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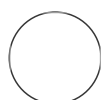
## Light Sheet Fluorescence Microscopy of Human Kidney Using Active Clearing with the SHIELD Reagent

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Human BioMolecular Atlas Program (HuBMAP) Method Development  
Community

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**Protocol status:** Working  
We use this protocol and it's working

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Light sheet fluorescence microscopy (LSFM) is a method to cover micro-mesoscale ( $\mu\text{m}$ - $\text{cm}$ ) areas of tissue while achieving depth of several millimeters to enable 3D-volumetric imaging of tissue structures or organs. By using fluorescence tagged primary or secondary immunofluorescence it is capable of provides cellular resolution and anatomical maps.

Compared to traditional laser confocal microscopy imaging LSFM, LSFM provides rapid acquisition of images over large optically cleared tissues while maintaining high resolution. As compared to serial 3D reconstruction, *In toto* imaging by LSFM provides a more accurate 3D dataset that captures the spatial relationships between structures and cells. Application of LSFM to adult human tissues, particularly solid tissues such kidney, is challenging due to its complex anatomical organization and composition, heterogeneity and interstitium. The relationships between extracellular and extrarenal structures have not been defined. This knowledge is critical to understand how different functional tissue units of the kidney are organized in 3D as key interactions and signaling necessary for its physiological roles cannot be correctly derived from 2D images. Here we describe a protocol we developed to aid in 3D visualization of kidney cells, functional tissue units and their relationship with nerves, vasculature and lymphatics as a goal in HuBMAP consortium to create 3D reference maps. After testing several protocols, here we describe a clearing method on tissue slices in conjunction with immunofluorescence staining o visualize deep kidney volumes (1-3mm depth) when imaged by light sheet microscope. We provide several tips during tissue preparation for clearing, clearing procedure and an example of imaging the tissue using LSFM. We also briefly discuss various outputs of imaging and metadata files that can be used for data submission or for publishing or hosting on external servers.

The imaging section contains the following: 1)Refractive Index (RI) matching tissue with Refractive Index Matching Solution (RIMS), 2) Capturing a full-volume 5x image, 3) Capturing 20X images of regions of interest (ROIs), 4) Stitching and down sampling images prior to analysis, 5) Different file formats. All imaging was completed using a Zeiss Lightsheet 7 microscope.

## MATERIALS

### FOR CLEARING AND IMMUNOSTAINING

Solutions:

Blocking reagent (PBS-BB)

1% BSA (Jackson ImmunoResearch  
001-000-162)

0.2% Skim milk

0.3% Triton X-100 (Sigma T8787)

1X PBS

LifeCanvas SHIELD  
kit

LifeCanvas EasyIndex  
solution

LifeCanvas  
Delipidation Buffer and Conduction Buffer

0.1% Sodium Azide (Sigma S2002)

## **FOR IMAGING**

### **Equipment And Reagents:**

The following equipment was used in imaging:

Zeiss Lightsheet  
7 for imaging.

Zeiss Lightsheet  
7 mounting equipment, including mounting hook and T-shaped mounting stub from

Zeiss. Quick curing epoxy was used to affix the mounting stub to a copper electrical clip (<https://www.amazon.com/Toothless-Alligator-Copper-Plated-Microscopic/dp/B012RHZJWC>) to pick up the sample.

Zeiss 5x/0.16 foc  
and Clr Plan-Neofluar  
20x/1.0 Corr n = 1.53 detection lenses.

Two Zeiss LSFM  
5x/0.1 foc and Zeiss LSFM 10x/0.2 foc illumination lenses.

Zeiss Large  
n=1.33-1.58 R.I. 5x and n=1.35-1.58  
R.I. 20x imaging chambers.

Clay Adams Gold  
Seal 24x12 mm Cover Glass.

Loctite Super  
Glue Professional Liquid for gluing sample to glass coverslip.

### **The following reagents were used in imaging**

LifeCanvas 1.52  
RI EasyIndex solution (<https://lifecanvastech.com/products/easyindex/>) to refractive index match sample.

