BIOLOGICAL IMAGING DEVELOPMENT COLAB

UCSF University of California San Francisco

Pod B: Leica THUNDER Microscope

Computational Clearing Widefield Microscope

Kyle Marchuk Austin Edwards Mohammad Naser

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1 Introduction

The Leica Thunder is an inverted computational-clearing microscope configured with nine illumination options and a wide range of emission filters. The system is enclosed with an incubation unit to regulate experimental temperature and air gas (5% CO2) for live cell imaging.

The objective turret houses six lenses including 2.5x Air, 5x Air, 10x Air, 20x Air, 40x Air, and 63x Oil. The stage is motorized for large tiled stitching or marking and storing positions.

The Thunder microscope can collect a large amount of data in a short amount of time. Be sure to factor in time for computational clearing and data transfer within your reservation.

All new users need to be trained by a BIDC staff member before independent use.

Sign up for time using the **iLab** microscope scheduler.

Note: For live-sample imaging, turn on the environmental chamber 30 min in advance to warm up the chamber. For the system to reach thermal equilibrium you will need to turn on the system 2 hours in advance.

The available objectives are:

Mag	Immersion	Numerical Aperture	Working Distance	ID Number
2.5x	Air	0.07	11.2 mm	506304
5x	Air	0.12	14.0 mm	506303
10x	Air	0.32	11.1 mm	506522
20x	Air	0.40	7.5-6.2 mm	506243
40x	Air	0.60	3.3-1.9 mm	506203
63x	Oil	1.40	0.14 mm	506349

2 Start-Up

1. Turn on the computer with the computer power button.
2. Turn on the power strip located back and right of the computer monitor. See Figure: 1.

Figure 1: The power supply switch located behind and right of the computer monitor.

3. If you plan on doing live imaging:
   (a) Place the necessary sample holder on the stage.

(b) Ensure the environmental enclosure box is sealed.

(c) Turn on the control boxes located behind the microscope. See (left) Figure: 2.

(d) Set the temperature for the environmental control box.

(e) Turn on the heater box located back and right of the microscope. See (right) Figure: 2.

Note: We recommend turning on the temperature control at least 30 min before the start of your experiment. To reach thermal equilibrium you will need to turn on the system 2 hours in advance. The carbogen gas can be opened once your sample is prepped.

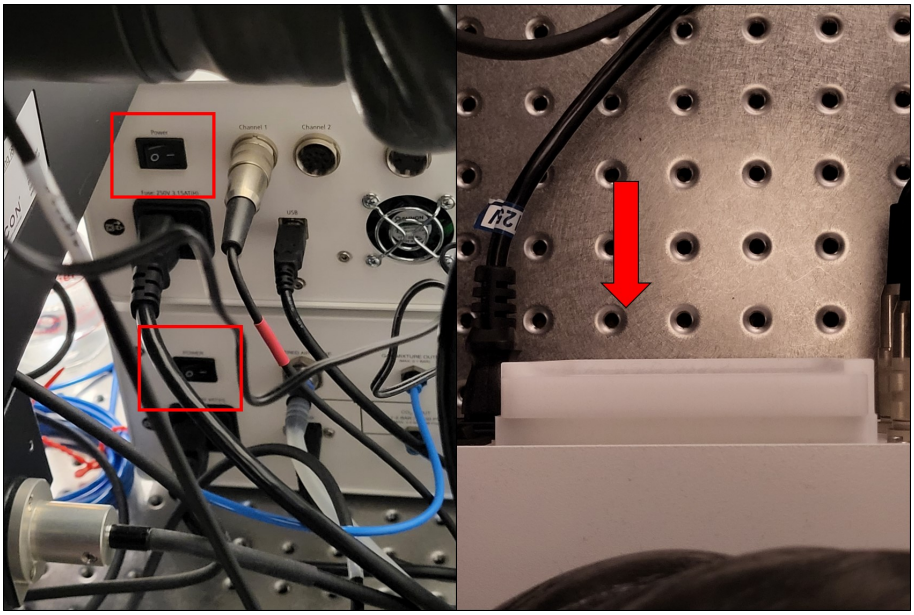

Figure 2: **Left:** Power switches for control boxes. **Right:** Power switch for heater unit.

