Chapter 7
Peptide Arrays

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7.1 General Presentation of Peptide Arrays with Some Examples of Implementation

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7.1.1 Introduction

The completion of the human genome sequencing project has led to a major shift in biomedical research from pure genomics to functional genomics and proteomics, aiming at better understanding of the function of the genes or more appropriately their protein products. Although the DNA/oligonucleotide microarray technology

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has proved to be unparalleled in its power in profiling gene expression and identifying single nucleotide polymorphisms (SNPs) in high-throughput manner, there is a poor correlation between mRNA and protein expression and activity. This arises from regulation of mRNA translation, for example, with microRNAs, and from the involvement of posttranscriptional and posttranslational modifications (PTMs), protein–protein interactions, and differences in subcellular localizations. These confounding factors have driven the development and the use of array technologies to directly study proteomes and have set the stage for the arrival of microarray-based proteomics.

Similarly to DNA/oligonucleotide microarrays, arrays for proteomics studies feature a wide range of molecules including recombinant proteins, complex protein samples, antibodies, peptides, or small molecules that are assembled in an addressable fashion on planar surfaces to allow parallel interrogations for activity and interactions associated with biomolecules at the protein level. Among various proteomics array types, protein microarrays and antibody microarrays have attracted most of attention in the field in the past decade, as illustrated by the rapid technological advancements and broad applications in numerous basic research and clinical studies (reviewed in Chap. 6).

Even though synthetic peptides together with oligonucleotides were among the first to be explored as molecular probes in array format, the development and application of peptide arrays has been sluggish and has lagged far behind arrays of many other types (Frank et al. 1983; Geysen et al. 1984). One of the concerns with the use of peptides as opposed to proteins as molecular probes is the potential loss of information as a result of the missing structural context (Mahrenholz et al. 2010). There is a much higher degree of entropy in the structures of peptides than with macromolecules such as proteins which are much more constrained. Arrays of proteins have the distinct advantage in being able to mimic their physiological counterparts through presenting full-length proteins that are likely in their native 3D conformations and with proper PTMs and associated proteins, to facilitate investigation of protein–protein interactions and assay of physiological activities. However, the molecular cloning and expression of tens of thousands of protein-coding genes with optimal covalent modifications and in combination with activating subunits has been proven to be technologically challenging, which significantly hampers the development and application of protein microarrays.

In contrast, the chemistry of solid-phase peptide synthesis (SPPS) was well defined about half a century ago (Merrifield 1965a, b). Further technological advancement in peptide synthesis over the years coupled with the recent influx of genome sequencing information upon the completion of a number of genome-wide sequencing projects has made designing and synthesizing peptides with defined sequences corresponding to the segments of a protein easier than ever. Furthermore, given the fact that the biological activity of a protein is often carried out through the coordinated actions of individual protein domains, it is reasonable to expect that the various functions of the protein can be recapitulated through the study of its constituent peptides representing individual functional domain sequences.

Since its advent about two decades ago, the peptide array has been implemented in a broad range of applications including antibody screening and epitope mapping, characterization of molecular interactions, and enzymatic activity profiling and has since become an increasingly important and versatile tool for proteomics research. More recently, with the improvements in array production techniques, peptide microarrays with higher peptide density and diversity can be produced en masse using the peptides generated from the parallel synthesis approaches such as the SPOT technology. The utility of these peptide microarrays has been demonstrated in some large-scale systems biology studies on the dynamics of protein interactions in cell signaling networks as well as kineome (also known as kinome) activity profiling. However, the clinical application of peptide arrays is still at its infancy despite potential and promises shown in early exploratory work.

This chapter summarizes the development of peptide array technology and highlights the progress made toward its applications in proteomics research in recent years. A perspective of its future implementation in clinical practice is also presented.

7.1.2 Development of Peptide Arrays

Despite the fact that the concept of the array-format peptide synthesis was introduced about three decades ago, it was not until the introduction of the SPOT synthesis of peptides in the early 1990s that the peptide array finally took its shape (Geysen et al. 1984; Frank 1992). Currently, there are two main strategies (in addition to very new approaches, see Sect. 7.2) being used to produce peptide arrays: in situ parallel on-chip synthesis or immobilization of presynthesized peptides on the array surface. Each has its own advantages and shortcomings. Hence, the choice of the approach for peptide array production is largely determined by the downstream applications of the resulting arrays.

7.1.2.1 Peptide Arrays by In situ Synthesis

The in situ on-chip synthesis of peptides is affordable as a result of the requirement of small amounts of reagents and of the fact that no purification of individual peptides is required. However, by the same token, the resulting peptides from in situ synthesis may suffer from low purity due to the variability in coupling efficiency between amino acid residues, especially in the case of long peptides (>20 residues) or those with residues such as Cys or Met, or containing multiple hydrophobic residues or phosphorylated amino acid residues. The two most common techniques for in situ synthesis that have been described and routinely used are SPOT synthesis and photolithography.

The SPOT synthesis was introduced by Ronald Frank back in 1992 and is essentially a stepwise synthesis of peptides through sequentially delivering small

amounts of activated amino acids on functionalized cellulose or polypropylene membranes using standard Fmoc-based peptide chemistry (Frank 1992). The resulting membrane-based arrays are usually at low to medium density and can be used directly for downstream assays such as antibody epitope mapping. Recently, improvements have been introduced allowing peptides either to be cleaved from the membrane using strong bases or to be recovered from individual spots as soluble peptides. For this purpose, they are synthesized on acid-labile cellulose membranes (e.g., trifluoroacetic acid (TFA)-soluble), allowing postsynthesis printing of the recovered peptides on selected array surfaces at a higher density (Zander et al. 2005; Hilpert et al. 2007).

Another technique used in preparing peptide arrays in situ is the photolithographic synthesis developed by Fodor et al. (1991a, b) on the basis of the addressable surface activation concept. Compared to SPOT synthesis, photolithographic synthesis of peptides on array surface is more suitable for generating high-density arrays. However, it is both laborious and expensive. It requires the use of special photolabile protected amino acid derivatives as building blocks and of photomasks through which a laser is then used to activate specific areas on the array to cleave photolabile protecting groups. Even though the technique was initially developed for peptide synthesis, it was more easily adopted for the production of oligonucleotide arrays, due to the complexity of making masks for each of the 20 amino acids for every coupling cycle, as opposed to only four bases in oligonucleotide array production (Pease et al. 1994; McGall and Fidanza 2001). In recent years, a number of modifications have been introduced, including the uses of conventional amino acids and photogenerated reagents, resulting in the improvement of efficiency and the reduction in cost for array production (Singh-Gasson et al. 1999; Gao et al. 2003; Bhushan 2006; Shin et al. 2010).

7.1.2.2 Peptide Arrays by Postsynthesis Printing

Thanks to technological advances in microarray printing and array substrate production in the field of genomics, it has become a common practice to spot presynthesized peptides onto reactive planar array surfaces. The approach is particularly useful when multiple copies of the same array with high density are required. Furthermore, peptides can be purified after synthesis and prior to array printing to avoid any complications that may stem from peptide impurity. Various chemistries have been utilized for immobilizing peptides onto the array surfaces. One of the popular approaches is to attach N-terminal biotinylated peptides onto avidin/streptavidin-coated microarray slides (Lesaicherre et al. 2002). Covalently immobilizing peptides through a terminal Cys onto a surface functionalized with maleimide groups or disulfide is also a method that is being used quite commonly (Inamori et al. 2008). The suitability of immobilization chemistry varies according to the nature of the peptide as well as the downstream applications of the array. In our hands, attaching peptides through the N-terminal free amino groups to a surface functionalized with epoxide groups worked very well for assaying protein kinase

activity (see below). Thus, it is very important and necessary to determine which strategy should be used for preparing peptide arrays already prior to peptide synthesis, according to the intended application of the resulting arrays.

7.1.3 Application of Peptide Arrays in Proteomics Studies

Since the advent of the technology more than two decades ago, peptide arrays have been applied in a broad range of investigations including antibody epitope mapping, protein domain-mediated interaction screening, and enzymatic activity profiling. In recent years, the utility of peptide arrays has been further extended to system-wide proteomics studies, fuelled by the advances in genomics and proteomics. This can be exemplified by their roles in cell signaling studies.

7.1.3.1 Enzymatic Activity Assays

Kineome Activity Profiling

Reversible phosphorylation and dephosphorylation of proteins mediated by protein kinases and protein phosphatases, respectively, are recognized as one of the most important and widespread molecular mechanisms in regulating cell signaling pathways involved in cell proliferation, division, differentiation, adherence, angiogenesis, and apoptosis (Brognard and Hunter 2011). According to our most recent tallies, the total number of phosphorylation sites in the human proteome is estimated to exceed 650,000, which encompass over 100,000 phosphosites that have been experimentally characterized and those predicted based on evolutionary conservation (http://www. phosphoNET.ca). The biological importance and potential clinical significance of protein phosphorylation, exemplified by the implication of deregulation of both kinase and phosphatase activity in a wide range of human diseases including cancer, autoimmune diseases, neurodegenerative diseases, and diabetes, has driven the development of strategies for the identification of physiological substrates for each of over 500 protein kinases within the human kineome, as well as for systematic profiling of kinase activity in biological samples.

