Supporting Information for

Multiplexed Microfluidic Blotting of Proteins and Nucleic Acids by Parallel, Serpentine Microchannels

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Materials

Ammonia persulfate, skim milk powder, salmon DNA, 1×TE buffer, 50×Denhardt buffer were purchased from Sangon Biotech (Shanghai) Co., Ltd. Acrylamide/bisacrylamide (30%, 29:1), acrylamide/bisacrylamide (40%, 19:1), phosphate buffered saline (PBS), 1.0mol/L Tris·HCl (pH 6.8), 1.5 mol/L Tris·HCl (pH 8.8), laemmli protein sample loading buffer were purchased from Bio-Rad Laboratories, Inc. Sodium chloride, potassium chloride, sodium hydrate, disodium monohydric phosphate, potassium dihydrogen phosphate, acetic acid, hydrogen chloride, glycerol, boron acid, ethylenediaminetetraacetic acid (EDTA), urea were purchased from Beijing Chemical Works. Glycine, tris(hydroxymethyl)aminomethane (*abbr.* Tris), sodium dodecyl sulfate (*abbr.* SDS), polyoxyethylene (20) sorbitan monolaurate (*abbr.* Tween-20) were purchased from Amresco, Inc. Triton X-100, phenylmethanesulfonylfluoride (abbr. PMSF), β-mercaptoethanol (*abbr.* β-ME), formamide were purchased from Sigma-Aldrich. Tetramethylethylenediamine (*abbr.* TEMED), bromophenol blue (*abbr.* BB) were purchased from BioBasic Inc.

The primary antibodies, such as β-actin antibody (Santa Cruz, sc-47778), pan-14-3-3 antibody (Santa Cruz, sc-133233), Annexin II antibody (Santa Cruz, sc-28385), GAPDH antibody (Santa Cruz, TA-09) were purchased from Santa Cruz Biotechnology and the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (A-11029) was purchased from Invitrogen. The Mini-Protean tetra cell (Catalog #165-8025) was purchased from Bio-Rad Laboratories, and it was used for all the electrophoresis and transfer experiment, either in protein or DNA.

Fabrication of the chip

First, we designed the silicon wafer mold by standard photolithography. Briefly, we designed the photomask using CAD software (AutoCAD, Autodesk) and printed it to a film by a high-precision laser photoplotter (SilverWriter 8850, Barco ETS). We made the master by hard contact photolithography (Mask Aligner MJB4, SUSS MicroTec) using negative photoresist (SU-8 2150, MicroChem) with speed of 1000 rpm in spin coating to achieve high height. Figure S1 showed the images of design for 5-lane system, whose sizes accurately match conventional

combs (Bio-Rad, Catalog # 165-3352) used in western blot. The width and the height of one channel are 400 micron and 500 micron, respectively. It can carry out 7 targets detection in one experiment from 5 samples.

The fabricated silicon wafer was subjected to surface tethering with 1H,1H,2H,2H-perfluorodecyltriethoxysilane in order to minimize the surface adhesion with subsequently procedures.

We obtained the PDMS microfluidic chip by replica molding against the silicon wafer. We mixed the PDMS pre-polymer and the curing agent with a ratio of 20:1 and cured the mixture for 30 min at 80 ^{o}C. The resulting chip was soft and slightly sticky so it should be covered with scotch tape to get rid of contamination.

Cell lysates preparation

We collected NIH-3T3 cells at a roughly estimated population of 10^7 and lysed them using 0.87 mL mono-surfactant lysis buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 0.15 M NaCl, and phenylmethanesulfonylfluoride100 μg/ml) for 1 h with ice bath. The solution turned opaque after 1 h lysis. We centrifuged the solution at 12000 rpm at 4 ^{o}C and carefully collected the supernatant to extract total proteins out of the cells. The protein concentration in the supernatant was determined by the commercial BCA kit (Beyotime Biotechnology, Beijing, Catalog # P0010S). After quantification, the protein samples were stored at -20 ^{o}C for long-term use and should be subjected to a heating process of 5 min at 95 ^{o}C and above with equal volume of laemmli protein sample loading buffer before electrophoresis.

Preparation of protein-loaded PVDF membranes

We used the Mini-Protean tetra cell (Bio-Rad, Catalog #165-8025) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The parameters of the SDS-PAGE system used in our study were listed as follows:

Gel size: 8 cm (width) × 7.3 cm (height) × 0.75 mm (thickness).

Denaturing discontinuous SDS-polyacrylamide gel: 5% for stacking gel and 12% for resolving gel.

