

# Micropatterned agarose gels for stamping arrays of proteins and gradients of proteins

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We describe a method for repetitive and rapid formation of planar microarrays and gradients of proteins using patterned agarose stamps. It demonstrates: (i) micropatterning of agarose gels with feature sizes as small as 2  $\mu\text{m}$ ; (ii) inking of posts (diameter 50–1000  $\mu\text{m}$ ) on patterned agarose stamps with one or multiple (here, eight) proteins and repetitive stamping of patterns ( $>100$  times in the case of one protein) and arrays (20 times in the case of eight proteins) without the need for intermediate re-inking; (iii) transferring spots of proteins with good homogeneity in surface coverage to glass slides; (iv) applying this technique to surface-based immunoassays; (v) stamping that requires only sub-nanomolar amounts of protein (typically  $\sim 3 \mu\text{g}$  in  $\sim 0.6 \mu\text{L}$  of solution); (vi) stamping without the need for drying of the proteins, as opposed to stamping with stamps made of poly(dimethylsiloxane); and (vii) patterning gradients of proteins by allowing two proteins to diffuse toward each other in an agarose stamp, followed by printing the protein gradients onto a surface.

**Keywords:** Hydrogel / Micropatterning / Protein gradients / Protein microarrays / Stamping

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## 1 Introduction

We present a method for repetitive, rapid, and parallel patterning of microarrays and gradients of proteins on surfaces over large areas ( $>10 \text{ cm}^2$ ) using microfabricated agarose stamps. We establish a procedure for preparing mechanically stable stamps of agarose having bas-relief patterns with dimensions as small as 50  $\mu\text{m}$ . In this procedure, we deposit solutions of protein in spatially distinct sections of these agarose stamps. The hydrogel stamps have six characteristics as tools with which to pattern arrays of proteins on planar substrates: (i) they have the capability to generate patterns of a single protein with a density of up to 2500 spots  $\text{cm}^{-2}$ ; (ii) they make it possible to transfer, in parallel, different proteins in lower density ( $\sim 25$  spots  $\text{cm}^{-2}$ ); (iii) they generate multiple arrays of proteins with small quantities of protein ( $\sim 50 \text{ pmol}$ , or  $\sim 3 \mu\text{g}$  in the case of BSA); (iv) they absorb excess solution during inking; (v) they provide

good homogeneity of the transferred spots of proteins; and (vi) they store proteins in a non-denaturing environment.

Microarrays of proteins on surfaces are used increasingly for high-throughput assays in drug-discovery, diagnostics, and proteomics [1–11]. For these applications, slides with many (ideally  $>100$ ) different proteins are desirable [5, 8]. Methods presently employed to fabricate arrays of proteins use ink-jet printing [12], electrospray through a dielectric grid mask [13], and direct application of solutions of proteins using microfluidic networks [14] or robots [1, 2, 15–17]. Since successive loading and dispensing of solutions of many proteins is time-consuming and can lead to cross-contamination and loss of protein due to frequent wash steps [15, 18, 19], it is desirable to transfer protein arrays in a parallel, rather than a serial, format.

Microcontact printing provides parallel transfer of material to a surface and makes it possible to stamp thousands of microspots of the same molecule simultaneously over areas larger than  $1 \text{ cm}^2$  [20–22]. Bernard *et al.* [23–29] and other groups have demonstrated patterning of proteins by printing with poly(dimethylsiloxane) (PDMS) stamps. Here, we demonstrate the utility of topographically patterned hydrogels [30, 31] to pattern arrays of either the same or different proteins repetitively. Markowitz *et al.* [32, 33] reported the use of a capillary filled with

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**Abbreviations:** PDMS, poly(dimethylsiloxane); TRITC, tetramethylrhodamine isothiocyanate

a synthetic hydrogel to transfer individual spots of proteins to a surface in a serial process. We explored the possibility of microfabricating a commercially available hydrogel – agarose [34, 35] – to develop a simple procedure for generating arrays of proteins from a biocompatible material. We found that we can fabricate stamps containing an array of posts (with diameters of 50–1000  $\mu\text{m}$ ) in agarose; we demonstrate serial inking of solutions of proteins to individual posts, and subsequent transfer of an array of proteins to a surface, in a single stamping event. We show a single, serially inked stamp can generate more than 100 copies of the same pattern of one protein and at least 20 copies of an array of eight proteins on glass substrates without the need for intermediate re-inking of the stamp.

In addition to stamping arrays of proteins, we demonstrate the capability of hydrogels to form gradients of proteins within the gel by diffusion; we show that we can transfer these gradients of proteins onto flat substrates. This approach is simpler than many other methods of generating gradients on surfaces [36–41], and requires no specialized setup or equipment. Micropatterned, mechanically robust agarose stamps are easy to fabricate and to use. They extend the concept of soft lithography to water-based stamps [21, 30, 31, 42] and aqueous “inks” (here, solutions of proteins). Since many hydrogels are biocompatible, patterned hydrogels should be particularly useful for biological and biochemical applications [30, 43, 44].

## 2 Materials and methods

### 2.1 Chemicals

All chemicals were purchased and used as received. We used deionized, purified water (MilliQ system, 18.2 M $\Omega$  cm; Millipore, Bedford, MA, USA).

### 2.2 Fabrication of agarose stamps

We used two types of masters to prepare agarose stamps: microfabricated masters and a PDMS replica (positive) of a PDMS replica (negative) of a standard 1536-well plate (polystyrene) with flat bottoms (Corning, Cambridge, MA, USA). We used photolithography [21] to prepare masters containing arrays of posts of SU8 photoresist with (i) 50  $\mu\text{m}$  diameter,  $\sim$ 25  $\mu\text{m}$  height, and a distance of 150  $\mu\text{m}$  between the posts; (ii) 200  $\mu\text{m}$  diameter, 130  $\mu\text{m}$  height, and a distance of 200  $\mu\text{m}$  between the posts; and (iii) 700  $\mu\text{m}$  diameter, 700  $\mu\text{m}$  height, and a distance of 300  $\mu\text{m}$  between the posts. The actual masters that we used for molding the agarose stamps were replicas (negatives) of these photolithographically prepared masters in PDMS. Repetitive casting of hot agarose solu-

tions onto masters with SU8 features gradually damaged the masters (the SU8 features lifted off the silicon wafer substrate).

