**Department of Medicine – Gastroenterology
Standard Operating Procedures:** Intestinal Organoid Culture and Applications
**Confidential Property of: Ian Williamson, Ph.D.
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This is a “living” document that is edited/expanded regularly, check for updates.

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| Reagent Overview**Common reagents** **Common solutions** |  |  |  |  |  |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Reagent Name | Suppliers | Cat. number | Stock solution | Storage | Expansion media (EM) | IESC media (SCM) | Differentiation Media (DM) | Epithelial Buffer(EB) | Intestinal Dissociation Buffer | Colon Dissociation Buffer | Crypt Shake Buffer | Single Cell Dissociation buffer | Collagen I Hydrogel |
| L-WRN conditioned medium | Made in-house |  |  | -20C | 50% | 50% | 0% |  |  |  |  |  |  |
| Advanced DMEM/F-12 | ThermoFisher | 12634-010 |  | 4C | 50% | 50% | 100% |  |  |  |  |  |  |
| GlutaMax | ThermoFisher | 35050061 | 100× | 4C | 1x | 1x | 1× |  |  |  |  |  |  |
| HEPES | ThermoFisher | 15630-080 | 1 M | 4C | 10 mM | 10 mM | 10 mM |  |  |  |  |  |  |
| Primocin | InvivoGen | ant-pm-1 | 50 mg/mL | -20C | 100 µg /mL | 100 µg /mL | 100 µg /mL |  |  |  |  |  |  |
| B27 | ThermoFisher | 12587010 | 50× | -20C | 1x | 1x | 1× |  |  |  |  |  |  |
| N-acetyl cysteine | MP Bio | 194603 | 1 M in PBS | -20C | 1 mM | 1 mM | 1 mM |  |  |  |  |  |  |
| Murine EGF | Peprotech | 315-09 | 250 µg/mL in 0.1% BSA | -20C | 50 ng/mL | 50 ng/mL | 50 ng/mL |  |  |  |  |  |  |
| Nicotinamide | Sigma | N0636-100G | 1 M in PBS | -20C | 10mM | 10mM | 10mM |  |  |  |  |  |  |
| Gastrin | Anaspec | AS-64149 | 1 mM in 0.1% BSA | -20C | 10 nM | 10 nM | 10 nM |  |  |  |  |  |  |
| Prostaglandin E2(PGE2) | Cayman Chemicals | 14010 | 1 mM in DMSO | -20C | 10 nM | 10 nM | 10 nM |  |  |  |  |  |  |
| A8301  | Sigma | SML0788 | 5 mM in DMSO | -20C |  |  | 500 nM |  |  |  |  |  |  |
| SB202190 | LC Laboratories | S-1700 | 30 mM in DMSO | -20C | 3 µM | 3 µM |  |  |  |  |  |  |  |
| Y27632 | ApexBio | A3008-200 | 10 mM in PBS | -20C | 10 µM\* | 10 µM |  |  | 10 µM | 10 µM | 10 µM | 10 µM |  |
| JAG-1 peptide | Anaspec | AS-61298 |  |  |  | 1 µM |  |  |  |  |  |  |  |
| Na2HPO4 | Sigma | S7907 |  | RT |  |  |  | 5.6mM | 5.6mM | 5.6mM | 5.6mM | 5.6mM |  |
| KH2PO4 | Sigma | P5655 |  | RT |  |  |  | 8mM | 8mM | 8mM | 8mM | 8mM |  |
| NaCl | Sigma | S5886 |  | RT |  |  |  | 96.2mM | 96.2mM | 96.2mM | 96.2mM | 96.2mM |  |
| KCl | Sigma | P5405 |  | RT |  |  |  | 1.6mM | 1.6mM | 1.6mM | 1.6mM | 1.6mM |  |
| Sucrose | Fisher | BP220-1 |  | RT |  |  |  | 43.4mM | 43.4mM | 43.4mM | 43.4mM | 43.4mM |  |
| d-sorbitol | Fisher | BP439-500 |  | RT |  |  |  | 54.9mM | 54.9mM | 54.9mM | 54.9mM | 54.9mM |  |
| EDTA | ThermoFisher | 15575-038 | 0.5M | RT |  |  |  |  | 3mM | 2mM |  |  |  |
|  1,4-Dithiothreitol (DTT) | Sigma | 10197777001 | 2M in H2O | -20oC |  |  |  |  |  | 20mM |  |  |  |
| DNAse I | Sigma | DN25 | 5.0 mg/mL in 0.15 M NaCl | -20oC |  |  |  |  |  |  |  | 50 ng/mL |  |
| Dispase | Corning | 354235 |  | -20oC |  |  |  |  |  |  |  | 10U/mL |  |
| 10x PBS | Gibco | 70011044 | 10X | RT |  |  |  |  |  |  |  |  | 1X |
| HEPES |  | 15630080 | 1M | RT |  |  |  |  |  |  |  |  | 20mM |
| NaHCO3 | ThermoFisher | 25080094 | 7.5% | RT |  |  |  |  |  |  |  |  | 53mM |
| NaOH | Sigma | 93065 | 1M | RT |  |  |  |  |  |  |  |  | 7.675mM |
| Ultra-Pure H2O | ThermoFisher | 10977015 |  | RT |  |  |  | Solvent pH to 7.4 |  |  |  |  | Solvent |
| Collagen I, Rat Tail, 100mg | Corning | 354236 | Variable | 4oC |  |  |  |  |  |  |  |  | 1mg/mL |
| Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix | Corning | 354230 |  | 4oC |  |  |  |  |  |  |  |  |  |
| TrypLE™ Express Enzyme (1X) | LifeTechnologies | 12605036 |  |  |  |  |  |  |  |  |  |  |  |
| FBS USDA  | MEDIATECH | ST-0000002130 |  |  |  |  |  |  |  |  |  |  |  |
| DPBS | Gibco | 14190250 |  |  |  |  |  |  |  |  |  |  |  |
| Collagenase IV | Fisher | NC9919937 | 5000units/mL in HBSS |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |

\*= not essential for all applications. Further details listed in relevant SOPs

# SOP: Dissect Mouse Intestine

Up to 3 mice may be processed in parallel. If more than 3 mice are necessary, keep intestines on ice at the end of this procedure before moving to next steps.

