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Fluidigm Based Single-cell Gene Expression Library Preparation from Patient-derived Small Intestinal Organoids

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[Abstract] In this protocol, we describe our methods to isolate crypts from patients biopsy samples and to culture human intestinal stem cells as it's called "organoid." Beyond that, we describe how to dissociate organoids cells into single cells for single-cell analysis as a further application. This protocol should provide investigators sufficient tools to generate human organoids from biopsy samples and to accomplish a stable *in-vitro* assay system.

Keywords: Organoid, Intestinal stem cell, Single-cell analysis, Endoscopic biopsy, Multiplex PCR

[Background] The intestinal epithelium is a multifunctional tissue that orchestrates homeostasis and forms a physical barrier. Each intestinal epithelial cells (IECs) arising from intestinal stem cells (ISCs) renew this epithelium every 4-5 days (Crosnier *et al.*, 2006). ISCs are located at the bottom of the crypts and express specific markers as previously reported by various papers (Muñoz *et al.,* 2012; Clevers, 2013). Studies suggested that malfunctions of proper renewals of stem cells are related to intestinal disorders, and understandings of ISCs dynamic may elucidate the pathogenesis of various disorders including Inflammatory Bowel Disease (IBD) (Okamoto *et al.*, 2016).

However, the studies of intestinal stem cell properties had been challenging due to the lack of efficient models that recapitulates physiological intestinal epithelial layers. The epic introduction of "organoid" has overcome various obstacles (Sato *et al.*, 2009 and 2011). Organoids can be established from a single ISC *in vitro*, and faithfully retain the physiological and pathological features of their tissue of origin (Middendorp *et al.*, 2014). Organoids have been used to dissect underlying pathologic changes in various gastrointestinal disease (Fatehullah *et al.*, 2016; Noben *et al.*, 2017) and has shown potentials to reflect complexed mechanisms of organs.

Also, recent advances in molecular biology techniques allow us to study single-cell modalities (Stuart and Satija, 2019). These techniques developed to have insights into each single cell diversities yet had known to be homogenous populations. Studies have shown that a heterogeneous group of cells share these ISC properties, and constitute a hierarchy within the ISC population (Smith *et al.*, 2016). Furthermore, organoids can be one of the ideal tools that are consist of mostly stem cells and transit-amplifying cells under the undifferentiated culture. In the previous report, a single-cell analysis displayed this heterogeneity among the mouse small intestinal stem cells (Li *et al.*, 2014). Combining organoid

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culture technique and single-cell analysis has the potential to open a new horizon toward the understandings of the dynamics of human intestinal stem cells. In this protocol, we describe in detail the work-flow of human intestinal organoids establishment and dissociation into single cells for further various applications. Compared to those protocols using scRNA-seq (Biton *et al.*, 2014), the present protocol using multiplex PCR enables acquiring single cell profiles in a low-cost, short-time basis, while the number of cells and genes will be limited by the capacity of the microfluid chip format.

Materials and Reagents

A. Organoid culture
1. 24-well Tissue Culture Plate (Corning, Falcon, catalog Number: 353226)
2. 50 ml and 15 ml Conical centrifuge tubes (Corning, Falcon, catalog numbers: 352096, 352070)
3. 15 ml STEMFULL™ low cell adhesion tube (Sumitomo Bakelite Co., catalog number: MS-90150Z)
4. 1.5 ml Eppendorf tube (Eppendorf Safe-Lock Tubes, catalog number: 0030121023)
5. 70 µm Cell strainer (Corning, Falcon, catalog number: 352350)
6. Patient intestinal biopsy samples obtained by endoscopies
7. Phosphate Buffered Saline (PBS) (Sigma, catalog number: D8537-500ML) (stored at 4 °C)
8. Matrigel (Corning, catalog number: 356231) (stored at 4 °C)
9. Cell recovery solution (Corning, Thermo Fisher Scientific, catalog number: 354253) (stored at 4 °C)
10. Advanced-DMEM (Thermo Fisher Scientific, catalog number: 12491015) (stored at 4 °C)
11. Penicillin/Streptomycin (Nacalai Tesque, catalog number: 26253-84) (stored at 4 °C)
12. 1 mol/l-HEPES Buffer Solution (Nacalai Tesque, catalog number: 17557-94) (stored at 4 °C)
13. GlutaMAX™-I (100x) (Gibco, Thermo Fisher Scientific, catalog number: 35050-061) (stored at 4 °C)
14. 0.5 M EDTA ((Thermo Fisher Scientific, catalog number: AM9260G)
15. Trypan blue solution (Thermo Fisher Scientific, catalog number: 15250061)
16. N-acetylcysteine (Sigma, catalog number: A9165-5G) (stored at -20 °C, 1 M,10 ml aliquot)
17. Gastrin I (Sigma, catalog number: 3006) (stored at -20 °C, 100 µl, 1,000 µl aliquot)
18. N2 supplement (R&D Systems, catalog number: AR009) (stored at -20 °C,100x, 1 vial)
19. B27 supplement (R&D Systems, catalog number: AR008) (stored at -20 °C, 50x, 1 vial)
20. Recombinant mouse EGF (R&D Systems, catalog number: 2028-EG-200) (stored at -20 °C, 20 µg/ml, aliquot 1,000 µl)
21. Recombinant mouse Noggin (R&D Systems, catalog number: 1967-NG-025/CF) (stored at -20 °C, 20 µg/ml, aliquot 250 µl)
22. Recombinant human R-spondin-1 (R&D Systems, catalog number: 4645-RS) (stored at -20 °C, 100 µg/ml, aliquot 500 µl)

