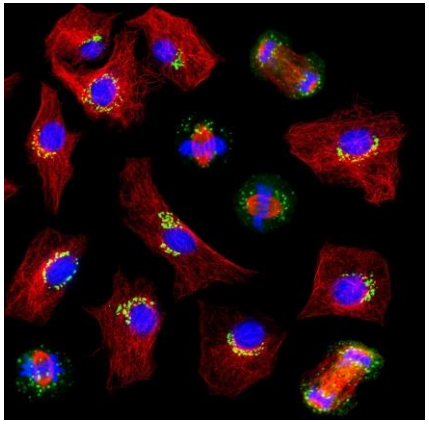
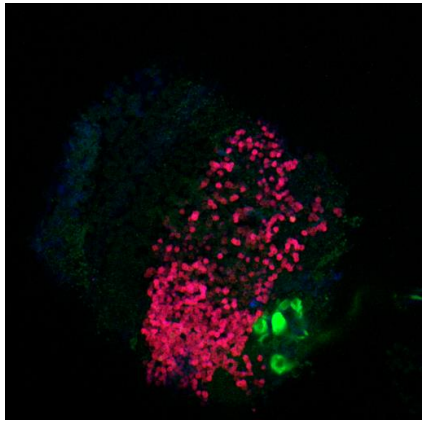
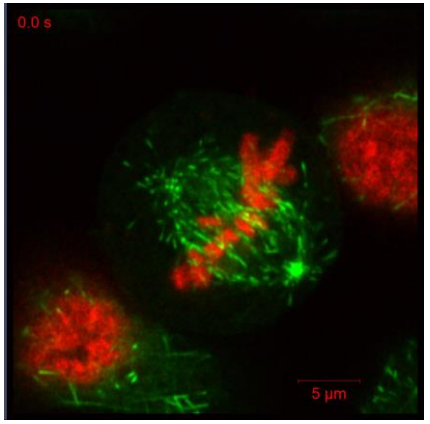
激光共聚焦显微镜的基本原理——LSM 900 Airyscan2

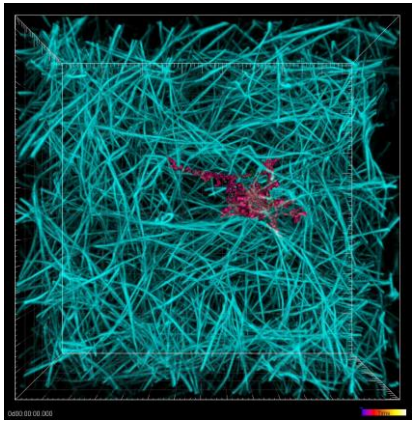
Reporter: Li Zehui (李泽惠） Application Specialist
Date:July 1, 2020

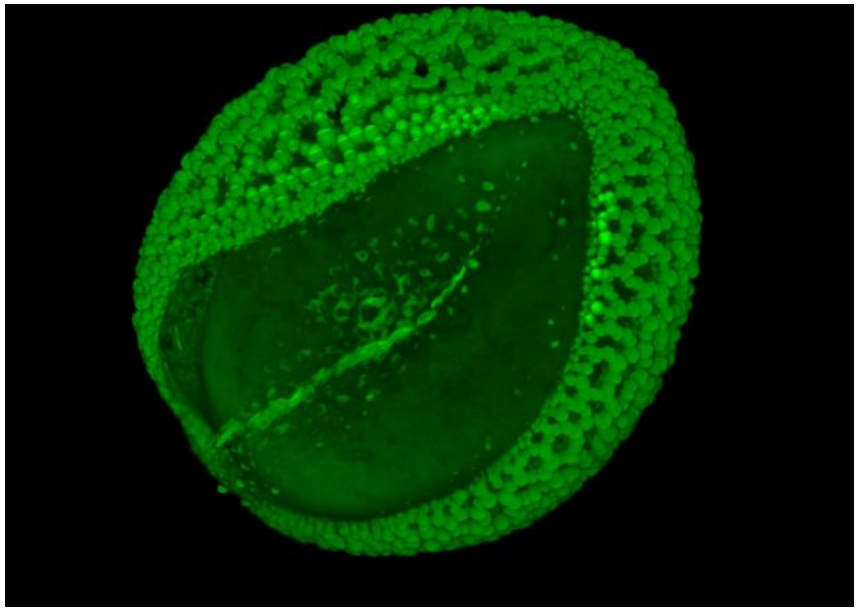
高质量、多维度、大视野显微图像

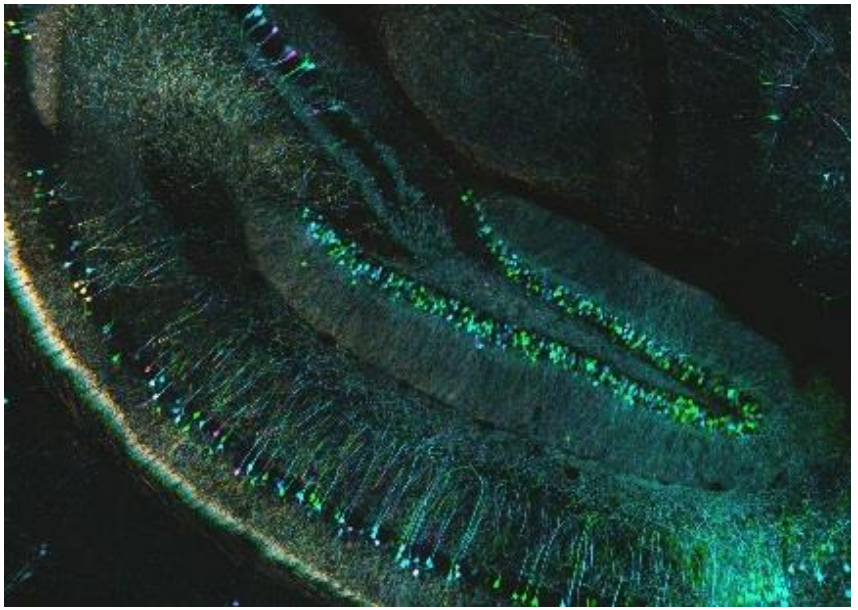












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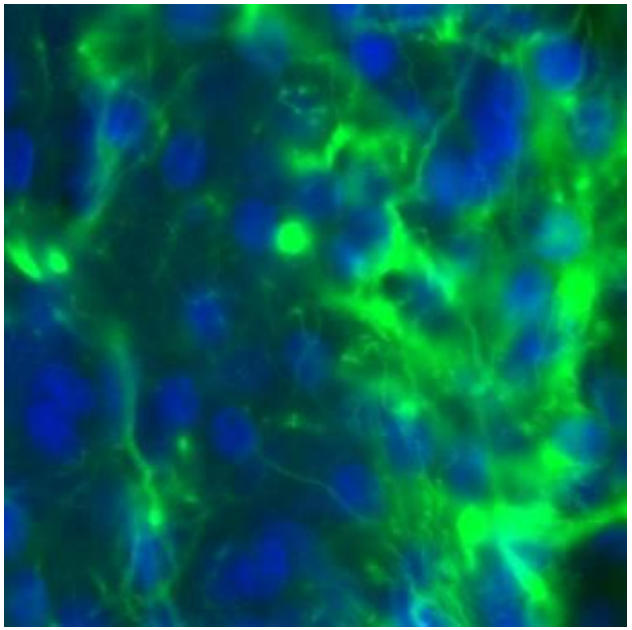
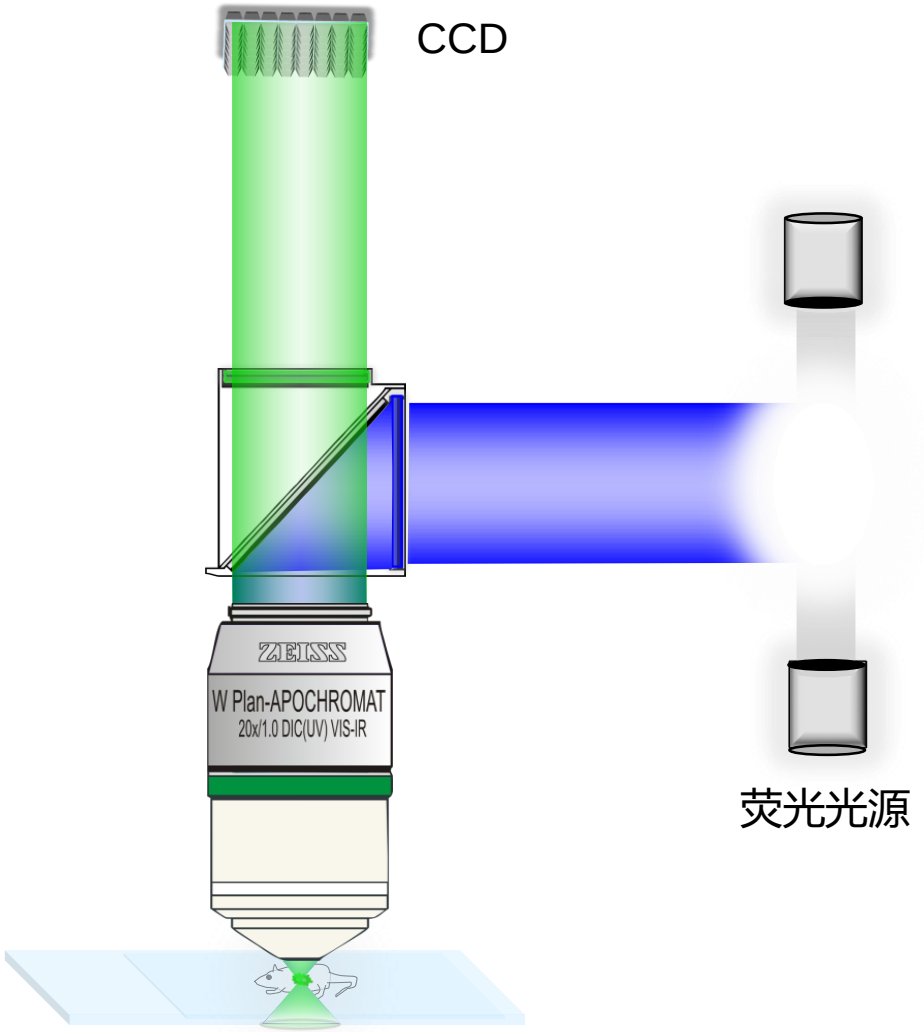
2. 激光共聚焦显微镜的重要组成
3. 如何获取一张高质量的图像
4. 激光共聚焦显微镜的应用
5. Airyscan2的成像原理

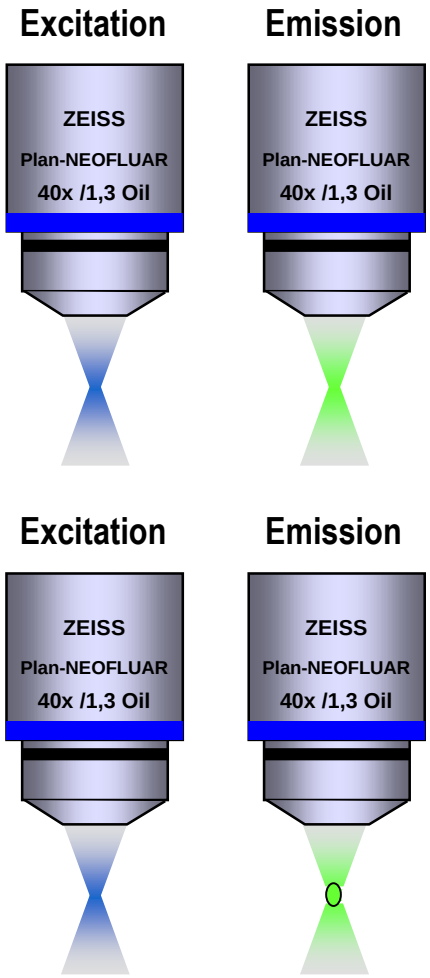
宽场荧光显微镜光路

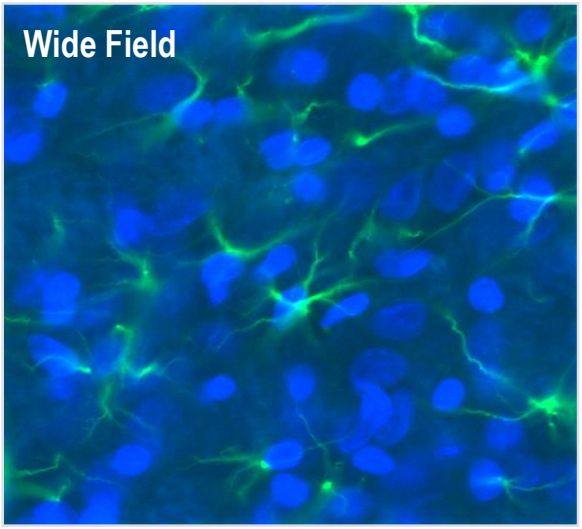


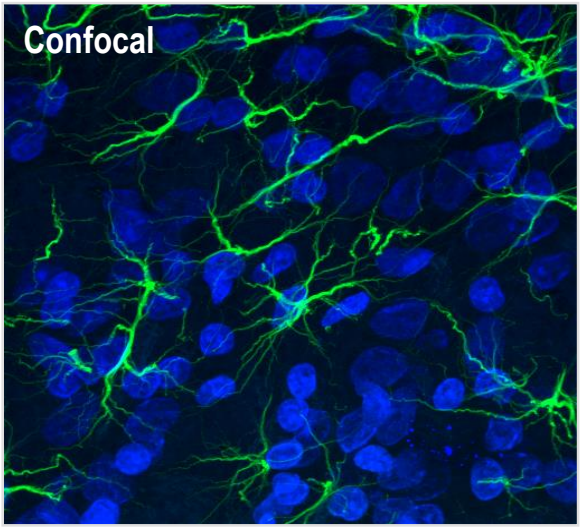
Rat Brain, Double labelling:
Green: Neurons, Blue: Nuclei



