The Basics: Confocal Laser Scanning Microscopy

Dr. Daniel Koch

07/05/2012

Agenda

1. Confocal Principle
2. Innovative Beam Path Technology
3. Confocal Imaging: Images and Z-Stacks
4. Scanning Strategies
5. Resolution: Point Spread Function
6. Evaluating an Image

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1	Confocal Principle
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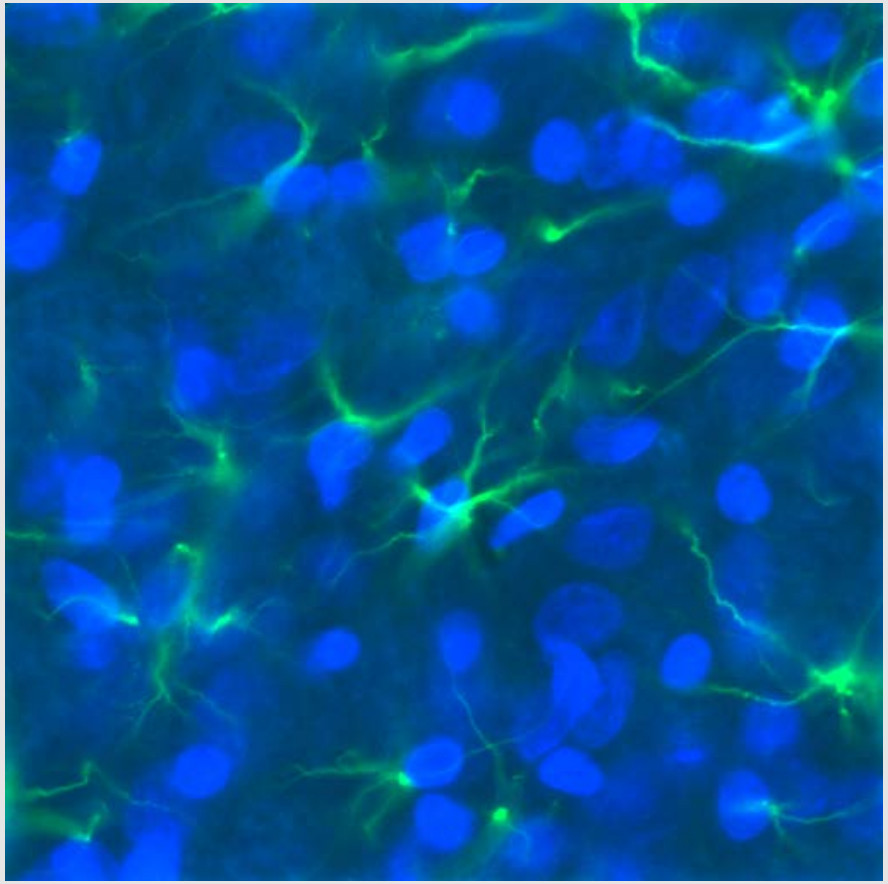
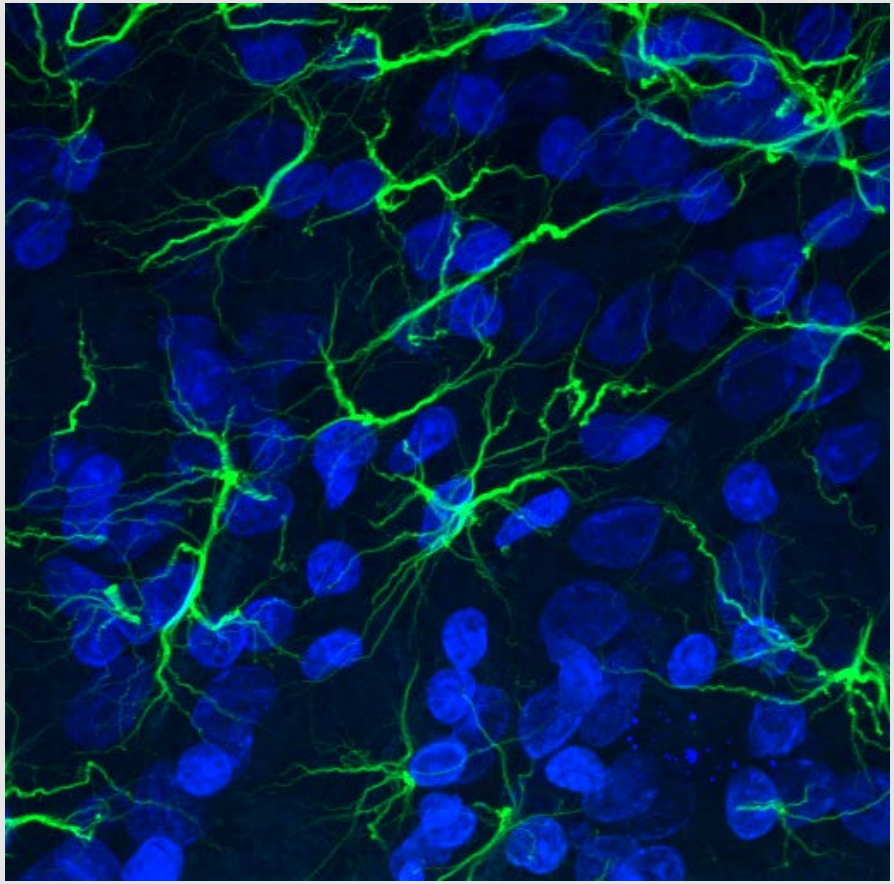
Confocal Principle

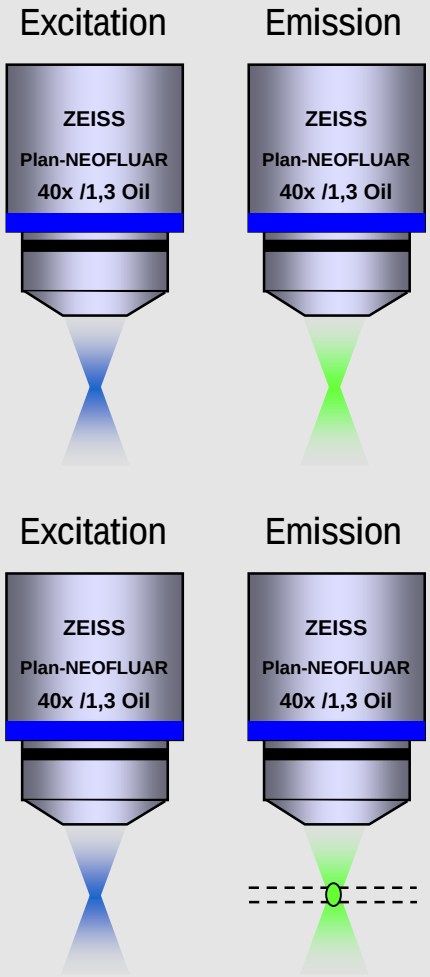


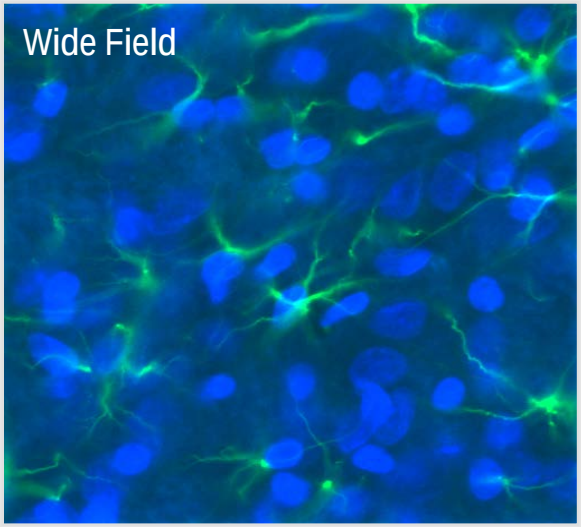
Widefield

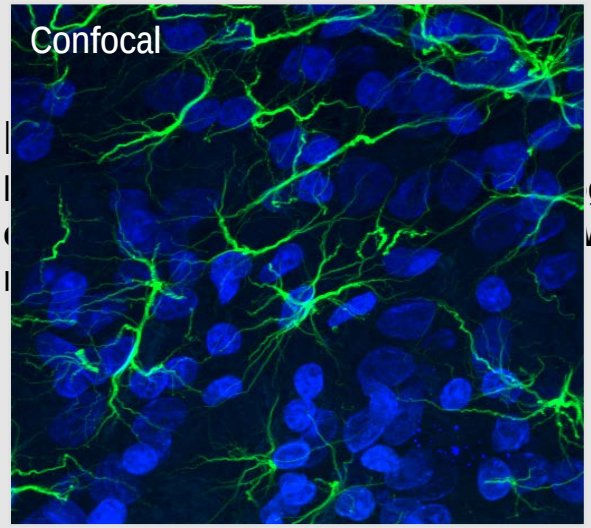


Confocal

Confocal Principle



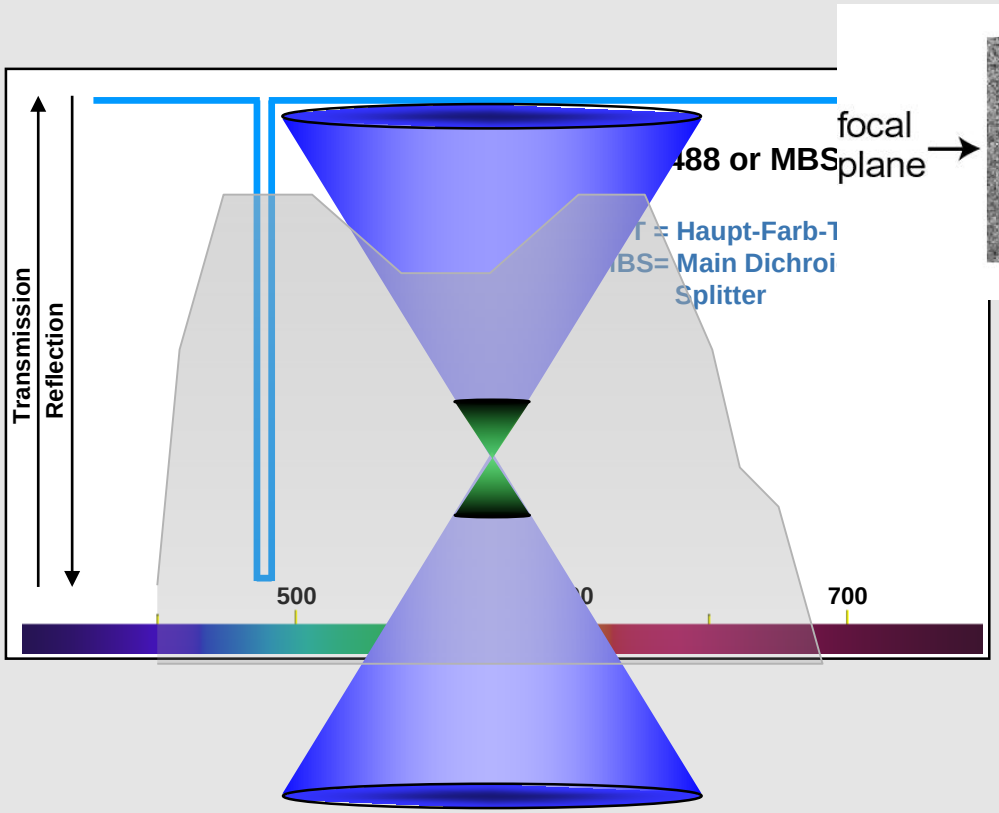


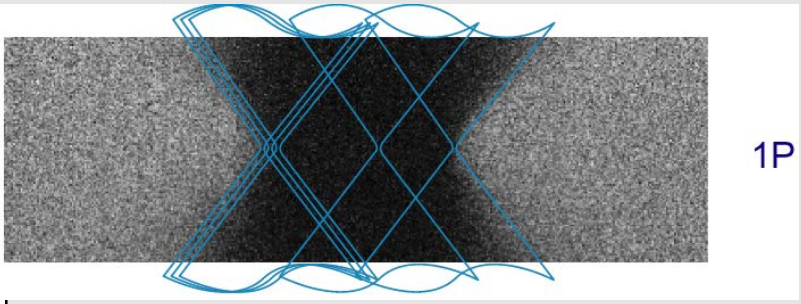


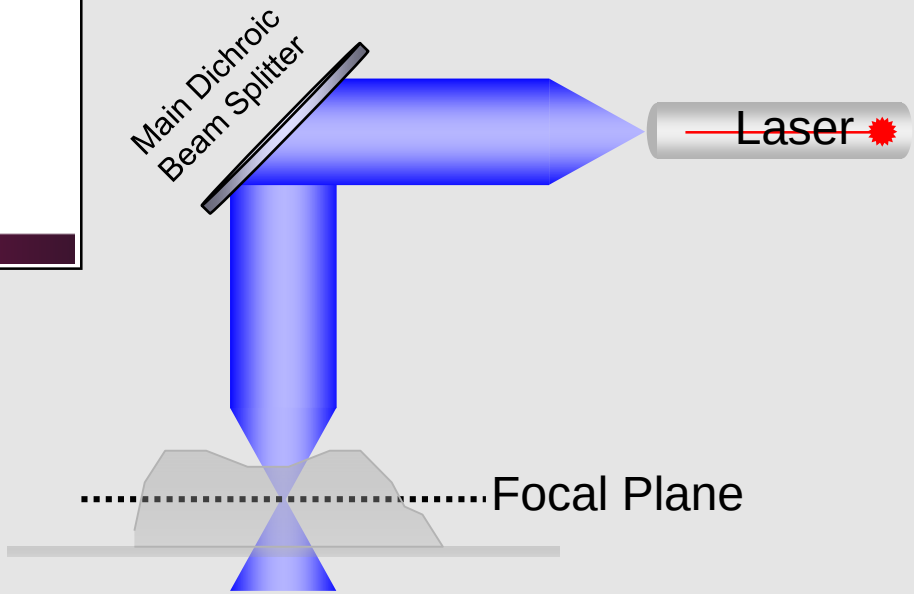
gether with out-
wide-field

Confocal Principle

Excitation Beampath



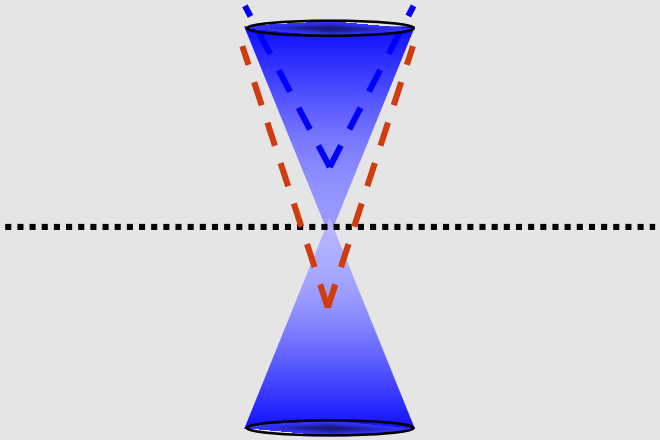


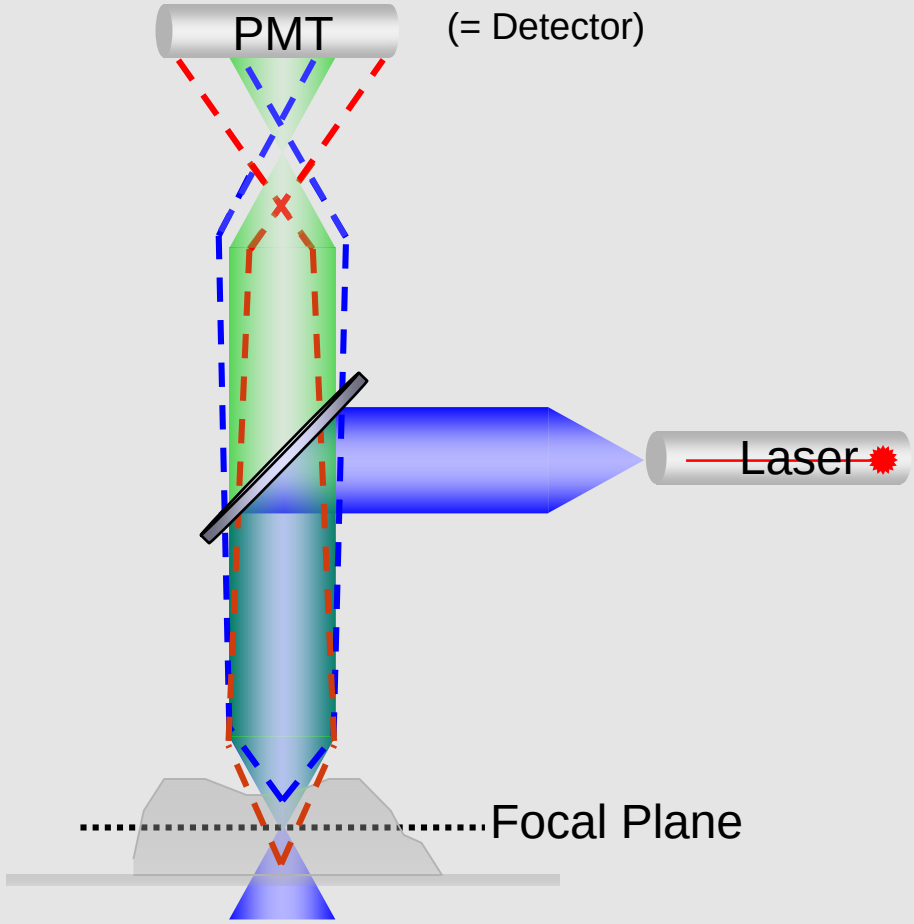


Confocal Principle

Emission Beampath

Using a Laser for excitation does not solve the problem alone:





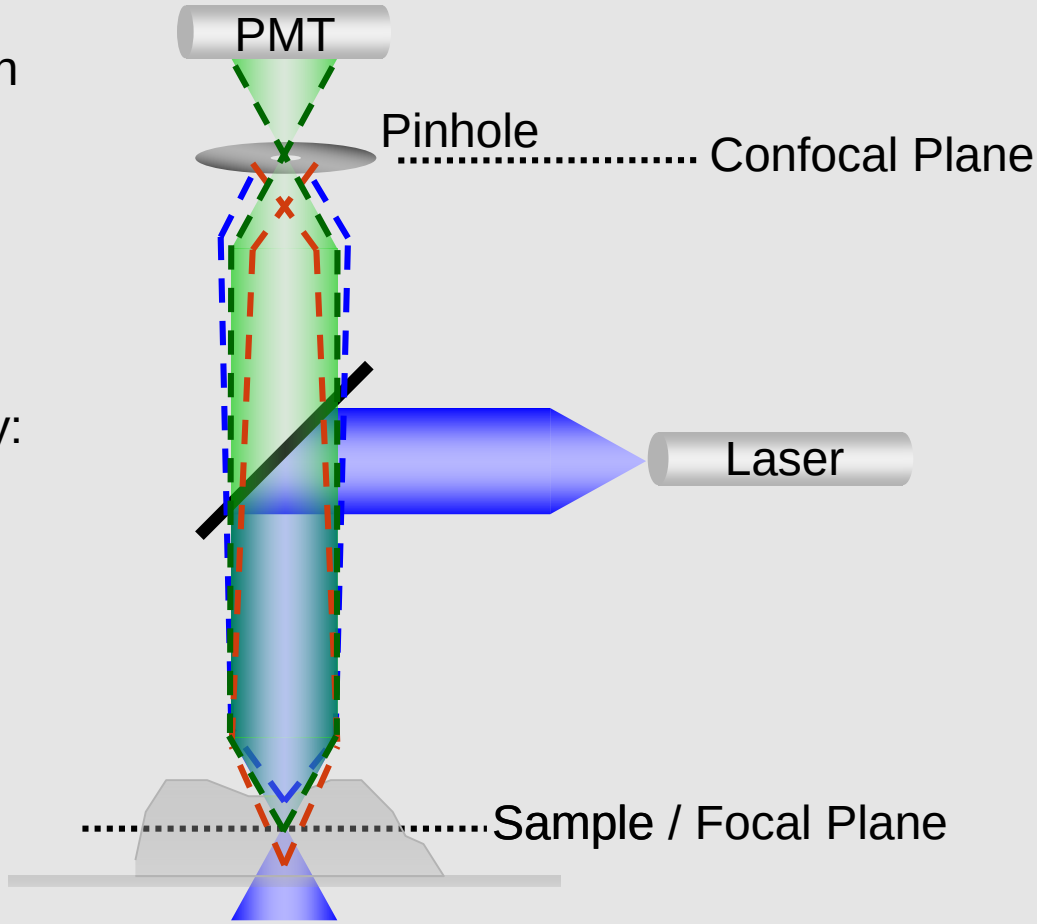
Confocal Principle

The Power Of Optical Sectioning

A minute diaphragm, situated in a conjugated focal plane, prevents out of focus light from being detected.

The thickness of an **optical section** is directly controlled by:

- Numerical aperture of objective lens
- Wavelength
- Pinhole diameter



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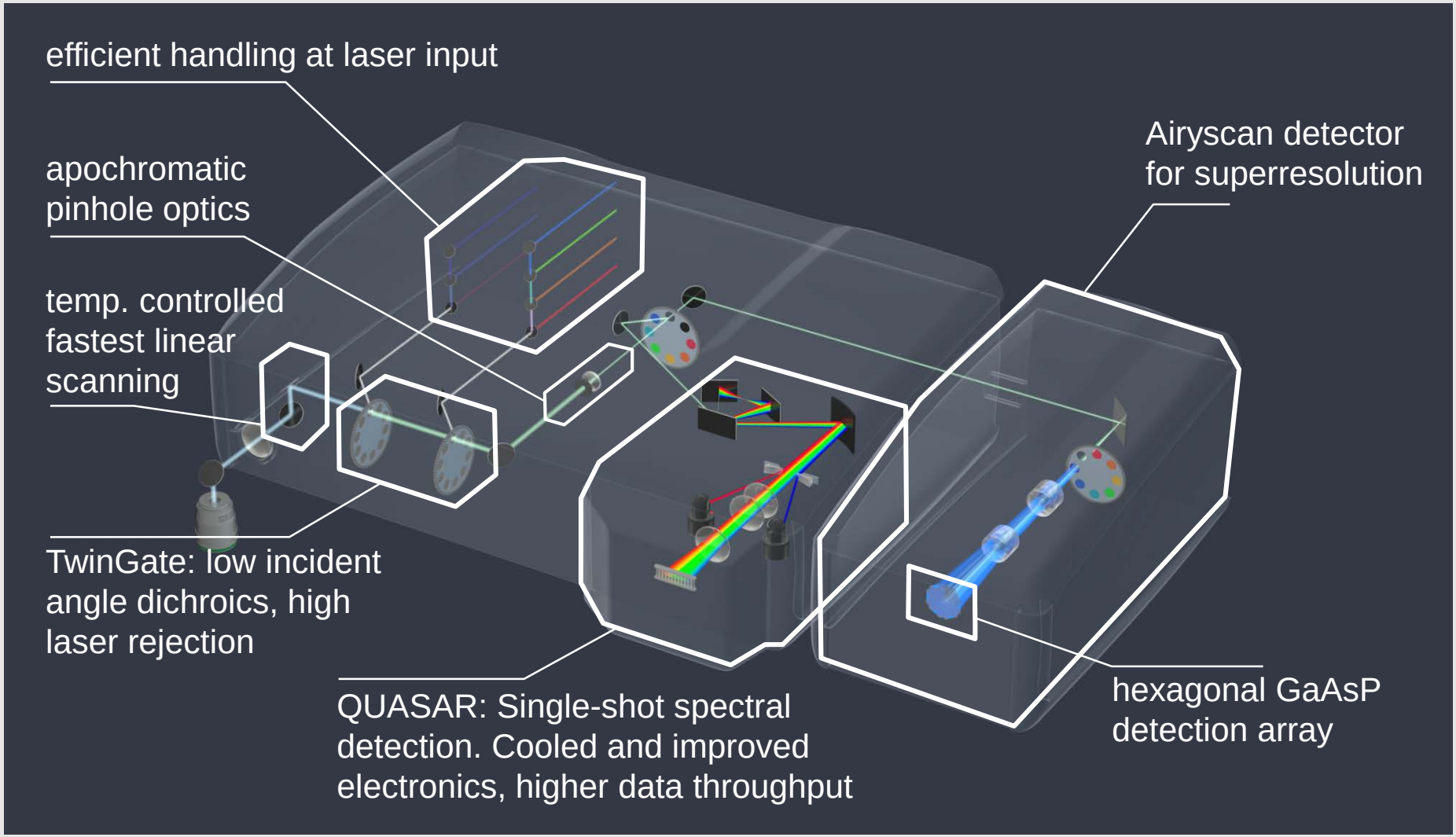
NEW: LSM 880

Our latest Member of the LSM 8 Family with GaAsP Detectors

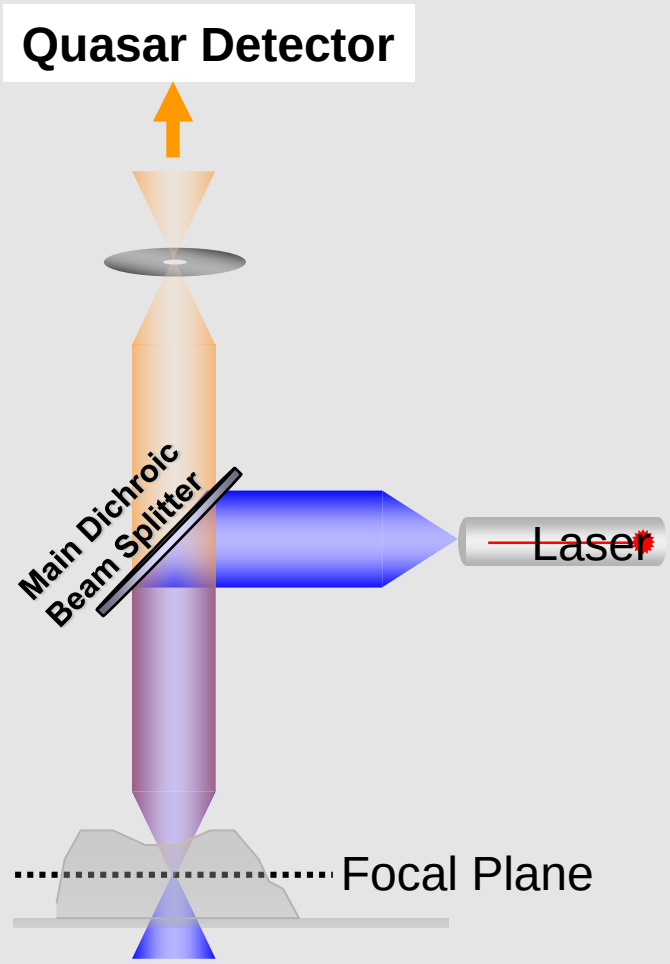
Innovative Beam Path Technology

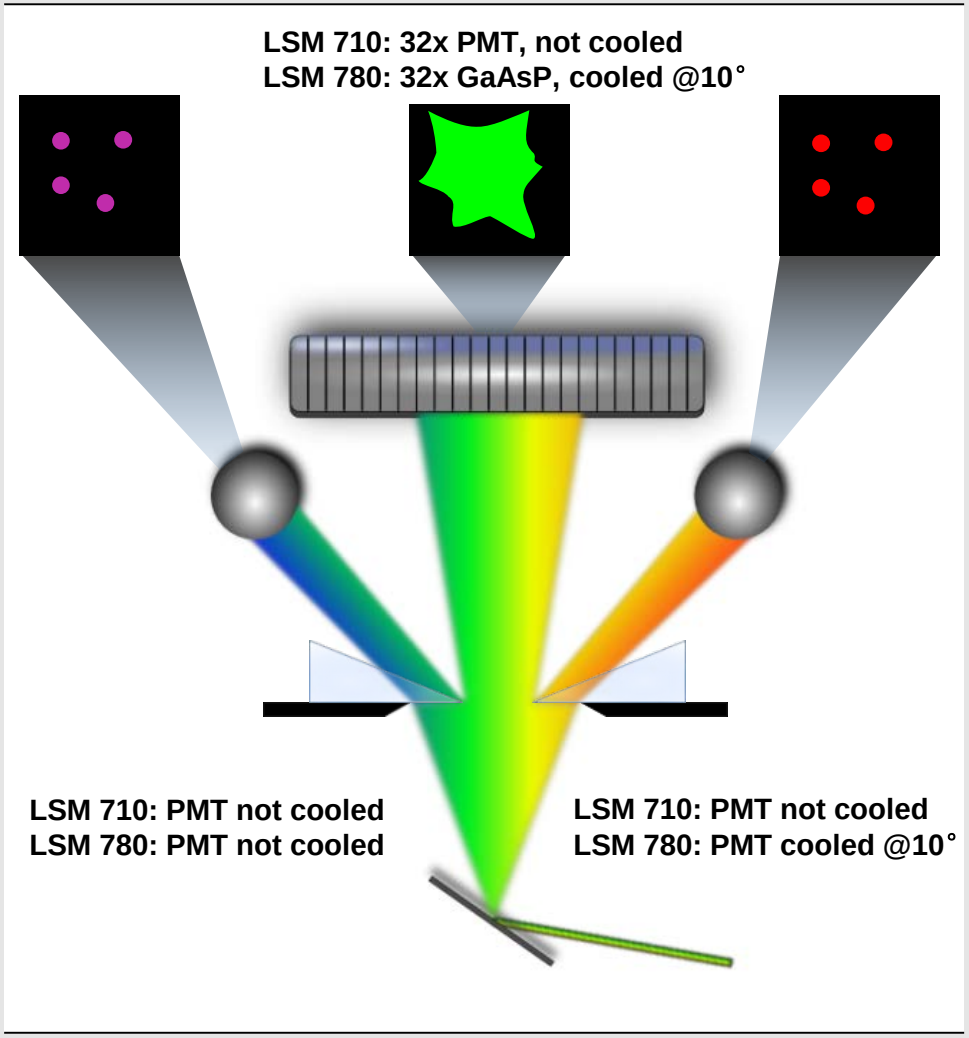
LSM 880



Innovative Beam Path Technology

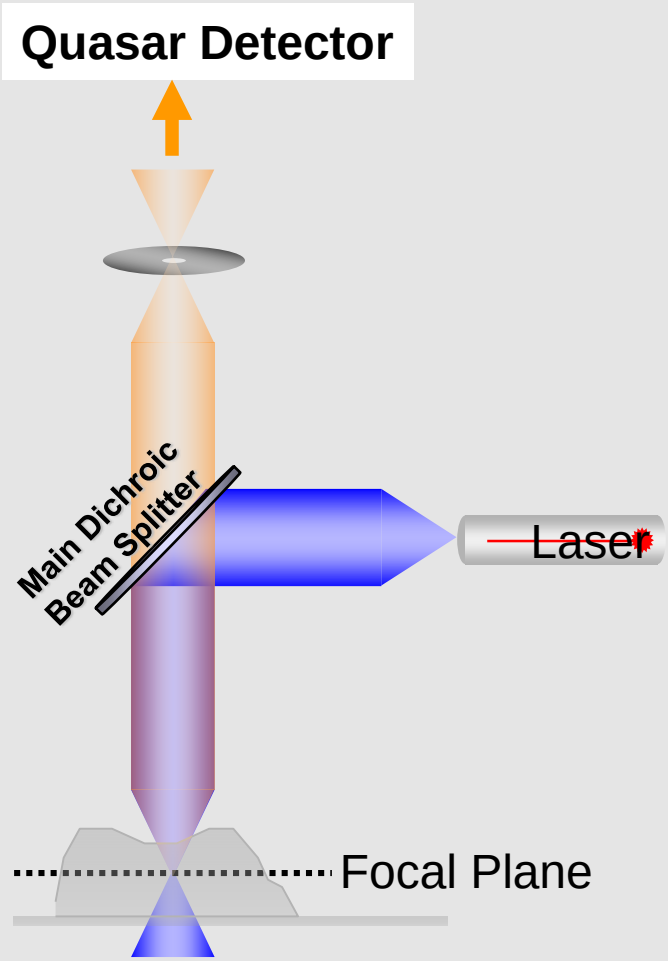
Emission Pathway: QUASAR Detection Unit





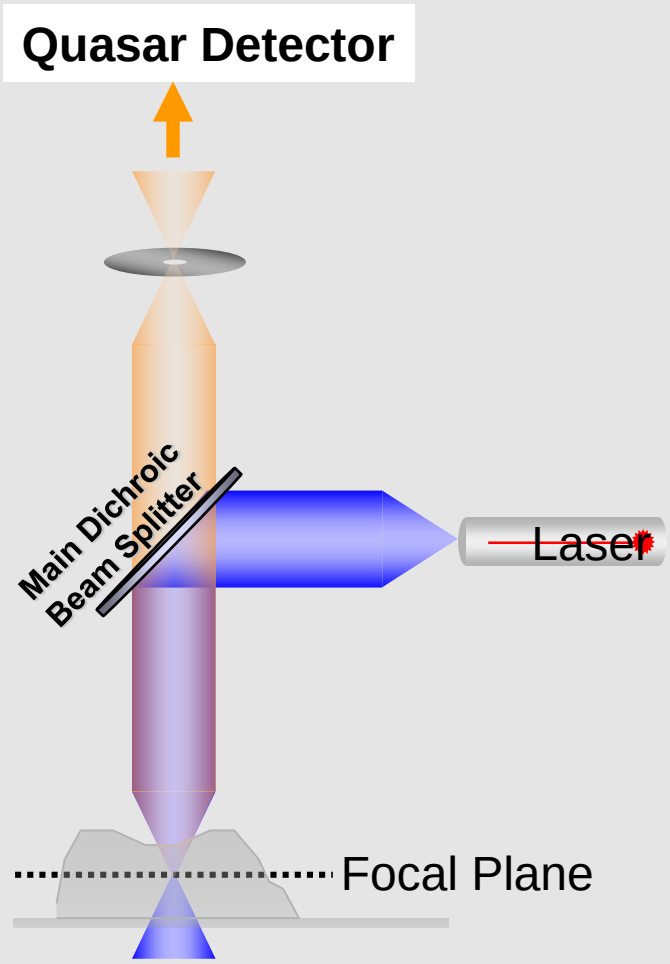
Innovative Beam Path Technology

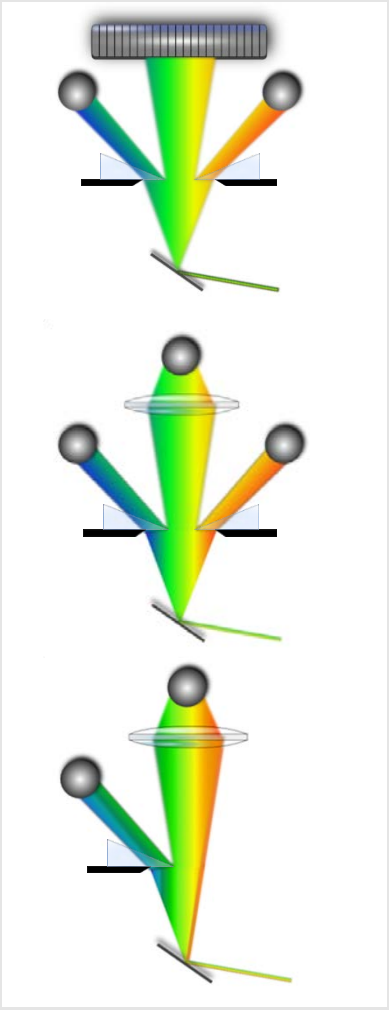
Emission Pathway: QUASAR Detection Unit



Innovative Beam Path Technology

Emission Pathway: QUASAR Detection Unit





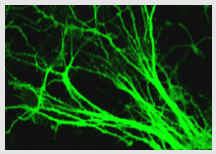
34 Channel QUASAR

3 Channel QUASAR

2 Channel QUASAR

Photomultiplier Tube / PMT Detectors

How does a PMT work?



