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Title: Label-Free, High-Resolution 3D Imaging and Machine Learning Analysis of Intestinal Organoids via Low-Coherence Holotomography

Authors and Affiliations:

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

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

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

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here:

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 35
Number of Shots: 55

Introduction

1.1. **Jimin Cho:** We aim to establish a real-time, label-free imaging method for monitoring biophysical changes in live organoids during development and in response to drugs **[1].**

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.6.1.*

How will your findings advance research in your field?

1.2. **Jimin Cho:** This protocol facilitates streamlined, scalable imaging and non-invasive drug testing in live organoids, supported by AI-driven segmentation and quantitative feature extraction for biomedical research **[1].**

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.12.1, 3.13.1.*

What research questions will your laboratory focus on in the future?

1.3. **Jimin Cho**: We plan to further integrate label-free 3D imaging and AI analysis for high-resolution, non-invasive organoid studies in disease modeling and precision medicine **[1].**

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

Protocol

NOTE: Time codes are added as provided by the authors. The writer has not reviewed the footage for regular (talent) shots.

SCREEN timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Balamurugan) for queries.

2. Passaging Small Intestinal Organoids

Demonstrator: Jimin Cho

2.1. To begin, aspirate the spent medium from each well of a 48-well plate containing the extracellular matrix **[1]**. Add 200 microliters of cell recovery solution to each well **[2]** and incubate at 4 degrees Celsius for 30 minutes **[3]**.

2.1.1. WIDE: Talent aspirating the medium from each well using a pipette. Author provided timecode: 68529_2.1.1.MP4 00:00-00:15

2.1.2. Talent dispensing cell recovery solution into each well. Author provided timecode: 2.1.2.MP4 00:00-00:23

2.1.3. Talent placing the plate on ice. Author provided timecode: 68529_2.1.3.MP4 00:00-00:10

2.2. Using a pipette, gently collect the organoid suspension and transfer it into a microcentrifuge tube **[1]**. Centrifuge the tube at 150 *g* for 3 minutes **[2]**, then carefully remove the supernatant **[3]**.

2.2.1. Talent collecting organoids by gentle pipetting and transferring them into a microcentrifuge tube. Author provided timecode: 68529_2.2.1.MP4 00:00-00:30

2.2.2. Talent placing the tube in the centrifuge. Author provided timecode: 68529_2.2.2.MP4 00:00-00:20

2.2.3. Talent removing the supernatant. Author provided timecode: 68529_2.2.3.MP4 00:00-00:24

2.3. To dissociate the pellet mechanically, resuspend it in 200 microliters of culture medium and pipette the suspension up and down 20 to 30 times using a P200 *(P-two-hundred)* pipette **[1]** before centrifuging it for 3 minutes **[2].**

2.3.1. Talent adding culture medium to the pellet in the microcentrifuge tube,

resuspending the pellet, and mixing the suspension by pipetting up and down. Author provided timecode: 68529_2.3.1.MP4 00:20-01:00

2.3.2. Talent placing the tube in the centrifuge. Author provided timecode: 68529_2.3.2.MP4 00:00-00:23

2.4. After centrifugation and removal of the supernatant, add medium and fresh extracellular matrix or ECM *(E-C-M)* in a 1 to 4 ratio to the pellet and gently pipette to mix thoroughly **[1]**. Dispense 15 microliters of the mixture per dome into each well of a 48-well plate **[2]**.

2.4.1. Talent adding ECM and medium to the pellet and pipetting to mix thoroughly. Author provided timecode: 68529_2.4.1.MP4 00:00-00:30

2.4.2. Talent dispensing the mixture per dome into the wells of a 48-well plate. Author provided timecode: 68529_2.4.2.MP4 00:00-00:15

2.5. Place the plate upside down in a 37-degrees Celsius, 5 percent carbon dioxide incubator for 1 hour to allow polymerization of the ECM **[1]**.

2.5.1. Talent inverting the plate and placing it inside the incubator. Author provided timecode: 68529_2.5.1.MP4 00:00-00:19

2.6. After polymerization, add 200 microliters of fresh culture medium to each well **[1]** and fill the empty outer wells with PBS **[2-TXT]**.

