Confocal microscopy course

7 HEC!

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Uppsala BioCenter

2014

Results of the quiz for the lecture 3 (CARS)

1. Comments about the lecture

good	too difficult/ more about applications
57%	43%

2. would you like more/less information in the lecture?

the same
100%

3. Average level of boredom (scale 0-10)

2.3 varying form 5 to 0

4. Will the information from the lecture be useful for you

no	maybe	yes
14%	29%	57%

5. Should CARS be included into the course?

no	maybe	yes
21%	8%	71%

Results of the quiz for the lecture 4 (High resolution microscopy)

1. Comments about the lecture

good	a bit too difficult	a bit slow/needs a break
70%	10%	20%

2. would you like more/less information in the lecture?

less	the same
10%	90%

3. Average level of boredom (scale 0-10)

1

4. Will the information from the lecture be useful for you

no	maybe	yes
10%	20%	70%

5. Should high resolution microscopy be included into the course?

no	yes
20%	80%

Results of the quiz for the seminar 1 (basics in confocal microscopy)

1. Comments about the lecture

great/very good/good	a bit slow
92%	8%

2. would you like more/less information in the lecture?

the same
100%

3. Average level of boredom (scale 0-10)

0.7!

4. Will the information from the lecture be useful for you

yes	maybe/some of it
92%	8%

Results of the quiz for the seminar 1 (basics in confocal microscopy)

5. what would you teach differently

too much time for the last discussion

not enough time for the last discussion

summarise the homework for all the groups

will do during the next seminar

6. would you like to be your student

yes	no!
85%	15%

there was not enough time for homework

Monday morning

most of you realised,
that you can't draw a
proper bicycle

a handful of facts

+ common sense

never ever consider yourself stupid

Wednesday morning

you could draw a
proper confocal
laser scanning
microscope

?

LSM T-PMT

LSM 780

VISION IsoSTATION™

Newport

it says "LSM T-PMT"

what is LSM?

what is PMT?

what is "T"?

why is it on the top of the microscope?

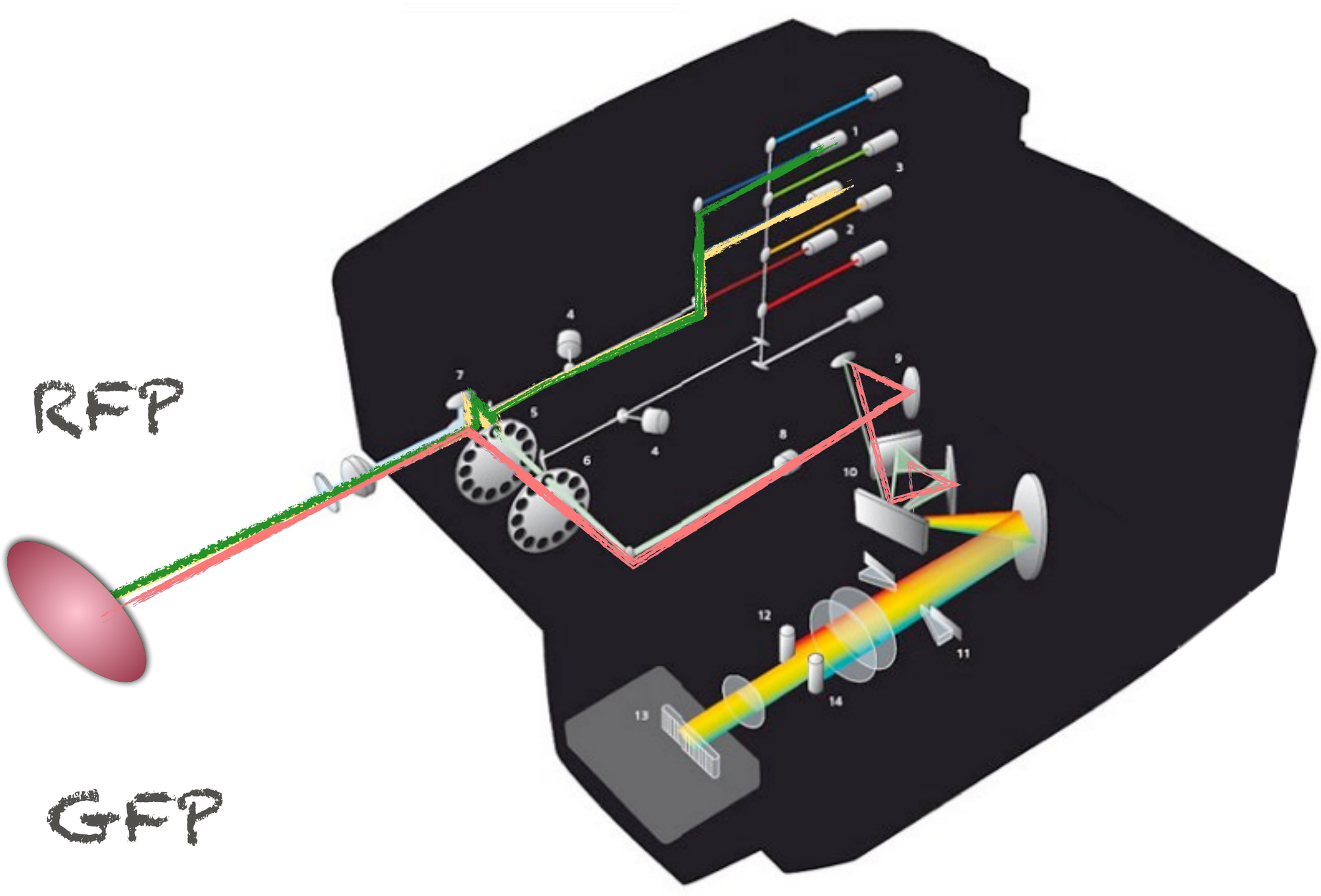
where is the pinhole?

will it give you a confocal image?

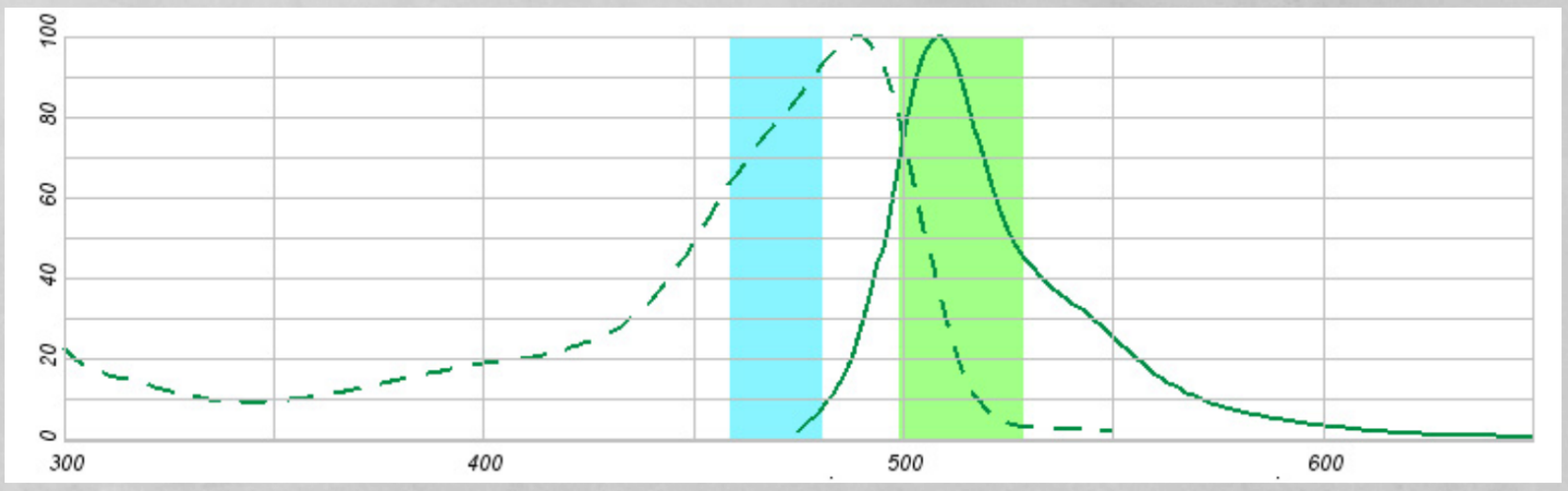
will it be a colour image?

Simultaneous/sequential scanning

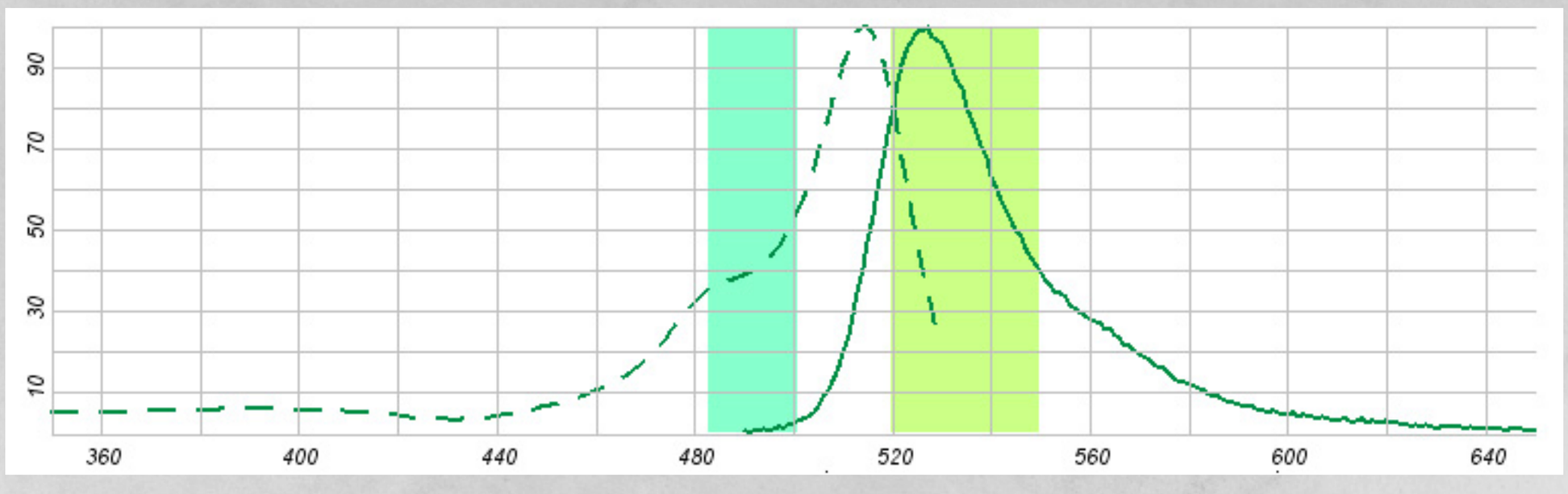
Insides of our confocal



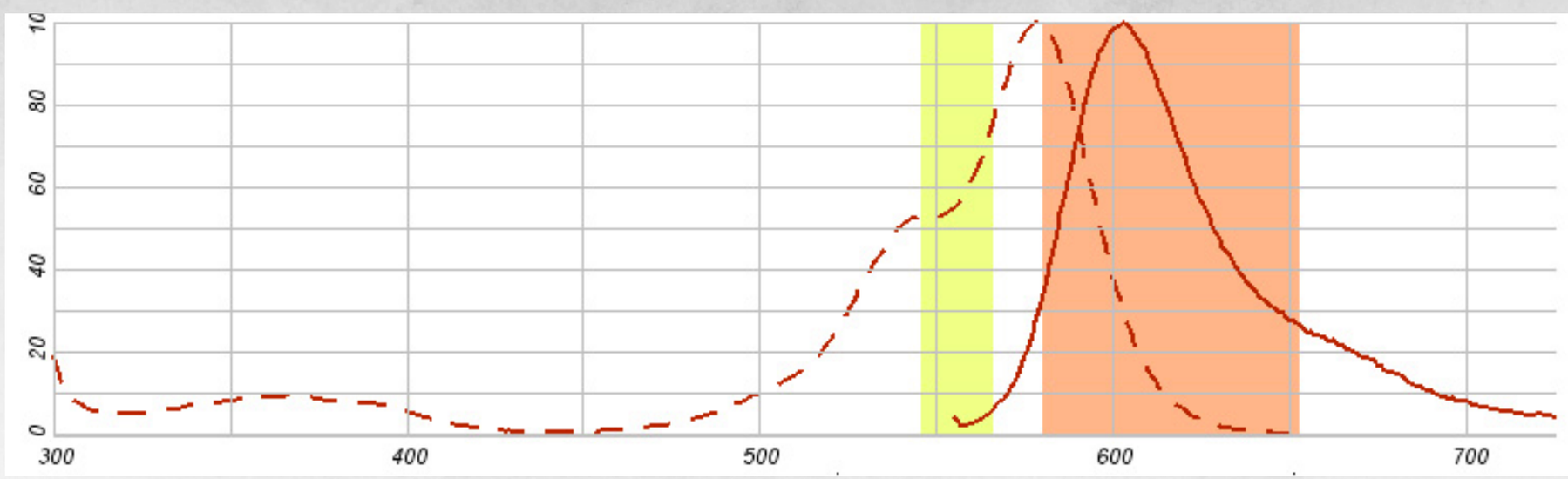
GFP


0
20
40
60
80
100
300
400
500
600

YFP


10
30
50
70
90
360
400
440
480
520
560
600
640

RFP


0
20
40
60
80
100
300
400
500
600
700

Sequential scanning for GFP/YFP

TRACK1:
Channel 1(GFP):
excitation 488 nm->on
detection 510-540 nm->on
->excitation/detection off

switching between tracks after each frame

switching between tracks after each line

TRACK2:
Channel 1(YFP):
excitation 514 nm->on
detection 520-550 nm->on
->excitation/detection off

please discuss:

1. what parts of the hardware will be switched
2. which switching method is preferential in what case
3. is it possible to have more than one channel in each track?

Simultaneous scanning for GFP/YFP

TRACK1:

Channel 1(GFP):
excitation 488 nm->on
detection 510-540 nm->on

Channel 2 (YFP):
excitation 514 nm->on
detection 520-550 nm->on

please discuss:

1. what parts of the hardware will be on during the scanning
2. what is the advantage of such set up?
3. what is the disadvantage of such set up
4. in what case is simultaneous scanning applicable?

Experimental part

For those of you who have some experience -> make connection between what you've learned and reality, try a bit of new stuff

software and report seminar

For those of you who have no experience -> make connection between what you've learned and reality

to use our confocal you need an SLU AD account

your user account: vbsg2

password: Sh6a6eZw

Graphics computer
nl-focal 2

To edit and store images
(each user has up to 100Gb of space)

file transfer to save created images

automatic back-up

Confocal computer
nl-focal

only to create images

file transfer

Remote Desktop Control

Back-up server
nl-focal bu

only to store images
(mirrors the Graphics computers' files)

no access

users computer

no access

To book the Graphics computer

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences
Uppsala BioCenter

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Start SLU / Faculty of Natural Resources and Agricultural Sciences / About the Faculty / Cluster / Uppsala BioCenter / Resources / Confocal microscopy

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  - Booking of rooms
  - **Confocal microscopy**
  - Course in confocal microscopy
  - Course in qPCR
  - Janitor
  - Plans
  - Webpage and Signage
- News
- Calender

Confocal microscopy

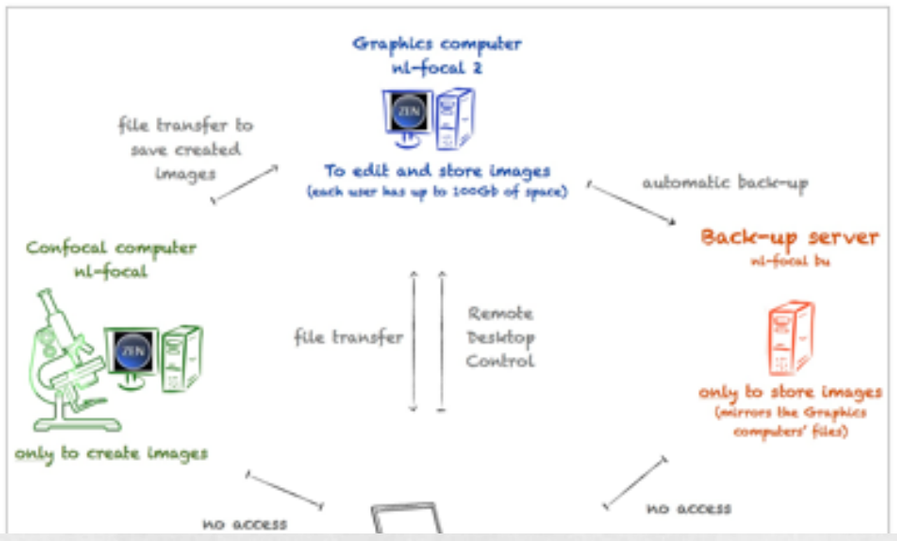
Before you start

Before you use the confocal microscope for the first time, you must have an introduction.

To have an introduction please contact a person responsible for the confocal microscope at your department:

BioCenter and Department of Plant Biology: Alyona Minina (alena.minina@slu.se, 0700405231)

The system



Booking

login into the boooking calendars with your AD username and password

To create images

Book the confocal microsocpe here

(175 SEK/ hour)

1. you need to know your groups KST number and also project number from which the fees will be paid

2.before making a booking please read the brief manual

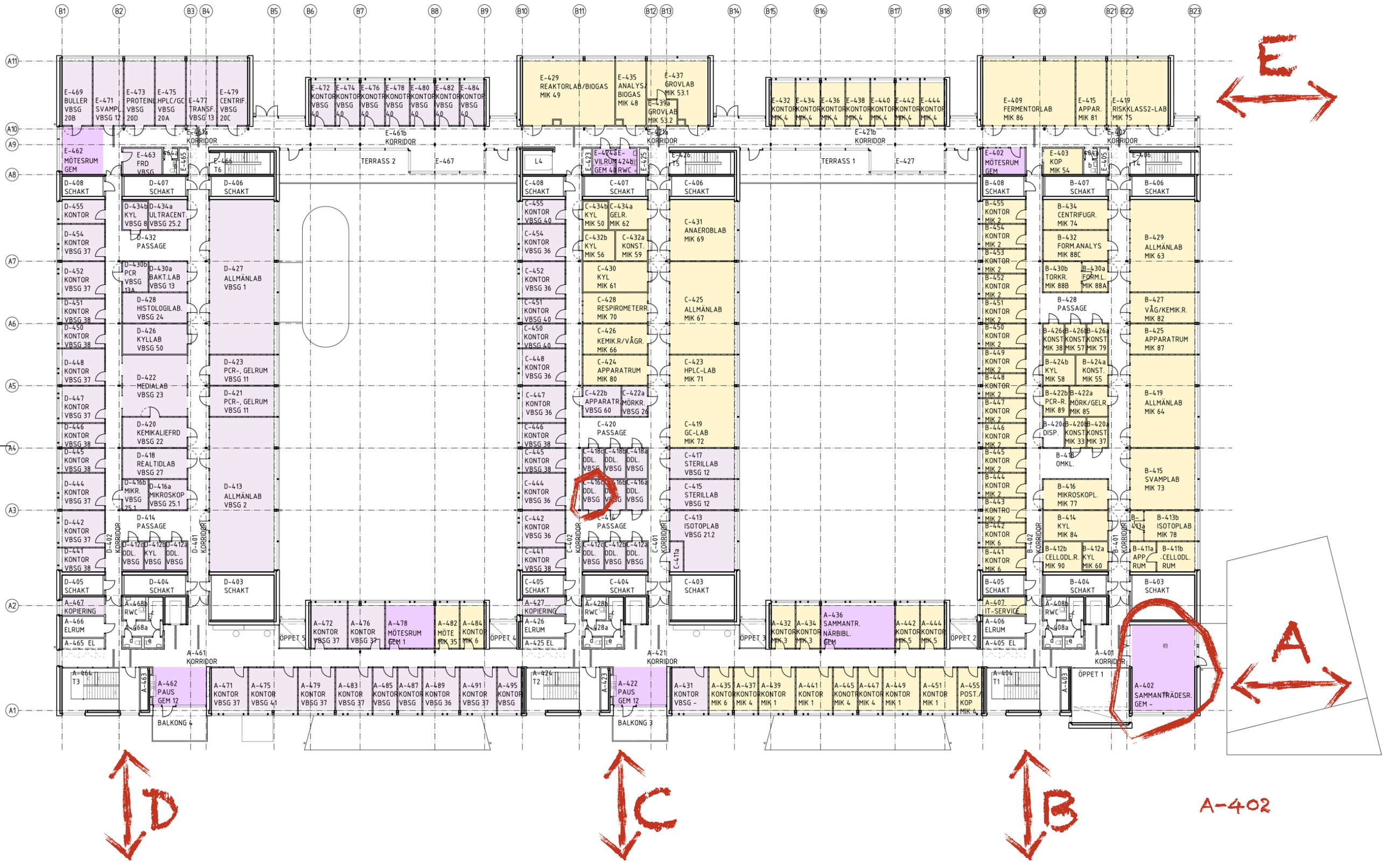
To edit images

1. you can use ZEN software on the common Graphics computer via Remote Desktop Connection (RDC)

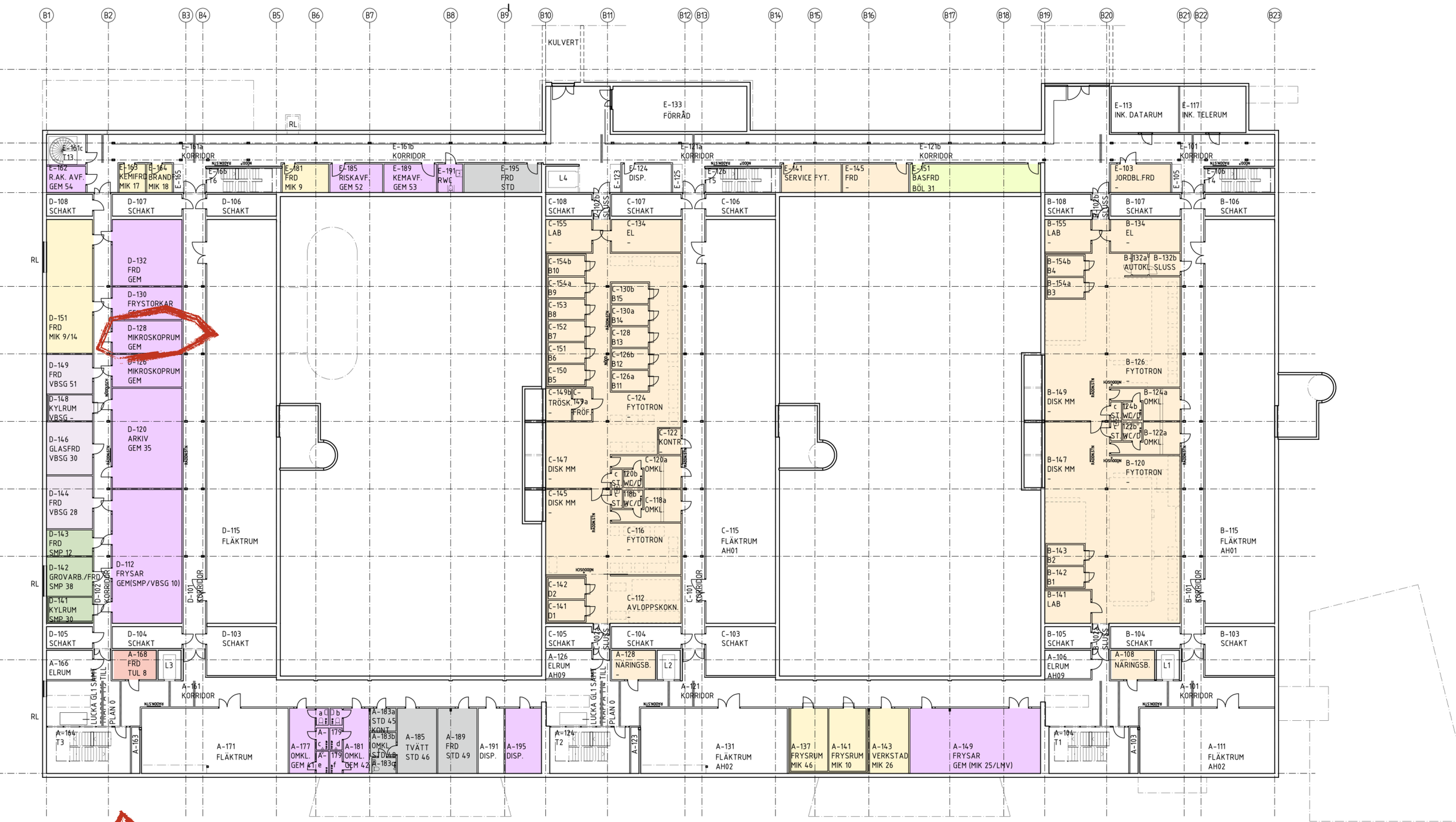
Book the Graphics computer here

(free of charge)

your samples are on the 4th floor, room C-416C



confocal is in the basement. 1st floor, room D-128



Your schedule

18 participants	November 2014 Tue 11		Wed 12		Thu 13	Fri 14	Tue 18		Thu 20
	9:00 AM - 1:00 PM	1:00 PM - 5:00 PM	9:00 AM - 1:00 PM	1:00 PM - 5:00 PM	1:00 PM - 5:00 PM	9:00 AM - 1:00 PM	9:00 AM - 1:00 PM	1:00 PM - 5:00 PM	1:00 PM - 5:00 PM
Reza				✓					
Ylva Sjunnesson						✓			
Eva Hellmén		✓							
Leonor Gouveia					✓				
Catarina				✓					
Tania Tajrin						✓			
Abdul Halim			✓						
JING LU								✓	
Maria Karlsson							✓		
Daniel Johansson							✓		
Masud Parvage			✓						
Shirin Akhter					✓				
Xue Zhao								✓	
Anna Carlson	✓								
Rita Batista									✓
Clément Lafon-Place									✓
Mohammad Jaber A		✓							
Panisara	✓								

before performing the experiment

1. please discuss with your pair what you are going to do
2. make sure you have each other's contacts
3. agree where you meet
4. pick a Petri dish with your names from the room C-416c
5. go to the confocal room D-128 (D-corridor, basement)
6. use your 4 hours to perform as many tasks as you can

while performing the experiment

1. DO NOT PANIC!!

2. Don't make any sudden movements, please be kind and patient with the hardware and software

3. make sure you saved all your data on the Graphics computer

4. If something goes wrong Alyona's phone can be found:

   in all e-mail she sent

   on the door of the confocal room

   on the walls of the confocal room

   alena.minina@slu.se

after performing the experiment

1. You can analyse your data individually (follow the tasks from the course site)

2. Book time on the Graphics computer (only one person at a time can use the computer)

3. Use Remote Desktop Connection to edit your images

4. Follow the instructions to analyse your data

You will be all by yourself

11th-25th of November

Homework

1. Draw a VERY thorough scheme of a Zeiss-like confocal connected to an upright microscope. Including a T-PMT. Make it as 3D as you can.

