Zeiss LSM Pascal confocal microscopy system
Manual and reference
Lady Davis Institute room 320
LDI Zeiss Pascal manual and reference v0610 © Judith Lacoste, 2010. All rights reserved.
Refer to the Carl Zeiss Microscopy document entitled "Pasc_C1-8.pdf" for complete instructions.
Equipment overview

- Argon laser (fan on top)
- Transmitted light detector
- Condenser
- Halogen-Tungsten lamp (housing)
- Mercury fluorescent arc lamp (housing)
- FD, FA and ND sliders for fluorescent light path
- Microscope on/off switch
- Mercury fluorescent arc lamp (controller)
- Filter cubes selector
- Argon laser controller
- Optical fibers (FRAGILE)
- Confocal head
- Table floating section
- Shutter for mercury arc
- Shutter for Halogen-Tungsten lamp
- Fine/Coarse focusing knob

miacellavie.com Zeiss Pascal confocal LSM reference and manual v0610
## Start up procedure

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.</strong> Turn on the mercury lamp (white box named &quot;mbq 52 ac&quot; to the right of the microscope).</td>
<td><strong>2.</strong> Turn on the power bar (115151, located on the right back of the left table).</td>
<td><strong>3.</strong> Turn on the required lasers:</td>
</tr>
<tr>
<td><img src="image1" alt="Mercury Lamp" /></td>
<td><img src="image2" alt="Power Bar" /></td>
<td><img src="image3" alt="Lasers" /></td>
</tr>
<tr>
<td><strong>3a:</strong> Argon laser (458, 488 and 514 nm)</td>
<td><strong>3b:</strong> HeNe laser (543 nm)</td>
<td><strong>3c:</strong> HeNe laser (633 nm)</td>
</tr>
</tbody>
</table>
| - Turn on the black **power enable switch** (top right of the black box under the left table).  
  - Turn on the **key** (below the **power enable switch**).  
  - Let laser warm up until light (inside the Argon laser) turns from **ORANGE TO PURPLE**.  
  - Flick the **silver switch** up from "stand by" to the "**RUN**" position.  
  - Slowly turn the **knob** clockwise until the arrow points to the mark. | Turn on the key of the lower unit (white boxes sitting at the left back of the left table). | Turn on the key of the upper unit (white boxes sitting at the left back of the left table). |
|  | ![Argon Laser](image4) | ![HeNe Laser](image5) |
| **4.** Turn on the computer. | **5.** - Ensure sample holder is inserted properly and leveled. Adjust screws if necessary.  
  - Ensure table is floating and leveled.  
  - Remove excess immersion oil from objective. | - Ensure microscope is turned on.  
  - Start LSM Pascal software.  
  - Click on "Vis".  
  - Adjust condenser with test slide and objective to be used.  
  - Evaluate objective performance (qualitatively), and clean IF necessary. |
| ![Computer](image6) | ![Sample Holder](image7) |  |
**Shut down procedure**

<table>
<thead>
<tr>
<th>1:</th>
<th>2a: Log off</th>
<th>2b: Complete shut down</th>
</tr>
</thead>
</table>
| - Exit the LSM Pascal program.  
- Use Nero software (multi-sessions with necessary) to save data.  
- Do not use any re-writable media such USB drives or CD-RW. | - If someone is using the system after you, log off your account.  
- Go to step 6.  
- Confirm with the next user that the system will be turned off at the end of the day. | - If nobody is coming after you, shut down computer. |

<table>
<thead>
<tr>
<th>3a: Argon laser (458, 488 and 514 nm)</th>
<th>3b: HeNe laser (543 nm)</th>
<th>3c: HeNe laser (633 nm)</th>
</tr>
</thead>
</table>
| - Slowly turn the knob anti-clockwise.  
- Flick the silver switch down from "run" to the "STAND BY" position.  
- Turn off the key (below the power enable switch).  
- Let laser cool off until fan stops (~ 3 minutes).  
- Turn off the black power enable switch. | Turn off the key of the lower unit. | Turn off the key of the upper unit. |

<table>
<thead>
<tr>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>
| Turn power bar off. | - Switch off the mercury lamp. | - Lower the objective turret.  
- Wipe excess oil from surface of objective lens and from its sides.  
- Turn objective turret to an empty position.  
- Log in the log book.  
- If the system was completely shut down, put the blue cover on the microscope and the large plastic cover over the entire system, making sure they don't touch the mercury lamp housing. |
Condenser alignment (Köhler illumination)

Looking at a test slide using bright field can provide an assessment of the objective quality. Proper alignment of the condenser is crucial to evaluate the objective, but also for proper image formation in all transmitted light contrast modes (bright field, phase contrast, DIC etc). Condenser alignment needs to be done for each sample-objective combination.

