**LSM 710 Confocal Microscope Standard Operation Protocol**

**Basic Operation**

**Turning on the system**

1. Check Compressed Air supply for the air table
2. Switch on Main power switch ①
3. Switch on System / PC power button ②
4. Switch on Components power button ③
5. Switch on X-Cite fluorescent lamp ④
6. Sign on log sheet (and record the Fluoro Start time from X-Cite lamp)
7. (Skip this step if 458/488/514nm (yellow to green) excitation is not needed) Turn on Standby/On key switch for argon laser ⑤ and Flip the Run-idle-switch to Run for argon laser ⑥
8. (Skip this step if two photons excitation is not needed) Turn On Standby/On key switch for MaiTai laser ⑦
9. (Please keep MaiTai laser off if you do not use it)
10. Turn On Computer ⑧
11. “Green” button on “MP-285” stage controller ⑨

**Starting the ZEN software**

1. Log on computer with “LSM User”
2. Double click the ZEN 2011 icon on desktop
3. Choosing Start System for acquiring new images
Setting up the microscope and Locating the specimen

1. **Use 10X objective first** by turning the objective turret manually (wait several seconds for the automatic objective position adjustment) and lower the stage by using coarse focus adjustment knob.
2. Put the sample on the mount on the stage (coverslip face up)
3. Move the area to be observed right under the objective lens (in X – direction by moving the sample mount or stage controller and in Y – direction by the stage controller)
4. Turn the shift knob to move the objective into the WORK position.
5. In the Zen software, click on the **Locate** tab to access the microscope controls and press the **Online** button
   - click on **DAPI** tab for blue emission channel observation
   - click on **GFP** tab for green emission channel observation
   - click on **DsRed** tab for red emission channel observation
   - click on **BF** tab for transmitted light observation
6. Focus with coarse and then fine adjustment knob
7. Move the desired area to the center of view for further magnification with higher power objectives or for later image acquisition
8. Select a suitable high magnification objective by turning the objective turret manually (wait several seconds for the automatic objective position adjustment). Add corresponding refracting medium needed and do not move the stage in any Z-direction if you have already used 10 X objective to focus.
   **Note:** Please make sure there is **no oil and water** on your slide when you are using any air objective and **no oil** on your slide when you are using water objective. Any inappropriate medium will damage these objectives)
9. Turn the shift knob **slowly and carefully** to lower the objective into the WORK position. Focus with the fine adjustment knob only.
   **Note:** Please be careful when you are focusing the sample. You may damage the objective and break your sample you keep moving objective down!
10. Check all the emission signals from different fluorophores in your sample. Center the view of interest and then click **Offline** in the Locate Panel to turn off all the light and go to **Acquisition** tab.
Setting up the Scanning Track/Channel

Method 1 (only applied to single photon laser)

1. In Acquisition tab, click on the Smart Setup button
2. Click on the Arrow in the Dye list and choose the fluorophore in the specimen, add one by one down the rows
3. Choose the Pseudo Color according to you need
4. In Proposal, choose
   - Fastest (simultaneous scanning) for fast live image acquisition but with (if any) excitation/ emission crosstalk among your fluorophore, or;
   - Best signal (sequential scanning) to minimize crosstalk among each fluorophore but slow image acquisition or;
   - Best Compromise (combined mode scanning) to simultaneously scan non-cross talking fluorophore to speed up while other cross talking fluorophore are scan sequentially when necessarily
5. Press Apply

Method 2

Load a predefined configuration of combination of dyes from the Load configuration list box.

Method 3

Open any previous image with desire setting and click Reuse button in Dimension panel.

Method 4

Setting up the configuration manually
Optimizing scanning parameters

1. Select one Tracks in the Channels tool
2. Select Live for continuous fast scanning
4. Set the Pinhole to 1 AU (Airy Unit). For colocalization studies, adjust the pinhole of each channel to the same Optical Slice Thickness.
5. To get optimal intensity and background signal, adjust
   a. Increase Detector Gain (Maximum of 800) until a few red pixel (indicating saturation) appear in the image;
   b. Increase the Laser Power (too high cause bleaching), if increasing detector gain cannot achieve the saturation or desired intensity;
   c. Fine-tune the focus with the fine adjustment knob to the brightest or preferred z-position. And then adjust the detector gain and laser power to optimize the signal intensity
   d. Decrease the Digital Offset to reduce background signal until the desired background region is filled with blue pixel
   e. Increase Digital Gain to increase signal amplification if necessary.
6. Stop the Live scan process and uncheck the Range Indicator.
7. Repeat step 1-6 for other tracks one by one
8. Check all the channel boxes when finish adjusting.
Setting the scanning parameters

1. Select the Acquisition Mode tab in the middle column.

2. Select a suitable Area and Zoom Power to capture image of interest (or use Crop function in Dimension under the image container after a Live image).

3. Choose a Frame Size (change with Pixel size, thus resolution). 1024x1024 usually produces good results for general propose. Click on the Optimal button for best resolution which depending on objective N.A. and λ.

4. Adjust the scan speed with the Speed slider. 6 or 7 usually produce good results. For weak signal samples with high background noise, choose Speed 4 or 5.

5. Select the number of Average. Averaging (usually in 2 or 4) improves the image by increasing the signal-to-noise ratio but it also increases the scan time.

6. Select the dynamic range of 8 or 12 or 16 Bit (per pixel) in the Bit Depth. 12 or 16 Bit is recommended when doing quantitative measurements.

