An optimized protocol to identify keratinocyte subpopulations in vitro by single-cell RNA sequencing analysis

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Protocol

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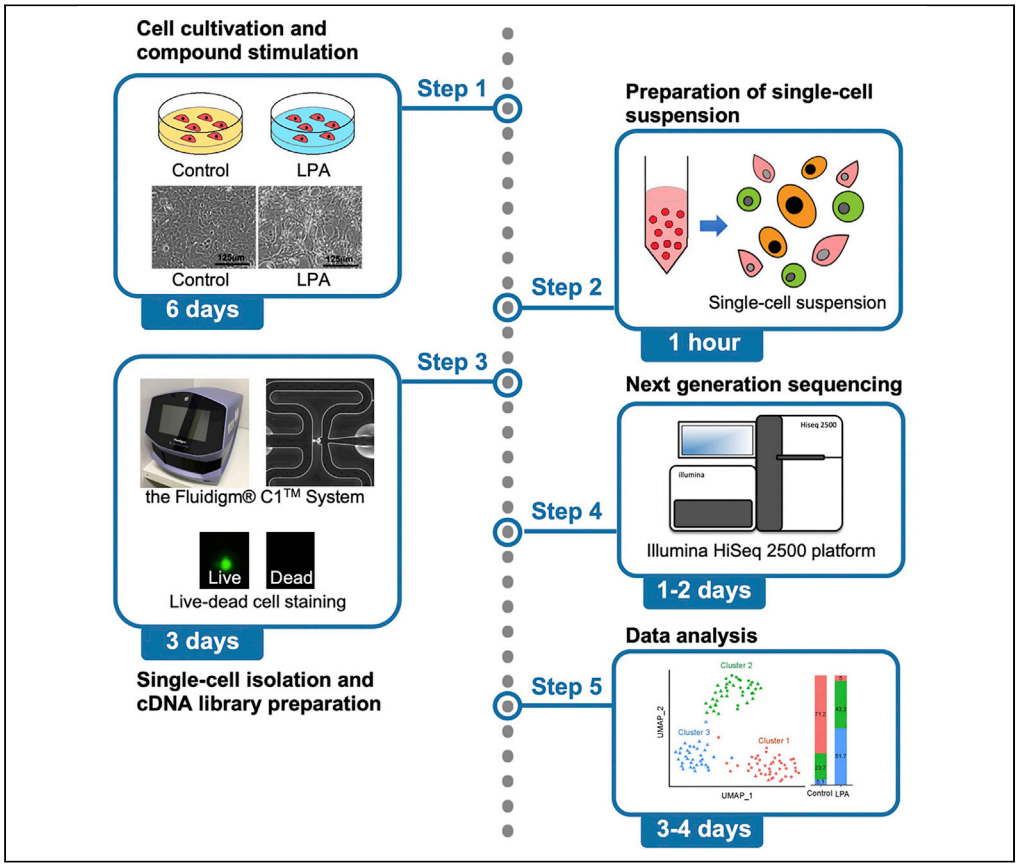

Here, we describe a protocol for single-cell isolation from the primary culture of normal human epidermal keratinocytes derived from neonatal foreskin. The cell culture conditions have been optimized for inducing expression of keratinocyte differentiation markers. Cells are cultured in the absence or presence of a bioactive lipid lysophosphatidic acid (LPA). Single cells are isolated by Fluidigm C1 system. This is followed by cDNA library preparation using Takara SMART-Seq v4 Ultra and Illumina Nextera XT kit for RNA sequencing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized cell culture protocol for single-cell isolation of human keratinocytes

Isolation of normal human epidermal keratinocytes *in vitro* with Fluidigm C1 system

Single-cell cDNA library preparation for RNA sequencing

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An optimized protocol to identify keratinocyte subpopulations *in vitro* by single-cell RNA sequencing analysis

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SUMMARY

Here, we describe a protocol for single-cell isolation from the primary culture of normal human epidermal keratinocytes derived from neonatal foreskin. The cell culture conditions have been optimized for inducing expression of keratinocyte differentiation markers. Cells are cultured in the absence or presence of a bioactive lipid lysophosphatidic acid (LPA). Single cells are isolated by Fluidigm C1 system. This is followed by cDNA library preparation using Takara SMART-Seq v4 Ultra and Illumina Nextera XT kit for RNA sequencing.

For complete details on the use and execution of this protocol, please refer to Siriwach et al. (2022).[1]

BEFORE YOU BEGIN

First time cell cultivation and stock preparation

Timing: approximately 3 days

1. Normal Human Epidermal Keratinocytes (NHEKs, KURABO) are used at passages 1–2 and maintained in an undifferentiated state in the HuMedia-KG2 media (KURABO).
2. The following protocol is based on the comprehensive instruction from KURABO. The manual (in Japanese) is available for download at: https://www.kurabo.co.jp/bio/support/download.php?M=PL&CID=6.
3. Frozen NHEKs are thawed and cultured in a T-25 flask (bottom area 25 cm^2).
   a. Warm up the HuMedia-KG2 medium containing 150 μM $CaCl_2$ at 37°C in a water bath approximately 10–15 min prior to seeding.
   b. Under aseptic conditions (on a clean bench), dispense 4 mL of the medium into a 15 mL centrifuge tube and store it in a refrigerator.
   c. Add 4 mL of the culture medium to each T-25 flask, slightly loosen the cap, place in a cell incubator (37°C, 5% CO_2, humidified) and incubate for at least 30 min.
   d. Thaw frozen NHEKs.
      i. Immerse the bottom of the stock vial in the water bath and gently shake it from side to side.
      ii. When the frozen cells are almost thawed (about 2 min), remove the vial from the water bath,
      iii. Wipe the surface, disinfect areas around the vial with 70% ethanol and place it on a clean bench.
      iv. Carefully remove the cap and gently mix by pipetting.

e. Count the cell number.
   i. Aliquot 20 μL of cell suspension from the vial.
   ii. Dilute with 20 μL of trypan blue for cell counting.
f. Immediately add the remaining cell suspension (about 1 mL) from (e) to the centrifuge tube containing the medium described in (b) and keep in a refrigerator during cell counting.
g. Count the number of viable cells (e) per 1 mL of cell suspension with a hemocytometer.
h. Transfer the cell suspension (e) into prewarmed flasks (c). Recommended cell density is 2,500 cells/cm^2, thus the total number of viable cells in (g) for one T-25 flask should be around 62,500 cells.

Note: The following is an example of seeding calculation.

Total number of viable cells counted in (g): 500,000 cells.
4 mL of the dispensed medium (b) + 1 mL of the cell suspension (e) = 5 mL of total cell suspension.
Number of viable cells required for one T-25 flask: 2,500 × 25 cm^2 = 62,500 cells.
Concentration of cell suspension: 500,000 ÷ 5 mL = 100,000 cells / mL.
Required amount of cell suspension per flask: 62,500 ÷ 100,000 / mL = 0.625 mL (625 μL).
Number of T-25 flasks: 5 mL ÷ 0.625 mL = 8 flasks.

i. Tighten the cap and then gently shake the flask to ensure even plating density.
j. Place T-25 flasks containing seeded cells in the cell incubator, loosen the cap, and incubate for 24 h.
k. After 24 h, observe the cells under a microscope.

Note: Cells should be attached and spread evenly on the flask surface in the form of single individual cells or small clusters.

   i. Aspirate the culture medium to remove the remaining DMSO (DMSO is included in the cryopreservation buffer).
   ii. Add new HuMedia-KG2 medium.
l. After 72 h of seeding, observe the cells under a microscope.

Note: Numerous cell divisions should be observed, indicating that cells have been recovered to an active proliferative state. If cell attachment and division are observed, then cell thawing and seeding are successful.

m. Confirm that cells reach confluency of 70%–90% before proceed to the next step.

Note: The remaining cells can be kept as cryopreservation stock and use within passage 2.

Cell size measurement

Timing: 3 days (for step 4)

Cell size of NHEKs is measured to determine the optimum size of C1 Single-Cell for Auto Prep IFC (integrated fluid circuit) plate (Fluidigm).

4. Cell cultivation and compounds for stimulation are prepared in advance of cell size measurement. In our study, NHEKs were seeded on 24-well plate for LPA stimulation. For more details, please refer to step-by-step method details.

5. NHEKs at optimum confluency (70%–90%) are stimulated using EpiLife medium (Invitrogen) without growth factors, containing 1.2 mM $CaCl_2$ (Gibco) and 30 ng/mL recombinant human IL-4 (Peprotech), in the absence or presence of 10 μM LPA (Avanti) for control vehicle- and LPA-treated conditions, respectively. Each condition is performed in triplicates.

   Note: Triplicate is the minimum well number used for each condition to minimize technical errors such as pipetting.

