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TITLE:

Investigating Intestinal Barrier Breakdown in Living Organoids

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KEYWORDS:
inflammatory bowel disease, interferon gamma, intestinal organoids, barrier permeability, functional analysis, live organoid microscopy

SUMMARY:
Here we describe a technique to quantify the barrier integrity of small intestinal organoids. The fact that the method is based on living organoids enables the sequential investigation of different barrier integrity modulating substances or combinations thereof in a time-resolved manner.

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.

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

1.1. Isolate organoids as described previously[3]. The procedure is briefly described below.

1.1.1. Collect the small intestines from mice.

1.1.2. Open the small intestines longitudinally and remove villi tips by scraping the inner intestinal tissue with a coverslip.

1.1.3. Cut the intestinal tissue in small pieces using scissors.

1.1.4. Wash pieces 5x in cold phosphate-buffered saline (PBS) by pipetting the pieces 10x up and down with a 25 mL pipette.

1.1.5. 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.

1.1.6. Replace the EDTA solution with PBS buffer once the tissue pieces settle at the bottom. Discard the supernatant and add 20 mL of PBS.

1.1.7. Release the intestinal crypts from the tissue by vigorously pipetting 10x up and down with a 10 mL pipette.

1.1.8. 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.

1.1.9. Repeat steps 1.1.6–1.1.8 until the number of intestinal crypts in the collected supernatant decreases.

1.1.10. Pass the fractions containing the most crypts through a 70 µm cell strainer.

1.1.11. Centrifuge the crypt suspension at 300 x *g*, 4 °C for 5 min.

1.1.12. 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.

1.1.13. 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.

1.1.14. Incubate the organoids at 37 °C, 5% CO_2, for 20 min to allow the cell matrix solution to solidify.

1.1.15. Cover the organoids with 300 µL of murine organoid medium per well.

1.1.16. Culture the organoids at 37 °C, 5% CO_2, changing the medium every 2–3 days.

1.1.17. Use the organoids for experiments after 7 days of culture.

1.2. Prepare the organoids for the barrier integrity measurement.

1.2.1. 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.

1.2.2. Thaw the cell matrix solution and organoid culture medium on ice.

1.3. 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).

1.4. Centrifuge the organoid suspension at 300 x *g* at 4 °C for 5 min.

1.5. Carefully discard the supernatant and resuspend the pellet with 1 mL of cold PBS.

1.6. Centrifuge the organoid suspension at 300 x *g*, 4 °C for 5 min.

1.7. 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.

1.8. 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.

1.9. Place 40 µL of the organoid-cell matrix solution suspension in the center of each well of an 8 well chambered coverslip.

1.10. 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.

1.11. Incubate for 20 min at 37 °C and 5% CO_2 to enable polymerization of the organoid-cell matrix blob.

1.12. 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.

1.12.1 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.

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

2. Organoid permeability assay

2.1. 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.

2.2. Prepare a 100 mM solution of LY in PBS. Store on ice protected from light.

2.3. Prepare a 200 mM solution of EGTA in PBS. Store it on ice.

2.4. 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.

2.5. 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.

2.6. 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.

2.7. 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.

2.8. 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.

2.9. 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.

2.10. 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.

2.11. 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.

2.12. 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.

2.13. 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.

2.14. Discard everything according to local safety regulations.

NOTE: The protocol can be paused here.

3. Data analysis

3.1. Only analyze the results of the organoids that took up LY after EGTA addition.

3.2. Results can be quantified with Fiji ImageJ.

3.3. 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**.

3.4. Open the region of interest (ROI) manager by clicking **Analyze** | **Tools** | **ROI Manager**.

3.5. 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.

3.6. Repeat the steps for three representative areas outside of the organoid.

3.7. Click on **Analyze** in the menu bar and select **Set Measurements**. Enable only **Mean Gray Value** and disable any other measurement. Then click **OK**.

3.8. 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**.

3.9. 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.

3.10. Calculate the mean intensity value of the three ROIs outside of the organoid for each timepoint.

3.11. 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.

3.12. 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-$\gamma R2^{\Delta IEC}$, **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 AND TABLE LEGENDS:

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; size bar = 20 µm). This figure has been modified from Bardenbacher et al.[8].

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].

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.

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.

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[4–7], 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].

