Video Article

Investigating Intestinal Barrier Breakdown in Living Organoids

Marco Bardenbacher[1], Barbara Ruder[2], Natalie Britzen-Laurent[1], Elisabeth Naschberger[1], Christoph Becker[2], Ralph Palmisano[3], Michael Stürzl*[1], Philipp Tripal*[1,3]

[1]Division of Molecular and Experimental Surgery, Department of Surgery, Translational Research Center, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg and Universitätsklinikum Erlangen

[2]Department of Medicine 1, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg and Universitätsklinikum Erlangen

[3]Optical Imaging Centre Erlangen (OICE), Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg

*These authors contributed equally

Correspondence to: Michael Stürzl at Michael.Stuerzl@uk-erlangen.de

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Abstract

Organoids and three-dimensional (3D) cell cultures allow the investigation of complex biological mechanisms and regulations in vitro, which previously was not possible in classical cell culture monolayers. Moreover, monolayer cell cultures are good in vitro model systems but do not represent the complex cellular differentiation processes and functions that rely on 3D structure. This has so far only been possible in animal experiments, which are laborious, time consuming, and hard to assess by optical techniques. Here we describe an assay to quantitatively determine the barrier integrity over time in living small intestinal mouse organoids. To validate our model, we applied interferon gamma (IFN-γ) as a positive control for barrier destruction and organoids derived from IFN-γ receptor 2 knock out mice as a negative control. The assay allowed us to determine the impact of IFN-γ on the intestinal barrier integrity and the IFN-γ induced degradation of the tight junction proteins claudin-2, -7, and -15. This assay could also be used to investigate the impact of chemical compounds, proteins, toxins, bacteria, or patient-derived probes on the intestinal barrier integrity.

Video Link

The video component of this article can be found at https://www.jove.com/video/60546/

Introduction

Integrity of the epithelial barrier is maintained by the apical junctional complex (AJC), which consist of tight junction (TJ) and adherence junction (AJ) proteins[1]. The polarized structure of the AJC is crucial for its function in vivo. Dysregulation of the AJC is present in various diseases and is suspected to be an important trigger of inflammatory bowel pathogenesis. Loss of intestinal barrier function represents the initiating event of the disease. The following translocation of commensal bacteria and inflammatory responses are the painful consequences[2].

Various in vitro and in vivo models have been developed to investigate the regulation of the AJC. The Transwell assay is based on two-dimensional (2D) cell monolayers that were derived from tumor cell lines. These systems are good to assess by optical and biochemical methods and enable the analysis of many samples at the same time but lack many features of primary cells and the differentiation processes present in vivo. Investigating the barrier integrity is also possible in animal models. In terminal experiments, the effects of specific treatments in vivo on the permeability of the whole intestine can be quantified. However, these models require a large number of animals, and they do not allow detailed visualization of the underlying molecular processes. Nowadays improved 3D in vitro models are available that closely recapitulate cell differentiation processes, cell polarization, and represent the crypt-villus structure of the intestine[3]. The application of 3D intestinal organoids for functional analyses requires the adaptation of available methods from 2D models. Here we describe a model to investigate intestinal barrier integrity in living small intestinal mouse organoids. The assay was established to investigate the effect of IFN-γ on the barrier integrity and respective tight junction proteins[8].

In contrast to the technique applied by Leslie[4], Zietek[5], or Pearce[6], which measures fluorescence after removing lucifer yellow (LY) from the medium, our approach allows quantification of the luminal uptake of the fluorophore over time. Therefore, the result represents a dynamic uptake kinetic and our assay enables the application of additional stimuli or inhibitors during the course of the experiment. The fact that both assays measure the uptake from the outside basolateral side to the inside apical surface is in clear contrast to the situation in vivo. In a model described by Hill et al.[7], this topic was explored. Upon microinjection of the fluorophore into the organoid's lumen, the fluorescence was quantified. The direction of diffusion represents the direction present in vivo. The technical effort of microinjection clearly reduces the throughput of this method. In contrast to the model described here, the microinjection method enables the measurement of effects that require biological activation on the apical epithelial surface.

The organoid barrier integrity model presented here is based on live cell microscopy and enables the analysis of dynamic changes within the AJC regulation over time. The setup can be applied to test the pharmacological impact of substances inducing and inhibiting the integrity of the intestinal barrier. Furthermore, organoid-based models help reduce the number of animals used for pharmacological studies.

