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3D Protein Microarrays: Performing Multiplex Immunoassays on a Single Chip

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The enzyme-linked immunosorbent assay (ELISA) is typically applied in the format of microtiter plates. To increase throughput and reduce consumption of precious samples, efforts have been made to transfer ELISA to the microchip format using conventional microarrays, microfluidic systems, and chips bearing microwells. However, all three formats lack the possibility to screen several analytes on several immobilized binders at a time or require complicated liquid handling, surface modifications, and additional equipment. Here, we describe an immunoassay performed on a standard microscope slide without the requirement for wells or tubes to separate the samples using standard surfaces and machinery already available for microarray technology. The new multiple spotting technique (MIST) comprises immobilization of a binder onto a surface and subsequent spotting of the second compound on the same spot, on top of the immobilized binder. We show that the analytes bind their ligands immediately within the confined space of separate droplets on the chip surface, thereby eliminating the need for extra incubation time. We illustrate the feasibility of the new technique by spotting dilution rows of proteins or monoclonal and polyclonal antibodies on top of their immobilized binders. Moreover, we demonstrate specificity by applying a mixture of antibodies in a multiplex format and demonstrate that the technique is compatible with conventional microarray protocols, such as total incubation. Finally, we indicate that the technique is capable of quantifying as little as 400 zmol (240 000 molecules) of analyte.

ELISA was originally developed in the format of microtiter plates.[1] The wells can be individually addressed to immobilize the components to be analyzed in the assay, thereby introducing the flexibility to screen ligands against each other in all combinations. This is well established in the 96 well format, but many efforts have been made to further increase the amount of different samples to be analyzed simultaneously and to reduce the consumption of precious samples.[2]

To further minimize the sample amount and space for high-throughput screening, microarrays in the format of microscopic glass slides have been developed.[3,4] The surfaces of such slides have been modified in various ways to optimize immobilization.[5,6] Several thousand different samples can be accommodated on a single microchip surface. However, the major drawback of this format is that the entire chip can be exposed to only one analyte at a time, and a simultaneous analysis of different analytes on one chip is not possible. First attempts to overcome this problem have been undertaken with FASTPAK Multiple Array Slides (Schleicher & Schuell Biosciences Inc., Keene, NH), but the technology is still limited to screening 16 different analytes simultaneously.

To be able to address all sample components freely, microfluidic chips and chips bearing microwells have been developed. Microfluidic chips consist of a network of storage and reaction chambers, which are connected by tubing. Those chambers can be produced by either chemical means, such as etching,[7] or boring of the surface.[8] By control of the flow inside the system, multicomponent reactions can be performed. Microwell chips consist of slides in which wells have been introduced.[9] In contrast to microfluidic chips, the wells are not connected, and the samples are applied as in microtiter wells.

To overcome complicated liquid handling and arduous surface modifications, the ability to address the same spot on the chip surface reproducibly could not just be used to immobilize the first binding partner on the surface, but also allows multiplex analysis by the addition of several analytes to the immobilized binder. Since the volumes are small, each spot can form a separate droplet on the chip surface, constituting high-density microarrays suitable for high-throughput studies. In addition to a two-dimensional array

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in conventional microarray technology, a third dimension is added by the possibility of adding different samples to each individual spot.

EXPERIMENTAL SECTION

Materials. Monoclonal (Clone 1C8) anti-HSA antibody was purchased from DPC Biermann Diagnostika GmbH (Bad Nauheim, Germany) and polyclonal anti-fibrinogen antibody was obtained from Calbiochem-Novabiochem Corp., San Diego, CA. Mouse monoclonal anti-human serum albumin (HSA) antibodies have been previously evaluated for functionality and cross-reactivity in ELISA and on microarrays.[10] HSA and fibrinogen were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Cy3-labeled anti-mouse IgG, Cy5-labeled anti-mouse IgG, and Cy5-labeled anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). HSA was labeled with Cy5 dye (Amersham Biosciences, Freiburg, Germany), as recommended by the manufacturer. Unconjugated dye was removed using Ultrafree-0.5 centrifugal filters (10kD cutoff, Millipore, Bedford, MA).