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23. Recombinant mouse Wnt-3a (R&D Systems, catalog number: 1324-WN-010/CF) (stored at -20 °C, 10 µg/ml, aliquot 1,000 µl)
24. Nicotinamide (R&D Systems, catalog number: 4106) (stored at -20 °C, 1 ml, aliquot 1,000 µl)
    Note: Stored in the RT shelf as powder. Nic 6.1 g + ddH_2O 50 ml, aliquot 10 ml each into 15 ml Falcon tubes. When you thaw one tube, aliquot 1.5 ml eppentubes and store.
25. A83-01 (Sigma-Aldrich, catalog number: 2939) (stored at -20 °C, 10 mM, 100 µl aliquot)
    Note: Protect from light. Aliquot 10 mM dilution into Eppendolf tubes and label them with x20, further dilute 10 mM Eppendolf tube with DMSO and aliquot 100 µl each.
26. SB202190 (Sigma-Aldrich, catalog number: 1264) (stored at 4 °C, 150 µl aliquot)
    Note: SB202190 10 mg + DMSO 3,018 µl. Aliquot 150 µl each and freeze at -20 until use.
27. Y-27632 (R&D Systems, catalog number: 1254) (protect from light, stored at -20 °C, 10 mM, 150 µl aliquot)
28. Human intestinal basal culture medium (see Recipes)
29. Human small intestinal organoid growth media (see Recipes)

B. Single-cell analysis
    1. C1 preamp IFC (10-17 µm, Fluidigm, catalog number: 100-5480)
    2. 48 x 48 IFC chip (Fluidigm, catalog number: BMK-M-48.48)
    3. TrypLE Select (Thermo Fisher Scientific, catalog number: 12563011) (stored at 4 °C)
    4. C1 Single-Cell Reagent Kit for Preamp (including Module 1, Module 2: Fluidigm, catalog number; 100-5319) (stored at -20 °C)
    5. Single Cell-to-CT kit (Thermo Fisher Scientific, catalog number: 4458236) (stored at -20 °C)
    6. LIVE/DEAD viability/cytotoxicity kit (Thermo Fisher Scientific, catalog number: L3224) (stored at -20 °C)
    7. Pooled primers (see Recipes)
    8. Lysis final mix (see Recipes)
    9. RT final mix (see Recipes)
    10. PreAmp final mix (see Recipes)
    11. LIVE/DEAD cell staining (see Recipes)

C. Biomark HD
    1. 2x Assay Loading Reagent, 1.5 ml (Fluidigm, catalog number: 100-7611)
    2. 20x TaqMan Gene Expression Assay (Thermo Fisher Scientific, catalog number: 4351372)
    3. 2x Master Mix (Thermo Fisher Scientific, catalog number: 4369514)
    4. 20x GE Sample Loading Reagent (Fluidigm, catalog number: 100-7610)