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

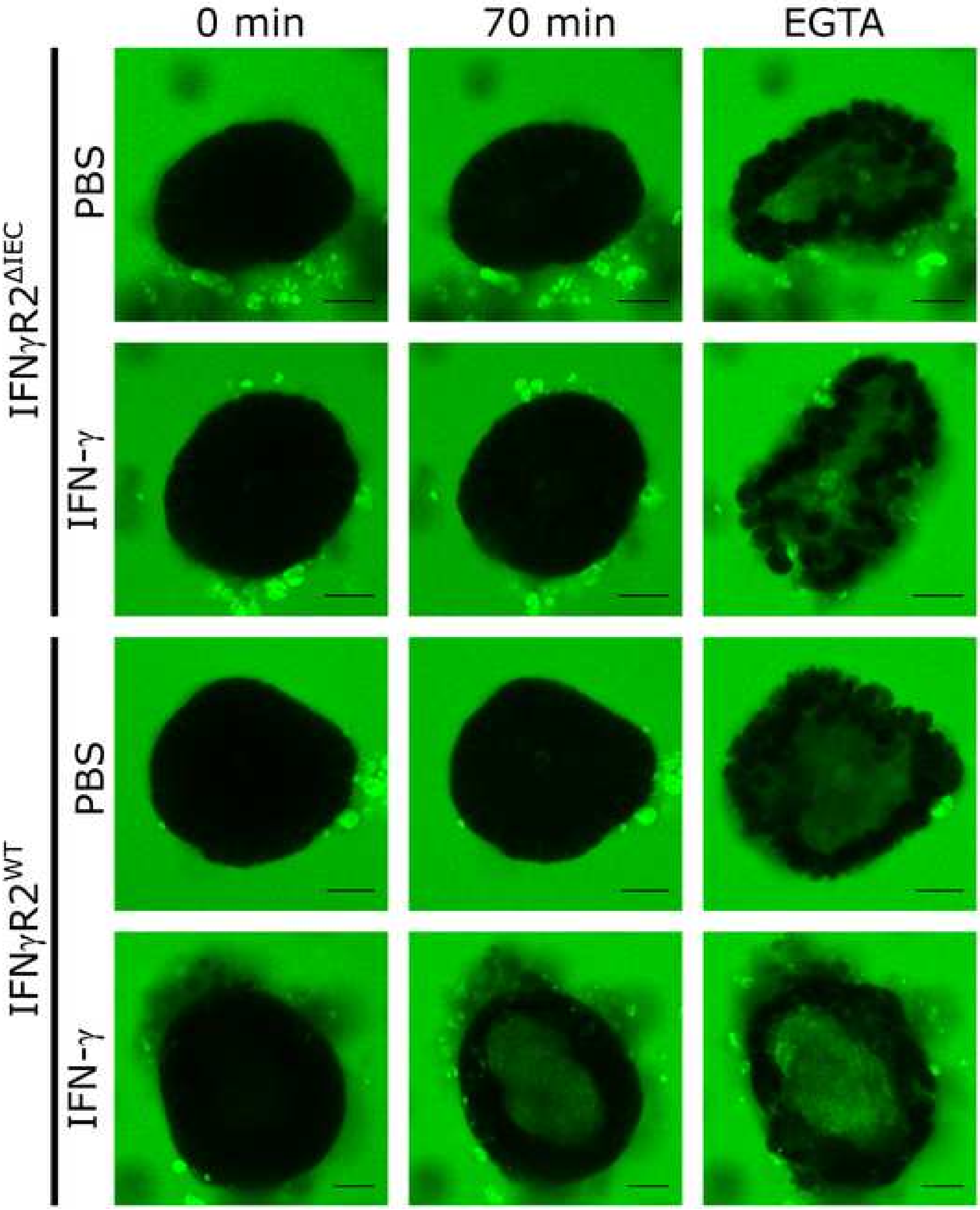

Figure 2

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