Leica DMI AF6000LX

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The Leica AF6000LX microscope is controlled via software LAS (Leica Application Suite). Below is a brief step-by-step guide to help new users get started smoothly.

**Start-up procedure**

1. Turn on the **Microscope**
2. Switch on the **Camera**
3. Turn on the Mercury lamp for fluorescence
   Or alternatively
3’. Turn on the monochromator (for experiments where speed is important)
4. Choose an intensity level (0-4) for the mercury lamp
5. Log as Administrator. Password: **admin**
6. double click LAS AF icon to start the software

7. Choose a configuration:

   - **AF6000LX** to work with the mercury lamp
   - **AF6000LXMono** to work with the monochromator
   - **SimulatorAF6000** to open existing images

8. Click OK

9. When prompted to initialize the stage choose **Yes**
IMPORTANT
Before inserting your specimen, check the microscope to make sure it is clean and free of oil. You are responsible for leaving it this way. Before using an unknown objective, be sure to use the correct immersion medium. It is very easy to damage an objective by putting for example oil on a dry objective. Please, ask if you are in doubt.
Always lower stage before starting software and inserting specimen

The microscope stand

1. Switches between camera port and eyepieces
2. Shutter
3. Fluorescence cubes switchers

Z-focus fast UP/DOWN buttons – to set objective top limit
4. Transmission / Epifluorescence switching
5. Aperture diaphragm - open or closes the Aperture Diaphragm
6. Field diaphragm - open or closes the Field Diaphragm
7. Light intensity
8. Objective switchers
9. Fine/Coarse Z-focus chooser – Coarse to find the specimen, fine for adjustments
10. Stage Y travel
11. Stage X travel
12. Z focus

Electronic stage with exchangeable holders

Objective turret with Z-piezo motor on position 6

Remote control module
Objectives

Objectives positions are exchangeable depending on which lens needs to be used in association with the Z-piezo (for stacks).

<table>
<thead>
<tr>
<th>LEN</th>
<th>N.A.</th>
<th>IMMERSION MEDIUM</th>
<th>WORKING DISTANCE</th>
<th>XY RESOLUTION</th>
<th>Z RESOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X</td>
<td>0.2</td>
<td>DRY</td>
<td>20 mm</td>
<td>1.5 um</td>
<td>12.85 um</td>
</tr>
<tr>
<td>10X</td>
<td>0.3</td>
<td>DRY</td>
<td>11 mm</td>
<td>1.05 um</td>
<td>5.7 um</td>
</tr>
<tr>
<td>20 X</td>
<td>0.7</td>
<td>Multi immersion</td>
<td>170 um (Gly)</td>
<td>0.45 um</td>
<td>0.483 um</td>
</tr>
<tr>
<td>40 X</td>
<td>0.55</td>
<td>DRY</td>
<td>3.3-1.9 mm</td>
<td>0.57 um</td>
<td>1.7 um</td>
</tr>
<tr>
<td>63 X</td>
<td>1.30</td>
<td>GLYC 37°C</td>
<td>280 um</td>
<td>0.24 um</td>
<td>0.44 um</td>
</tr>
<tr>
<td>100 X</td>
<td>1.40</td>
<td>OIL</td>
<td>90 um</td>
<td>0.22 um</td>
<td>0.31 um</td>
</tr>
</tbody>
</table>

XY resolution is calculated with the following formula:
\[
\gamma = \frac{0.61\lambda}{NA}
\]

Z resolution is calculated with the following formula:
\[
D = \frac{n\lambda}{NA^2}
\]

The 40X 0.55 does not have a very good resolution (the 20X is better) but is a long distance objective. This can be useful for thick sample or for working through a plastic dish.

The 4X is from Nikon and is longer so the black compensation ring must be removed. It cannot be used with the piezo (position 6).

**IMPORTANT**
- Use the correct immersion medium for each objective
Starting with LAS (software) and setting up the microscope stand

LCS has 4 function modules. The default module at start is the **Acquire** module. It is the module you need for acquiring new images.

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Acquire</th>
<th>Process</th>
<th>Quantify</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>Setup</td>
<td>Acquisition</td>
<td></td>
</tr>
</tbody>
</table>

**Acquire module**

It has 3 sub-modules:

- **Experiments**
  where you can see all images that have been acquired during the session. That’s where you will **rename, delete, save** your images and/or **export** them.

- **Setup**
  You should not really need to change parameters in there.

- **Acquisition**
  Where you set all parameters for the acquisition (lightpath, filtercubes, acquisition time, gain, time series, z-stacking, multipositions, …) and where you acquire the images.

