

**I. System description:**

- Upright confocal microscope with Airyscan2 module
- Motorized XY piezo stage for multiple position imaging
- Incubation chamber
- Objectives:
  - Plan-Apochromat 2.5x / NA 0
  - Plan-Apochromat 20x / NA 1.0 Water dipping
  - Plan-Apochromat 40x / NA 1.
  - Plan-Apochromat 63x / NA 1.4 Oil Korr
  - Plan-Apochromat 20x / NA 0.8 air (on the desk)
  
- Laser lines: 405, 488, 561, 640 nm
- Detectors:
  - 2 GaASP 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 Airyscan2 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)
  - A T-PMT detector for transmitted light.
  
- Simultaneous detection of 3 dyes possible, more than 3 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. **IMPORTANT: before you start the software, make sure that the currently selected objective is retracted. Not doing so results in serious hardware damage during stage calibration!**
6. Open **ZEN Blue 3.1** 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"
8. Once the software started up, control if the objective shown as active in the 'Microscope' tab on the left side corresponds with the actually selected objective in the nosepiece. If not, restart the whole system (microscope and PC).

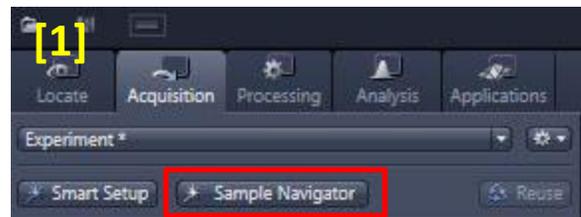
### III. Visual examination of the sample

1. Select the 'Locate' tab in the main microscope menu
2. You can change objectives manually at the nosepiece.
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' tab
5. For visual examination of fluorescence, press the buttons DAPI, EGFP, RFP or FarRed
6. If the sample appears too dim, increase light intensity by pressing the 'Colibri 5/7' icon in the 'Microscope control' panel 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 the 'Colibri 5/7' symbol and pressing 'Deactivate all LEDs'.

### IV. Sample navigator

The new Sample Navigator acquires the whole sample area quickly with a 2.5x objective and allows the selection of ROIs in the Tiles and Position menu for subsequent acquisition with higher resolution and/or Airyscan mode.

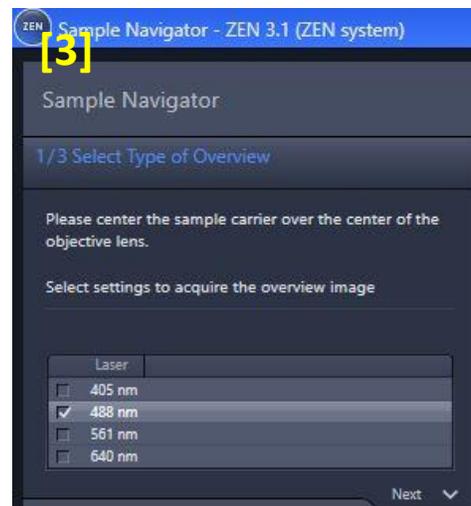
1. Click on 'Sample Navigator' in the 'Acquisition' tab.
2. If the message 'Coded component: 4x Coded Nosepiece' pops up, select the 2.5x objective (position 3) in the nosepiece. In case you have already done so, no window will pop up at this point.



3. Select the laser line matching the excitation wavelength of your fluorescent marker and click 'Next'

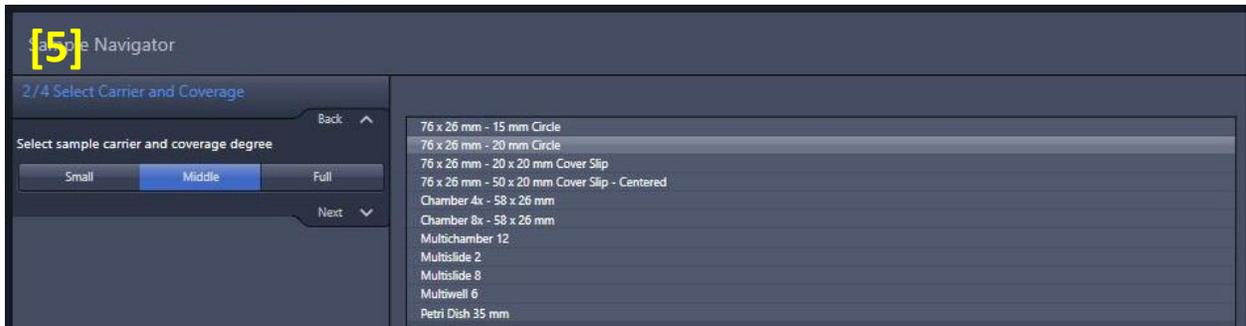
Choose something, that will give you a good overall signal throughout the whole sample.