Photon

p

Photoelectric effect:
Photocathode

Electron

e^-

Dynodes

Anode

Output
Meter

-

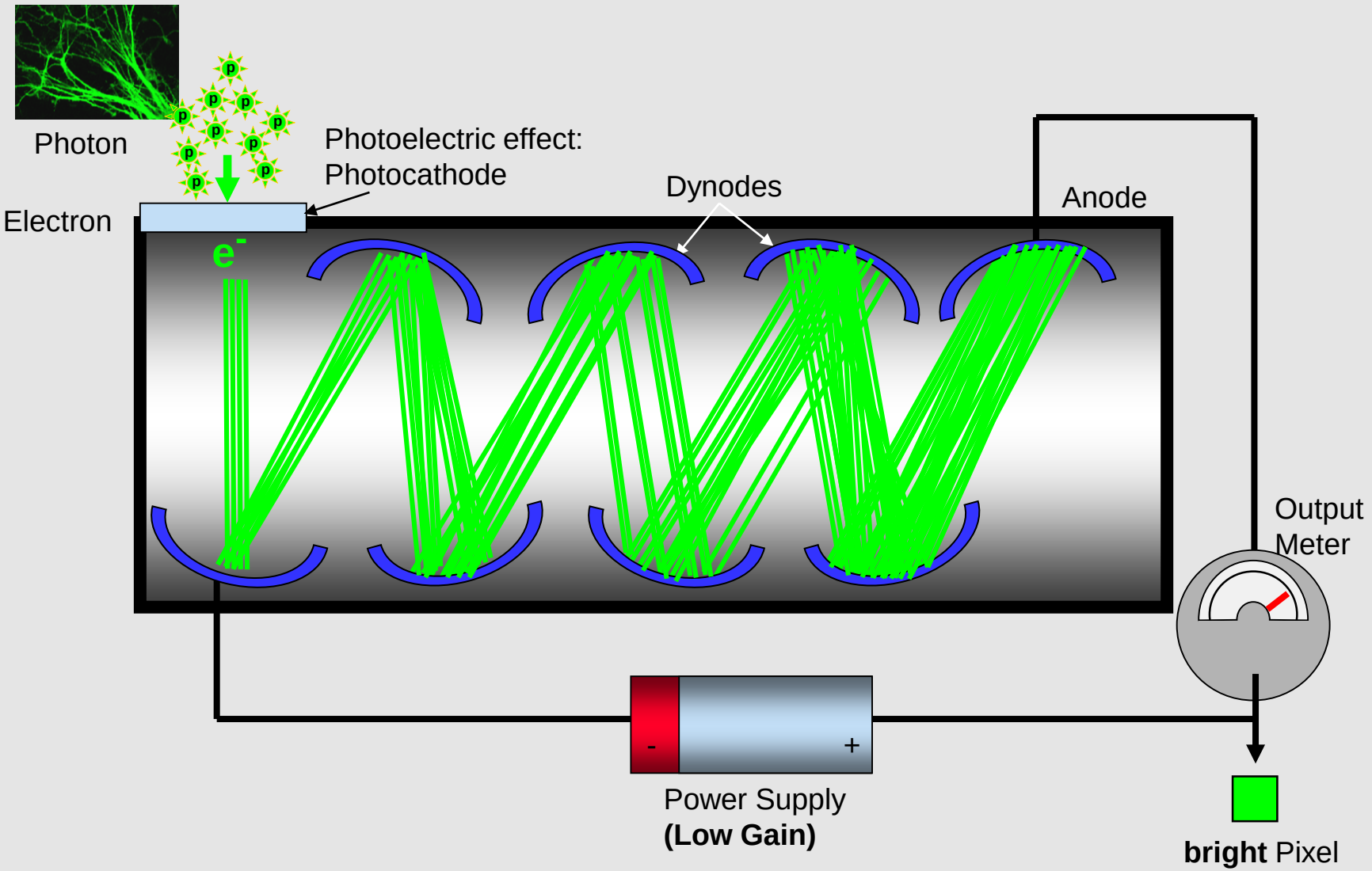
+

Power Supply
(Gain = Volts)

+ - bright Pixel

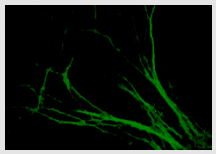
Photomultiplier Tube / PMT Detectors

Assuming a bright sample



Photomultiplier Tube / PMT Detectors

Assuming a dark sample



Photon

Photoelectric effect:
Photocathode

Electron

Dynodes

Anode

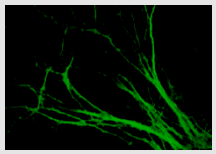
Output
Meter

Power Supply
(Low Gain)

dark Pixel

Photomultiplier Tube / PMT Detectors

Increase a dark sample´s signal with more Gain



Photons

Photoelectric effect:
Photocathode

Dynodes

Anode

Electrons

e-

Output
Meter

-

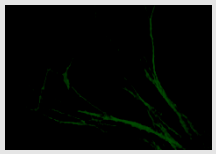
+

Power Supply
(Higher Gain)

bright Pixel

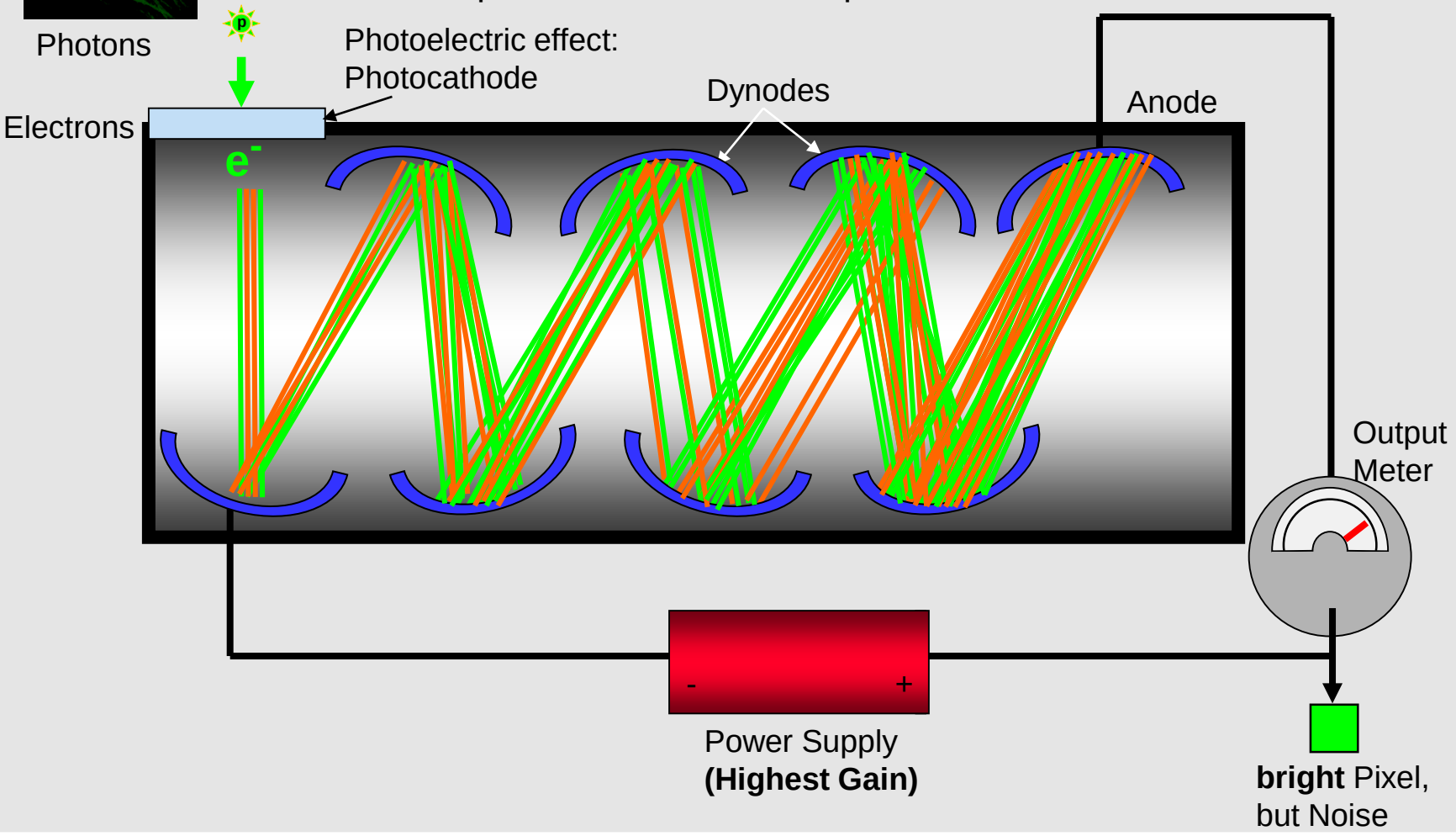
Assume a really dimm sample -

Extreme Gain values result in Noise



Photons

Red lines indicate electrons that were generated but have not come from the photons within the sample



Classical “PMT” Detectors

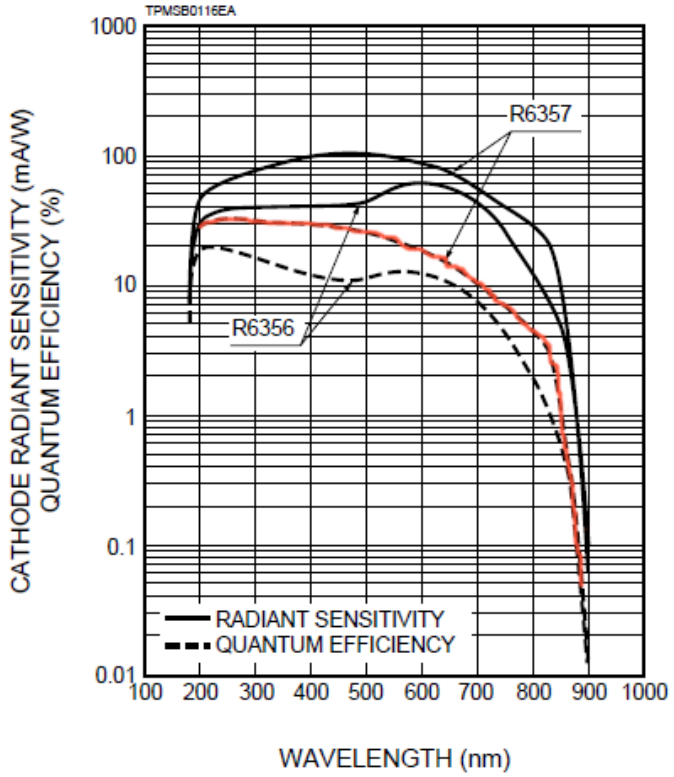
Confocal “standard” PMT detectors are based on photocathode material with a **wide spectral response from ultraviolet to near-infrared**

Photocathode Material:

“Multialkali” , MA (Na-K-Sb-Cs)

Sodium (AlkaliMetal)
Potassium (AlkaliMetal)
Antimony (Metalloid)
Cesium (AlkaliMetal)

Figure 4: Typical Spectral Response of High Sensitivity MA



Modified from Hamamatsu

32 Channel Spectral GaAsP Detector

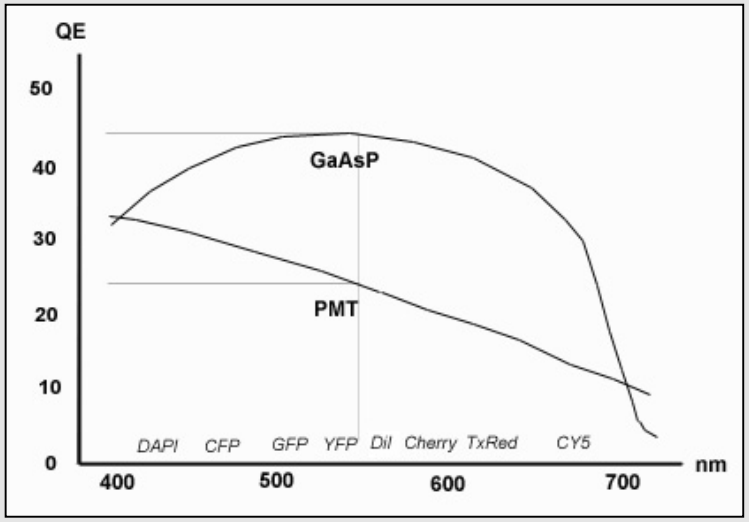
Unmatched sensitivity

GaAsP (*Gallium Arsenide Phosphide*) is a semiconductor material with ideal characteristics for converting photons into electrical signals.

Benefits of GaAsP detectors:

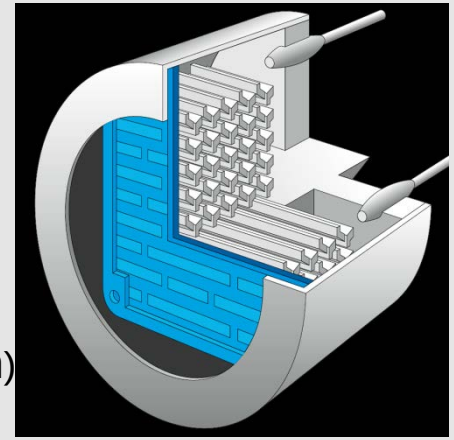
Two times better Quantum efficency than PMTs (resulting in higher sensitivity, better image quality, and higher acquisition speed).

GaAsP detectors can be operated in integration mode as well as in photon counting mode.



Typical sensitivity of detectors

Gallium-Other Metals
Arsen - Metalloids
Phosphor-non Metal



GaAsP detector
(schematic illustration)

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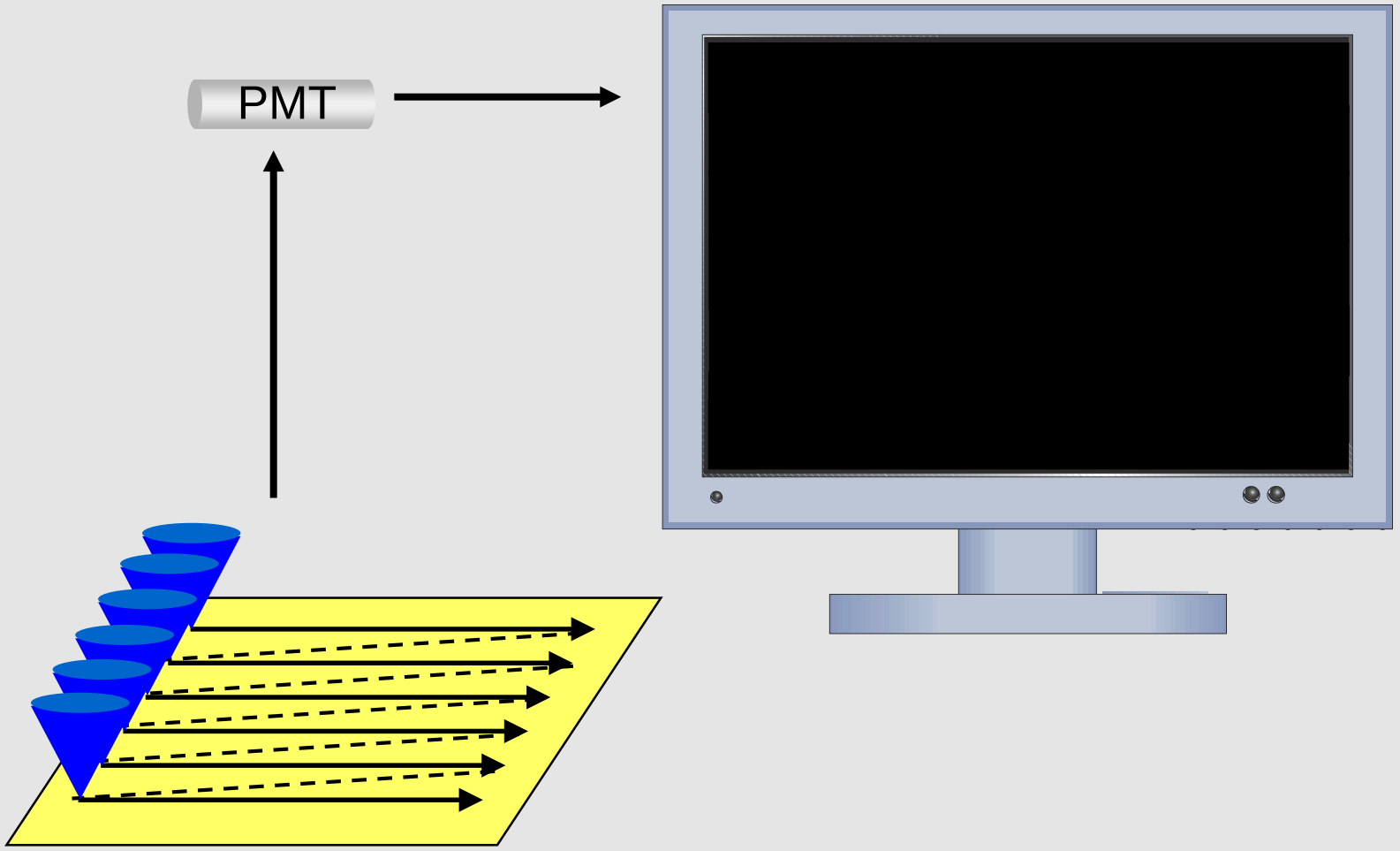
Confocal Imaging

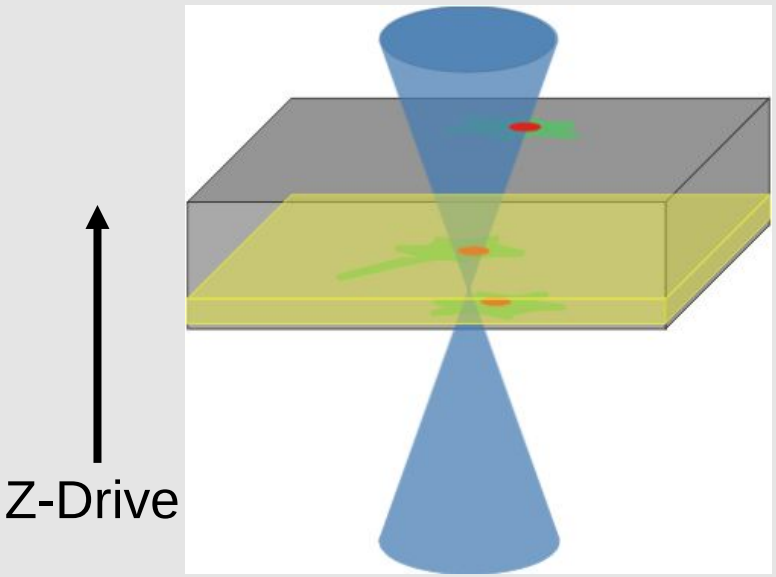
From Point to Image

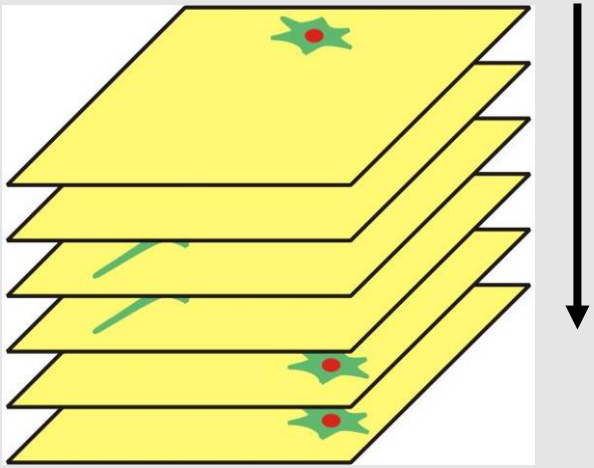


Confocal Imaging

From Image to 3D Information

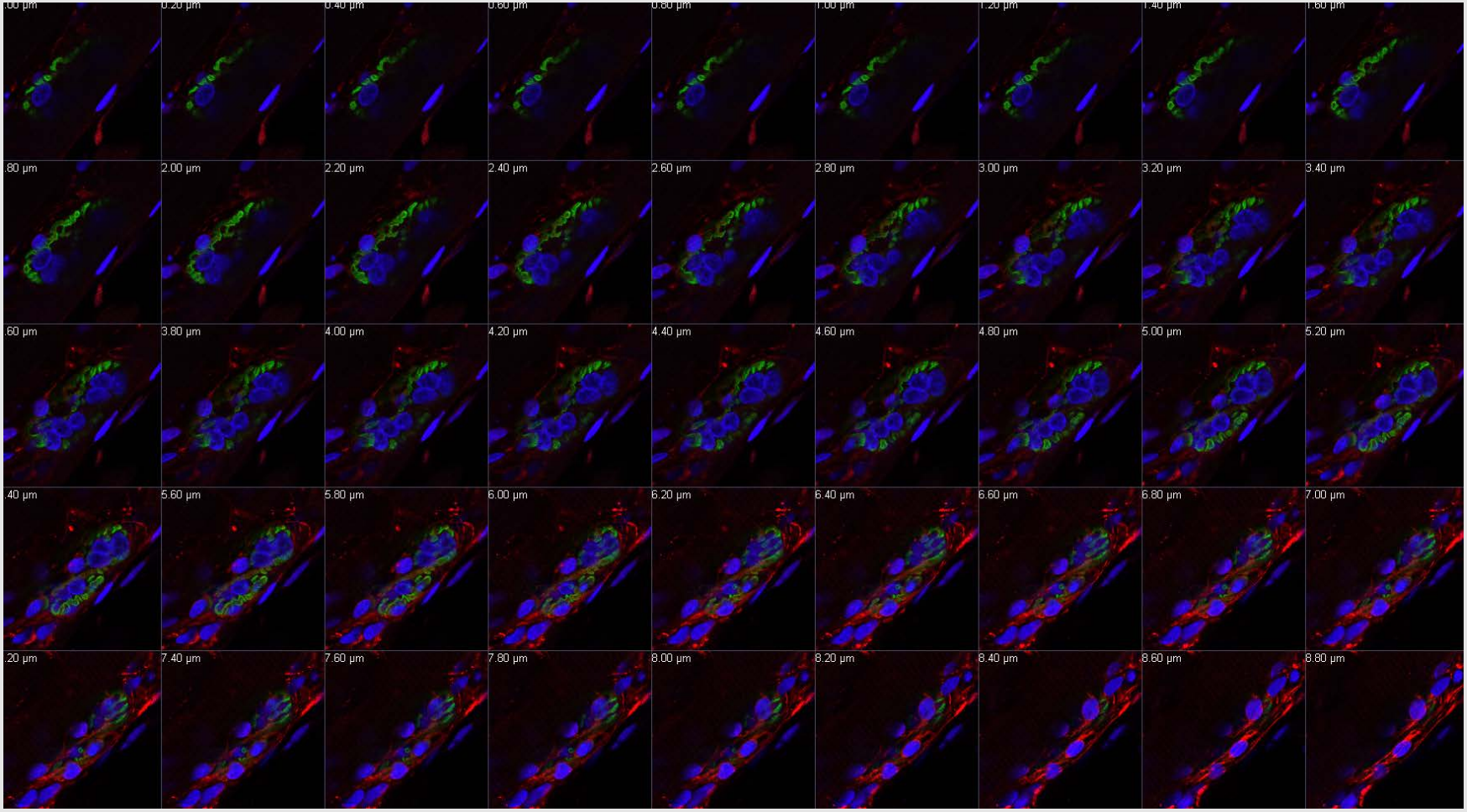
How is a X/Y/Z Stack produced?





Confocal Principle

Acquisition of 3D Image Stacks



8µm confocal Z-stack, displayed in gallery mode

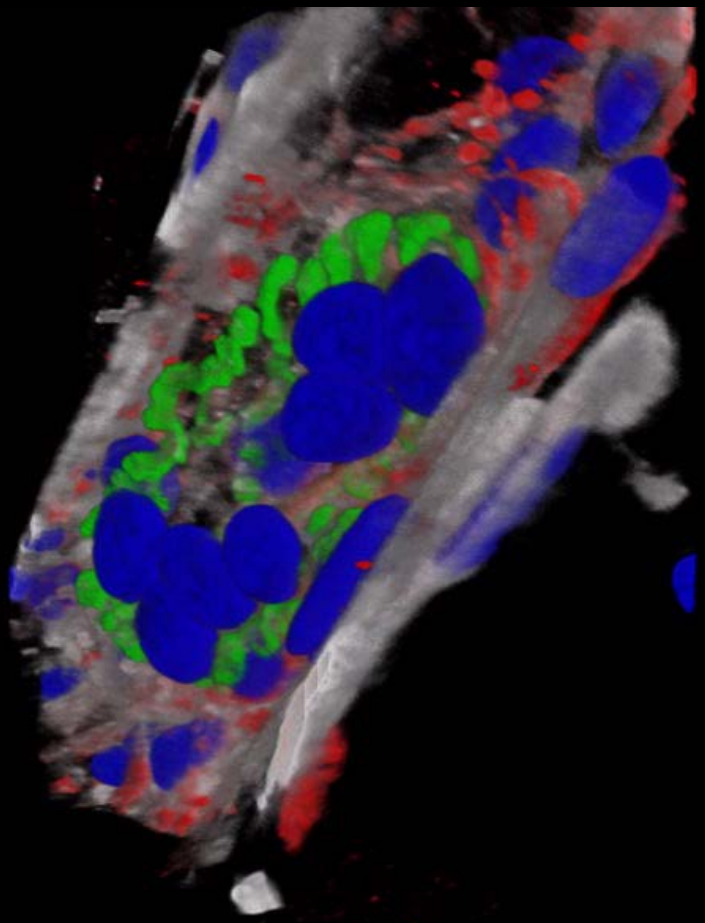
Confocal Principle

3D Rendering and Animation

Sample:

Rat Neuromuscular Junction

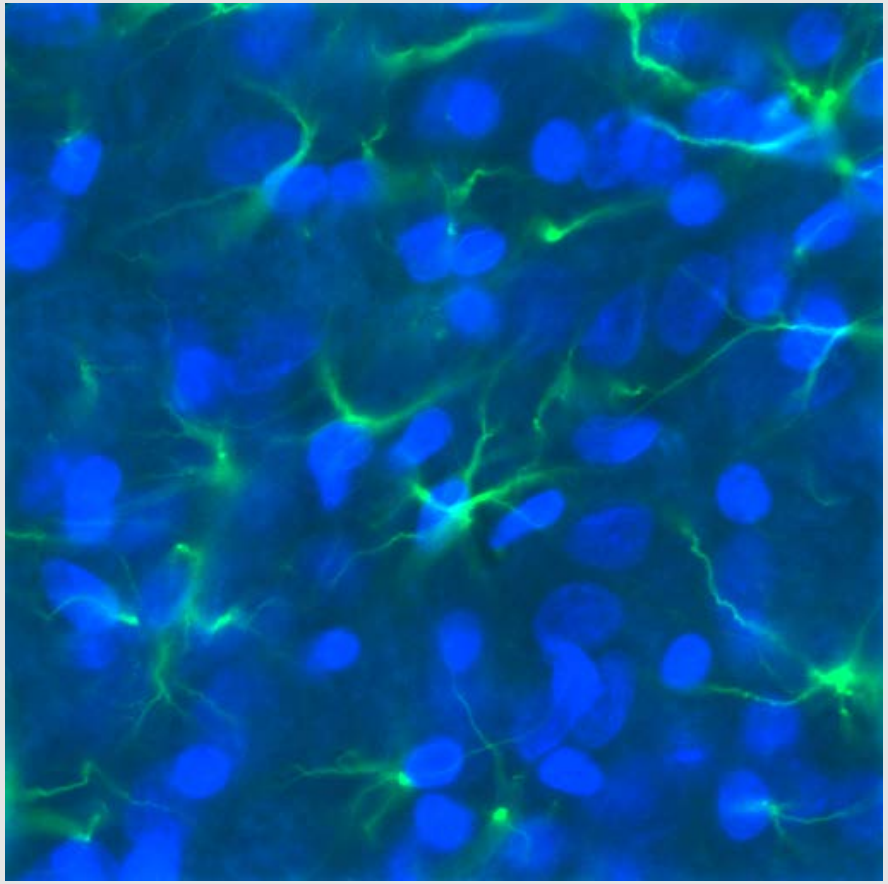
- green - Acetylcholine receptors (alpha-Bungarotoxin / Alexa 488)
- red- Schwann cell (S100 protein / Alexa 555)
- blue - Nuclei (DAPI)
- white - CD44 adhesion molecule / Atto 647N



