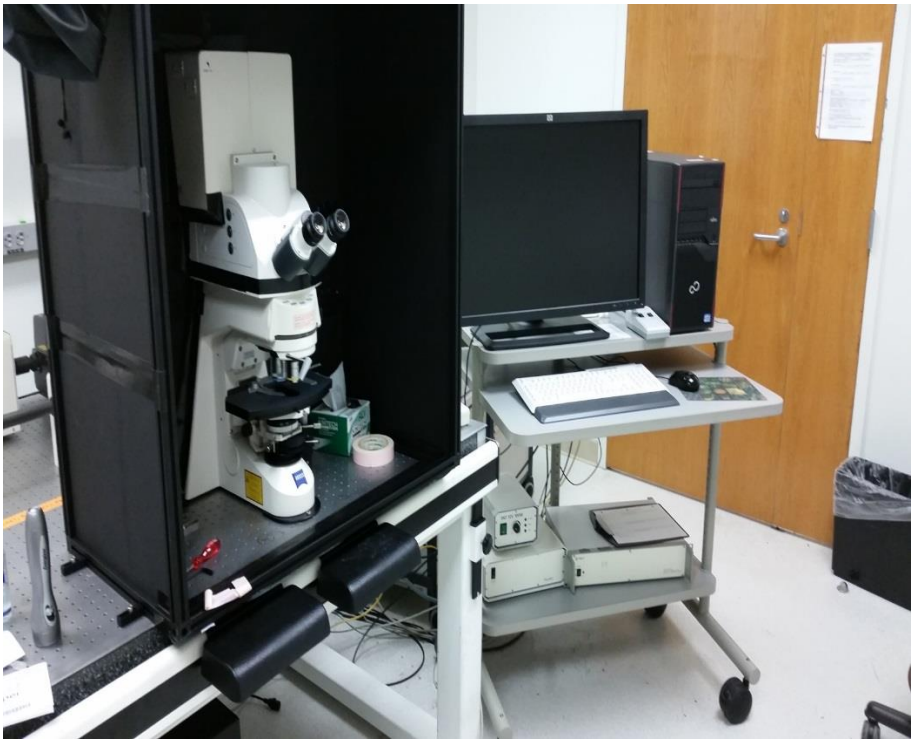
Zeiss LSM 510 Multiphoton Confocal Microscope

User Guide



LSU Health Sciences Center-Shreveport

Research Core Facility

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1. SAFETY

- The system contains components with **dangerous voltage**. The system must not be opened by anybody else other than authorized Carl Zeiss Service staff.

- Maintenance, repair, modification, removal or exchange of components, or other interference with the equipment beyond the operations described in this manual may only be carried out by the manufacturer Carl Zeiss or by persons expressly authorized by Carl Zeiss to do so. This applies especially to the microscope system, the laser scanning module, lasers, the PC system, the power supply units, cable connections and other system components.

- The openings for ventilation must not be covered.

- There are hot surfaces on the HBO and HAL lamp.

- The LSM 510 and LSM 510 META are laser hazard class 3 B instruments. If equipped with a Ti:Sa Laser, the LSM 510 and LSM 510 META are devices that belong to laser hazard class 4. These moderate and high-risk classes embrace medium-power and high power lasers. You must take care not to expose yourself to the radiation of such lasers. In particular, never look into the laser beam! Only personnel who have been instructed on laser safety are allowed to operate the system.

- If used properly, the LSM 510 and LSM 510 META will not pose any laser radiation risks for operating staff. Nevertheless, you should observe the following warnings:

    - Do not place any reflecting objects into the beam path.

    - Never open any covers or panels.

    - Never look into the laser beam, not even to simply view the specimen, whether with the aid of optical instruments or without. Otherwise you risk going blind!

    - Do not leave any empty objective positions of the nosepiece uncovered.

- If a class 4 laser is attached to the system, already stray light can impose danger to the operator.

- With class 4 lasers take special care of fire protection requirements. Do not use or store flammable or explosive solids, fluids or gases in the vicinity of the system.

- Class 4 lasers can ignite flammable materials like cloth or paper. Do not put such materials into the beam path.

- For NLO systems equipped with a specific push and click filter for NDD imaging be aware that the NDD reflector cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the ocular lens. When observing the specimen through the ocular lens the use of the NDD reflector cube should be avoided. The light flash is not harmful but unpleasant. The reflex of closing the eyelid is sufficiently protective. To completely avoid this situation an additional filter (#1261-345) can be mounted into the NDD reflector cube which prevents the back reflection of the HBO light in the ocular plane.

2. TURN ON THE SYSTEM

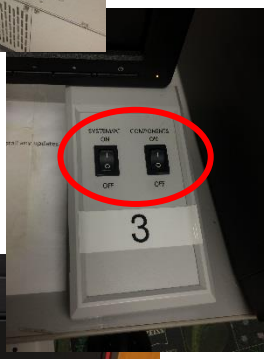
Always turn on in the order of 1->4. Numbers are labeled on the machines

1) Turn on the FluoArc mercury arc power supply (No. **1**).

2) Wait **two minutes** until ignition is complete on the FluoArc timer/controller. Then you may select the percent lamp intensity by pressing F1 or F2. The 50% intensity level will cause less photobleaching while you are observing your sample. (Record the bulb time in the sign-up sheet)

3) Turn on the main power switch on the laser cart, turning 90 degrees clockwise, to vertical (No. **2**).

4) Press the Components and System/PC switches to ON, one at a time (No. **3**).



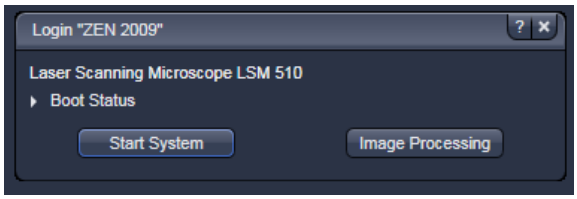
5) Press the **On** button to turn on the computer. Log in with your LSUHSC ID and password. Make sure the domain is set to **LSUMC-MASTER**.

6) If you are going to use the Chameleon multiphoton laser, turn the key on the front of the control panel from **Standby** to **On.**

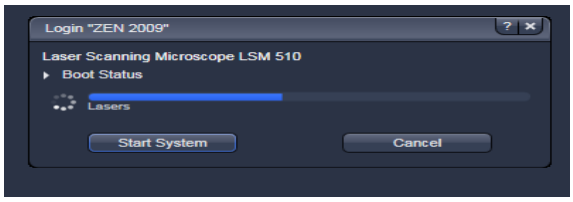
3. START UP THE ZEN SOFTWARE

Double-click on the ZEN 2009 desktop icon to start the program.

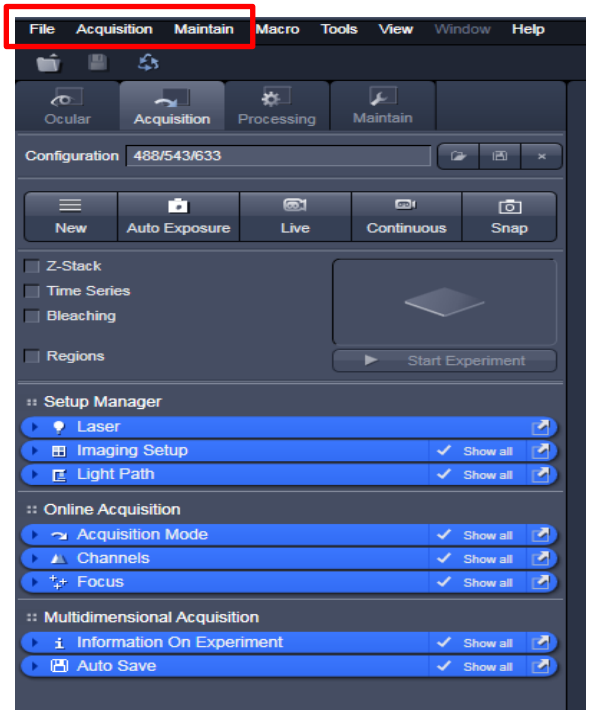
The Login "Zen 2009" window appears. Select **Start System** to initial microscope system for acquisition. If you want to only analyze your images, select **Image Processing**.



