Zeiss LSM Confocal Microscope SOP

START THE SYSTEM

1. Switch on the **Main** switch
2. Switch **System/PC** (provides power to the computer and allows the use of the computer and ZEN software offline)
3. Turn ON computer (wait till letters appear on the screen, means fully loaded)
4. Switch the **Components** switch to **ON** (this starts the other components and the complete system is ready to be initialized by the ZEN software)
5. Turn on the Ar-ML laser turn the key (to 3 o'clock) The laser is automatically kept in standby mode for 5 minutes to warm up.
6. Switch the idle-run-switch to run (flick up) (It takes about 50s until the laser has reached the set output power)
7. Turn on the X-Cite lamp (for reflective light illumination, fluorescence) and switch the intensity to one. (This is always turned down after every user!)

START THE ZEN SOFTWARE

1. Login: .\LSMUser and password: qblood
2. Double click the **Zen 2009** icon on the WINDOWS desktop (starts the Carl Zeiss LSM software)
3. Click ONCE on the **Start System** button on the LSM 710 Startup window screen
   (To acquiring new images)
4. After the start up, the ZEN Main Application Window opens and you can click **Online** (this allows the light to go to the microscope) under the **Ocular** tab.

FIND YOUR SAMPLE

1. Click **Ocular** tab (control for direct observation through the eyepieces)
2. Pull back the head of the microscope carefully and place your sample (slide faced down). Bring down the "tilt back arm" piece to start viewing.
3. Click **Online** (to use the microscope)
4. Select **BRIGTHFIELD**
5. Under **Ocular** tab click the Empty Position and select your desired objective.
6. Using the joystick move your sample to correct location and focus on your sample with the coarse and fine focus knobs. (Knobs are below the stage and on the side of the microscope)
7. Select a filter to view fluorescent image. (DAPI, GFP, DSRED)
   -once you have selected your filter the fluorescence shutter should automatically turn ON and the shutter to the transmitted light should close.
8. Make sure you click shutter OFF after you are ready to acquire

CONFIGURE SETTINGS

1. Click **Acquisition** tab
2. If you are going to use lasers 561 or greater you need to turn the lasers on. Click the **show manual tools**, click on the **Laser** tab. Turn on the lasers that you need.
3. Click **Smart Setup**
4. Click arrow under **Dye**
   -Choose your dyes which you used to stain your sample
5. Under Proposals choose the configuration that best suits your sample
   -Fastest: for single labeling, simultaneous scanning

(Pro: fastest mode, Con: possible bleed-through between dyes)

-**Best Signal**: for multiple labeling, sequential scanning
(Pro: reduces bleed-through due to turning a laser and detector one at a time, Con: acquisition image is slower) BEST RESULTS!

-Best Compromise:

-Linear Unmixing: automatically set the system in the lambda mode

6. Click **Apply**
-software will automatically configure the parameters for the chosen dyes.

PREPARATORY IMAGE

1. Go to **Channels** window under acquisition tab and select a track optimize (if doing multi-tracks optimize one at a time)
2. Set **Pinhole** to 1 Airy Unit
-Click 1 AU (for best compromise between detection efficiency and depth discrimination)
very important when testing co-localization; therefore, adjust pinhole so that each channel has the same optical slice thickness
3. Click **Auto Expose** (automatic pre-adjustment of gain and detector)
4. Click **Live** (continuous fast scanning)
-Optimize your image
-Under "Dimension" tab go to Channels and click the color box (this will show you the amount of saturation (red pixels) and black (blue pixels)
-**Gain (Master):** detector sensitivity
-**Digital Offset**: default is "0" but adjust the blue pixels (background)
-**Digital Gain**: default "1" signal amplification
5. Now click on your next track and uncheck the track that you just optimized (make sure you are adjusting the right track!)
-Repeat Step 4 and set the AU to match you previous track)
6. Turn ON the two tacks (by checking both boxes)
7. Under **Acquisition Mode** select your parameters
-**Frame Size**: click Optimal for optimal resolution, recommend 1024x1024
-**Scan Speed**: 7 (fast speed reduces scanning time, lower speed improves signal-noise ratio)
-**Bit Depth**: 16 Bit (intensity measurement)
-**Average Number**: 4
8. Click **Snap** (records a single image)
9. Save image on D: Drive
-(D:) → DATA → your folder
-ALWAYS SAVE AS LSM 5 (*lsm)

MAKE Z-STACK (optical sectioning)