2.6.1. Talent pipetting culture medium into the wells. Author provided timecode: 68529_2.6.1-2.6.2.MP4 00:13-00:53

2.6.2. Talent filling the empty outer wells with PBS. **TXT: Replace the culture medium every 2 – 3 d and passage the organoids weekly** Author provided timecode: 68529_2.6.1-2.6.2.MP4 01:31-02:09

3. Low-Coherence Holotomography Imaging

Demonstrators: Jimin Cho and Jaehyeok Lee

3.1. To prepare the sample for imaging, dispense 15 microliters of organoid-ECM dome onto a number 1.5 coverslip-bottom imaging dish and incubate it at room temperature for 1 minute **[1]**.

3.1.1. WIDE: Talent dispensing organoid-ECM mixture onto a coverslip-bottom dish. Author provided timecode: 68529_3.1.1.MP4 00:00-00:30

3.2. Place the plate upside down in a 37-degrees Celsius, 5 percent carbon dioxide incubator for 1 hour **[1]**. Then, gently add enough culture medium to fully submerge the organoids **[2]**.

3.2.1. Talent inverting the dish and placing it in the incubator. Author provided timecode: 68529_3.2.1.MP4 00:00-00:23

3.2.2. Talent pipetting culture medium over the ECM domes to submerge them. Author provided timecode: 68529_3.2.2.MP4 00:00-00:11

3.3. At 5 days post-passage, wash the sample 2 to 3 times with PBS immediately before imaging **[1]**.

3.3.1. Talent washing the sample with PBS. Author provided timecode: 68529_3.3.1.MP4 00:00-00:24

3.4. Next, for imaging, turn on the environmental controller. The chamber controller unit will automatically set the temperature to 37 degrees Celsius and carbon dioxide to 5 percent **[1]**.

3.4.1. Talent pressing the power button on the environmental controller and the chamber controller unit automatically sets the temperature and carbon dioxide percentage. Author provided timecode: 68529_3.4.1.MP4 00:00-00:06

3.5. Press the door button to open the door **[1]**. Add water to form a thin layer inside the chamber well **[2]**.

3.5.1. Talent pressing the door button to open the door. Author provided timecode: 68529_3.5.1.MP4 00:00-00:03

3.5.2. Talent pipetting water to create a thin layer inside the chamber well. Author provided timecode: 68529_3.5.2.MP4 00:00-00:10

3.6. Place the imaging dish in the vessel holder, insert it into the imaging chamber **[1]**, and secure it using a pin to prevent movement **[2-TXT]**.

3.6.1. Talent placing the dish in the holder and inserting it into the imaging chamber. Author provided timecode: 68529_3.6.1.MP4 00:00-00:04

3.6.2. Talent securing the dish to the holder with a pin. **TXT: Ensure the dish is firmly attached to the vessel holder without tilting** Author provided timecode: 68529_3.6.2-3.7.1.MP4 00:00-00:06

3.7. Close the door to prevent external light interference **[1]**.

3.7.1. Talent gently closing the door using door button. Author provided timecode: 68529_3.6.2-3.7.1.MP4 00:06-00:08

3.8. Now, launch the TomoStudio X *(Tomo-Studio-ex)* software and log in **[1]**. Click **Start** to open the main window, and then click **Add Project** in the top-left corner and assign the experiment. Confirm that the correct medium type is selected for appropriate refractive index usage **[2]**.

3.8.1. SCREEN: 68529_screenshot_1.mp4 00:03-00:10; 00:20-00:22 *Video editor: Speed up as needed*

3.8.2. SCREEN: 68529_screenshot_1.mp4 00:22-00:26 **and** 68529_screenshot_2.mp4 00:01-00:05; 00:10-00:20; 00:27-00:44 *Video editor: Speed up as needed*

3.9. Click on the desired well in the panel and then click **Create** at the top to register the well as a specimen **[1]**.

3.9.1. SCREEN: 68529_screenshot_2.mp4 00:45-00:58

3.10. Then, click **ROI Setup** *(R-O-I Setup)* in the top-right corner to define the region of interest in the dish **[1]**. Once set, click **Run Experiment** in the bottom-right corner to open the image acquisition window **[2]**.

3.10.1. SCREEN: 68529_screenshot_3.mp4 00:00-00:38 *Video editor: Speed up as needed*

3.10.2. SCREEN: 68529_screenshot_3.mp4 00:40-00:50

3.11. Click **Load Vessel** in the top-right corner to display a brightfield image **[1]**. Adjust the Z-position using the **+Z** *(plus Z)* and **-Z** *(minus Z)* buttons to bring the image into focus **[2]**.

