25. This resonance does not seem to be due to physically dissolved CO, which appears as a singlet near 180 ppm in the strong Lewis acid SbF_5 and in the superacid HSO_3F-SbF_5. The solubility of CO in HSO_3F-SbF_5 is known to be very low (<0.01 M) (*20*) whereas the concentration of the ^{13}C-containing materials in the CO-HF-SbF_5 system is calculated to be at least two orders of magnitude greater.
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28. Selective continuous wave–decoupling (~200 Hz wide) was stepped through the HF-SbF_5 proton resonance (12.5 ppm) in 200-Hz steps, and the intensities of the ^{13}C resonances were monitored.
29. We used two systems to obtain the IR spectra: (i) A ReactIR-1000 System (ASI, Millersville, MD) with a SiComp probe that was mounted to the bottom of a stainless steel pressure cell (volume, 2 ml) fitted with a gas inlet for introduction of CO. (ii) A stainless steel pressure "circle" cell for attenuated total reflectance IR (Spectratech) with a Si rod crystal and fitted with a gas inlet for introduction of CO (cell volume, 2 ml). In both cases, HF-SbF_5 (1 ml) was added to the reactor under anhydrous conditions and the reactor was then charged with ^{13}CO (CIL Isotec, 99%) or ^{12}CO (MG Industries, research grade) from lecture bottles in quantities determined by a pressure gauge. Repeated pressurization-depressurization with ^{13}CO allowed exchange of ^{12}CO with ^{13}CO.
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Patterned Delivery of Immunoglobulins to Surfaces Using Microfluidic Networks

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Microfluidic networks (μFNs) were used to pattern biomolecules with high resolution on a variety of substrates (gold, glass, or polystyrene). Elastomeric μFNs localized chemical reactions between the biomolecules and the surface, requiring only microliters of reagent to cover square millimeter–sized areas. The networks were designed to ensure stability and filling of the μFN and allowed a homogeneous distribution and robust attachment of material to the substrate along the conduits in the μFN. Immunoglobulins patterned on substrates by means of μFNs remained strictly confined to areas enclosed by the network with submicron resolution and were viable for subsequent use in assays. The approach is simple and general enough to suggest a practical way to incorporate biological material on technological substrates.

The immobilization of ligands on surfaces is a first step in many bioassays, a prerequisite in the design of bioelectronic devices, and a valuable component of certain combinatorial screening strategies. Existing approaches typically expose macroscopic areas of a substrate to milliliter quantities of solution to attach one type of molecule, sometimes using light and specialized chemistries to carry out localized reactions (*1*–*7*). We have explored an alternative approach, namely the use of μFNs to guide nanoliter quantities of reagent to targeted areas on a substrate with submicron control.

We used patterns in an elastomeric support to define a network of conduits for fluids (the μFN) along the surface of a substrate (Fig. 1A) (*8*, *9*). Three walls of these conduits corresponded to molded features in a poly(dimethylsiloxane) (PDMS) rubber (*10*). The fourth wall was the surface of the substrate after it came in contact with the PDMS. Brief exposure of the PDMS to an oxygen plasma before this contact rendered the surface of the conduits hydrophilic and thus allowed a positive capillary action on a liquid introduced at the openings of the conduits (*11*). A tight seal precluding flow between adjacent, noncommunicating capillaries occurred where the PDMS touched the substrate (*12*); spontaneous adhesion between the elastomer and surface maintained this seal without requiring additional pressure. We applied the elastomer to Au, glass, and Si-SiO_2 surfaces previously activated by formation of a hydroxylsuccinimidyl ester to achieve chemical coupling with pendant amino groups common to proteins. These substrates had enough reactivity so that monolayer quantities of immunoglobulin G (IgG) were readily fixed to the surface, preventing their detachment in the ensuing washing steps (*13*). We followed the attachment of IgGs on the surface by ellipsometry (*14*) and waveguide techniques (*15*) over the large areas (~1 mm^2) probed by these methods to confirm the extent of reaction and the quality of attachment.

