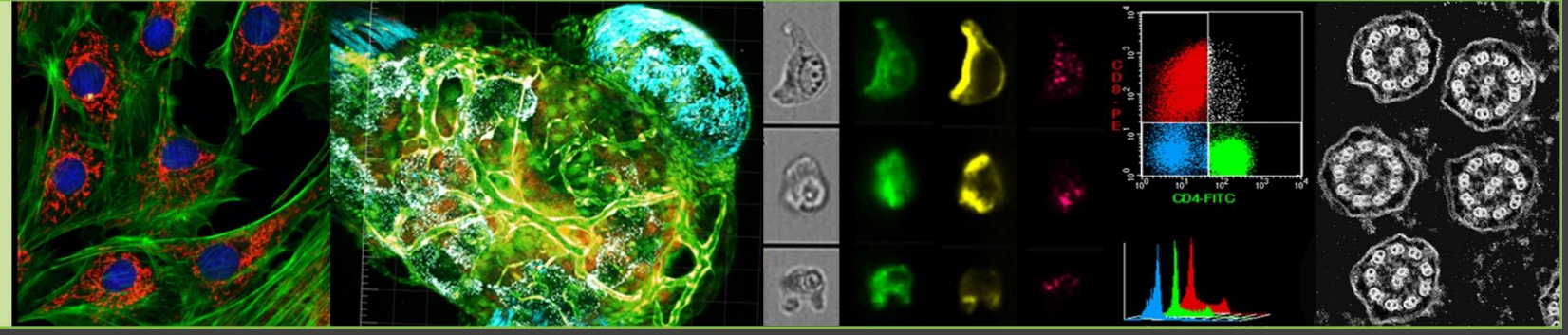
UPPSALA UNIVERSITET SciLifeLab BioVis Facility



Poaching through the Forest of ZEN
A graphic Novel

Dirk Pacholsky , BioVis

ZEN BLACK poaching through the program

Enter the arena – Things to know b4 the imaging

The Image –Information on image

6kg of Light – Intensity measurement

The big view – 2D 3D imaging

In the mix – Linear unmixing

Exit strategy –Options for exporting data

The process against light – processing

Don´t be rude – The F´Word techniques

Anybody else here? – Colocalization

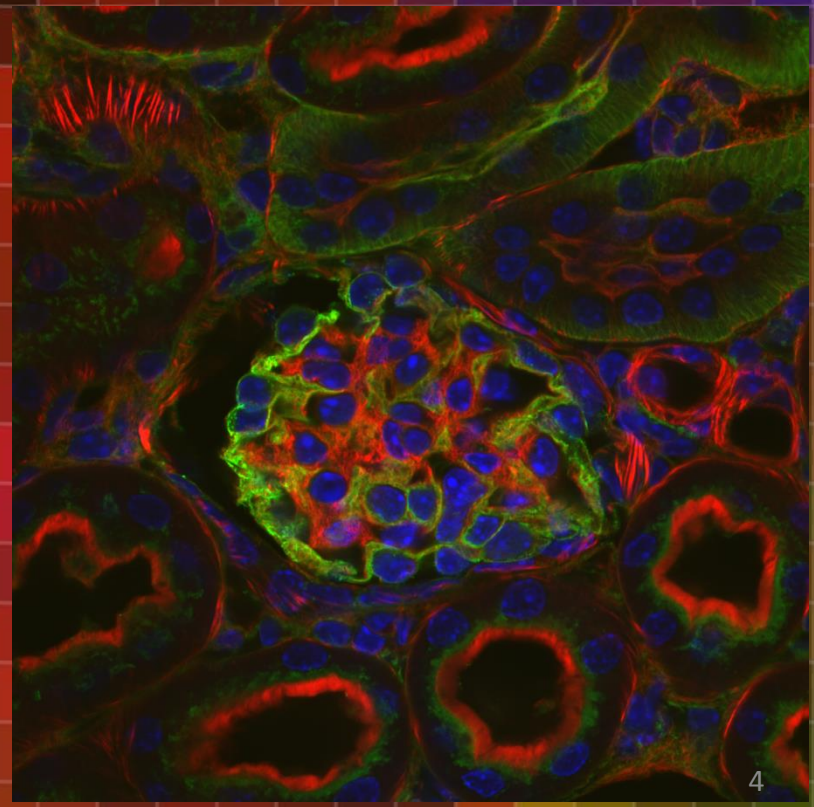
Enter the arena

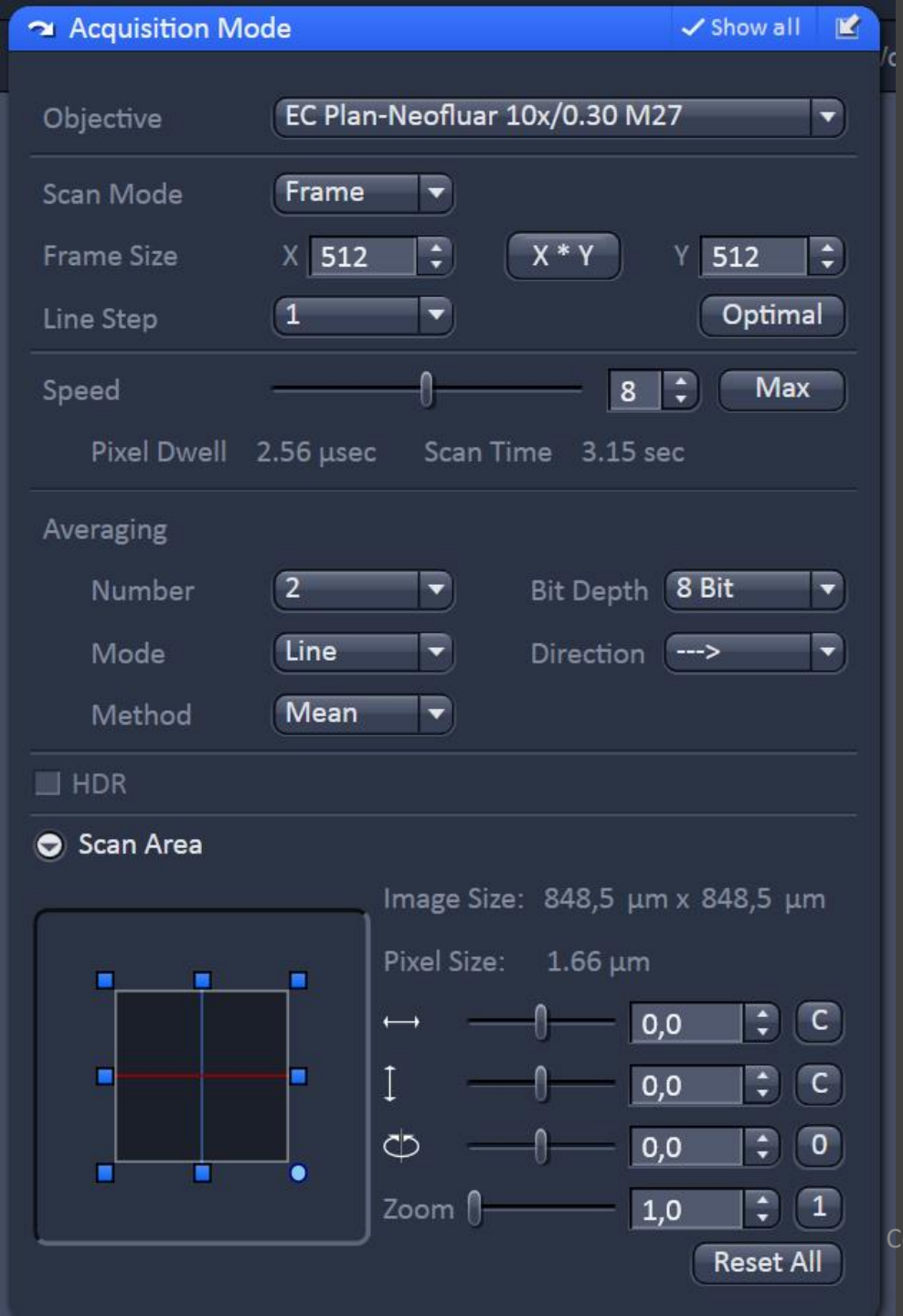
Things to know before the imaging

Exposure to light coming from Sample

→ For all pixels at same time : Camera based system

→ for each pixel separately: Scanning based system





Choose...

Many parameter are interconnected.

- Objective & optimal frame size, speed
- Speed and pixel dwell time (Image quality)
- averaging, bit, direction ....

ZEN BLACK poaching through the program

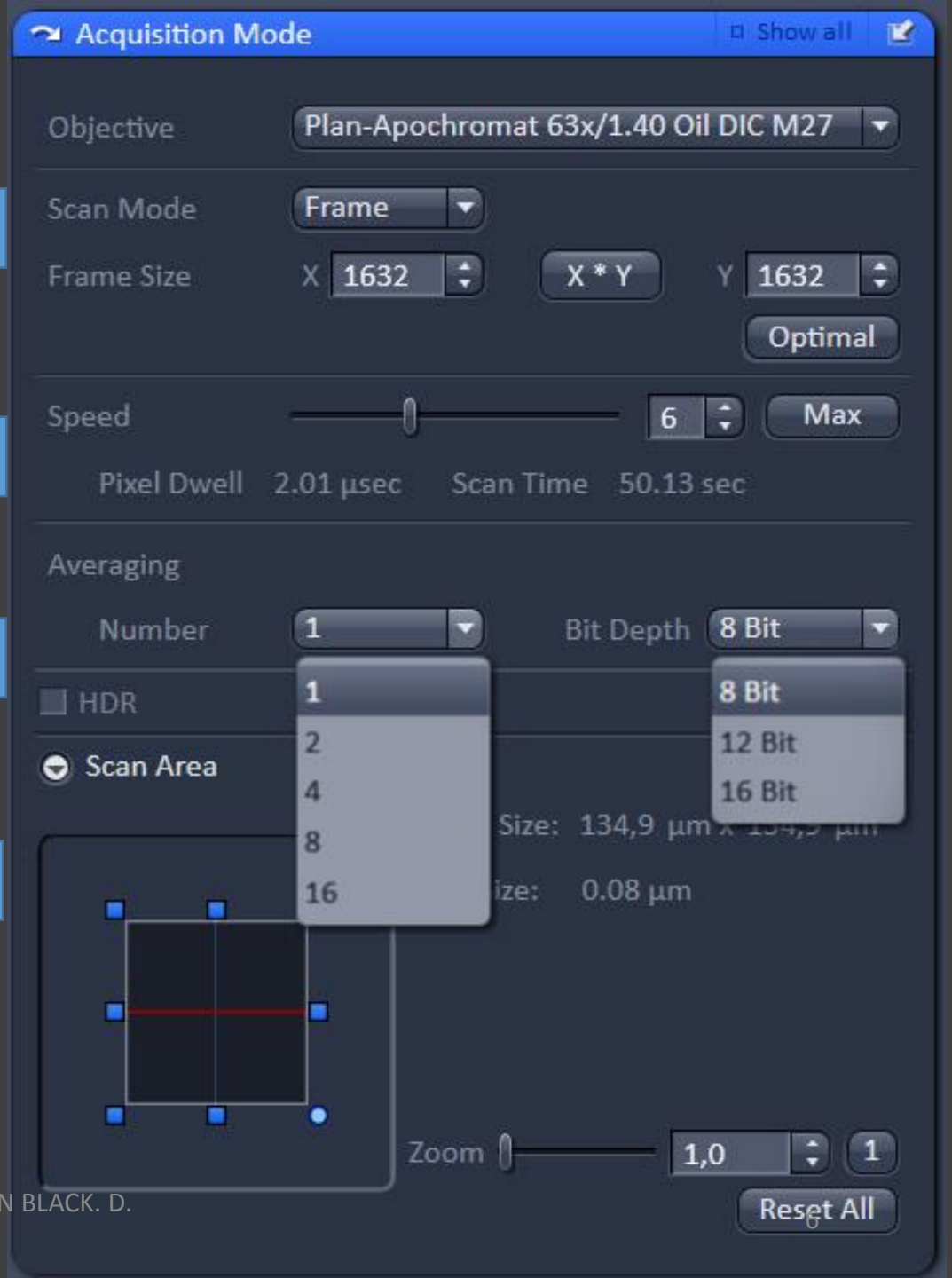
Frame size, i.e. Pixel size and number dependent on experiment (high resolution needed?)
Nyquist related BUT! ZEISS >LEICA>Nyquist

Speed ”Acquisition time”, do not exceed 9*,
7-8 does fine

Averaging , read 2 times and average out etc.
Quality versus speed and bleaching

Bit depth, for most applications 8 bit is fine,

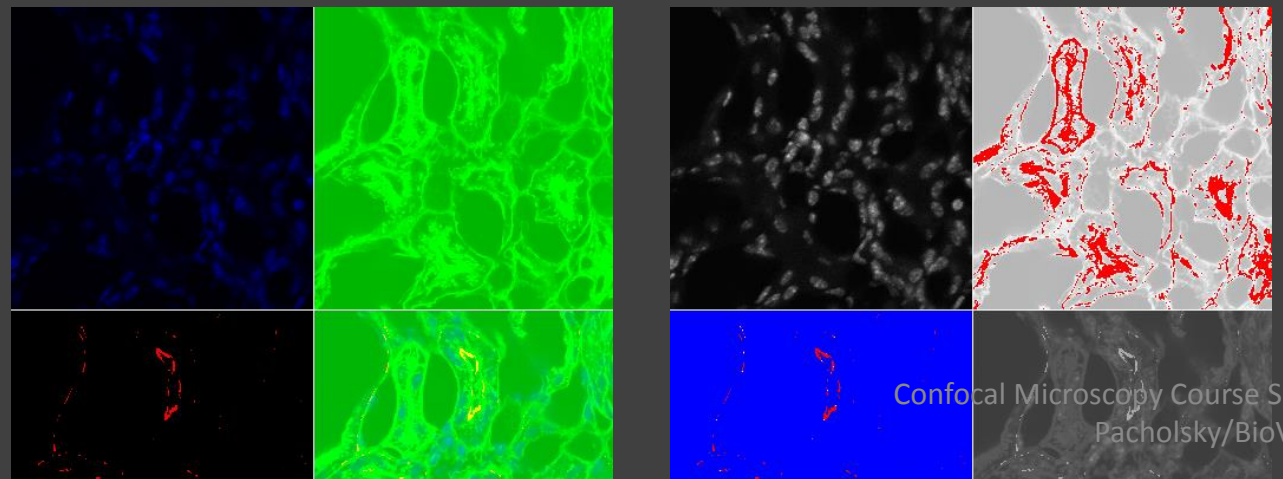
9 = very fast movement of scanning mirror
→ Mechanical stress for motor etc

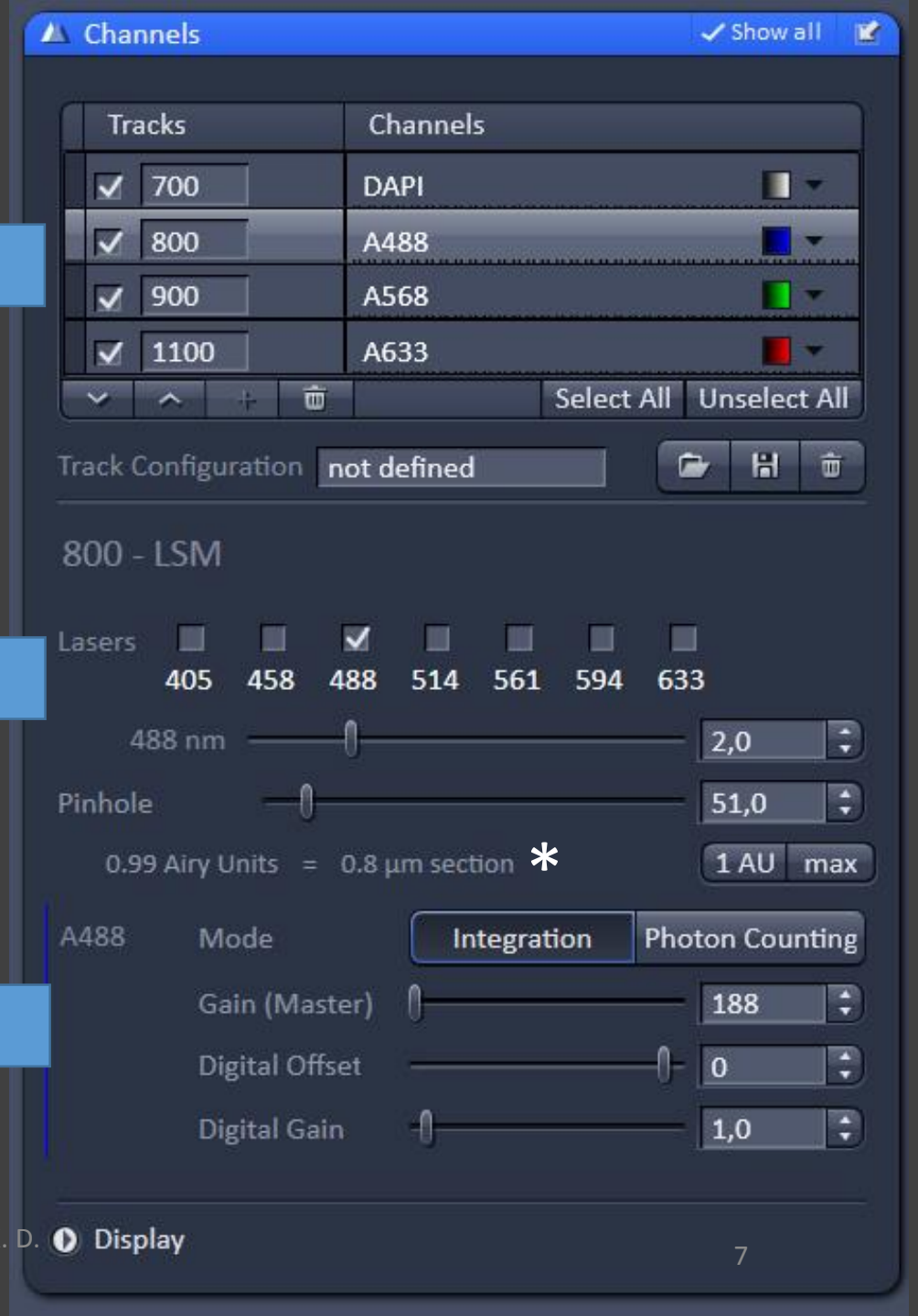


Channels.
using 1AU for each particular channel (i.e. wavelength)
results in different Z resolution
(here 700 to 1100 nm)*
Use same/similar Z resolution
(Optical section)

Use lowest Laserpower possible (bleaching...)

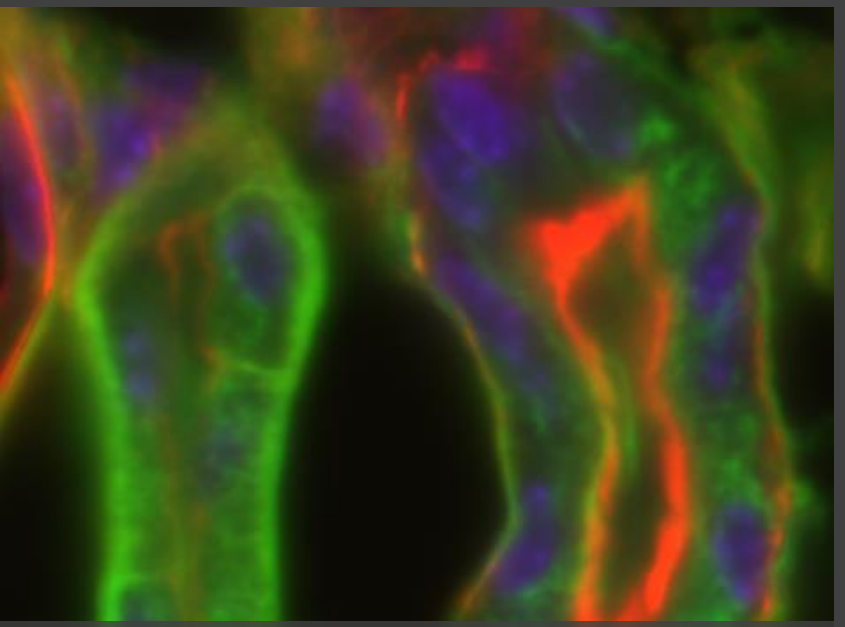
Cross check gain and offset with image



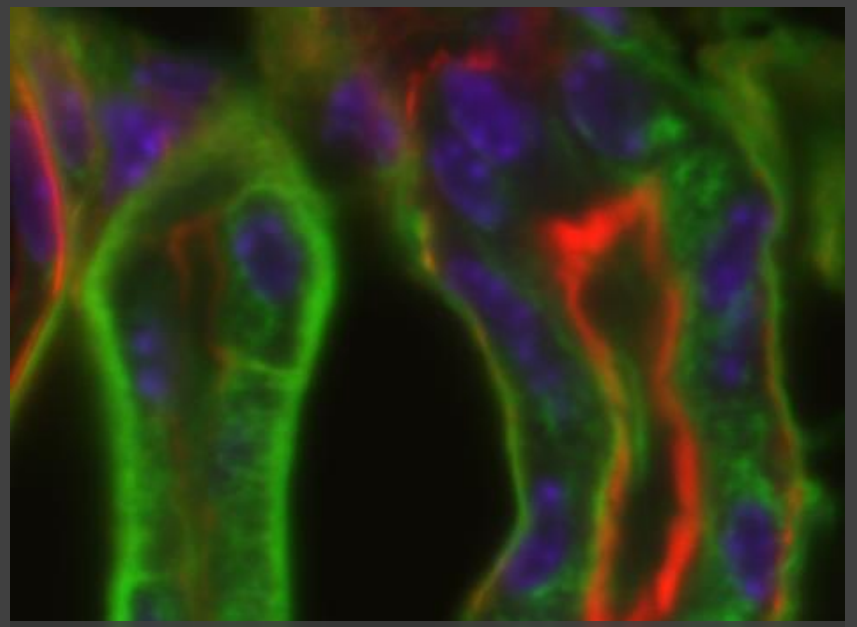


Better resolution by Pinhole size

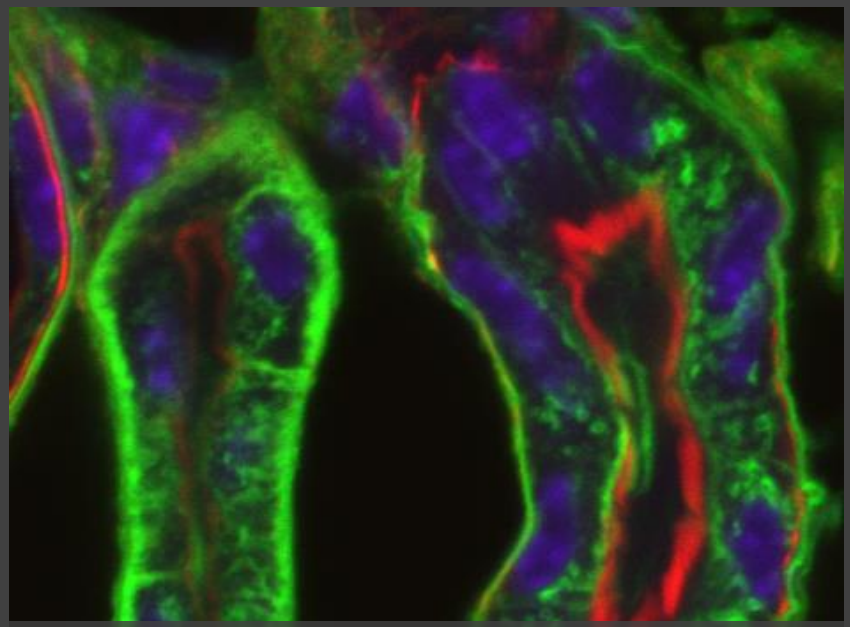
Pinhole fully open



half closed (7 AU)



closed to 1 AU



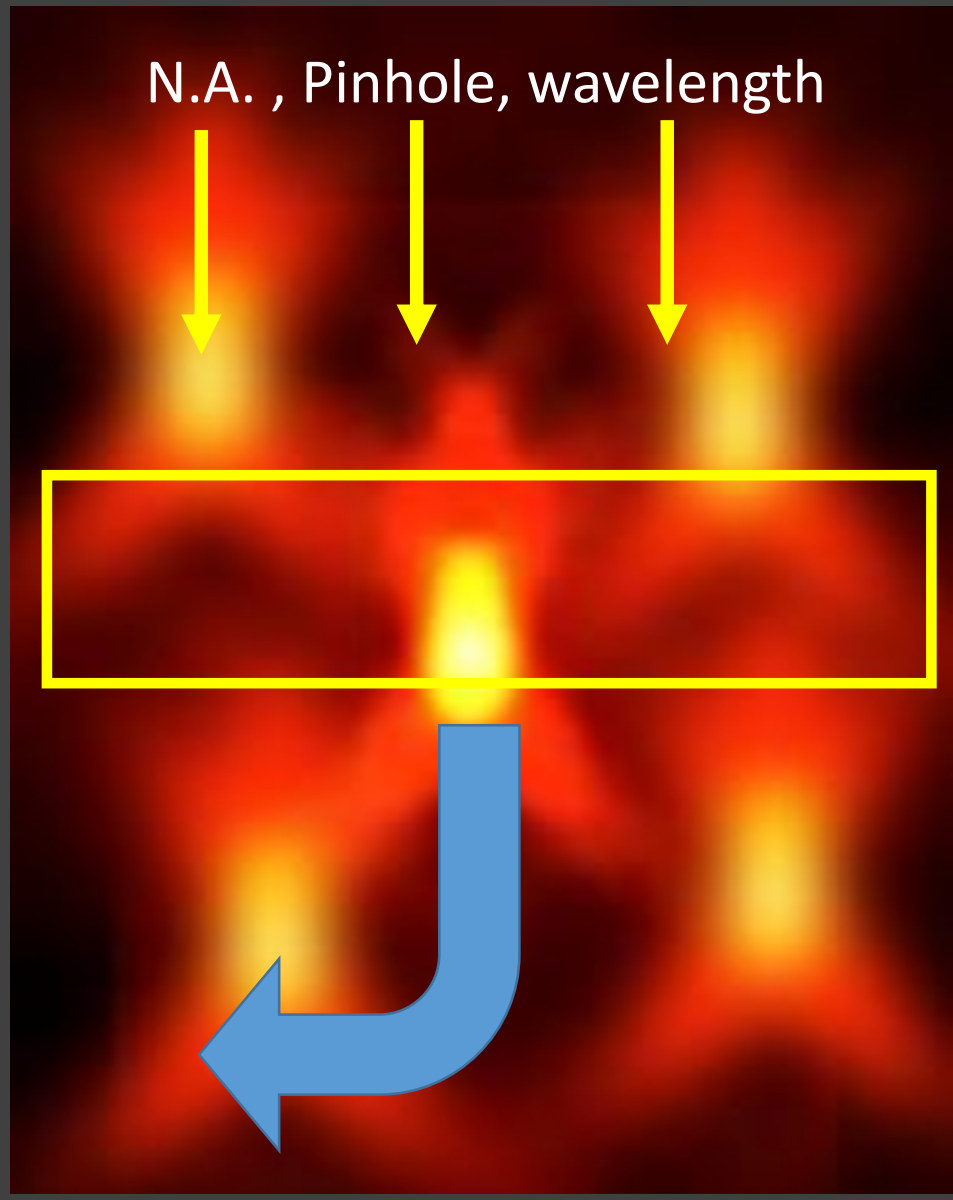
By closure of the pinhole, light coming from above/below the focal Plane will not get collected by the detector.

! The smaller the pinhole the smaller the actual focal plane from which light gets collected – good staining is needed !

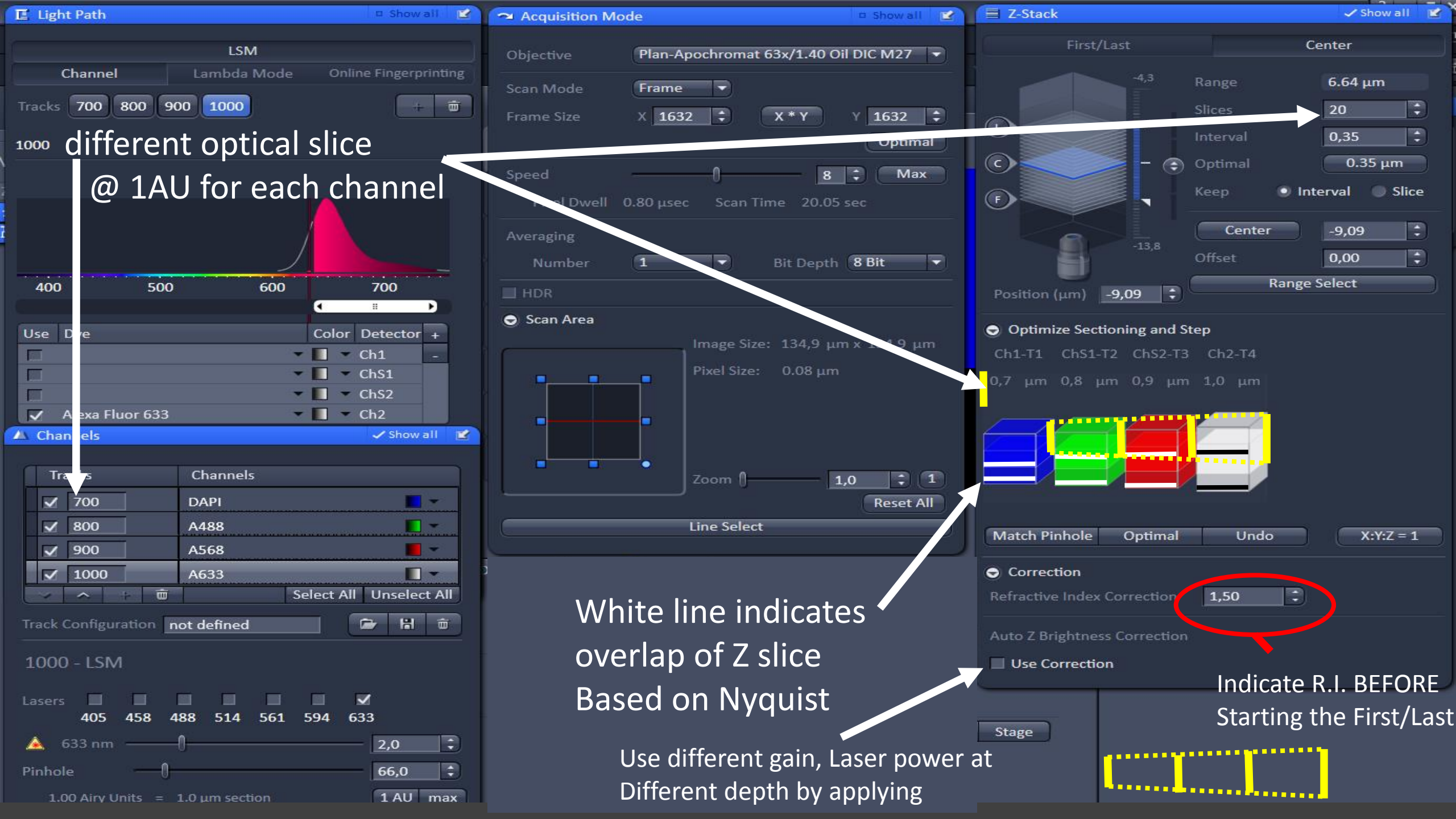
Objective Magnification - NA - optical section

The maximum resolution is app. 0.15 µm lateral
0.40 µm axial

Objective Magnification	NA	pinhole size < 1mm	pinhole size 7 mm
60x	1.40	0.40 µm	1.90 µm
40x	1.30	0.60 µm	3.30 µm
25x	0.80	1.40 µm	7.00 µm
4x	0.20	20.0 µm	100 µm
		Resolution in Z	



Optical slices not start/end suddenly at certain Z depth.
Due to intensity distribution along optical axis, there is a continuous transition from object information suppressed and such made visible


Light Path
LSM
Channel
Lambda Mode
Online Fingerprinting
Tracks 700 800 900 1000
1000 different optical slice
@ 1AU for each channel
400 500 600 700
Use Dye Color Detector
Ch1
ChS1
ChS2
Alexa Fluor 633 Ch2
Channels
Tracks Channels
700 DAPI
800 A488
900 A568
1000 A633
Select All Unselect All
Track Configuration not defined
1000 - LSM
Lasers 405 458 488 514 561 594 633
633 nm 2,0
Pinhole 66,0
1.00 Airy Units = 1.0 µm section
1 AU max
Acquisition Mode
Objective Plan-Apochromat 63x/1.40 Oil DIC M27
Scan Mode Frame
Frame Size X 1632 X * Y Y 1632
Optimal
Speed 8 Max
Pixel Dwell 0.80 µsec Scan Time 20.05 sec
Averaging
Number 1 Bit Depth 8 Bit
HDR
Scan Area
Image Size: 134,9 µm x 134,9 µm
Pixel Size: 0.08 µm
Zoom 1,0 1
Reset All
Line Select
Z-Stack
First/Last Center
-4,3
-13,8
Range 6.64 µm
Slices 20
Interval 0,35
Optimal 0.35 µm
Keep Interval Slice
Center -9,09
Offset 0,00
Range Select
Position (µm) -9,09
Optimize Sectioning and Step
Ch1-T1 ChS1-T2 ChS2-T3 Ch2-T4
0,7 µm 0,8 µm 0,9 µm 1,0 µm
Match Pinhole Optimal Undo X:Y:Z = 1
Correction
Refractive Index Correction 1,50
Auto Z Brightness Correction
Use Correction
Stage
White line indicates
overlap of Z slice
Based on Nyquist
Use different gain, Laser power at
Different depth by applying
Indicate R.I. BEFORE
Starting the First/Last

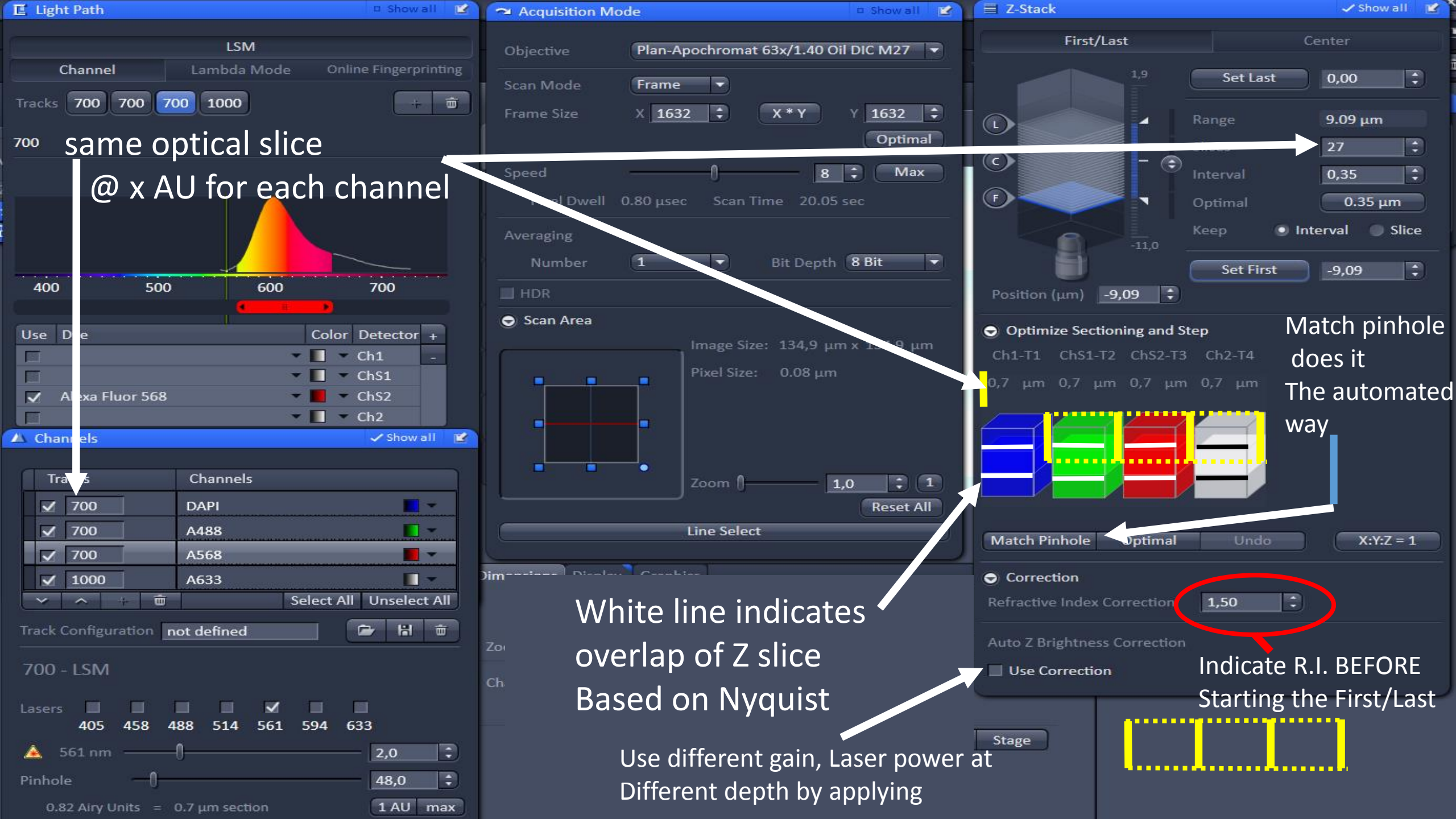
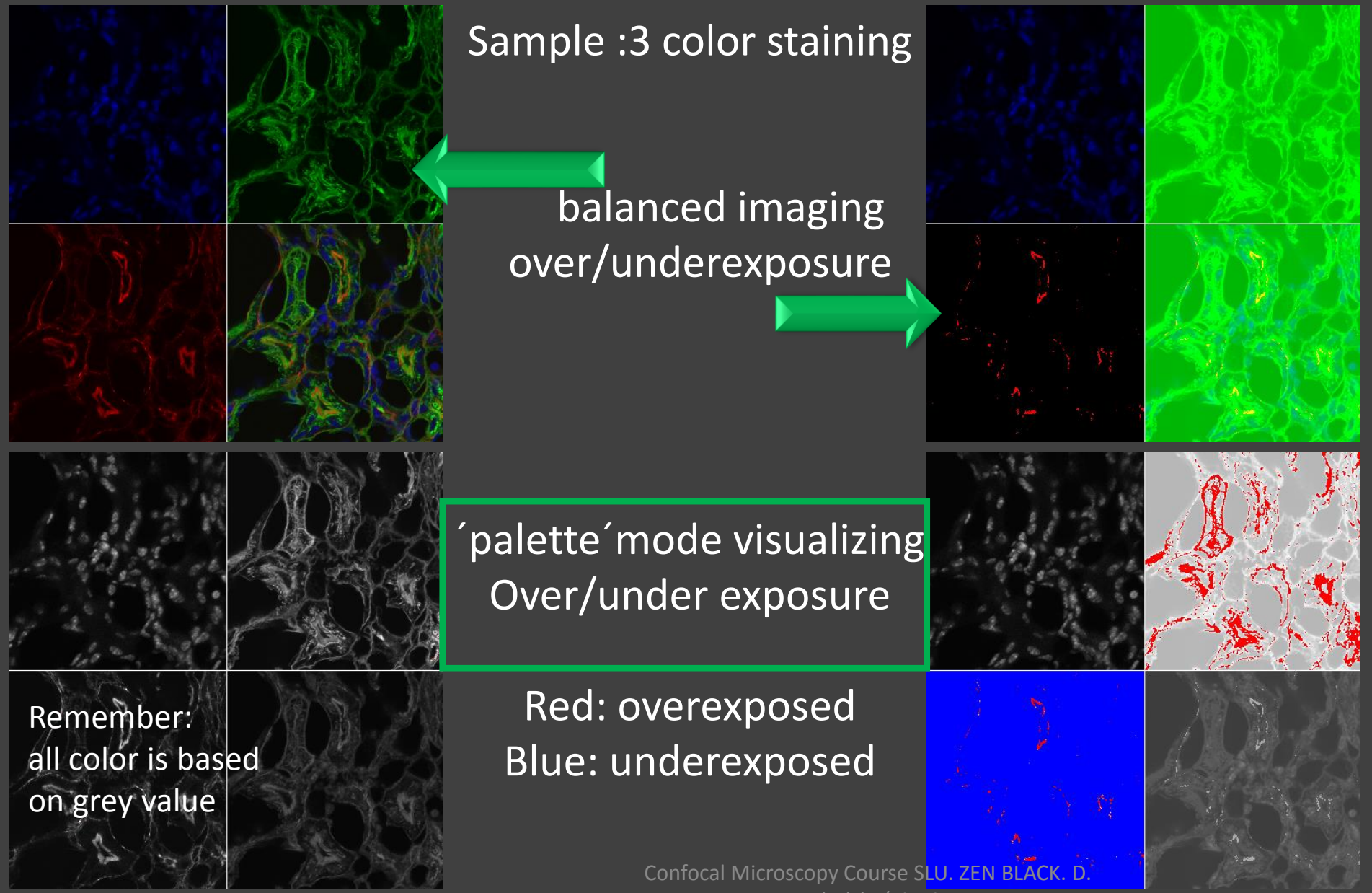

