3: Methods modification

Collaboration note:

I am very grateful to David Goulding for his help in developing and trialling the EM methods for imaging of the CL3 pathogens.

Some of the methods described in this chapter have been published as: “Using Human Induced Pluripotent Stem Cell-derived Intestinal Organoids to Study and Modify Epithelial Cell Protection Against Salmonella and Other Pathogens” (Lees et al. 2019).

A number of methods used in this study were modified from existing procedures in order to increase efficiency, or to attempt new methods of investigating infections in the iHO system. New methods employed in this study are outlined below.

3.1 Growth and differentiation of iHO

The method outlined in 2.1.2 streamlines the differentiation process of hiPSCs compared to previously published work.[1] Previously used methods required the transfer of hiPSCs from other hiPSC culture systems (e.g., feeder-dependent hiPSC culture) to chemically-defined medium–polyvinyl alcohol (CDM-PVA). This transfer to CDM-PVA typically took 2–3 weeks and required daily feeding of the hiPSCs. This protocol was also not consistently effective, with some differentiations failing; therefore, this study trialed differentiation using the same growth factors but starting with hiPSCs grown in Essential 8 Flex medium (Gibco) on Vitronectin XF (Stem Cell Technologies) coated plates, rather than in CDM-PVA on gelatin/MEF-coated plates, as well as replacement of CDM-PVA with Essential 8 Flex medium during differentiation days 0–3. This was successful for the five independent hiPSC lines trialed thus far, making the differentiation process much more rapid and efficient. This also allowed weekend-free culturing of hiPSCs prior to differentiation, allowing more flexibility with the hiPSC culture. iHO lines produced by this method were phenotyped via light microscopy (**Figure 3.1**), qPCR for cell markers (**Figure 3.2**), immunostaining (**Figure 3.3**) and

TEM as described in 2.1.5 and appeared phenotypically indistinguishable from iHO produced using the previous protocol, with markers for differentiated IECs being clearly demonstrable via RT-qPCR and imaging following embedding and passage of iHO.

In addition, following seeding, iHO usually require at least 4 weeks of routine passaging, with splitting every 4–7 days to facilitate maturation and clearing of contaminating cells from the culture. There is always a degree of variation in the speed of iHO development depending on the iPSC line used and the density of the initial culture. During the first few passages, there were visibly contaminating cells which were not iHO, which eventually died, leaving a clean culture of spherical and, after approximately 4-6 weeks, budded iHO. For recent differentiations, the use of an in-hood imaging system (EVOS XL; Thermo-Fisher) allowed acceleration of the cleaning of the culture, as it was possible to select iHO with the desired morphology and remove them from the contaminating material. These combined modifications have reduced the time required to differentiate and mature iHO from 3 months to 4-6 weeks in total.

