

# Setting up the Zeiss Elyra for TIRF imaging

Follow the start-up procedure described elsewhere. If you require Temperature and  $CO_2$  control follow the relevant procedure.

TIRF imaging should be done with the 63x Oil NA 1.46 lens. This has a correction collar which will



need to be adjusted when working at 37°C.

For the adjustment use the additional mark on the objective barrel. The small black mark (left image below) on the correction rings refers to the RT setting and the red mark is for 37°C. The additional mark (thicker Edding mark, no photo) on the correction ring refers to the setting for 27°C, the standard temperature we run the microscope at. <u>You</u> <u>should set the correction collar back to this</u> <u>thicker mark at the end of the session, when</u> <u>setting back the temperature.</u>





## **Adjusting Zen Black controls**

ZEN 3.0 SR	
File View Acquisition Maintain Macro Tools Window Help	
Locate Acquisition Processing Maintain	
	Acquisition Parameter
	D. E. Smarimant Darisman
New	
AF O I O O Find Focus Set Exposure Live Continuous Snap	Objective alpha Plan-Apochromat 63x/1.46 Oil Korr M.
- 7 One	Frame Size X 1280 🗘 X*Y Y 1280 🗘
Time Series	
Bleaching	Averaging
Tile Scan 9.38 MB	Number 1 Det Death 15 Bit
Regions Start Experiment	
# Setup Manager	image Size: 123.9 μm x 123.9 μm
Ser	Pixel Size: 0.10 µm
Switch track every Frame	
Tarda	Reset All
Irack1	
🐔 TV 2 🐔 TV 1	
SRS 10 560	🕨 🍰 Incubation 🛛 🖉
	🕨 🛥 Online Processing Options 💿 Show all 📝
Lens 1.6x	✓ ▲ Channels
SIM Empty	Tracks Channels
ransmission 🔊 👘	Track1 TV1
alpha Plan-Apochromat 63x/1.46 Oil Korr	ン A + 市 Expand All Collapse All
M27 Var2	Taraha Lattice Cita
	Irack1 - Lattice SIM
Incubator Stage Focus	Lasers
	405 488 561 642
	Exposure Time [ms] 50.00 \$
Aperture 0.55	O Display
	() 🎲 Focus 🗢 Show all 🐉
<b>₽</b>	💌 🛟 Definite Focus 🗆 Show all 🛃
Off 79 %	
	Offset 0.0 µm
	Find Surface
	Store Focus Recall Focus
	Interval 0 s
	Periodic
	Focus Devices and Strategy Show all  Strategy
CPU 0% Free HD 10 TB	

On the *Acquisition tab* arrange and open the following windows as shown to the left if they do not appear after the software has started.

Under Setup Manager

Imaging Setup

Under Acquisition Mode

- Acquisition Mode
- Channels
- Definite Focus

This will help you when setting up the controls and while imaging.

In the next steps you will make modifications in most of those windows to enable TIRF imaging.



👻 🔲 Imaging Setup		🗆 Show all 🛛 🔂
Tracks Track1		+ 1
WF Apotome	Lattice S	SIM Laser WF
Switch track every Frame		8
Track1		
<b>1</b>	TV 2	TV 1
		SBS LP 560
		SPr
		Lens 1,6x
TIRF	Angle: 0.0°	
		LBF 405/488/561/6 42
Pos: 18	815	3D
alpha Plan-Apochromat 6 Korr M27 Var2	3x/1.46 Oil	
Incubator Stage	Focus	
Аре	rture 0.55	
Off 79 %		

In the *Imaging Setup* window adjust the following:

Activate the *Laser WF* tab, this is required to enable TIRF modalities in the light path.

At the top, activate the cameras you want to use – *TV1* and *TV2* can be used simultaneously. When using *SBS LP 560* you can detect either BP 420-480 or BP 495-550 on TV2, on TV1 you can detect either BP570-620 or LP655 – this will be set automatically when choosing *SBS LP560*. You can set up the LUTs accordingly at the cameras/ *TV1* and *TV2* to help discriminate the channels.

Make sure to use the Lens 1,6x!

Move the *LBF 405/488/561/642* filter into place.

Next to the laser symbol you change the second symbol from the left to *TIRF*. This will change the *Pos values* under the box next to this. This value refers to the collimator settings which you will control from the Channels window (see further down).

A quicker way to set the *Imaging setup*: <u>while on the *Laser WF* tab</u>, go to the *Experiment Manager* section at the very top and load *Basic TIRF two channels gr* (gr = green red).



👻 🛥 Acquisition I	Mode	🗆 Show all 🛃
Objective	alpha Plan-Apochromat 63x/1.4	6 Oil Korr M: 🔻
	X 1280 🗘 X*Y	Y 1280 🗘
	128 x 128	
	256 x 256	
	1 512 x 512	Bit 🔻
HDR	1024 x 102 1280 x 128	24
 Noise Filter	TIRF or WF imaging (denoising o	n)
🕤 Scan Area		
	Image Size: 80.1 μm x	80.1 μm
	Pixel Size: 0.06 μm	
·		Reset All

In the *Acquisition Mode* you need to make sure that the *Noise Filter* is set to *TIRF or WF imaging (denoising on).* 

Here you can also control which field of view you use in your acquisition by choosing a suitable value from the drop-down menu. You cannot choose or draw another *Frame Size*/ ROI beside what is suggested.





In the *Channels* window you can select which lasers you need to use for excitation. This should obviously match your sample labels and camera selection. You can change the LUT table color here or in the Imaging Set up.

You can control the amount of illumination power for each laser individually.