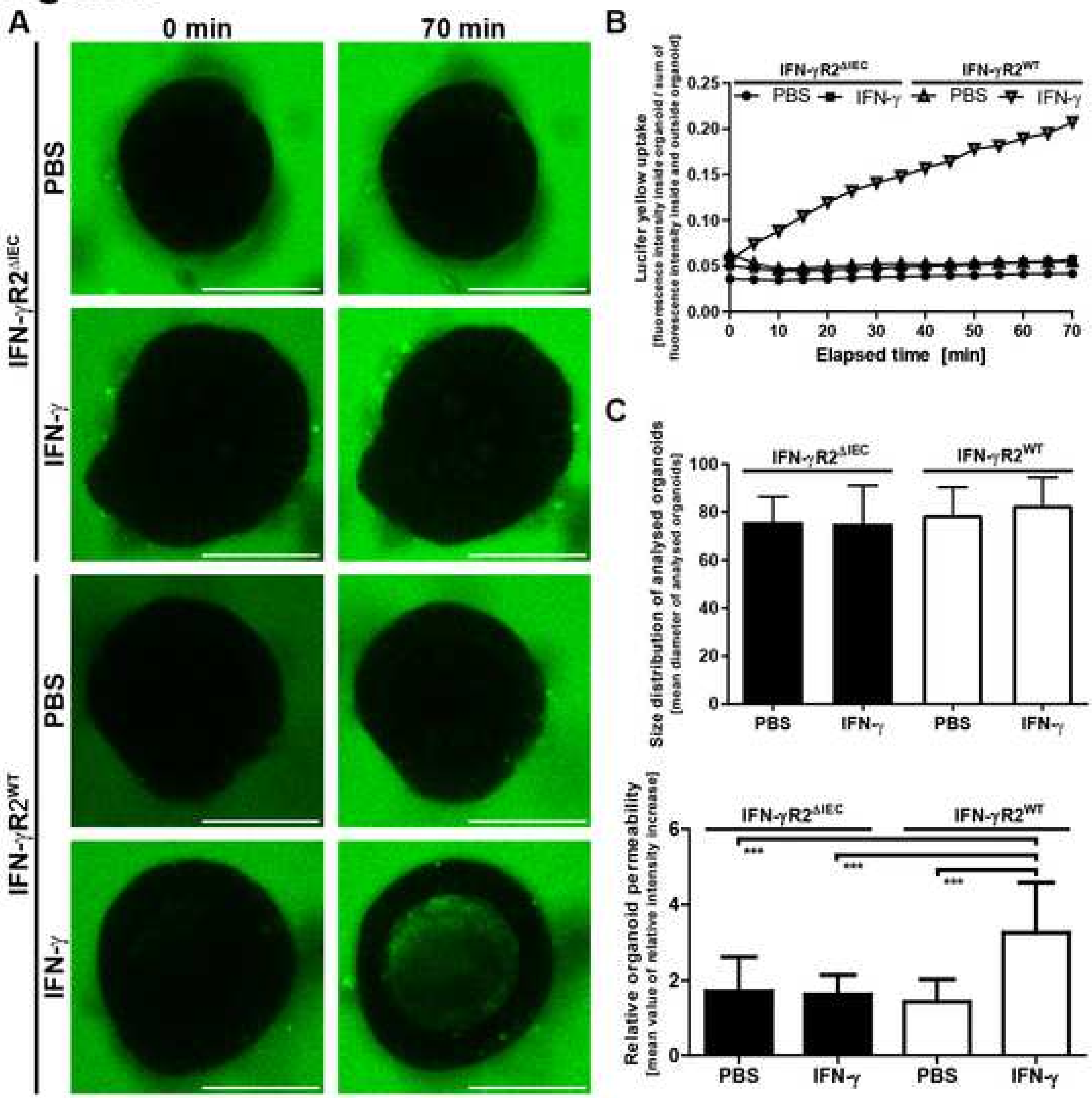
Figure 2



A

Organoid extraction

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Plating of organoids to microscope chamber plate