2. Use your scheme to modify it into a Two-Photon microscope

3. Please bring both of your schemes on the 25th of November

Homework

1. Analyse your data the best you can
   - try Zen Black (RDC to the Graphics computer)
   - ZEN Blue (RDC to the Graphics computer)
   - ImageJ (download from the http://fiji.sc/Fiji)

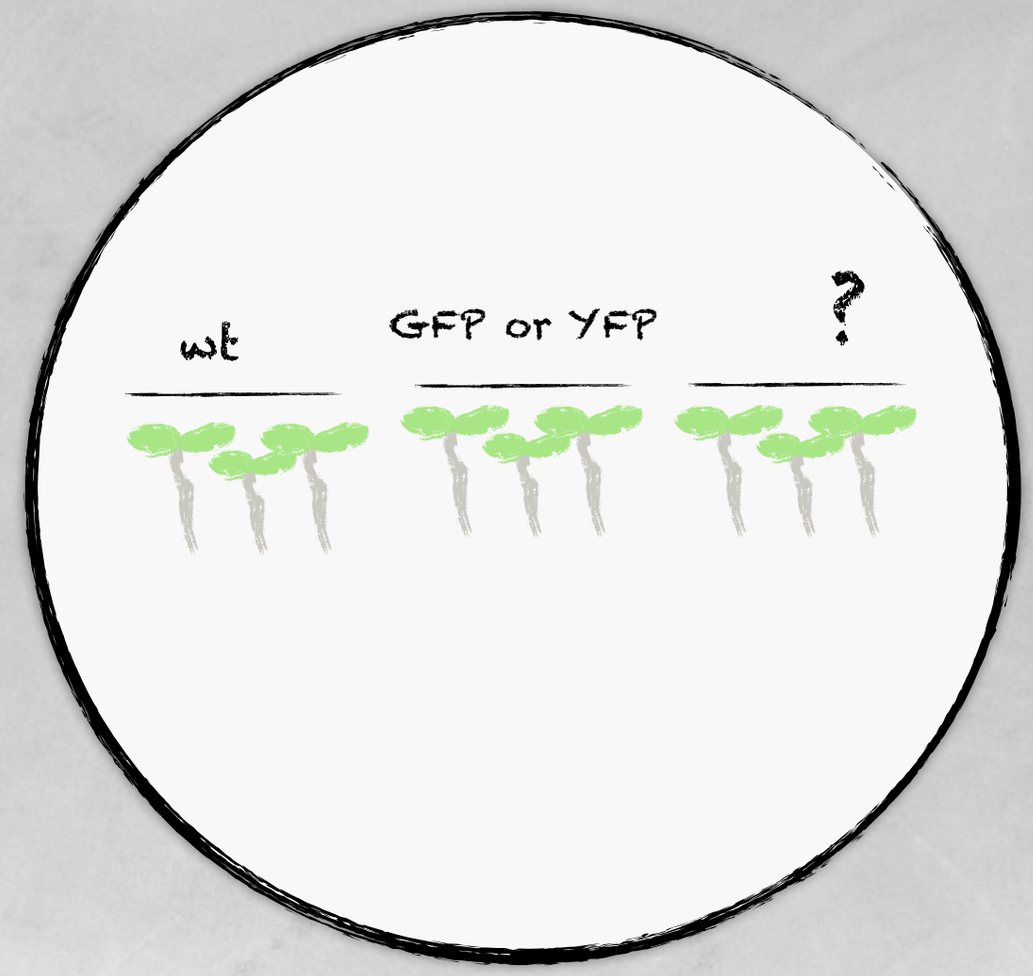
2. Write down ALL your questions about the softwares

3. Bring all your questions on the 27th, 28th of November and the 1st of December

Samples

Each group will have a Petri dish with 3 types of plants:

1. wild type
2. plants expressing GFP or YFP-tagged protein (depending on the group)
3. plants expressing protein(s) tagged with unknown fluorophore(s)



obligatory and optional tasks:

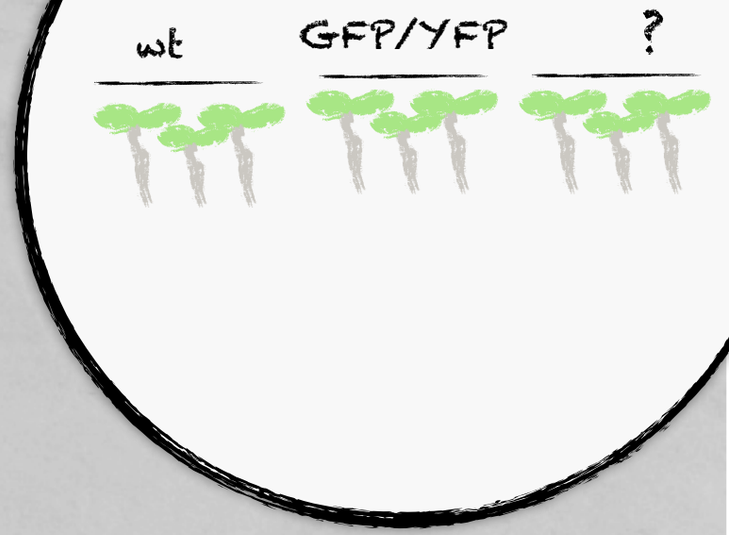
must

1. find out where GFP or YFP is localised
2. make a very nice representative image of localisation

should

3. figure out what fluorophore(s) you have in the plant type 3
4. where are these fluorophores localised
5. make a very nice representative image of localisation

Sample preparation

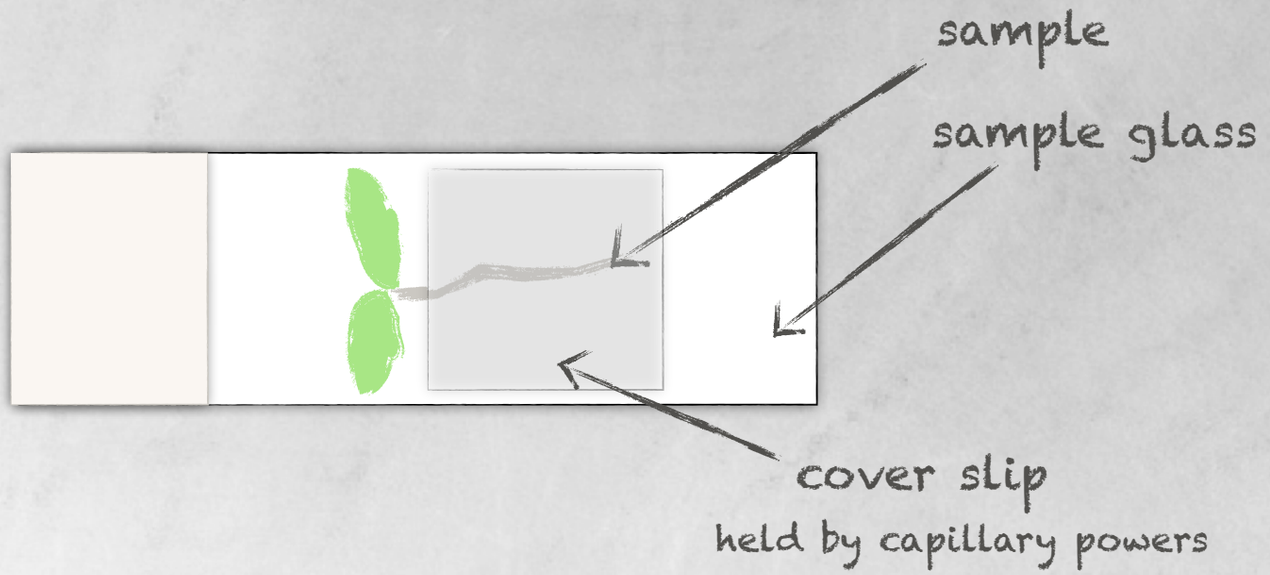


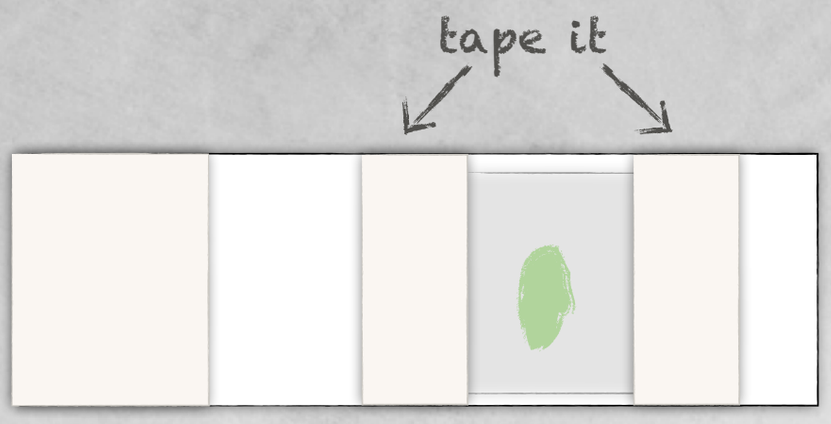
1. cover slip should be 0.17 mm (#1.5) why?

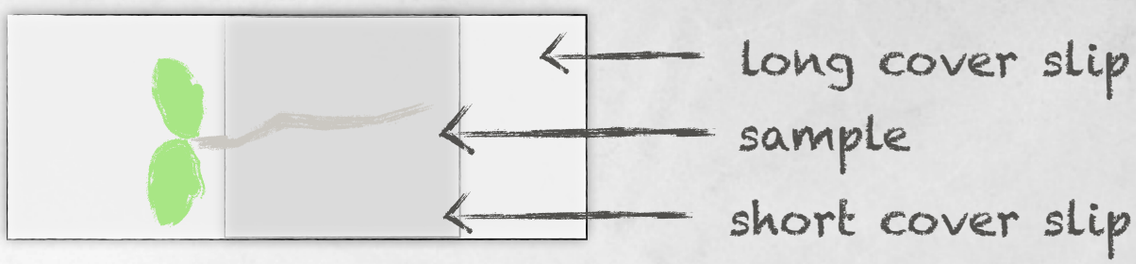
2. cover slip should ALWAYS face the lens! why?

3. cover slip and sample should not fall off or move during scanning why?

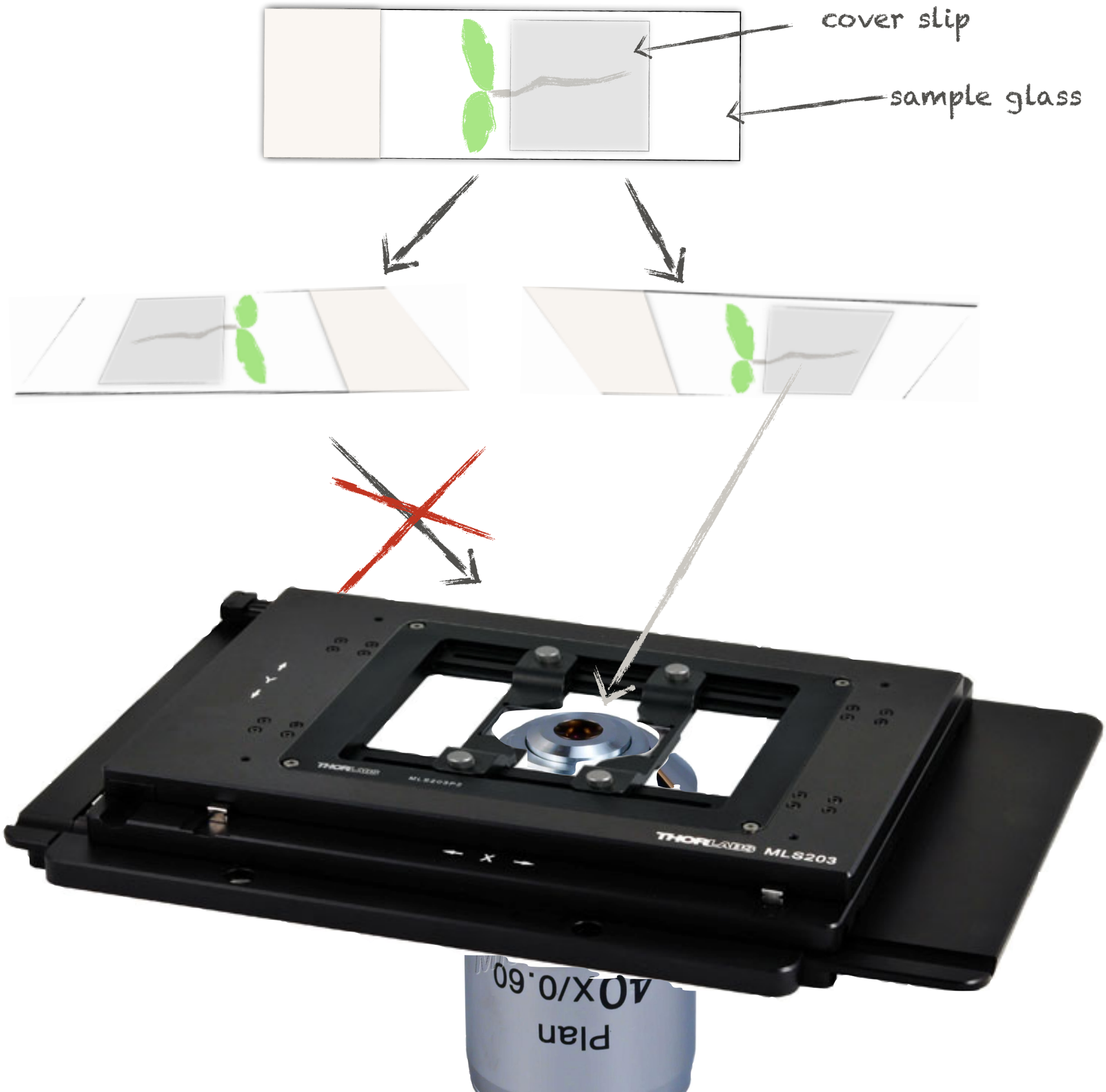
4. sample should be as flat as you can persuade it to be why?

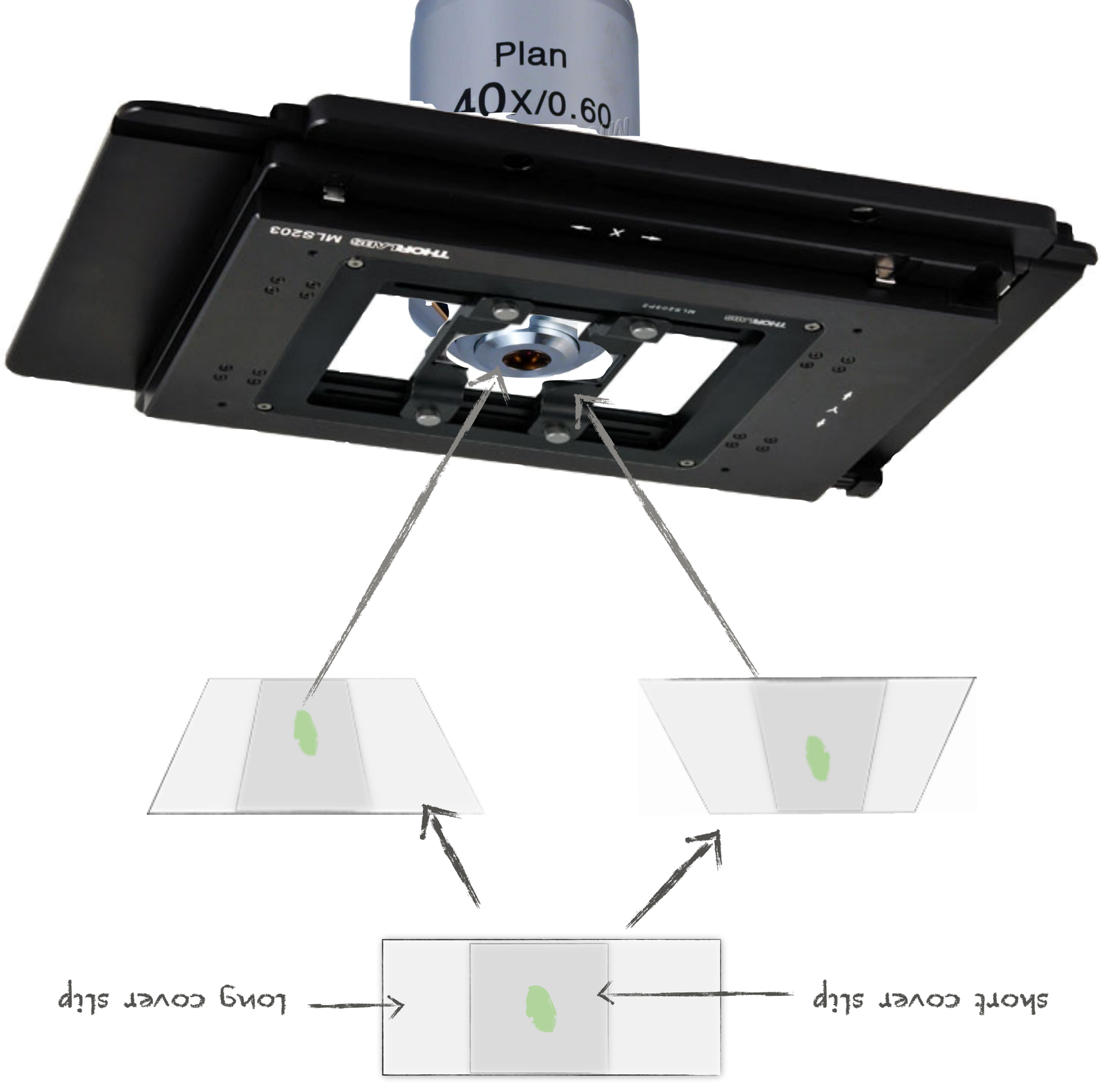






to prevent the sample from drying


cover slip
sample glass
THORLABS ML8203
Plan
40X/0.60


Plan
40X/0.60
THORLABS ML8203
long cover slip
short cover slip

Sample preparation

1. What sample are you going to use for your research:
    - living
    - fixed

2. For how long are you going to store your sample:
    - only for scanning
    - forever

3. What immersion/objective will you need for your sample
    - no immersion
    - oil
    - glycerol
    - water

Zeiss 780

stage of the heating module

Mercury "UV" lamp

power remote switch

remote control for the microscope

lasers rack

argon laser "run/ idle switch"

argon laser power supply

computer for hardware = real time computer (RTC)

computer for software.

start up

1. Turn on the power switch (the key must always be in the position "on"):
   - main switch
   - computer switch
   - components switch

2. Press the power button on the software computer

3. Turn on the Mercury lamp if you need it

4. If you need the Argon laser (458nm (CFP), 448nm (GFP), 514nm (YFP))
   a. turn the key on the Power supply of the Ar laser
   b. flip the tiny metal run/idle switch to the run mode
   c. wait until the green LED goes on
   d. Do NOT change the light control currency reel!! if you did find Alyona!
5. Find a user account vbsg2 use the password: Sh6a6eZw
6. Switch on the ZEN 2011 and click on START SYSTEM
7. if it doesn't boot:
   - close the ZEN 2011, make sure it is not "hanging" in the Task Manger
   - open the door on the Hardware computer and press the RTC-reset button
   - wait for 30sec before starting the software again

Zeiss 780

argon laser "run/
idle switch"

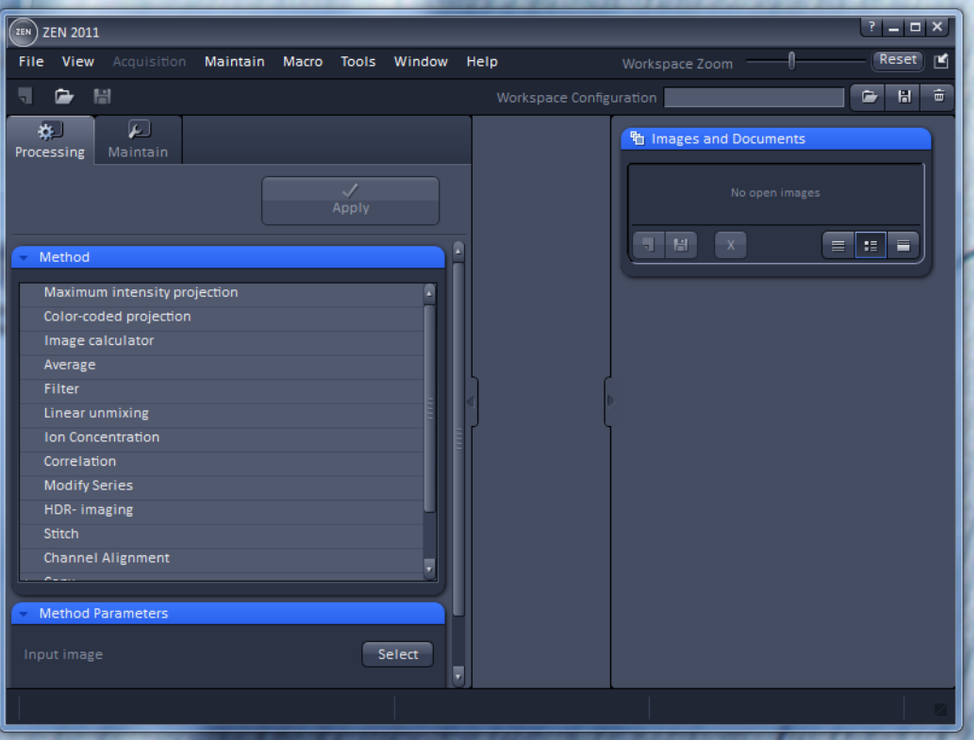
Mercury "UV" lamp

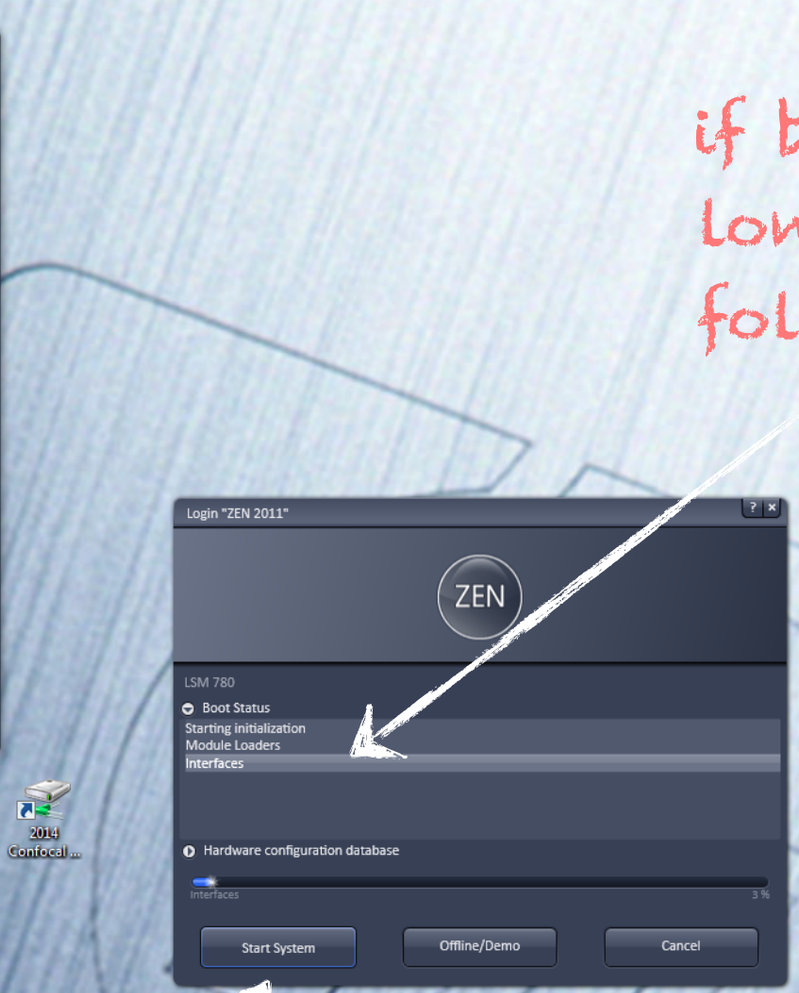
argon laser
power supply

30 minutes rule for the UV lamp

the same day rule for the Ar laser

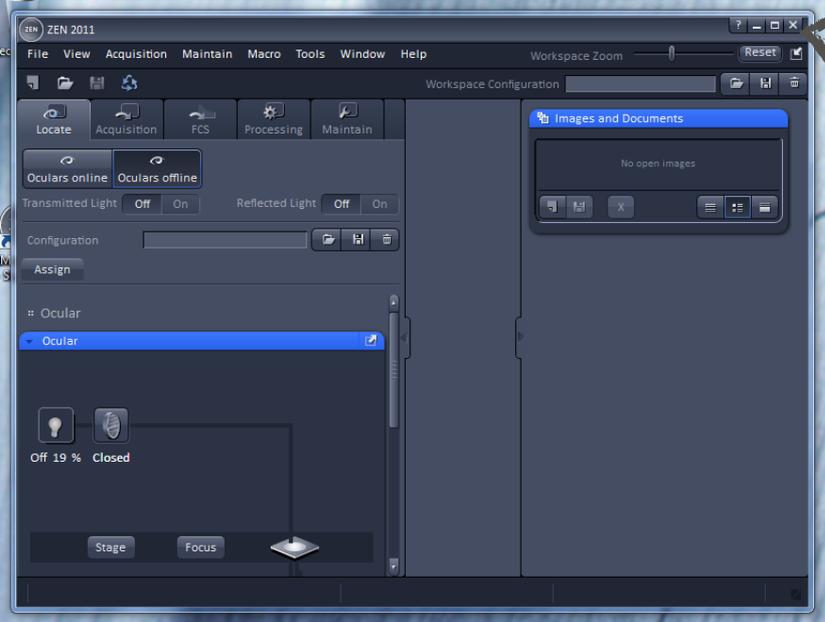

Recycle Bin
ZEN
AIMApplic... - Shortcut
2014 Confocal ...
ZEN
ZEN
open the ZEN software
View messages in Action Center
Click to view messages about your computer.
EN
12:56 PM
11/10/2014





if booting is stuck for longer than a minute, follow the instruction for RTC reset

chose "start system"

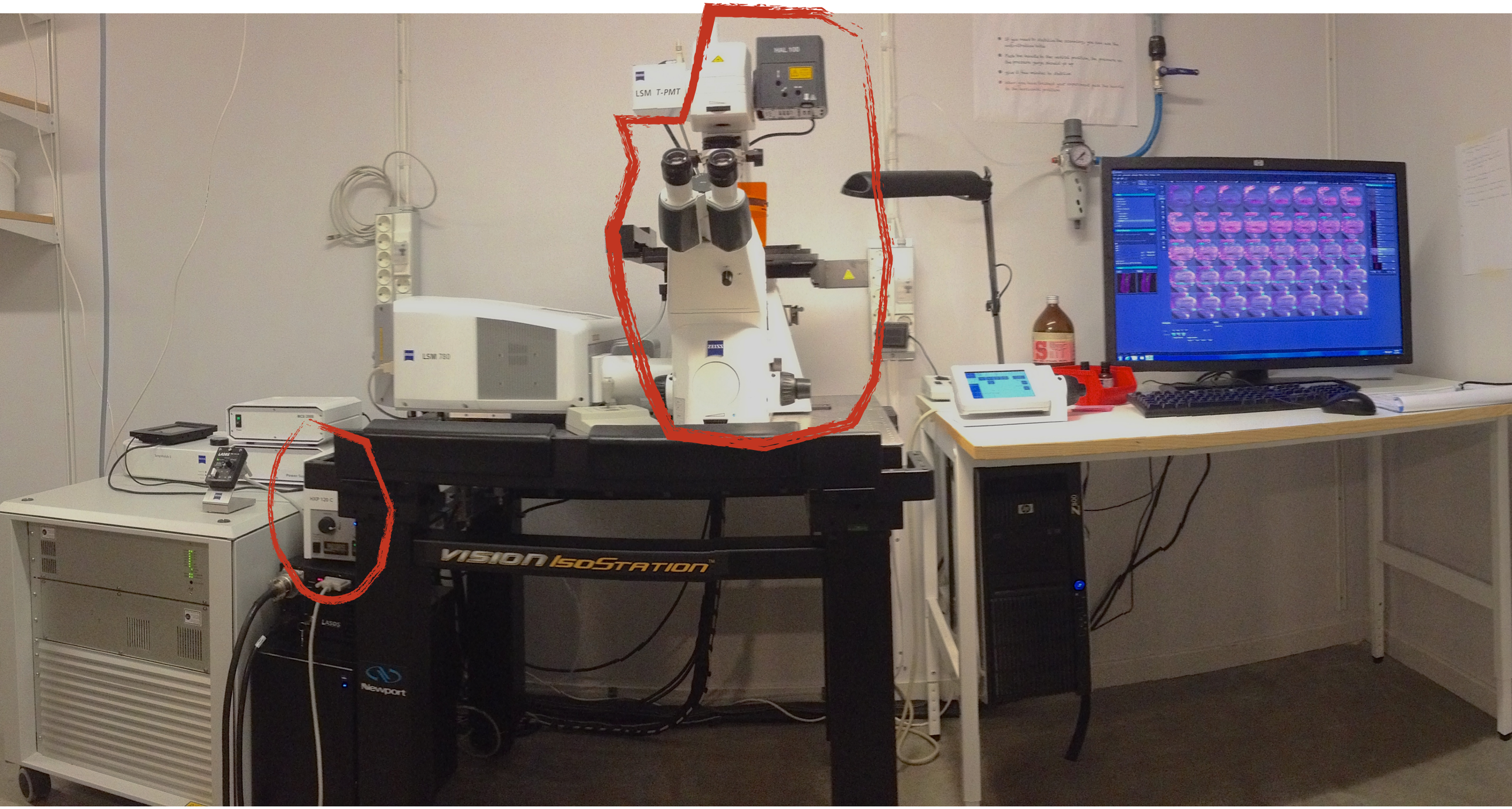


Maximize the window

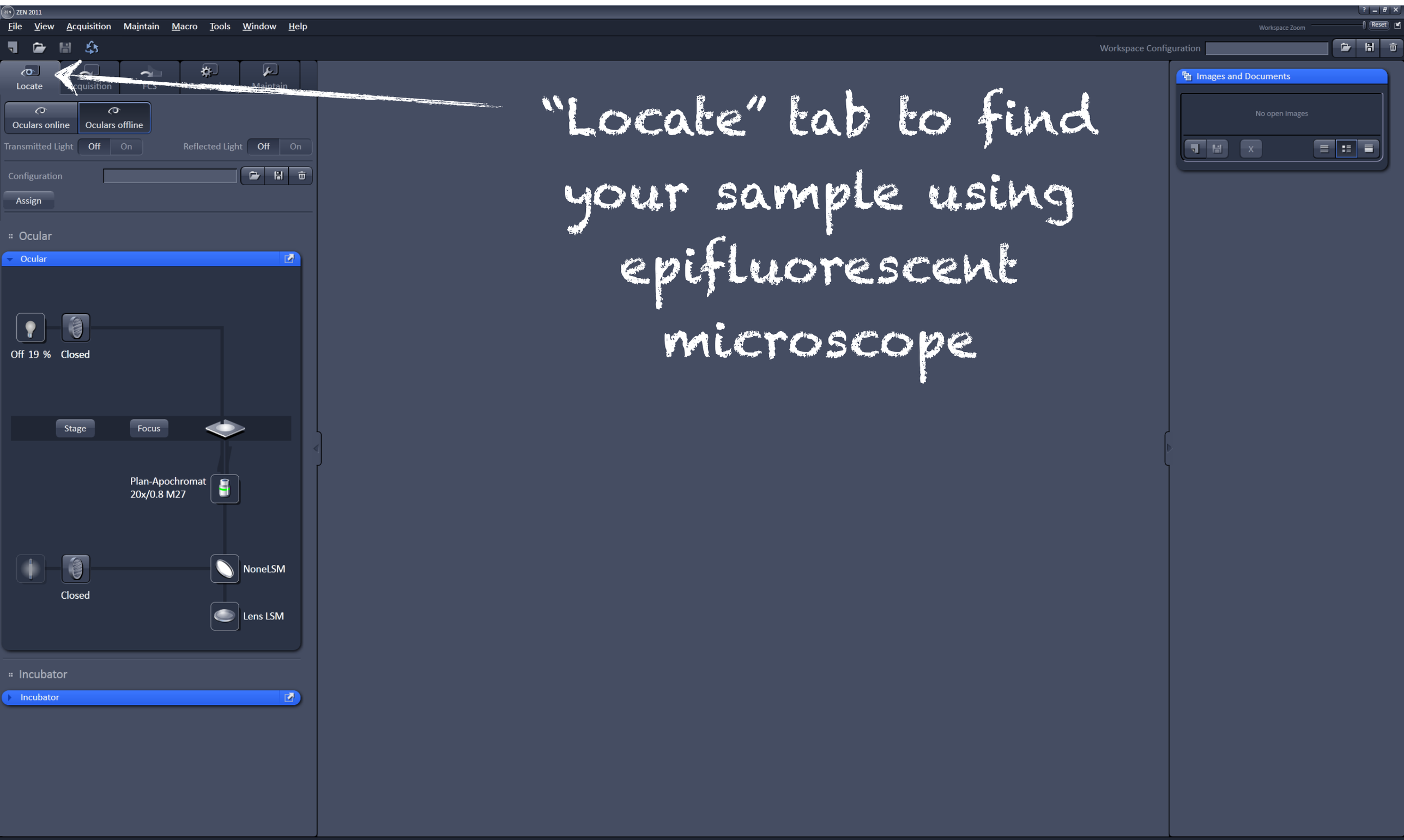
this is what you will see
when the software is
ready to use