Methods. *(1) Pin Coefficient Microarrays.* To investigate the homogeneity of the spotting pins, a dilution of 1:500 of Cy3-labeled anti-mouse antibodies was spotted onto poly(L-lysine) slides and incubated for 4 h at 4 °C. Poly(L-lysine) slides were prepared as described.[3] Spotting was performed in sets of 16 spots per pin with a spot-to-spot distance of 1.1 mm using a QArray spotting robot (Genetix, Hampshire, U.K.). After rinsing with TBS, the slides were washed in TBST for 30 min and spun dry by centrifugation before scanning and analysis. Scanning was performed using a ScanArray 4000 scanner (Perkin-Elmer Wellesley, MA). GenePix Pro 4.1 software was used to analyze the scan data.

(2) Dilution Row Microarrays. Dilutions of the species being immobilized were prepared using 1× PBS/0.1% (w/v) NaN_3. Dilution rows of the analyte used in the second spotting step were prepared in 1× PBS/0.1% NaN_3 (w/v) applying final concentrations of 0.4 mg/mL BSA and 8.7% (v/v) glycerol. For dilution row microarrays, shown in Figure 3, monoclonal anti-HSA antibodies, HSA, and fibrinogen were dissolved in PBS to a concentration of 100 μg/mL and spotted. After spotting, the slides were placed in a box at 4 °C for 90 min before being rinsed with TBS and blocked in 3% (w/v) fatfree milk powder/TBS-T for 90 min. Slides were then rinsed with TBS and spun dry by centrifugation. The analyte was spotted onto the dry slides at exactly the same position as the immobilized binding partner. Then all slides were stored for 90 min in a humidified chamber at 4 °C and rinsed with TBS. Protein microarrays were incubated for 30 min at 4 °C in either 1.6 μg/mL Cy3-labeled anti-mouse IgG (Figure 3C) or 1 μg/mL Cy5-labeled anti-rabbit IgG (Figure 3D). After rinsing with TBS, the slides were washed in TBST for 30 min and spun dry by centrifugation, scanned, and analyzed.

(3) Multiplex Microarray. The multiplex microarray was prepared as described for the dilution row microarray with the following exceptions: HSA concentrations of 90, 181, 451 and 903 amol/spot dissolved in PBS were spotted. Following spotting, the slides were placed in a box at 4 °C for 45 min. After blocking, equimolar concentrations (10, 21, 42, and 83 amol/spot) of polyclonal anti-fibrinogen antibodies and monoclonal anti-HSA antibodies were spotted onto the slides. Slides were rinsed immediately with TBS and then washed twice in TBST for 15 min each.

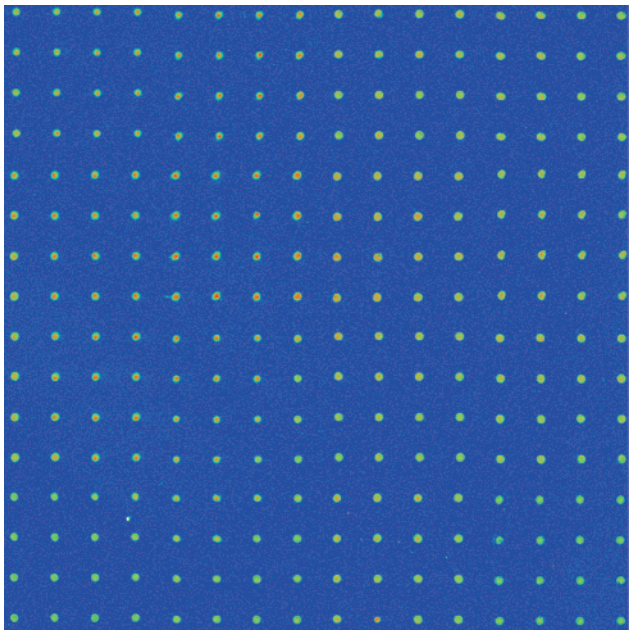

Figure 1. Pin coefficient microarray. Scan of one microarray used for the determination of the pin coefficients for the 150-μm solid flat pins. Sixteen spots were spotted by each pin, and the mean for each pin was calculated and compared.

