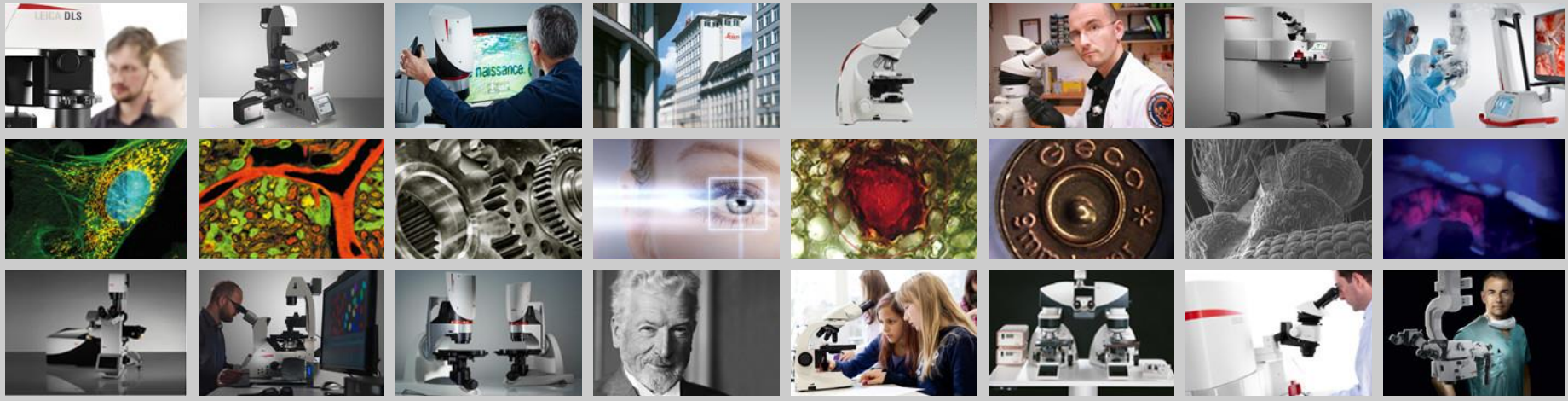
From Eye to Insight

Leica MICROSYSTEMS

如何在科研中用好激光共聚焦



徕卡显微系统（上海）贸易有限公司

方策 7/18/2018

内容

1、荧光成像原理及其应用

2、激光共聚焦的成像原理及应用

3、不同扫描模式的应用及其参数设置

4、高级成像应用及图像后期处理

5、样品制备注意事项及技巧

6、新技术简介

徕卡显微系统 – 悠久的历史

169年显微镜世家

“With the user, for the user”

—— Ernst Leitz

1849
Carl Kellner’s
Optical Institute

1967
Image
Analysis

1849 **1914** **1967** **1986** **1990** **1998** **2005**

一个品牌 - 三个独立营运的公司

◆徕卡照相机公司

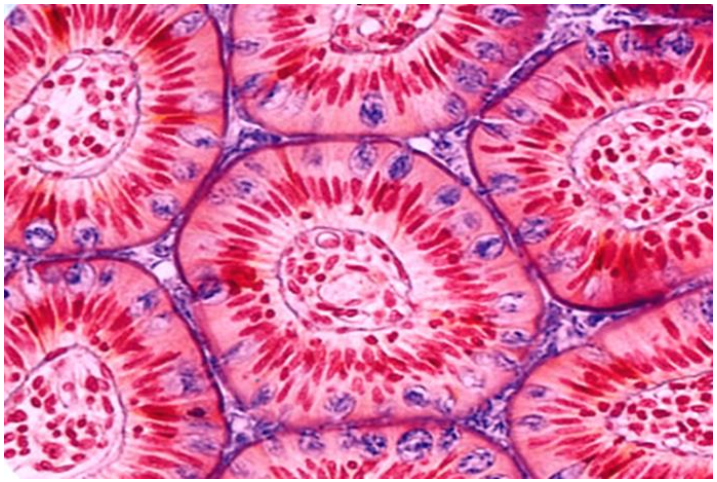
◆徕卡测量系统公司

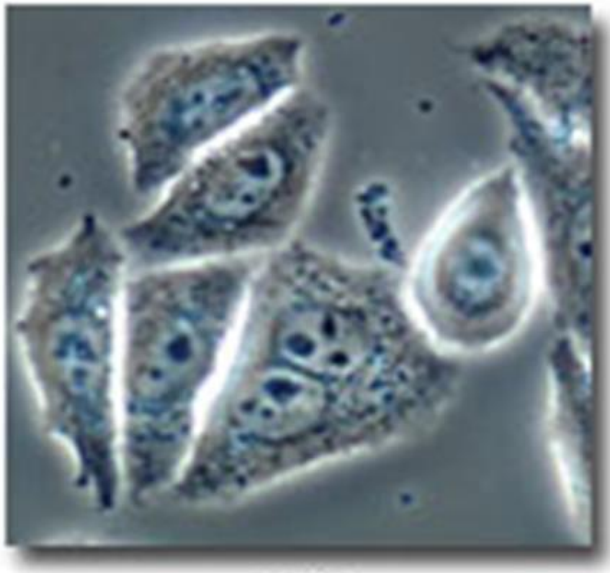
◆徕卡显微系统公司

对于这三家有着悠久历史和文化遗产并已独立的公司，徕卡品牌是三家公司之间的唯一连接。徕卡显微系统是徕卡商号和商标的拥有者，并可授权其他公司使用。

显微镜的不同观察方式

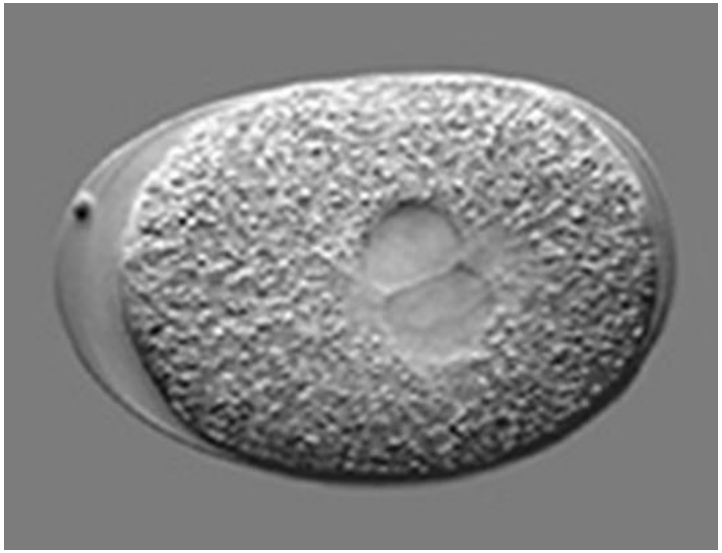
明场



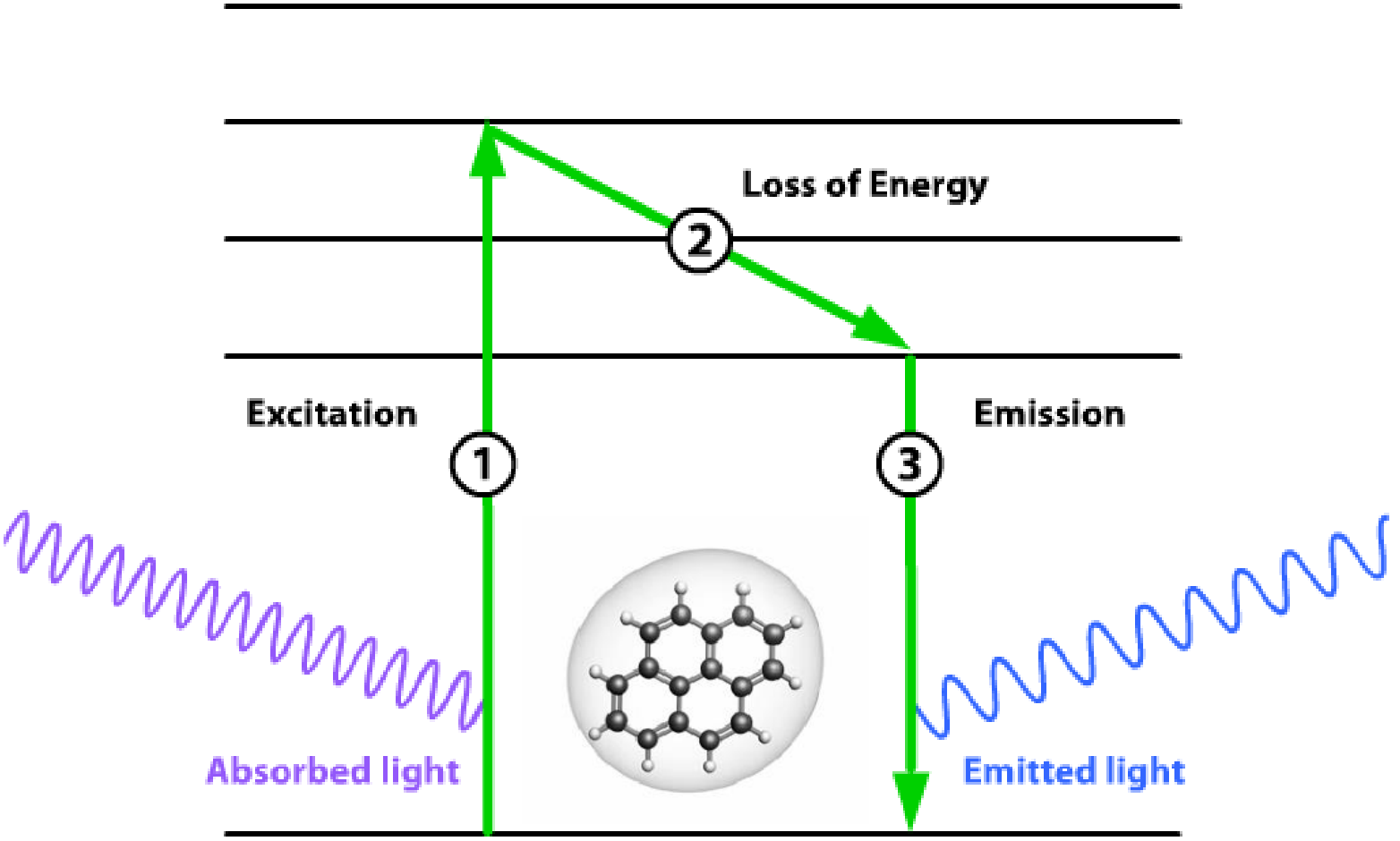


相差

DIC

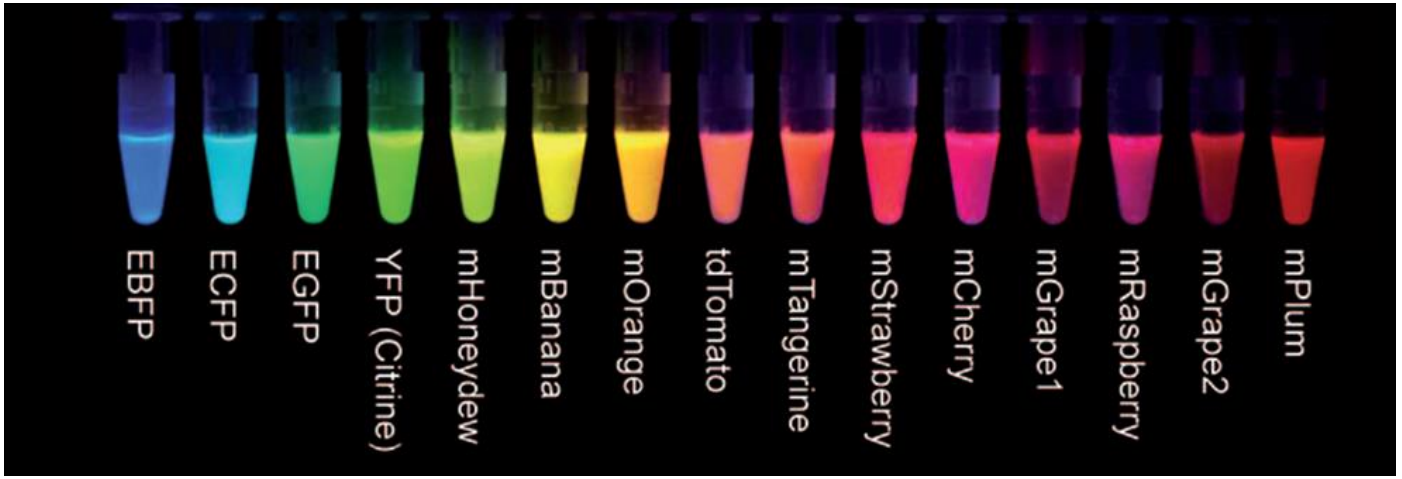


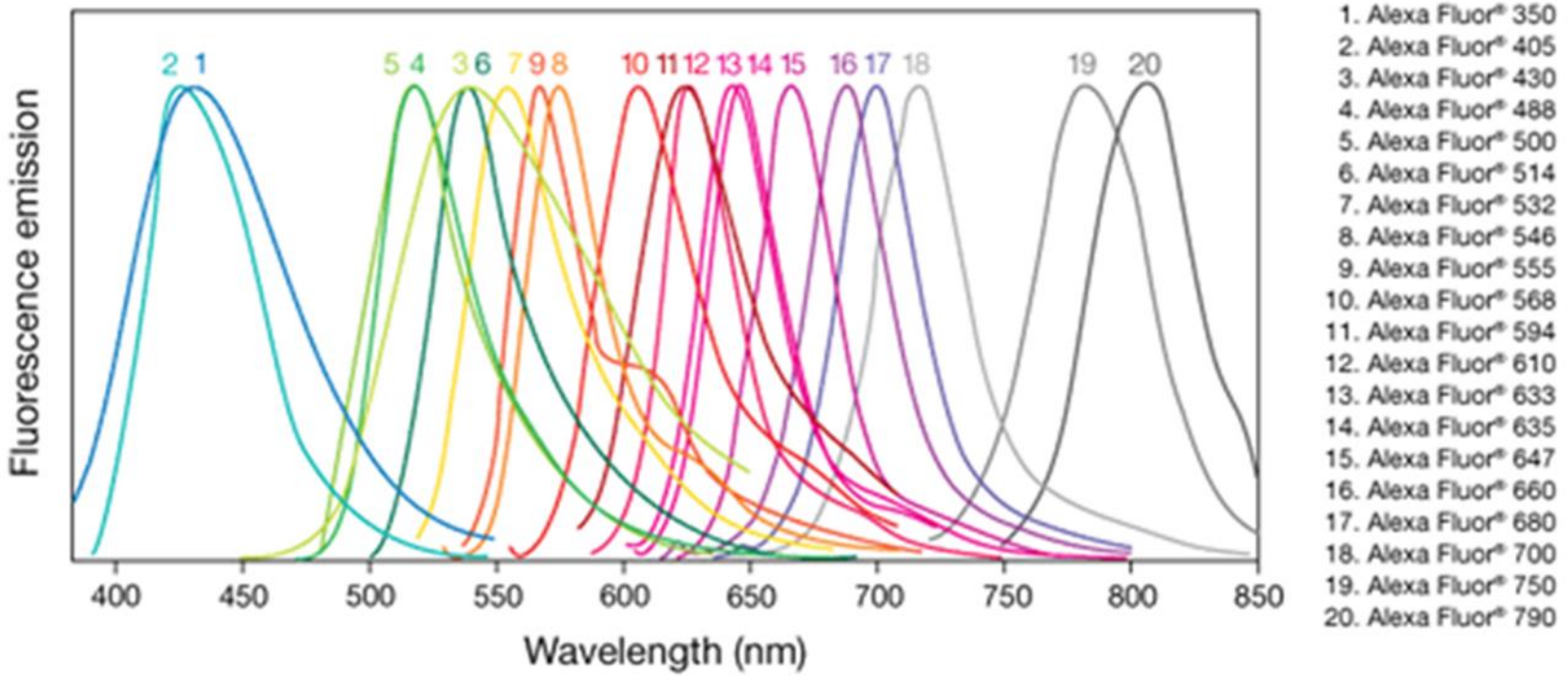
共聚焦最主要的观察对象：荧光信号



荧光蛋白或荧光染料

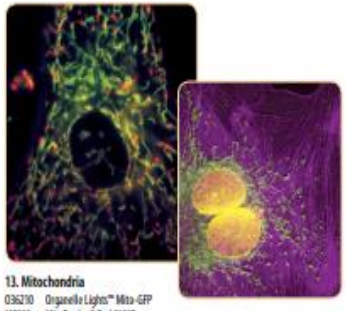
荧光蛋白与荧光染料


EBFP
ECFP
EGFP
YFP (Citrine)
mHoneydew
mBanana
mOrange
tdTomato
mTangerine
mStrawberry
mCherry
mGrape1
mRaspberry
mGrape2
mPlum


Fluorescence emission
2 1
5 4
3 6
7 9 8
10 11 12 13 14 15
16 17
18
19
20
400
450
500
550
600
650
700
750
800
850
Wavelength (nm)
1. Alexa Fluor® 350
2. Alexa Fluor® 405
3. Alexa Fluor® 430
4. Alexa Fluor® 488
5. Alexa Fluor® 500
6. Alexa Fluor® 514
7. Alexa Fluor® 532
8. Alexa Fluor® 546
9. Alexa Fluor® 555
10. Alexa Fluor® 568
11. Alexa Fluor® 594
12. Alexa Fluor® 610
13. Alexa Fluor® 633
14. Alexa Fluor® 635
15. Alexa Fluor® 647
16. Alexa Fluor® 660
17. Alexa Fluor® 680
18. Alexa Fluor® 700
19. Alexa Fluor® 750
20. Alexa Fluor® 790

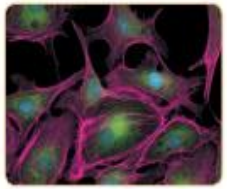
The Illuminated Cell

Invitrogen Cellular Analysis—biology in context



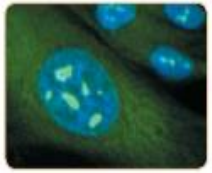
13. Mitochondria

O36210	Organelle Lights™ Mito -GFP
M7512	MitoTracker® Red CMXRos
M7514	MitoTracker® Green FM
M7510	MitoTracker® Orange CMTMRos
T3168	JC-1
	anti-OxPhos antibodies



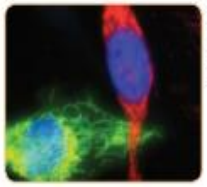
14. Nucleus

O36219	Organelle Lights™ Nuc-YFP
O36220	Organelle Lights™ Nuc-OFP
S7576	SYTO® 14
S11341	SYTO® 59
D1306	DAPI
H3570	Hoechst 33342
S34859	SYTOX® Red
S7010	SYTOX® Green
T3605	TO-PRO®-3 iodide



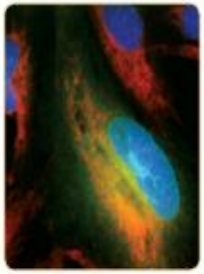
1. Nucleoli

S32703 SYTO® RNASelect™ green fluorescent cell stain



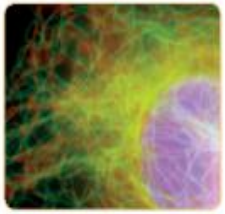
2. Endoplasmic Reticulum

E34251	ER-Tracker™ Green
E34250	ER-Tracker™ Red
O36223	Organelle Lights™ ER-OFP
S34200	SelectFX® Alexa Fluor® 488 Endoplasmic Reticulum Labeling Kit



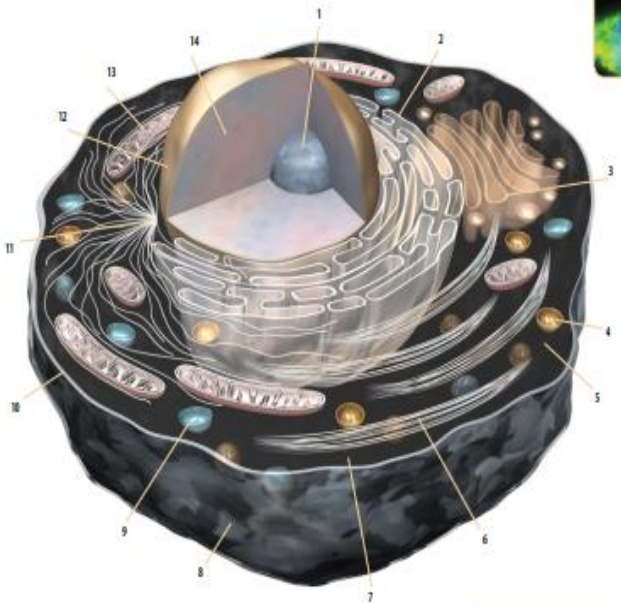
12. Nuclear Envelope

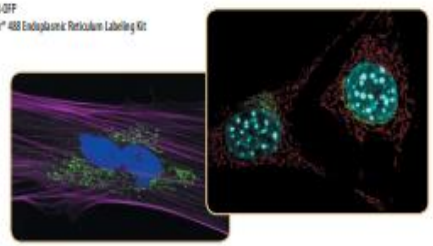
O36213 Organelle Lights™ NE-GFP



11. Cytoskeleton/Tubulin

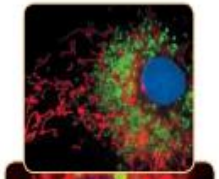
T34075	TubulinTracker™ Green
T34461	TC-FlAsH™ mammalian TC Tag Gateway® expression vectors
A11126	anti-α-tubulin

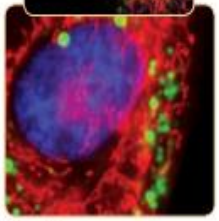




3. Golgi Complex

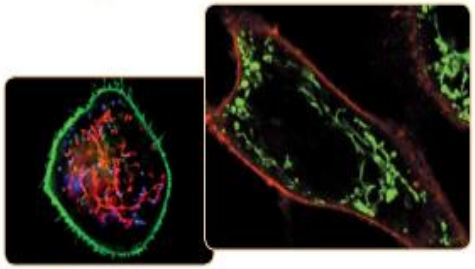
O36215	Organelle Lights™ Golgi-GFP
O36222A	Organelle Lights™ Golgi-OFP
A21270	anti-golgin-97
N22651	NBD C_6-ceramide complexed to BSA
B22650	BODIPY® FL C_5-ceramide complexed to BSA
B34480	BODIPY® TR C_5-ceramide complexed to BSA





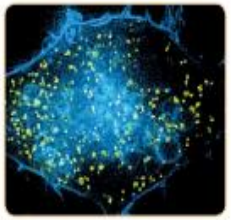
4. Lysosomes

L7538	LysoTracker® Red DND-99
L7526	LysoTracker® Green DND-26
L7545	LysoSensor™ Yellow/Blue DND-160



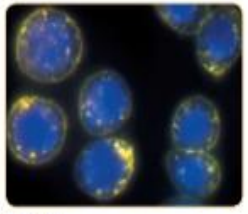
10. Plasma Membrane

F34653	FM® 4-64FX *fixable analog of FM® 4-64 membrane stain*
F35355	FM® 1-43FX *fixable analog of FM® 1-43 membrane stain*
O36216	Organelle Lights™ PM-CFP
O36217	Organelle Lights™ PM-YFP
O36226	Organelle Lights™ PM-OFP
C10045	CellMask™ Orange plasma membrane stain
C10046	CellMask™ Deep Red plasma membrane stain
W11262	Alexa Fluor® 594 wheat germ agglutinin



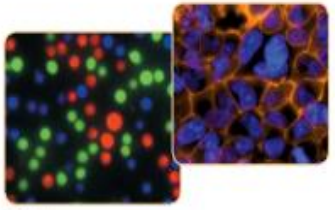
9. Peroxisomes

O36215	Organelle Lights™ Peroxi-GFP
O36225	Organelle Lights™ Peroxi-OFP
S34201	SelectFX® Alexa Fluor® 488 Peroxisome Labeling Kit



8. Lipid Rafts

B13950	BODIPY® FL C_5-ganglioside G_{M1}
V34402	Vybrant® Alexa Fluor® 488 Lipid Raft Labeling Kit
V34404	Vybrant® Alexa Fluor® 555 Lipid Raft Labeling Kit
V34405	Vybrant® Alexa Fluor® 594 Lipid Raft Labeling Kit

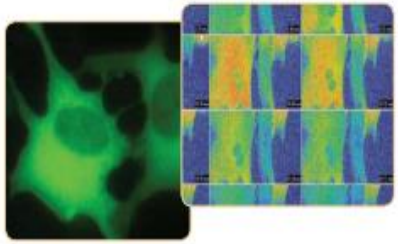


7. Cytosol

H32711	HCS CellMask™ Red cytoplasmic/nuclear stain
H34568	HCS CellMask™ Deep Red cytoplasmic/nuclear stain
H34558	HCS CellMask™ Blue cytoplasmic/nuclear stain
O36227	Organelle Lights™ Cyto-GFP
C2927	CellTracker™ Orange CMTMR
C2925	CellTracker™ Green CMFDA
C34552	CellTracker™ Red CMTPX
Q25021	Qtracker® 655 Cell Labeling Kit
C3100MP	calcein, AM

6. Cytoskeleton/Actin

A12379	Alexa Fluor® 488 phalloidin
R415	rhodamine phalloidin
A12381	Alexa Fluor® 594 phalloidin



5. Biosensors

Cytosolic Ca^{2+}

F36207	Cameleon
F36205	Fluo-4 NW Calcium Assay Kit
I1223	indo-1, AM
F1221	fura-2, AM
F14201	fluo-4, AM
R1245MP	rhod-2, AM

Cytosolic Mg^{2+}

M1426	mag-fluo-4, AM
M1292	mag-fura-2, AM

Zn^{2+}

F24195	FluoZin™-3, AM
R36351	RhodZin™-3, AM

Cytosolic pH

B1150	BCECF, AM
C1272	SNARF®-1, acetoxymethyl ester, acetate

Cytosolic ROS

M36008	MitoSOX™ Red mitochondrial superoxide indicator
I36007	Image-iT® LIVE Green Reactive Oxygen Species Detection Kit
A36005	APF
C6827	CM-H$_2$ DCFDA [ROS general]
D11347	dihydroethidium (hydroethidine) [superoxide]

Cytosolic RNS

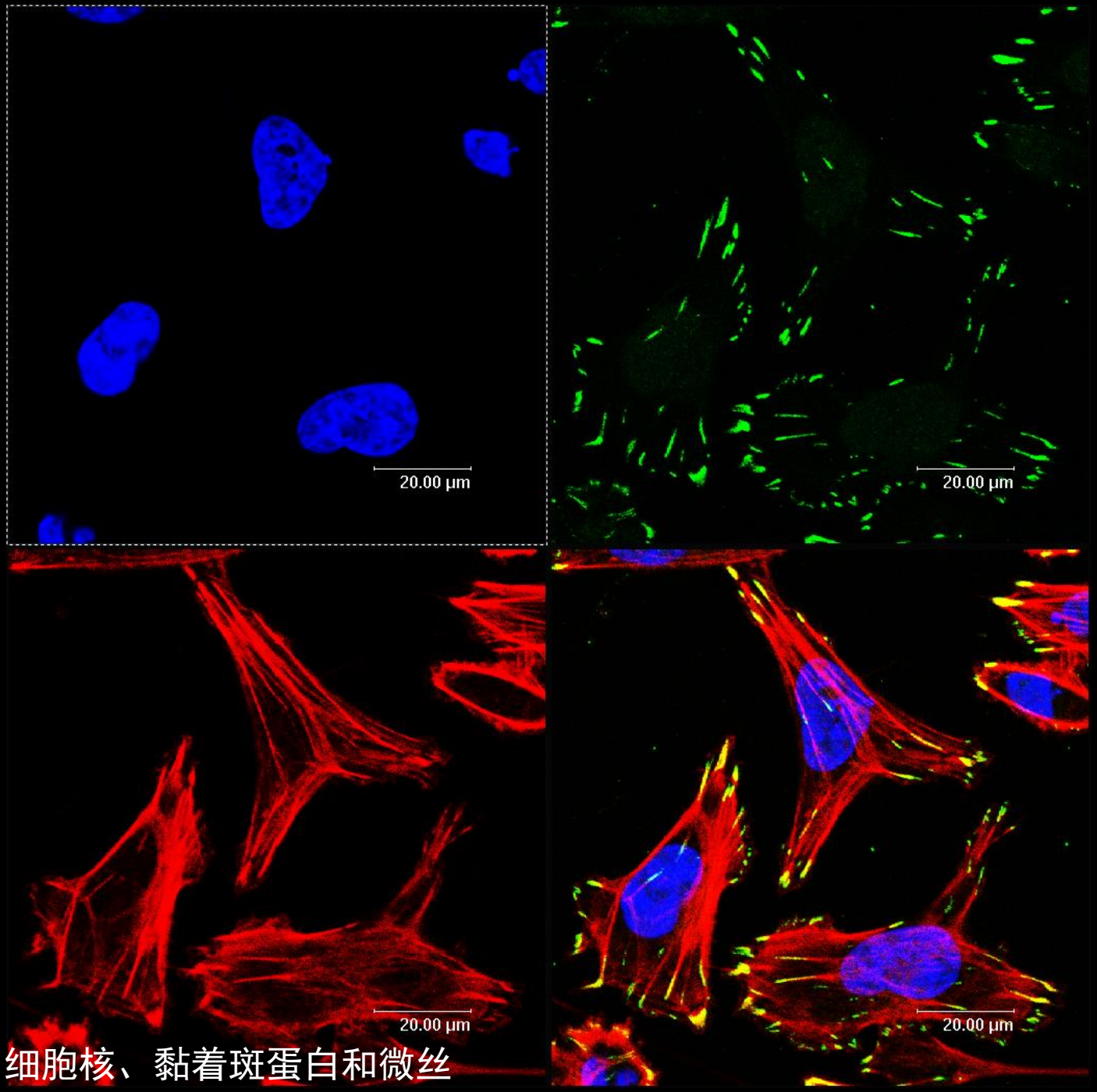
A21285	anti-nitrotyrosine, rabbit IgG fraction
D23842	DAF-FM diacetate

invitrogen™

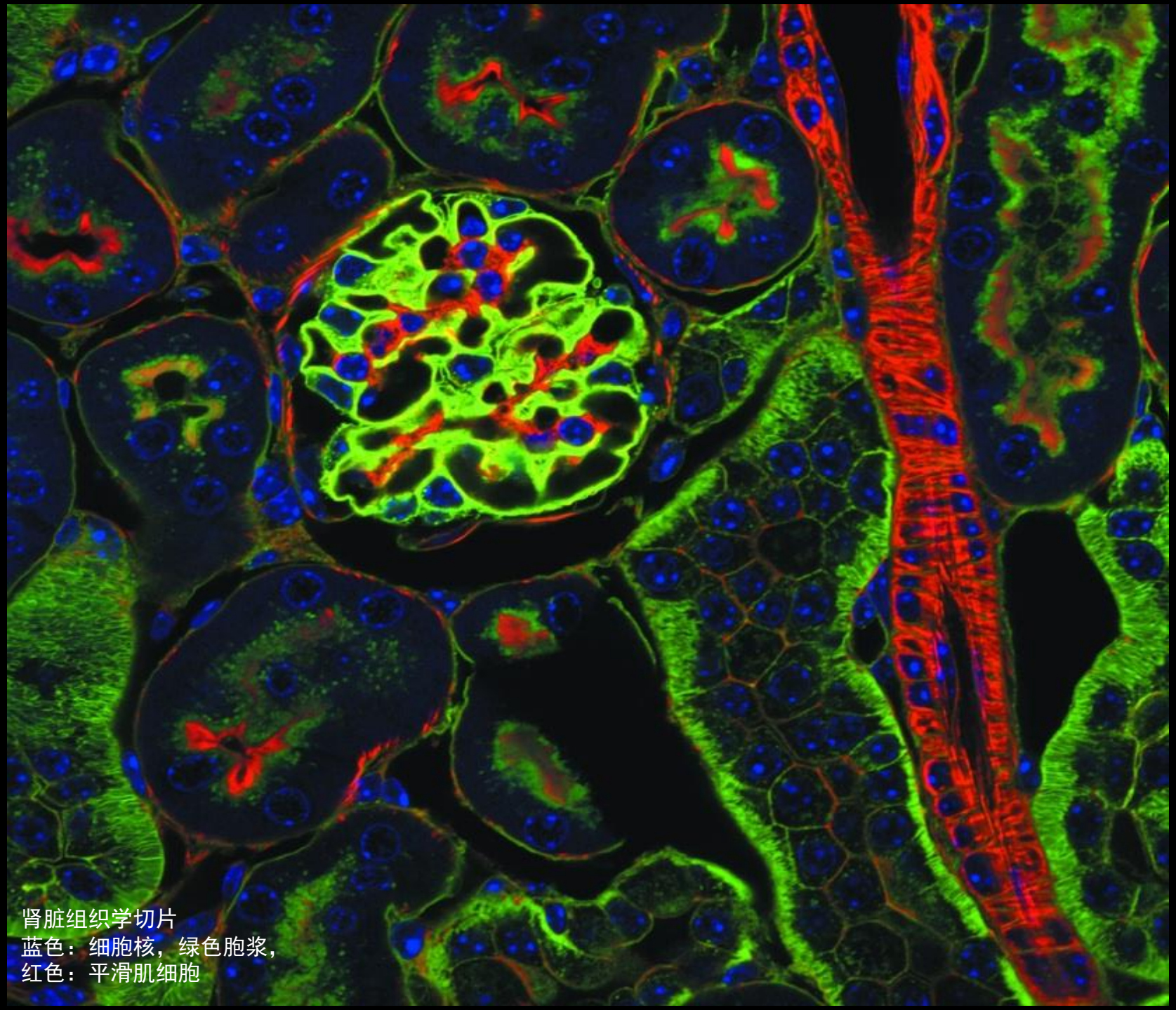
www.invitrogen.com

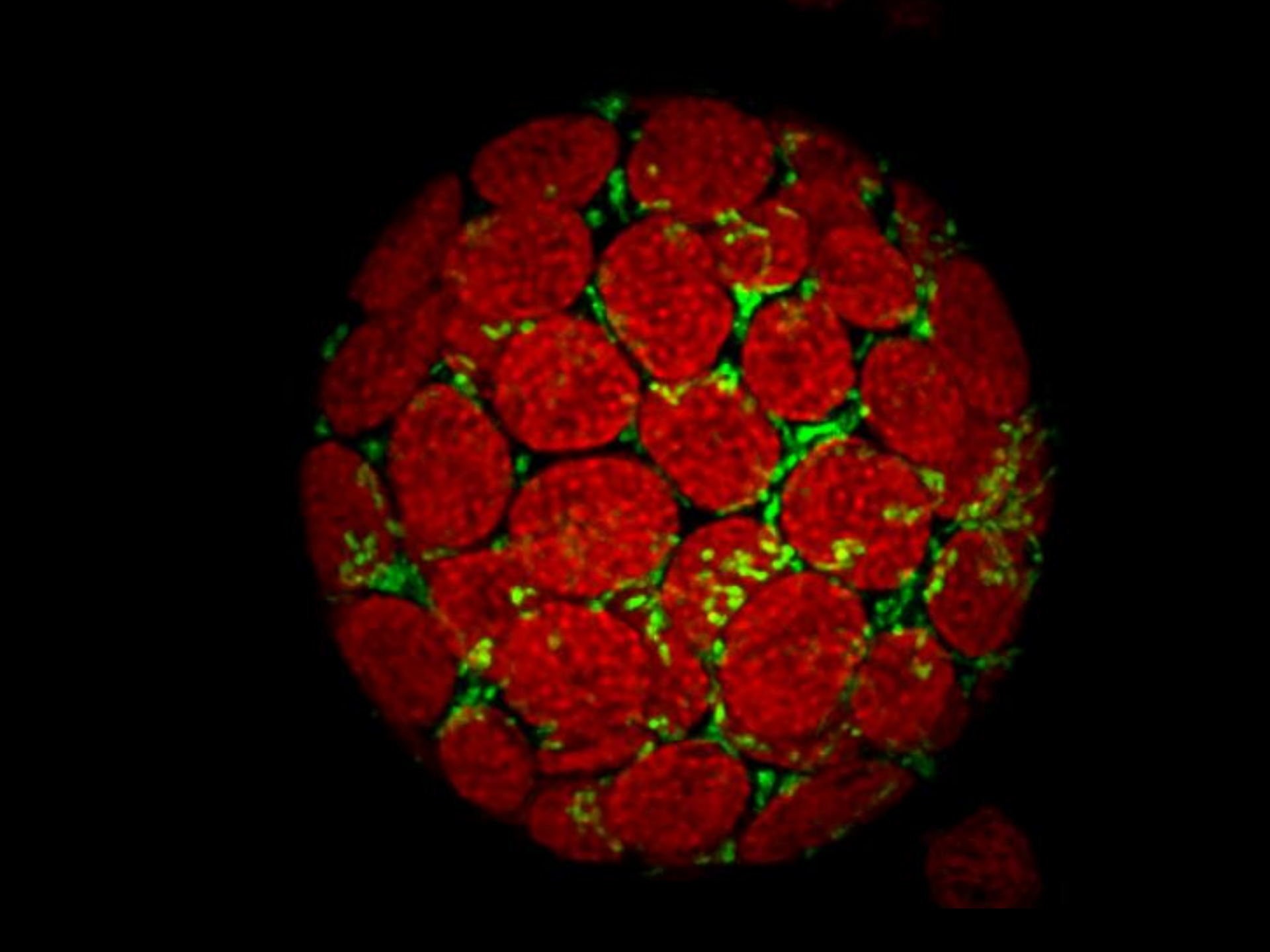
Molecular Probes®

invitrogen detection technologies



细胞核、黏着斑蛋白和微丝


肾脏组织学切片
蓝色：细胞核，绿色胞浆，
红色：平滑肌细胞



信号分子探针

Ca^{2+} Fluo-3, Fluo-4, Calcium Green, Fura2等

pH BCECF AM

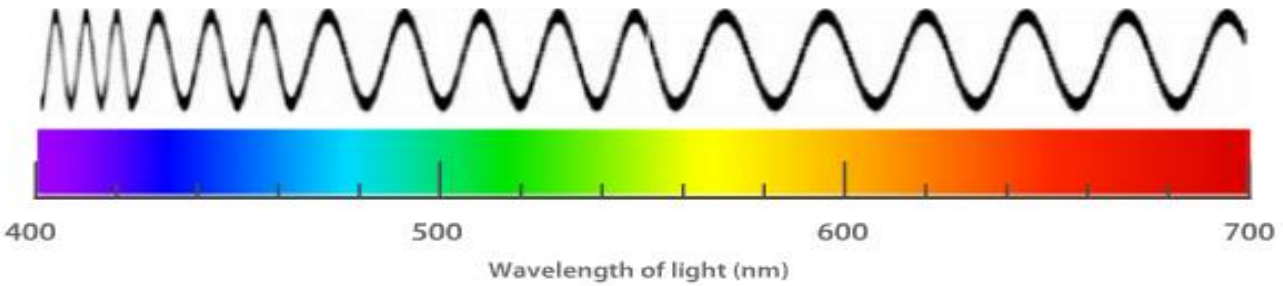
ROS H_2DCFDA (DCF)

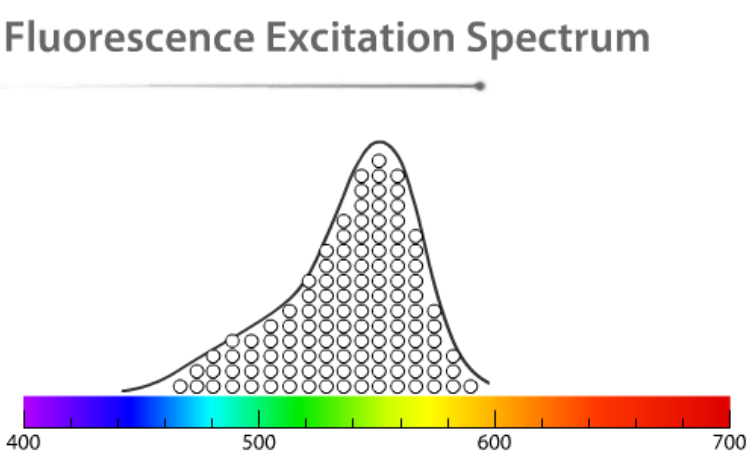
Singlet Oxygen Sensor Green

NO DAF

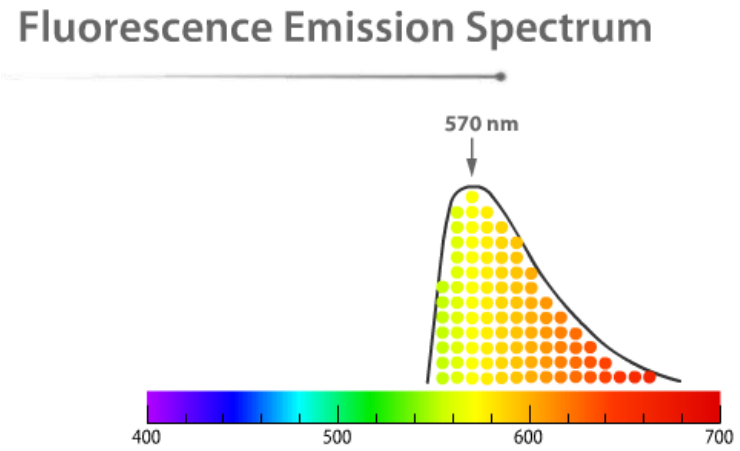
膜电势 JC-1, $DiOC_6(3)$

激发光谱和发射光谱



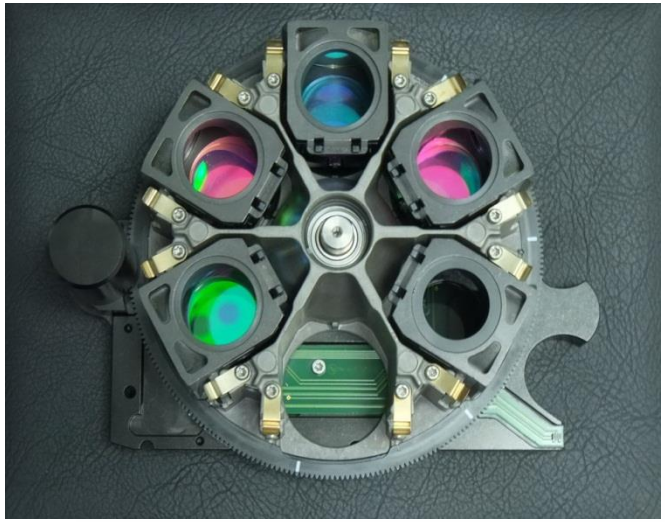


激发光谱

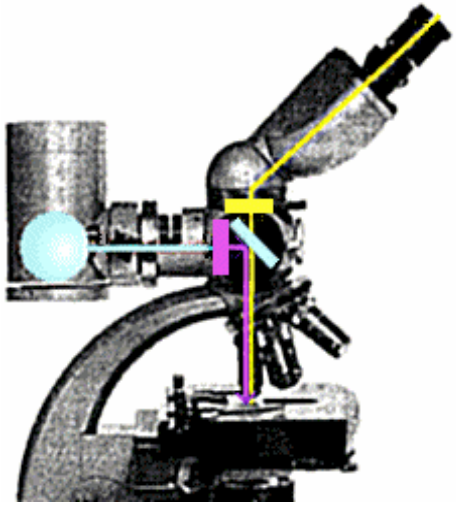


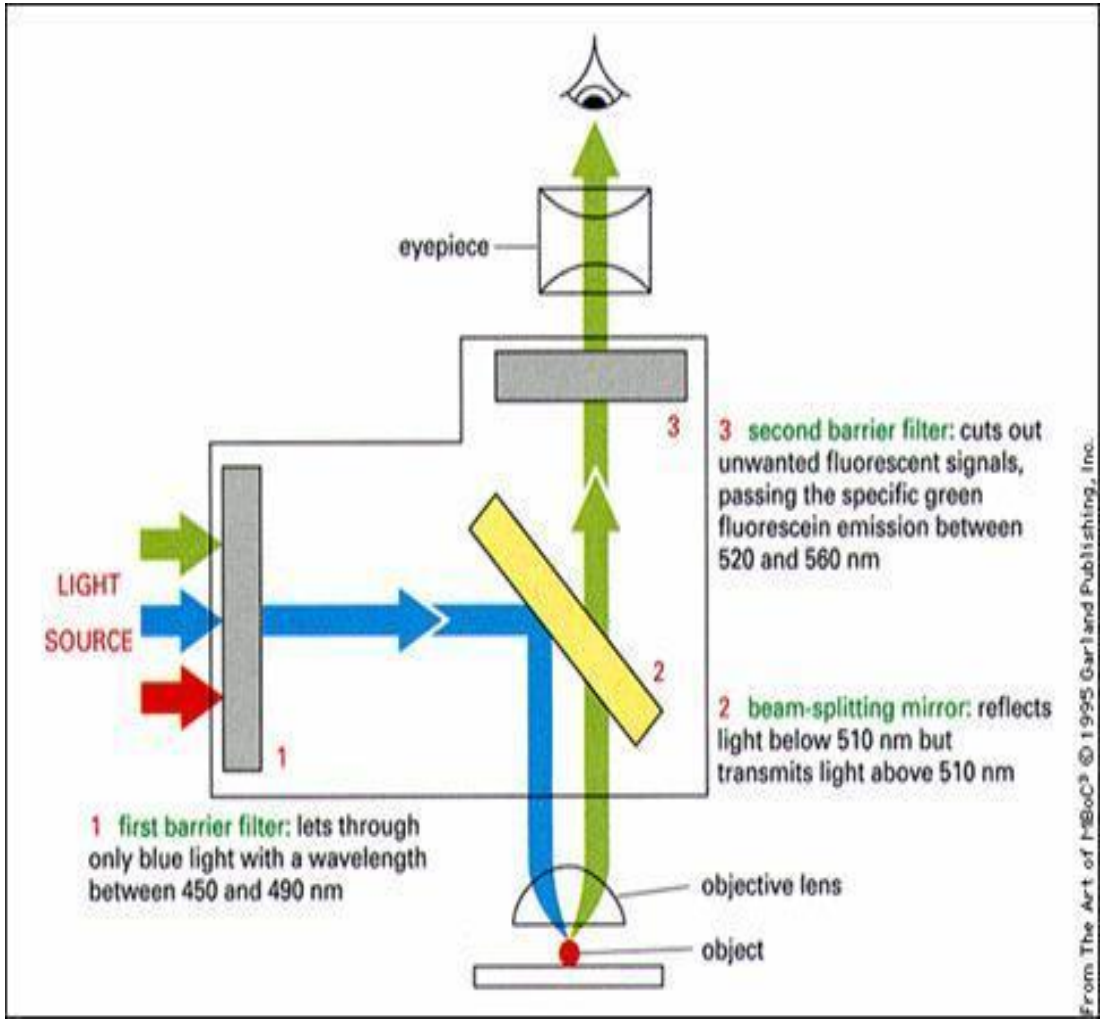
发射光谱

荧光显微镜



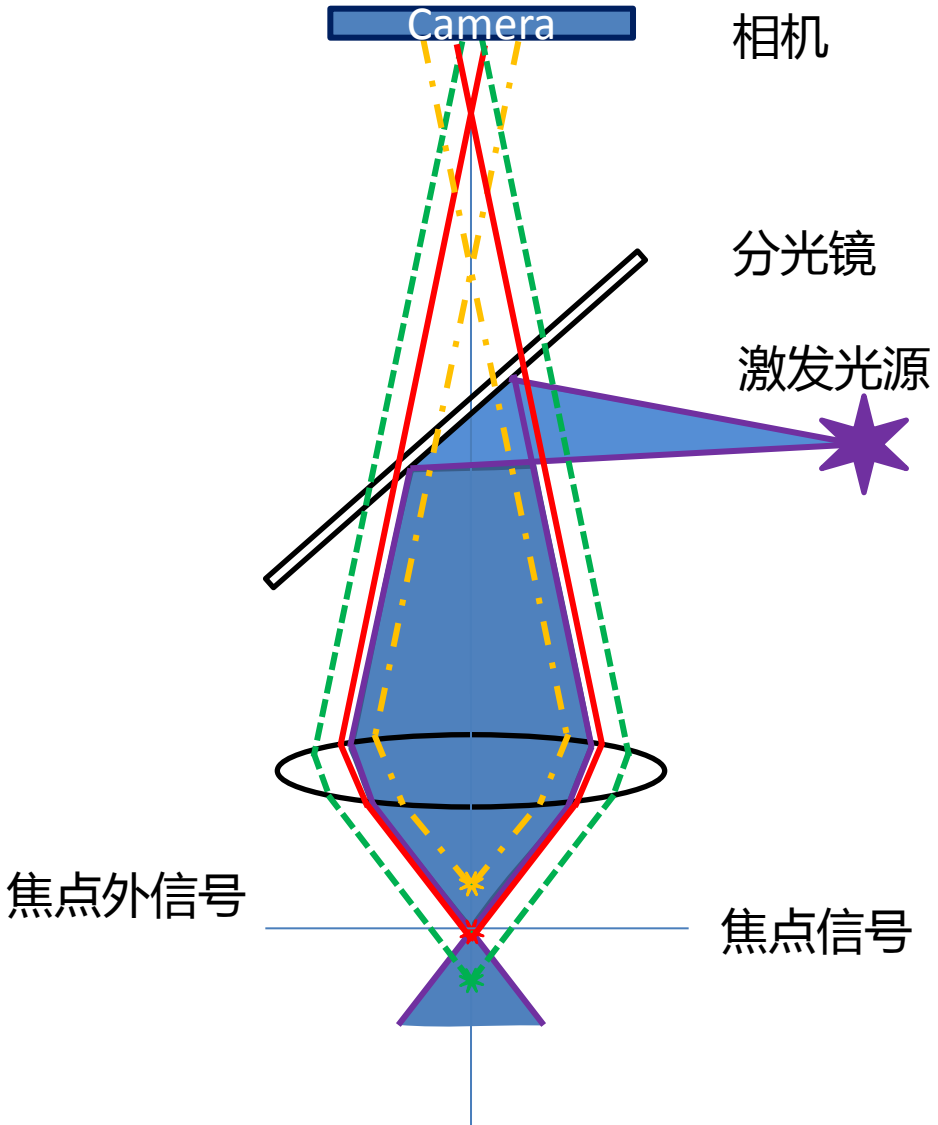
荧光滤块转轮



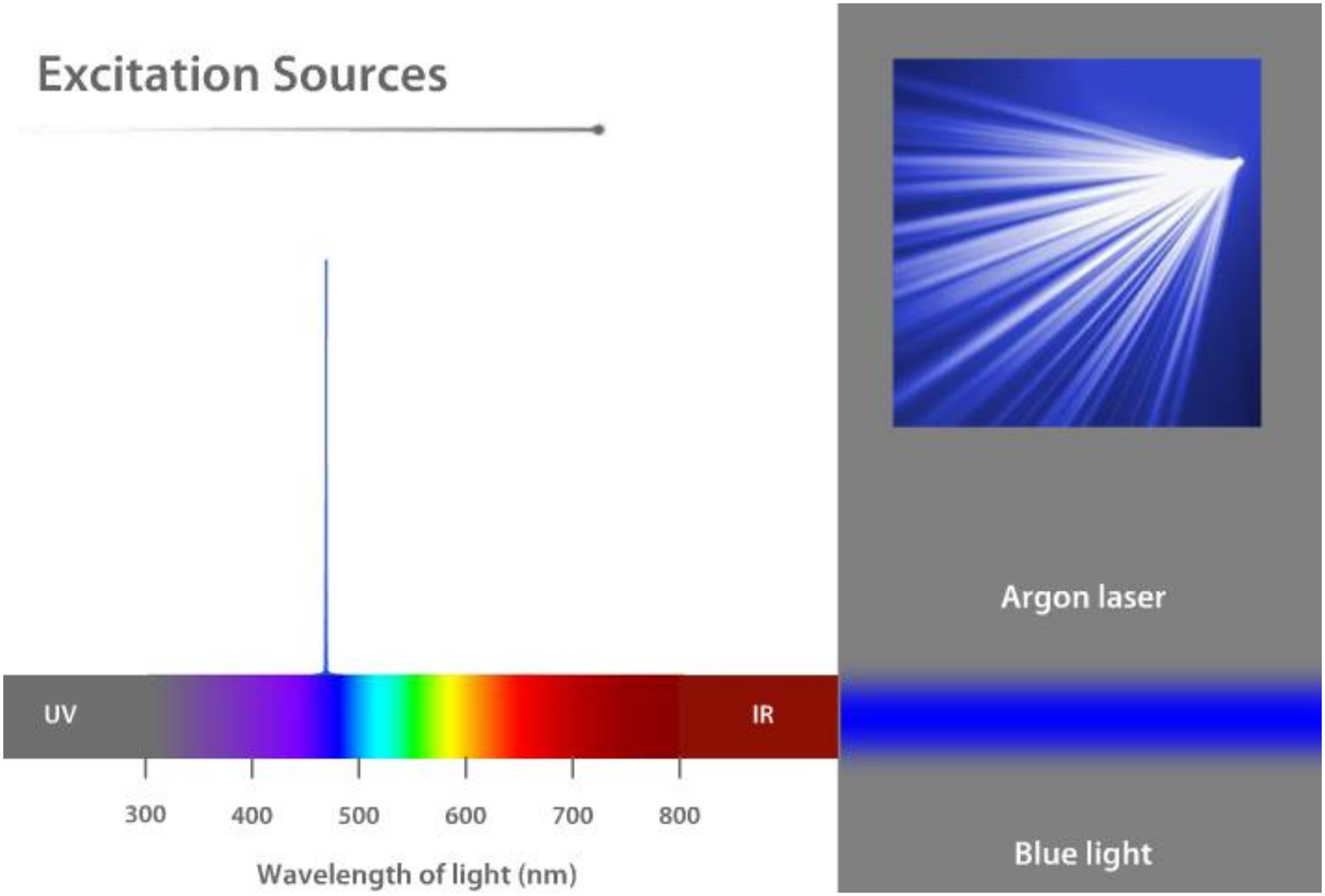


荧光显微镜光路图

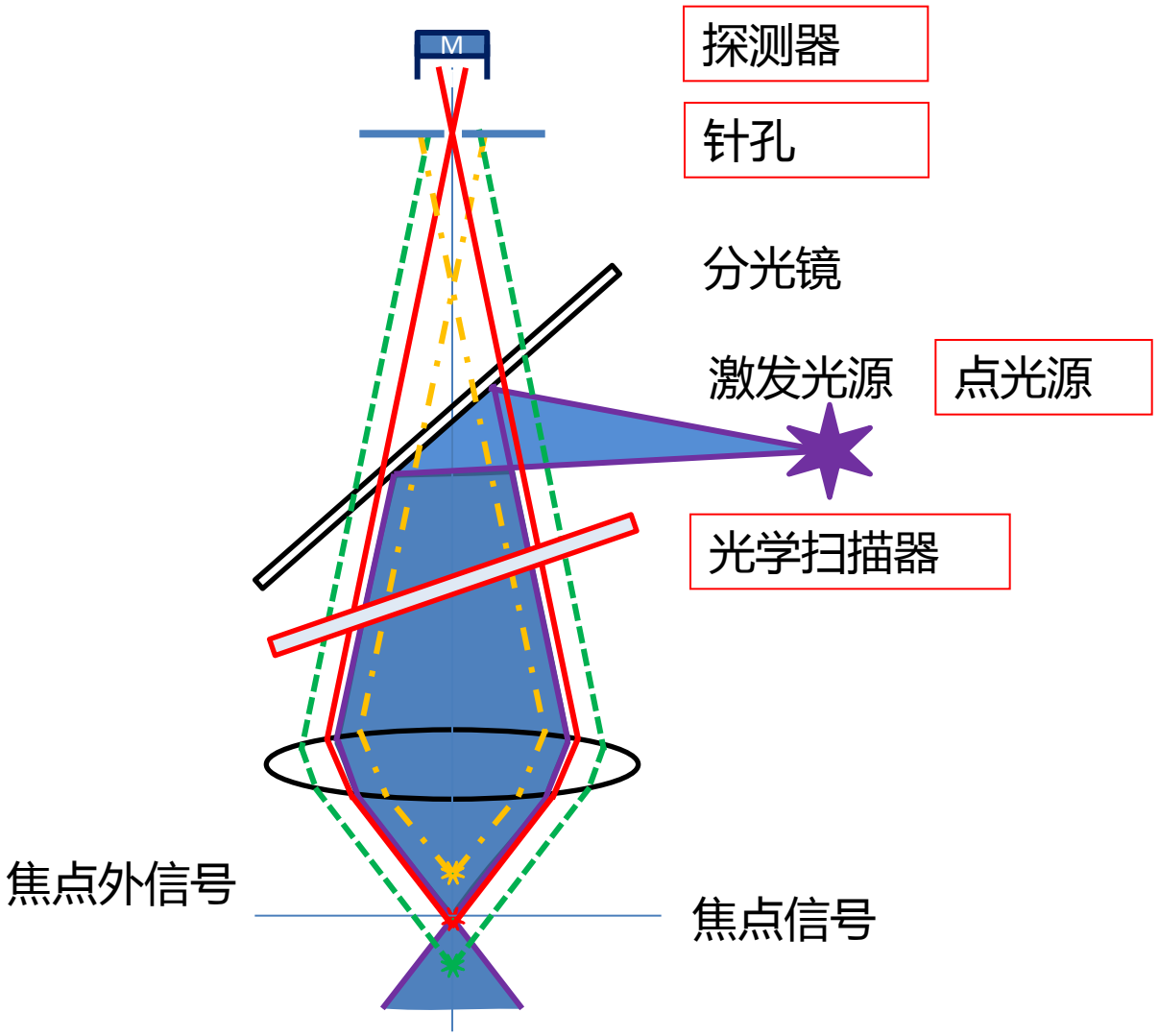
宽场荧光显微镜的局限



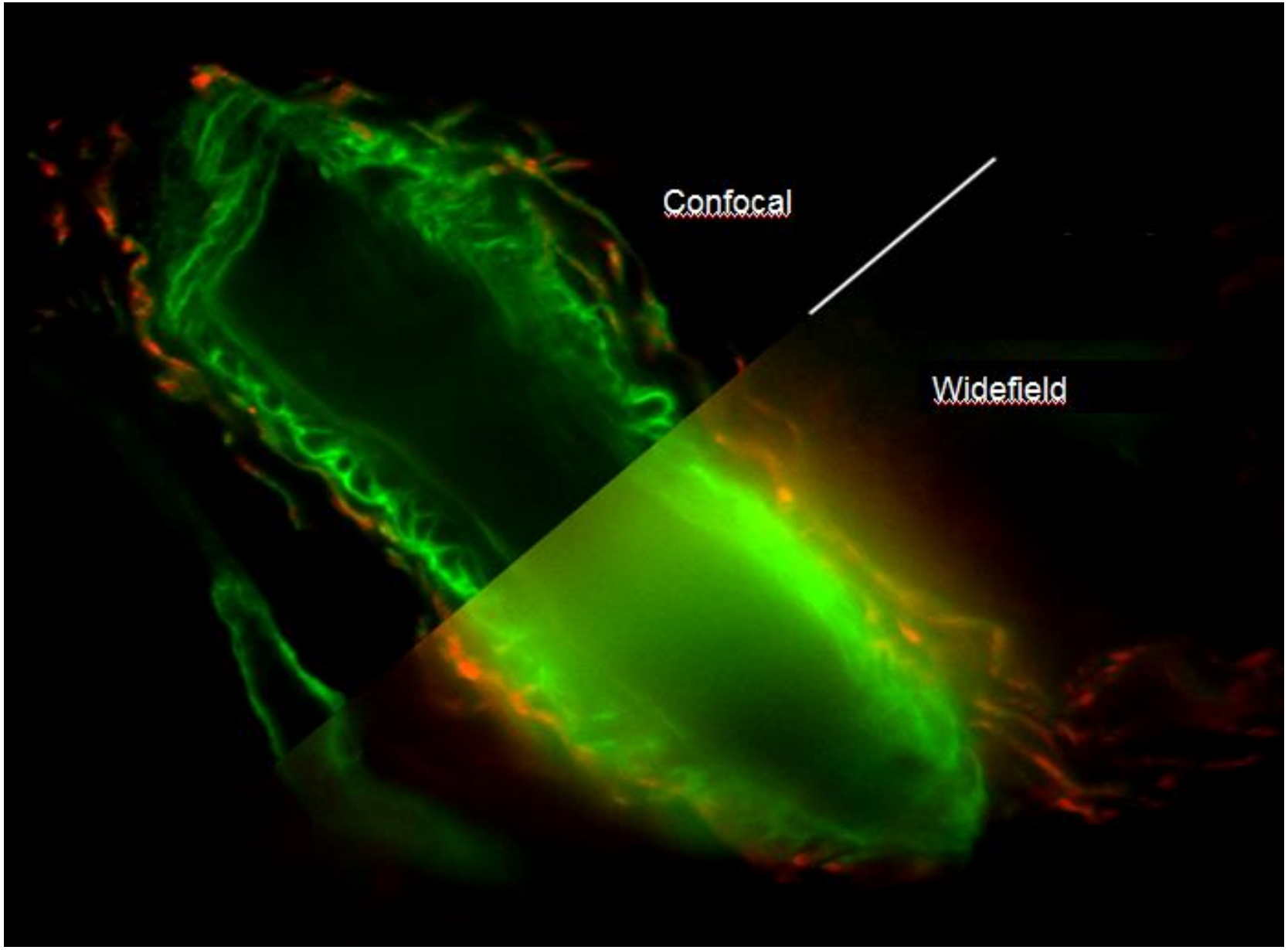
激光共聚焦显微镜


Excitation Sources
UV
IR
300
400
500
600
700
800
Wavelength of light (nm)
Argon laser
Blue light