1. Mount your sample and locate the place you want to image

Use epifluorescent microscope to locate a pretty place on your sample

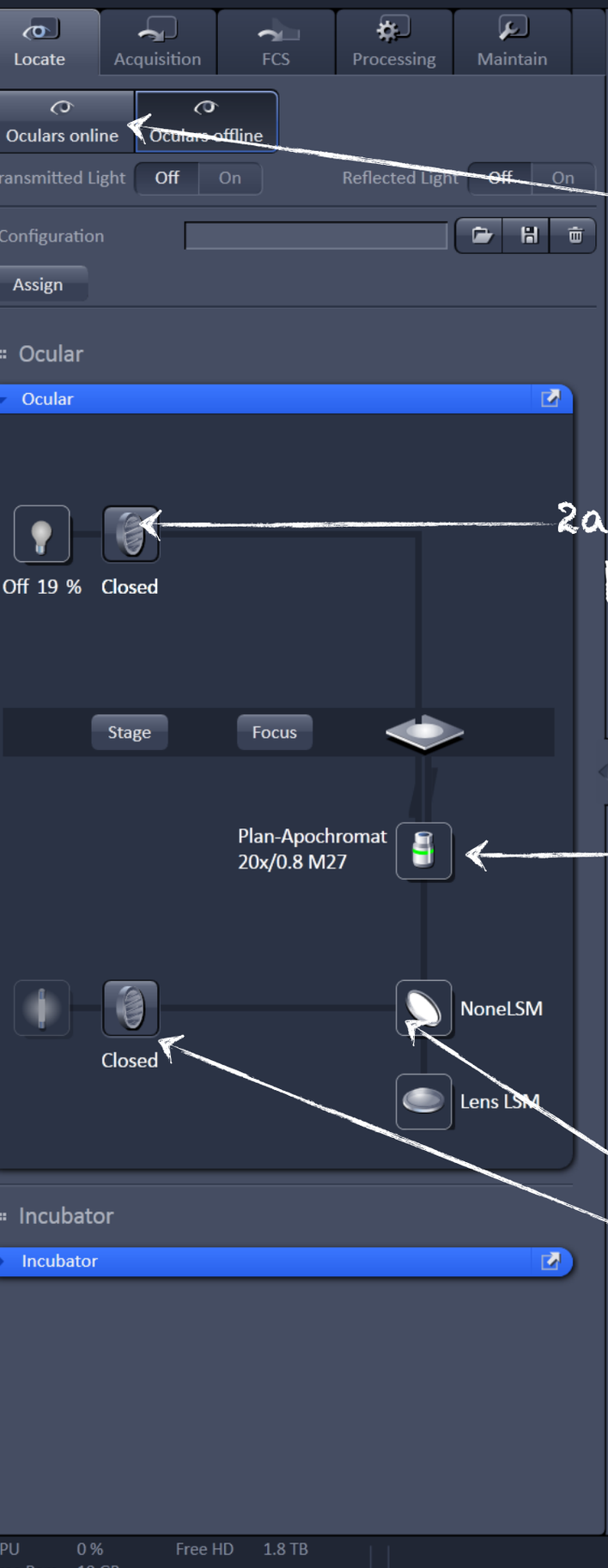


you can use transmitted light (HAL lamp) and excitation light from a UV (mercury) lamp


"Locate" tab to find your sample using epifluorescent microscope
ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Zoom
Reset
Workspace Configuration
Locate
Acquisition
FCS
Maintain
Oculars online
Oculars offline
Transmitted Light Off On
Reflected Light Off On
Configuration
Assign
Ocular
Ocular
Off 19 %
Closed
Stage
Focus
Plan-Apochromat
20x/0.8 M27
Closed
NoneLSM
Lens LSM
Incubator
Incubator
Images and Documents
No open images
CPU 0 %
Free Ram 10 GB
Free HD 1.8 TB
SV
12:59 PM
11/10/2014

Locating your sample

1. to see anything oculars should be in the Online mode



2a. to use transmitted light open the shutter and adjust the brightness of the HAL lamp by clicking on the light bulb

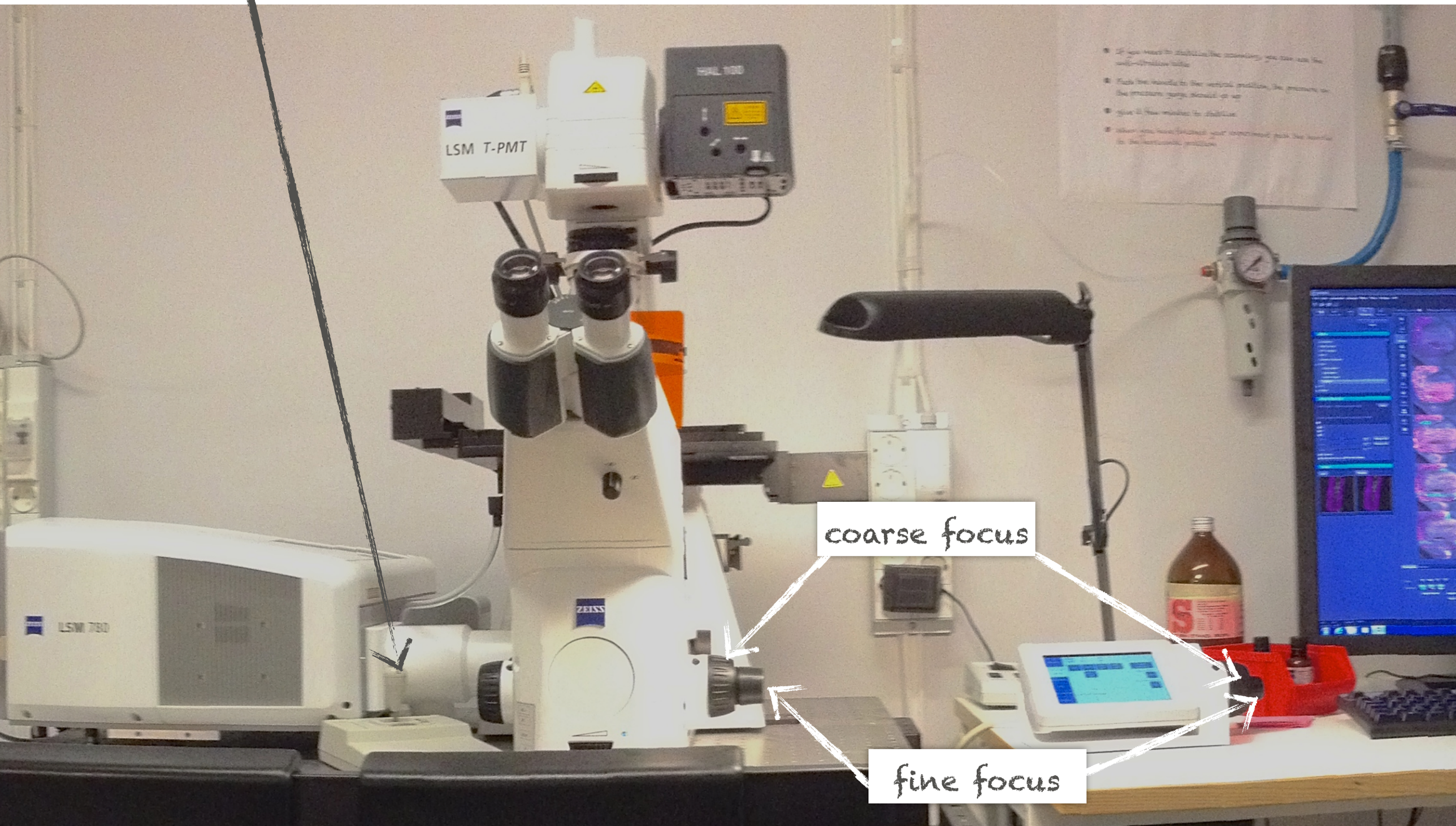
3. select the objective you want to use.make sure you have the correct immersion

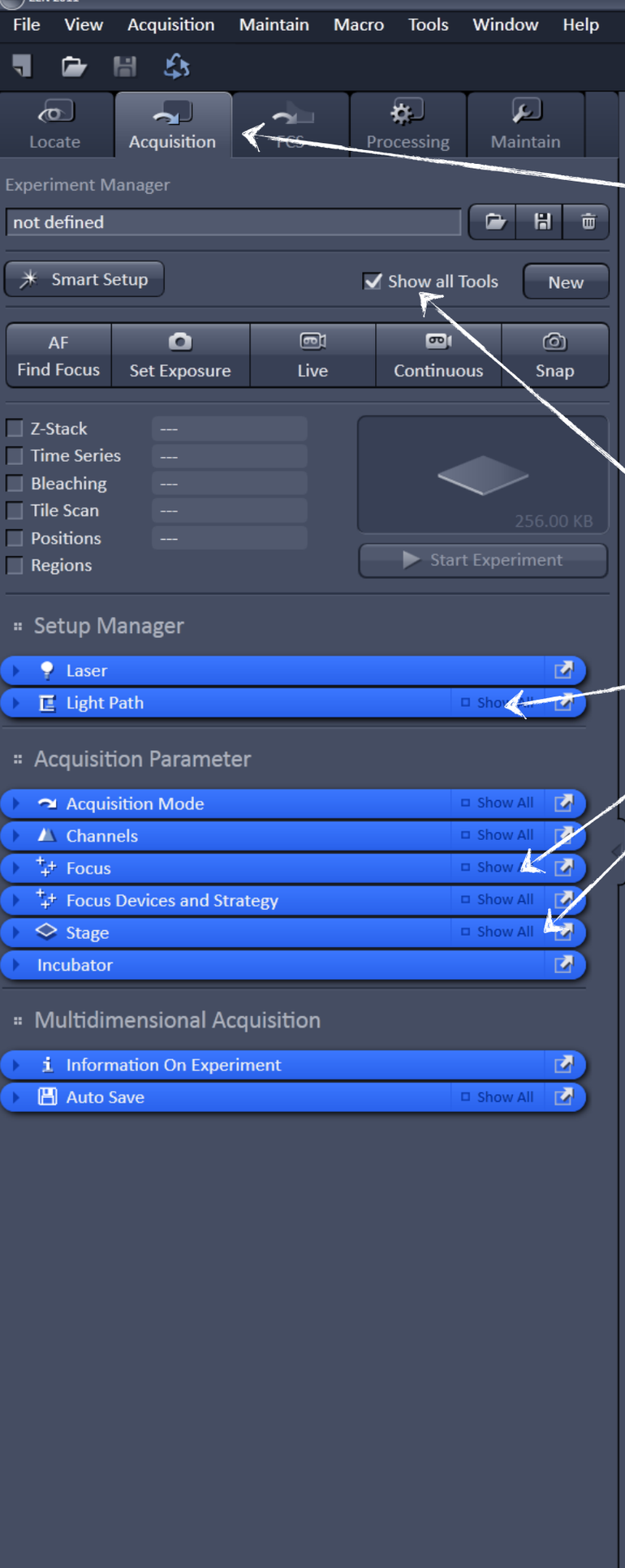
2b. to use fluorescent light click to open the shutter and select the filter you need (violet=DAPI, Green=GFP, Red=RFP)

Make sure the shutter of the HAL lamp is closed

Use the joystick to move the stage
the button on its top switches from
fast to slow movement

Use the focusing knobs to adjust the focus





after you located your sample click on the acquisition tab

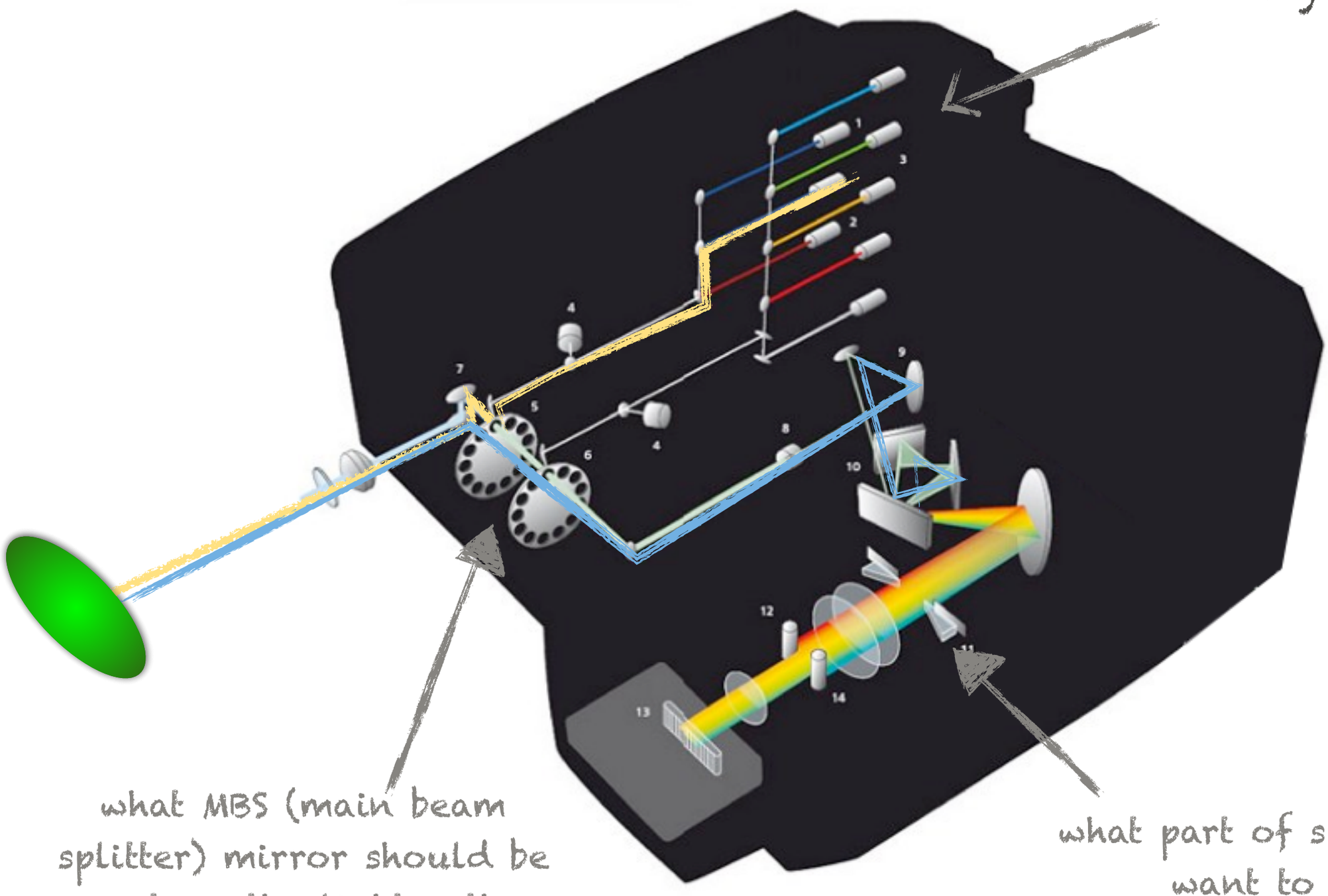
click on "show all tools", do the same in each tab you are going to use

1. Mount your sample and locate the place you want to image

2. Tell software what fluorophore are you going to scan?

confocal needs to know

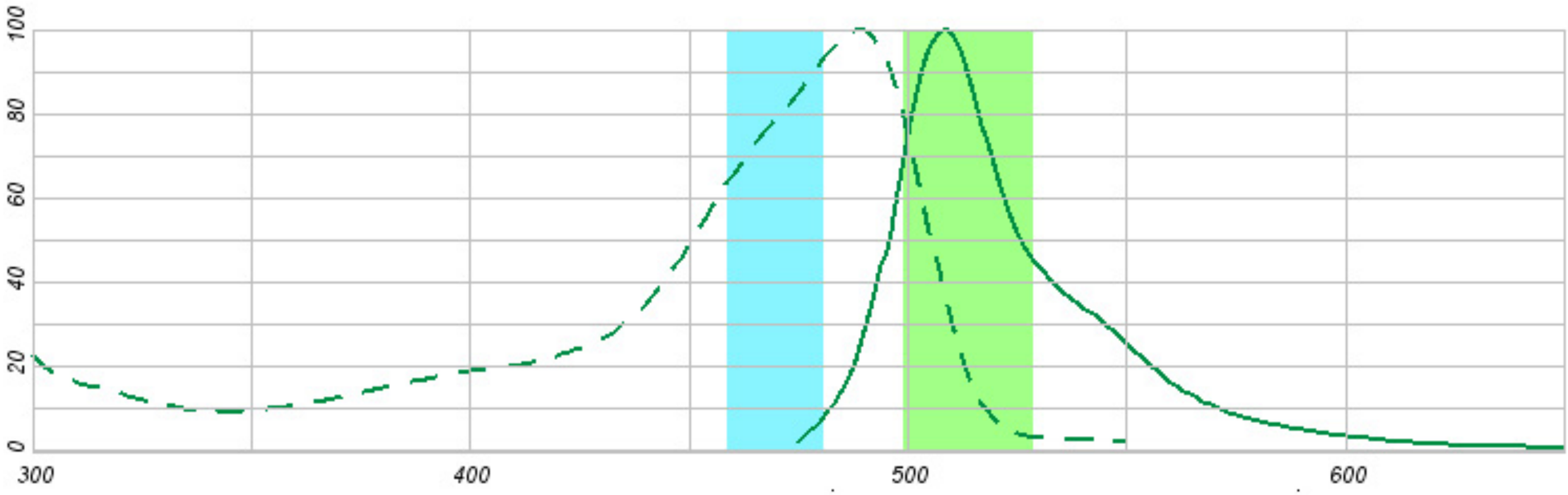
what laser you need to use



what MBS (main beam splitter) mirror should be put on the light path

what part of spectrum you want to detect

Green Fluorescent Protein

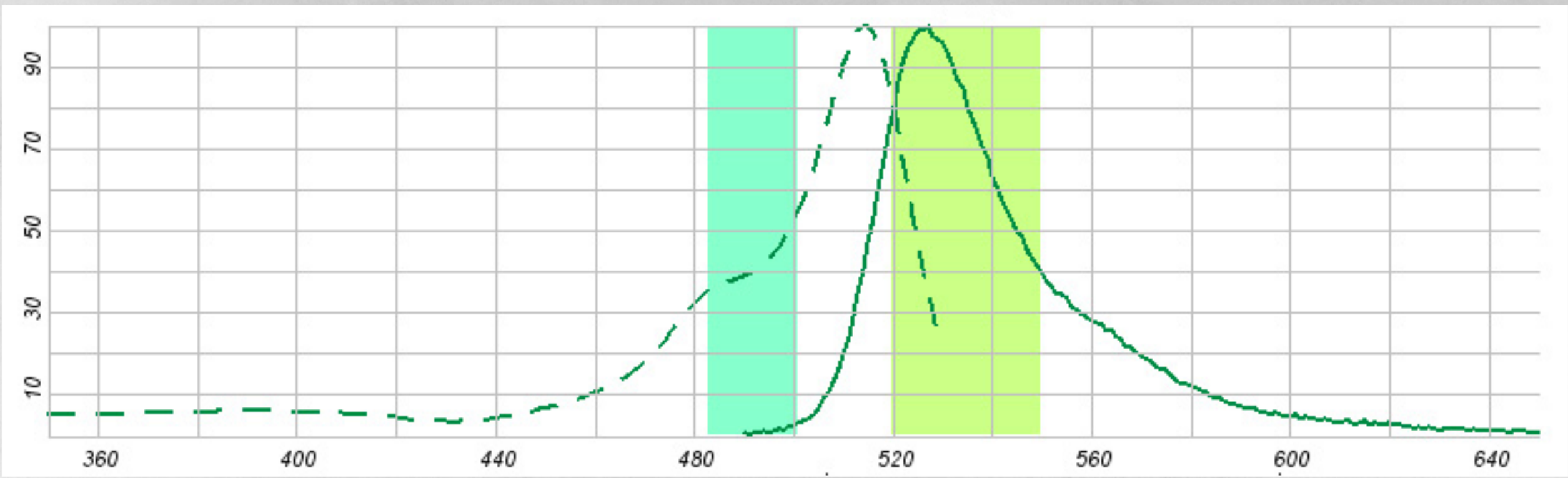


excitation

emission

please discuss what will be in the light path for GFP

Yellow Fluorescent Protein



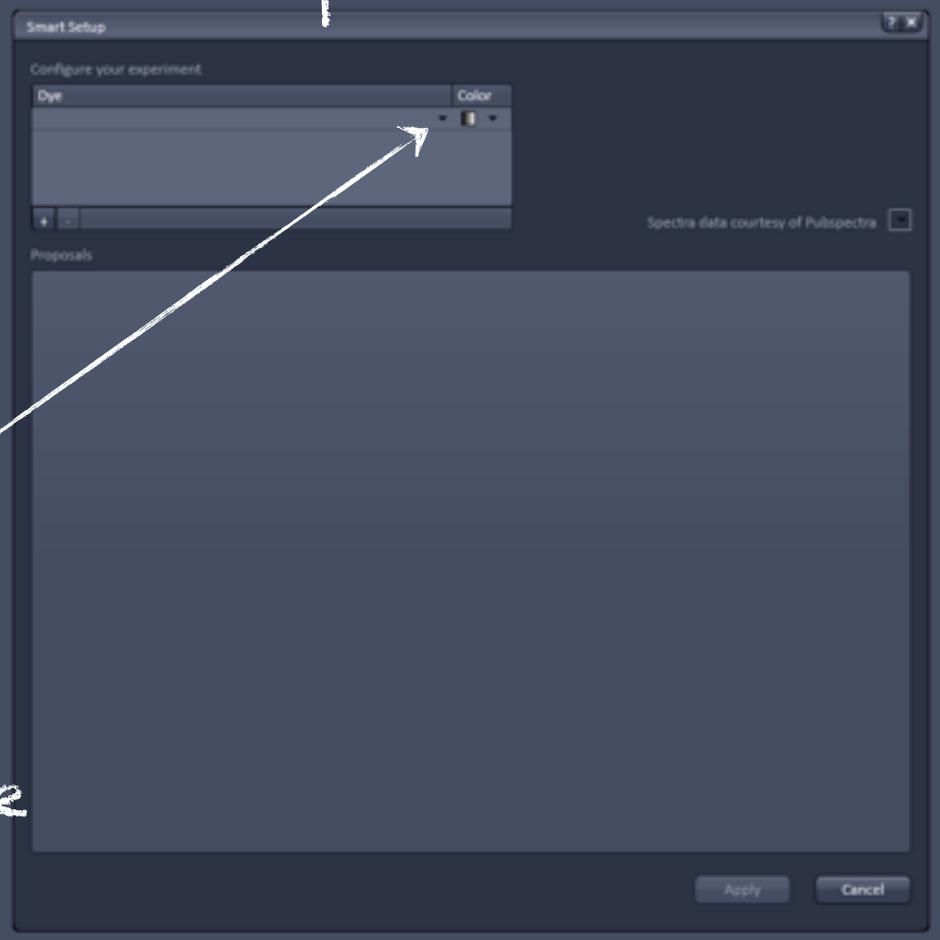
excitation emission

please discuss what will be in the light path for YFP

If you want to set up the light paths automatically

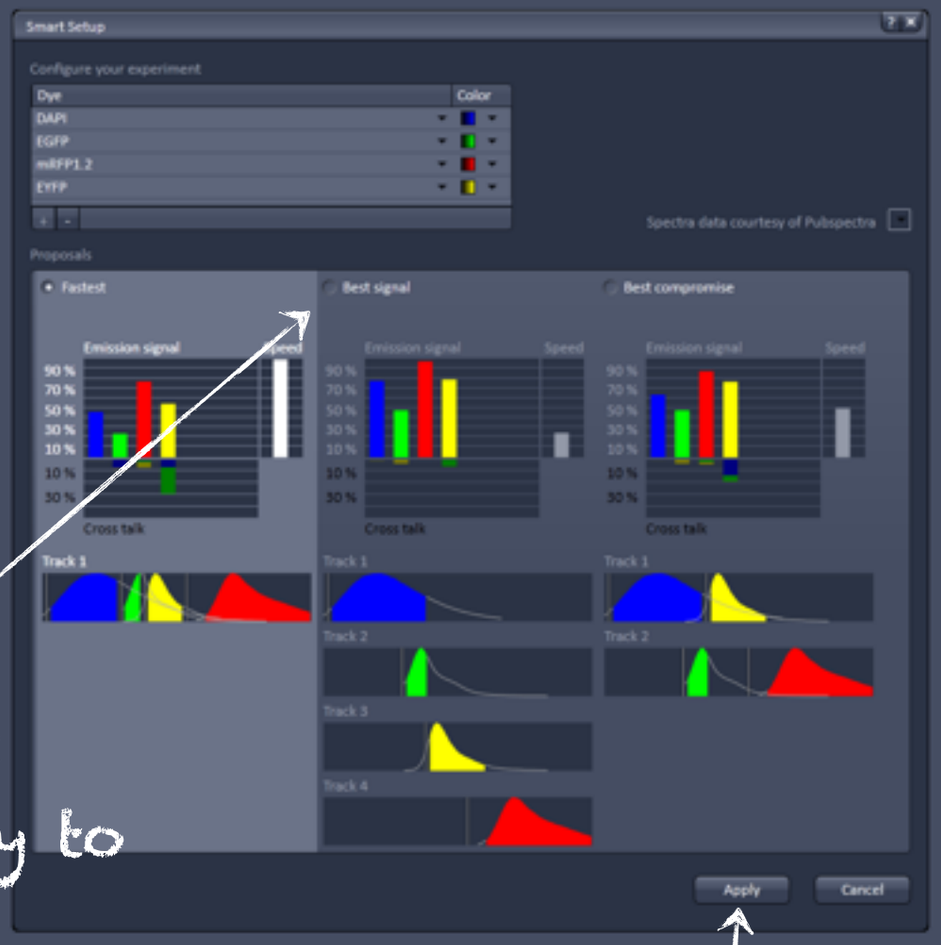
1. click on "smart setup"

2. select from the database list fluorophores you are going to detect



channel = scanning of one fluorophore

you can scan your channels in one track or in multiple tracks



please discuss

1. which option is sequential scanning
2. which option is simultaneous scanning
3. why do you think the best compromise looks like this

3. pick the most suitable for you way to scan

4. click apply

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Workspace Configurati

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack ---
Time Series ---
Bleaching ---
Tile Scan ---
Positions ---
Regions

256.00 KB

Start Experiment

Acquisition Parameter

- Acquisition Mode — Show All
- Channels — Show All
- Focus — Show All
- Focus Devices and Strategy — Show All
- Stage — Show All
- Incubator

