Mouse intestinal organoid time-course experiments from single cells

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Abstract

Organoids recapitulate the self-organizing capacity of stem cells and the tissue organization of the original organ in a controlled and trackable environment. Intestinal organoids, in particular, can develop from a single cell into a fully-grown structure that contains most of the cell types, patterns, and morphogenetic properties of the adult intestine. Here we present a protocol for high-throughput organoid culture in multi-well plate format combined with high content immunofluorescence imaging and RNA extraction. Our protocol allows recording and analysis of thousands of organoids during several days of development.

Introduction

Mouse intestinal organoids grow from single cells over days to form 3D structures composed of crypts and villi containing all cell types present in the mouse intestine[1]. They represent an example of self-organization where the different cell types occupy precise locations and establish spatial functional relationships with each other. To achieve such a level of organization, single cells undergo a series of morphological and functional changes relaying on gene expression networks, cell migration and cell-cell interactions.

Comprehensive investigation of such processes requires a systematic approach combining different experimental techniques. Moreover, the ability to investigate hundreds or thousands of organoids in parallel is fundamental for systems that present an intrinsic degree of variability. Here, we report a workflow to study intestinal organoid development by immunofluorescence, high-throughput microscopy and RNA extraction. Our protocol describes a droplet organoid culture method on 96 well-plate format compatibles with multiple rounds of multiplexing immunofluorescence staining[2]and optical clearing[3]. The same platform can be used for RNA extraction. This approach is particularly powerful to characterize the development of organoids that grow over several days and allows to link morphological changes and cell interactions to the underlying gene regulatory networks.

Many in-vitro model systems are now emerging which can be used to study self-organization starting from few cells[4,5,6]. The protocol presented here is not limited to intestinal organoids and can be adapted to other in-vitro organoid systems. We believe that a systematic and quantitative analysis of such multi-cellular systems will be essential for interpretation of experiments across different laboratories, especially when used in perturbation experiments and for personalized medicine.

Reagents

Culture maintenance medium:

· IntestiCult Organoid Growth Medium (STEMCELL Technologies, 06005)

· 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, 15140122)

ENR medium:

· DMEM/F-12 with 15 mM HEPES (STEM CELL Technologies, 11330032)

· 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, 15140122)

· 1 × GlutaMAX (Thermo Fisher Scientific, 35050061)

· 1 × B27 Supplement (Thermo Fisher Scientific, 17504044)

· 1 x N-2 Supplement (Thermo Fisher Scientific, 17502048)

· 1 mM N-Acetyl-L-cysteine (Sigma, A7250)

· 100 ng/ml murine EGF (R&D Systems, 2028-EG-200)

· 500 ng/ml R-Spondin (Recombinant murine R-spondin, for example can be purified from Cultrex HA-R-Spondin1-Fc 293T Cells, R&D systems, 3710-001-01)

· 100 ng/ml Noggin (PeproTech, 250-38-500ug)

Sample preparation and culturing

· PBS without Ca^{++} and Mg^{++} (for example Thermo Fisher Scientific, 10010023)

· Wnt3a-conditioned medium (Wnt3a-CM) can be produced from Wnt3a L-cells (for example ATCC, CRL 2647)

· ROCK inhibitor Y-27632 (STEMCELL Technologies, 72304)

· CHIR99021 (GSK3B inhibitor, STEMCELL Technologies, 72054)

· TrypLE (Thermo Fisher Scientific, 12604021)

· Matrigel (Corning, 356231)

Fixation and Immunostaining

· Paraformaldehyde, 16% Solution, EM Grade (Electron Microscopy Sciences, 15710)

· Triton X-100 (Sigma Aldrich, T9284)

· Donkey Serum (Sigma Aldrich, D9663-10ML)

- DAPI (Thermo Fisher Scientific, D1306)
- CellTrace Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific, A-20006)
- $NaHCO_3$(Sigma Aldrich,S5761-500G)
- Na_2CO3 (Sigma Aldrich, S7795-500G)

