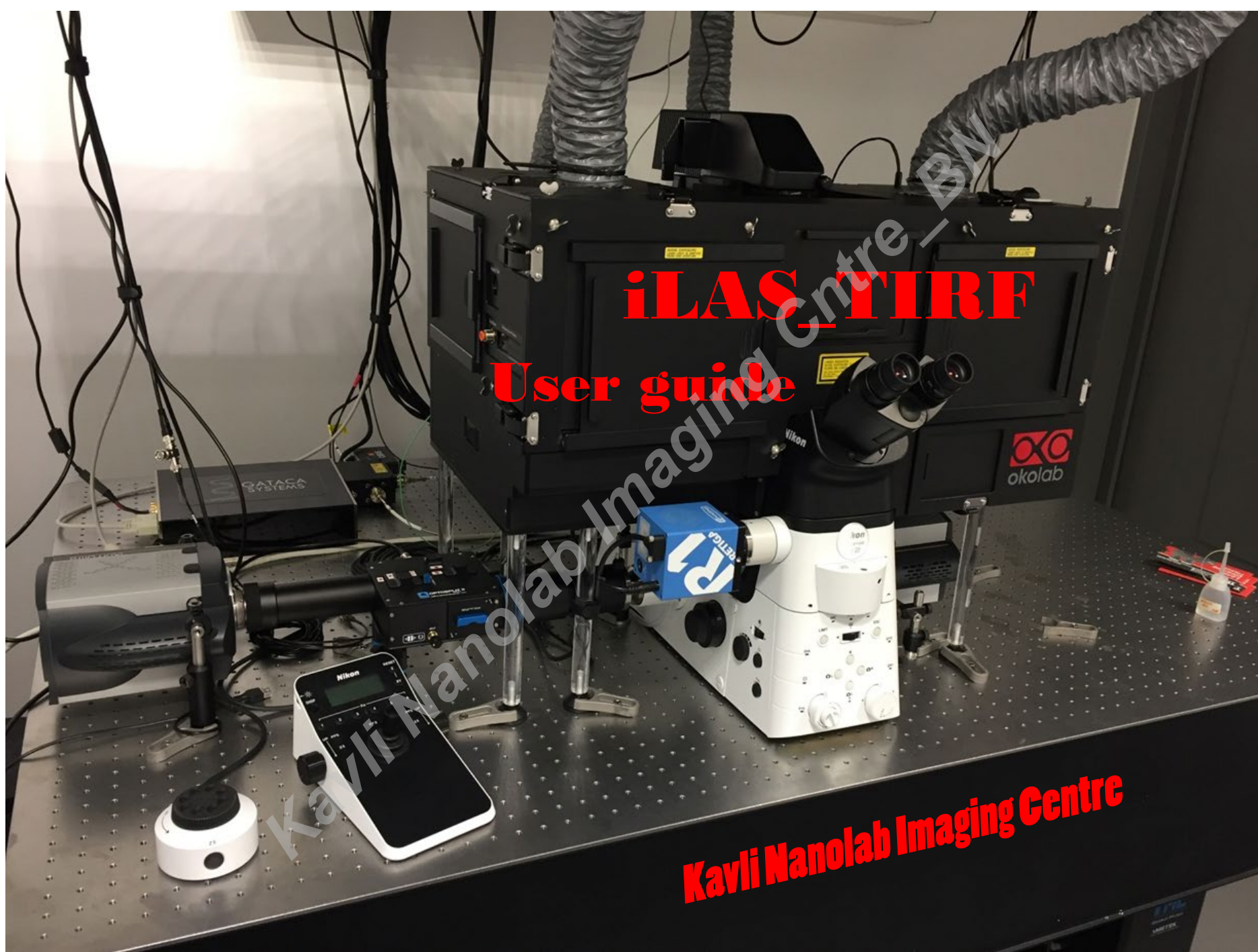


**iLAS TIRE**

**User guide**

**Kavli Nanolab Imaging Centre**

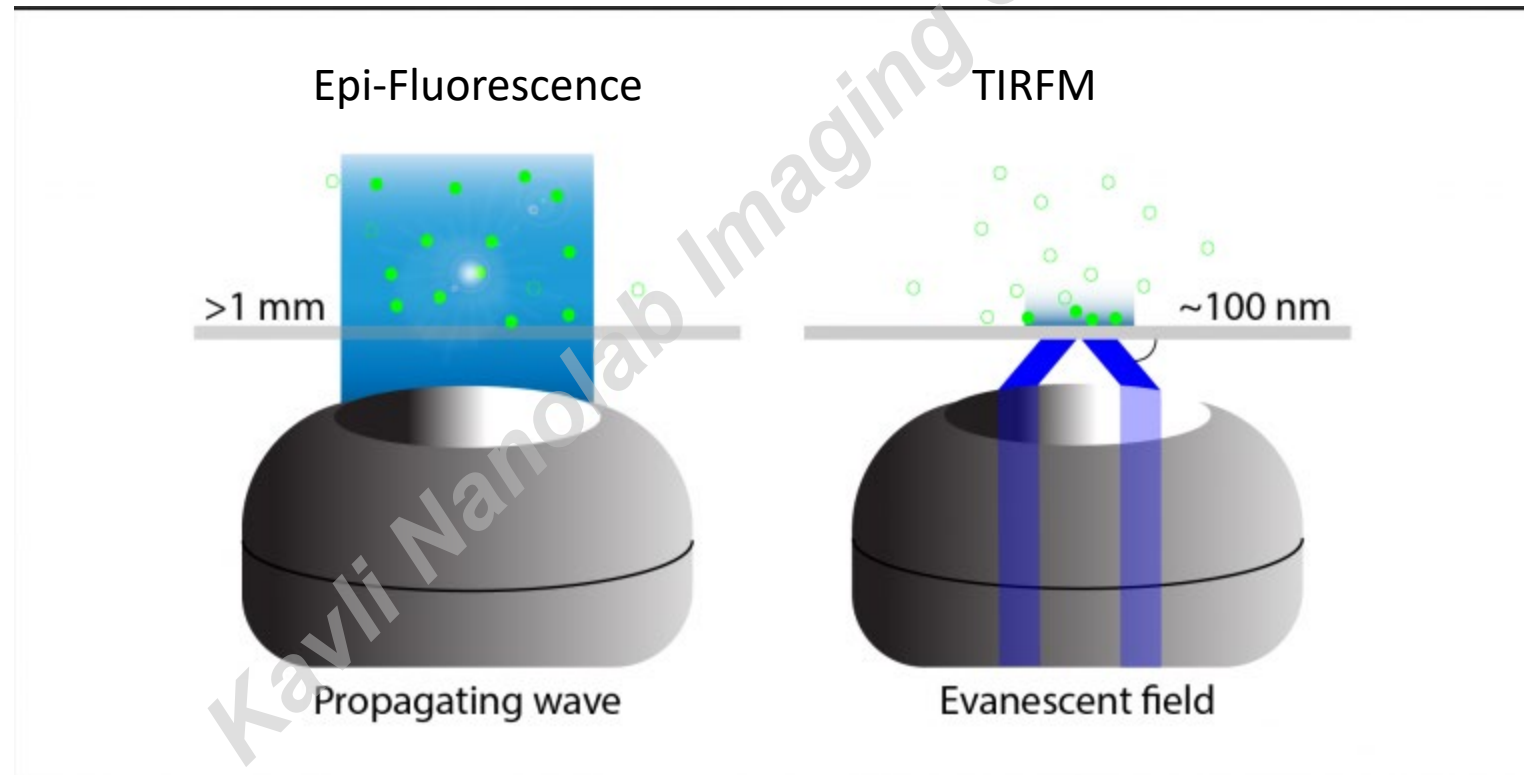


## Snell's law for the critical angle of light

$$\theta_c = \sin^{-1}(n_1/n_2)$$

When light hits an interface beyond the critical angle it is completely reflected, this is called Total Internal Reflection:

In TIRF microscopy, the light is presented to the slide-sample interface beyond this critical angle, either through the **objective** or using prisms. This produces an electromagnetic field at the interface called **the evanescent field** or wave

