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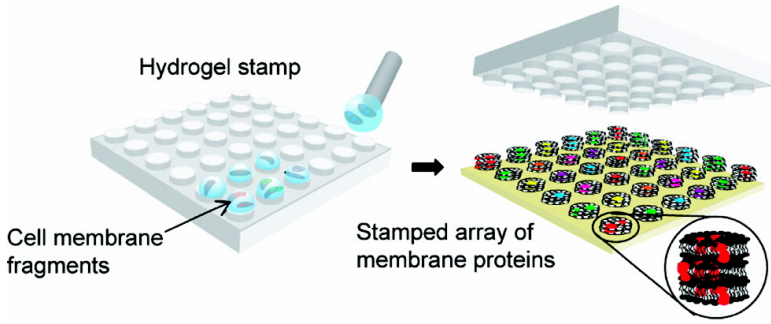

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Generating Arrays with High Content and Minimal Consumption of Functional Membrane Proteins

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Abstract: This paper introduces a widely accessible and straightforward technique for fabricating membrane protein arrays. This technique employs topographically patterned agarose gels to deliver various membrane preparations to glass substrates in a rapid and parallel fashion. It can fabricate more than 30 identical copies of a membrane protein array while requiring only femtomoles of protein. Taking advantage of on-stamp preconcentration, it is able to pattern arrays of multilayered membrane spots with more than 20-fold increased content of membrane proteins compared to existing methods.

Introduction

Membrane proteins play a prominent role in cellular function and therefore attract strong interest as therapeutic targets;[1−5]a great portion of the currently marketed therapeutic drugs target membrane proteins.[1,2,4] To identify new drug candidates by high-throughput screening, the pharmaceutical industry would benefit from arrays that display functional membrane proteins.[1−5] In addition, supported membranes[6−15] as well as arrays of membranes,[16−25] membrane proteins,[1−4,26] or native vesicles[5,27]

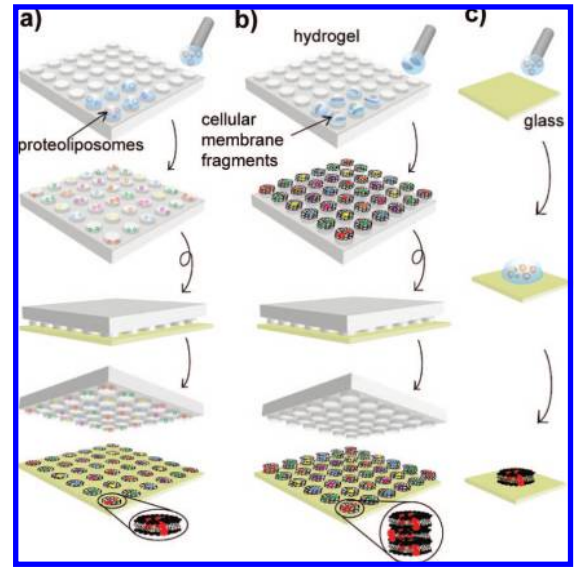

Figure 1. Cartoon comparing hydrogel stamping to spotting of membranes. (a) Storage of small proteoliposomes inside the posts of a stamp affords multiple printing of *single* lipid bilayers with embedded membrane proteins without intermediate reinking. (b) Preconcentration of relatively large membrane fragments on the posts of the stamp affords patterning of arrays of *multilayered* cell membrane fragments with high protein content. (c) Preparation of droplet-derived membrane spots by deposition (spotting) of a droplet of a suspension of membrane preparations onto substrate. This droplet was incubated for 1 h in a humid chamber to avoid drying by evaporation. Note, the resulting membrane arrays or spots were immersed in an aqueous solution immediately after their generation.

