Introduction: The past decade has witnessed tremendous progress in surface micropatterning techniques for generating arrays of various types of biomolecules. Multiplexed protein micropatterning has tremendous potential for drug discovery providing versatile means for high-throughput assays required for target and lead identification as well as diagnostics and functional screening for personalized medicine. However, ensuring the functional integrity of proteins on surfaces has remained challenging, in particular in the case of membrane proteins, the most important class of drug targets. Yet, generic strategies to control functional organization of proteins into micropatterns are emerging.

Areas covered: This review includes an overview introducing the most common approaches for surface modification and functional protein immobilization. The authors present the key photo and soft lithography techniques with respect to compatibility with functional protein micropatterning and multiplexing capabilities. In the second part, the authors present the key applications of protein micropatterning techniques in drug discovery with a focus on membrane protein interactions and cellular signaling.

Expert opinion: With the growing importance of target discovery as well as protein-based therapeutics and personalized medicine, the application of protein arrays can play a fundamental role in drug discovery. Yet, important technical breakthroughs are still required for broad application of these approaches, which will include in vitro “copying” of proteins from cDNA arrays into micropatterns, direct protein capturing from single cells as well as protein microarrays in living cells.

Keywords: cell patterning, membrane protein, protein array, protein immobilization, protein interaction, surface micropatterning
Article highlights.

- Microarray-based technology is a cornerstone for high-throughput assays in modern drug discovery. Protein microarray techniques are gaining importance not only for target and biomarker identification and validation, but also for developing protein-based pharmaceuticals.
- The key challenge in protein microarray technology is to maintain the functional integrity of immobilized proteins, which requires the substrate surface to be engineered protein compatible as well as site-specific protein conjugation under physiological conditions.
- Multiplexed protein immobilization on biocompatible surface is very important for high-content, high-throughput screening. For this purpose, a variety of bioorthogonal conjugation methods and multiplexed micropatterning techniques have been developed.
- More sophisticated surface architectures are required for fabricating membrane protein microarrays, since the functions of membrane proteins are inextricably linked to their native lipid environment.
- Live-cell protein microarray techniques are emerging, which exploit the tremendous progress in single-cell and single-molecule analysis and promises ultrasensitive drug screening to single cell-single molecule level under native conditions.

This box summarizes key points contained in the article.

oligonucleotides, carbohydrates, peptides and proteins. Due to the diversity of samples to be immobilized on different substrate materials, multifarious surface chemistries and microfabrication methods have been developed to ensure the specificity and efficiency of interaction screening. Thus, highly multiplexed oligonucleotide and peptide microarrays are routinely produced with very high quality and reproducibility.

However, more than 80% of the therapeutic targets are proteins, which include membrane receptors, ion channels and enzymes. Moreover, therapeutic proteins have tremendously gained importance during the past decade, which often require intensive further engineering efforts before being suitable for application. Traditional technologies for generating microarray are often not compatible with proteins as their functional integrity is not sustained under the immobilization conditions. The metastable nature of three-dimensional protein structures, which is inextricably linked to their function, as well as the highly diverse physicochemical and functional properties of proteins demand dedicated solutions for engineering protein-compatible surface architectures. This is particularly challenging for membrane proteins which constitute a major fraction of the most relevant drug targets, yet often require a specific lipid environment for maintaining their structural and functional integrity. In this review, we focus on emerging strategies for spatially resolved surface functionalization suitable for functional organization of proteins on surfaces. We present chemical and biochemical aspects of functional protein immobilization on solid support as well as micropatterning methodologies for spatial controlled functionalization under physiological conditions. Recent applications of using micropatterned protein arrays for drug discovery and screening are highlighted. Given the significant advances of live-cell micropatterning and single-cell analysis, recent applications of micropatterned protein surfaces for mapping signaling pathways and protein–protein interaction in live cells are presented and their potential in drug discovery is discussed.

2. Surface chemistries and surface architectures

A variety of substrate materials have been used for functional protein micropatterning, including nitrocellulose, polydimethylsiloxane (PDMS), glass, silicon wafer and gold-coated silicon/glass. Glass is widely used due to its excellent compatibility with optical detection techniques, including highly sensitive fluorescence imaging. Glass or silicon supports coated with gold or other metals are also very commonly used, as they support functionalization via self-assembled monolayers (SAMs) as well as the application of label-free detection by surface plasmon resonance. For sensitive assays, functional interfacing of proteins with these substrate materials is a major prerequisite. Except for very few robust proteins such as antibodies, direct interaction of proteins with the bare substrate mostly results in loss of their functions because the interactions with the substrate surface may block active sites or even denature the native protein structure. In order to minimize such nonspecific interactions, functional protein immobilization requires coating with organic polymers for rendering the surface protein repellent. In turn, site-specific attachment of the protein to the surface is needed in order to ensure their presentation at the surface in a functionally homogenous manner. During the past 15 years, substantial progress in “soft” surface engineering has been made, providing a versatile spectrum of surface chemistries and surface functionalities suitable for functional protein immobilization (summarized in Table 1). Choosing an appropriate strategy not only depends on the substrate material, but also requires careful considerations, which will be elaborated in this section.