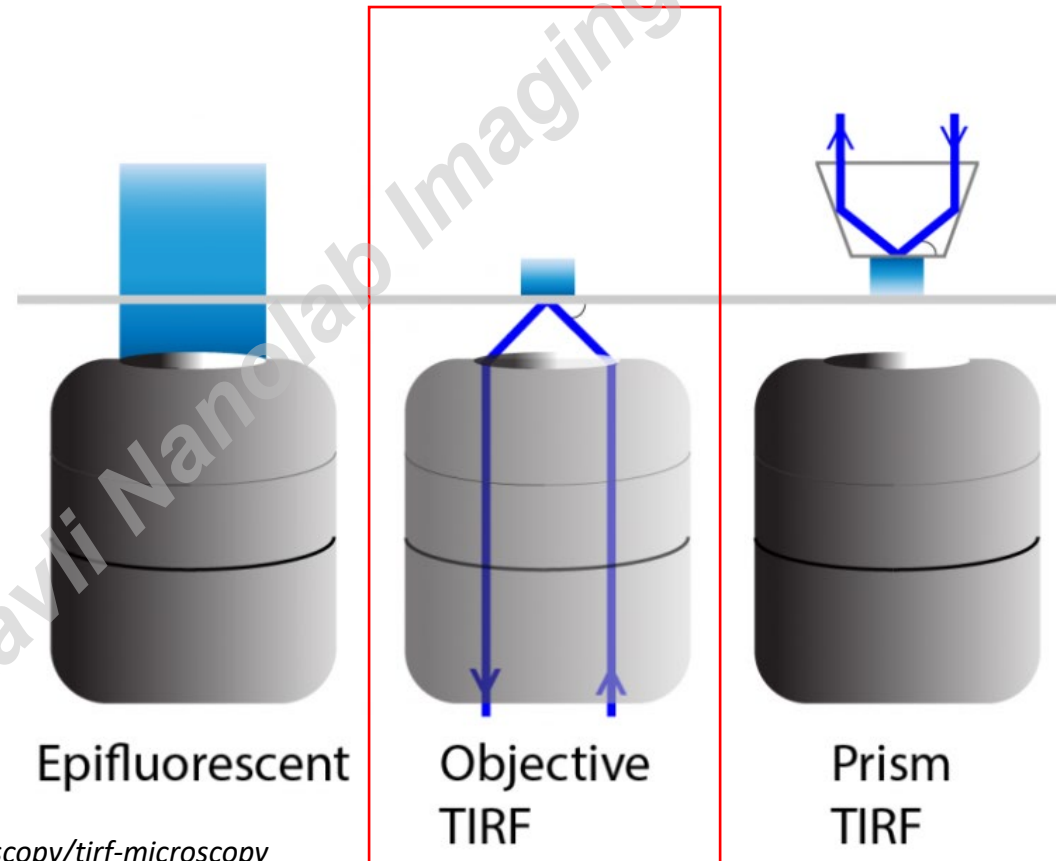


**Objective based TIRF:** the laser light source entering the back-pupil of the objective off-axis. The angle of incidence leaving the lens correlates with the extent of off-axis input light.

Pay attention- your objective should have:

**High NA** ( $> 1.49$ ) to provide shallow field

**Correction collar for temperature-** compensates for change in oil RI



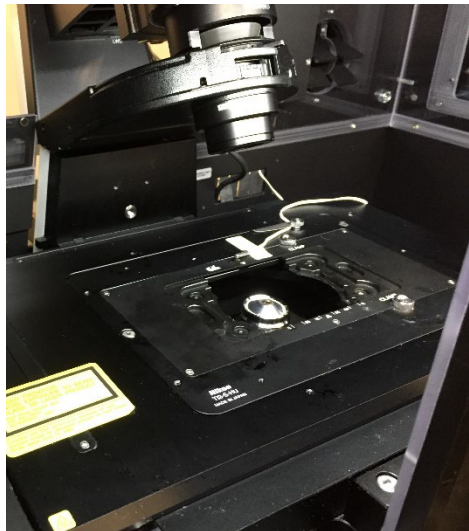


# StartUp:

- 1, Turn ON the 2 main switches
- 2, Turn ON PC and Metamorph software
- 3, Locate your sample (BF only):
  - 1, place oil on 100X objective, place path to eyepiece
  2. Turn on BF light and turn the focus knob to move objective UP until the oil spreads on the coverglass (Slow!)
  - 3, Pay attention- when reaching focus the PFS beeps- view with eyepiece (you always see the glass if moving stage). Activate PFS
  - 4, Change to Camera port (left)- choose Bypass/Split, place filters and align if needed
- 4, View with "Live" button

## Hardware- General:

3



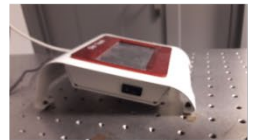
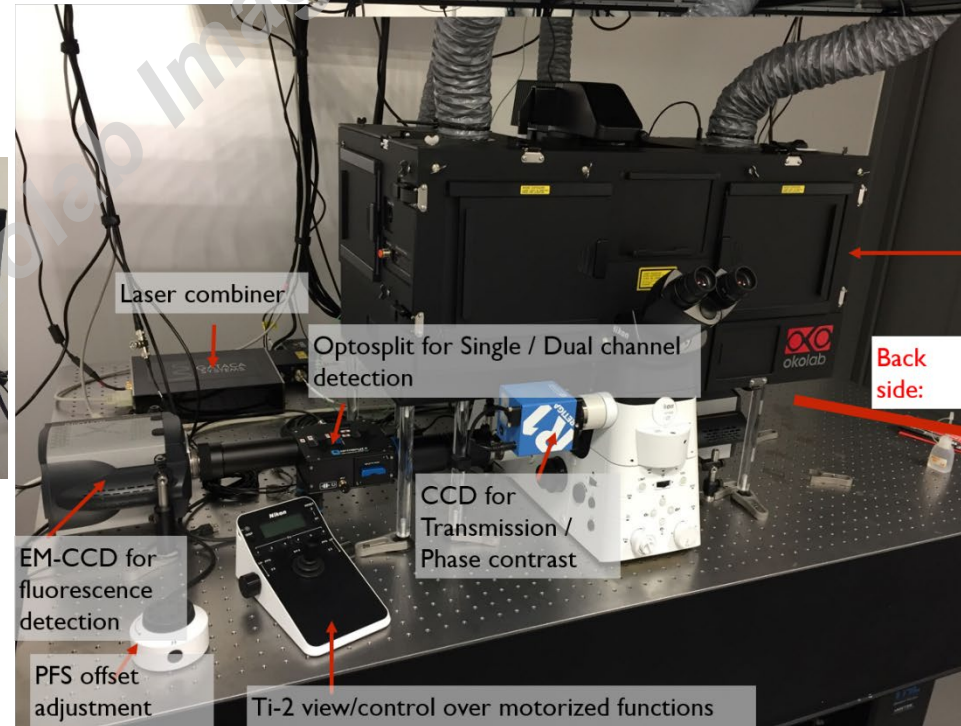
2

PC



1

2 main switches



Okolab incubation box

Optical fibers for TIRF and FRAP laser illumination



Laser power range adjustment

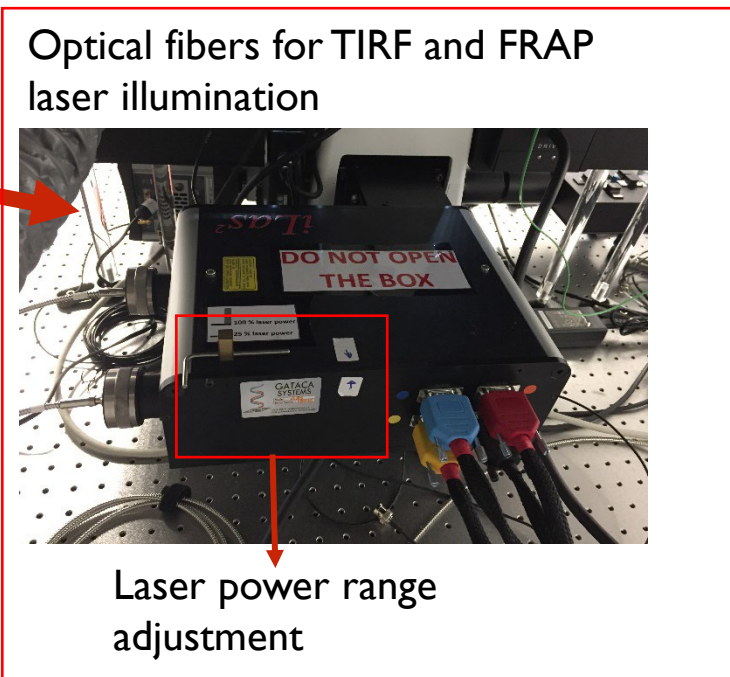
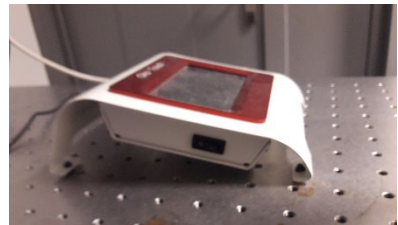
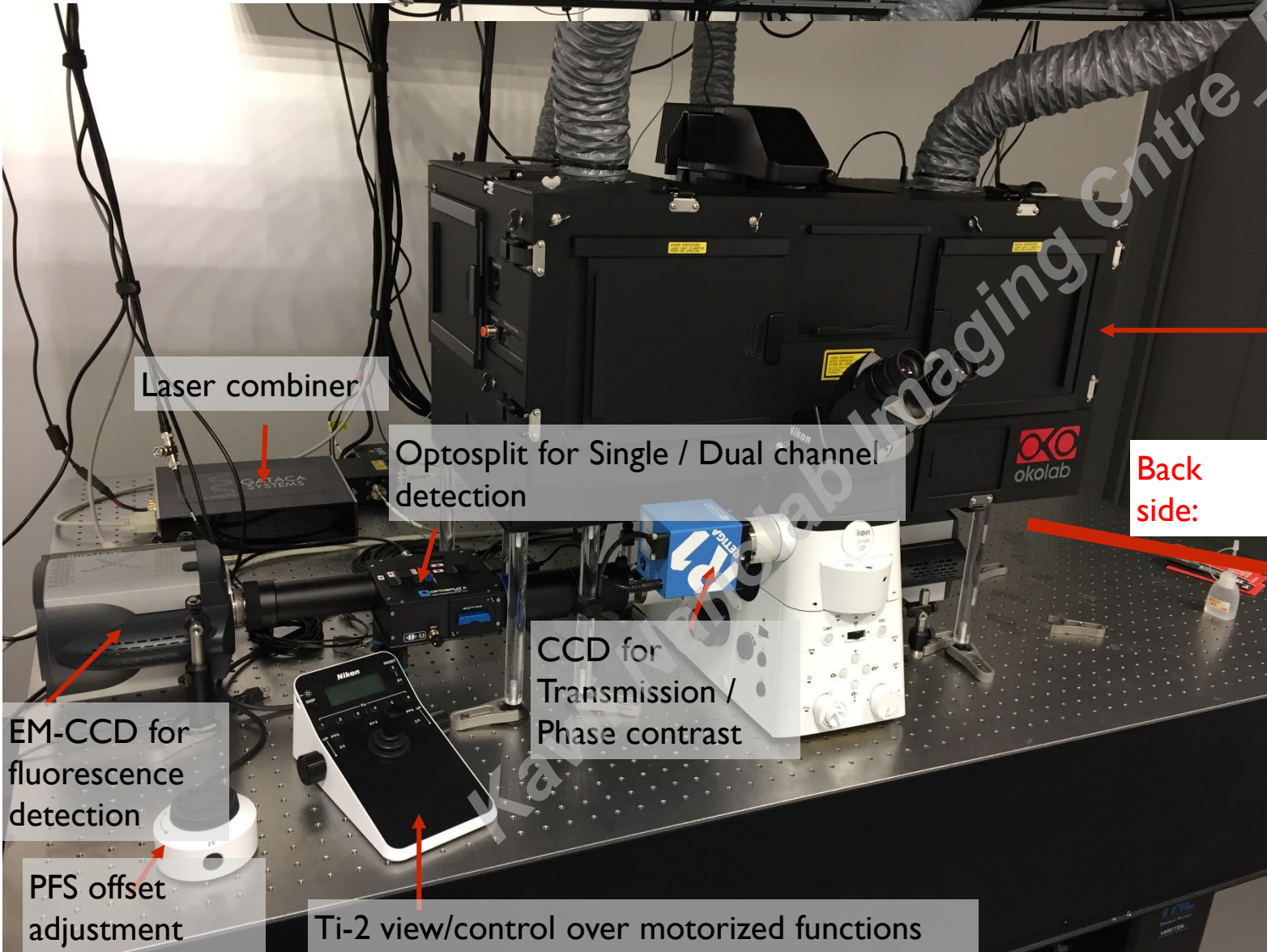
## Shut-Down:

