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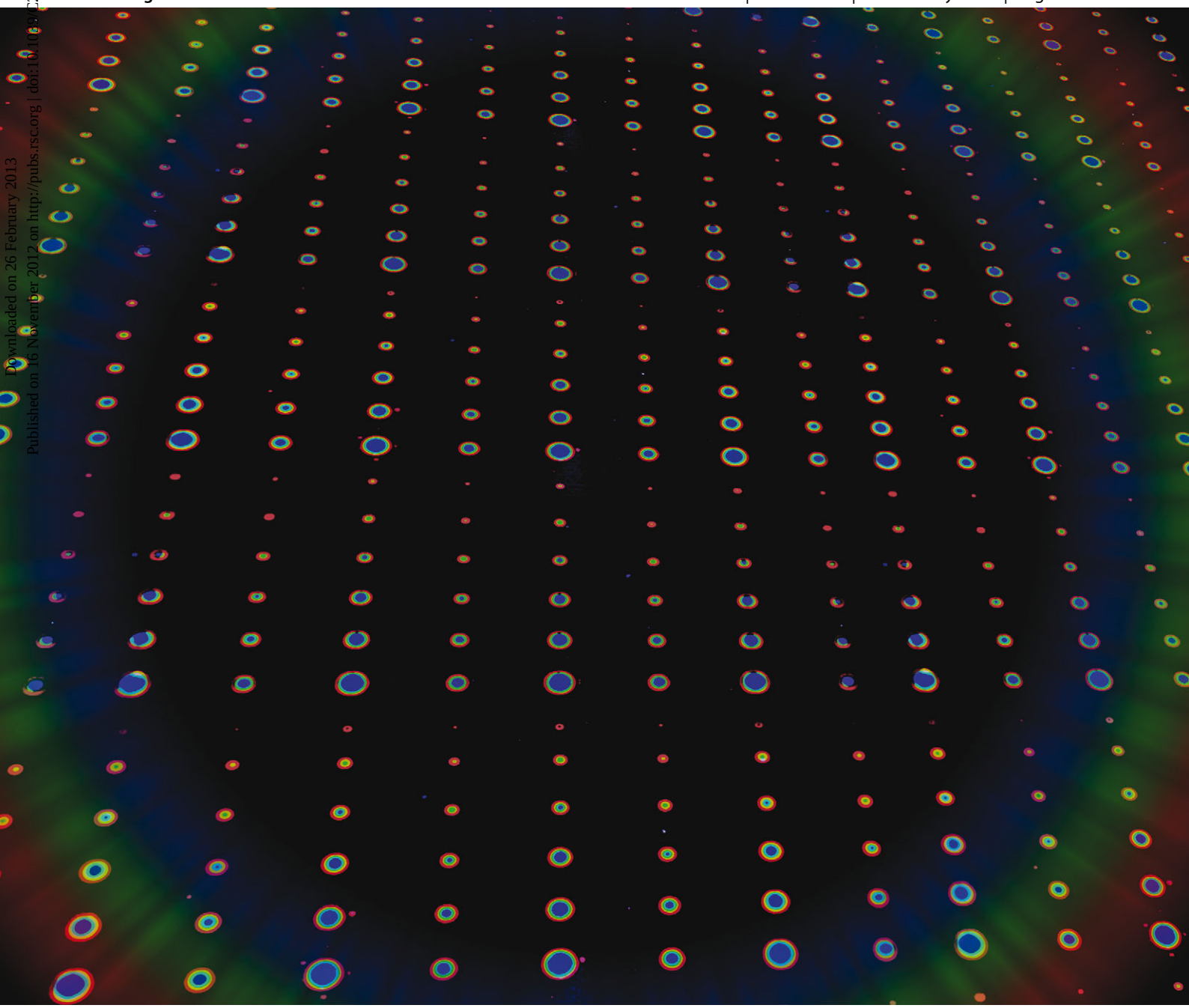

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We report the flow-based *in situ* patterning of multiple proteins in microfluidic channels by simply tuning the actuation pressure of a microfluidic button-membrane. We show that a single button-membrane can pattern several concentric protein annuli, and we apply this patterning approach to a multiplexed immunoassay.

