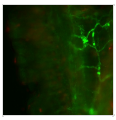

An organotypic slice model for ex vivo study of neural, immune, and microbial interactions of mouse intestine

Luke Schwerdtfeger[1], Elizabeth Ryan[1], Stuart Tobet[1]

[1]Colorado State University

dx.doi.org/10.17504/protocols.io.xjvfkn6

Luke Schwerdtfeger
Colorado State University

ABSTRACT

Organotypic tissue slices provide seminatural, three-dimensional microenvironments for use in ex vivo study of specific organs and have advanced investigative capabilities compared with isolated cell cultures. Several characteristics of the gastrointestinal tract have made in vitro models for studying the intestine challenging, such as maintaining the intricate structure of microvilli, the intrinsic enteric nervous system, Peyer's patches, the microbiome, and the active contraction of gut muscles. In the present study, an organotypic intestinal slice model was developed that allows for functional investigation across regions of the intestine. Intestinal tissue slices were maintained ex vivo for several days in a physiolog- ically relevant environment that preserved normal enterocyte struc- ture, intact and proliferating crypt cells, submucosal organization, and muscle wall composure. Cell death was measured by a membrane- impermeable DNA binding indicator, ethidium homodimer, and less than 5% of cells were labeled in all regions of the villi and crypt epithelia at 24 h ex vivo. This tissue slice model demonstrated intact myenteric and submucosal neuronal plexuses and functional intersti- tial cells of Cajal to the extent that nonstimulated, segmental contrac- tions occurred for up to 48 h ex vivo. To detect changes in physio- logical responses, slices were also assessed for segmental contractions in the presence and absence of antibiotic treatment, which resulted in slices with lesser or greater amounts of commensal bacteria, respec- tively. Segmental contractions were significantly greater in slices without antibiotics and increased native microbiota. This model ren- ders mechanisms of neuroimmune-microbiome interactions in a com- plex gut environment available to direct observation and controlled perturbation.

EXTERNAL LINK

https://www.physiology.org/doi/full/10.1152/ajpgi.00299.2015

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Schwerdtfeger LA, Ryan EP, Tobet SA. An organotypic slice model for ex vivo study of neural, immune, and microbial interactions of mouse intestine. *Am J Physiol Gastrointest Liver Physiol*. 310:G240-G248, 2016.

2016_Organotypic gut slice model AJP_Schwerdtfeger1.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS

NAME	CATALOG #	VENDOR
Sodium bicarbonate	View	P212121
Copper Sulfate	View	P212121
Triton-X100		
Phosphate Buffered Saline	28374	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
Bovine Serum Albumin (BSA)	A7906	Sigma Aldrich
1 Liter TBS [10X] (Tris buffered saline) (100mM Tris.HCl, 1.5M NaCl, pH 7.5)	R029	G-Biosciences
250 mg 5-Ethynyl-2'-deoxyuridine	orb340617	biorbyt
25 mg Azide-Fluor 488	orb64204	biorbyt
250 mg Nicardipine HCl	orb340258	biorbyt
Collagen	C8919	Sigma
Low Melt Agarose	A-204	Gold Biotechnology
60 mm Center Well Organ Culture Dish	353037	Corning
Culture media (e.g., Hibernate-A)		
Glycine	50046	Sigma
Krebs Buffer	View	
Vibrating Microtome	View	Leica Biosystems
Penicillin-Streptomycin	SV30010	HyClone
35mm Glass Bottom Culture Dish	354077	Corning
Neurobasal-A Medium	10888022	
B-27 Supplement	17504044	Gibco - Thermo Fischer
Ethidium Homodimer-1	E1169	

Intestinal Tissue Preparation

1 Adult mice were deeply anesthe- tized with isoflurane and killed by cervical dislocation. The small intestine was removed from the pylorus-duodenal junction to the distal ileum, and the colon, excluding the cecum, was removed. Tissue was placed immediately in 4°C 1× Krebs buffer (in mM: 126 NaCl, 2.5 KCl, 2.5 CaCl2, 1.2 NaH2PO4, 1.2 MgCl2), and dissected free from external vasculature and remaining mesenteric attachments. Successful cutting was notably dependent on minimizing mesenteric remnants prior to embedding in agarose.

Tissue Slicing

2 Slices were prepared from 1- to 3-mm sections of jejunum, ileum, and colon that were cut from the whole intestine and submerged in 8% agarose (type VII-A; Sigma; 39°C). The tissue spent a total of 7 min in the agarose: 5 min on a room temperature shaker, and 2 min in 4°C to ensure polymerization. Agarose encapsulated the entire tissue but did not penetrate the luminal space. Once the agarose was hardened, the tissue was cut at a thickness of 250 µm on a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany). Slices were collected in 4°C Krebs buffer.