Wait for the Initialization to complete.

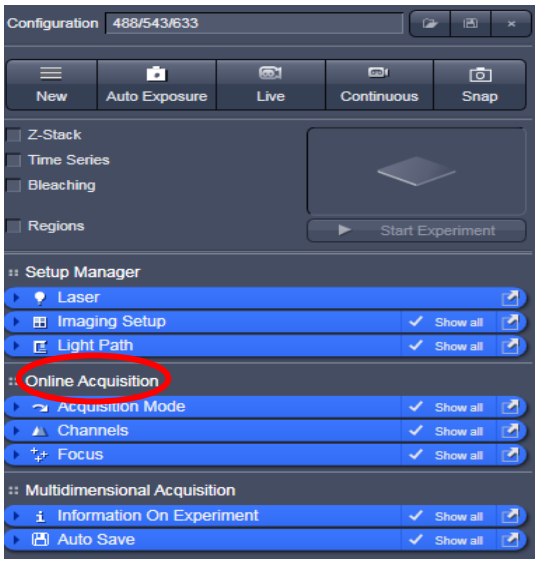


The main menu opens. This guide will only cover features from the **File, Acquire**, and **Maintain** submenus. There are many other very useful features which are explained in the Zeiss Operating manual, located in the Help menu.

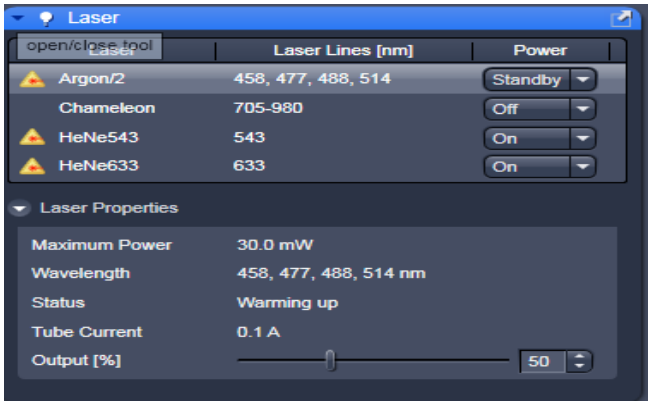


4. TURN ON THE LASERS

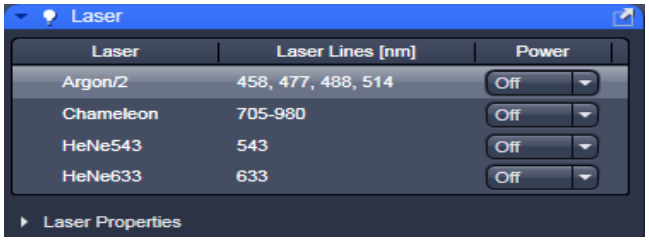
From the **Setup Manager** submenu, click on the **Laser** menu**.**



In the **Laser** window, click on the name of the laser to activate it.
For the argon laser, click **Standby**. When the status goes from **warming up** to **ready**, click **On** and then use the slider to set output to 50%. If you exceed 50%, you will get a warning. Above 50 % is only used for intentional photobleaching.

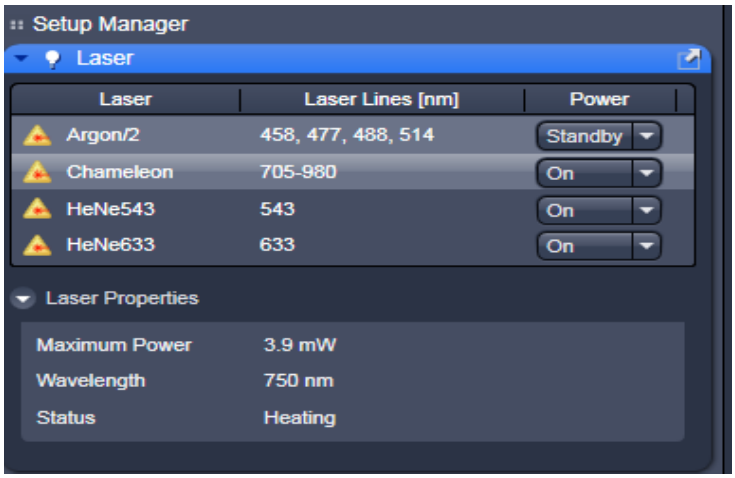


For the HeNe 543 and 633, select **On.**

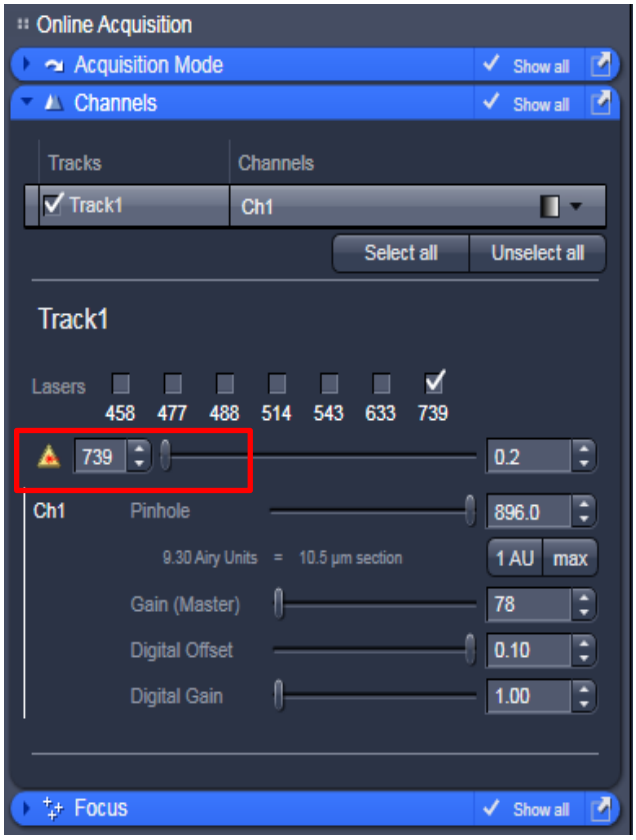


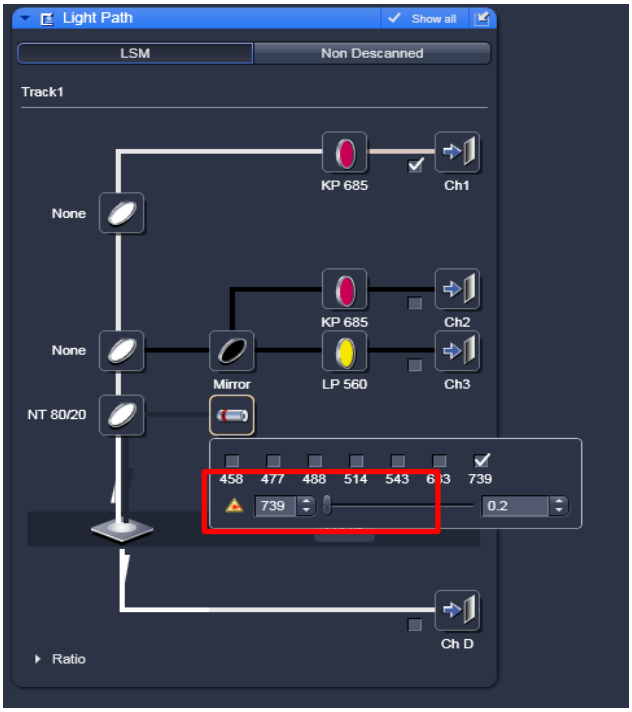
If you log out of the system, the argon laser will go to **standby**.