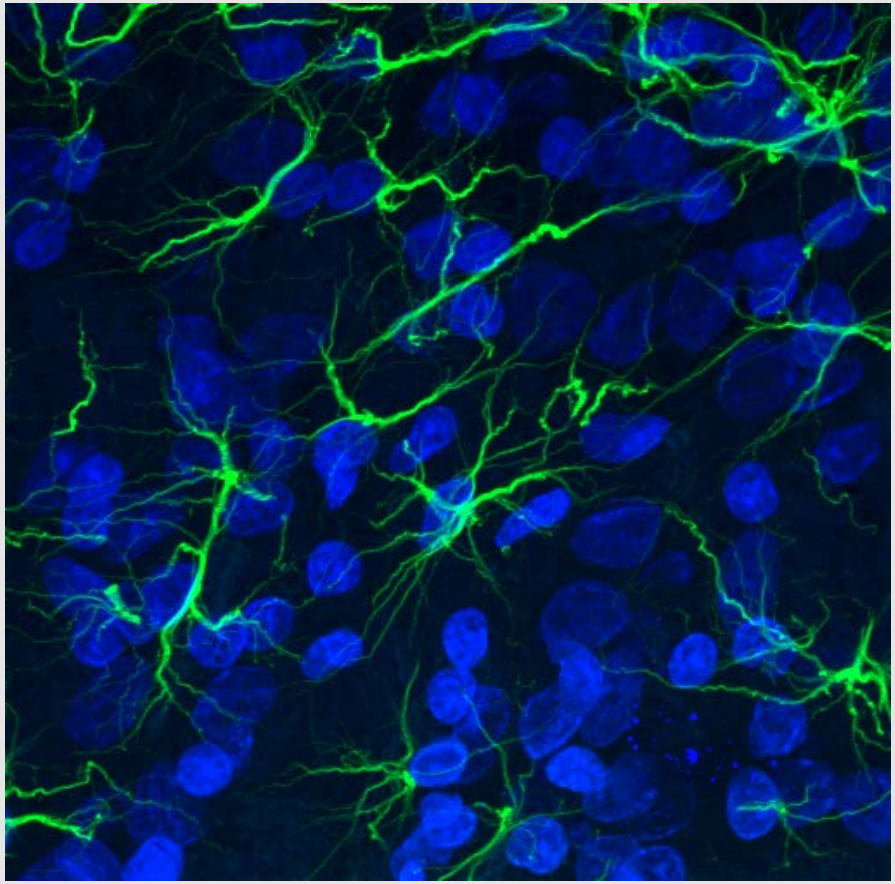
Sample Courtesy:

Dr. Grzegorz Wilczynski
Nencki Institute
Warsaw, Poland

Confocal Principle



Widefield



Confocal

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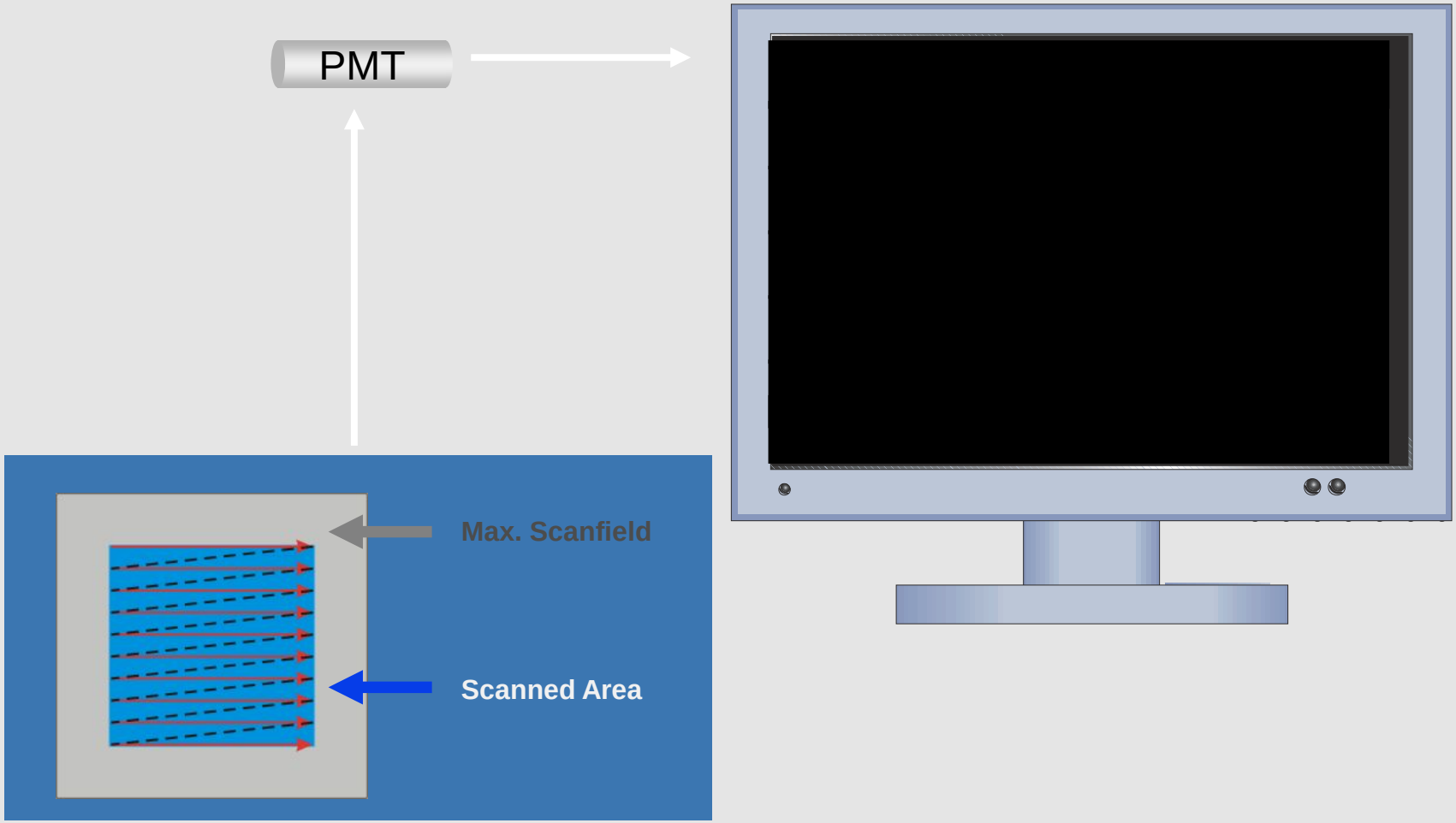
Confocal Imaging

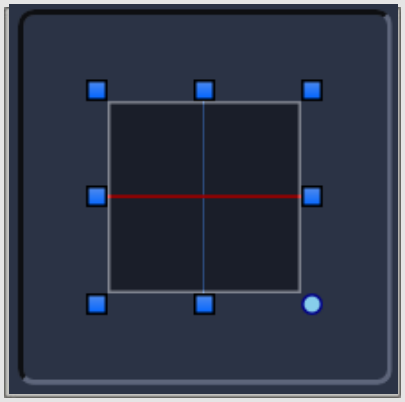
Image acquisition: From Points to Image



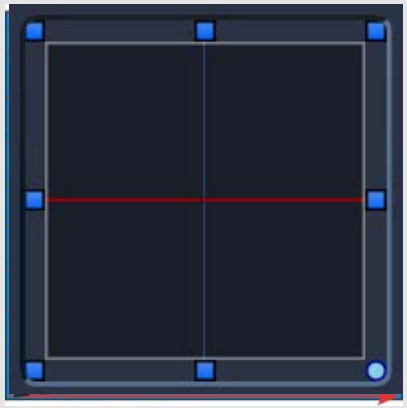
Scanning Strategies

Uni- and birectional mode, Zoom, & Rotation

Unidirectional Scan



Fly Back Blanking, Zoom 1



Fly Back Blanking, Zoom 0.7

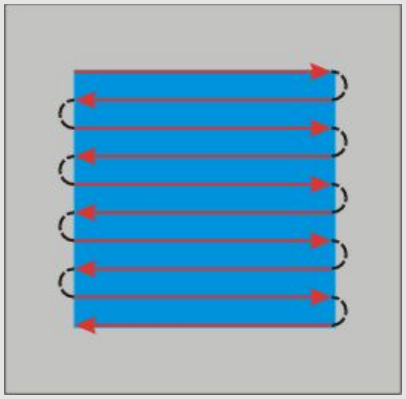
Fly Back Blanking, Zoom 1, Rotation 45°

Fly Back Blanking, Zoom 2, Rotation 45° , X,Y Offset

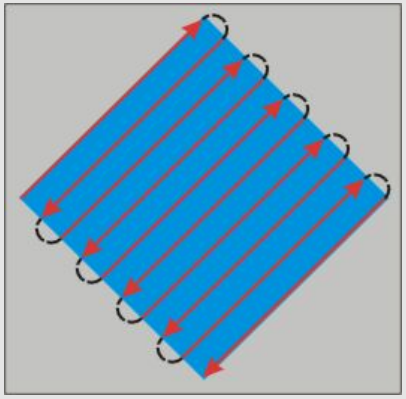
Real Time Processor synchronizes:

- AOTFs
- Scanner mirrors
- Data acquisition

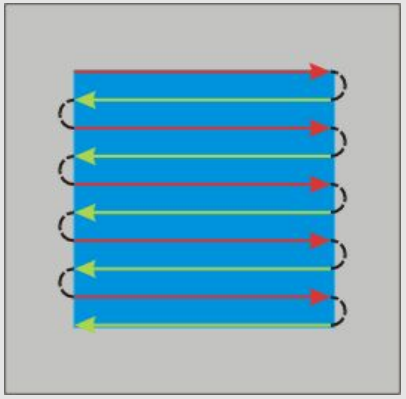
Bi-directional Scan



Bi-directional Scan, Zoom 1



Bi-directional Scan, Zoom 1 Rotation 45°

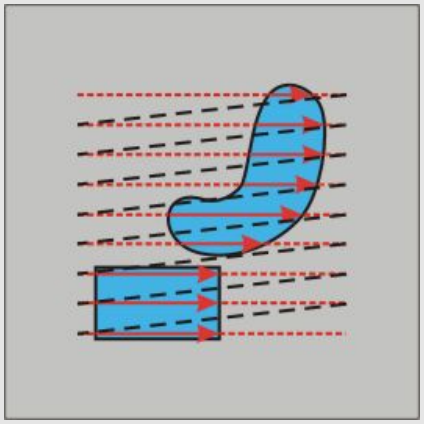


Bi-directional Scan, **Multitrack Configuration (!drawing!)*

Scanning Strategies

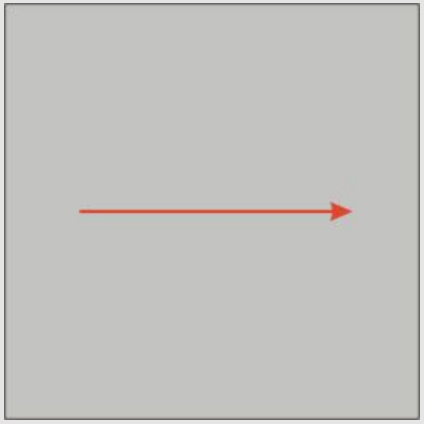
Regions of Interest, Line- and Spline scan, Tile scan

ROI Scan

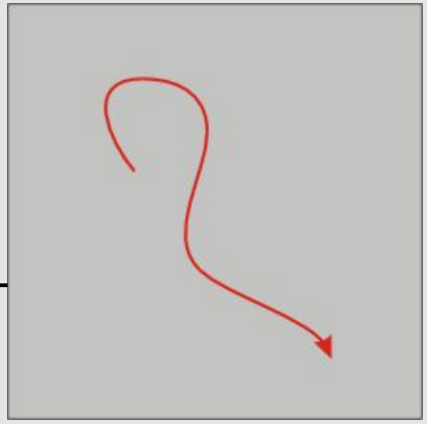


Selective excitation, bleaching, activation uncaging and data acquisition from user defined ROIs

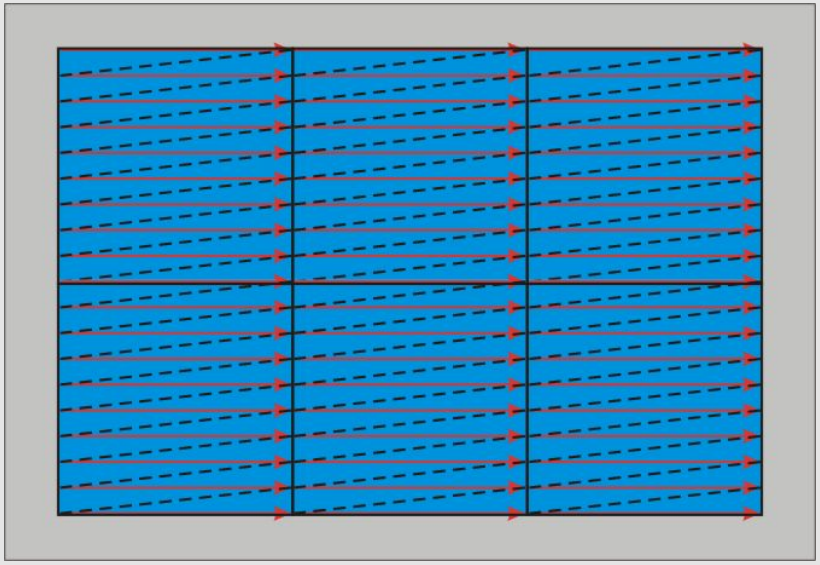
Line Scan



Spline Scan



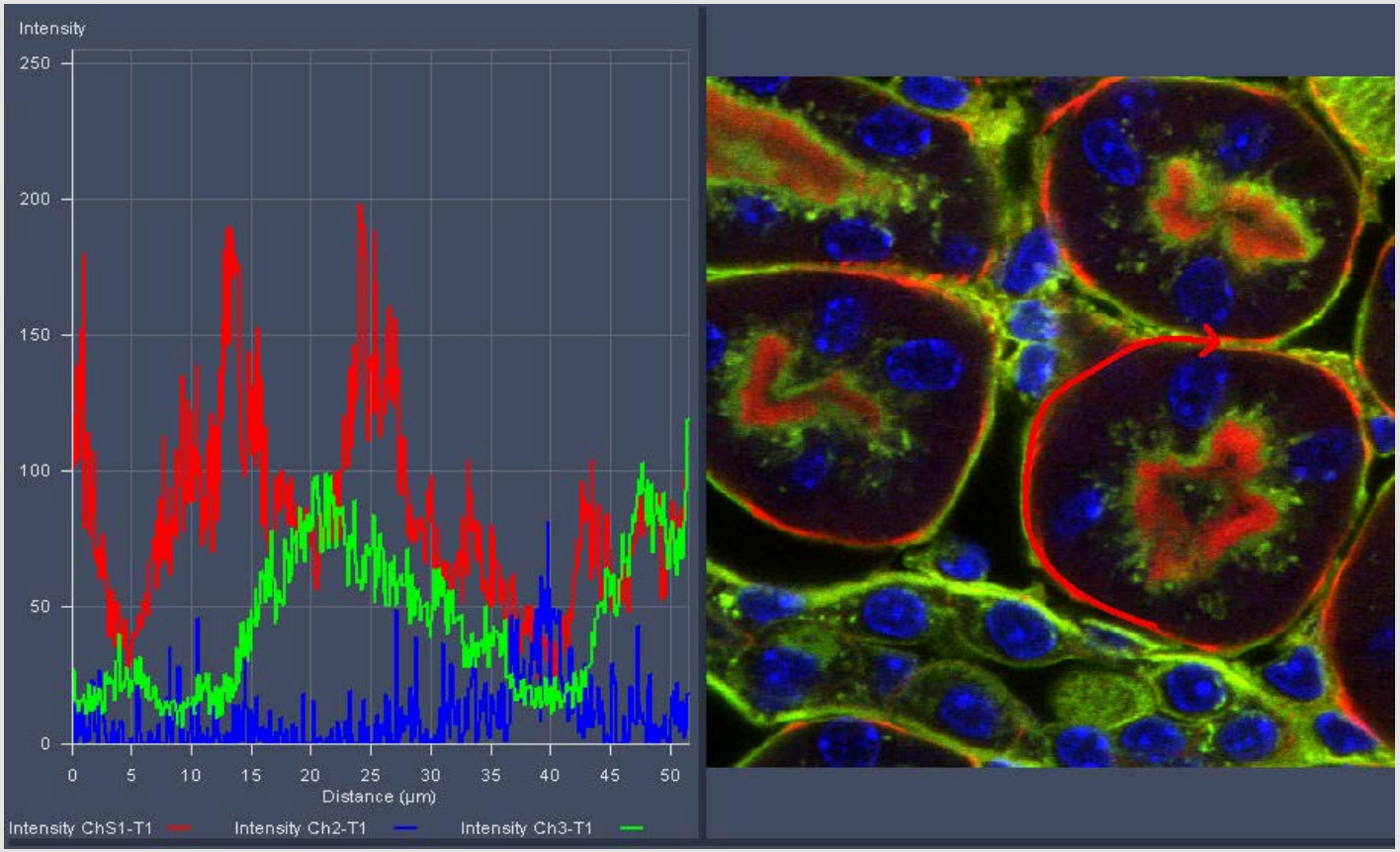
Tile Scan



With a motorized scanning stage single XY frames can be patched together for an overview image that exceeds the traditional single field of view

Scanning Strategies

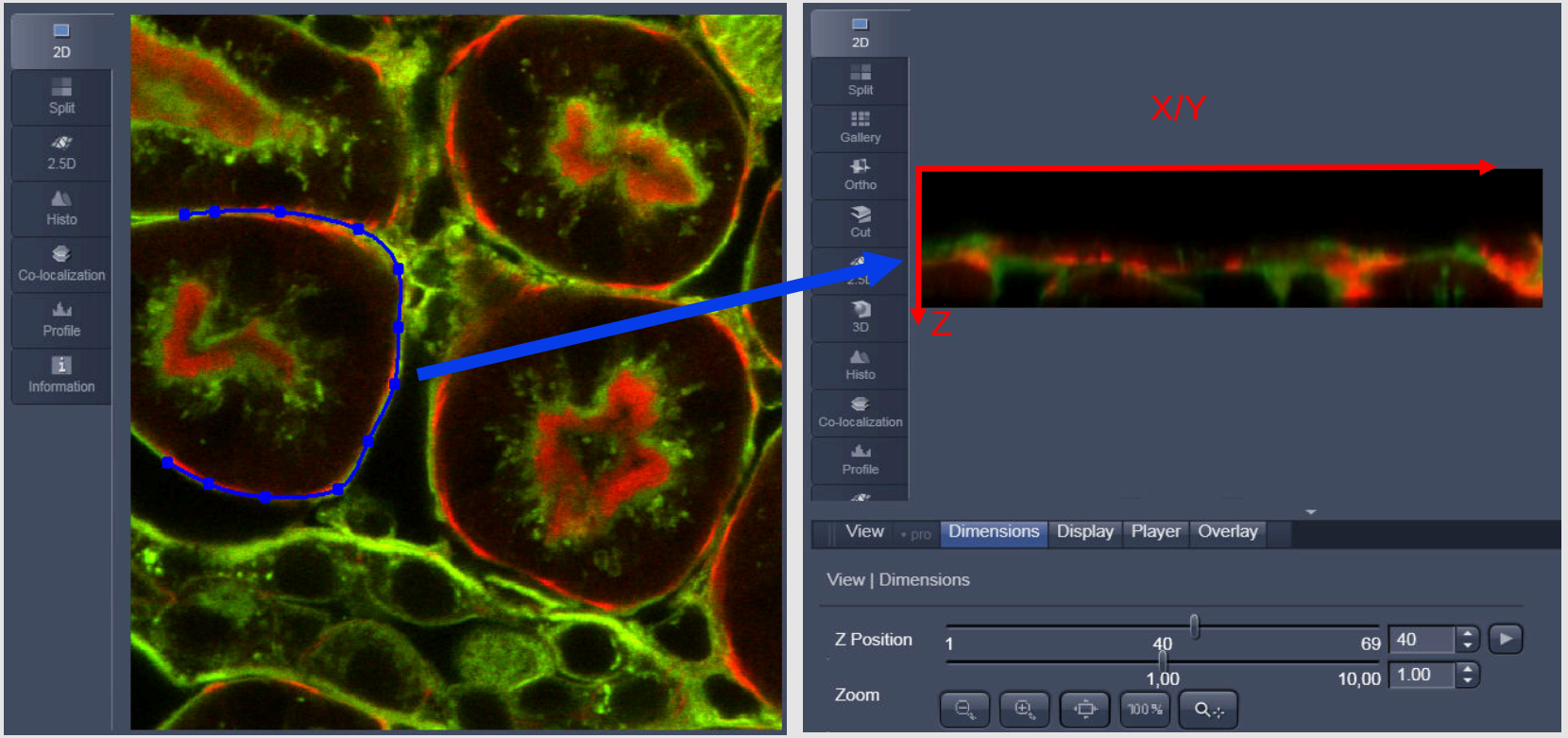
Regions of Interest, Line- and Spline scan, Tile scan



Spline Scan

Scanning Strategies

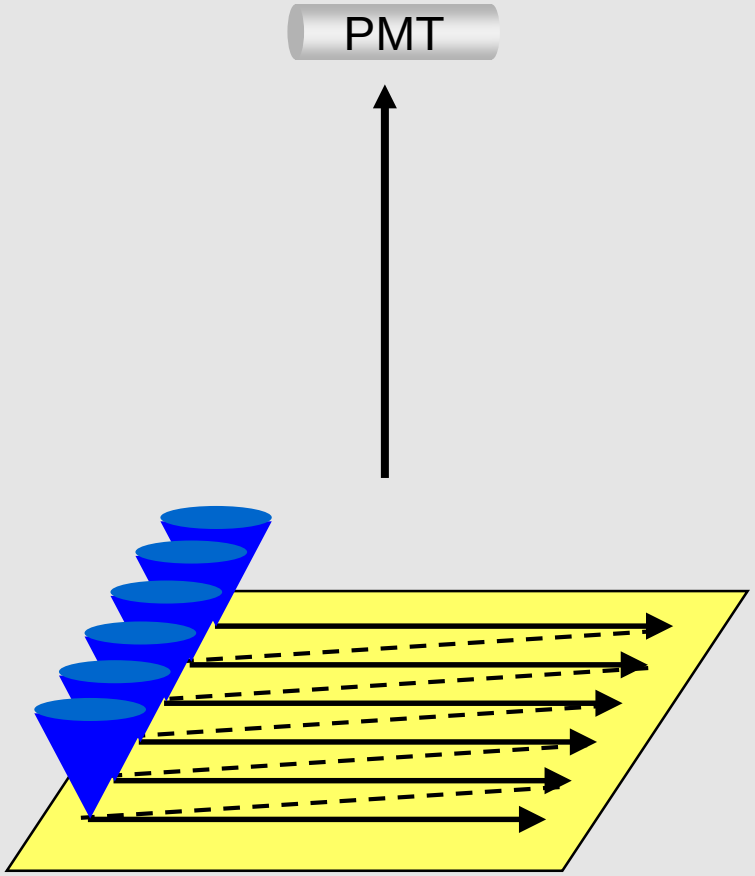
Regions of Interest, Line- and Spline scan, Tile scan



Spline Scan in Z

Scanning Strategies

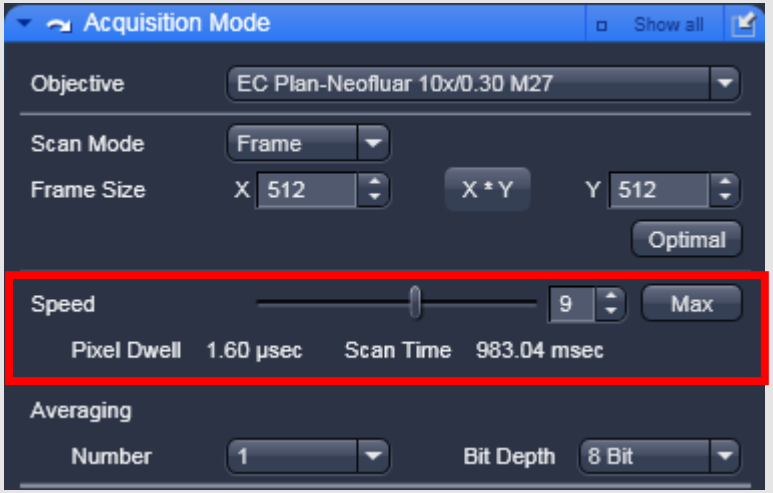
Speed and Averaging



Speed:

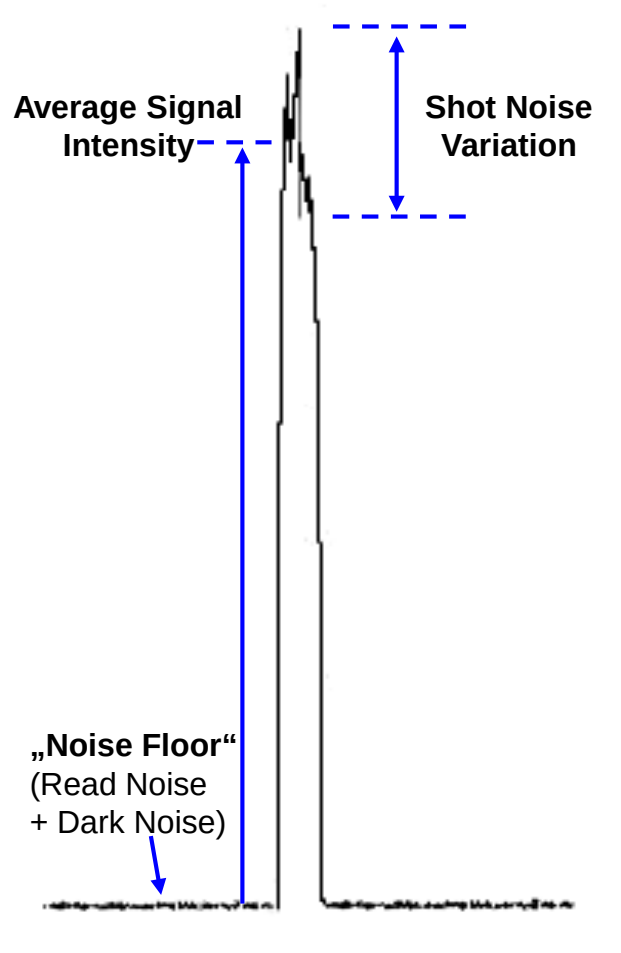
Application: Frames per second
→ Temporal Resolution

Image Quality: Pixel Dwell Time
→ How much time to collect photons for each image pixel



Scanning Strategies

Speed and Averaging

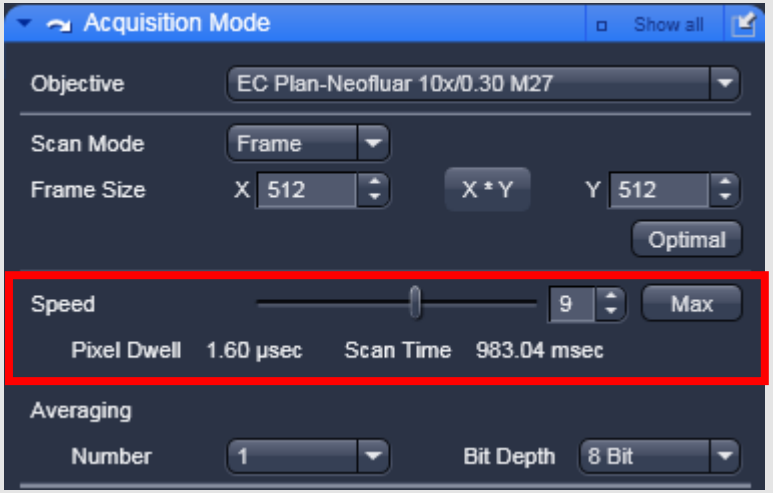


The lower the pixel dwell time (fast scan), less photons can be collected.

→ The Image becomes noisy, due to **shot noise**
→ Shot noise can be described with poisson statistics:

$$\textbf{Shot noise} = \sqrt{\textbf{Signal}}$$

→ Electronic noise is independent of scan speed

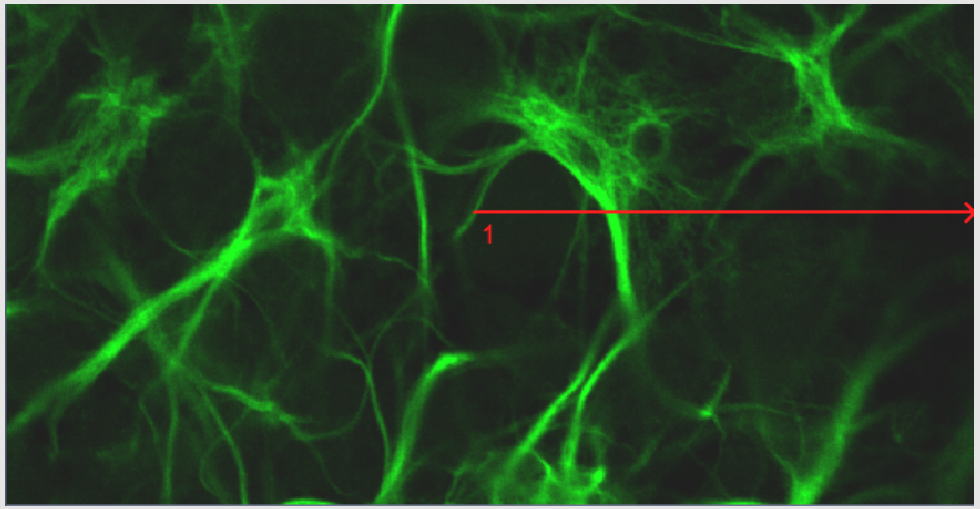


→ Detector Read Out: **40 MHz** (40 events / µsec) = Oversampling
→ Scan Speed influences SNR

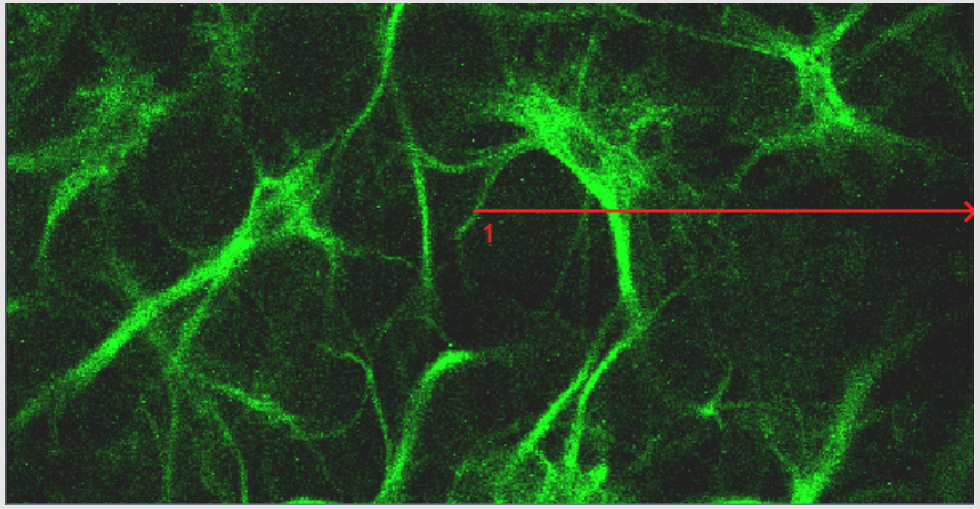
Scanning Strategies

Image Noise: What does it look like?