Protocol

All steps were completed in accordance and compliance with all relevant regulatory and institutional animal care guidelines.

1. Plating of Organoids

1. **Isolate organoids as described previously[3]. The procedure is briefly described below.**
    1. Collect the small intestines from mice.
    2. Open the small intestines longitudinally and remove villi tips by scraping the inner intestinal tissue with a coverslip.
    3. Cut the intestinal tissue in small pieces using scissors.
    4. Wash pieces 5x in cold phosphate-buffered saline (PBS) by pipetting the pieces 10x up and down with a 25 mL pipette.
    5. Incubate the tissue pieces in cold 2 mM EDTA solution on ice for 30 min on a horizontal shaking platform. Allow the tissue pieces to sediment.
    6. Replace the EDTA solution with PBS buffer once the tissue pieces settle at the bottom. Discard the supernatant and add 20 mL of PBS.
    7. Release the intestinal crypts from the tissue by vigorously pipetting 10x up and down with a 10 mL pipette.
    8. Collect the supernatant in centrifugation tubes and inspect it by phase contrast microscopy. To do this, add a drop of the supernatant to a 96 well cell culture plate. Keep centrifugation tubes on ice.
    9. Repeat steps 1.1.6–1.1.8 until the number of intestinal crypts in the collected supernatant decreases.
    10. Pass the fractions containing the most crypts through a 70 µm cell strainer.
    11. Centrifuge the crypt suspension at 300 x *g*, 4 °C for 5 min.
    12. Discard the supernatant and resuspend the pellet in cold PBS in order to wash the crypts. Then repeat the centrifugation step as described in 1.1.11.
    13. Resuspend the pellet in a total of 25 µL per well of a 1:1 mixture of cell matrix solution and murine organoid culture medium and plate the organoids in a 48 well cell culture plates.
    14. Incubate the organoids at 37 °C, 5% CO_2, for 20 min to allow the cell matrix solution to solidify.
    15. Cover the organoids with 300 µL of murine organoid medium per well.
    16. Culture the organoids at 37 °C, 5% CO_2, changing the medium every 2–3 days.
    17. Use the organoids for experiments after 7 days of culture.

2. **Prepare the organoids for the barrier integrity measurement.**
    1. Precoat all the centrifugation tubes that will be used for storing the organoids during the plating process with bovine serum albumin (BSA) by adding enough of a 0.1% BSA solution in PBS to cover all plastic surfaces. Then remove the BSA solution again and store the centrifugation tubes on ice.
    2. Thaw the cell matrix solution and organoid culture medium on ice.

3. To separate the organoids, carefully remove the culture medium and resuspend the organoids from one well of a 48 well plate in 1 mL of cold PBS. Dissolve the cell matrix by vigorous pipetting. Always keep the organoid suspension in centrifugation tubes precoated with BSA and always keep on ice.
   NOTE: The density, size, and position of the organoids within the chambered coverslip slide are influenced by the split ratio, cell matrix solution concentration, and handling of the organoid-cell matrix suspension. It is recommended to practice the handling of the cell matrix solution in advance. Usually eight well chambered glass coverslips are suitable for the assay. Organoids derived from one well of a confluent 48 well plate can be split into two wells of an eight well chambered coverslip (40 µL of the organoid-cell matrix pellet per well).
4. Centrifuge the organoid suspension at 300 x *g* at 4 °C for 5 min.
5. Carefully discard the supernatant and resuspend the pellet with 1 mL of cold PBS.
6. Centrifuge the organoid suspension at 300 x *g*, 4 °C for 5 min.
7. Discard the supernatant completely and resuspend the organoids derived from one well from a 48 well plate in 40 µL of cold medium. Fragment large organoid structures by pipetting the organoid suspension 5x through a 10 µL pipette tip to collect structures with a size of 40–60 µm for seeding.
   NOTE: Use the 10 µL tip on a 100 µL pipette tip for the fragmentation of the organoid structures, and practice step 1.7 in advance to ensure consistent results. Control the size of the organoids by phase contrast microscopy within the centrifugation tube. Ensure that there are no more multibranched organoids present and that the organoid fragments are roughly 40–60 µm long.
8. Once the organoids have obtained the desired size, mix them with 40 µL of the cell matrix solution (medium:cell matrix solution = 1:1).
   NOTE: The medium to cell matrix solution ratio must be kept constant to achieve consistent results. The dilution of the cell matrix solution reduces the stiffness of the organoid blob and impacts its diffusion properties. Use precooled pipet tips (-20 °C) for all suspensions containing cell matrix solution.
9. Place 40 µL of the organoid-cell matrix solution suspension in the center of each well of an 8 well chambered coverslip.
10. Keep the slide on an ice pack for 5 min. This preserves the cell matrix organoid suspension liquid and increases the organoid concentration at the coverslip surface by gravity.
11. Incubate for 20 min at 37 °C and 5% CO_2 to enable polymerization of the organoid-cell matrix blob.
12. **Add 150 µL of organoid culture medium per well and incubate for 24 h at 37 °C and 5% CO_2 prior to proceeding with the experimental treatment.**
    1. Use this period to treat the organoids and modulate their barrier integrity according to the corresponding scientific hypothesis. For the positive control, treat the organoids for 48 h with IFN-γ in order to investigate IFN-γ associated tight junction degradation and