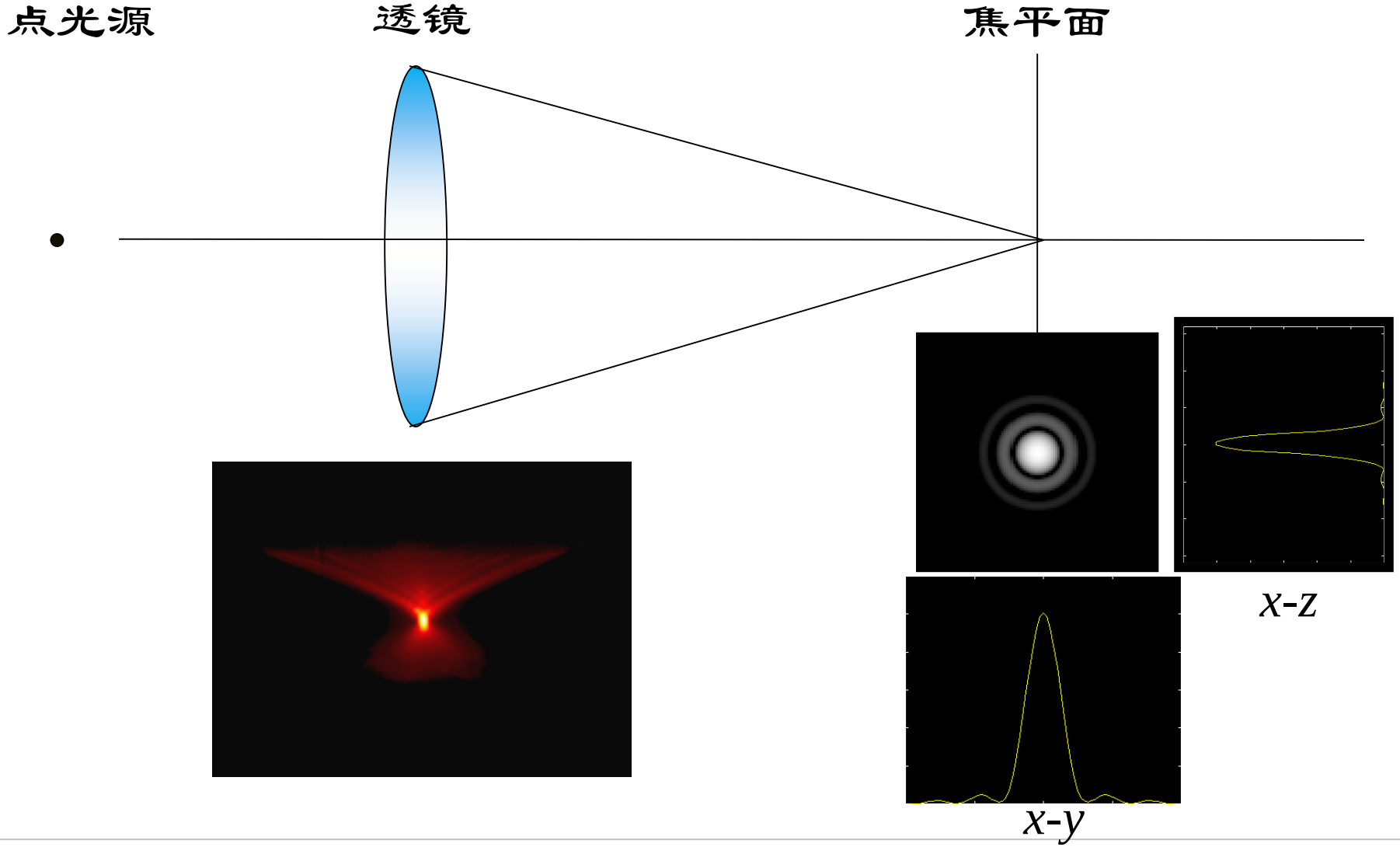
宽场显微镜与共聚焦显微镜的比较



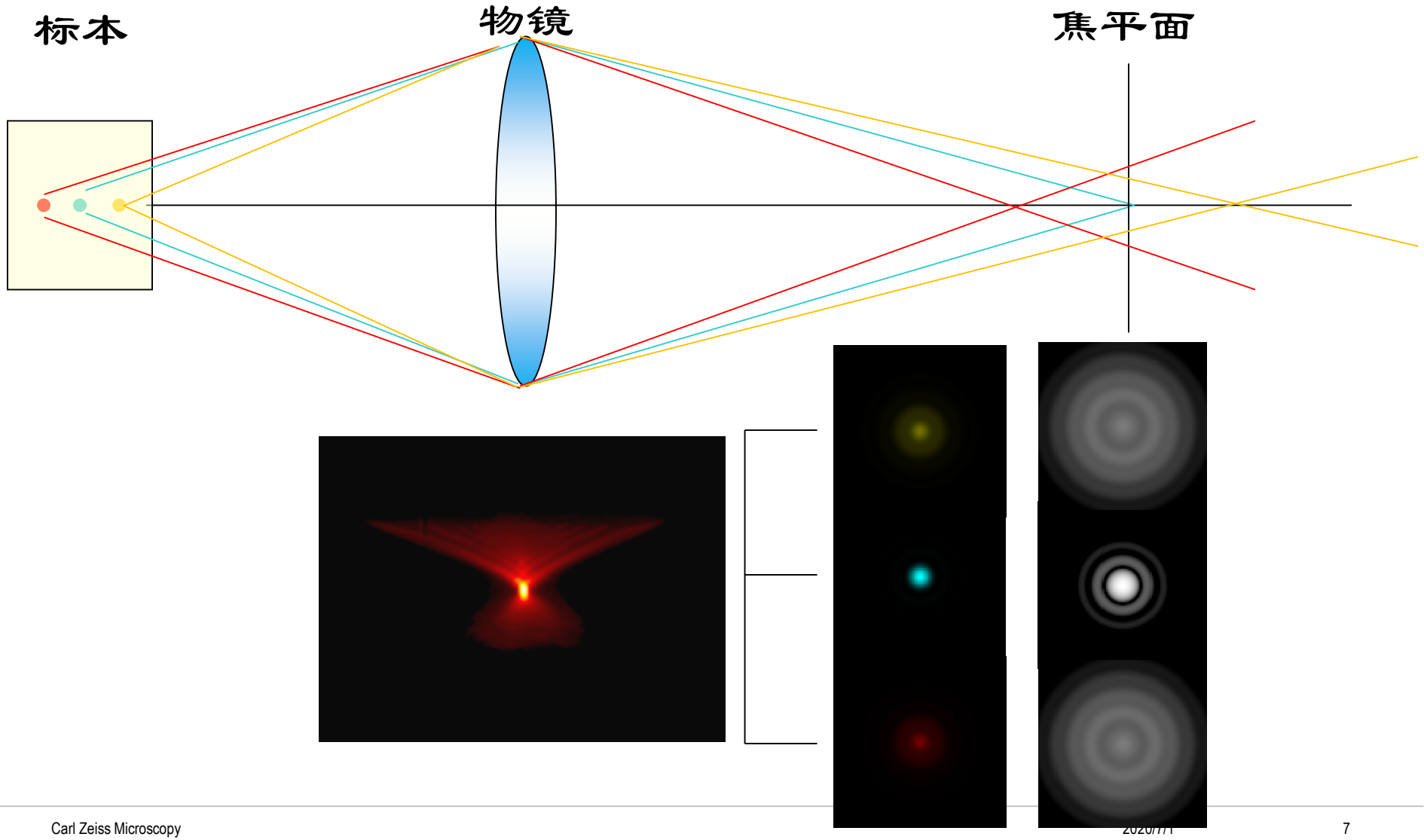




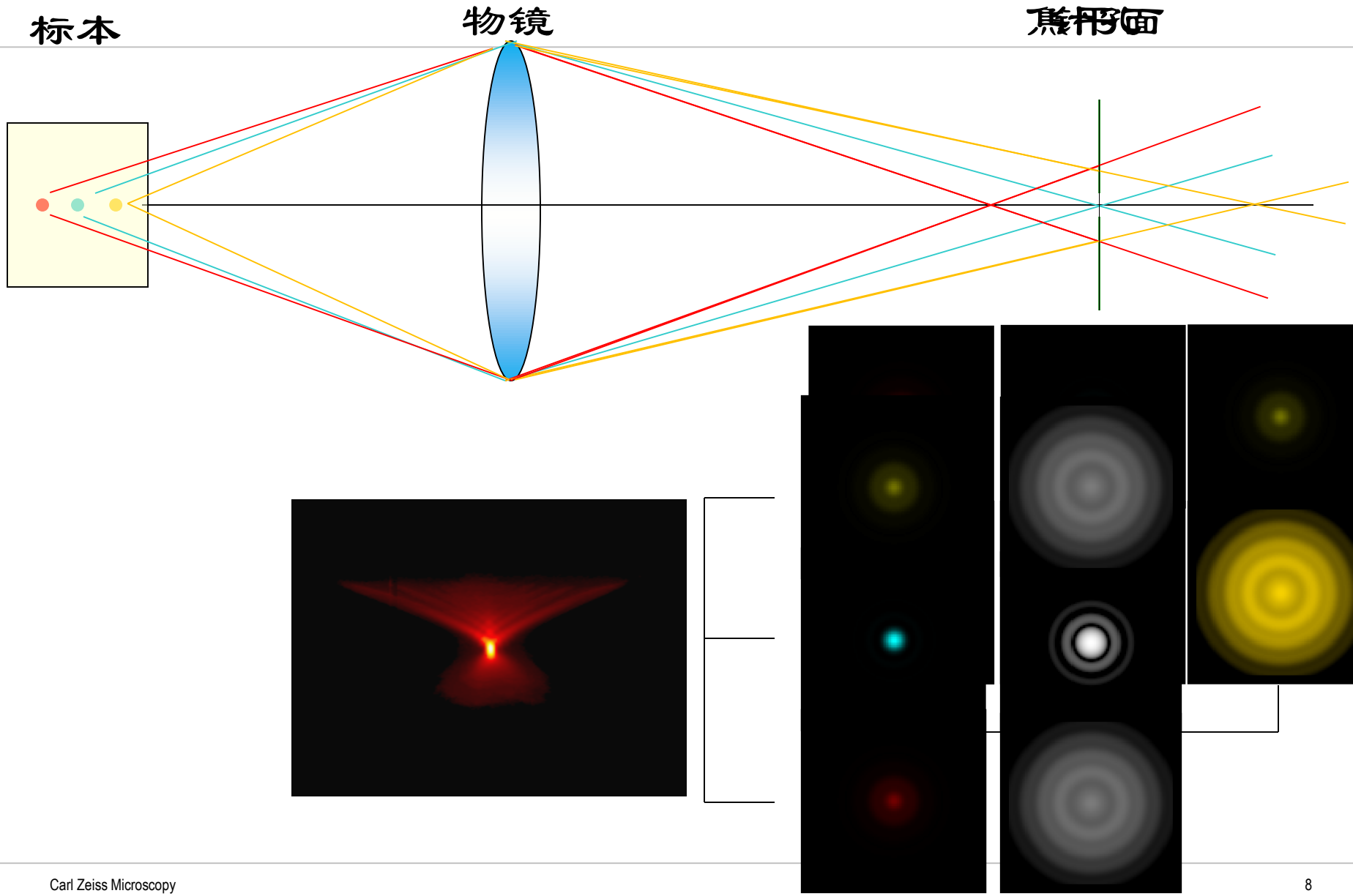
光学显微镜特性



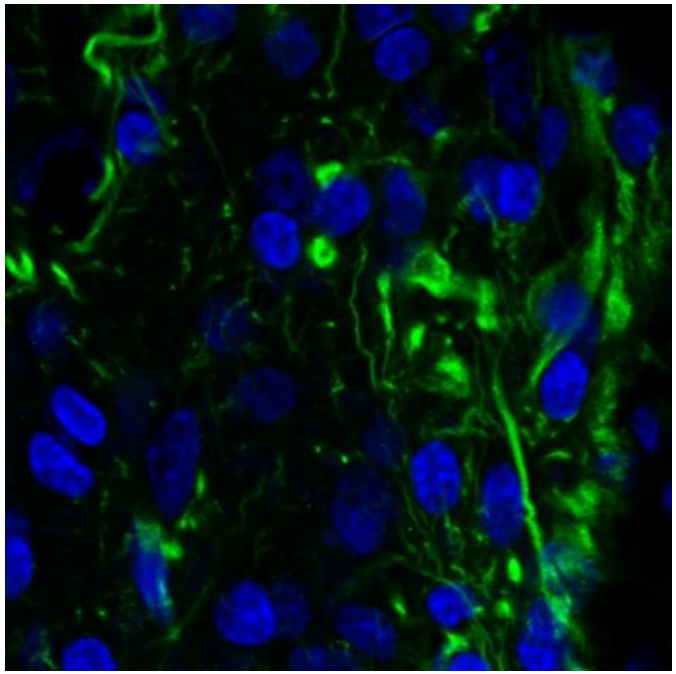
宽场荧光显微镜成像



共聚焦显微镜成像



激光共聚焦显微镜光路



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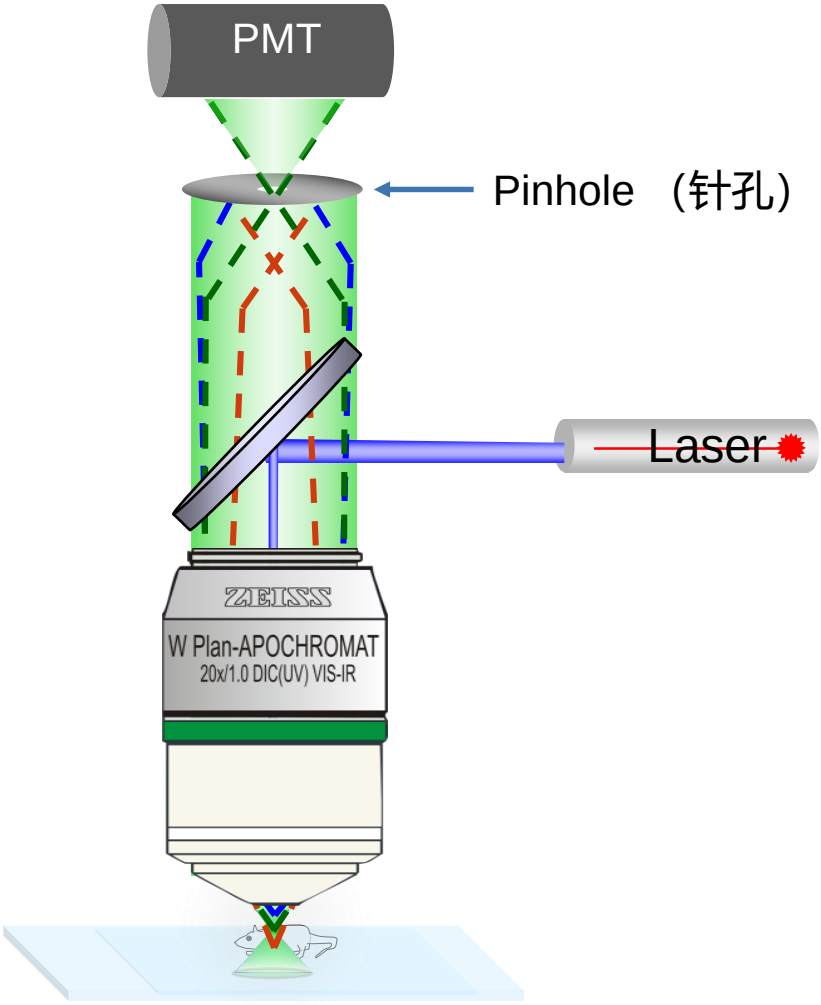
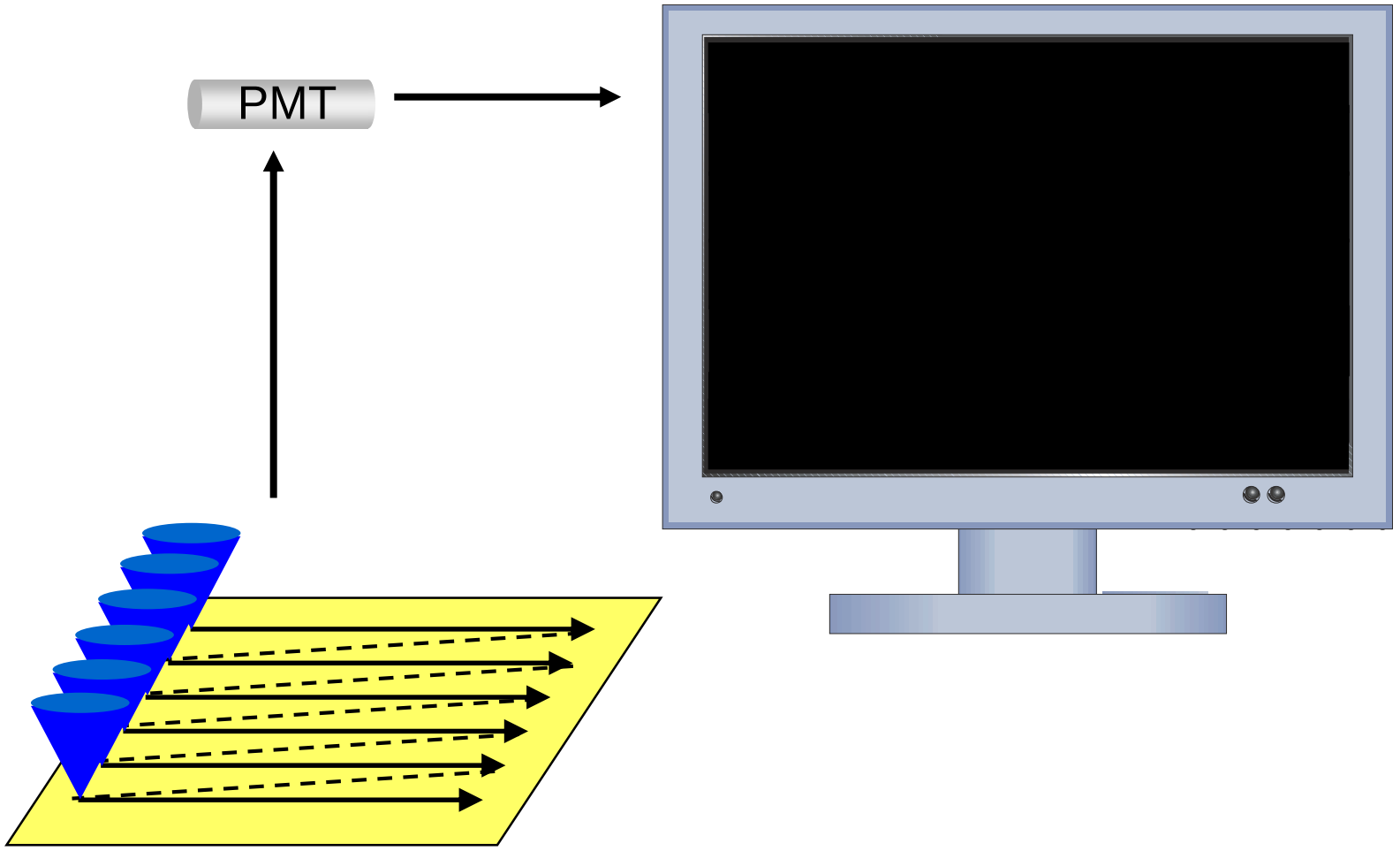


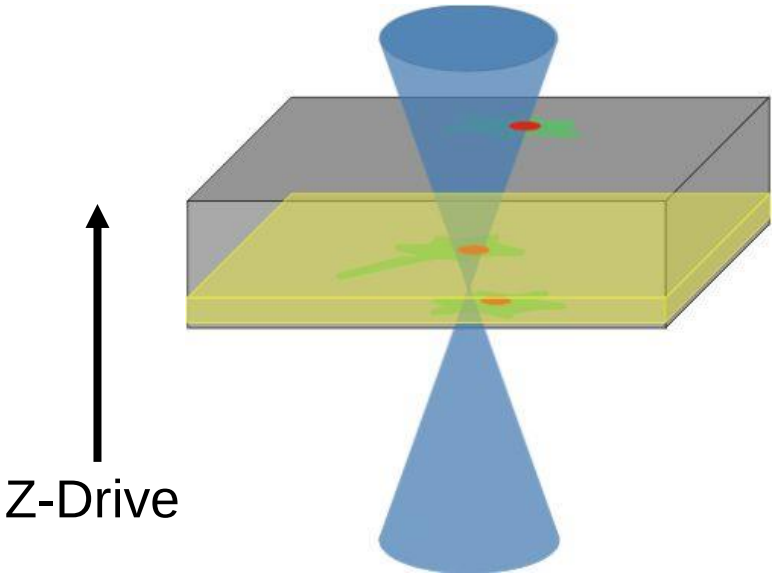
X-Y平面扫描方式

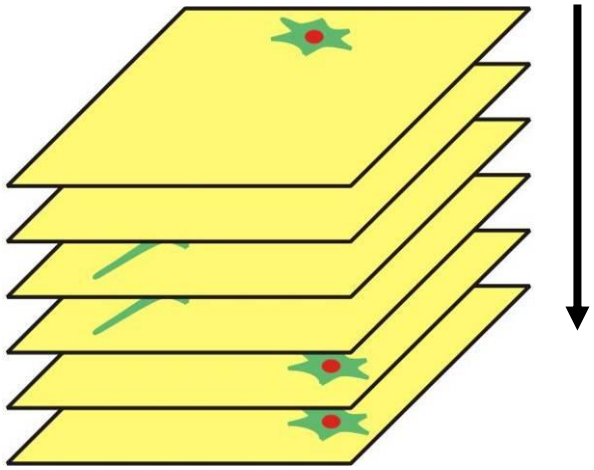
逐点扫描 点 — 线 — 面



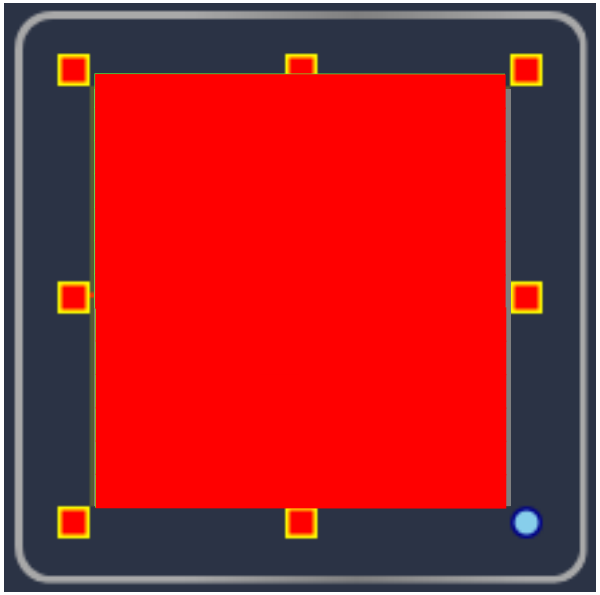
Z轴扫描方式
逐层扫描

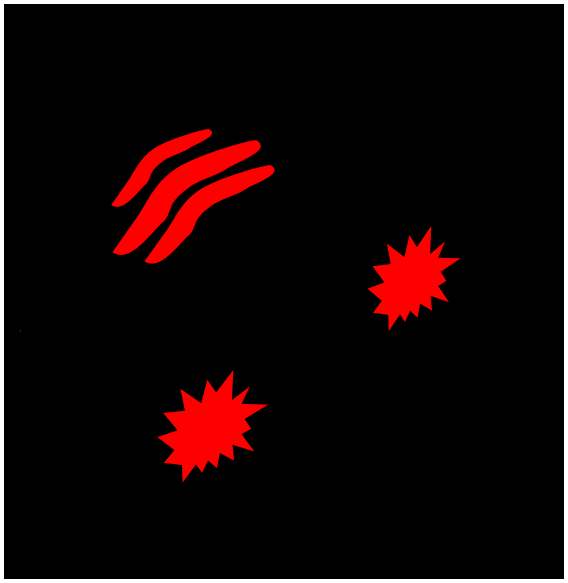
How is a X/Y/Z Stack produced?





Sequential Image Acquisition: Framewise
连续扫描：帧切换



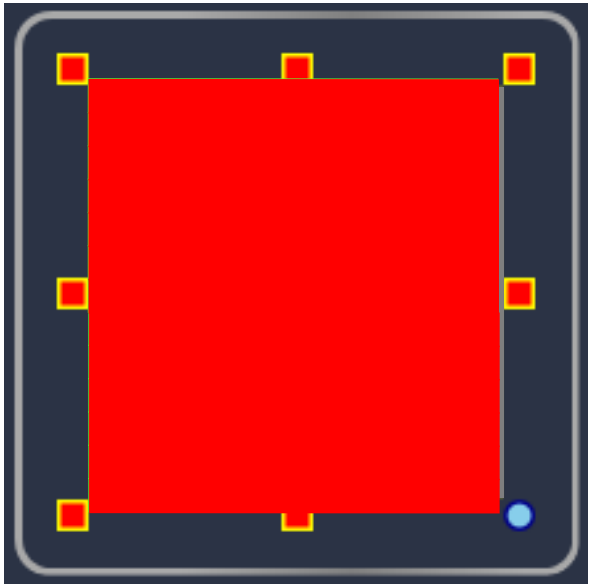


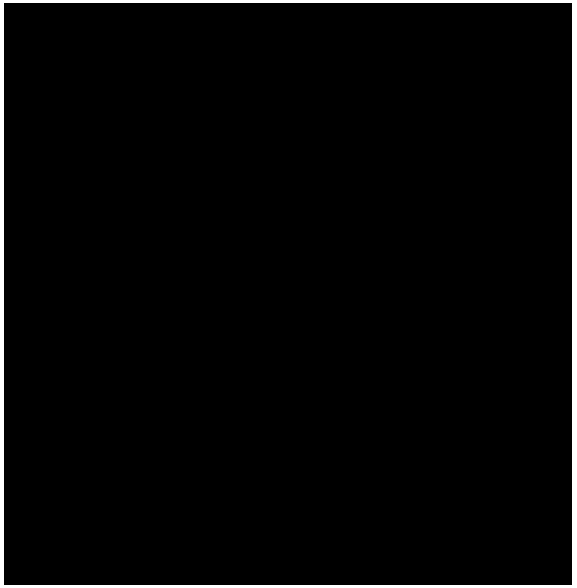
Switch Track every Frame

+ **不同通道的硬件参数设置可以改变 （如，针孔大小，分光片等）**

- **样品的运动 会对最终成像带来影响**

Sequential Image Acquisition: Linewise
连续扫描：行切换





Switch Track every Line

+ 减少样品运动的影响
+ 快速图像获取
- 不同通道的硬件参数设置不可以改变

Smart Setup
通道设置

- **Fastest:**
All channels in one track
- simultaneous mode

- **Best Signal:**
Each channel in a track
- sequential mode

- **Smartest:**
Combination of sequential and simultaneous mode

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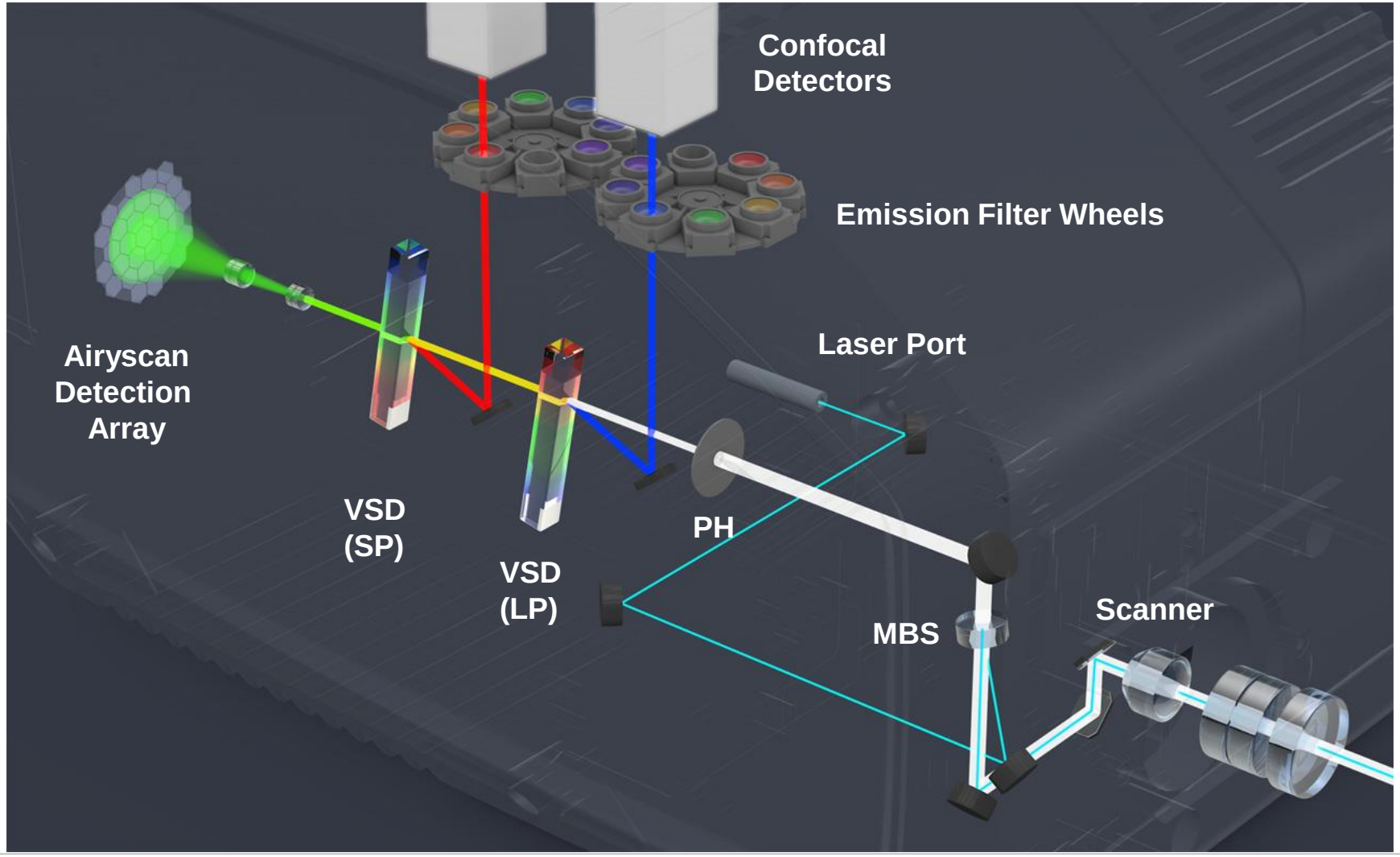
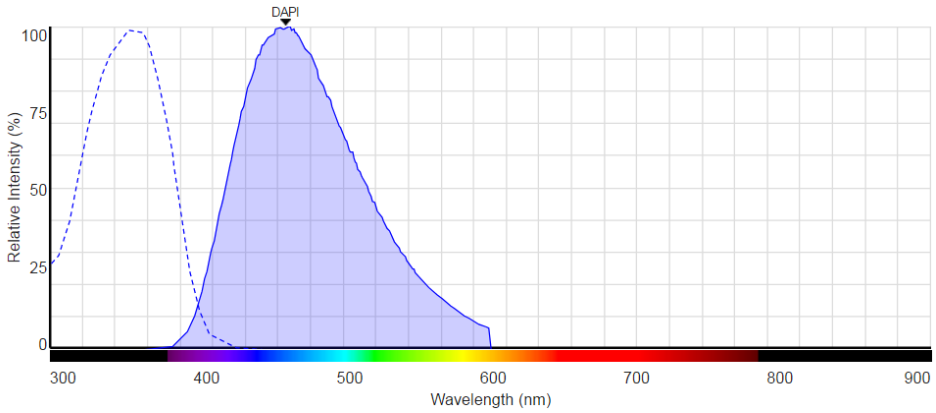
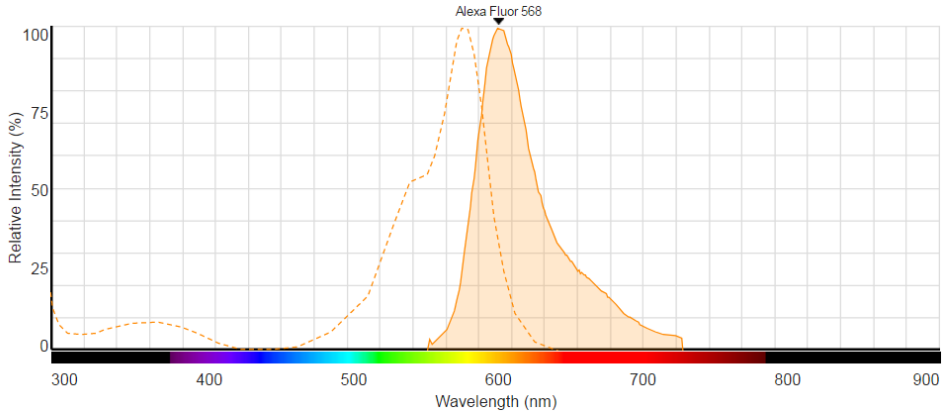
LSM 900 光路