“Good” Image

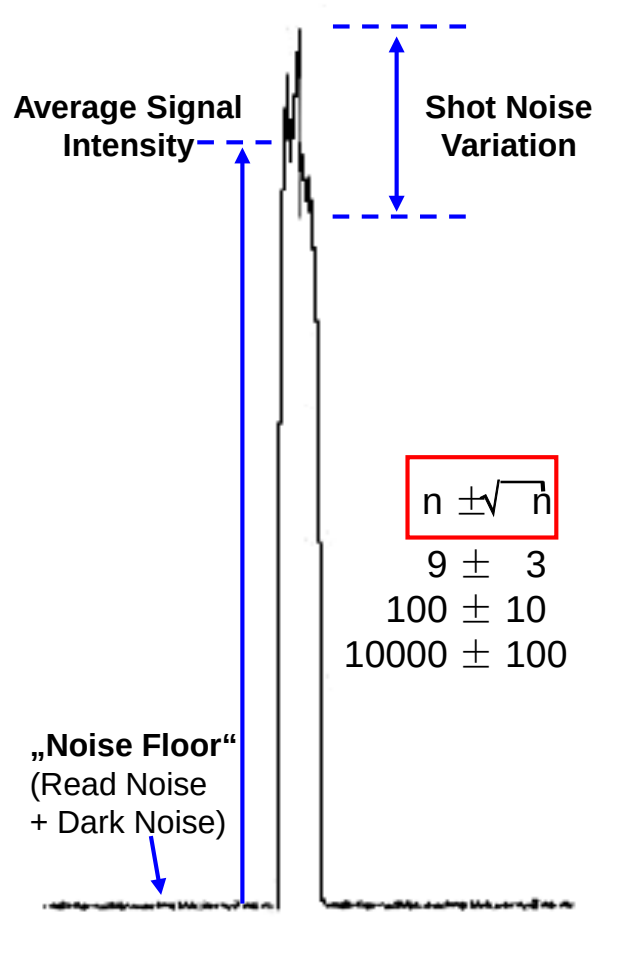


“Bad” Image



Scanning Strategies

Speed and Averaging



Shot noise = $\sqrt{\text{Signal}}$

→ Shot noise will increase with signal intensity, BUT Signal / Shot noise ratio will improve also

$$\textbf{Signal / Shot noise} = \frac{\textbf{Signal}}{\sqrt{\textbf{Signal}}}$$

→ say 9 photons fall within a pixel during a given pixel dwell time; from pixel to pixel the Shot noise uncertainty is 3

$$\textbf{Signal / Shot noise} = \frac{9}{\sqrt{9}} = 3$$

→ significant improvement of Signal / Shot noise if 100 photons fall within a pixel

$$\textbf{Signal / Shot noise} = \frac{100}{\sqrt{100}} = 10$$

Scanning Strategies

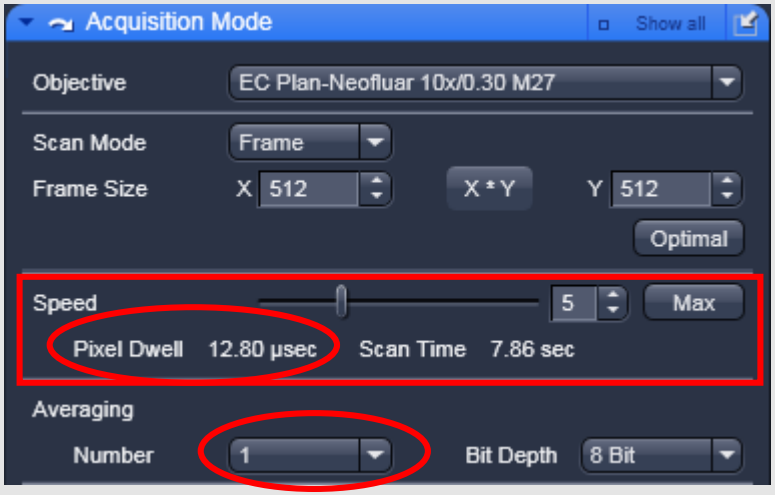
Speed and Averaging

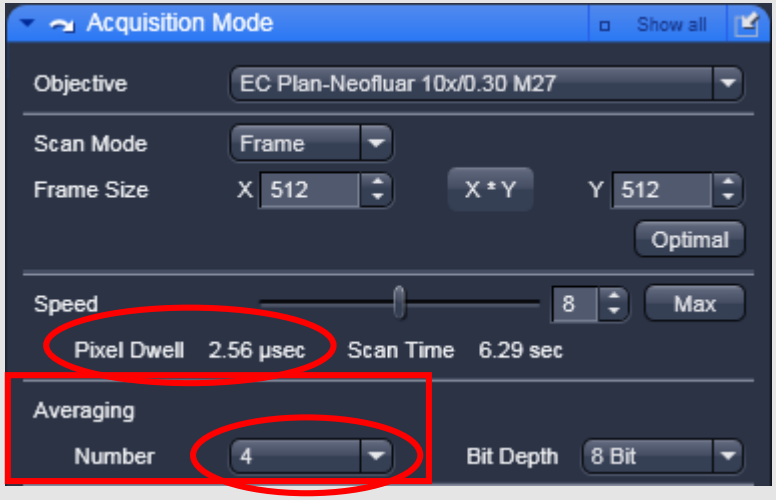
To decrease the effect of noise, more photons (signal) must be collected:

1) **Slower Scan Speed**

2) **Averaging**
   Scan the image x-times and take the average signal for each pixel
   -> addition of photons from several scanning runs

Which of the two setups will lead to better image quality?

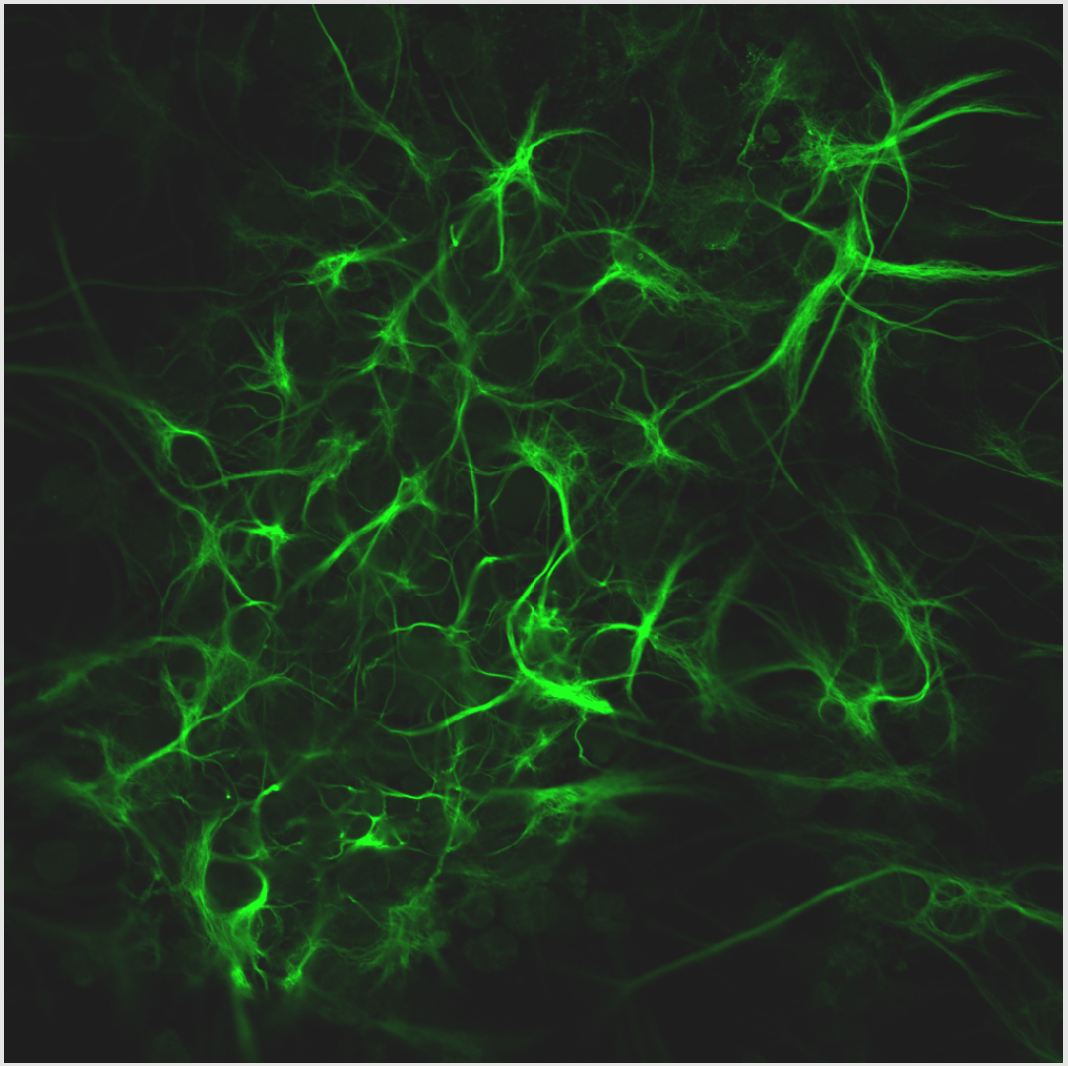




How to set up the optimal values

A good illuminated image

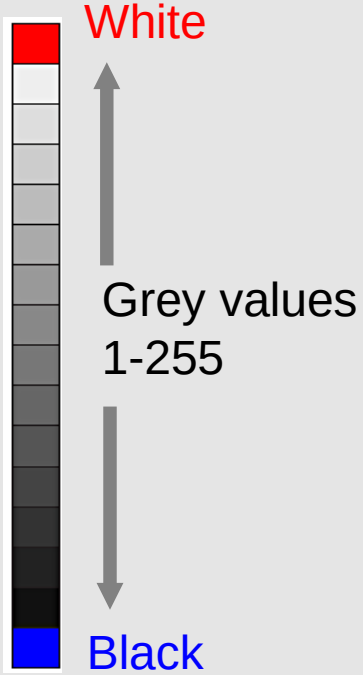
Illumination:
Not too bright,
Not too dimm



Range Indicator

How to evaluate the dynamic range the best

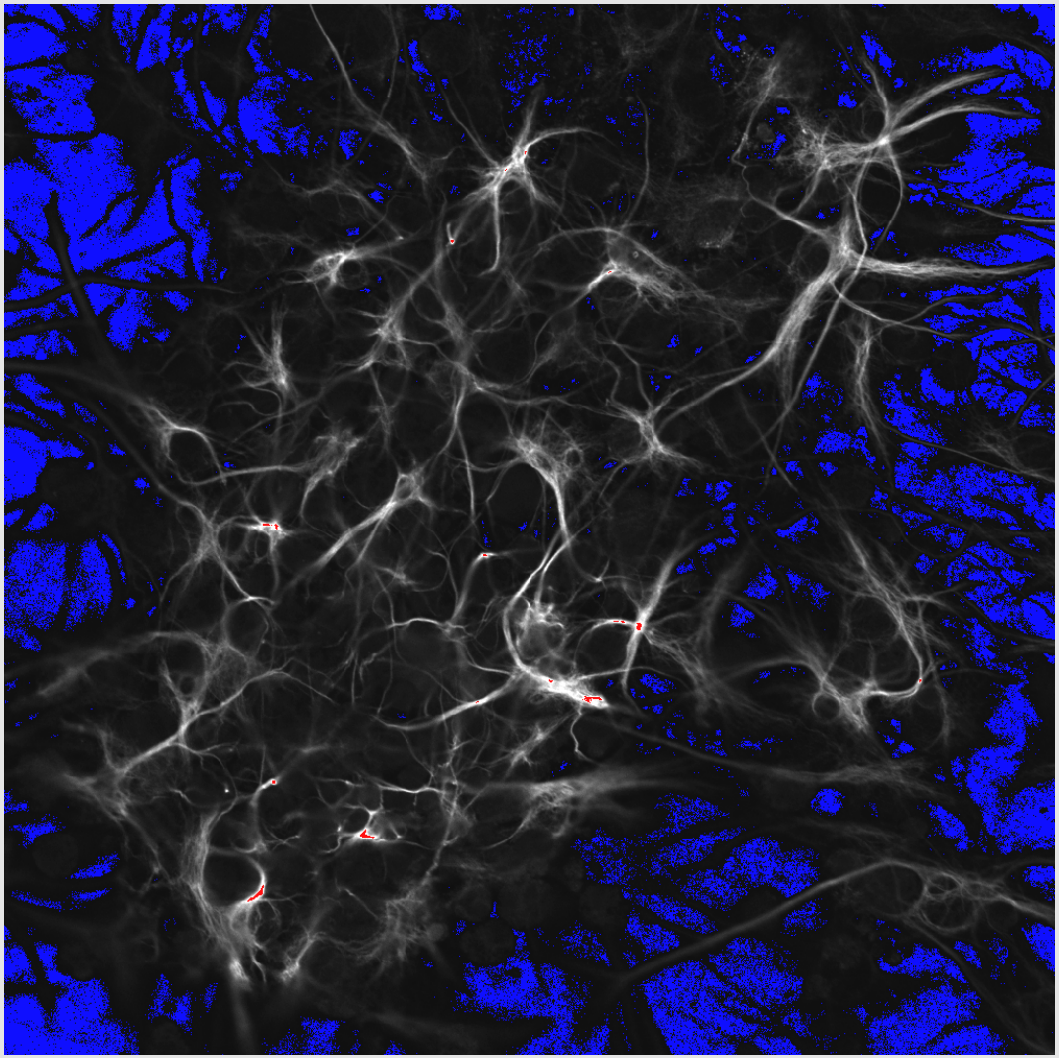
Look-up table
Range Indicator (8bit)



Grey value 256
White

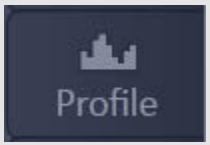
Grey values
1-255

Black
Grey value 0

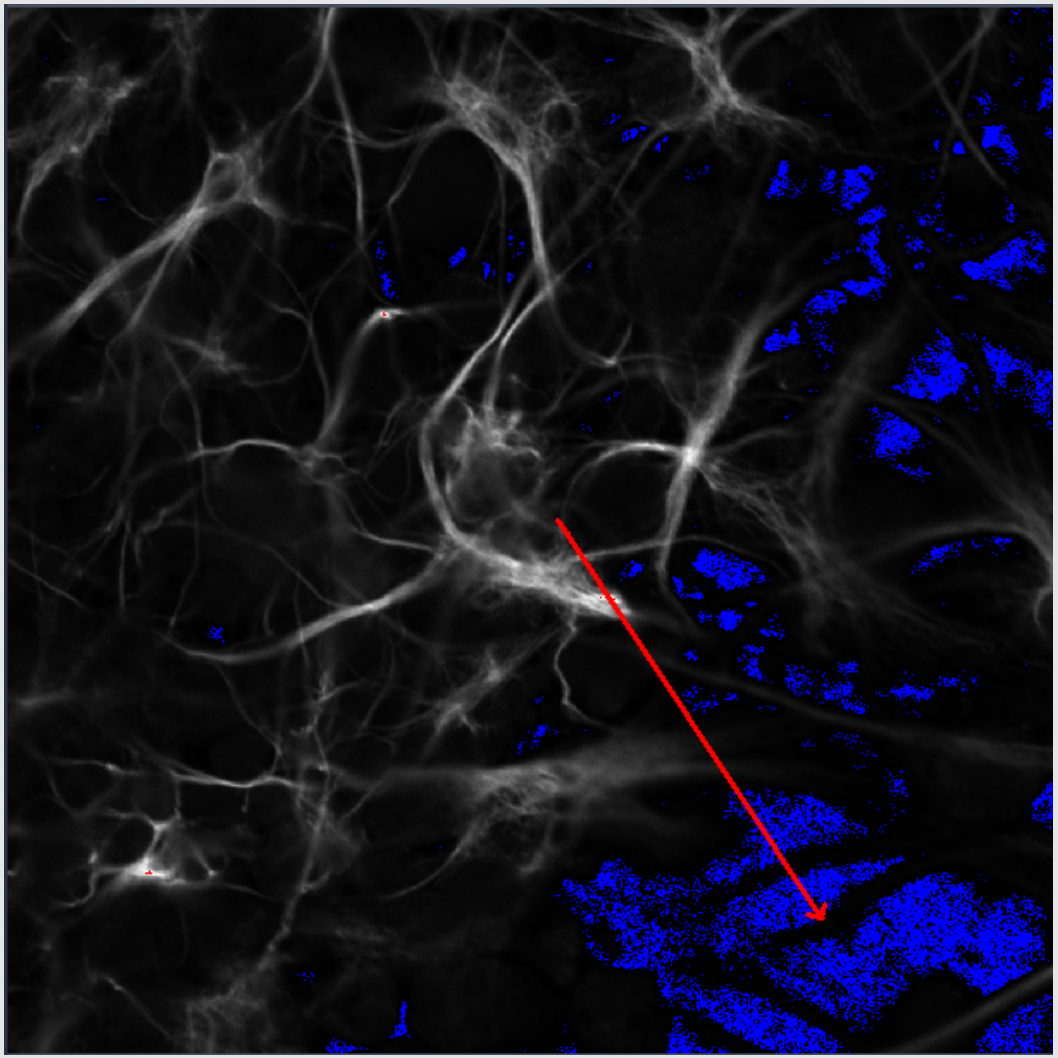


Profile

How to measure the dynamic range

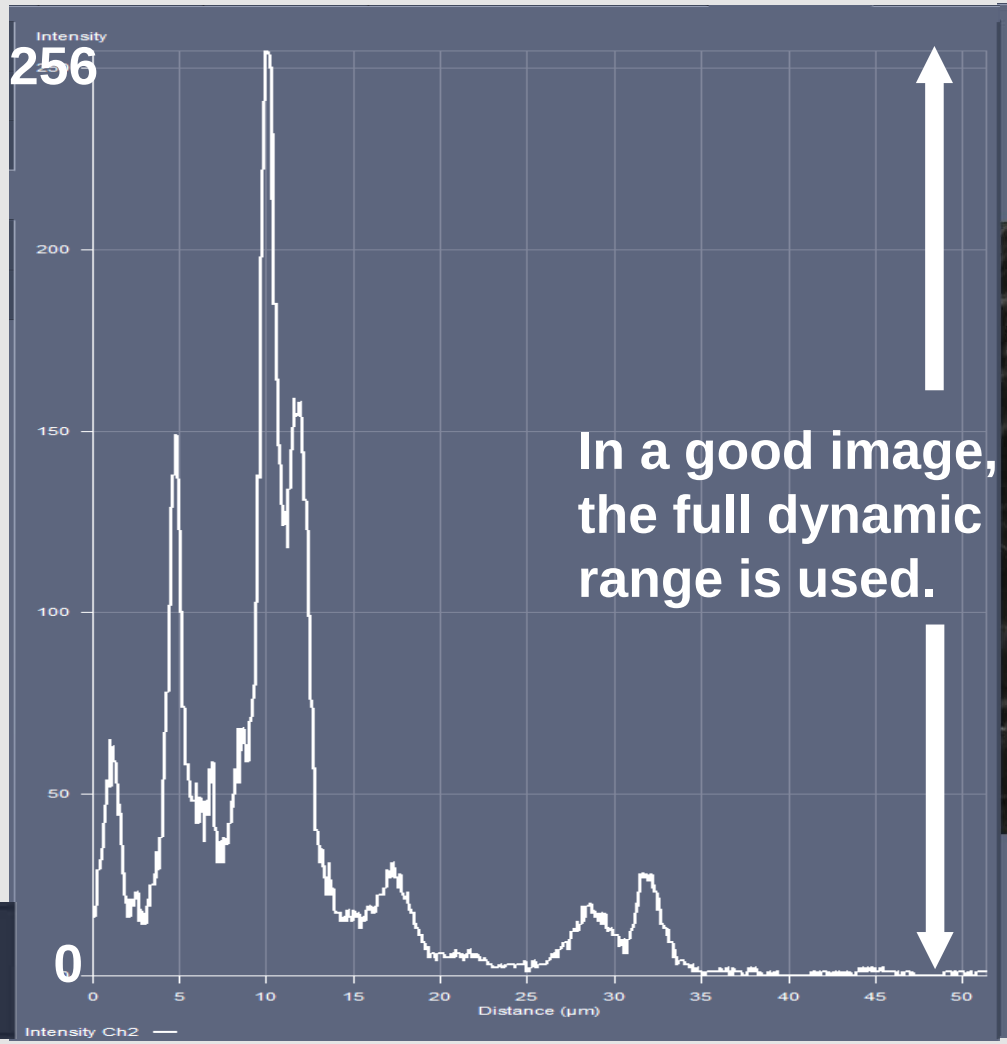


= gives measurements of grey values along a line

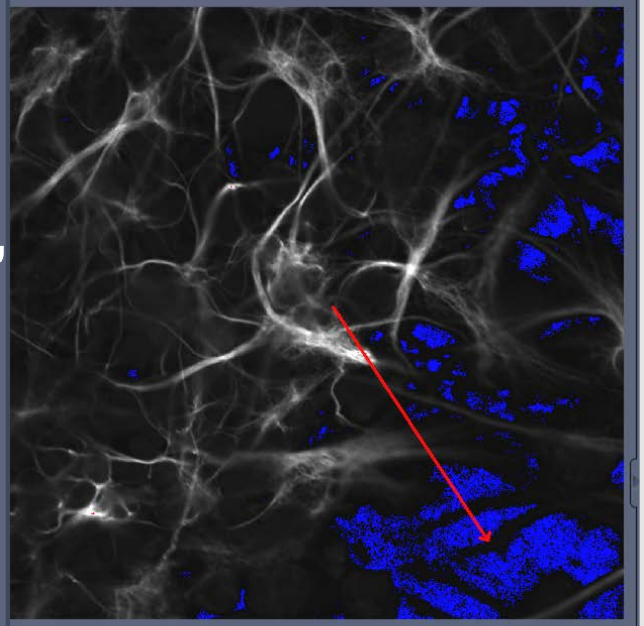


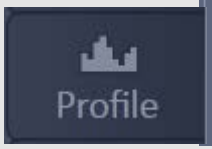
Profile

How to measure the dynamic range



In a good image, the full dynamic range is used.



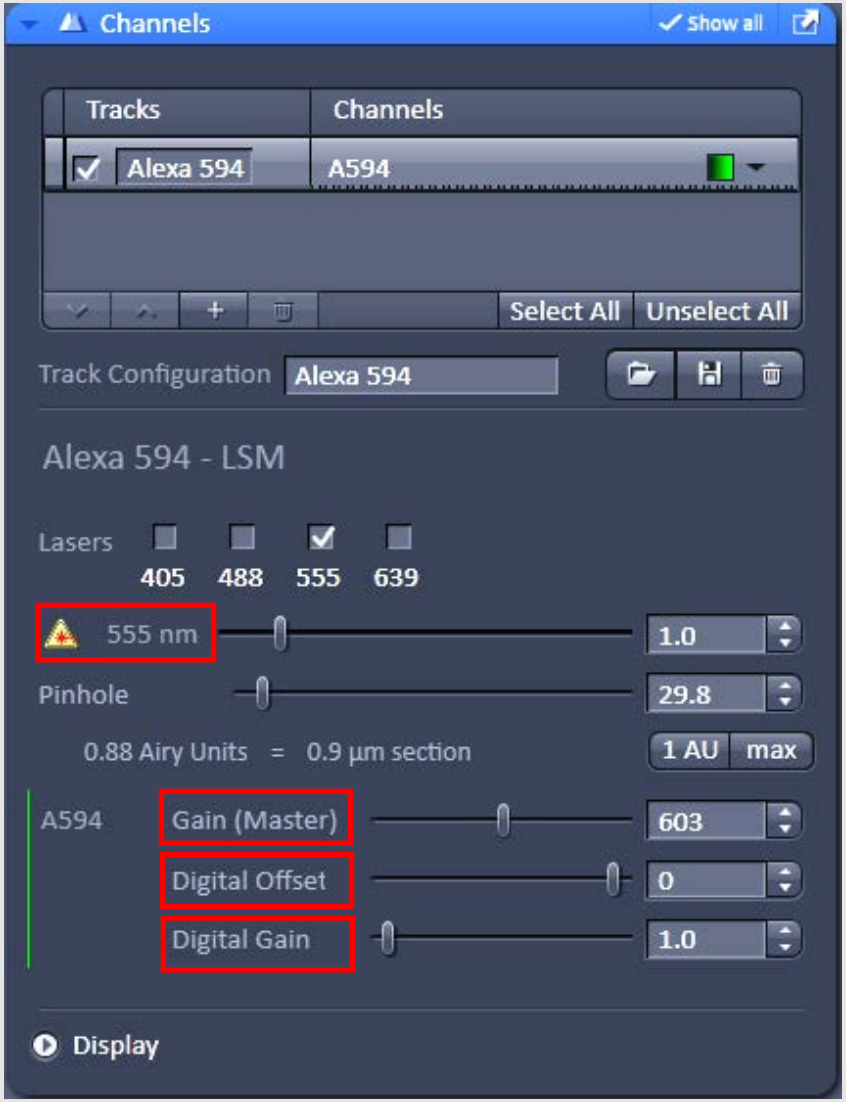


Setting up the perfect Image

The Detector and Laser settings

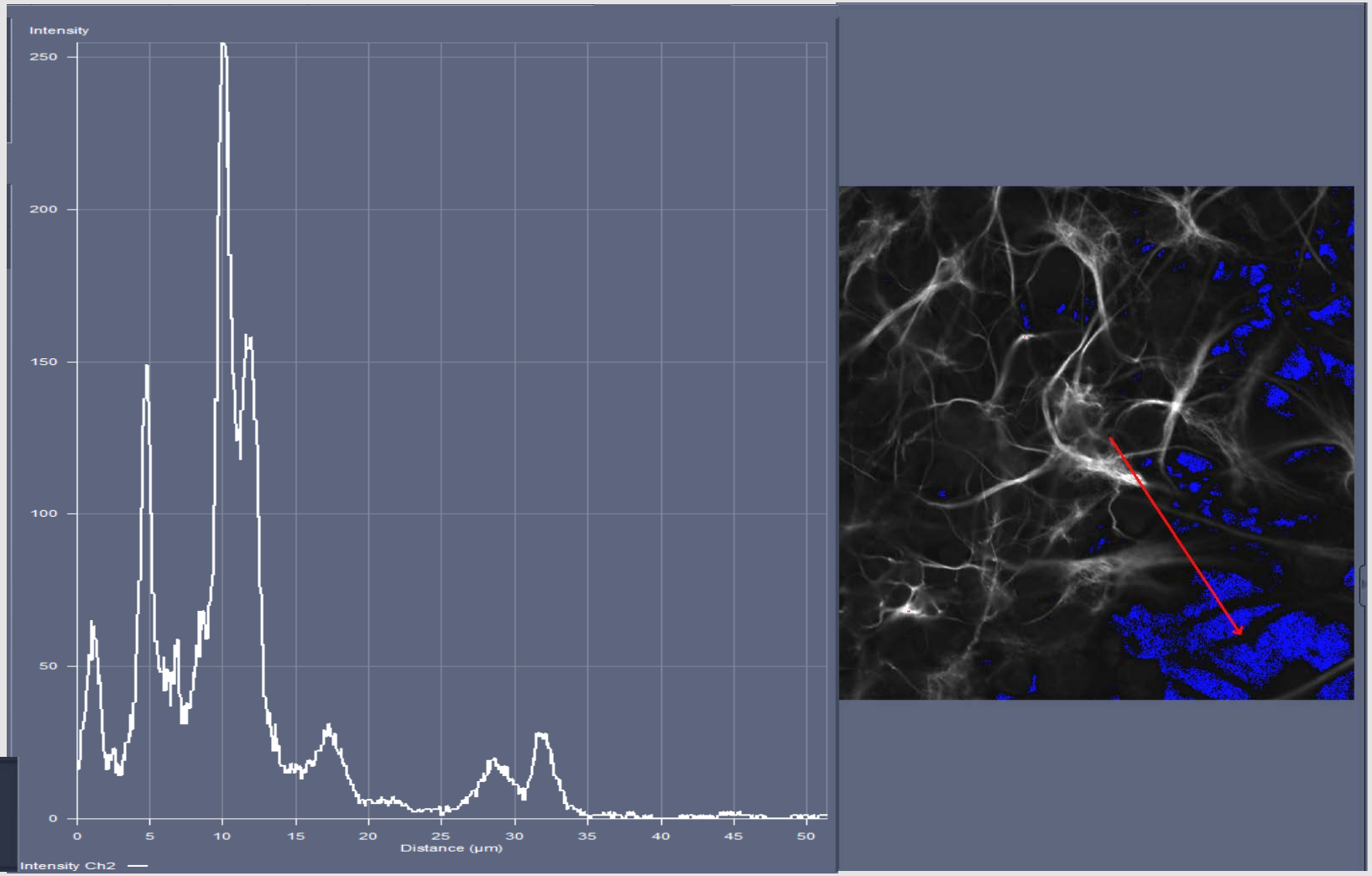
The main tools are:

- Laser power
- Gain (Master)
- Digital Offset
- Digital Gain



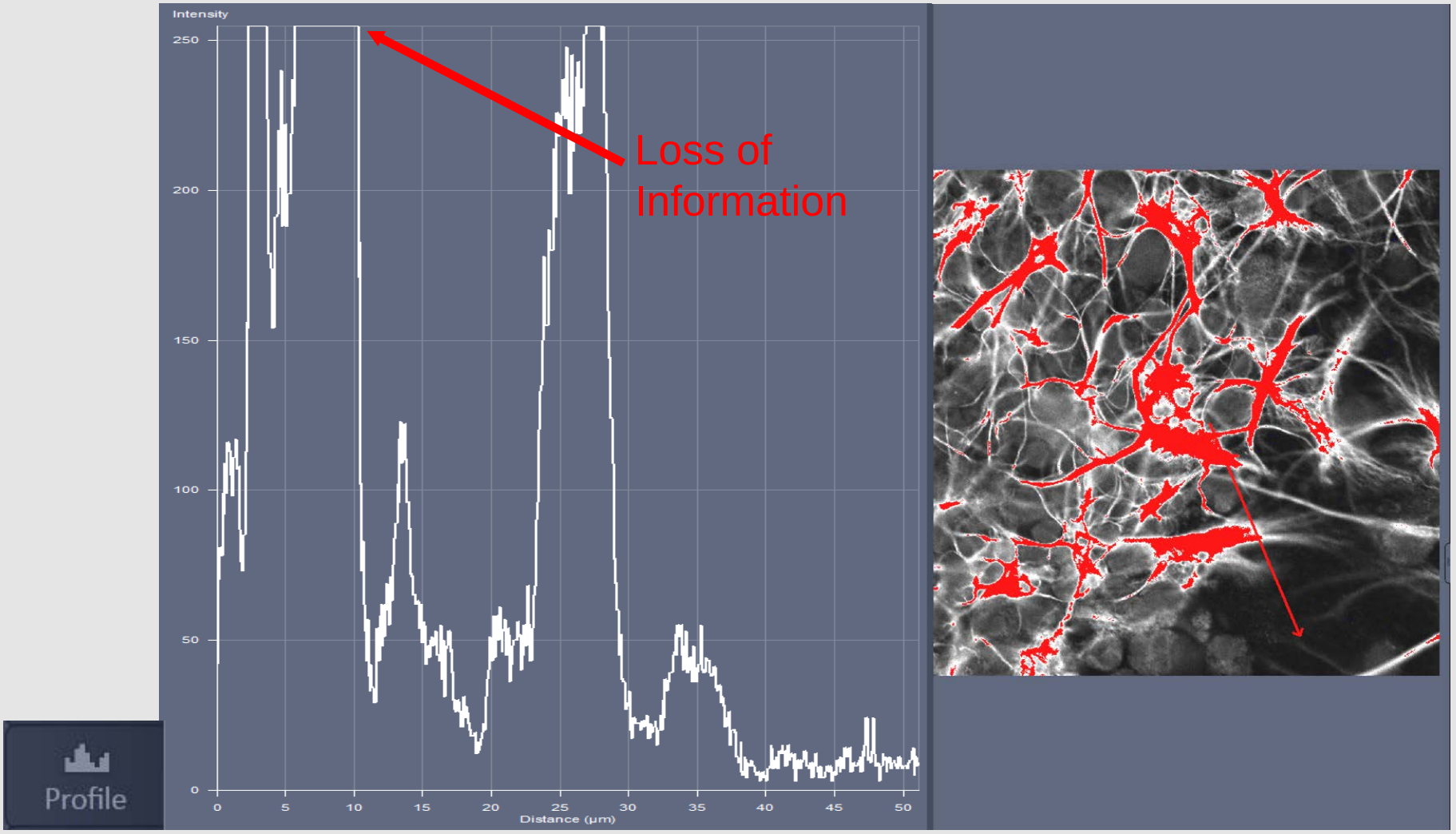
Gain (Master)