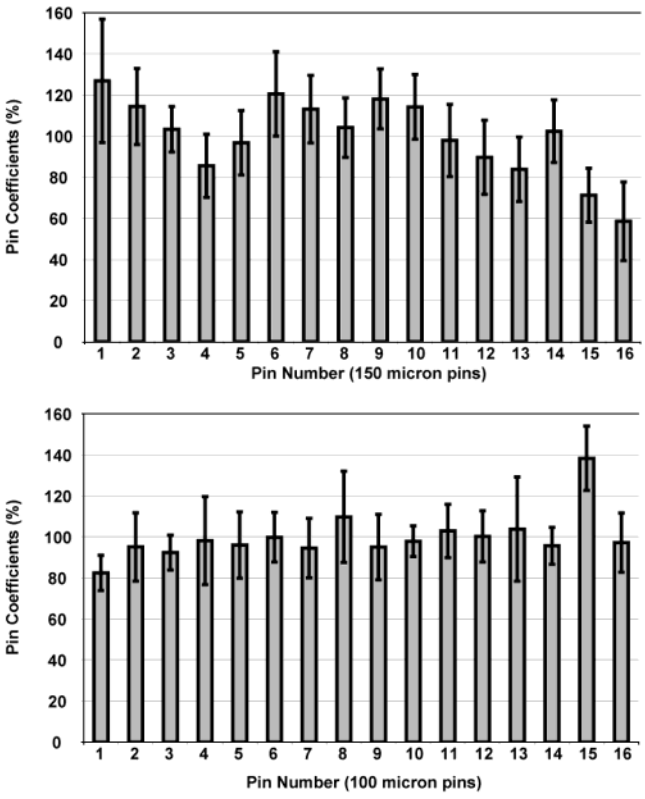

Figure 2. Diagrams of pin coefficients. Diagrams of the relative pin coefficients for robot head 1 (150-μm solid-tip pins) and robot head 2 (100-μm solid-tip pins) with standard deviation error bars. 100% represents the mean signal intensity of all measured signals.

