# 3D intestinal organoids in metabolic research: Virtual reality in a dish.

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**Short Title**

**Intestinal organoids in metabolic research**

**Highlights**

- **Organoids can be generated from adult stem cells or iPSCs.**
- **Organoids exhibit all intestinal epithelial cell types including functional EECs.**
- **Organoids can be generated from patient specific biopsies.**
- **Organoids are amenable to numerous molecular and genetic techniques.**
- **Current organoid usage in transdifferentiation, cell fate and function of EECs.**

**Abstract**

The advent of near physiological organoid technology has produced a step change in our understanding of stem cells and has provided the research community with a powerful new cell based tool to model human physiology and disease. We review the pros and cons of intestinal organoid culture systems. The molecular and genetic tools to manipulate them and how they are being used to answer fundamental questions in metabolic research, including the function of enteroendocrine cells in health and disease.

**Introduction**

The enteroendocrine system is a key player in the regulation of metabolism. It controls how the body responds to a meal and fine tunes a large number of physiological responses to ensure optimal fuel absorption, use and storage [1]. This is achieved by enteroendocrine cells, which have the capacity to integrate complex nutrient signals and to respond by releasing peptide hormones which communicate information regarding the current state of energy balance to the brain and other tissues [2] [3]. Gut hormones exhibit actions ranging from the local control of gut motility and secretion to the regulation of appetite, energy expenditure and glucose homeostasis[4]. These features have made the enteroendocrine system an excellent target for the development of anti-obesity and anti-diabetic therapies and related metabolic disorders[5]. Producing successful therapeutics will hinge on understanding the regional roles and functions of different endocrine cell types under both physiological and pathophysiological conditions.

**Modelling gut endocrine cells**

Enteroendocrine cells (EECs) are scattered throughout the length of the gastrointestinal epithelium [6,7]. This feature has made them uniquely difficult to study in comparison to other metabolically relevant cell types which are often found in large numbers grouped together. As such, *in vitro* models that faithfully reproduce enteroendocrine physiology have proven difficult to develop. EECs have historically been modelled using cell lines and explanted tissues, both of which have limitations [8]. Cell lines are transformed, raising questions regarding their ability to mimic physiology. Indeed, the two rodent cell lines most commonly used in enteroendocrine research, STC-1 and GLUTag cells, and the only available human-derived cell line, NCI-H716, all have limitations in this regard and therefore lack some

predictive power[9]. Additionally, traditional 2D clonal cultures lack a normal cellular environment and cannot reproduce the integrative physiological interplay present *in vivo*. On the other hand, explant cultures exhibit organotypic features such as a 3D structure and cellular heterogeneity and offer the opportunity to investigate physiologically relevant primary cells [10]. However, their use is limited by their short-term nature, the need for multiple tissue donors and the relative difficulty in direct genetic manipulation. The recent development of *in vitro* organoid culture systems offers the opportunity to address these limitations. We review the different methodologies for gut organoid culture, the tools available for their manipulation and how these are being used to answer fundamental questions of gastrointestinal physiology in relation to EEC's function and metabolic disease.

**Organoids derived from resident adult tissue stem cells (Figure 1)**

In 2009, Clevers and colleagues established a culture system which allowed the long-term growth and expansion of intestinal epithelia from either purified intestinal crypts or single Lgr5+ stem cells, often called mini-guts or enteroids [11,12]. This was made possible by the identification of critical components of the intestinal stem cell niche. Enteroids are grown in 3D, in laminin-rich Matrigel, with a defined set of niche factors including epidermal growth factor (EGF), noggin and R-spondin [13]. Within days, crypts or single stem cells form cystic single-cell epithelial structures with a central lumen. These then form crypt-like budding structures which ultimately organize into multiple discrete budding crypts, harbouring intercalated stem and Paneth cells at their base and associated villus-like regions comprising the various differentiated cell lineages, absorptive enterocytes, secretory goblet and EECs [11,14]. These cultures have been grown and passaged (every 5-7 days) continuously for over 2 years in various laboratories, without exhibiting chromosome rearrangement [15]. They exhibit normal polarisation, where the apical membrane has microvilli and faces the central lumen, whilst the basolateral side faces outward [16]. They can be generated from any segment of the gut; duodenum, jejunum, ileum and colon. Interestingly, enteroids retain their regional identity in culture. Small intestinal enteroids can be grown in the minimally effective media described above, likely due to the sufficient production of Wnt3A required for stem cell self-renewal by their resident Paneth cells [17]. This is not the case for mouse colonoids or for human enteroids and colonoids, which all require exogenous Wnt3A for their long-term maintenance [14]. In the presence of Wnt3A these organoids more closely resemble the cells