Set Correctly



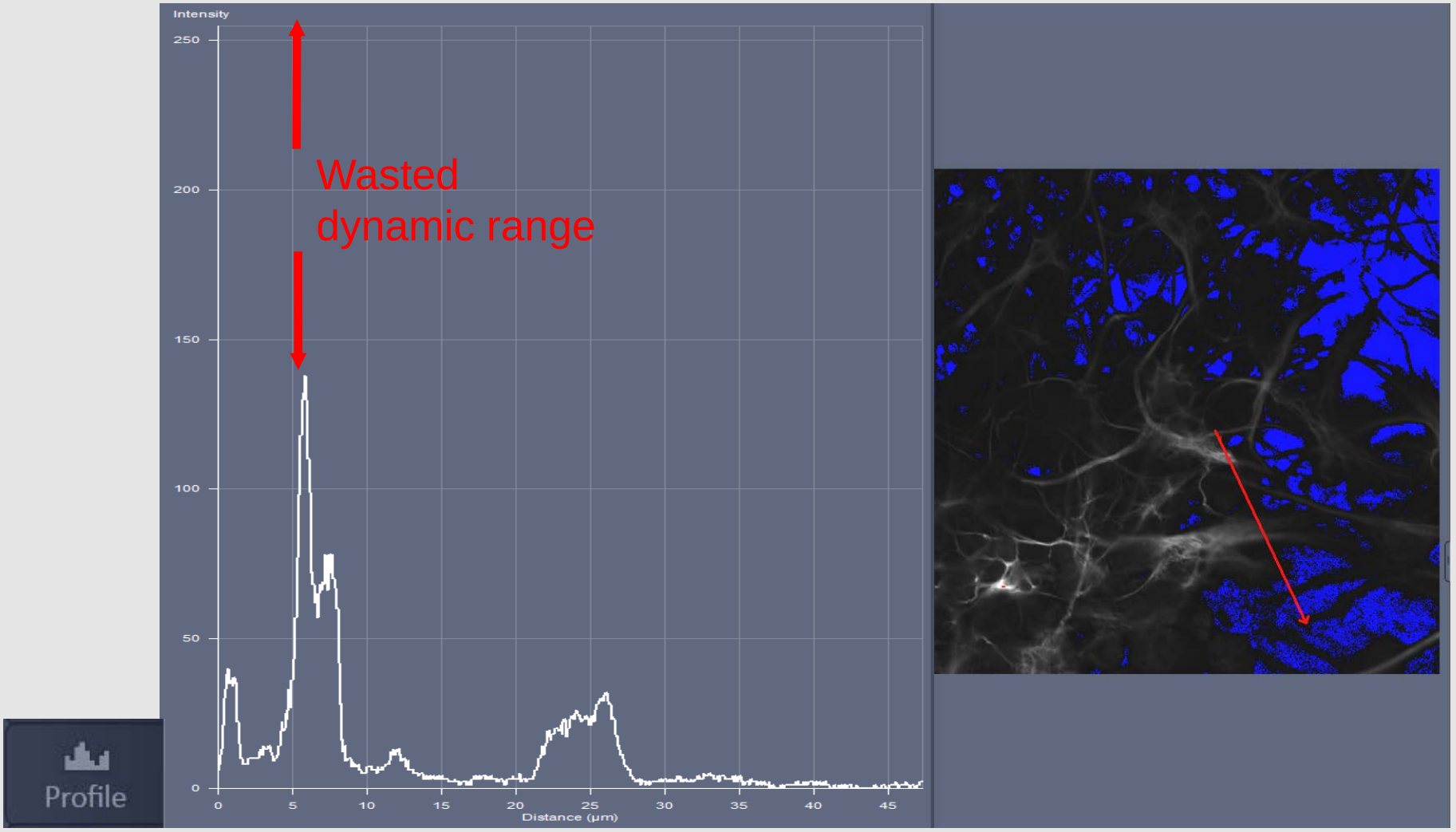
Gain (Master)

Set too high



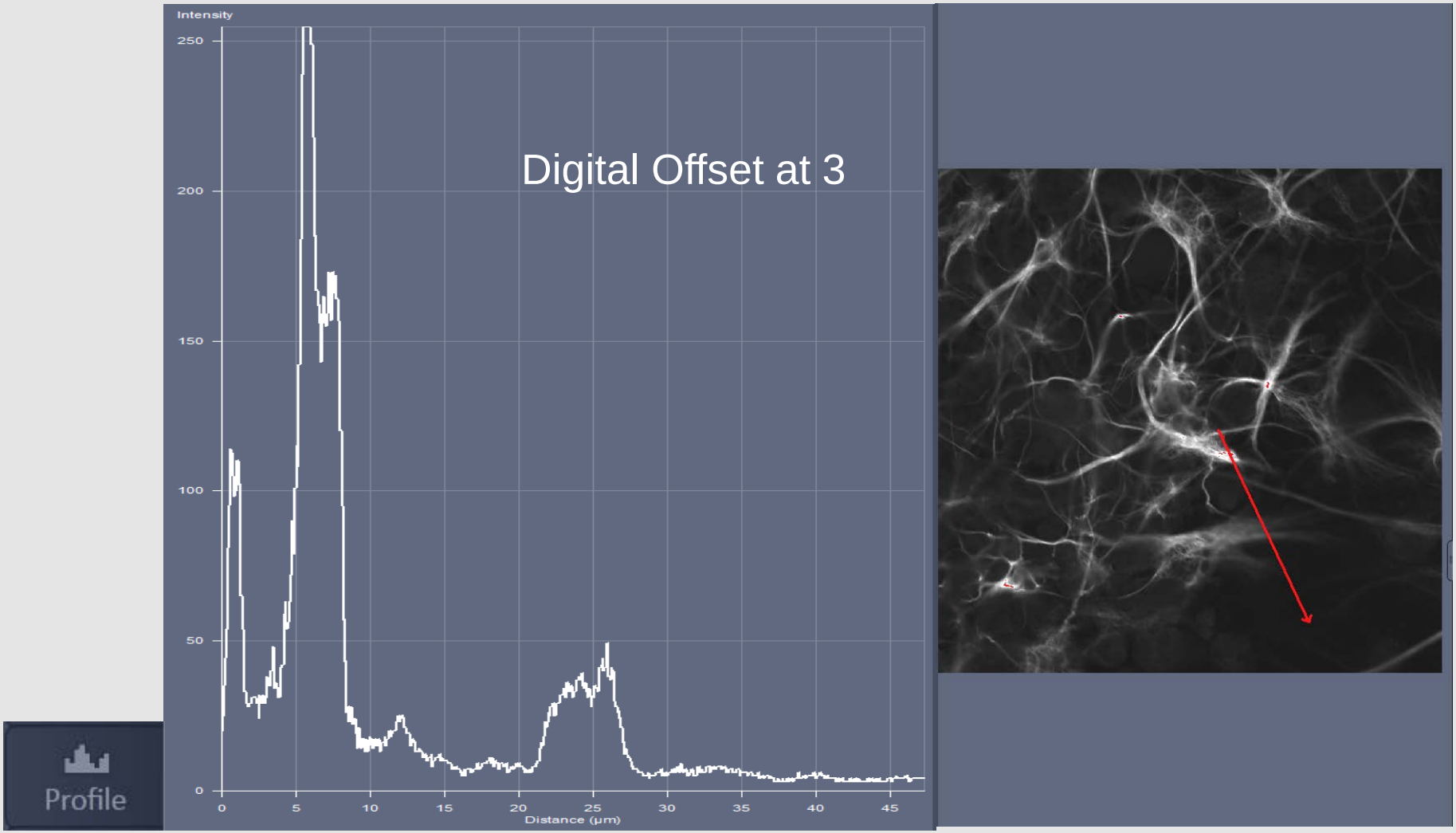
Gain (Master)

Not enough



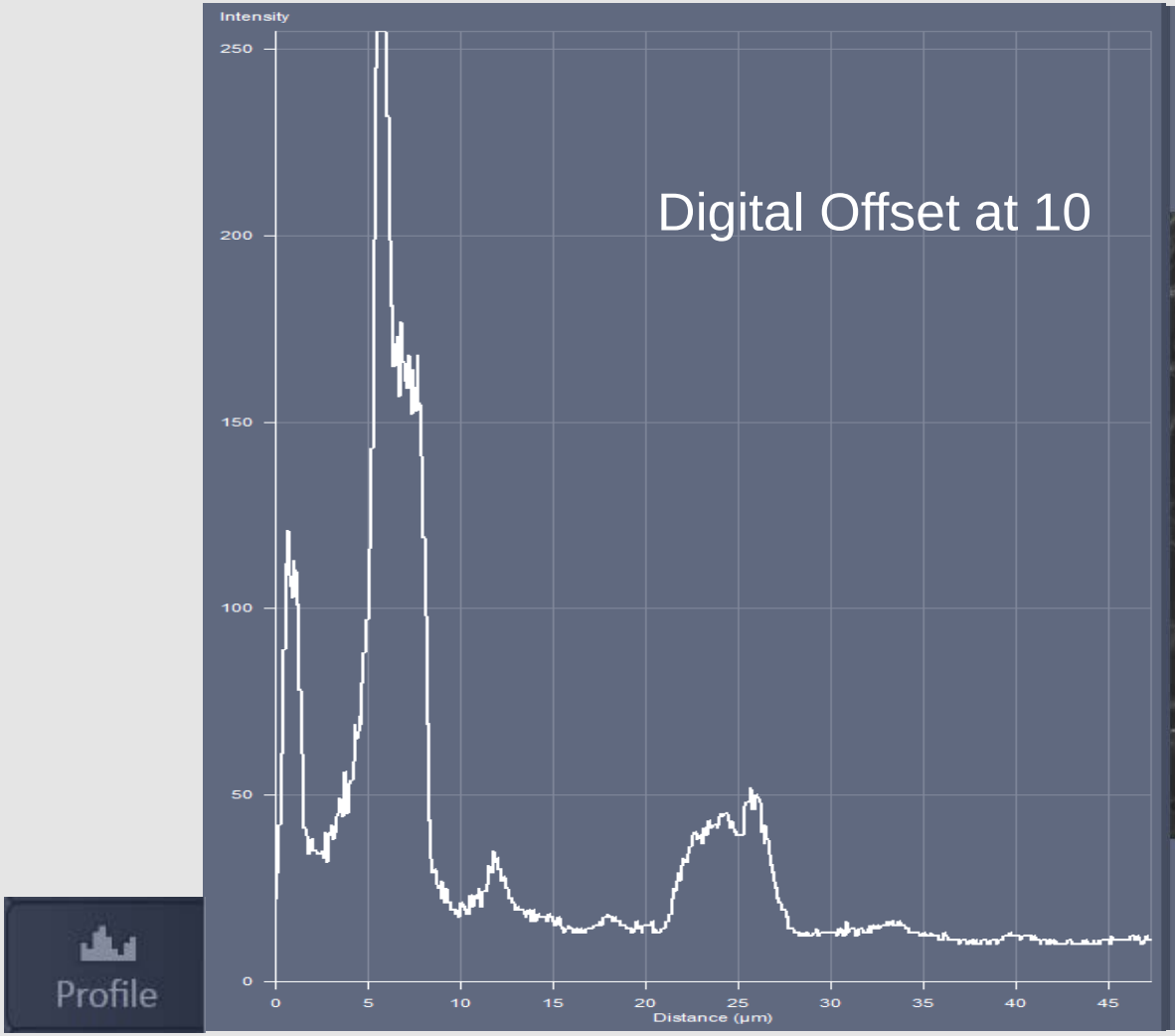
Use of Digital Offset

Enhances less bright details

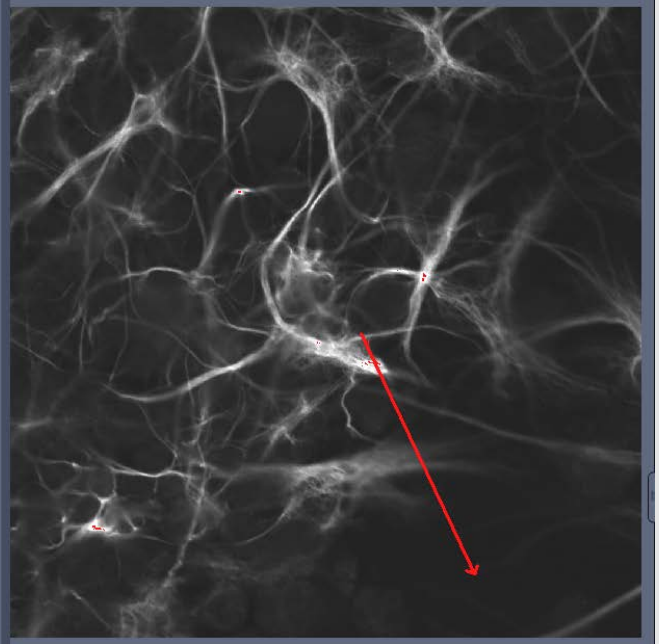


Use of Digital Offset

Enhances less bright details

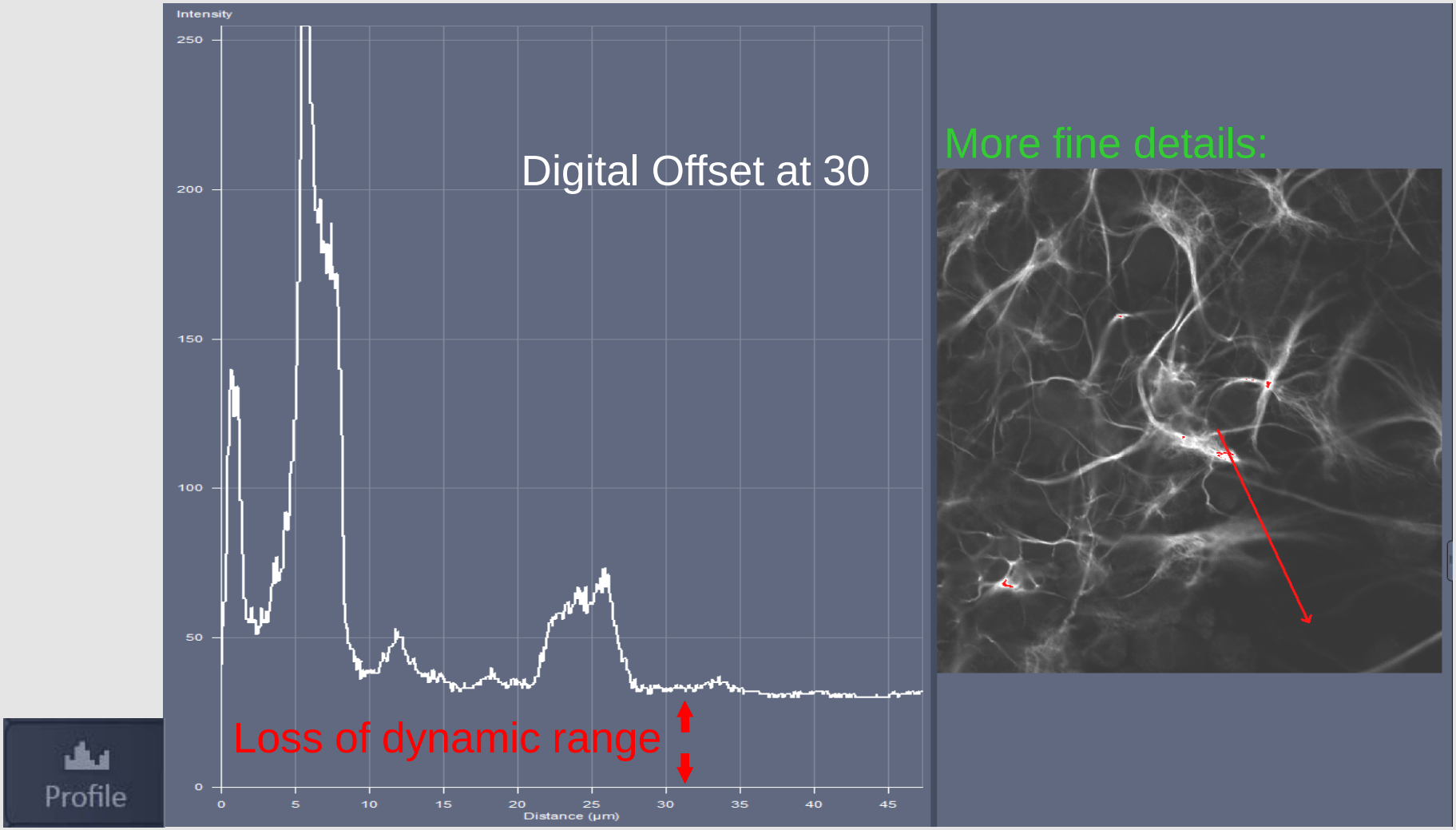


More fine details:



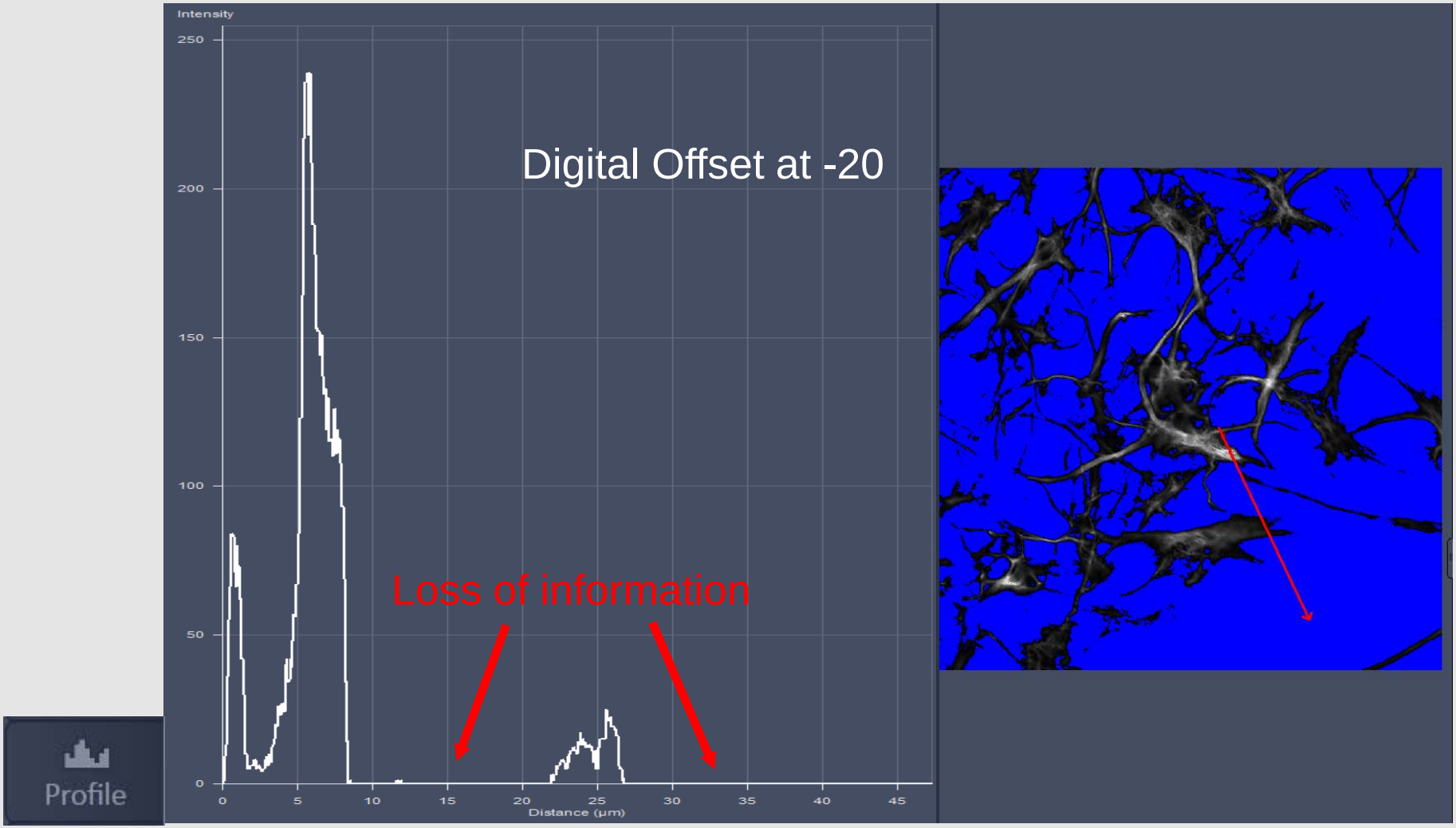
Use of Digital Offset

Too high



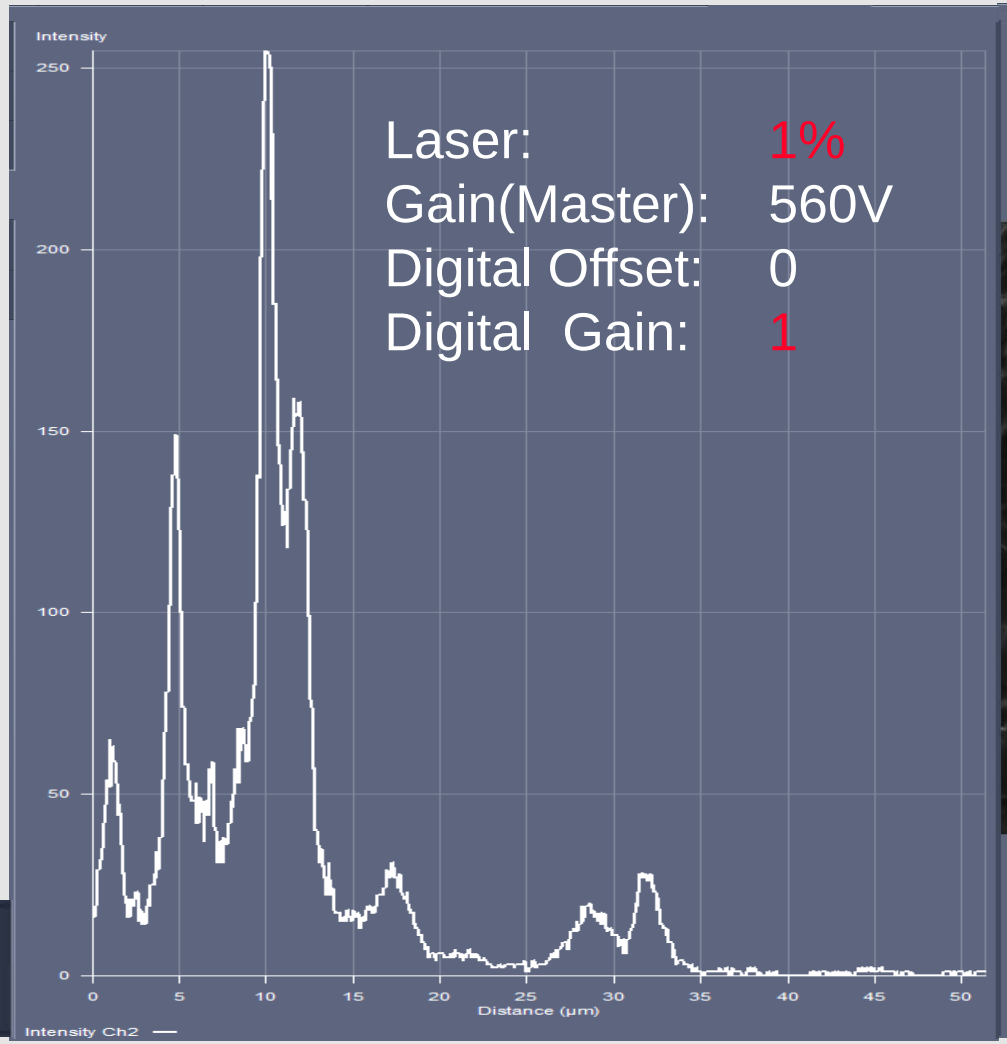
Use of Negative Digital Offset

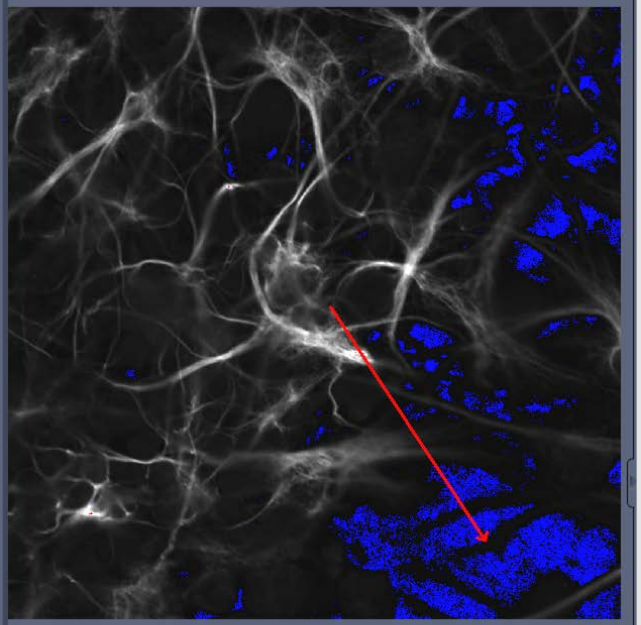
Reduces Background

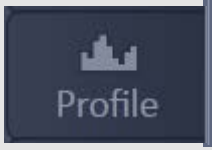


Use of Digital Gain

Increases the Signal (and saves Laser Power)







Use of Digital Gain

Increases the Signal (and saves Laser Power)

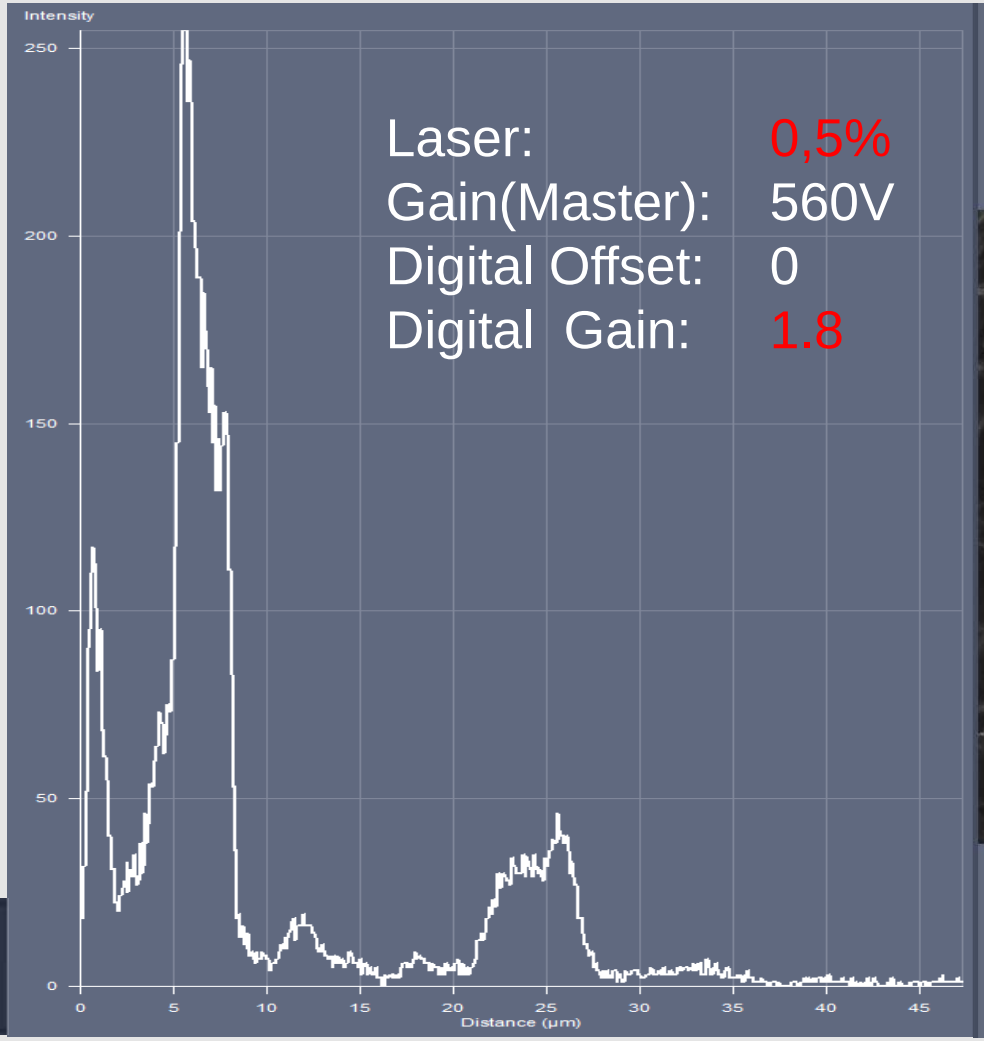
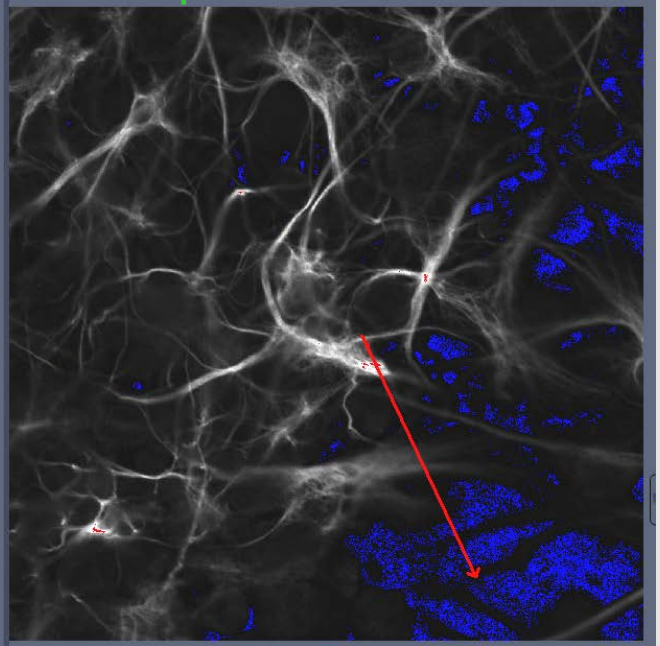