Multidimensional Acquisition

- Information On Experiment
- Auto Save — Show All

Smart Setup

Show All

Configure your experiment

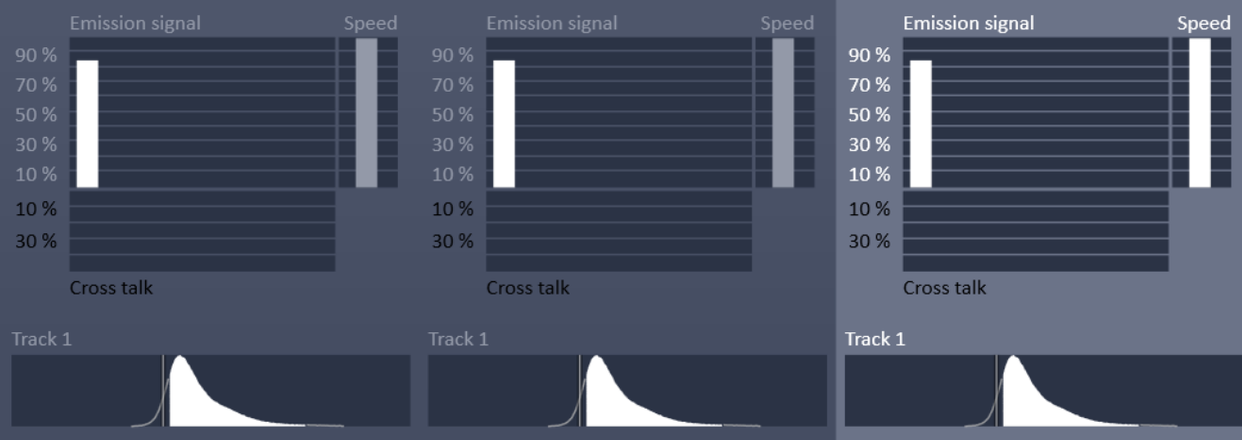
Dye	Color
EYFP	

Spectra data courtesy of Pubspectra

Current | Quality | Speed | Standard | Widefield Like

Proposals

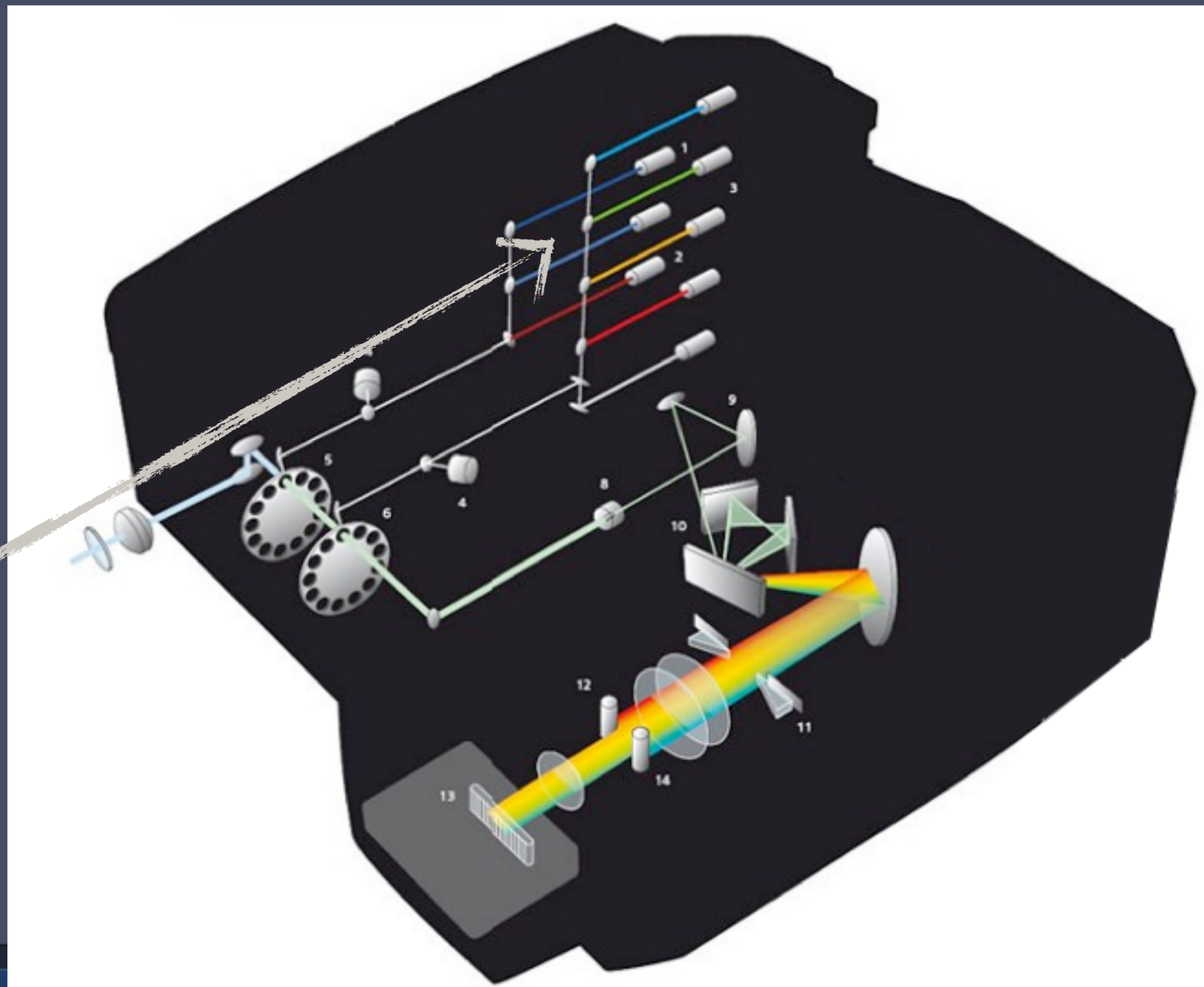
Fastest | Best signal | Smartest



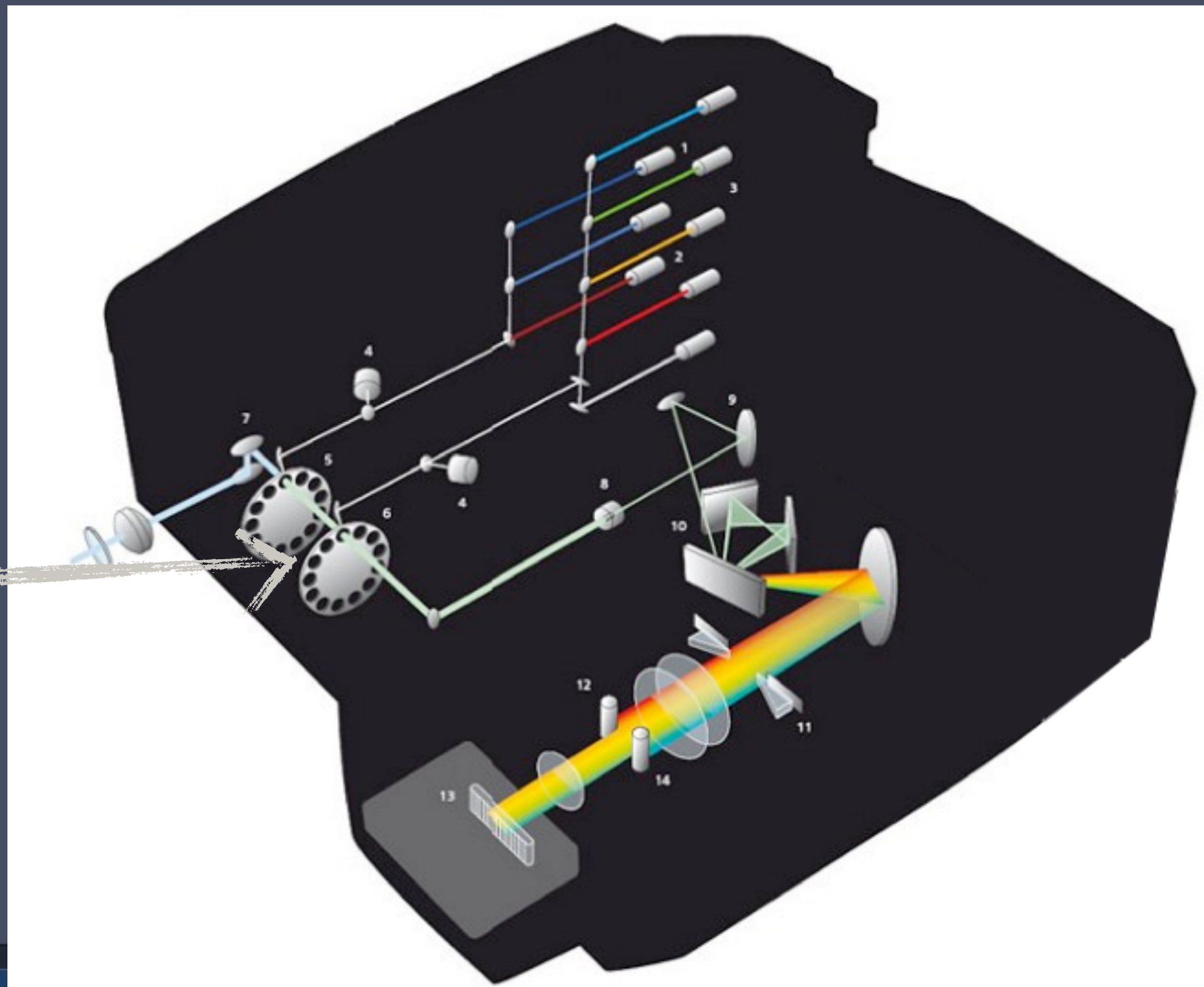
Apply | Cancel

CPU 0 % Free HD 1.8 TB
Free Ram 10 GB

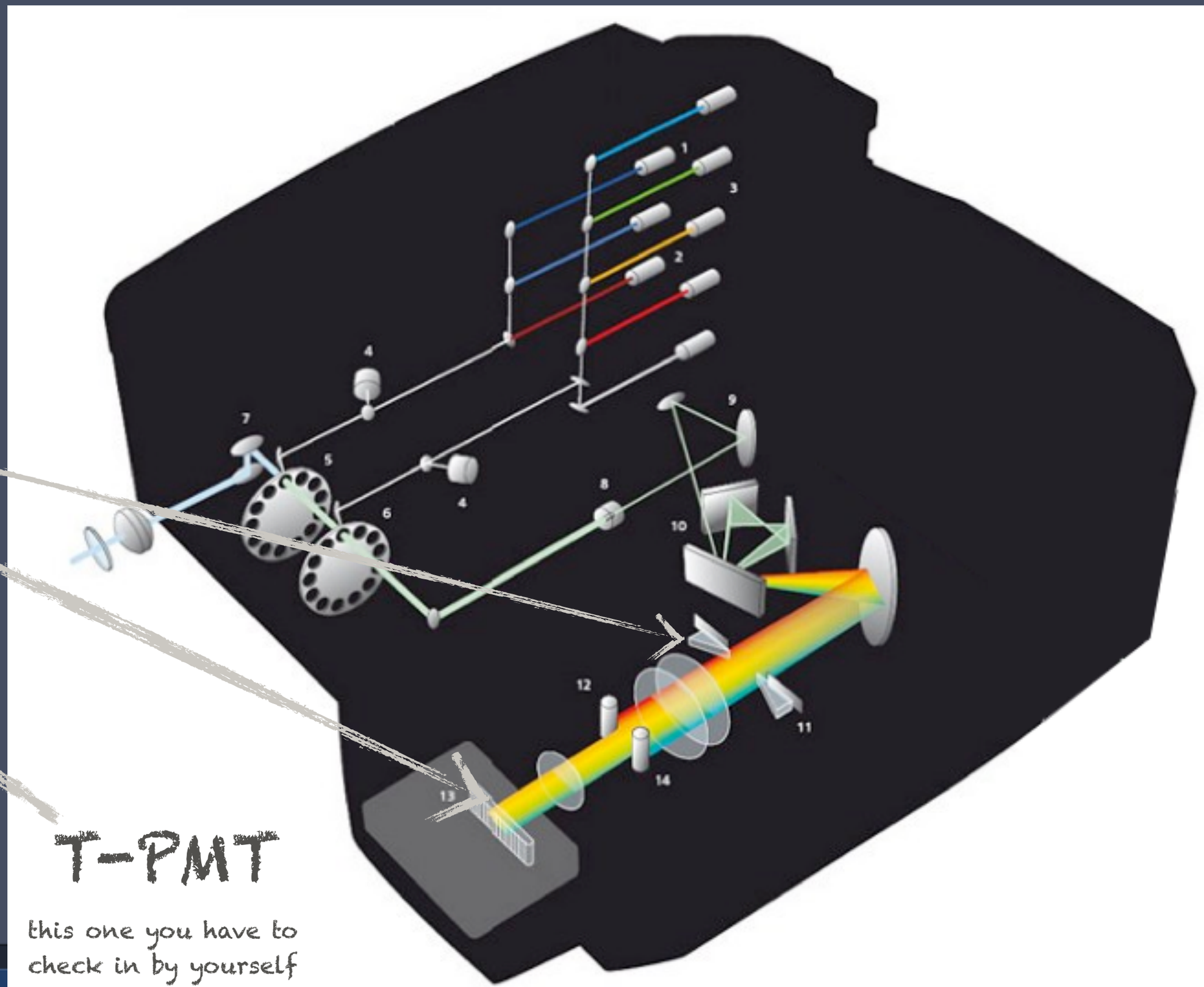
Smart Setup sets up for you the Light path



Smart Setup sets up for you the Light path

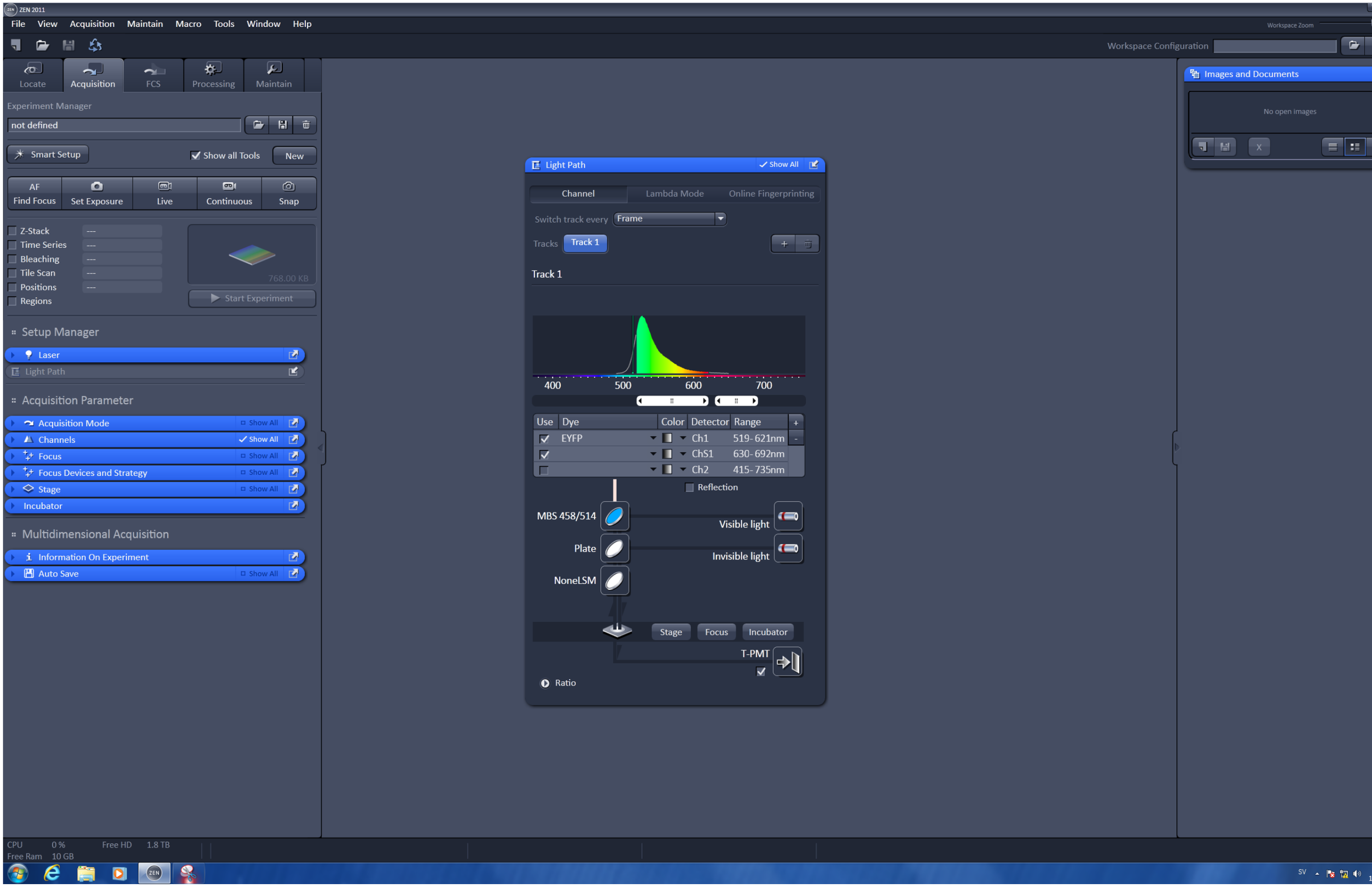


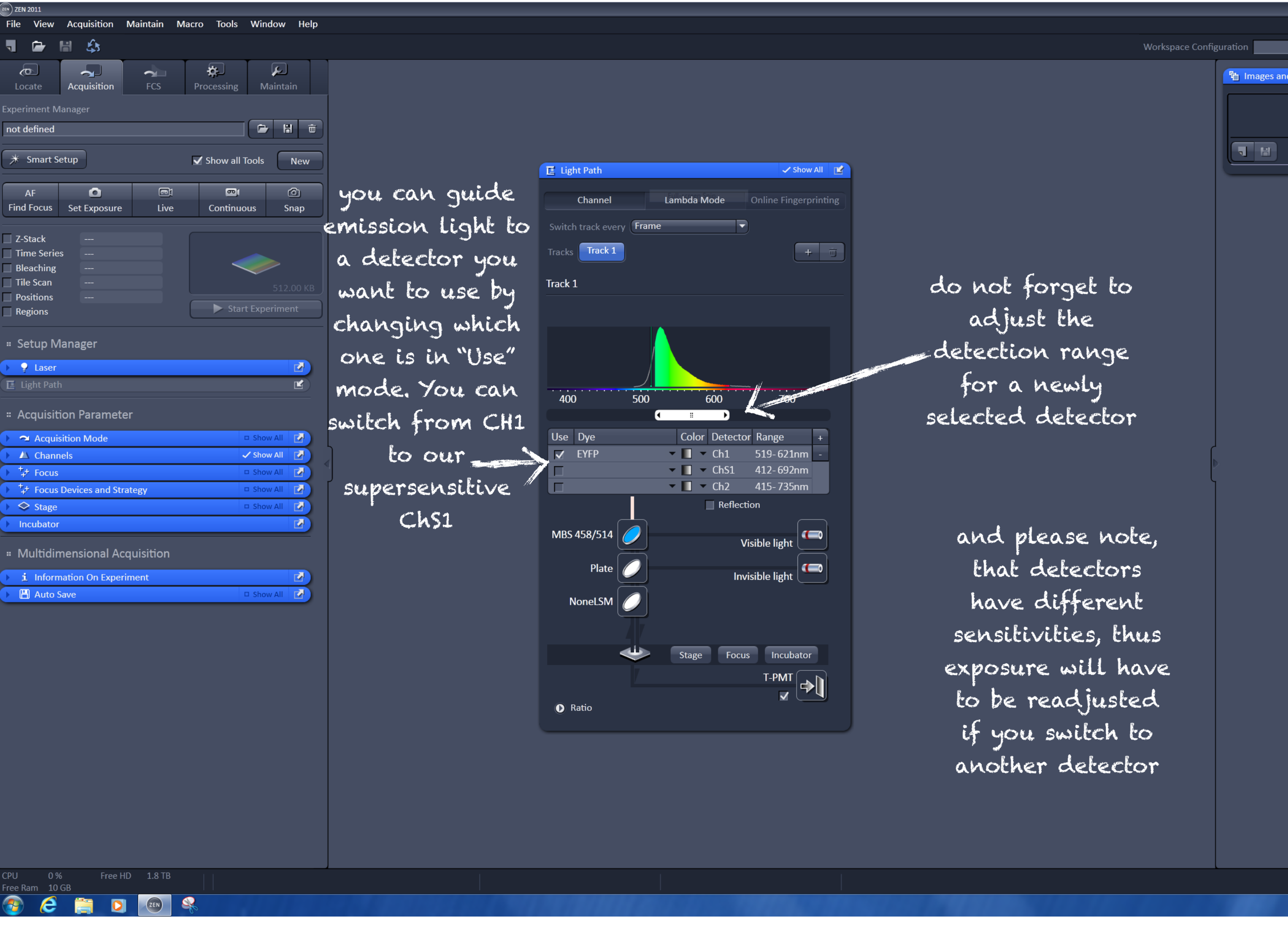
Smart Setup sets up for you the Light path



T-PMT

this one you have to check in by yourself

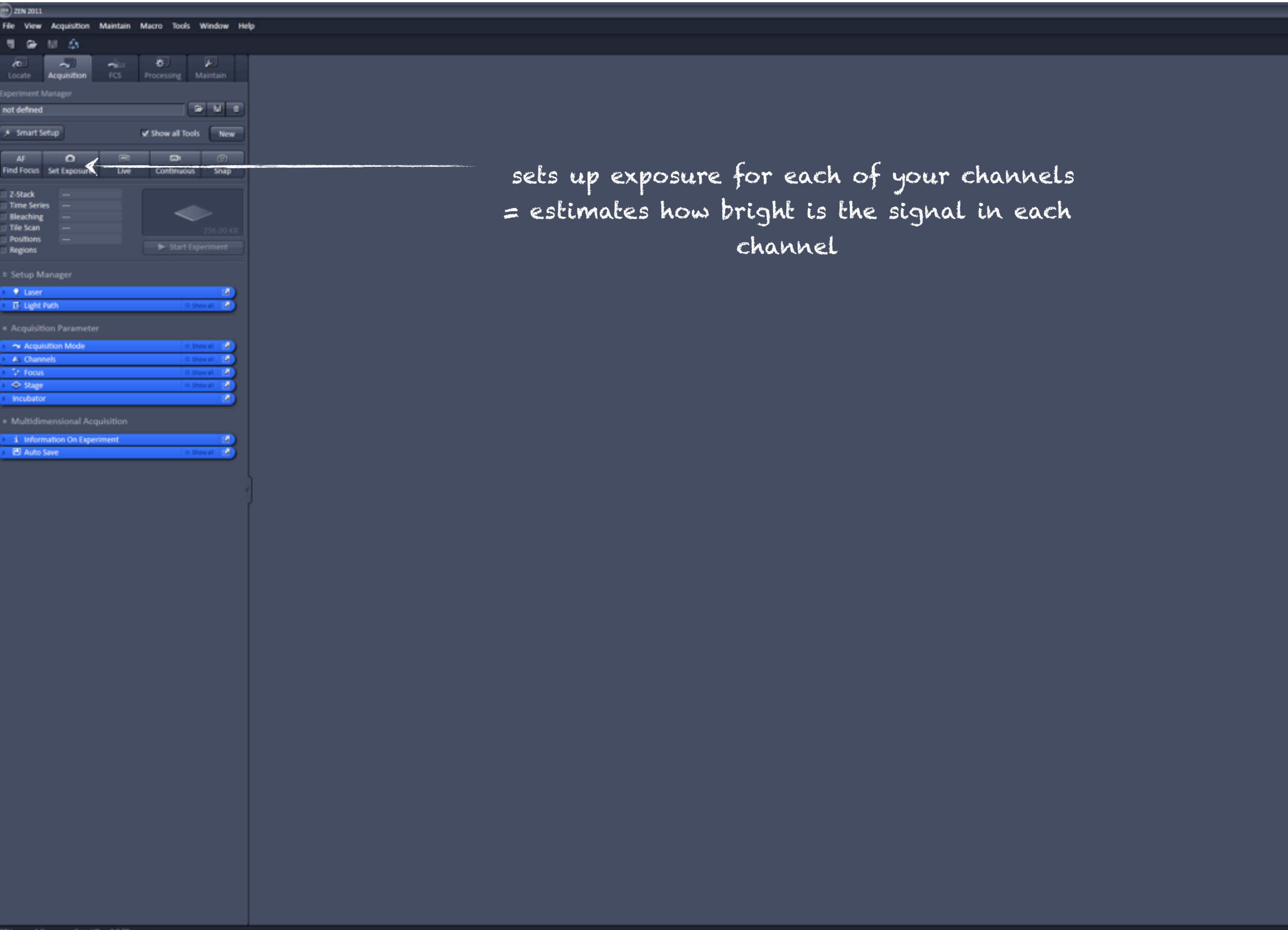

ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Zoom
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
768.00 KB
Start Experiment
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Focus Devices and Strategy
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
Show All
Light Path
Show All
Channel
Lambda Mode
Online Fingerprinting
Switch track every
Frame
Tracks
Track 1
Track 1
400
500
600
700
Use
Dye
Color
Detector
Range
EYFP
Ch1
519-621nm
ChS1
630-692nm
Ch2
415-735nm
Reflection
MBS 458/514
Plate
NoneLSM
Visible light
Invisible light
Stage
Focus
Incubator
T-PMT
Ratio
Images and Documents
No open images
CPU 0 %
Free Ram 10 GB
Free HD 1.8 TB
SV


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
512.00 KB
Start Experiment
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Focus Devices and Strategy
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
Show All
Light Path
Show All
Channel
Lambda Mode
Online Fingerprinting
Switch track every
Frame
Tracks
Track 1
Track 1
400
500
600
Use Dye Color Detector Range
EYFP Ch1 519-621nm
ChS1 412-692nm
Ch2 415-735nm
Reflection
MBS 458/514
Plate
NoneLSM
Visible light
Invisible light
Stage
Focus
Incubator
T-PMT
Ratio
you can guide emission light to a detector you want to use by changing which one is in "Use" mode. You can switch from CH1 to our supersensitive ChS1
do not forget to adjust the detection range for a newly selected detector
and please note, that detectors have different sensitivities, thus exposure will have to be readjusted if you switch to another detector
CPU 0 %
Free Ram 10 GB
Free HD 1.8 TB

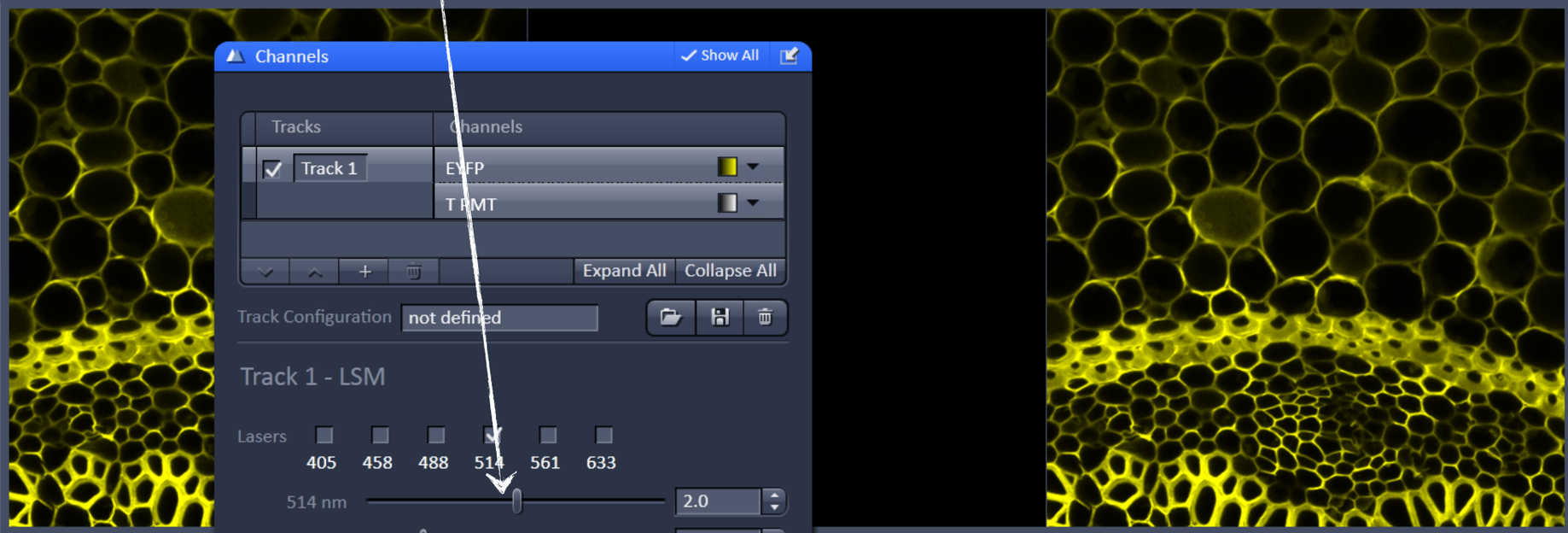
1. Mount your sample and locate the place you want to image