激光共聚焦显微镜原理

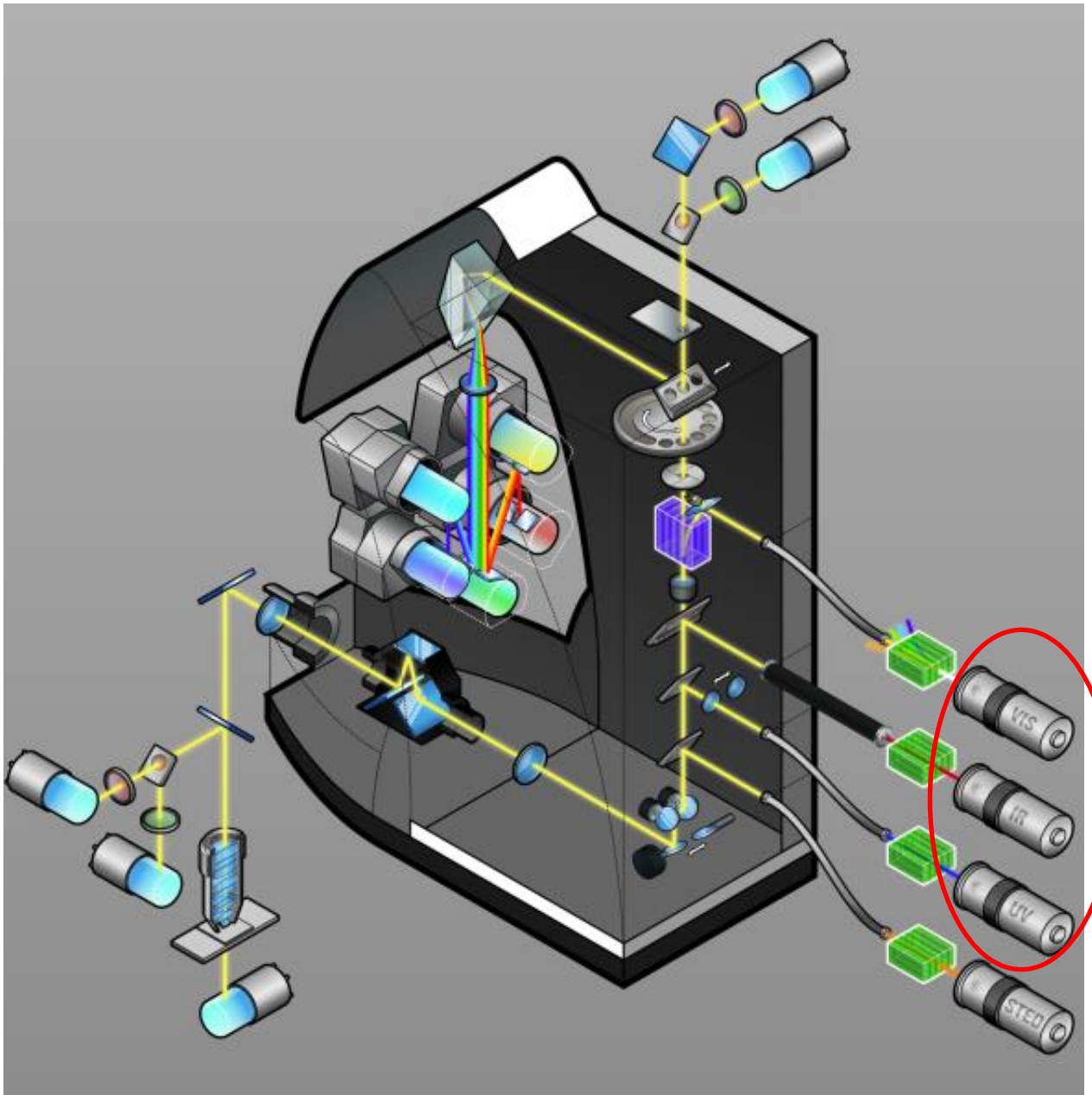


共聚焦 vs 宽场显微镜



徕卡SP8扫描光路图

Leica TCS SP8 扫描头光路图



激光器

固体激光器

1、405nm 激光器50mW：
DAPI，Hoechest 33342蓝色荧光

2、 488nm 激光器20mW：
FITC，GFP，Alexa 488等绿色荧光

3、 514nm激光器20mW：
YFP，Venus等黄色荧光

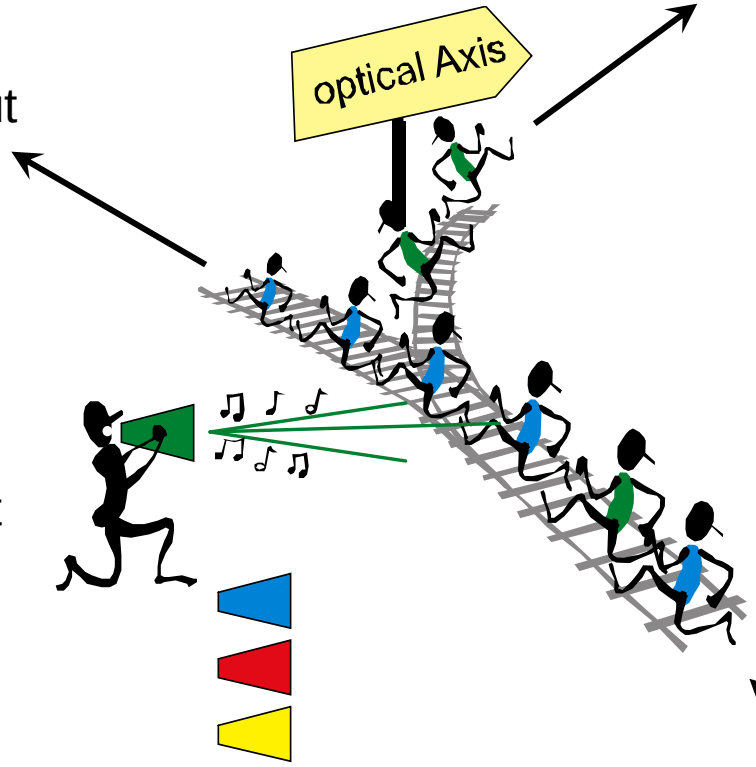
4、552nm 激光器20mW：
RFP，mCherry，Cy3，Alexa 555等红色荧光

5、 638nm 激光器30mW：
Cy5，Alexa 647等近红外荧光

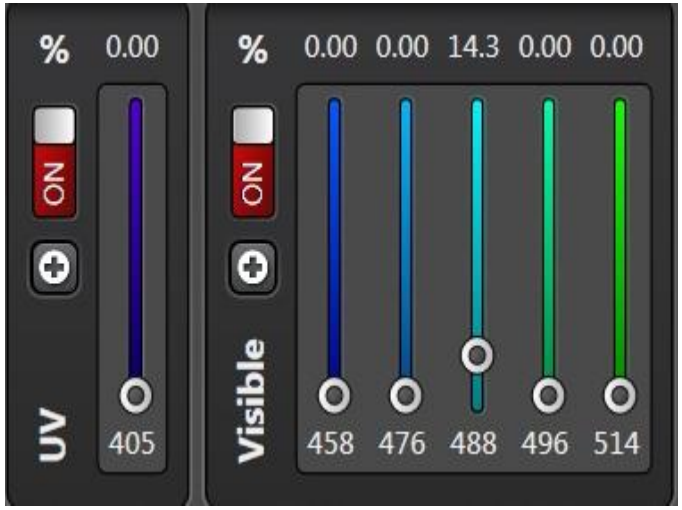
AOTF：精确控制激光能量

Desired lines in
(to scan device)

Undesired lines out
(to photon dumpster)

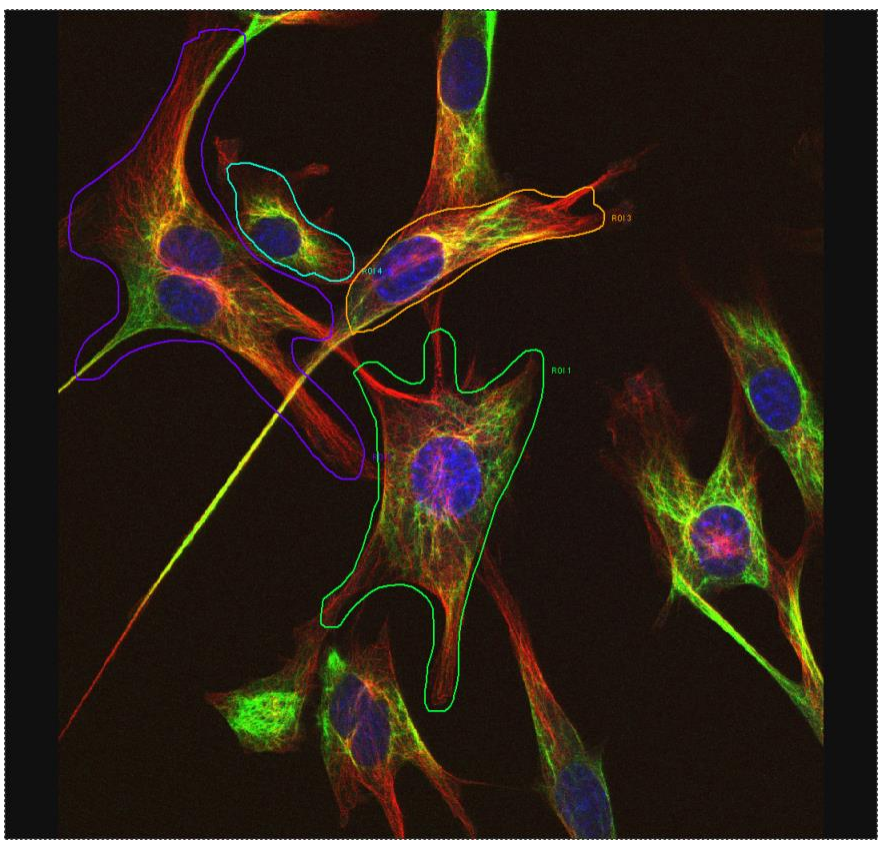


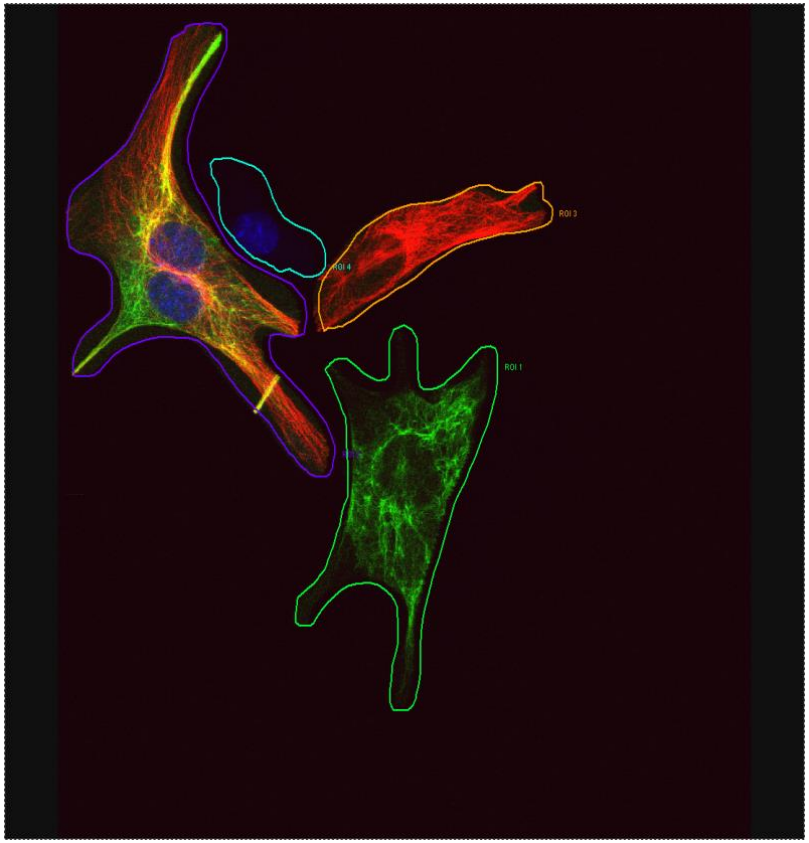
Acoustic Scout
(activation by sound)



All lines in
(from laser crowd)

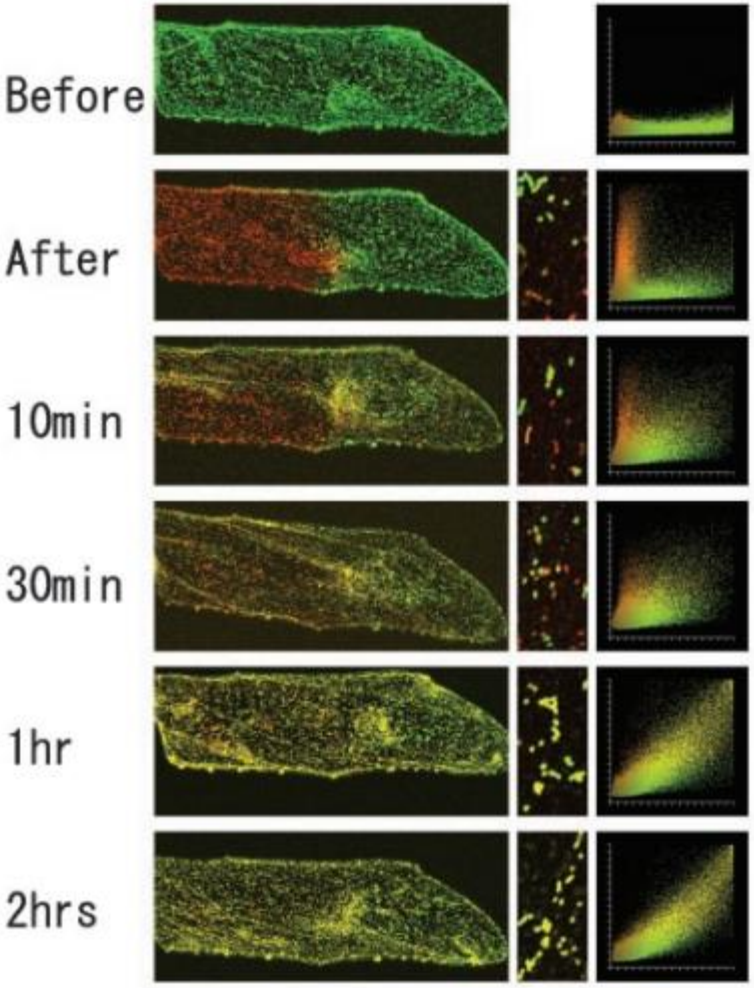
激光控制: AOTF - ROI 扫描

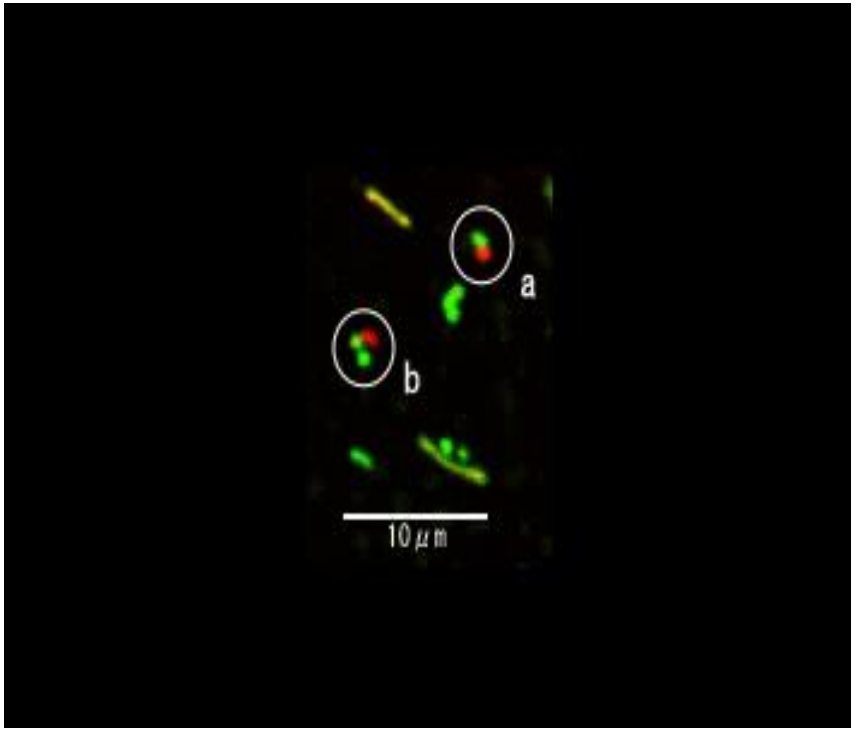




ROI1: 488nm for FITC only. (Green colour)
ROI2: 405nm for DAPI, 488nm for FITC, and 552nm for TRITC
ROI3: 552nm for TRITC only (Red colour)
ROI4: 405nm for DAPI only (Blue colour)

光转化荧光蛋白Kaeda

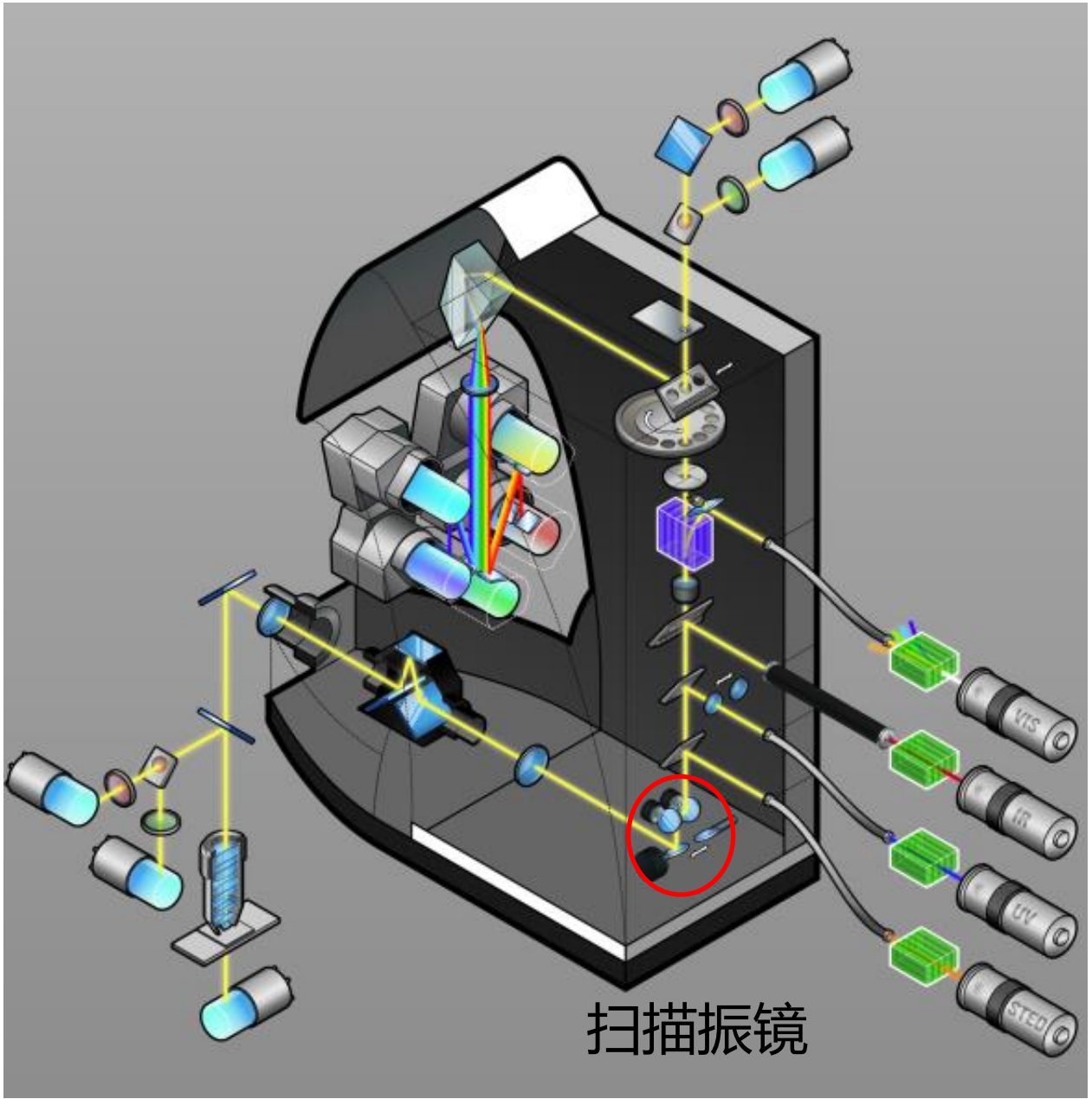




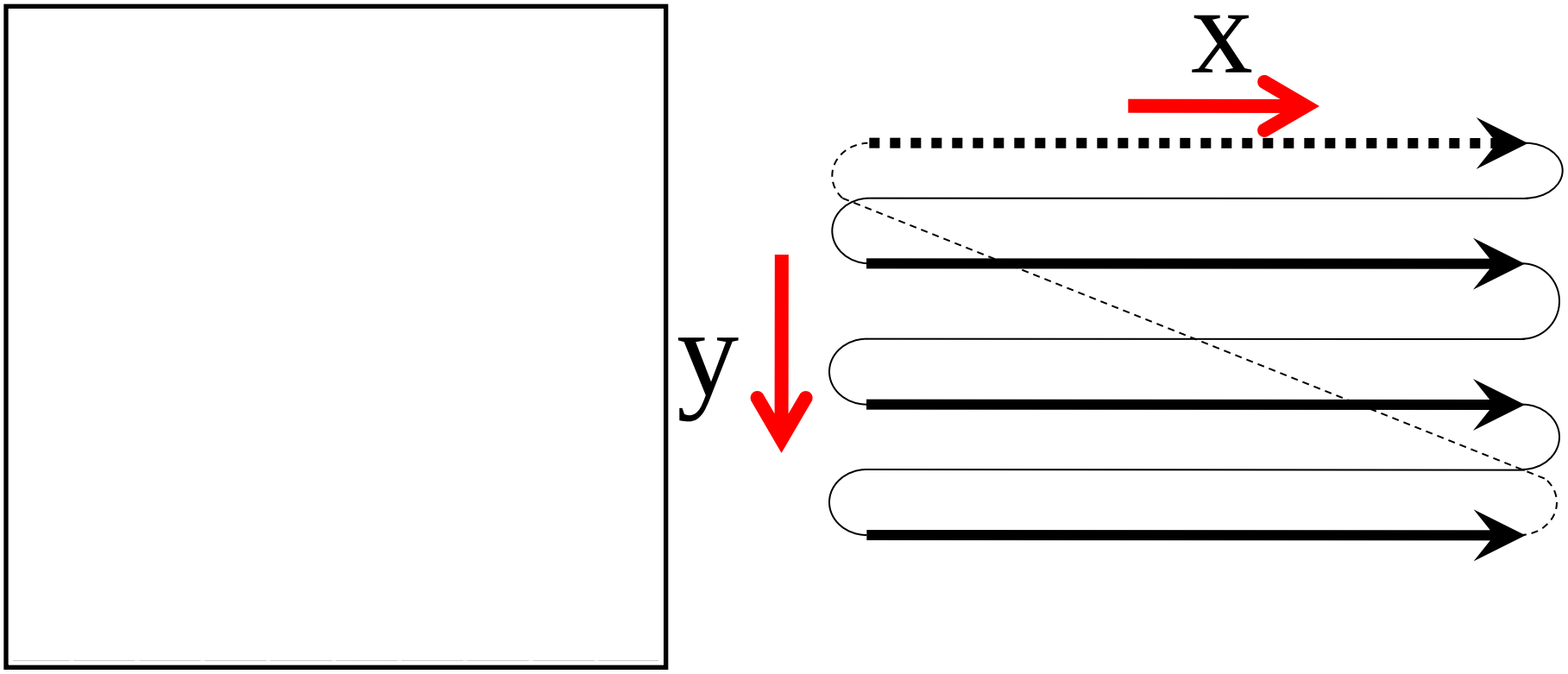
Fusion of mitochondria in single onion bulb epidermal cells transformed with Kaede fusion proteins.

Arimura S *et al.*, PNAS, 2004

Leica TCS SP8 扫描头



扫描振镜的速度决定了共聚焦成像的快慢

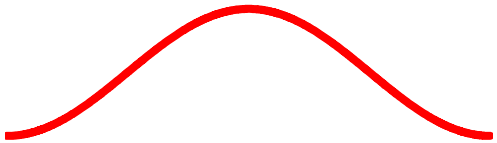


共聚焦扫描过程

由于使用点光源和探测针孔，共聚焦显微镜每次只能探测一个特定的点的信息。为了能够得到一个二维的图像，必须要在x- 和 y-方向移动采样点。

线性扫描：相同扫描速度下的图像信噪比更好

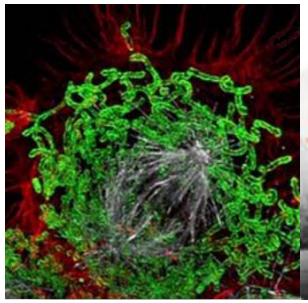
传统的正弦扫描

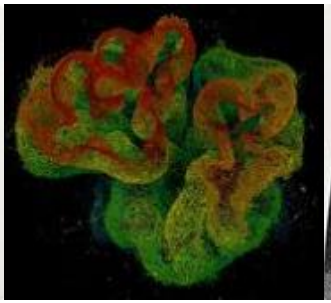


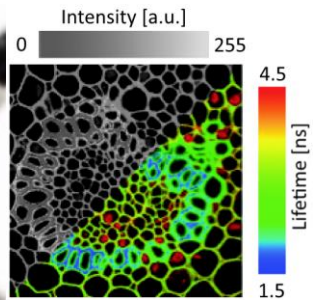
非对称的线性扫描

Dwell time	1.20µs	3.05 µs

512*512 @ 400Hz







- Lightning
- STED
- FALCON

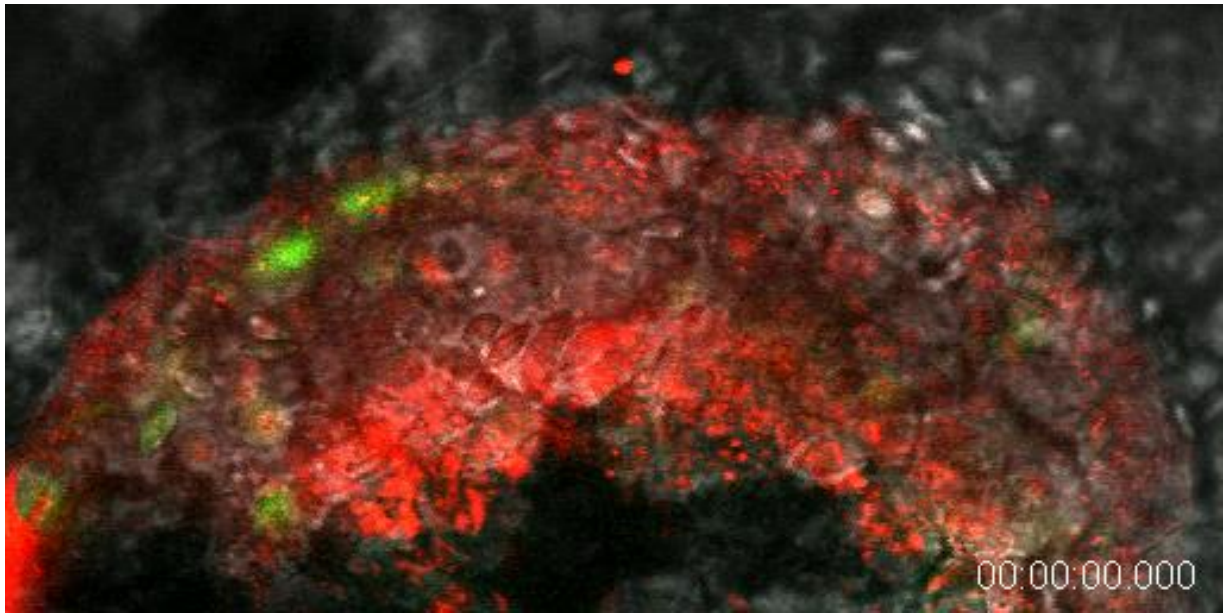
光子计数模式： 2.5倍的亮度提高

普通模式： 58% 信噪比提升

徕卡SP8 – 双扫描头

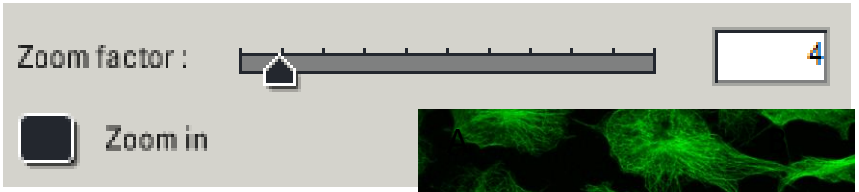
同一系统内两套扫描振镜，分别实现高分辨率扫描 + 高速扫描

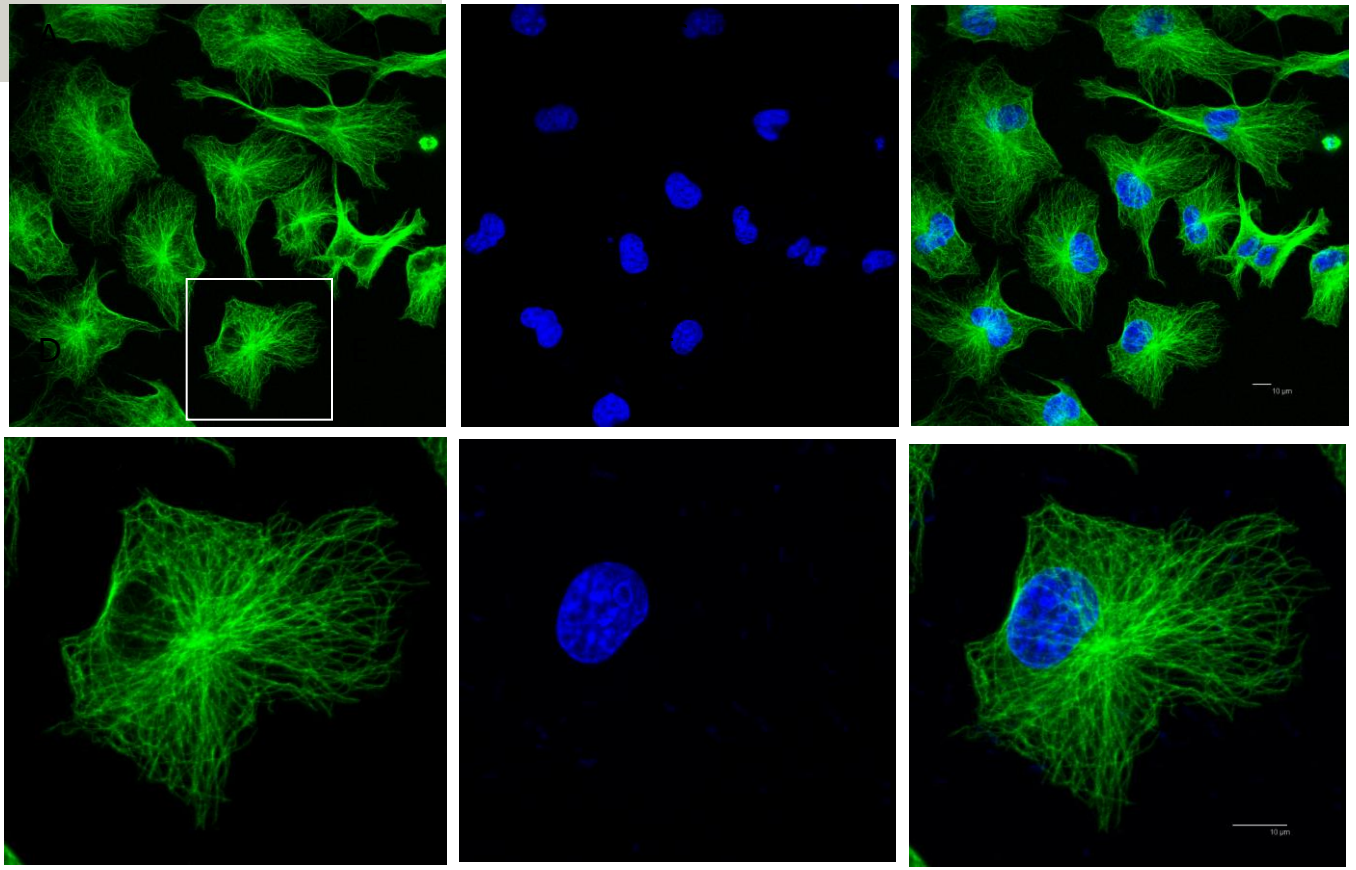
扫描器	扫描速度 @ 512×512
全视野扫描器	7 fps
共振扫描器 8K	28 fps



血细胞流动

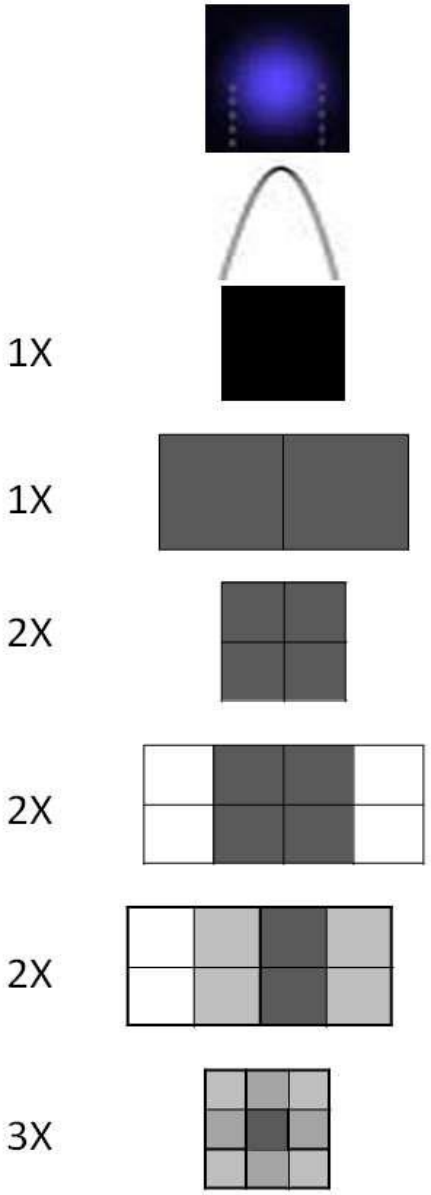
激光点扫描成像光学放大





上方为63×物镜下不放大扫描图像，下方为zoom放大图像

Nyquist采样定律



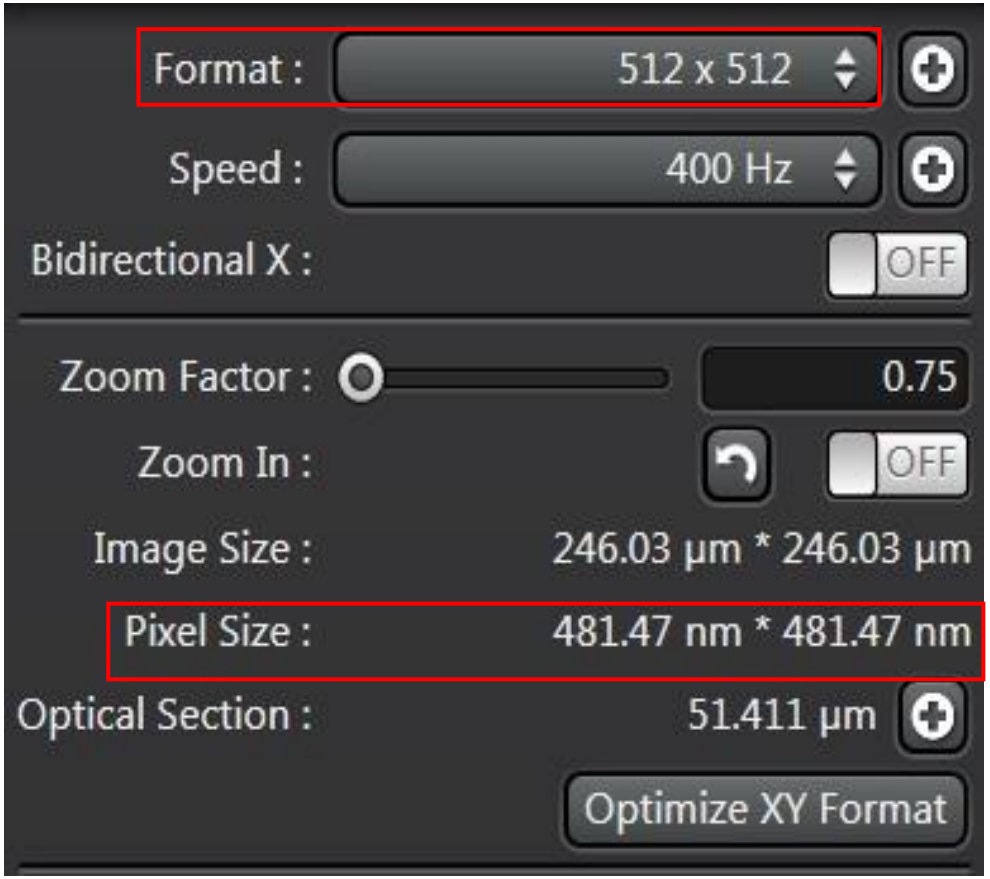
采样频率必须要达到信号频率的2-3倍。

$$d_{pix} = \frac{1}{3} \times r_{xy} = \frac{1}{3} \times \frac{0.61 \times \lambda ex}{NA}$$

为了获得最佳分辨率，需要达到“像素点尺寸(pixel size)”=“当前所使用物镜的横向光学分辨率大小”的1/2 – 1/3。

Magnification	*63*	*40*	*10*
Numerical Aperture	1.4	1.25	0.4
Optical Resolution [µm]	0.14	0.16	0.5
Field (Edge) [µm]	238	375	1500
# Resel (Field / Resolution)	1700	2344	3000
2 x Oversampling	3400	4688	6000
3 x Oversampling	5100	7031	9000

扫描图像参数设置



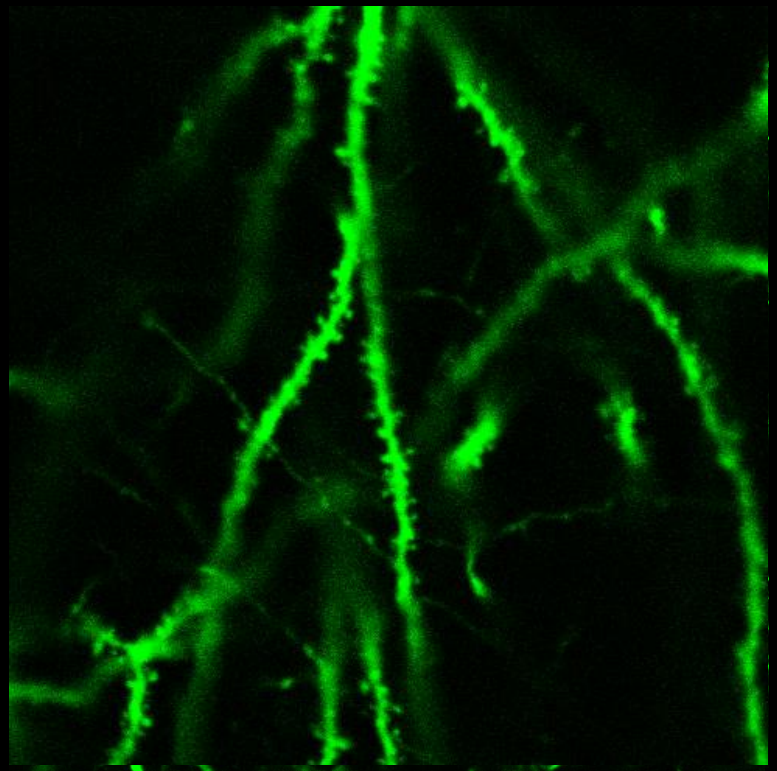
HCX Apochromat 40x/1,25 oil
At ca 550nm wavelength

lateral resolution: ≈ 0,2 µm
axial resolution: ≈ 1,0 µm

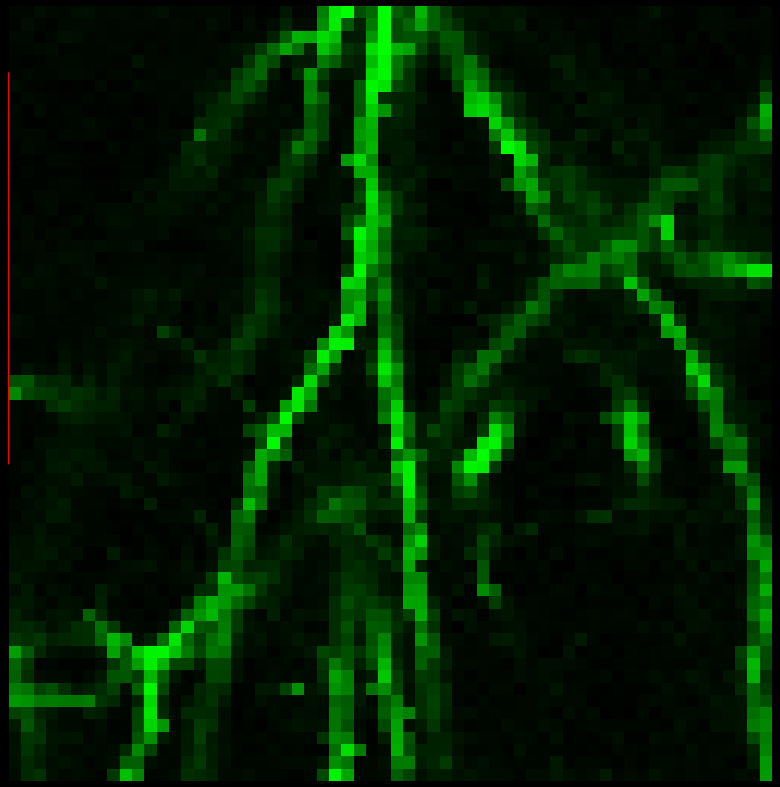
高分辨率要求：**pixel size < 0.5 * xy侧向分辨率**

但扫图分辨率越高，光毒性和淬灭越强

20x/0.7 NA Objective + 1024x1024 pixels = loss of detail



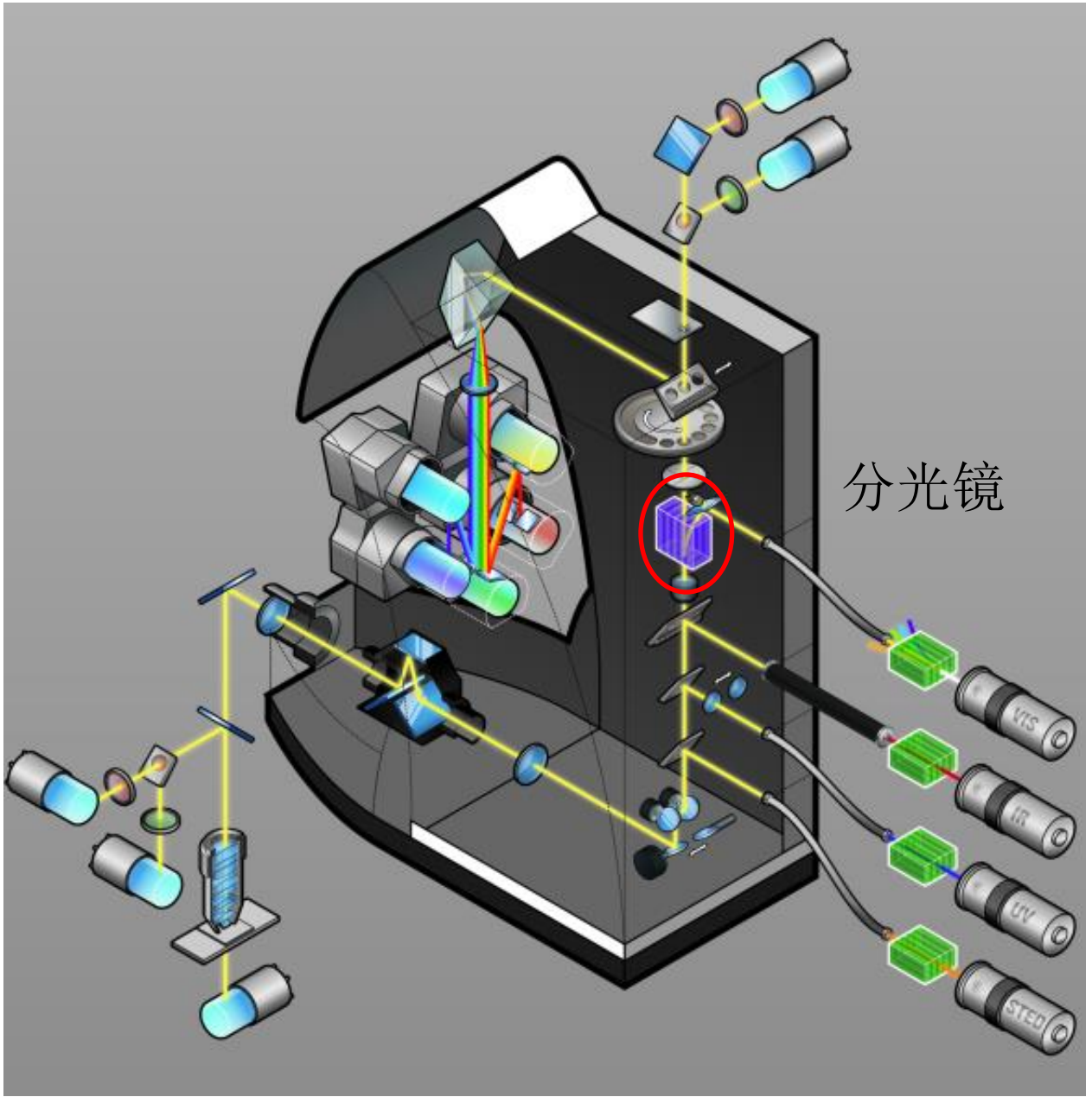
8192x8192 detail



1024x1024 detail

Courtesy: Dr. Michael E. Calhoun, Department of Cellular Neurology, Hertie Institut for Clinical Brain Research, Tübingen, Germany

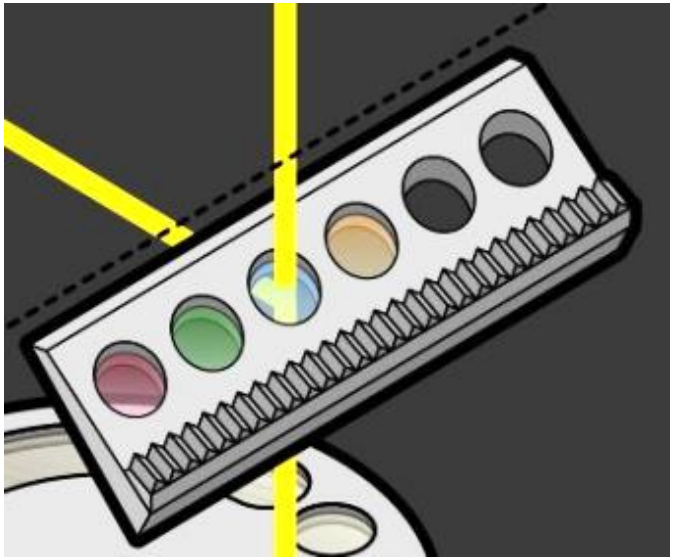
Leica TCS SP8 扫描头



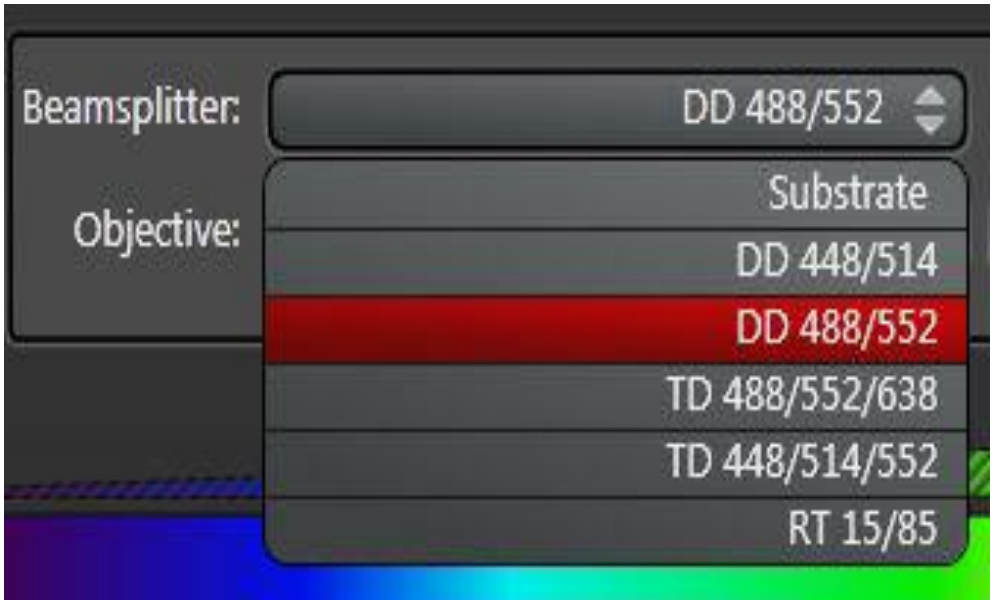
分光镜

荧光

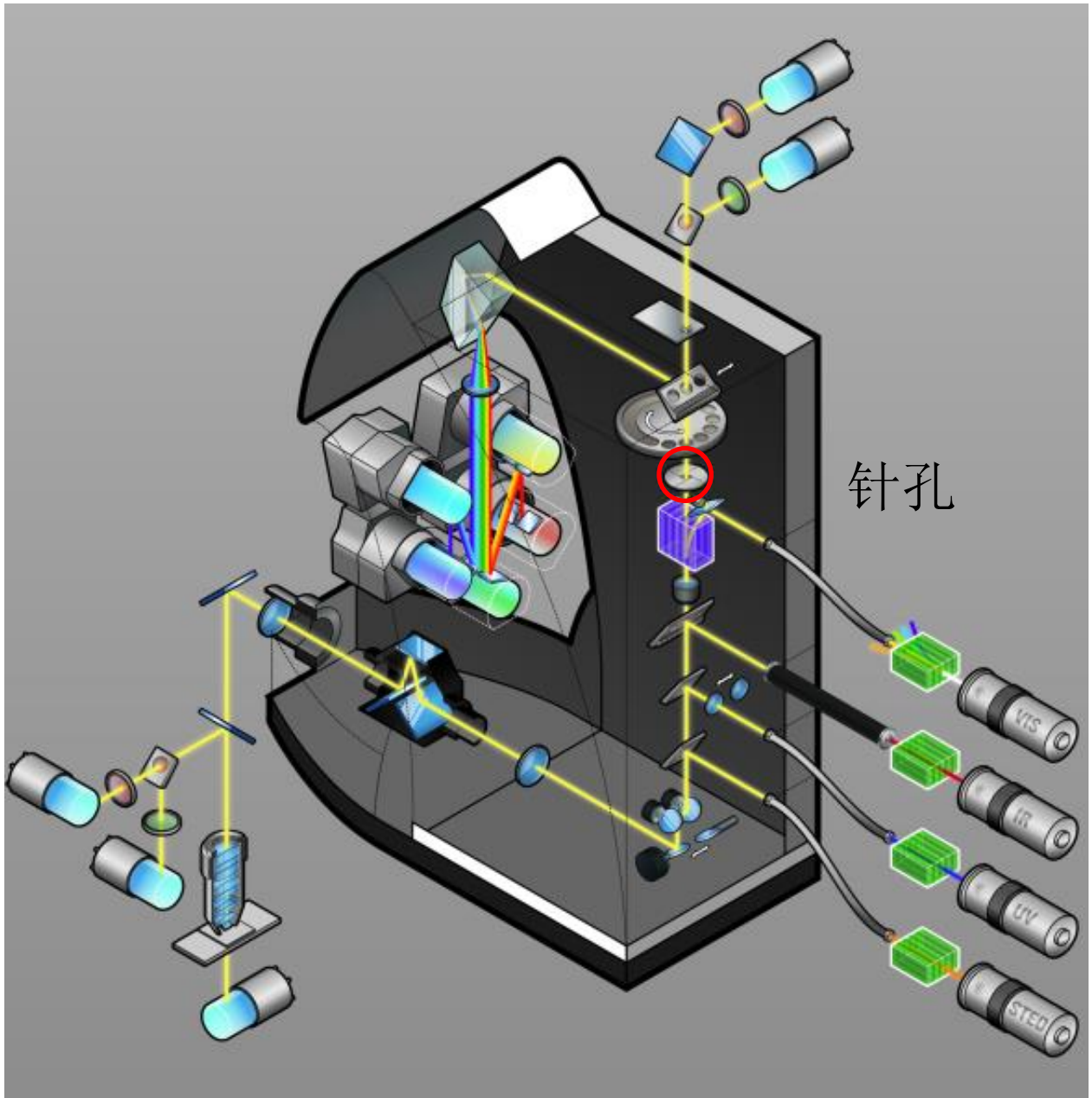
激光



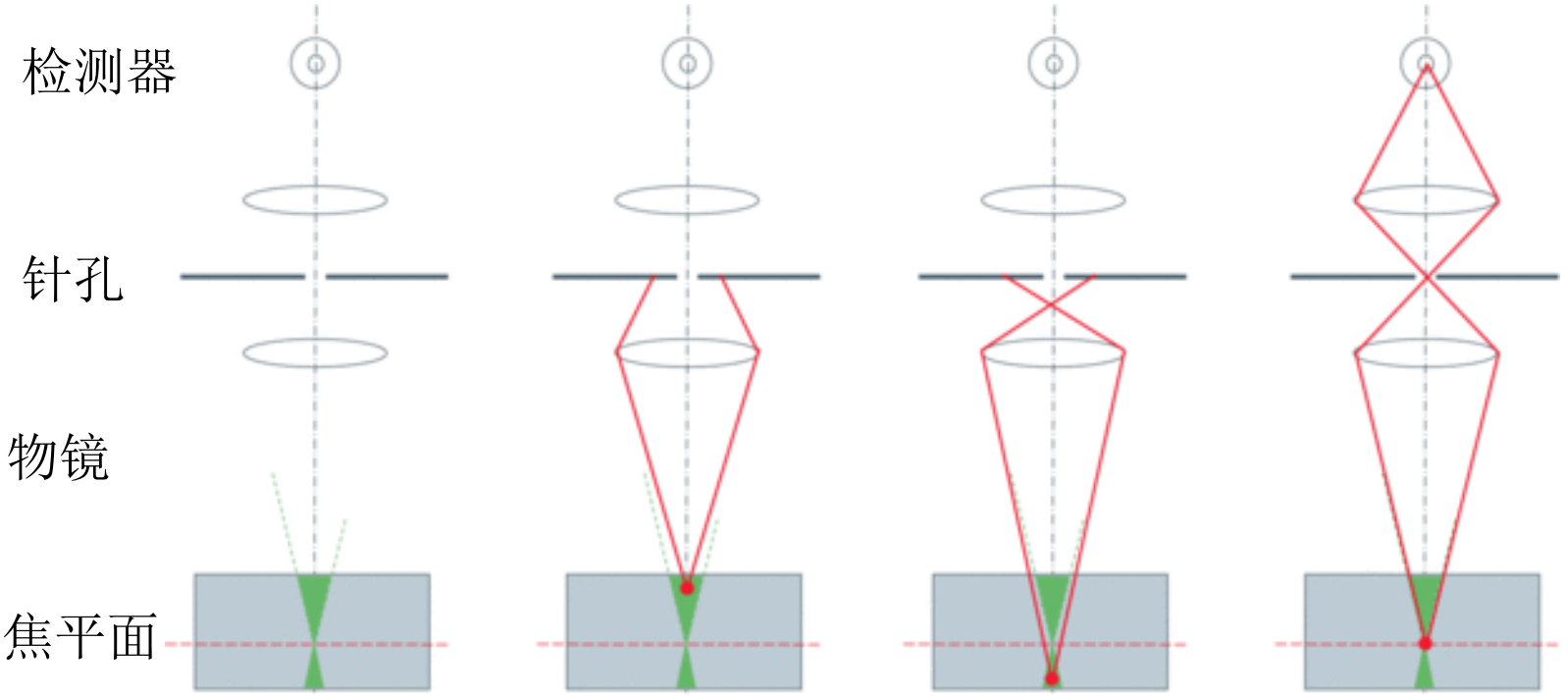
激光 + 荧光



Leica TCS SP8 扫描头

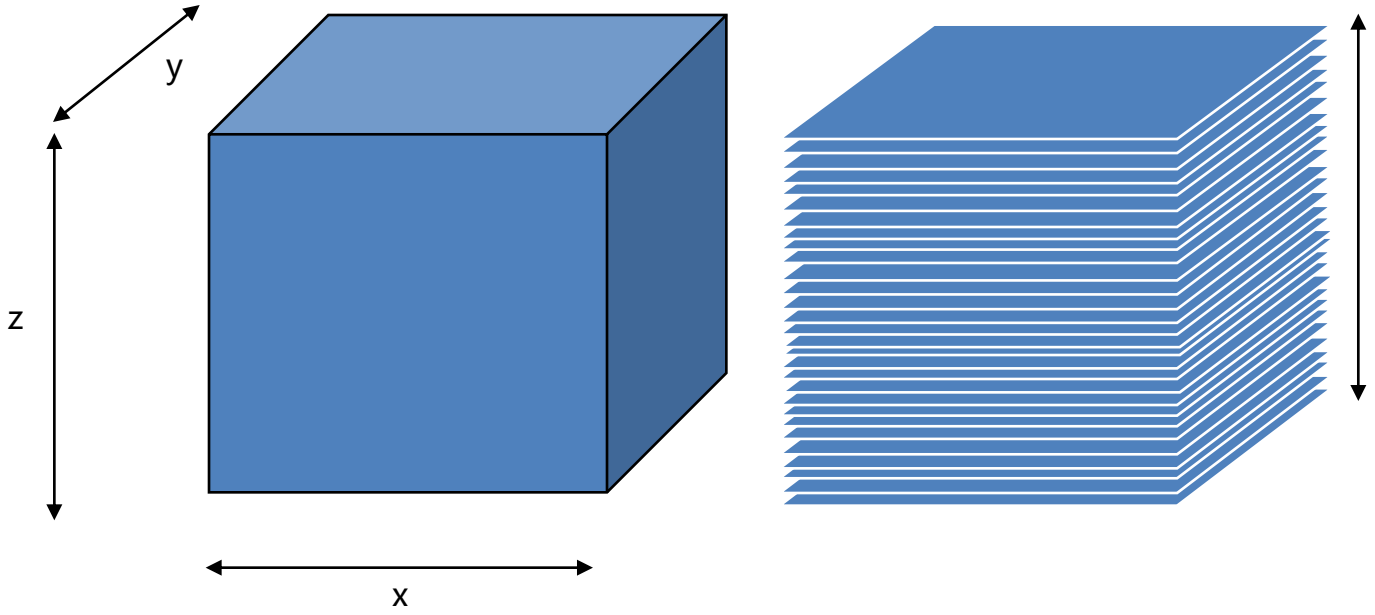


共聚焦针孔（pinhole）

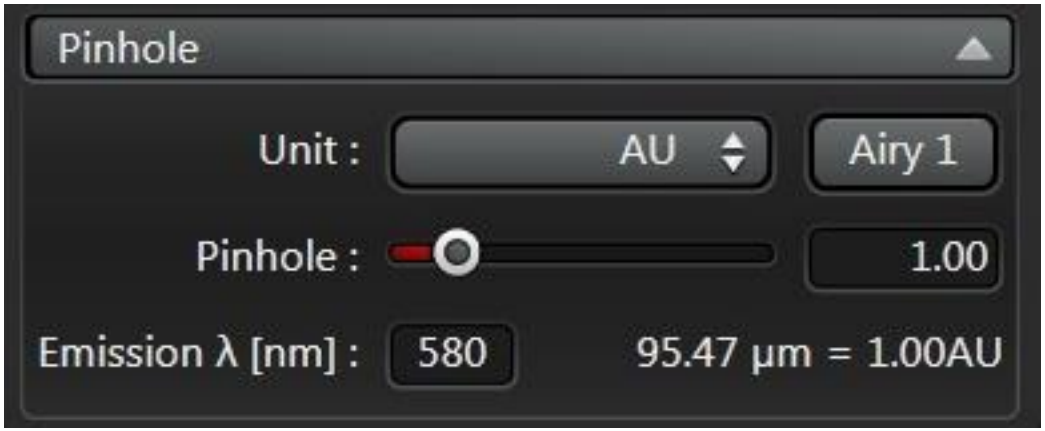


三维成像

光学层切



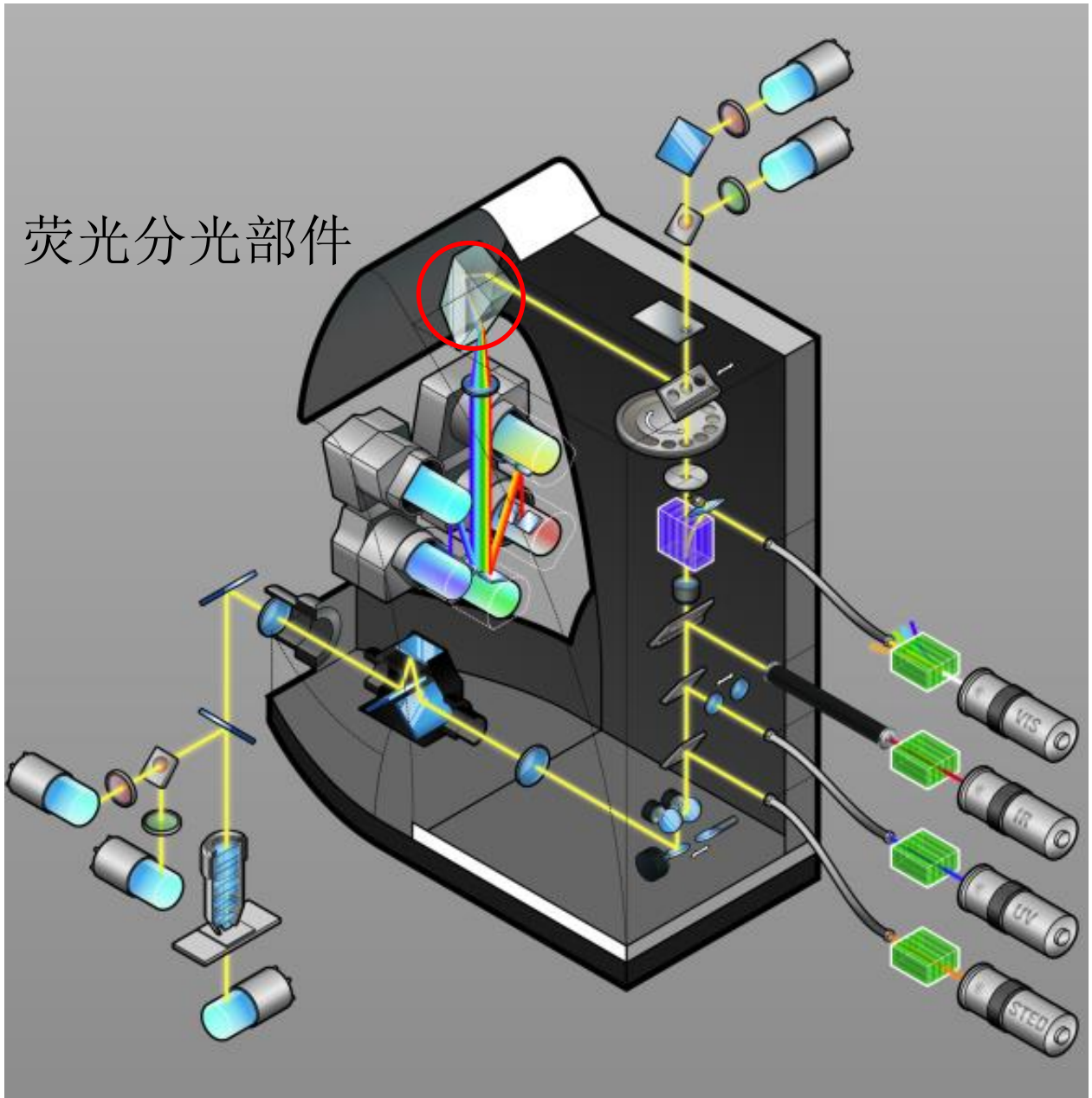
共聚焦针孔参数设置



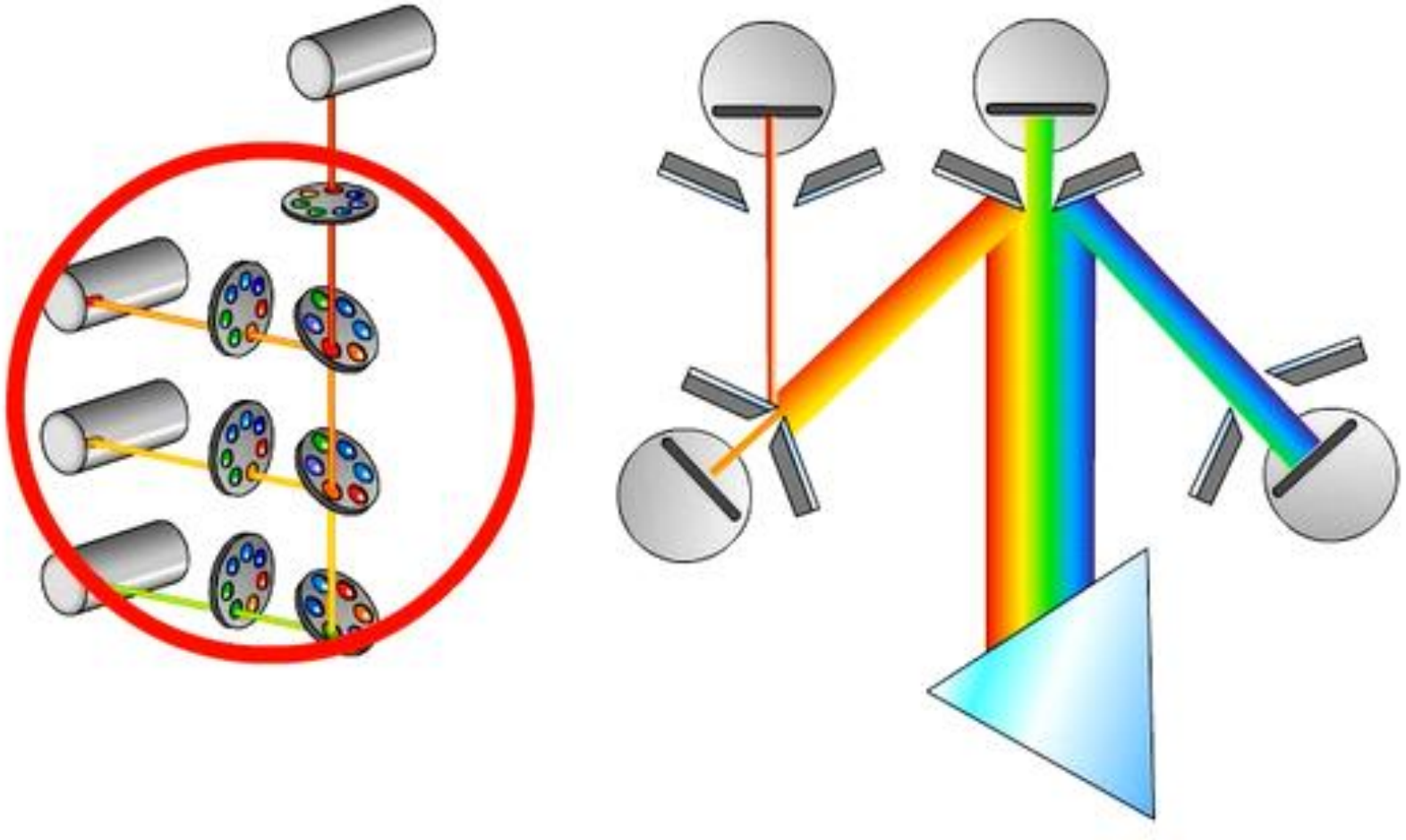
针孔越大，信号越强，但分辨率越低，
针孔越小，信号越弱，但分辨率越高，
各种性能最平衡的针孔大小为1 AU；