When you turn the Chameleon laser on, all you are doing is opening a shutter and activating the software control. The laser is actually turned on with the key on the front of the Chameleon control panel. The status will read **Key Locked,** if you have not turned the key, and **Heating** if only a few minutes have passed since you turned the key. It is ready to use when it says **Modelocked.** Note: Please inform staff when the chameleon laser has problem going into the **Modelock** status.



You can tune the wavelength of the Chameleon laser from 705nm to 980nm by clicking on **the laser icon in the Light Path tab or in the Channels** window by entering the wavelength of choice. The status will read **Busy** while the mirrors are adjusted, and then it will read **Modelocked.** (This is indicated with a red box over the laser wavelength for **Busy** and disappears when in **Modelocked**.) Please modify the wavelength in multiple steps if you are moving it through a large portion of the range, i.e. three 50nm steps to go from 800nm to 950nm.





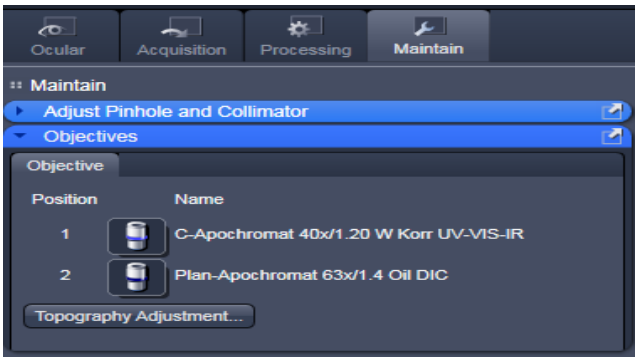
For quantitative work, the lasers should be on for one hour before collecting data.
Note: The tuning process should only take a few seconds to complete, when there is a delay or takes longer time please inform staff.

5. SELECT AND MOUNT THE OBJECTIVES

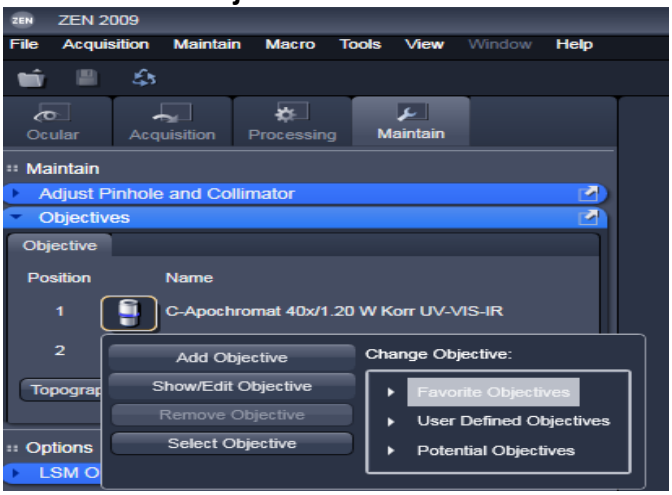
The microscope holds only two objectives, and the ones you plan to use need to be activated in the software because many of the optimal settings for imaging are related to the objective chosen and the software needs to be told what you are using.

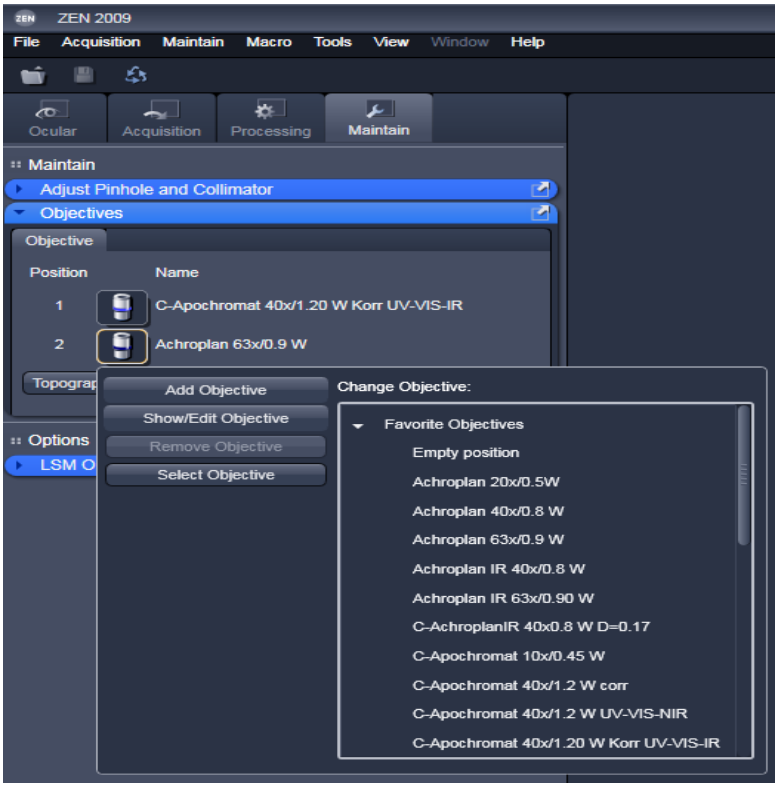
From the main menu, select the **Maintain Tab,** then select **Objectives.**

In the **Objectives** window, two objectives will be displayed. They may/may not be the actual objectives that are screwed into the turret mount.



To change the objective, in the software, click on the objective icon. See if the desired objective is already in the software by clicking one of the change objective options: **Favorite, User-Defined, or Potential**. Find the correct objective and then click on **Select Objective**.





Before changing objectives, use the **↑ focus button** to move the objective carrier to the highest up position, to prevent the objectives hitting the stage while operating.

To mount the objective(s), turn the front silver lever clockwise and raise the objectives. Push or pull the lever gently to move the objectives out of the load position. Then unscrew the mounted one(s) clockwise. Screw in the one(s) you want counterclockwise and tighten slightly.

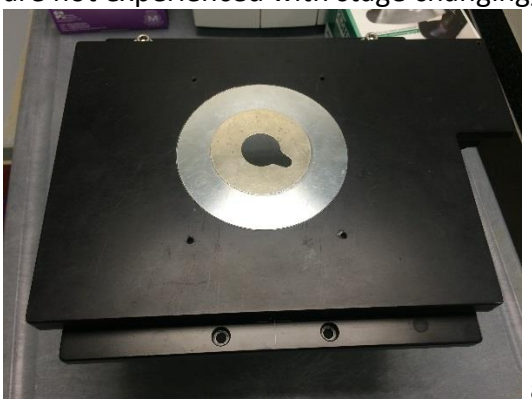
To use a selected objective, Turn the front silver lever clockwise to raise the objectives, then push or pull the lever gently to put the objective of choice in the load position.