2.1 Protein-compatible surface modification

As introduced above, the “protein compatibility” of a surface is largely equivalent to its minimized nonspecific interaction affinity toward proteins, and strategies, therefore, highly depend on the class of target proteins. Thus, soluble proteins typically require hydrophilic but largely uncharged surfaces. In contrast, membrane proteins are preferably integrated into lipid bilayers as the lipid environment often is critical for their activities. In general, molecular layers of non-charged,
hydrophilic polymers such as dextran hydrogels [31] and dense layers of poly(ethylene glycol) (PEG) [32] have been proven efficient for shielding surfaces against nonspecific protein binding (FIGURE 1A). While PEGs with a molecular mass of 2000–5000 g/mol are commonly used in many cases, highly ordered SAMs formed on gold surface by alkyl thiolates with short PEG chains of 3–6 repeat units also efficiently shield the substrate surface.[33] Simple, yet highly efficient surface coating with PEG layers is achieved by a layer-by-layer assembly, in which a poly-l-lysine-graft-PEG (PLL-PEG) copolymer is directly deposited on negatively charged surfaces.[14] Noncovalent assembly of lipids and lipid-like molecules into solid-supported lipid bilayers has been successfully applied for rendering surface biocompatible, which is important for functional reconstitution of membrane proteins on surfaces [16] (FIGURE 1B–E). A more sophisticated form of this surface architecture suitable for the integration of transmembrane proteins are polymer-supported membranes, where lipid bilayers are tethered above a thin polymer cushion to further minimize nonspecific interactions with the solid substrate.[18]

2.2 Surface biofunctionalization for protein immobilization

On the basis of protein-repellent surfaces, further functionalization with anchoring moieties is essential for efficient protein immobilization. Amine-reactive coupling via aldehydes or activated carboxyl groups have been used for covalent tethering proteins to surfaces through lysine residues presented on protein surfaces. However, these approaches yield heterogeneously orientated attachment, which likely results in heterogeneous functionality due to blocking of relevant residues of protein.[34] Therefore, site-specific techniques for tethering proteins to surface are desired. Several chemical reactions such as Staudinger ligation, Diels–Alder reaction, azide-alkyne click reaction and thiol-ene reaction

Figure 1. Surface engineering of micropatterned protein arrays. A. Generic surface architecture of functional protein micropatterning including a coating for rendering the substrate biocompatible (cyan layer) as well as spatially resolved functionalization (orange grids) for specific conjugation with biological components. Several commonly used materials for biocompatible layer are depicted. B–E. Surface modification for biocompatibility layers on different substrates. The reactive sites for introducing further functionalization are denoted by orange circles. B. Molecular scheme of PEGylated surface for glass (silica) and other metal oxide support. C. Self-assembled monolayer for ultrathin coatings of noble metal surfaces. D. Surface coating with functionalized poly(-)-lysine graft poly(ethylene glycol) (PEG) on a negatively charged surface (material independent). E. Formation of lipid bilayer on surface provides the crucial lipid environment for keeping the activity of membrane proteins. F. Site-specific protein capturing on streptavidin-functionalized surfaces. A biotinylated antibody was used for multiplexed protein immobilization. G. Site-specific protein capturing onto tris-NTA-functionalized surfaces. H. Site-specific protein capturing based on self-immobilizing protein tags. As an example, the HaloTag/HaloTag® ligand (HTL) reaction is shown.