3.11.1. SCREEN: 68529_screenshot_4.mp4 00:00-00:10; 00:36-00:40; 00:51-00:55; 01:17-01:20; 01:35-01:38 *Video editor: Speed up as needed*

3.11.2. SCREEN: 68529_screenshot_5.mp4 00:03-00:16; 00:20-00:32*Video editor: Speed up as needed*

3.12. In the **Single Imaging** tab, adjust the ROI size. Capture organoids in a 160-micrometer by 160-micrometer field of view and acquire stacks up to 140 micrometers deep **[1-TXT]**.

3.12.1. SCREEN: 68529_screenshot_6.mp4 00:00-00:10 **TXT: For larger organoids, check**

the Tile Imaging box to stitch multiple tomograms

3.13. Navigate to the **Time Lapse Imaging** tab to set up long-term imaging and set the desired duration and interval time **[1]**.

3.13.1. SCREEN: 68529_screenshot_7.mp4 00:00-00:30 *Video editor: Speed up as needed*

3.14. Click the **Scan** icon to capture the current ROI location **[1].** ~~and center the ECM dome using brightfield imaging **[2].**~~ Click the **BF** button to adjust the intensity and exposure values for brightfield imaging **[2]**. NOTE: VO is struck through for the removed shot.

3.14.1. SCREEN: 68529_screenshot_8.mp4 00:02-00:07; 00:14-00:19; 00:39-00:41: 04:38-04:41 *Video editor: Speed up as needed*

~~3.14.2. SCREEN: To be provided by authors: Centering the ECM dome using brightfield imaging.~~ NOTE: Shot removed by the author

3.14.3. SCREEN: 68529_screenshot_10.mp4 00:00-00:11

3.15. Move the ROI box in the Preview panel to select the ROI. Once the desired ROI is selected, click **Add Point** at the bottom **[1].** The imaging point list will be created **[2]**.

3.15.1. SCREEN: 68529_screenshot_11.mp4 00:02-00:05; 00:08-00:10; 00:16-00:20; 00:28-00:30 *Video editor: Speed up as needed*

3.15.2. SCREEN: 68529_screenshot_11.mp4 00:32-00:34; 00:50-00:55 *Video editor: Speed up as needed*

3.16. Now, click **Acquire** to begin imaging and to acquire the raw image data **[1]**.

3.16.1. SCREEN: 68529_screenshot_12.mp4 00:03-00:11; 00:40-00:43; 01:07-01:09 *Video editor: Speed up as needed*

3.17. Launch the **HTX processing server** by clicking the desktop icon **[1].** Drag and drop raw image files to the HTX processing server. Click **Process** to generate a TCF file from the raw image file **[2]**.

3.17.1. SCREEN: 68529_screenshot_13.mp4 00:00-00:20 *Video editor: Speed up as needed*

3.17.2. SCREEN: 68529_screenshot_13.mp4 00:20-00:30; 01:55-02:09 *Video editor: Speed up as needed*

3.18. Launch **TomoAnalysis Viewer** by clicking the desktop icon **[1]**. Load the processed TCF *(T-C-F)* files by dragging and dropping them into the Viewer window **[2]**.

3.18.1. SCREEN: 68529_screenshot_14.mp4 00:00-00:07

3.18.2. SCREEN: 68529_screenshot_14.mp4 00:07-00:17

3.19. Double-click a file thumbnail to open the RI *(R-eye)* tomogram **[1]**. Examine the 2D view by navigating through the XY-Z planes using zoom, pan, and scroll controls **[2]**.

3.19.1. SCREEN: 68529_screenshot_15.mp4 00:00-00:07

3.19.2. SCREEN: 68529_screenshot_15.mp4 00:07-00:11; 00:18-30; 00:41-00:48 *Video editor: Speed up as needed*

3.20. Click the **MIP Rendering View** icon on the left to switch to 3D rendering mode. Navigate the 3D view by rotating, zooming, and panning the image **[1]**.

3.20.1. SCREEN: 68529_screenshot_16.mp4 00:00-00:08; 00:19-00:26; 00:36-00:42 *Video editor: Speed up as needed*

4. Image Analysis

Demonstrator: Jimin Cho

4.1. For the machine learning-based image segmentation, export the TCF *(T-C-F)* image file to HDF5 *(H-D-F-five)* format to ensure the data is in a multi-dimensional format compatible with ilastik **[1]**.

4.1.1. SCREEN: 68529_screenshot_4.1.1.mp4 00:35-00:55 *Video editor: Speed up as needed*

4.2. Open ilastik and navigate to **New Project [1]**. Select **Pixel Classification** under the **Segmentation Workflows** section and save the project in a designated folder **[2]**.

