Membrane Assisted on-line Renaturation for Automated Microfluidic Lectin Blotting

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Supporting Information: Table of Contents

Reagents and Materials

30% acrylamide (29:1 acrylamide/bisacrylamide ratio) stock solution, 99% pure acrylamide powder and bisacrylamide powder, sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Premixed 10× Tris–glycine native electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Premixed 10× Zymogram renaturation buffer (contains 2.5% Triton X-100) and streptavidin-acrylamide were purchased from Invitrogen (Carlsbad, CA). The water-soluble photoinitiator 2, 2-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Alexa Fluor 568-conjugated human serum albumin (HSA, 68 kDa), myosin heavy chain (200 kDa), β-galactosidase (114 kDa), phosphorylase B (96 kDa) were purchased from Invitrogen

(Carlsbad, CA). Alexa Fluor 488-conjugated bovine serum albumin (BSA, 66 kDa) and trypsin inhibitor (21 kDa) were purchased from Abcam (Cambridge, MA). Recombinant full-length Aequorea victoria GFP (27 kDa) and biotinylated goat polyclonal anti-GFP were purchased from Abcam (Cambridge, MA). Biotinylated lectin from Helix aspersa (HAA) was purchased from sigma (St. Louis, MO). The proteins were fluorescently labeled in-house using Alexa Fluor 488 protein-labeling kits per the supplier's instructions (Invitrogen, Carlsbad, CA). Briefly, 50-100 µg antibody (~1mg/mL) was mixed with the commercial provided reactive dye in 1M sodium bicarbonate solution (pH~8.3). Incubate the solution for 1 hour at room temperature. Every 10 – 15 minutes, gently invert the vial several times in order to mix the two reactants and increase the labeling efficiency. Extra free dye was removed by using micro-spin column (30kDa cut-off). Labeled proteins were stored at 4°C in the dark until use.

IgA1 Sample Preparation

Naturally galactose-deficient IgA1 myeloma protein was isolated from plasma of a patient with multiple myeloma[1]. Plasma was precipitated with ammonium sulfate (50% saturation). The precipitate was then dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) prior to fractionation by ion-exchange chromatography on DEAE-cellulose. The purity of the IgA1 preparations was assessed by SDS-PAGE and Western blotting using an IgA-specific monoclonal antibody and IgA concentration was measured by ELISA. The molecular form of the IgA1 proteins was assessed by size-exclusion chromatography, SDS-PAGE under non-reducing conditions, and Western blots developed with anti-IgA antibody[2, 3].

Pooled single-donor normal human serum (freshly collected, IPLA-CSER) was purchased from Innovative Research (Novi, MI). Normal human serum IgA1 was purified by using Slide-A-Lyzer dialysis (Thermo Scientific), followed with Peptide M/Agarose column (InvivoGen).

Microfluidic Chip Fabrication

Glass microfluidic chips were designed in-house and fabricated using standard wet etch processes by Caliper Life Sciences (Hopkinton, MA). Chip layouts consist of a cross injector and a 0.5×2 mm^2 rectangular chamber connected to the reservoirs (3 µL volume each) *via* microchannel arrays (mask design: 20 µm deep and 10 µm

wide; actual width: ~50 μm due to isotropic etching) on each side (Figure 1). A pack of side channels with interval spacing 100 μm, 50 μm, or 10 μm connected to microchamber was used to form the membrane-enclosed individual compartment for protein on-line renaturation. The rectangular chamber housed the separation and blotting gels. The microchannel arrays were designed to yield uniform electric fields over the large chamber in vertical and horizontal dimensions. The chip geometry was chosen to be compatible with CCD-based imaging on the described epi-fluorescence microscopy system. Prior to the introduction of precursor solutions for gel fabrication, the glass chip was first incubated for 30 minutes with a 2:2:3:3 mixture of silane, acetic acid, methanol and water. This silanization step was critical in linking the polyacrylamide to the glass so that the gel would not shift under the extended application of an electric field.

