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Supplementary Materials:

Cytokine Analysis at Countable Number of Molecules from Living Single Cell on Nanofluidic Device

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Pressure-Driven Flow Control of The Micro/Nano-Integrated Fluidic Device

In order to operate the micro/nano-integrated fluidic device, liquid flow was controlled using a pressure-driven flow control method developed by our group.[1] Furthermore, liquid-air interfaces were inserted into the channels as necessary to prevent sample dispersion and unintended mixing of reagents via mutual diffusion. These liquid-air interfaces were regulated using Laplace valves that exploit wetting on hydrophobic surfaces.[2]

By controlling the balance of external pressures, liquid can be driven in the desired direction. External pressures for controlling the movement of fluids in the nanochannels were determined based on the Hagen-Poiseuille equation:

$$\Delta P = \frac{8\mu L}{\pi r^4} Q \tag{S1}$$

where Q represents the flow rate, ΔP represents the pressure drop across the channel, μ represents the viscosity of the solvent, L represents the length of the channel and r represents the radius of the channel. As the cross-section of the nanochannels is rectangular, we used a modified version of the Hagen-Poiseuille equation for rectangular channels:[3]

$$\Delta P = \frac{8\mu L}{ab^3 X} Q \tag{S2}$$

where a represents the width of the channel, b ($< a$) represents the depth of the channel, and X represents the following parameter:

$$X = \frac{16}{3} - \frac{1024b}{\pi^5\ a}\left[\tanh\frac{\pi a}{2b} + \frac{1}{27}\tanh\frac{3\pi a}{2} + \cdots\right] \tag{S3}$$

when we define the fluidic resistance as

$$R\ = \frac{8\mu L}{ab^3 X}, \tag{S4}$$

the Hagen-Poiseuille equation is transformed to

$$\Delta P = RQ \tag{S5}$$

In the case of micro/nano-integrated fluidic device, fluidic resistance is described as

$$R = R_{\text{cap}} + R_{\text{micro}} + R_{\text{nano}} \tag{S6}$$

where R_{cap}, R_{micro} and R_{nano} represent the fluidic resistances of the capillary, microchannel, and nanochannel respectively. As values of R_{cap} and R_{micro} are smaller than that of R_{nano} by more than 1000-fold, R_{cap} and R_{micro} can be neglected.

In order to control injection of the 9 reagents into nanochannels through microchannels as illustrated in Figure. S2, the fluidic resistances of the nanochannels R_{nano} were calculated using the width, depth and length evaluated by scanning electron microscopy and a nanoscale optical profiler (WYKO NT9100A, Bruker Corp., MA, USA) (see Table S1). The designed values of external pressure used for fluidic operation are shown in Table S2.

To operate the fL-volumetric pipette and pL-flask, a Laplace valve was utilized as illustrated in Figure S3. The value of Laplace pressure (P_L) used in the Laplace valve was determined using the Young-Laplace equation:

$$P_L = -2\gamma cos\theta\left(\frac{1}{a}+\frac{1}{b}\right) \quad \text{(S7)}$$

where γ represents the surface tension and θ represents the surface contact angle. When the surface wettability is hydrophobic ($\theta > 90°$), P_L takes a positive value and functions as a pressure barrier against introducing liquid. We implemented 2 different Laplace valves as shown in Figure S3. Laplace valve 1 exploits the difference in surface wettability (hydrophobic versus hydrophilic) to halt flow of the sample solution when no pressure is applied. Laplace valve 2 was placed in the main channel to control the sampling volume. In order to precisely control sampling volume on a fL-scale level, we chose to control the Laplace pressure via channel size. A pL-flask of a circular shape was designed so that it can smoothly accommodate and transfer the sample via pneumatic pressure.

Surface Modification of The Micro/Nano-Integrated Fluidic Device

To realize comprehensive integration of entire chemical processing of living single-cell protein analysis, it was necessary to incorporate the following four different functional surfaces into the nanochannels via surface chemical modifications: octadecylsilane (ODS) for hydrophobicity, silanol (OH) for hydrophilicity, capture antibody for selective capture of IL-6, and polyethylene glycol (PEG) for preventing non-specific adsorption.

