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Live imaging of bacteria-cell interactions in genetically modified intestinal organoids

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Summary

We present an improved protocol for microinjection of bacteria into mouse small intestinal organoids that recapitulates the natural route of bacterial entry and infection of cells from the lumen. To visualize bacteria-cell interactions, we introduce live imaging of infected organoids using a light sheet microscope. Additionally, to study essential gene functions, we present an improved method to generate doxycycline-inducible expression of mutant proteins in organoids. These protocols can be applied independently as required.

Intraluminal delivery of bacteria is referred from Nigro et al. ¹. The technical method of organoid transduction described here is modified from ² and ³. For complete details on the use and execution of this protocol, i.e., live imaging of organoids carrying intraluminally microinjected bacteria and generation of doxycycline-inducible organoids to regulate essential gene function, please refer to ⁴.

Graphical abstract

Before you begin

Intestinal organoids are powerful tools as they mimic *in vivo* intestines in a dish ⁵. One of the challenges of using this system to study enterobacteria-intestine interactions is the closed lumen into which bacteria must be introduced. Although organoid-derived 2D monolayers ^{6,7} and organoids with inside-out reversed topology have been developed ⁸, intraluminal microinjection is a very useful method as it does not perturb the morphology and integrity of the organoids and allows to control the composition of the basal compartment (extracellular matrix, cells from the *lamina propria*...). Due to the volume of organoids, live 3D imaging using classical approaches is time-consuming and leads to phototoxicity. The light sheet microscopy presented here combines both advantages to overcome these drawbacks: fast-deep imaging with limited phototoxicity. Finally, genetic manipulation of organoids is not feasible with most methods based on liposome-based (transient) transfection of 2D cultured cells. Lentivirus transduction can therefore be used to introduce stable genetic modifications into mini-organoids or organoid stem cells.

Here we present improved protocols for intraluminal microinjection of bacteria, live imaging with a specific light sheet microscope (ZEISS Z1) and lentivirus transduction of intestinal organoids. For efficient microinjection and viral transduction, it was crucial for us to first obtain organoids with a visible central lumen enriched in stem cells. Therefore, the trick we used was to generate stem cell-enriched organoids with a spherical morphology (see the section “Generation of organoids with a spherical lumen to facilitate successful microinjection”). These stem cell-enriched organoids are used for lentiviral transduction. For microinjection of bacteria, we differentiate these spheroids into regular organoids containing blobs of differentiated intestinal epithelial cells. Here, we introduce intraluminal microinjection followed by live imaging and then lentiviral transduction in a separate part (with a new timing). Both approaches can be combined in a single experiment, by microinjecting bacteria into genetically modified organoids.

This protocol can be adapted to any project aimed at understanding the role of lumenally localized compounds (chemicals, microbes, viruses...) in an *ex vivo* relevant model of the intestinal epithelial barrier. Combined with retroviral transduction, it allows the characterization of the host molecular mechanisms involved in the phenotypes studied.

This protocol is designed for organoids growing in a 24-well plate using 50 μ L of Matrigel[®] and 500 μ L of medium per well. Therefore, the volumes should be adjusted according to the user's scale. To generate one well of transduced organoids, 3-4 wells of stem cell-enriched organoids were pooled (a total of 400-600 stem cell-enriched organoids). All materials for organoids should be kept at the proper temperature before the experiment. For example, the centrifuge, 1 mL, and 200 μ L pipet tips should be ready at 4°C. Liquid phase Matrigel[®] has to be prepared before manipulating the organoids. As intestinal crypt isolation and organoid derivation are well described ^{5,9} and 293T cell transfection and virus concentration are routine methods in the cell biology labs, this part is not included in this manuscript.

[Generation of organoids with a spherical lumen to facilitate successful microinjection]

Timing: ~30 mins

1. Prepare stem cell (SC)-enriched medium (500 μ L/well, Materials and equipment, Table 2).
 - a. Add a final concentration of 100 ng/mL of Wnt3a, 1 M of Nicotinamide, and 10 μ M of CHIR99021 in the EGF Noggin R-Spondin (ENR) media and filter through 0.22 μ m.

Alternatives: Use a 2:1 ratio of ENR media and 50% L-WRN conditioned media¹⁰, see Key Resource Table.

- b. Incubate the media at 37°C.
2. Organoid passage (100-200 organoids/well).
 - a. Aspirate the medium and add 500 μ L/well of cold Cell Recovery Solution.
 - b. Liquefy the Matrigel[®] at 4°C (e.g., in a refrigerator or on ice, around 20 mins).
 - c. Place the plates on ice. Using 1 mL cold pipet tip, harshly pipet up and down to physically dissociate the organoids into small pieces.
 - d. To dissociate further, a 26-gauge needle in a 1 mL syringe can be used. Suck the organoids to pass through the needle and collect them into a 15 mL Falcon tube.
 - e. Add 2-3X volume of cold Advanced DMEM/F12 to the Falcon tube.
 - f. Tap or invert the tube so that the liquid Matrigel[®] is homogenously mixed.
 - g. Centrifuge at 4°C, 300g for 5 mins.
 - h. Using a 10 mL pipet, carefully discard the supernatant containing dead cell debris.

Note: Before discarding the supernatant, drop ~ 100 μ L and check roughly the amount of dead cells/cell debris. This allows to know if the washing is enough (Figure 1A). When using a 10 mL pipet to collect the supernatant, ~100 μ L of the media is left over. Leave this volume and follow step i.