permeability increase. Stimulate the positive control with 10 U/mL (10 ng/mL) recombinant murine IFN-γ. Leave the organoids of one well untreated.

13. Culture organoids at 37 °C and 5% CO_2 for up to 48 h.

2. OrganoidPermeability Assay

1. Bring the incubation chamber of the microscope to 37 °C at least 2 h before starting the experiment to reduce thermal drift while imaging the organoids.
2. Prepare a 100 mM solution of LY in PBS. Store on ice protected from light.
3. Prepare a 200 mM solution of EGTA in PBS. Store it on ice.
4. Transfer the chambered coverslip including the organoids into the incubation chamber of an inverted confocal microscope and turn on the CO_2 incubation (5%). Make sure the chambered coverslip is tightly locked within the stage of the microscope.
5. Using the organoids in one well as a reference, adjust the imaging settings of the microscope. Add LY (3 µL of 100 mM LY in 150 µL of medium) to obtain a final volume of 1 mM LY in 300 µL of medium. Incubate on the microscope for 1 h and adjust the focus for the imaging of the organoids' lumen. Define the required laser energy for LY excitation (488 nm) and respective detection sensitivity of the instrument and try to image the LY fluorescence at 30–40% of the available dynamic range of the instrument being used.
   NOTE: Adjust the laser excitation energy and detection efficiency on untreated organoids 70 min after the addition of LY. Ensure that the excitation energy is high enough to get a well exposed image. To avoid saturation of LY fluorescence within the microscopic images, it is recommended to adjust these settings after the LY diffusion reaches a steady state.
6. Define the position of the organoids by differential interference contrast (DIC) live imaging. Try to image organoids with comparable diameters (80 ± 30 µm) and focus on the central slice of the organoids to image their lumen. Define roughly 10 organoids per well and try to image only organoids close to the coverslip surface with a spherical structure.
   NOTE: The number of organoids that can be imaged per run depends on the speed of the microscope. It is recommended to image the organoids within a 5 min interval. On a regular laser scanning microscope, 40 positions in total are a reasonable starting point.
7. Record the DIC and the LY fluorescence of every position to document the organoid's shape and autofluorescence before adding the LY to the wells, used for the barrier integrity assay.
8. Do not image organoids that display high autofluorescence. This is due to the accumulation of dead cells within the organoid's lumen, and the results of autofluorescent organoids are hard to analyze afterwards.
9. Dilute 3 µL of the prepared LY solution (100 mM LY in 150 µL of medium) and add this carefully to each well without touching the chambered coverslip. The recommended concentration of LY per well is 1 mM. The final volume per well should be 300 µL.
10. Quickly check the focus of the defined positions and correct if needed.
    NOTE: LY diffuses quickly through the cell matrix. Therefore, the confocal imaging must be started within 3 min after the addition of the fluorophore.
11. Start a time-lapse imaging on the microscope. Take a fluorescence image of every position every 5 min for a total of 70 min.
    NOTE: The organoids were imaged in 5 min intervals to visualize the LY uptake over time. To measure the intestinal barrier breakdown, it is sufficient to record the fluorescence before and 60 min after LY addition and once again 10 min after the addition of EGTA.
12. Add 3 µL of the prepared EGTA solution per well without touching the chambered coverslip. The recommended concentration within the chambered coverslip of EGTA is 2 mM. The total volume per well should be 300 µL.
13. Start a second time-lapse. Record the fluorescence of the defined organoids again with an interval of 5 min for a total of 30 min.
14. Discard everything according to local safety regulations.
    NOTE: The protocol can be paused here.