Confocal
Detectors
Emission Filter Wheels
Laser Port
Airyscan
Detection
Array
VSD
(SP)
VSD
(LP)
PH
MBS
Scanner

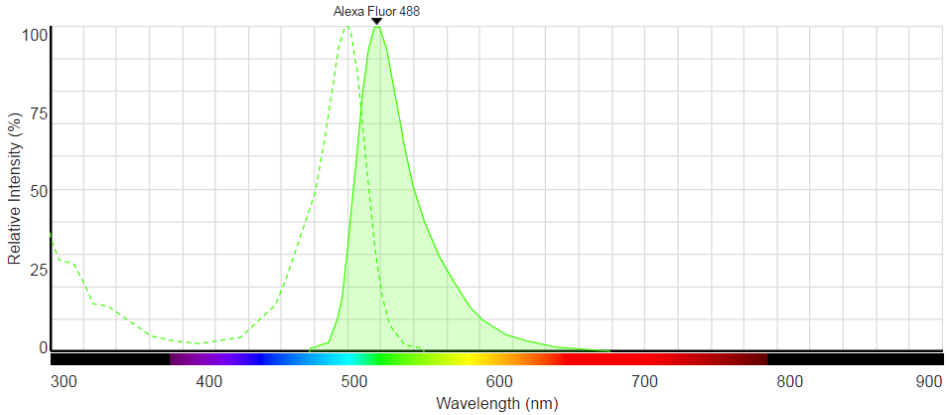
激光
颜色：选择与荧光基团匹配的激光



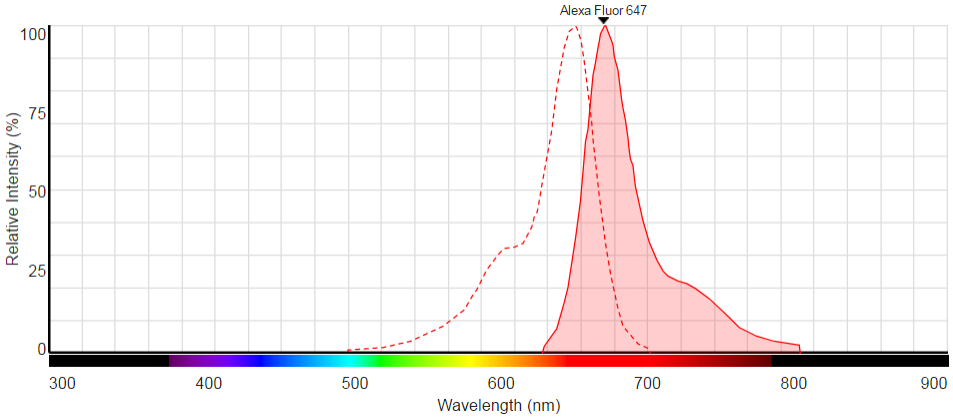
405 nm　DAPI / Hoechst



561nm RFP / Alexa Flour 568 / Cy3



488nm GFP / Alexa Flour 488 / FITC

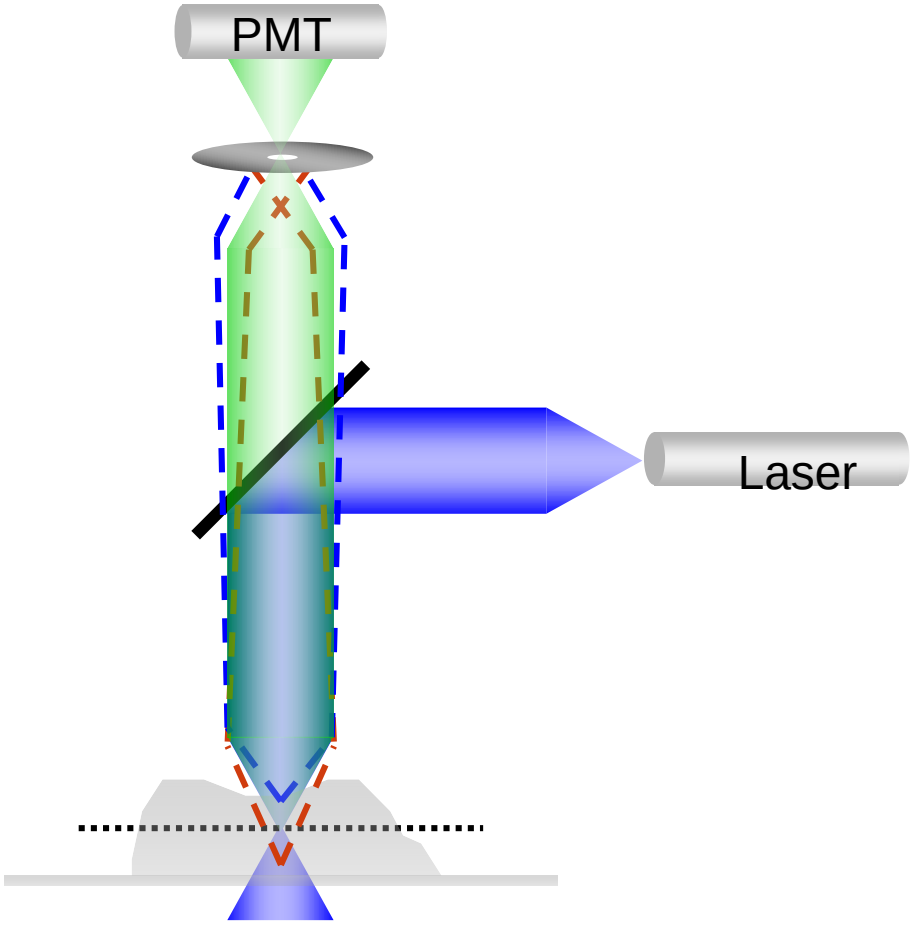


633nm Alexa Flour 647 / Cy5

针孔

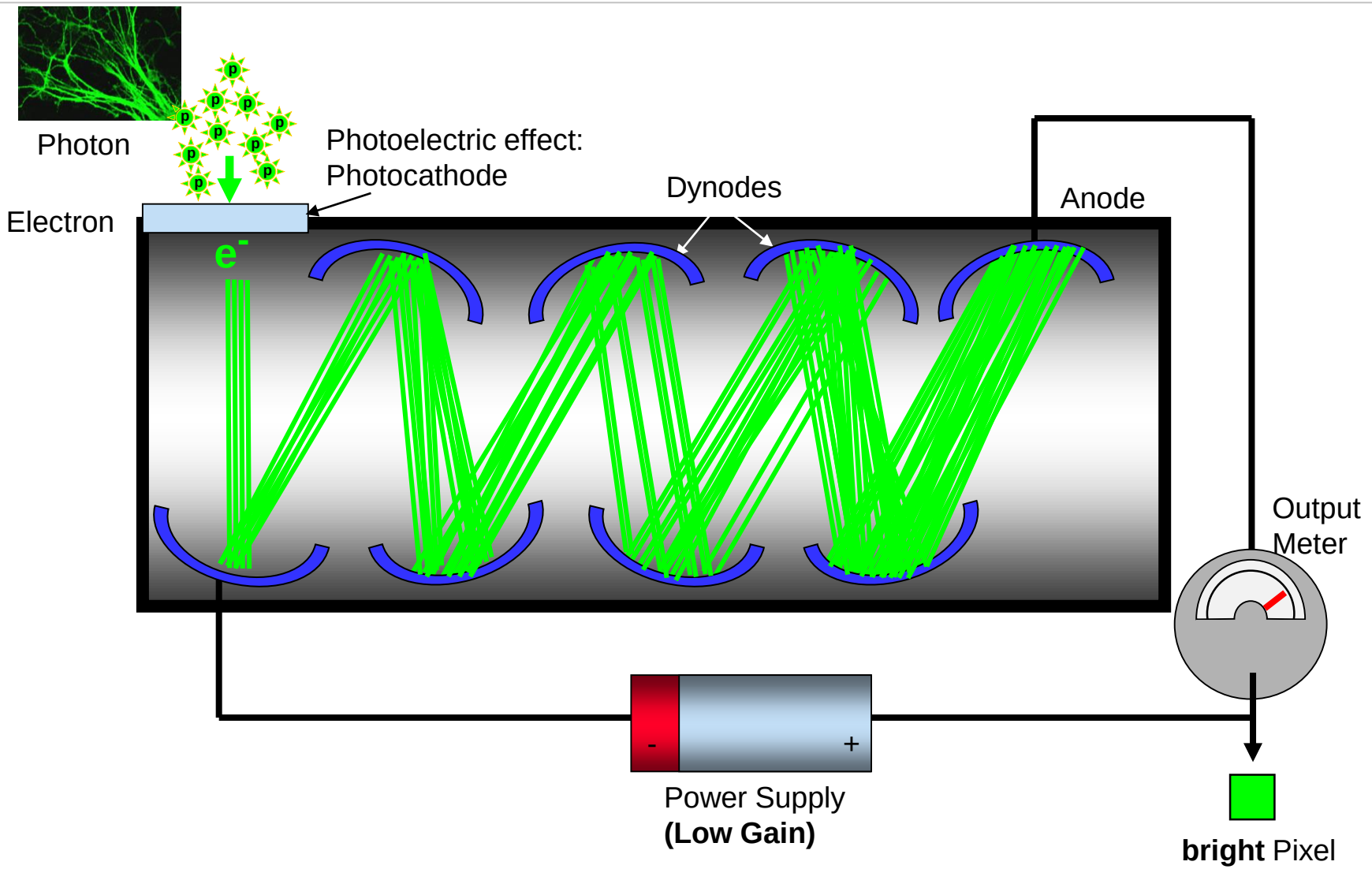
- 屏蔽非焦平面信号
  - 针孔增加，光通量增加，非焦平面信号增加
  - 针孔减小，光通量减小，非焦平面信号减小
- 影响光切厚度
  - 针孔增加，光切厚度增大，Z轴分辨率变差
  - 针孔减小，光切厚度减小，Z轴分辨率增加

一般情况，针孔大小为1AU



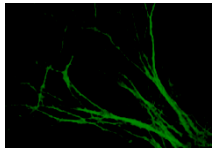
PMT Detectors / Photomultiplier Tube （光电倍增管）

Assuming a bright sample



PMT Detectors / Photomultiplier Tube

Assuming a dark sample



Photon

Electron

Photoelectric effect:
Photocathode

Dynodes

Anode

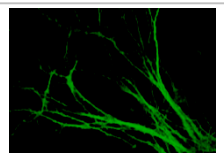
Output
Meter

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

-

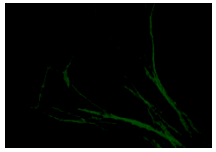
+

Power Supply
(Higher Gain)

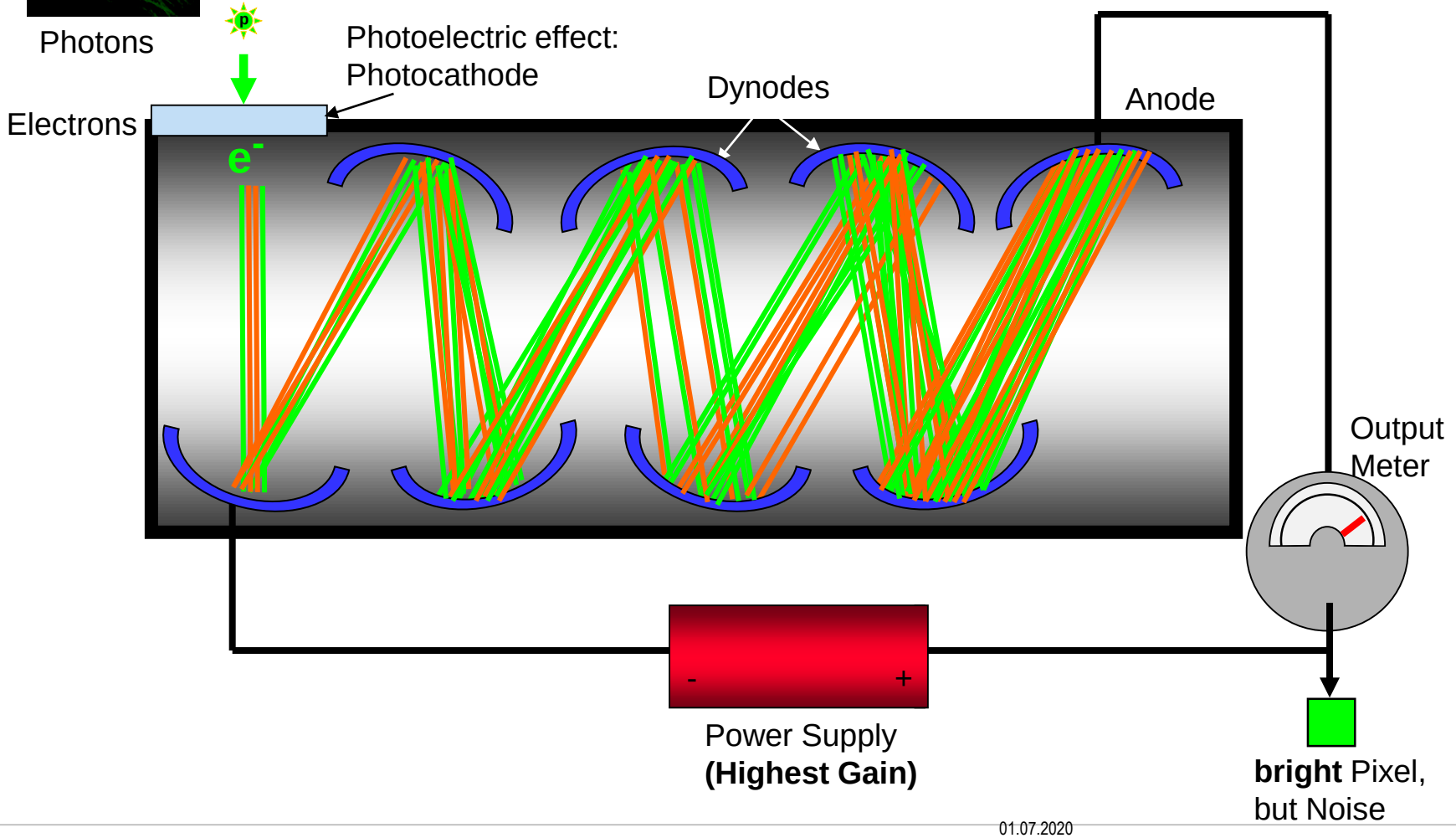
bright Pixel

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



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什么是高质量的图片?

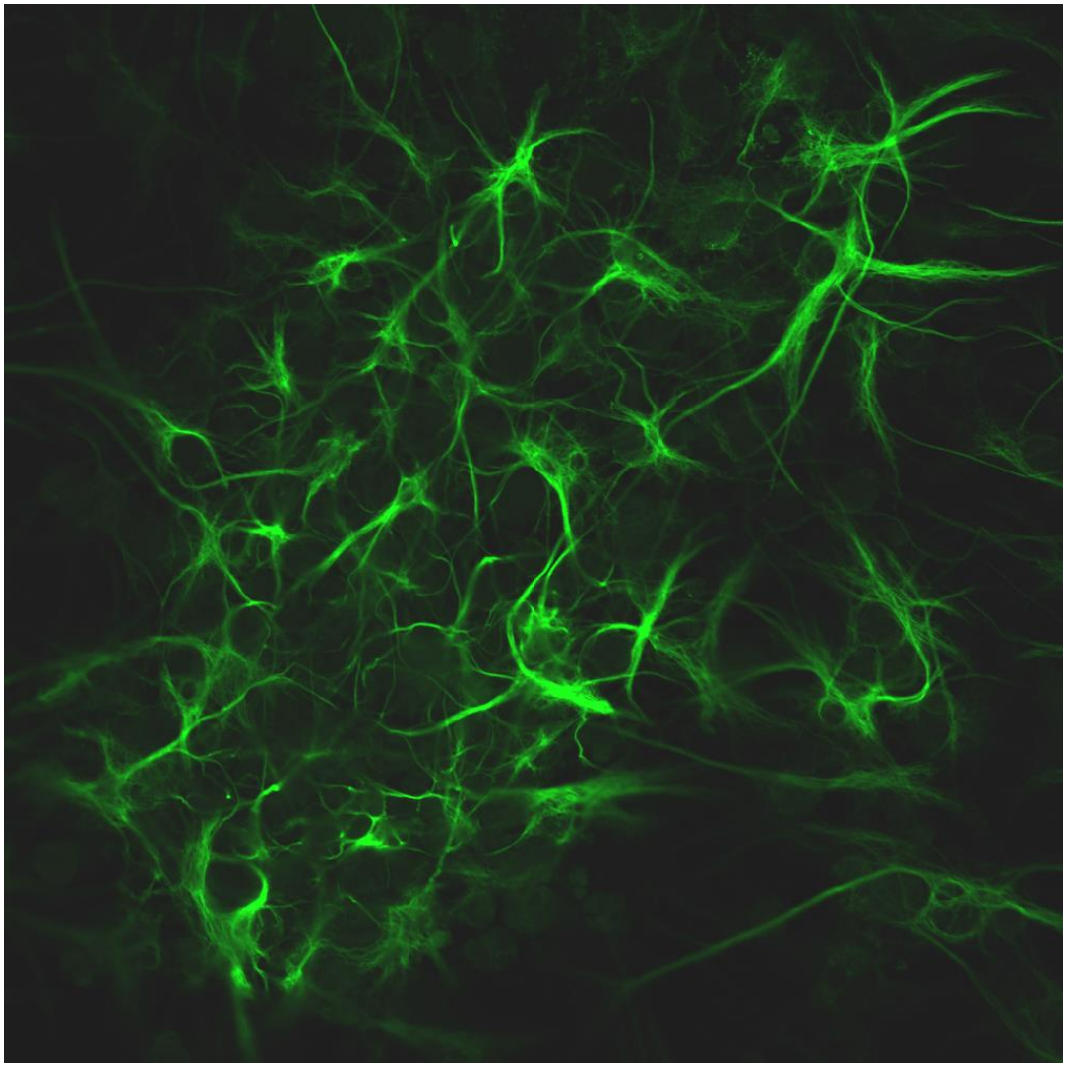
当我们谈到图像质量，我们说的是……

信号强度
信噪比
分辨率

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)