- i. Resuspend the pellet with the remaining Advanced DMEM/F12. You can gently tap the tubes and see that the cells are no longer pellet and are mixed with the remaining Advanced DMEM/F12.
 - j. Add 4 mL of cold Advanced DMEM/F12 and centrifuge at 4°C, 150g for 1-2 mins.
 - k. Repeat 2-3 times steps h – j.

Note: Check the supernatant as in Note step h. Do the wash until there is few dead cells/cell debris (Figure 1A).

- l. Discard the supernatant as much as possible, but do not disturb the pellet. Gently tap the tube to prevent aggregation of organoid pieces.
 - m. Using a cold pipet tip, add the proper amount of liquid phase Matrigel[®] (50 μ L/well). Gently mix. We usually passage at a 1:3 or 1:4 ratio depending on the organoid density.

- n. Distribute 50 μ L in Ibidi μ -Dish 35 mm, low Grid-500 for microinjection or 50 μ L/well in a new 24-well plate for lentivirus transduction.
 - o. Place the dish or the plate into a 37°C incubator for at least 5 mins to solidify the Matrigel®.
 - p. Supplement SC-enriched organoid medium (1 mL in Ibidi μ -Dish 35 mm, low wall, grid 500 Ibidi-Treat, 500 μ L per well of 24 well plates).
 - q. Incubate at 37°C, 5 % CO₂ incubator. After 1-2 days, the organoids should appear with spheroid-like morphology.
3. Grow 2-3 days until the spheroid becomes big with few dead cells in the lumen (Figure 1B).

Key resources table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--------------------------------|-------------|
| Bacterial and virus strains | | |
| <i>Li</i> -GFP (InIA+) | This paper and Kim et al, 2021 | MBHL 366 |
| rLV.EF1.Tet3G-9 | Takara | 631311 |
| Chemicals, peptides, and recombinant proteins | | |
| N2 Supplement | Gibco Invitrogen | #17502048 |
| B27 Supplement | Gibco Invitrogen | #17504044 |
| N-Acetylcysteine | Sigma Aldrich | #A9165-5G |
| Human recombinant R-spondin 1 (final 500 ng/ml) | R&D systems | #4645-RS250 |
| Mouse recombinant Noggin (final 100 ng/ml) | Peprtech | #250-38-20 |
| Mouse recombinant EGF (final 50 ng/ml) | Invitrogen | #PMG8044 |
| Y-27632 (final 10 μ M) | Sigma Aldrich | #Y0503-1MG |
| Mouse recombinant Wnt3a (final 100 ng/ml) | Millipore | #GF160 |
| Nicotinamide (final 10 mM) | Sigma Aldrich | #N0636-100 |
| CHIR99021 (final 10 μ M) | Stemgent | #248040004 |
| Doxycycline (final 2 μ g/ml) | Takara | #631311 |
| TransDux MAX™ | System Bioscience | LV860A-1 |
| Matrigel® | Corning | 356231 |
| Cell recovery solution | BD | 354253 |
| Advanced DMEM/F12 (AdvDMEM/F12) | Gibco Invitrogen | 12634010 |
| DMEM/F12 phenol red-free | Gibco Invitrogen | 21041025 |
| GlutaMAX | Gibco Invitrogen | 35050038 |
| 1M HEPES | Gibco Invitrogen | 15630056 |
| Penicillin-streptomycin (PenStrep) | Gibco Invitrogen | 15140163 |
| Puromycin (final 1 μ g/ml) | Sigma | 540411-25MG |
| Neomycin (Geneticin, final 1X) | Gibco Invitrogen | 10138031 |
| Cell mask | Invitrogen | C10046 |
| TrypLE™ Express | Gibco Invitrogen | 12604-013 |
| Ultrapure™ LMP (low melting point) Agarose | Gibco Invitrogen | 16520-050 |
| Tet-advanced FBS | Takara | |
| Experimental models: Cell lines | | |

| | | |
|--|--------------------------------|-----------------|
| L-WRN cell | ATCC | #CRL-3276 |
| Experimental models: Organisms/strains | | |
| Mouse intestinal organoid | This paper and Kim et al, 2021 | N/A |
| Recombinant DNA | | |
| pLVS-TREG3-IRES | Takara | #631362 |
| Software and algorithms | | |
| Arivis Vision 4D 3.0. | Arivis | N/A |
| ZEN 2014 SP1 | ZEISS | N/A |
| Microscope & Microinjection equipment & Other | | |
| Light sheet fluorescent microscope | ZEISS Z1 | N/A |
| X40 objective W Plan-APO 40x/1.0 D | ZEISS | 421462-8210-000 |
| Zeiss L710 or any inverted fluorescent microscope | ZEISS L710 | |
| X10 objective EC Plan-Neoflaur 10x/0.30 M27 | ZEISS | 440330-9902 |
| X20 objective Plan-Apochromat 20x/0.8 Ph2 M27 | ZEISS | 420651-9911 |
| Glass capillary #3 (green) | BRAND | 701908 |
| Plunger | BRAND | 701998 |
| InjectMan & FemtoJet microinjection system | Eppendorf | |
| IVF needle | Vitromed | V-INJ-S3-35 |
| Microloader pipet tips, 20 μ L | Eppendorf | 5242956003 |
| Ibidi μ -Dish 35 mm, low Grid-500, ibidi-Treat plates | Ibidi | 80156 |
| BHI | | |
| 10 mL Syringe | BD | 320912 |
| 1 mL syringe | BD | 303172 |
| 26-Gauge needle | BD | 300300 |
| 0.22 μ m filter | Fisher | 15181499 |