3. Data Analysis

1. Only analyze the results of the organoids that took up LY after EGTA addition.
2. Results can be quantified with Fiji ImageJ.
3. Open dataset in ImageJ by clicking **File** |**Open** and select image data. In the following BIO-Formats Import options dialog select **View stack with: Hyperstack**.
4. Open the region of interest (ROI) manager by clicking **Analyze** | **Tools** | **ROI Manager**.
5. Draw an oval ROI by clicking on the **Oval selection** button in the ImageJ menu bar. Draw a selection containing the inner lumen of the organoid. Then press **Add** in the ROI Manager.
6. Repeat the steps for three representative areas outside of the organoid.
7. Click on **Analyze** in the menu bar and select **Set Measurements**. Enable only **Mean Gray Value** and disable any other measurement. Then click **OK**.
8. Make sure that all ROIs are selected in the ROI Manager. In the ROI Manager, click **More** | **Multi measure**. In the option dialog select **Measure all […] slices** and **One row per slice**. Then click **OK**.
9. Select all the values in the **Results** window and copy them into a spreadsheet application for further analysis.
   NOTE: If the position of the organoid moved during the time-lapse imaging, the ROI must be adjusted accordingly. To do so, select the correct ROI in the ROI manager and move it to the new position. Then click **Update** in the ROI manager. Perform the measurement for each timepoint individually by clicking **Measure** in the ROI manager, then switch to the next timepoint in the image window using the bar on the bottom. Collect all measurements in a spreadsheet. The individual shape and the movement of organoids during the imaging period requires the analysis of the data in a manual manner.
10. Calculate the mean intensity value of the three ROIs outside of the organoid for each timepoint.
11. Divide the intensity of the ROI inside the organoid's lumen by the mean intensity of the ROI outside and the mean intensity inside the organoid.
12. In order to calculate the relative increase of luminal organoid fluorescence, divide the relative fluorescence (see step 3.11) at each timepoint imaged by the minimal relative fluorescence.

NOTE: Use the minimal relative fluorescence, because sometimes the diffusion of the fluorophore can be slow at the beginning of the experiment.

Representative Results

To validate the application of 3D small intestinal mouse organoids as a model to quantify the effect of compounds regulating the intestinal barrier integrity, we applied IFN-γ. To do so, we isolated and cultured organoids derived from IFN-γ responsive wild type and IFN-γ-receptor-2 knockout mice, which do not respond to IFN-γ[8]. Upon treatment for 48 h with IFN-γ or PBS (control), all organoids were exposed to LY and imaged by confocal spinning disc live cell microscopy in 5 min intervals for a period of 70 min. The functional integrity of the intestinal barrier in this model resulted in exclusion of LY from the organoid's lumen while intraluminal accumulation of LY signified destruction of the TJ. The representative fluorescence microscopic images after 70 min of incubation with LY clearly demonstrate that intraluminal LY fluorescence was only visible in organoids from wild type animals treated with IFN-γ. In unstimulated (PBS) controls nor in organoids derived from knock out animals (IFN-γR2$^{\Delta IEC}$, **Figure 1**), no intraluminal LY fluorescence was present after 70 min.

The addition of EGTA causes an unspecific breakdown of the intestinal barrier integrity by sequestering TJ cofactors. This control was always utilized at the end of the experiment to demonstrate the ability of the respective organoid to take up LY (**Figure 1**). If no intraluminal LY fluorescence was detected upon EGTA treatment, the organoid was excluded from the experiment.

For the quantitative evaluation of the microscopic results, LY fluorescence was measured within the organoid's lumen and outside of the organoid. Relative intensity values were calculated (fluorescence inside/ fluorescence outside + inside) and are shown for each time point imaged. It is recommended to avoid imaging of organoids of varying sizes. We chose to focus on organoids with a diameter of 80 ± 30 µm (**Figure 2**). A schematic of the protocol with representative images is shown in **Figure 3**. Some major problems and troubleshooting techniques are shown and discussed in **Figure 4**.

