Zeiss Confocal Microscope - LSM880

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Date: 8/30/2019

Agenda

Training Contents

- **Talk:**

Principle and Application cases of LSM880 Confocal Microscopy

- **Hands on:**

Introduction of Hardware components and laser safety
Startup and shutdown the system
Acquisition of 2D images
Transmission light images acquisition
Save and export 2D images
Z-stack images acquisition
Time series images acquisition
Tile images acquisition
Airyscan imaging and processing
Fast Airyscan imaging and processing
Image analysis

- **Q & A:**

Hands on by customers with their own samples
Answer the customers' questions

Agenda

	August 28th (Wednesday)	August 29th (Thursday)	August 30th (Friday)
AM	9:30 ——11:00 Talk: Principle of Confocol Microscopy	9:00 —— 12:00 Hands on 6-8	9:00 —— 12:00 Hands on 11
PM	13:30 —— 17:00 Hands on 1-5	13:30 —— 17:00 Hands on 9-10	13:30 —— 16:00 Hands on, Q & A

Agenda

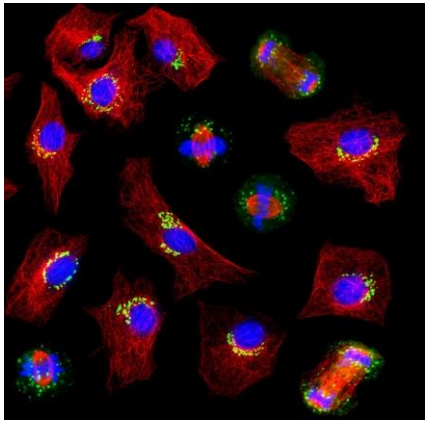
	August 28th (Wednesday)	August 29th (Thursday)	August 30th (Friday)
AM	9:30 ——11:00 Talk: Principle of Confocol Microscopy	9:00 —— 12:00 Hands on 9-11 Group 1	9:00 —— 12:00 Hands on 9-11 Group 2
PM	13:30 —— 17:00 Hands on 1-8 Group 1	13:30 —— 17:00 Hands on 1-8 Group 2	13:30 —— 16:00 Q & A

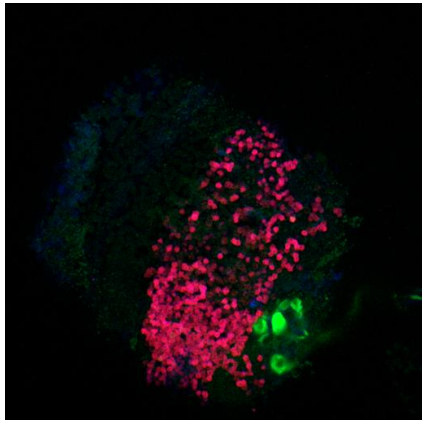
Agenda

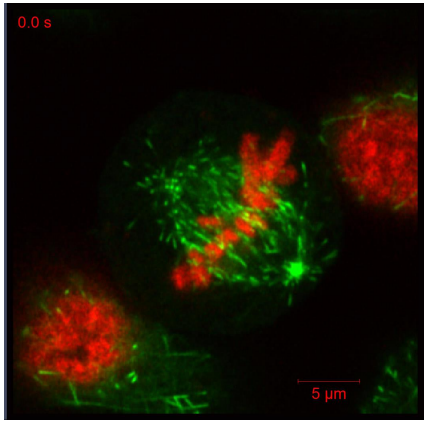
1	Applications of Confocal Microscope
2	Principle of Confocal Microscope
3	Light path components of LSM880
4	How to acquire a high quality image?

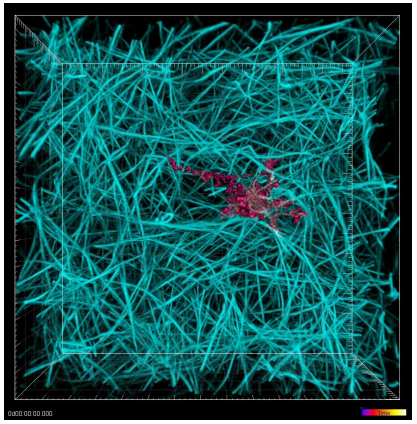
LSM880 Confocal Microscopy

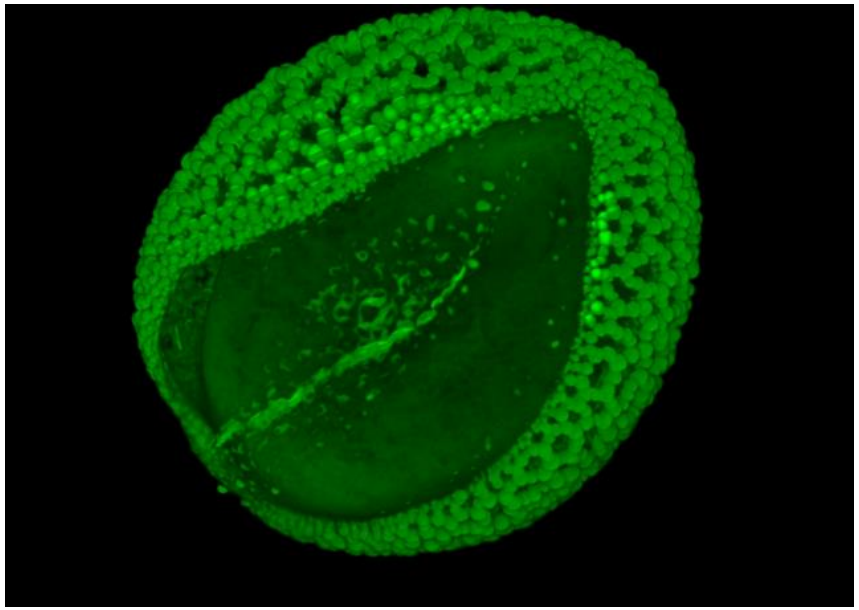
High Quality, Multi-dimensions, Large Field of View

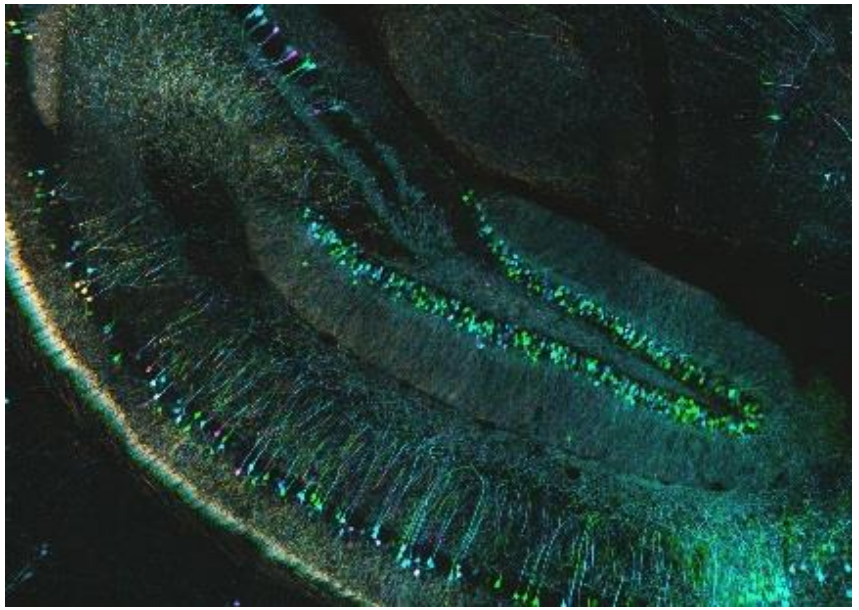








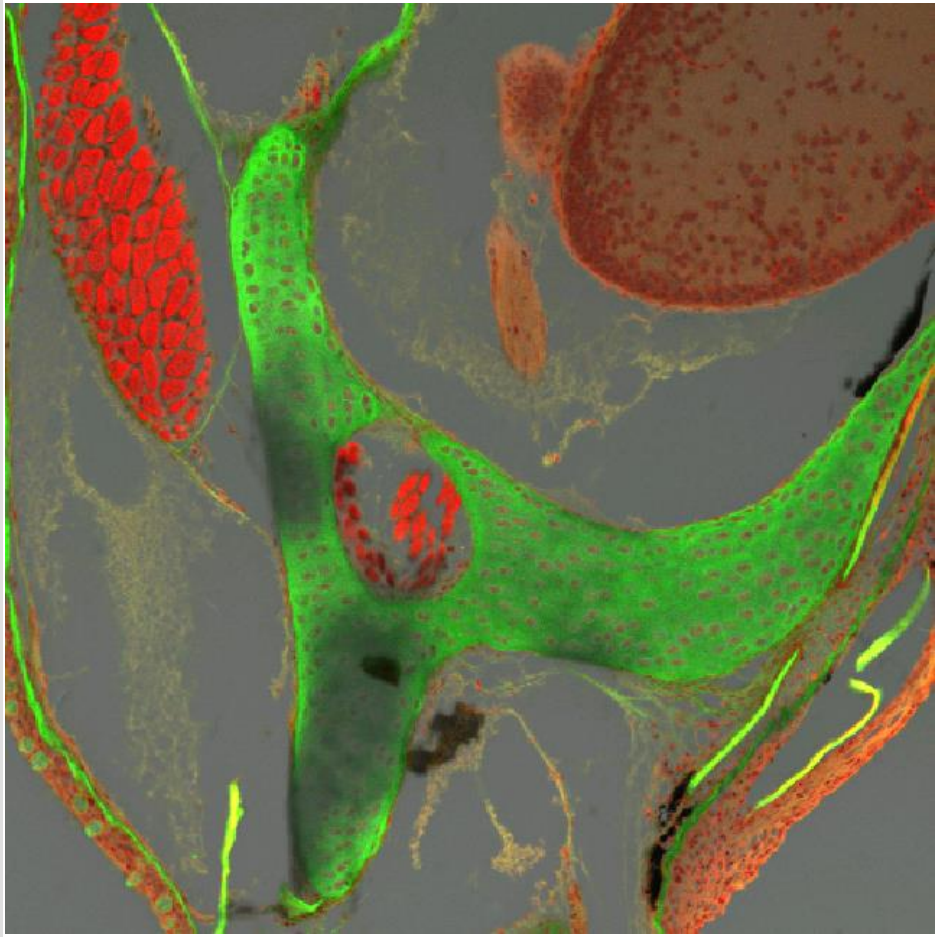




Confocal: widely used in almost all field of Biological and Medical Research

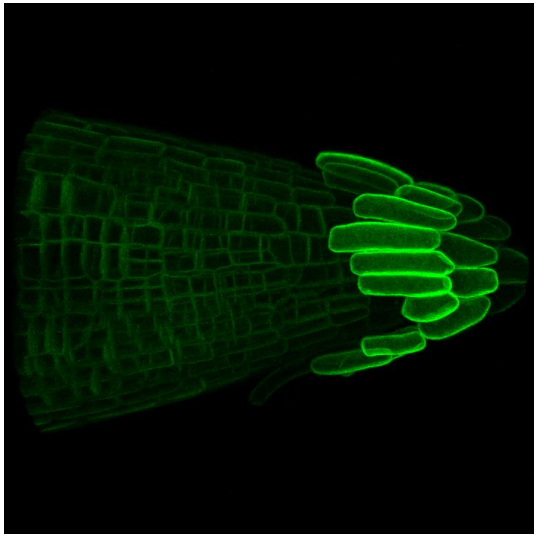
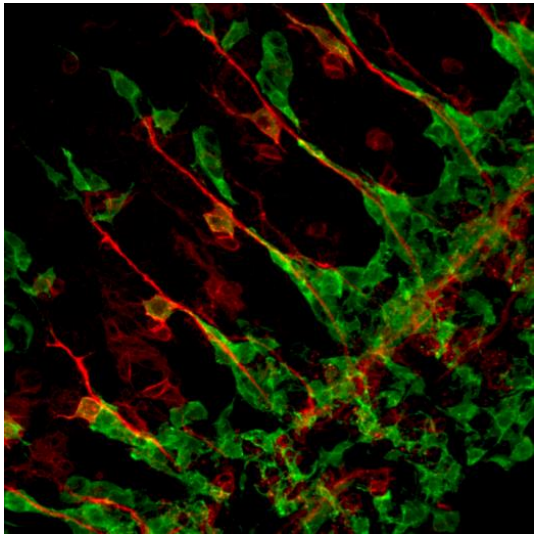
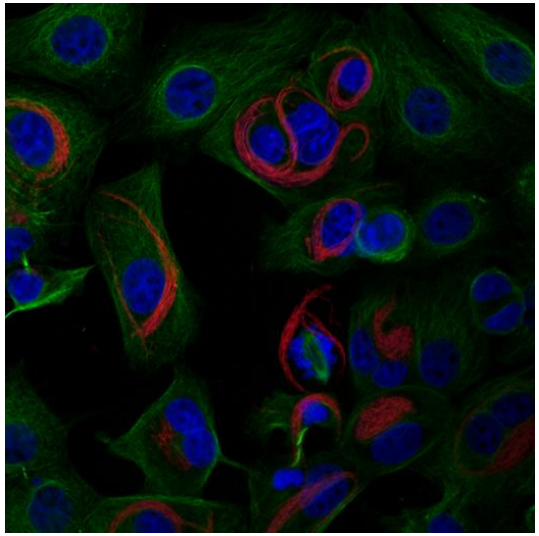
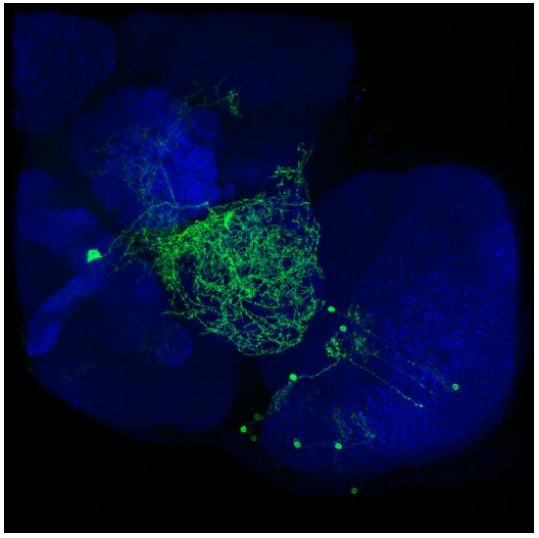
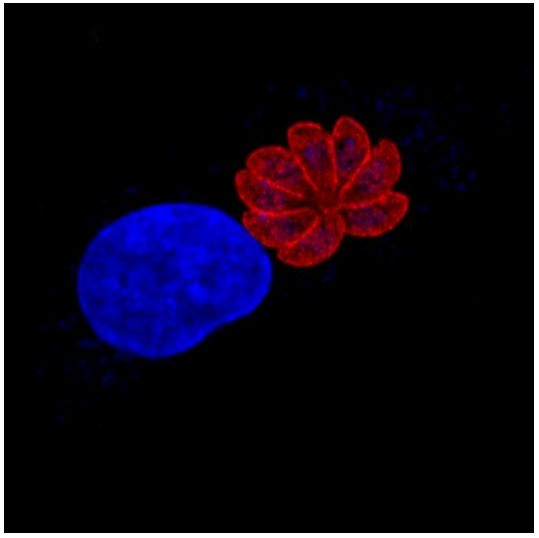
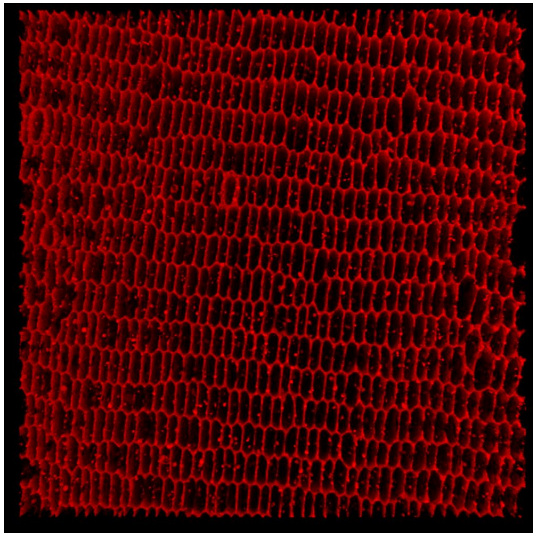
Nearly all fields of Science...

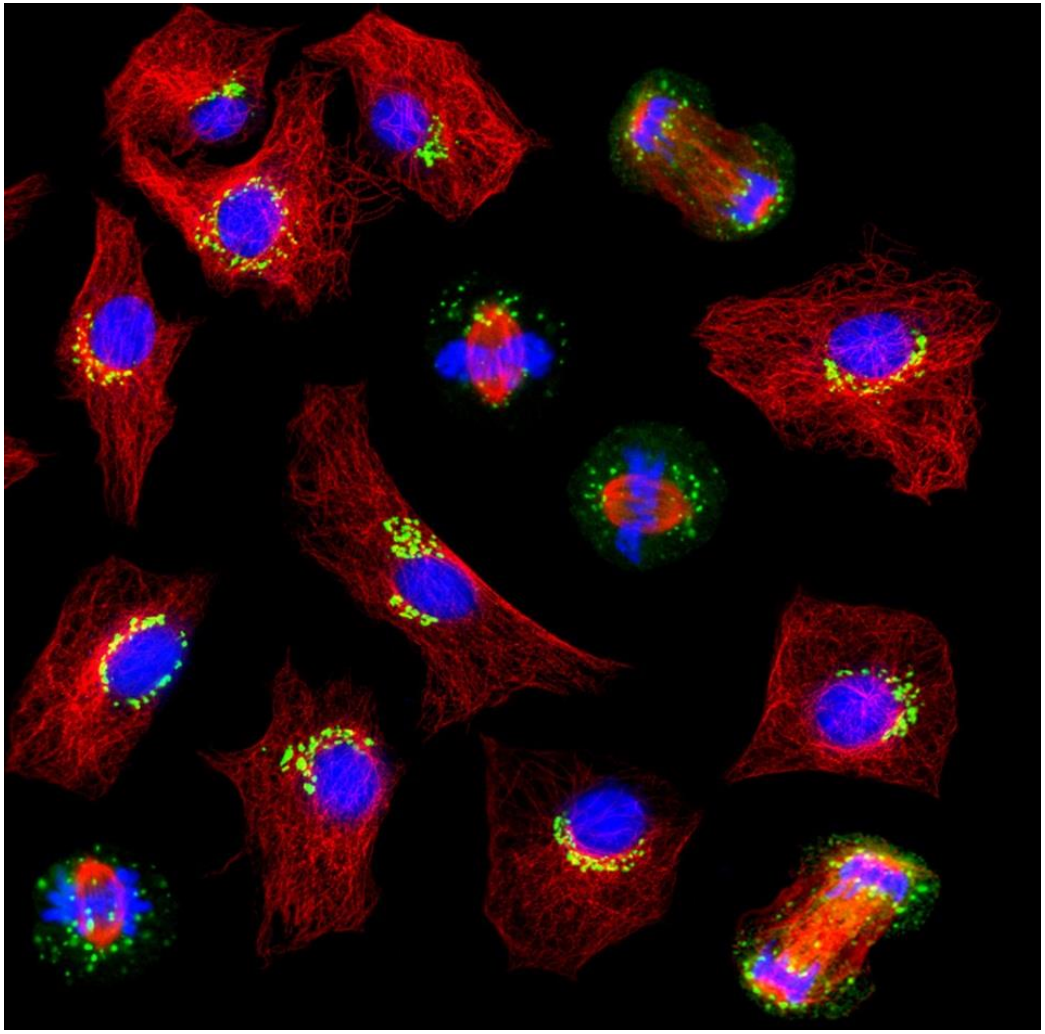
Agriculturual Research,
Alzheimer, Cancer,
Cell Science, Biochemistry,
Botany, Immunology,
Developmental Biology, Ecology,
Epidemiological Deseases,
Evolutionary Biology,
Food design, Genetics, HIV,
Material Quality Control,
Material Sciences, Medicine,
Membrane Research,
Neurobiology , Parasitology,
Pharmacology, Physics,
Plant Biology, Proteomics,
Signal Transduction, Virology...