Equipment

1. Pipettes (5 ml, 10 ml, 25 ml, 50 ml), micro-pipettes (10 µl, 20 µl, 200 µl, 1,000 µl)

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2. Multi-channel pipette (Mettler Toledo, RAININ, model: E8-20 XLS+, catalog number: 17013798)
3. Centrifuge
4. Vortex mixer
5. Hemacytometer (Burker-Turk, Fujirika Co., A114)
6. 37 °C, 5% CO_2 cell culture incubator
7. Fluorescence microscope (Keyence, model: BZ-X700,)
8. C1 single cell auto prep system (Fluidigm, San Francisco, CA, USA)
9. C1 single cell auto prep array IFCs (MX) (Fluidigm, San Francisco, CA, USA)
10. Biomark HD system (Fluidigm, San Francisco, CA, USA)

Software

1. Singular Analysis Toolset Software v3.5.2 (Fluidigm, San Francisco, CA, USA)
2. R software
3. The Partek Genomic Suite (Version 6.6-6.16.0812, Partek, Chesterfield, MO, USA)

Procedure

Part I: Crypt isolation and epithelial organoid culture

A. Procedure to isolate and culture small intestinal organoids from patient-derived biopsy samples
   1. Small intestinal enteroscopic biopsy samples are obtained from patients undergoing evaluation for diseases such as small intestinal tumors, occult bleeding, or Crohn's disease. Up to 8 biopsies from each patient are taken from a region approximately 100 cm proximal to the ileocecal valve. The Ethics Committee approval and written informed consent should be obtained from each patient.
   2. Using a 50 ml Falcon tube, wash the fragments with cold PBS until the supernatant is clear.
   3. Incubate the samples in 15 mM EDTA/PBS solution (rotating) at 4 °C for 30 min (10 ml PBS + 300 µl 0.5 M EDTA).
   4. After removing the EDTA buffer, add 10 ml of PBS and voltex for 2 min to isolate intestinal crypts (Figure 1).

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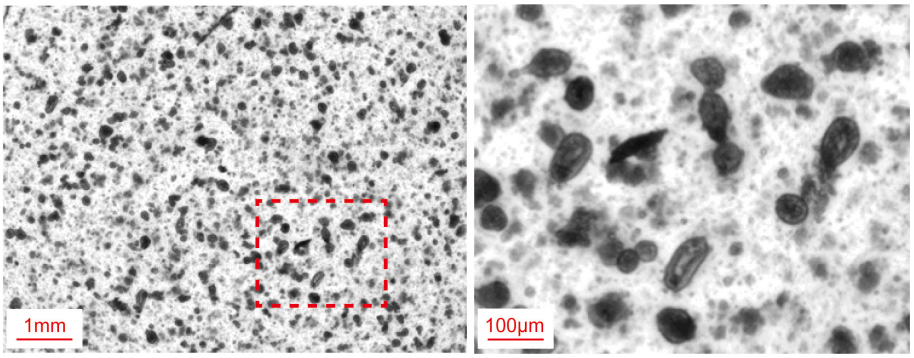

Figure 1. Freshly embedded of small intestinal crypts from patients. Scale bars = 1 mm (left) and 100 μm (right).

5. Confirm intact crypt isolation by observation under a microscope (see Notes).
6. After settling down the specimen in the tube, collect the supernatant and transfer to a 50 ml Falcon tube filtering through a 70 μm filter.
7. Repeat the Step A5 of Part I about 2-3 times till an approximately 30 ml volume of crypts containing solution is collected.
8. Centrifuge the tube at 300 *x g* for 3 min to pellet.
9. Resuspend the cell pellet with10 ml of Advanced-DMEM/F12 and transfer to one STEMFULL™ tube.
10. Centrifuge the tube at 300 *x g* for 3 min to pellet.
11. After examining the pellet under a microscope, carefully aspirate the supernatant and add the desired amount of Matrigel (30-50 μl per well) to embed the crypts (20-30 crypts per well).
12. After gently pipetting within the Matrigel, gently apply 30 μl of Matrigel on the 24-well plate. Incubate 30-60 min to allow Matrigel to polymerize.
13. Add the 500 μl of human intestinal growth medium to each well. Add 10 μM of Y-27632 to the culture medium for the initial 3 days.

B. Procedure to maintain and passage small intestinal organoids
1. After isolation, change the small intestinal growth medium every two days.
2. After 13 days of isolation, you will be able to see the growth of organoids, and they start to form crypt-like structures called "budding" (Figure 2). In order to subculture, remove the growth media and add 800 μl of cold cell recovery solution to the well. Using cell recovery solution, scrape off the Matrigel containing organoids by 1,000 μl pipette.