Stimulation cocktails are prepared as follows;

   a. Prepare 1 mL of the EpiLife/DMSO solution by adding 10 μL of DMSO in 990 μL of EpiLife medium.
   b. Prepare 3 mL of the EpiLife/IL-4/Ca^{2+} by adding 1.3 μL of 30 ng/mL recombinant human IL-4 and 5.1 μL of 1 M $CaCl_2$.
   c. Prepare 1 mL of 100 μM LPA by mixing 50 μL of LPA (1 mM) with 450 μL of Epilife/DMSO.
   d. Prepare stimulation cocktails for control and LPA conditions:
      i. Control condition: mixing of 480 μL of the Epilife/DMSO + 1,120 μL of the Epilife/IL-4/Ca^{2+}= 1.6 mL in total.
      ii. LPA condition: mixing of 160 μL of 100 μM LPA + 320 μL of the Epilife/DMSO + 1,120 μL of the Epilife/IL-4/Ca^{2+} = 1.6 mL in total.

6. At 72 h post stimulation, single-cell suspension from either control or LPA stimulation is trypsinized to measure the cell size.
   a. Thaw the frozen trypsin/EDTA solution (KURABO) and HEPES buffer (KURABO), and prewarm necessary amounts at 37°C.
   b. For each condition, dispense 1.5 mL of trypsin neutralizing solution (KURABO) into a 15 mL-centrifuge tube and prewarm at 37°C.
   c. For each condition, dispense 0.5 mL of culture medium in a 1.5 mL centrifuge tube.
   d. Prewarm the culture medium at 37°C in a water bath. It will be used later for cell resuspension.
   e. Remove culture medium from each well by aspiration.
   f. Gently wash adherent cells with 500 μL of HEPES buffer for ~30 s.
   g. Remove HEPES buffer by aspiration.
   h. Treat the cells with 100 μL of the pre-warmed trypsin/EDTA solution.
   i. Leave the 24-well plate at room temperature (20°C–25°C).

      ⚠ CRITICAL: Do not incubate the cells in a 37°C incubator.

   j. Confirm that the cells are completely detached under a microscope. The cell shape should appear round and does not form large cell aggregate.
   k. Five to eight mins after trypsinization, when approximately half of the cells should be detached, tap the side of the plate to further detach the remaining cells from the culture surface.
   l. Observe the cells under a microscope. Confirming that cells are completely detached from the culture surface.
      i. Add 500 μL of HEPES buffer and gently mix with a pipette.
      ii. Collect them by combining 3 wells from each condition together into a 15 mL-centrifuge tube containing trypsin neutralizing solution (6b).
   m. Centrifuge at 220 *g* for 5 min at room temperature (20°C–25°C).
   n. Discard the supernatant by aspiration.
   o. Resuspend cells in the prewarmed medium (6d).

7. For each condition, measure the diameters of ~100 cells using the ruler function of the Nikon camera control unit DS-L3 (Figure 1) and calculate the average diameter of NHEKs.

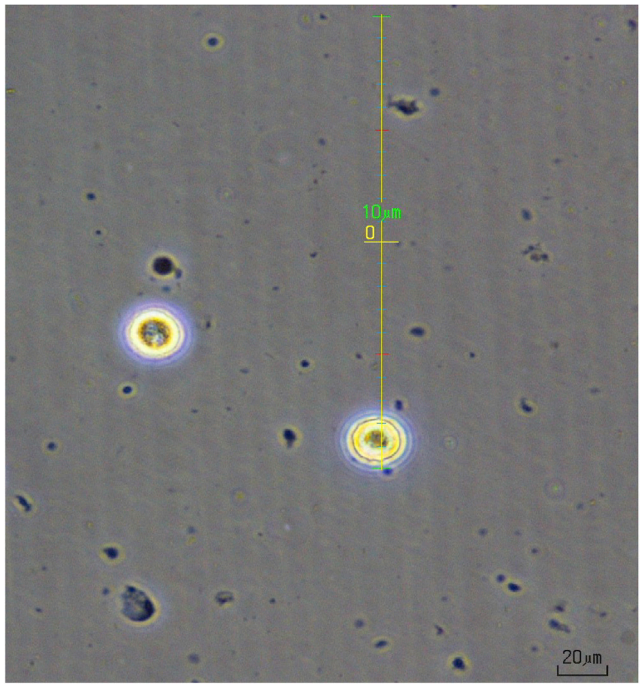


Figure 1. Measurement of the cell size under a microscope
A phase contrast image of the keratinocytes is shown. Scale bar 20 μm.

Note: In our study, the optimal IFC size was 17–25 μm in diameter.[1]

Software preparation

8. Download and install the following software for data analysis.
   a. FastQC v0.11.8[2] (https://github.com/s-andrews/FastQC).
   b. Cutadapt v2.3[3] (https://github.com/marcelm/cutadapt).
   c. STAR v2.7.0f[4] (https://github.com/alexdobin/STAR).
   d. featureCounts v1.6.4[5] (http://bioinf.wehi.edu.au/featureCounts).
   e. R v3.6.3 (https://cran.r-project.org).

   Note: Statistical analysis in R can be performed on R studio or Jupyter Notebook.

   f. Seurat v3.1.5[6] (https://satijalab.org/seurat/articles/install.html).
   g. Harmony v1.0[7] (https://github.com/immunogenomics/harmony).

   Note: Newer versions of software might be available. The user might use the latest version of the software with their own compatibility testing.

9. Download the reference human genome: hg38 (NIH); https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.40.
10. Download an annotation file: GENCODE human; https://www.gencodegenes.org/human/.

    Note: We used the reference human genome: hg38 (Genome Reference Consortium Human Build 38 patch release 12; GRCh38.p12) and GENCODE human Release 30. We recommend using the latest version of the reference genome and the GENCODE annotation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Recombinant human IL-4	PeproTech	Cat# 200-04
LPA 18:1	Avanti Polar Lipids	Cat# 857130C
HEPES	KURABO	Cat# HK-3320
Trypsin/EDTA	KURABO	Cat# HK-3120
Trypsin neutralizer	KURABO	Cat# HK-3220
HuMedia-KG2	KURABO	Cat# KK-2150S
EpiLife	Invitrogen	Cat# MEPI500CA
Critical commercial assays		
C1 Single-Cell for Auto Prep IFC (17–25 μm)	Fluidigm	Cat# 100-8135
SMART-Seq® v4 Ultra® Low Input RNA Kit for the Fluidigm® C1™ System	Clontech	Cat# 635025
Nextera XT DNA Library Preparation Kit	Illumina	Cat# FC-131-1096
Nextera XT Index Kit	Illumina	Cat# FC-131-1002
Quant-iT™ PicoGreen® dsDNA Assay Kit	Thermo Fisher Scientific	Cat# P11496
High Sensitivity DNA Kit (chips and reagents)	Agilent Technologies	Cat# 5067-4626
Agencourt AMPure® XP	Agencourt BioScience Corp.	Cat# A63880
Experimental models: Cells		
Normal human epidermal keratinocytes (NHEKs), neonatal, primary, male, Asian/Caucasian, lot number 04228	KURABO	Cat# KK-4009
Deposited data		
Genome Reference Consortium Human Build 38 patch (hg38; GRCh38.p12)	NIH	https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.38/
GENCODE human v30	GENCODE	https://www.gencodegenes.org/human/release_30.html
scRNA-seq data	Siriwach et al.[1]	GEO: GSE167056
Software and algorithms		
FastQC v0.11.8	Andrews[2]	https://github.com/s-andrews/FastQC
Cutadapt v2.3	Martin[3]	https://github.com/marcelm/cutadapt
STAR v2.7.0f	Dobin et al.[4]	https://github.com/alexdobin/STAR
featureCounts v1.6.4	Liao et la.[5]	http://bioinf.wehi.edu.au/featureCounts/
Seurat v3.1.5	Stuart et al.[6]	https://github.com/satijalab/seurat
Harmony v1.0	Korsunsky et al.[7]	https://github.com/pardeike/Harmony
Other		
T-25 flask	Corning	Cat# 431463
24-well plate	Corning	Cat# 3526
Water bath (SDminiN)	Titec	Cat# 0068750-000
Serological pipettes: 10 mL	Iwaki	Cat# 7154-010
Single-channel pipettes: 10 μL, 20 μL, 200 μL, and 1000 μL	Eppendorf	Cat# 3123000020, 3123000098, 3123000055, and 3123000063
Pipette tips: 10 μL, 200 μL, and 1000 μL	Corning	Cat# 4135, 4138, and 4140
Microcentrifuge tube 1.5 mL	Watson	Cat# 131-815C
Centrifuge tubes: 15 mL	Falcon	Cat# 352096
Disposable hemocytometer	Waken	Cat# WC2-100
Cell culture hood	Sanyo	Cat# MHE-130A
Swing rotor centrifuge	Tomy	Cat# AX-511
CO_2 cell incubator (Forma Steri-Cycle 370)	Thermo Fisher Scientific	Cat# TH-370
Inverted light microscope (Eclipse TS100)	Nikon	Cat# ECLIPSE TS100
High-definition color camera (DS-Fi2)	Nikon	Cat# DS-Fi2
Standalone camera control unit (DS-L3)	Nikon	Cat# DS-L3
Vortex(Vortex-Genie 2)	Scientific Instruments	Cat# SI-0236
Fluidigm® C1™ system	Fluidigm	Cat# 100-7000

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Inverted fluorescence microscope (IX81)	Olympus	Cat# IX81
96-well PCR plate	BMBio	Cat# MB-Q96-S
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific	Cat# 4306311
8-channel pipette: 10 μL	Eppendorf	Cat# 4922000013
Thermal cycler Veriti 96 wells	Applied Biosystems	Cat# 4375305
Single-Cell mRNA seq PicoGreen Template	Fluidigm	Cat# 100-6260
Fluorometer (EnVision 2103)	PerkinElmer	Cat# EnVision 2103
12-channel pipette: 10 μL	Eppendorf	Cat# 4926000026
8-tube strip	Nacalai	Cat# 18668-14
Bioanalyzer 2100	Agilent	Cat# G2939BA
HiSeq 2500 System	Illumina	https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html

MATERIALS AND EQUIPMENT

- Cell culture medium:

HuMedia		
Reagent	Final concentration	Amount
HuMedia-KG2	N/A	13 mL
1 M $CaCl_2$	150 μM	1.95 μL
Total	**N/A**	**13 mL**
Freshly prepare and store at 4°C before use.		