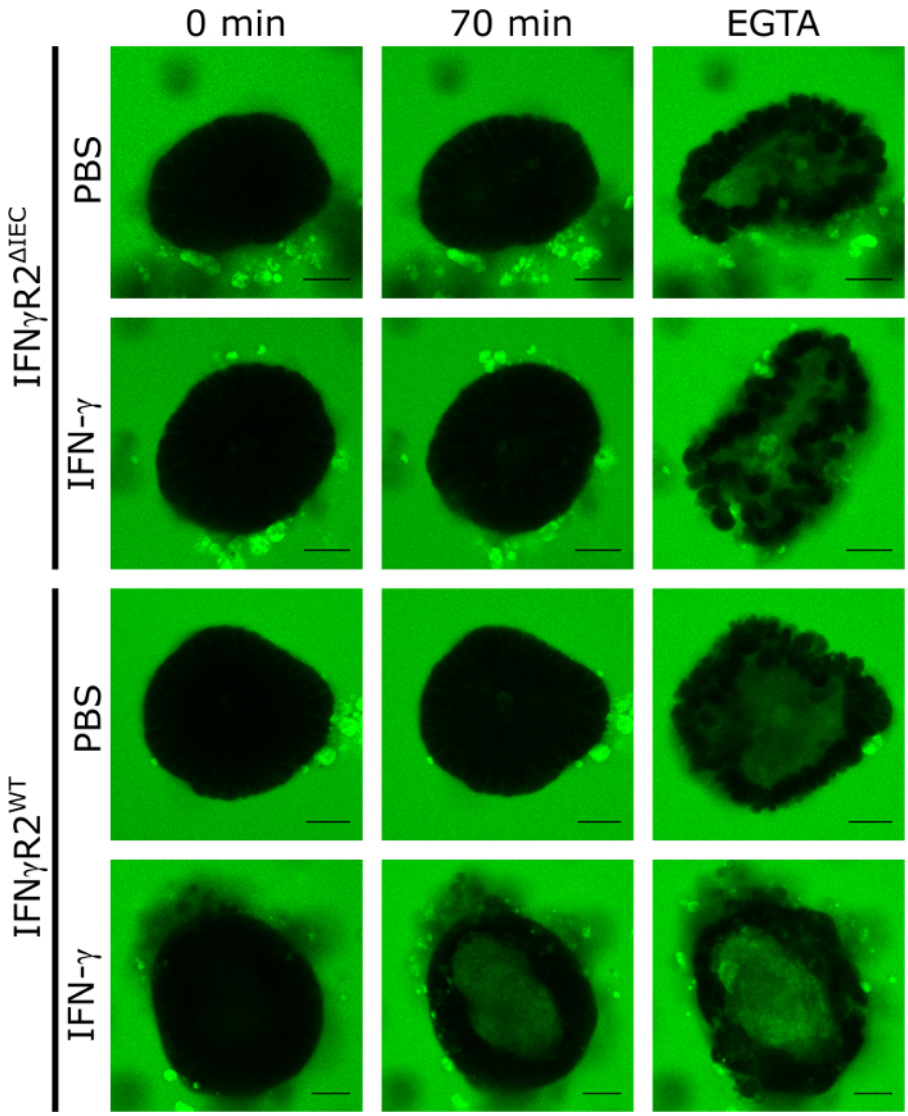

Figure 1: Intestinal barrier integrity can be analyzed in mouse organoids. Intestinal organoids from IFN-γR2^{WT} and IFN-γR2$^{\Delta IEC}$ were cultured in the presence of IFN-γ for 48 h or left untreated. To investigate the integrity of the intestinal barrier, LY (457 Da) was added and confocal fluorescent images were captured in 5 min intervals for a total of 70 min. Representative images at time point 0 min, 70 min, and after addition of EGTA are shown (green = Lucifer yellow; Scale bar = 20 µm). This figure has been modified from Bardenbacher et al.[8]. Please click here to view a larger version of this figure.