2. Tell software what fluorophore are you going to scan?

3. Ask confocal to estimate how bright your signal is


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF
Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
256.00 KB
Start Experiment
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
sets up exposure for each of your channels
= estimates how bright is the signal in each
channel
CPU 1 %
Free Ram 10 GB
Free HD 2.0 TB

be careful adjusting the laser power!
You can fry and/or bleach your sample



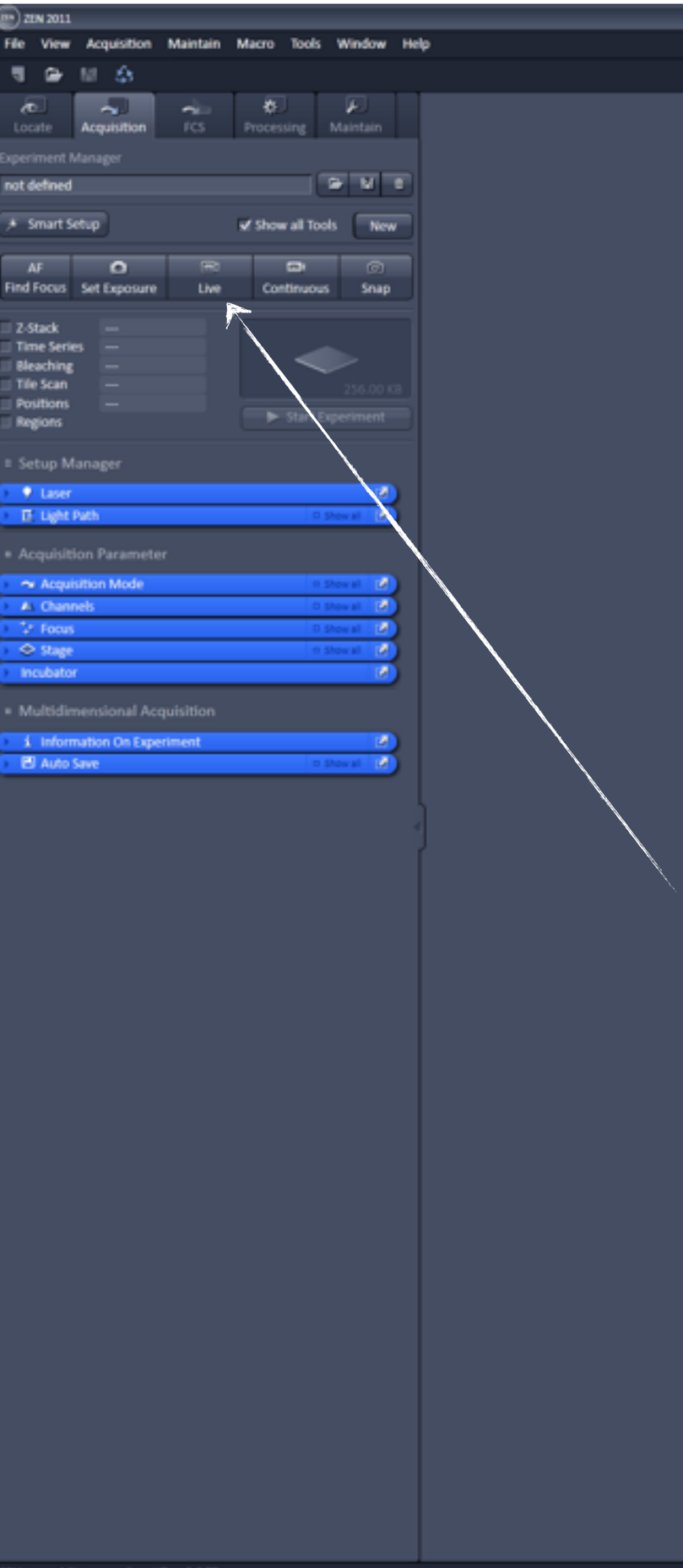
you can adjust pinhole here

you can adjust master gain, digital offset and gain for each of your channels

Keep Master Gain <900 if possible

Set Exposure sets up for you the Channels

ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Image 1
2D Split 2.5D Histo Coloc Profile Info
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Stop
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
512.00 KB
Start Experiment
Setup Manager
Laser
Light Path
Show All
Acquisition Parameter
Acquisition Mode
Channels
Focus
Focus Devices and Strategy
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
Channels
Show All
Tracks
Channels
Track 1
EYFP
T PMT
Expand All
Collapse All
Track Configuration
not defined
Track 1 - LSM
Lasers
405 458 488 514 561 633
514 nm
2.0
Pinhole
32.6
1.00 Airy Units = 1.8 µm section
1 AU
max
EYFP
Mode
Integration
Photon Counting
Gain (Master)
544
Digital Offset
0
Digital Gain
1.2
T PMT
Gain (Master)
344
Digital Offset
0
Digital Gain
1.1
Display
Dimensions
Display
Graphics
Zoom
100 %
71
Channels
ChS1
T PMT
Single Channel
Range Indicator
Merged
Reuse
Crop
Positions
Stage
You can see the effect of your adjustments in Live scanning
CPU 2 %
Free Ram 10 GB
Free HD 1.8 TB
Cancel
Grabbing



LIVE. very fast continuos scan in all channels at resolution 512x512 (this is shitty resolution)
This option is basically replaces oculars for you

while scanning in the LIVE mode you can:
change the focus
move the sample
adjust parameters in Channels
set borders for Z-stack

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Workspace Configurat

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions

512.00 KB

Start Experiment

Setup Manager

Laser
Light Path — Show All

Acquisition Parameter

Acquisition Mode — Show All
Channels — Show All
Focus — Show All
Focus Devices and Strategy — Show All
Stage — Show All
Incubator

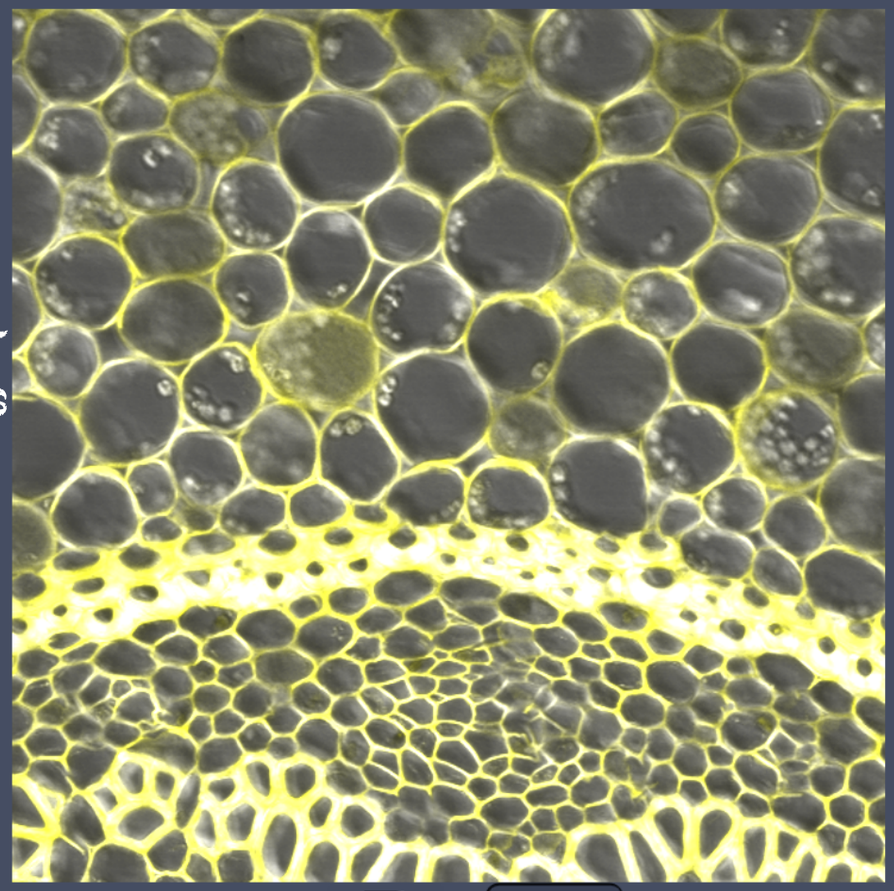
Multidimensional Acquisition

Information On Experiment
Auto Save — Show All

Image 1

2D
Split
2.5D
Histo
Coloc
Profile
Info

overlay of all your channels



Show All

Dimensions | Display | Graphics

Zoom 100 % 143

Channels ChS1 T PMT

Single Channel | Range Indicator

Reuse | Crop | Positions | Stage

CPU 0 % Free HD 1.8 TB
Free Ram 10 GB

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Workspace

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions

512.00 KB

Start Experiment

Setup Manager

Laser
Light Path — Show All
Channels — Show All
Focus — Show All
Focus Devices and Strategy — Show All
Stage — Show All
Incubator

Acquisition Parameter

Acquisition Mode — Show All

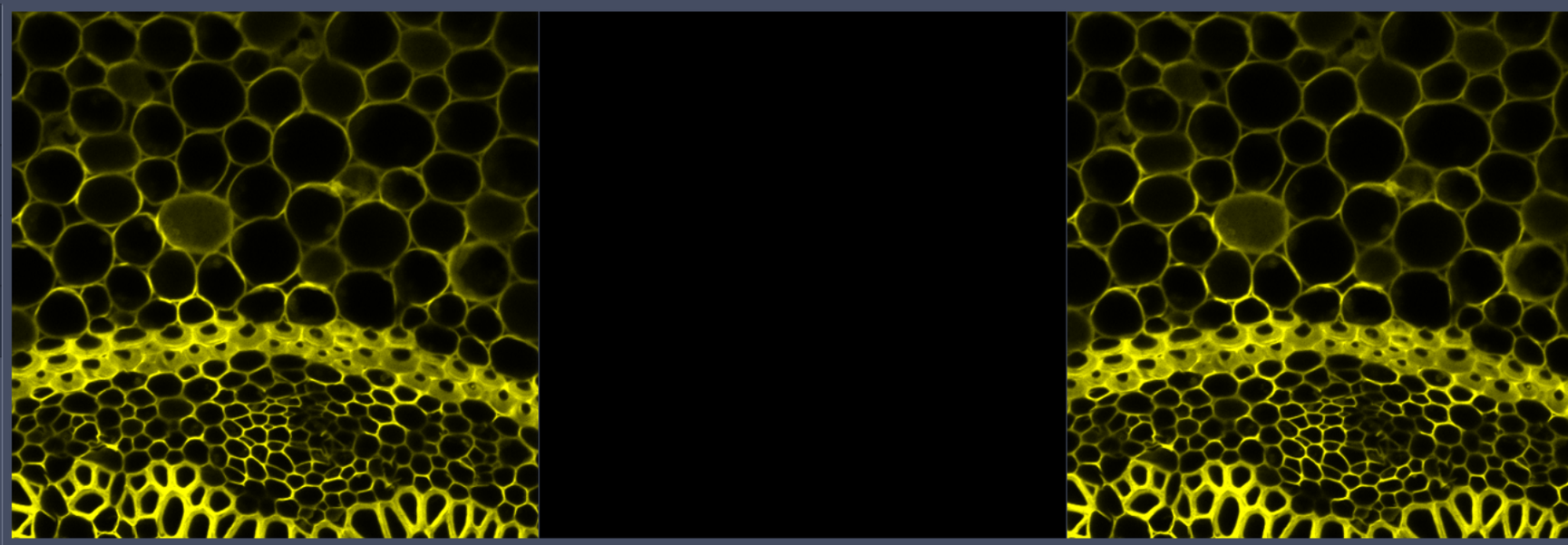
Multidimensional Acquisition

Information On Experiment
Auto Save — Show All

Image 1

2D
Split
2.5D
Histo
Coloc
Profile
Info

each channel can be seen individually



Show All

Dimensions | Display | Graphics

Zoom 100 % 92

Channels ChS1 T PMT

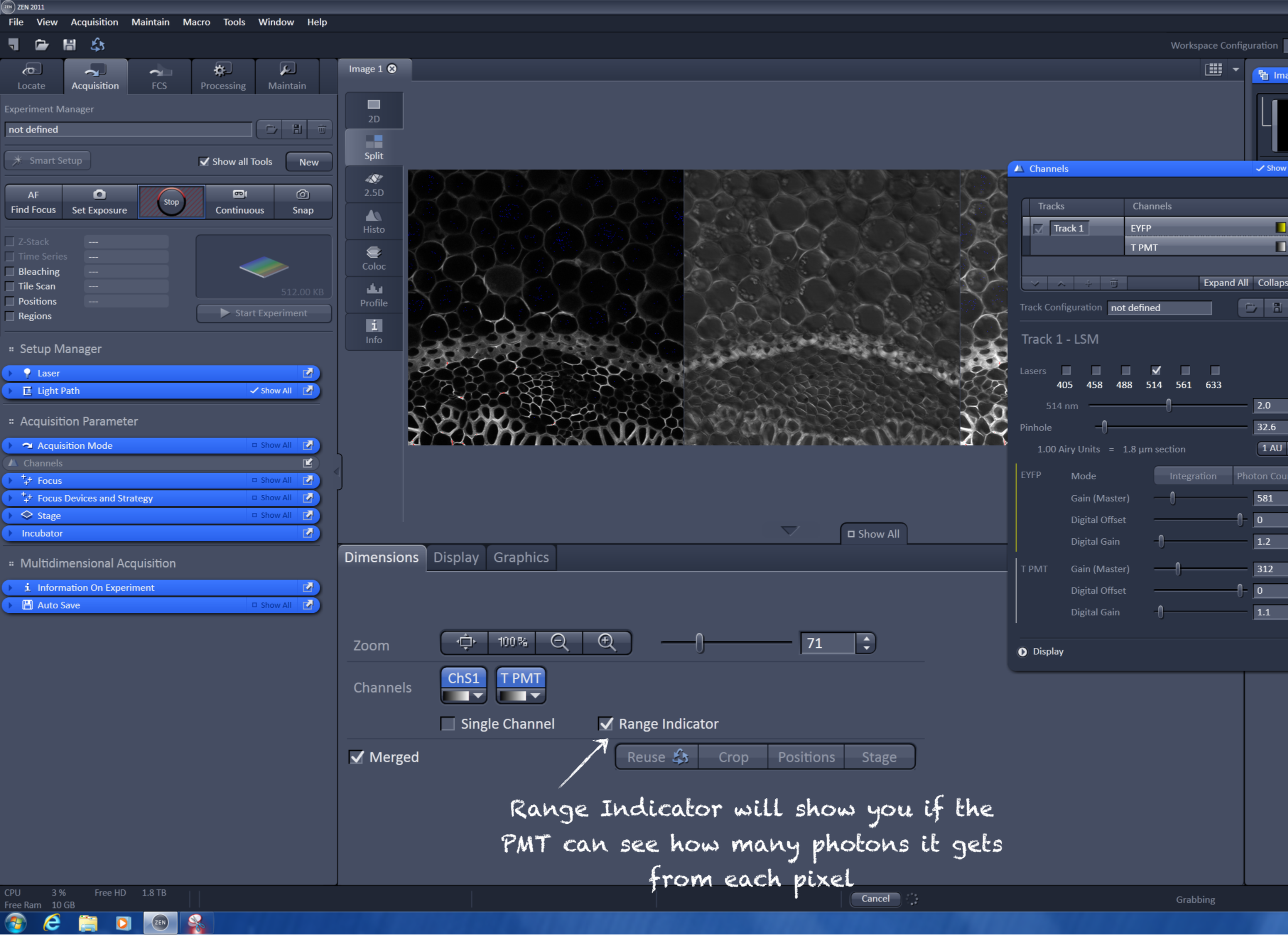
Single Channel | Range Indicator

Merged

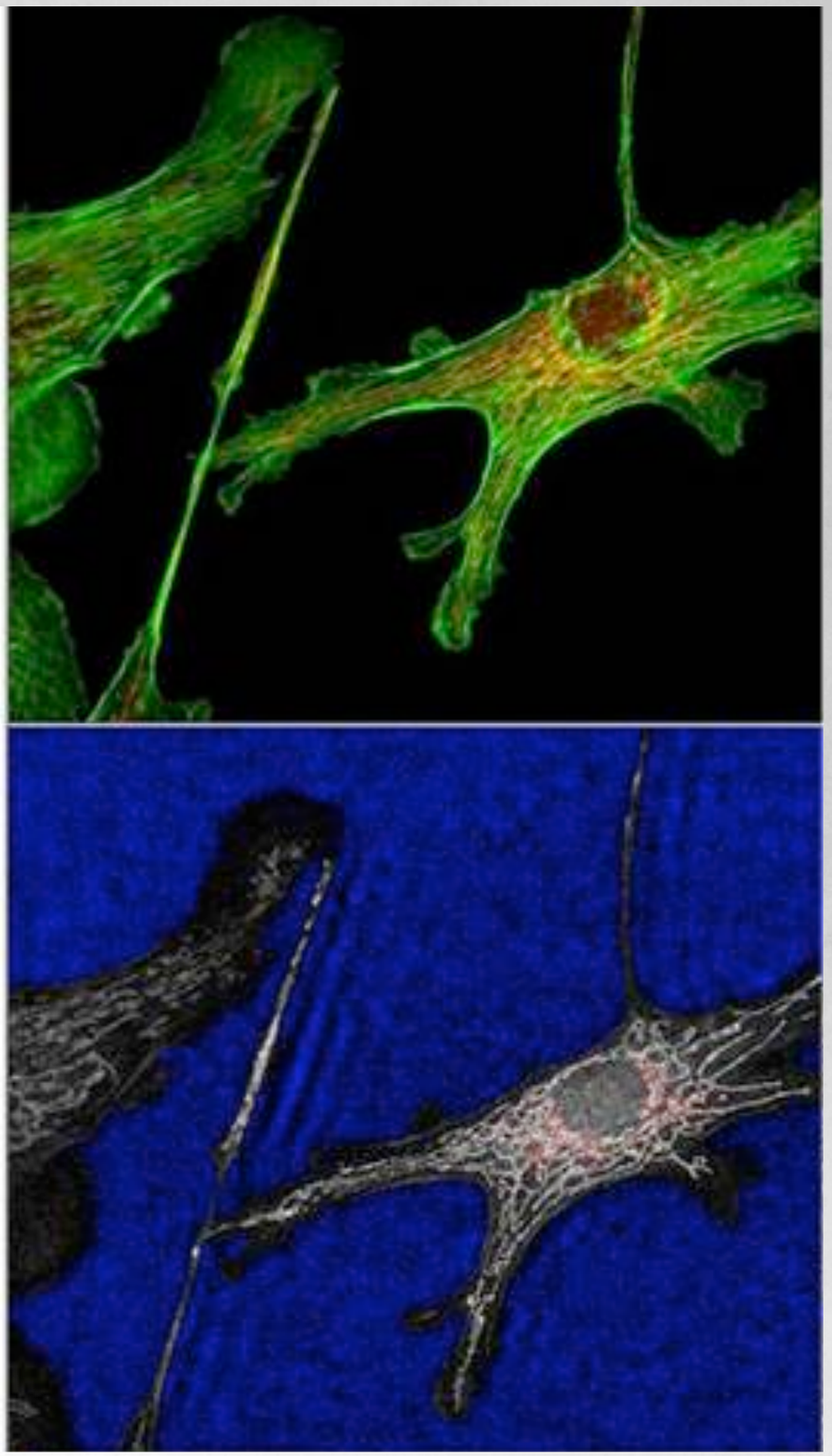
Reuse | Crop | Positions | Stage

channels which are shown to you are marked blue,
you can select/deselect channels which you want to see

CPU 1 % Free HD 1.8 TB
Free Ram 10 GB


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Image 1
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Stop
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
512.00 KB
Start Experiment
2D
Split
2.5D
Histo
Coloc
Profile
Info
Setup Manager
Laser
Light Path
Show All
Acquisition Parameter
Acquisition Mode
Channels
Focus
Focus Devices and Strategy
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
Channels
Tracks
Channels
Track 1
EYFP
T PMT
Expand All
Collaps
Track Configuration
not defined
Track 1 - LSM
Lasers
405 458 488 514 561 633
514 nm
2.0
Pinhole
32.6
1.00 Airy Units = 1.8 µm section
1 AU
EYFP
Mode
Integration
Photon Cou
Gain (Master)
581
Digital Offset
0
Digital Gain
1.2
T PMT
Gain (Master)
312
Digital Offset
0
Digital Gain
1.1
Display
Show All
Dimensions
Display
Graphics
Zoom
100 %
71
Channels
ChS1
T PMT
Single Channel
Range Indicator
Merged
Reuse
Crop
Positions
Stage
Range Indicator will show you if the PMT can see how many photons it gets from each pixel
CPU 3 %
Free Ram 10 GB
Free HD 1.8 TB
Cancel
Grabbing

Range indicator color-codes pixels which are within and out of the range of the PMT



it is difficult for a human eye to see what is the intensity of each pixel here

please discuss what colours do you want to see on the range indicator image in what case

the PMT can not detect any photons

the PMT can count how many photons it catches

the PMT gets too many photons, can't count them anymore

1. Mount your sample and locate the place you want to image

2. Tell software what fluorophore are you going to scan?

3. Ask confocal to estimate how bright your signal is

4. See the result in LIVE scanning mode. If required adjust pinhole, Master Gain etc in Channels

1. Mount your sample and locate the place you want to image

2. Tell software what fluorophore are you going to scan?

3. Ask confocal to estimate how bright your signal is

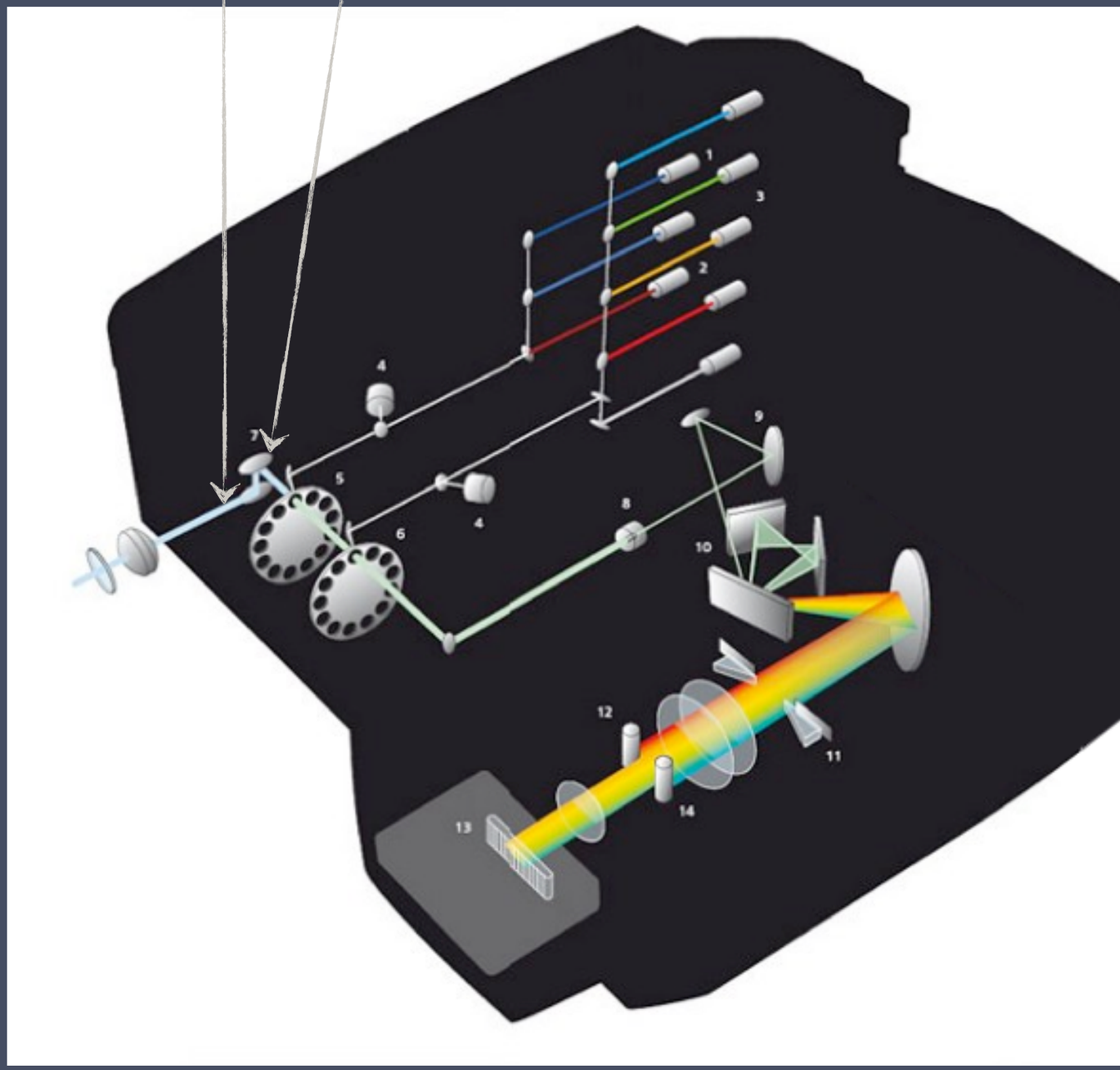
4. See the result in LIVE scanning mode. If required adjust pinhole, Master Gain etc in Channels