7. Other setting can be kept as defaults (as in the right) which will be good enough for general propose.

8. Click Snap to acquire a single frame (multi-channel) image.
Storing and exporting image data

1. To save the acquired or processed images, click on that image to highlight it and then click on the Save Data button in the Images and Documents panel in the right or Click Save or Save As button in File menu.

   Warning Icon indicated unsaved or modified image

2. Create or choose your own folder in D:/user, enter a file name, select format as LSM 5 and click on the Save button

3. After saving the raw data as LSM5 format, Image can be export as various Image format and series images can be export as video. Go to File > Export, and choose the Format and Data as followed. Adjust Frames per seconds if necessary. Click Select file name and save and save in your own folder.

<table>
<thead>
<tr>
<th>Single Frame</th>
<th>Format</th>
<th>Date</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tagged Image File</td>
<td>Raw data – single plane (without any overlay graphics)</td>
</tr>
<tr>
<td></td>
<td>JPEG File Interchange Format</td>
<td>Contents of image window – single plane (with overlay; as displayed as in image container; compressed)</td>
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<tr>
<td></td>
<td>Portable Network Graphics</td>
<td>Full resolution image window – single plane (with overlay; as displayed as in image container; uncompressed)</td>
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<table>
<thead>
<tr>
<th>Series (Gallery of images; Time series movie or 3D animation)</th>
<th>Format</th>
<th>Date</th>
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<tbody>
<tr>
<td></td>
<td>Video of Window</td>
<td>Raw data – series (without any overlay graphics)</td>
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<td></td>
<td>Apple Quick Time</td>
<td>Contents of image window – series (with overlay; as displayed as in image container; compressed)</td>
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<td>Full resolution image window – series (with overlay; as displayed as in image container; uncompressed)</td>
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Scanning a Z-Stack

1. Check the Box for Z-Stack in the main tools area.
2. Open the Z-Stack tool panel by clicking on the Z-stack tab.
3. Choose a channel that have signal throughout the interested volume and then Click on Live.
4. Use the focus knob to locate one end of the specimen and click on the Set First button.
5. Then use the focus to locate another end of the specimen and click on the Set Last button.
6. Stop the Live. Select all the channel needed for the Z-stack and then click the arrow for Optimize sectioning and Step.
7. Set the Z-stack Interval between each frame, users are suggested to set the interval less than the thickness of 1 Optical Section of the channel of the shortest wavelength (so that you can sample all the space in your specimen). Optimal value is calculated according to the emission wavelength, objective lens, and the pinhole diameter. Using the Optimal thickness mean double sampling (interval = ½ thickness of 1 optical section) and thus provide the best 3D image reconstruction.
8. For colocalization studies, adjust the Pinhole of each channel in the Channels panel to the same Optical Slice thickness (so that each voxel contain data from the same volume of the specimen).
9. Click on the Start Experiment button to start the recording of the Z-Stack.
10. After saving the LSM 5 format, a Maximum Projection images can be made. Image in all frames will projected onto a single plane to visualize all the signal for the specimen in 2D Click Create image to generate the projection.
11. A video of rotating 3D image can also be made. Activate the Series tap, select the rotating axis, number of frame and the rotation range (= Total Frame × Difference angle). Click Apply and the series images will be generated in a new tab. Go to File > Export, choose video for Windows and then choose a suitable series Adjust the playing speed by Frame per Second and save in your own folder.
Setting up Time Series Experiment

Check the Box for Time Series in the main tools area.

1. Open the Time Series tool panel by clicking on the Time Series tab.

2. Set the number of Cycle and time Interval between each frame. (The scanning of each frame is included in the countdown of the Interval, therefore Interval time should ≥ scanning time of one multi color frame.)

3. Set the channel and acquisition parameter if necessary and then click Start Experiment.

4. To quantifying changes in signal intensity after acquisition, click Mean ROI tab and create a ROI region with the drawing tool for your region of interest.

5. The intensity profile along the experiment duration will be shown in the graph while corresponding data will be shown in the table below.

6. After saving the LSM 5 format, video for the time series images can be exported. Go to File > Export, choose video for Windows and then a suitable series. Adjust the playing speed by Frame per Second and Select file name and save in your own folder.
Turning off the system

Please check if the equipment will be used / booked by other users. Please switch off the system if no one is using within two sessions (1 hr) afterward.

1. Turn off MaiTai laser in software (if it is ON)
2. Exiting ZEN Software, transfer data through the Faculty Core Facility network storage server and then Shut down PC
3. Turn key switch 90° anti-clockwise for MaiTai (2 photon) laser to Standby
4. Flip the toggle of Run-idle-switch of argon laser to Idle
5. Turn key switch 90° anti-clockwise for argon laser to Standby

Wait for 10 min to let Laser Fan OFF!!!

While waiting, please
- Remove your sample and clean the stage
- Clean oil objective (with lens cleaning tissue only but NOT Kimwipe)
  1. Remove residue oil from the objective lens by a dry lens cleaning tissue
  2. Clean the lens with a new lens cleaning tissue with 100% absolute ethanol
  3. Dry the lens with another new lens cleaning tissue
- Clean water objective (with lens cleaning tissue soaked with autoclaved Milli-Q water)
- Change objective to 10x;
- Record the fluorescent lamp hour and Sign on log sheet according to Actual usage time

6. Turn off X-Cite fluorescent lamp
7. Components button
8. System / PC button
9. Main power switch *(Only after Argon Laser Fan OFF)*
10. Compress Air (if necessary)