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have been exploited for site-specific protein immobilization. [30] These reactions, however, require not only coupling-specific anchoring groups on surface but also modifications of the protein, for example, by incorporation of non-natural amino acids,[35] which is not yet routinely used in most laboratories. Moreover, these chemical reactions are relatively slow, thus requiring high protein concentrations, which in turn increase the background. For these reasons, quasi-irreversible noncovalent interactions mediated via genetically encoded tags are currently favored as they allow highly efficient and specific protein capturing to surfaces. A large proportion of noncovalent protein immobilization approaches relies on either the very rapid and extremely high-affinity interaction between biotin and streptavidin (or the related proteins), or the complexation of immobilized transition metal ions by oligohistidine-tagged proteins.[21] In the first case, the surface is functionalized with streptavidin or related proteins, and the protein of interest modified with biotin by means of chemical or biochemical biotinylation are readily immobilized to the surface (FIGURE 1F). For site-specific immobilization of oligohistidine-(His)-tagged proteins, functional moieties of iminodiacetic acid or nitrioltriacetic acid (NTA) are coupled to surfaces for immobilizing transition metal ions such as Zn(II), Cu(II), Ni(II) and Co (II).[36] More stable and stoichiometrically defined tethering of histidine-tagged protein was achieved by using multivalent chelators with two to four NTA moieties grafted onto different scaffolds,[24] with tris-NTA exactly matching the stoichiometry of the frequently applied hexahistidine-tag (FIGURE 1G). A broad spectrum of surface architectures functionalized with tris-NTA have been successfully implemented, yielding quasi-irreversible, site-specific protein immobilization.[24] These stable complexes, however, can be rapidly dissociated by competitors of imidazole at millimolar concentration, providing means for rapidly switching this interaction under mild conditions.

Driven by the increasing demand for site-specific protein-labeling techniques, protein immobilization via enzymatic reactions has gained much attention. In these methods, small-molecule ligands are covalently immobilized onto the
Spatially resolved protein immobilization assembly of protein arrays. For the assembly of protein arrays, highly multiplexed protein immobilization is required, and therefore orthogonal coupling approaches are desired. For this purpose, antibodies against the proteins of interest have been most frequently applied. For instance, monoclonal antibodies modified with biotin were chosen for site-specifically anchoring a variety of target proteins on the surface. However, antibody binding to proteins often blocks active sites and thus considerably bias functional assays. In other attempts, the huge multiplexing capability of oligonucleotide pairing has been exploited. Single-stranded DNA with different oligonucleotide sequences were coupled to the surface in a spatially resolved manner and proteins modified with the complementary strands were captured to the surface via sequence-specific DNA hybridization, thus enabling in situ assembly of protein arrays.

3. Spatially resolved protein immobilization

The repertoire of techniques for spatial organization of proteins on surfaces in micrometer and nanometer dimensions is rapidly expanding in line with the rapidly growing application of protein arrays. Micro- and nanopatterned surface modification can be achieved by self-assembly as exploited for block copolymer micelle nanolithography. While such bottom-up techniques are powerful for reaching nanometer resolution, the ability to control and vary geometries is rather limited. Therefore, top-down approaches are desired, which can be achieved either by local deposition of materials on the surface (soft lithography techniques) or by photochemical manipulation (photolithography techniques). Most frequently used techniques are briefly presented here as comprehensive in-depth background has been reviewed recently.

3.1 Local deposition by soft lithographic techniques

Piezoelectric dispensing by exploiting ink-jet technology has been a very straightforward approach to the assembly of spatially resolved surface modification, which has been exploited for generating the first-generation protein arrays. While this technology is low-cost, robust and mature, features below 10 μm can only be obtained with relatively high technological efforts. In the late 1990s, the concept of soft lithography for spatially resolved deposition of (bio)molecule onto surfaces with submicrometer resolution was introduced by George M Whitesides and coworkers to cover a set of microfabrication methods using molds, stamps or channels made by elastomeric materials. Soft lithography is highly versatile as very different compounds can be directly transferred onto surfaces. The most commonly used elastomeric material is PDMS. The currently most broadly applied soft lithographic technique is microcontact printing (μCP), where the patterns of a master PDMS stamp coated with ink are transferred to the substrate surface through face-to-face contact (Figure 2A). By using stamps with different featured sizes, patterning resolution down to tens of nanometers has been obtained by μCP. μCP has been very broadly applied for patterning proteins and other biomolecules in very different formats. Applications include direct deposition of proteins, for example, antibodies or streptavidin onto reactive or adhesive surfaces. However, this approach is not well compatible with most proteins as the printing process is prone to protein denaturation. Therefore, μCP of functional groups for site-specific protein capturing is preferred, which has been extensively applied to alkythiolates on gold surfaces to form micropatterned SAM. Likewise, μCP of biotin to an activated PEG polymer brush on glass surface has been implemented to prepare protein microarrays. Approaches for Ni-NTA-based protein patterning by μCP of reactive alkyl thiols followed by chemical coupling of NTA as well as direct μCP or by backfilling of NTA derivatives have been described.