Comb types: (1) Number of wells: 5; width per well: 12.7 mm, thickness: 0.75 mm, (Bio-Rad, Catalog # 165-3352); (2) Number of wells: 10; width per well: 5 mm; thickness: 0.75 mm, (Bio-Rad, Catalog # 165-3354)

We run the electrophoresis at 80 V until the proteins stacked as a thin line at the boundary of stacking gel and the resolving gel and resolve them at 120 V for 70 min to resolve them at room temperature.

After electrophoresis separation, the gels were blotted onto a Millipore PVDF-FL membrane (Millipore Immobilon-P, Catalog# IPVH00010, pore size: 0.45 μm) for 1.5 h at 100 V using a Mini TRANS-BLOT electrophoresis transfer cell (Bio-Rad, Catalog # 165-8025). We immediately marked the position of each lane and each molecular weight standard by a ball pen. After that, the blotted PVDF membrane was incubated at 37 ^{o}C for 1 h to dry. It should be noted that the PVDF membrane must be dried directly after the electric transfer without treatment with BSA, skim milk or Tween-20 to the membrane because it will deprive the hydrophobicity of the PVDF membrane and cause leakage in subsequently procedures. The dried membrane could be stored at 4 ^{o}C for long-term use and should be dried again to remove any possible vapor before use.

Assembly of the PDMS microfluidic chip and PVDF membrane

We attached the PDMS microfluidic chip onto the protein-loaded PVDF membrane. A clamp with base pad and cover pad was used to hold the chip and membrane together tightly and they were fastened by six screws (Figure S2). The cover pad has two symmetric crescent-shaped dents leaving for the inlets and outlets of the microfluidic channels, in order to guide the sample introduction by a pipette. The assembling process was quite simple and the clamp enabled the analytical tool to be a robust hand-held device (Figure S3).

Microfluidic Immunoassay

The primary antibodies we used here were:

β-actin antibody (Santa Cruz, sc-47778),Pan-14-3-3 antibody (Santa Cruz, sc-133233),Annexin II antibody (Santa Cruz, sc-28385),GAPDH antibody (Santa Cruz, TA-09), and the dilution for each primary antibodies was described specially below.

The secondary antibody we used was:

Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A-11029) and the dilution of the secondary antibody were unified as 1:2000.

The effect of size reduction on the improvement of spatial efficiency

We first loaded different amount of cell lysates into four electrophoretic lanes: 20 μg, 10 μg, 5 μg, 2.5 μg, and immobilized them on the PVDF membrane by electrophoresis and transfer. For conventional blotting, we cut every lane into seven pieces along the direction of electrophoresis and incubated them separately with designated antibodies. For MMB, we placed a chip on the membrane and introduced different amount of antibodies into the channels (Figure S3). Again, we selected the Annexin as the model protein.

The effect of size reduction on the improvement of temporal efficiency

For conventional blotting, we prepared two pieces of PVDF membranes and cut off seven identical small pieces, each of which contained a single lane loaded with 20 μg cell lysates. We sequentially placed these seven pieces into the incubation chamber at settled time points. For MMB, we loaded 20 μg cell lysates into one electrophoresis lane and transferred them onto one piece of PVDF membrane. A chip was placed on the membrane and antibodies were introduced into the channels, also sequentially at different time points. The primary antibody was Annexin

antibody and the dilution was 1:100. Figure S5 shows the fluorescence intensity for different time intervals in these two scenarios.

We can use calculation to explain the trend for the curve. It has been reported that the adsorption of antibody on antigen-immobilized surfaces in both microfluidic systems and bulk systems was dominated by the mechanism of diffusion. Therefore, we can employ Fick's second law to compare the diffusion/incubation time between MMB and conventional blotting systems:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2}\right) \qquad \textbf{(1)}$$

In equation ***(1)***, t is the time, C is the concentration of the antibody, and x, y are the width and depth of the reaction space. In our MMB system, x was 400 μm and y was 500 μm for one channel, while in the conventional system, x was 2 cm and the y was 5 mm for a common incubation chamber used in the comparative studies (For details in mathematical reasoning, see supporting information). The above equation clearly indicates that the velocity of binding is directly proportional to the cross-sectional area of the reaction spaces when the concentration of antibodies is the same. That is, the time required for diffusion/incubation will be longer if the cross-sectional area of the incubation chamber is larger. Since the cross-sectional area of the MMB system is several orders of magnitude smaller than their conventional counterparts (Figure S6), we could therefore dramatically decrease the diffusion/incubation time, which was also confirmed by the experimental results (Figure S5). In other words, the antibody-antigen recognition process should be subjected to a profile of sigmoidal curve, and we reasoned that the turning point at sigmoidal curve in MMB should appear much earlier than that at conventional blotting. We recoded the signal intensity at settled different time intervals for the two scenarios and found that the results coincided with our prediction. The signal increment in conventional blotting as a function of time was almost linear even when the incubation time was extended to 90 min. Our MMB system, by contrast, exhibits a clear sigmoidal binding kinetics within 90 min. Particularly, the signal intensity would almost approach its saturation within 20 min, which was in excellent agreement with previously reported results. Therefore, with our MMB system, we could shrink all the incubation process into one hour, including primary antibodies and secondary antibodies. This was a significant reduction of incubation time that was meaningful for the rapid, practical operations of blotting.

