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

Light Sheet Fluorescence Microscopy of Human Kidney Using Clearing with CUBIC

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

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ABSTRACT

Light sheet fluorescence microscopy (LSFM) is a method to cover micro-mesoscale (µm-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.

PROTOCOL REFERENCES

Young-Gyun
Park, Chang Ho Sohn, Ritchie Chen, Margaret McCue, Dae Hee Yun, Gabrielle T Drummond,
Taeyun Ku, Nicholas B Evans, Hayeon Caitlyn Oak, Wendy Trieu, Heejin Choi, Xin Jin, Varoth Lilascharoen, Ji Wang, Matthias C Truttmann, Helena W Qi, Hidde L Ploegh, Todd R Golub, Shih-Chi Chen, Matthew P Frosch, Heather J Kulik, Byung Kook Lim & Kwanghun Chung.
Protection of tissue physicochemical properties using polyfunctional crosslinkers, ***Nature Biotechnology***, 2018 Dec 17.

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:

- Clearing with CUBIC (Matsumoto, K., Mitani, T.T., Horiguchi, S.A. et al. Advanced CUBIC tissue clearing for whole-organ cell profiling. Nat Protoc 14, 3506–3537 (2019). https://doi.org/10.1038/s41596-019-0240-9) -infiltrated samples takes around fourteen days to complete.

- CUBIC clearing is a straightforward clearing method that involves incubation of the sample in the CUBIC clearing solution for fourteen days to induce delipidation and allow refractive index matching.

- Caution: Reagent 1 can erase labeling on the tube if it comes in contact with the outside surface, so open the tubes carefully and have alternate ways to label or secondary containers and track if multiple

samples are being done at the same time.

- Tip: Reagent 1 is light sensitive so wrap the tubes in foil.

CLEARING

2 Either of the following solutions may be used for clearing. Please mix the listed reagents together prior to beginning clearing:
Reagent-1 A:
25 % (wt/v)Urea (Sigma U5378)
25 % (wt/v) Quadrol (N,N,N',N'-tetrakis (2-hydroxypropyl) ethylendiamine, Sigma S2002)
15 % (wt/v) Trition X-100 (Sigma T8787) in DDW

Reagent-1/CHAPS:
25 % (wt/v)Urea (Sigma U5378)
25 % (wt/v) Quadrol (N,N,N',N'-tetrakis (2-hydroxypropyl) ethylendiamine, Sigma S2002)
15 % (wt/v) Trition X-100 (Sigma T8787) in DDW
10 % (wt/v) CHAPS(Sigma C5849) in DDW

3 Human kidney sections that have been fixed (4% PFA), rinsed and stored in PBS at 4 degrees C until 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. Penetration of antibody is better with thinner sections, ideally less than 2mm.

4 Remove the sample from PBS and incubate the prefixed specimen in Reagent-1 or Reagent-1/CHAPS at 37 degrees C with rotation for 14 days.

5 Wash with 1X PBS at room temperature with rotation for 3 hours x2, then overnight.

6 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

7 Incubate the CUBIC-cleared specimen with primary antibodies in PBS-BB)/ 0.1% Sodium Azide:

7.1 at room temperature with rotation for 7 days.

7.2 at 37 0C for 2 days, then room temperature overnight.

8 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.

9 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.

10 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.

11 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.

12 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.

13 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.

14 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.

15 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 cubic RIMS. First transfer sample into new 5mL Eppendorf tube and incubate sample in the CUBIC RIMS solution for one day at RT. It will now be ready for imaging. See Figs 3 and 4 below for a before and after image of the sample.

16 

Fig. 1: Sample before clearing.

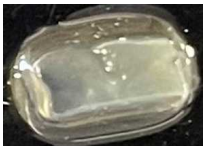
17 

Fig. 2: After clearing and RI-matching, the sample should appear transparent.

Refractive Index Matching Procedure for Cleared Samples

18 *Tip: If the sample is being stored in PBS/0.1% Sodium Azide, it must be refractive index matched prior to imaging.*
First create CUBIC RIMS by mixing the following reagents until homogenous:

40g Histodenz (Sigma D2158)
30 ml 0.02M PBS with 0.1% Triton X-100 and 0.01% sodium azide, pH 7.5

19 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 CUBIC RIMS and leave it at room temperature for one day.

20 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 around 1.46 and appear transparent, although this value should be verified with a refractometer prior to imaging.

5x Imaging Procedure

21 **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.