Traditional μCP is designed to generate binary micropatterns. Substantially higher multiplexing has been achieved by using microscopic or nanoscopic stamps (“pens”) for locally depositing compounds (“inks”). This has been initially implemented by dip-pen nanolithography (DPN) using the tip of an atomic force microscope (AFM) as a pen to directly create patterns with inks on the substrate. With the options of using different proteins as inks, this method offers a great flexibility for making multiplexed protein arrays with length scale down to 50 nm on gold surface. Since different types of tips and substrates are implemented lately, it is also named as scanning probe lithography. DPN had been used for protein nanopatterning, for example, depositing antibodies on hydrophilic surface by DPN has been successfully demonstrated as well as specific capturing of His-tagged proteins into nanopatterns. By the combination of block copolymer micelle nanolithography and DPN, single-molecule protein arrays were obtained.

Several further techniques based on nano-manipulation via the AFM tip have been devised, including native protein nanolithography. However, preparation of protein nanoarrays by this method is slow since writing proteins with nanometer profiles has to be carried out one by one. To overcome this limitation, functional immobilization of proteins on PEG-modified SAM surfaces by parallel DPN was developed, which helps improving the throughput. More recently, Si-based nanostencil was introduced to make protein nanoarrays as “nanostencil lithography.” This method can be deemed as a variation of parallel DPN. Instead of using tips to dip the ink, a nanostencil with ink reservoir was applied on the surface for allowing protein inks being patterned on substrate through apertures. Multiplexed DPN of lipid bilayer was realized on glass substrates with subcellular...
dimensions (down to 200 nm), which was functionalized with proteins for T-cell activation.[56] An even more rigorous development into parallel deposition has been achieved by polymer pen lithography (PPL).[57] Rather than AFM tips, this technique employs elastomeric polymer molds comprising an array of pyramid-shaped "pens" (Figure 2D). After inking these pens with the desired compounds, these are transferred onto surfaces by means of a piezoelectric system, which allows control of lateral position as well as the size of deposited features via the axial force. Thus, huge throughput of the fabrication process was achieved with 150,000 features per second for sub-100-nm structures.[57] In combination of multiplexed inking of polymer pen arrays, highly multiplexed deposition of relatively complex features has been demonstrated.[50,58]

3.2 Photolithographic techniques
Photolithographic protein micropatterning is typically based on a photosensitive molecular layer directly grafted on a biocompatible surface coating. Typical components are nitroveratryl group, azide, tetrazine, C=C bond containing compounds and maleimide. Photolithographic modification by means of illumination through a photomask or spatially controlled focused light is employed for generating micro-patterns. Due to the diffraction limitation of light, conventional photolithography obtains patterns down to a length scale in the range of hundreds of nanometers. By exploiting the concept of stimulated emission depletion, resolution beyond the diffraction limit down to ~40 nm was achieved,[59] which has been used for fabricating protein nanoarrays.[60]

The simplest approaches for photolithographic micropatterning are based on photodestruction of functional groups on the surface. This can be done by directly destroying capturing groups such as NTA, which can be photodegraded by a light-induced Fenton reaction.[61] Alternatively, chemical groups for coupling of functional groups can be eliminated by light. Thus, photolithographic patterning of maleimide-modified surface has been introduced [13], which are used for surface functionalization via thiols.

Figure 2. Examples of soft lithography and dip-pen nanolithography (DPN) for preparing protein microarrays. A. Scheme of microcontact printing. Polydimethylsiloxane (PDMS) stamp soaked with poly-(l)-lysine-PEG-HaloTag® ligand (PLL-PEG-HTL) contacts a plasma-cleaned glass substrate (i), thus transfer the PLL-PEG-HTL patterning to the surface (ii). Backfilling with PLL-PEG-OMe blocks the uncoated regions (iii). The obtained micropatterned surface is used for affinity-capturing HaloTag-fused proteins (iv). B. Scheme of nanocapto printing. A planar PDMS stamp is incubated with a protein solution, blow-dried (i), and quickly brought into contact with a photopolymer (Norland Optical Adhesive 63) replica that was plasma activated, and separated to lift-off proteins in the contact areas (ii). The flat PDMS stamp is then printed onto a glass slide (iii) and separated, producing protein nanopatterns on the slide (iv). (Adapted with permission from John Wiley and Sons.[47]) C. Surface nanopatterning by DPN. Top: principle of surface deposition of chemical “ink” adsorbed onto an atomic force microscope tip. (Reprinted with permission from the American Association for the Advancement of Science.[48]) Bottom: functional protein nanoarray fabricated by DPN and protein capturing. (Reprinted with permission from the American Chemical Society.[49]) D. Multiplexed protein immobilization by polymer pen lithography (PPL). Top: scheme depicting the deposition of different biomolecules adsorbed onto a polymer pen array. Bottom: multicolor micropatterns generated by PPL with fluorescent lipids. (Reprinted with permission from John Wiley and Sons.[50])
A strikingly high signal to background was achieved, which facilitated single-molecule studies in microarrays requiring high contrast (Figure 3B). Maleimide patterning also obtained very homogenous patterning of polymer-supported membranes that allowed assessing diffusion and interaction of reconstituted transmembrane proteins on the single molecule level without biasing their diffusion and interaction properties as resolved by single molecule imaging [62] (Figure 3C, D).