**Tools and Consumables**

* IACUC approved isofluorane container for euthanasia (*ex: bell jar*)
* Chemical hood
* Dissection tools
	+ Large forceps
	+ Large scissors
	+ Small forceps
	+ Small scissors
* Dissection surface (*ex: lid of extra Styrofoam box*)
* 4 needles to pin mouse for dissection. (*18-25 gauge work well*)
* 10 cm ruler
* (Optional) 10 mL syringe fitted with P200 tip trimmed from wide end to fit syringe
* 10 cm petri dish (1 per mouse)
* Conical tube (1 per mouse)

**Reagents**

* Isofluorane
* Ice-cold Epithelial Buffer (~50 mL per mouse) (*PBS may be substituted*)
* 70% EtOH in spray bottle
1. **Euthanize mouse in chemical hood with isoflurane followed by cervical dislocation** in accordance with IACUC-approved protocol.
2. **Dissect intestine.**
	1. Mount euthanized animal on dissection plate and secure in supine position with needles.
	2. Spray abdomen with 70% EtOH to wet and sterilize fur.
	3. Using large scissors and large forceps, make an incision into abdominal skin ~ 2 cm above the anus.
	4. Insert scissors below skin and open the blades to separate skin from muscle.
	5. Cutting in a V-pattern, free the skin, lifting up and to the side to expose the abdominal muscle. Spray scissors with 70% EtOH to remove any fur that may be stuck on the blades.
	6. Perform laparotomy to open abdominal cavity
	7. Using small scissors and forceps to shift visceral organs, locate the stomach underneath the liver.
	8. Holding the stomach with forceps at pyloric antrum, cut through the stomach immediately proximal to the forceps.
	9. Still holding the stomach, gently pull upward and to the left to remove intact intestine from duodenum to rectum.
		1. *IMPORTANT: While pulling, use small scissors or a second pair of forceps to release pancreatic adhesions and mesenteric plexus.*
	10. Transfer to glass plate and stretch intestine to full length.
3. **Collect tissue from desired intestinal regions**.
	1. Trim small intestine by cutting immediately distal to stomach and proximal to cecum.
		1. Duodenum: 1st 2-5 cm distal to pyloric sphincter
		2. Jejunum: Middle third of the intestine
			1. (*NOTE: Most small intestinal preps are made from jejunum*)
		3. Ileum: distal 8-10 cm intestine
	2. Trim large intestine by cutting distal to cecum and 10 mm proximal to anus. Remove any remaining non-intestinal tissue.
		1. (*NOTE: avoid collecting rectum and anus by omitting the distal ~0.5 cm from collection*)
		2. Proximal colon: Cecum to end of wide curvature
		3. Distal colon: end of curvature to ~0.5 cm proximal to the anus
4. **Remove luminal debris**
	1. (optional) Flush intestinal contents with ice-cold EB using a 10 mL syringe fitted with a trimmed P200 tip.
	2. Fillet open the intestine by passing scissors through the lumen, starting at the proximal end of the tissue. The intestine may be handled directly or with forceps.
	3. Rinse briefly in 10 cm petri dish by agitating filleted intestine in 5-10 mL ice-cold EB.
	4. Transfer to conical tube with 5-25 mL of cold EB.
	5. Shake to remove any remaining debris.
	6. Keep tube on ice until proceeding

# SOP: Tissue Fixation for Histology

This protocol uses paraformaldehyde (PFA) chemical fixative to preserve tissue proteins for histological analysis. *Note: Prepare and work with PFA solution inside of a chemical fume hood. Using ice cold fluids drastically improves tissue quality.*

 **Tools and Consumables**

* 15mL/ 50mL Conical tubes
* Chemical hood
* Dissection tools
	+ Small forceps
	+ Small scissors
* Dissection surface (*ex: lid of extra Styrofoam box*)
* 10 mL syringe fitted with P200 tip trimmed from wide end to fit a syringe

**Reagents**

* Epithelial buffer
	+ To flush intestine segments
* PFA solution
	+ ~10mL to flush intestine segments
	+ 20mL to fix intestine segments overnight
* Sucrose solution
	+ ~10mL to wash intestine segments
	+ 20mL to post-fix intestine segments overnight
1. **Remove remaining lumen debris**
	* Flush intestinal contents with ice-cold EB using 10 mL syringe fitted with a trimmed P200 tip.
	* Repeat as necessary until all observable debris is removed
2. **Fix overnight in PFA at 4oC**
	* Complete inside of a chemical fume hood
	* Flush intestinal contents with ice-cold PFA using 10 mL syringe fitted with a trimmed P200 tip.
	* Submerge intestine segment in 20mL of ice-cold PFA in a 50mL tube
		1. Removing residual adipose and lumen air bubbles if tissue floats
	* Refrigerate overnight
3. **Post-fix overnight in Sucrose at 4oC**
	* Wash intestine segment in ice-cold sucrose solution briefly
	* Submerge intestine segment in 20mL ice-cold sucrose in a 50mL tube
	* Refrigerate overnight

# SOP: Murine Small Intestine Epithelial Dissociation

The final prep will beintact crypt/villus units. Crypts may separate from villi, which is desirable if planning to *enrich* for crypts. Expected contaminating cell types including circulating cells with little contamination from mesenchymal cell sources. Crypt/villi units isolated with this protocol can be fixed, cultured, or further dissociated into single cells.

*IMPORTANT: If crypts will be plated in Matrigel, begin thawing Matrigel several hours before starting dissociation, as per SOP: Plate in Matrigel.*

**Tools and Consumables**

* Small forceps
* Small scissors
* 15mL/ 50mL Conical tubes (4 per mouse)
* MACSmix™ Tube Rotator
* Bacterial plate

**Reagents**

* **Intestinal Dissociation Solution**: Ice-cold
	+ 2 tubes per sample
		- Containing 5mL per small intestinal segment
		- 10mL sufficient for the entire small intestine
* **Crypt Shake Solution**:
	+ 2 tubes per sample
		- Containing 5mL per small intestinal segment
		- 10mL sufficient for the entire small intestine
1. **Loosen epithelium**
	1. Use forceps to transfer the intestine from ice-cold EB to the first tube of Intestinal Dissociation Solution.
	2. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in a cold room at 80 rpm for 15 minutes
	3. Use sterile forceps to transfer the intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution
2. **Deplete damaged epithelium**
	1. Use sterile forceps to transfer the intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution
	2. Shake sample at 2.5 cycles per second for 2 minutes
	3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
		1. The solution should contain villi fragments with very few crypt units
		2. Keep conical for comparison to final preparation
	4. Use sterile forceps to transfer the intestine to the second tube of Intestinal Dissociation Solution
	5. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in a cold room at 80 rpm for 30 mins
		1. Crypt yield from distal small intestinal section may be improved by extending to 45 mins
3. **Isolate epithelial units**
	1. Use sterile forceps to transfer the intestine from the second tube of Intestinal Dissociation Solution to the second tube of Crypt Shake Solution
	2. Shake sample at 2.5 cycles per second for 2 minutes
		1. Can be extended for large samples containing multiple regions or pooled animal samples
			1. Tissue fragments float when depleted of epithelium
				1. Adipose attached to the intestine can cause premature floating
	3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
		1. The solution should contain intact villus/crypt units as well as intact villi and intact crypts
			1. The solution can be compared to the contents of the first shake tube
		2. Use forceps to transfer a piece of digested intestine to the bacterial plate and examine with a tissue culture microscope. The remaining crypts appear as dense circles in the epithelium.
		3. *NOTE: If crypt yield is sub-optimal and many crypts are visible in the remaining tissue, repeat the shaking step using reserved tissue. To avoid damaging crypts isolated from the first round of shaking, transfer tissue to a new tube with Epithelium Shake. Pool crypts before proceeding to the next step.*
	4. **Remove digested tissue** using forceps for a sterile cotton swab.
		1. Using sterile forceps, transfer digested intestine to a storage tube.
			1. *Optional: Reserve on ice in the event of catastrophic failure at downstream steps*.
			2. *Optional: Process intestinal fragments for histology to examine dissociation efficiency.*
	5. **Proceed to compatible SOP**
		1. SOP: Single Cell Dissociation
		2. SOP: Plate Crypts in Matrigel
		3. SOP: Plate Crypts on Collagen HydrogelSOP: Plating Crypts in Matrigel for Organoid Culture

# SOP: Embedding Cypts in Matrigel for Organoid Culture

This protocol is adapted from the Matrigel product sheet and associated literature. Read the Matrigel product sheet for more detailed handling instructions.

