

I. System description:

Inverted (vertical) confocal microscope

Motorized stage for multiple position imaging

Objectives:

- Objective EC-Plan Neofluar 2.5x / 0.075 Air
- Objective Plan-Apochromat 10x / NA 0.45 Air (WD=2.1mm)
- Objective Plan-Apochromat 20x / NA 0.8 Air
- Objective C-Apochromat 40x / NA 1.2 Water Korr UV-VIS-IR
- Objective Plan-Apochromat 40x / NA 0.95 Korr

- Laser lines: 405, 488, 555, 639 nm

2 PMT detectors with free choice of the spectral range. (Uses a tunable short pass and bandpass filter in front of the PMTs)

An ESID detector for transmitted light.

Simultaneous detection of only two dyes possible, more than two have to be acquired sequentially

II. Starting up:

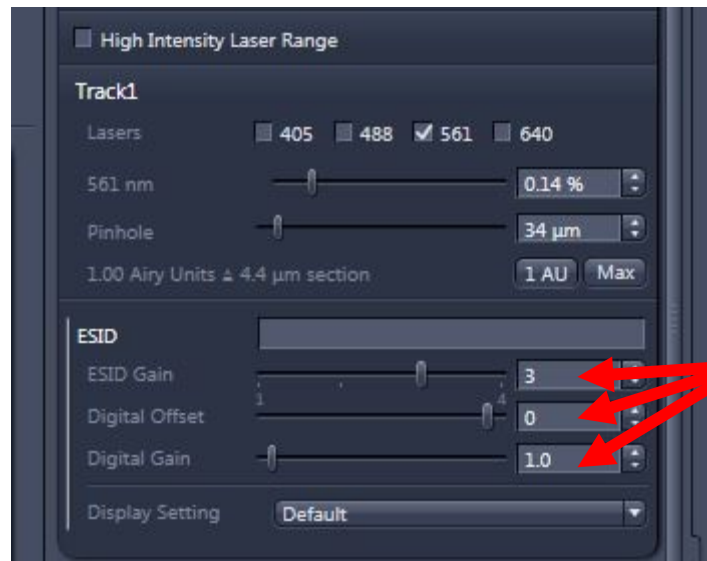
1. Switch on the multiple socket (1). Provides power to microscope controllers
2. Switch on the system(2) and Components (3)
3. Switch on the computer (4)
4. Login: BIF-User (Useme!11)
5. Switch on the fluorescent lamp (5), if you need it for visual examination
6. Open **ZEN Blue** Software and press "Start System" ("Image Processing" is for offline analysis of your images)
7. If Pop-up appears about stage calibration, press "calibrate now"

III. Visual examination of the sample

1. Press “Locate” in the main microscope menu and “Online”
2. You can change objectives automatically on the small touch screen attached on the right side of the microscope by selecting “microscope” menu and “objectives”
3. Please make sure the chosen objective corresponds to the one shown in the software. If this is not the case please restart the system. (Otherwise your pinhole and pixel sizes as well as the sectioning thickness are all incorrect.)
4. If you wish to view your sample in Brightfield press the TL button in the “Locate” menu
5. For visual examination of fluorescence, press the buttons DAPI, GFP or RFP

IV. Acquiring confocal images

- Select “Acquisition” in the main menu of Zen blue
- Go to “Smart Setup” choose your dyes (set the LUT) and configuration, you can choose between simultaneous (fastest), Best compromise (Smart) and sequential (best signal) scan, press “Apply”
- If you are using the detector for transmitted light (ESID): activate (ESID) represented in the light path scheme. Adjust intensity in the ESID Gain, then adjust digital offset and digital gain of the ESID detector in order to “stretch” the histogram and use the whole dynamic range of the detector.



Make sure Köhler illumination is set properly if you are getting bad bright field images!

- Press the “AF” (Autofocus) button to find your focus, alternatively go to “Live” acquisition modus and find it manually
- Press the “Auto exposure” button and the software will adjust the gain of the PMTs but not the laser power, according to the brightness of your dyes.

- What Auto exposure does is to increase the PMT voltages until about 1% of all pixels are overexposed. If the resulting gain is below 400 V decrease the laser power. If the gain is above 900 V increase the laser power.
- Set pinhole to Airy Unit (AU) 1 in the channel dialog to achieve confocality.
- In the channel dialog you can adjust the pinhole, gain and laser power manually
- To zoom in your image, go to “dimensions” below the acquisition window. With “Crop” you can select the desired area of your image, then press “Live” or “Snap” to apply, “Reset all” will remove the zoom again. Depending on the scan speed, the lowest zoom is 0.5 and not 1.0.
- Before taking an image, press “optimal” in the “acquisition mode” to adjust optimal number of pixels and the pixel size according to Nyquist sampling criteria.
Note: More pixel than “optimal” almost never make sense but feel free to decrease the pixel number to increase acquisition speed at the cost of resolution.
- To capture single images, click on ‘Snap’