Slice Culturing

3 Immediately after sectioning on the vibrating microtome, slices were transferred to 5 ml of Hibernate media (Life Technologies, Grand Island, NY) with 1% penicillin-streptomycin (PS; HyClone Laboratories, Logan, UT) in a 60-mm plastic-bottom dish (Corning, Corning, NY) and left at 4°C for at least 15 min. After Hibernate media, samples were transferred into 5 ml of Adult Neu- robasal media (ANB; Life Technologies) with 1.3% PS and 5% B-27 supplement (Life Technologies; https://www.thermofisher.com/order/ catalog/product/17504044) and incubated at 37°C for 35 min. Once initial media treatments were completed, the samples were plated on 35-mm-diameter plastic- (Corning) or glass (MatTek, Ashland, MA)- bottom dishes, with excess media being siphoned from the dish surface. Tissue was left at 37°C to adhere to the dish surface for 10 min before being covered by a thin layer of collagen [vol/vol: 10.4% 10× MEM, 1.9% PS, 4.2% sodium bicarbonate, and 83.5% collagen (PureCol; Inamed, Fremond, CA)]. Finally, the tissue was incubated at 37°C for 20 min to allow the collagen solution to polymerize before a final addition of 1 ml of ANB with PS and B-27 prior to being left in 37°C in a 5% CO2 incubator until visualization or experimentation. Fresh media changes were performed every 2 days.

Imaging Slices

4 Samples were imaged at 0, 24, 48, 72, 96, 124, and 148 h ex vivo on a Nikon Te2000-U inverted microscope (×4 and ×10 Plan-Fluor objectives) with a Quantix 57 frame-shift camera (Photometrics, Tucson, AZ) and UniBlitz shutter system (Vincent Associates, Rochester, NY). Time-lapse video microscopy was used for samples that were contracting, with images collected at 500-ms intervals. A single contraction count was measured as an intestinal contraction and subsequent relaxation to equal one count. Contrac- tions per minute were recorded and analyzed with Metamorph Mi- croscopy Automation and Image Analysis Software (Molecular De- vices, Sunnyvale, CA).

Contraction Blocking

5 Calcium ion channel blocker, nicardipine (Sigma- Aldrich, St. Louis, MO) was used to clarify the origins of the slice contractions. Nicardipine was diluted in distilled H2O from an initial concentration of 10 mM for use at 1, 3, and 10 µM. Contractions per minute were measured by time-lapse video microscopy, with all contraction counting performed by a researcher blinded to treatment condition. Video images were collected before drug treatment and again 30 min after drug treatment. Only slices that showed contrac- tions were used for drug treatment. Distilled H2O vehicle (10 µl) was used as a control. In addition, samples were washed for 1 h, four times in ANB×B-27×PS, and allowed to sit for an additional 2 h after initial washes, prior to a second drug addition. Dishes were varied for the second drug addition compared with initial treatments and were again allowed to incubate for 30 min after treatment prior to imaging. Data were collected from all dishes, including those given a second drug addition postwashing, and contraction rates were remeasured postwashing to ensure the tissue had recovered to predrug contraction rates prior to the second drug addition.

Cell Death Labeling

6 Cell death was estimated by using the membrane- impermeable red fluorescent DNA marker ethidium homodimer (EtHD; Biotium, Hayward, CA). EtHD was added to the media at a concentration of 2.5 µM, achieved with a volume of 1 µl of EtHD per 1 ml of media (ANB×PS×B-27) for 45 min, and was then washed out. Slices were then imaged on the Nikon Te2000-U inverted microscope setup at 0-, 24-, 48-, and 72-h intervals. Analysis of cell death was performed with ImageJ Image Processing and Analysis software (NIH) to determine the area of EtHD fluorescence within defined regions of interest (ROIs). Three regions were defined based on the anatomy of apical and basilar villi and the adjacent crypt regions. These regions were analyzed independently via the “analyze particles” tool on a threshold image.