1.52  
Refractive-Index Cargille Oil (Catalog: Immersion Liquid Number 5040) for immersing sample during imaging.

## Tips

### 1 Tips:

- Active clearing of SHIELD (Park et al., 2018; PMID: 30556815; PMCID: PMC6579717; DOI: 10.1038/nbt.4281) -infiltrated samples takes around seven days to complete.
- The first phase uses a polyepoxy matrix dubbed Stabilization under Harsh conditions via Intramolecular Epoxide Linkages to prevent Degradation (SHIELD) to infiltrate throughout the sample using the LifeCanvas SHIELD kit ([https://lifecanvastech.com/shop/tissue-preservation-shield-solution/?utm\\_term=&utm\\_source=adwords&utm\\_medium=ppc&utm\\_campaign=Campaign+%231&hsa\\_acc=9064444419&hsa\\_grp=140304471241&hsa\\_src=g&hsa\\_cam=1045314231&hsa\\_tgt=dsa-1655710008190&hsa\\_ad=598029723340&hsa\\_net=adwords&hsa\\_mt=&hsa\\_kw=&hsa\\_ver=3&gclid=EAlaI QobChMI0bD63p\\_JgQMVtujjBx2Hdw\\_qEAAYASAAEgJQDvD\\_BwE](https://lifecanvastech.com/shop/tissue-preservation-shield-solution/?utm_term=&utm_source=adwords&utm_medium=ppc&utm_campaign=Campaign+%231&hsa_acc=9064444419&hsa_grp=140304471241&hsa_src=g&hsa_cam=1045314231&hsa_tgt=dsa-1655710008190&hsa_ad=598029723340&hsa_net=adwords&hsa_mt=&hsa_kw=&hsa_ver=3&gclid=EAlaI QobChMI0bD63p_JgQMVtujjBx2Hdw_qEAAYASAAEgJQDvD_BwE)), and requires 3 days infiltration at 4 0C and one day polymerization at 37 0C with rotation.
- The second phase delipidates the sample using the LifeCanvas SmartBatch+ system (<https://lifecanvastech.com/products/tissue-clearing-labeling/>), which requires 1 day incubation at 45 0C with shaking and 30 hours active delipidation with the SmartBatch+ ETC system.

## CLEARING

- 2 Human kidney sections that have been fixed (4% PFA), rinsed and stored in PBS are ready for clearing. We have typically used full depth cortex-medulla of the kidney up to 3 mm in depth, 2.5 cm in length and 5 mm in width.
- 3 Remove the sample from PBS and place it in 20 mL of SHIELD-Off solution, consisting of 10 mL LifeCanvas SHIELD-Epoxy, 5 mL LifeCanvas SHIELD-Buffer and 5 mL deionized water in a 20 mL scintillation vial and incubate on a shaker at 4 0C for 3 days.
- 4 Switch to incubation in 20 mL of LifeCanvas SHIELD-ON solution at 37 0C with rotation for 1 day.
- 5 Move samples out of scintillation vials and into mesh bags that come with the SmartBatch+ delipidation kit for delipidation using the LifeCanvas SmartBatch+ system. Load bags with samples into metal ring holder within cup (Fig. 1), both from Smartbatch+ kit. Fill cup with LifeCanvas delipidation buffer until

samples are submerged, close the lid, and incubate at 45 0C while rotating for one day.



Fig. 1: SmatBatch+ sample loading kit consisting of mesh bags, metal ring holder, and cup.

- 6 Remove samples from delipidation cup and transfer them to the LifeCanvas SmartBatch+ dilipidation chamber (Fig. 2) for 30 hours of active clearing using LifeCanvas delipidation buffer and conduction buffer.



Fig. 2: SmartBatch+ Delipidation Chamber

***Note: After delipidation as above the sample will appear almost white, step 5 time may vary depending on tissue type, size.***

- 7 Wash with 1X PBS at room temperature with rotation for 3 hours X2, then overnight.
- 8 Block with 1%BSA/0.2%skim milk/0.3% Triton X-100 in 1X PBS (PBS-BB)/ 0.1% Sodium Azide at room temperature on rotator for 2 days.

## IMMUNOSTAINING

- 9** Incubate the shield-cleared specimen with primary antibodies in PBS-BB)/ 0.1% Sodium Azide:
- 9.1** at room temperature with rotation for 7 days.
- 9.2** at 37 °C for 2 days, then room temperature overnight.
- 10** Note: For the kidney we routinely use anti-CD31 (mouse), anti-Tuj1 (rabbit), anti-AQP2 (goat) and anti-NPHS1 (sheep), each at 1:100 dilution.
- 11** 9. Remove antibodies and wash with 0.3% Triton X-100 in 1X PBS/0.1% Sodium Azide at room temperature with rotation for 3 hours X2, then overnight.
- 12** 10. Incubate the specimen with fluorescence conjugated secondary antibodies (in 0.3% Triton X-100 in 1X PBS/0.1% Sodium Azide at room temperature with rotation for 4 days.
- 13** Note: For primary antibodies listed in step 10, we have used anti-mouse alexa-488, anti-rabbit-cy3 and anti-goat-cy5 and anti-sheep-cy5. Samples are always covered with aluminum foil during all incubation steps to preserve fluorescence from this step onwards.
- 14** 11. Wash with 0.3% Triton X-100 in 1X PBS/0.1% Sodium Azide at room temperature with rotation for 3 hours X2, then overnight at RT.
- 15** 12. Incubate with DAPI 1:1000 in 0.3% Triton X-100 in 1X PBS/0.1% Sodium Azide at room temperature for one day to label nuclei.