Blocking buffer: 0.1% TritonX-100, 3% Donkey Serum, diluted in PBS

Permeabilization buffer: 0.5% TritonX-100 diluted in PBS

CellTrace buffer (for 10 ml): 8 ml ddH_2O, 1.95 ml 0.5 M $NaHCO_3$, 50 µl of 0.5 M, Na_2CO_3

Multiplexing:

- Methanol (Sigma Aldrich 322415)
- N-Acetyl-L-cysteine(Sigma Aldrich A7250)
- Ammonium chloride (NH_4Cl, Sigma Aldrich, A9434)
- Glycine (Sigma Aldrich G8898)
- Urea (Sigma Aldrich U5128)
- Guanidinium chloride (Sigma Aldrich G4505)
- TCEP-HCl (Sigma Aldrich C4706)

Multiplexing blocking buffer: 3% Donkey Serum with 200 mM NH_4Cldiluted in PBS.

Imaging Buffer: 700 mM*N*-Acetyl-Cysteine diluted in ddH_2O, pH adjusted to 7.4.

Elution Buffer: 0.5 M Glycine, 5 M Urea, 5 M Guanidinium chloride, 70 mM TCEP-HCl, all in ddH_20 with pH adjusted to pH2.5.

Optical clearing:

- 60% Iodixanol solution (Sigma Aldrich D1556)
- N-methyl-D-glucamine (Sigma Aldrich M2004)

- Diatrizoic acid (Sigma Aldrich D9268)

Optical clearing solution: For 10 ml, 9.2 ml 60% iodixanol solution, 4 g N-methyl-D-glucamine, 5 g diatrizoic acid, dissolved in 6.3 ml of ddH_20

Compound treatment:

- Verteporfin (Sigma Aldrich, SML0534)
- DMSO (Sigma Aldrich, D8418)
- Doxycycline hyclate (Sigma Aldrich, D9891)
- 4-Hydroxytamoxifen (Sigma Aldrich, H6278)
- DAPT (Stemgent, 04-0041)
- Ly411575 (for example STEMCELL Technologies, 72792)
- MK-0752 (for example MedChemExpress, HY-10974)
- CHIR99021 (GSK3B inhibitor, STEMCELL Technologies, 72054)
- IWP-2 (Porcupine Inhibitor, STEMCELL Technologies, 72124)
- EREG (R&D System, 1068-EP-050)

RNA extraction bulk for bulk RNA sequencing:

- b-Mercaptoethanol (Sigma Aldrich, M6250-10ML)
- Ethanol (Merck, 1070172511)
- Single Cell RNA Purification Kit (Norgen Biotek Corporation, 51800)
- RNase-Free DNase I Kit (Norgen Biotek Corporation, 25710)

Plastics:

- Cell culture microplate, 96 well, black (Greiner, 655090)

- Tissue Culture Plates 24 well (TPP, Z707791-126EA)
- Cell strainer, CellTrics 30 µm (Sysmex, 04-004-2326)
- Falcon 15 ml Polystyrene Centrifuge Tube (Corning 352095)
- Eppendorf Safe-Lock microcentrifuge tubes 1.5 ml (Eppendorf, 0030 120.086)
- Tubes for FACS sorting (for example Corning 352063 5 ml round bottom Falcon tubes)

Equipment

• Refrigerated centrifuges for 15 ml/50 ml falcon tubes and 1.5 ml Eppendorf tubes

• Refrigerated centrifuge for 96 well-plates (for example Eppendorf 5430R)

• Cell culture incubator (37°C, 5% CO2)

• Vortex mixer (for example, Scientific Industries, Vortex-Genie 2)

• Plate shaker (for example, Heidolph TITRAMAX 100)

• BSL-1 biosafety cabinet

• Automated spinning disk confocal microscope able to image 96 well-plates (for example Yokogawa, CV7000S)

• **Optional:**FACS sorter (for example BD FACSAria cell sorter, Becton Dickinson)

Procedure

Organoid cultures prior to time course experiments

Organoids are cultured in 50 µl/well of 1:1 Matrigel/culture maintenance medium mix within wells of 24 well-plates and in the presence of 500 µl of culture maintenance medium. Organoids are split mechanically every 5-7 days.

