# Micromosaic Immunoassays

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# Micromosaic Immunoassays

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**Immunoassays are widely used for medical diagnostics and constitute the principal method of detecting pathogenic agents and thus of diagnosing many diseases. These assays, which are most often performed in well plates, would be greatly improved by a practical method to pattern a series of antigens on a flat surface and to localize their binding to many analytes. But no obvious method exists to expose a planar surface successively to a series of antigens and analytes. Here, we present miniaturized mosaic immunoassays based on patterning lines of antigens onto a surface by means of a microfluidic network (*μ*FN). Solutions to be analyzed are delivered by the channels of a second *μ*FN across the pattern of antigens. Specific binding of the target antibodies with their immobilized antigens on the surface results in a mosaic of binding events that can readily be visualized in one screening using fluorescence. It is thus possible to screen solutions for antibodies in a combinatorial fashion with great economy of reagents and at a high degree of miniaturization. Such mosaic-format immunoassays are compatible with the sensitivity and reliability required for immunodiagnostic methods.**

Having originated in the microelectronic industry, microfabrication is now being used in other fields as well. In biotechnology, for instance, it has been applied to microanalytical devices,[1−4] microreaction chambers,[5] microarrays,[6] combinatorial synthesis,[7] microelectromechanical systems (MEMS),[8] and micrototal chemical analysis systems (*μ*-TAS).[9] Genetic analysis in particular benefits from lithographically prepared DNA chips.[10−13] Unlike for DNA, a microarray-based technology has not yet been adopted for protein-based assays. Protein assays in general, and immunoassays in particular, are widespread and essential for the diagnosis of many diseases, but appear difficult to integrate into smaller, highly sensitive, practical formats. The most common format to perform a binding assay for diagnostic purposes is the enzyme-linked immunosorbent assay (ELISA), which is conducted in plastic microtiter plates. This technique involves coating the plastic wells with ligands and then blocking them with "neutral" proteins such as bovine serum albumin (BSA) to prevent nonspecific adsorption of proteins during subsequent steps. Coating the wells requires their incubation with ∼100 *μ*L of solution with the appropriate ligand for up to 1 h. The wells are subsequently rinsed and filled with the sample containing the putative target analyte. Binding between the ligand and the analyte, usually an antigen and an antibody, can be transduced when the latter is complexed in a final step with an enzyme-conjugated antibody. Alternatively, fluorescence-labeled antibodies can serve as the means to detect and quantify binding.

We propose in this article a new format for immunoassays. In practice, a microfluidic network (*μ*FN) patterns a series of antigens as narrow stripes onto a planar substrate, Figure 1A. After a blocking step with BSA, Figure 1B, the antigens in each line may be recognized by specific analytes from a sample solution also guided over the substrate with a second *μ*FN, Figure 1C. We call this assay a micromosaic immunoassay (*μ*MIA) because it places a series of ligands and analytes along micrometer-wide intersecting lines, thus providing a mosaic of signals from cross-reacted zones, Figure 1D. The resulting binding pattern can then be readily evaluated when analytes are tagged (two-step immunoassays) or can develop by binding a fluorescent- or enzyme-conjugated antibody to the analyte (sandwich-type immunoassay).

Microfluidic networks have high-resolution and high-contrast capabilities for simultaneously patterning lines of proteins onto a surface.[14,15] There are several techniques to displace fluids in microchannels, and a variety of materials can be structured to