Materials and equipment (optional)

- Organoid culture

[Table 1. Organoid growth medium (ENR medium)]

| Reagents | Stock Concentration | Final Concentration (1X) |
|-------------------------------|---------------------|--------------------------|
| Advanced DMEM/F12 | n/a | n/a |
| GlutaMax | 100X | 1X |
| 1 M HEPES | 100X | 1X |
| Penicillin-Streptomycin | 100X | 1X |
| N2 supplement | 1000X | 1X |
| B27 supplement | 500X | 1X |
| N-Acetylcysteine | 500 mM | 1 mM |
| Human recombinant R-spondin 1 | 500 μ g/mL | 500 ng/mL |
| Mouse recombinant Noggin | 100 μ g/mL | 100 ng/mL |

| | | |
|-----------------------|------------|--------------------|
| Mouse recombinant EGF | 50 µg/mL | 50 ng/mL |
| Y-27632 | 10 mM | 10 µM |
| Total | n/a | 500 µL/well |

- **Medium to enrich stem cells**

[Table 2. SC-enriching organoid medium]

| Reagents | Stock Concentration | Culture (1X) | Infection (2.5X) |
|-------------------------------|---------------------|--------------------|--------------------|
| Advanced DMEM/F12 | n/a | 474.5 µL | 174.5 µL |
| GlutaMax | 100X | 5 µL | 5 µL |
| 1 M HEPES | 100X | 5 µL | 5 µL |
| Penicillin-Streptomycin | 100X | 5 µL | 5 µL |
| N2 supplement | 1000X | 0.5 µL | 0.5 µL |
| B27 supplement | 500X | 1 µL | 1 µL |
| N-Acetylcystein | 500 mM | 1 µL | 1 µL |
| Human recombinant R-spondin 1 | 500 µg/mL | 0.5 µL | 0.5 µL |
| Mouse recombinant Noggin | 100 µg/mL | 0.5 µL | 0.5 µL |
| Mouse recombinant EGF | 50 µg/mL | 0.5 µL | 0.5 µL |
| Y-27632 | 10 µM | 0.5 µL | 0.5 µL |
| Mouse recombinant Wnt3a* | 100 µg/mL | 0.5 µL | 0.5 µL |
| Nicotinamide* | 10 M | 5 µL | 5 µL |
| CHIR99021* | 10 mM | 0.5 µL | 0.5 µL |
| Total | n/a | 500 µL/well | 200 µL/well |

* Additional supplements added

Alternatives: Stem cells can also be enriched with L-WRN cell-conditioned media¹⁰. Use a 2:1 ratio with regular ENR media and 50% L-WRN conditioned media.

- **Lentivirus mixture solution**

[Table 3]

| Lentiviral solution | Final Concentration (1X) | Amount |
|--|--------------------------|---------------|
| Lentivirus 1 (Tet-activator solution)* | n/a | 5 µL |
| Lentivirus 2 (pTREG3-mutant gene) | n/a | 192.5 µL |
| TransDuxMAX™ | 1X | 2.5 µL |
| Enhancer (in the TransDuxMAX™ kit) | 1X | 100 µL |
| Total (for 1 well) | n/a | 300 µL |

* Tet-activator virus particle is purchased from Takara (Key Resource Table). Resuspension has been done according to the user's manual. If one makes their own Lentivirus 1, the volume needs to be adjusted with Lentivirus 2, so that the total volume can be 300 µL for one 24 well.

CRITICAL: Lentivirus handling should be done in BSL 2.

Alternatives: The volume of Lentiviruses can differ depending on the viral titer of the production. Instead of TransDuxMAX™, polybrene can be used but in our hands, TransDuxMAX™ gave the best transduction efficiency based on the antibiotic selection (more than 2-fold).

Step-by-step method details

Intraluminal microinjection of bacteria

This step allows bacteria to be delivered into the lumen of the organoids. The method is based on the use of an Eppendorf InjectMan and a FemtoJet microinjection system (Key Resource Table). We use bacteria grown in exponential phase (optical density at 600 nm (OD_{600}) = 0.8 for *Listeria*).

For intraluminal microinjection, having organoids with a round-centered visible lumen, but in a well-differentiated state facilitates the success of microinjection. Once the organoids develop a round lumen with few dead cells (generation of organoids with spherical lumen to facilitate the success of microinjection), we replace the media with regular ENR media for at least 2 days (Figure 1B). The correct differentiation can be verified by the thickening of the epithelium (cells gain their height therefore the epithelium becomes thick), the formation of blobs (Figure 1C), and the immunostaining of differentiated cells such as goblet cells and enteroendocrine cells. The following protocol is used when the organoids reach this stage. We switch between 10X and 20X objectives to get a global view and to get a fine adjustment, respectively.

Day 1.

Timing [5-10 mins]

1. Change the organoid media without antibiotics
 - a. Carefully remove the ENR media with a 1 mL pipet tip.
 - b. Wash 3 times with pre-warmed AdvDMEM/F12 to thoroughly wash out antibiotics.
 - c. Replace with pre-warmed ENR media without PenStrep.
2. Inoculate bacteria for overnight culture
 - a. 3 mL of liquid culture in a round bottom 15 mL tube (BHI for *Listeria*).
 - b. Grow them overnight at 37 °C, 200 rpm.

Day 2.

