

Submission ID #: 70512

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Title: A Two-Dimensional Organoid Culture on Invasin or Basement Membrane Extract to Study Host-Bacteria Interactions

Authors and Affiliations:

AUTHORS AND AFFILIATIONS:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 16

Number of Shots: 53

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Theodore Grenier:** We use human intestinal organoids to study how bacteria infect the gut and how gut cells respond to infection.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Theodore Grenier:** We culture the organoids in a polarized 2D monolayer to enable access to the cells' apical side.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Theodore Grenier:** This protocol allows researchers to study interactions between bacteria and non-tumoral, differentiated human intestinal cells.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Theodore Grenier:** An important consideration while performing this protocol is to let the organoid cells reach confluency before infecting them with bacteria. Then, bacteria need to grow properly during infection.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Theodore Grenier**: This protocol can be adapted to study other organs, bacterial species, and interactions between bacteria and different cell types.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What problem in your field of research does your JoVE article address?

- 1.6. **Theodore Grenier, Post-Doctoral Researcher, Hubrecht Institute for Developmental Biology and Stem Cell Research: Hubrecht Institute, Oncode Institute, Royal Netherlands Academy of Arts and Science (KNAW), University Medical Center Utrecht:** (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

What value do you expect this video article to have for other researchers working in your field?

- 1.7. **Theodore Grenier, Post-Doctoral Researcher, Hubrecht Institute for Developmental Biology and Stem Cell Research: Hubrecht Institute, Oncode Institute, Royal Netherlands Academy of Arts and Science (KNAW), University Medical Center Utrecht:** (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Please film the testimonials in both English and Dutch

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at the Hubrecht Institute

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Videographer's NOTE: All shots handled with some extra CU and some shots divided in two or three.

2. Coating Transwell Inserts with Invasin or Basement Membrane Extract

Demonstrator: Theodore Grenier

2.1. To begin, place the required materials inside a clean and sterile tissue culture hood [1]. Prepare a 5 microgram per milliliter solution of invasin in sterile PBS [2]. Add 100 microliters of this solution to the upper compartment of a 3-micrometer pore polyester cell culture insert placed in a 24-well plate [3]. Ensure that the membrane is completely covered [4] and incubate overnight at 4 degrees Celsius [5].

2.1.1. WIDE: Talent arranging materials inside a tissue culture hood.

2.1.2. Talent adding the invasin to PBS in a sterile container.

2.1.3. Talent pipetting 100 microliters of solution into the upper compartment of a cell culture insert in a 24-well plate.

2.1.4. Shot of the solution fully covering the membrane.

2.1.5. Talent placing the plate into a refrigerator set to 4 degrees Celsius.

2.2. Alternatively, if using Basement Membrane Extract for coating, prepare a 10 percent solution in ice-cold DMEM [1]. Add 100 microliters of the diluted solution to the upper compartment of the 3-micrometer pore cell culture insert [2]. Ensure complete coverage of the membrane surface [3] and incubate for 1 hour at room temperature [4].