of the crypt and are therefore in a relatively non-differentiated state. Removal of Wnt3A inhibits stem cell proliferation, allowing a more differentiated state to be achieved, including increases in the number of EECs [17]. Further manipulation of cell fate can be achieved by the addition of specific factors to the media which inhibit Wnt or Notch signalling or various combinations thereof [18]. A paradigm for manipulating the endocrine population has recently been described. Inhibition of Wnt and Notch signalling combined with either reduced EGFR signalling or inhibition of Mitogen-associated protein kinase (MAPK) signalling produced large numbers of enteroendocrine cells with high purity [19]. These endocrine cells demonstrated heterogeneity and region specificity like their endogenous counter parts. Manipulation of cell fate in enteroids provides an impressive degree of flexibility and the ability to increase the number of EECs will make their study more accessible. The main advantages of using adult stem cell-derived enteroids include segment specification, ease of genetic manipulation, simplicity and speed of generation.

**Organoids generated from pluripotent stem cells (Figure 1).**

Human intestinal organoids (HIOs) are generated from two different sources, iPSCs or embryonic stem cells. The conversion of stem cells into HIOs was pioneered in a series of publications by James Wells and colleagues who described a directed differentiation protocol that mimics stages of *in vivo* intestinal differentiation [20,21]. In this protocol, 2D cultures of iPSCs are differentiated into definitive endoderm using low serum media and activin A. Subsequent specification to hind gut endoderm is achieved by the addition of Wnt3A and FGF4 which also spontaneously transforms the endoderm into 3D spheroids [22,23]. Culture of these spheroids in Matrigel in the presence of EGF, noggin and R-spondin-1 results in their growth and eventual differentiation into HIOs. HIOs' exhibit Lgr5+ crypt-like domains, inter-villus regions, villus-like projections and microvilli brush borders. Architecturally, the epithelium contains all known intestinal cell types including enterocytes, goblet cells, Paneth cells and chromogranin A positive endocrine cells. In contrast to organoids derived from adult stem cells, HIOs also contain a laminated mesenchyme with myofibroblasts, circular and longitudinal smooth muscle and endothelial cells [24]. Functionally, like their adult stem cell derived counter parts, HIOs demonstrate some absorptive and secretory functions and can be passaged for over 1 year [21]. Despite their morphological similarities to endogenous tissue, HIOs are considered foetal in nature, and unlike enteroids do not reproduce segment

specificity, but form a mixed population of cells exhibiting proximal and distal intestinal cell signatures [21]. These short comings have been overcome, but at the cost of a more complex protocol. Engraftment of HIOs under the kidney capsule in mice induces their vascularisation by host endothelial cells and after 6 weeks produces adult intestinal units between 1 and 2 cm in size [24]. The resulting tissue has mature structure and function, exhibits highly differentiated adult epithelial cell types and maintains a tendency toward a small intestinal identity. Further regional specificity has been achieved by using a short induction of BMP signalling during the directed differentiation protocol, which induces colonic patterning [25]. *In vitro* human colonic organoids (HCOs) express colonic markers and contain colonic specific cell types. *In vivo* maturation of HCOs produces tissue that has molecular, cellular and morphological properties of the human colon [25]. Importantly, with respect to metabolic research, HIOs and HCOs each have a distinct set of EECs that mirror those found in the adult small intestine and colon respectively.

**Tools**

Beyond their obvious advantage of representing a more physiological long term model of the gut, organoids offer a multitude of other opportunities. Two key features which underpin their power are the ability to culture human patient-derived organoids, which offers great potential in translational and personalised medicine, and their genetic and molecular tractability [26,27]. For example, numerous established molecular techniques such as genetic manipulation technologies [28,29], mass spectrometry, flow cytometry[19], single-cell RNA sequencing [30], high resolution and high content microscopy [31] can all be brought to bear on the organoid platform (figure 2).

