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Supporting Information

High-Throughput Isolation of Cell Protrusions with Single-Cell Precision for Profiling Subcellular Gene Expression

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Materials and Methods

Cell culture

MDA-MB-231/GFP (ATCC), SUM159 (Asterand), F27 melanoma, and NIH 3T3 fibroblasts (ATCC) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Corning) supplemented with 10% fetal bovine serum (FBS; Corning) and 1% penicillin-streptomycin (Corning) at 37°C in a humidified atmosphere with 5% CO_2. The F27mel cell line was generously provided by Dr. Rongfu Wang (Center for Inflammation and Epigenetics, Houston Methodist Research Institute, Houston, TX 77030, USA). U2OS cells (ATCC) were cultured in McCoy's 5a medium (Corning) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. HMEC-1 cells (ATCC) were cultured in MCDB131 medium (without L-Glutamine) (Gibco) supplemented with 10 ng/mL epidermal growth factor (Gibco), 1 μg/mL hydrocortisone (Sigma), 10 mM glutamine (Gibco), 10% FBS, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The 661W cell line was generously provided by Dr. Muayyad Al-Ubaidi (Department of Biomedical Engineering, University of Houston, Houston, TX, USA) and cultured in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic (Gibco), 40 ng/mL hydrocortisone, 40 ng/mL progesterone (Sigma), 0.032 mg/mL putrescine (Sigma), and 0.004% (v/v) β-mercaptoethanol (Sigma) at 37°C in a humidified atmosphere with 5% CO_2.

Design and fabrication of the PG-Chip

All PG-Chips were designed using AutoCAD software, printed out as 5-inch glass photomasks (Photo Sciences Inc.), and fabricated using standard photolithography and elastomer molding. Briefly, SU-8 3025 photoresists (MicroChem Corp.) were spin-coated onto a 5-inch silicon wafer (Silicon Quest International Inc.) at 3000 rpm for 1 min (Laurell Technologies Corp.) to form a 20-μm thick film.

After soft baking at 65ºC for 3 min and hard baking at 95ºC for 10 min, the spin-coated wafer was exposed to UV light with the printed photomasks at an exposure dose of 150 mJ/cm^2 for 5 s. The wafer was then baked at 95ºC for 3 min and developed. After a second exposure for 20 s and hard baking at 135ºC for 30 min, the wafer with the designed structures was fabricated and served as the template for polydimethylsiloxane (PDMS) devices. Before pouring the PDMS mixture (10A:1B; Sylgard 184 kit from Dow Corning Corp.), the wafer was modified with trimethylchlorosilane for 30 min to make the surface hydrophobic. After degassing in a vacuum, the wafer with PDMS mixture was cured at 80ºC for 30 min. Then, the PDMS replica was peeled off, cut to the appropriate size, and punched. The devices were assembled by directly placing the fabricated PDMS replica onto a commercial petri dish surface (Fisher Scientific) for imaging or a PEN membrane frame slide coated with various biomolecules (ThermoFisher) for LCM, without thermal or oxygen plasma treatment. All assembled devices were kept in a vacuum container for at least 30 min before use to avoid bubbles during the loading of cells. The PEN membranes were first treated using oxygen plasma (1 min at 20 sccm oxygen flow rate, 500 mTorr chamber pressure, and 50 W power) and then covered with FN solutions (5 μg/mL in PBS; Sigma), BME solutions (150 μg/mL in DMEM medium; R&D), or Poly-L-lysine solution (0.01% in water; Sigma) at 4ºC overnight.

Generation of cell protrusion arrays in the PG-Chip

The cell-free medium was loaded into channels before cells were loaded. Cell suspensions (10 μL; approximately 5×10^6 cells/mL) were placed in the inlet and pumped into the channels. The flow was maintained by connecting the outlets to a negative-pressure control system, and flow rates were kept to less than 100 μm/s to avoid damaging the cells. The use of negative pressure for loading cells in our experiments could preserve the integrity of the assembled chips and prevent the leakage of mediums.

After capturing, free cells were washed away by replacing the cell suspensions with culture medium and applying a slightly larger negative pressure. The negative pressure was then turned off to allow *in situ* cell adhesion/growth and protrusion extension at 37°C in a humidified atmosphere with 5% CO_2 for the following times: 4 h for NIH 3T3 and HMEC-1 cells; 5 h for U2OS, F27mel, and 661w cells; and 6 h for MDA-MB-231/GFP and SUM159 cells.

Isolation of cell protrusions by LCM technique

After the cell protrusion array, the PDMS mold was carefully peeled off, and the cell protrusion array was left on the PEN membranes. After washing twice with PBS, the cell protrusion arrays were fixed in a paraformaldehyde solution (4% in PBS) on ice for 15 min and then either stored at 4°C by covering the arrays with RNA*later*® RNA Stabilization Solution (Ambion) or immediately processed by LCM. For LCM isolation, fixed cell protrusion arrays on PEN membrane frame slides were loaded onto the laser capture microscope stage (ArcturusXT Laser Capture Microdissection System). A Capsure Macro LCM cap (Thermo Fisher) was then placed automatically over the chosen area of the cell protrusion arrays. Regions of interest were highlighted using the accompanying software and attached to the cap using an infrared capture laser. The selection of cutting boundary follows two principles: (1) the isolated cell protrusions must be close to the leading edge of cells and (2) as many as cell protrusions are selected in one area so that we can isolate enough cell protrusion in a short time, because mRNAs are extremely sensitive to RNase that is ubiquitous in the surrounding environment. Finally, a UV cutting laser was employed to excise the desired cell protrusions. After the cap was removed from the PEN membrane frame slide, cell protrusions were collected on the cap surface. As controls, the corresponding cell bodies left on the PEN membrane were also isolated using another cap.