Bacteria preparation

Timing [2-3 hours]

3. Dilute 20-fold of overnight bacterial culture.
 - a. Put 0.5 mL of liquid culture in 9.5 mL of new media in a 50 mL falcon tube.
 - b. Grow them at 37 °C, 200 rpm until the OD_{600} reaches 0.8. For *Listeria*, this takes around 2 hours.

Note: OD₆₀₀ 0.8 is based on microinjection of *Listeria monocytogenes* but may be adapted to the species tested if different. *Listeria monocytogenes* is a BSL2 pathogen.

Note: During this time, turn on the microscope and set the temperature (37 °C) and CO₂ (5 %). Install the Eppendorf InjectMan and FemtoJet microinjection system. If live imaging follows subsequently, prepare the 1.5 % agar solution (step 19). Turn on the microscope and equilibrate the temperature and CO₂ (step 21).

4. Wash the bacteria 3 times in PBS, 5000 rpm, 5 mins.
5. Resuspend bacteria with 100 µL of phenol red-free DMEM/F12. Put the bacteria on ice.

Note: Resuspending volume can differ depending on the experimental purpose. This is based on microinjecting 3000-3500 CFU of *Listeria* per pulse (200 hPa, 1 second).

Microinjection

Timing [~30 mins to inject ~15 organoids/Ibidi µ-Dish 35 mm]

6. Set the injection pressure (psi) and injection times (t_s). For *Listeria*, we use 200 hPa and $t_s = 1$.
7. Turn on the InjectMan and FemtoJet, and raise the capillary holder to a maximum Z (Figure 2).
8. Place the Ibidi µ-Dish with organoids in the microscope. Using the 10X objective, position the organoid to inject (Figure 3A).

Note: Only the organoids located at the edge of the Matrigel can be easily injected.

9. Using Eppendorf 20 µL microloader tip, load 10 µL of bacteria to the V-INJ-S3-35 injection needle.
10. Install the injection needle into the capillary holder of the InjectMan.
11. Carefully lower the capillary holder so that the microinjection needle can go into the Ibidi µ-Dish 35 mm. Adjust the cantilevered portion of the needle to be parallel to the XY plane (Figure 3A).
12. Using the joystick, adjust the X, Y, and Z of the needle so that the Z of the needle is at the same level as the Z of the organoid epithelium to be injected. Using 20X objective helps to fine-adjust.
13. Slowly move the needle horizontally and poke into the epithelium. Check that the needle is in the lumen.
14. Press the injection button of the InjectMan. Successful injection should be verified. Wait until bacteria no longer exit from the needle (Figure 3B). **Troubleshooting 2.**

Note: It is important to verify successful injection as the bacterial solution can be stuck within the needle without ejection. We use bacteria expressing a fluorescent protein for visualizing injection (Figure 3). If there is no fluorescent reporter, one can also add fluorescent dye when resuspending bacteria after the final wash in step 5.

15. Carefully take the needle out from the organoid. Before retrieving the needle across the epithelium, verify that nothing is exiting from the needle (Figure 3B).

16. Turn the Ibidi μ -Dish 35 mm (carefully, by hand) and position the next organoid to continue to inject.
17. Continue the steps from 12.
18. After the microinjection is finished, infected organoids can be incubated and fixed for further studies or undergo live imaging.

Live imaging of microinjected organoids

This step allows the host-bacteria interaction to be imaged in real time. To achieve successful live imaging at the scale of both organoids and bacteria with the least phototoxicity, it is important to use light sheet fluorescence microscopes or equivalents that have fast acquisition with the least phototoxicity. Below is the protocol adapted for the Zeiss Z1 light sheet fluorescence microscope. Therefore, sample preparation and detailed operation of the microscope should be adapted to the user's instrument. Both bacteria and organoids should express fluorescence to facilitate imaging. We use membrane tomato or Muc2 organoids expressing mCherry and bacteria expressing GFP. If there is no endogenous fluorescence in the organoids, the cell membrane can be stained with Cell Mask directly in the media for at least 30 min (Key Resource Table, Figure 2). We visualize both the bacteria and the organoids using an x40 N.A.1.0 objective (Key Resource Table). More detailed microscope features can be found on the company website.

Live imaging of organoids may be a separate entity, depending on the purpose of the experiments. In this case, start with step 19.

Day 2 (In continuous from microinjection)

Sample preparation (Videos S1, S2, S3, S4)

Timing [~10 mins]

19. Prepare 1.5 % low-melting agar solution in PBS containing Ca^{2+} and Mg^{2+} . Dissolve the agar and filter through a 0.22 μm pore unit and put it in a 37°C water bath to preserve the liquid phase until imaging.
20. Install x10 illumination/ x40 detection objectives in ZEISS Z1.
21. Turn on the microscope and equilibrate the temperature to 37°C and CO_2 to 5 %.
22. Fill the sample chamber with organoid medium (~35 mL) made with phenol red-free DMEM/F12 (Video S1). The medium needs to be equilibrated to 37°C and 5 % CO_2 before live imaging.
23. Prepare the glass capillary and plunger. For mouse intestinal organoids, we use either black or green capillary/plunger set, the size of which is compatible with the size of mouse small intestinal organoids (Both BRAND 701904/701997 and 701908/701998 are compatible with the size of the organoids).

Note: The choice of syringe/plunger set is dependent on the size of the sample. The black capillary/plunger set has an inner diameter of ~1 mm, whereas the green capillary/plunger set is ~1.5 mm. Both can contain mouse small intestinal organoid samples. We use green capillary/plunger set for our experiment.