For equation (***1***), we can get it by considering how the size is related to time while ignoring other parameters (Figure S7).

1. Define *x, y, z* as the width, height and length of the incubation chamber. In two scenarios of conventional blotting and MMB, the *x* and *y* are different while *z* is the same.

2. Define *N(x)* as the amount of molecules in the plane whose vertical position is *x*.

3. M(x) as the net transport amount of molecules across this plane.

4. Assume that the antibody molecules would move Δx towards the PVDF substrate in the time interval of Δt .

As a result, the antibody-antigen reaction in the incubation process can be re-phrased as a plane y of antibodies that have to from the liquid bulk phase to the solid PVDF membrane with a net displacement of *x*. We have

$$M(x) = \frac{1}{2}[N(x-\Delta x) - N(x)] = -\frac{1}{2}\Delta x \frac{d[N(x)]}{dx}$$

$$\frac{[N(x-\Delta x) - N(x)]}{\Delta x} = -\frac{[N(x) - N(x-\Delta x)]}{\Delta x} \to -\frac{d[N(x)]}{dx}$$

Define *J* as the diffusive flux,

$$\Rightarrow J = \frac{M(x)}{S*t} = \frac{M(x)}{yz*\Delta t} = \frac{-\frac{1}{2}\Delta x \frac{d[N(x)]}{dx}}{yz*\Delta t} = \frac{-\frac{\Delta x}{2}*\frac{d[c(x,t)*V]}{dx}}{yz*\Delta t} = \frac{-\frac{\Delta x}{2}*\frac{d[c(x,t)*\Delta x*yz]}{dx}}{yz*\Delta t}$$

$$= -\frac{(\Delta x)^2 * yz * d[c(x,t)]}{2yz*\Delta t*dx} = -\frac{(\Delta x)^2 * d[c(x)]}{2\Delta t*dx}$$

$$= -D\frac{d[c(x,t)]}{dx}$$

$$\Rightarrow J = -D\frac{d[c(x,t)]}{dx}$$

again we have

$$\frac{d[N(x)]}{dt} = [yz * J(x - \frac{\Delta x}{2}) - yz * J(x - \frac{\Delta x}{2})]$$

$$\Rightarrow \frac{d[c(x,t) * \Delta x * yz]}{dt} = -\Delta x * yz \frac{dJ}{dx}$$

$$\Rightarrow \frac{d[c(x,t)]}{dt} = -\frac{dJ}{dx}$$

$$\Rightarrow \frac{d[c(x,t)]}{dt} = -\frac{d}{dx}\{-D\frac{d[c(x,t)]}{dx}\} = D\frac{d^2[c(x,t)]}{d^2x}$$

$$\Rightarrow \frac{\partial[c(x,t)]}{\partial t} = \frac{\partial^2[c(x,t)]}{\partial x^2}$$

or more generally, $\frac{\partial[c(x,y,t)]}{\partial t} = \frac{\partial^2[c(x,t)]}{\partial x^2} + \frac{\partial^2[c(y,t)]}{\partial y^2}$

or it can be simplified as $\frac{\partial C}{\partial t} = D(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2})$, which is equation ***(1)*** where D is just a constant as the coefficient. In this equation we can clearly see that the time require for completing the reaction inside a chamber is proportional to the square of the size of the chamber. This is the solid mathematically interpretation of why the reaction in the small microfluidic channels reaches equilibrium much faster than that in the conventional blotting.

Next, we examined some extreme reaction conditions for MMB. We dictated combination of different parameters including incubation time, antibody dilution and blocking to the seven channels. The results were shown in Figure S8.

Identification of neighboring bands

We loaded 20 μg cell lysates onto an electrophoresis lane and separated the proteins with only 10 min. In this case almost all the protein bands were merged together. For conventional blotting,

we applied the mixture of four antibodies that targeted β-actin, Annexin, Pan-14-3-3 and GAPDH, respectively. For MMB, we introduced different antibodies into separate channels.

