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Project Page Link: <https://review.jove.com/account/file-uploader?src=21977738>

Title: Live-Cell Imaging and Single-Cell Tracking to Monitor Clonal Dynamics and Fate Transitions in Human and Murine Intestinal Organoids

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? **Yes**

2.3.5, 2.5.1

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes.**

Videographer: Please film the computer screen

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. **No**

Current Protocol Length

Number of Steps: 12

Number of Shots: 31

Interviews

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

Authors: Answers are slightly edited in accordance with journal guidelines. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

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

- 1.1. Xuan Zheng: Our research focuses on studying how individual cells make fate decisions and coordinate their behavior within self-renewing intestinal epithelia.
 - 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. Daniel Krueger: Light microscopy cannot attribute a cell's membrane machinery to that cell or its neighbors, but mosaic organoids address this limitation.
 - 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. Daniel Krueger: This protocol allows researchers to study how a single cell remodels its cytoskeleton during migration, division, or extrusion within intact tissue.
 - 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. Xuan Zheng: The important consideration is choosing the labeling ratio, as sparse reporter cells isolate single-cell signals while sparse unlabeled cells reveal neighborhood responses.
 - 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. Xuan Zheng: Future studies can combine fate reporters with mosaic labeling in one organoid to link lineage commitment to single-cell cytoskeletal dynamics.
- 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.

Ethics Title Card

This research has been approved by the UMCU ethical committee and conducted in accordance with the Declaration of Helsinki and Dutch law at the University Medical Center Utrecht. Informed consent was obtained from all the patients

Protocol

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

2. Generation of Mosaic Intestinal Organoids

Demonstrator: Daniel Krueger

2.1. To begin, obtain the required reagents [1] and validated murine and human fluorescent reporter intestinal organoid lines [2]. Passage both organoid lines into Wnt (*Wnt*)-conditioned medium 3 days before mosaic organoid generation to promote stem cell expansion and maximize cell viability during dissociation [3].

2.1.1. WIDE: Talent sitting with a tray of required reagents. ***Videographer:** Please obtain CU shot as well for this shot.*

2.1.2. TEXT ON A PLAIN BACKGROUND:

Murine: H2B-mCherry, H2B-iRFP, Myosin-II-mNeonGreen

Human: MUC2-mNeonGreen, and DEFA5-dsRed

2.1.3. Shot of passaging both organoid lines into Wnt-conditioned medium.

2.2. Prepare 5% BME (*B-M-E*) in DMEM (*D-M-E-M*) [1]. Coat each well of a 48-well plate with 20 microliters of the prepared BME to provide a thin adhesive layer for cell attachment [1]. Then, incubate at 37 degrees Celsius for 1 hour [2]. **NOTE: The VO is edited for the additional shot**

Added shot: Talent preparing 5%BME in DMEM.

2.2.1. Talent coating the wells of a 48-well plate with 5% BME in DMEM.

2.2.2. Talent placing the 48-well plate into the incubator.

2.3. Next, dissolve the BME by adding ice-cold base medium [1]. Collect organoids from each line separately by centrifugation at 300 g for 5 minutes at 4 degrees Celsius [2]. Dissociate organoids into single cells by incubating them in an enzymatic cell-detachment solution [2] for 5 to 7 minutes in a water bath at 37 degrees Celsius [3]. Using a P1000 pipette, repeatedly pipette the suspension to aid mechanical dissociation [4]. Monitor the dissociation under a brightfield microscope [5].

2.3.1. Talent adding ice-cold base medium to dissolve the BME

2.3.2. Talent placing separate organoid samples into the centrifuge.

2.3.3. Talent placing the organoid suspension in the enzymatic cell-detachment solution into a water bath. **NOTE: 2.3.3 is filmed in two parts, first is talent suspending organoids in the enzymatic cell-detachment solution and 2nd is Talent placing the organoid suspension into a water bath.**

