Supplementary Information

Microfluidic Cell Unroofing for the *In Situ* Molecular Analysis of Organelles Without Membrane Permeabilization

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1. Materials and Methods

1.1 Materials

Slide glass and sticky Slide 0.1 Luer (ib10812 and ib81128) were purchased from ibidi GmbH (Gräfelfing, Germany). Collagen (Cellmatrix Type A) was from Nitta Gelatin Co. Ltd. (Osaka, Japan). MilliQ water was from Nihon Millipore Ltd. (Tokyo, Japan). Dulbecco's phosphate-buffered saline (PBS) was from Shimadzu Diagnostics (Tokyo, Japan). Dimethyl Sulfoxide (DMSO), Bovine Serum Albumin (BSA), RPMI-1640, Penicillin-Streptmycin, and Paraformaldehyde (PFA) were from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). 25% glutaraldehyde in aqueous solution was from Electron Microscopy Sciences (Hatfield, PA). 4% Aqueous osmium tetroxide (OsO_4) in aqueous solution were from Nisshin EM Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was from Nichirei Bioscience (Tokyo, Japan). Recombinant mouse interleukin-3 (IL-3), 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) and Anti-Rabbit IgG (H+L) secondary antibody, Alexa Fluor 488 labeled were from Thermo Fisher Scientific K. K. (Tokyo, Japan). Calcein-AM, Hoechst 33342, MT-1 and MitoMP Detection kit were from Dojindo Laboratory (Kumamoto, Japan). Anti-TOMM20 antibody (Cat. # ab78547) was from Abcam Japan (Tokyo, Japan). Triton X-100 (TX-100) was from Sigma-Aldrich Japan (Tokyo, Japan). Photoactivatable PEG-lipid was synthesized same as described in previous report.[S1]

1.2 Microfluidic Device and Photoactivatable Cell Anchoring Surface

Microfluidic devices and photoactivatable cell anchoring surfaces were prepared as reported in our previous work.[S1] Briefly, slide glasses were first washed with an alkaline solution and organic solvents, and then incubated in a collagen solution (0.3 mg/ml, pH 3) overnight at room temperature. After rinsing with MilliQ water and air drying, the collagen-coated glass was combined with a sticky-slide, establishing a microfluidic device that holds a microchannel with a width, length and height of 5 mm, 48.5 mm and 0.1 mm. The microfluidic device was securely fastened with a handmade clip to prevent solution leakage (Fig. S1). The microchannel is first placed on the bottom plate equipped with screws. Next, the top plate, which includes cushioning material, is placed over the microchannel and fixed with clamps. When a syringe pump is used to flow the solution

at high speed, high pressure is generated inside the microchannel. Without securing the microchannel with this clip, water leakage may occur between the top cover of the microchannel and the glass slide.

Then, the collagen-coated bottom surface of the channel was modified with photoactivatable PEG–lipid **1** via the amine coupling reaction by the treatment of a phosphate-buffered saline (PBS) solution of **1** (100 μM compound **1**, including 50% DMSO). After incubation for 3 h at 37 °C with light sheilded, the microchannel was washed with PBS.