For the identification of physiological substrates of kinases, a protein microarray featuring all the proteins representing the entire proteome would seemingly be an ideal platform. However, it is technically challenging to create such a comprehensive array encompassing all of the proteins encoded by about 23,000 genes in the human genome with current cloning and expression technologies. The most comprehensive protein microarray currently available commercially, trademarked as ProtoArray® by Life Technologies (Carlsbad, CA, http://www.lifetechnologies.com), consists of only 9,000 human proteins that have been expressed and purified from a baculovirus-based expression system. Moreover, issues with protein conformation, autophosphorylation, and stability on the array surface, as well as

complications in data interpretation as a result of the presence of multiple phosphorylation sites within a protein potentially targeted by different kinases, have limited the practicality of this approach.

As a result, a number of peptide-based strategies have been devised including peptide libraries and peptide arrays, based on the notion that the substrate specificity of the kinase is largely defined by the flanking linear amino acid sequences around its target phosphorylation site(s) on the substrates. Based on the consensus recognition sequences for protein kinases derived from the peptide-based studies, one can deduce potential physiological substrates for each of the kinases in a proteome, coupled with information about protein–protein interactions, subcellular colocalization, and correlations in expression or activation.

Back in 1995, Luo and colleagues originally used the peptide array approach to identify and optimize substrate sequences for protein kinase A (PKA) and transforming growth factor (TGF) β receptors (Luo et al. 1995). Since then, the substrate specificities for a number of protein kinases have been elucidated and refined sequentially using peptide arrays (Schutkowski et al. 2005). There are two main strategies to be deployed for determining consensus phosphorylation sequences for protein kinases. On the one hand, peptide macroarrays featuring combinatorial peptide libraries or random peptide libraries such as those on the SPOT cellulose membranes were indispensable tools for elucidating the recognition sequences targeted by the kinases for which little information on their physiological substrates is available. The availability of expanding collections of recombinant active protein kinases in the past several years has facilitated the effort in this front. On the other hand, incorporation of increasing numbers of physiological phosphorylation sites uncovered through recent large-scale mass spectrometry-based phosphoproteomics studies into peptide microarrays has also significantly improved the efficiency of the substrate peptide screening process.

Currently, many large-scale peptide microarrays comprising a large number of experimentally verified phosphosites as well as those identified and optimized using the peptide library approach are readily available through various commercial sources such as PepStar™ from JPT Peptide Technologies (JPT Peptide Technologies GmbH, Berlin, Germany, http://www.jpt.com), PamChip® from PamGene (PamGene International B.V., Hertogenbosch, The Netherlands, http://www.pamgene.com), and PepChip™ from Pepscan Presto (Pepscan Presto, Lelystad, The Netherlands, http://www.pepscanpresto.com), either as products or services, for profiling kinase activities in biological samples. Various experimental protocols based on the same approach have been developed (Schutkowski et al. 2005; Thiele et al. 2010). The approach was also adapted to kineome activity profiling in bovine samples by utilizing information gathered through bioinformatics analysis of the phosphorylation sites conserved in evolution (Jalal et al. 2009).

However, inferring endogenous kinase activities based on the data from such peptide microarrays is less straightforward than initially thought. One of the main issues associated with the current approach is the overlapping specificity among protein kinases dictated by the promiscuity in substrate recognition, especially for the kinases from the same or related families. Phosphorylation of a peptide on each

spot may represent the sum of activity of all the kinases targeting this particular peptide. Indeed, a specific phosphosite sequence may be optimized through evolution to be recognized by a panel of kinases and phosphatases and not be optimized for an individual kinase or phosphatase. Thus, under most circumstances, it is impossible to directly correlate the level of peptide phosphorylation on the array with the activity of a specific kinase. It is even more challenging when the activity of kinases in crude cell or tissue lysates is to be assessed, where the cell compartmentalization has been destroyed and the proper subcellular localization of proteins cannot be maintained.

In light of these challenges, we set out to identify the optimal peptide substrate sequences unique to each kinase by combining the high-throughput capability of peptide microarrays with the power of a proprietary kinase–substrate prediction algorithm developed at Kinexus (Fig. 7.1). The algorithm was built based on the information gathered through manual analysis of close to 10,000 confirmed kinase–substrate pairs for 229 typical kinases. Coupling with the alignment of the primary amino acid sequences of the catalytic domains of protein kinases, the specificity-determining residues (SDRs) were identified, and the position-specific scoring matrix (PSSM) was generated for each of the kinases for predicting their respective recognition sequences around the phosphorylation site. The PSSMs were then used to derive the optimal substrate peptide sequences. In total, 445 15-mer peptides corresponding to the predicted sequences with a single phosphorylatable residue (Ser, Thr, or Tyr) in the middle were synthesized and immobilized onto an epoxysilane-coated glass microarray surface. The resulting peptide microarray was made up of four identical subfields to allowing four kinase assays to be run in parallel. Phosphorylation of the peptides on the array was carried out by applying active protein kinases individually into each field under their respective assay conditions, and the extent of peptide phosphorylation was then detected with Pro-Q Diamond (Life Technologies), a fluorescent dye that had been validated to bind specifically to phosphorylated residues including Ser, Thr, and Tyr, regardless the context of sequences they are in. So far, over 200 protein kinases have been assayed. Many highly reactive and selective peptides have been identified as substrates for the kinases tested. While most of the sequences conformed to those reported previously, some novel motifs were also uncovered. Detailed analysis of the "hit" peptide sequences is expected to reveal the prototype optimal substrate peptides unique to each kinase, which will then be further optimized for their reactivity and selectivity using an oriented peptide library approach. It is expected in the near future a peptide microarray spotted with substrate peptides that are preoptimized to each of the kinases will become available from Kinexus for kineome profiling in complex biological samples.

Protein Phosphatase Activity Fingerprinting

Compared to protein kinases, protein phosphatases have been less well characterized with respect to their regulation and physiological substrates. This can be attributed to the misconception that phosphatases are promiscuous in substrate recognition and