- 2.3.4. Talent repeatedly pipetting the suspension with a P1000 pipette.
- 2.3.5. SCOPE: Shot of the dissociated organoid suspension under a brightfield microscope.
- 2.4. Stop the reaction by adding excess ice-cold base medium when predominantly single cells or small organoid fragments of 2 to 10 cells are visible [1]. Then, pellet the cells by centrifugation at 300 g for 5 minutes at 4 degrees Celsius [2].
 - 2.4.1. Talent adding excess ice-cold base medium to the dissociated cell suspension.
 - 2.4.2. Talent placing the cell suspension into the centrifuge.
- 2.5. Assess cell viability and count the viable cells using trypan blue exclusion [1]. Combine the two cell populations at the desired ratio to obtain a total of 300,000 cells in 500 microliters of Wnt-conditioned medium supplemented with 10 micromolar Y-27632 (*Y-two-seven-six-three-two*) dihydrochloride for murine mosaic organoids or human Expansion Medium for human mosaic organoid cultures [2]. Mix thoroughly by gentle pipetting [3].
 - 2.5.1. SCOPE: Shot of viable and non-viable cells during trypan blue exclusion and cell counting.
 - 2.5.2. Talent combining the two cell populations in Wnt-conditioned medium supplemented with Y-27632 dihydrochloride for murine mosaic organoids or in human Expansion Medium for human mosaic organoid cultures.
 - 2.5.3. Shot of pipetting the mixed cell suspension.
- 2.6. Then, remove the coating medium from the 48-well plates [1], and seed the cell suspension into the coated wells [2]. Centrifuge the plate at 200 g for 5 minutes at room temperature [3] and incubate at 37 degrees Celsius [4]. **NOTE: The VO is edited for the additional shot**
Added shot: Shot of removing the coating medium in the 48-well plate.
 - 2.6.1. Shot of adding the cell suspension into the coated 48-well plate.
 - 2.6.2. Talent placing the 48-well plate into the centrifuge.
 - 2.6.3. Talent placing the plate into the incubator.
- 2.7. After 24 hours, mechanically detach the cell layer by scraping with a P200 pipette tip and flushing thoroughly with ice-cold base medium [1]. Collect the detached aggregates by centrifugation at 300 g for 3 minutes at 4 degrees Celsius without additional mechanical shearing [2]. Resuspend the aggregates in BME [3] and plate into imaging-compatible sample carriers [4].
 - 2.7.1. Shot of scraping the cell layer with a P200 pipette tip and flushing with ice-cold base medium.
 - 2.7.2. Talent placing the tube into the centrifuge.

- 2.7.3. Shot of resuspending the aggregates in BME.
- 2.7.4. Shot of plating the aggregates into imaging-compatible sample carriers.
- 2.8. Culture the plated aggregates in ENR medium for Murine and human Expansion Medium for human mosaic organoids [1].
 - 2.8.1. Talent adding ENR or human expansion medium to the plated aggregates.

3. Live Imaging and Single-Cell Tracking of Organoids

Demonstrator: Xuan Zheng

- 3.1. Use glass-bottom imaging chambers to ensure optical compatibility with high numerical aperture confocal objectives [1].
 - 3.1.1. Talent preparing a glass-bottom imaging chamber.
- 3.2. Plate the organoid-BME suspension as a thin dome near the center of the glass bottom [1]. Incubate the plate at 4 degrees Celsius for 5 to 10 minutes to allow the organoids to sink toward the glass bottom by gravity [2].
 - 3.2.1. Shot of plating the organoid-BME suspension as a thin dome near the center of the glass bottom.
 - 3.2.2. Talent placing the plate into the refrigerator.
- 3.3. Transfer the plate to 37 degrees Celsius and allow the BME to solidify for 20 to 30 minutes [1]. Add 500 microliters of pre-warmed growth medium to each well [2]. Allow the organoids to recover and establish their morphology for at least 24 hours before beginning live imaging [3].
 - 3.3.1. Talent placing the plate into the incubator.
 - 3.3.2. Shot of adding pre-warmed growth medium to a well.
 - 3.3.3. Shot of organoids established in culture with recovered morphology.
- 3.4. Acquire long-term time-lapse images and perform single-cell tracking and lineage reconstruction for downstream analysis [1].
 - 3.4.1. Display acquisition of long-term time-lapse images *Videographer: Please film the computer screen*

Results

4. Results

- 4.1. Successful mosaic formation was characterized by either a salt-and-pepper distribution [1] or defined boundaries between the two populations, depending on the degree of dissociation used [2].
 - 4.1.1. LAB MEDIA: Figure 3A. *Video editor: Highlight an organoid in the row labelled 'single cell dissociation' where green cells are dispersed among the magenta cells at the single-cell level.*
 - 4.1.2. LAB MEDIA: Figure 3A. *Video editor: Highlight an organoid in the row labelled 'small fragment dissociation' where green and magenta cells form separate coherent patches.*
- 4.2. Thorough single-cell dissociation produced salt-and-pepper mosaics [1], while gentle fragmentation preserved small clonal clusters [2].
 - 4.2.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the row labelled 'single cell dissociation'.*
 - 4.2.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the row labelled 'small fragment dissociation'.*
- 4.3. When unlabeled neighbors surround an individual cell co-expressing a reporter of interest, its cytoskeletal dynamics during migration, division, or extrusion can be unambiguously attributed to that cell [1].
 - 4.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the white boxed region.*
- 4.4. Nuclear H2B reporters facilitated the identification of informative cell constellations within the mosaic [1], and per-cell measurements, such as lifetime, division rate, and reporter intensity dynamics, were extracted from the tracking data [2].
 - 4.4.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the magenta nuclei in the white boxed region.*
 - 4.4.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the green curve in the graph labelled 'Myosin-II intensity'.*
- 4.5. Goblet cells activated and maintained high MUC2 (*mewk-two*)-mNeonGreen expression throughout the imaging period [1], while Paneth cells displayed initial MUC2-mNeonGreen activation [2] followed by progressive signal decrease [3] and subsequent DEFA5 (*D-E-F-A-five*)-dsRed activation [4].
 - 4.5.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the green MUC2 signal that*

remains visible across the time points from 0 h to 50 h.