4.2.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:00-00:01

4.2.2. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:01-00:03

4.3. To load the HDF5 file, navigate to the **Input Data** tab, click **Add New File**, select the appropriate H5 dataset, and verify the correct image channel assignments **[1]**.

4.3.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:06-00:08

4.4. Now, navigate to the **Feature Selection** tab to choose features like **Color, Intensity**, **Edge**, and **Texture** to optimize segmentation **[1]**.

4.4.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:08-00:16

4.5. In the **Training** tab, label the organoid and non-organoid regions using different color brush strokes **[1-TXT]**.

4.5.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:17-00:49 *Video editor: Speed up as needed* **TXT: Carefully annotate regions around boundaries**

4.6. Click **Live Update** to preview segmentation results and make adjustments if necessary **[1]**.

4.6.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 00:49-00:51

4.7. Once done, go to the **Prediction Export** tab, select **Simple Segmentation** as the source to export labeled predictions, and click **Export All**. Set the export format to **.h5** *(H-five)* or **.tiff** *(T-I-F-F)* based on the analysis requirements **[1]**.

4.7.1. SCREEN: 68529_screenshot_4.2.1-4.7.1.mp4 01:56-02:08

4.8. For the quantitative analysis, open the supplementary coding file 2. Designate the appropriate folder paths for the mask file and the corresponding TCF file within the script **[1]**. Run the code to initiate quantitative analysis **[2]**.

4.8.1. SCREEN: 68529_screenshot_4.8.1-4.8.2.mp4 00:26-00:33

4.8.2. SCREEN: 68529_screenshot_4.8.1-4.8.2.mp4 00:43-00:53

4.9. The code will calculate the organoid volume, protein density, and total protein content for each dataset **[1]**.

4.9.1. SCREEN: 68529_screenshot_4.9.1.mp4 00:00-00:04

Results

5. Results

5.1. This figure illustrates the high-resolution three-dimensional refractive index reconstructions used to visualize the overall morphology of small intestinal organoids **[1]**. The rendered reconstructions revealed distinct structural differences between **[2]** vehicle-treated **[3]** and cisplatin-treated organoids **[4]** across all three optical section depths **[5]**.

5.1.1. LAB MEDIA: Figure 3A, 3B.

5.1.2. LAB MEDIA: Figure 3A, 3B.

5.1.3. LAB MEDIA: Figure 3A, 3B. *Video Editor: Highlight A.*

5.1.4. LAB MEDIA: Figure 3A, 3B. *Video Editor: Highlight B.*

5.1.5. LAB MEDIA: Figure 3A, 3B. *Video Editor: Highlight the six small images in the bottom row.*

5.2. Cisplatin-treated organoids showed significantly higher volume **[1]**, lower protein density **[2]**, and higher total protein contents **[3]** compared to vehicle-treated organoids at 10 minutes post-treatment **[4]**, indicating early structural swelling and altered protein composition **[5]**.

5.2.1. LAB MEDIA: Figure 3C, 3D, 3E. *Video Editor: Highlight the blue bar in C when the VO says "Higher volume".*

5.2.2. LAB MEDIA: Figure 3C, 3D, 3E. *Video Editor: Highlight the blue bar in D.*

5.2.3. LAB MEDIA: Figure 3C, 3D, 3E. *Video Editor: Highlight the blue bar in E.*

5.2.4. LAB MEDIA: Figure 3C, 3D, 3E. *Video Editor: Highlight the pink bars in all three graphs.*

5.2.5. LAB MEDIA: Figure 3C, 3D, 3E.

5.3. Time-lapse imaging over 24 hours showed that vehicle-treated organoids maintained structural integrity **[1]**, while cisplatin-treated organoids experienced progressive structural degradation **[2]**, including crypt collapse and increased cell dissociation, suggesting time-dependent cytotoxic damage **[3]**.

5.3.1. LAB MEDIA: Figure 4A.

5.3.2. LAB MEDIA: Figure 4B.

5.3.3. LAB MEDIA: Figure 4B. *Video Editor: Emphasize the **two** images in the bottom row (24 h).*

5.4. Quantitative tracking confirmed that **[1]** vehicle-treated organoids increased in volume and protein contents over time **[2]**, while cisplatin-treated organoids showed a time-dependent decline in both parameters **[3]**, indicating that cisplatin suppressed growth and induced cellular degradation **[4]**.