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are increasingly employed in academic research laboratories for studies of interactions between proteins and lipids,[28,29] proteins and membrane proteins,[1,2,4,26] as well as between therapeutic drugs and biomembranes.[21,22,30]

Despite their significance, membrane proteins are notoriously difficult to prepare in sufficient quantities and in correctly folded, functional, and pure form.[2,3,15,31] Fabrication of arrays of these proteins has, therefore, remained challenging and limited to a few expert research groups.[1−4,11,26] Among the fabrication techniques developed to date,[1−3,26] robotic spotting is most common.[2−4] In this method, a robotic pin printer deposits small droplets of membrane suspensions onto substrates to create an array of membrane proteins.[2] Despite its usefulness, this method requires a robotic system and is hence not accessible to most academic research laboratories. In contrast, microcontact printing[20,32−35] is an accessible, simple, and well-established arraying technique that has been applied for fabrication of a variety of arrays, including arrays of supported lipid bilayers[20,34] and arrays of soluble proteins.[32−34] This technique has not, however, been adopted for direct fabrication of arrays of membrane proteins, presumably because stamping with poly(dimethylsiloxane) (PDMS) stamps requires drying of the ink on the posts of the stamp in order to preserve the fidelity of the micropattern. In the case of preparations of fragile membrane proteins, this drying step can result in protein denaturation and is therefore typically avoided.[3]

Here we introduce a novel, straightforward, and efficient method that employs hydrogel-based microcontact printing[21,28,36−41] to fabricate arrays of various membrane proteins in a parallel and rapid fashion. The unique characteristics of hydrogel stamps provide a hydrated and biocompatible environment that makes it possible to employ microcontact printing for direct and rapid fabrication of membrane protein arrays. We demonstrate the capability of this technique to fabricate these arrays by two distinct approaches with complementary benefits. In one approach, hydrogel stamps store small proteoliposomes and deliver them onto glass substrates. This procedure consumes a minimal amount of precious membrane preparations while fabricating multiple copies (at least 30) of a membrane protein array. In the other approach, which addresses the challenge of fabricating arrays from low abundance membrane proteins, hydrogel stamps preconcentrate and deliver relatively large membrane fragments (from mammalian cells) to bare or chemically activated glass substrates. This procedure patterns multilayered membrane structures with more than 20-fold increased protein contents per area of the spot compared to existing methods. We employed the resulting membrane protein arrays to carry out ligand-binding assays with a fluorescently labeled ligand and demonstrated excellent signal to background ratio of fluorescence.

Recently, we introduced a method that used topographically patterned agarose gels to fabricate arrays of various lipid bilayers with up to 600 spots cm^{-2}.[21] We demonstrated that this method can create more than 100 copies of an array of functional[28] and fluid supported lipid bilayers[42] while using only picomoles of lipids.[21] This method was, however, limited to generating spots of lipid bilayers; it did not generate membrane spots that contained embedded transmembrane proteins. Here we expand the scope of this method to the important application of generating arrays of membrane proteins that are embedded functionally in lipid membranes. In addition, we demonstrate a novel aspect of hydrogel stamps, namely, preconcentration of large membrane fragments, that made it possible to generate arrays of multilayered membranes with high content of functional membrane proteins.

Results and Discussion

In order to generate arrays of membrane proteins, we used detergent dialysis to reconstitute an integral membrane protein, human tissue factor (TF), into small liposomes with a protein to lipid ratio of 1:5000 (containing 3% fluorescently labeled lipids) and used the resulting proteoliposomes to ink an agarose stamp (Figure 1a). The small size of these proteoliposomes (diameter $\sim$ 50 nm)[43] allowed them to diffuse into agarose stamps where these proteoliposomes were stored in a biocompatible and hydrated environment. Consequently, the inked stamp could be used to pattern 100 copies of a membrane protein array without intermediate re-inking (Figure S2 in the Supporting Information). Comparison of the fluorescence intensity and fluidity of fluorescently labeled lipids in the resulting patches of supported membranes showed no significant difference between these 100 arrays (Figure S2 in the Supporting Information). In order to confirm the presence of TF proteins in these arrays, we added a primary antibody (pAb) against TF followed by exposure to a fluorescently labeled secondary antibody (sAb*). Figure 2a shows that the fluorescent signal (and hence the content of TF in the membranes) remained close to constant during the first 30 stamped arrays. We also compared the fluorescent signal of these stamped membrane spots with control spots of supported membranes that we prepared by placing small droplets of solutions of membrane preparations on glass or γ-aminopropylsilane (GAPS)-coated substrates (Figure 1c; this technique is similar to the method of robotic spotting,[2] and we refer to the resulting spots as "droplet-derived spots"; see Supporting Information for details). This comparison showed no significant difference between the stamped membrane spots and droplet-derived membrane spots (Figure 2b). We examined the specificity of antibody binding to the TF-containing membrane spots by exposing one of these membrane spots only to the sAb* and detected no fluorescent signal in the absence of

