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TITLE:

Functional Assessment of Intestinal Tight Junction Barrier and Ion Permeability in Native Tissue by Ussing Chamber Technique

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SUMMARY:

Intestinal epithelium confers not only nutrient absorption but protection against noxious substances. The apical-most epithelial intercellular junction, i.e., the tight junction, regulates paracellular solute and ion permeability. Here, a protocol for the preparation of mucosal sheets and assessment of the ion selectivity of tight junctions using Ussing chamber technique is described.

ABSTRACT:

The Ussing chamber technique was first invented by the Danish scientist Hans Ussing in 1951 to study the transcellular transport of sodium across frog skin. Since then, this technique has been applied to many different tissues to study the physiological parameters of transport across membranes. The Ussing chamber method is preferable to other methods because native tissue can be used, making it more applicable to what is happening *in vivo*. However, because native tissue is used, throughput is low, time is limited, and tissue preparation requires skill and training. These chambers have been used to study specific transporter proteins in various tissues, understand disease pathophysiology such as in Cystic Fibrosis, study drug transport and uptake, and especially contributed to the understanding of nutrient transport in the intestine. Given the whole epithelial transport process of a tissue, not only transepithelial pathways, but also paracellular pathways are important. Tight junctions are a key determinant of tissue specific paracellular permeability across the intestine. In this article, the Ussing chamber technique will be used to assess paracellular permselectivity of ions by measuring transepithelial conductance and dilution potentials.

INTRODUCTION:

The Ussing chamber method was first developed by the Danish scientist Hans Ussing. Ussing first used it to measure the short-circuit current of sodium transport across frog skin after it

was observed that NaCl could be transported across the skin against a steep concentration gradient[1]. His system consisted of the frog skin mounted between two chambers with access to either side of the skin. Each chamber contained Ringer's solution which was circulated and aerated. Two narrow agar ringer bridges situated near the skin and connected to saturated KCl-calomel electrodes measured the potential difference as read by a potentiator. A second pair of agar ringer bridges were situated at the opposite end of each chamber connected to beakers with saturated KCl saturated with AgCl to apply an electromotive force provided by a battery. A potential divider was used to adjust the voltage so that the potential difference across the skin remained zero, thus creating short-circuit conditions. A microampere meter was also connected to read the current passing through the skin (see the figure in ref.[1] for original chamber design).

Over the past 70 years, this technique has been applied to many different tissues, particularly intestinal tissue, to study nutrient and ion transport. For example, the mechanism of cholera-induced diarrhea was studied by mounting rabbit ileum in these chambers, and it was found that cholera toxin-induced diarrhea is mediated by cAMP[2]. In addition, these chambers were also used to study the mechanism underlying glucose transport via Na^+-Glucose cotransporter 1 (SGLT1)[3]. Our lab focuses on transcellular and paracellular transport in intestinal epithelial cells. Using the Ussing chamber method, peptide transport was assessed in Claudin 15 knockout mice, which have impaired paracellular sodium transport, using Ussing chambers to measure the absorption of the nonhydrolyzable dipeptide glycylsarcosine. It was found that luminal Na^+ homeostasis is important for proton-coupled peptide transport[4]. In addition, these chambers were also used to investigate anion secretion in the murine cecum in response to submucosal activation of proteinase activated receptor 1 by the serine protease trypsin[5].

Ussing chambers have also recently been used to assess the paracellular pathways in epithelial tissue. Paracellular pathways are regulated by tight junctions, which are complexes of proteins that form at the point where two or more cells meet[6]. The barrier function and ion selectivity (whether anions or cations are selectively able to pass through the tight junction) is determined by the presence of claudin family proteins; some of which act as barriers (claudin 3 and 7), anion pores (claudin 10a), or cation pores (claudin 2, 10b, and 15)[7]. Other methods have been used to assess the paracellular pathway, such as oral gavage of FITC accompanied by blood plasma FITC concentration[8], or EDTA-Cr[9]; however, these techniques are of lower resolution and cannot assess ion selectivity or a specific section of the sections of the intestinal tract. Ussing chambers, however, can be used to assess the dilution potential of target ions, and, therefore, determine the ion selectivity of the tight junctions. For example, with NaCl, the selectivity of the tight junctions for Na^+ and Cl^- can be calculated by diluting one side of the membrane (usually the mucosal side) and measuring the change in transepithelial potential difference. The relative permeabilities of Na^+ and Cl^- can be estimated by the Goldman-Hodgkin-Katz equation[10] and the selectivity of the tight junction can be estimated using the Kimizuka-Koketsu equation[11]. These chambers, therefore, have the advantage of measuring the electrophysiological parameters of tissue and as a result provide more information about the passage of ions through the tight junctions than other lower resolution methods.

The Ussing chamber method is not only limited to the intestinal tract, although it is widely used

in studies concerning the intestine, it has many other applications as well. For example, these chambers have been used to study Cystic Fibrosis, and specifically the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR)[12]. Cystic Fibrosis is caused by a mutation in CFTR[13], which results in impaired chloride secretion and fluid transport by respiratory epithelial cells, and a resulting thicker, drier mucous layer[14]. Study of airway epithelial CFTR has been performed with these chambers to not only understand the disease, but to discover ways to treat the disease. For example, in patients with rare mutations causing Cystic Fibrosis, analysis of patient respiratory epithelial cells has been used to test therapies such as Orkambi and an amplifier co-therapy[15].

Ussing chambers have also been used to study routes of drug delivery, such as with human biopsy tissue to study drug uptake and pharmacokinetics[16]. Intestinal uptake is not the only route of drug delivery. These chambers have also been used to study nasal drug delivery systems[17]. Drug delivery studies with Ussing chambers have also been performed for the eye. In the rabbit cornea, permeability and uptake studies were conducted with Labrasol, a drug that is designed to increase the absorption of drugs across tissues[18]. Another study examined the effect of benzylalkonium chloride on transscleral drug delivery in the rabbit sclera[19].

The Ussing chamber method is useful because native tissue can be used. As such, it is preferable over *in vitro* models such as Caco-2 cell lines. However, the technique requires skill and time to prepare specimens, so it is not suitable for high throughput applications. The electrophysiological properties of cell monolayers can be studied using cell culture inserts in these chambers. Recent discoveries have allowed for the culture of organoids which are mini-organs grown in culture from the harvest of epithelial or endothelial stem cells[20]. Organoid culture can be manipulated to be grown in a monolayer, thereby making it possible to mount organoids in an Ussing chamber[21]. Organoids of various epithelial and endothelial tissues can be studied, lowering the number of animals required, as organoid culture can be maintained long term. This will also increase the throughput since time consuming and laborious tissue dissection and preparation steps will not be needed. In the future, Ussing chamber studies will continue to be very useful for studying tissue transport and they will be especially important in the field of personalized medicine.

The following protocol demonstrates the application of the Ussing chamber method to assess the permselectivity and barrier function of the tight junctions in the small intestine of Claudin 15 knockout (*Cldn15*$^{-/-}$) mice and wild type (WT) controls by measuring the dilution potential of NaCl. Tight junctions (TJ) are formed at the point where two or more cells meet in epithelial and endothelial tissue. Bicellular tight junctions (bTJ), particularly the claudin family proteins found within the bTJ, are thought to determine the barrier function and permselectivity of TJ[7]. *Cldn15*$^{-/-}$ mice have a mega small intestine[22] and reduced nutrient uptake capability due to the loss of intestinal Na^+ recycling that occurs via claudin 15[4,23,24]. *Cldn15*$^{-/-}$ mice have impaired Na^+ homeostasis, which makes them an interesting model for studying the permselectivity of the TJ. The following protocol assesses the permeability of the TJ to NaCl by measuring the dilution potential of NaCl (P_{Na}/P_{Cl}) in the middle small intestine. Briefly, the change in membrane potential difference that occurs by diluting one side of the membrane (M side or S side, both

are measured in the below protocol) can be used to calculate the permeability of Na^+ (P_{Na}) and Cl^- (P_{Cl}), and the dilution potential (P_{Na}/P_{Cl}) will show whether the tight junction has a cationic or anionic selectivity.

The experiments in this protocol were conducted using a customized Ussing chamber (**Figure 1A**), which consists of two halves, between which the intestinal preparation is mounted vertically), voltage clamp amplifier, electrical recorder, electrodes, salt bridges, Ringer's solution, HEPES buffer (150 mM NaCl), diluted HEPES buffer (75 mM NaCl), intestinal preparation (for details about equipment see the **Table of Materials**).