当荧光很弱时，可适当调大针孔直径

Leica TCS SP8 扫描头

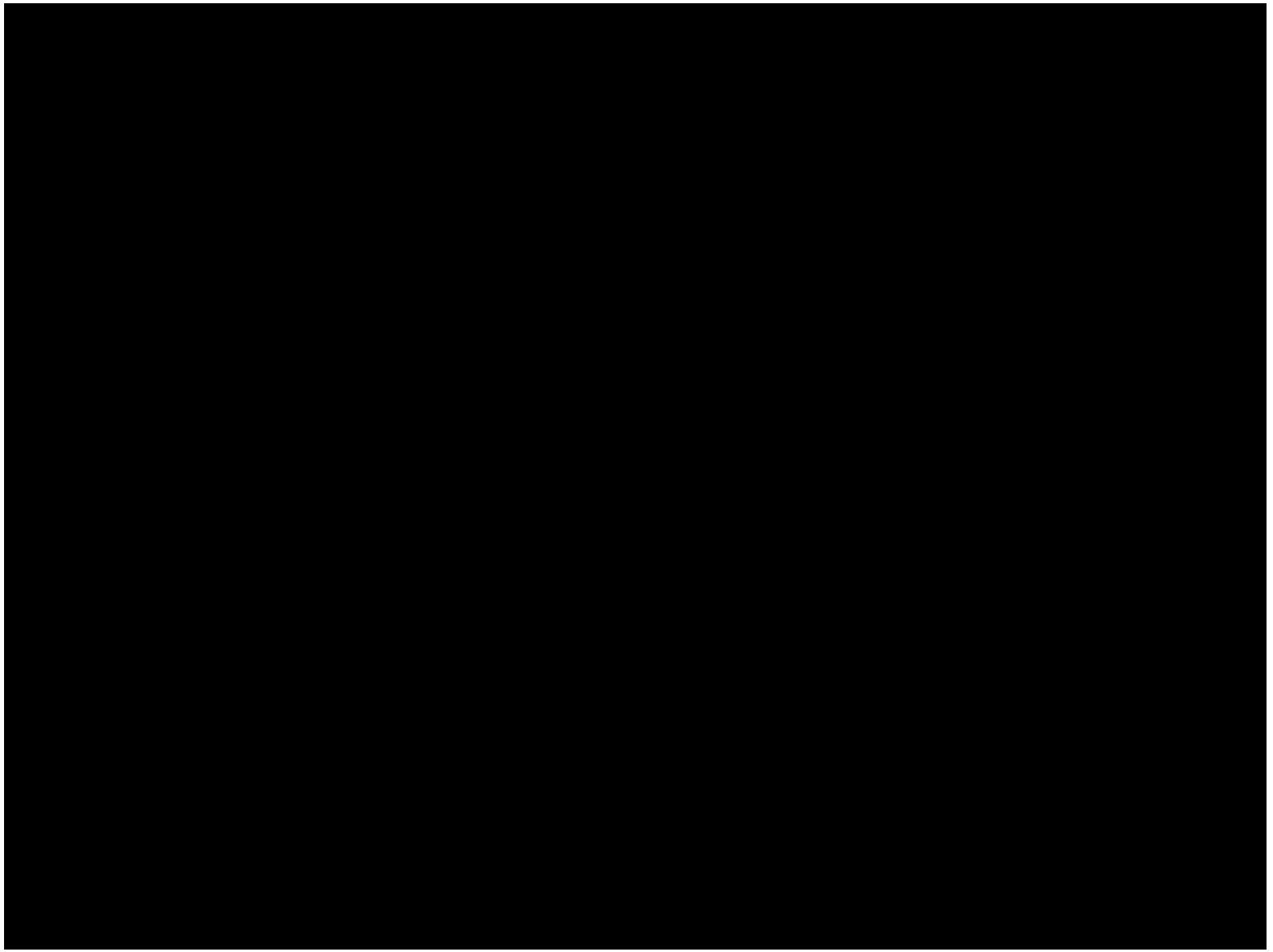


滤色片分光与棱镜分光

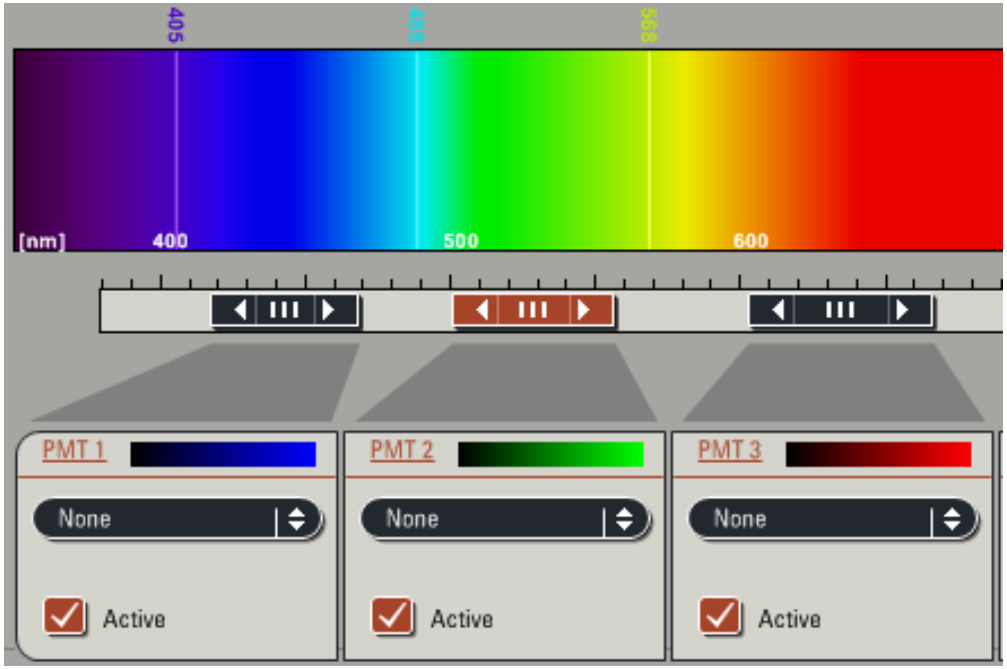


Leica SP2 – SP8

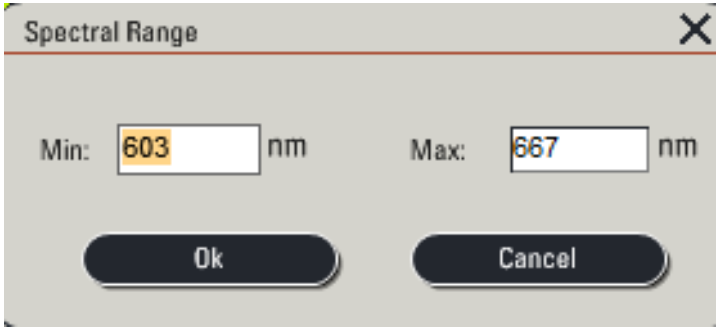
棱镜分光，狭缝检测



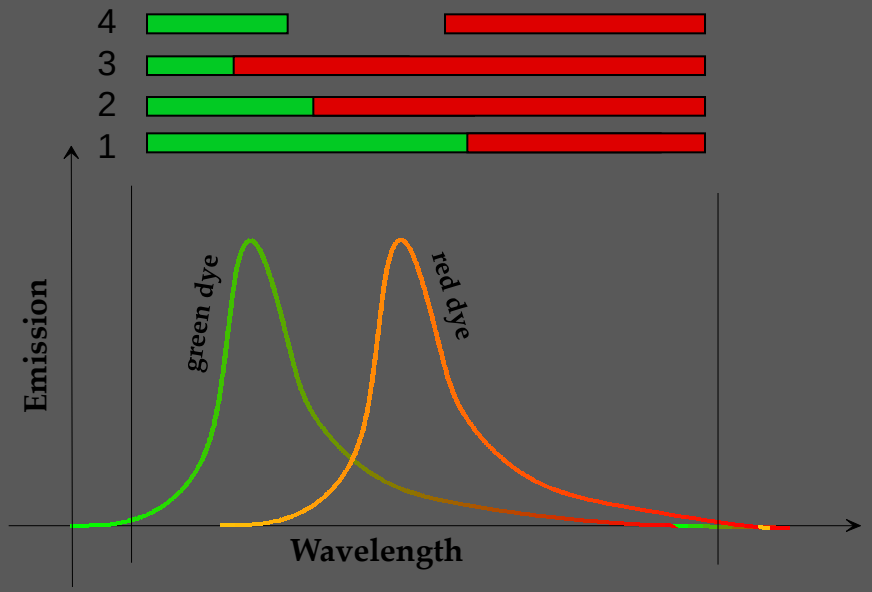
荧光接收范围设置

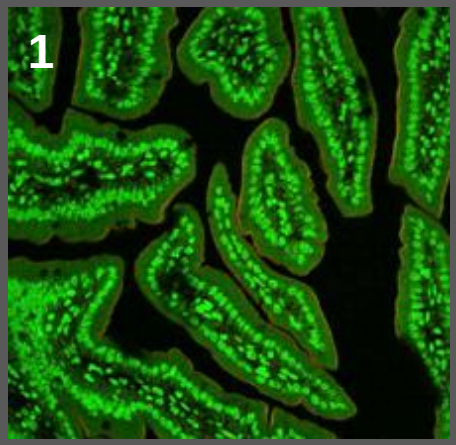


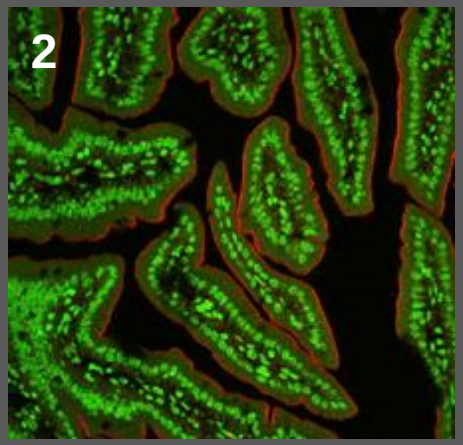
双击滑条可手动更改波长范围

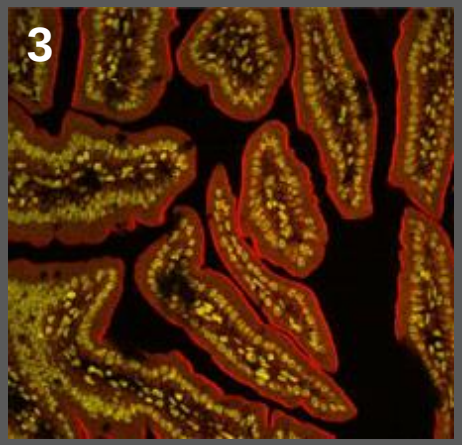


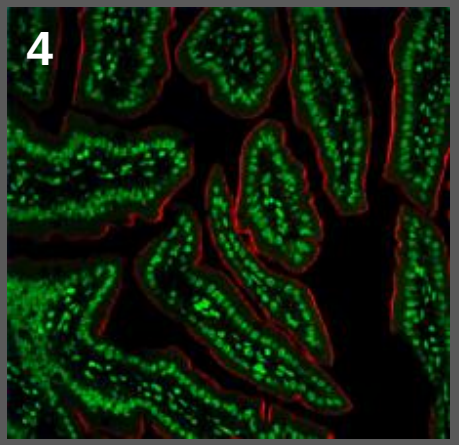
光谱检测器的参数调节





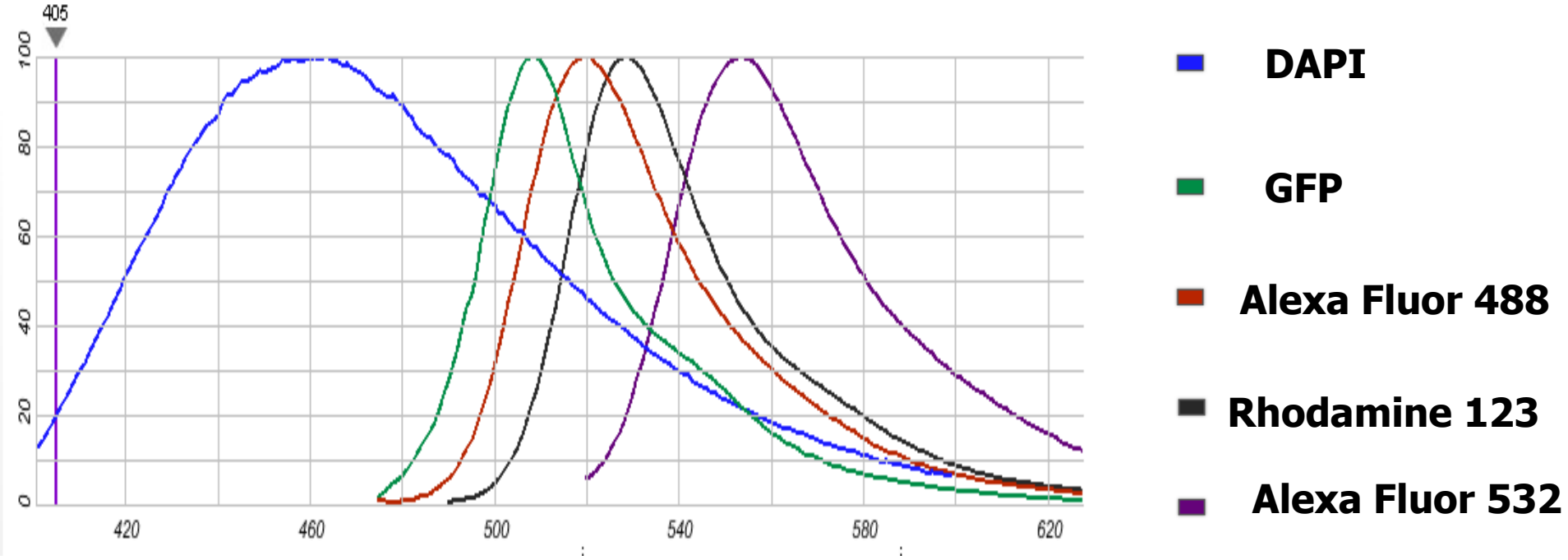






序列扫描

DAPI与其他染料之间的窜色



由于染料之间的荧光发射光谱的交叉（窜色），
所以多色标记时经常需要通过序列扫描进行成像。

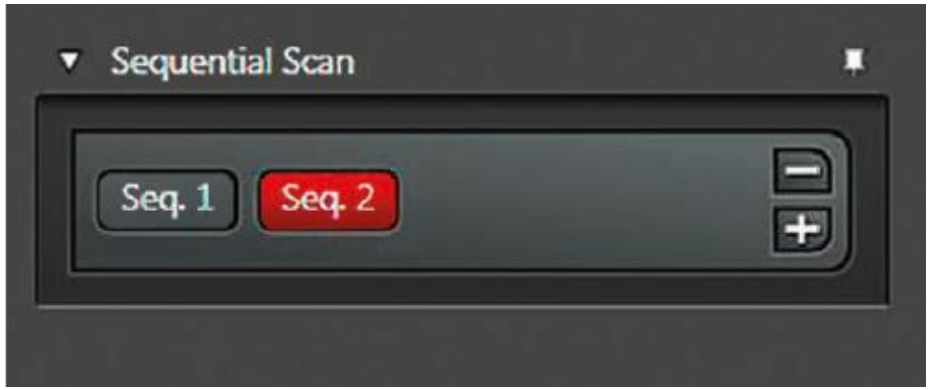
序列扫描

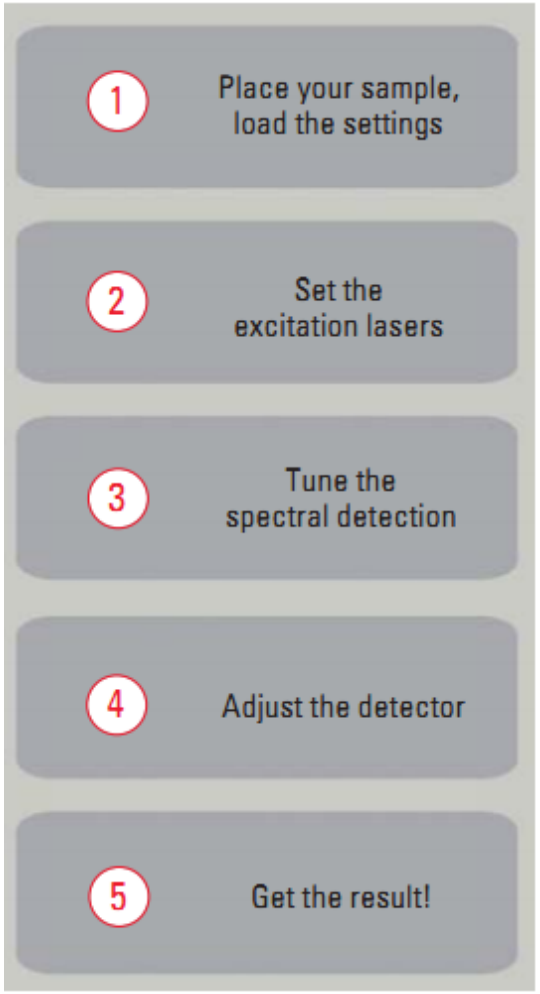
一个序列中只开一根激光谱线和一个检测器

以下另类实验 强烈建议使用序列扫描：

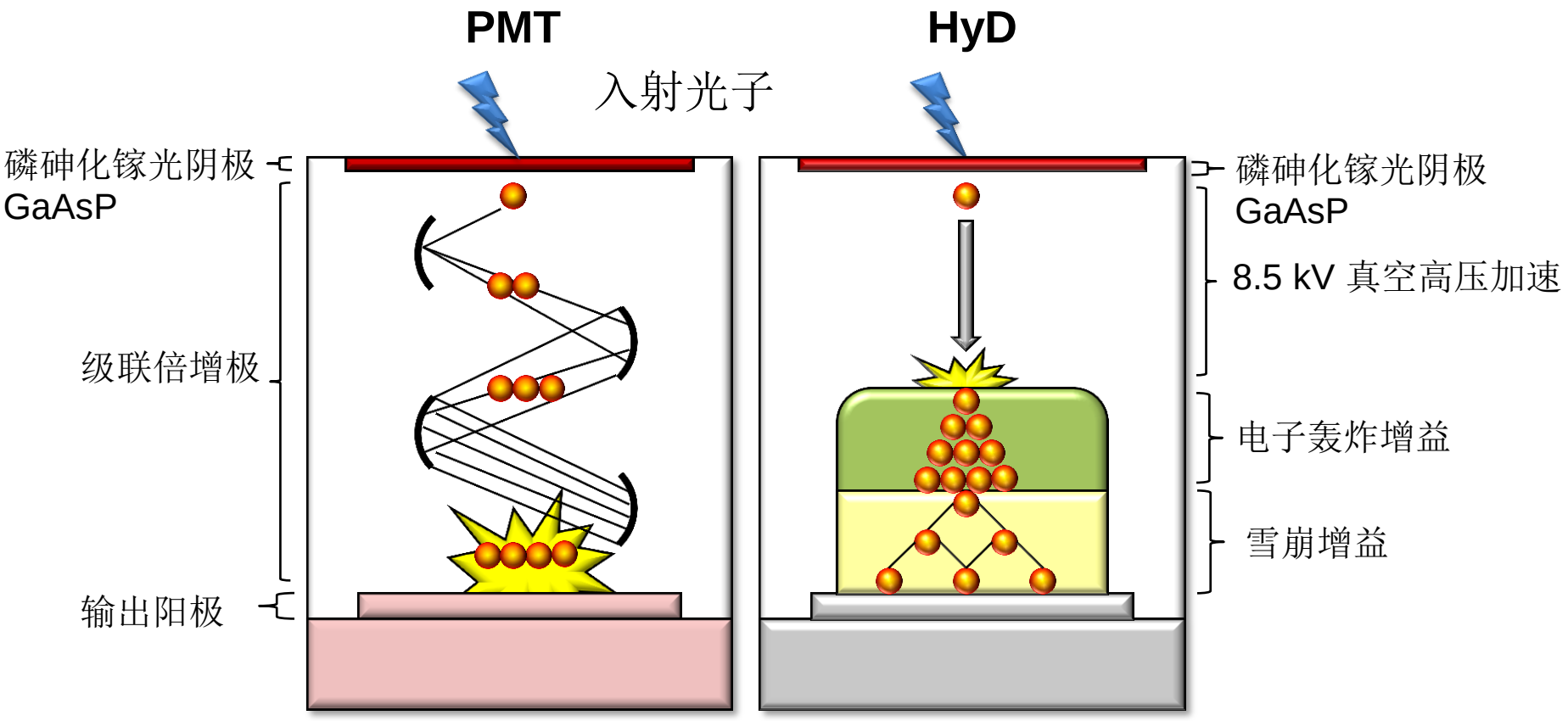
1、与DAPI组合的多色成像

2、共定位分析





检测器



HyD是Hybrid Detector的简写，指的是整合了PMT和APD结构的混合型检测器。

PMT：动态范围大，但是灵敏度较低，背景噪音较高
APD：灵敏度很高，噪音很低，常用于单分子荧光检测
HyD综合了以上两者的优点：高灵敏度，低噪音和大动态范围。

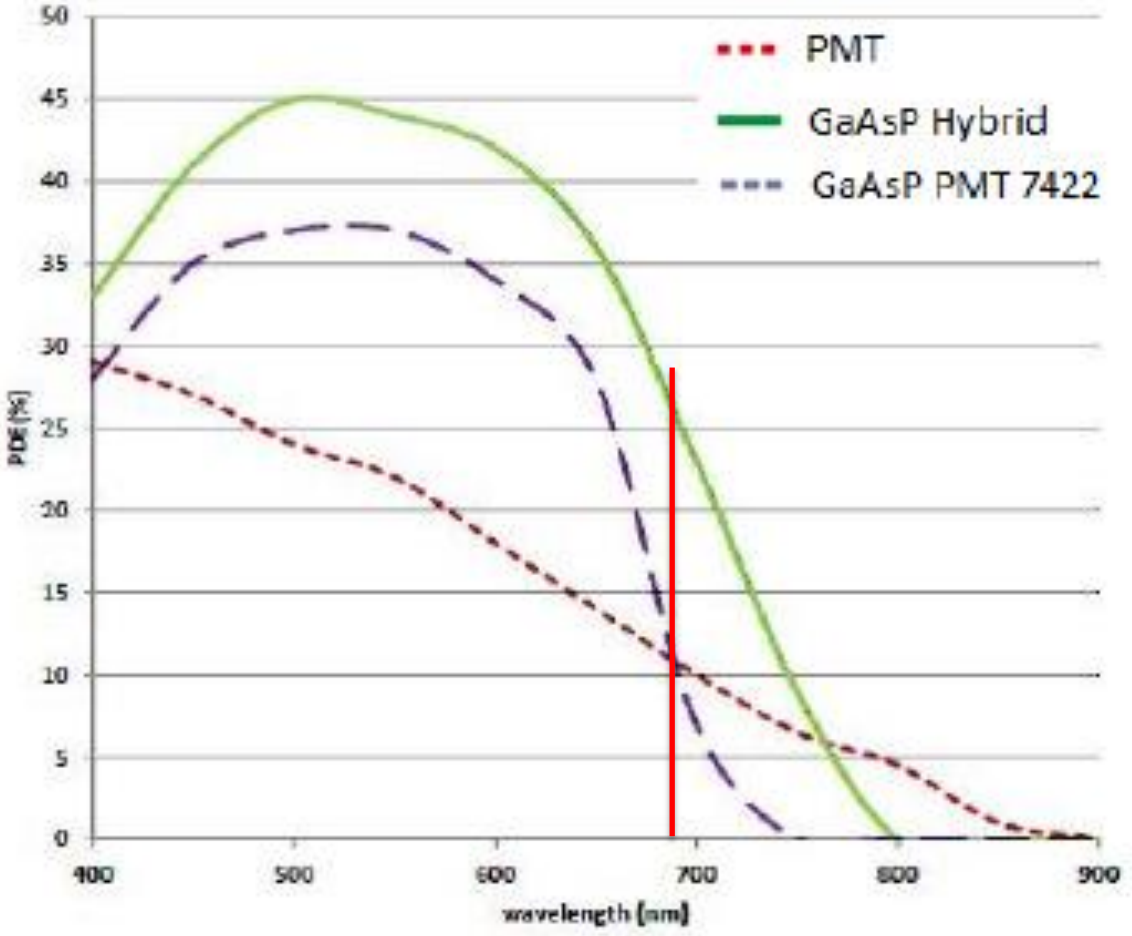
HyD具有更高的光子检测效率

HyD适用于

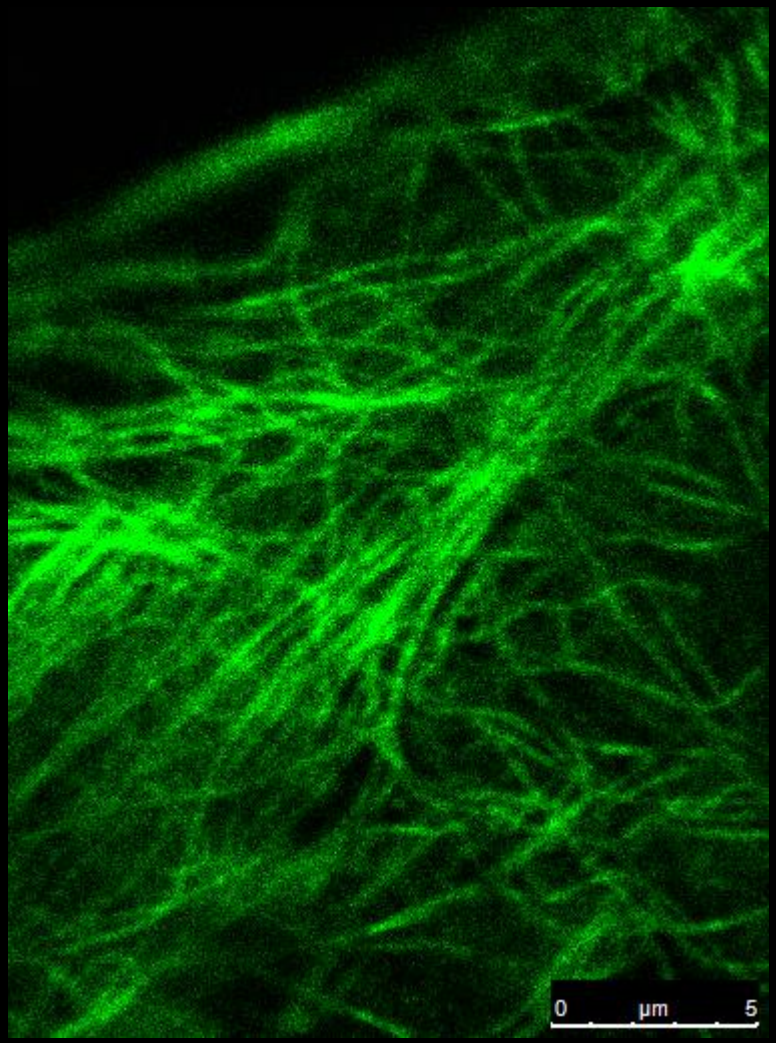
1、弱荧光成像

2、低光毒性活细胞实验

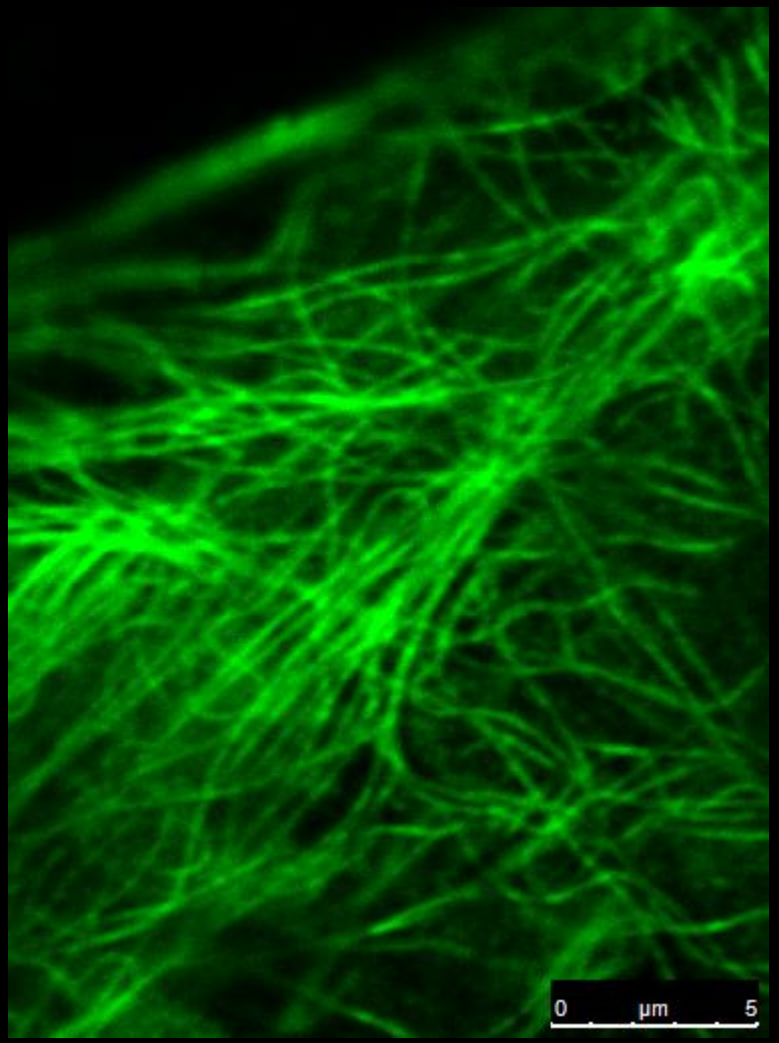
3、深度成像



标准模式

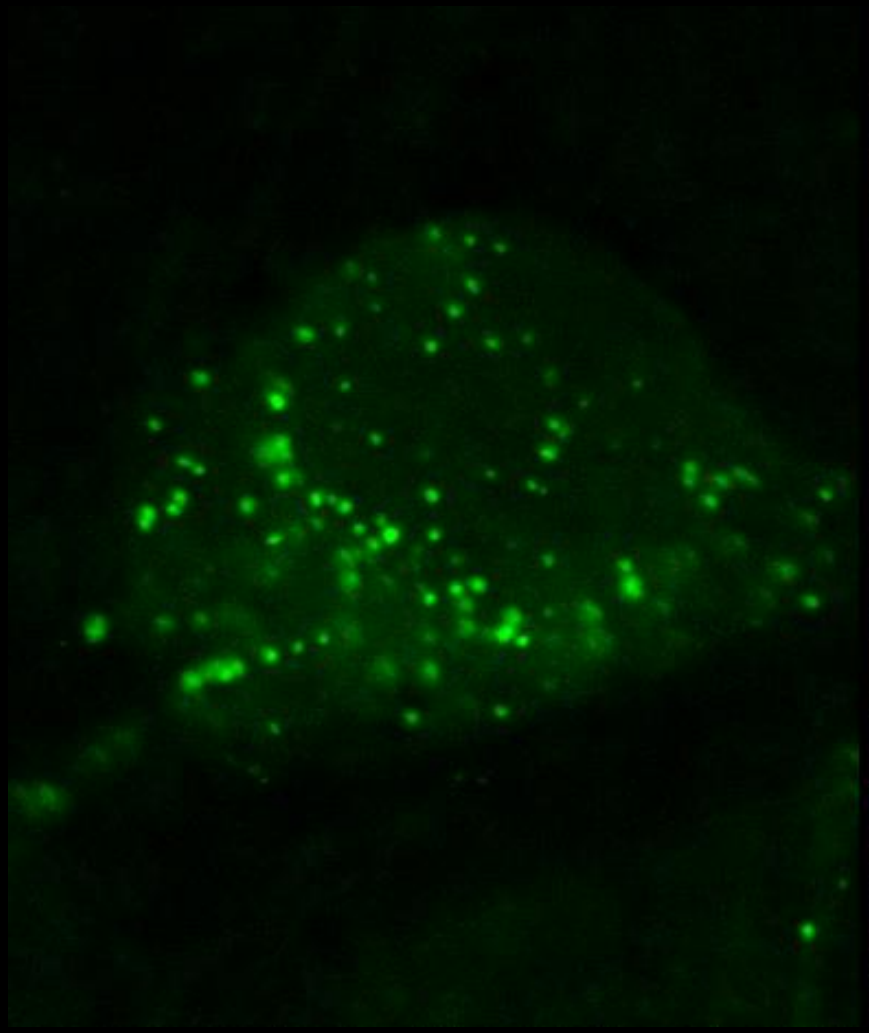


PMT

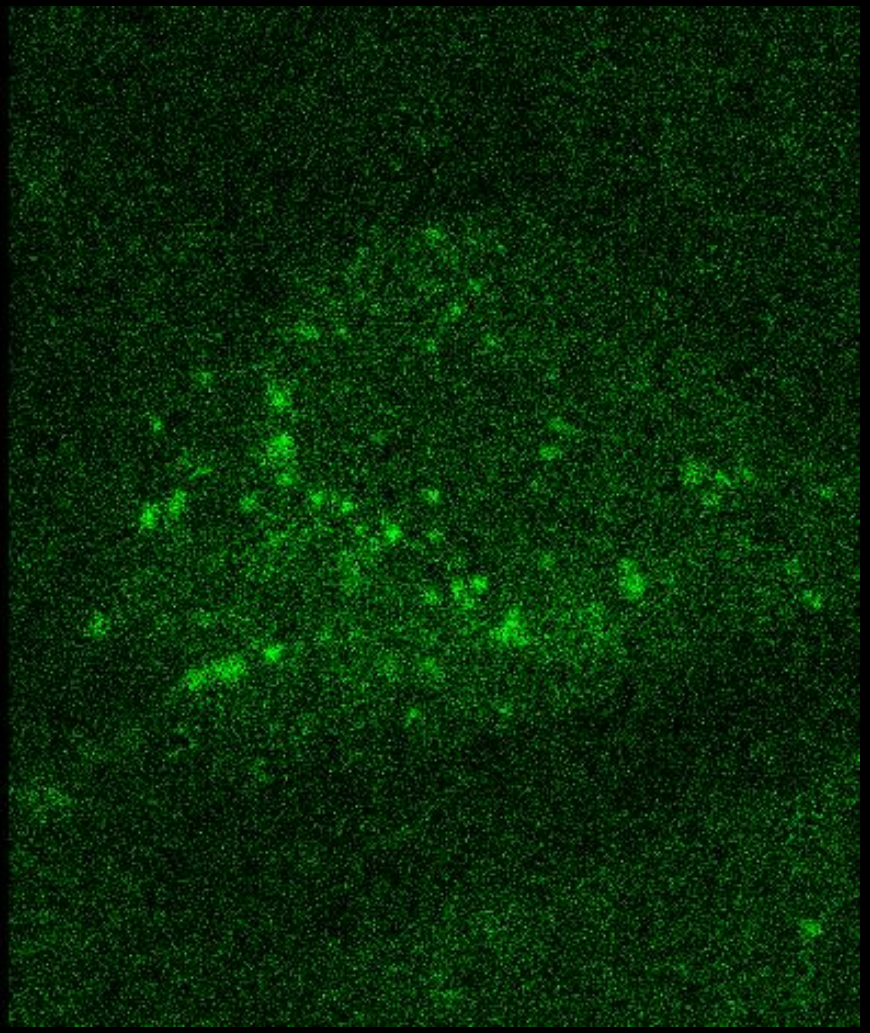


HyD

光子计数模式十分适用于内源表达弱荧光成像

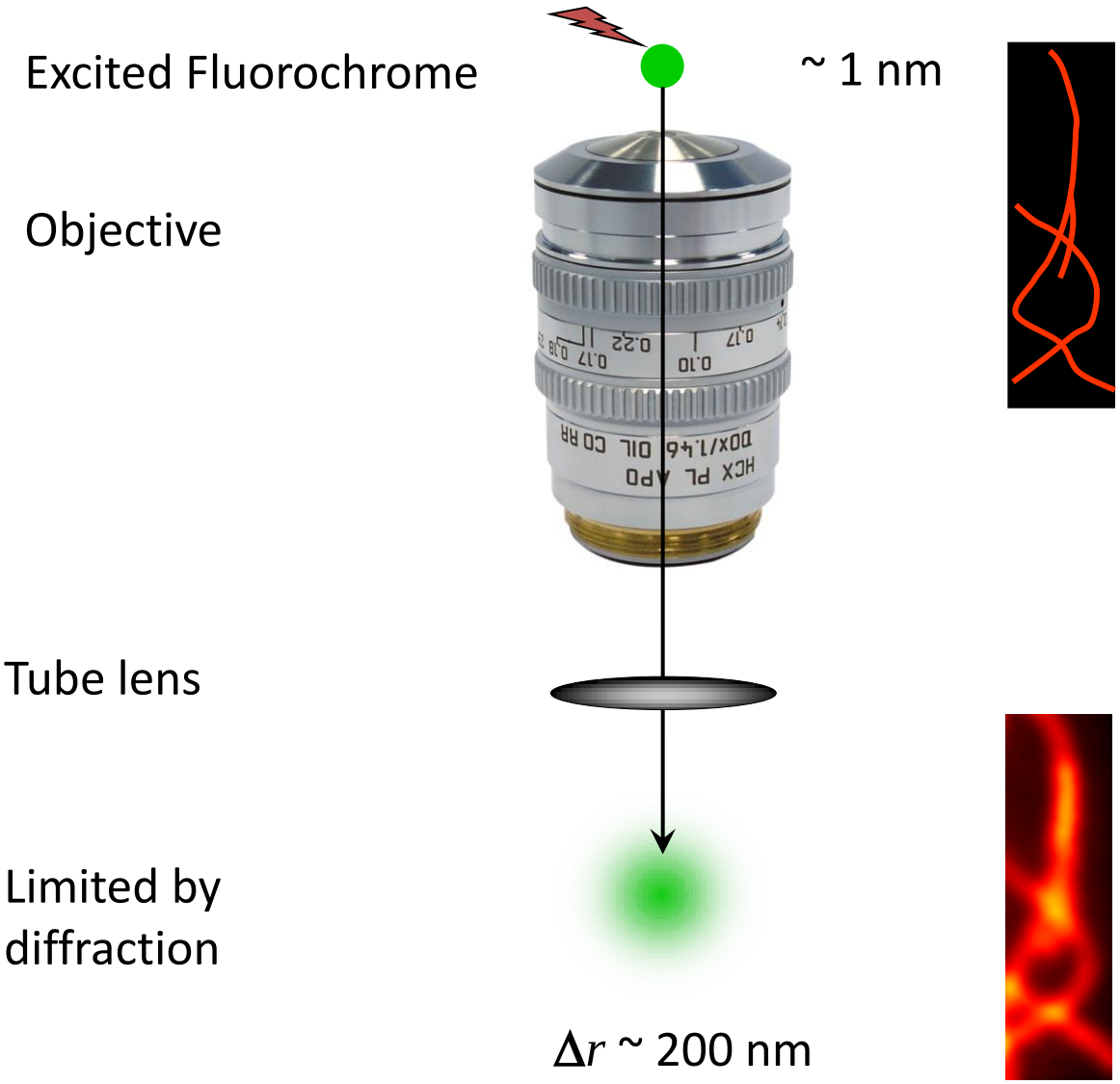


HyD

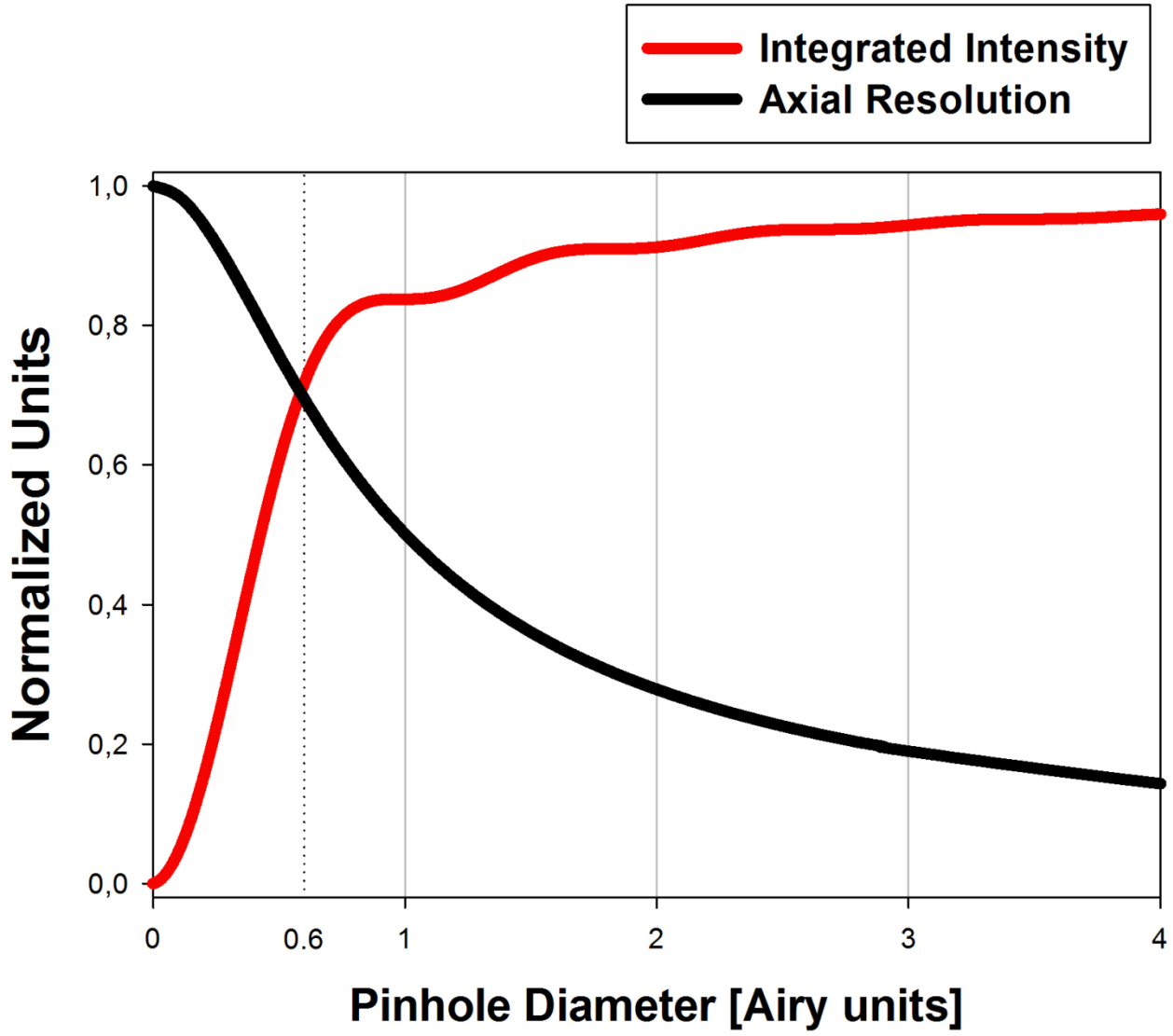


PMT

受衍射限制的成像分辨率



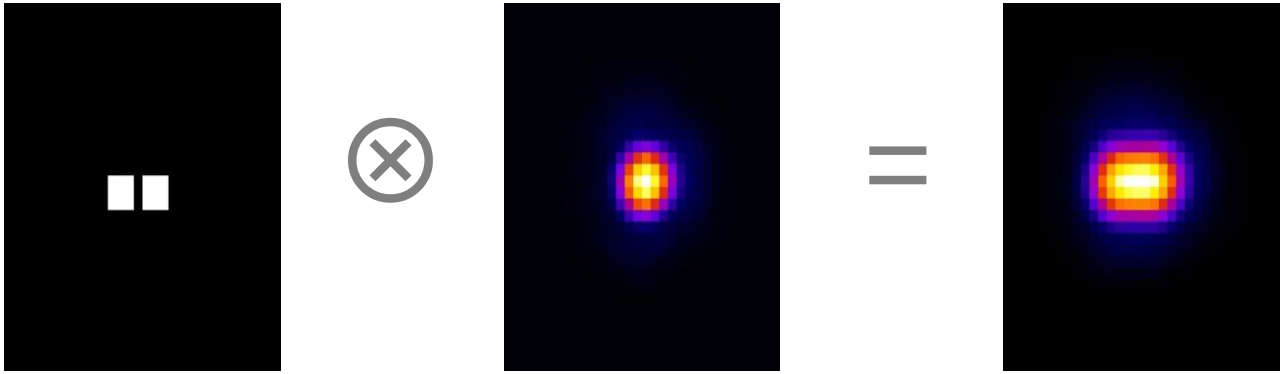
关小针孔可提高分辨率，但是信号强度会减弱，图像信噪比会变差



Convolution and Deconvolution
卷积与反卷积

- Convolution of 2 objects with PSF

Convolution



Convolution and Deconvolution

- 反卷积能提高图像信噪比，同时提高分辨率

Deconvolution

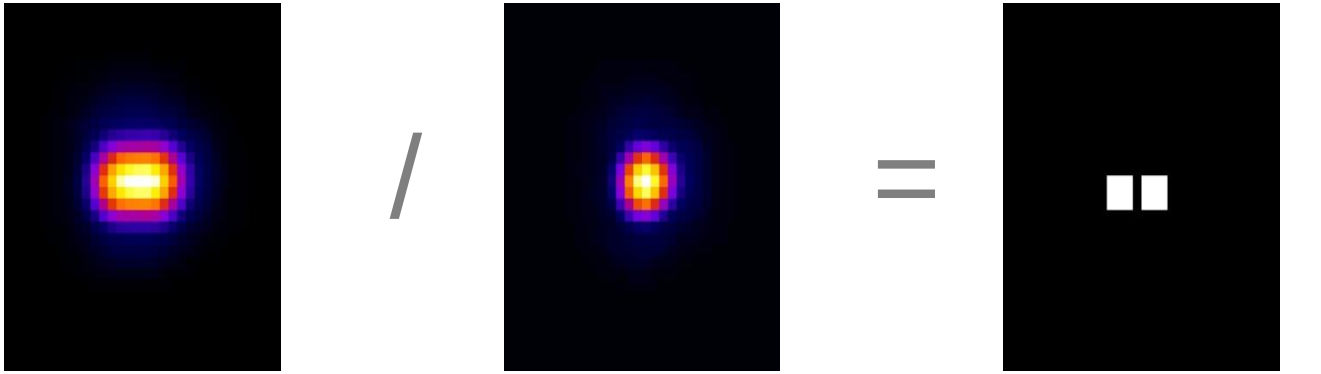
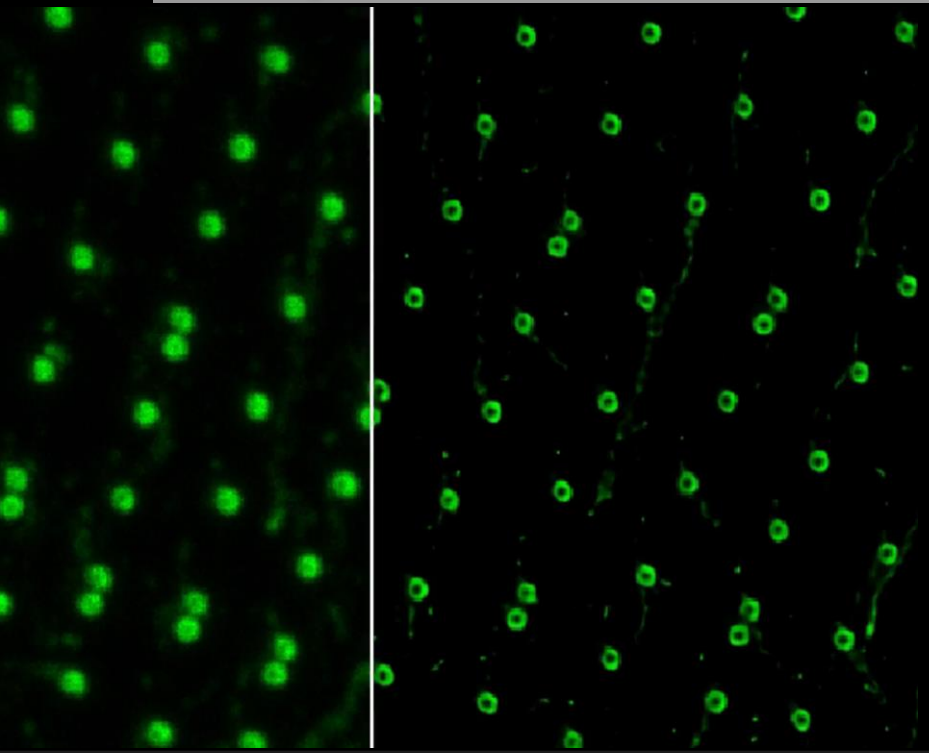