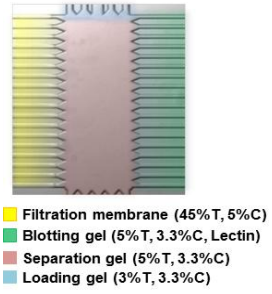
Multifunctional Polyacrylamide Gel Photopatterning[4]

Spatial control over polyacrylamide gel properties provides a crucial tool for integrating the lectin blotting functionality. Several functional gel regions were sequentially photopatterned within the microchamber and side channels using a four-step process. The lithography was accomplished *via* a UV objective (UPLANS-APO 4×, Olympus) in combination with a transparency film mask and epi-fluorescence microscope system (Nikon Diaphot 200, Japan). A Hamamatsu LightningCure LC5 UV light source (Hamamatsu City, Japan) with variable intensity control was used for photopatterning of the gels. The UV beam from the LC5 was directed along the light path of a Nikon Diaphot 200 inverted microscope and up through a UV-transmission objective lens.

The blotting gel was fabricated by exposing a region filled with a 5%T, 3.3%C precursor solution (diluted by 1× Tris–glycine native electrophoresis buffer containing 0.4 mg/mL streptavidin-acrylamide and 1 mg/mL biotinylated lectin) to UV excitation (~12.5 mW/cm^2) for 330 s. The notation %T and %C indicate the percentage of total acrylamide and cross-linker, respectively. Here, covalently bonded streptavidin in the gel matrix was used to immobilize biotinylated antibodies or lectins for immunoblotting[5]. In Step 2, mask alignment to the chip was performed using a manual adjustable x-y translation stage on the microscope to subsequently photopattern an array of 500 μm wide renaturation membranes across a pack of side channels. The membrane interface was determined by observing through the microscope eye piece and aligned at the junctions of side

channels with microchamber. The composition of the membrane precursor solution is 45%T and 5%C, prepared by dilution of 99% pure acrylamide and bisacrylamide powders using 1× Tris–glycine native electrophoresis buffer. The exposure was performed at UV intensity of ~40 mW/cm^2 for 85 s. The PAGE separation gel had a composition and structure similar to the blotting matrix albeit with no immobilized antibodies (5%T, 3.3%C, diluted by 1× Tris–glycine native electrophoresis buffer containing 0.01% TitronX-100, ~10 mW/cm^2 for 300 s). Titron X-100 in separation gel was used to match the buffer strength with sample buffer in high SDS concentration. Finally in Step 4, a larger-pore-size loading gel was formed using 3%T, 3.3%C acrylamide solution and an 8-min flood exposure of the chip to a filtered mercury lamp (300-380 nm, 10 mW/cm^2, UVP B100-AP, Upland, CA) with cooling fan. Each functional region was indicated in figure below rendered with false color. The photopolymerization times reported were determined empirically based on the intensity of each UV light source, composition of acrylamide precursor solution, and desired pore-size to achieve optimal gel performance for the desired function.

Each precursor solution was introduced by pressure-flushing the previous un-polymerized solution away. Each precursor contains 0.2% (w/v) VA-086 photoinitiator. Quiescent conditions were necessary inside the large chamber to ensure a high-resolution photopatterning process and were established by applying 5% HEC drops onto each reservoir after precursor loading. A 10-min equilibration period was implemented before UV exposure. After use, the glass chip housings were regenerated through removal of the cross-linked gels[5]. Used chips containing polyacrylamide gels were soaked in a 2:1 mixture of perchloric acid (Sigma, ACS grade, 70 wt%) and hydrogen peroxide (Sigma, ACS grade, 30 wt%) at 75℃ overnight. Care should be taken when working with the perchloric acid and hydrogen peroxide solution, including all proper protective equipment and other safety controls. After gel dissolution, channels were flushed using 0.1 M sodium hydroxide for 30 min.