Basic Function：2D Fluorescent Images

High quality images of Single or Multiple channels



Kidney cell of Rat

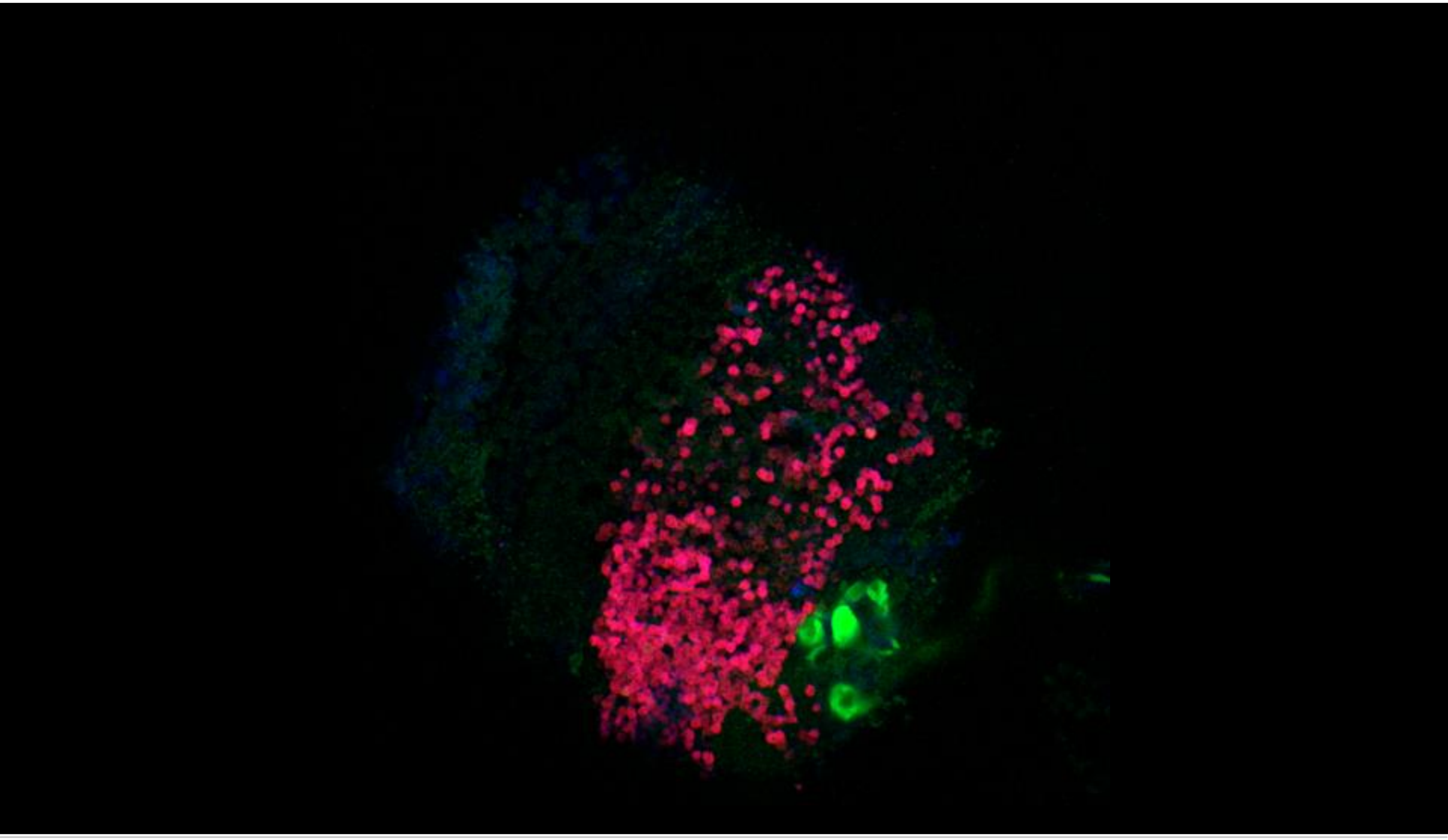
Green：GFP fusion protein，Golgi body

Red：antibody, microtubule

Blue：DAPI，cell nucleus

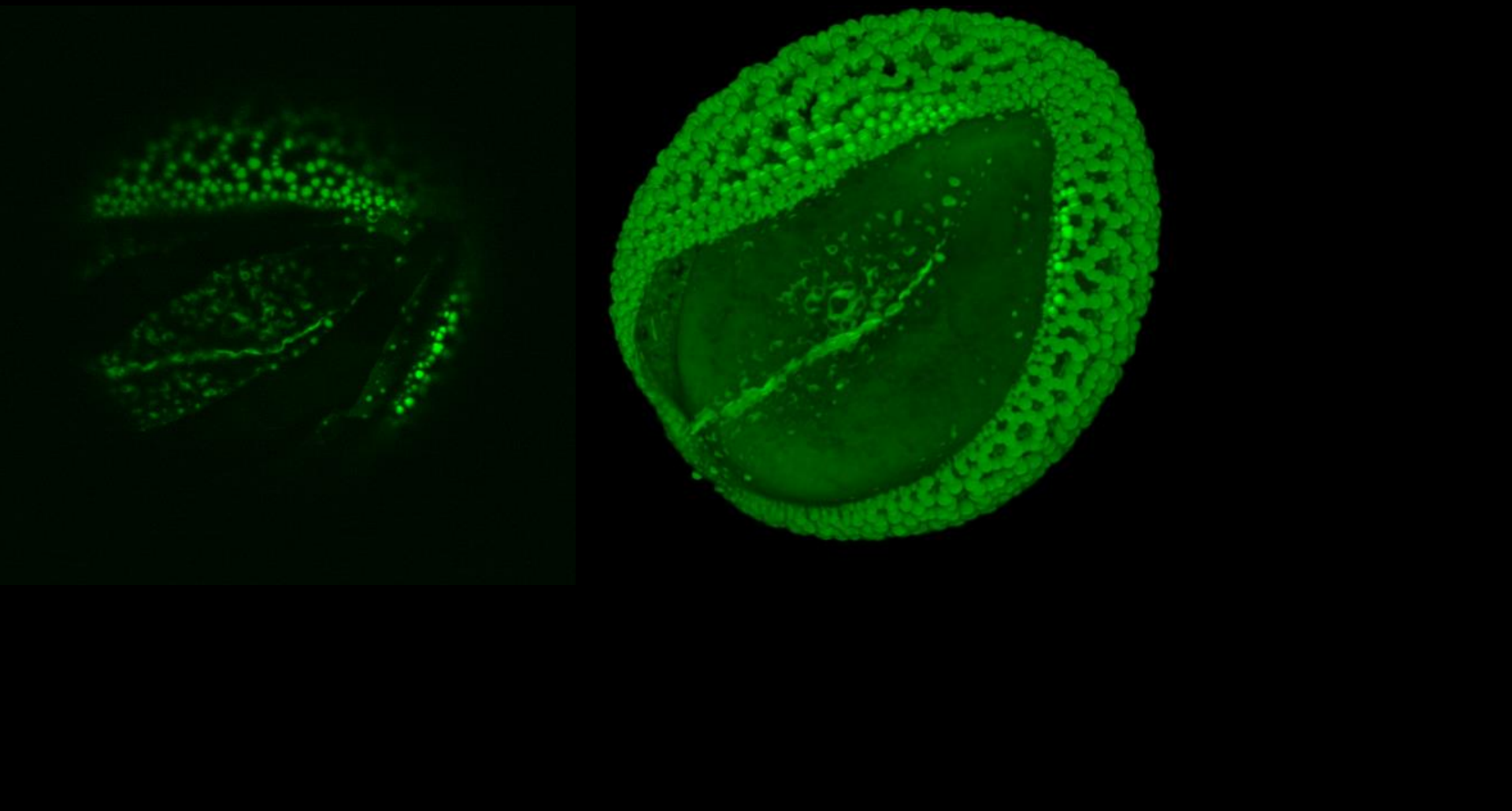
Basic Function: Z-stack

Drosophila Brain– different neuron cluster

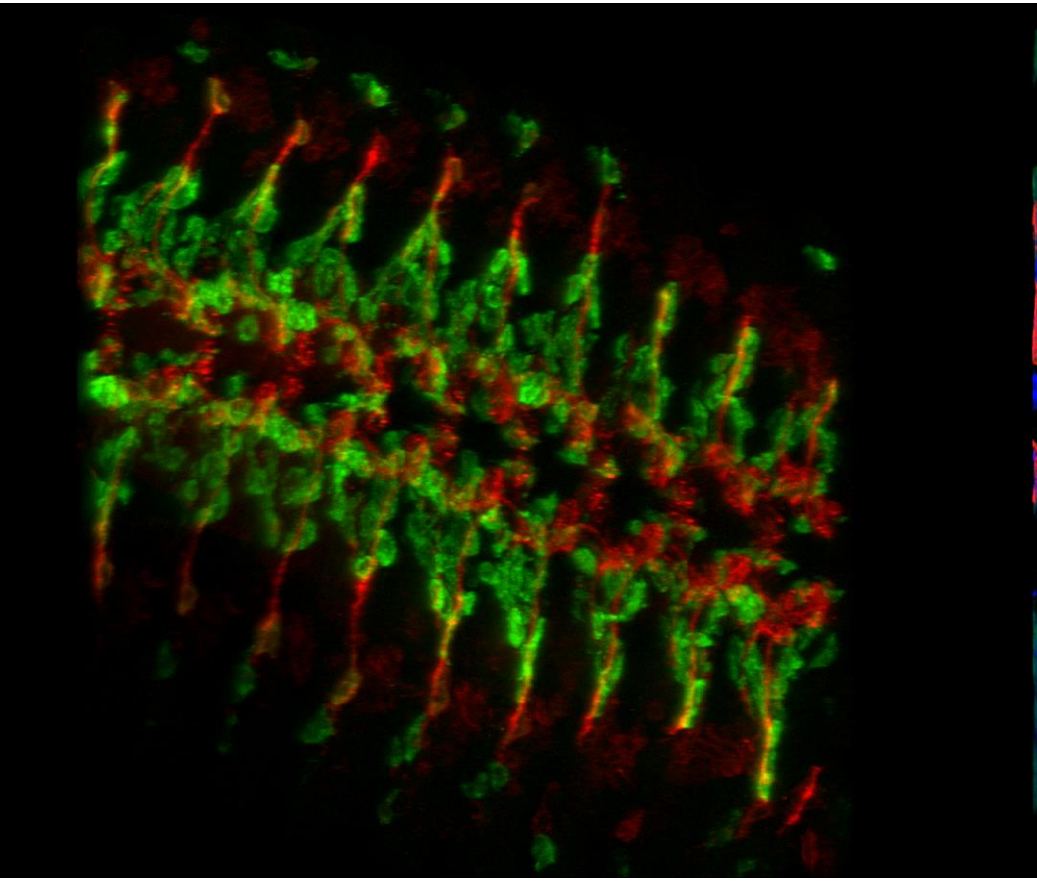


Z-stack

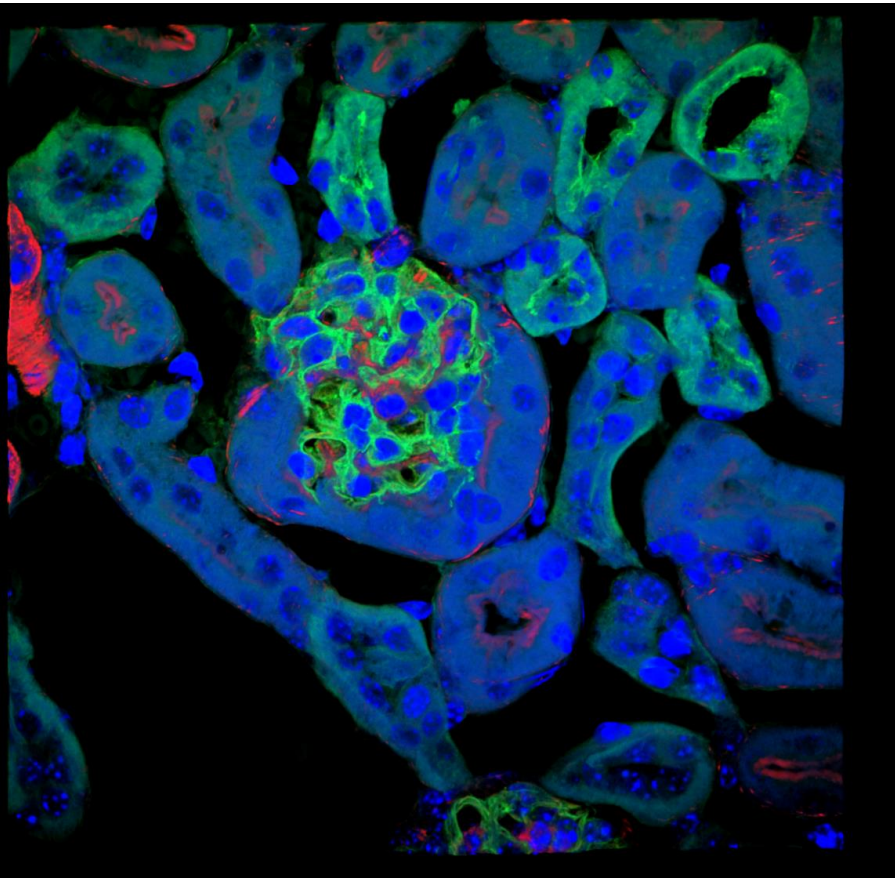
3D reconstruction



Drosophila Nerve System

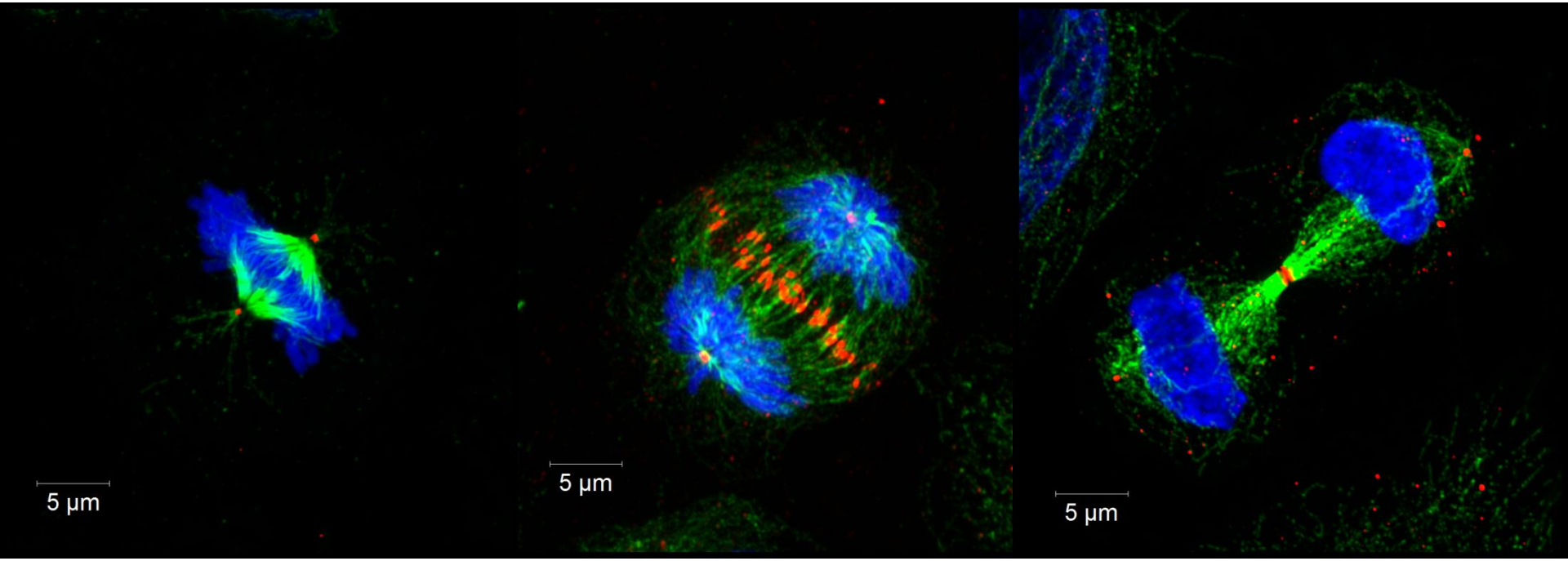


Glomeruli of Rat Kidney



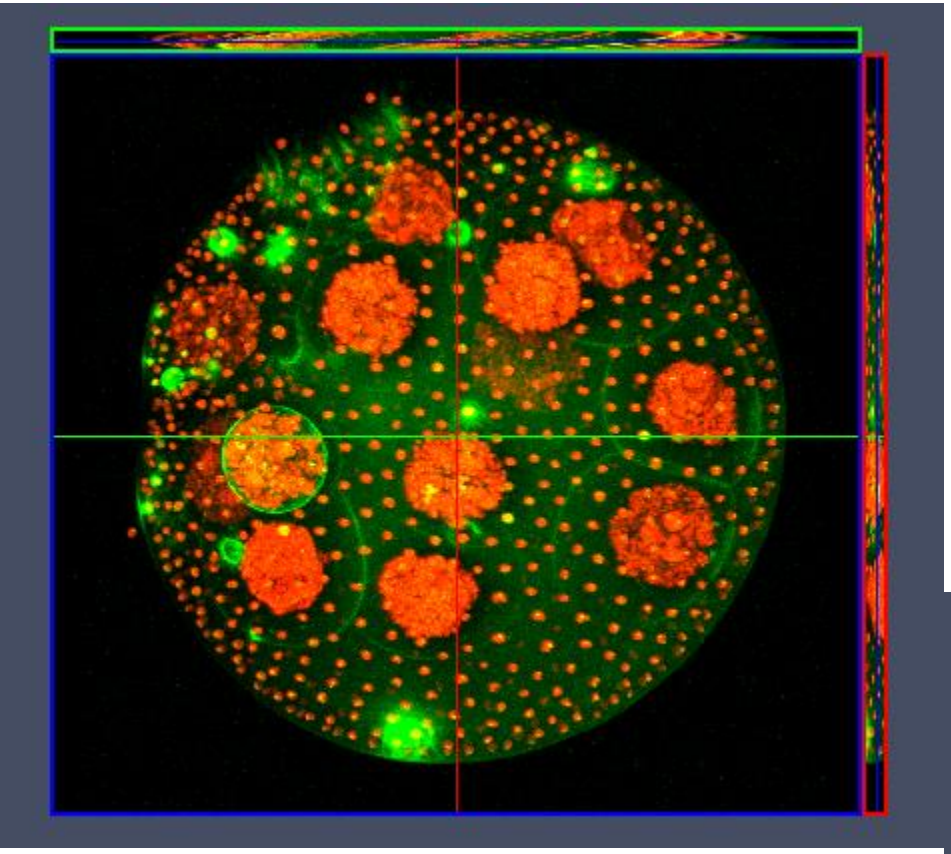
Z-stack

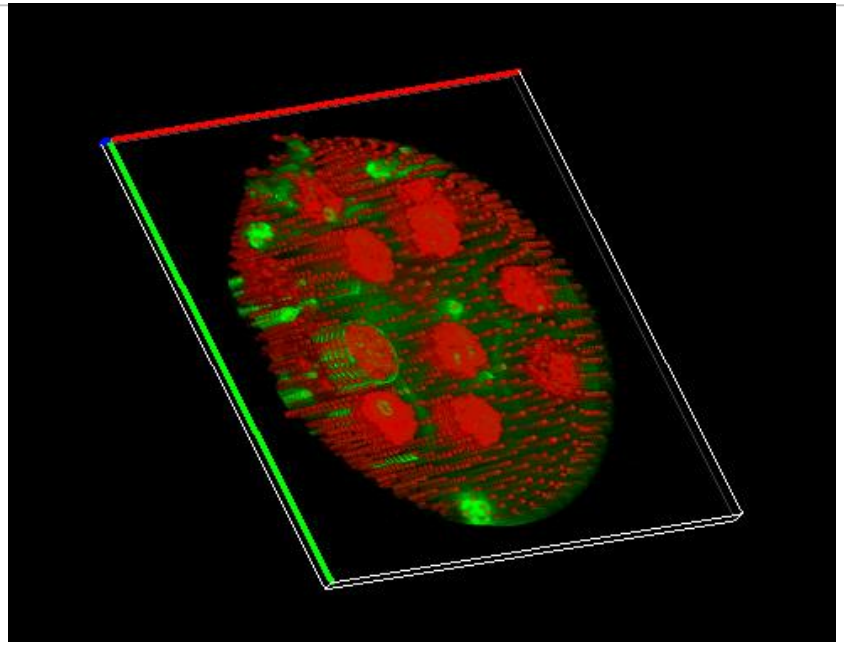
Maximum intensity projection (MIP)

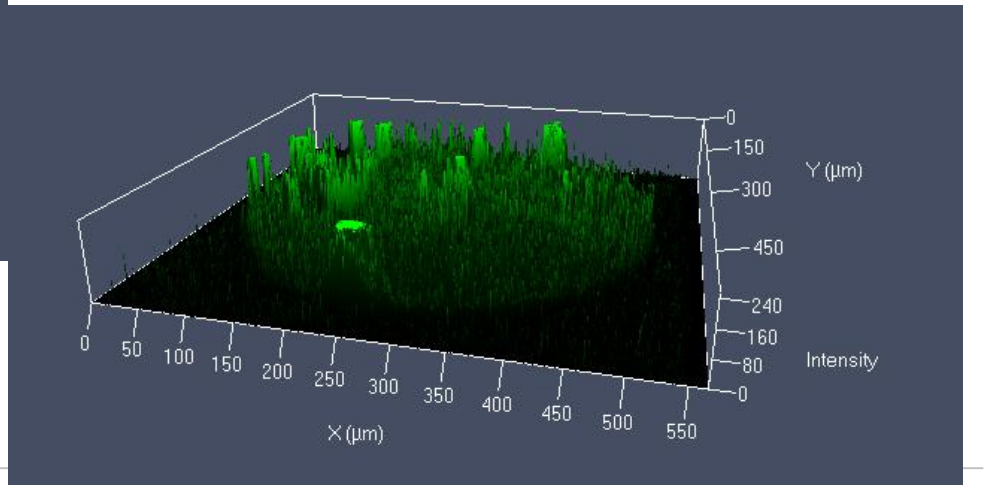


- Objective: Plan-Apo 63x/1.4 Oil
- MIP

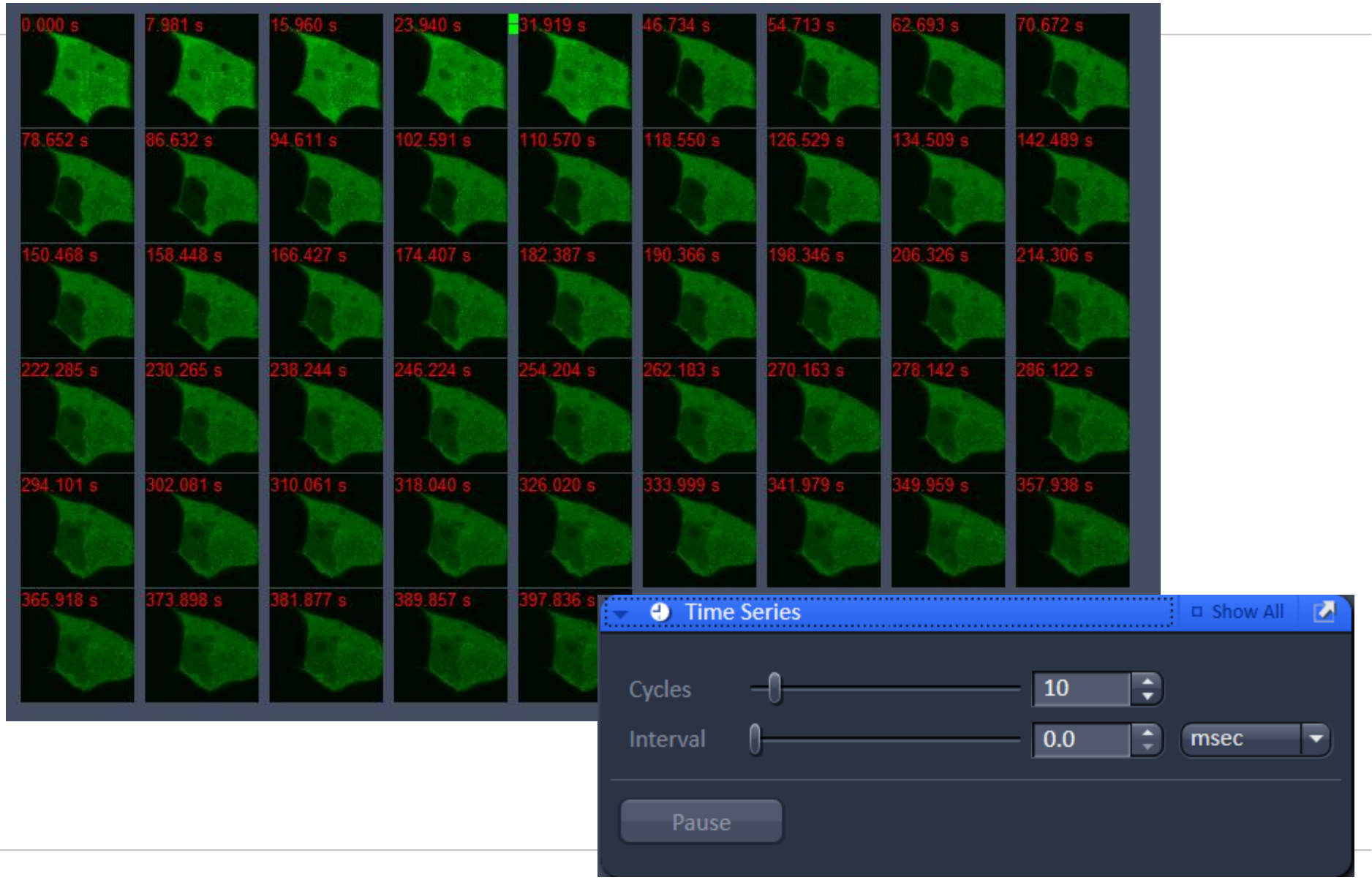
Ortho Function： X-Z、Y-Z cross section



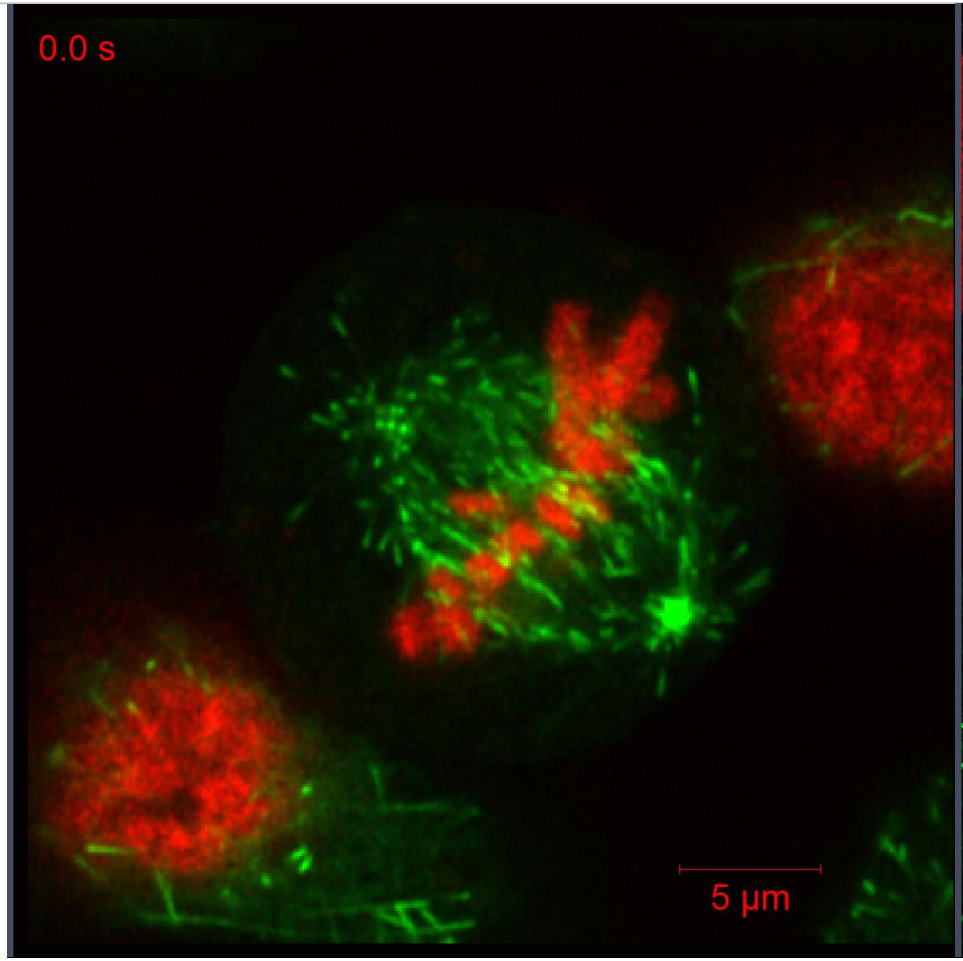


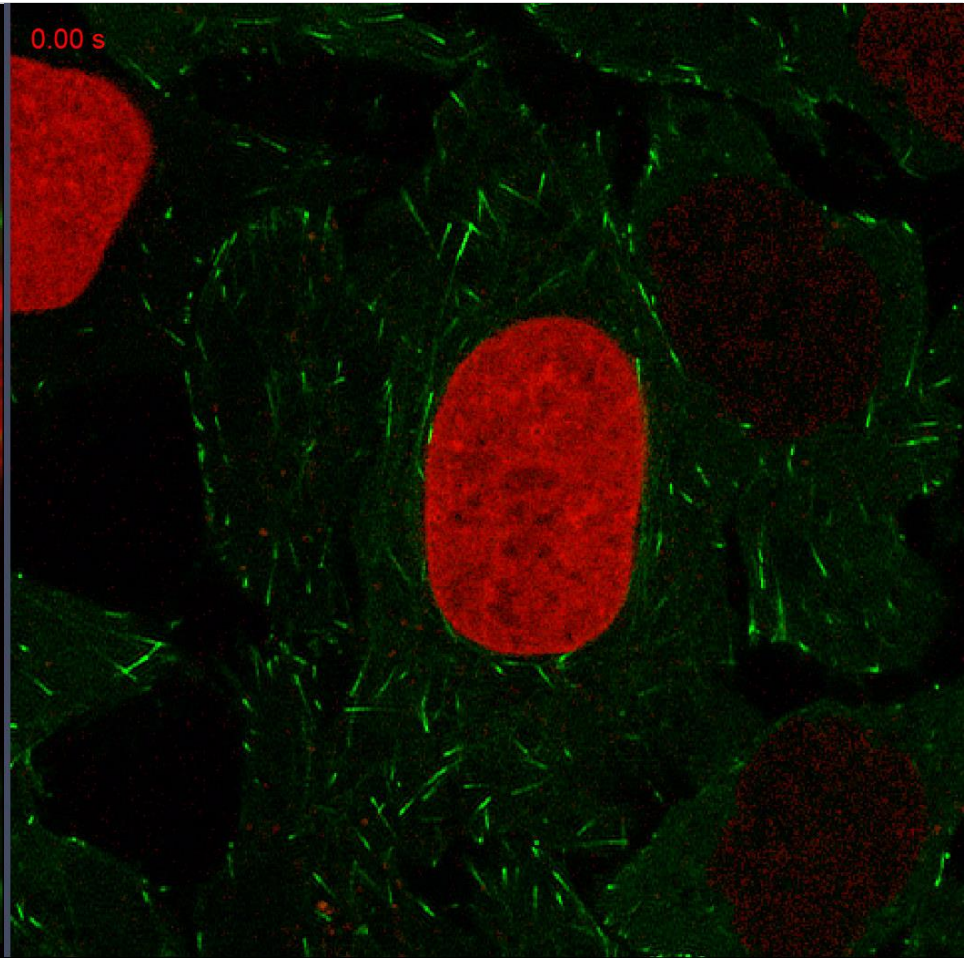


Basic Function： Time series



Time series imaging



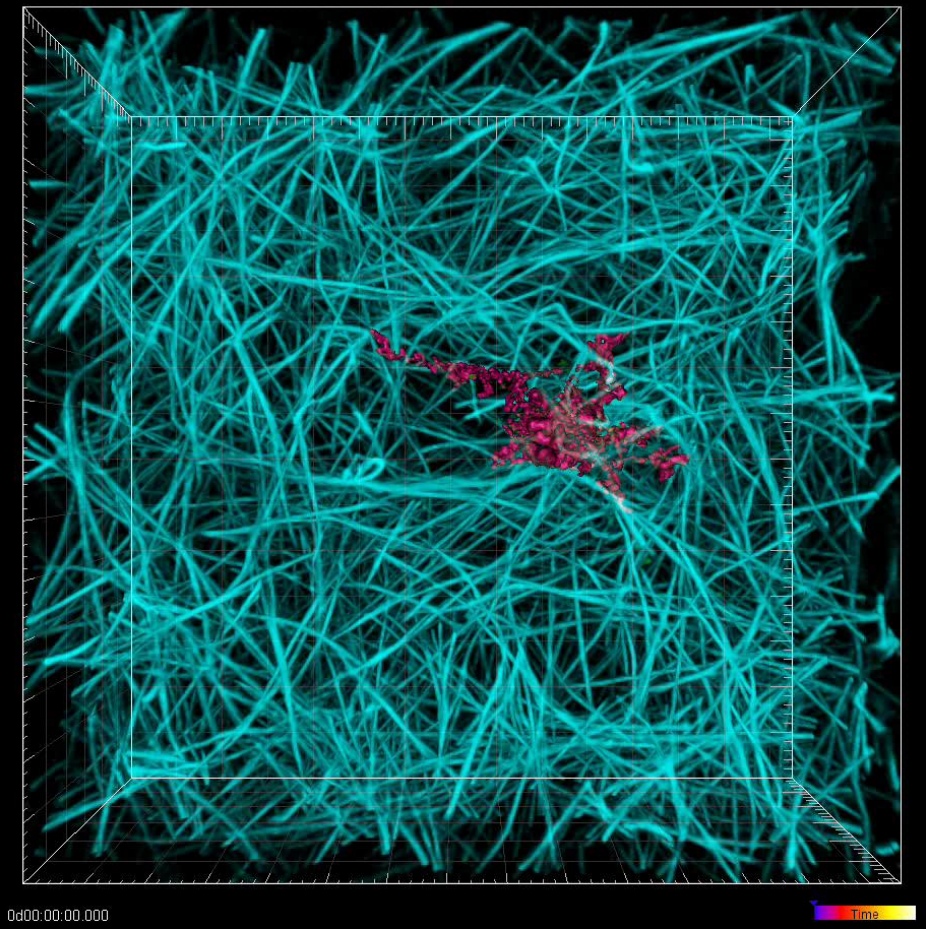


Mitosis in HeLa-Kyoto cell line during mitosis
Video showing Histone 2B (H2B, red, mCherry) and microtubule end-binding protein 3 (EB3, blue, EGFP)
Sample courtesy of: Jan Ellenberg, EMBL, Heidelberg.

4D dynamic structure

Magration of the cancer cell

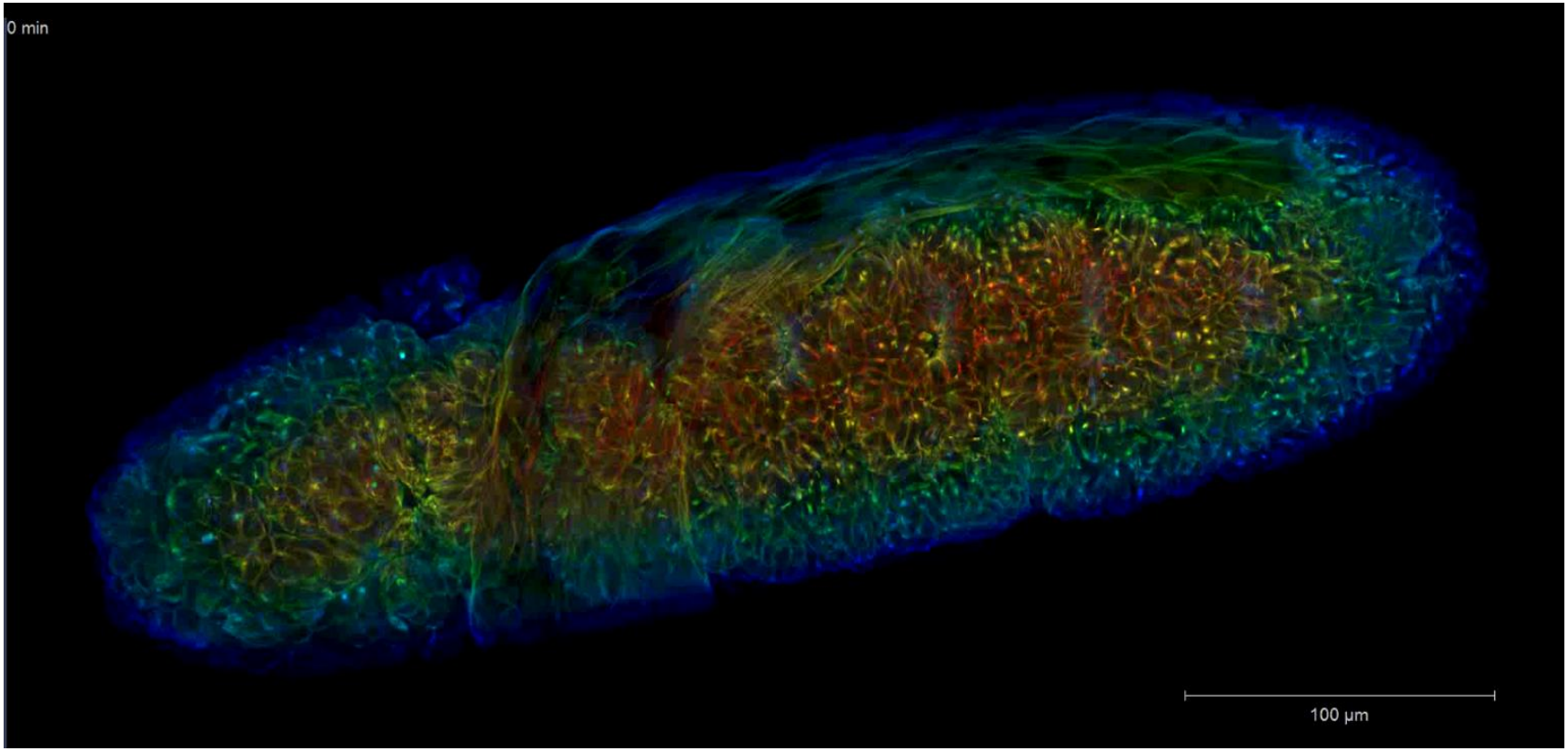
样品：MDA231细胞
胶质 GFP
基质金属蛋白酶 Alexa 647



来源:
Institute of Cancer, Fudan University
Shanghai, China

4D dynamic structure

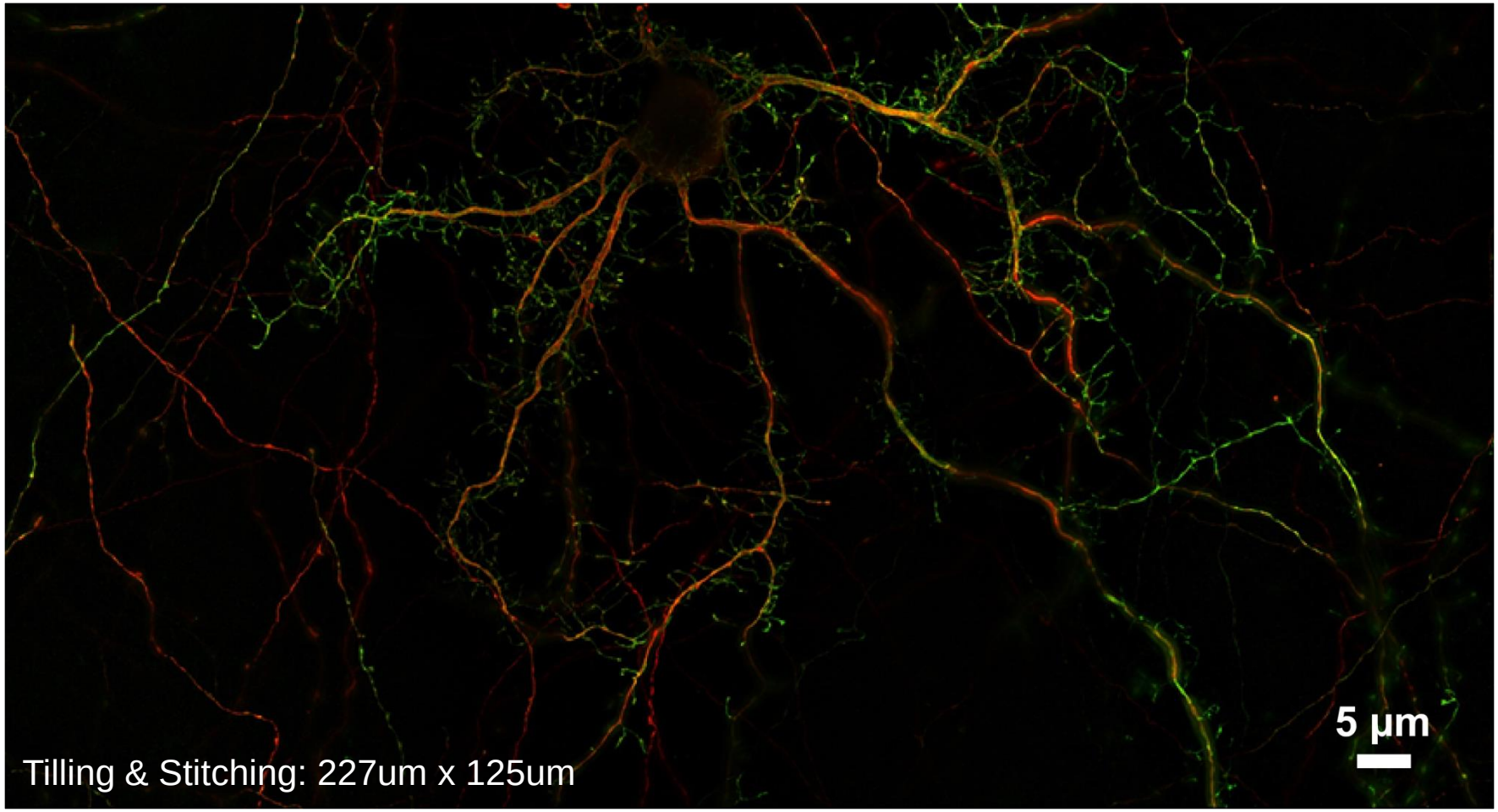
Development of drosophila embryo



Drosophila melanogaster embryo, Jupiter-GFP (microtubules)
Z-stack depth coded: 72 slices, Every 15 min, 11.5 h
Sample courtesy of Balazs Erdi, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter

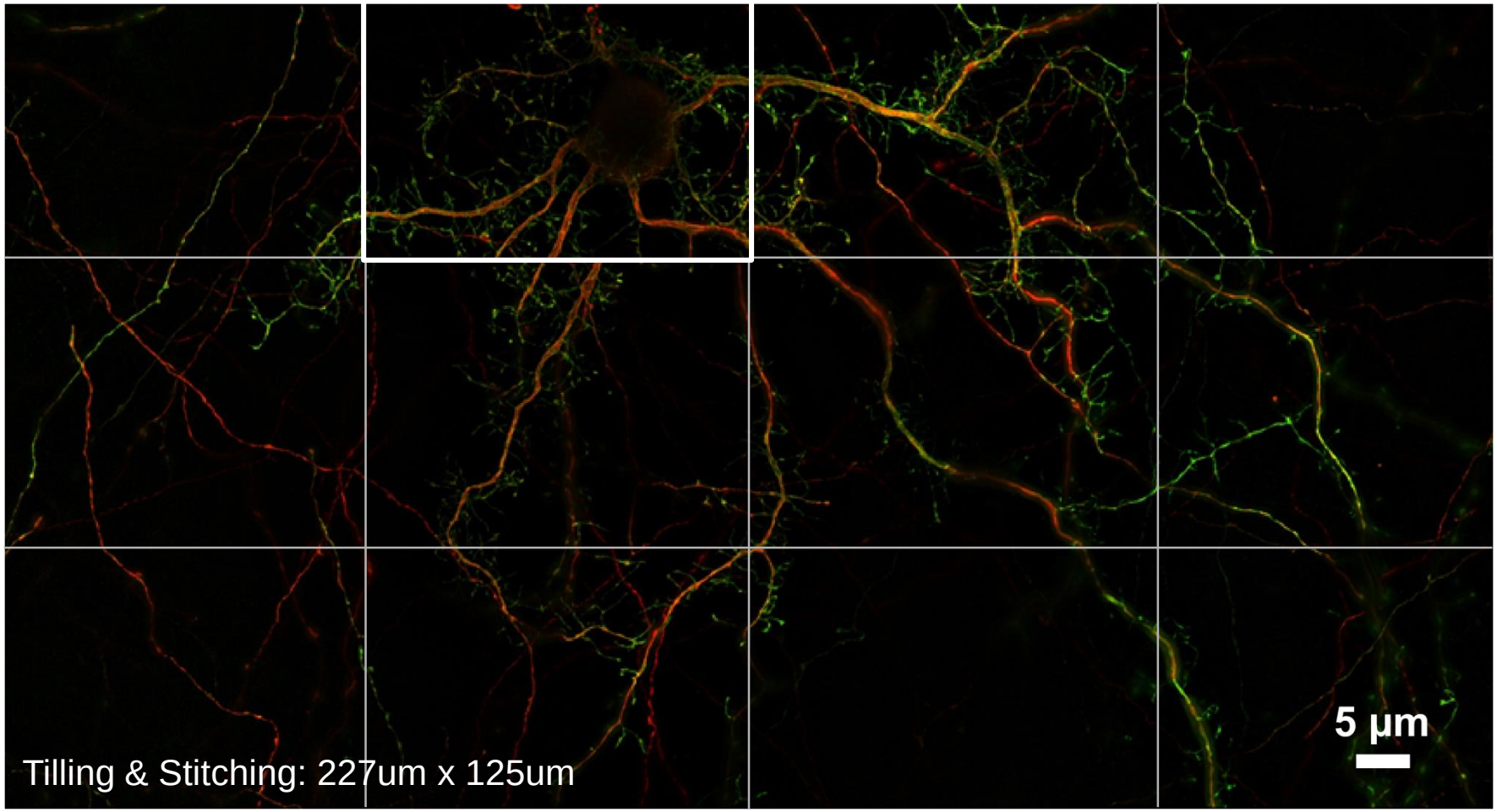
Basic Function：Tile scan

expend field of view

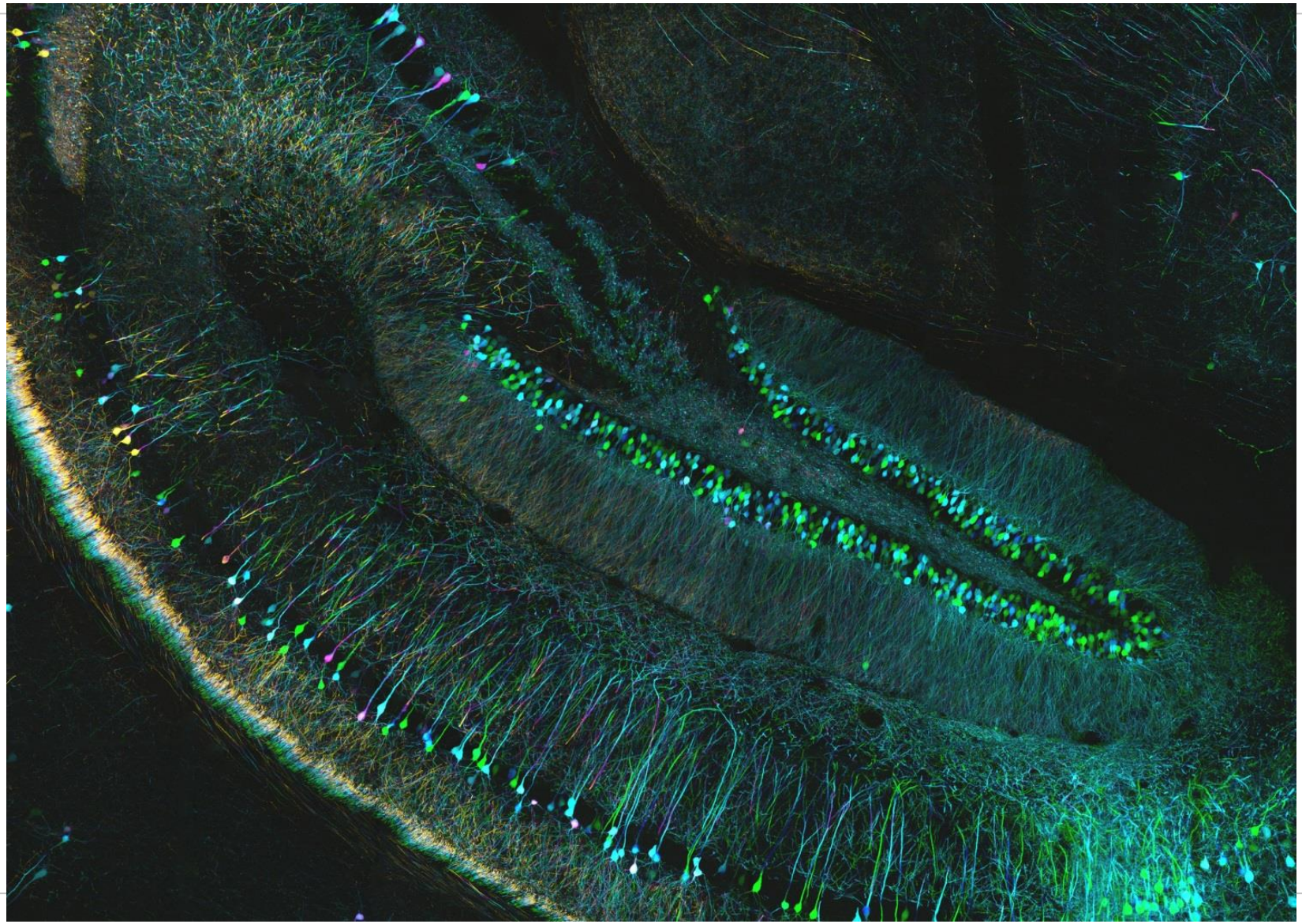


Basic Function： Tile scan

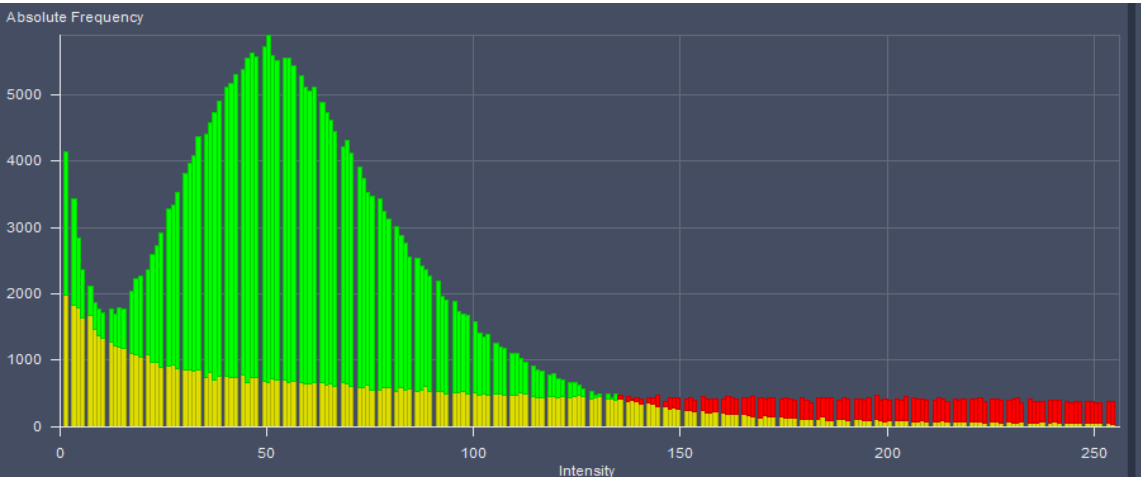
expend field of view

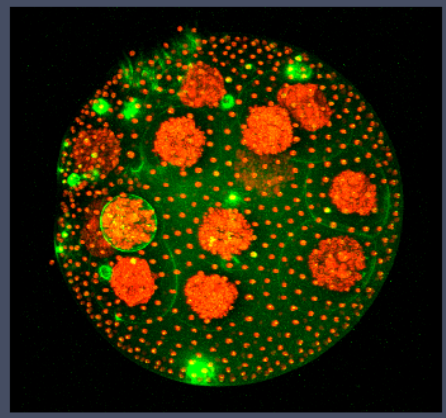


Brain section of Rat (Z-stack, Tiled, MIP)



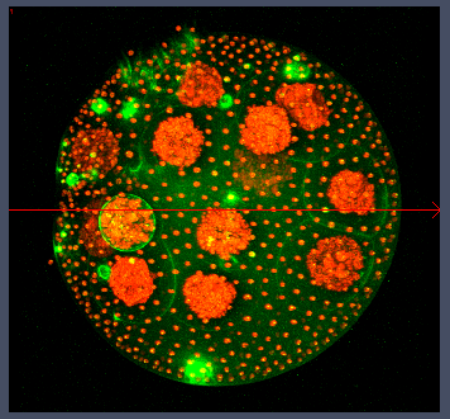
Measurement and analysis: Intensity





	Ch1	Ch2	Ch2
Mean Intensity	43.45529	37.38946	37.38946
Standard Deviation	84.16126	44.23883	44.23883
Pixels	522900	522900	522900
Area [µm x µm]	299038.87	299038.87	299038.87

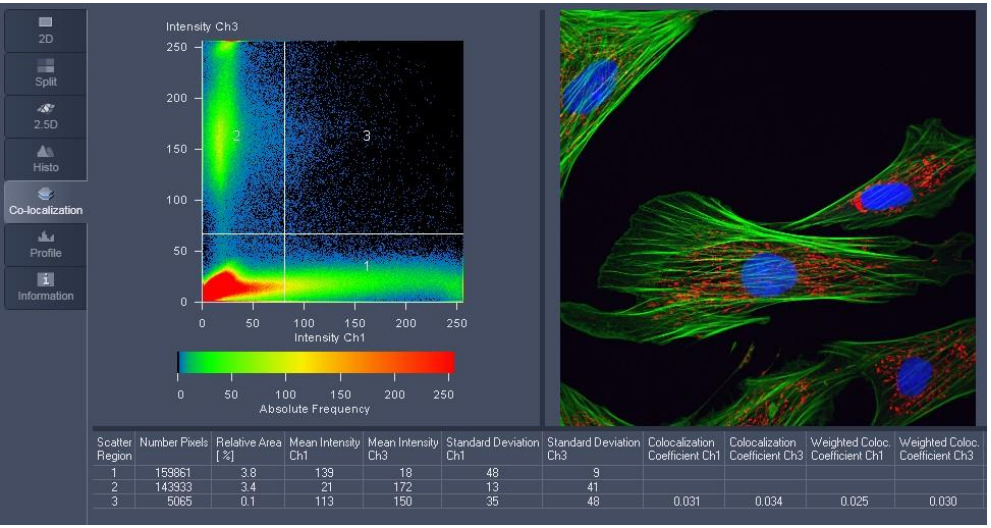
Profile
Analyze intensity distribution of a line



Used to analyze the distribution of specific markers, when using medicine to stimulate bioplast or suspension cells

co-localization

quantity colocalization analysis of two fluorescents signal



Scatter Region	Number Pixels	Relative Area [%]	Mean Intensity Ch1	Mean Intensity Ch3	Standard Deviation Ch1	Standard Deviation Ch3	Colocalization Coefficient Ch1	Colocalization Coefficient Ch3	Weighted Coloc. Coefficient Ch1	Weighted Coloc. Coefficient Ch3
1	159861	3.8	139	18	48	9				
2	143933	3.4	21	172	13	41				
3	5065	0.1	113	150	35	48	0.031	0.034	0.025	0.030

Directly show the co-localization coefficent

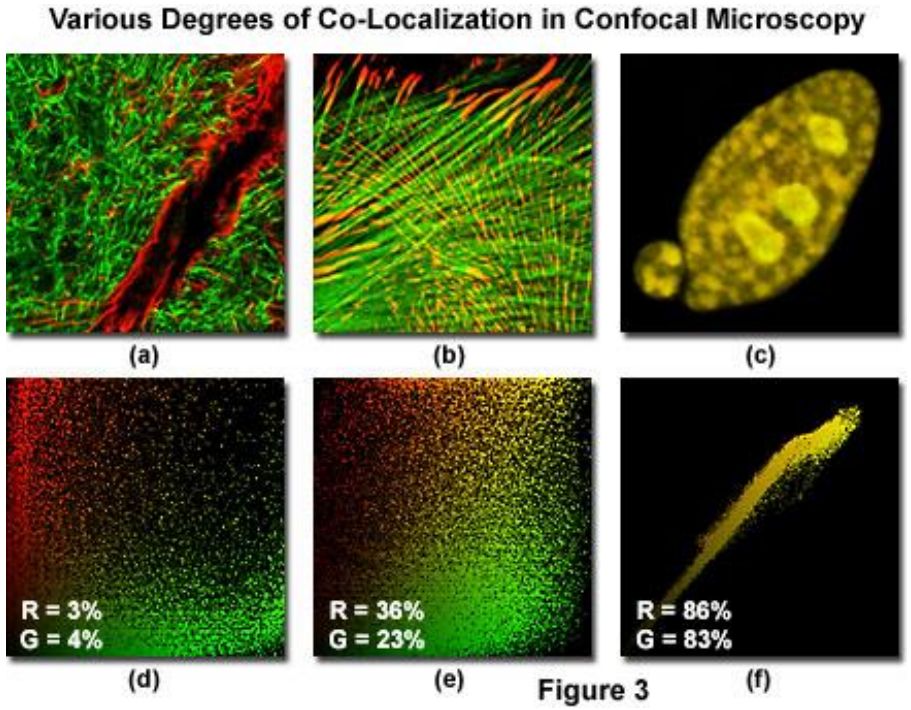
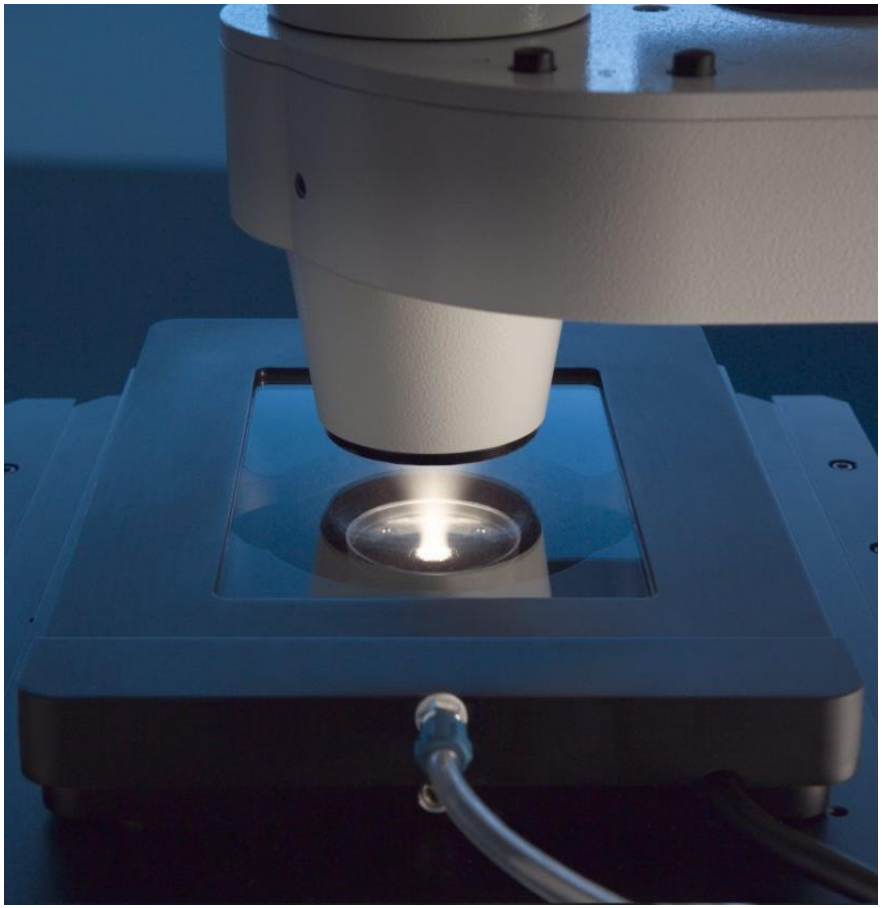


Figure 3

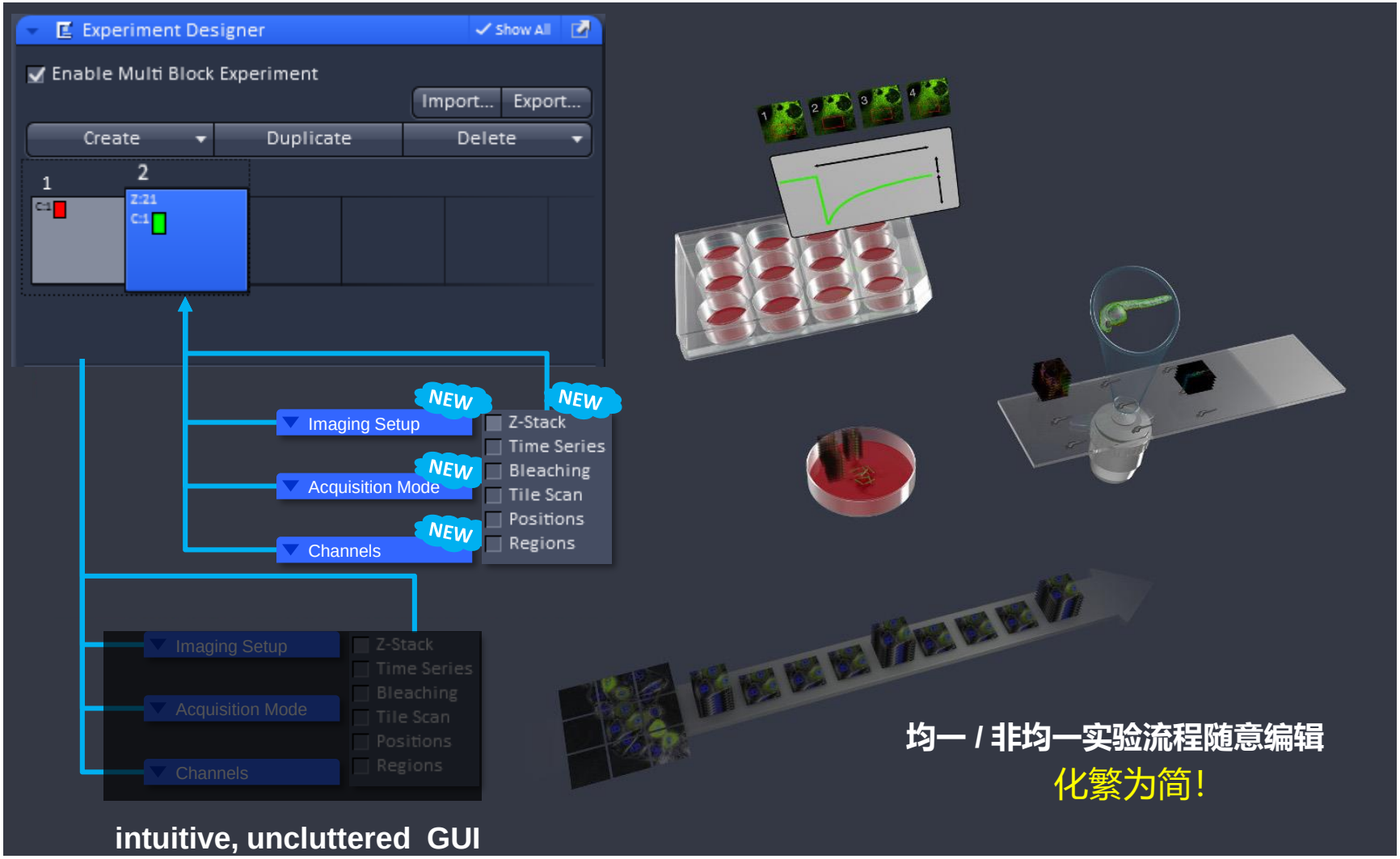
$$R_r = \frac{\sum_i (S1_i - S1_{aver}) \bullet (S2_i - S2_{aver})}{\sqrt{\sum_i (S1_i - S1_{aver})^2 \bullet \sum_i (S2_i - S2_{aver})^2}}$$

Expanding Function：Live cell Incubator

Culture and imaging of live cells

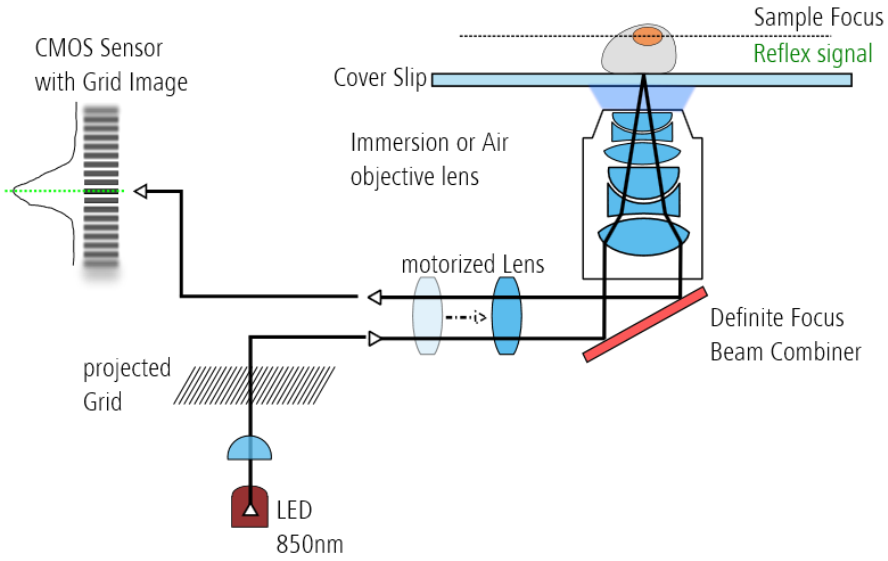


Experiment designer
6D long term live cell imaging



稳定快速的焦距控制 - Definite Focus.2

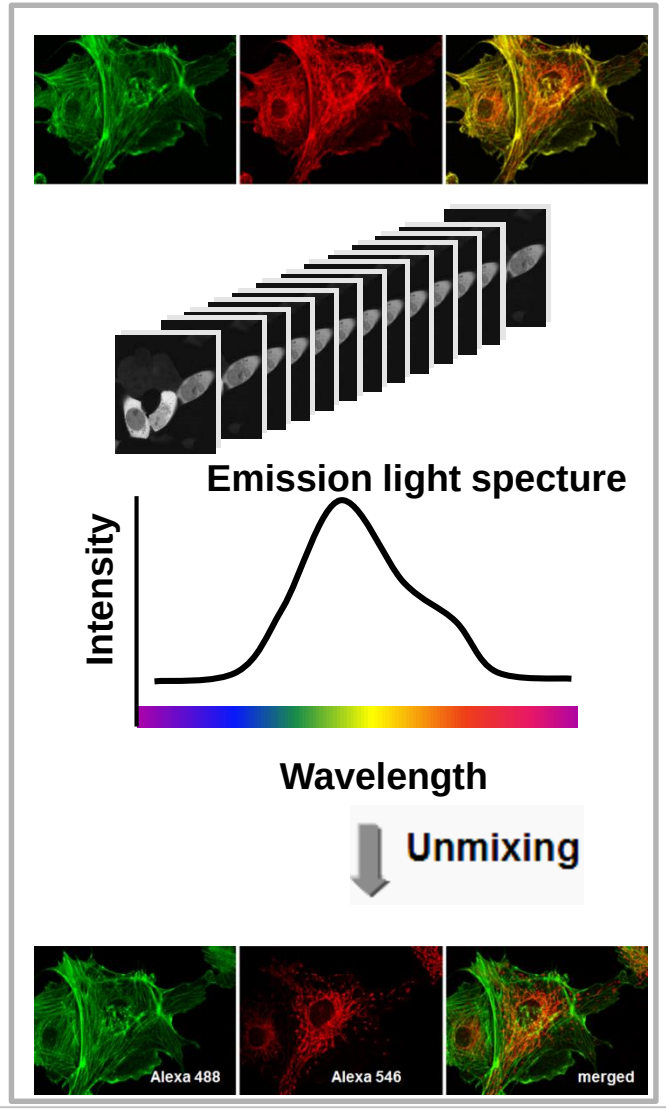
长时间活细胞成像



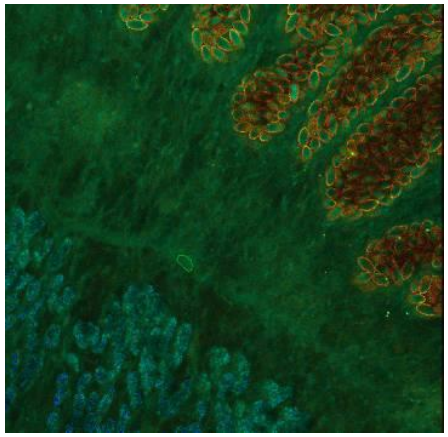
- 高达**200HZ的高速稳定模式**确保实时成像的稳定性
- 使用了数字控制的压电陶瓷Z轴来实现高分辨成像的稳定性（适用于Zen Black 2.1及以上版本）
- 具备**多位点完美聚焦**补偿，可确保每一个位置的细胞都完美的聚焦
- 重新设计的DF.2软件控制功能(Zen Blue/Black)
- 物镜和显微镜兼容性更好

Expanding Function：Linear unmixing

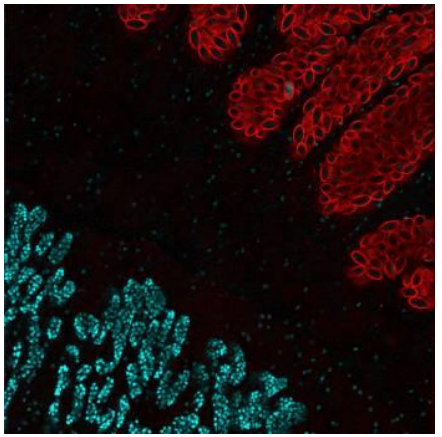
最大程度避免荧光串色的影响

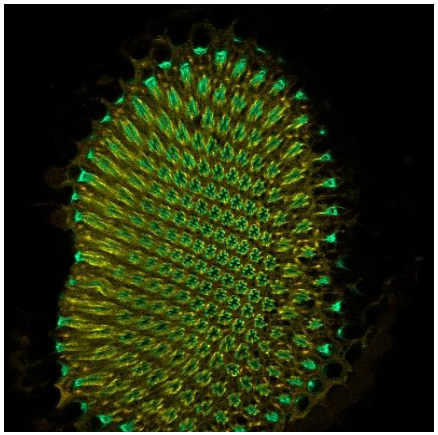


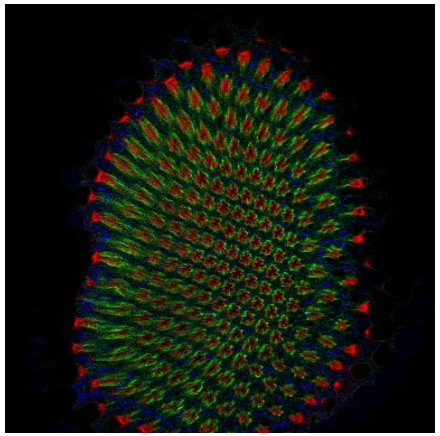
Before unmixing



After unmixing







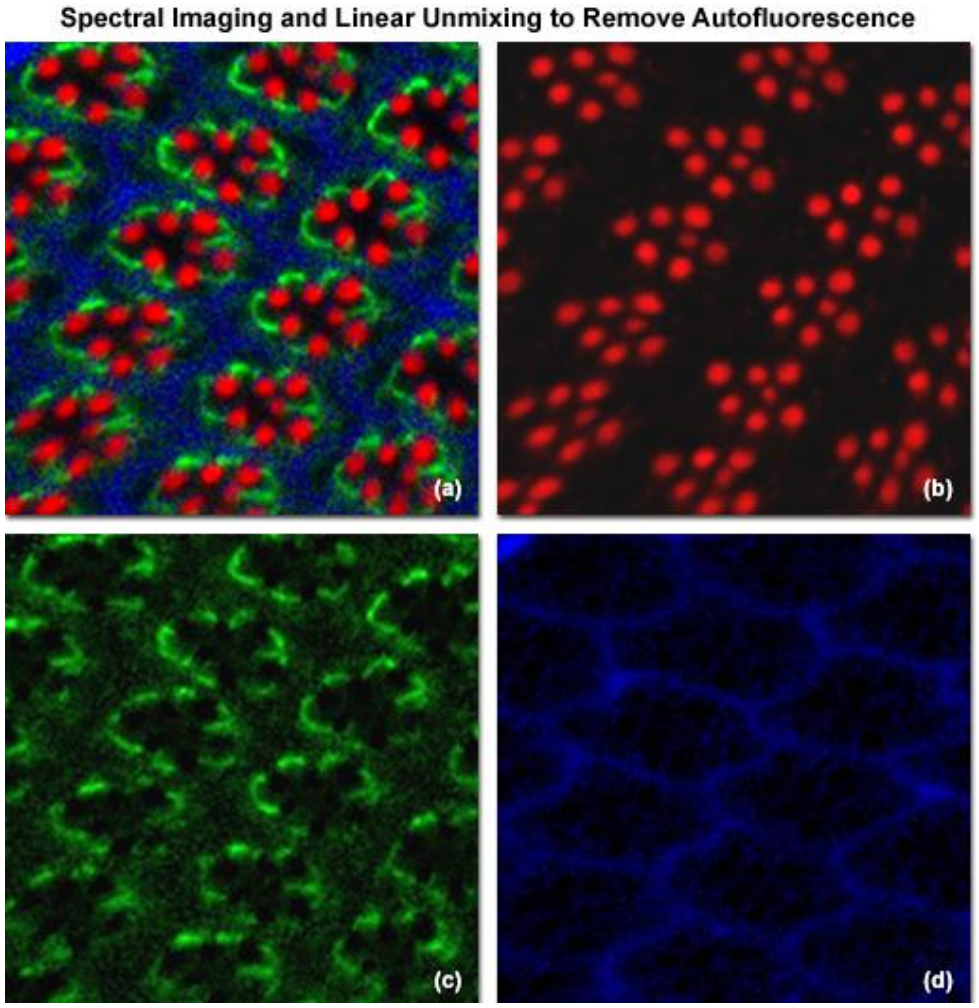
光谱拆分
去除自发荧光

果蝇眼

Green: AF488, actin

Red: Cy3, Crumbs

Blue: autofluorescence



Picture from Zeiss Campus
http://zeiss-campus.magnet.fsu.edu /articles/
spectralimaging/considerations.html

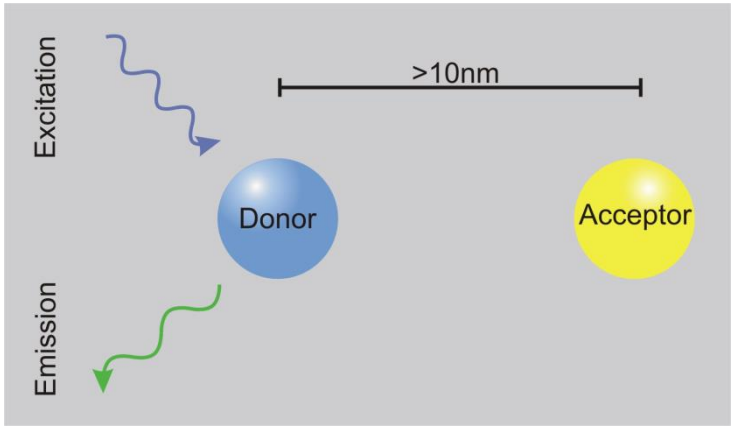
Expending Function：FRET

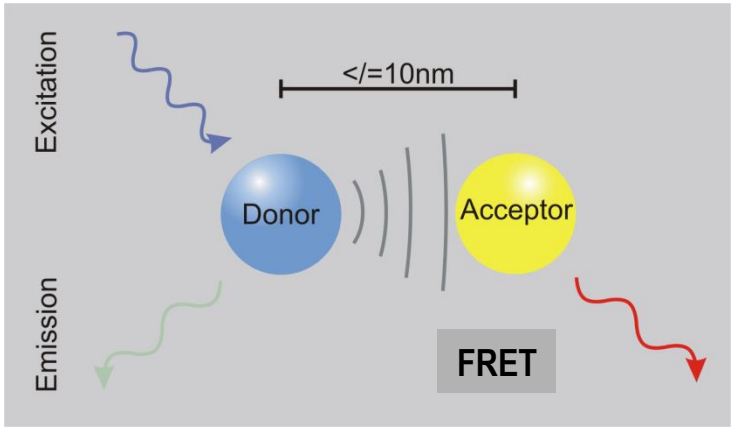
Fluorescence Resonance Energy Transfer 荧光共振能量转移