Image still fine with half Laser power



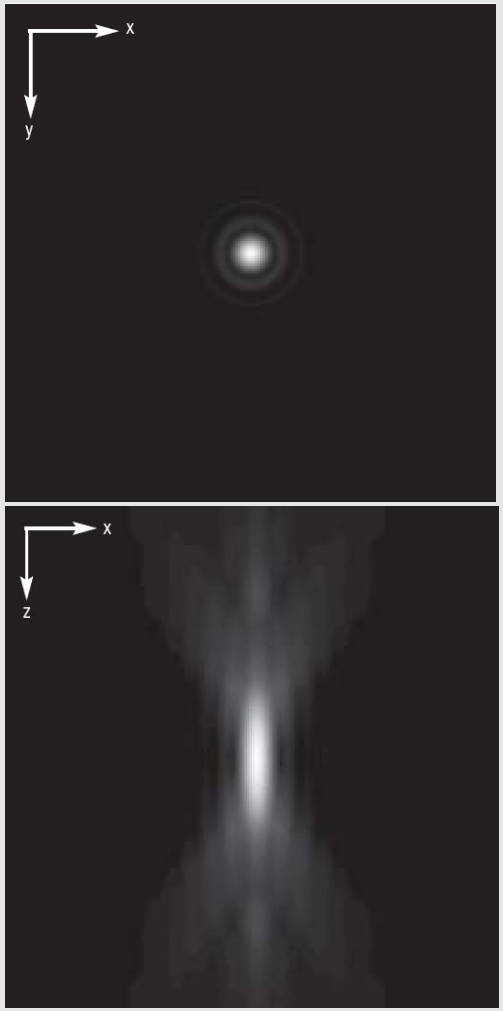
Profile

Agenda

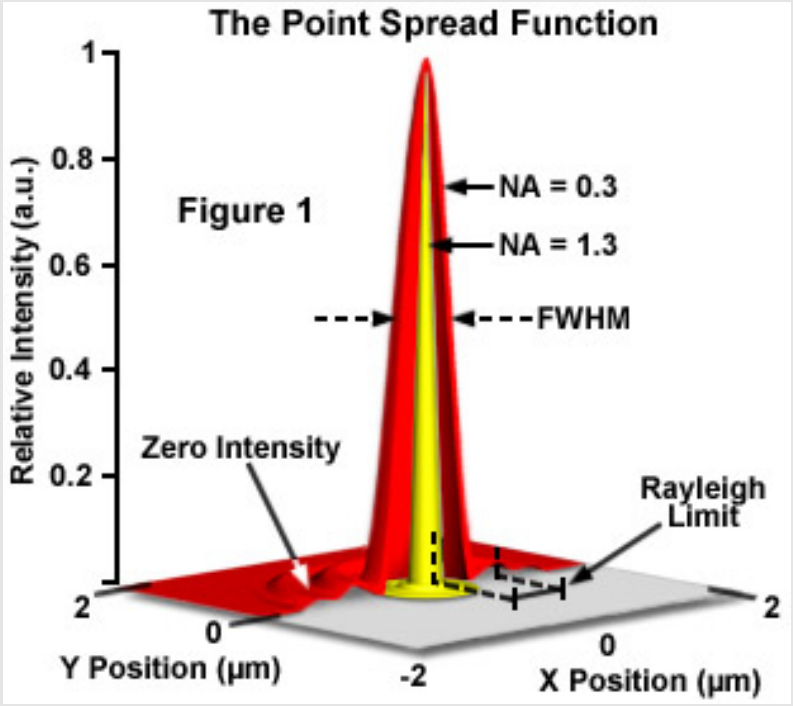
1	Confocal Principle
2	Innovative Beam Path Technology
3	Confocal Imaging: Images and Z-Stacks
4	Scanning Strategies
5	Resolution: Point Spread Function
6	Evaluating an Image

Resolution: Point Spread Function

3D Diffraction Pattern of Light



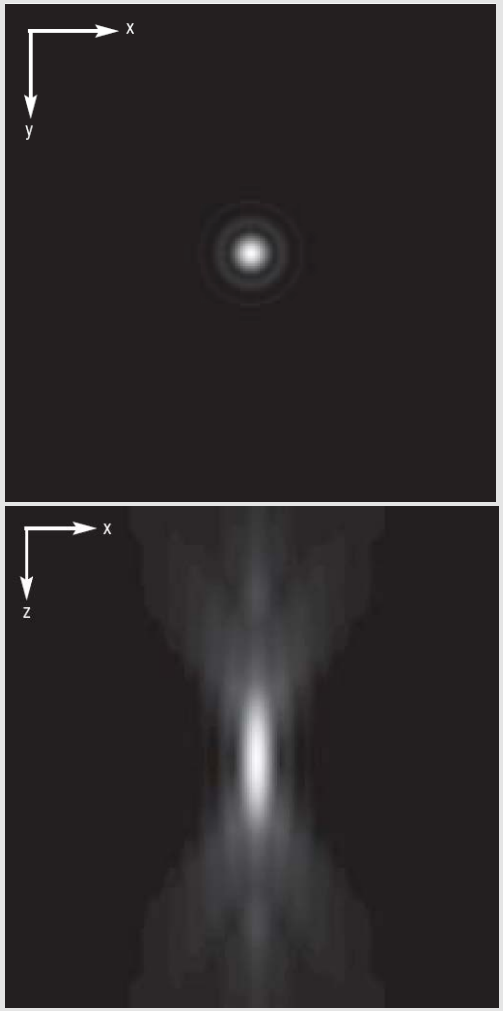
Three-Dimensional Intensity Distribution

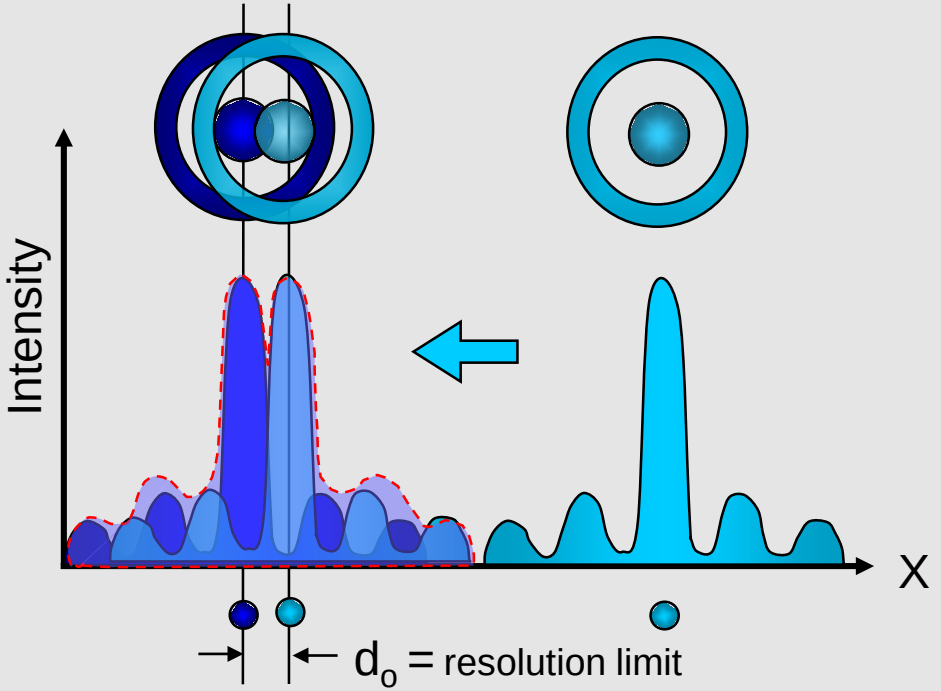


PH is set optimally if it matches the diameter of the Airy Disc.

Resolution: Point Spread Function

The limit up to which two small objects are seen as separate



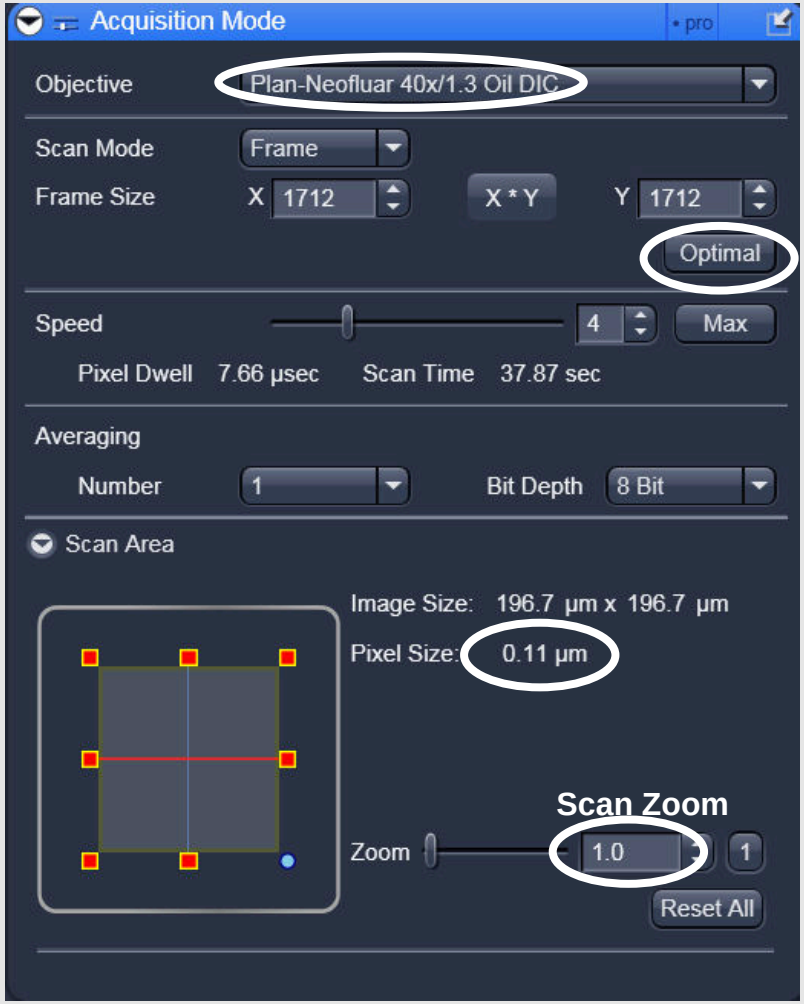


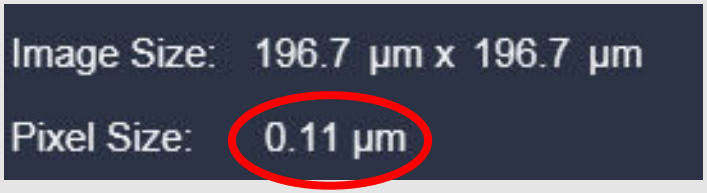
$$FWHM_{ill,lat} = \frac{0.51 * \lambda_{em}}{NA}$$

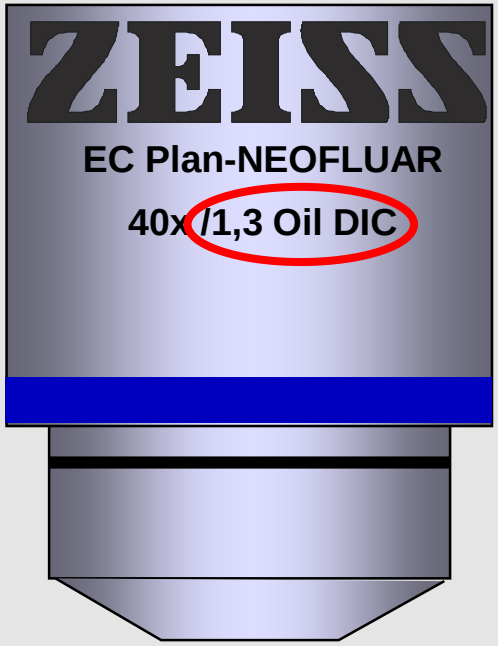
FWHM = Lateral Resolution [µm]
NA = Objective Numerical Aperture
λ_{em} = Emission Wavelength [nm]

Resolution

Information given in the Software





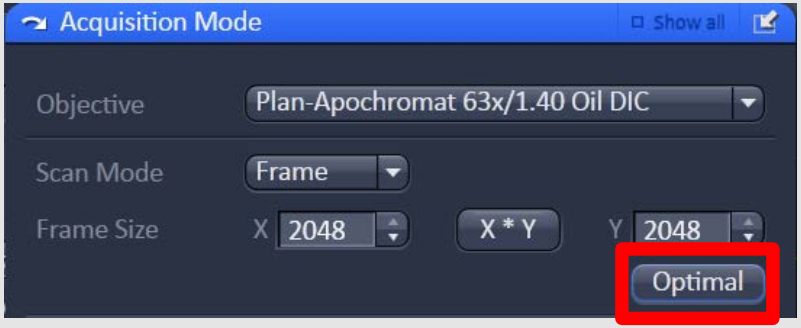


Resolution and Application

Empty Magnification

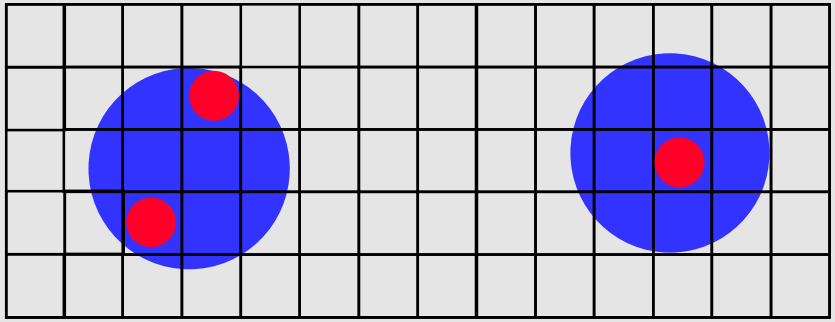
Frame size defined in the software:

Sets the frame size according to the achievable resolution
The frame size depends on Zoom factor and Objective



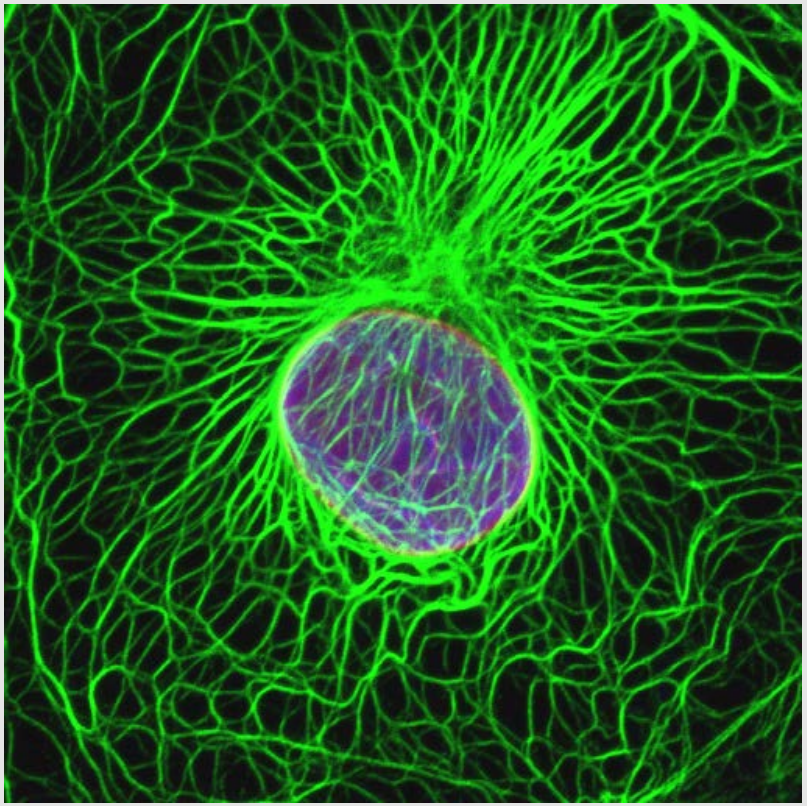
+ No information is lost
+ Optimal sampling achieved
- Scan Time takes longer

Pixel size is **optimal** for optical resolution
→ but what about the structure?

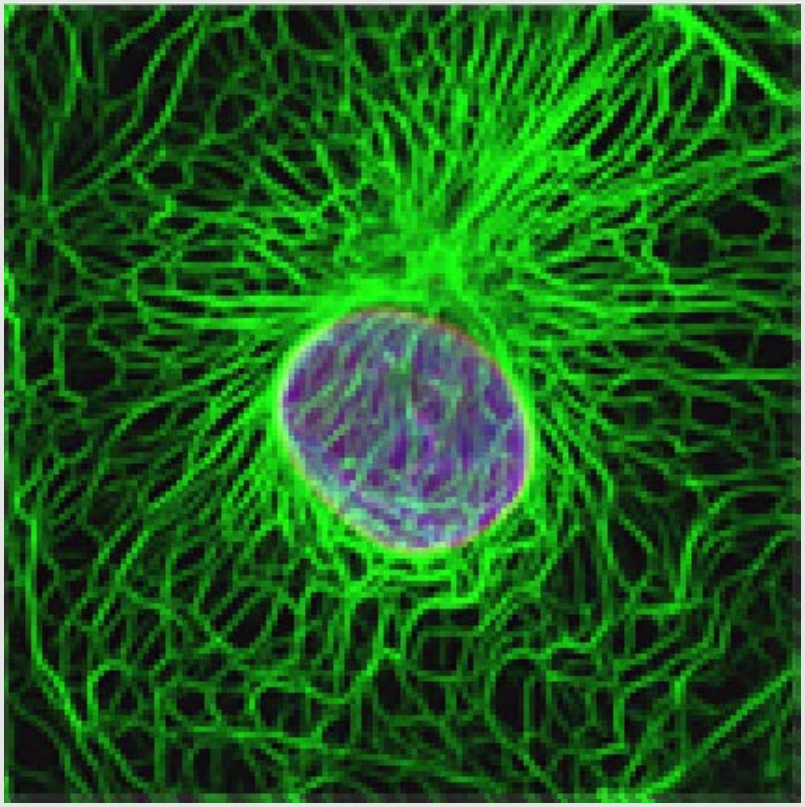


Hunting for Details?

Choose the right Resolution



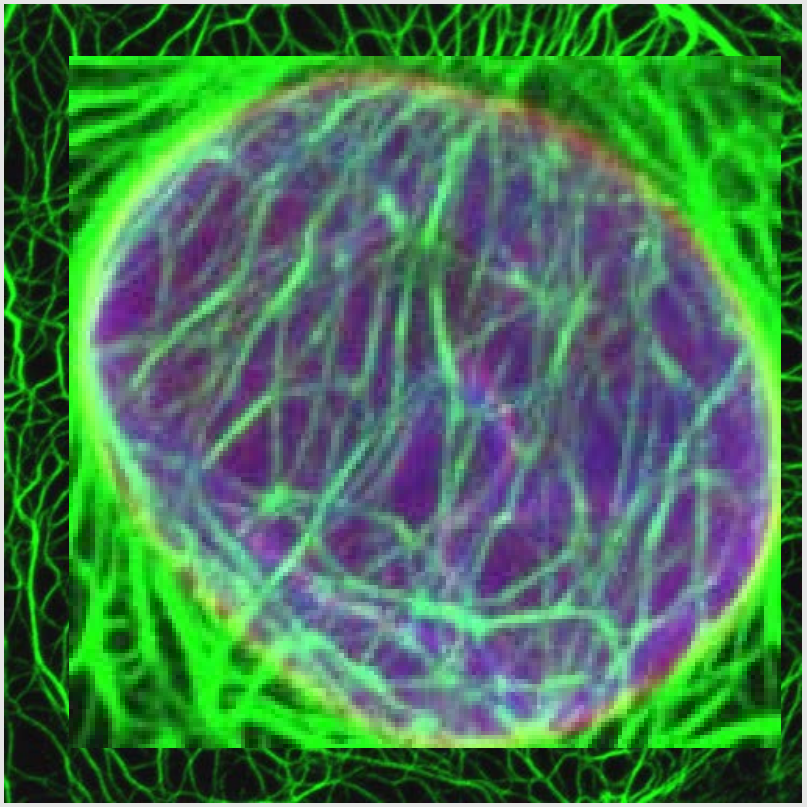
Resolution okay



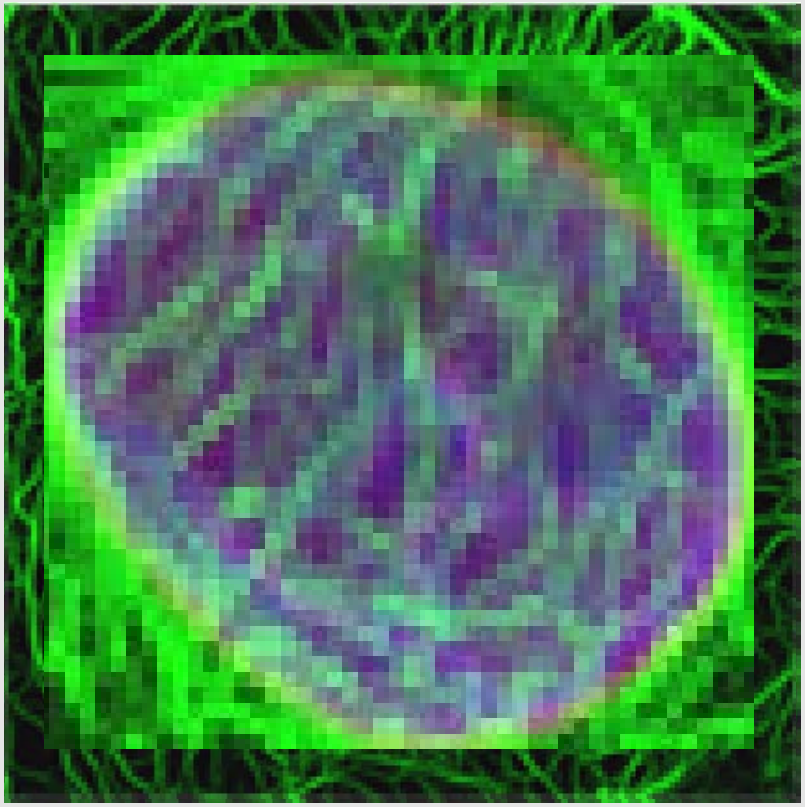
Resolution too low

Hunting for Details?

Choose the right Resolution



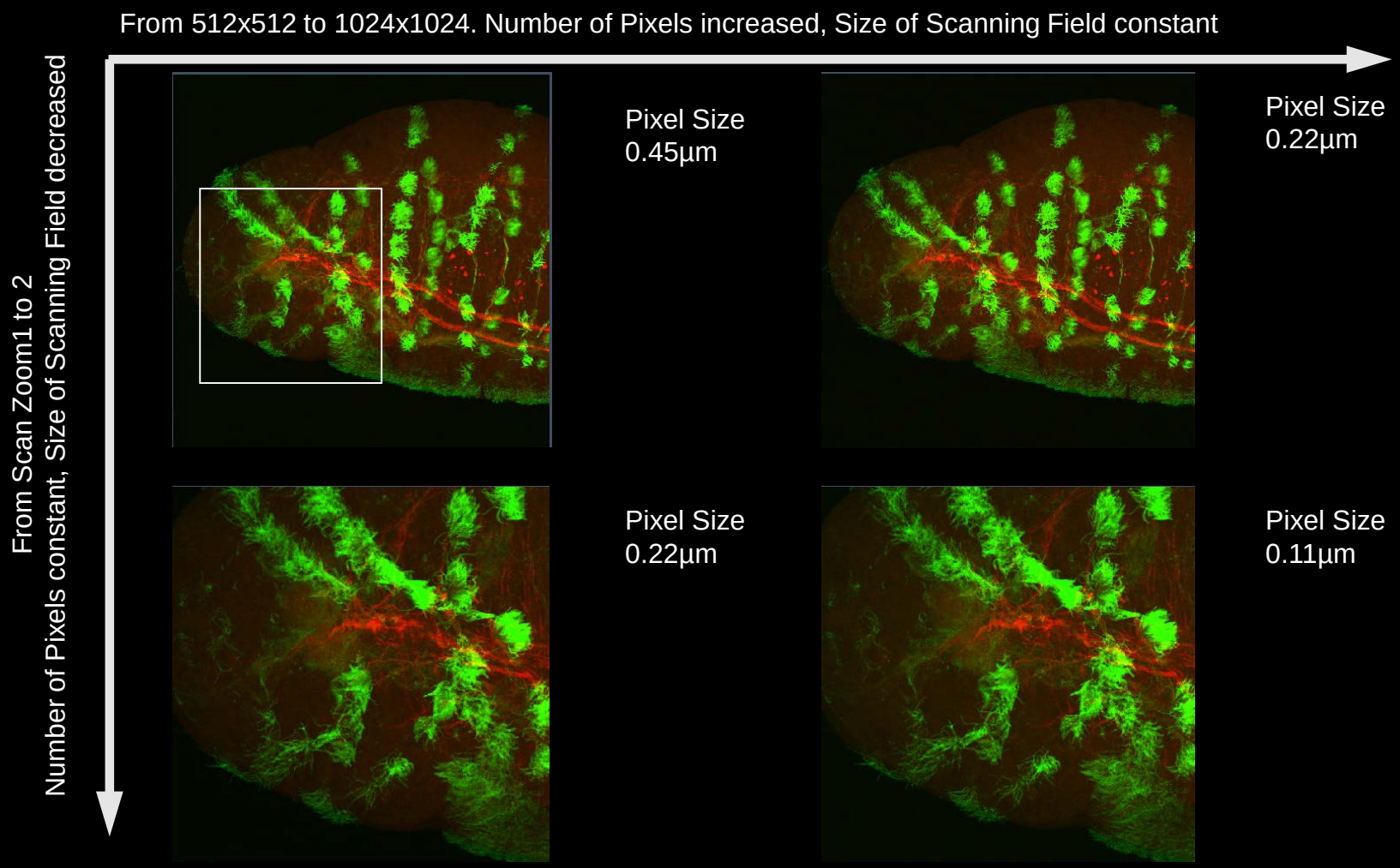
Resolution okay



Resolution too low

Resolution in the Confocal Scan

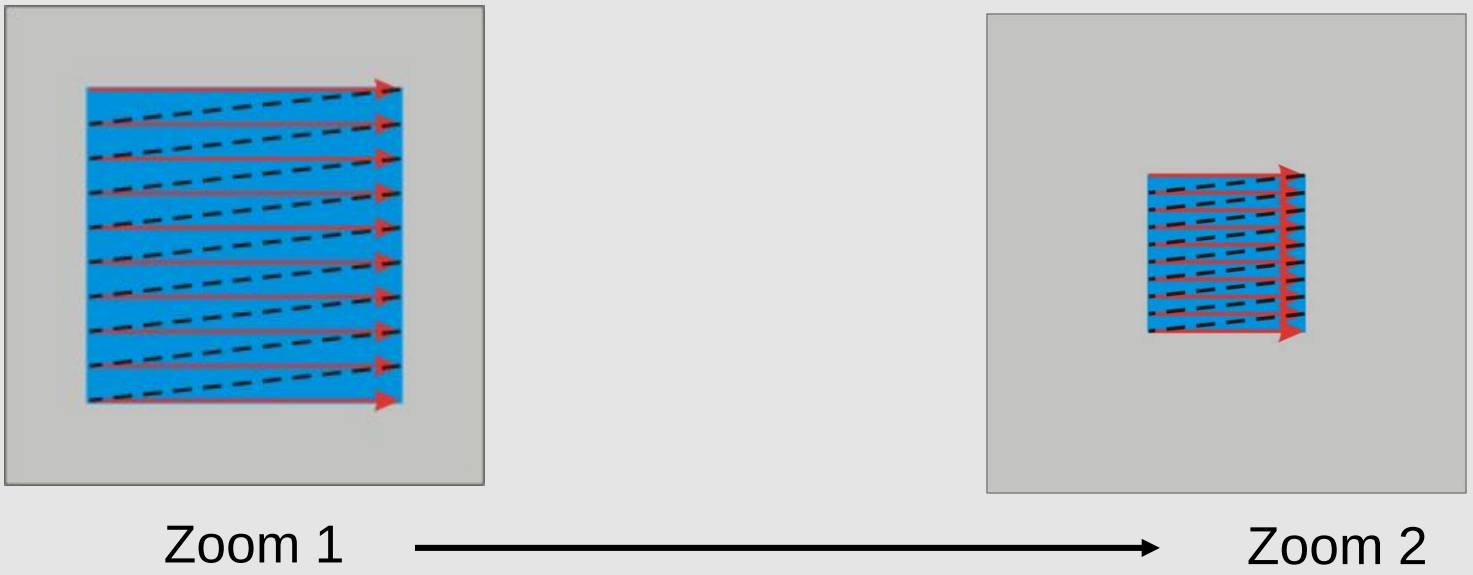
Pixel Resolution and Optical (Scan) Zoom



Resolution in the Confocal Scan

Pixel Resolution and Optical (Scan) Zoom

Laser Energy Density can be increased by zooming



Optical Slice Thickness

Calculation of Optical Slice Thickness

Optical Slice Thickness
(1AU < PH < ∞)

$$\approx \sqrt{\left[\frac{0.88 * \lambda_{em}}{(n - \sqrt{n^2 - NA^2})}\right]^2 + \left[\frac{\sqrt{2} * n * PH}{NA}\right]^2}$$

Wave-optical Term

Geometric-optical Term

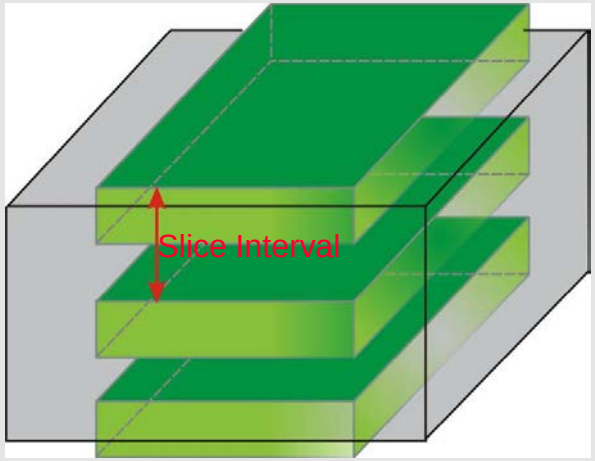
The Optical Slice thickness is controlled by:

NA = Numerical Aperture of Objective
λ_{em} = Emission Wavelength [nm]
PH = Pinhole diameter [µm]
n = Refractive Index of Immersion liquid

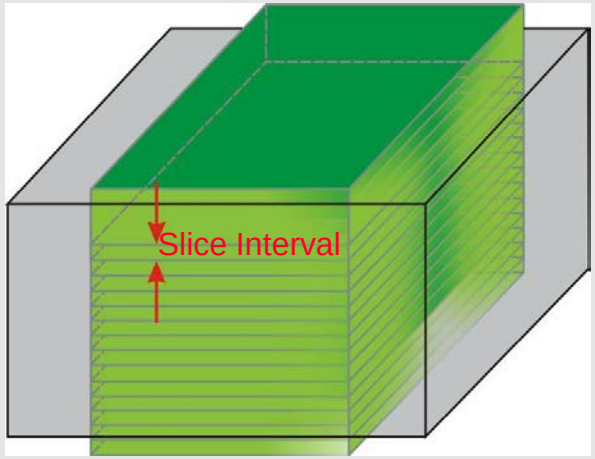
Having enough slices ensures also good quality for 3D rendering

Optical Slice Thickness

Overlap between Optical Slices



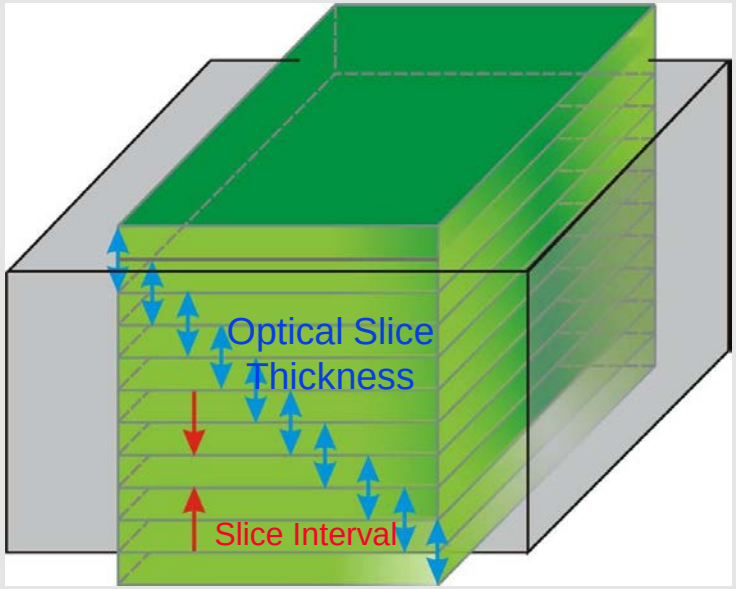
With this setting, the object structures between the slices cannot be detected.