**Setting the lightpath**

1. Click on the Acquisition sub-module
2. Click on + to add a channel (if you click on – the active channel is deleted)

3. Click on the little pallet icon to choose a LUT for your channel and click OK to confirm
4. Give a name to your channel (here: GFP)

5. Select a contrast method (TL-BF: transmission light – Brightfield, TL-DIC: DIC contrast, FLUO: Fluorescence)

6. Select a filter cube (no filter can be selected if TL-BF was chosen and Ana (analyzer) if TL-DIC).
### List of available filtercubes

<table>
<thead>
<tr>
<th>Filter Cube</th>
<th>Excitation Range</th>
<th>Excitation Filter</th>
<th>Dichromatic Mirror</th>
<th>Suppression Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>UV</td>
<td>BP 360/40</td>
<td>400</td>
<td>BP 470/40</td>
</tr>
<tr>
<td>Analyzer cube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Analyzer</td>
</tr>
<tr>
<td>B/G/R</td>
<td>UV</td>
<td>BP 420/30;495/15;570/20</td>
<td>415;510;590</td>
<td>BP 465/20;530/30;640/40</td>
</tr>
<tr>
<td>CFP</td>
<td>Violet / blue</td>
<td>BP 436/20</td>
<td>455</td>
<td>BP 480/40</td>
</tr>
<tr>
<td>CFP/YFP/DsRED</td>
<td>Violet / blue</td>
<td>BP 436/8;495/12;580/20</td>
<td>445;510;595</td>
<td>BP 460/25;535/35;630/55</td>
</tr>
<tr>
<td>Empty system</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GFP</td>
<td>Blue</td>
<td>BP 470/40</td>
<td>500</td>
<td>BP 525/50</td>
</tr>
<tr>
<td>I3</td>
<td>Blue</td>
<td>BP 450-490</td>
<td>510</td>
<td>LP 515</td>
</tr>
<tr>
<td>RFP</td>
<td>Green</td>
<td>BP 546/12</td>
<td>560</td>
<td>BP 605/75</td>
</tr>
<tr>
<td>Y5</td>
<td>Red</td>
<td>BP 620/60</td>
<td>660</td>
<td>BP 700/75</td>
</tr>
<tr>
<td>YFP</td>
<td>Blue</td>
<td>BP 500/20</td>
<td>515</td>
<td>BP 535/30</td>
</tr>
</tbody>
</table>

7. For this step you will need to have your sample in focus and to see it on the Live display. You will see (next page) how to work with the **Image Preview window**.
   - adjust **exposure**
   - adjust **gain** (1 if possible, 2 if the signal is too weak)
   - adjust **EM gain** (Electron Multiplication gain):
     
     Easily up to 3000-3200 without too much increasing the noise
8. go through steps 2-7 for each new channel

9. once the channels have been defined, they can be **saved** so that they can be **loaded** again later

---

**Setting the microscope stand**

1. Select a channel
2. Click on **live**
3. Push button [ ] to switch between camera and eyepieces (the little camera drawing is then replaced by an eye)
   Sometimes you will need to push open the shutter
4. Push the UP button until you come in the immersion medium with the lens or until you are near focus

5. On the remote control module select Coarse

Then find the focal plane by turning the Z position wheel on the remote control or the manual Focus wheel on the microscope stand

6. On the remote control module select Fine

7. Fine adjust the focus
8. Once the sample is in focus, push the SET button \[14\] and while still holding it down, push twice the UP button to set the focal plane as 0

9. Now go back to the computer

10. Click \textbf{Stop}

11. Click \textbf{Live} again to have the image through the camera port \((\rightarrow \text{On the screen})\)

\textbf{Working with the Live Preview window}

1. Select a channel

2. Click on \textbf{live}

3. The image should now appear on the right screen. If it is still black, the exposition parameters or the histogram need to be adjusted
4. Click on the Histogram icon

5. Adjust the sliders to get a readable image
If you get the following image (with black blotches where it should be very bright) that means the histogram adjustment is not correct:

6. You can also click on the Auto Histogram Button ![Auto Histogram Button](image) to have the sliders automatically adjusted. This won’t work correctly if you have very bright objects on your field (dead fluorescent cells, dust, …)

7. Click on the Overlay button to show the overlay image. You can display/hide a channel by clicking/unclicking it’s number. Select a channel by double clicking on it (double click again to see the other channels again).

8. You can Zoom in/out or see the image at the 1:1 scale
Histograms

The Roper Cascade produces 16 bits images. That means there are 65'000 intensity values between Black (0) and white (65'000). Producing a histogram extending all the way from 0 to almost 65'000 is nice but not necessary. For printing purpose, an 8 bit file is enough (only 256 intensity values) and our eyes cannot even see all the variations in an 8 bit image. That means that you can have a fairly small histogram and as you move the sliders together, still get more intensity variation than you would have in an 8 bit image.