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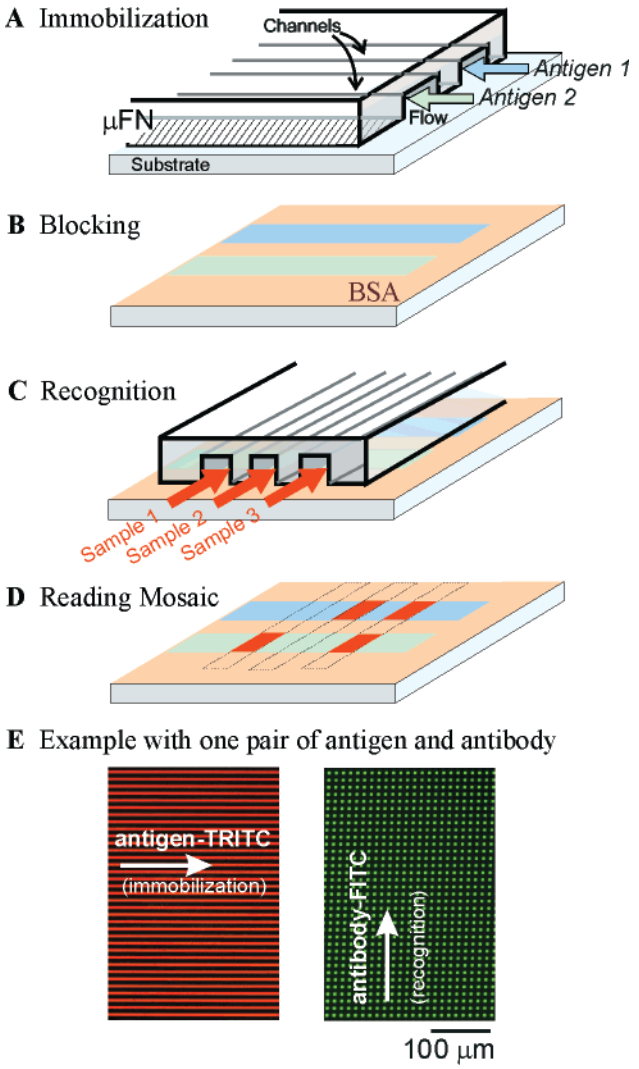


Figure 1. Strategy for performing a $\mu$MIA on a surface with $\mu$FN cross-delivery of a series of antigens and one of antibodies. (A) A $\mu$FN patterns different antigen molecules along single lines on a substrate. (B) The area of the substrate left unpatterned during (A) is blocked with BSA to prevent nonspecific binding of proteins in subsequent steps. (C) Antibodies flowing through the channels of a second $\mu$FN locally bind to the patterned antigens. (D) Reading the binding mosaic reveals the amount of antibodies present in the samples. (E) A mosaic can be read using a fluorescence microscope. First, TRITC-labeled rabbit IgGs were deposited on PDMS from a 200 $\mu$g mL$^{-1}$ solution using a Si-$\mu$FN having parallel channels that emerge from a common filling pad. After the substrate is blocked with BSA, a second $\mu$FN positioned across the lines of antigens exposed the substrate to a solution of FITC-labeled anti-rabbit-IgGs so that immunobinding occurred over an array. The arrows indicate the direction of flow of the protein solution in the $\mu$FN.

fabricate the necessary $\mu$FNs. The flow of a liquid[16] in small channels can be induced by capillary electrophoresis,[17,18] mechanical syringes or pumps,[19] or electrochemical principles.[20] In this work, fluids fill the microstructures of $\mu$FNs owing to the capillary pressures generated by the small dimensions of the channels and the hydrophilicity of their walls.[21] Consequently, $\mu$FNs are simple devices to draw fluids across a surface because they are "passive" systems, and more importantly, they can be removed and used repetitively to pattern the same region of a surface. For this we used silicon $\mu$FNs and substrates made of elastomeric poly(dimethylsiloxane) (PDMS). PDMS is soft enough to seal the cavities formed when the two parts are assembled; furthermore, it is a hydrophobic material prone to the adsorption of proteins from solution in a manner similar to the polystyrene of titer plates.[22]

## MATERIAL AND METHODS

**Preparation of $\mu$FNs.** Silicon $\mu$FNs were fabricated by contact photolithography and reactive ion etching (RIE) of Si wafers (for details of fabrication, see ref 23). Cleaning and hydrophilization of the $\mu$FNs were done with either a strongly oxidizing solution or an rf-induced $O_2$ plasma for 10−20 s. The dimensions of the Si $\mu$FNs used in this study were as follows: channel width 20 $\mu$m, gap between the channels 20 $\mu$m, height 10 $\mu$m, length 5 mm, macroscopic filling and flow-promoting pad for each channel 1.5 by 1.5 mm$^2$.