There are several options for using and generating genetically manipulated organoids. The simplest being the culture of organoids from transgenic and knockout mice which does not require the maintenance of a colony, only the transfer of donor tissue or frozen organoid cultures between labs, making primary genetically altered material very accessible. Novel genetic manipulations can be introduced most simply by using gymnosis, a process which introduces locked nucleic acid (LNA) antisense oligonucleotides in the absence of carriers or conjugation, to produce sequence specific gene silencing [32]. Reagents can be applied directly into the culture medium and/or Matrigel to achieve targeted gene knockdown. This is in contrast to the techniques described below which require dispersing organoids or

manipulation of iPSCs before differentiation into organoids. Viral vectors can be used to easily introduce stable genetic alterations. There are published protocols for standard retroviral transduction [33] and an elegant inducible model which allows temporal control of gene expression [34]. More recently, the need for viral packaging has been removed by combining electroporation and transposon based systems such as PiggyBAC, making the process of genetic manipulation safer, cheaper and more efficient [35]. Bacterial artificial chromosome (BAC) payloads are large, enabling whole gene loci to be carried, which can be used to direct precise endogenous transgene expression patterns [36]. BAC transgenesis has been applied to produce fluorescent reporter organoids without the need to generate modified mice. CRISPR technology has been shown to work well in epithelial organoids [28,37]. Currently there are few published studies, none of which focus on metabolism or EEC function. However, some of the possibilities are highlighted by a study in which cystic fibrosis patient-derived enteroids had their CFTR gene defect corrected using CRISPR technology [38]. CRISPR technology can also be combined with HIOs by manipulating iPSCs before differentiation into organoids [39]. Apart from CRISPR and BAC technologies, the techniques described above result in global alterations, potentially a major drawback when studying individually scattered EECs. However, combinations of techniques can be applied to specifically target EECs. For example, we have combined cre recombinase inducible genes of interest and the PiggyBAC transposon system, with gut hormone cre driver lines to access specific endocrine cell populations (Figure 2). This suite of tools provides endless opportunities for the modification of intestinal organoids, and researchers will only be limited by their imagination and the questions they wish to answer.

**Considerations for organoid use.**

There are several general shortcomings for intestinal organoids. They lack essential components of the living digestive tract, such as the enteric nervous system, the vascular system, lymphatic system and functional adaptive and innate immune systems. *In vivo* maturation of HIOs induces some vascularisation albeit host derived [24], whilst other protocols are emerging which attempt to add the missing components. For example, co-culture techniques, allow the addition of specific immune cells into the culture, but only in the short term [40,41]. Furthermore, the complexity of the HIO model has recently been

increased to include a functioning enteric nervous system by incorporating human iPSC-derived neural crest cells into the differentiation protocol[42].

However, maturity is an issue with both HIOs and enteroids. *In vitro* enteroids are relatively more mature than HIOs but are not as mature as HIOs engrafted and matured *in vivo*. The later representing the model which most closely resembles adult tissue but with the disadvantage of being complex and time consuming to generate; 35 days *in vitro* and 6 weeks engraftment.

Other general disadvantages include inconsistencies within each culture and access to the luminal side of the epithelium. Organoid 3D architecture is not regular, the crypt-villus structures are variable in size and shape from one organoid to another, as is the degree of differentiation. Luminal access is restricted, which has implications for microbiome and nutritional research, and requires microinjection of agents of interest into the lumen of each individual organoid, this is time consuming and technically challenging [43]. Luminal access can be achieved by fragmenting organoids and growing them on semi-permeable supports enabling controlled access to both apical and basolateral surfaces. In 2D, organoid monolayers exhibit all major cell types of the epithelium. This model allows apical exposure to microbiota, pathogens and nutrients, for example, and may offer a more reproducible paradigm for measuring gut hormone release compared to whole organoid preparations [44].