**Tools and Consumables**

* Clinical centrifuge in a cold room
* (Optional) cold pipette tips
* Conical centrifuge tube
* Tissue culture plate
* (Optional) 100 µm cell strainer
* (Optional) 70 µm cell strainer

**Reagents**

* Growth factor-reduced Matrigel
* Organoid Growth Media (several options)
	+ **Expansion media** (recommended)
	+ *Note: Many different media combinations have been used to grow organoids from crypts. The original media formulations required expensive recombinant growth factors. Refer to the Media Formulation sheet for additional recipes. Current protocols replace Noggin, R-spondin, and/or Wnt3 growth factors with conditioned media made from L-cells that produce and secrete these factors into culture media.*
		- IntestiCult™ Organoid Growth Medium (Mouse)
			* Stem Cell Technologies cat: 06005
			* Add supplement A and supplement B
		- 50% L-WRN conditioned media
			* Produced according to <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/>
		- Antibiotic supplement (several options)
			* Pen/Strep, Gibco: 15140-122
			* Primocin, InvivoGen: ant-pm-1
* **(Optional) Crypt Shake Solution**:
	+ 1mL in tubes to collect filter flow through
1. **(Optional) Separate Crypts from isolated whole epithelial units**
	1. Filter isolated epithelial unit solution produced by SOP: Small Intestine Epithelial Dissociation through a 100 µm cell strainer
		1. Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.
		2. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
			1. The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size
	2. Filter the flow-through using a 70 µm cell strainer
		1. Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.
		2. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
			1. The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size
2. **Pellet crypts for plating**
	1. Remove 3 separate 5 µL aliquots of the filtered crypt solution and examine by phase microscopy
		1. Quantify the average crypt contents of the aliquots and extrapolate to determine the total crypt yield
	2. Mix the filtered crypt solution by flicking and transfer an adequate volume to a microcentifuge tube
		1. Volume is determined by the amount of Matrigel being utilized
			1. Transfer 5 crypts/1 µL Matrigel for plating
		2. well then aliquot enough crypts for 5 crypts/1 ul Matrigel to 1.5 mL centrifuge tube or up to 5000 single cells per well
	3. Centrifuge on benchtop centrifuge in a cold room at 2000g for 5 minutes
	4. Remove supernatant and examine an aliquot by phase microscopy to ensure crypts are pelleted efficiently
		1. *NOTE: >80% of intact crypts will form organoids. Contaminating single cells that include stem cells can also form organoids.*
3. **Embed in Matrigel**
	1. Centrifuge Matrigel at max speed for 15-30 sec in benchtop centrifuge in a cold room.
		1. This will pellet any insoluble collagens that may be in the Matrigel.
		2. The amount of insoluble material varies by Matrigel lot.
	2. Re-suspend pellet directly into the appropriate volume of Matrigel. Mix very well by pipetting ~25-50 times.
	*\*Note: Cells and crypts may be plated in up to a 1:1 dilution in Matrigel.*
		1. Use pre-chilled tubes and tips.
		*\*Optional: Perform pipetting steps on ice.*
		2. When pipetting Matrigel from aliquot, pipet up and down one time to coat the inside of the tip. Or, push the pipette plunger slightly past the stop so that no bubbles will be added to the Matrigel when the depressing plunger
		3. DO NOT introduce bubbles into the Matrigel.
	3. Plate by adding Matrigel as a hemispheric ‘dome’ to the center of each well in a pre-warmed plate
		1. For 48 well plate, use 25-50 uL Matrigel
		2. For 96 well plate, use 8-10 µL Matrigel
		*\*Note: Place pipette tip on the bottom of the middle of the well, lift slightly, then depress to first stop to plate Matrigel in hemispheric droplet*
	4. Carefully transfer the plate to a 37°C incubator and allow the Matrigel to polymerize for 30 minutes
4. **Overlay Media**
	1. Remove the plate from the incubator
	2. Add 10-20x Matrigel volume of desired Culture Media
		1. Continue to change culture media on an appropriate schedule
			1. Typically, every 2-3 days
	3. Transfer into a standard tissue culture incubator
		1. Organoids are ready for passage after 6-12 days or when the lumens begin to burst and expel contents into the Matrigel

# SOP: Passage Organoids Grown in Matrigel

This protocol is adapted from Matrigel product sheet and associated literature. Read the Matrigel product sheet for more detailed handling instructions.

**Tools and Consumables**

* Clinical centrifuge (in cold room)
* (Optional) cold pipette tips
* Conical centrifuge tube
* Tissue culture plate

**Reagents**

* Growth factor-reduced Matrigel
* Organoid Growth Media (several options)
	+ **Expansion media** (recommended)
	+ *Note: Many different media combinations have been used to grow organoids from crypts. The original media formulations required expensive recombinant growth factors. Refer to the Media Formulation sheet for additional recipes. Current protocols replace Noggin, R-spondin, and/or Wnt3 growth factors with conditioned media made from L-cells that produce and secrete these factors into culture media.*
		- IntestiCult™ Organoid Growth Medium (Mouse)
			* Stem Cell Technologies cat: 06005
			* Add supplement A and supplement B
		- 50% L-WRN conditioned media
			* Produced according to <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/>
		- Antibiotic supplement (several options)
			* Pen/Strep, Gibco: 15140-122
			* Primocin, InvivoGen: ant-pm-1
* **(Optional) Crypt Shake Solution**:
	+ 1mL in tubes to collect filter flow through
* TrypLE express +10µM Y27632
	+ 4x matrigel volume being passaged
	+ Contained in a conical tube of appropriate volume
* FBS
	+ Cold
	+ 10% of TrypLE volume employed
1. **Digest the matrigel and fragment organoid units**
	1. Remove culture media from wells being passaged
	2. Wash wells in DPBS containing matrigel cultures to remove residual media
		1. Add 1.5x culture media volume removed in step 1
		2. Remove DPBS and discard
	3. Add a small volume of TrypLE +y27 to each well being passaged
		1. Add 2x the volume of matrigel in TrypLE +y27
	4. Transfer organoids in matrigel for digestion
		1. Fragment the matrigel domes in each well using a pipette tip
		2. Transfer the fragmented matrigel in the conical tube containing the full volume of TrypLE +y27 being employed
	5. Digest the matrigel fragments at 37oC for 5 mins
		1. Place the tube upright in a water bath
	6. Quench the TrypLE digestion with cold BS
		1. Add 10% of the TrypLE solution volume and mix by inverting*.*
2. **Embed organoid fragments in Matrigel**
	1. Centrifuge Matrigel at max speed for 15-30 sec in benchtop centrifuge in a cold room.
		1. This will pellet any insoluble collagens that may be in the Matrigel.
		2. The amount of insoluble material varies by Matrigel lot.
	2. Pellet organoid fragments for plating
		1. Centrifuge digestion solution containing organoid fragments at 2000gs for 5 mins
		2. Discard supernatant
	3. Re-suspend organoid fragments in the appropriate volume of Matrigel.
		1. Typically 4x the original matrigel volume is used
		2. Mix very well by pipetting (~40x) without adding bubbles to the system
	4. Plate organoid fragments in hemispheric matrigel ‘domes’ in the center of each culture well
		1. Plate matrigel domes in the center of wells in a new tissue culture plate
			1. For 48 well plate, use 25-50 uL Matrigel
			2. For 96 well plate, use 8-10 µL Matrigel
			*\*Note: Place pipette tip on the bottom of the middle of the well, lift slightly, then depress to first stop to plate Matrigel in hemispheric droplet*
		2. Transfer plate into tissue culture incubator
		3. Incubate at 37oC for 45 mins to polymerize matrigel
3. **Overlay Growth Media on Matrigel Domes**
	1. Remove the plate from the incubator
	2. Add 10-20x Matrigel volume of desired Culture Media
		1. Continue to change culture media on an appropriate schedule
			1. Typically, every 2-3 days
	3. Transfer into a standard tissue culture incubator