Light Path
Show all
LSM
Channel
Lambda Mode
Online Fingerprinting
Tracks 700 700 700 1000
700
same optical slice
@ x AU for each channel
400 500 600 700
Use Color Detector
Ch1
ChS1
Alexa Fluor 568
ChS2
Ch2
Channels
Show all
Tracks Channels
700 DAPI
700 A488
700 A568
1000 A633
Select All
Unselect All
Track Configuration not defined
700 - LSM
Lasers
405 458 488 514 561 594 633
561 nm 2,0
Pinhole 48,0
0.82 Airy Units = 0.7 µm section
1 AU max
Acquisition Mode
Show all
Objective Plan-Apochromat 63x/1.40 Oil DIC M27
Scan Mode Frame
Frame Size X 1632 X * Y Y 1632
Optimal
Speed 8 Max
Dwell 0.80 µsec Scan Time 20.05 sec
Averaging
Number 1 Bit Depth 8 Bit
HDR
Scan Area
Image Size: 134,9 µm x
Pixel Size: 0.08 µm
Zoom 1,0 1
Reset All
Line Select
Stage
Z-Stack
Show all
First/Last
Center
1,9
-11,0
Set Last 0,00
Range 9.09 µm
27
Interval 0,35
Optimal 0.35 µm
Keep Interval Slice
Set First -9,09
Position (µm) -9,09
Optimize Sectioning and Step
Ch1-T1 ChS1-T2 ChS2-T3 Ch2-T4
0,7 µm 0,7 µm 0,7 µm 0,7 µm
Match Pinhole
Optimal
Undo
X:Y:Z = 1
Correction
Refractive Index Correction 1,50
Auto Z Brightness Correction
Use Correction
Match pinhole does it
The automated way
White line indicates overlap of Z slice
Based on Nyquist
Use different gain, Laser power at
Different depth by applying
Indicate R.I. BEFORE
Starting the First/Last

Image quality – over/underexposure



Sample :3 color staining

balanced imaging
over/underexposure

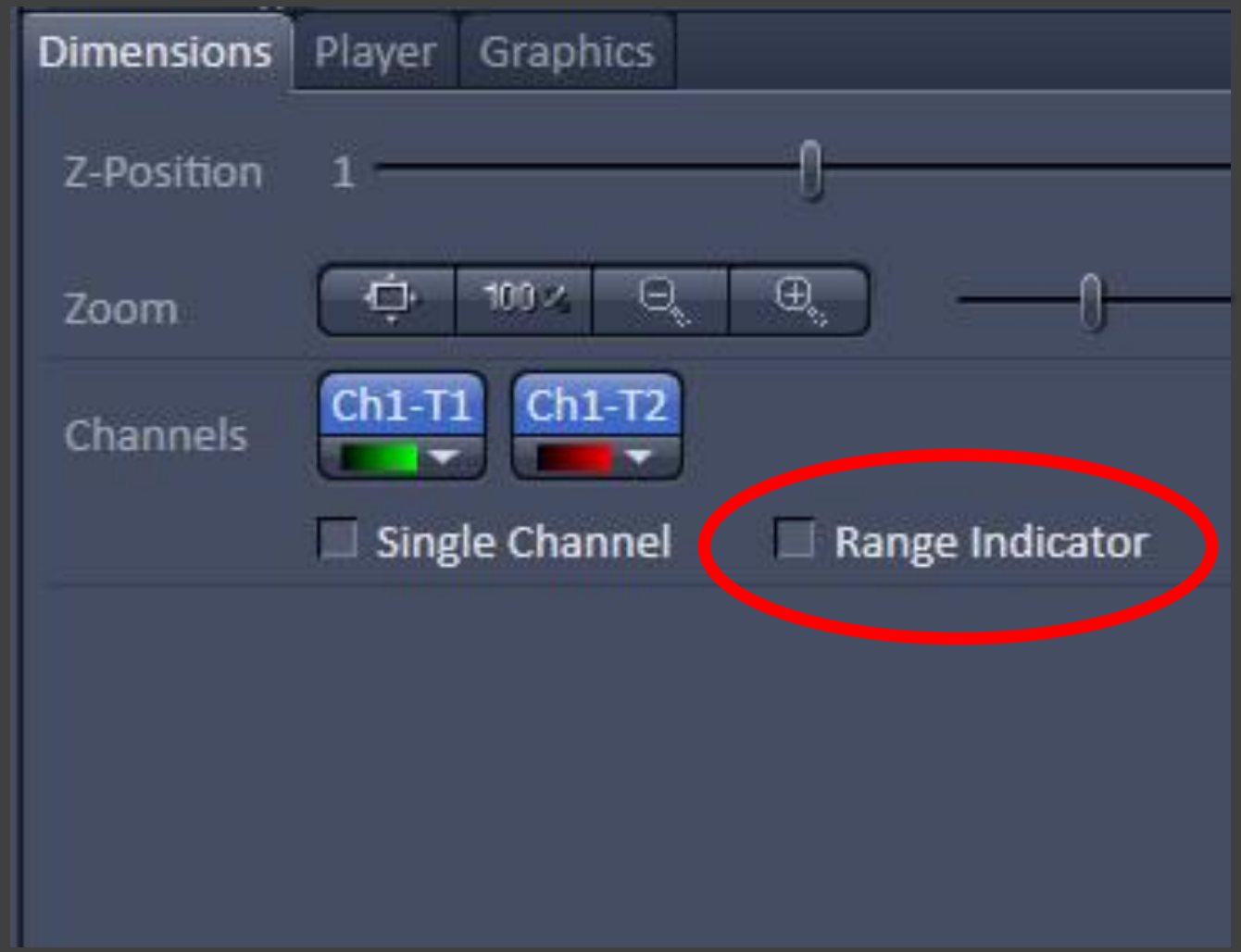
Blinded by the light
Or lost in the dark

´palette´mode visualizing
Over/under exposure

Red: overexposed
Blue: underexposed

Remember:
all color is based
on grey value

Image quality – over/underexposure



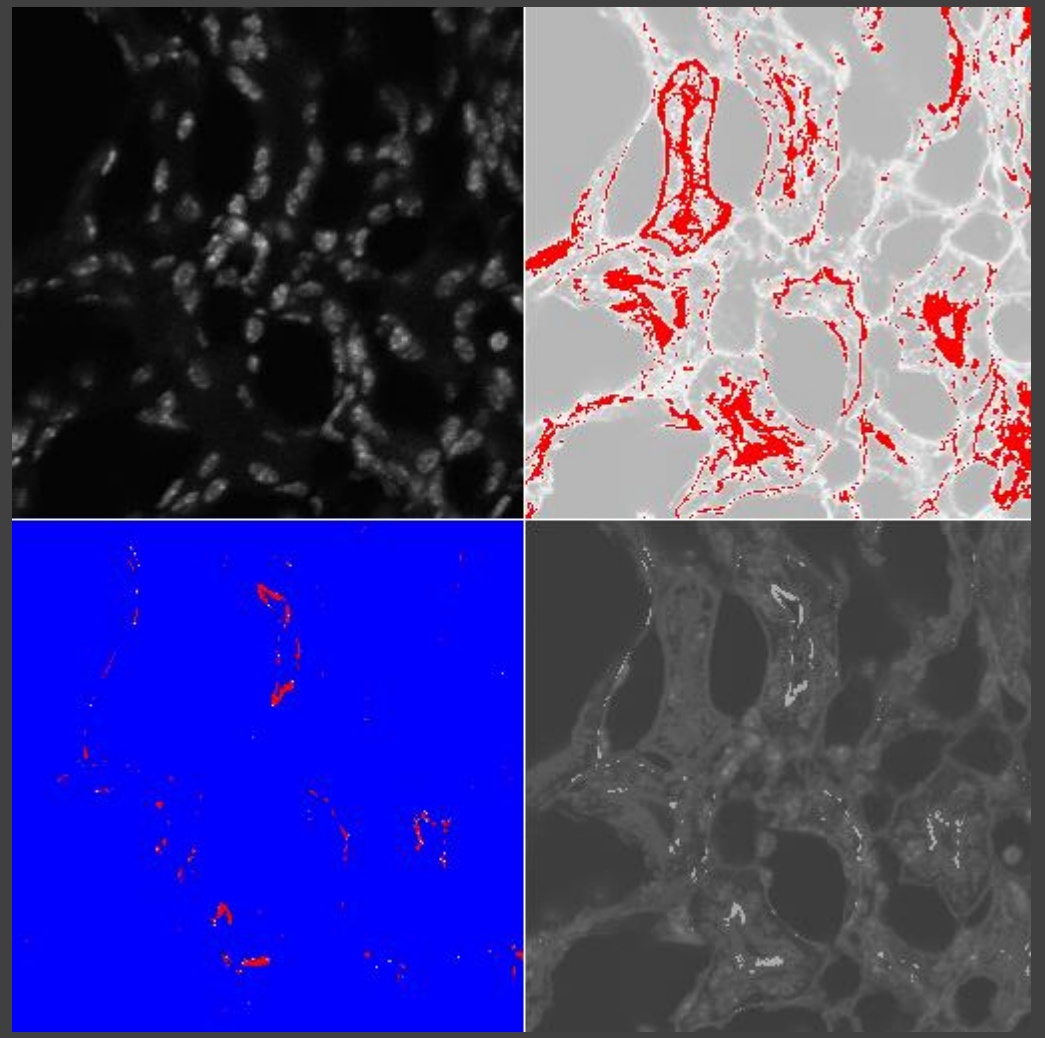
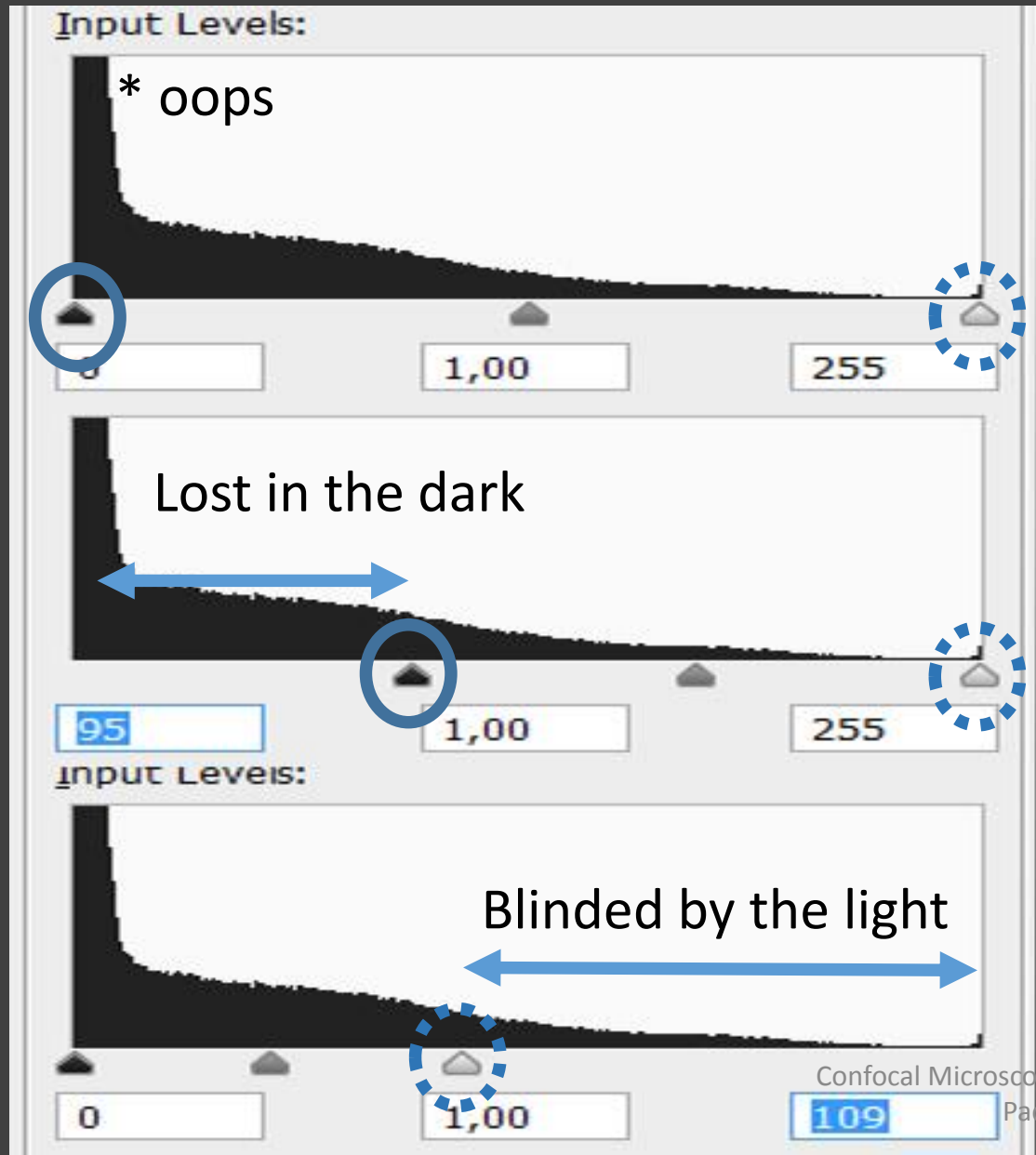
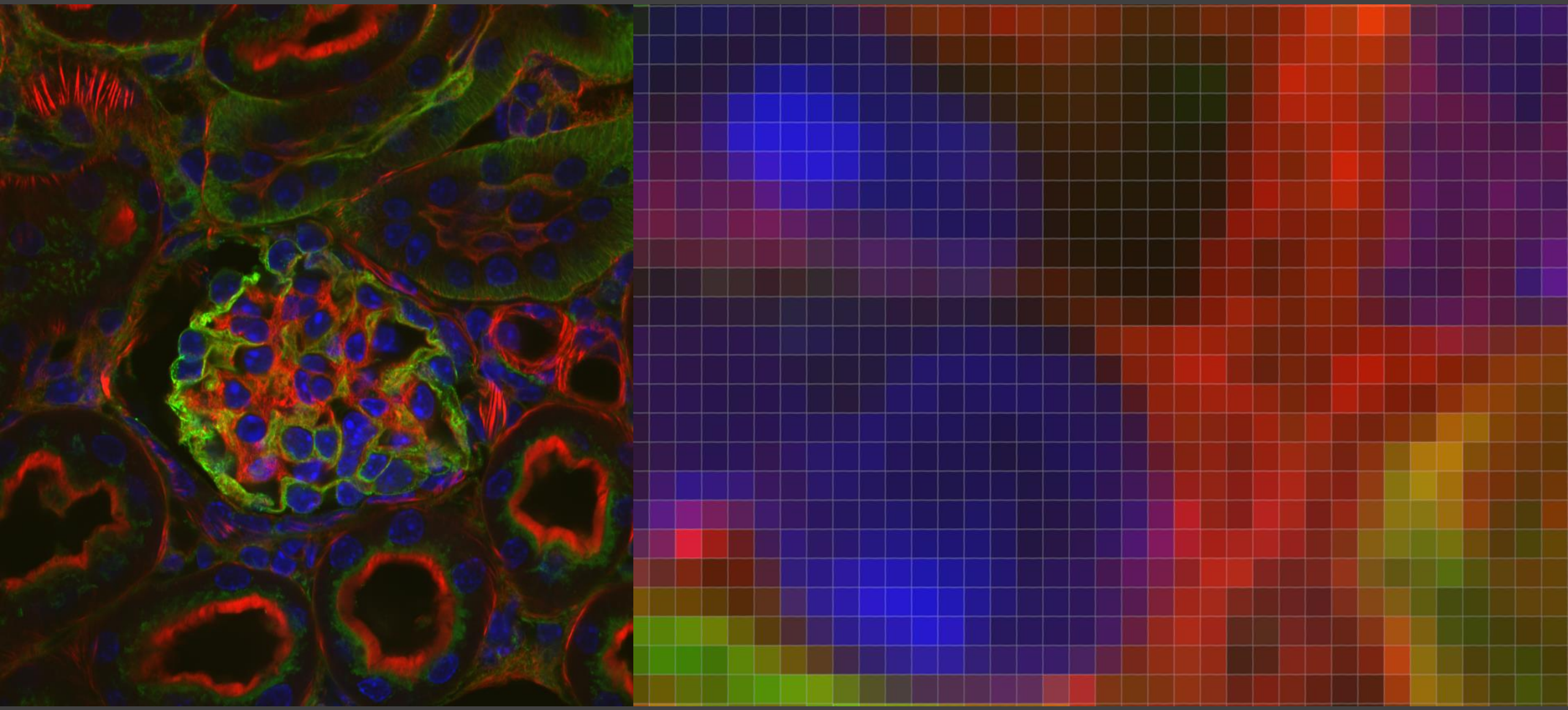


Image quality – over/underexposure

Blinded by the ligh
Or lost in the dark



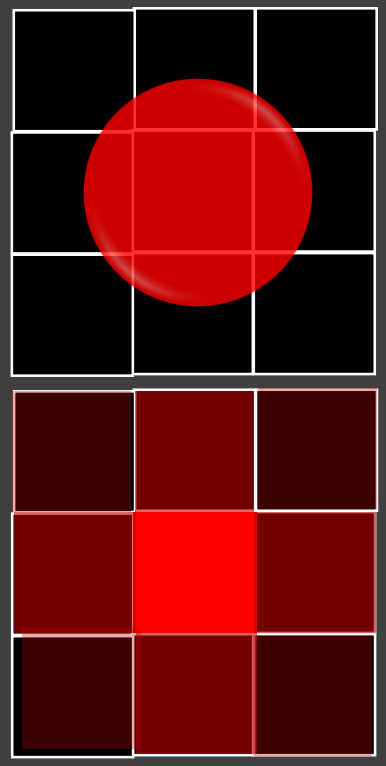
Biology into pixel → Nyquist theorem



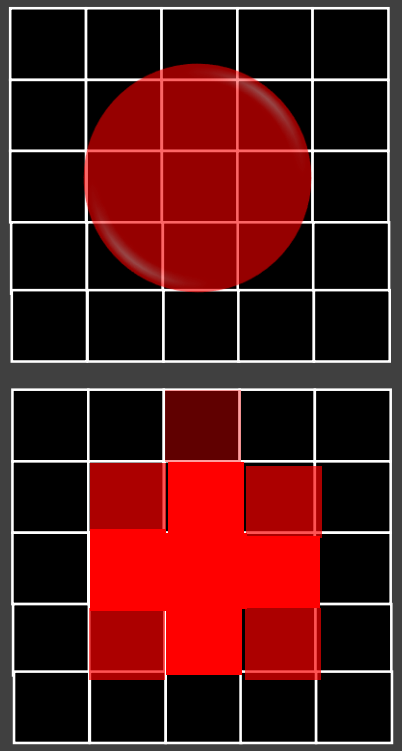
Imaging – Nyquist theorem in 2D

Sampling of the signal

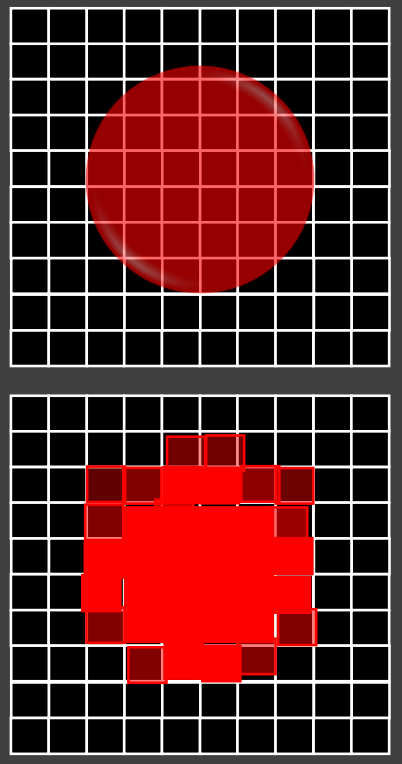
Generated image



Undersamplig, wrong information gained



Optimal sampling (2.3 times), correct information gained



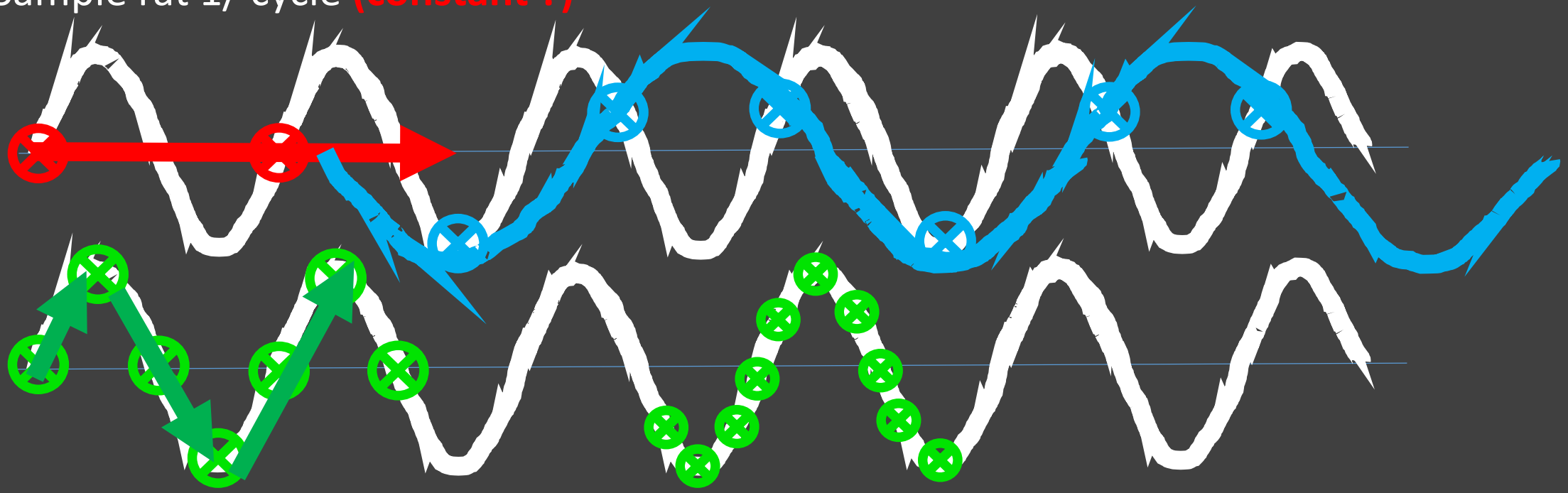
Oversampling?, same information gained like with optimal sampling, but more time, computer power, storage etc. Involved, photons per pixel might be too low...

Imaging – Nyquist theorem

Nyquist found that in order to reconstruct a pure sine wave, it must be sampled at least twice during each cycle of the wave, or at two-times the temporal frequency.

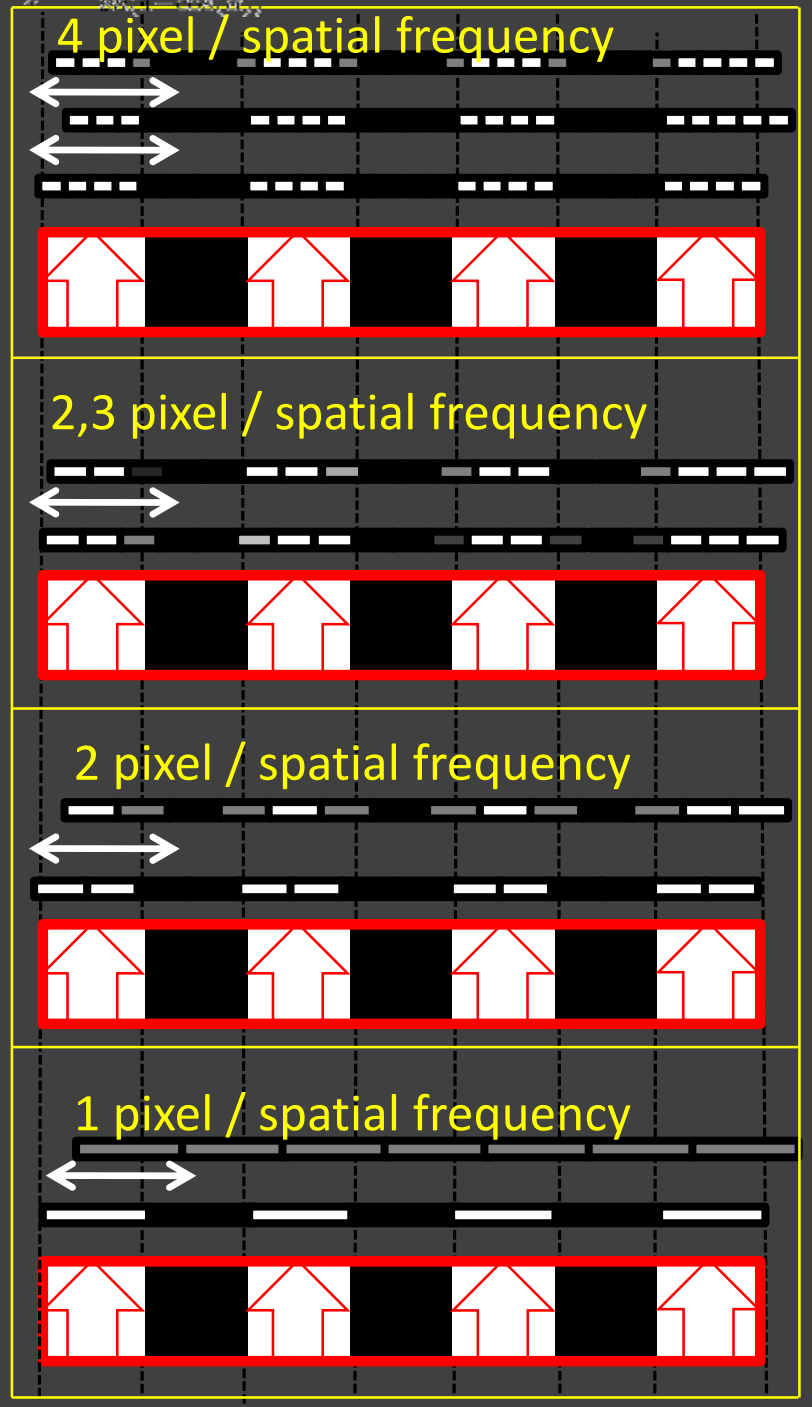
Sample rat 1/ cycle **(constant ?)**

Sample rat 1,5/ cycle **(lower frequency?)**



Sample rat 2/ cycle **(jagged ?)**

Sample rat > 2/ cycle **(a sine wave !)**



Imaging – Nyquist theorem

light pattern of Specimen versus different pixel size

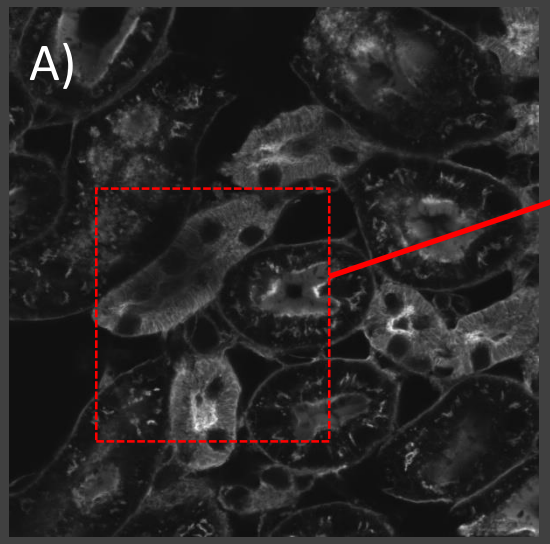
BUT:
Nyquist calculator by Huygens* calculates for
63x/1.4 N.A. and Refr.Index (n) 1.5, CONFOCAL

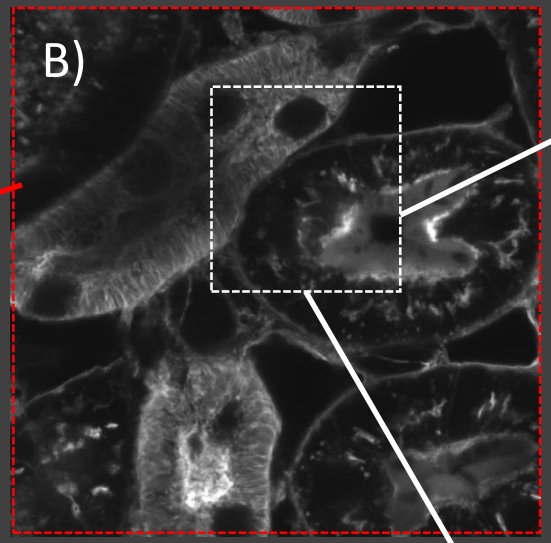
Huygens:	XY:	42,42,
LEICA:	XY:	63,63,
ZEISS:	XY:	96,96,

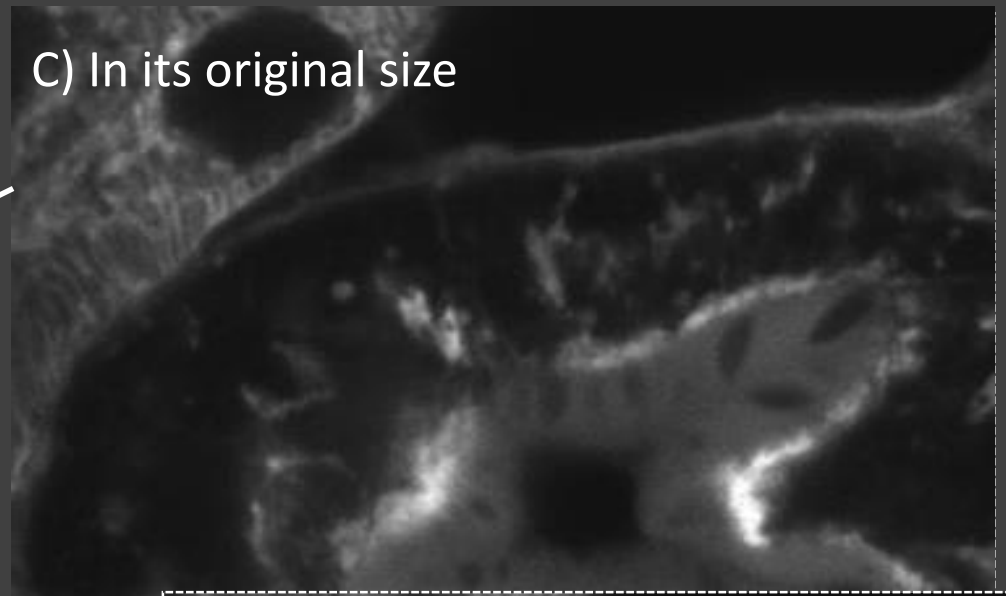
e.g. $dx = \Lambda ex / 8n \sin(NA)$ (Confocal) = 41,2
$dx = \Lambda em / 4n \sin(NA)$ (widefield) = 87,2

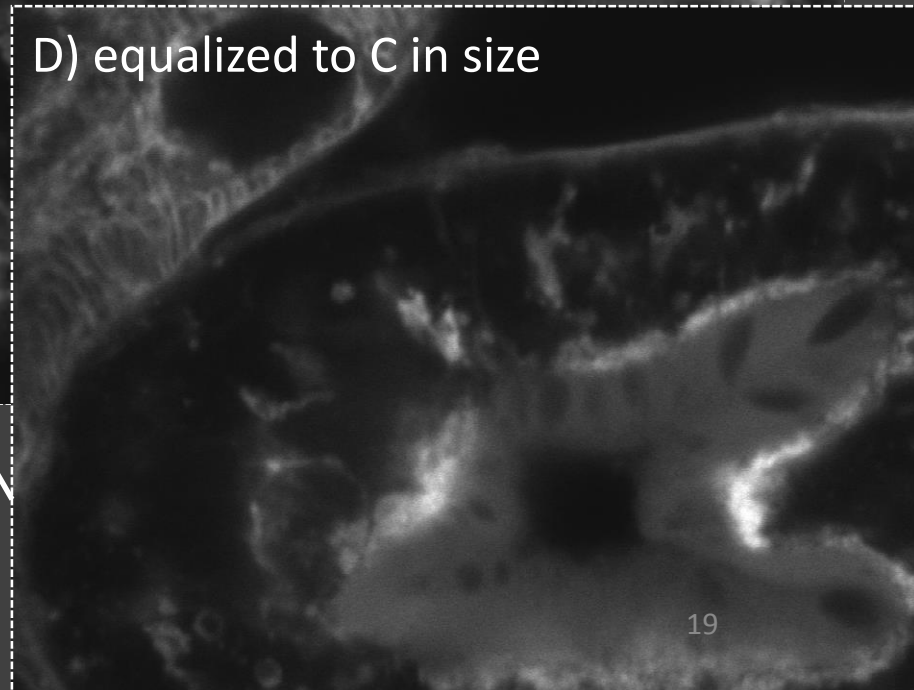
For Z formula and more information see
Source:
http://www.svi.nl/NyquistRate

Image quality: pixel size and optical zoom









Images taken according to Nyquist,
and scan speed 7, avg 2, 63x/NA1.4
A) Zoom 1.0, 2048x2048px, scan : 16 sec
B) Zoom 2.0, 756x756px, scan: 8.0 sec
C) Zoom 4.0, 376x376px, scan : 2.8 sec
D) Zoom 4.0, 1024x1024px, scan : 7.8 sec
→A, B, C – normal sampling
→ D, oversampling but not better resolution

Image quality - Average

Average mode: instead of ”reading the speciment” only once, one can ”read it” 2, 4, 8, 16 times. And average these 2 or 16 images into one.Better signal to noise ratio → better image. ...BUT Bleaching might occur...

The ”average” can be combined with ´pixel dwelling time´ - the timeof how long the laser excites the pixel/voxel and hence how much time. There is for the objective /PMT to gather emitted photons. ... BUT Bleaching might occur ...