- 4.5.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the green MUC2 signal at 0 h and 10 h in the sequence.*
- 4.5.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the green MUC2 signal at 50h*
- 4.5.4. LAB MEDIA: Figure 4A. *Video editor: Highlight the magenta DEFA5 signal that appears and increases across the time points.*
- 4.6. Single-cell quantification confirmed this sequential transition at the level of individual cells [1].
 - 4.6.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the graph showing the green 'MUC2' curve and the magenta 'DEFA5' curve.*
- 4.7. Averaged intensity curves across multiple tracked cells confirmed that this pattern was consistent at the population level [1], with Paneth cell precursors displaying a characteristic inverse relationship between MUC2-mNeonGreen and DEFA5-dsRed signals [2].
 - 4.7.1. LAB MEDIA: Figure 4C. *Video editor: Highlight the averaged fluorescence intensity plots labeled 'Paneth' and 'Goblet'.*
 - 4.7.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the 'Paneth' plot showing the green MUC2 curve decreasing while the magenta DEFA5 curve increases.*

1. Murine
Pronunciation link: <https://www.merriam-webster.com/dictionary/murine>
IPA: /'mjʊr, aɪn/
Phonetic Spelling: myoor·ine
2. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊ'res.ənt/
Phonetic Spelling: floor·ess·uhnt
3. Intestinal
Pronunciation link: <https://www.merriam-webster.com/dictionary/intestinal>
IPA: /ɪn'tes.tən.əl/
Phonetic Spelling: in·tes·tuh·nuhl
4. Organoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/organoid>
IPA: /'ɔ:r.gə,nɔɪd/
Phonetic Spelling: or·guh·noyd
5. Mosaic
Pronunciation link: <https://www.merriam-webster.com/dictionary/mosaic>

- IPA: /moʊˈzeɪ.ɪk/
Phonetic Spelling: moh·zay·ik
6. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /dɪˌsoʊ.siˈeɪ.ʃən/
Phonetic Spelling: dih·soh·see·ay·shuhn
 7. Myosin
Pronunciation link: <https://www.merriam-webster.com/dictionary/myosin>
IPA: /ˈmaɪ.ə.sɪn/
Phonetic Spelling: my·uh·sin
 8. Enzymatic
Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>
IPA: /ˌen.zəˈmæt.ɪk/
Phonetic Spelling: en·zy·mat·ik
 9. Centrifugation
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>
IPA: /ˌsen.trəˈfjuːˈɡeɪ.ʃən/
Phonetic Spelling: sen·truh·fyoo·gay·shuhn
 10. Trypan blue
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypan%20blue>
IPA: /ˈtrɪp.ən bluː/
Phonetic Spelling: trip·uhn bloo
 11. Dihydrochloride
Pronunciation link: <https://www.merriam-webster.com/dictionary/dihydrochloride>
IPA: /daɪˌhaɪ.droʊˈklɔːr.aɪd/
Phonetic Spelling: dye·hy·droh·klor·ide
 12. Aggregate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aggregate>
IPA: /ˈæɡ.rɪ.ɡət/
Phonetic Spelling: ag·rih·guht
 13. Numerical aperture
Pronunciation link: <https://www.merriam-webster.com/dictionary/aperture>
IPA: /nuːˈmer.ɪ.kəl ˈæp.ə.tʃər/
Phonetic Spelling: noo·mair·ih·kuhl ap·er·cher
 14. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kənˈfoʊ.kəl/
Phonetic Spelling: kon·foh·kuhl
 15. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔːrˈfɑːlə.dʒi/
Phonetic Spelling: mor·fol·uh·jee
 16. Lineage
Pronunciation link: <https://www.merriam-webster.com/dictionary/lineage>

- IPA: /'lɪn.i.ɪdʒ/
Phonetic Spelling: lin·ee·ij
17. Clonal
Pronunciation link: <https://www.merriam-webster.com/dictionary/clonal>
IPA: /'kloʊ.nəl/
Phonetic Spelling: kloh·nuhl
18. Cytoskeletal
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoskeleton>
IPA: /,saɪ.toʊ'skel.ə.təl/
Phonetic Spelling: sy·toh·skel·uh·tuhl
19. Extrusion
Pronunciation link: <https://www.merriam-webster.com/dictionary/extrusion>
IPA: /ɪk'struː.ʒən/
Phonetic Spelling: ik·stroo·zhuhn
20. Unambiguously
Pronunciation link: <https://www.merriam-webster.com/dictionary/unambiguous>
IPA: /,ʌn.æm'biɡ.ju.əs.li/
Phonetic Spelling: un·am·big·yoo·uhs·lee
21. Goblet
Pronunciation link: <https://www.merriam-webster.com/dictionary/goblet>
IPA: /'ɡɑː.blət/
Phonetic Spelling: gob·luht
22. Paneth
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: pan·eth
23. MUC2
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: myook·two
24. DEFA5
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: dee·ee·ef·ay·five
25. Quantification
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>
IPA: /,kwɑːn.tə.fə'keɪ.ʃən/
Phonetic Spelling: kwon·tuh·fuh·kay·shuhn