Isolation and culture of adult intestinal, gastric and liver organoids for Cre-recombinase mediated gene deletion.

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1. Introduction

The ability to identify, isolate and culture gastrointestinal stem cells as 3Dmini-tissue organoids has permitted intensive research into processes governed by stem cells such as embryonic development, lineage specification, tissue homeostasis and response to injury. Coupled with the power of transgenic mouse models that allow tissue and cell specific manipulation of genes, organoid-based technology has advanced our understanding of the molecular mechanisms that lead to human disease, such as cancer and host-microbe interactions. Although for many decades human and mouse continuous cell lines have provided the bedrock for interrogations into the molecular underpinnings of cell and tissue function, it is now recognised that many processes cannot be adequately modelled in continuous cell lines or short-term primary cultures [i.e. 2-dimensional (2D) culture]. Almost all cells *in vivo* are surrounded by other cells and extracellular matrix in a 3D fashion, and thus 2D cultures do not adequately represent this important aspect of their biology. 2D cultures also adhere to the plastic flasks they are grown in and thus not only represent a poor model of the *in vivo* 3D context, but also have significant changes in their biology and thus they do not closely resemble the living tissue they are meant to model (ADD REF Pampaloni et al Nature Reviews Molecular Cell Biol. 2007 volume 8; page 839). This caveat is not confined to processes that are complex and multifaceted, such as organogenesis, but investigations into cell-intrinsic behaviour, particularly of non-transformed cells, which require a 3D context such as *in vivo* models. Thus, much of what we understand about mammalian tissue biology is derived from *in vivo* mouse models. This presents issues surrounding access to internal organs for analysis and the cost of generating transgenic mouse models and maintaining mouse colonies. The seminal discovery of Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) as a specific marker of adult stem-cells (Barker, van Es et al. 2007, Barker, Bartfeld et al. 2010) has facilitated successful isolation and cultivation of these stem cells, which generate mini-tissue organoids, faithfully recapitulating the characteristics of the intact tissue of origin (Sato, Vries et al. 2009, Barker, Huch et al. 2010, Huch, Dorrell et al. 2013). Thus, organoids hold great promise for

translational research, bridging an important gap between 2D cultures and *in vivo* mouse/human models. They provide more physiological relevance than 2D culture systems, and are easier to generate and more amenable to genome editing and biochemical manipulation than *in vivo* models. Here we describe robust protocols for the isolation and culture of adult mouse intestinal, stomach and liver organoids and their application to Cre-recombinase mediated gene deletion as a means to study gene function. Organoids are established from compound mice that carry transgenes for a tissue or cell type specific, inducible Cre-recombinase and the gene of interest that is flanked by DNA sequences (called LoxP sites) that are recognised by the Cre-recombinase enzyme. Once the organoids are established, Cre-mediated recombination to delete the intervening DNA sequence between the two LoxP sites is induced *in vitro,* thus manipulating the gene of interest. For the gastrointestinal epithelium this allows the study of deleting or activating a gene of interest in a model that faithfully recapitulates the cell types and organisation of the intact epithelium (Flanagan, Phesse et al. 2015). For the liver, differentiation of the expanding organoid cultures faithfully mimics the functional characteristics of adult hepatocytes (Huch, Dorrell et al. 2013, Huch, Gehart et al. 2015).

2. Materials

Breed mice that harbour the relevant alleles or transgenes. Diligently follow ethical guidelines as outlined by your institution or governing body. Follow aseptic techniques throughout the procedure from harvesting the tissue until the organoids are harvested for further analysis using standard cellular and molecular techniques. Prepare and store tissue culture reagents in volume aliquots necessary to make the final media or at a concentration for easy dilution to working concentrations. Use molecular biology and tissue culture grade reagents and materials.

2.1 Tissue dissection

1. Female or male mice
2. Autoclaved forceps and dissection scissors, sterile scalpel
3. Phosphate buffered saline (PBS), pH 7.3, no magnesium or calcium (Invitrogen). Store at 4°C, use ice cold.
4. Orbital nutator
5. Bench top centrifuge