mRNA extraction from LCM samples

Briefly, the LCM cap with collected samples was covered with 5 μL of lysis buffer (1 μL PKD buffer [Qiagen] in 15 μL of proteinase K solution [Qiagen]) and incubated at 56°C for 1 h in an oven to perform the reverse-crosslinking and lysis steps. Next, the lysates were transferred to PCR tubes and placed on ice. Oligo dT25 magnetic beads (Life Technologies) were prepared by washing three times with 1× hybridization buffer (2× SSPE, 0.05% Tween 20, and 0.25% RNasin Plus) and then re-suspended in half the original volume of 2× hybridization buffer. Five microliters washed dT25 beads (0.05 mg of beads) were added to reverse cross-link samples, and the samples were heated to 56°C for 1 min, incubated at room temperature for 10 min to allow mRNA hybridization, and then incubated on ice for 1 min. The beads were then washed three times in 100 μL ice-cold 1× hybridization buffer and once in ice-cold 1× PBS with 0.25% RNasin Plus. Any traces of PBS were removed to avoid inefficient elution. The dried beads were re-suspended in 2.8 μL RNase-free water, incubated at 80°C for 2 min, and then immediately pelleted on a room temperature magnet. The supernatant containing mRNA was rapidly transferred to a new PCR tube and stored at −80°C.

RNA-Seq and data processing

Following mRNA extraction, we performed cDNA synthesis and amplification (SMART-Seq v4 Ultra Low Input RNA Kit; Takara Bio USA), followed by purification (Agencourt AMPure XP beads; Beckman Coulter). cDNA was quantified using a High Sensitivity DNA Chip (Agilent) on an Agilent 2100 Bioanalyzer. RNA-Seq libraries were generated from cDNA using the Illumina Nextera XT DNA Sample Preparation Kit. The quality of the cDNA libraries was assessed using an Agilent 2100 Bioanalyzer.

RNA-Seq was conducted on the Illumina HiSeq4000 platform in the 150 bp pair-end configuration using service from LC Sciences (Houston, TX, USA). Raw reads were processed using an established bioinformatics pipeline for mapping and assembling to yield gene expression levels of all transcripts. Reads were normalized to 20 million total reads for each sample and were converted to transcripts per million (TPM). Differential gene expression analysis on protrusion vs. cell body was carried out using the "limma" ($p < 0.05$) and "samr" (pairwise comparison and FDR < 0.1) R packages and the results were combined with genes having at least two-fold increased expression in protrusions.

Validation of RNA localization

RT-qPCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad), and reactions were run on the Applied Biosystems StepOnePlus™ Real-Time PCR System. RT-qPCR was performed on cDNA with equal amounts from cell protrusion and cell body samples. Briefly, the reaction solutions consisted of 4 ng cDNA, 0.5 μM primers, and 5 μL Supermix in a 10 μL final volume. Each cycle (total 40 cycles) consisted of denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. The experiment was performed in triplicate for each gene. β-actin was used as an endogenous control to normalize each sample. The localization ratio was defined as the ratio between the mean expression in the cell body fraction and the cell protrusion fraction. Primer sequences are shown in Table S1.

For FISH, MDA-MB-231/GFP cells were incubated within the PG-Chips, which were attached on fibronectin-coated glass coverslips for 6 h and subsequently fixed in 4% formaldehyde for 10 min. FISH was performed with QuantiGene ViewRNA ISH Cell Assay kit (Affymetrix) according to the manufacturer's instructions. The Affymetrix probe sets used were *RAB13* cat#VA1-12225-VC and *PINK1* cat#VA1-3001255-VC. Nuclei were stained using 4',6-diamidino-2-phenylindole.

Cell staining

For the imaging of single-cell arrays and cell protrusion arrays, cells (except the GFP-labeled MDA-MB-231/GFP cells) were stained with 2 μM CellTracker Green (Invitrogen) for 30 min before loading into the PG-Chips. To image the cytoskeletal components, the cell protrusion arrays were first fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and then treated with 3% BSA (Sigma) for 30 min. F-actin was labeled with rhodamine phalloidin or Alexa Fluor 488 phalloidin (150 nM in PBS; Life Technologies) for 20 min. For imaging tubulin, cells were immunostained with α/β tubulin mouse monoclonal antibody (2 μg/mL in PBS containing 0.2% BSA) (Invitrogen) overnight at 4°C and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (1 μg/mL in PBS containing 0.2% BSA) (Invitrogen) for 45 min at room temperature. For other conditions, cell nuclei were labeled with Hoechst 33342 (5 μg/mL in PBS) (Invitrogen) for 10 min at room temperature and an anti-HDAC1 antibody-conjugated Alexa Fluor 555 (2 μg/mL for 1 h at room temperature) (Invitrogen).