Image　　Optics　　Restored Image

Lightning

Adaptive Multicolor Super-Resolution

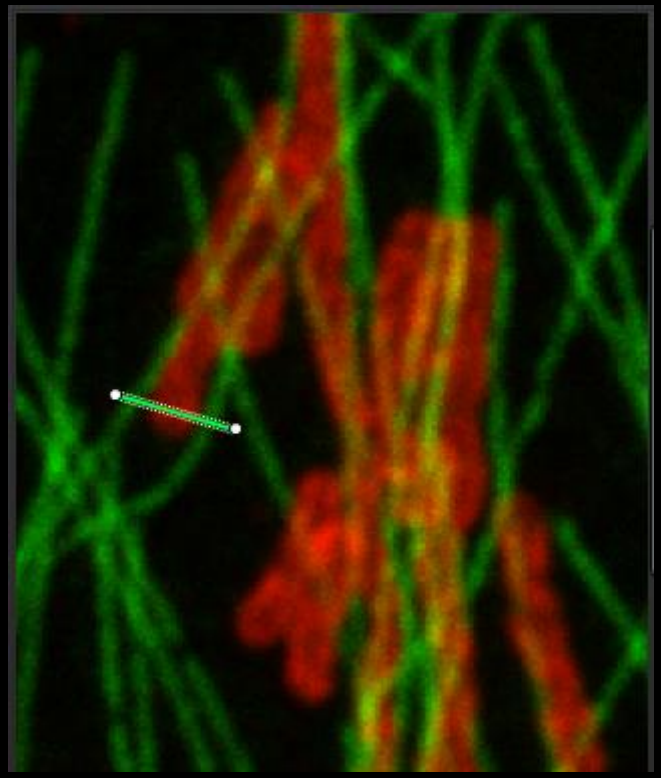


Confocal | STED | MP

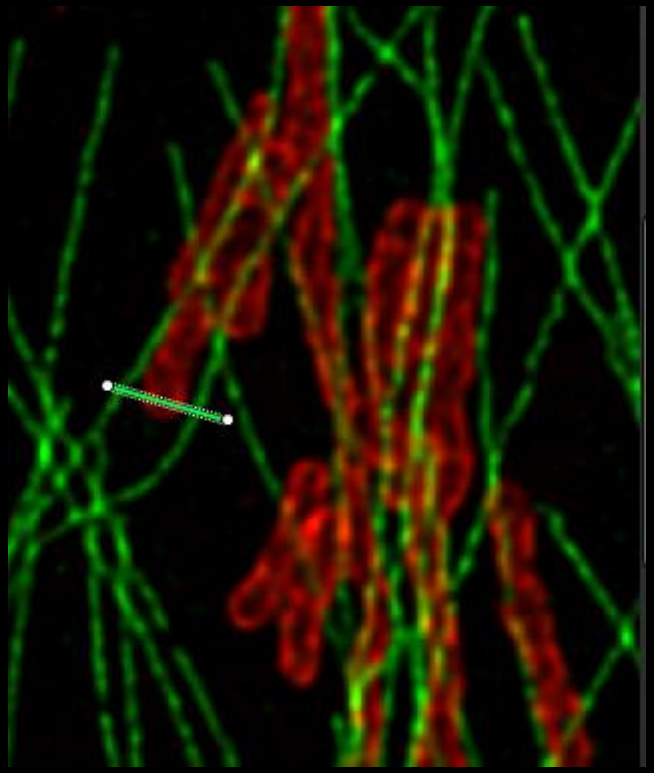
Including every imaging modality

Lightning超高分辨率系统

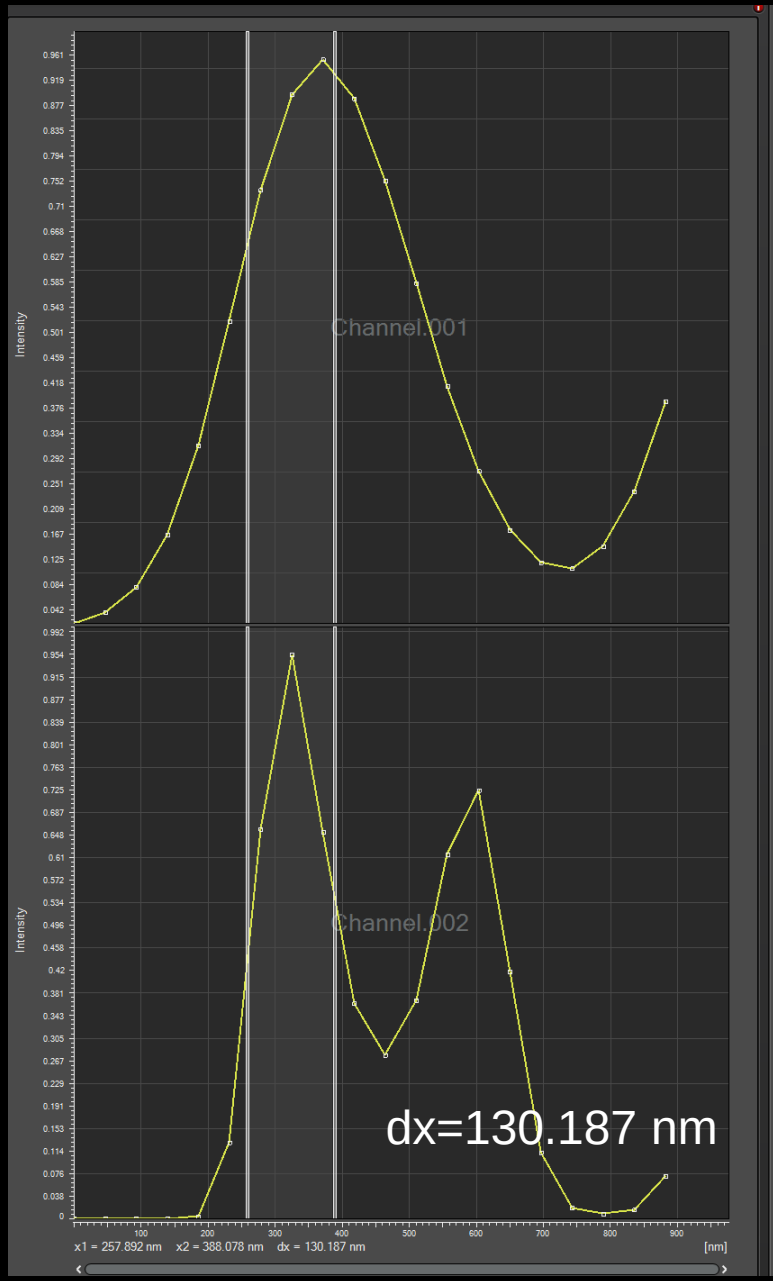
Confocal

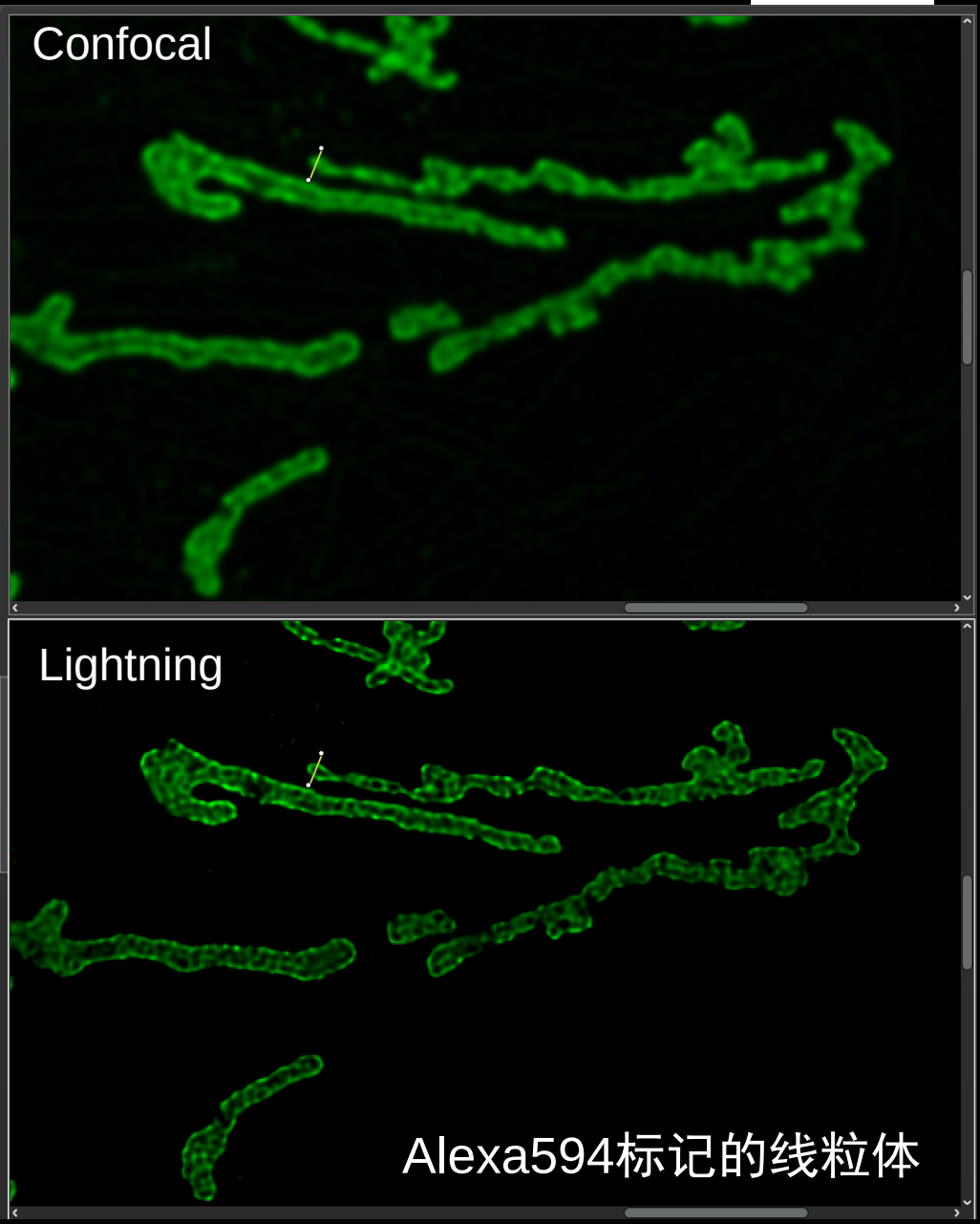


Lightning

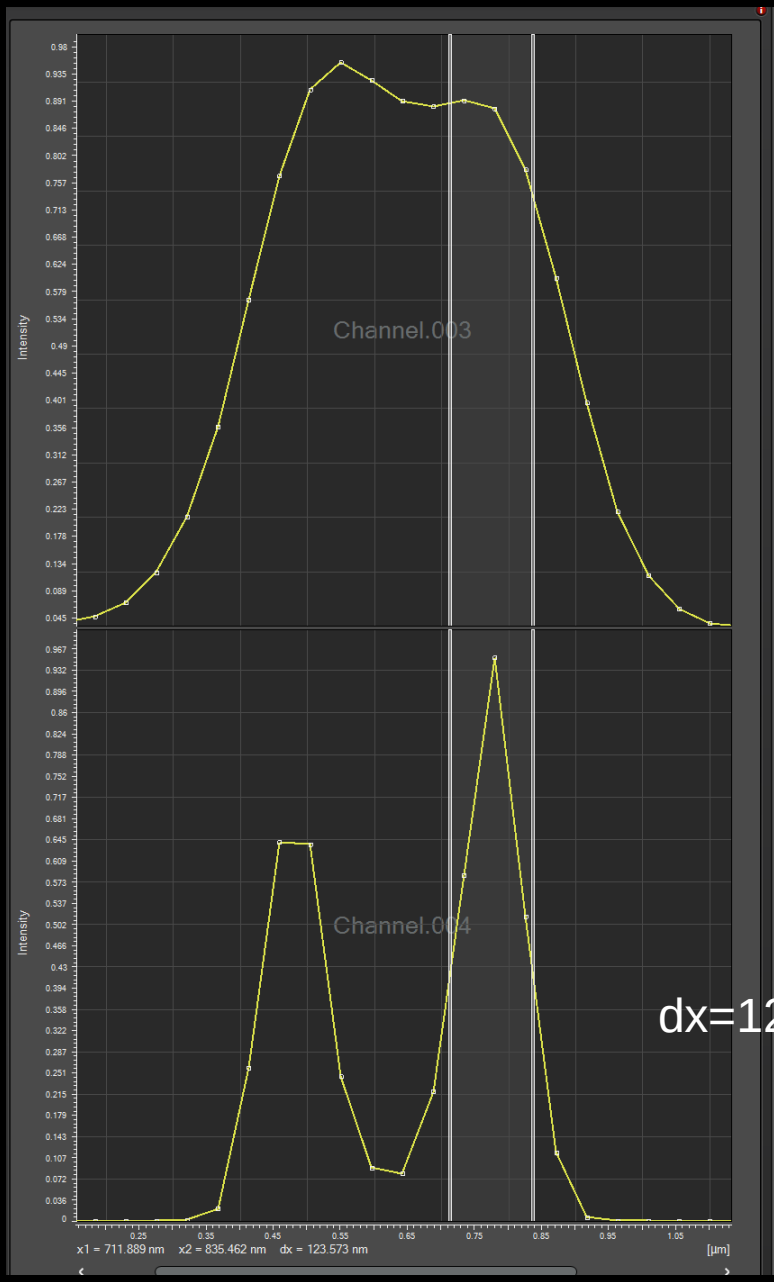


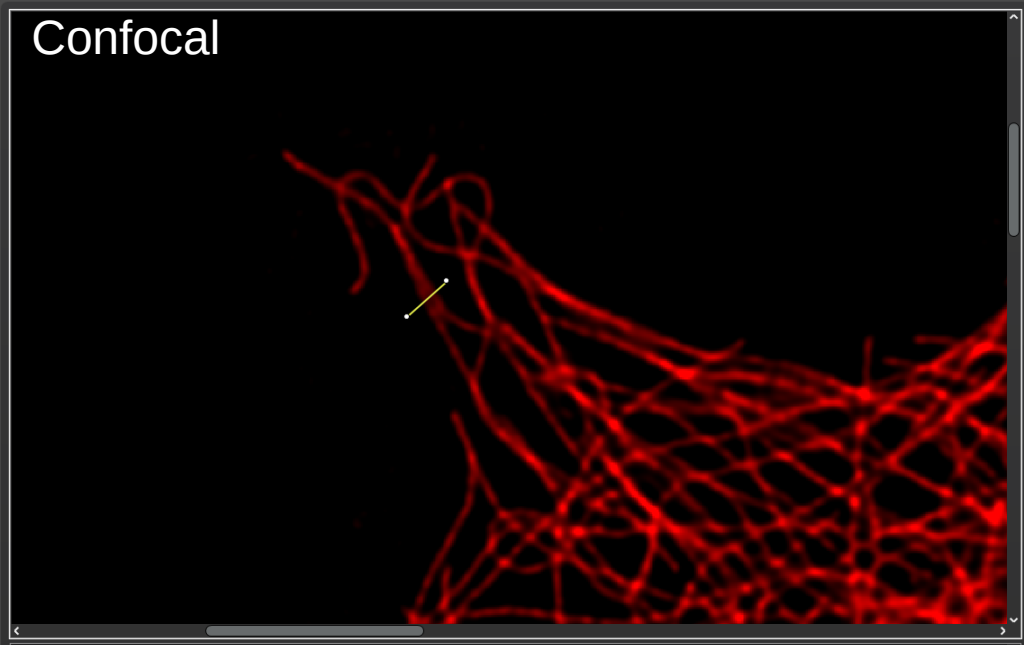
Lightning超高分辨率系统





Lightning超高分辨率系统

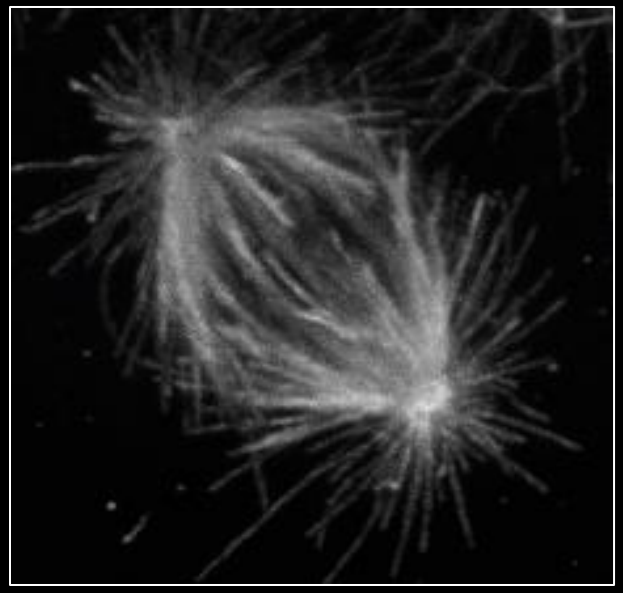




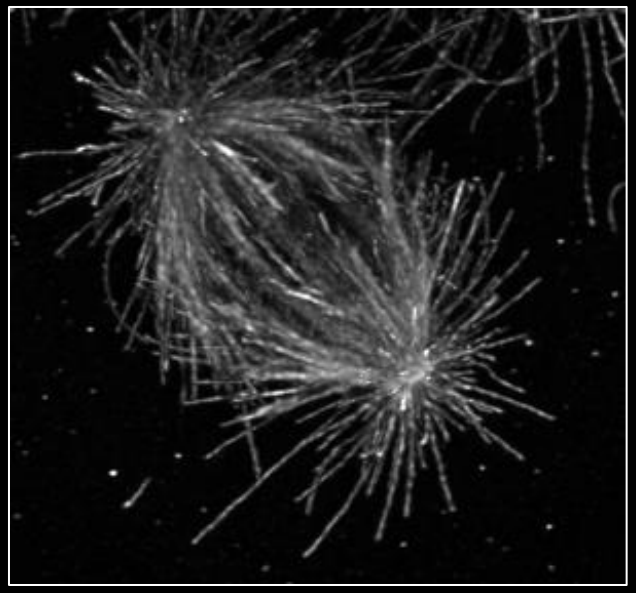
dx=123.573 nm

Lightning

SP8

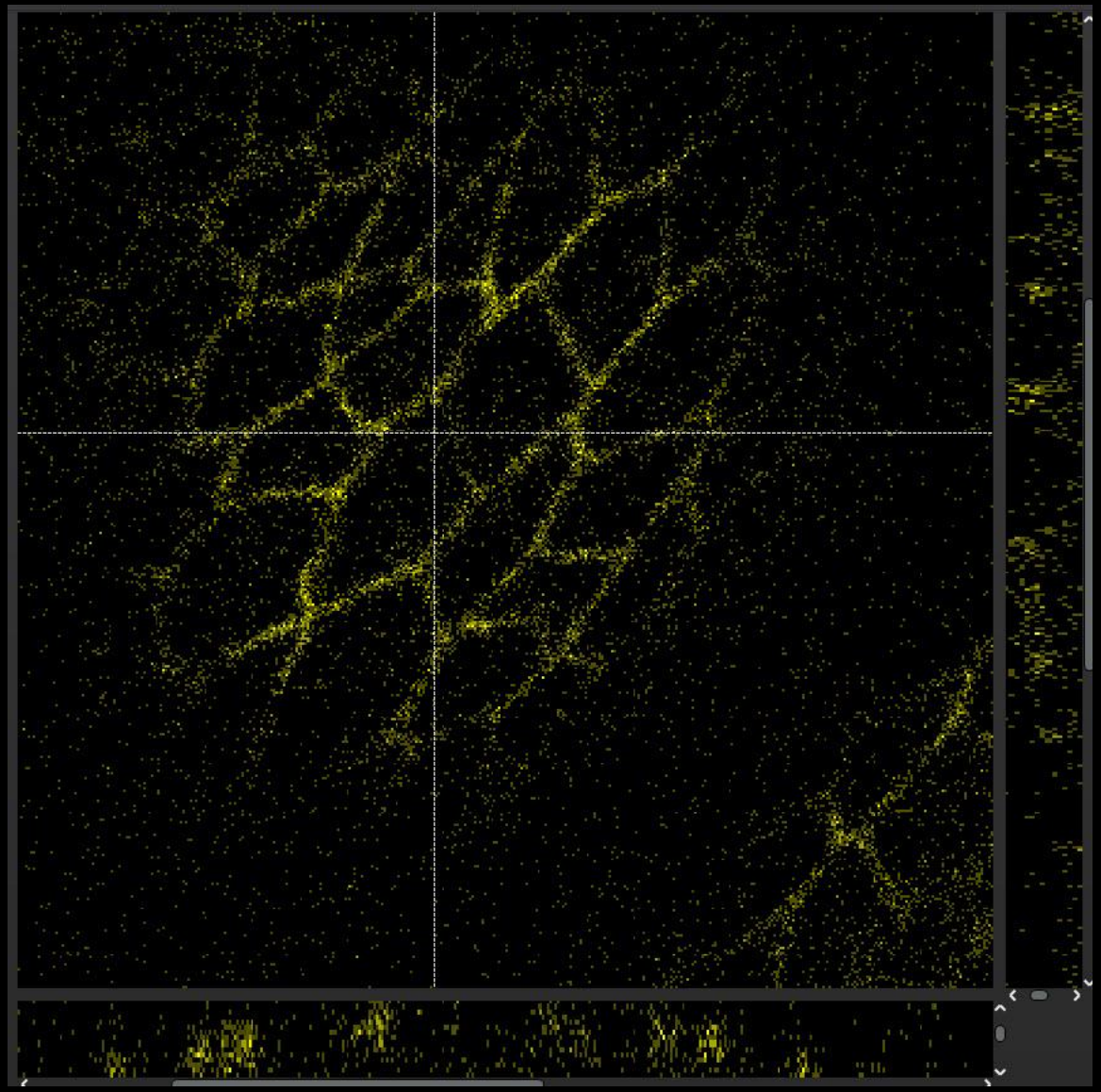


Lightning



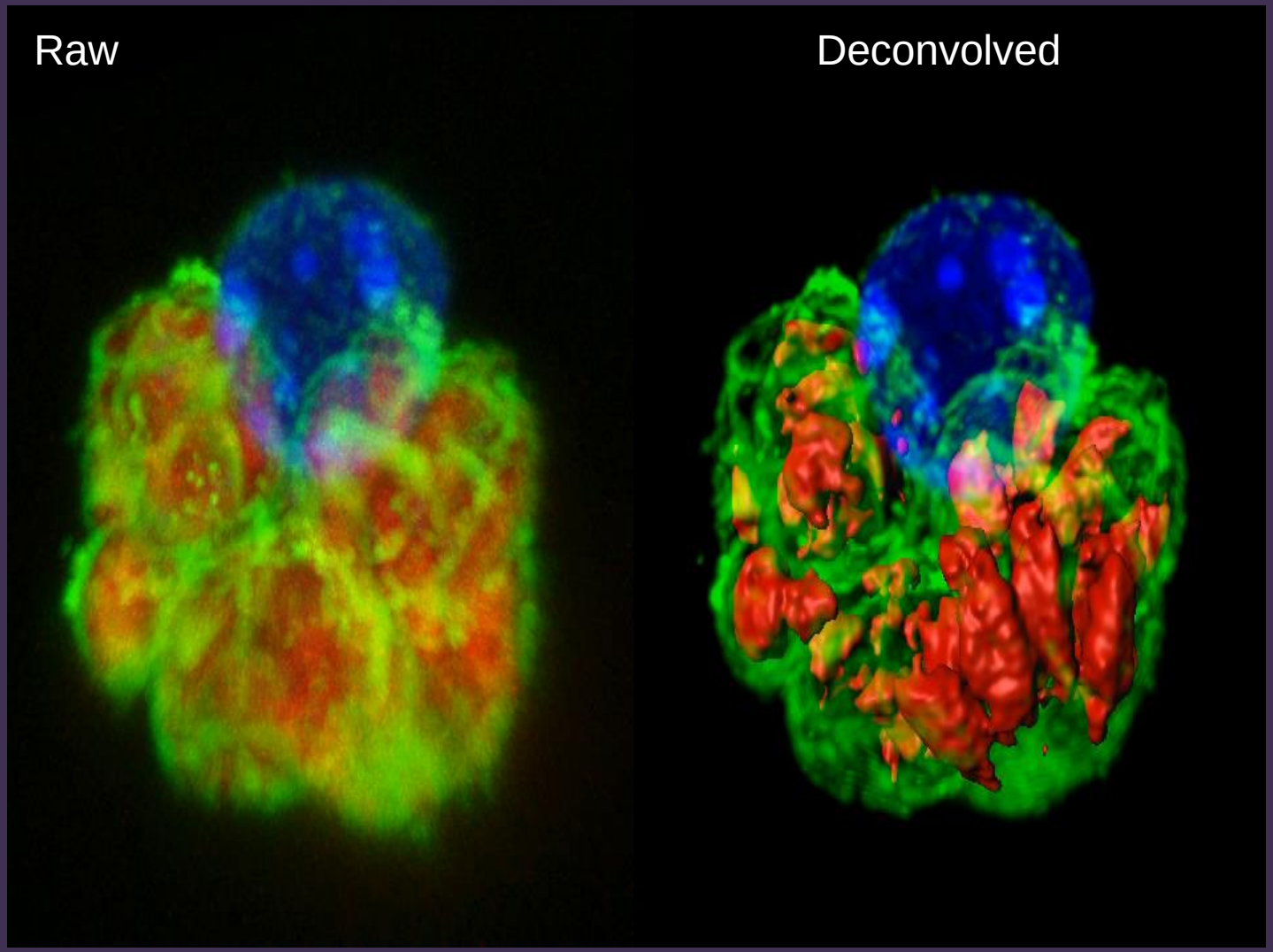
COS-7 cells. Sample: courtesy of Dr. Jana Doehner, Center of Microscopy and Image Analysis, University of Zurich, Switzerland.

Lightning能大幅提高图像信噪比



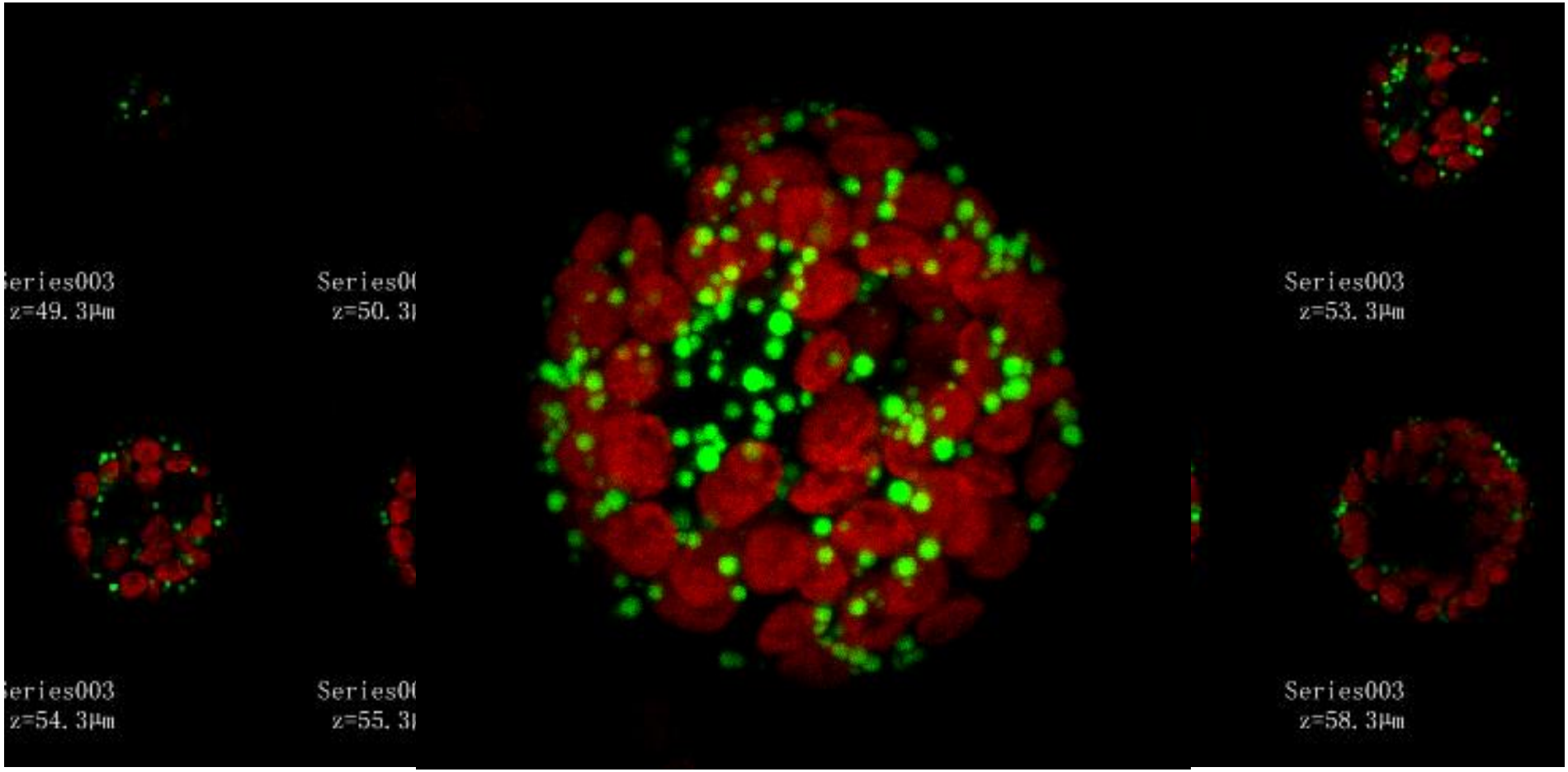
Imaginal leg disc of Drosophila showing the distribution of the **canoe-YFP** fusion protein, acquired in living sample with the resonant system, without or with deconvolution. Courtesy of Magali Suzanne, LBCMCP, University of Toulouse III

Lightning可提高三维重构通透感



成像模式

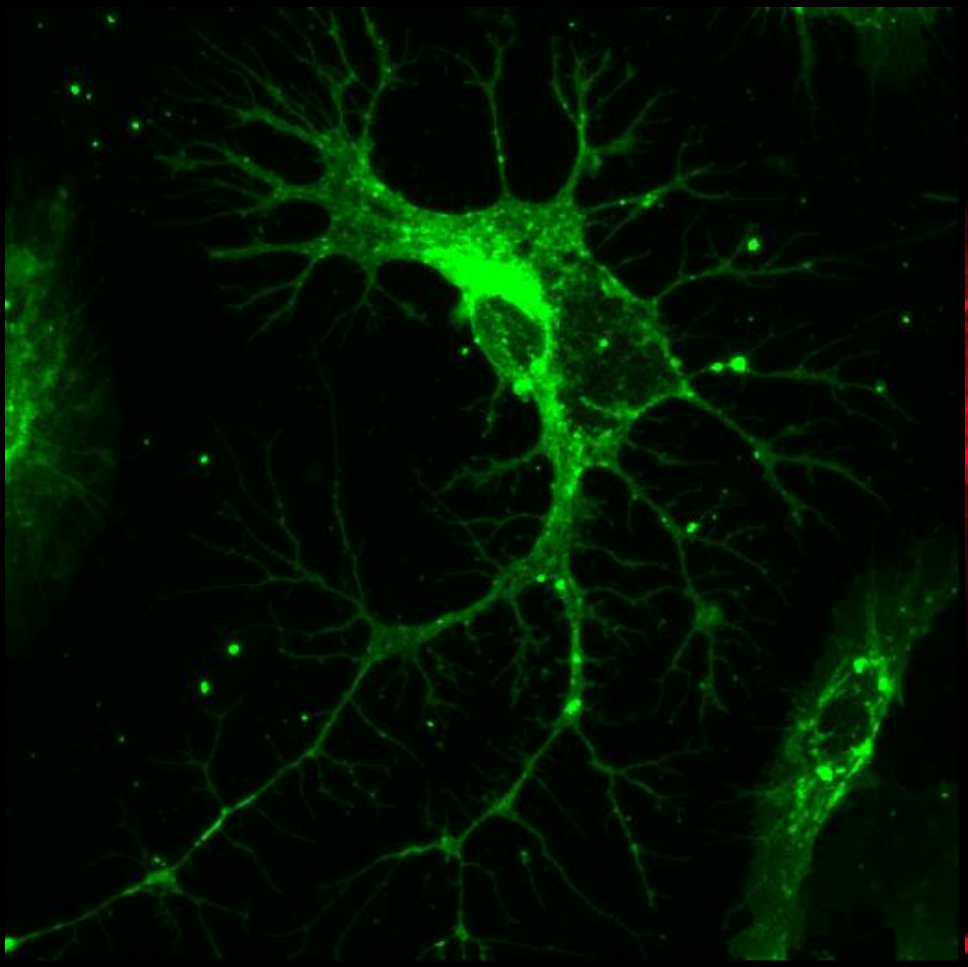
xyz 层切扫描

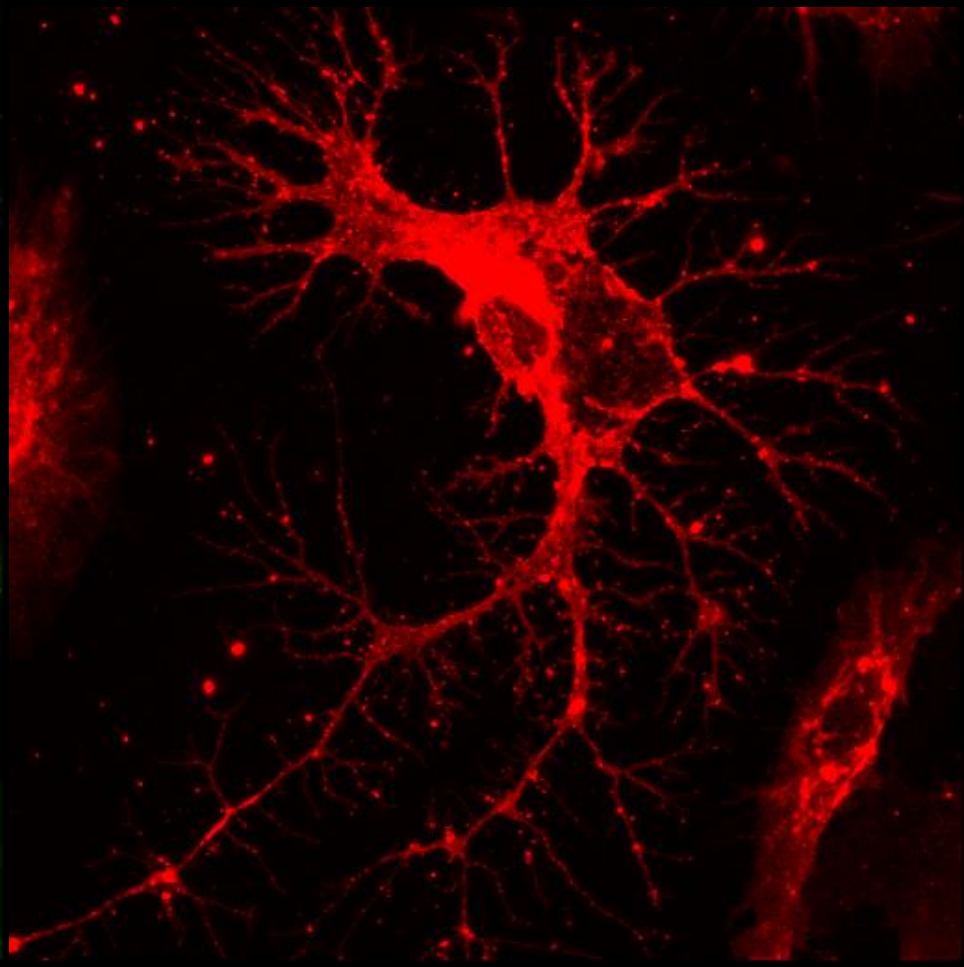


最大强度投影

成像模式

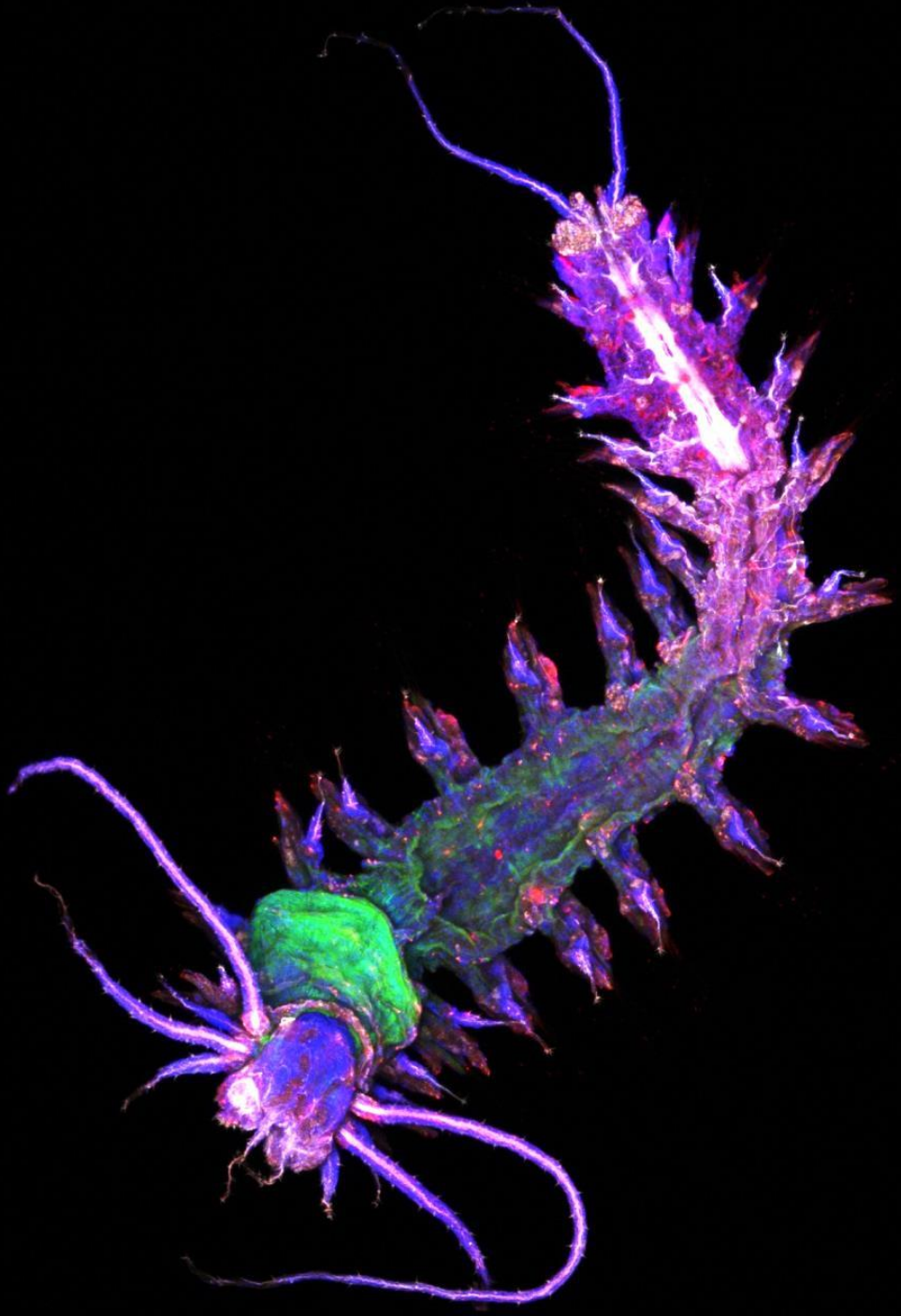
XYZ扫描

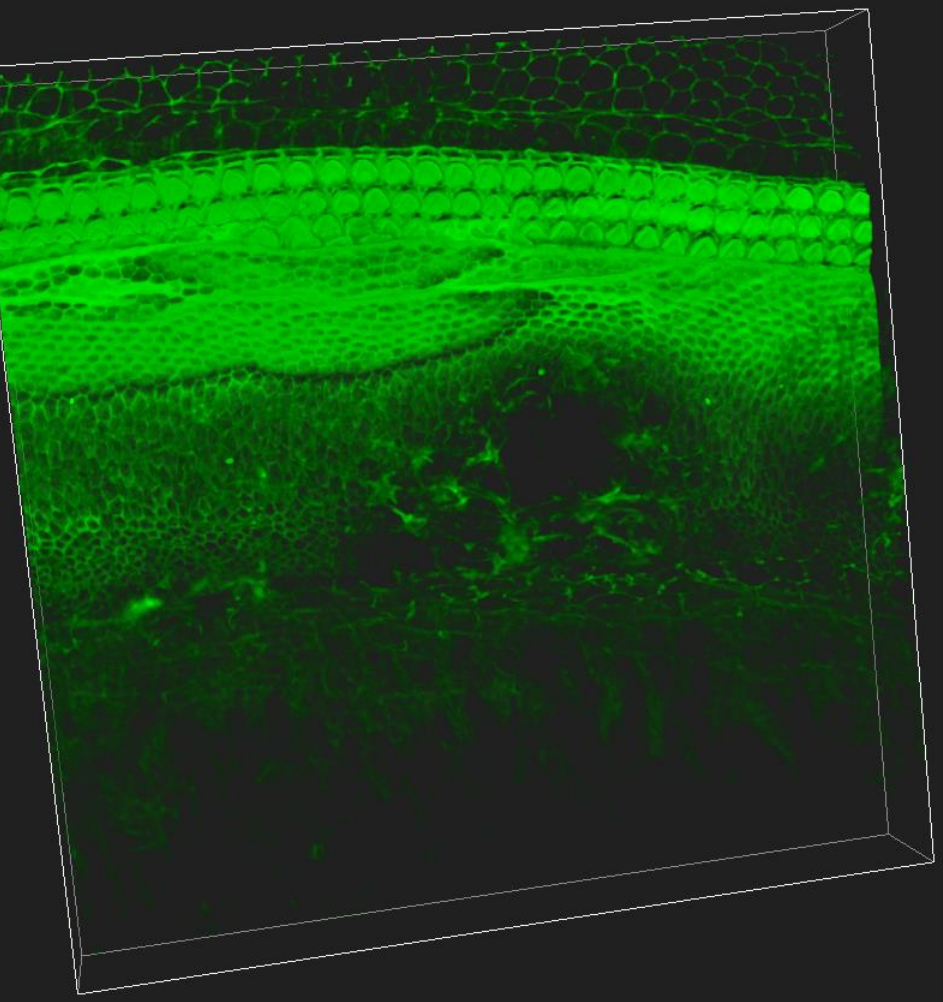




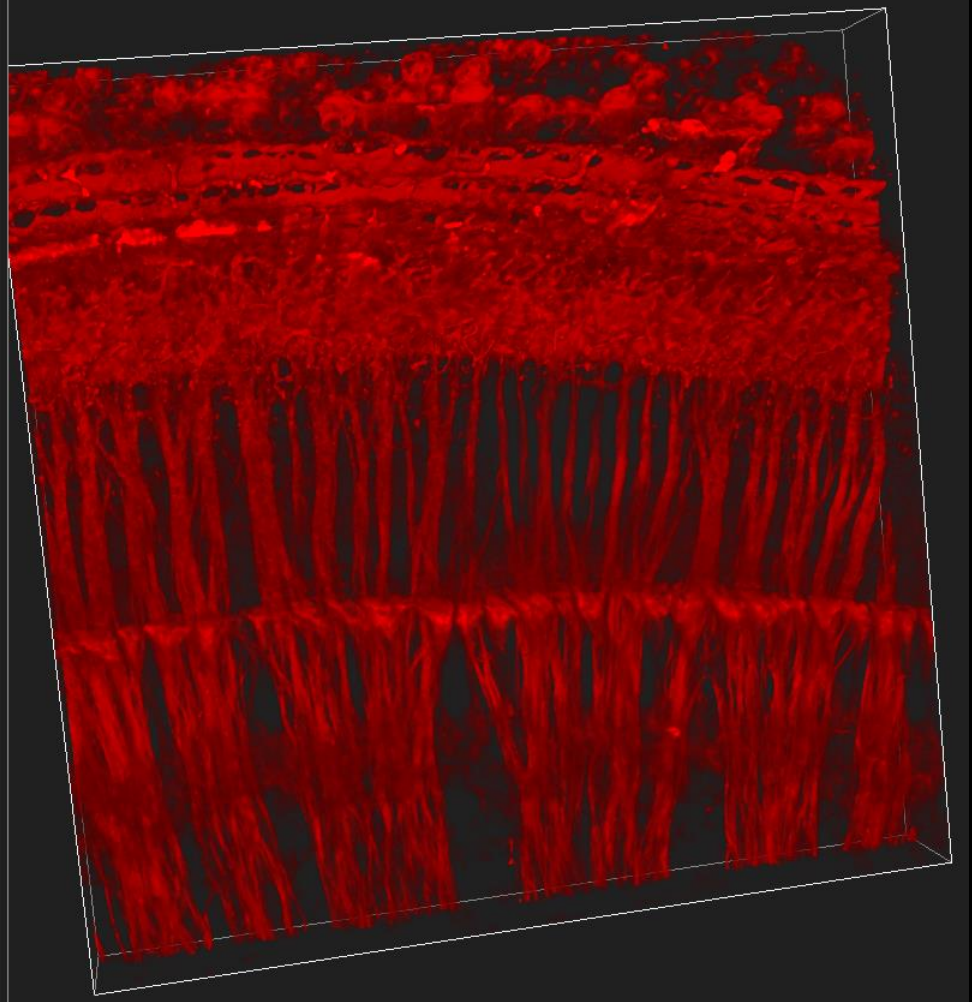
MIP最大强度投影

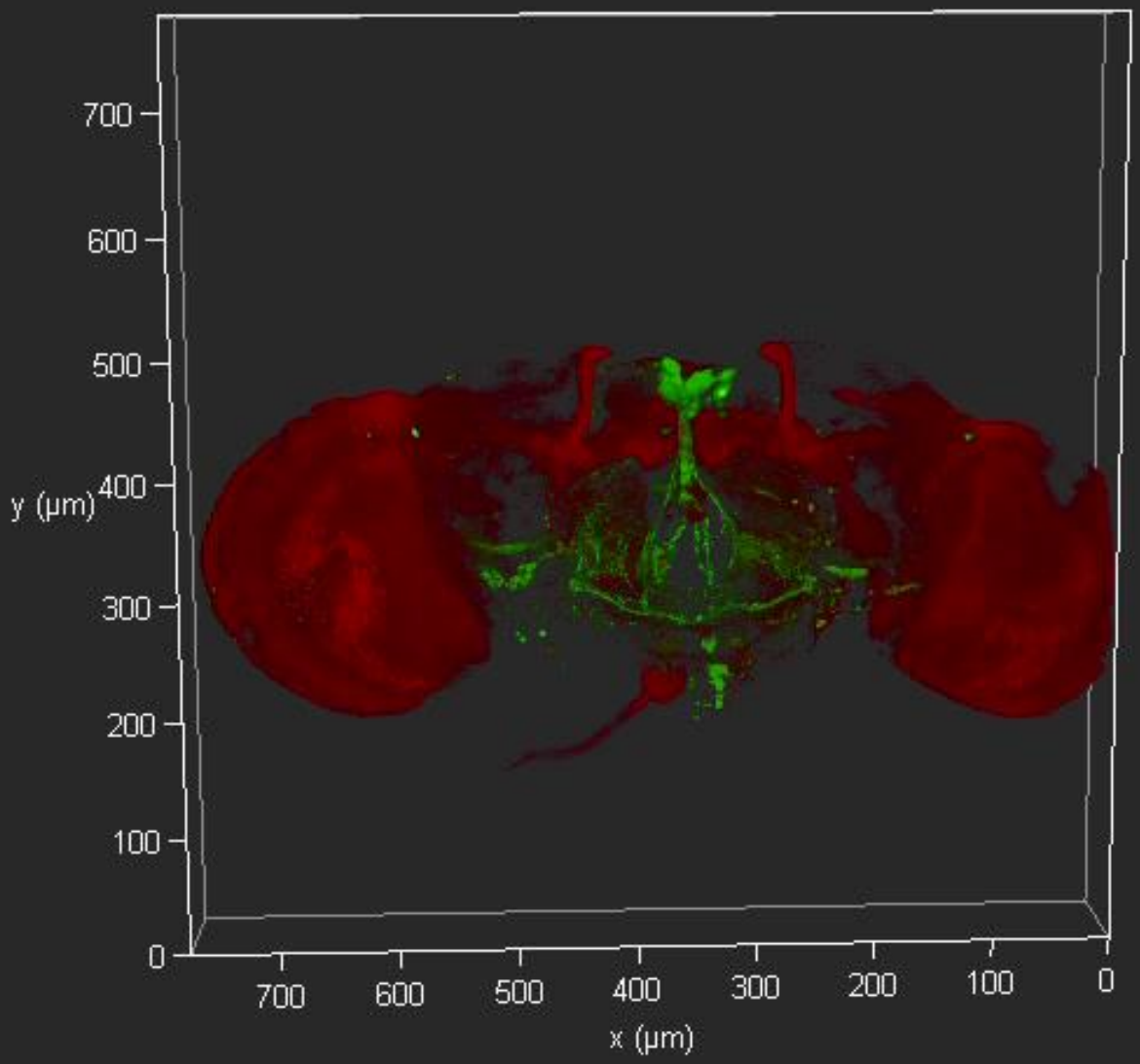
神经细胞



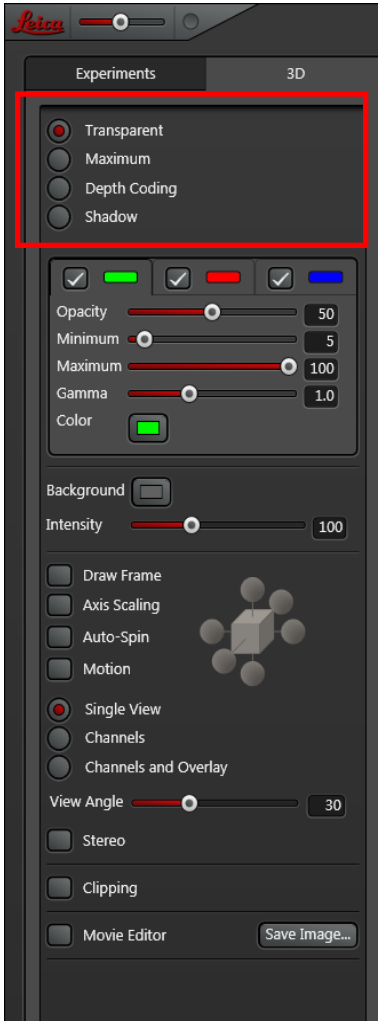


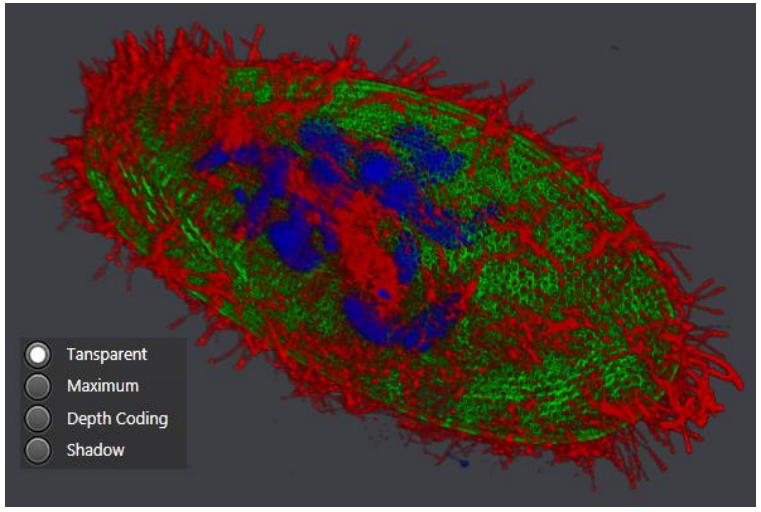
毛细胞

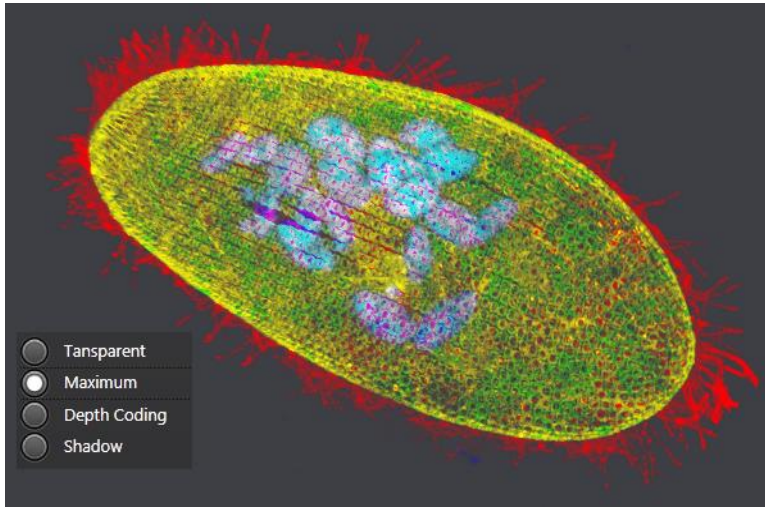


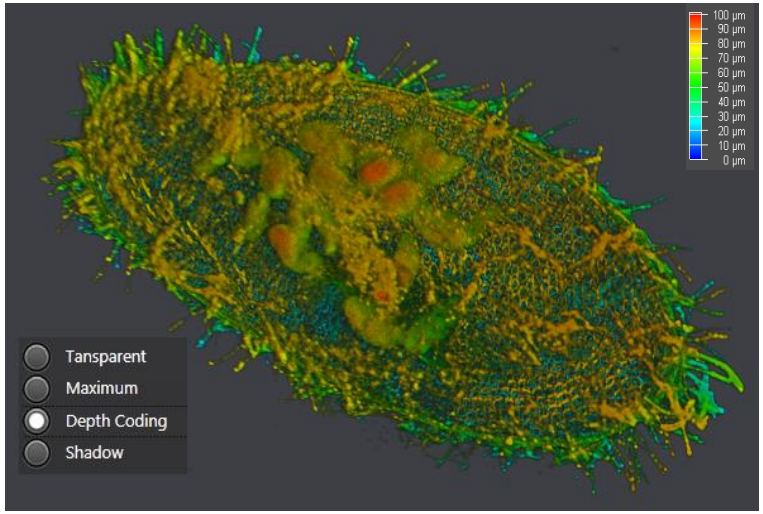

700
600
500
y (µm) 400
300
200
100
0
700
600
500
400
300
200
100
0
x (µm)

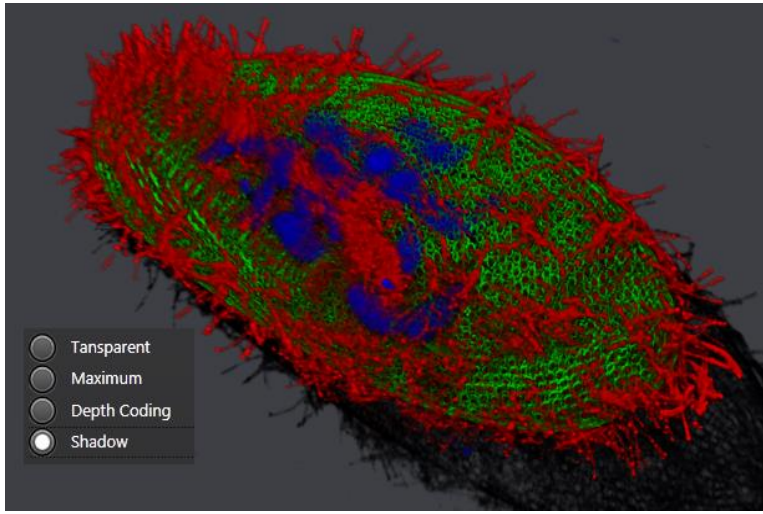
三维重构：4种渲染模式

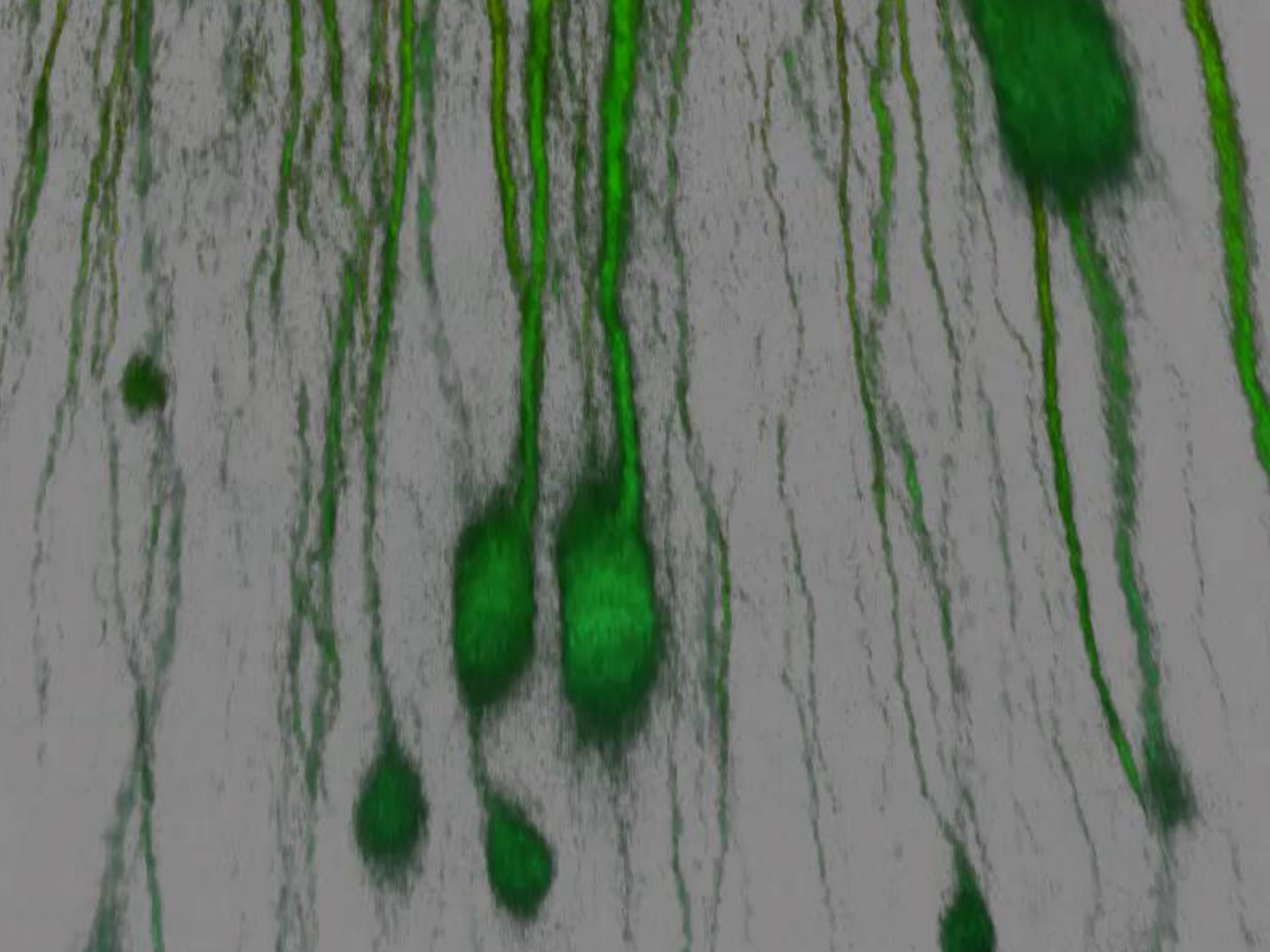

Experiments
3D
Transparent
Maximum
Depth Coding
Shadow
Opacity 50
Minimum 5
Maximum 100
Gamma 1.0
Color
Background
Intensity 100
Draw Frame
Axis Scaling
Auto-Spin
Motion
Single View
Channels
Channels and Overlay
View Angle 30
Stereo
Clipping
Movie Editor
Save Image...


Tansparent
Maximum
Depth Coding
Shadow


Tansparent
Maximum
Depth Coding
Shadow


100 µm
90 µm
80 µm
70 µm
60 µm
50 µm
40 µm
30 µm
20 µm
10 µm
0 µm
Tansparent
Maximum
Depth Coding
Shadow


Tansparent
Maximum
Depth Coding
Shadow



如何提高成像穿透深度

1、使用更长波长的近红外染料；

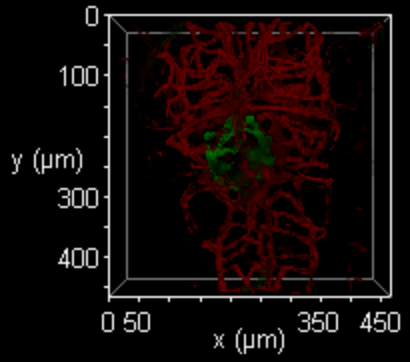
2、使用高灵敏度的HyD检测器；

3、通过提高激光能量和检测器增益进行深部组织荧光亮度的补偿（z compensation）；

4、对样品进行透明化处理。

三维重构模块

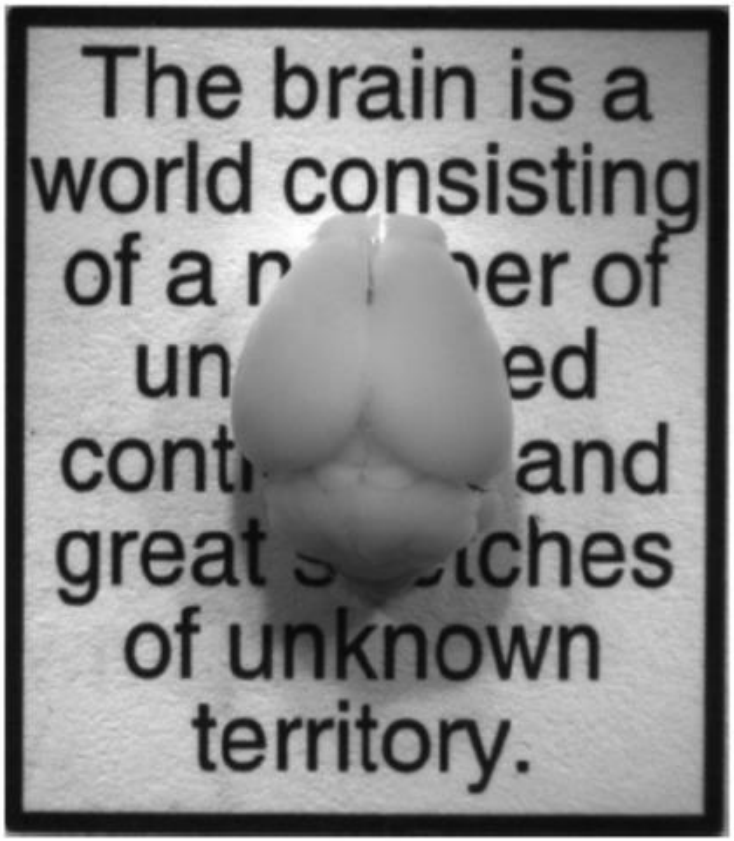

斑马鱼头部原始数据
GFP标记肿瘤细胞
mCherry标记血管
z compensation
z=5098.3μm
0 μm 50



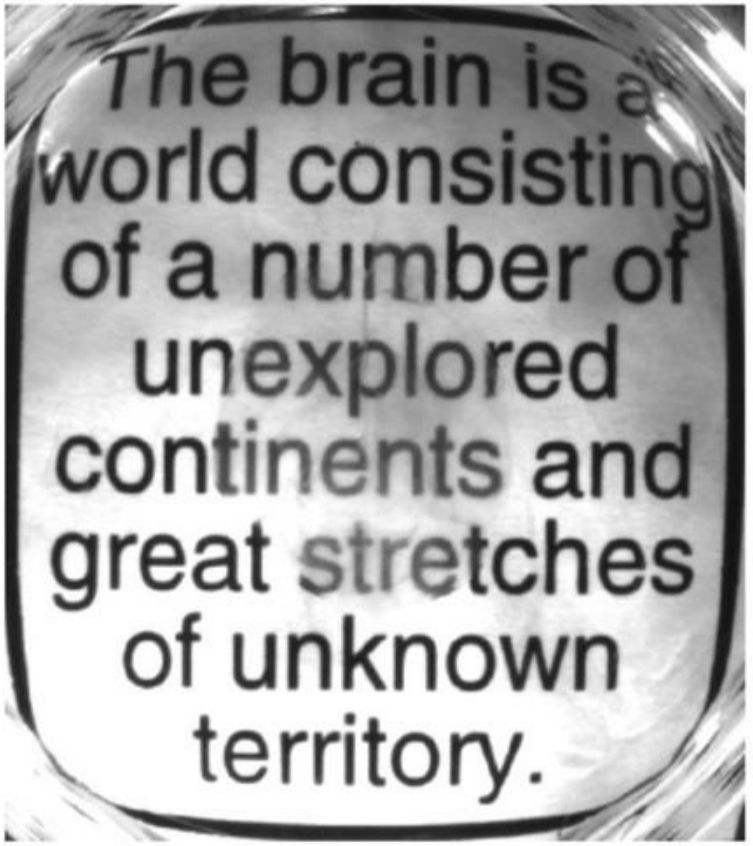
斑马鱼头部
GFP标记肿瘤细胞
mCherry标记血管

Clarity透明化样品

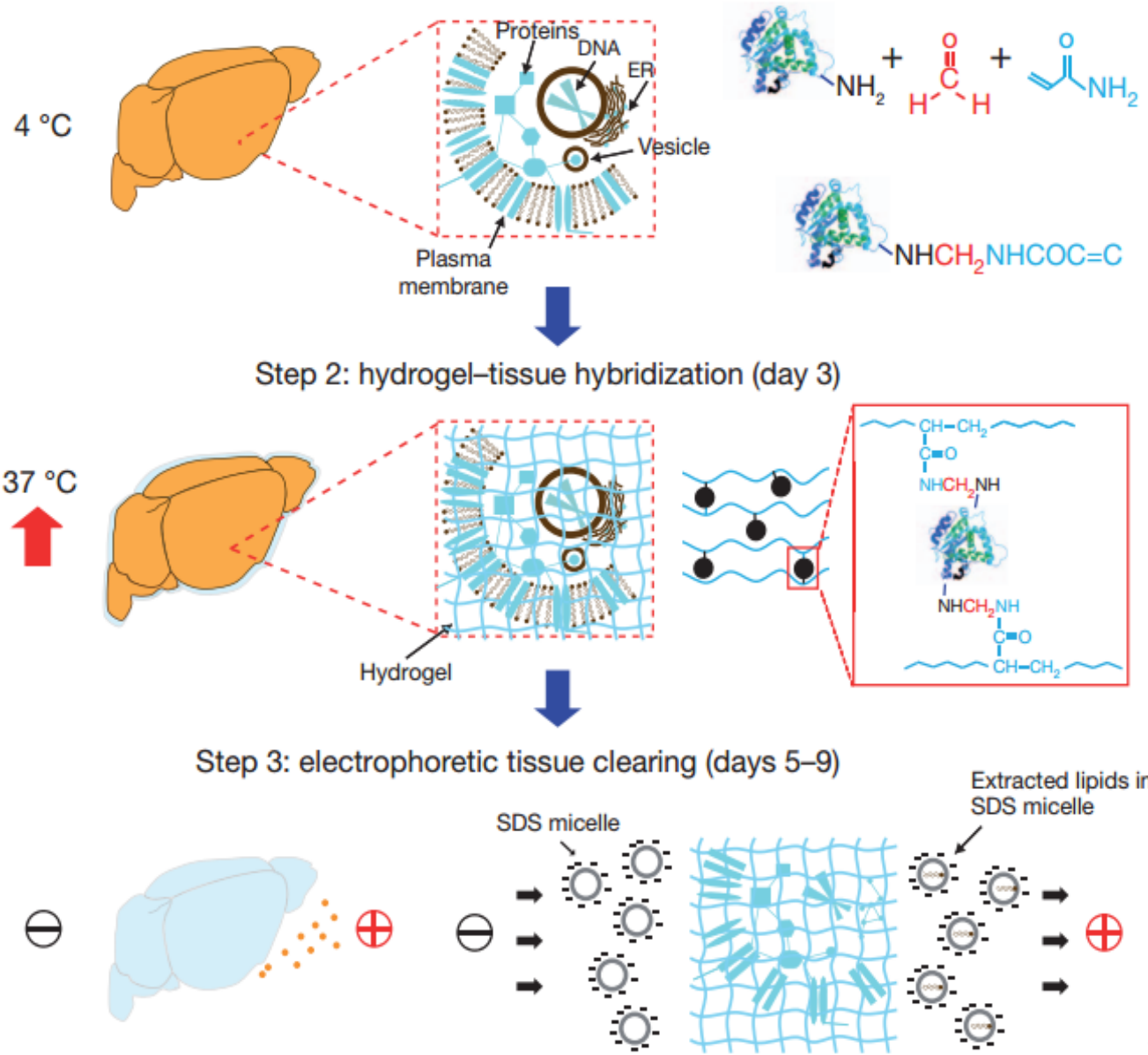
Before

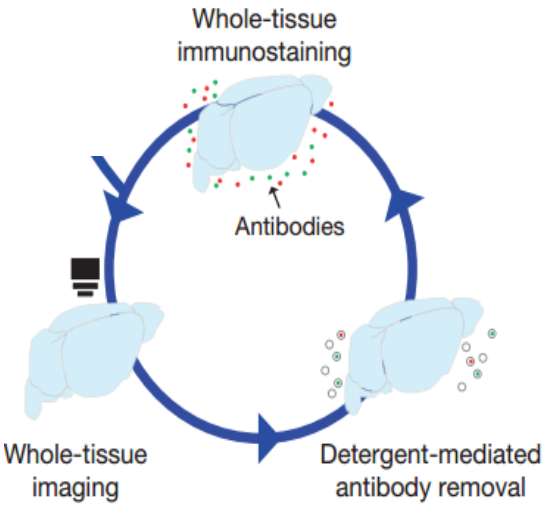


After



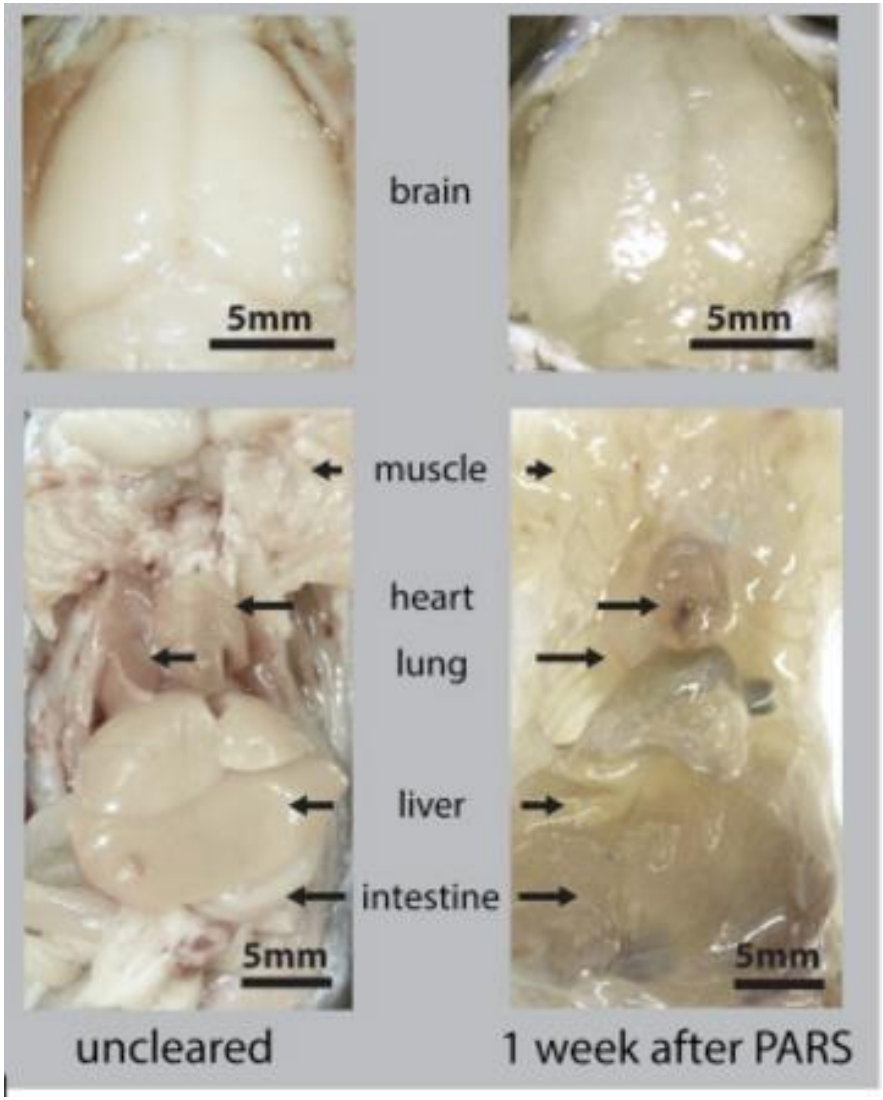
Clarity





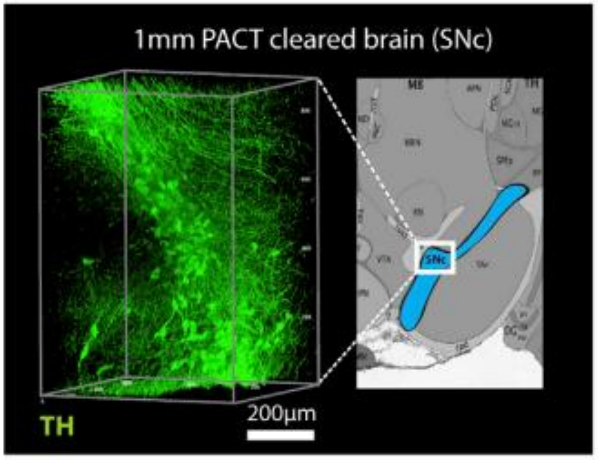
Chung K et al., Nature, 2013

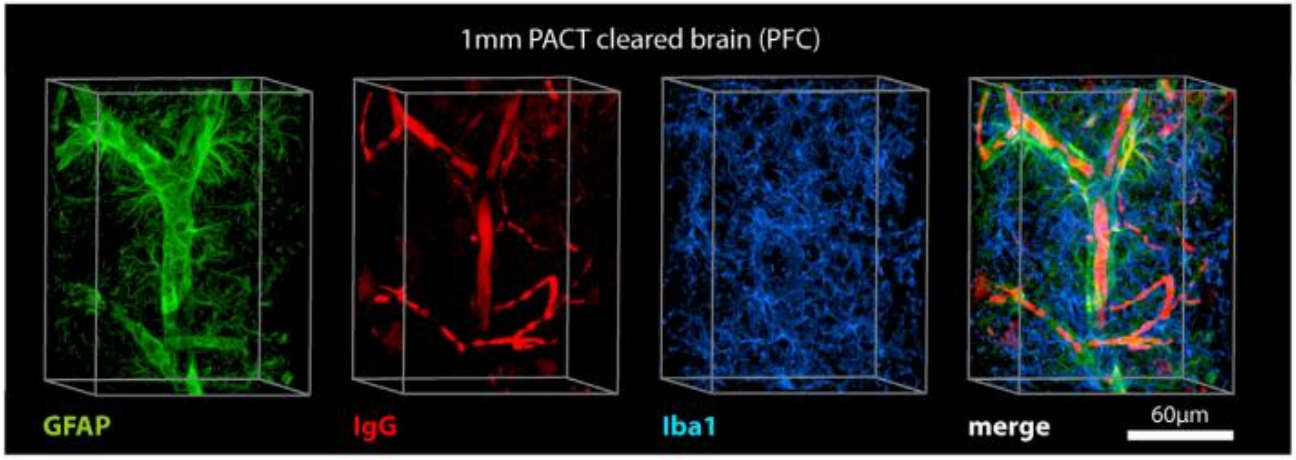
Single-Cell Phenotyping within Transparent Intact Tissue through Whole-Body Clearing