PROTOCOL:

All animals used in these experiments were maintained in the animal care facility at the University of Shizuoka and the experiments were conducted according to the guidelines for animal research set out by the University of Shizuoka. All experiments were carried out with approval from the Animal Care and Use Committee at the University of Shizuoka (Permits #205272 and #656-2303).

1. Preparation of NaCl electrodes

NOTE: The electrodes used in these experiments consist of concentrated NaCl or KCl. The KCl/calomel electrodes are purchased commercially. Before starting the experiment, ensure that all electrodes are filled to the top with concentrated NaCl or KCl solution.

1.1 Prepare small glass jars with plastic lids (volume 20 mL).

1.2 Drill two holes in the plastic lids, one for the NaCl salt bridge (2.5 mm diameter), and the other for silver wire (1 mm diameter; **Figure 1C**, NaCl electrode).

1.3 Fill the glass jar with saturated NaCl solution (about 15 mL, until full).

1.4 Insert silver wire (0.8 mm diameter, 7 cm long) into the jar, but ensure that the wire portion outside the jar can be connected via alligator clips (small size) to the amplifier system.

1.5 When not in use, wrap the electrodes and ensure the holes are covered, with parafilm to prevent drying.

2. Preparation of salt bridges

NOTE: Prepare salt bridges at least a day before the experiment to provide adequate time to solidify. Salt bridges can be used repeatedly but use after 2 months is not recommended.

2.1 NaCl salt bridges

2.1.1 Prepare #7 polyethyl tubing (outer diameter 2.3 mm, inner diameter 1.3 mm), 19 G needle and lock-type syringe, 200 mL of 1 M NaCl solution, 2 g agar, sealable plastic container for salt bridge storage.

2.1.2 Prepare appropriate number of salt bridges by cutting tubing to the size necessary for the Ussing chamber set up (each chamber requires two salt bridges).

2.1.3 Before injection of agar, make a U shape with the tubes and place them in a beaker of warm water (to create an easy shape for setting up salt bridges).

2.1.4 Make 200 mL of 1 M NaCl by dissolving 11.688 g of NaCl in 200 mL in deionized water.

2.1.5 Split 1 M NaCl into 100 mL portions: Make 100 mL of 2% agar in 1 M NaCl (mix 2 g agar in NaCl, heat in microwave to dissolve).

2.1.6 Using 19 G needle and locking syringe, fill the syringe with 1 M NaCl/agar solution. Gently begin to expel solution drop by drop and while doing so insert the needle into one end of the tube and fill until the mixture comes out from the other side.

2.1.7 Slowly withdraw the needle while still expressing the solution and repeat until all the required salt bridges have been made. (If the solution solidifies in the syringe or needle, briefly warm it in hot water until solution can be expressed again.)

2.1.8 Check salt bridges to ensure there are no bubbles and store in remaining 1 M NaCl solution in a sealable container.

2.2 KCl salt bridges

NOTE: Thinner tubing is used for the KCl agar bridges to avoid the increment of K^+ concentration in the buffer, as the salt bridge tips can dissolve and K^+ can leak into the buffer.

2.2.1 Prepare #3 polyethyl tubing (outer diameter 1.0 mm, inner diameter 0.5 mm), 23 G needle and lock type syringe, 200 mL of 1 M KCl solution, 2 g agar, sealable plastic container for salt bridge storage.

2.2.2 Prepare appropriate number of salt bridges by cutting the tubing to the size necessary for the Ussing chamber set up (each chamber requires two salt bridges).

2.2.3 Make 200 mL of 1 M KCl by dissolving 14.91 g of KCl in 200 mL of deionized water.

2.2.4 Split into two 100 mL portions: Make 100 mL of 2% agar in 1 M KCl (mix 2 g agar in KCl, heat in a microwave to dissolve).

2.2.5 Using a 23 G needle and locking syringe, inject tubing with 2% agar 1 M KCl mixture (ensure that the tubes are completely filled and there are no bubbles) in the same manner as with the NaCl salt bridges.

2.2.6 Check salt bridges to ensure there are no bubbles and store in the remaining 1 M KCl solution in a sealable container.

3. Preparation of Ringer's solution and HEPES buffer

NOTE: Depending on the tissue mounted in the Ussing chamber, the components of Ringer's solution may differ. The recipes presented here are specific for the small and large intestine.

3.1 Make Ringer's solution fresh on the day of the experiments as described in **Table 1.**

3.2 Bubble the solution with 95% O_2/5% CO_2 to provide O_2 to the tissue and a buffering capacity.

[Place **Table 1** here]

3.3 Make HEPES buffer fresh on the day of experiment as described in **Table 2** by mixing ingredients in de-ionized water.

3.4 Do not adjust to the final volume of buffer until after pH adjustment.

3.5 Warm HEPES buffer to 37 °C and adjust the pH to 7.4 by slowly adding drops of 1 M Tris solution while stirring.

3.6 Adjust to the final volume by adding the appropriate amount of deionized water.

[Place **Table 2** here]

4. Ussing chamber setup

NOTE: The Ussing chambers used in this protocol are custom-made continuous perfusion chambers. To assess mouse intestinal barrier function or nutrient uptake, chambers with a 4 or 5 mm diameter opening is recommended[25] (**Figure 1A-C**).

4.1 To reduce edge effect[26] and help seal the chambers, attach 4 or 5 mm hole punched paraffin film (about 4 cm^2) before setting up (**Figure 1B**).

4.2 Set up in open circuit conditions for dilution potential measurement. Set in current clamp mode. Set the output as current and set current pulse to ±20 µA.

4.3 When setting up in short circuit conditions for the measurement of short circuit current and transmucosal resistance, set in voltage clamp mode. Set the output as voltage and set voltage pulse to ±5 mV.

4.4 Ensure 37 °C water is circulating in the water jacket.

4.5 Fill each chamber with Ringer's solution or HEPES buffer (amount depends on the system used, the chambers used here require 5 mL for each side) and ensure there are no leaks.

4.6 Connect salt bridges and electrodes.

4.7 Ensure voltage is 0 and stable, pulse current to ensure that salt bridges and electrodes are properly set up.

4.8 Allow system and Ringer solution temperature to equilibrate for at least 20 min.

4.9 After equilibration, correct asymmetrical voltage difference between KCl electrodes and compensate for fluid resistance by changing it to zero (check the manual for the Ussing chamber system used to determine the correct way).

5. Dissection of intestinal tissue

NOTE: All animal experimentation must be carried out within the regulations set by the country and the university.

5.1 Before taking the intestinal tissue, prepare fresh, ice-cold Ringer solution and bubble with 95% O_2 and 5% CO_2 for 15 min (step 3).

5.2 Anesthetize mice according to guidelines governing the use of animals in research. For this experiment, mice were anesthetized with 2%–3% isoflurane administered by an anesthetizer. Check for the proper anesthesia by pinching toes and ensuring there is no pain response.

5.3 Make an incision in the abdomen with scissors from the pelvis to the diaphragm; locate the stomach and cut the pyloric end of the stomach.

5.4 Grip the stomach portion attached to the small intestine with forceps and gently pull the small intestine while cutting away the mesenteric attachments. Be careful not to cut or damage the intestinal tissue in any way.

5.5 Continue dissecting the intestine all the way to the anus. For the complete removal of large intestine, cut the pelvic bones to reveal the distal portion of the large intestine and carefully remove the rest of the intestine by cutting away the attachments.

5.6 Measure the length of the intestine and divide into desired segments. For this experiment, divide the small intestine into three segments and use the middle segment.

5.7 Place the desired segments into ice cold, bubble Ringer's solution; then, open each segment longitudinally by cutting along the mesenteric attachments. Trim away fat and connective tissue.

5.8 Return the segments to the ice-cold Ringer's solution and wash thoroughly (even in the ice-cold solution, oxygenation of the luminal epithelium is important to maintain epithelial function).

6. Stripping the muscle layer and preparation of the intestinal sheet

NOTE: Removal of the serosa (muscle layer) is important for transport studies using the intestine. If the serosa remains, the intestinal tissue can be subject to random muscular contractions that will distort the electrophysiological data, and transport may be inhibited. Unstripped tissue rapidly deteriorates when mounted in Ussing chambers, since the serosa is a significant diffusion barrier for substrate and oxygen. In some special cases, it may be necessary to keep the muscle layer, so the decision is up to the researcher and the experimental design. The intestinal sheets can be prepared in two ways depending on which layer is removed (**Figure 2**). For this experiment, mucosa and submucosal preparations are required (**Figure 2**, 2nd panel).