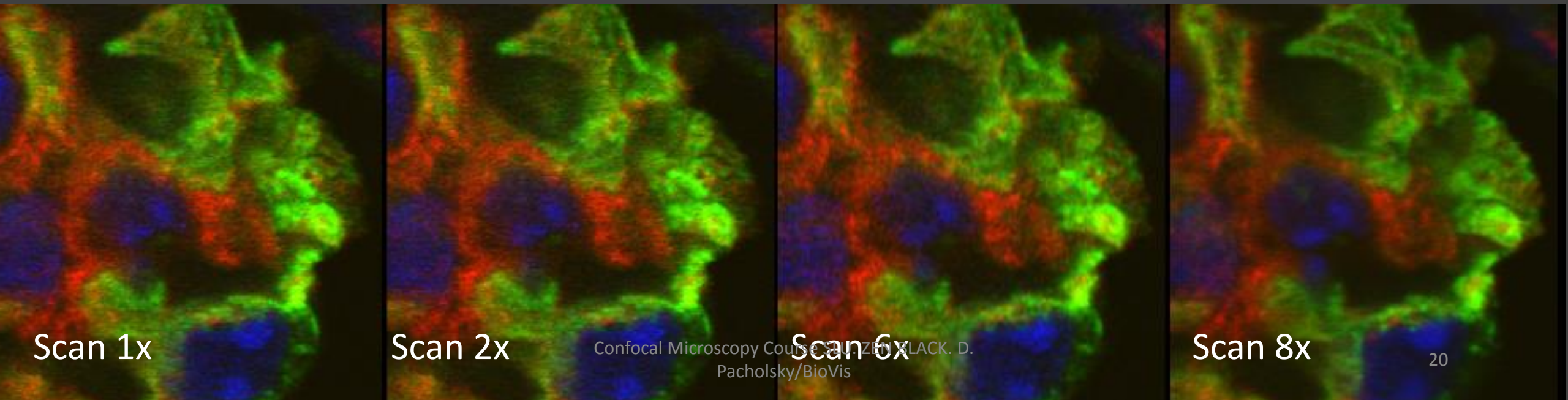


Image quality: bit depth

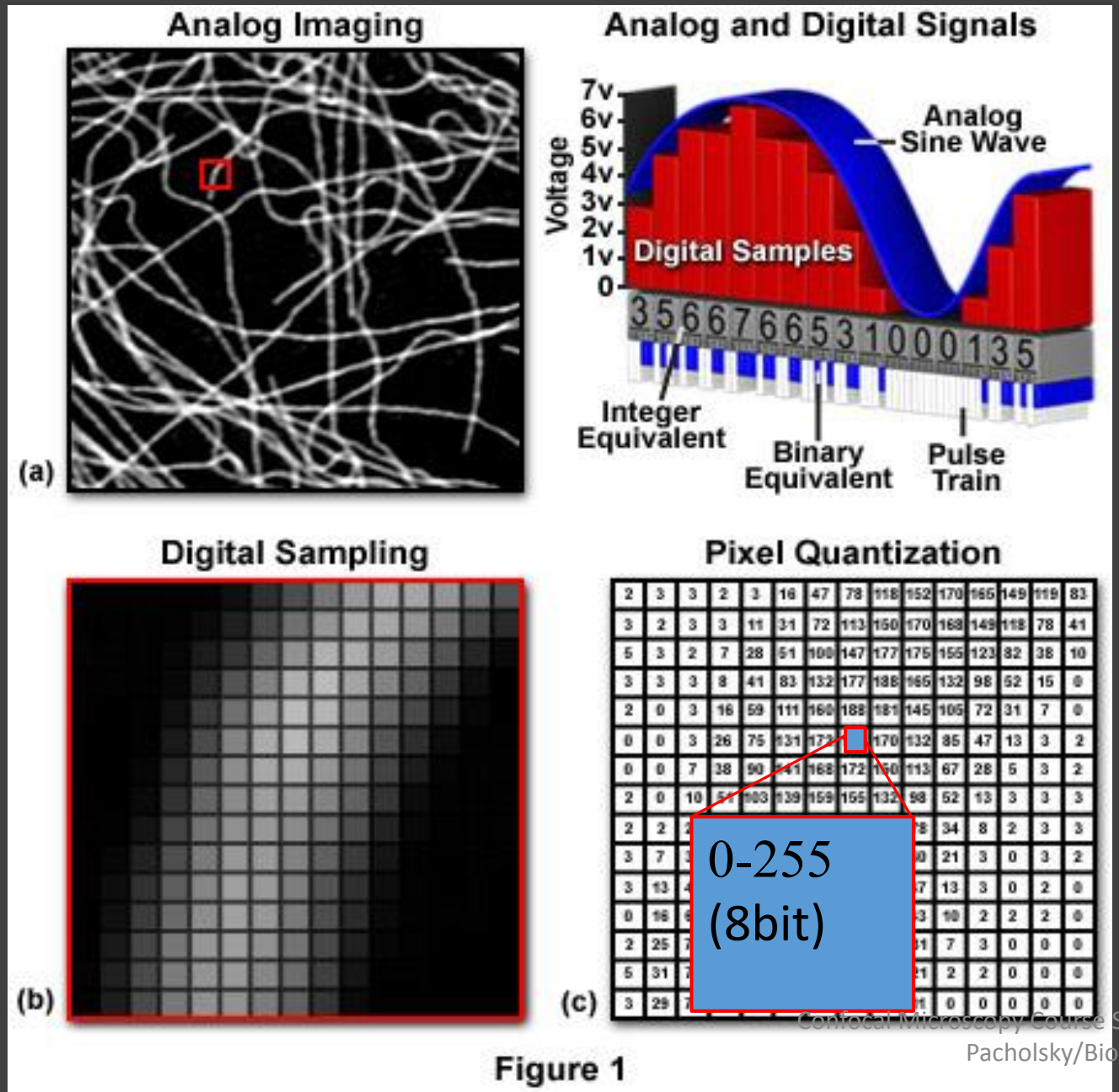


Figure 1

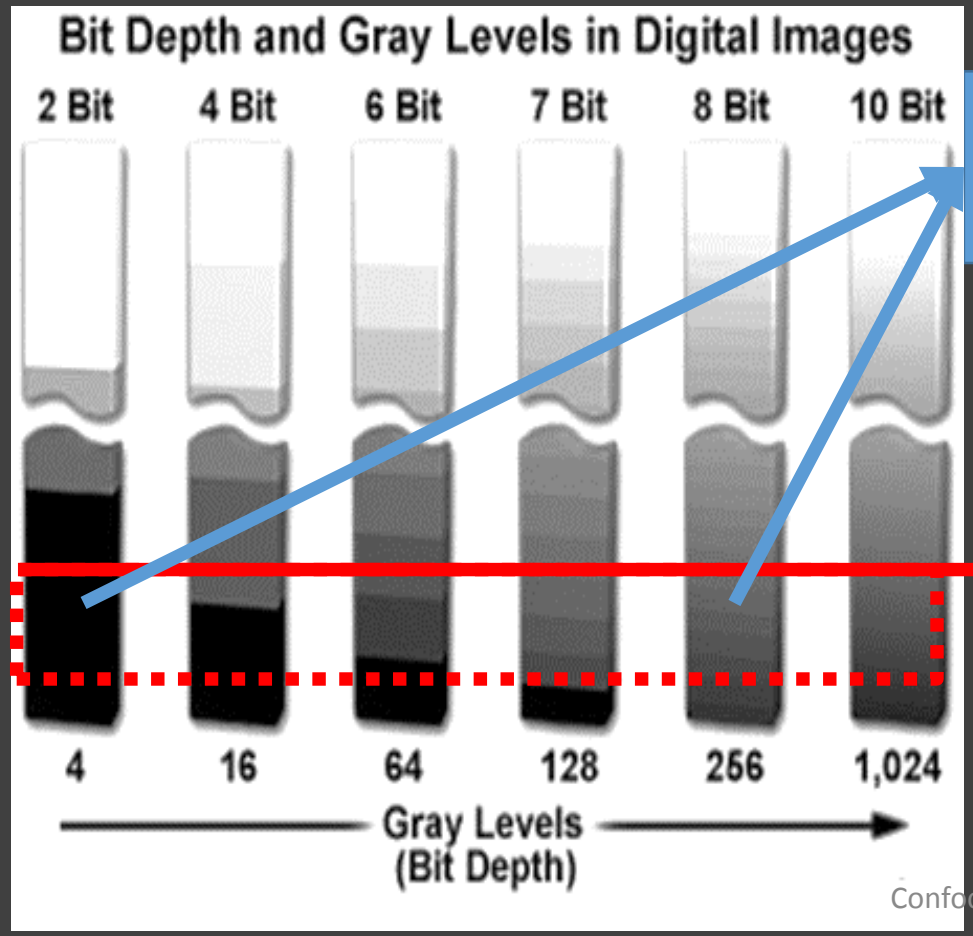
How detailed can we represent the different intensities of light?

Where is the limit to detect?
- ”x” Photons per pixel
- Background noise of detector

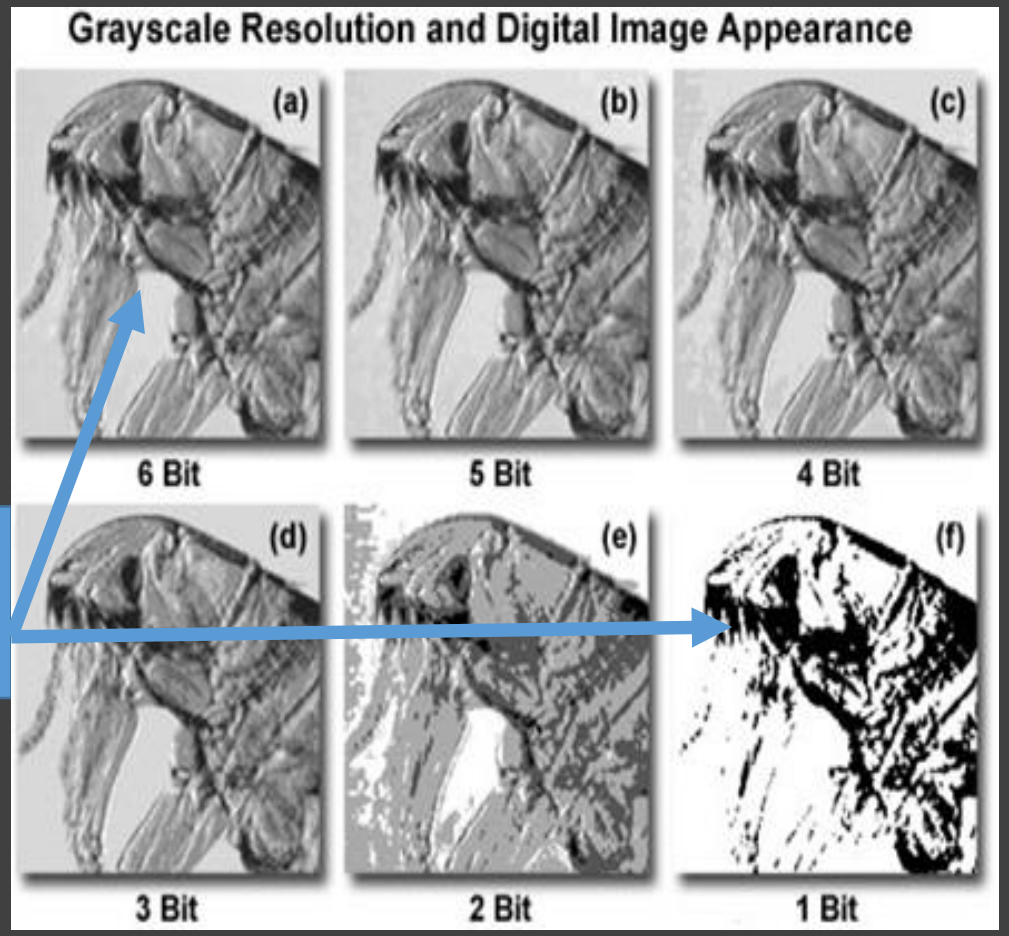
What is needed in detail/sensitivity?

Image quality: bit depth

Acquire your image with appropriate grey level (8bit at least) to represent different intensity levels .



Apportionment of photons

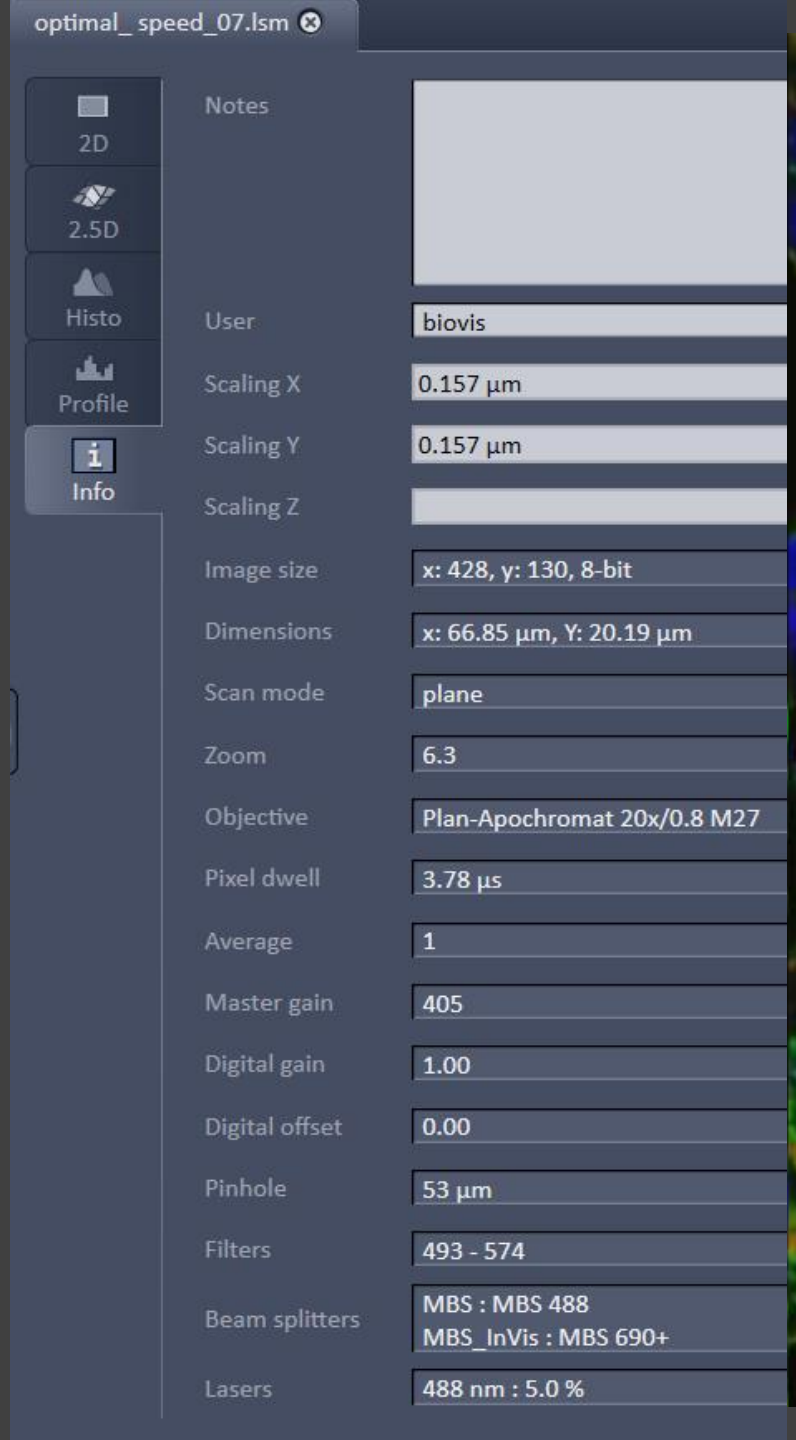


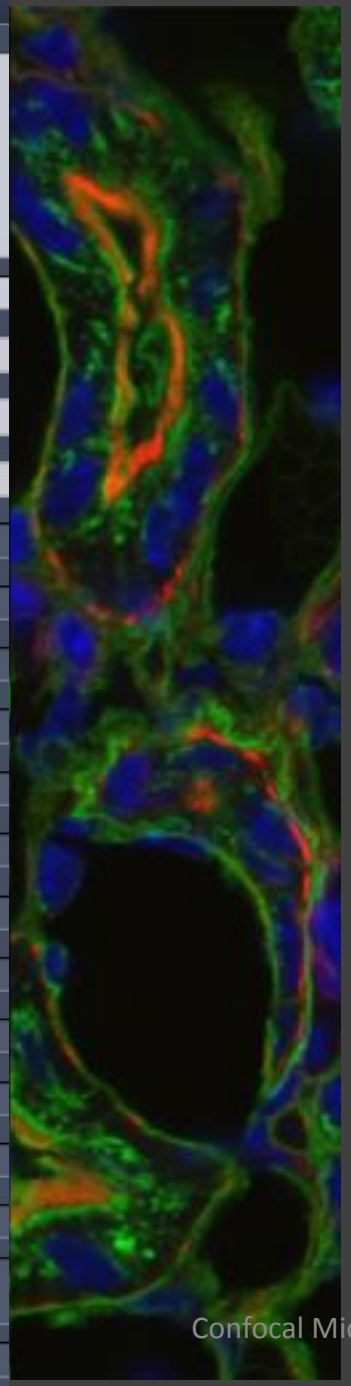
→12 or 16 (4096 – 65536 grey levels)bit images would also allow you e.g. to enhance features lying in the dark to stretch them into the light...
→ High bit might also result in more information Coming from noise not signal

The Image

Information on image

Image / Channel information

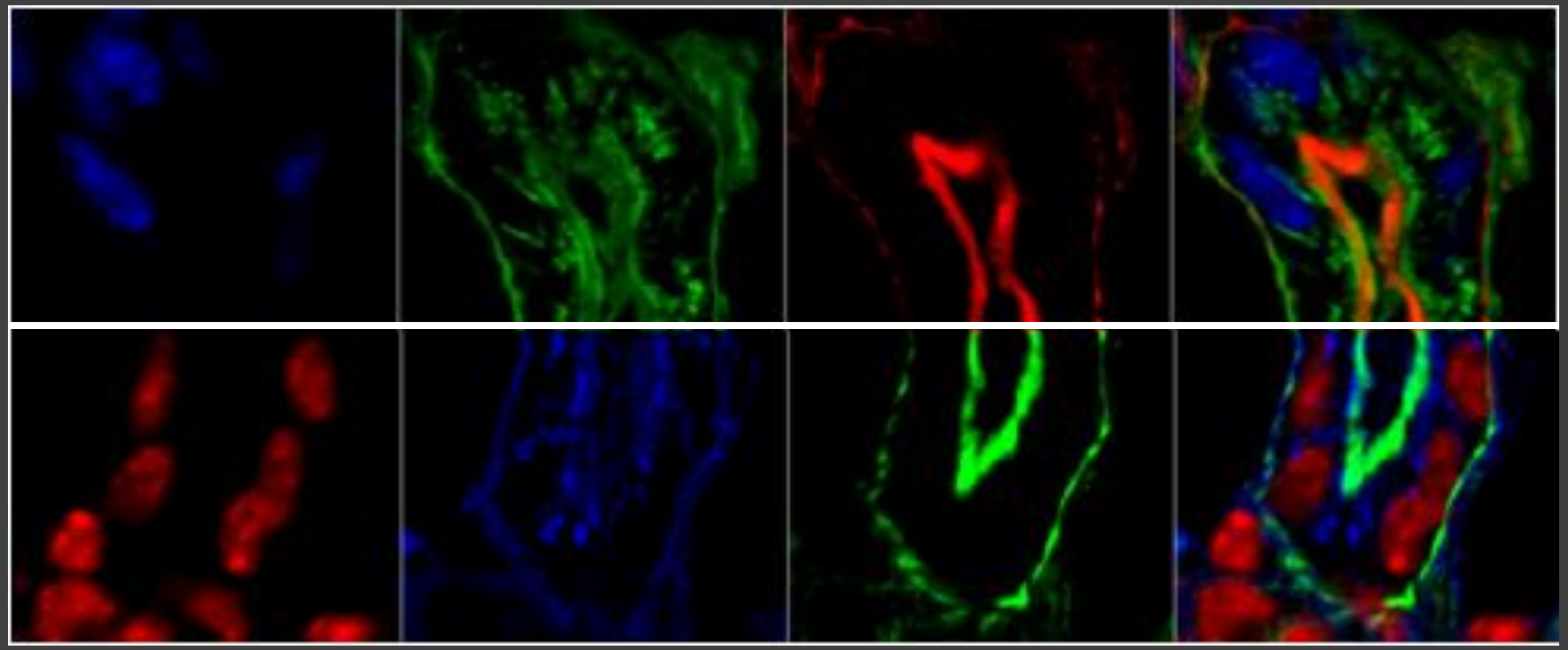




original Images stores meta-data: information about pixel/voxel size, image mode, user, Laser/filter, opbjective used.

RE:USE this info for future application
On same or related instrument
(do not try to reuse settings from LSM510 on LSM780...)

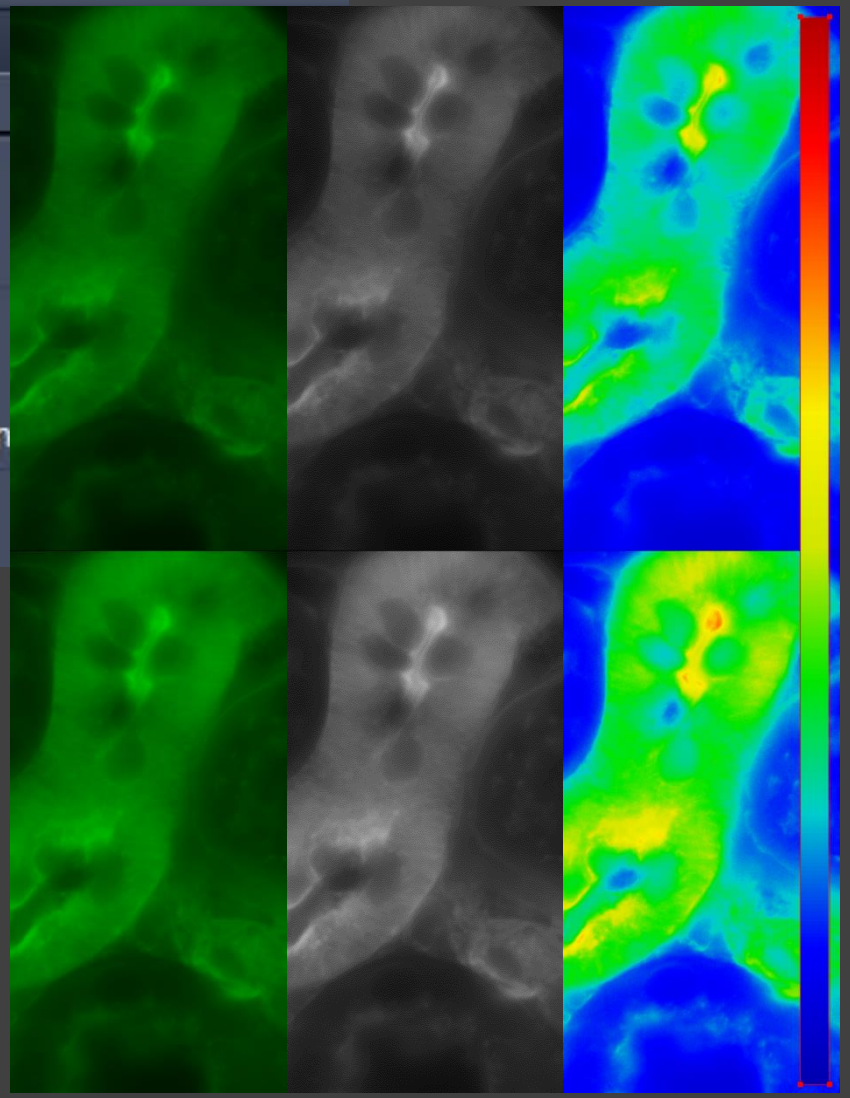
Image presentation: Colors



Channel Split → free choice of color per channel
... Same brightness in GREY might appear NOT the same in different Colors for human eye....

visualize Comparing channels in contrasting color pairs Red vs Green

Image presentation: Look Up Table LUT



Bleaching before and after
100x imaging same area with
Widefield microscopy.
Test sample is a strong stain and so
bleaching might be subtle
and only clearly be see in
LUT (look-up-tables)

Intensities of emission are shown in LUT
Black to white
LUT= Blue, over green, yellow, red

You might not see the subtle changes
But would like to compare Intensities
Between image 1 and 2?

Be aware...

6kg of Light

Intensity measurement

Comparing different slides for intensity:

- Sample I : no staining
- Sample II: secondary Ab (incl Fluorophore) only, to define background
- Samples III: your actual sample
- Imaging: NO over, or under exposure
- Imaging: same settings on instrument (Gain, Laserpower etc.)
- Make notes IF something needs to be changed for later normalizations
- Use ROI to define e.g. nucleus and integrated intensity

for more details on proper image analysis :

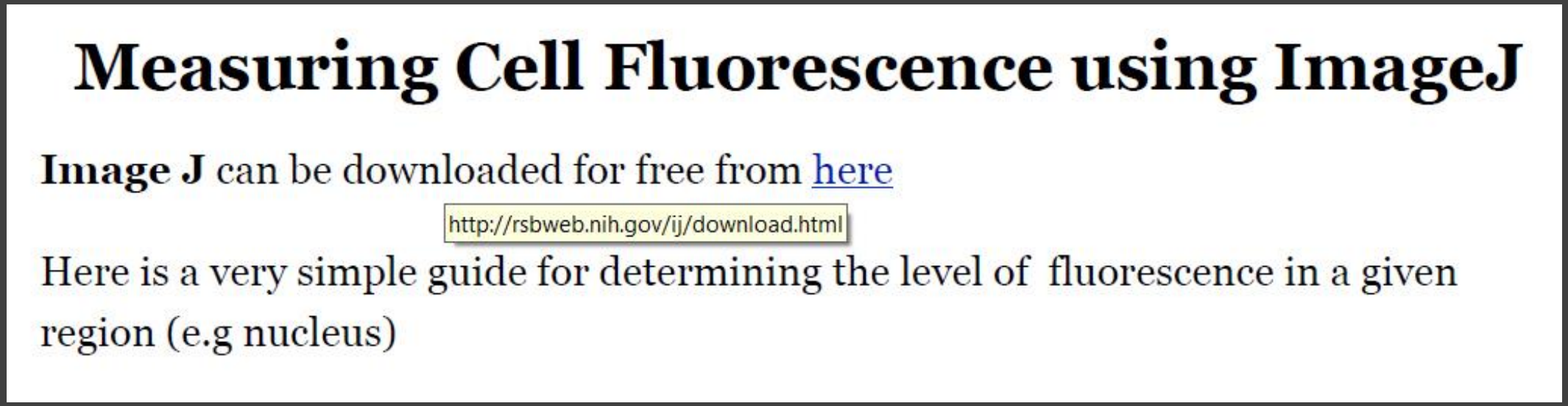
→ Centrum För Bildanalys (Carolina Wählby)

→ see also .pdf about Image J link:

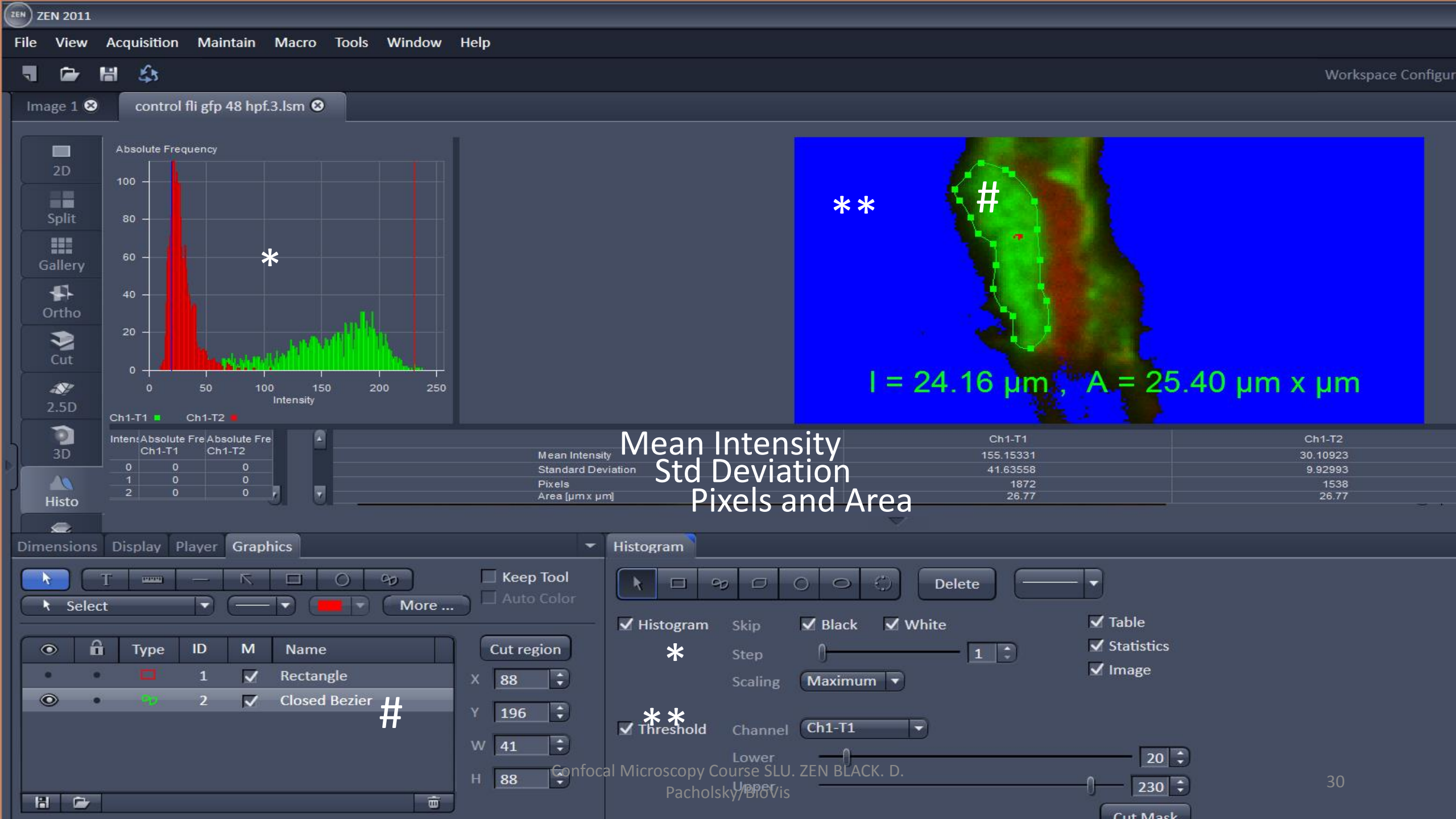
→ Other sources Imaris, Amira

→ as for ZEN, see next slide

http://sciencetechblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej



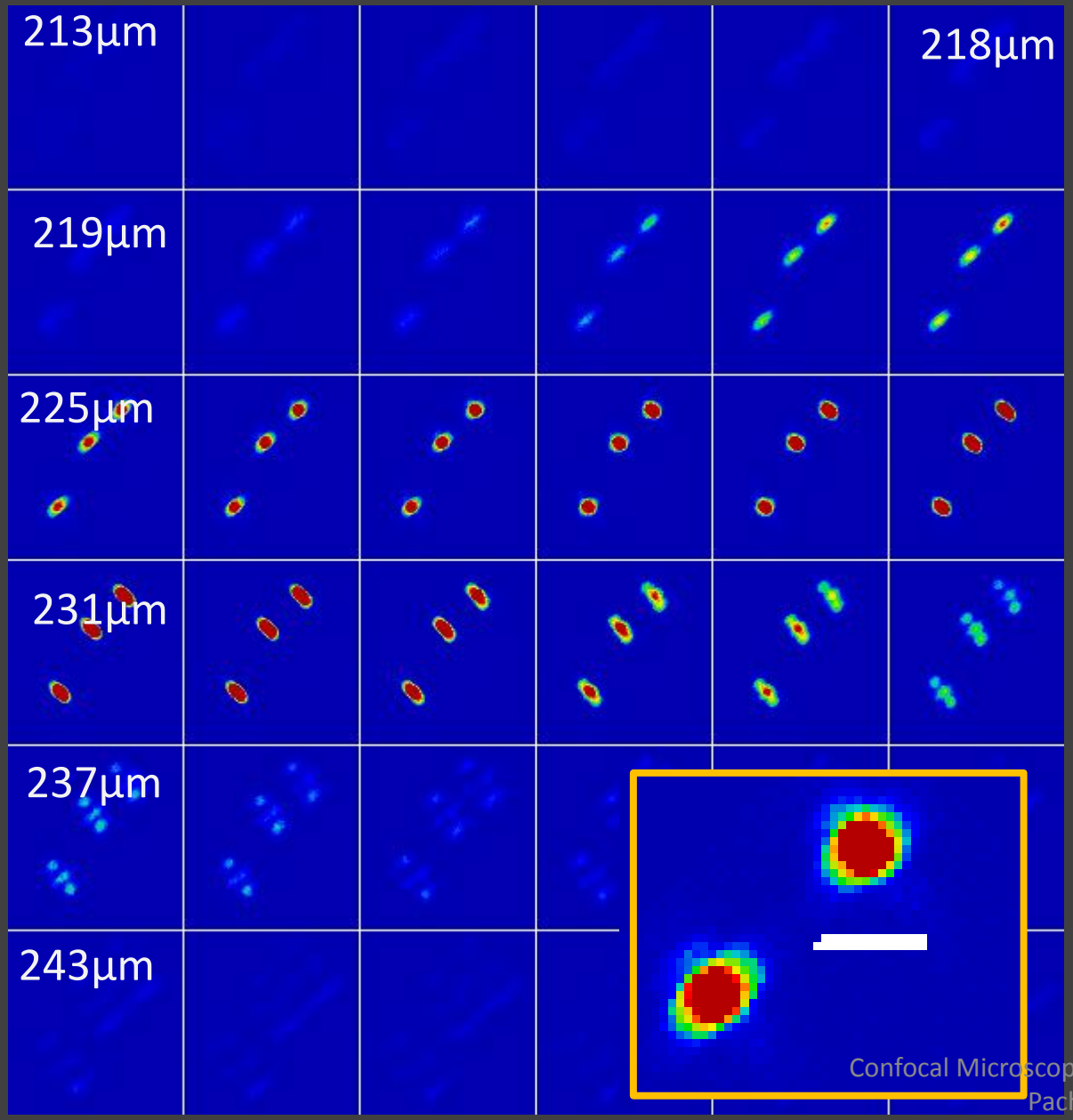
http//rsbweb.nih.gov/ij/download.html

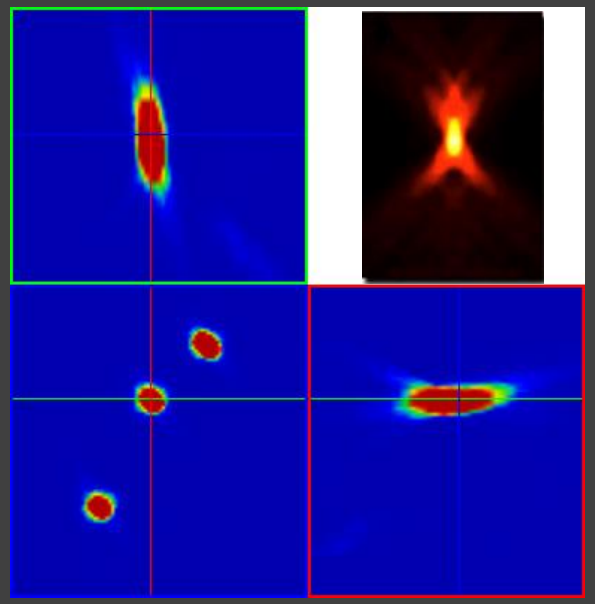

ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Configur
Image 1
control fli gfp 48 hpf.3.lsm
2D
Split
Gallery
Ortho
Cut
2.5D
3D
Histo
Absolute Frequency
100
80
60
40
20
0
0 50 100 150 200 250
Intensity
*
Ch1-T1
Ch1-T2
Intens Absolute Fre Ch1-T1 Absolute Fre Ch1-T2
0 0 0
1 0 0
2 0 0
**
#
l = 24.16 µm , A = 25.40 µm x µm
Mean Intensity
Std Deviation
Pixels and Area
Ch1-T1 Ch1-T2
Mean Intensity 155.15331 30.10923
Standard Deviation 41.63558 9.92993
Pixels 1872 1538
Area [µm x µm] 26.77 26.77
Dimensions Display Player Graphics
Keep Tool
Auto Color
Select
More ...
Type ID M Name
1 Rectangle
2 Closed Bezier
#
Cut region
X 88
Y 196
W 41
H 88
Histogram
Delete
Histogram
Skip
Black
White
*
Step
1
Scaling
Maximum
**
Threshold
Channel
Ch1-T1
Lower
20
Upper
230
Cut Mask
Table
Statistics
Image

