

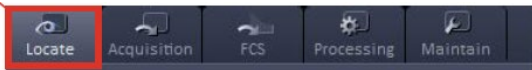
## IMAGE ACQUISITION ON ZEISS LSM FOR SPINE/DENDRITE ANALYSIS

### TURN ON MICROSCOPE AND FIND COVERGLASS

- To boot up system, first turn on the big power switch, followed by the 2 smaller power switches.
- Open Zen black software. Click 'Start System'.
- In Zen black software go to 'Acquisition' main tab



- Go to 'Laser' blue tab and turn on power for all laser lines (they take a little time to warm up).
- On the touchscreen focus adjuster press 'Home' → 'Load position'. This will move the objective to the lowest z-position. Press 'Set Work Position'. Press 'Microscope' and select objective (10x). This is an air objective, don't use oil.
- Tilt back microscope head unit and place microscope slide onto holder inside incubator. Place microscope head back in position. *Always place the slide in upside down, with the label to the right (so the same cells can be found again later if necessary). Clean the coverglass with tissue and lens cleaner (clear spray bottle).*
- Go to 'Definite Focus' blue tab at bottom right of control panel → Click 'Find Surface'. This will adjust the focus automatically to find the coverglass.
- Go back to 'Locate' main tab.



- Click A488 to activate green epifluorescence.
- On microscope air table, find the epifluorescence power dial and press dial down once to turn on lamp. Look through the eyepieces. The coverglass should be in view. Use the joystick to search around and look for GFP transfected neurons. Coarse and fine focus dials are on the right of the microscope (and also on the right of the touchscreen pad). Scan the coverglass to make sure the cell health/transfections look good. Turn off the lamp after you are done looking through the eyepiece by pressing the dial down once.

### LOAD THE ACQUISITION PARAMETERS

- At the top of the control panel under 'Experiment Manager', Click the open file icon to load a set of pre-saved acquisition parameters. Here we will use 'ANGIE\_FastAiry 488, 543, 633'. *Note: 488nm is the wavelength of blue laser light that will excite GFP or other green fluorophores. 543nm green light will excite RFP or other red fluorophores. 633nm red light will excite far-red fluorophores.*
- In the 'Channels' blue tab, you will see three checked boxes, one for each laser. Uncheck all boxes except 488. This will allow us to look only at the GFP transfected neurons.

Click 'Live' and find a transfected neuron on the coverglass by moving the joystick and focus.

### MAKE A TILESCAN OVERVIEW OF THE ENTIRE COVERGLASS

- Under the 'Experiment Manager', open '\_10x\_Confocal-Overview'
- Also in experiment manager, check the 'Tile Scan' box. This will add a new blue tab entitled 'Tile Scan' at the bottom right of the control panel. Click to expand.
- Go to 'Convex Hull'. Hit 'Live' to see neuron.
- Use the joystick to find the top of the coverglass. In the Tile Scan tab click 'Add'. Use the joystick to find the bottom of the coverglass, click 'Add'. Do the same for the left and right edges of the coverglass. A diamond will appear in the Tile Scan window showing the area within which a scan will be made.
- In 'Acquisition Mode' blue tab, set 'Frame Size' to 512x512 'Speed' to max.
- Hit 'Start Experiment'. Save as (Date\_Slide#\_coverglass#\_Overview). An overview image of the coverslip will be generated (this takes ~5 mins).
- Right click below side tabs to left of image and click '2 containers'. This will allow us to view 2 windows simultaneously.
- Drag the Overview into the window on the right.
- Below the overview image in the 'Dimensions' tab, click 'Stage'.
- Click the left hand window and hit 'Live'.
- You can now click anywhere in the overview image and it will take you to that position on the coverglass.

