Published in final edited form as:

Methods Mol Biol. 2019 ; 1576: 171–181. doi:10.1007/7651_2016_8.

Murine Colonic Organoid Culture System and Downstream Assay Applications

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Abstract

Colonic organoids, three-dimensional colonic crypts grown in vitro that show realistic microanatomy, have many potential applications for studying physiology, developmental biology, and pathophysiology of intestinal diseases including inflammatory bowel disease and colorectal cancer. Here, we describe detailed protocols for mouse colonic crypt isolation, organoid culture, and downstream applications. Specific culture strategies including growth factor enriched Matrigel and Wnt and R-spondin conditioned media serve as key factors for enhancing the growth and cost efficiency of colonic organoid cultures.

Keywords

Niche-independent crypt expansion; Stem cells; In vitro primary culture

1 Introduction

The ability to grow intestinal tissue-derived cultures in vitro, broadly referred to as organoid culture, has allowed the gastrointestinal research field to move away from transformed cell lines to a more physiologically relevant “mini-gut” model. For example, the unique three-dimensional culture system allows the crypt, the basic functional unit of the intestine, to expand to generate organoids, comprising multiple crypt-like domains surrounding a central lumen lined by an epithelium, similar to the basic crypt physiology in vivo. Significant research has highlighted the use of organoid systems to characterize the adult stem cell niche and gut morphogenesis. This organoid primary culture system is being applied to study intestinal physiology and pathophysiology with great expectations for translational applications, including regenerative medicine [1, 2]. Interestingly, despite the fact that small intestinal cancer is fairly rare compared to colorectal cancer, most studies utilize organoids derived from small intestine. Few studies have been conducted in colonic crypt-derived organoids, or colonoids. This may be due to the fact that colonic organoids are more difficult to culture and maintain.

Building on the protocol developed in the Clevers lab [3, 4], our laboratory has refined the culture system with unique features suited to colon tissue, resulting in higher growth efficiency and lower culture maintenance costs. Furthermore, several downstream applications are included in this chapter to provide insight into potential usage of this unique in vitro culture system.

2 Materials

Most ingredients for organoid culture should be prepared as stock solutions, aliquoted (see **Note 1**) and kept at -20°C or -80°C for prolonged storage.

2.1 Commercially Available Materials

Advanced DMEM/F-12 medium (ADF) (Gibco #12634-010); DMEM, high glucose with GlutaMAX (Gibco #10569-010); nonessential amino acids (Gibco #11140-050); Zeocin (Gibco #R25001); GlutaMAX (Gibco 35050-061); Ca/Mg free Dulbecco's PBS (Gibco #14190-144); growth factor reduced basement membrane matrix (Matrigel) (Corning #356231); recombinant mouse epidermal growth factor (EGF) (Gibco #PMG8041, working stock [100 $\mu\text{g}/\text{mL}$ in PBS + 0.5 % FBS]); LDN-193189 (Cellagen Technology #C5361-2s, working stock [0.2 mM in PBS + 0.5 % FBS]), (see **Note 2**); N2 supplement, 100 $\times$, (Gibco #17502-048); B27 supplement minus vitamin A, 50 $\times$, (Gibco #12587-010); N-acetyl-L-cysteine (Sigma #A9165, working stock [400 μM in water]); Y-27632 (Sigma #Y0503, working stock [10 mM in ADF]) (see **Note 3**). ADF+: ADF medium, supplemented with 2 mM GlutaMAX, 100 units/mL penicillin–streptomycin, and 10 μM HEPES.

2.2 Wnt Conditioned Medium Generation

1. L-Wnt3A cell medium: DMEM high glucose with GlutaMAX supplemented with 10 % FBS, 100 units/mL penicillin–streptomycin, and 1 $\times$ nonessential amino acids.
2. Grow L-Wnt3A cells (ATCC #CRL-2647) (see **Note 4**) in one T-75 flask. Add Zeocin to the culture medium at a final concentration of 125 $\mu\text{g}/\text{mL}$.
3. When confluent, split cells into two T-175 flasks. Add fresh Zeocin to the culture medium at a final concentration of 125 $\mu\text{g}/\text{mL}$.
4. When confluent, split each flask into four T-175 flasks with 25 mL medium per flask, *without* addition of Zeocin.
5. After 96 h of culture, collect media and centrifuge at $200 \times g$ for 5 min to remove cells and debris. Collect the supernatant and filter through 0.22 μm filter. Note that the cells will become over-confluent during the growth period.
6. This is the first batch of Wnt conditioned medium. Aliquot and store at -80°C .
7. (Optional): Fresh medium can be added to the same flasks, and the culture continued (without Zeocin) for another 5 (batch 2) or 10 days (batch 3). Collect the conditioned medium as described in **step 5** (see **Note 5**).