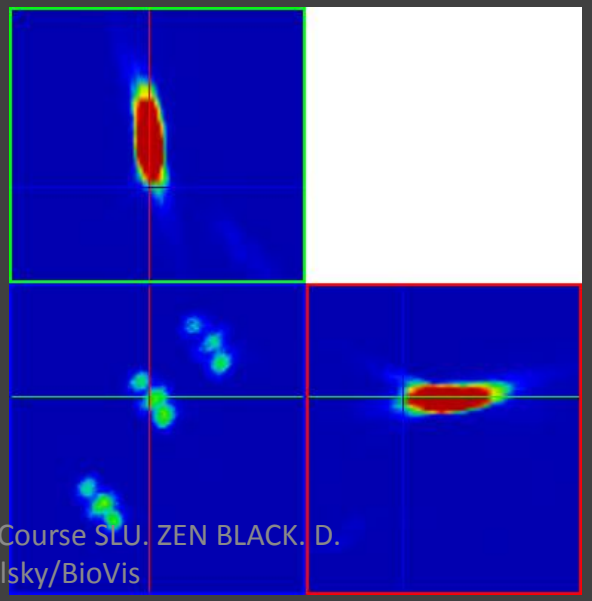
The big view

2D 3D imaging

6µm beads in agarose gel w water objective ...PSF







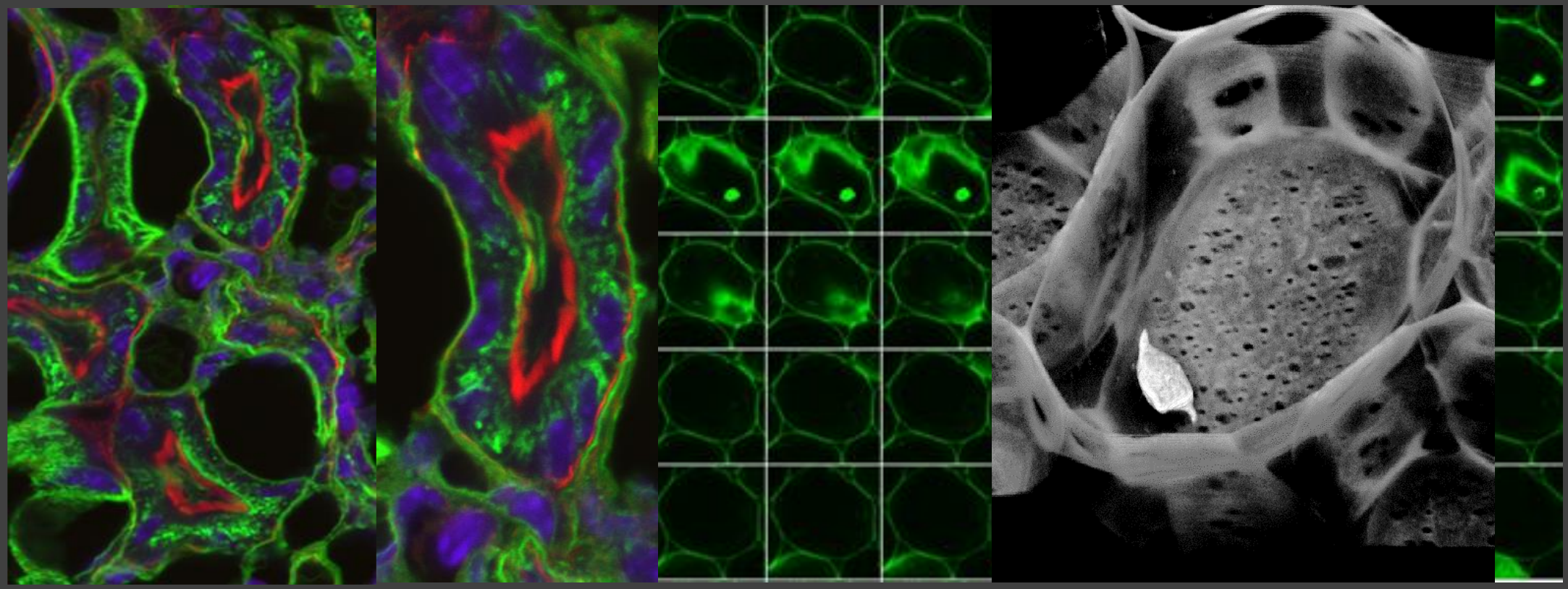
Point Spread Function
PSF is real

sample preparation
microscopic set up*
are parameter
who usually worsen
it

*

- Wavelength
- N.A.
- Ref.Index
- and more

Imaging with LSM



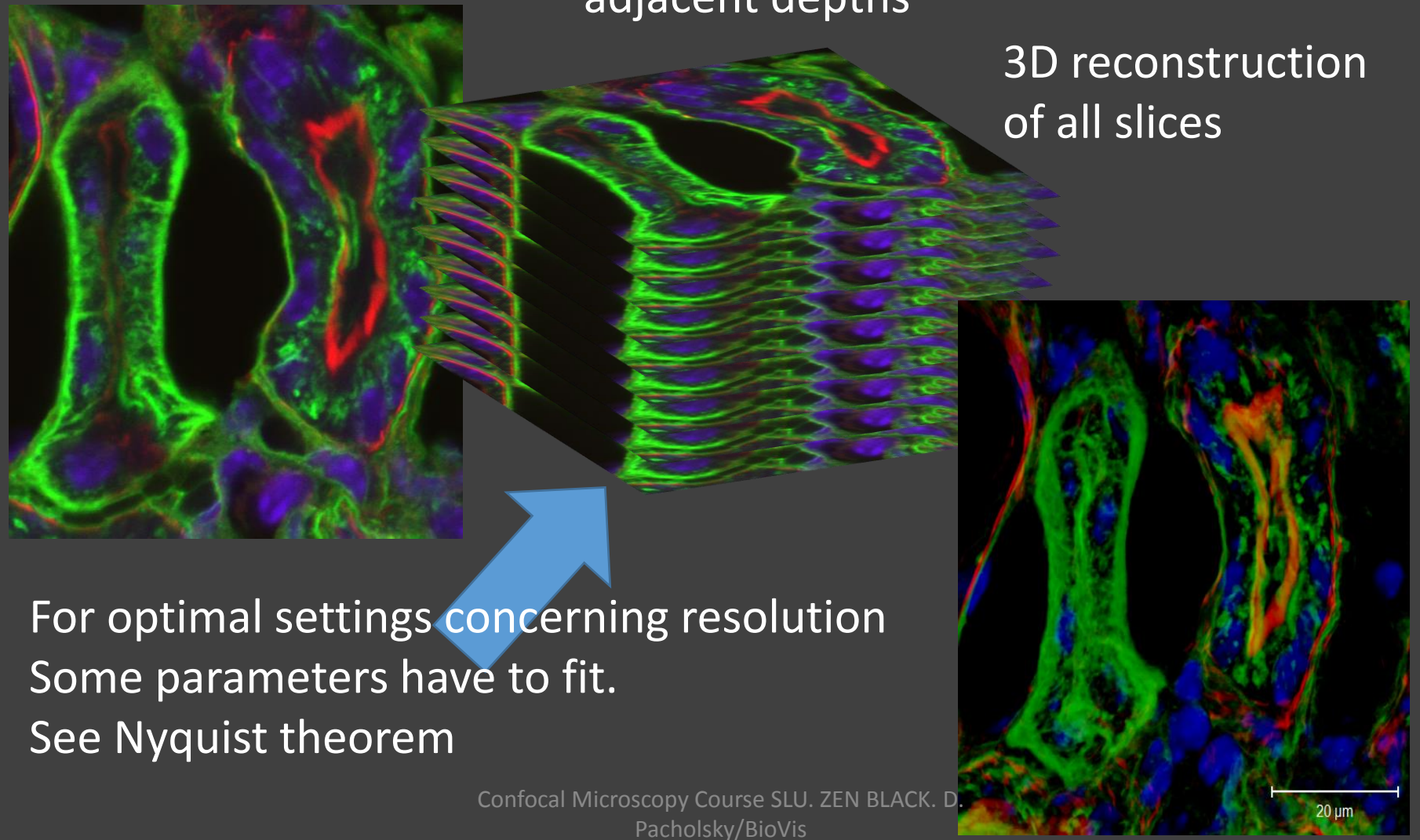
possibilities

From Optical sectioning to 3 D reconstruction

Optical slice from certain depth in sample

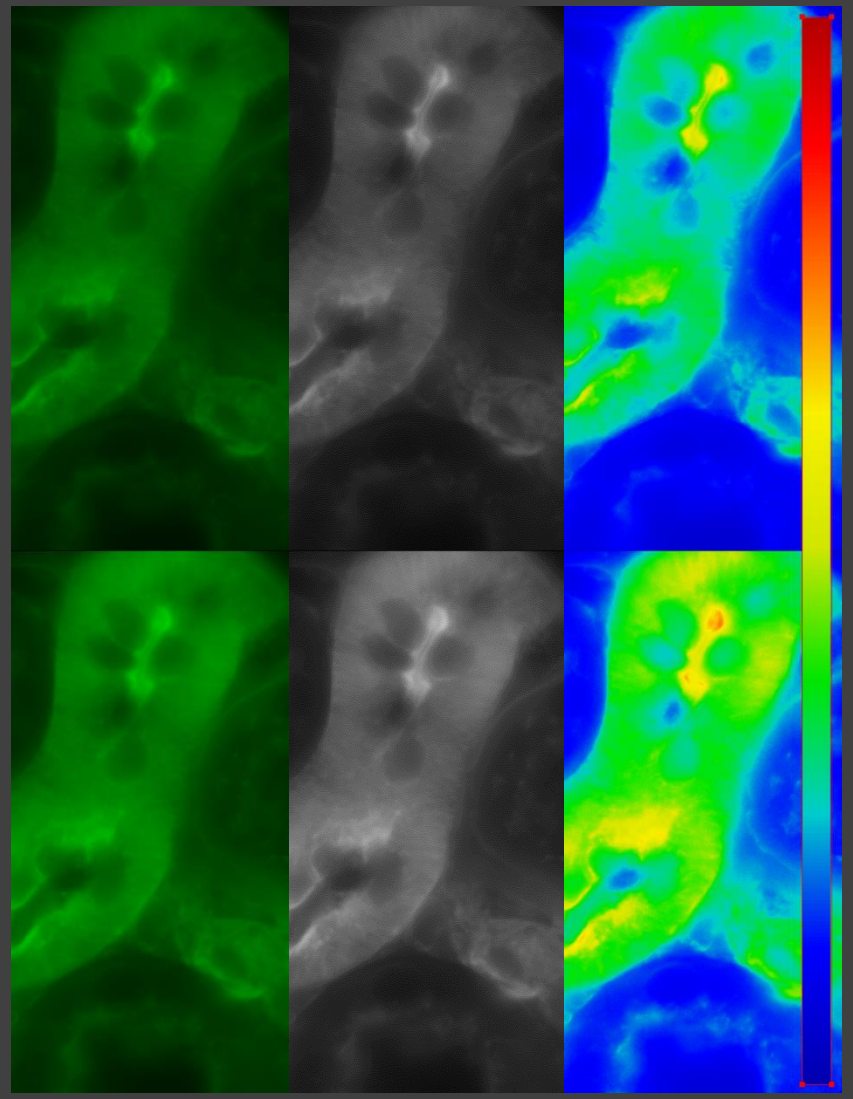
Many slices from adjacent depths

3D reconstruction of all slices



For optimal settings concerning resolution
Some parameters have to fit.
See Nyquist theorem

Bleaching



Bleaching before and after
100x imaging same area with
Widefield microscopy.
Test sample is a strong stain and so
bleaching might be subtle
and only clearly be see in
LUT (look-up-tables)

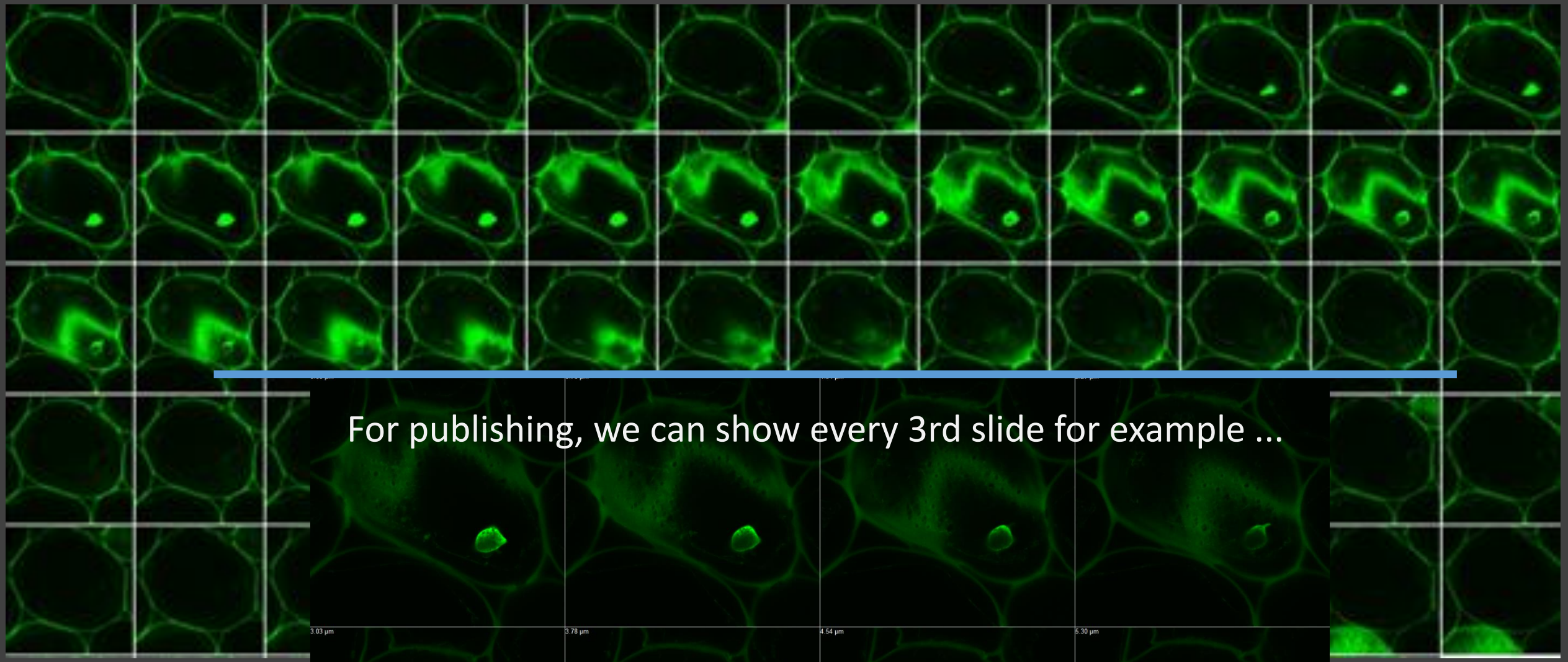
Intensities of emission are shown in LUT
Black to white
LUT= Blue, over green, yellow, red

You might not see the subtle changes
But would like to compare Intensities
Between image 1 and 2?

Be aware...

Gallery view of optical slices from LSM images

Part of gallery of 107 optical slides through plant stem



For publishing, we can show every 3rd slide for example ...

Processing Tab → Method → Copy → Subset

For result press Apply

Subset

Method parameter

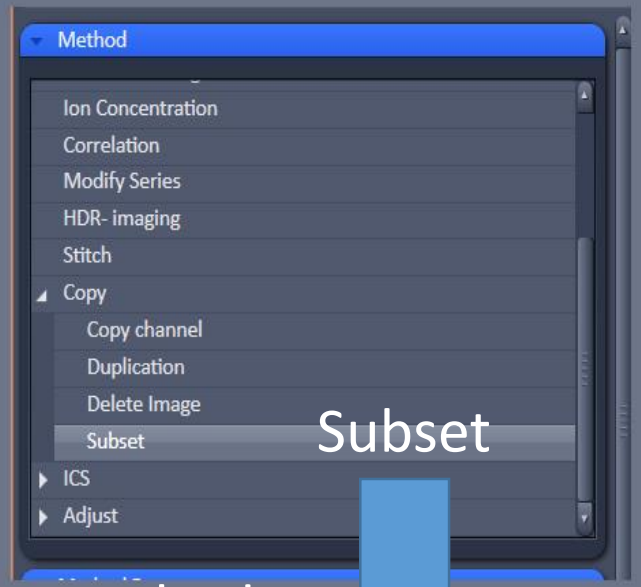
- seen if image is 3D , or time lapse

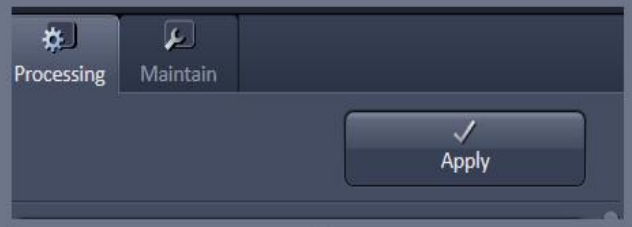
* - seen if image is 3D

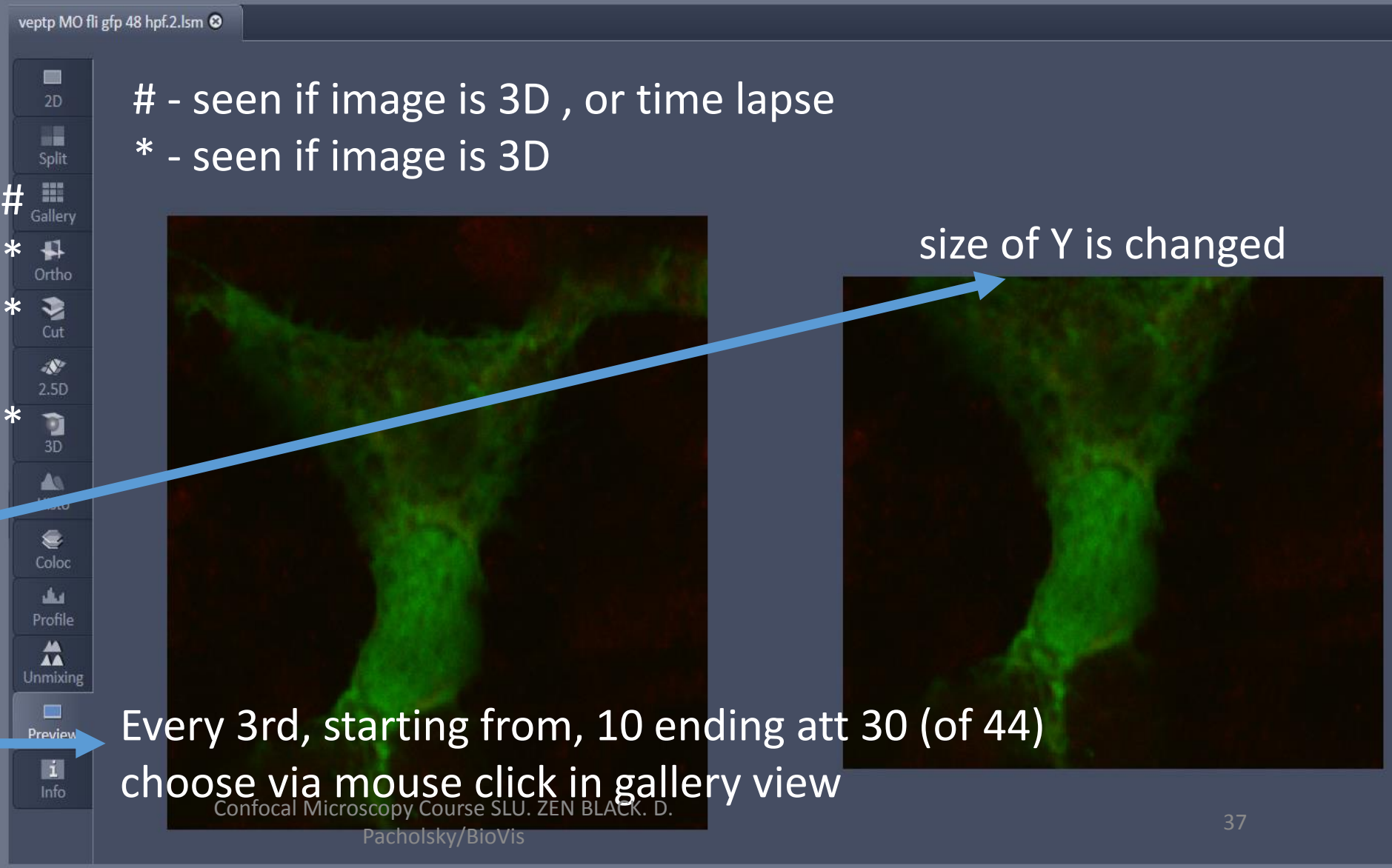
size of Y is changed

Every 3rd, starting from, 10 ending att 30 (of 44)

choose via mouse click in gallery view

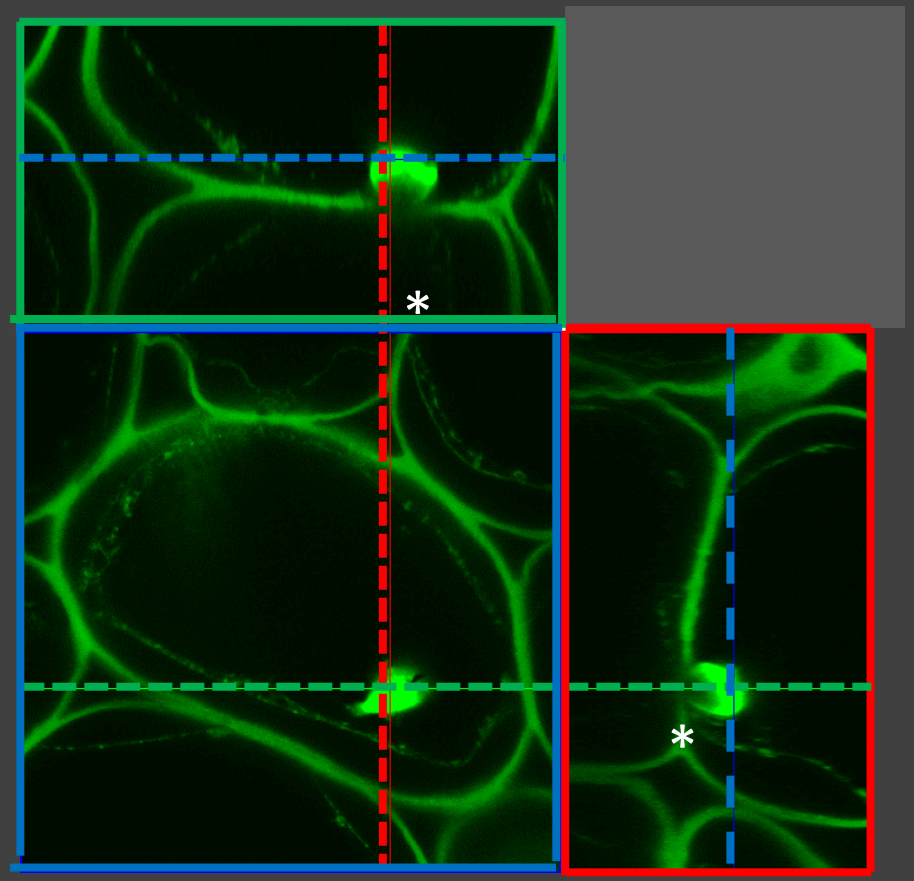






3D information from LSM images

Orthogonal view



3D information , dashed lines in blue, red, green indicate position in ZXY and are movable

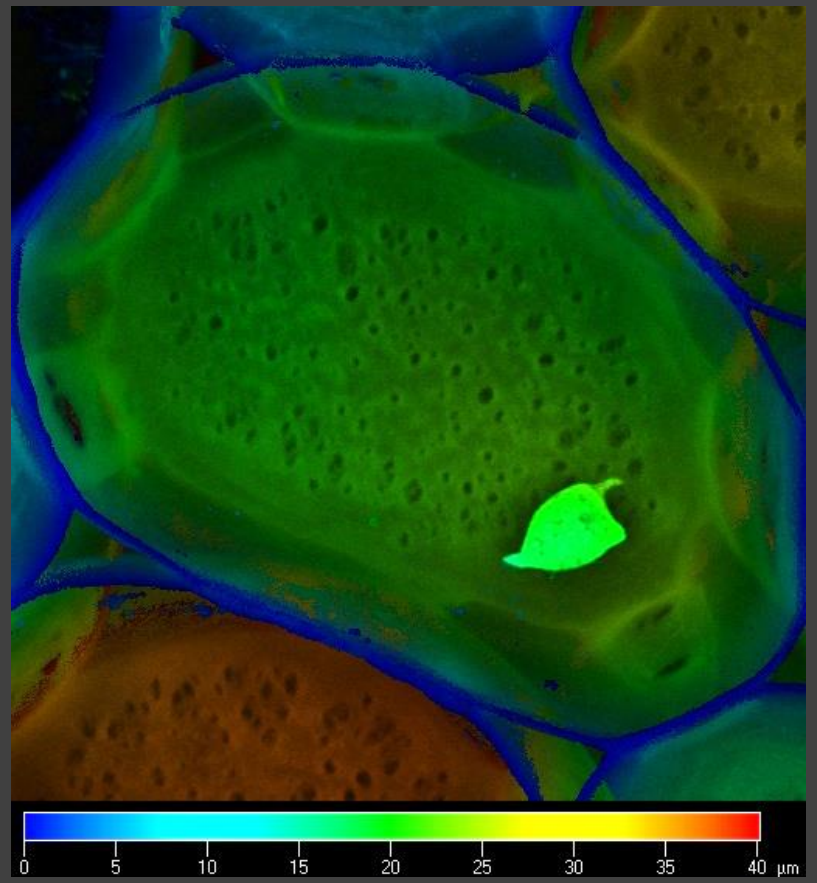
Observe that light could not´penetrate´ material on certain areas*

3D surface reconstruction

3 dimensional reconstruction of image

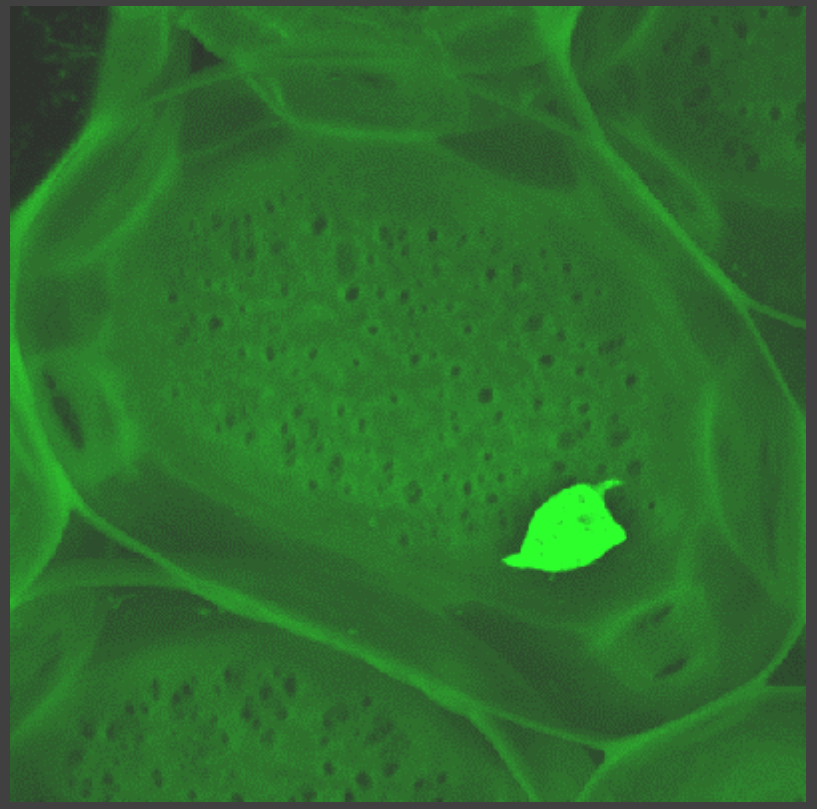
3D information from LSM images

Color depth coding



Overlay of all (or selected) optical slices into one merged image with colors indicating their depths

Maximum Intensity Projection (MIP)



Overlay of the maximum intensity voxels of all (or selected) optical slices into one merged image

Processing | Maintain

Processing

Apply

- Maximum intensity projection
- Color-coded projection
- Image calculator
- Average
- Filter
- Linear unmixing
- Ion Concentration
- Correlation
- Modify Series
- HDR- imaging
- Stitch
- Copy

Method Parameters

Input image: full scan kidney 1AE — Select

Output Image: full scan kidney 1AE_Maximum inter

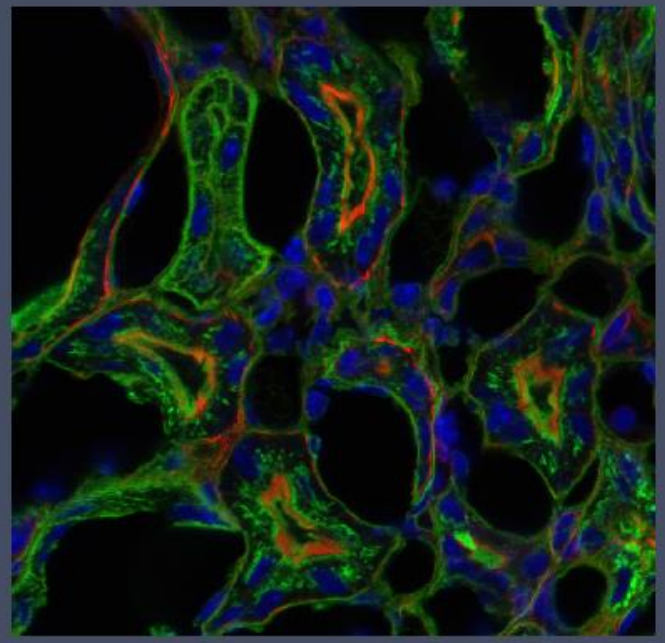
Z

Preview

Input | Preview

fullscankidney1AE.lsm

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



Dimensions | Display

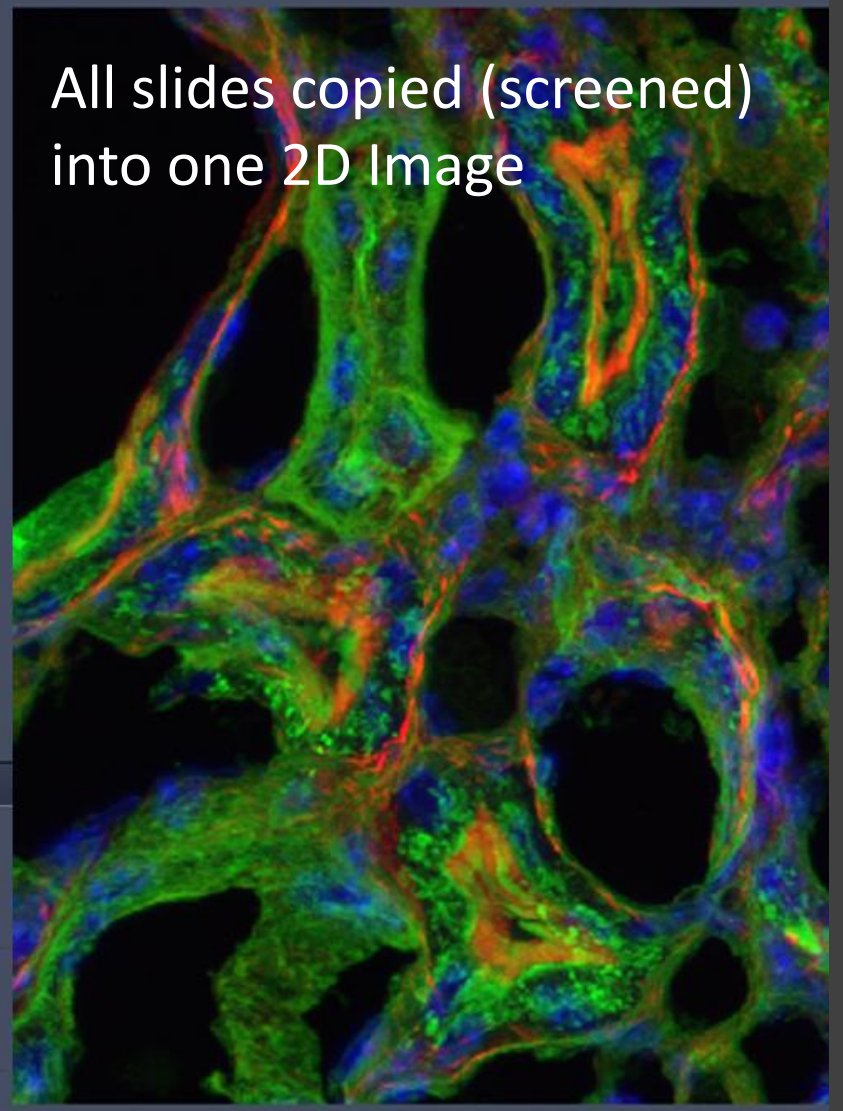
Z-Position 1 — 38 — 20

Zoom 100% — 38

Channels: Ch2-T1 | Ch2-T2 | Ch3-T3

Single Channel | Range Indicator

Reuse | Crop



All slides copied (screened) into one 2D Image

Processing

Maintain

Processing

Apply

- Color-coded projection
- Image calculator
- Average
- Filter
- Linear unmixing
- Ion Concentration
- Correlation
- Modify Series
- HDR- imaging
- Stitch
- Copy

Method Parameters

Input image OCABrainPhal2PMhigh Select

Output Image OCABrainPhal2PMhigh_Color-coded

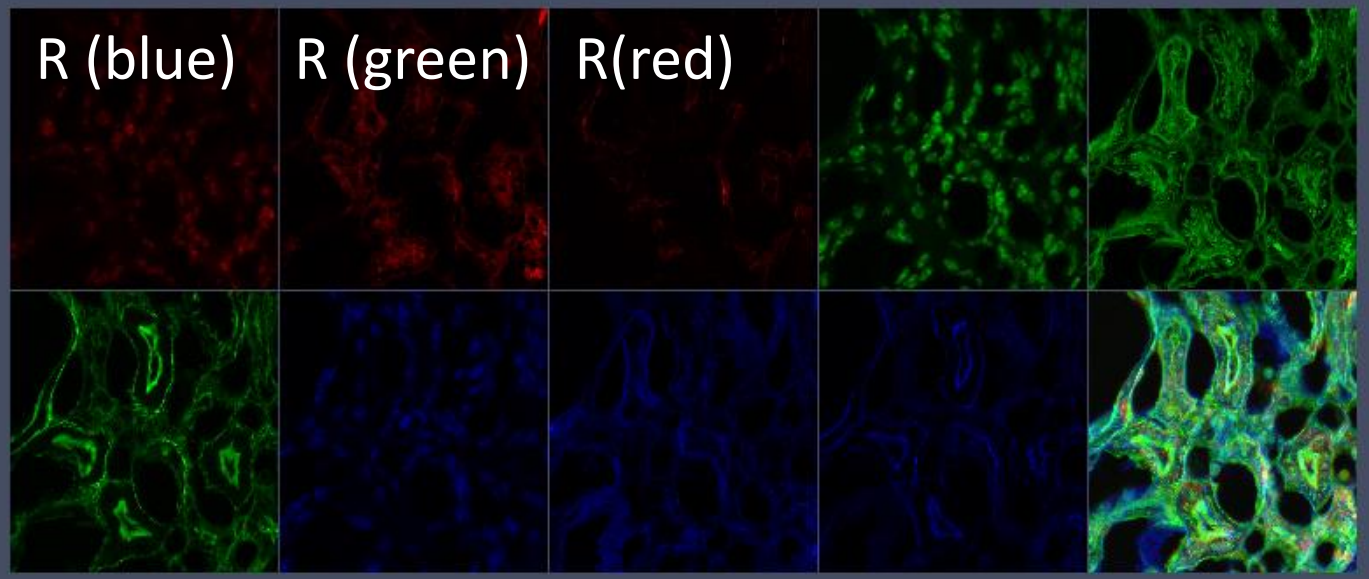
Z

0 316467 632934 ni

Preview

fullscankidney1AE.lsm | full scan kidney 1AE_Co...jection | OCABrainPhal2PMhigh.lsm

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



Depth is color coded via RGB coding.
Signal on surface : pur red
Signal in lower middle: greenish red
→ It appears in red and green in different intensity

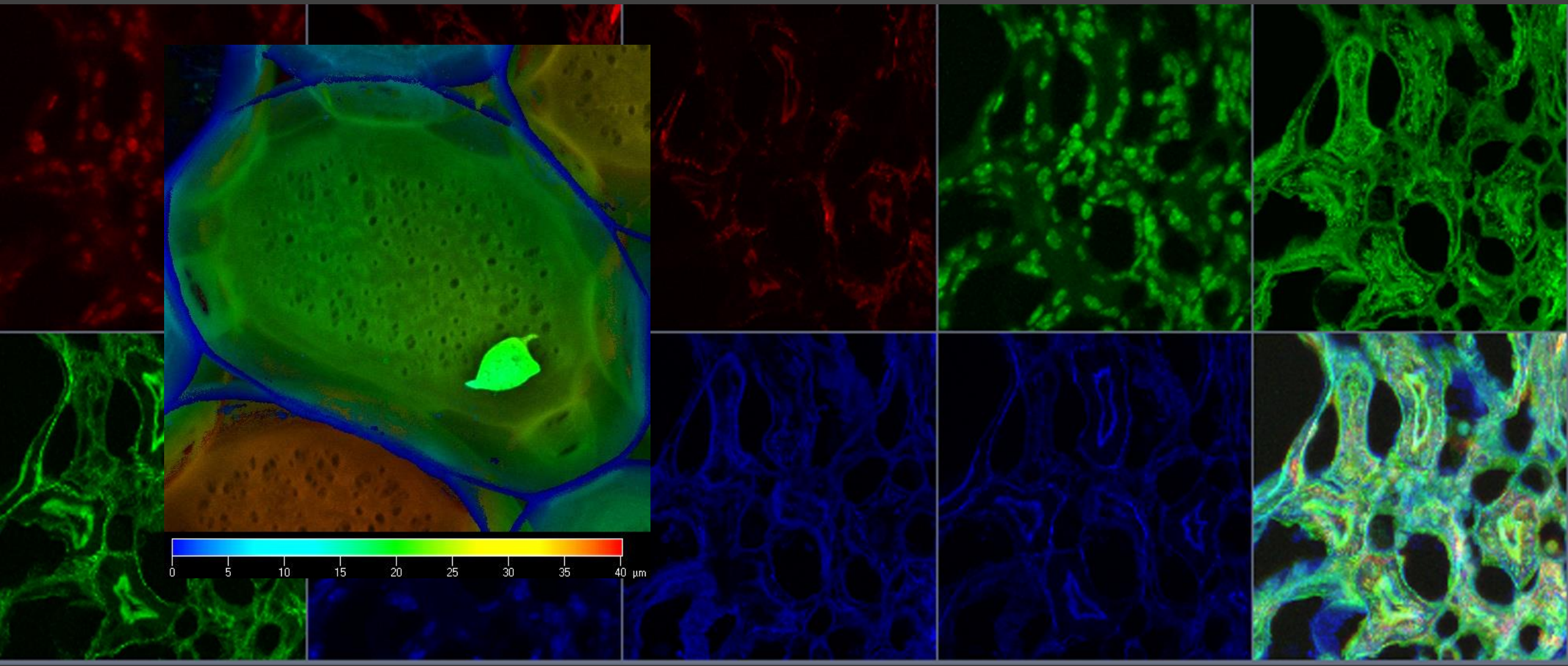
Dimensions | Display | Graphics

Zoom 100 % 15

R G

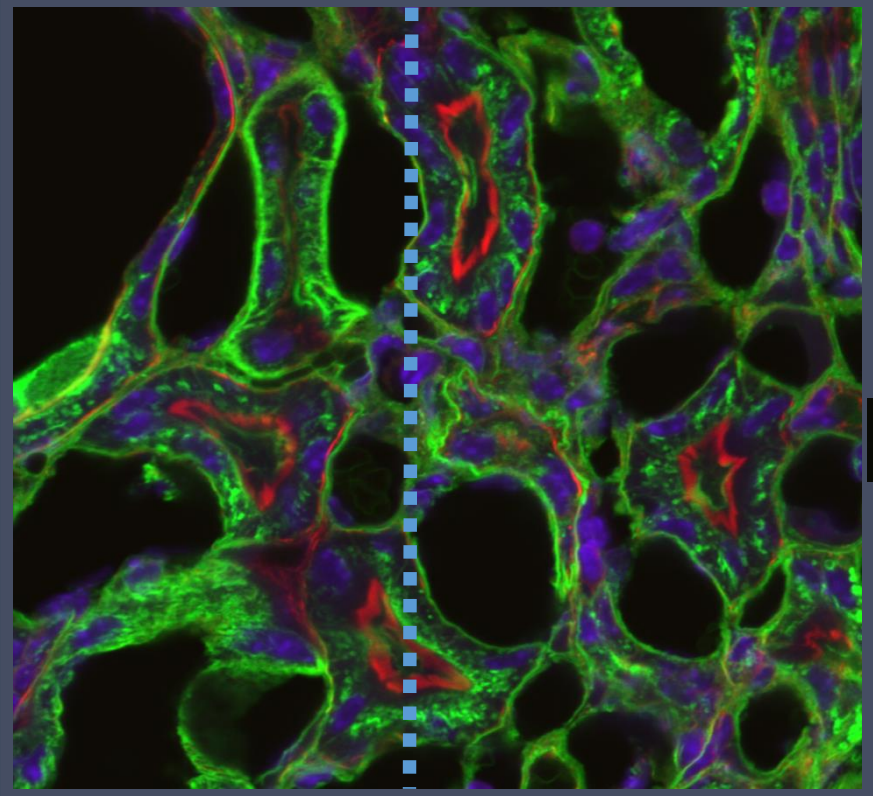
Channels Ch2-T1-R | Ch2-T2-R | Ch3-T3-R | Ch2-T1-G | Ch2-T2-G | Ch3-T3-G

Single Channel | Range Indicator

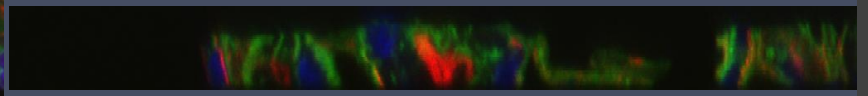


How to get an indicator what is deep (and how deep) and what is surface

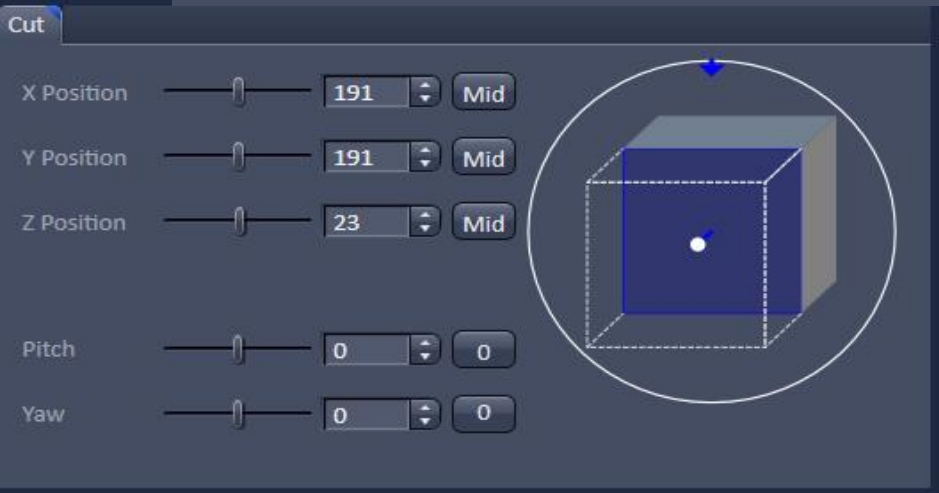
... Let me know, i just remember it wa s complicated in the 3.2 software ...