Between the three illumination modes *EPI*, *HILO and TIRF* you can toggle and this will change the values in the *TIRF Angle* slider. The green mark on the *TIRF Angle* slider marks the theoretical optimal

value for the angle. Close to this setting the software will show the calculated penetration depth for the given angle and used excitation wavelength. This will appear in the TIRF mode and also in HILO if

👻 🖄 Chan	nels			🗸 Sh	ow all 🛛 📝
Tracks		Channels	\$		
🗸 Tra	ick1	TV1			-
		TV2			<b>I</b> -
			Exp	and All Colla	apse All
	iguration no	t defined		🖻 li	1 <del>w</del>
Track1 -					
Lasers 40	J5 488 5	✓ □ 61 642			
🔺 488 i	nm ——(	]		_	2.0 🛟
🔺 561 i	nm ——(	)		-	2.0 🛟
FOV					
	n mode	PI HILO	TIRF	3D	SMLM
			- <b>J</b>	66.9	3
			0	1704	:
	n: 488	nm : 120.6	nm 561	nm : 135.4 ni	n
Save TIRF a	angle Reset	Si	ave collimat	or position(s)	Reset
TV1				50.0	0 🛟
TV2		ne [ms]		50.0	0
	Camera Tem				
Laser Tr	igger				
Display					

you have moved the TIRF angle slider closer to the green mark.

How to use and control the *Collimator slider* will be discussed in the training. You should <u>never</u> use the *Save* buttons, neither for TIRF nor Collimator position.

Further below the camera exposure can be controlled. If you are using both cameras they will use the same *Exposure time*, it cannot be set independently.



#### Additional Zen Black controls

#### Positions

Activating the tick box at *Positions* (left from Start Experiment) will give you an additional window in the *Multidimensional Acquisition* list.

" Multidimensional Acquisition					
👻 🚹 Positions			🗆 Show all 📝		
Position List		Sample	Carrier		
Add Upda	ate	Remove	Remove All		
Move to Up [	Down				
Scan overview image					

You can make this window freely movable by using the top right white arrow. This allows you to take it over to the Locate tab, where you could use it to find a number of potential imaging positions.

Just click *Add* and the positions will appear in the list.

As you can see xy and z positions are saved. You can visit the positions with the *Move to* function.

- 🕆 P	ositions			🗆 Show all 🔂	
	Position Li	st		nple Carrier	
No.	x [µm]	y [µm]	z [µm]	offset [µm]	
1	-0.100	-0.100	87.790	-0.000	
	١dd	Update	Remove	Remove All	
Movet		Jp Down			
	Scan overview image				

If you use <u>this make sure to untick</u> <u>positions in the top menu before</u> <u>you say Start experiment</u> – the TIRF angle for each position will vary slightly hence in TIRF you should only ever acquire one position per time-lapse/ experiment.



## **Time-Series**

For a time-lapse you will need to activate the tick box for *Time Series*.

In the times series control window, you can define how many cycles you want to run the time-lapse for, and how long the interval should be.

• Time Series	s		□ Show all	Ľ
Cycles – Interval 🌔	-0	25 <b>*</b> 0.0 <b>*</b>	ms	
Pause				

*Interval* refers in this case to the time from the <u>start of an image to the start of the next one</u>, you can choose ms, s and min from the drop-down.

Example:

- exposure time is 50ms,
- read/ transfer time is ~2ms (in this example!) and
- the interval time is 100ms.

So, the wait time (no illumination) between images will be ~48ms.

You need to make sure that you enter a reasonable interval time; there will be no warning if your experimental set up requires more time as your planed interval and the system will just image as fast as possible without any wait time.

*Cycles* refers to how often your experiment will be repeated. If you have a defined timespan you want to run the experiment for, you need to calculate how many cycles you need:

Using the above example:

100ms interval => 10 images/s (1000ms = 1s and 60s = 1min) => 600 images/min

This means if you want to image for 10 minutes you need 6000 cycles.

Another examples:

No interval/ 52ms/image (including exposure time and read out) => 19 images/s and 1153/ min

For 15 minutes you will need 17367 cycles.



## **Definite Focus**

The control window for the *Definite Focus* system can be found under *Acquisition Parameters*. The *Definite Focus* works stable when using the TIRF lens and a TIRF sample (e.g. a sample in aqueous solution).

▼ <sup>+</sup> + Defi	inite Focus			✓ Show all	
Status:					
		Offset	0.0 µm		•
		Find St	urface		
	Store Focus			Recall Focus	
		Interval	0	s s	
		Peri	odic		
Use Z	Piezo				

Make sure to have Show all activated to be able to see the Status bar. If you have moved the objective close to the focus/into the oil you can click Find Surface and in the Status bar you will see a green blinking message while the Definite Focus works. Once it is finished Status should read Reflex found.

Only after the system has found the reflex you can use the Definite Focus system to stabiles the focus during a time-lapse experiment.

			🗆 Show all 🛃
	Offeet	0.0	
	Oliset	0.0 μm	
	Find St	ırface	
Store Focus		Recall Fo	ocus
	Interval	0	s
	Perio	odic	
	d Strategy		🗆 Show all 📝
Autofocus Mode		Definite Focu	s 🔻
🖌 Autofocus every n T	imepoint	1	

One needs to define in *Focus Devices and Strategy* that the *Definite Focus* is to be used via the drop-down menu. You will need to define how often the *Autofocus Mode* will be activated during a time-lapse experiment. Any time the Autofocus runs it will create a small delay before the next image can be acquired.

If you want to avoid this delay in TIRF imaging you can use the Periodic function to have the focus stabilization continuously (with an interval of 0) running during a timelapse. In this case for *Focus Devices and Strategy* select *None*!

Just active *Periodic* by clicking on it, to stop it click again.

BioOptics Light Microscopy Facility