### SELECT NEURONS FOR IMAGING

- Switch the objective to the 20x (this can be done on the touchscreen unit or in the 'Acquisition Mode' blue tab. Don't add oil to 20x objective.
- Re-load the '\_FastAiry 488, 543, 633' settings. In 'Channels' blue tab, uncheck everything but 488.
- In the Experiment Manager tab, check 'Positions'. This will allow us to choose multiple cells for batch imaging.
- A new 'Positions' blue tab will appear at the bottom right of the control panel. Click to expand.
- Go to 'Definite Focus' blue tab at bottom right of control panel → Click 'Find Surface'. This will adjust the focus automatically to find the coverglass.
- Click left hand window and hit 'Live'.
- You can now move around coverslip by clicking on overview image and look for pyramidal neurons.
- When you find a cell you would like to use, center the soma in the field of view and adjust focus so that dendrites/spines are clear.
- In the 'Positions' blue tab, click 'Add'. These coordinates will be saved.

## OPTIMIZE CHANNELS FOR IMAGE ACQUISITION, ALIGN THE AIRYSCAN DETECTOR

- Switch now to the 63x objective: On the microscope touchscreen control unit, press 'Load Position' then press '63x oil' objective. This is an **oil** objective.
- Using the joystick, move the objective away from the slide so that the objective is visible and oil can be applied. Add one drop of oil to the objective.
- In the 'Positions' blue tab, select one of the neurons chosen and click 'Move To'.
- Hit live and re-focus until the soma and dendrites are visible.
- Using the overview, choose cells that you would like to image and click 'Add' to positions. *Look for branching dendrites and spines.*
- Repeat until you have saved the coordinates of ~10 cells.

### How to get optimal signal-to-noise

- Starting with the 488 channel, we now will optimize the frame size, sampling rate, capture speed, laser intensity and detector sensitivity (master gain) for each channel to make sure we can generate the best images possible.
- In the 'Acquisition Mode' blue tab, adjust the 'Zoom' to 1 and click 'FS' in the sampling tab and set the speed to 'Max'. This will automatically set the 'Frame Size', 'Nyquist' and 'Speed fps'. 'Bit Depth' should be 8 Bit and the 'Direction' should be set to '<- ->'
- In the 'Channels' blue tab, click on the 488 channel to make sure it is highlighted. You can now alter the laser power and master gain. As a good starting point, set the laser power for this channel to 6 and the gain to 720.
- Hit 'Live' and adjust the focus so that you can see dendrites/spines. Hit 'Stop'. Below the image in the 'Display' tab is a histogram reporting on the intensity of each pixel within the image. X-axis shows intensity, y-axis shows number of pixels. The goal now is to alter the **laser power** and **master gain** to set a good dynamic range for each channel. Ideally, all channels should reach at least 50% of *dynamic range*.
- In the 'Dimensions' tab below the image, check the 'Range Indicator' box. This will show us if any pixels are saturated (this means the intensity is too high and out of the dynamic range of the detector, thus relative intensity measurements cannot be made for these pixels). Saturated pixels are displayed in red. *Notes: It is important to adjust so that none of the structures we will be analyzing are saturated or too dim. If the soma is a little saturated it is ok for this particular experiment, as we will not be taking measurements in the soma.*
- Uncheck the 488 channel and select the next channel. Repeat the above process to find a good dynamic range for each channel.
- *Notes: If the laser power is too high, the fluorophores will begin to bleach, too low and we won't excite enough photons to resolve the image properly. Each channel must be adjusted separately due to different excitation properties of fluorophores. For example, 543nm excitation will emit a lower quantity of photons from the corresponding fluorophore and therefore can require a higher laser intensity.*

## ALIGN THE AIRYSCAN DETECTOR

Before acquiring any images, the AiryScan detector needs to be aligned.

### **Alignment for Airyscan Fast:**

1. Select only the green channel.
2. In the 'Acquisition Mode' blue tab, click Sampling at 'FS', and Speed fps at 'Max'
3. Hit 'Live' icon and focus the sample until you receive a high signal. Hit 'Stop' to exit live mode.
4. Hit 'Continuous' icon (only use for alignment) and then click the AiryScan tab in the side tabs to the left of the image.
5. Below the image, in the AiryScan grey tab, click "Detector View" to see the detector alignment. The whitest sections of the detector should be centered. Click 'Stop' to exit continuous when aligned.
6. To check that the detector is properly adjusted, go back to 'Continuous' mode and click the 'Maintain' tab at the top left of the control panel (near the Locate and Acquisition tabs). Expand the 'AiryScan' blue tab by clicking on it. There will be a 'Quality and Status' message that is updating. When it says 'Good (Waiting)', the detector is aligned.