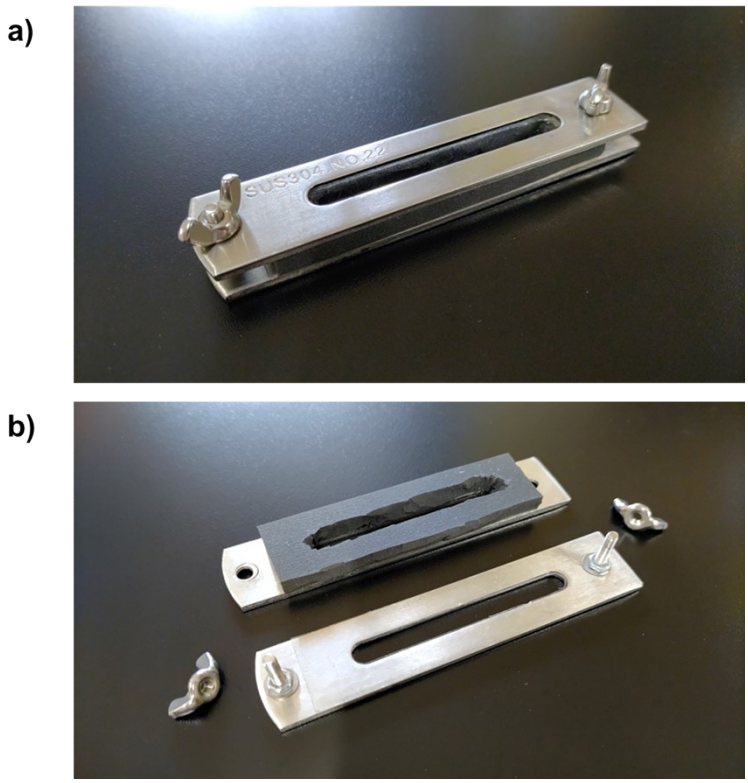

Figure S1. Photograph of handmade stain-less steal clips to prevent solution leaks. (a) The overall views of the clips when assembled, and (b) their three main components.

1.3 Light-induced Cell Anchoring

Light-induced cell anchoring was performed as described in our previous work.[S1] IL-3-dependent mouse pre-B-cell line Ba/F3 was obtained from the JCRB Cell Bank (Osaka,

Japan). Briefly, Ba/F3 cells were collected from the culture medium (RPMI-1640 medium supplemented with 10% FBS and 1 ng/ml IL-3) by centrifugation and washed with RPMI-1640 medium without any supplement (RPMI (FBS-)) by resuspension and centrifugation. Finally, cells were suspended in RPMI (FBS-) to prepare cell suspensions at the desired concentrations. In parallel, spots of ultraviolet (UV) light (wavelength: 365 ± 5 nm, light dose: 1.5 J/cm^2) was irradiated using a UV irradiator (Rex-250, from Asahi Spectra, Japan) on the modified surface through a contacted photomask, on which an array of transmissive spots (various diameters: 8, 10, 14 μm, various grid sizes: from 25 μm × 25 μm to 200 μm × 200 μm) was printed. After washing the microchannel, a cell suspension (over 1×10^7 cells/mL, in RPMI (FBS-)) was loaded into the microchannel. After incubation for 10 min at room temperature, non-anchored cells were removed by washing with RPMI (FBS-). Preparation of single cell arrays with intended various intervals was confirmed by observing with a fluorescence microscope (IX83, from Olympus Co., Tokyo, Japan) (Fig. S2).

1.4 Microfluidic Unroofing

Microfluidic unroofing was performed as reported in our previous work.[S2] Briefly, the microchannel was connected to a syringe pump (YSP-301, from YMC Ltd., Kyoto, Japan) after preparation of single-cell arrays. PBS was injected into the flow path at velocities of 50 mL/min, for 1 min, to induce cell fracture via laminar flow. The Reynolds number of this microchannel is less than 500, indicating a laminar regime even at the maximum flow rate of 50 mL/min.[12] Cells were fluorescently stained with DiI for biomembrane, Calcein-AM for cytoplasm and Hoechst 33342 for nucleus according to the manufacturer's protocol. These multiple stained cells were observed before and after unroofing with a fluorescence microscope equipped with 4× objective lenses and with a confocal laser-scanning microscope equipped with 63× objective lenses (TCS SP8, from Leica Microsystems, Wetzlar, Germany). Image analysis was conducted using the open-source software Fiji, which is just ImageJ (NIH, Bethesda). The cell position was identified as 15 μm-diameter circles with TrackMate of Fiji plugin.[S3,S4] The red fluorescence images of cell membrane were employed for the identification of cell position.

1.5 Fluorescence microscopy imaging of mitochondrial membrane potential

Cells were simultaneously stained with Calcein-AM, Hoechst 33342 and MT-1. The protocol followed the manufacturer's manual, except for MT-1, where the concentration was reduced to one-tenth of the specified amount, and the treatment time was shortened to half of the recommended duration. As a control, the permeabilized sample was prepared by treating the anchored cells with fixation and permeabilization instead of unroofing. Fixation was conducted by loading 3% PFA in PBS into the microchannel for 15 minutes and washing with PBS. Permeabilization was conducted by loading 1% TX-100 in PBS into the microchannel for 15 minutes and washing with PBS. After each treatment, the solution in the microchannels were exchanged to the imaging buffer of MitoMP Detection Kit. Finally, cells were observed by confocal fluorescence microscopy as described above.