Microfluidic chips that fit for ten-well combs

We still used Mini Protean electrophoresis system but only replaced the five-well comb with the ten-well comb. The width of a well was measured to be 4 mm. We designated the channel number as seven, each of them with a length of 50 cm, width of 200 μm, height of 400 μm, and distance between neighboring channels of 400 μm. We also introduced serially diluted antibodies that targeted Annexin into seven channels and molecular weight markers into other three lanes. The chip was proved to function well (Figure S8). In addition, as the width of one lane in the five-lane system is 12.7 mm, three times wider than that of one lane in the ten-lane system, we therefore inferred that the maximum channel number for the five-lane system was $7 \times 3 = 21$ channels.

Data acquisition

Fluorescence signals were recorded using a Typhoon Trio Variable Mode Imager (Amersham, Bioscience) with the following parameter settings:

Laser: 488 nm

Emission filter: 520 nm

PMT: 480 V

Pixel size: 25 μm

All data points in this paper represent an average of three to six measurements. The fluorescence images are analyzed with the software of ImageQuant (Molecular Dynamics). All signal intensities were normalized by subtracting the non-specific baseline signals.

Table S1. Data obtained from the Figure S10**,** showing the interaction between different subunits in a single blot.

Antigen / Antibody	R3β	R2	R1	R3α	4C	Negative Control	Positive Control
4C	√	√	√	√	√		√
R3α		√		√			√
R1							√
R2	√	√		√			√
R3β	√	√					√

Table S2. two series of sequences that were used in the nucleic acid blotting experiment.

Name		Sequence (5'~3')	Modification
Hepatitis B virus surface antigen gene	Target 1 (T-HVB-EG18)	AGAAAGAGGAGTTAA	EG18
	Target 2 (T-HVB-A10)	AAAAAAAAAAAGAAAGAGGAGTTAA	/
	Detection Probe (DP-HVB)	TTAACTCCTCTTTCT	5'6-FAM
Variola virus (VV)	Target 3 (T-VV-EG18)	CTGATTACTATTGCA	EG18
	Target 4 (T-VV-A10)	AAAAAAAAAACTGATTACTATTGCA	/
	Detection Probe (DP-VV)	TGCAATAGTAATCAG	5'6-FAM

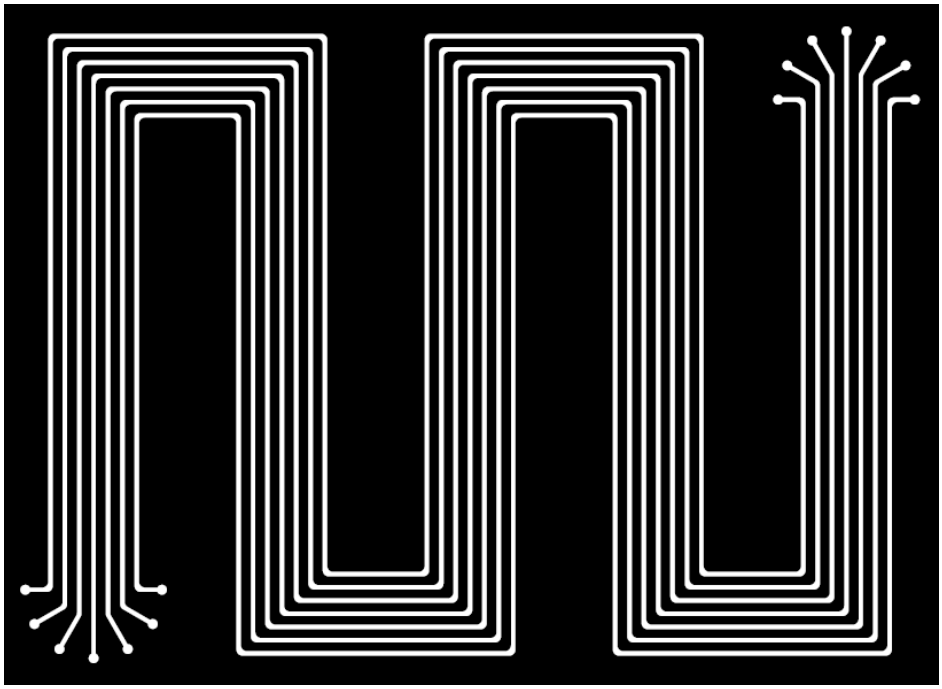


Fig. S1 Serpentine profiles of the microfluidic chip used in this study. We designed the microfluidic channels for 5-lane western blot. A noteworthy factor is that we should consider the actual size of comb and silicon wafer, simultaneously. In designing for the microfluidic channel, we got rid of wells in both ends since the limited diameter of 4 inch of silicon wafer. In addition, we found that the commercial products of combs are not the same distance between each lane, so the actual measuring one by one is critical for this work.