4. Launch the **LAS X** software from the desktop.

5. Press **OK** to confirm the configuration settings. See Figure: 3.

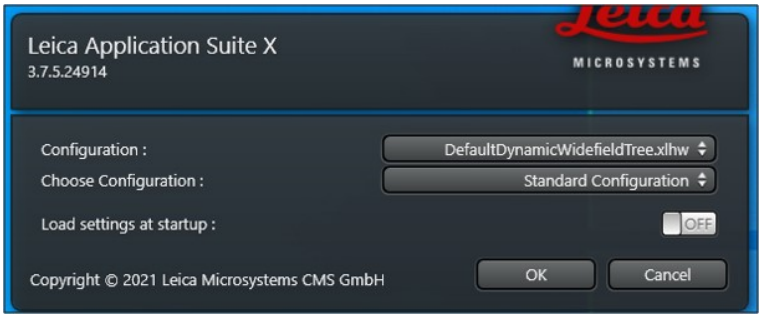

Figure 3: Confirm the proper settings are selected at startup.

6. Turn on the lamp within the environmental chamber using the knob located on the top-right of the chamber. See Figure: 4.

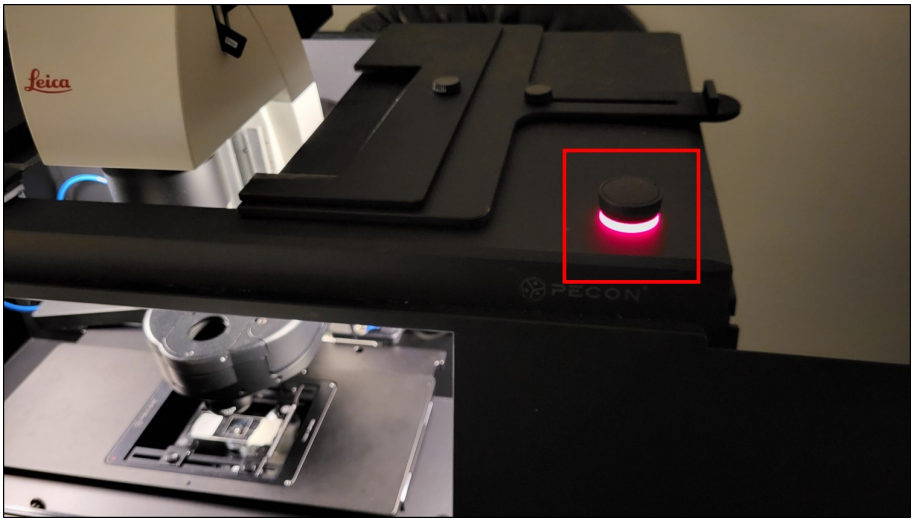

Figure 4: Lamp knob located on the top-right of the environmental chamber.

7. Using the control panel located on the front of the microscope, select the objective you want to use and add immersion media if necessary. See **Blue Box** Figure: 5.
8. Place sample on holder and gently add the holder to the stage.
9. Select the **Eyepieces** port on the control panel. See **Red Box** Figure: 5.
10. Select a channel to illuminate the sample in software and press **Live** or skip to **Setting up and Experiment**.