Image acquisition and analysis

The morphology of the micro-hook arrays was characterized using a Nova Nano scanning electron microscopy 230 instrument (SEM, high vacuum, HV = 5 kV). Confocal immunofluorescence images were obtained using a Nikon confocal microscope. The images were analyzed with ImageJ (National Institutes of Health).

Data availability

The RNA-seq data has been deposited at GEO under the accession code GSE130271. All other data are available upon reasonable request from the corresponding author (L.Q.).

SI Figures (Figures S1–S10)

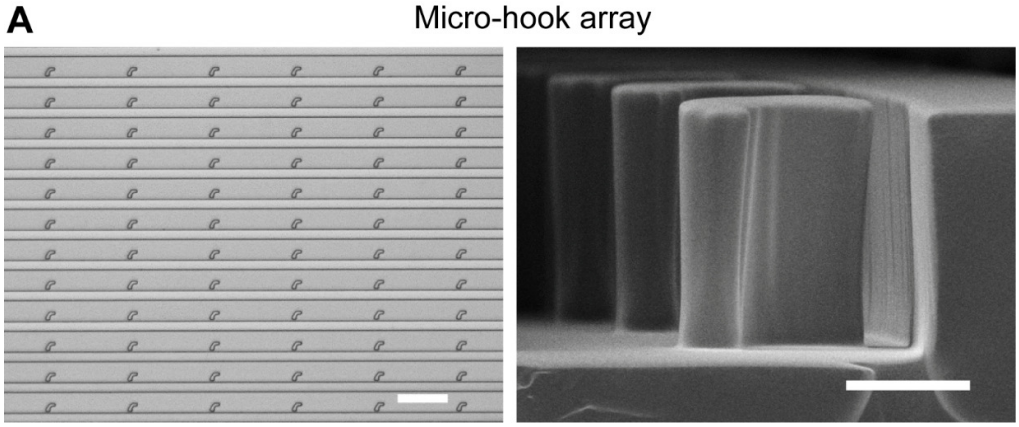

Figure S1. PG-Chip for generating large-area single cell arrays. (A) Representative image of a micro-hook array (left) and side view of the micro-hooks (right). The micro-hooks are located along one side of the flow channels, with 3-μm gaps, and are arrayed to trap single cells. To accommodate the size of MDA-MB-231/GFP cells, we adjusted the width (14 μm) and height (20 μm) of the micro-hooks and the width (43 μm) of the flow channels. These parameters could be designed to achieve high loading efficiency based on the diameter of cells. Scale bars, 100 μm (left) and 10 μm (right). (B) Image of the generated large-area array of MDA-MB-231/GFP single cells. Scale bars, 1 mm.