- 1, Save your data to Bulk folder
- 2, Turn OFF PC and Metamorph software
- 3, Remove your sample:
  - 1, Lower the objective height to minimum
  2. Remove your sample and **clean the objective** with 2-propanol (see instructions)
  - 3, Change back to 20X air objective
  - 4, Close Oko-cage doors and clean your environment.
- 4, Turn OFF the 2 main switches

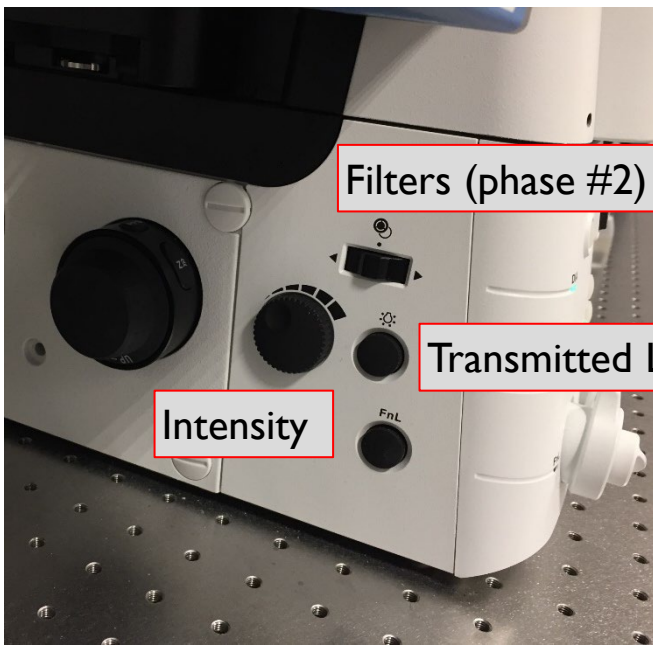
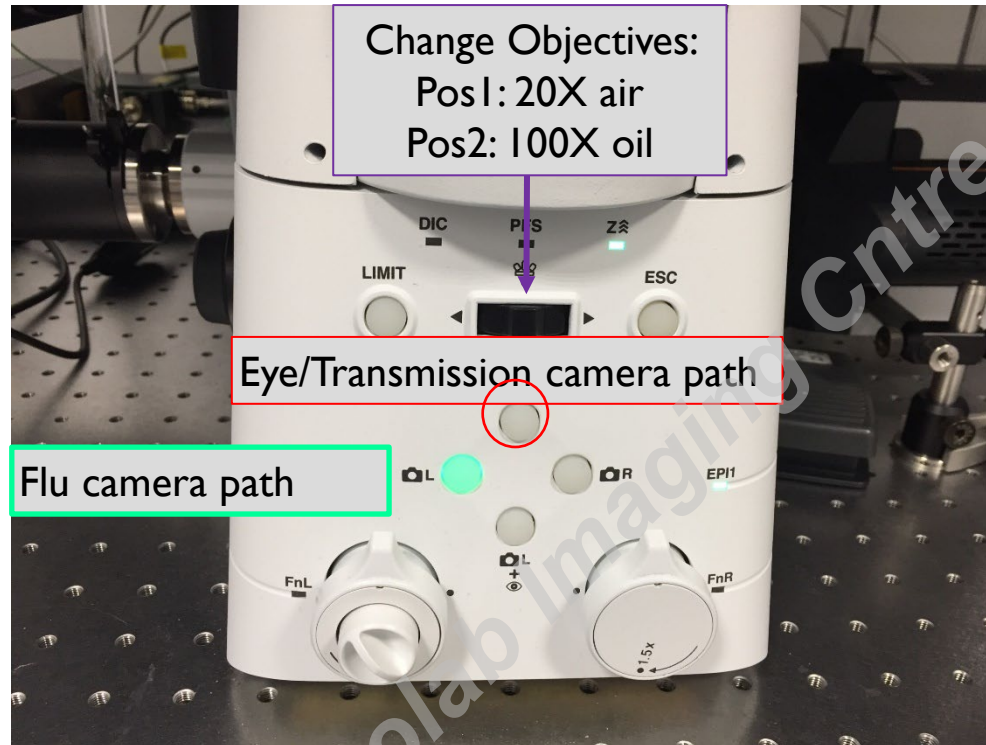
Kavli Nanolab Imaging Centre - BN



# Hardware- General:



# Ti-2 body:



Turn PFS ON

Turn on light in the OKO cage

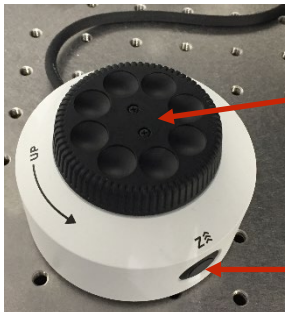
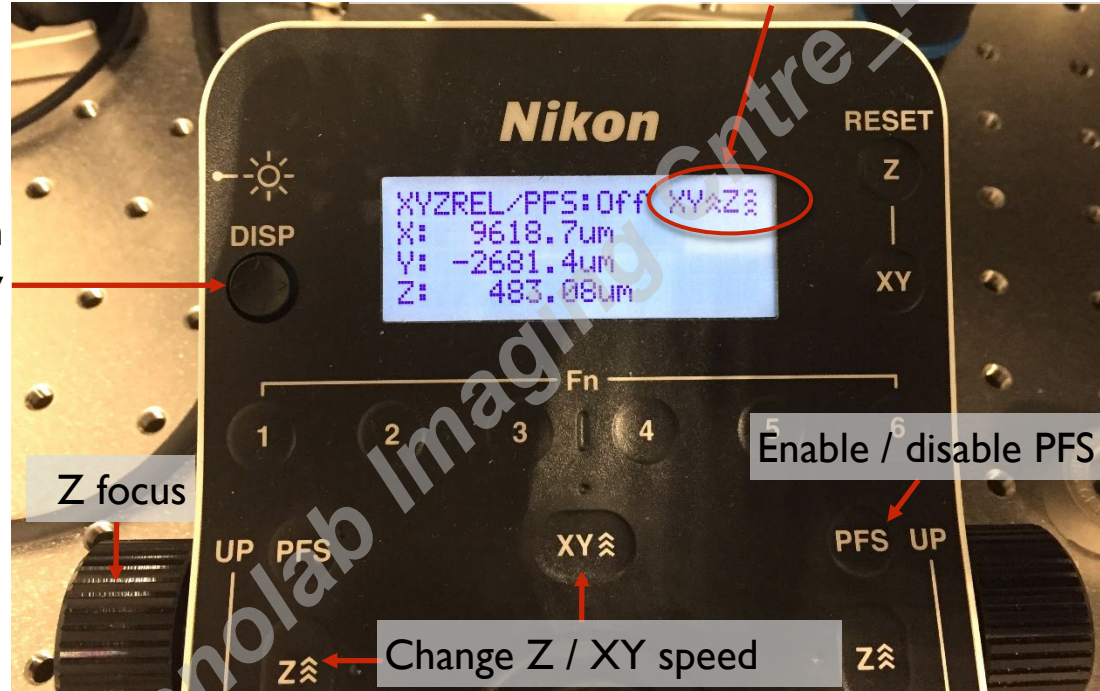