¹Aliquot growth factors to avoid repeated freeze–thaw cycles. We typically do not freeze–thaw the growth factors more than 3 times.

²LDN-193189 is a synthetic, highly potent bone morphogenic pathway inhibitor. It can be used as a more economical replacement for Noggin [6] while providing similar growth support for the organoid culture.

³Y-27632 is a highly potent, cell-permeable, reversible, and selective ROCK inhibitor [7]. It is required for growing organoids from single colonic cells, but optional for organoid culture from crypts.

⁴The L-Wnt3A cell line is commercially available from ATCC (#CRL-2647). A new cell line is now available which produces Wnt-3A, R-spondin 3, and noggin (ATCC #CRL-3276). This cell line was not tested for this manuscript.

⁵Multiple batches of Wnt-conditioned media can be collected from the cultures. In our experience, the 1st batch of conditioned medium (5 days culture) consistently provides superior growth support compared to media collected from 10 or 15 days cultures.

2.3 R-Spondin Conditioned Medium Generation (See Note 6)

1. Initial growth medium: DMEM high glucose with GlutaMAX supplemented with 10 % FBS and 100 units/mL penicillin–streptomycin.
2. Grow RSPO1 cells (HA-R-Spondin1-Fc, Trevigen #3710-001-01) in one T-175 flask in initial growth medium. Add fresh Zeocin to the culture medium at a final concentration of 125 µg/mL.
3. When confluent, split cells into six T-175 flasks, culture in initial growth medium *without* addition of Zeocin.
4. When confluent, aspirate initial growth medium, change to ADF+ medium (50 mL/flask). Continue incubation for 7 days (*see Note 7*).
5. Collect media and centrifuge at $200 \times g$ for 5 min to remove cells and debris. Collect the supernatant and filter through 0.22 µm filter. Aliquot and store at -80°C .

3 Methods

3.1 Isolation of Colonic Crypts for Organoid Culture

1. Thaw Matrigel *on ice*, keep at 4°C . Prechill 200 and 1000 µL pipet tips in a 4°C refrigerator the day before crypt isolation (*see Note 8*).
2. Prepare complete organoid medium by adding the following growth factors to ADF⁺ medium at the final concentrations given: EGF [50 ng/mL], LDN-193189 [0.2 µM], N2 supplement [1×], B27 supplement [1×], N-acetylcysteine [1 µM], R-Spondin conditioned medium [10× dilution] and Wnt conditioned medium [2× dilution]. 500 µL of complete media is needed per organoid well.
3. Prepare growth factor enriched Matrigel (GF-Matrigel) by adding the following growth factors to Matrigel (*see Note 9*): EGF [50 ng/mL], LDN-193189 [0.2 µM], N2 supplement [1×], B27 supplement [1×], N-acetylcysteine [1 µM], R-Spondin conditioned medium [10× dilution], and Y-27632 [10 µM]. Keep on ice. 50 µL of growth factor enriched Matrigel is needed per well of organoids to be plated. Due to the viscosity of Matrigel, significant loss occurs in the pipet tips. Therefore, make allowance for approximately 15 % extra volume for plating.
4. Make fresh 20 mM EDTA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS, adjust to pH 7.4. Warm to 37°C in a water bath.

⁶R-Spondin can be purified from the conditioned media using Protein A Agarose via the Fc tag on the protein. However, in our hands, conditioned media supports growth of colonic organoids equivalent to that of organoids grown with the purified protein.

⁷RSPO expressing cells will become very confluent and will lift in patches but will continue to grow in suspension.

⁸Prechilled pipet tips are used for pipetting the liquefied Matrigel, helping to avoid solidification of Matrigel during the plating procedures.

⁹The addition of growth factors to the Matrigel, modified from Ahmad et al. [8], greatly enhances the viability and growth of organoids. Without addition of growth factors, the viability of colonic organoids 1 day after plating is ~10 % compared to 30–50 % with the addition of growth factors to the Matrigel. The organoids also grow larger and with more structural complexity.