1. **Setting up Z-stack:**

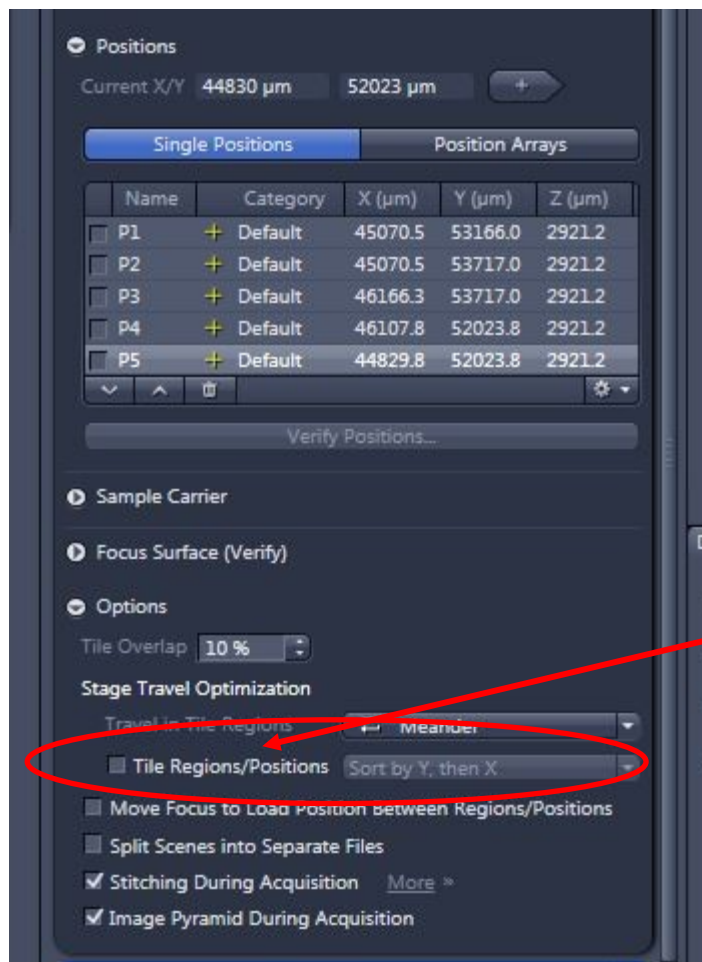
- Enable Z-Stack and the Z-Stack dialog will appear
- Press “Live” and set your first and last slice
- The software will suggest optimal slice number and interval which you can change manually
- Press “Start experiment”

2. **Setting up time series:**

- Enable “time series”
- Time series dialog will open
- Set cycle number and interval time
- Press “start experiment”

3. **Tile Scan and (in this mode you have: Tiles, Multipositions):**

- Enable “tile scan”
- Tile scan dialog will open
- Set desired number of horizontal and vertical tiles
- You can choose different regions to scan. Choose regions and press button “+” to add it.
- Press “start experiment”
- **Advanced Set up** allows you to do preview scan of region of interest.
- **You can also add multipositions in this tab:**
Important: to make sure that the software images the positions in the order you added them, make sure that you DISABLE the feature “tile regions/positions” in the options menu of the Tiles bar (see picture below)



Disable this option to keep the order of positions during imaging!

4. Data Saving:

Save your data to D: data folder. Note that this computer is not backed up. Please move your data as soon as possible using portable storage devices. Since this is a Demo instrument it's not being connected to our network. You can also use "Auto save", your experiment will go directly to your folder.

V. Switching off:

1. Close the ZEN Blue software
2. Switch off fluorescent lamp (5)
3. Shut down the computer (4)
4. Switch of the components (3) and system(2)
5. Switch off multiple socket (1)

!!! IMPORTANT !!!