FRET是指当两个荧光基团满足一定条件时发生的一种非辐射性的、偶极-偶极配对过程，借此过程，能量从激发态荧光供体以非常接近的波长转移到荧光受体。

FRET发生的条件：

- 供体与受体的距离在2-10nm
- 供体的发射波长与受体的激发波长一致
- 供体与受体的极性一致
- 供体与受体有足够的荧光寿命



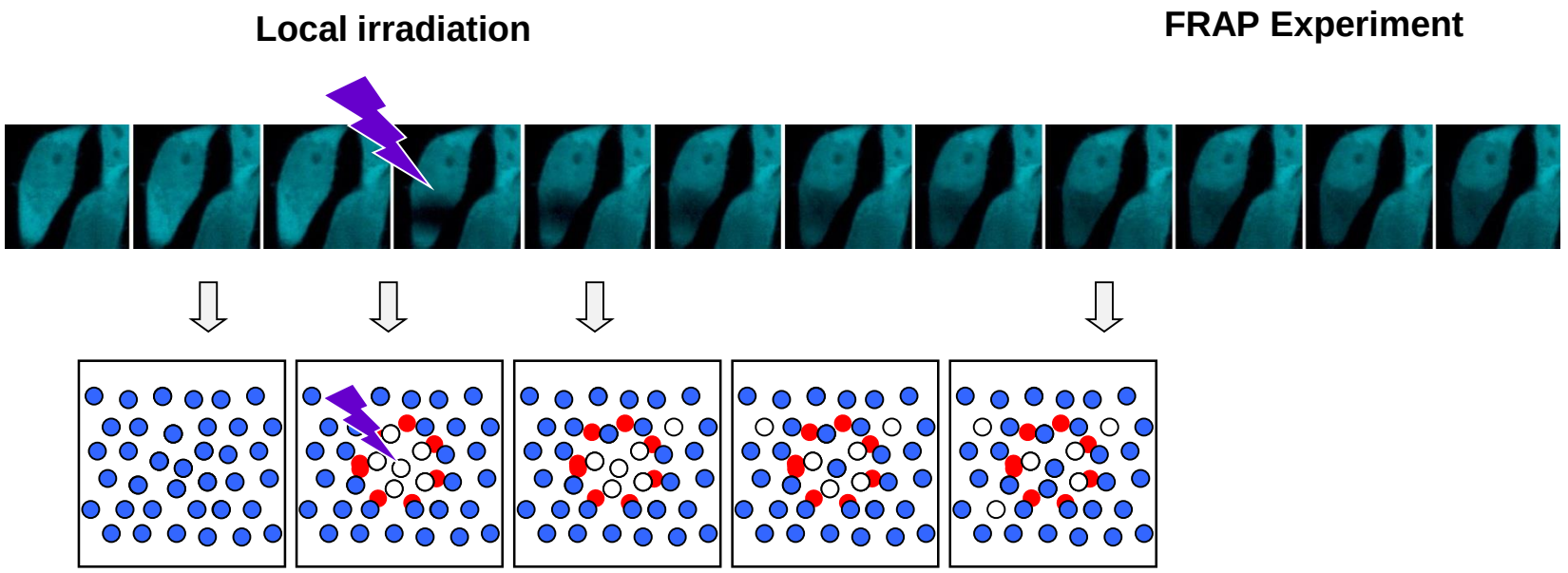


常用的FRET组合：

CFP/YFP
CFP/dsRED
BFP/GFP
GFP/dsRED
YFP/dsRED
Cy3/Cy5
Alexa488/Alexa555
Alexa488/Cy3
FITC/TRITC
YFP/TRITC
YFP/ Cy3

Expanding Function: FRAP

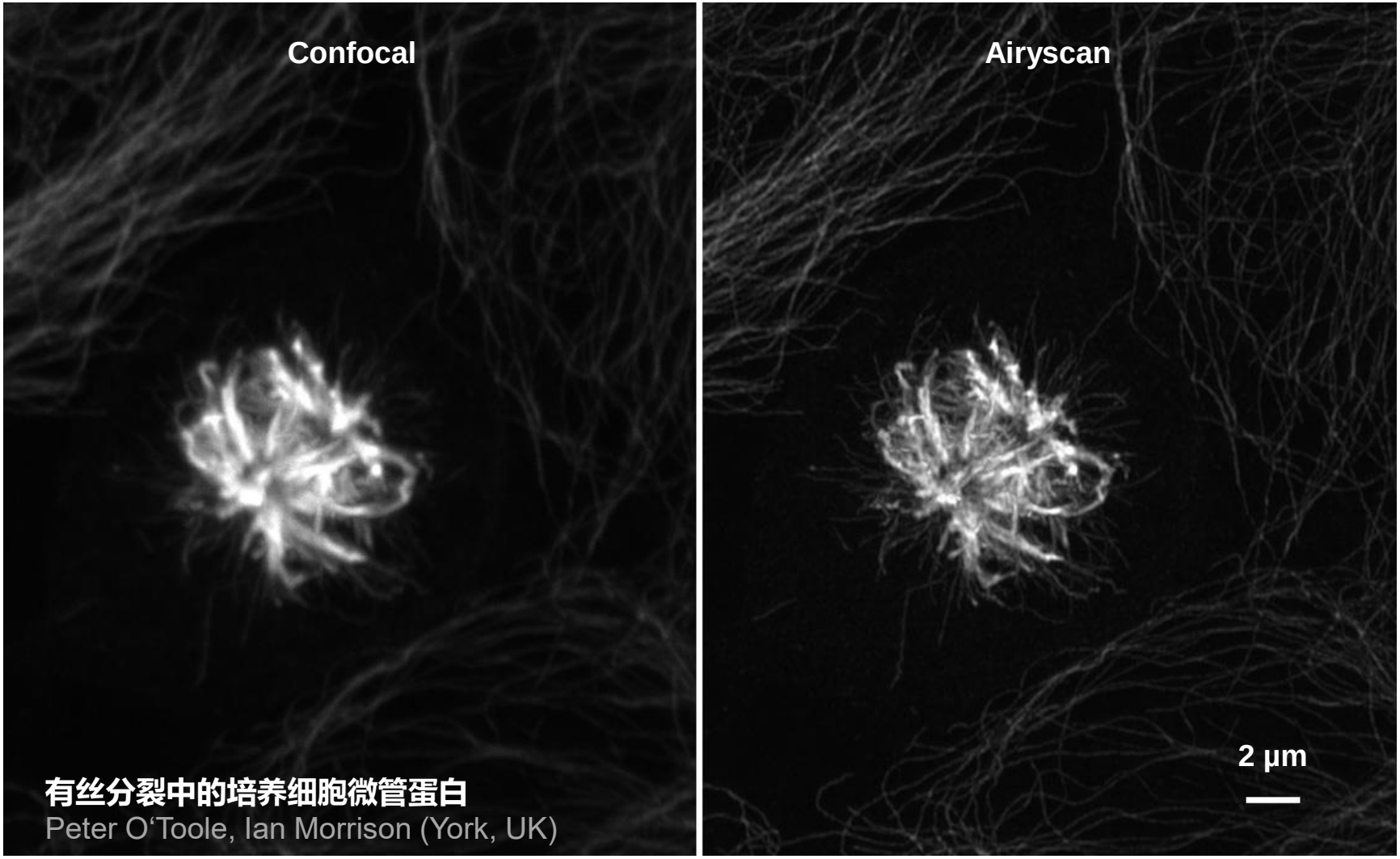
Fluorescence Recovery After Photobleaching 荧光漂白后恢复实验



对分子的移动性进行量化

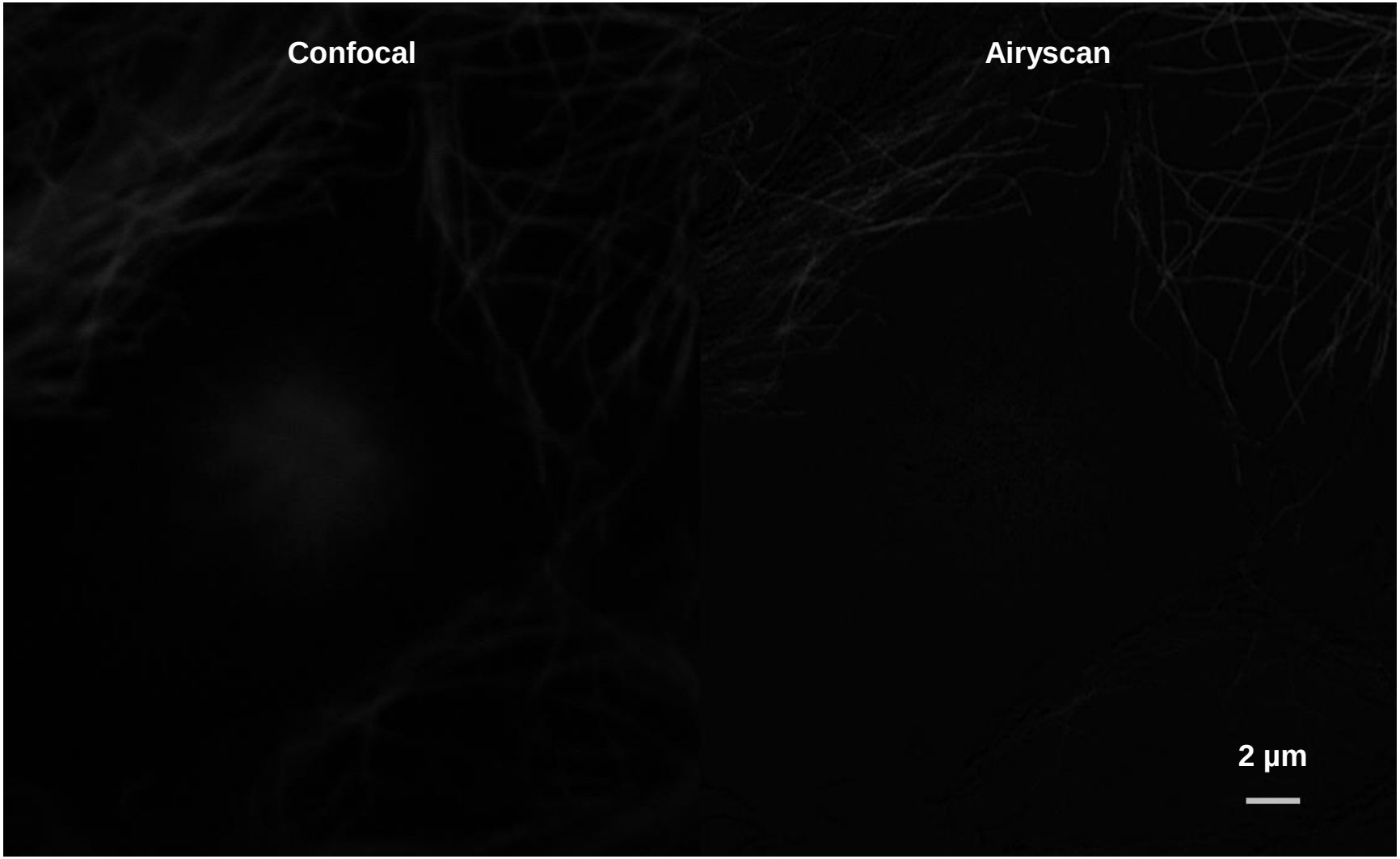
Expanding Function：Super Resolution Microscopy

Airyscan



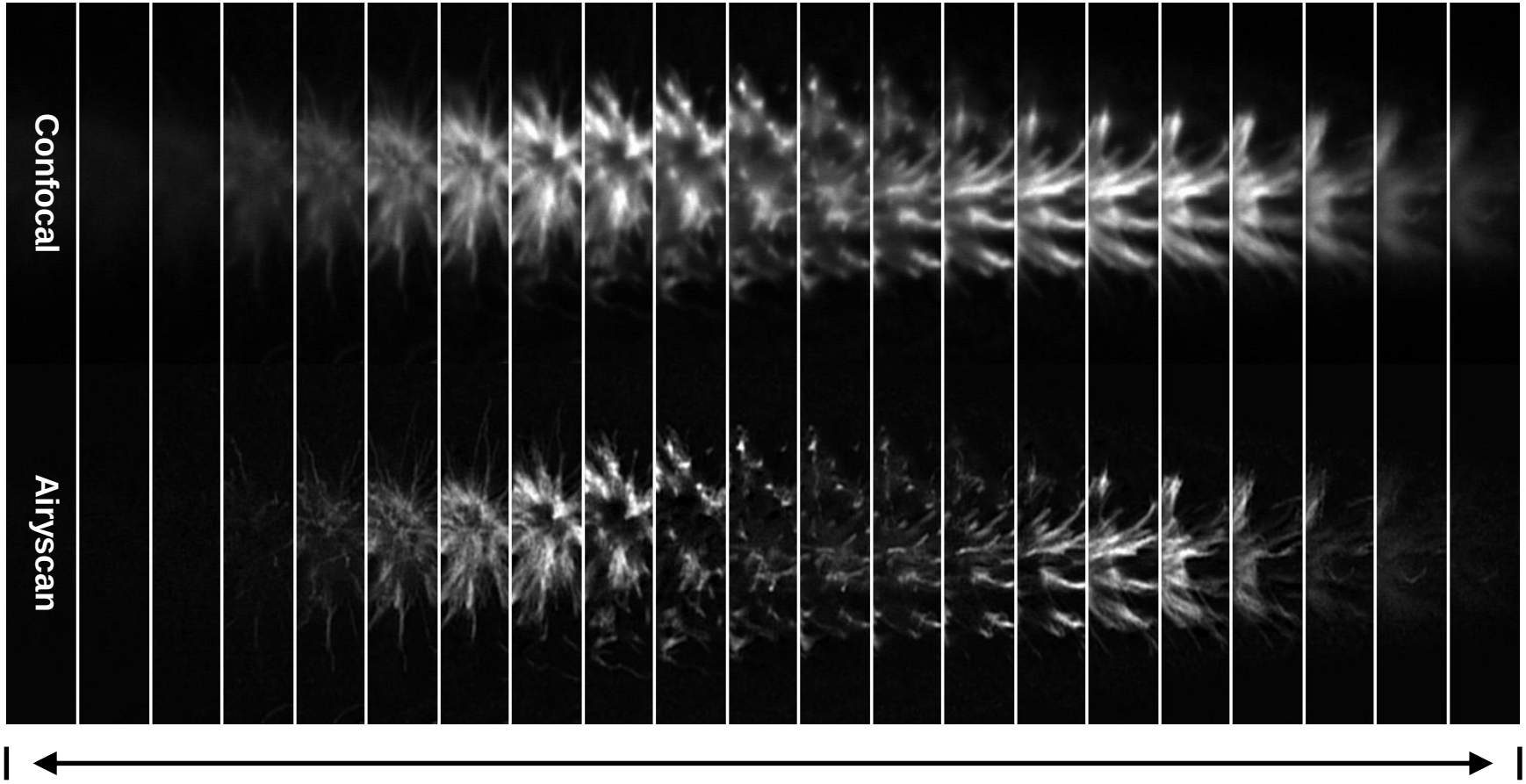
有丝分裂中的培养细胞微管蛋白
Peter O'Toole, Ian Morrison (York, UK)

Expanding Function：Super Resolution Microscopy
Airyscan



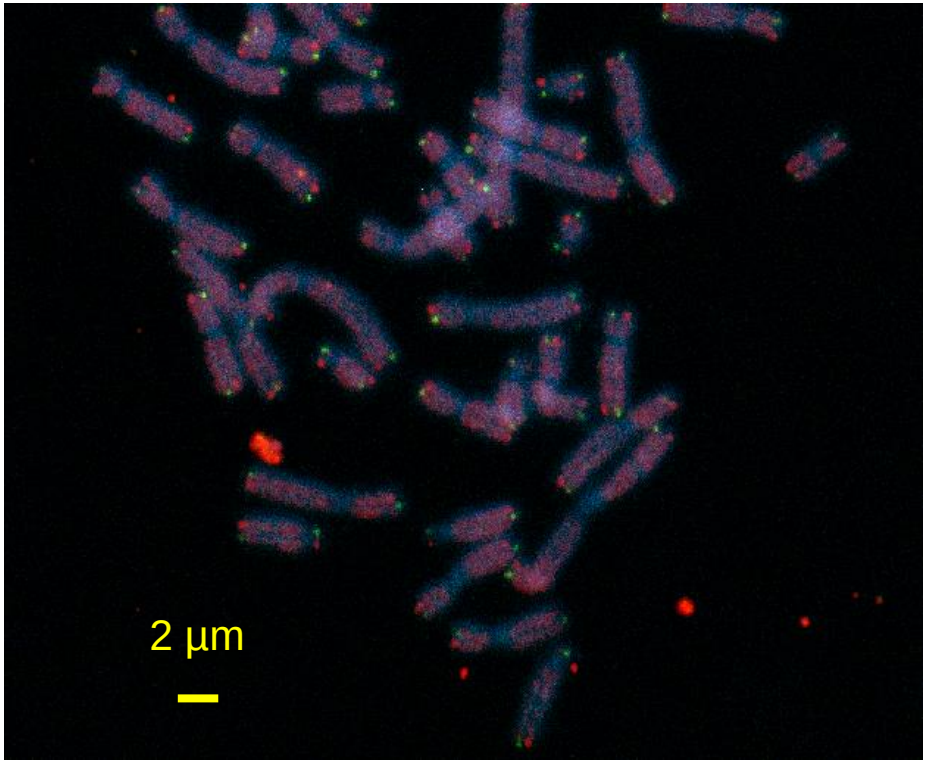
Expanding Function：Super Resolution Microscopy

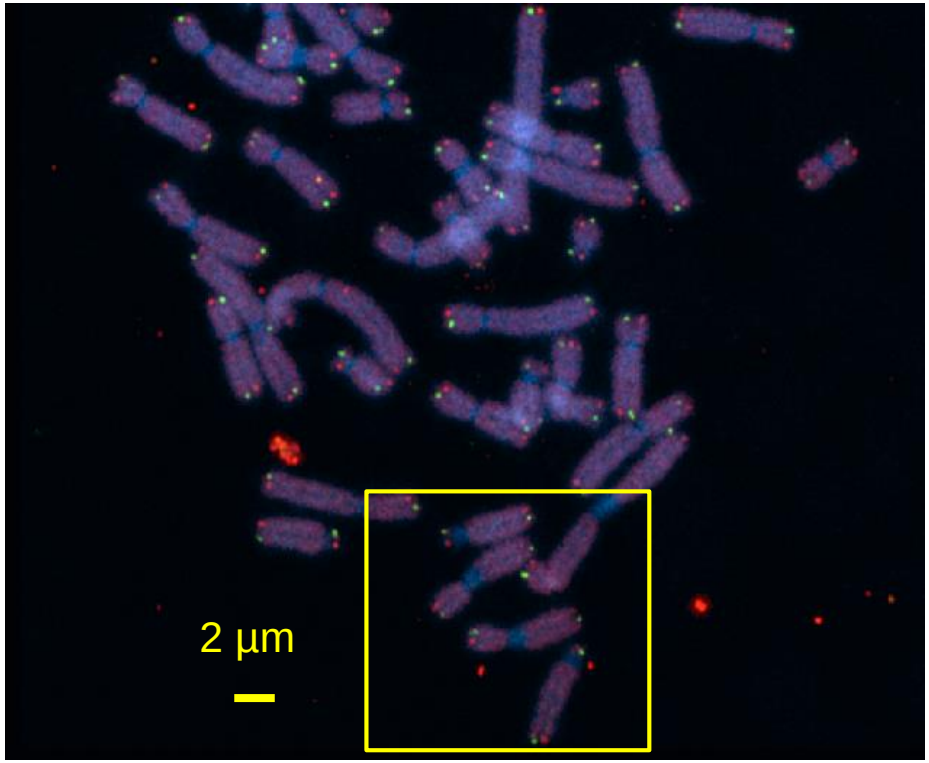
Airyscan improves resolution in lateral and axial direction



Z-stack: 3.98 µm; 步进: 200 nm (Plan-Apochromat 63x/1.4 Oil)
有丝分裂中的培养细胞微管蛋白

Airyscan：高分辨率，高信噪比成像

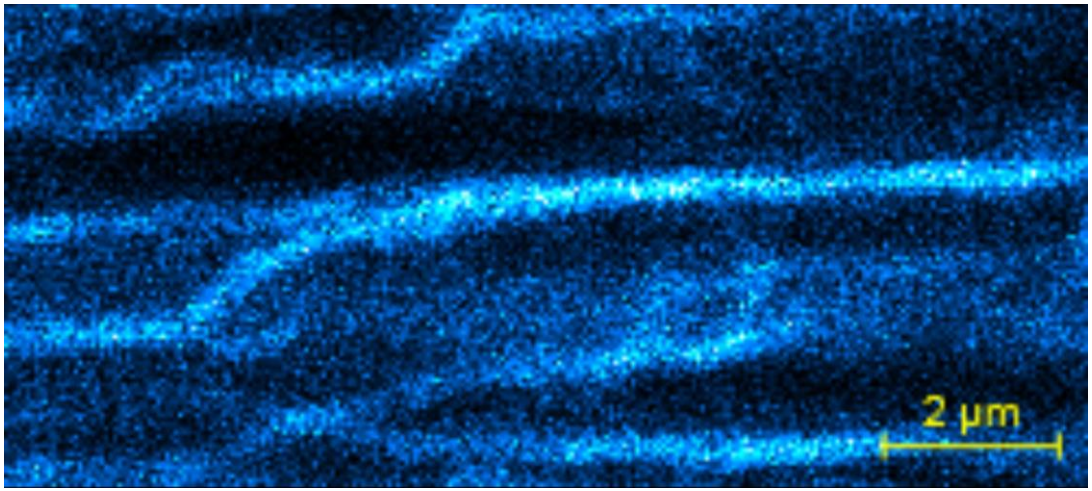




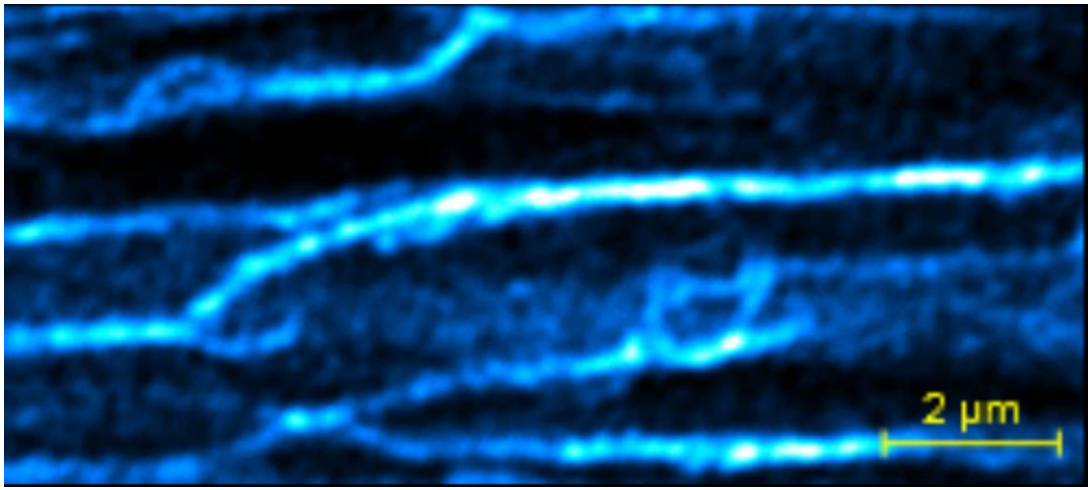
Telomere replication without RTEL1: Stalled forks and telomere breakage visualized as doubled dots using Airyscan. Resolution is meaningless without good SNR.

Courtesy: J. Karlseder Ph.D. (Molecular and Cell Biology Laboratory) and J. Fitzpatrick Ph.D. (Director, Waitt Advanced Biophotonics Core), The Salk Institute, La Jolla, USA.

扩展功能：快速 超高分辨率成像
Fast Airyscan



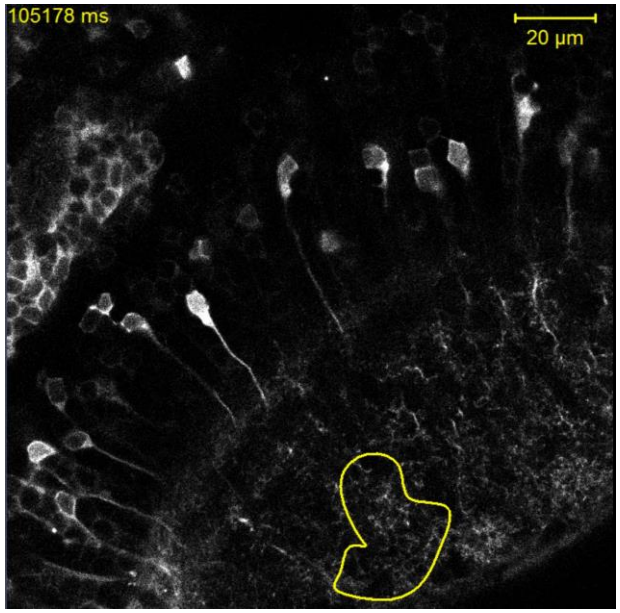
Standard Confocal – **30 FPS**

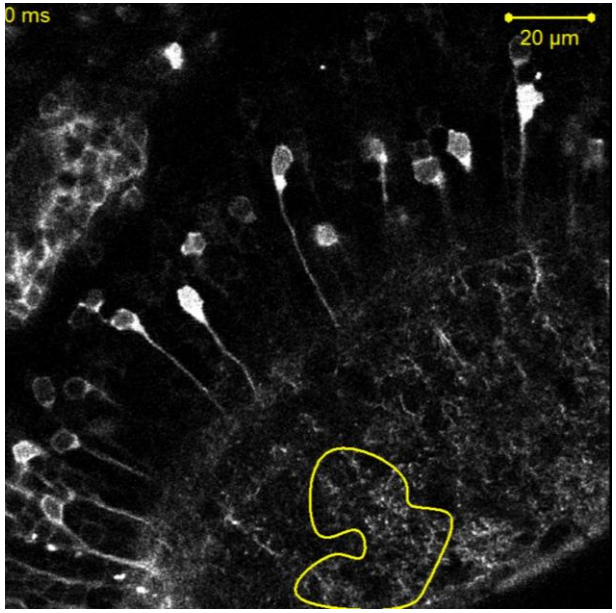


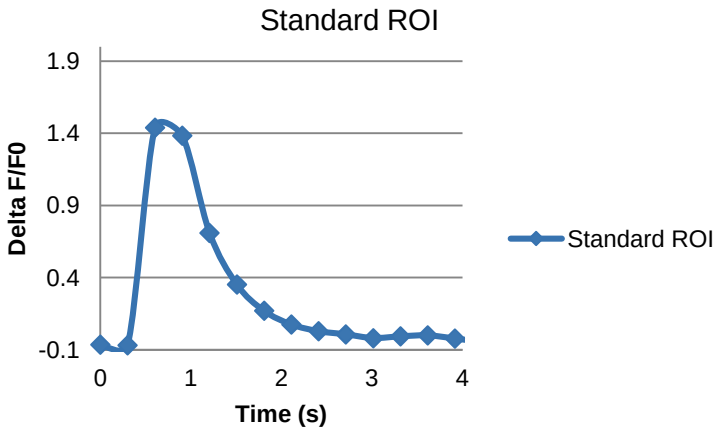
Airyscan – **30 FPS**

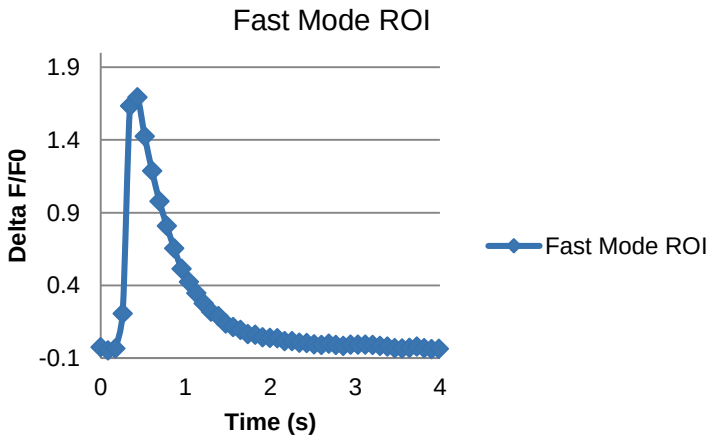
Cardiomyocyte Cells with SiR Tubulin to measure microtubule buckling,
Robison et Al, “Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes”, Science April 2016

Fast Airyscan 应用
Calcium Signaling in Zebrafish-GCaMP6









20x 1.0 W Plan Apo ; Sample Courtesy of Claire Oldfield Univ of California Berkeley

LSM880 with Fast Airyscan Configuration

Hardware：

Axio Observer 7—— Inverted Microscopy with scanning stage

4 Lasers—— 405，Argon (458/488/514)，561，633

3 Detectors—— 2 PMT + 1 GaAsP

Objectives ——10x/0.45，20x/0.8，40x/1.4 oil，63x/1.4 oil，100x/1.4 oil

Super resolution modual—— Airyscan，Fast Airyscan

Software：

Tile and Position

Experiment Designer

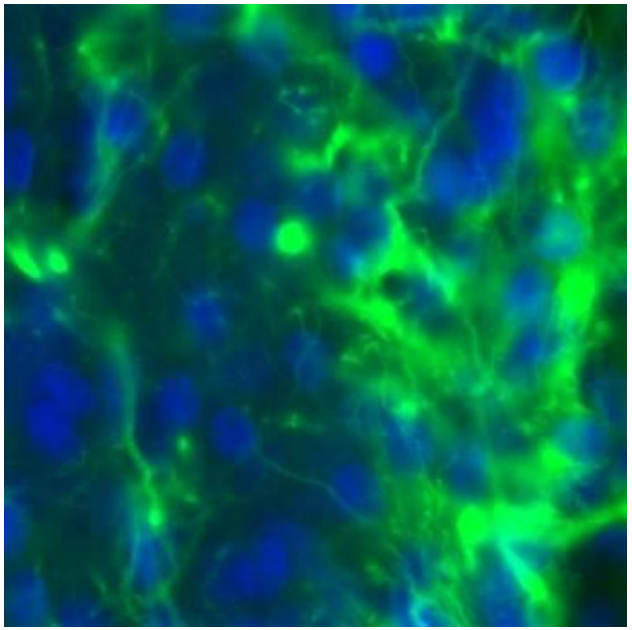
FRET

FRAP

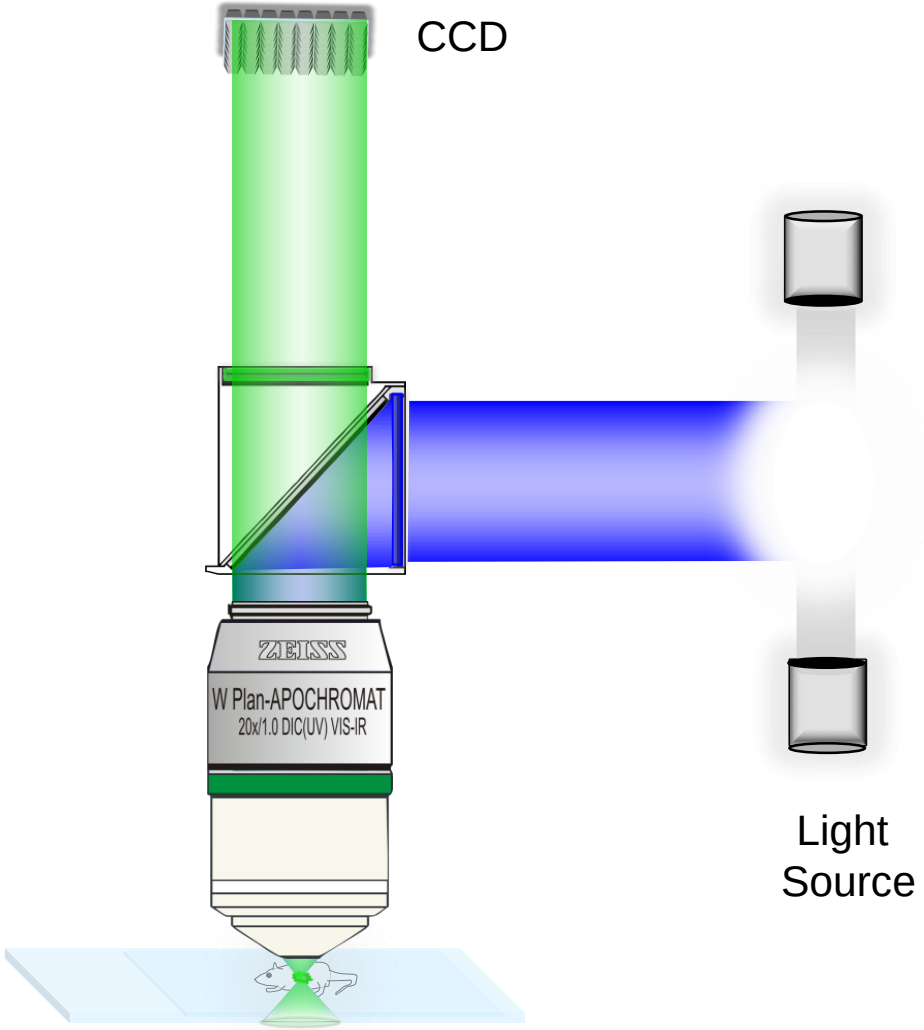
Agenda

1	Applications of Confocal Microscope
2	Principle of Confocal Microscope
3	Light path components of LSM880
4	How to acquire a high quality image?