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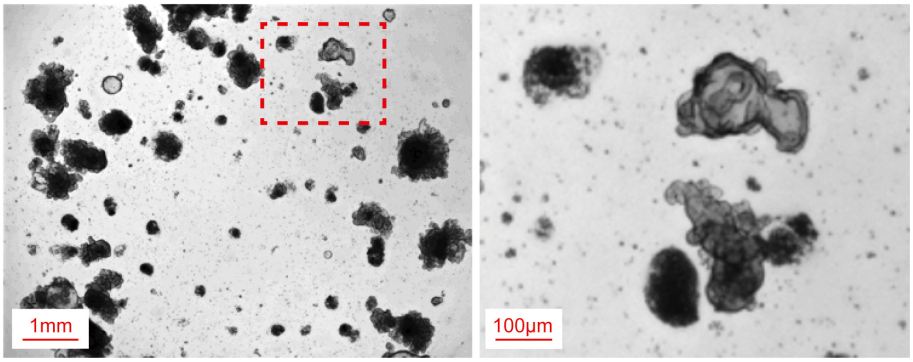

Figure 2. Establishment of small intestinal organoids from patients. Scale bars = 1 mm (left) and 100 μm (right).

3. Transfer the resuspension to a 15 ml STEMFULL™ tube. Using a P100 pipette, pipette up and down 50-100 times vigorously to mechanically disassociate the organoids into smaller fragments. After examining the fragments under the microscope, add 9 ml of cold Advanced-DMEM/F12 to the mixture.
4. Centrifuge the cells at 300 *x g* for 3 min.
5. Carefully aspirate the supernatant and resuspend the pellet in Matrigel. Usually, split at a 1:5-6 ratio. On the around day 13, established organoids start budding.

Part II: Single cell-level gene expression analysis

A. Single-cell dissociations of cultured organoid cells
   1. Remove the entire volume of the growing medium from each well (use 2-3 wells to obtain a sufficient number of cells).
   2. Using the same method as its described in passing cultured organoids, collect the organoids in a 15 ml STEMFULL™ tube, pipette up and down 50-100 times vigorously to mechanically disassociate the organoids into smaller fragments. Add 9 ml of cold Advanced-DMEM/F12 to the mixture.
   3. Centrifuge at 300 *x g* for 3 min and aspirate the supernatant.
   4. Add 5 ml of TrypLE select to the pellet and vortex for 10 min at 600 rpm (add Hoechst 10 μl).
   5. After 10 min, shake the tube vigorously for 10 times to further dissociate them into single cells.
   6. Centrifuge at 500 *x g* for 3 min and confirm the palette.
   7. Resuspend with 1,000 μl of Advanced-DMEM/F12 and transfer them to a 1.5 ml Eppendorf tube and count the live cell number by using conventional Trypan Blue staining (optimized cell concentration: 3 x 10^5 cells/ml, expected cell viability > 70%).
   8. Take out the desired amount of cells containing solution and centrifuge at 500 *x g* for 3 min and aspirate the supernatant.
   9. Resuspend with 1,000 μl of PBS and proceed to C1 protocol.

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B. Loading single cells into the chip (C1 pre-amplification process)

1. Prepare single-cell suspension for C1 loading by mixing previously prepared cell suspension (A9) and C1 Cell suspension reagent while thawing other reagents.

Components	**Volume (µl)**
Cells 166-250K/ml	30 µl
C1 Cell Suspension Reagent (Fluidigm)	20 µl
Total	50 µl

2. C1 chip priming
   a. Turn on the C1 machine, place a new C1 single-cell auto prep IFC and apply each reagent as shown in the below (Figure 3).