Patterning of proteins at the microscale has become a useful tool in the study of cell biology, in the development of biosensors, and in the advancement of tissue engineering.[1–4] Different techniques have been developed for protein immobilization such as photolithography-based patterning,[5] microcontact printing (μCP),[3] microchannel laminar-flow pattering,[3] autonomous capillary systems (ACSs),[6] inkjet printing,[1] spot arraying,[1] and for features smaller than 100 nm, dip-pen nanolithography.[7] Given the scales at which micropatterning operates, integration with microfluidics can enrich the number and nature of applications. For example, in the area of diagnostics, Fan *et al.*[8] used microchannel flow patterning to enable the simultaneous detection of 12 biomarkers in whole blood. In cell biology, Khademhosseini *et al.*[9] demonstrated that microcontact printing facilitated mammalian cell capture in an array format. However, the fabrication of these devices requires the use of one of the above-mentioned techniques to pattern the biomolecules of interest on a substrate, followed by aligning and bonding of a microfluidic device on top of the patterned substrate, thereby increasing fabrication complexity, in addition to requiring a separate device to pattern the biomolecules.

Given the flexibility and advantages of microfluidics it should be possible to combine both, patterning and fluidic, operations in the same device, thus increasing functionality and simplifying assembly. Such attempts have been made, but are often limited to the patterning of a single type of biomolecule.[10,11] Here, we introduce an *in situ* microfluidic approach to patterning multiple proteins per assay unit. By varying the pressure of a microfluidic button-membrane and sequential loading of biomolecules, it is possible to create multiplexed patterns in the form of concentric annuli (Fig. 1).

We fabricated our devices by multi-layer soft-lithography, consisting of a polydimethylsiloxane (PDMS) control layer and flow layer (ESI‡).[12] Circular button-membranes, of different diameters ranging from 100 to 300 μm, were designed on the control layer and aligned with flow channels 300 μm and 400 μm wide (Fig. 1a). Upon pressurizing the control layer, the thin membrane separating the control and flow channels deforms downwards, until it contacts the glass surface (Fig. 1b), effectively trapping any molecules

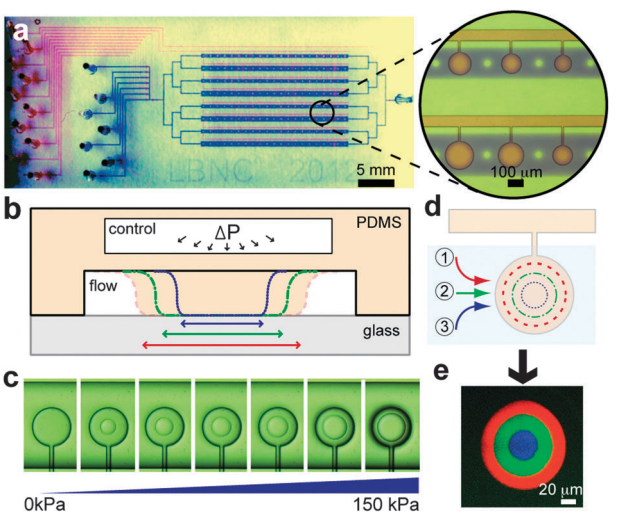

Fig. 1 Protein patterning with a microfluidic button-membrane. (a) Photograph of the device. The inset shows button-membranes (red) aligned with flow channels (blue). (b) Schematic cross-section of a button-membrane and flow channel. The PDMS membrane is deflected downwards with increasing pressure. (c) A series of photographs show the area in contact with the substrate at various pressures (0–150 kPa). (d) Multiple proteins can be immobilized in the form of concentric annuli. The first protein is introduced into the chip with the valve actuated at the highest pressure, followed by a washing step. The pressure is decreased slightly and the next protein introduced. These steps are repeated successively for the rest of the proteins. (e) Fluorescence image of three different types of fluorescently-labeled neutravidin patterned using this approach.

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between the substrate (glass) and PDMS, but also preventing molecules in solution from entering this protected circular area.[10] By varying the pressure in the control layer, the circular area in contact with the substrate can be modulated proportionally (Fig. 1c). This programmability in defining the contact area can be exploited to pattern multiple biomolecules in the form of concentric annuli. To do so, the button is actuated at its highest pressure and the flow channel is passivated to prevent non-specific absorption in the channel except for the area protected by the pressurized button (Fig. 1d). The pressure is then slightly decreased to reveal the first non-passivated annulus, and the first biomolecule is flown through the channel, creating the first and outermost annulus.

Repeating these steps (pressure reduction and biomolecule introduction) allows patterning of multiple biomolecules in the form of concentric annuli (Fig. 1e).

To investigate the range of diameters that can be patterned, we characterized the flow button diameters by bright field microscopy as a function of pressure (Fig. 1c). Fig. 2a shows that the relationship for each control button follows an exponential curve that eventually plateaus at pressures above 140 kPa. Curve fits for the two microfluidic channel widths (300 and 400 μm) are identical, suggesting independence of channel width. From each curve two parameters can be derived that prove useful in the design of future devices: the minimum closing pressure (*i.e.* the minimum pressure at which the membrane contacts the substrate) and the maximum contact diameter (*i.e.* the largest diameter possible to pattern on the substrate). The minimum closing pressure follows an exponential curve (Fig. 2b), with smaller buttons requiring an exceedingly high pressure (110 kPa for 100 μm) to contact the substrate, while for button diameters larger than 175 μm very low pressures are sufficient. The maximum flow button diameter relation fits a straight line (Fig. 2c) with a slope of ~1 and a slope-intercept of 67.3 μm. The maximum button diameter is thus ~70 μm smaller than its corresponding control button diameter.