# Ti-2 Joystick

View speed:  
XY: ^^ = fast / ^ = slow  
Z: ^^^ = fast / ^^ = fine / ^ = extra-fine

Change information display



When the PFS is enabled, use this knob to adjust the focus

Keep pressing this button to speed up the focus adjustment

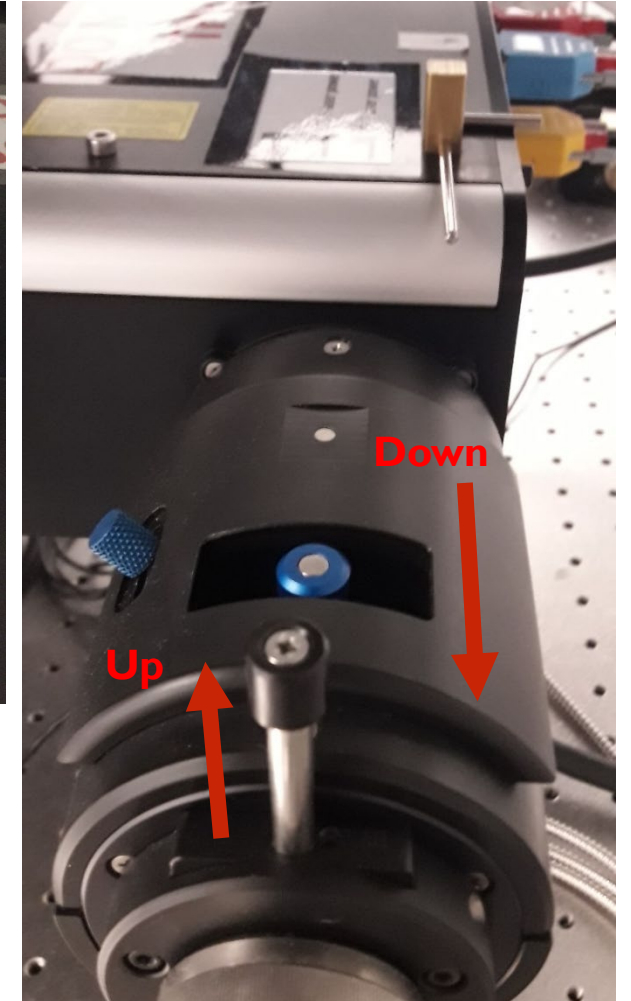


# To modify laser intensity- Hardware

High laser Intensity

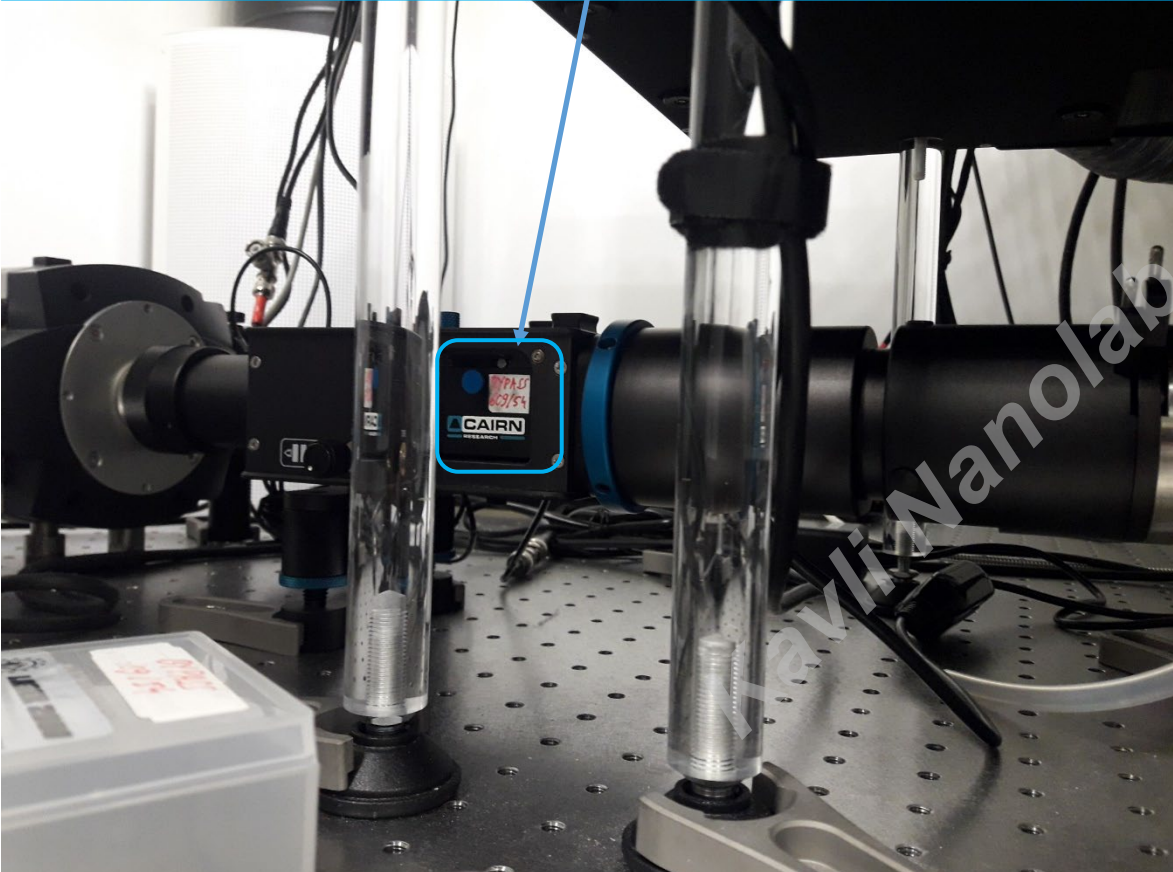


Low laser Intensity



# Optosplit for single/dual flu imaging- Emission path

2, Make sure you have the **filter cube** inserted for **Bypass/Split** with desired emission band (*2<sup>nd</sup> drawer left*)



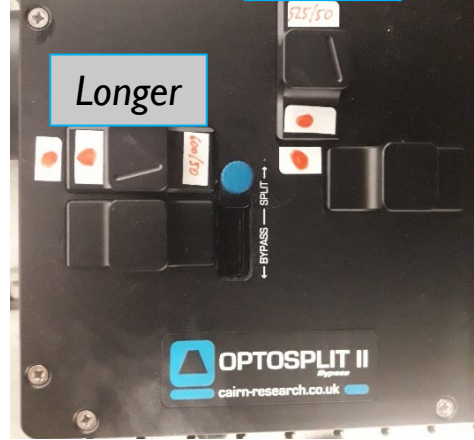
1, Use Bypass path to image only one channel  
Shift knob to split path to image 2 channels