1.6 Fluorescence microscopy imaging of mitochondria

Cells were fluorescently stained with Hoechst 33342 as described above. After unroofing, TOMM20 on the mitochondria was immunestained using a primary anti-TOMM20 rabbit polyclonal antibody and a secondary anti-Rabbit IgG (H+L) donkey polyclonal antibody AF488 conjugate. To block nonspecific binding, cells were pre-incubated with a 2% BSA solution in RPMI (FBS-), followed by successive treatments of the primary and secondary antibody solutions. Each antibody treatment lasted for 5 min. The doubly stained cells were then observed by confocal fluorescence microscopy as described above.

1.7 Computational Fluid Dynamics (CFD) simulation

The CFD simulation was performed using the Flowsquare software[S5] (Nora Scientific, Japan) to model the flow direction and rate during the cell fracture. The models comprised a rigid wall representing the top cover of the microchannel and the glass bottom, through which the laminar flow get through from left to right. Additionally, the cells were regarded as solid sphere with a diameter of 15 μm, placed on bottom glass. The

simulations were performed in two dimensions for simplification. The model dimensions were set to a height of 100 μm to match the actual length and 200 μm along the axis of flow. The mesh size of the models in any direction was 0.39 μm, and they contained approximately 131,000 active cells. The fluid density of the CFD experiment was set to 1000 kg/m^3, and the velocity was calculated from the liquid volume pushed out by the syringe. The delta t factor and time step were set as 100 and 400 respectively.

1.8 SEM Observation

Unroofed cells were prepared as described above, and after washing with PBS, they were chemically fixed with 2.5% glutaraldehyde in PBS at room temperature for 1 h. After washing again with PBS, the glass slides at the bottom of the microchannel were cut out using a glass cutter. The unroofed cells on the glass pieces were then fixed with 1% OsO_4 in phosphate buffer (75 mM, pH7.4) at room temperature for 1 h. After washing with the same buffer and distilled water, the samples were dehydrated stepwise in series of gradient alcohol solutions. Subsequently, they were dried with a critical point dryer (EM CPD 300, from Leica Microsystems, Wetzlar, Germany) and coated with OsO_4 using an osmium coater (Neoc-Pro, from Meiwafosis Co., Ltd., Tokyo, Japan). Samples were subsequently observed with a scanning electron microscope (SU8220, from Hitachi Ltd, Tokyo, Japan).

2. Wide-field images of cell arrays before and after unroofing

The overall view of the arranged cells in a single-cell array pattern is shown in the images below (Fig. S2a). Applying microfluidic laminar flow to these cell arrays from left to right results in their unroofed (Fig. S2b, c).