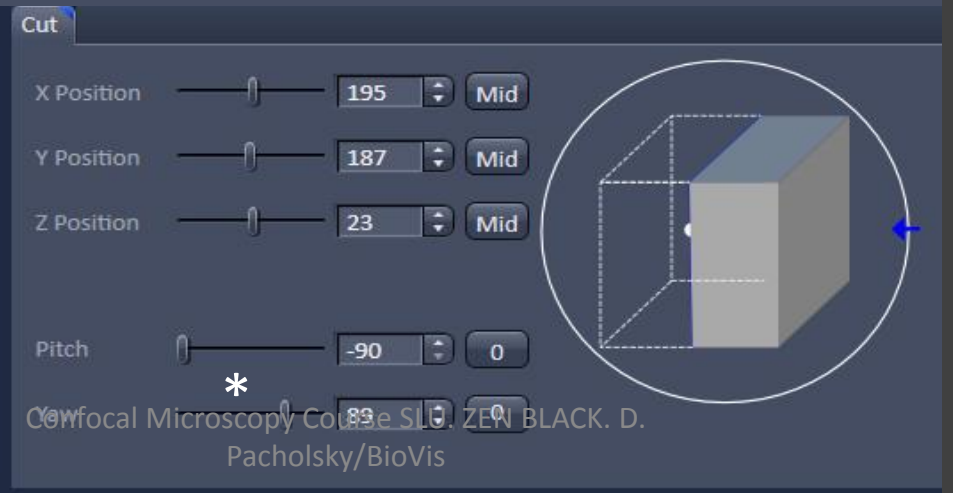


Create inside views by using Cut
Or do this directly whilst imaging in
”line mode” plus ”Z-Stack”



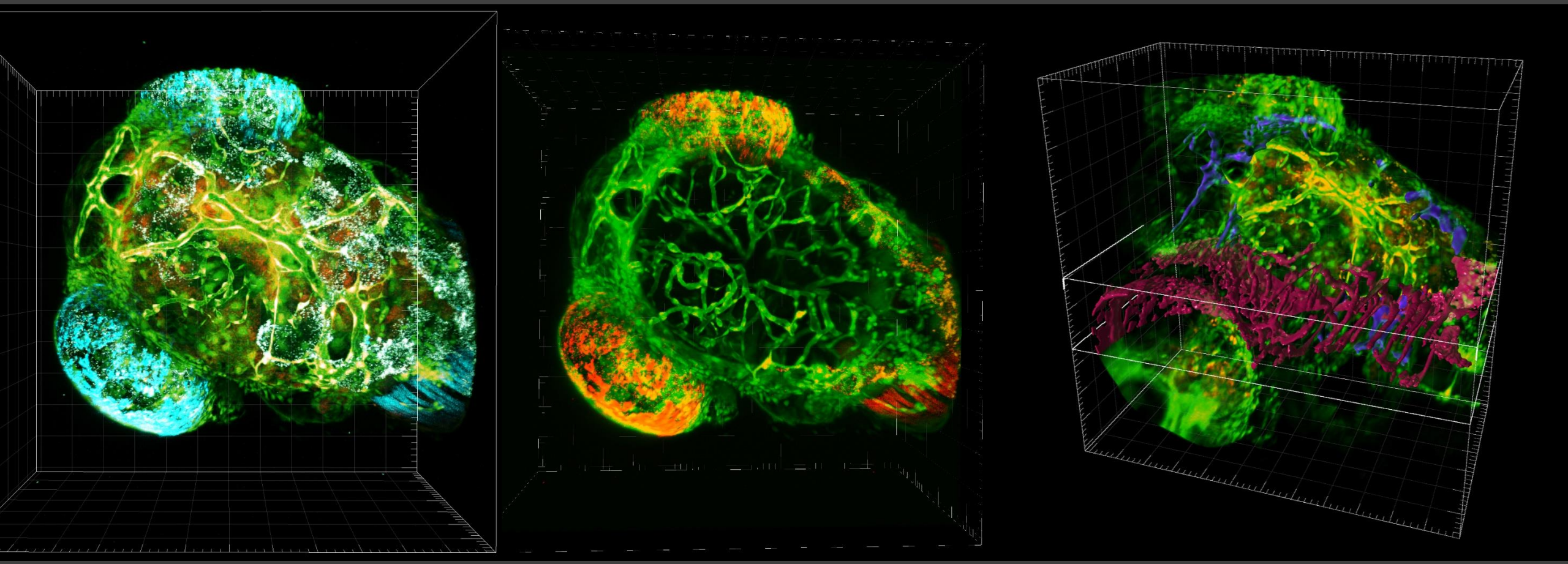
Cut allows for inside view, in case you
Know how to orientate yourself
In your sample
Try 90 onPitch and Yaw *





*

You want great, quick an easy 3D , 4D Reconstruction and image analysis on the fly
→ IMARIS (or AMIRA, which has a steeper learning curve

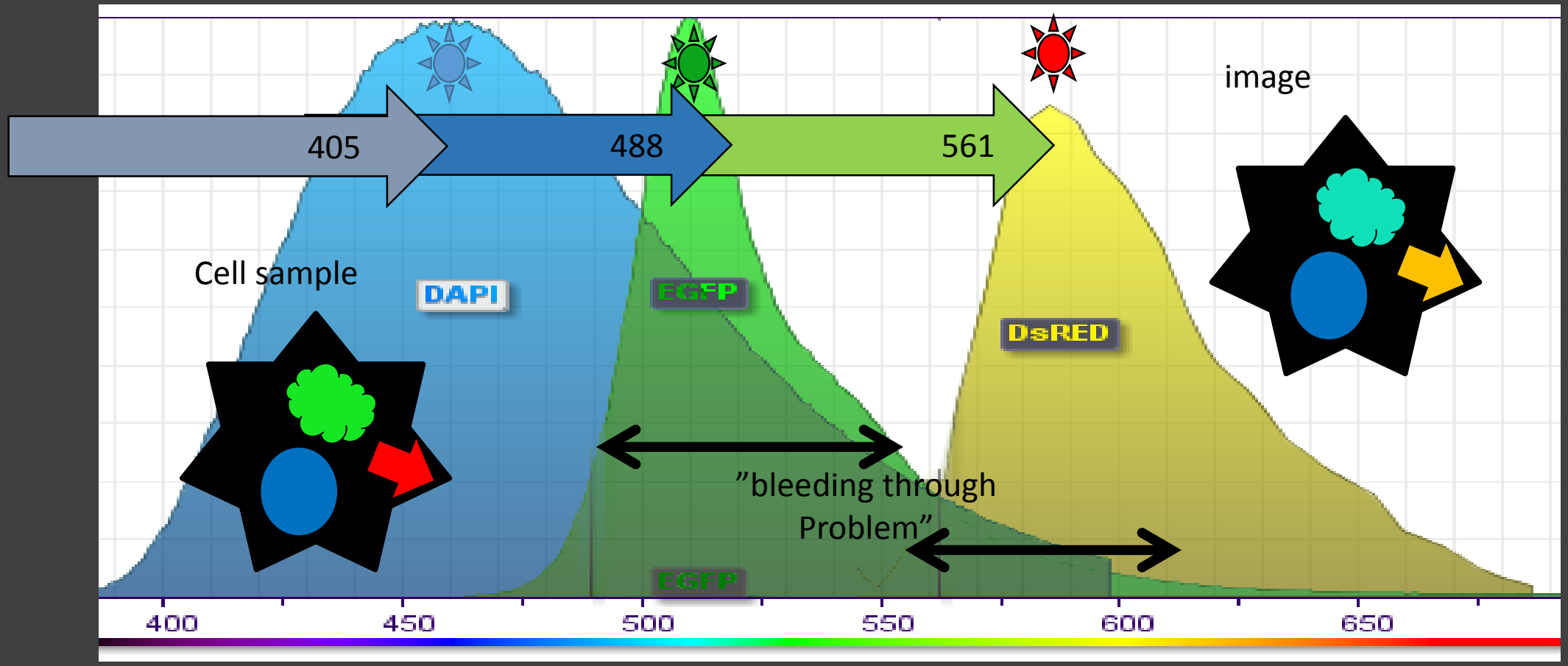


→ December 3, Rudbeck Lab, Fårheus Salen, IMARIS Seminar 09:30 – 11:00 and workshop

In the mix

Linear unmixing

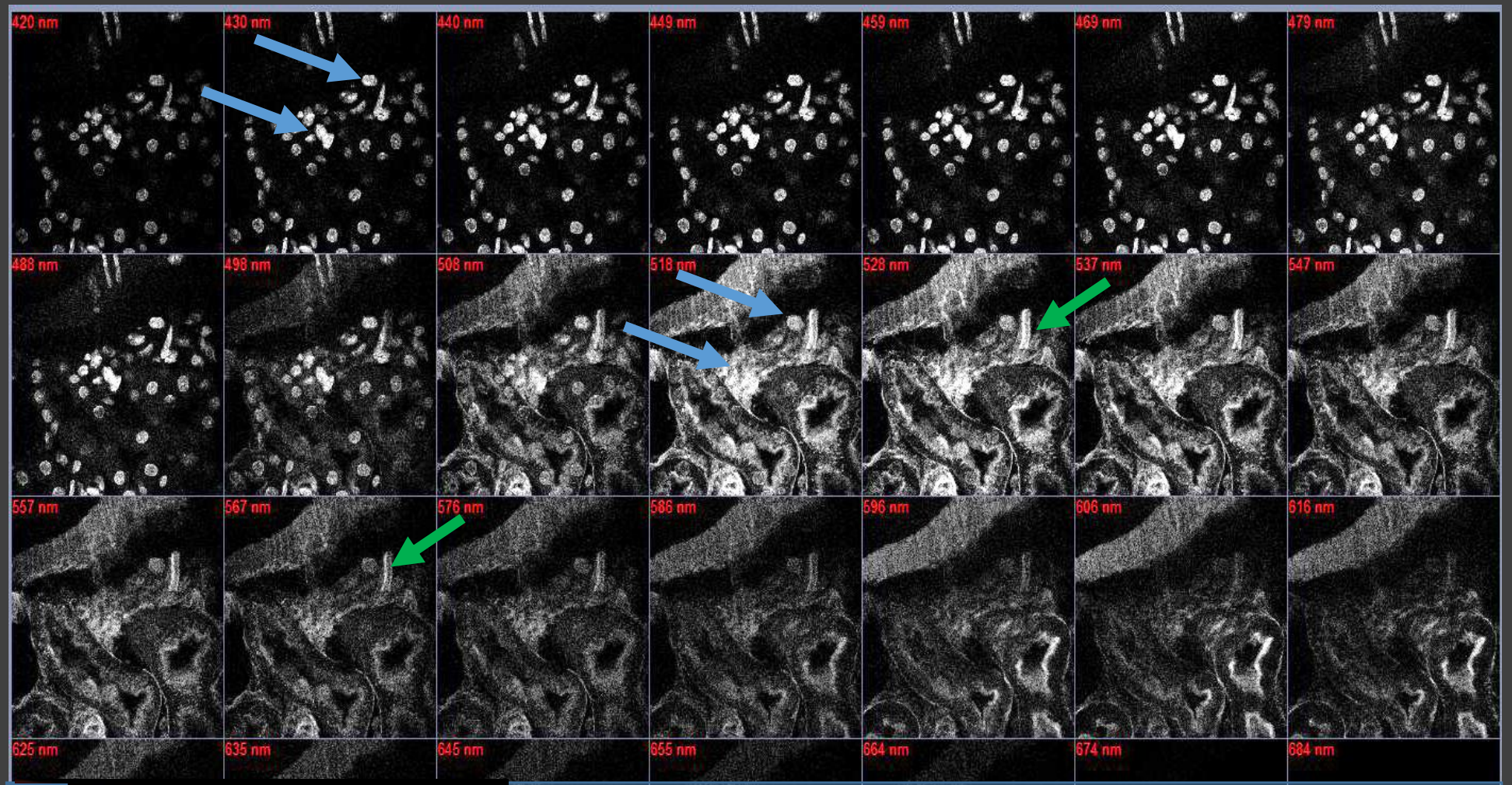
Dealing with fluorescence

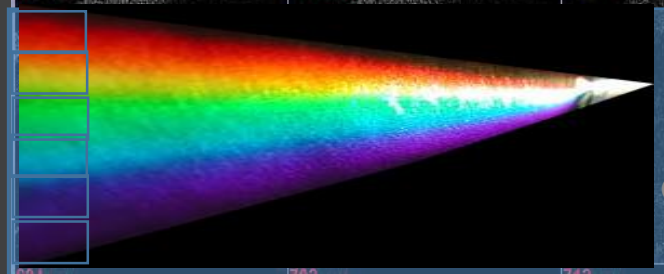


Simultaneous exitation of all three fluorophores or ”unspecific” emission filter setiings might lead to cross excitation and bleeding through Solution : 1. separatable fluorophore combination
2. sequential scan and optimal filter setting
3. ”linear unmixing” for ”Blue/Green/Red”

Lambda Scan with 2PM via QUASAR detector of LSM710

Exitation
405
488
633



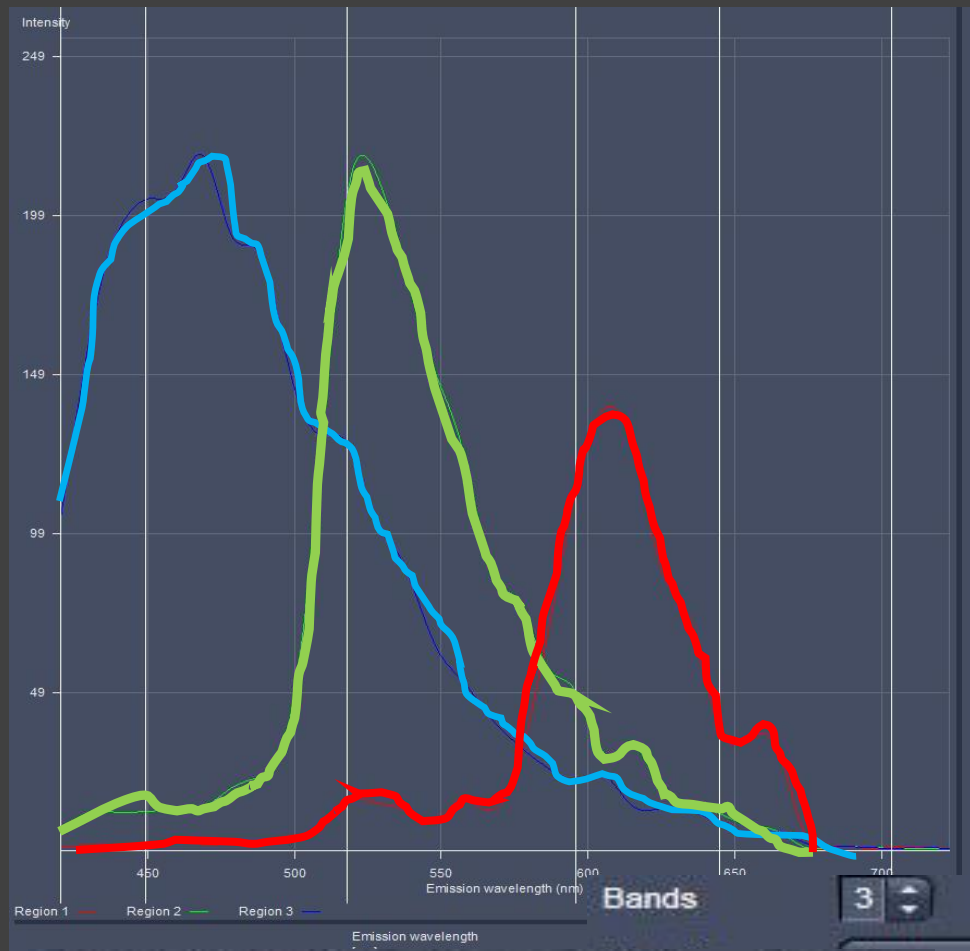


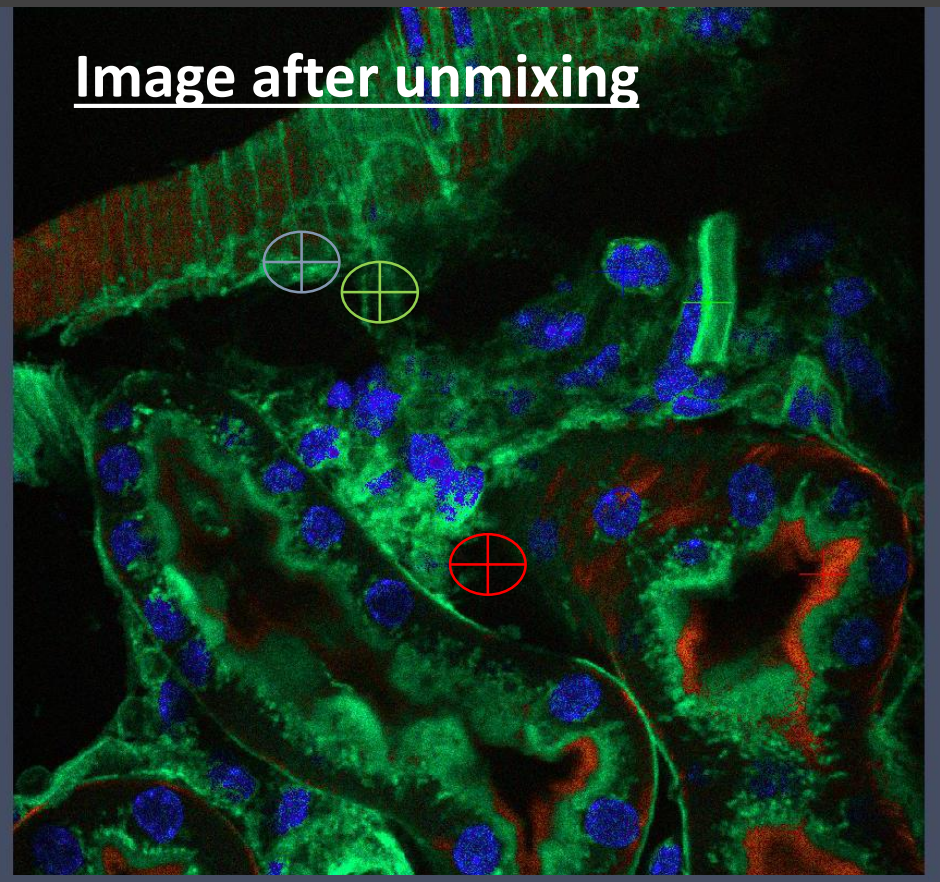
- Optical grid disperses incoming light into wavelength
-QUASAR has 32 highly sensitive (GaAsP) detectors
- Each single QUASAR-detector collects ”its range”.

Lambda Scan with 2PM via QUASAR detector of LSM710

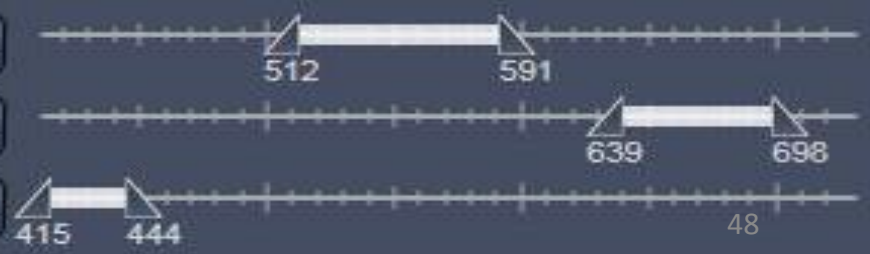
Linear Unmixing

determines relative
contribution
of each fluorophore
for every pixel
of the image.
recalculates
image for
fluorophores used





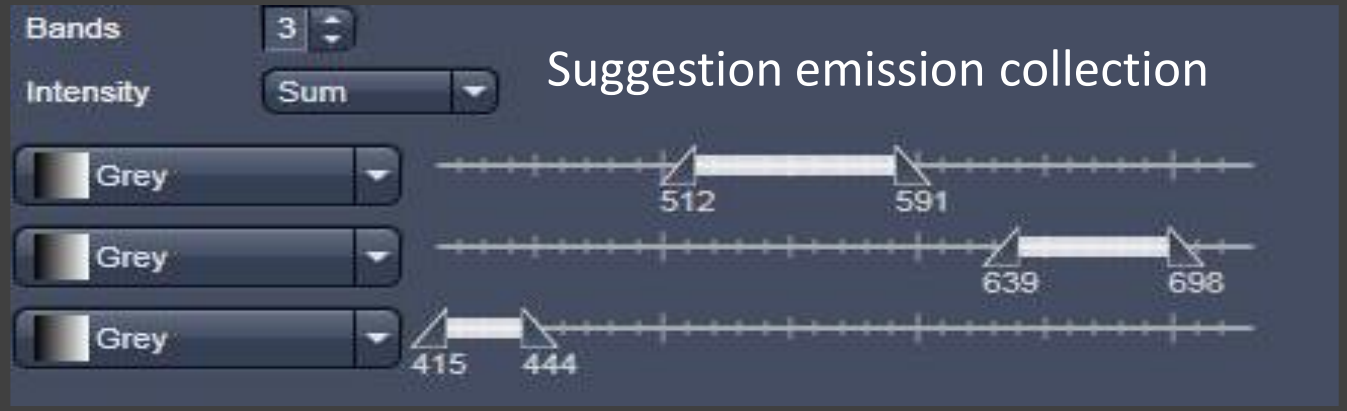
Suggestion emission collection

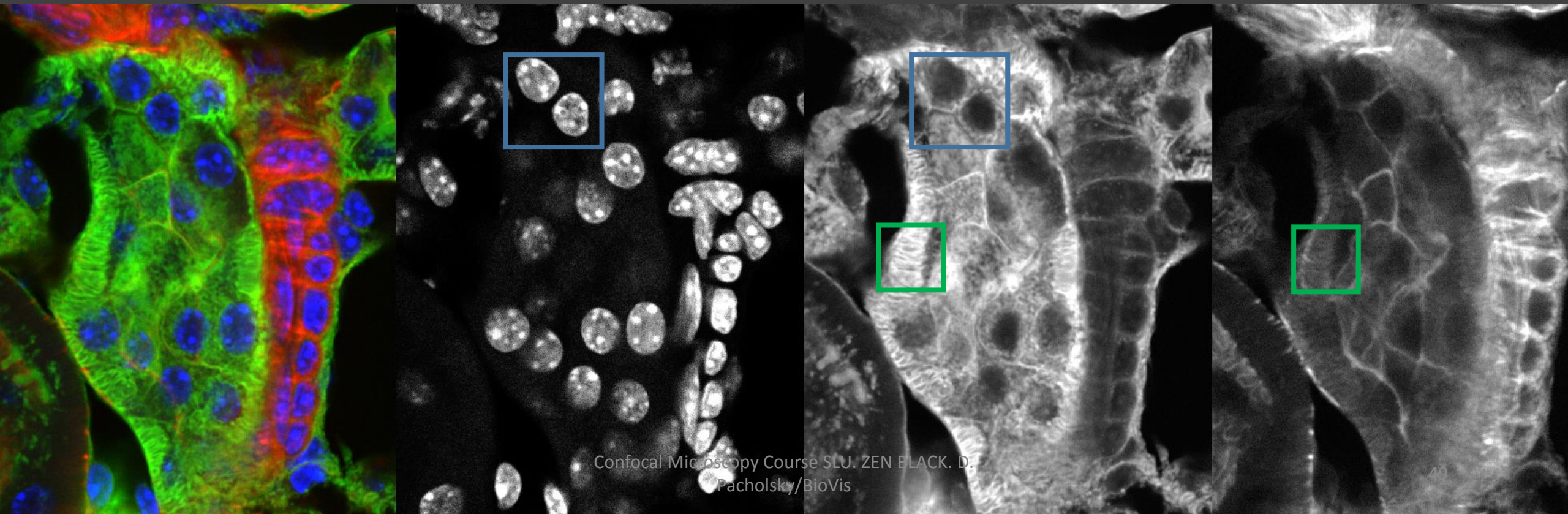


Lambda Scan with 2PM/LSM – linear unmixing

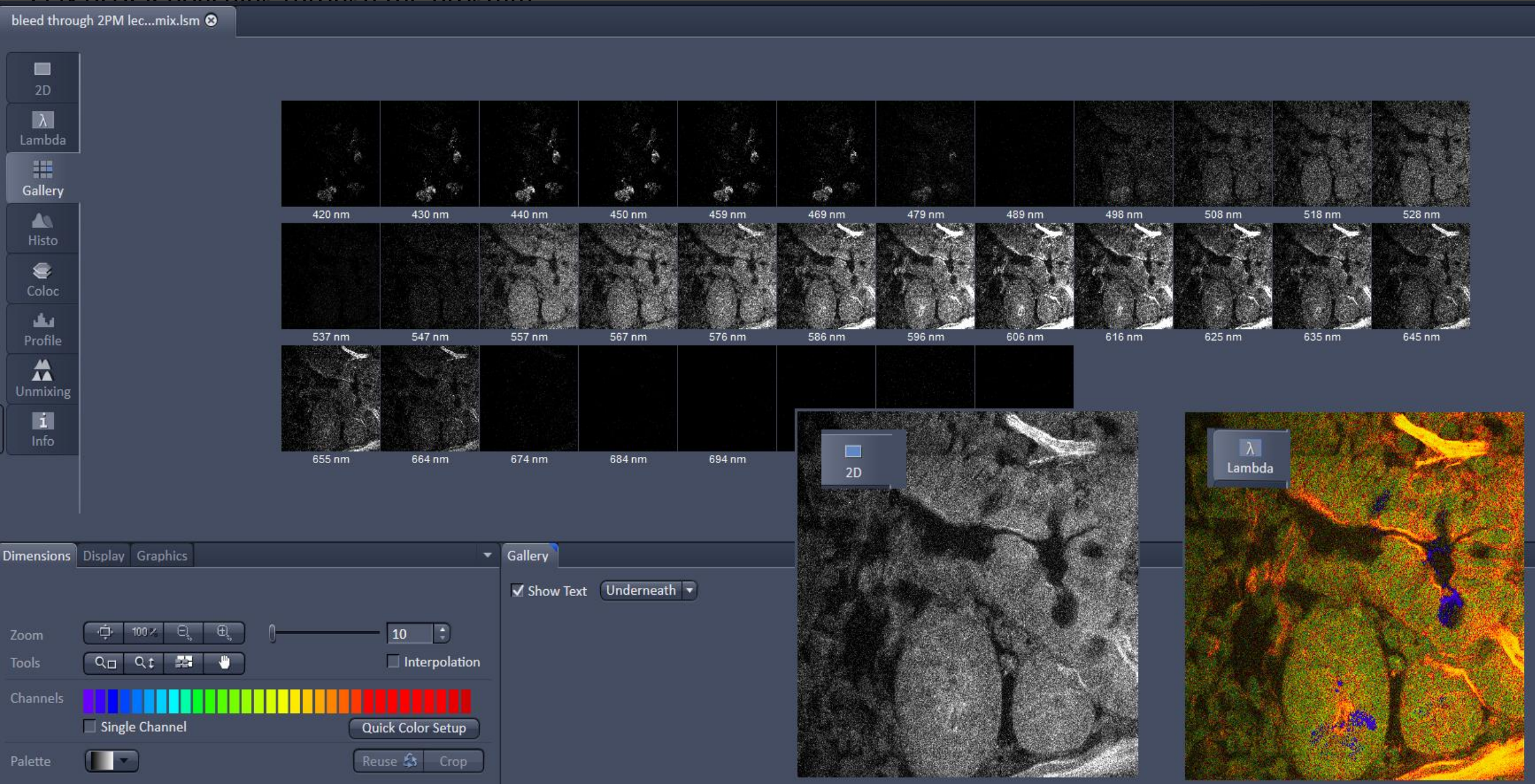
2PM Excitation 800nm

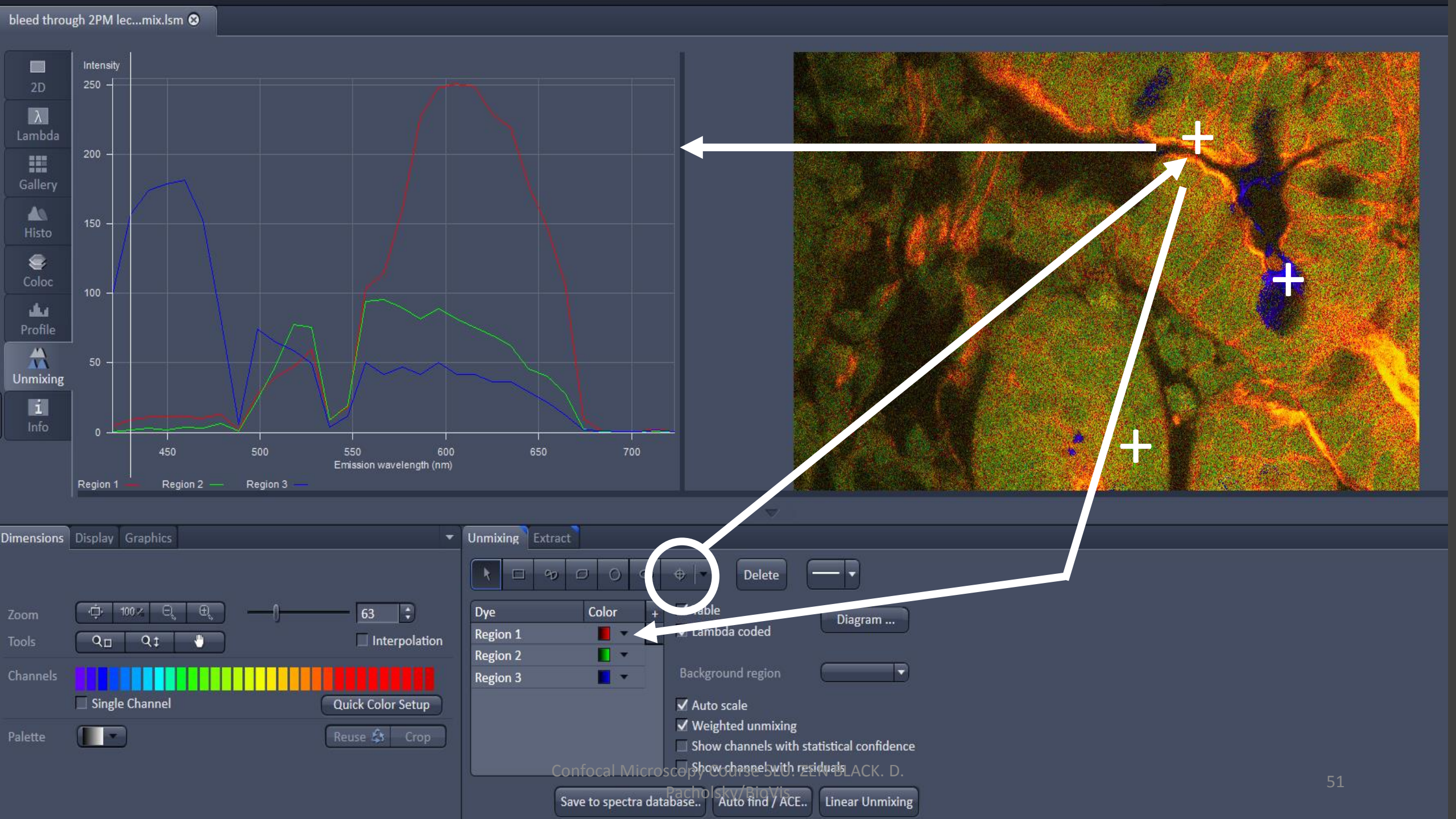
Image over-contrasted for viewing purpose





