Light Sheet Fluorescence Microscopy Image Acquisition

Seth Currlin[1], Marda Jorgensen[1], Jerelyn Nick[1]
[1]University of Florida

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1 Works for me
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Human BioMolecular Atlas Program (HuBMAP) Method Development Community
Tech. support email: Jeff.spraggins@vanderbilt.edu

Seth Currlin

ABSTRACT

The Zeiss Lightsheet 7 is used to quickly and efficiently scan large tissue volumes using laser light sheet illumination. The focused laser sheets and imaging plane are static while the tissue is moved throughout the selected volume for three dimensional image acquisition. This protocol will cover the procedures used for the day of light sheet image acquisition.

Setting the acquisition volume: Large scanning volumes are composed of multiple z-stack being stitched back together based on overlapping edges of the scanning window. This requires setting an overall z-stack, which must include the entirety of tissue as covered by adjoining z-stacks. This becomes tricky when considering irregularly shaped tissues and their orientation relative to the various laser and imaging objectives.

Dual-side light sheet alignment: To obtain a strong and clear image from large tissues it is helpful to illuminate the sample with both the left and right laser. Each laser sheet can be angled slightly within the focal plane to account for differences in the R.I. of the tissue and R.I. media (TDE). This is a manual tuning process and is not intended for major R.I. differences.

Protocol Overview:
- Refractive index matching of cleared tissue samples.
- Mounting tissue onto sample holder.
- Image acquisition in Zen 3.1 LS (black edition), including:
  - Setting the acquisition volume
  - Dual-sided light sheet alignment

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KEYWORDS

Lightsheet, microscopy, acquisition, Zen, 3D

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MATERIALS TEXT

MATERIALS

Isopropanol **Contributed by users**

Forceps (tweezers), 12.5cm, Blunt End **Bio Basic**

Inc. Catalog #FC003.SIZE.1

10 x PBS Solution (Phosphate Buffered Saline) **Bio Basic**

Inc. Catalog #PD8117.SIZE.4L

KimWipes **Fischer Scientific**

Cyanoacrylate adhesive, optical grade

Vwr Catalog #19806-00-1

22'-Thiodiethanol **Sigma**

Aldrich Catalog #166782-500G

Software:
Zen Blue acquisition software
ImageJ

SAFETY WARNINGS

2,2'-Thiodiethanol can cause severe eye irritation. Eye protection and nitrile gloves should be worn when handling the solid material.

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Refractive Index (R.I.) Matching

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1 Refractive index matching solution (RIMS)

After tissue clearing, using procedures such as CLARITY, a final matching of refractive index of the tissue with imaging medium is required. Acquisition system settings and objectives determine what refractive index to adjust for.

This three step incubation series in higher concentration TDE solutions minimizes tissue deformation and improves refractive index matching. Use a refractometer to check that 63% TDE solution refractive index is n = 1.45.

For a 1 cm^3 piece of tissue use at least 25 ml volume of TDE solution om a 50 ml conical tube. Prepare a 20%, 47%, and 63% TDE solution with phosphate buffered saline (PBS), pH 7.2. Mix gently to avoid bubbles.

2 RIMS incubation 4h

Incubate a tissue sample in 25 ml 20% TDE with gentle rocking at 37ºC. When tissue sinks to bottom of tube transfer to 47% TDE. Again, wait for tissue to sink then transfer to 63% TDE solution. Let tissue incubate in 63% TDE for an additional hour. The tissue should be near invisible to the eye.

The tissue is ready to mount of the Zeiss Z.1 tissue holder.

The tissue should be okay for a day in 63% TDE. After that it is best to preserve tissue in PBS or clearing solution.

3 Prepare Z.1 for imaging 10m

Initialize the microscope and fill the sample imaging chamber with 63% TDE solution. Insert the imaging chamber.

Tissue Mounting

4 This tissue mounting setup is for a Zeiss Z.1 light sheet microscope. This system moves the tissue through the illumination plane to acquire large volume scans in very little time. There are many ways to mount cleared tissues and should be optimized for particular tissue needs. This protocol uses the capillary and plunger components included with the Z.1 microscope to create a vacuum upon a small portion of tissue to hold it while imaging. If a sample can fit within the glass capillary, without significant tissue deformation, that is an appropriate mounting method.

Prepare the metal capillary holder by inserting a fitted glass capillary and tighten screw to secure. Insert fitted plunger into glass capillary all the way.

5 Place tissue on clean surface (weigh boat) and orient the tissue so that a flat area is in a convenient direction to approach with glass capillary. 2m

While holding capillary end against flat tissue surface slowly retract plunger to create small vacuum, causing the tissue to barely enter the capillary. Slowly lift the suction-adhered sample up and if satisfied with stability then proceed to next step. Otherwise, remount the tissue as needed.

