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Generation of 3D Printed Biological iMolds for Optically Transparent Tissue Specimens

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TITLE:

Generation of Three-dimensional Printed Biological Innovative Mold for Optically Transparent Tissue Specimens

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SUMMARY:

Tissue innovative Molds (iMolds) have been developed to reduce specimen movement, structurally support the specimen being imaged, and allow for repeated imaging on precise anatomical locations using optically-transparent samples.

ABSTRACT:

Histology continues to evolve in modern day experimentation. Recently, groups have been able to make tissue and bones transparent by removing lipids and other biological species that prevent deep tissue imaging. Now, groups can robustly “clear” the tissue or specimen and perform a wide array of microscopy such as single-photon, multiphoton, and light-sheet to evaluate their biological target of interest. This has eliminated the need to freeze, cyro-slice, and cyro-preserve tissue, greatly limiting human prone error. However, current tissue clearing methods still lack a robust system to mount and structurally support unique specimens. This leads to issues with tissue movement during imaging, the lack of reliability when re-staining and re-evaluating specimens, and working with abnormally shaped specimens such as tumors. To overcome these obstacles, the innovative Mold (iMold) was generated. iMolds are first created from images of the specimen followed by three-dimensional (3D) printed molds that support the unique tissue structure being imaged and re-imaged. This leads to a dramatic reduction in tissue movement while imaging and allows for scientists to robustly re-evaluate areas of interest that have been re-stained or need to be re-imaged. Furthermore, iMolds can be created in a short time period and generated for multiple samples in any imaging format (*i.e.* slide, cell culture plate). This allows users to scan multiple organs or specimens on one slide or cell culture dish. In

closing, iMolds can be utilized by any research laboratory studying cleared specimens while also reducing costs, time, and tissue movement compared to other current methodologies.

INTRODUCTION:

Whole tissue clearing methods have recently become a widely-used method in histology. First the tissue and organ are cleared resulting in the specimen becoming optically-transparent, then this is followed by staining and imaging. With these techniques scientists are able to image at high resolution entire organs and thick specimens. In addition, many tissue clearing methodologies allow for repeated staining. This provides users the ability to develop 3D renderings of structural and functional relationships across a large volume of area.

The 3D renderings that are produced have application in many fields of science. For example, scientists can now clear and generate a 3D model of a tumor at cellular and subcellular resolution[1]. This allows scientists the ability to study the heterogeneity of cells, create 3D renderings of entire organs to examine cell interactions, vascularization, and environment niches. Furthermore, using systems like this also allows a whole organ evaluation after drug screenings and other therapeutic interventions. The benefits of tissue clearing make it imperative that there is a rapid and reliable method to structurally support any specimen being imaged to the micron level and also provide a method to place multiple organs on one imaging platform, to allow for screening of multiple specimens.

For these reasons, the tissue iMold has been generated[2]. iMolds are made to be utilized by researchers evaluating large tissue specimens that are optically transparent. The iMold restricts all tissue movement during prolonged and repeated imaging. Current methodologies lead to specimen movement during imaging and re-imaging[3]. In addition, current methods make it near impossible for sequential staining of the same cellular environment, due to relocating precise anatomical locations. These caveats are resolved with the generation of the tissue specific iMolds.

iMolds can be generated by almost all 3D printers and used by any research laboratory. First, they are generated at the micron level with the material of choice, such as PLA plastic. Then the iMold is mounted and can be used for repeated imaging. iMolds also allow the user to image multiple organs on one imaging platform during one imaging session, or repeated sessions. They are inexpensive to generate and reliable to use. The development of the iMold and representative results are detailed in this protocol.

PROTOCOL:

1. Clear the Tissue or Bone Specimens Following Established Clearing Methods

1.1. Follow this protocol or another established tissue-clearing method: for passive CLARITY begin by making hydrogel monomer (HM) solution included of 1% (wt/vol) acrylamide, 0.0125-0.05% (wt/vol) bisacrylamide, 4% paraformaldehyde, 1x PBS, deionized water, and 0.25% of the thermal initiator into a 50-mL tube[3].

1.2. Perform a trans-cardial perfusion with HM and incubate the tissue overnight or 2 days at 4 °C with the HM solution in a 50-mL tube[3].

Note: Add enough HM solution to completely incubate the tissue, typically 10-15 mL is sufficient.