22 Mount the imaging objectives in the Light sheet microscope.

23 Adjust collar of 5x detection objective to ~1.46 (verify the RI of the CUBIC RIMS). Mount the 5x detection objective (Fig. 6) into back of Zeiss Light sheet 7 microscope.

Fig. 3: Zeiss 5x/0.16 foc lens used as 5x detection objective.

24 Adjust collars of dual 5x imaging objectives to 1.46. Screw 5x illumination objectives (Fig. 7) onto both walls inside the light sheet microscope, perpendicular to detection objective.

Fig. 4: Zeiss LSFM 5x/0.1 foc lenses used as illumination objectives.

25 Prepare imaging chamber for focusing the sample.

26 Fill 5x imaging chamber (Fig. 8) past port windows with 1.46 Refractive-Index CUBIC RIMS. Slide filled 5x imaging chamber into Light sheet microscope and lock chamber in place and close the front door.

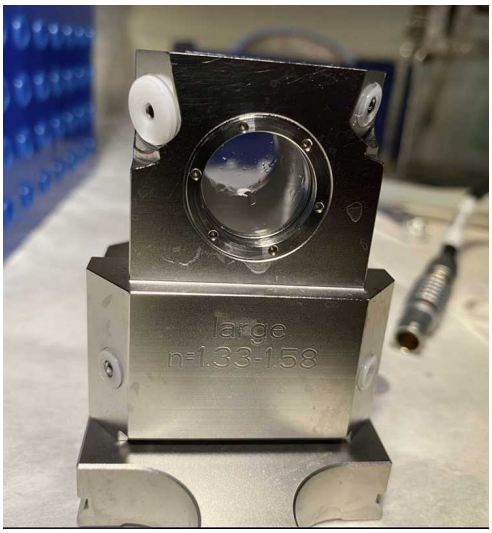


Fig. 5: Large n=1.33-1.58 R.I. 5x Imaging Chamber.

27 Start ZEN Black software used to imaging.

Focusing Light sheet for 5x imaging

28 Specify correct imaging and detection objectives in ZEN Black.

29 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.

30 Focus light sheet.

31 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 1.46. The following steps will need to be repeated to focus both the left and right-side illumination objectives:

32 Focus light sheet in Y.

33 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”.

34 Focus light sheet in X.

35 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.

36 Focus light sheet in Z.

37 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

38 Obtain a 24x12 mm glass coverslip.

39 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. 6: Super Glue used to glue sample to glass cover slip.

40 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.

41 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. 7: 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.

42 Place mounting apparatus with sample in imaging chamber.

Remove the 5x imaging chamber filled with 1.46 CUBIC RIMS 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.

43 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. 8: 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.

44 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

45 In the Acquisition ribbon, enable laser lines in the far-red (647), red (561), green (488), and blue (405) spectrums.

46 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).

47 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.

48 Input starting Z-offsets for all lasers for dual-side imaging.

49 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.

50 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.

51 Repeat the above step for all four laser lines.

Setting up Image Acquisition Volume – 5x Imaging

52 Under the Acquisition ribbon, enable Z-stack and tiling.

53 Enable only one of the four laser lines and press continuous to begin scanning.

54 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”.

55 Set up the Z-stack.

56 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

57 Set acquisition settings to “dual-side while experiment” to gather the view from both illumination optics if both sides provide only partial illumination of each side of the tissue. If one side is notably stronger, single side imaging from that side alone may provide better results.

58 Enable “online dual-side fusion” to fuse the view from both illumination optics into one image.

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

Initiating Imaging – 5x

60 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.

61 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.

62 Initiate Imaging:
Select “start experiment”. Find an appropriate directory, name image file, and press “save”.

63 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

64 Mount proper lenses in the Light sheet microscope:
Adjust the collar of the 20x detection objective to 1.46 (verify CUBIC RIMS). 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 1.46. 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.