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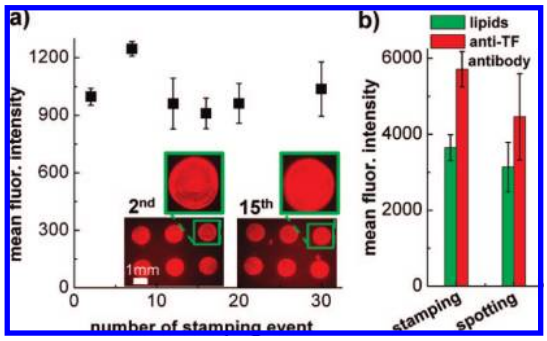

Figure 2. Multiple stamping of a membrane protein array using a stamp that was inked once and comparison of stamped membranes with droplet-derived membranes. (a) Mean fluorescence intensity of antibodies bound to TF proteins in membrane spots of arrays as a function of the number of stamping events. Error bars represent the standard error of the mean intensity of several spots in each array. Insets show fluorescent micrographs of two of these arrays. (b) Comparison of the fluorescence intensity of labeled lipids (green) and antibodies bound to TF (red) in stamped membranes with droplet-derived (spotted) membranes. Error bars represent the standard deviation of mean fluorescence intensities.

pAb against TF (Figure S1 in the Supporting Information). Stamping of small proteoliposomes, thus, made it possible to generate rapidly up to 30 copies of a membrane protein array while using a stamp that was inked only once with a total amount of 0.4 pmol of TF per post of the stamp (i.e., ~13 fmol corresponding to ~470 pg of TF per spot). After approximately 30 stamping events, the fluorescence intensity decreased in a linear fashion (Figure S3 in the Supporting Information); we attribute this decrease to a depletion of proteoliposomes in the posts of the stamp (see Supporting Information for details).

In order to extend this patterning method to membrane preparations that are typically used for drug binding studies,[2] we obtained membrane fragments from mammalian cells that contained the human nicotinic acetylcholine receptor (AChR) and reconstituted this transmembrane receptor into small liposomes. We inked agarose stamps with these proteoliposomes and stamped multiple arrays of membranes. Using epifluorescence microscopy, we found close to constant fluorescence intensity from the fluorescent lipids that we included during the reconstitution procedure in the membranes of these arrays. Immunofluorescence assays with pAb against AChRs and sAb*, however, revealed no detectable AChR in these membranes. We attribute the low fluorescent signal to the low initial concentration of AChRs in the original preparation of membrane fragments (<1 nM).

In order to maximize the protein concentration in the membrane preparation while simplifying the procedure and keeping the manipulation of membrane proteins to a minimum, we used cell membrane fragments directly and omitted the dialysis reconstitution step. We, thus, inked individual posts of agarose stamps with suspensions of membrane fragments that contained either AChRs or human dopamine transporters (DATs) and printed several membrane arrays on glass or GAPS-coated slides. Figure 3a,b shows fluorescent micrographs of two arrays that were exposed to pAb against either AChR or DAT (both pAbs were from rat), followed by incubation with antirat sAb*. The remarkably strong fluorescent signal from only one group of membrane spots in each of these arrays illustrated the specificity of binding interactions; pAb against AChR did not bind to DAT, and pAb against DAT did not bind to AChR (Figure 3a,b). We compared the fluorescent signal of these stamped membrane spots with droplet-derived membrane spots.

