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Review

Protein micropatterns printed on glass: Novel tools for protein-ligand binding assays in live cells

Micrometer-sized patterns of proteins on glass or silica surfaces are in widespread use as protein arrays for probing with ligands or recombinant proteins. More recently, they have been used to capture the surface proteins of mammalian cells seeded onto them, and to arrange these surface proteins into pattern structures. Binding of small molecule ligands or of other proteins, transmembrane or intracellular, to these captured surface proteins can then be quantified. However, reproducible production of protein micropatterns on surfaces can be technically difficult. In this review, we outline the wide potential and the current practical uses of printed protein micropatterns in a historical overview, and we detail some potential pitfalls and difficulties from our own experience, as well as ways to circumvent them.

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1 Introduction: The production and use of protein patterns so far

Patterned protein surfaces that interact with cell surface proteins were first developed for shaping and positioning cells (or parts of cells, such as neurites) with the aim of engineering tissues and specific microenvironments, for example to study the growth of cells in response to specific patterns and microstructures ([1]; for reviews see [2–5]). One convenient way to achieve this is a soft lithography method called microcontact printing [6], in which a polydimethylsiloxane (PDMS) stamp is inked with the protein solution and then stamped onto the surface (for example, a glass coverslip). Recently, researchers have decreased the sizes of individual pattern elements (i.e., dots or squares) that can be printed to the lower micrometer to nanometer range. Such small pattern elements allow the redistribution of cell surface proteins, such as receptors, into the shape of the printed pattern: if the printed

protein is an antibody that binds to the extracellular domain of the cell surface protein of interest, then cells can be seeded onto the glass slide that contains the pattern, and the surface protein will be captured by the printed antibody into the shape of the pattern (Fig. 1A). This technique has allowed researchers to study cell adhesion and phagocytosis [7,8], signaling [9,10], cellular protein–protein interactions [11–14], and protein–ligand interactions [15,16]. The advantage of using printed protein patterns to measure protein interactions is that the cell surface protein is in its native environment and composition, and that the assay can be performed in the living cell.

In this review, we focus on the use of printed protein patterns for single-cell applications, more specifically on proteins that are printed directly onto the glass surface. We take the perspective of the researcher with a background in cell biology who is entering this exciting field. There are good reviews available about protein arrays for *in vitro* studies [17–20], so we do not include these. We mention, but do not review in detail, the significant recent developments in protein micropatterns that are generated by indirect immobilization or sophisticated attachment chemistry [13,21,22].

2 The uses of protein patterns at the single cell level

When proteins that were printed in a pattern onto a glass slide interact with a surface protein of a single cell, then the isotropic distribution of that surface protein is altered, and it becomes rearranged into the printed pattern. In contrast to proteins bound

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Abbreviations: AF, Alexa Fluor; BSA, bovine serum albumin; cLSM, confocal laser scanning microscopy; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFP, green fluorescent protein; H-2K^b, the murine MHC class I protein H-2K^b; mAb, monoclonal antibody; MHC, major histocompatibility complex; MW, molecular weight; NHS, N-hydroxysuccinimide; oligo, oligonucleotide; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; SEM, scanning electron microscopy; TAMRA, tetramethylrhodamine; TIRF, total internal reflection fluorescence

Figure 1. (A) Schematic of a cell surface GFP fusion protein binding to a printed protein micropattern in a ligand binding assay. The peptide receptor H-2K^b, a type I transmembrane major histocompatibility complex (MHC) class I protein, is produced in epithelial cells with the green fluorescent protein (GFP; green) fused to its C terminus. The cells are seeded onto a glass surface that is printed with a micropattern of the monoclonal antibody (mAb) Y3, which recognizes H-2K^b. Upon binding to Y3, the H-2K^b-GFP proteins on the cell surface are recruited into the printed patterns. In the insert, two H-2K^b-GFP proteins are shown binding their natural peptide ligand (red), here labeled with a fluorophore. (B) A cell spreads out over several pattern elements to allow assessment of surface protein patterning. A pattern of mAb Y3 labeled with Alexa Fluor 647 (red; pattern element diameter 10 μ m, interspace 15 μ m) was printed onto a glass coverslide using a PDMS stamp. Vero (African green monkey kidney epithelial) cells expressing H-2K^b-GFP (green) were then seeded onto this pattern and grown for 24 h. In the phase contrast picture (right), the cell is outlined in white, and the nucleus is labeled with the letter n. Yellow arrows show the circular patterning of H-2K^b-GFP by the antibody pattern, as depicted in A. The intracellular stain of H-2K^b-GFP (overlaid with the blue letters ER) is in the endoplasmic reticulum. Bar, 25 μ m. The technique is described in detail in [16].

