

System description

Inverted confocal microscope, with the following components:

- Zeiss Axio observer inverted microscope.
- Confocal scanner head for 3 descanned spectral channels: one GaAsP PMT (green) and two multialkali PMTs (blue and red). Up to three channels detected simultaneously.
- A transmitted light (T-PMT) detector.
- An AiryScan detector for resolutions beyond the diffraction limit (120 nm xy).
- Fluorescence Correlation Spectroscopy (FCS) software option.
- An Ar laser lines: 458nm, 488nm, 514nm
- Violett (405nm), Green 561nm, red (633nm) diode lasers.

Objectives:

- **Pos 1** Plan-Apochromat 10x / NA 0.45 Air (WD=2.1mm)
- **Pos 2** Plan-Apochromat 20x / NA 0.8 Air
- **Pos 3** Plan-Apochromat 40x / NA 1.3 Oil
- **Pos 4** Plan-Apochromat 63x / NA 1.4 Oil
- **Pos 5** C-Apochromat 40x / NA 1.1 Water LD
- **Pos 6** C-Apochromat 63x / NA 1.3 Water

Startup

1. Turn the **main switch** (1) on.
2. Turn the **SYSTEM/PC** switch (2) on. This powers the PC, but doesn't start it.
3. Turn on the PC and log into Windows. Login: **LSMUser** (password: **Useme!11**).
4. Switch on the **COMPONENTS** switch (3).
5. Switch on the HXP fluorescent lamp, *if you need it* for visual examination and don't forget to switch it off when you don't need it!
6. Make sure that lasers are connected. Laser key should *always* be in position on (key horizontal) (4).
7. Open ZEN Software and select the **Start system** option. *Image Processing* is for image analysis only. The startup might take up to a couple of minutes, depending on the hardware.
8. Swap to the *Acquisition* tab and set the Ar laser lines to standby if you are going to use them. Swap back to *Locate*.

Shutdown

9. Retract the objective and remove the sample.
10. Close the software. **Turn off all the lasers when requested in the software.**
11. The Ar laser needs **15 minutes** to cool down before the system is completely turned off. Cooling fan stays on automatically
12. Turn off the computer.
13. Turn off the LED lamp if it's running.
14. Turn off the switches, in reversed order: First **COMPONENTS** (3), then **SYSTEMS/PC** (2) and finally the **main switch** (1).
15. Clean the objective(s).

Note: it is not necessary to leave the FL lamp running, but lack of communication generates some error messages that can be ignored. The software retrieves the control of the lamp any time it is turned on again.

Widefield / visual examination

The widefield modes are only to let users find the focus and check dye and sample integrity. For these purposes the eyepieces will be used, and no records can be made.

By default the Zen software opens in the **Locate tab**. Here the widefield configurations can be chosen (fig. 1).