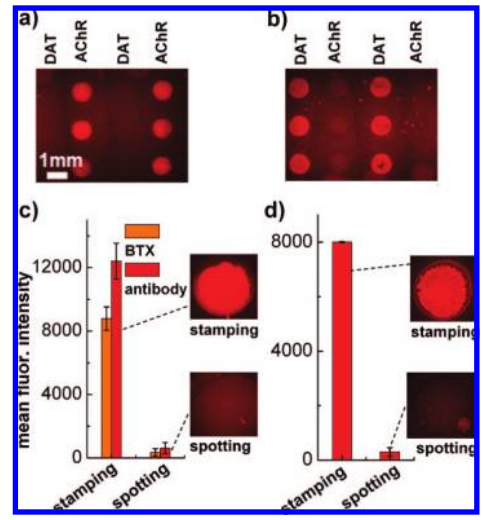

Figure 3. Stamped arrays of two different membrane proteins and comparison of stamped spots with droplet-derived spots. Fluorescence micrographs of arrays with alternating columns of DAT-containing and AChR-containing membranes after exposure to fluorescent antibodies against (a) AChR and (b) DAT. (c) Comparison of fluorescence intensity from antibodies (red) or BTX (orange) bound to AChRs in stamped membranes with droplet-derived membrane spots. (d) Comparison of fluorescence intensity of antibodies bound to DATs in stamped membranes with droplet-derived (spotted) membrane spots. Bar graphs show fluorescence intensities (after background subtraction) of stamped spots and droplet-derived spots. Error bars represent standard deviation of mean fluorescence intensities.

This comparison showed that hydrogel-stamped membrane spots containing AChR resulted in at least 20 times stronger fluorescence intensity upon binding of labeled antibodies against AChR compared to the intensity from droplet-derived spots (Figure 3c). Figure 3d illustrates the fluorescence intensities from a similar comparison for DAT-containing membrane spots, revealing, again, at least 20-fold higher content of DAT in stamped spots compared to droplet-derived spots. The remarkably strong fluorescence intensity from antibodies bound specifically to AChRs or DATs in membranes of these stamped arrays (Figure 3a,b) suggested the presence of multilayer membranes with concomitant high protein contents in the stamped arrays. We hypothesized that the large size of membrane fragments impeded their diffusion into the network of the agarose gel (pore diameter of 243 ± 5 nm for a 4% gel)[44] and that membrane fragments thus preconcentrated at the surface of the posts of the agarose stamps while excess liquid from the membrane preparation adsorbed into the gel (Figure 1b). Once we brought the inked stamps into contact with clean glass slides for 5−20 s, the agarose posts transferred the preconcentrated membrane fragments onto the substrate, forming multilayer membrane structures with embedded membrane proteins (Figure 1b). We confirmed the multilayered structure of these membranes by confocal microscopy (Figure 4) (see Experimental Section for details). Immunofluorescence assays revealed that the employed stamp delivered almost all membrane fragments during the first stamping event; almost no membrane fragments were delivered during the following stamping events. This result confirms the hypothesis of preconcentration and subsequent multilayer transfer.

For applications in the pharmaceutical industry, it is important that membrane protein arrays can be used to carry out functional

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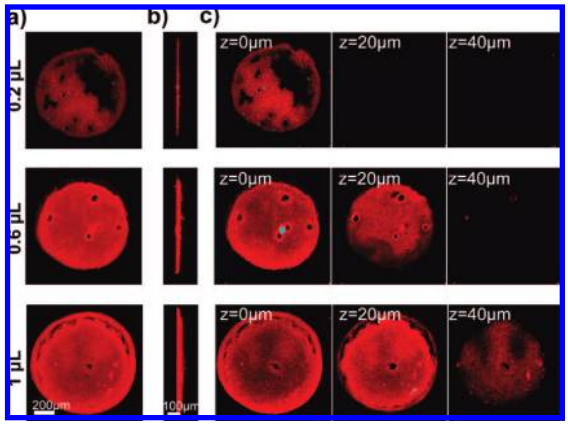

