1.System description

Total Internal Reflection (TIRF) microscope, with the following parts:
- Olympus IX83 fluorescence inverted microscope,
- Olympus Cell^TIRF module for TIRF illumination,
- Four different Fiber-Coupled Lasers, (405nm, 488nm,561nm and 640nm).
- There's no FL-lamp. TIRF and wide field fluorescence imaging modes share the same laser light source.
- W-VIEW Gemini Image splitting optics provide one pair of dual wavelength images separated by a dichroic mirror onto a single camera
- Hamamatsu EM-CCD C9100-13 camera, 512x512 pixels, 16µm pixel size
- Incubator and CO_2 supply for live cell imaging

Objectives:
- Olympus UAPON OTIRF 100x/1.49 OIL
- Olympus Uplan SApo 100x/1.40 OIL
- UAPON 0340 40/1.35 OIL, Bright field imaging only.
- TIRF Tool 0.5x, Facility only

Filtersets:

Quad filter cube:

Quad Line Beamsplitter 405/488/561/640, see specs here: https://www.chroma.com/products/parts/zt405-488-561-640rpcEmission filter

Quad emission filter BrightLine Multiband 446-523-600-677, specs
http://www.laser2000.co.uk/semrock_filter.php?code=FF01-446/523/600/677-25

GFPsingle filter cube: Dichroic LP491 , Bandpass 525/45

W-VIEW filtersets (green/red; green/far red; red/far red) see appendix1

2. Laser safety

1. An Interlock shuts off the lasers if the eyepiece shutter is open.

2. TIRF cannot work unless the TIRF lid is placed above the sample. When the lid is present two red LEDs on the Cell^TIRF module report that the lasers are available.

3. The interlock box allows to shut off lasers selectively and to switch between *user* and maintenance modes. Maintenance mode bypasses some interlocks and is devoted to technical maintenance only, **users should never work in maintenance mode**.

4. Black incubator box must be closed at all times during laser operation

2. Startup

Please follow this sequence:

1. Turn on the switches from the box, from left to right:

   - Real time controller (RTC).
   - Control box.
   - **Switch on the needed lasers only to save their runtime!!!**
   - The options are 405nm, 488nm, 561nm and 640nm.

2. Switch on the needed lasers: On the laser boxes turn keys to *on* position and then press the *start* button. Repeat the operation for the used lines.
3. Log into the computer. Login: BIF-user   Password: Useme!11
4. Turn on the microscope touch-screen by pressing the button on its rear side.
5. Switch on Camera Controller
6. Start the *cellSense Dimensions* software.
7. The touch screen switches from *start operation* to its default state
8. The stage needs an calibration upon every software start, click yourself through the wizard

3. Setting Up Acquisition

3.1. Brightfield (BF) Illumination visual

1. Go to **Microscope Control** and choose **BF** observation method.

2. Switch the **BF** lamp on. Set a low illumination power (e.g. 3). 12 is the maximum.

3. Select the objective. Objectives switch automatically.

4. Select the prism. The options are *Camera*, *Eyepieces* or *50/50* (touchpad).

5. To free the light path for visual examination you have to pull out the shutter below the eyepieces.
   This disables laser illumination. Use the external focus drive for focusing. Push in eyepiece shutter afterwards to enable laser modes again.

6. The bright field mode is not Laser-based and may work either with or without the interlock lid. The lid dims the light from the condenser, that powers around 7 are needed.
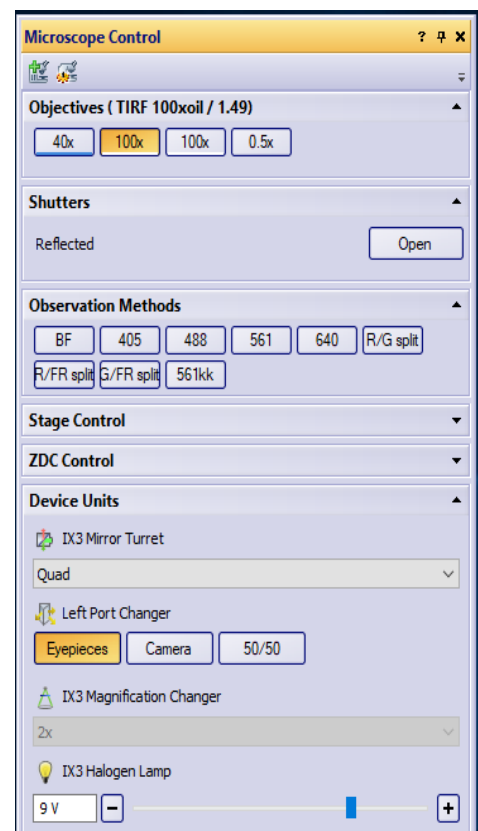