**Intestinal organoid use in metabolic research.**

Relatively little is known about the regulatory networks which specify the identity of terminal differentiated EECs, particularly in humans. Organoids offer the opportunity to unpick the fine details of this process. Genetic manipulation of *Neurog3* in HIOs has corroborated data from mouse studies [21,45]. Viral overexpression of NEUROG3 increases EECs number whilst partial knockdown using lentiviral delivered shRNAs dramatically reduces the number of EECs [21]. Similarly, knockdown of ARX reduces the number of CKK and secretin cells just as its targeted deletion does in mice [46]. These studies demonstrate the utility of organoids for exploring the regulation of human EEC specification.

An increasingly important feature of EECs is their plasticity. For example, until recently it was thought the magnitude of the gut hormone response to nutrients was determined solely by the cells capacity to produce and release these hormones. Increasing evidence

suggests that the plasticity of the gut epithelium can be hijacked to alter the density and characteristics of EECs to augment the anorectic potential and/or the incretin response of the gut and improve metabolic health. A number of recent studies highlight the use of organoids in this area. Comparison of the microRNA repertoire of endocrine cells resident in the crypt versus villus regions of the duodenum, identified a handful of targets which were upregulated in both compared to non-endocrine cells. Of these, mir-375 was found to be a key regulator of EEC fate in mouse enteroids [47]. Prebiotics and their fermentation products, short chain fatty acids (SCFA), have been thought to alter transcription factors involved in the regulation EEC fate. Exposure of both mouse and human enteroids to SCFAs increases the expression of key transcription factors and increases the density of GLP-1 producing cells [48]. We recently found the anorectic potential of the gut may be determined by the density of PYY producing cells and this could be harnessed by supplementing the diet with a prebiotic. We used mouse enteroids to explore the mechanism and found the density of PYY cells in enteroids increased in response to a free fatty acid receptor 2 agonist, one of the endogenous receptors for SCFAs [49]. An elegant study by Petersen *et al.* provides the best proof-of concept evidence demonstrating the utility of gut remodelling in the treatment of metabolic disease. Inhibition of notch by dibenzazepine increases the density of EECs including those expressing GLP-1 in both mouse and human enteroids [50]. Furthermore, in a model of diabetes, notch inhibition augmented GLP-1 cell density, the incretin response and improved glucose homeostasis [50].

Both HIOs and enteroids have also been used to investigate the transdifferentiation of EECs into insulin-producing cells. The gut is a particularly attractive source of cells for the treatment of insulin dependent diabetes given the paucity of islet donor tissue. Domenico Accili's lab demonstrated that *Foxo1* ablation in mice, increased the NEUROG3 positive endocrine progenitor pool and caused the appearance of functionally responsive insulin producing cells. Inhibition of FOXO1 expression using a dominant negative mutant or lentiviral encoded shRNA produced functional insulin positive EECs in HIOs [51]. When transplanted under the skin they survived but did not produce detectable concentrations of c-peptide, a surrogate marker of insulin secretion, likely due to the low number of insulin cells. A second study by Zhou and colleagues reprogrammed stomach antrum EECs to produce insulin, by expressing the insulin reprogramming factors NPM (Neurog3, Pdx-1, MafA) specifically in Neurog3 positive endocrine cells. Gastroids derived from these mice produced insulin and when transplanted under the kidney capsule rescued STZ-induced diabetes [52].

In addition to cell fate decisions, intestinal organoids offer the ability to investigate the basic functional characteristics of EECs in a more physiological setting. Small intestinal mouse enteroids enable concurrent investigations of nutrient and drug transport, nutrient sensing and hormone secretion as well as fluorescent live-cell imaging of intracellular signalling processes, making them the new *in vitro* gold standard [31,53]. The transformative power of organoids will be most evident when genetic modifications are combined with functional studies in human derived intestinal organoids to investigate EEC physiology.

**Conclusions**

Intestinal organoids provide an unprecedented opportunity to understand the physiology and pathophysiology of EECs. This will enable us to begin to answer the many remaining fundamental questions regarding EEC specification, the triggers for hormone release and how diet, the microbiome and disease states feedback to alter specification of and secretion from EECs. Studying organoids harvested from patients with metabolic diseases or before and after weight loss surgery will be particularly powerful. Moreover, the organoid platform lends itself to high throughput screening [54], particularly for visual readouts, which will aid metabolic drug discovery programmes aimed at targeting the EEC system. Finally, it is easy to envisage the future combination of organoids derived from different metabolically relevant tissues on a microfluidics chip to model obesity and diabetes [55]. The future looks brighter in 3D.