**Preparation of Substrates.** Flat PDMS substrates resulted from curing Sylgard 184 following the procedures of the manufacturer (Dow Corning) on the bottom of polystyrene dishes (Falcon, Optilux 1001, Becton Dickinson) for >12 h at 60 °C and cutting the PDMS layers to the desired dimensions. The substrates were then sonicated in a 2:1 solution of water and ethanol for ~3 min and dried with a stream of $N_2$ prior to use.

**Chemicals and Reagents.** All species antibodies were purchased from Sigma Immunochemicals as a lyophilized IgG fraction of reagent grade. Stock solutions of these antibodies were prepared at a concentration of 4 mg mL$^{-1}$ in PBS. The fluorescence-labeled anti-species antibody solutions (Sigma Immunochemicals) were freshly diluted before use in PBS containing 1% BSA (Sigma). ELF-97 phosphate was obtained from Molecular Probes and used as a 50 $\mu$M solution in glycine buffer (10 mM, pH 8.6, containing 100 mM NaCl, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$).

**Fluorescence Imaging.** Fluorescence images were obtained with a microscope (Nikon Labophot-2) equipped with optical filters and a charge-coupled camera cooled to 0 °C (ST-8, SBIG, Santa Barbara, CA) and then captured and analyzed using the software SkyPro (Software Bisque, Golden, CO).

**Preparation of the Micromosaic Assay.** A $\mu$MIA generally involved the following steps: (i) the PDMS substrate was applied over the open channels of a plasma-hydrophilized Si-$\mu$FN; conformal contact was established between the two materials by gentle manual pressure and the channels were sealed; (ii) ports of the microchannels were filled with less than 1 $\mu$L of antigen solution; (iii) 3 min later, the substrate was removed from the $\mu$FN in a solution of BSA in PBS; (iv) the substrate surface was blocked for nonspecific binding with BSA (10% in PBS) for 10 min; (v) the substrate was rinsed with water and dried under a stream of $N_2$; (vi) a second $\mu$FN was applied to the substrate across

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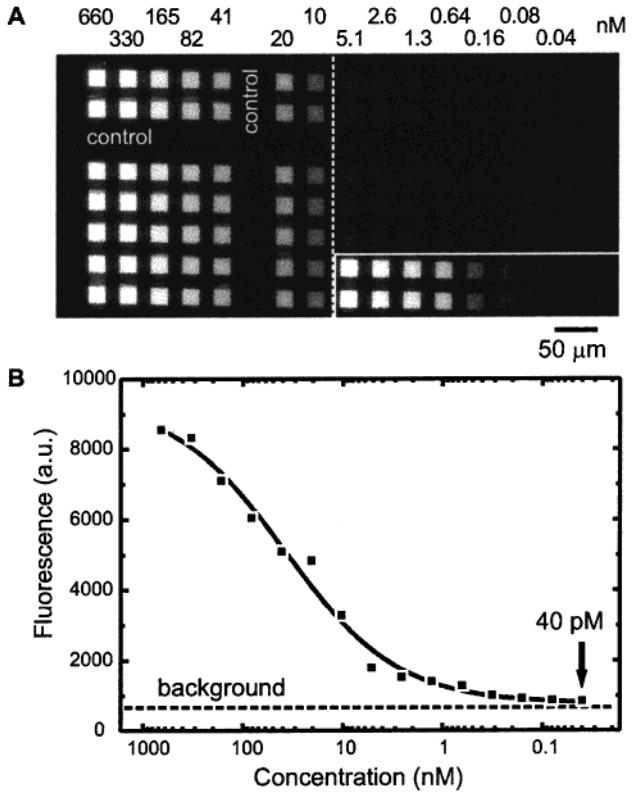


Figure 2. Study of an antibody−antigen interaction to assess the detection limits of $\mu$MIA. (A) Immunofluorescence image of TRITC-labeled anti-guinea pig antibody bound to lines of guinea pig IgGs on the surface. Solutions of the labeled antibodies filled the second $\mu$FN with concentrations from 660 nM (100 $\mu$g mL$^{-1}$) down to 40 pM (6 ng mL$^{-1}$). The image is composed of two frames acquired from adjacent regions of the sample (dashed line); part of the second frame is displayed with a higher brightness (inset). (B) Average fluorescence in the squares is plotted against the concentration of the anti-guinea pig IgGs. The dashed line indicates the measured background fluorescence.