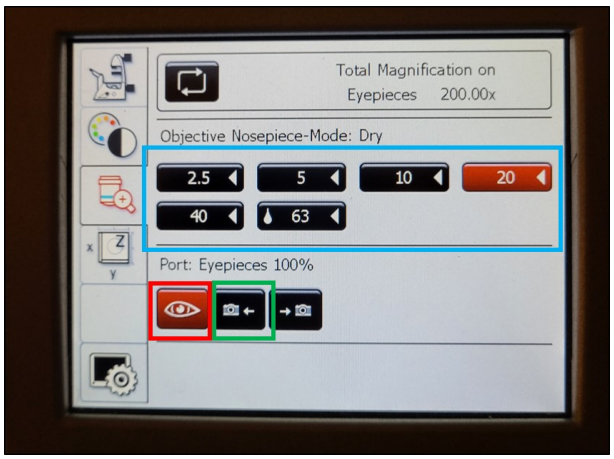

Figure 5: Microscope control panel located on the front of the microscope.

3 Setting up an Experiment

The Leica Thunder has many features to perform complex experiments. You may not need to set all of the following parameters.

3.1 Adding Channels

1. In the main window of the **Acquire** tab press the **'+'** button to add as many channels as you will image. See **Red Box** Figure:6.

2. Press the **Dropdown Menu** for each channel to select the appropriate filters. See **Yellow Box** Figure:6.

3. For each channel, ensure only the illumination line you want is selected and add some illumination power using the slider. See **Blue Box** Figure:6.

4. If you need to remove a channel, select the channel and press the **'-'** button. **DO NOT** right click and press **remove** as this will delete the channel from the configuration.

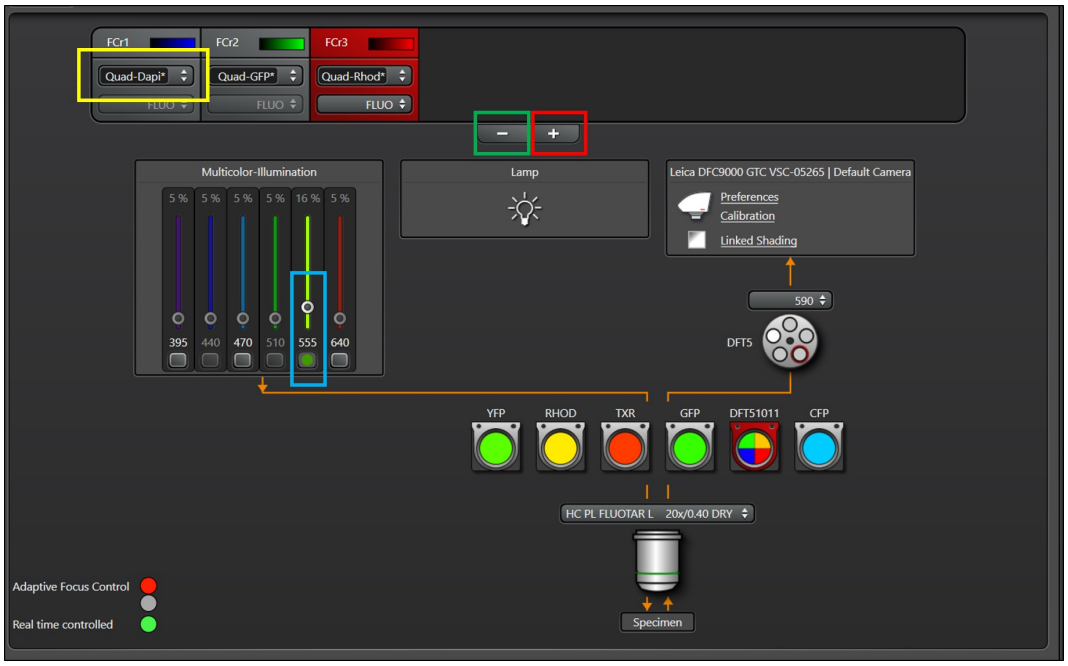

Figure 6: Main display.