EpiLife/Ca^{2+}		
Reagent	Final concentration	Amount
EpiLife	N/A	17 mL
1 M $CaCl_2$	60 μM	1 μL
Total	**N/A**	**17 mL**
Freshly prepare and store at 4°C before use.		

EpiLife/IL-4/Ca^{2+}		
Reagent	Final concentration	Amount
EpiLife	N/A	7 mL
30 ng/mL recombinant human IL-4	N/A	3 μL
1 M $CaCl_2$	1.2 mM	12 μL
Total	**N/A**	**7 mL**
Freshly prepare and store at 4°C before use.		

- Buffer and solutions

EpiLife/DMSO solution		
Reagent	Final concentration	Amount
EpiLife	N/A	1 mL
DMSO	100%	10 μL
Total	**N/A**	**1 mL**
Freshly prepare and store at 4°C before use.		

100 μM LPA		
Reagent	Final concentration	Amount
EpiLife/DMSO solution	N/A	900 μL
1 mM LPA	100 μM	100 μL
Total	**N/A**	**1 mL**

Freshly prepare and store on ice.

1 mM LPA in HEPES buffer		
Reagent	Final concentration	Amount
2.5 mg LPA	1 mM	lyophilzed
1 M HEPES	10 mM	55 μL
5 M NaCl	500 mM	550 μL
Distilled water	N/A	4.895 mL
Total	**N/A**	**5.5 mL**

LPA is provided by the manufacturer as clear solution in chloroform. Chloroform should be evaporated with N_2 gas before reconstitution. In brief, transfer LPA to a 50 mL round bottom glass flask and evaporate chloroform solvent by blowing a slow stream of nitrogen gas for approximately 5 min. Then, add HEPES buffer at the desired amount to reconstitute LPA. Make aliquots of 100 μL. Store at −20°C and use within one month. Avoid repeated freezing and thawing.

STEP-BY-STEP METHOD DETAILS

Cell cultivation and compound stimulation

Seeding

Timing: approximately 1 h

1. The procedure of the *in vitro* NHEKs culture is described in Figure 2. From the seeding step, cells are cultured in a 24-well plate (surface area 1.9 cm^2).

Note: The 24-well plate is used, thus consistent with the other experiments in Siriwach et al.[1]

a. Observe cells cultured in T-25 flask under a microscope. Confirm that those cells reach a good confluency of around 70%–90%.
b. Prepare a 24-well culture plate.
c. Thaw the frozen trypsin/EDTA solution and HEPES buffer, and prewarm necessary amounts at 37°C.
d. Dispense 5 mL of trypsin neutralizing solution into a 15 mL-centrifuge tube and prewarm at 37°C.
e. Prewarm the HuMedia-KG2 medium containing 150 μM $CaCal_2$ to 37°C. It will be used later for cell resuspension.
f. Remove the culture medium from the flask by aspiration.
g. Gently wash adherent cells with 2 mL of HEPES buffer for ~30 s.
h. Remove HEPES buffer by aspiration.
i. Treat the cells with 2 mL of trypsin/EDTA solution for about 1 min at room temperature (20°C–25°C).
j. Remove the trypsin/EDTA solution by aspiration, but leave approximately 0.5 mL of the solution. Tighten the cap to prevent cells from drying out.
k. Leave the flask at room temperature (20°C–25°C).

⚠ CRITICAL: Do not incubate the cells in a 37°C incubator.

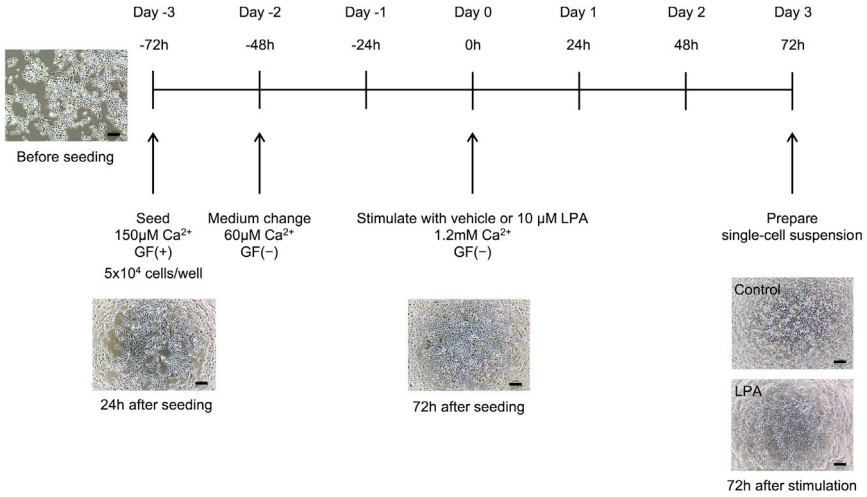


Figure 2. Protocol for cell culturing and stimulation of NHEKs
Protocol was designed based on our previous study.[8] Scale bars = 200 μm.

l. Occasionally tilt the flask to spread trypsin/EDTA solution over the entire cell layer. Gently tap the side of the flask approximately every 2 min to further detach the cells.
m. Observe the cells under a microscope at 5–8 min after trypsinization. If attached cells could still be observed, strongly tap the side of the plate to further detach the remaining cells from the culture surface.
n. After confirming that cells are completely detached from the culture surface, add 5 mL of HEPES buffer, gently pipette mixing 1–2 times using a 10-mL pipette, and then collect them into the centrifuge tube containing trypsin neutralizing solution (d).
o. Centrifuge at 220 *g* for 5 min.
p. Remove the supernatant by aspiration.
q. Resuspend cells with 5–8 mL of the prewarmed medium (1e) and count the number of cells with a hemocytometer.
r. The seeding density is 2,500 cells/cm^2. Number of viable cells required for one well in 24-well plate is 2,500 × 1.9 cm^2 = 4,750 cells and the medium volume per well is 500 μL. Adjust the concentration of cell suspension accordingly before transferring into the 24-well plate, 500 μL of cell suspension per well.
s. Close the lid and gently shake the 24-well plate to ensure even plating density.
t. Incubate the plate for 24 h.

⚠ CRITICAL: At 24 h after seeding, observe the cells under a microscope. Cells should be attached and spread evenly on the culture surface in the form of single individual cells or small clusters (Figure 2).

Medium change

Timing: approximately 30 min

2. At 24 h after seeding, change the culture medium to EpiLife (Invitrogen) containing 60 μM $CaCl_2$ (Gibco) in the absence of growth factors for 48 h.
   a. Prewarm EpiLife containing 60 μM $CaCl_2$ at 37°C in a water bath approximately 10–15 min.
   b. Under aseptic conditions, discard the culture medium by aspiration and then immediately add 500 μL of EpiLife containing 60 μM $CaCl_2$ per well.

⚠ CRITICAL: Avoid pipette tips touching cells directly by placing the tips at the sidewall of the wells.

c. Put the 24-well plate back into the 37°C, 5% CO_2 incubator and further incubate for 48 h.

LPA stimulation

Timing: approximately 1 h

3. At 48 h after medium change, cells are stimulated with EpiLife, in the absence of growth factors, containing 1.2 mM $CaCl_2$ (Gibco) and supplemented with 30 ng/mL recombinant human IL-4 in the absence or presence of 10 μM LPA for control vehicle- and LPA-conditions, respectively. Stimulation cocktails are prepared for 12 wells per each condition.
   a. Prewarm EpiLife medium containing 1.2 mM $CaCl_2$ at 37°C in a water bath, approximately 10–15 min beforehand.
   b. Prepare 3 mL of the EpiLife/DMSO solution by adding 30 μL of DMSO in 2,970 μL of EpiLife medium.
   c. Prepare 7 mL of the EpiLife/IL-4/Ca^{2+} by adding 3 μL of 30 ng/mL recombinant human IL-4 and 12 μL of 1 M $CaCl_2$.
   d. Prepare 1 mL of 100 μM LPA by dissolving 100 μL of LPA (1 mM)/HEPES with 900 μL of EpiLife/DMSO.
   e. Prepare stimulation cocktails for LPA and control conditions.
      i. Control condition: mixing of 1,440 μL of the EpiLife/DMSO + 3,360 μL of the EpiLife/IL-4/Ca^{2+}= 4.8 mL in total.
      ii. LPA condition: mixing of 480 μL of 100 μM LPA + 960 μL of the EpiLife/DMSO + 3,360 μL of the EpiLife/IL-4/Ca^{2+} = 4.8 mL in total.
   f. Prewarm the cocktails at 37°C in a water bath approximately 10–15 min prior to stimulation.
   g. Place the 24-well plate in the clean bench. Under aseptic conditions, discard the culture medium by aspiration and then immediately add 500 μL of the stimulation cocktails (3e). Start from 12 wells of control condition to 12 wells of LPA condition.
   h. Put the 24-well plate back into the 37°C, 5% CO_2 incubator and further incubate for 72 h.