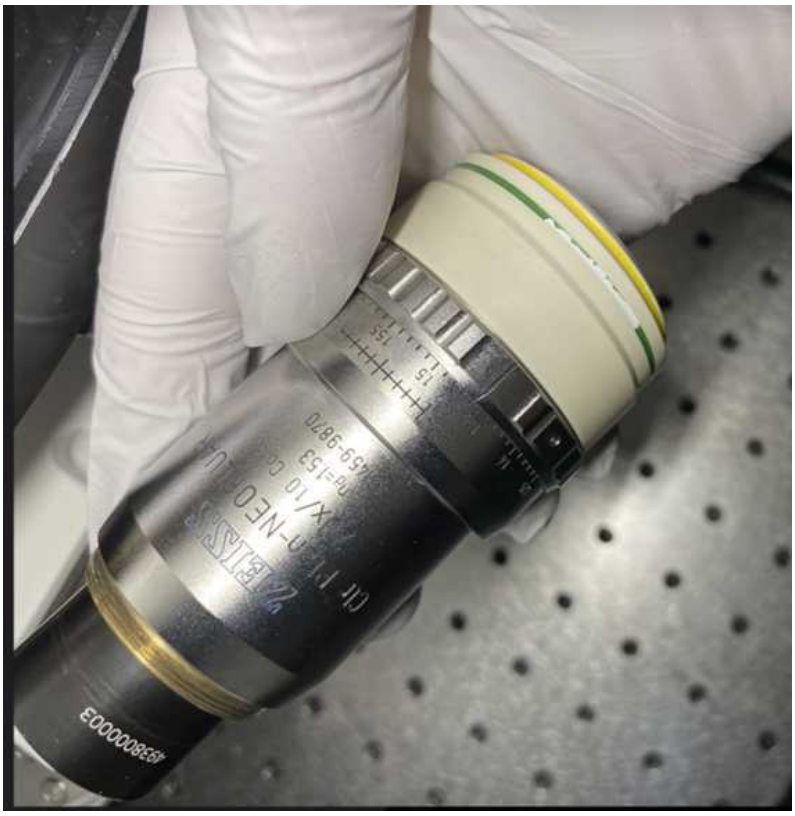


Fig. 9: Zeiss Clr Plan-Neofluar 20x/1.0 Corr n = 1.53 detection objective.

Fig. 10: Zeiss LSFM 10x/0.2 foc illumination objectives.

65 Start ZEN Black software.

Tissue Mounting – 20x Imaging Procedure

66 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.

67 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.

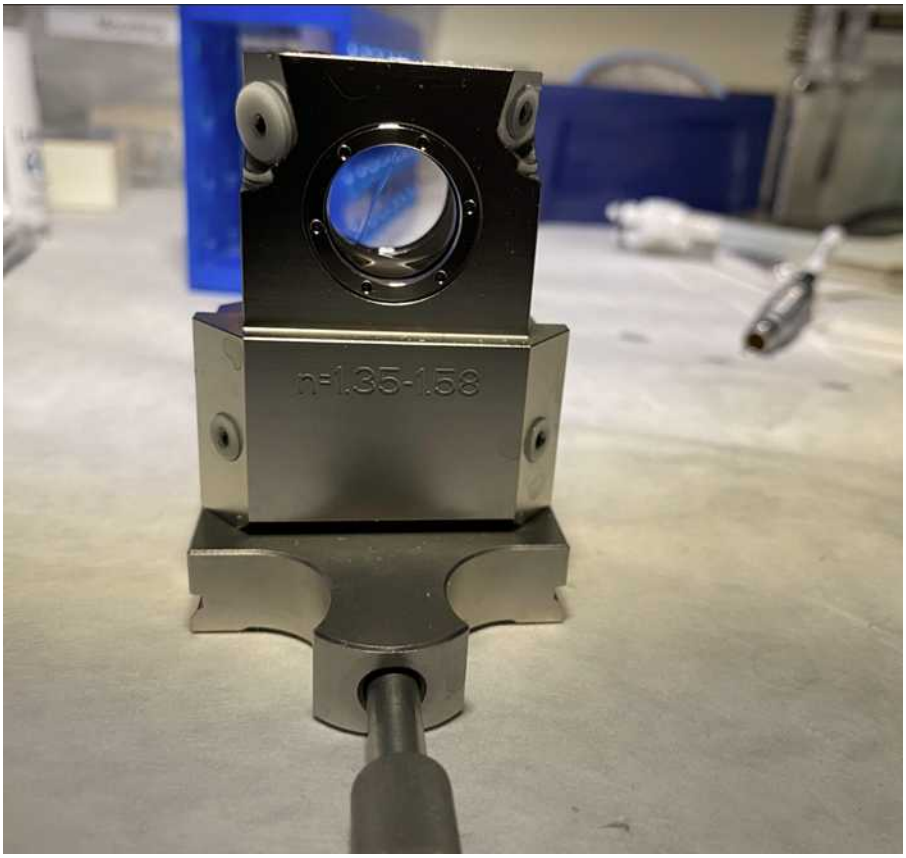


Fig. 11: n=1.35-1.58 R.I. 20x Imaging Chamber. Note that the rear port window is open to allow for imaging with an immersion lens.

