Video Article

Flow-pattern Guided Fabrication of High-density Barcode Antibody Microarray

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Abstract

Antibody microarray as a well-developed technology is currently challenged by a few other established or emerging high-throughput technologies. In this report, we renovate the antibody microarray technology by using a novel approach for manufacturing and by introducing new features. The fabrication of our high-density antibody microarray is accomplished through perpendicularly oriented flow-patterning of single stranded DNAs and subsequent conversion mediated by DNA-antibody conjugates. This protocol outlines the critical steps in flow-patterning DNA, producing and purifying DNA-antibody conjugates, and assessing the quality of the fabricated microarray. The uniformity and sensitivity are comparable with conventional microarrays, while our microarray fabrication does not require the assistance of an array printer and can be performed in most research laboratories. The other major advantage is that the size of our microarray units is 10 times smaller than that of printed arrays, offering the unique capability of analyzing functional proteins from single cells when interfacing with generic microchip designs. This barcode technology can be widely employed in biomarker detection, cell signaling studies, tissue engineering, and a variety of clinical applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/53644/

Introduction

Antibody microarrays have been widely used in proteomic studies for decades to examine the presence of targeted proteins, including protein biomarkers[1-3]. Although this field is currently facing great challenges from other high-throughput technologies such as mass spectrometry (MS), there is still plenty of room for the utility of antibody microarrays, mainly because these devices afford simple data interpretation and easy interface with other assays. In recent years, the integration of microarrays into microchip scaffolds has provided the antibody microarray a new opportunity to thrive[4-7]. For instance, the barcode microarray integrated into a single-cell microchip has been used in cell communication studies[8,9]. This technology has distinctive advantages over other available microarray technologies. It features array elements at 10-100 µm, much smaller than the typical 150 µm size used in conventional microarray elements. The construction of smaller array elements is achieved using systematic flow-patterning approaches, and this gives rise to compact microarrays that can detect single-cell secreted proteins and intracellular proteins. Another advantage is the use of a simple, instrument-free setup. This is particularly important, because most laboratories and small companies may not be able to access microarray core facilities. Such barcode antibody microarrays feature enhanced assay throughput and can be used to perform highly multiplexed assays on single cells while achieving high sensitivity and specificity comparable with that of conventional sandwich enzyme-linked immunosorbent assay (ELISA[8]). This technology has found numerous applications in detecting proteins from glioblastoma[9-11], T cells[12], and circulating tumor cells[13]. Alternatively, barcode DNA microarrays alone have been utilized in the precise positioning of neurons and astrocytes for mimicking the *in vivo* assembly of brain tissue[14].

This protocol focuses only on the experimental steps and build-up blocks of the two-dimensional (2-D) barcode antibody microarray which has potential applications in the detection of biomarkers in fluidic samples and in single cells. The technology is based on an addressable single-stranded, one-dimensional (1-D) DNA microarray constructed using orthogonal oligonucleotides that are patterned spatially on glass substrates. The 1-D pattern is formed when parallel flow channels are used in the flow-patterning step, and such a pattern appears as discrete bands visually similar to 1-D Universal Product Code (UPC) barcodes. The construction of a 2-D (*n* x *m*) antibody array — reminiscent of a 2-D Quick Response (QR) matrix code — needs more complex patterning strategies, but allows for the immobilization of antibodies at a higher density[8,15]. The fabrication requires two DNA patterning steps, with the first pattern perpendicular to the second. The points of intersection of these two patterns constitute the *n* x *m* elements of the array. By strategically selecting the sequences of single-stranded DNA (ssDNA) utilized in flow-patterning, each element in a given array is assigned a specific address. This spatial reference is necessary in distinguishing between fluorescence signals on the microarray slide. The ssDNA array is converted into an antibody array through the incorporation of complementary DNA-antibody conjugates, forming a platform called DNA-encoded antibody library (DEAL[16]).

This video protocol describes the key steps in creating *n x m* antibody arrays which include preparing polydimethylsiloxane (PDMS) barcode molds, flow-patterning ssDNA in two orientations, preparing antibody-oligonucleotide DEAL conjugates, and converting the *3 x 3* DNA array into a *3 x 3* antibody array.

Protocol

Caution: Several chemicals used in this protocol are irritants and are hazardous in case of skin contact. Consult material safety data sheets (MSDS) and wear appropriate personal protective equipment before performing this protocol. The piranha solution used in Step (1.1.1) is highly corrosive and should be prepared by adding the peroxide slowly to the acid with agitation. Handle this solution with extreme caution in a fume hood. Use appropriate eye protection and acid-resistant gloves. Trimethylchlorosilane (TMCS) is a corrosive, flammable chemical used in an optional step after (1.1.6). Handle this chemical in a fume hood.

Note: Perform the barcode slide fabrication and critical flow-patterning procedures in a clean room to minimize contamination by particulate matter. Dust particles may block the ports and microchannels of PDMS molds and interfere with flow-patterning.