1. Use your saved 2D image
-Press the "**Reuse**" button under Dimension tab that's on the bottom of the image box
(This allows you to use the same parameters and settings from previous image)
2. Click **Z-Stack** box under the scan control window
3. Open Z-Stack tab under "Multidimensional Acquisition"
4. Before setting your first and last turn OFF one track (it will be faster to scan)
5. Click **Live** (always press Live before moving the knobs and setting your first and last)
6. Using the "Keypad Controller" scroll down with the fine focus until you barely see your sample

then press "Set First" then scroll to the top of your sample and press "Set Last" (always go to bottom to top!)
7. Click **Stop**
8. Click **Optimal** (this will set the number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter)
9. Turn **ON** all you tracks!
10. Click **Start Experiment**

MAKE A 3D OF A Z-STACK
(Several two-dimensional optical sections at different focal planes, a XYZ image stack)

1. After obtaining your z-stack you can go to the Process windows on the left monitor and choose visualization 3D Projection
2. You will see a rendering of your Z stack. Click and move your mouse in this window to view different angles of your image.
3. You can optimize the view of your 3D image to maximum, average, transparent or color-coded
4. If you want to save this projection, press the Apply button (bottom right of left monitor) and a Projection of Series image will appear in the Experiment tab.
5. Click **Create Movie** box if you want to make a movie of the rotation of your 3D projection
6. Set the start and end angles by dragging your image to the desire angles or by inserting numbers
7. You can also optimize the slice thickness, the number of frames, and different modes
8. Press **Apply** to create movie

MAKE A TILE SCAN

Entire sample:
1. Follow the same steps for configuring and optimizing your sample (tile scan is created around the starting position of your image- therefore, center your sample)
2. Click **Tile Scan** under **Acquisition** tab
3. Under **Multidimensional Acquisition** put down the tile scan tab and click **Center grid**
4. Set your dimensions you desire
   -number of tiles in horizontal and vertical direction (ex: 4x4, 5x5, etc.)
5. Click **overlap** to 20% (this creates a tile scan with overlap that stitches single 2D images or Z-Stacks 3D)
6. If you have more than one track make sure you turn all of them ON
7. Click **Start Experiment**

From certain region:
1. Under the **Tile Scan** tab click **Bounding grid**
2. Using **Add** mark the regions of interest (ex: mark the four corners of the tumor that's inside your brain tissue) this will define the dimensions of your tile scan.
3. Click the box in the regions of your sample the click add, then go to another region of your sample with the joystick and click add, after you mark all the regions (extreme edges of your sample) you want **Start Experiment**

Fast Overview:

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1. Under **Tile Scan** and **Bounding grid** click **Scan overview image** (this will open a new window were you can select the number of tiles, the objective, and zoom factor)
2. After you define your properties click **Scan** (this starts the tile scan that navigates your sample)

*TIPS: Since the objectives are not flat and have fall over in the edges is best to do a **zoom of 1.2** or more so when the image is scanned it won't have grid boxes. Also ALWAYS do an **overlap.** If you don't then you will have a hard time stitching your large image at the end!

HOW TO IMAGE AT 63X OIL

When using this objective you have to be very careful not to crash it into your slide because it has a very small working distance. If you are using the 63x or 100x oil immersion objectives you should NOT switch to a lower objective because you will contaminate the other objectives with immersion oil. If you are on 63X oil and have to use a lower objective you must take off your slide and properly remove all oil from the coverslip! This is an oil immersion objective so it is only used for stained and fixed samples. NO LIVE IMAGING!

1. Always start with **5X** objective to focus your sample
2. Go to **10X** and focus
3. Go to **20X** and focus
4. Go to **63X oil** and set your objective to **Load Position** from the remote keypad
5. Carefully pull back the "tilt back arm" so you can remove your slide
6. ONLY STAFF: Place a very small drop of immersion oil (ONLY Zeiss Immersol 518F!) onto the top lens of objective (NOTE: Using a large amount of oil will loosen the lens of the objective which will decrease image resolution and the life of the objective)
7. Place your slide on the stage and bring down the "tilt back arm"
8. On the remote keypad set the objective back to viewing mode
9. Focus your image (IMPORTANT: Only use fine focus for high mag objectives!)
10. After your done using the 63X oil:
    -Set to loading position
    -Pull back the "tilt back arm"
    -Remove your slide and clean all the oil from the coverslip! Very important because if you leave some oil and go use another microscope you might contaminate it and get bad images. DO <u>NOT</u> clean objectives! Only Staff

SHUT DOWN

Please check Google calendar if another user is going to come in within an hour! If there IS a user coming in then just switch the Argon laser on standby mode (toggle switch DOWN)

If you are the last user:

1. Move the objective to an empty position
2. Switch the Argon laser on standby mode
3. Turn the Argon key OFF (from 3 o'clock to 12 o'clock) WAIT FOR 10min TO COOL BEFORE SHUTING OFF! Meanwhile…
4. Backup your data and save your images in your own folder on the LSM710 WORKSTATION D: Drive ONLY SAVE AS LSM5 (.lsm)!
5. You can also download your data onto a USB drive or external hard drive
6. If you used the 561nm laser turn it OFF (in the laser control window)