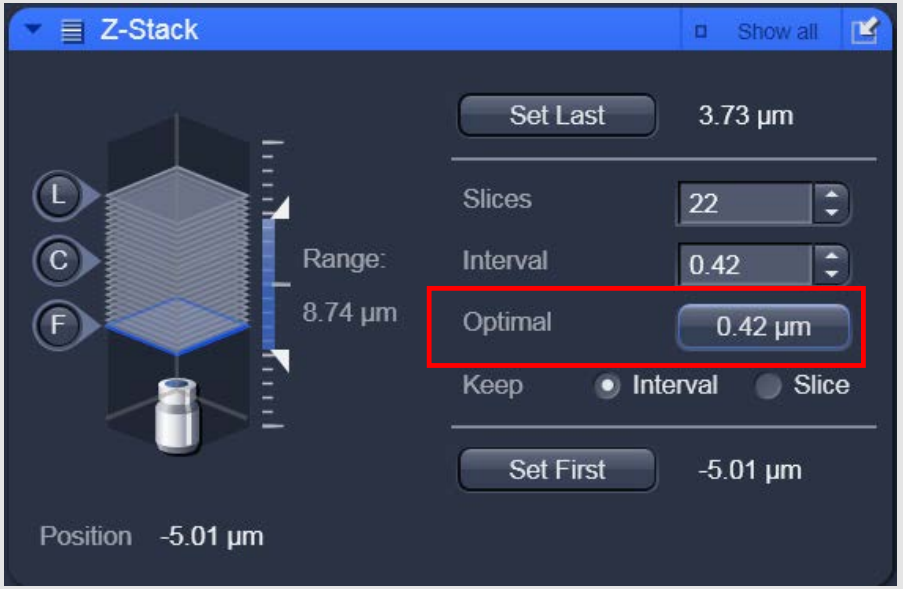


At very small intervals a lot of additional data without additional information is generated.

Optical Slice Thickness

Overlap between Optical Slices





The optimal overlap is fulfilled at **"Nyquist"** or "Sampling Theorem" conditions.

→ Sampling frequency (slice interval) must be the double of the information frequency (z-resolution or optical slice thickness).

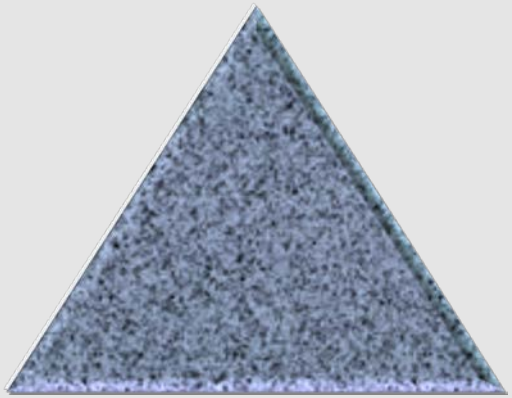
To achieve these conditions just press *Optimal Interval* in *Z-Stack* dialog. Then, the slices overlap by half of their thickness (no missing information @ minimal number of sections).

Life is Full of Compromises:

A good compromise has to be found for every imaging task!

„Non-invasive“ Data Recording

- low photobleaching
- low cytotoxicity (laser irradiation)



High Temporal Resolution

- fast acquisition speed
- high number of different time points

High Spatial Resolution

- high resolution images
- high resolution Z-Stacks
- optimal S/N

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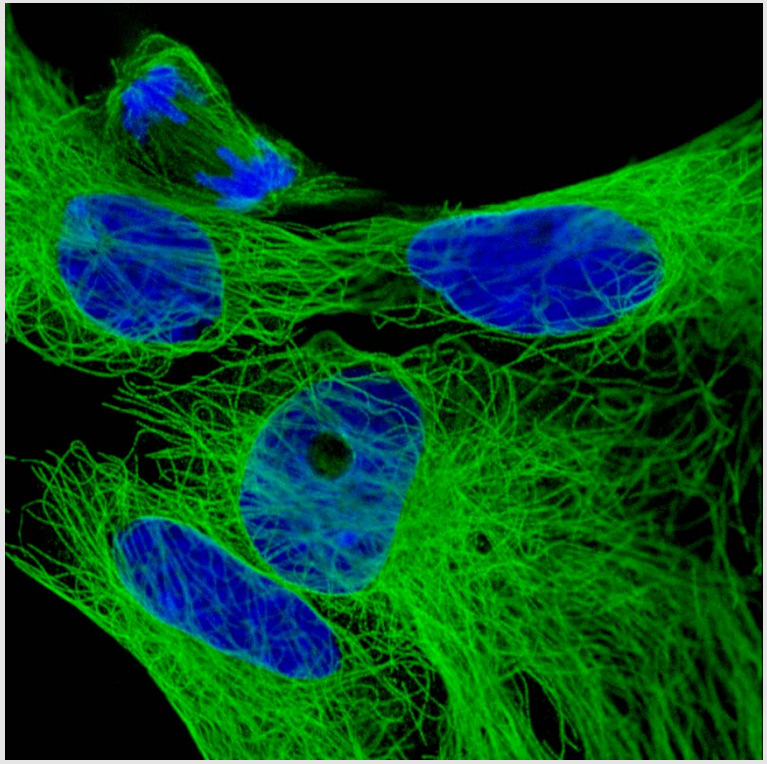
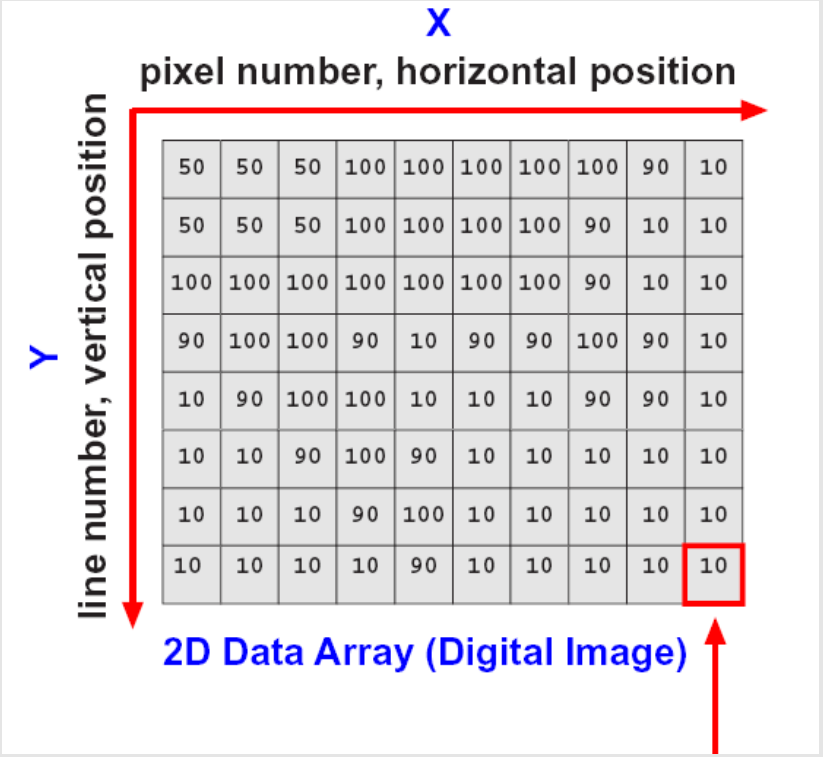
Evaluating an Image

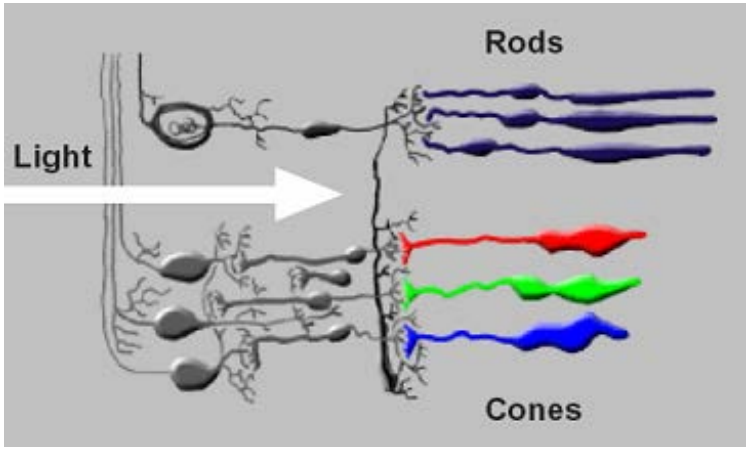
Displayed Image



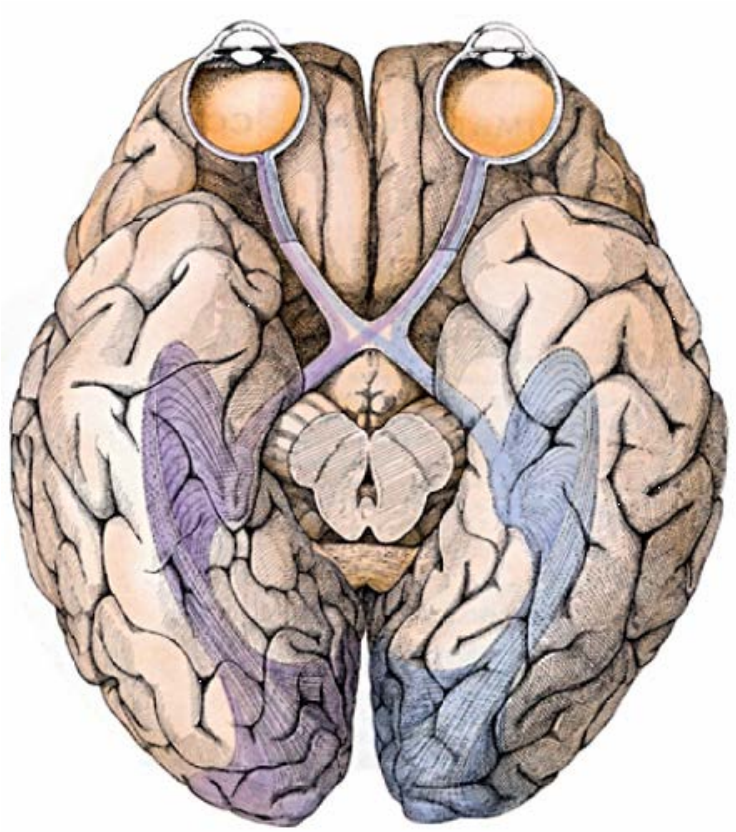
Digital Image



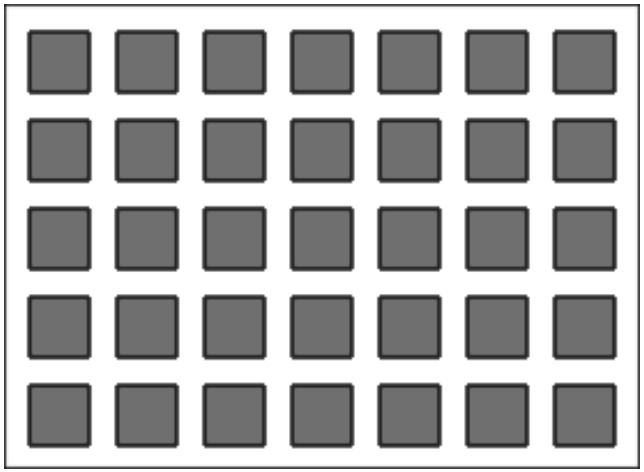
Visualizing Biology
Human visual perception and the problem of subjectivity

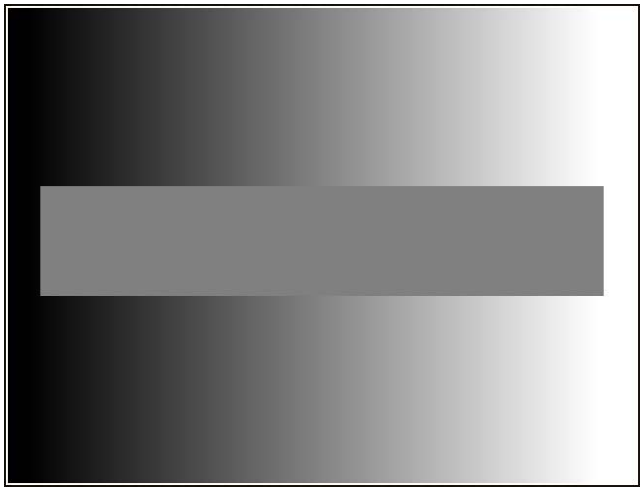


A perfect imaging system ?

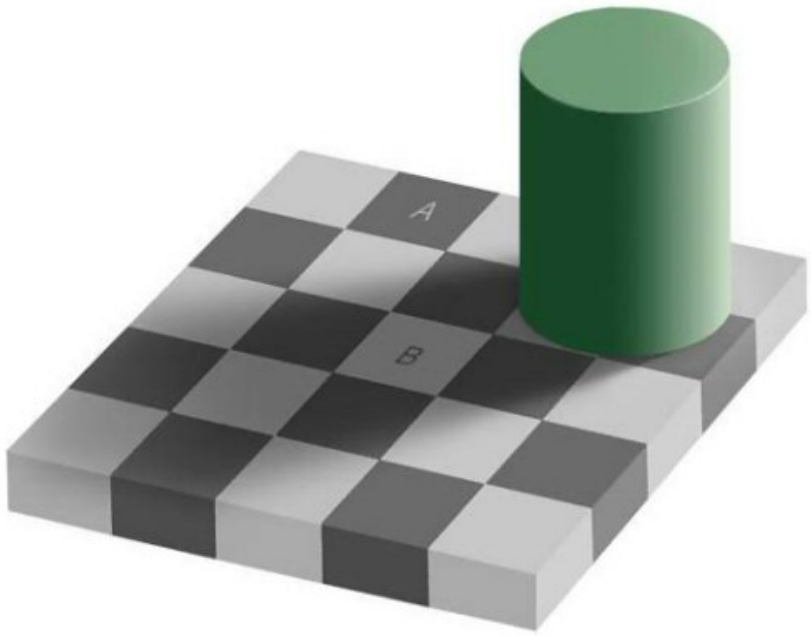


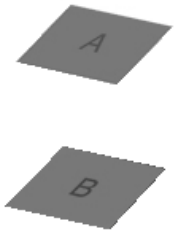
Visualizing Biology
Optical illusions reveal imperfections of human vision





Visualizing Biology
Optical illusions reveal imperfections of human vision

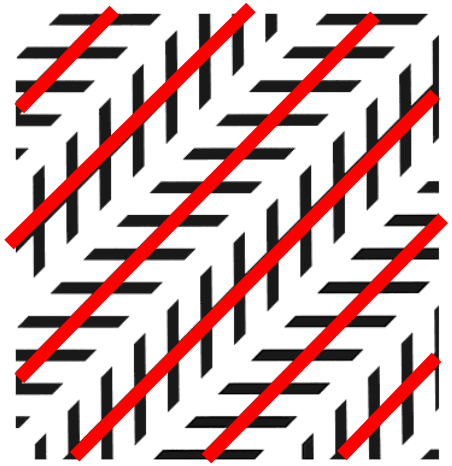


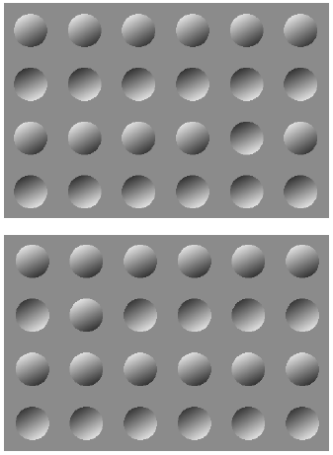


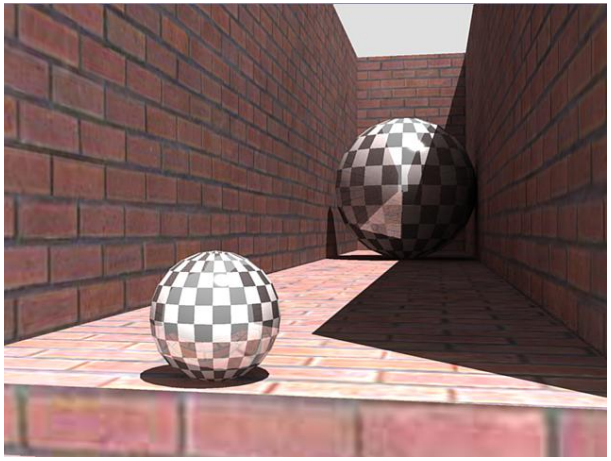
Edward H. Adelson

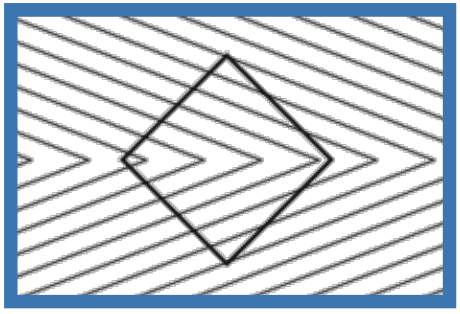
Visualizing Biology

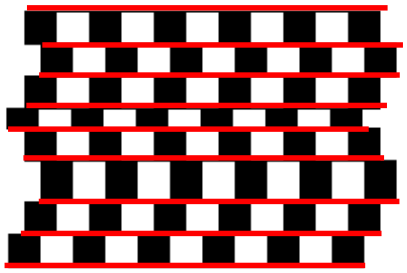
Optical illusions reveal imperfections of human vision

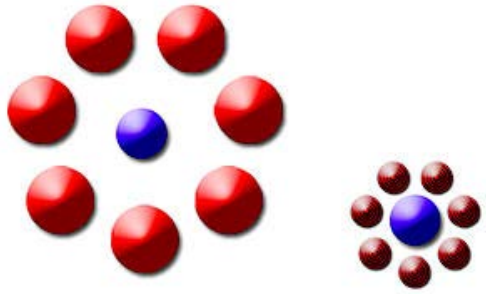




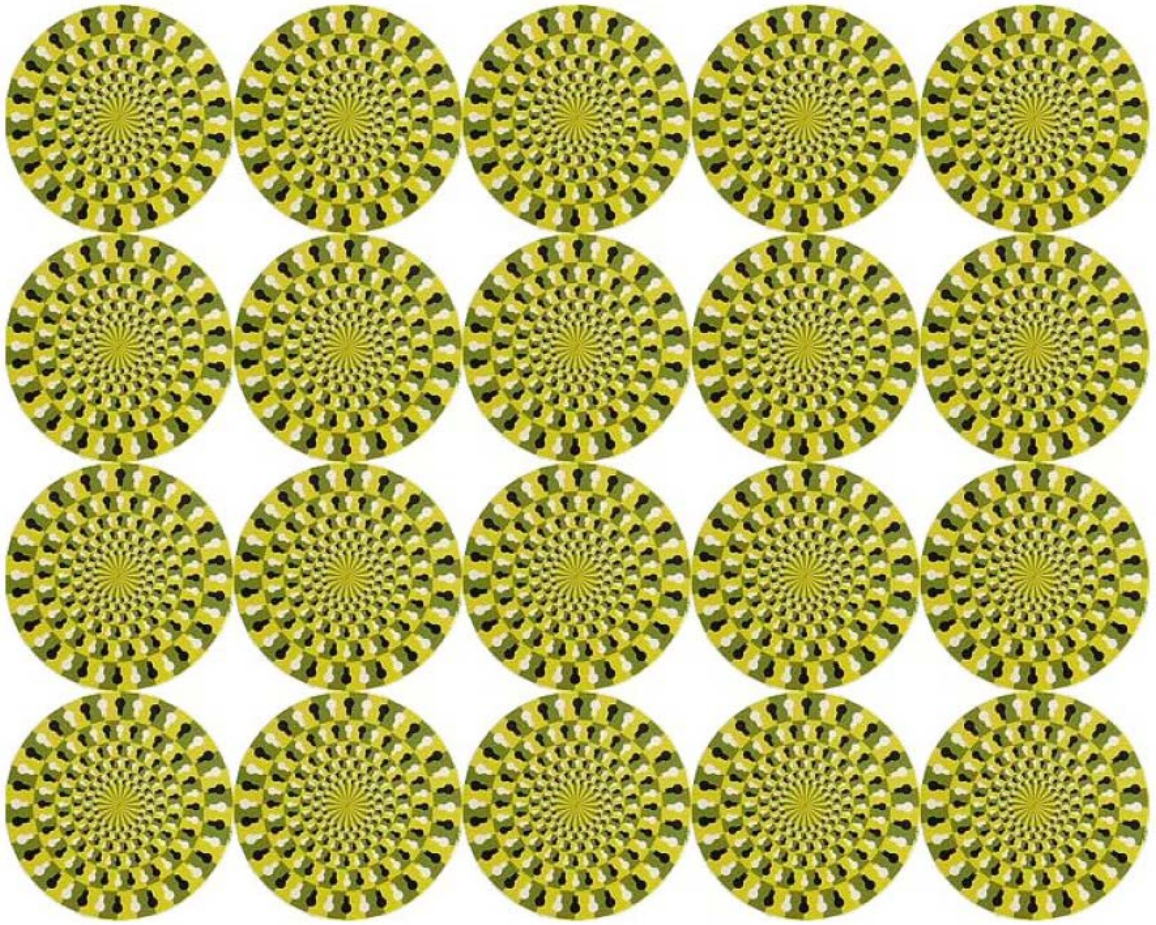








Visualizing Biology
Optical illusions reveal imperfections of human vision



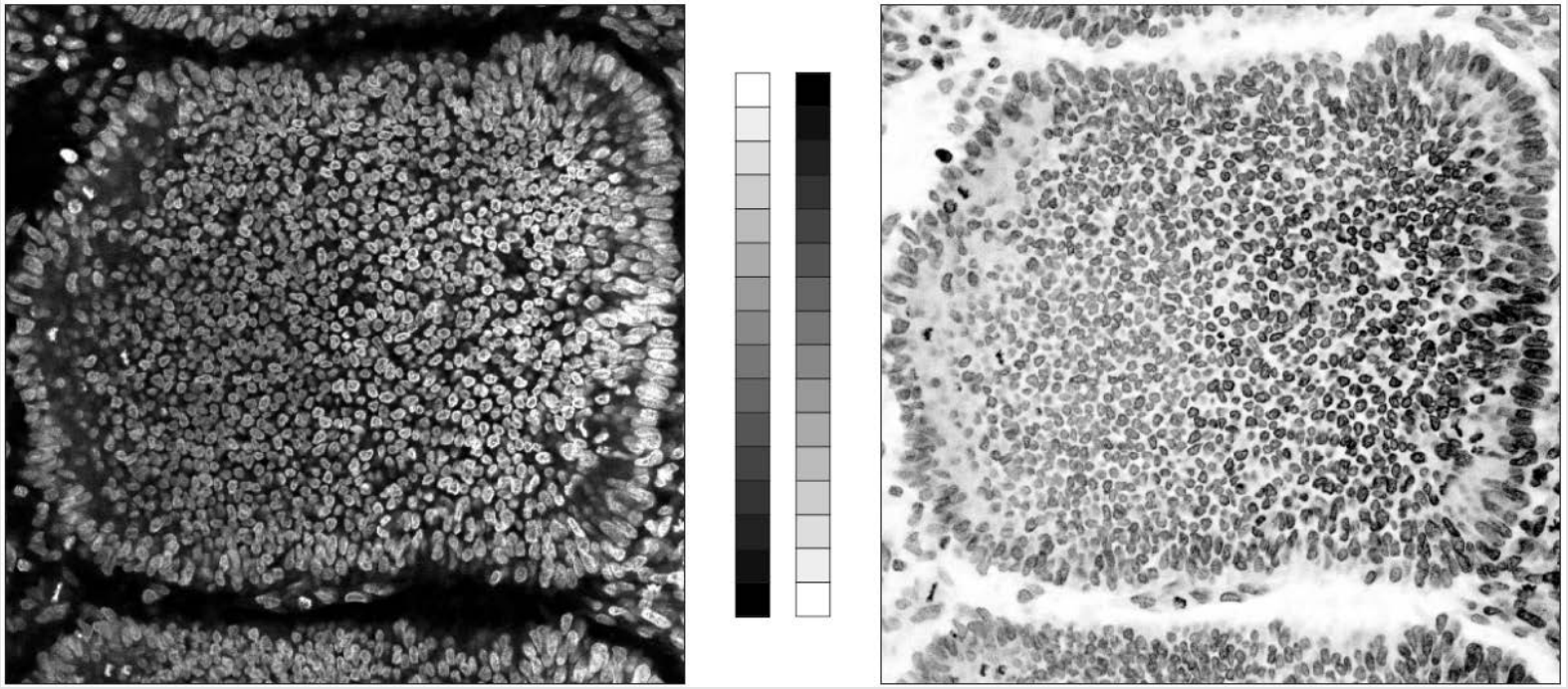
Visualizing Biology
Optical illusions reveal imperfections of human vision

Concepts for displaying image data

Lookup tables (LUTs)

Black to white

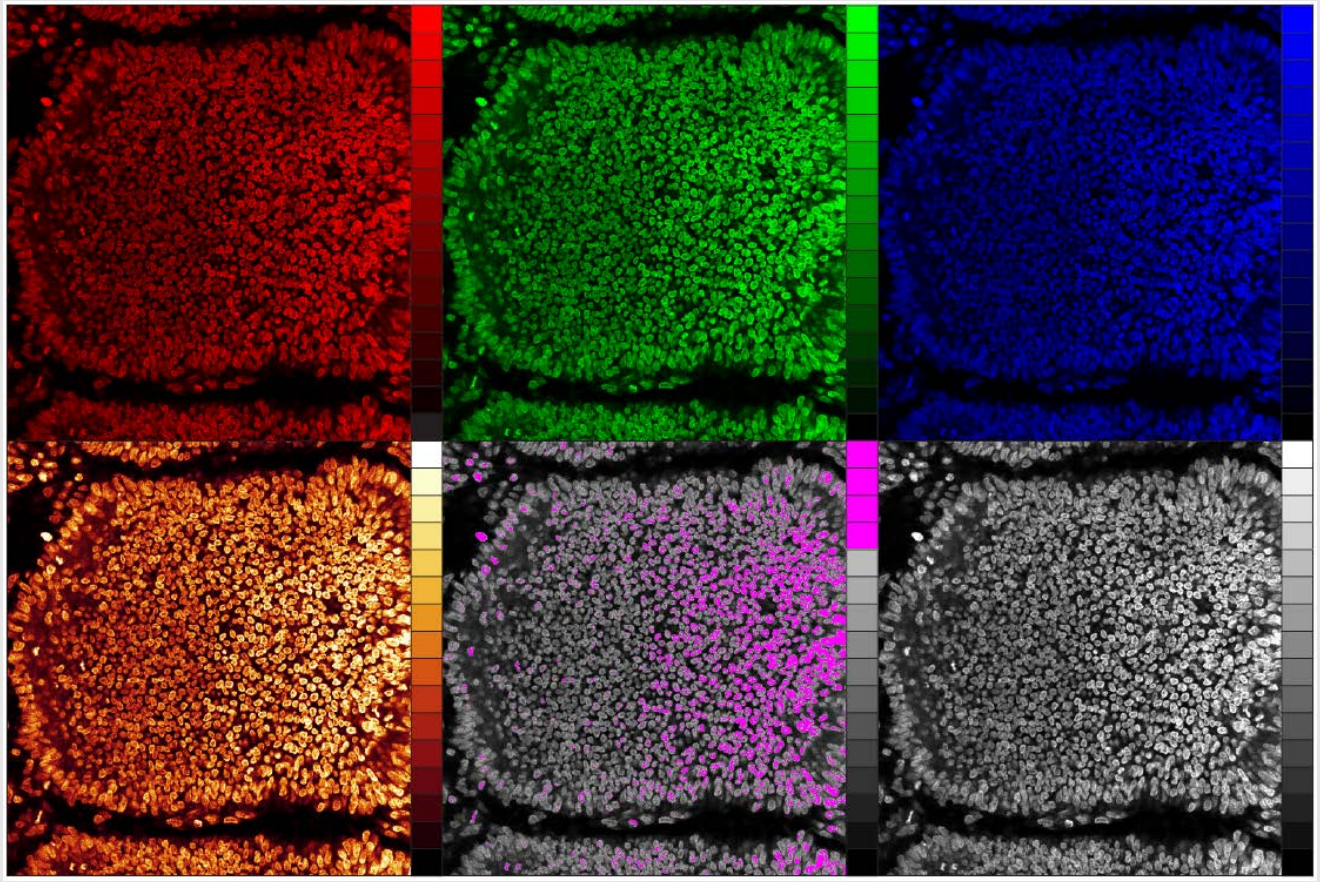
White to black



Concepts for displaying image data

Lookup tables (LUTs)

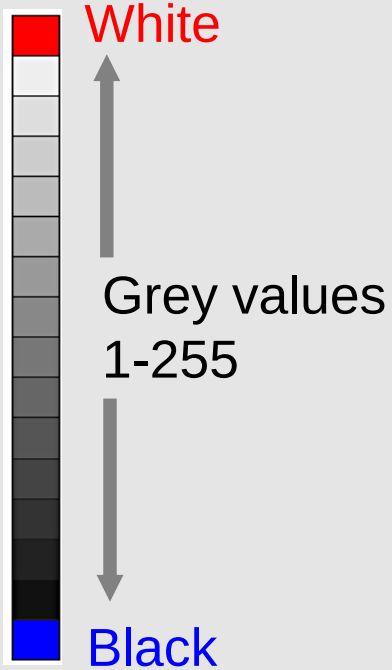
Using LUTs Small Differences in Images can be seen much better

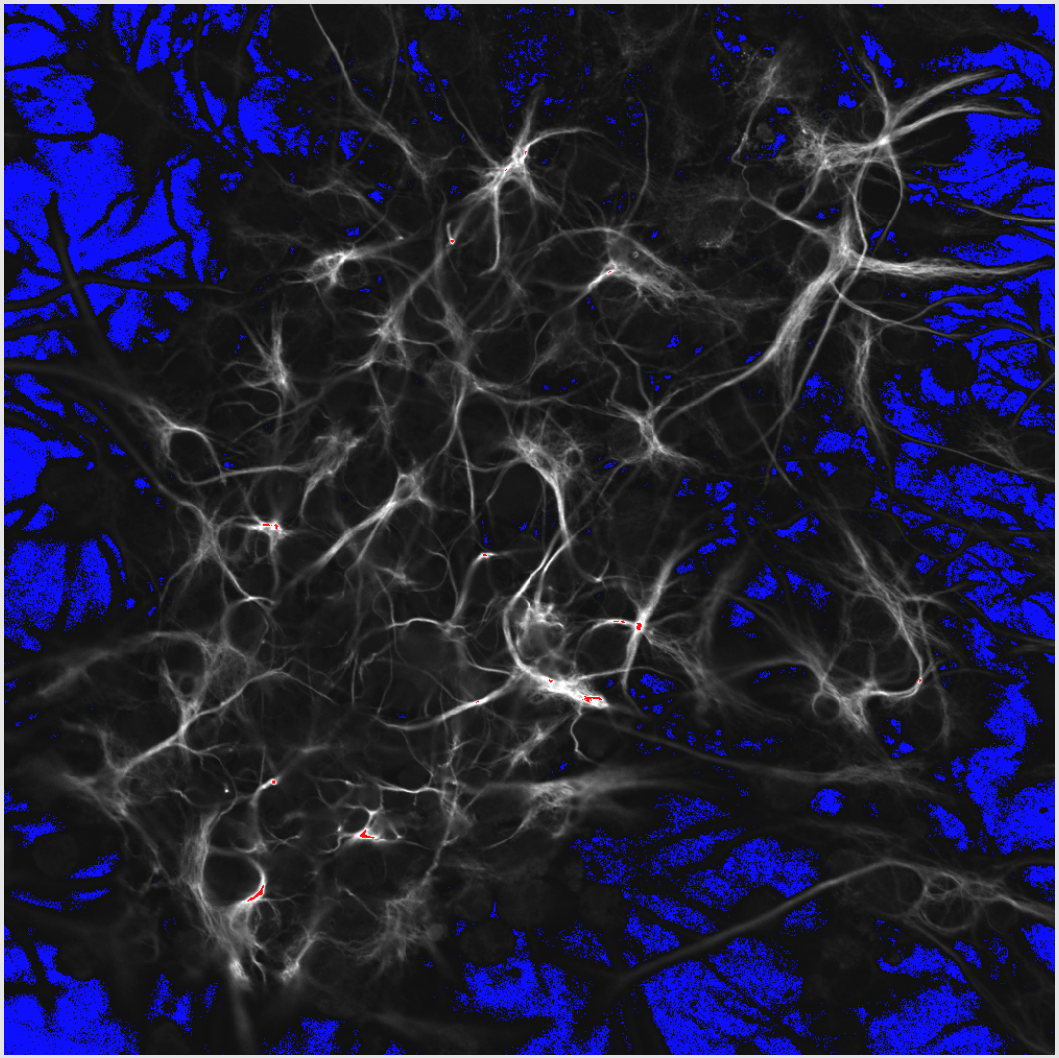


Range Indicator

How to evaluate the dynamic range the best

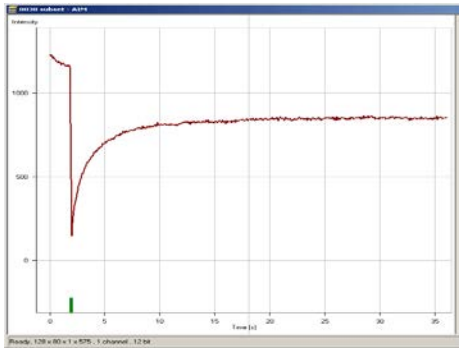
Look-up table
Range Indicator (8bit)