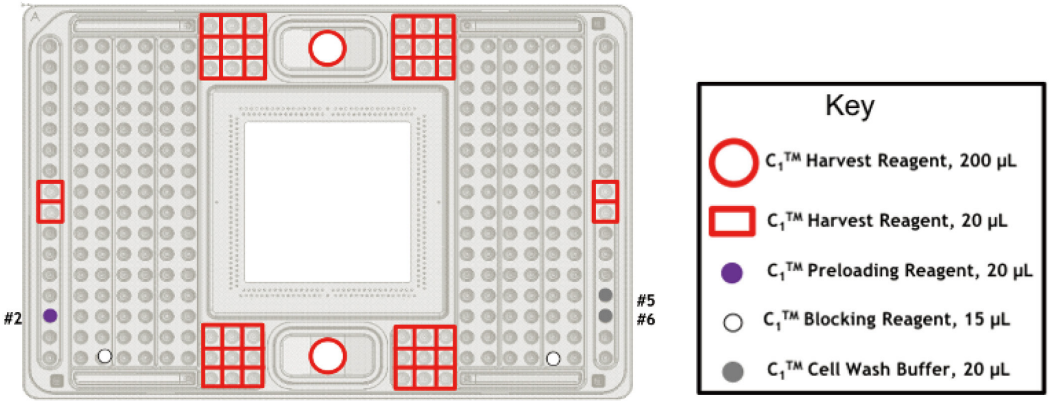

Figure 3. Application of a plate priming (Fluidigm C1 to Taqman Primers protocol, PN100-6117)

   b. After loading each reagent, place the place on the stage and select Prime program and run (it takes 10min).
   Important: After the priming, apply the sample within 1 h to prevent the priming reagents from drying out.
3. While priming the plate, prepare Lysis Final Mix, Reverse Transcription (RT) Final Mix, and PreAmp Final Mix and keep them on ice (Recipes 4-6).
4. Cell application and staining
   a. After the priming, remove the blocking solution as shown below (Figure 4).
   b. Pipet prepared cell mix for 10 times and apply 20 µl to the blue coded well.
   c. Prepare the C1 LIVE/DEAD (see Recipes) and apply 20 µl to the pink coded well.

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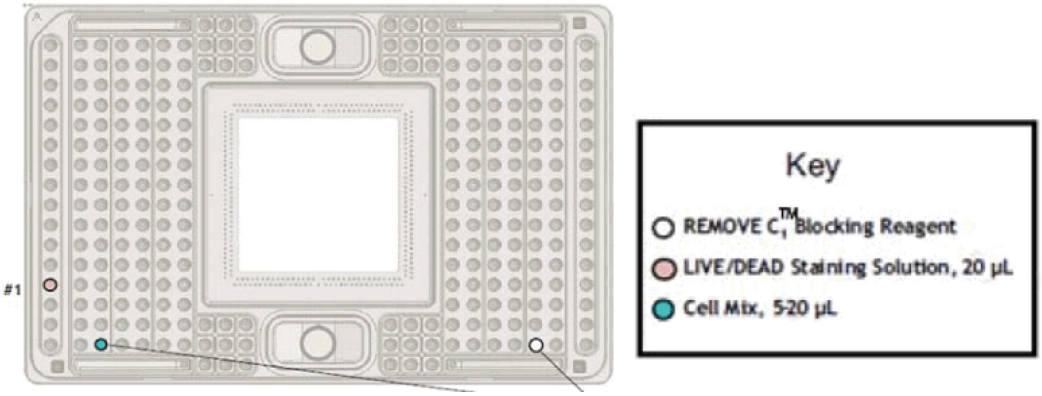

Figure 4. Cell application and staining to a C-1 plate (Fluidigm C1 to Taqman Primers protocol, PN100-6117)

d. Set the plate on the stage, select Cell Load & Stain program and run (this process takes 60-80 min)
e. After the program is over, take out the plate and confirm the single-cell capture (Figure 5) and cell viabilities of 96 wells under a microscope (BX-X700, Keyence, Osaka, Japan).