For manual inking of individual posts, we used agarose stamps with posts with  $\sim$ 1 mm diameter,  $\sim$ 1.5 mm height, and  $\sim$ 1 mm distance between the posts; we fabricated these stamps from a PDMS replica (positive) of a 1536-well plate. To prepare the stamps, we heated an aqueous solution of high-gel strength agarose (OmniPur; Merck, Darmstadt, Germany) or low-melting temperature agarose (GibcoBRL, Life Technologies, Grand Island, NY, USA) in a microwave oven to the boiling point and cast the solution (to a height of  $\sim$ 3 cm) onto the PDMS masters. Casting agarose solutions with a thickness of 3 cm had the advantage of extended time of gelation for removal of trapped air bubbles from the wells of the master (by repeatedly pulling and releasing a vacuum) and easy handling during stamping. Finally we allowed the agarose solution to gel at room temperature and ambient pressure. The next step of the fabrication process depended on the diameter of the posts on the agarose stamp. In the case of stamps with posts with diameters of  $\geq$  200  $\mu\text{m}$ , we peeled off the elastomeric PDMS master, cut the stamp to the desired dimensions (*e.g.*, 3 cm wide and 4 cm long) with a scalpel, inked the stamps, and used them for patterning. In the case of stamps with 50  $\mu\text{m}$  posts, however, we embedded the stamps in PDMS by placing the master with the agarose stamp in a dish, pouring PDMS prepolymer over and around the stamp (such that the stamp was covered by PDMS prepolymer), and curing at room temperature for  $>$ 48 h. Before pouring the PDMS prepolymer, we slanted the sidewalls of the gel by cutting it such that the side opposite to the posts had slightly bigger dimensions than the patterned side of the stamp; the slanted sidewalls kept the gel in the PDMS shell. Before use, we peeled the stamp (with its PDMS shell) off the master. The PDMS shell improved the handling of the stamps in three ways. First, the stamps did not slide on the surface of the substrate during stamping because the PDMS frame provided tight, conformal contact. Second, the PDMS protected the gel stamps from drying. Third, the additional PDMS shell provided mechanical stability. Stamps with posts with diameters  $\geq$  200  $\mu\text{m}$  did not require a PDMS shell; spots with these dimensions were less sensitive to sliding in the micrometer range than spots with 50  $\mu\text{m}$  diameter.

### 2.3 Storage of agarose stamps

We stored agarose stamps by immersing the side of the stamp without features (the side opposite to the posts) in a bath of deionized water (depth  $\sim$ 5 mm). To avoid drying during repetitive stamping events we added  $\sim$ 200  $\mu\text{L}$  of

deionized water in a cavity that we cut into the top of the stamp (the side opposite to the posts). We added water sufficiently often such that the cavity did not dry out.

## 2.4 Inking and stamping procedure

Depending on the size of the posts on the agarose stamps, we inked the stamps in different ways. We inked posts with a diameter of 50  $\mu\text{m}$  by turning the stamps upside down (such that the posts faced upwards) followed by covering the exposed agarose with protein solution for at least 1 h (by repeatedly adding 100  $\mu\text{L}$  of solution of protein). We used the following protein solutions: 100  $\mu\text{g}\cdot\text{mL}^{-1}$  fluorescein isothiocyanate (FITC)-labeled collagen (type I from bovine skin) or 15  $\mu\text{g}\cdot\text{mL}^{-1}$  tetramethylrhodamine isothiocyanate (TRITC)-labeled bovine serum albumin (BSA) in phosphate-buffered saline (PBS; both proteins were purchased from Molecular Probes, Eugene, OR, USA). We removed the remaining solution of protein with deionized water followed by a stream of nitrogen. Before stamping on a surface, we performed up to ten stamping events on glass to remove excess solution of protein from the stamp. We inked the posts with  $\sim 1$  mm diameter individually with a different protein by pipetting  $\sim 0.2$   $\mu\text{L}$  of a solution of protein (typically at a concentration of 5  $\text{mg}\cdot\text{mL}^{-1}$  in deionized water or PBS) onto the top of each post. After the stamp absorbed the solution (usually within less than 5 min), we added another droplet of protein solution. We repeated this cycle two to four times. After the stamp had absorbed all the solution, we used it for patterning; no wash step was necessary. We explored stamping on ethanol-cleaned glass, amine-modified glass (SuperAmine slides; Telechem International), glass carrying aldehyde groups (SuperAldehyde slides; Telechem International), aldehyde glass slides with immobilized neutravidin™ (from Pierce, Rockford, IL, USA), and on flat PDMS substrates.

## 2.5 Preparation of arrays of proteins for immunoassays

We used concentrations of 5  $\text{mg}\cdot\text{mL}^{-1}$  of protein (Sigma, St. Louis, MO, USA) (in PBS pH 7.4 from Sigma or in deionized water) for the inking step except for the monoclonal mouse anti-dinitrophenyl (DNP) immunoglobulin E (IgE) antibody (in this case, we used the original solution from Sigma). We placed the stamp in contact with amine-modified glass slides for 2 min, and patterned 20 arrays with the same stamp without intermediate re-inking. After removing the stamp from the glass slides, we immersed the slides immediately in a filtered (0.45  $\mu\text{m}$  filter) solution of 2  $\text{mg}\cdot\text{mL}^{-1}$   $\beta$ -casein (Sigma) in deionized water to block the areas of the slides that were not in contact with the

stamp. We incubated the slides for at least 30 min in the casein solution and then washed with PBS. In order to perform the surface-based immunoassays, we immersed each slide in a solution containing a different primary antibody. For this step, we diluted the original solutions of the primary antibodies from the supplier (see below) five- to tenfold in PBS, except for the anti-ubiquitin antibody, which we used undiluted. We purchased the monoclonal mouse anti-ubiquitin antibody (IgG1, kappa), the monoclonal mouse anti-myoglobin antibody (IgG1) and the rabbit anti-lysozyme antibody from Zymed Laboratories (San Francisco, CA, USA). The anti-BSA antibody was from Sigma and the anti-ovalbumin antibody from Biotest International (Saco, ME, USA; we used this antibody at a concentration of 1  $\text{mg}\cdot\text{mL}^{-1}$  in PBS). After incubation of the slides overnight at 4°C in the solutions containing the primary antibodies, we washed all slides thoroughly with PBS. In order to detect bound primary antibodies from the first incubation step, we incubated the slides with rhodamine-labeled secondary antibodies (from Zymed Laboratories). We immersed those slides that we incubated in the first step with primary antibodies from mouse with a solution of a TRITC-labeled, secondary, anti-mouse antibody from goat, and those slides that we incubated in the first step with a primary antibody from rabbit with a solution of TRITC-labeled, secondary, anti-rabbit antibody from goat. We used 0.15  $\text{mg}\cdot\text{mL}^{-1}$  concentrations of these secondary antibodies in PBS and we incubated the slides overnight at 4°C. We washed the slides with PBS followed by a brief wash with deionized water before drying in a stream of nitrogen, or we left the slides under PBS buffer before microscopic observation.