4. A popup message will tell you to adjust focus. Move down the 2.5x objective and use the focus knobs to go close to your sample. Once done, click 'Objective is now close to the sample'

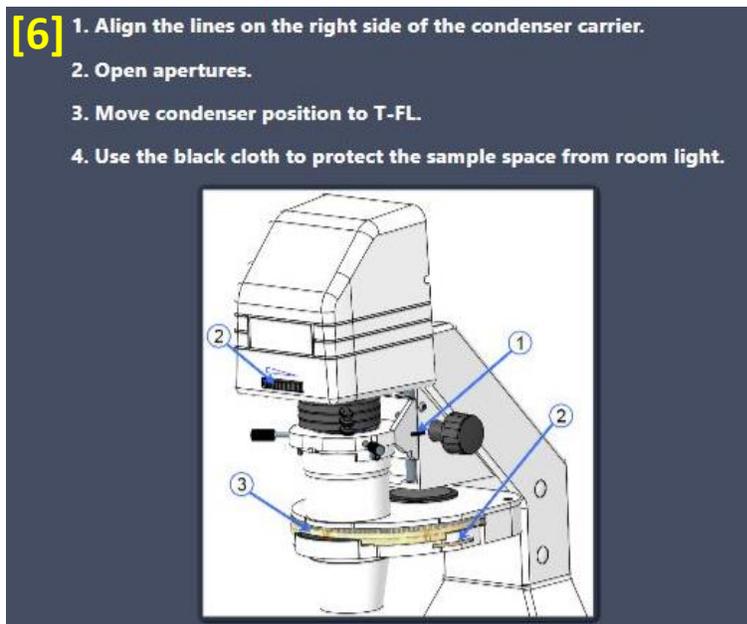


5. Select the type of carrier you are using. Depending on the size of sample/cover slip, select the appropriate coverage (small, middle or full). Click 'Next' when ready.

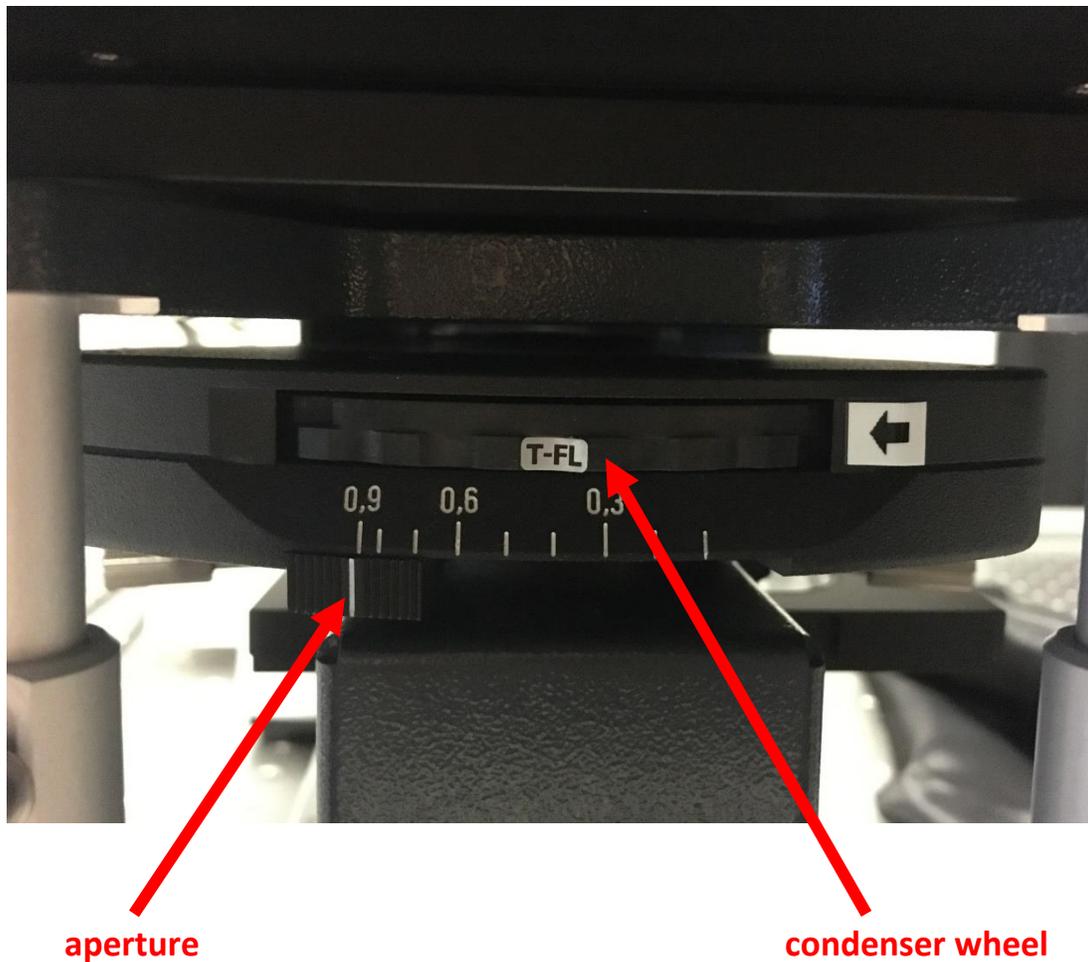
**Important: ONLY slides will work on an upright system, please do not attempt to scan dishes, well-slides and well plates. The walls of them are too high and will crash with the objective!**