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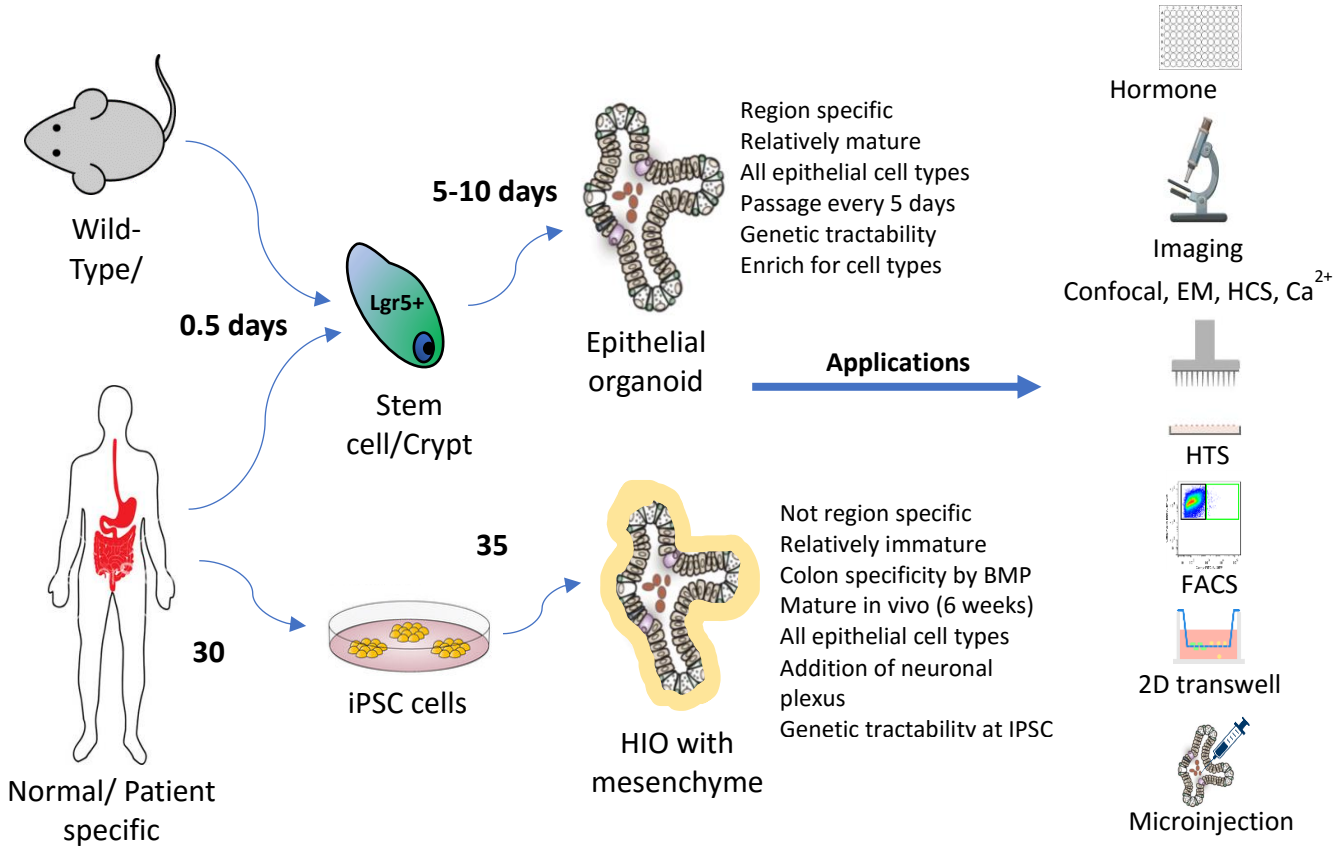


**Figure 1: Comparison of gut organoid methodology and examples of applications.**
Two main types of gut organoid can be generated, epithelial only or those also including a mesenchyme. Epithelial only organoids can be generated from mouse or human biopsies and grown from single Lgr5+ stem cells or from disassociated crypts. The process is quick and requires specific niche factors. These enteroids are region specific and can exhibit all epithelial cell types. Human organoids derived from iPSC's are comparatively slower to produce, lack region specificity and are relatively immature. However, modifications to the protocol allow colonic specification the addition of a nervous system and in vivo maturation under the kidney capsule, which represents the most adult like model. Both types of organoids can be utilised in many downstream methods as shown (this list is not exhaustive). HTS- high throughput screening, HCS – high content screening, EM- electron microscopy, FACS- fluorescence activated cell sorting