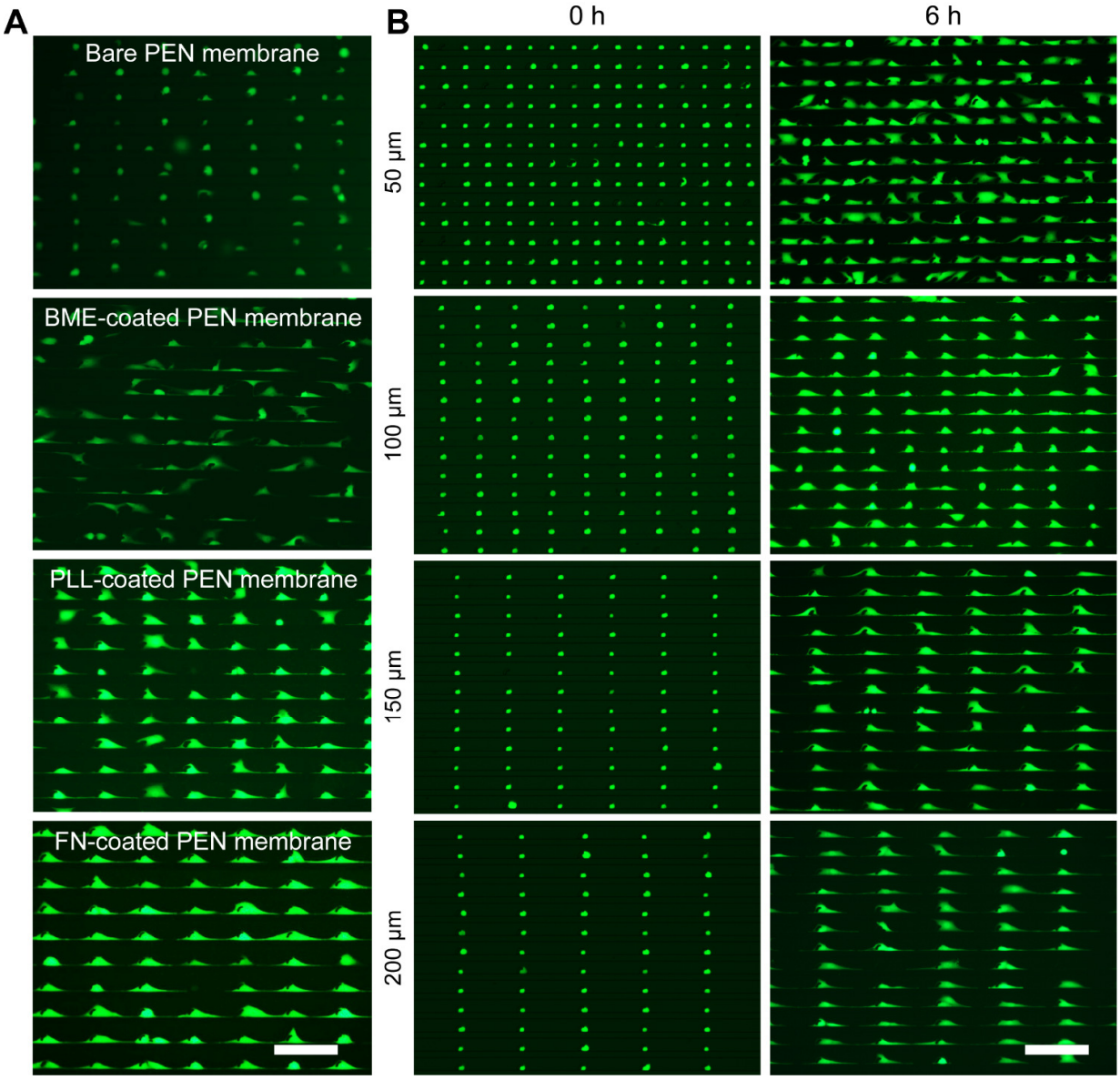

Figure S2. The influence of PEN membranes coating and distance between adjacent micro-hooks on the morphology of MDA-MB-231/GFP cells. (A) Images illustrating MDA-MB-231/GFP cell protrusions on uncoated or BME-, PLL-, or FN-coated PEN membranes. (B) Images illustrating MDA-MB-231/GFP cell protrusions over 6 h at micro-hook distances of 50 µm, 100 µm, 150 µm, and 200 µm. Scale bars, 200 µm.