# SOP: Plating Crypts on Collagen Hydrogel for Organoid Culture

This protocol is adapted from the publication demonstrating the growth of organotypic monolayers on collagen hydrogels. Review the paper and subsequent publications from the Allbritton group <http://dx.doi.org/10.1016/j.jcmgh.2017.02.011>

**Tools and Consumables**

* Clinical centrifuge in a cold room
* Pipette tips
* Conical centrifuge tube
* Tissue culture plate
* (Optional) 100 µm cell strainer
* (Optional) 70 µm cell strainer

**Reagents**

* Collagen hydrogel scaffolds
	+ Prepared in different well formats in accordance with Collagen hydrogen SOP
* Organoid Growth Media (several options)
	+ **Expansion media** (recommended)
	+ *Note: Many different media combinations have been used to grow organoids from crypts. The 2D organoid culture was originally described using mixtures of Noggin, R-spondin and Wnt3 conditioned media made from L-cells that produce and secrete these factors into culture media. Current protocols use a single L-cell line to produce all required factors.*
		- 50% L-WRN conditioned media
			* Produced according to <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/>
		- Antibiotic supplement (several options)
			* Pen/Strep, Gibco: 15140-122
			* Primocin, InvivoGen: ant-pm-1
* **(Optional) Crypt Shake Solution**:
	+ 1mL in tubes to collect filter flow through
1. **Prepare Hydrogel scaffolds for culture**
	1. Transfer collagen hydrogel scaffolds into a tissue culture hood
	2. Remove residual PBS from each well being used for culture
		1. Carefully remove with a sterile pipette without disturbing the collagen hydrogel in the bottom of each well
	3. Wash each well in PBS 2x
	*\*Note: Do not store collagen scaffolds for extended periods (>10mins) without PBS covering the scaffolds*
	4. Add half of the desired culture volume of Organoid Growth Media to each well
2. **(Optional) Separate Crypts from isolated whole epithelial units**
	1. Filter isolated epithelial unit solution produced by SOP: Small Intestine Epithelial Dissociation through a 100 µm cell strainer
		1. Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.
		2. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
			1. The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size
	2. Filter the flow-through using a 70 µm cell strainer
		1. Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.
		2. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
			1. The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size
3. **Pellet crypts for plating**
	1. Remove 3 separate 5 µL aliquots of the filtered crypt solution and examine by phase microscopy
		1. Quantify the average crypt contents of the aliquots and extrapolate to determine the total crypt yield
	2. Mix the filtered crypt solution by flicking and transfer an adequate volume to a microcentifuge tube
		1. Volume is determined by the plate size and number of wells
			1. Transfer 1000 crypts/cm2 of plating surface
				1. One mouse intestinal section is generally plated onto one 6-well plate for expansion.
	3. Centrifuge on benchtop centrifuge in a cold room at 3000g for 5 minutes
	4. Remove supernatant and examine an aliquot by phase microscopy to ensure crypts are pelleted efficiently
	\**Note: >80% of intact crypts will form organoids. Contaminating single cell that include stem cells can also form organoids.*
4. **Plate Crypts on Collagen Hydrogel**
	1. Re-suspend pellet directly into the appropriate volume of Organoid Growth media.
		1. First, resuspend the crypt pellet in 1mL of Organoid Growth media
			1. Mix very well by pipetting~25-50 times.
			2. Resuspend the remaining half of the culture volume on the cumulative culture volume
	2. Plate by adding crypts in the remaining half of the culture media of each well
	3. Transfer into a standard tissue culture incubator

# SOP: Passaging Organoids Grown on Collagen Hydrogel

This protocol is adapted from the publication demonstrating the growth of organotypic monolayers on collagen hydrogels. Review the paper and subsequent publications from the Allbritton group <http://dx.doi.org/10.1016/j.jcmgh.2017.02.011>

**Tools and Consumables**

* Clinical centrifuge in a cold room
* Pipette tips
* Conical centrifuge tube
* Tissue culture plate