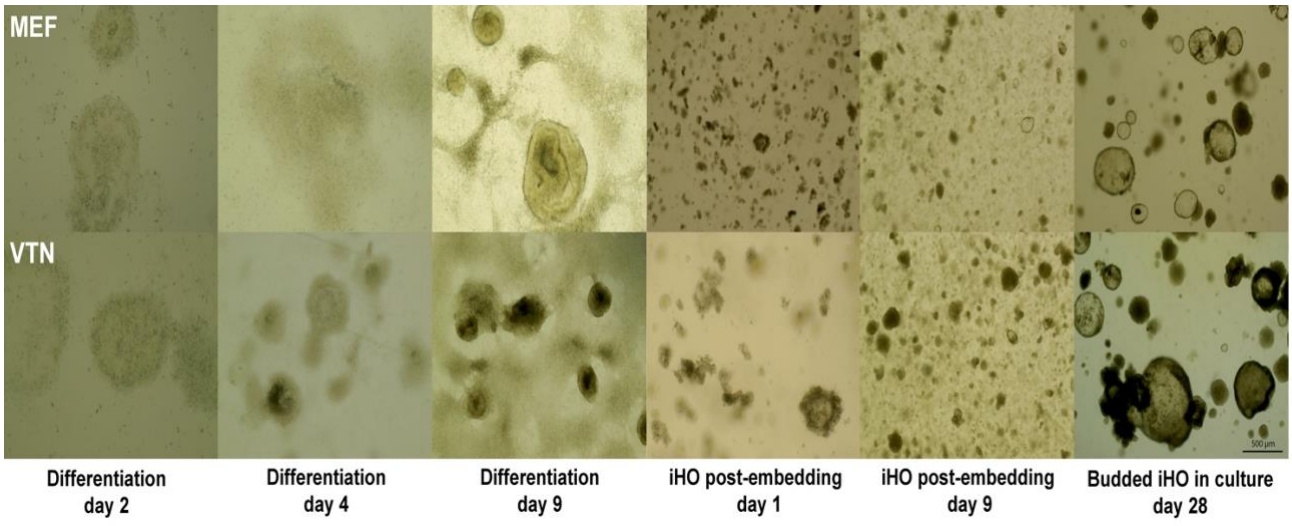

Figure 3.1 - Comparative differentiation and embedding of iHO lines using new method (Sojd2 hiPSC line as example). Cytokine supplementation during differentiation was as described in 2.1.2, however Essential 8 Flex medium (Gibco) was used as a base on days 0-3 rather than CDM-PVA. Plates were coated either in Vitronectin XF (VTN), or gelatin/MEF media (MEF). For gelatin/MEF coating, plates were incubated at 37°C for 30 minutes in gelatin (1mg/ mL gelatin in water for embryo transfer; Sigma-Aldrich), followed by 24 hours incubation with MEF media (Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), 10% foetal bovine serum (Biosera), 1% 200 mM L-glutamine (Invitrogen), and 1% penicillin-streptomycin (10,000 U ml^{-1} ; Invitrogen). No macroscopic differences between iHO were seen with use of alternative media / plate coating. Images taken on Thermo-Fisher EVOS XL imaging system at 4x / 10x magnification.

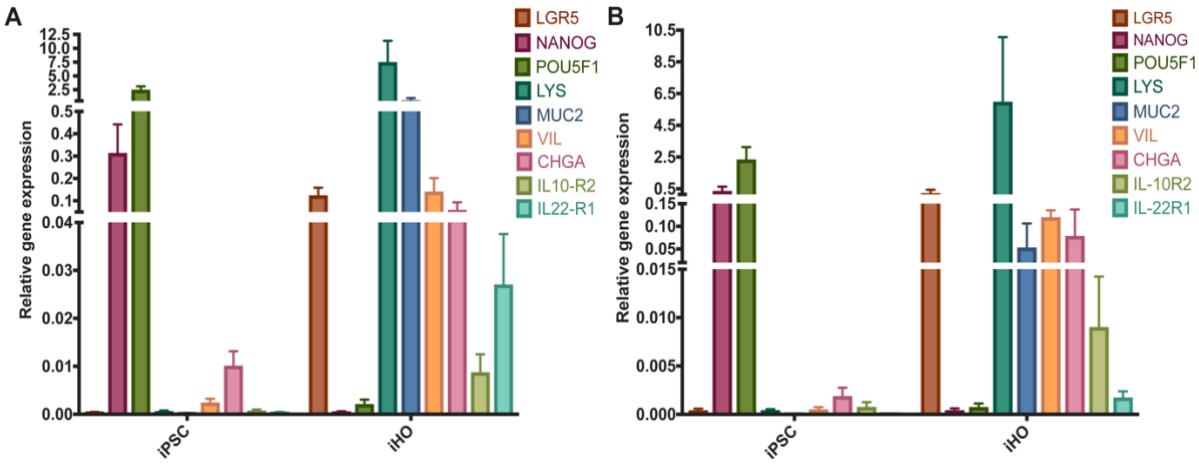

Figure 3.2: Differences in relative gene expression between iPSC and iHO from Rayr2 and Sojd2 cell lines. Data presented are from 4 technical replicates, using cDNA at 1:100 concentration, with assays repeated 3 times using paired iPSC/iHO of different batches for Rayr2 (**A**) and Sojd2 (**B**) cell lines. Data were analysed using the comparative cycle threshold (C_T) method, with GAPDH as an endogenous control.