- 16 13. Wash with 0.3% Triton X-100 in 1X PBS/0.1% Sodium Azide at room temperature with rotation for 3 hours X2, then overnight at RT.
- 17 14. After staining, the sample may be stored in foil in 1x PBS/0.1% Sodium Azide prior to imaging. When preparing to image, the sample must be refractive index matched with EasyIndex. First transfer sample into new 5mL Eppendorf tube and incubate sample at RT in a 50/50 mixture of LifeCanvas EasyIndex solution for one day. Next, incubate sample in LifeCanvas EasyIndex at RT for one day. It will now be ready for imaging. See Figs 3 and 4 below for a before and after image of the sample.



Fig. 3: Sample before clearing.



Fig. 4: After clearing and RI-matching, the sample should appear transparent.

## Refractive Index Matching Procedure for Cleared Samples

- 20 *Tip: If the sample is being stored in PBS/0.1% Sodium Azide, it must be refractive index matched prior to imaging.*

Mix EasyIndex

Thoroughly shake bottle of Lifecanvas

EasyIndex solution (Fig. 5) and wait thirty minutes for bubbles to settle.



Fig. 5: LifeCanvas EasyIndex bottle for Refractive Index Matching to 1.52.



- 21 Prepare a solution of 50/50 EasyIndex and deionized water.
- 22 Incubate the sample in the 50/50 solution.
- 23 When samples are ready to image, transfer them from their storage buffer after immunolabeling (typically in 2 ml round bottom Eppendorf tubes willed with PBS wrapped in a foil) into a clean, 5 mL Eppendorf tube filled with the 50/50 EasyIndex/DI water solution and leave it at room temperature for one day.
- 24 Mix EasyIndex
- 25 The next day, shake EasyIndex as in step 1 and let it settle for thirty minutes.
- 26 Incubate the sample in EasyIndex.
- 27 Fill a clean, 5 mL Eppendorf tube with EasyIndex and leave the sample immersed inside for one day at room temperature and covered with foil. The sample will now be RI-matched to 1.52 and appear transparent.

## 5x Imaging Procedure

- 28 **NOTE:** The full-volume of the sample is first imaged at an effective 5x FOV resolution for gross-analysis and to identify regions of interest for 20x imaging, using the following procedure:  
*Tip: It is advisable to have the microscope calibrated by Zeiss at least annually to ensure dual camera alignment and laser power. Stage movements are software modulated and monitored and will be automatically reinitialized by the system if out of range.*

**29** Mount the imaging objectives in the Light sheet microscope.

**30** Adjust collar of 5x detection objective to 1.52. Mount the 5x detection objective (Fig. 6) into back of Zeiss Light sheet 7 microscope.



Fig. 6: Zeiss 5x/0.16 foc lens used as 5x detection objective.

**31** Adjust collars of dual 5x imaging objectives to 1.52. Screw 5x illumination objectives (Fig. 7) onto both walls inside the light sheet microscope, perpendicular to detection objective.



Fig. 7: Zeiss LSFM 5x/0.1 foc lenses used as illumination objectives.

**32** Prepare imaging chamber for focusing the sample.

**33** Fill 5x imaging chamber (Fig. 8) past port windows with 1.52 Refractive-Index Cargille Oil (Immersion Liquid Number 5040, Fig. 9). Slide filled 5x imaging chamber into Light sheet microscope and lock chamber in place and close the front door.

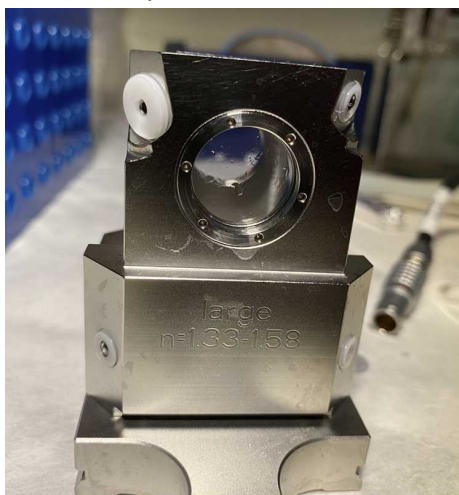


Fig. 8: Large  $n=1.33-1.58$  R.I. 5x Imaging Chamber.

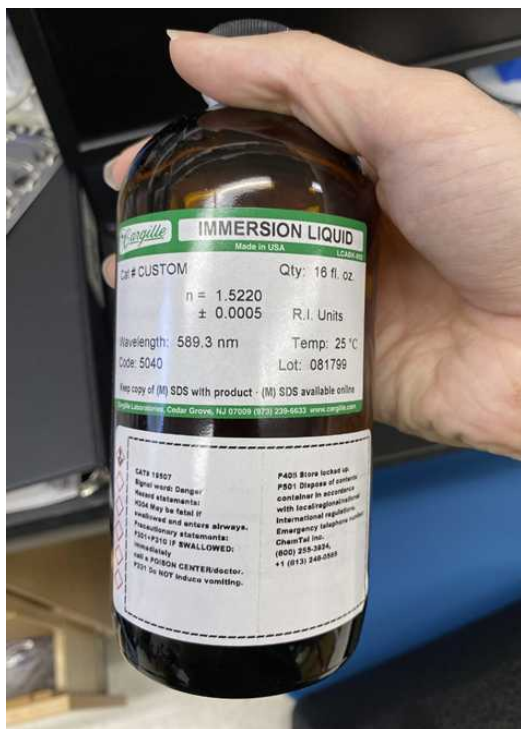


Fig. 9: R.I. = 1.52 oil used for sample immersion during imaging.