To demonstrate the feasibility of this approach we patterned three different fluorescently-labelled proteins in 20 μm wide concentric annuli using the 175 μm button membrane. A microfluidic device was initially bonded to an epoxy-coated glass slide (ESI‡).[10] First, button valves were actuated at 170 kPa and a solution of 1% bovine serum albumin (BSA) and 1% casein in phosphate buffer saline (PBS, pH 7.4) were flowed through the chip to passivate the channel, except for the protected area (~120 μm diameter) beneath the control button. Channels were then washed with PBS/Tween (0.005%). Next, the pressure was released and biotinylated BSA was flowed through the chip, which is bound to the area previously protected by the button. The button valve was actuated again, this time at 62 kPa (~80 μm diameter), and a 650-fluor conjugated neutravidin (650-NA) was flowed through the chip, followed by a washing step. The button pressure was then reduced to 27 kPa (~40 μm diameter) and 550-NA was flowed through the chip, again followed by a washing step. Finally, the pressure was completely released, 488-NA was loaded onto the chip followed by a final washing step. The resulting fluorescence microscope image is shown in Fig. 3a for 6 buttons of different diameters. Fig. 3b and c shows the resulting surface patterning for the 175 μm button, and the corresponding normalized fluorescence intensity plot.

We used a similar patterning protocol to demonstrate the multiplexed detection of three cytokines (IL6, TNFα, and IL12p70) using a fluorescent sandwich immunoassay (Fig. 4a, ESI‡).

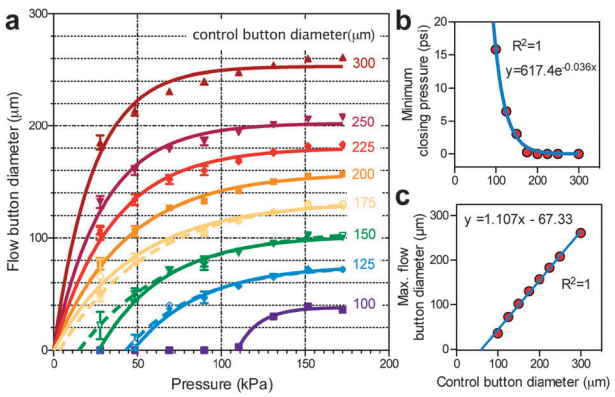

Fig. 2 Physical characterization of pressure-modulated button-membranes. (a) Change of contact diameter as a function of pressure for different control button diameters. Solid lines and dashed lines represent fitted curves for 400 and 300 μm wide channels. Error bars represent standard deviations of three measurements. (b) Minimum closing pressures and (c) maximum contact diameter as a function of button size. Values derived from curve fits from (a).

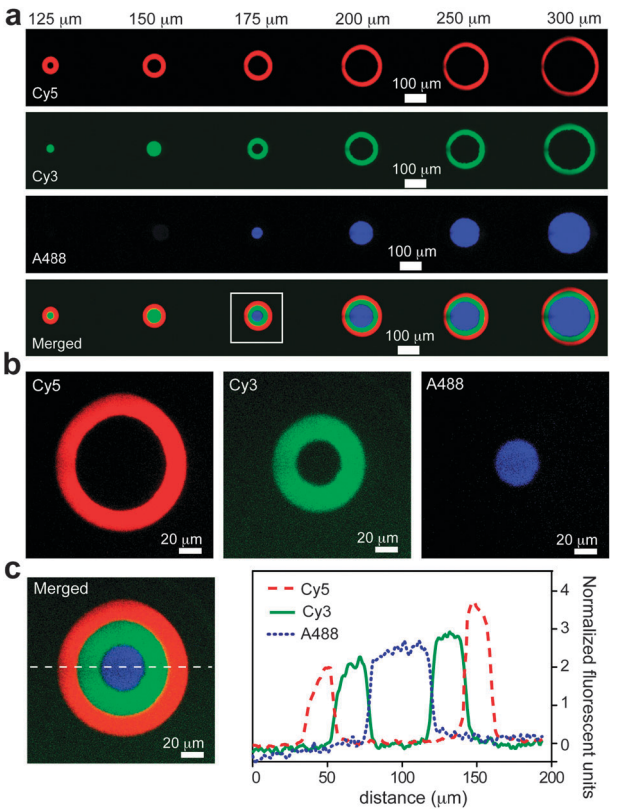

Fig. 3 Multiplexed patterning of biomolecules. (a) Fluorescent intensity images of a section of the chip showing six different buttons after surface functionalization; each annulus is labelled with a fluorescently labelled neutravidin molecule. Labels on top indicate the button diameter. Each image corresponds to a different channel, from top to bottom: Cy5, Cy3, and A488 channels, and merged image. (b) Fluorescence microscopy images of the different channels for the boxed button in (a). (c) Merged image and corresponding normalized intensities across the dotted line.