5. Set up a colon eversion station by positioning a disposable 10 mL syringe upright in a clamp and attach a flexible disposable feeding needle (such as Soloman Scientific #FTP-20-38) onto the syringe (Fig. 1a).
6. Fill a 5 mL syringe with cold PBS and attach a reusable animal feeding needle (such as Popper & Sons, #7922).
7. For each tissue, prepare three 50 mL conical tubes containing 30 mL cold PBS (see **Note 10**) supplementing one tube with 100 units/mL penicillin–streptomycin.
8. Euthanize the mouse with CO₂ asphyxiation followed by cervical dislocation.
9. Remove the colon and place in a cup of cold PBS.
10. Remove excess fat from the outside of the colon using a forceps.
11. Using the preloaded 5 mL syringe from **step 6**, perfuse the colon in order to flush out feces.
12. Gently thread the proximal end of the colon (wider end) onto the disposal gavage needle. Once the entire colon passes through the tip of the needle (Fig. 1b), tie the distal end onto the needle with a piece of thread (Fig. 1c) and cut off the extra length. Evert the tissue by placing thumb and index finger on each side of the colon (Fig. 1d) and gently pulling upward. The colon will easily slide over itself and evert. Be careful to not damage the now-exposed inner lining of the colon.
13. Place the tissue, attached to the gavage needle, into the conical tube containing 30 mL cold PBS + penicillin–streptomycin. Keep on ice.
14. Vortex this tube at 80 % of maximum, 5×, 5 s each, to remove remaining fecal material, swirling the tube to untangle the colon between vortexing cycles.
15. Use a forceps to transfer the colon/needle to another conical tube containing 30 mL cold PBS. Vortex at maximum speed 3×, 5 s each, again detangling colon after each cycle.
16. Transfer colon/needle to the pre-warmed 20 mM EDTA in PBS in a 50 mL conical tube. Incubate at 37 °C in a water bath for 35 min.
17. Meanwhile, warm a 24-well culture plate (such as Costar #3524) in a 37 °C cell culture incubator (see **Note 11**).
18. Following incubation, transfer the colon still on the gavage needle to a conical tube containing 30 mL cold PBS and vortex at maximum speed 8×, 5 s each to release crypts, untangling the colon after each cycle by swirling tube. (*The PBS should turn cloudy, from crypts released from the colon tissue*). Figure 2a shows an aliquot of freshly released crypts viewed under the microscope.

¹⁰Keep the volume to 30 mL as recommended. Subsequent vortexing steps do not work efficiently if the volume is over 40 mL.

¹¹Pre-warming of the culture plate will facilitate the solidification of Matrigel after plating the crypts.

19. Remove colon/needle and discard. Add 0.6 mL FBS to the tube containing crypts to yield a final concentration of 2 % and spin down the crypts at $125 \times g$ for 3 min.
20. Aspirate solution and resuspend crypts with 10 mL cold ADF⁺ and transfer to a 15 mL conical tube.
21. Centrifuge at $70 \times g$ for 2 min. This step helps remove single cells.
22. Remove supernatant and gently resuspend pellet with 10 mL ADF⁺ to repeat the wash.
23. Pipet a 10 μ L aliquot onto a slide and count crypts in the entire 10 μ L volume. (*Typical yield ~ 80–120,000 crypts from one mouse colon which is 80–120 crypts in the 10 μ L aliquot*).
24. Transfer the required number of crypts (750 crypts/well to be plated) into a 15 mL conical tube, fill the tube with ADF⁺ to ~10 mL in order to resuspend the crypts well, and spin down at $100 \times g$ for 3 min.
25. Thoroughly discard the supernatant and keep the crypts cold at all times. All buffer must be removed from the crypt pellet to avoid Matrigel becoming diluted. Thinning the Matrigel with buffer will cause it to spread when plated, rather than forming a mound. If the pellet becomes loose and the remaining buffer cannot be removed, spin the tube again for 30 s at $70 \times g$. Carefully aspirate the remaining buffer with a P-200 tip.
26. Place the *prechilled* pipet tip boxes on ice and use the chilled tips to gently but thoroughly resuspend the crypt pellet in GF-Matrigel at the density of ~750 crypts/50 μ L Matrigel. Avoid bubbles. *See Note 12*.
27. Using the prechilled pipet tips and holding the pipet perpendicular to the well, seed 50 μ L of Matrigel–crypt mixture to the center of the wells of the pre-warmed 24-well plate, and incubate for 5–10 min in 37 °C incubator until solidified. (*The droplet of Matrigel should remain in the center of the well and not spread*).
28. During the Matrigel solidification step, prewarm the complete organoid medium to 37 °C.
29. Once the Matrigel is solidified, add 500 μ L warm complete organoid medium to each well without touching the Matrigel mound. (*The medium should barely cover the Matrigel–crypt mound*).
30. Incubate at 37 °C in a CO₂ incubator. The plate is now ready for application of ex vivo treatments. Change complete medium every 2–3 days as needed.