We designed the network as a system of two pads, each with lateral dimensions of 3 mm by 1 mm, connected by 100 channels, each 3 mm long, 3 μm wide, and separated by 0.8 μm (Fig. 1B). The channels were 1.5 μm deep, which provided an aspect ratio that allowed the formation of well-defined and stable capillaries in the PDMS. Deeper capillaries proved prone to collapse, either spontaneously (because of gravity) or during one of the processing steps; substantially shallower capillaries tended to block, provide poor mass transport of proteins, or deform onto the surface (*16*). With a μFN of the above dimensions, delivery of proteins onto the substrate could be homogeneous over distances of a few millimeters while still providing practical quantities of covalently attached material for convenient screening using enzyme-linked immunosorbent assay (ELISA) methods or ordinary fluorescence microscopy. The independence of capillaries in a network also allows simultaneous attachment of different biomolecules in each zone of flow (Fig. 1C). The topology of the network ensures a minimal use of solutions needed to derivatize the surface and can concentrate zones of flow into small fields of view without compromising their integrity.

Depletion of proteins from a dilute solution confined in small volumes can result from the loss of material onto the walls of the conduits or its incorporation into the bulk part of the PDMS (*17*). Flow through the capillaries into a second, hydrophilic pad avoided such loss of material available for the coupling step, where diffusion of the dilute protein from the filling pad might be insufficient. Depletion could also be cir-

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cumvented, at least in part, by using concentrated solutions of ligands or by passivating the walls of the capillaries with polyethylene glycols (*18*) or bovine serum albumin (BSA) (*13*). Filling of the capillaries was fast (the speed of filling was >1 mm s^{-1} with the geometries and buffers used here) and homogeneous as long as the μFN remained hydrophilic (*19*). We allowed the fluid to remain confined in the μFN and on the surface of the substrate for times similar to those needed to carry out the reaction on macroscopic areas (typically 1 hour). Reactions longer than several hours could be carried out before removal of the μFN from the substrate with no evident loss of coupling efficiency or resolution, underscoring the quality of the seal between adjacent capillaries on the surface (*12*). The elastomeric μFN was peeled away from the substrate under a flow of buffer to rapidly dilute and flush away the remaining unattached material from the substrate; this procedure avoided a general contamination of the surface. Procedures that flush the capillaries are also possible, but these proved to be more cumbersome than practical. Depending on the subsequent use of the patterned surface, sites that remained unreacted on the surface were quenched chemically (with an aminoglycol) or blocked with BSA.

The spatially controlled deposition of chicken IgGs was visualized indirectly at high resolution (Fig. 2A). The contrast in the scanning electron microscopy (SEM) image correlates with the amount of protein present on the surface (*20*). We measured 5 nm of attached IgGs (~1 monolayer) (*14*) by ellipsometry on large exposed areas of the substrate. The observation of a constant contrast even for the smallest features in the image demonstrates that the attachment of IgGs was independent of the geometry of this μFN. The Au substrate used in Fig. 2A can be replaced by other types of material such as glass, silicon wafer, or plastics commonly used for ELISA or other immunoassays because the elastomeric μFN had sufficient deformability to make the tight contact necessary to seal it on the substrate.

Immunoglobulin G's attached to glass by means of a μFN remained sufficiently intact to allow their specific recognition by an antispecies antibody (Fig. 2B). Placement of the patterned surface, derivatized as in Fig. 2A, into a solution of the secondary antibody resulted in specific attachment of these rhodamine-tagged IgGs only where their binding partner was present on the surface. This attachment survived subsequent washing steps that removed more weakly bound material. Fluorescence originating from regions defined by the μFN was the same over the length of the capillary, and its intensity was similar to that from larger regions of the substrate; this observation indicated similar yields of reaction and recognition in both environments. This method of detection was convenient and sensitive. The high, constant contrast in the image confirmed the success of our procedure to direct the attachment of IgGs reliably and to prevent their adventitious adsorption onto unwanted areas.