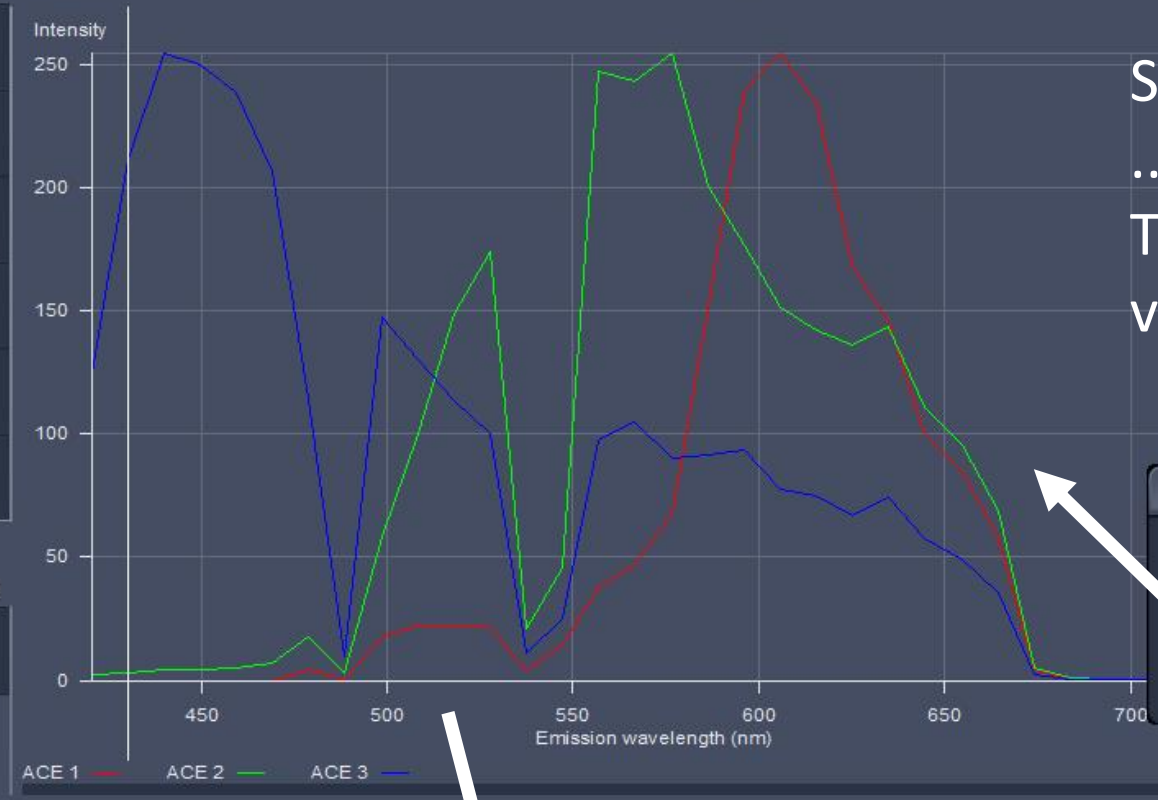
ZEN BLACK poaching through the program



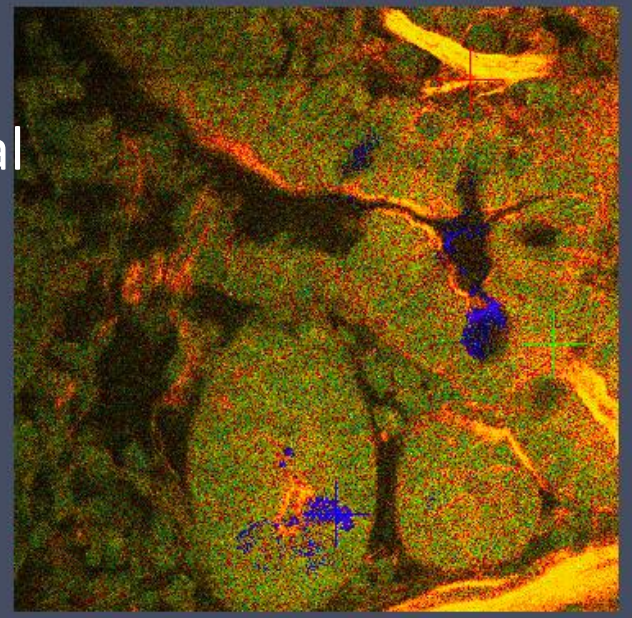

bleed through 2PM lec...mix.lsm
2D
Lambda
Gallery
Histo
Coloc
Profile
Unmixing
Info
Intensity
250
200
150
100
50
0
450
500
550
600
650
700
Emission wavelength (nm)
Region 1
Region 2
Region 3
Dimensions
Display
Graphics
Zoom
100%
63
Tools
Interpolation
Channels
Single Channel
Quick Color Setup
Palette
Reuse
Crop
Unmixing
Extract
Delete
Dye
Color
Region 1
Region 2
Region 3
Lambda coded
Diagram ...
Background region
Auto scale
Weighted unmixing
Show channels with statistical confidence
Show channel with residuals
Save to spectra database..
Auto find / ACE..
Linear Unmixing

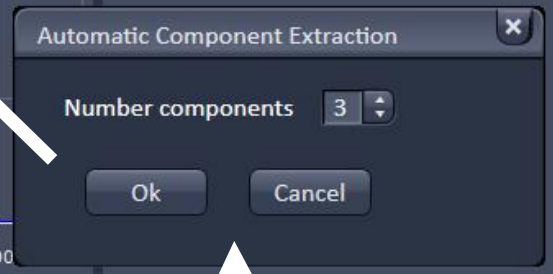
bleed through 2PM lec...mix.lsm | bleed through 2PM lec...nmixing

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



Sit back relax
... And compare
The spectra made manual versus automated





Dimensions | Display | Graphics

Zoom | 100% | 30 | Interpolation

Tools

Channels | Single Channel | Quick Color Setup

Palette | Reuse | Crop

Unmixing | Extract

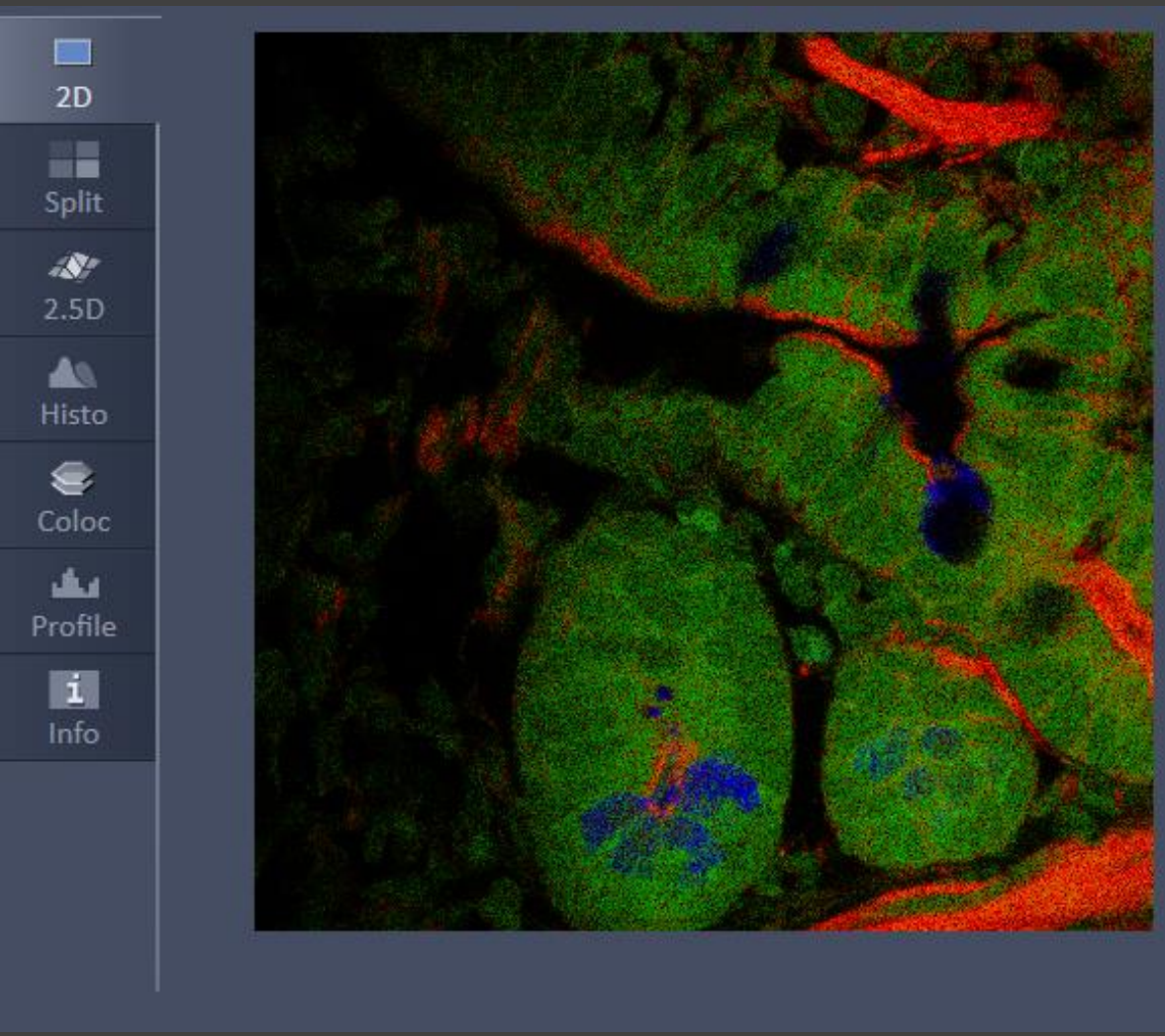
Delete

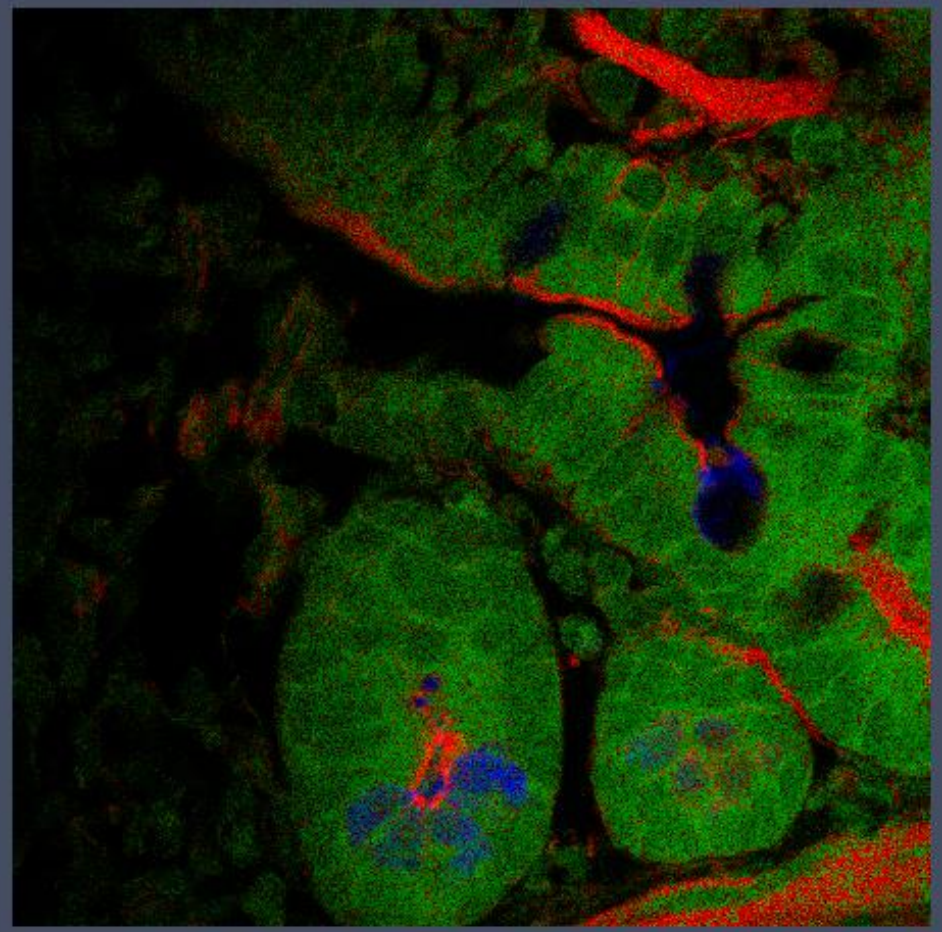
Dye	Color
ACE 1	
ACE 2	
ACE 3	

Table
Lambda coded
Diagram ...
Background region
Auto scale
Weighted unmixing
Show channels with statistical confidence
Show channel with residuals

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

ZEN BLACK poaching through the program





Automated

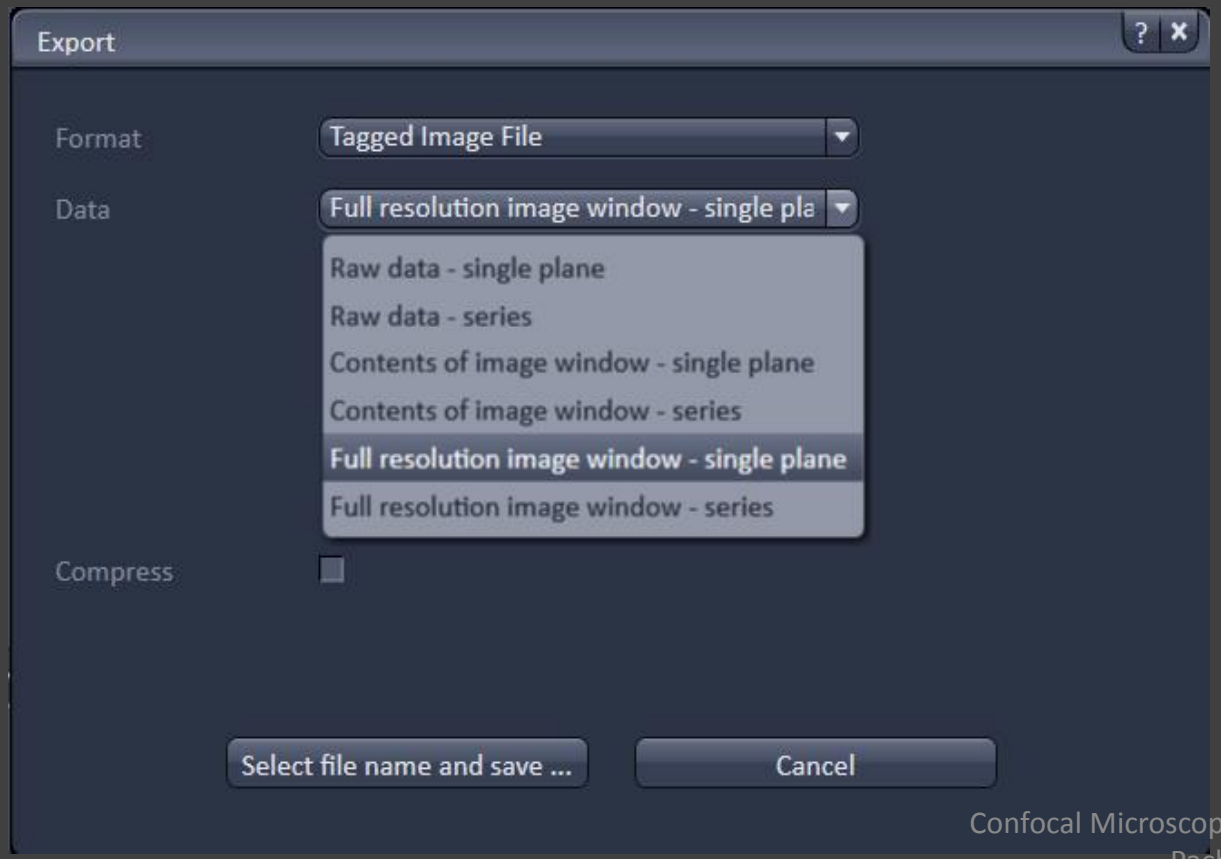
Manual

Exit strategy

Options for exporting data

Options on Exporting data

- 1. full resolution (→ full resolution with graphics)
- 2. raw data (→ full resolution , no graphic)
- 3. contents of image window (as you see it on screen size)
- export options general:
  - → A) file format
  - → B) single plane (one image out of a stack)
  - → C) series (all images from e.g. stack)



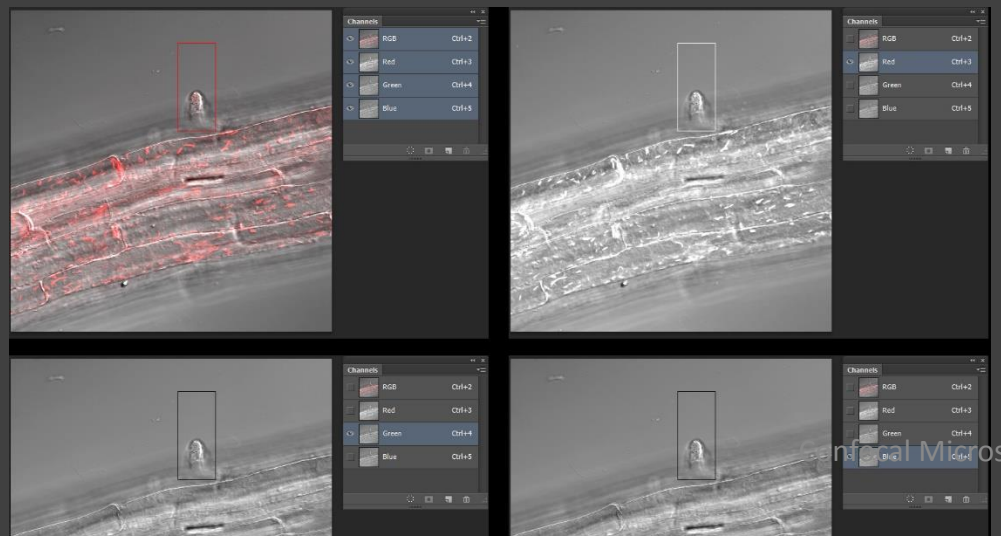
- specific options for 1.2.3. see slides
- “specific option” for stack/series:
  - open stack in ZEN as gallery view
  - export as gallery by using 1.2.3.
  - in combination with B

ZEN BLACK poaching through the program

full resolution

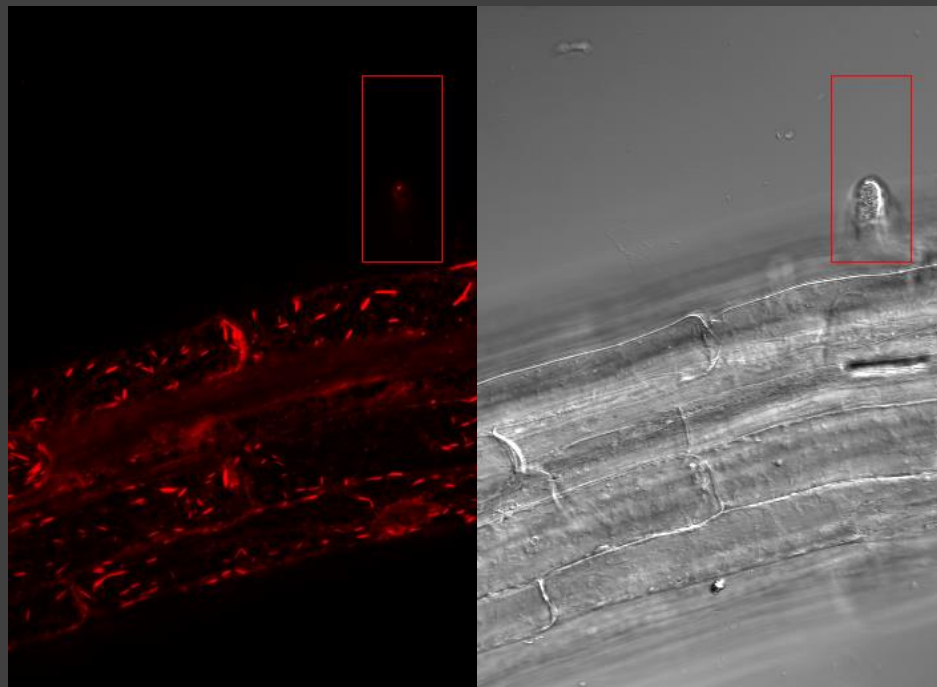
→ Exports image as seen - in full resolution
→ in file format you choose
→ as RGB (RED GREEN BLUE channel)
→ any graphics will be exported (measure bar)

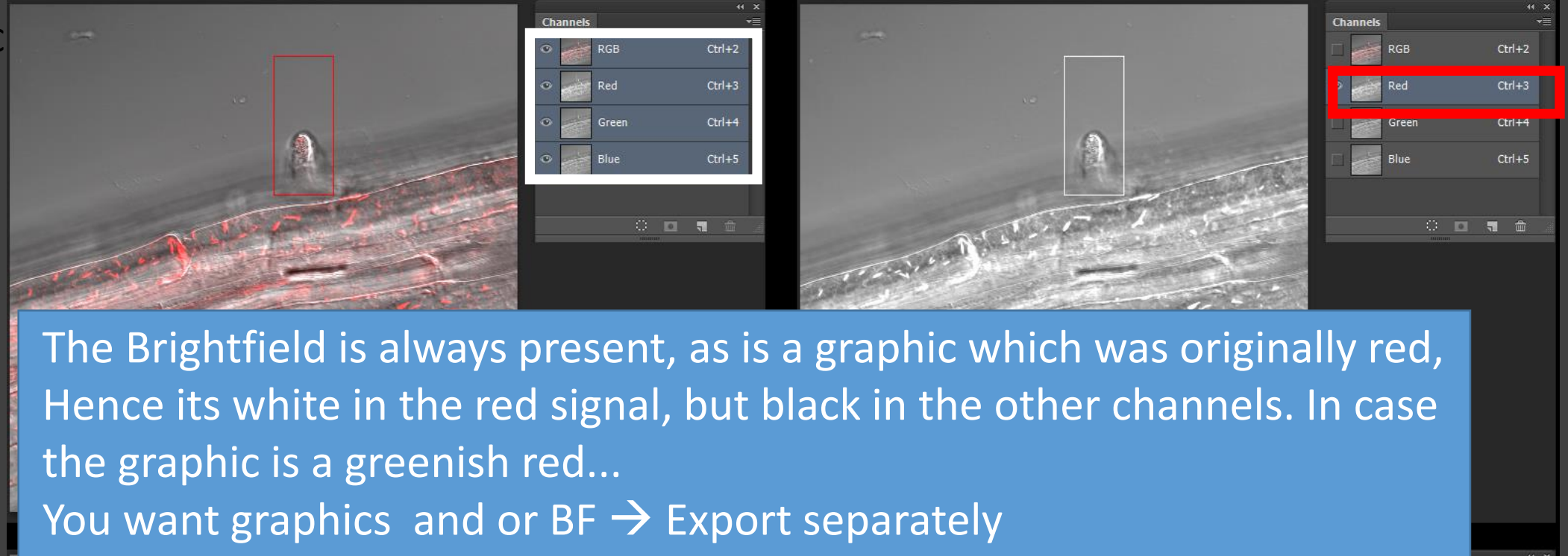
→ Problem: more then 3 colors (4th color appears in all channels of RGB
→ problem: BF is overlaid to all RGB channels
→ any graphics appears in all RGB channels



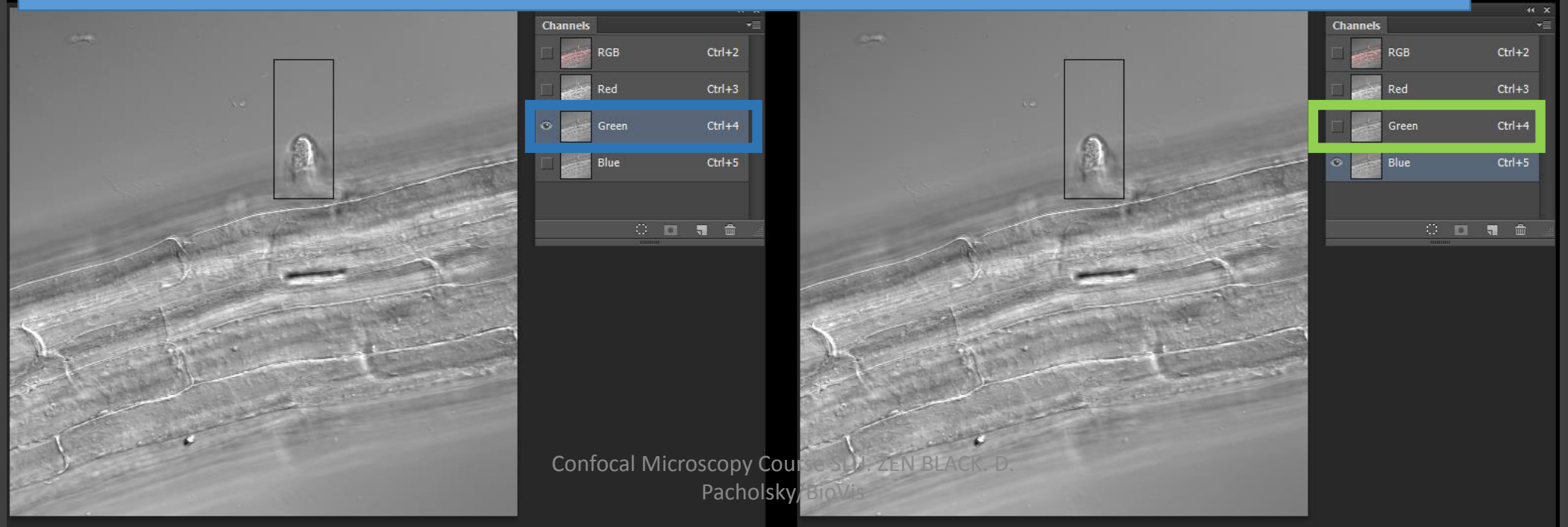
Solution to the problems:
Join 3 colors as RGB on screen in ZEN
Take away grphics
Export as RGB
take image with graphics, 4th color or BF
Export it as RGB

Work on in Photshop in combining or
Cut/paste measure bar





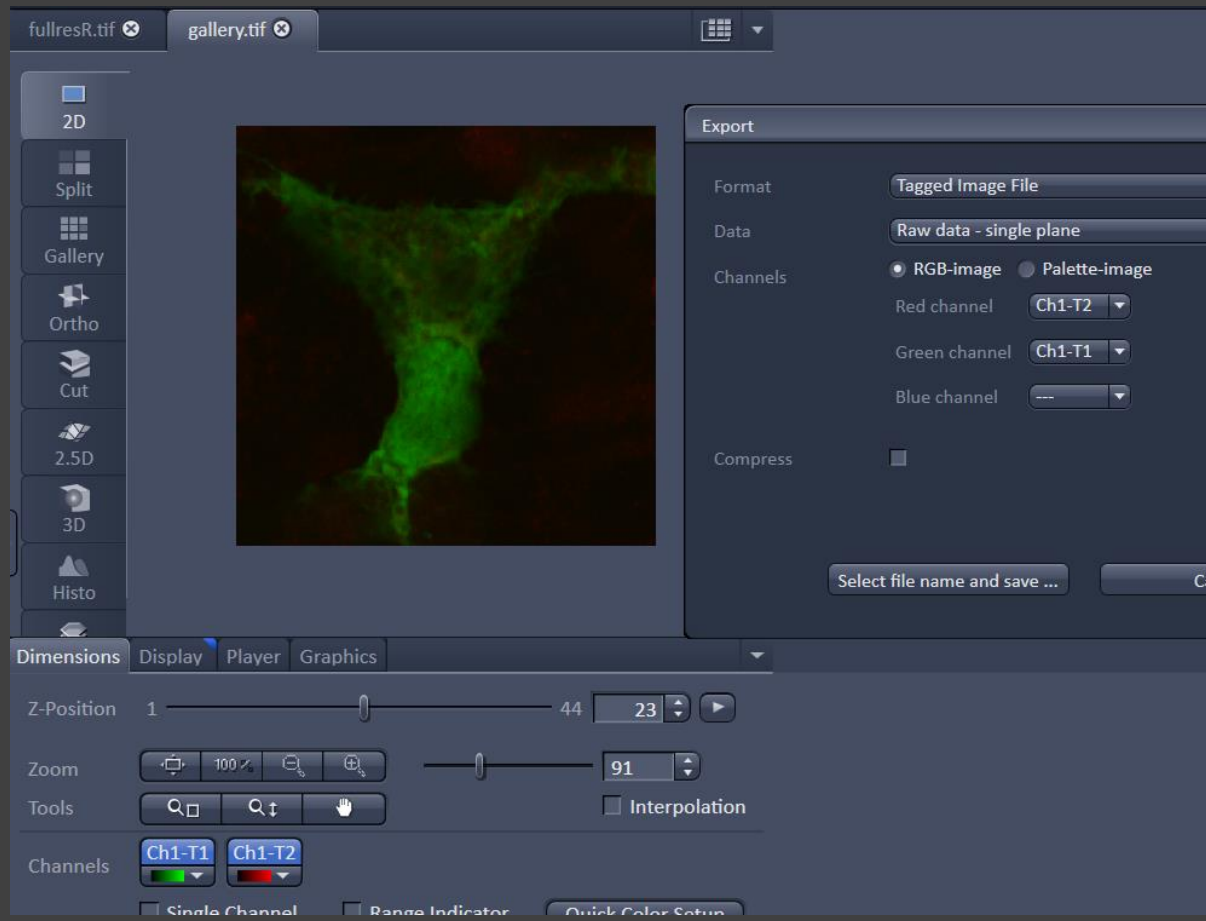
The Brightfield is always present, as is a graphic which was originally red, Hence its white in the red signal, but black in the other channels. In case the graphic is a greenish red...
You want graphics and or BF → Export separately



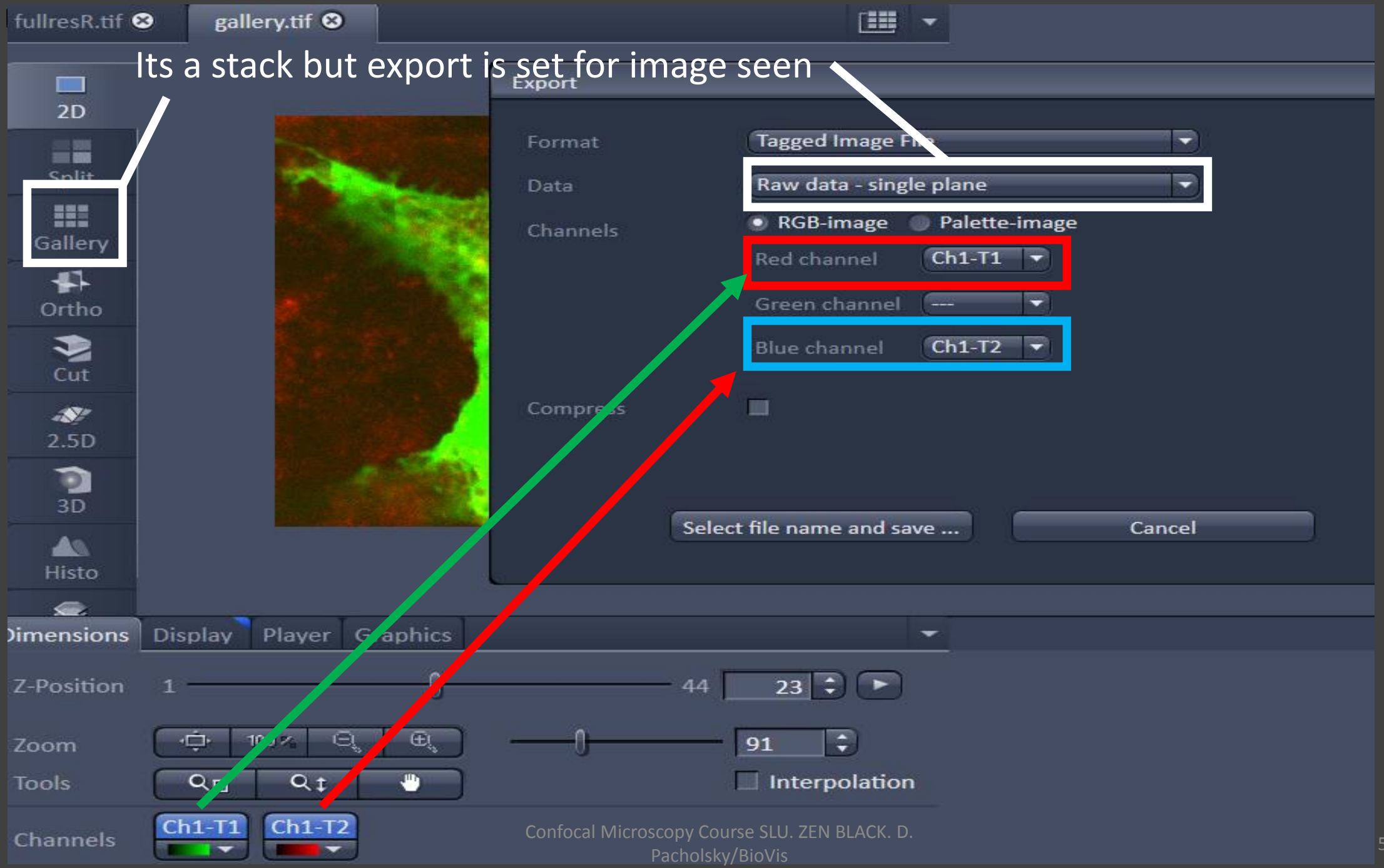
RAW DATA

→ Exports image as seen - in full resolution
→ in file format you choose
→ OPTION : choose which ZEN channel
is on which RGB channel
→ NO graphics will be exported (measure bar)

→ Problem: only 3 colors get exported per export
(RGB)



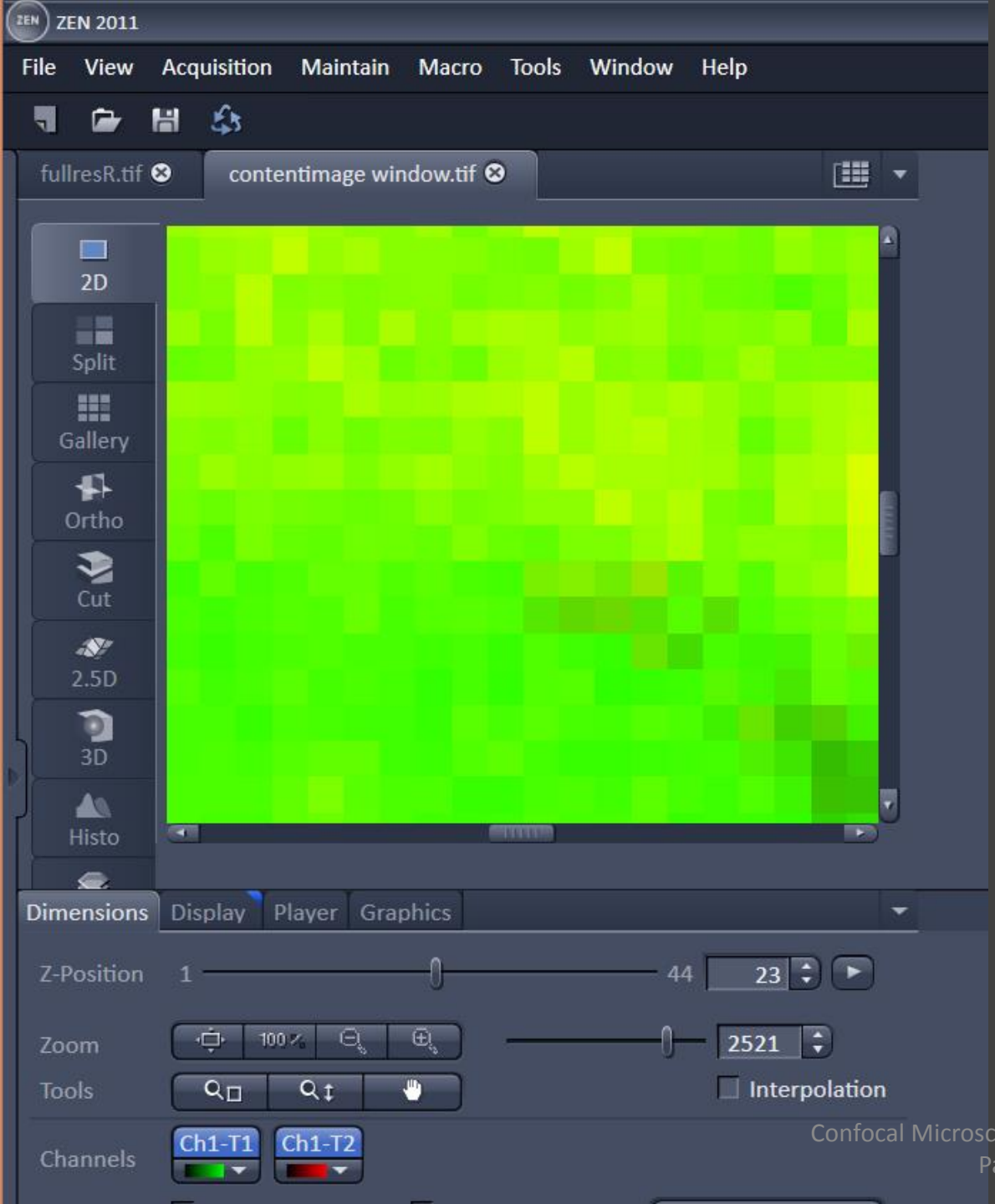
Solution to the problems:
Choose the ZEN channels for RGB export
any more color export /separate/combined
For measure bar export choose full resolution export