¹²Manipulation of Matrigel takes some practice. When resuspending crypts, take care not to push air into the Matrigel. If bubbles do occur within your Matrigel mound, however, the organoids will grow with no problem. If you are plating an entire 24-well plate, pipet half of the wells, place the plate in the incubator to solidify, then complete the plating in the rest of the wells. This helps avoid sedimentation of the crypts to the plate surface while the Matrigel is liquid, where they will attach and grow in two dimensions.

Representative images of organoid culture in basal vs. GF enriched Matrigel are shown in Figs. 2b, c and 3.

3.2 Organoid Harvest for Immunohistochemistry (IHC) Analysis

1. Place organoid culture plates on ice. Carefully aspirate and discard culture medium, add 0.5 mL ice cold ADF⁺ and mechanically break up the Matrigel by pipetting up and down multiple times using a 1000 μ L pipet. Transfer the dissociated Matrigel into a 15 mL conical tube and keep on ice. Typically, several wells of organoids (~5–8) are pooled for one embedded sample in order to obtain sufficient organoid pieces on each slide cut from the block.
2. Add additional cold ADF⁺ to the tube to ~10 mL to dissociate and wash the organoids from the Matrigel.
3. Centrifuge at $100 \times g$ for 3 min.
4. Remove supernatant and wash once in cold PBS. Centrifuge at $100 \times g$ for 3 min.
5. Resuspend the pellet in 2 mL 4 % PFA in PBS for 10 min at room temperature for fixation. Stop reaction by adding 4 mL 0.1 M glycine in PBS.
6. Centrifuge at $100 \times g$ for 3 min. Remove supernatant and wash once in PBS.
7. Dehydrate the samples in a series of ethanol washes: Resuspend the pellet in 2 mL 30 % ethanol on ice for 10–20 min. Centrifuge at $100 \times g$ for 3 min. Repeat with 50 % ethanol and then 70 % ethanol. (*Can be stored at 4 °C in 70 % ethanol until ready to embed*).
8. After removal of 70 % ethanol, resuspend the organoids in 2 mL of 96% ethanol then transfer the fixed, washed organoids to a flat bottom glass vial (VRW, #66011-041) using a plastic disposable pipet.
9. Add an equal volume of 0.1 % eosin in 96 % ethanol to the vial, which will aid in visualization of the organoids in subsequent steps. Let sit for 30 min at RT.
10. For the following washes, allow the organoids to settle to the bottom of the vial and aspirate the solution with the disposable pipet, tipping the vial at an angle so the organoids collect in the corner.

Wash with 2 mL fresh 96 % ethanol, 10 min.

Wash with 100 % ethanol, 3 $\times$, 10 min each.

Finally, wash with n-butanol, 3 $\times$, 10 min each.
11. Heat a dry block to 58 °C. Place a glass dropper into the wells of heater to warm (FisherBrand #14-955-216)
12. Aspirate butanol from organoids and add liquid paraffin to the vial of organoids.
13. Keep vial at 58 °C to dry off residual butanol. Leave to embed for 30 min.
14. Perform two more changes of paraffin, 30 min each.

15. Using a warm glass dropper, suck out organoids in paraffin and transfer into a 1 × 1 cm metal embedding mold at room temperature.
16. Use a heated metal poker to push organoids to the center of the mold.
17. Briefly chill the mold on cold plate until the organoids adhere, then cover with a tissue cassette and add additional liquid paraffin so the mold is full and let set at room temperature.
18. The organoid blocks are now ready for sectioning onto slides for IHC applications. If using reporter mice, such as the Lgr5-EGFP-IRES-creER² transgenic line [5], co-localization of GFP-positive stem cells can be done with other targets of interest.

3.3 Single Cell Harvesting from Organoid Culture for Further Analyses (See Note 13)

1. Harvest organoids as described in Section 3.2, **steps 1–3**.
2. Carefully remove supernatant and wash again with 10 mL cold ADF⁺. Spin down and aspirate media.
3. Resuspend organoids in 1 mL of 0.25 % Trypsin-EDTA (Gibco #25200-056) at 37 °C for 5 min.
4. After incubation, gently pass suspension through a 20-gauge needle 3× to aid dissociation. Incubate for 1 min at room temperature and pass through needle one more time.
5. Add 5 mL ice-cold 5 % FBS in ADF⁺ to stop reaction. Spin down at 500 × *g* for 4 min.

The cell pellet is now ready for resuspending in appropriate buffers/reagents for further analyses, such as fluorescence activated cell sorting.