5.4.1. LAB MEDIA: Figure 4C, 4E.

5.4.2. LAB MEDIA: Figure 4C, 4E. *Video Editor: Highlight the pink plots.*

5.4.3. LAB MEDIA: Figure 4C, 4E. *Video Editor: Highlight the blue plots.*

5.4.4. LAB MEDIA: Figure 4C, 4E.

5.5. Protein density remained stable in vehicle-treated organoids **[1]**, but progressively decreased in cisplatin-treated organoids over the 24-hour period **[2]**, suggesting a breakdown in cellular structure and increased extracellular space due to exfoliation **[3]**.

5.5.1. LAB MEDIA: Figure 4D. *Video Editor: Highlight the pink plot.*

5.5.2. LAB MEDIA: Figure 4D. *Video Editor: Highlight the blue plot.*

5.5.3. LAB MEDIA: Figure 4D.

Pronunciation Guide:
aspirate
Pronunciation link: https://www.merriam-webster.com/dictionary/aspirate How To Pronounce+2How To Pronounce+2
IPA (US): /ˈæs.pəˌreɪt/ (verb)
Phonetic spelling: AS-puh-rayt

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supernatant
Pronunciation link: https://www.merriam-webster.com/dictionary/supernatant Merriam-Webster
IPA: /ˌsuː.pərˈnæt.ənt/
Phonetic spelling: soo-pur-NAT-uhnt

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microcentrifuge
Pronunciation link: https://www.howtopronounce.com/microcentrifuge How To Pronounce+1
IPA: /ˌmaɪ.kroʊˈsɛn.trəˌfjuːdʒ/
Phonetic spelling: MY-kroh-sen-TRI-fyoohj

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organoid
Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/organoid youtube.com+1
IPA: /ˈɔːrgəˌnɔɪd/
Phonetic spelling: OR-guh-noyd

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extracellular
Pronunciation link: https://www.merriam-webster.com/dictionary/extracellular Merriam-Webster
IPA: /ˌɛk.strəˈsɛl.jə.lər/
Phonetic spelling: eks-truh-SEL-uh-ler

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matrix
Pronunciation link: https://www.merriam-webster.com/dictionary/matrix Merriam-Webster
IPA: /ˈmeɪ.trɪks/
Phonetic spelling: MAY-triks

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polymerization
Pronunciation link: https://www.merriam-webster.com/dictionary/polymerization Merriam-Webster
IPA: /pəˌlɪm.ə.rəˈzeɪ.ʃən/
Phonetic spelling: puh-li-MER-uh-zay-shuhn

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resuspend

Pronunciation link: No confirmed link found
IPA: /ˌriː.səˈspɛnd/
Phonetic spelling: ree-suh-SPEND

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dissociate
Pronunciation link: No confirmed link found
IPA: /dɪˈsoʊ.si.eɪt/
Phonetic spelling: dih-SOH-shee-ayt

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passage (as in cell culture)
Pronunciation link: Merriam-Webster “passage” available but obvious; not provided
IPA: /ˈpæs.ɪdʒ/
Phonetic spelling: PAS-ij

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microliter (microlitre)
Pronunciation link: No confirmed link found
IPA: /ˈmaɪ.kroʊˌliː.tər/
Phonetic spelling: MY-kroh-LEE-tər

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environmental (as in controller)
Pronunciation link: Merriam-Webster “environmental” available but obvious
IPA: /ɪnˌvaɪ.rənˈmɛn.təl/
Phonetic spelling: in-VY-ruhn-MEN-tuhl

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controller
Pronunciation link: Merriam-Webster “controller” available but obvious
IPA: /kənˈtroʊ.lər/
Phonetic spelling: kuhn-TROH-lər

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tomogram (as in RI tomogram)
Pronunciation link: No confirmed link found
IPA: /ˈtoʊ.məˌgræm/
Phonetic spelling: TOH-mə-gram

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18. brightfield
Pronunciation link: No confirmed link found
IPA: /ˈbraɪtˌfiːld/
Phonetic spelling: BRYTE-feeld

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tomostudio (software name)
Pronunciation link: No confirmed link found
IPA: /ˌtoʊ.moʊˈstjuː.di.oʊ/
Phonetic spelling: TOH-moh-STYOO-dee-oh

ilastik (software)
Pronunciation link: No confirmed link found
IPA: /ɪˈlæstɪk/
Phonetic spelling: ih-LAS-tik