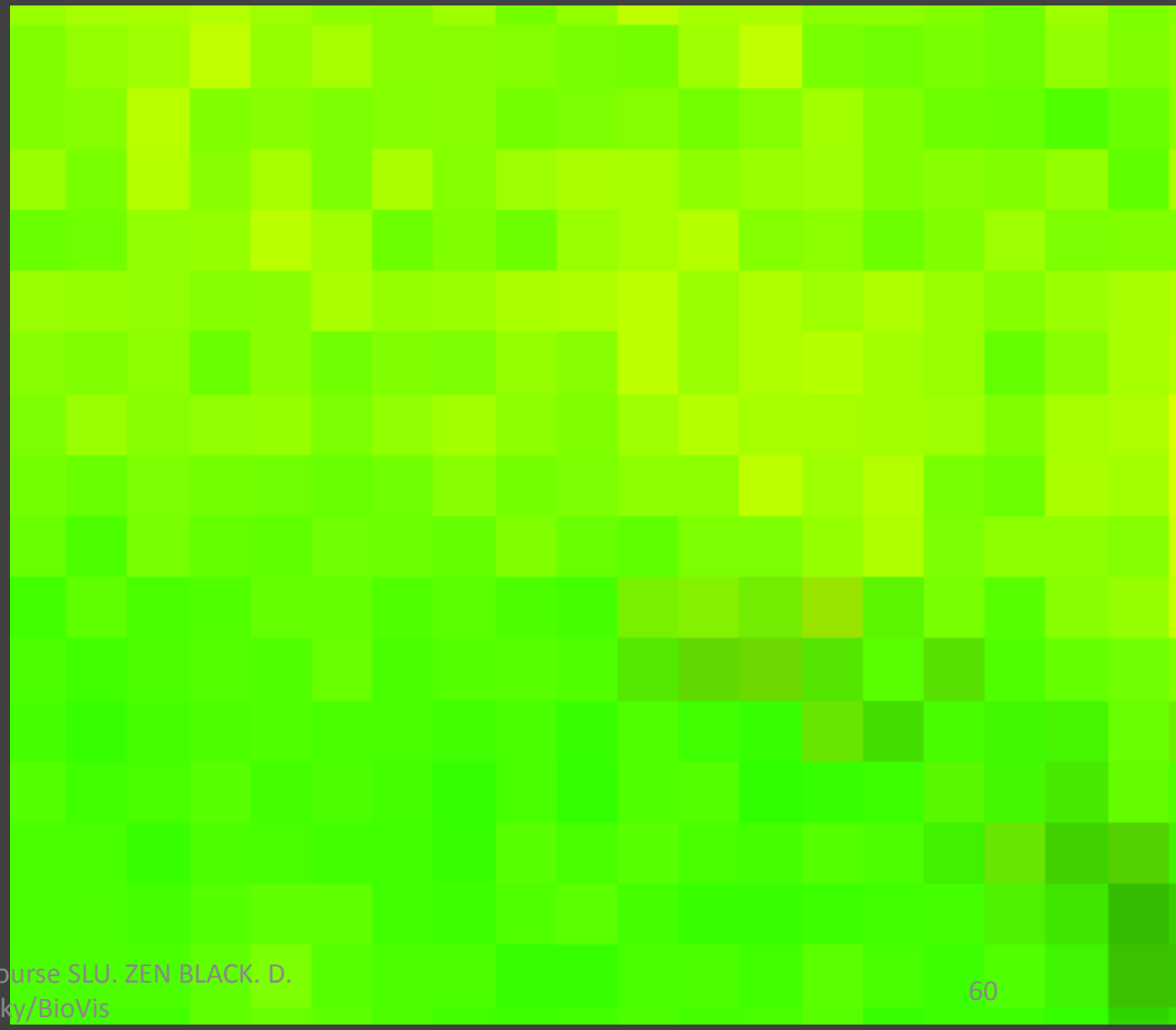

fullresR.tif
gallery.tif
Its a stack but export is set for image seen
2D
Split
Gallery
Ortho
Cut
2.5D
3D
Histo
Export
Format
Tagged Image File
Data
Raw data - single plane
Channels
RGB-image
Palette-image
Red channel
Ch1-T1
Green channel

Blue channel
Ch1-T2
Compress
Select file name and save ...
Cancel
Dimensions
Display
Player
Graphics
Z-Position
1
44
23
Zoom
91
Tools
Interpolation
Channels
Ch1-T1
Ch1-T2

Image window content

→ Exports image as seen

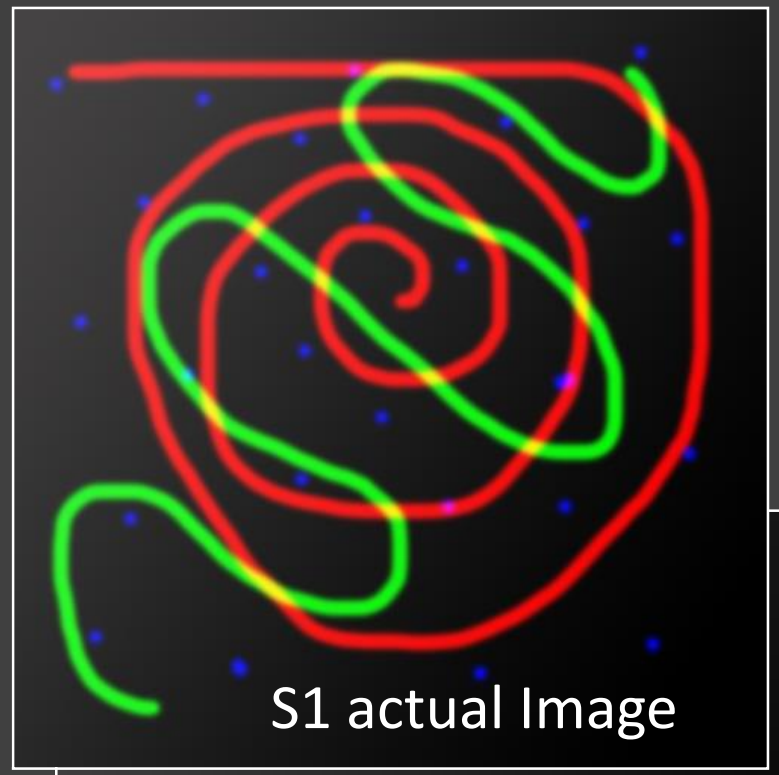




The process against light

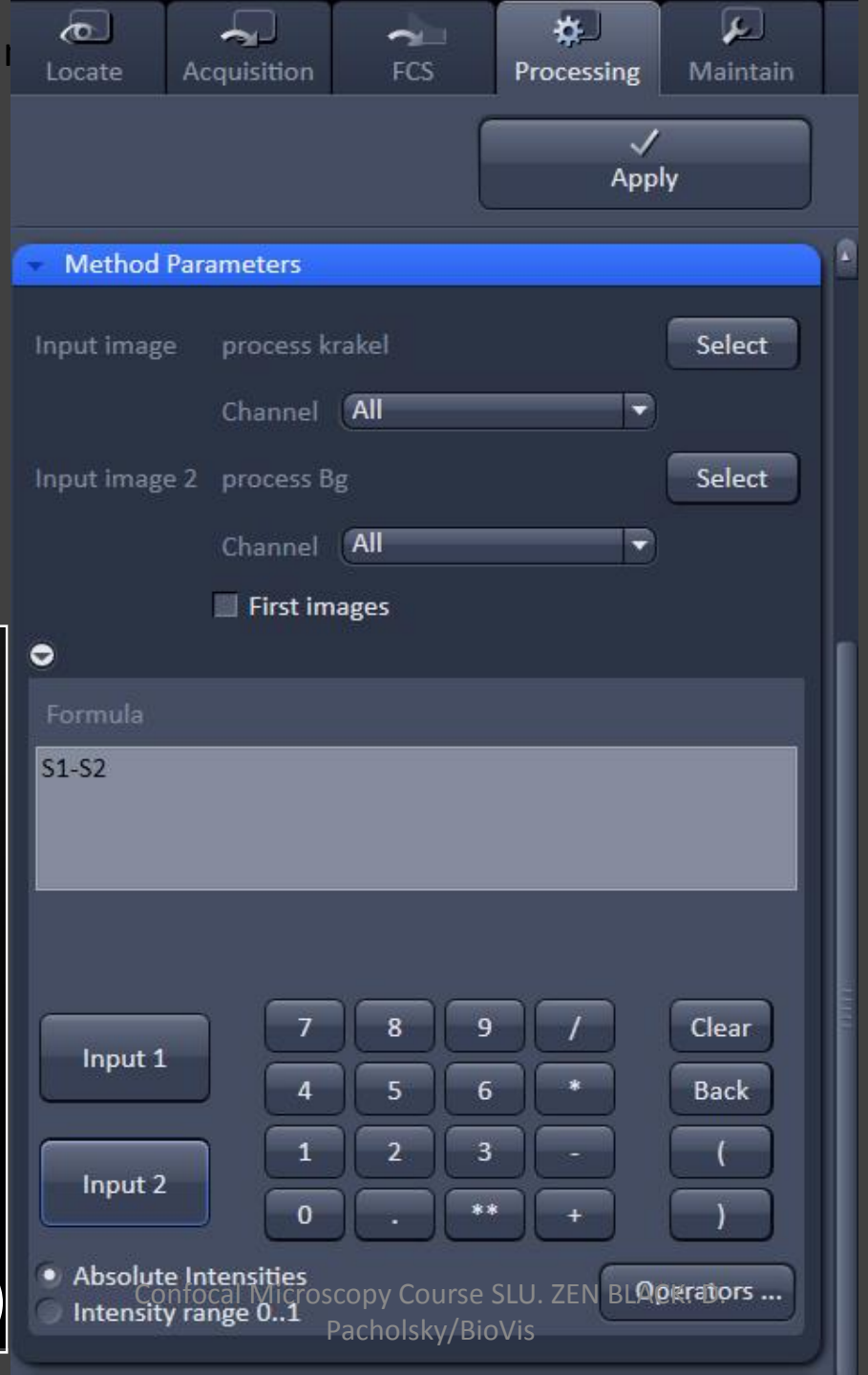
processing

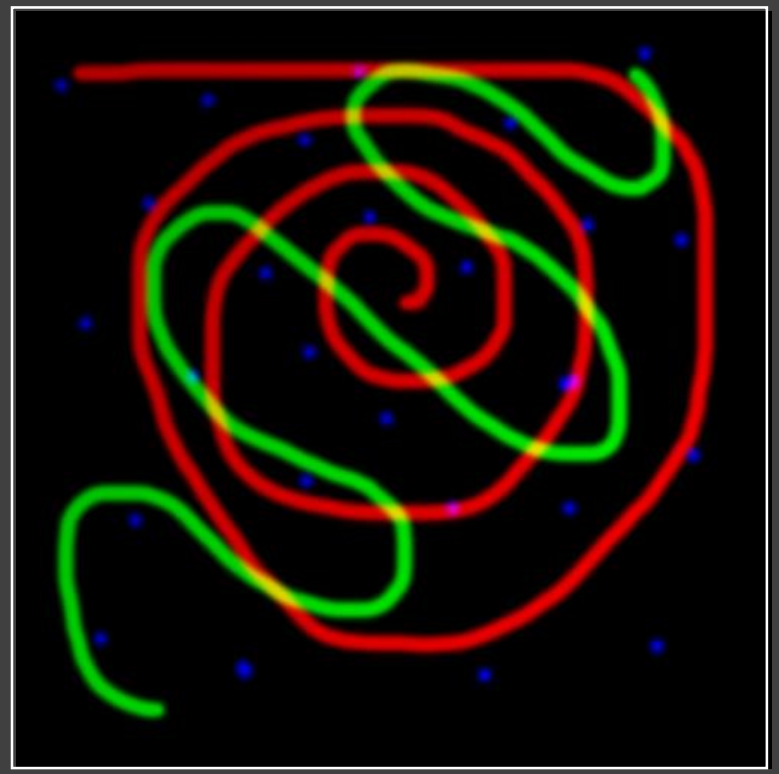
ZEN BLACK poaching through the progra



S1 actual Image

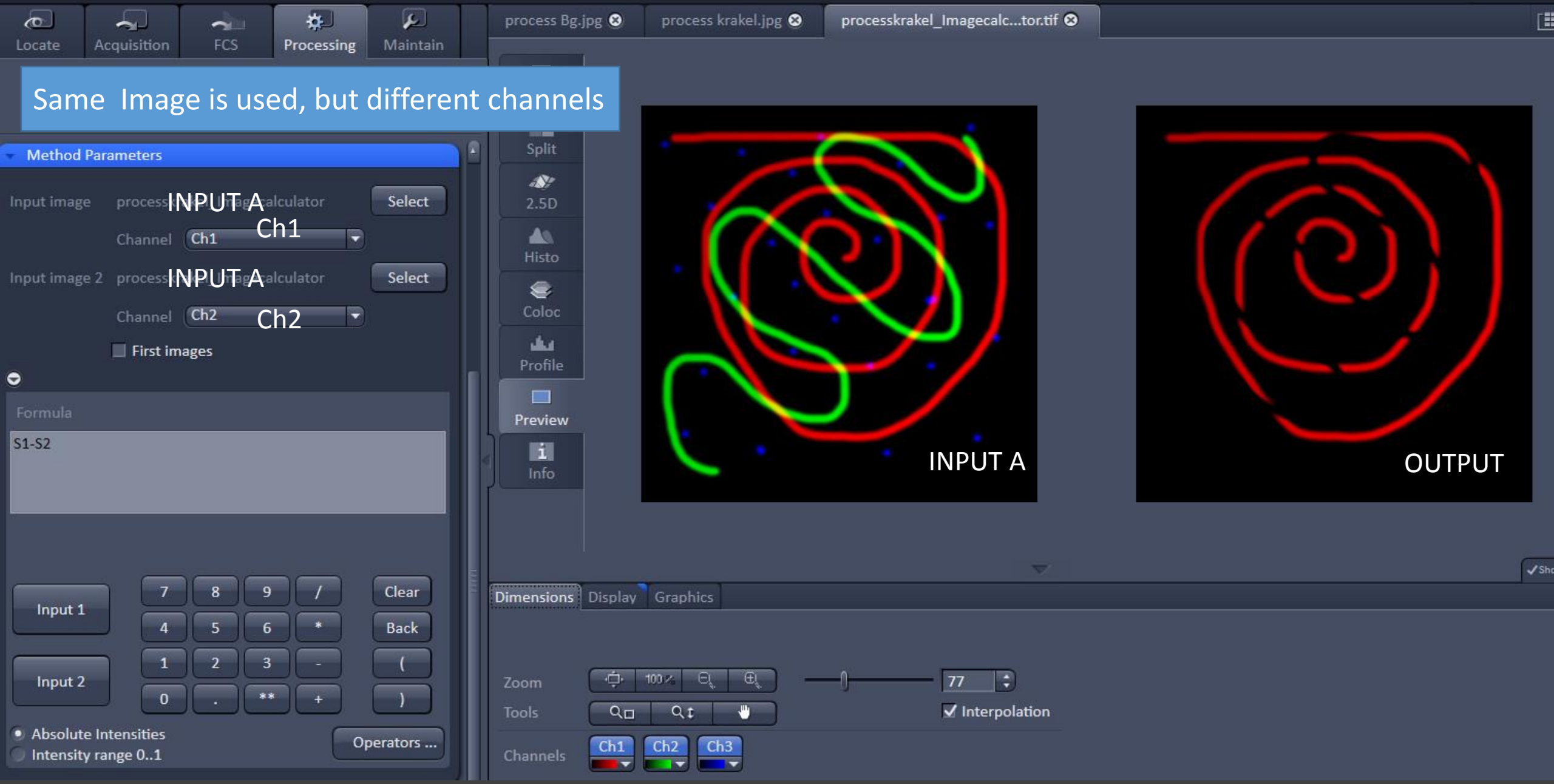
S2 background (all channels)





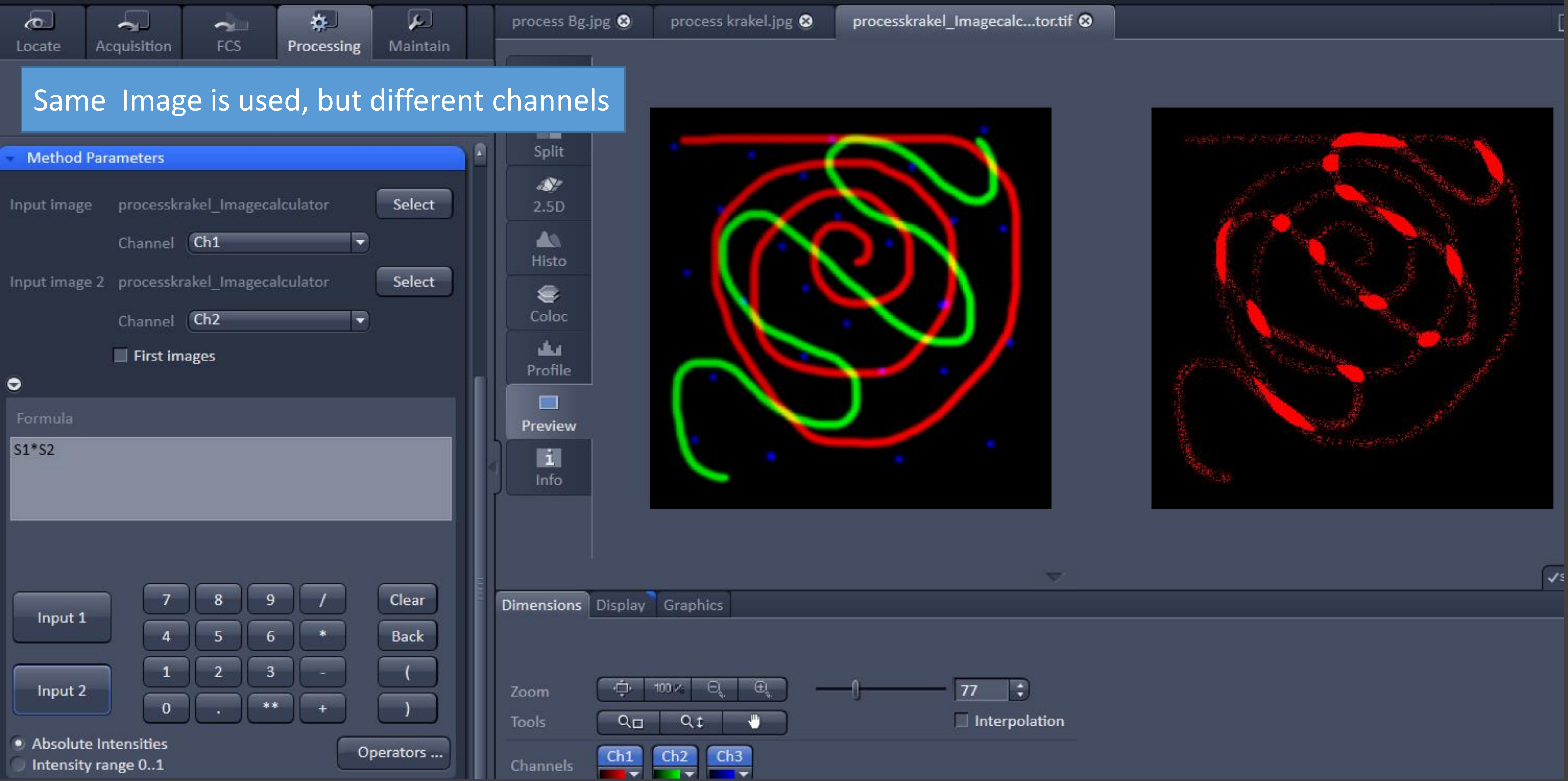
S1-S2 removes background

Same Image is used, but different channels

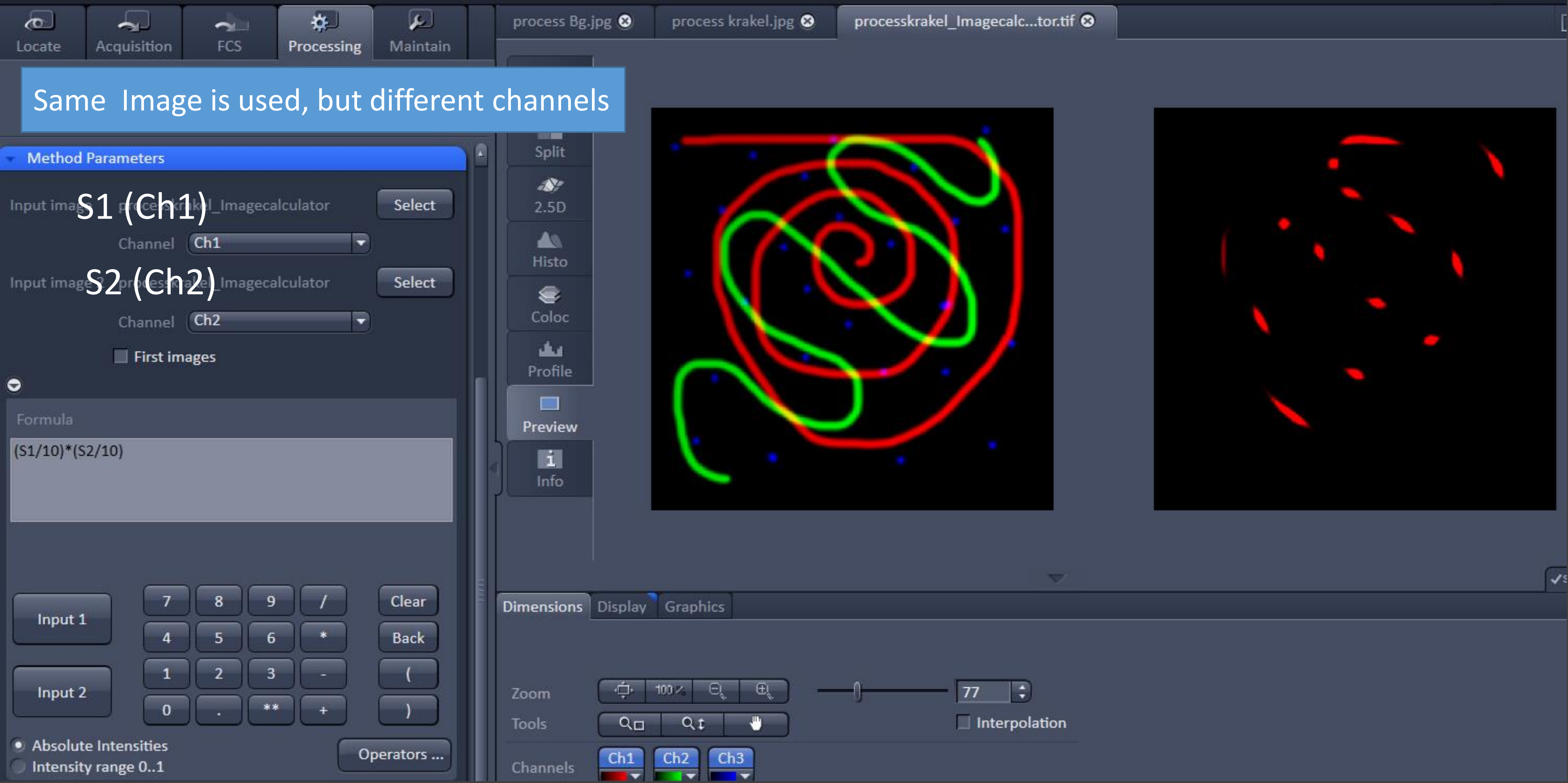


S1 (Ch1) - S2 (Ch2): how many red sections are made by green onto red

Same Image is used, but different channels

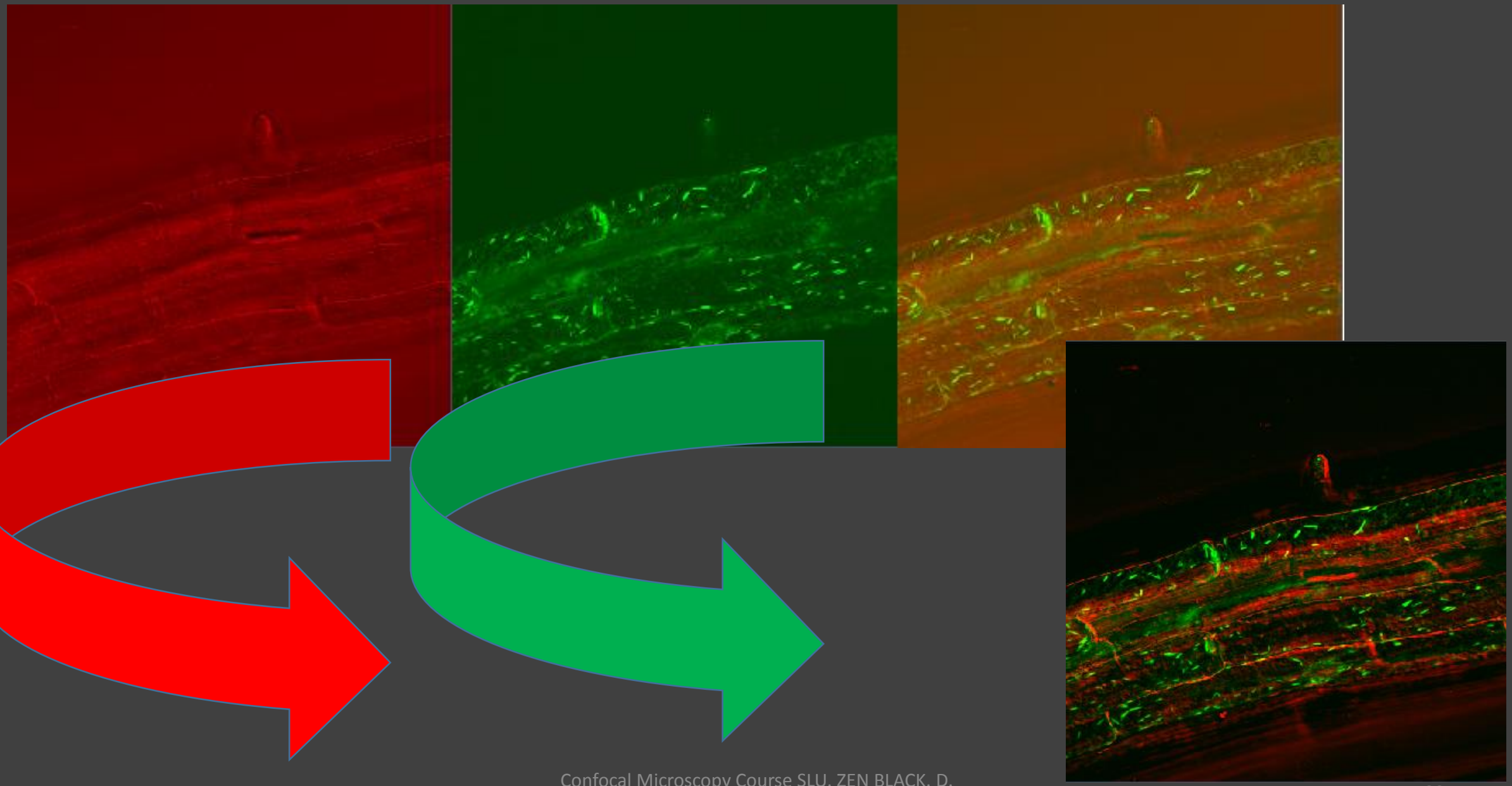


S1 (Ch1) * S2 (Ch2): how contact points have green and red?, needs to be refined...



(S1 /10) * (S2/10): how contact points have green and red?, REFINED

ZEN BLACK poaching through the program



S1

S2

Method Parameters

Input image GFP tile scan_m08 Select

Channel Ch1

Input image 2 GFP tile scan_m12

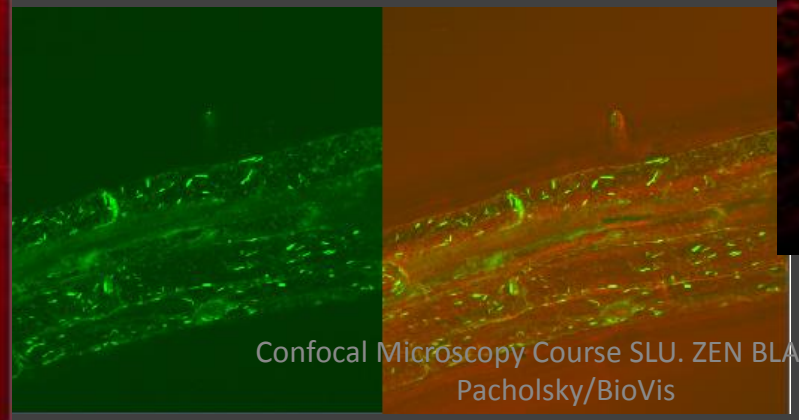
Channel Ch1

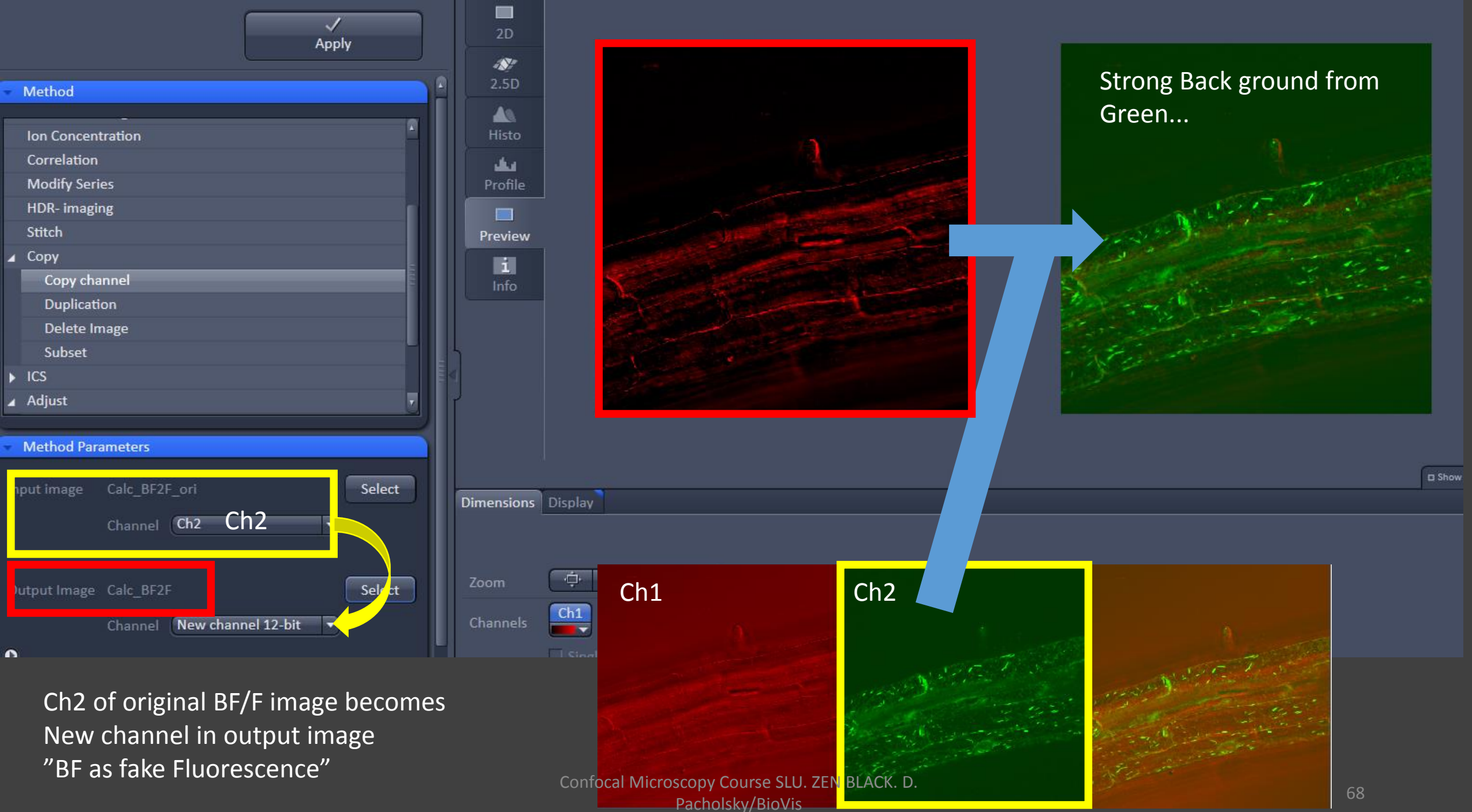
First images

Formula

S2-S1

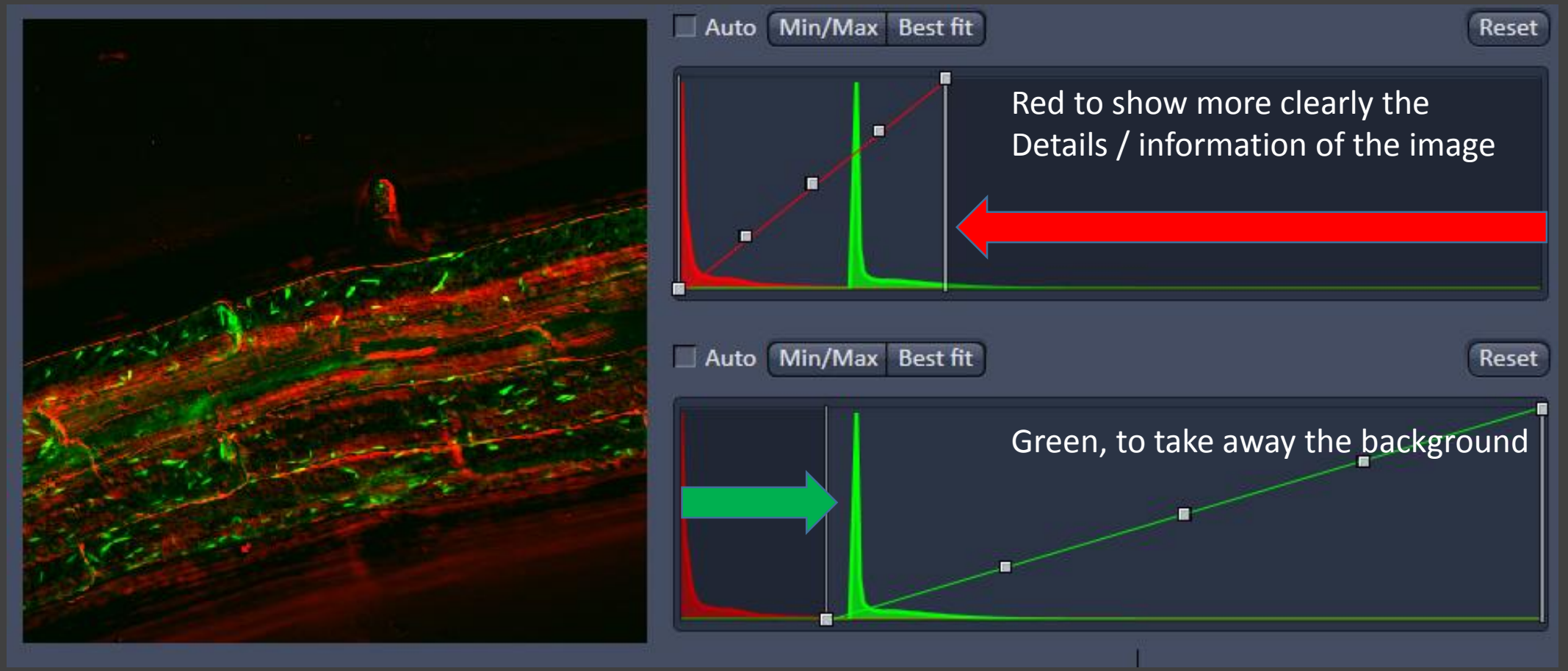
New generated image,
”fake FL of BF”
to be combined with the green channel





Ch2 of original BF/F image becomes
New channel in output image
”BF as fake Fluorescence”