2.2 Organoid Culture and Cre-recombinase induction

1. Sterile 15 mL and 50 mL polypropylene centrifuge tubes (Greiner)
2. Cell strainers (Becton Dickinson), 70 µM and 100 µM pore size
3. Petri dish(Greiner), small (6 cm) and large (10 cm)
4. Chelation buffer: PBS with 2.5 mM EDTA (stomach), 2.0 mM EDTA (intestine). Make freshly by adding EDTA pH 8.0 to PBS and keep on ice.
5. Collagenase:dispase enzyme digestion**:** Add 12.5 mg collagenase type XI (Sigma) and 12.5 mg dispase (Gibco) to 100 mL of DMEM supplemented with 1% (w/v) fetal bovine serum.
6. Advanced DMEM/F12 base medium (ADF): Advanced DMEM/F12 (Invitrogen) supplemented with 10 mM HEPES (*N*-2-hydroxyl piperazine-*N'*-2-ethane sulphonic acid), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and 0.1% (w/v) bovine serum albumin. Make 500 mL at a time and store at -20°C in 50 mL tubes with 48.5 mL/tube.
7. Organoid culture medium base (ADF+): ADF supplemented with N2 and B27 (Invitrogen).
8. Organoid expansion culture medium for **intestine** (ADF-I): ADF+ supplemented with 50 ng/mL epithelial growth factor (EGF, Peprotech), 500 ng/mL R-spondin (R&D) (*see* **Note 1**) and 100 ng/mL Noggin.
9. Organoid expansion culture medium for **stomach** (ADF-S): ADF+ supplemented with 50 ng/mL epithelial growth factor (EGF, Peprotech), 500 ng/mL R-spondin (R&D) (*see* **Note 1**), 100 ng/mL Noggin, 50 ng/mL human fibroblast growth factor-10 (FGF-10, Peprotech), 100 ng/mL murine Wnt3a (Peprotech) (*see* **Note 2**), 10 nM [Leu^{15}]-Gastrin (Sigma-Aldrich) and 1 µM N-acetylcysteine (Sigma-Aldrich).
10. Organoid expansion culture medium for **liver** (ADF-L). ADF+ supplemented with 50 ng/mL epithelial growth factor (EGF, Peprotech), 500 ng/mL R-spondin (R&D) (*see* **Note 1**), 100 ng/mL Noggin, 100 ng/mL recombinant murine Wnt3a (Peprotech) (*see* **Note 2**), 50 ng/mL recombinant human fibroblast growth factor-10 (FGF-10, Peprotech), 50 ng/mL human growth factor (HGF, Peprotech), 10 nM [Leu^{15}]-Gastrin (Sigma-Aldrich), 10 mM nicotinimide (Sigma-Aldrich) and 1 µM N-acetylcysteine (Sigma-Aldrich).
11. Organoid culture medium for **liver differentiation** (ADF-LD): ADF+ supplemented with 100 ng/mL recombinant human fibroblast growth factor-10 (FGF-10, Peprotech), 10 nM [Leu^{15}]-Gastrin (Sigma-Aldrich), 10 mM nicotinimide (Sigma-Aldrich), 1 µM N-acetylcysteine (Sigma-Aldrich), 50 nM glycated human albumin (Sigma, A8301) and 10 µM DAPT (GSI-IX, LY-374973,

N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). This medium is used for the first 9-12 days of differentiation and then replaced with ADF-LD supplemented with 3 µM dexamethasone (Sigma-Aldrich).

12. Matrigel basement membrane matrix, growth factor reduced, phenol red-free (BD).
13. 24-well adherent tissue culture dish (Greiner)
14. β-Napthoflavone (BNF): Dissolve 1mg of 5-6 Benzoflavone in 10.4 ml dimethylsulphoxide (DMSO) to make 180 µM stock solution .Store at -20°C.
15. 4-Hydroxytamoxifen (4OHT): Dissolve 1mg of (Z)-4-(1-[4-(Dimethylaminoethoxy)phenyl]-2-phenyl-1-butenyl)phenol, (Z)-4-OHT in 10.3ml of 100% ethanol to make 250 µM stock solution. Store at -20°C.

3. Procedure

Thaw Matrigel on ice and keep it on ice throughout the procedure until culture incubation as it will solidify irreversibly at room temperature. Keep tissue and reagents on ice and work on ice as much as possible, for example during dissection, place petri dish onto a bed of ice. Place 24 well tissue culture plates into the 37°C incubator to warm up, and keep them in there until ready to plate cultures. Prepare chelation buffer, digestion buffer and organoid culture media fresh each time. The procedures for stomach and liver organoid culture are variations of the protocol used for intestinal epithelial crypt organoid culture.