Figure 4. Confocal images of stamped membrane fragments containing AChR on a glass slide after exposure to fluorescent antibodies. These membrane spots were transferred from a stamp on which the posts were inked with 0.2 μL (first row of images), 0.6 μL (second row of images), or 1 μL (third row of images) of a solution of cell membrane fragments. (a) Top view and (b) side view of rendered *z*-scans of the stamped membrane spots. (c) Confocal scans of these membrane spots at different distances (in *z*-direction) from the surface of the glass substrate confirming the multilayered nature of the spots. Note the exceptionally strong signal to background ratio of these multilayered membrane spots.

binding assays of ligands, agonists, or antagonists.[2−4] We investigated the suitability of the stamped membrane arrays for these assays by probing the binding of a fluorescently labeled derivative of the neurotoxin α-bunguratoxin (BTX) to AChRs. Figure 3c compares the fluorescent signal from BTX bound to AChRs in stamped membranes with the signal from BTX bound to droplet-derived spots. The fluorescence intensities from bound BTX were in good agreement with the results from antibody binding and demonstrated at least a 20-fold higher content of AChR in stamped membrane spots compared to droplet-derived spots (Figure 3c). Moreover, this approach (i) directly employed cell membrane fragments with the benefit of minimized processing of membrane preparations, (ii) patterned multilayer spots of membranes with high protein contents, and (iii) minimized undesired and potentially denaturing interaction of membrane proteins with the supporting substrate[1,2,4,8−11,45] due to the presence of multilayer membranes.

Conclusion

In conclusion, we present the first demonstration of microcontact printing of proteoliposomes and cell membrane fragments by taking advantage of the storage and preconcentration capability of biocompatible hydrogels. The method presented here is remarkably simple, efficient, and cost-effective, which requires only standard laboratory equipment and chemicals to fabricate membrane protein arrays in any laboratory within less than 3 h. We carried out ligand-binding assays and showed that the resulting supported membrane proteins were functional and retained their binding activity. We employed this method for two distinct approaches to fabricate arrays of membranes with integral membrane proteins. One approach takes advantage of the storage capability of agarose stamps and minimizes the required time and amount of membrane proteins by generating multiple copies of a membrane protein array. This approach is particularly beneficial when membrane proteins can be reconstituted in relatively high concentrations and when fabrication of several copies of a membrane protein array is desirable. The other approach preconcentrates membrane fragments to generate arrays of multilayered membranes with high contents of embedded proteins and, thus, achieves more than 20-fold enhanced detection sensitivity while requiring only femtomoles of membrane proteins. This second approach is most beneficial when membrane proteins are sensitive to reconstitution or can be obtained only in low concentrations. The advantageous characteristics of these two complementary approaches make biocompatible hydrogel stamping compelling for fabrication of arrays of precious membrane proteins. We expect hydrogel-based microcontact printing of membrane protein preparations to be useful for the steadily growing interest in drug−membrane interactions and drug−protein interactions in industrial and academic research.

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Experimental Section

Materials. We obtained high-gel strength agarose powder from OmniPur (Merck, Darmstadt, Germany). All the lipids, including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), porcine brain L-α-lysophosphatidylserine (L-α-PS), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). We purchased recombinant human tissue factor (TF) and mouse monoclonal anti-TF antibody from Calbiochem (San Diego, CA, USA). We obtained cell membrane fragments containing human dopamine transporter (DAT) (transporter concentration of ∼14.5 nM) and membrane fragments containing human acetylcholine receptor (AChR) (receptor concentration of ∼1 nM) from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). We purchased rat monoclonal anti-DAT antibody from Abcam (Cambridge, MA, USA) and rat monoclonal anti-AChR antibody from Sigma-Aldrich (St Louis, MO, USA). Alexa-fluor 555 goat antimouse antibody and Alexa-fluor 555 goat antirat antibody were purchased from Molecular Probes (Eugene, OR, USA). We purchased the detergent *n*-octyl-β-D-glucopyranoside (OG) from Sigma-Aldrich (St Louis, MO, USA) and the blocking solution of casein in phosphate buffered solution (PBS) from Pierce (Rockford, IL, USA). Buffer A solution contained 100 mM NaCl, 20 mM Hepes/NaOH buffer, pH 7.5, and 0.02% (w/v) sodium azide.

