL, Winssinger N (2005) PNA encoding (PNA=peptide nucleic acid): from solution-based libraries to organized microarrays. Chemistry 11:6792–6801
52. Winssinger N, Ficarro S, Schultz PG, Harris JL (2002) Profiling protein function with small molecule microarrays. Proc Natl Acad Sci USA 99:11139–11144
53. Debaene F, Mejias L, Harris JL, Winssinger N (2004) Synthesis of a PNA-encoded cysteine protease inhibitor library. Tetrahedron 60:8677–8690
54. Urbina HD, Debaene F, Jost B, Bole-Feysot C, Mason DE, Kuzmic P, Harris JL, Winssinger N (2006) Self-assembled small molecule microarrays for protease screening and profiling. ChemBioChem 7(11):1790–1797
55. Winssinger N et al. (2004) PNA-encoded protease substrate microarrays. Chem Biol 11:1351–1360
56. Harris J et al. (2004) Activity profile of dust mite allergen extract using substrate libraries and functional proteomic microarrays. Chem Biol 11:1361–1372
57. Sano S, Tomizaki KY, Usui K, Mihara H (2006) A PNA-DNA hybridization chip approach for the detection of beta-secretase activity. Bioorg Med Chem Lett 16:503–506
58. Diaz-Mochon JJ, Bialy L, Keinicke L, Bradley M (2005) Combinatorial libraries – from solution to 2D microarrays. Chem Comm 7:1384–1386
59. Bryan MC et al. (2002) Saccharide display on microtiter plates. Chem Biol 9:713–720
60. Fazio F, Bryan MC, Blixt O, Paulson JC, Wong CH (2002) Synthesis of sugar arrays in microtiter plate. J Am Chem Soc 124:14397–14402
61. Studer A et al. (1997) Fluorous synthesis: a fluorous-phase strategy for improving separation efficiency in organic synthesis. Science 275:823–826
62. Ko K-S, Jaipuri FA, Pohl NL (2005) Fluorous-based carbohydrate microarrays. J Am Chem Soc 127:13162–13163
63. Gosalia DN, Diamond SL (2003) Printing chemical libraries on microarrays for fluid phase nanoliter reactions. Proc Natl Acad Sci USA 100:8721–8726
64. Bailey SN, Sabatini DM, Stockwell BR (2004) Microarrays of small molecules embedded in biodegradable polymers for use in mammalian cell-based screens. Proc Natl Acad Sci USA 101:16144–16149
65. Kumaresan PR, Lam KS (2006) Screening chemical microarrays: methods and applications. In: Bartlett P, Entzeroth M (eds) Exploiting chemical diversity for drug discovery. RSC, UK, pp 291–312
66. Kukar T et al. (2002) Protein microarrays to detect protein–protein interactions using red and green fluorescent proteins. Anal Biochem 306:50–54
67. Sielaff I et al. (2006) Protein function microarrays based on self-immobilizing and self-labeling fusion proteins. Chem Bio Chem 7:194–202

68. Kawahashi Y et al. (2003) In vitro protein microarrays for detecting protein–protein interactions: application of a new method for fluorescence labeling of proteins. Proteomics 3:1236–1243
69. Koehler AN, Shamji AF, Schreiber SL (2003) Discovery of an inhibitor of a transcription factor using small molecule microarrays and diversity-oriented synthesis. J Am Chem Soc 125:8420–8421
70. Kato R, Kunimatsu M, Fujimoto S, Kobayashi T, Honda H (2004) Angiotensin II inhibitory peptide found in the receptor sequence using peptide array. Biochem Biophys Res Commun 315:22–29
71. Reddy MM, Kodadek T (2005) Protein fingerprinting in complex mixtures with peptoid microarrays. Proc Natl Acad Sci USA 102:12672–12677
72. Uttamchandani M et al. (2004) Microarrays of tagged combinatorial triazine libraries in the discovery of small-molecule ligands of human IgG. J Comb Chem 6:862–868
73. Tegge W, Frank R, Hofmann F, Dostmann WR (1995) Determination of cyclic nucleotide-dependent protein kinase substrate specificity by the use of peptide libraries on cellulose paper. Biochemistry 34:10569–10577
74. Dostmann WR et al. (2000) Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Iα inhibit NO-induced cerebral dilation. Proc Natl Acad Sci USA 97:14772–14777