1. Construction of the One-dimensional DNA Barcode Slide

1. Preparation of the SU-8 master for barcode flow patterns
   Note: The drawings for the perpendicular flow patterns (**Figure 1A-B**) are created using a computer-aided design (CAD) software. These patterns are rendered on a chrome photomask. Transparent areas of the mask correspond to the features of the SU-8 master.
   1. Clean a silicon wafer (100 mm diameter) thoroughly in a mixture of 3 H_2SO_4: 1 30% H_2O_2 (piranha solution) heated to 96 °C. Wash the wafer with deionized water and isopropyl alcohol, followed by drying with a nitrogen blow gun.
   2. Pour about 4 ml of SU-8 2025 photoresist on the wafer. Use a programmable spin coater to uniformly spread the photoresist on the wafer for 10 sec at 500 rpm, then 30 sec at 3,000 rpm. This creates a photoresist layer with a thickness of ~25 µm. Gradually allow spinning to slow down before stopping — this is to maintain an even coating on the wafer's surface.
   3. Bake the coated wafer on a hotplate for 1 min at 65 °C, then for 5 min at 95 °C. This step allows the coating to solidify. Cool to RT for 5 min.
   4. Place the chrome mask (**Figure 1C**) on the photoresist coat. Expose the mask features to near-UV light (350-400 nm, exposure energy 150-160 mJ/cm^2) for 20 sec.
      Note: The design on the chrome mask contains 20 channels, each of which are 20 µm wide with 50 µm pitch. The channels are winding from one end of the pattern to the opposite end. Altogether, the 20 channels cover a rectangular area with a length of ~40 mm and a width of ~20 mm. Each channel is flanked by two circular features which correspond to an inlet and an outlet. Inlets and outlets are interchangeable.
   5. Bake the exposed wafer on a hotplate for 5 min at 95 °C. Cool to RT gradually.
   6. Immerse the wafer in SU-8 developer with agitation for 5 min. Wash the wafer with a small portion of fresh SU-8 developer, followed by isopropyl alcohol. Dry the wafer using a nitrogen blow gun. Hard-bake the wafer on a 200 °C hotplate for 30 min, and allow the wafer to cool gradually to RT.
      Note: Development may be carried out for a longer time if a white film is observed after washing in this step.
      Optional: Silanize the SU-8 master by exposing it to trimethylchlorosilane vapor in a closed Petri dish for 10 min.

2. Preparation of the PDMS barcode mold
   1. Combine 40.0 g silicone elastomer base with 4.0 g curing agent. Stir the prepolymer mixture vigorously for 10 min. Degas for 20 min under vacuum.
      Note: As a general rule, use a 10:1 (base: curing agent) mass ratio.
   2. Pour the prepolymer into a Petri dish containing a silicon wafer with the SU-8 master of the barcode pattern. The height of the PDMS mixture should be about 7.5 mm or above. Degas the mixture in the Petri dish for a second time to remove any remaining bubbles, then bake the mixture for 1 hr at 75 °C to allow PDMS to cure.
      Note: It is important to maintain enough thickness of the PDMS slab at ~7.5 mm or above to avoid adding too much tension to the PDMS-glass bond upon insertion of pins/tubing through holes in the PDMS mold in step (1.3.3.1)
   3. Using a scalpel, carefully cut around the area of the PDMS slab that contains the barcode mold features and peel the slab from the wafer.
   4. Trim the edges of the slab to attain the desired shape of the PDMS barcode mold. Punch 20 holes (1.0-mm diameter) through the mold using a biopsy punch with plunger. Ensure that the holes are aligned with the circular features of the barcode pattern. These holes serve as the inlets and outlets.

3. One-dimensional patterning of poly-L-lysine (PLL)
   1. Remove any dust on the surface of a poly-L-lysine coated glass slide using a nitrogen blow gun. Attach the PDMS mold to the clean glass slide. Ensure that the edges of the mold and the slide are aligned.
   2. Bake for 1.5 hr at 75 °C to strengthen the bond between the PDMS mold and the PLL-coated slide. Meanwhile, prepare 20 pieces of flexible polyethylene tubing (3- to 4-inch pieces, with an inner diameter of 0.5 mm and an outer diameter of 1.5 mm).
      Note: The number of pieces of tubing corresponds to the number of inlets in the PDMS barcode mold. The tubing serves to couple the channels on the PDMS barcode mold to a nitrogen gas tank equipped with a pressure regulator.
   3. To one end of each piece of tubing, attach a stainless steel hollow pin (1-mm diameter). Aspirate sterile-filtered poly-L-lysine solution through the pin, until at least 1 cm of the tubing is filled with the solution.
      1. Fasten the pins (connected to solution-filled tubing) to the inlets of the PDMS barcode mold (**Figure 1E**). Attach the other end of the tubes to a pressure-regulated nitrogen tank set-up. Allow the solution to flow through the mold using a pressure range of 0.5-1 psi for at least 6 hr.

Note: Refer to step (1.3.3) for all flow-patterning procedures.

4. One-dimensional patterning of ssDNA [14]
   Note: The phosphate buffered saline (PBS) used in subsequent steps is prepared from 137 mM NaCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4. The pH of the buffer is 7.4.
   1. Prepare 2 mM bis(sulfosuccinimidyl)suberate (BS3) solution in PBS. Prepare 300 µM solutions of **A, B,** and **C** ssDNA (5'-amine modified, 80 nucleotides) in PBS or ultrapure water.
      Note: Use the BS3 solution within about 30 min after preparation because the *N*-hydroxysuccinimide ester easily undergoes hydrolysis.
   2. Combine 2.5 µl of 300 µM A, B, or C ssDNA with 2.5 µl of 2 mM bis(sulfosuccinimidyl)suberate in PBS for each channel. **A, B,** and **C** DNA are designated to channels 1, 2, and 3, respectively. This order is also applied to remaining sets of 3 channels (**Figure 2A**).
   3. Perform step (1.3.3). This time, aspirate the 5-µl BS3/DNA solutions through stainless steel pins and into the polyethylene tubing, then couple the PDMS mold to the nitrogen source. Allow the BS3/DNA solution to flow through the barcode mold for about 40 min or until at the channels are filled.
   4. Stop the flow once all channels are filled, then incubate the BS3/DNA solution in the barcode at RT for 2 hr. Do not allow the solution to dry up. Bake the PDMS mold with attached barcode slide for 1 hr at 75 °C.
      Note: To facilitate alignment in subsequent flow-patterning steps, the edges of the channel pattern on the barcode slide may be outlined. This is done by carefully scratching the glass surface using a diamond scribe to generate alignment markers on the bottom of the glass slide. Alignment in later stages of the protocol may be checked under a microscope.
   5. Remove the PDMS mold from the barcode slide. Wash the slide gently with 0.01% SDS once and three times with ultrapure water.
      1. Dry the barcode slide using a microscope slide spinner. Store the barcode slide in a clean 50-ml centrifuge tube for subsequent use.