Fig.1 Microscope Control

3.2. Setting up TIRF imaging

1. Go to the **Microscope Control** window and choose 405, 488, 561 or 640 observation method dependant on your laser excitation.
2. Select 100xOTIRF objective
3. **Note:** Focusing with eyepieces on the sample is only possible in BF mode using the selected objective.
4. Set *Left port changer* to *camera*. Make sure that the eyepiece shutter is closed.
5. Turn off BF Lamp
6. Go to **Camera control** (Fig.2.) window and press **Live**
7. Adjust exposure time manually or press **Auto** (clock symbol).
8. Adjust EM gain to improve signal to noise ratio, maximum value is 200
9. Adjust Laser Power of the corresponding laser line in the **Single Laser** window
10. Always check gray values and histogram in the **Adjust Display** Window on the right. Images are displayed in 16 bit.
11. Click on **Auto contrast** to get live gray values and automatic scaling of the image
12. **DO NOT OVEREXPOSE THE CAMERA!!!**
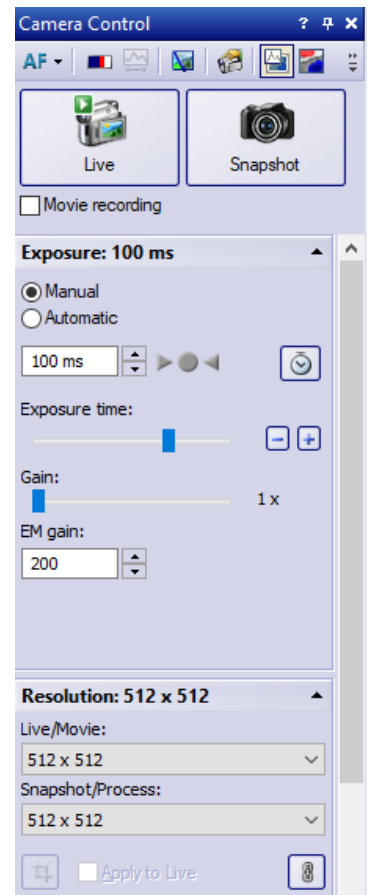13. The *online histogram* can also be toggled when you click on **Fixed scaling**.

Fig.2 Camera Control

14. Fig. 3 In the **Fiber Adjustment** window one can access the TIRF angles (fiber positions) of each individual laser line. Fast change between widefield and critical angle is possible with the respective buttons below the sliders.
15. Type in the required penetration depth (e.g. 100 nm) and press **Apply** to get TIRF illumination.
16. The **Setup Parameters** remain the same unless you are using an imaging medium with a different refractive index than water (please change).
17. In **Illumination Parameters** you can adjust the Incident Angle with an offset without changing the penetration depth, to adapt the TIRF illumination to your sample.
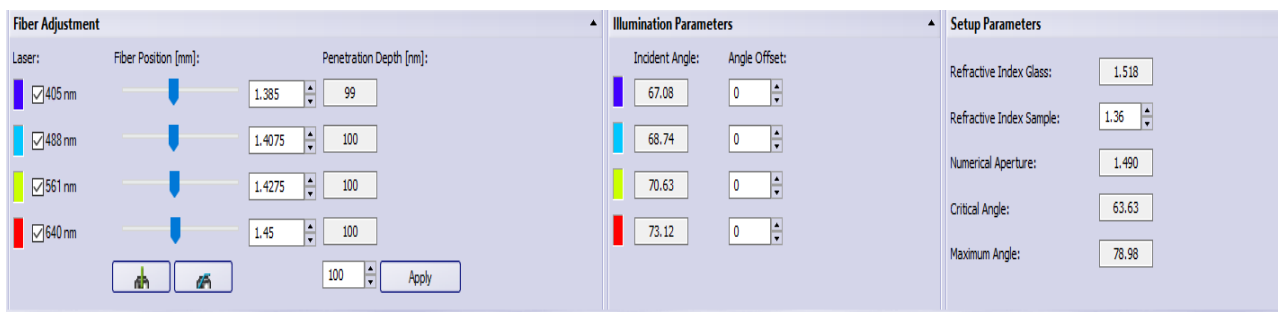
Fig. 3 TIRF Setup