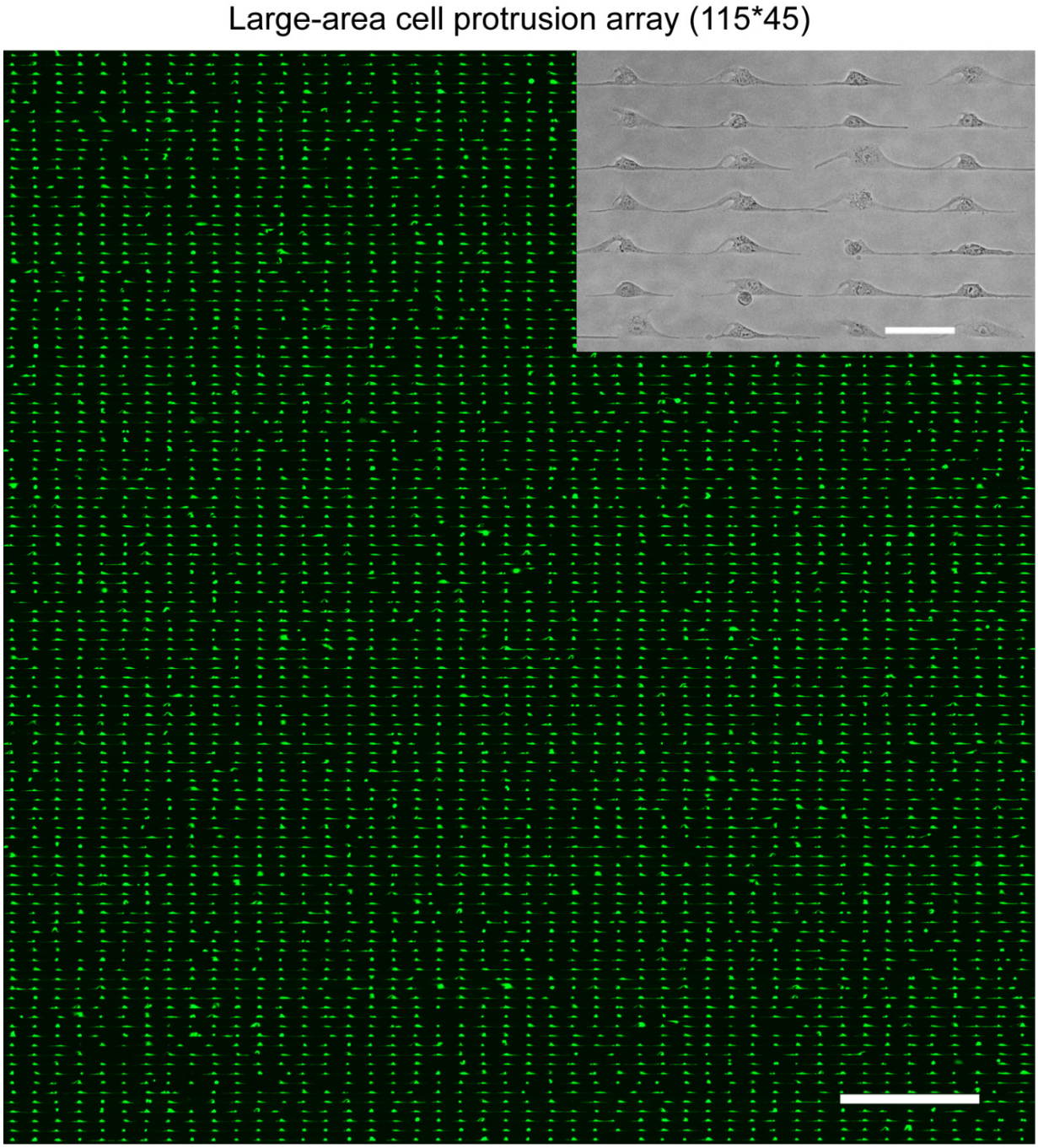

Figure S3. Large-area protrusion array of MDA-MB-231/GFP cells. Image illustrating cell protrusions in approximately 5000 cells, generated using the PG-Chips. Scale bar, 1 mm. Insert image shows the uniformity of cell protrusion arrays. Scale bar, 100 μm.

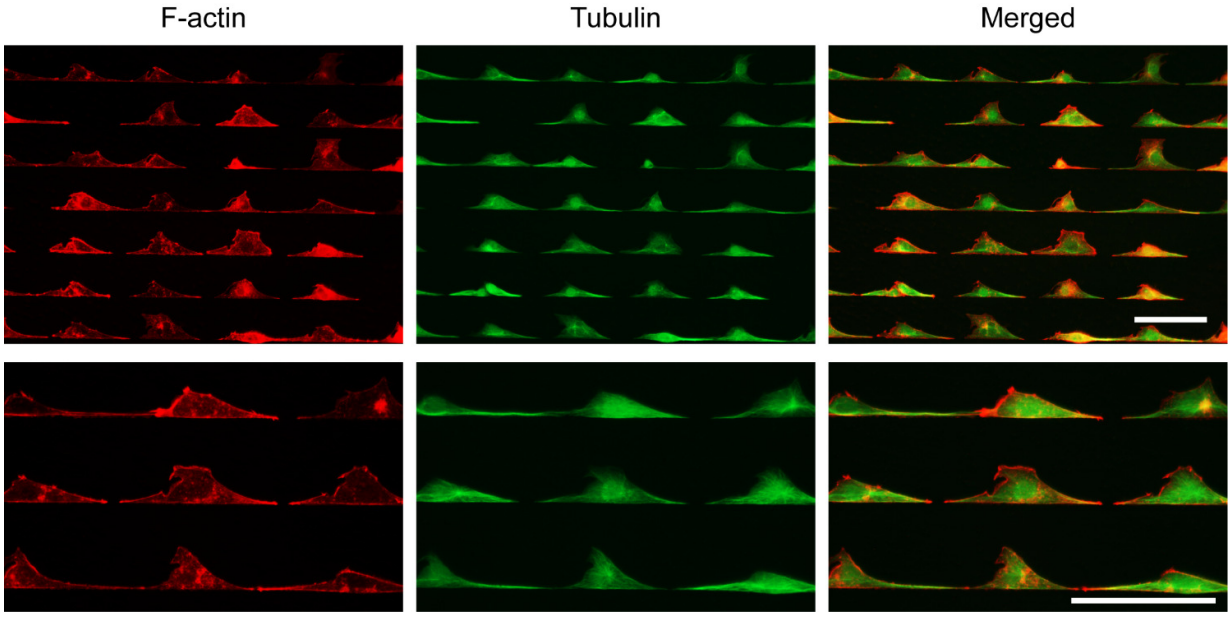

Figure S4. Characterization of the composition of cytoskeletal components of cell protrusion arrays. F-actin was labeled with rhodamine phalloidin (red). Tubulin was immunostained with an α/β tubulin mouse monoclonal antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (green). Scale bars, 100 μm.