24. In the plate with microinjected organoids, remove the medium so that the organoids embedded Matrigel® are exposed. Gently pour the 37°C low-melting agar to cover the Matrigel®.
25. Using the sample syringe/plunger, first suck the agar outside the Matrigel® about ~ 3 mm, then gently suck directly the Matrigel® with organoids until the plunger is close to the color-coded part of the syringe (Video S2).

26. Once the plunger is reaching the color-coded part, stop sucking the organoid, and suck the agar about ~ 3 mm so that the agar fills the top & bottom part of the sample (Video S2).

Note: As the organoids are hung while imaging, having the agar at the top/bottom will help to stabilize the sample from trivial movement. This step must be finished before the agar solution solidifies (Video S2).

27. Cool the agar at room temperature (2 – 3 minutes).
28. When the agar becomes solid, install the syringe/plunger to the sample holder and install the sample holder to the microscope (Video S3).

Microscope operation: Locating the sample (Videos S4)

Timing [10 – 30 mins]

29. Click “Locate.”
30. Click “Locate Capillary.”
31. **[Specimen Navigator]** Using the mouse, roughly adjust the X and Y position to locate the bottom of the syringe visible at the center top of the detection lens. Roughly focus (Z).
32. Open the lid of the sample box and gently push the plunger and check the monitor that the agar is coming out from the syringe.
33. Continue to carefully push the plunger until the organoids are visible. When the desired sample is found, stop “Locate Capillary” (Video S4).
34. Switch to “Locate Sample.”
35. **[Camera]** As the x40 detection objective is installed, set the minimal zoom with 0.5% light intensity. To adjust the light, click “min/max” icon to have an optimal contrast automatically.
36. Adjust the fine position & focus using the joystick.
37. Depending on the size of the organoid sample, adjust the zoom factor. Click “mean/max” to adjust the light if necessary.

Microscope operation: Acquisition & Setting

Timing [desired length of the live imaging]

38. Click “Acquisition.”
39. **[Experiment Manager]** Click Z-Stack and Time Series.
40. **[Setup Manager, Laser]** Turn on the proper laser.
41. **[Setup Manager, Light Path]** Select the laser and detecting filter sets depending on the fluorescent being imaged.
42. **[Acquisition Parameter, Acquisition Mode]** Click the pivot scan. Choose a single side light sheet (either left or right). During the imaging, this can be changed to the other side and/or dual side if needed. The last setup will be used for the acquisition.
43. **[Acquisition Parameter, Channels]** Set the tracks depending on the lights being used. Depending on the signal intensity, first start with the minimal light (e.g., 0.2%) and default exposure time for every track.

Note: Both bacteria and organoids should express enough fluorescence to facilitate imaging.

44. **[Experiment Manager]** Click “Continuous” and check the sample on the monitor. Adjust the light intensity and exposure, first by clicking “min/max” to automatically adjust the best contrast. If better adjusting is needed, change the light intensity, exposure, and/or focus with Z and/or rotate the sample to find a good area.
45. **[Acquisition Parameter, Acquisition Mode]** Test both left and right side illumination and use the better illumination. If both illuminations are required, choose dual-side illumination.

Note: Dual-side illumination doubles the data size. Therefore, if single-side illumination gives enough resolution/quality of the image, dual side illumination is not necessary. If dual-side illumination is used, it creates two-time series imaging with each side illumination unless online dual side fusion is used. These two sets of illuminations need to be merged (Processing, fusion).

46. **[Multidimensional Acquisition, Z-Stack]** Set the first and last plane of the sample. Clicking “Optimal” will create the maximum slices with the least interval. Click “continuous drive.”
47. **[Multidimensional Acquisition, Time Series]** Once the area of interest is set, set the live imaging parameters and start live imaging. See Video S5 as an example.

Note: The data size of live imaging can range up to Terabytes depending on the time interval and z thickness. Therefore, proper image analysis software is necessary to handle such files. We use Arivis Vision 4D to manage big files.

CRITICAL: Since the sample is suspended in the low percentage agar, it is possible to lose the sample. This can happen if you take an image with multiple organoids in different positions as the sample is moved around. We wanted to live image the fast bacterial translocation event across the epithelium, so we did not use a multilocation function to avoid missing the fast event.

Lentivirus-mediated organoid transduction

To study the molecular mechanisms of bacteria-epithelium interaction, it may be necessary to manipulate host genes/proteins. The following protocol allows for the modification of the organoid genome if a mouse with the desired genetic background is not available or if it takes time to generate such mice.

The advantage of the Tet-on system presented here is that it induces the expression of a gene that could be detrimental to organoid growth if constitutively expressed¹¹. In this protocol, a bicistronic expression vector was used for to combine both the gene of interest and a gene encoding for a fluorescent reporter protein on the same vector. These two genes are linked by an internal ribosomal entry site (IRES) are under the control of the same promoter that is activated by the doxycycline-responsive transactivator. Transduced organoids will then express the fluorescent protein along with the protein of interest (Figure 4)¹². Other lentiviral vectors can be used, depending on the question being asked.

Once the lentiviral vectors deliver a gene of interest into the genome of stem cells, it is expected that all progeny cells will contain the gene. Therefore, to achieve effective transduction, organoids are enriched with the stem cell population prior to the experiment (generation of organoids with a spherical lumen to facilitate microinjection). It is important to have a high titer of virus. We use fresh virus mixtures prepared immediately before (or during) the procedure. All reagents/materials should

be kept at 4°C. Cells derived from $\sim 10^3$ organoids are incubated with a virus mixture and plated in 1 well. Viruses must be handled in a BSL2 environment.