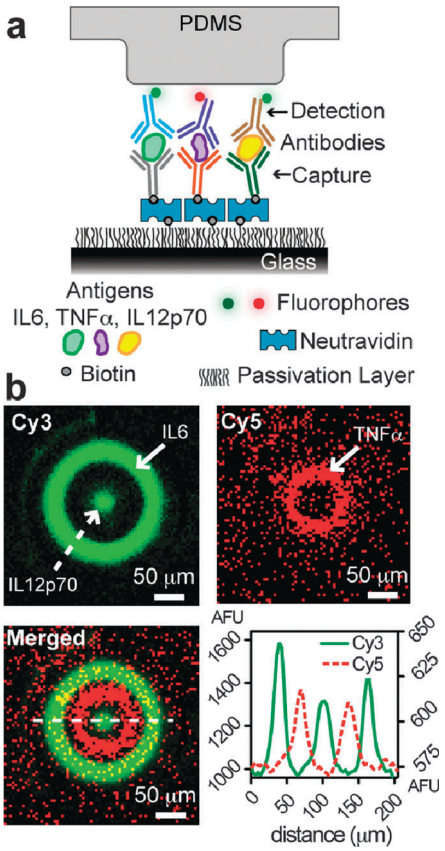

Fig. 4 Multiplexed sandwich immunoassay. (a) Schematic of the fluorescent sandwich immunoassay. Three cytokines, IL6, TNFα, and IL12p70, are captured on primary biotinylated antibodies immobilized under a button-membrane. Fluorescently labelled antibodies bound to their target antigens forming a sandwich immunocomplex. (b) Top row, Cy3 and Cy5 channel fluorescence intensity images of one button. Bottom row, merged image of both channels and measured fluorescent intensities across the dotted line. AFU, Arbitrary Fluorescence Units.

Biotin-BSA and neutravidin are first immobilized on the flow button. Next, the different primary biotinylated antibodies are introduced onto the chip in a similar sequence as with the fluorescently labelled neutravidin variants: first anti-IL6, followed by anti-TNFα, and finalized with anti-IL12p70. After immobilization of all primary antibodies, a cocktail of the three cytokines each at a concentration of 100 pM was flowed through the chip for 20 min. and captured by its corresponding antibody. Next, a solution containing three fluorescently-labelled secondary antibodies against each of the cytokines (anti-IL6 and anti-IL12p70 conjugated with phycoerythrin and anti-TNFα conjugated with Cy5) was loaded onto the chip. Fig. 4b shows the detection of these three complexes using a fluorescence microarray scanner.

In summary, we fabricated and characterized a microfluidic device that allows patterning of multiple biomolecules in small circular areas in the form of concentric annuli by tuning the pressure of a deformable button-membrane. We showed the potential of the approach by implementing a multiplexed sandwich immunoassay. Micropatterning of more than one biomolecule has proven to be challenging and is an active area under investigation.[1,13–15] Here, we demonstrated a fast and simple approach to multicomponent patterning by embedding a programmable 'stamp' in the control layer of our device, which also facilitates alignment of the patterning steps. In addition, the ability to pattern annuli of different widths could be used to produce diverse pattern configurations that may be useful in cell biology, to pattern specific areas where cells could attach, to study immunological synapse structure formation in T cells,[4] or to pattern different biomolecules residing in close proximity to each other. More immediately this technique will increase the throughput of current microfluidic platforms that employ the MITOMI button-membrane for measuring molecular interactions.[10,16–20] Generating 3 concentric annuli per button membrane and multiplexing the detection of two biomarkers per annulus through the use of fluorophores with two distinct spectral characteristics (Cy3 and Cy5 for example) would provide a 6-fold increase in throughput. Given that 4–5 concentric annuli are likely achievable with this approach, in combination with multiplexed detection with up to 4 distinct fluorophores, a 20-fold increase in throughput is likely possible. For current MITOMI devices containing 2400 unit cells this translates into a throughput increase from 2400 to 48 000 measurements per device.

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