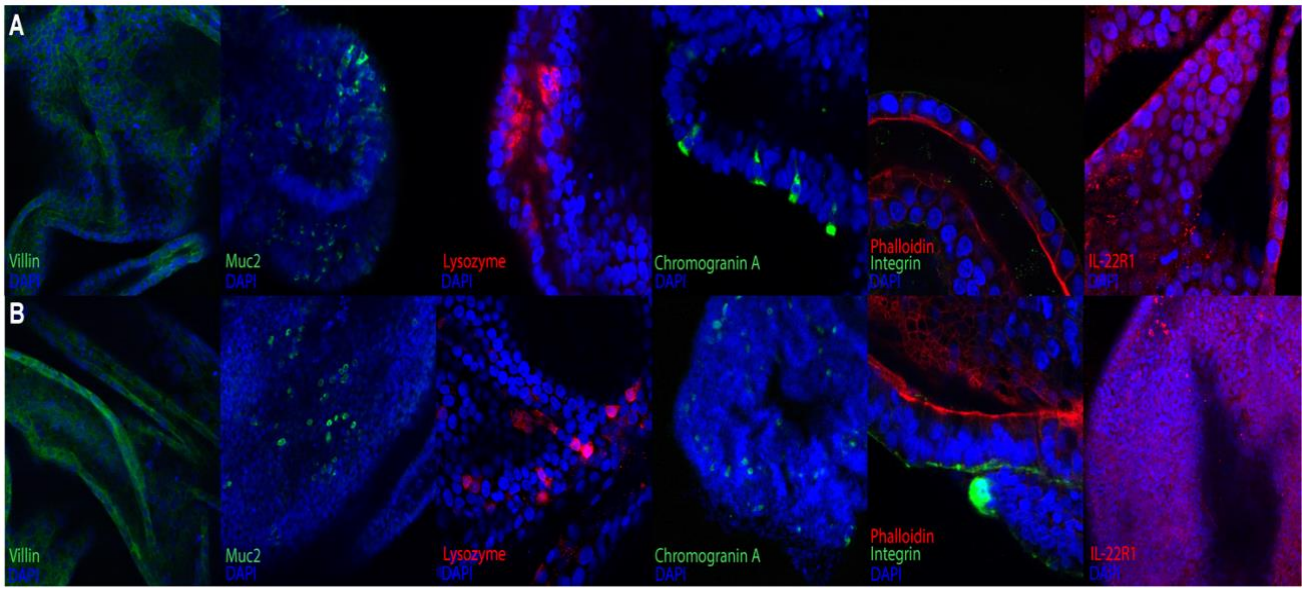

Figure 3.3: Immunohistochemistry of iHO lines generated with new method. Presence of constituent intestinal cell types demonstrated in iHO from Rayr2 (**A**) and Sojd2 (**B**) cell lines; namely goblet, Paneth, enteroendocrine cells and enterocytes, with a polarised epithelium and widespread presence of IL-22R1 on the basal surface of iHO generated using the modified method. Images taken on Zeiss LSM 510 Meta confocal microscope at 20x (Villin, Muc2, Lysozyme, Chromogranin A and IL-22R1 panels) or 40x (Phalloidin panel) magnification.

3.2 Establishing multiplicity of infection (MOI) in the iHO model

Establishing MOI in the iHO model is not as simple as in the macrophage model of infection, wherein it is possible to know how many cells are in a given well based on counting cells and seeding at specific densities. There is heterogeneity of iHO size and maturity within iHO cultures, which is why it is important to inject a relatively large number of iHO for

experiments, in order to reduce the impact of this variable. During routine splitting, attempts were made to establish the average number of cells in an iHO as follows.

iHO were released from Matrigel using Cell Recovery Solution. Five iHO were selected and transferred into a Falcon tube containing DPBS. iHO were washed, supernatant was removed and iHO resuspended in 1 mL of TrypLE, then incubated at 37 °C for 5 minutes to produce a single cell solution. 10 µL of cells were mixed with 10 µL Trypan Blue (Sigma Aldrich), and 10 µL of the resulting solution was added to a disposable C-Chip haemocytometer (NanoEnTek). Cells were counted, averaged and the number of cells per organoid determined. This was repeated 3 times on 3 separate occasions. The mean number of cells per iHO was 46,000 (SEM = 8413 cells).

It was also important to determine the inoculum of bacteria being injected into the closed lumen of the iHO. Two methods were trialled to achieve this. Luminal assays using microinjection (as described in 2.3) were set up, and 25 iHO per plate injected, but instead of incubating prior to harvesting, iHO were released from Matrigel immediately after injection, and their contents diluted and plated to give CFU injected per plate. This was repeated 3 times on 3 separate occasions to obtain an average CFU injected per plate (**Figure 3.4**).