2.2.1. Talent preparing the 10 percent solution in ice-cold DMEM.

2.2.2. Talent pipetting 100 microliters of the diluted solution into the upper compartment of a cell culture insert.

2.2.3. Talent pointing to the insert to confirm complete membrane coverage.

2.2.4. Talent placing the plate on the bench for incubation at room temperature.

3. Preparation and Seeding of Human Intestinal Organoid Cells onto Coated Transwell Inserts

- 3.1. Remove the human intestinal organoids derived from colorectal cancer surgical waste from the incubator three to five days after splitting [1]. After removing the spent medium [2], resuspend the organoids in 1 milliliter of ice-cold DMEM [3]. Transfer the suspension to a 15-milliliter conical tube containing 4 milliliters of ice-cold DMEM [4].
 - 3.1.1. Talent removing the culture plate from the incubator.
 - 3.1.2. Talent aspirating culture medium from organoid plates inside a tissue culture hood.
 - 3.1.3. Talent adding ice-cold DMEM to the organoids and resuspending them.
 - 3.1.4. Talent transferring the suspension into a 15 milliliter conical tube containing ice-cold DMEM.
- 3.2. Centrifuge the tube for 5 minutes at 500 *g* at 4 degrees Celsius [1]. Then, remove the supernatant completely [2].
 - 3.2.1. Talent placing the conical tube into a centrifuge.
 - 3.2.2. Talent aspirating and discarding the supernatant from the tube.
- 3.3. Resuspend the pellet in 3 milliliters of recombinant trypsin substitute [1]. Pipette up and down using a P1000 pipette to mechanically disrupt the organoids [2]. Incubate at 37 degrees Celsius for 15 to 20 minutes until single cells or small clusters of 3 to 4 cells are obtained [3].
 - 3.3.1. Talent adding recombinant trypsin substitute to the pellet and resuspending it.
 - 3.3.2. Talent pipetting the suspension up and down using a P1000 pipette.
 - 3.3.3. Talent placing the tube into a waterbath an incubator set to 37 degrees Celsius.
Author's Note: We used water bath set ad 37°C, not into an incubator.
- 3.4. Now, add 5 milliliters of ice-cold DMEM [1]. Centrifuge for 5 minutes at 500 *g* at 4 degrees Celsius [2] and remove the supernatant [3].
 - 3.4.1. Talent adding ice-cold DMEM to the tube.
 - 3.4.2. Talent placing the tube into a centrifuge.

- 3.4.3. Talent aspirating and discarding the supernatant.
- 3.5. Resuspend cells in expansion medium at 100 microliters per cell culture insert [1]. Count cells using a hemocytometer [2] and adjust the concentration to approximately 2×10^6 cells per milliliter [3].
 - 3.5.1. Talent resuspending the cell pellet in expansion medium.
 - 3.5.2. Talent loading the sample into a hemocytometer/Malassez slide.
 - 3.5.3. Talent adjusting the cell suspension concentration using medium.
- 3.6. Next, remove the coating solution from the inserts [1]. Add 100 microliters of cell suspension containing 2×10^5 cells to the upper compartment [2] and then dispense 500 microliters of expansion medium to the lower compartment [3].
 - 3.6.1. Talent aspirating coating solution from the cell culture inserts.
 - 3.6.2. Talent pipetting cell suspension into the upper compartment of the insert.
 - 3.6.3. Talent adding expansion medium to the lower compartment of the well plate.
- 3.7. Culture inserts at 37 degrees Celsius with 5 percent carbon dioxide until confluent [1], then differentiate with appropriate media and maintain for 5 days with regular media changes [2]. Measure transepithelial electrical resistance of the cells with suitable electrodes [3].
 - 3.7.1. Talent placing the cell culture plate into an incubator set to 37 degrees Celsius with 5 percent carbon dioxide.
 - 3.7.2. Talent aspirating and replacing medium in both compartments of the insert.
 - 3.7.3. Talent inserting the electrodes into the transwell compartments.
- 4. **Infecting the Differentiated Organoid Monolayers with Adherent-Invasive *Escherichia coli* (AIEC)**
 - 4.1. To begin, inoculate fluorescent adherent invasive *Escherichia coli* in 3 milliliters of Luria-Bertani medium inside a hood [1] and incubate overnight at 37 degrees Celsius with shaking [2].
 - 4.1.1. Talent inoculating fluorescent bacterial culture into a tube containing Luria-Bertani medium inside a tissue culture hood.
 - 4.1.2. Talent placing the culture tube into a shaking incubator.

4.2. After incubation, centrifuge 500 microliters of culture for 5 minutes at 1500 *g* [1]. Resuspend the pellet in 1 milliliter of Advanced DMEM supplemented medium [2]. Measure the optical density of the suspension at 600 nanometers [3] and dilute the cells to approximately 1×10^8 colony forming units per milliliter in Advanced DMEM supplemented medium [4].

4.2.1. Talent placing a microcentrifuge tube into a centrifuge.

4.2.2. Talent resuspending the bacterial pellet in medium.

4.2.3. Talent placing the sample in a spectrophotometer.

4.2.4. Talent diluting the bacterial suspension to the required concentration by adding media.

4.3. Now, remove media in both compartments of the transwell harboring differentiated organoid monolayers [1]. Add 500 microliters of fresh differentiation medium to the lower compartment [2] and then introduce 100 microliters of bacterial suspension to the upper compartment for the infected condition [3]. Add 100 microliters of Advanced DMEM supplemented medium to the upper compartment of the control insert for the non-infected condition [4]. Incubate the inserts at 37 degrees Celsius with 5 percent carbon dioxide and calculate the colony forming units per insert after plating on LB agar 2.5 hours post-infection [5].