Figure 2

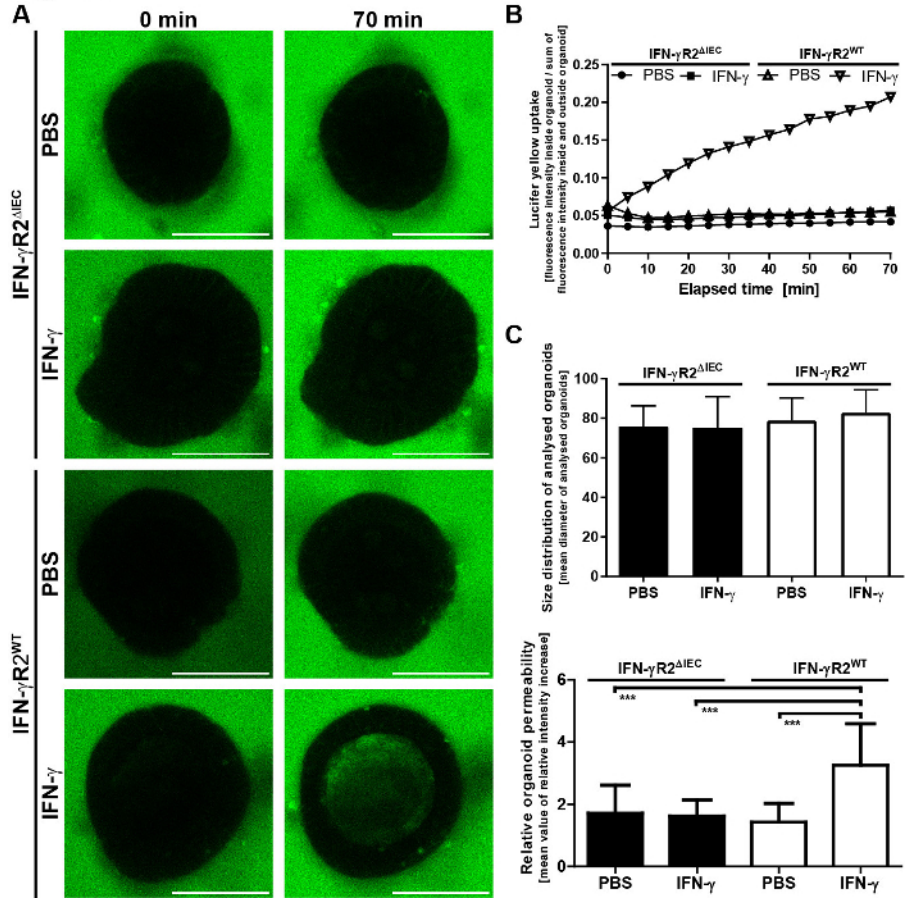

Figure 2: Small intestinal organoid barrier integrity model provides quantitative results. (**A**) LY fluorescence was determined inside and outside the organoid. Relative intensity values were calculated (inside/fluorescence outside + inside) relative to the initial relative intensity + SEM and are shown for each time point. (**B**) Size distribution of analyzed organoids. To reduce the standard deviation and errors due to changes in the surface-to-volume ratio, we only analyzed organoids with a diameter of 80 ± 30 µm. Mean values of the respective organoid diameters are shown + SD (IFN-γR2^{WT}, n = 20; IFN-γR2$^{\Delta IEC}$, n = 18). The mean diameter values did not vary significantly between the different groups (one-way ANOVA). (**C**) The permeability of the organoids was determined 70 min after the addition of LY. It was defined by dividing the intraluminal fluorescence intensities after 70 min by the minimal relative fluorescence intensities measured during the observation period. Each bar represents mean values + SD, measured in 10 organoids derived from two independent experiments (IFN-γR2^{WT}, n = 20; IFN-γR2$^{\Delta IEC}$, n = 18). IFN-γ significantly increased the LY uptake only in IFN-γR2^{WT} organoids. ***p-value <0.001 in the Student's t-test. This figure has been modified from Bardenbacher et al.[8]. Please click here to view a larger version of this figure.