Split filters



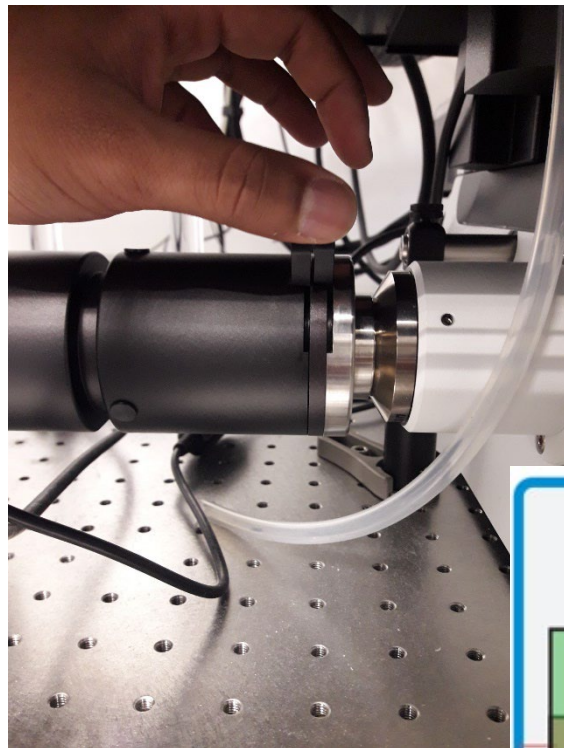
Shorter





# Optosplit for single/dual flu imaging- Alignment

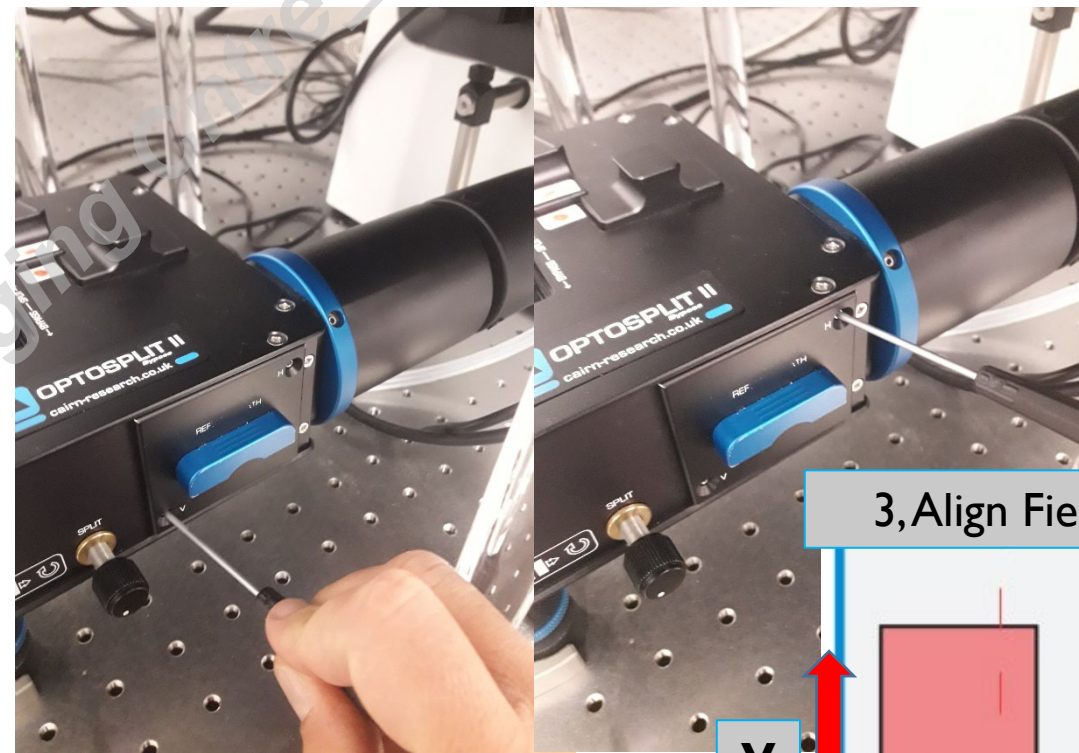
1, Use the knobs to reduce Field size



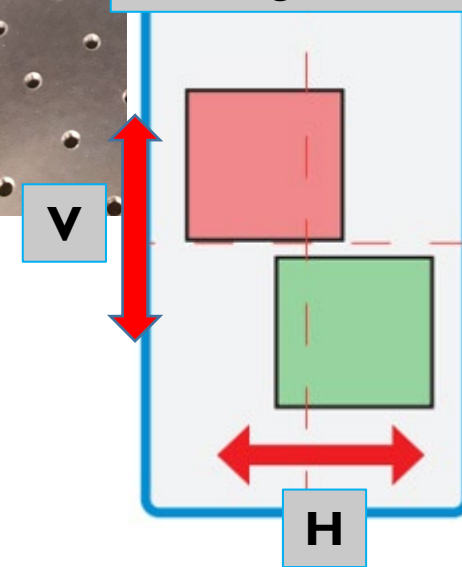
2, Separate Fields (anti-clockwise)



3, Align Fields



3, Align Fields







Your illumination should match the laser line used

# MetaMorph

Snap

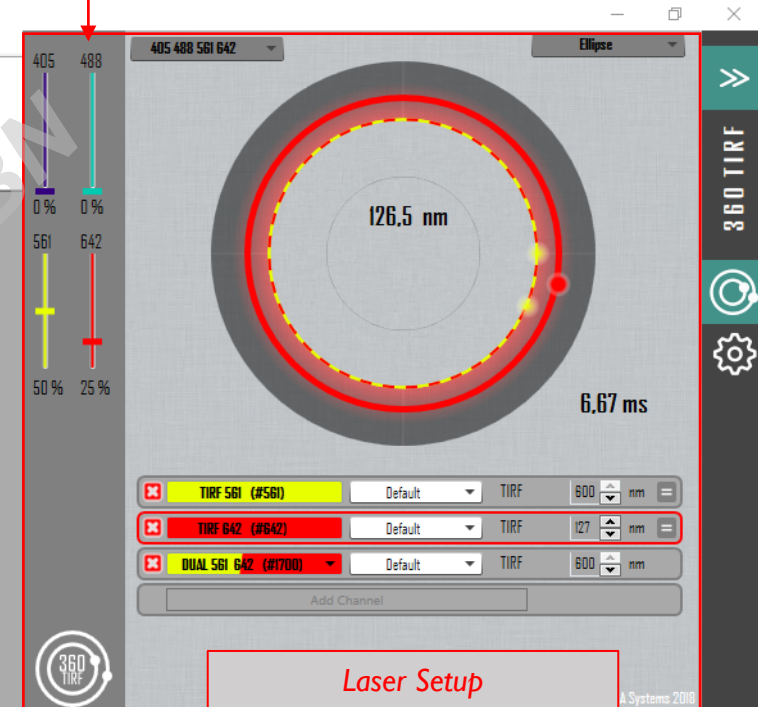
Live

Multi Dimensional Acquisition

In Metamorph, you can work with:  
**Journals** (Acquisition scripts)  
 or  
**Multi-Dimensional Acquisition**

Automatic acquisitions	
Stream TIRF 405	PhaseC + Stream Fluo 488 nm
Stream TIRF 488	Fast PhaseC + StreamFluo + ...
Stream TIRF 561	PhaseC + PALM_561 + Strea...
Stream TIRF 642	PhaseC + ALEX_561_640 + ...
Stream_TIRF_488-561_ALEX	Trans + ALEX_561_640 + Str...
Stream_TIRF_561-640_ALEX	PhaseC + sptPALM_561 + St...
Stream_TIRF_DUAL 561-640	-
Stream_TIRF_488-640_ALEX	-

Quick Setups

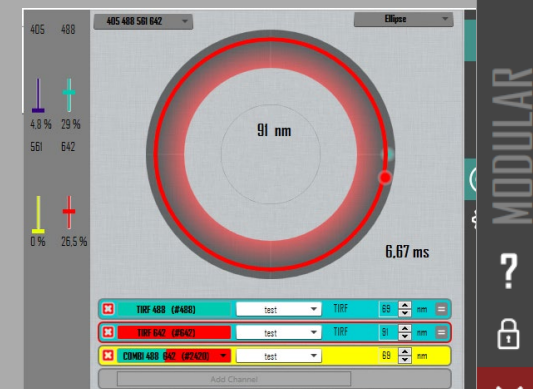


Laser Setup

**TIRF line (#nm1)- illuminate 1 wavelength at a time**

**Dual (#nm1 #nm2)- illuminate 2 wavelengths simultaneously.**  
 Both are restricted to the same TIRF angle

**Combi (#nm1 #nm2)- illuminate 2 wavelengths sequentially, within the exposer time set (faster than acquisition).**  
 Both can be set to different TIRF angles  
 !You need the two individual lasers defined above the combi setup



# Adjust TIRF illumination

## point

405 488  
0 % 0 %  
561 642  
50 % 25 %

405 488 561 642

Point

126.5 nm

2 ms

360 TIRF

TIRF 561 (#561) Default TIRF 600 nm =

TIRF 642 (#642) Default TIRF 127 nm =

DUAL 561 642 (#1700) Default TIRF 600 nm =

Add Channel

360 TIRF

Copyright GATACA Systems 2018

## Ellipse

405 488  
0 % 0 %  
561 642  
50 % 25 %

405 488 561 642

Ellipse

126.5 nm

6.67 ms

360 TIRF

TIRF 561 (#561) Default TIRF 600 nm =

TIRF 642 (#642) Default TIRF 127 nm =

DUAL 561 642 (#1700) Default TIRF 600 nm =

Add Channel

360 TIRF

Copyright GATACA Systems 2018

# Journals view

MetaMorph

File Edit Regions Stack Acquire Devices Display Process Log Measure Journal Apps Window Help

illum: TIRF 561 Mag: 100x TIRF OIL X: -2130.90 Y: 511.70 Z: -2145.92

### Journal Editor

File Edit

Builtin Functions | Recorded Journals | Actions | Journal: C:\MM\app\mmproc\journals\lri\_variables.jnl

View: Alphabetical

- 3D Reconstruction
- Acquire
  - Acquire - Acquire Background
  - Acquire - Acquire Shading
  - Acquire - Auto Expose
  - Acquire - Get Flatfield
  - Acquire - Load Background
  - Acquire - Load Setting
  - Acquire - Load Shading
  - Acquire - Measure Black Balance
  - Acquire - Measure White Balance
  - Acquire - Save Setting
  - Acquire - Start Live
  - Acquire - Stop Live
  - Acquire Background
  - Acquire Color
  - Acquire Image
  - Acquire Shading
  - Acquire Timelapse
  - Acquire Z Series
- Add Plane
- Adjust Digital Contrast
- Adjust Focus
- Align Stack
- Analog Auto Adjust
- Annotate Image
- Annotate Log File
- Application Link
- Arithmetic
- Arrow
- As Displayed
- Assign Time Reference
- Assign Variable

Functions | Descriptions

Initiation variables for automatic acquisition

-----

Camera EM-CCD IXON

-----

Assign To Variable

Assign To Variable

Assign To Variable

Assign To Variable

-----

Camera CCD R1

-----

Assign To Variable

Assign To Variable

-----

Assign To Variable

Variable: Exp\_time\_IXON

Expression: 10

Disable

Save Run Journal Exit

### Automatic acquisitions

Stream TIRF 405	PhaseC + Stream Fluor 488 nm
Stream TIRF 488	Fast PhaseC + StreamFluo + ...
Stream TIRF 561	PhaseC + PALM_561 + Strea...
Stream TIRF 642	PhaseC + ALEX_561_640 + ...
Stream_TIRF_488-561_ALEX	Trans + ALEX_561_640 + Str...
Stream_TIRF_561-640_ALEX	PhaseC + sptPALM_561 + St...
Stream_TIRF_DUAL 561-640	-
Stream_TIRF_488-640_ALEX	-

405 488 561 642

Point

54°

2 ms

4.9% 7.5% 100% 0%

TIRF 488 (#488) Default TIRF 88 nm

TIRF 561 (#561) Default HILO 54°

TIRF 405 (#405) Default HILO 54°

DUAL 405\_561 (#1310) sptPALM 561 HILO 54°

Add Channel

Copyright BATAKA Systems 2018

### Acquire

Acquire Image: IXON

Save Image Save to: C:\MM\...Acquired001.tif Set Save...