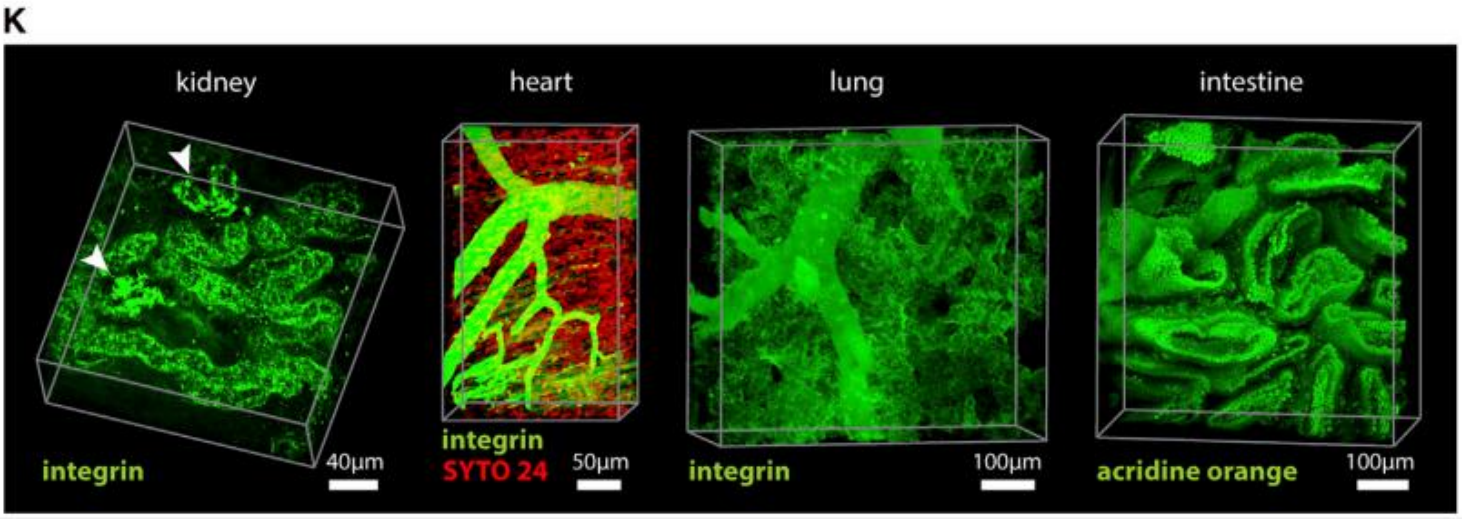


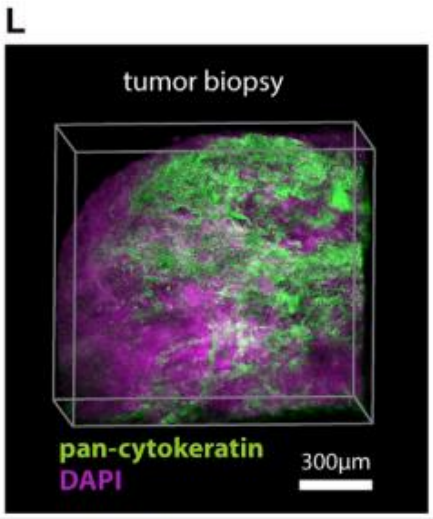
Yang *et al*, Cell, 2014

Single-Cell Phenotyping within Transparent Intact Tissue through Whole-Body Clearing

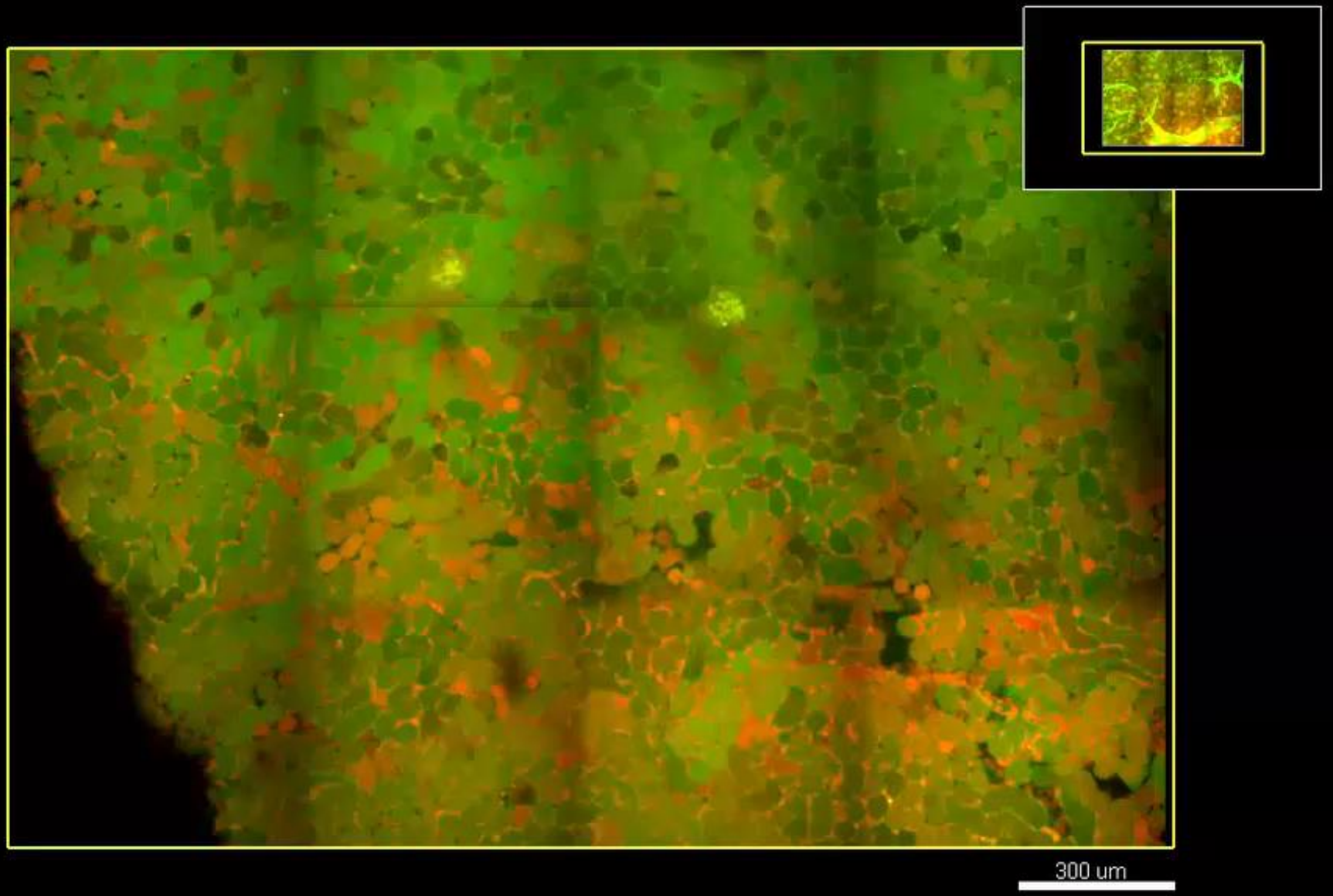


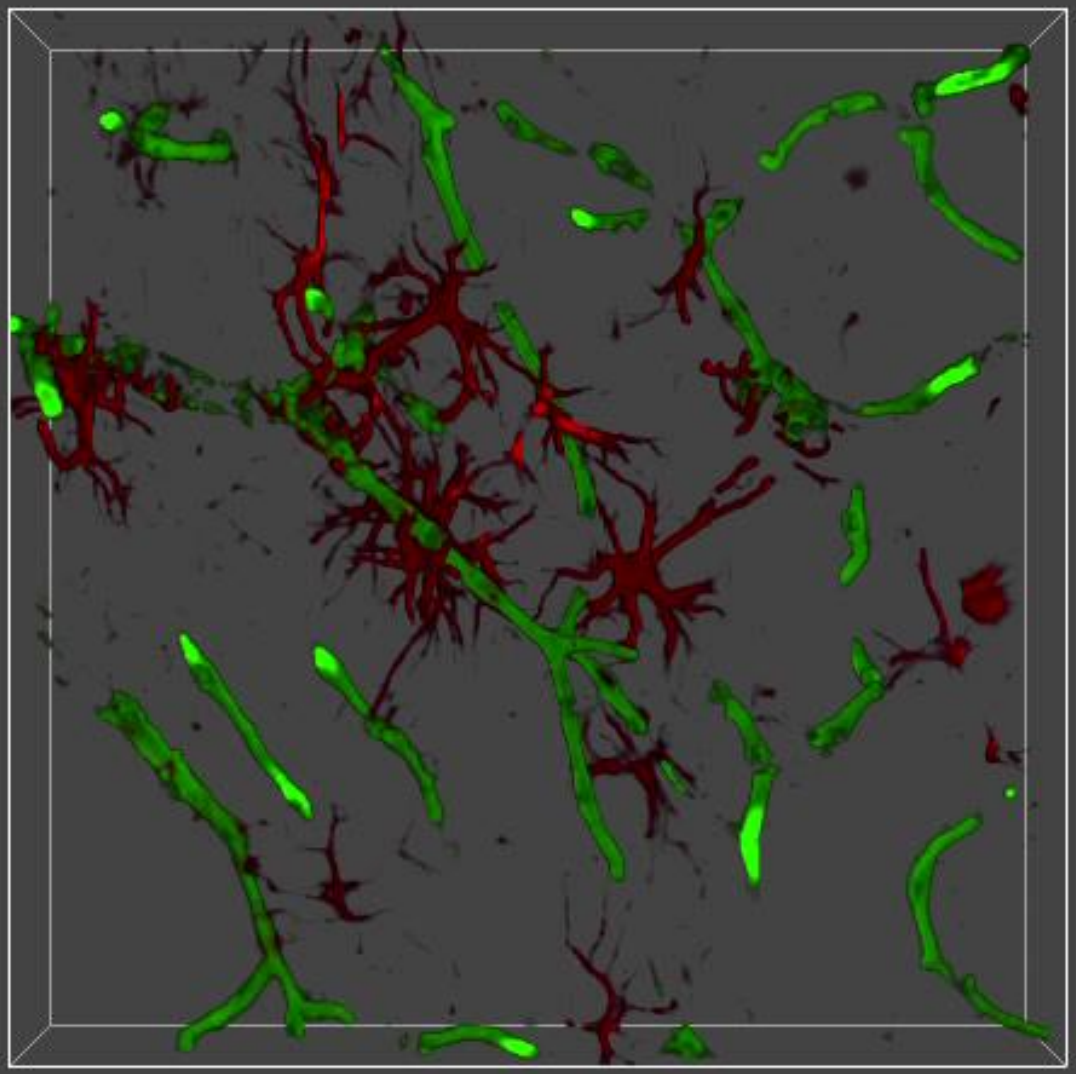






Yang *et al*, Cell, 2014


300 um



透明化的小鼠大脑

脑血管 + 星形胶质细胞

100 μm

层切厚度设置

Leica MICROSYSTEMS

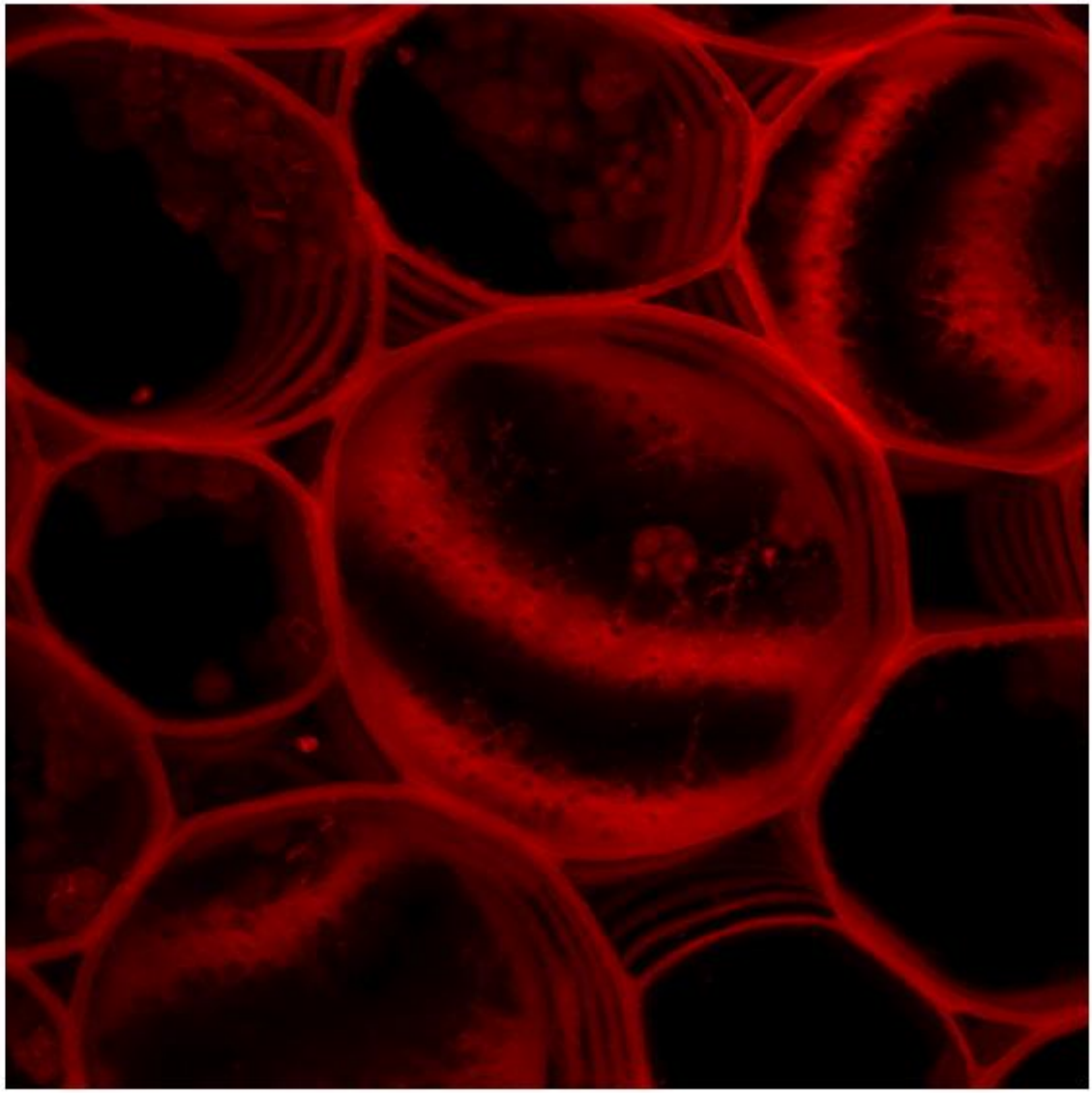
Stack recording

axial res.: ≈ 1,0 µm

Z-distance: 6,4 µm

HCX Apochromat 40x/1,25 oil
At ca 550nm wavelength

lateral resolution: ≈ 0,2 µm
axial resolution: ≈ 1,0 µm



Z轴层切厚度需要满足过采样

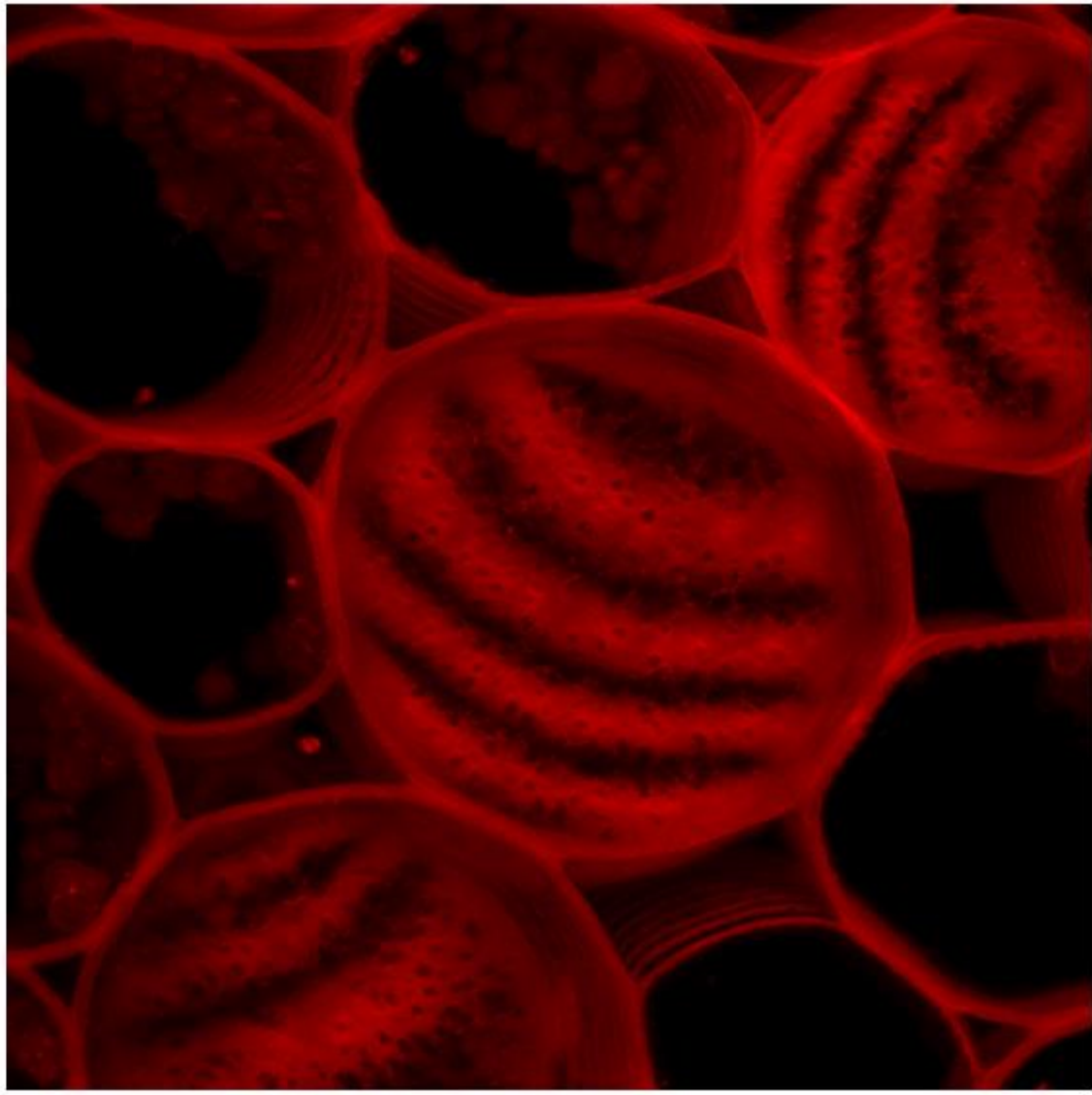
层切厚度设置

Leica MICROSYSTEMS

Stack recording

axial res.: ≈ 1,0 µm

Z-distance: 3,2 µm



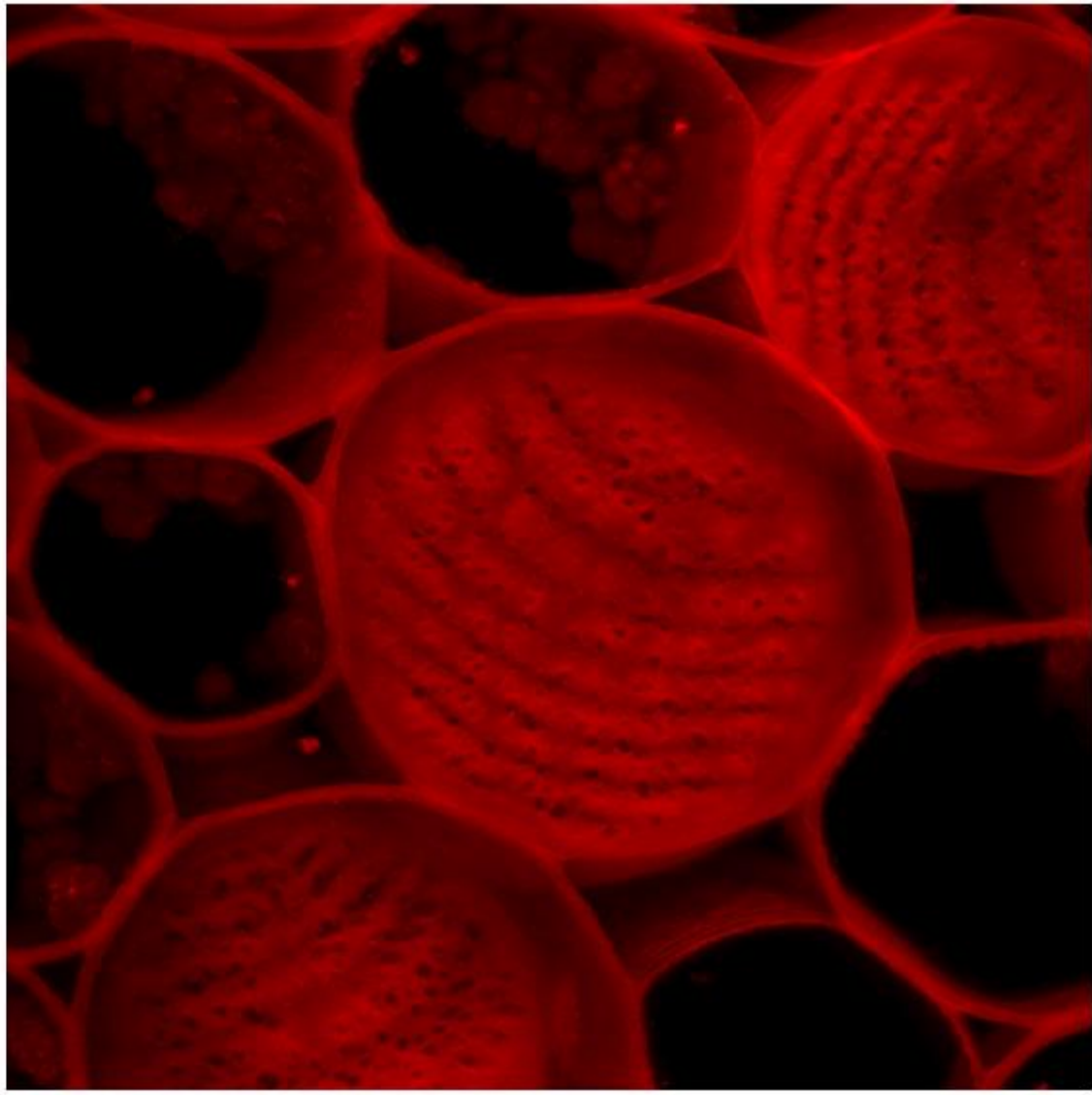
层切厚度设置

Leica MICROSYSTEMS

Stack recording

axial res.: ≈ 1,0 µm

Z-distance: 1,6 µm



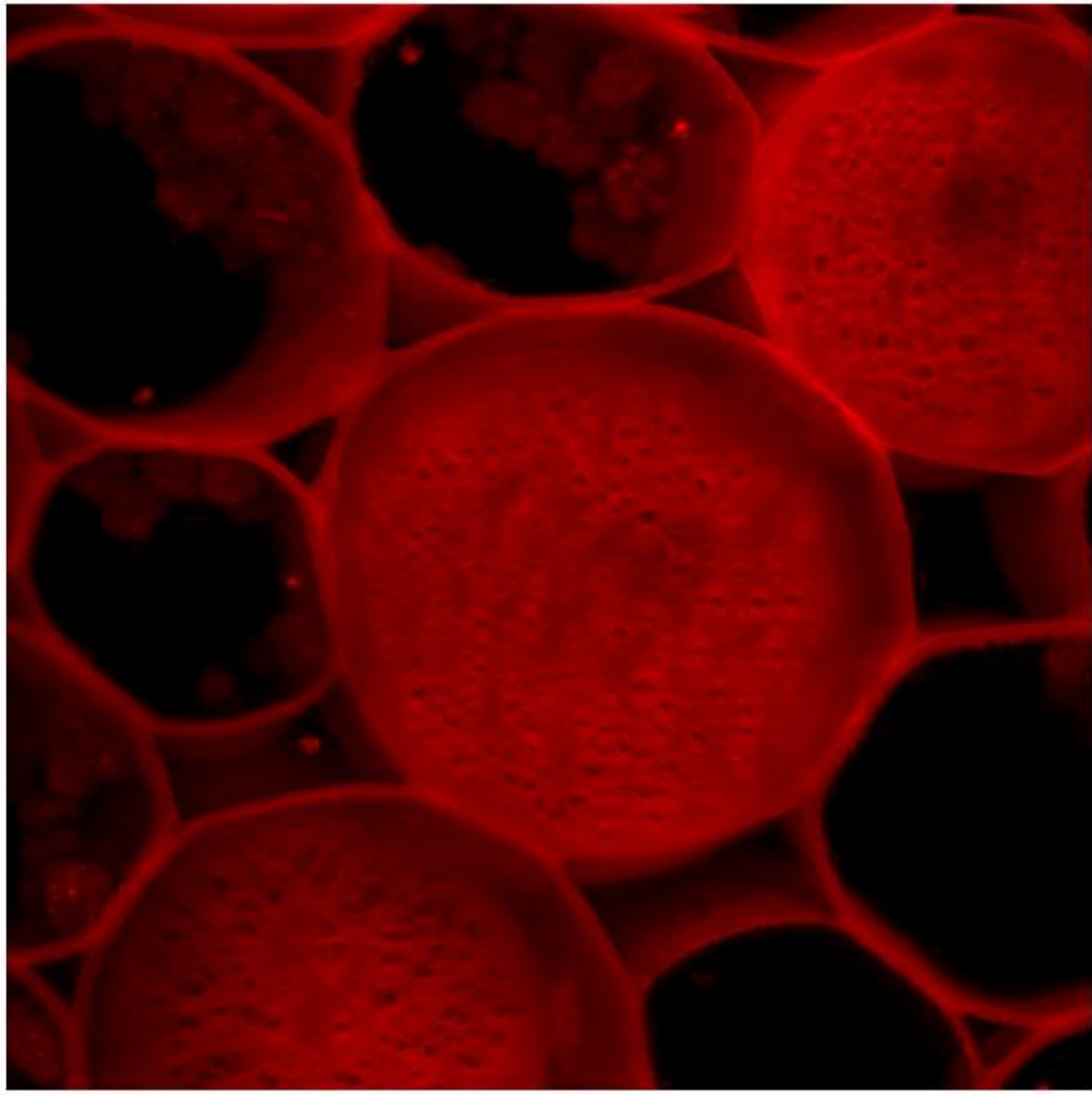
层切厚度设置

Leica MICROSYSTEMS

Stack recording

axial res.: ≈ 1,0 µm

Z-distance: 0,8 µm



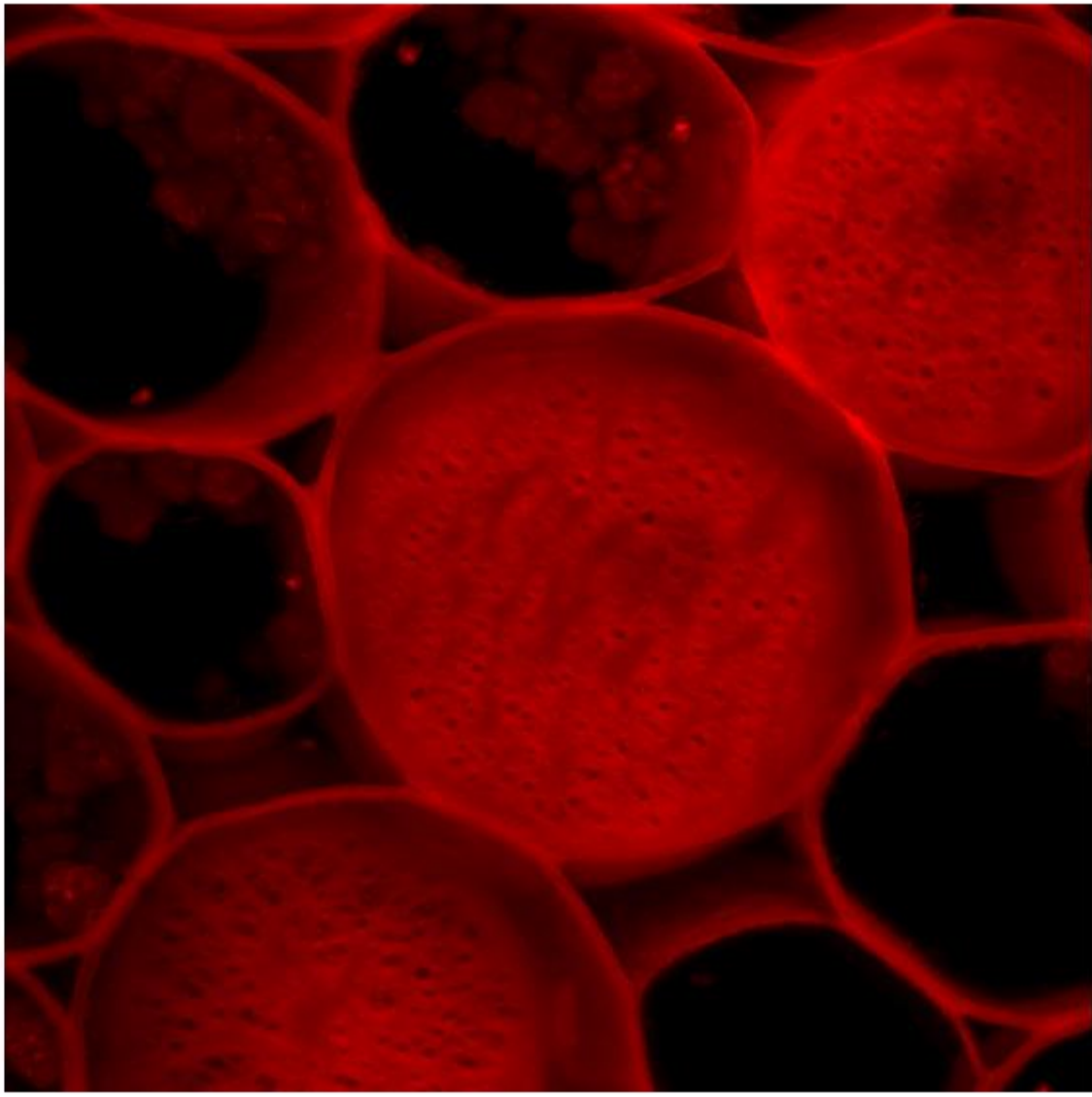
层切厚度设置

Leica MICROSYSTEMS

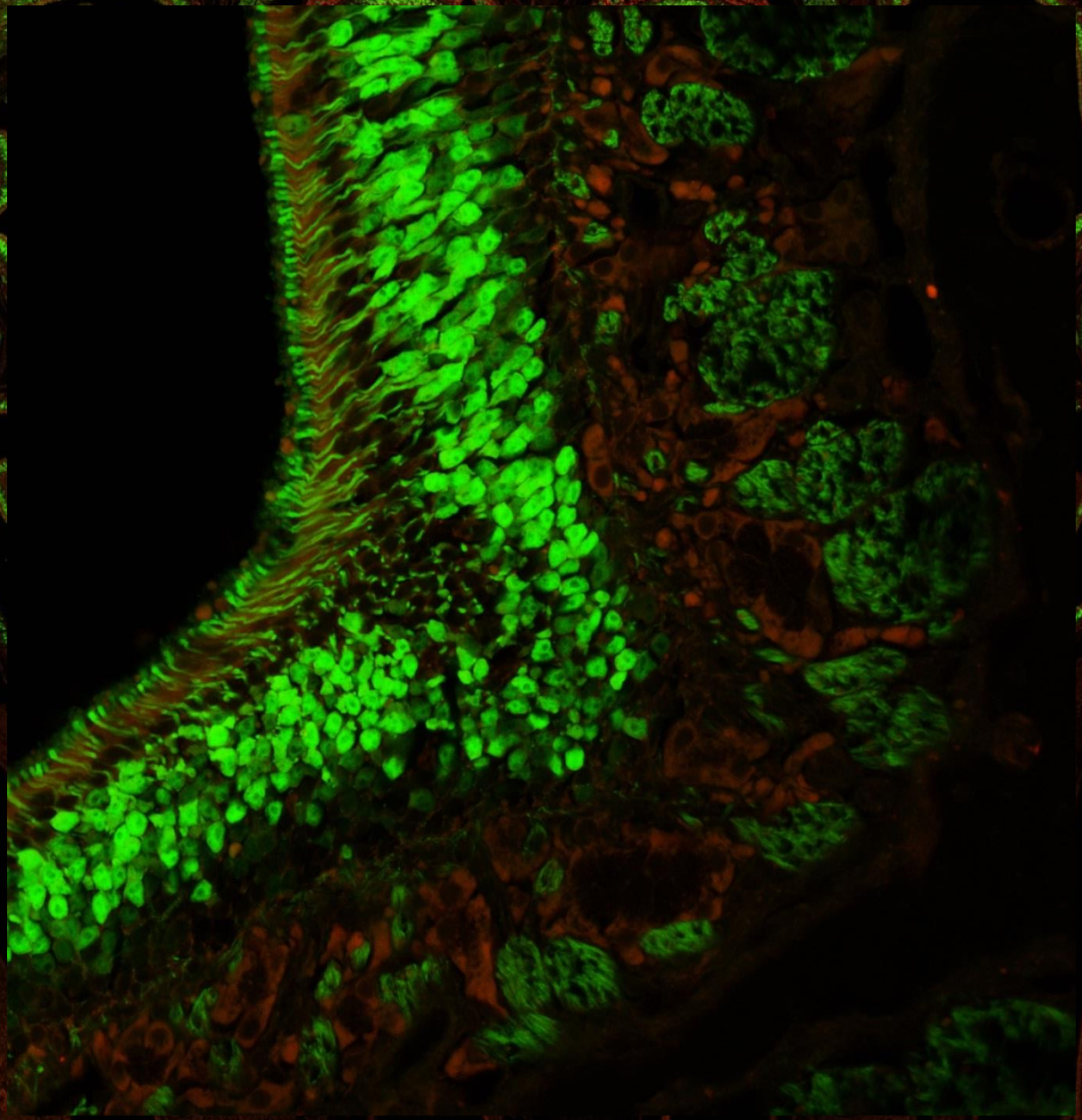
Stack recording

axial res.: ≈ 1,0 µm

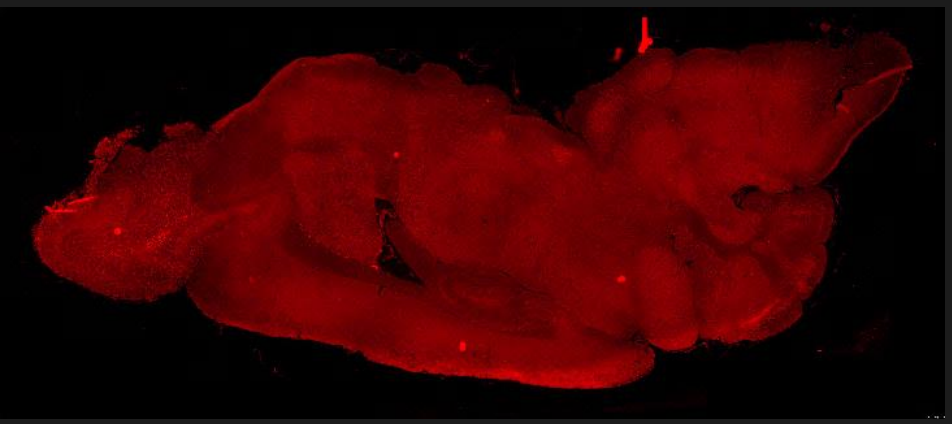
Z-distance: 0,4 µm

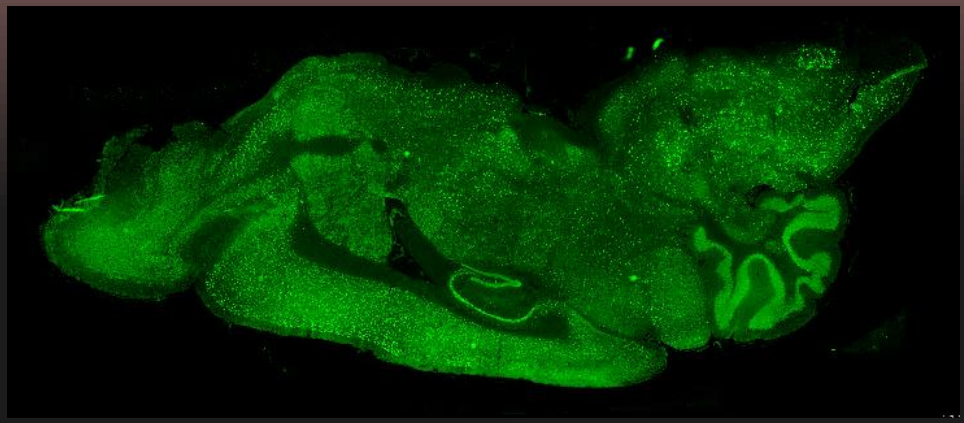


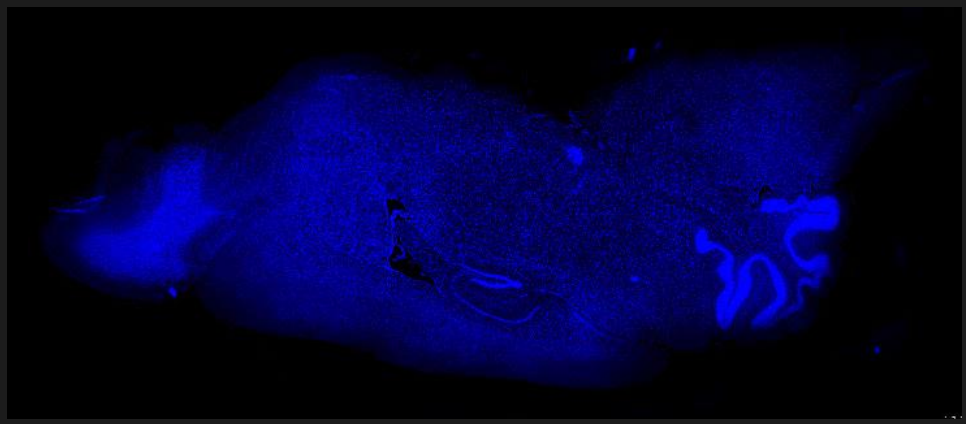
xy大视野拼图



xy大视野拼图



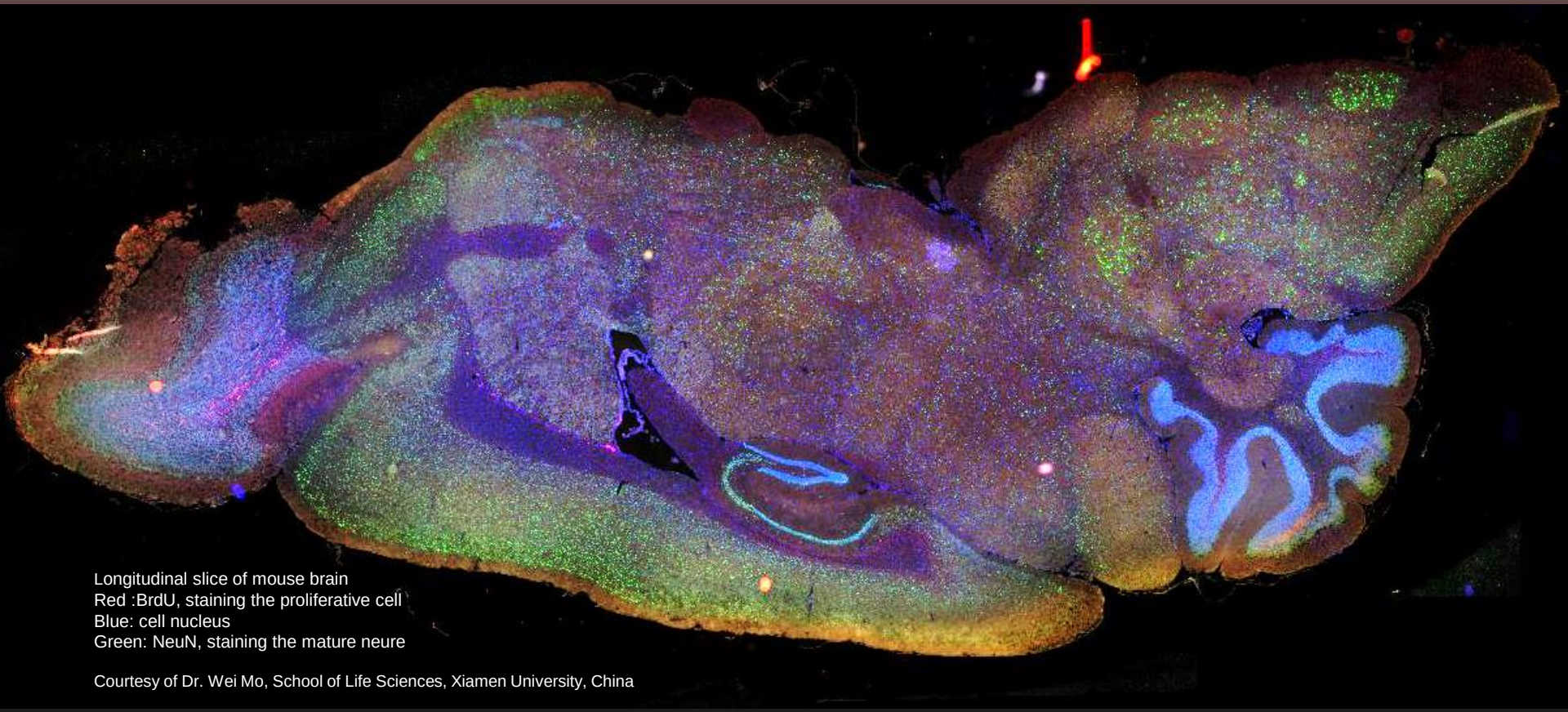




Longitudinal slice of mouse brain
Red :BrdU, staining the proliferative cell
Blue: cell nucleus
Green: NeuN, staining the mature neure

Courtesy of Dr. Wei Mo, School of Life Sciences, Xiamen University, China

xy大视野拼图

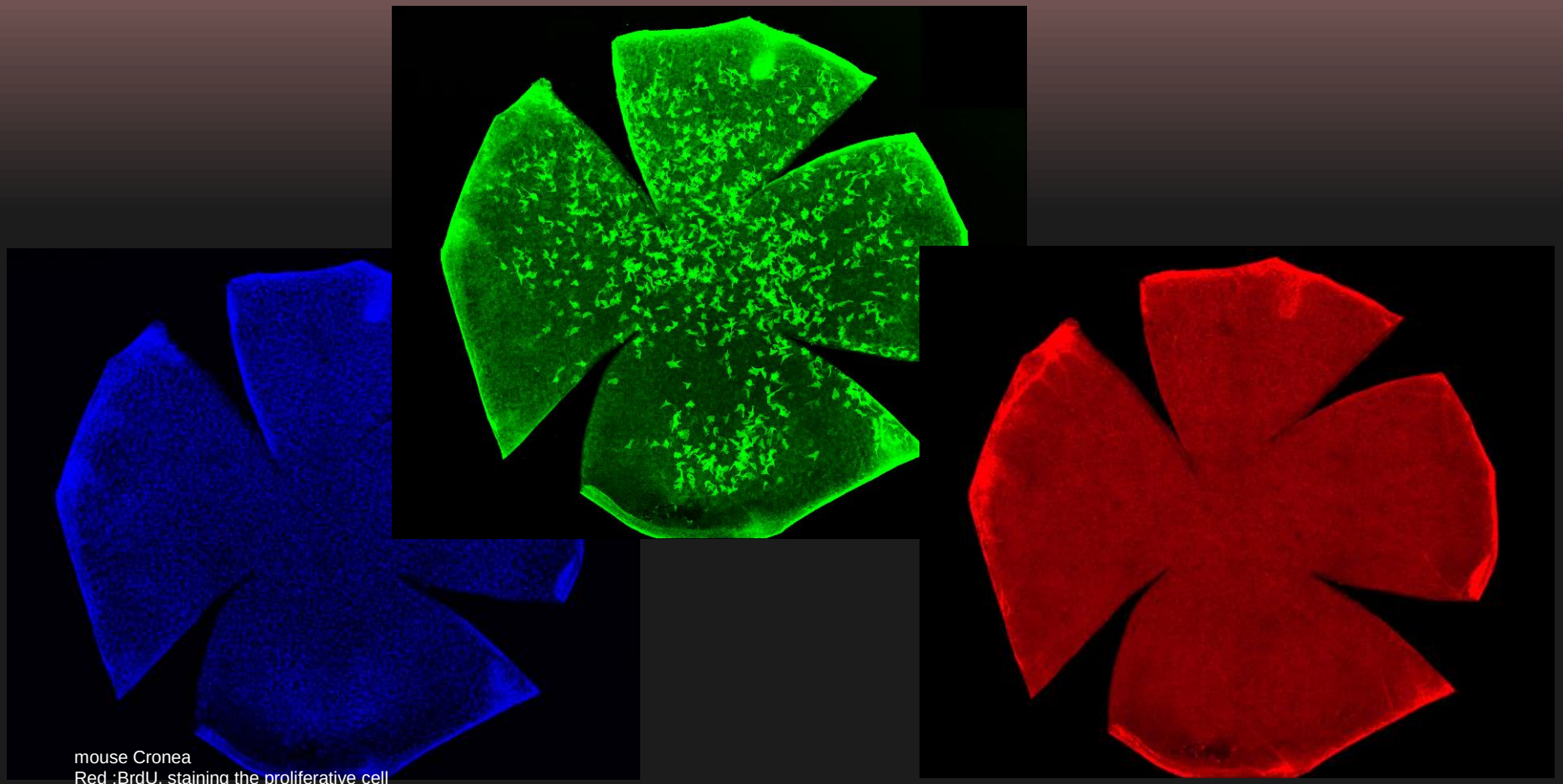


Longitudinal slice of mouse brain
Red :BrdU, staining the proliferative cell
Blue: cell nucleus
Green: NeuN, staining the mature neure

Courtesy of Dr. Wei Mo, School of Life Sciences, Xiamen University, China

3D拼图

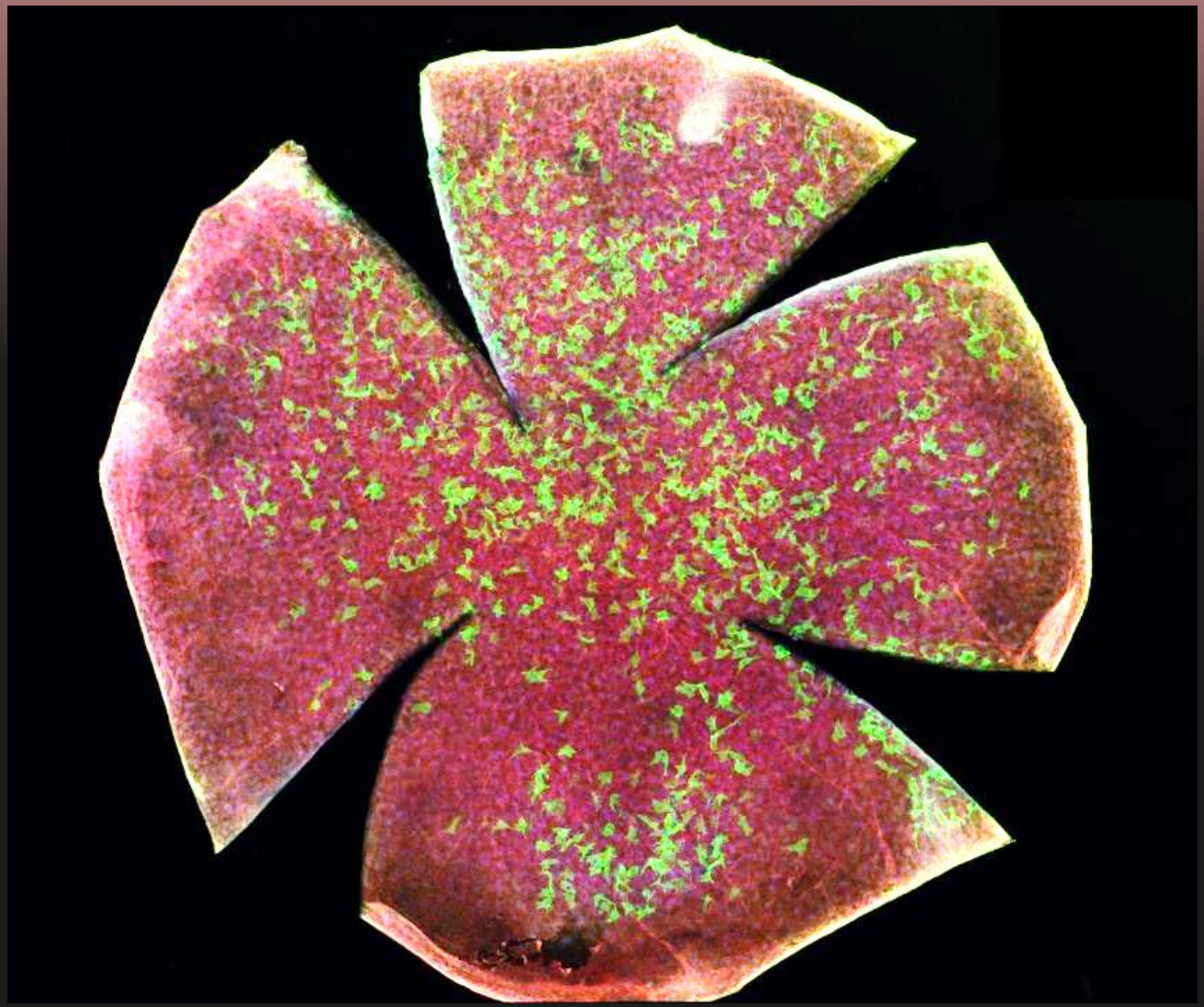
Mouse cnea tissue, multicolor immunofluorescent labeling,
Z-stack 9, Tile scan 6x5 in 20X objective with shading correction. Captured by SP8+LAS-X



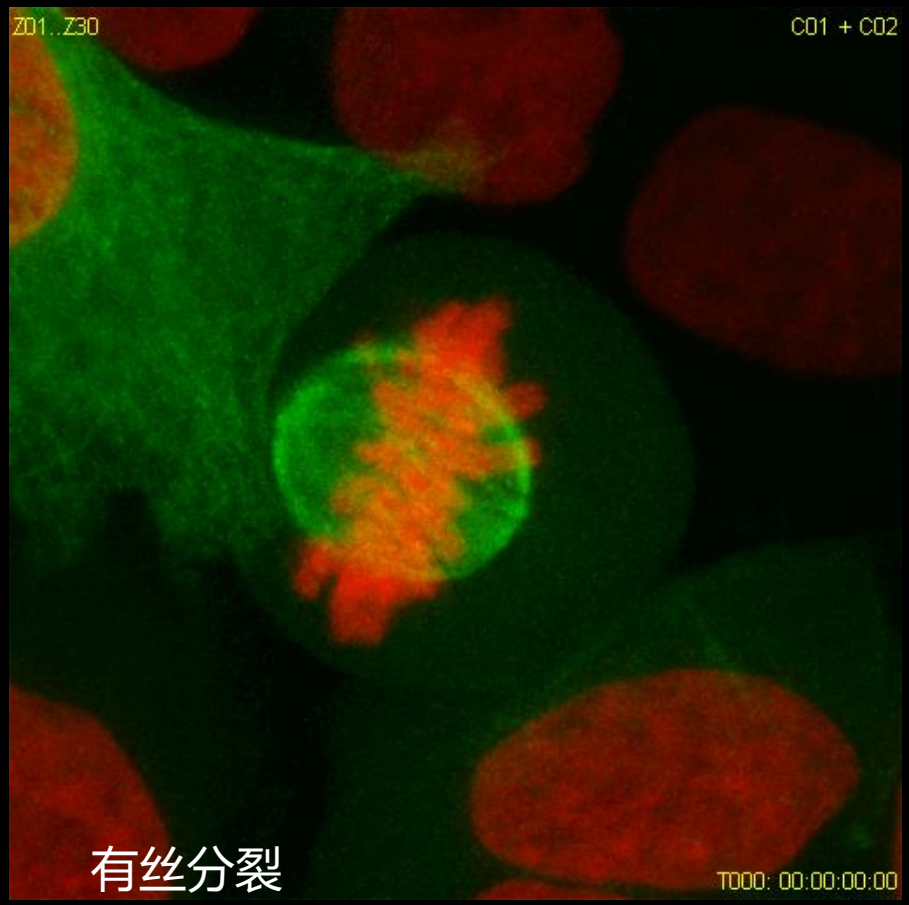
mouse Cronea
Red :BrdU, staining the proliferative cell
Blue: cell nucleus
Green: NeuN, staining the mature neure

Courtesy of Dr. Zhizuo Chen, School of Life Sciences, Xiamen University, China

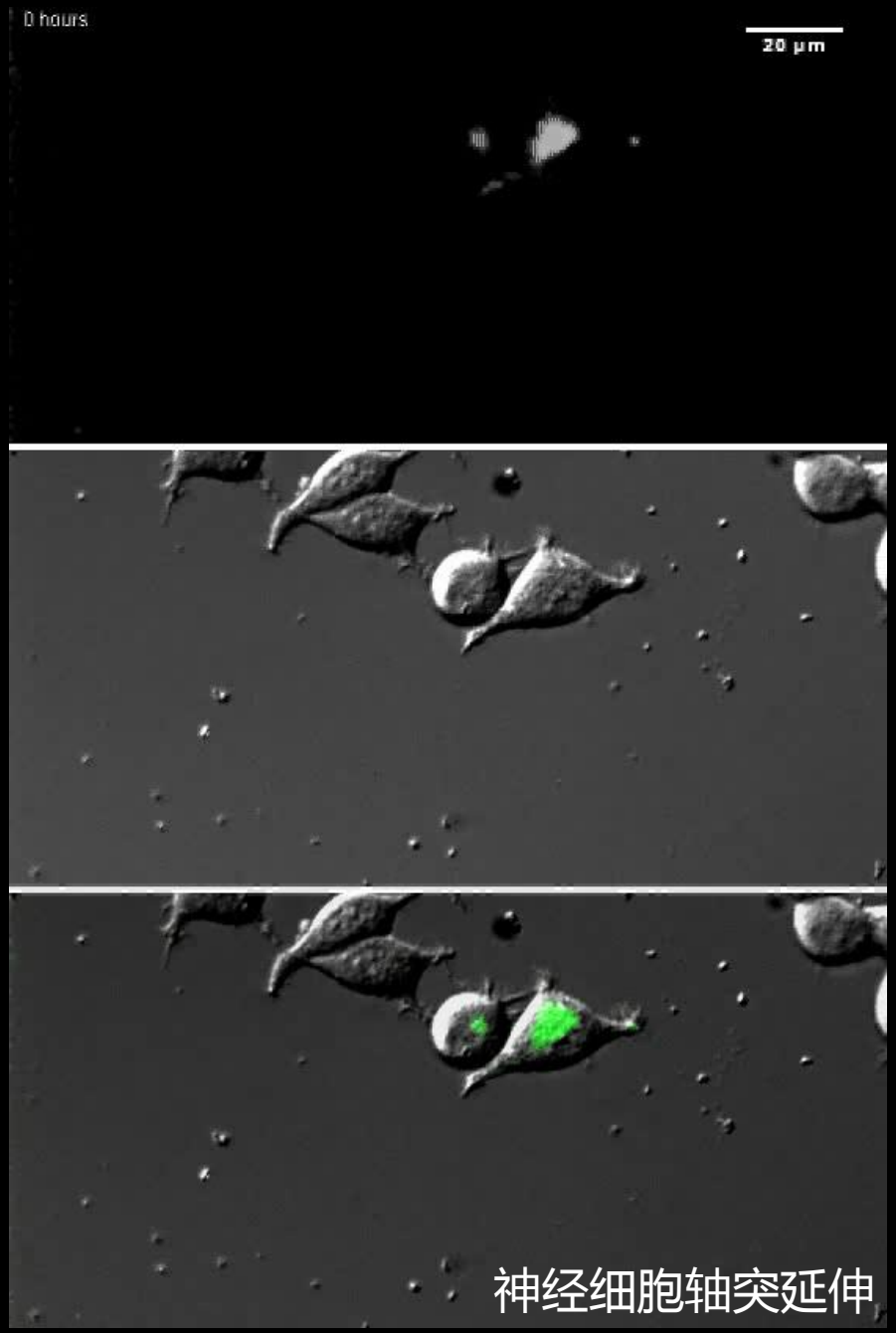
3D拼图z轴投影



xyt 扫描



有丝分裂



神经细胞轴突延伸

活细胞成像

小鼠胚胎异染色质的形成需要关键的组蛋白变体H3.3.

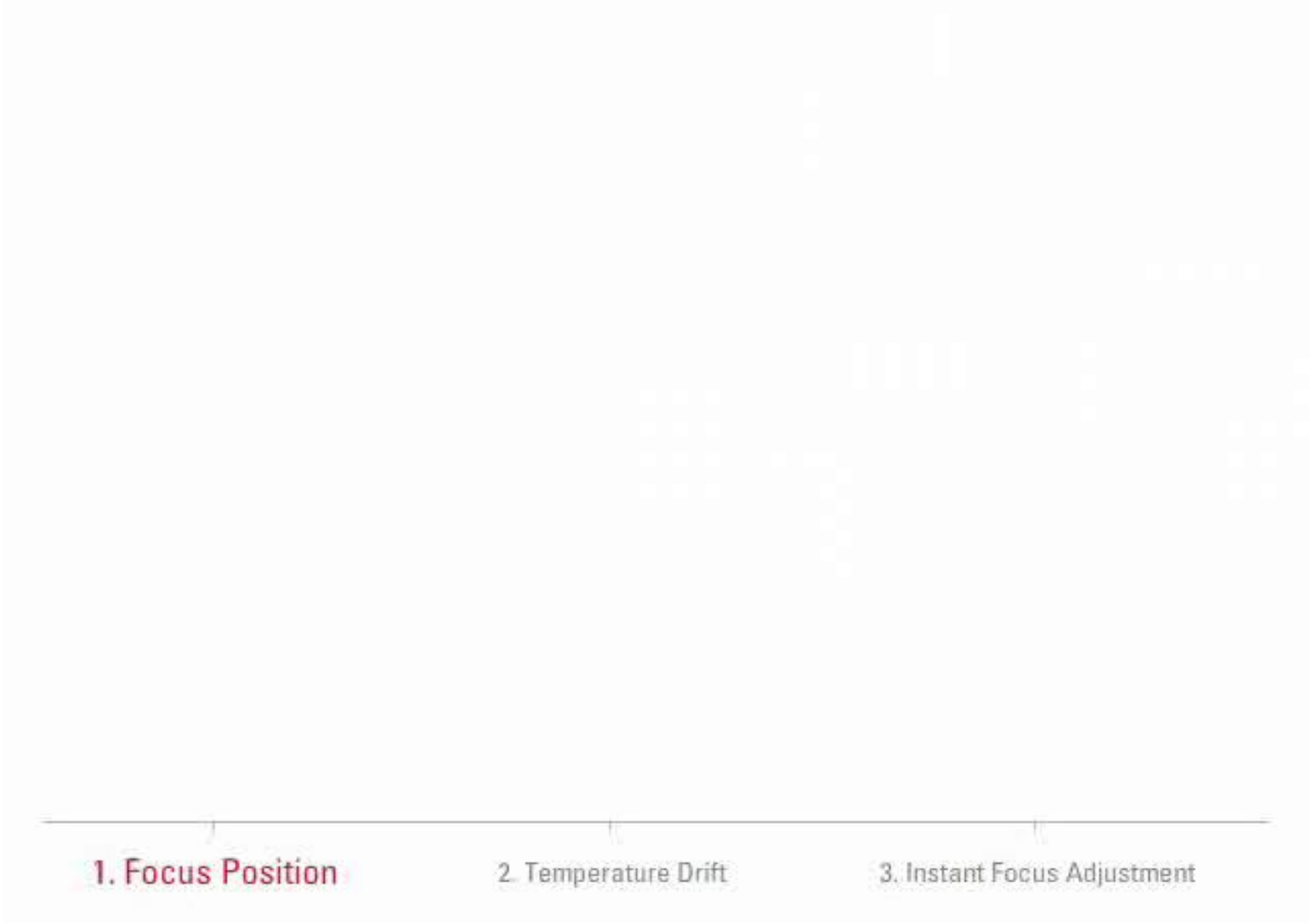
Courtesy of ME Torres-Padilla (Team L. Tora) & Marc Koch (Imaging Centre IGBMC).