Note: Lower the objective slowly by turning the lever counterclockwise, but do not let it drop down into the complete down position. Since the stage can be (and often is) manually raised or lowered to different positions, you may not have enough working distance to fully lower the objectives.

Available objectives are in a box on the shelf labeled: Zeiss LSM Objectives. All objectives are listed on the RCF ->microscopy ->Zeiss Confocal webpage.

6. CHANGE STAGE AND MOUNT SAMPLES

Slide-samples and live animals use different stages. You need to change the stage according to your need. If you are not experienced with stage changing, ask for assistance.



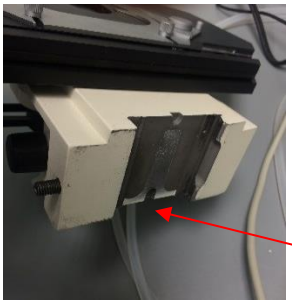
animal stage

slide stage

To change stage, screw or unscrew the stage holder as shown in the image. Both slide-stage and animal-stage have magnatic legs to fix on the stage.

For the slide stage, you need to fit the groove on the back of the stage to the microscope, and make sure the groove sits on the knob of the microscope.

stage holder

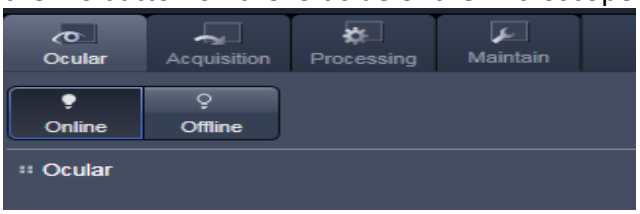


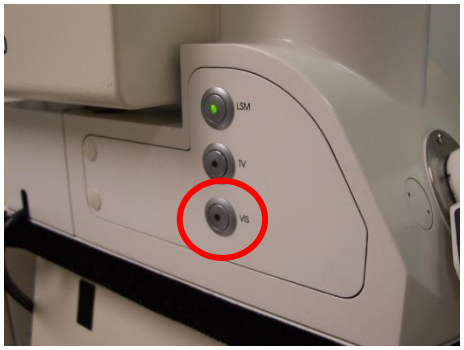
Knob on the microscope

Place the slide, coverslip facing up, in the slide carrier on the stage.
If you are using an immersion objective, place a VERY small drop of the appropriate immersion fluid on the coverslip. Be sure to only use the immersion fluids located next to the microscope. If you accidentally use the wrong fluid, please contact staff right away so they can clean the objective.

7. VIEW SAMPLES THROUGH EYEPIECE

To observe your sample through the eyepieces, click the **Online** button on the **Ocular** submenu or press the **Vis** button on the left side of the microscope.





Manually turn the turret to the desired filter cube with its number in front and center.

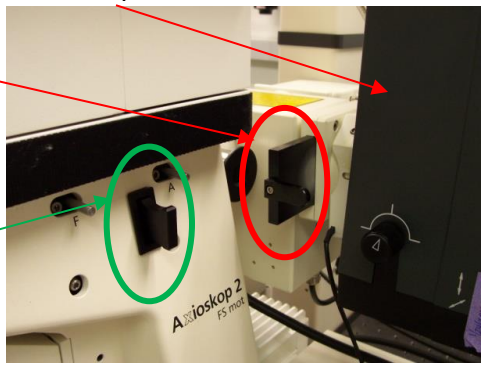
1 = scanning
2 = DAPI (EX 365,EM LP 420)
3 = FITC (EX BP 470/40, EM BP 525/50)
4 = TRITC (EX BP 546/12, EM LP 590)
5 = Brightfield or NDD (for 2-photon)

HBO lamp

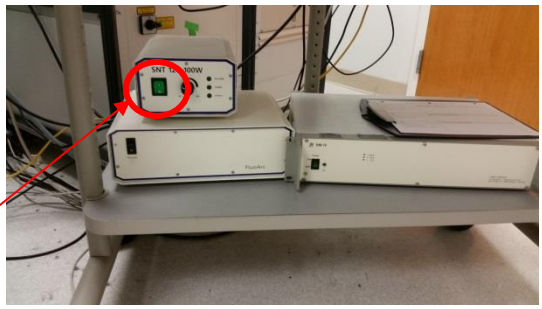
For fluorescence excitation, use **position 2,3, or 4** on the turret, and make sure that the **manual shutter to the HBO lamp** is pulled out, and the **manual slider** on the right side of the microscope is pulled out to the first stop (open position).

When performing a scan, the **manual slider** on the the microscope needs to be pushed in all the way (close position), and the filter turret turned to **position 1**.

Note: The HBO lamp (mercury bulb) only provides fluorescence light for eyepiece observation. For confocal scanning, the laser are involved.



For Bright field observation, make sure that the Bright field filter cube is loaded (**position 5)**. The #5 position is also used with another filter cube in place for observation with the **NDD detectors**, and you must confirm that a previous user has not left the NDD cube in place. You will be shown how to do this during training.

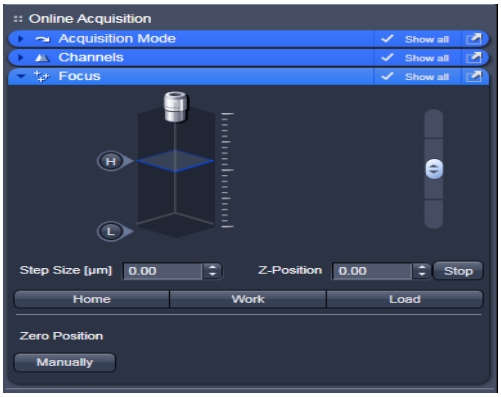


Turn on the **HAL illumination power supply**, with bulb intensity adjusted. (HAL is the light source for bright field transmitted light)

When you are doing fluorescence scanning, you will need to have the HAL bulb intensity turned to zero, or it may add background signal to your images.

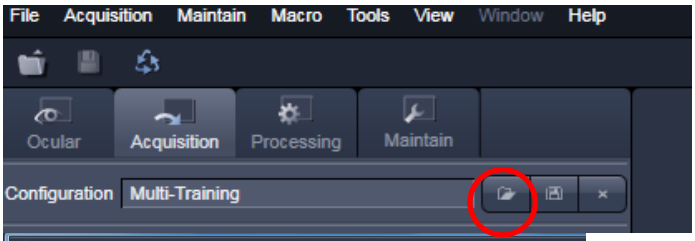
To focus on your sample, use the focus control module.
Slow = fine focus
Speed = fast focus
Turn wheel = ultra-fine focus (CW ↑, CCW ↓)
↑ = moves the objective carrier up
↓ = moves the objective carrier down

You can also focus using the software by selecting **Focus** menu from the **Online Acquisition** submenu.