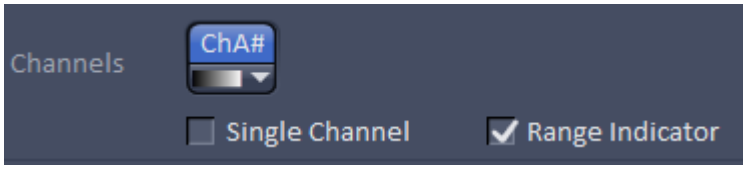
Grey value 256

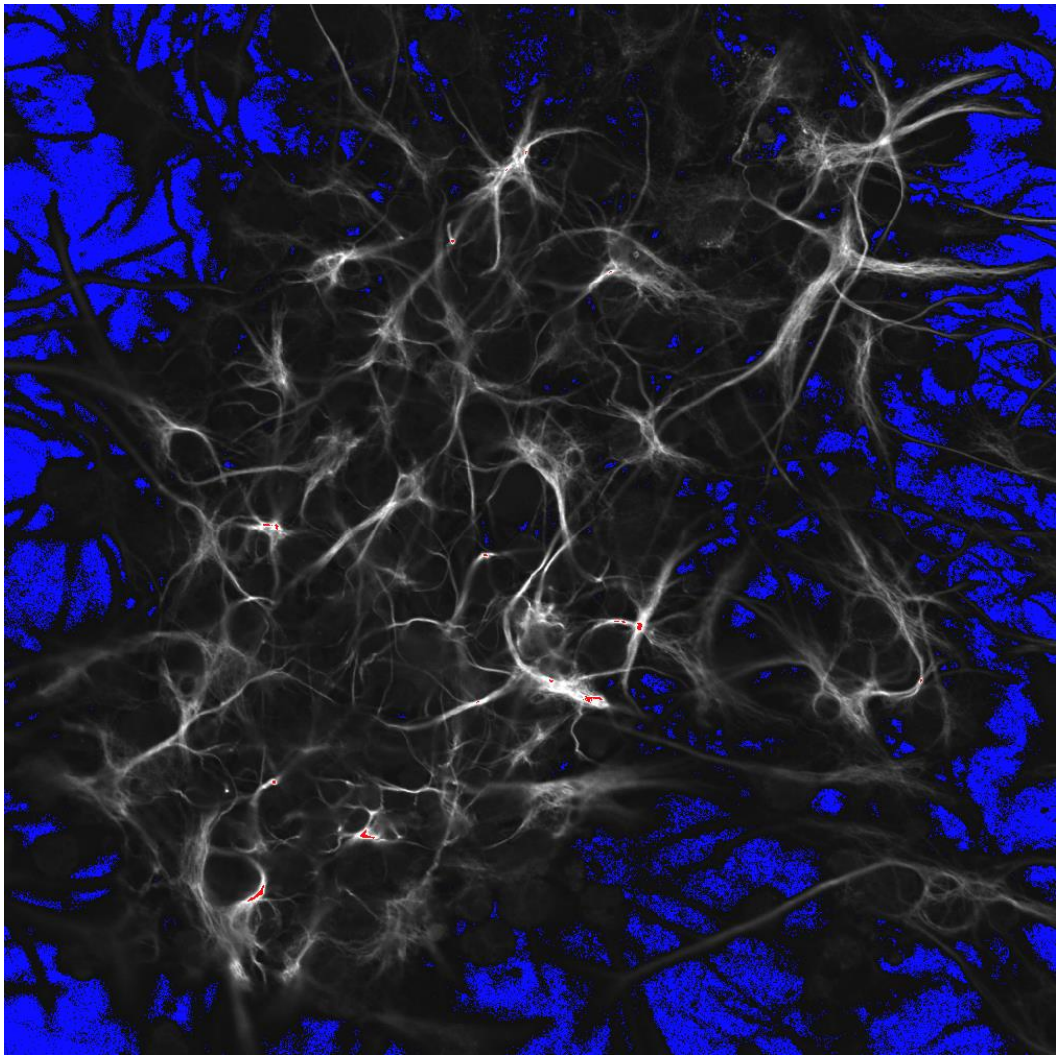
White

Grey values
1-255

Black

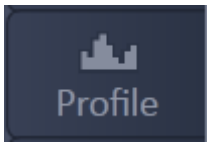
Grey value 0



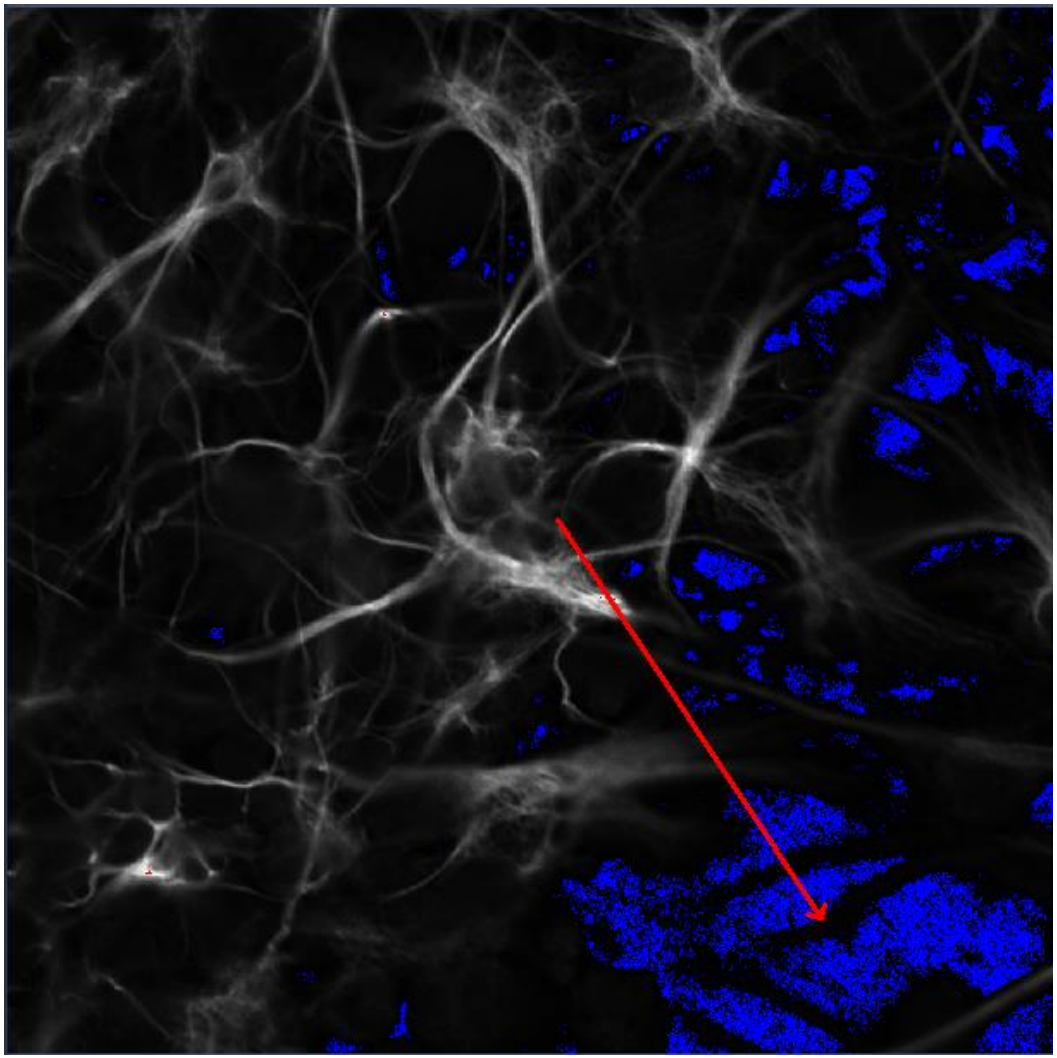


Profile

How to measure the dynamic range



= gives measurements of grey values along a line



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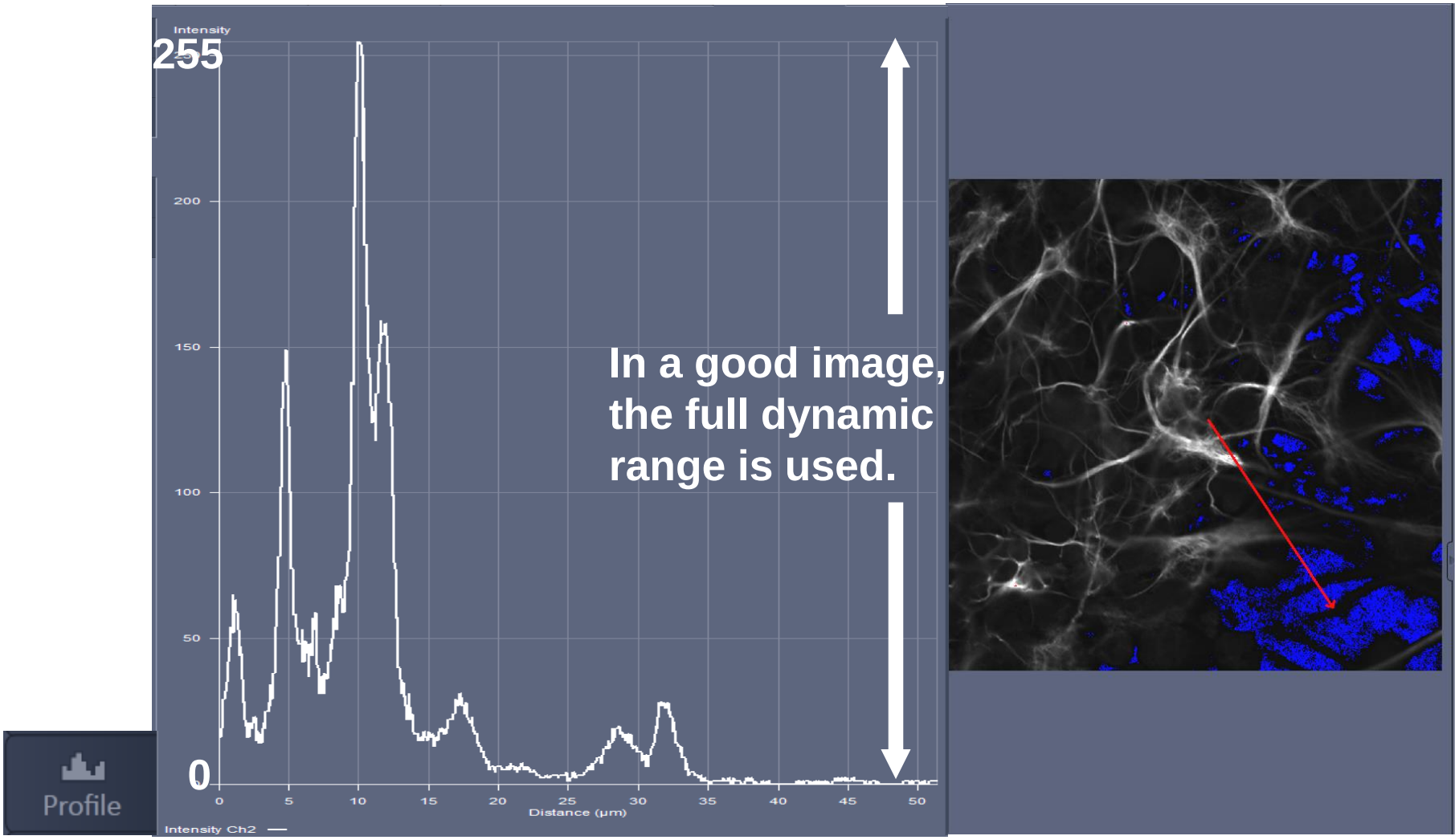
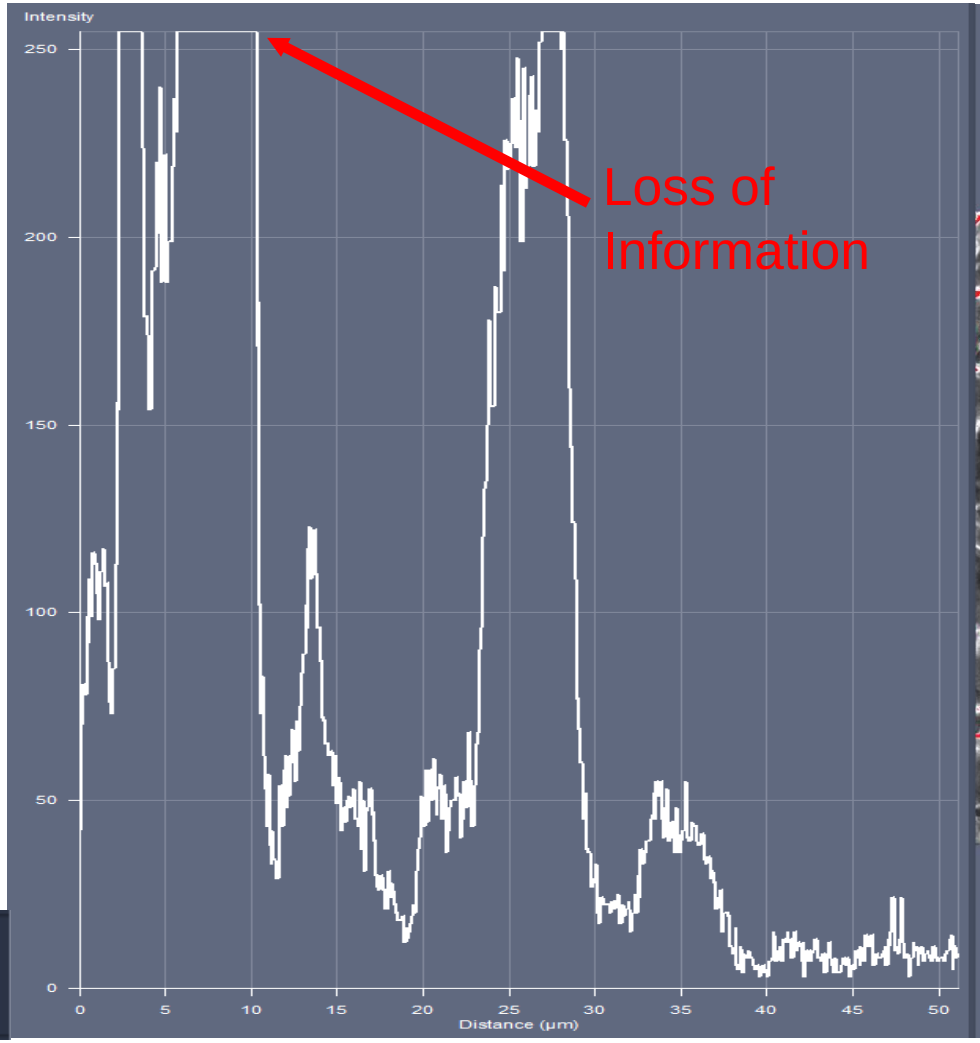
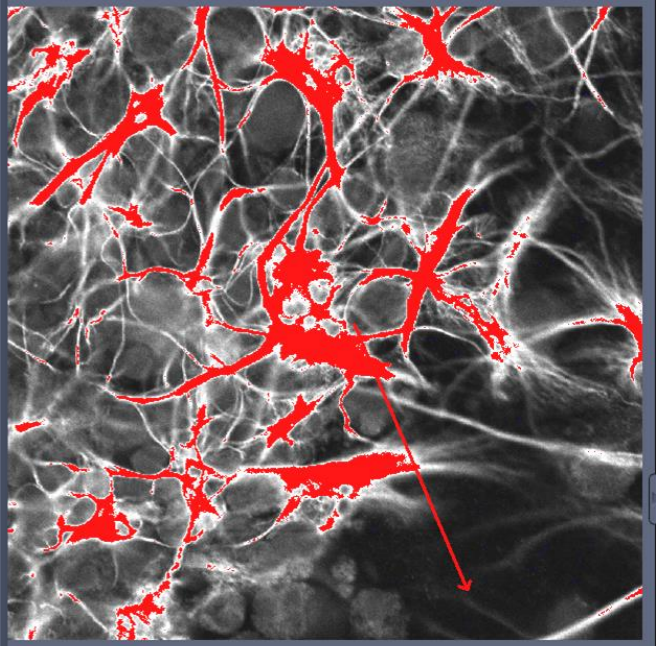


Gain (Master)

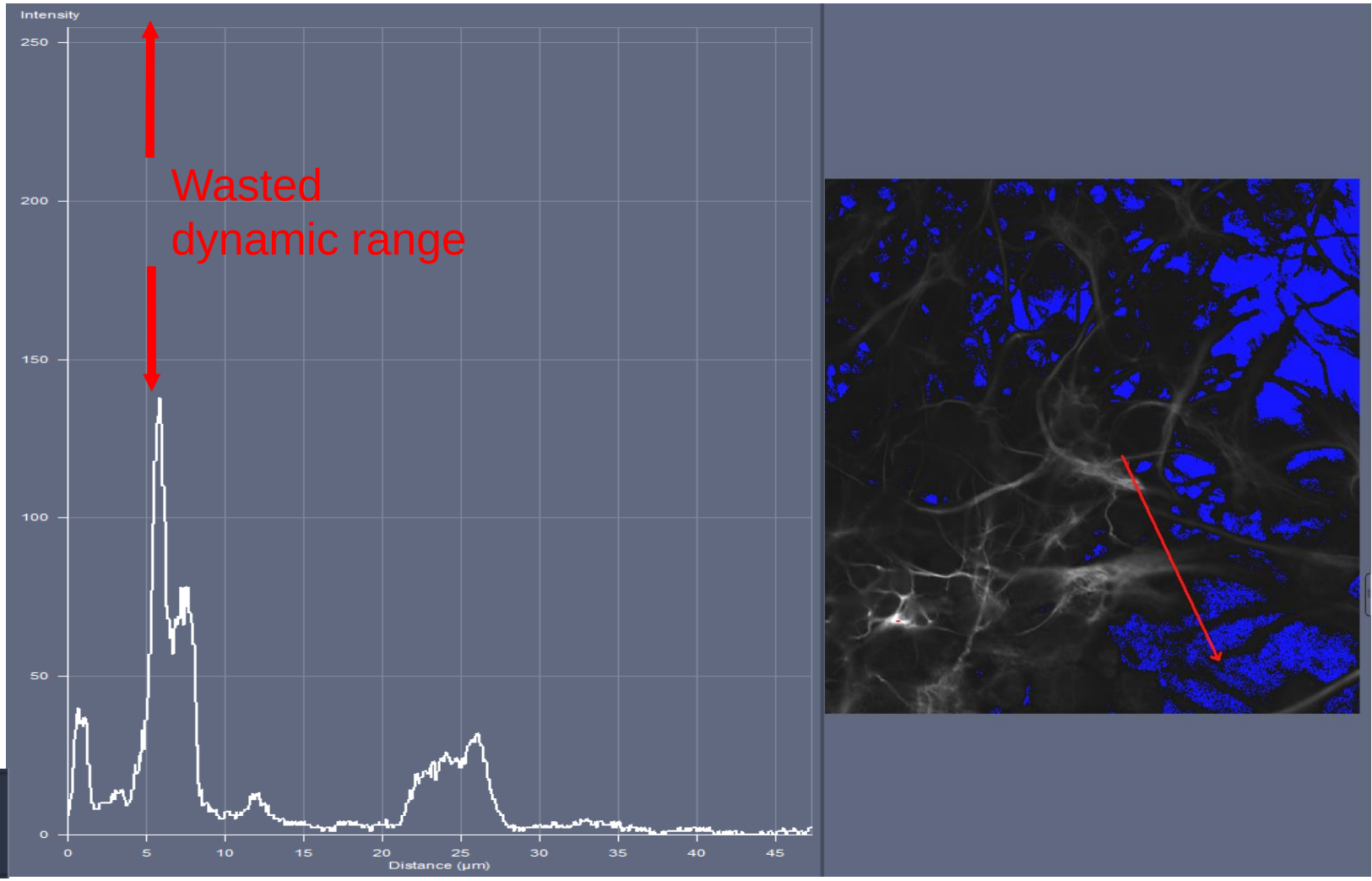
Set too high





Gain (Master)

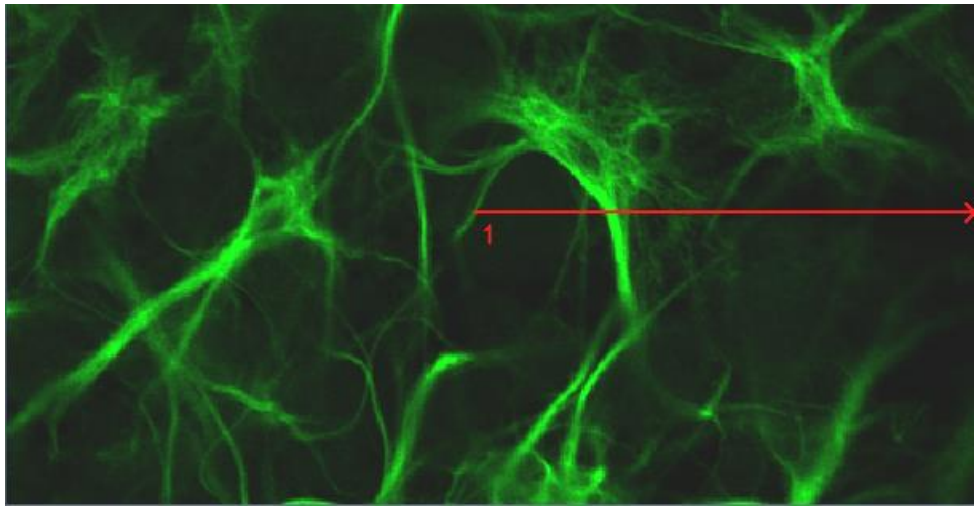
Not enough



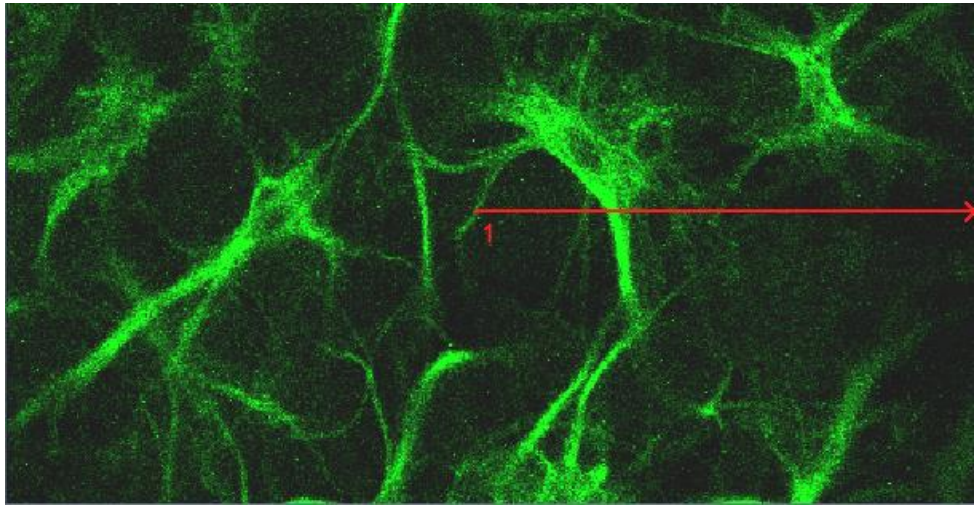
Scanning Strategies

Image Noise: What does it look like?

