

Quick Start

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1.1 D0 Collect fish

- Raise in 1.5X PTU

1.2 D2-3 Presort fish

- Try to have 10-30 double fluorescent fish
 - Randomly pick 8 on day of imaging to mount

1.3 D6 Larvae mounting:

- Apply tricaine to pre-sorted fish and wait 3 minutes
- Clean image chambers with 70% ethanol and insert culture chamber inserts
- Take 2.5% LMP agarose out of 55C incubator and wait 2-4 minutes
- Load single larva into a small bore glass pipette, then pipette 2-3 drops of cooled agarose into a cell of the culture chamber insert and transfer larva with a small volume of E3 into the center of the chamber insert; pushing down to the bottom, positioning ventral side up and with tail to the right using a truncated thin plastic pipette tip.
- Repeat for additional fish in other insert cells, wait 5 minutes for LMP to set, add additional E3 with tricaine and then transfer to scope.
- Clean bottom of image chamber with 70% ethanol prior to imaging

1.4 Software configuration:

- Start Leica software as instructed in Leica start-up protocol
- Load LAS AF and if “Check configuration: machine” is the last line displayed, click “OK”; otherwise click “Configuration” and select “machine”
- Tilt condenser back and click “Yes” when asked to initialize stage.
- Once loaded, click on “Configuration.”
- Under “Hardware Configuration” Click on “Laser,” click checkboxes for the “Argon” and “DPSS 561 laser,” and adjust the Argon power to 20%.
- Click on Stage and change Overlap manual stitching to 25um for Y.
- Click on Settings and change Resolution to 12 Bit
- Click on “Acquire,” Input Beam Path Settings:

Input the following:

Laser Power: 561 @ 5%, DD 488/561

HyD 1 - Leica/Kaede (Green), BrightR - 25%, 500-550nm

HyD 2 - Leica/DSRED, BrightR - 25%, 571-700nm

- Under Acquisition, expand the “XY” menu and change the “Format” dropdown box to “More..” X: 616 & Y: 496”,
- Set the “Speed” dropdown box to “More..” 600 Hz
- Check Bidirectional X, and change Phase correction X to -34.5.

1.5 Choosing samples to scan:

- Change objective to 25X water
- Add 2-3 drops of water to the objective and place slide insert with sample into slot
- Examine all mounted samples under the epifluorescence of the confocal and pick the 3 to scan based on how representative and well oriented they are
 - It is suggested to create a google spreadsheet to keep organized during scanning

1.6 Setting boundaries

- Of the 3 best samples to scan put one under the objective and make sure it is in focus
- Under Z-Stack, set both the Begin and z-Position to -200um.
- In the image window menu, Click “Quick LUT” and then click the “Live” button
- Double-click on the reference channel (2nd window down) to expand, center the brain (use joystick) , and find the dorsal (top) end of the reference channel
- Adjust the z-Wide knob (on microscope) so that the dorsal aspect of the brain is just above the Begin position
- Click on “Show Tile Scan Panel”
- Click on “Scaling” to Bring up a cross-hair
- Move the Z-Galvo up until the olfactory epithelium is visible and use this plane to set the rostral scanning boundary and to compensate for any rotation present
- Make note of any rotation
- Change the intensity of the 561 laser to 20% and scan up until you are just past the fluorescence of the ventral aspect (bottom) of the brain. This should give you a z-Volume of 300-400um.
- Click stop then click on Go to “Begin”
- Click on “Show Tile Scan Panel”
- Expand the “Tile Scan” window and deselect “Auto-Stitching” and “Merge Images” then click “Mark pos.” twice
- Ensure that the ScanField lists 2x1 and that the ‘tiling box’ overlaps completely with ‘actual view’ box and that the settings boundaries are correct.

1.7 Configuring Prescan in order to properly define Z-Stack laser Compensation:

- Turn Laser 561 laser to 0% and 488 laser to 10%
 - weak fluorescent lines (e.g., xa24) - 20%
 - strong fluorescent lines (e.g., xa381) - 10%
- click z-step size radio button and change step-size to 5um, turn off the cross-hair (Click “Scaling” button), and double-click on reference channel to see both channels

Click start (this should take ~1min and give you a good idea where your pattern is and how you will need to compensate it)

Click Show overlay and Maximum projection

Z-stack laser compensation:

- Define laser compensation parameters by clicking on “compensation” in the “z-stack” box
- Next change from “No compensation” to “Linear compensation by AOTF”
- Knowing the areas from the prescan that may be oversaturated adjust lasers accordingly
 - We tend to do interpolation points around every 70 um

1.8 High-resolution image: Once the laser compensation has been adjusted it is time to do a high resolution scan.

- Change step-size to 2um
- Change frame average to 10

Click “start” (scans should take ~25 minutes)

Repeat for samples 2 and 3

1.9 Image Processing

Dye separation: use same LAS AF software as with image acquisition. This step can be done right after acquiring the images just make sure to save the original file before proceeding as the dye separation step alters the files.