There are two diaphragms to be adjusted: the field diaphragm (FD) and the field aperture (FA).

Adjustment of the Field diaphragm (FD)
If necessary, switch the light path to the oculars.
Switch on the halogen lamp and adjust light intensity for eye comfort.
Make sure no fluorescence filters is engaged in the light path.
Set the condenser "H".
Focus on the test slide.
Close the field diaphragm (FD) until it (or a black shadow) appears in the field of view.

Focus the field diaphragm (i.e. adjust condenser's height) using the black knob on either sides of the pillar.

Center the field diaphragm using the two screws at the front of the condenser
Open the field diaphragm just enough to have it out of the field of view:

Adjustment of the field aperture (FA)
Remove an eyepiece and look for the aperture in the back focal plane of the objective.

Open or close the field aperture to find the diameter that matches the whole field of view (i.e. 100%).

Adjust the diameter of the field aperture to 80%.
LSM AIM Pascal acquisition software

Make sure the microscope is already on. Double click on the "Pascal" icon. Select "Online Mode". Click "Start". Use the "Offline Mode" when using the software only.

Acquisition menu
In the acquisition menu, toggle between VIS mode and LSM mode to switch the light path to the eyepieces (VIS) or to the confocal detectors Ch1, Ch2 and ChD (LSM).

In VIS mode, transmitted and fluorescence images are directed to the eyepieces.

In LSM mode, transmitted and fluorescence images are constructed by the computer and displayed on the monitor.
Microscope Control

The "Micro" (Microscope) button presents an overview of the light paths going to the eyepieces.

Halogen shutter and intensity controls.

The mercury (Hg) lamp shutter. Click to open or close.

Objectives available in the system.

Filter cubes available for regular epifluorescence.

Eyepieces
Configuration control

After finding your sample in the "VIS" mode, switch to "LSM" mode (laser scanning microscopy). In LSM mode, the lasers are used as the light source for both transmitted and reflected light microscopy.

Ch1 and Ch2 are photomultiplier tubes (PMT) detectors for reflected (fluorescence) signals.

Lasers light source for both fluorescence (Ch1 and Ch2) and transmitted (ChD) light.

ChD PMT detector for transmitted signals.

Multi track configurations are optimally designed for multi-color acquisition, one track for each wavelength (or color). It is better to first acquire the tracks with the longest wavelength. Thus first acquire the far-red emission, then orange/red emission, green emission and blue emission. The tracks are selected/deselected as needed. Single track configurations are designed to collect the maximum amount of emitted light by one single signal. It is preferable not to use them for multi-color acquisition.

A track is also used to acquire transmitted light signals in the ChD. Make sure the DIC analyzer and polarizer are out of the light path during the acquisition. Use the 405nm or 488nm lasers.

Note that the PMT detectors collect black and white images that are pseudocolored by the user.

Security warning: Always click before "stop" putting your eyes through the eyepieces.
Define the acquisition settings at the resolution to be used:

e.g. For 63x/1.4NA objective, use 1024x1024 frame size, scan speed 6, 12-bit, number 2.

*Parfocality adjustments

Run Fast YX to adjust the focus on the monitor, to set the eyepiece-PMT parfocality (and diopter), and to adjust XY positioning. Stop the lasers as quickly as possible.

With the crop tool, fine tune XY positioning, rotate, zoom.
Scan control box: Channel

While running continuously "Cont.", one track at a time, look at the image with the palette, using the "range indicator". Pixels that are overexposed appear as red. Those underexposed appear as blue.

While using the range indicator, adjust laser intensities, gain ("red" pixels) and offset ("blue" pixels). Repeat the operation for each track.

Use the lowest possible intensity of laser.

Guidelines:
- Argon 488nm: first try 5%, 10% max.
- HeNe 543nm: first try 80%, 100% max.
- HeNe 633nm: first try 50%, 100% max.