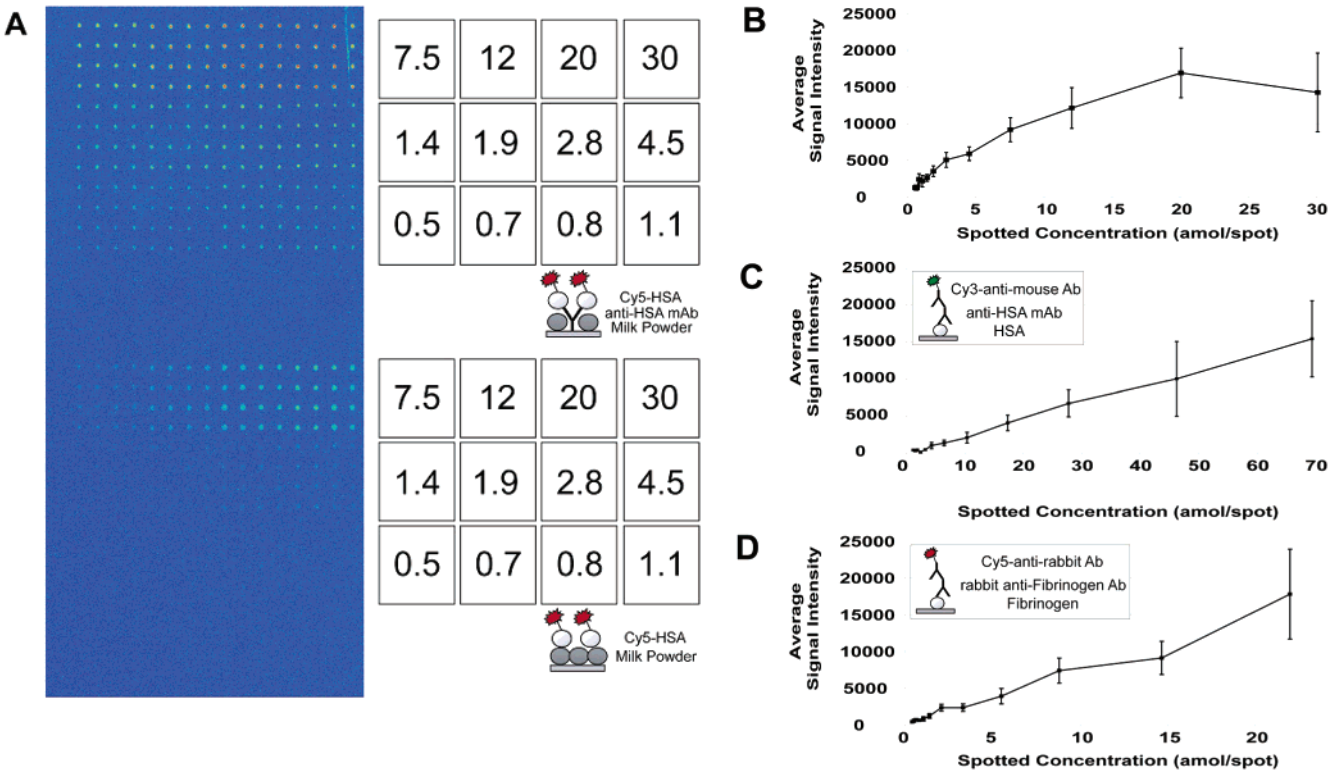

Figure 3. Dilution row chips. (A) Left: scan of an antibody chip (excitation at 649 nm, emission at 670 nm). Right: scheme of the second spotting with Cy5-labeled HSA and illustration of the molecular assembly on the chip. Numbers in squares indicate spotted amount in attomoles per spot. All concentrations were spotted in 4 × 4-spot squares. On the upper half of the chip, specific interaction between immobilized anti-HSA Ab and Cy5-labeled HSA has occurred, whereas on the lower half, an unspecific concentration-dependent interaction between milk powder (blocking reagent) and Cy5-labeled HSA is seen. (B) Average signal intensity versus spotted concentration for the microarray shown in A, with standard deviation error bars. (C−D) Average signal intensity versus spotted concentration for protein microarray with monoclonal and polyclonal antibodies.

RESULTS AND DISCUSSION

To assess the quality of printing, a uniform dilution of 2 μg/mL of Cy3-labeled anti-rabbit IgG was spotted multiple times using two different spotting heads with 16 pins each. The robot heads have solid-tip pins with 150- and 100-μm diameters, respectively. The arithmetic mean of all signal intensities produced by each pin was calculated, and the average signal intensity of all 16 mean values was defined as 100%. For both heads, a graph indicating the relative deviations for each pin from this mean was obtained (Figures 1, 2). Deviations in signal intensities of up to 41% were observed. In all further experiments the 150-μm pins were used for spotting, and the obtained signal intensities were corrected by the corresponding pin coefficient to reduce the effects of external factors on the results.

Different assays were performed applying the MIST alone (Figure 3A,B) and in combination with a standard total incubation (Figure 3C,D). For all assays, a negative control was performed on the same chip, in which 3% (w/v) fatfree milk powder was immobilized instead of a binder. The signal intensities of the interactions were calculated by

$$\frac{\text{(average signal intensity of analyte} - \text{average signal intensity of negative control)}}{\text{pin coefficients}}$$

Diagrams of the calculated signal intensities versus spotted concentration were drawn (Figure 3B−D), and the obtained coefficients were used to normalize the obtained data. Subtraction of the negative control signal intensities was necessary, since high concentrations (above 10 amol/spot) result in concentration-dependent unspecific binding (Figure 3A). Figure 3B displays the corresponding diagram, which shows a saturation of signal intensity with increasing antigen concentration on the antibody array. Quantification of 1.1 amol of Cy5-labeled HSA with immobilized anti-HSA antibodies was achieved.

Figure 3C,D shows characteristic linear signal-to-concentration ratios on protein microarrays between immobilized antigens and monoclonal or polyclonal antibodies, respectively. On these protein microarrays, as little as 2.4 amol monoclonal anti-HSA and 400 zmol of polyclonal anti-fibrinogen antibody could be detected using labeled secondary antibodies in a total incubation.