## 2.6 Preparation of gradients of proteins

We used flat agarose gels to form and stamp gradients of proteins. We cast hot agarose solution (high gel-strength, 3% in deionized water) onto a silicon wafer (Silicon Sense, Nashua, NH, USA) and waited for the solution to gel at room temperature. We peeled the stamp (thickness  $\sim 3$  cm) from the wafer and turned it such that the flat side (that previously faced the wafer) faced upwards. We placed a strip of PDMS (width 1 mm, height  $\sim 0.7$  mm, length  $\sim 1$  cm) onto the flat side of the stamp. We prepared these strips of PDMS either by cutting a flat film of PDMS or, if we needed strips with precisely defined width, we used a microfabricated master [21] with a groove with the width of the desired thickness of the strip that we wanted to use. We then cast PDMS prepolymer into the groove to obtain a strip with defined width. In the next step we pipetted 5  $\mu\text{L}$  of a solution of 5  $\text{mg}\cdot\text{mL}^{-1}$  FITC-BSA on one side of the PDMS strip and 5  $\mu\text{L}$  of a solution with 5  $\text{mg}\cdot\text{mL}^{-1}$  TRITC-BSA on the opposite side of the

strip. After the protein solutions absorbed into the gel we removed the PDMS strip and placed a glass cover slide onto the stamp to prevent the formation of wrinkles on the flat surface of the agarose stamp. We placed the stamp into a bath (depth  $\sim 5$  mm) of deionized water to prevent drying. To form overlapping gradients of FITC-BSA and TRITC-BSA in the stamp, we allowed the proteins to diffuse toward each other for 4–7 h at room temperature. We peeled off the glass cover slide, removed excess liquid on the stamp by a stream of nitrogen, and placed the stamp onto a glass slide (carrying aldehyde groups) for 1 min to transfer the gradient of proteins to the slide.

## 2.7 Fluorescence microscopy and image treatment

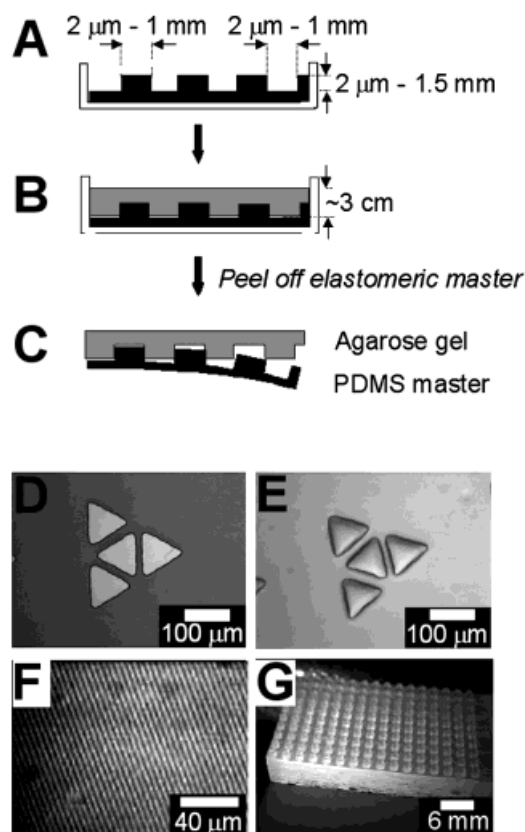
We used an inverted microscope (Leica DMIRB) equipped with a  $1.6\times$  fluotar lens with a numerical aperture (NA) of 0.05 from Leica and a digital camera (ORCA-ER) from Hamamatsu for all microscopy. We captured all images using Metamorph software (Universal Imaging Corporation) employing an exposure time of 1 s and binning 8 (adding the intensity of eight pixels and representing it as one pixel with increased intensity). We enhanced the contrast of the images using Photoshop software (Adobe).

## 3 Results and discussion

### 3.1 Micropatterning of agarose gels

We patterned agarose gels by a casting and molding procedure using PDMS masters [21]. Figures 1A–C illustrate the procedure: We first poured a boiling solution of agarose (in phosphate-buffered saline (PBS), pH 7.4 or in deionized water) over a PDMS master. Subsequent gelling of the solution at room temperature fixed the features of the master in the gel (see Section 2 for details). We peeled the PDMS masters from the agarose gels to release the stamps; the elastomeric properties and hydrophobic surface of PDMS facilitated removal of the master from the stamp.

Figures 1D–G show images of a microfabricated master and of patterned agarose gels. The smallest features generated in the gel were lines with a width of  $2\ \mu\text{m}$  spaced by  $2\ \mu\text{m}$  and a height of  $\sim 2\ \mu\text{m}$ . The stability of patterns in agarose gels depended on the type of gel (high gel-strength *versus* low-melting temperature agarose) and on its concentration. With high gel-strength agarose, we obtained stable features at concentrations of 2–5% w/v agarose in PBS (pH 7.4) or deionized water; with low-melting temperature agarose, we used 5–8% w/v agarose. Gels made from lower concentrations of agarose lost the