Apparatus and Imaging Analysis for On-chip Assays

Image collection was performed by using a CCD camera (CoolSNAP^{TM} HQ2, Roper Scientific, Trenton, NJ) equiped with a shutter system and an inverted epi-fluorescence microscope (IX-70, Olympus, Melville, NY) with a 10x objective (UPlanFL, N.A. = 0.3). Camera exposure time was 400 ms with a 10 MHz frequency. This resulted in a full-field image representing a ~1 mm x 1.34-mm field of view. Use of full-field imaging allows all analytes to be simultaneously observed during protein separation, renaturation, transfer and final blotting in illumination shutter control. Light from a mercury arc lamp was filtered through XF100-3 or XF111-2 filter sets (Omega Optical, Brattleboro, VT) for illumination of AlexaFluor 488- and 568-labelled proteins, respectively. Two-color images were compiled from individual red and green wavelength image sequences taken in two separate runs upon the same device. Identical conditions and timing were maintained for both runs. Image analysis was performed using ImageJ and regions of interest corresponding to the separation, renaturation and blotting regions were selected and consistently applied. The fluorecence profile was plotted using ImageJ across the regions of interest. The fluorescent signal was normalized to background. Separation resolution (*SR*) between protein bands is defined as $SR = \Delta L/4\sigma$, where L is the distance between adjacent band centers and represents the average characteristic band width (Gaussian distribution fitting with OriginLab).

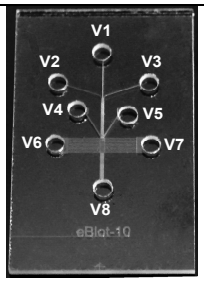
Electrical Control Program with Buffer Exchange

After sample addition to the chip, assay operation was programmable and controlled via a power supply equipped with platinum electrodes (Caliper Life Sciences). The sample (V3), sample waste (V2), buffer (V1, V4, V5, V7), and buffer waste (V6, V8) reservoirs are indicated in Table S1. Applied current control was used, as listed in Table S1.

Buffer exchange was performed during renaturation process as indicated in Table S1. After SDS-treated proteins were transferred into individual renaturation compartments, the electric flow was stopped. Each renaturation compartment physically confines the resolved protein without sample dispersion. By pipetting ~10 μL 1× Zymogram renaturation buffer (contains 0.25% Triton X-100) into reservoirs 7 and 6, the running buffer

was replaced to renaturation buffer. Electric field was applied again in lateral direction for 10 s. Then, buffer was replaced in reservoirs 7 and 6, and the electric current control was applied again. The renaturation buffer was flushed for another 50 s. This renaturation process is effective for cleaning SDS from proteins in concentration up to 12% (w/v) under investigated condition without reducing agents. As demonstrated from both on-chip and slab-gel SDS-PAGE of standard molecular weight ladders, 3% and 5% SDS concentrations are sufficient to cover large proteins (200 kDa) with uniform charge and produce smooth linear correlation for molecular-weight calibration.

Table S1 Programmable electric control sequences over chip layout. The chip reservoirs 1to 8 are labeled in the inset chip image.

 **Applied current control (μA) in each chip reservoir/ Duration (s)**			**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**
① **sample loading/ 60 s**			-3	9	-3	0	0	0	0	-3
② **separation/ 30-60 s**			-5	0.5	0.5	-4	-4	0	0	12
③ **transfer for renaturation/ 10-30 s**			0	0	0	0	0	2	-2	0
④ **renaturation**	**Stop electric flow for buffer exchange /20 s**		0	0	0	0	0	0	0	0
	Flush renaturation buffer /50 s		0	0	0	0	0	2	-2	0
	Oscillating voltage (repeat five circles)/40 s	**4 s**	0	0	0	0	0	-0.5	0.5	0
		4 s	0	0	0	0	0	0.5	-0.5	0
⑤ **transfer to blot/ 30-60 s**			0	0	0	0	0	-2	2	0

The applied electric field in the vertical and horizontal dimensions within the designed geometry was simulated by using COMSOL Multiphysics (Version 4.0a, COMSOL AB) in Figure S1A. The straight and uniform electric field distribution [6] provided the basis for precisely manipulating the sample during separation, renaturation, transfer and blotting in three directions. Both experiments and simulation showed a well controlled electric field distribution in the vertical and horizontal dimensions within the designed geometry (Figure S1). In Figure S1b, a

continuous loading of GFP stream vertically was used to prove the horizontal transfer process in less dispersion. The distortion of the stream shape after crossing separation gel twice is below 5% (see Figure S1b).