Save w/Sequence

Display | Acquire | Correct | Annotate | Special | Andor | Live Replay

Exposure Time: 100 ms

AutoExpose

Binning: 1

Camera Area: Full Chip

Center Quad

Use Active Region

Show Live

Live Bin: 1

Temp: ~ -70 c

Setting [Modified]: IXON

Close Less << Setting: Load ... Save Save As...

Digitizer: 16-bit (17 MHz, EM Gain)

Enable EM Gain 200

Vertical Shift Speed: 3.33 MHz (0.30 usec/pixel)

Vertical Clock Voltage: +4

Pre-Amplifier Gain: Gain 3

Camera Shutter: Always Open

Info...

Cooler On  External Trigger

Camera State: Non-Overlapped

Baseline Offset: 0

Show Focus Indicator  Baseline Clamp

Frames To Avg: 1 Reset



## Work on Ini\_Variables Journal

Select a journal to Edit

Look in: journals

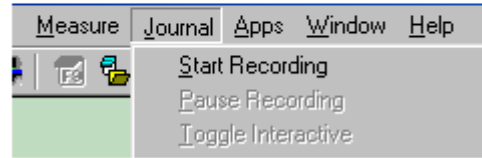


Name	Date modified	Type	Size
Micellaneous	18-12-2020 15:50	File folder	
ModularJournals	18-12-2020 15:50	File folder	
Aswin_timelapse.JNL	31-5-2022 17:04	JNL File	22 KB
avgthresh.jnl	11-1-2013 09:38	JNL File	4 KB
avgtime.jnl	11-1-2013 09:38	JNL File	3 KB
centerplane.jnl	11-1-2013 09:38	JNL File	1 KB
disablemdamontage.jnl	11-1-2013 09:38	JNL File	1 KB
enablemdamontage.jnl	11-1-2013 09:38	JNL File	1 KB
Fast PhaseC + StreamFluo + StreamPhaseC .JNL	31-5-2022 15:23	JNL File	60 KB
Fast_TIRF_561-640_ALEX.JNL	16-1-2019 16:47	JNL File	44 KB
Ini_variables.JNL	19-10-2022 15:03	JNL File	2 KB
invert16.jnl	11-1-2013 09:38	JNL File	5 KB
invert16stk.jnl	11-1-2013 09:38	JNL File	7 KB
IXON.JNL	10-9-2019 16:57	JNL File	4 KB
IXON_int_trigger.JNL	11-12-2018 10:44	JNL File	4 KB
loadrgns.jnl	11-1-2013 09:38	JNL File	2 KB
mdanostacquire.inl	11-1-2013 09:38	INI File	10 KB

Kavli Nanolab Imaging Centre - BN

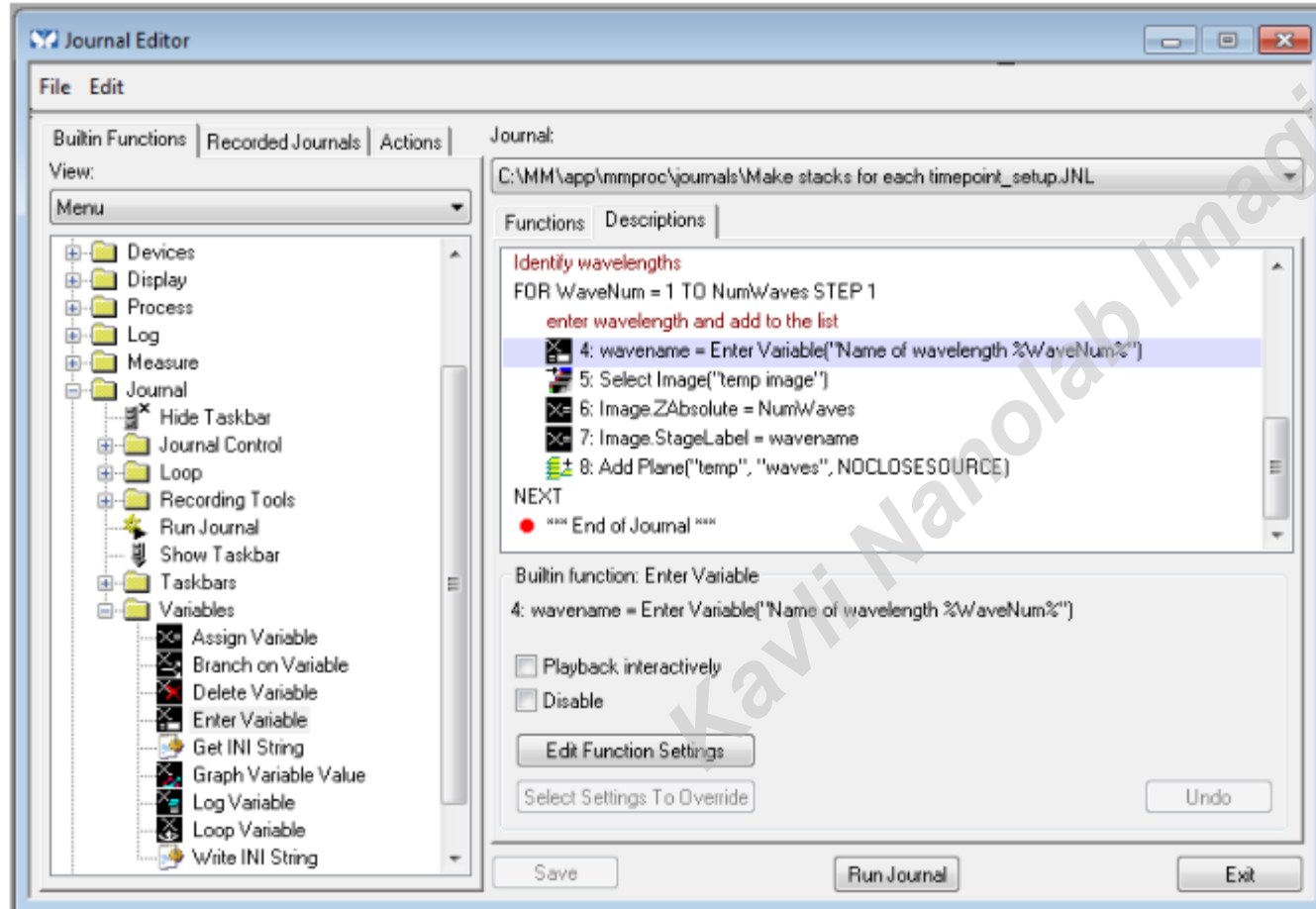
## How do I create and edit a journal?

To record a journal, select **Start Recording** from the **Journal** menu.



## The Journal Editor

To edit a journal or create a new journal, select **Edit Journal** from the **Journal** menu.

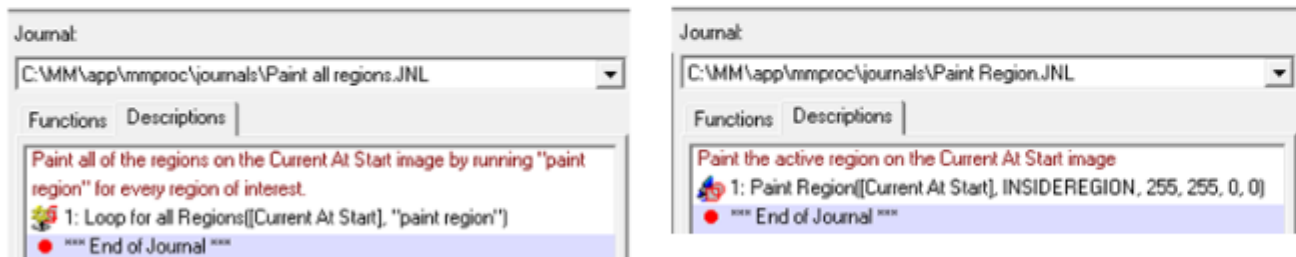


## Running sub-journals and looping journals

A sub-journal is a journal that is run within another journal.

### Sub-journals

If several steps in a procedure need to be repeated, you can reduce the complexity of a journal by placing the repeated steps into a separate journal that is executed multiple times from the main journal. For example, suppose we are writing a journal in which we want to paint every region on an image. This journal could be written to select region 1, paint the image, select region 2, paint the image, and so on for as many regions as needed. It is much simpler to use the built-in journal function **Loop for all Regions** to run a second journal. The second journal paints the active region of interest.



Sub-journals can also simplify the use of image selectors. If the journal asks the user to select an image interactively or to open an image file, you cannot predict the name of the result active image. In this case, you could request that the user make the image active once, and then run a sub-journal. When you run the sub-journal, the active image window becomes "Current At Start" while it is running.