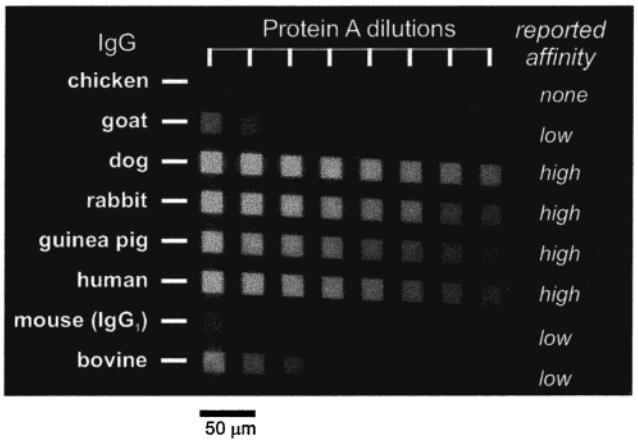


Figure 3. Fluorescence image of a $\mu$MIA examining the binding affinity between protein A and antibodies from different species. Eight types of IgGs were immobilized from 250 $\mu$g mL$^{-1}$ solutions in PBS onto a PDMS substrate with a $\mu$FN (horizontal axis). Solutions of protein A in PBS with decreasing concentrations from 80 $\mu$g mL$^{-1}$ (left vertical row) to 625 ng mL$^{-1}$ (right vertical row) were drawn across the initial pattern with a second $\mu$FN. Binding specificities taken from the literature[24] are given and provide values for a qualitative comparison.

the first pattern; (vii) its ports were filled with samples and controls; (viii) the substrate was separated from the $\mu$FN after 3 min; and (ix) the substrate was rinsed with water and dried. The mosaic on the substrate was then ready for imaging. Alternatively, an ELISA-type assay was conducted; (x) in this case, the substrate was incubated with a solution of an alkaline phosphatase immunoconjugate to bind the immobilized analytes. Finally, after rinsing, (xi) a solution of fluorogenic substrate was added to the surface to accumulate locally its insoluble, fluorescent product, revealing the mosaic.

## RESULTS AND DISCUSSION

**Fluorescence-Labeled $\mu$MIA.** We used two labeled binding partners, rabbit TRITC-IgGs and anti-rabbit FITC-IgGs, to demonstrate the simplest case of a $\mu$MIA, Figure 1E. One set of parallel microchannels in Si patterned rabbit IgGs as lines on a PDMS substrate, and after a blocking step with BSA, a second $\mu$FN exposed the immobilized rabbit IgGs to their antibodies from solution. Binding only occurred where the patterns of the two networks intersected, yielding an array of green fluorescence. Fluorescence imaging furthermore reveals the excellent and homogeneous localization of proteins in the pattern. In addition, the small dimensions of the patterns permit "one-shot" screening of the results using a standard fluorescence microscope at moderate magnification. The small dimensions also allow the results to be readily duplicated.

Immunoassays performed as $\mu$MIA must in essence be sensitive and specific. We therefore prepared a substrate with lines of guinea pig IgGs (antigens) and delivered solutions of anti-guinea pig IgGs (analytes; tagged with TRITC) with decreasing concentration to this surface, Figure 2A. The fluorescence associated with the analyte bound to the surface reveals that their binding and detection occurred over a wide range of concentrations. The graph in Figure 2B reflects a "sigmoidal binding behavior" between the two partners as is usually observed in a standard ELISA analysis. The fitting of the titration curve in Figure 2B yields a dissociation constant of 38 ± 12 nM, which is a typical value for the affinity of polyclonal antibodies such as the ones used here.[24] We did not attempt to optimize the $\mu$MIA for high sensitivity. For lower concentrations of analytes, better antibodies and a sandwich-type assay format are preferred. A further increase of sensitivity is possible by employing a competitive assay scheme.[25] The inter-channel reproducibility per test was excellent, namely, well below 5% for higher concentrations of analyte and ~10% for the lower ones tested. It is important to note that no depletion of the analyte solutions occurred as they flowed along the microchannels, even though some of the solutions were very diluted: the high surface-to-volume ratio of the channels requires that the design of the $\mu$FNs promotes the flow of solutions to supply sufficient protein reactants to the PDMS substrate.[15]

**Functional Mosaic Assays.** Protein A is a protein from *Staphylococcus aureus* that binds the Fc region of immunoglobulins G. The IgG-binding specificity of protein A varies substantially with the origin of IgG, i.e., the species from which it is derived.