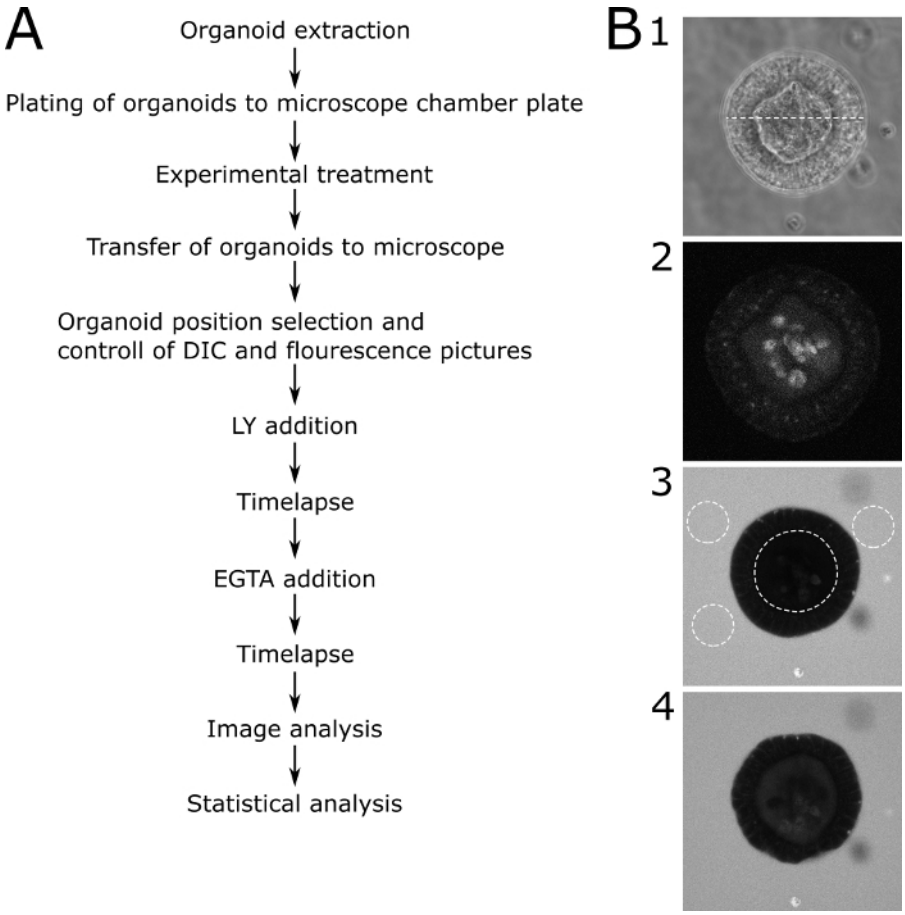

Figure 3: Schematic protocol with representative images. (**A**) Schematic description of the main steps of the protocol. (**B**) Representative pictures of the major steps of the protocol. (**B1**) DIC microscopy image of a central slice through a suitable organoid that was selected for permeability analysis. The dotted line represents a width of 89 µm. (**B2**) Fluorescence microscopy picture of the same organoid in (**B1**) before adding LY. The image shows the autofluorescence of the organoid. (**B3**) An organoid 70 min after the addition of LY. The depicted organoid shows no uptake of LY and therefore an intact barrier function. Dotted lines show the ROIs for further analysis. The inner lumen of the organoid and three representative areas around the organoid are marked. (**B4**) An organoid after the addition of EGTA. The organoid is usable for further analysis because it shows LY uptake after the EGTA treatment. Please click here to view a larger version of this figure.

A

-Organoids are to big/multibranched (1) -No spherical organoids	-Increase intensity of the cropping step (1.7.) by increasing the number of pipetting steps -Pick only spherical organoids with a consistent size to ensure a constant volume to surface ratio
-Organoids show high autoflourescence before adding LY (2)	-Replace organoid if high autoflourescence occurs -Decrease experimental treatment time after plating organoids as over time more cells are released to the inner lumen of the organoid -Some basal autoflourescence is normal (3)
-Organoids show no LY uptake after the addition of EGTA (4)	-Most of the times due to a wrong vertical image plane which does not include the inner lumen of the organoid -Organoids with no LY uptake after LY addition must be excluded from analysis

B

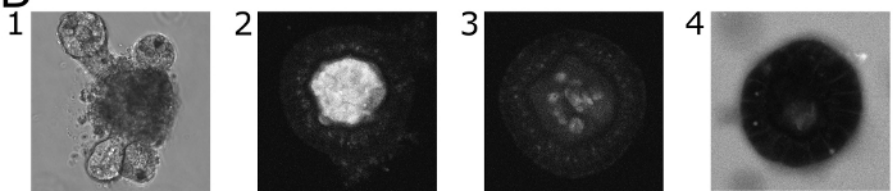

Figure 4: Troubleshooting of common problems. (**A**) Table with common problems and solutions. (**B**) Exemplary images. (**B1**) DIC image of a large multibranched organoid that is not suitable for this assay. (**B2**). Confocal image of an organoid displaying high autofluorescence before LY was added to the medium. The organoid was excluded from quantification. (**B3**) Confocal image of an organoid displaying low autofluorescence before LY was added to the medium. The fluorescence was quantified in this case. (**B4**) Organoid showing no LY uptake from the medium 30 min after addition of EGTA and therefore excluded from quantification. Please click here to view a larger version of this figure.