68 Fill the 20x Imaging Chamber with 1.46 CUBIC RIMS.
Once the 20x Imaging Chamber is locked in place in the Light sheet, use a syringe to extract 1.46 CUBIC RIMS and fill the 20x Imaging chamber until the RIMS is above the port window. This is most easily performed with a large 60 mL syringe fitted with corresponding tubing.

69 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.

70 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).

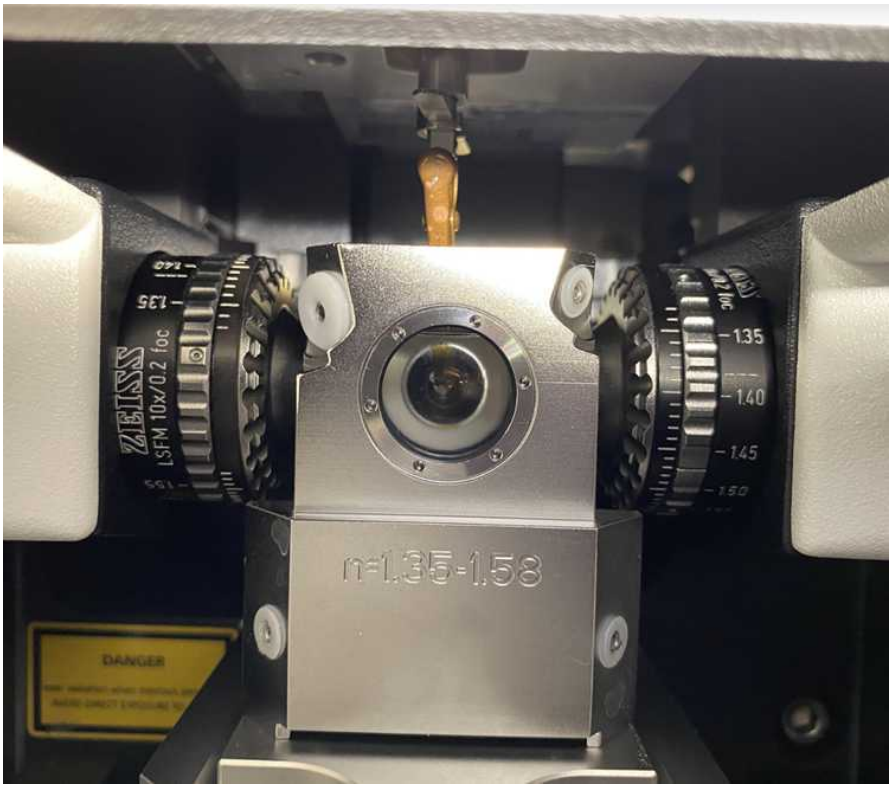


Fig. 12: How the sample should be mounted prior to imaging (20x). The sample should be directly facing the detection objective and perpendicular to the dual illumination objectives. The glass cover slip should be facing towards the front port window. 5x mounting will look similar, but with its respective chamber and lenses.

Focusing the Light sheet microscope for 20x imaging

71 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.

72 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.

73 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 1.46. The following steps will need to be repeated to focus both the left and right-side illumination objectives:

74 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”.

75 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.

76 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.

77 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

78 In the Acquisition tab, enable laser lines in the far-red (647), red (561), green (488), and blue (405) excitation wavelengths.

79 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).

80 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.

81 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.

82 Repeat the above step for all four laser lines.

Setting up Image Acquisition Volume – 20x Imaging

83 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 mm3.

84 Under the Acquisition ribbon, enable Z-stack and tiling.

85 Enable only one of the four laser lines and press continuous to begin scanning.

86 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”.

87 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

88 Set acquisition settings to “dual-side while experiment” to gather the view from both illumination optics if needed. Otherwise, single side imaging is fine.

89 Enable “online dual-side fusion” to fuse the view from both illumination optics into one image.

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

Initiating Imaging – 20x

91 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.

92 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.

93 Initiate Imaging.
Select “start experiment”. Find an appropriate directory, name image file, and press “save”.

Image Processing for 5x and 20x Images

94 Open image in ZEN Blue.
Open Zen Blue software, and open 5x or 20x image files into Zen Blue.

95 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.

96 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 imaged will have to be saved manually.