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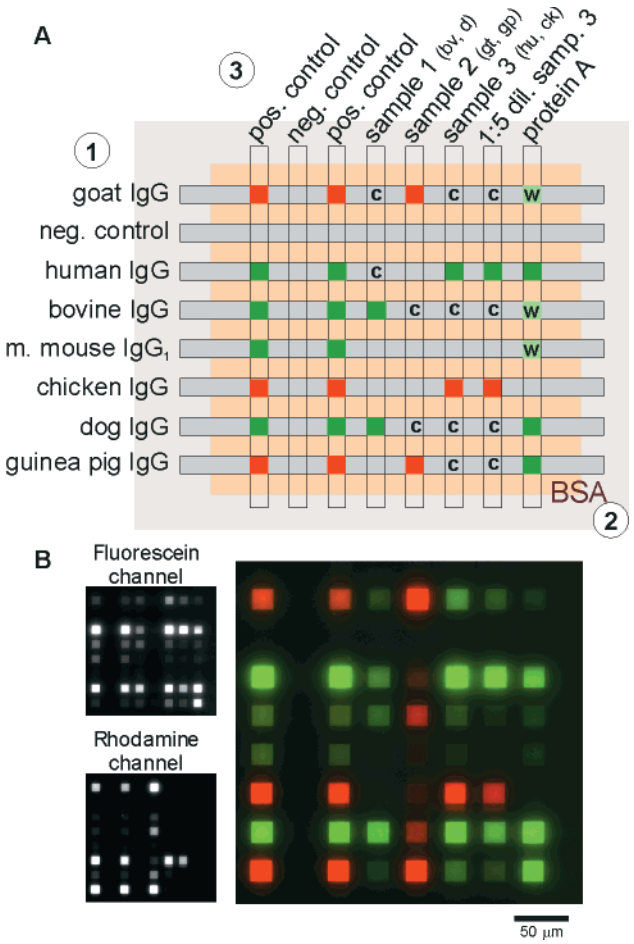


Figure 4. Eight-by-eight $\mu$MIA representing the experiments and their outcome as revealed simultaneously in the immunofluorescence color channels. (A) Different species antibodies are immobilized as lines onto a PDMS substrate using a $\mu$FN (1). The BSA blocking step (2) is done before the cross-delivery of solutions providing the positive, negative controls, four samples, and FITC-labeled protein A (3). As all antibodies in the samples and protein A are labeled with either FITC or TRITC, it is possible to predict the mosaic pattern, represented by colored squares. "w" and "c" denote the expectation of weak binding and cross-reactivity, respectively, between the antigens and the delivered proteins. (B) The mosaic corresponds to the recombination of the FITC and TRITC channel images (left side) acquired with a fluorescence microscope. The mosaic is consistent with the diagram and reveals nonanticipated cross-reaction.

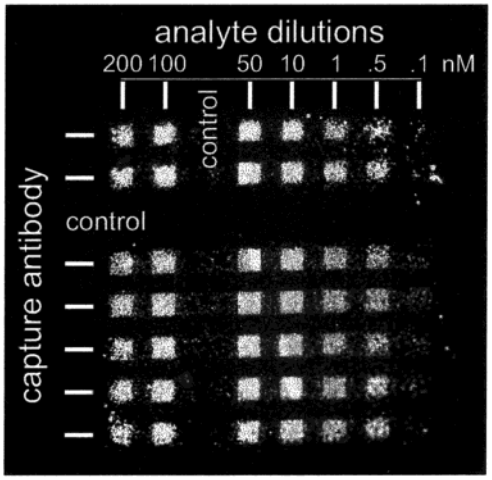


Figure 5. Fluorescence image of a sandwich-type ELISA $\mu$MIA using alkaline phosphatase to convert ELF-97 phosphate into its fluorescent, insoluble product to reveal the binding mosaic. Anti-chicken antibodies were patterned with a $\mu$FN and used to capture antigens (chicken IgG in PBS with 1% BSA) from solutions of decreasing concentration, which were delivered by another $\mu$FN. The entire surface was then incubated with an anti-chicken phosphatase-conjugated antibody. After the addition of the fluorogenic substrate, green fluorescence developed within a few seconds as precipitating clusters at the sites of enzymatic turnover.