3.3   Setting up 2 simultaneous channels using the W-View Gemini

The W-VIEW GEMINI is an image splitting optic which provides one pair of dual wavelength images separated by a dichroic mirror onto a single camera. Simultaneous image acquisition of dual wavelength images allows you high speed ratio metric imaging and other multiple fluorescence applications. We have Red/Green; Red/Far Red and Green/Far Red bandpass filter sets and the corresponding dicroic mirrors available.

3.3.1   Set up the Hardware (Please wear gloves !!!)

1. Put in the dual color bandpass filterset
2. Put in the Dicroic Mirror
3. Put in the mask 3.9
4. Switch from bypass to dual color mode by pushing the button on top of the Gemini

3.3.2   Setup the Software

1. Go to Microscope Control and choose one of the following observation methods: G/R split (488, 561); R/FR split (561, 640); G/FR split (488, 640). These options will split the field of view and enable the corresponding dual laser excitation.

Please note: Alignment of the two images is very stable and can only be done by BIF. Please contact our team in case the alignment needs further correction.

3.4 Using ZDC Hardware Focus

1. Slide in Dicroic mirror on the left side of the microscope next to eyepieces
2. Go to **Microscope Control** window to **ZDC Control** dialog
2. Select **Continuous ZDC** mode from drop down menu
3. Focus your sample with the Focus drive and press **Find Offset**
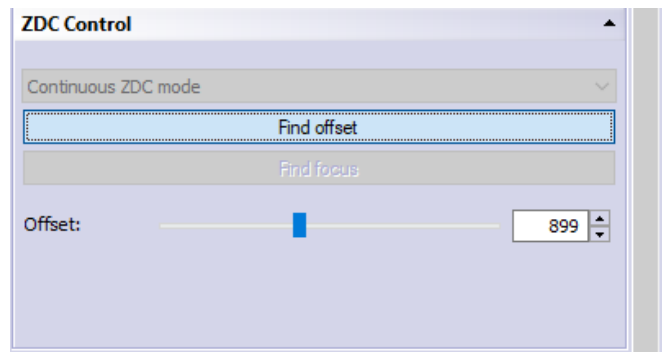4. Focus will be kept and Focus drive will be disabled

Fig.4 ZDC Hardware Autofocus

3.5   Using Experiment manager

The experiment manager (fig. 4) is a graphical tool for configuring experiments. The experiments can be configured by establishing sequences of tasks, or nesting them. These tasks are:

- image acquisitions
- time series
- Z-stack
- multiple position

3.5.1 Guidelines

- The upper icons list the available operations. The first one corresponds to the acquisition of a single image.
- The edit boxes for the parameters associated with the selected object appear in the **Experiment Manager** dialog box.
- For image acquisitions the *channel*, *objective*, *exposure time* and *EM-gain* have to be specified. Press [get settings] to load the current values from the software. Click on the image acquisition icon in your experiment and press [apply settings] to load the image parameters into the software.
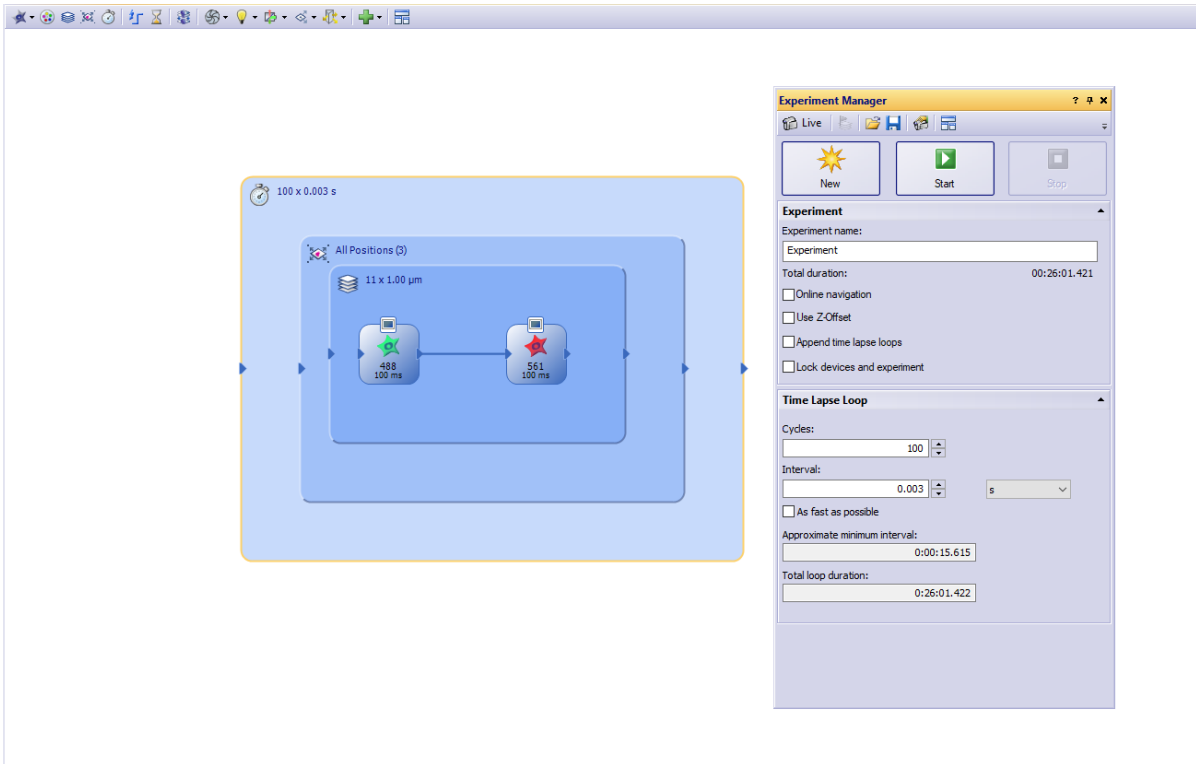

<div align="right">Fig.4 Experiment Manager</div>

3.5.2 Execution threads

The operations cannot take place in parallel, so the task boxes have to be connected to specify the execution order. Wires are used to connect these elements, and sequential operations are performed from left to right.

3.5.3 Loops (Fig.4)

The elements enclosed by a task frame are iterated according to the *nesting hierarchy*.

- Two image acquisition elements enclosed in a time series frame will be executed as two channels over time with n time points (Fig.4)
- For instance, a multiposition frame needs to enclose at least one image acquisition element otherwise the experiment would have an error (iterating nothing).
- A z-stack enclosed by a time series box will acquire the full stacks at every time time point

3.5.4 Setting up Experiments

- Press **New** in the **Experiment Manager** dialog and create your channels.
- If you want to view your images during acquisition, press every individual image icon, go to **Experiment Manager** dialog and activate **Online Display**.

3.5.4.1   Multiple positions

Multiple position experiments require the storage of a list of positions:
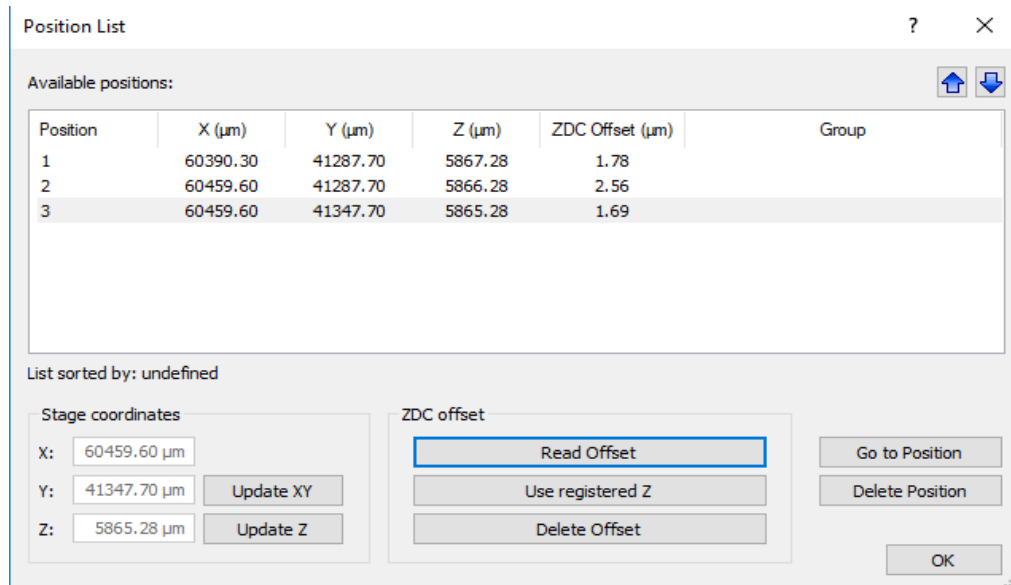