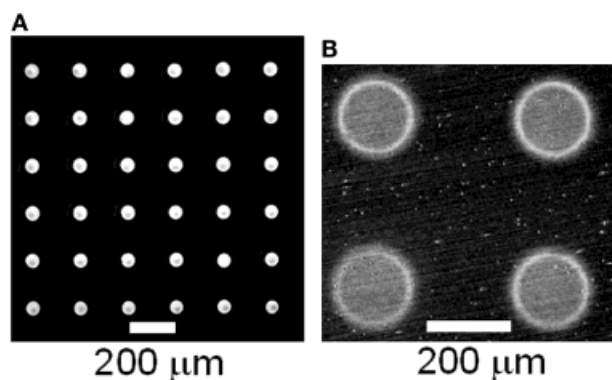
**Figure 1.** Fabrication of micropatterned agarose gels. (A) We used either microfabricated masters made out of PDMS with feature sizes from  $2\ \mu\text{m}$  to  $700\ \mu\text{m}$ , or PDMS replicas of standard polystyrene plates with 1536 wells (diameter 1 mm). (B) After pouring a boiling solution of agarose onto the mold, we let the solution gel at room temperature. (C) Peeling the elastomeric master from the patterned gel resulted in an agarose stamp with imprinted features. (D) Image of a microfabricated master. (E) Corresponding replica in 5% w/v high-gel strength agarose. (F) Agarose gel with grooves with a width, depth, and pitch of  $2\ \mu\text{m}$ . (G) Agarose stamp made from a PDMS replica of a 1536-well plate.

integrity of the imprinted features over time and were too fragile to serve as a stamp. Features made with low concentrations of agarose ( $<2\%$  w/v) were soft and tipped over when the aspect ratio was  $>1$ ; sharp edges (e.g.,  $90^\circ$  angles) became smooth over time.

### 3.2 Using patterned agarose gels for stamping of proteins

To test the feasibility of using patterned agarose gels for stamping patterns of proteins, we made agarose stamps from PDMS masters with  $50\ \mu\text{m}$  wells and  $200\ \mu\text{m}$  wells. Figure 2A shows a micrograph of a pattern of one protein





**Figure 2.** Fluorescence micrographs of patterns of proteins obtained by printing with agarose (7% w/v, low melting temperature) stamps. (A) Pattern of  $\sim 50 \mu\text{m}$  spots of TRITC-BSA printed onto a hydrophobic surface (PDMS) using a stamp with posts with  $50 \mu\text{m}$  diameter. The density of spots in the array is  $\sim 2500 \text{ cm}^{-2}$ . (B) Pattern of spots of FITC-collagen printed onto a glass slide using a stamp with posts with  $200 \mu\text{m}$  diameter. Due to spreading of the protein solution on glass, the spot size is  $215\text{--}235 \mu\text{m}$ . The density of spots in the array is  $\sim 625 \text{ cm}^{-2}$ .

obtained after contact with a stamp; we inked this stamp with a solution of rhodamine-labeled BSA (TRITC-BSA). Figure 2B shows a pattern prepared with a stamp that we inked with a solution of fluorescein-labeled collagen (FITC-collagen). These micrographs demonstrate that stamping with patterned agarose gels can (i) form high-density arrays of protein microspots with diameters as small as  $50 \mu\text{m}$  and (ii) produce high contrast in fluorescence between the spot and the background. Although we show only arrays with circular spots of proteins in this work, hydrogel stamps, in principle, make it possible to stamp noncircular two-dimensional shapes (e.g., triangles from a stamp as the one shown in Fig. 1E). Triangular and square-shaped spots have been used to manipulate the shape of mammalian cells in culture and to test the effect of shape-restrictions on the architecture of the cytoskeleton. Standard spotting techniques are limited to the fabrication of circular spots [45]. Figure 2 also illustrates the homogeneity of the fluorescence intensity within single spots, and from spot to spot. We quantified the homogeneity by evaluating the mean value and the standard deviation of the fluorescence intensity of all pixels in a fluorescent spot. Analysis of all spots ( $N = 40$ ) in Figs. 2A and B showed the average standard deviation of the fluorescence intensity of the pixels within any individual spot was  $<9\%$ . The standard deviation of the mean fluorescence intensity from spot to spot was  $<6\%$ . We tested if the stamping procedure would also work after inking the stamps with solutions of TRITC-BSA prepared in 40% glycerol and 0.1% Tween 20. Under these conditions the average standard deviation of the fluorescence intensity

of the pixels within individual spots was  $<12\%$ . The standard deviation of the mean fluorescence intensity from spot to spot was  $<10\%$  (we used bare, amine-coated, and aldehyde-coated glass slides for these experiments). This good homogeneity [46] might be due to diffusion of proteins from within the posts of agarose to the surface while the stamp was in contact with the surface.

We investigated the effect of the concentration of the protein in the posts and the effect of the time of contact between the stamp and the substrate on the amount of protein that transferred. We inked the posts of a stamp with different concentrations of TRITC-BSA, and varied the time of contact of the stamp with a glass substrate (carrying amine groups). We compared the fluorescence intensities  $I$  (unitless) of the spots with the intensity  $I_{\text{max}}$  of a spot that we obtained by leaving a droplet of  $10 \text{ mg} \cdot \text{mL}^{-1}$  of TRITC-BSA on the same substrate overnight (we set the intensity of this spot to 100%). Inking the stamp with concentrations  $\geq 15 \mu\text{M}$  ( $1 \text{ mg} \cdot \text{mL}^{-1}$ ) TRITC-BSA, and using times of contact  $\geq 10 \text{ s}$ , resulted in 93% of the maximum fluorescence intensity. In order to reach  $\sim 90\%$  intensity with an inking solution of  $1.5 \mu\text{M}$  TRITC-BSA, we increased the time of contact to  $\geq 2 \text{ min}$ . The increase of the fluorescence intensity  $I$  of the spots that we obtained with posts that were inked with  $0.1 \text{ mg} \cdot \text{mL}^{-1}$  TRITC-BSA over time  $t$  (s) could be fitted well ( $r^2 = 0.97$ ,  $N = 8$ ) with a hyperbola:  $I/I_{\text{max}} = t/(45.8 \text{ s} + t)$ . The lowest concentration of inking solution that we tried was  $151 \text{ nM}$  TRITC-BSA; in this case, we reached 74% fluorescence intensity after 30 min of contact between the stamp and the substrate. Interestingly, the homogeneity of the fluorescence intensity of the pixels within the spots was independent of the time of contact between the stamp and the substrate. We obtained good homogeneity [46] for all times of contact that we tested (5 s to 30 min) when we used TRITC-BSA concentrations  $\geq 1.5 \mu\text{M}$ .