**Reagents**

* Collagen hydrogel scaffolds
	+ Prepared in different well formats following Collagen hydrogen SOP
* Organoid Growth Media (several options)
	+ **Expansion media** (recommended)
	+ *Note: Many different media combinations have been used to grow organoids from crypts. The 2D organoid culture was originally described using mixtures of Noggin, R-spondin and Wnt3 conditioned media made from L-cells that produce and secrete these factors into culture media. Current protocols use a single L-cell line to produce all required factors.*
		- 50% L-WRN conditioned media
			* Produced according to <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/>
		- Antibiotic supplement (several options)
			* Pen/Strep, Gibco: 15140-122
			* Primocin, InvivoGen: ant-pm-1
* **(Optional) Crypt Shake Solution**:
	+ 1mL in tubes to collect filter flow through
* Collagenase IV solution
	+ Fisher NC9919937
	+ 5000units/mL in HBSS with Calcium chloride
* DPBS
1. **Prepare Hydrogel scaffolds for culture**
	1. Transfer collagen hydrogel scaffolds into a tissue culture hood
	2. Remove residual PBS from each well being used for culture
		1. Carefully remove with a sterile pipette without disturbing the collagen hydrogel in the bottom of each well
	3. Wash each well in PBS 2x
	*\*Note: Do not store collagen scaffolds for extended periods (>10mins) without PBS covering the scaffolds*
	4. Add half of the desired culture volume of Organoid Growth Media to each well
2. **Digest Collagen Hydrogel**
	1. Remove 75% of the culture media from each well and discard
	2. Disrupt the collagen hydrogel with a pipette tip to release from the bottom of the tissue culture plate
		1. Hydrogel should lift off of the plate bottom when a pipette tip is run around the circumference of the well
	3. Transfer the collagen hydrogel and remaining media to a centrifuge tube
		1. Pool all wells of each sample being passaged
		2. Further disrupt hydrogels by pipetting repeatedly with a 5-10mL pipette
	4. Digest the collagen hydrogels by adding collagenase IV solution @ 10% of the pooled volume
		1. Incubate in 37oC water bath for 12 total mins
			1. Further disrupt collagen hydrogel by pipetting repeatedly with a 5-10mL pipette every 4 mins during 12 min incubation
	5. Quench collagenase activity with cold FBS
		1. Add 10% of the pooled volume in ice-cold FBS
3. **Inactivate Collagenase IV and further dissociate organoid fragments**
	1. Pellet organoid fragments following quenching @3000g for 5 mins
		1. Remove supernatant and examine a sample to ensure efficient pelleting
	2. Resuspend organoid fragments in 1-2mL DPBS +0.5µM EDTA
		1. Mix well by pipetting repeatedly
	3. Incubate in 37oC water bath for 4 minutes
		1. Mix by flicking once during incubation
	4. Remove EDTA from the cell sample
		1. Dilute the EDTA content of the solution by adding 10mL DPBS
			1. Pellet cell fragments at 100g for 5 min
				1. Remove supernatant and examine a sample to ensure efficient pelleting
		2. Wash Cell fragments in DPBS
			1. Resuspend the crypt pellet in 1mL of DPBS
				1. Mix very well by pipetting~25-50 times.
			2. Resuspend in an additional 9mL of DPBS
4. **Pellet cell fragments for plating**
	1. (Optional) Quantify the cellular content of the diluted cell solution
		1. Remove 3 separate 5 µL aliquots of the filtered crypt solution and examine by phase microscopy
			1. Quantify the average crypt contents of the aliquots and extrapolate to determine the total crypt yield
	2. Mix the filtered cell fragment solution by flicking and transfer an adequate volume to a microcentifuge tube
		1. Volume is determined by the plate size and number of wells
			1. Transfer 5000 cells/cm2 of plating surface
				1. *\*Note Organoid cultures are generally expanded at 1:3 by culture surface area as quantifying the cellular content of fragments is technically difficult*
	3. Centrifuge on benchtop centrifuge in a cold room at 3000g for 5 minutes
	4. Remove supernatant and examine an aliquot by phase microscopy to ensure crypts are pelleted efficiently
5. **Plate Organoid Fragments on Collagen Hydrogel**
	1. Re-suspend pellet directly into the appropriate volume of Organoid Growth media.
		1. First, resuspend the cell fragment pellet in 1mL of Organoid Growth media
			1. Mix very well by pipetting ~25-50 times.
			2. Resuspend the remaining half of the culture volume on the cumulative culture volume
	2. Plate by adding cell fragments in the remaining half of the culture media of each well
	3. Transfer into a standard tissue culture incubator

# SOP: Making Hydrogel Scaffolds

This protocol is adapted from the publication demonstrating the growth of organotypic monolayers on collagen hydrogels. Review the paper and subsequent publications from the Allbritton group <http://dx.doi.org/10.1016/j.jcmgh.2017.02.011>

**Tools and Consumables**

* Clinical centrifuge in cold room
* Pipette tips
* Conical centrifuge tube
* Tissue culture plates
	+ Chill in -20oC

**Reagents**

* Rat tail Collagen I
	+ Corning #356236 in 20 mM acetic acid
* 10x PBS
	+ Gibco 70011044
* HEPES
	+ Gibco 15630080
* NaHCO3
	+ ThermoFisher 25080094
* NaOH
	+ Sigma 93065
* Ultra-Pure H2O
	+ ThermoFisher 10977015
* Rat Tail Collagen I 100mg
	+ Corning 354236
* DPBS
	+ Gibco 14190250

**Prepare Collagen Hydrogel Precursor**

* Calculate the hydrogel composition using the included [SOP Collagen Hydrogel Composition](https://drive.google.com/open?id=1C2Mop2KRQOm_gkkcOwPfND_MGno1SRx1&authuser=iawilli3@ncsu.edu)
	+ Enter the concentration of the Rat Tail Collagen I received from Corning in cell D2
		- 
	+ Enter the cell culture plates the hydrogel will be added to in cell E7:E11
		- 
	+ Enter a total volume greater than the minimum volume listed in cell F12 into cells F13 and G22
		- 
	+ The composition of the collagen hydrogel precursor will be displayed in cells B15:H21
		- 
* Mix the neutralizing components of the precursor in a microfuge tube and ice for 5 mins
	+ Inert components include all reagents excluding the rat tail collagen and are listed in cells G16:G20
		- 
	+ Mix components inside a cell culture hood to retain hydrogel sterility
	+ Ice tube for 5 minutes before proceeding

**Coat cell culture plates in collagen hydrogel scaffolds**

* Transfer cold cell culture plates into the biosafety cabinet
* Add rat tail collagen to the neutralizing components and mix thoroughly by pipetting
	+ Do not introduce bubbles during the mixing process
	+ Keep rat tail collagen and neutralization components on ice as much as possible
* Dispense appropriate amounts of collagen hydrogel precursor to each well of the cold cell culture plates

|  |  |  |
| --- | --- | --- |
| **Plate Setup** | Vol-plate | Vol-well |
| 96-Well | 7.2mL | 70mL |
| 48-Well | 9.6mL | 150µL |
| 6-Well | 6mL | 1mL |
| 24-Well | 7.2mL | 300µL |
| 12-Well Transwell | 2.4mL | 300µL |

* + Spread the gel precursor across the entire surface of the culture well using the pipette tip and swirling
	+ Spin down plates @300gs for 2 minutes to pop any bubbles in the collagen gel
* Thermally polymerize the collagen hydrogel at 37oC for 1 hour in a standard tissue culture incubator

**Store collagen hydrogel-coated plates for future use**

* Transfer collagen hydrogel-coated plates into a tissue culture biosafety cabinet
	+ Collagen hydrogel forms an opaque gel in the bottom of each well
		- No residual liquid should be present on the hydrogel surface
		- Turn cell culture plates to verify that the hydrogel has bonded to the culture well surface
* Cover the collagen hydrogel in DPBS to prevent dehydration
	+ Add 2x the collagen hydrogel volume to each well
* Store collagen hydrogel scaffold plates in a sterile, humidified environment until use
	+ *\*Note: sealing bags that tissue culture plates are packaged in are suitable for long-term storage*

# **SOP: Colon Epithelial Dissociation**

The final prep will be intact crypt units. Expected contaminating cell types including circulating cells with little contamination from mesenchymal cell sources. Crypt units isolated with this protocol can be fixed, cultured, or further dissociated into single cells.