2. Validation of the One-dimensional Pattern on the Barcode Slide

Note: This validation protocol may also be adapted for use in assessing the quality of subsequent flow patterning steps.

1. Blocking of the slide
   1. Prepare 1% bovine serum albumin (BSA) in PBS. Filter this solution through a 0.45 µm syringe filter before use. Using a gel-loading tip, apply 50 µl of 1% BSA solution to one edge of the barcode slide.
   2. Incubate the 1% BSA solution for 1 hr at RT.

2. Incubation with Cyanine 3 (Cy3)-conjugated complementary DNA
   1. Prepare a cocktail of **A'**, **B'**, and **C'** oligonucleotides conjugated to Cy3 at one end. The sequences of **A'**, **B'**, and **C'** are complementary to those of **A**, **B**, and **C**, respectively. The working concentration of the Cy3-DNA is 0.05 µM in 0.1% BSA.
   2. Remove the BSA solution from the slide by pipetting, then apply 30 µl of the DNA cocktail solution on the same edge of the slide. Incubate the Cy3-DNA cocktail on the slide at RT for 1 hr. Perform this incubation step in the dark to protect the Cy3 moiety from photobleaching.

3. Analysis of fluorescence intensity
   1. Pipette out the Cy3-DNA cocktail and wash the slide in 1% BSA, PBS, and finally in diluted PBS (1 part PBS with 50 parts ultrapure water). Dry the slide using a spinner.
   2. Observe the fluorescence (**Figure 2B**) using a fluorescence microscope or a microarray scanner. When using a microarray scanner, set the laser emission wavelength to 532 nm, the pixel size at 5 microns, photomultiplier tube (PMT) gain at 450 and power at 15%.

3. Fabrication of the 2-dimensional (3x3) DNA Array[14]

1. Preparation of the PDMS barcode mold
   1. Perform procedure (1.1) to construct another SU-8 master, but this time use a chrome mask with a flow pattern perpendicular to that of the first design. Perform procedure (1.2) to construct a new PDMS mold with the perpendicular pattern.

2. Two-dimensional flow patterning of ssDNA
   1. For a *3 x 3* array, prepare 150 µM stock solutions of **A'-i, B'-ii, C'-iii, A'-iv, B'-v, C'-vi, A'-vii, B'-viii**, and **C'-ix** DNA in 3% BSA/PBS. These oligonucleotides serve as "bridging sequences" (**Figure 2A**). Combine the oligonucleotide solutions (Solutions 1 to 3) such that the working concentration is 50 µM for each oligonucleotide. Use the guide provided in **Table 1**.
   2. Flow 3% BSA/PBS blocking solution into all 20 channels for 1 hr at 0.5-1 psi.
   3. Flow Solutions 1, 2, and 3 into channels 1, 2, and 3, respectively. Follow this order for subsequent sets of 3 channels. Flow is usually completed in around 40 min. Incubate the DNA solutions at RT for 2 hr to allow the DNA to hybridize.
   4. Flow 3% BSA/PBS blocking solution into all 20 channels for 1 hr at 0.5-1 psi to remove unhybridized DNA. Peel off the PDMS slab, and wash the resulting DNA microarray slide by dipping the glass slide into 3% BSA/PBS once and PBS twice, followed by diluted PBS (1 part PBS and 50 parts ultrapure water). Dry the slide using a microscope slide spinner.
   5. Perform a second validation step (**Figure 2C**) similar to that in section 2. Use oligonucleotides **i'** to **ix'** that are Cy3-conjugated. Store the *3 x 3* array slide in a 50-ml centrifuge tube for subsequent use.
      Note: The DNA microarray can be stored at RT in a desiccator for months.