To enhance the image (always state if doing so) the histogram was stretched

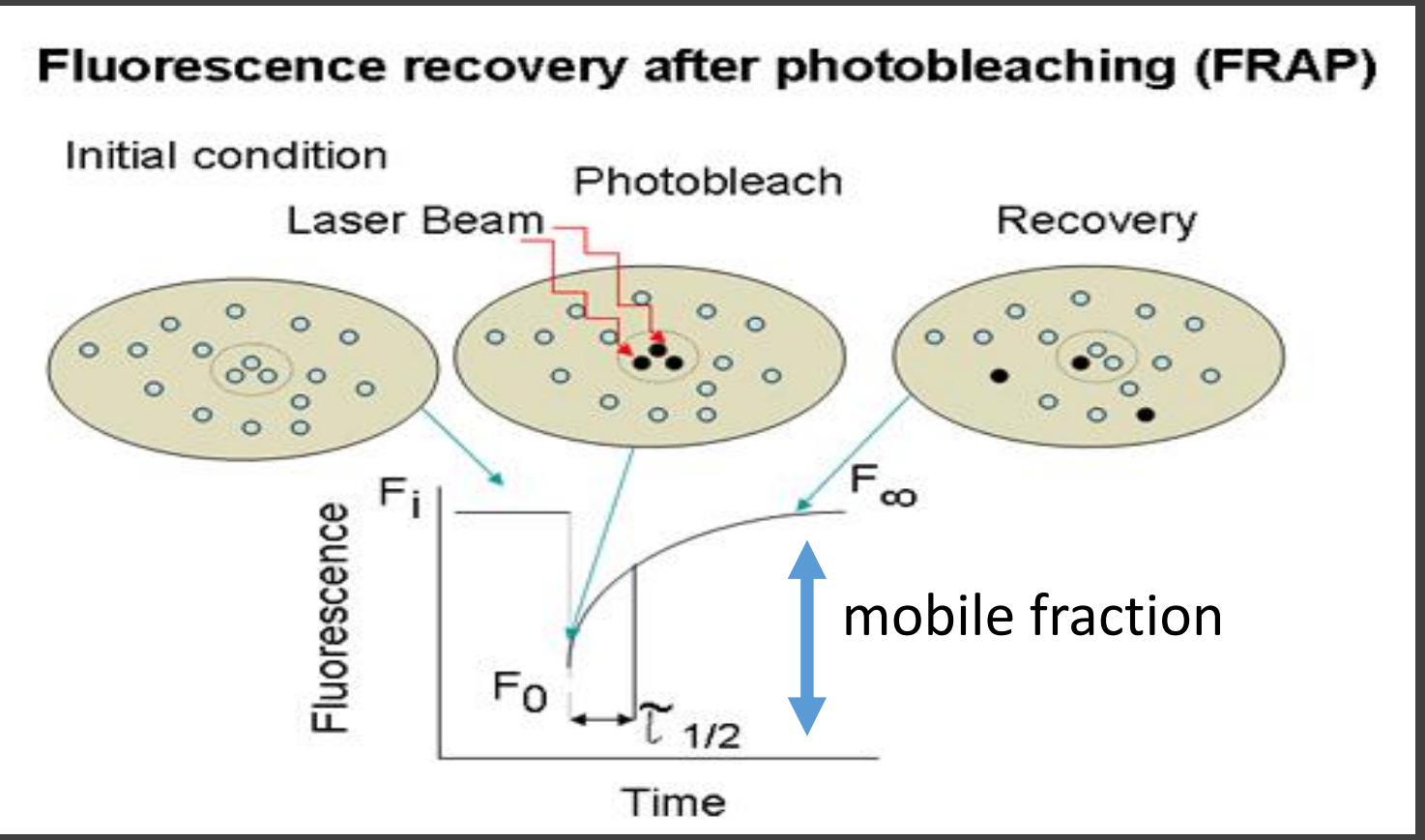


Don´t be rude

The F´Word techniques

FRAP, iFRAP, FLIP, FLAP, FRET, FLIM, ”F”otoactivation/inactication, FCS

All techniques beside FRET are Live Cell imaging techniques



FRAP

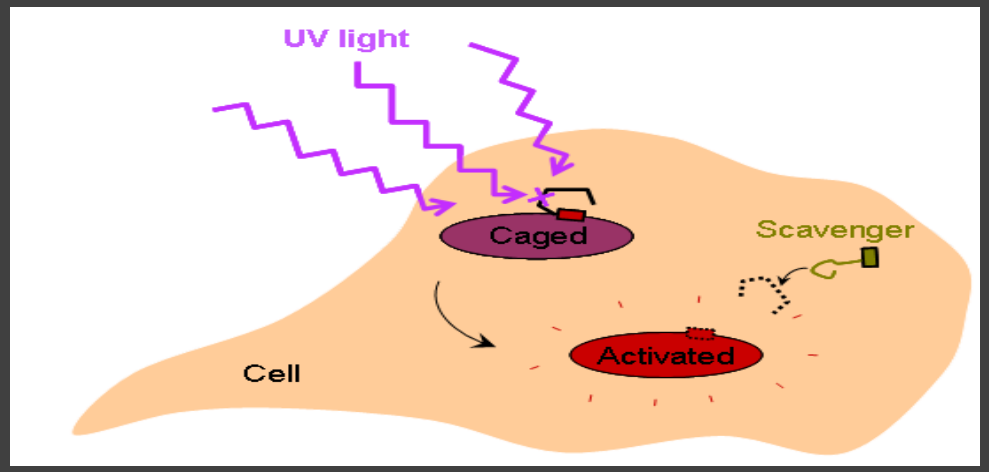
1. Stained cell,
2. Time lapse (TL) start
3. ROI drawn
4. ROI bleached
5. TL ends when bleached
   ROI reaches equilibrium

→ Check for generel bleaching during TL (neighbouring cells)

I= (I∞-Io)/(Ii-Io) = mobile fraction,

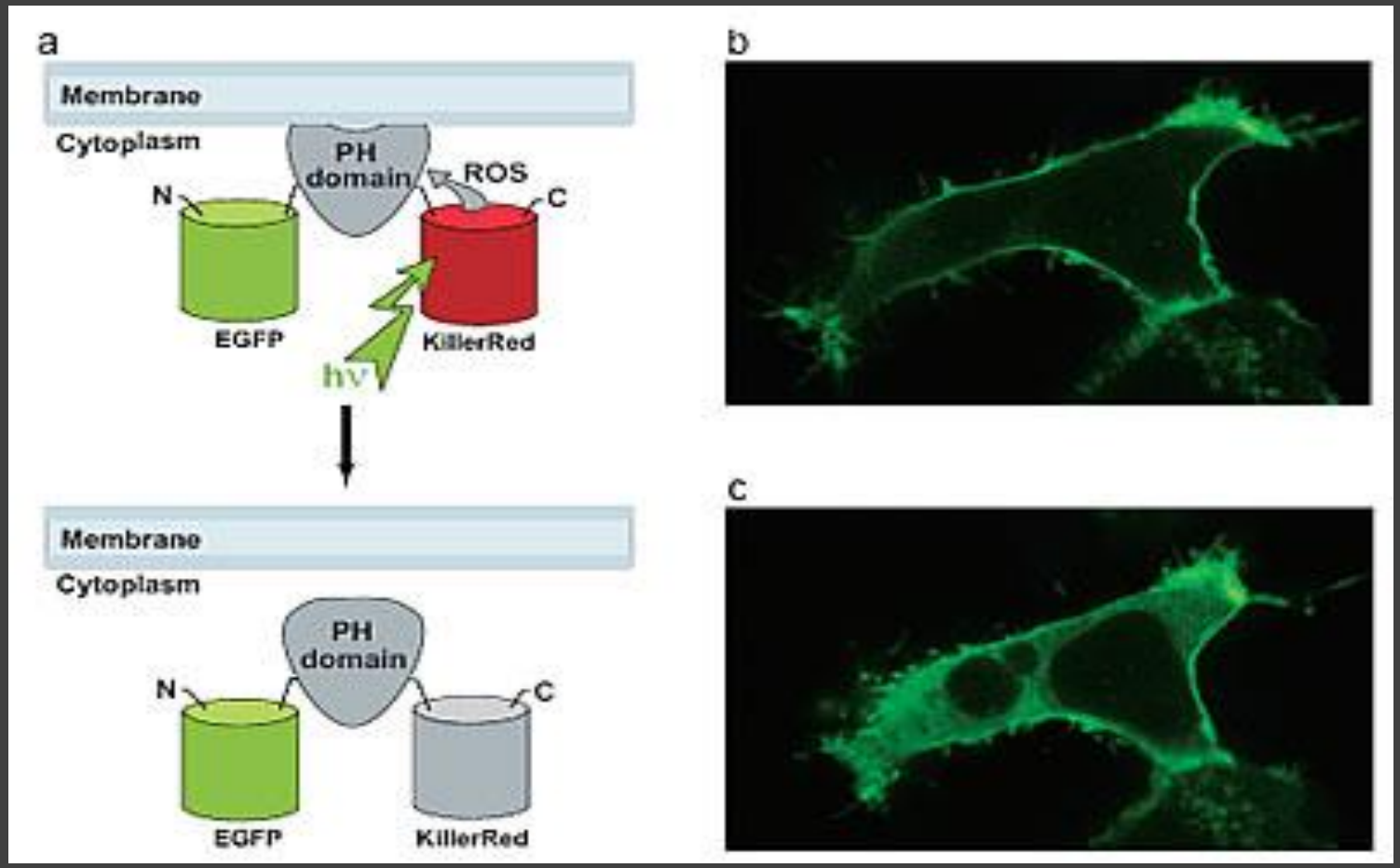
Kinetic analysis (rate constant, t (half) needs curve fittings and module...

Photoactivation /uncaging

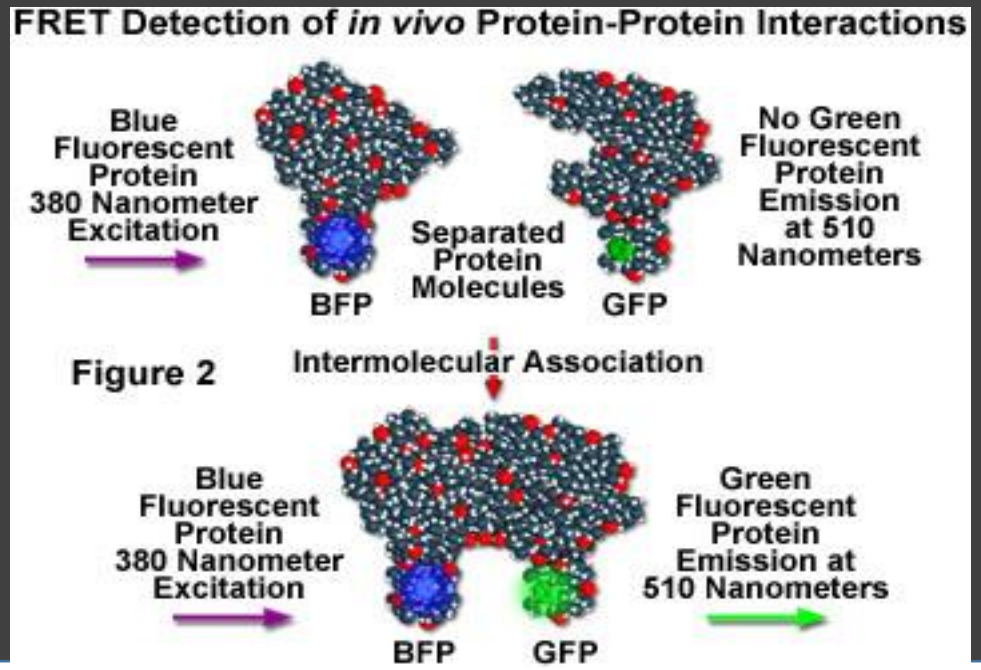


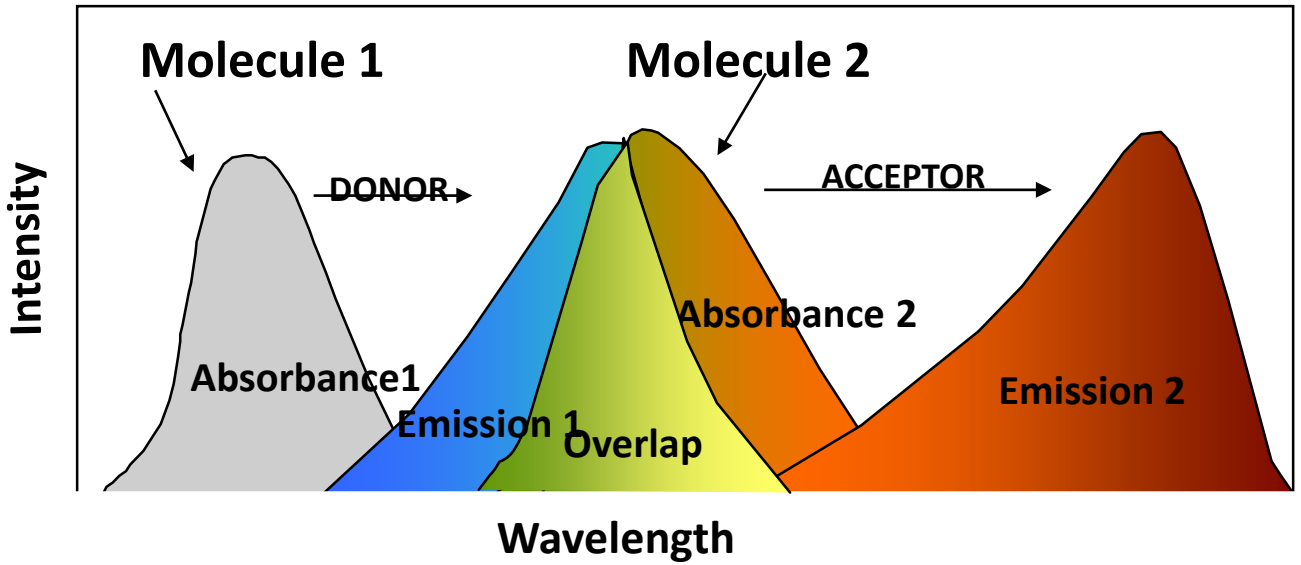
CALI Chromophore Assisted Light inactivation

Such ”effect” might happen per se during live cell imaging



http://www.cellmigration.org/resource/imaging/imaging_approaches_photomanipulation.shtml





FRET, in general

1. Samples:
   - FTRET ”pos.pair”
   - FRET ”neg. Pair”
   - FRET donor ”single”
   - FRET aceptor ”single”
2. Imaging setup
   - Ex Donor - Em Donor
   - Ex Aceeptor - Em Acceptor
   - Ex Donor - Em Acceptor

Analysis

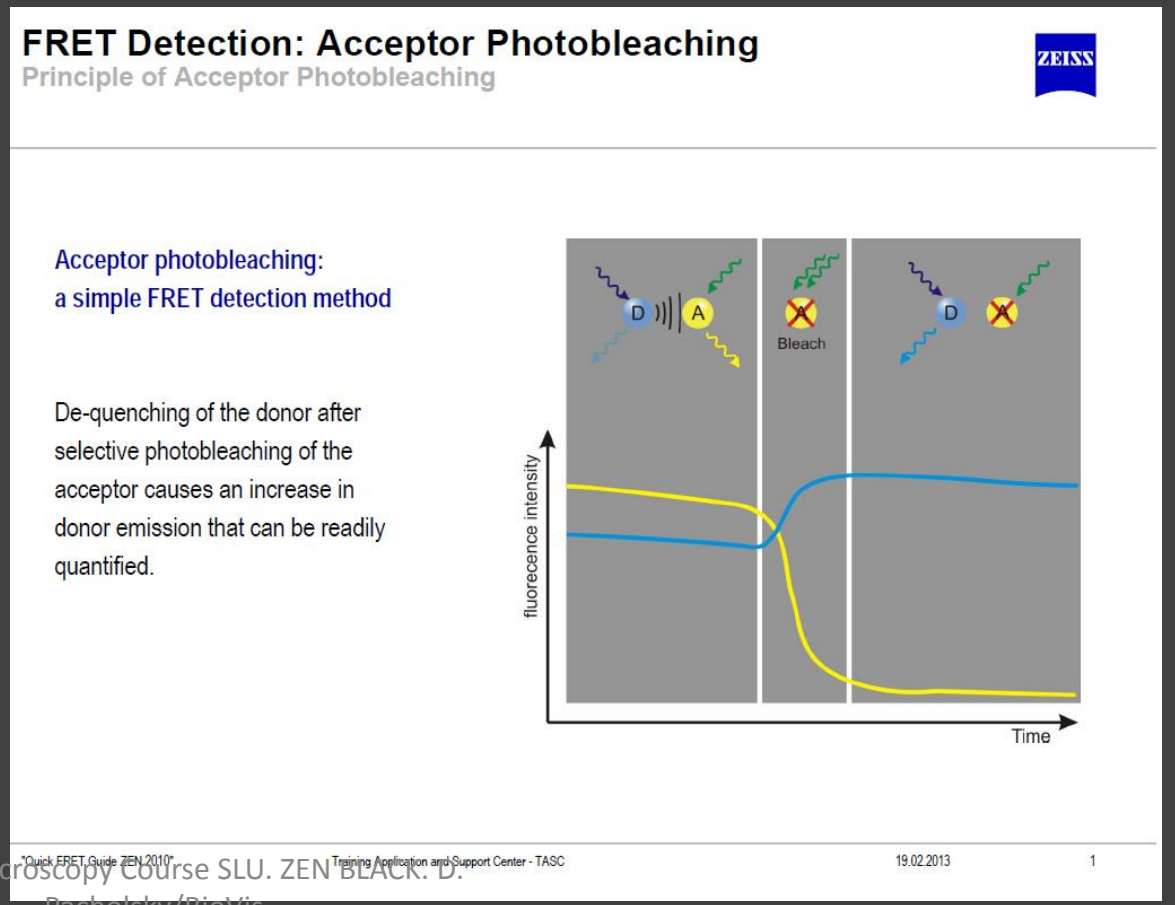
See pdf FRETLSM780 for acceptor photobleaching, simple FET approcah

FRAP

www.jove.com/video/2568/fluorescence-recovery-after-photobleaching-frap-fluorescence-tagged

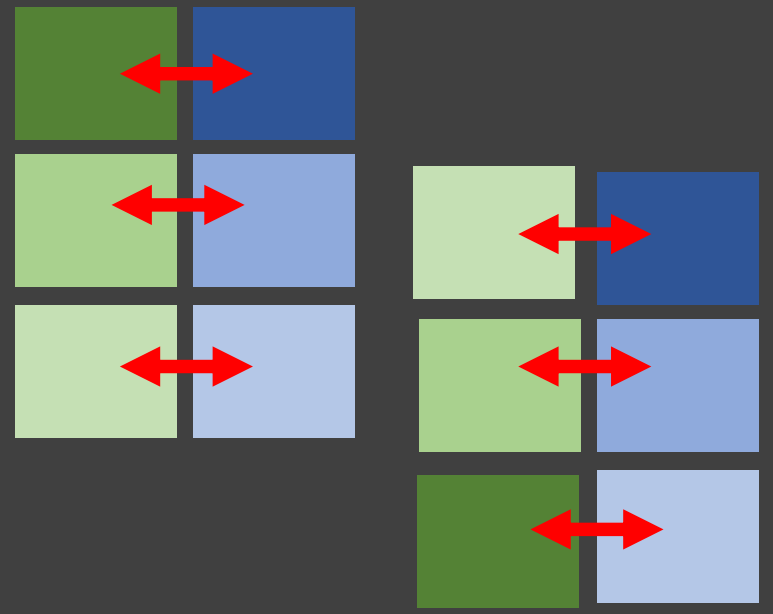
www.ncbi.nlm.nih.gov/pmc/articles/PMC3339873/

.



Anybody else here?

Colocalization



Brief overview to colocalization

Many names and coefficients. Which one to use?

Correlation = Pearson Coefficient ;

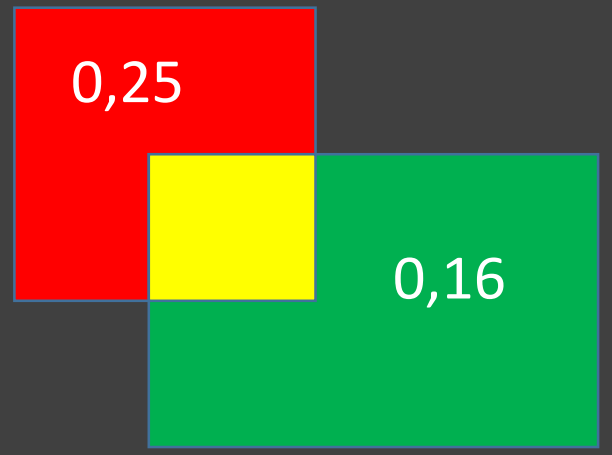
→ linear relationship between pixel intensities

→ Ch1 up & Ch2 up or Ch1 down & Ch2 down

→ Ch1 up & Ch2 down = Exclusion

→ r, R

→ values between 1 (Correlated), 0 (random), -1 (Exclusion)



Meanders coefficient

→ ratio (%) of how much of Ch1 is overlapping with Ch2

→ do not confuse it with Meander overlap coefficient MOC...

Many coefficients on the market and very confusing names often used wrongly in literature...

ZEN BLACK p

ORIGINAL ARTICLE

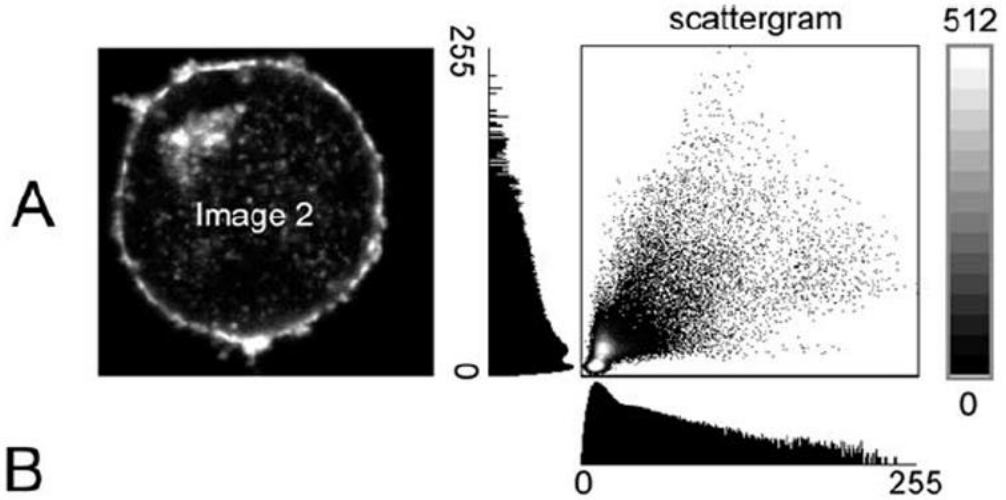
Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Quantifying Colocalization by Correlation: The Pearson Correlation Coefficient is Superior to the Mander's Overlap Coefficient

Jeremy Adler, Ingela Parmryd*

Noise and Colocalization in Fluorescence Microscopy: Solving a Problem

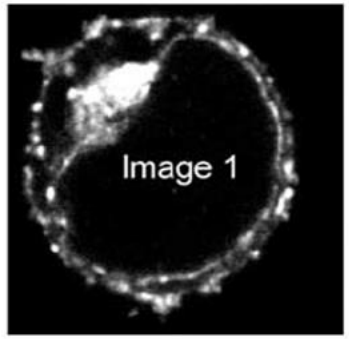
Jeremy Adler,[1] Fredrik Bergholm,[2] Stamatis N Pagakis,[3] and Ingela Parmryd.[1]
1. Cell Biology, The Wenner-Gren Institute, Stockholm University, Sweden. 2. Dept. of Numerical Analysis and Computer Sciences, KTH, Stockholm, Sweden. 3. Biological Imaging Unit, Biomedical Research Foundation Academy of Athens, Athens, Greece.



B

ROI	PCC	SRC	MOC	ICQ
All	.71	.76	.85	.31
Threshold	.61	.60	.85	.29
Cell	.63	.53	.85	.30
Nucleus	.00	.00	.94	.01
Plasma mmb	.45	.52	.91	.21
Perinuclear	.44	.52	.92	.18

Background	min	max	mea	SD
Image 1	2	20	9.5	3.0
Image 2	0	13	5.8	1.9



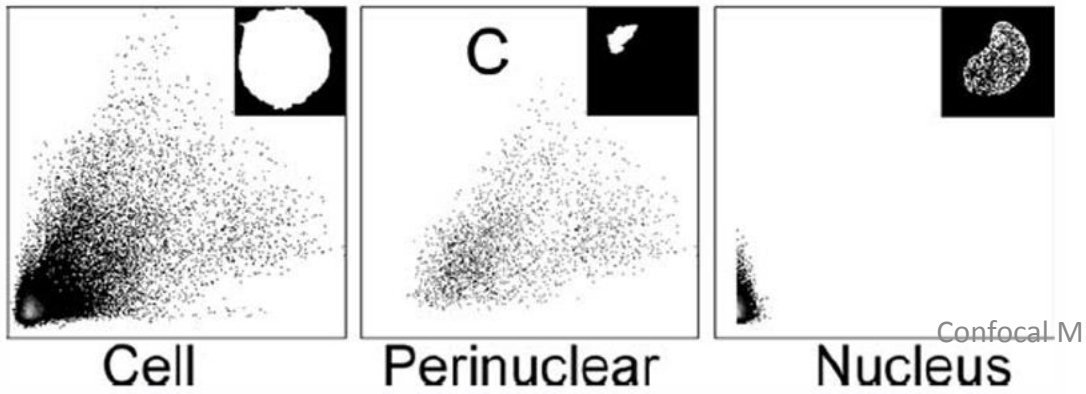
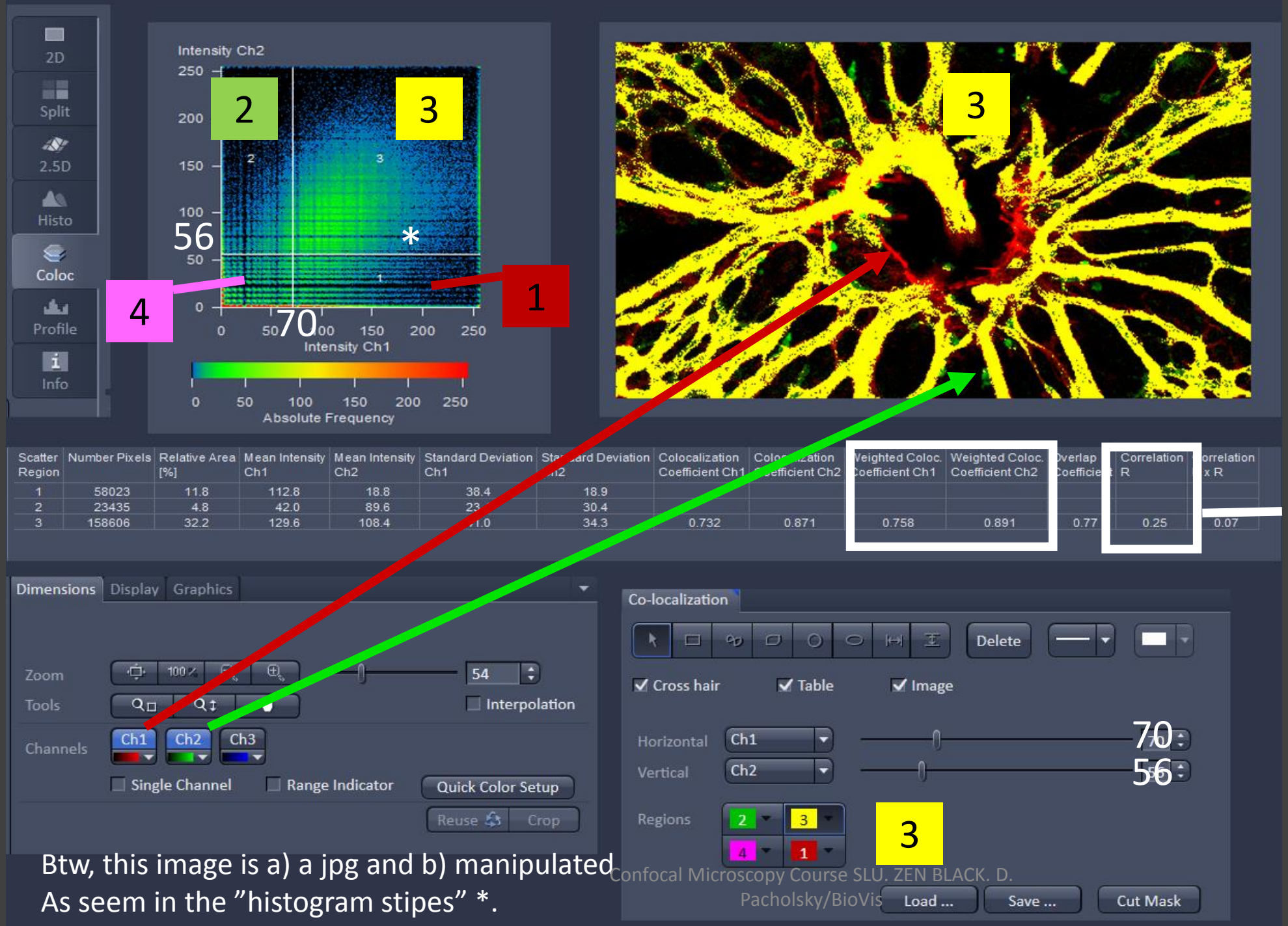


Figure 1. Colocalization, scattergrams, and regions of interest (ROI). **A**: Two images and their log frequency distribution histograms. For each pixel in the pair of fluorescent images, the two intensities are used as the coordinates of an entry in the scatterplot. This shows the relationship between the two fluorophores. Pixels from the whole area, including areas outside the cell are included. A grayscale look up table shows the frequency of occurrence of each pairs of intensities. Note that a white background has been used for the scattergrams and that the fluorescent images have been contrast stretched for display purposes, but that the histograms and scattergrams show the original distribution. **B**: Correlation measurements and the background intensity. **C**: Scattergrams for different ROIs (inserted top left), showing which pixels were included in the analysis: the nuclear ROI is speckled because an intensity threshold (mean plus twice the standard deviation) was also employed.



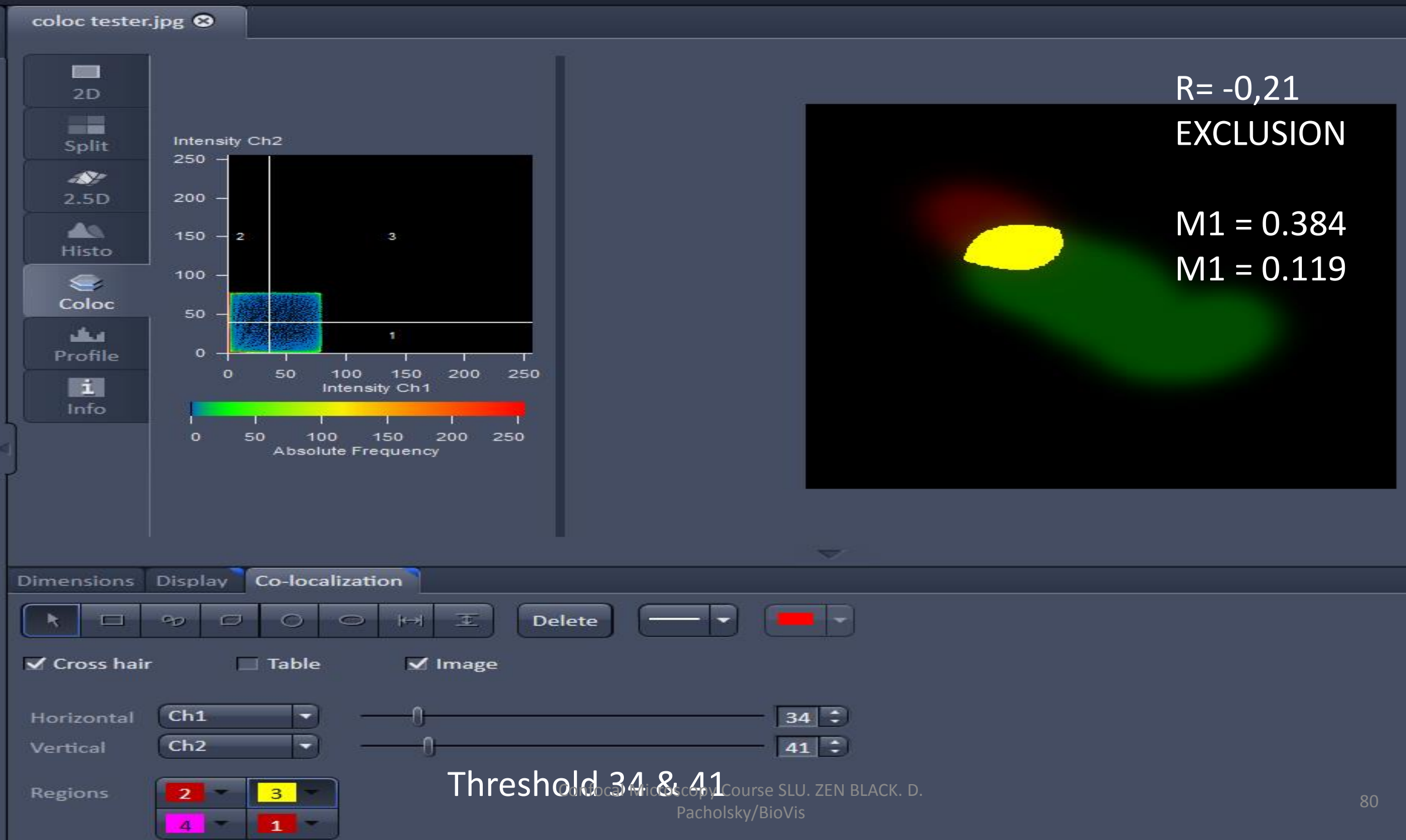
ZEN vs Image J
Same image
and threshold

Pearson Coefficient
Corellation Coef.
R

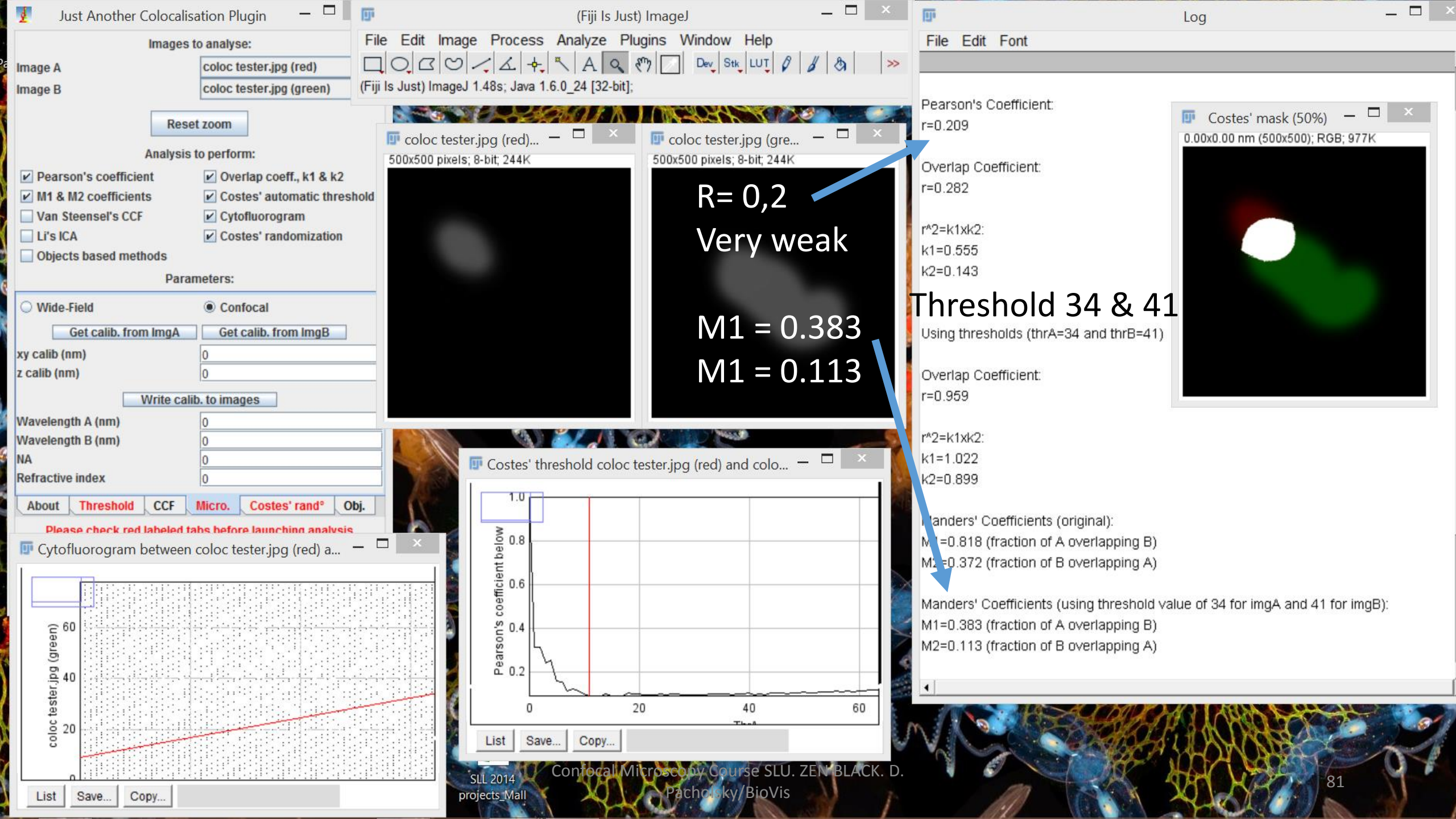
ZEN: 0.25
M1:0.75, M2:0.89
Image J: 0.698
M1 & M2 0.9

So what?
Zen not working
With 3D
More options in
Image J

Btw, this image is a) a jpg and b) manipulated
As seem in the ”histogram stipes” *.



Threshold 34 & 41


Just Another Colocalisation Plugin
Images to analyse:
Image A
coloc tester.jpg (red)
Image B
coloc tester.jpg (green)
Reset zoom
Analysis to perform:
Pearson's coefficient
Overlap coeff., k1 & k2
M1 & M2 coefficients
Costes' automatic threshold
Van Steensel's CCF
Cytofluorogram
Li's ICA
Costes' randomization
Objects based methods
Parameters:
Wide-Field
Confocal
Get calib. from ImgA
Get calib. from ImgB
xy calib (nm)
z calib (nm)
Write calib. to images
Wavelength A (nm)
Wavelength B (nm)
NA
Refractive index
About
Threshold
CCF
Micro.
Costes' rand°
Obj.
Please check red labeled tabs before launching analysis
(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 1.48s; Java 1.6.0_24 [32-bit];
coloc tester.jpg (red)...
500x500 pixels; 8-bit; 244K
coloc tester.jpg (gre...
500x500 pixels; 8-bit; 244K
R= 0,2
Very weak
M1 = 0.383
M1 = 0.113
Log
File Edit Font
Pearson's Coefficient:
r=0.209
Overlap Coefficient:
r=0.282
r^2=k1xk2:
k1=0.555
k2=0.143
Threshold 34 & 41
Using thresholds (thrA=34 and thrB=41)
Overlap Coefficient:
r=0.959
r^2=k1xk2:
k1=1.022
k2=0.899
anders' Coefficients (original):
=0.818 (fraction of A overlapping B)
=0.372 (fraction of B overlapping A)
Manders' Coefficients (using threshold value of 34 for imgA and 41 for imgB):
M1=0.383 (fraction of A overlapping B)
M2=0.113 (fraction of B overlapping A)
Costes' mask (50%)
0.00x0.00 nm (500x500); RGB; 977K
Cytofluorogram between coloc tester.jpg (red) a...
coloc tester.jpg (green)
List
Save...
Copy...
Costes' threshold coloc tester.jpg (red) and colo...
Pearson's coefficient below
List
Save...
Copy...

Anybody else still awake?

The End