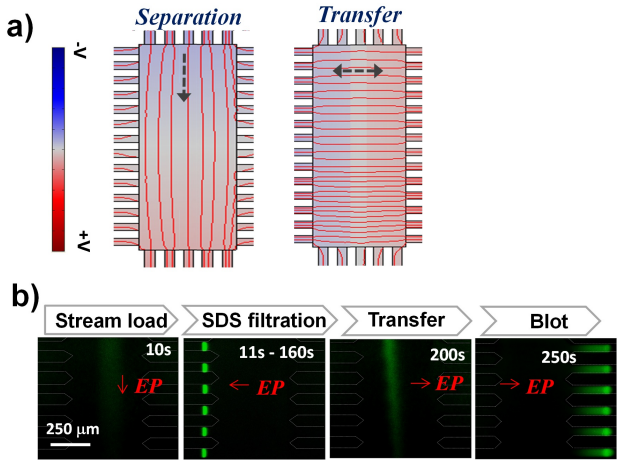


Figure S1. a) COMSOL simulation shows the electric field distribution within the chip chamber geometry during the separation and lateral transfer process. b) Fluorescence micrographs illustrate the four-step renaturation of 5% SDS treated GFP in the microchamber flanked by (left) small pore-size filtration membranes and (right) antibody-laden blotting membranes. Renaturation took place after stream loading between 11 s to 160 s. The straight stream shape was preserved after multi-direction transfer.

Helix aspersa (HAA) Binding Affinity

For comparison to on-chip lectin binding efficiency in cross-linked PA blotting gel, the dissociation constant was measured by using 96-well plate with active amine surface functionality (round plate, Corning). Following the standard direct ELISA protocols, the biotin-conjugated HAA was immobilized onto well surfaces. The PBS buffer and SuperBlock T20 (PBS) Blocking Buffer (Thermo scientific) were used for washing and blocking steps. Series diluted IgA1 (galactose-deficient) solutions in 488 Alexa Fluor labeling were incubated in well for 2 h at room temperature. The emission fluorescence was read by using TECAN plate reader (Infinite V200 pro). For on-chip dissociation constant measurement, the blotting gel was fabricated by exposing a region filled with a 5%T, 3.3%C precursor solution (diluted by 1× Tris–glycine native electrophoresis buffer containing 0.4 mg/mL streptavidin-acrylamide and 1 mg/mL biotinylated HAA) to UV excitation (~12.5 mW/cm^2) for 330 s. Series diluted IgA1 (galactose-deficient) solutions with 488 Alexa Fluor labeling were electrophoresed into chip and

bind to blotting gel plug[5]. The bound fluorescence on blotting gel was measured. The dissociation constant was obtained by fitting the response curves in binding equation: B = Bmax * C/(Kd+C), where B is the signal from binding complex, and C is the antigen concentration. Compared from Figure S2, the on-chip 3D porous polymer networks produce a heterogeneous phase and yield a high surface-area to volume ratio structure, allowing more efficient binding. In conjunction with the lectin-decorated 3D polymeric materials, we utilize directed transport (electrophoretic) to bring protein in proximity to HAA binding sites, thus facilitate the binding process.

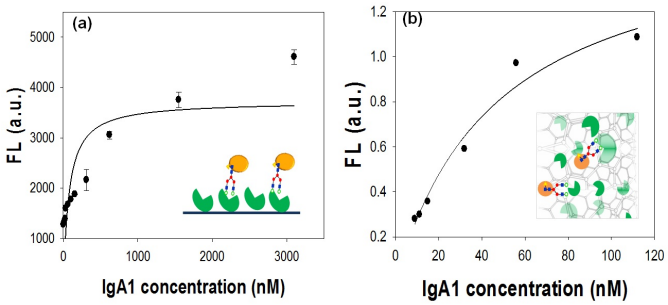


Figure S2. The dissociation constant of HAA binding to galactose-deficient IgA1 measured by ELISA (a) and on-chip lectin blotting gel (b). The Kd measured by ELISA through surface immobilization is 73.1 nM (a). The Kd measured through on-chip lectin blotting gel is 47.2 nM (b).