Widefield Microscope

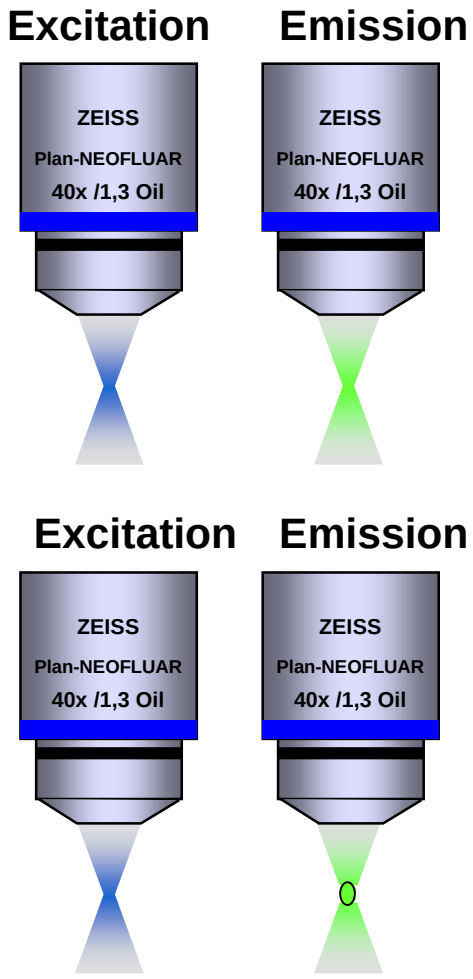


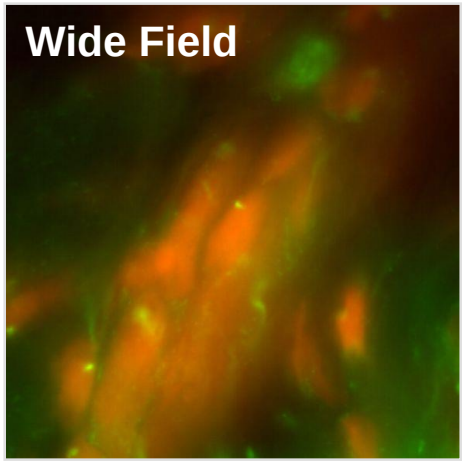
Rat Brain, Double labelling:
Green: Neurons, Blue: Nuclei

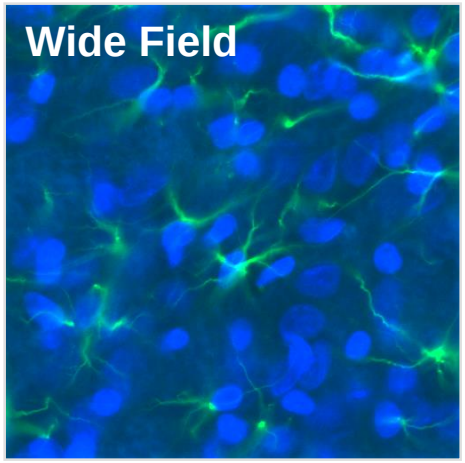


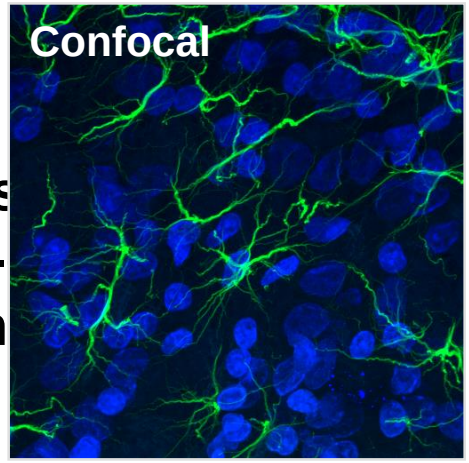
Confocal

Only detect in-focos information

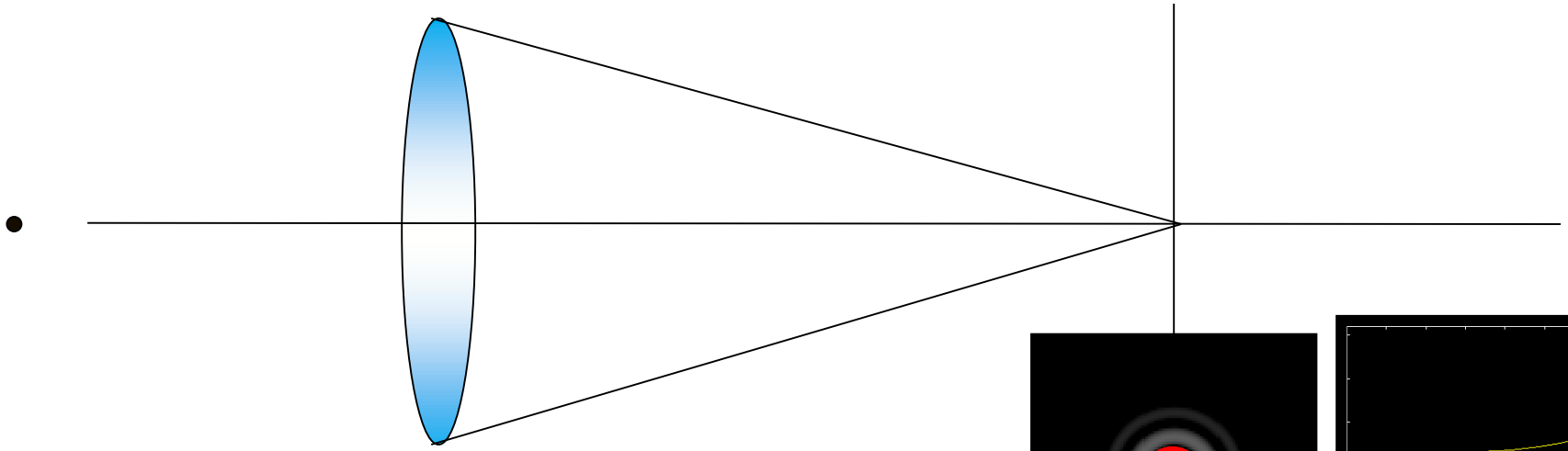


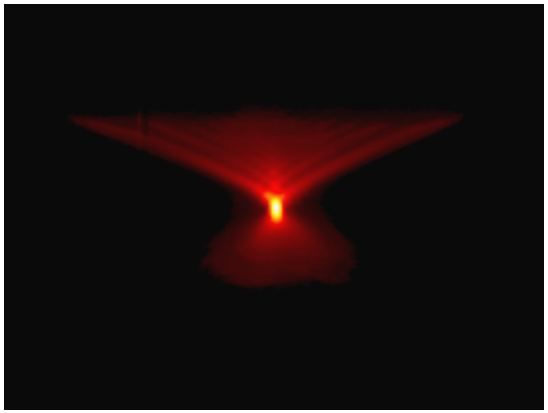




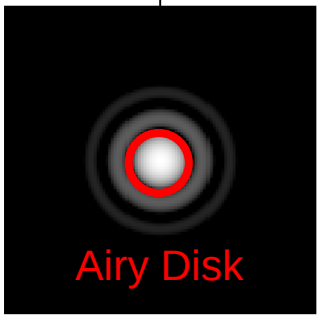


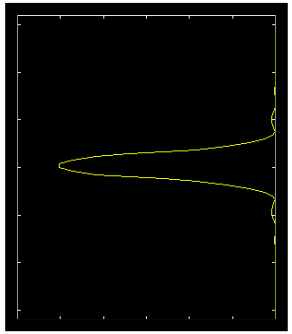
Property of Light Microscope



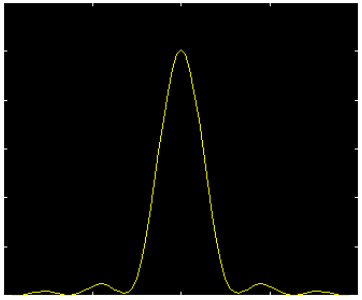


Point Spread Function (PSF)



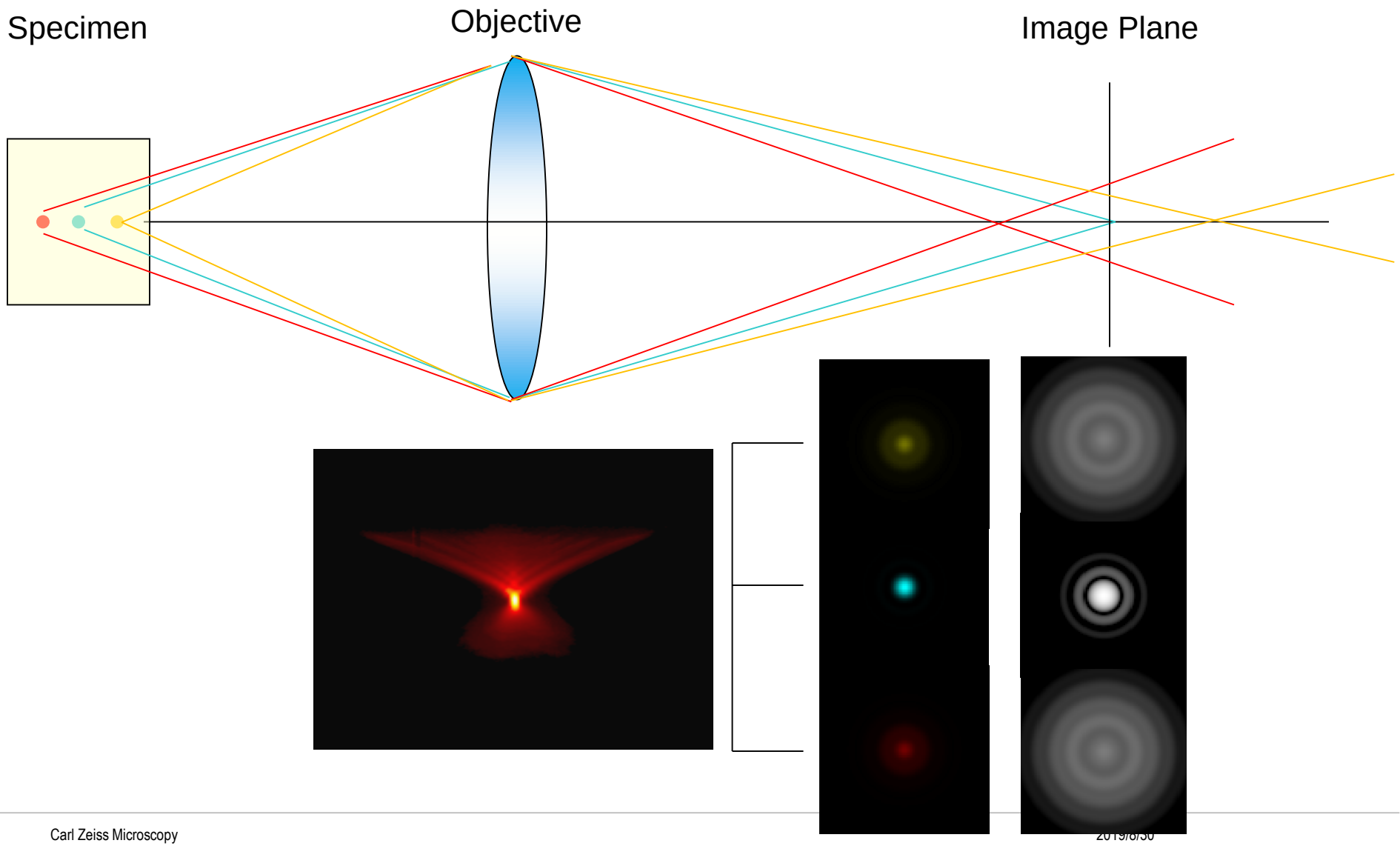


x-z

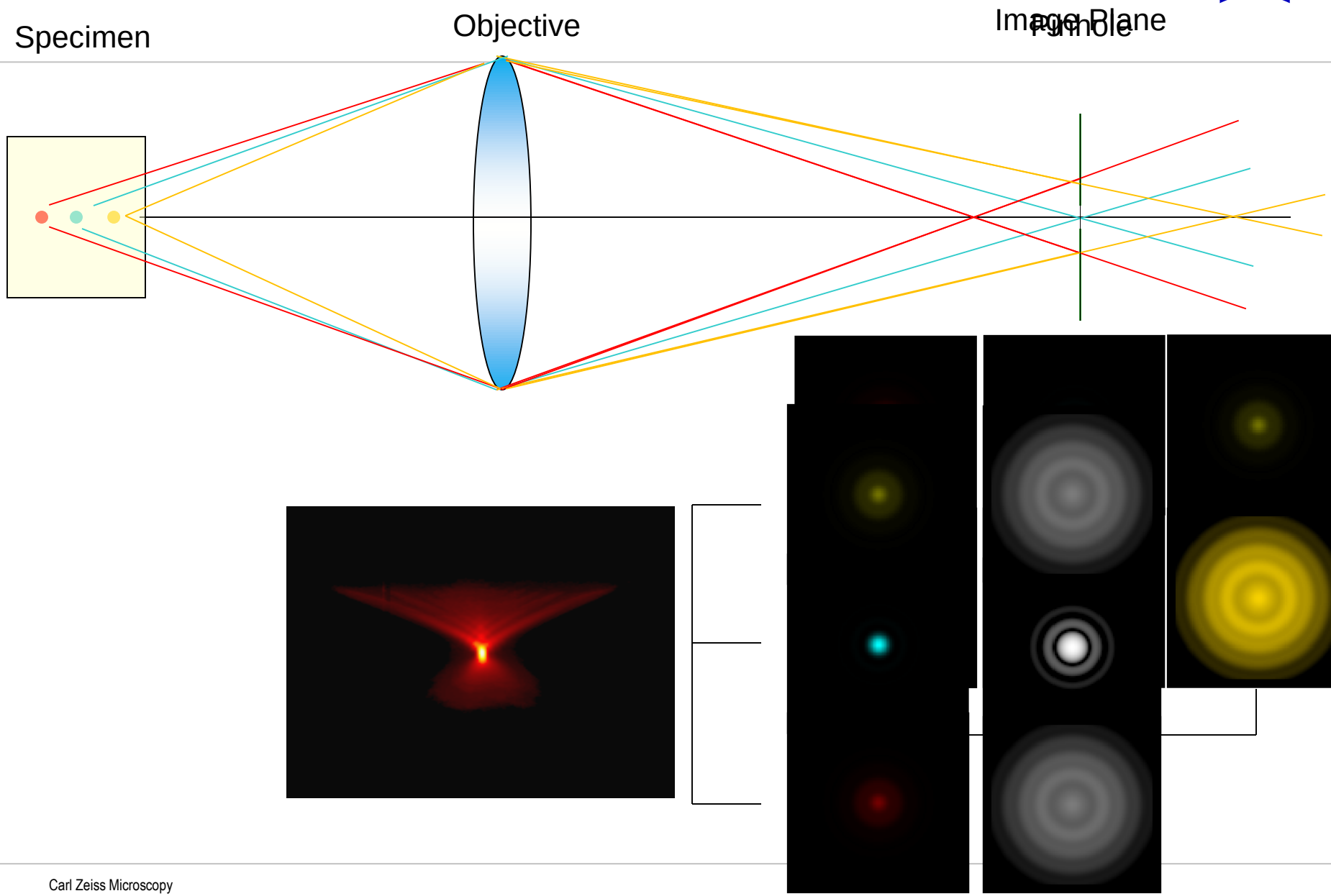


x-y

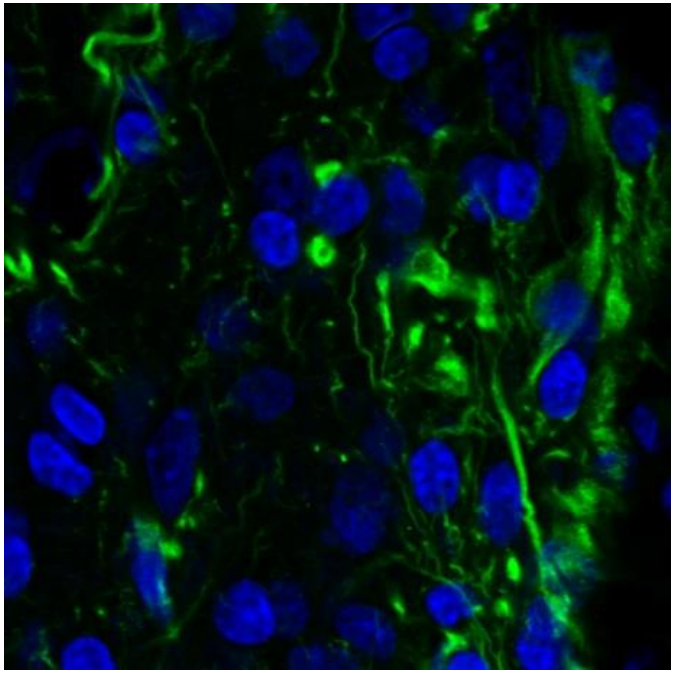
Widefield Microscopy



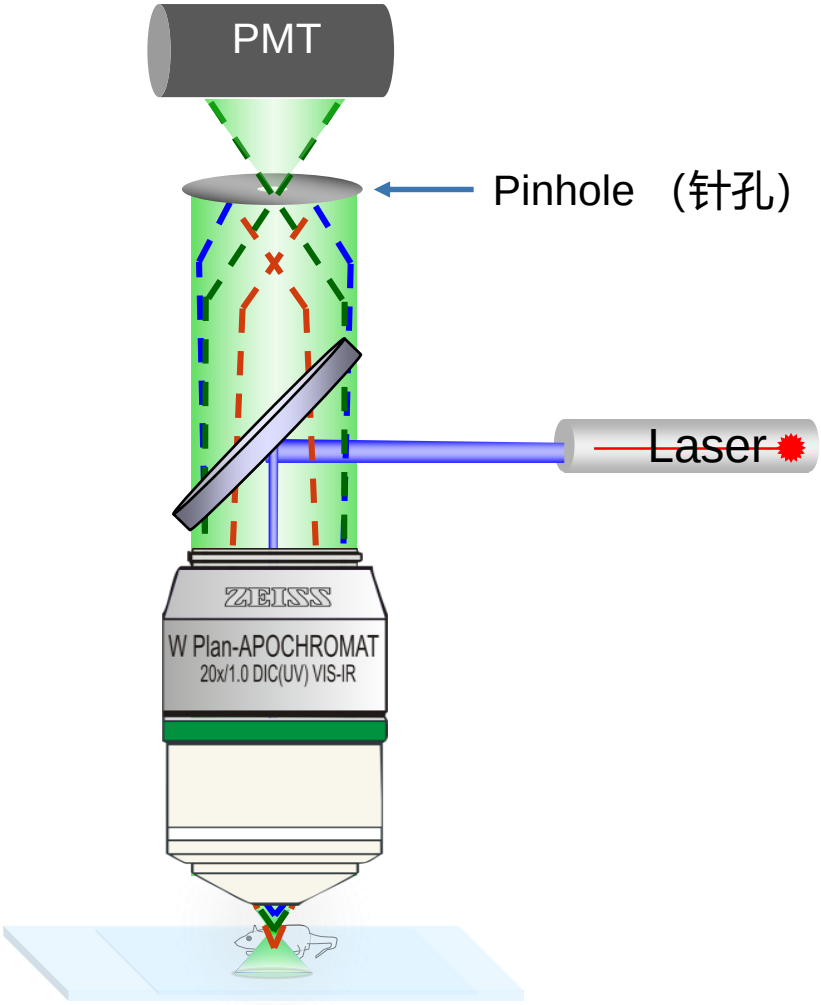
Confocal Microscopy



Confocal Microscopy

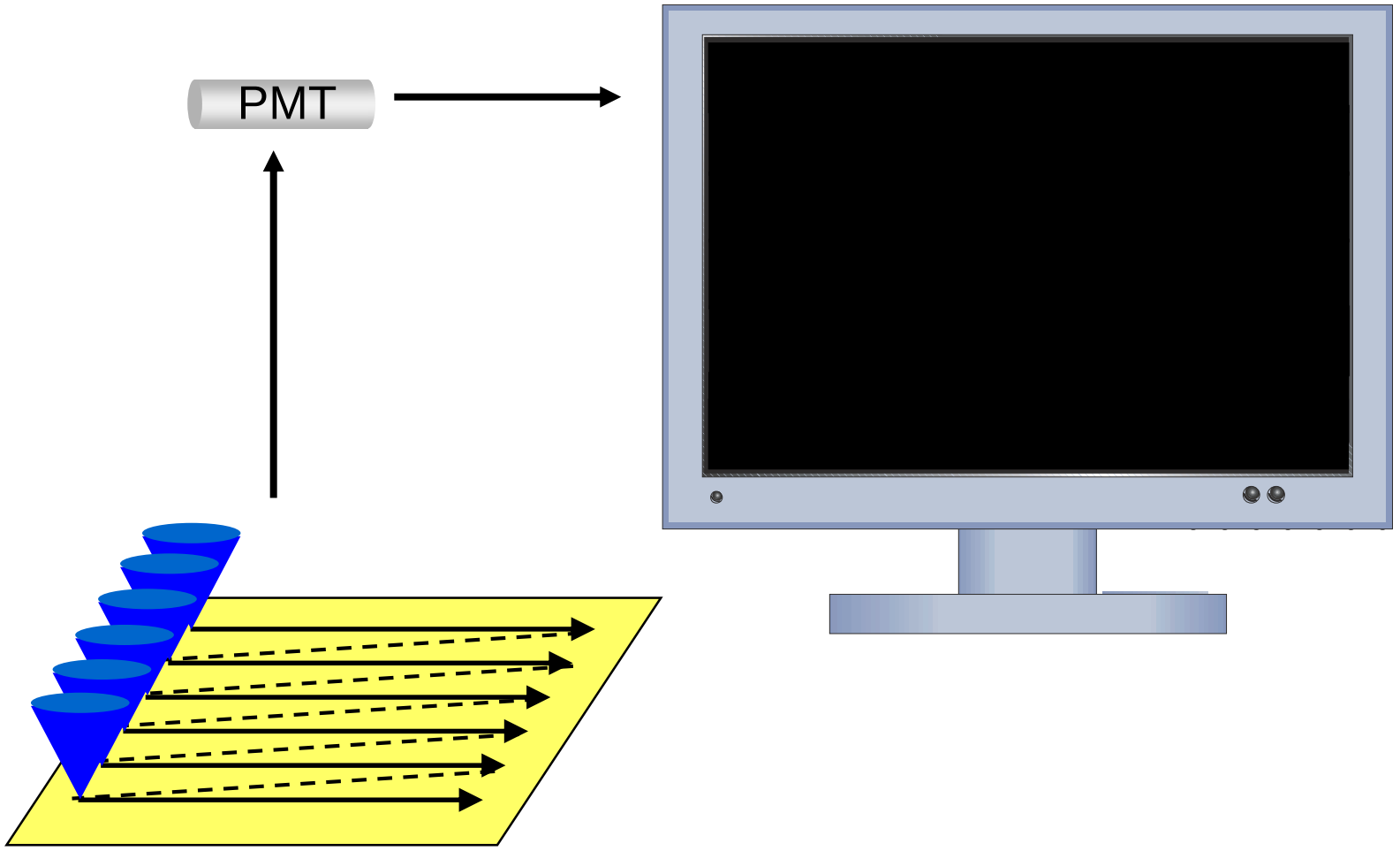


Rat Brain, Double labelling:
Green: Neurons, Blue: Nuclei



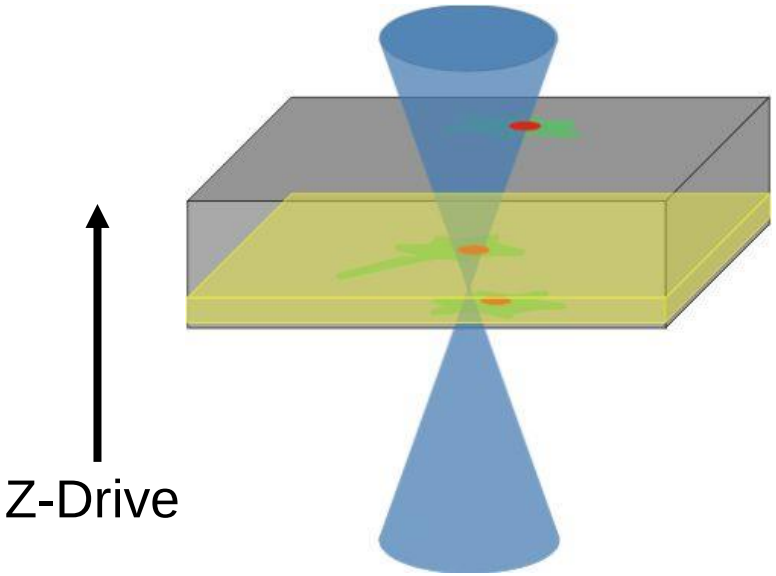
Scanning Strategy in X-Y plane

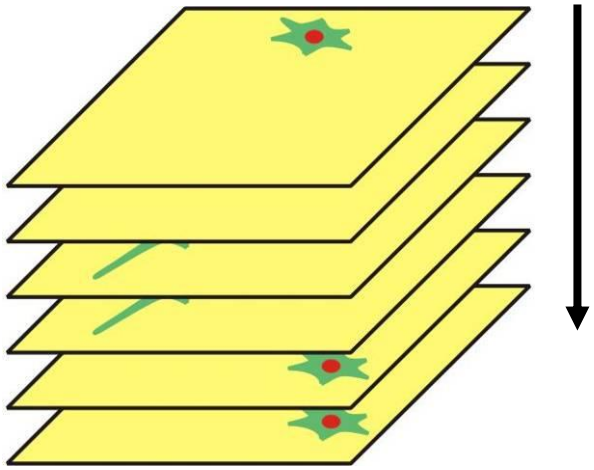
Ponit – Line - Frame



Scanning Strategy in Z-Stack
Frame by Frame

How is a X/Y/Z Stack produced?

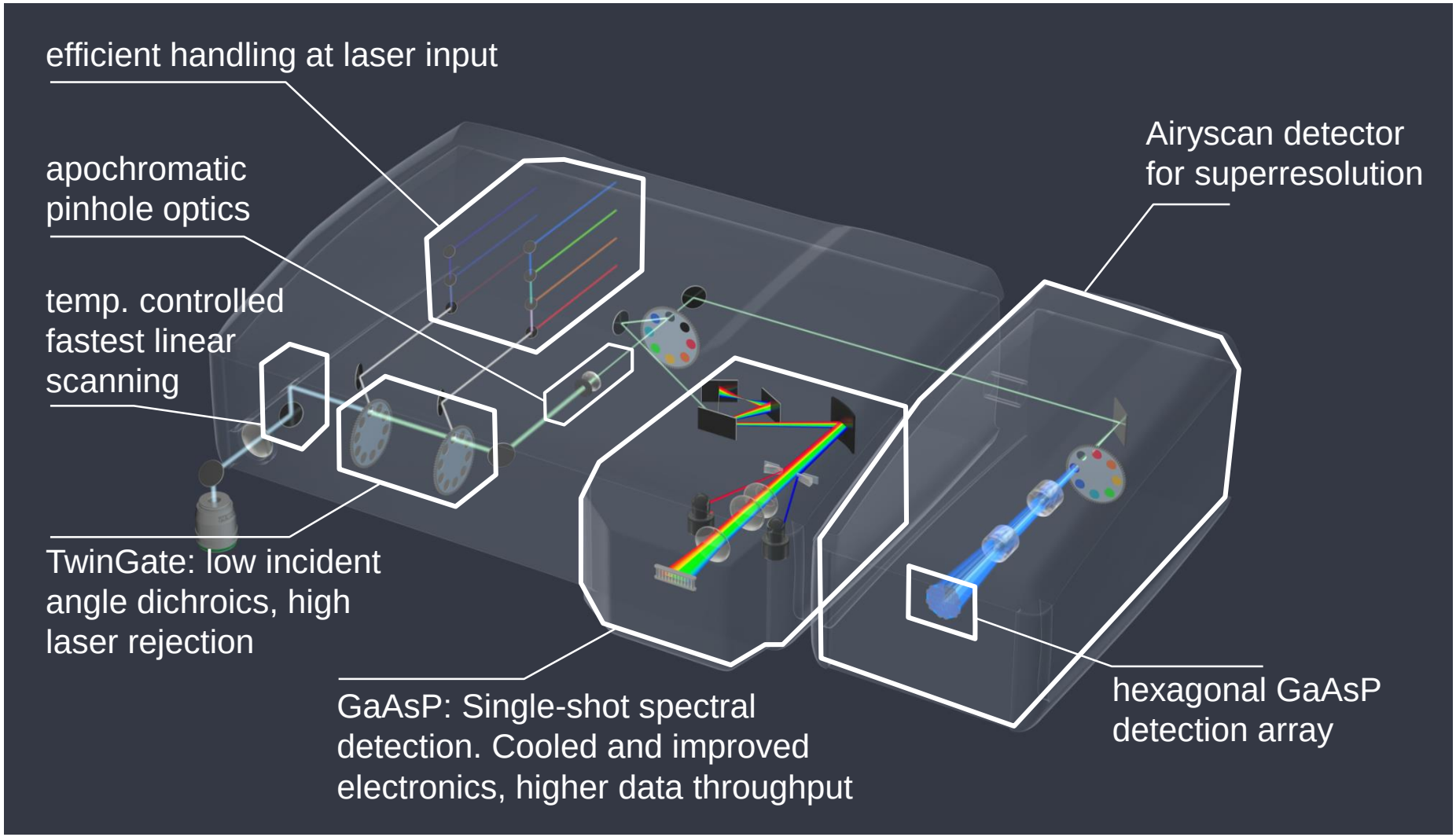




Agenda

1	Applications of Confocal Microscope
2	Principle of Confocal Microscope
3	Light path components of LSM880
4	How to acquire a high quality image?