We also used a μFN to couple two different IgGs to a glass substrate with high spatial resolution (Fig. 3). Each IgG solution flowed from a macroscopic filling pad into a converging set of channels by capillary action (as in Fig. 1C). After attaching the IgGs, removing the μFN, and blocking underivatized areas with BSA, we covered the entire substrate with a solution containing a cocktail of fluorescently tagged IgGs. Its removal from solution was followed by a thorough rinsing of the substrate with buffer. We only observed color corresponding to the anticipated emission from tagged IgGs specific for each region; light from other fluorescently tagged IgGs present in the cocktail, but without specific binding partners on the surface, did not appear in our images. These results illustrate the power of the μFN approach to bringing different pro-

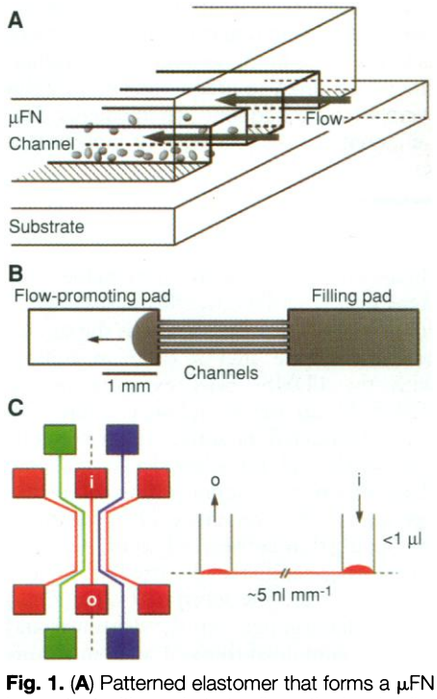

Fig. 1. (**A**) Patterned elastomer that forms a μFN by contact with a substrate allows the local delivery of a solution of biomolecules to the substrate. (**B**) Flow of liquid between the filling pad and an opposite pad fills the array of microchannels that constitute the strategic part of this device. (**C**) Assembly of different zones of flow on the surface results from the independence of capillaries, each requiring only a small volume (~1 μl) of liquid to fill the zone and derivatize the underlying substrate. Left panel, top view; right panel, side cut along the channel.