3.1 Intestinal epithelial crypt isolation and tissue culture

1. Euthanize the mice using an appropriate ethically approved method, and then isolate the small intestine as an entire organ by standard surgical procedures.
2. Place the intestine in a 10 cm petri dish with ice cold PBS. Using surgical scissors, cut the intestine longitudinally (*see* **Note 3**) and rinse it clear of luminal contents in petri dish with PBS on ice. Transfer the clean tissue to a 5 mL sterile tube containing PBS and take to tissue culture on ice. Continue procedure in a Class I or Class II hood.
3. Carefully scrape villi from the small intestine using a glass cover slip (*see* **Note 4**) and rinse the tissue free of villi with PBS in a petri dish on ice.
4. Over a 50ml sterile tube, cut the intestine with dissection scissors into small (~5mm) pieces and incubate in 2 mM EDTA in PBS at 4ºC for 30 min.

5. Allow the tissue to settle to the bottom and discard the EDTA/PBS. Add 20 mL PBS to the tissue pieces.
6. Release the crypts from the EDTA treated tissue by either pipetting up and down using a 10 mL serological pipette (5 mm tissue pieces) or shaking the intact tissue in 20 mL PBS in the 50 mL tube.
7. Crypts are separated from the remaining tissue by allowing the large pieces of tissue to settle at the bottom and transferring the supernatant to a new tube (keep the supernatant and label it fraction 1). Step 6 is typically repeated 3-4 times (fraction 2 and so on). Transfer a drop of each fraction onto a clean petri dish and check under an inverted microscope. Pool the crypt-rich fractions and pellet by centrifuging at 1200 rpm/306rcf for 5 min. Discard the supernatant PBS.
8. Resuspend the crypts in 10 mL ADF and pass the crypts through a 70 µM cell strainer into a 50 mL centrifuge tube. Wash the cell strainer with 5 mL ADF and transfer the flow-through to a 15 mL centrifuge tube (*see* **Note 5**).
9. Wash the crypts 2-3 times in ADF and centrifugation at 600 rpm/76rcf for 2 min (*see* **Note 6**).
10. Count the crypts by transferring 20 µL of the crypt suspension to a petri dish during washes. On the final centrifugation, discard as much of the ADF as possible (*see* **Note 7**).
11. Resuspend the crypts at 2000 crypts/mL of Matrigel on ice and seed plates by adding 50 µL of the crypt/matrigel suspension per well in a pre-warmed 24-well tissue culture plate (*see* **Note 8**).
12. Allow the Matrigel to set by placing the tissue culture plate into the 37°C incubator for 10-15 min.
13. Add 500 µL of ADF+ culture medium to each well carefully along the side of the well to avoid dislodging the Matrigel, and incubate at 37°C, 10 %CO_2, humidified incubator. Typically, the crypts form cysts within a day and start to bud new crypts after 2-3 days after culture.
14. Add fresh EGF, R-spondin and Noggin every other day, and change the whole culture medium twice per week once the organoids are established.
15. After 7-10 days culture the organoids need to be passaged. Discard culture medium and mechanically disperse the organoids by pipetting the organoids/Matrigel with 1000 µL pipette tip. Transfer the dissociated organoids/Matrigel into a 15 mL centrifuge tube and dilute it with 2 mL ADF. Centrifuge with 600 rpm/76 rcf for 2-3 min. This will separate ADF, Matrigel and

organoids into discrete layers, enabling the Matrigel to be removed from organoid pieces. Remove as much supernatant as possible without disturbing organoid pellet. Repeat the wash with ADF and mechanically disperse the pellet with 200 µL pipette tip until the organoids are dispersed and the released dead cells washed away.

16. Re-seed by repeating steps 10-14.

3.2 Antral stomach gland isolation and tissue culture

1. Euthanize the mice using an appropriate ethically approved method, and then isolate the stomach as an entire organ by standard surgical procedures.

2. Transfer the stomach with forceps to a flat surface and use scissors to cut stomach along greater curvature and wash twice in cold PBS to remove mucus and stomach contents (*see* **Note 9**). The following steps are performed in a Class I or Class II tissue culture cabinet.

3. Transfer stomach with forceps to petri dish on ice, gently flatten with backside of forceps and dissect out antral stomach with scalpel (paler than corpus,).

4. Transfer antral stomach with forceps to 50 mL tube containing 20 mL of chelation buffer and place on orbital nutator for 1.5 hrs at 4°C. Use this time to prepare culture reagents.

5. Whilst securing the antral stomach to the side of tube with forceps, pour off chelation buffer and add 10 mL of ice cold PBS to the tube.

6. Release the antral glands from the underlying stromal tissue by shaking the tube vigorously ~12-15 times (*see* **Note 10**).

7. Transfer 20 µL of gland suspension to a slide or petri dish and check under microscope for quality and amount of glands. Quality (intact) antral stomach glands will resemble a U-shaped sock. If there's less than 10 glands in the 20 µL droplet, repeat step 6.