Prepare single-cell suspension

Timing: approximately 1 h

4. At 72 h post-stimulation, prepare 300 cells/μL of single-cell suspension from either control or LPA -treated conditions.
   a. Prepare two of 1.5 mL centrifuge tubes.
   b. Thaw the frozen trypsin/EDTA solution and HEPES buffer, and prewarm necessary amounts at 37°C.
   c. For each condition, dispense 6 mL of trypsin neutralizing solution into a 15 mL-centrifuge tube and prewarm at 37°C.
   d. For each condition, transfer 500 μL of the stimulating culture medium (3e) in a 1.5 mL centrifuge tube. EpiLife/DMSO/IL-4/Ca^{2+} for control condition and EpiLife/DMSO/IL-4/Ca^{2+}/LPA for LPA stimulation condition.
   e. Prewarm the medium (4d) to 37°C in a water bath. Later they will be used for cell suspension.
   f. Remove culture medium from the 24-well plate by aspiration.
   g. Gently wash the cells with 500 μL of HEPES buffer for about 30 s.
   h. Remove HEPES buffer by aspiration.
   i. Treat cells with 100 μL of trypsin/EDTA solution.
   j. Leave the 24-well plate at room temperature (20°C–25°C).

⚠ CRITICAL: Do not incubate the cells in a 37°C incubator.

k. Confirm that cells detach, turn into round shape and don't form large cell aggregate under a microscope.
l. Five to eight mins after trypsinization, when approximately half of the cells are detached, tap the side of the plate to further detach the remaining cells from the culture surface.
m. After confirming that cells are completely detached from the culture surface, add 500 μL of HEPES buffer, gently pipette mixing and then collect them by combining wells of the same condition together into a 15 mL-centrifuge tube containing trypsin neutralizing solution (4c).
n. Centrifuge at 220 *g* for 5 min at room temperature (20°C–25°C).
o. Discard the supernatant by aspiration.
p. Resuspend cells in 200 μL of the prewarmed medium (4e) and count the cell number with a hemocytometer.
q. Adjust the cell concentration to 300 cells/μL.
r. Store the cell suspension on ice.

⚠ CRITICAL: Cell suspension should be prepared as quickly as possible before the cell capture step using the Fluidigm C1 system. As least 200 μL of cell suspension for each condition is required for subsequent steps. In our study, we used the IFC size 17–25 μm. The number of cells for loading was 600 cells.

Note: The recommendation of cell concentration is vary depending on the size of IFC. Please check the SMART-Seq® v4 Ultra® Low Input RNA Kit for the Fluidigm® C1™ System (Clontech), IFCs user manual. The protocol is available for download at https://www.takarabio.com/documents/User Manual/SMART/SMART-Seq_v4 (Ultra Low Input RNA Kit for the Fluidigm C1 System IFCs User Manual_032416.pdf).

Single-cell isolation, cDNA library preparation, and next generation sequencing

Timing: 3 days

5. The procedure of single-cell isolation, cDNA library preparation and next generation sequencing are described in Figure 3. Conduct experiment according to the SMART-Seq® v4 Ultra® Low Input RNA Kit for the Fluidigm® C1™ System (Clontech), IFCs user manual. The protocol is available for download at https://www.takarabio.com/documents/User Manual/SMART/SMART-Seq_v4 (Ultra Low Input RNA Kit for the Fluidigm C1 System IFCs User Manual_032416.pdf).
6. Single cell suspension of 300 cells/μL from either control or LPA stimulation is loaded onto the IFC for single-cell isolation on the Fluidigm C1 Autoprep system (hereafter, C1 machine). In our study, the IFC size was 17–25 μm. Perform cell capturing according to the Fluidigm C1 protocol.
   a. Prepare the C1 reagent mixtures.
      i. Prewarm buffers and reagents from the C1 Single-Cell Auto Prep Reagent Kit to room temperature (20°C–25°C) prior to use. These include Cell Wash Buffer, C1 Preloading Reagent, C1 Blocking Reagent, and C1 Harvest Reagent. Thaw C1 Suspension Buffer on ice and vortex well before use.
      ii. Thaw 5× Ultra Low First-Strand Buffer.
      iii. Prepare Lysis Mix.
      iv. Prepare Reverse Transcription (RT) Mix.
      v. Prepare PCR Mix.
   b. Apply priming solution into the IFC according to the loading mapping in Figure 4A.
      i. Apply 200 μL of C1 Harvest Reagent into the inlets marked with large red circles for total 2 inlets. After application, swirl the IFC gently to distribute the reagent evenly.

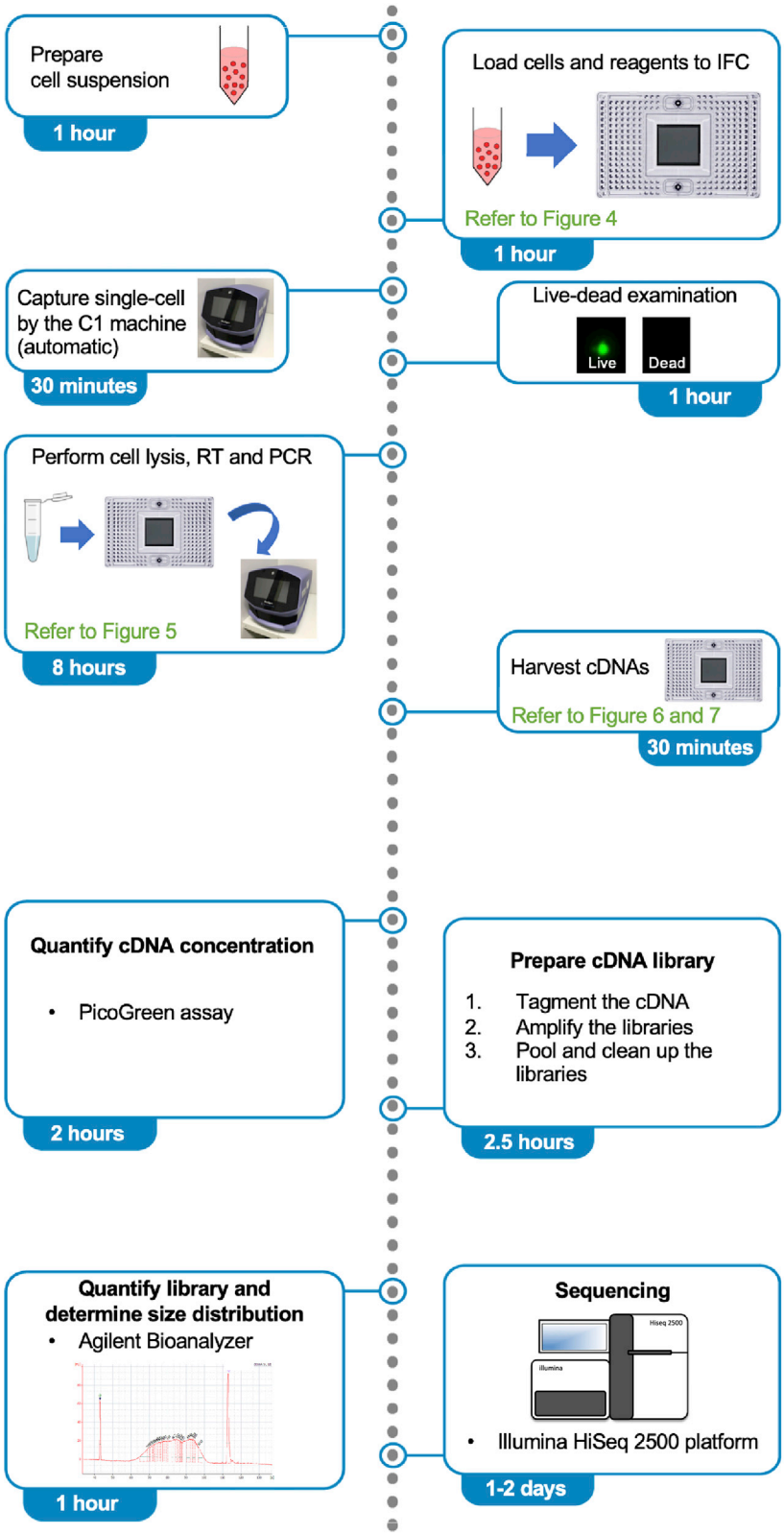


Figure 3. Step-by-step procedure of single-cell isolation, cDNA library preparation, and next generation sequencing