*IMPORTANT: If crypts will be plated in Matrigel, begin thawing Matrigel several hours before starting dissociation, as per SOP: Plate in Matrigel.*

**Tools and Consumables**

* Small forceps
* Small scissors
* 15mL/ 50mL Conical tubes (4 per mouse)
* MACSmix™ Tube Rotator
* Bacterial plate

**Reagents**

* **Colon Dissociation Solution**: Ice-cold
	+ 2 tubes per sample
		- Containing 5mL per small intestinal segment
* **Crypt Shake Solution**:
	+ 2 tubes per sample
		- Containing 5mL per small intestinal segment
		- 10mL sufficient for the entire small intestine
1. **Loosen epithelium**
	1. Use forceps to transfer the intestine from ice-cold EB to the first tube of Colon Dissociation Solution.
	2. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in a cold room for 15 minutes
	3. Use sterile forceps to transfer the intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution
2. **Deplete damaged epithelium**
	1. Use sterile forceps to transfer intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution
	2. Shake sample at 2.5 cycles per second for 2 minutes
	3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
		1. Solution should contain very few crypt units
		2. Keep conical for comparison to final preparation
	4. Use sterile forceps to transfer intestine to the second tube of Colon Dissociation Solution
	5. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in cold room at 80 rpm for 60 mins
		1. Crypt yield from large samples may be improved by extending to 75 mins
3. **Isolate epithelial units**
	1. Use sterile forceps to transfer the intestine from the second tube of Intestinal Dissociation Solution to the second tube of Crypt Shake Solution
	2. Shake sample at 2.5 cycles per second for 4 minutes
		1. Can be extended for large samples or pooled animal samples
			1. Tissue fragments float when depleted of epithelium
				1. Adipose attached to the colon can cause premature floating
	3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
		1. The solution should contain intact villus/crypt units as well as intact villi and intact crypts
			1. The solution can be compared to the contents of the first shake tube
		2. Use forceps to transfer a piece of digested intestine to the bacterial plate and examine with a tissue culture microscope. The remaining crypts appear as dense circles in the epithelium.
		3. *NOTE: If crypt yield is sub-optimal and many crypts are visible in the remaining tissue, repeat the shaking step using reserved tissue. To avoid damaging crypts isolated from the first round of shaking, transfer tissue to a new tube with Epithelium Shake. Pool crypts before proceeding to the next step.*
	4. **Remove digested tissue** using forceps for a sterile cotton swab.
		1. Using sterile forceps, transfer digested intestine to a storage tube.
			1. *Optional: Reserve on ice in the event of catastrophic failure at downstream steps*.
			2. *Optional: Process intestinal fragments for histology to examine dissociation efficiency.*
	5. **Proceed to compatible SOP**
		1. SOP: Single Cell Dissociation
		2. SOP: Plate Crypts in Matrigel

# SOP: Human Intestinal Biopsy Dissociation

The final result will be intact crypt/villi units. Expected contaminating cell types including circulating cells with little contamination from mesenchymal cell sources. Crypt units isolated with this protocol can be fixed, cultured, or further dissociated into single cells.

*IMPORTANT: If crypts will be plated in Matrigel, begin thawing Matrigel several hours before starting dissociation, as per SOP: Plate in Matrigel.*

**Tools and Consumables**

* Small forceps
* Small scissors
* 15mL/ 50mL Conical tubes (4 per mouse)
* 100mm Dish

**Reagents**

* **Collagenase IV Solution**: Ice-cold
	+ 2mL per sample
* **Crypt shake buffer**:
	+ 15-20mL per sample

**Collagenase digestion**

1. Transfer biopsies to a 100mm culture dish containing 2ml Collagenase IV solution +10µM y-27632
2. Finely mince biopsies into <1mm segments with razor blades
3. Transfer the minced tissue and collagenase solution to a centrifuge tube using a p1000 pipette tip
4. Incubate for 30 minutes at 37oC
	1. Vigorous pipet tissue solution for 30 seconds every 5 minutes.
	2. Observe a sample of the solution under a phase microscope after every shake for liberated crypts.
5. Quench the collagenase solution by adding 1mL cold FBS
	1. Mix thoroughly
6. Centrifuge at 500g for 5 minutes
	1. Remove supernatant and observe a sample of the solution under a phase microscope
		1. If crypts are present increase the speed of centrifugation
7. Wash crypts with 5mL Crypt Shake Buffer
8. Centrifuge at 500g for 5 minutes
	1. Remove supernatant and observe a sample of the solution under a phase microscope
		1. If crypts are present increase the speed of centrifugation
9. Re-suspend liberated crypts in 1-5mL of Epithelial buffer +10µM y-27632
	1. Proceed to the appropriate organoid culture or single cell SOP

# **SOP: Epithelial Single Cell Dissociation**

Use this protocol to dissociate intact crypt or villi units into single cells for cytometry applications. Expected contaminating cell types, depending on starting material, including circulating cells with little contamination from mesenchymal cell sources.

*IMPORTANT: If cells will be plated in Matrigel or analyzed by flow cytometry, begin thawing Matrigel and preparing equipment several hours before starting dissociation, as per SOP: Plate in Matrigel and SOP: Sorting Sox9EGFP Populations.*

**Tools and Consumables**

* Large scissors
* 15mL/ 50mL Conical tubes (4 per mouse)
* MACSmix™ Tube Rotator
* Bacterial plate
* Cell strainers(optional)
	+ 100µm, 70µm, and 40µm

**Reagents**

* **Epithelial buffer**: Ice-cold
	+ + 10µM y27
	+ 10-20mL per sample
* **Epithelial buffer**: warm
	+ + 10µM y27
	+ 10-20mL per sample
		- Whole murine intestine
		- 3g of human/porcine mucosa
* **FBS**: ice cold
	+ 1mL per sample
* **Dispase**
	+ ~100uL/ sample
* **DNAseI**
	+ ~100uL/ sample
1. **Separate crypt/villi units (optional)**
	1. *Note: The purified crypt and villi samples can be isolated by size exclusion to study each component exclusively*
		1. *A significant amount of material is lost in this process*
	2. Resuspend/mix isolated crypt/villi units in a large volume of cold EB +y27
	3. Filter crypt/villi solution through a 100µm cell strainer into a clean centrifuge tube
		1. Observe flow through under phase microscopy to determine crypt purity
		2. Save filter containing villi units
	4. Filter flow through using a 70µm cell strainer into a clean centrifuge tube
		1. Observe flow through under phase microscopy to determine crypt purity
		2. Save filter containing villi units
	5. Backflush the 100µm and 70µm cell strainers into a clean centrifuge tube with cold EB +y27 to release villi units
2. **Dissociate Crypt villi units**
	* 1. Pellet isolated crypt villi units @3000g for 5 mins at 4oC
			1. Observe supernate under phase microscopy for the absence of crypt/villi units
		2. Resuspend pelleted units in 10mL warm EB +y2
			1. First, resuspend in 1mL of warm EB +y2 then transfer to the remaining 9mL of warm EB +y2
		3. Add Dispase and DNAse to resuspended crypt/villi units
			1. Keep enzymes cold until addition to the solution
		4. Incubate the digestion solution at 37oC for 4 minutes
		5. Shake digestion solution at 5 cycles/second for 30 seconds
			1. Observe a 10-20µL aliquot under phase microscopy for single cells
			2. If >70% of cells are singlets precede to quenching
		6. Incubate the digestion solution at 37oC for 2 minute
		7. Shake digestion solution at 5 cycles/second for 30 seconds
			1. Observe a 10-20µL aliquot under phase microscopy for single cells
			2. If >70% of cells are singlets precede to quenching
		8. Repeat 37oC incubation and shaking until singlets are achieved
			1. The maximum incubation time of 16 minutes
3. **Quench the dissociation reaction**
	1. When dissociation has reached >70% singlets quench the reaction using cold FBS
		1. Filter dissected cells through a 40µm cell strainer into a centrifuge tube containing 1mL of cold FBS
4. **Proceed to compatible SOP**
	1. SOP: Single Cell Dissociation
	2. SOP: Plate Crypts in Matrigel
	3. SOP: Plate Crypts on Collagen HydrogelSOP: Plating Crypts in Matrigel for Organoid Culture