to supported lipid bilayers [9], protein patterns have a defined distribution, and they do not allow the captured cell surface protein to diffuse further. Its rearrangement can thus be conveniently read out by microscopy in the fixed or even live cell (Fig. 1B). Sometimes, the distribution of the surface protein is identical to that of the pattern, but sometimes, the protein is more concentrated on the edges of the pattern elements, as one would expect if proteins on the plasma membrane are freely diffusing and becoming mechanically trapped as they encounter the edges of the pattern elements and bind to the printed proteins (Fig. 1B, yellow arrows). Consequently, single-cell experiments are especially well suited to detect interactions of the patterned protein with other proteins, or with ligands, for microscopic readout.

One example for this are protein-ligand binding assays [13], our own work being a recent example [16]. We print a monoclonal antibody called Y3 that binds to a cell surface peptide receptor of the adaptive immune system, a major histocompatibility complex (MHC) class I protein called H-2K^b. When cells that contain a green fluorescent protein (GFP) fusion of H-2K^b, H-2K^b-GFP, are seeded onto the glass slides with the Y3 patterns, then the H-2K^b-GFP protein is arranged on the cell surface in the shape of the pattern (Fig. 1). Binding of the peptide ligand can be assessed by labeling it with a fluorescent dye and adding the labeled peptide to the cells that are growing on the patterns. When the peptide ligand binds to its receptor H-2K^b-GFP, then the peptide fluorescence also becomes visible in the shape of

the pattern, but not in the interspaces of the pattern elements (Fig. 2). We have shown that by competition with a fluorescently labeled index peptide, the binding affinity of any prospective ligand to H-2K^b-GFP can be measured (for details see [16]). This competition assay principle might be applicable to other cell surface receptors whose ligands can be fluorescently labeled, and its special advantages are that the affinity measurement is done on the receptor in its natural condition and environment (e.g., with all of its subunits, auxiliary proteins, and the native lipid environment present).

Another advantage of optical ligand binding assays with printed antibody patterns is that conformation- or assembly-specific antibodies can be used to pattern particular assembly states or conformations of the receptor, and that ligand binding measurements are therefore conducted only on this form of the receptor and not on any of its other forms that are present in the same membrane at the same time. For example, in our experiment with the H-2K^b peptide receptor, the printed antibody Y3 only recognizes the heterodimeric form (heavy chain + light chain) of H-2K^b, but not for example the single heavy chain. This ensures that in the binding assay, only the heterodimeric contributes to the observed binding of the ligand. In principle, ligand association and dissociation kinetics can also be measured in such assays [13].

In addition to binding their ligands, cell surface proteins also interact with other proteins, which may also be membrane-bound, or else cytosolic and soluble. Several groups have used

Figure 2. Binding of the peptide ligand FL9-TAMRA to the MHC class I receptor H-2K^b captured by printed antibody. A pattern of mAb Y3 was printed onto a glass coverslide with a PDMS stamp as in Fig. 1, but this time, the antibody was not fluorescently labeled. STF1 (human fibroblast) cells were then seeded onto this pattern and grown for 16 h. Then, a specific peptide ligand binding to H-2K^b (the nonapeptide FAPK_{TAMRA}NYPAL (FL9-TAMRA), labeled on the lysine side chain with the fluorescent dye TAMRA) was added (1 μ M final concentration), and cells were incubated at 37°C for 4 h. The cells were then washed with phosphate-buffered saline (PBS), fixed, and imaged. STF1 cells stably expressing H-2K^b without GFP (center panels) show only the red pattern of the peptide bound to the patterned H-2K^b, while STF1 cells expressing H-2K^b-GFP show in addition the green fluorescent patterns of the protein (right panels; this is the situation shown in the insert of the schematic in Fig. 1A). As a control, STF1 cells not expressing H-2K^b show no patterning of peptide or protein (left panels). Bar, 25 μ m. The peptide binding assay is described in more detail in [16].