↓

Experimental treatment

↓

Transfer of organoids to microscope

↓

Organoid position selection and
controll of DIC and flourescence pictures

↓

LY addition

↓

Timelapse

↓

EGTA addition

↓

Timelapse

↓

Image analysis

↓

Statistical analysis

B 1 2 3 4

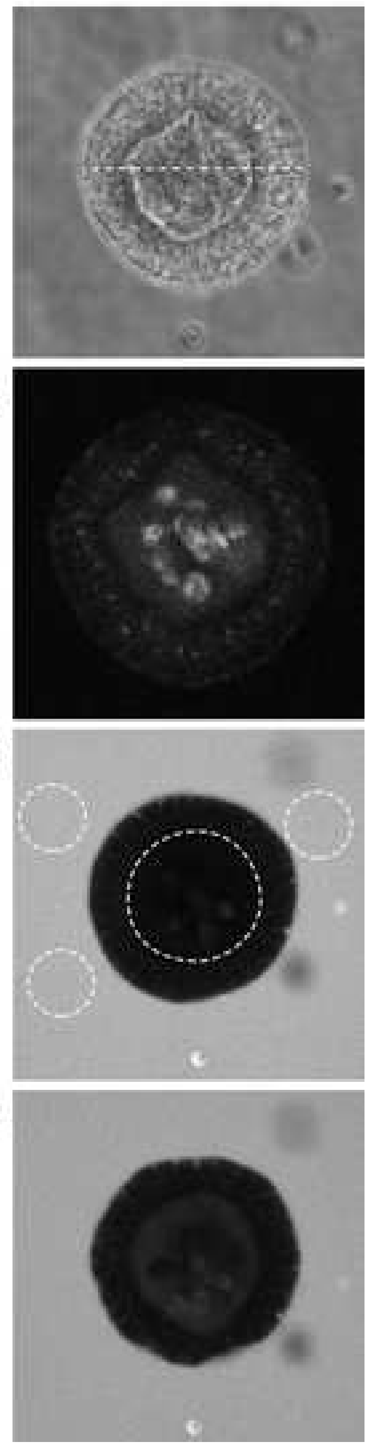
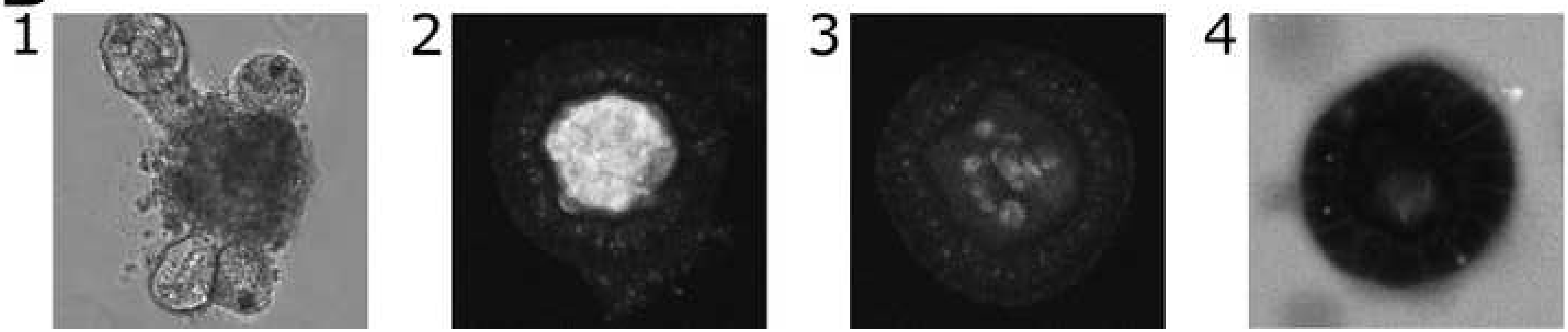


Figure 4

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



Name of Material/Equipment	Company	Catalog Number	Comments/Description
48-well culture plate	Thermo Fisher Scientific	#150687	
8-well chamber slides	Ibidi	#80826	
96-well culture plate	Greiner Bio-One	#655101	
Axio Observer.Z1 - spinning disc	Zeiss		excitation laser 488 nm / emissi
Bovine serum albumin	Sigma-Aldrich	A3059-100G	
Cell strainer	Falcon	352350	
Centrifugation tube 15 ml	Thermo Fisher Scientific	11507411	
Centrifugation tube 50 ml	Thermo Fisher Scientific	10788561	
EDTA	Sigma-Aldrich	431788-25g	
EGTA	Sigma-Aldrich	431788	
Lucifer Yellow CH dilithium salt	Sigma-Aldrich	L0259	
Matrigel, growth factor reduced, phenol red free	Corning	356231	Cell matrix solution
Mice	The Jackson Laboratory	M. musculus C57/Bl6	
Microscope coverslip		24x60 mm	
Organoid Growth Medium mouse	Stemcell Technologies	#06005	
Phosphate buffered saline	Biochrom	L182-05	
Recombinant murine IFN-γ	Biolegend	Cat#575304	

on filter 525/25

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Title of Article: INVESTIGATING INTESTINAL BARRIER BREAKDOWN IN LIVING ORGANOIDS

Author(s): BARDENBACHER / RODER / BRITZEN-LAURENT / NASCHBERGER / BECKER / STÜRZL / TRIPAL

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JoVE Editorial Office

Erlangen, 9 October 2019

Dear Editors,

Hereby we wish to submit the revised version of our manuscript "Investigating intestinal barrier break-down in living organoids". Thank you for your helpful comments.

We tried to address all the suggested improvements in a point by point manner:

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

    Proofreading was performed.