5. Adjust the resolution of your image in Acquisition mode

Acquisition mode

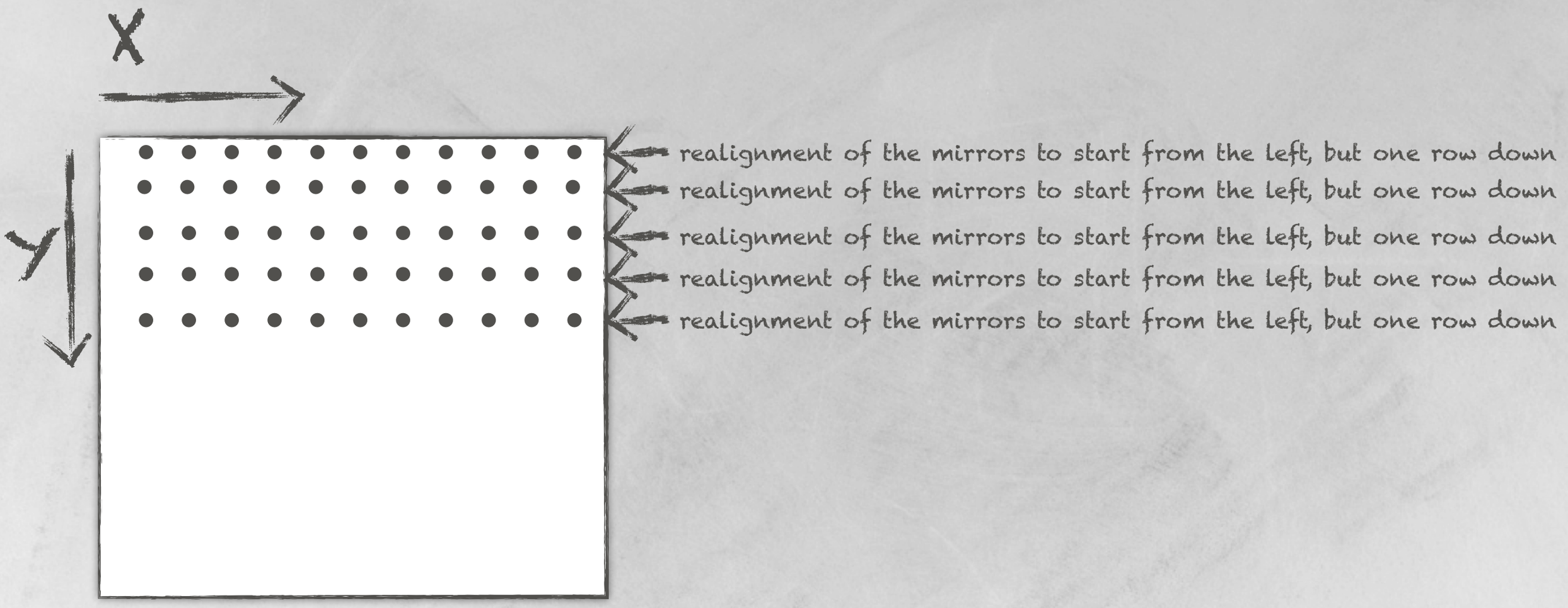
galvanometer mirrors

bi-directional scanning is twice faster

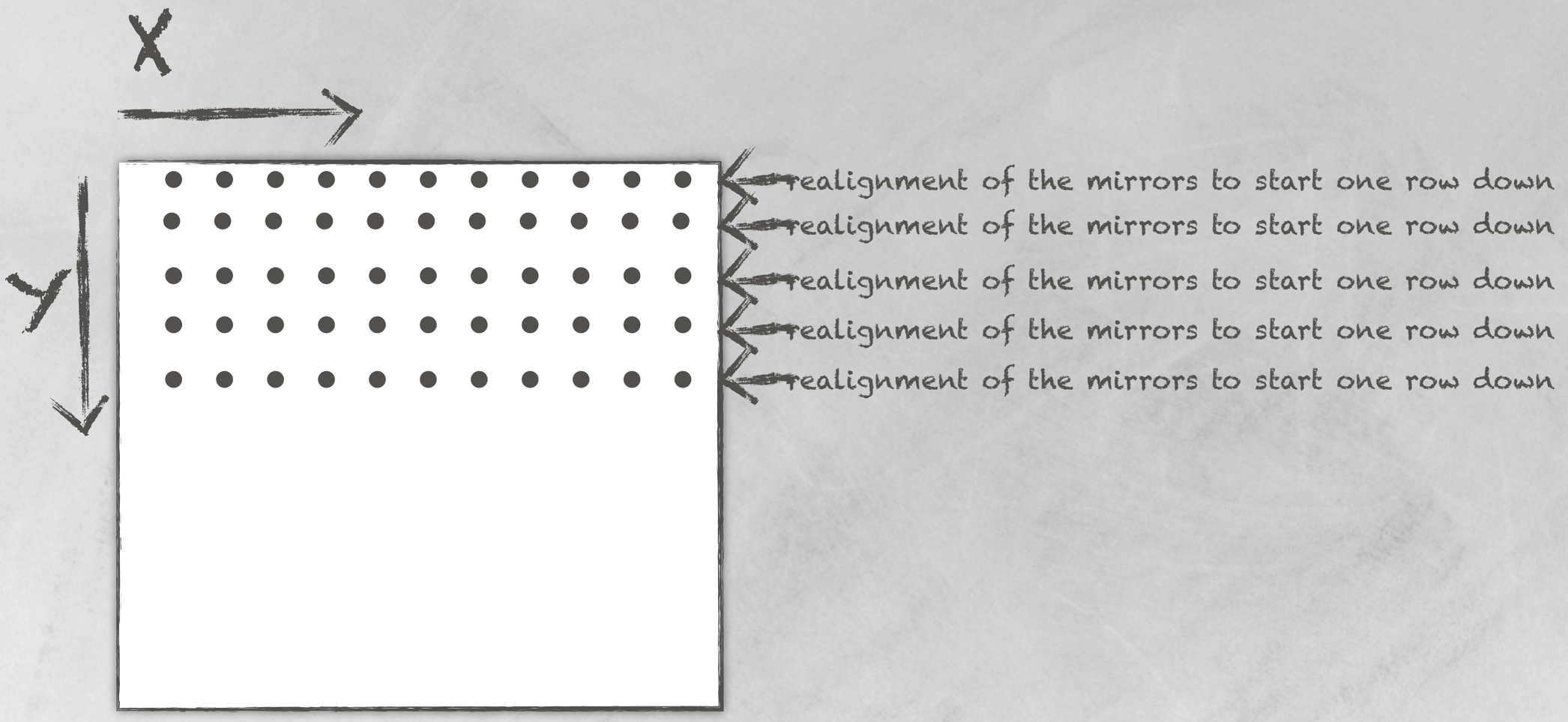


you can drag the blue dot to rotate area of scanning, zoom in and out

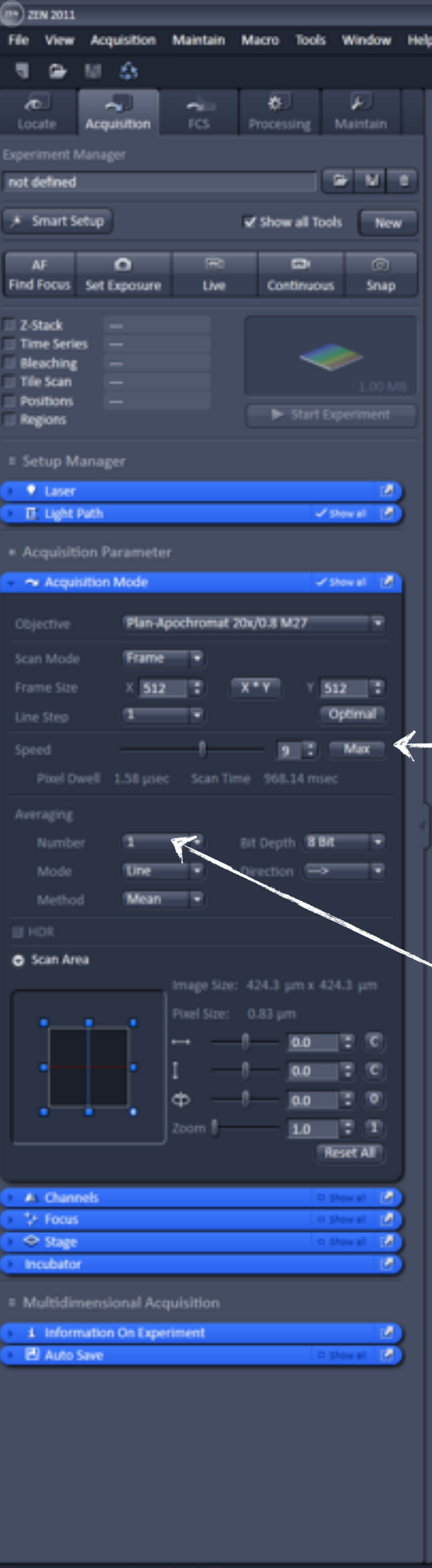
Single directional scanning



Bidirectional scanning



Acquisition mode



scanning speed/dwelling time

amount of time a PMT can spend "looking" at each pixel

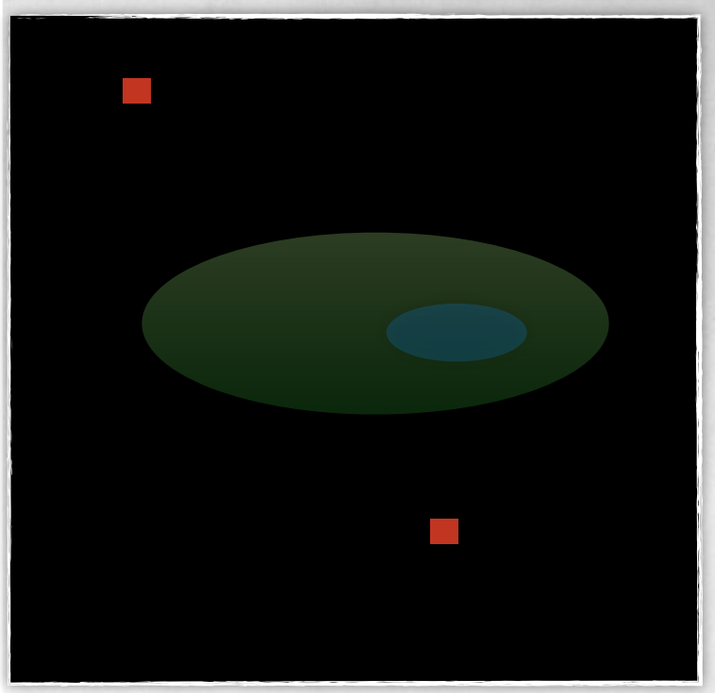
increasing number of scanning for each line makes picture much sharper

Speed of scanning

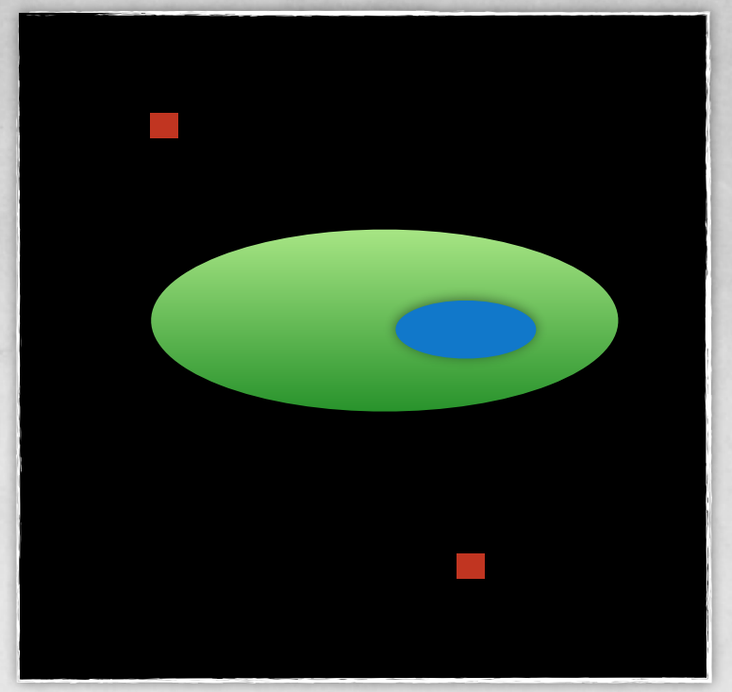
at high speed PMT doesn't have time to catch a lot of catches photons
=> true signal will not be very strong, comparable with the background noise

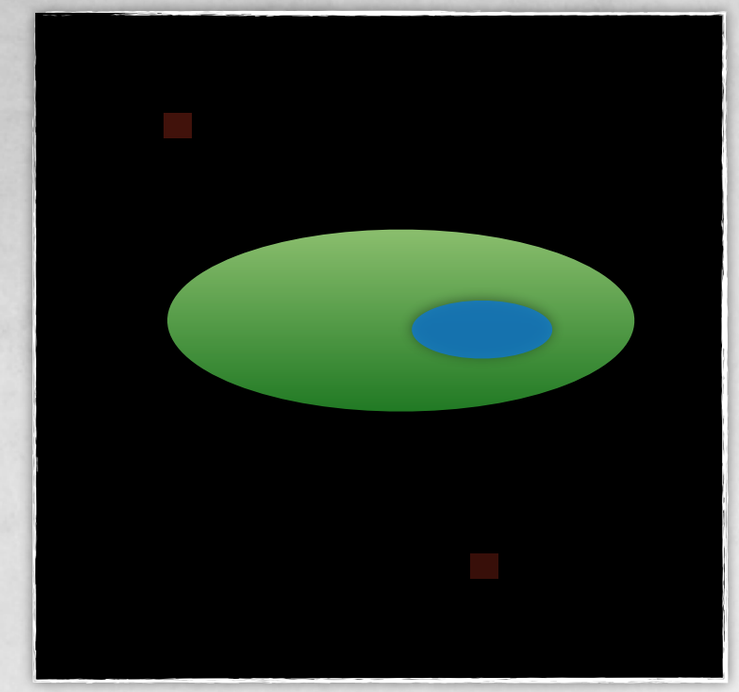
at low speed PMT can catch a lot of photons from your fluorophore
=> true signal will not be very strong, background noise can be thresholded away

11 = high speed



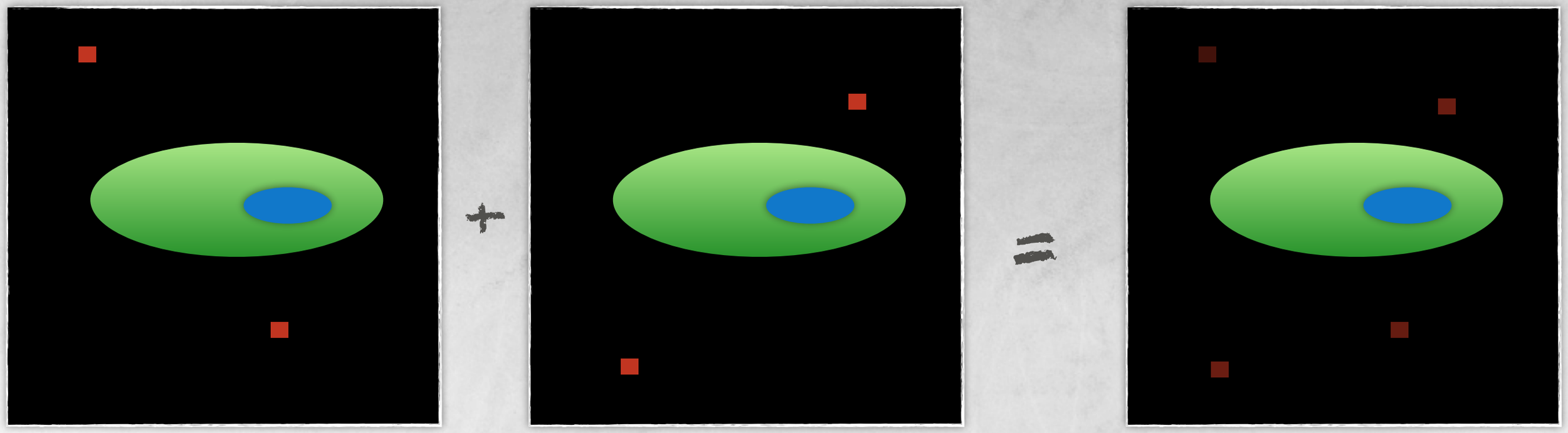
3 = low speed





Averaging

- noise pixels appear in random positions

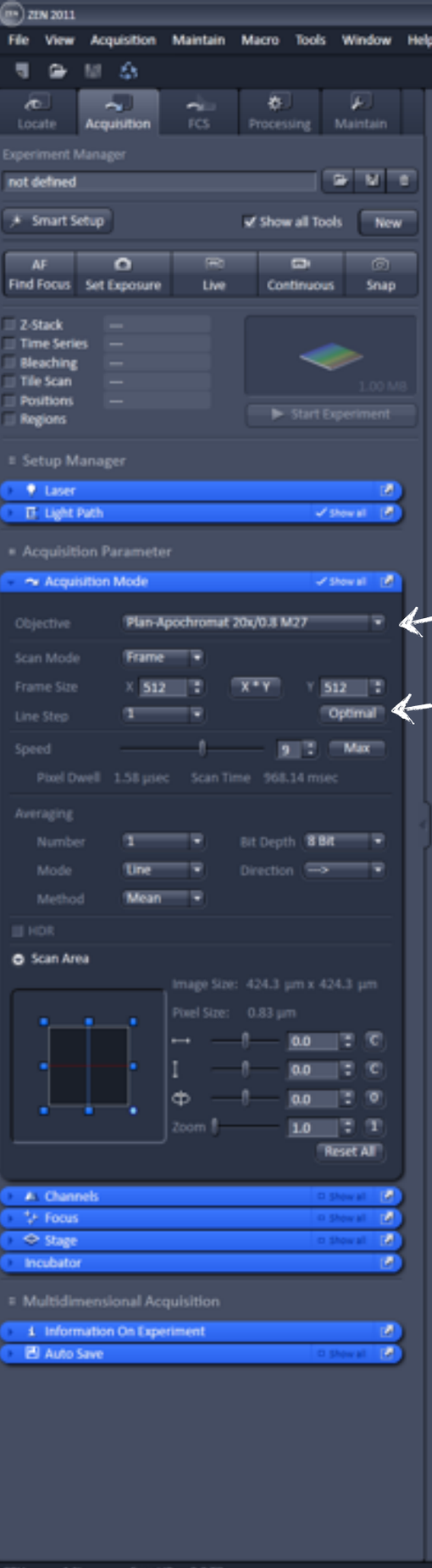


Averaging vs. Speed of scanning

you need to detect vesicles which move very fast,
which one to change?

you need to detect fluorophore which bleaches
extremely fast which one to change?

Acquisition mode



objective you are using

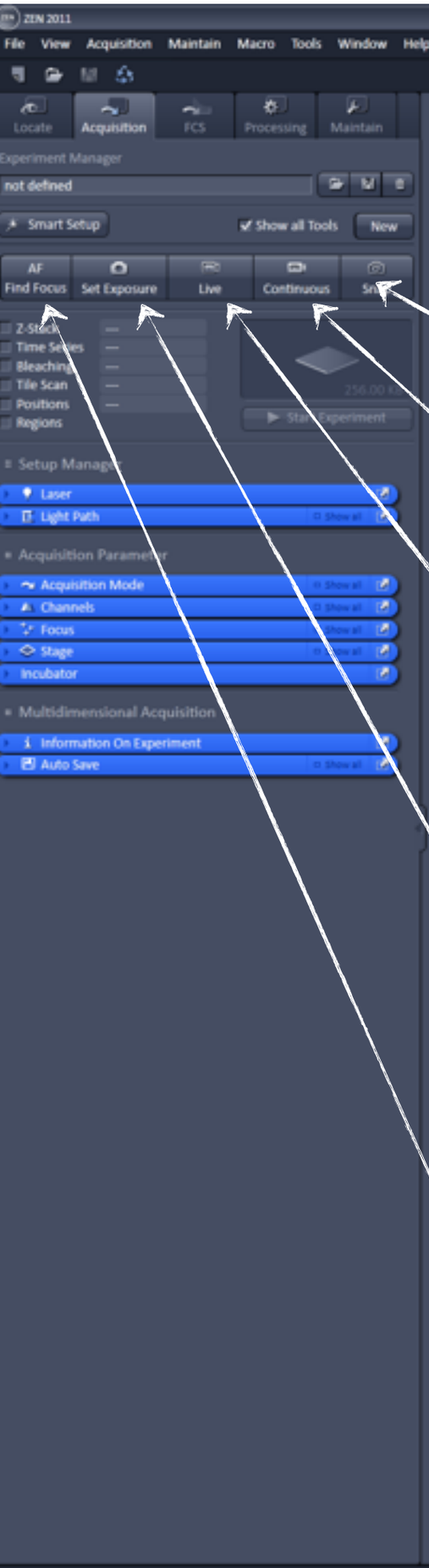
optimal resolution for each objective/fluorophore

DO NOT use higher than Optimel (highest) resolution

Changes made in the Acqusition Mode can be seen in Continuous scanning mode

Live replacement of oculars

Continuous shows you what the scanned image will look like



SNAP. makes a snap picture in all channels at resolution/ speed you selected in the acquisition mode

CONTINUOS. continuos scan in all channels at resolution/speed you selected in the acquisition mode

LIVE. very fast continuos scan in all channels at resolution 512x512

SET EXPOSURE. estimates signal intensity in each of your channels

FIND FOCUS. makes a quick vertical scan and focuses on the brightest place

1. Mount your sample and locate the place you want to image

2. Tell software what fluorophore are you going to scan?

3. Ask confocal to estimate how bright your signal is

4. See the result in LIVE scanning mode. If required adjust pinhole, Master Gain etc in Channels

5. Adjust the resolution of your image in Acquisition mode

6. Activate z-stack and set up parameters for it. Start the Experiment

PMT

Z stack

X

Y

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Locate Acquisition FCS Processing Maintain

Experiment Manager

not defined

Smart Setup Show all Tools New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack

Time Series

Bleaching

Tile Scan

Positions

Regions

256.00 KB

Start Experiment

Setup Manager

Laser

Light Path

Acquisition Parameter

Acquisition Mode

Channels

Focus

Stage

Incubator

Multidimensional Acquisition

Information On Experiment

Auto Save

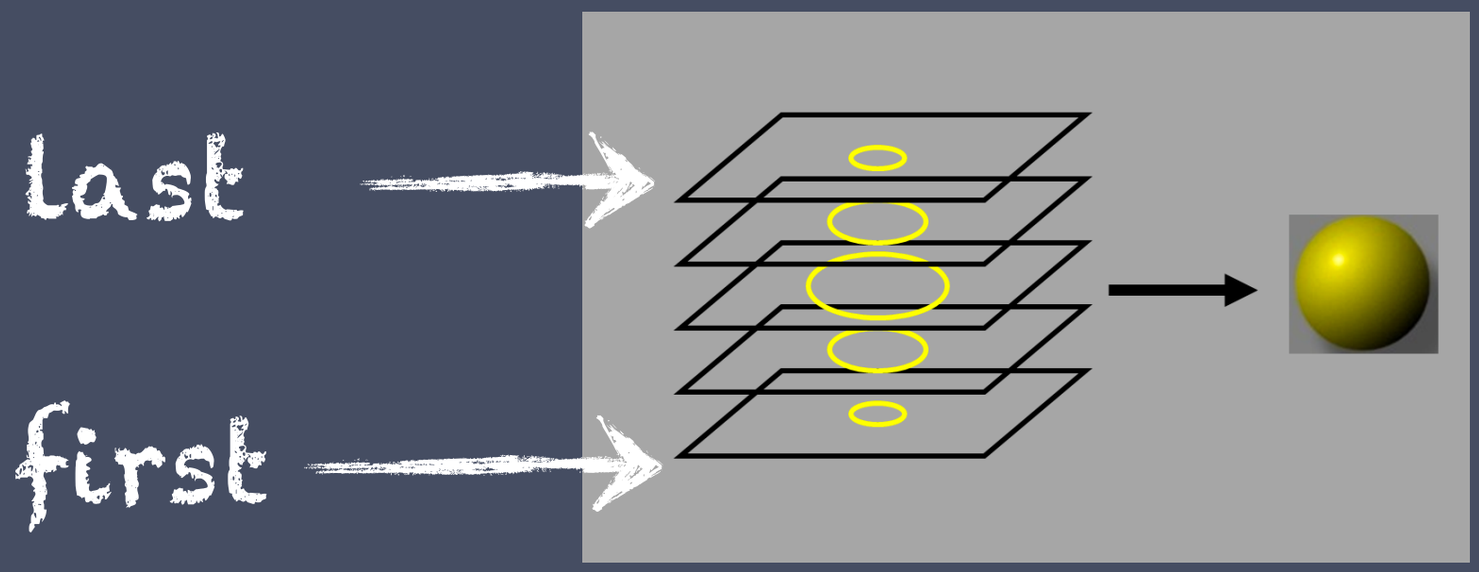
CPU 1 % Free HD 2.0 TB

Free Ram 10 GB

z-stack

The software needs to know:

where should be the top optical slice
where should be the bottom optical slice
how many optical slice you want to make



Z-stacks

3. using micro-focus select the lower (click "set last") and upper border (click "set first")

4. stop fast scan and click on "optimal slice" or type in manually the amount of slices

2.turn on fast scan

1.activate Z-stack

START EXPERIMENT to make a z-stack

5. you can correct channel settings for different slices of Z-stack

Please discuss:

what does define optimal size of the pinhole

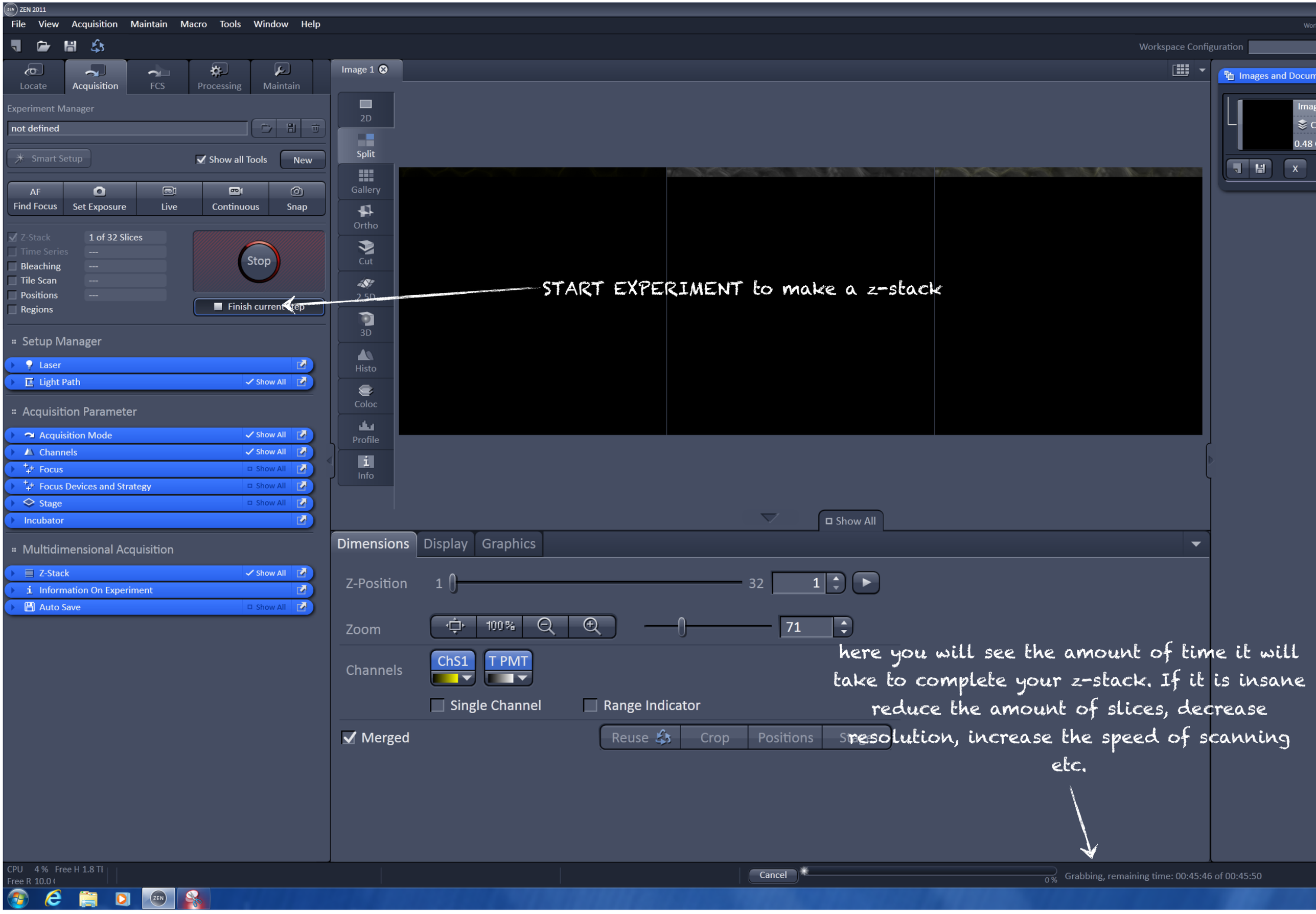
why to use optimal pinhole size

what would happen t your z-stack if you use pinhole larger than optimal

what would happen t your z-stack if you use pinhole smaller than optimal

would you use the same size of pinhole for GFP and RFP channels during simultaneous scanning

would you use the same size of pinhole for GFP and RFP channels during sequential scanning


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Image 1
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
1 of 32 Slices
Stop
Finish current Step
START EXPERIMENT to make a z-stack
2D
Split
Gallery
Ortho
Cut
2.5D
3D
Histo
Coloc
Profile
Info
Images and Docum
Setup Manager
Laser
Light Path
Show All
Acquisition Parameter
Acquisition Mode
Channels
Focus
Focus Devices and Strategy
Stage
Incubator
Multidimensional Acquisition
Z-Stack
Information On Experiment
Auto Save
Dimensions
Display
Graphics
Z-Position
1
32
Zoom
100 %
71
Channels
ChS1
T PMT
Single Channel
Range Indicator
Merged
Reuse
Crop
Positions
here you will see the amount of time it will take to complete your z-stack. If it is insane reduce the amount of slices, decrease resolution, increase the speed of scanning etc.
CPU 4 % Free H 1.8 TI
Free R 10.0
Cancel
0 %
Grabbing, remaining time: 00:45:46 of 00:45:50

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

- [x] Z-Stack — 32 Slices
- [ ] Time Series — ---
- [ ] Bleaching — ---
- [ ] Tile Scan — ---
- [ ] Positions — ---
- [ ] Regions

492.29 MB

Start Experiment

Setup Manager

- Laser
- Light Path — Show All

Acquisition Parameter

- Acquisition Mode — Show All
- Channels — Show All
- Focus — Show All
- Focus Devices and Strategy — Show All
- Stage — Show All
- Incubator

Multidimensional Acquisition

- Z-Stack — Show All
- Information On Experiment
- Auto Save — Show All

Image 1

2D | Split | Gallery | Ortho | Cut | 2.5D | 3D | Histo | Coloc | Profile | Info

Save As

Save in: 2014 Confocal course (\\10.0.0.1) (Z:)

Recent Places | Desktop | Libraries | Computer | Network

Name	Date modified	Type	Size
1. Anna & Panisara	11/10/2014 12:45 ...	File folder	
2. Eva & Mohammed	11/10/2014 12:46 ...	File folder	
3. Abdul & Masud	11/10/2014 12:46 ...	File folder	
4. Reza & Catarina	11/10/2014 12:46 ...	File folder	
5. Leonor & Shirin	11/10/2014 12:47 ...	File folder	
6. Ylva & Tania	11/10/2014 12:47 ...	File folder	
7. Maria & Daniel	11/10/2014 12:47 ...	File folder	
8. Jing & Xue	11/10/2014 12:48 ...	File folder	
9. Rita & Clement	11/10/2014 12:48 ...	File folder	
Alyona	11/10/2014 1:06 PM	File folder	

File name: Image 1 — Save

Save as type: Carl Zeiss Image (*.czi;*.zisraw) — Cancel

SAVE your files into your groups folder. Please save your images as .czi (carl zeiss image). The file will contain ALL the metadata