printed protein patterns to demonstrate interactions between cell surface proteins (which they call ‘baits’) and intracellular (cytoplasmic) non-membrane proteins (‘preys’) [13, 15]. In the simplest instance, which is technically a one-hybrid assay, the cell surface ‘bait’ protein (which is unmodified) is arranged by a printed antibody pattern to catch the intracellular ‘prey’ protein (which is fused to a fluorescent protein such as GFP). The arrangement of the intracellular ‘prey’ protein in the shape of the pattern is then observed by microscopy. Ideally, in live cells, one can even follow in real time the association and dissociation kinetics of the intracellular ‘prey’ protein [12], such as the recruitment of elements of the signal cascade to the plasma membrane in a kinetic study [23]. Grinstein and collaborators have used this approach to investigate in an elegant study how the formation of the phagocytic cup is regulated [8]. It is also possible to lyse the cells and then follow the dissociation of the cytosolic ‘prey’ protein from the surface ‘bait’ protein that is still attached to the antibody pattern [14]. The assay might also be converted to a two-hybrid assay by printing antibodies against an universal epitope tag such as the hemagglutinin (HA) tag and fusing that tag to the extracellular domain of the cell surface ‘bait’ protein.

Interactions of surface proteins with other surface proteins can be measured in the same way [13]. For example, if the question is asked whether two membrane proteins that are located in the plasma membrane of the same cell interact with each other (*‘in cis’*), one protein is fused to the epitope tag (such as HA), and the other to GFP. Cells are transfected with both fusion (hybrid) proteins and seeded onto anti-HA patterns. Arrangement of the GFP-fused protein then demonstrates that it interacts,

in the natural membrane environment of the live cell, with the HA-tagged protein [24].

3 Technical implementations: Challenges and the way ahead

In the following, we would like to point out some important technical issues in the implementation of assay systems based on protein patterns, focusing especially on those that we have encountered during recent work with single-cell investigations [16], with the aim of perhaps making others, who wish to enter this exciting field, aware of potential problems and how to avoid them.

3.1 Attaching proteins to the surface

The simplest way of attaching proteins to a glass surface is to directly print them. A PDMS stamp is inked with the protein solution (usually in phosphate-buffered saline, PBS) and lowered gently onto the glass surface, or else the stamp is laid down with the inked side up, and the coverslip is laid onto it. After some minutes, the contact is broken (and the stamp discarded or re-used), and the printed protein pattern is used further. The presence and patterning of the printed proteins can be assessed by scanning force microscopy [25], by fluorescence microscopy (after additional antibody labeling), or by gold labeling and electron microscopy [16]. The native character of the printed protein then needs to be shown through its functionality. Printed antibodies, for example, should be tested for binding to their antigen.

Indeed, it was shown by Graber and collaborators that many antibodies can be printed directly, in this way, onto untreated glass coverslips without loss of specificity of binding [25–27].

It is not entirely clear what holds proteins on glass surfaces, though it is fair to speculate that the ‘adsorption forces’ are a mixture of ionic interactions and hydrogen bonds, depending on the surface and the protein [28]. We have found that patterns of antibodies that are printed directly onto untreated glass with PDMS stamps are resistant to wash-off (Fig. 3).

Unfortunately, we as well as others have encountered problems with this direct printing method, such that some printed proteins were present on the glass but nonfunctional. For example, we have reproducibly obtained patterns of the MHC class I-specific monoclonal antibody (mAb) Y3 in its functional form [16] (as judged by its ability to bind MHC class I in the overlaying cell), but this was not achieved for another monoclonal antibody, 25-D1.16 (Dirscherl, unpublished). The most likely reason for this is that when the protein is bound directly to the glass surface, the surface-protein adsorption forces lead to the denaturation (unfolding) and subsequent loss of function of the protein. Whether this will occur with a given protein, or not, is currently impossible to predict. Another possibility is that an epitope in the protein antigen that is accessible to the soluble antibody might not be accessible to the immobilized one, for sterical reasons.