Fabrication of Agarose Stamps. We prepared agarose stamps freshly with a concentration of 2−4% (w/v), as previously described.[21,39] We used 2% agarose stamps (pore size of 364 ± 8 nm[44]) for multiple stamping applications and 4% agarose stamps (pore size of 243 ± 5 nm[44]) for preconcentration applications. Briefly, we added agarose powder to a solution of 150 mM KCl, 10 mM Hepes, pH 7.4 and heated the mixture (in a microwave oven) to the boiling point, while shaking the beaker occasionally, until the agarose powder was completely dissolved. Then we immediately poured the hot solution onto a patterned PDMS master (placed inside a Petri dish) and quickly degassed the solution in a desiccator that was connected to a diaphragm vacuum pump to remove all air bubbles from the wells of the PDMS master (such bubbles can result in defective posts on the resulting stamp and must be removed). The PDMS master which contained wells with 1 mm diameter (and was used as the mold for agarose stamps) was a replica (positive) of a PDMS replica (negative) of a standard 1536-well plate (polystyrene) with flat bottoms (Corning, Cambridge, MA, USA).[21,39] The advantage of using a PDMS replica of a 1356-well plate, instead of the plate itself, is that the PDMS mold is elastomeric, which facilitates removal of the agarose stamp from the mold. We allowed the gel to form at 4 °C for 1 h and peeled off the PDMS master to obtain topographically patterned agarose stamps.

Preparation of Small Proteoliposomes. We prepared small proteoliposomes containing human tissue factor (TF) by a detergent

dialysis method as described by Neuenschwander et al.[46] Briefly, we generated a lipid film by depositing a total of 0.65 μmol of lipids composed of 77% (mol %) POPC, 30% L-α-PS, and 3% NBD-PE dissolved in chloroform in a round-bottom flask followed by pulling a vacuum while rotating the flask. We hydrated this lipid film with 100 μL of a freshly prepared solution of 100 mM OG in buffer A. In order to obtain a TF to lipid ratio of 1:5000, we added 189 μL of 1 mg mL^{-1} TF in deionized (DI) water to this mixture. The resulting mixed micelles containing lipids, TF, and OG were incubated at room temperature for 30 min followed by dialysis with a dialysis cassette (0.1−0.5 mL, 10 000 molecular weight cutoff) from Pierce in 1 L of buffer A. We replaced the buffer solution every 24 h for 72 h.

Preparation of Glass Substrates. We cleaned all microscope glass slides (precleaned slides from Corning Inc., Corning, NY, USA) by immersing them in a freshly prepared, hot Piranha solution (2:1 concentrated sulfuric acid and 30% hydrogen peroxide) for ∼10 min. We rinsed these slides with copious amounts of DI water and stored them in DI water until use. Immediately before use, we dried the glass slides with a stream of gas.

Glass slides coated with γ-aminopropysilane (GAPS II)[47,48] were purchased from Corning Inc., and we used them as obtained.