6.1 Prepare dissection plates (10 cm diameter) covered with silicone rubber, pins (small acupuncture needles), 5 mm punched filter paper and parafilm squares (2 cm x 2cm; may not be necessary for other systems).

6.2 Pour fresh, ice-cold, bubbled Ringer's solution into the dissection plate (enough to cover the tissue, about 2–3 mL).

6.3 Under a stereomicroscope, pin the ends of intestinal tissue (mucosal side down).

6.4 Using fine forceps, bluntly dissect the muscle layer from the underlying mucosa.

6.5 Be careful not to tear or introduce any holes into the tissue.

6.6 Once the muscle layer is removed, cut a piece large enough for a 5 mm diameter opening. When preparing the small intestine, removal of the serosa-muscle layer should be done within 10 min, since luminal oxygenation is difficult under these conditions.

6.7 Wet 5 mm punched filter paper square in Ringer's solution and place the intestinal tissue on it with mucosal side down, since submucosal preparations spontaneously wrap around with mucosal side outside.

6.8 Ensure the opening is completely covered by the intestinal tissue and no wrinkles are present. Use a black board underneath the preparation to examine whether the opening is completely covered.

6.9 Repeat this procedure for the required number of mucosal preparations (in this experiment two preparations are required: one preparation will be used to measure dilution potential, and the other will be used to measure baseline electrical parameters).

7. Mounting intestinal preparations in Ussing chambers

NOTE: Set up will depend on the type of the Ussing chamber system and recording system used.

7.1 Suction out Ringer solution/HEPES buffer from the Ussing chamber.

7.2 Disassemble the Ussing chamber and lay the filter paper with the intestinal preparation mucosal side down on the mucosal side chamber and adjust so that the chamber's window aligns with the hole of the filter paper (**Figure 1A**, black marking around the chamber window is useful for alignment of the preparations).

7.3 Carefully place the Serosal side chamber on to Mucosal side chamber and close tightly but be sure that the intestinal sheet has not moved during connection.

7.4 Quickly refill both chambers with Ringer's solution or HEPES buffer, and place bubbling wands (Ringer's solution: 95% O_2/5% CO_2; HEPES buffer: 100% O_2) at the opposite end of the chambers, away from the membrane (bubbling too close to the preparation could have an effect on the measurements).

7.5 Reconnect salt bridges and check whether the voltage is stable and pulse current to ensure the connections are okay (**Figure 1C**).

7.6 Repeat for each intestinal preparation.

7.7 Let the system equilibrate for about 15 min. If using a recording system, let the conductance and I_{sc}/membrane potential difference stabilize before starting the experiments.

8. Dilution potential experiment (open circuit conditions)

8.1 Wash both sides of the chamber by suctioning the HEPES buffer and adding 5 mL of fresh pre-warmed HEPES buffer to each side.

8.2 Turn the recording system on. Set range to 250 mV, (the system used here amplifies output voltage 10x), set marker positions, and set recording system to measure.

8.3 Turn Ussing chamber systems to clamp mode and start measuring. Once membrane potential has stabilized (~15–20 min), the assessment can begin.

8.4 Suction the HEPES buffer from the Mucosal side and quickly replace with 5 mL of warmed dilution HEPES buffer containing 75 mM NaCl.

8.5 Once the membrane potential has peaked (5–10 min), remove the dilution buffer from the "Mucosal" side and replace with HEPES buffer.

8.6 If necessary, repeat step 3 for the Serosal side, adding dilution HEPES buffer to the Serosal side.

8.7 To ensure that the tissue is viable, add the adenylate cyclase activator Forskolin (final concentration 10 µM) to the Serosal side.

8.8 Once the membrane potential difference has reached a peak and has started to decline, the experiment is over.

9. Measurement of transepithelial electrical conductance and baseline I_{sc} (short-circuit conditions)

9.1 Wash both sides of the chamber by suctioning the Ringer's solution and adding 5 mL of fresh bubbled Ringer's solution to each side.

9.2 Turn the recording system on. Set range to 2.5 V (the system used here amplifies output voltage 10x), set marker positions, and set recording system to measure.

9.3 Turn Ussing chamber systems to clamp mode and start measuring; once I_{sc} and conductance have stabilized (~15–20 min), baseline measurements can be obtained.

9.4 To ensure the tissue is viable, add the adenylate cyclase activator Forskolin (final concentration 10 µM) to the Serosal side.

9.5 Once the membrane potential difference has reached a peak and has started to decline, the experiment is done.

10. Analyzing results

10.1 Under open-circuit conditions, calculate transmucosal conductance from the change of voltage in response to current pulses according to Ohm's law. Determine the equivalent short circuit current (I_{sc}) from transmucosal voltage and conductance applying Ohm's law.

10.2 Use the dilution potential of NaCl for calculating the relative ionic selectivity (P_{Na}/P_{Cl}) with the Goldman-Hodgkin-Katz equation[10].

10.3 Estimate the absolute selectivity of the tight junction for each ion using the Kimizuka-Koketsu equation[11].

10.4 Calculate P_{Na}/P_{Cl} using the Goldman-Hodgkin-Katz equation from dilution potentials, and determine absolute permeabilities P_{Na} and P_{Cl} from the Kimizuka-Koketsu equation as described by Yu et al.[10] as follows:

$$V = \frac{RT}{F} \ln\left[\frac{(\alpha + \beta)}{1 + \alpha\beta}\right]$$

$$\beta = \frac{PCl}{PNa} = \frac{\alpha - x}{\alpha x - 1}$$

$$x = e^{-VF/RT}$$

$$PNa = \frac{RT}{F^2} \frac{GM}{\alpha(1 + \beta)}$$

where, V: Dilution potential (mV); α: Activity ratio. The calculated activity of NaCl in the HEPES buffer divided by the calculated activity of NaCl in the dilution HEPES buffer (For this experiment it was calculated as 1.8966); e: Mathematical constant, 2.71828; GM: Transmucosal conductance (mS/cm^2); F: Faraday constant (96,485.3329 C/mol); R: Gas constant (8.314 J/mol K);T: Temperature (310.15 K)

REPRESENTATIVE RESULTS:

The results shown in this paper are results that were part of larger project that has been completed (see ref.[4,23,24]).

Transepithelial electrical conductance of the small intestine is decreased in *Cldn15*$^{-/-}$ mice.

The baseline transmucosal conductance (under short circuit conditions) of the middle small intestinal segment in *Cldn15*$^{-/-}$ mice was lower than that measured in wild type mice (**Figure 3A**; 22.3 mS/cm^2 and 48.7 mS/cm^2 in *Cldn15*$^{-/-}$ and wild type mice, respectively). Baseline short circuit current (I_{sc}) was also measured in *Cldn15*$^{-/-}$ mice (**Figure 3B**). Baseline I_{sc} was increased (since Na-dependent glutamine transport is upregulated, see reference[23]) in *Cldn15*$^{-/-}$ mice compared to controls (**Figure 3B**; 36.0 µA /cm^2 and 26.9 µA/cm^2 in *Cldn15*$^{-/-}$ and wild type mice, respectively).

Dilution potential is decreased in *Cldn15*$^{-/-}$ mice

In order to assess the selectivity of the small intestinal mucosa to NaCl in *Cldn15*$^{-/-}$ mice, the dilution potentials were measured with a concentration gradient (60 mmol/L NaCl on the mucosal side and 119 mmol/L NaCl on the serosal side). Upon luminal NaCl dilution, a positive potential difference (9.2 mV) with respect to serosal side was observed in WT mice (**Figure 3C**). However, this positive dilution potential was decreased in *Cldn15*$^{-/-}$ mice (**Figure 3C**; 0.8 mV).

The relative permeability of NaCl (P_{Na}/P_{Cl}) was calculated as above and it was found that it was decreased in *Cldn15*$^{-/-}$ mice, similar to the decreased potential difference (**Figure 3D**; 1.1 and 3.5, *Cldn15*$^{-/-}$ and WT mice, respectively). $P_{Na}/P_{Cl} > 0.7$ means that Na^+ and Cl^- diffuse via a cationic selective pore (see Figure 4 in ref.[27]). From this data, it can be said that the cationic selectivity of the middle small intestine in *Cldn15*$^{-/-}$ mice is decreased. This agrees with the conductance data (**Figure 3A**), which shows a reduction for *Cldn15*$^{-/-}$ mice, meaning that the paracellular pathways have decreased.