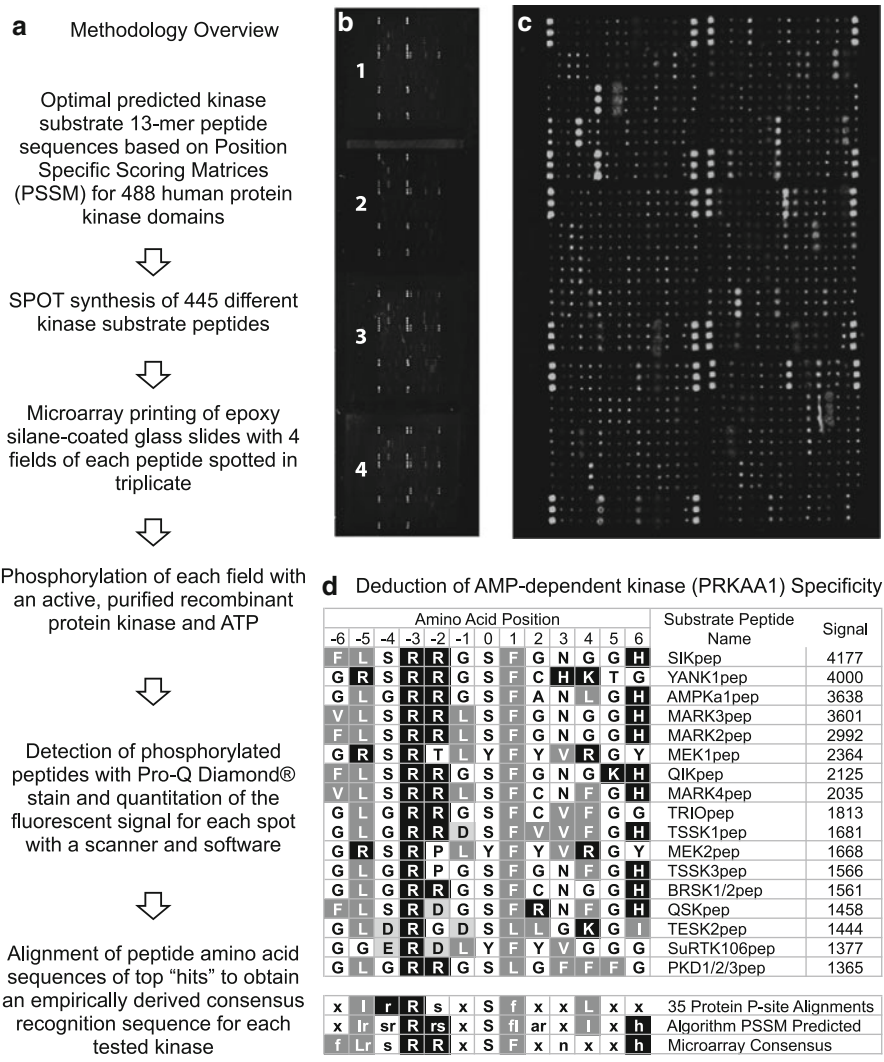

Fig. 7.1 A bioinformatics algorithm-guided identification of the optimal peptide substrates unique to each kinase on peptide microarrays. *Panel* a. Schematic description of the workflow from peptide substrate sequence prediction using the Kinase Predictor 1.0 Algorithm developed by Kinexus to select test peptides for phosphorylation by kinases on the peptide microarray and, finally, to the deduction of the optimal substrate sequences for individual kinases. *Panel b*. Scanned image of the full Kinex™ Kinase Substrate Peptide Microarray phosphorylated with three different kinases. The second field was incubated with ATP in the absence of added kinase as a control. Each peptide featured a phosphorylatable residue (Ser, Thr, or Tyr) in the middle. The strong spots common among all four fields are the orientation markers designed for easy peptide localization. *Panel* c. Close-up scanned image of one field of the Kinex™ Kinase Substrate Peptide Microarray. *Panel* d. Alignment of the top phosphorylated peptides detected following incubation with AMP-dependent protein kinase alpha 1. Peptides were ranked according to their respective phosphorylation signal intensity, and an optimal substrate peptide sequence is shown in the *bottom row*

regulated in less stringent fashion in vivo, which might have arisen from that observation that a relatively small number of protein-serine/threonine- (Ser/Thr-) specific phosphatases are able to catalyze a myriad of dephosphorylation events, and that most protein phosphatases have not been found to recognize well-defined linear sequences or consensus motifs within their substrates so far.

Despite prevailing evidence that short synthetic phosphopeptides are poor phosphatase substrates compared to their parent proteins (Zhao and Lee 1997), as supported by the role of regulatory subunits in forming the substrate-binding sites required for substrate recognition according to crystallography studies (Virshup and Shenolikar 2009), several phosphopeptide-based studies have been reported that aimed at the delineation of substrate preferences using either activity- or interaction-based approaches (Sun et al. 2009; Wang et al. 2002).

Among the two main classes of protein phosphatases, protein-tyrosine (Tyr-) phosphatases (PTPs), not protein-Ser/Thr phosphatases, had been the focus of early studies on substrate specificities, due to the availability of better characterized phospho-Tyr antibodies than phospho-Ser/Thr antibodies. In those studies, phosphatase substrate specificities were commonly delineated using individually synthesized phosphopeptides (Cho et al. 1993; Zhang et al. 1994). In recent years, peptide arrays, peptide microarrays in particular, have been demonstrated for their utility in protein phosphatase specificity mapping and activity profiling.

In 2008, Waldmann's and Yao's groups independently used phosphopeptide microarrays for large-scale, high-throughput characterization of PTP and protein-Ser/Thr phosphatase substrate specificities, respectively (Köhn et al. 2007; Sun, et al. 2008), for the first time. While a fluorescently labeled phospho-Tyr antibody was employed to monitor dephosphorylation of tyrosine in the peptides in Waldman's study, Yao and coworkers used Pro-Q Diamond dye to detect dephosphorylation of Ser/Thr, circumventing the detection problem as a result of the lack of well-characterized generic antibodies for phospho-Ser/Thr. The dye has recently extended to the detection of dephosphorylation of tyrosine in place of anti-phospho-Tyr antibodies on peptide microarray by the same group (Gao et al. 2010). A phosphopeptide microarray featuring the most evolutionarily conserved human phosphorylation sites is now being explored for its potential for the determination of phosphatase specificities and activity profiling in our laboratory.

Protease Activity Profiling

In addition to protein kinases and phosphatases, peptide microarrays have also been successfully used to characterize protease specificity, based on the notion that proteolytic cleavage can be monitored by the changes in fluorescent signals on fluorogenic peptides immobilized on the array upon the action of proteases. Salisbury et al. (2002) used a fluorogenic peptide substrate array with 361 spatially addressable peptides to decipher the specificity of thrombin. Gosalia and colleagues employed a solution-phase fluorogenic peptide microarray, in which peptides were spotted as spatially separate nanodroplets, to reveal the evolutionary conservation

of substrate specificity of thrombin from human, bovine, and salmon (Gosalia and Diamond 2003). The approach was also applied to determine substrate preferences of 13 serine and 11 cysteine proteases (Gosalia et al. 2005). Winssinger et al. (2004) generated a library of 192 peptides tagged with peptide nucleic acid (PNA) molecules and incubated it with protease in solution, followed by spatial deconvolution on a DNA microarray to profile the substrate specificities of thrombin, plasmin, and caspase-3.

The peptide array-based protease specificity profiling approach has now become an essential part of protease characterization platform, complementary and synergistic to other proteomic approaches used to detect alterations of substrate abundance and to identify and quantitate proteolytically generated neo amino- or carboxy-termini (auf dem Keller and Schilling 2010).

7.1.3.2 Domain-Mediated Protein–Protein Interaction Mapping

The application of peptide arrays for protein–protein interaction characterization has been well documented since the advent of SPOT peptide synthesis. It is applicable to characterizing protein–protein interactions where the interface between the two interacting proteins can be recapitulated by linear peptide sequences derived from the parent proteins. It is even more advantageous compared to other proteomics techniques such as protein arrays when the protein–protein interactions mediated by PTMs such as phosphorylation are concerned, as amino acids carrying corresponding PTMs can be readily incorporated into specific sites during peptide synthesis. Not only can the peptide array-based approach be used to map the consensus sequences recognized by these domains, it can also provide dynamic information on signal-dependent change in molecular networks for proteins defined by the peptides on the array and the proteins for which the binding is monitored (Sinzinger and Brock 2010).

Intracellular signal networks are organized through the interactions of proteins, which are often mediated by a group of diverse modular protein interaction domains (PIDs) with defined specificity. Among them, the Src homology 2 (SH2) domains are the largest family recognizing tyrosine phosphorylated sequences, and thus play pivotal roles in relaying information flow emanating from receptor protein-Tyr kinases (Pawson 2007). A phospho-Tyr-oriented peptide library with only one amino acid introduced at the defined positions at a time, and a mixture of amino acids at the randomized position, was spotted on the array and was interrogated with 120 bacterially expressed human SH2 domains, and the phosphotyrosine-containing peptide sequence motifs for 76 of them were defined (Huang et al. 2008). Combining the power of phage-displayed libraries, SPOT technology, and bioinformatics, the peptide array-based approach was also successfully used to deduce the consensus sequences of yeast SH3 domains (Tonikian et al. 2008, 2009). A peptide microarray featuring peptides with inverted configuration representing 6,223 C-terminal sequences of human proteins was probed with a PDZ domain to screen for putative interaction partners (Boisguerin et al. 2004).

With the knowledge of consensus sequences for the PIDs, peptide microarrays that carry peptides corresponding to known sequences recognized by SH2, SH3, PDZ, and other PIDs have been employed to profile the binding of proteins from complex biological samples to detect the differences in molecular interactions between different physiological states (Stoevesandt et al. 2005; Sinzinger and Brock 2010).

A peptide microarray populated with peptides, in which kinase consensus sequences and caspase cleavage recognition motifs (identified through a search of the human proteome) are overlapping, was employed in a study to investigate the role of phosphorylation in the regulation of caspase signaling pathways. Protein kinase CK2 emerged as the kinase with the most number of substrates that contained kinase consensus sequences that overlapped with caspase-3 cleavage motifs, indicating a role of phosphorylation in the inhibition of caspase-mediated apoptosis signaling pathways (Duncan et al. 2011).

7.1.4 Potential of Peptide Arrays in Clinical Applications

As a natural extension to their classical application in antigenic epitope mapping, peptide arrays displaying a collection of biologically active synthetic peptides have been demonstrated in recent years to be a very versatile tool for profiling the antibody repertoire in complex biological samples such as serum, urine, saliva, and other types of body fluids for the diagnosis of pathogen infections, allergy reactions, and autoimmunity, based on the notion that the immune response to pathogens, allergens, or autoantigens can be captured by the presence or absence of specific populations of antibodies. Hence, serological mapping has become one of the most sought after applications of the peptide array technology, as it appears to have the greatest clinical potential. Peptide libraries featuring fragments derived from autoantigens, allergens, or viral proteins presented on either the SPOT membrane-based peptide macroarrays or glass slide-based peptide microarrays have been used for antibody profiling in clinical samples. The clinical potential of such analyses have been shown by their use for antibody spectrum profiling in the sera from patients infected with hepatitis B and C, with a simian–human immunodeficiency virus (SHIV), the severe acute respiratory syndrome (SARS) corona virus, and with herpes virus. This provides crucial information not only for infection diagnostics but also for the development of vaccines (Neuman de Vegvar et al. 2003; Duburcq et al. 2004; Guo et al. 2004; Andresen and Grötzinger 2009).