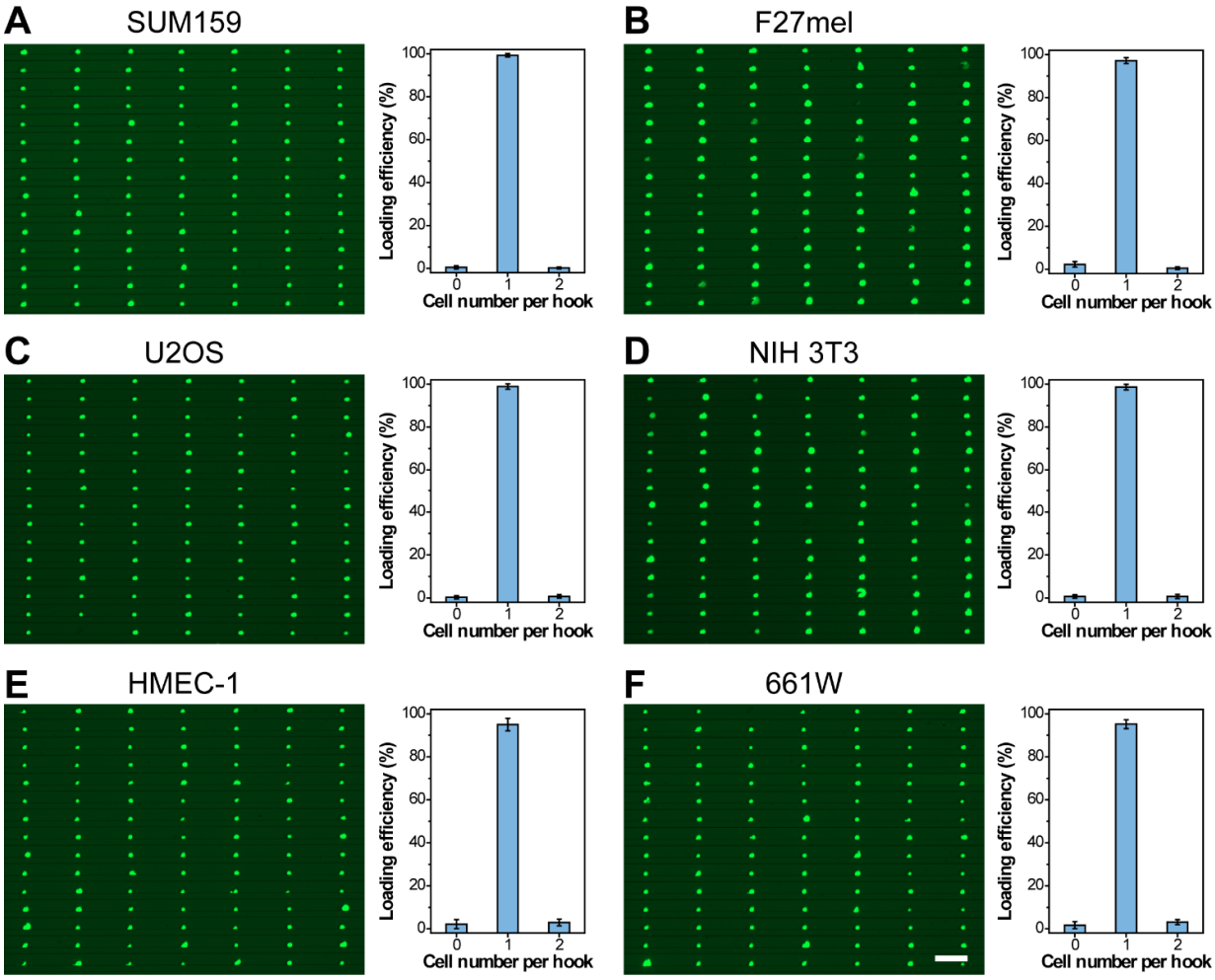

Figure S5. PG-Chip for generating single-cell arrays of various cell types. Images and quantification of loading efficiency of (A) SUM159 breast cancer cells, (B) F27 melanoma cells, (C) U2OS osteosarcoma cells, (D) NIH 3T3 fibroblast cells, (E) HEMC-1 endothelial cells, and (F) 661W photoreceptor cells were loaded onto the PG-Chips. Data are expressed as mean ± SD. All cells were stained with 2 μM CellTracker Green for 30 min. Scale bars, 100 μm.