Show All

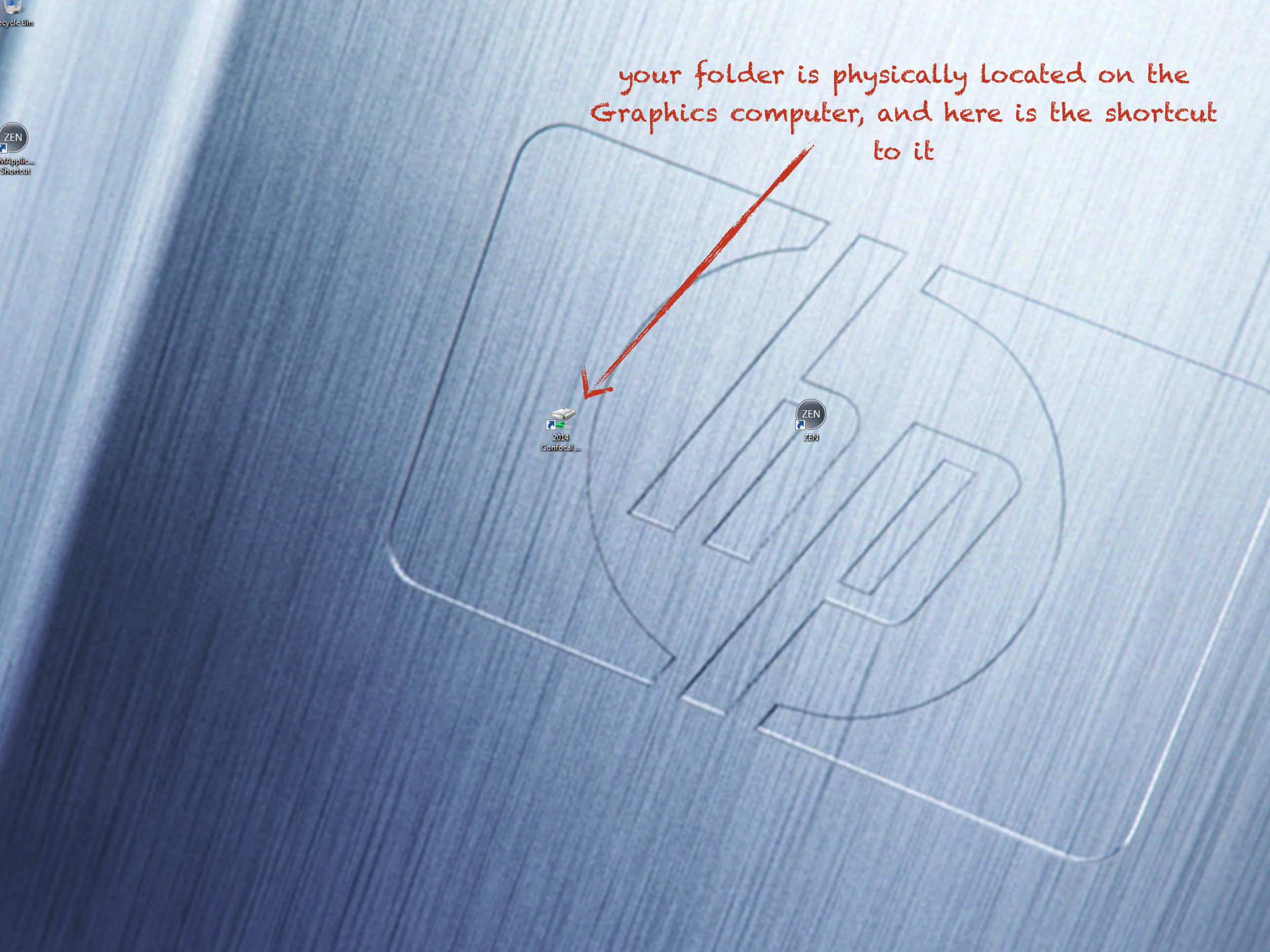
Dimensions | Display | Graphics

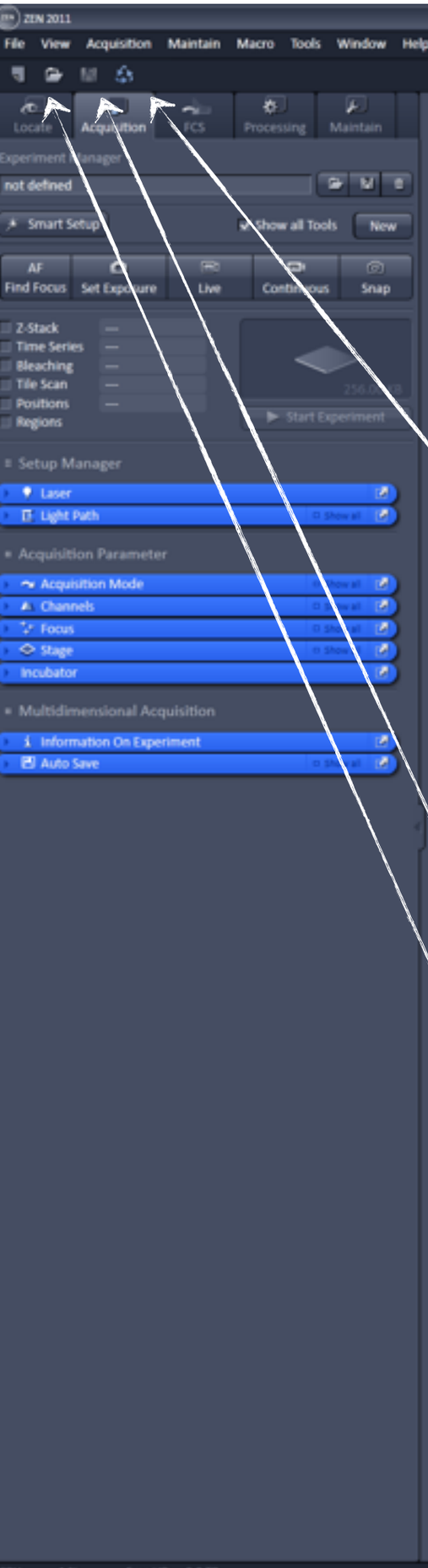
Z-Position 1 — 32 — 17

Zoom — 100% — 17

Channels — ChS1 | T PMT

your folder is physically located on the Graphics computer, and here is the shortcut to it

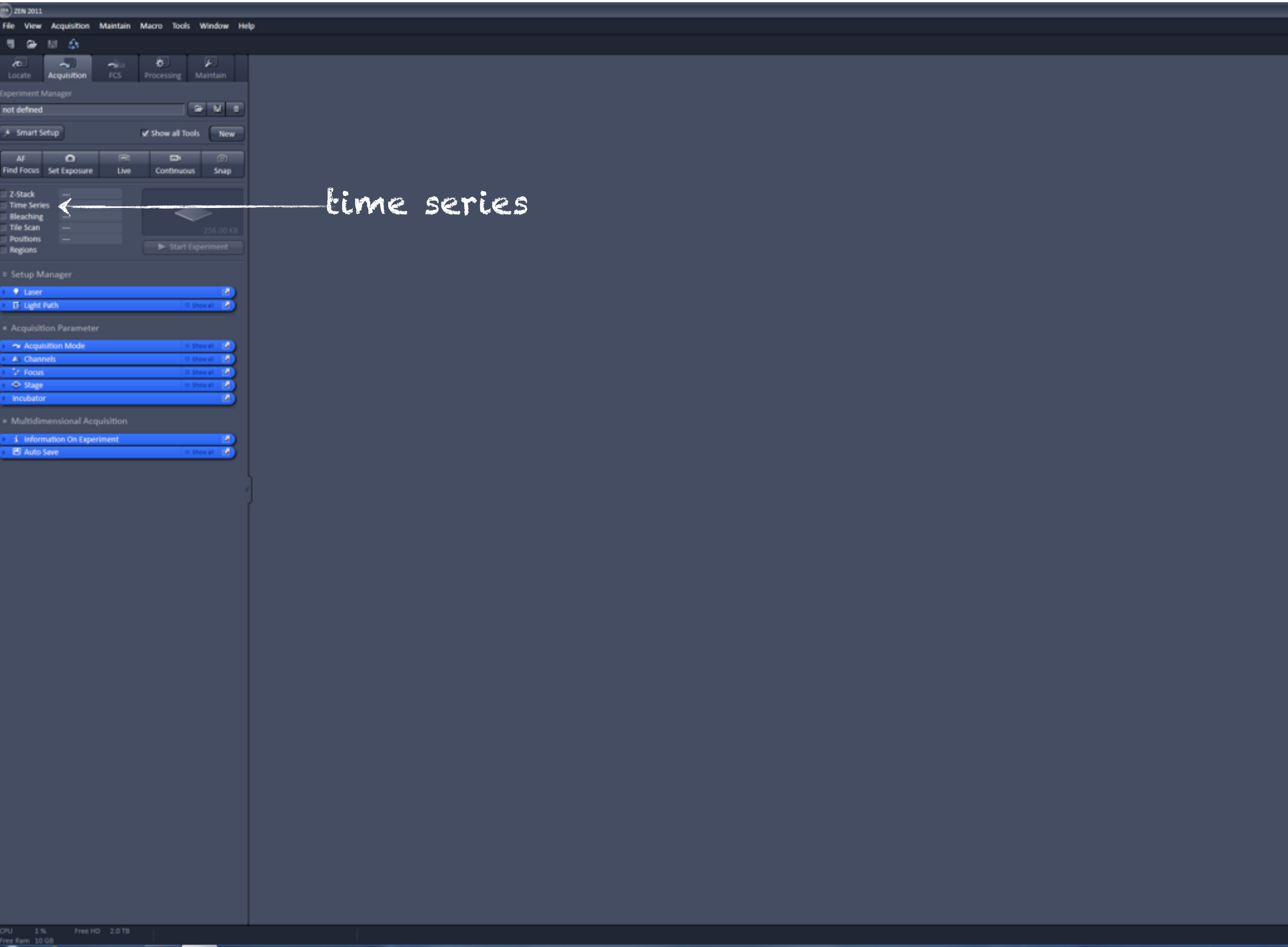


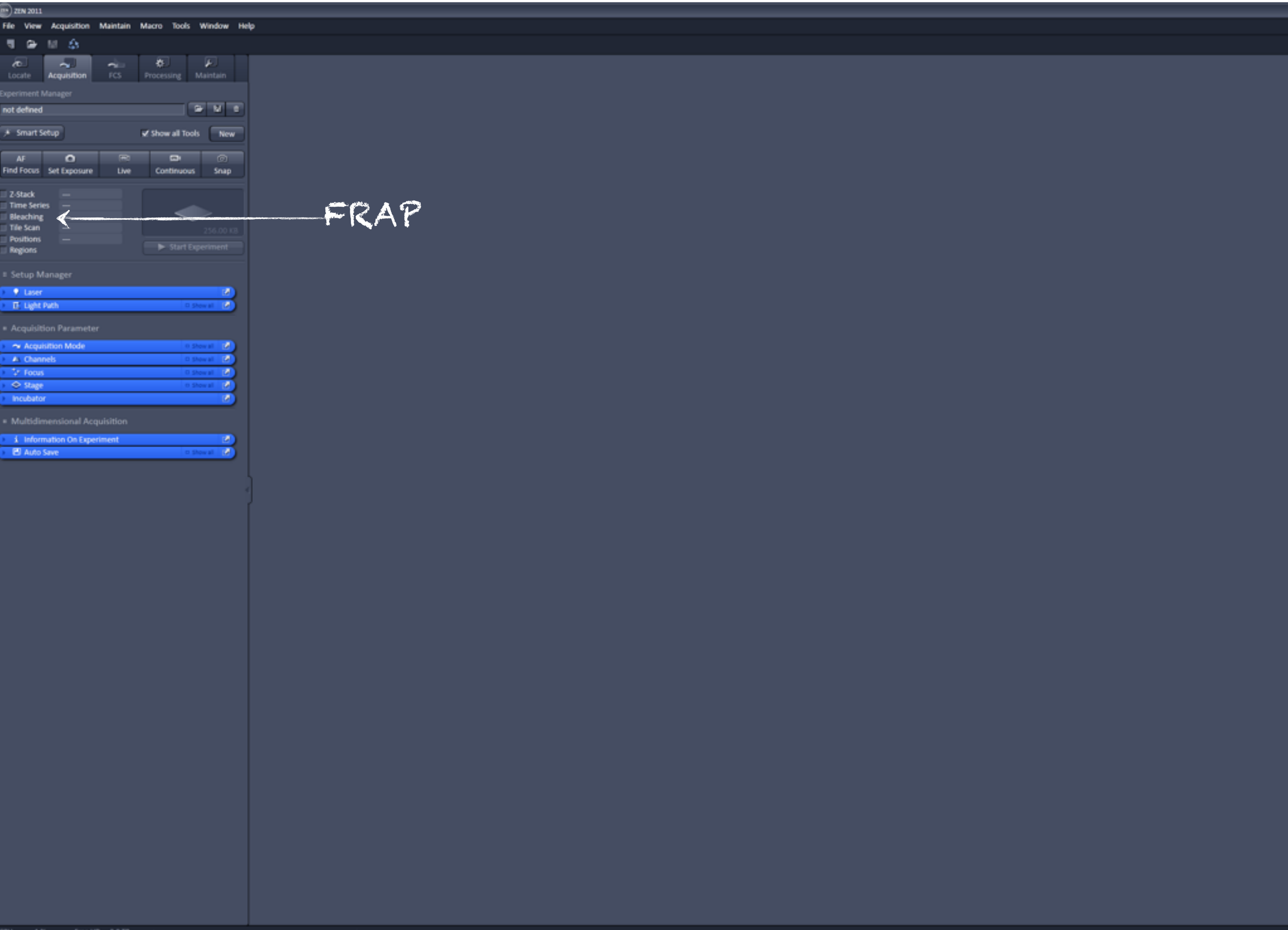


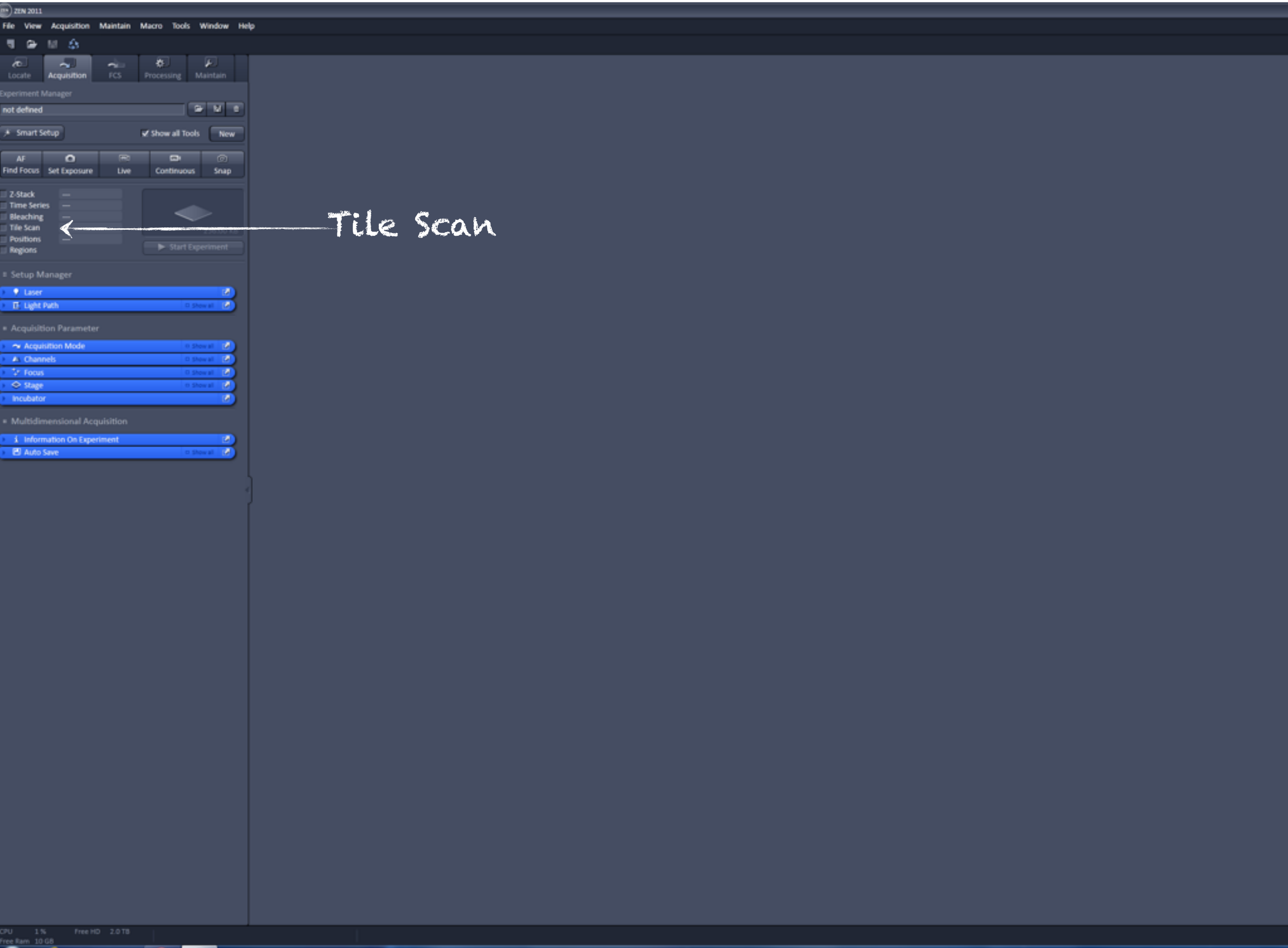
REUSE. you can open an old image and click on reuse. The software will reuse ALL the settings from this image

SAVE. Please save your images as .czi (carl zeiss image). The file will contain ALL the metadata

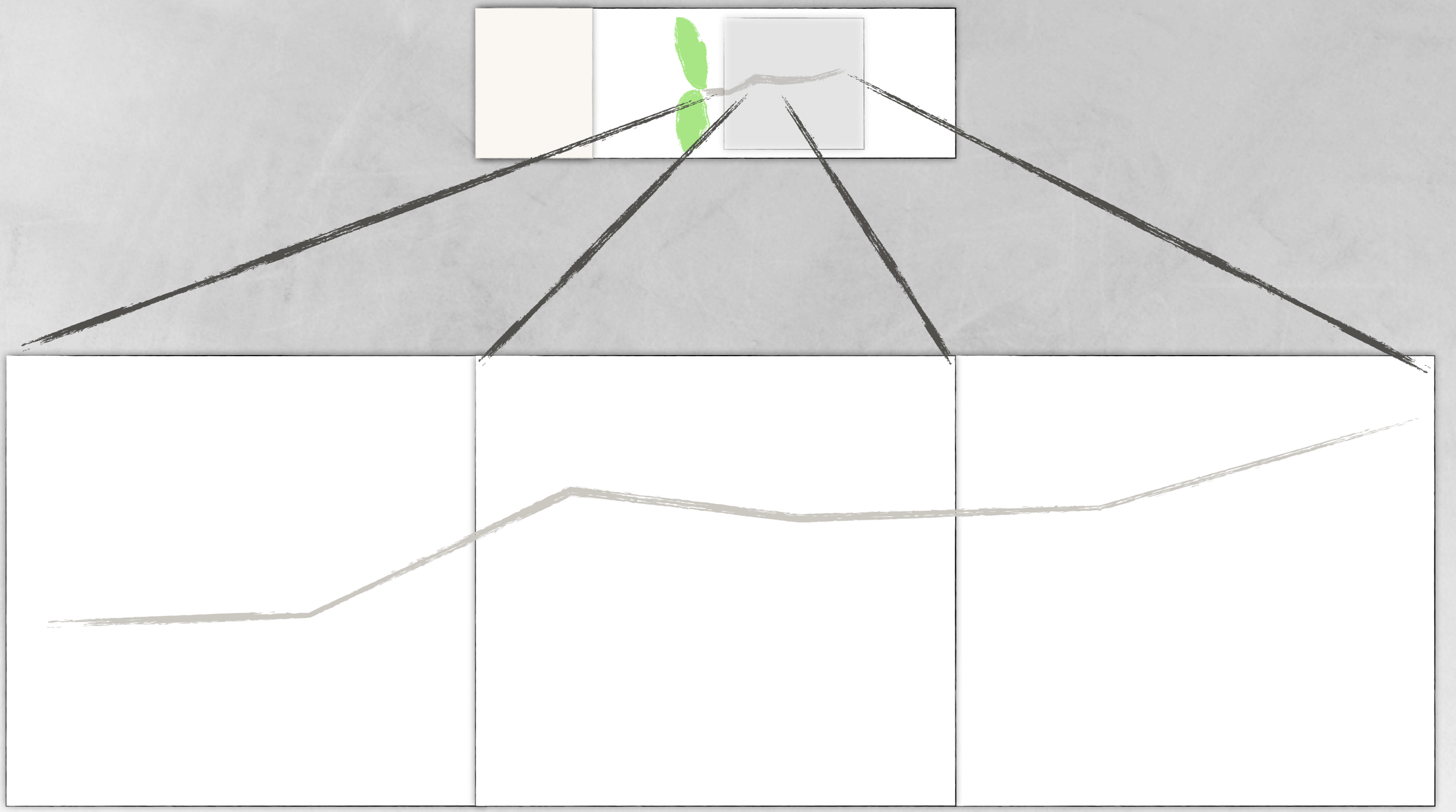
OPEN. open old file


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
256.00 KB
Start Experiment
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
time series
CPU 1 %
Free Ram 10 GB
Free HD 2.0 TB


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
256.00 KB
Start Experiment
FRAP
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
CPU 1 %
Free Ram 10 GB
Free HD 2.0 TB


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
Start Experiment
Setup Manager
Laser
Light Path
Acquisition Parameter
Acquisition Mode
Channels
Focus
Stage
Incubator
Multidimensional Acquisition
Information On Experiment
Auto Save
Tile Scan
CPU 1 %
Free Ram 10 GB
Free HD 2.0 TB

Tile Scan



some % of overlap for stitching precision

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack ---
Time Series ---
Bleaching ---
Tile Scan 19 of 25 Tiles
Positions ---
Regions

Stop

Finish current step

Setup Manager

Laser
Light Path Show All

Acquisition Parameter

Acquisition Mode Show All
Channels Show All
Focus Show All
Focus Devices and Strategy Show All
Stage Show All
Incubator

Multidimensional Acquisition

Tile Scan Show All

Centered grid | Bounding grid | Convex hull

	Tiles	Pixels	Size
Horizontal	5	14	2125.48 µm
Vertical	5	14200	2125.48 µm

Overlap 0 % | Add | Remove | Remove all

Rotation 0.0000 °

Bi-directional
Online stitching

Marked positions

Scan overview image ...

Information On Experiment
Auto Save Show All

Image 1 | Image 2 | Image 2_Linear unmixing

2D | Split | 2.5D | Histo | Coloc | Profile | Info

this is the position you were focused on

now the tile scan makes 5x5 tiles around this position
please note, that there is 0% overlap between the tiles

Show All

Dimensions | Display | Graphics

Select | More ...

Keep Tool
Auto Color

Type | ID | M | Name

Cut region

X 0
Y 0
W 0
H 0

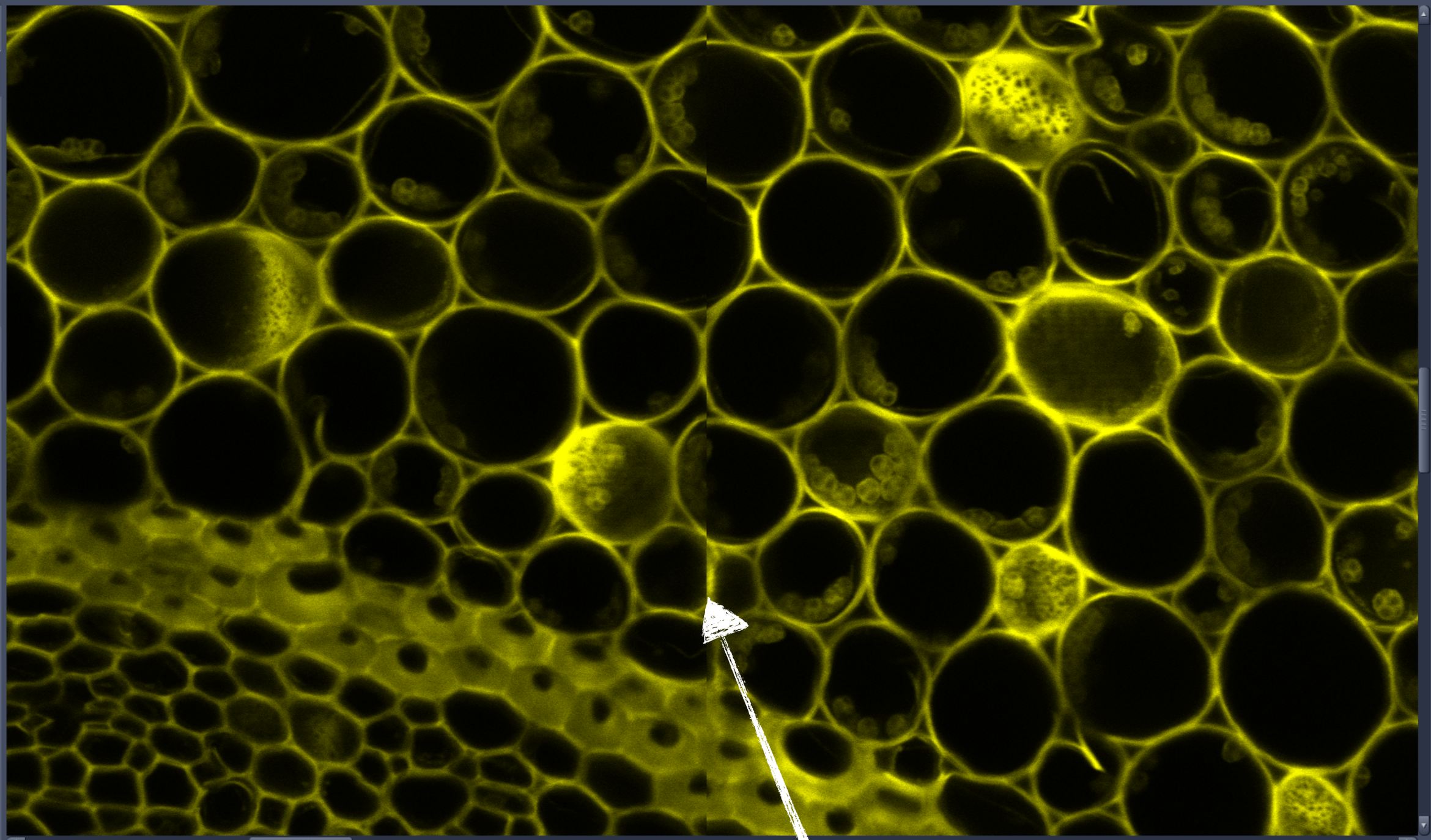
CPU 16 % Free HD 1.8 TB
Free Ram 7.3 GB

Cancel 76 % Grabbing, remaining time: 00:00:22 of 00:01:40

Workspace Config

Image 1 | Image 2 | Image 2_Linear unmixing

2D
Split
2.5D
Histo
Coloc
Profile
Info



Show All

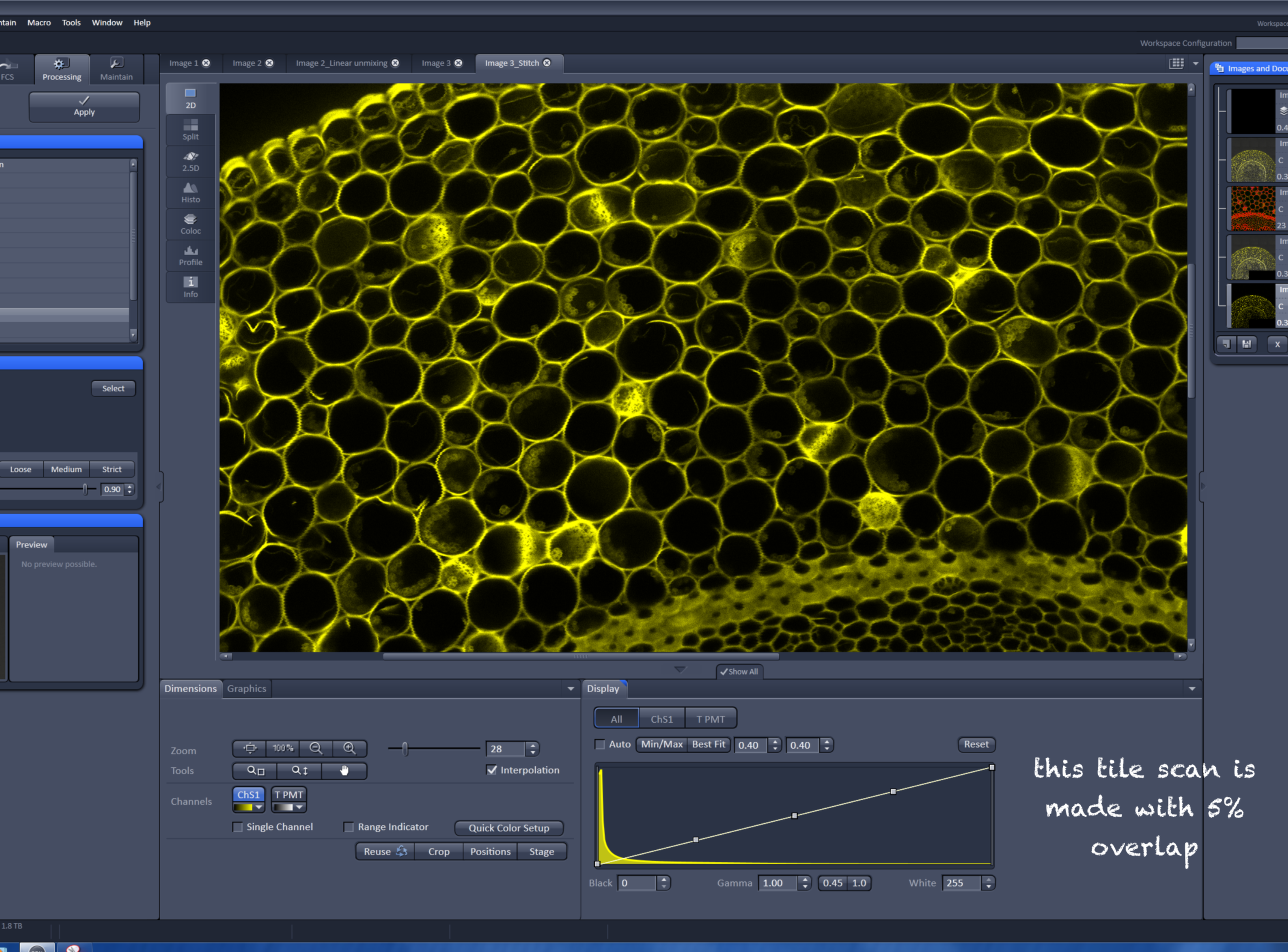
Dimensions | Display | Graphics

Zoom 100% 50
Tools
Interpolation

Channels ChS1 T PMT

Single Channel | Range Indicator | Quick Color Setup

you can see, that the tiles are not stitching well together


Macro
Tools
Window
Help
Workspace Configuration
FCS
Processing
Maintain
Apply
Image 1
Image 2
Image 2_Linear unmixing
Image 3
Image 3_Stitch
Images and Docu
2D
Split
2.5D
Histo
Coloc
Profile
Info
Select
Loose
Medium
Strict
0.90
Preview
No preview possible.
Show All
Dimensions
Graphics
Zoom
100%
28
Tools
Interpolation
Channels
ChS1
T PMT
Single Channel
Range Indicator
Quick Color Setup
Reuse
Crop
Positions
Stage
Display
All
ChS1
T PMT
Auto
Min/Max
Best Fit
0.40
0.40
Reset
Black
0
Gamma
1.00
0.45
1.0
White
255
this tile scan is made with 5% overlap
1.8 TB
ZEN