LSM880 Light Path

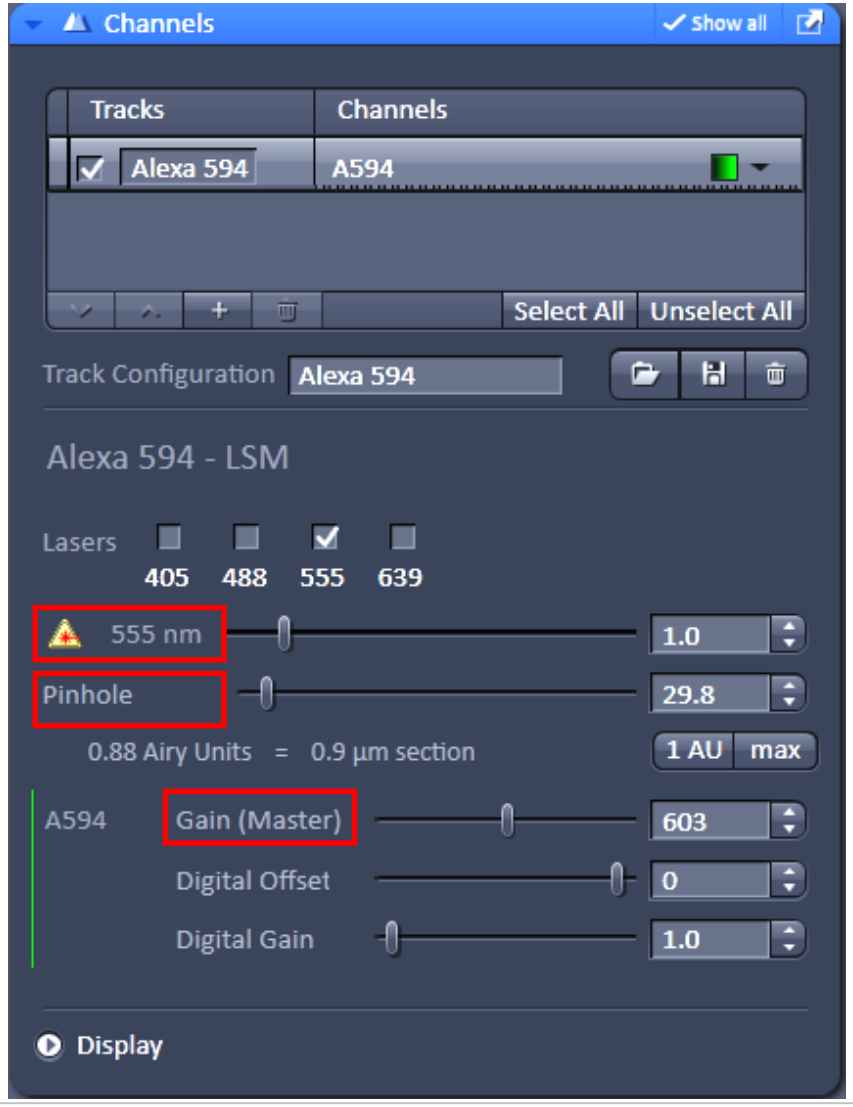


To acquire a high quality image, we should focus on …

Laser Pinhole PMT

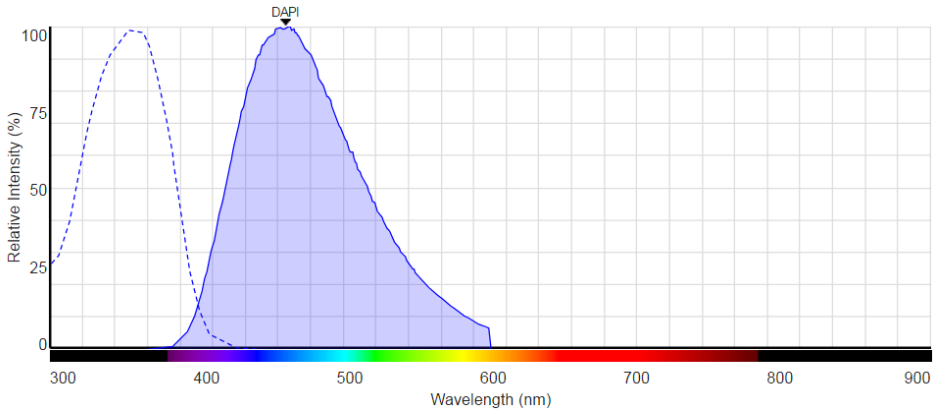
The main tools are:

- Laser power
- Pinhole
- PMT (Gain)

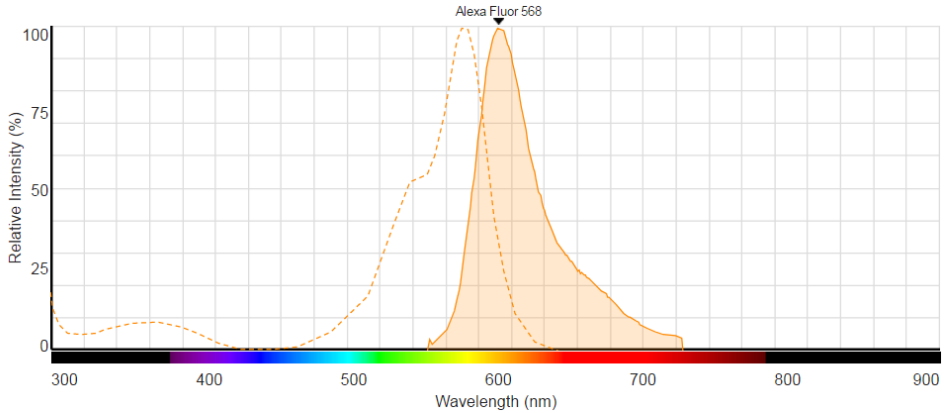


Laser

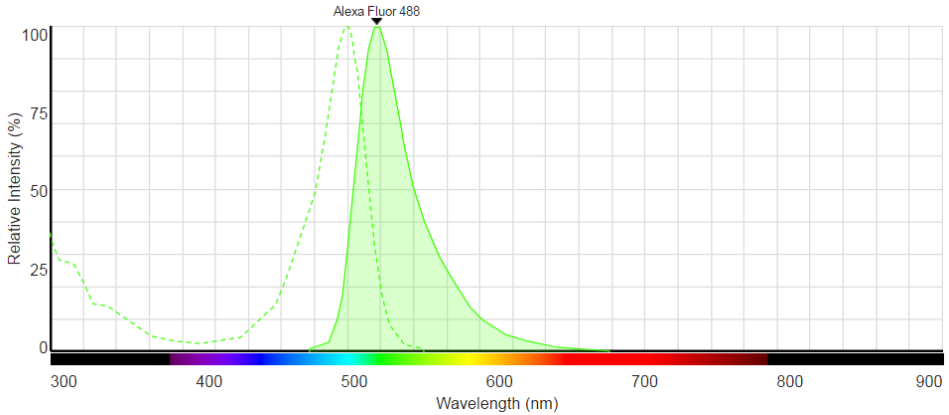
Color： the laser color should match the fluorophore



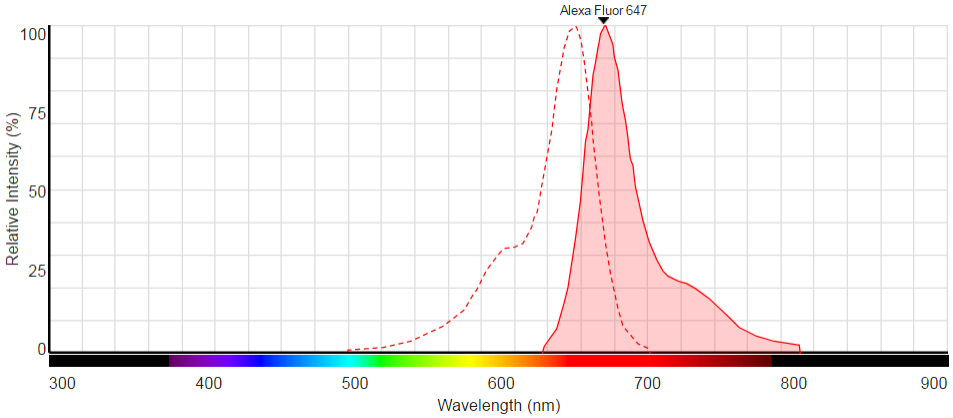
405 nm　DAPI / Hoechst



561nm RFP / Alexa Flour 568 / Cy3



488nm GFP / Alexa Flour 488 / FITC



633nm Alexa Flour 647 / Cy5

Laser

Power： High laser power bleached the sample signal

Sample:

Cultured mammalian cell expressing GFP (live cell)

Imaging Strategy:

Time series of 1000 images acquired at low laser power but relatively high gain setting.

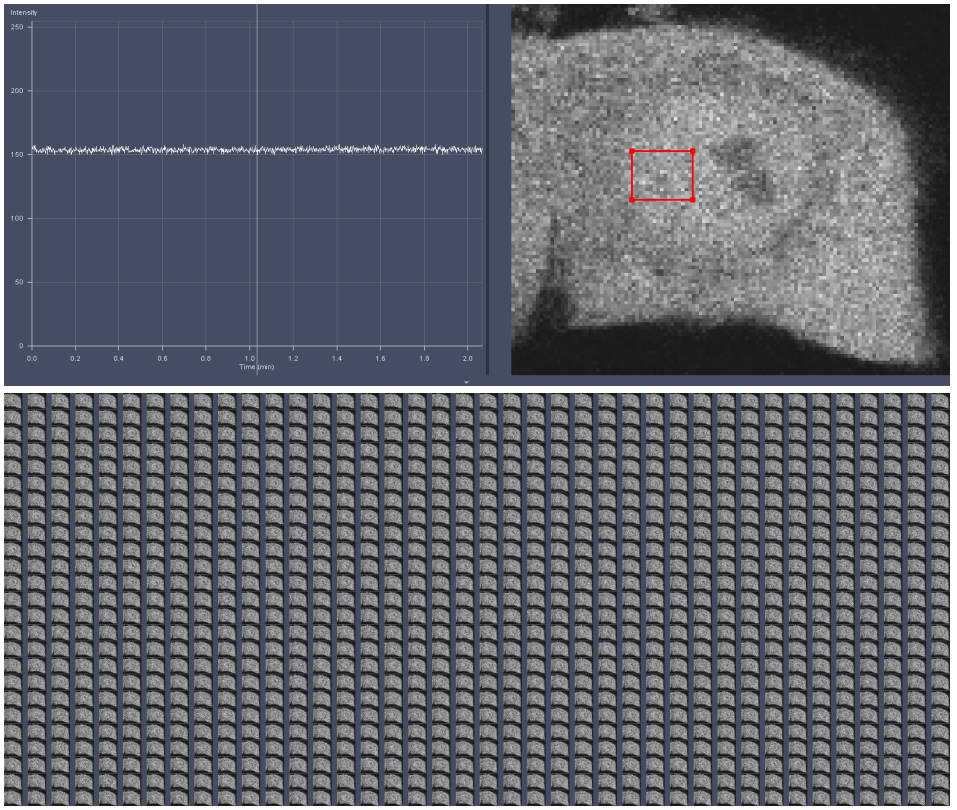
(excitation at 488 nm with just 0.5% of the max. power).

Result:

Hardly any photobleaching !

Intensity Profile of time series

Cultured mammalian cell expressing GFP

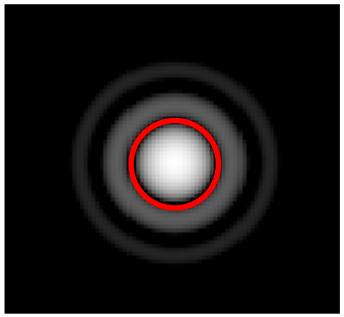


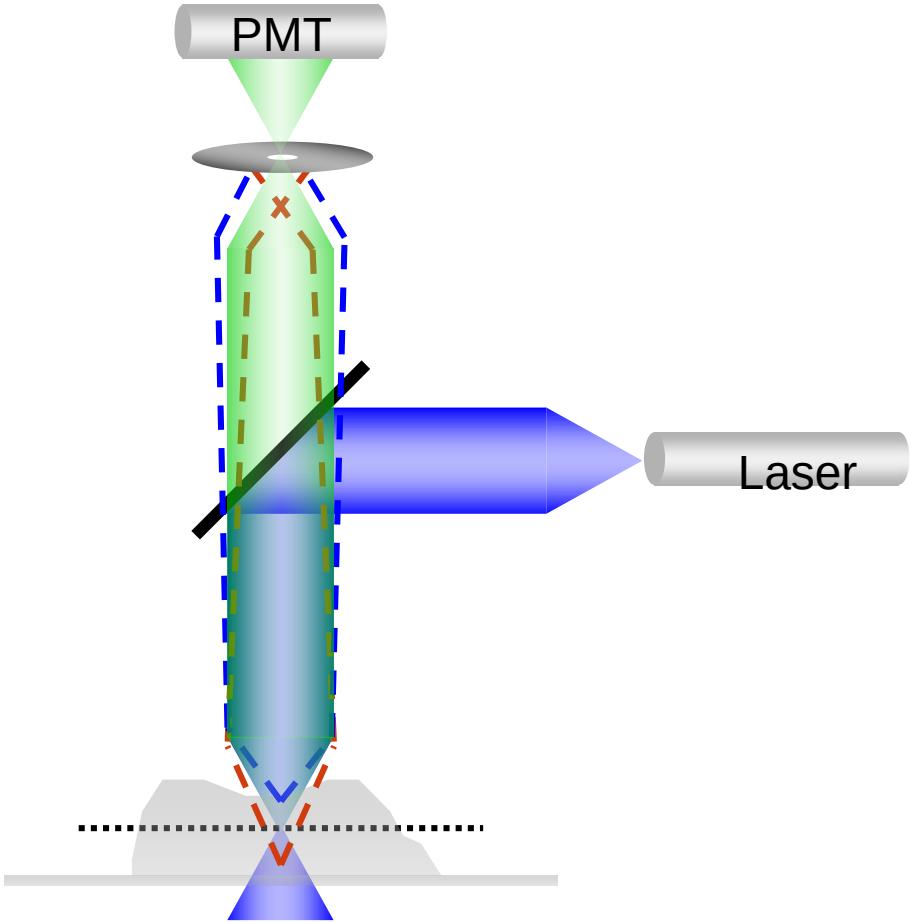
Time series: 1000 Images (!)

Pinhole

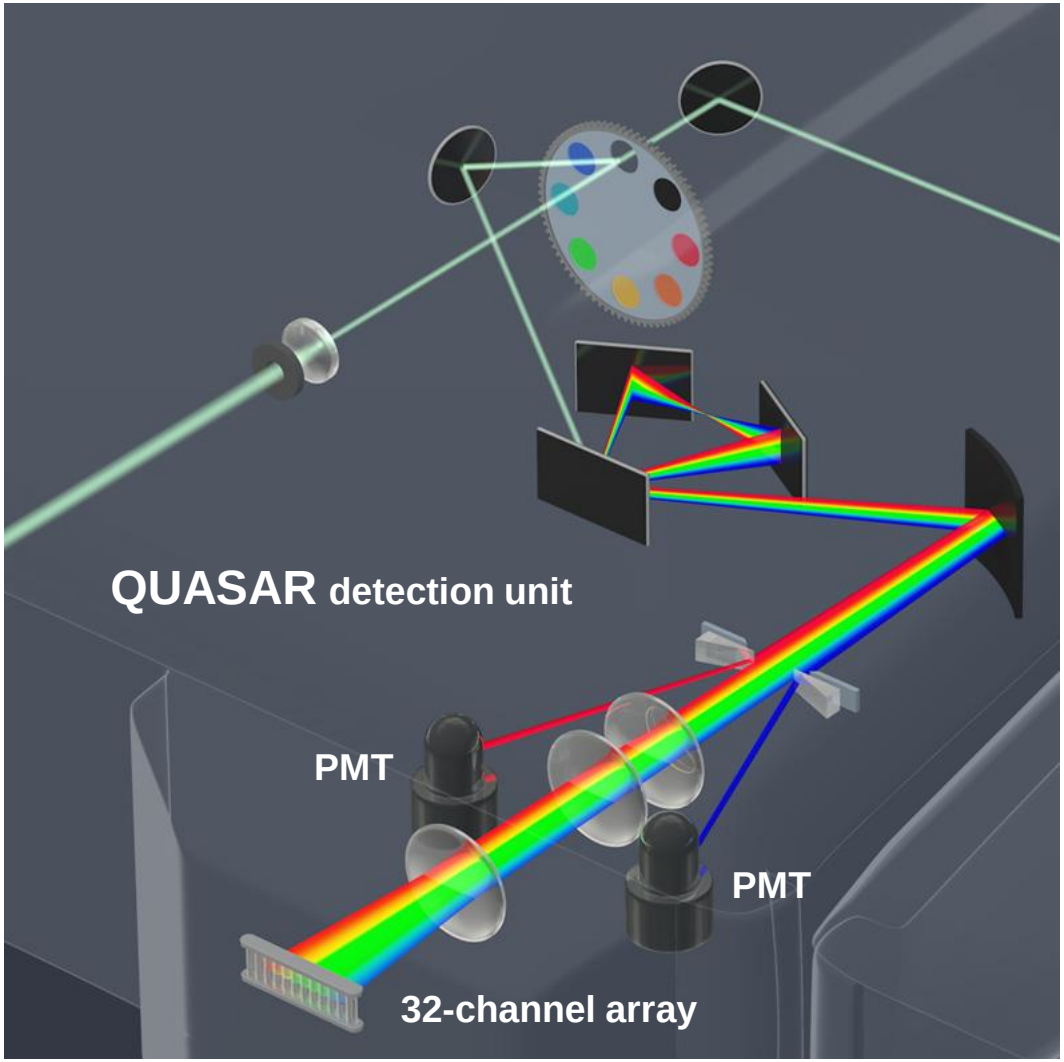
- Block out of focus signals
  - The larger the pinhole, the more light pass through, the more out of focus signal
- The thickness of optic section
  - The larger the pinhole, the thicker the optic section, the better Z axis resolution

Generally, the pinhole is 1AU



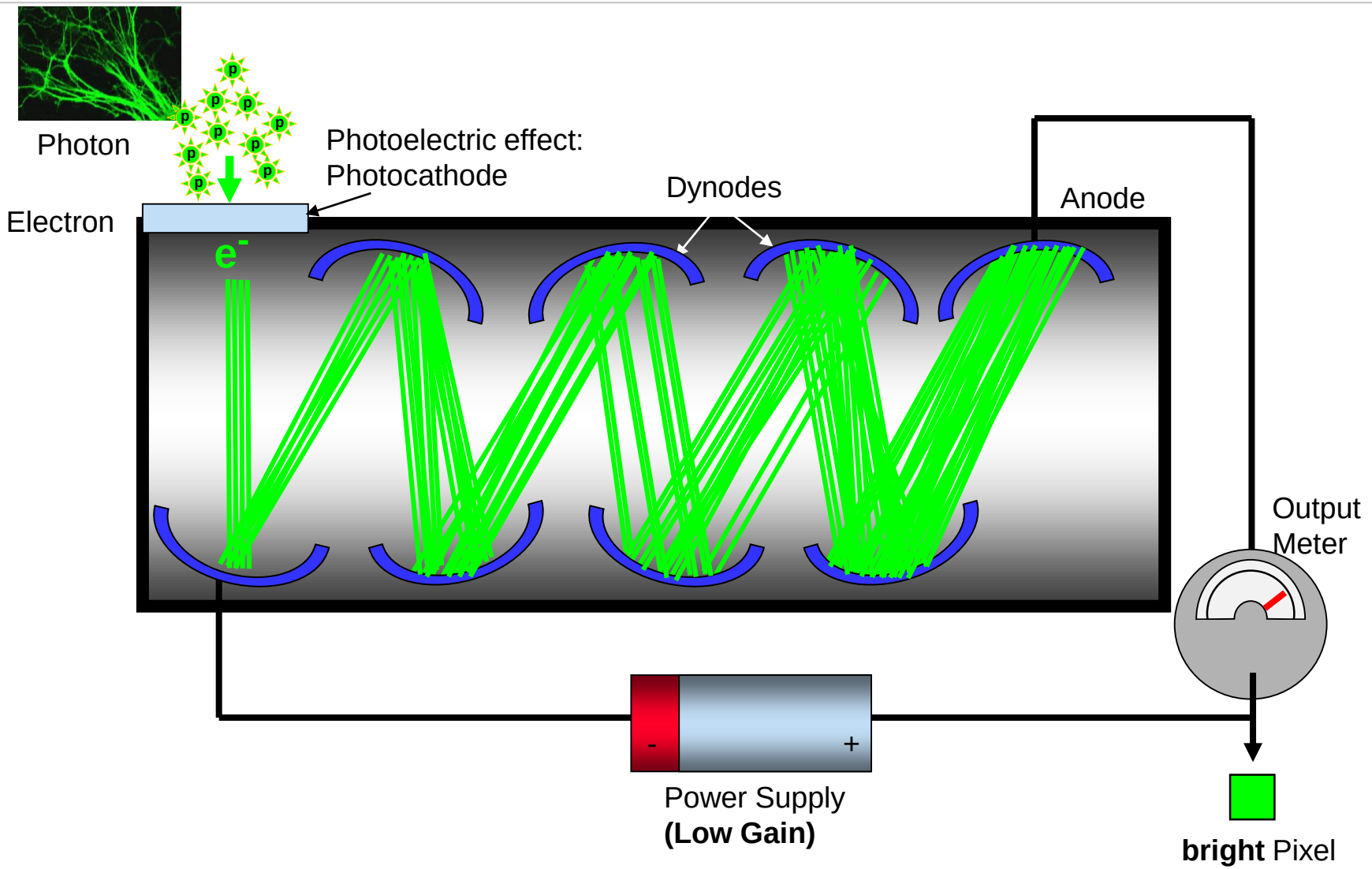


Detector



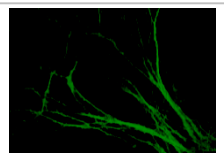
PMT Detectors / Photomultiplier Tube （光电倍增管）

Assuming a bright sample



PMT Detectors / Photomultiplier Tube

Assuming a dark sample



Photon

Electron

Photoelectric effect:
Photocathode

Dynodes

Anode

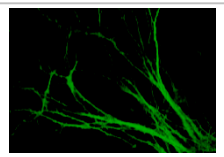
Output
Meter

Power Supply
(Low Gain)

dark Pixel

PMT Detectors / Photomultiplier Tube

Increase a dark sample´s signal with more Gain



Photons

Photoelectric effect:
Photocathode

Dynodes

Anode

Electrons

e-

Output
Meter

-

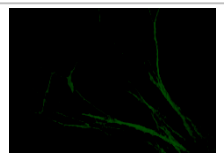
+

Power Supply
(Higher Gain)

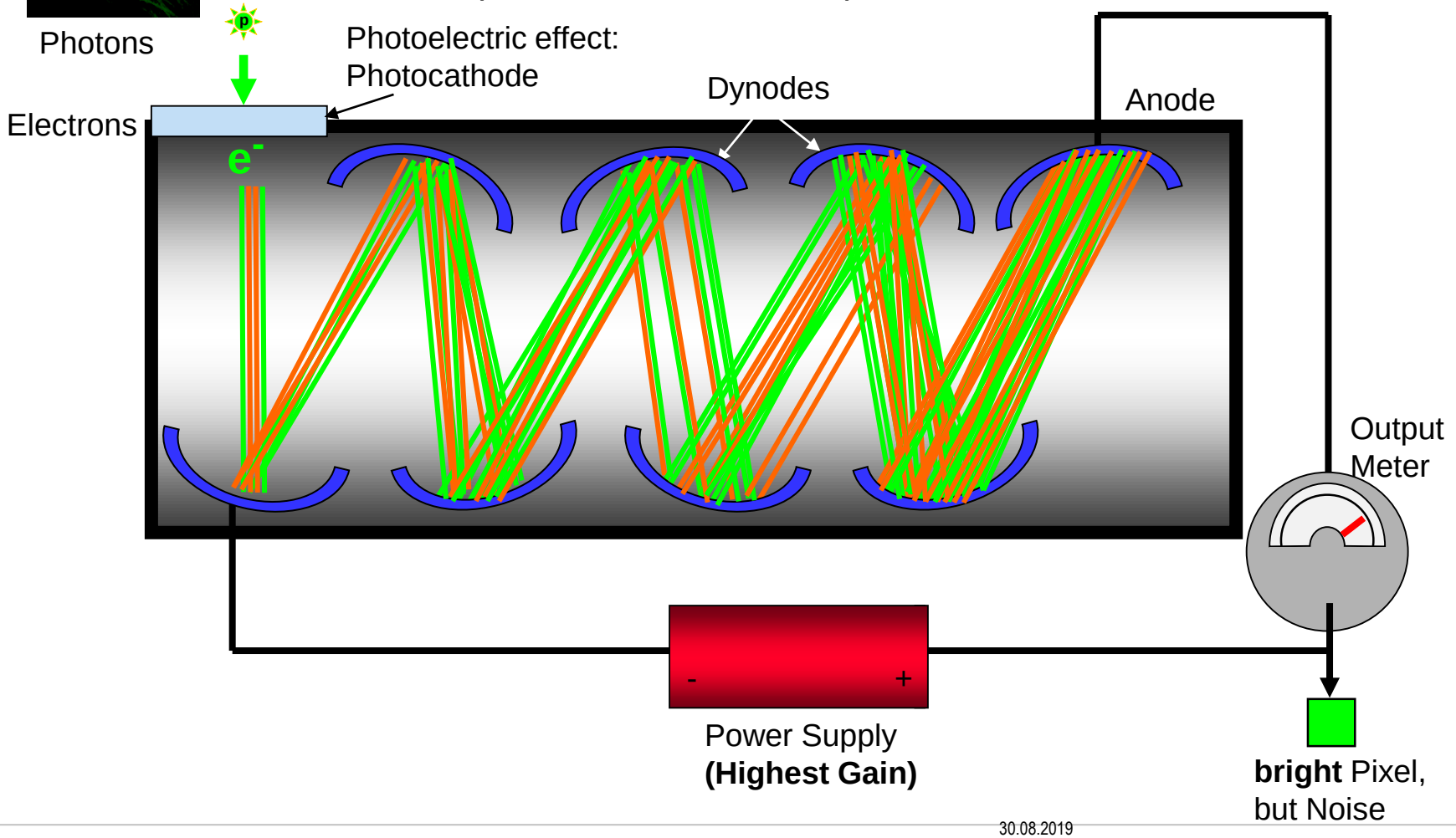
bright Pixel

Assume a really dimm sample -

Extreme Gain values result in Noise



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



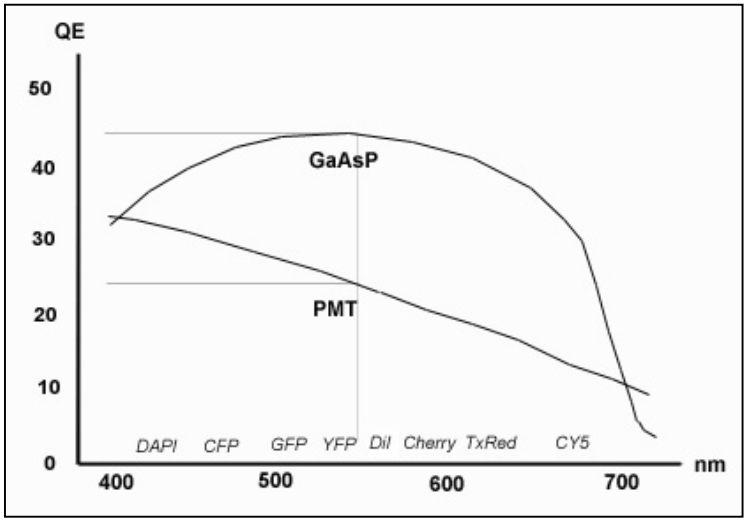
GaAsP Detector

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

Benefits of GaAsP detectors:

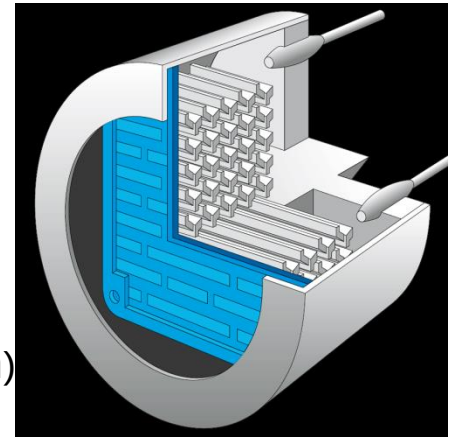
Two times better Quantum efficency than PMTs (resulting in higher sensitivity, better image quality, and higher acquisition speed).