# SOP: Human/Porcine Intestinal Tissue Dissociation

The final product will be intact crypt units. Expected contaminating cell types including circulating cells with little contamination from mesenchymal cell sources. Crypt units isolated with this protocol can be fixed, cultured, or further dissociated into single cells.

*IMPORTANT: If crypts will be plated in Matrigel, begin thawing Matrigel several hours before starting dissociation, as per SOP: Plate in Matrigel.*

**Tools and Consumables**

* Small forceps
* Small scissors
* 15mL/ 50mL Conical tubes (4 per mouse)
* 100mm Dish
* 20.5g needle at 10mL syringe

**Reagents**

* **Colon Dissociation Solution**
	+ 20mL per 5g sample
* **Crypt shake buffer**:
	+ 15-20mL per 5g sample

**Mucosectomy**

1. Transfer intestinal tissue to a 100mm culture dish containing 1ml cold epithelial buffer
2. Load the 20.5g needle with ~10mL cold epithelial buffer
3. Insert the needle through the mucosa into the lamina propria at an angle tangential to the mucosa surface
4. Inflate the lamina propria with EB to separate the two layers
5. Peel away the mucosa and discard the muscularis mucosa layer
6. Measure the mass of the resected mucosa
	1. Use 3-5g per sample

Epithelial dissociation

1. mince mucosa into ~1mm x 1mm segments with razor blades
2. Transfer the minced tissue to a centrifuge tube containing 20mLs colon dissociation solution
3. Incubate for 60 minutes rocking at 4oC
4. Transfer tissue segments to a centrifuge tube containing 20mLs crypt shake solution
5. Vigorously shake tissue solution for 4 minutes at 2.5 cycles per second.
	1. Observe a sample of the solution under a phase microscope after every shake for liberated crypts.
6. Proceed to the appropriate organoid culture or single-cell SOP

# SOP: Growing Confluent Differentiated Organoid Transwells

Use this protocol to grow confluent organoid monolayers from established proliferative organoid monolayers. Expect 70-80% success forming these confluent monolayers.

*IMPORTANT: Collagen hydrogel weakly binds to the PET transwell surface. Use care when manipulating these cultures are the gel is unstable and mechanical disruption of the organoid will affect confluence.*

**Tools and Consumables**

* Proliferative apical organoid culture
	+ Preferably grown according to SOP: Plating Crypts on Collagen Hydrogel for Organoid Culture in **Expansion media**
	+ Cells are plated at high density to form confluent organoid cultures
		- 100000 cells/cm2
			* Typically, 1 well of a 6-well plate is passaged to 2 permeable transwells in a 12-well plate

**Reagents**

* Collagen hydrogel scaffolds on porous transwells
	+ Prepared in different well formats following SOP: Making Hydrogel Scaffolds
* **Expansion media** (recommended)
	+ *Note: Using other media compositions may work but has not been validated using this protocol.*
* **Collagenase IV solution**
	+ Fisher NC9919937
	+ 5000units/mL in HBSS with Calcium chloride
1. **Prepare Hydrogel scaffolds for culture**
	1. Transfer collagen hydrogel scaffold transwells into a tissue culture hood
	2. Remove residual PBS from each well being used for culture
		1. Carefully remove with a sterile pipette without disturbing the collagen hydrogel in the bottom of each well
	3. Wash each well in PBS 2x
	*\*Note: Do not store collagen scaffolds for extended periods (>10mins) without PBS covering the scaffolds*
	4. Add the desired volume of Organoid Growth Media to the basal media reservoir of each transwell
2. **Digest collagen Hydrogel to release organoid fragments**
	1. Remove 75% of the culture media from each well and discard
	2. Disrupt the collagen hydrogel with a pipette tip to release it from the bottom of the tissue culture plate
		1. Hydrogel should lift off of the plate bottom when a pipette tip is run around the circumference of the well
	3. Transfer the collagen hydrogel and remaining media to a centrifuge tube
		1. Pool all wells of each sample being passaged
		2. Further disrupt hydrogels by pipetting repeatedly with a 5-10mL pipette
	4. Digest the collagen hydrogels by adding collagenase IV solution @ 10% of the pooled volume
		1. Incubate in 37oC water bath for 12 total mins
			1. Further disrupt collagen hydrogel by pipetting repeatedly with a 5-10mL pipette every 4 mins during 12 min incubation
	5. Quench collagenase activity with cold FBS
		1. Add 10% of the pooled volume in ice-cold FBS
3. **Inactivate Collagenase IV and further dissociate organoid fragments**
	1. Pellet organoid fragments following quenching @3000g for 5 mins
		1. Remove supernatant and examine a sample to ensure efficient pelleting
	2. Resuspend organoid fragments in 1-2mL DPBS +0.5µM EDTA
		1. Mix well by pipetting repeatedly
	3. Incubate in a 37oC water bath for 4 minutes
		1. Mix by flicking once during incubation
	4. Remove EDTA from the cell sample
		1. Dilute the EDTA content of the solution by adding 10mL DPBS
			1. Pellet cell fragments at 100g for 5 min
				1. Remove supernatant and examine a sample to ensure efficient pelleting
		2. Wash Cell fragments in DPBS
			1. Resuspend the crypt pellet in 1mL of DPBS
				1. Mix very well by pipetting ~25-50 times.
			2. Resuspend in an additional 9mL of DPBS
4. **Pellet cell fragments for plating**
	1. (Optional) Quantify the cellular content of the diluted cell solution
		1. Remove 3 separate 5 µL aliquots of the filtered crypt solution and examine by phase microscopy
			1. Quantify the average crypt contents of the aliquots and extrapolate to determine the total crypt yield
	2. Mix the cell fragment solution by flicking and
	3. Transfer an adequate volume to a microcentifuge tube
		1. Volume is determined by the plate size and number of wells
			1. Transfer 100000 cells/cm2 of plating surface
	4. Centrifuge on benchtop centrifuge at 4oC at 3000g for 5 minutes
	5. Remove supernatant and examine an aliquot by phase microscopy to ensure cells are pelleted efficiently
5. **Plate Crypts on Collagen Hydrogel**
	1. Re-suspend pellet directly into the appropriate volume of Organoid Growth media for the apical compartment f each transwell to be plated
		* 1. 500µL per 12-well transwell
		1. First, resuspend the crypt pellet in 1mL of Organoid Growth media
			1. Mix very well by pipetting ~25-50 times.
		2. Add the remaining culture media volume to the cell fragment solution
	2. Plate by adding organoid fragments to apical media compartment of each transwell to be plates
	3. Transfer into a standard tissue culture incubator
6. **Grow organoid fragments to a confluence and differentiate**
	1. Change the organoid growth media in both compartments every 48 hours with fresh, warmed **Expansion Media** until a confluent organoid monolayer is formed
		1. Typically, 48-72hr post-plating
		2. Confluence can be estimated visually or measured by transepithelial electrical resistance
	2. Once confluence is reached, change the organoid growth media in both compartments with fresh, warmed **Differentiation Media**
		1. *Note: The DM described above will differentiate the majority of organoid cells toward absorptive lineages. Other media formulations may be more applicable to studies focusing on other post-mitotic cell types*
	3. Change the organoid media in both compartments 24 hours later with fresh, warmed **Differentiation Media** to remove residual growth factors from the system
		1. *Note: Hydrogels typically retain media and may release growth factors from the expansion phase of culture into the differentiation media*
	4. Incubate organoids in **Differentiation Media** an additional 24 hours before use in transport assays