Simple treatments of the glass surface, such as cleaning with ethanol or acid (e.g., ‘piranha’ solution of sulfuric acid and hydrogen peroxide), are sometimes used to generate a uniform and defined glass surface for printing proteins. In reports from the literature [29] and in our own experience, acid treatments alter the glass surface to decrease, or to increase, protein denaturation, depending again on the type of protein. The same may be said for plasma cleaning [30]. Thus, even though the surface treatments presumably generate reproducible surface conditions, a given treatment may or may not work for the protein of interest. In our experience, since fabrication-fresh coverslips are usually fat- and dust-free, pre-treatments are not generally necessary.

In addition to the danger of protein denaturation, direct printing of proteins onto glass has the theoretical disadvantage that the protein is printed in a random orientation, which may obscure its binding site for the cellular interaction partner.

Figure 3. Directly printed antibody patterns are stable over time. PDMS stamps were inked with mAb Y3-AF647 in PBS for 15 min and printed onto untreated glass coverslips for 10 min. Patterns were washed and incubated in PBS for the times indicated, then imaged by cLSM. Bar, 50 μ m.

Whether this actually poses a problem has, to our knowledge, never been systematically investigated. One possible solution to both issues, denaturation and random orientation, is the directed attachment of the proteins of interest to other proteins that are themselves adsorbed to the glass (‘anchor proteins’). For example, antibodies may be printed as anchor proteins, which then hold the protein of interest. Or, secondary antibodies may be printed, which then hold the antibody of interest that should interact with the cellular surface protein. Other possible anchor proteins for antibodies are the *S. aureus* protein A or the *Streptococcus* protein G, both of which bind the Fc regions of antibodies with high affinity. It is obvious that this strategy depends on being able to print the anchor proteins themselves in a functional state; for us, direct printing of protein A or G onto glass has been fraught with difficulties. Still, this path is worth pursuing in the future, since indirectly coupled antibodies might be less randomly oriented and better positioned for binding to their ligands.

In addition to printing proteins directly onto glass, and to attaching them to anchor proteins, more elaborate systems that use the very tight biotin-streptavidin interaction have been developed. Printing of streptavidin as an anchor protein [31] allows the attachment of proteins that are biotinylated in vitro (using a biotinylating enzyme) or in vivo. Since streptavidin has several binding sites for biotin, it can itself be used in a sandwich mode: biotin is attached to the glass surface, streptavidin is bound to the immobilized biotin, and then biotinylated proteins are bound to the free binding sites of streptavidin, resulting in a directed-attachment protein pattern. The patterned attachment of biotin to the glass is not trivial, but several ingenious ways have been developed: Rapp and collaborators have used projection lithography to create biotin patterns by light-triggered covalent coupling of biotin–fluorescein to BSA-coated glass surfaces [21]. Proteins can also be indirectly attached to the glass *via* nucleic acids; this allows multiplexing [15, 32] or the creation of nanometer-sized patterns on DNA origami structures [33].

3.2 Differentiating between pattern elements and interspaces

A related issue is the differentiation between pattern elements and interspaces, that is, to make sure that the interspaces are free of protein and do not present a highly adhesive surface in the assay. In protein arrays that are subsequently incubated with other proteins (for example, for interaction studies), this is a serious problem that must be overcome by blocking the interspaces. For single-cell experiments with directly printed protein patterns, untreated interspaces may be less of a problem if the cell culture medium (as usually) contains 5–10% fetal bovine serum (FBS), which may accomplish the blocking without the need for further steps. But if the protein pattern is built up in several steps, for example by printing protein A and then binding an antibody to it (see above), then blocking of the interspaces is a necessity. Popular blocking (or ‘passivation’) reagents are grafted polyethylene glycol, block copolymers, and bovine serum albumin [2].

An interesting alternative approach that at the same time patterns the protein of interest and blocks the interspaces has been developed by Schütz, Weghuber and collaborators

[12, 23]. In one example [23], they use commercially available streptavidin-coated glass slides and print the interspaces of the pattern elements with BSA-Cy5-biotin, blocking them from further interaction. The protein to be patterned, a biotinylated antibody, is then simply added to the prepared slides and binds to the pattern elements only, since the interspaces are blocked.