“Good” Image



“Bad” Image



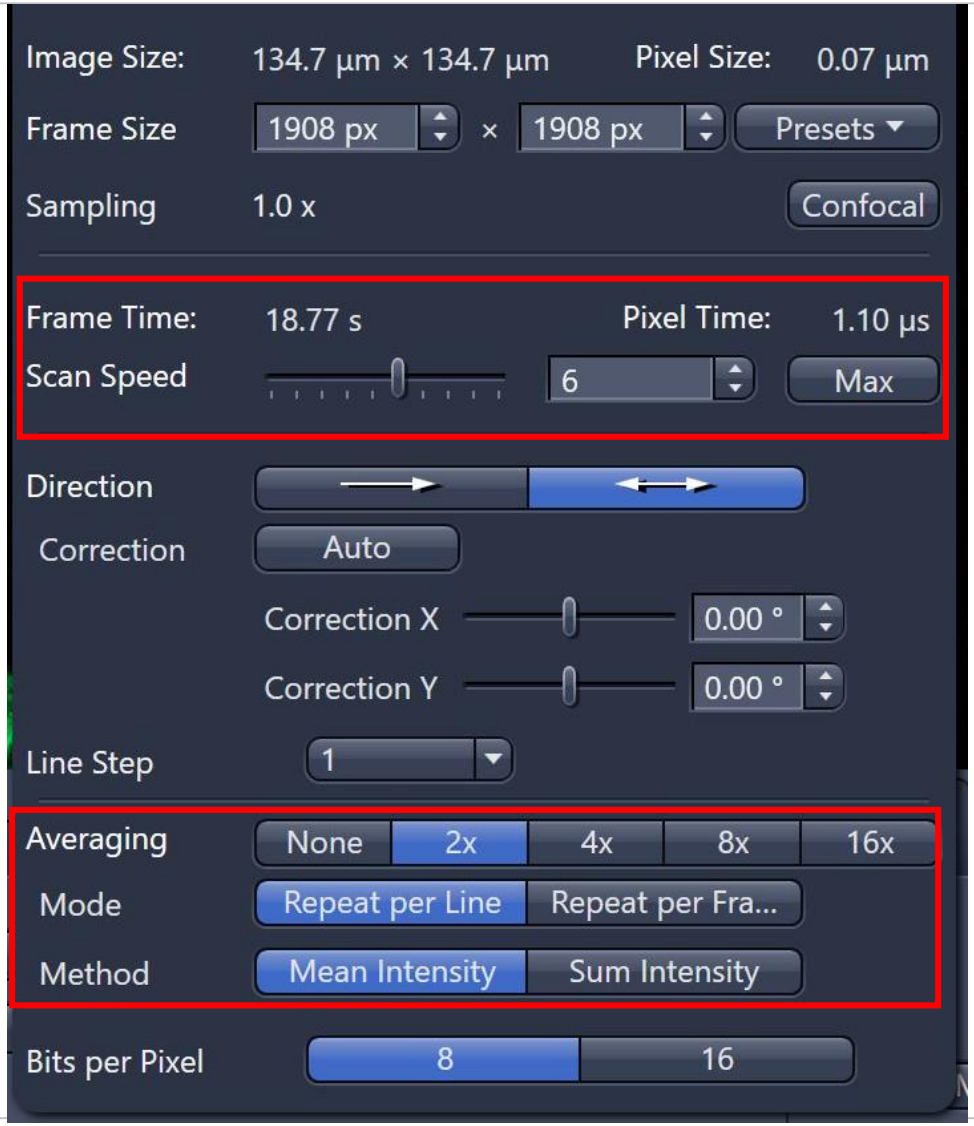
Scanning Strategies

Speed and Averaging

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

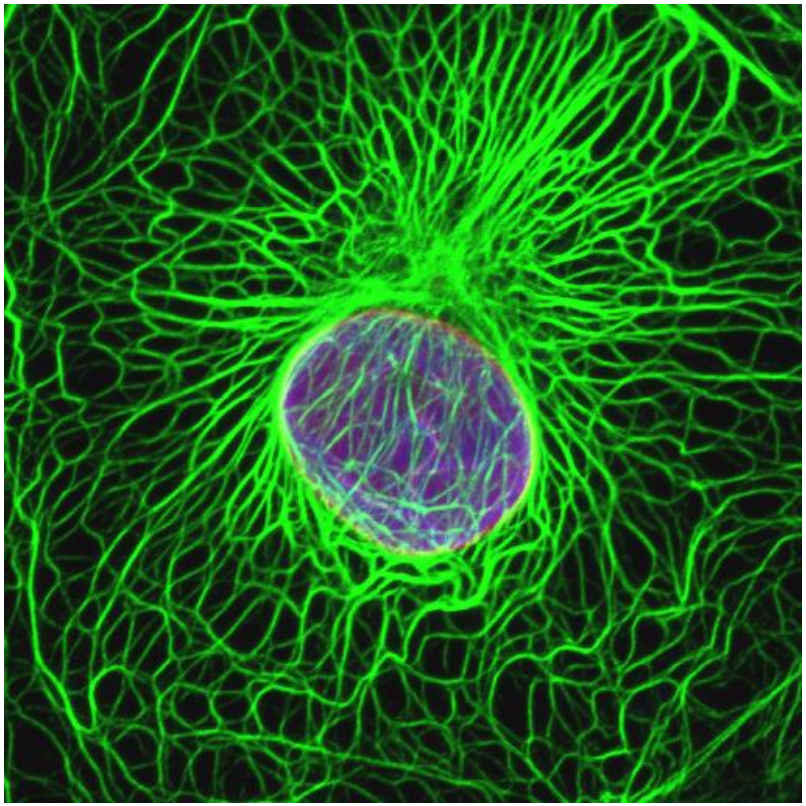
1) **Slower Scan Speed**

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

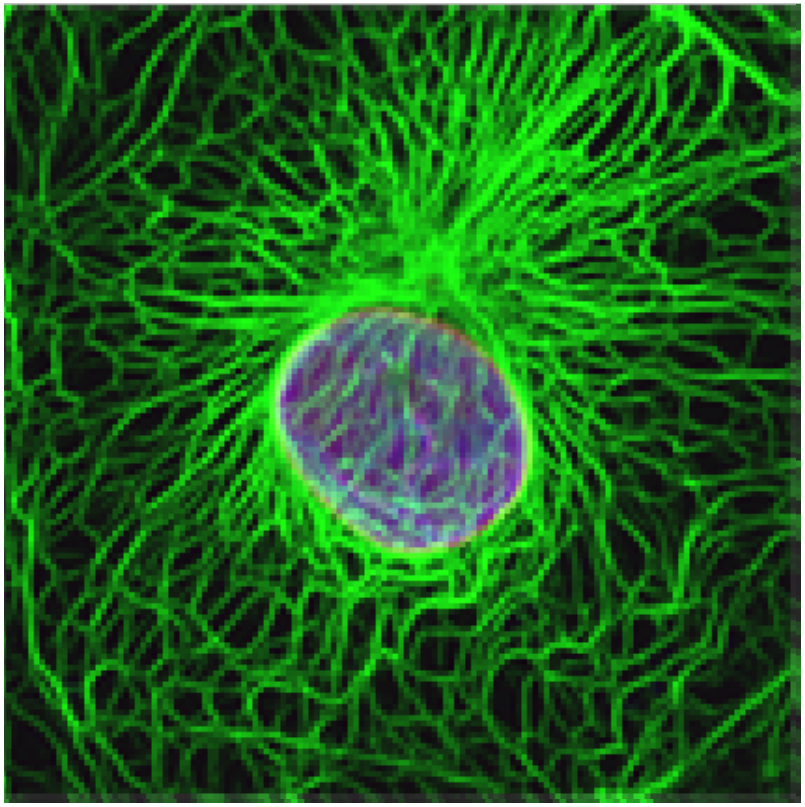


Hunting for Details?

Choose the right Resolution



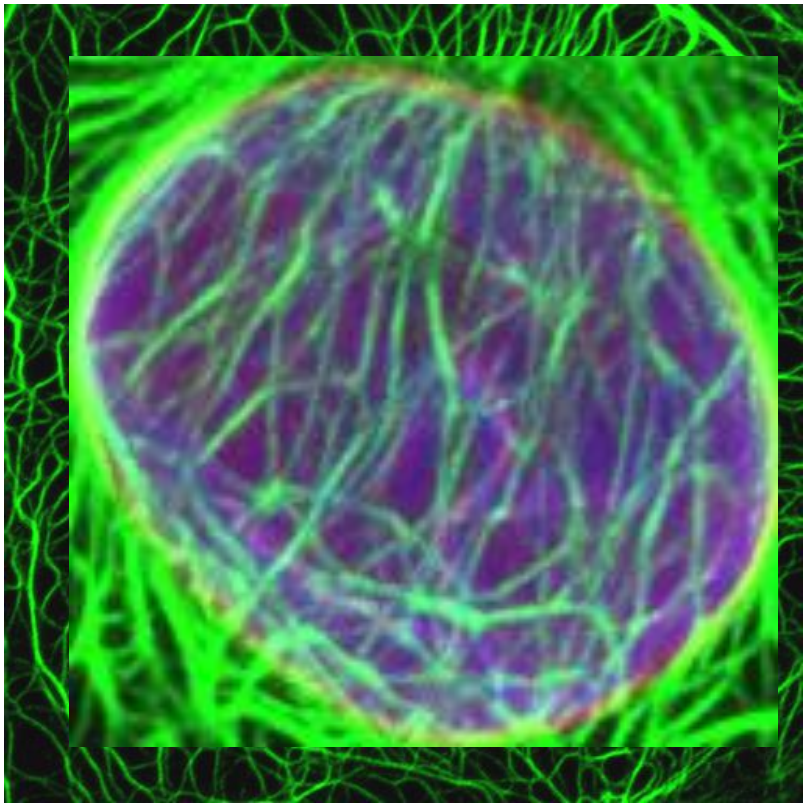
Resolution okay



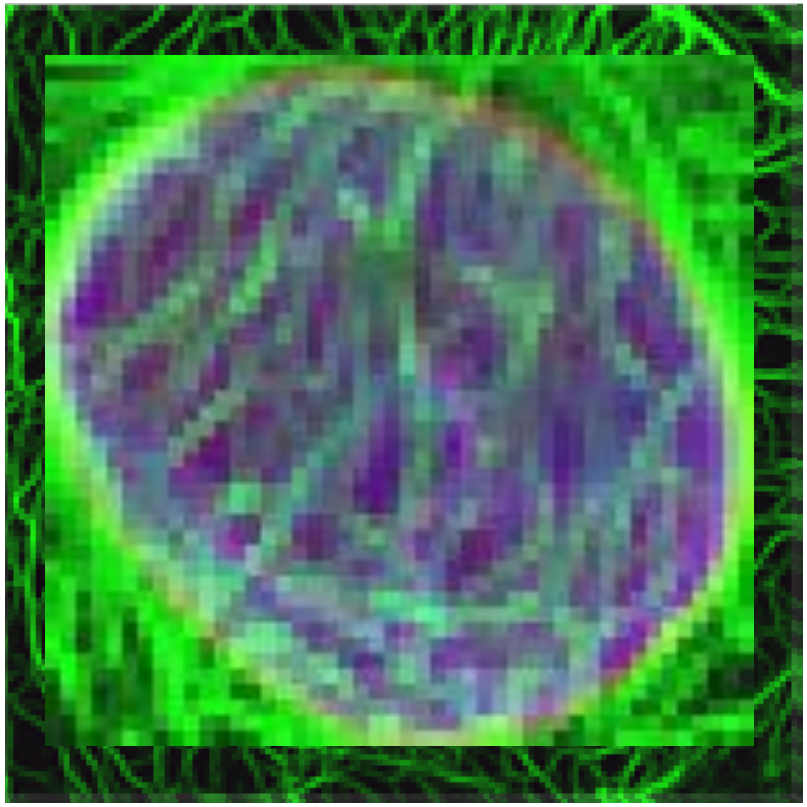
Resolution too low

Hunting for Details?

Choose the right Resolution



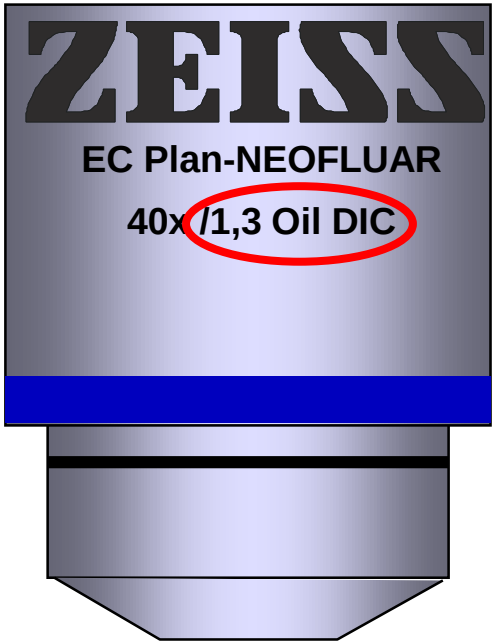
Resolution okay

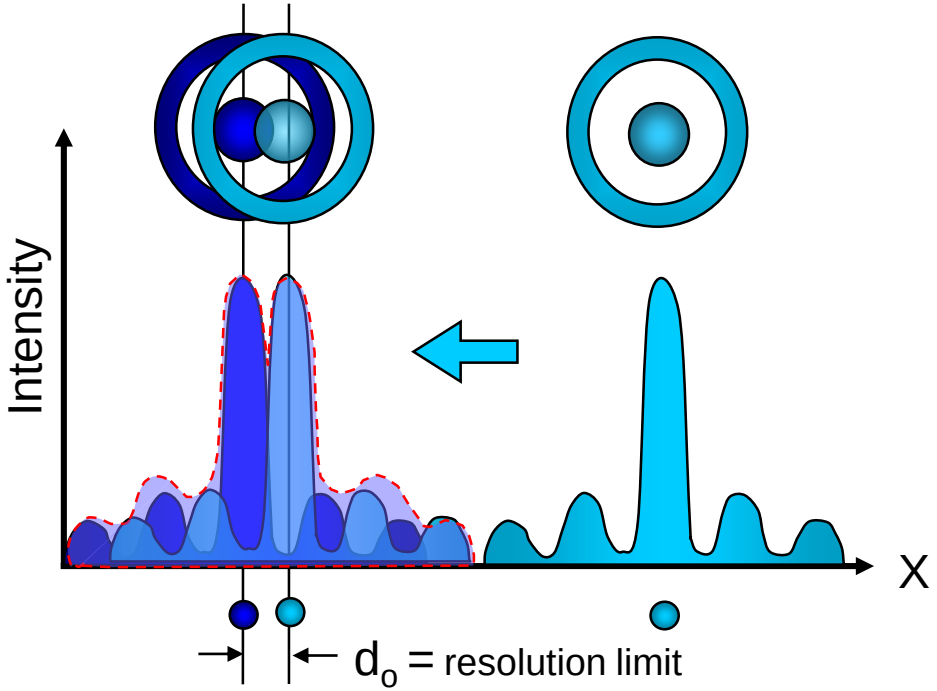


Resolution too low

Resolution: Point Spread Function

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



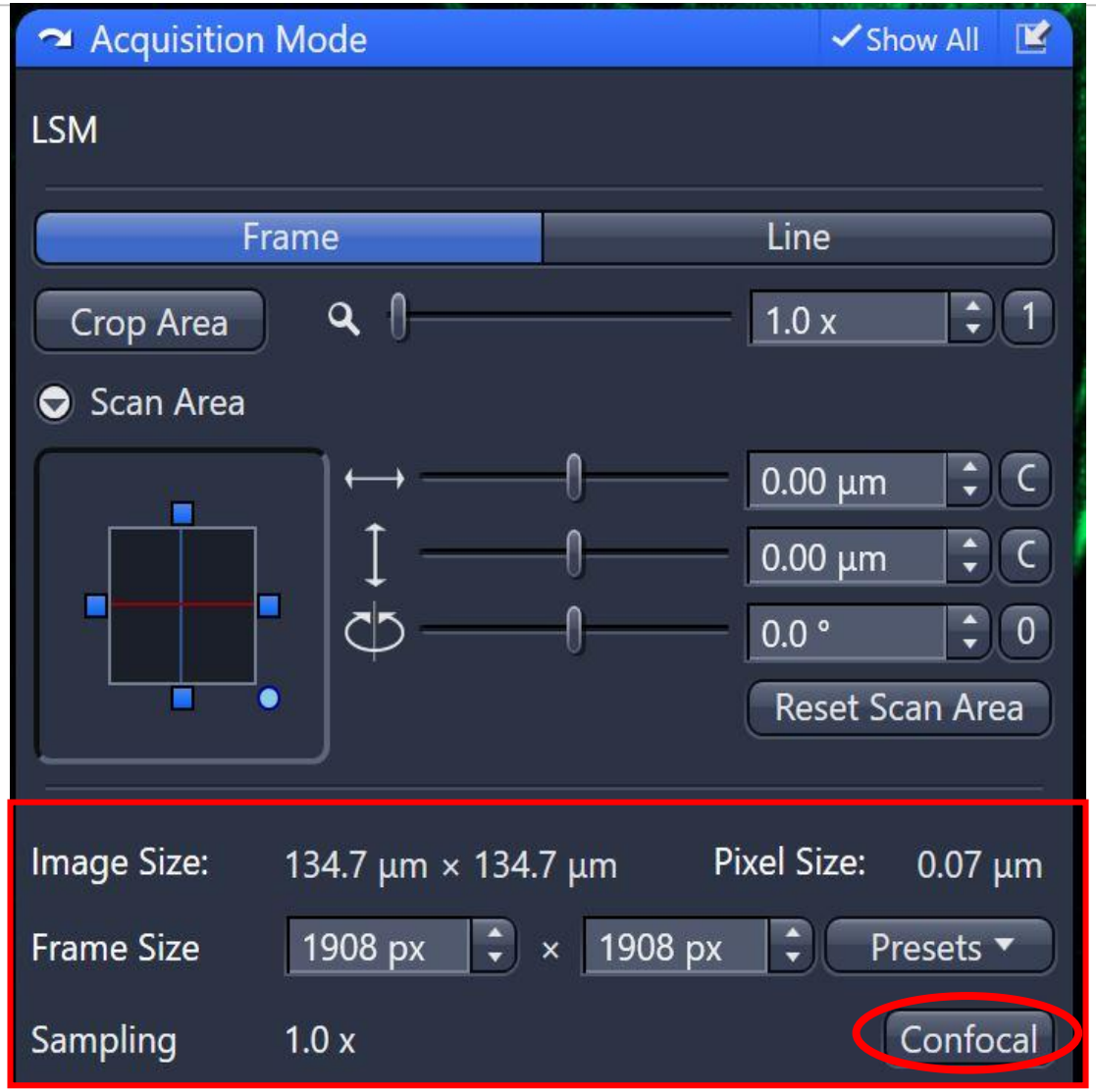


$$FWHM_{i11,lat} = \frac{0.51 * \lambda_{em}}{NA}$$

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

Resolution

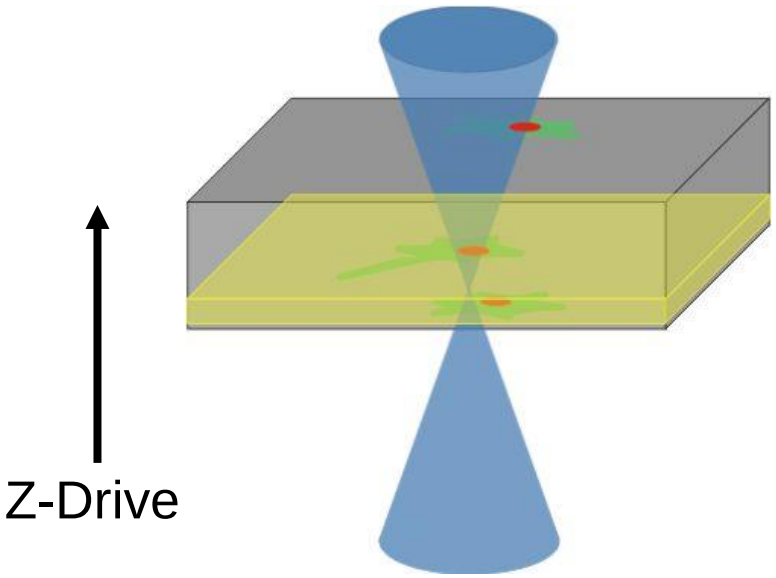
Information given in the Software

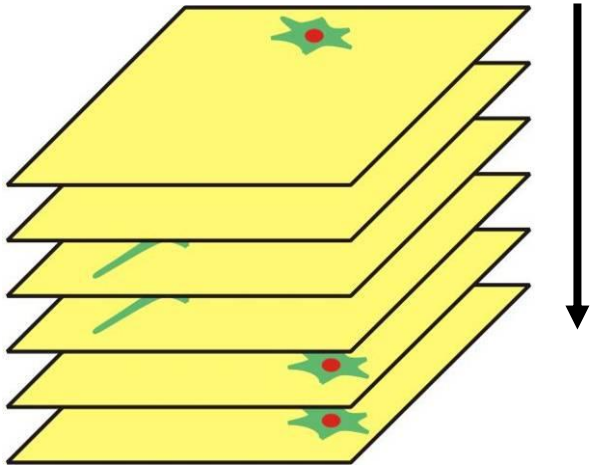


Confocal Imaging

From Image to 3D Information

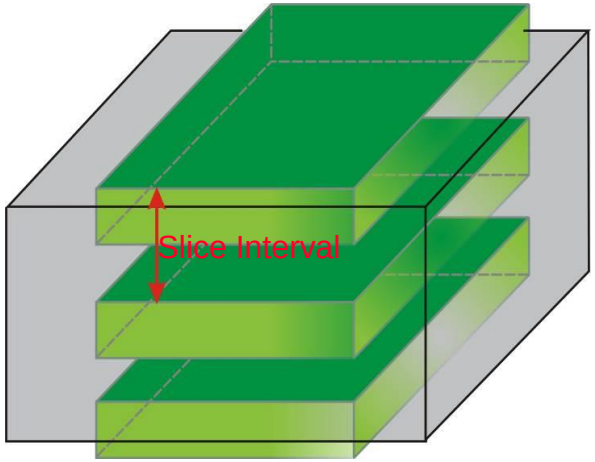
How is a X/Y/Z Stack produced?