Organoid time course experiment from single cells

Sample preparation and single cell seeding

1. Remove the medium from the wells and collect Matrigel droplets containing organoids with ice cold PBS into a falcon tube (use 0.5 ml of PBS per well). Centrifuge the tube for 5 min at 400 rcf and 4°C to pellet the organoids

Note: Number of wells to collect depends on the type of experiment conducted (number of replicates and number of plates to prepare). For a normal time course experiment, we usually collect between 12 and 24 wells

2. Discard supernatant and re-suspend pellet in Tryple (add 100 µl of Tryple with 10 µM of Rock inhibitor for each collected well)

3. Incubate 20 min at 37°C with harsh pipetting every five minutes

4. AddDMEM/F-12 (~3 times the amount of used Tryple) and centrifugefor 5 min at 400 rcf and 4°C

5. Discard supernatant and re-suspend in 0.5 ml of cold PBS (PBS plus 10 µM Rock inhibitor)

6. Filter cell suspension through a 30 µm cell strainer directly into a FACS sorting tube

7. Keep samples on ice until sorting (ideally this should be done within 30 min)

8. Prepare a 1.5 ml Eppendorf tube with 200 µl of ENR medium (add 10 µM Rock inhibitor to ENR medium) to collect the sample (collection tube, store on ice)

9. FACS sort single alive cells into prepared collection tube

10. After sorting, centrifuge collection tube for 5 min at 800 rcf and 4°C

11. Discard supernatant carefully, re-suspend in 1:1 ratio of ENR medium and Matrigel

Note: Dilute pellet with a 1:1 ratio of ENR medium and Matrigel to obtain a final concentration of ~3000 cells/5 µl droplet. Depending on batch to batch variability of the medium compounds, organoid forming efficiency can show some variation. For this reason, adjustment of seeding densities might help (usually in a range between 2500 and 5000 cells/5 µl droplet). A final density of 100-200 organoid/well represents a good number for further analysis

12. Seed 5 µl droplet of the prepared cell suspension for each well of a 96 well-plate

Note: Number of plates and number of wells per plate to prepare depends on the experiment. Usually we prepare 6 plates with multiple wells containing organoids which we fix after 3h, 24h, 48h, 72h, 96h and 120h after plating

13. Incubate the plate for 15-20 min at 37°Cin an incubator to let the droplets solidify.

14. Add 100 µl/well of ENR medium supplemented with 20% Wnt3a-CM, 10µM of Rock inhibitor and 3µM of CHIR99021

Remark: If FACS sorting is not possible, after step 6, count the cells and continue with step 10 to seed them. However, FACS sorting ensures that only single alive cells are in the starting population

15a. For experiments without perturbations:

1. After 24h replace medium with 100 µl of ENR medium with addition of 20% Wnt3a-CM and 10µM of Rock inhibitor

2. After 72h replace medium with 100 µl of ENR medium only

15b. For experiments with perturbation by small compounds:

Note: Depending of the experiment compounds can either by added directly after seeding or at later time points

Verteporfin treatment:

1. Remove ENR medium

2. Add 100 µl/well of 5 µM Verteporfin or DMSO diluted in ENR medium at time point of interest

3. Change medium daily adding fresh compound and supplement as under **15a** until fixation

Inducible hYap1 overexpression:

1. Remove ENR medium

2. Add 100 µl/well of 0.05 µg/ml Doxycycline hyclate or ddH_2O diluted in ENR medium at time point of interest

3. Change medium daily adding fresh compound or ddH_20 and supplement as under **15a** until fixation

Inducible Lats1/2 double knock-out:

1. 24 h before single cell isolation culture organoids growing in a 24-well plate in 500 µl/well of 1 µg/ml 4-Hydroxytamoxifen or DMSO diluted in ENR medium

2. Grow single cells in normal ENR medium as described in **15a** until fixation

DAPT treatment:

1. Remove ENR medium

2. Add 100 µl/well of 10 µM DAPT or DMSO in ENR medium at the time point of interest

3. Change medium daily adding fresh compound and supplement as under **15a** until fixation

Ly411575 or MK-0752 treatment:

1. Remove ENR medium

2. Add 100 µl/well of 0.5 µM Ly411575 or MK-0752 or DMSO in ENR medium

3. Change medium daily adding fresh compound and supplement as under **15a** until fixation

CHIR99021 or IWP-2 treatment:

1. Remove ENR medium

2. Add 100 µl/well of 5 µM CHIR99021 or 2 µM IWP-2 or respectively 5 and 2 µM DMSO diluted in ENR at time point of interest

3. Change medium daily adding fresh compound and supplement as under **15a** until fixation

EREG treatment:

1. Remove ENR medium

2. Add 100 µl/well of 0.5 µg/ml EREG or PBS diluted in ENR at time point of interest

3. Change medium daily adding fresh compound and supplement as under **15a** until fixation

Fixation

1. Spin the 96-well plate at 3000 rpm for 10 min in a pre-cooled centrifuge at 10°C prior to fixation

2. Wash each well once with 100 µl of PBS before adding 100 µl of 4% PFA (diluted in in PBS) for 45 min at room temperature

3. Wash 3 x with PBS (100 µl/well)

Note: After fixation, plates can be stored for weeks in the fridge (add 200 µl of PBS into each well). Plates should be sealed with Parafilm to prevent evaporation of PBS

(General) Immunostaining*:* After fixation

1. Add 100 µl of permeabilization buffer, incubate for 1h at room temperature (shake plate at 300 rpm)

2. Wash 3 x with PBS (100 µl/well)

3. Add 100 µl of blocking buffer for 1h at room temperature (shake plate at 300 rpm)

4. Dilute the primary antibody to an appropriate concentration in blocking buffer and add 60 µl/well for 2 h at room temperature or overnight at 4°C depending on the used antibody

5. Wash 3 x with PBS (100 µl/well)

6. Dilute the secondary antibody 1:500 in blocking buffer and add 100 µl/well for 1 h at room temperature (shake plate at 300 rpm)

7. Wash 3 x with PBS (100 µl/well)

8. Stain cell nuclei with 100 µl/well DAPI (20 µg / ml diluted in ddH_20) for 15 min at room temperature

9. Wash 3 x with PBS (100 µl/well)

10. To stain cell outlines, add CellTrace dye to the CellTrace buffer (final concentration 1 µg/ml) and immediately add 100 µl/well for 10 min at room temperature

11. Wash 3 x with PBS (100 µl/well)

Note: Plates are now ready for imaging or can be stored in the fridge at 4°C for several weeks. Plates should be sealed with Parafilm to prevent evaporation of PBS and covered with aluminum foil to prevent bleaching of the fluorophores

12. Optional: If confocal z-stacks of organoids are acquired, optical penetration depth ofthe sample can be improved by optical clearing of the sample[3]. Each well is incubated in 100 µl of optical clearing solution at room temperature for 20 min (sample should become transparent). After incubation, the optical clearing solution is replaced with 100 µl of fresh optical clearing solution and the sample is imaged in optical clearing solution

Note: Optical clearing solution can lead to bleaching of the fluorescence signal over time. For this reason, the solution should be removed after imaging by washing with PBS

(Multiplexed) Immunostaining*:* After fixation, the 4i (iterative indirect immunofluorescence imaging)[2] protocol can be used instead of the (General) Immunostaining protocol

1. Permeabilize with 100/well µl of -20 °C Methanol for 30 min at -20°C

2. Wash 3 x shortly with PBS (100 µl/well) followed by 3 x 10 min washes with PBS (200 µl/well, shake plate at 300 rpm)

3. Add 100 µl/well of multiplexing blocking buffer for 1 h at room temperature (shake plate at 300 rpm)

4. Dilute the primary antibody to an appropriate concentration in multiplexing blocking buffer and add 60 µl/well for 2 h at room temperature or overnight at 4°C depending on the used antibody

5. Wash 3 x with PBS (100 µl/well)

6. Dilute the secondary antibody with a concentration of 1:500 in multiplexing blocking buffer and add 100 µl/well for 1 h at room temperature (shake plate at 300 rpm)