### Types of loops

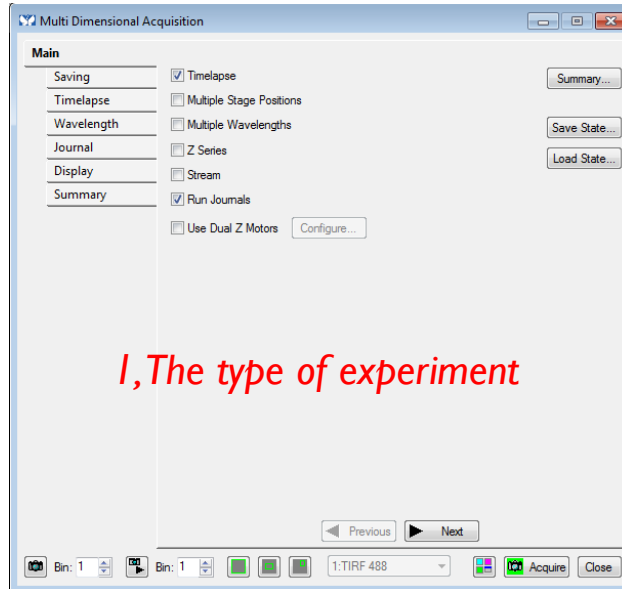
The MetaMorph Software provides a variety of ways to loop and run a journal. The loop functions all execute a selected journal a specified number of times. Some of the loop functions include:

- **Loop a Journal:** Runs a journal a specified number of times and can include a time interval between loops.
- **Loop for all Regions:** Makes each region of interest on the image active and runs a journal.
- **Loop for all Planes:** Makes each plane of a stack active and runs a journal.
- **Loop for all Images:** Makes each image window active and runs a journal
- **Loop for all Images in Directory:** Loads each image in a directory and runs a journal.
- **Run Journal for Multi Dimensional Data:** Loads each time point, Z position, and/or stage position, and runs a journal.

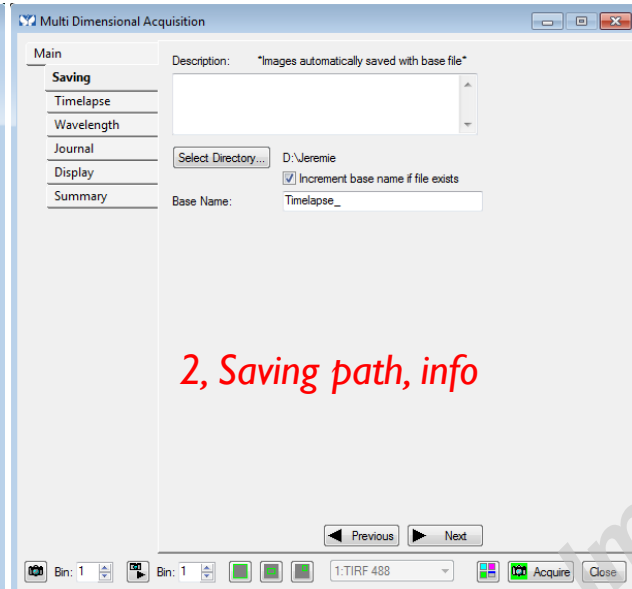


# Multi Dimensional Acquisition view- Step by Step

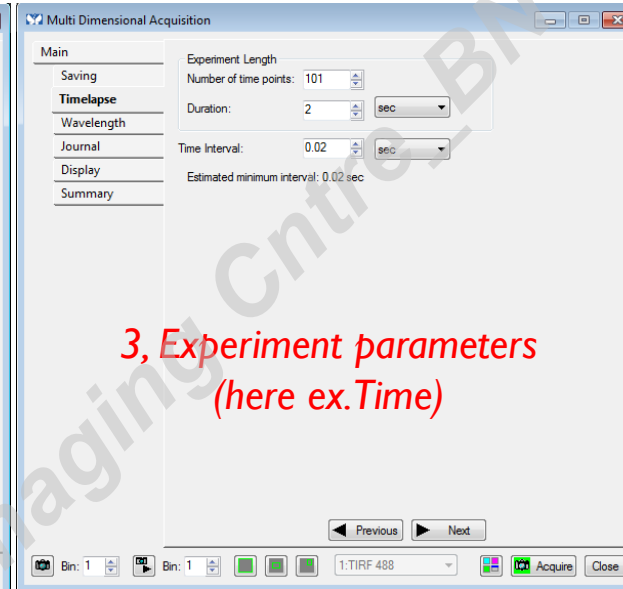
Choose:



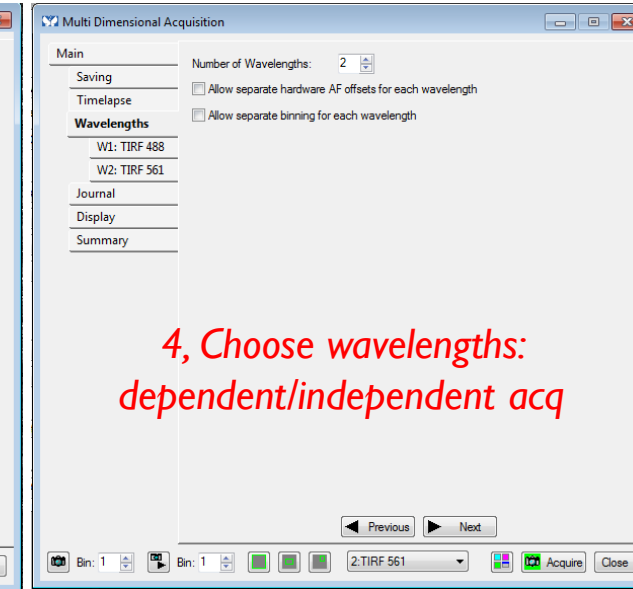
1, The type of experiment



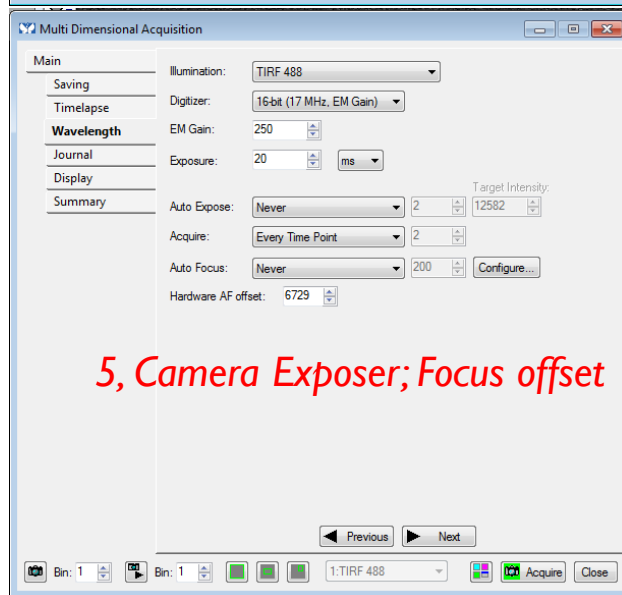
2, Saving path, info



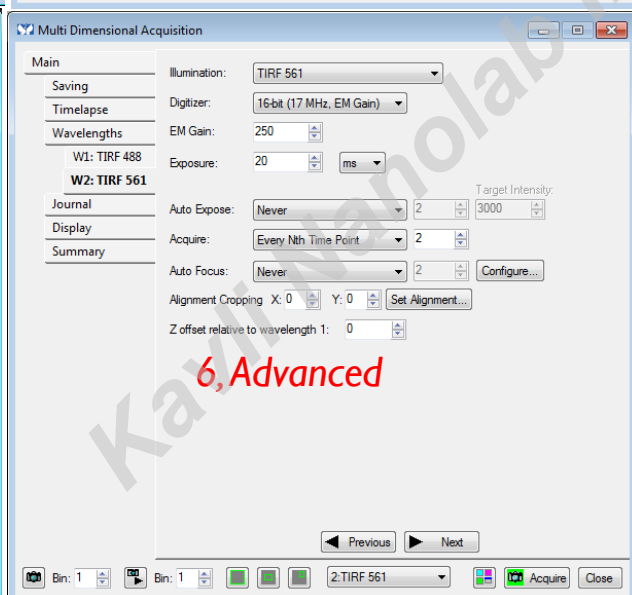
3, Experiment parameters  
(here ex. Time)



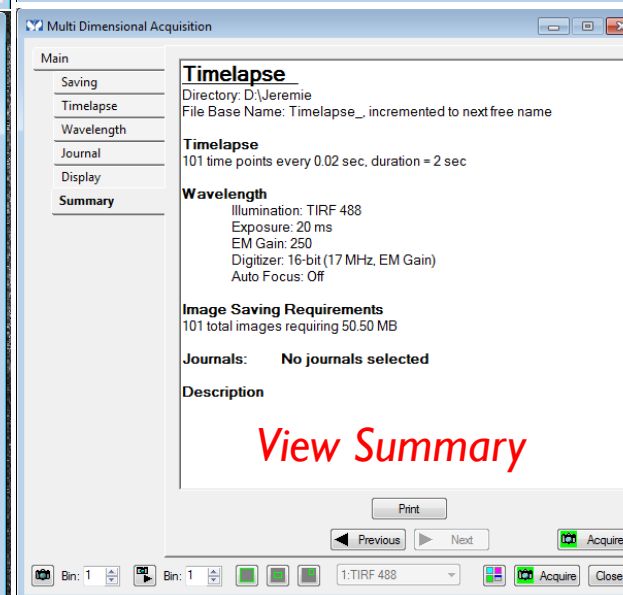
4, Choose wavelengths:  
dependent/independent acq