3.2 Find the Sample by Eye

1. Using the **XY Stage Controller**, move the sample over of the objective. See Figure: 7

2. Select the channel of the brightest and most ubiquitous fluorophore (this is typically DAPI).

3. Using the **Z Stage Controller**, raise the objective to focus on your sample. See Figure: 7

4. When you have focused on your sample, press the **Left Camera Port** button on the front control panel. See **Green Box** Figure: 5.

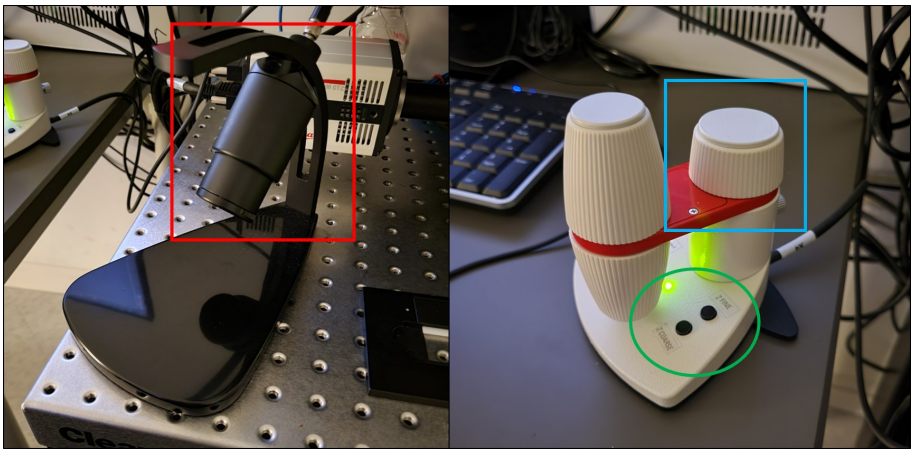

Figure 7: **Left:** XY stage controller. **Right:** Z stage controller.

3.3 Camera Settings

The most common settings to change on the camera are **Image Format** and **Exposure**. The **Exposure** should be set in conjunction with the laser power for each channel to optimize the signal. See Figure:8

Image Format: Full image format utilizes all the pixels of the camera. **Binning** combines pixels into 1 pixel. This reduces resolution and image size, but increases signal and data transfer.

Exposure: The amount of time the shutter is open per image. The longer the exposure, the higher the signal, with the drawback that the sample is illuminated for longer.

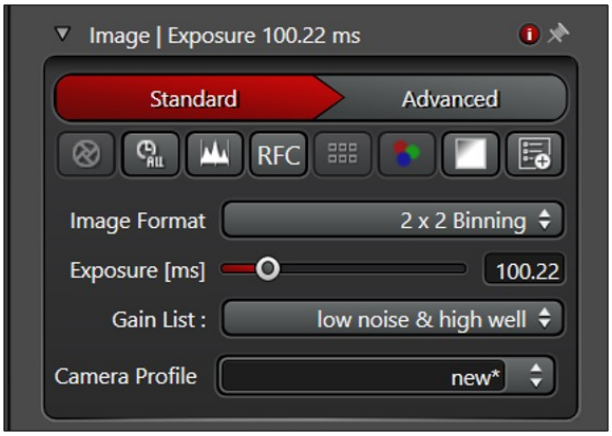

Figure 8: Image/Exposure Tab.

3.4 Acquisition Mode

Use the **Acquisition Mode** tab to add dimensions to the experiment as well as add the **Tiling** and **Navigator** modules. See Figure: 9

1. Press **'Z'** to add volumes to the experiment.
2. Press **'t'** to enable timelapse imaging.
3. **Red Box** icon to access **Tiling**.
4. **Blue Box** icon to access the **Navigator** module (see below).

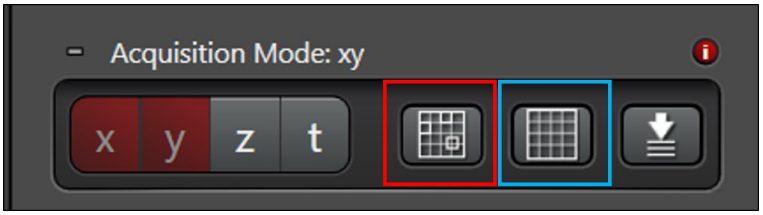

Figure 9: Add dimensions to the experiment.