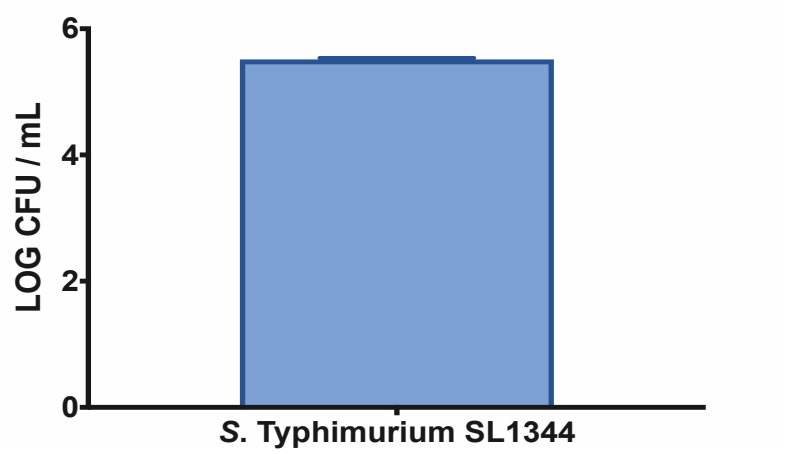

Figure 3.4 - Luminal contents at Time 0 after microinjection. LOG CFU/mL shown for microinjections with 25 x iHO per plate which were harvested immediately post-injection. Results plotted are for 3 biological replicates, each comprising 3 technical replicates. Mean LOG CFU/mL was 5.51; error bar indicates SEM.

This was compared with the expected CFU injected per iHO, by injecting the bacterial / Phenol red solution into a droplet of oil, and using a stage micrometer to measure the diameter (and therefore calculate the volume) of the injection. This was repeated 3 times on 3 separate occasions to obtain an average volume of inoculum.

At an OD_{600} of 1, counts of *S*. Typhimurium would be 1.6×10^9 CFU/mL, therefore:

Volume of inoculum: 3.59×10^{-6} mL
CFU per injection: $3.59 \times 10^{-6} \times 1.6 \times 10^9 = 5744$ CFU per injection
CFU per iHO (3 injections per iHO): 5744 x 3 = 17,232 (LOG 4.23)
CFU per plate (25 iHO per plate): 430,800 (LOG 5.63)

Having established cell counts in iHO and bacterial counts at injection, it is therefore possible to work out that MOI for experiments at the above settings was ~0.4.

3.2 Alternative antibiotic protection assays, to study gentamicin-resistant bacteria

It was of interest to study clinically relevant strains of *S*. Typhimurium to establish how different serovars interact with the epithelium. We consequently used a collection of Vietnamese ST34*, S*. I:4,[5],12:i:− (*S.* Typhimurium variant) clinical isolates, all from the same BAPS (Bayesian Analysis of Population Structure) cluster, which had been found in the blood or stool of patients presenting with salmonelloses (**Table 3.1**):[2]

Strain number:	Flagellar status:	Isolated from:	Presenting feature:	Gentamicin MIC:
VNB1779	Biphasic	Blood	Bloodstream infection	5
VNB2140	Biphasic	Blood	Bloodstream infection	7
VNB2315	Monophasic	Blood	Bloodstream infection	0.094
VNS20081	Biphasic	Stool	Diarrhoea	4
VNS20101	Monophasic	Stool	Diarrhoea	0.25

Table 3.1: Vietnamese clinical strains used in iHO, with flagellar status, location of isolation and minimum inhibitory concentration (MIC) for gentamicin highlighted.

Given that a number of these strains were gentamicin-resistant, results of antibiotic sensitivity assays run on the Vitek 2 system (Biomérieux) were examined to establish whether there was an alternative antibiotic that could be used to replace this in the modified gentamicin protection assay used for intracellular invasion assays in the iHO. There were only 3 antibiotics which all strains were sensitive to: Cefepime, Meropenem and Ertapenem. Cefepime is a 4th generation cephalosporin with particular action against Enterobacteriaceae. Cephalosporins disrupt the synthesis of the peptidoglycan layer of the bacterial cell wall, and are able to accumulate in the cytoplasm to a degree (far less in epithelial cells than phagocytic cells), but do not efficiently penetrate lysosomes.[3] Variable concentrations of Cefepime over the MIC were used until bacterial growth was inhibited. This concentration was then trialled as the antibiotic agent in the intracellular invasion competition assay in the iHO (for full protocol, see 2.1.7), allowing these assays to be performed with gentamicin-resistant bacteria.