*Tip: Oil is used for imaging over RIMS because the refractive index of EasyIndex changes when exposed to air over moderate durations.*

- 34 Start ZEN Black software used to imaging.

## Focusing Light sheet for 5x imaging

- 35 Specify correct imaging and detection objectives in ZEN Black.
- 36 In ZEN Black, under the "Maintain" ribbon, open the objectives window and set the detection objective to the 5x detection objective. Set the illumination objective to the 5x illumination objectives in the Light sheet microscope.
- 37 Focus light sheet.

- 38** Under the Maintain ribbon, open the Adjustment window and select “Adjust Light Sheet”. When prompted to select laser line, select 488 nm for the best accuracy.. When prompted for refractive index, input 1.52. The following steps will need to be repeated to focus both the left and right-side illumination objectives:
- 39** Focus light sheet in Y.
- 40** Move the light sheet up or down in Y until the beam is in the center of the target. You may need to select “find light sheet” for broad focusing if the light sheet is not on screen, before tuning it under “fine adjustment”.
- 41** Focus light sheet in X.
- 42** Without exiting the focusing window, open the light sheet and manually adjust the correction collar of the corresponding illumination objectives until the thinnest part of the light sheet, denoted by a red line, is in the center of the target. Because the laser will shut off while the light sheet is open, adjusting the X focus may require periodically shutting the door of the Light sheet microscope to check progress.
- 43** Focus light sheet in Z.
- 44** Move Z slider up or down until light sheet is as thin as possible. Record the numbers for the Z-focus offsets for both the left and right illumination objectives, as they will need to be inputted later.

## Tissue Mounting - 5x Imaging Procedure

- 45** Obtain a 24x12 mm glass coverslip.

- 46** Place glue on the coverslip.  
Place a dab of super glue (Fig. 10) into a petri dish. Using the wooden end of a cotton swap applicator, apply a small amount of super glue on the center of the glass coverslip, ideally 1-2 microliters to avoid glue causing RI mismatch.



Fig. 10: Super Glue used to glue sample to glass cover slip.

- 47** Glue the sample to the coverslip.  
Holding the sample with either forceps or gloved fingers, carefully place the sample over the glue, centered on the coverslip. The side glued to the coverslip should be perpendicular to the shortest dimension of the sample. Line up the remaining short and long dimensions with the short and long sides of the glass coverslip, respectively. Wait one minute for the glue to dry.
- 48** Pick up sample with mounting apparatus.  
Mount the sample so it will be able to be maneuvered in front of the 5x detection objectives. In this case, the sample was picked up by a generic copper electric wire clip glued with epoxy resin to a T-shaped mounting stub that came with the Light sheet 7 microscope. Use the wire clip to hold the center of the short dimension of the glass coverslip (Fig. 11).



Fig. 11: Mounting Apparatus with Sample on Coverslip. The T-shaped mounting stub was glued to a copper electrical clip with fast drying epoxy. The clip may be used to pick up the glass coverslip with sample as shown. The sample was glued to the center of the coverslip as shown.

- 49** Place mounting apparatus with sample in imaging chamber.  
Remove the 5x imaging chamber filled with 1.52 oil from the Light sheet microscope. Lay the T-shaped mounting stub with the attached sample onto the grooves of the 5x chamber. Place the 5x chamber with sample back in the Light sheet microscope.
- 50** Pick up the mounting apparatus with hook.  
Using either automatic docking or manual controls, use mounting hook (Fig. 12) from Light sheet 7 kit to pick up T-shaped mounting stub. Lock the mounting hook when the sample has been picked up. After this step, all movement while the sample is in the Light sheet microscope will be done using the ZEN Black to control the imaging stage.



Fig. 12: The hook used to mount samples in the Light sheet 7. The hook may be inserted through the top of the Light sheet to grab the T-shaped mounting stub from above.

- 51 Maneuver the sample to face the detection objective.  
Rotate the sample so that it is facing directly at the detection objective. The glass coverslip should be perpendicular to the detection objective. Maneuver the sample so it is directly in the center of the detection objective's FOV. See Figure 12 in the 20x imaging procedure for an approximate depiction of how the sample should be mounted.

## Setting up Lasers

- 52 In the Acquisition ribbon, enable laser lines in the far-red (647), red (561), green (488), and blue (405) spectrums.
- 53 Set each laser to image at 50 ms, and set a temporary, low laser power for each laser for focusing Z-offsets (1-5%, for example).
- 54 Choose appropriate zoom.  
Depending on the size of the tissue, it may be appropriate to zoom out before imaging to avoid overly-large files and long imaging times, at the cost of resolution. If zoom-out is used, the acquisition FOV



must be decreased in Y or the light sheet will not be wide enough to illuminate the entire acquisition area. For example, if a zoom of 50 is used, in the Acquisition Mode window, change acquisition area frame size from 1920x1920 pixels to 1920x1000.