1.3. De-gas the tissue in the 50-mL tube to replace the oxygen with nitrogen, followed by incubation at 37 °C for 3-4 h for hydrogel polymerization.

Note: De-gas following the established protocol[3]. Remove the oxygen by placing an aspirator into the 50-mL tube. With the aspirator in place, put a line of nitrogen gas directly into the solution, causing it to bubble. Perform this action for 5 min followed by polymerization.

1.4. Clear the tissue in a 37 °C shaking incubator with a buffered clearing solution consisting of 4% (wt/vol) sodium dodecyl sulfate and 0.2 M boric acid (pH 8.5) with solution replacement every one to two days. After this step, wash off the clearing solution with a 0.2 M boric acid buffer (pH 8.5)/0.1% Triton X-100. Typically, 10-15 mL of solution is sufficient, ensure the tissue is completely submerged.

1.5. For storage, place specimens in 1X PBS or imaging solution until it is time to image.

2. Generate the Tissue iMold Image File

2.1. Place the specimen onto a flat surface (*i.e.* lab bench).

2.2. Measure the length, width, and thickness of the specimen.

Note: Measuring the samples before clearing the specimen is preferred, as cleared tissue may be challenging to measure due to its transparency. However, measuring after clearing should also be performed, if the user notices tissue structure deformations post-tissue clearing.

2.3. Take a photo of the specimen with the measurements (*e.g.* cell phone camera).

2.4. Double-click to open a sterolithography (.STL) file creator.

2.5. Upload the current photo into the software by selecting "**file**" and select "**open**".

2.6. Now with the uploaded image, click and hold to select the image and drag the current image onto the current z-plane.

2.7. Click the image and scroll towards the image corner to shrink or outwards to maximize the image.

Note: It is easier for measurements if the user makes the initial imported photo small and then proceed to adjust it after tracing.

2.8. On the toolbar select the “**freehand**” drawing tool.

2.9. With the freehand tool draw around the region or specimen of interest.

Note: Write down the current dimensions to be used to speed up the process of making the current image.

2.10. Click the “**pointer**” button on the toolbar and select the image that was just traced.

2.11. Press “**delete**” on the keyboard and delete the original image (now there is a sketch of the specimen).

2.12. Select the “**pointer**” button on the toolbar and click the original image face.

2.13. Select the “**scale**” tool in the toolbar.

2.14. Select the specimen image face with the scale tool. Type in the proper width or length into the scale measurement box at the bottom right corner of the screen.

2.15. Repeat the prior step to complete both the width and the length of the specimen.

Note: Now there will be an image face file of the exact same dimensions of the specimen being traced and used.

2.16. Click and select a region outside of the specimen face.

2.17. Click and select the “**rectangle**” tool.

2.18. With the rectangle tool draw a rectangle or square of the size of the slide or plate that is planned to be imaged on.

Note: Slides are commonly used and are 25 mm × 75 mm. However, if the imaging platform is designed for cell culture plates, then design based on those measurements.

2.19. Select the “**pointer**” button on the toolbar and click and select the face of the specimen image.

2.20. Click and select the “**move**” tool from the toolbar.

2.21. Click the specimen and move the specimen face onto the face of the rectangle (same z-plane) by dragging the image.

Note: Place the specimen in the innermost area of the iMold to allow for the gluing step to be performed without mixing of imaging medium and glue.

2.22. Select the "**pointer**" button on the toolbar and re-select the specimen face.

2.23. Press "**delete**" on the keyboard to remove the specimen face.

Note: Now the rectangle will have an empty space for where the specimen will be placed.

2.24. In the toolbar select the "**push/pull**" tool.

2.25. Click and select the rectangle face with the specimen area previously deleted.

2.26. In the button left hand corner type in the measurement to be the thickness of the rectangle (*i.e.* the thickness of the tissue).

Note: Now there will be a rectangle the size of a slide or cell culture plate to be imaged with a missing place for the specimen to be placed within. However, it is suggested to add 0.5 mm to each side of the specimen to allow for imaging media to be placed with the specimen, but this is not required.

2.27. In the "**file**" tab select the file and export it as a .STL.

3. Printing the .STL file iMold

3.1. Open the saved .STL file in the 3D printer software. Go to the "**file"** tab and select "**open"**.

3.2. Select "**print**" from library on the button of the screen.

3.3. Select the .STL file.

3.4. Print the file at optimal resolution (*i.e.* 150 μM) with the highest quality setting.

Note: It typically takes between 1-4 h depending on the specimen and imaging platform. PLA is commonly used for its flexibility but other plastics such as ABS also work for iMold generation. 3D printers with heated stages are preferred for improved printing results.