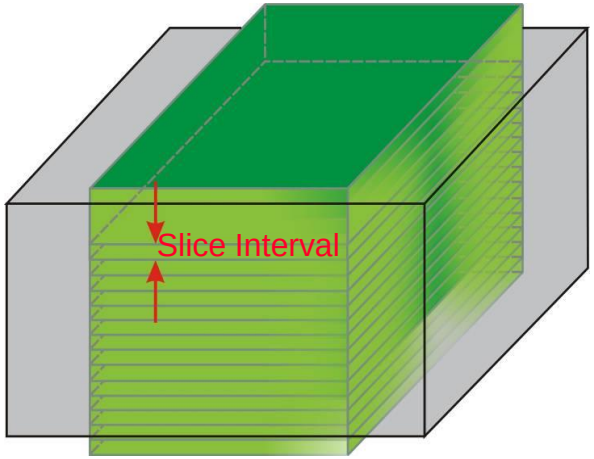


Optical Slice Thickness

Overlap between Optical Slices



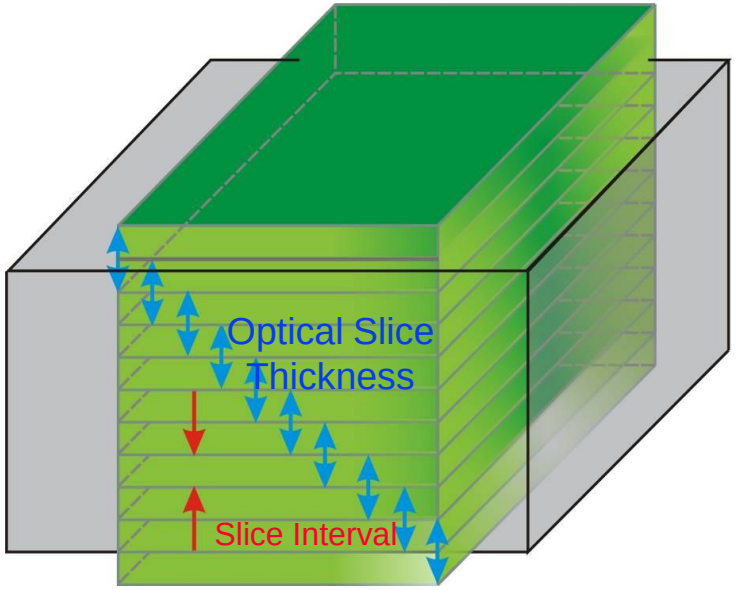
With this setting, the object structures between the slices cannot be detected.

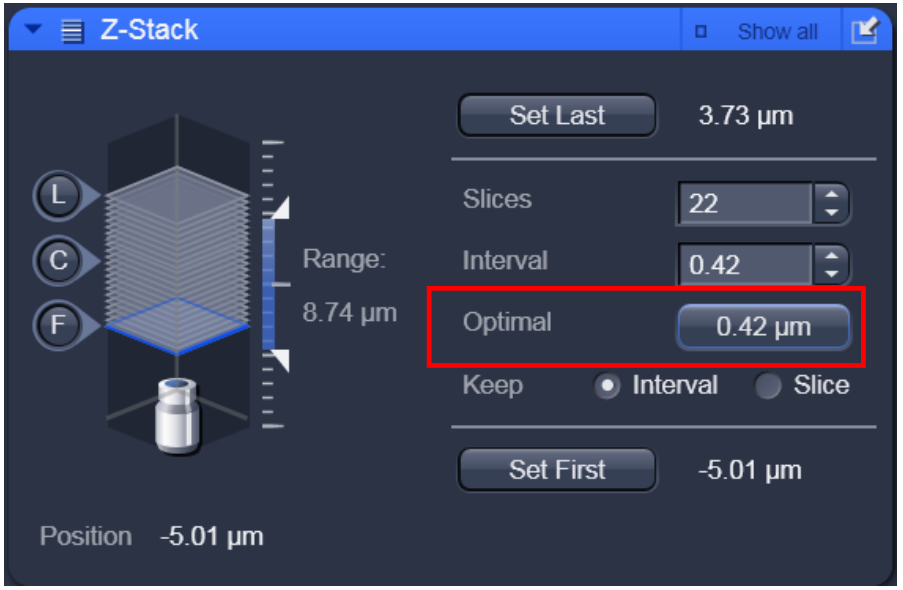


At very small intervals a lot of additional data without additional information is generated.

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).

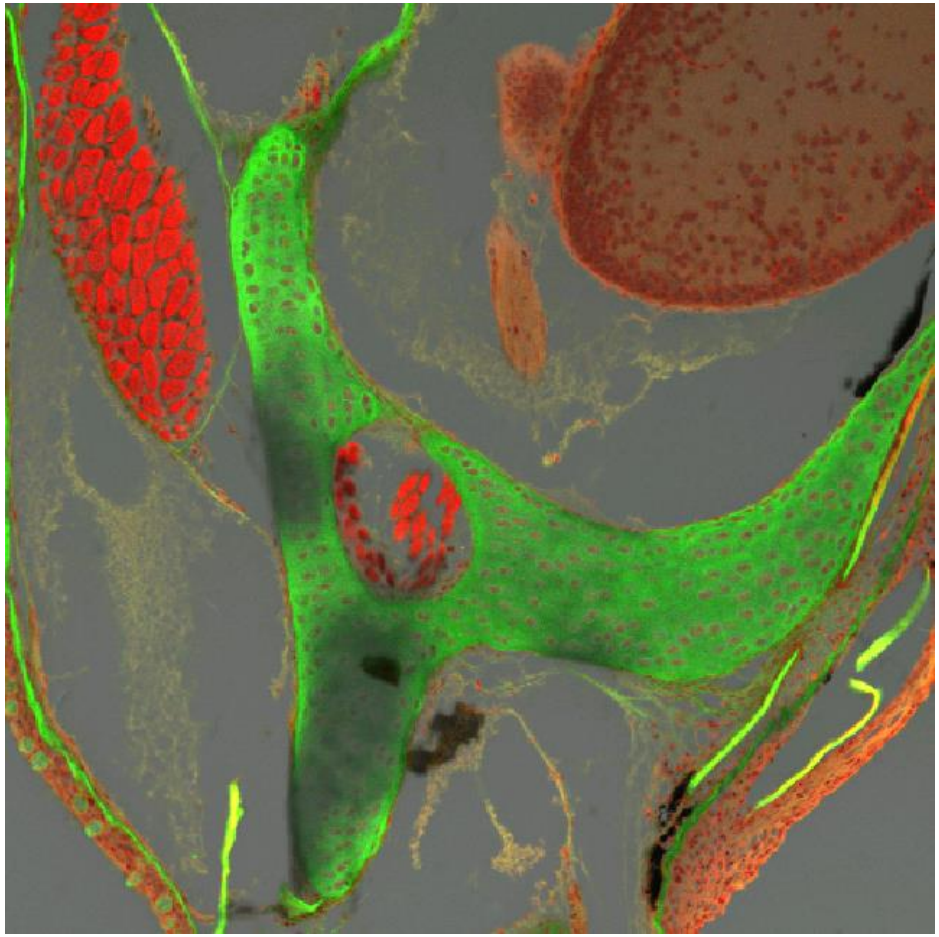
Agenda

1	激光共聚焦显微镜的原理
2	激光共聚焦显微镜的重要组成
3	如何获取一张高质量的图像
4	激光共聚焦显微镜的应用
5	Airyscan2 的成像原理

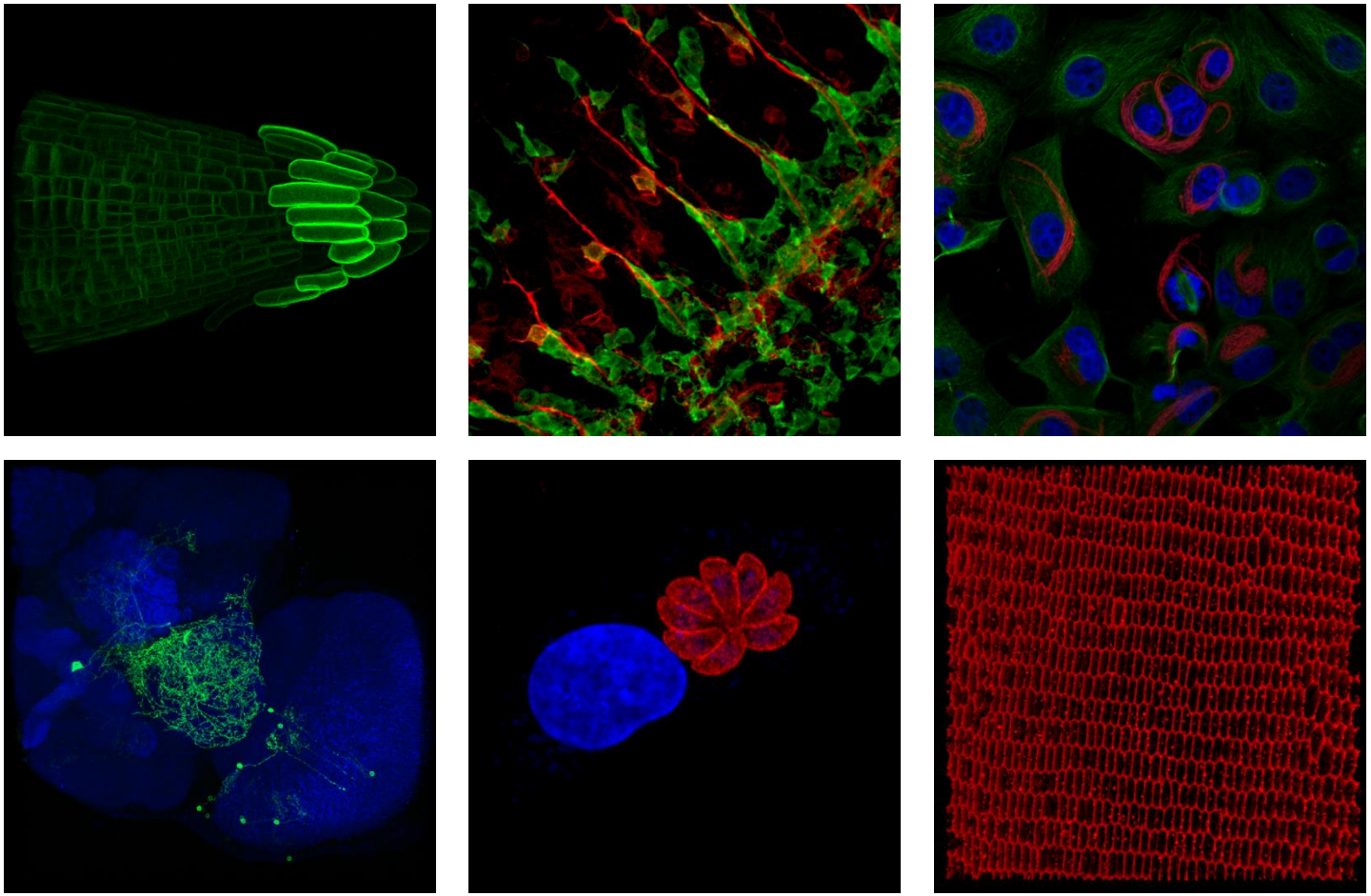
共聚焦广泛应用于各领域

Nearly all fields of Science...

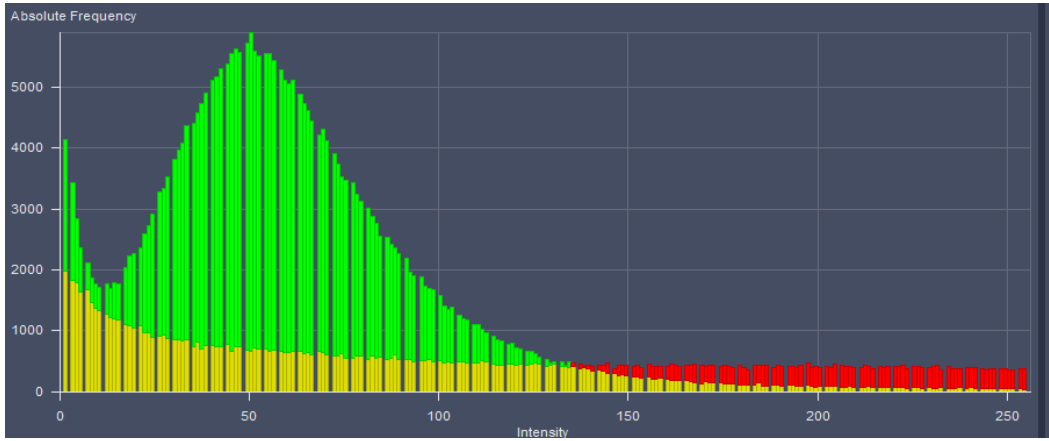
Agriculturual Research, Alzheimer, Cancer, Cell Science, Biochemistry, Botany, Immunology, Developmental Biology, Ecology, Epidemiological Deseases, Evolutionary Biology, Food design, Genetics, HIV, Material Quality Control, Material Sciences, Medicine, Membrane Research, Neurobiology , Parasitology,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

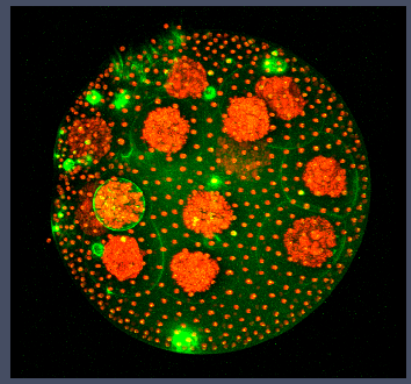


基本功能
拍摄高质量的单通道或多通道荧光照片



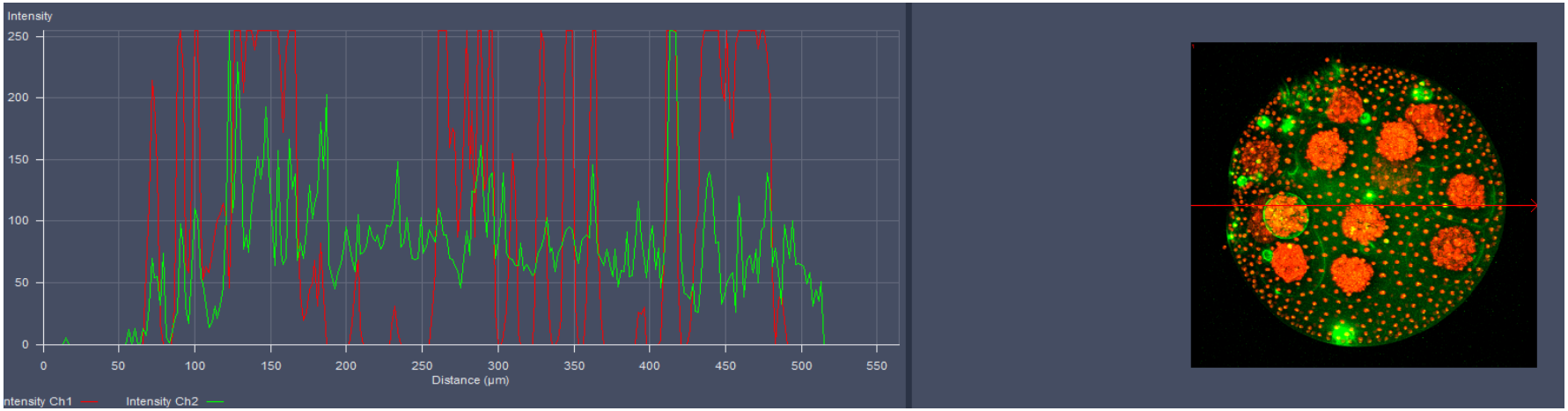
荧光强度的分析





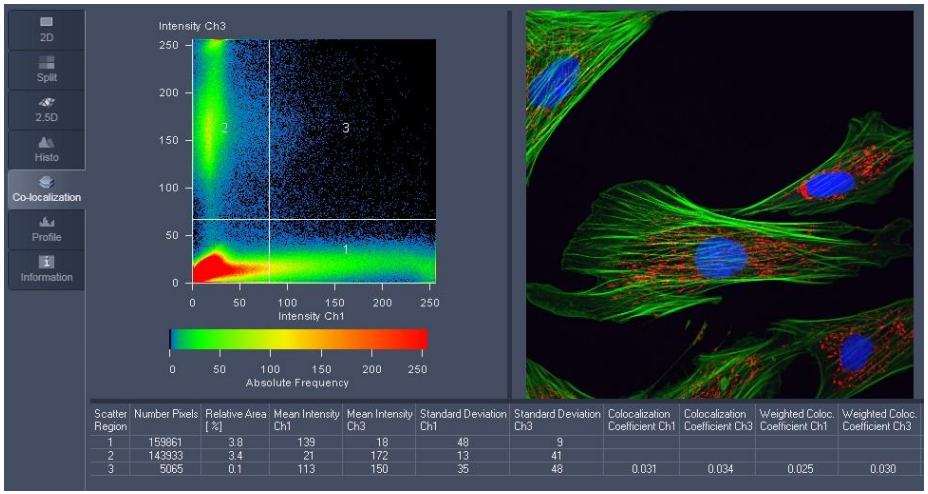
	Ch1	Ch2
Mean Intensity	43.45529	37.38946
Standard Deviation	84.16126	44.23883
Pixels	522900	522900
Area [µm x µm]	299038.87	299038.87

Profile功能
用户分析一条线上的荧光强度的分布



可用于分析药物刺激原生质体或者悬浮细胞在加药前后，某特定标记物的在细胞内外的分布

共定位Co-localization
两种荧光信号的共定位定量分析



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

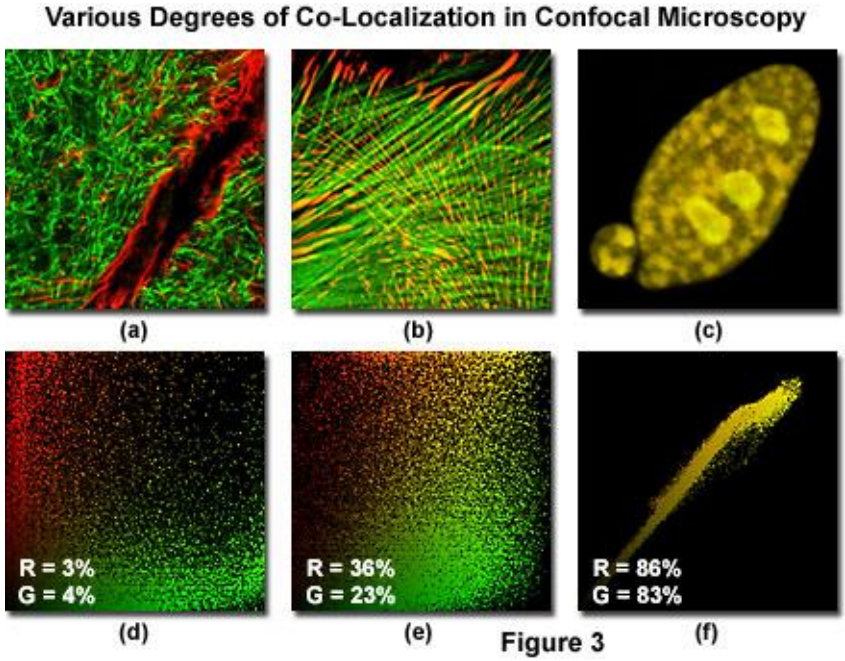
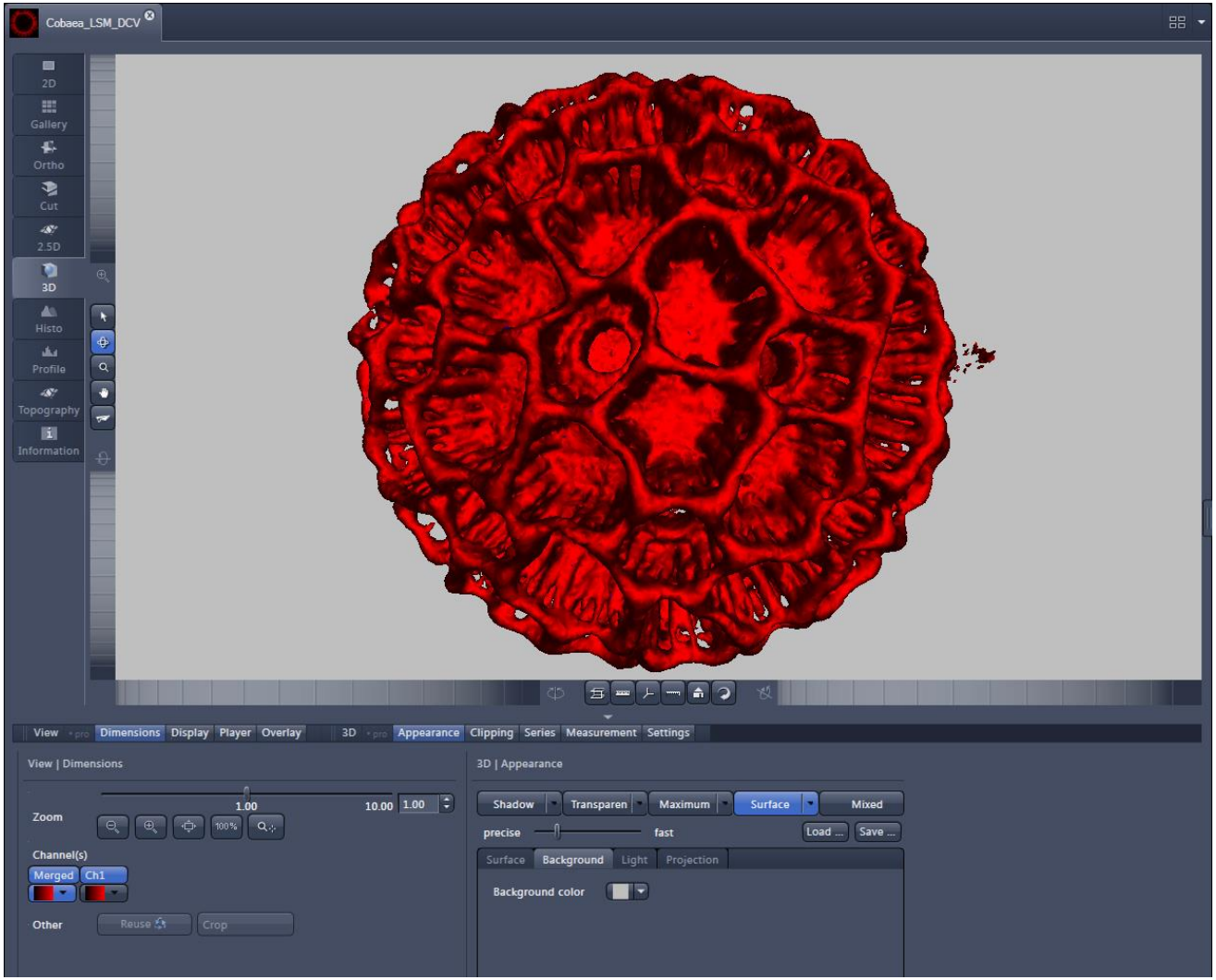