显微注射oregon green后细胞的生存实验

Courtesy of Adrien Eberlin (Team L Tora) & Marc Koch (Imaging Centre IGBMC).

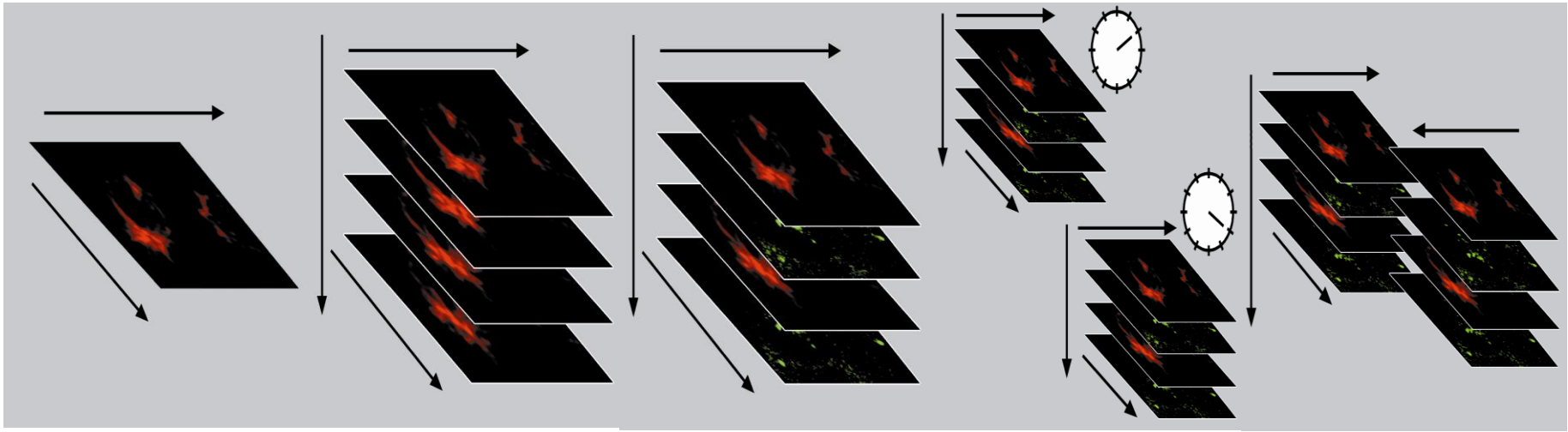
AFC硬件支持自动稳焦系统

AFC采用850nm LED，对细胞损伤更低、对近红外染料成像无影响

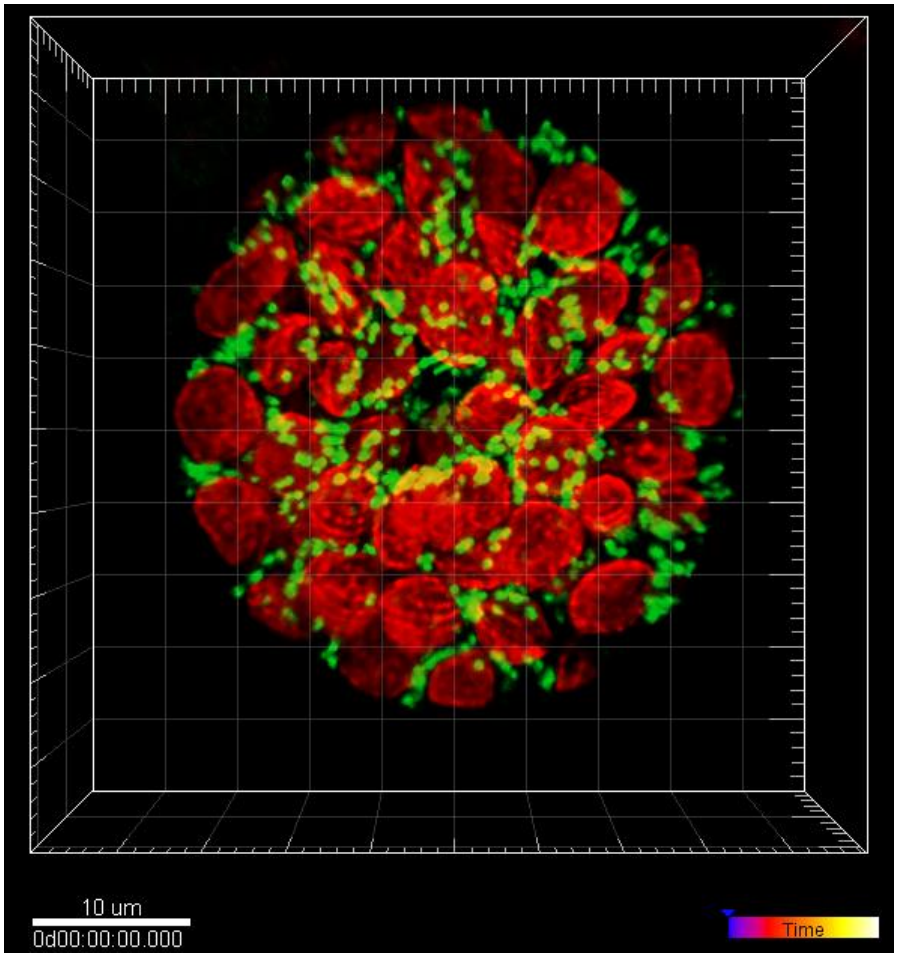


AFC硬件支持自动稳焦系统

xyzt 扫描

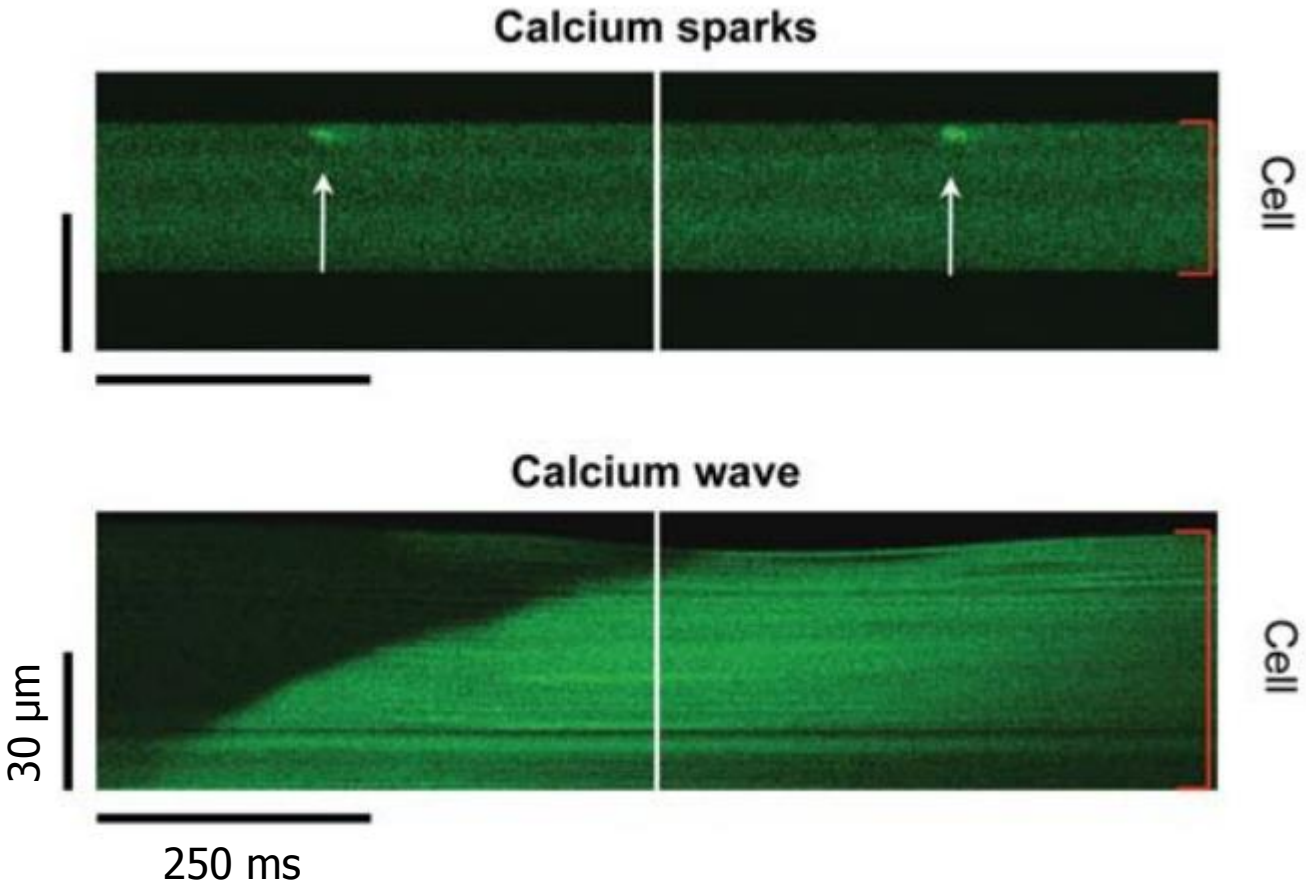


细胞器运动三维追踪



Arabidopsis thaliana protoplast
Monitoring mitochondrial (GFP-green) and chloroplast (autofluorescence-red) movement.

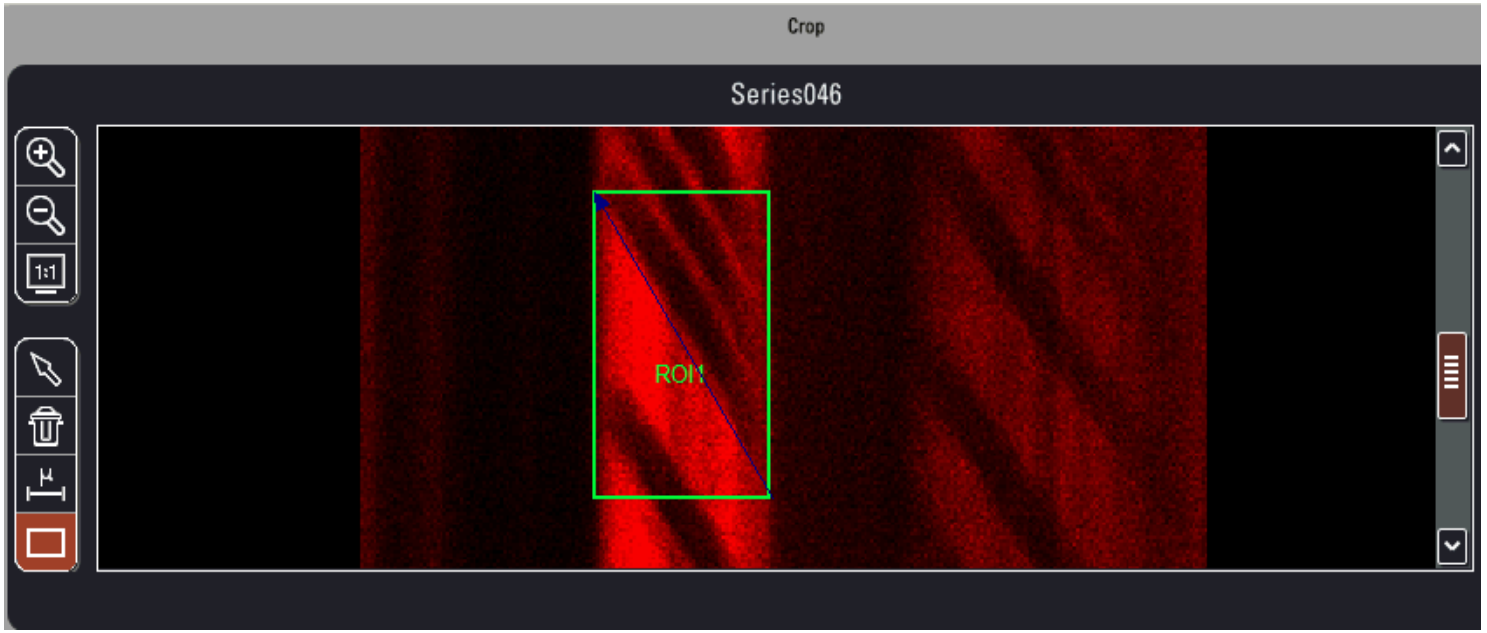
xt扫描



Consecutive line-scan images from HAMs showing two calcium sparks and a calcium wave.

Anna Llach *et al.*, European Heart Journal (2011)

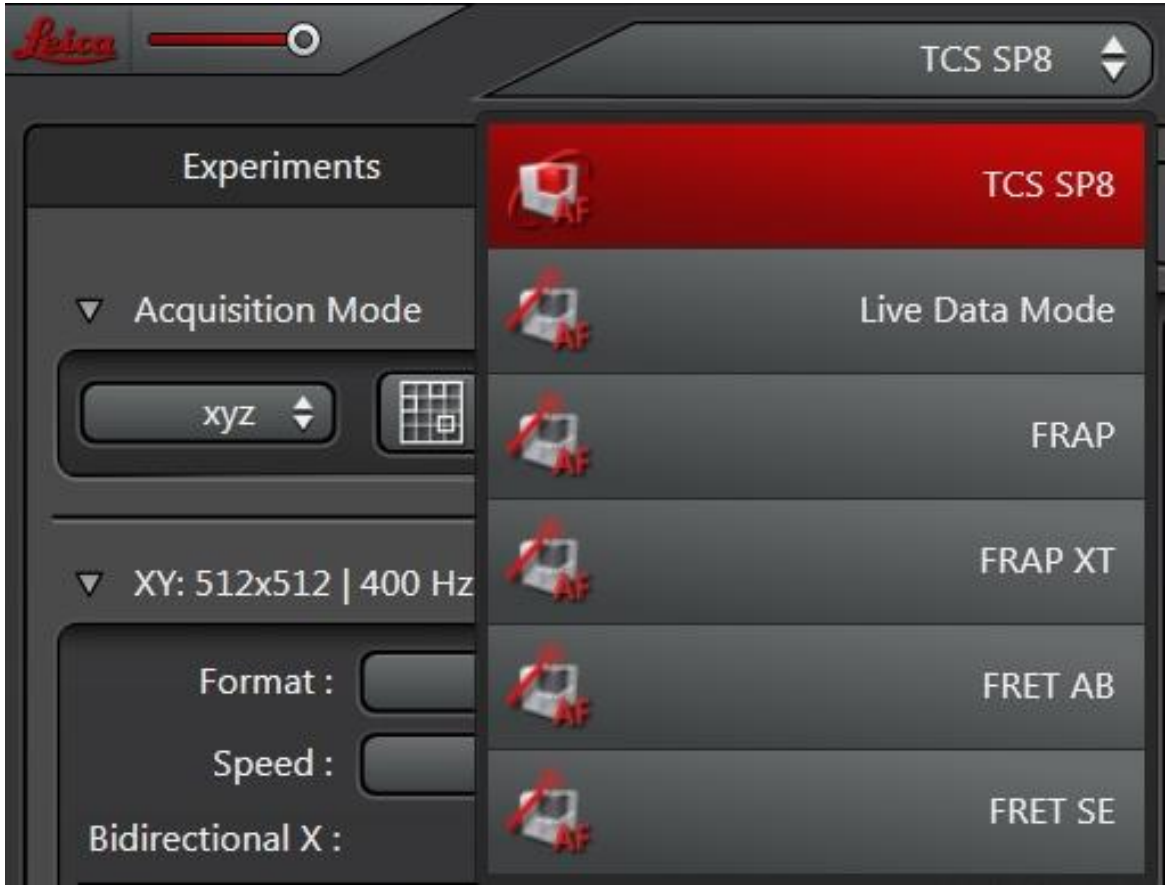
xt扫描：血流速度测量



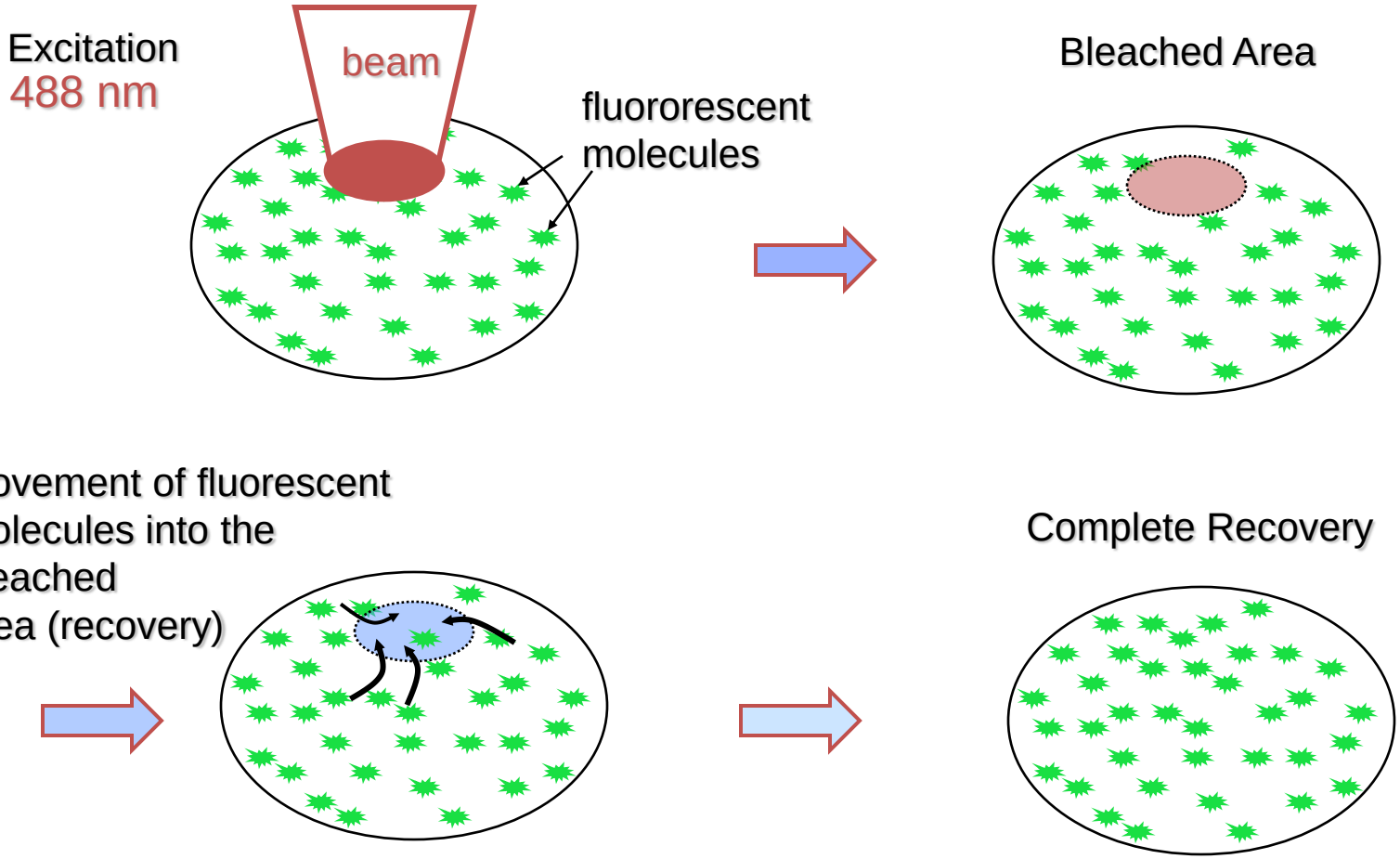
Dimensions

Dimension	Logical Size	Physical Length	Physical Origin
X	53	23.61 µm	74.46 µm
T	86	0.061s	0.230s

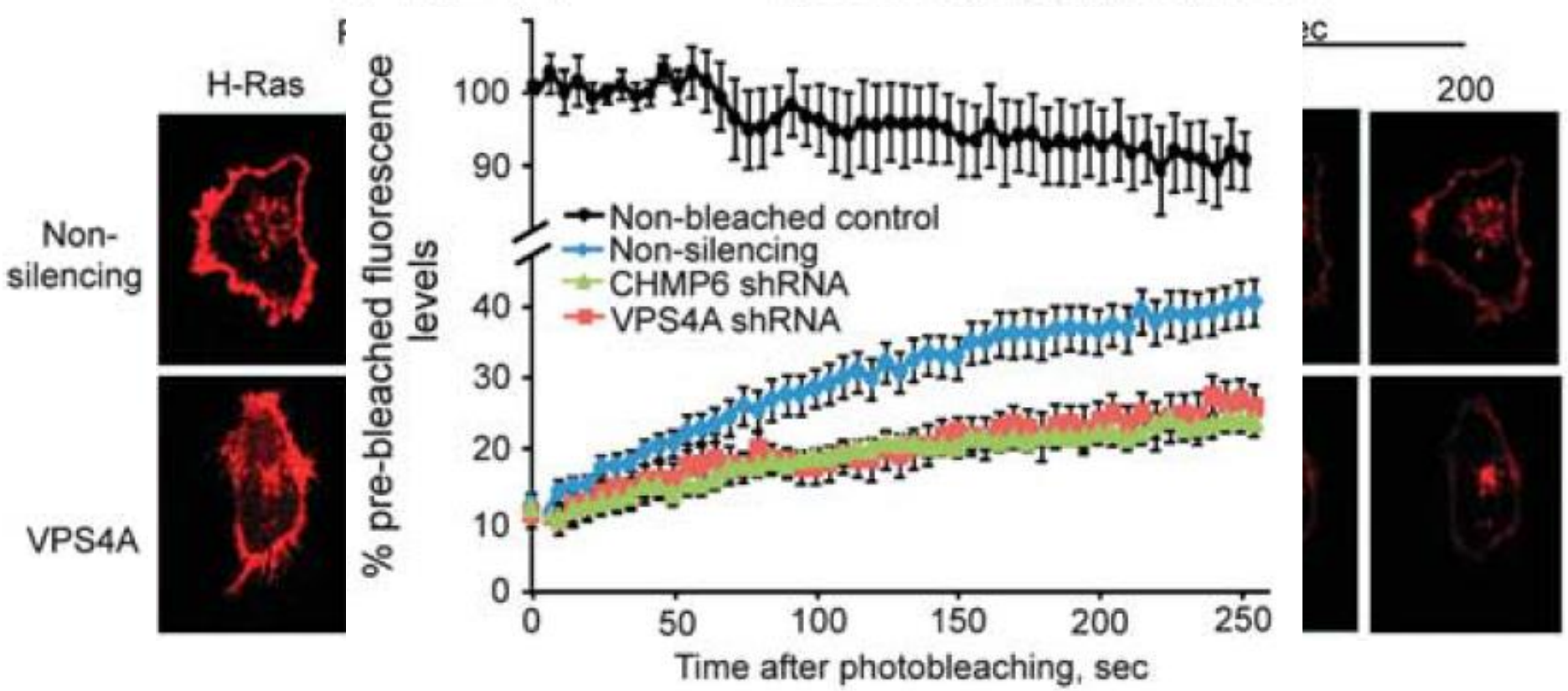
高端应用成像模块


TCS SP8
Experiments
Acquisition Mode
xyz
XY: 512x512 | 400 Hz
Format :
Speed :
Bidirectional X :
TCS SP8
Live Data Mode
FRAP
FRAP XT
FRET AB
FRET SE

荧光漂白后恢复 FRAP

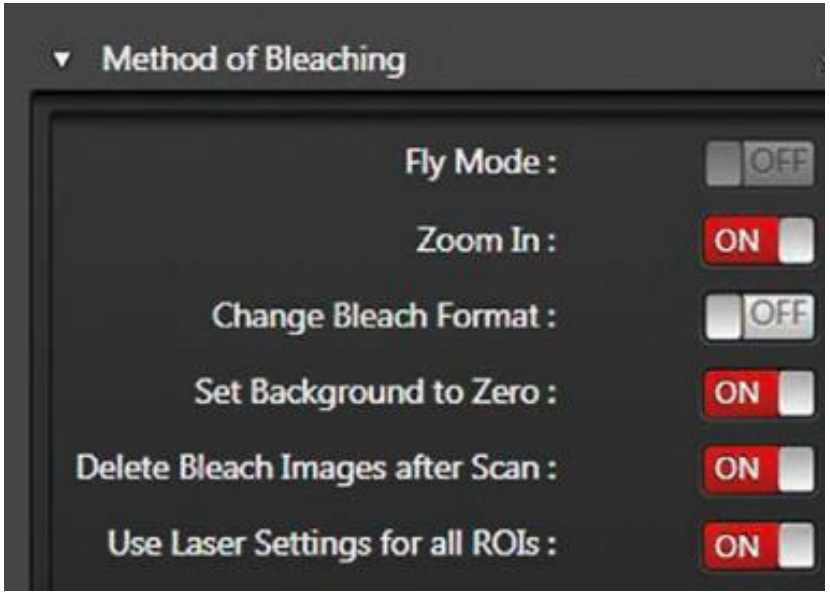


荧光漂白后恢复 **FRAP**



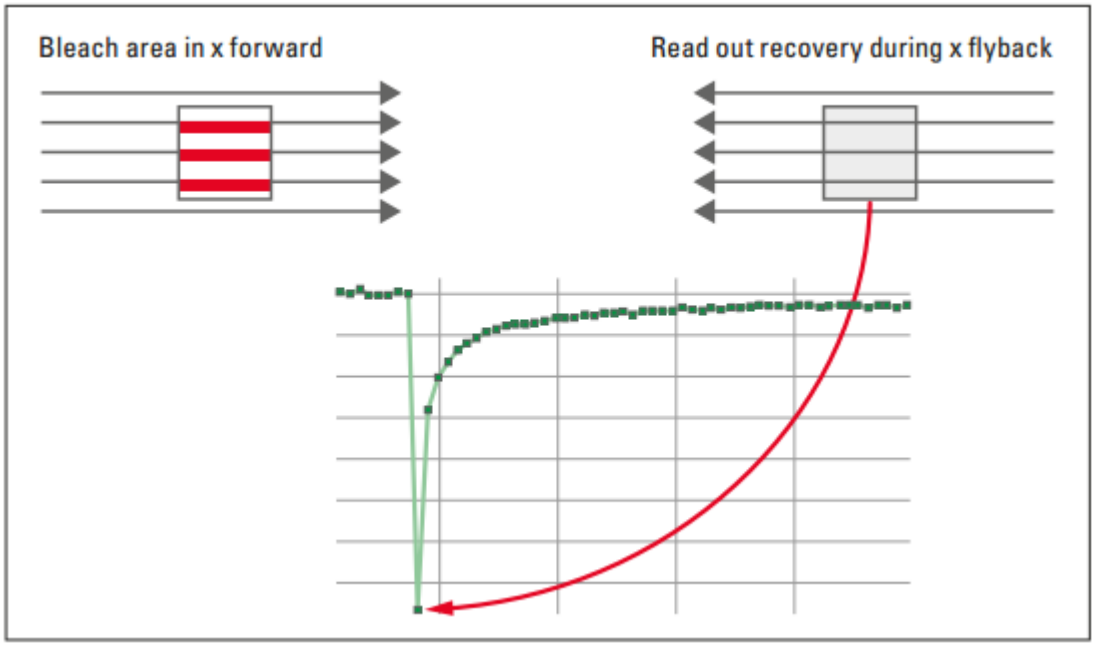
Z-Y Zheng *et al*., Oncogene, 2012

FRAP参数设置



Zoom in：
可大幅增强漂白效果

Flymode：
结合双向扫描的方式，
适用于快速恢复实验



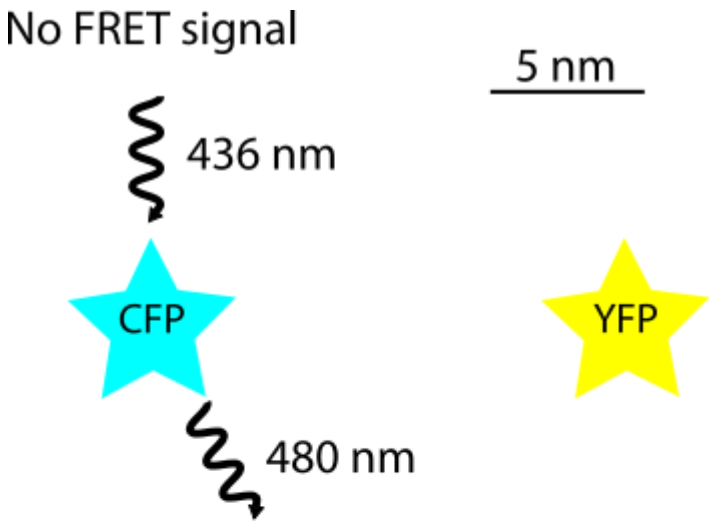
荧光共振能量转移 FRET

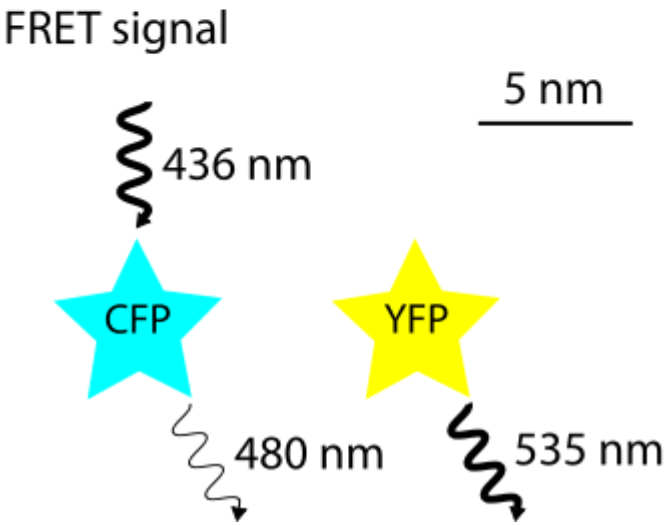
应用：

蛋白与蛋白相互作用

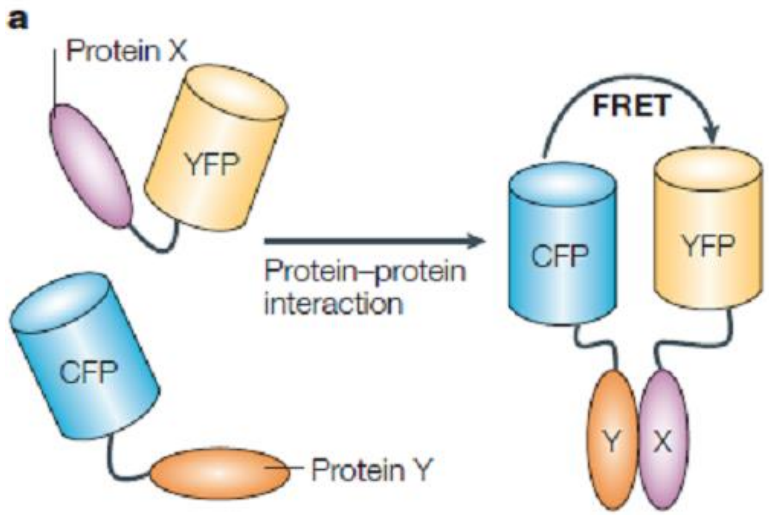
Cameleon测钙

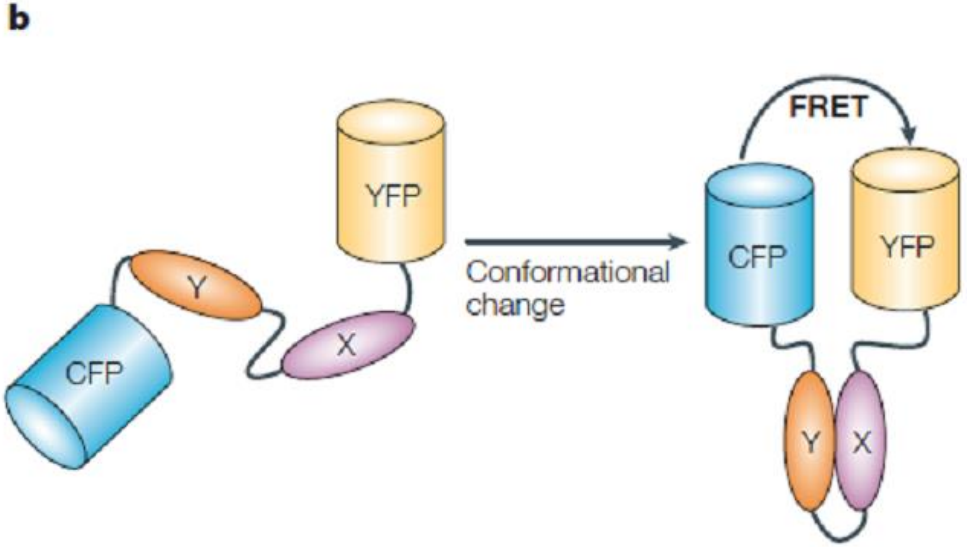
蛋白构象变化

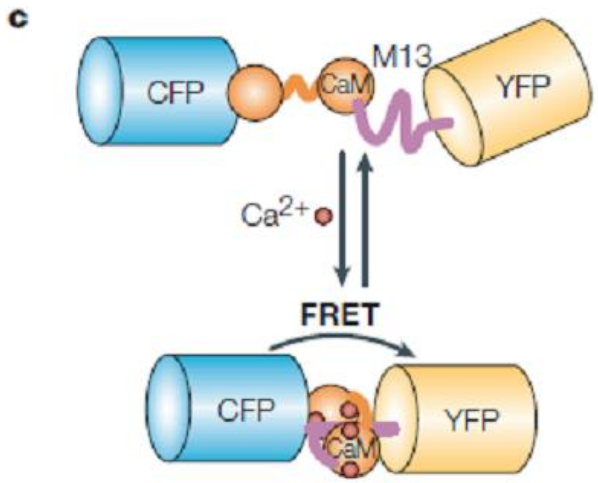


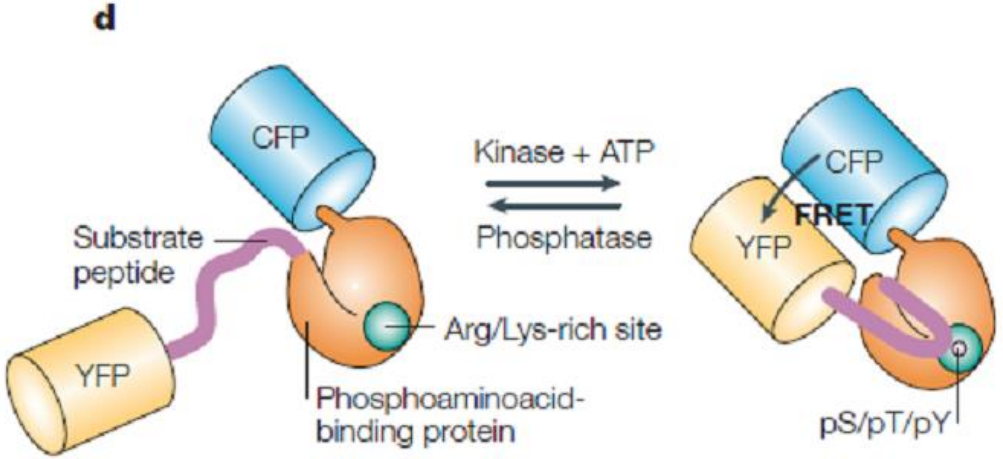


FRET应用


a
Protein X
YFP
CFP
Protein Y
Protein–protein interaction
FRET
CFP
YFP
Y
X


b
YFP
Y
X
CFP
Conformational change
FRET
CFP
YFP
Y
X

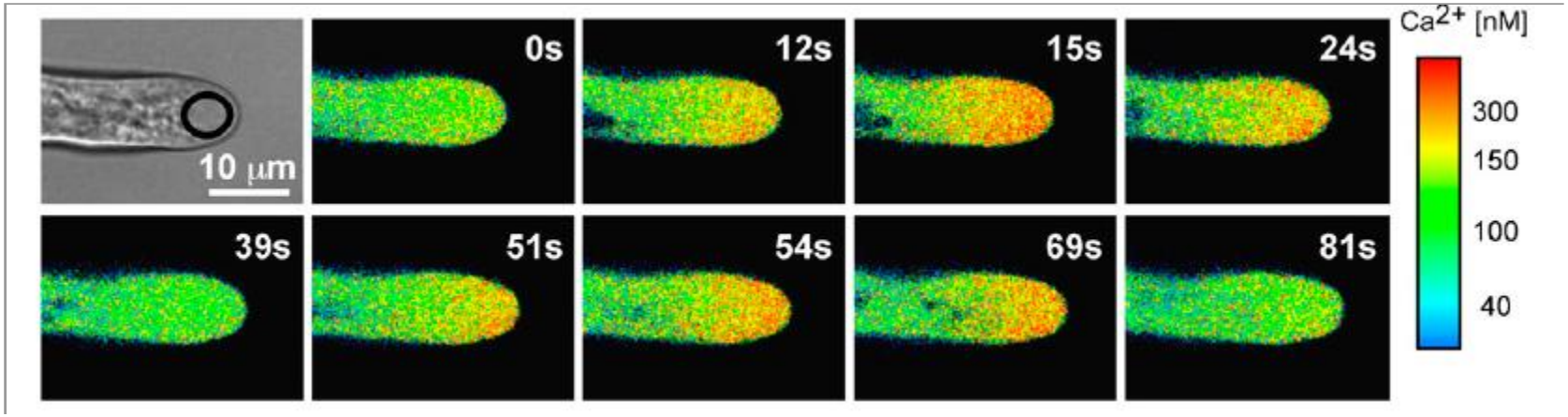

c
CFP
M13
CaM
YFP
Ca2+
FRET
CFP
CaM
YFP

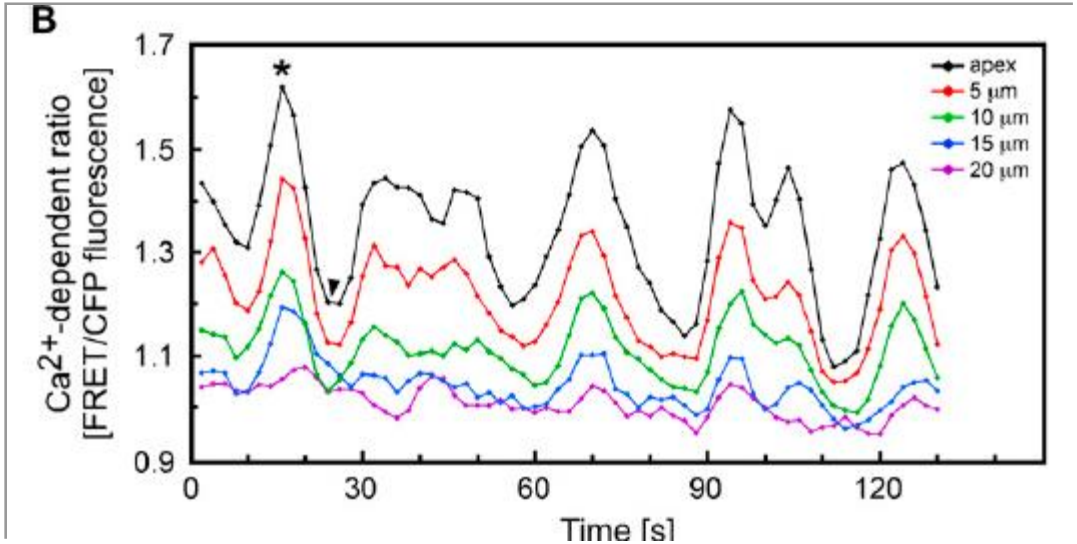

d
CFP
Kinase + ATP
Phosphatase
Substrate peptide
YFP
Arg/Lys-rich site
Phosphoaminoacid-binding protein
CFP
FRET
YFP
pS/pT/pY

FRET Pairs

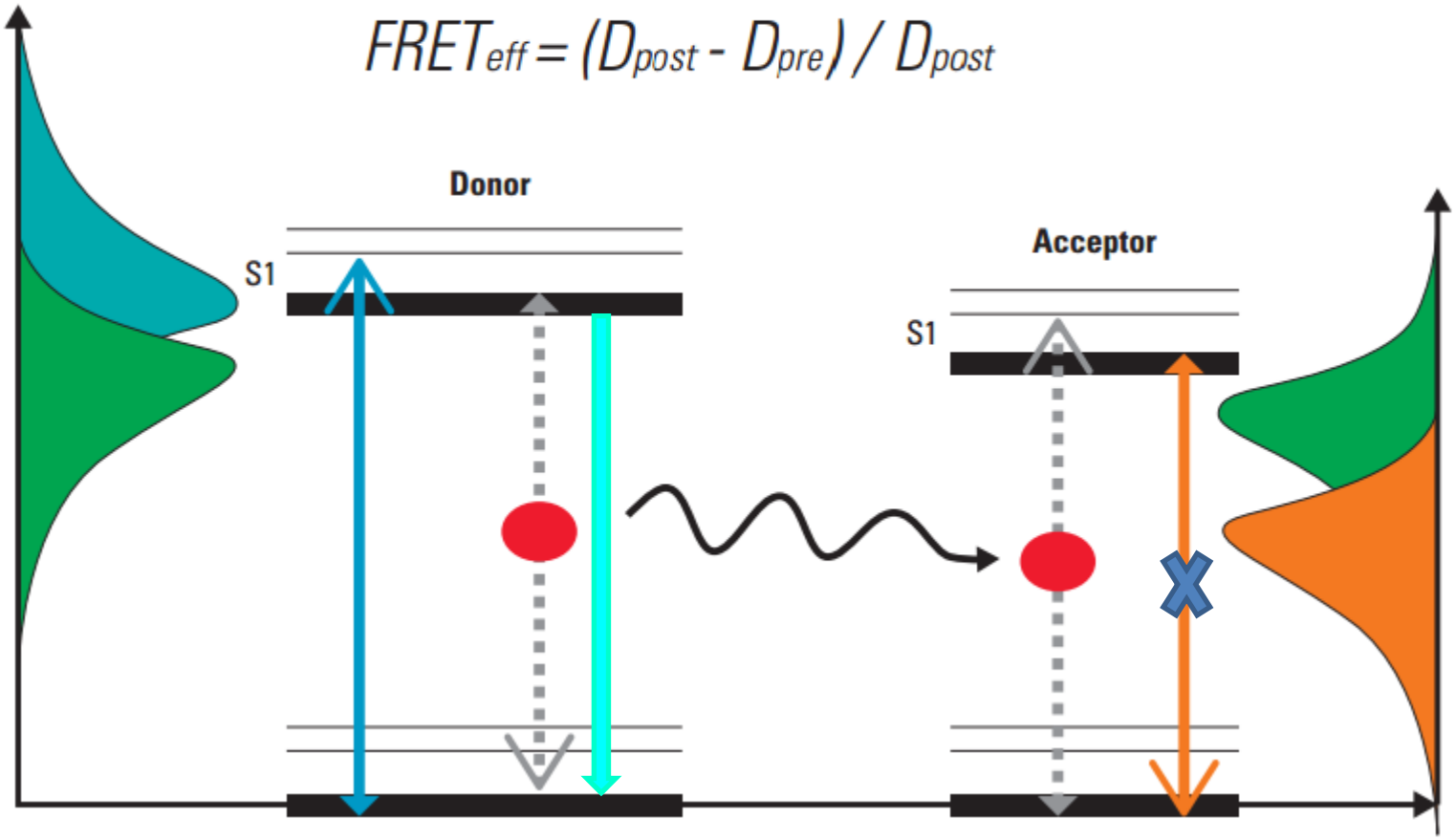
Protein (Acronym)	Ex (nm)	Em (nm)	EC × 10^{-3} M^{-1} cm^{-1}	QY	Relative Brightness (% of EGFP)[a]	Use as FRET probe
Aequorea-based FPs						
EBFP2	383	448	32.0	0.56	53	Donor to GFP/YFP
mCerulean3	433	475	40.0	0.87	103	Donor to YFP
mTurquoise	435	477	35.0	0.51	53	Donor to YFP
EGFP	488	507	56.0	0.60	100	Donor to OFP, RFP
mVenus	515	528	92.2	0.57	156	Acceptor for CFP, Donor to RFP
mCitrine	516	529	77.0	0.76	174	Acceptor for CFP
T-Sapphire	399	511	44.0	0.60	79	Long Stokes shift donor
mAmetrine	406	526	45.0	0.58	78	Long Stokes shift donor
REACh	515	528	92.2	0.04	1	Strong absorber, weak emitter Acceptor for FLIM studies
Coral FPs						
Midoriishi Cyan	472	495	27.3	0.90	73	Donor to mKO
mTFP1	462	492	64.0	0.85	162	Donor to YFP, OFP, RFP
Kusabira Orange2	551	565	63.8	0.62	118	Acceptor for CFP
mCherry	587	610	72.0	0.22	47	Acceptor for GFP
TagRFP-T	555	584	81.0	0.41	99	Acceptor for GFP
mRuby	558	605	112.0	0.35	117	Acceptor for GFP

YC3.6 细胞内钙浓度测定



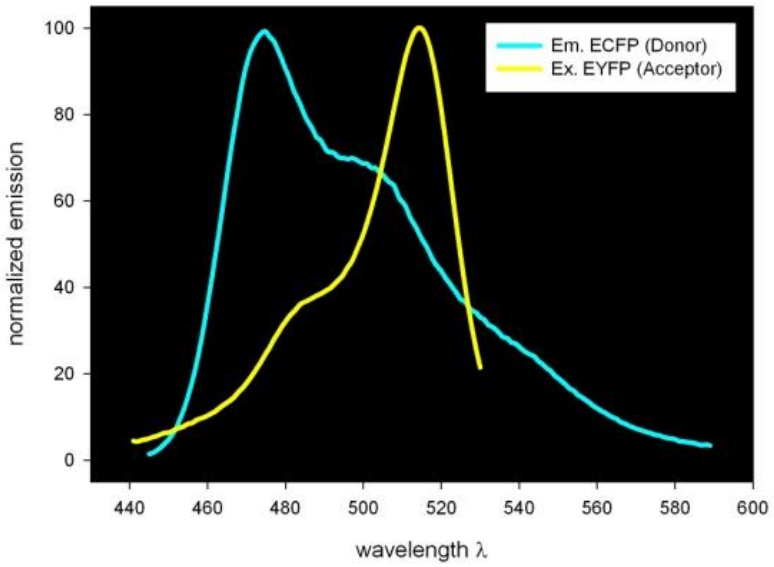


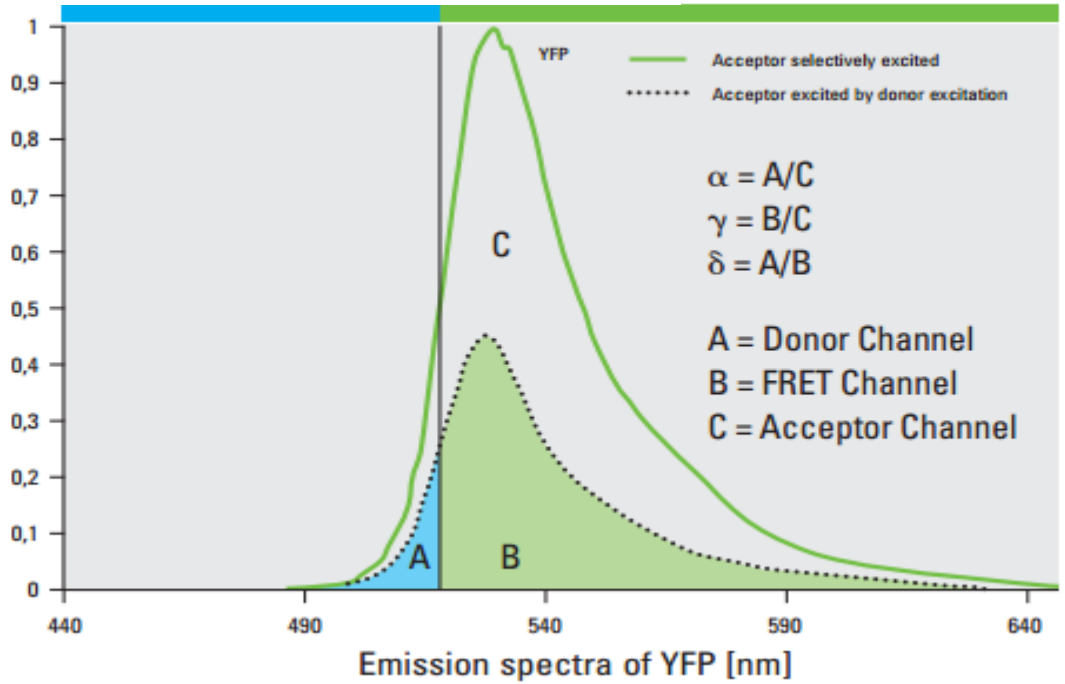
FRET AB（Acceptor Bleach）受体漂白法



FRET SE（Sensitized Emission）敏化发射法

	Channel 1 (A)	Channel 2 (B)	Channel 3 (C)
Specimen: FRET	Signal (Donor)	Signal (FRET)	Signal (Acceptor)
Specimen: Donor only	Signal (Donor)	Signal < Channel 1 (x-talk)	No Signal
Specimen: Acceptor only	Very little to no Signal (x-excited x-talk Acceptor)	Signal < Channel 3 (x-Excitation)	Signal (Acceptor)





FRET SE（Sensitized Emission）敏化发射法

Method 1:

$$E_A(i) = \frac{B - A \times \beta - C \times \gamma}{C}$$

Ref. Wouters et al., TRENDS in Cell Biology, Vol 11, No.5, May 2001: 203-211

Method 2:

$$E_A(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$$

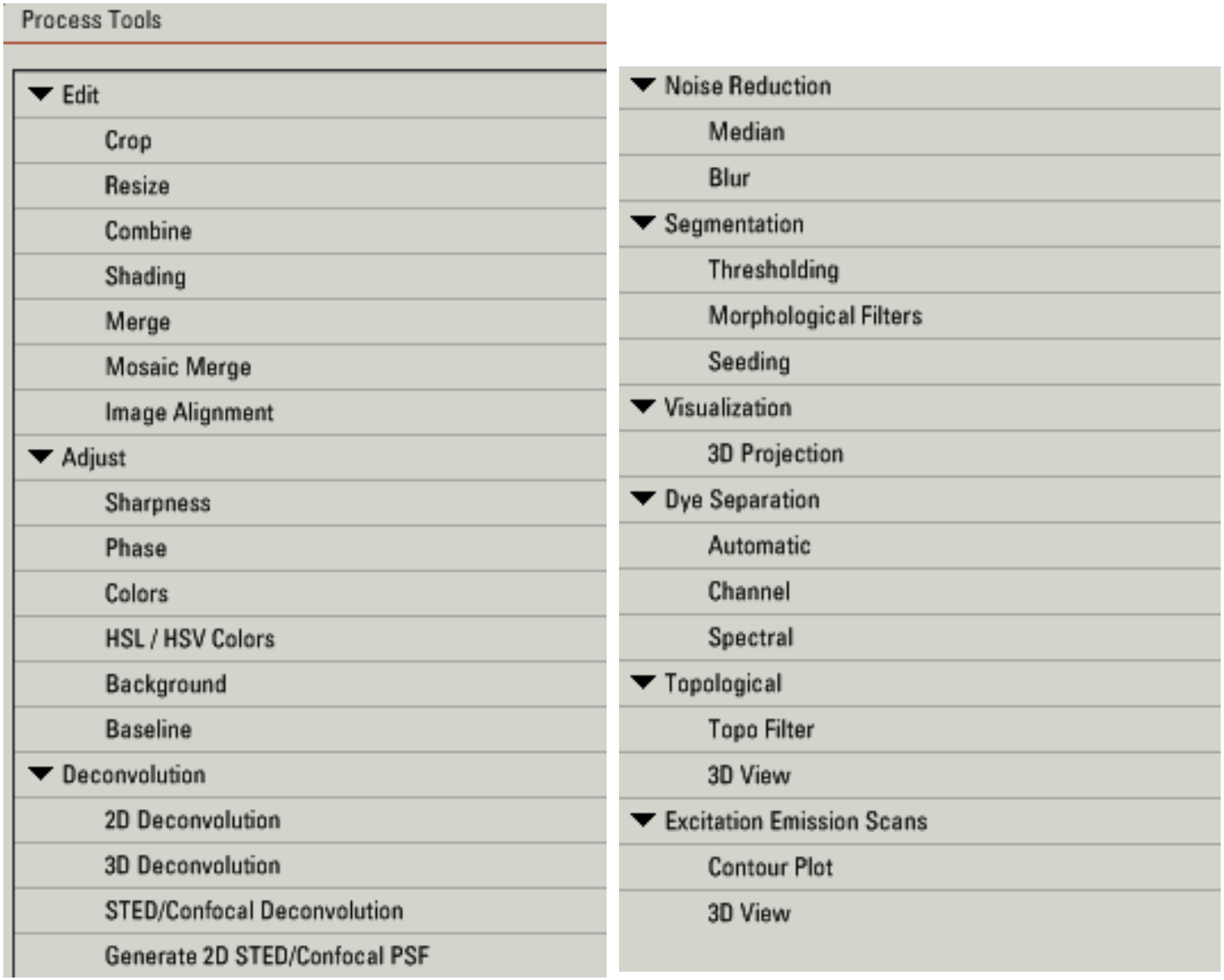
Ref. Van Rheenen, J., M. Langeslag, K. Jalink: Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission. Biophysical Journal, Vol. 86, April 2004: 1-13.

Method 3:

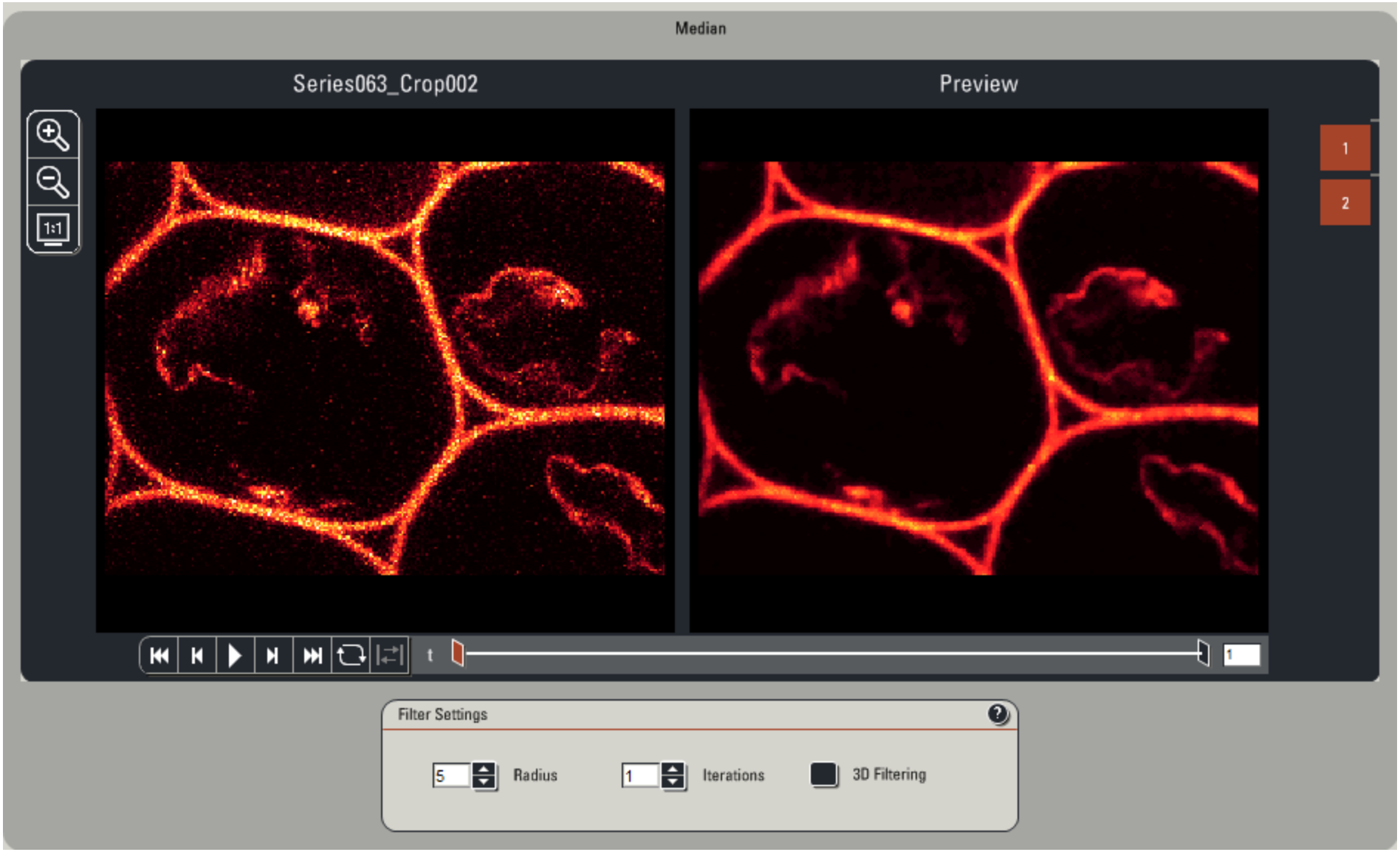
$$E_A(i) = \frac{B}{A}$$

The Ratiometric Calculation is used in samples with a fixed stochiometry (1:1) of donor and acceptor (e.g. Cameleons).