Fig. 5 Position List

1. Enclose your channels with a stage loop (stage icon in the upper panel, Fig.4).
2. Go to life mode in the **Camera Control** window
3. In **Experiment Manager** dialog go to **Stage Loop** dialog and add your positions [+]
4. Open the *position list* window to view your list of positions Fig.5). There's an icon for it in the **Stage Loop** dialog in **Experiment Manager** dialog.
5. If you need an Autofocus over time for each position set ZDC to *single position mode* in the **Microscope Control→ZDC Control** settings
6. In the **Position list** go to each positon and press **Read Offset** (value will be displayed)

3.5.4.2   Time series

1. Enclose the desired operations with a time lapse Loop box (clock symbol icon in the upper panel of **Experiment Manager**, Fig.4).
2. Specify Cycle number and Interval time in the **Time lapse Loop** Dialog (Fig.4)
3. *Aproximate minimal Interval* time and the *Total loop duration* of your timelapse are displayed.

3.5.4.3   Z-Stack

1. Enclose your channel icons with a **Z-stack Loop** (Z-stack icon in the upper panel, Fig.4).
2. Choose between **Range** or **Top and bottom** in the **Z-Stack Loop** settings (Fig.6)
3. Go to life mode in the **Camera Control** window (Fig.2)
4. Set the top and bottom of your Z-stack
5. Set the number of layers
6. For Nyquist sampling **Apply** the *Recommended Step Size*

4   Data Saving

- *All recorded images will appear in folders in the main display*
- **means images are not saved yet*
- *Save over File→Save as, choose .vsi or .tif fileformats, Both will preserve metadata*

Note: Pixel numbers and scale are only correct in. vsi!!!

Fig.6 Z-stack Dialog

4.1   Auto Save

- Go to **Experiment Manager** Dialog and press the icon **Acquisition settings**
- Select **Saving** and choose **movie** or **Snapshot**
- In **Destination** choose **File System**
- *In Directory select your path on the D:drive*
- **Apply to All** will activate auto saving everything to the respective folder

5   Shut Down

1. Transfer your data to the fileserver
2. Close CellSense software
3. Shutdown the Computer
4. Turn Laser Keys
5. Turn Off Buttons on the box from left to right (RTC, Control box, Lasers)
6. Turn Off camera controller
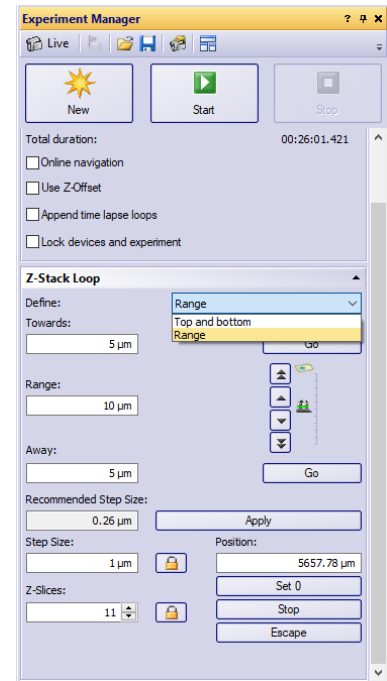7. Close program on the microscope touch screen, only then press the button