Cell Proliferation Labeling

7 The incorporation of 5-ethynyl-2=-deoxyuridine (EdU; Invitrogen, Eugene, OR) was used to indicate the synthesis of new DNA in presumptive dividing cells ex vivo. Mice were injected (25 mg/kg ip) 24 h prior to euthanasia and used for intestinal slice visualization between 0 and 24 h ex vivo. Some slices were exposed to 5 µl of EdU per 1 ml of media, in vitro. Slices were then incubated for 24 or 48 h before being visualized. The EdU visualization procedure began with three phosphate-buffered saline (PBS) washes for a total of 30 min. Next, samples were placed in glycine (Fisher Scientific, Pittsburgh, PA) for 30 min before being again washed in PBS (in mM: 42.98 Na2 HPO4 , 7.25 NaH2 PO4 , 145.45 NaCl) for 10 min (1 change). Samples were then blocked with 3% bovine serum albumin buffer (BSA; Lampire Biological, Pipersville, PA) and 0.5% Triton-X (Tx) for 2 h. Samples were subsequently washed two times with 3% BSA buffer. Next, click-IT cocktail (1× click-IT Reaction Buffer, CuSO4, Alexa-Fluor azide, 1× reaction buffer additive; In- vitrogen) was added for 2 h. Finally, slices were washed in 3% BSA buffer and 0.02% Tx three times for a total of 1.5 h and left overnight in 3% BSA before being mounted, coverslipped with Aqua-Poly/ Mount (Polysciences, Warrington, PA), and imaged by confocal microscopy (Zeiss Meta 510; Carl Zeiss). Quantification of EdU incorporation was done in ImageJ (NIH) as noted for EtHD. Individ- ual villi and crypt regions were selected from slices that demonstrated normal ileal villi structure (tall and fingerlike), with three ROIs being quantified: apical villi, basilar villi, and crypt regions.

Whole-mount Immunohistochemistry

8 Following live viewing and image collection, slices were immersion fixed in 4% paraformalde- hyde for 15 min, and washed in 0.05 M PBS (pH 7.5), prior to immunohistochemical studies. Tissue processing was similar to that previously described (25). Once fixing and PBS washes were com- plete, the slices were incubated at 4°C in 1% sodium borohydride for 2 h. Slices were then washed in PBS for 10 min prior to incubation in block containing PBS with 5% normal goat serum (NGS; Lampire Biological, Pipersville, PA), 3% hydrogen peroxide and 0.3% Tx for 2 h with a change of solution at 1 h. Slices were placed into primary antisera; anti-NeuN (a neuronal nuclear marker) for neuronal pheno- typing (Cell Signaling Technologies, Danvers, MA), neuronal nitric oxide synthase (nNOS; ImmunoStar, Hudson, WI), CD3 and CD79a antibodies (to cell surface markers for T and B cells, respectively; Novus Biologicals, Littleton, CO), and anti-c-Kit (ACK2; Novus Biologicals), with PBS containing 5% NGS and 0.3% Tx for 6 days. ACK2 was added at 2 µg/ml to live slices 90 min prior to fixation and then processed as noted for the other antisera below. NeuN primary antibody was used at 1:1,000 (bright field) and 1:200 (fluorescence) concentrations, as well as a blank for control, with all other antibodies being done at either 1:300 (CD3 and CD79a) or 1:10,000 (nNOS) concentrations. Six days following primary antibody addition, slices were washed at 4°C in PBS with 1% NGS and 0.2% Tx four times at 30-min intervals. Slices were incubated for 24 h in a biotinylated secondary antiserum (anti-rabbit, 1:2,500 or anti-rat 1:1,000 for ACK2 rat monoclonal antibody; all secondary antisera from Jackson ImmunoResearch, West Grove, PA) specific to the species of the primary antibodies and were constituted in PBS with 1% NGS and 0.32% Tx. Slices were washed for 2 h in room temperature PBS with 0.02% Tx four times before being placed in either their tertiary conjugated antibody (Cy-3) for 3 h before being washed in PBS and mounted, or Avidin-Biotin Complex (ABC; Vector Laboratories, Burlingame, CA) with 0.32% Tx/PBS for 3 h. After the ABC incubation, slices were washed in PBS at room temperature for 2 h with four changes before being placed into 0.025% diaminobenzidine (DAB; Sigma-Aldrich) in PBS; 15 min after the addition of DAB, 1% H2O2 was added for 20 min. Finally, the slices were washed three times with PBS prior to being mounted on slides and coverslipped with an aqueous mounting medium (Aqua-Poly/Mount, Polysciences, Warrington, PA).

Fluorescent Gram Staining

9 Fluorescent staining was performed by using LIVE BacLight Bacterial Gram stain (Life Technologies). Equal volumes of SYTO 9 (component A) and hexidium iodide (component B) were mixed thoroughly on the day of use to create the final staining solution, and 3 µl of the staining solution was added to 1 ml of ANB-B-27 without PS. Samples were incubated in the dark for 15 min at room temper- ature, with subsequent media washes occurring two times for 5 min each prior to imaging. Because of technical limitations of accurately assessing the bacterial load with captured images, the density of bacteria was evaluated by using a subjective scale, with the researcher being blinded to treatment. Subjective ratings ranged from 1 (virtually no bacteria) to 4 (extremely dense bacteria).