4. Conversion of the 3 x 3 DNA Array into an Antibody Array

1. Preparation of antibody-oligonucleotide (DEAL) conjugates
    1. Prepare solutions of 200 mM succinimidyl-4-formylbenzoate (S-4FB) and 40 mM succinimidyl-6-hydrazinonicotinamide (S-HyNic) in anhydrous *N,N*-dimethylformamide (DMF).
    2. Prepare up to 7 separate solutions of capture antibodies (1 mg/ml) in PBS.
    Note: If the antibody stock solutions contain sodium azide bacteriostat, perform buffer exchange with PBS using spin desalting columns with 7 kDa molecular weight cut-off (MWCO).
    3. Prepare separate 400 µM solutions of **i', ii', iii', iv', v', vi',** and **vii'** DNA. Assign each capture antibody to one oligonucleotide sequence. Combine 40 µl of 400 µM DNA with 12.25 µl of DMF in microcentrifuge tubes and spin down to mix thoroughly.
        1. To each DNA/DMF solution, add 2.3 µl of 200 mM S-4FB in DMF. For every 100 µg of capture antibody, add 2.25 µl of 40 mM S-HyNic in DMF.
    4. Incubate the antibody/S-HyNic and DNA/S-4FB solutions for 4 hr at RT.
    5. In preparation for the antibody-DNA coupling reaction, prepare new spin desalting columns (one for each DNA solution and one for each antibody) by washing them with citrate buffer at pH 6.
    6. After 4 hr of incubation, perform buffer exchange for each antibody/S-HyNic and DNA/S-4FB solution. Combine the S-4FB-conjugated **i', ii', iii', iv', v', vi'** and **vii'** oligonucleotides with the antibody-S-HyNic conjugates. Incubate the mixtures for 2 hr at RT.
    7. Incubate the reaction O/N at 4 °C.

2. Purification of antibody-oligonucleotide (DEAL) conjugates
Note: To purify the antibody-oligonucleotide conjugates, perform fast protein liquid chromatography (FPLC) on a standard FPLC system equipped with a Superose 6 10/300 GL column.
    1. Set the wavelength of UV detector of the FPLC system to 280 nm. Use isocratic flow of PBS (pH 7.4) at 0.3 ml/min to separate the antibody-oligonucleotide conjugates from the excess S-4FB-DNA.
    2. Pool the fractions containing the conjugate and concentrate the fractions to a volume of 150 µl using spin filters with a 10 kDa MWCO.

3. Two-dimensional patterning of antibodies
    1. Prepare another PDMS mold with microchambers or micro-wells using fabrication procedures in step (1.2). The PDMS mold may contain microliter to nanoliter wells for immunoassays.
    Note: For general biomarker detection in fluidic samples, a PDMS mold with multiple wells is mated with the array slide. The features of the PDMS mold depend on the system being studied. For instance, the detection of proteins from single cell experiments can be performed with a PDMS mold containing microchambers with 0.15-nl volumes.
    2. (Optional) Subject the PDMS mold to plasma cleaning (18 W) for 1.5 min to render its patterned surface hydrophilic. Prior to plasma cleaning, use adhesive tape to block all other surfaces that will be directly bonded to the *3 x 3* array slide.
    Note: Step (4.3.2) is optional but is usually performed when the PDMS mold is intended for use in single cell experiments.
    3. Mate the PDMS mold with the *3 x 3* array slide, then block the slide with 1% BSA in PBS. Incubate for 1 hr at RT. Meanwhile, prepare a cocktail (200 µl final volume) of antibody-oligonucleotide conjugates. The working concentration of each conjugate is 10 µg/ml in 1% BSA/PBS.
    4. Add the antibody-oligonucleotide cocktail, then incubate for 1 hr at 37 °C to allow the oligonucleotide moiety of the conjugates to hybridize with specific spots on the *3 x 3* arrays, thereby converting the DNA array to an antibody array.
    5. Repeat step (3.2.4) to clean and dry the slide. The highest sensitivity can be achieved if the antibody array is used immediately. Prolonged storage may result in loss of sensitivity.

4. Detection of proteins
    1. Reconstitute recombinant proteins or prepare a filtered cell sample.
    2. Mate the microarray with a custom-designed chip, and block the surface with 3% BSA in PBS for 1 hr. Then remove the BSA solution, and apply samples and incubate for 2 hr.
    3. Wash off the samples using 3% BSA in PBS three times, and then add detection antibodies at a concentration provided by the product datasheet. Incubate for 2 hr.
    4. Wash off the detection antibodies by 3% BSA in PBS three times, and then add Cyanine 5(Cy5)-streptavidin at 1 µg/ml, followed by incubation for 1 hr.
    5. Repeat step (3.2.4) to clean and dry the slide for scanning.

Representative Results

The designs for the PDMS molds (**Figure 1A-1B**) were drawn using a CAD program (AutoCAD). Two designs shown feature channels for flow patterning, one horizontal and one vertical. The left and right parts of each design are symmetric; either of them could be inlets or outlets. Each of 20 channels is winding from one end all the way to the other end. Each design is printed on a chrome photomask (**Figure 1C**). The fabricated SU-8 master on a wafer is shown in **Figure 1D**. To facilitate the flow-patterning of PLL or DNA, the PDMS mold was coupled to a nitrogen gas flow set-up (**Figure 1E**).

There are three flow-patterning steps used in this protocol. The first step immobilizes PLL on the glass substrate, while the succeeding steps both introduce oligonucleotide solutions. In **Table 1**, the oligonucleotide compositions of Solutions 1-3 are given. The working concentration of each oligonucleotide is 50 µM and the total volume of each solution is 39 µl. These solutions are prepared by combining 13 µl of each oligonucleotide stock solution (150 µM).

The patterned DNA microarray units are repetitive and adjacent across the whole glass slide. For *3 x 3* microarrays, the maximum density is about 400,000 spots on one glass slide. Side-by-side comparison with commercial DNA microarray reveals that our technology is about 5 times more sensitive when binding to Cy3 tagged complementary DNAs. The patterned DNAs are relatively uniform with ~5% variation across multiple repeats. Those features promise the high quantification ability of our technology when measuring protein contents from biological samples. In addition, the orthogonality of DNAs in combination with the flow channel design offer the flexibility of patterning in a variety of geometries (**Figure 2D**).