4. Using the .STL iMold

4.1. Place the printed iMold on the working lab bench.

4.2. Place a few lines of super glue around the specimen area.

Note: Glue the outermost area of the iMold to avoid mixing of superglue with the specimen. In addition, using superglue that can be purchased in a pen style to allow a finer width to be applied.

4.3. Place coverglass onto the superglue.

Note: Allow sufficient time for the glue to dry, if using an upright microscope then super glue must be applied to both sides with coverglass once the specimen and liquid has been placed in the iMold.

4.4. Place the specimen into the iMold.

4.5. Add the appropriate amount of liquid of clearing solution to fill the chamber of the iMold but do not add excess as this will mix with the superglue.

Note: The clearing solution refractive index should match the refractive index of the objective being used to image for optimal imaging results.

4.6. Proceed to imaging (step 5).

Note: If re-staining, remove the specimen, clear the prior antibodies following the previously established protocols, re-stain, and replace the specimen into the iMold and re-image. If the iMold becomes structurally altered, then re-print a new iMold from the saved .STL file.

5. Imaging the iMold

5.1. Place the tissue iMold into the appropriate microscope (*i.e.* single-photon confocal or multi-photon) platform (*i.e.* plate holder, slide holder). Add imaging solution until the entire iMold chamber is filled, typically less than 200 µL is needed.

5.2. Find the location of interest and image using the ideal microscope settings.

Note: Imaging using the z-stack tile allows for a large area to be imaged through multiple z-planes. In addition, ensure proper controls when using new antibodies.

REPRESENTATIVE RESULTS:

The benefits of this method, in combination with tissue clearing methods is the ability to control movement of tissue to avoid shifting during long term imaging and/or the re-staining of tissue. Therefore, it is critical that the user designs a properly fitted iMold, have proper tissue-clearing, and have the validation of the immunofluorescent target (*i.e.* RNA, Protein). Other methods include using putty or iSpacers to maintain the tissue, however this leads to the issues previously discussed (*i.e.* tissue movement, relocation of targets of interest in the specimen).

For this experiment, the brain was used from the BAC-Glt1-eGFP mouse model, which labels all grey-matter astroglia with eGFP (**Figure 1A**). The eGFP reporter is strongly expressed throughout

all astroglia soma and major astroglial processes and with minor eGFP-fluorescence distributed on the minor processes in the neuropil[4]. To achieve detailed images of these minor processes would require the usage of higher magnified objectives during imaging, and have been previously shown with super resolution[5]. **Supplemental Figure 1** illustrates taking a specimen and designing an iMold for imaging.

After clearing a thick coronal section of the brain, the mouse cerebral cortex was imaged using single photon with 40x objective and numerical aperture of 0.8 (**Figure 1B**, **1C**, **Movie 1**). Imaging results display highly abundant astroglia distribution throughout the entire cortical column imaged (**Figure 1B**, **1C**). Next, using prior published method that allows users to transform fluorescence signal into voxels. Each voxel represents a cell body. Using this method, the software calculated a total of 463 astroglia in the 0.9-mm tall section (**Figure 1B**)[5].

Next, astroglia are known to physical connect with other astroglia[6]. In support of this, representative images found astroglia in direct connection with neighboring astroglia (**Figure 2A**). Furthermore, astroglia are highly important in the maintenance of the blood-brain-barrier and extend major processes that connect with the cerebral arteries, known as endfeet[7]. Using the same microscopy parameters as before, it is shown that astroglia are harnessing a cerebral artery around 500 μm deep (**Figure 2B**, **2C**).

Studies of the central nervous system using tissue clearing methods are continuing to advance the understanding of cellular and molecular biology. With the current study, it is shown that astroglia can be automatically counted using iMolds and can be imaged during an extended time period without movement. Furthermore, it is shown that a cellular interface between astroglia with other astroglia and cerebral arteries can be imaged and investigated with this methodology. Lastly, the representative data show that minor processes in neuropil can also be imaged with the eGFP signal, as it can be seen distributed throughout the neuropil, appearing as fragmented eGFP-fluorescence (**Movie 1**, **Figure 2A-2C**).