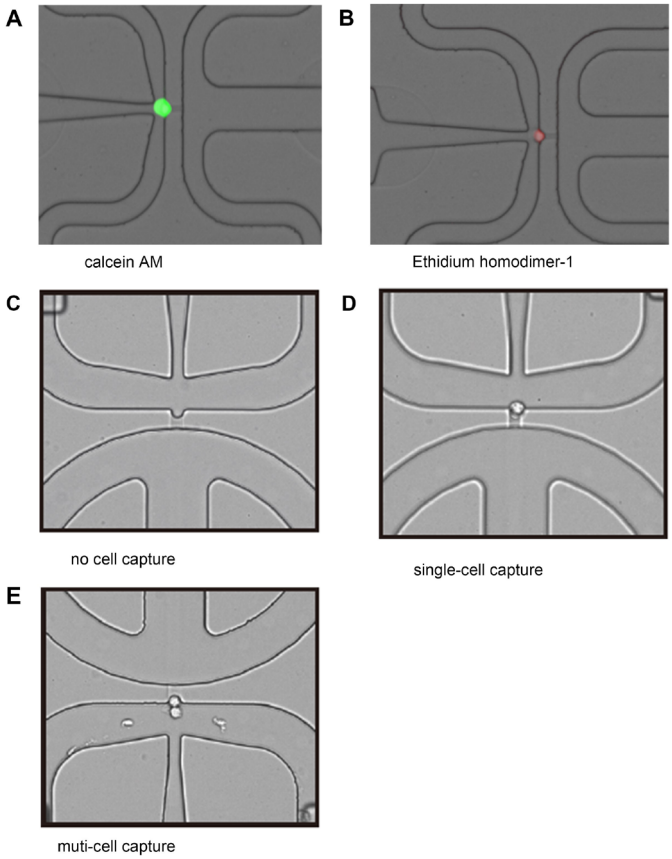

Figure 5. Examples of a viable single-cell capture positively stained with calcein AM and a dead cell stained with Ethidium homodimer-1. Scale bar = 100 µm.

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5. After confirming the cells, apply each solution as shown in the below (Figure 6) (Recipes 4-6)

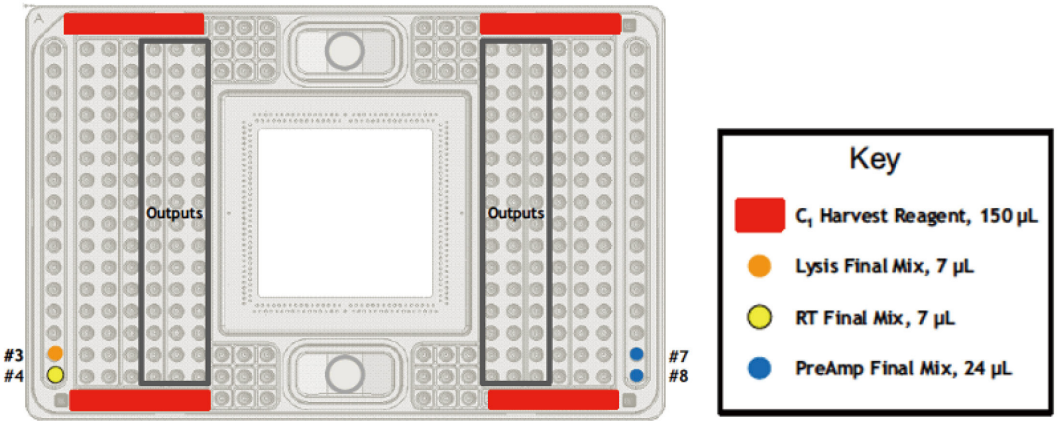

Figure 6. Application of pre-amplification solutions onto a C-1 plate (Fluidigm C1 to Taqman Primers protocol, PN100-6117)

6. Set the plate on the stage and select the PreAmp program and set the estimated end time (it is an over-night process).
7. It is important to collect the sample within an hour of completion; otherwise, the samples will be evaporated.
8. Prepare a new 96-well plate and aliquot 25 µl of C1 DNA Dilution reagent into each well of the labeled Diluted Harvest Plate
9. Harvest the Pre-Amplified products showed in the below and mix with each well with DNA dilutions using an 8-channel pipette (each well has approximately 3.6 µl) (Figure 7).
10. Store the harvested plate in -20 °C.

Components	**Volume (µl)**
C1 DNA Dilution Reagent (Fluidigm)	25
C1 harvest amplicons	3
Total	28