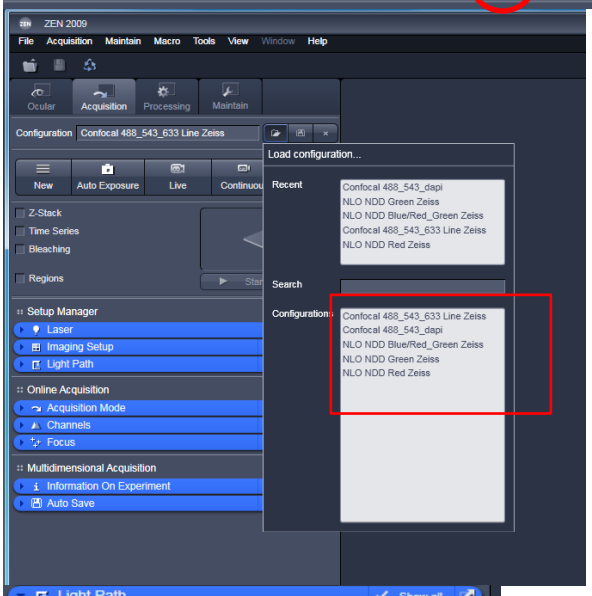


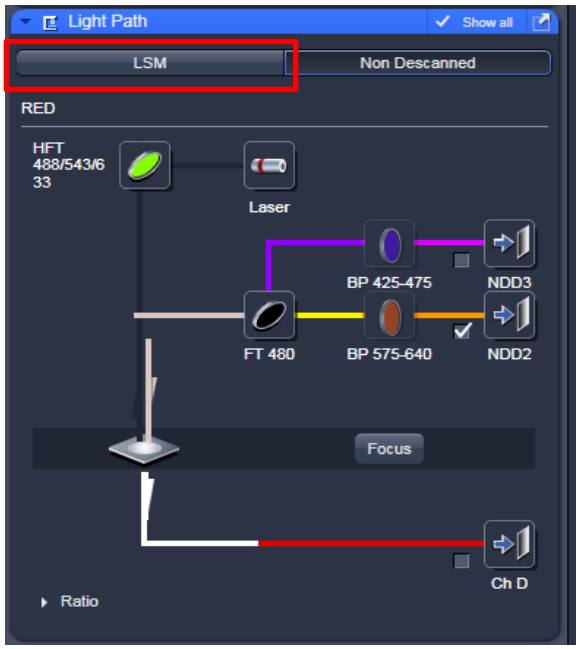
8. CONFIGURE THE BEAM PATH

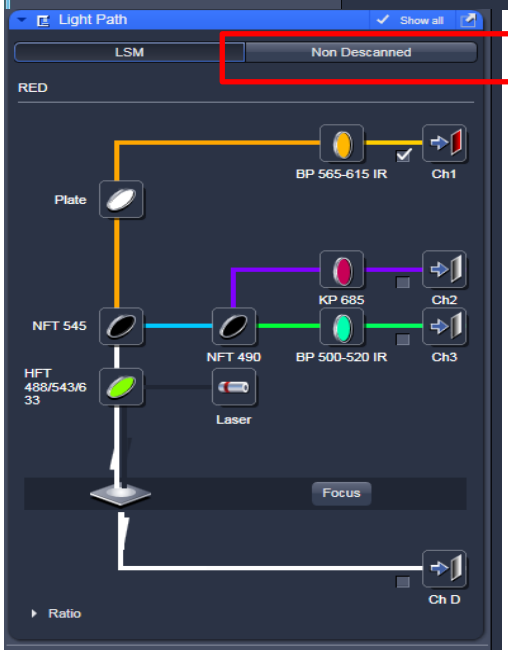
Click on the folder icon under to add or change an experiment **Configuration**. This allows you to load predetermined configurations for the light path and for tracks used during acquisition.



From the opened pull down menu, select the cofiguration that meets your needs. A custom configuration can also be written and saved to the list through setting the **Light Path**. Any method that has NDD in the name uses the NDD detectors, and should only be selected if you are using them.





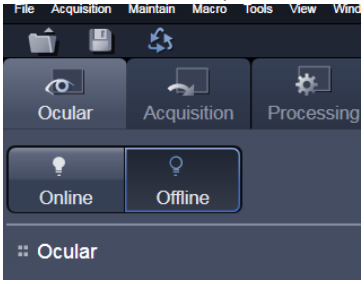


Examples of Light Path configurations using the standard confocal detectors, LSM, and the Non Descanned Detectors, NDD.

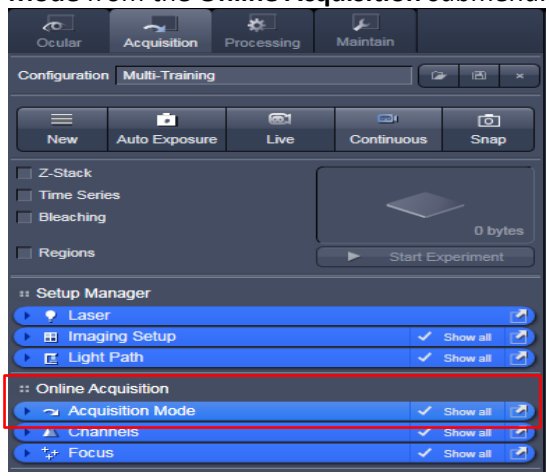
The light path is initially difficult to understand, and you should not write your own methods until you do understand it. Please ask staff for assistance in writing a custom method, and they will explain the positions of dichroic mirrors and emission filters.

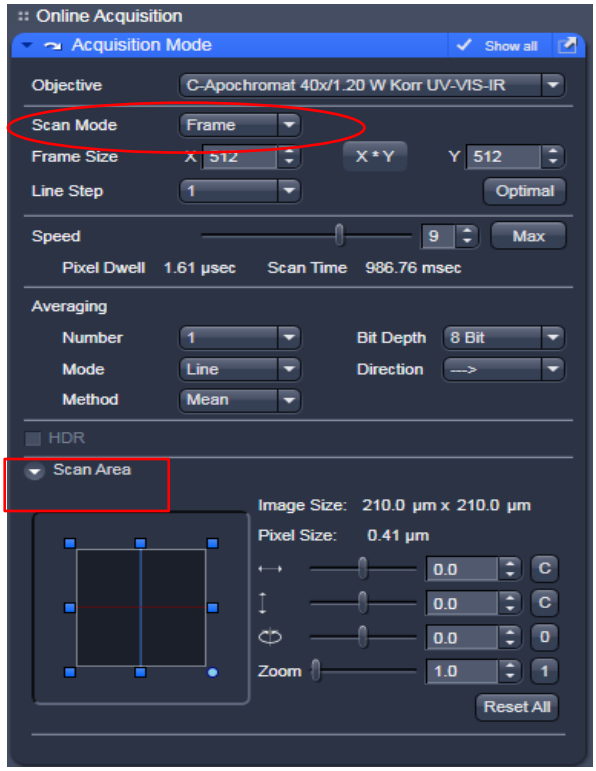
9. SET THE PARAMETERS FOR SCANNING

- **Before any scanning, <u>turn the filter cube turret to Scan (1)</u> and push in the manual slider on the right side of the microscope (see picture page 12).**
- In the **Ocular** tab, select **Offline** to switch from eyepiece to software.



- In the **Acquisition Tab**, click on **Acquisition Mode** from the **Online Acquisition** submenu.