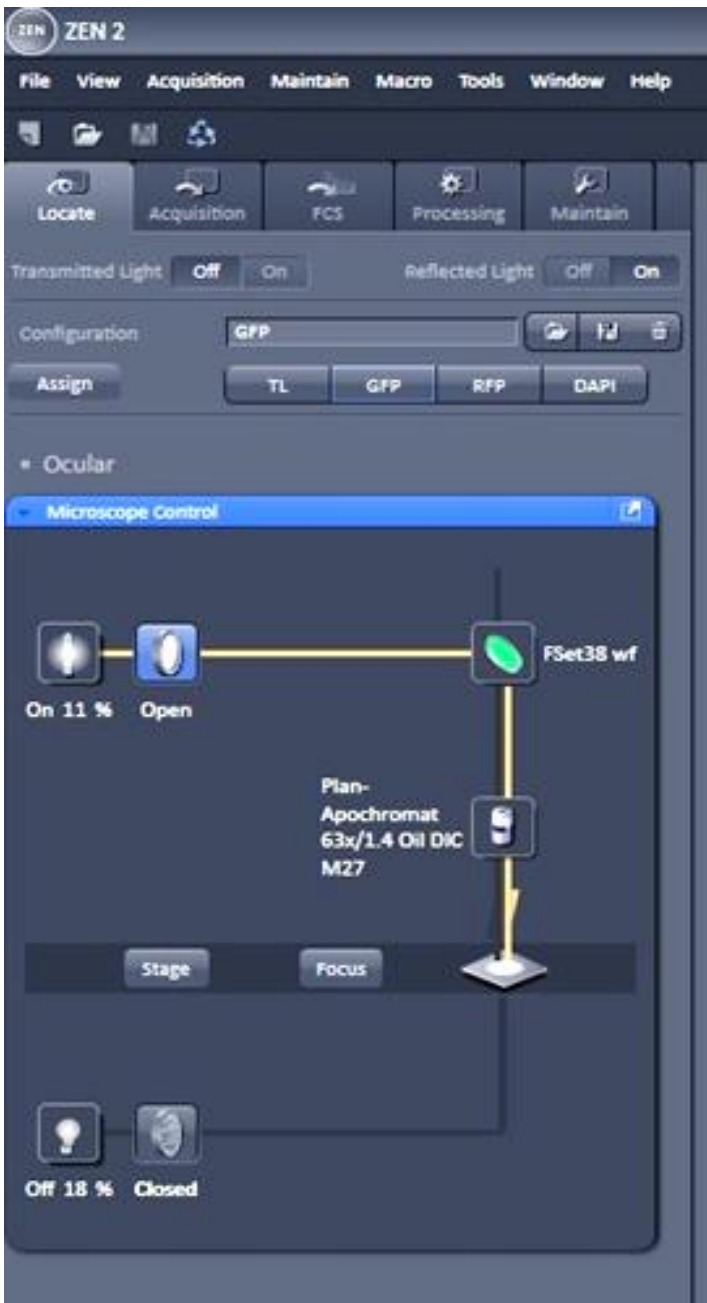


Figure 1: Locate tab and widefield mode selection.

Objectives/Joystick

- The objectives can be changed automatically using the touchpad.
- Every objective has a different stage speed assigned to it. The joystick stage movement is much faster using lower magnifying objectives than with high magnifying objectives

Widefield mode selection

Select one of the configurations available as shortcuts:

- TL¹ for transmitted light
- GFP / RFP / DAPI for epifluorescence

The different light sources, shutters and filtercubes are selected accordingly (see fig.1)

Confocal microscopy

First, select the **Acquisition tab**. The optical path to the eyepieces closes.

I. Channels and tracks:

1. **Smart setup** (upper left corner). The *smart setup* button opens a menu where different dyes can be added. The software shows different options, according to resources and dyes: *fastes (simultaneous scan)*, *best signal (sequencial scan)* or *smartest (line scan)*. Select an option and accept to **turn on the lasers** when requested. The confocal map upgrades accordingly (bottom left corner). See figure 2 for details.

Note: In line scan, only lasers are switching between lines, no hardware movement (same dichroic mirror, PMT and pinhole settings for all lines).

2. For transmitted light activate the **T-PMT** detector on the map. This detector collects the laser light that traverses the sample while acquiring some confocal track.
 - It's better to choose a track at which only one dye is being imaged, so only one laser is turned on. Otherwise the sum of the powers reaches the T-PMT.
 - In case of uneven illumination make sure that the Köhler illumination is set properly.

II. Preliminary settings:

3. Press **AF/Find focus** to readjust the focus automatically. This works if the focus was found already at the *locate* mode. It's also possible to focus at the **Live** mode.
4. Press **Set exposure** to adjust the gain of the detectors according to the current laser power and pinhole settings, and to the sample brightness. **These values are just preliminary.**

¹The transmitted light intensity can be very strong and harmful for the observer. The safe way to start it is to select the TL mode first, then to regulate the TL power, and only after, to watch through the eyepieces.

III. Image quality: Acquisition mode

The next steps optimize the digital resolution for a given optical configuration.

5. In the acquisition mode window select **Optimal** to adjust the pixel resolution according to the Nyquist criterion.

6. Undersampling makes imaging faster and files lighter, and it's fine if the features of interest are still resolvable on the images. Oversampling is in general not recommended.

Press **Optimal** again every time you zoom in or out. For a given pixel size (e.g. the optimal), the pixel count depends on the imaged area.

7. In the scan area, the image can be zoomed in, rotated or moved within the field of view.

Please note: zooming will also change the optimal pixel number so just press optimal button again after zooming.

IV. Dynamic range: Channels

Images are optimal when the histogram spreads across the whole dynamic range of the detectors. (typical is a bit depth of 8 (256 grey values)). This can be guaranteed when the first saturated and underexposed pixels start to appear, although **saturation and underexposure must be avoided**. To see the saturation map enable the **range indicator**, on the *dimensions tab*, below the image window.

The **digital gain** enhances images artificially, interfering with the next steps. Set these gains to their minimum (0.3) to get optimal results.

8. For confocal imaging set the Pinhole diameter to **1AU** (one Airy unit). This discriminates the out-of-focus light optimally, allowing the finest 3D reconstruction. Less light reaches the detectors so **laser intensities and detector gains must be readjusted**.

9. Above all, **avoid saturation**. Under the **continuous mode** follow these steps:

- Set a low laser power (**see the note below**) as a starting point.
- Start at no gain and increase it slowly until the sample becomes visible.
- If at high gains images are still too dim, raise the laser power. If at low gains images are too bright, decrease the laser power.
- Adjust the laser power and the gain until the first saturated and underexposed pixels become visible.

High laser powers at lower gains: good SNR but high bleaching ratios.