3.5 z-Stack

Used for creating the volumes of a 3D stacked image. See Figure: 10

Begin: Using the **Z Position** knob, move the image plane to the 'Top' of your sample and press this button to assign this position.

End: Using the **Z Position** knob, move the image plane to the 'Bottom' of your sample and press this button to assign this position.

Trash Can Icon: Deletes your position assignments.

Stack Direction (Z): Scanning direction for the volume acquisition. Typically want to move the stage in the **Down** direction to scan from the Top of your sample to the Bottom.

Number of Steps: Within the given volume, determines z-step size by number of image planes.

Z-Step Size: Within the given volume, determines the number of steps by step size.

System Optimized: Determined by the objective; the smallest step size before over-sampling the volume.

Z-Compensation: Is used to associate laser power and gain with a z-position. Typically used to compensate for scattering in a thick sample.

Travel Range: Travel range of the selected z stage.

Figure 10: Z-Stack Tab.

3.6 Tiling

The **Tiling Module** will create a 2D grid for acquisition based upon saved stage positions.

1. Locate an initial corner position (upper right, lower left, etc) for your grid either through the eyepiece or while **Live**.
2. Press the **Add Position** button to save that position.
3. Move the stage to another edge of the ROI and add that position.
4. A grid will appear in the **Stage** window. **Note:** move the Zoom slider if you do not see a grid appear.
5. To increase the grid size, add more points outside the current grid.
6. To decrease the grid size, add points within the current grid, and delete the unecessary point by selecting that point in the drop down menu and pressing the **Trash Can Icon**.
7. **Turn Merge Images**, **Auto Stitching**, and **Linear Blending** to **ON** to automatically create a stitched imaged at the end of acquisition.
8. To **Use Focus Map** a focus map will need to be made in the Navigator (See Below).

3.7 Time

Used for setting up timelapse experiments. See Figure: 11

Time Interval: The amount of time it will take to run one iteration of the experiment. If set to longer than the amount of time the instrument needs to run the iteration, the microscope will sit idle until the interval expires.

Acquire Until Stopped: The experiment will continue until the user presses the **Stop** button.

Duration: The experiment will run continuously until the timer expires.

Cycle: How many iterations of the experiment to run, regardless of the time it takes.

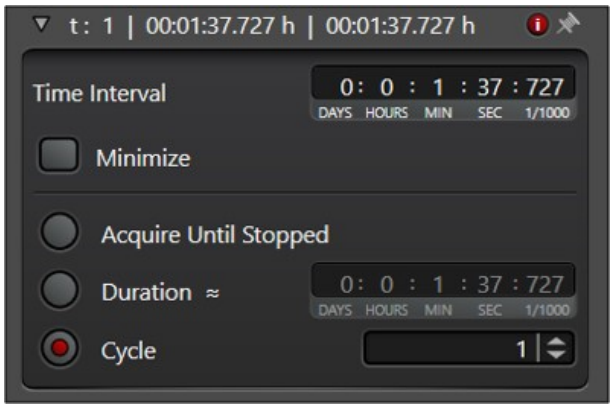

Figure 11: Time Tab.

4 Navigator Module

The **Navigator Module** is module useful for exploring and defining large volumes for imaging.

4.1 Exploration

Point-and-Click: Double clicking on the screen will move the stage to that position and take an image.

Live: Continuously images with the selected channel. This is useful for adjusting the z-position.

Spiral: The stage will move in a spiral pattern stitching images together in an expanding pattern.

Draw ROIs: ROIs can be drawn for directed exploration. Choose a shape from the bottom task bar and select the region on the stage to image. Press the **Arrow Icon** to select, adjust, or move the previously created ROIs.

