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In vitro and in vivo approaches to determine intestinal epithelial cell permeability

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TITLE:

In Vitro and *In Vivo* Approaches to Determine Intestinal Epithelial Cell Permeability

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Intestinal barrier function, intestinal permeability, transepithelial electrical resistance, FITC-dextran, Caco-2BBE cells, gavage

SUMMARY:

Two methods are presented here to determine intestinal barrier function. An epithelial meter (volt/ohm) is used for measurements of transepithelial electrical resistance of cultured epithelia directly in tissue culture wells. In mice, the FITC-dextran gavage method is used to determine the intestinal permeability *in vivo*.

ABSTRACT:

The intestinal barrier defends against pathogenic microorganism and microbial toxin. Its function is regulated by tight junction permeability and epithelial cell integrity, and disruption of the intestinal barrier function contributes to progression of gastrointestinal and systemic disease. Two simple methods are described here to measure the permeability of intestinal epithelium. *In vitro*, Caco-2BBE cells are plated in tissue culture wells as a monolayer and transepithelial electrical resistance (TER) can be measured by an epithelial (volt/ohm) meter.

This method is convincing because of its user-friendly operation and repeatability. *In vivo*, mice are gavaged with 4 kDa fluorescein isothiocyanate (FITC)-dextran, and the FITC-dextran concentrations are measured in collected serum samples from mice to determine the epithelial permeability. Oral gavage provides an accurate dose, and therefore is the preferred method to measure the intestinal permeability *in vivo*. Taken together, these two methods can measure the permeability of the intestinal epithelium *in vitro* and *in vivo*, and hence be used to study the connection between diseases and barrier function.

INTRODUCTION:

Intestinal epithelial cells are not only responsible for the absorption of nutrients, but also form an important barrier to defend against pathogenic microorganisms and microbial toxins. This intestinal barrier function is regulated by tight junction permeability and epithelial cell integrity^{1,2,3}, and dysfunction of the epithelial barrier function is associated with inflammatory bowel disease (IBD). The perijunctional actomyosin ring (PAMR) lies within the cell that is closely contiguous to the tight junctions. The contraction of the PAMR, which is regulated by the myosin light chain (MLC), is crucial for the regulation of tight junction permeability⁴⁻¹⁰. Tumor necrosis factor (TNF) is central to intestinal barrier loss by upregulating intestinal epithelial MLC kinase (MLCK) expression and inducing occludin internalization¹¹⁻¹³.

Ions such as Na⁺ and Cl⁻ can cross the paracellular space by either the pore or leak pathway¹⁴. In a “leaky” epithelium, changes in TER primarily reflect altered tight junction permeability. TER measurement is a commonly used electrophysiological approach to quantify tight junction permeability, primarily to Na⁺ and Cl⁻, based on the impedance of cell monolayers. Diverse cell types, including intestinal epithelial cells, pulmonary epithelial cells, and vascular endothelial cells, have been reported for TER measurements. Advantages of this method are that TER measurements are non-invasive and can be used to monitor live cells in real-time. In addition, the TER measurement technique is useful for drug toxicity studies¹⁵.

Caco-2BBE cells are human epithelial colorectal adenocarcinoma cells with a structure and function similar to the differentiated small intestinal epithelial cells: for example, these cells have microvilli and enzymes associated with small intestinal brush border. Therefore, cultured Caco-2BBE monolayers are utilized as an *in vitro* model for testing barrier function.

In mice, one way to study intestinal paracellular permeability is by measuring the ability of FITC-dextran to cross from the lumen into the blood. Thus, the intestinal permeability can be assessed by gavaging FITC-dextran directly into mice and measuring the fluorescence within the blood. The following protocol describes two simple methods to assess intestinal epithelium permeability both *in vitro* and *in vivo*.

PROTOCOL:

This study was approved by the Animal Care and Use Protocol of Cambridge-Suda Genomic Resource Center (CAM-SU), Soochow University.