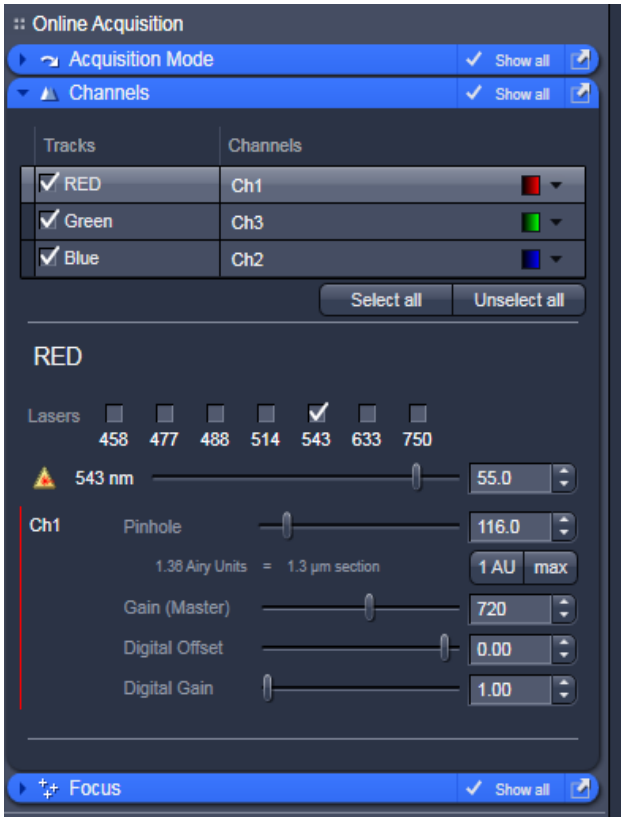
- Choose your objective from the pull down menu.
- In the **Scan Mode** menu icon, select **Frame.**
- Select the frame size by clicking on one of the predefined selections, define your own, or click on **Optimal** to select the appropriate frame size for your objective N.A. and λ. Pixel size is very important for successful imaging. Pixels smaller than optimal result in oversampling, more photobleaching, and longer scan times. Pixels larger than optimal produce images with reduced spatial resolution. High resolution may not be important to you – you decide. Remember that more pixels will result in a larger image file.
- Use the slider to set **Scan Speed**. Use a fast speed to focus on your sample, center it, and do other basic adjustments. Use a slower speed for scanning. Fast scanning produces a lot of undesirable noise in the image.
- Select the **Data Depth** as 8 bit or 12 bit. 12 bit is recommended for publication and quantitative imaging.
- Set **Scan Direction** as unidirectional or

Scan Area: The default zoom position is **1**. With **Zoom** set at 0.7, you are using the entire scanning field. With zoom >0.7, the scan area is decreased, but the frame size (number of pixels in x,y) remains the same. Thus the pixel size is smaller. Click **Optimal** under **Frame Size** to readjust the pixel size. The red-blue box representing the scan field can be moved by dragging it with the mouse or clicking the **Offset** arrows. Pressing **Reset** re-centers the scan field.

bidirectional. Bidirectional is twice as fast, and is mostly used for live sample scan.

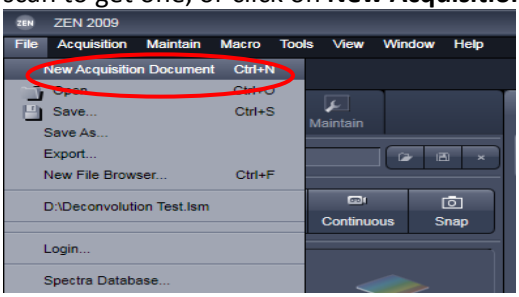
- Set **Scan Average**, which will improve the signal to noise ratio. You can avg. line by line (smoother image) or frame by frame (less photobleaching). You can either average the number of scans you choose, or sum them for weak signals.

- Select **Channels** from the **Online Acquisition** submenu**.**
- Set the **Pinhole** size to 1 (Airy Unit) by clicking on the **1.** Pinhole adjustment changes the optical slice thickness, the z thickness from which signal is collected. When collecting multichannel images, adjust the pinholes so that each channel has the same optical slice thickness. This is important for colocalization studies.
- Set the **Detector Gain,** for each channel, between 600 and 800.
- Make sure a laser is checked for each channel (clicking on each channel name will display the **Excitation** pane for each channel. Select a usual Transmission % to start (488nm – 5%, 543nm - 50%, 633nm – 20%, and the Chameleon laser – 2%).
- Select **Snap** to acquire a single image.
- Select **Continuous** for continuous scanning with the selected scan speed.
- Select **Stop** for stopping the current scan procedure.
- The best settings for gain, offset, and laser power are determined while scanning in continuous mode at a medium speed, and adjusting the sliders to optimal positions while using the range indicator from the Color Palette window.

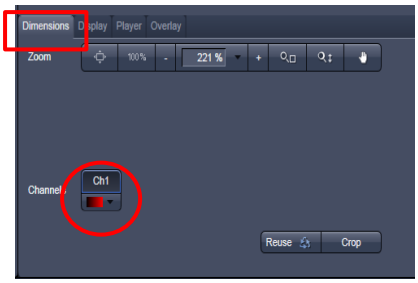


10.OPTIMIZE IMAGE ACQUISITION

If you do not already have an image window open, do a fast scan to get one, or click on **New Acquisition Document.**

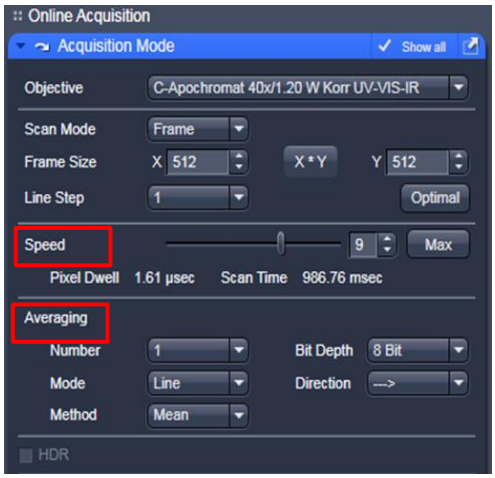


From the bottom panel of the image window, select **Dimensions.**

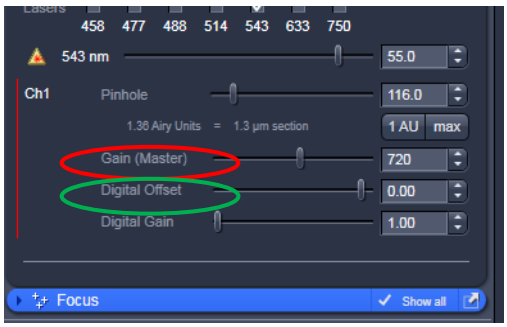


Then click on the display indicator below the channel indicator. This will display a list of possible choices, choose **Range Indicator**. This false-color LUT colors saturated pixels red (255 intensity value for 8 bit and 4095 for 12 bit images) and black pixels (0 intensity value) are colored blue. This will help you set laser power, gain, and offset correctly so that there are almost no oversaturated red pixels and only a few blue pixels.

Set the **Scan Speed** to a medium speed, around 6-7.
To reduce noise in the image, you can reduce scan speed, and/or use average successive scans (**Mean**) by Line or Frame. To increase signal you can **Sum** successive scans or increase laser power or detector gain.