Another ‘negative patterning’ technique (*i.e.*, that first derivatizes the interspaces and then the pattern elements, [2]) that does not use printing at all was described by Piehler and collaborators [13]. They uniformly coat the glass slide with a maleimide-functionalized polymer and then attach integrin-binding arginine-glycine-aspartate (RGD) peptides to the interspaces only by a photolithographic reaction. The remaining maleimide groups, which are only in the pattern elements, are then used to attach a HaloTag ligand [34]. The resulting patterns of HaloTag ligand are used to pattern cell surface receptors that are fused to the HaloTag sequence.

3.3 Binding of cells to the pattern elements

In single-cell experiments, the printed proteins usually interact with a component of the cell membrane (usually also a protein), arranging it into a pattern of similar shape (see above) [16]. For this to occur, it must be ensured that the cells spread evenly over the pattern, covering the pattern elements and the interspaces (the areas between the pattern elements) alike. To a cell, however, the pattern element, rich in printed protein, and the interspace, with a glass surface or a different coating on it, may provide very different attachment opportunities. We have seen cells clinging preferentially to the pattern elements or to the interspaces (Fig. 4). The different cell densities achieved on different pattern scales in a competitive setting suggested to us that cells may especially like substrates with alternating surface structure of a certain scale (Fig. 5), as observed by others [35–37]. These properties varied from one cell line to the other, and with the growth state of the cells, and it is likely that with primary cells, such preferential adhesion will be a major problem. Here again, special treatment (passivation) of the interspaces with an agent that is cell-compatible without encouraging too tight binding, for example with polylysine-polyethylene glycol (PLL-PEG), may be helpful [38].

To make sure that cells spread out evenly over the pattern, both elements and interspaces must have an appropriate size and shape, such that they are too small for cells to exclusively occupy. We have found it most convenient to use 10 μm dots or squares as pattern elements and interspaces of 5 μm (or wider) between the dots (Fig. 1). This fits with the pattern element sizes between 3 and 10 μm given in the literature for similar approaches [13, 15, 39]. For even spreading, treatment of the interspaces with a protein (*e.g.*, BSA) or other coating (see 3.2.) may also be necessary.

The other important concern in single-cell experiments with protein patterns is that the cell membrane is not deformed above the pattern elements. For example, tight binding of non-specific cell surface adhesion proteins to the patterned protein might hold the cell membrane very close to the glass surface right over the pattern elements, whereas in between, the cell membrane

Figure 4. Cells may prefer pattern elements or interspaces, or spread across both. Patterns of mAb Y3 stained with fluorescent secondary antibody (left) and seeded with HeLa wt cells for 60 h (right). Widths of pattern elements and of interspaces (in *italics*) are indicated.

Figure 5. Cells may prefer structured over unstructured areas, and some pattern sizes over others. The same experiment as in Fig. 4, larger area shown. Bar, 50 μm .

might arch upwards because the repulsion between the charges on the plasma membrane and whatever is used to passivate the interspaces, or because of the natural urge of the cell to move or to undulate its plasma membrane. In such cases, the plasma membrane will acquire pattern-shaped protuberances that are closer to the glass surface than the remainder of the cell surface, and thus in a different focal plane in the confocal laser scanning microscope (cLSM), or more prominent in total internal reflection fluorescence (TIRF) microscopy. This might mislead the investigator to believe that an accumulation of the protein of interest has occurred over the pattern elements through specific interaction, when in reality, the protein is still evenly distributed on the

plasma membrane. It is therefore very important to use negative controls such as test proteins that are not bound by the printed protein, and printed proteins that do not bind to the test protein of interest. We think that it is essential to show that such plasma membrane deformations do not occur [16], for example by staining with rhodamine phalloidin (which binds to the cortical actin cytoskeleton), anti-actin antibody, wheat germ agglutinin, which binds to the protein- and lipid-linked glycans of the plasma membrane, or perhaps a fluorescent lipid that is inserted into the membrane.