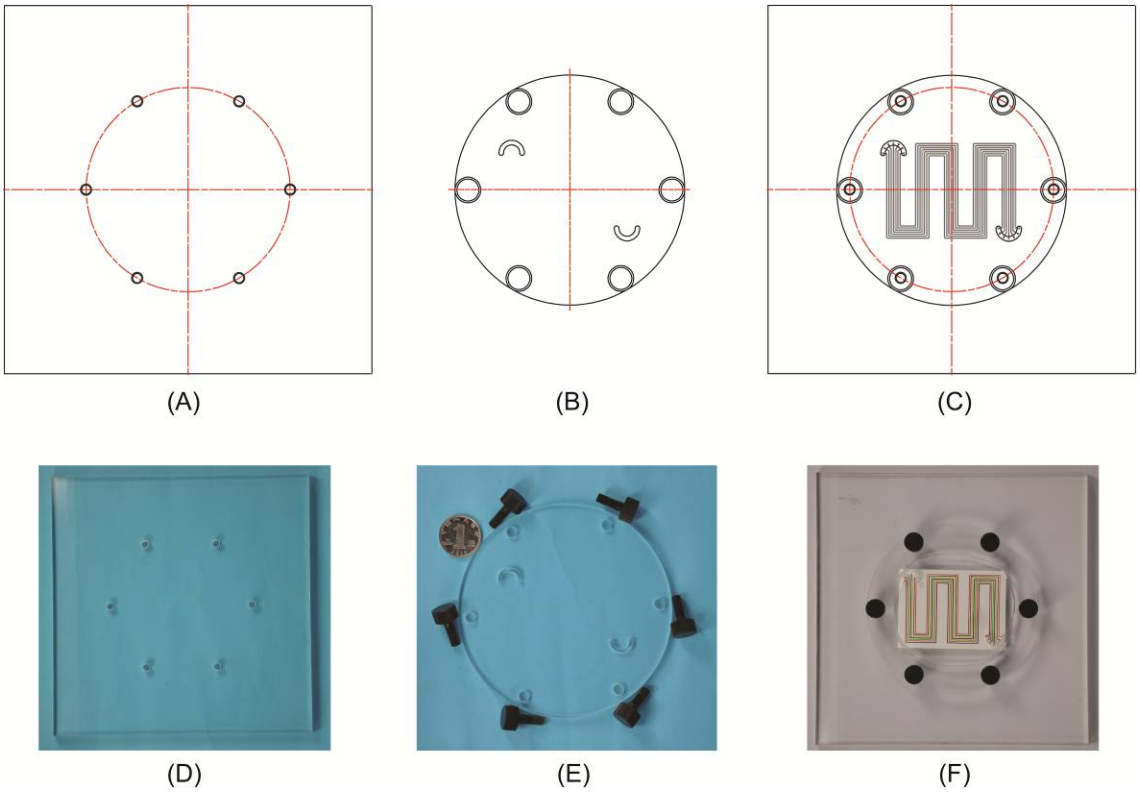


Fig. S2 Schematic illustration for the fabrication and use of the clamp. (A) The base pad of the clamp with six identical, symmetric holes for immobilization. (B) The cover pad of the clamp also with corresponding holes for immobilization and two crescent holes for sample introduction at the inlets and outlets of the microfluidic channels. (C) Screws are used to fasten the base pad and the cover pad, clamping the PDMS chip and the PVDF membrane tightly. (D), (E) and (F) are the corresponding digital images of the (A), (B) and (C), respectively.