6. Filter setup step: the cartoon in the software displays an inverted system, but the principles are the same.
  - 1) If the Köhler-illumination is set correctly, there is no need to align the condenser carrier.
  - 2) and 3) The aperture and the condenser wheel can be found below the stage -> see photo on the next page. It is CRUCIAL that the condensor is on position T-FL!



- 4) Close all doors of the incubator chamber -> there's no need to use an additional cloth. Press 'Next' when done.



#### V. 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 are using the detector for transmitted light (T-PMT): activate (T-PMT) represented in the light path scheme of the Track of your choice. Adjust transmitted light signal intensity in the T-PMT gain only after first finishing the setup of fluorescent channels.

**Make sure that Köhler illumination is set properly if you intend to use the T-PMT 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. Auto exposure increases 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 a Z-stack:

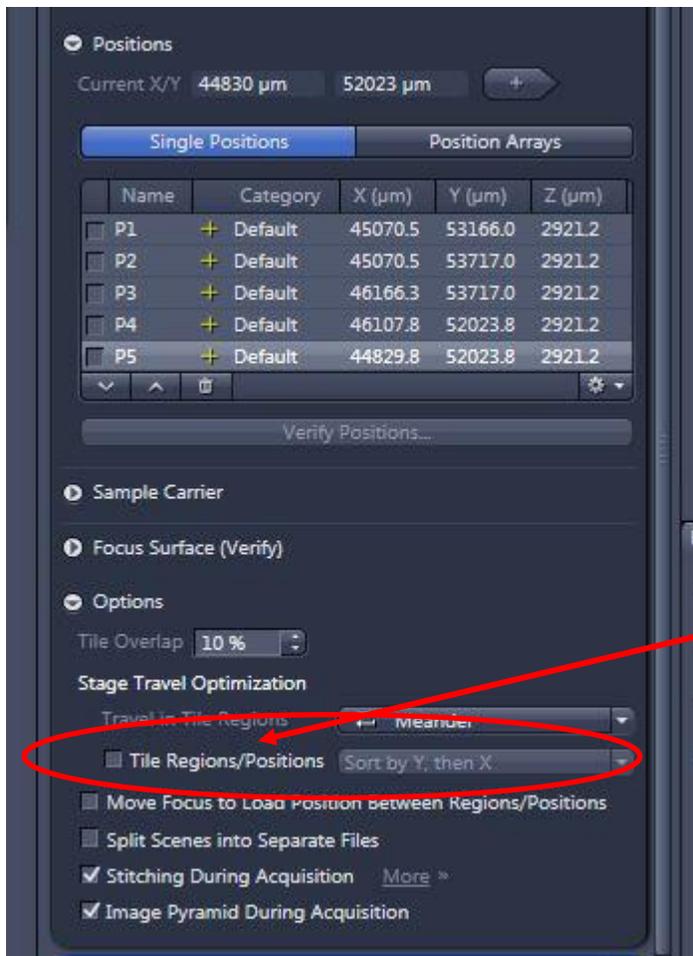
- Enable Z-Stack and the Z-Stack dialog will appear
- Press "Live" and set your first and last slice
- The software will suggest optimal slice number and interval which you can change manually
- Activate the  'Optimize sectioning and step' checkbox to see slice overlapping. Here you can again set an 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 a time series:

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

### 3. Tile Scan and Positions:

- Enable “Tiles”
- The Tiles and Positions 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 Setup** 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\**. You can also use the 'Auto save' option to automate file saving – however, make sure to configure the destination folder first!

Note that this computer is not backed up. Please move your data as soon as possible by uploading it to the fileservers. Just like on all other BIF systems, the use of any portable storage devices is strictly forbidden.

Never use too long folder and/or data names -> it will lead to problems with the ZEN software and during data processing for instance in Imaris. Save experiment info in a separate .txt file if needed.

#### **VI. Switching off:**

1. Close the ZEN Blue software
2. Shut down the computer (4)
3. Switch of the components (3) and system(2)
4. 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.