As shown in Figure S4, the four different functional surfaces were incorporated using partial surface modification methods by combination of optical patterning and low temperature bonding[4, 5] and flow of liquid reagents within the channels.[6]

The optical patterning method involves photo decomposition of the self-assembled monolayer (SAM) modified on a glass substrate by irradiation of vacuumed ultra violet (VUV, λ = 172 nm) light as shown in Figure S4. VUV light converts oxygen to ozone, and the ozone cleaves the covalent bond between the SAM and the surface silanol group on glass. A photomask is used for patterning of the decomposition. Only the part of the monolayer protected by the photomask remains on the surface while the unprotected monolayer is decomposed.

Two methods to facilitate flow of liquid reagents in the channels were developed: liquid/liquid flow and liquid/gas flow. In the liquid/liquid flow method, as shown in Figure S4, when a modification reagent is introduced into the nanochannel, a solvent is also introduced via another channel. The liquid/liquid interface is maintained by balancing the pressure at the interface and only the place where the regent made contact is modified. The precision of partial modification is expected to be the distance of diffusion of the modification reagent (~10 μm, dependent on the flow velocity). In contrast, in the liquid-gas flow method, a gas is introduced instead of the solvent, and the liquid/gas interface is maintained. The precision of partial modification is expected to be the size of the meniscus of the liquid (~μm). We chose liquid/liquid flow method for modification with organic solvents and the liquid/gas flow method for modification with aqueous solvents, as maintenance of the liquid/gas interface is difficult when the surface tension is low.

The details of the modification procedure for the device, which consists of (step1) pre-modification of the ELISA channel, (step 2) hydrophobic/hydrophilic-modification of the fL-volumetric pipette, (step 3) hydrophobic/hydrophilic-patterning of the single-cell chamber and (step 4) antibody modification of

the ELISA channel, as illustrated in Figure S5, are described below:

Step1. Pre-modification of the ELISA channel for scaffolding of antibody and blocking (Figure S5(a))

1-1. To form a pattern of amino group as scaffolding for the capture antibody in the ELISA channel, an optical patterning method was utilized. First, a SAM of 3-amino-propyl triethoxysilane (APTS, Sigma Aldrich, St. Louis, MO, USA) was formed on the substrate by gas-phase modification. The substrate was then washed with toluene, ethanol and water, and dried with N_2 gas. Next the substrate was irradiated with VUV (Ushio Inc., Tokyo, Japan) to decompose the SAM of APTS in unwanted areas. The substrate was bonded with another substrate with nanochannels to form the confined nanochannels. The bonded substrates were heated at 110°C for 3h at a pressure of 5000 N to enhance the bonding using a custom-built system (Bondtech, Co. Ltd, Kyoto, Japan).

1-2. The remainder of the channel surface was modified with PEG for blocking using the liquid/liquid flow method. A solution of trimethoxysilane-PEG (molecular weight = 5000, NANOCS, New York, NY, USA) (0.1% [wt/wt] in water/ethanol [5/95]) was used as the PEGylation reagent. PEG solution was introduced into the nanochannels by pressure-driven flow, while ethanol was also introduced to form the liquid/liquid interface in the pL-flask. The formed interface was maintained for 2 hours at room temperature for the modification reaction. After modification, the channels were washed with ethanol for 15 min at 100-200 kPa. The ethanol inside the device was then replaced with acetone, and subsequently toluene by applying pressure at 100-200 kPa for 20-30 min.

Step 2. Hydrophobic/hydrophilic-patterning of the fL-volumetric pipette (Figure S5(b))

2-1. To partially modify the fL-volumetric pipette with ODS to render the surface hydrophobic, a liquid/liquid flow method was utilized. Octadecyldimethyl-N,N-diethylaminosilane (ODS-DEA) was synthesized from chlorodimethyl-N-octadecylsilane (Alfa Aesar, Haverhill, MA, USA) and diethylamine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to a previous study.[7, 8] For modification, 20 %(v/v) of ODS-DEA/toluene solution was introduced into the nanochannels by pressure-driven flow while toluene was introduced from other nanochannels to form the liquid/liquid interface in the pL-flask. The liquid/liquid interface was maintained for 4 h at 70°C to facilitate modification. After modification, all of the microchannels and nanochannels were washed with toluene for 30 min at 70°C. The toluene inside the device was then replaced with acetone, ethanol and water (in that order), and finally, the channels were dried with air.