Applying the multiple spotting technique, a multiplex screening approach was conducted on a protein microarray using an equimolar mixture of two different antibodies simultaneously (Figure 4). Detection of the spotted primary antibodies was performed by a total chip incubation with differently labeled secondary antibodies simultaneously. Scanning the chip at respective wavelengths (Figure 4A,B) revealed specific concentration-dependent binding of the monoclonal anti-HSA antibody on immobilized HSA, while no unspecific binding of polyclonal anti-fibrinogen antibody was observed. The obtained signal intensities match the theoretical expectations and display linear relationships between binder and analyte and, hence, allow antibody quantification.

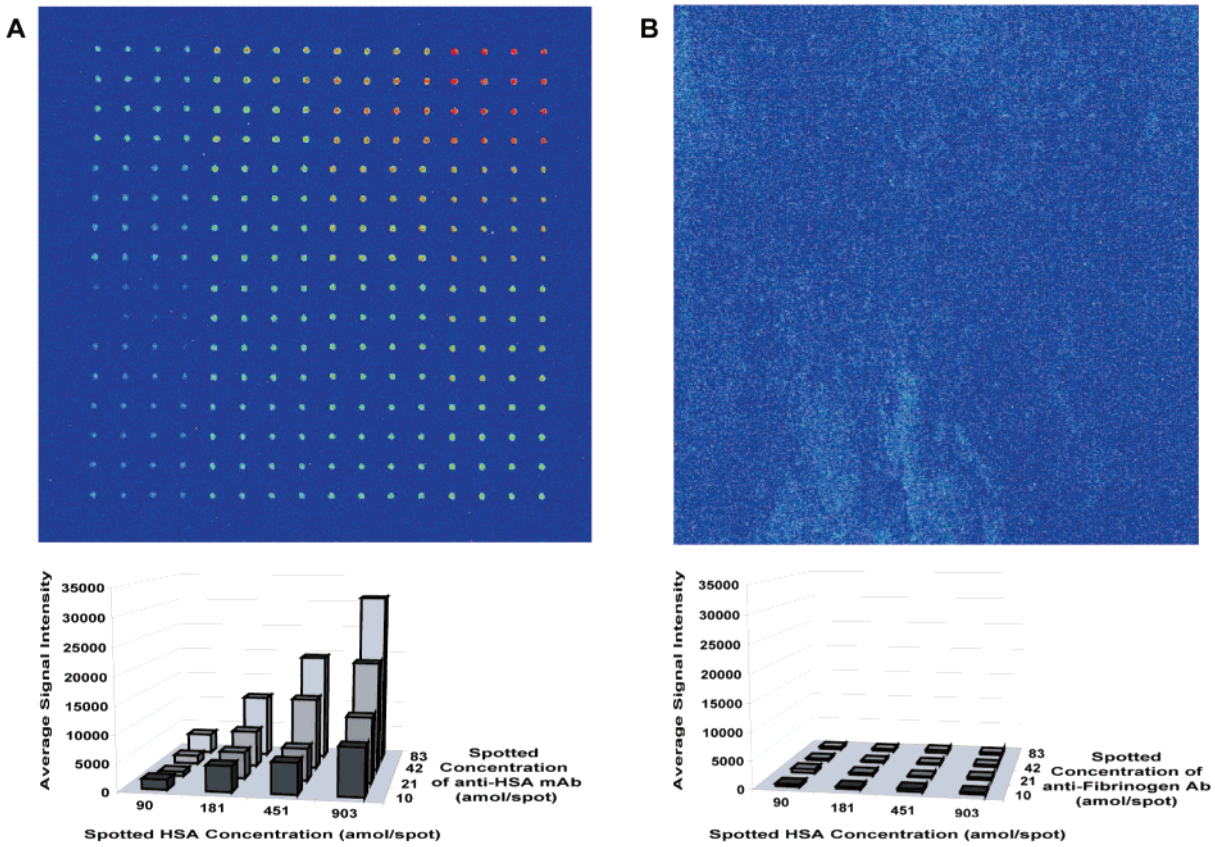

Figure 4. Multiplex chip. Scan and quantification of spotted monoclonal anti-HSA and polyclonal anti-fibrinogen antibodies in equimolar ratio on a protein chip. (A) Scanning at the respective wavelength for bound anti-HSA antibodies (Cy5-labeled anti-mouse antibody; excitation at 649 nm, emission at 670 nm). (B) Scanning at the respective wavelength for bound anti-fibrinogen antibodies (Cy3-labeled anti-rabbit antibody; excitation at 550 nm, emission at 570 nm). HSA and antibody concentrations are indicated on the *y*- and *x* axes of the diagrams, respectively, and the signal intensities obtained are shown on the *z*-axis. Signal intensities are illustrated in the same spatial arrangement as on the chip.

The results clearly demonstrate the feasibility of this technique for high-throughput multiplex screening on a single chip, allowing for direct comparison of different analytes without deviation of the results by interchip variations. This enables more accurate comparisons, since those variations can range between 12 and 60%, depending on the coating of the microarray.[10] In addition to the accuracy of the measurements, this investigation revealed high sensitivity and demonstrates that specific recognition of proteins by their antibodies is possible, using the multiple spotting technique. In addition, MIST consumes extremely small amounts of sample, since the volumes of spotted solution are, depending on the pins, only 0.19 nL or 0.6 nL.[11] Moreover, the need for extra incubation times of the analyte is eliminated and, hence, allows a more rapid screening, as compared to conventional microarray technology. Multiple spotting technique might permit the introduction of new methodologies to microarray science, such as screening of compounds that lose their activity upon immobilization. This is possible by immobilization of more stable interacting partners and the subsequent application of the labile compound by MIST. We have tested this possibility by screening scFv's selected from large phage display libraries for specific binding on their respective targets (data not shown). Following selection and analysis, the binding of the scFv's was confirmed by ELISA. Since many scFv's lose their ability to bind their respective targets upon immobilization, a screening