Photolithographic patterning based on azide functionalization has gained considerable attention nowadays as the azide is a small, photosensitive moiety and widely used in azide-alkyne click reaction. Extracellular matrix (ECM) proteins containing RGD or REDV peptides as well as the unnatural amino acid azidophenylalanine (pN₃-Phe) were spreaded on glass coverslides and cross-linked by UV-illumination through a photomask. By washing with 6 M guanidine hydrochloride, the protein was selectively removed in the non-illuminated regions, yielding guided cell adhesion these micropatterned surfaces.[63] Direct photopatterning of biomolecules via azide groups, that is, positive photolithography, were successfully demonstrated.[64] With the ability to site-specifically introduce amino acids with clickable side chains into recombinant proteins, exciting applications of these approaches for protein patterning can be envisaged.

More versatile and sophisticated surface modification can be achieved by photocaging of functional groups. o-Nitroveratryloxycarbonyl (NVOC) is a very common photosensitive moiety applied for caging amine groups on the surface [65] (Figure 3E). A general photopatterning strategy for binary surface functionalization using NVOC contains two steps: uncaging the NVOC-protected amino groups with a photomask under UV irradiation, followed by chemical or biochemical functionalization of the amine groups in the uncaged areas for capturing proteins (Figure 3F).[66] This approach has been successfully applied for site-specific non-covalent and covalent protein immobilization [25,66] (Figure 3G). Likewise, photolithographic writing of SNAP-tag fusion proteins was achieved using caged benzylguanaine.[28]
While these photolithographic techniques are powerful for spatially organizing one or two different proteins on surfaces, higher multiplexing requires techniques for directly targeting proteins into microscopic structures. Photolithographic “writing” of proteins has been achieved by different approaches. Nitroveratryl groups have also been applied for photocaging biomolecules. By local uncaging of surface Ni-NTA complexed with photocleavable oligohistidine peptides, in situ micropatterning of His-tagged proteins on surfaces by laser lithography was achieved with diffraction-limited resolution.[67] Likewise, caged glutathione was employed for micropatterning of proteins fused to glutathione S-transferase,[68] a common protein used for affinity purification. These techniques very elegantly allow multiplexed writing and erasing of proteins on surfaces, yet suffer from instabilities and relatively high background due to the non-covalent nature of the interactions. More robust laser lithography is achieved by photocatalysed formation of covalent bonds. Thus, protein micropatterning by means of photo-initiated thiol-ene click reactions has been implemented.[69] Another click reaction based on tetrazine-norbornene chemistry was recently reported for forming cell-laden hydrogels. Duplexed protein patterning in this “click gel” was realized by taking advantage of orthogonal thiol-ene photoreaction and Diels–Alder reaction of tetrazine-norbornene.[12]

Related to photolithographic techniques, also electrons provide versatile means for spatially resolved chemical manipulation. Electron beams (e-beams) can be focused to spot sizes of a few nanometers, making e-beam lithography a powerful way for fabricating nanoarrays. By e-beam-controlled reduction and cross-linking of aromatic SAMs, nanopatterned amino groups on gold surfaces were obtained for further biofunctionalization and nanopatterning of biomolecules at the submicron scales.[70] Multiplexed protein patterning with nanometer scale by e-beam lithography was achieved by depositing functionalized PEG dendrimers, yielding a line width of ~100 nm.[71] Alternatively, electron-beam-induced deposition has been used for depositing carbon nanostructures onto a PEG polymer brush for subsequent adsorption of proteins with very high contrast.[72] It should be noted, however, that application of these methods is limited to conductive substrates.