The use of peptide arrays in kineome profiling has also inspired the exploration of their application in the studies of human diseases. As increasing numbers of kinase substrate peptides have been identified in recent years, peptide microarrays with the capability of screening a broad range of protein kinases have been established and used to profile the aberrant kinase activity in clinical samples as well as for monitoring the response to kinase inhibitory compounds in a high-throughput manner. This underscores the potential of peptide arrays in disease diagnosis and drug discovery (Piersma et al. 2010). Among the handful of studies

reported so far in this area, Schrage et al. (2009) recently reported the activation of multiple pathways in relation to AKT/GSK3β, PDGFRB, and Src protein kinases in chondrosarcoma cells on a kinase substrate peptide array containing 1,024 peptides. Supplemented with the cell viability data in vitro, the study indicated that the Src inhibitor dasatinib is a potential treatment option for patients who are inoperable (Schrage et al. 2009). Tuynman and colleagues investigated the molecular mechanism underlying anticarcinogenic activity of celecoxib (Celebrex), a selective cyclooxygenase-2 (COX-2) inhibitor, against colorectal cancer (CRC) using a kinase substrate peptide array with 1,176 different kinase substrate consensus sequences and found that celecoxib represses c-Met-dependent signaling, which in turn led to downregulation of oncogenic Wnt signaling in CRC, supporting the potential of targeting c-Met and Wnt signaling in CRC therapy (Tuynman et al. 2008).

Recently, a cellulose membrane-based peptide array of 70 peptides derived from p160 peptide, a cancer cell targeting peptide identified by phage display, was employed to optimize the affinity of the peptides for human cancer cells using peptide-whole cell interaction assay (Ahmed et al. 2010). The binding of the three peptides with the highest affinity and selectivity for cancer cells was further confirmed using fluorescence imaging and flow cytometry. The study revealed the potential of the peptide array-based whole cell binding assay for screening and identifying cancer cell targeting peptides for cancer diagnosis and drug targeted delivery.

7.2 Peptide Arrays for Kinase Activity Assays

Rob Ruijtenbeek

7.2.1 Application Fields for Peptide Arrays Measuring Activity

7.2.1.1 Functional Proteomics

Peptide microarrays broaden a new field of research and applications often referred to as functional proteomics (Thiele et al. 2011). While DNA and protein arrays mostly focus on determination of abundance of RNA or protein molecules, peptide arrays allow the *functional* analysis of multiple proteins or protein families (Fig. 7.2). By functional analysis, we mean the detection of protein activity. Clear examples are the detection of enzymatic activities, for example, of kinases, phosphatases, and proteases in lysates from cells or tissues. However, nonenzymatic functions, like the responses to hormone binding of nuclear receptors in terms of specific coregulator protein recruitment, are also currently being studied on peptide arrays (Heneweer et al. 2007; Koppen et al. 2009). Peptide arrays enable the miniaturization and multiplexing of activity-based assays.

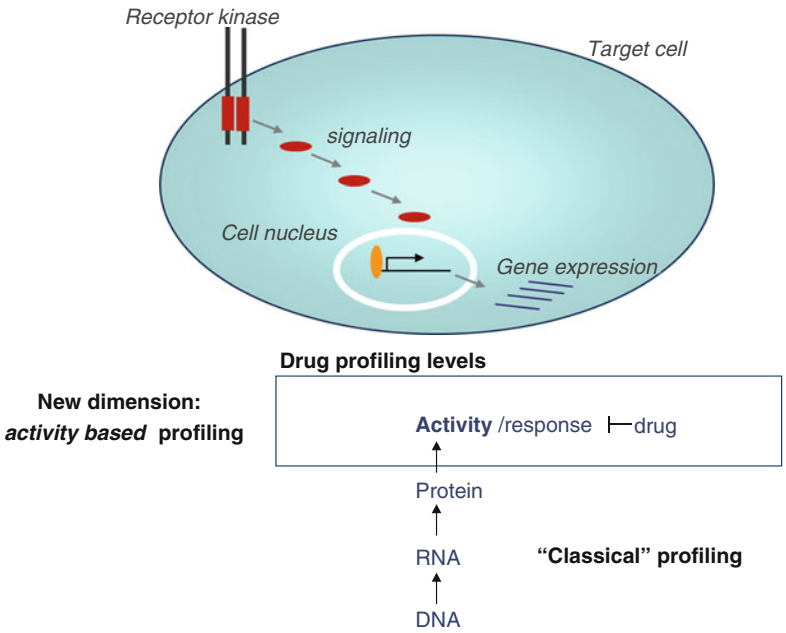

Fig. 7.2 Molecular profiling aimed at identification of molecular biomarkers often involves detection of DNA mutations, or measurements of RNA or protein abundance levels. Functional proteomics approaches, however, profile the *activities* of proteins instead. Kinase activity profiling is an example in which the enzymatic activities of kinases, playing a central role in signal transduction—in many cases leading to gene and protein expression—are measured

In the context of pharmaceutical research, and in the field of translational medicine in particular, such array-based approaches are emerging. This makes sense since the majority of the drugs being developed target protein activity and function. These new and more targeted drugs act by effecting protein function rather than targeting DNA or RNA or interfering with the modulation of protein levels. Because functional profiling of the interaction of drugs with cellular or tissue samples is of specific interest in pharmaceutical research, peptide microarrays are proving to be very useful with their ability to profile protein activity and its modulation by drugs.

7.2.1.2 Kinase Drug Targets

We focus here on the drug class of kinase inhibitors which have been reshaping the oncology field due to their high success rate. These molecules inhibit kinase function by reducing kinase activity, which can be monitored on a peptide array.

Kinases play a pivotal role in cellular biology by being the key regulators of signal transduction. Signals being detected by a membrane-bound receptor are transduced to the inner parts of the cell to result in an appropriate response.

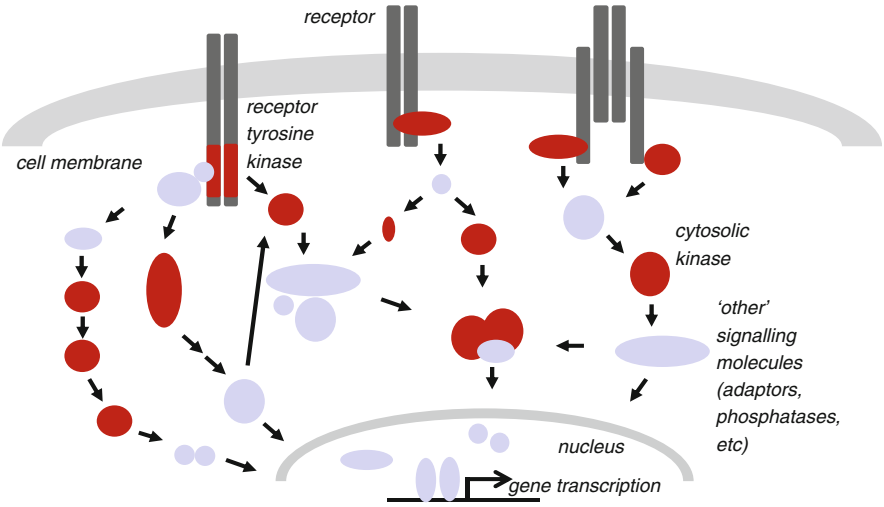

Fig. 7.3 Kinases in signal transduction cascades. In this figure, the complexity of signal transduction is represented by the interaction of multiple proteins of which the kinases are shown in *red*

This happens via highly complex cascades of events in which the signal is received and propagated using transphosphorylation reactions (Fig. 7.3). These reactions are catalyzed by protein kinases together with the crucial ATP molecule. ATP is important as not only does it provide the kinase's energy source, it also supplies the phosphate moiety, vital to the whole signal transduction cascade. A kinase becomes activated and places this phosphate group on a substrate protein; this being the subsequent link in the signal transduction pathway. Often this substrate protein is a kinase as well. A signal transduction event can be compared to a relay in athletics, where each kinase gets activated by an upstream event and subsequently passes on the baton to the next member downstream in the pathway. Most protein kinases have distinct preferences for the aromatic hydroxyl groups of tyrosine residues or for the aliphatic serines or threonines. It is this characteristic which divides this family of more than 500 members into two kinase subfamilies: protein-tyrosine kinases (PTKs) and protein-serine/threonine kinases (STKs).