Adjust the signal intensity with the detector gain (red pixels). Best image quality is obtained with gain values ranging 600-800. For dim samples, gain may need to be pushed to 1250 but image quality will suffer. In that case, try averaging by a number of 4, 8 or 16 (scan control box: mode).

Adjust the background levels with the offset (blue pixels). The maximum value usually works well.

The amplifier gain should be left at a value of 1.
Scan control box: Channel

The pinhole diameter of the middle track (usually the red emission) should be set to 1.00 Airy units. Look at the optical slice of the other tracks and adjust them to the same thickness.

Confocal vs widefield:
When the pinhole diameter is opened at the maximum "Max", confocality is lost but more light hits the PMT detector, constituting "widefield" microscopy. It may be useful to open the pinhole when trying to focus on dim features. If a confocal image is to be taken however, the pinhole has to be reset to the desired optical slice.
Scan control box: Z Settings

Before taking a Z stack, look at the optical Z Slice and make sure that it is identical for all tracks to be acquired. The quality of 3D reconstruction and subsequent deconvolution will be improved by selecting the optimal interval option.

There are two methods for acquiring Z stacks:

Mark first/mark last method: click on “XY cont.” and adjust the fine focus to find the top (“Mark First”) and bottom (“Mark Last”) Z position of your sample. Then stop “XY cont.” and click “start” to acquire the entire stack. This method is useful when the thickness of the sample is unknown. However, it implies exposing the sample for some time while establishing the Z positions.

Z Sectioning method: use this method when the required Z thickness is already known. Focus the sample on the features of interest. Select the number of slices and the interval. Select “C” when the current Z position is to be the center of the stack. Click “start”. This method is better to avoid photobleaching but it works best if the parfocality has been well adjusted.
**Miscellaneous**

**Controls**
Use single positive controls to adjust settings. Also use them as negative controls for the other channels to check for bleedthrough and crosstalk.

**3D view/Projection**
Suggested 3D rendering settings:
- turning Axis: X, First Angle: 0, Number Projections: 64, Difference Angle: 2

**Scale bars**
Scale bar is added by clicking "overlay" and then "1um".

**Image display**
Images of different tracks are automatically overlaid. One can toggle between the split view and the overlay view.
Parfocality adjustment

This adjustment will reduce photobleaching of the sample. It is essential for the proper functioning of the Z sectioning method to acquire Z-stacks.

1. In visible mode, transmitted light bright field, focus on the test slide.
2. Switch to LSM mode, channel D, and create a transmitted light image on the monitor.
3. While continuously scanning, use the fine focus knob to focus an easily recognizable fine feature on the monitor.
4. Switch back to visible mode and look for the same feature down the eyepieces. It may be out of focus.
5. Refocus on the feature using the left eye and the left ocular.
6. Repeat the last step with the right side.
Possible spectra on Zeiss LSM Pascal confocal: LSM mode

GFP, FITC, AF488  AF 546-594, TR, TRICT, Cy3  AF647, MTDR, Draq5, To-Pro3

Fluorophore
1: Alexa Fluor 488 (antbody conjugate)  ✓ ex ✓ em
2: Alexa Fluor 647 (antbody conjugate)  ✓ ex ✓ em
3: Alexa Fluor 568 (antbody conjugate)  ✓ ex ✓ em
4: None  ✓ ex ✓ em
5: None  ✓ ex ✓ em

Excitation
Laser (nm): 488
Filter / Bandpass:
518 / 13
543 / 633
650 / 28
633 /

Emission
Filter / Bandpass:
518 / 13
588 / 28
650 /

Fluorophore
1: YFP  ✓ ex ✓ em
2: CFP  ✓ ex ✓ em
3: None  ✓ ex ✓ em

Excitation
Laser (nm): 514
Filter / Bandpass:
458 / 514
500 / 514
553 / 48

Emission
Filter / Bandpass:
553 / 48
500 / 25

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Possible spectra on Zeiss LSM Pascal confocal: VIS mode

Fluorophore
1. Alexa Fluor 488 (antibody conjugate)  ex  em
2. DAPI  ex  em
3. Alexa Fluor 568 (antibody conjugate)  ex  em
4. None  ex  em

Excitation
Laser (nm): None

Emission
Filter / Bandpass
365 / 40
434 / 40
546 / 50

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Reflected (fluorescence) light mode:

Filter set FITC/Bodipy/Fluo/Dia: FSet 01
for GFP, AF488, FITC

Filter set TRITC: FSet 15
for AF546, AF594, Cy3, Texas red, rhodamine, mCherry.