4. Applications for drug discovery

4.1 Profiling protein–protein interaction networks

Early applications of large-scale protein arrays have been focused on protein interaction analyses, which have had substantial impact in proteomic research for drug discovery and, in particular, for personalized medicine.[73] Compared to affinity-capturing techniques coupled with mass spectrometry (AC/MS) and two-hybrid technologies, the advantage of protein microarrays is that they contain nearly all proteins coded by whole genome, and they can be assayed simultaneously in vitro. The first proteome chip contained 94% (~5800 of 6200) of the yeast proteins with N-terminal GST-His-tag tandem affinity purification tag.[34] Since then, this technology has become an important tool in several fields of drug discovery [74] and protein microarrays of kinases, phosphatases and proteases have become available.[75] High-throughput global identification of protein kinase substrates and protein kinase–phosphatase interaction networks have emerged.[76,77] Such global network mapping is very important for clinical diagnosis because the anomaly regulation of protein kinase activity is strongly related to diseases such as cancer, and the inhibitor or activators for these interactions are important drug candidates. Fluorescence detection techniques are currently the preferred readout methods as they provide unrivaled signal-to-background ratios with a sensitivity down to the level of single molecules.[78] However, recent development of ultrasensitive label-free detection by LSPR offers interesting options,[15,79] as often the labeling is a critical and time-consuming step in assay development.

A key shortcoming of state-of-the-art protein array technology is the necessity to produce and purify proteins prior to deposition on the surface, a very demanding and time-consuming process. Owing to the fragile folding, storage of proteins and protein arrays often leads to loss of function and therefore in situ assembly of protein arrays is highly desired. For this reason, current developments in the field aim for closer linking protein production and assembly into arrays, for example, by local in vitro translation on micropatterned support [80,81] or by in situ protein capturing from cells.[29] For plasma membrane receptors, which are key drug targets, yet are often very difficult to isolate, live-cell protein capturing has been implemented [22] (also see Section 4.3). Recently, this technology has been adapted to allow multiplexed protein interaction analysis inside a living cell.[82] To this end, an antibody array fabricated by DPN was used to micropattern cytosolic proteins through an engineered transmembrane linker, allowing for monitoring activation of protein kinase A upon G-protein-coupled receptor (GPCR) stimulation.

4.2 Screening GPCR ligand binding

GPCRs represent the largest family of therapeutic targets.[5] These receptors are the targets of more than 50% of the current pharmaceutical agents on the market, with a global market reaching ~100 billion USD in 2013.[83] Over a long time, GPCR drug discovery by high-throughput screening (HTS) for hit identification has remained the major focus of drug discovery worldwide.[84] Commonly used HTS assays for GPCR ligand binding and functional assays include the key detection points in GPCR signaling pathway, such as detection of ligand binding, GTPγS binding, cAMP, IP3/IP1, Ca2+ flux, β-arrestin recruitment, receptor internalization and receptor dimerization.[85]
Over the past decade, versatile techniques for functional micropatterning of membrane proteins have been established, which facilitate in vitro HTS of GPCR ligand binding. [16,86] GPCR arrays of the β1-adrenergic receptor, the neurotensin receptor (NTR1) and the dopamine receptor based on microspotted lipid membrane have been successfully fabricated. Specific ligand binding to these GPCR microarrays was confirmed and IC_{50} value of neurotensin to NTR1 down to 2 nM was measured by competitive ligand chasing. [87] In the following work reported by Pfizer Inc. (New York, NY, USA) and Corning Inc. (New York, NY, USA), multiplexed GPCR microarrays of α-adrenergic receptors were developed for ligand-binding assays based on [125]I-labeled agonist clonidine. A 200- to 400-fold decreased sample preparation was sufficient for analysis, which is very useful for HTS. [88] Another type of GPCR microarray was achieved by embedding membrane proteins into liposomes prepared from Sf9 cellular membrane extracts. Site-directed immobilization of the liposomes on protein repelling surface was achieved by hybridization of cholesterol-modified oligonucleotides between the liposomes and the surfaces. The functionalities of GPCR models in liposomes, for example, H1-histamine receptor and the M2-muscarinic receptor, were confirmed by ligand-binding assays and G-protein signaling assays under a microarray reader format. [19,20] This liposome-based strategy yielded impressive performance and can be very efficient for HTS of potential GPCR ligands since the proof of concept of single-liposome arrays has been established by using µCP. [89] To further simplify the sample preparation of GPCR microarray and effectively screen GPCR drugs in native cellular environments, live-cell microarrays in the format of well-plate, micro-patterned surface, and microfluidic systems have been developed. [90]

4.3 Probing signal transduction in live cells

Large-scale monitoring of signal transduction pathways stands as the largest challenge in drug discovery, as almost all known diseases exhibit dysfunctional aspects in signal transduction networks. [91] and therefore the vast majority of drug target are part of these. [5] Many signaling pathways have been reasonably mapped at molecular level, uncovering a highly complex network of subtle and interdependent cross talk. The integration of this complexity into cellular decision making is still largely unclear. Therefore, the overall potency of a drug has to be verified under physiological conditions. Monitoring signaling pathways and screening the perturbations of drugs to these pathways with multiplexed assays thus has unique potential to bridge the gap between molecular-level assays and the animal tests.