2. Please provide an email address for each author.

    All email addresses were filled into the form.

3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

    172 words – goal of the protocol: measuring of intestinal barrier integrity

4. Please do not cite any references in the abstract.

    Citation was removed from the abstract.

5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Also number the references in the reference section in the order.

    References were formatted according to the journal's guidelines.

6. Please ensure the Introduction contains all of the following with citation:
a) A clear statement of the overall goal of this method

    The assay was established to investigate the effect of IFN-γ on the barrier integrity and respective tight junction proteins[8].

b) The rationale behind the development and/or use of this technique

    The application of 3D intestinal organoids for functional analyses requires the adaptation of available methods from 2D models.

c) The advantages over alternative techniques with applicable references to previous studies

    In contrast to the technique applied by Leslie[4], Zietek[5] or Pearce[6]....

d) A description of the context of the technique in the wider body of literature

Various models have been developed to investigate the regulation….

e) Information to help readers to determine whether the method is appropriate for their application

We tried to present the advantages and disadvantages of the available methods. This should enable the readers to define the method, that matches their requirements.

7. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Falcon, Matrigel, etc.

All commercial language was removed from the manuscript.

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

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

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We changed the text of the protocol section to the imperative tense.

10. The Protocol should contain only action items that direct the reader to do something.

We moved additional information as "Notes".

11. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We removed personal pronouns from the protocol

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

We tried to be as short as possible…

13. Please ensure you answer the "how" question, i.e., how is the step performed?

We tried to answer all "how" questions within the protocol section

14. 1.1: What kind of organoids are used in this experiment? Please briefly describe the organoid culture protocol to make this a stand-alone manuscript.

We increased the information for the culture of organoids.

15. 1.7: Crop to what size?

Crop large organoid structures by pipetting the organoid suspension 5 times through a 10 µl pipet tip to receive structures with a size of 40-60 µm.

16. 1.13: What kind of barrier integrity question was asked in your experiment. Please be as specific as you can with respect to your experiment.

The assay was established to investigate the effect of IFN-γ on the barrier integrity and respective tight junction proteins[8].

Within the cited publication, we showed that the breakdown of the observed barrier integrity was a consequence of proteasomal degradation of the tight junction proteins Claudin-7, -12 and -15.

17. 2.5-2.6: Please include how is this done. Please provide all the button clicks, knob turns etc. For steps involving software usage, please include a click by click instruction. For example, Click Live to image the cells, then turn the knob on the right side of the microscope to adjust the focus.

With respect to the variability within microscopic setups, we can only describe the required conditions or settings. The position of the buttons is usually specific for each user or instrument.

18. Line 162: Adjust to what?

The power of the laser is individual for each instrument. Therefore, we tried to describe the way to do this according to good scientific practice:

Adjust the imaging settings of the microscope. Add LY to one well of your organoid culture, prepared for the assay. Incubate within the microscope for one hour 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 your instrument. Try to image the LY fluorescence at 30-40% of the available dynamic range of your instrument.

19. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted (yellow) the 2.5 pages, which include the filmable content.

20. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We removed all figures from the manuscript and uploaded the figures according to your suggestion.

21. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

All figure legends are within the manuscript test at the end of the results.

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Figures are not identical to the figures published in Bardenbacher et al., 2019. We added "This figure has been modified from [citation]." within the respective figure legend. In addition, I applied for a license to use figures from the previous publication:

Licensee:	Optical Imaging Centre Erlangen
Order Date:	Oct 7, 2019
Order Number:	4683690863563
Publication:	Stem Cell Research
Title:	Permeability analyses and three dimensional imaging of interferon gamma-induced barrier disintegration in intestinal organoids
Type of Use:	reuse in a journal/magazine

23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Within the discussion we present technical solutions to reduce the challenges of the live organoid based imaging technique.

b) Any modifications and troubleshooting of the technique

We added additional figure to enable troubleshooting.

c) Any limitations of the technique

Each of the established methods to measure barrier integrity have advantages and disadvantages. Within the Discussion we tried to compare our assay with previously used methods.

d) The significance with respect to existing methods

In contrast to methods previously described, our method allows to quantify barrier function over time. This allows to expose organoids to additional stimuli over the course of the experiment.

e) Any future applications of the technique

The fact, that the method is based on living organoids, offers opportunity to discover substances, inhibiting the breakdown of the intestinal barrier integrity. This passage was added to the manuscript:

We applied the assay to investigate the function of IFN-γ on the tight junction of small intestinal mouse organoids. The fact, that we 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 which 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[11].