2-2. To form a surface pattern of OH groups in the fL-volumetric pipette and render the surface partially hydrophilic by removing ODS, liquid/gas flow of NaOH solution and air was utilized. A solution of 1 M NaOH was introduced into a nanochannel while air was introduced from other nanochannels, and the liquid/gas interface was formed at a position between the single-cell chamber and fL-volumetric pipette. The interface was maintained for 15 min for patterning by removing the modified-ODS. All of the channels were then washed with water at 100-200 kPa for 15 min. This procedure was conducted twice to complete the patterning. The water in the nanochannels was then replaced with ethanol, acetone, and toluene in that order.

Step 3. Hydrophobic/hydrophilic-patterning of the single-cell chamber (Figure S5(c))

3-1. To ODS-modify the microchannel in front of the single-cell chamber to render the surface hydrophobic, 20 % (v/v) ODS-DEA/toluene solution was introduced into the microchannel. To prevent ODS-DEA solution from penetrating into the nanochannels, toluene was introduced from the nanochannels, and a liquid/liquid interface was formed in the single-cell chamber. The interface was maintained for 1 h at 70°C to complete the modification. After modification, all of the microchannels and nanochannels were washed with toluene at 20-100 kPa for 30 min at 70°C. The toluene inside the device was then replaced with acetone, ethanol and water (in that order), and the channels were dried with air.

3-2. To render the surface inside the single-cell chamber hydrophilic with OH groups to prevent non-specific adsorption, liquid/gas flow of NaOH solution and air was utilized. The NaOH solution was introduced into the single-cell chamber via the stimulation nanochannel (nanochannel 3 & 4), while air was introduced from other nanochannels and microchannel to control the position of the liquid/gas interface. The interface was maintained for 15 min for patterning. All of the channels were then washed with water at 100-200 kPa for 15 min. This procedure was conducted twice to complete the patterning. Finally, the water in the nanochannels was replaced with ethanol, acetone, and toluene.

3-3. To render the microchannel for stimulation hydrophobic to prevent liquid leakage from the single-cell chamber, 20 % (v/v) ODS-DEA/toluene solution was introduced into the microchannel. To prevent ODS-DEA solution from leaking into the nanochannels, toluene was introduced from the nanochannels and a liquid/liquid interface was formed. The interface was maintained for 1 h at 70°C to complete the modification. After modification, all of the microchannels and nanochannels were washed with toluene at 20-100 kPa for 30 min at 70°C. The toluene inside the device was replaced with acetone, ethanol and water (in that order), and the channels were dried with air.

Step 4. Modification of the ELISA channel with capture antibody (Figure S5(d))

4-1. To modify the ELISA channel with capture antibody, liquid/gas flow was utilized. The gas/liquid interface was formed between the fL-volumetric pipette and pL-flask to prevent antibody solution from leaking into the fL-volumetric pipette. The amino groups introduced in step 1 were bound to the amino groups of the antibodies via a cross linker (glutaraldehyde). The procedure was based on a previously reported protocol.[4] A 2.5% solution of glutaraldehyde in borate buffer pH 7.2 was introduced into the nanochannel for 1 h. After washing with pure water for 10 min, a 25 µg/mL solution of anti-IL-6 antibody (MAB206-500, clone 6708, R&D Systems, Inc., Minneapolis, MN, USA) in phosphate-buffered saline (PBS) was introduced at 100 kPa for 1 h. After washing with PBS for 10 min, 0.5 M ethanolamine was introduced at 100 kPa for 10 min for capping of the unreacted sites. Next, 2 % (wt/wt) bovine serum albumin in PBS was introduced at 100 kPa for 30 min for blocking of the surface of the nanochannel. The liquid/gas interface was maintained at a position between the fL-volumetric pipette and pL-flask throughout the modification process to prevent leakage of modification reagent into the fL-volumetric pipette.