5, Camera Exposer; Focus offset



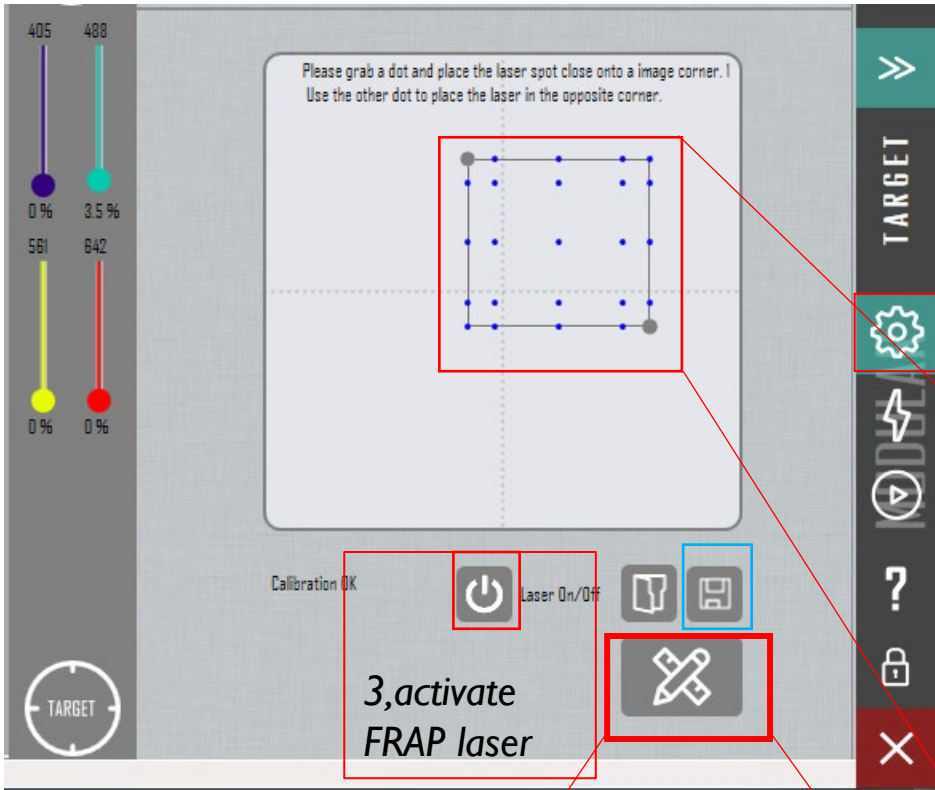
6, Advanced



View Summary

# FRAP calibration

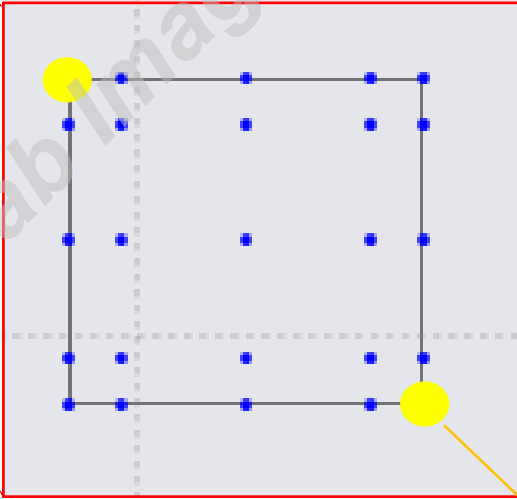
To calibrate the FRAP illumination with your field of view, you need to place a calibration slide (Ask us for it) or an empty position on your own sample (no signal)



1, Find the sample's focus, adjust laser intensity and camera exposer

2, Click on the calibration tab

3, activate FRAP laser



4, click on the top left circle- you should see a bright laser spot : place it on the top left corner in your view. Then do the same for the bottom right circle. By this you define the area to FRAP.

5, Click on "calibrate" to illuminate a matrix of laser points in your defined area.

Make sure the laser spot is seen round and small size (lower laser intensity if needed)



You can save and load your calibration settings

# FRAP on the fly\*- check your calibration

The screenshot displays the MetaMorph software interface. On the left, the 'Acquire' panel shows settings for an IXON camera, including exposure time (50 ms), binning (1), and various hardware parameters. The central window shows a live image of a sample with a pink rectangular ROI. On the right, a control panel features a circular diagram with a yellow ROI and a diameter of 112.3 nm. Below this, channel settings are listed: TIRF 561 (#561) and TIRF 488 (#488). A 'LIVE' button is highlighted with a red box. At the bottom, a status bar shows system information like '1 pixel = 0.125 um' and '27.7 GB physical memory'.

*\*Useful when you want to bleach without setting a full experiment*

**To verify calibration:** Increase number of repetitions and laser intensity, press "live" and check if the laser spot is on your ROI



# FRAP experiment- setup

**Use this to record pre and post imaging (camera is OFF while bleaching)**

1, Preview and adjust your sample's view. Snap an image at good SNR

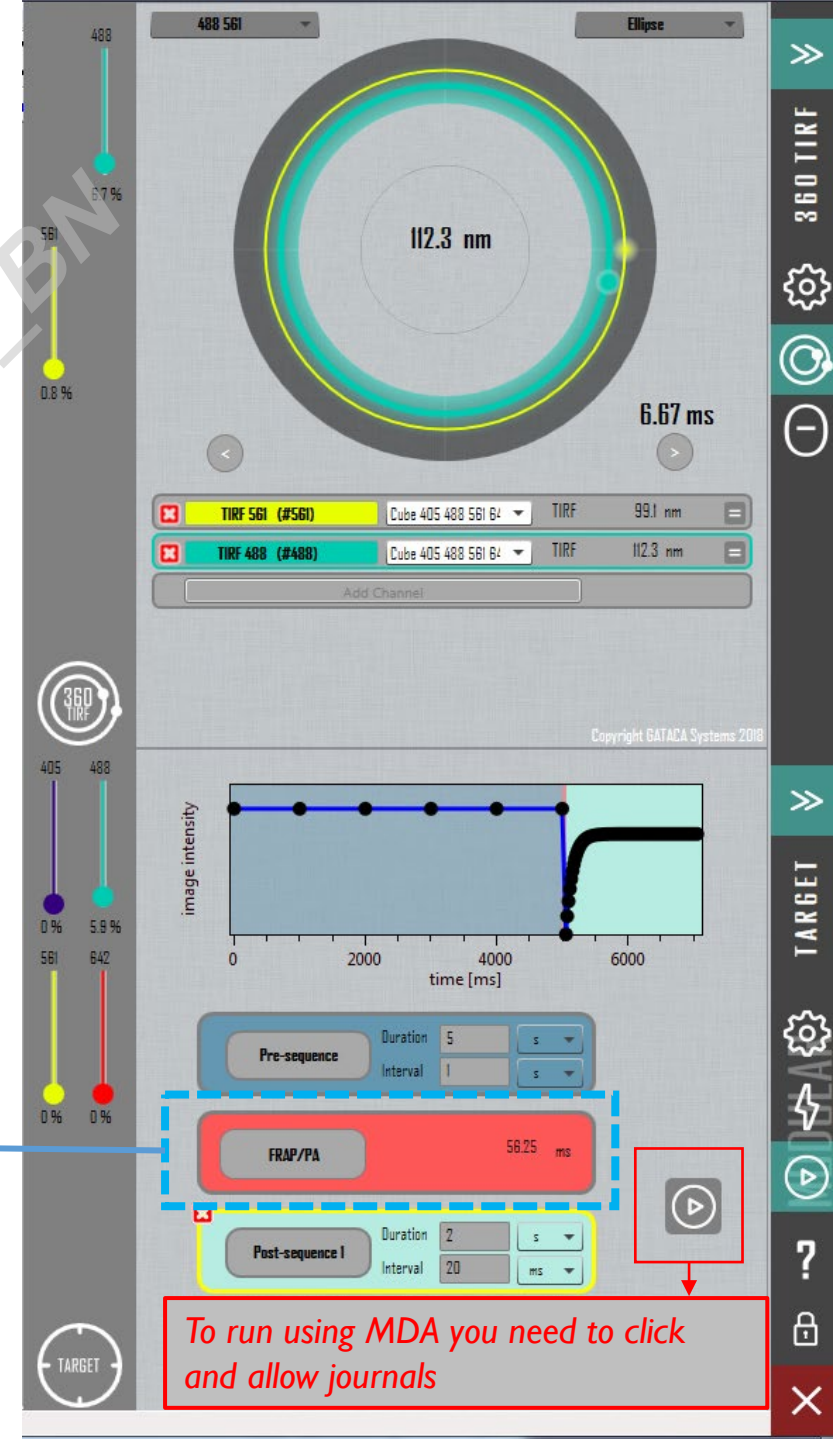
2, Mark your ROI(s) on the snap 

3, Adjust bleaching parameters: #repetitions; laser power (On the fly\*)

4, Click on laser activation to test it

5, Setup interval and duration for pre and post FRAP acquisitions.

*\*the bleaching parameters correspond to your "FRAP on the fly" settings*



488 561 Ellipse

112.3 nm

6.67 ms

TIRF 561 (#561) Cube 405 488 561 642 TIRF 99.1 nm

TIRF 488 (#488) Cube 405 488 561 642 TIRF 112.3 nm

Add Channel

360 TIRF

405 488 561 642

0% 5.8% 0% 0%

image intensity

0 2000 4000 6000

time [ms]

Pre-sequence Duration 5 s Interval 1 s

FRAP/PA 58.25 ms

Post-sequence I Duration 2 s Interval 20 ms

TARGET

To run using MDA you need to click and allow journals