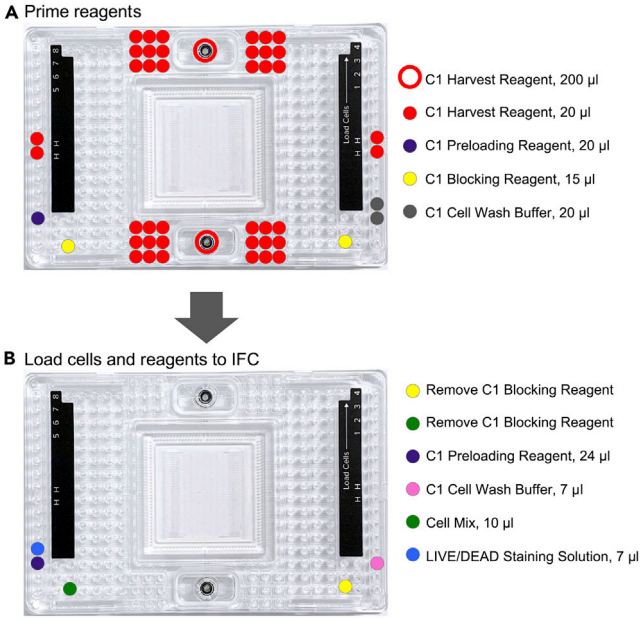


Figure 4. Pipetting map

(A) Priming step.

(B) Cell loading step. Figure is generated based on the Fluidigm's protocol "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing". Protocol is available for download at https://www.fluidigm.com/support/instrument-support/c1-support.

ii. Apply 20 µL of C1 Harvest Reagent into the inlets marked with red dots for total 36 inlets.
iii. Apply 20 µL of C1 Harvest Reagent into 2 pairs of inlets marked with red dots in the middle of the outside columns for total 4 inlets.
iv. Apply 15 µL of C1 Blocking Reagent into the inlets marked in yellow for total 2 inlets.
v. Peel off the white tape at the IFC bottom.
vi. Place the IFC in to the C1 machine and press the LOAD button. Run the SMART-Seq v4: Prime (1861×/1862×/1863×) script.
vii. When the prime run finishes, press EJECT to remove the primed IFC from the C1 machine.

⚠ CRITICAL: Avoid bubbles in IFC by placing pipette tips at the bottom of inlets and slowly dispensing the reagents.

c. Prepare the LIVE/DEAD cell staining solution.
i. Vortex the dyes for 10 s and spin down.
ii. Prepare the LIVE/DEAD staining solution (see LIVE/DEAD cell staining solution Table).

LIVE/DEAD cell staining solution

Reagent	Final concentration	Amount
Cell Wash Buffer	1×	1,250 µL
Ethidium homodimer-1	2 mM	2.5 µL
Calcein AM	4 mM	0.625 µL
Total	**N/A**	**1,253.125 µL**

Freshly prepare, protect from light and store at room temperature (20°C–25°C) before use.

iii. Vortex the solution thoroughly before transferring into the IFC.

d. Apply cells into the IFC according to the loading mapping in Figure 4B.
   i. Prepare the Cell Mix by combining the cell suspension with C1 Suspension Reagent at 3:2 ratios according to the Cell Mix table. Slowly pipet up and down for thorough mixing. Do not vortex the cells. Keep the cell suspension on ice.

Cell Mix

Reagent	Final concentration	Amount
The cell suspension (300 cells/μL)	N/A	20 μL
C1 Suspension Reagent (2.5×)	1×	30 μL
Total	**N/A**	**50 μL**

Freshly prepare and store on ice.

   ii. Remove blocking reagent from the IFC inlets marked in green and yellow by aspiration.
   iii. Set a pipette to 12 μL, then slowly pipet the Cell Mix up and down 5–10 times to mix thoroughly and dissociate any cell clumps.

Note: Do not vortex the Cell Mix. Avoid bubbles when mixing.

   iv. Apply 24 μL of C1 Preloading Reagent into the inlet marked in purple.
   v. Apply 7 μL of Cell Wash Buffer into the inlet marked in pink.
   vi. Apply 10 μL of the Cell Mix into the inlet marked in green.

Note: You may pipet up to 20 μL of Cell Mix, but only 5 μL will enter the IFC.

   vii. Vortex the LIVE/DEAD staining solution well, then apply 7 μL of the solution into the inlet marked in blue.
   viii. Place the IFC into the C1 machine. Run the SMART-Seq v4: Cell Load & Stain (1861×/1862×/1863×) script.

Note: Run IFCs of control and LPA-stimulated cells in parallel on two C1 machines.

   ix. When the run finishes, press EJECT to remove the IFC from the C1 machine.

7. The capturing efficiency can be evaluated under a microscope and only live single cells should be used for library preparation.
   a. Examine all 96-capture sites on the IFC using an inverted fluorescent microscope. Record the number of captured cells in each capture site. Check also the viability of the cells using LIVE/DEAD staining.
8. Proceed to cDNA library preparation using "SMART-Seq v4" (Clontech).
   a. Apply Lysis Mix, Reverse Transcription and PCR reagents into the IFC according to the loading map in Figure 5.
      i. Discard the remaining solution in both the flow through outlet marked in yellow, and in the inlet marked in purple.
      ii. Apply 180 μL of C1 Harvest Reagent into each of the reservoirs marked with red rectangles for total 4 total reservoirs.
      iii. Apply 7 μL of Lysis Mix into the inlet marked in orange.
      iv. Apply 8 μL of Reverse Transcription (RT) Mix into the inlet marked in green.
      v. Apply 24 μL of PCR Mix into the inlets marked in blue for total 2 inlets.
      vi. Apply 24 μL of Preloading Reagent into the inlet marked in purple.
      vii. Place the IFC into the C1 machine and run the SMART-Seq v4: Sample Prep (1861×/1862×/1863×) script. Run the script overnight (8 h).

Note: The running time is 8 h.

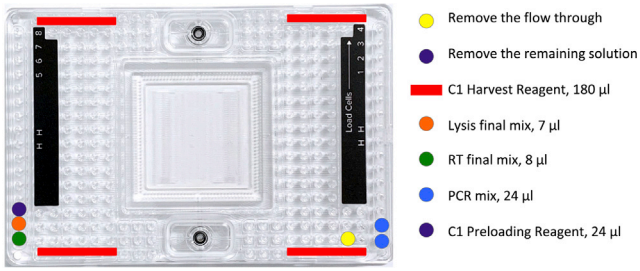


Figure 5. Pipetting map for cell lysis, revere transcription, and PCR steps
Figure is generated based on the Fluidigm's protocol "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing".

⚠ CRITICAL: Harvest should be performed as soon as the Sample Prep script run finishes. Otherwise, small volumes are prone to loss by evaporation. If the elapsed time between the start of sample prep and harvest (for example, when running the script overnight) is over 8 h, adjust the finishing time in the script by sliding the orange bar in the dialog box to "Select when the script should finish."

b. Harvest cDNAs from the C1 IFC.
   i. Prewarm C1 DNA Dilution Reagent to room temperature (20°C–25°C).
   ii. When the SMART-Seq v4: Sample Prep script run completes, press EJECT to retrieve the IFC from the C1 machine.
   iii. Label a new 96-well plate "Diluted Harvest" along with the experiment name, and date.
   iv. Apply 10 µL of the C1 DNA Dilution Reagent into each well of the "Diluted Harvest" plate.
   v. Carefully pull back the four pieces of tape covering the IFC harvesting inlets using a plastic removal tool.
   vi. Use an eight-channel pipette to transfer 3.5 µL of the harvested amplicon from each collection site according to Figure 6 into the Diluted Harvest plate (see Harvest amplicon dilution Table).

Harvest amplicon dilution

Component	Volume (µL)
C1 DNA Dilution Reagent (Fluidigm)	10 µL
C1 harvest amplicons	~3 µL
Total	**~13 µL**

   vii. Transfer the entire volume of C1 harvest amplicons from the IFC into each well of the diluted harvest plate containing 10 µL of C1 DNA Dilution Reagent. Follow step 1–4 in Figure 7.

⚠ CRITICAL: It is critical to follow the instruction map while transferring the cDNAs from the IFC to the "Diluted Harvest" plate in order to trace the sample IDs.

   viii. Seal and vortex the "Diluted Harvest" plate for 10 s at medium speed and then spin it down.

Pause point: Samples can be stored for up to one week at 4°C or at –20°C for long term.

9. Quantify harvested cDNAs using PicoGreen® assay.
   a. Determine cDNA concentration.
      i. Use Single-Cell mRNA Seq PicoGreen Template for quantifying the cDNA library.