FIGURE AND MOVIE LEGENDS:

Figure 1: Z-stack section of the entire volume imaged displaying the abundance of astroglia. (**A**) iMold with coronal section. (**B**) Each astroglia was replaced as a cylindrical voxel and computationally counted. (**C**) the tissue section imaged with single-photon microscopy top view.

Figure 2: Cellular interactions of astroglia in the cerebral cortex. (**A**) z-slice image of astroglia physically connecting. (**B**) z-slice image of astroglia harnessing a cerebral artery. (**C**) End-foot processes on a cerebral artery of Glt1-eGFP positive astroglia. Arrows represent arrows of contact. Red dots represent an astroglia soma. Images were taken around 500 μm deep.

Movie 1: Imaging processing through each z-slice. A video of one imaging session of an entire section of mouse cerebral cortex from pia to corpus callosum.

Supplemental Figure 1: iMold generation for a specimen, measurements, computational modeling of the iMold, and result of 3D printing the iMold for imaging. Modified from Miller S.J. and Rothstein J.D.[2]

DISCUSSION:

Optical clearing methods are continuing to increase the understanding of environmental niches and cellular heterogeneity. The tissue is maintained and stored in a hydrogel complex that allows biomacromolecules to permeate the matrix. This allows for groups to study immunofluorescence of proteins and nucleic acids either by endogenous fluorescence or post-hoc labeling, across large volumes of tissue.

However, a major caveat in the system comes when attempting to image the tissue. In current methods, the tissue is placed onto a slide and surrounded by an adhesive material such as putty. Then the tissue is imaged and removed. Next time the user images, they have to generate a new putty structure to maintain the tissue, leaving large gaps between each imaging session[3]. Another caveat is that during long imaging sessions the tissue is likely to move, due to being imaged in a semi-aqueous optical solution. These are variables that make CLARITY and other tissue clearing methods challenging to re-image and generate additional 3D renderings on the same cellular microenvironment.

With the generation of the iMold, users can stop movement of the tissue being imaged by using an optical slide or plate which is 3D printed and allow for re-imaging of the same environment with the same parameters as the original imaging session. This allows for groups to essentially, setup their imaging parameters and walk away from the system while it images. This can be done by combining multiple organs or tissue onto one slide or plate. This allows screening of multiple organs much easier.

Additionally, iMolds do allow for storage of the tissue or specimen. Please note that each iMold will vary slightly based on gluing for how long tissue can be kept inside before leaking. iMolds have lasted for months in the same iMold for storage but note that the medium must be changed every week to preserve tissue integrity. In addition, when imaging tissue or specimens that are not flat on the glass surface, simply adjust your z-plane until you acquire fluorescent signal then proceed with z-stack tiling.

In the future, iMolds can be used to study tissue in all fields of medicine and biology. In tumor studies, groups can reconstruct the entire tumor with multiple stainings to generate a true 3D picture of the cellular and subcellular environment. For neuroscience, the entire central nervous system can be imaged and reconstructed, evaluating neural circuitry and cellular and subcellular changes. An additional approach is investigating bone, which can be cleared as previously shown, to be able to study metastasized cancer cells or microenvironments. The usage of using tissue clearing methods with iMolds is endless and will provide scientists with the ability to quickly evaluate their tissue of interest thoroughly without being concerned about movement or re-imaging or being unable to identify exact anatomical locations. For these reasons, the iMold

provides a perfect, affordable, user-friendly approach to use in combination with tissue clearing methodologies.