1. Plating and Maintenance of Caco-2bbe on Porous Polycarbonate Membranes

1.1. Grow cells in a T75 flask with media (DMEM containing 10% FBS). Flasks should be fed regularly, depending on the cell density.

Note: For optimal plating, cells should divide rapidly and have a flat “fried-egg” shape, which indicates that cells are in the growth phase.

1.2. Once cells are 80% confluent, take the flask out of the incubator and remove the media. Rinse any residual media with 1 - 2 mL of sterile PBS (without Ca^{2+}). Pipet 1.5 mL Trypsin-EDTA into the flask and gently rock the flask; then, place the flask in the 37 °C incubator for 20 min without rocking.

1.3. While the cells are trypsinizing, place inserts containing porous polycarbonate membranes (pore size, 0.4 μm ; surface area, 0.33 cm^2 ; see **Table of Materials**) into 24-well plates. Add 1.0 mL of culture media in the basal chamber (the lower space of the membrane).

1.4. Pipet 5 mL of media into the flask and vigorously pipet the cells against the side of the flask 5 - 10 times to achieve loose, individual cells or 2 - 3 cell clumps.

1.5. Plate 0.166 mL of cells (achieving a 1:8 dilution ratio) into the apical chamber (the upper space of the membrane). Incubate at 37 °C for up to 3 weeks.

1.5.1. Feed cells three times weekly by carefully aspirating the media from the basal compartment of each well using a pressure pump. Gently drip 1 mL of media into the apical chamber of each insert.

2. Use of Epithelial Meter (Volt/Ohm) for Measuring TER

Note: After about 3 weeks of culture on polycarbonate membranes, Caco-2BBE cells are ready for TER measurement.

2.1. For cytokine studies, one day before measurement, replace the basal media with media containing 10 ng/mL of $\text{IFN}\gamma$. On the day of the experiment, replace the media with HBSS containing 2.5 or 7.5 ng/mL of TNF.

Note: $\text{IFN}\gamma$ treatment increases expression of TNF receptor 2 (TNFR2)¹⁶.

2.2. To correct the meter, insert the correction electrode into the Input port, and choose “Ohm” mode. Adjust the R Adj screw with a screwdriver until the meter displays a reading of 1,000 Ω .

2.3. Sterilize the electrodes by placing them in 70% ethanol for 15 - 30 min, and then allow them to air dry for 15 s. Rinse the electrode in the experimental cell culture media.

2.4. Turn on the power, and choose the “Ohm” mode. Carefully place the long ends of the electrode bridges into the basal chamber and the short ends into the apical chamber. Ensure

that the longer electrodes touch the bottom of the dish, while keeping the shorter electrodes below the surface of the media but above the tissue culture inserts. Keep the electrodes vertical.

2.5. Measure the resistance of the sample inserts and blank inserts (*i.e.*, the culture inserts without cells but with HBSS) at 0, 1, 2, 3, 4 h after cytokine treatment. Record the resistance.

2.6. To achieve consistency across different plate formats, calculate the product of the resistance and the effective membrane area:

$$\text{Unit Area Resistance } (\Omega \cdot \text{cm}^2) = \text{Resistance } (\Omega) \times \text{Effective Membrane Area } (\text{cm}^2)$$

For 24-well inserts, the effective membrane area is 0.33 cm².

3. Murine Model of Dextran Sulphate Sodium (DSS)-induced Colitis

3.1. Add DSS to autoclaved water to a final concentration of 3.5% (wt/vol). Replace drinking water in each mouse cage with the DSS solutions. Administer 3.5% DSS to 8-week-old male C57BL/6 mice for a total of 7 days. Give regular drinking water without DSS to control mice.