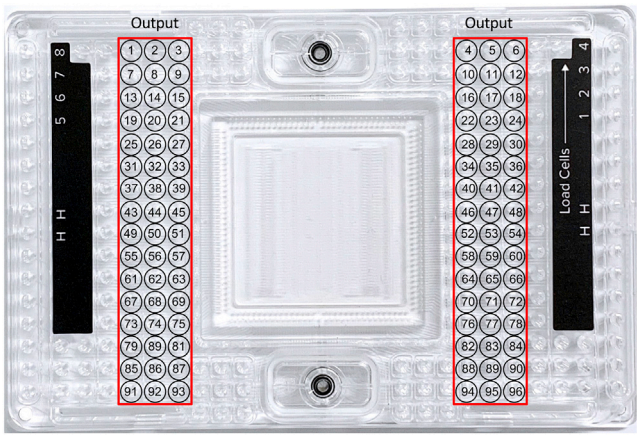


Figure 6. Output map
Figure is generated based on the Fluidigm's protocol "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing".

ii. Input values into the Concentration Estimate Table on the "Example Results" tab in the template.

b. Dilute each sample based on an optimal dilution determined from the Concentration Estimate Table, which should fall within the optimal range of concentrations for Nextera XT library preparation as below.

Note: The Nextera XT protocol is optimized for 1 ng of input DNA. The optimal cDNA concentration for the Nextera XT DNA Library Preparation is 0.10–0.3 ng/μL.

i. Label a new 96-well PCR plate as "Diluted for Sequencing."
ii. Apply an appropriate amount of C1 Harvest Reagent into each well of the "Diluted for Sequencing" plate according to the determined dilution (see Harvest amplicon dilution Table):

Harvest amplicon dilution

cDNA sample dilution	Volume of C1 harvest reagent (μL)
1:2	2
1:3	4
1:4	6
1:5	8
1:6	10
1:8	14
1:10	18
1:12	22

iii. Transfer 2 μL of each harvest sample from the "Diluted Harvest" plate to the "Diluted for Sequencing" plate.
iv. Seal the "Diluted for Sequencing" plate with adhesive film.
v. Vortex at medium speed for 20 s and centrifuge at 350 *g* for 1 min.

10. Perform library preparation using the Nextera XT DNA Library Preparation Kit (Illumina) and Nextera XT Index Kit (Illumina).

Note: In our study, we used the Nextera XT Index kit.[1] However, a new version, V2 kit has been released. We recommend using this new kit version following the manufacturer's instructions.

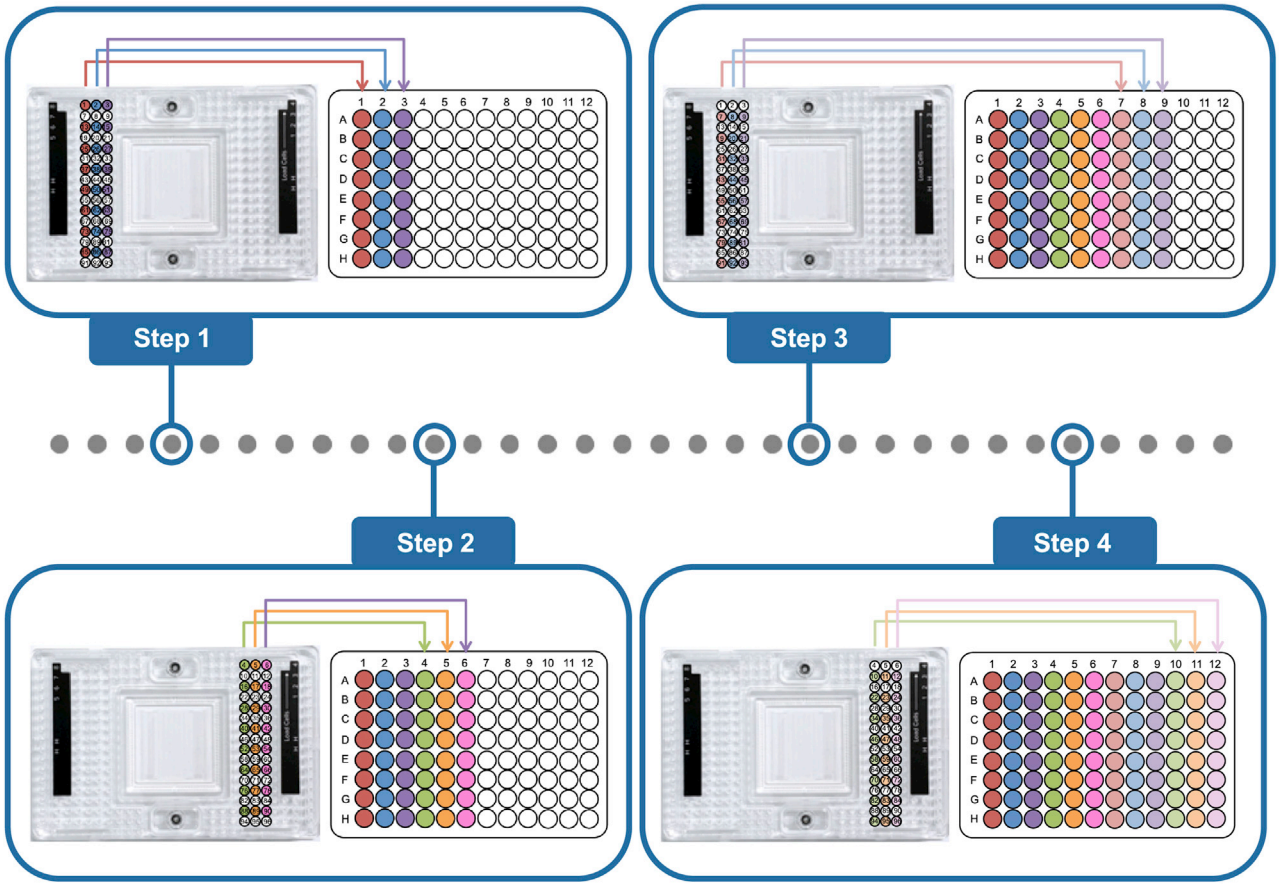


Figure 7. Output harvest map
Figure is generated based on the Fluidigm's protocol "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing."

a. Prepare cDNA for Tagmentation.

⚠ CRITICAL: Warm up Tagment DNA Buffer and NT Buffer to room temperature (20°C–25°C). Confirm there is no precipitation in NT Buffer. If there are any precipitates, vortex until they are completely dissolved.

i. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief centrifugation.
ii. Label a new 96-well PCR plate "Library Prep."
iii. Prepare tagmentation premix in a 1.5 mL centrifuge tube. Calculate the required amount of the premix based on the sample number, or prepare for 96 samples (see below tables).

PCR reaction mix for 1 sample

Reagent	Amount (μL)
Tagment DNA Buffer	2.5
Amplification Tagment Mix	1.25
Diluted sample	1.25
Total volume of premix	5

Freshly prepare and store on ice.

PCR reaction master mix for 96 samples*	
Reagent	Amount (μL)
Tagment DNA Buffer	300
Amplification Tagment Mix	150
Diluted sample	–
Total volume of premix	–

*Calculation based on a 25% overage.
Freshly prepare and store on ice.

iv. Vortex gently for 20 s and spin down the tube.
v. Aliquot equal amounts of the premix into each tube of an 8-tube strip.
vi. Transfer 3.75 μL of the premix into each well of the "Library Prep" plate using an 8-channel pipette.
vii. Transfer 1.25 μL of each diluted sample from the "Diluted for Sequencing" plate to the "Library Prep" plate.
viii. Seal the plate and vortex at medium speed for 20 s. Centrifuge at 2,000 *g* for 5 min to remove bubbles.
ix. Place the "Library Prep" plate in a hot-lid thermal cycler and run the following program:

PCR cycling conditions	
Temperature	Time
55°C	10 min
10°C	Hold

x. Aliquot equal amounts of NT Buffer into each tube of an 8-tube strip. Calculate the required amount of NT Buffer based on the sample number, or prepare for 96 samples (see below tables).

PCR reaction mix for 1 sample	
Reagent	Amount (μL)
Library Prep sample	5
NT Buffer	1.25
Total volume	6.25

Freshly prepare and store on ice.

PCR reaction master mix for 96 samples*	
Reagent	Amount (μL)
Library Prep sample	–
NT Buffer	150
Total volume	–

*Calculation based on a 25% overage.
Freshly prepare and store on ice.

xi. Once the thermal cycler reaches 10°C, apply 1.25 μL of NT Buffer into every tagmented samples for neutralization.
xii. Seal the plate and vortex at medium speed. Centrifuge at 2,000 *g* for 5 min.

b. Amplify the tagmented cDNA.

Note: Carefully read the Illumina protocol, "Nextera XT DNA Library Prep Reference Guide" for index primer selection criteria before proceeding to PCR amplification of the tagmented cDNA.

i. Aliquot equal volumes of Nextera PCR Master Mix (NPM) into each tube of an 8-tube strip. Calculate the required amount of NPM based on the sample number, or prepare for 96 samples (see below tables).

PCR reaction mix for 1 sample	
Reagent	Amount (μL)
Library Prep sample	6.25
NPM	3.75
Total volume	10
Freshly prepare and store on ice.	