75. Mah AS et al. (2005) Substrate specificity analysis of protein kinase complex Dbf2-Mob1 by peptide library and proteome array screening. BMC Biochem 6:22
76. Panse S et al. (2004) Profiling of generic anti-phosphopeptide antibodies and kinases with peptide microarrays using radioactive and fluorescence-based assays. Mol Divers 8:291–299
77. Rychlewski L, Kschischo M, Dong L, Schutkowski M, Reimer U (2004) Target specificity analysis of the Abl kinase using peptide microarray data. J Mol Biol 336:307–311
78. Schutkowski M et al. (2004) Automated synthesis: high-content peptide microarrays for deciphering kinase specificity and biology. Angew Chem Int Ed 43:2671–2674
79. Schutkowski M, Reineke U, Reimer U (2005) Peptide arrays for kinase profiling. ChemBioChem 6:513–521
80. Lesaicherre ML, Uttamchandani M, Chen GY, Yao SQ (2002) Antibody-based fluorescence detection of kinase activity on a peptide array. Bioorg Med Chem Lett 12:2085–2088
81. Martin K et al. (2003) Quantitative analysis of protein phosphorylation status and protein kinase activity on microarrays using a novel fluorescent phosphorylation sensor dye. Proteomics 3:1244–1255
82. Su J, Bringer MR, Ismagilov RF, Mrksich M (2005) Combining microfluidic networks and peptide arrays for multi-enzyme assays. J Am Chem Soc 127:7280–7281
83. Salisbury CM, Maly DJ, Ellman JA (2002) Peptide microarrays for the determination of protease substrate specificity. J Am Chem Soc 124:14868–14870
84. Gosalia DN, Salisbury CM, Maly DJ, Ellman JA, Diamond SL (2005) Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays. Proteomics 5:1292–1298
85. Gosalia DN, Salisbury CM, Ellman JA, Diamond SL (2005) High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays. Mol Cell Proteomics 4:626–636
86. Park S, Lee MR, Pyo SJ, Shin I (2004) Carbohydrate chips for studying high-throughput carbohydrate–protein interactions. J Am Chem Soc 126:4812–4819

87. Park S, Shin I (2002) Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. Angew Chem Int Ed 41:3180–3182
88. Adams EW et al. (2004) Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology; glycan-dependent gp120/protein interactions. Chem Biol 11:875–881
89. Houseman BT, Mrksich M (2002) Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem Biol 9:443–454
90. Schwarz M et al. (2003) A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody. Glycobiology 13:749–754
91. Kohn M et al. (2003) Staudinger ligation: a new immobilization strategy for the preparation of small-molecule arrays. Angew Chem Int Ed 42:5830–5834
92. de Paz JL, Noti C, Seeberger PH (2006) Microarrays of synthetic heparin oligosaccharides. J Am Chem Soc 128:2766–2767
93. Tully SE, Rawat M, Hsieh-Wilson LC (2006) Discovery of a TNF-alpha antagonist using chondroitin sulfate microarrays. J Am Chem Soc 128:7740–7741
94. Love KR, Seeberger PH (2002) Carbohydrate arrays as tools for glycomics. Angew Chem Int Ed 41:3583–3586
95. Manimala JC, Roach TA, Li Z, Gildersleeve JC (2006) High-throughput carbohydrate microarray analysis of 24 lectins. Angew Chem Int Ed 45:3607–3610
96. Simmons G et al. (2005) Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci USA 102:11876–11881
97. Yeo W-S, Min D-H, Hsieh RW, Greene GL, Mrksich M (2005) Label-free detection of protein–protein interactions on biochips. Angew Chem Int Ed 44:5480–5483
98. Becker CF et al. (2005) Direct readout of protein–protein interactions by mass spectrometry from protein-DNA microarrays. Angew Chem Int Ed 44:7635–7639
99. Inamori K et al. (2005) Detection and quantification of on-chip phosphorylated peptides by surface plasmon resonance imaging techniques using a phosphate capture molecule. Anal Chem 77:3979–3985
100. Kanoh N et al. (2006) SPR imaging of photo-cross-linked small-molecule arrays on gold. Anal Chem 78:2226–2230