# SOP: Human/Porcine Sub-epithelial Cell Preparation

 Use this protocol to isolate sub-epithelial cell populations from full-thickness large animal tissues.

*IMPORTANT: Collagen hydrogel weakly binds to the PET transwell surface. Use care when manipulating these cultures are the gel is unstable and mechanical disruption of the organoid will affect confluence.*

**Tools and Consumables**

* 2cm segment of full-thickness intestine from human or porcine
* Razer blades
* Epithelial buffer
* Collagenase IV (5mL)
* 5mL syringe with 30.5g
1. **(Optional) Preform Mucosectomy to Separate Tissue Layers**
	1. Fill multiple 5mL syringes with ice-cold EB and attach 30.5g needles
	2. Access the submucosal space by inserting the needle nearly parallel to the tissue
		1. Do not penetrate through the entire thickness of the tissue
	3. Inflate the submucosa space with cold EB by injecting EB through all syringes
	4. Cut and pull the mucosa and submucosa layers apart through the inflated submucosa space.
2. **Mince tissue into 2mm segments**
	1. Transfer the tissue layer of interest (generally mucosa) to a petri dish on ice.
	2. Mince the tissue into 2mm2 segments using sterile razor blades.
3. Enzymatically digest tissue to release cells
	1. Transfer tissue segments to a 15-mL centrifuge tube containing 5mL of warm Collagenase IV solution
	2. Incubate the tissue segments at 37oC while rotating for 40 minutes
		1. Mechanically disrupt the tissue fragments every 10 minutes by pipetting the solution up and down 20x with a 10mL strip pipette
4. **Quench dissociation and purify isolated cells**
	1. Filter solution through a 100um cell strained into 1mL of cold FBS
	2. Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.
	3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
		1. The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size
	4. Back flush filter to retrieve tissue fragments for additional digestion.

# SOP: Plating Single Cells in Matrigel for Organoid Culture

This protocol is adapted from the Matrigel product sheet and associated literature. Read the Matrigel product sheet for more detailed handling instructions.

**Tools and Consumables**

* Clinical centrifuge in a cold room
* (Optional) cold pipette tips
* Conical centrifuge tube
* Tissue culture plate
* (Optional) 100 µm cell strainer
* (Optional) 70 µm cell strainer

**Reagents**

* Growth factor-reduced Matrigel
* Stem Cell Growth Media (several options)
	+ **IESC media (SCM)** (recommended)
	+ *Note: Many different media combinations have been used to grow organoids from crypts. The original media formulations required expensive recombinant growth factors. Refer to the Media Formulation sheet for additional recipes. Current protocols replace Noggin, R-spondin, and/or Wnt3 growth factors with conditioned media made from L-cells that produce and secrete these factors into culture media.*
		- IntestiCult™ Organoid Growth Medium (Mouse)
			* Stem Cell Technologies cat: 06005
			* Add supplement A and supplement B
		- 50% L-WRN conditioned media
			* Produced according to <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/>
		- Antibiotic supplement (several options)
			* Pen/Strep, Gibco: 15140-122
			* Primocin, InvivoGen: ant-pm-1
1. **Pellet single cells for plating**
	1. Remove 3 separate 5 µL aliquots of the filtered single-cell solution and examine by phase microscopy
		1. Quantify the average cell contents of the aliquots and extrapolate to determine the total cell yield
	2. Mix the filtered single-cell solution by flicking
	3. Transfer an adequate volume to a microcentifuge tube
		1. Volume is determined by the amount of Matrigel being utilized
			1. Transfer 25-100 single cells/1 µL Matrigel for plating
		2. Transfer to 1.5 mL centrifuge tube
	4. Centrifuge on benchtop centrifuge at 2000g for 5 minutes at 4oC
	5. Remove supernatant and examine an aliquot by phase microscopy to ensure crypts are pelleted efficiently
2. **Embed in Matrigel**
	1. Centrifuge Matrigel at max speed for 15-30 sec in benchtop centrifuge in a cold room.
		1. This will pellet any insoluble collagens that may be in the Matrigel.
		2. The amount of insoluble material varies by Matrigel lot.
	2. Re-suspend pellet directly into the appropriate volume of Matrigel. Mix very well by pipetting ~25-50 times.
	*\*Note: Cells and crypts may be plated in up to a 1:1 dilution in Matrigel.*
		1. Use pre-chilled tubes and tips.
		*\*Optional: Perform pipetting steps on ice.*
		2. When pipetting Matrigel from aliquot, pipet up and down one time to coat the inside of the tip. Or, push the pipette plunger slightly past the stop so that no bubbles will be added to the Matrigel when depressing the plunger
		3. DO NOT introduce bubbles into the Matrigel.
	3. Plate by adding Matrigel as a hemispheric ‘dome’ to the center of each well in a pre-warmed plate
		1. For 48 well plate, use 10-50 uL Matrigel
		2. For 96 well plate, use 3-10 µL Matrigel
		*\*Note: Place pipette tip on the bottom of the middle of the well, lift slightly, then depress to first stop to plate Matrigel in hemispheric droplet*
	4. Carefully transfer the plate to a 37°C incubator and allow the Matrigel to polymerize for 30 minutes
	*\*Optional: invert the plate to prevent contact with the plate.*
3. **Overlay Media**
	1. Remove the plate from the incubator
	2. Add 5-20x Matrigel volume of desired Culture Media
		1. Continue to change culture media on an appropriate schedule
			1. Typically, every 2-3 days
	3. Transfer into a standard tissue culture incubator
		1. Organoids are ready for passage after 6-12 days or when the lumens begin to burst and expel contents into the Matrigel

# SOP: FACs Sorting Lgr5EGFPHi Small Intestine Cells