7.2.2 Technology and Applications

7.2.2.1 Kinase Activity Profiling Technology

While in classic kinase assays the activity is detected by the phosphorylation of a single substrate, multiple substrates can now be immobilized and monitored on a microarray. Instead of placing multiple protein substrates on a chip, only the phosphosites (the sites within the protein which become phosphorylated) are

immobilized in a peptide microarray. Thus, the peptides represent the protein substrates. As has been discussed in the previous chapters, this can be done in a variety of ways, but all are based on a solid support. In most cases, the sequences are derived from known phosphorylation sites in the human proteome. As the human proteome is estimated to comprise more than a million proteins, of which more than two-thirds can be phosphorylated, this indicates the huge amount of different phosphosites that can be investigated by peptide arrays.

The principle of the assay is that the kinase activities in the sample of interest phosphorylate the peptides. The phosphorylation event is detected by either radiography or fluorescence imaging of the array. In radio assays, the peptide is phosphorylated using radioactive ATP as the phosphate source. This approach is increasingly being replaced by the use of fluorescence assays. In the latter case, the phosphorylation of the peptide is detected by a fluorescently labeled molecule which is either a chelator (e.g., phosphotag) or an antibody. Ideally, the antibody needs to detect the phosphoamino acid in all the available peptide sequences on the chip equally well and independently of the adjacent amino acids. Antibodies like PY20 work very well in detecting tyrosine phosphorylated peptides, but for serine/threonine phosphorylated peptides, cocktails are needed for full coverage of detection.

The first peptide microarray applications for kinase profiling used glass as a solid support and radioactivity for readout at a single time point. Later, protocols were developed based on the fluorescent readout of labeled antibodies (or cocktails of antibodies) binding to phosphorylated peptides. A second generation of this technology was developed by researchers in the Netherlands and is referred to as the PamChip® technology (Lemeer et al. 2007; Hilhorst et al. 2009; Versele et al. 2009) (Fig. 7.4). With this technology, antibody-based fluorescence detection has been combined with a change of solid support from glass slides to a porous ceramic. In this format, the sample is pumped up and down through the porous aluminum oxide ceramic sheets, in which the peptides are immobilized at designated spots. Each spot comprises thousands of separated pores with diameters of 0.2 μm in which the peptides are site-specifically immobilized. Each time the sample is below the solid support, the degree of phosphorylation is monitored by imaging the fluorescence intensities caused by the antibody binding to the phosphorylated peptides alone. These time curves, or kinetic readouts, appear to be instrumental in the enzymatic studies; a kinase is after all an enzyme which catalyzes the rate (the kinetics) of a phosphorylation reaction. In addition, the kinetic and multi(time) step readout for each of the 144 or 256 peptides on each single array allows much more comprehensive statistical analysis of the signals than data from a single time point per peptide spot on a glass array (Thilakarathne et al. 2011).

The application of peptide arrays in biological, pharmaceutical, and medical studies often requires the analysis of many samples under variable conditions. For example, lysates from cells should be analyzed using a range of time points, varying concentrations, and with multiple different drugs. For this reason, a system has been developed which has the capability of analyzing 96 arrays at once. This latest technology for kinase activity profiling is based on a 96-well plate format, in

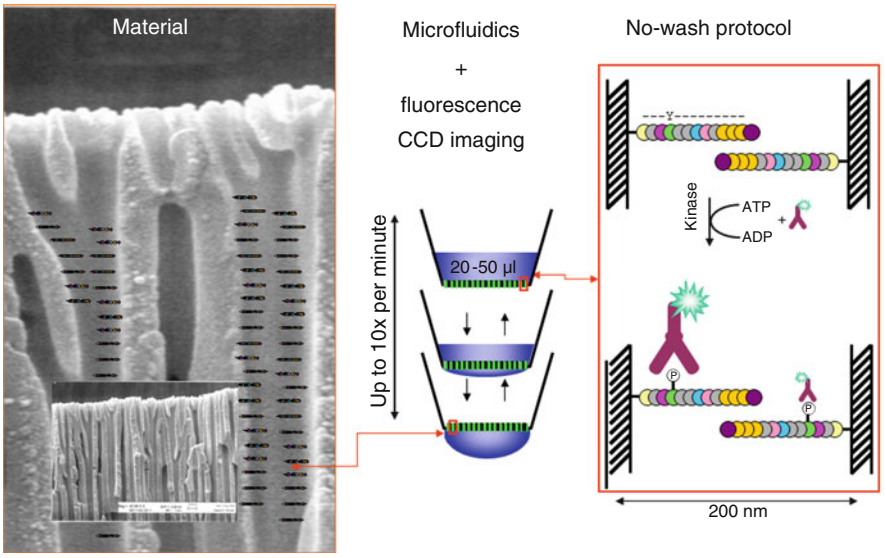

Fig. 7.4 PamChip® technology is based on a porous substrate made of aluminum oxide, in which pores the peptides are immobilized (*left panel*). Due to this porosity, the sample can be pumped up and down through this solid support. Every time when the sample is positioned below the microarray, an image is taken of the microarray by a CCD camera (*middle panel*). Via this real-time imaging of the microarray, the signal, developing in the peptide spots due to binding of fluorescent antibody detecting peptides phosphorylated by the kinases, can be monitored

which each well comprises a peptide microarray. Bioinformatics for analysis of the vast datasets from such studies has been evolving in parallel. Thilakarathne et al. (2011) developed a new method based on semiparametric mixed linear models to further enhance the amount of information that can be obtained from the multiparallel kinetic readouts from each microarray.

7.2.2.2 Applications

A straightforward application is substrate identification using recombinant kinases. Such studies have indicated that different kinases have their own preferences for the peptide sequences they phosphorylate. Clear differentiation between the PTKs and STKs has been confirmed, although dual specificity kinases have also been found. In addition, it has become apparent that although each kinase has a preference for particular peptide sequences, they can also be promiscuous, resulting in multiple peptides being phosphorylated to different degrees in diverse peptide sets. In short, the degree of phosphorylation by purified kinases varies from peptide to peptide and can be profiled in hundreds per array, resulting in phosphorylation *fingerprints*.

Substrate profiling studies have revealed important biological information as described in the paper by Schutkowski et al. where they showed that for optimal recognition by GSK3b, a peptide substrate should be prephosphorylated or primed

(Schutkowski et al. 2004). Another interesting application was explored by Poot et al. who identified an optimal substrate for PKC isozymes and coupled this peptide to an ATP-binding site inhibitor to generate a bisubstrate (Poot et al. 2009; van Ameijde et al. 2010). The peptide microarrays were subsequently used to evaluate the resulting inhibitors, which were potent and selective toward the theta isozyme.

With the development of protocols for profiling cell lysates of tissue homogenates, the application area has been broadened to signal transduction and pathway studies [well reviewed by the group of Schutkowski (Thiele et al. 2011)]. The effect of a stimulus or a kinase-inhibiting drug on cultured cells can now be investigated at the complexity level of a cell, where multiple kinases can be active in the context of the interacting networks that exist. At this point, peptide arrays provide a welcome extension to classical methods like (phospho) Western blots and ELISAs, which monitor drug effects on a (single) kinase by detecting the variation in abundance of the downstream phosphorylated substrate. The peptide arrays monitor the enzyme activity of multiple kinases at once and not only the end result of this activity.

An interesting feature of functional proteomics is found in the ability to study *direct effects* of the investigative drug. Because activities of kinases can be monitored in cell lysates or tissues, drugs can be characterized in a complex, and probably more realistic, context than in classical single readout (singleplex) assays. This latter type of assay is limited as it can only investigate the activity of the isolated drug target. Drug selectivity profiling is a clear example of an application which benefits from the combination of multiplexing and miniaturization (Fig. 7.5).

Another example of an application area is the unraveling of a drug's cellular mechanism of action. In this application, the peptides on the chip represent multiple different proteins involved in complex cellular pathways and signal transduction networks. In a small lysate sample, derived from just 10,000 to 100,000 cells or less than a tenth of a cubic millimeter of tissue, multiple diverse interactions can be studied at once.