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Figure

Figure 1

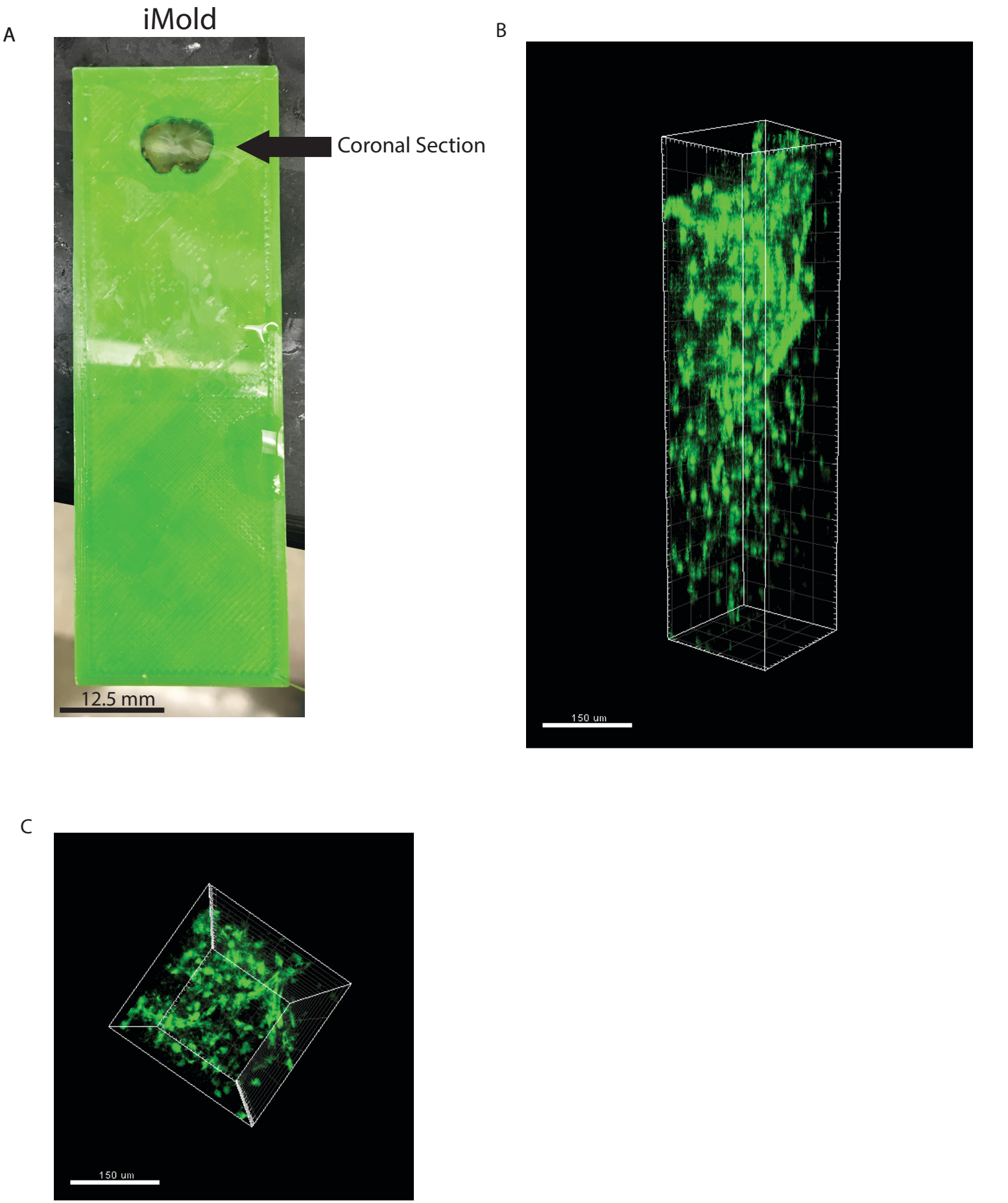

Figure Figure 2

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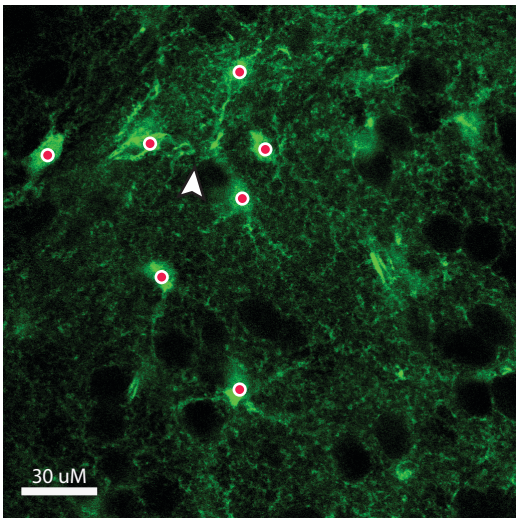

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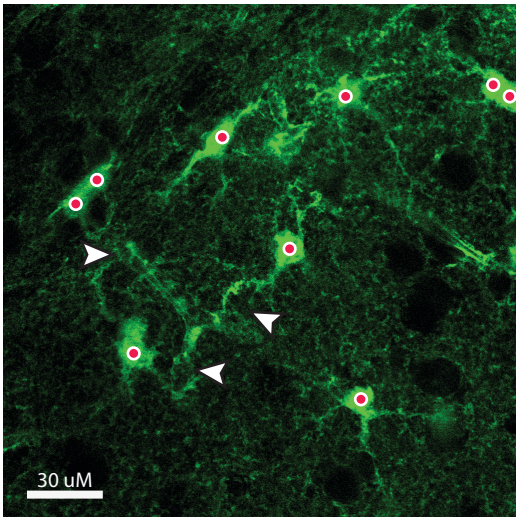