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)

Get More Results With GaAsP Detectors

Applications Benefit from Improved Sensitivity in Many Ways

Better image quality

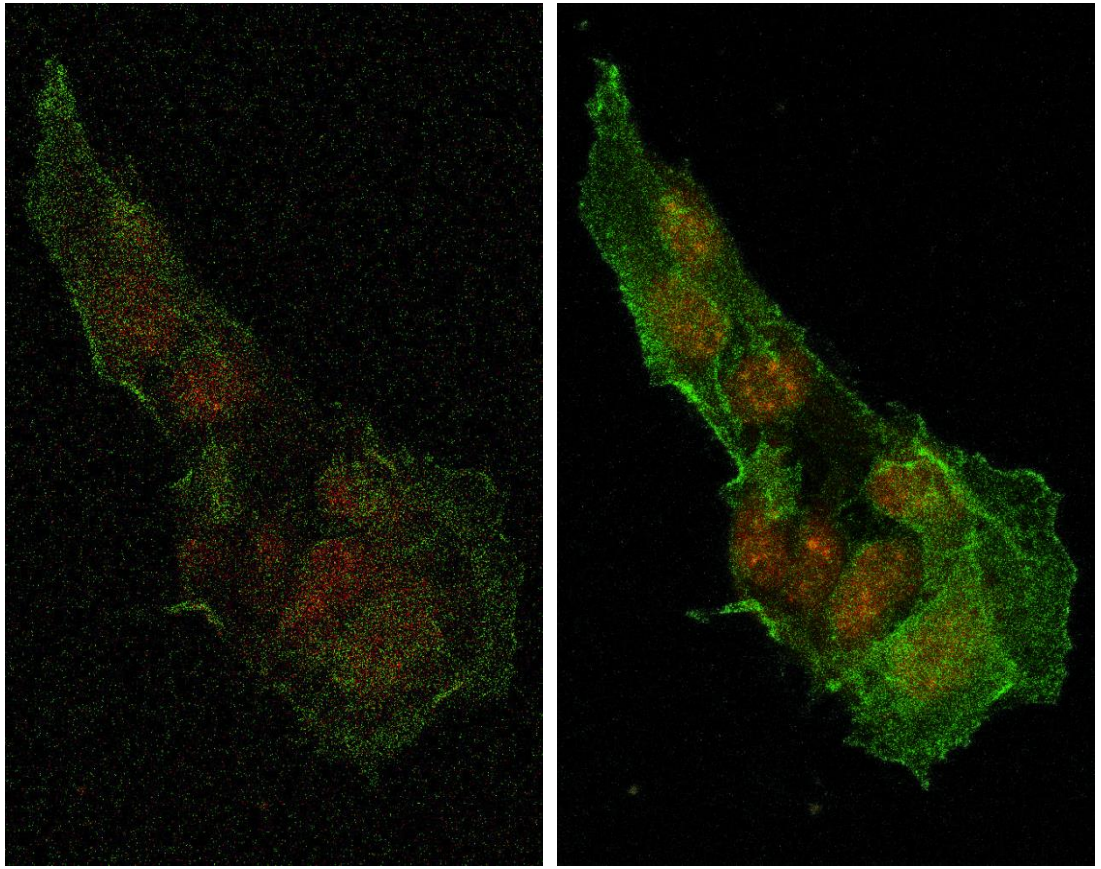
- Higher sensitivity equals better signal-to-noise ratio (detection of faint signals)

Faster scanning

- Data recording at shorter pixel times
- Need for averaging strategies largely reduced

Longer imaging

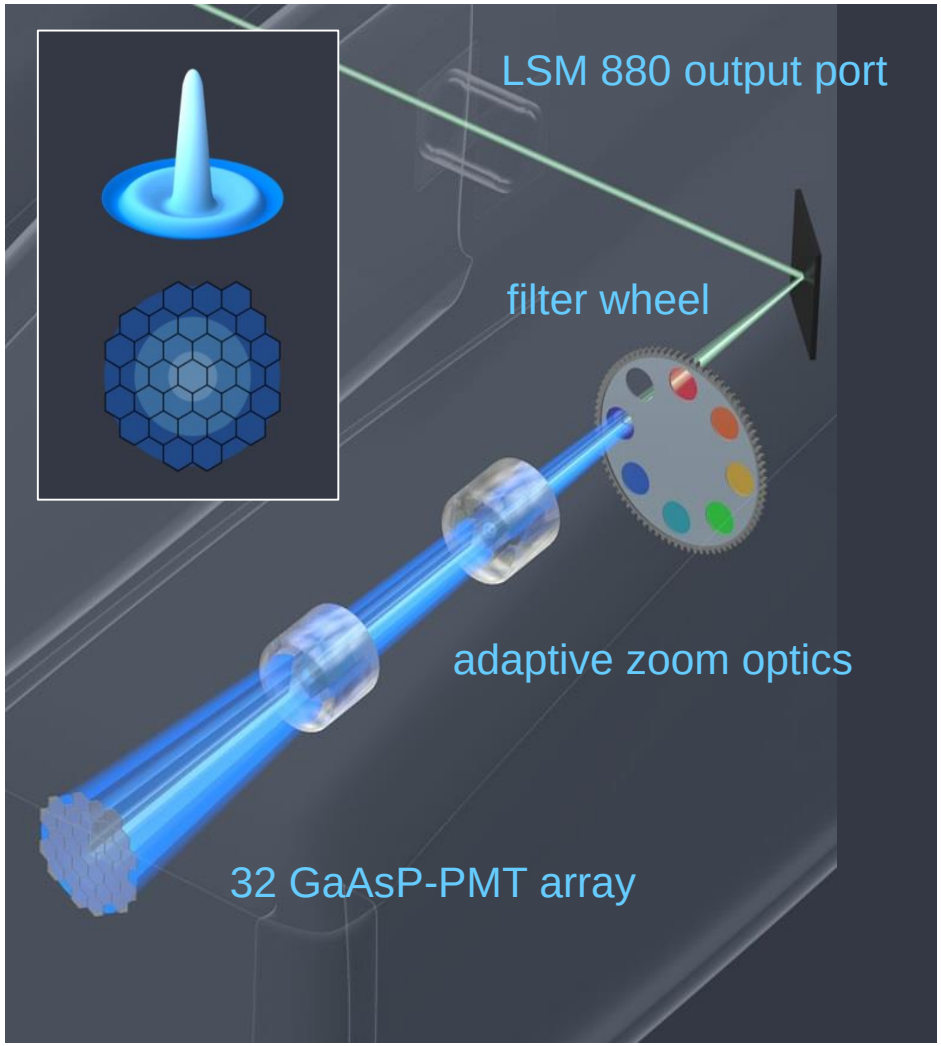
- Data recording at lower laser power (reduced bleaching and photo-toxic effects in live cell imaging)



Cultured 2h8 cells labeled with extremely low expressing GFP and mCherry. Courtesy A. Bruckbauer Cancer Research, London, UK

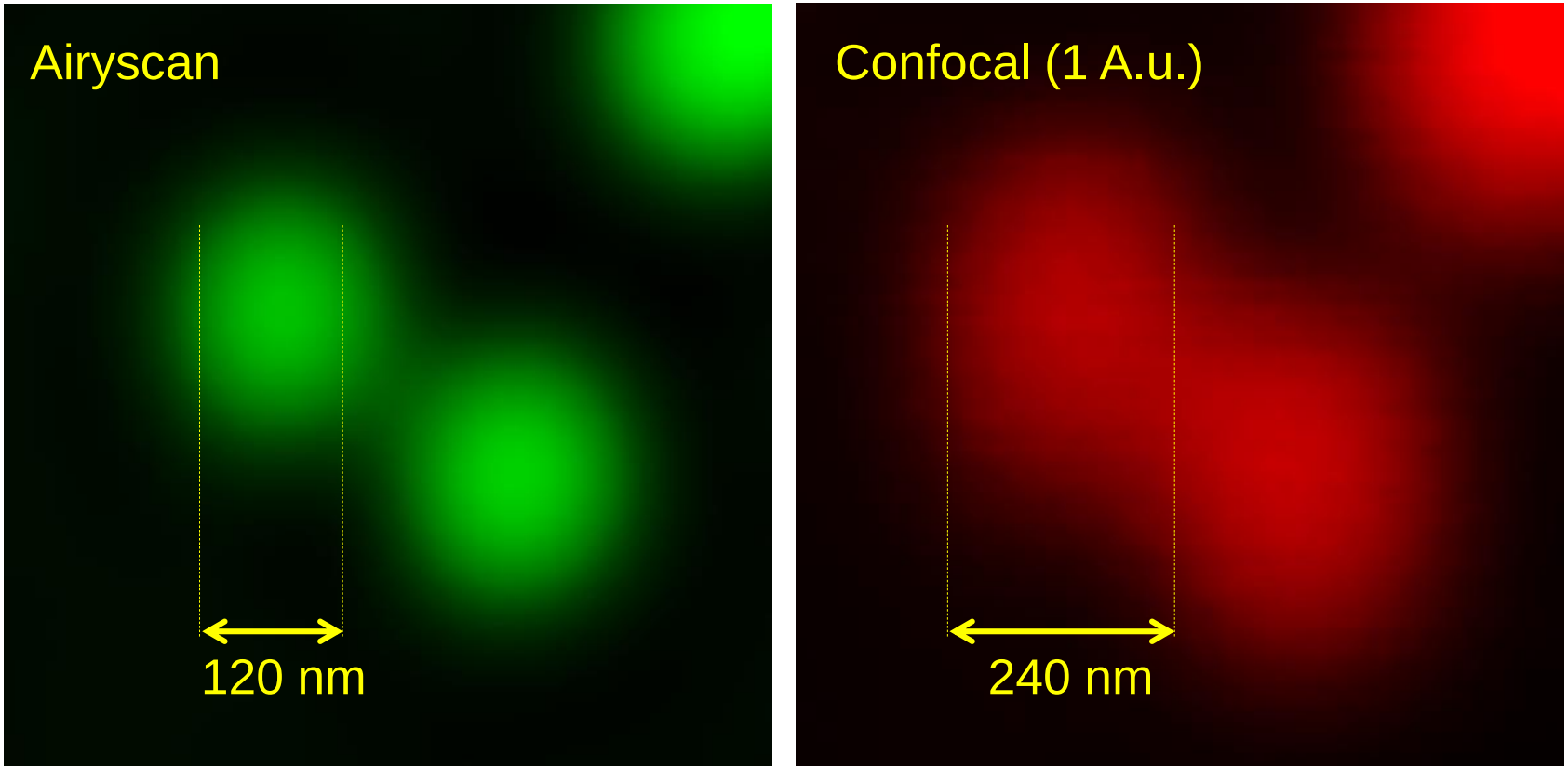
LSM 880 – Airyscan Detection

Zoom optics and a unique 32 GaAsP-PMT design



Airyscan enhances resolution up to 2 fold

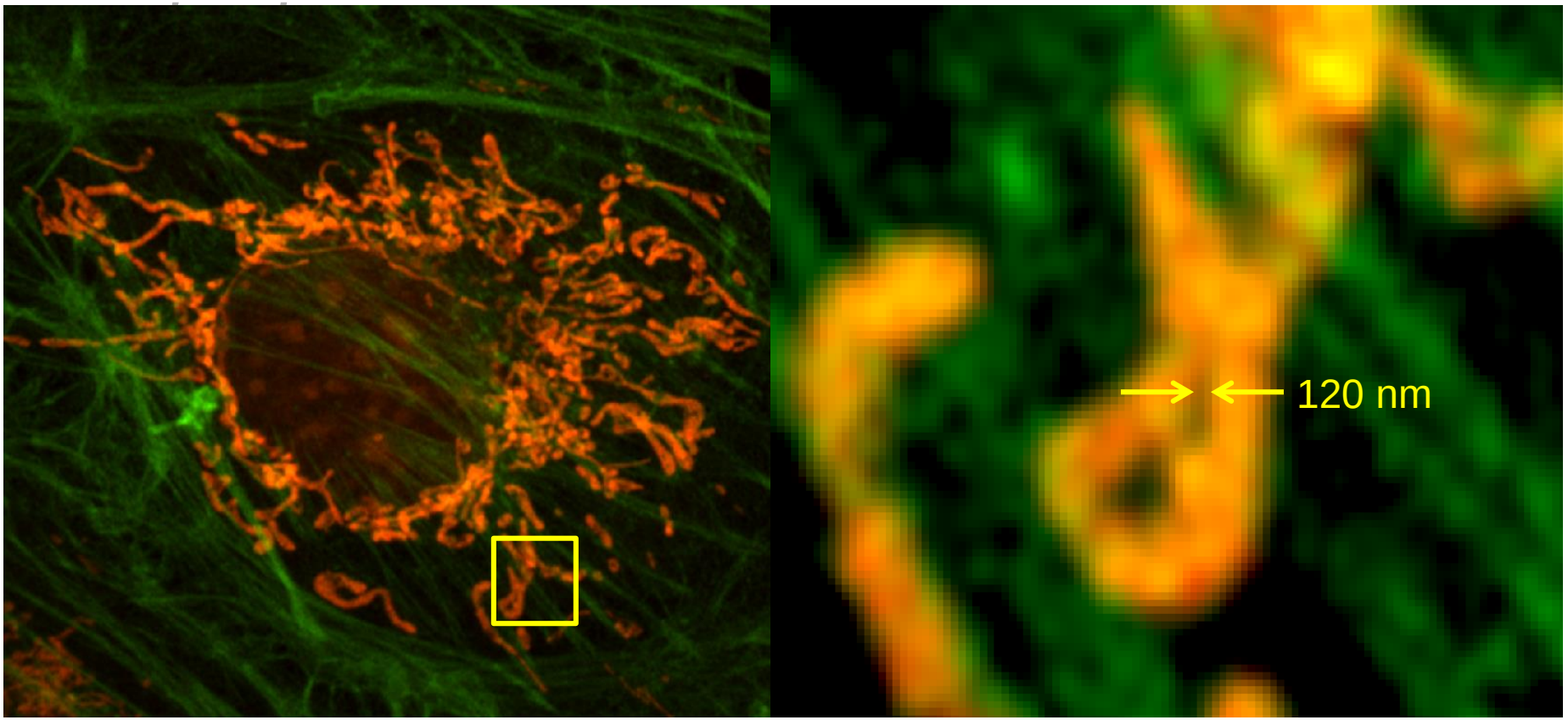
The comparison is made with confocal using PH = 1 A.u.



Improvement 2x: Measured using 40 nm beads (excitation at 488 nm)
Same acquisition time, same laser power.

LSM 880 – Airyscan: Resolution, SNR, and Flexibility

Choose zoom-in ROI's, orientations, laser



Use Airyscan just as an additional detector. Scan speeds, ROIs, field orientations, laser wavelengths, multitracking, z-stacks, time series etc. are setup as always. Sample handling and fluorophore selection don't need rethinking

Agenda

1	Applications of Confocal Microscope
2	Principle of Confocal Microscope
3	Light path components of LSM880
4	How to acquire a high quality image?

How to evaluate the quality of a image?

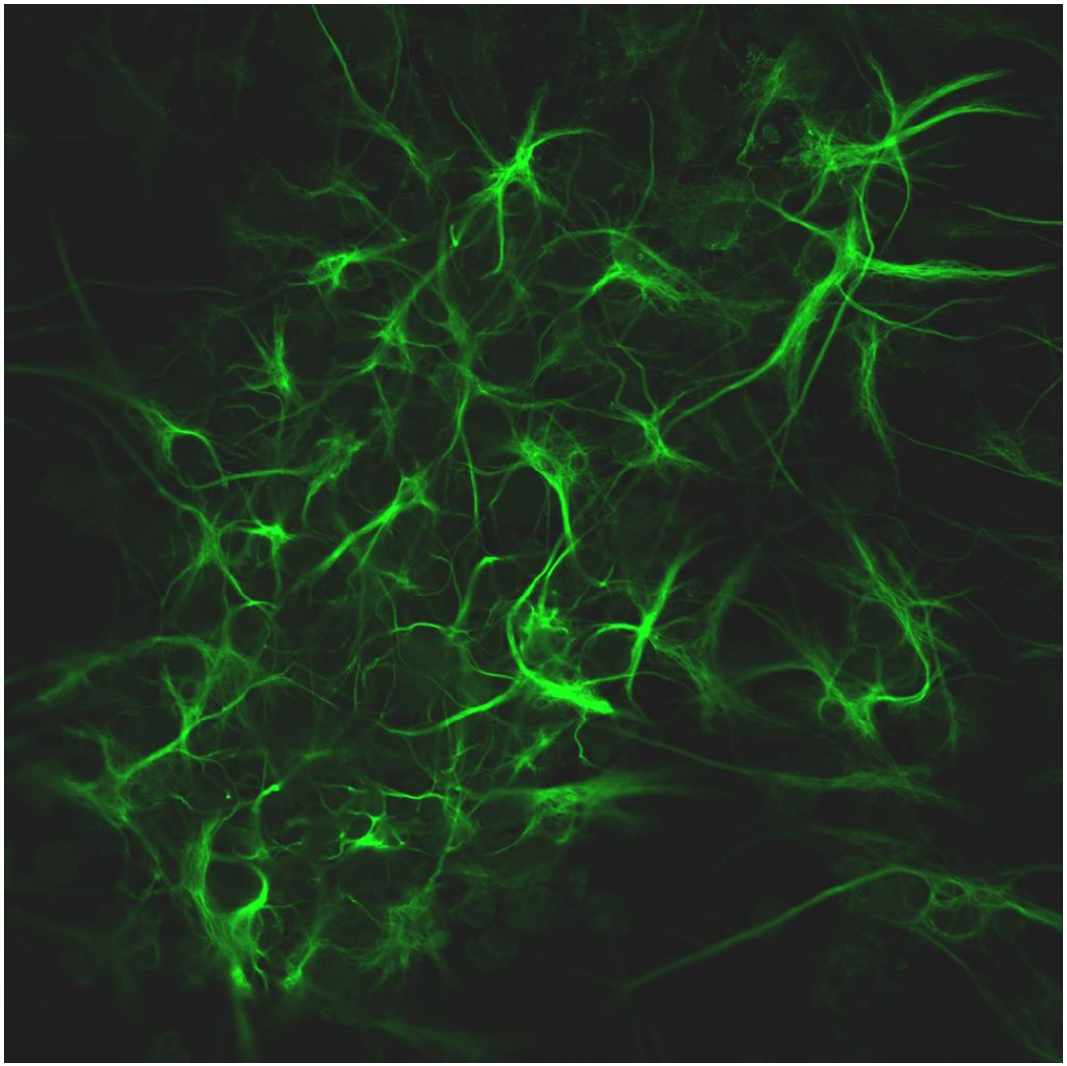
When we are talking about the quality of a image, we are actually talking about…

Intensity
SNR
Resolution

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)

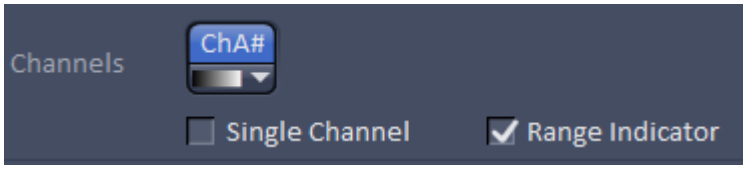
Grey value 255

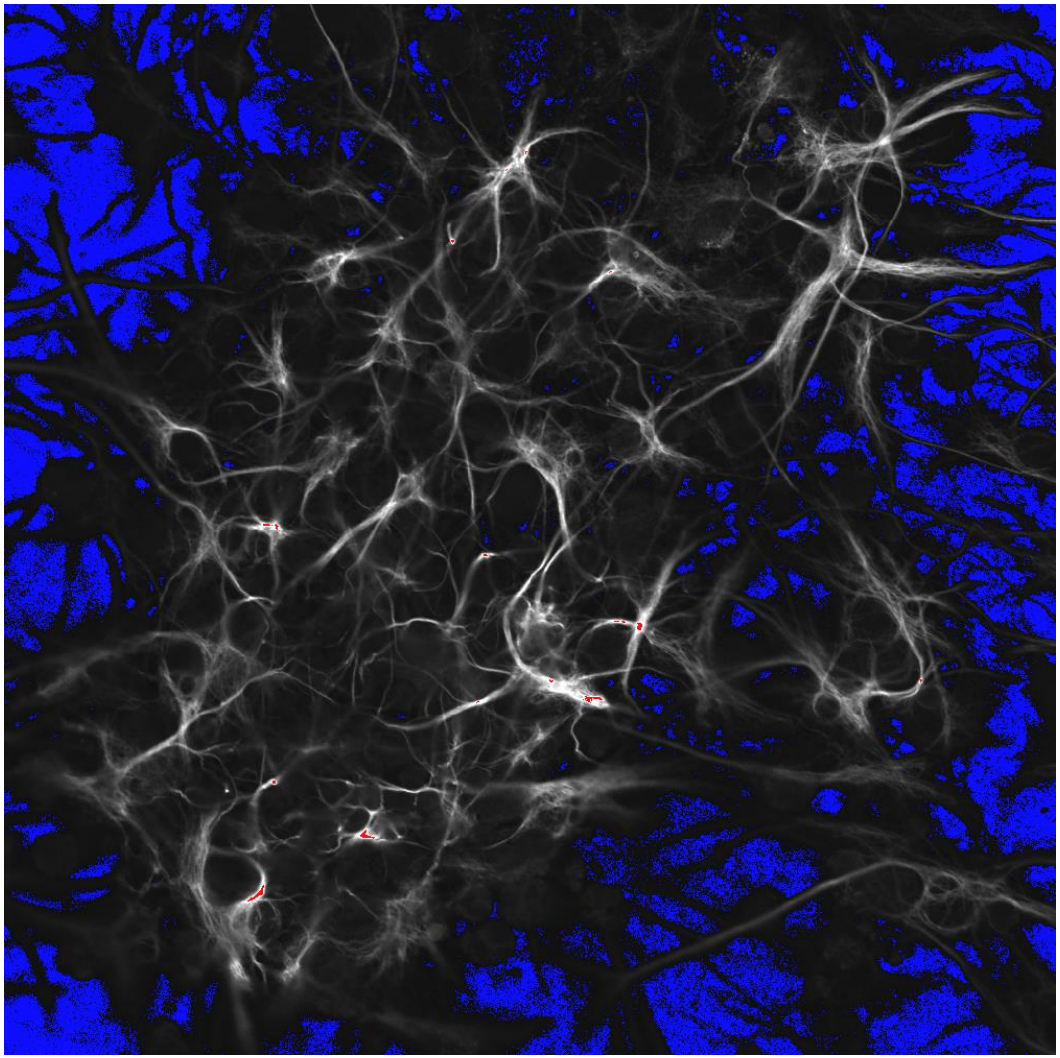
White

Grey values
1-255

Black

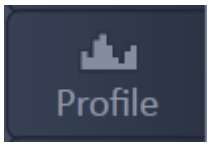
Grey value 0



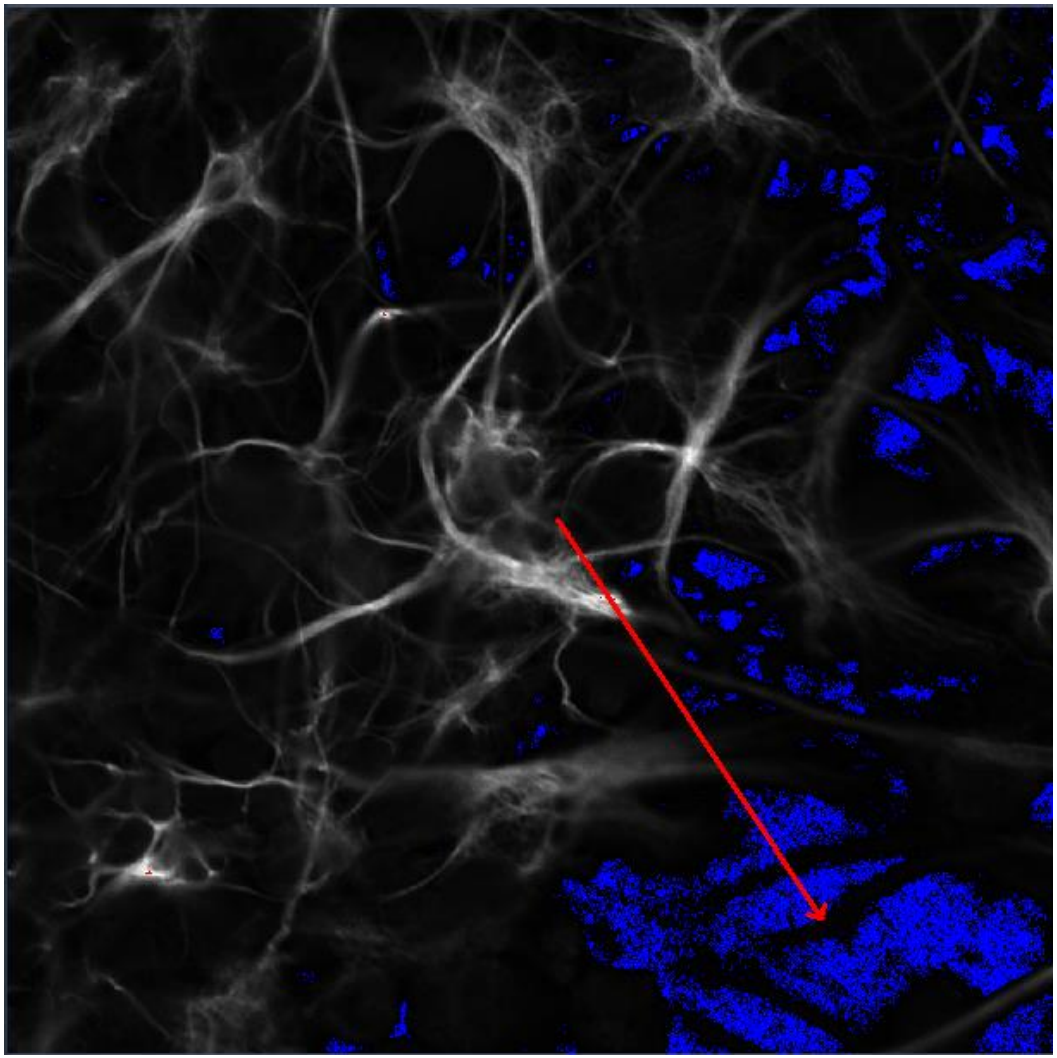


Profile

How to measure the dynamic range

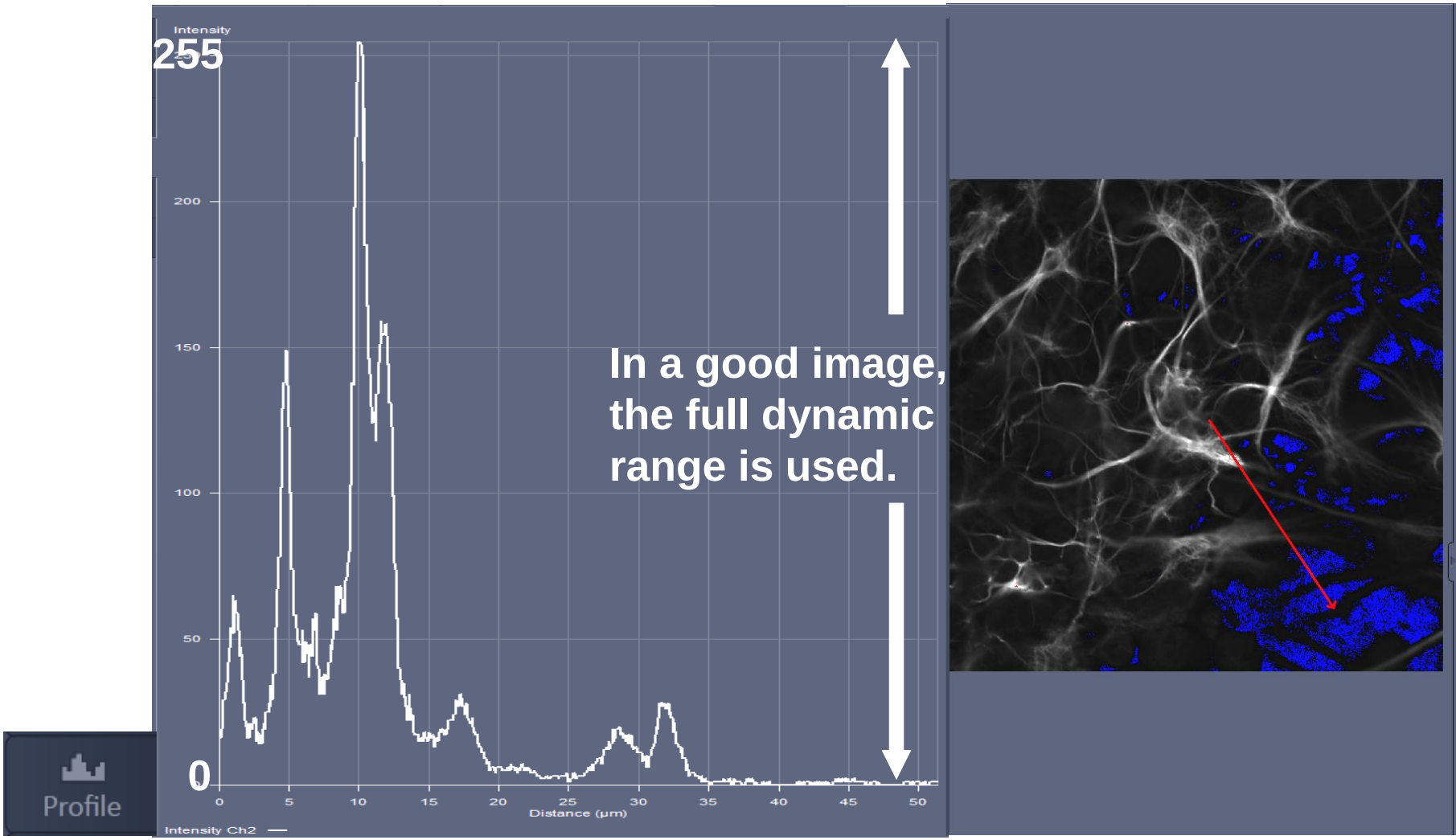


= gives measurements of grey values along a line



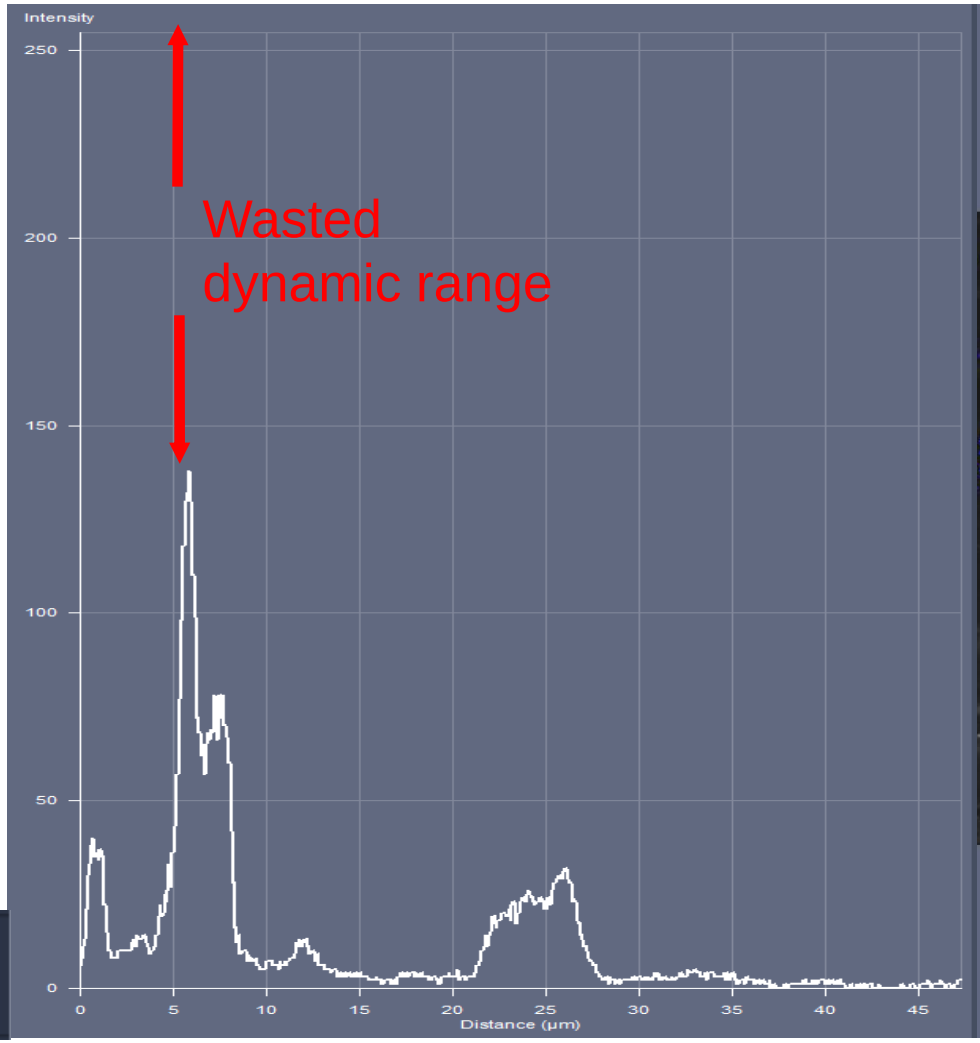
Profile

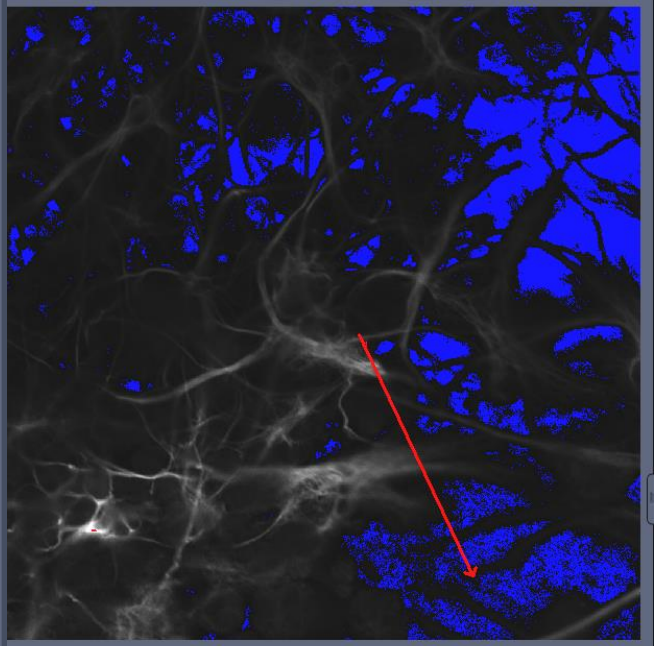
How to measure the dynamic range

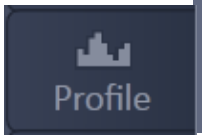


Gain (Master)