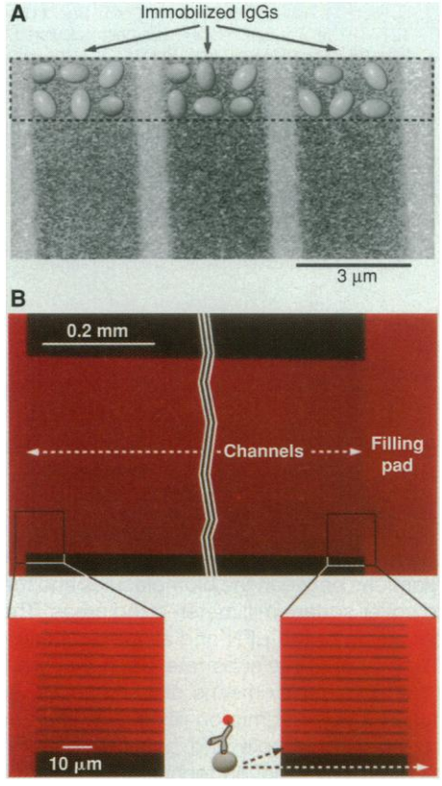

Fig. 2. The use of a μFN allowed IgGs to be attached to Au or glass with high spatial definition while preserving their antigenicity. (**A**) This SEM image reveals a pattern of chicken IgGs on gold [see (*13*)]. Bright regions in the image have little, if any, deposition of IgG and correspond to 0.8-μm gaps where the PDMS μFN contacted the surface and separated adjacent channels. Darker zones correspond to regions where reaction between IgGs in the filled μFN and the surface occurred, leaving approximately one monolayer of attached IgGs, shown schematically within the dashed box on the image. The fine texture in the image results from the surface roughness of the polycrystalline Au film (20 nm thick). (**B**) A fluorescence micrograph shows light from tagged antibodies to chicken IgG that bound chicken IgGs patterned using a μFN as depicted in Fig. 1B. After attachment of the chicken IgGs and removal of the μFN from the substrate, underivatized parts of the sample were blocked with BSA and the entire sample was exposed to a solution of antibodies to chicken IgG for 20 min in accordance with the instructions of the supplier (Sigma). A flow of PBS buffer (~10 ml) flushed away the solution, and unbound antibodies to chicken IgG were further eliminated under a continuous flow of Tween 20 in PBS (0.5%, ~10 ml) followed by 10 ml of deionized water. The sample was dried and the fluorescence was observed with a Leica DMRXE microscope equipped with oil-immersion lenses.

teins into the same region of space with high definition while preserving their specificity in a recognition experiment. We were also able to carry out ELISA-type assays on the patterned IgGs and watch the local appearance of a colored substrate indicative of enzymatic turnover at the targeted points of attachment.

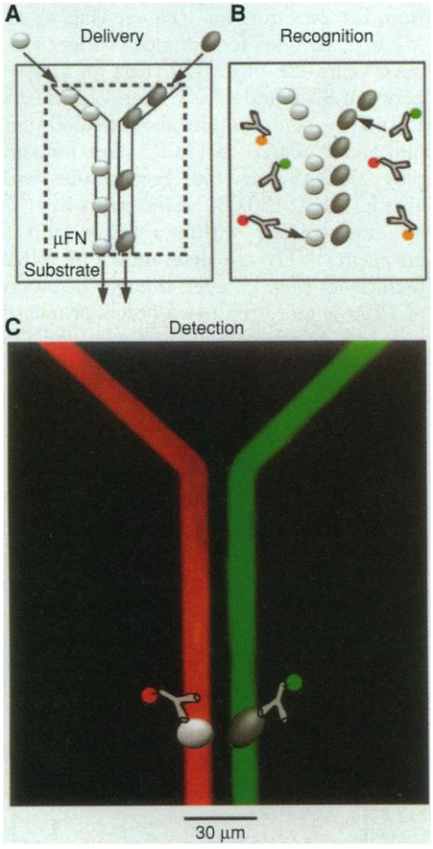

Fig. 3. Schemes for the delivery and attachment of two different IgGs using a μFN (**A**) followed by an immunoassay for the attached proteins after removal of the μFN (**B**). A composite digital image shows light emitted from fluorescently tagged antispecies IgGs, each specifically recognizing its binding partner previously patterned on a glass surface (**C**). The sample was handled as in Fig. 2B, except that this immunoassay was carried out with a heterogeneous solution of IgGs: tetramethyl rhodamine isothiocyanate–conjugated antibody to chicken IgG (red), fluorescein isothiocyanate–conjugated antibody to mouse IgG (green), and R-phycoerythrin–conjugated antibody to goat IgG (orange-red), each diluted 1:300 from their concentrated solutions (obtained from Sigma). The left stripe comprises chicken IgGs and the right stripe comprises mouse IgGs. No light was evident from nonspecific deposition of antibodies to goat IgG anywhere on the surface. There was no green fluorescence on the left channel or red fluorescence on the right channel; each color channel was collected independently so that such emission, resulting from cross-reactivity between the antibodies or their uncontrolled deposition, would have been easy to detect.

Our method for making patterns of biomolecules by attaching them using chemical reactions within μFNs has several practical benefits. It is simple, inexpensive, and economic of reagents. The μFN approach is compatible with many existing chemistries and substrates already used to attach macromolecules to surfaces, and it is compatible with new forms of covalent coupling requiring light activation because the μFN is transparent well into the ultraviolet. The deposited material binds to the surface in solution so that ligands are not exposed to denaturing conditions. The patterning step is local, that is, exposure of biomolecules to the surface occurs only on targeted areas. Simultaneous reactions in adjacent flow channels are possible without the introduction of cross-interferences, even where different coupling chemistries are needed. The method has high spatial definition and is inherently general, so that many assay formats in current use can be readily miniaturized without requiring access to standard lithographic equipment because formation of the μFN proceeds from its direct replication of a master. The use of μFNs requires only environments typical of biological and chemical laboratories and needs no extraordinary care or preparation. Therefore, μFNs may find applications in many tasks involving the formation of active biological interfaces.