7.2.2.3 Reversed Translational Medicine and Biomarkers

A recent development of peptide microarrays has been the application of new drugs in pharmaceutical research and clinical development. In the field of oncology in particular, fundamental progress has been made by so-called *targeted medicine*. Previously, anticancer drugs were targeting cellular processes, like cell division, more globally. New insights into cell signaling and signal transduction cascades have changed the way novel oncological drugs are being developed, and it is the kinase enzyme class which is playing a crucial role in this progress. Many of its members play a pivotal role in the mechanisms of tumor genesis, and some kinases are even the active protein products of oncogenes. Examples of successful cancer drugs targeting protein kinases—or the signaling pathways they are involved with—are imatinib, erlotinib, gefitinib, and the previously mentioned sunitinib

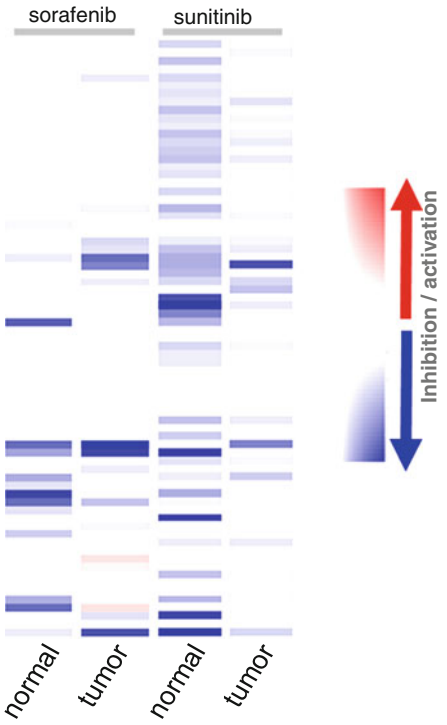

Fig. 7.5 Selectivity profiling of kinase inhibitor drugs using peptide microarrays. Here sorafenib inhibition profiles are compared with sunitinib in extracts from both normal and tumor tissue from a renal cell carcinoma patient

and sorafenib. These are all molecules that block the catalytic activity of protein kinases. A related class of therapeutics is antibodies, which intervene in a different way with cellular signaling: they act by blocking the initiation of receptor signaling. Examples of the latter are trastuzumab and bevacizumab, which block EGFR kinase and VEGFR signaling, respectively.

These drugs can be studied comprehensively with peptide arrays. In these studies, two formats are currently being used. Using cell line models, the cells are either treated with the drug in culture or on the chip. In the first case, lysates are prepared from the cells before and after treatment and profiled for activities on the chip. In the second case, lysates can be treated directly by spiking the drug into the solution just before application onto the chip. In the latter instance, cell lines, tissue homogenates from animal models, or even clinical samples can all be used. The effects of the inhibitors on the kinase activities in these samples can all be directly assessed. Although the highly important context of the cellular architecture is lost, which is surely a downside, the potential to profile all detectable, full-length kinases—with their relevant posttranslational modifications—in the same sample, opens up vast new fields of applications.

In drug discovery, researchers screen for kinase-inhibiting compounds in chemical libraries. During such studies, they often use an abstracted model, the purified protein, but this protein is frequently truncated to its domain only. A major disadvantage of this approach is the absence of other domains, including those with a

regulatory function. In the recently developed protocols for peptide array analysis of kinases in cell and tissue lysates, the drug target can now be studied more naturally as a full-length protein, in the way it is actually expressed in cells or tissues. At first, this was shown in a model system, but interestingly, this approach appears to be translatable to patient-derived tissues. This means that the kinase drug targets can now be studied in the same form as they are expressed in a patient's tumor, thus full length, fully decorated with all relevant posttranslational modifications and in the presence of stabilizing or activating cofactors (e.g., heat-shock proteins). In addition, they can be studied in the presence of all other kinases expressed in the cell or tissue being investigated. Such analysis of patient-derived tumor samples can result in the identification of tumor-specific kinase activities. When linked to pathological, diagnostic, and/or clinical data, this can lead to the identification of diagnostic or prognostic biomarkers.

While the on-target effects are being monitored, the researchers can also obtain insights into the drug's effect(s) on other kinase targets, which are either intended—in the case of multitarget inhibitors—or unintended and can putatively cause side effects. The latter opens up new opportunities for the toxicologist in investigating and understanding adverse drug reactions leading to toxicological biomarkers.

A very typical feature of activity-based assays is the capability of drug testing. With peptide microarray-based kinase assays, not only can multiple kinases in a patient sample be studied at once, their response to their inhibiting drugs can also be studied. This possibility links the presence and activity of the kinase drug targets to their responsiveness to the drug.

7.2.2.4 Personalized or Precision Medicine and Drug Response Prediction

Drug response is a leading parameter in the clinical development of a drug. In the development of kinase inhibitors in cancer, the response rates are often very low, even in case of effective drugs. These drugs are developed against specific kinase targets, but these targets are not always equally present or active in the whole treated population. Furthermore, in a subset of nonsensitive or resistant patients, the role of this target in tumorigenesis and growth or metastasis is not essential and can be overruled by other mechanisms. In order to identify the patient subpopulation that is likely to respond, tests need to be developed that match the right patient to the right drug and vice versa as more drugs are being developed.

There are already examples of such companion diagnostic tests. For the prediction of response to trastuzumab (Moelans et al. 2011), targeting the receptor tyrosine kinase Her2/Neu, patients are tested for the presence of this target on their tumor cells, before they receive this breast cancer therapy. A recent example is the test for the ELM4-ALK translocation to select patients for pharmacotherapy with crizotinib (Kwak et al. 2010), an ALK kinase inhibitor. If the whole lung cancer patient population would have been treated, only an extremely low percentage would have shown a clinical response because only 4% have this mutation. The availability of the companion diagnostic test was therefore essential for the success of the clinical trial.

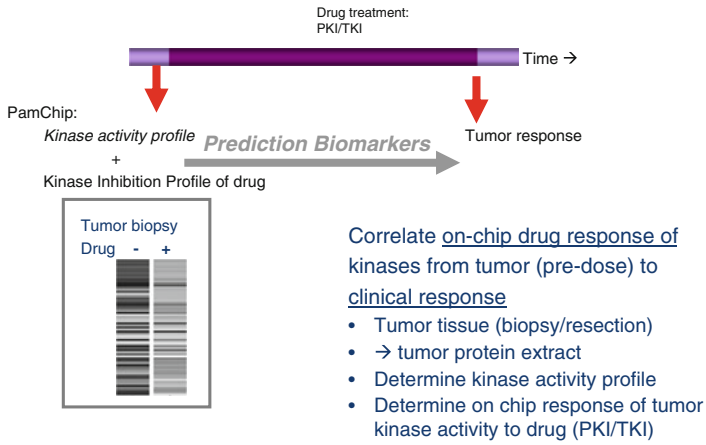

Fig. 7.6 Strategy for prediction of a patient's drug response using pretreatment biopsies on kinase substrate microarrays

The identification of predictive biomarkers appears to become essential in many drug development programs. The classical technologies for biomarker discovery are based on testing for DNA mutation or RNA or protein expression levels. Molecular data are obtained in biopsies taken before the patient is treated. If these data can be correlated or associated to the therapy response, this can be the start of generating a companion diagnostic test. Peptide microarrays are also currently being used in this effort. While classical methods cannot involve the drug of interest in predose tissue samples, kinase microarrays can, as discussed above. In addition, the drug can actually be used in the test. This means that drug-specific data and information can be generated using predose biopsies. Proof of concept of this approach was shown by Versele et al. in a multiple cell line study. Analogous to the way it is aimed to work in a clinical setting, they profiled the lysate of a cell line on a peptide microarray in the presence and absence of their drug candidate. The inhibition profiles were used to predict the response of the cell line to drug treatment in culture. From these profiles, they could identify a set of peptide phosphorylations of which the response (inhibition) on the chip was predictive for the tumor cell proliferation (Versele et al. 2009). This concept (Fig. 7.6) is now being explored in clinical studies by my research group in collaboration with the Netherlands Cancer Institute (NKI), the VU Medical Center, and other cancer centers in both the USA and Japan. In a study presented at ASCO in 2011 on neoadjuvant treatment of non-small-cell lung cancer with the EGFR kinase inhibitor erlotinib, we showed that candidate biomarkers could be identified. On-chip peptide phosphorylations and inhibitions were correlated to clinical responses. With no information on the pathological assessment of the resection tissues available to the testers, a model built on those profiles could still predict the pathological response (Hilhorst et al. 2011). It should be noted that resection tissue was used and not pretreatment biopsies which is needed to make this into a

companion diagnostic test. Nonetheless, this shows a promising new application of peptide microarrays.

7.2.3 *Conclusion and Future Applications in Personalized Medicine*

It could be possible to apply this principle of drug testing on patient-derived tissues to other targeted therapies as well. In addition, if other protein classes are targeted, for example, phosphatases, proteases, nuclear receptors, acetyltransferases, histone deacetylases, and methyltransferases, the target responses in patient-derived samples could be tested using a peptide microarray.

The nonfocused, nonbiased, and global profiling nature of the arrays allows parallel monitoring of drug targets and class-related nontargets. These nontargets can be functional proteins involved in the mechanisms of resistance and are therefore possibly very useful markers for predicting resistance to targeted therapies.

Finally, nontargeted therapies such as chemoradiation could also be accompanied in the future by such testing methods, as was shown in a recent publication by a Norwegian group. They generated kinase activity profiles of tens of biopsies taken before patients were treated and could identify peptide phosphorylation patterns that correlated to the tumor regression grade after therapy. They generated a response prediction model that could predict the responses of a newly tested set of patients with promising accuracy (Folkvord et al. 2010).