Figure 3

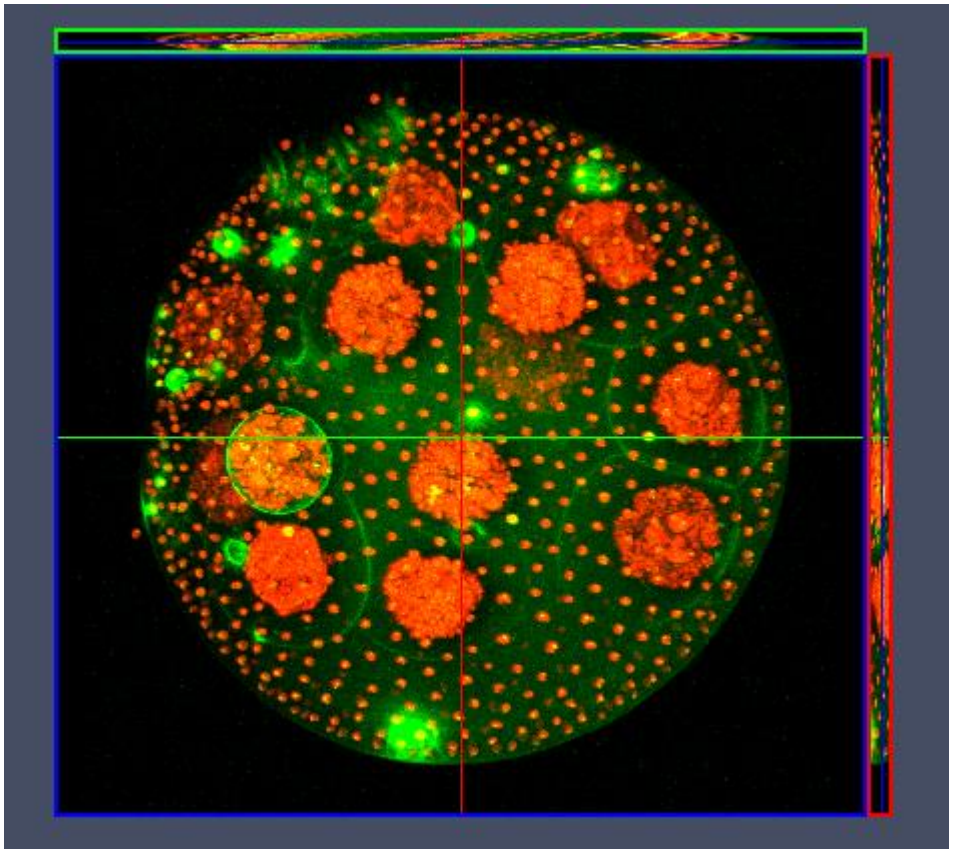
直接显示所选区域荧光信号的共定位系数

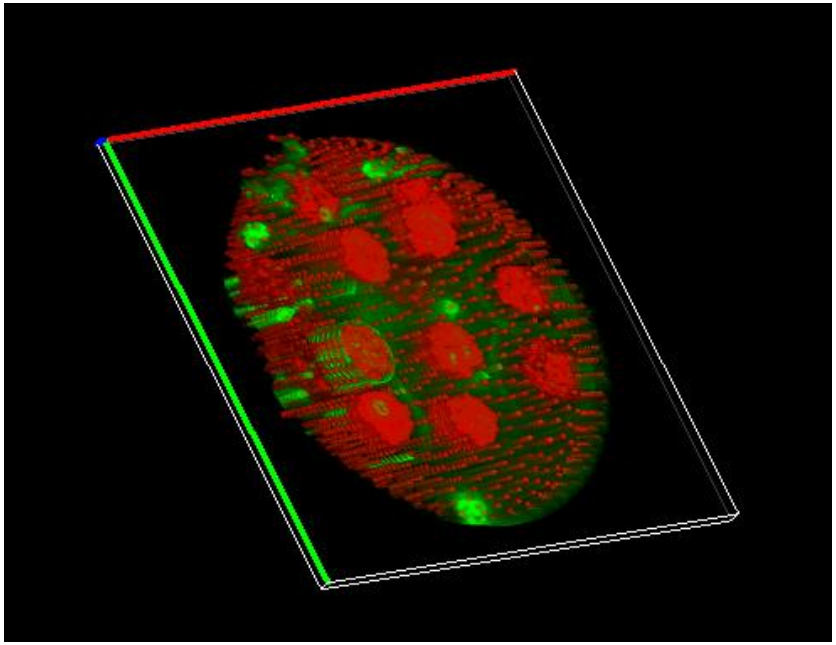
$$R_r = \frac{\sum_i (S1_i - S1_{aver}) \bullet (S2_i - S2_{aver})}{\sqrt{\sum_i (S1_i - S1_{aver})^2 \bullet \sum_i (S2_i - S2_{aver})^2}}$$

3D功能：Z-Stack
三维结构分析和测量 (需3D模块)

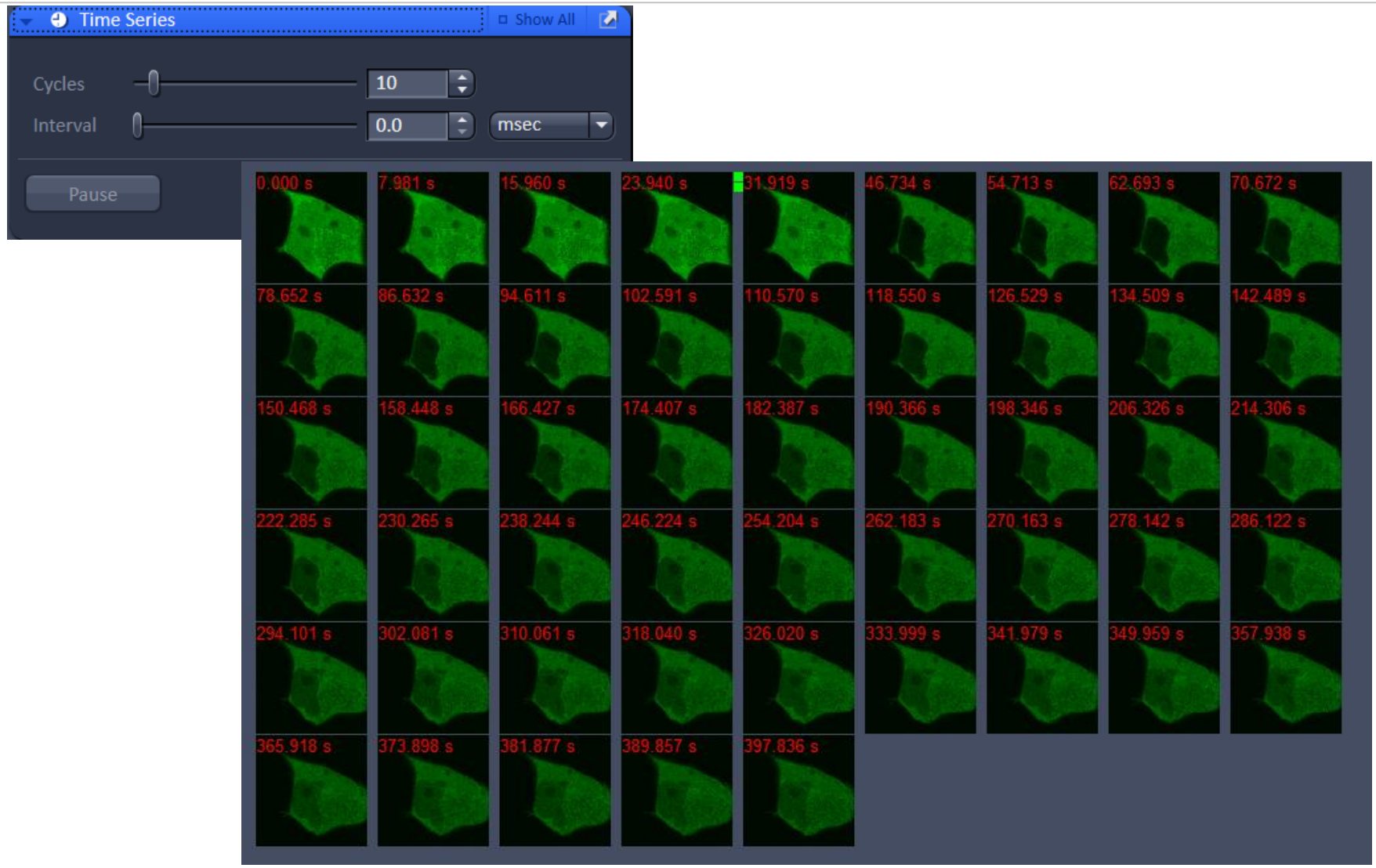


3D功能：Z-Stack
三维结构分析和测量 (需3D模块)



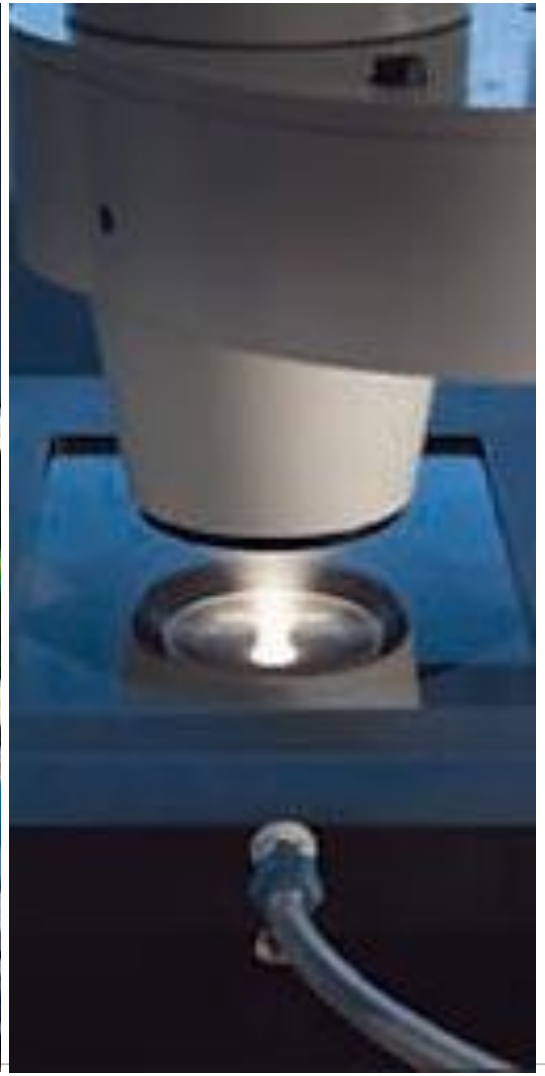


时间序列


Time Series
Show All
Cycles
10
Interval
0.0
msec
Pause
0.000 s
7.981 s
15.960 s
23.940 s
31.919 s
46.734 s
54.713 s
62.693 s
70.672 s
78.652 s
86.632 s
94.611 s
102.591 s
110.570 s
118.550 s
126.529 s
134.509 s
142.489 s
150.468 s
158.448 s
166.427 s
174.407 s
182.387 s
190.366 s
198.346 s
206.326 s
214.306 s
222.285 s
230.265 s
238.244 s
246.224 s
254.204 s
262.183 s
270.163 s
278.142 s
286.122 s
294.101 s
302.081 s
310.061 s
318.040 s
326.020 s
333.999 s
341.979 s
349.959 s
357.938 s
365.918 s
373.898 s
381.877 s
389.857 s
397.836 s

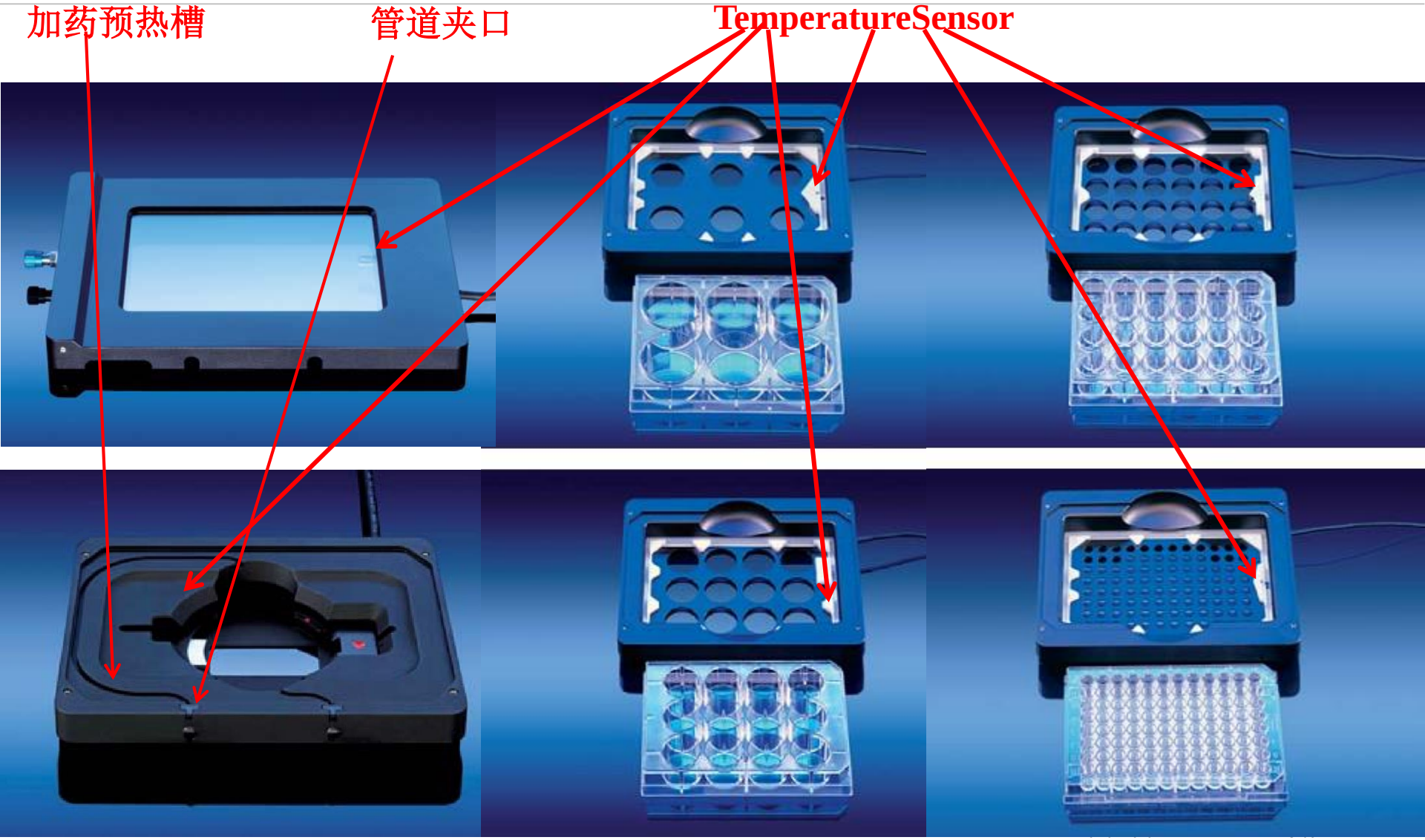
扩展功能： 活细胞工作站 Incubator

活细胞培养和观察拍照



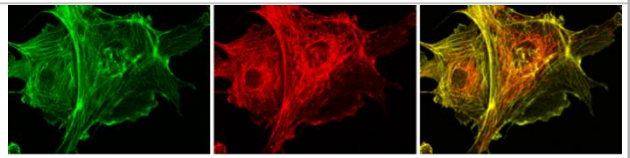
一体化整合的活细胞工作站

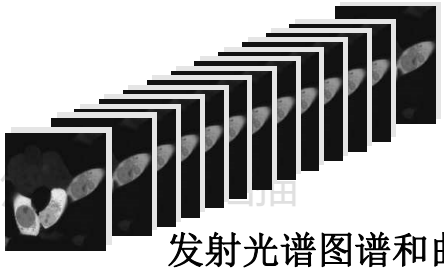
- 四通道温度精确控制，范围室温至 60° C，精度：0.01° C
- CO_2浓度设定范围：0-8%，精度：0.01



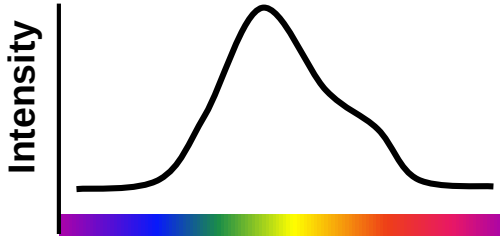
扩展功能： 光谱扫描，线性拆分

最大程度避免荧光串色的影响

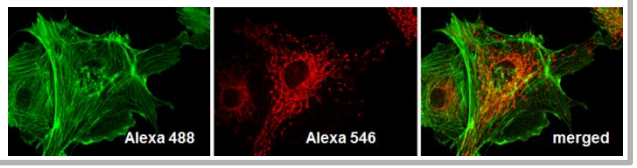




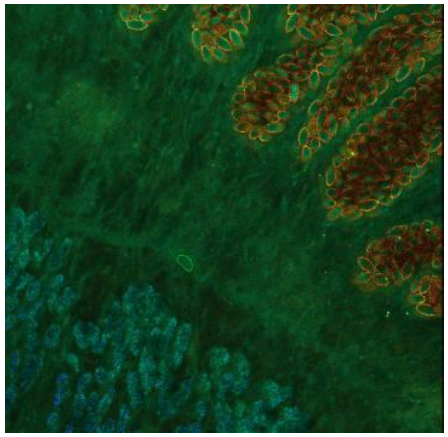
发射光谱图谱和曲线



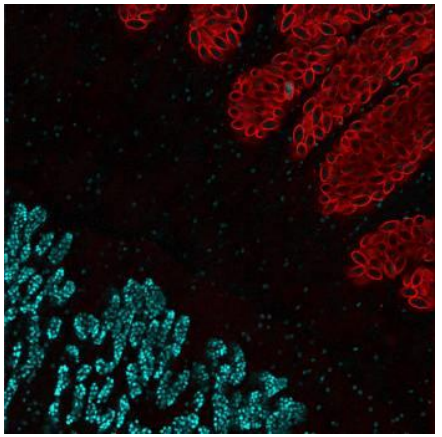
Unmixing

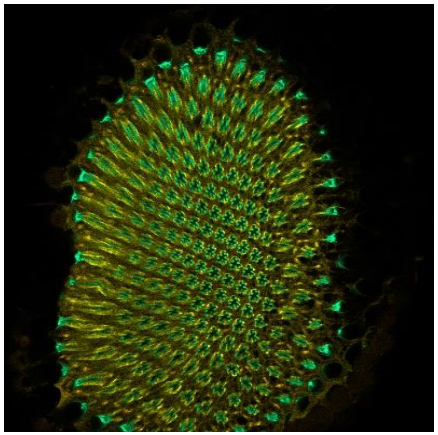


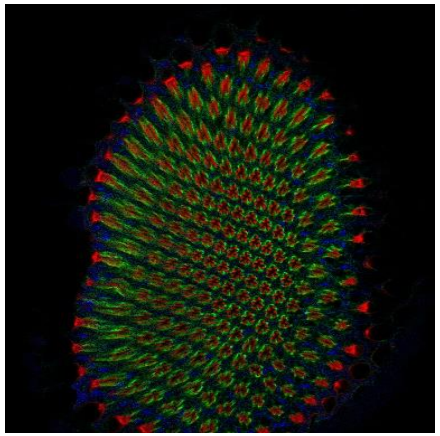
光谱扫描和拆分之前



光谱扫描和拆分之后





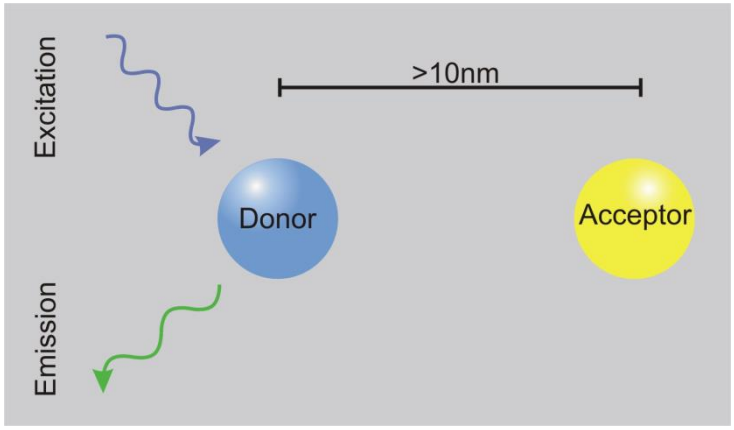


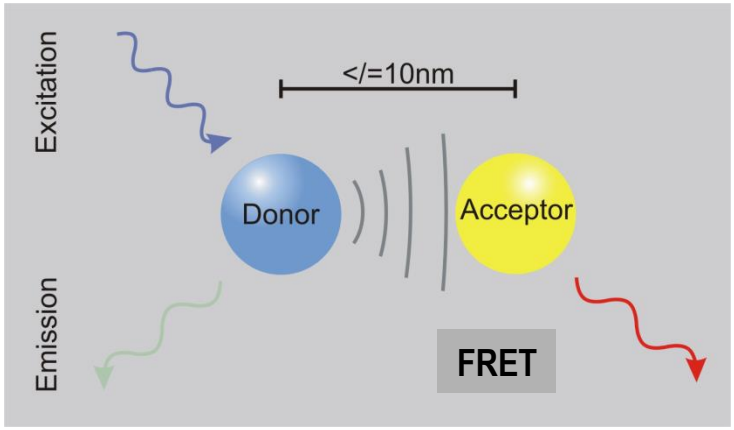
扩展功能：Fluorescence Resonance Energy Transfer
荧光共振能量转移