4.3.1. Talent aspirating media in both compartments of the cell culture insert.

4.3.2. Talent adding differentiation medium to the lower compartment of one insert.

4.3.3. Talent pipetting bacterial suspension into the upper compartment of the insert.

4.3.4. Talent pipetting medium into the upper compartment for the control insert.

4.3.5. Talent placing the inserts into an incubator set to 37 degrees Celsius with 5 percent carbon dioxide.

5. Post-Infection Fixation and Fluorescent Imaging of Infected Epithelial Monolayers

5.1. At 5 hours post-infection, remove media and fix the cells with 4 percent paraformaldehyde inside a fume hood [1]. Incubate the cells for 30 minutes [2] and wash them three times with PBS [3].

5.1.1. Talent aspirating media and adding paraformaldehyde to the cell culture insert inside a fume hood.

5.1.2. Talent leaving the plate aside.

5.1.3. Talent washing the sample by adding PBS.

5.2. Excise the membrane insert and place cells facing upward in a well plate [1]. Stain with

fluorescent phalloidin for 1 to 2 hours [2] and wash three times with PBS [3].

5.2.1. Talent carefully cutting out the membrane insert and placing it in a well plate with cells facing upward.

5.2.2. Talent adding fluorescent phalloidin solution to the membrane.

5.2.3. Talent washing the membrane by adding PBS.

5.3. Stain nuclei with DAPI for 10 minutes [1]. After washing the sample three times with PBS [2], mount it on a glass slide with mounting medium and apply a coverslip [3]. Then, seal the coverslip with nail polish [4].

5.3.1. Talent adding 4',6-diamidino-2-phenylindole stain to the sample.

5.3.2. Talent washing the sample with PBS.

5.3.3. Talent placing the membrane on a glass slide, adding mounting medium, and applying a coverslip.

5.3.4. Talent sealing the edges of the coverslip with nail polish.

5.4. Finally, acquire and process confocal z-stack images to visualize nuclei, actin, and bacteria, including cross-sectional views [1]. Assess epithelial response by extracting RNA, and performing RT-qPCR to quantify gene expression [2].

5.4.1. Talent working at the confocal imaging system.

5.4.2. Talent placing the sample in a qPCR machine.

Results

6. Results

6.1. After reaching confluency, human intestinal cells on the cell culture insert displayed a mosaic-like pattern without visible gaps in the monolayer [1].

6.1.1. LAB MEDIA: Figure 1B.

6.2. Transepithelial electrical resistance values above 500 ohms square centimeters were observed, consistent with the formation of a tight epithelial monolayer [1].

6.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the data points positioned above the 500 ohm square centimeter level on the vertical axis*

6.3. The quantity of adherent invasive *Escherichia coli* in the basolateral compartment increased over time after infection [1].

6.3.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the upward trend of the plotted points from left to right*

6.4. Confocal imaging demonstrated a polarized monolayer with nuclei localized basally [1] and an apical enrichment of the actin network [2]. Additionally, the bacterial cells were visualized [3].

6.4.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the DAPI image*

6.4.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the magenta-stained Phalloidin image*

6.4.3. LAB MEDIA: Figure 3B. *Video editor: Highlight the AIEC image*

6.5. High magnification imaging revealed intracellular adherent invasive *Escherichia coli* [1] and extracellular bacteria attached to the apical membrane [2].

6.5.1. LAB MEDIA: Figure 3C. *Video editor: Zoom in on the bacteria marked with * an asterisk inside the cells*

6.5.2. LAB MEDIA: Figure 3C. *Video editor: Zoom in on the bacteria marked with \$ dollar symbols along the outer top edge of the cells*

6.6. Tumor necrosis factor alpha expression was significantly upregulated in response to adherent invasive *Escherichia coli* infection [1] compared to non-infected control [2].