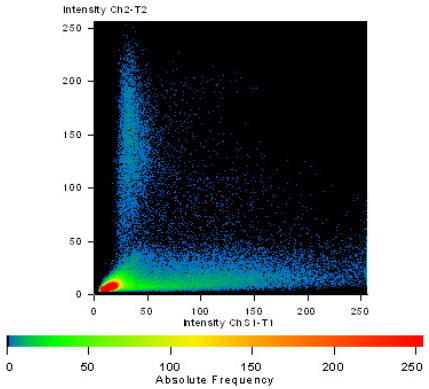


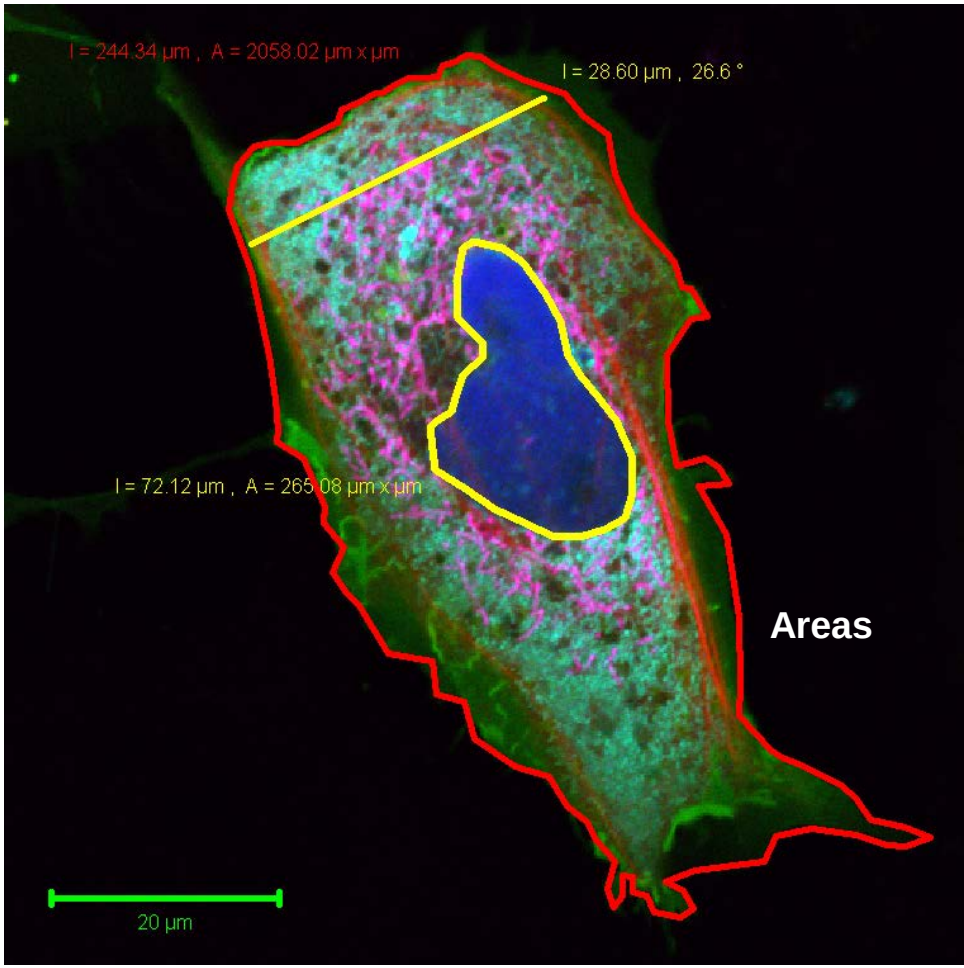
Eliminating subjectivity by quantifying image information

Signal intensity plot (time series)



Scatter diagram





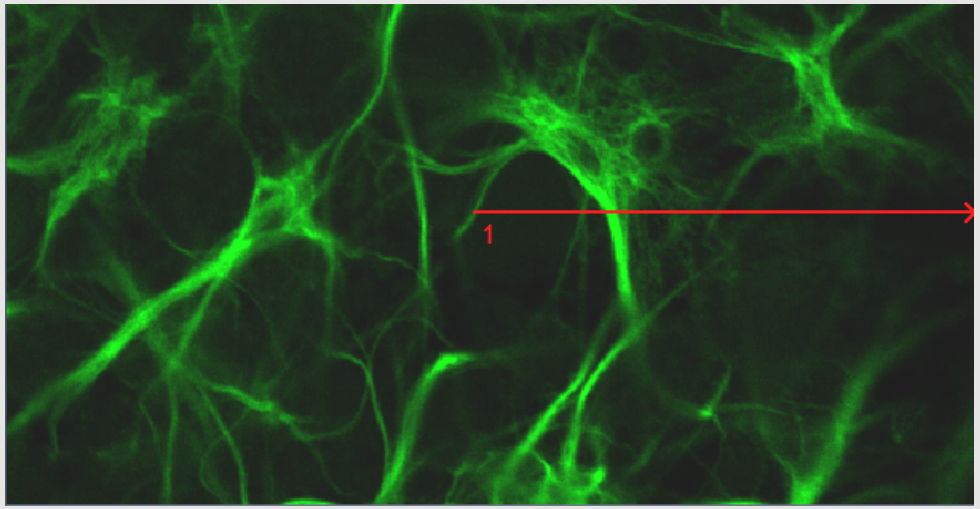
Measuring distances and areas

We make it visible.

Image Noise

What does it look like?

“Good” Image



“Bad” Image

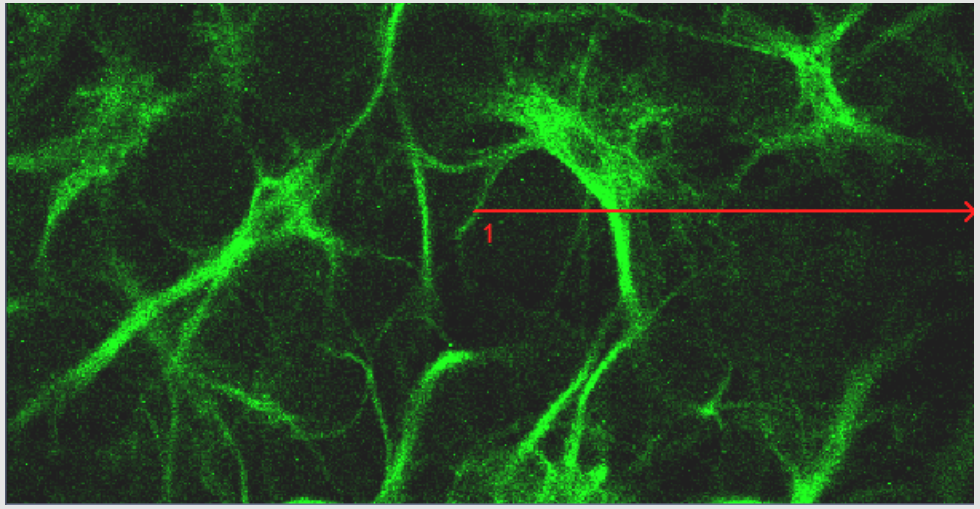


Image Noise

How to measure it using the Profile

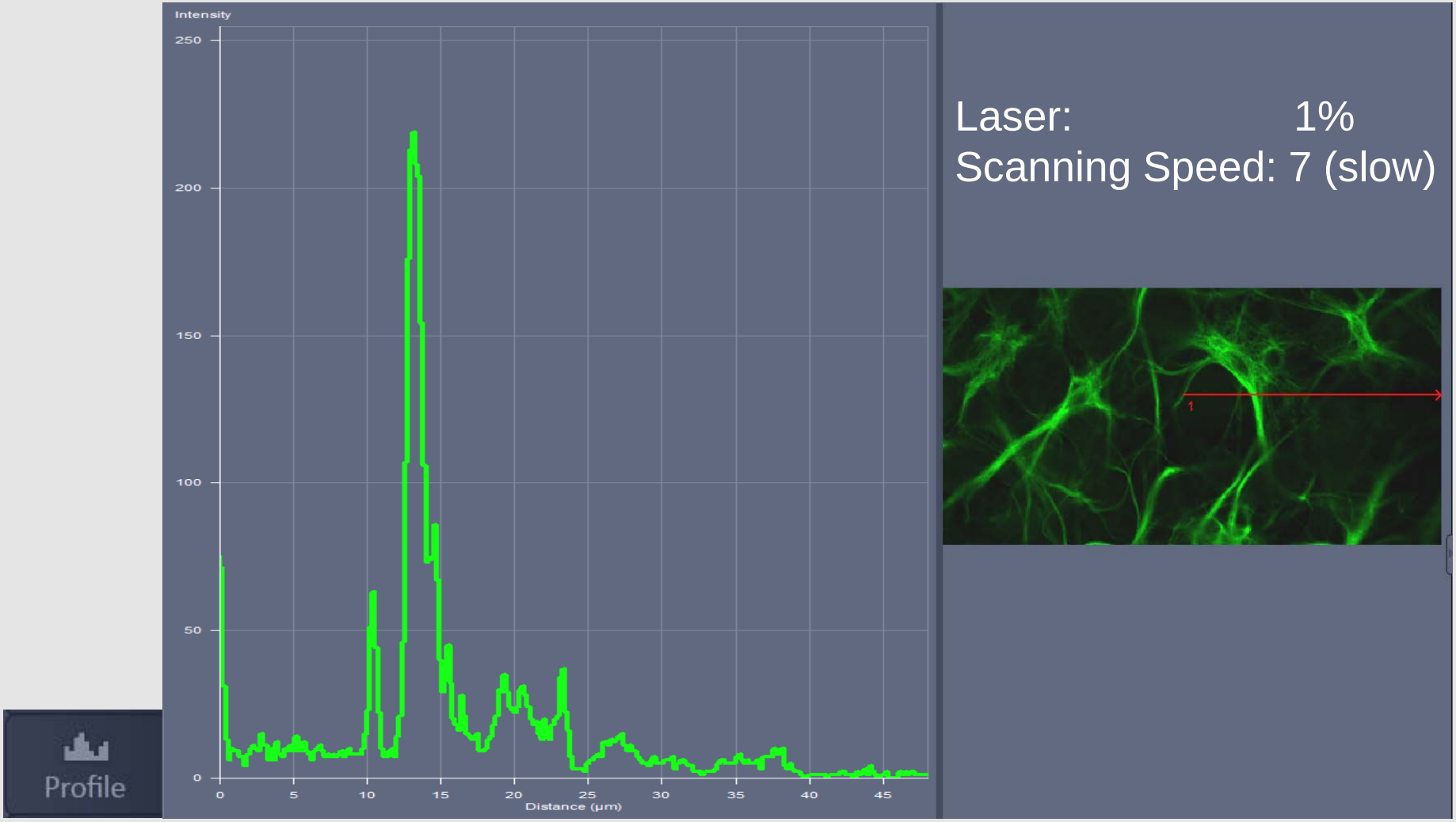


Image Noise

Noise increases with speed and less signal

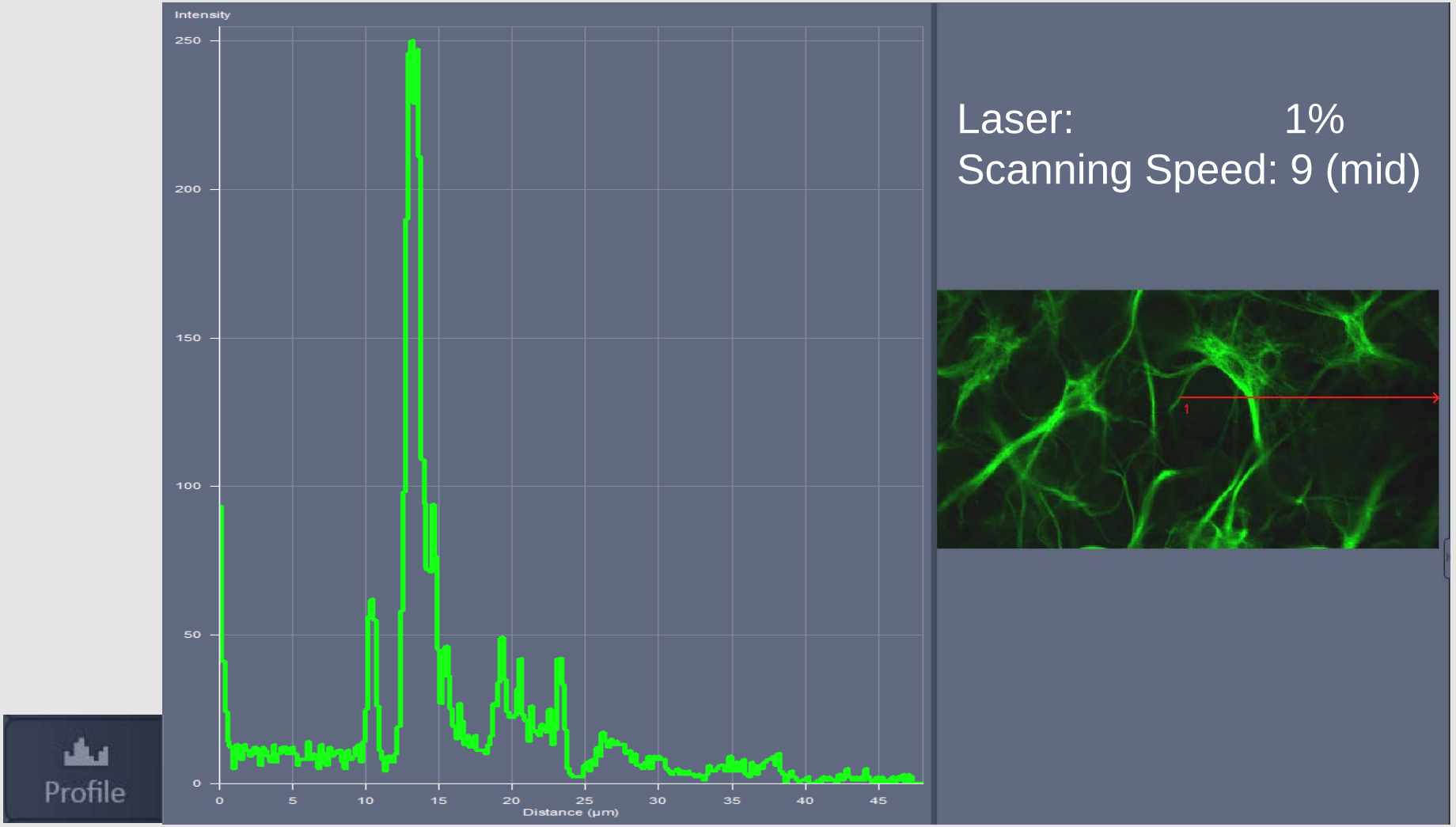
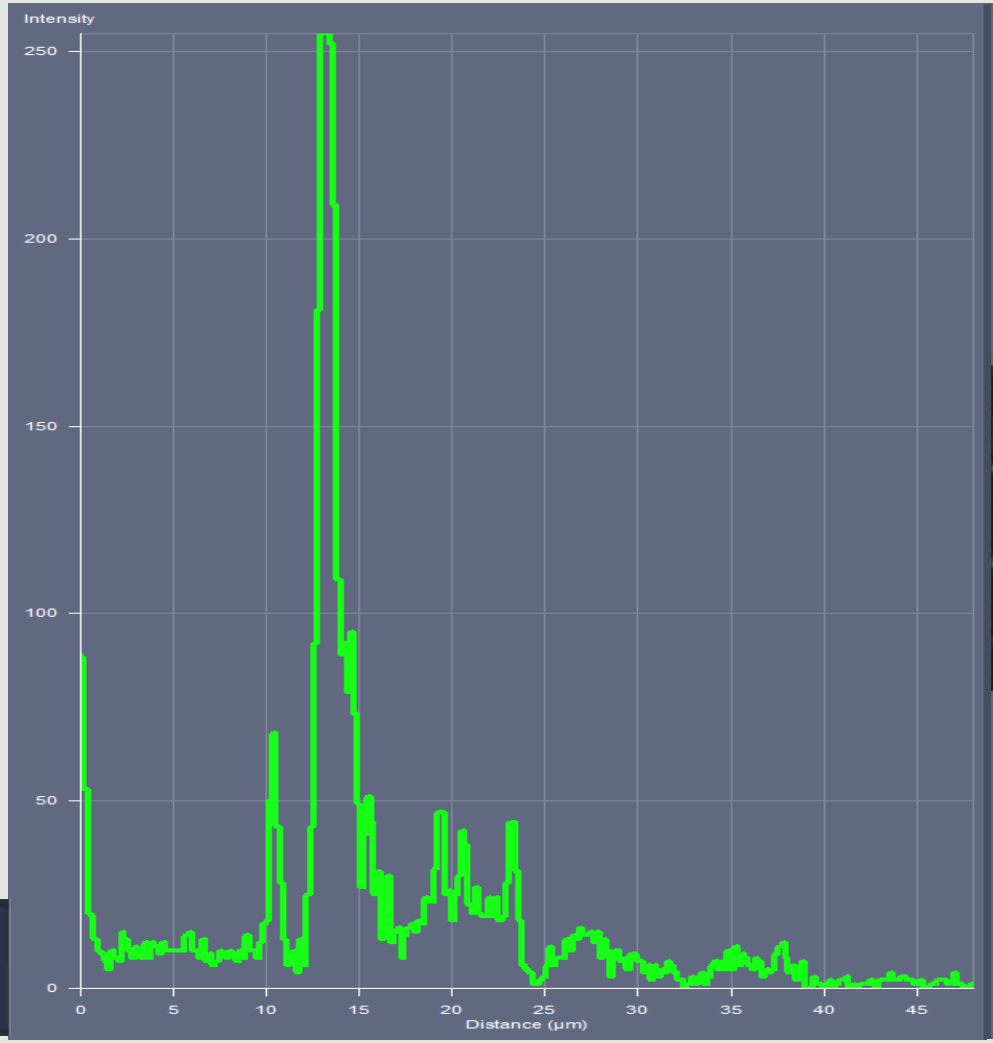
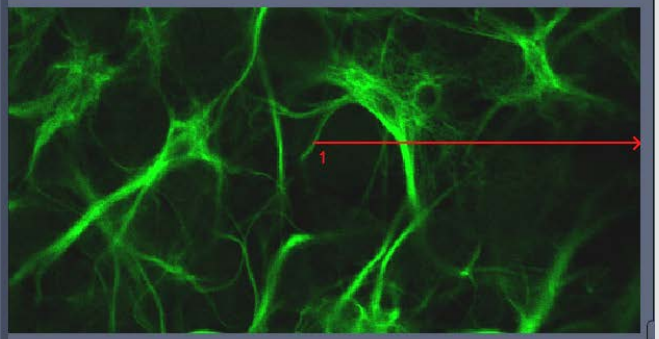


Image Noise

Noise increases with speed and less signal



Laser: 1%
Scanning Speed: 10 (fast)



Profile

Image Noise

Noise increases with speed and less signal

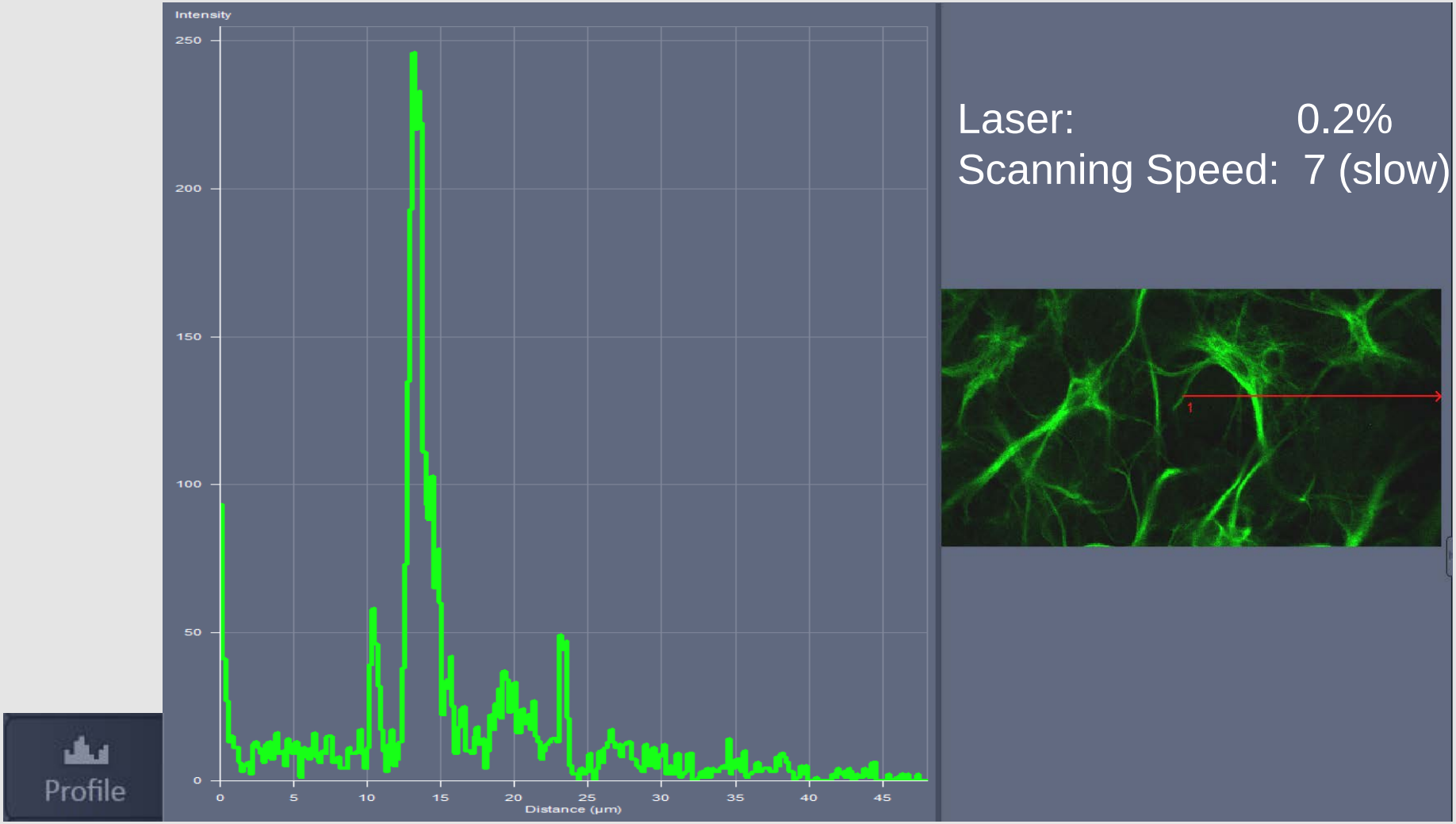


Image Noise

Noise increases with speed and less signal

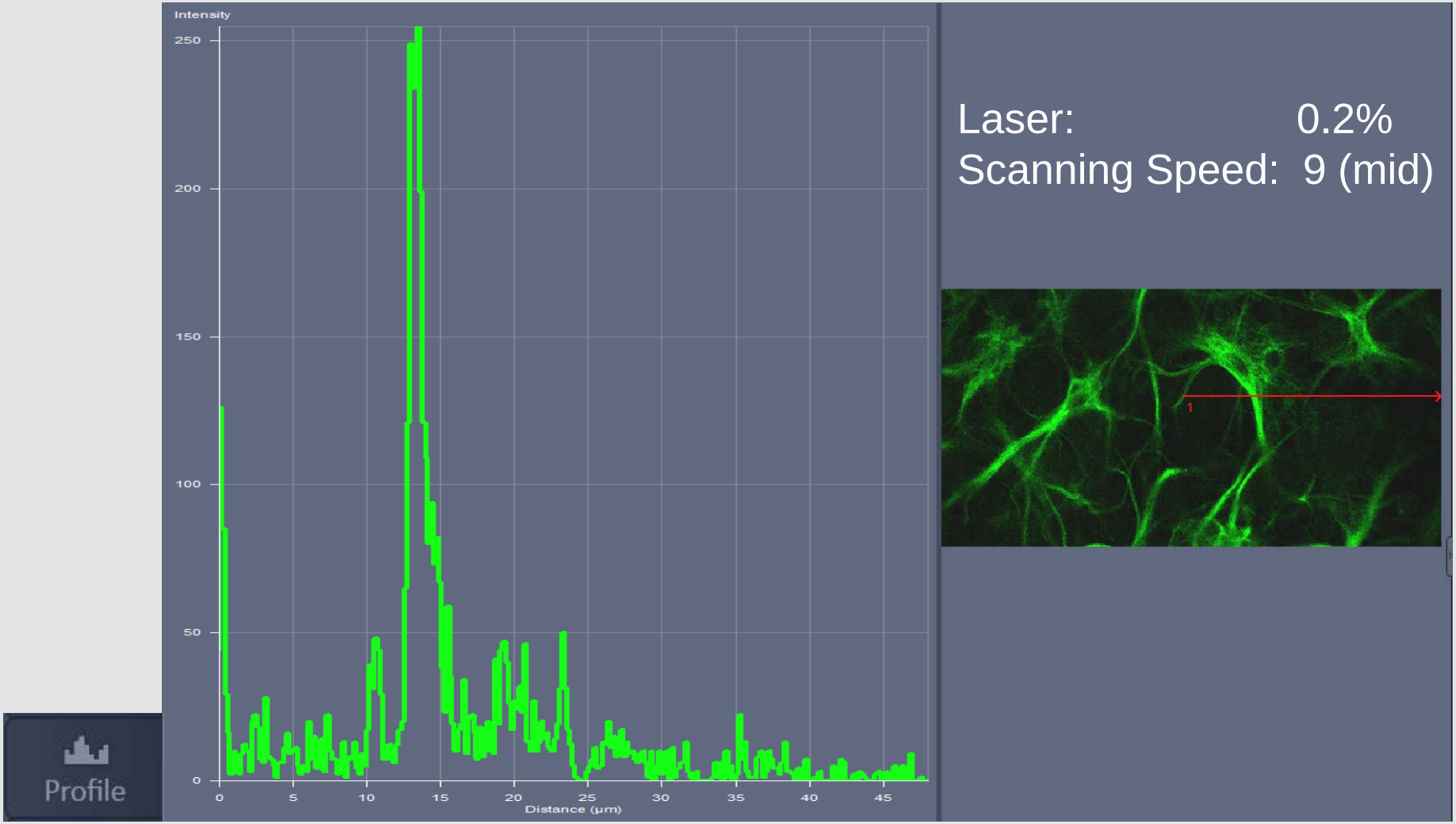
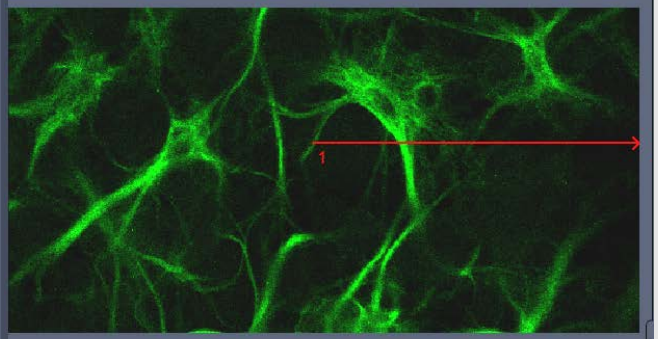


Image Noise

Noise increases with speed and less signal

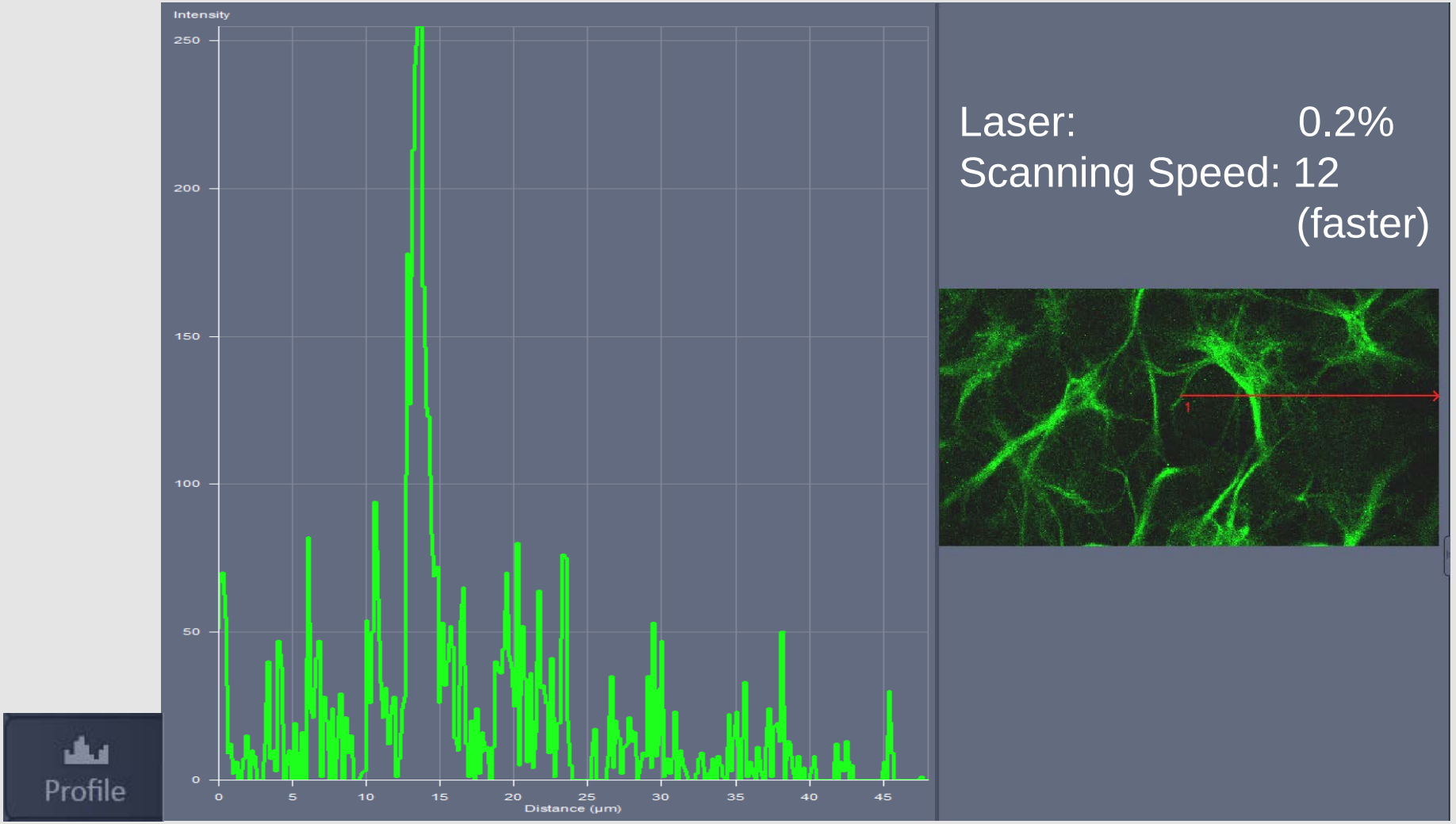
Laser: 0.2%
Scanning Speed: 10 (fast)



Profile

Image Noise

Noise increases with speed and less signal



Use of Digital Gain

Increases the Signal (and saves Laser Power)

Artificial Example of the Amplification Step of the Digital Gain