

***I. System description:***

Inverted (vertical) confocal microscope with Airyscan module

Motorized stage for multiple position imaging

Objectives:

- Objective Plan-Apochromat 10x / NA 0.45 Air (WD=2.1mm)
- Objective Plan-Apochromat 20x / NA 0.8 Air
- Objective W-Plan-Apochromat 40x / NA 1.0 water dipping DIC VIS-IR
- Objective C-Apochromat 63x / NA 1.2 W Korr
  
- Laser lines: 405, 488, 561, 640 nm

Detectors:

- 2 PMT detectors with free choice of the spectral range. (PMT 1: tunable short pass and various bandpass filters in front of the PMT, PMT 2: tunable long pass and various bandpass filters in front of detector)
- 1 GaASP PMT for the Airyscan detector that can function as a normal detector in confocal mode (detection range adjustable by a tunable short pass and a tunable long pass filter, no bandpass filters)
- An ESID detector for transmitted light.

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

***II. Starting up:***

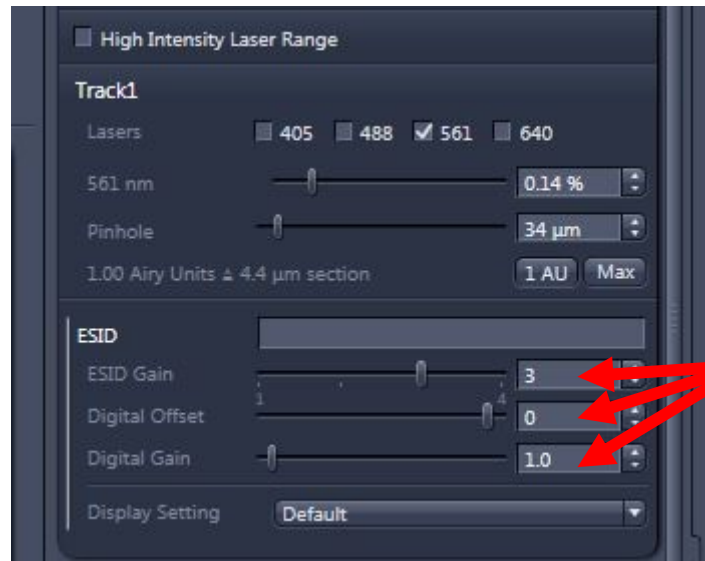
1. Switch on the multiple socket (1). Provides power to microscope controllers
2. Switch on the system(2) and Components (3)
3. Switch on the computer (4)
4. Login: BIF-User (Usemel11)
5. Open **ZEN Blue** Software and press "Start System" ("Image Processing" is for offline analysis of your images)
6. 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, RFP or FarRed
6. If sample appears too dim, increase light intensity by pressing the ‘Colibri 5/7’ button and adjust the LED intensity slider.
7. Before switching to acquisition mode, close transmitted and reflected light shutters by pressing ‘off’ and turn off the LED light source by clicking on ‘Colibri 5/7’ and pressing ‘Deactivate all LEDs’.

### ***IV. Acquiring confocal images***

- Select the ‘Acquisition’ tab 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 line scanning mode (Smart) and sequential (best signal) scan, press “Apply”.
- **If you use ‘Smart setup’, please make sure that you DO NOT have any track using “Ch2”, which is the Airyscan GaASP detector!** You’ll have to manually switch the channels that are assigned to it to a different detector: low wavelengths to ‘Ch1’ and high wavelengths (from mCherry on) to ‘Ch3’. Due to the arrangement of dichroic mirrors in the scanhead, it won’t work the other way round!
- 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 intend to use the ESID detector (for bright field images)!**

- Press the “AF” (Autofocus) button to find your focus, alternatively go to “Live” acquisition modus and find it manually
- Set pinhole to Airy Unit (AU) 1 in the channel dialog to achieve confocality. Note, that activating the ‘1AU’ button by clicking on it once (gets light blue background color) fixes the pinhole to 1AU. In this case, the match pinhole function in the Z-stack setup will not change pinhole sizes.
- In the ‘Channels’ dialog you can adjust the pinhole, gain and laser power 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.
- To zoom in your image, go to ‘Acquisition mode’ and adjust the slider accordingly. 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’ tab to adjust optimal number of pixels and the pixel size according to Nyquist sampling criteria.  
Note: More pixel than suggested by the software as ‘optimal’ almost never make sense. On the other hand, 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:**

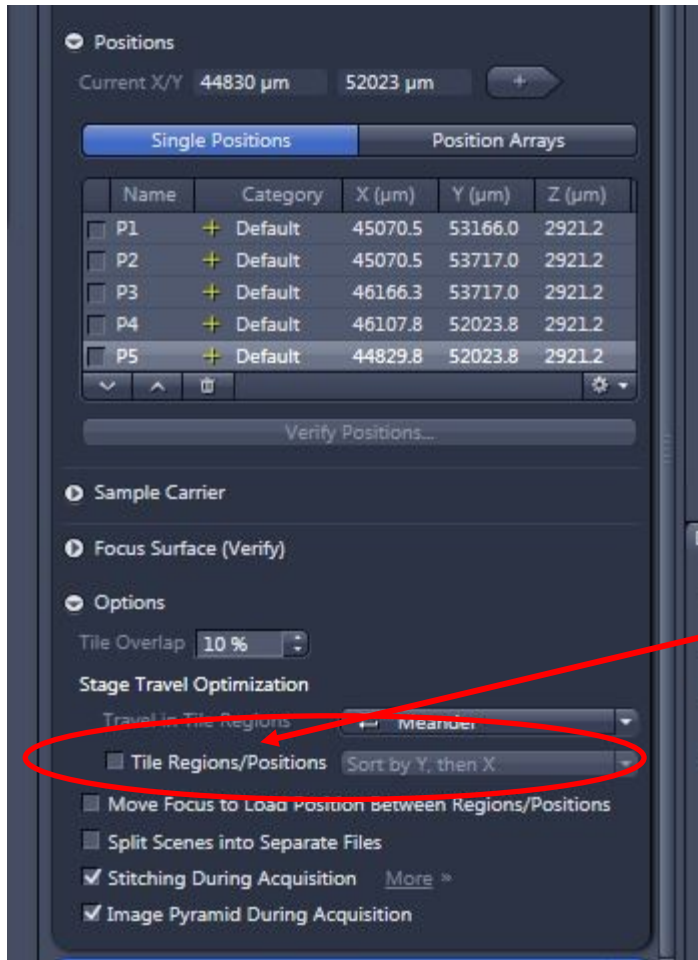
- Enable Z-Stack and the Z-Stack dialog will appear
- Press “Live” and set your first and last slice
- The software will suggest optimal slice number and interval which you can change manually
- Activate the  ‘Optimize sectioning and step’ checkbox to see slice overlapping. Here you can again set an optimal interval to ensure ~50% overlap between sections.
- If you wish to use the match pinhole function, make sure that the ‘1AU’ button in the ‘Channels’ menu is deactivated on all tracks (no blue background of button). This function will match pinhole sizes to the optimal pinhole size of the lowest wavelength of your imaging setup and ensure that all sections have the same thickness.
- 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 on the next page)



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)

## ***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.