As the process described below is itself an independent method, we use a separate timing.

1. Preparation of organoid stem cells for viral transduction

This step allows the generation of small pieces of organoid fractions containing stem cells, which are the targets of viral transduction.

Day 1. Matrigel® coating

Timing 1: [~ 10 mins]

1. Coat the 24 wells plates with 100 μ L Matrigel® to evenly cover the wells.
 - a. Wash the wells with 500 μ L of cold PBS so that the Matrigel® can be evenly spread.
 - b. Using a cold tip, evenly spread 100 μ L/well of Matrigel® and incubate at 37°C to solidify the Matrigel®.

Day 1. Organoid stem cells for virus infection

Timing 1: [~ 30 mins]

2. Liquefy the Matrigel® containing SC-enriched organoids (Generation of stem cell-enriched organoids to facilitate microinjection).
 - a. Aspirate the medium and add 500 μ L/well of Cell Recovery Solution.
 - b. Incubate the plate at 4°C (e.g., in the fridge).
3. After 5~10 mins, place the plate on ice. Using cold 1 mL pipet tips, scratch the Matrigel® several times to break it down.
4. Put back the plate at 4°C until the Matrigel® is dissolved. This takes total of ~ 15 mins.
5. Place the plates on ice. Using 1 mL cold pipet tip, harshly pipet up and down to physically dissociate the organoids into small pieces.
6. Collect the organoids in a 15 mL Falcon tube on ice.
7. Add at least 3X volume of cold Advanced DMEM/F12 and mix well to be homogenous.
8. Centrifuge at 4°C, 300g for 5 mins.
9. The cell pellet should be visible after the centrifuge. Carefully discard the supernatants. This is to remove the Matrigel® component.
10. Resuspend well the cell pellet by adding 4 mL of cold Advanced DMEM/F12.
11. Centrifuge at 4°C, 150g for 1 min.
12. Carefully discard the supernatants to completely remove Matrigel® and tap the tube to prevent aggregation of the pellet.
13. Add 500 μ L of 37°C pre-warmed TrypLE™ Express. Gently pipet once or twice to mix homogeneously.
14. Incubate for 3 mins in the 37°C water bath.
15. Add warm Advanced DMEM/F12 up to 4 ml and centrifuge at 300g for 5 mins.
16. Carefully discard the supernatants containing dead cell debris and TrypLE™ Express.
17. Resuspend the pellet by adding 4 ml of warm Advanced DMEM/F12.
18. Centrifuge at 200g for 2 mins to completely wash out TrypLE™ Express.

19. Discard the supernatants as much as possible, but do not disrupt the pellet.
20. Tap-resuspend the cells with the residual Advanced DMEM/F12.
21. Label the tube with what will be infected. The virus mixture (Table 2) and the 2.5X concentrated SC-enriching organoid medium (Materials and equipment, Table 2) will be added to this tube (see the next step).

2. Virus infection

This step allows the infection of the organoid stem cells with lentivirus mixture to deliver *i*) the Tet-activator and *ii*) the pTREG3-mutant gene of interest into the genome. Prepare the virus mixture fresh, immediately before or during the infection. If the high titer virus has been previously frozen, it can also be thawed and used. In this case, thaw the virus vial at room temperature, not directly in the 37°C water bath. Once the stem cells are infected with the virus, they are placed between the two layers of Matrigel® (top and bottom, Graphical Abstract), which will eventually grow as transduced organoids (Figure 4). A negative control for infection is performed by replacing the viral mixture with culture media. This control is important for the antibiotic selection of successful transduction.

Day 1.

Timing 2: [~1 hr]

21. Prepare virus solution from the virus mixture (Materials and equipment, Table 3).
 - a. Add 100 µL of Enhancer to the Lentivirus mixture to become the final 297.5 µL.
 - b. As the enhancer is viscous, gently tap-resuspend to mix homogeneously (or very gently pipet up and down).
 - c. Add 2.5 µL of TransDuxMAX™, gently tap-resuspend to mix homogeneously
22. Carefully pipet the virus solution of 300 µL into the tube with organoid stem cells from Step 20.
23. Gently tap the tube so that the cells and virus mix homogeneously.
24. Add the 200 µL of 2.5X medium (pre-warmed, Table 2) so that the final [cell + virus mixture] will be 1X. Gently pipet mix.
25. Incubate the tube in a 37°C water bath for 1 hour. Within this 1-hour incubation, gently tap the tube and mix the cell and the virus every 15 mins.
26. Carefully pipet the cell-virus mixture and place them in the Matrigel® coated well.
27. Incubate at 37°C for 1 day. During this 1-day incubation, viable cells will be attached to the Matrigel®.

Day 2. Infected stem cells being sandwiched by the Matrigel®

Timing: [20 mins]

28. Prepare 1X SC-enriching organoid medium (500 µL/well).
29. Take out the plate of step 27 from the incubator and carefully discard the medium containing the virus and dead cells. [Troubleshooting 4](#) (*)
30. Place an additional 100 µL/well of liquid Matrigel® on the plate so that the viable cells are located at the center between the top and the bottom layer of Matrigel® (forming a Matrigel® sandwich, Figure 4).
31. Place the plate in the 37°C incubator for 5 mins to solidify the newly added Matrigel®.

32. Take out the plate and add the 1X SC-enriching organoid medium to cover the Matrigel®.
33. Place back the plate into the 37°C incubator and grow the stem cells to form organoids.