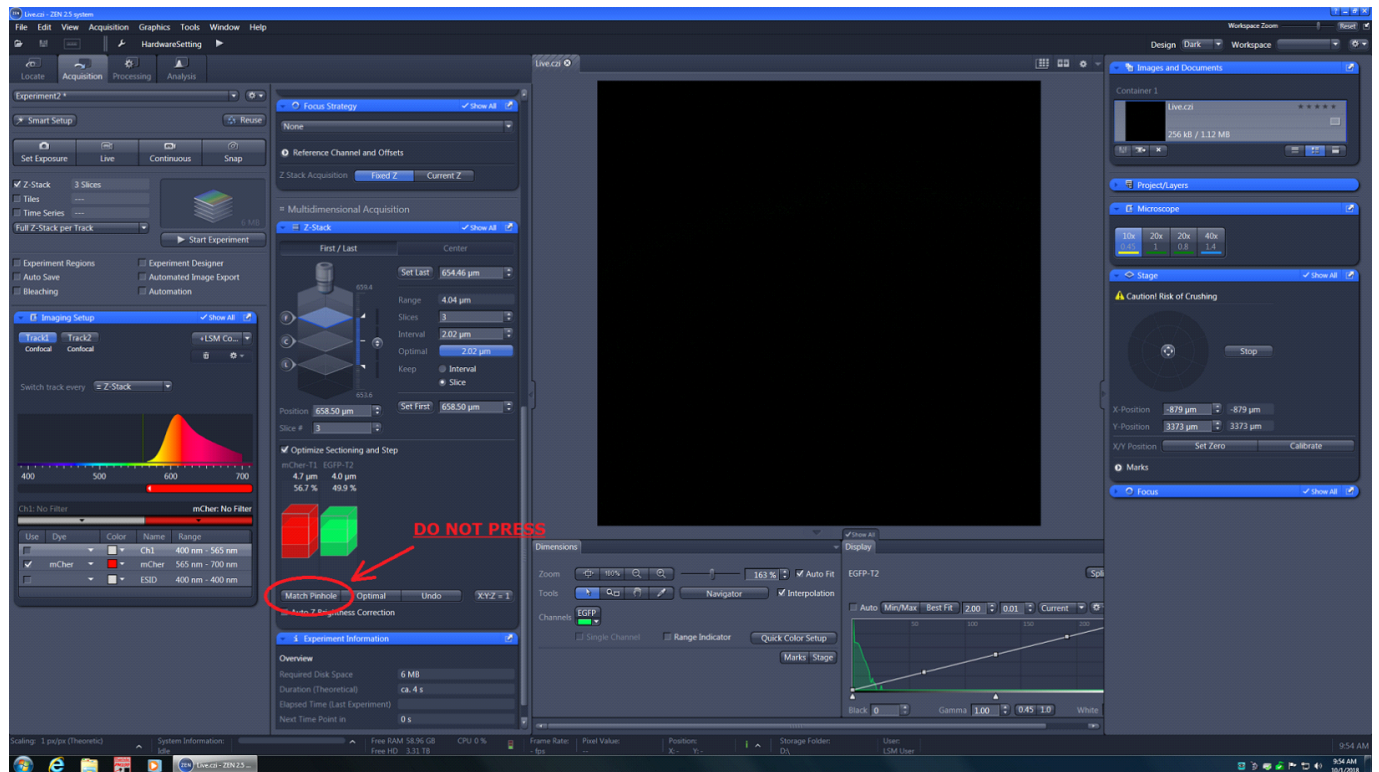
Dear Users!

Due to a bug found in our new ZEN Blue software, the option „**Match Pinhole**“ should not be used until further notice. (Please see the image below)

When setting up z-stacks you should do it with optimal z-sampling, otherwise if you want to change number of slices/interval, it won't have a proper overlap (50%)!

Please write/call us in case of any questions.

Please also report us any further bugs or problems you find!



!!! IMPORTANT !!!

Please make sure that the chosen objective corresponds to the one shown in the software.

**If this is not the case, restart the system.
(Otherwise the pinhole and pixel sizes, as well as the sections thicknesses are all incorrect)**

Safety hazard warning concerning fluorescence lamps with mercury bulbs

We state that mercury-containing lamps are safe and that they have electrical and interlock protections. Nevertheless, mercury vapors are extremely toxic and a self-protection remainder might be necessary:

- Every mercury lamp is labeled with a warning sticker. The lamps without sticker are halogen and these instructions don't apply to them.
- Before turning on the Hg-lamp, check the lamp housing to confirm that the lamp has cooled down → wait if not cooled down yet
- In the unlikely event of mercury vapor release, avoid breathing and leave the laboratory. An indication might be that ***the light disappears suddenly, with or without noise***. In this case the lamp might have suffered damage, although other causes are possible. For safety, **immediately turn off the lamp and leave the lab quickly, keeping sure that the other users also leave the room.**
- **Inform BIF immediately** so we can react properly and confirm if mercury was released or not. **If you think that you could have been exposed to the vapors, contact EHS.**
- **After any kind of accident (shock, spill, etc.) the mercury lamps have to be tested by the Biolmaging Facility before being used again.**
- If ***you turn on the lamp and it doesn't work***, don't insist. A damaged lamp might be inside. Ask BIF and we will check it. ***Don't try to open or manipulate the lamp.*** At room temperature, mercury is in the liquid state, so there's no danger if the lamp enclosure remains closed.
- ***After your session***, if nobody else is going to use the lamp within the next 30 minutes, ***turn the lamp off***. Never leave these lamps running overnight or unattended.

In case of any incidence concerning the mercury lamps, the Biolmaging Facility has to be contacted immediately via:

→ group.imaging@list.ist.ac.at

or personally inform the Biolmaging Facility:

→ Bertalanffy Foundation Building, I04.EG.037

→ Lab Building East, I06.EG.107

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.