7. Wash 3 x with PBS (100 µl/well)

8. Stain cell nuclei with 100 µl/well of DAPI (20 µg / ml diluted in ddH_20) for 15 min at room temperature

9. Wash 3 x with PBS (100 µl/well)

10. To stain cell outlines, add CellTrace dye to the CellTrace buffer (final concentration 1µg/ml) and immediately add 100 µl into each well for 10 min at room temperature

11. Wash 3 x with PBS (100 µl/well)

Note: Plates are now ready for imaging of the first round or can be stored in the fridge at 4°C for several weeks

12. Imaging: Add 200 µl/well of imaging buffer and image

13. Antibody Elution: Wash3 x 10 min with elution buffer (100 µl/well) at room temperature (shake plate at 300 rpm)

14. Wash 3 x shortly with PBS (100 µl/well) followed by 3 x 10 min washes with PBS (200 µl/well, shake plate at 300 rpm)

15. Re-block the plate by adding 100 µl/well of multiplexing blocking buffer for 1 h at room temperature (shake plate at 300 rpm)

16. Staining for next round of imaging: Dilute next set of antibodies to appropriate concentration in multiplexing blocking buffer and apply for 2 h at room temperature or overnight at 4°C depending on the used antibody (add 60 µl/well)

17. Wash 3 x with PBS (100 µl/well)

18. Dilute the secondary antibody with a concentration of 1:500 in multiplexing blocking buffer and add 100 µl/well for 1h at room temperature (shake plate at 300 rpm)

19. Wash 3 x with PBS (100 µl/well)

20. Re-stain nuclei by adding 100 µl/well of DAPI (20 µg/ml diluted in ddH_20) for 15 min at room temperature

21. Wash 3 x with PBS (100 µl/well)

22. Imaging: Add 200 µl/well of imaging buffer

Note: Steps 13-22 can be repeated iteratively for multiple rounds with new antibodies

Imaging, segmentation and feature extraction

Code to automatically segment and extract features for organoids stained and imaged as described under **Immunostaining**can be found under https://github.com/fmi-basel/glib-nature2018-materials. We recommend to use a spinning disk confocal microscope for acquisition of maximum intensity projections together with z-stack images for each organoid.

RNA extraction for bulk RNA sequencing

For sample preparation follow: *Sample preparation and single cell seeding.*

Note: for each time point plate 5000 cell/well in 30 wells of a 96-well plate.

1. Remove the culture medium and wash the wells with 100 µl of PBS

2. Scrape the cells embedded in Matrigel with 100 µl/well of Buffer RL + b-mercaptoethanol (10 µl b-mercaptoethanol for each 1 ml of Buffer RL required) and collect them in a 15 ml falcon tube

3. Add 30 µl/well of ethanol 97% to the lysate and mix by vortexing

4. Follow the protocol of the Single Cell RNA Purification Kit from Section 2. Total RNA Purification from All Types of Lysate

a. **Note:** DNase treatment is an Optional step described in Appendix A

b. **Note:** Elute RNA in 20 µl of Elution Solution A

Troubleshooting

Organoids do not show anticipated growth pattern or the efficiency of organoid formation is low.

Medium and Matrigel components can show some batch to batch variability. Using a different batch might improve culture conditions. Organoid cultures decrease in efficiency after several passages. It is recommended to use low passage organoid lines.

Antibody staining is not working.

A good antibody staining needs some optimization. Parameters which can be optimized are: antibody dilution, incubation time, permeabilization and blocking conditions.

RNA yield is low.

Depending on the time point of RNA extraction yields can vary. For early time points of organoid growth, it may be necessary to use more wells.

Time Taken

To allow maturation of mouse intestinal organoids time course experiments last 5-6 days. Sample preparation and single cells seedingtypically take 1-2 hours. Medium change takes around 30 minutes. A single round of immunostaining takes one or two days depending on the antibody incubation times. RNA extraction takes 1-2 hours.

Anticipated Results

In a time-course experiment without perturbation the following stereotypic organoid growth stages are observed: day 1 - single-cell state; day 2 – small spheres in which a polarized epithelial cell layer surrounds a lumen; day 3 – larger spheres; day 4 - asymmetric polarized spheres in which the initiation of a bud is visible; and day 5 - organoids with fully formed crypts.

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