Next, the absolute permeabilities of Na^+ (P_{Na}) and Cl^- (P_{Cl}) were calculated. P_{Na} was found to be decreased in *Cldn15*$^{-/-}$ mice (**Figure 3E**; 3.28 x 10^{-4} cm/s and 10.92 x 10^{-4} cm/s, *Cldn15*$^{-/-}$ and WT mice, respectively); however, P_{Cl} did not seem to change (**Figure 3F**), showing that the decrease in the relative permeability is due to a decrease in the permeability of Na^+. This result makes sense since *Cldn15*$^{-/-}$ mice have lost a Na^+ pore in claudin 15, meaning the pathways available to Na^+ have been decreased.

Assessment of viability in intestinal preparation

Finally, to check the viability of intestinal preparation, the adenylate cyclase activator, forskolin, was added to the serosal side, which activates CFTR chloride channel, resulting in increases of short circuit current (I_{sc}). There is no big difference between *Cldn15*$^{-/-}$ and WT mice (**Figure 3G**; 341.5 μA/cm^2 and 320.1 μA/cm^2, *Cldn15*$^{-/-}$ and WT mice, respectively). Since the intestinal segments responded to serosal application of forskolin, the membrane preparation is considered to be viable.

FIGURE AND TABLE LEGENDS:

Table 1: Ringer's Solution Recipe. To make Ringer's solution, mix all components together with de-ionized water. Ringer's solution is best made fresh before experiments. Keep in the refrigerator or on ice until use. Before using, gas with 95% O_2/5% CO_2.

Table 2: HEPES Buffer Recipe. To make HEPES buffer and dilution HEPES buffer, dissolve all ingredients in de-ionized water. Solutions must be pH adjusted with 1 M Tris solution, so do not add full volume of water (e.g., when making 1 L, dissolve all ingredients in about 800 mL of water). Then heat the solution to 37 °C, adjust the pH to 7.4 and then adjust the final volume.

Figure 1: Ussing chamber set up. (**A**) Ussing chamber consists of two halves and the intestinal preparation is mounted vertically. (**B**) Close-up view of the chamber openings (5 mm), which have parafilm punched with a 5 mm hole attached. (**C**) Assembled apparatus. Calomel electrode is used for potential difference measurements, which are connected to the chamber via KCl salt bridges. Ag-AgCl electrode is used for I_{sc} measurement, which passes across the mucosal preparation and is connected to the chambers via NaCl salt bridges. The water jacket is continuously circulating water at 37 °C powered by a water pump.

Figure 2: H&E stained representative image of an Ussing chamber preparation from mouse colon. The first panel shows a whole thickness (unstripped) preparation. The second panel

shows a mucosa and submucosal preparation (serosa-muscle layer has been stripped). The third panel shows a mucosal preparation. Scale bar represents 100 µm, magnification 20x.

Figure 3: The effect of deletion of claudin 15 on electrophysiological parameters. (**A**) Baseline transmucosal conductance in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 11, respectively). (**B**) Baseline short circuit current (I_{sc}) in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 11, respectively). (**C**) Dilution potential of NaCl in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 4, respectively). (**D**) The relative permeability of NaCl in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 4, respectively). The absolute permeabilities of Na^+ (**E**) and Cl^- (**F**) in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 4, respectively). (**G**) Forskolin-induced I_{sc} increment to measure tissue viability in *Cldn15*$^{-/-}$ and WT mice (n = 2 and 7, respectively). Values represent the mean ± SEM (where applicable).

DISCUSSION:

In this experiment, Ussing chambers were used to measure the baseline electrical parameters and the dilution potential of NaCl in the small intestine of *Cldn15*$^{-/-}$ and WT mice. It is very important when doing Ussing chamber experiments to verify that the membrane preparation used in the experiments is viable. This is usually done by adding glucose or the adenylate cyclase activator forskolin and seeing whether there is an appropriate rise in I_{sc} (100-300 µA/cm^2 in mice). Another way of assessing whether the intestinal preparation was acceptable for use is by looking at the conductance of the tissue. Damaged tissue will often have higher than normal conductance (normal conductance: ~20–60 mS/cm^2 in mouse), while tissue in which the muscle layer has not been adequately stripped will tend to have a lower-than-normal conductance (**Figure 2**). The most critical step in all Ussing chamber protocols, is the tissue preparation, and ensuring there is no damage to it. In addition, with the small intestine, ideally membrane preparations should be mounted in the chambers within 10 min of dissection to avoid break down of the tissue. After mounting the tissue, it is not a good idea to try and readjust the preparation, as damage may occur. During the experiments, when removing buffer from the chambers or adjusting the salt bridges or bubbling wands, avoid touching the intestinal preparation at all costs. Another important point for Ussing chamber experiments is proper heating of the chambers and adequate bubbling of the buffer solutions. When using Ringer solution, bubbling with 95% O_2/5% CO_2 is very important for the buffering system. In addition, bubbling that is too weak will not mix well enough and if the bubbling is too strong, the membrane and electrical measurements may be affected. If there is a hole in the intestinal preparation, dilution potential experiments will likely not have a large membrane potential difference (PD) after a dilution. If the PD is small after a dilution, it can be due to a hole in the intestinal preparation or it can indicate the tissue was not mounted properly in the chamber.

When performing Ussing chamber experiments, a number of things can go wrong. Most commonly, a bubble develops at the tip of a salt bridge. When this occurs, if it is the thin KCl salt bridge that is affected, the I_{sc} or the membrane PD will likely become very erratic and the amplifier may become over loaded. In this situation, first turn off the clamp mode. Then, using forceps, gently pinch the tip of the salt bridge until the bubble is removed. Be very careful not to touch the membrane while doing this. If the bubble develops at the tip of the thicker NaCl salt bridge, the conductance measurement will be affected. The procedure to remove the

bubble is the same. It is very important to monitor the recording of the data, to ensure there are no abnormalities in the data. In addition, it is important to realize that the parameters will not suddenly change without a stimulus, so if the data recording has sudden drastic changes, check the salt bridges and the connections of the electrodes.

Measuring the barrier function and ion selectivity of the tight junctions is not possible by other commonly used methods to assess paracellular permeability. A commonly used protocol to estimate paracellular permeability is oral gavage of FITC and measurement of plasma FITC at a later time[8]. This method measures the overall permeability of the entire intestinal tract to FITC. Moreover, FITC is likely transported via the leak pathways. Paracellular transport is believed to include the leak pathway and the pore pathway[28]. The leak pathway is a non-selective low capacity pathway for larger charged or uncharged molecules to pass through the tight junctions, while the pore pathway is a selective, high-capacity pathway that allows molecules to pass based on their size and charge[28]. FITC and other macromolecule tracers can assess the leak pathway, but they reveal no information about the ion or charge selectivity of a tissue. Oral gavage of FITC or other macromolecular tracers also does not give information about where in the intestine leakiness is occurring. In contrast, the method presented here can be used to assess the ionic selectivity of a specific segment of tissue, and specifically, it can estimate the relative permeability of specific ions. The leakiness of a tissue can be assessed by measuring the baseline conductance, and in leaky tissues such as the small intestine, >90% of the conductance is considered to be due to the contribution of the paracellular pathway[29]. Thus, the transepithelial conductance measurement gives information about the amount of paracellular ion movement, but it is not specific and does not reveal whether a tissue has cationic or anionic selectivity. To understand the permselectivity of the tight junctions, measurement of dilution potential is necessary. Here, *Cldn15*$^{-/-}$ mice were used, which lack the paracellular Na^+ pore-forming claudin 15. By measuring the membrane potential difference in response to dilution of NaCl, the ionic selectivity as well as the permeabilities of Na^+ and Cl^- can be calculated. As expected, *Cldn15*$^{-/-}$ mice had a decreased potential difference, and decreased P_{Na}/P_{Cl} (**Figure 3D**) compared to WT mice. Knockout of claudin 15 results in a lower cationic selectivity (P_{Na}/P_{Cl}) and decreased relative permeability of Na^+ (P_{Na}) (**Figure 3E**). In addition, the baseline electrical conductance was decreased in *Cldn15*$^{-/-}$ mice (**Figure 3A**), revealing that the TJ have become tighter, and the barrier function has increased.