PCR reaction master mix for 96 samples*	
Reagent	Amount (μL)
Library Prep sample	–
NPM	450
Total volume	–
*Calculation based on a 25% overage.	
Freshly prepare and store on ice.	

ii. Apply 3.75 μL of the aliquoted NPM into each well of the "Library Prep" plate using an 8-channel pipette.

iii. Select appropriate Index 1 (N7xx) and Index 2 (S5xx) primers required for all samples. Each Index 1 Primer corresponds to a column of the 96-well plate and each Index 2 Primer corresponds to a row.

Apply 1.25 μL of Index 1 Primers (N7xx) into the corresponding wells in row A of the "Library Prep" plate using a 12-channel pipette. As a result, 12 wells in row "A" will contain different Index 1 Primers.

Note: If a 12-channel pipette is not available, use an 8-channel pipette instead.

Apply 1.25 μL of Index 2 Primers (S5xx) to the corresponding wells in column B of the "Library Prep" plate using an 8-channel pipette. As a result, each of the 8 wells in column "B" will contain different Index 2 Primers.

iv. Seal the plate with adhesive film and vortex at medium speed for 20 s.
Centrifuge at 2,000 *g* for 2 min.

v. Place the "Library Prep" plate into a thermal cycler and perform PCR amplification.

PCR amplification		
Temperature	Time	Cycles
72°C	3 min	1
95°C	30 s	1
95°C	10 s	12 cycles
55°C	30 s	
72°C	60 s	
72°C	5 min	1
10°C	∞	

Pause point: The PCR products can be remained on the thermal cycler overnight (8 h), or be stored at 2°C–8°C for up to 2 days or at –20°C for long term.

c. Library pooling, cleanup, and quantification.

PCR-amplified, tagmented cDNA is purified by immobilization on AMPure XP beads (Agencourt).

Note: Before starting, incubate bead aliquots at room temperature (20°C–25°C) for at least 30 min and mix well to disperse.

i. Determine the number of samples to be pooled based on desired sequencing depth and sequencer throughput.

Note: If preferred, samples can be cleaned up individually prior to pooling.

ii. Incubate AMPure XP beads at room temperature (20°C–25°C) and vortex for 1 min.
iii. Prepare the library by pipetting the appropriate volume from each sample into a 1.5-mL tube, according to the desired sample number.

Number of samples to be pooled	Volume per sample (μL)	Total library volume (μL)	AMPure bead volume* (μL)
8	4	32	29
12	4	48	44
16	2	32	29
24	2	48	44
32	1	32	29
48	1	48	44
96	1	96	87

iv. Add the required amount of AMPure XP beads to the pooled library according to the table above.
v. Mix well by vortexing or pipetting the entire mixture up and down 5 times.
vi. Incubate the bead mix at room temperature (20°C–25°C) for 5 min to let the cDNA libraries bind to the beads.

Note: The beads are viscous. Pipette the entire volume and dispense slowly.

vii. Spin down before placing the tube on a magnetic stand for 2 min. Confirm that supernatant appears completely clear without any beads left.
viii. Keep the sample on the magnetic stand and discard the supernatant.
ix. Add 180 μL of 70% ethanol without disturbing the beads, incubate for 30 s and carefully remove the supernatant. cDNA libraries will remain bound to the beads during this washing step.

Note: Prepare fresh 70% ethanol.

x. Repeat the above step (ix).
xi. Spin down and place the sample again on magnetic stand for 30 s before removing all remaining ethanol with a pipette.
xii. Allow the sample to air-dry for 10–15 min.

⚠ CRITICAL: Be sure that the pellet after drying should look matte with no shine. Insufficient drying or over-drying may reduce the recovery yield.

xiii. Once the pellet is dry, elute the pooled, purified library by adding a required volume of C1 DNA Dilution Reagent based on the number of pooled samples.

Number of pooled samples	C1 Dilution Reagent volume* (μL)
8	32
12	48
16	32
24	48
32	32
48	48
96	96

*C1 Dilution Reagent volume is equal to the original pool volume.

xiv. Remove the sample from the magnetic stand and vortex the tube for 3 s to mix thoroughly. Incubate at room temperature (20°C–25°C) for 2 min.
xv. Spin down and place the sample back on magnetic stand for 2 min or longer, until the solution is completely clear.
xvi. Transfer the entire volume of clear supernatant containing purified cDNA library to a new 1.5-mL centrifuge tube.
xvii. Repeat steps iv – xii.
xviii. Elute the library by adding a required volume* of C1 DNA Dilution Reagent according to the number of pooled samples.

Number of libraries pooled	C1 Dilution Reagent volume* (μL)
8	48
12	72
16	48
24	72
32	48
48	72
96	144

*C1 Dilution Reagent volume is 1.5 times larger than the initial library volume.

xix. Remove the tube from the magnetic stand and vortex the tube for 3 s.
xx. Incubate at room temperature (20°C–25°C) for 2 min.
xxi. Place the tube on the magnetic stand for 2 min until the supernatant is completely clear.
xxii. Carefully transfer the supernatant to a new 1.5-mL centrifuge tube labeled as "Cleaned Lib."

11. Quantify the cDNA library and check its quality using a Bioanalyzer 2100 (Agilent) with a High Sensitivity DNA kit (Agilent) in triplicate (see Figure 8).
12. Refer to the "Nextera XT DNA Library Preparation Guide" to determine the appropriate library concentration for sequencing.
13. Proceed to next generation sequencing in single-end mode using Illumina HiSeq 2500 platform (Illumina) at the read depth of 1 million reads per cell.

Sequencing, mapping, and data analysis

Timing: approximately 1 week

14. Evaluate raw sequencing data using FastQC.[2]
15. Remove adapter sequence using Cutadapt.[3]
16. Map sequence reads to the reference genome (hg38) together with the GENCODE human annotation by using STAR.[4]
17. Generate a count matrix using featureCounts.[5]
18. Conduct downstream analysis using Seurat (v3.1.5) on R.[6]

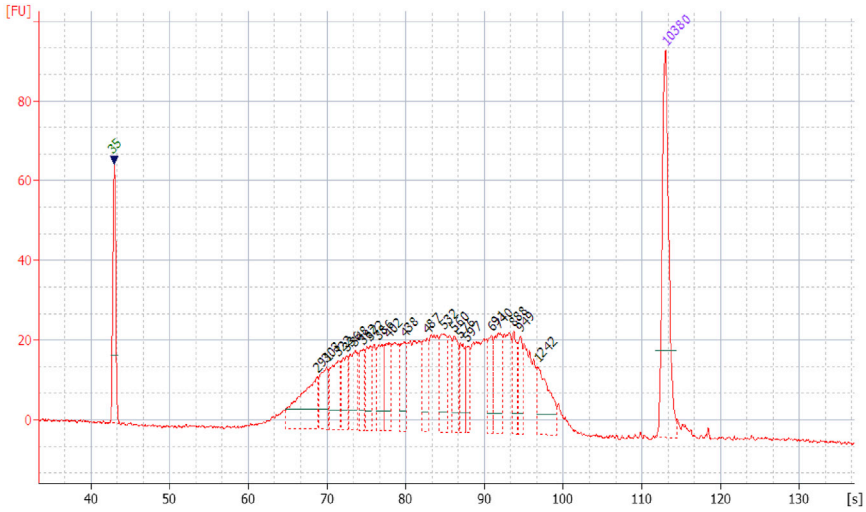


Figure 8. Quantitative of the single-cell cDNA library using Bioanalyzer

The representative quantification data of control NHEKs single cell cDNA library with Bioanalyzer is shown.

Note: Cells with poor quality should be filtered out based on 3 major criteria: total count number, total detected gene number, and percentage of mitochondrial genes. Given significant batch differences in stimulated cell preparation, cDNA library preparation, and sequencing between the 2 conditions, it is critical to use a suitable batch-effect correction method to remove batch effects before further analysis. For our data, Harmony was used (v1.0).[7] More analysis details are available in Siriwach et al.[1]

EXPECTED OUTCOMES

Based on this protocol, live single NHEK cells stimulated by LPA, can be isolated from the cell culture for subsequent scRNA-seq analysis. This protocol could also be implemented to isolate single NHEK cells from other compound stimulation. However, it should be noted that the cell size should be carefully determined to select the appropriate size of the IFC for single-cell isolation.

The transcription profiles of each NHEK cell in the culture *in vitro* could be obtained using this protocol. In our study, we subjected 600 cells from each condition into the 96 wells IFC plate.[1] The single cell isolation and cDNA synthesis were performed on the C1 system. The concentration of cDNA was adjusted to the range of 0.10–0.3 ng/μL and the libraries were then generated using 1 ng of cDNA using Nextera XT DNA Library Preparation. cDNA libraries were subsequently quantified using a Bioanalyzer 2100. The size distribution of our libraries were ~300–1,000 bp. The cDNA libraries were sequenced using Illumina HiSeq 2500 platform at the read depth of 1 million reads per cell. After alignment and quality control, we obtained the transcription profiles of 59 NHEK cells from the control group and 60 NHEK cells from the LPA-treatment group. Two-dimensional visualized with uniform manifold approximation and projection (UMAP) of integrated scRNA-seq data (n=119) using Seurat revealed three different subpopulations of NHEK cell (Figure 9).[6] We found that while most of NHEK cells from the control group belong to cluster 1, NHEK cells from the LPA treatment group largely belong to cluster 2 and 3. This result suggests that LPA facilitates the differentiation of a relatively homogenous NHEK population to two different cell populations.