3.1.1. Switch the DSS-containing water to regular drinking water after day 7.

3.2. Weigh mice and assess clinical scores of each mouse every day. Scores are defined according to the disease severity by four parameters: rectal prolapse (0 - 2), stool consistency (0 - 2), bleeding (0 - 2), and activity (0 - 2)⁵. Sum the scores from these parameters for a final clinical score.

3.3. To analyze the histopathological condition of colon tissue, euthanize mice by intraperitoneal (i.p.) injection with 1.2% (vol/vol) Avertin (0.6 mL/10 g body weight) 7 days post-DSS treatment.

3.4. Isolate the colon and cecum, and measure the length of the colon⁵.

3.5. Cut 0.5 cm segments from the distal colon and fix in a 15 mL falcon tube containing 10 mL of 10% formalin overnight. Wash the fixed tissues with graded ethanol (75, 95, and 100%) and xylene. Embed the tissues in paraffin and cut 6 mm sections for the hematoxylin & eosin staining⁸.

4. Measuring the Epithelial Barrier Permeability in DSS-induced Colitis Mice

4.1. Measure the barrier permeability 7 days after the start of DSS administration.

4.2. On the day of the assay, fast mice for 3 h.

4.3. Autoclave a gavage needle to ensure sterility, then gavage mice with 150 µL 80 mg/mL 4

kDa FITC-dextran in sterile water, and keep the unused FITC-dextran to measure the standard curve after serum collection. Weigh the mice for the permeability calculation.

Note: The FITC-dextran solution should be made in water.

4.4. After 4 h, anesthetize the mice by i.p. injection of 1.2% (vol/vol) Avertin (0.3 mL/10 g body weight). Confirm proper anesthetization by the lack of reflexes and whisker movement. Place the mice on a heat block for 5 min.

4.5. Clip a 1 cm piece of the tail by a razor blade, and collect 100 μ L of blood from the tail into serum collection tubes. Spin the collected blood at 10,000 x g for 10 min at room temperature.

4.6. Dilute the serum 1:4 in water. To make a standard curve, dilute unused FITC-dextran with water at 1:300, 1:1,000, 1:3,000, 1:10,000, 1:30,000, 1:100,000, 1:300,000, 1:1,000,000, and 1:3,000,000. Add 100 μ L/well of the serum and the standard curve samples into 96-well plates.

4.7. Read the fluorescence in a plate reader with 485 excitation/528 emission. Calculate the permeability values based on the standard curve, and multiply by 4 to correct for the dilution.

4.8. Divide the concentration of FITC-dextran by the weight to normalize the values (this helps normalize the difference in FITC-dextran delivery if mice are sick and have lost weight).

REPRESENTATIVE RESULTS:

In culture, Caco-2BBE cells grow as a monolayer and slowly differentiate into mature absorptive enterocytes that have brush borders. In this protocol, Caco-2BBE cells were plated with a high density on polycarbonate membranes, and cells reached 100% confluency one day after seeding. However, cells are undifferentiated at this stage: To fully differentiate the cells, the media is changed every 2 - 3 days for 3 weeks. Cells were stained with nuclei and F-actin stains to show the differences between undifferentiated and differentiated cells. Stress fibers are clearly seen in undifferentiated cells. Compared with undifferentiated cells, differentiated cells have a smaller volume, larger nuclei, and fewer stress fibers (**Figure 1**).

TNF is central to intestinal barrier loss through MLCK-dependent tight junction regulation. As shown in **Figure 2**, TNF significantly decreases the TER of the Caco-2BBE monolayer in a dose-dependent manner, suggesting that TNF increases epithelial permeability.

Administration of DSS induces acute colitis in mice. Mice treated with DSS lose weight significantly compared to their initial body weight (**Figure 3A**). The severity of colitis is scored by rectal prolapse, stool consistency, bleeding, and activity (**Figure 3B**). Cross sections of colonic tissues stained with hematoxylin & eosin show colonic mucosal damage in DSS treated mice (**Figure 3C**). The length of colon crypt is decreased in DSS treated mice (**Figure 3D**). The colon length is shortened in DSS treated mice (**Figure 3E**). As seen in **Figure 3F**, there is approximately a 2-fold increase in FITC-dextran levels in DSS treated mice compared to control mice.