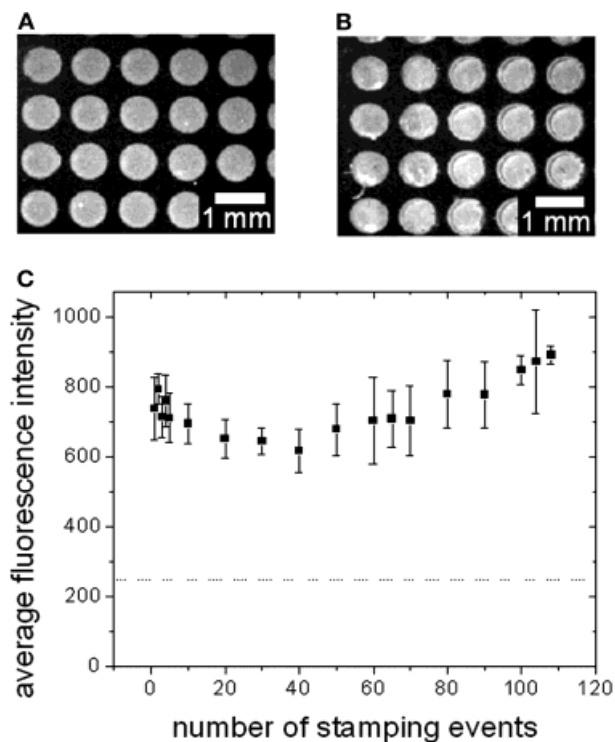
Figure 2 also demonstrates that the size of the protein spots depended on the diameter of the posts of the agarose stamp and on the wetting of the substrate by the aqueous solutions of proteins stored in the posts. Spots of proteins stamped from posts with a diameter of  $50 \mu\text{m}$  onto a PDMS substrate were  $\sim 50 \mu\text{m}$  in diameter because the hydrophobic surface of PDMS limited spreading of the protein solution during stamping. When we used the same stamp on clean glass slides, the resulting spots were  $\sim 70 \mu\text{m}$  in diameter because the protein solution stored in the gel posts spread to the area in the vicinity of the posts. Stamping with  $200 \mu\text{m}$  posts onto glass slides resulted in spots with diameters  $\leq 235 \mu\text{m}$  (see Fig. 2B). Clean, unmodified glass slides caused the most pronounced spreading of the solutions of proteins during stamping. Glass substrates carrying (i) amino, (ii) aldehyde, or (iii) covalently immobilized neutravidin mole-

cles resulted in spot sizes that were similar to the diameter of the posts. On these substrates, spots from posts with 200  $\mu\text{m}$  diameter were  $\leq 210 \mu\text{m}$ , spots from posts with 700  $\mu\text{m}$  were  $\leq 744 \mu\text{m}$ , and spots from posts with 1 mm diameter were  $\leq 1.03 \text{ mm}$ .

### 3.3 Repetitive stamping of proteins without intermediate inking

The capability of hydrogels to store proteins in solution in the network of the gel makes it possible to use agarose stamps for repetitive stamping without the need for intermediate re-inking. We inked an agarose stamp by immersing the posts (diameter 700  $\mu\text{m}$ ) for 5 h in a solution of  $10 \text{ mg} \cdot \text{mL}^{-1}$  TRITC-BSA and performed 108 successive stamping events. We also prepared twenty arrays with a stamp that was inked with eight different proteins (see section on stamping arrays of different proteins). Figures 3A and B show the spots of TRITC-BSA obtained

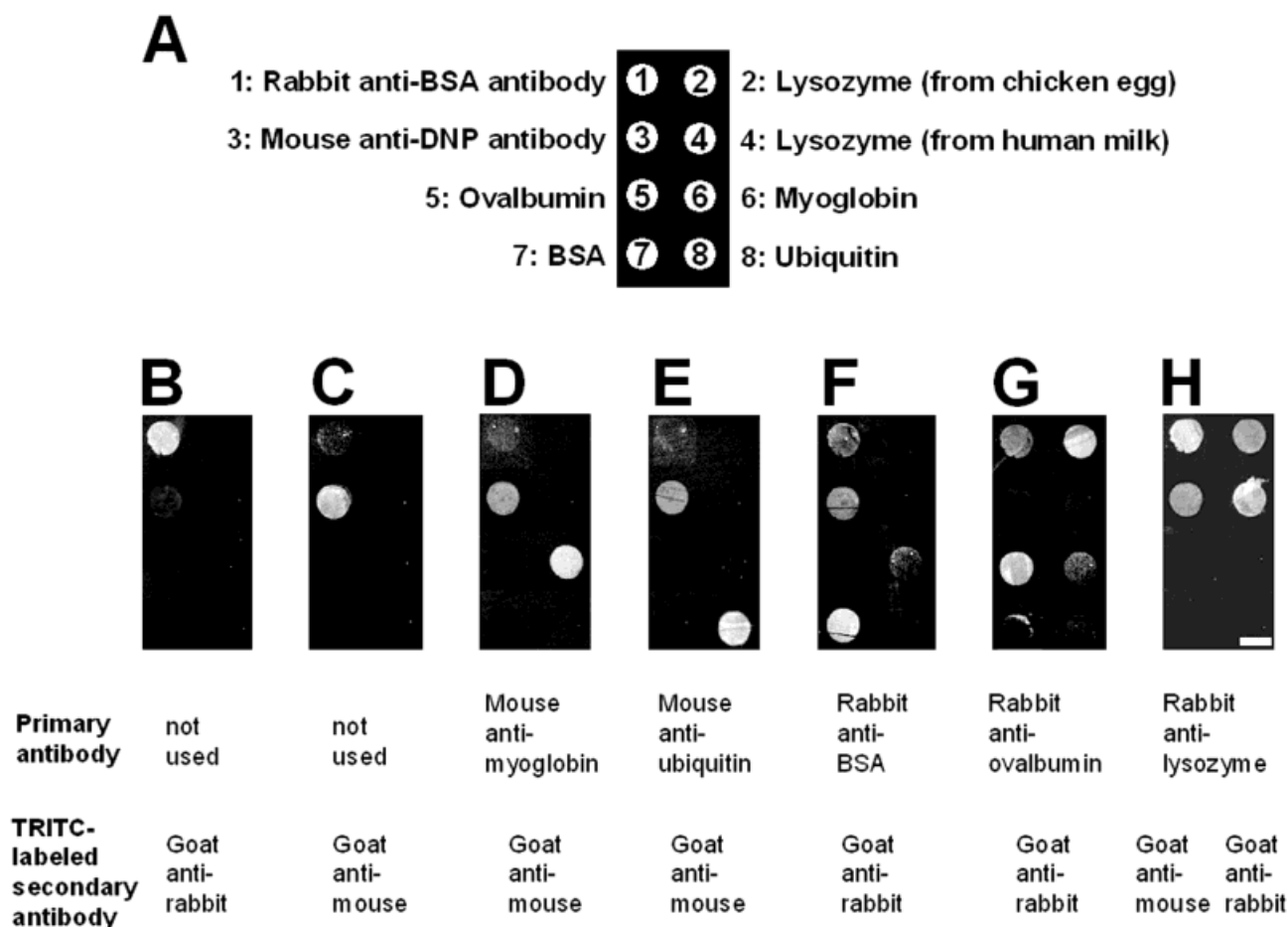
after the first and after the 100<sup>th</sup> stamping event (we kept microscope and camera settings constant during this experiment). Figure 3C is a plot of the average fluorescence intensity of the TRITC-BSA spots as a function of the number of stamping events. Overall the fluorescence intensity of the spots varied by  $< \pm 17\%$  during 108 stamping events. A comparison of Figs. 3A and B shows that the homogeneity of the fluorescence of the spots decreased with increasing number of stamping events. Also, after 100 stamping events, the edges of the spots were not defined as well as after the first stamping event. We attribute this loss in homogeneity to a transfer of small amounts of agarose from the posts of the agarose stamp to the surface (employing cross-linked hydrogels for stamping reduces unwanted material transfer [30]). Once inked, agarose stamps are therefore useful for rapid replication of more than 100 protein arrays.