After converting the DNA array into an antibody array through hybridization with DNA-antibody conjugates (**Figure 3B**), the resulting antibody panel is used in multiplexed detection, mainly through sandwich ELISA platform as shown in **Figure 3C**. In sandwich ELISA, biotinylated detection (secondary) antibodies bind to the captured proteins, and subsequent labeling with fluorophore-conjugated streptavidin allows for fluorescence-based detection (**Figure 3C-E**). Detection of proteins shows <10% variation from one end to the other end of the glass slide (**Figure 3D**). With the *3 x 3* array, we are able to detect up to 7 proteins, while assigning one array element as reference (Cy3 labeled) and another element as negative control. For instance, in a single-cell experiment performed for tumor studies, the proteins interleukin-6 (IL-6), matrix metallopeptidase 9 (MMP9), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and macrophage migration inhibitory factor (MIF) from a single cell can be detected simultaneously (**Figure 3E**).

We also calibrated the system using recombinant proteins at various concentrations. In **Figure 3F**, calibration curves for the recombinant proteins interferon-γ(IFN-γ), tumor necrosis factor α (TNF α), interleukin-13 (IL-13), tumor necrosis factor β (TNF β), granzyme B, interleukin-4 (IL-4), and interleukin-8 (IL-8) are shown. The sensitivity of detection is quite similar to that of conventional sandwich ELISA on well plates even with higher loading of DNAs and possibly antibodies on the substrate.

The barcode antibody microarray can be used to detect biomarkers in fluidic samples as well as in single cells. Since single-cell analysis is not the focus of this protocol, we simply exemplified the application of the *3 x 3* antibody microarray in the detection of cytokines. Through antibody-surface marker interactions, cluster of differentiation 8 (CD8)-positive cells were captured on one of the microarray elements (**Figure 3G**; a PDMS chip on the top), and the rest of the elements were used to detect secreted cytokines (**Figure 3H**). With this cell capture method, "cell arrays" can be formed. This array capture technique also allows for control over the number of cells subjected to each ELISA experiment. The cells were physically isolated in the microchambers so that the detected proteins pertained to particular single cells. In **Figure 3H**, it is shown that each microchamber is equipped with 16 microarray elements to ensure encapsulation of a complete *3 x 3* microarray set.

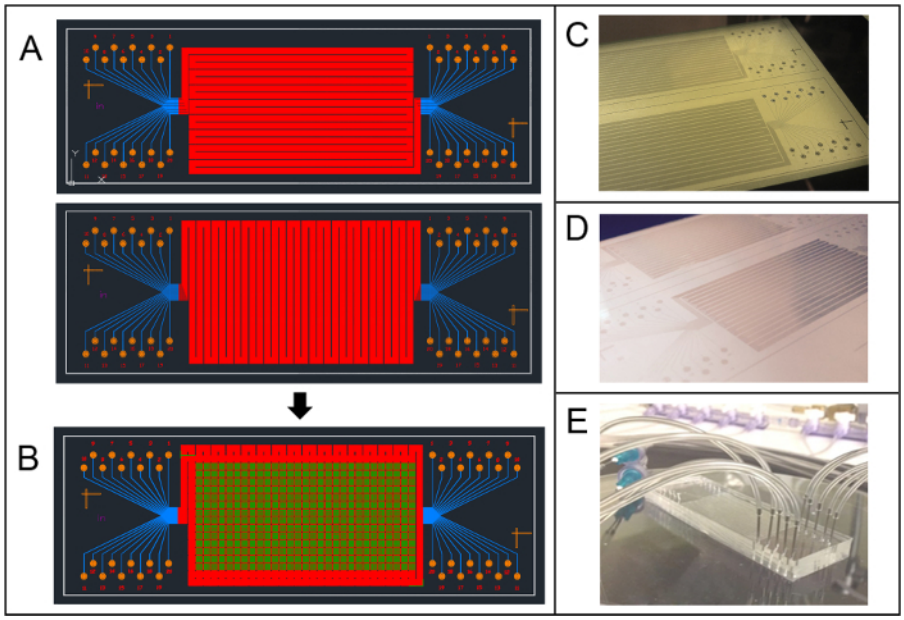

Figure 1. Design and fabrication of PDMS barcode molds for flow-based DNA patterning. Panel (**A**) shows the AutoCAD drawings for horizontal and vertical one-dimensional barcode patterns. Panel (**B**) shows the superimposed horizontal and vertical barcode patterns from (A). Green boxes enclose areas patterned with discrete *3 x 3* arrays. (**C**) Chrome mask for fabricating the SU-8 master. (**D**) SU-8 master fabricated on a silicon wafer. (**E**) Set-up for flow patterning. Please click here to view a larger version of this figure.