of scFv's with labeled selection target as analyte was not possible with common microarray techniques. Moreover, MIST holds the promise to solve the problem that not all antibodies remain functional after immobilization,[10] which is a major bottleneck of antibody microarray technology. Immobilization of the protein mixture followed by the administration of antibodies or scFv's by MIST could solve this problem in some applications. Furthermore, this technique could also be able to bypass the shortcomings of complex sandwich antibody microarrays. Since a multitude of secondary antibodies in a total incubation would supersede the specific signals, MIST would be able to apply the matching pair for each antigen directly without interfering with neighboring samples. This would circumvent the problem of labeling complex solutions with defined stoichiometric ratios and allow the application of antibody microarrays for the quantitative measurement of nonlabeled antigens. Additionally, combinatorial synthesis and functional screening of chemical compounds on the chip is potentially possible by multiple additions of several reagents on the same spot. An additional advantage is that MIST remains compatible with the standard techniques applied in microarray science in which the entire array is incubated and washed.

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CONCLUSION

In conclusion, we have introduced a new multiple spotting technique (MIST), which enables the performance of multiplex immunoassays on a flat surface without the requirement for wells or tubes to separate the samples. The technique relies on the formation of separate droplets on the chip surface without the need for onerous modifications, such as etching or casting. Additional benefits of this technique are the extremely small consumption of sample and the elimination of incubation times. We demonstrated that machinery already available for microarray technology can be used for this purpose and that the technique is capable of detecting zeptomoles of analyte with a linear signal to concentration relationships. Additionally, this technology can be combined with protocols already existing for conventional microarray applications and can enhance protein microarrays to live up to the foreseen expectations.[12−15] Limitations in terms of accuracy could be overcome by introducing pin coefficients to correct variations in sample transfer of different pins. In the initial experiments, spots of nonspherical shape have been found using the MIST and could by explained by imperfect overlap of sample transfer to the same coordinates. A more careful alignment of the microchips to the grid of the spotting robot could greatly avoid such artifacts. Though only proteins and antibodies were used exclusively in these experiments, we foresee no obstacles for utilizing other interacting elements, such as DNA, peptides, Fab's or drugs. With proper adjustment of the technique, microarrays should be capable of being fabricated from all these substances.

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