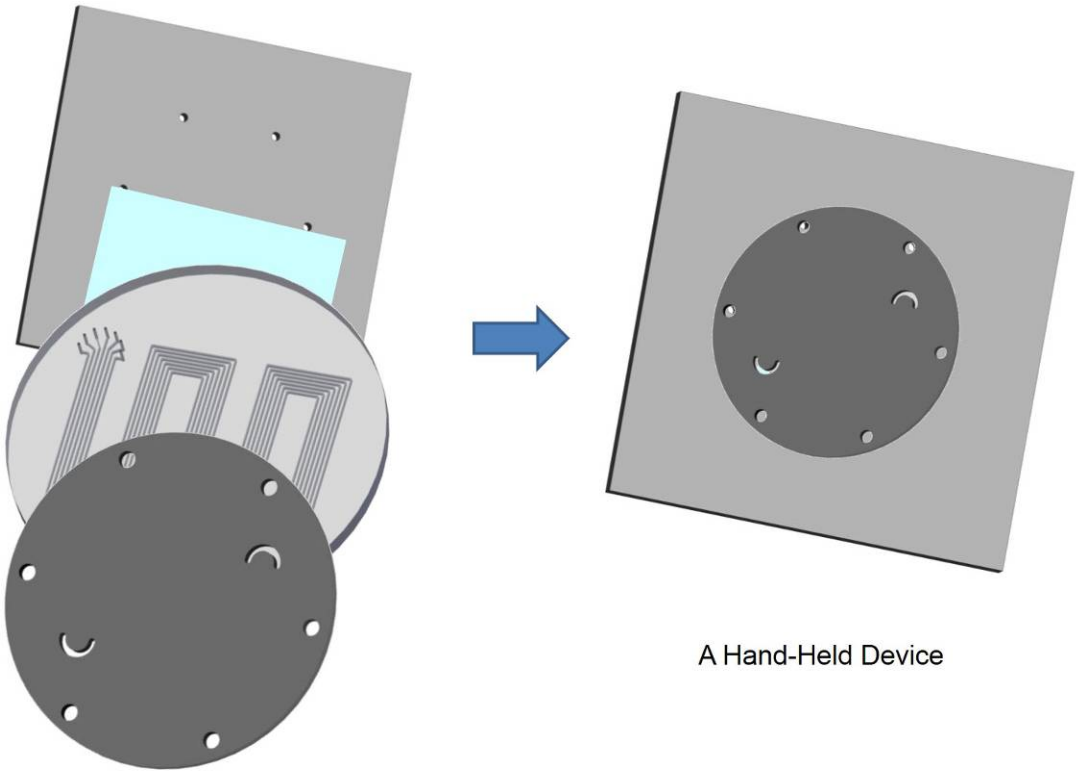
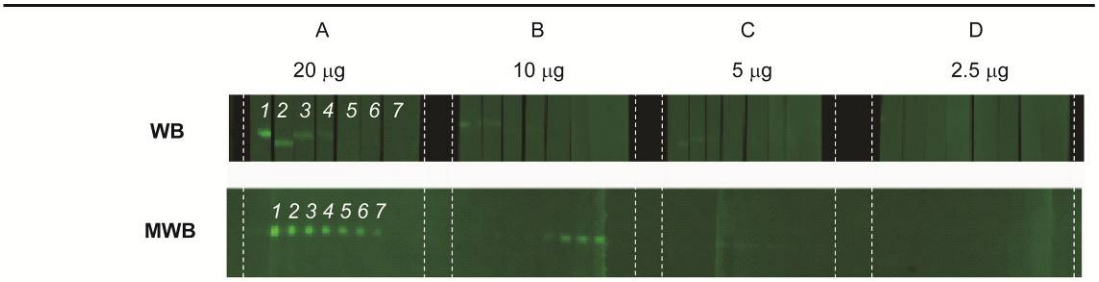


Fig. S3 Schematic illustration for assembling different components of the clamp. From bottom to top, they are the base pad, the PVDF membrane loaded with protein samples, the PDMS microfluidic chip, and the cover pad. They are aligned and fastened with screws.



		A		B		C		D	
		WB	MWB	WB	MWB	WB	MWB	WB	MWB
1	0.2 μg	√	√	√	√	√	√		√
2	0.1 μg	√	√	√	√	√	√		
3	0.05 μg	√	√	√	√		√		
4	0.025 μg	√	√		√		√		
5	0.0125 μg		√		√				
6	0.00625 μg		√						
7	0.003125 μg		√						

Fig. S4 Comparison of sensitivity between the conventional blotting and MMB. We compared their performances point by point with a serial of dilutions of antigens and antibodies.