3.4 Readout of cell-based assays

In all publications known to us, readout of single-cell experiments with protein patterns is by cLSM or TIRF microscopy. The intracellular portion of the protein of interest is fused to a fluorescent domain (most frequently the green fluorescent protein, GFP), and in the experiment, the arrangement of the GFP into the printed pattern is followed. In principle, such patterning may be followed in live cells, such that changes can be observed over time; in this case, special care must be taken to avoid the bleaching of the fluorescent protein domains, which easily occurs.

An alternative detection method is the staining of the protein of interest with antibodies, and subsequent detection by immunofluorescence microscopy. In our experience, the gap between the glass surface and the cell is very narrow, and antibodies (molecular weight (MW) 155 000) do not easily penetrate into it, and cells must be permeabilized or lysed before proteins of interest can be stained. In our experiments, the glass-cell gap did admit fluorescently labeled nonapeptides (MW 1200).

Any serious use of single-cell experiments with protein patterns, such as *in cis* interaction assays or ligand screening in a high throughput format, will require quantification of the microscopic readout. This is not trivial, since intracellular background signals can occur. For example, our model protein H-2K^b-GFP is present to only about 50% at the cell surface, whereas the remainder is in the endoplasmic reticulum (ER). This ER background is visible in addition to the surface-patterned protein in our experiments, which are done with cLSM (Fig. 1B, label e). Thus, simply integrating over one pattern element and comparing to an interspace area of similar size (similar to what one would do when, for example, quantifying a band in a protein gel) does not yield reproducible results. Instead, the information from many pattern elements must be processed. We have found it most convenient to compare the spatial distribution of the pattern elements (*i.e.*, the fluorescence of the printed protein) and the protein of interest by means of the Pearson coefficient [16, 40]. To this end, it is best if the printed antibody is directly covalently labeled with a fluorescent dye (Fig. 1A); this can easily be achieved with N-hydroxysuccinimide (NHS) chemistry. Pearson coefficient analysis has the added advantage that it can be standardized between experiments and even be automated for high-throughput analysis. Alternatively to this analysis, TIRF images may, if the cell type is right and the ER is sufficiently far above the plasma membrane, avoid the background signal altogether.

4 Concluding remarks

Single-cell experiments with protein patterns are useful for basic research, assay, and screening applications. Their special advantage is that the cell surface proteins that are investigated are in their native protein and lipid environment, and as such, they might be a significant extension to conventional printed protein arrays that are probed not with cells but with ligands or recombinant proteins, with exciting future development opportunities.

5 Materials and methods

Patterns of antibodies on glass surfaces were produced, incubated with cells, and evaluated by microscopy as described [16]. The same publication also lists the cell lines, antibodies, and reagents.

For the experiment in Fig. 1, patterns of fluorescently labeled mAb Y3 (Y3-AF647) were PDMS-printed for 15 min on glass coverslips. Then, Vero cells stably transduced with H-2K^b-GFP were seeded on the Y3 patterns, incubated at 37°C for 24 h, fixed, and imaged by confocal laser scanning microscopy (cLSM).

For the experiment in Fig. 2, patterns of mAb Y3 were PDMS-printed for 15 min on glass coverslips. STF1 wt cells, either untransduced or stably transduced with H-2K^b(-GFP), were seeded on the Y3 patterns and incubated at 37°C until they adhered, then shifted to 25°C and incubated overnight. The next day, fluorescently labeled peptide-ligand (1 μM FL9-TAMRA) was added and cells were incubated at 37°C for 4 h, washed with PBS, fixed, and imaged with cLSM.

For the experiment in Fig. 3, fluorescently labeled mY3 (AF647) was printed for 10 min and the protein pattern was then incubated in PBS for 2 min or 48 h at 4°C, then rinsed with PBS and imaged by cLSM.

For the experiment in Figs. 4 and 5, patterns of mAb Y3 were PDMS-printed for 60 min on plasma-cleaned glass coverslips, the interspaces were blocked with 5% milk for 30 min, and the patterns were stained for 60 min with goat anti-mouse antibody conjugated with Alexa Fluor 488 (gαm-AF488). Then, HeLa wt cells were seeded on the Y3/gαm-AF488 patterns and incubated at 37°C for 60 h and imaged with cLSM.

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