Preview: This option will image the ROIs in the **Task List** with the current z-position with the current channel selected.

4.2 Focus Map

The **Focus Map** is useful for compensating for uneven (non-flat) samples.

4.3 Carrier

The software comes preloaded with a wide range of sample holders. The **Carrier** module allow for quick selection of individual wells or other ROIs of interest.

4.4 Acquisition

The **Start** button will run the experiment as set up so far. If you have used the **Navigator** to set up ROIs, you will need to launch the experiment from here.

5 Thunder Processing

While an excellent widefield microscope, the Thunder microscope's main strength is the ability to do **Computational Clearing**. The software mathematically removes out-of-focus light using know parameters of the microscope and the sample during acquisition.

1. Navigate to the **Process** page and down to the **Lightning & Thunder process** tool. See **Blue Box** and **Green Box** Figure: 12.

2. Go to the **Open projects** tab and select the file you want to process.

3. Select the Method to computationally clear the sample. See **Red Box** Figure:12.

   Instant Computational Clearing: Fuzzy structures resulting from out-of-focus blur, for example, are removed. No PSF is calculated. No 3D information is required for this method.

   Small Volume Computational Clearing: The image is developed using a Leica internal deconvolution process. Lightnings Decision Mask Technology can be used to separate signals from noise and reconstruct them optimally. This method is particularly suitable for thinner or less strongly marked samples.

   Large Volume Computational Clearing: The image is developed using a Leica internal deconvolution process. This method is particularly suitable for denser and thicker samples. Unwanted signals are removed before deconvolution, resulting in faster results on these samples than with Small Volume Computational Clearing.

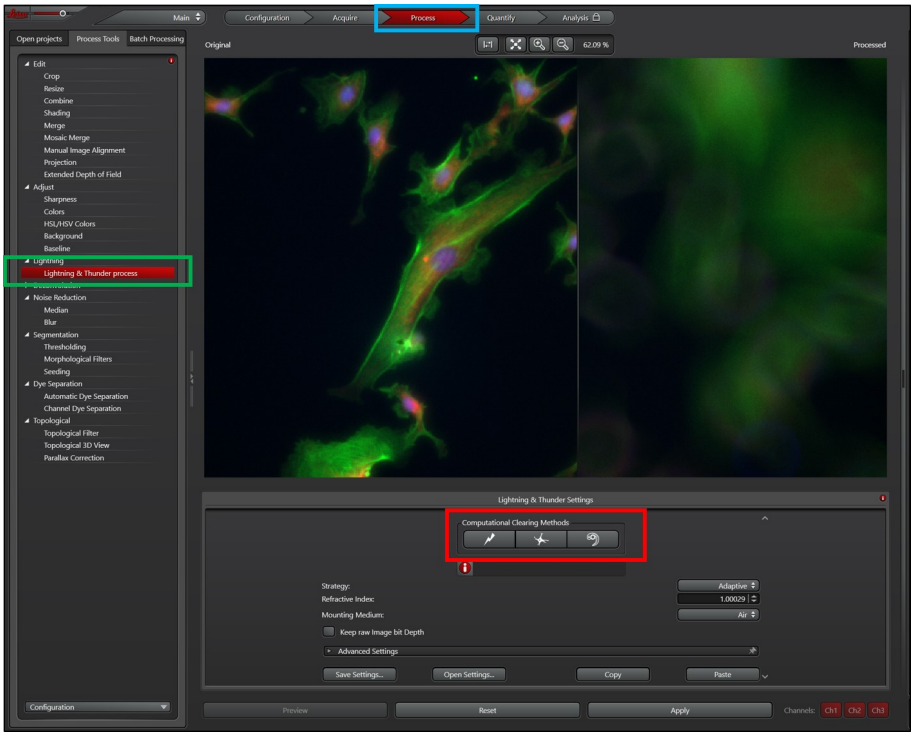

Figure 12: Time Tab.