While dilution potential is a very useful tool for assessing the permselectivity of the tight junctions as well as defining the role of claudin proteins, it has limitations. A major limitation of this technique is that it is an average of the tissue. Within the small intestinal epithelia, there are villi and crypts, and the parameters measured with Ussing chambers do not assess the contribution of villi and crypt epithelia separately. This could be a factor when assessing the role of certain selectively expressed claudins, for example, claudin 2 is also considered a cation pore, but it is only expressed in the crypts[24]. Another limitation is that due to the presence of several different claudin proteins in the tight junctions, the measurements cannot be specifically attributed to a certain protein. Although, dilution potentials have been very useful in exploring the roles of claudin family proteins in exogenous claudin expressing cell models[10,30]. Finally, Ussing chamber techniques use *ex-vivo* tissue, and this protocol uses intestinal

preparations without the muscle layer, meaning that the conditions are not the same as *in vivo* conditions. Preparing the intestine for Ussing chambers requires skill and time, so experiments in native tissue are often difficult to complete successfully, are low throughput, and take a lot of time.

The Ussing chamber is a very important tool that can be applied to many different epithelial and endothelial tissues. A major benefit is that it uses native tissue, which is much more reliable than cell line models, although cell monolayers and organoid monolayers can also be assessed in Ussing chambers. Ussing chambers have contributed to many great findings in membrane physiology, and they will continue to be an important tool in the future.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no potential conflicts of interest to disclose.

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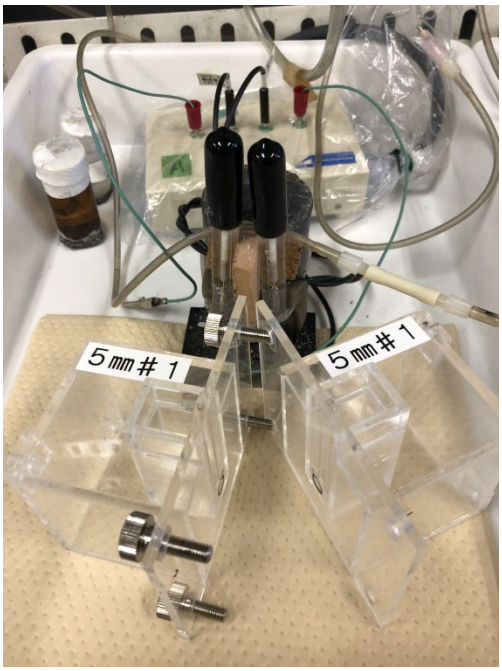
A



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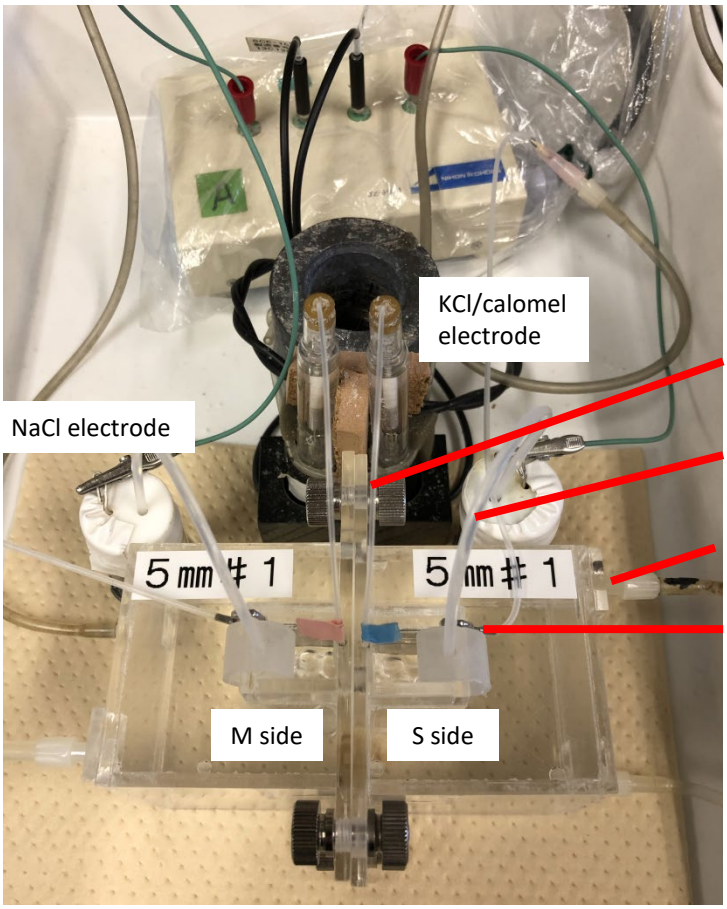
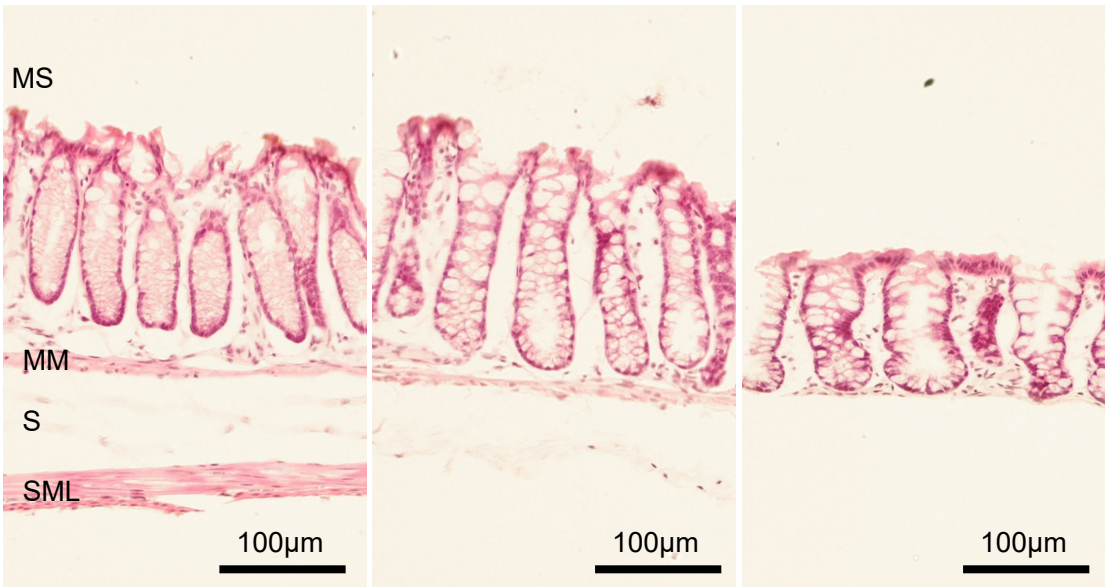


Figure 2



Whole thickness preparation

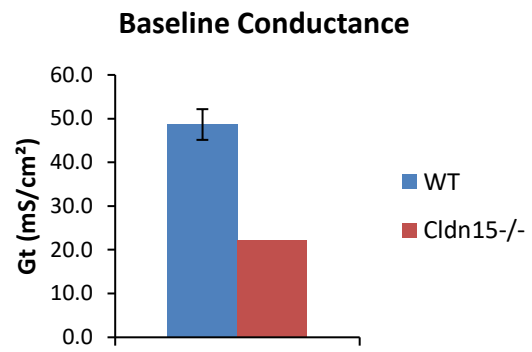
Mucosa and submucosal preparation

Mucosal preparation

MS: mucosal side
MM: muscularis mucosae
S: submucosa
SML: serosa-muscle layer

Figure 3

A



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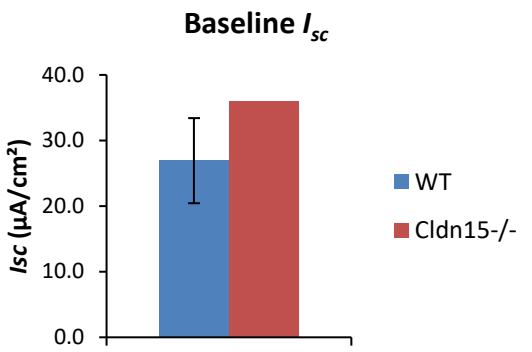

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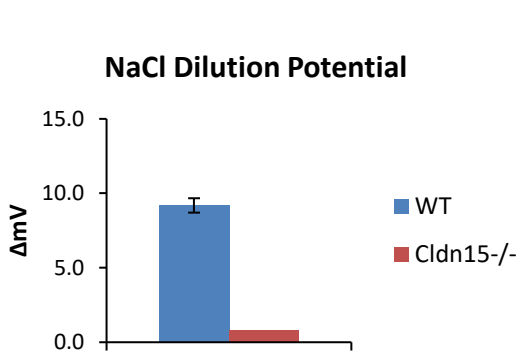