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10. The PDMS mold is formed by curing its prepolymer (Sylgard 184, Dow Corning) on a fluorinated master having a negative pattern of the μFN formed in photoresist (Hoechst 6612, 1.5 μm thick) on its surface [Y. Xia *et al.*, *Science* **273**, 347 (1996)].
11. We used a plasma treatment with O_2 (oxygen pressure ≈ 0.8 torr, load coil power ≈ 200 W; Technics Plasma, Florence, KY) for 15 s to render the μFN hydrophilic, although many other conditions of reaction are possible, including wet chemical oxidation of the surface.
12. We found that capillaries remained effectively isolated from each other for long periods of time (>1 hour) except in the case where both the surface and the μFN had contact angles for the fluid of ~10° or less. In that case, the capillary force at the point of contact between the PDMS and substrate is high and evidently disrupts conformal contact between them. Wherever we encountered this problem, we used selective oxidation of the μFN, an oxidation confined to its interior walls, to keep the liquid pinned in the capillaries (E. Delamarche, H. Schmid, B. Michel, H. Biebuyck, A. Bernard, unpublished data).
13. After cleaning for 10 min with H_2SO_4/H_2O_2 (3:1) and a copious rinse with deionized water (resistivity > 18.2 ohm cm^{-1}), glass or silicon wafers with native oxide were aminopropylated by reaction for 3 hours at 80°C with 10% aminopropyltriethoxysilane (Fluka) in a sodium acetate–acetic acid buffer (10 mM, pH 5.0). The aminopropylated substrates were rinsed with deionized water and dried at 120°C for at least 3 hours before they were activated with a *N*-hydroxysuccinimide ester cross-linker (BS^3, Pierce) to promote the adhesion of the IgGs to the surface by amide bond formation. Alternatively, the self-assembly of a monolayer of an alkyl disulfide terminated by *N*-hydroxysuccinimide esters on Au provided an equivalent type of activated surface. In either case, coupling of the IgGs to the surface proceeded in the μFN for 1 hour with the use of a 1 mg/ml solution of IgG [chicken polyclonal IgG (Sigma) or mouse monoclonal IgG prepared at the University of Zürich, clone X7B3] in acetate buffer. Conditions for coupling were controlled by monitoring the amount of deposited IgG with an ellipsometer or an optical grating coupler with nonpatterned samples. After coupling, the μFN was carefully removed from the substrate under a flow of phosphate-buffered saline (PBS, ~10 ml) to prevent spreading of IgGs from their channels into adjacent channels or other parts of the surface. The patterned substrate was rinsed three times with 1 ml of PBS, three times with 1 ml of 0.5% Tween 20 in PBS, and three times with 1 ml of deionized water and finally dried. Regions of the surface outside the μFN (and therefore free of IgGs), or regions only partially derivatized by IgGs, were blocked by their exposure to a 1% solution of BSA in PBS for 1 hour to prepare the sample for immunoassays.
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17. We noticed a complete absence of deposited IgGs, as measured by SEM or fluorescence from a tagged IgG, in micrometer-sized capillaries only 50 μm downstream from the filling pad when concentrated solutions of IgG (~1 mg/ml) filled, but did not flow through, these capillaries. This result emphasizes the competition between flow through the network, adsorption at interfaces, and the poor recovery of concentration of proteins by their diffusion from the bulk reservoir into narrow capillaries. Thus, simple filling is usually not adequate for efficient chemical reaction with dilute solutions in μFNs.
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