Inking and Stamping Procedure. We performed the inking and stamping procedure as previously described.[21] Briefly, once the agarose gel formed on the PDMS master, we peeled off the PDMS master and placed the resulting agarose stamp in a Petri dish containing water (with the posts facing upward) such that ∼2/3 of the height of the stamp was immersed in DI water. We inked individual posts of the stamp by manually pipetting small droplets (∼0.1 μL) of the desired solutions on top of each post. We often inked each post 2−4 times to supply adequate amounts of material (particularly for multiple stamping applications). Once no more excess liquid was visible on the surface of the posts, we employed the stamp to pattern an array of membranes. After removal of the stamp, we immediately immersed the patterned glass or GAPS II slides in water or buffer. For the multiple stamping experiments, when we inked the stamps with small proteoliposomes, we carried out the stamping procedure in a room with a humidity of >65% to avoid possible dehydration. As opposed to stamped arrays from small proteoliposomes, we found that stamped arrays from relatively large membrane fragments were not sensitive to humidity and we carried out these experiments in a standard laboratory without humidity control. We think that the transfer of a multilayer of membranes retained adequate humidity to prevent dehydration. This characteristic of the novel concept of stamping multilayered membrane preparations is advantageous because it simplifies the procedure for stamping multilayered membrane arrays while preserving function.

Microscopy and Imaging. To carry out imaging, we used an upright E600FN Nikon microscope equipped with an XCite 120 lamp (EXFO Life Sciences, Ontario, Canada) and a Coolsnap camera (Photometrics, Tucson, AZ). We acquired images using Metamorph 7 software (Universal Imaging, Downingtown, PA). All of the fluorescent micrographs presented in this work are false color images. We acquired images of NBD labeled lipids with filter settings for fluorescein and images of Alexa-fluor 555 labeled antibodies with filter settings for rhodamine. We performed confocal microscopy with an inverted TE2000-U Nikon microscope equipped with an argon 488 laser and a helium−neon 543 laser and a Nikon scan head. Confocal images were acquired by Nikon EZ-C1 3.5 software (Image Systems, Inc., Columbia, MD).

Immunofluorescence Assays. As mentioned previously, patterned glass or GAPS II slides were immersed in PBS immediately after removal of the stamp. After performing epifluorescence microscopy on these arrays (to probe the quality of lipids in stamped membranes by imaging the fluorescent lipids in these preparations), we incubated these arrays in a solution of casein in PBS for 1 h to block the bare glass. We then incubated these arrays in PBS solutions containing either anti-TF antibody (1.5 μg mL^{-1}), anti-DAT antibody (5.8 μg mL^{-1}), or anti-AChR antibody (0.75 μg mL^{-1}) followed by a fluorescently labeled secondary antibody; we used Alexa-fluor 555 goat antimouse antibody (5.5 μg mL^{-1}) or Alexa-fluor 555 goat antirat antibody (5 μg mL^{-1}). We carried out the incubation with antibodies at room temperature for 5−15 h with the primary antibody and for 1−3 h with the secondary antibody. We did not rinse the slides between these incubations but rinsed them with PBS prior to imaging.

Characterization of Multilayered Membranes by Confocal Microscopy. In order to confirm the multilayer structure of stamped membrane fragments from cell preparations, we performed confocal microscopy on these membranes and used rendered *z*-scans of stamped membrane spots to probe the thickness of these spots. Figure 4 shows confocal images of stamped membrane spots that were transferred from a stamp on which each post had been inked with different amounts of membrane preparations. The membrane spots shown in Figure 4a−c were transferred from posts that were inked with 0.2, 0.6, and 1 μL of solution of AChR-containing membrane fragments, respectively. In order to image one membrane spot completely in each image, we employed a 10× objective for this series of confocal images. Figure 4c shows *z*-scans of each of these three membrane spots at different distances from the glass surface and demonstrates the difference in thickness of these membrane spots. This figure clearly illustrates an increase in thickness of stamped membrane spots with increasing amount of membrane preparations used to ink the corresponding posts on the stamp. These results confirm the multilayer structure of the stamped membrane spots.

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Supporting Information Available: Experimental details of multiple stamping of membrane protein arrays, droplet-derived membrane spots, immunofluorescence assays, physical properties of membrane arrays from stamping small proteoliposomes, and complete author list of ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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