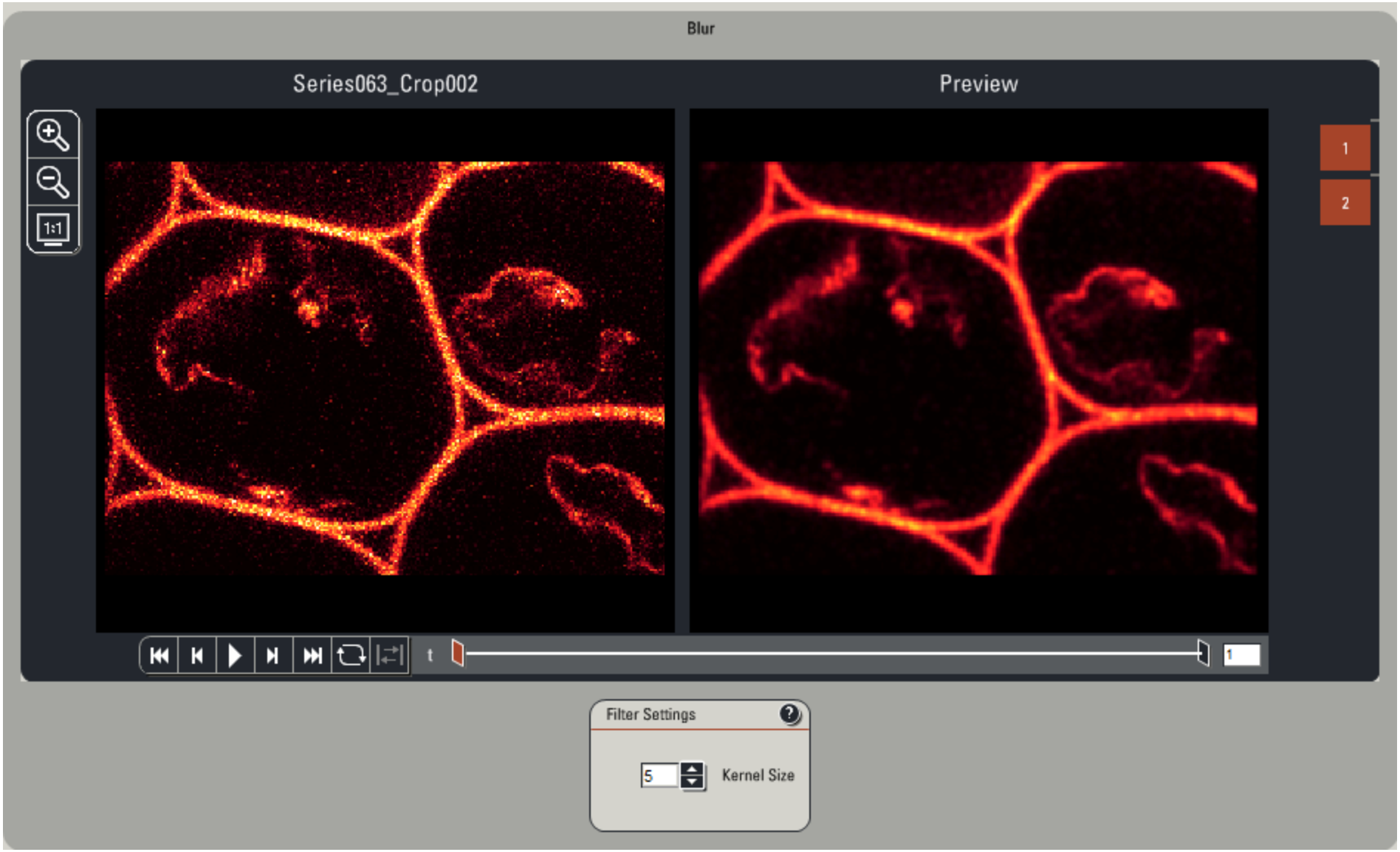
图像处理功能


Process Tools
Edit
Crop
Resize
Combine
Shading
Merge
Mosaic Merge
Image Alignment
Adjust
Sharpness
Phase
Colors
HSL / HSV Colors
Background
Baseline
Deconvolution
2D Deconvolution
3D Deconvolution
STED/Confocal Deconvolution
Generate 2D STED/Confocal PSF
Noise Reduction
Median
Blur
Segmentation
Thresholding
Morphological Filters
Seeding
Visualization
3D Projection
Dye Separation
Automatic
Channel
Spectral
Topological
Topo Filter
3D View
Excitation Emission Scans
Contour Plot
3D View

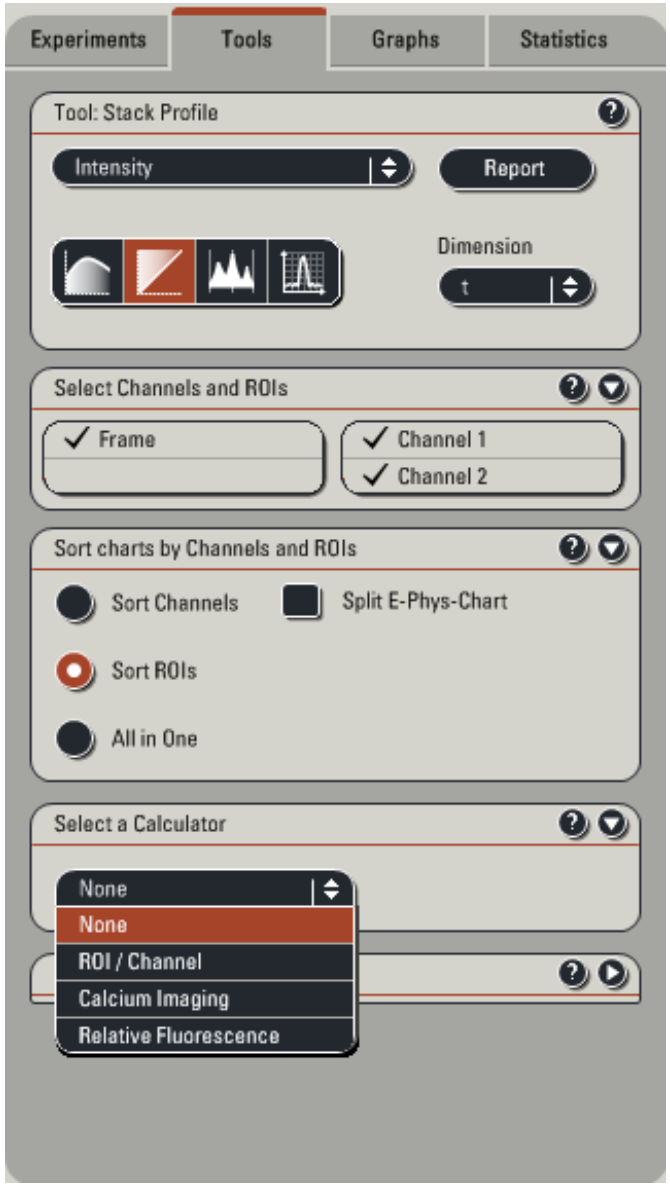
降噪：中值滤镜


Median
Series063_Crop002
Preview
1:1
1
2
t
1
Filter Settings
5
Radius
1
Iterations
3D Filtering

降噪：高斯滤镜


Blur
Series063_Crop002
Preview
1:1
1
2
t
1
Filter Settings
5
Kernel Size

荧光强度测量及计算器



测量维度

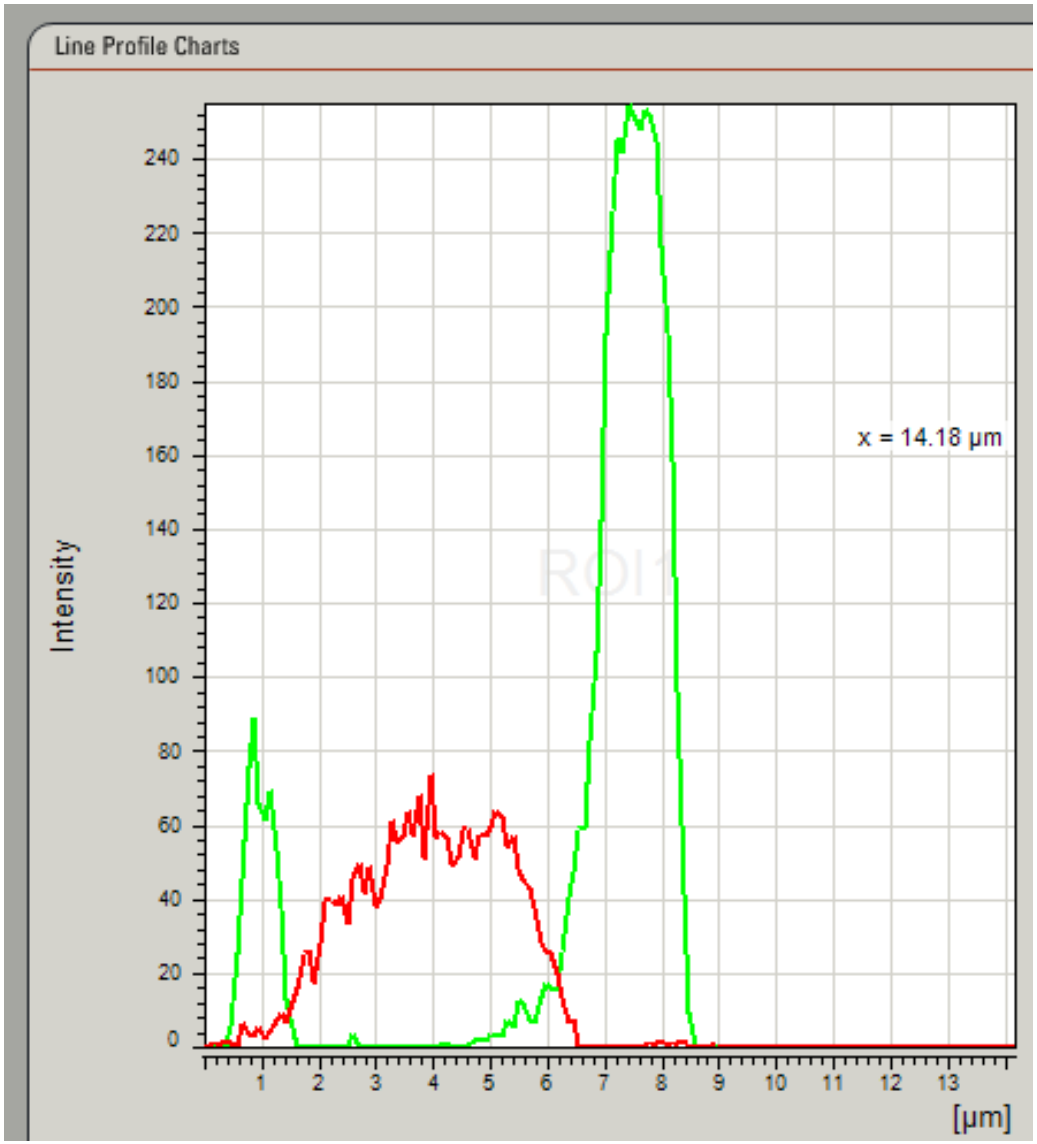
数据显示及分类方式设置

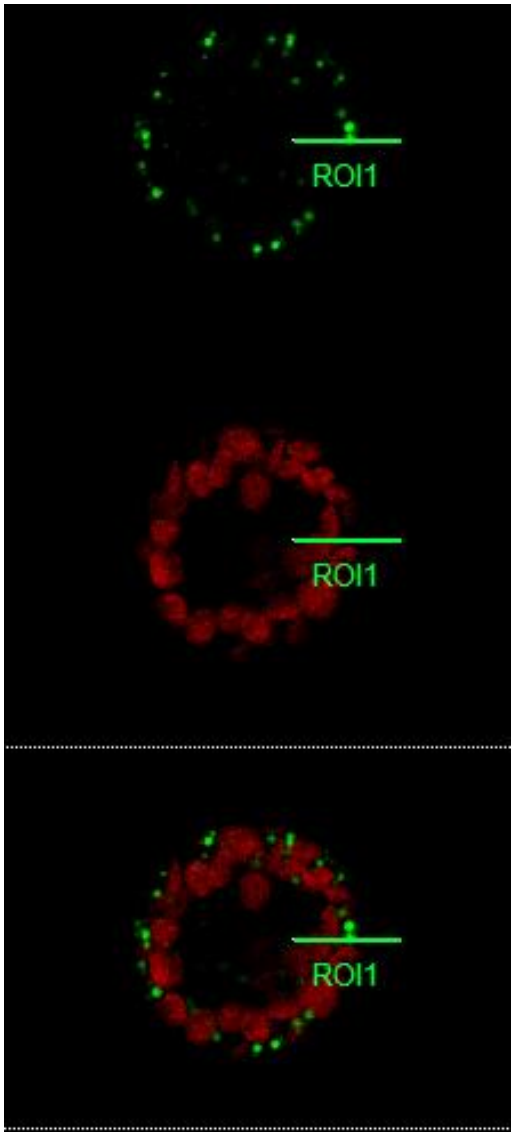
通道比率计算

钙离子浓度计算

相对荧光强度计算

一维测量


Line Profile Charts
Intensity
240
220
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
[µm]
x = 14.18 µm


ROI1
ROI1
ROI1

二维测量

Leica MICROSYSTEMS

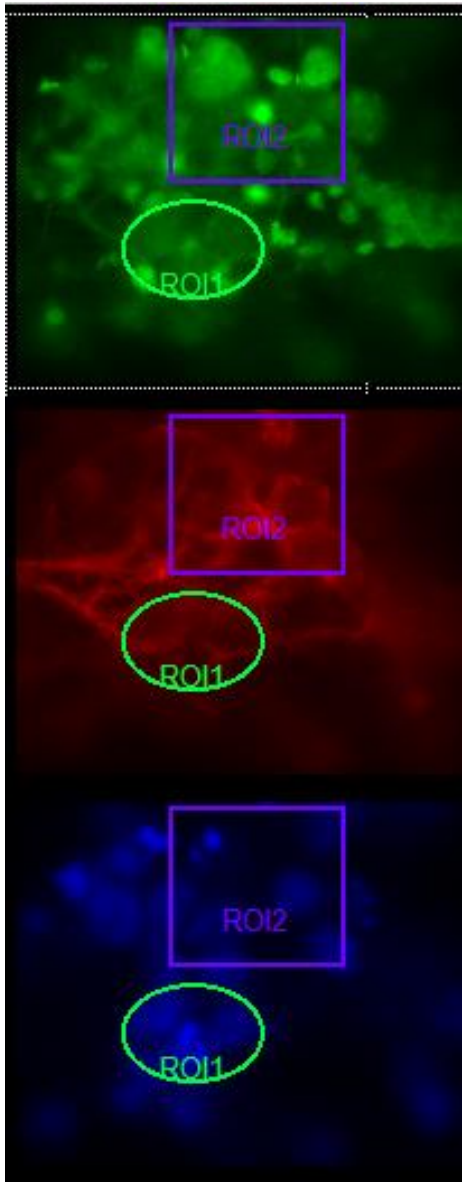
Experiments | Tools | Graphs

Stack Profile Statistics

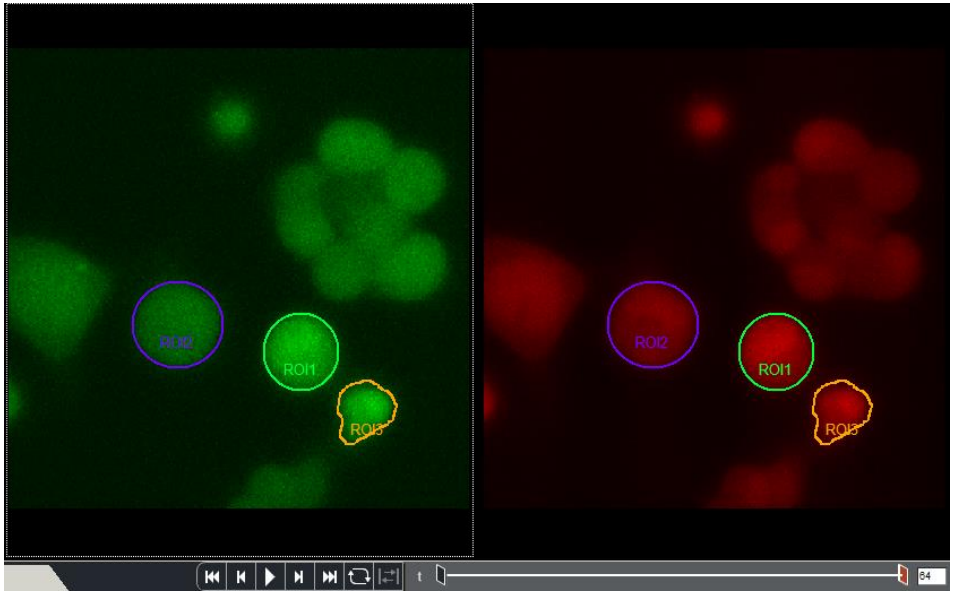
ROI1	Channel 1	Channel 2	Channel 3
Mean Value	1500.19	1162.85	1509.43
Pixel Count	117884	117884	117884
Pixel Sum	176.85 10E6	137.08 10E6	177.94 10E6
Length	17.64 µm	17.64 µm	17.64 µm
Frame Count	52	52	52
Variance	6816.4	530.5	25506.97
Standard Deviation	82.56	23.03	159.71
Average Deviation	62.79	17.12	138.57
Max Amplitude	1594.52	1185.93	1731.57
Max Position	9.34 µm	8.65 µm	9.68 µm
Min Amplitude	1282.98	1096.86	1194.42
Min Position	17.64 µm	17.64 µm	17.64 µm
Center Of Mass Pos.	8.68 µm	8.75 µm	8.85 µm

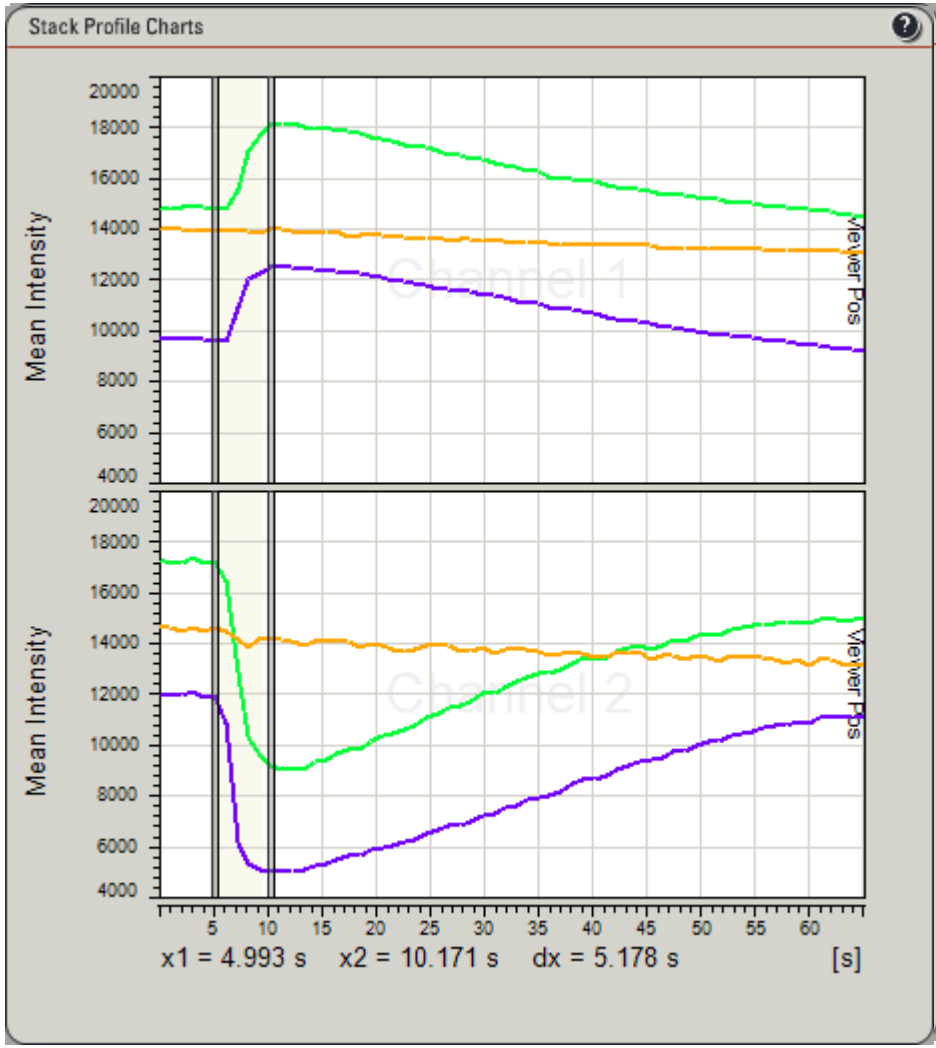
Export

ROI2	Channel 1	Channel 2	Channel 3
Mean Value	1513.31	1425.93	871
Pixel Count	303680	303680	303680
Pixel Sum	459.56 10E6	433.03 10E6	264.5 10E6

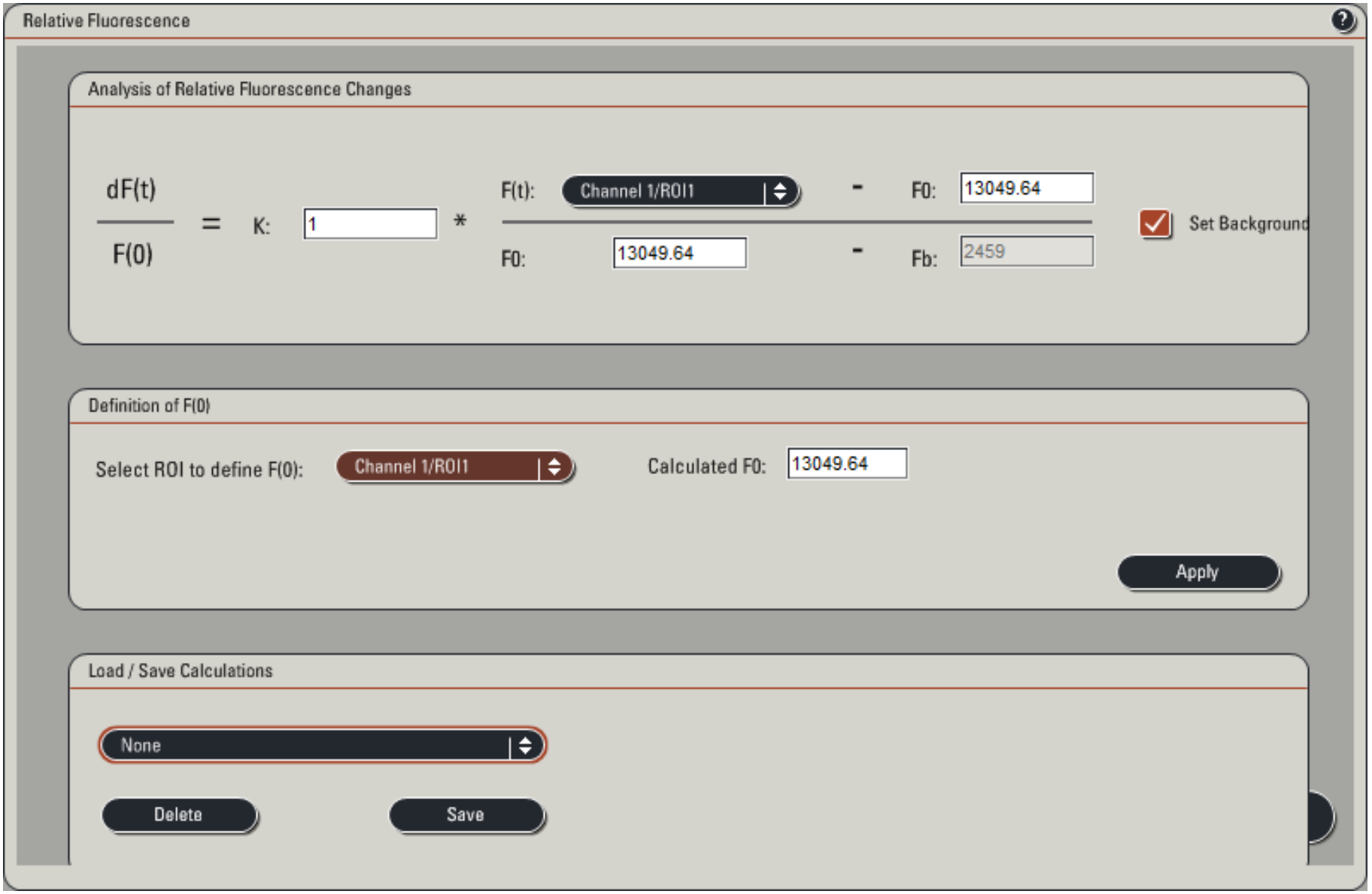


二维时间序列测量

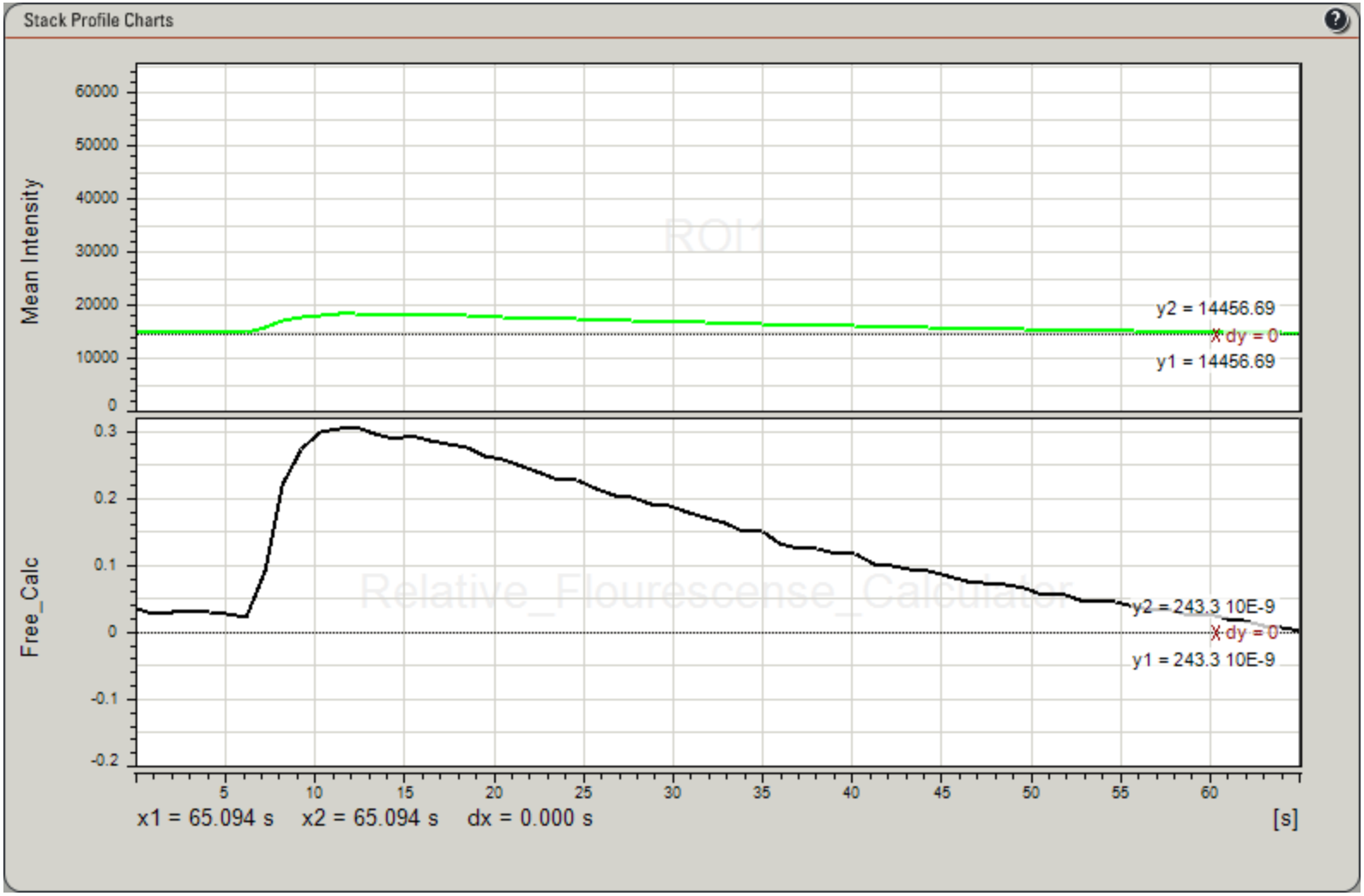

ROI2
ROI1
ROI3
ROI2
ROI1
ROI3


Stack Profile Charts
Mean Intensity
20000
18000
16000
14000
12000
10000
8000
6000
4000
Viewer Pos
Channel 1
Mean Intensity
Viewer Pos
Channel 2
5
10
15
20
25
30
35
40
45
50
55
60
x1 = 4.993 s
x2 = 10.171 s
dx = 5.178 s
[s]

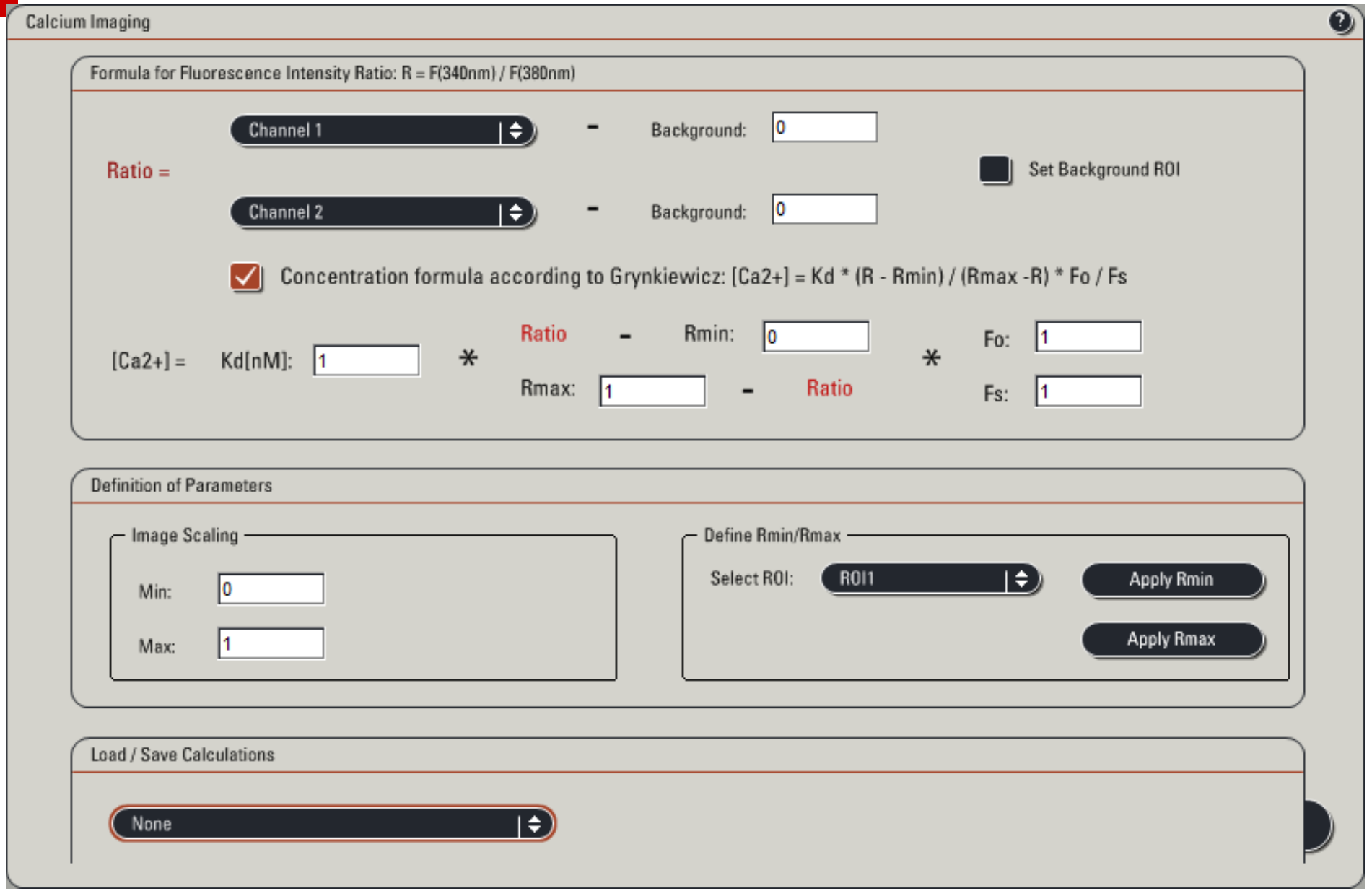
相对荧光变化计算


Relative Fluorescence
Analysis of Relative Fluorescence Changes
dF(t) / F(0) = K: 1 * F(t): Channel 1/ROI1 - F0: 13049.64 / F0: 13049.64 - Fb: 2459
Set Background
Definition of F(0)
Select ROI to define F(0): Channel 1/ROI1
Calculated F0: 13049.64
Apply
Load / Save Calculations
None
Delete
Save

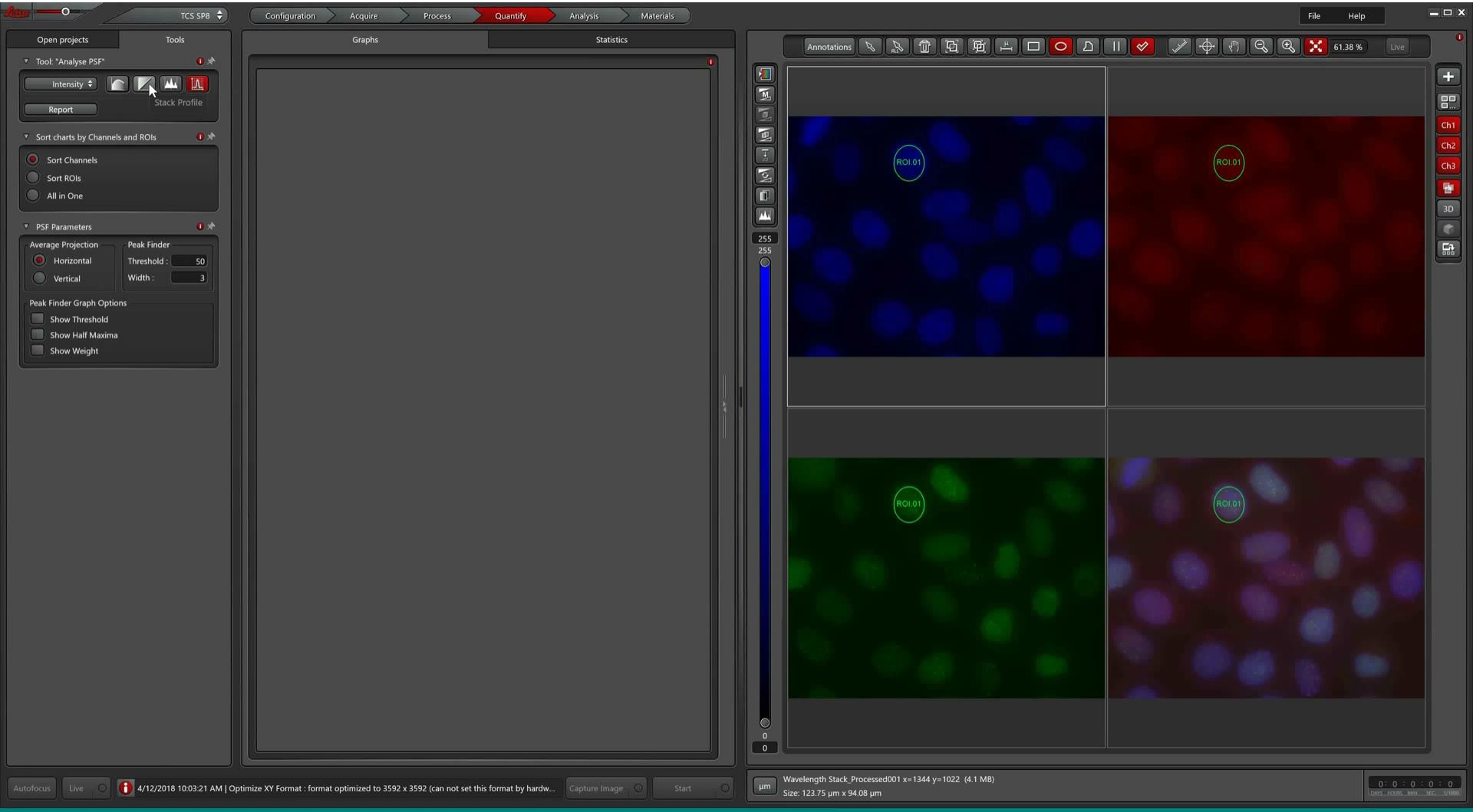
相对荧光强度变化


Stack Profile Charts
Mean Intensity
60000
50000
40000
30000
20000
10000
0
ROI1
y2 = 14456.69
dy = 0
y1 = 14456.69
Free_Calc
0.3
0.2
0.1
0
-0.1
-0.2
Relative_Flourescense_Calculator
y2 = 243.3 10E-9
dy = 0
y1 = 243.3 10E-9
5
10
15
20
25
30
35
40
45
50
55
60
x1 = 65.094 s
x2 = 65.094 s
dx = 0.000 s
[s]

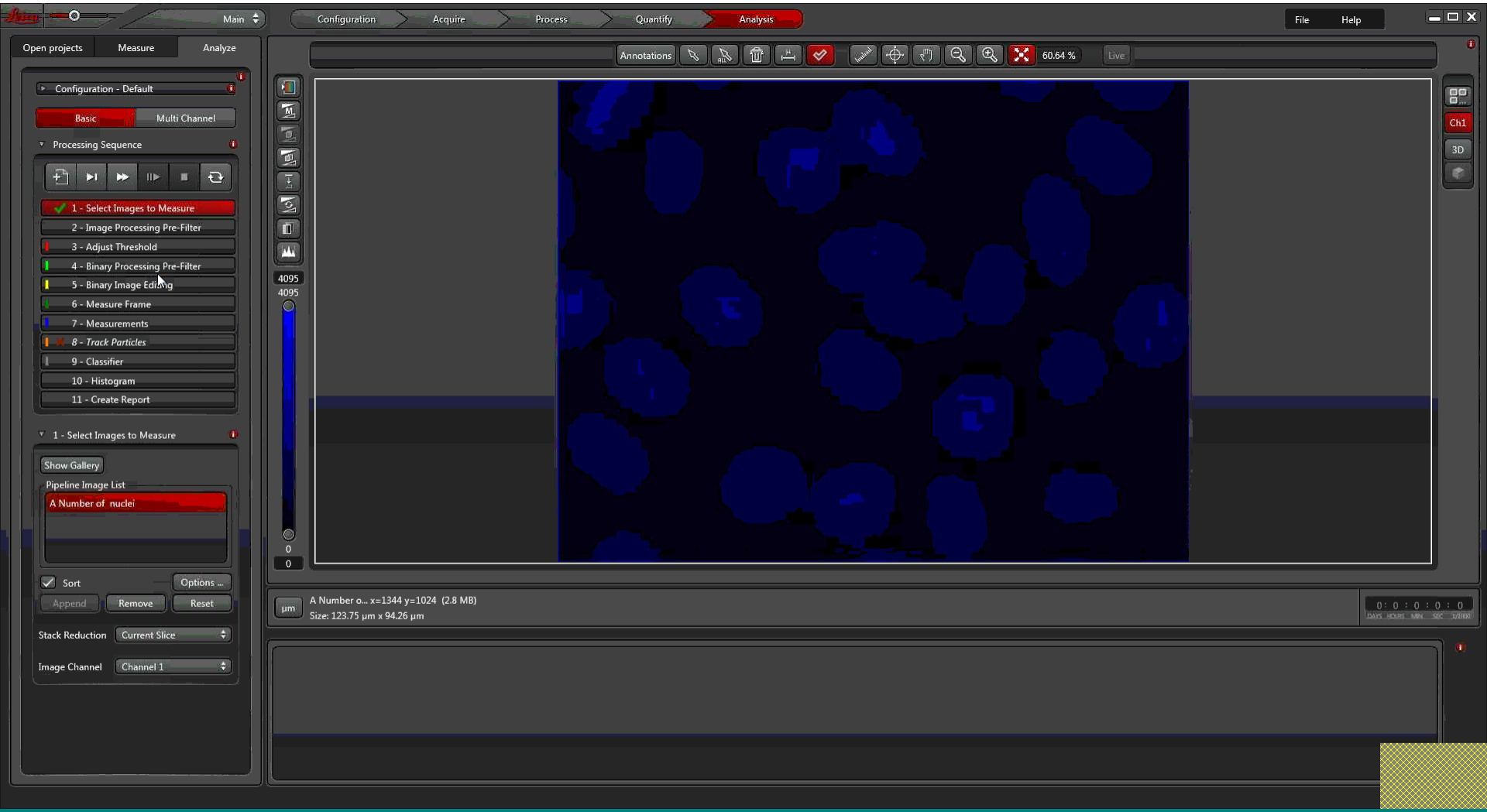
钙离子溶度计算


Calcium Imaging
Formula for Fluorescence Intensity Ratio: R = F(340nm) / F(380nm)
Channel 1
Background: 0
Ratio =
Set Background ROI
Channel 2
Background: 0
Concentration formula according to Grynkiewicz: [Ca2+] = Kd * (R - Rmin) / (Rmax -R) * Fo / Fs
[Ca2+] = Kd[nM]: 1
Ratio
Rmin: 0
Fo: 1
Rmax: 1
Ratio
Fs: 1
Definition of Parameters
Image Scaling
Min: 0
Max: 1
Define Rmin/Rmax
Select ROI: ROI1
Apply Rmin
Apply Rmax
Load / Save Calculations
None

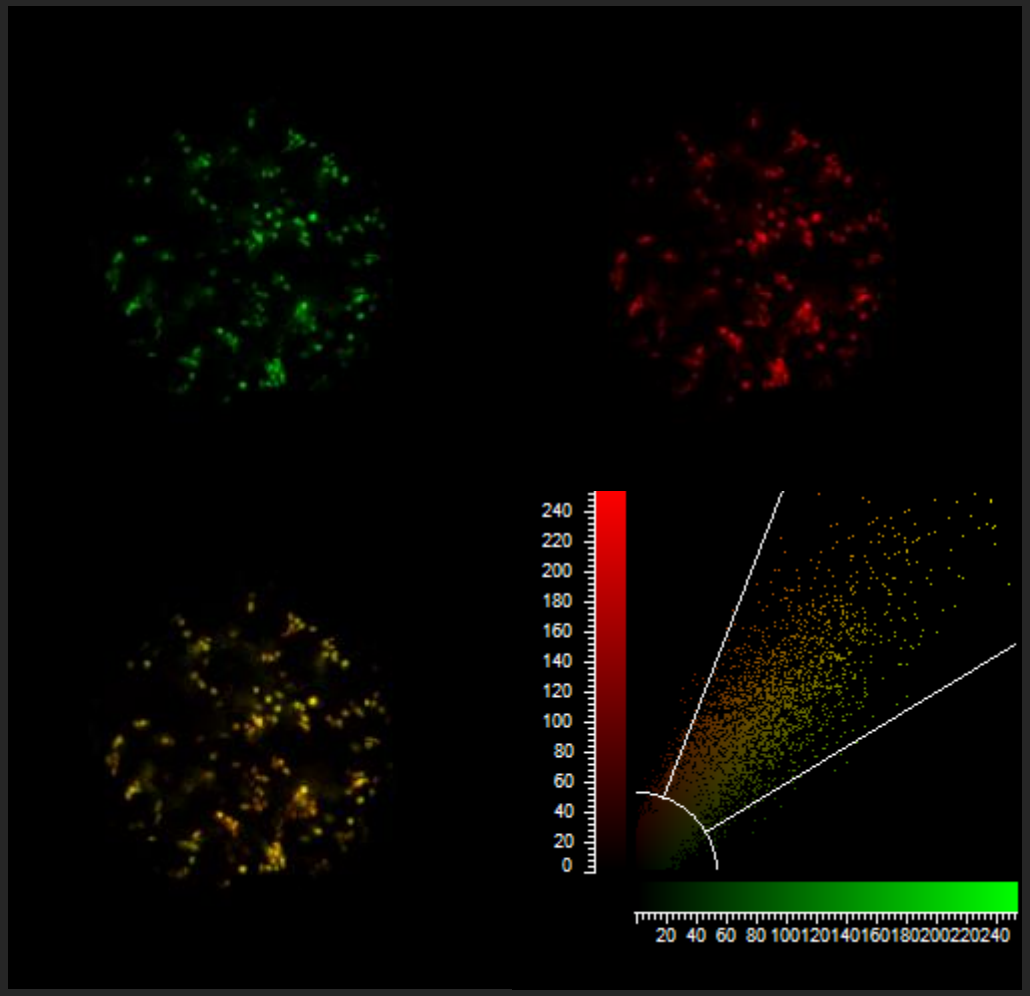
比率计算、钙离子浓度计算



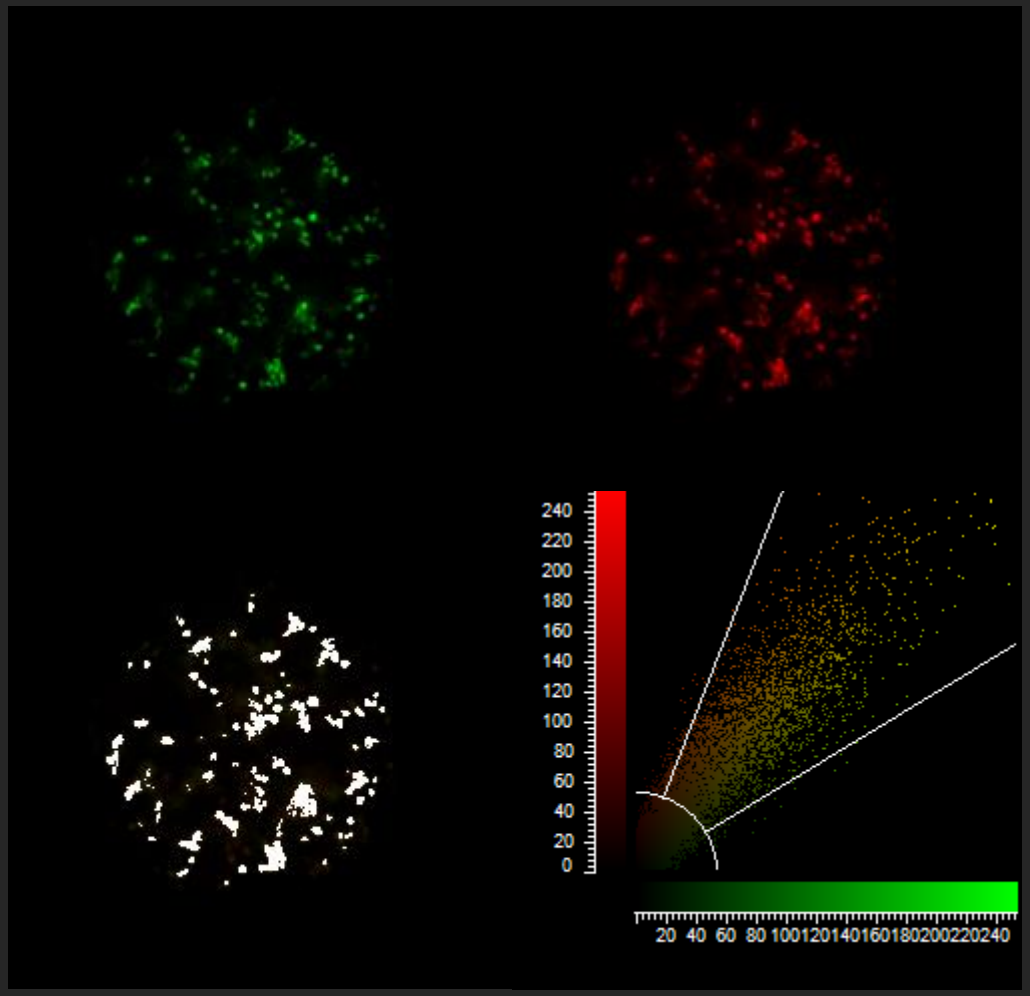
图像数据自动分析


Main
Configuration
Acquire
Process
Quantify
Analysis
File
Help
Open projects
Measure
Analyze
Configuration - Default
Basic
Multi Channel
Processing Sequence
1 - Select Images to Measure
2 - Image Processing Pre-Filter
3 - Adjust Threshold
4 - Binary Processing Pre-Filter
5 - Binary Image Editing
6 - Measure Frame
7 - Measurements
8 - Track Particles
9 - Classifier
10 - Histogram
11 - Create Report
1 - Select Images to Measure
Show Gallery
Pipeline Image List
A Number of nuclei
Sort
Options ...
Append
Remove
Reset
Stack Reduction
Current Slice
Image Channel
Channel 1
Annotations
60.64 %
Live
Ch1
3D
4095
4095
0
0
A Number o... x=1344 y=1024 (2.8 MB)
Size: 123.75 µm x 94.26 µm
µm

散点图法共定位分析


240
220
200
180
160
140
120
100
80
60
40
20
0
20 40 60 80 100120140160180200220240

散点图法共定位分析


240
220
200
180
160
140
120
100
80
60
40
20
0
20 40 60 80 100120140160180200220240

共定位分析统计数据

	Colocalization
Pearson's Correlation	0.9405
Overlap Coefficient	0.9435
Colocalization Rate	94.92 %
Colocalization Area	65.25 µm²
Area Image	2421.26 µm²
Area Foreground	68.74 µm²
Area Background	2352.52 µm²

	Channel 1	Channel 2
Mean Intensity Image	3.01	4.27
Mean Intensity Colocalization	68.94	87.66
Intensity Sum Image	790274	1119142
Intensity Sum Colocalization	487006	619201

倒置显微镜成像耗材

盖玻片底35mm培养皿
（共聚焦成像专用小皿）

Chambered Coverglass

底部厚度**0.170mm**容器可用油镜观察

培养皿规格及类型

	Glass Bottom Culture Dishes	
❶	**Dish diameter (35 or 50 mm)**	
	P35	35 mm
❷	**Coating**	
	G	Uncoated
	GC	Poly-d-lysine coated
	GCOL	Collagen Coating
❸	**Coverslip Thickness**	
	No. 0	0.085 - 0.13 mm
	No. 1.0	0.13 - 0.16 mm
	No. 1.5	0.16 - 0.19 mm
	No. 2.0	0.19 - 0.23 mm
❹	**Glass Diameter**	
	10	Glass diameter (10, 14, 20 or 30 mm)

耗材供应商

MatTek Corporation

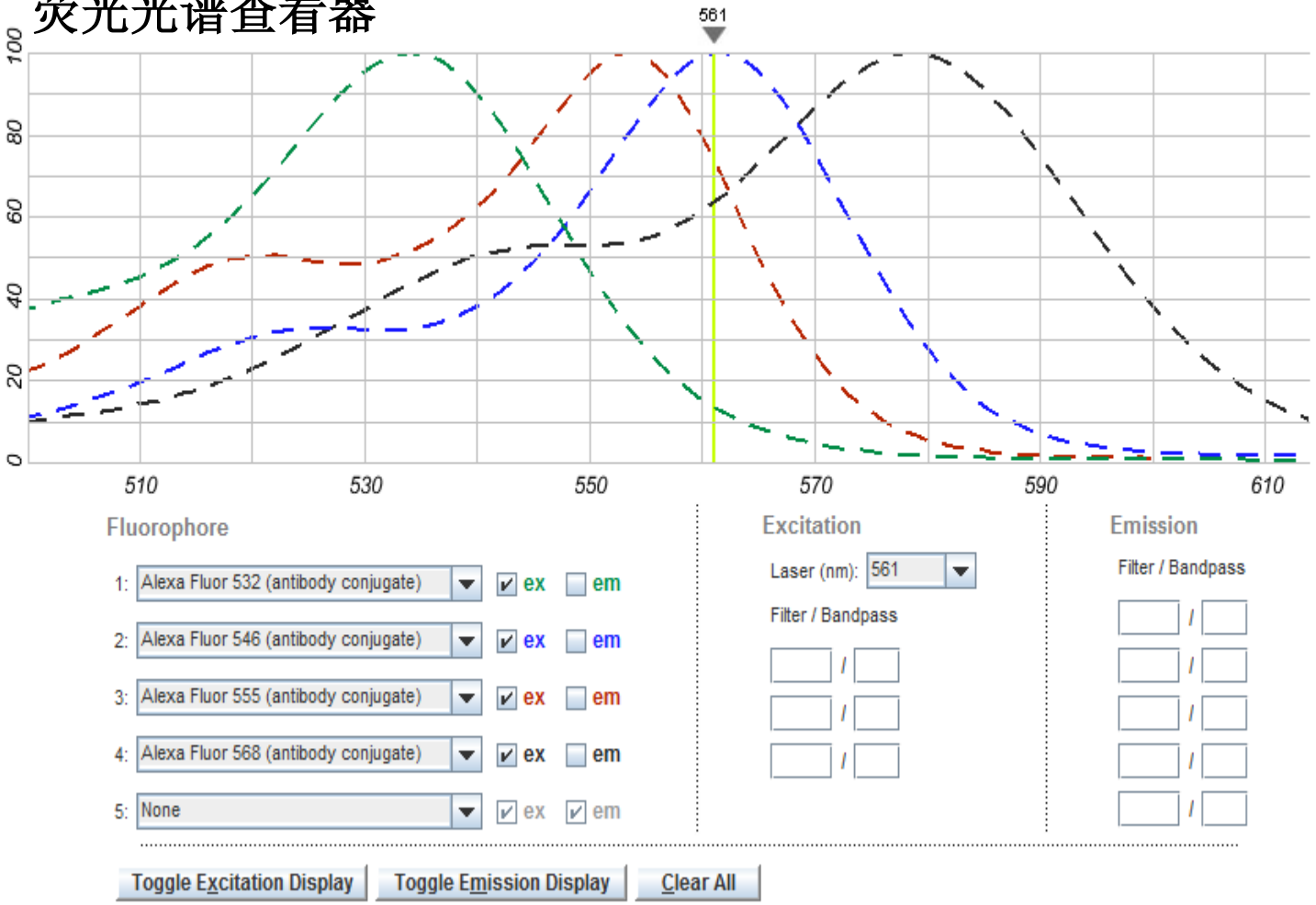
Thermo Scientific

Willco Wells

Ted Pella, Inc.

荧光染料的选择

荧光光谱查看器



http://www.thermofisher.com/cn/zh/home/life-science/cell-analysis/labeling-chemistry/**fluorescence-spectraviewer**.html

（一）、荧光染料选购原则

1. 根据现有的激光器进行染料选择；

2. 多色荧光成像时，要尽量避免染料之间的窜色，同时还要避开样品自发荧光的影响；

3. 染料的物理化学性质，优先考虑稳定性和抗淬灭性强的染料，离子荧光染料尽量选择Km值大的染料，对细胞内的离子浓度缓冲作用小；

4. 尽量选择负载后不会改变细胞的生理生化状态，或对细胞无毒副作用的染料；

5. 根据自己的实验需求是染活细胞还是固定细胞，选择相对应的染料，有时还要考虑染料能否经受醛类物质的处理；

6. 包装形式：很多染料厂商会提供粉末和溶液两种形式，优先选择粉末形式的，粉末的稳定性和保质期一般要比溶液长很多，而且尽量选择多管分装的粉末；

7. 厂商选择：首选Molecular Probes，其次再考虑Sigma，Roche等其他公司。

（二）、荧光染料配制及操作注意事项

1. 详细阅读厂商提供的说明书，了解该染料的详细信息，严格参照操作指南进行配制；

2. 如果是多管分装的粉末，每次配一管，配成适当高浓度的母液，然后再小管分装，每管10~20微升，小管封口，避光，低温保存，尽量避免反复冻融，每小管依照次序用完后再另开新的小管；

3. 用母液配制的工作液尽量现配现用，染色过程尽量避光；

4. 第一次使用某染料时，必须根据说明书或参考文献，进行染色浓度和染色时间摸索，以确定最佳染色条件；

5. 为了增强染料的负载效率，可适当进行抽真空，或者添加微量的表面活性剂（如0.005% silwet，Triton X-100等等）；

6. 染完色后，用培养液或缓冲液洗涤几次，以降低背景荧光强度；

7. 染色完成后及时进行观察，适时使用些抗淬灭剂以增强染料的光稳定性。

徕卡SP8可扩展的高端成像技术

STED 3X 超高分辨率

WLL 白激光

HCS-A 高内涵筛选

Hi Speed 高速扫描

SMD 单分子检测

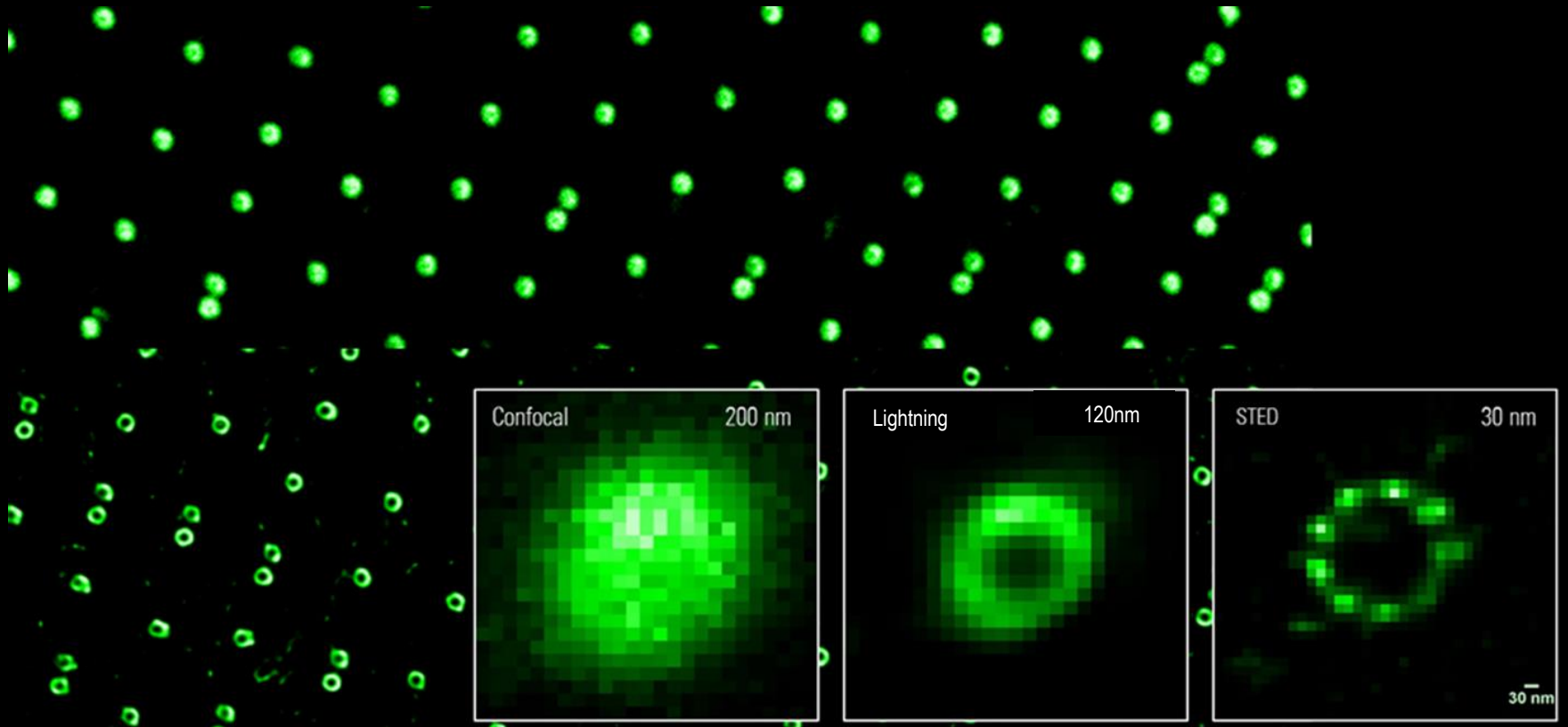
DIVE 光谱型多光子

CARS

相干反斯托克斯拉曼散射

Lightsheet 光片

不同分辨率的成像效果对比



The Nobel Prize in Chemistry 2014

***"for the development of super-resolved fluorescence microscopy"*.**

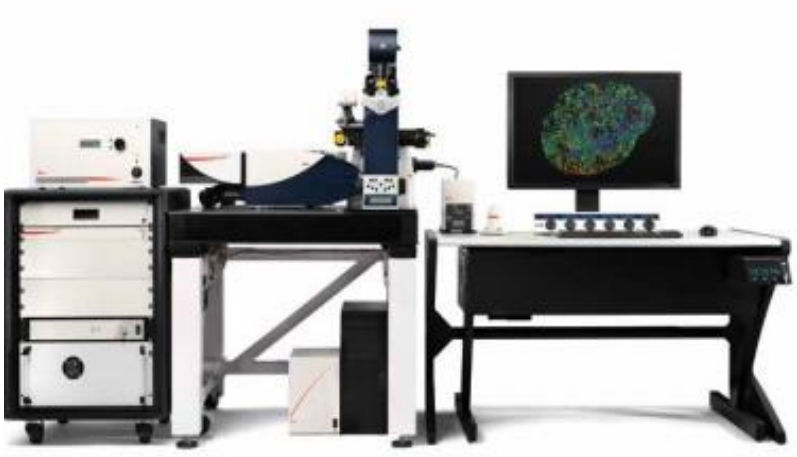
Eric Betzig **Stefan W. Hell** William E. Moerner

Leica 4Pi, STED, GSD & RESOLFT

徕卡--超高分辨率显微技术领航者

Leica MICROSYSTEMS

TCS 4PI — 2004
TCS STED — 2007
TCS STED CW — 2009
Dual color for TCS STED (CW) — 2010
SR GSD — 2011
TCS SP8 STED and gated STED — 2012
SR GSD 3D — 2013
TCS SP8 STED 3X — 2014
HyVolution — 2015
TCS SP8 STED ONE — HyVolution 2 — 2016

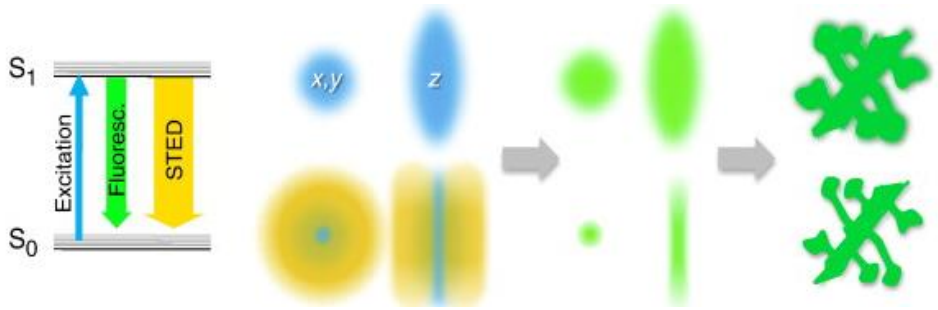


SP8 STED 3X: Confocal super-resolution

SR GSD 3D: WF super-resolution

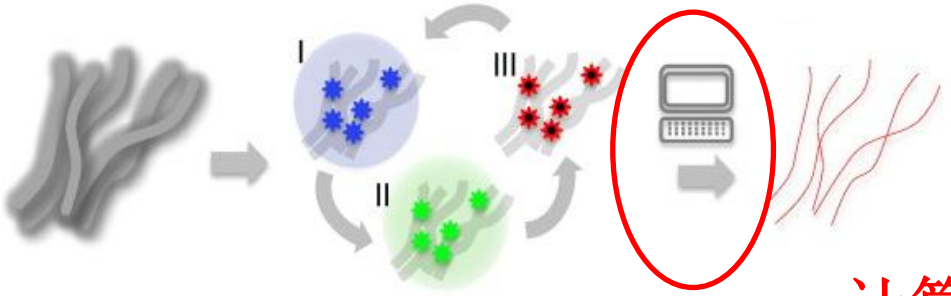
超高分辨率显微技术类型（纳米显微镜Nanoscopy）

STED
xy 50nm
z 130nm



纯光学方法

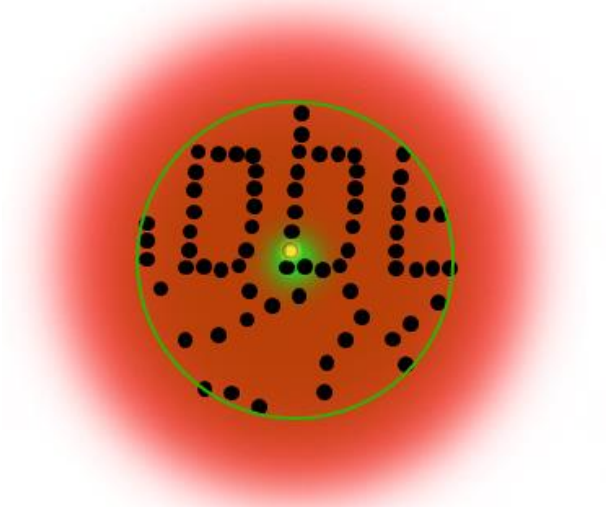
Localization Microscopy
(GSD, PALM, STORM)
理论值 xy 20nm，z 50nm
实测值 xy ~40nm，z ~100nm



计算方法

超高分辨率显微镜技术对比

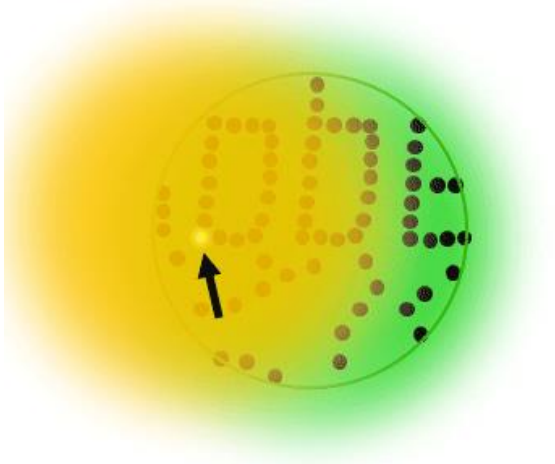
Confocal共聚焦



Leica SP8 STED ＜50nm

唯一纯光学实现超高
可使用常规染料
可进行活细胞快速成像
棱镜分光+光谱检测
穿透深度大40-160微米
升级扩展空间大

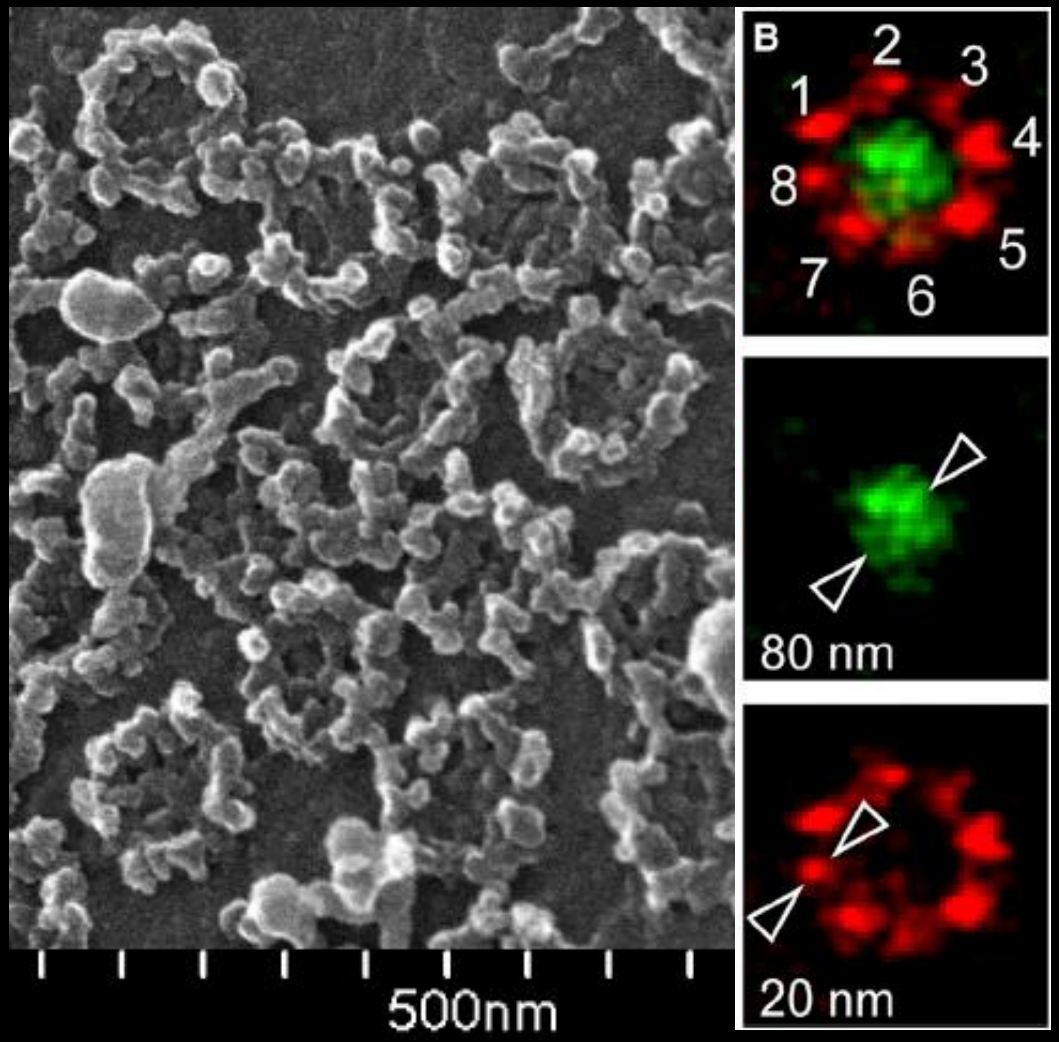
Widefield宽场显微镜



GSD, PALM & STORM
理论20nm，实测40nm

需要特殊染料及制样
成像速度慢（0.5-20min）
无法进行活细胞成像
采用滤片分光
穿透深度低1~4微米
无升级空间

STED 技术原理



STED reveals immunolabeled subunits in amphibian NPC.