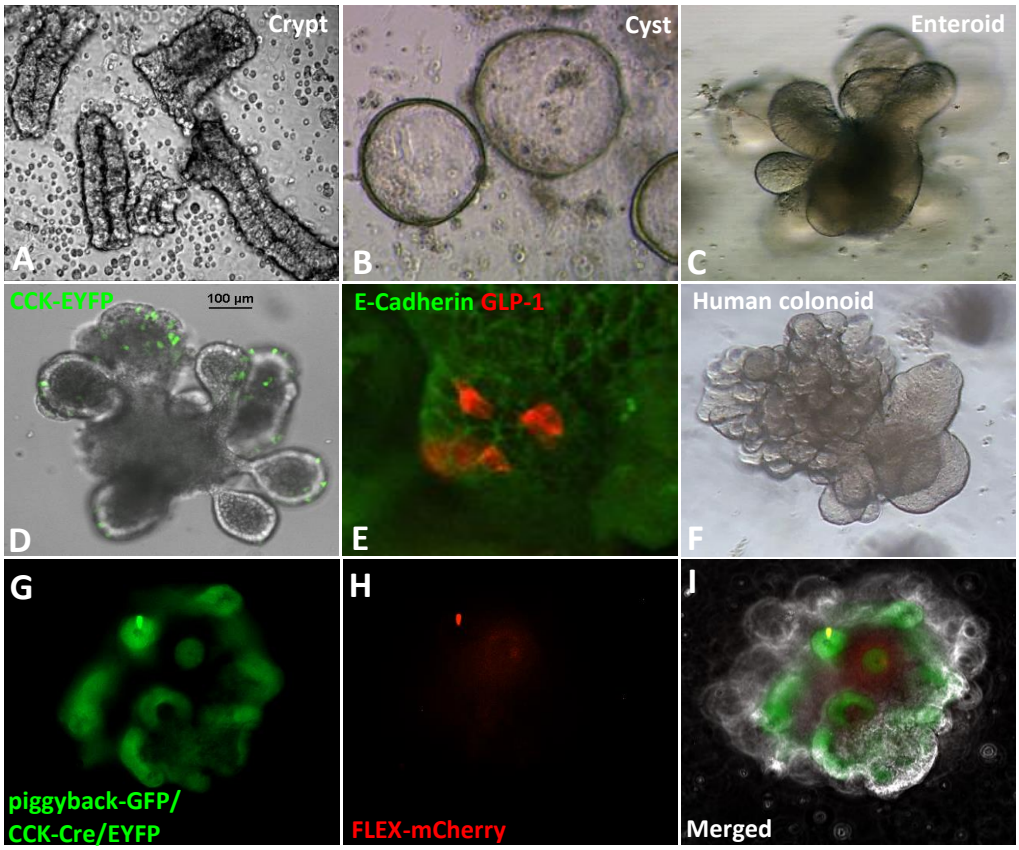


**Figure 2: Examples of Gut epithelial enteroids derived from mouse or human biopsies. A,** Crypts directly following isolation from mouse small intestine. **B,** in 24-48 hours post isolation crypts form spheroid cysts. **C**, by days 5 -7 enteroids form budding crypt like structures around a central lumen reminiscent of adult gut structure. **D**, duodenal enteroid derived from CCK-cre/EYFP. **E**, whole mount wild-type organoid stained for glucagon like peptide-1 (red) and E-Cadherin (green). **E,** example of a day 12 colonoid derived from human gut biopsy. **G-J,** example demonstrating genetic access to EEC cells. A piggyback transposon harbouring a cre dependent FLEX switch for mCherry and a EF1alpha driven GFP construct to identify transgenic organoids was introduced into stem cells from CCK-cre/EYFP mice. This resulted in mCherry expression in CKK cells which co-localised with EYFP, and ubiquitously expressed GFP from the EF1alpha construct.

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