Acknowledgments

This work was supported by funding from NIH R35CA197707 and P30ES023512. We would like to thank Drs. Hans Clevers, Nick Barker, and Toshiro Sato for initial guidance in organoid culture technique.

References

1. Sato T, Clevers H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science*. 2013; 340:1190–1194. [PubMed: 23744940]
2. Zachos NC, Kovbasnjuk O, Foulke-Abel J, In J, Blutt SE, de Jonge HR, et al. Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. *J Biol Chem*. 2016; 291:3759–3766. [PubMed: 26677228]

¹³Organoid cultures can be digested to a single cell suspension, which can be used for flow cytometry [9], bioenergetic profiling [10], or other assays. In addition, harvested organoids can be incubated with Annexin V before single cell isolation, for apoptosis measurement by flow cytometry [11]. Proliferation assays can also be performed by treating the organoid culture 4 h prior to harvest with 10 μM EdU (Molecular Probes Click-It kit #C10340) and following kit instructions after removing the organoids from the Matrigel (Section 3.2, **steps 1–4**). Use of a stem cell reporter line, such as Lgr5-EGFP-IRES-creER² will allow identification of GFP-positive stem cells in the assays described above.

3. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009; 459:262–265. [PubMed: 19329995]
4. Sato T, Clevers H. Primary mouse small intestinal epithelial cell cultures. *Methods Mol Biol*. 2013; 945:319–328. [PubMed: 23097115]
5. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007; 449:1003–1007. [PubMed: 17934449]
6. Zhao J, Li S, Trilok S, Tanaka M, Jokubaitis-Jameson V, Wang B, et al. Small molecule-directed specification of sclerotome-like chondroprogenitors and induction of a somitic chondrogenesis program from embryonic stem cells. *Development*. 2014; 141:3848–3858. [PubMed: 25294938]
7. Ohata H, Ishiguro T, Aihara Y, Sato A, Sakai H, Sekine S, et al. Induction of the stem-like cell regulator CD44 by Rho kinase inhibition contributes to the maintenance of colon cancer-initiating cells. *Cancer Res*. 2012; 72:5101–5110. [PubMed: 23002207]
8. Ahmad AA, Wang Y, Gracz AD, Sims CE, Magness ST, Allbritton NL. Optimization of 3-D organotypic primary colonic cultures for organ-on-chip applications. *J Biol Eng*. 2014; 8:9. [PubMed: 24690469]
9. Fan YY, Davidson LA, Callaway ES, Goldsby JS, Chapkin RS. Differential effects of 2- and 3-series E-prostaglandins on in vitro expansion of Lgr5+ colonic stem cells. *Carcinogenesis*. 2014; 35:606–612. [PubMed: 24336194]
10. Fan YY, Davidson LA, Callaway ES, Wright GA, Safe S, Chapkin RS. A bioassay to measure energy metabolism in mouse colonic crypts, organoids, and sorted stem cells. *Am J Physiol Gastrointest Liver Physiol*. 2015; 309:G1–G9. [PubMed: 25977509]
11. DeClercq V, McMurray DN, Chapkin RS. Obesity promotes colonic stem cell expansion during cancer initiation. *Cancer Lett*. 2015; 369:336–343. [PubMed: 26455770]

Fig. 1. Colon eversion station for colonic crypt isolation. (a) Disposable gavage needle on a 10 mL syringe. Note the white tip which will help hold the colon in place. (b) Mouse colon situated on the needle. (c) Thread is tied tightly around the colon, just under the white tip of the plastic needle. (d) By gently pulling upward, the colon everts on itself and can be turned completely inside out, remaining tied to the needle

Fig. 2.

Crypts and organoids at various stages during isolation and growth. Note that crypts and organoids are three dimensional and, hence, the single plane images shown can appear out of focus in some areas. (a) An aliquot of freshly detached crypts is shown at $\times 10$ magnification. As can be seen, the crypts are isolated as mainly intact units. (b) Growth of representative organoids in basal Matrigel (no growth factors added) and (c) GF enriched Matrigel ($\times 20$ magnification). By day 4, both basal and GF Matrigel groups show maturing organoids especially around the edges of the Matrigel mound, with the 100 % GF group exhibiting more highly developed organoids compared to the basal group. (d) Examples of highly developed organoids at day 6 of growth in the GF enriched group ($\times 20$ magnification)

Basal Matrigel

GF enriched Matrigel

Fig. 3.

Whole well organoid growth. The entire 50 μ l Matrigel mound is shown 6 days after plating. Note that more vigorous growth occurs around the edges of the mound. Significantly increased organoid number and size can be seen in the growth factor enriched Matrigel at right ($\times 4$ magnification)