24. Please do not abbreviate the journal titles in the references section.

We removed all journal title abbreviations from the reference section.

25. Please number the citations in the reference section.

Citations were numbered within the text and the reference section.

26. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Table of materials was revised and indicated symbols were removed.

Reviewers' comments:

Reviewer #1:

* Summary

In the manuscript "Investigating intestinal barrier breakdown in living organoids", Barenbacher et al. describe a method of quantitatively measuring non-specific permeability of the intestinal epithelial barrier in tissue-derived murine intestinal organoids. The authors present data demonstrating that IFN-induced permeability is abrogated in IFN receptor deficient intestinal organoids. The authors suggest that this method offers improved throughput compared to other previously reported methods. Overall, the protocol is clearly described and the data is well presented.

* Major issues

The authors make it a point to refer to this method as "medium throughput", however there are several steps that I think significantly impair throughput and rely on subjective or manual interpretation.

The term “medium throughput” was removed from the manuscript.

- First, step 2.7 advises to replace organoids with high autofluorescence. High autofluorescence is not defined and no examples are given. How is this done uniformly across experiments? More guidance is needed on this issue.

We added fluorescent images to enable troubleshooting and to depict autofluorescence within the sample.

- Second, data analysis (3) in ImageJ is completely dependent on manual operation of the software. Working with the recommended 5 minute intervals and considering biological and technical replicates, even a short 70 minute experiment with just a few groups such as that the example given would take a significant amount of time to compile. Larger screens would be prohibitively time consuming. ImageJ has an entire macro programming language for automating these kinds of analyses. This type of approach not only greatly enhances throughput, it also provides a structure for documented reproducibility and consistency of application in image analysis. The authors should strongly consider developing an ImageJ macro for automating image analysis, and share source data demonstrating this approach.

The 5 min intervals were chosen to follow the accumulation of the fluorescent marker over time. For the measurement of barrier integrity, it is sufficient to measure fluorescence before and 60 minutes after the addition of the dye. This reduces the number of images and also the effort for image analysis. We added this helpful comment to the protocol:

NOTE: We imaged the organoids with an interval of 5 minutes, to visualize the LY uptake over time. To measure intestinal barrier breakdown, it is sufficient to record the fluorescence before and 60 minutes after LY addition and once again 10 min after the addition of EGTA.

The lateral movement of the organoids within the imaging period of 1 hour and changes of the structure, required the positioning of the respective ROIs by hand. This prevented us from developing an automated ImageJ macro for the automated analysis. This would clearly increase the throughput of our method.

* Minor Issues

- The authors should justify why they used Lucifer Yellow. Could other fluorescent markers be used?

We discussed this issue within the text. The selection of the dye should be done in accordance with the scientific question.

- In a previous publication by Leslie et al. Infect Immun. 2015 Jan;83(1):138-45, a highly similar "outside in" strategy for measuring epithelial barrier permeability in organoids was applied. The authors should make mention of this paper and draw contrasts with their method.

We discussed our results in the context of Leslie et al., 2015:

In contrast to methods previously described[4–7] our method allows to quantify barrier function over time. This allows to expose organoids to additional stimuli over the course of the experiment.

- Line 32: The statement "monolayer cell cultures are dissected" is unclear.

We removed the statement:

Moreover, monolayer cell cultures are good in-vitro-model systems but do not represent the complex cellular differentiation processes and functions, which rely on the three-dimensional structure.

- Line 55: The statement "These systems are good to assess and enable medium throughput but lack many features of primary cells..." is poorly worded. It's not at all clear what makes these systems "good to assess" or what you mean by "medium throughput".

We changed the statement:

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 differentiation processes, present *in vivo.*

The statement medium throughput was removed from the manuscript.

- Line 75: It is not clear that microinjection produces long term injury. Prior studies (included the ones you site i.e. Hill et al.) demonstrate that microinjected organoids retain fluorescent dyes for an extended period of time in the absence of chemical or biological perturbation.

We changed the statement:

The technical effort of microinjection clearly reduces the throughput of this method. In contrast to the here described model, the microinjection enables the measurement of effects that require biologic activation on the apical epithelial surface.

- Line 156: Why 5 minute intervals? This generates high resolution data, but given the effort required to process these images I'm not sure that the same conclusions would not be possible at 10-20 minute intervals with dramatically less effort.