Discussion

This assay offers a technique to study the intestinal barrier integrity within living organoids. The whole assay is based on small intestinal mouse organoids and confocal live cell microscopy. Therefore, it is mandatory to practice the proper handling of organoids in advance. Upon isolation, organoids can be routinely split and stored by cryofreezing[3,9]. For this assay we recommend splitting the organoids 48 h before the treatment is started. This period gives the organoids the chance to totally close and form spherical structures. The seeding of the organoids for the experiment is a critical step within the assay. To reduce individual handling variations, we recommend a routine procedure for the seeding process. This step is crucial, and a routine handling protocol clearly reduces experimental variations.

During the seeding procedure (step 1.7) the organoids get fragmented by repetitive passaging through a standard 10 µL pipette tip. The pore size of this product varies from company to company. This procedure should be practiced in advance, and the result should always be checked by phase contrast microscopy. Once the organoids obtained reach the desired size, do not change the procedure.

The seeding of the organoids must be optimized and adapted for the available microscopic setup. To be able to culture and image organoids for at least 48 h, an incubated microscope chamber is absolutely required. Choose a chambered coverslip that matches your requirements. When seeding the organoids, make sure to concentrate the organoids on the coverslip surface. This is possible by keeping the chambered coverslip on an ice pack for 5 min after placing the cell matrix-organoid suspension. This step is important to increase the quality of confocal live cell imaging. The axial resolution and working distance of confocal microscope lenses is especially limited. The closer you bring the sample to the lens, the better you can image it and the less laser energy is needed to excite the LY fluorescence.

Phototaxis is an important issue when it comes to live cell microscopy. Within this assay we exclude this option. A functional AJC is visible by exclusion of LY from the organoid's lumen (**Figure 1**, PBS). The addition of EGTA at the end of the experiment causes sequestering of bivalent ions, which are cofactors for AJC proteins. LY is excluded from the organoid's lumen only in vital organoids with a functional AJC complex.
In general, fluorescent molecules can be used to measure the integrity of the intestinal barrier. We chose LY instead of other commonly used fluorophores such as fluorescein labeled dextran because those are transported transcellularly in intestinal cells from the basal to the apical compartment[9]. We also chose LY because of its small size. LY has a molecular weight of 457 Da and therefore facilitates the investigation of the barrier permeability for small molecules. The fluorescent molecule has to be chosen depending on the scientific question investigated. Because phototoxic AJC defects are present, laser excitation energy has to be reduced or the imaging interval extended. The optimal confocal imaging technique for this assay is spinning disc microscopy. Respective instruments enable confocal imaging with short exposure time at low laser power.

Different models have already been developed to study intestinal barrier integrity in vitro. While the use of assays based on cell line monolayers or experiments in vivo are declining, organoid-based methods increasing. In contrast to methods previously described[4,5,6,7], our method allows quantification of barrier function over time. This allows exposure of the organoids to additional stimuli over the course of the experiment. Here we apply EGTA as a second stimulus at the end of the experiment as a positive control.

In contrast to the situation in vivo, in our assay LY is added into the medium and penetrates the organoid from the outside basolateral epithelial side towards the inside apical lumen. The LY is small and is only used to visualize the tightness of the intestinal barrier. Molecules and stimuli

that modulate the epithelial layer at the apical surface need to be injected into the organoid's lumen[7]. To reduce the experimental effort and to be able to measure the barrier integrity of many organoids at the same time, we chose to apply the fluorescent dye from the outside.

We used the assay to investigate the function of IFN-γ on the tight junction of small intestinal mouse organoids. The fact that we were able to analyze the barrier integrity in living organoids offers future possibilities to apply this technique to describe inhibitors for the inflammation-induced breakdown of the intestinal barrier. Substances that counteract the impaired barrier function caused by IFN-γ could be candidates for the treatment of inflammatory bowel diseases, in which impaired barrier function is one of the pathogenic factors[10].

Disclosures

The authors have nothing to disclose.

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