Optional: * Step 29. Viable, transduced stem cells can still be present floating in the medium in Step 27. This medium can be placed in the Matrigel®-coated well prepared in supplement and incubated at 37°C (500 µL/well). On the next day, proceed from Step 28. See [Troubleshooting](#), Problem 4.

Note: The concentration factor of the infection medium and the volume of the virus mixture can be adjusted. The final [stem cells + virus] are in 1X SC-enriched organoid medium with 1X TransDuxMAX™ & Enhancer.

3. Antibiotic selection

This step allows the selection of organoids transduced with both the tet-activator (neomycin-resistant) and the gene of interest (puromycin-resistant). The concentration of antibiotics can be optimized to obtain the doses necessary to kill all the non-transduced organoids. In our hand, 1 µg/mL of puromycin and 100 µg/mL of neomycin are the minimum concentrations that completely kill the non-transduced organoids.

Day 4-5.

Timing: [~10 mins]

34. 2-3 days later, when the transduced stem cells form small but closed organoids, change the medium with fresh SC-enriched organoid medium with antibiotics (1 µg/mL puromycin + 100 µg/mL Neomycin).
35. Grow for 2-3 days more.

CRITICAL: In parallel, perform the control test by treating the non-transduced organoids with the antibiotics. The control organoids have undergone the same procedure throughout, except that the viral infection that is replaced by culture media. Each antibiotic should kill the non-transduced organoids.

Optional: If the Matrigel® is overcrowded with dead cells, remove the organoids from the Matrigel® sandwich and replace them with fresh Matrigel®, resulting in a droplet-based culture. To do this, discard the medium, put 500 µL/well of Cell recovery solution, and incubate at 4°C to liquefy the Matrigel®. Using a 1 mL cold pipet, gently pipet the liquid Matrigel® and collect it to the 15 mL falcon tube on ice (no harsh pipetting to minimize organoids breakdown). Wash the well with cold Advanced DMEM/F12 and collect them together in the 15 mL falcon tube. Resuspend to 4-5 mL with cold Advanced DMEM/F12 and centrifuge at 4°C, 300g for 5 mins to pellet the organoids. Discard the supernatant and wash once with 4 mL cold Advance DMEM/F12 by centrifugation at 4°C, 150g for 2 mins. Discard the supernatant, mix the pellet with fresh liquid Matrigel® and add 50 µL droplet/well. Solidify the Matrigel® at 37°C for 5 mins and add antibiotics to the SC-enriched organoid medium.

Day 6 >

Timing: [~10 mins]

36. The viable organoids should be visible. [Troubleshooting 5](#)
37. Replace the medium with a regular ENR medium containing Neomycin and Puromycin.
38. Grow until the organoids become big in presence of antibiotics. Change the medium every 2 days.
39. When the organoids become sufficiently big with dead cells accumulating in the lumen, passage the organoids in a regular ENR medium containing Neomycin and Puromycin.
40. Change the medium every 2-3 days (ENR medium + antibiotics)
41. Amplify/grow in sufficient amounts for the maintenance and experiments.

Note: As the organoids underwent simultaneous infections, the survival in the presence of the two antibiotics can be lower than that of a single infection. Sequential infection can also be done.

4. Doxycycline induction

To detect successful induction, we used a bicistronic expression of the fluorescent reporter protein (Figure 4).

Day >20

Timing: [~10 mins]

48. Passage the organoids for dox-induction and control (no addition of dox)
49. When the organoids grow sufficiently (~ 4-5 days), add 2 $\mu\text{g}/\text{mL}$ doxycycline in the dox-induction well.
50. Treat doxycycline for at least 16 hours.
51. Test the induction based on the detection method.

Note: A sufficient amount of doxycycline is required as it must penetrate the Matrigel[®] to reach the organoid cells. The concentration and duration of doxycycline treatment can be optimized depending on the experiment. We recommend performing a test of the serial concentration of doxycycline. In our hand, 2 $\mu\text{g}/\text{mL}$ of doxycycline of at least overnight treatment was required.

Expected outcomes

Intraluminal microinjection of bacteria

The shape of the organoids is important for successful microinjection (clear visible lumen, ~ 300 μm in diameter), and microinjection skill improves with practice. If everything runs smoothly, ~ 20 organoids can be successfully microinjected in 30 mins.

Live imaging of microinjected organoids

With the ZEISS Z1, it is possible to live image organoids of ~ 400 μm diameter every 6 mins for more than 8 hours (in a single view, single side illumination). However, the endogenous fluorescence becomes weaker during this process.

Lentivirus-mediated organoid transduction

Successful simultaneous viral transduction results in around 30 – 40 % survival of the seeded stem cells in the presence of both antibiotics. Single transduction results in around 60% survival in the presence of the antibiotic. Depending on the number of organoids used for transduction, several days to weeks are required to obtain a sufficient number of transduced organoids for the experiments and the maintenance (see the Note after Step 41). 2 $\mu\text{g}/\text{mL}$ of doxycycline overnight induction results in ~90% of successful induction despite mosaicism.

Limitations

Successful microinjection varies significantly with experience.

The live imaging method here is restricted to ZEISS Z1. Therefore, the detailed microscopic operation differs depending on the microscope used. However, the principles of live imaging of the organoids remain similar: fast acquisition with minimal photodamage in a thick specimen. Even though the light sheet method causes the least phototoxicity to the sample, it can still damage the organoids, especially when using multiple illumination lasers with a short time interval/long process. Therefore, it is important to find the optimal imaging and timing conditions depending on the biological question being addressed.