- 55 Input starting Z-offsets for all lasers for dual-side imaging.
- 56 To ensure illumination objectives are illuminating their optimal plane and are aligned with each other, the Z-offset of the laser from each illumination objective will need to be adjusted. For each laser, input the numbers obtained while focusing the light sheet in Z into the left and right Z-offsets of the illumination objectives.
- 57 Fine-tune Z-offset.  
**Enable only one track.** Press continuous to begin scanning. Move the tissue into view if nothing is seen on the screen. Once the tissue is in view, move the Z-offset for that laser up or down for either the left or right illumination objective until the displayed image looks as crisp as possible. Switch to the other illumination objective and adjust the Z-offset until it is focused on the same plane as the first illumination objective. When finished adjusting, stop the scanning.
- 58 Repeat the above step for all four laser lines.

## Setting up Image Acquisition Volume - 5x Imaging

- 59 Under the Acquisition ribbon, enable Z-stack and tiling.
- 60 Enable only one of the four laser lines and press continuous to begin scanning.
- 61 Set up the lateral plane boundaries.  
Under tiling, select "bounding grid". Maneuver the sample and add tiles along its edges until the entire tissue is contained in the bounded region. In tiling, set the overlap to 5%. Set the delay to 5 seconds to ensure the captured image is synchronized with sample movement in Z. Select "bidirectional".
- 62 Set up the Z-stack.

- 63** Maneuver to the face of the tissue closest to the detection objective until the sample can no longer be seen. Select “Last” in the Z-stack window. Maneuver to the back of the tissue furthest away from the detection objective, glued to the coverslip, until the tissue can no longer be seen. Select “First” in the Z-stack window.

## Other Settings - 5x Imaging

- 64** Set acquisition settings to “dual-side while experiment” to gather the view from both illumination optics.
- 65** Enable “online dual-side fusion” to fuse the view from both illumination optics into one image.
- 66** Enable “pivot scanning” to improve signal to noise compared to continuous illumination.

## Initiating Imaging - 5x

- 67** Determine Laser Power:  
Enable all four lasers. Click “Snap” to take a single image at that power setting. Adjust laser power for appropriate brightness in final image. A good starting point is 5% laser power in all channels.
- 68** Set Z-Step size:  
The lightsheet thickness and corresponding optimal Z-step size will change depending on the excitation wavelength. Under the Z-stack window, select “optimal” to automatically adjust Z-step size appropriate to laser settings.
- 69** Initiate Imaging:  
Select “start experiment”. Find an appropriate directory, name image file, and press “save”.
- 70** Storing sample for 20x imaging:  
After the sample has been imaged, leave the sample glued to the same glass coverslip for 20x imaging.

Store the sample on the coverslip away from light, in a 5 mL Eppendorf tube filled with EasyIndex.

## Setting up the Light sheet for 20x Imaging

### 71 Mount proper lenses in the Light sheet microscope:

Adjust the collar of the 20x detection objective to 1.52. Mount 20x detection objective (Fig. 13) into the back of the Zeiss Lightsheet 7. Adjust correction collars of dual 10x illumination objectives (Fig. 14) to 1.52. Screw 10x imaging objectives onto both walls inside the Light sheet microscope, perpendicular to the detection objective. 10x illumination objectives are used alongside a 20x detection optic to widen the lightsheet and better illuminate the entire plane.



Fig. 13: Zeiss Clr Plan-Neofluar 20x/1.0 Corr n = 1.53 detection objective.



Fig. 14: Zeiss LSM 10x/0.2 foc illumination objectives.

- 72 Start ZEN Black software.

## Tissue Mounting - 20x Imaging Procedure

- 73 Pick up sample with mounting hook.  
Mount the sample still glued to the same coverslip so it will be able to be maneuvered in front of the 5x detection objective. In this case, the sample was picked up by a generic copper electric wire clip glued with epoxy resin to a T-shaped mounting stub that came with the Light sheet 7 microscope. Use the wire clip to hold the center of the short dimension of the glass coverslip.
- 74 Place mounting apparatus with sample in imaging chamber.  
Lay the T-shaped mounting stub with the attached sample onto the grooves of the empty 20x chamber (Fig. 15). Place the empty 20x chamber with sample in the Light sheet microscope.



Fig. 15:  $n=1.35-1.58$  R.I. 20x Imaging Chamber. Note that the rear port window is open to allow for imaging with an immersion lens.