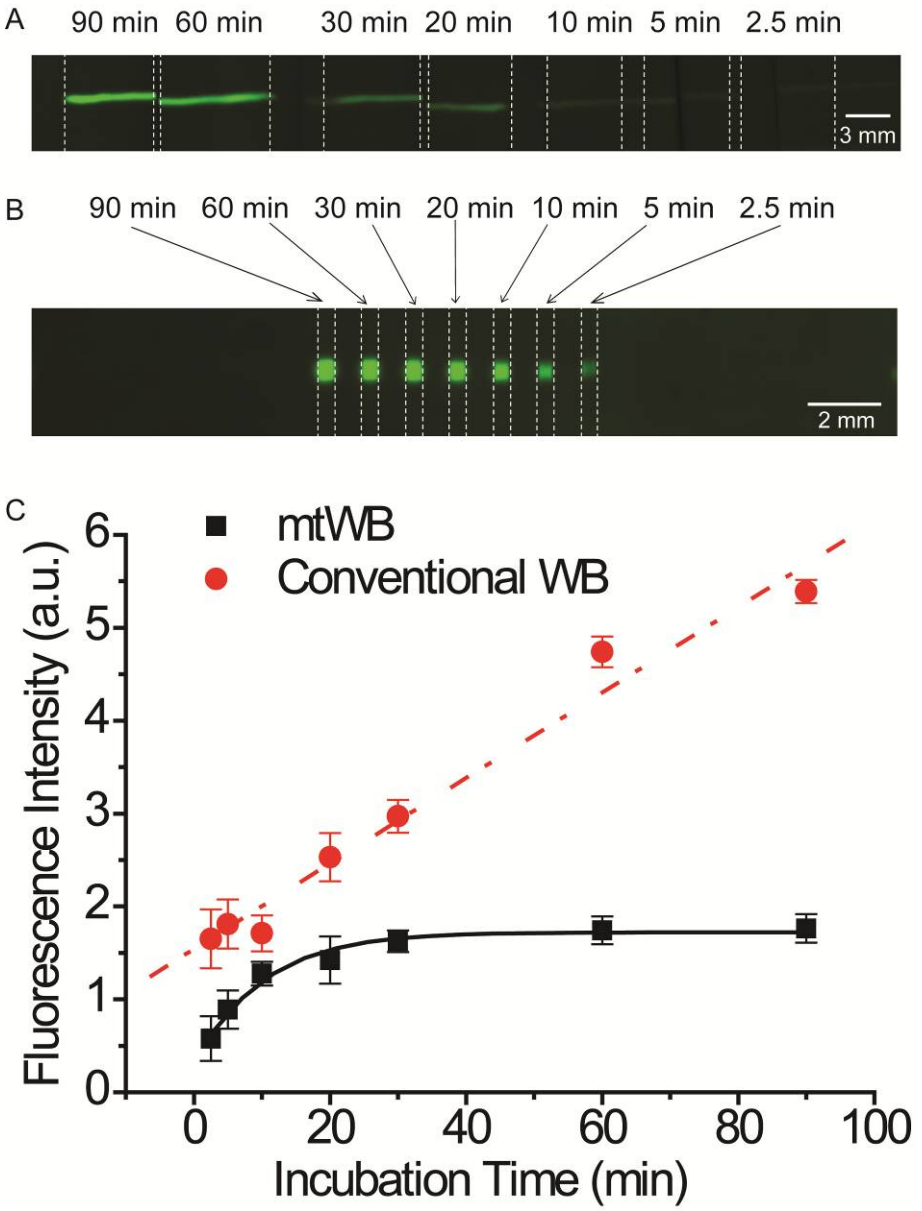


Fig. S5 Comparison of mass transfer between conventional blotting and MMB. (A) The time-dependent fluorescence change for conventional blotting system. The incubation time for the primary antibodies were set to be 2.5 min, 5 min, 10 min, 20 min, 30 min, 60 min and 90 min, respectively. (B) The time-dependent fluorescence change for MMB by using the same concentration of primary antibodies. (C) The quantitative comparison between the total fluorescence intensity in the two scenarios. The fluorescence intensity for MMB was weaker than that in conventional blotting in each step because the antibodies available in the micro-channels were much less than that in bulk incubation chambers. However, the reaction in MMB reached its plateau much sooner than conventional blotting because of the confinement from the tiny sizes of the micro-channels.

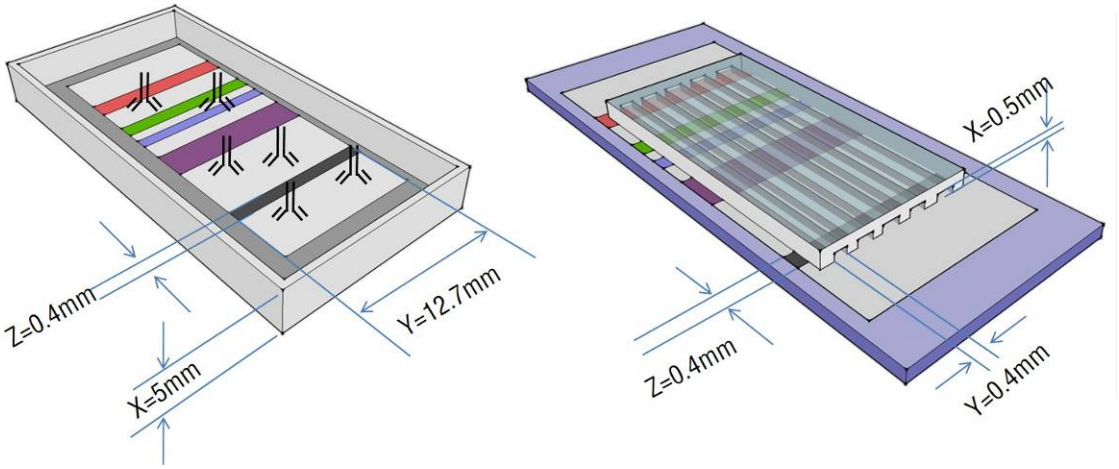


Fig. S6 Schematic illustration of the size of chambers where the reactions take place. The *z* of the conventional blotting and the MMB are the same while the *x* and *y* are different.