8. Discard underlying stromal layer and centrifuge PBS/gland suspension 1500 rpm/450 rcf for 5 mins. Carefully discard supernatant with pipette to avoid disturbing the pellet.

9. Resuspend pellet in 1 mL ice cold ADF and pass the suspension through a 100 µM cell strainer and collect the flow through in a new 50 mL centrifuge tube (*see* **Note 5**). Remove 20 µL of ADF/gland suspension and count the number of glands on slide/dish under microscope.

10. Transfer the glands to be cultured into a new tube and centrifuge PBS/gland suspension 1500 rpm/450 rcf for 5 mins. On this final centrifugation, discard as much of the ADF as possible (*see* **Note 7**).

10. Resuspend the glands at 1000-2000 glands/mL of Matrigel on ice and seed plates by adding 50 µL of the gland/matrigel suspension per well in a pre-warmed 24-well tissue culture plate (*see* **Note 8**).

11. Allow the Matrigel to set by placing the tissue culture plate into the 37°C incubator for 10-15 min.

12. Add 500 µL of ADF-S culture medium to each well carefully along the side of the well to avoid dislodging the Matrigel, and incubate at 37°C, 10 %CO_2, humidified incubator. Typically the organoids will begin to bud around 5-7 days following plating.

13. Discard medium and replace with 500 µL fresh ADF-S every other day.

14. After 7-10 days culture the organoids need to be passaged (*see* **Note 11**). To passage the gastric organoids, follow step 15 in section 3.1 (*see* **Note 12**) and re-seed following steps 10 to 12, Section 3.2.

3.3 Establishing organoids from liver ductal tissue

1. Euthanize the mice using an appropriate ethically approved method, and then isolate the liver as an entire organ by standard surgical procedures.

2. Rinse the tissue in PBS or DMEM and transport to a tissue culture cabinet on ice. The following steps are performed in a Class I or Class II tissue culture cabinet.

3. Place the tissue in a 10 cm petri dish on ice so that it is immersed in DMEM. Cut the tissue into small pieces and transfer it to a 50 mL centrifuge tube.

4. Clean the tissue pieces by pipetting up-down with a 10 ml serological pipette to remove some of the attached fat (*see* **Note 13**). Allow the tissue to settle and discard the supernatant.

4. Add sufficient collagenase-dispase media to immerse the tissue and incubate at 37°C in water bath.

5. Every 20 min, pipette up-down with a 10 mL serological pipette, allow the tissue pieces to settle to the bottom and collect fractions by transferring the supernatant to a new tube. Add fresh collagenase-dispase media and return the tube to the water bath to continue digestion.

6. After collecting each fraction, check the digest by transferring a drop onto a slide or petri dish. The aim is to isolate ductal structures. During the first hour the supernatant fraction only contains hepatocytes and very few ductal cells. These fractions are discarded.

7. Continue digestion by repeating steps 4-6 until the whole tissue is disrupted (*see* **Note 14**).

8. Pool the fractions containing the ductal structures by centrifuging 300 rpm/rcf for 2-3 min (*see* **Note 6**).

9. It might be necessary to incubate again with collagenase-dispase to further disrupt the ducts. In that case, incubate for 10 min intervals at 37°C with fresh collagenase-dispase and check after pipetting up and down.

10. Once the collagenase-dispase digestion is complete, collect the ducts individually under the dissection microscope using a pipette and transfer them to a 15 mL centrifuge tube containing 10 mL ADF.

11. Count and seed the ducts in Matrigel following steps 9-12, Section 3.1.

12. Add 500 µL of ADF-L culture medium to each well carefully along the side of the well to avoid dislodging the Matrigel, and incubate at 37°C, 10 %CO_2, humidified incubator (*see* **Note 15**). Typically, the ductal cells form cysts within 1-2 days and begin to bleb/bud 3-4 days after culture.

13. Ductal version of re-feeding

14. After 7-10 days culture the organoids need to be passaged (*see* **Note 11**). To passage the ductal organoids, follow step 15 in section 3.1 (*see* **Note 12**) and re-seed following steps 9 to 12, Section 3.1.

3.4 Differentiating liver ductal oragnoids into hepatocytes

1. Seed the ductal organoids into the required number of wells for an experiment following steps 9 to 12, Section 3.1, noting that once the organoids are differentiated to hepatocytes, they cannot be expanded further.

2. Add 500 µL of ADF-L culture medium to each well carefully along the side of the well to avoid dislodging the Matrigel, and incubate at 37°C, 10 %CO_2, humidified incubator (*see* **Note 15**).