7.3 Peptide Microarrays by Laser Printing

Thomas Felgenhauer, Ralf Bischoff, Frank Breitling, and Volker Stadler

7.3.1 *Introduction*

Several sophisticated methods are in use worldwide to produce peptide microarrays. Each of these methods has its special advantages and drawbacks. High amounts of identical oligomers are achievable on cellulose supports via SPOT synthesis (Frank 1992; Dikmans et al. 2006), but spot densities are very low due to droplet handling. With photochemical methods where chain growth is induced by a laser beam, very small spot sizes and high spot densities are possible (Fodor et al. 1991a, b; Lipshutz et al. 1999). In this case, the drawback is in the sequential use of monomer solutions which might be acceptable in DNA synthesis (four monomers), but the yield is dramatically reduced when the number of needed

coupling cycles increases as in the case of standard peptide synthesis where minimum 20 individual cycles are needed to complete a fully combinatorial layer.

The use of a laser printer as synthesis machine makes it possible to overcome the obstacles of the methods described above. Solid particles (toners) carrying the reactive building blocks are printed in parallel in high resolution to a desired support. A full combinatorial layer is developed—like a color picture printout—at once, and the coupling cycles are reduced from 20 to a single one per layer (Stadler et al. 2008).

7.3.2 Technical Aspects of Commercial Laser Printers

A commercial laser printer uses small solid toner particles (~10 μm) that are triboelectrically charged by friction inside a toner cartridge drum system. Because of the materials involved, this procedure leads to strong electrical charges on the particle surfaces, which enables the directional movement of the particles within electrical fields. A laser beam or an LED row translates 2D light patterns into electrical patterns on top of an organic photoconductor drum. These images are developed with the charged toner particles that are finally transferred to the support. At office applications, a color laser printer system delivers four different color toners (black, cyan, yellow, and magenta) on a sheet of paper with a resolution of 1,200 up to 2,400 dpi. The polymer-based toner particles are fixed to the cellulose support by heat.

7.3.3 Combinatorial Synthesis with Laser Printers

The main challenge in combinatorial synthesis is to deliver different kinds of monomers with high accuracy to their designated reaction partner or reaction site. Whereas a color laser printer delivers only four toners, a peptide synthesizer based on the xerographic technique should be able to handle at least 20 different building blocks for basic peptide synthesis or other feasible monomers for the production of peptide mimetics (amino acids in D-form, methylated, phosphorylated derivatives, nonnatural versions).

In addition to the great flexibility of the synthesizer, an exact positioning of consecutively printed layers is the basic requirement for the parallel elongation of combinatorial assembled oligomer chains. With increasingly better printing accuracy, the spot density also increases, as well as the diversity of synthesized peptides.

7.3.4 Solid Particles as Reaction Units

To benefit from the laser printer as delivery machine for monomers in combinatorial chemistry, the toner particles (delivery packages) have to be modified for this chemical purpose. In addition to their properties as solid, electrically charged particles, they also need the attributes of a solvent once melted. This change of properties happens after the particles have been addressed to their designated reaction site, where they are transformed into a liquid sphere simply by melting. Thereby, activated monomers are mobilized, which allows them to diffuse to their reaction partner for chain elongation. These very special solid/liquid characteristics of the toner particle depend on the choice of the appropriate matrix material. On the one hand, this material should withstand the harsh mechanical treatment inside the printer (e.g., friction, charging, transport); on the other hand, the liquefaction at moderate temperatures ($<100^{\circ}C$) is fundamental in order to perform as a solvent for a chemical reaction. In addition, the matrix material should protect the reactive monomers from ambient conditions during long-term storage in cartridges, and finally, the material itself must be inert toward the components inside.

Since all the different monomer particles are addressed in parallel by the printer, all the different activated amino acid derivatives within a completed layer of amino acid particles are activated at once in a single melting step. This feature is the main advantage of our technique. Washing and deprotection steps that follow after the coupling step finish the cycle, and result, if repeated, in the combinatorial synthesis of a peptide array (Fig. 7.7).

Our method uses conventional Fmoc chemistry (Chan and White 2000) and differs from the SPOT synthesis only in the solvent we employ: it is solid at room temperature, which allows for the intermittent immobilization of chemically highly activated amino acid derivatives within particles. This activation is due to a C-terminal pentafluorophenyl ester in combination with N-terminal Fmoc protection and standard side chain protecting groups. Surprisingly, when embedded in particle matrix, these very reactive chemicals proved to be stable for months at room temperature, an exception being Fmoc-arginine-OPfp that shows a decay of 4% per month. However, if compared to the much faster decay of activated arginine esters in solution, this decay is negligible.

7.3.5 Surface Coatings

The surface-coated solid support must provide free amino groups that react with preactivated amino acid derivatives. It must stand harsh conditions during peptide synthesis (solvents, bases, strong acids during final cleavage of side chain protecting groups) and postsynthesis; it must allow for the incubation of arrays

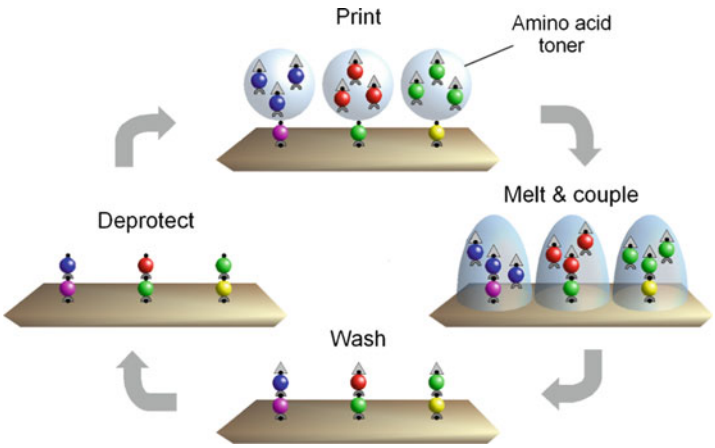

Fig. 7.7 Combinatorial synthesis with a peptide laser printer. (Print): a laser printer addresses different reactive monomers embedded in a solid matrix material in parallel to a support that displays previously synthesized peptide fragments with reactive groups. (Melt and Couple): once printed, the particles are melted. This frees the monomers to diffuse and couple to the growing chains on the support. Different reaction spheres are separated from each other due to surface tension, which constricts melted particles to small individual hemispheres. (Wash): a cycle of synthesis is completed when excessive monomers are washed away, and (Deprotect): the Fmoc protection group is removed. Repetitive coupling cycles yield a peptide array. In contrast to lithographic synthesis methods, this is done with only one coupling reaction per layer

with an analyte, for example, an antibody solution. We employ 30-100 nm thick 3D polymer coatings that have a high loading capacity (high density of amino groups). Alternatively, essentially 2D layers are used as solid supports for peptide synthesis. These are generated from functionalized silanes that also stand the conditions during peptide synthesis and, dependent on the used assay system, sometimes perform better when compared to the polymer coating. Such surfaces are described in detail elsewhere (Beyer et al. 2006; Stadler et al. 2007).

7.3.6 The Peptide Laser Printer

The peptide laser printer at PEPperPRINT GmbH (see Fig. 7.8a, b) has 24 printing units assembled in a row. Twenty of these toner cartridges that are based on the Oki system are equipped with Fmoc-amino acid esters; the four remaining cartridges are used for nonstandard amino acid particles. The printer works with micron resolution which currently allows for the synthesis of 270,000 peptides on a (20 × 20) cm^2 glass substrate. This corresponds to a spot density of ~800 spots per cm^2. Currently, the machine is used for the synthesis of up to 20meric peptides.

Fig. 7.8 (**a**) The peptide laser printer. (**b**) Horizontal setup of the 24 development units (printing cartridges) assembled in a row

7.3.7 Applications

Our particle-based synthesis method makes available to the scientific community, for the first time, very high-density peptide arrays. This is, in technical terms, the main novelty of this method (Table 7.1). As such, this method certainly soon will approach the number of different molecules that Nature's screening systems employ. These use, for example, millions of randomly generated antibodies to screen for binders against virtually any target molecule, among them nonnatural molecules that have never been encountered by evolution. Peptide arrays with *natural* amino acids (L-form) have been used in the past mainly as protein fragment libraries in proteome research, or as diagnostic tools for serum screening and for antibody profiling. For the development of novel therapeutics, often *nonnatural* amino acids (e.g., D-amino acids) were integrated at critical positions within the peptide sequence in order to increase the metabolic stability of the peptides. However, due to the use of only low-density peptide arrays, up to now, such screens usually had to rely on extensive previously available knowledge. Typically, a peptide sequence that was already known to bind to the target was then modified to improve the stability or the binding affinity, or an already known antigenic protein sequence was used to generate many overlapping peptides in order to narrow down an antibody's binding epitope.