6 The sample can be briefly dipped into 80% isopropanol in PBS to remove any small bubbles. A 2-3 second dip while gently flicking the sample holder should be sufficient. These bubbles will create massive shadow artifacts across the tissue otherwise. 1m

7 The sample is now ready to lower into the light sheet imaging chamber, which should be pre-filled with 63% TDE.

Image Acquisition - Setting Acquisition Volume

8 Acquisition considerations

The Z.1 light sheet microscope can move the tissue in the x, y, and z direction as well as rotate 360º within the imaging

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chamber. The focal plane is static and laser light sheets can illuminate this focal plane from either side.

Every piece of tissue will require some exploration to find the best alignment. Vessels, capsular segments, or artifacts within the prepared tissues may cause light scattering and reduced focus over the region of interest.

The following settings can be used for any laser frequency available on the microscope system that suits your tissue labeling scheme.

9 Set z-axis range 15m

Progress through tissue to locate the furthest plane that is in focus along the z-axis, and select it as the first plane.

Scroll back through the tissue to locate the final focal plane in the other z-direction that is in focus and set it as the last plane.

10 Multiview scan for multiple z-stacks 10m

A bounding region needs to be designated in which the multiple z-stacks will be sequentially acquired. Determine the upper and lower y-axis values, recording them from the specimen navigator panel. Repeat this process for each x-axis value.

11 Assign top left and bottom right bounds

Use the specimen navigator panel to bring sample to the top-left most region, using the upper y-axis and left-most x-axis values. Add this position to the multi-view panel.

Repeat this procedure for the lower right-most region of the tissue.

Be sure to determine these bounds keeping in mind the z-stack scanning range of tissue specimen.

12 Load and generate Multiview z-stacks 10m

Save the multiview positions and load into Zeiss Tile Stitcher, a supplemental script used to populate multiview positions for every z-stack required to capture the entire tissue volume within the top-left to bottom-right bounds just set.

Within the Zeiss Tile Stitcher select the bounding box feature, set to meander mode, and fill in field of view parameters (image size, number of planes, % overlap). The Tile Stitcher program will auto-populate the many z-stack multiview coordinates which can be saved and loaded into the multiview panel within the acquisition software. Make note of how many z-stacks run along the x and y axes.

Image Acquisition - Laser Alignment 15m

13 Adjust single side laser sheet angle 15m

Depending on quality of tissue preparation some scattering of light entering the sample may not line up exactly with the objective focal plane. To account for small differences in refractive index the laser objectives can be tuned by adjusting the angle offset at which the light sheet enters the tissue.

Select either left or right side laser illumination and adjust the corresponding laser offset in the Illumination Parameters panels. Visually inspect the live feed and adjust the laser offset until the clearest image is observed. Repeat this for the other side's laser offset settings.

Make note of the alignment angle and explore other regions in the tissue within the designated z-stack range to confirm laser offset is appropriate for the bulk of tissue.

Reconcile the dual-sided laser illumination 10m

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To capture the clearest image illumination from each laser should have the same focal plane so that the same tissue features appear in the same location when illumination occurs from either side. This may require some fine tuning of the left and right laser alignment and should be checked within various regions of the tissue volume.

Image Acquisition - Other Settings

15 Laser Settings

Laser settings are determined by tissue autofluorescence and or the specific labeling scheme applied to the sample. For any laser used it is helpful to check that the laser offset, as covered above, is appropriate throughout the imaging region.

16 Acquisition Settings

Check on 'dual-sided acquisition", 'online dual-sided fusion', and 'Pivot scan'.

17 Start Experiment

The experiment is ready to begin. Click Start Experiment, designate a file name and directory location, then save.

The duration of this step is dependent on tissue size and imaging parameters. Acquisition time is mainly determined by the volume of the scanning region and the number of optical slices along the z-axis.

Saving imaged sample

18 Once image acquisition is complete the tissue can be removed from the imaging chamber.

The samples can be returned to a large volume of PBS overnight to wash out TDE. Specimens can be stored in a fresh aliquot of PBS and kept cold, or can be returned to clearing solution and kept at room temperature.

Be sure to protect the samples from ambient light to preserve fluorophore intensity.

Data Management

19 The Zeiss acquisition software, Zen, produces a *.czi file which can be viewed with a variety of programs (i.e. ImageJ).

Files intended for HuBMAP include the original *.czi file, an OME-TIFF file exported from Zen, and a metadata *.csv extracted from the *.czi file using ImageJ.

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