- 75 Fill the 20x Imaging Chamber with 1.52 oil.  
Once the 20x Imaging Chamber is locked in place in the Light sheet, use a syringe to extract 1.52 Cargille oil and fill the 20x Imaging chamber until the oil is above the port window. This is most easily performed with a large 60 mL syringe fitted with corresponding tubing.
- 76 Pick up the mounting apparatus with sample.  
Using either automatic docking or manual controls, use mounting hook from the Lightsheet kit to pick up T-shaped mounting stub. Lock the mounting hook when the sample has been picked up. After this step, all movement while the sample is in the Light sheet microscope will be done using the ZEN Black to control the imaging stage.
- 77 Maneuver the sample to face the detection objective.  
Rotate the sample so that it is facing directly at the detection objective. The glass coverslip should be perpendicular to the detection objective. Maneuver the sample so it is directly in the center of the detection objective's FOV (Fig. 16).

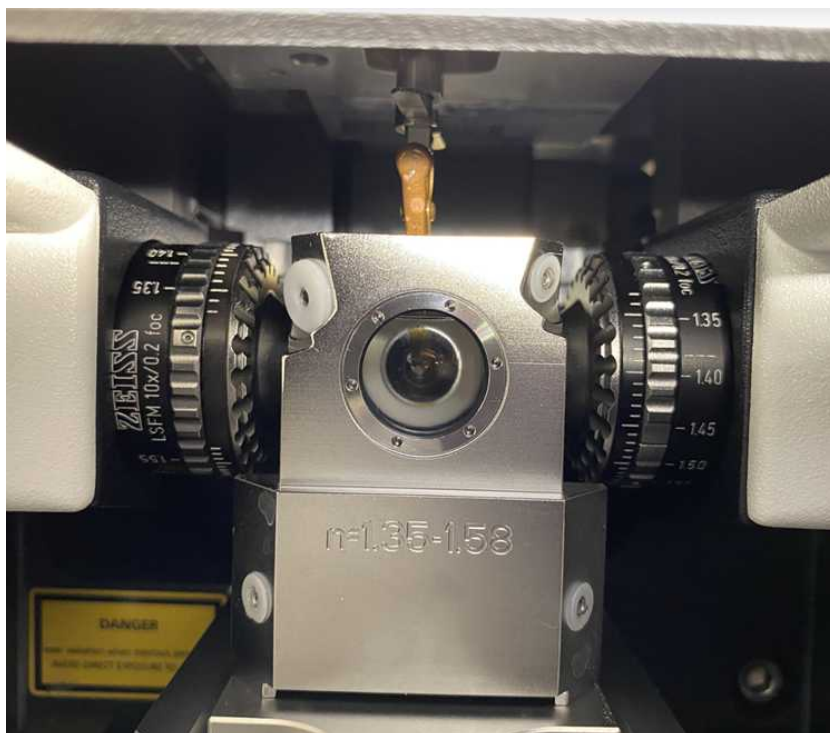


Fig. 16: How the sample should be mounted prior to imaging (20x). The sample should be directly facing the detection objective and perpendicular to the dual illumination objectives. The glass cover slip should be facing towards the front port window. 5x mounting will look similar, but with its respective chamber and lenses.

## Focusing the Light sheet microscope for 20x imaging

- 78 Specify correct detection and illumination objectives.  
In ZEN Black, under the "Maintain" ribbon, open the objectives window and set the detection objective to the 20x detection objective in the Light sheet microscope. Set the illumination objectives to the 10x illumination objectives in the Light sheet microscope.
- 79 Lift sample out of chamber.  
Lift the sample as high as it can go in the Y dimension, making sure it is out of the way of the light sheet before it is focused.
- 80 Focus light sheet.  
Under the Acquisition ribbon, make sure "online dual-side fusion" is not selected during focusing. Under the Maintain ribbon, open the Adjustment window and select "Adjust Light Sheet". When prompted to select laser line, select 488 nm for the best accuracy. When prompted for refractive index, input 1.52. The following steps will need to be repeated to focus both the left and right-side illumination objectives:

- 81** Focus light sheet in Y.  
Move the light sheet up or down in Y until the beam is in the center of the target. If you cannot see the lightsheet at all, you may need to select “find light sheet” to zoom out for broad focusing, bringing the lightsheet closer to the target before tuning it under “fine adjustment”.
- 82** Focus light sheet in X.  
Without exiting the focusing window, open the light sheet and manually adjust the correction collar of the corresponding illumination objectives until the thinnest part of the light sheet, denoted by a red line, is in the center of the target. Because the laser will shut off while the light sheet is open, adjusting the X focus may require periodically shutting the door of the Light sheet to check progress.
- 83** Focus light sheet in Z.  
Move Z slider up or down until light sheet is as thin as possible. Record the numbers for the Z-focus on scrap paper for both the left and right illumination objectives, as they will need to be inputted later.
- 84** Lower sample into chamber.  
Once finished focusing, lower the sample back into the chamber until it is in front of the detection objective.