3.3 Use of IncuCyte for observation of progress of infection in iHO

In order to help establish the fate of bacteria in the lumen of the organoids, it was beneficial to image them at regular time points during infection assays. This process was automated by use of the IncuCyte S3 Live-Cell Analysis system (Sartorius). Use of the TIMER^{bac}-*Salmonella* SL1344, which contains a DsRed S197T variant called TIMER that acts as a growth rate reporter by fluorescing at different wavelengths depending on replication stage,[4] allowed observation of bacterial replication and changes to the structure of the organoid in the hours following microinjection. In bacterial cells which are not growing, both green (rapidly maturing) and orange (slowly maturing) TIMER particles accumulate, with the predominant fluorescence being orange. In contrast, in dividing cells, rapidly maturing green particles are the predominant source of fluorescence, since whilst both molecules are diluted by cell division before they have matured, this disparately affects the orange molecules as they are slower to accumulate. Images were taken every 10 minutes, using the 10 x objective. Videos were produced from the collation of images, and red versus green fluorescence intensity over time plotted using the Incucyte S3 software.

3.4 Development of CryoEM methods for CL3 pathogens

Having reviewed TEM images from iHO injected with *S*. Typhi, and seen that a number were remaining in the lumen of the organoid at 3 hours post-injection, we wished to better visualise what was happening in the lumen and obtain better definition of the luminal contents, namely the mucus layer lining the epithelium. We therefore decided to trial high pressure freezing of the iHO to facilitate this.

iHO were microinjected with the pathogen of interest as described in 2.1.7 and incubated at 37 °C for 3 hours. The bottom of an aluminium planchette was lined with 1-Hexadecen 92% (Sigma-Aldrich), and a single iHO placed into the planchette using a 20 μL pipette. The planchette was placed into the sample holder and the sample holder was then placed directly into a 250 mL conical flask, which was sealed with Parafilm M (Bemis), sprayed with 70 % ethanol and removed from the CL3 laboratory as rapidly as possible.

Once in the CL2 facility, the sample holder was placed directly into the high pressure freezer (Bal-Tec HP010), and the HPF programme engaged and completed. Following this, the sample holder containing the planchette was held under liquid nitrogen, then the planchette removed, opened and placed into a 2 mL cryovial (Corning) and stored under liquid nitrogen. After freezing, the planchette was opened and placed in an automated Leica FS100 freeze-substitution unit at -200 °C, then gently warmed to -90 °C in 0.1% tannic acid with 0.5% glutaraldehyde in acetone for 6 hours, followed by 0.2% uranyl acetate (Sigma-Aldrich) and 1% osmium tetroxide (Acros Organics) in acetone (Sigma-Aldrich) for 72 hours. The samples were then rinsed in acetone and the temperature raised slowly to 4 °C. Acetone was then replaced with an epon resin/acetone mix overnight, followed by neat epon resin (Epoxy Embedding Medium kit, Sigma-Aldrich) for 24 hours. The sample was removed from the planchette, embedded in epon resin and cured at 65 °C for 48 hours. 500nm semi-thin sections were cut on a Leica UCT ultramicrotome and stained with toluidine blue on a microscope slide. Images were recorded on the Zeiss Axiovert CCD camera and areas selected for ultrathin 50nm sectioning. Ultrathin sections were collected onto copper grids and contrasted with uranyl acetate and lead citrate before viewing on a FEI 120kV Spirit BioTWIN TEM and recording CCD images on an F4.15 Tietz charge-coupled device camera.

References:

1. Forbester JL, Hannan N, Vallier L, Dougan G. Derivation of Intestinal Organoids from Human Induced Pluripotent Stem Cells for Use as an Infection System. *Methods Mol Biol.* 2016.
2. Mather AE, Phuong TLT, Gao Y, et al. New Variant of Multidrug-Resistant Salmonella enterica Serovar Typhimurium Associated with Invasive Disease in Immunocompromised Patients in Vietnam. *MBio.* 2018;9(5).
3. Hof H. Listeriosis: therapeutic options. *FEMS Immunol Med Microbiol.* 2003;35(3):203-205.
4. Claudi B, Sprote P, Chirkova A, et al. Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. *Cell.* 2014;158(4):722-733.