4.3.1 Cell adhesion and integrin signal transduction

The interaction and migration of cells within tissues play a key role in stem cell differentiation and organogenesis, but also in tumor development and metastasis. Promising new drug targets such as the Wnt/β-catenin pathway involved in controlling these processes are currently emerging. [92] Micro- and nanopatterned surface functionalization offers unique means to mimic and study these extremely complex processes in vitro and thus to identify key molecular and cellular determinants and their potential manipulation by drugs. [93] Cell adhesion and apoptosis on micropatterned RGD peptides [94] and ECM proteins [95] have been examined to elucidate the mechanism of integrin signal transduction, which have shown determinant functions for regulating cell survival, differentiation and growth. By using copolymer micelle nanolithography, induced cell polarization and migration were examined in detail by spacing adhesive ligands in a gradient in nanoscale. [94] (Figure 4A). Interestingly, these experiments revealed that inducing cell adhesion on ordered RGD nanopattern requires the RGD peptide being spaced below 70 nm. This critical distance confirms that the formation of stable focal adhesion and actin fiber networks have a distance threshold of 70 nm. Hierarchical RGD micro-/nanopattern designed using a similar strategy was recently used for investigation of stem cell differentiation. [96] Strikingly, growth of mammalian cells on micropatterned surface showed preserved left–right patterning, a phenomenon termed as “chirality”. [97] Chirality has also been detected for other single-cell types and multicellular structures, which highlights the possibility of chiral morphogenesis during stem cell differentiation.

4.3.2 Receptor tyrosine kinase signal transduction

Receptor tyrosine kinases (RTKs) are major regulatory hubs controlling cell growth and differentiation, and therefore play a prominent role in tumor development. For this reason, several RTKs have become key drug targets. [100] Closer studies revealed an enormous complexity of RTK activation, [101] demanding new tools for testing the molecular mechanisms responsible for inhibition. Live-cell micropatterning (cf. Section 4.1) has opened unique avenues to study RTK signaling in situ. For this purpose, cells are cultured on micropatterned monoclonal antibodies against the target receptor, which will be captured and thus spatially rearranged within the plasma membrane. [22] Thus, interactions with other receptor subunits and/ or cytosolic effector proteins can be readily monitored. [22,98,102] By using tag-specific surface capturing rather than antibodies, generic and robust surface micropatterning for probing assembly and signaling of RTK and related receptors in living cells was recently implemented. [98] These surfaces allow control of cell attachment via RGD peptides and assembly of signaling complexes via receptors fused to the HaloTag (Figure 4B, C). Thus, protein–protein interactions involved in receptor dimerization and recruitment of cytosolic effector proteins could be quantified. [98] By using micropatterned lipid bilayers, cell–cell
interactions can be mimicked to identify the determinants of cellular recognition processes. Thus, immune synapse formation on surfaces was achieved on micropatterned surface which mimics the antigen-presenting cells and signaling pathways involved in T-cell activation were probed.[17] The spatial control of EphA2 RTK signal activation during cell–cell contact formation was quantitatively explored by a similar approach [99], revealing a striking correlation between spatial receptor organization and invasion potential of different mammary epithelial cell lines (FIGURE 4D). Further exciting possibilities are opened by combination of these approaches with spatial control of cell adhesion using RGD nanopatterns.[103]

5. Conclusions

In the past decade, spatially controlled protein immobilization has become a versatile and reliable technology with numerous existing and potential applications in drug discovery. Key challenges for combining biocompatible surface architectures and bioorthogonal coupling reactions with functional micropatterning have been resolved. To this achievement, development of affinity tags and bioconjugation techniques for site-specific protein modification has been instrumental. Thus, maximized protein functionality has been achieved in artificial environments for reliably probing the interactions between proteins and protein substrates.
The sensitivity of surface-based detection techniques down to the single-molecule level by both fluorescence-based and label-free imaging techniques nowadays allows enormous miniaturization and parallelization for quantitative binding and activity assays. With these tools, the scope of drug discovery is profoundly expanded to reach whole proteomes. Yet, manufacturing of high-density multiplexed protein arrays remains challenging because of the limited stability of proteins. Moreover, membrane proteins as a key class of drug targets are typically not well covered by whole proteome analysis techniques as they require dedicated approaches toward functional immobilization.