## SETUP TILESCAN AND Z-STACK TO TAKE IMAGE OF FULL ARBOR

- In Experiment Manager check 'Z-Stack'.
- Go to the 'Z-Stack' blue tab that now appears at the bottom right of the control panel and click to expand.
- Select 'Center'.
- Set the number of planes to 9 and the interval to 0.4  $\mu\text{m}$ .
- This will acquire 9 z-images at intervals of 0.4  $\mu\text{m}$ , centered around the coordinates we added in the 'positions' tab.
- Below the laser path diagram, set the 'change track' to 'every z'.
- Go to the 'Tile Scan' blue tab that now appears at the bottom right of the control panel and click to expand.
- With the 63x objective, set up a 5x5 scan with 10% overlap.

## CHECK IMAGE QUALITY AND ACQUIRE THE MULTI-NEURON TILED Z-STACKS

- Now that all channels are optimized, make sure that they are all checked in the 'Channels' blue tab.

### **To check that the images will be of sufficient quality (optional):**

- We now need to perform a test tiled z-stack to ensure that the images generated will be of sufficient quality for analysis.
- Choose any neuron and check only 'Z-stack' and 'Tile Scan' and click 'Start Experiment'.
- Go to the 'Processing' Tab at the top left of the control panel.

- Highlight 'Airyscan Processing' in the 'Method' blue tab.
- Click 'Select' in the 'Method Parameters' blue tab. This will select the neuron to be analyzed, which will be open in the window (the neuron you have just captured). Click 'Apply'.
- Highlight 'Maximum Intensity Projection' in the 'Method' blue tab.
- Click 'Select' in the 'Method Parameters' blue tab (to select AiryScan processed image). Click 'Apply'.
- Highlight 'Stitch' in the 'Method' blue tab.
- Click 'Select' in the 'Method Parameters' blue tab. Click 'Apply'.
- Inspect the processed image by zooming on spines and puncta, to ensure sufficient resolution is being achieved.
- If it's good, in the experiment manager, check 'Z-Stack', 'Tile Scan' and 'Positions'.

**Start experiment:**

- Go through each of your saved coordinates one last time in 'positions' blue tab and check that the z-positions are all good. Focus then 'update' if necessary.
- Streaming allows you to save the images directly. Make sure tile/position tabs are unchecked (otherwise each tile / position is saved as individual files).
- With 'z-stack', 'tile scan' and 'positions' all checked, in the Experiment Manager tab, hit 'start experiment'. Select location to save your files. Observe the images being acquired for a minute or two to make sure everything is running as expected.
- Come back in a few hours to see your lovely images.
- Save 63x images as "Date\_Slide#\_coverglass#\_tiledzstacks" and overview as "Date\_Slide#\_coverglass#\_overview".

**TURNING OFF THE SYSTEM**

- On touch screen control unit, press 'Load position' to safely lower the objective.
- Remove slides from microscope and store in a slide folder in the fridge.
- If the 63x objective was used, the oil must be cleaned from the objective. Use Kim-Wipes (black) and lens cleaner (clear bottle). Make sure the objective is clean and dry.
- When all files are saved, close the Zen black software. You will see a prompt to power down the lasers. Turn all lasers to Off.
- Turn off the 2 small power switches, followed by the large power switch.
- Unprocessed image files are extremely large, AiryScan processed images are ~5% of the size. Remove all unprocessed files from the computer at the end of each session (either onto a hard disc or delete unprocessed and save processed).
- Leave the computer on.
- Check the microscope to make sure you haven't left anything.
- Make sure the door is locked when you leave the room.