**Figure 3.** Multiple stamping of a single protein (TRITC-BSA) onto glass slides. (A) Patterns of TRITC-BSA obtained after the first and (B) after the 100<sup>th</sup> stamping event. The stamp was inked with  $10 \text{ mg} \cdot \text{mL}^{-1}$  TRITC-BSA and contacted with each glass substrate for  $\sim 5 \text{ s}$ . (C) Graph illustrating the change of the mean fluorescence intensity of the spots with increasing number of stamping events (without intermediate re-inking). The error bars reflect the standard deviation of the fluorescence intensity of the pixels within the spots. The dotted line represents the fluorescence intensity of the background.

### 3.4 Stamping arrays of different proteins

Standard procedures to form microarrays of proteins typically employ serial processes to deliver solutions of different proteins to a slide [15]. For screening applications, it is advantageous to obtain replicas of the same array of proteins in a parallel and repetitive fashion. Here, we explore the feasibility of delivering solutions of different proteins, not directly to a slide, but to a stamp, of storing the proteins in the posts of the stamp, and then using the stamp for patterning replicas of the same array without the need for intermediate re-inking. We inked the posts of a stamp (fabricated from a PDMS replica of a commercially available master with 1536 wells) by pipetting  $\sim 0.6 \mu\text{L}$  of different protein solutions manually onto individual posts. After the posts absorbed the deposited solution, we used the stamp to transfer the same protein array twenty times without intermediate re-inking. The density and homogeneity of the proteins in the spots did not vary significantly over 20 stamping events. Although we only performed twenty stamping events, it might be possible to obtain more than 20 copies without the need for intermediate re-inking. Figure 4 shows fluorescence micrographs of seven amine-modified glass slides [47] each carrying an array of eight proteins that we used for a surface-based immunoassay (we describe the procedure for stamping and immunostaining in detail in the experimental section). The identity and position of each of these eight proteins is illustrated in Fig. 4A. We obtained the images in Figs. 4B–H after incubating each slide with a different primary antibody solution followed by a solution of TRITC-labeled secondary antibody (the two lines below Figs. 4B–H specify the primary and secondary antibody we used to label the spots fluorescently).



**Figure 4.** Arrays of eight different proteins produced by stamping with the same agarose stamp and use of the arrays for surface-based immunoassays. (A) Schematic representation of the array of proteins. (B)–(H) Fluorescent micrographs of glass slides carrying the array depicted in (A) upon incubation of the slides first with a primary antibody (listed below each micrograph) and then with a rhodamine-labeled secondary antibody (listed below the primary antibody). Scale bar: 1 mm.

Figure 4 shows that we identified the expected fluorescent spots of protein with high selectivity and good contrast from the background. Two spots showed significant nonspecific immunolabeling (out of a total of 56 spots from 7 slides with 8 spots). The spot composed of mouse anti-DNP antibody in Fig. 4F (spot 3) fluoresced after incubation with rabbit anti-BSA antibody followed by TRITC-labeled goat anti-rabbit antibody. Since we could barely detect the same spot when we incubated only with TRITC-labeled goat anti-rabbit antibody (spot 3 in Fig. 4B), we infer that the rabbit anti-BSA antibody bound either nonspecifically to the mouse anti-DNP antibody or to contaminants (*e.g.*, BSA) in the sample of the mouse anti-DNP antibody. The second spot that showed nonspecific binding was the spot composed of lysozyme from chicken egg when incubated with rabbit anti-ovalbumin followed by TRITC-labeled goat anti-rabbit antibody (Fig. 4G, spot 2). Since the lysozyme sample originated

from egg white, the sample may have contained ovalbumin as contaminant making binding of the anti-ovalbumin antibody plausible. In the same array the spot of lysozyme from human milk was not labeled (spot 4) supporting the hypothesis that lysozyme from egg white contained an impurity of ovalbumin. We obtained all micrographs in Fig. 4 with an objective with very low numerical aperture ( $NA = 0.05$ ). We expect that a microscope lens with higher numerical aperture would increase the local light intensity of the excitation wavelength and therefore increase the sensitivity of the assays.