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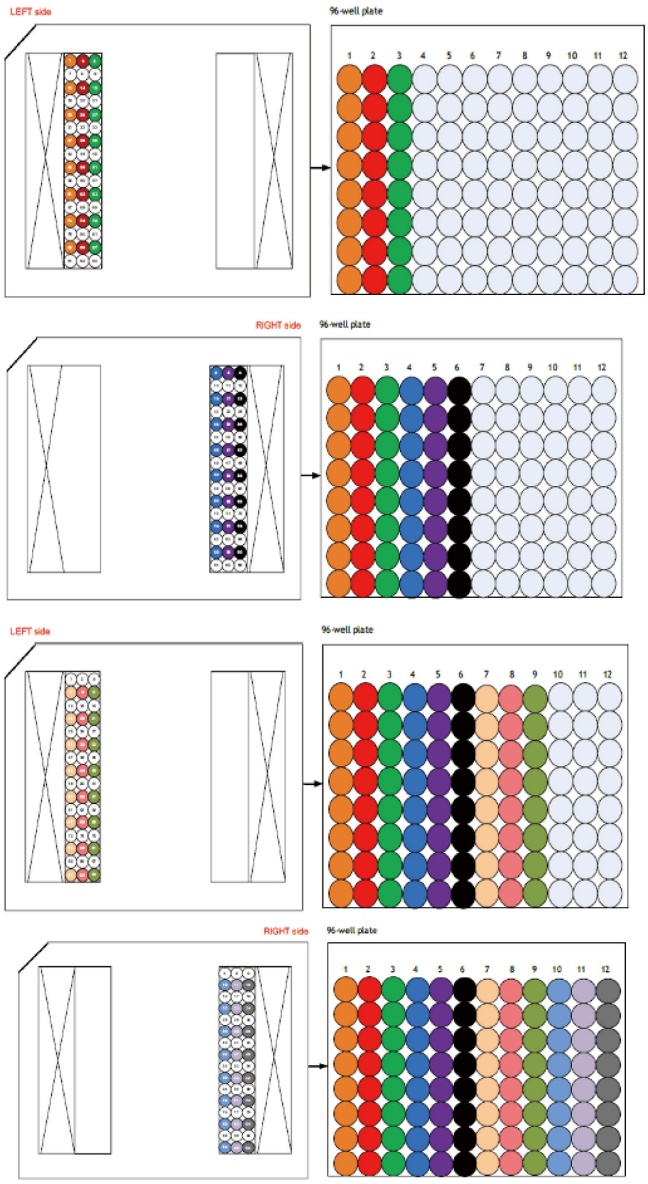

Figure 7. Pipetting steps of pre-amplified products from a C1-plate to a 96-wells plate (Fluidigm C1 to Taqman Primers protocol, PN100-6117)

C. Biomark HD workflow

Further amplification and data acquirement of the multiplex quantitative PCR analysis will be performed using the Biomark 48.48. Dynamic array IFC and Biomark HD system (Fluidigm, San Francisco, CA, USA). Dynamic array IFC system operates nanofluidic IFC circuits that automatically combines harvested samples with sets of gene assays.

1. Preparing
   a. Prepare both “10x assay mix” and “Sample mix” as they are shown below.

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TaqMan primers 10x assay mix

Components	Volume per Inlet (µl)	Volume per Islet with Overage (µl)	Volume for 48.48 (µl)	Volume for 96.96 (µl)
2x Assay Loading Reagent	2.5	6.0	X 60 = 360	X 120 = 600
20x TaqMan Gene Expression Assay	2.5	6.0		
Total volume	5.0	6.0	300.0	600.0
Final concentration at 10x	Primers: 9 µM Probe: 2.5 µM			

Store at -20°C

Sample Mix

Components	Volume per Inlet (µl)	Volume per Islet with Overage (µl)	Volume for 48.48 (µl)	Volume for 96.96 (µl)
2x Mater Mix	2.5	3.0	X 60 = 180.0	X 120 = 36.0
20x GE Sample Loading Reagent	0.25	0.3	X 60 = 18.0	X 120 = 36.0
cDNA	2.25	2.7		
Total	5.0	6.0	198	396.0

2. Priming IFC and sample loading

   Make sure to use IFC Controller MX for 48.48 Dynamic Array IFC, and IFC Controller HX for 96.96 Dynamic Array IFC.

   a. Inject control line fluid into each accumulator on the IFC.
   b. Remove and discard the protective film from the bottom of the IFC.
   c. Place the IFC into the appropriate controller, then run the appropriate “Prime” script to prime the control line fluid into the IFC. Each IFC type corresponds to a distinct script number, and the plate ID number will be identified by the controller (Priming takes up to 30 min).

      Important: *Please make sure to run “Load” within 1 h after completing the priming as the plate will dry out.*
   d. Remove the IFC from the controller, and pipette samples and assay solutions into the inlets on the IFC (5 µl each).
   e. Return the IFC to the controller.
   f. Run the appropriate “Load Mix” script. Air pressure forces samples and gene assay solutions into the IFC where they mix.
   g. Remove the loaded IFC from the controller.
   h. Place the IFC into the Biomark HD instrument, making sure the A1 corner on the IFC aligns with the A1 on the instrument tray.