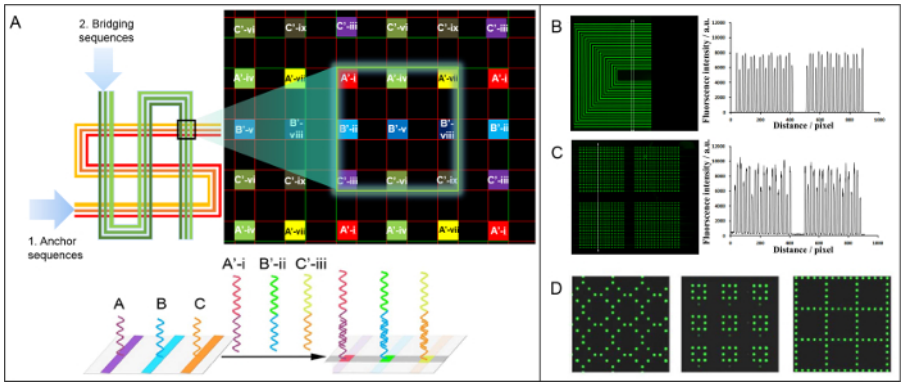

Figure 2. Construction and validation of a *3 x 3* DNA array. This figure has been modified from "Quantitating Cell-Cell Interaction Functions with Applications to Glioblastoma Multiforme Cancer Cells," by Wang, J. *et al*., 2012, *Nano Lett* 12. Copyright 2012 by the American Chemical Society. Adapted with permission. (**A**) Scheme for the DNA flow-patterning steps. (**B**) Fluorescence intensity profile of 20 channels in a selected area (traced by a vertical line) of the 1D barcode. Cy3-conjugated oligonucleotides were hybridized with the DNA arrays for validation. (**C**) Fluorescence intensity profile of arrays validated with Cy3-conjugated DNA after completing the second DNA patterning step. (**D**) Alternative fluorescent patterns from different flow-patterning strategies. Please click here to view a larger version of this figure.

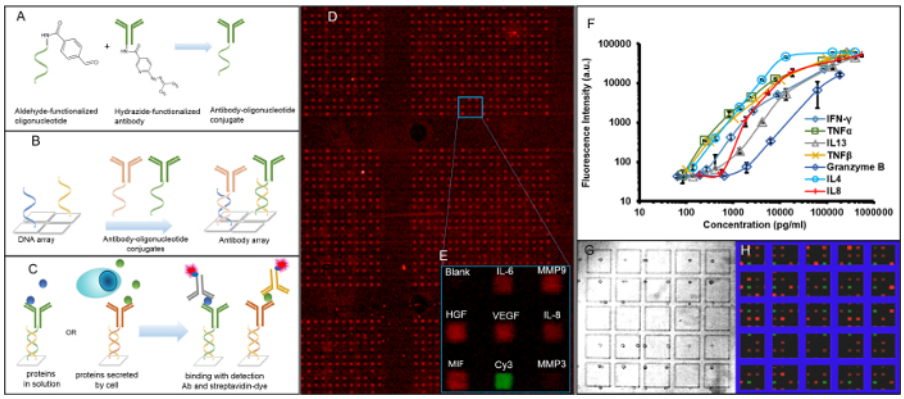

Figure 3. Applications of the *3 x 3* microarray. (**A**) Scheme for the synthesis of antibody-oligonucleotide DEAL conjugates. (**B**) Scheme for the conversion of the DNA array into an antibody array through hybridization with antibody-oligonucleotide conjugates. (**C**) Scheme for utilizing the *3 x 3* microarray in a sandwich ELISA platform. This figure has been modified from "Quantitating Cell-Cell Interaction Functions with Applications to Glioblastoma Multiforme Cancer Cells," by Wang, J. *et al*., 2012, *Nano Lett* 12. Copyright 2012 by the American Chemical Society. Adapted with permission. Panel (**D**) shows a fluorescence readout generated under a Cy5 channel. This corresponds to a sandwich ELISA experiment that detected 6 proteins. Panel (**E**) is a zoomed-in profile of a *3 x 3* array readout under Cy3 and Cy5 channels. Panel (**F**) shows the sandwich ELISA calibration curves for recombinant proteins. Panel (**G**) shows a "cell array" formed by capturing cells through binding with antibodies on the array. Panel (**H**) shows the detection result superimposed with microchambers in a microchip. Please click here to view a larger version of this figure.

Solution	Oligonucleotide Composition
1	A'-i, B'-ii, C'-iii
2	A'-iv, B'-v, C'-vi
3	A'-vii, B'-viii, C'-ix

Table 1. Composition of Solutions 1-3 for DNA Flow Patterning.

Anchoring sequences	
A	ATCCTGGAGCTAAGTCCGTA-AAAAAAAAAAAAAAAAAAAA-ATCCTGGAGCTAAGTCCGTA-AAAAAAAAAAAAAAAAAAAA
B	GCCTCATTGAATCATGCCTA-AAAAAAAAAAAAAAAAAAAA-GCCTCATTGAATCATGCCTA-
	AAAAAAAAAAAAAAAAAAAA
C	GCACTCGTCTACTATCGCTA-AAAAAAAAAAAAAAAAAAAA-GCACTCGTCTACTATCGCTA-AAAAAAAAAAAAAAAAAAAA
Bridging sequences	
A'-i	TACGGACTTAGCTCCAGGAT-AAAAAAAAAAAAAAAAAAAA-ATGGTCGAGATGTCAGAGTA
B'-ii	TAGGCATGATTCAATGAGGC-AAAAAAAAAAAAAAAAAAAA-ATGTGAAGTGGCAGTATCTA
C'-iii	TAGCGATAGTAGACGAGTGC-AAAAAAAAAAAAAAAAAAAA-ATCAGGTAAGGTTCACGGTA
A'-iv	TACGGACTTAGCTCCAGGAT-AAAAAAAAAAAAAAAAAAAA-GAGTAGCCTTCCCGAGCATT
B'-v	TAGGCATGATTCAATGAGGC-AAAAAAAAAAAAAAAAAAAA-ATTGACCAAACTGCGGTGCG
C'-vi	TAGCGATAGTAGACGAGTGC-AAAAAAAAAAAAAAAAAAAA-TGCCCTATTGTTGCGTCGGA
A'-vii	TACGGACTTAGCTCCAGGAT-AAAAAAAAAAAAAAAAAAAA-TCTTCTAGTTGTCGAGCAGG
B'-viii	TAGGCATGATTCAATGAGGC-AAAAAAAAAAAAAAAAAAAA-TAATCTAATTCTGGTCGCGG
C'-ix	TAGCGATAGTAGACGAGTGC-AAAAAAAAAAAAAAAAAAAA-GTGATTAAGTCTGCTTCGGC
Cy3-conjugated oligonucleotides for validation	
A'	TACGGACTTAGCTCCAGGAT
B'	TAGGCATGATTCAATGAGGC
C'	TAGCGATAGTAGACGAGTGC
i'	TACTCTGACATCTCGACCAT
ii'	TAGATACTGCCACTTCACAT
iii'	TACCGTGAACCTTACCTGAT
iv'	AATGCTGGGGAAGGCTACTC
v'	CGCACCGCAGTTTGGTCAAT
vi'	TCCGACGCAACAATAGGGCA
vii'	CCTGCTCGACAACTAGAAGA
viii'	CCGCGACCAGAATTAGATTA
ix'	GCCGAAGCAGACTTAATCAC

