Detailed protocol for intestinal culture.

1. **Preparation of collagen gel matrix (Cellmatrix type I-A, Nitta Gelatin Inc.).** Mix the following solutions and keep on ice until use: A, Cellmatrix I-A; B, 10 X concentrated sterile culture medium (Ham's F-12); C, sterile reconstitution buffer (2.2g $NaHCO_3$ in 100ml of 0.05N NaOH and 200mM HEPES). Mix ice-cold A, B, and C at a ratio 8:1:1. The order of mixing is very important. After mixing A and B well, add C and mix well again. Do not make bubbles through the procedure. Hereafter, this mixture is called the reconstituted collagen solution. Keep the reconstituted collagen solution on ice (4°C) to prevent gel formation.
2. **Creation of the acellular bottom layer in the inner dish**. This culture system maintains the cultured cells embedded in the collagen gel under an air-liquid interface environment. The following procedure is done using Cellmatrix type I-A (Nitta Gelatin Inc.) as type I collagen gel, however, other products are able to use as an extracellular matrix, such as matrigel. The inner dish should have permeable or pored membrane bottom, such as a cell culture insert. We typically use Millicell culture plate inserts (PICM03050, Millicell-CM, Millipore) or Falcon cell culture inserts (BD) as the inner dish. All the following material scale/volume are variable and should be selected in accordance with the intended use. Before preparing the tissue, an inner dish with collagen gel bottom layer should be made. Pour 1 ml of reconstituted collagen solution described above into a 30-mm diameter inner dish in the tissue culture hood and allow to solidify (30 min) either in the hood or in a 37°C incubator. The inner dish is ready to use after the gel solidifies (see below).
3. Small or large intestine is removed from neonatal mice, with aseptic procedure. Postnatal

day 0–2 intestine grows most vigorously, although we have had success with samples from small and large intestine up to 26 weeks of age. For adult tissues we recommend treatment with recombinant R-spondin1 (see below).

4. The removed tissue (typically 1 cm) is immediately immersed in ice-cold PBS or other culture media/tissue preservative solution such as Ham's F12 medium without serum.
5. The small intestine or colon are opened lengthwise and washed in ice-cold PBS (or other solution mentioned above) to remove all luminal contents.
6. The washed tissue is minced by iris scissors etc. on ice-cold plate such as a tissue culture plate lid. The final minced tissue has heterogenous size, but under 0.3 mm^3 is suitable for culture. The tissue should be minced extensively so as to have an almost viscous and homegenous appearance. This mincing procedure should not exceed 5 minutes to avoid cell damage and drying the tissue.
7. **Creation of the cell-containing top layer.** The minced tissue is mixed by pipetting in ice-cold, reconstituted collagen solution (1.0 ml per dish to be prepared). Pour 1.0 ml of the cell-containing collagen gel onto the inner dish prepared in step 2. The now-completed inner dish will now have a total volume of 2.0 ml and is then placed in a new empty 60 mm outer dish. After covering the 60 mm dish, the gel of the inner dish solidifies to gel in a 37°C incubator within 30 minutes.
8. After solidifying the cell-containing gel, 1.0 ml culture media (Ham's F12 supplemented with 20% fetal calf serum and 50 $\mu g\ ml^{-1}$ gentamicin) is poured into the outer dish. At this point, the cultured cells should not be immersed in culture media. The cellular gel layer should exist above the medium level to create the air-liquid interface microenvironment. Variable substances such as protein (i.e. R-spondin1-Fc, 500 $ng\ ml^{-1}$) or test compounds can

be added in the culture media.

9. The culture assembly is carried out over 30 to > 350 d at 37 °C in a humidified atmosphere of 5% CO_2 in air. Medium is changed every 7 d, but the frequency may depend on cell numbers and if labile test growth factors are being added.
10. Living culture cells can be observed by phase-contrast microscopy or stereo microscopy.
11. For histological analysis, the culture assembly can be fixed with variable solutions such as 4% PFA and embedded in paraffin. Deparaffinized cross sections can be stained with variable staining methods such as hematoxylin and eosin. Deparaffinized sections are able to be use for immunohistochemistry for variable antibodies. Generation of frozen sections is also possible.
12. For ultrastructural analysis by transmission electron microscopy, the culture assembly can be fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated with alcohol, and embedded in epoxy resin.

Technical comments for intestinal culture.

- Culture growth was most prolonged with cultures from postnatal d 0–2, and this would be an optimal starting point for researchers just familiarizing themselves with this technique. We have been able to culture small and large intestine from mice up to 26 weeks of age although success of culture appears to decline with age.

- The air-liquid interface was absolutely essential for growth in this system and thus it is important that the level of the medium in the outer dish (which equilibrates within the collagen

gel in the inner dish) be low enough as to permit the upper layer of the collagen gel to be exposed to air without concomitant culture medium.

- Extensive and rapid tissue mincing is critical in our experience. All tissue handling should be performed on ice. The mincing should be performed as soon as possible after harvesting and should be done at varying angles and to a degree where the tissue appears viscous and homogenous. This step is dependent on the operator and on the amount of starting material.

- In our experience, the two most common causes for the inability to culture neonatal tissue are (1) Too much delay between the initial tissue harvest and the subsequent mincing and embedding in collagen. Speed is of critical importance. (2) Mincing is insufficient and tissue fragments are too large.

- The duration of successful culture in our experience is consistently >30 d using P0-P2 tissue. Approximately 50% of our cultures continue to grow for >100 d. The cultures should form cysts around day 7 and enlarge thereafter. From our experience teaching this technique to other researchers, culture durations of 4–10 weeks should be routinely achievable by those new to the method.

- Culture growth and yield of progenitor cells can be stimulated by inclusion of R-spondin1-Fc (500 ng ml^{-1}). This expression construct is freely available from our laboratory.