Supplementary Figures

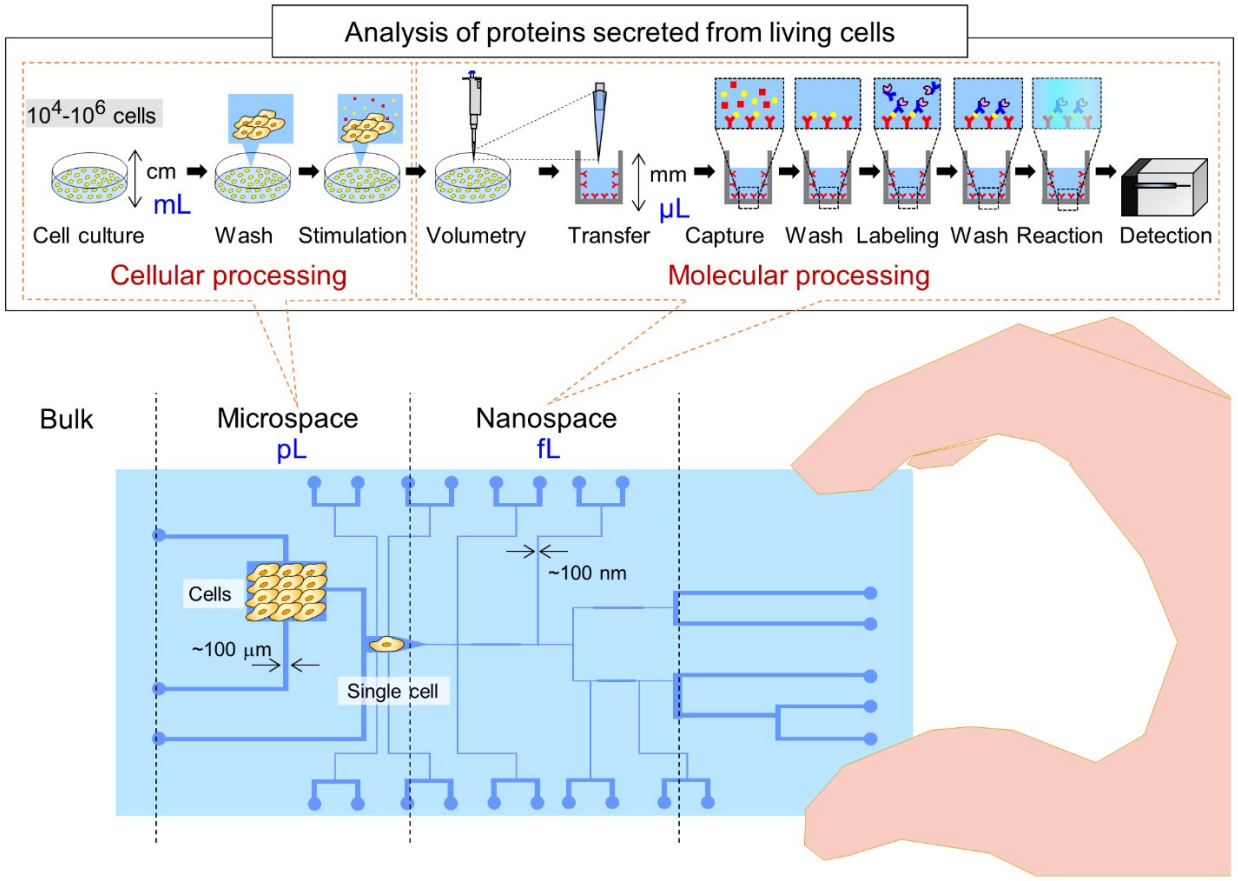

Figure S1. Schematic illustration of the concept of the micro/nano-integrated fluidic device for living single-cell protein analysis by comprehensive integration of cellular processing into microspaces (pL scale) and molecular processing into nanospaces (fL scale).