The 5 min intervals were chosen to follow the accumulation of the fluorescent marker over time. For the measurement of barrier integrity, it is sufficient to measure fluorescence before and 60 minutes after the addition of the dye. This reduces the number of images and also the effort for image analysis. We added this helpful comment to the protocol:

NOTE: We imaged the organoids with an interval of 5 minutes, to visualize the LY uptake over time. To measure intestinal barrier breakdown, it is sufficient to record the fluorescence before and 60 minutes after LY addition and once again 10 min after the addition of EGTA.

- Line 216: Does the use of minimal relative fluorescence bias your interpretation? I expect there will be some variation in the amount of external fluorescence between wells.

The relative quantification is used to reduce the impact of external fluorescence. We choose confocal imaging to reduce well specific differences.

- Line 334: the claim that the fluorescent dye has no function should be cited.

The statement was removed:

The fluorescent dye LY is small (457 Da) and is only used to visualize the tightness of the intestinal barrier.

Reviewer #2:

Major Concerns:

Parts of the protocol need more detail to be able to be followed by a user not experienced with organoids. i.e. line 94: "vigorous pipetting": How many times? which size pipette tip?

The materials list seems to lack material needed to follow the protocol, such as BSA, PBS,.....

I recommend showing an image with an example of outside ROI and organoid ROI indicated

We substantiated the protocol and the respective materials list. Thank you for the idea to show an image to explain ROI positioning. We added a figure to clarify this:

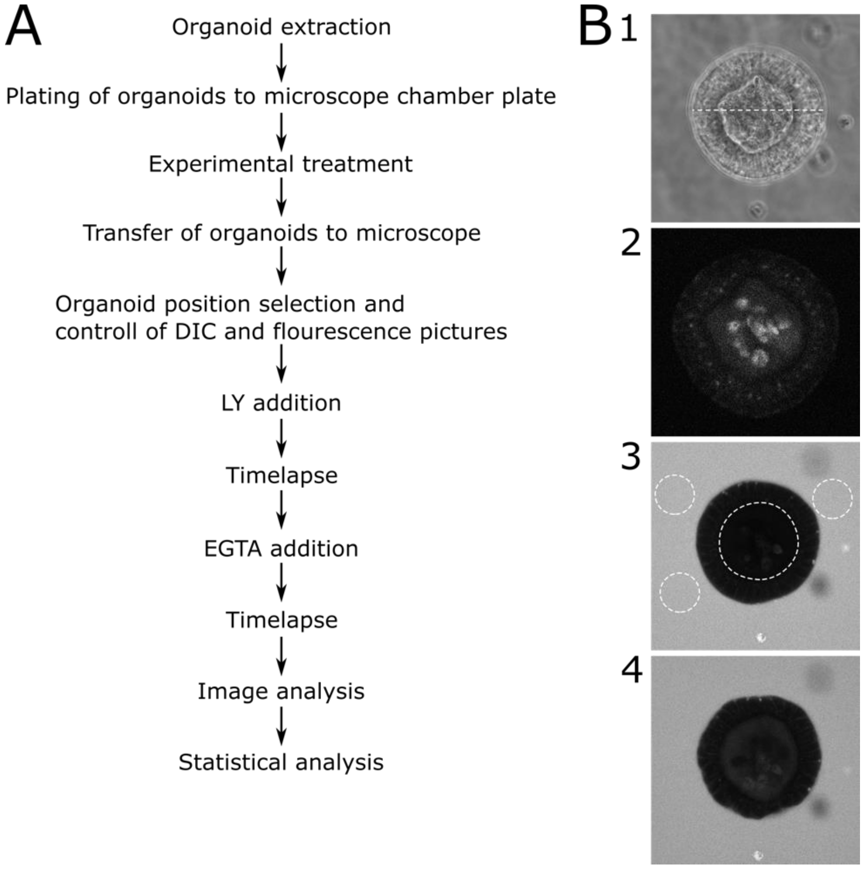

Fig. 3: Schematic protocol with representative images

(A) Schematic description of the main steps of the protocol **(B)** Representative pictures of major steps of the protocol. **(1)** DIC microscopy image of a central slice through a suitable organoid which was selected for permeability analysis. The dotted line represents a width of 89 µm. **(2)** Fluorescence microscopy picture of the same organoid as in (1) before adding LY. The image shows an autofluorescence of the organoid. **(3)** Picture 70 minutes after the addition of LY. The depicted organoid shows no uptake of LY and therefore an intact barrier function. Dotted lines depict the ROIs for further analysis. The inner lumen of the organoid and 3 representative areas around the organoid are marked. **(4)** Organoid after the addition of EGTA. The organoid is valid for further analysis as it shows LY uptake after EGTA treatment.