6. Expert opinion

During the past decade, drug discovery has become a much more demanding and multifaceted process. Key challenges include more efficient target identification, development, and testing macromolecular drugs as well as coping with the complex demands of personalized medicine. Robust and user-friendly protein-based assay technologies providing high level of multiplexing and flexible design will play a key role in addressing these challenges. Conventional protein arrays are, meanwhile, commercially available from various suppliers. These are typically designed for proteome-wide screens with the aim to cover a large proportion of a given proteome. For drug discovery, however, more complex, dedicated and adaptable assay formats will be requested for functional analysis of a subset of interesting protein candidates. Implementation of such assays will profit from several major biotechnological innovations, which are currently emerging.

One major current limitation in multiplexed protein assay development results from the limited availability of specific binders with well-characterized protein recognition properties. While in the past monoclonal antibodies has been the solution of choice, alternative approaches for generating high-affinity binders are now available, which have the advantage of being much more readily accessible. Next to recombinant antibody fragments, which are directly obtained by in vitro selection, simpler scaffolds such as nanobodies or DARPins have been designed,[104] which can be readily produced by recombinant protein expression or by in vitro translation. As a fully synthetic alternative, aptamer technology has the potential to play an important part in this field.[105] However, considerable and concerted efforts will be required to provide a large and validated repertoire of recombinant or synthetic binders to the scientific community.[106] As a second important technology for site-specific protein attachment, incorporation of non-natural amino acids into recombinant protein is coming of age, being available for different cell types and providing a large variety of possible modifications. After further optimization of the efficiency and selectivity of coupling reactions, this tool will be the key for generic site-specific immobilization and labeling of proteins on very small scale.

Functional reconstitution and micropatterning of membrane proteins – the most important class of drug targets – on surfaces has been achieved by membrane architectures such as vesicles and solid-supported membranes, as well as nanodisk technology.[107] These approaches depend on isolation of membrane proteins by detergent solubilization, which requires time-consuming identification of a suitable detergent for a specific membrane protein. As the functions of membrane proteins are intrinsically linked to lipid environment [7], appropriate selection of lipids for functional reconstitution is required. Currently, techniques for more direct transfer of membrane proteins to surfaces are emerging, which do not require the extraction from their lipid environment. Promising strategies include budding of proteoliposomes from plasma membranes [89] as well as extraction of membrane proteins within their lipid environment by amphiphatic polymers (lipodisp).[108] Successful extraction of GPCRs along with endogenous lipids in lipodisps was recently demonstrated in a detergent-free manner,[109] highlighting the potentials for a wide application in drug discovery. As a promising alternative strategy, cell-free production of membrane proteins by in vitro translation opens exciting possibility to in situ reconstitute GPCR into suitable membrane architectures.[110]

In general, more closely interfacing protein production and array fabrication will be an important trend in this field. Next to generating protein arrays by local in vitro translation of cDNA libraries (cf. Section 4.1), strategies for capturing proteins directly from cells offer promising opportunities. In particular for diagnostic screening required for personalized drug selection, assays need to be performed from minute sample quantities, ideally from a single cell. Single-cell analysis, moreover, opens new avenues to deconvolute the cellular heterogeneity.[111] Cell-dependent variations of stem cell signaling and differentiation responses were examined by large scale (~2000 cells) single-cell western blotting (scWestern) using an open-microwell array.[112] This approach has already been applied to demonstrate the possibility for quantifying cell-to-cell variance to pharmaceutical stimuli.[113] Alternative strategies aim for performing protein assays directly on the cell culture substrate. In case of plasma membrane protein such as receptors and transporters, capturing to surfaces within living cells opens exciting possibilities (cf. Section 4.3.2). In-cell micropatterning of soluble proteins is possible [82], yet requires genetic manipulation. For exploiting the growing availability of high-affinity binders and the very high sensitivity of surface-sensitive detection, single-cell pull-down assays are highly promising, which have been recently demonstrated for recombinant proteins [29] (Figure 4E). By taking advantage of efficient cell capturing to surface in combination with local high-density arrays of binders in close proximity to captured cell, this approach allows efficient isolation of proteins from primary cells. Thus, a broad application of ultrahigh-sensitive HTS in the format of single-cell single molecule can be envisioned, which ultimately will fuel drug development for personalized medicine.
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