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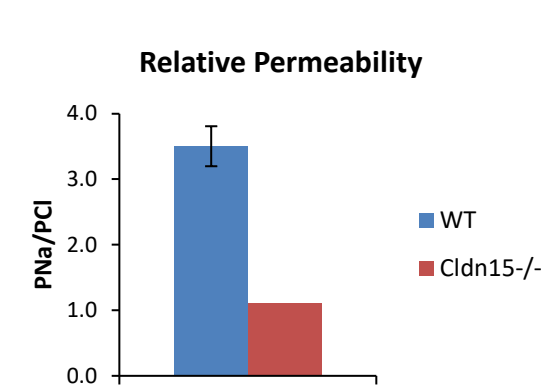

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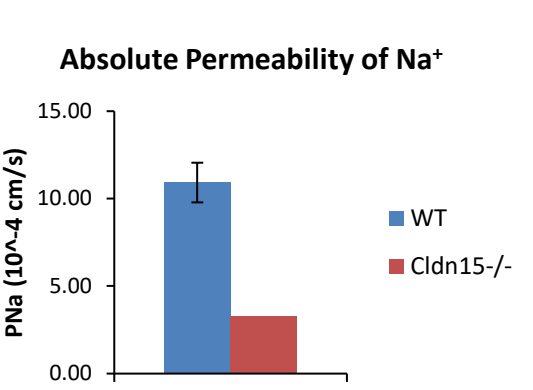

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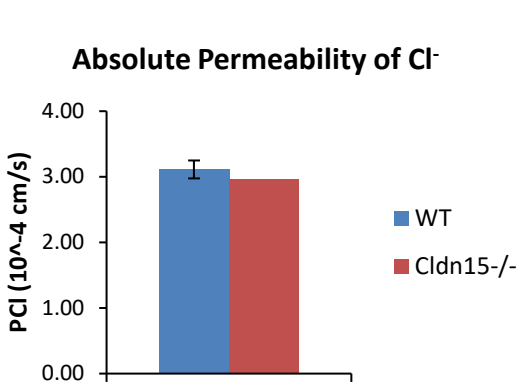

G

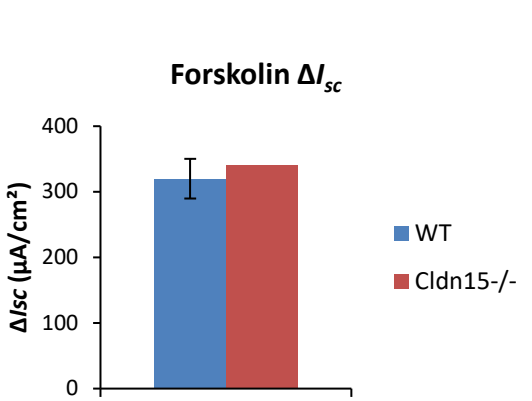

Table 1

Ringer solution (small Intestine)	**Ringer solution (large intestine)**
$NaHCO_3$ – 21.0 mM	$NaHCO_3$ – 21.0 mM
K_2HPO_4 – 2.4 mM	K_2HPO_4 – 2.4 mM
KH_2PO_4 – 0.6 mM	KH_2PO_4 – 0.6 mM
NaCl – 119.0 mM	NaCl – 119.0 mM
$MgCl_2$ – 1.2 mM	$MgCl_2$ – 1.2 mM
$CaCl_2$ – 1.2 mM	$CaCl_2$ – 1.2 mM
Indomethacin – 10 µM (Make 1 mM stock in 21 mM $NaHCO_3$, add 10 mL of stock for 1 L of Ringer solution)	Indomethacin – 10 µM (Make 1 mM stock in 21 mM $NaHCO_3$, add 10 mL of stock for 1 L of Ringer solution)
1 mM Glutamine (0.146 g/L)	10 mM Glucose

Table 2

HEPES Buffer	**Dilution HEPES Buffer**
HEPES – 10 mM	HEPES – 10 mM
Glucose – 10 mM (Large intestine) 1 mM Glutamine (0.146 g/L) (Small intestine)	Glucose – 10 mM (Large intestine) 1 mM Glutamine (0.146 g/L) (Small intestine)
NaCl – 150 mM	NaCl – 75 mM + 120 mM mannitol (to adjust for osmolality differences)
$MgCl_2$ – 1 mM	$MgCl_2$ – 1 mM
$CaCl_2$ – 2 mM	$CaCl_2$ – 2 mM
Indomethacin – 10 µM (Make 1 mM stock in 21 mM $NaHCO_3$, add 10 mL of stock for 1 L of Ringer Solution)	Indomethacin – 10 µM (Make 1 mM stock in 21 mM $NaHCO_3$, add 10 mL of stock for 1 L of Ringer Solution)
Adjust to pH 7.40 (37°C) using 1 M Tris	

Materials

Click here to access/download;Table of Materials;JoVE Materials list.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#3 polyethyl tubing	Hibiki		outer diameter 1.0 mm; inner diameter 0.5 mm
#7 polyethyl tubing	Hibiki		outer diameter 2.3 mm; inner diameter 1.3 mm
10 mL locking syringe	Terumo	SS-10LZ	Locking syringes are necessary to prevent the needle from dislodging during filling
19 g needle	Terumo	NN-1938R	Please use caution when working with needles and dispose of in sharps container
23 g needle	Terumo	NN-2332R	Please use caution when working with needles and dispose of in sharps container
5 mm punch	NA	NA	Use to punch holes in filter paper and parafilm
acupuncture needles	Seirin	NS	Used as dissection pins to pin tissue to dissection plate
Agar	Fujifilm Wako	010-15815	
Alligator clips	NA	NA	Connects the electrode to the amplifier
$CaCl_2$	Fujifilm Wako	038-00445	
D(-)-Mannitol	Fujifilm Wako	133-00845	This is used to correct for the osmolality difference in dilution HEPES buffer
D(+)-Glucose	Fujifilm Wako	049-31165	
Dissection kit			You will need, scissors and curved forceps
Dissection plates			We used 10 cm cell culture plates and covered with silicon rubber
DMSO	Sigma	472301-500ML	For making forskolin stock
Electrical recorder	TOA Electronics	PRR-5041	Other equivalent electrical recorders are available commercially
Epithelial voltage clamp amplifier	Nihon Kohden	CEZ9100	Other equivalent amplifiers are available commerically
filter paper, cut into squares	NA	NA	Punched with a 5 mm punch, used to hold intestinal preparation
fine forceps	Fast Gene	FG-B50476	For blunt dissection of the muscle layer
Forskolin	Alomone Labs	F-500	Make 10 mM stock in DMSO, final concentration will be 10 µM
HEPES	Sigma	H4034-1KG	
Indomethacin	Sigma	I7338-5G	Make a 1 mM stock in 21 mM NaHCO3, final concentration is 10 µM
K_2HPO_4	Fujifilm Wako	164-04295	
KCl	Fujifilm Wako	163-03545	
KCl/calomel electrode	Asch Japan Co.	SCE-100	
KH_2PO_4	Kanto chemical	32379-00	
L(+)-Glutamine	Fujifilm Wako	074-00522	
$MgCl_2$	Fujifilm Wako	135-00165	

Mixed Gas (95% O_2/5% CO_2)	Shizuoka Oxygen Company		Used for bubbling Ringer solution and chambers when using Ringer solution
NaCl	Fujifilm Wako	191-01665	
NaCl electrode	NA	NA	Handmade electrodes which require concentrated NaCl and Silver wire
$NaHCO_3$	Fujifilm Wako	191-01305	
O_2 Gas	Shizuoka Oxygen Company		Used for bubbling chambers when using HEPES buffer
parafilm	Bemis	PM-996	Used to help seal Ussing chambers
pH meter	DKK-TOA Corp	HM-305	HEPES buffer needs to be adjusted to pH 7.4 at 37 °C
pH meter electrode	DKK-TOA Corp	GST-5311C	
silicone rubber	Shinetsu Chemical	KE-12	Used to fill dissection plates
silver wire			Used for making NaCl electrodes
Small jars w/ plastic lids	NA	NA	Use for NaCl electrodes
stereomicroscope	Zeiss	Stemi 305	A stereomicroscope allows you to see depth, so you can dissect the tissue more easily
Tris (Trizma base)	Sigma	T1503-1KG	Make a 1M solution to adjust pH of HEPES buffers
Ussing chambers	Sanki Kagaku Kougei		These chambers are custom made continuous perfusion Ussing chambers with a window diameter of 5 mm
Water pump and heating system	Tokyo Rikakikai Co. Ltd.	NTT-110	

February 18, 2021

Dr. Vidhya Iyer
Review Editor
JoVE

Thank you for your letter regarding our manuscript submission ID JoVE62468 (EMID: 35bbf668817f8d97) "Functional assessment of intestinal tight junction barrier and ion permeability in native tissue by Ussing chamber technique". We have read the editorial board's and Reviewers' comments carefully and modified the text according to their suggestions. The changes to the manuscript in the revised version are indicated by red-colored characters.