In the near future, we will certainly see that very high-density and affordable peptide arrays will be used to find binders without extensive previous knowledge about the sequence of a potential binder. Similar to Nature's screening systems, a vast number of different peptides should be sufficient to find binders against nearly any target molecule, and different from surface display techniques, the array format will allow for an easy and unequivocal discrimination of specific from nonspecific binders. Very high-density peptide arrays will be used for such screens that have been cleared from all those peptides that were found to bind to more than a few different target proteins. It is practically impossible to avoid such nonspecific binders in all the surface display methods. Thus, the high combinatorial diversity given by the laser printer method should increase the possibility to discover potent

Table 7.1 Peptides are covalently bound to a glass slide microarray substrate and available for a wide variety of binding assays including:

• Biomarker discovery
• Antibody profiling
• Epitope mapping
• Immunoassays
• Protein interactions
• Kinase screening
• Protease screening
• Phosphatase screening
• Affinity profiling
• Protein fingerprinting

lead structures. Moreover, fast follow-up synthesis of all possible permutations of these structures due to affinity maturation should reduce the timescale and the costs in *lead-to-hit* development dramatically.

7.3.7.1 Serum Profiling

Low-density peptide arrays have been used previously to narrow down the binding epitope(s) of binding antibodies by synthesizing many overlapping peptides derived from the sequence of a known antigen that has been used, for example, to immunize an animal; however, such experiments used to be prohibitively expensive. High-density peptide arrays are cheaper—they simply allow more of these experiments. It should be feasible in the future, for example, to routinely monitor the kind of antibodies that evolved in a mouse that was immunized with a protein. The experimenter could then use only those mice that evolved an antibody specific for an especially interesting epitope within the protein that was used for immunization, thus saving a lot of time and money by using only selected mice for the generation of hybridomas. Shown as an example for this statement are the results obtained when we used four different rabbit sera that were immunized with protein A and closely related protein B (Fig. 7.9). Only one of two rabbits immunized with protein A or with protein B revealed that specific antibodies have been generated, while the other two rabbits did not generate protein A- or protein B-specific antibodies. The staining pattern revealed that rabbit 1 developed several antibodies that were specific for the C-terminal region of protein A, while rabbit 3 developed antibodies that targeted the N-terminal region of protein B, and when scrutinizing the peptide sequences, both productive sera did not reveal cross-reacting antibodies against determinants from closely related proteins #A and #B.

7.3.7.2 Biomarker Discovery: Follow-Up Synthesis, Permutation Screen

A biomarker is a substance that is used as an indicator of a biological state. The biomarker serves as an indicator to measure and evaluate normal or pathogenic biological processes, or pharmacologic responses to a therapeutic intervention.

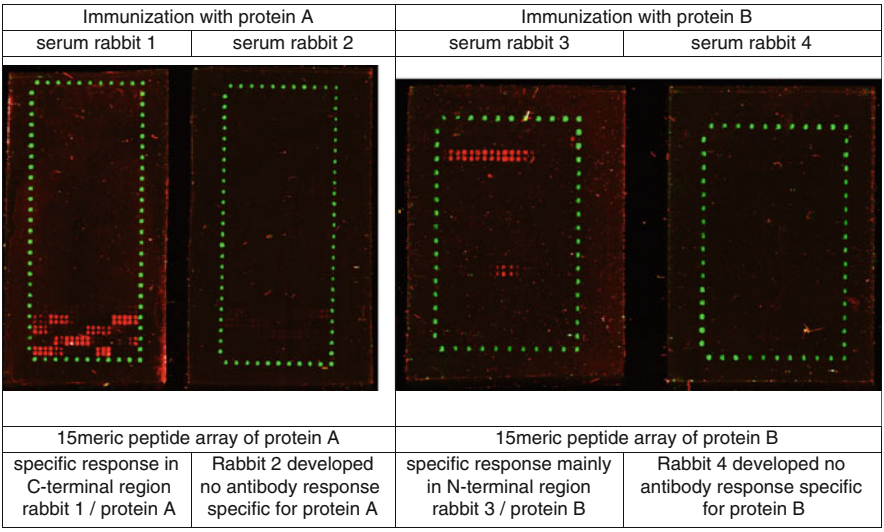

Fig. 7.9 Profiling of four rabbit sera against 15meric peptides derived from two antigens (A&B). Peptide spots are arranged in double rows and adjacent structures have an overlap of 14 amino acids. The arrays are framed by green control spots

Especially useful are biomarkers that are found in human sera. These are a rich and accessible source for the detection of diagnostic markers in human diseases. Serum antibodies have been used extensively for diagnosis, for example, of flu antibodies in a patient. Such antibodies often are found in a patient decades after infection and can easily be analyzed with peptide arrays. Especially interesting are those (peptide-)antigens (and their corresponding antibodies) that are targeted by an immune response. These are useful as biomarkers for drug discovery and diagnostics. However, the most interesting scientific question in biomarker discovery is as follows: can we find novel antigens and their corresponding antibodies without previous knowledge about the antigen? Figure 7.10a shows that such a scientific question could be answered with very high-density peptide arrays. A randomized high-density array of 15meric stochastically chosen peptides (only a detail view of ~5,000 structures is shown) was used to find peptide binders for the Flag M2 antibody with its known binding epitope NNN**DYK**NNN**D/E**NNN. Indeed, we could find six weak binders in a first screen. Sequences from all of these initial hits were then used in a follow-up screen that stained the completely permutated sequences from these initial binders. Figure 7.10b shows such a permutation screen that started with the sequence "ECWGDYKSMECADWH" found as an initial hit. This sequence, and all the other five hits from the initial screen, then revealed either the sequence NNN**DYK**NNN**E**NNN or NNN**DYK**NNN**D**NNN, i.e., the Flag M2 epitope. All amino acid positions depicted by "N" could be exchanged by other amino acids. It remains to be seen if such a screen could be employed to also find novel biomarkers when staining stochastically chosen very high-density peptide arrays with serum antibodies derived from patients with enigmatic diseases.

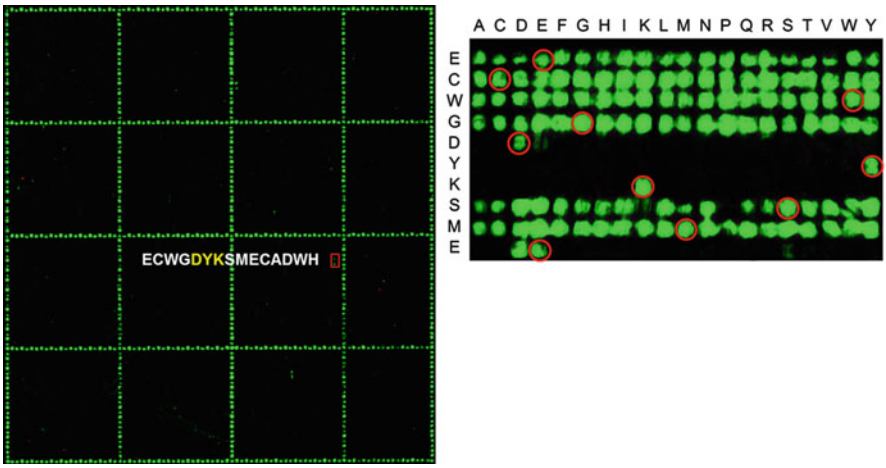

Fig. 7.10 (**a**) Lead structure "ECWG**DYK**SME**C**ADWH" found within a randomized array of 15meric peptides. (**b**) Follow-up synthesis; full permutation of the sequence ECWG**DYK**SM**E**. Wild-type residues are highlighted by a *red circle*

7.3.7.3 Drug Development

Many protein–protein interactions are mediated by short linear motifs. Some of these interactions are deregulated in diseases and thus are potential targets for modulating peptide-based drugs. These peptides should interfere with the protein–protein interactions by binding to one of the partners, either activating or inhibiting the signals that depend on the respective proteins. Historically, and as stated above, peptide drugs have been based, for example, upon the optimization of natural peptide hormones but, more recently, novel peptides are being developed that have been isolated from combinatorial recombinant libraries. The idea is to offer such a large number of different potential peptide-based binders that simply by chance any protein would "find" at least one binder among a plurality of different peptides. However, the current size limits of peptide arrays and the associated costs had made it, until now, unrealistic to conduct such comprehensive profiling screens.

7.3.8 Conclusion

The production of microarrays by laser printing uses a novel chemical concept where an activated monomer is encapsulated within solid particles that are sent to different "addresses" on a surface displaying reactive groups. Thereby, an established technology is used for the rapid construction of a densely spaced pattern of different kinds of particles. These comprise different building blocks that are

initially "frozen" at room temperature within solid particles. Thawing these particles at once leads to a single coupling step per layer, which is the main advantage of this method when compared to sequential coupling, for example, in lithographic methods. Our particle-based method is particularly well suited for automation and, thereby, results into drastically reduced cost per peptide spot. It brings affordable high-density peptide arrays within reach of normal laboratories and may have an impact similar to the one that high-density oligonucleotide arrays had in the field of genomics.

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