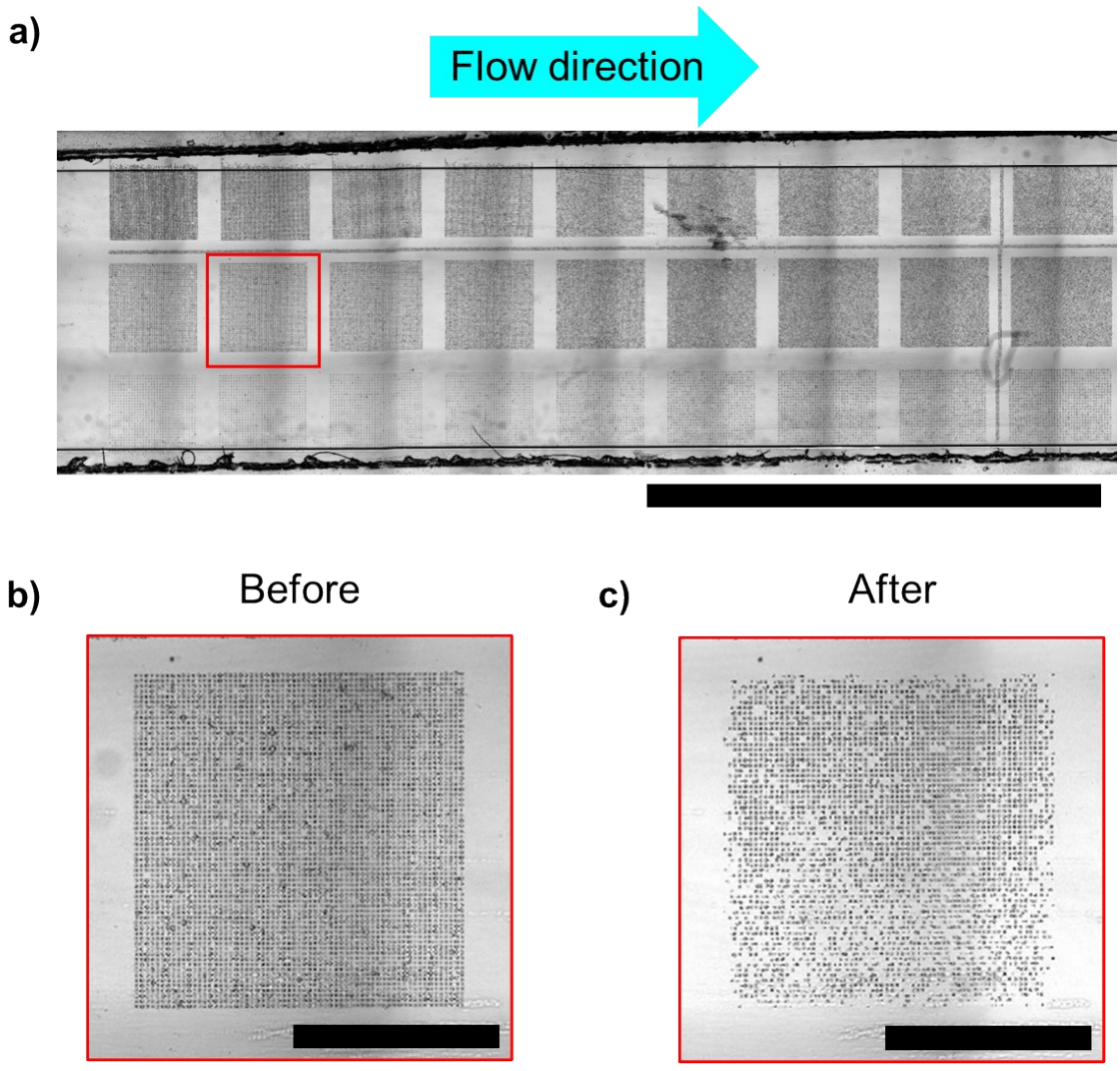

Figure S2. Overview of the microscopic images of single-cell arrays before and after unroofing. (a) The Whole view of the single-cell arrays in a microchannel before unroofing. Scale bar: 8 mm. (b, c) Enlarged images of the red squared area in (a) were obtained (b) before unroofing and (c) after unroofing. Scale bars: 1 mm.

3. Scanning electron microscopy (SEM) imaging of intact cells

As a control SEM image for the unroofed cells shown in Fig. 5, a wide-view SEM image of whole intact cells was obtained and is presented in Fig. S3.

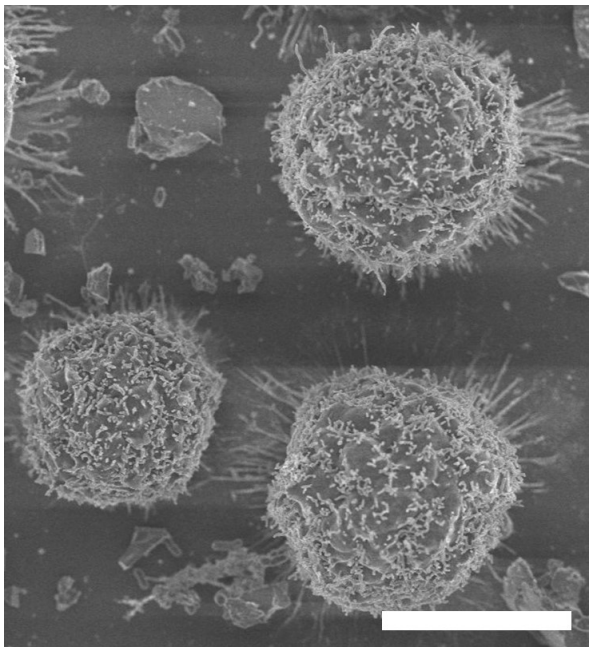

Figure S3. A SEM image of whole intact cells. Scale bar, 10 μm.