- 6.6.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the green bar labelled AIEC*
- 6.6.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the non-infected bar*

Pronunciation guides:

1. Transwell
Pronunciation link: <https://www.howtopronounce.com/transwell>
IPA: /'trænz,wəl/
Phonetic Spelling: tranz·wel
2. Invasin
Pronunciation link: <https://www.howtopronounce.com/invasin>
IPA: /ɪn'veɪzɪn/
Phonetic Spelling: in·vay·zin
3. Polyester
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyester>
IPA: /,pɑːli'ɛstər/
Phonetic Spelling: paa·lee·es·ter
4. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪkroʊ,li:tər/
Phonetic Spelling: my·kroh·lee·terz
5. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪnkjə,bert/
Phonetic Spelling: in·kyuh·bayt
6. Basement
Pronunciation link: <https://www.merriam-webster.com/dictionary/basement>
IPA: /'beɪsmənt/
Phonetic Spelling: bays·ment
7. Extract
Pronunciation link: <https://www.merriam-webster.com/dictionary/extract>
IPA: /'ɛk,strækt/ (noun), /ɪk'strækt/ (verb)
Phonetic Spelling: ek·strakt
8. DMEM
Pronunciation link: <https://www.howtopronounce.com/dmem>
IPA: /,diːɛmɛm/
Phonetic Spelling: dee·em·em
9. Organoids
Pronunciation link: <https://www.howtopronounce.com/organoids>
IPA: /'ɔːrgənɔɪdz/
Phonetic Spelling: or·guh·noydz
10. Centrifuge

- Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
 IPA: /'sentrəˌfjuːdʒ/
 Phonetic Spelling: sen·truh·fyooj
11. Supernatant
 Pronunciation link: <https://www.howtopronounce.com/supernatant>
 IPA: /ˌsuːpərˈneɪtənt/
 Phonetic Spelling: soo·per·nay·tuht
12. Recombinant
 Pronunciation link: <https://www.merriam-webster.com/dictionary/recombinant>
 IPA: /rɪˈkɑːmbənənt/
 Phonetic Spelling: ree·kaam·buh·nuht
13. Trypsin
 Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
 IPA: /ˈtrɪpsɪn/
 Phonetic Spelling: trip·sin
14. Hemocytometer
 Pronunciation link: <https://www.howtopronounce.com/hemocytometer>
 IPA: /ˌhiːməsaɪˈtɑːmɪtər/
 Phonetic Spelling: hee·moh·sy·taa·muh·ter
15. Transepithelial
 Pronunciation link: <https://www.howtopronounce.com/transepithelial>
 IPA: /ˌtrænzˌɛprɪˈθiːliəl/
 Phonetic Spelling: tranz·ep·ih·thee·lee·uhl
16. Escherichia coli
 Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia%20coli>
 IPA: /ˌɛʃəˈrɪkiəˈkoʊˌlaɪ/
 Phonetic Spelling: esh·uh·rik·ee·uh koh·ly
17. Luria-Bertani
 Pronunciation link: <https://www.howtopronounce.com/luria-bertani>
 IPA: /ˈlʊriə bərˈtɑːni/
 Phonetic Spelling: loo·ree·uh ber·taa·nee
18. Optical
 Pronunciation link: <https://www.merriam-webster.com/dictionary/optical>
 IPA: /ˈɑːptɪkəl/
 Phonetic Spelling: aap·tih·kuhl
19. Nanometers
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
 IPA: /ˈnænəˌmiːtər/
 Phonetic Spelling: nan·uh·mee·terz
20. Paraformaldehyde
 Pronunciation link: <https://www.howtopronounce.com/paraformaldehyde>
 IPA: /ˌpærəfɔːrˈmældəˌhaɪd/
 Phonetic Spelling: pa·ruh·for·mal·duh·hyde
21. Phalloidin
 Pronunciation link: <https://www.howtopronounce.com/phalloidin>

IPA: /ˌfælərˈdɪn/

Phonetic Spelling: fal·oy·din

22. Diamidino-phenylindole

Pronunciation link: <https://www.howtopronounce.com/diamidino-phenylindole>

IPA: /daɪˌæmɪˈdiːnoʊ ˌfɛnɪlˈɪndoʊl/

Phonetic Spelling: dye·am·ih·dee·noh fen·uhl·in·dohl

23. Confocal

Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>

IPA: /kənˈfoʊkəl/

Phonetic Spelling: kuhn·foh·kuhl

24. RT-qPCR

Pronunciation link: <https://www.howtopronounce.com/rt-qpcr>

IPA: /ˌɑːrˈtiː kjuː piː siː ɑːr/

Phonetic Spelling: ar·tee kyoo·pee·see·ar