An itemized list of the specific changes and additions made is detailed below:

Editorial comments:

1. As requested by the editor, we have thoroughly proofread the manuscript and are satisfied that spelling and grammar issues are at a minimum.
2. Lines 386-388 were revised to avoid previously published work. We changed it from "To determine the ion selectivities of the *Cldn15*$^{-/-}$ mice, we measured the dilution potentials across the small intestinal mucosa under a chemical gradient of NaCl (60 mmol/L NaCl at the mucosal side to 119 mmol/L NaCl at the serosal side)" to "In order to assess the selectivity of the small intestinal mucosa to NaCl in *Cldn15*$^{-/-}$ mice, the dilution potentials were measured with a concentration gradient (60 mmol/L NaCl on the mucosal side and 119 mmol/L NaCl on the serosal side)", which can be found in line 482-4 (red font) in the new document.
3. The text has been rewritten without personal pronouns.
4. Company names and commercial language have been removed from the manuscript.
5. Protocol numbering has been adjusted as per the JoVE instructions for authors.
6. An ethics statement has been inserted in the protocols section. It reads:

"Statement of Ethics:

All animals used in these experiments were maintained in the animal care facility at the University of Shizuoka and the experiments were conducted according to the

guidelines for animal research set out by the University of Shizuoka. All experiments were carried out with approval from the Animal Care and Use Committee at the University of Shizuoka (Permits #205272 and #656-2303)" and can be found in red text in lines 141-6.

7. Lines 136-140 have been removed and the information is listed in the table of materials.
8. Protocol steps have been revised to include more details about how to do the steps. Particularly in protocol step 2 and 3 (see text in red).
9. Information about glass jar size was inserted (see line 161). List of materials was also updated with the information.
10. The size of the holes drilled into the jar lids (line 163-4, red text) was added.
11. The volume of concentrated NaCl to add to the jar was specified (line 166, red text).
12. Wire dimensions and alligator clip size were specified in the protocol (line 168-9, red text) and the supplementary list of materials was also updated.
13. Lines 155-158 were shortened into a note, and lines 300-313 were moved to the last paragraph of the introduction (lines 125-36, red text).
14. Lines 195-200 were reformatted into discrete steps under section 3 of the protocol.
15. The anaesthetic agent, dose, and route of administration were specified in the protocol (Lines 294-7, red text) and the list of materials was updated.
16. The length of the incision cannot be specified as it is animal dependent, however the text was updated to say "Make an incision in the abdomen with scissors from the pelvis to the diaphragm, locate the stomach and cut the pyloric end of the stomach" (line 299-300, red text).
17. Stripping the muscle layer is a crucial step in the protocol, and it is very technically challenging and difficult, so we think that this explanation is an important preface to this step of the protocol and so we have turned it into a "note" (lines 322-330).
18. The size of the dissection plate and parafilm squares has been added to the protocol (line 332-4, red text).
19. The volume of Ringer solution has been specified (line 336-7, red text).
20. The units for "minutes" was changed as directed to "min".
21. Protocol has been highlighted in yellow, and the formatting of the protocol steps has been revised to include a one line space between each step.

22. The tables have been removed and will be resubmitted as table 1 and table 2. The descriptions of the tables have been added to the manuscript (lines 508-515, red text). Please note that "place table here" memos have been left in the place where they were before (protocol section 3).
23. References section has been changed to omit "&" and the journal names are no longer abbreviated.
24. Figure 1 has been removed and in its place a note directing the reader to the original publication (line 58-59, red text).
25. Scale bars have been added to figure 2 (formerly figure 3), and the legend has been updated (line 529, red text).

We trust that we have addressed the editorial comments adequately. Next we will respond to the Reviewers' comments.

Reviewer 1:

Minor Concerns: Reviewer #1 requested that we change our word usage from "make" to "preparation" and we have done so in lines 156 and 174-5 (red text). The reviewer also asked that we include the volume of the jars used for NaCl electrodes and we have done so in line 161 and 166 (red text).

We thank the reviewer for their thoughtful and useful comments.

Reviewer 2:

Specific Comments:

1. The reviewer commented about the size-selectivity properties of the tight junctions. Size selectivity can also be determined by dilution potential, but the protocol will differ from what we have presented here, which is a protocol for charge selectivity.
2. The reviewer commented that good hand skills are required to prepare a mucosal preparation. We agree with the reviewer that the instructions presented in this protocol are not adequate for the mucosal preparation. Our goal is the mucosal and submucosal preparation (middle panel). We have revised the protocol to make it more clear which preparation we are aiming to do in lines

328-330 (red text). Our intention is that this part of the protocol will be filmed to show the readers/viewers the serosal stripping technique.

3. The reviewer requested (if possible) that we provide a spread sheet with the Kimizuka-Koketsu and Goldman-Hodgkins-Katz equations. We are happy to comply with this request if the journal will accept it.

We thank Reviewer #2 for their valuable feedback and comments.

Having made the specified editorial changes and after addressing the reviewers' comment, we believe that the manuscript is now suitable for publication in JoVE.

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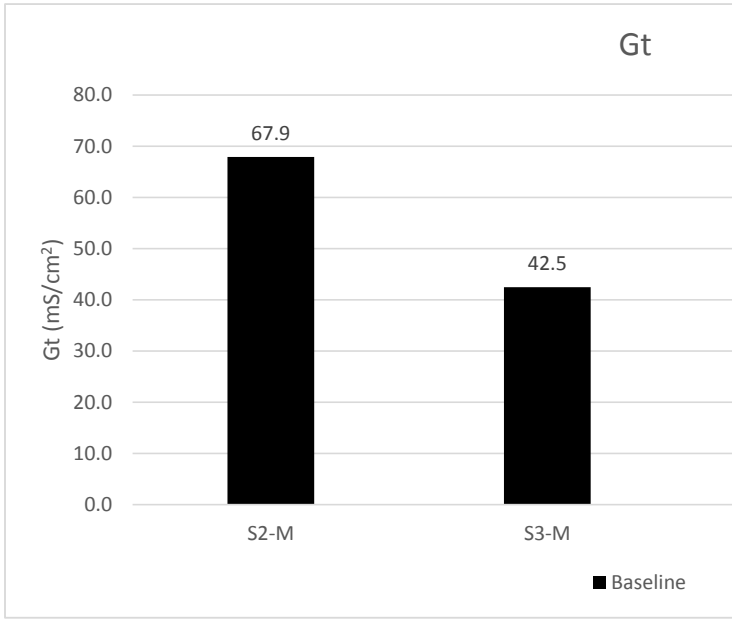
On behalf of all authors.

Date	Window area（cm²）→	0.19625	Pulse	V→	10	A→	40

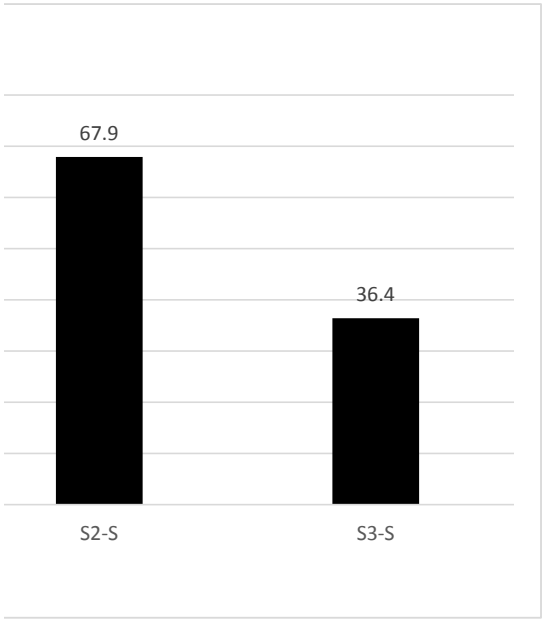
open

			baseline	
	S2-M	mV	3	
	S3-M		4.8	
	S2-S		3	
	S3-S		5.6	
	S2-M	mS	13.33333	
	S3-M		8.333333	
	S2-S		13.33333	
	S3-S		7.142857	
	0		#DIV/0!	
	0		#DIV/0!	↓Diameter(mm)
	S2-M	cm^2	0.19625	5
	S3-M		0.19625	5
	S2-S		0.19625	5
	S3-S		0.19625	5
	0		0.19625	5
	0		0.19625	5
	S2-M	mS/cm²	67.94	
	S3-M		42.46	
	S2-S		67.94	
	S3-S		36.40	
	0		#DIV/0!	
	0		#DIV/0!	