Table 2. Sequences used in DNA flow-patterning.

Discussion

Flow pattern design is the first critical step in fabricating the *2-D* microarray. To generate two overlapping DNA patterns on a glass substrate, the channel features of the first design should be perpendicular to those of the second (**Figure 1A-B**). The designs also consider the downstream applications of the microarray. In the case of single cell analysis, the microarray is used to detect proteins from single cells enclosed in microchambers, therefore the channel dimensions are made compatible with the microchambers that align with the *2-D* arrays. Each design is rendered on a photomask and standard photolithography techniques are used to fabricate the channel features in SU-8 on a silicon wafer (**Figure 1C-D**). This serves as the master for molding PDMS. Once the mold has been fabricated, it is bonded to a PLL-coated slide and then coupled with a nitrogen gas flow set-up (**Figure 1E**). The set-up we use is simple and has the advantage of being easily incorporated into any laboratory with a pressure-regulated nitrogen gas source. We use an assembly of inexpensive multiple 3-way valves connected to a nitrogen gas

tank. The air flow for patterning must be maintained at low pressures (0.5-1 psi) to avoid the delamination of the glass slide from the mold. Leaks within the PDMS mold can compromise the integrity of the patterns.

The DNA flow patterning steps in this protocol utilize ssDNA with carefully selected orthogonal sequences (**Figure 2A**). Prior to flow-patterning, the unique sequences on these oligonucleotides should be tested for the absence of cross-talk. A simple test for cross-talk is done by modifying the validation procedure we introduce in our protocol (Step 2). Instead of using a cocktail of Cy3-tagged complementary DNA, only one type of complementary sequence is used at a time for a selected area of the slide on which multiple DNA sequences are immobilized. In the absence of DNA cross-talk, only one stripe (for the 1-D DNA array) or one spot (for the 2-D DNA array) should be fluorescent under a Cy3 filter. Examples of thoroughly validated orthogonal sequences can be found in the Table of Materials and Equipment. The first DNA patterning step introduces three kinds of "anchor" oligonucleotides. These 80-nt sequences are comprised of two 20-mer poly-A regions that alternate with two unique 20-mer sequences. The 5'-amine modification on these anchor sequences is necessary for amine-to-amine cross-linking[17] with PLL mediated by BS3. The second DNA patterning step does not require a cross-linker. This step introduces "bridging" oligonucleotides that partly hybridize with the anchor sequences. Each 60-nt bridging sequence consists of a 20-mer region complementary to one anchor sequence, a 20-mer poly-A spacer, and a unique 20-mer sequence. Validation of the DNA arrays (**Figure 2B-C**) is performed after every DNA flow-patterning step to assess the quality of the patterning steps and to ensure no cross-contamination[14]. This is performed by introducing Cy3-conjugated oligonucleotide probes that hybridize with the DNA patterns. With the parameters specified in Step (2.3.2) for analyzing fluorescence intensity, the DNA patterns should exhibit fluorescence intensities of at least 40,000 a.u. to maximize the sensitivity of the downstream immunoassays performed using the microarray. Strategic use of different Cy3-DNA sets during validation gives rise to alternative patterns (**Figure 2D**), thus demonstrating the flexibility of the DNA patterning steps[8]. Failure to achieve uniform patterns could result from leaks or mechanical obstructions (such as dust particles) encountered in the flow-patterning steps.

The preparation of antibody-oligonucleotide DEAL conjugates is another critical step in this protocol. The choice of antibodies is dictated by the biological system under study. The oligonucleotides used in conjugation should have sequences complementary to those anchored on the DNA array. Stock solutions of the S-4FB and S-HyNic linkers should be freshly prepared and kept away from moisture to avoid hydrolysis. The incubation times used in our conjugation protocol are long enough to introduce the desired functionalities on the antibodies and DNA. The final coupling reaction is only quenched by removing the unreacted S-4FB-conjugated oligonucleotides via FPLC. Thus, FPLC purification should be performed immediately after completing the conjugation. When performing FPLC with the system described in our protocol, lower flow rates (0.2-0.25 ml/min) may also be used to improve resolution. The conjugates are eluted as a broad peak (elution volume of around 9-15 ml) that appears before a narrower and higher DNA peak (17-20 ml elution volume). We do not recommend storing solutions of conjugates with excess S-4FB-DNA because this can lead to loss of antigen-binding sites in the antibody upon conjugation with excess functionalized DNA. The sensitivity, specificity, and reproducibility of sandwich ELISAs using DEAL conjugates have been demonstrated in previous studies.[8,9,11,12,16] To assess the quality of antibody-DNA conjugates, the conjugates may be incubated on DNA arrays to generate the antibody array, which is subsequently used in sandwich ELISA experiments to generate calibration curves for protein detection (**Figure 3F**). To generate these calibration curves, recombinant proteins provided in a conventional sandwich ELISA kit may be used. The use of high-quality conjugates should give rise to linear ranges and lower limits of detection comparable with those of the kit. As an example, the lower limits for INFγ and TNFα for sandwich ELISA on our antibody array (**Figure 3F**) are ~50-100 pg/ml, and are consistent with the linear detection range of ~15-1,000 pg/ml indicated in the product datasheet for the conventional sandwich ELISA kit. Poor signal readout obtained in downstream sandwich ELISA experiments could be attributed to the low quality of DNA array, the interference of excess DNA in the conjugate solutions, or alternatively, incomplete conjugation of ssDNAs with antibodies.