FIGURE LEGENDS:

Figure 1. Caco-2BBE cells cultured on polycarbonate membranes. Cells cultured 1 day (undifferentiated) and 3 weeks (fully-differentiated) after plating are stained with Hoechst 33342 (blue) for the nucleus and Alexa Fluor 594 Phalloidin for F-actin (red). Scale bar = 20 μ m.

Figure 2. TNF reduces the TER of the Caco-2BBE monolayer. Cells are primed with 10 ng/mL of IFN γ overnight. On the next day, the culture medium is replaced with HBSS containing 2.5 or 7.5 ng/mL of TNF. TER is measured by an epithelial meter (Volt/Ohm) at the indicated time points. Values are mean \pm SEM, n = 4. Significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$), as compared to TER values of cells without TNF treatment, by two-tailed t -test.

Figure 3. DSS-induced colitis in mice. C57BL/6 male mice are given 3.5% DSS in drinking water for 7 days. Body weight (**A**) and clinical scores (**B**) are assessed daily for each group (mean \pm SEM, n = 4 for each group). Control mice received water without DSS. (**C**) Histological analysis of colonic tissue sections collected on day 7 post-DSS treatment. (**D**) Colon crypt lengths are measured on day 7 post-DSS treatment. (**E**) Colon lengths are measured on day 7 post-DSS treatment. (**F**) Permeability of colon epithelium is measured on day 7 post-DSS treatment by FITC-Dextran gavage. Values are mean \pm SEM, n = 4. Significant differences are indicated by * ($p < 0.05$ by two-tailed t -test).

DISCUSSION:

There are several critical steps in the protocol. Caco-2BBE (brush border-expressing) cells are always used for TER measurement, selected from the Caco-2 cell line for expression of brush-border proteins. Caco-2BBE cells have a villus absorptive phenotype when fully-differentiated (after about 3 weeks of culture post-confluence)¹⁷. It is necessary to avoid contamination during the measurement, and to sterilize the electrode. Because the procedure is non-sterile, measurements can only be performed for up to 8 h in most cases. During the measurement, we determined the resistance value of the two points inside and outside the insert. The resistance measurements at various sites can sometimes greatly differ because of differences in the cell state; This is a major limitation of the method. However, this variation can be significantly reduced by statistical methods and other methods. The TER method can be applied to other cells, such as determining the barrier integrity of pulmonary epithelial cells and vascular endothelial cells. In addition, TER measurements can be used in drug toxicity studies.

Administration of indigestible macromolecules, which enter the intestinal epithelium through diffusion, can be used to evaluate the intestinal permeability. The advantage of this method is that the barrier function of the intestine can be examined *in vivo*. There are several types of markers that can be used, including radioisotopes, polyethylene glycols, and sugars¹⁸. However, several factors should be considered for precise measurement of the permeability: (1) the position of gut barrier abnormality cannot always be precisely determined; and (2) the mucosal blood flow and gastrointestinal motility might affect the absorption and the excretion of the macromolecules.

In this study, 4 kDa FITC-dextran was administered intragastrically in mice and the amount of FITC-dextran in the mouse serum was measured. This method has numerous advantages. Oral gavage reduces possible risks that are associated with using enemas. For instance,

unintentional impairment to the colonic epithelium may be caused by rectal instillation, leading to a false-positive result. Additional effects caused by stress should also be eliminated. Permeability is known to increase when the mice are stressed. It is recommended to conduct a preliminary experiment at least one week prior to the actual experiment. Performing a practice experiment will help minimize experimental noise for the real experiment. A wild-type control (no treatment) should always be included.