Patterning arrays of proteins with agarose stamps requires only minute amounts of protein. To prepare the arrays shown in Fig. 4 we inked each post with a total of  $\sim 0.6 \mu\text{L}$  of protein solutions with a concentration of  $5 \text{ mg} \cdot \text{mL}^{-1}$ ; these numbers correspond to a total mass of  $\sim 3 \mu\text{g}$  of protein (equivalent to  $\sim 50$  picomoles in the

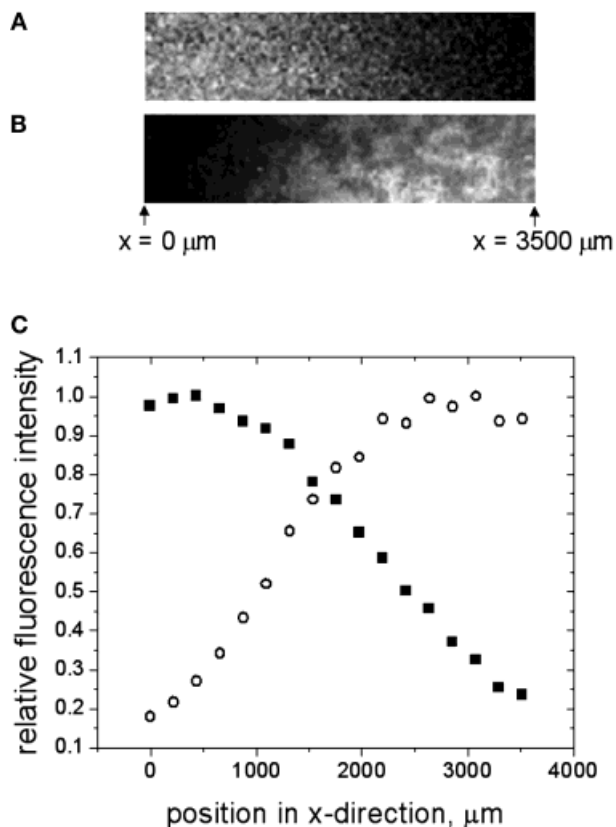
case of BSA). By pipetting the protein solutions onto the posts, practically no loss in protein (solution) occurs during the inking procedure. The gel absorbs excess solution within a few minutes (<5 min) and thereby circumvents spreading of excess solution during the stamping event. Stamping proteins with agarose stamps should therefore be particularly useful in preparing multiple (here, twenty) copies of arrays from a small library of proteins that are available in limited quantities.

Figure 4 shows arrays of proteins obtained by inking the posts of the agarose stamp manually. Due to the limited precision of manual pipetting, the density of the arrays was relatively low ( $\sim 25$  spots  $\text{cm}^{-2}$ ). We expect such low-density arrays to be useful for quick screening assays in research laboratories (one advantage of the spots with 1 mm diameter is that they were made from, and were thus compatible with, standard 1536-well plate formats and plate readers). Industrial high-throughput screening applications may require arrays with higher density. We expect inking 200  $\mu\text{m}$  spots on patterned agarose gels with a robotic spotting technique [2], followed by repetitive stamping, to be a strategy for the preparation of higher-density arrays of proteins.

Amine-modified slides worked well for stamping of the proteins shown in Fig. 4. For some applications, however, a more gentle interaction of the stamped molecules with the substrate is desirable [1]. To explore another strategy of immobilization that minimizes possible denaturation of fragile biomolecules, we stamped biotinylated proteins (in this case antibodies) onto neutravidin-coated glass slides (data not shown) [48]. Using 15 min contact time for each stamping event followed by incubating the arrays with secondary antibodies resulted in fluorescent spots that were specific for the given pair of biotinylated and TRITC-labeled antibody (e.g., we detected a spot of biotinylated goat anti-rabbit antibody when we incubated the array with TRITC-labeled rabbit anti-goat antibody but we did not find fluorescent spots when we used a TRITC-labeled goat anti-mouse or goat anti-rabbit). These results demonstrate that stamping proteins with agarose gels is amenable to gentle, biospecific immobilization protocols. In addition to biospecific immobilization we explored stamping of proteins onto aldehyde-coated glass slides that can form covalent bonds with amino-groups of amino acid side chains in proteins [2] (see Fig. 5).

### 3.5 Diffusion of proteins within the agarose stamp

In order to avoid cross-contamination of different proteins stored in neighboring posts of the stamp, it is important to complete the inking and stamping process before pro-



**Figure 5.** Overlapping gradients of two fluorescently labeled proteins on an aldehyde-functionalized glass slide. We obtained these gradients by diffusion of FITC-BSA and TRITC-BSA in a flat agarose stamp followed by stamping. (A) Area of  $3580 \mu\text{m} \times 800 \mu\text{m}$  observed with filter settings for fluorescein. The image shows a gradient with a high surface concentration of FITC-BSA on the left and a low surface concentration of FITC-BSA on the right side of the image. (B) The same area as in (A) captured with filter settings for rhodamine. The image shows a gradient with a high concentration of TRITC-BSA at the right side of the image and a low concentration of TRITC-BSA at the left side. (C) Relative fluorescence intensities of FITC-BSA (solid squares) and TRITC-BSA (open circles) as a function of the position in the stamped area. We evaluated the fluorescence intensities by dividing the area shown in (A) and (B) into 17 rectangles (with dimensions of  $210.6 \mu\text{m} \times 800 \mu\text{m}$ ) and computing the average intensity of all pixels in each rectangle. The relative fluorescence intensity shown in the graph reflects the average fluorescence intensity of the pixels in each of these 17 rectangles normalized to the intensity of the rectangle with the highest average intensity.

teins diffuse from the surface of one post to the surface of a neighboring post. The time  $t$  (s) required for a protein to diffuse to a neighboring post depends on the diffusion coefficient  $D$  ( $\text{cm}^2 \text{s}^{-1}$ ) and the total distance  $x$  (cm) that the protein has to cover from the surface of the post,



**Table 1.** Time for diffusion of TRITC-BSA from the surface of a post through an agarose stamp to the surface of the neighboring post as a function of the dimensions of the stamp

Dimensions of posts ( $\mu\text{m}$ )		Distance between posts, $x_1$ ( $\mu\text{m}$ )	Shortest distance, $x$ , from surface of post to neighboring post ( $\mu\text{m}$ )	Density of posts ( $\text{cm}^{-2}$ )	Time for diffusion, $t$ (min)	
Diameter	Height, $x_p$				theoretically	experimentally
50	25	150	200	2500	6	n. d.
200	130	200	460	625	30	$25 \pm 5$
700	700	300	1700	100	415	$480 \pm 90$
1000	1500	1000	4000	25	2300	$2400 \pm 240$

n.d. not determined

We used a diffusion coefficient of  $D_g = 2.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  as an estimate for TRITC-BSA in 3% agarose to calculate the theoretical time for diffusion.