Not enough

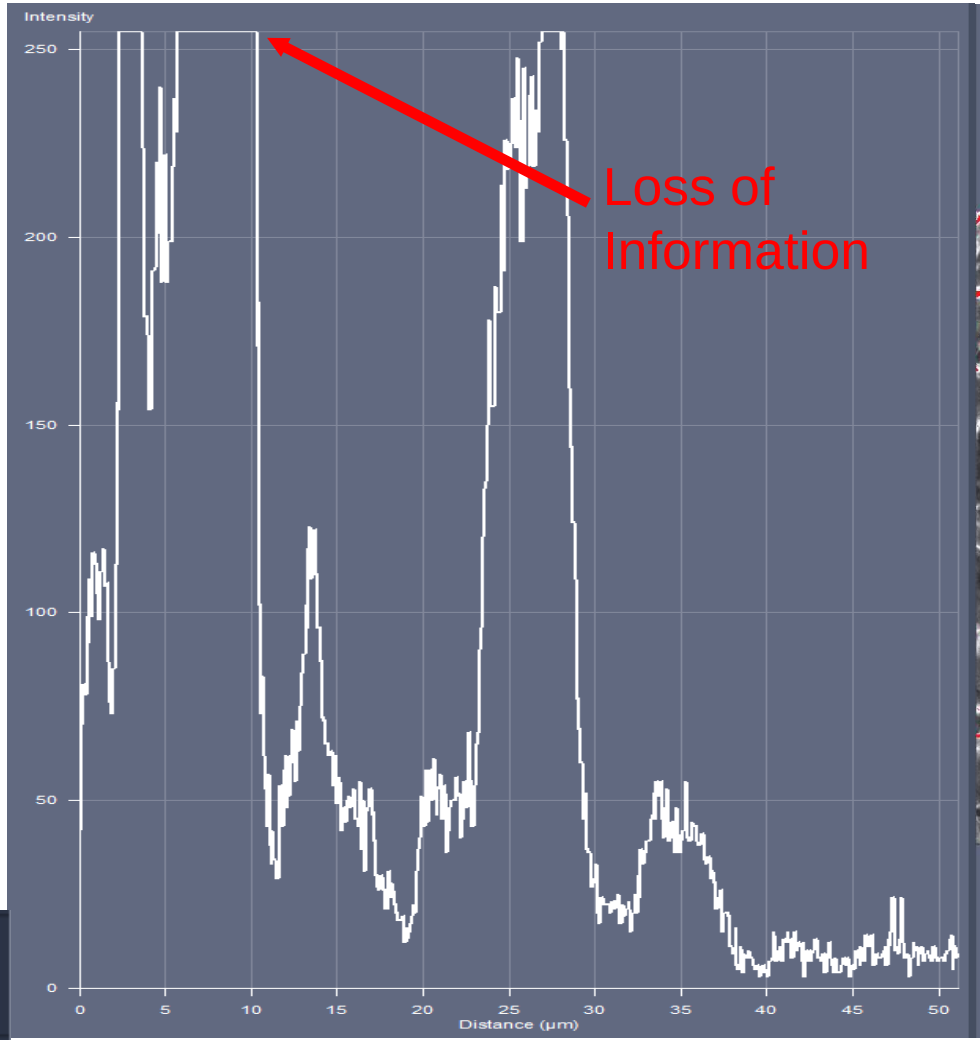


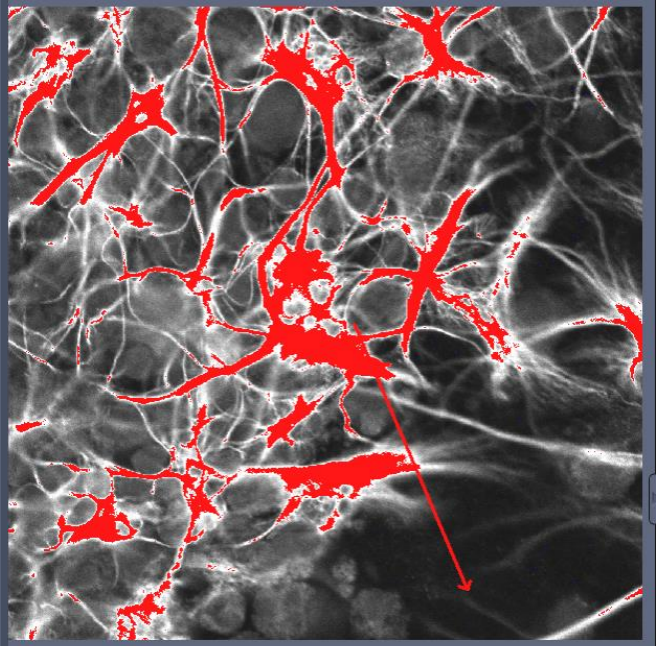




Gain (Master)

Set too high

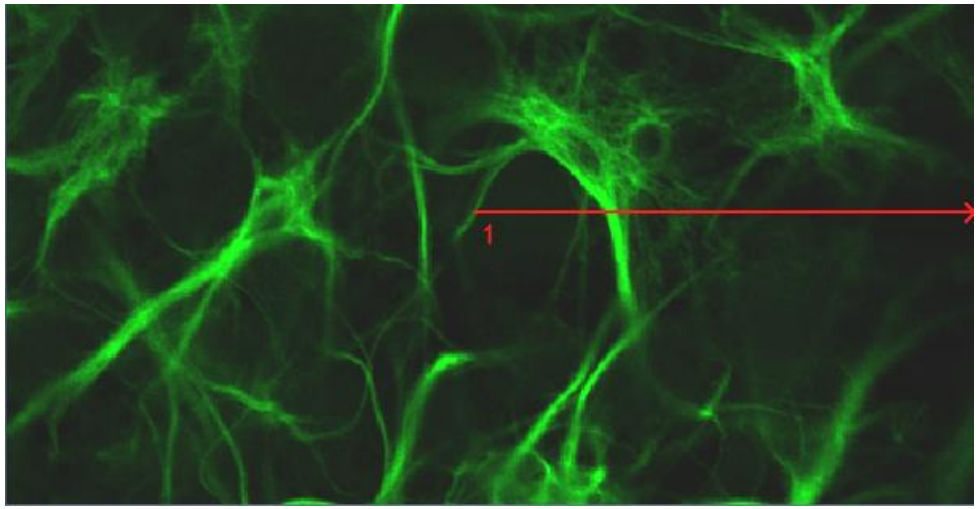




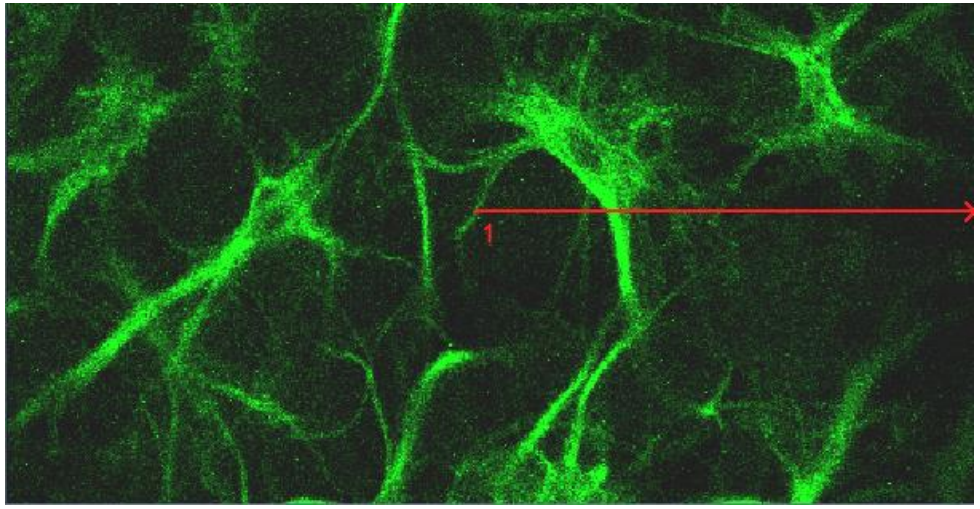
Scanning Strategies

Image Noise: What does it look like?

“Good” Image



“Bad” Image



Scanning Strategies

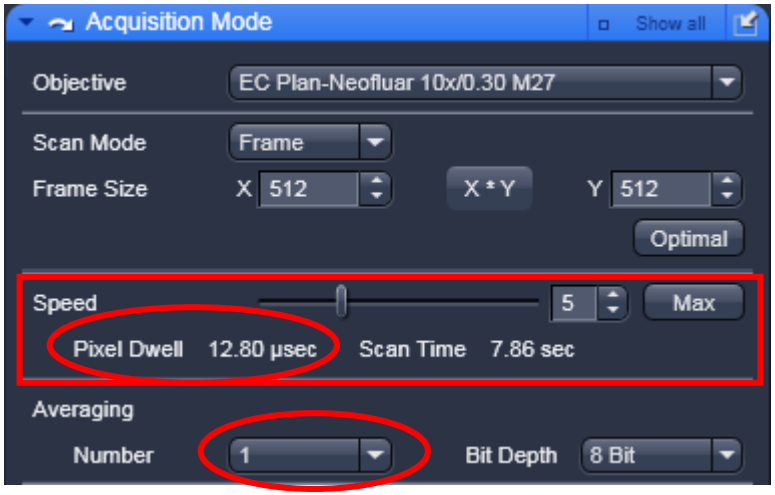
Speed and Averaging

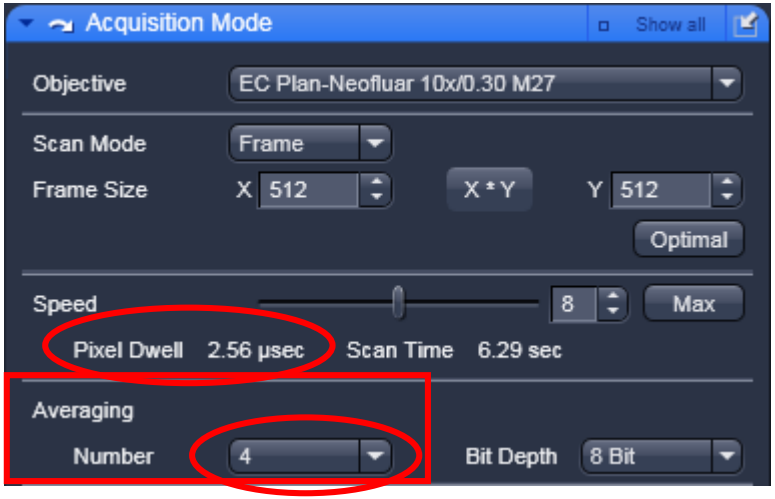
To decrease the effect of noise, more photons (signal) must be collected:

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

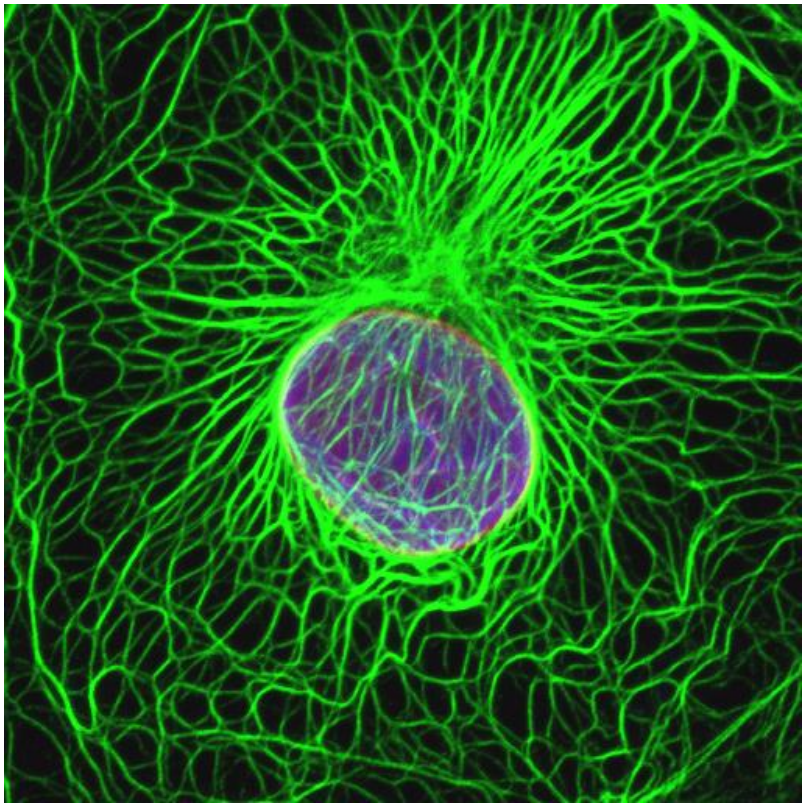
Which of the two setups will lead to better image quality?



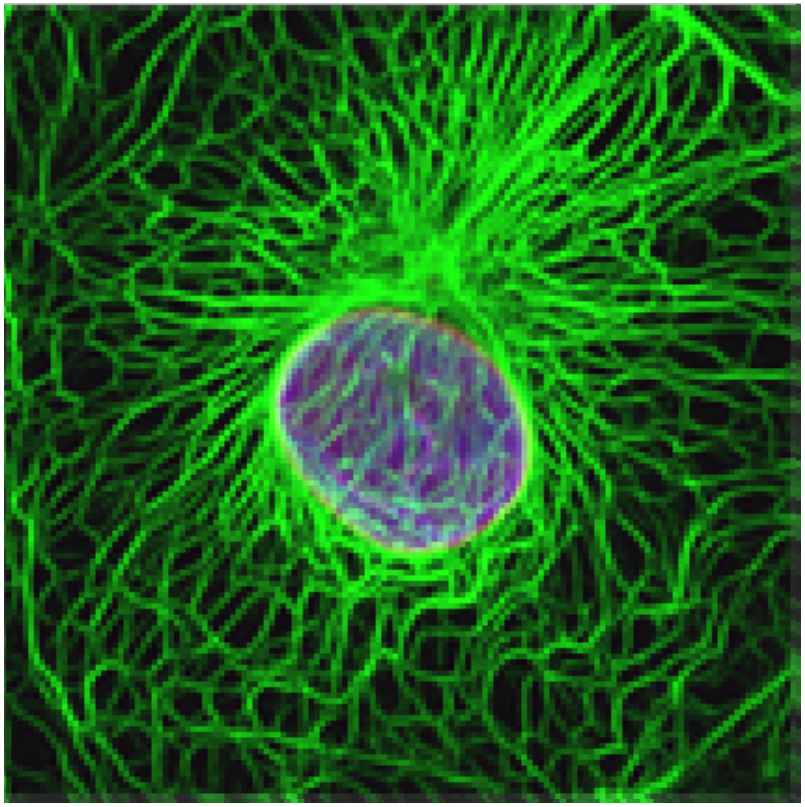


Hunting for Details?

Choose the right Resolution



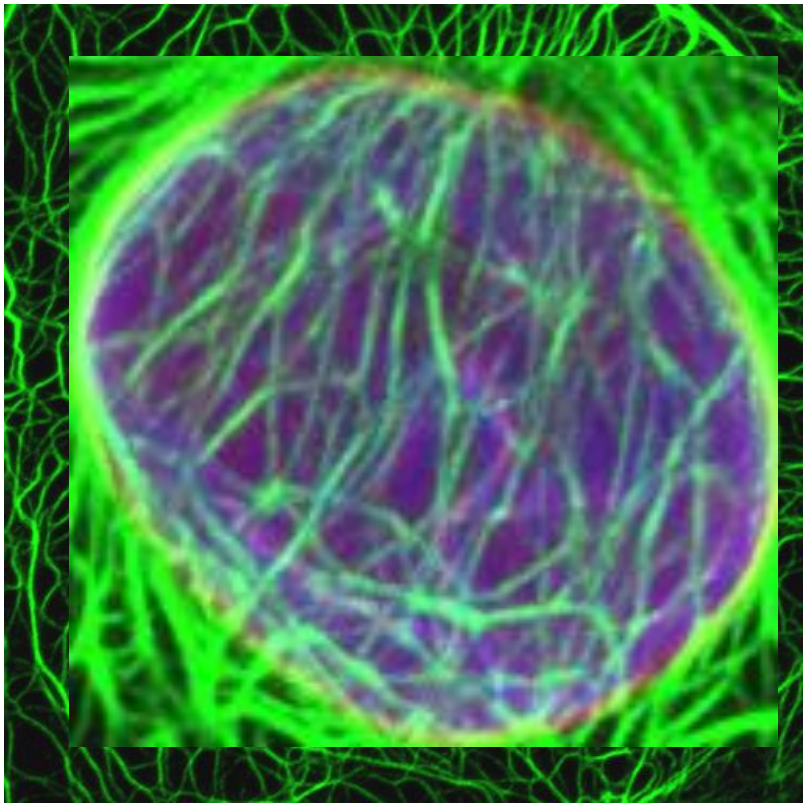
Resolution okay



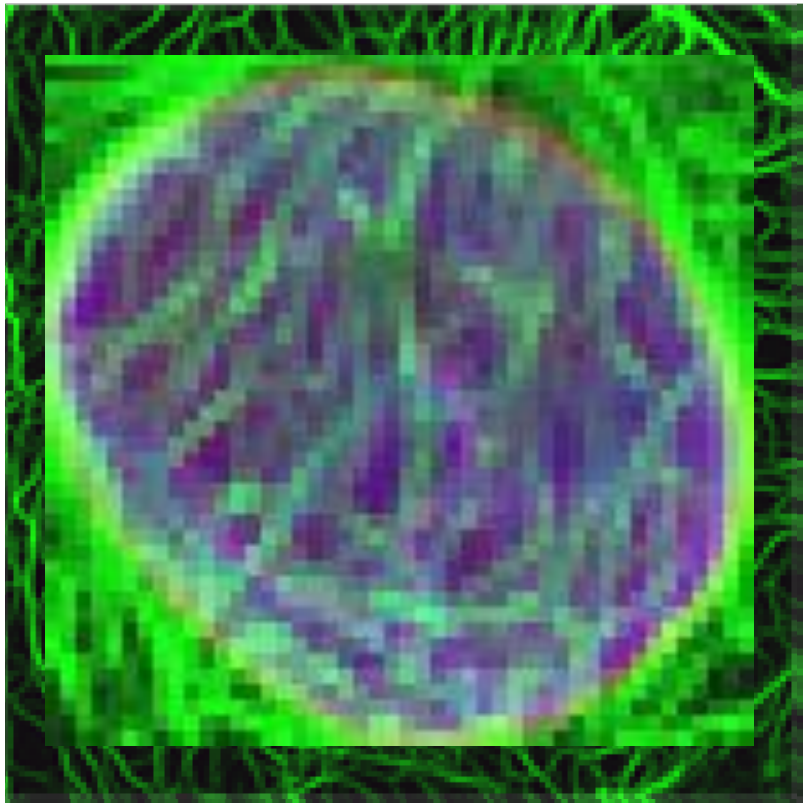
Resolution too low

Hunting for Details?

Choose the right Resolution



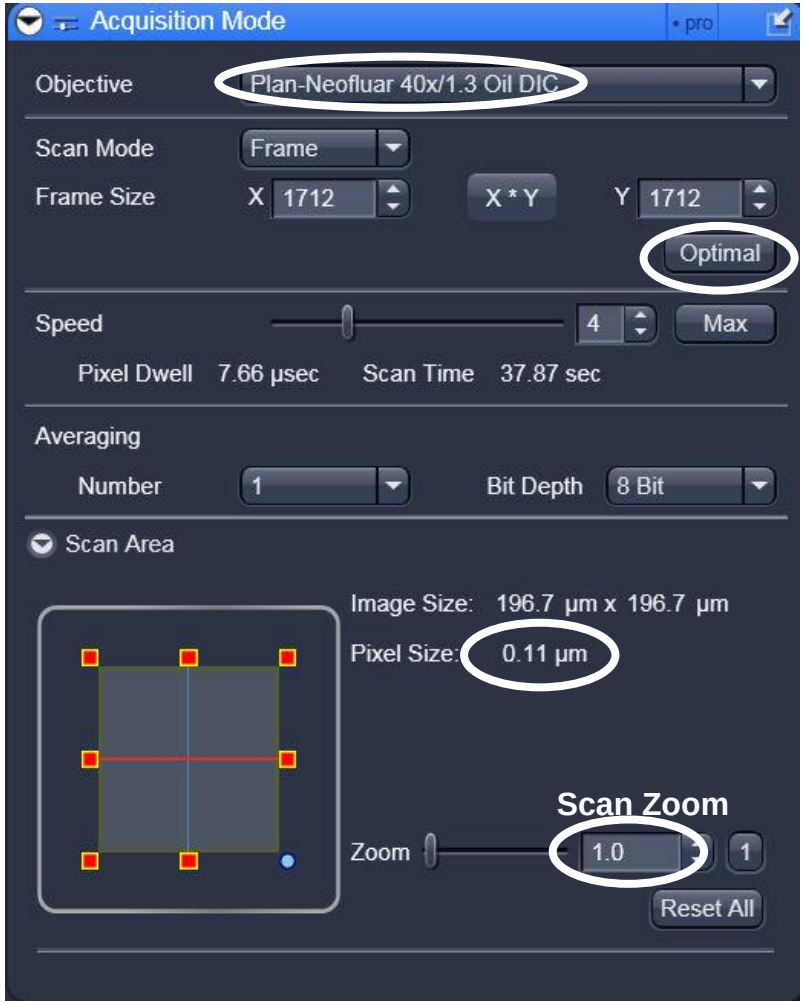
Resolution okay

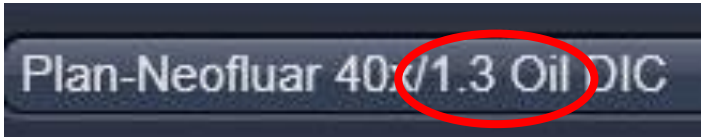


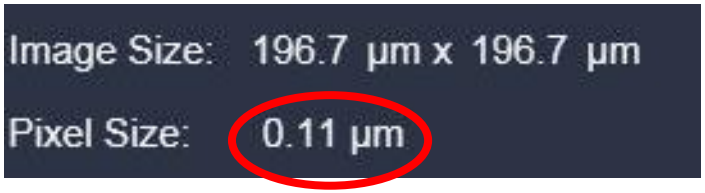
Resolution too low

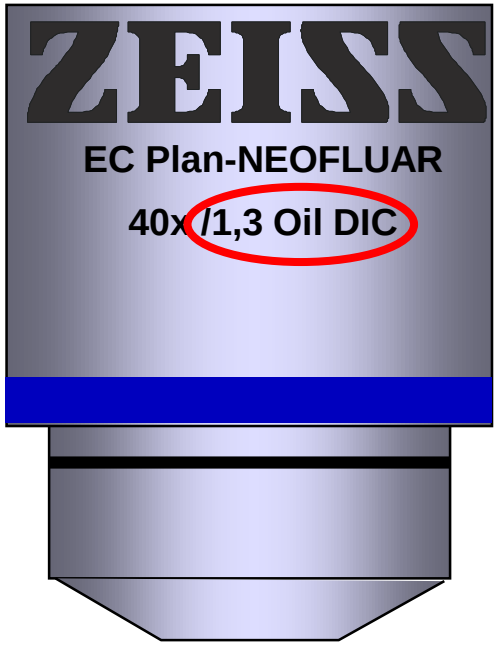
Resolution

Information given in the Software





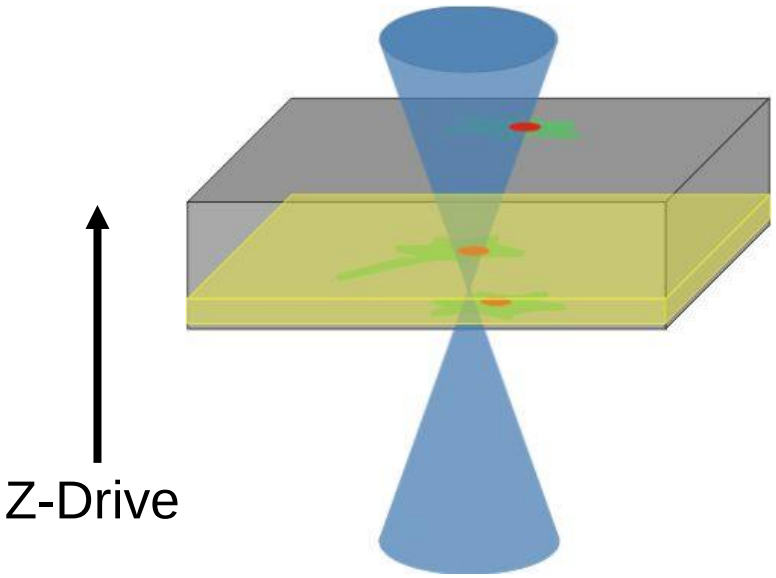


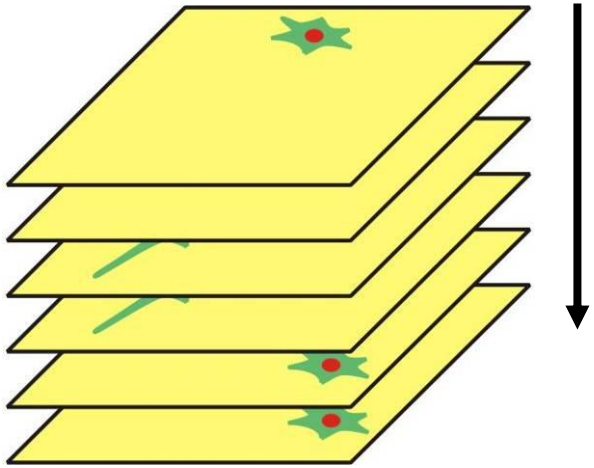


Confocal Imaging

From Image to 3D Information

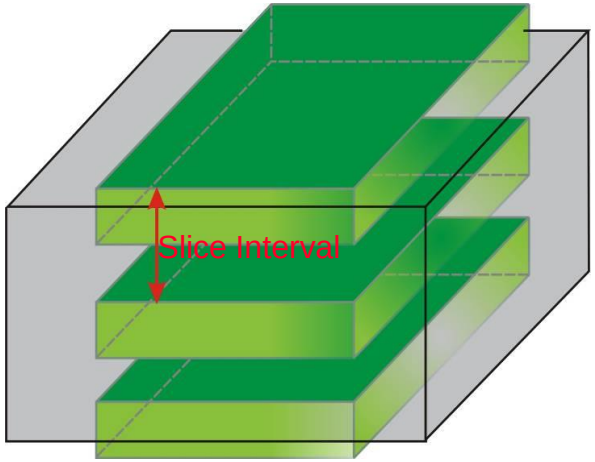
How is a X/Y/Z Stack produced?



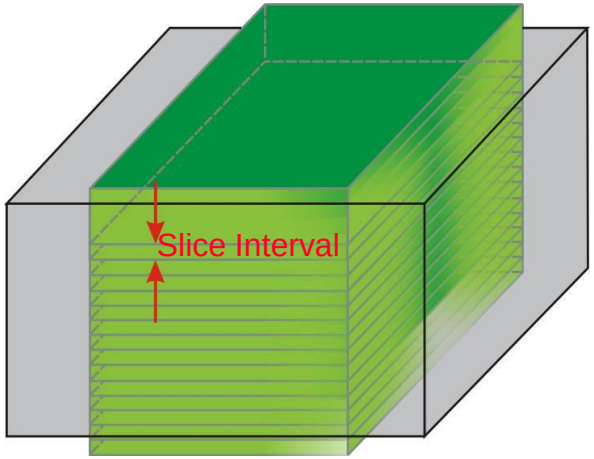


Optical Slice Thickness

Overlap between Optical Slices



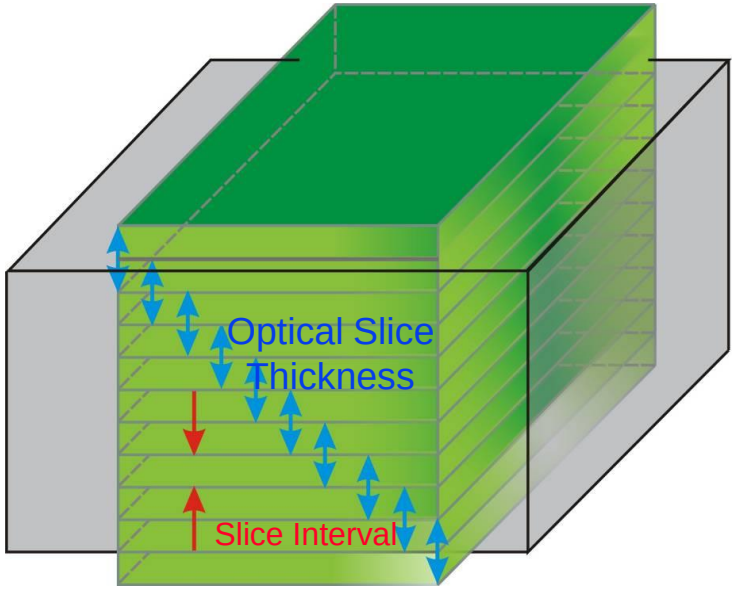
With this setting, the object structures between the slices cannot be detected.

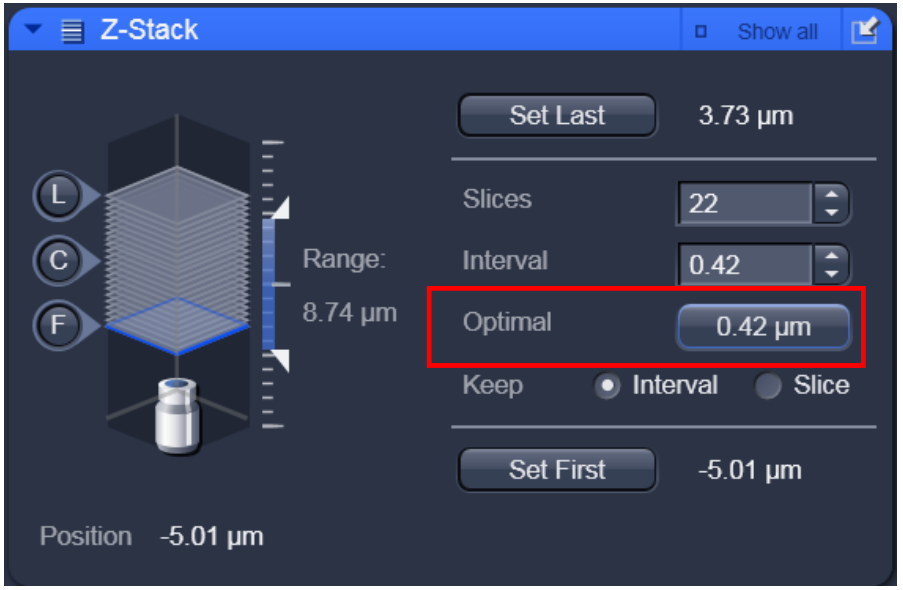


At very small intervals a lot of additional data without additional information is generated.

Optical Slice Thickness

Overlap between Optical Slices



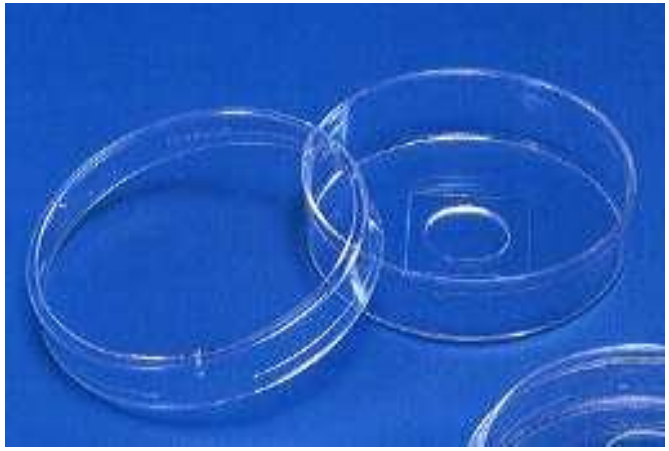


The optimal overlap is fulfilled at **"Nyquist"** or "Sampling Theorem" conditions.

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

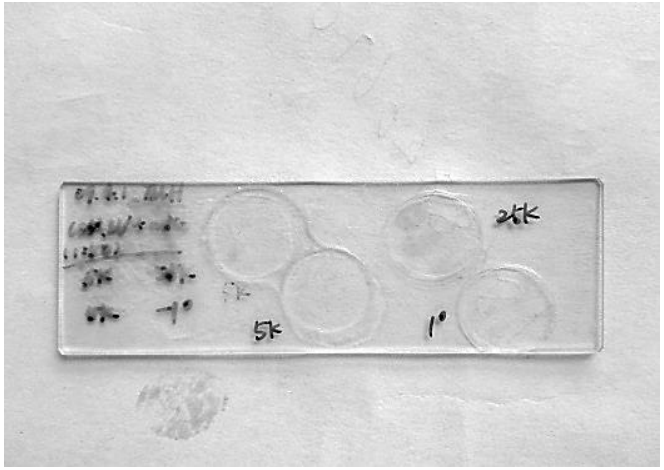
To achieve these conditions just press *Optimal Interval* in *Z-Stack* dialog. Then, the slices overlap by half of their thickness (no missing information @ minimal number of sections).

Consumptive materials of Confocal



Cover Glass:170um
Glass bottom dish

Cover Glass:170um
Cell Chamber Slides



We make it visible.