FITC-dextran travels through the small intestine and begins to enter the colon 3 h after gavaging. Thus, 3 h is an ideal time point for measuring small intestinal permeability. For measurement of colonic permeability, the time can be extended, but this then becomes a balance between the leakage of FITC-dextran into the lumen and clearance of these molecules from the blood stream. 4 h is generally accepted as a good time point for colonic measurements.

In conclusion, two separate methods were described to determine intestinal epithelial cell permeability both *in vitro* and *in vivo*. Whereas TER measurement and FITC-dextran gavage methods both measure paracellular permeability, they each provide independent and distinct properties of the paracellular permeability.

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DISCLOSURES:

The authors declare no competing financial interests.

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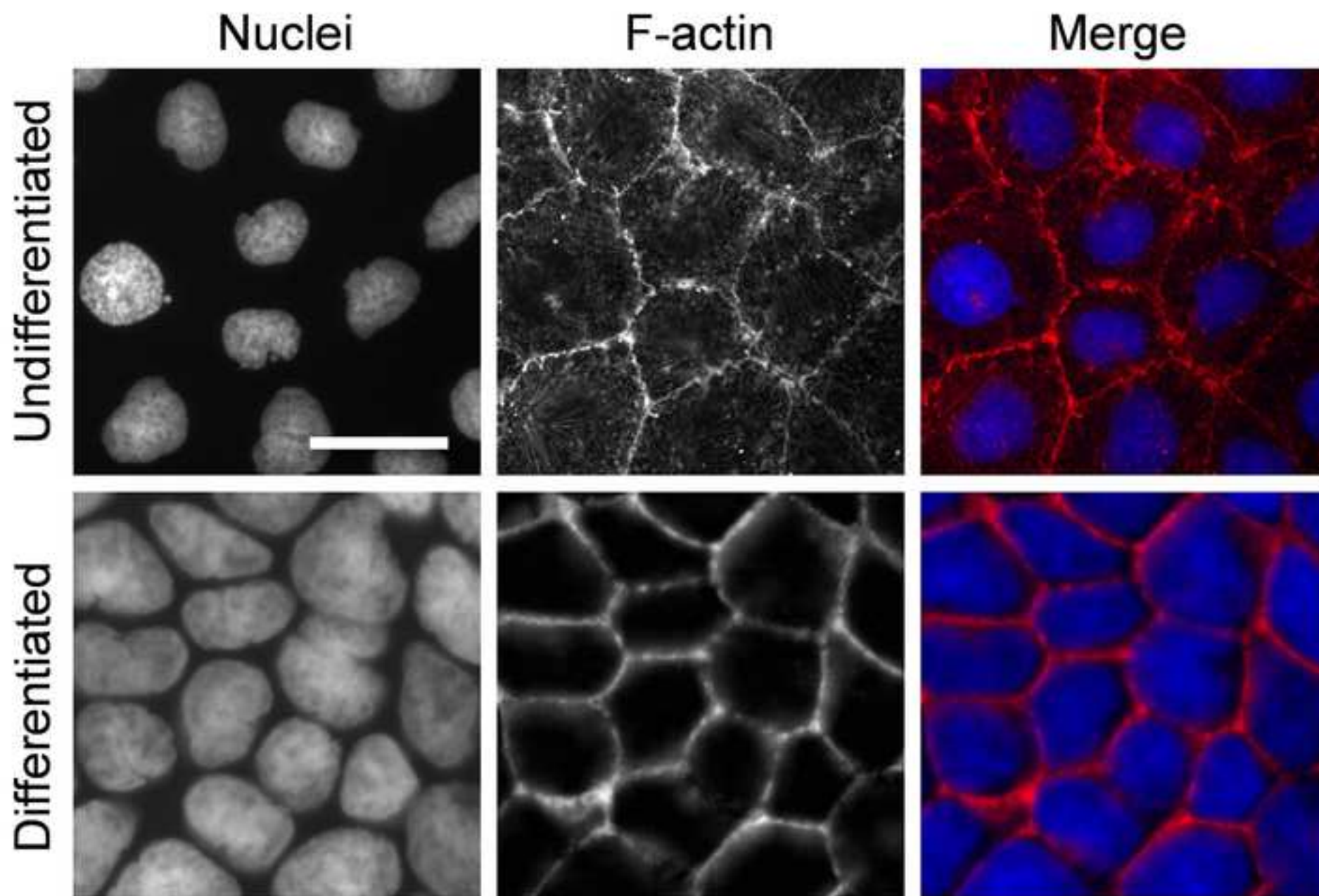
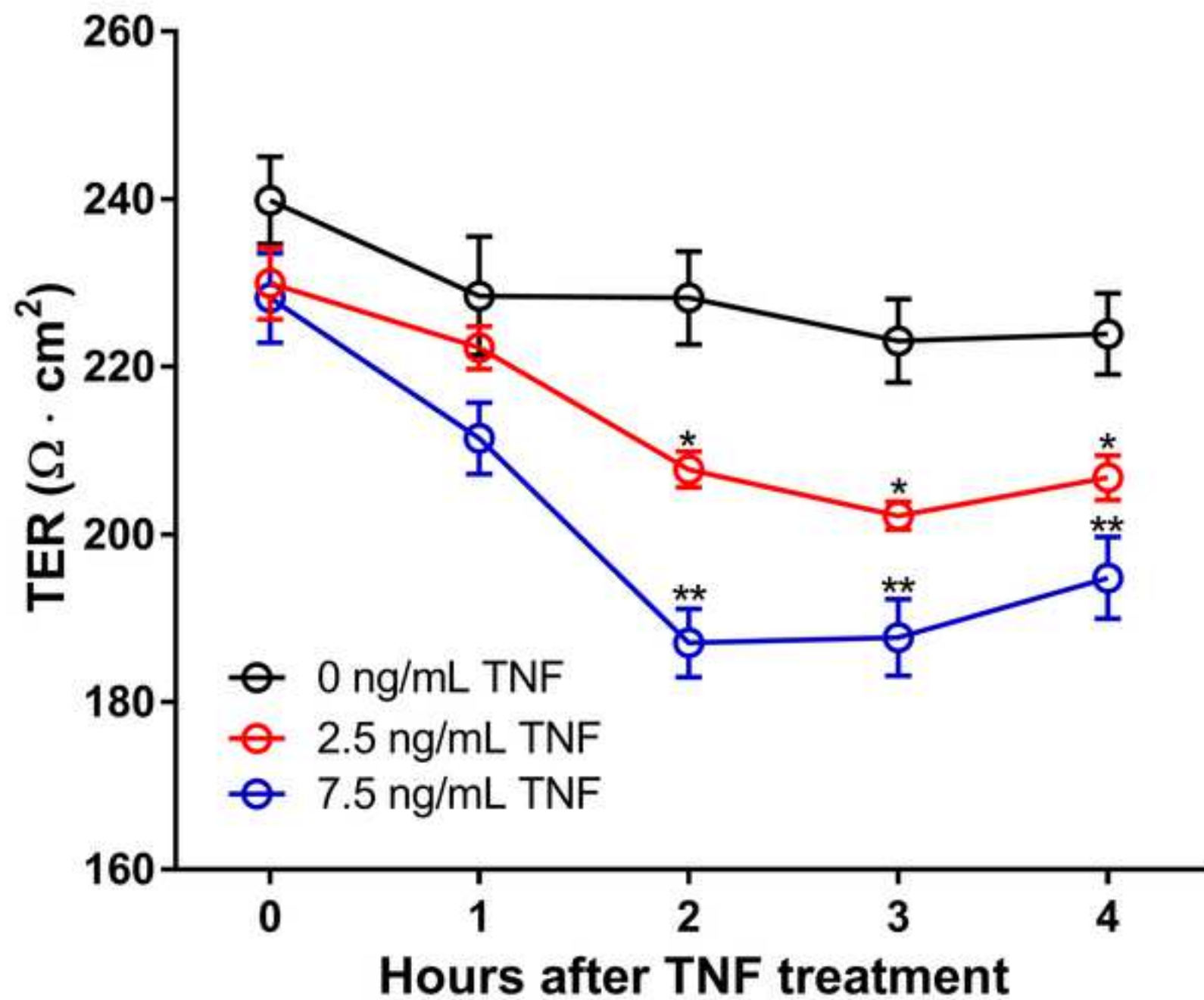
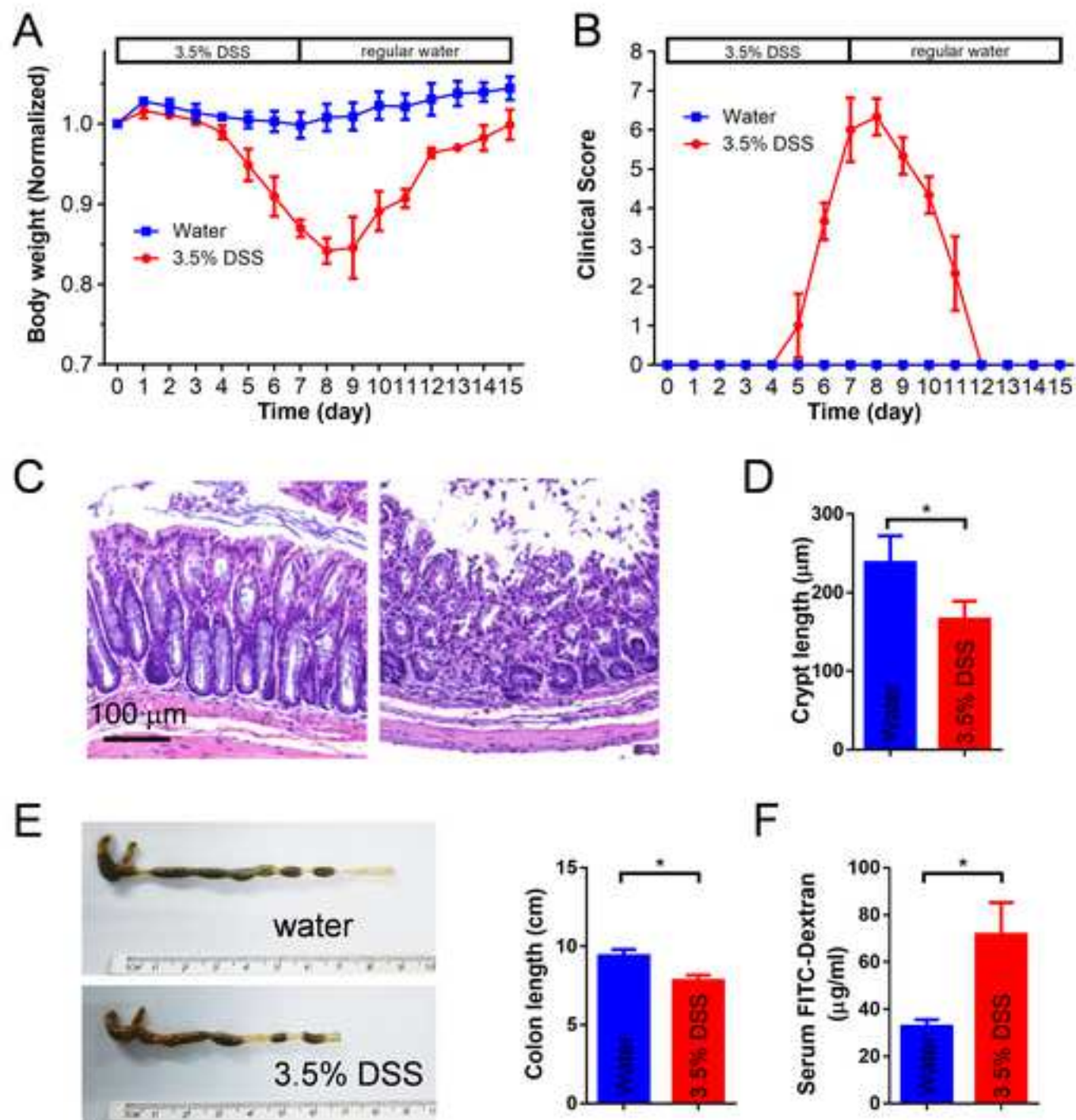