through the stamp, to the surface of the neighboring post. The time for diffusion can be estimated using Eq. (1) [49], where  $n$  specifies whether the diffusion occurs in one dimension ( $n = 1$ ), two dimensions ( $n = 2$ ), or three dimensions ( $n = 3$ ).

$$t = \frac{x^2}{2nD} \quad (1)$$

In Eq. (1),  $x$  represents the root mean square displacement of a particle. Here, we approximated the minimum distance that a protein spreads in a given time interval with the root mean square displacement [49]. The diffusion coefficient of BSA in free solution  $D_0$  is  $6.4 \pm 0.4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ , in 2% agarose gel the diffusion coefficient  $D_g$  is  $2.9 \times 10^{-7}$  to  $4.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  [50]. The minimum estimate of the total distance of diffusion  $x$  is the sum of the distance along the height of both posts,  $2 \times x_p$ , and the lateral distance between two neighboring posts  $x_1$ ;  $x = 2 \times x_p + x_1$ . We estimated the time  $t$  it takes for a protein on one post to reach the surface of a neighboring post by approximating the diffusional spreading with a two-dimensional random walk ( $n \cong 2$ ). Table 1 compares the estimated times for diffusion with experimentally determined values. We determined the times for diffusion by inking a limited number of posts in an agarose stamp with a solution of  $10 \text{ mg} \cdot \text{mL}^{-1}$  TRITC-BSA and leaving neighboring posts blank. Monitoring the onset of fluorescence in originally blank spots upon repetitive stamping made it possible to determine the time for diffusion of proteins from post to post. We estimate that agarose gels with  $200 \mu\text{m}$  high posts and a distance of  $200 \mu\text{m}$  between the posts will prevent cross-contamination of proteins from post to post over an interval of 50 min. We estimated the time until cross-contamination occurs by using the diffusion coefficient  $D_g$  of BSA. For larger proteins this time will increase proportionally to the diffusion coefficient, for smaller proteins it will decrease (Eq. 1). Insulin,

as an example for a very small protein, has a diffusion coefficient of  $D_0 = 11 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  in solution [51]. This value is about twice the value  $D_0$  of BSA in solution, therefore, cross-contamination with insulin will occur in approximately half the time that it takes with BSA (Table 1). If the inking procedure takes 20 min [52], and each stamping event a total of 20 s (10 s time of contact and 10 s to move to the next slide), an agarose stamp with  $200 \mu\text{m}$  posts ( $x_p = 200 \mu\text{m}$ ,  $x_e = 200 \mu\text{m}$ ), could pattern up to 90 slides with  $>600$  spots  $\text{cm}^{-2}$  before significant cross-contamination occurs. Table 1 illustrates that stamps made from agarose are not useful for patterning different proteins in densities higher than  $600$  spots  $\text{cm}^{-2}$  (diameter of posts  $200 \mu\text{m}$  and  $x_1 \leq 200 \mu\text{m}$ ). In order to increase the density of the posts above  $600$  spots  $\text{cm}^{-2}$  without the disadvantage of rapid cross-contamination, the diffusion coefficient of the proteins in the gel would have to be decreased. Chemically cross-linked hydrogels may offer this opportunity [30, 43, 44, 53, 54].

### 3.6 Agarose gels for stamping gradients of proteins

We made use of diffusion of proteins in hydrogel stamps to transfer gradients of proteins from a stamp to a surface. Figure 5 shows an example of overlapping, oppositely directed, gradients of FITC-BSA and TRITC-BSA on a glass surface patterned with an agarose stamp. We used a straightforward method to make these gradients (explained in detail in the experimental section) that relied on time-dependent diffusion (Eq. 1,  $n \cong 2$ ) of proteins in a flat stamp. To ink the stamp, we sealed a strip of PDMS (width 1 mm, length  $\sim 1$  cm, height  $\sim 0.7$  mm) conformally to the surface of the flat agarose gel and deposited a different protein solution on each side of the strip. After the protein solutions entered the gel, we allowed them to dif-

fuse for ~4 h and stamped the resulting gradient of proteins onto glass slides. By allowing the proteins to diffuse for a defined period of time (in combination with a well-defined initial separation using the strip of PDMS), we prepared multiple copies of overlapping protein gradients reliably and rapidly. We illustrate the profile of the gradients of FITC-BSA and TRITC-BSA over the same area of the substrate in Fig. 5C. As expected for diffusion of two proteins with similar molecular weight and properties, the concentration profiles of the gradients were bell-shaped and approximately 'mirror images' of each other.

Surfaces carrying gradients of proteins are useful for cell biology: e.g., cells can sense and move along such gradients [39, 55], and axons of nerve cells grow preferentially towards an increasing concentration of laminin on a surface [41]. In this work, we show that diffusion of proteins in agarose stamps provides a simple strategy for preparation of surfaces with gradients of proteins.

#### 4 Concluding remarks

Micropatterned agarose stamps can generate patterns of protein microspots with high contrast between the fluorescence of the spots and the background. The capability of the gel to absorb and store solutions of proteins makes it possible to ink individual posts with different proteins and to use such a stamp for at least twenty stamping events. Using a microspotter to ink posts with a diameter and a height of 200  $\mu\text{m}$  on an agarose stamp may allow parallel and repetitive formation of high-density arrays (up to 600 spots  $\text{cm}^{-2}$ ) containing a multitude of different proteins (and, perhaps, other biomolecules). Once a high-density stamp is inked, protein spots could be transferred quickly, reproducibly, and with good homogeneity. The resulting arrays of proteins may be useful for drug-discovery, diagnostics, and proteomics.

Flat agarose stamps provide a simple method for stamping gradients of proteins on a surface. By setting (i) the distance between two (or, presumably several) solutions of proteins in a stamp and (ii) the time for diffusional spreading, it is possible to prepare gradients of proteins with a defined profile in concentration on flat substrates. Such gradients of proteins may be useful for assays of cellular spreading and chemotaxis [41]. Stamping with hydrogels provides a straightforward, low-tech method for patterning biomolecules in low-density from aqueous solutions. Hydrogels provide a biocompatible environment, and the stamping process does not require drying of proteins; both properties minimize denaturation of proteins [1]. The technique should be particularly useful for screening of binding interactions (e.g., surface-based immunoassays or cross-reactivity studies) in academic

laboratories where the number and amount of available proteins is often limited, and where flexibility in the preparation of protein arrays is more important than high density for high throughput.

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