	Baseline
S2-M	67.9
S3-M	42.5
S2-S	67.9
S3-S	36.4

0	#DIV/0!
0	#DIV/0!


67.9
36.4
S2-S
S3-S

		baseline	Dilution	Difference
	Blank	0	0	**0**

Φ5mm

		mV			
		baseline	Dilution	Change	**-Blank**
mV	S2-M	0.3	-10	-10.3	**-10.3**
	S3-M	1.3	-9.5	-10.8	**-10.8**
	S2-S	-0.2	4.5	4.7	**4.7**
	S3-S	0.7	6.7	6	**6**

	E (mV)	BL Gt	Equivalent Isc
S2-M	0.3	67.94055	20.38
S3-M	1.3	42.46285	55.20
S2-S	-0.2	67.94055	-13.59
S3-S	0.7	36.39672	25.48

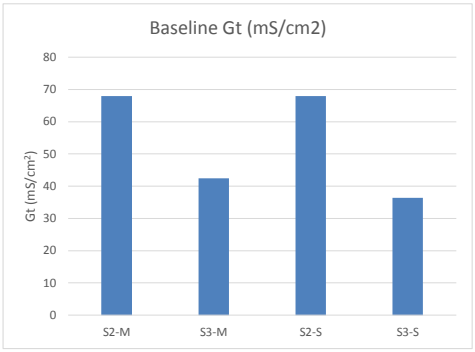
Concentration (mM)	Activity	Concentration×Activity
150	0.754	113.1
75	0.793	59.475
α	1.901639344	
F (C/mol)	96500	
R (J/K・mol)	8.314	
T (K)	310	←273+37
R*T	2577	

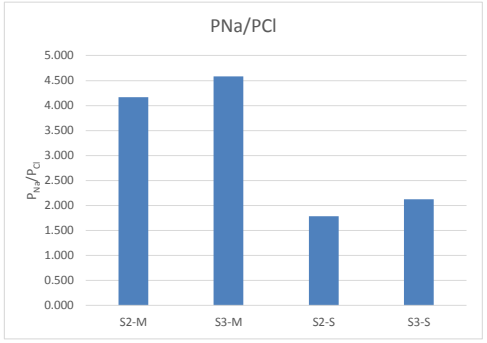
control

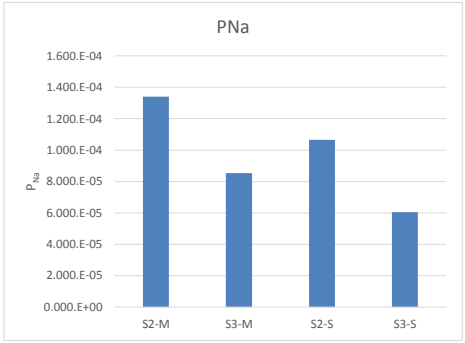
NaCl

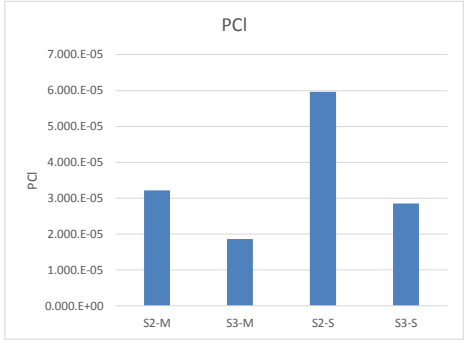
	S2-M	S3-M	S2-S	S3-S	0	0
baseline Gt (mS/cm^2)	67.94055202	42.46284501	67.94055202	36.39672429	#DIV/0!	#DIV/0!
Gt (S/m^2)	679.4055202	424.6284501	679.4055202	363.9672429	#DIV/0!	#DIV/0!
ΔV (mV)	-10.3	-10.8	-4.7	-6	0	0
V*F	-993.95	-1042.2	-453.55	-579	0	0
$e^{-vF/RT}$	1.470569213	1.498358832	1.192409448	1.251884756	1	1
PCl/PNa	0.240	0.218	0.560	0.471	1.000	1.000
PNa/PCl	4.168	4.586	1.787	2.125	1.000	1.000
numerator	175105.90	109441.19	175105.90	93806.73	#DIV/0!	#DIV/0!
denominator	1305935589	1282887526	1642527206	1548880460	2106430950	2106430950
PNa	1.341.E-04	8.531.E-05	1.066.E-04	6.056.E-05	#DIV/0!	#DIV/0!
numerator	175105.90	109441.19	175105.90	93806.73	#DIV/0!	#DIV/0!
denominator	5442509298	5882983976	2935517791	3291144058	2106430950	2106430950
PCl	3.217.E-05	1.860.E-05	5.965.E-05	2.850.E-05	#DIV/0!	#DIV/0!

Dilution	control					
	S2-M	S3-M	S2-S	S3-S	0	0
baseline Gt (mS/cm2)	67.94055202	42.46284501	67.94055202	36.39672429	#DIV/0!	#DIV/0!
ΔmV	-10.3	-10.8	-4.7	-6	0	0
PNa/PCl	4.168	4.586	1.787	2.125	1.000	1.000
PNa	1.341.E-04	8.531.E-05	1.066.E-04	6.056.E-05	#DIV/0!	#DIV/0!
PCl	3.217.E-05	1.860.E-05	5.965.E-05	2.850.E-05	#DIV/0!	#DIV/0!

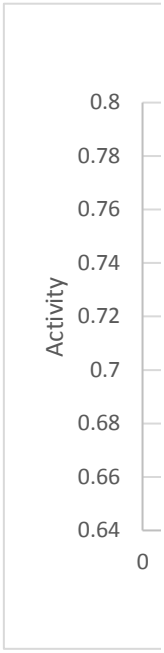


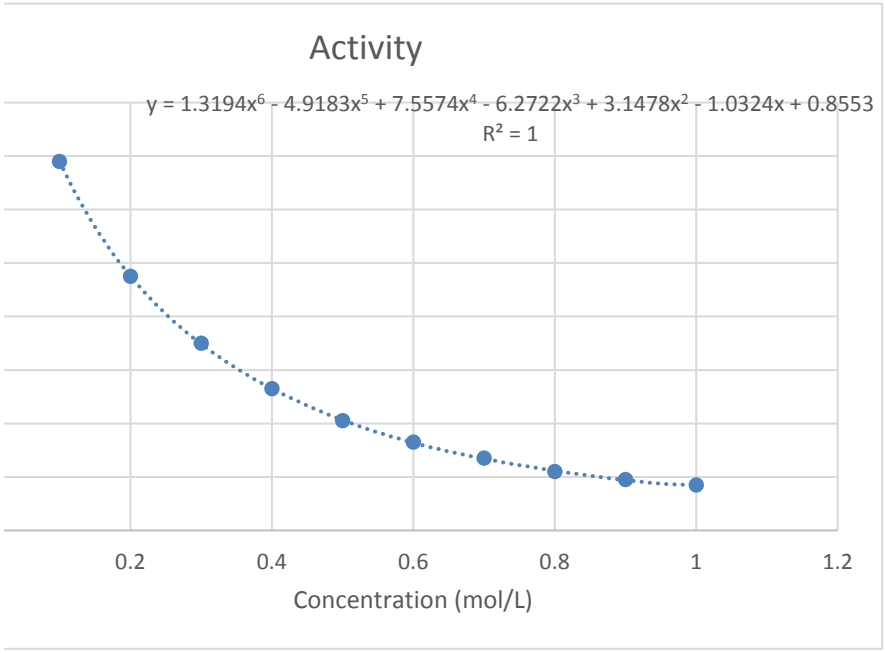






	x	y		
	Concentration (M)	Activity		
	0.1	0.778		
	0.2	0.735		
	0.3	0.71		
	0.4	0.693		
	0.5	0.681		
	0.6	0.673		
	0.7	0.667		
	0.8	0.662		
	0.9	0.659		
	1.0	0.657		
Concentration (mol/L)	0.119	0.060	0.075	0.150
Activity	0.768	0.803	0.793	0.754




Activity
y = 1.3194x⁶ - 4.9183x⁵ + 7.5574x⁴ - 6.2722x³ + 3.1478x² - 1.0324x + 0.8553
R² = 1
0.2
0.4
0.6
0.8
1
1.2
Concentration (mol/L)