for your sample

- ☐ Make sample using a root of a GFP/YFP expressing plant
- ☐ In "Locate" find a sample and decide which objective you want to use
- ☐ Find the place which shows you the typical localisation of the signal
- ☐ Use smart setup to detect GFP or YFP (depending on your sample)
- ☐ Activate T-PMT in the Light path
- ☐ Use SET EXPOSURE to see the sample
- ☐ Adjust settings in Channels to have no over/underexposed pixels, and ok Master Gain and a proper pinhole
- ☐ Adjust settings in Acquisition mode to have good resolution, low noise and reasonable scanning time
- ☐ Make a nice SNAP image and save it into your folder
- ☐ activate z-stack. set up the parameters and click START EXPERIMENT
- ☐ save your z-stack file
- ☐ uncheck z-stack and activate tile scan
- ☐ try to image a larger part of the root, select overlapping of the tiles 5%
- ☐ Save your tile scan file in your folder
- ☐ Think about your report, will you need an image of wild type for any of these scanning, if yes what settings will you use for it? Please make a corresponding image

for your sample

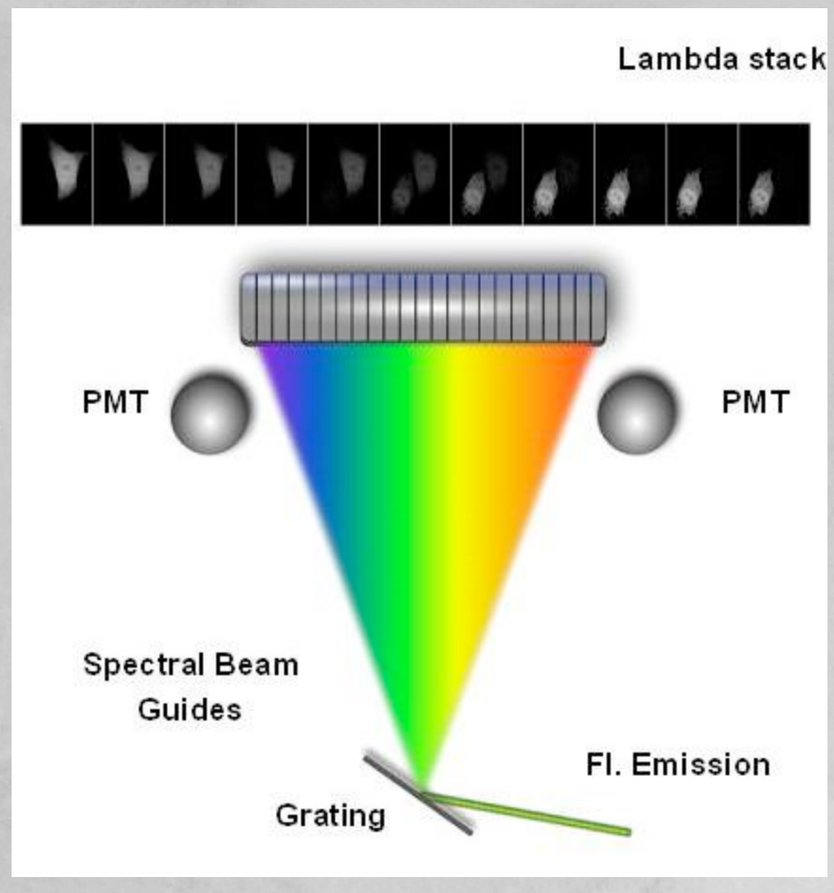
- ☐ Make sample using a leaf of a GFP/YFP expressing plant
- ☐ In "Locate" find a sample and decide which objective you want to use
- ☐ Find the place which shows you the typical localisation of the signal
- ☐ Use smart setup to detect GFP or YFP (depending on your sample) AND Chlorophyll A, select option with simultaneous scanning
- ☐ Activate T-PMT in the Light path
- ☐ Use SET EXPOSURE to see the sample
- ☐ Adjust settings in Channels to have no over/underexposed pixels and a proper pinhole
- ☐ Adjust settings in Acquisition mode to have good resolution, low noise and reasonable scanning time
- ☐ Make a nice snap image
- ☐ Save it in your folder
- ☐ What kind of control do you need for this image?

for your sample

- [ ] Use the same sample
- [ ] In smart setup select to detect GFP or YFP (depending on your sample), this time select the option with sequential scanning
- [ ] Activate T-PMT in the Light path and select switching between the tracks "every frame"
- [ ] Use SET EXPOSURE to see the sample
- [ ] Adjust settings in Channels to have no over/underexposed pixels and a proper pinhole
- [ ] Adjust settings in Acquisition mode to have good resolution, low noise and reasonable scanning time
- [ ] Make a nice snap image
- [ ] Save it in your folder
- [ ] Go to Light path and select switching between the tracks "every line"
- [ ] Make a nice snap image
- [ ] Save it in your folder
- [ ] What kind of control do you need for this image?

Optional experiments

- [ ] Make a sample using the root of a plant with unknown fluorophores
- [ ] Focus on a place where you think the signal is present
- [ ] In the Light path select "Lambda mode" tab
- [ ] click on Invisible light-> switch on 405
- [ ] click on MBS 405nm to put it on the light path
- [ ] In Channels:
    - [ ] open pinhole till 90
    - [ ] set Laser intensity on 0.2
    - [ ] Master Gain on 700
    - [ ] Digital offset on 0
    - [ ] Digital Gain on 1
- [ ] Set the step size for the lambda scan
- [ ] Click on SNAP
- [ ] Save the file in your folder
- [ ] Uncheck the 405 laser and repeat the experiment for all other lasers we have in the Visible light (selecting them one by one and putting the appropriate MBS on the light path, save all your files)
- [ ] Analyze the spectrum in each of your files:
    - [ ] In the Gallery tab of the image window you can see images for lambda scan. If you click on "text" you will see the nm
    - [ ] In Linear Unmixing tab of the image window use bottom tool bar to select a crosshair tool
    - [ ] The image is in the top right corner, click on the spot with the signal
    - [ ] in the top left corner a spectrum will appear
    - [ ] estimate at which nm you have the max, you know what excitation light you used. Google for fluorophores with similar excitation/emission parameters
- [ ] Select these fluorophores in smart set up, decide which you should use simultaneous or sequential scanning
- [ ] Make a nice SNAP image of the sample



Maintain Macro Tools Window Help

Workspace Configuration

FCS Processing Maintain

Show all Tools New

Live Continuous Snap

8.00 MB

Start Experiment

Show All

Show All

Show All

Show All

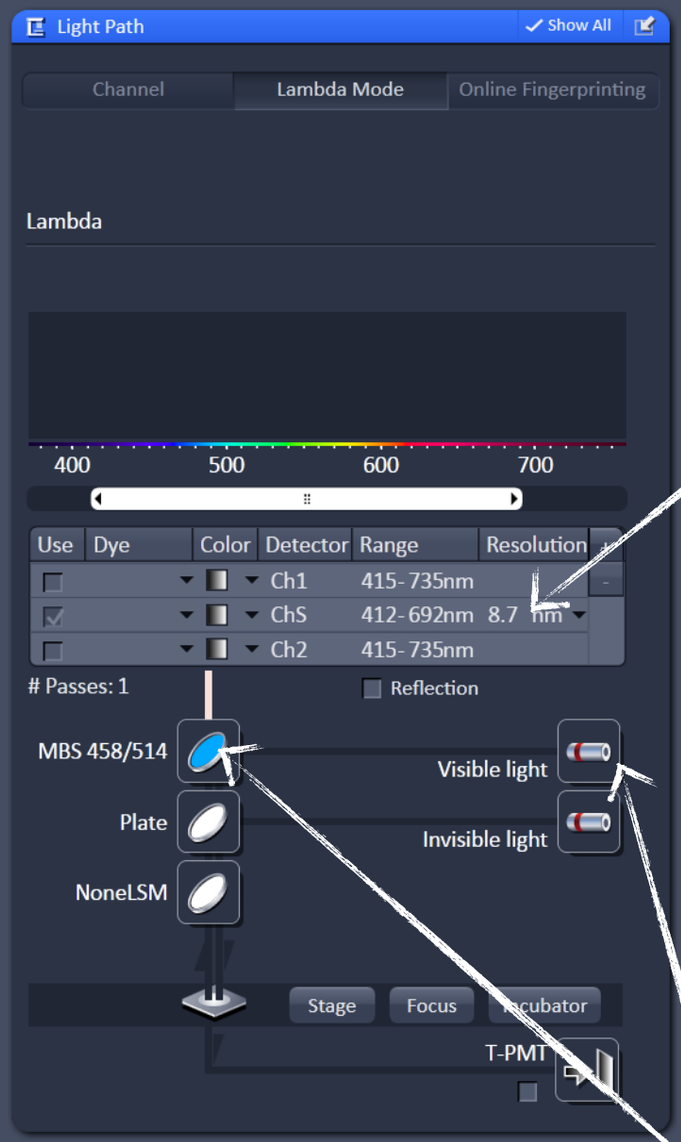
Show All

Images and Document

No open

X

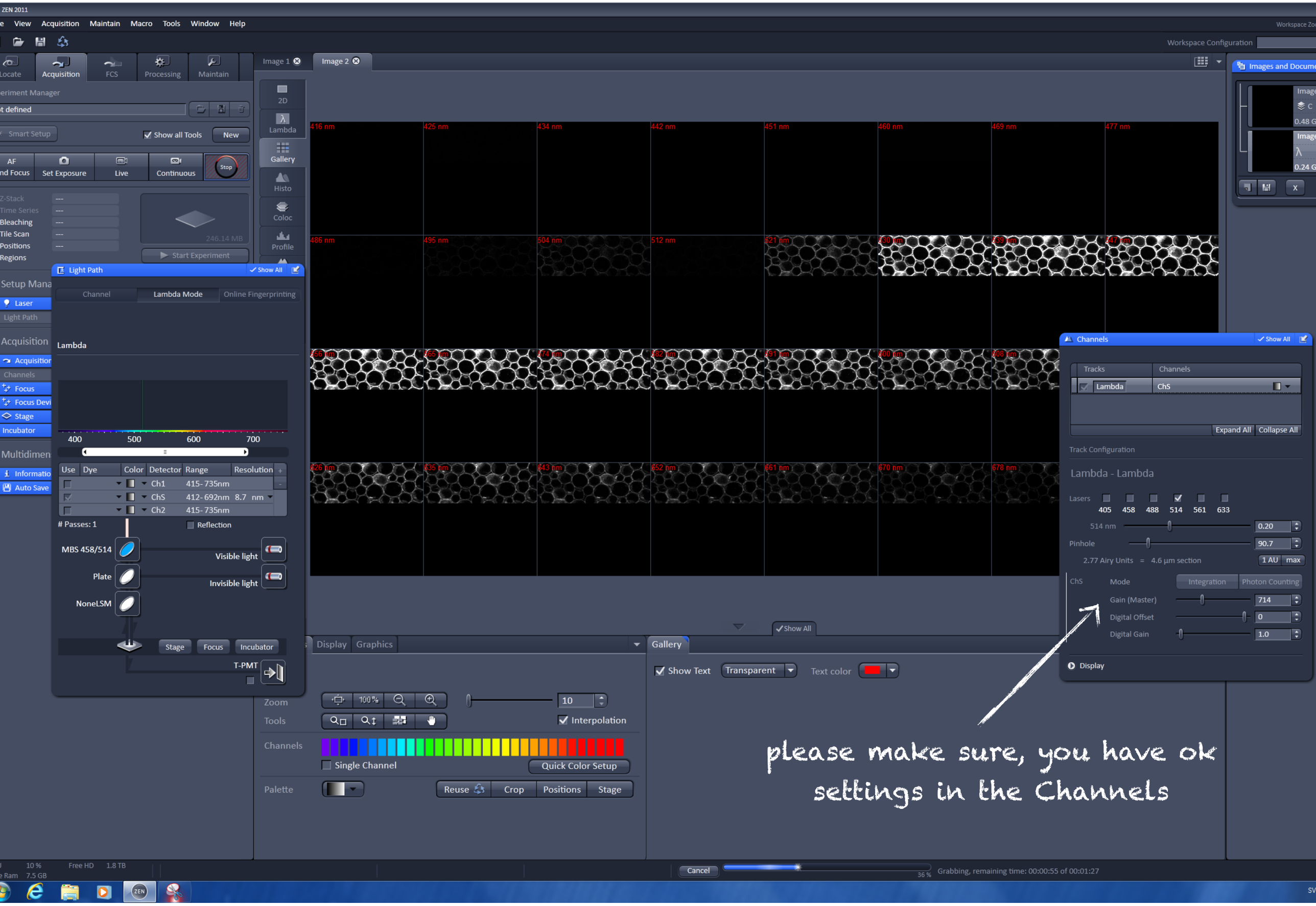
Lambda mode can be found in the Light Path tab



if you select here a smaller step it will take forever to scan, why?

please make sure, your MBS is matching the laser you selected

HD 1.8 TB


ZEN 2011
View Acquisition Maintain Macro Tools Window Help
Workspace Configuration
Locate Acquisition FCS Processing Maintain
Image 1
Image 2
Smart Setup
Show all Tools
New
AF
Set Exposure
Live
Continuous
Stop
Z-Stack
Time Series
Bleaching
Tile Scan
Positions
Regions
246.14 MB
Start Experiment
2D
Lambda
Gallery
Histo
Coloc
Profile
Images and Documents
416 nm
425 nm
434 nm
442 nm
451 nm
460 nm
469 nm
477 nm
486 nm
495 nm
504 nm
512 nm
521 nm
530 nm
539 nm
547 nm
Light Path
Show All
Channel
Lambda Mode
Online Fingerprinting
Lambda
400
500
600
700
Setup Mana
Laser
Light Path
Acquisition
Acquisitior
Channels
Focus
Focus Devi
Stage
Incubator
Multidimen
Use Dye Color Detector Range Resolution
Ch1 415-735nm
ChS 412-692nm 8.7 nm
Ch2 415-735nm
Passes: 1
Reflection
MBS 458/514
Visible light
Plate
Invisible light
NoneLSM
Stage
Focus
Incubator
T-PMT
Channels
Show All
Tracks
Channels
Lambda
ChS
Expand All
Collapse All
Track Configuration
Lambda - Lambda
Lasers
405 458 488 514 561 633
514 nm
0.20
Pinhole
90.7
2.77 Airy Units = 4.6 µm section
1 AU
max
ChS
Mode
Integration
Photon Counting
Gain (Master)
714
Digital Offset
0
Digital Gain
1.0
Display
Display
Graphics
Gallery
Show Text
Transparent
Text color
Zoom
100 %
10
Tools
Interpolation
Channels
Single Channel
Quick Color Setup
Palette
Reuse
Crop
Positions
Stage
Cancel
36 %
Grabbing, remaining time: 00:00:55 of 00:01:27
10 %
Free HD
1.8 TB
e Ram
7.5 GB
please make sure, you have ok settings in the Channels

ZEN 2011

File View Acquisition Maintain Macro Tools Window Help

Work

Locate | Acquisition | FCS | Processing | Maintain

Experiment Manager

not defined

Smart Setup | Show all Tools | New

AF Find Focus | Set Exposure | Live | Continuous | Snap

Z-Stack ---
Time Series ---
Bleaching ---
Tile Scan ---
Positions ---
Regions

246.14 MB

Start Experiment

Setup Manager

- Laser
- Light Path — Show All

Acquisition Parameter

- Acquisition Mode — Show All
- Channels — Show All
- Focus — Show All
- Focus Devices and Strategy — Show All
- Stage — Show All
- Incubator

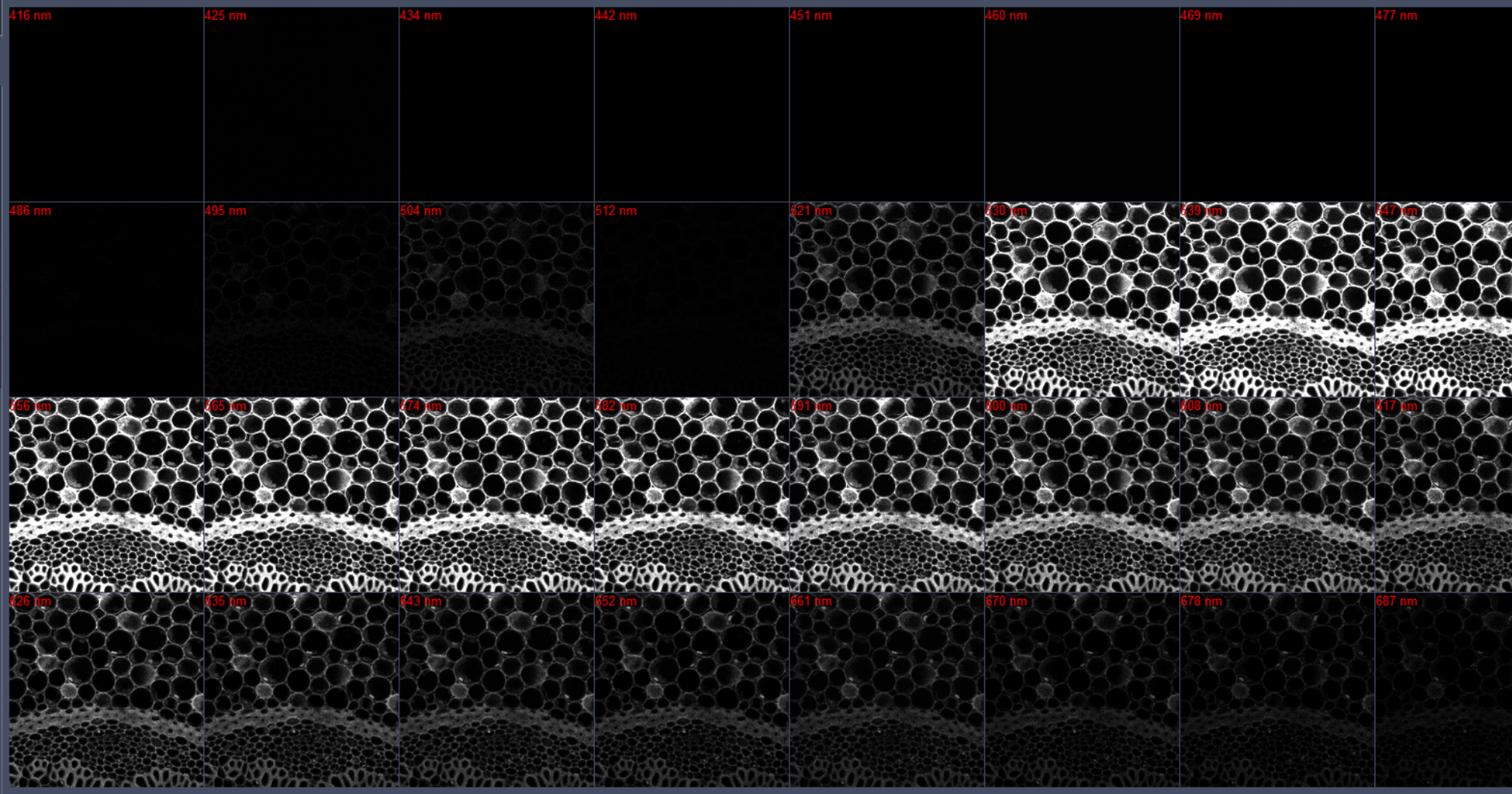
Multidimensional Acquisition

- Information On Experiment
- Auto Save — Show All

in gallery you can see image for each 8.7 nm (in this case) wide region of the spectrum

Image 1 | Image 2

2D | Lambda | Gallery | Histo | Coloc | Profile | Unmixing | FRET | Info



Show All

Dimensions | Display | Graphics

Zoom 100% 10

Tools Interpolation

Channels

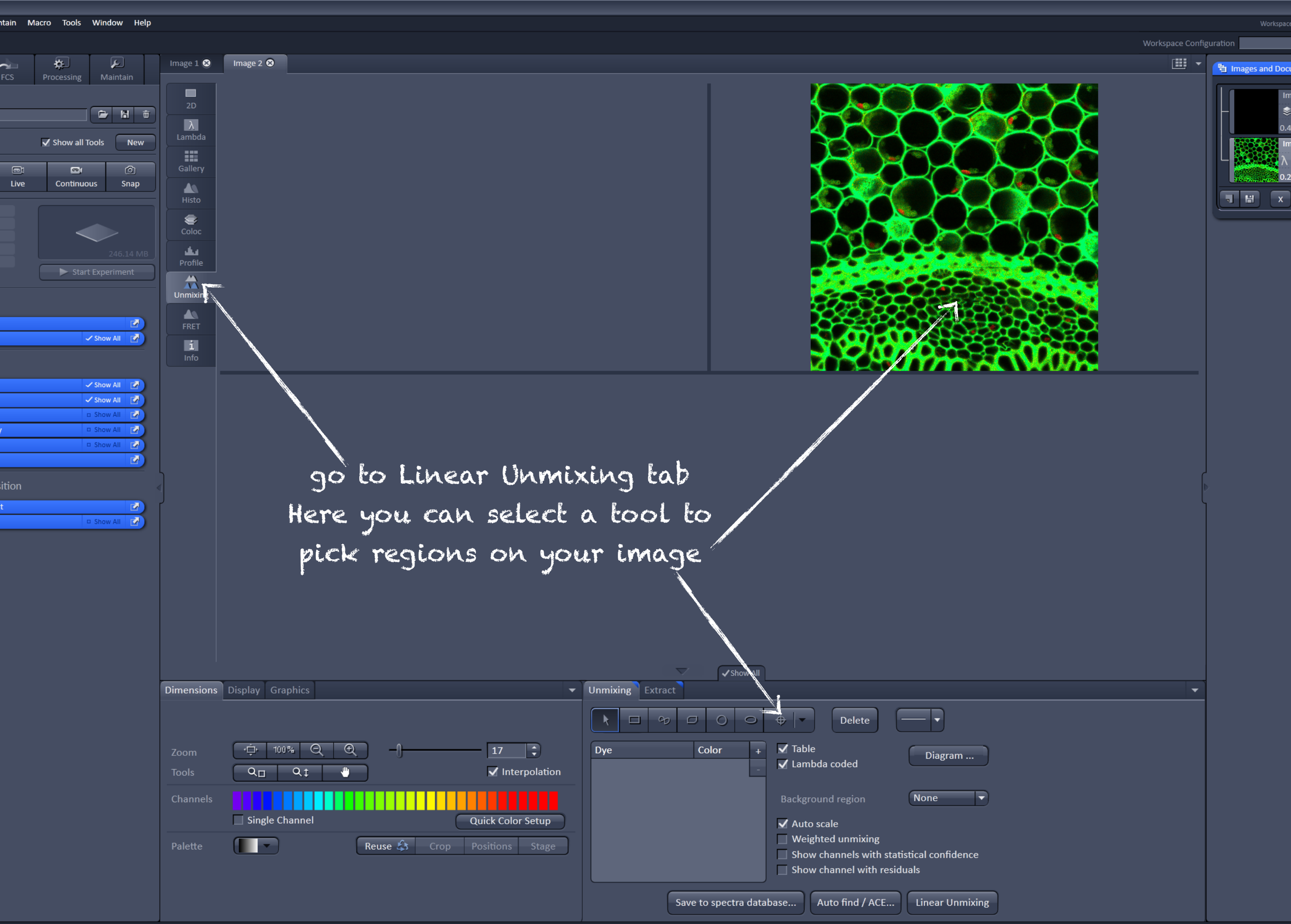
Single Channel | Quick Color Setup

Palette | Reuse | Crop | Positions | Stage

Gallery

Show Text | Transparent | Text color

CPU 1 % Free HD 1.8 TB
Free Ram 7.5 GB


Macro
Tools
Window
Help
Workspace Configuration
Processing
Maintain
Image 1
Image 2
2D
Lambda
Gallery
Histo
Coloc
Profile
Unmixing
FRET
Info
Show all Tools
New
Live
Continuous
Snap
246.14 MB
Start Experiment
Images and Docu
go to Linear Unmixing tab
Here you can select a tool to
pick regions on your image
Dimensions
Display
Graphics
Zoom
100%
17
Tools
Interpolation
Channels
Single Channel
Quick Color Setup
Palette
Reuse
Crop
Positions
Stage
Unmixing
Extract
Delete
Dye
Color
Table
Lambda coded
Diagram ...
Background region
None
Auto scale
Weighted unmixing
Show channels with statistical confidence
Show channel with residuals
Save to spectra database...
Auto find / ACE...
Linear Unmixing
1.8 TB

Macro Tools Window Help

Workspace Configuration

FCS Processing Maintain

Image 1 Image 2

2D Lambda Gallery Histo Coloc Profile Unmixing FRET Info

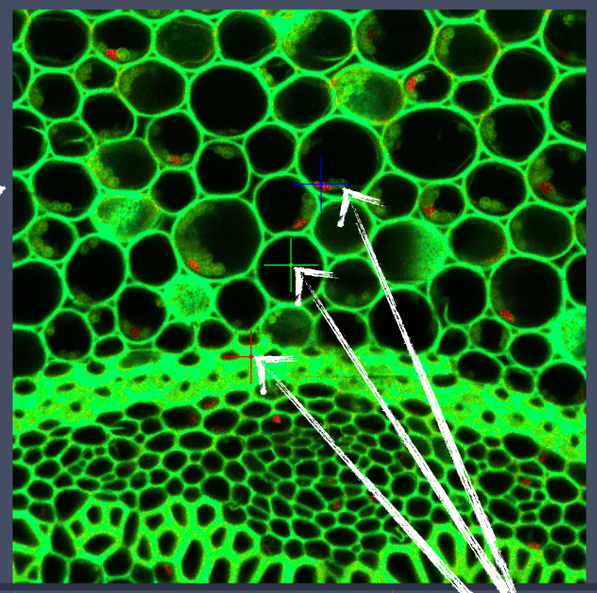
Intensity

250 200 150 100 50 0

450 500 550 600 650

Emission wavelength (nm)

Region 1 Region 2 Region 3



Images and Docu

Show all Tools New

Live Continuous Snap

246.14 MB

Start Experiment

Show All

you can see better where the max of emission is, if the image is not over saturated. How would you make it not oversatureated?

	Region 1	Region 2	Region 3
	0.4	0.0	0.2
	0.7	0.9	0.9
	0.1	0.0	0.1
	0.1	0.0	0.1
	0.1	0.0	0.0
	1.1	1.0	
	0.2	0.0	0.1
	0.2		
	1.5		
	8.7		
	26.4		
	4.6		
521	92.6		
530	255.0		
539	255.0		
547	255.0	2.5	105.1
556	255.0	2.1	115.0
565	255.0	2.4	127.2
574	254.7	2.4	149.9
582	253.0	1.7	163.0
591	240.2	3.4	176.2
600	205.8	1.6	184.0
608	177.5	2.6	195.2
617	139.8	0.9	189.9
626	115.2	0.9	179.4
635	90.8	0.6	162.1
643	72.1	1.3	149.0
652	58.3	0.4	135.6
661	48.5	2.4	118.4

I picked three spot on the image and got spectrum for each one of them

Show All

Dimensions Display Graphics

Zoom 100% 17

Tools Interpolation

Channels

Single Channel Quick Color Setup

Palette Reuse Crop Positions Stage

Unmixing Extract

Delete

Dye	Color
Region 1	
Region 2	
Region 3	

Table

Lambda coded

Diagram ...

Background region None

Auto scale

Weighted unmixing

Show channels with statistical confidence

Show channel with residuals

what happens if you click on this one?

Save to spectra database... Auto find / ACE... Linear Unmixing

1.8 TB

shut down

1. Make sure you saved all your data on the Graphics computer
2. Close the ZEN program
3. Check in the schedule if anyone is going to use confocal after you. If no, shut down the computer as usually. If yes, just log off your user account
4. If nobody is going to use the UV lamp within next 30 minutes, turn it off
5. If nobody will use the Argon laser after you within the same day

    a. flip the run/idle switch to the idle mode
    b. turn off the key of the Power supply of the Ar laser
    c. when the ventilator will stop proceed with switching off the system

6. If nobody will use the confocal after you turn off the power switch

    - computer switch
    - components switch
    - main switch

7. Make sure the anti-vibration table is off.

shut down