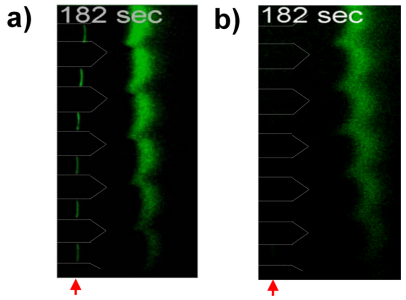
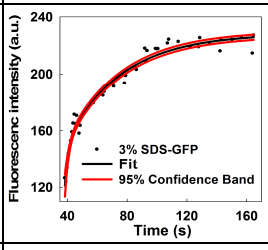
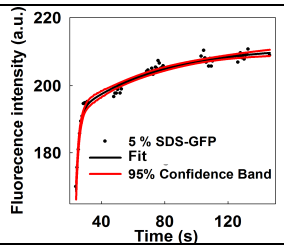
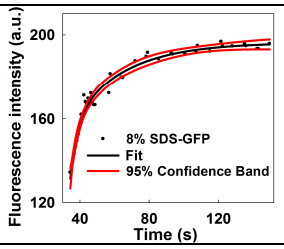
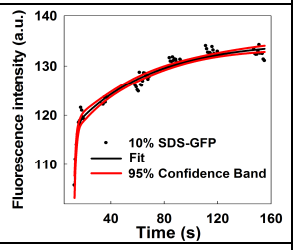


Figure S3. The CCD images showing the membrane interfaces after renaturation transfer for blotting. The red arrows indicate the membrane interface positions. The substantial protein residues were observed at membrane interfaces after lateral transfer, indicating the sample loss (a). Through applying oscillating voltage sequences at the end stage of renaturation, the post-renaturation transfer performance was improved; the sample loss was reduced by ~ 16.6%.

On-chip Renaturation Kinetics

Unlike that for pre-labeled proteins, the fluorescence of GFP is closely related to its native state. GFP denaturation process is reversible by showing the return of visible fluorescence. The fluorescence recovery makes GFP a good model for monitoring the renaturation kinetics. The recovery of renatured GFP is calculated by normalizing to corresponding fluorescence of non-denatured GFP at the same conditions. Plot the recapitulated fluorescence at a given time, leading to a kinetic trace obtained experimentally. Then this trace could fit to a double-exponential function: $\langle Q(t)\rangle = A_0 - A_1\exp(-k_1 t) - A_2\exp(-k_2 t)$, where Q(t) is the value of recapitulated fluorescence as a function of time, and A0, A1, A2, k1, and k2 are free parameters in the fitting, corresponding to the relative amplitudes and the rate constants of the phases, respectively. This fit allowed for the determination of the rate constant and half time in the kinetic mechanism. In this work, the renaturation process starts from transfer and continues during filtration through membrane. To avoid any enrichment-induced fluorescence growing, the progress-curves were the depiction of fluorescence after complete transfer. The secondary rate constant (k) from renaturation kinetics was used to express the half time according to the function: Half time (t)= ln2/k.

Table S2. Kinetic analysis of renaturation progress under different SDS concentration (w/v%) treatments

Kinetics	3% SDS-GFP	5% SDS-GFP	8% SDS-GFP	10% SDS-GFP
Regression curves				
Correlation	R^2=0.97	R^2=0.98	R^2=0.98	R^2=0.96
Rate constant (s^{-1})	0.0265	0.0189	0.0130	0.0112
Half time (s)	26.1	36.7	53.3	61.8