In a 2D cell culture system, stable cell lines usually show homogeneous induction & expression of the integrated gene. However, transgene induction in 3D organoids shows a mosaic pattern of expression. This may be due to the random insertion of the two transgenes, independently within the genome. Unlike the 2D cell lines, the stem cells of the organoids generate different cell subtypes, and it is possible that during lineage specification and differentiation, either one or both of the randomly inserted genes are regulated differently, resulting in various expression patterns. Alternatively, as the organoids are embedded within the Matrigel[®], through which the doxycycline penetrates to activate the Tet-transactivator, the doxycycline treatment may need to be further optimized.

Troubleshooting

Problem 1: Unsuccessful microinjection

Despite that the organoids are in good shape/location for the microinjection, the success of microinjection significantly varies on the experience of the experimenter. Sufficient practice and trial & error are required.

Problem 2: Bacteria is not ejected from the needle, related to intraluminal microinjection of bacteria, step 14.

Bacteria solution can stick to the needle and not be ejected even if you force the injection. Unfortunately, this can happen after several rounds of microinjections.

Potential solution:

- Try the “Clean” button to flush the needle.
- Remove the needle from the organoids and lift the capillary holder. Gently tap the metal thread above the screw holding the needle in place. Replace the capillary holder and test inject into PBS or a dish containing media before continuing with the microinjection. The initial number of bacteria ejected after tapping may vary, therefore, perform the test injection.

3. Change the needle.

Problem 3: Bacteria leaked from the needle.

Bacteria can leak out from the needle after the microinjection. If the bacteria are in the Matrigel, it is not a big problem as the Matrigel contains gentamicin that prevents overgrowth. Also, within the Matrigel, bacterial movement is extremely limited. If the organoid media is contaminated, gently remove the media, wash the plate with warm AdvDMEM/F12, and supplement the new media.

Problem 4: Too few organoids survive after virus infection within a Matrigel® sandwich, related to Lentivirus-mediated organoid transduction, virus infection step 29.

The transduced organoids stem cells may not adhere to the Matrigel® coating but still float in the medium.

Potential solution:

Prepare a new well with the Matrigel® coating. Do not discard the [virus + medium] but collect it and place it in the Matrigel® coating. Incubate for one day at 37°C. The next day, start the procedure from Step 29. Alternatively, incubate for two days at Step 27 and continue with the next steps.

Problem 5: Few viable organoids after antibiotic selection, related to Lentivirus-mediated organoid transduction, antibiotic selection step 36.

This indicates that the (double) transduction did not work and/or the treatment with two antibiotics was not optimal. Make sure that the organoids are used in stem cell-enriched conditions. We did not get good results when using frozen/thawed organoids.

Potential solution:

- Optimize the concentration of the antibiotics, as excessive antibiotics can be toxic. Since two antibiotics are used at the same time, optimize the concentration with uninfected organoids one at a time and two at a time.
- Perform sequential transduction. This will help to determine which virus transduces less efficiently. If necessary, reproduce the virus and use fresh virus for transduction. However, sequential transduction takes twice as long.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marc Lecuit (marc.lecuit@pasteur.fr).

Materials availability

This study did not generate new unique reagents

Data and code availability

This study did not generate/analyze datasets/codes.

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Author contributions

M.K. designed the protocol strategy, performed the experiment and wrote the manuscript. M.Lavina provided technical help. O.D. participated in the supervision of the study, writing & editing of the manuscript. M. Lecuit conceived the project, supervised and wrote & edited the manuscript.

Declaration of interests

The authors declare that there is no competing financial interest in this study.

References

Figure legends

Figure 1: Preparation of the organoids for microinjection. (A) Cell debris during washing. After wash, the dead cells are cleared. Scale bar, 100 μm . (B) Organoid preparation for microinjection. Stem cell-enriched organoids (day 3) are provided with ENR media for one day (day 4). This leads to thickening of epithelium and bud formation, indicating that the organoids start to differentiate. Scale bar, 500 μm . (C) Organoids grown in normal ENR media (left) and in stem cell-enriching media that is ready for lentivirus infection (right). Scale bar, 500 μm .

Figure 2: Image of the microinjection system (top left). FemtoJet (top right), joystick (bottom right), and needing ready for microinjection (bottom left).

Figure 3: Microinjection to the organoids. (A) Proper position of the microinjection needle (B) Organoid microinjection, during and after the injection. Goblet cells express mCherry ($\text{Muc2}^{\text{mCherry}}$), indicating proper differentiation. Bacteria express GFP. Scale bar, 50 μm .

Figure 4: Transduction of the organoids. pCMV: constitutive CMV (Cytomegalovirus) promoter. pTreg3G: TREG3G promoter activated by the Tet-Activator in presence of doxycycline. Gene: gene of interest. IRES: Internal Ribosome entry site. mCER: coding gene for the mCerulean fluorescent protein. Successfully transduced organoids are located between the two Matrigel[®] layers. Scale bar, 200 μm . Picture of induced organoids are reused with permission from ⁴

Methods Video S1: Filling the (phenol red-free) media into the sample chamber, related to live imaging of microinjected organoids step 22.

Video S2: Sample preparation for the microscope, related to live imaging of microinjected organoids step 23-26.

Video S3: Fixing the capillary into the sample holder, related to live imaging of microinjected organoids step 28.

Video S4: Sample loading to the microscope, related to live imaging of microinjected organoids step 29-33.

Video S5: An example of a bacterium crossing the epithelial barrier. The video is reused with permission from [\[ref 4\]](#).

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