High gains at low laser powers: improved sample viability but higher noise.

NOTE: Typical laser powers

Gains and laser powers must be adequate for the corresponding detector. The following table can be used as a starting point while setting them.

Using the locate mode the user can estimate how bright are the samples. For bright samples it's reasonable to start at the lowest laser powers; for dim samples, please try the maximal powers *mentioned here* before further increases.

	multialkali PMTs	GaAsP PMT and Airyscan detector
Typical laser power	2% - 5%	0.020% - 0.5%
Detector gain ranges	0V - 1100V	500V - 900V

Typical laser powers depending on the detector

Snap

To capture single images, click on **Snap**. Remember that snapshots are not saved automatically.

Press "New" to open a new image document, otherwise the snapshots will be overwritten by the next Acquisition.

Alternatives

These alternatives might generate not-far-from optimal settings. Some further adjustment is recommended.

A) To retrieve settings from another file: Imaging settings (everything except the objective selection) can be retrieved by opening a **czi** file and pressing the **“reuse”** button right below the main menu. **This applies only to configurations already set at the same microscope.** Problems:

- For different reasons the sample might have a different brightness.
- The optimal settings depend on the chosen objective, so they are not applicable to any other.

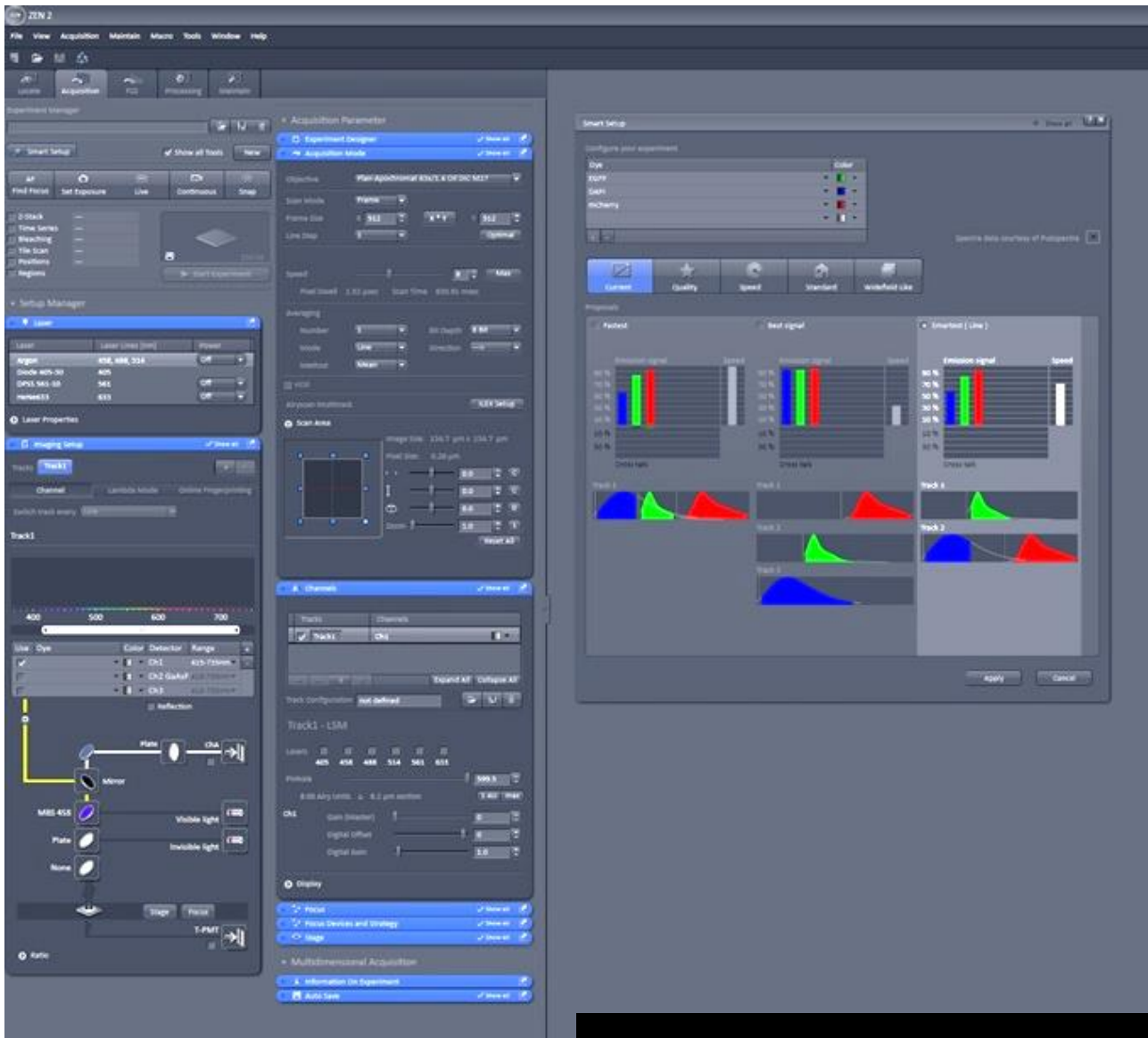
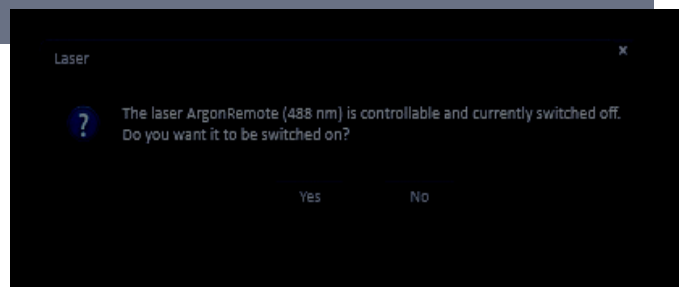