Major concerns for performing sandwich ELISA on the antibody array include cross-talk between reagents and whether the signal reflects the real biological events. The orthogonal DNAs we use in this protocol have been thoroughly validated to have less than 0.1% cross-talk. Therefore any cross-talk observed at the immunoassay stage is mainly from the antibodies and ELISA reagents. Testing for cross-talk among antibodies in the array should be performed prior to conducting assays on real samples. Once an antibody panel is constructed, a few sandwich ELISA control experiments should be performed with the following conditions: 1. Without antigens, 2. Without detection antibodies, and 3. Detection antibodies and labeling reagents only. If cross-talk is observed at the immunoassay stage, the antibody pairs and/or ELISA reagents should be replaced. It should be noted that the use of commercially available antibodies from conventional ELISA kits does not guarantee applicability to the array-based sandwich ELISA technique.

The merits of this technology include inexpensive manufacturing, miniature size, and flexibility of design. Our fabrication protocol does not need a microarray spotter that may not be available to many users of antibody arrays. The flow patterning set-up can be easily assembled in simple laboratories. For laboratories that are not equipped with instruments for photolithography, the CAD designs we provide in our protocol may be forwarded to companies that offer microfabrication services. Downsizing the conventional microarray into the compact array fabricated with our protocol allows for compatibility with microchips used in single-cell experiments. Our protocol features the production of the array as an ssDNA array first before conversion to an antibody array. Preliminary patterning with ssDNAs has a number of advantages. First, ssDNAs are chemically more stable compared to antibodies, thus the ssDNA-patterned slides can be stored in a desiccator for at least 6 months without significant degradation[8,16]. Second, our approach offers an end user the flexibility to choose the assays needed, by simply mixing the selective conjugates together in a solution without modification of the microarray; On the contrary, conventional protein/antibody arrays are pre-designed and fixed on a substrate, so the change of assays would require the patterning/printing of proteins/antibodies from the very beginning. Representative studies illustrate the use of the barcode antibody microarray in the high-throughput detection of multiple signaling proteins from single cells. By varying the flow pattern designs and/or the oligonucleotide patterning solutions used, a multitude of array layouts can be explored. As an example of this application, functional proteins from single cells may be studied. The chip for single-cell analysis encompasses as many as ~8,700 microchambers, each aligned with a complete *3 x 3* antibody array (**Figure 3H**). Such a set-up allows for high-throughput detection. Proteins secreted by cells cultured in microchambers can be detected by this array. The versatile microarray design also allows for multiplexed detection of proteins from serum, cell lysates, and single cells after combining the microarray with other generic components[8,15]. In addition to protein detection, the *3 x 3* antibody array is also useful in capturing cells (**Figure 3G**).

The main disadvantage of this approach is its added complexity compared with the manufacturing of conventional microarrays. Strategies for patterning and for array design must be carefully conceptualized while considering the specific applications of the fabricated array. This approach also requires a number of critical steps including validation procedures and tests for cross-talk. The *3 x 3* array constructed using our protocol is

limited to detecting 7 proteins at a time. However, this limit can be surpassed by creating larger arrays. The protocol featured here is not limited to the fabrication of *3 x 3* microarrays. We have been able to successfully fabricate *5 x 5* microarrays and other dimensions of array elements. In principle, other *n* x *m* arrays may be fabricated using similar techniques as long as the oligonucleotide sets used in creating the preliminary DNA-patterned array are orthogonal to avoid cross-talk between array elements. The creation of expanded arrays is achieved by flow patterning *n* types of ssDNA anchors for the first DNA patterning step, followed by *n* x *m* bridging ssDNA sequences for the second patterning step. For instance, to create a *5 x 5* array, anchor sequences A to E may be used, while bridging sequences A'-i to E'-xxv are utilized. To automate the flow patterning process, a robotics platform has been developed[18] although this has only utilized one DNA flow patterning step. In principle, the same platform can be extended to the second step of perpendicular flow patterning, while the switching between two steps may require manually peeling off the first PDMS mold after the first patterning step, followed by washing and drying the slide (this step would take about 10 min), before mating the slide with another PDMS mold to facilitate the perpendicularly oriented second patterning step.

We have discussed detailed procedures for fabricating an *n x m* array patterned with antibodies at high density, which holds great promise for future applications in proteomic studies and cell signaling. The protocol presented here may also serve as a guide for the construction of more elaborate DNA/antibody arrays for other purposes.

Disclosures

The authors have no competing interests to disclose.

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