Minor Concerns:
Please refrain from using colloquial terms such as "falcon" for research material

Commercial language was removed from the protocol.

I am not sure this protocol can be called medium throughput as the time spent per treatment seems to be quite long.

We removed the term medium throughput from the manuscript.

It should be mentioned that diffusion time for compounds inside matrigel is slower than in liquid medium and that effects of compounds reaching organoids at the borders of the blobs earlier than organoid in the middle have to be taken into consideration.

We described the seeding of the organoids in a fashion to reduce the axial extension of the matrix-organoid blob:

Keep the slide on an ice pack for 5 minutes. This preserves the cell matrix organoid suspension liquid and increases the organoid concentration at the coverslip surface by gravity.

The difference to the protocols published by Zieke and Pearce should be explained in more detail. A short discussion of Co et al 2019 is also recommended, especially as the topic of apical versus basolateral application is brought up.

In contrast to the technique applied by Leslie[4], Zietek[5] or Pearce[6], which measure fluorescence after removing LY from the medium, our approach allows to quantify the luminal uptake of the fluorophore over time.

lines 328-331: please rephrase to be more clear or remove Boj et al.,

Boj et al., established an organoid based method to study forskolin induced swelling for the individualized therapy of cystic fibrosis. This is independent of barrier integrity and we therefore removed the passage from the manuscript.

Reviewer #3:

Manuscript Summary:

The manuscript entitled "Investigating intestinal barrier breakdown in living organoids" is a protocol describing a new technique to quantify the barrier integrity of intestinal organoids. The protocol is well written, and the authors already published a more detailed study showing part of this method on "Stem Cell Research" journal (Bardenbacher et al., 2019).

Major Concerns:

To increase the value of this manuscript, the authors should include a troubleshooting section (ideally in table format) and include a schematic drawing of the protocol to provide a more accessible overview.

Thank you for this helpful suggestion. We added a figure for the schematic drawing (Fig. 3) and one for the troubleshooting (Fig. 4).

On page 2, line 117, where the authors wrote "as the organoids have obtained the desired size," a specific size range and representative picture should be included.

In Fig. 2 we show the size distribution of all organoids used for the quantification. Beside this information we added steps within the protocol to ensure seeding of organoids with sizes, that enable a standardized quantification of the intraluminal increase of LY fluorescence:

Discard complete supernatant and resuspend organoids, derived from one well (48-well) in 40 µl cold medium. Crop large organoid structures by pipetting the organoid suspension 5 times through a 10 µl pipet tip to receive structures with a size of 40-60 µm.

Note: Use the 10 µl tip on a 100 µl pipet tip to perform the cropping of the organoid structures. Practice cropping step 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 multi-branched organoids are present and that organoid fragments are roughly 40-60 µm long.

Reviewer #4:

In this manuscript, Marco Bardenbacher and colleges introduced a new medium throughput assay to determine the barrier function of tight junctional (TJ) in live mouse small intestinal organoids. As a validation of this method, the authors treated intestinal organoids with IFNg, and found the intraluminal accumulation of Lucifer yellow (LY), while LY was excluded from the organoid lumen by functional intestinal barrier in non-treated wild-type organoids or IFNgR2DIELeven after IFNgtreatment. This is an interesting technique, but has already been shown in their previous report (Bardenbacher M. et al. Stem Cell Res, 2019). Although the authors described the details of the technique and the tips for experimental success, the validation data is almost the same in the previous report, and did not bring any new scientific insights to this manuscript.

This protocol was written upon an invitation to publish the method in JoVE after we published the results in Stem Cell Research. New scientific insights were not the focus of this protocol.

Minor comment:

In the figure legend of Fig.1, EGTA(EGTA) in the last second line is hard to understand. EGTA in "()" should mean the column of representative pictures treated with EGTA. The authors should clarify this point.

The figure legend was changed to be clearer:

Representative images at time point 0 min, 70 min and after addition of EGTA are shown (green: Lucifer yellow, size bar = 20 µm). This figure has been modified from Bardenbacher *et al.*, 2019[8].

With the help of the reviewer's comments and the editor´s instructions, we could clearly increase the quality of the manuscript. We hope, that we were able to convince the reviewers of the validity of the presented assay and we were able to fulfil the formal requirements of the Journal.

Yours sincerely,

Philipp Tripal