Figure 2. Smart setup and confirmation dialog box.

Experiments can be performed alone or combined in any way, either simultaneously or sequentially. Each of the corresponding check boxes opens an additional settings window.

Once configured, press **run experiment** to start.



Data saving

The **Autosave** window allows to select the path and the way the files are stored. Choose your own path on the data drive (D). Snapshots can only be saved manually.

Z stack

- Check the Z-Stack box on the experiment menu. The Z-Stack dialog appears.
- Select the **Live mode** for interactive selection. With the focus knob select the upper and lower sections on the sample, and click them as *first* and *last*.
- Select **Optimal** to sample slices according to the Nyquist criterion.
- At SR mode the z-stacks cannot be shown in real time. This is done **later, under the processing tab**.

Time series

- Check the **time series** on the experiment menu.
- On the corresponding window choose the number of cycles and the time interval.
- It's also possible to set start and end times instead.

Tile scan

- Select **Tile Scan** on the experiment menu.
- Choose the desired number of tiles (rows and columns).
- Try setting some overlap if stitching is not perfect.

Multiposition

- Check **Multiposition** on the experiment menu.
- Start **Live mode** above, to select positions interactively.
- While on a desired spot, add the current position to the position list.
- Repeat the last step as many times as needed until the list is complete.
- Leave the Live mode.

Experiment planner

For sequences of experiments use the experiment planner. Different blocks can be used for **different types of experiments** or for **similar experiments with different e.g. z-stack settings**.

Each block corresponds to an image setting in ZEN.

Z-stack-multiposition using the experiment planner

For convenience set up one position per block, this allows you different z-stack settings for each position.

Note: Always save the center position of your z-stack in the position list!!!

Laser Safety Instructions

- During operation of class 3B and class 4 lasers red warning lights have to be switched on manually
- Red warning light at the door of the room, containing laser-based equipment, prohibits the entrance
- Optical path of the laser beam at all setups has to stay intact and should never be disassembled by a user. Users are never permitted to disconnect optical connections (pipes, fibers etc), remove protective coverings or disassemble any parts of the setups, especially those parts that are labeled with laser-warning signs.
- User has to make sure, that objectives mounts are blocked by objectives or light- blocking plugs, before switching the system on or starting the work
- Any cleaning activities (objectives, stage cleanings) as well as changing of objectives or filters have to be performed only after blocking of the laser light is ensured. This can be ensured by closing the scanhead shutter or switching off the laser.
- Laser class-specific warnings at each setup have to be observed and considered
- Eye contact with direct beam of Class 3B laser, or eye contact with mirror reflection from class 3B laser, should be avoided at all times
- Eye or skin contact with direct or diffuse light of Class 4 laser, should be avoided at all times
- Laser safety goggles are situated at all workspaces and should be used in any situation where potential contact of eyes with Laser light of the classes 3B or 4 is possible, according to the previous two points
- Laser safety goggles have to be worn at all times of operation of Laser Class 4
- Laser safety goggles have to be worn at all times of operation laser Class 3 at the optogenetic setups
- Laser safety goggles are assigned to each setup and matched to the corresponding laser wavelengths. Matching laser safety goggles should be used at all times, and should not be carried over between the setups
- Only one person is allowed to be in the corresponding compartment during Laser Class 4 operation and optogenetic setup operation
- Users are not allowed to wear any reflective objects (rings, watches etc) during laser operation
- Using of the equipment is only allowed after the introduction from a laser safety officer of IST Austria. Introduction has to be done individually for each setup.
- Changing experimental conditions, that involves changes in the laser application, have to be reported to the laser safety officer prior to the start of experiment

Users have to understand that any violations against the instructed rules and also withholding information leading to safety hazards will ultimately result in denial of admission to all laser equipped instruments at the IST Austria.