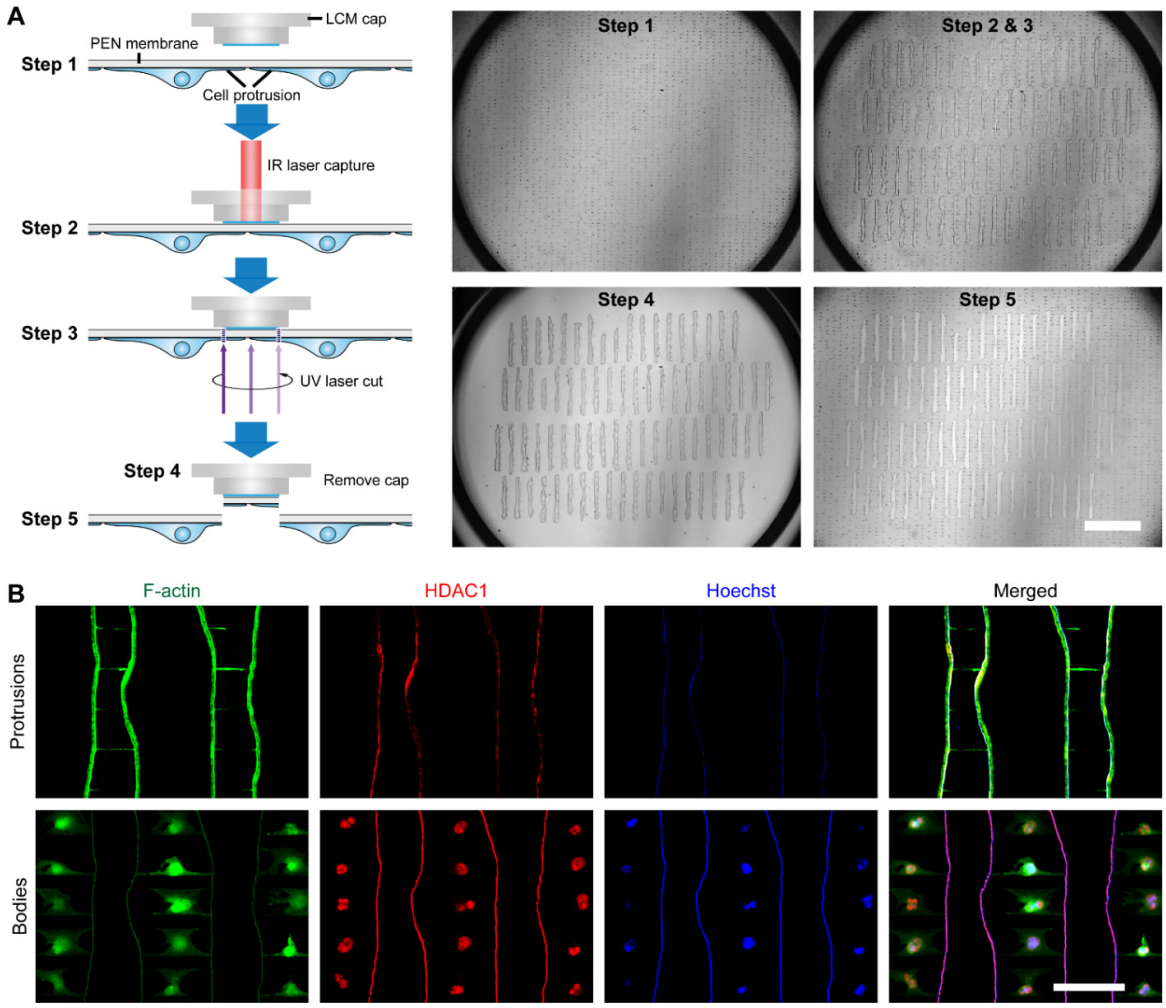

Figure S6. Precise isolation of cell protrusions using LCM. (A) Schematic illustration and corresponding images of the LCM process. Step 1: LCM cap was placed on the selected cell protrusion area. Step 2: The selected area was captured using an IR laser. Step 3: The selected cell protrusion area was cut using a UV laser. Step 4: Removal of the LCM cap; cell protrusions were collected on the surface of the LCM cap. Step 5: Cell bodies were left on the PEN membrane. Scale bar, 1 mm. (B) Representative images of isolated cell protrusions (top panel) and corresponding cell bodies remaining on the PEN membranes (bottom panel), indicating the high purity of the collected cell protrusions. Actin was stained with Alexa Fluor 488-phalloidin, and nuclei were stained with anti-HDAC1 antibody and Hoechst 33342. Scale bar, 100 μm.

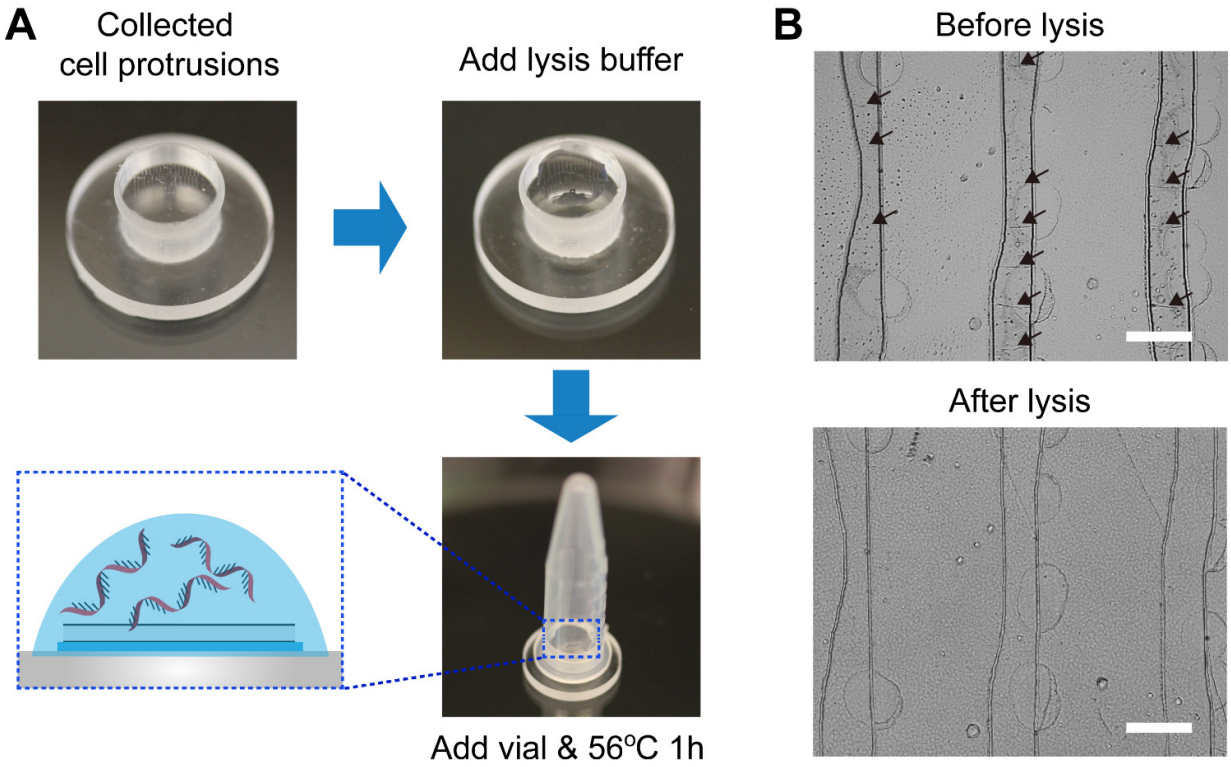

Figure S7. Enrichment of mRNAs from isolated cell protrusions. (A) Isolated cell protrusions on the surface of an LCM cap were lysed by directly adding lysis buffer. To avoid the evaporation of lysis buffer during lysis, a 0.5-mL vial was placed on the top of the LCM cap. mRNAs were released into the lysis buffer after incubation at 56°C for 1 h. (B) The bright field images show the cell protrusions disappeared after lysis. Scale bars, 100 µm.