- Click on “Process, “ Under “Dye Separation,” Select “Automatic
- Click on “Experiments,” Open files that need to be dye separated
- Click on “Manual, “ Then input matrix for which fluorophore used:
- eGFP (500-550)-DsRed-Express (571-700):

	Ch1	Ch2
Dy1	0.904795775	0.095204225
Dy2	0.029959552	0.970040448

- KaedeG (500-550)-DsRed-Express (571-700)

	Ch1	Ch2
Dy1	0.914432968	0.085567032
Dy2	0.029959552	0.970040448

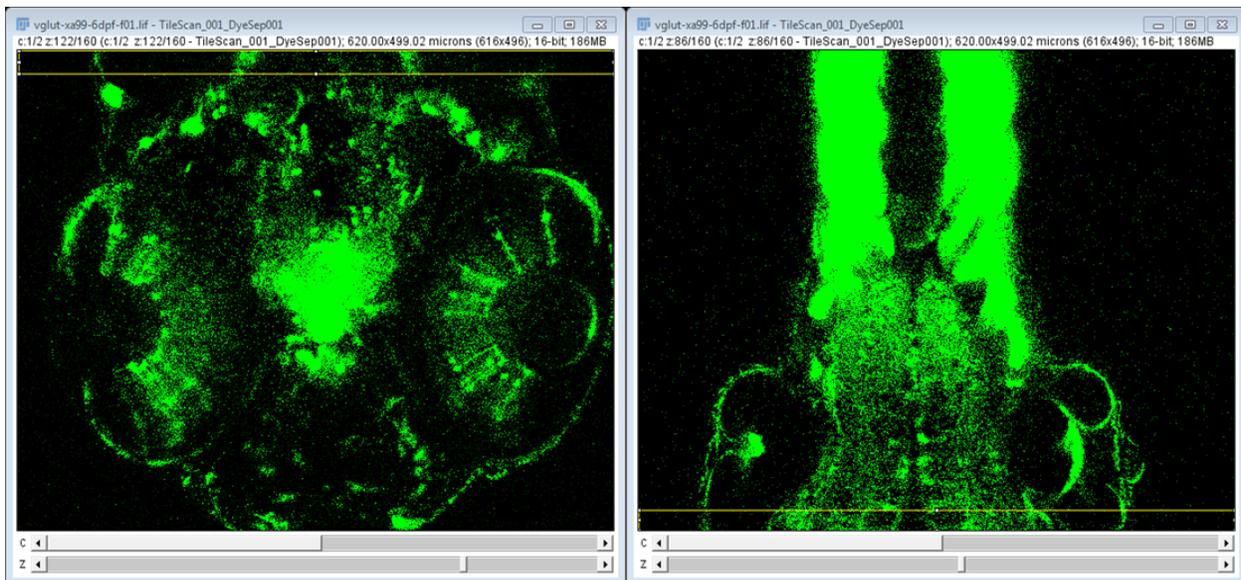
- Click “save, “ give proper fluorophore title
- Click “apply,” and exit matrix box
- Click “run”

- Repeat for additional files that need dye separated. Each time you must click on “manual” but the matrix should stay listed. If you dye separate samples at another time the save matrices will need to be uploaded.
- Make sure to delete the original files before saving the dye separated files as the original file should already be save in another location

Stitching: use Fiji

Manual stitching

- Drag dye separated file into Fiji
 - Two windows will appear and you want to create regions of interest around the 2 sides you want stitched. Marked in yellow. (It is helpful to adjust the brightness Image→Adjust→Brightness/Contrast)



- Plugins→Stitching→Pairwise stitching
- Select top file for First image (reference) and then select bottom file for Second image (to register)
- Click “Ok”
- Pairwise stitching box pops up
 - Fusion method: Linear Blending
 - Checked peaks: 5
 - Check all boxes (ignore zero values when fusing, display fusion, compute overlap, and subpixel accuracy)
 - Keep x, y, and z values at automated values
 - Registration channel image 1: click only channel 2
 - Registration channel image 2: click only channel 2
 - Click “okay”

- An r value is displayed in the “log” box. If this value is less than 0.8 re-do the stitch
- After the stitched has finished it needs to be transformed to the right:
image→transform→rotate 90 degree right
- Next, image→color→split channels
- Save as nrrd
 - Save reference as _01 and the other file as _02