The $\mu$MIA in Figure 3 is defined to identify in a single screening the specificity of interaction between protein A and eight IgGs from different species. In this case, a series of solutions of protein A with decreasing concentration flowed within microchannels across continuous stripes of IgGs immobilized on a PDMS substrate. Detection of the fluorescent tags coupled to protein A reveals its affinity for the respective IgGs and compares well with qualitative data from the literature.[26] The ease and speed necessary for performing this $\mu$MIA complements the simple readout of the results. The overall time to perform this test was less than 20 min, including the BSA blocking step of 10 min. However, the entire test can be subdivided into two independent parts: first the preparation involving the coating and blocking, and second the binding and reading. In our experiments, the second assay part can be performed much later (days) after the first one. The very short times for the coating and binding steps were a direct consequence of the small dimensions of the channels, which prevented mass transport limitations for the adsorption of the antigens and the binding of the partners.[25,27]

The experiment displayed in Figure 4 integrates many capabilities of $\mu$MIAs. In this eight-by-eight mosaic assay, a series of seven proteins is immobilized onto PDMS and acts as antigens for the potential capture of antibodies from liquid samples. This experiment includes negative and/or positive controls at both levels of the assay (patterning of antigens and delivery of samples); it also comprises screening a sample at two different concentrations and repeating the experiment using protein A, Figure 4A. Inspection of the 64 zones forming the mosaic in Figure 4B reveals that all controls behaved as expected. Binding FITC-protein A on the surface with the different antibodies present on the surface yielded results equivalent to those found with the previous $\mu$MIA. The antibodies present in the various samples were detected, but interestingly, some unexpected cross-reactions between species occurred, such as between dog IgGs and (rabbit) anti-guinea pig IgGs. Cross-reactions were independently confirmed by two-site ELISA immunoassays performed in single wells. This shows that, in a single experiment, the $\mu$MIA quickly provided information on the content of the samples and the behavior of their antibodies with regard to affinity and cross-reactivity.

**Enzyme-Linked $\mu$MIA.** Micromosaic immunoassays are not limited to the use of fluorescently tagged antibodies as the signal generator. The ELISA technique is equally able to reveal the binding mosaics. In Figure 5, we "sandwiched" the captured analytes with alkaline phosphatase antibodies and then used the

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enzymatic conversion of ELF-97 phosphate into its insoluble fluorescent product to detect analytes locally. Low levels of analytes can be detected due to the strong signal amplification of ELISA. Their detection was fast (∼10 s for the zones with the highest density of analytes), and the fluorescence scaled to the amount of analyte present on the surface as long as the amount of insoluble product was low enough not to obstruct the enzymatic activity. Consequently, it was optimal to follow the evolution of the fluorescence on the surface in real time to obtain a direct correlation between the analyte concentration and the developing fluorescence. Similar to the previous immunofluorescence mosaics, this one has a high contrast and accuracy for which the precipitation of the enzymatic product on its site of production was a key to keeping this mosaic localized. Again, the $\mu$MIA with ELF-97 was not optimized in terms of detecting low concentrations of analytes because only polyclonal antibodies with medium affinities were used here.

## CONCLUSION

In summary, changing the formats of immunotests, conventionally done in well-type reaction chambers, into $\mu$MIAs provides the opportunity to perform denser, parallel, and self-consistent immunoassays. These assays consume only nanoliter quantities of reagents and have incubation times of seconds to minutes. In light of the increasing demand for diagnostic assays with performances comparable to advanced clinical instrumentation, and which can be carried out in small laboratories, doctors' offices, and points-of-care, $\mu$MIAs open the door to miniaturized biodiagnostics.

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