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

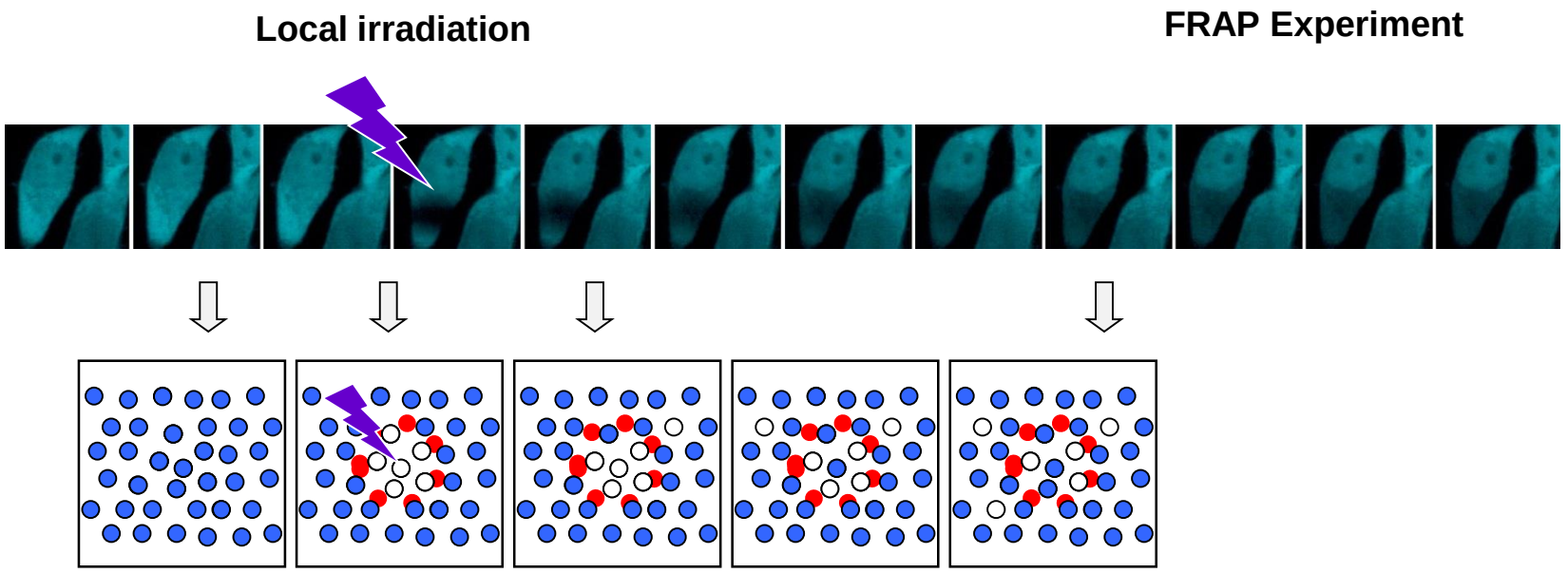




常用的FRET组合：

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

扩展功能：Fluorescence Recovery After Photobleaching
荧光漂白后恢复实验



对分子的移动性进行量化

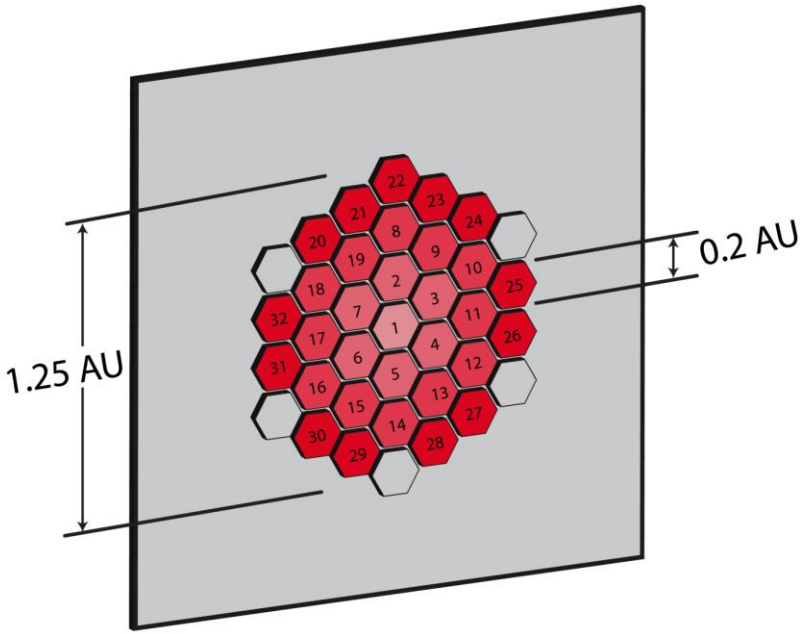
Agenda

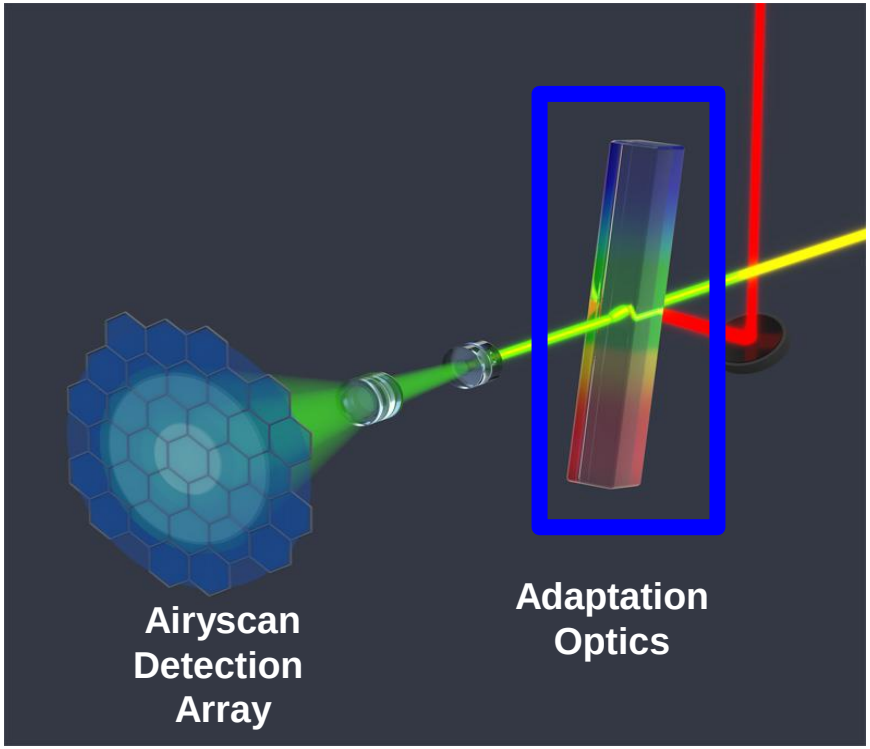
1	激光共聚焦显微镜的原理
2	激光共聚焦显微镜的重要组成
3	如何获取一张高质量的图像
4	激光共聚焦显微镜的应用
5	Airyscan2的成像原理

Ariyscan 2

-----更高的分辨率、灵敏度和信噪比

- 一个 32 通道平面探测器
- 每个探测器元件0.2 AU
- 同时收集一个Airy斑的所有信号





横向分辨率（X/Y）： 120 nm

轴向分辨率（Z）： 350 nm

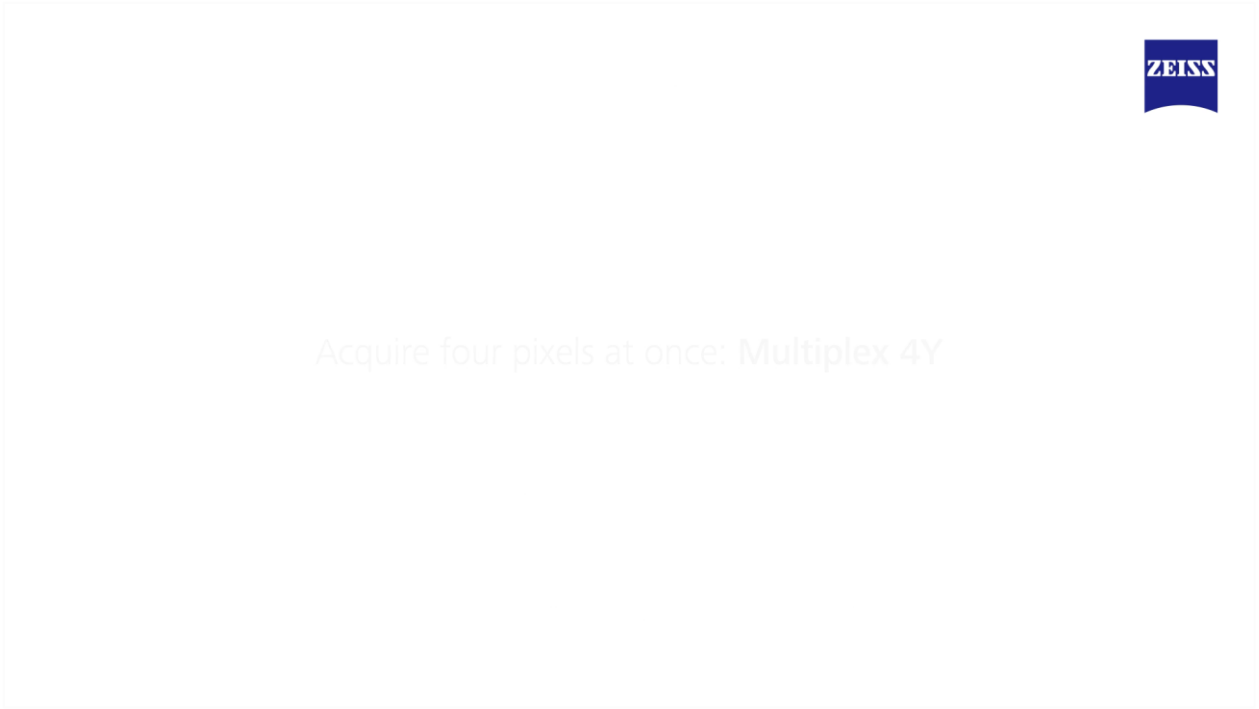
@488nm

Airyscan 2 灵活的Multiplex 模式
并行数据处理大大提高超高分辨率成像速度

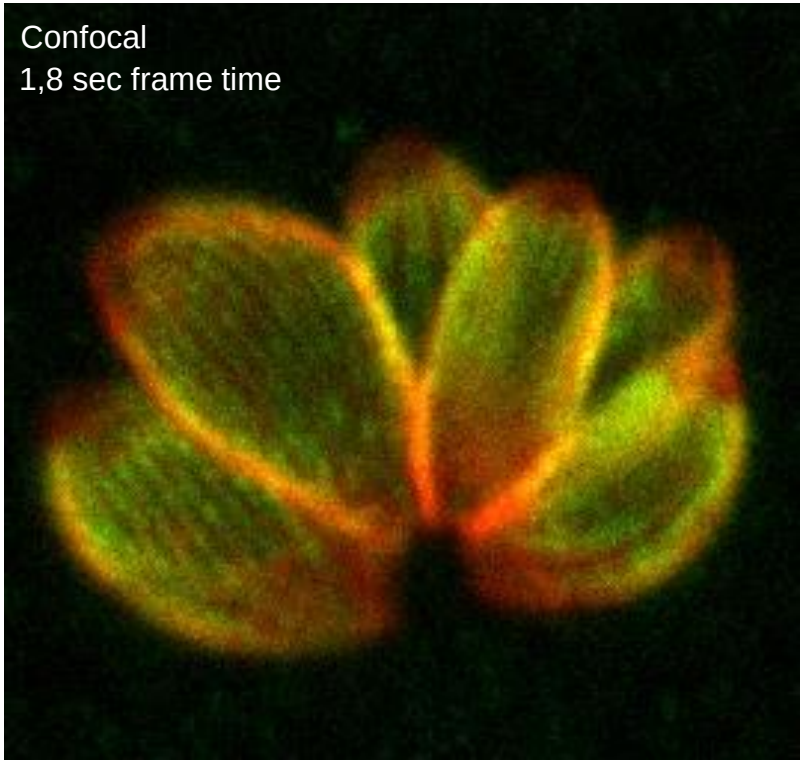
Airyscan 2 灵活的Multiplex 2Y模式
2倍并行数据处理大大提高超高分辨率成像速度

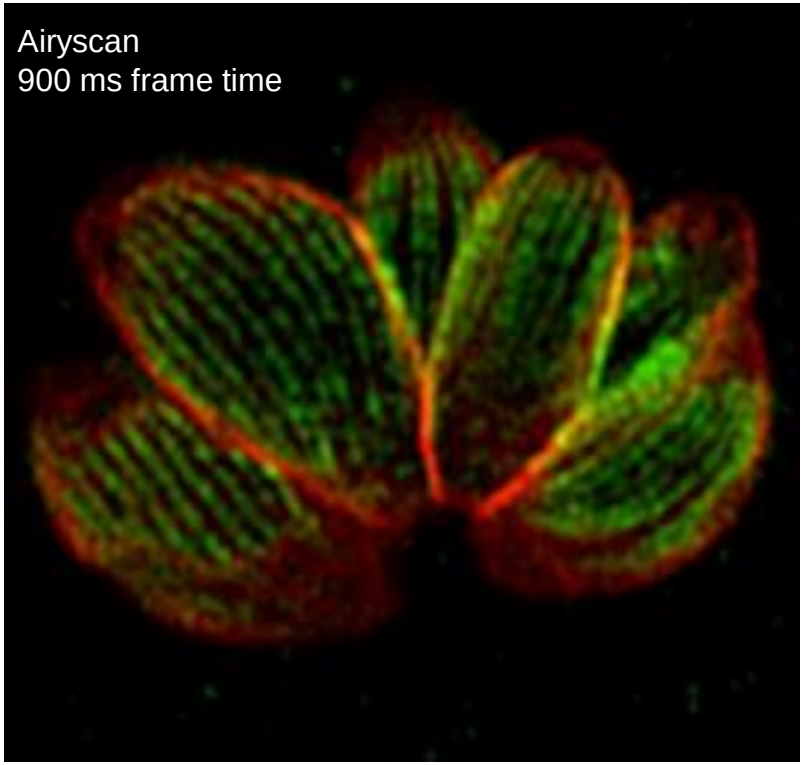
Acquire two pixels at once: **Multiplex 2Y**

Airyscan 2 灵活的Multiplex 4Y模式
4倍并行数据处理大大提高超高分辨率成像速度



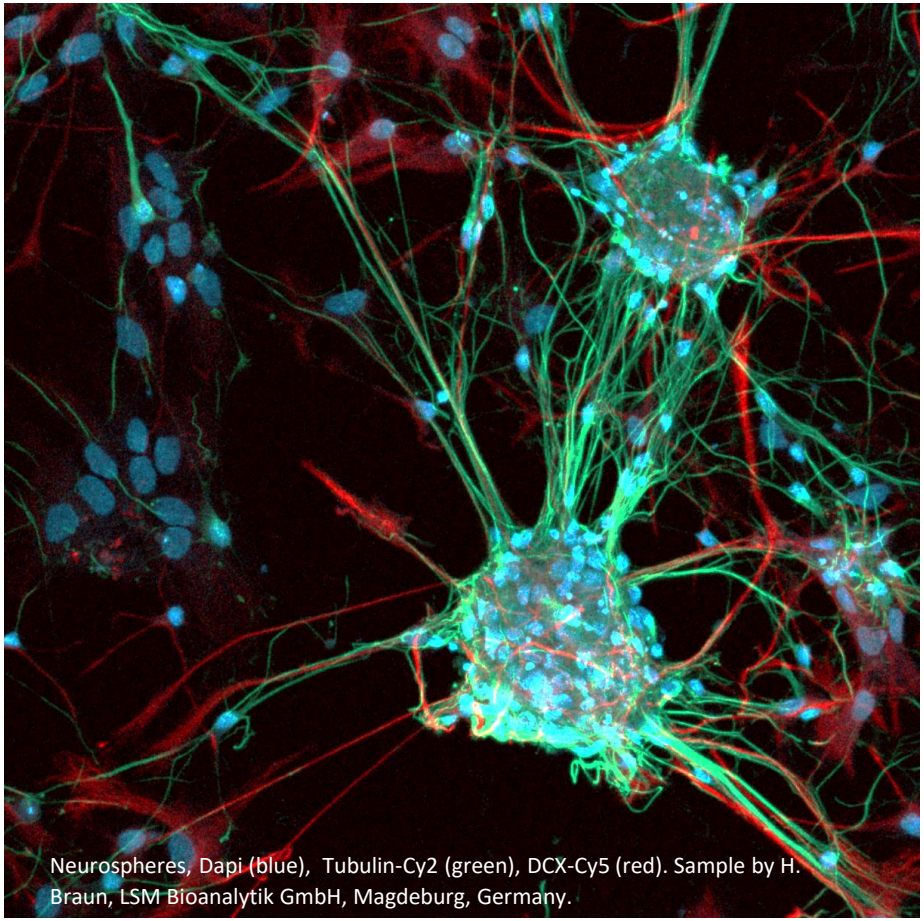
Classic Airyscanning
无需对速度，分辨率和信噪比做任何的妥协



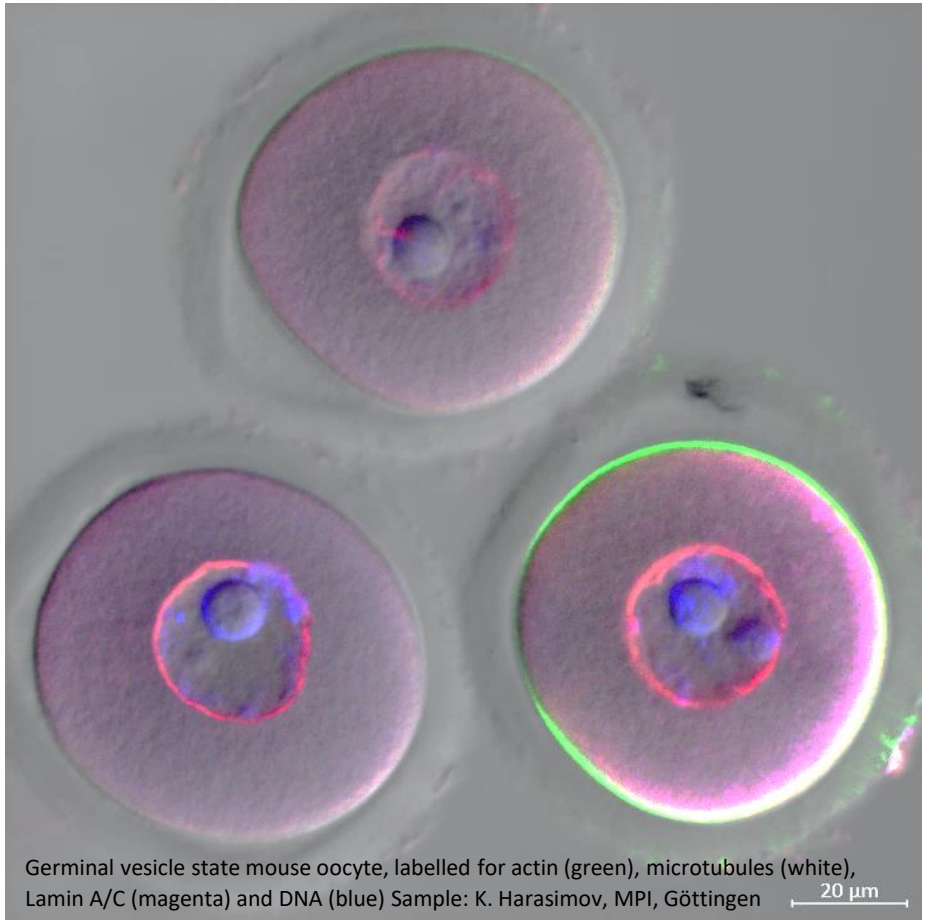


Toxoplasma gondii parasites, labelled microtubules in green (Alexa 488) and plasma membrane in red (Alexa 594). Sample: D. Jacot, MIMOL, University of Geneva

LSM 900
出色的三维成像

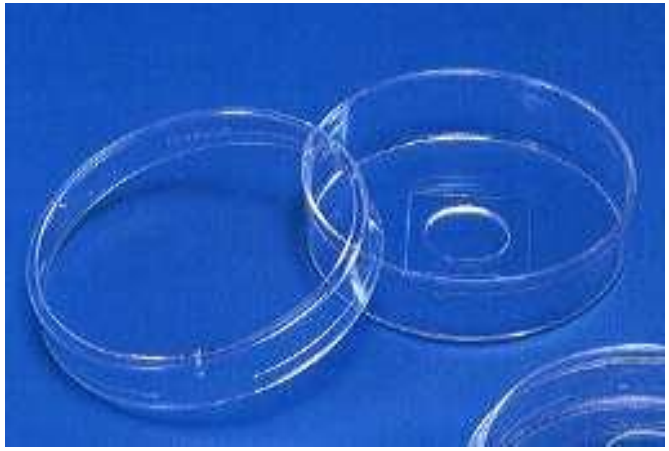


Neurospheres, Dapi (blue), Tubulin-Cy2 (green), DCX-Cy5 (red). Sample by H. Braun, LSM Bioanalytik GmbH, Magdeburg, Germany.



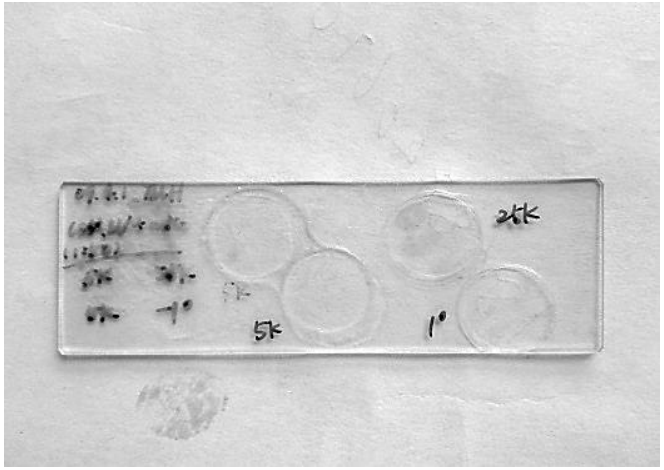
Germinal vesicle state mouse oocyte, labelled for actin (green), microtubules (white), Lamin A/C (magenta) and DNA (blue) Sample: K. Harasimov, MPI, Göttingen

共聚焦推荐使用的耗材



Cover Glass:170um
Glass bottom dish

Cover Glass:170um
Cell Chamber Slides



注意事项

显微镜室内温度控制在22℃，湿度在45-60%。

使用油镜时应使用专业油，不要用其它介质（如香柏），以免损伤物镜 。每次使用完油镜后，需使用无水乙醇将油镜擦拭干净（物镜及样品）.（倒置镜，油直接滴在物镜上）
禁止油镜直接切换低倍镜。

刻录图像数据资料：应使用刻录光驱刻录，实验前应准备好刻录光盘

不要戴手套操作显微镜。

定期打扫实验室，保证工作环境的清洁。避免灰尘。

每天工作完成后，请使用防尘罩遮盖显微镜及扫描头

Seeing beyond