## Setting up Lasers

- 85** In the Acquisition tab, enable laser lines in the far-red (647), red (561), green (488), and blue (405) excitation wavelengths.
- 86** Set each laser to image at 50 ms, and set a temporary, low laser power for each laser for focusing Z-offsets (1-5%, for example).
- 87** Input starting Z-offsets for all lasers for dual-side imaging.  
To ensure illumination objectives are illuminating their optimal plane and are aligned with each other, the Z-offset of the laser from each illumination objective will need to be adjusted. For each laser, input the numbers obtained while focusing the light sheet in Z into the left and right Z-offsets of the illumination objectives.
- 88** Fine-tune Z-offset.  
Enable only one laser. Press continuous to begin scanning. Move the tissue into view if nothing is seen on the screen. Once the tissue is in view, move the Z-offset for that laser up or down for either the left or right illumination objective until the displayed image looks as crisp as possible. Switch to the other illumination objective and adjust the Z-offset until it is focused on the same exact plane as the first illumination objective. When finished adjusting, press continuous again to end scanning.

- 89 Repeat the above step for all four laser lines.

## Setting up Image Acquisition Volume - 20x Imaging

- 90 Find desired ROI to image.  
Using the 5x full-volume image for reference, find a desired region to take an image that is approximately 1 mm<sup>3</sup>.
- 91 Under the Acquisition ribbon, enable Z-stack and tiling.
- 92 Enable only one of the four laser lines and press continuous to begin scanning.
- 93 Set up the tiles.  
Under tiling, select "tiled grid". Setup a tiled grid with the desired dimensions. In tiling, set the overlap to 5%. Set the delay to 5 seconds to ensure the captured image is synchronized with sample movement in Z. Select "bidirectional".
- 94 Set up the Z-stack.  
Maneuver to the front of the tissue closest to the detection objectives until the desired starting plane is in view. The starting plane should be near the surface of the tissue but need not be the very first plane that signal can be seen. Select "Last" in the Z-stack window. Maneuver towards the back of the tissue, reaching a plane around 1 mm deep. Select "First" in the Z-stack window.

## Other Settings - 20x Imaging

- 95 Set acquisition settings to "dual-side while experiment" to gather the view from both illumination optics.
- 96 Enable "online dual-side fusion" to fuse the view from both illumination optics into one image.



- 97 Enable “pivot scanning” to improve signal to noise compared to continuous illumination.

## Initiating Imaging - 20x

- 98 **Determine Laser Power.**  
Enable all four lasers. Select desired zoom. 1.0 zoom will offer the optimal depth of field and minimize zooming distortions. Click “Snap” to take a single image at that power setting. Adjust laser power for appropriate brightness in final image. A good starting point is 5% laser power in all channels.
- 99 **Set Z-Step size.**  
The light sheet thickness and corresponding optimal Z-step size will change depending on the excitation wavelength. Under the Z-stack window, select “optimal” to automatically adjust Z-step size appropriate to laser settings.
- 100 **Initiate Imaging.**  
Select “start experiment”. Find an appropriate directory, name image file, and press “save”.

## Image Processing for 5x and 20x Images

- 101 **Open image in ZEN Blue.**  
Open Zen Blue software, and open 5x or 20x image files into Zen Blue.
- 102 **Create 5x Image Pyramid if necessary.**  
Depending on Zen Blue version, 5x images will make the user create an image pyramid before any processing is possible, as ZEN Black will not create image pyramids during acquisition. The software will automatically prompt to make an image pyramid. This will process the image for improved 3D display within ZEN. If given the option, however, this step may be skipped.
- 103 **Stitching images.**  
Under the Processing ribbon, find the stitching method in the “Methods” window. Under the Image Parameters window, select desired 5x or 20x file as input. Under the parameters window, select new output and check “Fuse Tiles”. Press Apply to begin stitching. See Fig. 17 below for an image of these settings. After stitching, the stitched image will have to be saved manually.

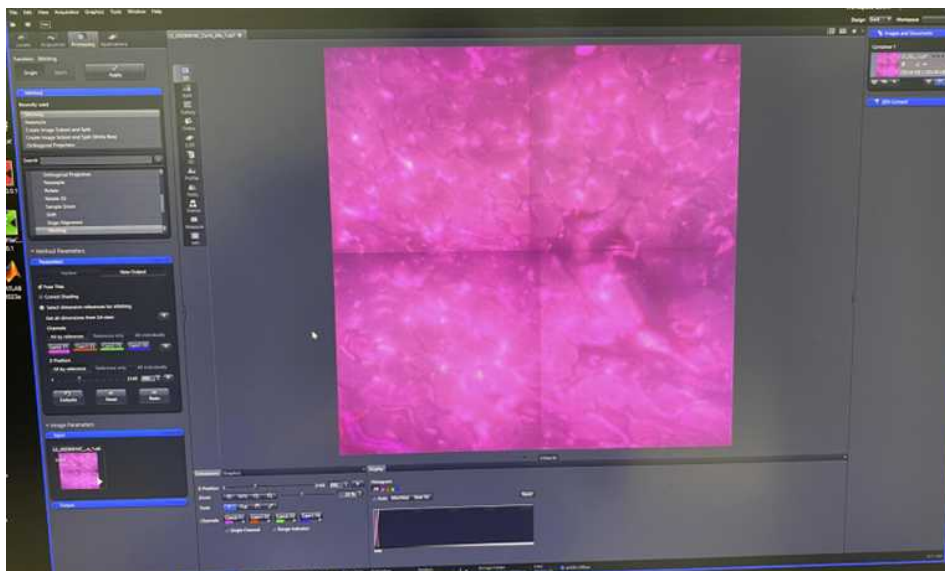


Fig. 17: The Correct Settings for Stitching in ZEN Blue.