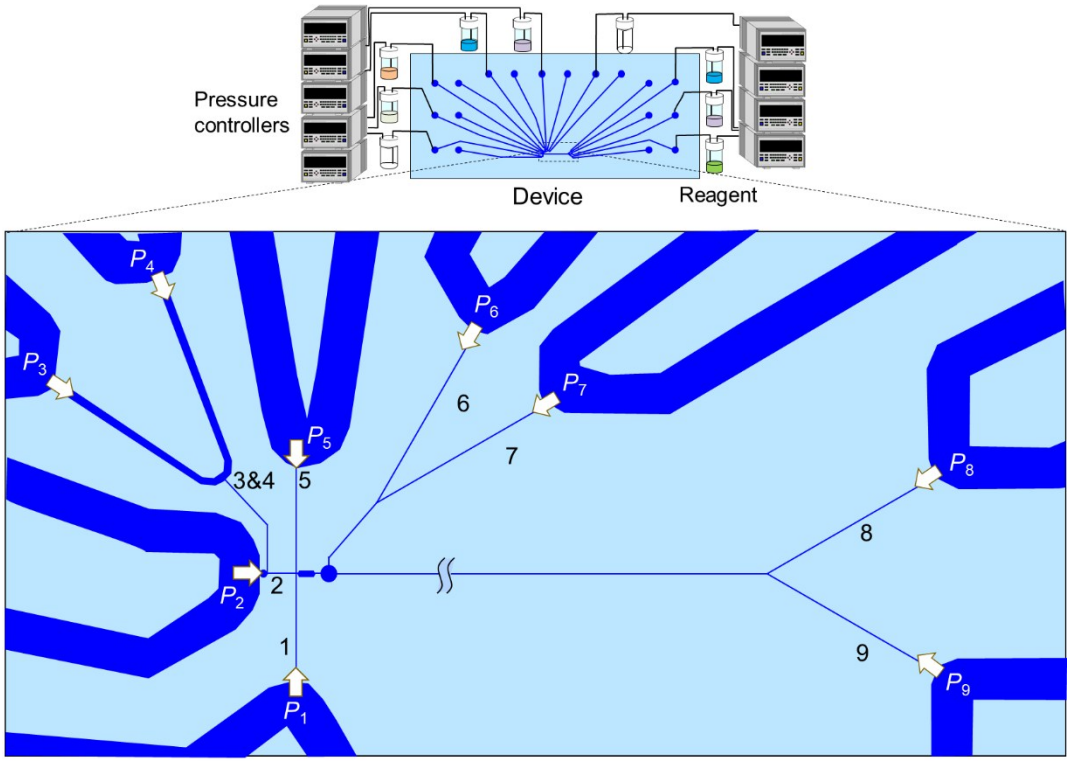

Figure S2. Layout of nanochannels and application of external pressure in the micro/nano-integrated fluidic device.

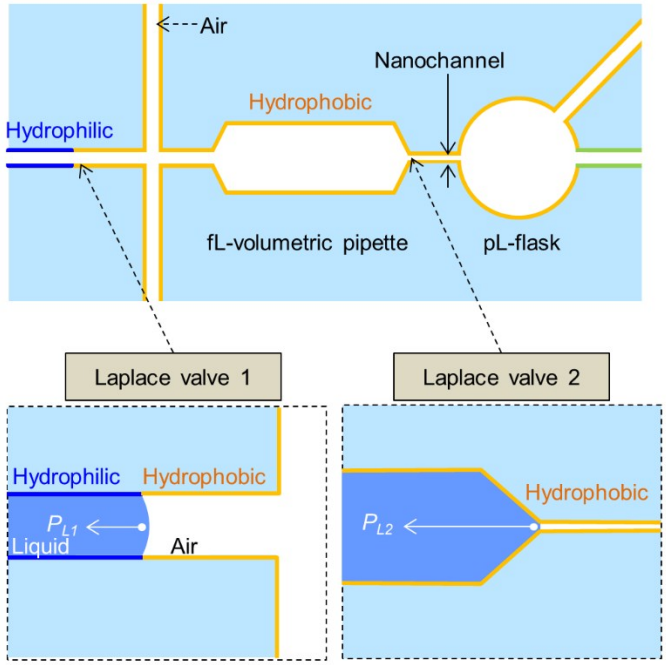

Figure S3. Structure of the fL-volumetric pipette and pL-flask.