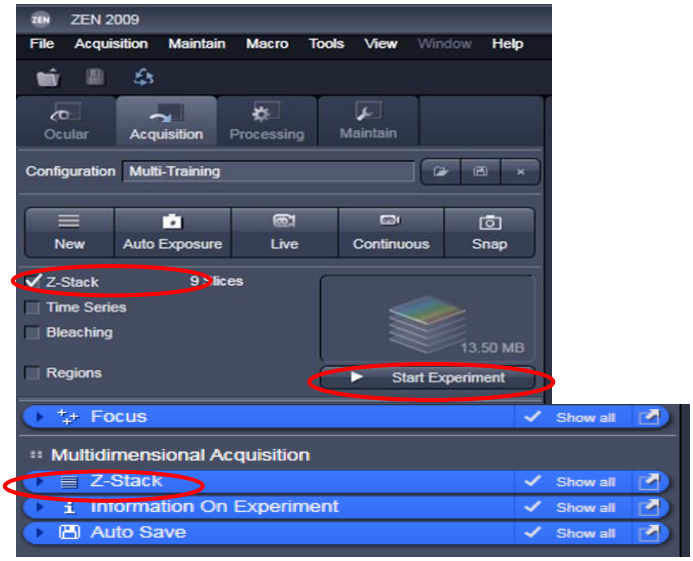


Click the **Continuous** button, which scans continuously. Set the **Detector Gain** (Master) to 800, and then adjust the laser power slider until you do not have any saturated pixels. Next, set the **Digital Offset** with the slider until you have a few blue pixels. When you are finished with your adjustments, stop the continuous scan by clicking on **Stop**, and then collect an image by clicking on **Snap.**



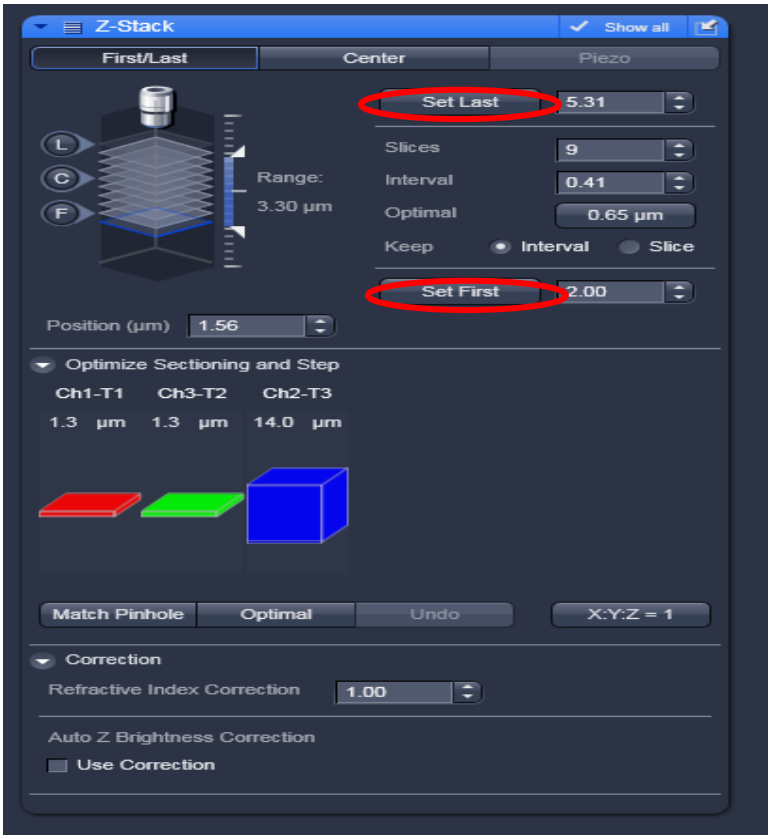
11. Z -STACK ACQUISITION

Select **Z Stack** in the **Scan Control** Window. This will insert a menu in the **Acquisition Tab** in the **Multidimensional Acquisition** submenu, which is circled.



To set the Z scan region**,** Click on **Continuous** or **Live** and focus to a lower position of the sample where the Z Stack is to start. Click **Stop**, then **Set First**. Repeat this procedure, focusing to the top of the sample and **Set Last.**

The **Interval** is the distance the motorized objectives will move with each step. On this microscope, the motorized objectives will only move in the up direction during a z stack. To determine the ideal interval size that will collect the most information without oversampling, click on **Optimal,** below the Interval number which is circled.



Click on **X:Y:Z = 1:1:1** to set the Z interval in such a way that the voxel has identical dimensions in x, y and z. This is sometimes important in 3D image reconstruction.

Refractive Index Correction is important if there is a difference in the refractive index of the objective immersion medium (n) and the mounting medium of the specimen (n'). Use Ratio = n/n' to determine the proper setting with the slider.

Auto Z Brightness Correction allows you to set different gains, offsets, and laser power at different positions of the stack and linearly interpolate between them.

Click **Start Experiment,** in the Acquisition Tab**,** to begin the Z Stack.

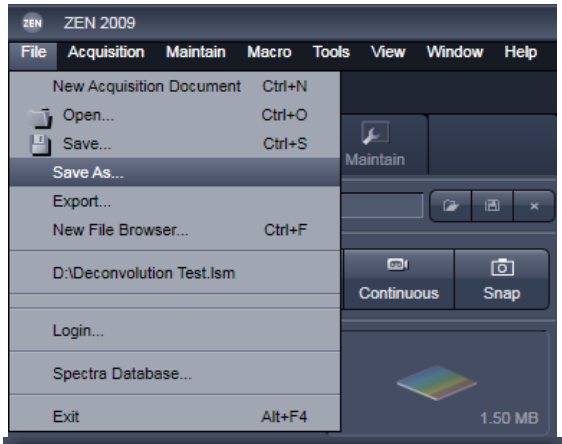
12.USING THE NDD DETECTORS-For Two photon

These instructions come from a Zeiss Applications Specialist. They cover the process from turning on the Chameleon laser to scanning your sample. Some pieces of these instructions have been covered in this guide already.

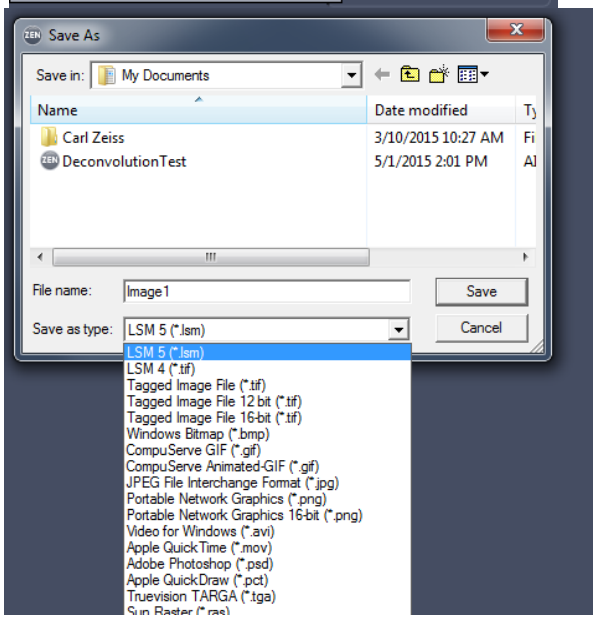
1. Normal startup of the system, plus turn the Chameleon from “Standby” to “On” via the key on the Chameleon power supply
2. Select the “Laser” button and click on Chameleon.
3. Click “On”. This opens the shutter on the Chameleon, and turns the AOM on for attenuation control of the laser.
   If you need to change the wavelength, click “Modify” in the laser window, then type in the wavelength desired and click “Store”.
4. After locating your sample as you normally would with the “Vis” button and proper filters, go back to the LSM mode, and open the Configuration window. Here, about half of the way down, you will see two tabs, “LSM 510”, and “Nondescanned”. Click “Nondescanned”
5. All of the configurations that use the NDDs have ndd in their descriptions. You have the choice of single or one multitrack. You can choose one of those, or make your own.
   - To make your own, click on the 4 position drop-down near the middle of the diagram, which includes 3 dichroic filter choices, and “HBO Mirror”
   - Choose one of the dichroics, and the corresponding filters for the 2 channels will be listed. Choose the channel that you want to use with a check mark.
   - Make sure that the primary dichroic in the upper left hand corner is on “KP 650”.
6. Click your laser to on in the laser window in the diagram. It should be at relatively low power when using a high NA objective when in the 750 to 800nm range, say 1 to 5%, and incrementally higher power away from this center of the range. Lower NA objectives pass less power, so will also require higher power settings.
7. Make sure the NDD dichroic is in the 5 position on the reflector turret at the front of the microscope. Select the 5 position to send the emission light to the NDDs.
8. If the HBO lamp is on, push the black slider that sticks out to the back of the tube that connects the lamp house to the closed position.
9. Make sure that the black slider on the right side of the microscope is in the middle or outermost position
10. Turn the lights off, and keep light minimally in the room. Cover the sample stage with the black vinyl cloth with Velcro strip. Consider turning the monitors off or covering them.
11. Go to scan control and use it as you would for normal imaging, keeping in mind that there is no pinhole.