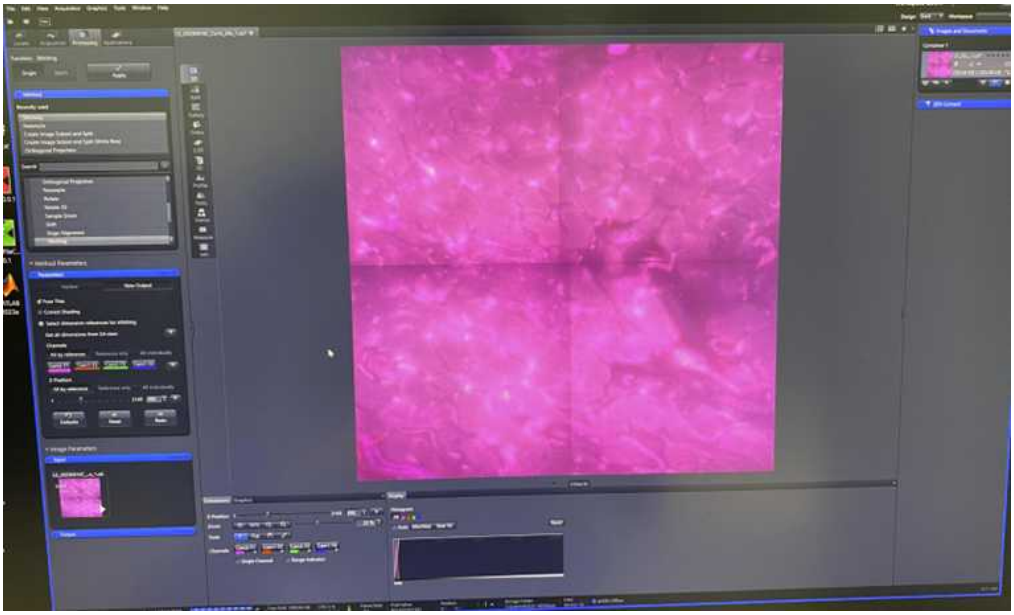


Fig. 13: The Correct Settings for Stitching in ZEN Blue.

97 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.

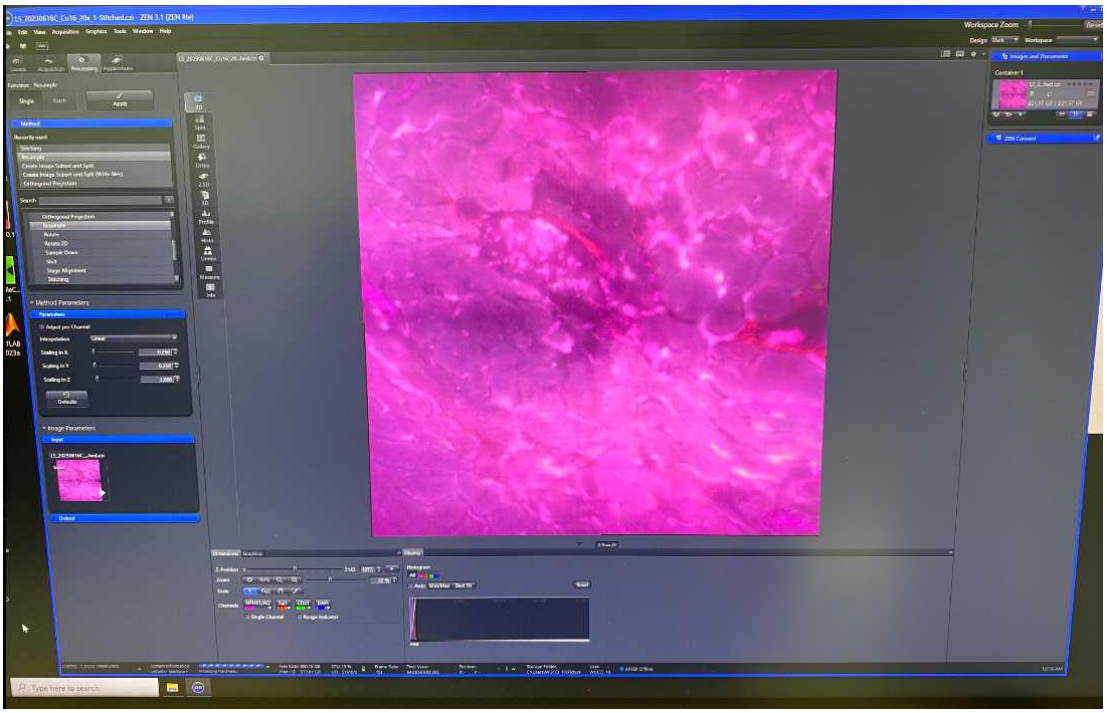


Fig. 14: The Correct Settings for Downsampling in ZEN Blue.

File Format Options for Analysis with Various Softwares

98 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:

99 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.

100 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.

101 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.

102 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.