4. Choose the strategy you want to employ.

   Adaptive: On the basis of the SNR, the settings for optimum image quality are determined automatically, precise down to the voxel. This prevents noise in the images from being assessed as signals. In addition, processing is completed once an optimum result is achieved. We recommend using this strategy because it usually delivers the best results.

Global: In this strategy, the parameters that you have configured under Advanced Settings are applied globally to every voxel of a channel. If there are high proportions of noise, a high number of iterations as well as a very low regularization can lead to the appearance of noise as signals/structure.

5. Choose your **Mounting Medium**.
6. Select **Advanced Settings**. See Figure: 13
7. Ensure the **Type:** is set to **Widefield**.
8. Adjust the **Feature Scale [nm]** for each channel. The lower the value, the finer the structures that remain after image optimization, refer to graphic. Do not set the value smaller than the size of the structures that you want to identify.
9. Set the **Strength [%]**. The higher the value, the stronger the clearing effect. To obtain an optimum deconvolution result, the value should be = 90%.
10. Press **Apply** to run the calculation. **Note:** This process is very resource heady. We recommend not taking images while the **Computational Clearing** is running.

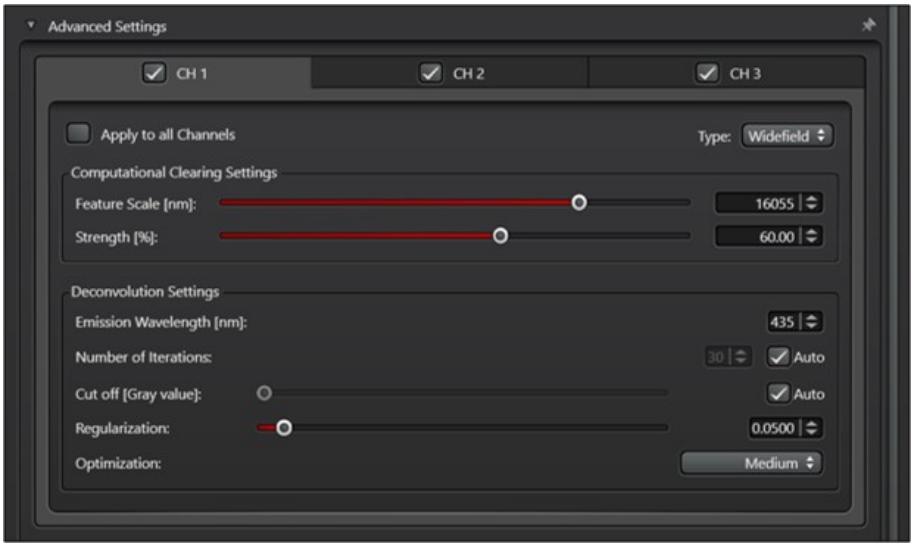

Figure 13: Time Tab.

6 Saving Acquired Images

1. Be sure to factor in enough time at the end of your experiment to save and transfer your files.
2. Click the **Open projects** tab to view all images taken.
3. Delete or Rename images as needed.
4. Click **Save Icon** to save the project as a .lif file in your hard drive or on Box.
5. Please remove old files from the Thunder computer once they have been saved on your hard drive.

7 Shut Down

1. After saving, close the **LAS X** software.
2. Turn off the environmental control and gas flow if used.
3. Shut down the computer using Shut Down option in the Start Menu.
4. Remove sample and clean objectives as needed.
5. Turn off the power switch behind the monitor.
6. Confirm the space is clean and ready for the next user.

Contact the BIDC

The BIDC office is located in Medical Sciences Building Room S1109.
The BIDC office phone number is 415-476-4550.
If you need immediate assistance and no one is available in the office, or it is after business hours, please call the **BIDC Hotline** at 415-745-2432.