FSet 02: FSet 02
for DAPI

FSet 25: FSet 25
DAPI/GFP/TRITC triple cube
<table>
<thead>
<tr>
<th>Fluorophore:</th>
<th>Source</th>
<th>Abs (nm)</th>
<th>Ems (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlexaFlour 405</td>
<td>Kr&lt;sup&gt;405&lt;/sup&gt;, BDL</td>
<td>401</td>
<td>421</td>
</tr>
<tr>
<td>AlexaFlour 350</td>
<td>Hg</td>
<td>346</td>
<td>442</td>
</tr>
<tr>
<td>EBFP</td>
<td>Kr&lt;sup&gt;405&lt;/sup&gt;</td>
<td>405</td>
<td>447</td>
</tr>
<tr>
<td>AMCA</td>
<td>Hg</td>
<td>350</td>
<td>450</td>
</tr>
<tr>
<td>DAPI</td>
<td>Hg, Kr&lt;sup&gt;405&lt;/sup&gt;</td>
<td>358</td>
<td>461</td>
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<tr>
<td>Hoechst 33342</td>
<td>Hg, Kr&lt;sup&gt;405&lt;/sup&gt;</td>
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<td>461</td>
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<tr>
<td>ECFP</td>
<td>Kr&lt;sup&gt;405&lt;/sup&gt;, HeNe&lt;sup&gt;488&lt;/sup&gt;, Hg</td>
<td>435</td>
<td>485</td>
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<tr>
<td>Cy2</td>
<td>Hg, Ar&lt;sup&gt;488&lt;/sup&gt;</td>
<td>492</td>
<td>510</td>
</tr>
<tr>
<td>MFG</td>
<td>Hg, Ar&lt;sup&gt;488&lt;/sup&gt;</td>
<td>490</td>
<td>516</td>
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<td>495</td>
<td>519</td>
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<tr>
<td>FITC</td>
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<td>520</td>
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<td>EGFP</td>
<td>Hg, Ar&lt;sup&gt;488&lt;/sup&gt;</td>
<td>492</td>
<td>520</td>
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<tr>
<td>Sytox-Green</td>
<td>Hg, Ar&lt;sup&gt;488&lt;/sup&gt;</td>
<td>503</td>
<td>524</td>
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<tr>
<td>EYFP</td>
<td>Hg, Ar&lt;sup&gt;514&lt;/sup&gt;</td>
<td>500</td>
<td>535</td>
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<tr>
<td>Lucifer Yellow</td>
<td>Ar&lt;sup&gt;488&lt;/sup&gt;</td>
<td>458</td>
<td>536</td>
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<tr>
<td>Fluoro-Gold</td>
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<tr>
<td>AlexaFlour 514</td>
<td>Ar&lt;sup&gt;514&lt;/sup&gt;</td>
<td>517</td>
<td>542</td>
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<tr>
<td>AlexaFlour 532</td>
<td>Nd&lt;sup&gt;3+&lt;/sup&gt;, YAG</td>
<td>532</td>
<td>553</td>
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<tr>
<td>AlexaFlour 555</td>
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<td>555</td>
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<tr>
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<tr>
<td>Cy3</td>
<td>Hg, HeNe&lt;sup&gt;543&lt;/sup&gt;</td>
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<td>570</td>
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<tr>
<td>TRITC (Rhodamine)</td>
<td>Hg, HeNe&lt;sup&gt;543&lt;/sup&gt;</td>
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<tr>
<td>AlexaFlour 546</td>
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<td>603</td>
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<tr>
<td>mCherry</td>
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<td>AlexaFlour 594</td>
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<td>690</td>
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<td>AlexaFlour 680</td>
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<td>AlexaFluor 700</td>
<td>FRDL, Xe</td>
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<tr>
<td>AlexaFluor 750</td>
<td>FRDL, Xe</td>
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<td>775</td>
</tr>
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</table>

AMCA, aminomethylcoumarin acetate  
DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride  
MFG, Mitofluor green

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