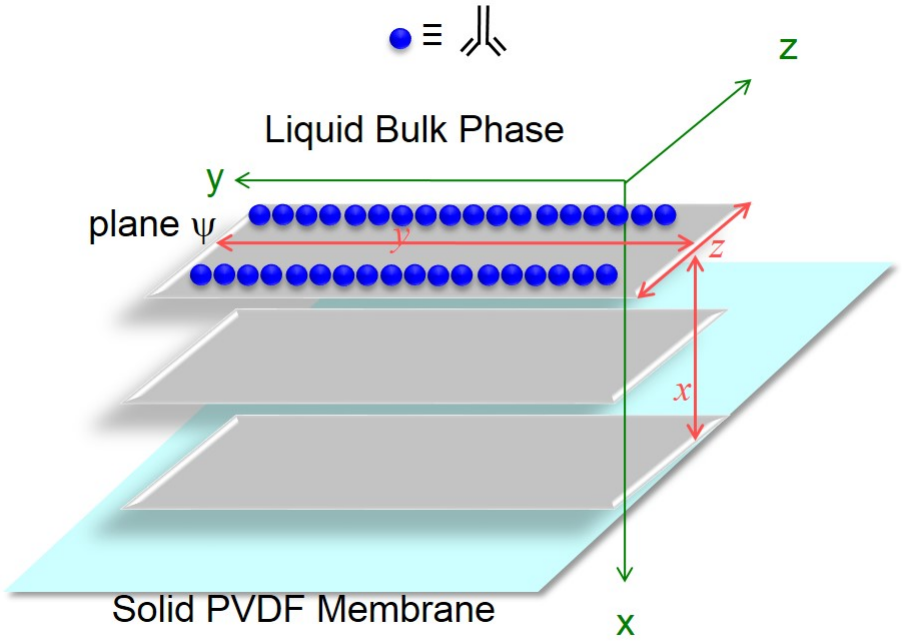


Fig. S7 Theoretical model of the relationship between the chamber size and reaction time. A plane *y* of antibodies has to go from the liquid bulk phase to the solid PVDF membrane with a net displacement of *x*.

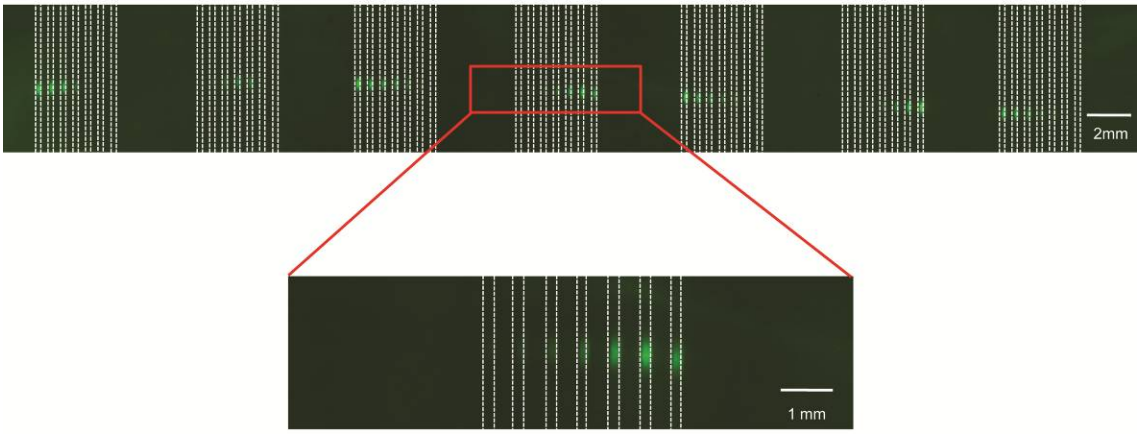


Fig. S8 A typical result for the assay from the microfluidic chip that fits the ten-lane system. There were seven lanes of cell lysates, and each of the lanes coordinated with seven channels.