3. Two days after seeding, change the medium to ADF-LD. Replace with fresh ADF-LD every other day for the next 7-9 days (*see* **Note 16**).

4. After 7-9 days incubation in ADF-LD, change medium to ADF-LD supplemented with 3 µM dexamethasone (Sigma-Aldrich) and continue incubation for another 3 days to further differentiate towards an adult hepatocyte phenotype.

3.5 Cre-recombinase mediated gene deletion

1. Seed the organoids while in an expansion phase in organoid expansion culture medium (ADF-I, ADF-S or ADF-L depending on the organoids) in the appropriate number of wells required for the experiment.

2. Once organoids are established, typically 3-4 days after seeding, induce the Cre-recombinase enzyme by adding xxBNF or 100nM 4OHT (depending on the Cre-recombinase transgene the compound mice harbour) for 16-18hrs at 37°C, 10 %CO_2 (*see* **Note 17**).

3. Following Cre-induction, process organoid cultures for cellular and molecular analyses as required for the experiment.

4. To harvest the organoids for protein, DNA or RNA extraction, the organoids are harvested in the same way as for passaging and the pellet is resuspended in the appropriate lysis buffer (*see* **Note 18**).

4. Notes

1. Recombinant R-spondin can be replaced by conditioned medium harvested from R-spondin producing cells, which is added at 20% (v/v).

2. Recombinant Wnt 3a can be replaced by conditioned medium harvested from Wnt3a producing cells, which is added at 50% (v/v).

3. Aim to not stretch the intestine while cutting it longitudinally.

4. A dissecting microscope can help with this step to better visualise scraping off of the villi without scraping off the crypts as well.

5. Passing the suspension through a cell strainer is essential as it removes larger pieces of tissue that has not dissociated properly.

6. The tube is centrifuged with lower speed (600 rpm/76 rcf for 2 min), so that single cells do not pellet.

7. To remove all of the supernatant ADF, take off as much as possible without disturbing the very lose pellet, then leave the tube on ice for several minutes and remove the remaining ADF with a filter tip. This is to ensure that the Matrigel is not diluted by ADF wash.

8. Keep the Matrigel suspension on ice while dispensing to the tissue culture plate and resuspend the suspension after 2-3 transfers to ensure the Matrigel suspension remains homogeneous.

9. Can use foam or cork board to pin out stomach with epidemiological pins to facilitate removing antral stomach.

10. Following the chelation buffer step, if vigorous shaking isn't suitable (physical strain etc.), you can alternatively chop the antral stomach into small pieces on petri dish (1-2mm) and place them into a 50 mL tube with 10 mL of cold PBS and using a serological 10 mL pipette, pipetting up and down 10-15 times, to dissociate glands from stromal tissue. This method often requires multiple (2-3) rounds of pipetting. Be sure to check/count how many glands are released following each round of pipetting.

11. The rate at which organoids grow and mature/bud will vary depending on how densely they are plated. However, if organoids are plated too densely this can be inhibitory to proper gastric organoid growth and maturation.

12. ***Optional* step**: pass through a 100 µM cell strainer when passaging the organoids.

13. This is a gentle wash to avoid destroying the tissue.

14. Typically it is necessary to incubate for about 2 hrs until ductal structures start to appear and then for another 2 hrs to release the ductal structures i.e. a total of approximately 4 hrs.

15. Wnt3a and Noggin are not necessary for long term survival but help with initiating and establishing the organoid cultures, is thus complete ADF-L is only added for the first 3 days and Wnt3a and Noggin are omitted after this time.

16. After 7 to 9 days, the ductal organoids should be differentiating to a hepatocytes phenotype and begin to express markers of adult hepatocytes such as albumin and cyp3a (cytochrome P450/family 3/subfamily A) (Huch, Dorrell et al. 2013, Huch, Gehart et al. 2015).

17. The agent used to induce the Cre-recombinse activity of the enzymes depends on which transgene is expressed. For example, 4OHT is used for *Villin-Cre^{ERT2}* while BNF is used for *AhCre* (Flanagan, Phesse et al. 2015). Parallel organoid culture are also treated with the carrier (DMSO or ethanol).

18. If the organoids are to be processed for immunohistochemical analysis, primary and secondary antibodies need to be pre-absorbed on Matrigel coated wells overnight at 4^{o}C. To coat the wells, add the minimum volume of Matrigel required to cover a 96-well flat bottom tissue culture tray, allow the Matrigel to set at 37°C, then add the antibody (Flanagan, Phesse et al. 2015).

5. References

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