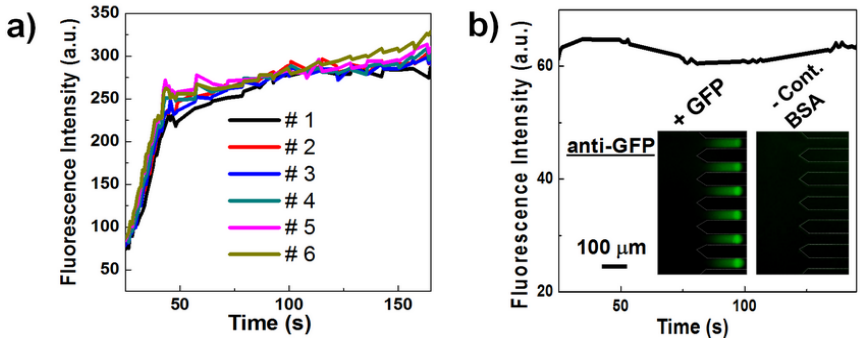
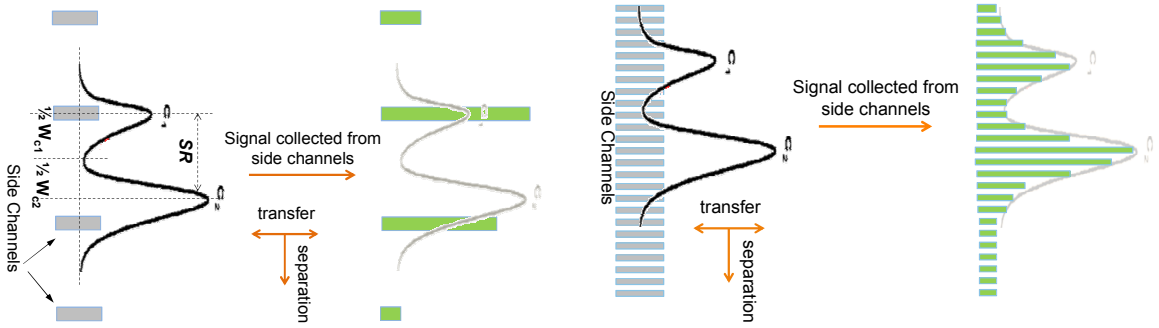


Figure S4. a) The performance of the on-chip renaturation compartment array. A stream loading of 5% SDS-GFP was distributed into each renaturation compartment. The recovered fluorescence was collected from each individual compartment, showing the consistent renaturation efficiency in parallel. Data were collected from 6 parallel renaturation channels indicated in different color. b) On-line renaturation progress-curve from 5% SDS-BSA as negative control. Inset shows the subsequent blotting profile after online renaturation. BSA exhibits non-binding to GFP blotting gel where anti-GFP antibodies are immobilized through streptavidin-biotin linkage.

Nyquist–Shannon sampling based protein collection by renaturation compartment array

According to the Nyquist–Shannon sampling theorem, in order to perfectly reconstruct a signal without any aliasing, the sampling frequency is at least equal or greater than the maximum frequency of the signal being sampled. The sampling theorem asserts that the uniformly spaced discrete samples are a complete representation of the signal if this sampling bandwidth (here the channel space) is equal or less than the bandwidth of signal being sampled (here the protein bandwidth). Based on this theorem, the sufficient condition for exact reconstructability from samples in a uniformly spaced channel array is: $Ws \leq Wp$. Here the Ws is the channel space interval in the side channel array, and Wp is the protein band width (see illustration in figure a and b below).



(a) Aliasing causes the decreased separation resolution

(b) High density of side channels produces well reconstructed signal without aliasing

The separation resolution is defined by $SR = \frac{C_2 - C_1}{1/2W_{C1} + 1/2W_{C2}}$. A baseline separation is *SR*=1 (see illustration in Figure (a) above). For two proteins with bandwidth of ~50 µm in baseline separation, the width between two peak centers is about 50 µm. The sufficient condition of a complete representation of the *SR* is that the interval of collection side-channel is equal or less than 50 µm.

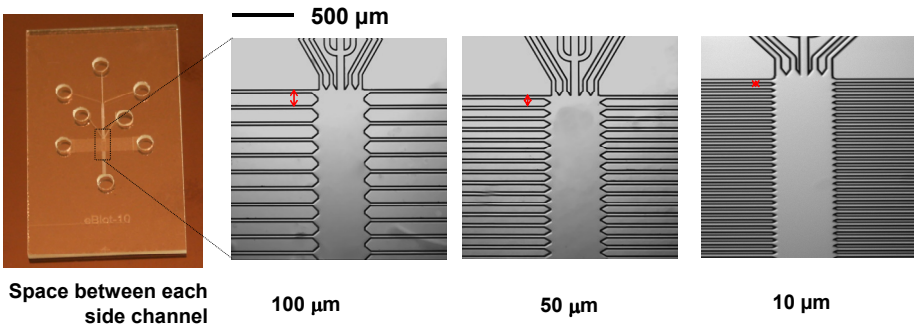


Figure S5: Bright field images of various array network designs in glass chips. Both the width of each side channel and the spacing between neighboring channels can be adjusted. Here the channels are ~50 um wide, with spacing between channels shown as: 100 µm, 50 µm, and 10 µm, indicated by the red arrows in Figure.