      Important: *Make sure to run Biomark HD within 1 h after completing the “Load Mix” script as the plate will dry out.*

3. Running Biomark HD

   a. Turn on the Biomark HD and the computer.
   b. Click the icon named “Biomark Data Collection”.

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c. Wait until the application shows “Ready” when the temperature of the CCD camera reaches to -5 °C.
d. Click “Start a New Run” of the default desktop. Confirm that the “Data Collection” screen appears in response.
e. Place the pre-loaded IFC on the tray and click “Load”. The tray retracts and the system scans the barcode and identifies the IFC.
f. Click “Next”. Browse and select a file that you desire to save.
g. Select the Gene Expression application. Select the Passive Reference (ROX). Select Probe and select “Next”.
h. Select Browse and Protocol file. Start the Run.

Data analysis

Data acquired from multiplex single-cell gene expression analysis were processed using the Singular Analysis Toolset Software v3.5.2 (Fluidigm, San Francisco, CA, USA) and the Partek Genomic Suite (Version 6.6-6.16.0812, Partek, Chesterfield, MO, USA) by following standard workflows.

Notes

Crypts that can be harvested from endoscopic biopsy samples are very limited in number. After step A4, take out 1ml of the crypt suspension and spread it onto a 24-well plate to observe under a microscope and confirm existence of viable crypts.

Recipes

1. Human intestinal basal medium

Components	Stock Con.	Final con.	/500 ml medium
Advanced-DMEM/F12		1x	500 ml
GlutaMAX-1	200 mM	2 mM	5 ml
HEPES	1 M	10 mM	5 ml
Penicillin/Streptomycin	10,000 U/ml	100 units/ml	5 ml
N-acetylcysteine	500 mM	1 mM	1 ml
Gastrin	100 μM	10 mM	50 μl
N2 supplement	100x	1x	5 ml
B27 supplement	50x	1x	10 ml

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2. Human small intestinal organoid growth media

Components	Stock Cons	Final Cons	Unit ml	1 well 0.5	2 wells 1
Basal Medium	-	-	ml	0.25	0.5
m EGF	20 µg/ml	50 ng/ml	µl	1.25	2.5
m Noggin	20 µg/ml	100 ng/ml	µl	2.5	5
m R-spondin-1	100 µg/ml	1 µg/ml	µl	5	10
m Wnt-3A	10 µg/ml	300 ng/ml	µl	15	30
Nicotinamide	1 M	10 mM	µl	5	10
A83-01	500 µM	500 nM	µl	0.5	1
SB202190	10 mM	10 µM	µl	0.5	1

3. Pooled primers

Components	Volume (µl)
1 µl each primer pair (100 µM each)	1.0 (x 93 = 93 µl)
Optional RNA Spike primers	1.0 (x 3 = 3 µl)
C1 DNA Dilution Reagent	104.0
Total	200.0
Can save up to 6 months (-20 °C)	

4. Lysis final mix

Components	Volume (µl)
C1 DNA Dilution Reagent (Fluidigm)	0.90
Single-Cell Lysis Solution (Thermo Fisher Scientific)	12.75
C1 Lysis Plus Reagent (Fluidigm)	4.35
Total	18.0

5. RT final mix

Components	Volume (µl)
Single-Cell VOLO RT Mix (Thermo Fisher Scientific)	5.84
Single-Cell SuperScript RT (Thermo Fisher Scientific)	3.62
Stop solution (Thermo Fisher Scientific)	1.94
C1 Loading Reagent (Fluidigm)	0.60
Total	12.00

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6. PreAmp final mix

Components	Volume (µl)
Single-Cell PreAmp Mix (Thermo Fisher Scientific)	12.0
C1 Loading Reagent (Fluidigm)	3.0
DNA-Free Water (DPEC)	30.0
Pooled Primers (500 nM)	15.0
Total	60.0

7. LIVE/DEAD cell staining

Components	Volume (µl)
C1 Cell Wash Buffer (Fluidigm)	1250.0
Ethidium homodimer-1 (LIVE/DEAD kit, Thermo Fisher Scientific)	2.5
Calcein AM (LIVE/DEAD kit, Thermo Fisher Scientific)	0.625
Total	1253.125

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Competing interests

The authors declare that they have no conflict of interest.

Ethics

The Ethics Committees of Tokyo Medical & Dental University and Yokohama Municipal Hospital approved our study (M2000-2093 and M2000-1176); and written informed consent forms were obtained from each patient.

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