Furthermore, the identity of NHEK cells in each cluster can be deduced by differential expression genes (DEGs) analysis. We found that cluster 1 cells expressed high level of basal keratinocyte markers, *KRT5* and *KRT14*, and therefore annotated them as basal-like keratinocytes (Figure 10A). For cluster 2 cells, we found that they expressed high level of early granular keratinocyte markers such as *TGM1* and *SCEL*, and therefore annotated them as granular-like keratinocytes (Figure 10B).

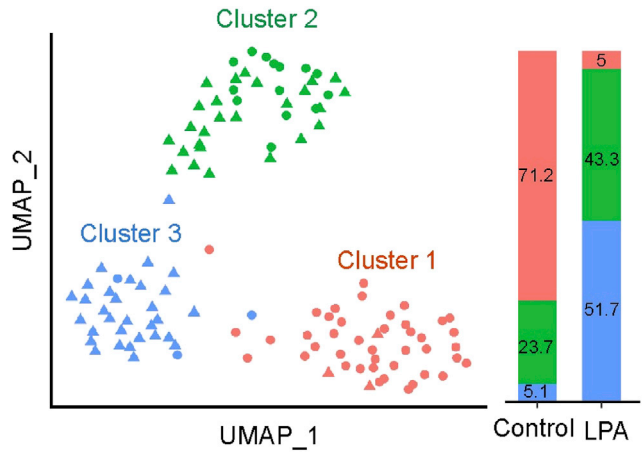


Figure 9. Clustering analysis results

Two-dimensional visualized with uniform manifold approximation and projection (UMAP) of cells that passed the quality control (n = 119), colored according to unsupervised clustering. Round mark (○) indicates control NHEKs and triangle mark (Δ) indicates cells treated with 10 μM LPA for 72 h. Bar chart on the right indicates the percentage of cells assigned to each cluster per condition, colored according to unsupervised clustering. Figure is reproduced from Siriwach et al.[1]

Notably, the top DEG of cluster 3 cells was *thrombospondin-1* (*THBS1*), a matricellular protein involved in tissue repair and cell migration (Figure 10C).

The above results suggest that this protocol can be implemented to evaluate the effect of other compound stimulations on the NHEK differentiation *in vitro*.

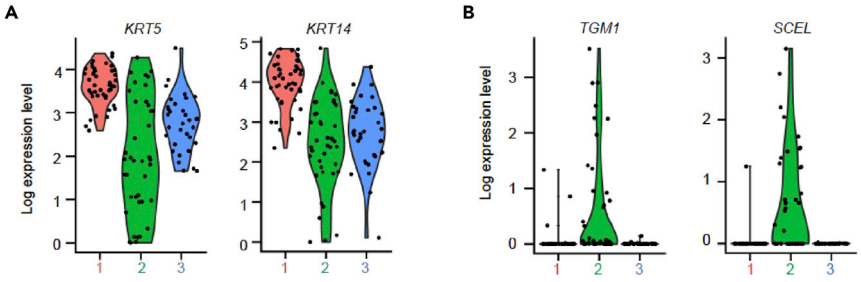
LIMITATIONS

First of all, it should be noted that although *in vitro* differentiation of the NHEK cells partially mimics *in vivo* differentiation of human keratinocytes, it is not physiological. Therefore, further validation of scRNA-seq analysis results with biological experiments are necessary. Regarding the Fluidigm C1 system, one limitation is the range of cell sizes. The cell sizes exceeding the size range of IFC will be filtered out. The other limitation is that the Fluidigm C1 system can process only small number of cells compared to the droplet-based scRNA-seq system. Therefore, cluster analysis should be conducted and interpreted with caution. We used Fliudigm C1 system for this experiment, because at the beginning time of our experiment, the droplet-based scRNA-seq system was just getting established and not widely available. We speculate that droplet-based system might also be applicable to our experiment. However, it should be noted that Fludigm C1-based scRNA-seq is capable to read more genes than other scRNA-seq system. Therefore, it is also possible that the data obtained by other scRNA-seq system (ex. Droplet-based system) might be different. It should also be noted that one more important limitation of the Fluidigm C1 system is cells from different experimental condition could not be processed at the same time using the same C1 machine. For example, in our study, we processed the control and LPA-stimulated NHEK cells on two different C1 machines in parallel. Therefore, researchers should also be careful about possible batch effect when running cells on more than one C1 machine. Finally, as a general limitation of scRNA-seq, the prevalence of genes with zero count are known. Due to this limitation, many low-expressed genes are rarely detected.

TROUBLESHOOTING

Problem 1

Response to LPA stimulation is weak. Please note that we used the expression level of the gene "*FLG*" by qPCR to determine the LPA response.[1,8] Given that FLG is a keratinocyte differentiation marker, high *FLG* expression reflects strong response to LPA stimulation.



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Rank	Gene	Average log fold change	P value
1	*THBS1*	1.927	3.90E-08
2	*RPL37*	0.732	4.32E-08
3	*RPL27A*	0.682	1.70E-06
4	*RPL28*	0.613	4.79E-06
5	*RPL13P12*	0.268	6.48E-06
6	*ADAMTS1*	0.773	7.64E-06
7	*FST*	1.431	9.01E-06
8	*EEF2*	0.729	9.43E-06
9	*ACTN1*	0.867	1.04E-05
10	*INHBA*	1.367	1.24E-05

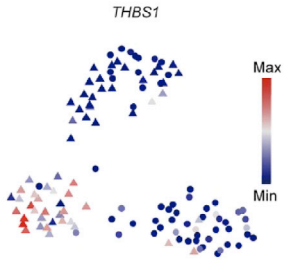

Figure 10. Annotation of cell clusters

(A) Violin plots showing expression of representative basal keratinocyte markers, Keratin 5 (*KRT5*) and Keratin 14 (*KRT14*).

(B) Violin plots showing expression of representative granular keratinocyte markers, Transglutaminase 1 (*TGM1*) and Sciellin (*SCEL*).

(C) Top 10 differential expression (DE) genes in cluster 3 ranked by p-value and Feature plot showing Thrombospondin-1 (*THBS1*) expression projected on UMAP plot as in Figure 9. Figures are reproduced from Siriwach et al.[1]

Potential solution

LPA is prone to oxidization and its biological activity can be easily lost. Therefore, LPA should be handled carefully. We suggest the use of freshly prepared LPA in every experiment. In addition, the condition of NHEK cells before seeding is critical. They should be kept in mitogenic and undifferentiation state. Furthermore, to obtain optimum results, lot check of NHEKs is recommended.

Problem 2

Bubbles in the IFC inlet when loading the reagents or cells after step 6.

Potential solution

Allow the bubble to float to the top of well, then remove by pipetting or remove all reagents in the inlet and reloading.

Problem 3

C1 System error due to cell clogging.

Potential solution

After generating single cell suspension, always confirm the quality under the microscope. Never subject cell clumps into the C1 system. If clumps are observed, then filtering with cell strainer might be helpful. Or alternatively, conduct FACS sorting. However, if there is no improvement, then modification of cell isolation protocol might be necessary.

Problem 4

At the quantification of cDNA library step by Bioanalyzer, the shape of the chromatogram curves indicating the size distribution might be different between different samples.

Potential solution

The shapes of library size distribution might be varied. It depends on the biological nature of the sample. Therefore, the cDNA libraries obtained from NHEKs stimulated with different compounds may have different shapes. However, it will not affect the sequencing.

Problem 5

Batch effect between control and stimulated NHEKs.

Potential solution

To avoid the technical batch effect, researchers should prepare the samples from 2 different conditions similarly and subject them to C1 system in parallel. For the data analysis, given that batch effect issue varies differently between experiments, we recommend trying different batch-effect correction computational methods such as Harmony[7] or SCTransformed available in the Seurat integration[9] and, choose the one that works the best for your data. In addition, we also recommend trying different parameter settings for each method. It should also be noted that overcorrection masks biological signals and therefore, attention should be paid to avoid overcorrection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dean Thumkeo (d.thumkeo@mfour.med.kyoto-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The raw and analyzed scRNA-seq data generated during this study are available at Gene Expression Omnibus: GSE167056.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.S. and D.T.; methodology, R.S. and D.T.; acquisition of data, R.S., A.Q.N., and D.T.; writing – original draft, R.S., A.Q.N., and D.T.; writing – review & editing, R.S., A.Q.N., S.N., and D.T.; supervision, S.N. and D.T.

DECLARATION OF INTERESTS

S.N. is a scientific advisor to Astellas Pharma Inc and Toray Co., Ltd.

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