Figure 2





Name of Material/ Equipment	Company	Catalog Number
22G gavage needle	VWR	20068-608
4 kDa FITC-dextran	Sigma	46944
Avertin	Sigma	T48402
Black 96-well plates for fluorescence	Fisher	14-245-197A
C57/B6 mice	Nanjing Biomedical Research Institute of Nanjing University	
Caco-2BBe cells	ATCC	CRL-2102
Dextran sulphate sodium	MP Biomedicals	2160110
DMED with high glucose and sodium pyruvate	Hyclone	SH30243.01B
Epithelial (Volt/Ohm) Meter	Millicell-ERS	MERS00002
Ethanol	Sinopharm Chemical Reagent	10009218
Falcon tube (15 mL)	Corning	430791
FBS	Gibco	10437-028
Fluorescence microscope	Olympus	FV1000
Fluorometer	Biotek	Synergy 2
HBSS		
IFN γ	PeproTech	315-05-20
Modular Tissue Embedding Center	Leica	EG1150H
Serum collection tubes	Sarstedt	41.1378.005
T75 flask	corning	430641
TNF	PeproTech	315-01A
Parraffin	Sigma	A6330-1CS

Polycarbonate membranes (Transwell)	Costar	3413
Pressure pump	AUTOSCIENCE	AP-9925
Rotary Microtomy	Leica	RM2235
Trypsin-EDTA	Gibco	25200-056
Xylene	Sinopharm Chemical Reagent	10023418

Comments/Description
138 mM NaCl, 0.3 mM Na ₂ HPO ₄ , 0.4 mM MgSO ₄ , 0.5 mM MgCl ₂ , 5.0 mM KCl, 0.3 mM KH ₂ PO ₄ , 15.0 mM HEPES, 1.3 mM CaCl ₂ , 25 mM glucose



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Dear Dr. Steindel,

We have responded with text editing to address the raised issues. Thanks for your careful reviews.

Kind regards,
Wei-Qi He
Soochow University

Editorial comments:

1. Note that some edits have been made to correct some language errors and streamline the protocol. Please proofread to remove any other errors.

[Response: We have proofread the manuscript.](#)

2. Protocol, 1.6: What vacuum? Something like a normal laboratory line?

[Response: Yes. It is a laboratory line \(pressure pump\). We have added this information in the Table of Materials.](#)

3. Protocol, 3.2: Please describe what these clinical scores mean (severity? What exactly does, say, 'activity' mean?) or provide a reference.

[Response: We have added a reference.](#)

4. Protocol, 3.4: Why is anesthetization confirmed here, if this is a euthanasia step? Should that action be in 4.4 instead? Also, should 'beard' be 'whisker'?

[Response: It should be in 4.4. We have amended this. Thanks.](#)

5. Protocol, 3.4/4.4: Why is the same amount of Avertin used for euthanasia and anesthesia?

[Response: We have modified the description after checking the amount of Avertin.](#)

6: Figure 2: Do the double stars in fact indicate what the single stars do? The legend has been edited as such.

[Response: We have corrected this error. Significant differences are indicated by * \(\$p < 0.05\$ \) and ** \(\$p < 0.01\$ \), as compared with TER values of cells without TNF treatment.](#)