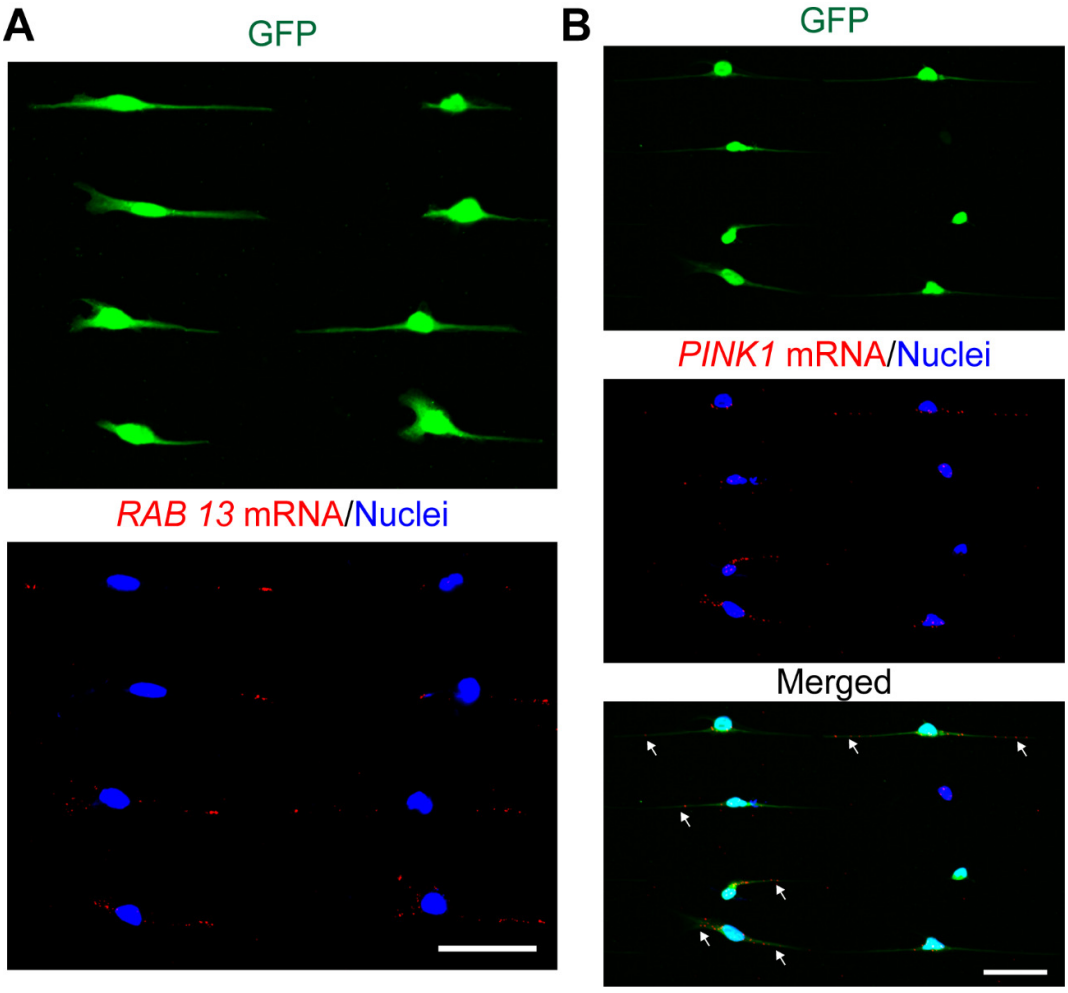

Figure S8. Validation of RNA-Seq results by FISH. Validation of the protrusion-localized (A) *RAB13* mRNAs and (B) *PINK1* mRNAs by FISH. White arrows indicate localized in cell protrusions. Nuclei were stained with Hoechst 33342. Scale bar, 50 μm.

Table S1. List of primers for RT-qPCR.

mRNA	**Forward primer**	**Reverse Primer**
ARPC3	ACATGGCACTGTTGCCTATC	GAAGAAGACATTGGCCTTGA
ACTB	TCTACAATGGCTGCGTGTG	AGCCTGGATAGCAACGTACA
RAB13	CCGCACTGTGGATATAGAGG	ATGGCTCCACGGTAGTAGG
CENPB	CTCCACTTGGAACACGATCT	CAGCTTTGATGTCCAAGACC
ZEB1	AGACATGTGACGCAGTCTGGGT	TGGGCATTCATATGGCTTCTCTCCA
ANP32B	CTGTTCGAGAACTTGTCTTGGAC	AGCTTGGGGAGATTTGAAACTG
CORO1A	CCAACATCGTCTACCTCTGTGG	CTCACACTTGTTCACCTCCAGG
PINK1	CCCAAGCAACTAGCCCCTC	GGCAGCACATCAGGGTAGTC