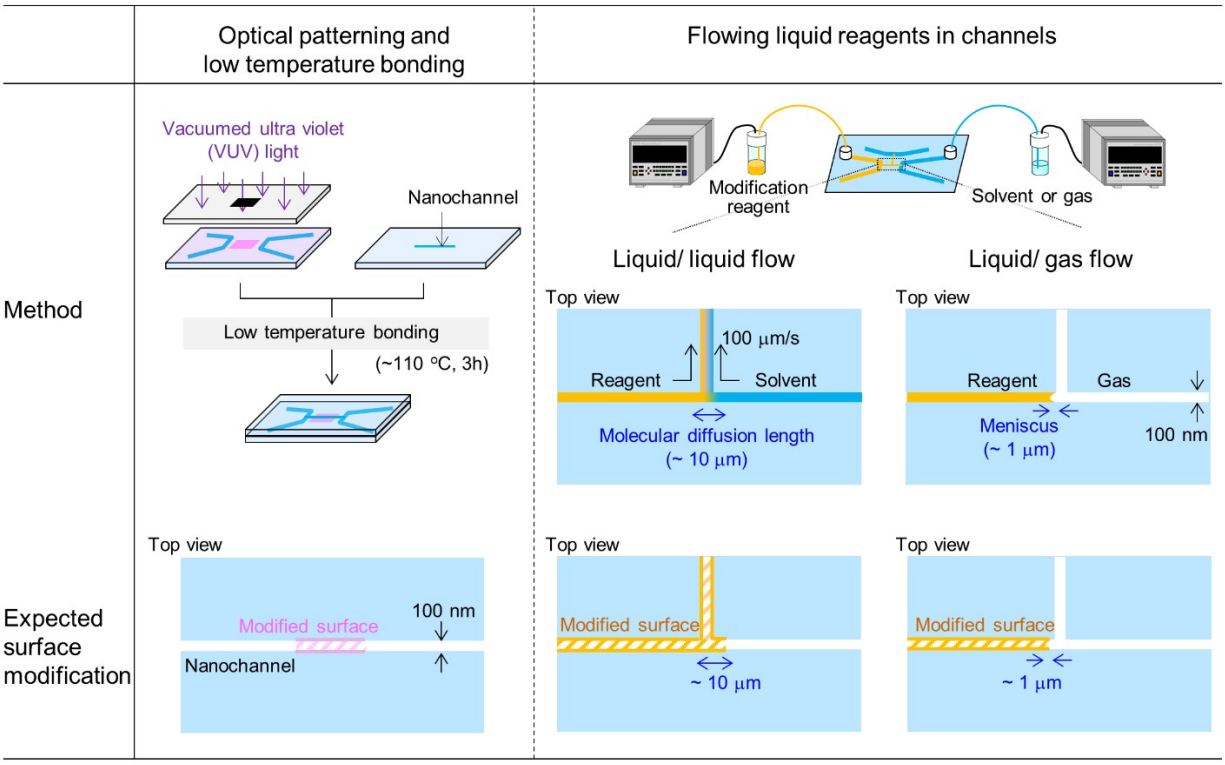

Figure S4. Principles of the partial surface modification methods.