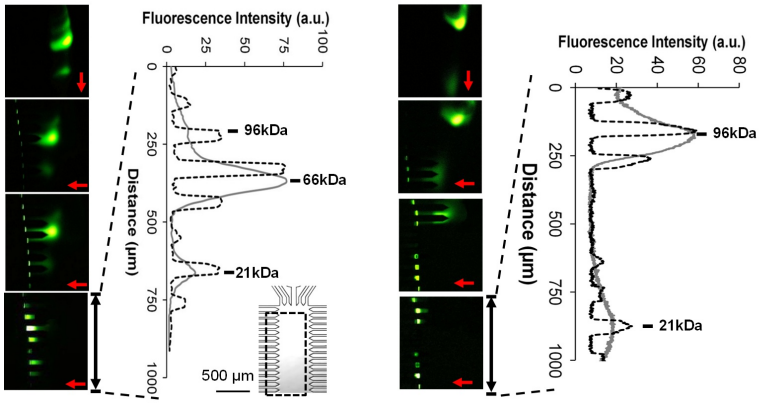


Figure S6. Fluorescence micrographs report time evolution of integrated assay for several model proteins (phosphorylase B 96 kDa, bovine serum albumin 66 kDa, trypsin inhibitor 21 kDa, 5% SDS treatment). Plots of fluorescence intensity distribution on separation axis (gray lines) are compared to fluorescence intensity distribution in both MWCO microfilter array (dashed black line) and blotting array (dashed black line). Arrows indicate the direction of electrophoresis. Array channel spacing is ~50 um. Chip design and imaging region are

shown in inset. The spaced discrete samples can completely represent the protein signal in PAGE separation region.

Table S3 The variance characterized from parallel side channel sampling during the lateral transfer process.

	MW shifting (kDa)[a]		SR variance (RSD%)	
	50-μm spacing microchannel array	10-μm spacing microchannel array	50-μm spacing microchannel array	10-μm spacing microchannel array
SR>1.5	~2	-	3.1%	-
SR=1~1.5	~8	~2	3.5%	3.2%
SR<1	~12	~5	9.3%	3.3%

a. MW shifting from MW calibration was based on center peak shifting

Slab PAGE Lectin Blotting and *Helix aspersa* (HAA) affinity

Slab gel SDS PAGE and lectin blotting were performed by using Tris-glycine pH 8.3, 4-12% precast polyacrylamide slab mini-gels with XCell *SureLock* Mini-Cell & XCell II Blot Module (Invitrogen Novex). For non-reduced SDS-PAGE, proteins were added to a 15 μl sample buffer containing 125 mM Tris, pH 8.3, 0.005% bromophenol blue, 20% glycerol, 12% SDS (gel loading volume ~ 25 μl). Relative molecular weights were estimated using protein standard ladders (All blue, BioRad). Slab PAGE was visualized by post-staining with a Colloidal Blue Staining Kit (Invitrogen). After electrophoresis at 125 V for 2.5 h, gels were electrotransferred to PVDF membranes (0.2 μm) at 25 V for 2.5 h. After twice washing with wash buffer (1×PBS, 0.5% Tween 20, pH 7.4), the PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 1% Tween 20 overnight at 4℃ with shaking. The solution of biotinylated HAA lectin (1 μg/mL) was added into blocking buffer for 2-h incubation with shaking at room temperature. After incubation, the membrane was washed 3 times for 10 min each. The Extravidin-HRP (Invitrogen, ELISA grade, 1.1 mg/mL) diluted 1:2000 in the blocking solution was added for 1-h incubation at room temperature with agitation. After that, the washing step was repeated three times, followed with the membrane rinse with water for 1 min. The membrane was subsequently developed with Novex Chromogenic Substrate Reagent (Invitrogen) until the desired band intensity was achieved. Imaging was performed by using ChemiDoc XRS (BioRad) with a proprietary filter. We have tested 1% nonfat dry milk and BSA as blocking solution, which produced a very high background for lectin blotting, probably due to the glycoprotein contamination in these blocking solutions.