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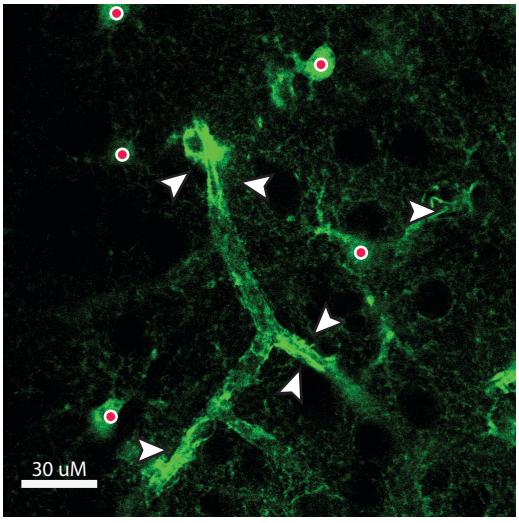

Animated Figure (video and/or .ai figure files)

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iMold JoVE Movie 1.wmv

Materials Table

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Acrylamide	Sigma	A9099	
bisacrylamide	VWR	0172-50G	
paraformaldehyde	Electron Microscopy Sciences	15714-S	
PBS	Quality Biological	119-069-131	
thermal initiator	Wako Chemicals	VA-044	
sodium dodecyl sulfate	Bio-Rad	161-0302	
boric acid	Sigma	B-7660	
Triton X-100	Sigma	X100	
focus clear	CEDARLANE	F101-KIT	
Google Sketchup			
Camera			we used an iPhone 7 camera
3D Printer	M3D	Micro +	
PLA plastic roll for 3D Printer	M3D	3D Ink	no catalog number listed, we use black PLA filament

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Signature:	 Date: 09/16/2017

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Dear Dr. Alaghemandi,

Thank you for your editorial comments, we have now modified the original article and addressed your concerns, listed in bold lettering. Please let us know if there is anything you need from our behalf.

Very Best,

Sean and Jeff

Dear Dr. Rothstein,

Your manuscript JoVE57347R1 "Generation of 3D Printed Biological iMolds for Optically Transparent Tissue Specimens" has been again editorially reviewed and the following comments need to be addressed. Please track the changes to identify all of the manuscript edits. After revising the submission, please also upload a separate document that addresses each of the editorial comments individually with the revised manuscript.

Your revision is due by **Jan 11, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Mohammad Alaghemandi, Ph.D.
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Editorial comments:

1. The manuscript has been modified. Please read it carefully and revise if necessary. Enclosed please find the top copy. Please apply your changes on the top copy. Please do not change the current format/font.

We have modified the manuscript to address your concerns and kept the formatting the same.

2. Please avoid using any abbreviation in the Title.

We have now changed the total to remove the abbreviation, thank you.

3. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Thank you for allowing us to improve the introduction. We have now modified it to address the concerns by including additional sentences and paragraph.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.).

We thoroughly went through the manuscript to ensure everything is in the imperative tense.

5. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added the "how" question and also added additional references for the users to be able to refer to.

6. For steps that involve software, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we're looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

We have highlighted all buttons to be clicked in bold with quotations to allow users to easily identify what buttons to be clicked. In addition, the software has a nice user interface that we plan to film.

7. Protocol: 1.1: Please use the imperative tense for all the sentences of the protocol steps.

We have addressed this, thank you.

8. Protocol: 1.2: How much of the solution is roughly enough?

We have added the information to address how much liquid is often used.

9. Protocol: 1.3: "De-gas" how? Using what? Please clearly describe the actions.

We have added a reference that explains thoroughly how to do this, in addition we have added more information.

10. Protocol: 1.4: How much solution is needed for clearing or washing?

We have added how much solution is roughly needed for both clearing and washing of the tissue. In addition, we have provided an additional reference.

11. Protocol: 1.5: How is that done?

We have added more detail to explain how this is performed.

12. Protocol: 2.4: “Open” where? How? Please include all the buttons clicked in the software.

We have added all the information needed to now know what buttons to click, these buttons are now highlighted in bold with quotations.