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7. Exit ZEN2009 software
8. Log Off your user account and Shut Down the computer from the Start Menu
9. After the Argon laser is cooled (you can feel the fan on the back of the LASOS box) if it's not blowing you can switch off the **System/PC** and the **Component** switch, followed by the **Main** switch.
10. Power Xcite lamp to zero and turn OFF.
11. Please clean after yourself! If you used oil make sure you clean and remove any oil from your slides! (DO NOT CLEAN OBJECTIVES! Monica is the ONLY person who can clean objectives!)
12. Remember to record your time in the sign-in sheet and note any problems you encountered. If there are any problems with the Confocal please report to Monica for assistance. Thank you!

ANALYSIS WORKSTATION

MAKING A STICH ON A Z-STACK

1. Turn on the ZEN 2009 and click on **Image Processing**
2. Under Processing tab click **Copy**
3. Then click **Subset** function
4. Open your image that you selected under file → open…
5. Click the **Z** tab (to select the slice of interest) to choose the z-plane Start and End select **Apply** (ex: you have 20 slices of an imaged z-stack and you only like slice 9 thru 11, selected them Start: 9 and End: 11. If you only like slice 9 then insert 9-9)
6. If you image has tiles then you would ask it to have all the tiles! Start 1 and End:(#of end tiles)
7. This will create a new image of just the one z-plane.
8. You can then stitch this new image by clicking on the **Stitch** and **Apply**

LIVE CELL IMAGING

(8 channel Automate Scientific programmable perfusion system. This type of imaging is very critical and needs to be SUPERVISED AT ALL TIMES! If not careful the valves can come off or the chamber can overflow! This can flood the microscope and cause damage to the objectives and the microscope itself!)

1. Turn on the Rainin peristaltic pump and flush the all the channel lines thru with HEPES (depending on how many solutions you are going to be using flush that many lines. Ex 4channels: Ch1-HEPES, Ch2-CCH, Ch3-PE, Ch4-Caffeine) You can select each channel by pressing the number on the Manual valve command controller.
2. **Stop** the pump
3. Insert one channel line per solution tube.
4. Pull back the "tilt back arm" and set objective in **load position**
5. Replace the mounting slide stage with the live cell adaptor plate.
6. Carefully set your specimen chamber on the stage and slide the security locks on the side

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7. Connect the fluid valve to the right side of the chamber- input port (make sure that its properly connected so the fluid won't come out)
8. On the left side of the chamber- export port, mount the vacuum syringe down with clay (make sure that you hold down the tubing with tape! Remember that the stage is going to be moving so you don't want the tubes hitting the back of the microscope. Also make sure that the perfusion chamber is not overflowing and that the water suctioning flow is consistent- important when analyzing your data)
9. Start the pump (flow going counterclockwise) and keep an eye on the perfusion chamber to see if the suctioning is working and not overflowing
10. Now that the perfusion channel is running you can start the **ZEN2009**
11. Go to O**cular** and click Online...focus your image with the brightfield (5X→ 10X→ 20X)
12. Click on **Acquisition** tab and select the **Smart Setup**
13. Select the fluo-4, and apply
14. Start adjusting your **pinhole** (1AU), **gain master**, **digital offset**, and **digital gain**
15. Click the box that says **Time Series** this will time your live imaging cycle.
16. Once the image is focused and optimized click **Start Experiment**
17. Next steps vary with each experiment: (remember that you have to manually change the channels by pressing the number on the Manual valve command controller)
    1. HEPES 5min→ record cycle___
    2. CCH 10min→ record cycle___
    3. HEPES 10min→ record cycle___
    4. Caffeine 5min→ record cycle___
    5. HEPES 5min
    6. Start bubble___ (you lift the channel line out of the solution and let air in and then place in solution again)
    7. End bubble___ (record when the start of the bubble gets to the perfusion chamber
18. Click **Stop** on the experiment run

*Now that you have acquired a **Time Series** of your live image you want to properly clean up! This is also very important because if you are not careful the perfusion chamber can overfill.

1. Stop the Rainin peristaltic pump
2. Wait until all the fluid from the perfusion chamber is suctioned
3. Put the objective on **Empty Position**
4. First remove the input fluid valve and then remove the export port vacuum syringe
5. Remove the perfusion adaptor insert and replace with the mounting slide stage.
6. Now place the Input fluid valve on an empty bottle and start Flushing each channel with water (manually flush 1,2,3, and 4 channels one at a time)
7. After you flush with water you need to run the channels without anything just air!
8. Turn off the Rainin peristaltic pump, the Manual valve command, and the aqua lifter.
9. Turn off the ZEN2009 system the same as you would when imaging!