13. SAVE

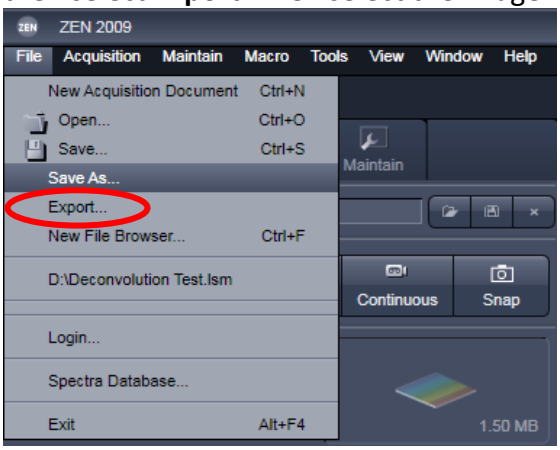
Click on the **Save** or **Save As** button in the Image window or in the **File** toolbar of the **Main** menu.

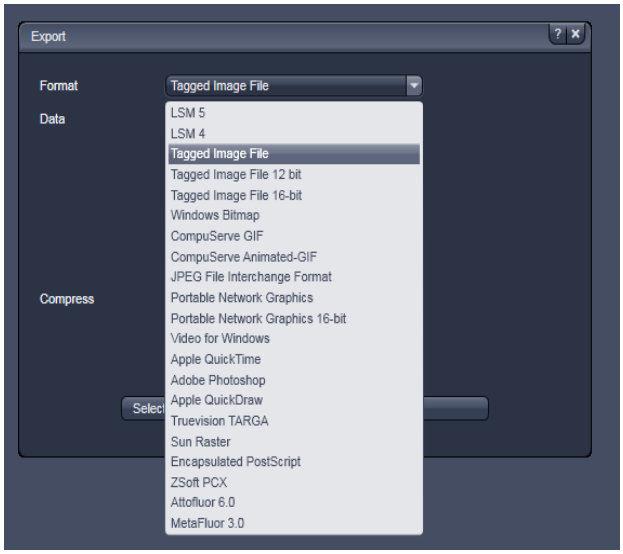


In the opened **Save As** window**,** select a file format. LSM 5 is the current software file format and LSM 4 should be openable by AIM or LSM software programs. **All files should be saved to the E drive.** Afterwards, enter a name for the file, type in any description and notes. Make sure the appropriate file is highlighted and click **OK.**



To save an image in a different data format, open the .lsm image, choose **File** from the main menu, then select **Export**. Then select the image **Format**.

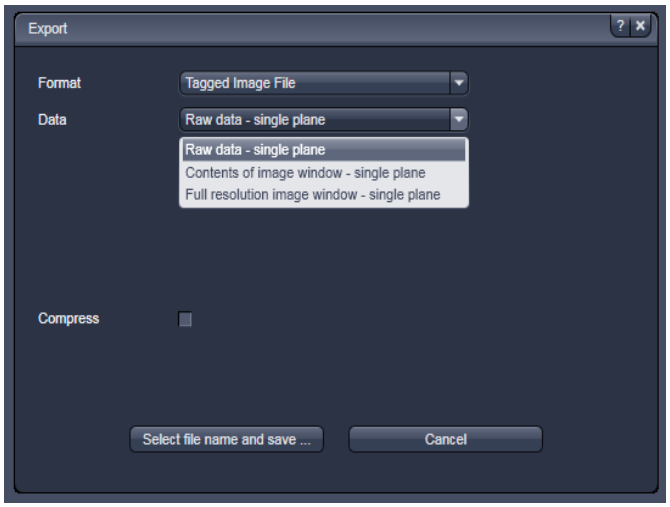




Raw data: single plane or series (single image or time series/z stack with no LUT or overlays)

Contents of image window: single plane or series (single image or time series/z stack as viewed on the monitor – easy way to save the panel image)

Full resolution image window: single plane or series (single image or time series/z stack with the pixel resolution as collected, 512 x 512, 1024 x 1024, etc.)



Be sure to save all images to the C Drive in a folder with your first and last name. This is only temporary storage. Transfer your files to external storage as soon as possible.

14. SHUTDOWN

If someone is signed up after you within two hours, **do not** turn off the system, but do turn the Multiphoton laser to standby with the key, then turn it off in the laser panel. Turn the argon laser to standby in the laser panel. Close the software, and log out. If no one else is signed up to use the system for 2 hours, shut the system down in the following order:

- Save your images to your database
- Click on the file button in the **Main Menu** of the ZEN software and then click **Exit.**
- In the pop up window, shut off all lasers that are running.
- Log off and shut down the computer.
- **Wait until the argon laser fan has switched off** – about 5 minutes. The fan is on top of the laser cart, and you can hear it running and feel the air flow. It is important for the life of the laser that it not be turned off prematurely. If you turn off the System/PC switch prematurely, it will shut the fan off.
- If the Chameleon multiphoton laser was used, turn the key on its front panel to standby.
- Turn off the Components and System/PC switches, one at a time (No. 3).
- Turn off the main power switch on the laser cart, turning 90 degrees counter-clockwise, to horizontal (No. 4).
- Record the Bulb time and time of day in 24 hour format in the sign-up sheet.
- Switch off the HBO 100 mercury lamp switch (No. 1).

15. SPECIFICATIONS FOR PUBLICATION

The system is a Zeiss LSM 510 NLO with a Coherent XR multiphoton laser. It has three standard Photomultiplier Tube (PMT) detectors, two Non-Descanned Detectors (NDD), and a transmitted light detector. The attached microscope is a Zeiss Axioscop 2 FS MOT with an upright configuration.

It has the following lasers and objectives:

LASERS

1. Argon (458,477,488,514nm)
2. Green HeNe (543nm)
3. Red HeNe (633nm)
4. Multiphoton Coherent Chameleon XR tunable pulsed laser (705– 980nm)

OBJECTIVES

10x /.3NA Fluar WD 12.5mm
25x/.8NA DIC LCI Plan Neofluar (for use with/without coverslips, water or glycerol immersion)
20x/0.5NA Acroplan WD 1.9mm, water dipping
40x/0.8NA Acroplan WD 3.6mm, IR corrected, water dipping
63x/0.9NA Acroplan WD 2.0mm, IR corrected, water dipping
63x/1.4NA DIC Plan Apochromat WD .18mm, oil immersion

16. ADDITIONAL INFORMATION ON SOFTWARE OPTIONS

This manual is only a quick start guide and does not cover many additional and useful features in the software. You can also access information on software features through the **Help** section, accessed from the **Main Menu.**