4. High-contrast images of immunostaining on mitochondria

Anti-TOMM20 antibody-derived fluorescence was enhanced by adjusting the brightness and contrast of the images in Fig. 7 to determine whether even a small amount of antibody had entered the permeabilized cells under the present staining condition. As a result, the antibody-derived green fluorescence became detectable in the permeabilized cells in Fig. S4, while no antibody-derived fluorescence was observed in the negative control intact cells, even after image adjustment. This clearly shows that antibody entered the permeabilized cells, although the amount was extremely small compared to the unroofed cells.

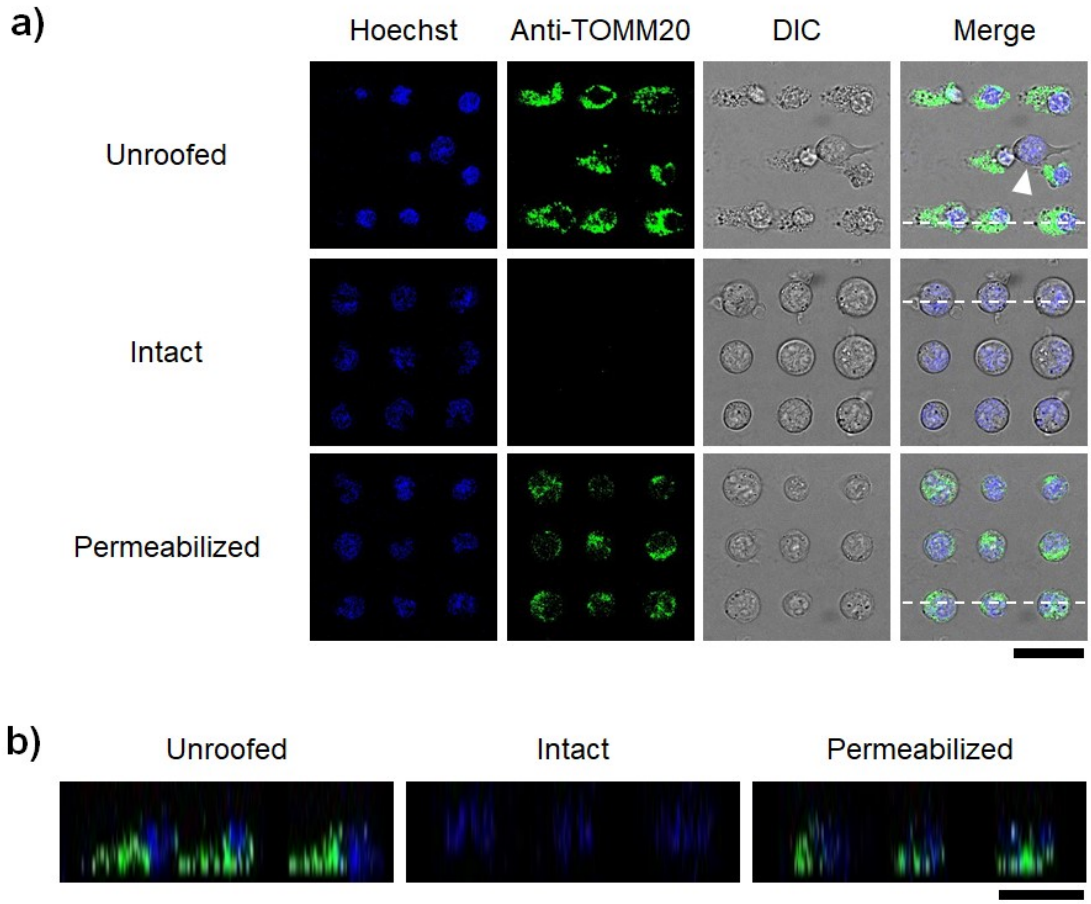

Figure S4. The high-contrast confocal microscopic images of immunostained cells. Anti-TOMM20 antibody-derived fluorescence was enhanced by adjusting the brightness and contrast of the images in Fig. 7. (a) XY cross section images of unroofed cells, intact cells and permeabilized cells. Cells were stained with Hoechst 33342 (blue) and AF488 conjugated anti-TOMM20 antibody (green). A white arrowhead indicates a cell that was not stained with anti-TOMM20 antibody. (b) XZ cross section images corresponding to the positions of each white dotted line in merge image of (a). Scale bar: 30 μm.

5. References

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