- 104** Downsampling images.
- Under the Processing ribbon, find the resample method in the “Methods” window. Under the Image Parameters window, select the stitched 5x or 20x file. Under the parameters window, set desired down sampling parameters in X, Y, and Z. See Fig. 18 below for an image of these settings for down sampling 4 times in both X and Y, which will minimize file size by 16x to allow for analysis. After down sampling, the down-sampled file will have to be saved manually.

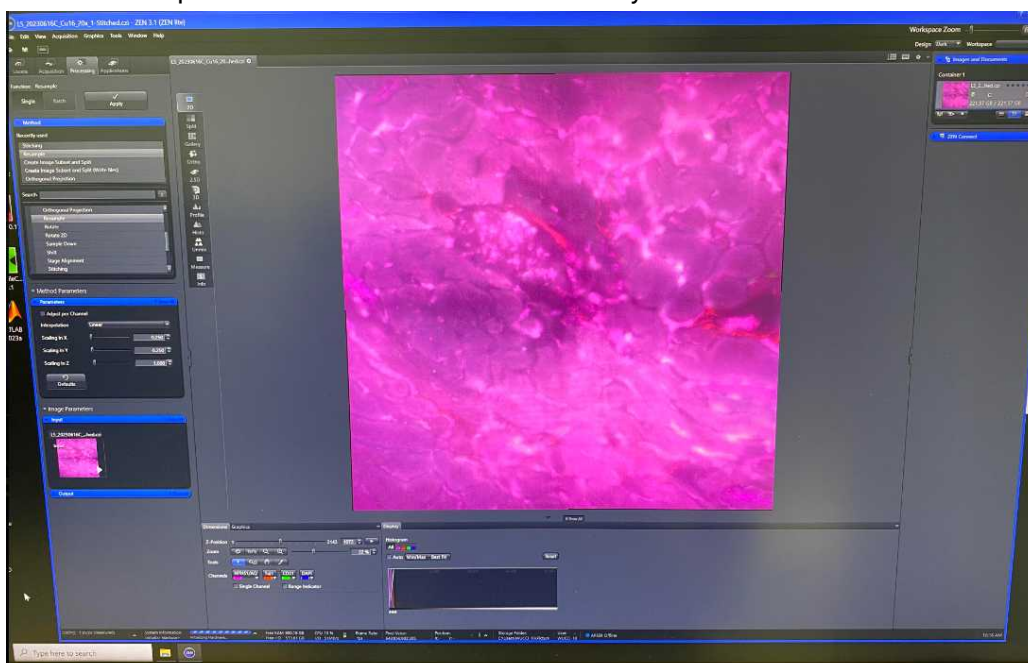


Fig. 18: The Correct Settings for Downsampling in ZEN Blue.

## File Format Options for Analysis with Various Softwares

- 105** Raw data will be in the .czi format, but may be converted to TIFF, OME.TIFF, or IMS file formats for analysis using the following steps:
- 106** Raw data and ZEN software .czi files.  
The raw data and all processing by ZEN Blue software will be in the .czi format. This format will maintain all metadata and may be viewed and processed by ZEN software. CZI files are proprietary to Zeiss, and therefore are not compatible with certain other imaging analysis software.
- 107** Creating a standard TIFF file for analysis in FIJI and various other software.  
Drag the stitch/downsampled .czi file into ImageJ/FIJI window. When prompted, selecting “split channels” will allow separate channel analysis. Each channel will open in a separate window. Select each window and choose “save as TIFF” in ImageJ. The advantage of TIFF is that it is nonproprietary and compatible with most image analysis softwares. The disadvantage is that converting to TIFF will lose associated metadata.
- 108** Creating OME.Tiff with rich OME-XML metadata.  
To create an OME-Tiff file that also contains an OME-xml with metadata, .czi files may be converted using the BioFormats command line tools, bftools.zip: (Bio-Formats Downloads | Open Microscopy Environment (OME)). An OME.TIFF will maintain an image’s metadata while offering the software compatibility of a TIFF file but requires this extra conversion step.
- 109** Creating an IMS file for analysis in Imaris.  
To analyze samples in Imaris, files can be converted to .ims format by running them through the “Imaris File Converter” software, or by opening them directly in Imaris. IMS files allow for image analysis within Imaris software, which offer many useful tools, but are proprietary and therefore not compatible with certain other analysis softwares.