This is more than perfect: Min=4725, Max=22'271 -> More than 17'000 grey values

If you would do that with an 8 bit image, you would end up with only a few intensity variation and get so called posterization:

In practice, if the histogram gets too small, smaller than 1/10 of its maximal size (extending from 0 to 6'500), the image will start to get noisy. So in case of a weak signal, it would be better to increase the light intensity or the exposition time. But this has the drawback of generating cell toxicity and photobleaching. So it is always a question of compromise. Sometimes it is worth compromising a little bit the quality to get the quantity (time series + stacks)!
Working with stacks

1. In the **acquisition** sub-menu of the **acquire** module, click on 
   The **Z-stack** window will open

![Z-stack window](image)

2. Click **Live**

![Live button](image)

3. Adjust z-focus to bring the middle section of your sample into focus

4. Click on **Set Plane** 
   The middle Z-plane is now set. 
   *If working in **Multiposition**, you won’t have to set the plane anymore*
5. Select **Fine focus** to use the piezo for precise stacking

6. Click and hold the middle plane and drag it up. You will see the focal plane changing on the **Live preview** screen (right monitor)

7. Once the UP position is OK, click on the little **Begin Arrow**
8. Then, drag the plane down to find the DOWN border of the Z-stack

9. Click on the little **End Arrow** to set the DOWN limit of the Stack:
   The stack volume is now defined
10. Choose **system optimized** or enter a **number of steps** or a **z-step size** for your stack.

11. Check your stack: While still in **Live**, use the **Go to** button to go to the **Begin**, **End** or **Middle Plane**.

12. If working in **Multiposition**, **Go to** the middle **Plane** before moving the stage to the next position. You won’t have to touch the **Z-Stack** window any more: the Stack will be constructed around this new position automatically.
Time Series

1. In the acquisition sub-menu of the acquire module, click on The Time window will open

2. Use minimize to set the Time interval as small as possible for this combination of Channels.

3. Alternatively, you can enter a time interval

4. Choose the desired number of cycles
5. Alternatively, enter the desired total duration of the Time Series

**Working with Multipositions**

1. Click on ![Mark and Find icon](image) to open the **Mark and Find** window

2. Click on ![View all positions icon](image) to view all positions on the Mark and find screen
3. Click to enlarge view

4. Click on Live and choose a channel to search through your sample

5. Find a XYZ position of interest, either viewing your sample through the oculars (see ‘Setting the microscope stand’) or using the Live Preview Screen. You will see the present position moving on the Mark and Find screen.

6. Click to mark the position
7. Repeat steps 5-6 until all positions have been stored. You can then go through all the stored positions to have a final check.

8. Stop the Live Preview (Click **STOP**) and click **Start**
**IMPORTANT**
It is important to define the Z-Stack first (before the first position) and then the Multipositions.

9. Other commands:
   - Delete a position
   - Delete all positions
   - Exchange a position with a new one
   - Load a positions file
   - Save defined positions

10. Define a time serie as explained in *Time Series* if you wish

**Doing a Tile Scan**

1. Click on

2. The Tile Scan window is the same as the Mark and Find window but you will only need to define the bottom right corner position of the scanning area (top left in the oculars) and then enter a grid size in the **ScanField** field
3. Click 🔄 to open the Stage Configuration window

Do not touch the **Orientation of stage axes** and **Tile Scan merge settings** parameters.
The **Overlaps** and **Origin Offsets** parameters need to be configured for each objective. We already determined the parameters for several lenses so please ask us.

4. Click on **Start**
Once all images have been taken, they will automatically be compiled to create a bigger image.

**IMPORTANT**
If the number of images is important (time serie, Z-stack), the software will need a (very) long time to compile the images so don’t think it has crashed and restart the computer!
Working with the Process Module

Click on the **Process** Module

Under **Tools**, you will see all available actions:

Use **Crop** to reduce frame size (deconvolution will be faster):
1. Draw a ROI
2. Select Channels to be included
3. Select Z range
4. Click Apply
3D Deconvolution

- Select an Image series (or the Crop of an Image series) in **Experiments**
- In **Tools** select **3D Deconvolution**
- Choose the desired number of iterations (10 is usually a good compromise)
- The refractive index of the immersion medium will be automatically selected

- Check Faster processing to have a faster Deconvolution (the quality of the resulting images will be reduced)
- Click Apply
3D Projection

Use 3D Projection to produce the maximum or average projection of a stack.

Changing objectives and filter cubes

See Objectives (page 5) to have a list of all available lenses. Only position 6 has a piezo-electric motor for fine Z-movements. So the objectives need to be exchanged at this position if you wish to use another magnification:

1. Exchange the objective
2. In the Configuration module, click on the Microscope Icon
3. Click Run LAS

4. You will get the following message. Click OK
5. The LAS program opens

![LAS program interface]

6. Go to the Setup module and choose **Nosepiece NOSEPI ECE (6 POS)** in the list. You will see the 6 positions as well as the complete list of all Leica objectives.
7. Click on the position where you changed the objective (6 for the piezo)

8. double click the line of the new objective in the complete list

9. If you are over with configuration, close the LAS conf software. You will get the following message: Click Yes

10. You will then be prompted if you want to save changes: Click OK

11. Click Yes to initialize the stage
You are now ready to work

**Filtercubes**

Filtercubes exchange is tricky and we ask the users not to change them by themselves. For information, Configuration window looks like the one for the objectives. You can find it under **IL-turret IL-TURRET (6 POS)** in the list.