13. Protocol: 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14, 2.15, 2.17, 2.18, 2.19, 2.20, 2.21, 2.22, 2.23, 2.26, 2.28, 3.31: How is that done? Please include all the buttons clicked in the software, and all the setting selected.

We have added all the information needed to now know what buttons to click, these buttons are now highlighted in bold with quotations.

14. Protocol: 5.2: Note 2: Please move the second Note to the Discussion.

We have now added this note to the discussion part of the manuscript.

15. After revising the protocol, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted roughly 2.5 pages for filming that we believe are essential components for the users to know how to perform to complete this protocol.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted the sub sections in addition to the steps.

17. Please avoid numbering Figures inside them, e.g., please remove “Figure 1” from Figure 1.

Thank you, we have removed this from the figure.

18. Figure 1: Panels (B) and (C): Please use SI units, please use “µm” instead of “um”.

Thank you, we have revised the figure.

19. Figure 2: Please use SI units, please use “µm” instead of “uM”.

Thank you we have revised the figure.

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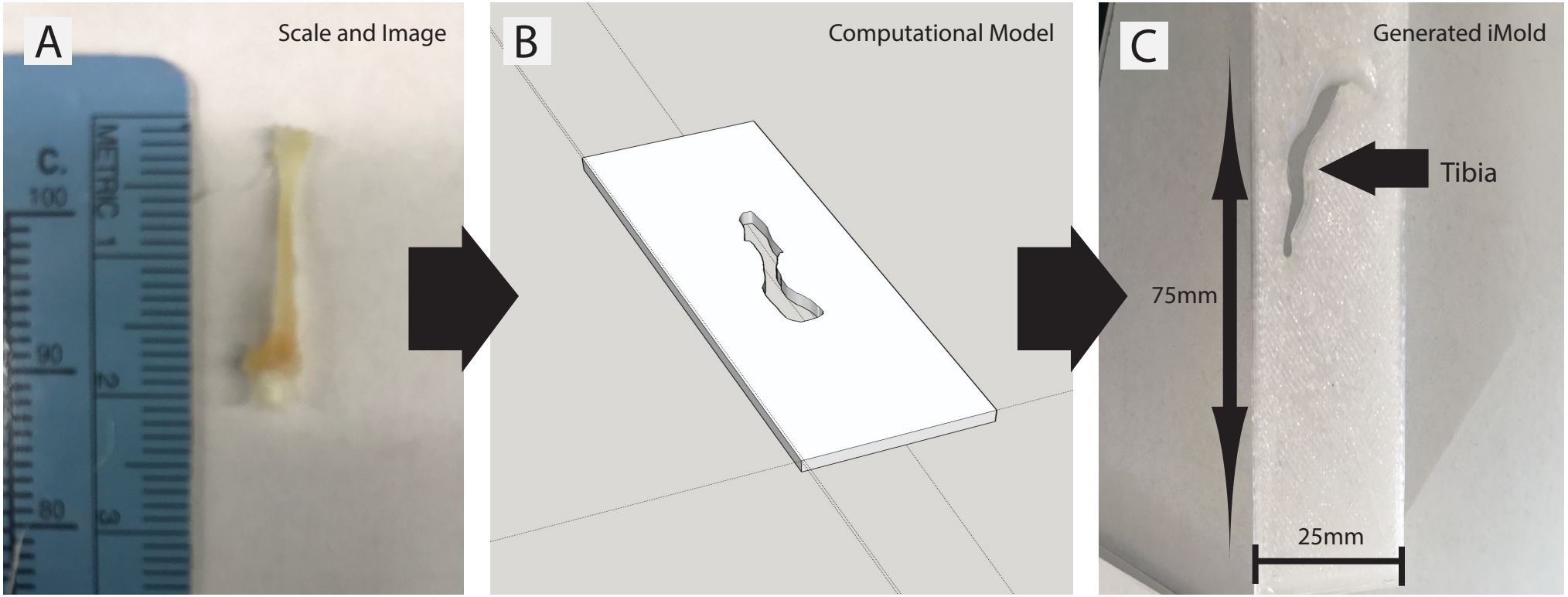
Supplemental Figure 1



modified from Miller S.J., and Rothstein. J.D. 3D printer generated tissue iMolds for cleared tissue using single- and multi-photon microscopy for deep tissue evaluation. Biol Proced Online, 2017. doi: 10.1186/s12575-017-0057-2.