To correlate the lectin reactivity of serum sample to its IgA1 molecular identity, Western blotting with anti-IgA antibody (α chain-specific) was performed following standard protocol. Biotin-conjugated goat $(Fab')_2$ anti-human IgA (α-chain-specific, Biosource) was used as primary antibody to identify the IgA band. This validation indicates that the lectin affinity was solely contributed by IgA1 protein. In antibody probing case, 1% nonfat dry milk blocking solution (Invitrogen) was used for blocking. The Extravidin-HRP (Invitrogen, ELISA grade, 1.1 mg/mL) was used as secondary antibody by adding to blocking solution for 1-h incubation at room temperature. The membrane was subsequently developed with Novex Chromogenic Substrate Reagent (Invitrogen) and Imaged by ChemiDoc XRS (BioRad).

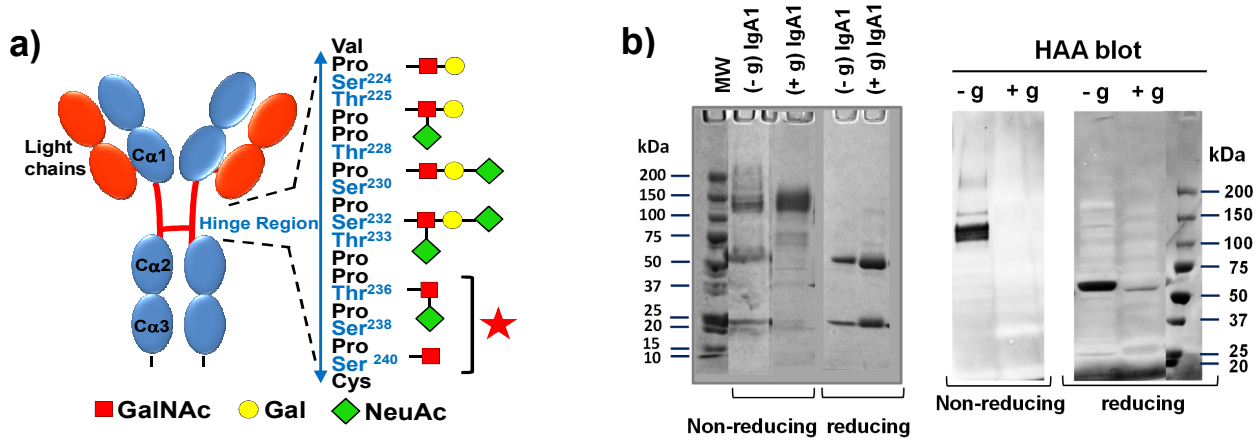


Figure S7. a) Possible *O*-glycan structures in hinge region of human IgA1, including aberrant glycosylation, *i.e.,* galactose-deficient variants (two bottom structures indicated by red star). Ser/Thr residues as potential sites of *O*-glycan attachment are in blue. Only up to 6 sites are usually glycosylated[2]. NeuAc, *N*-acetylneuraminic acid; Gal, galactose. b) Non-reducing SDS-PAGE of galactose-deficient IgA1 (-g) myeloma protein and IgA1 from normal human serum (+g), compared with reducing PAGE condition. HAA blotting of galactose-deficient IgA1 (-g) myeloma protein, compared with normal human serum IgA1 (+g). The weak positive response (non-specific) from normal human IgA1 under reducing condition was observed. Tris-glycine pH 8.3, 4-12% precast polyacrylamide slab mini-gel. Compared with reducing conditions that only showed H and L chains, IgA1

protein shows 160 kDa monomer form under non-reducing condition, which would be well resolved from IgG (150 kDa) .

References:

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