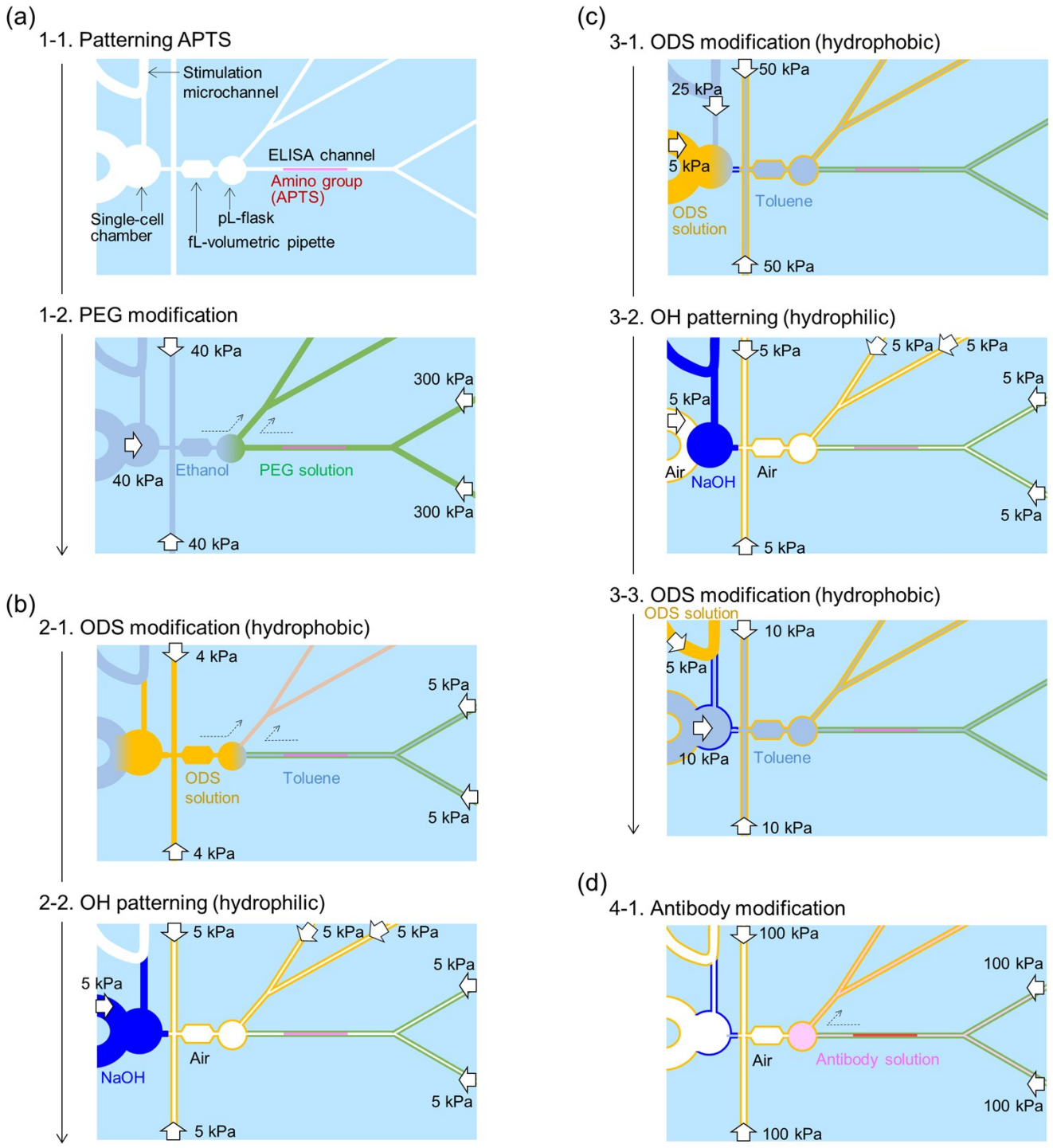

Figure S5. Procedure for surface modification of the micro/nano-integrated fluidic device: (a) Step1. Pre-modification of the ELISA channel with scaffolding for antibody and blocking. (b) Step 2. Hydrophobic/hydrophilic-patterning of the fL-volumetric pipette. (c) Step 3. Hydrophobic/hydrophilic-patterning of the single-cell chamber. (d) Step 4. Modification of the ELISA channel with capture antibody.

Supplementary Tables

Table S1. Width, depth and length of nanochannels 1-9

Nanochannel #	1	2	3&4	5	6	7	8	9
Width (nm)	2300	1700	2100	2300	2100	2100	2100	2100
Depth (nm)	770	770	770	770	770	770	770	770
Length (μm)	530	68	590	530	920	920	920	920

Table S2. Designed pressure values for fluidic operation of the micro/nano-integrated fluidic device

Unit operations		Values of external pressure at each channel (kPa)								
		P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9
1	Single-cell selection	100	10	0	0	100	100	100	0	0
2	Stimulation	100	0	100	0	100	100	100	0	0
3	Isolation	100	10	0	5	100	100	100	0	0
4	Incubation	100	10	0	0	100	100	100	0	0
5	Volumetry	100	180	0	0	100	100	100	0	0
6	Isolation	200	0	0	0	200	50	50	0	0
7	Dilution	100	0	0	0	100	25	25	0	0
8	Ag-Ab reaction	100	0	0	0	100	100	100	0	0
9	B/F separation	100	0	0	0	100	0	0	0	200
10	Ag-Ab reaction	100	0	0	0	100	10	60	10	10
11	B/F separation	100	0	0	0	100	10	10	10	60
12	Enzymatic reaction	50	0	0	0	50	0	0	0	0
13	Detection	100	0	0	0	100	0	0	50	0

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