Atto 590 + KK114

Gottfert *et al*., Biophysical Journal, 2013

中科院昆明动物所与徕卡合作发表的STED应用文章

Virology Journal

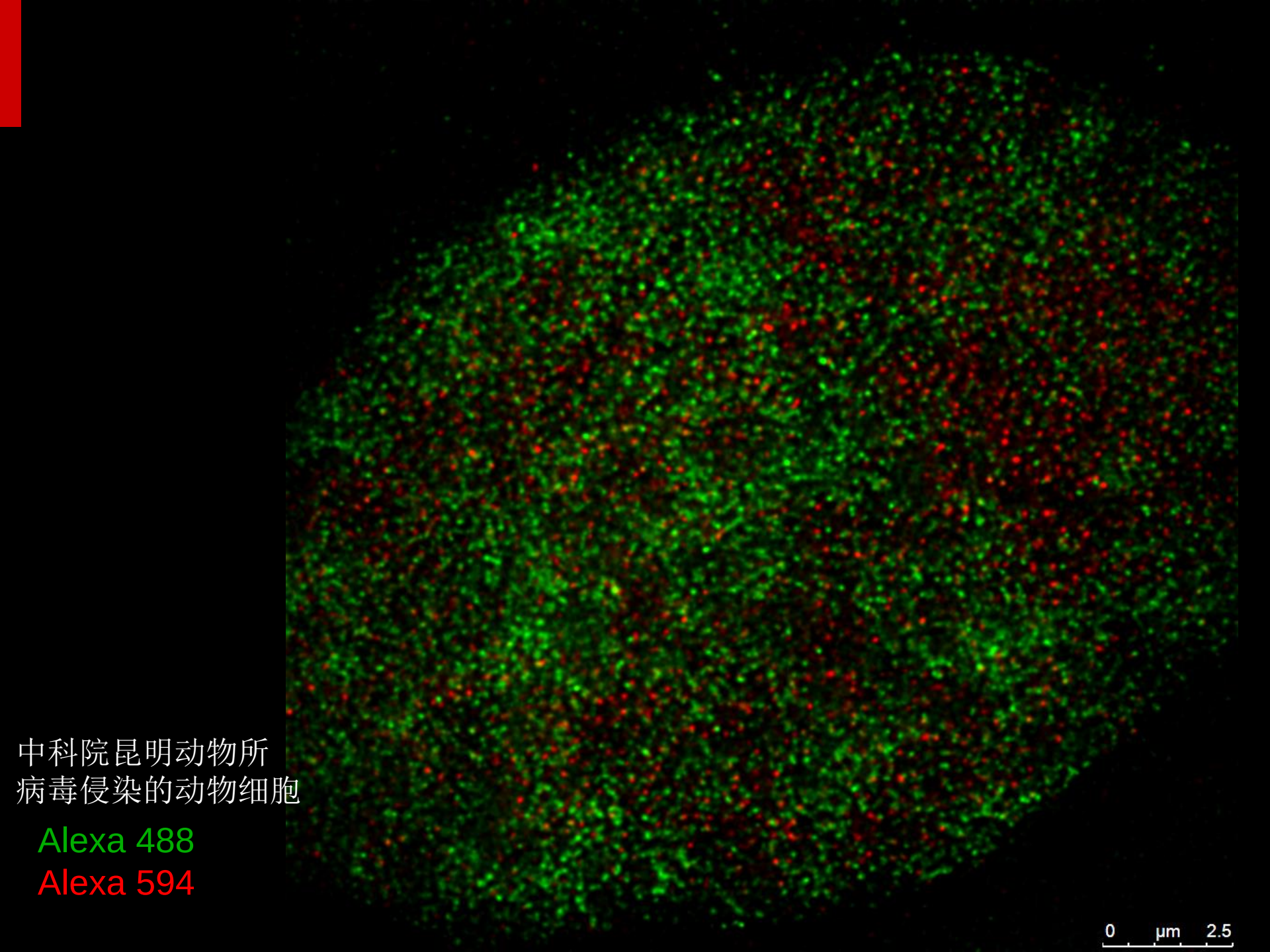
ABOUT | ARTICLES | SUBMISSION GUIDELINES

RESEARCH | OPEN ACCESS

Visualizing the replicating HSV-1 virus using STED super-resolution microscopy

Zhuoran Li, Ce Fang, Yuanyuan Su, Hongmei Liu, Fengchao Lang, Xin Li, Guijun Chen, Danfeng Lu and Jumin Zhou

95 °C for 4 min; finally, cells were incubated with antibody at room temperature for 1 h. Images were acquired using a Leica TCS SP8 STED 3× (Germany). The distance measuring software was Leica LAS X. Figures were analyzed with Image-Pro Plus 6.0 software (USA).


中科院昆明动物所
病毒侵染的动物细胞
Alexa 488
Alexa 594
0
μm
2.5

STED图像的精确距离测量

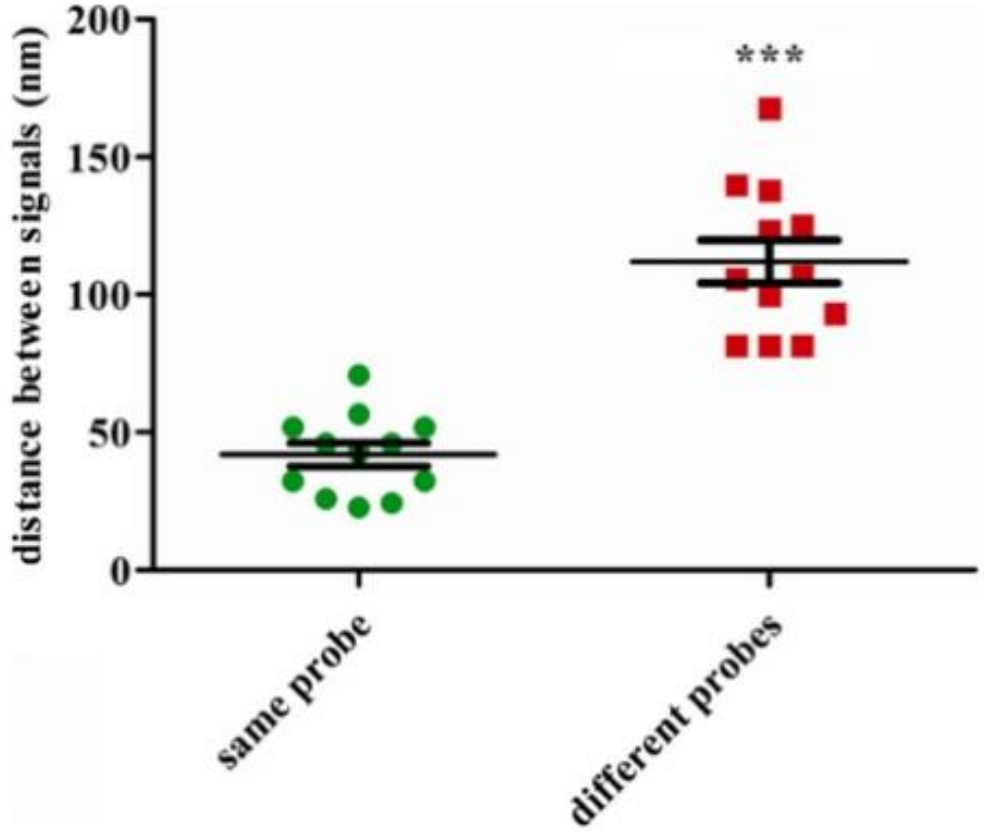
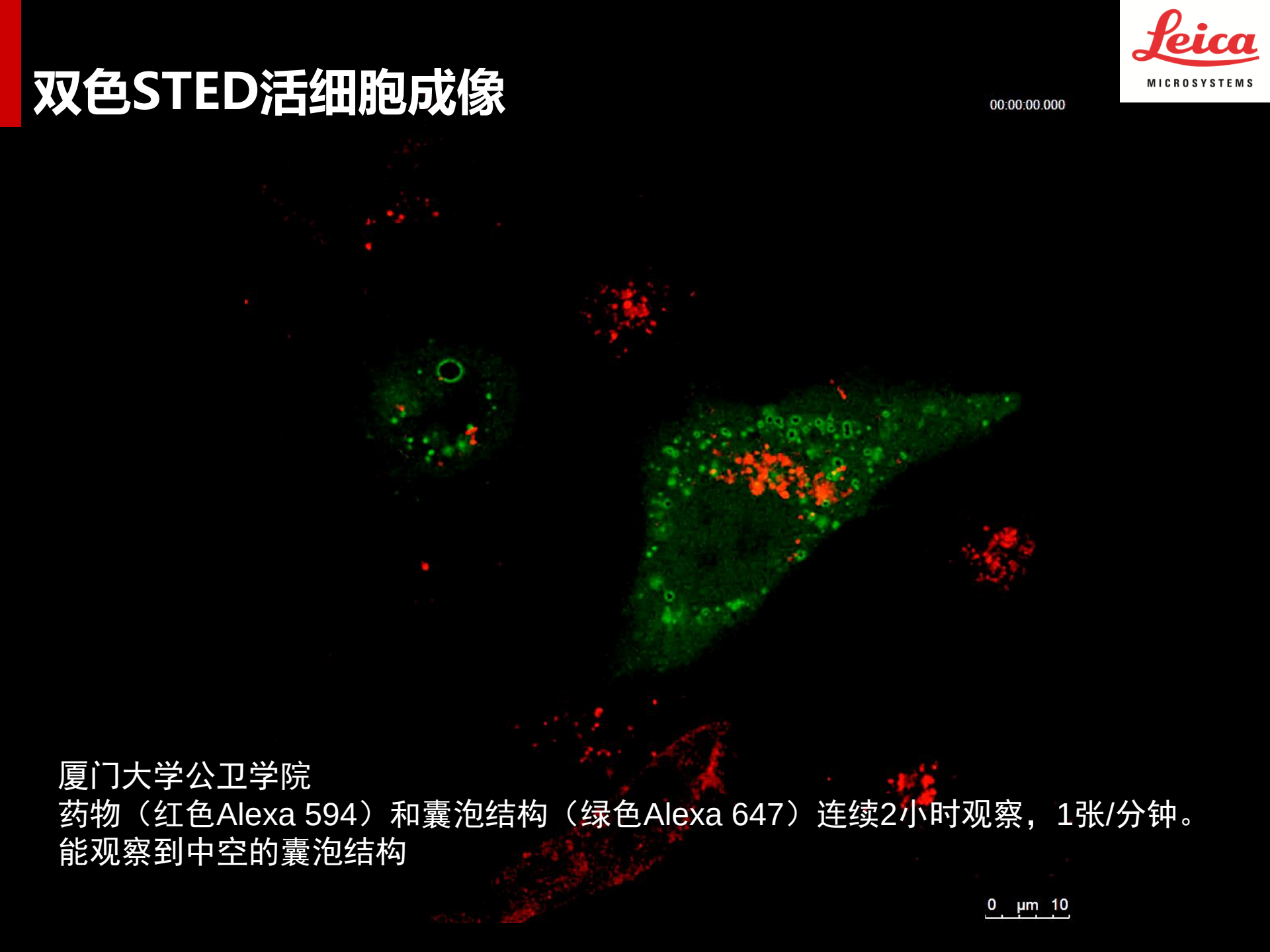


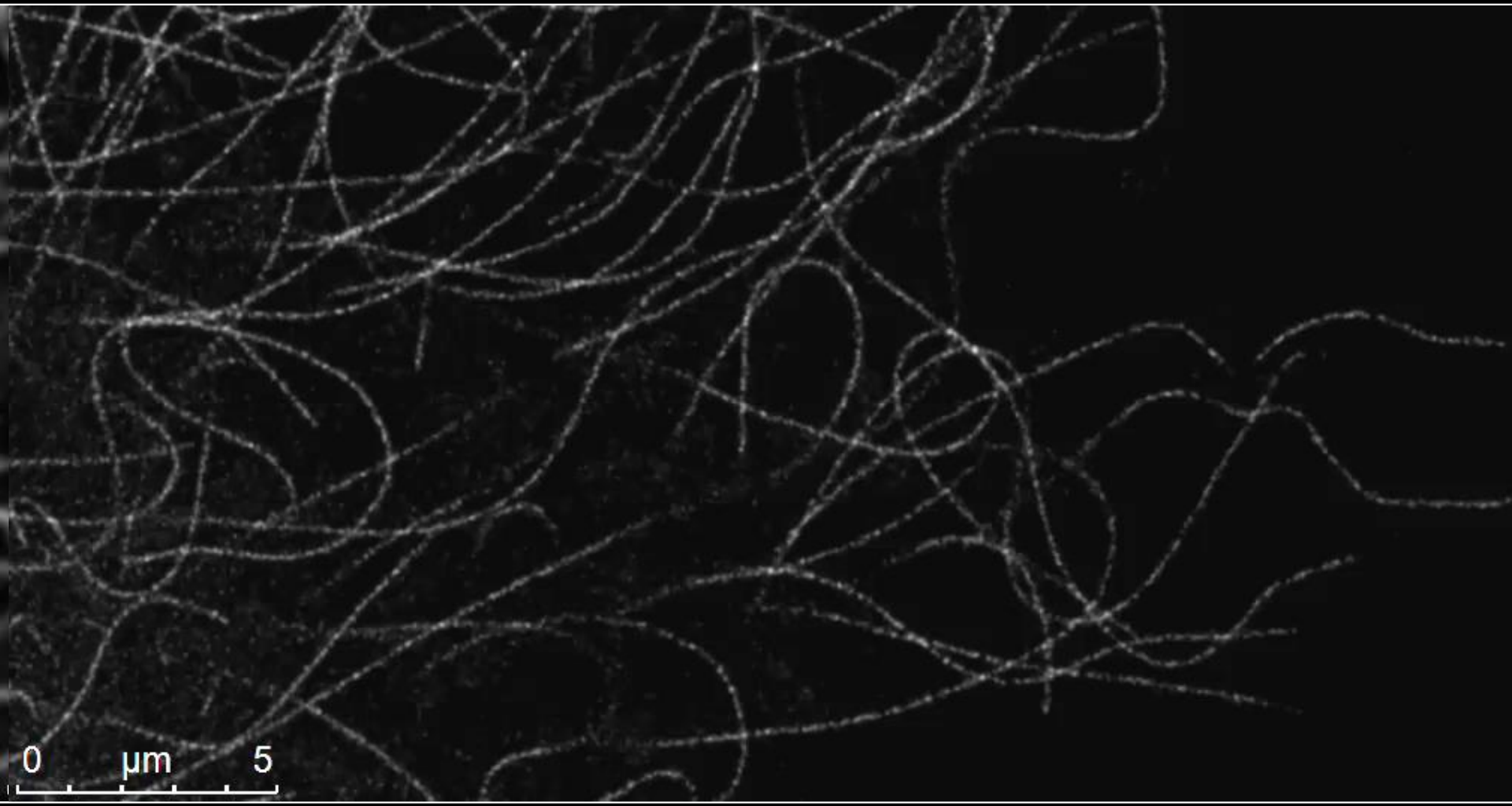
Fig. 3 Average distances of the same probe and different probes. Distances of the same probe and different probes were calculated under STED microscopy. The average distance of the same probe is 41.9 nm and that of different probes is 111.9 nm, which is 2.7-fold higher than the same probe, p value < 0.001 (***). The data were evaluated with the Students' t-test

双色STED活细胞成像



厦门大学公卫学院
药物（红色Alexa 594）和囊泡结构（绿色Alexa 647）连续2小时观察，1张/分钟。
能观察到中空的囊泡结构

SiR Tubulin 100 Frames



775 STED live:

Fluorophores courtesy of Spirochrome

Two-color STED Live Cell Imaging

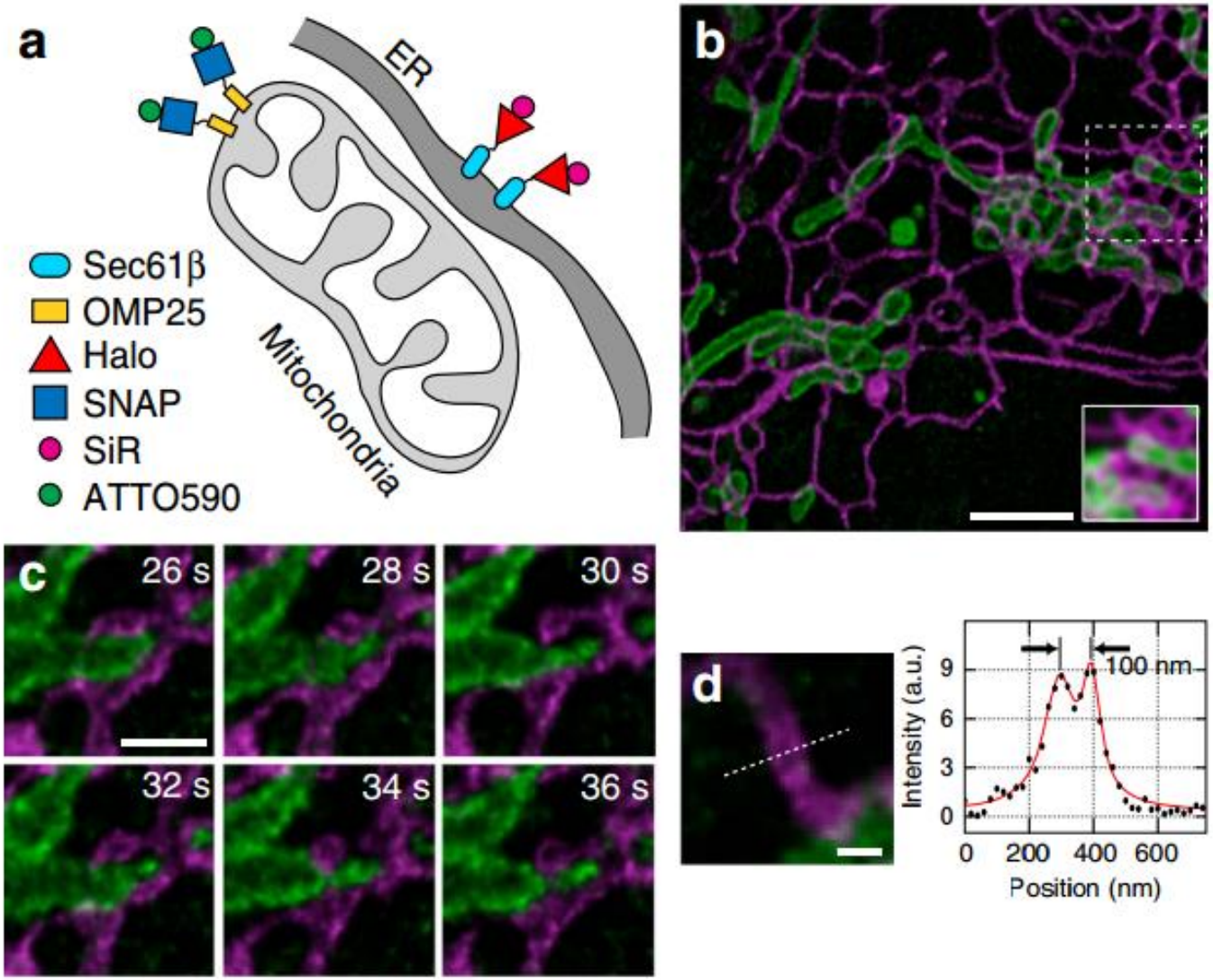


Figure 1 | STED nanoscopy of dynamic interactions between ER and mitochondria.

Bottanelli, *et al.* Nature Communication, 2016

Two-color STED Live Cell Imaging

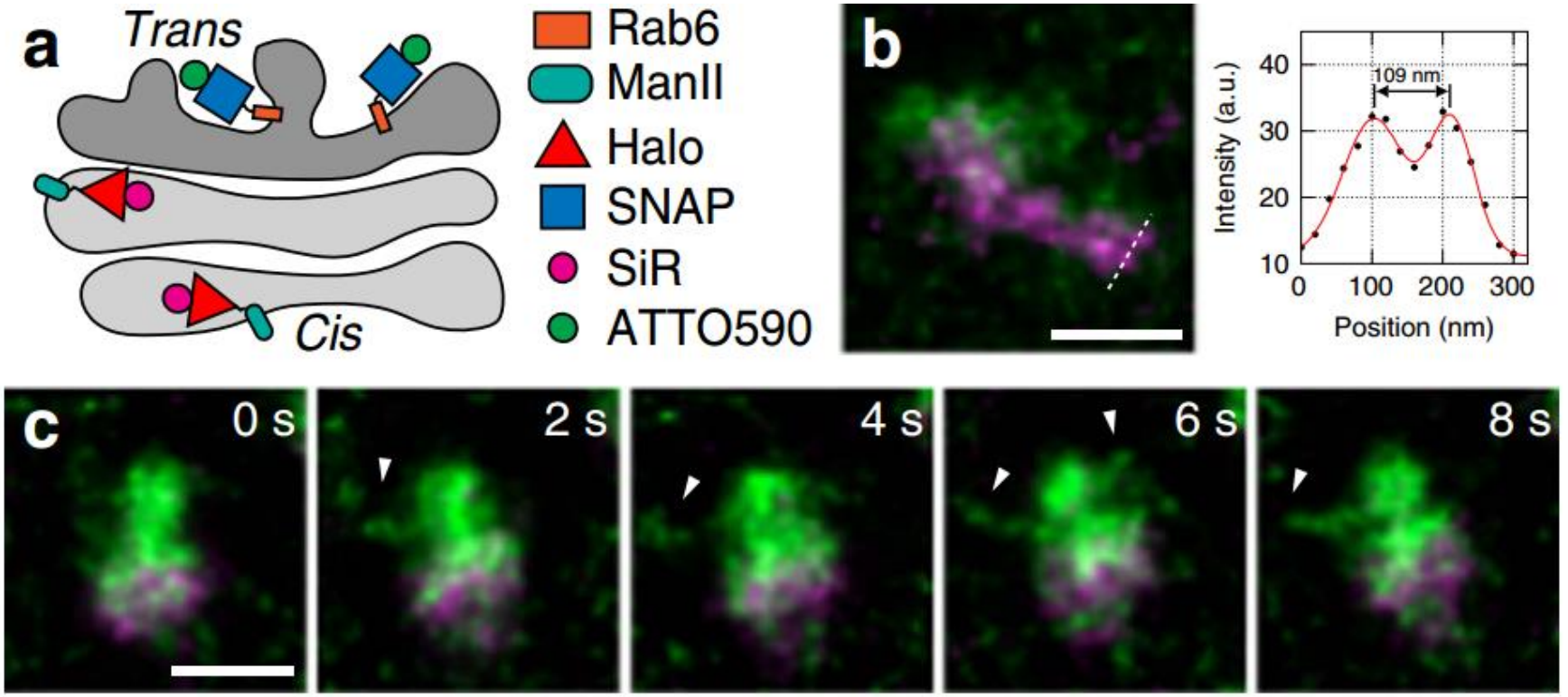
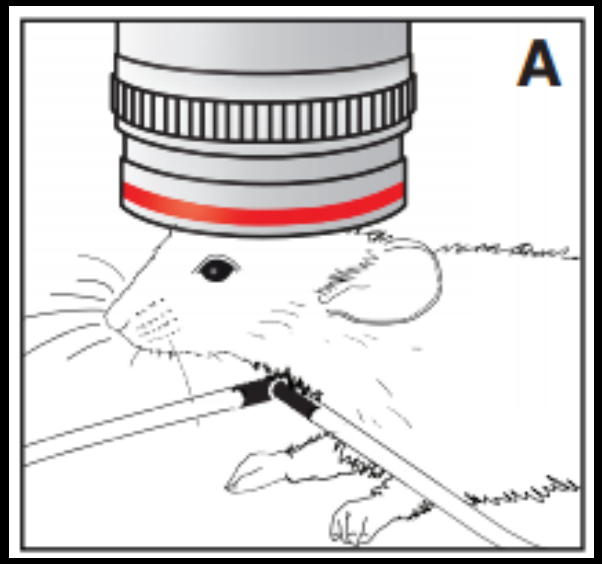
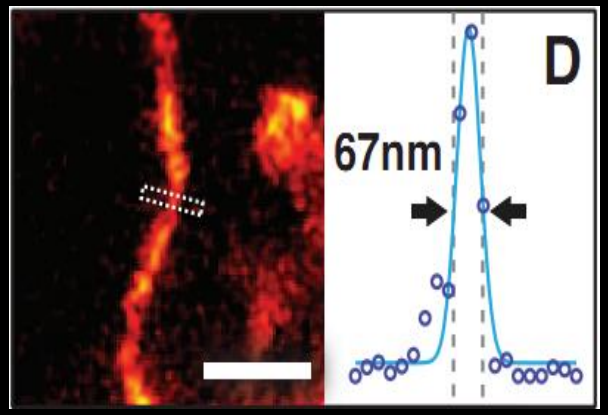


Figure 2 | Golgi protein dynamics imaged by live-cell STED nanoscopy.

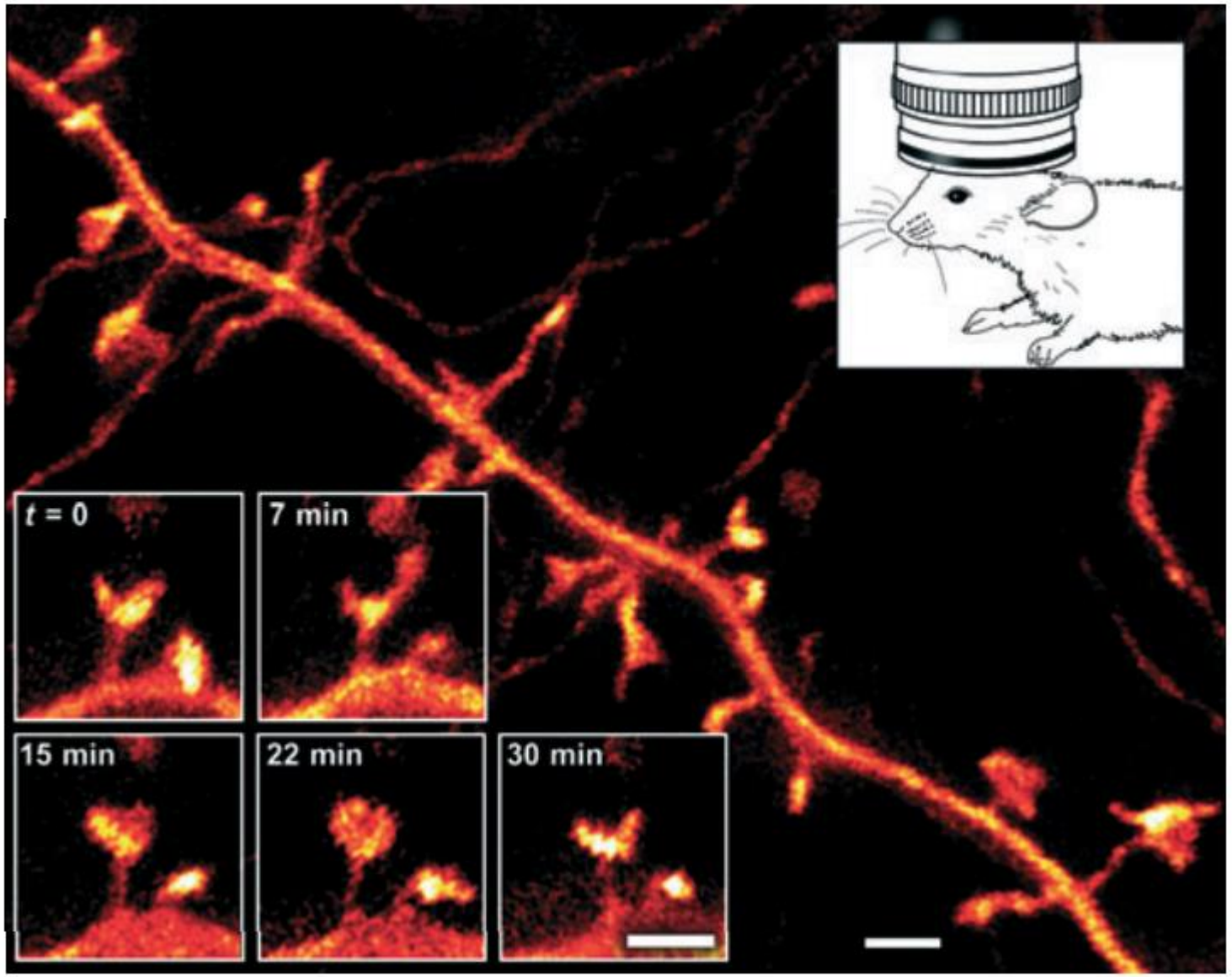
Bottanelli, *et al.* Nature Communication, 2016





STED microscopy in the molecular layer of the somatosensory cortex of a mouse with EYFP-labeled neurons.

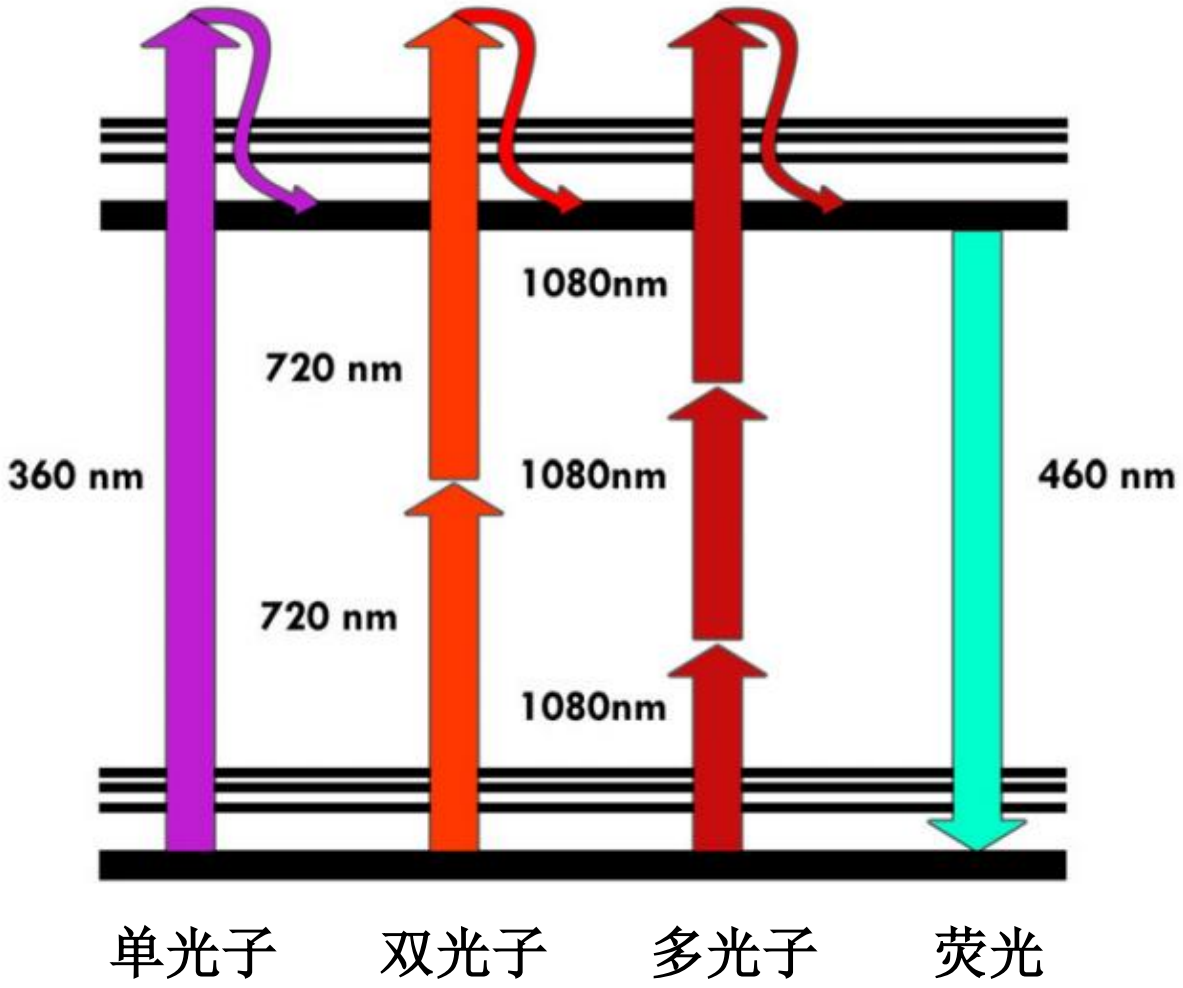
Sebastian *et al*, Science, 2012



STED imaging of the temporal dynamics of dendritic processes within the molecular layer of a TgN (Thy1-YFP) mouse, about 10–15 um below the surface.

Berning et al. 2012

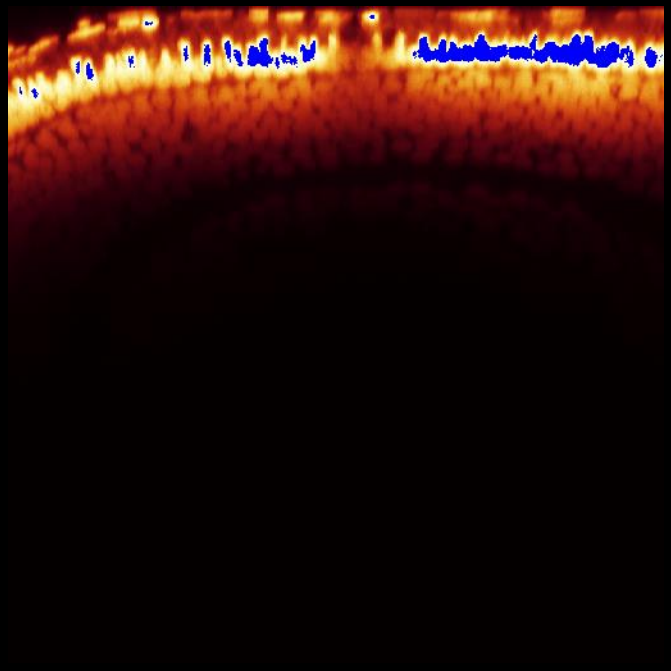
单光子与多光子



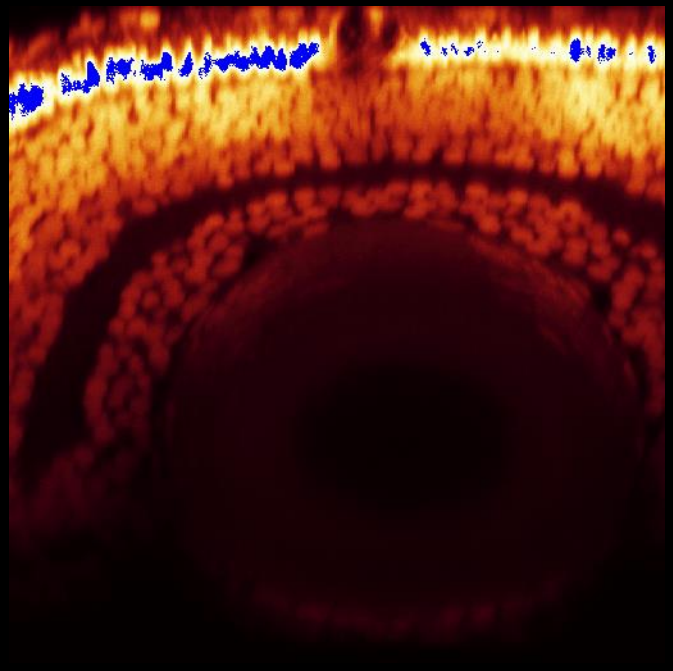
单光子 vs. 多光子

Eye of zebrafish embryo (stained with DAPI)

Image size (xz): 125 μm x 125 μm - Objective: 63x 1.2 Water - Detection range: 400nm – 500nm

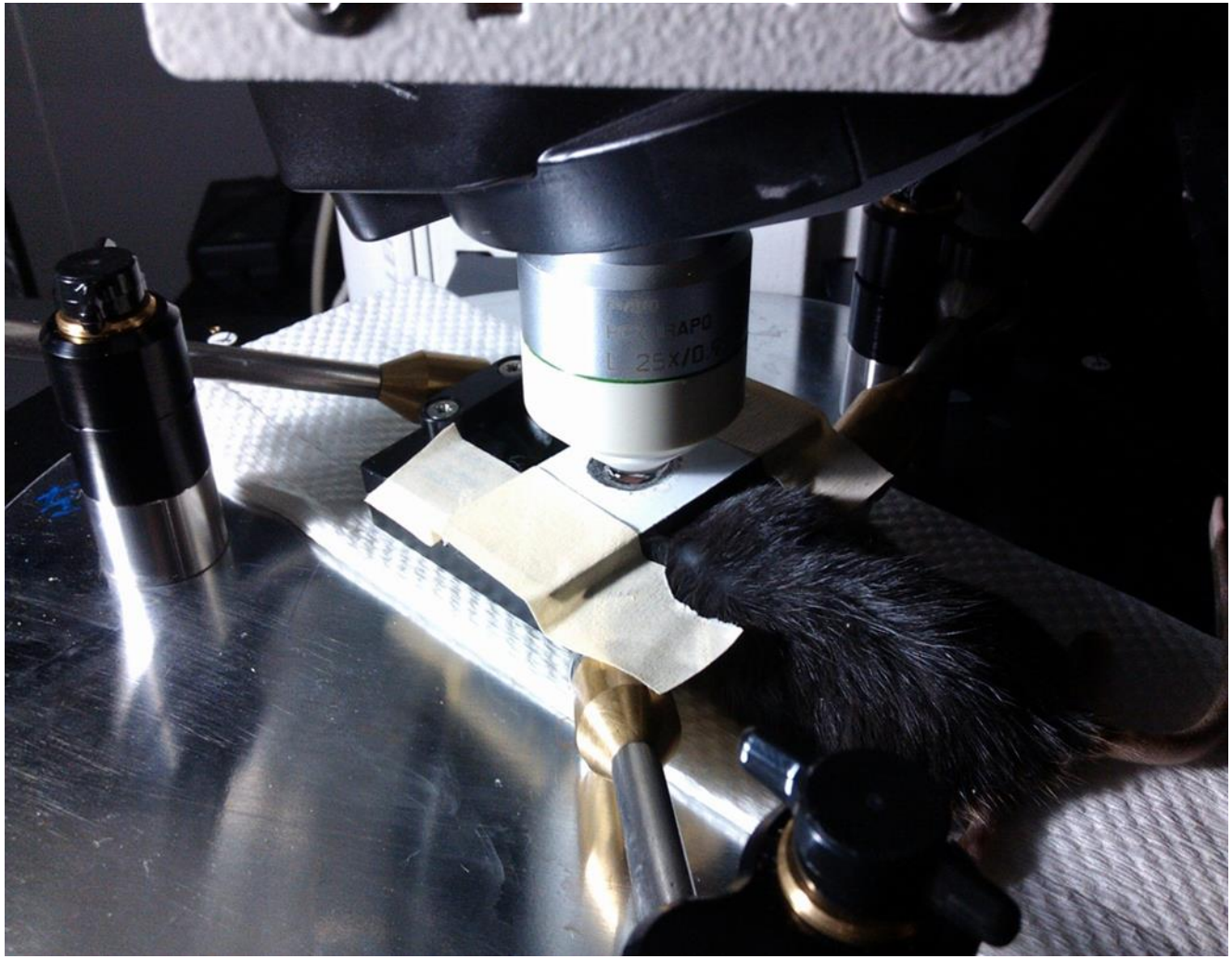


Ex: 405nm
PMT: 800V

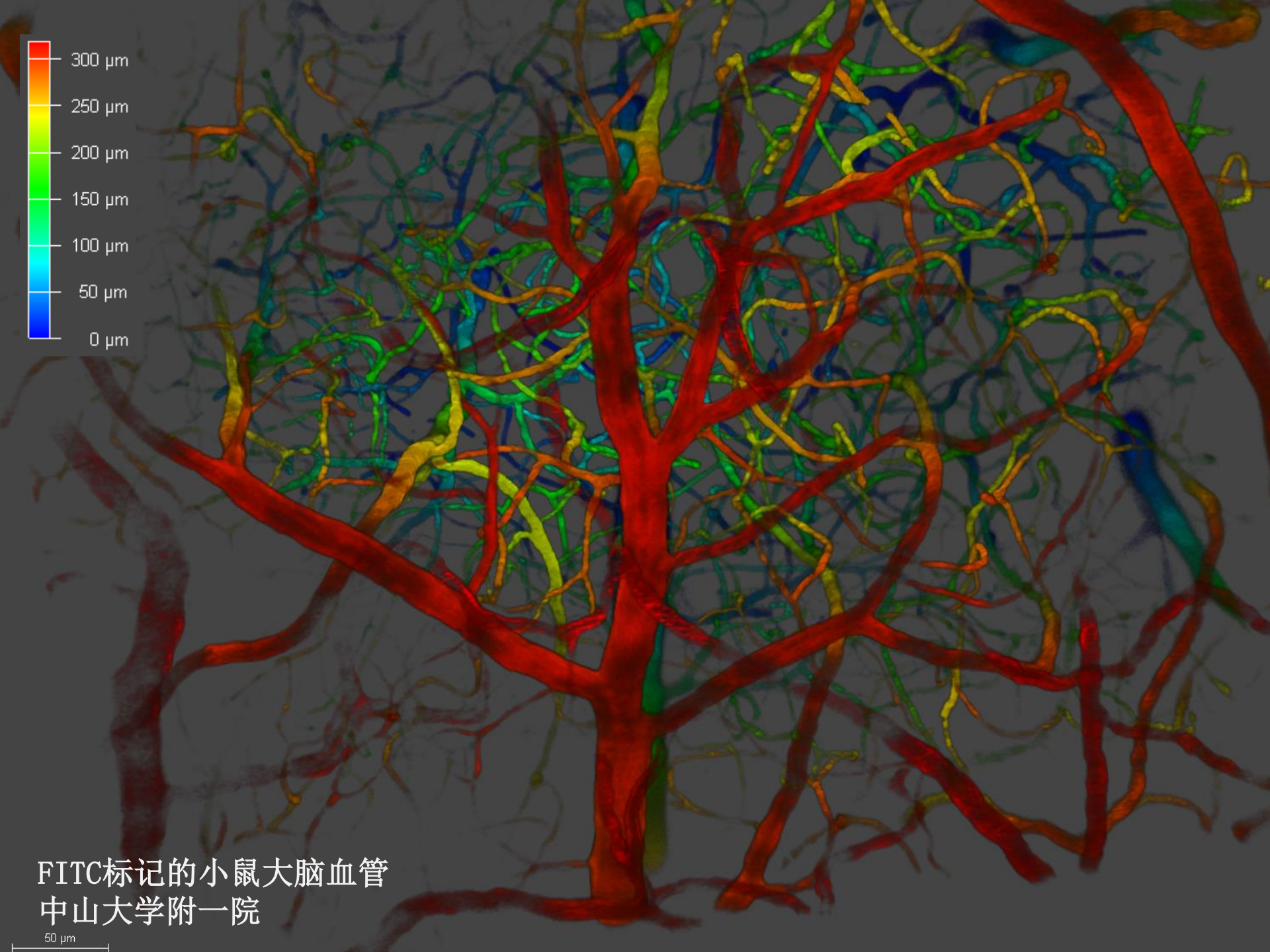


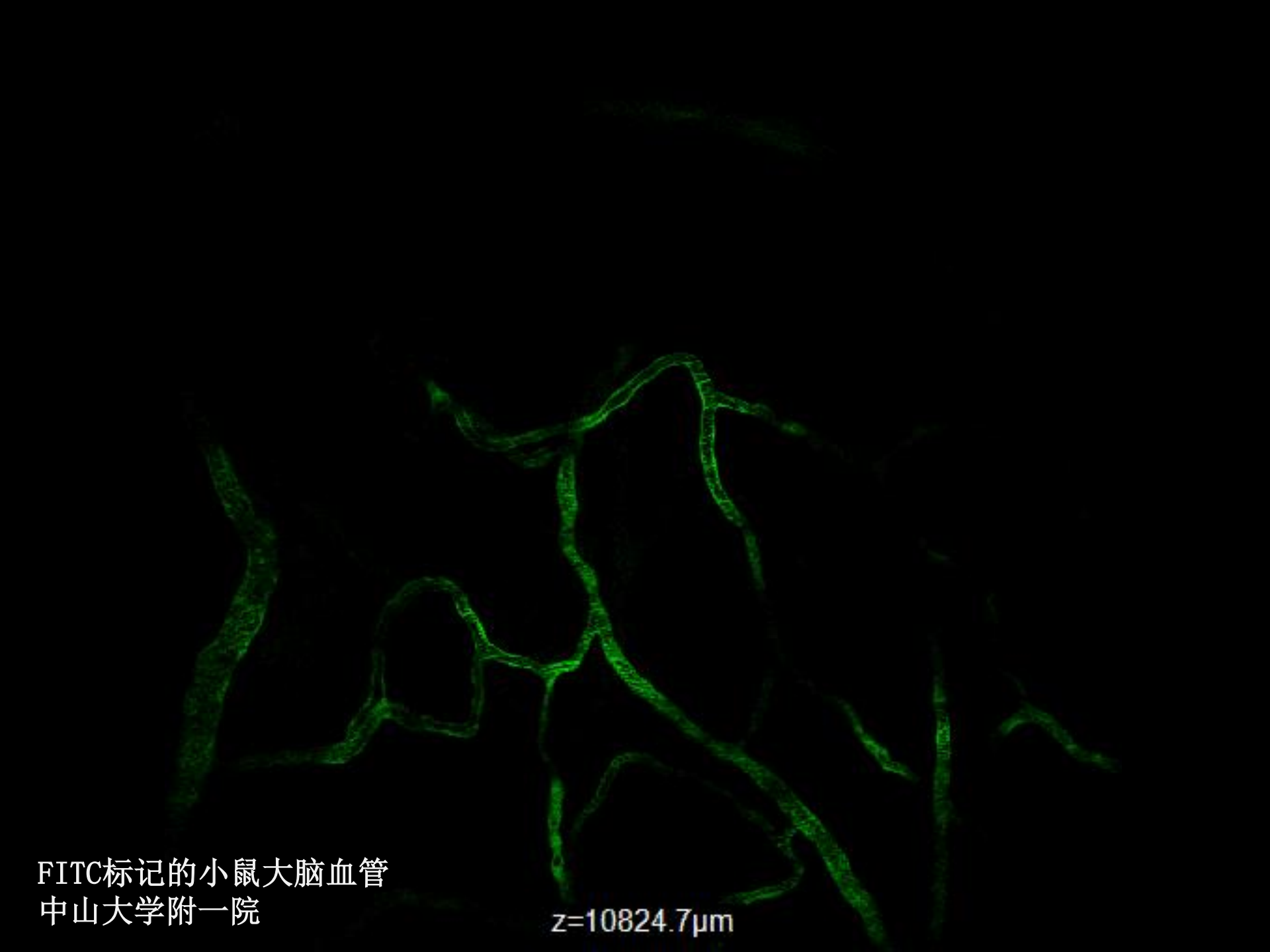
Ex: pulsed IR / 780 nm
PMT: 800V

多光子更适合活体成像

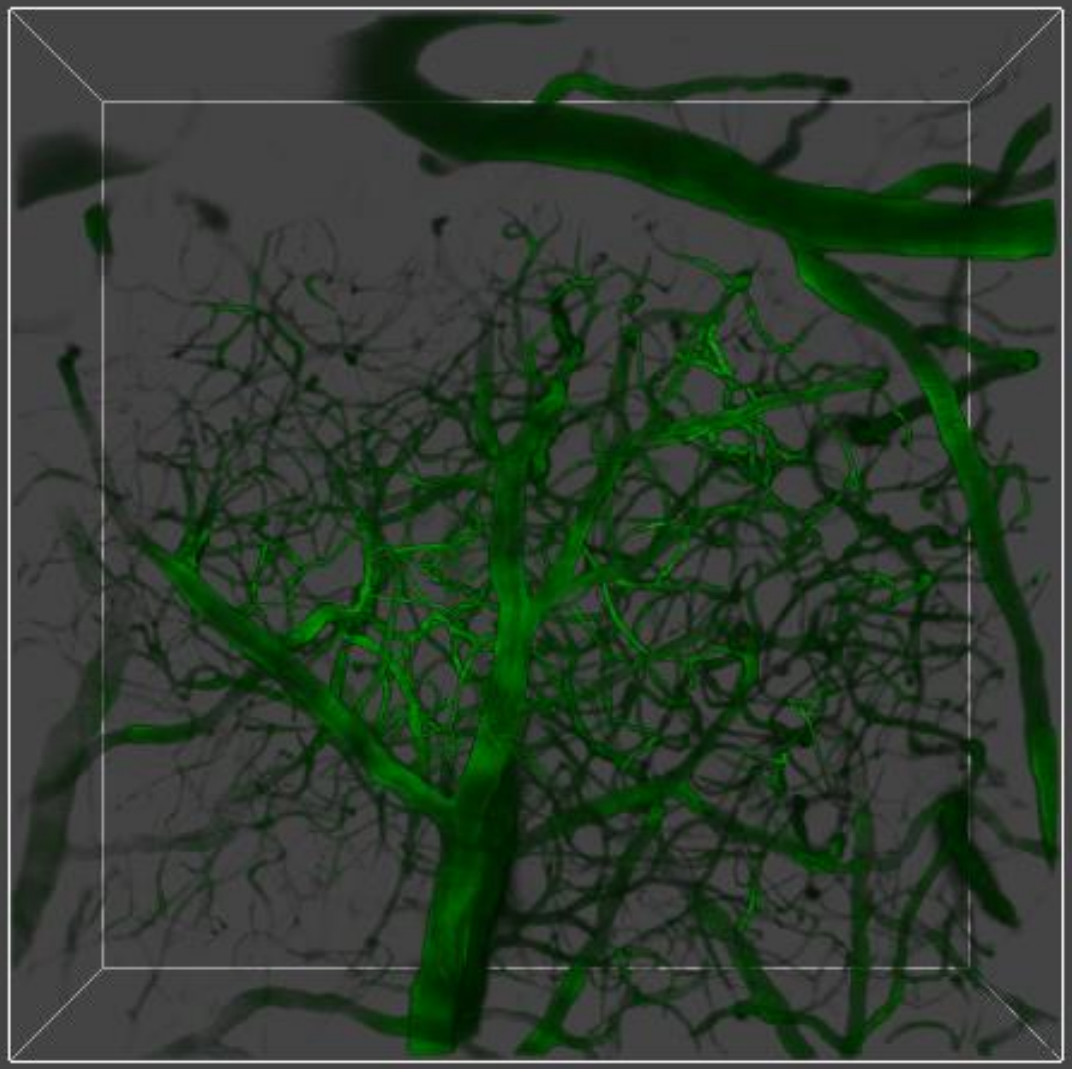


活体小鼠观察

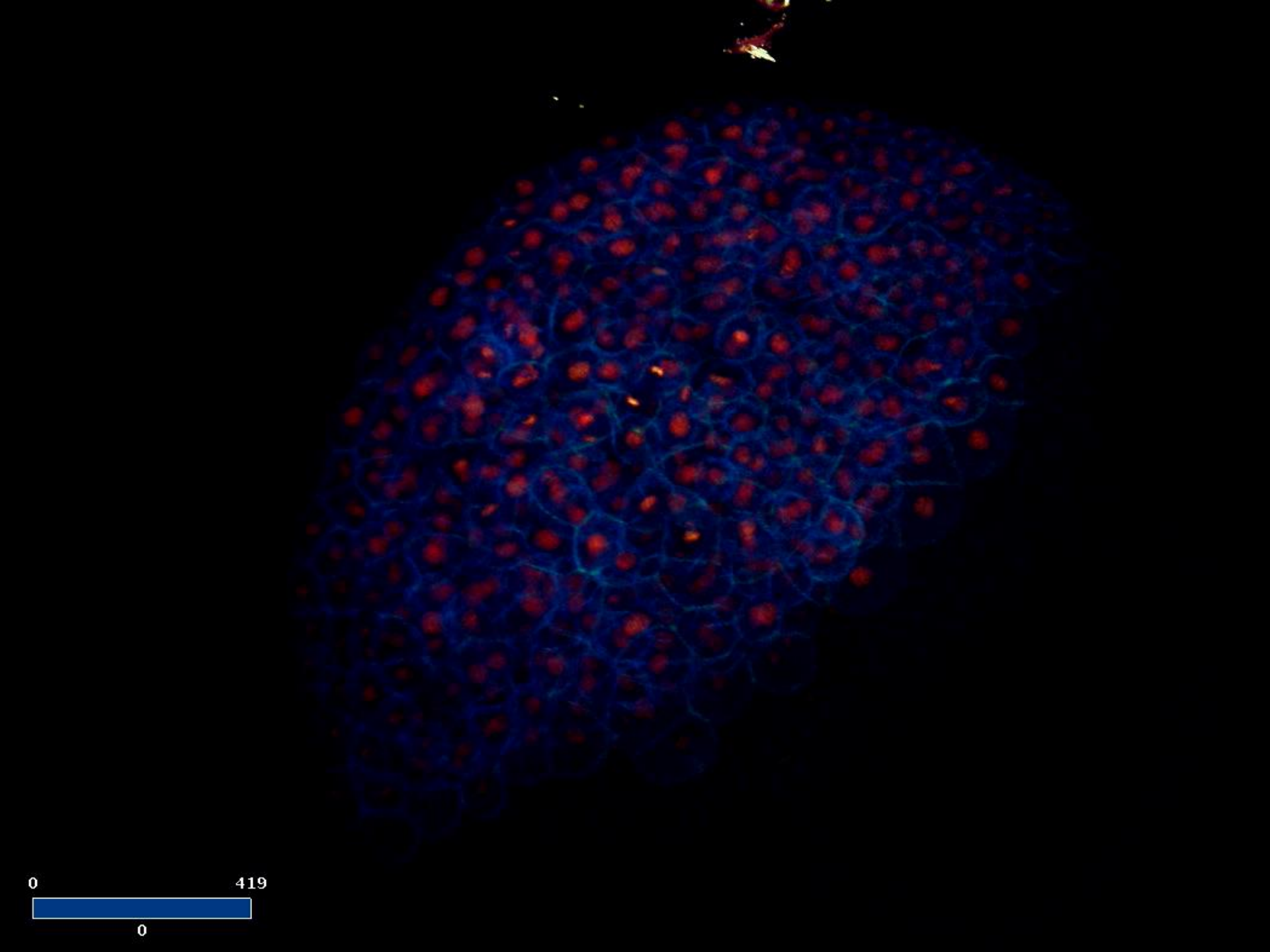

300 µm
250 µm
200 µm
150 µm
100 µm
50 µm
0 µm
FITC标记的小鼠大脑血管
中山大学附一院
50 µm

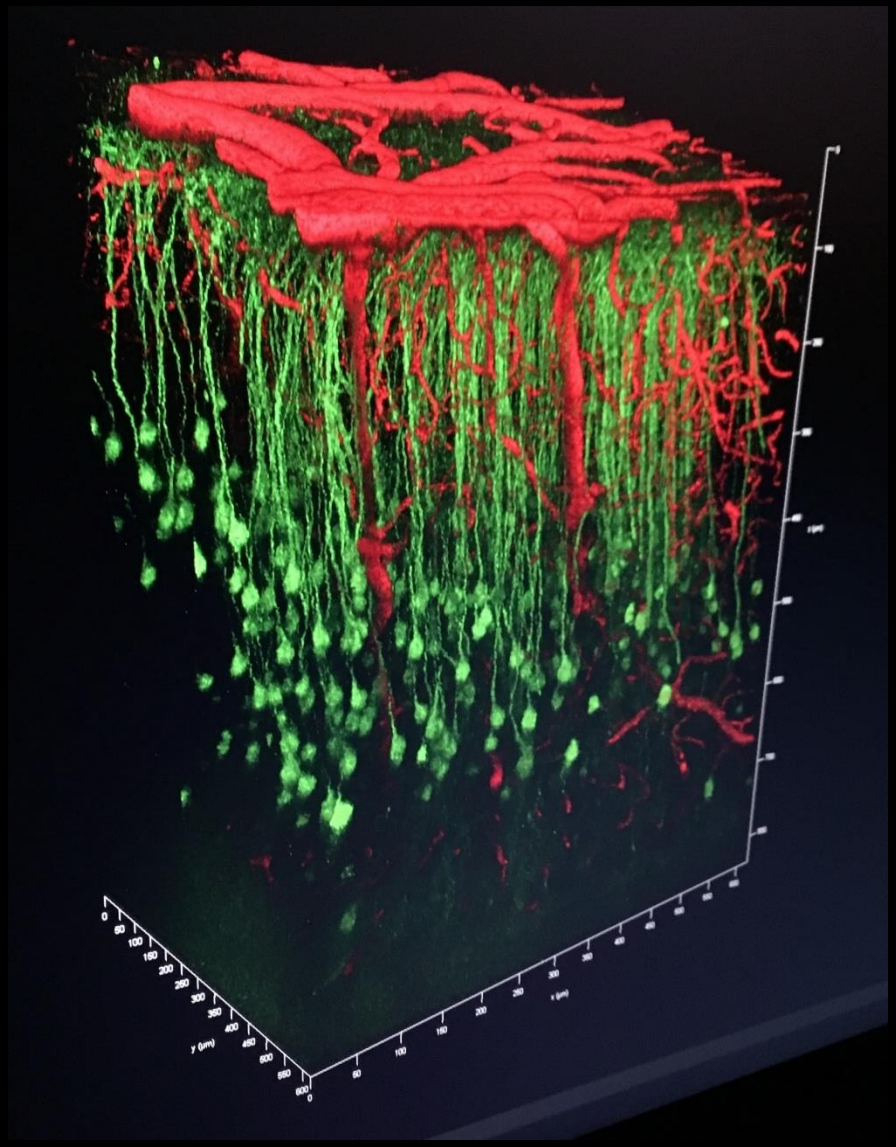


FITC标记的小鼠大脑血管
中山大学附一院



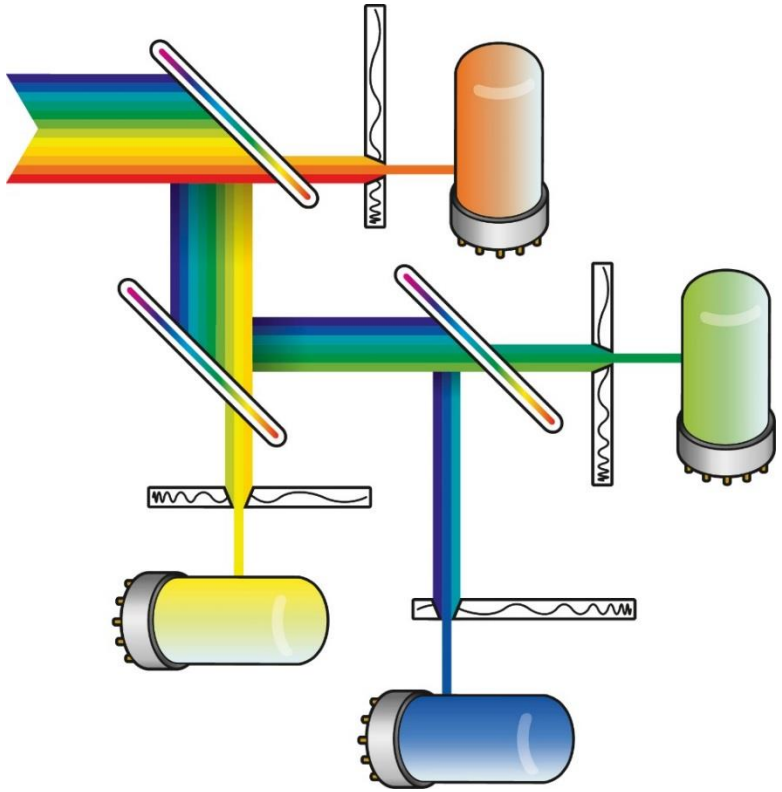
FITC标记的小鼠大脑血管
中山大学附一院


0
419
0



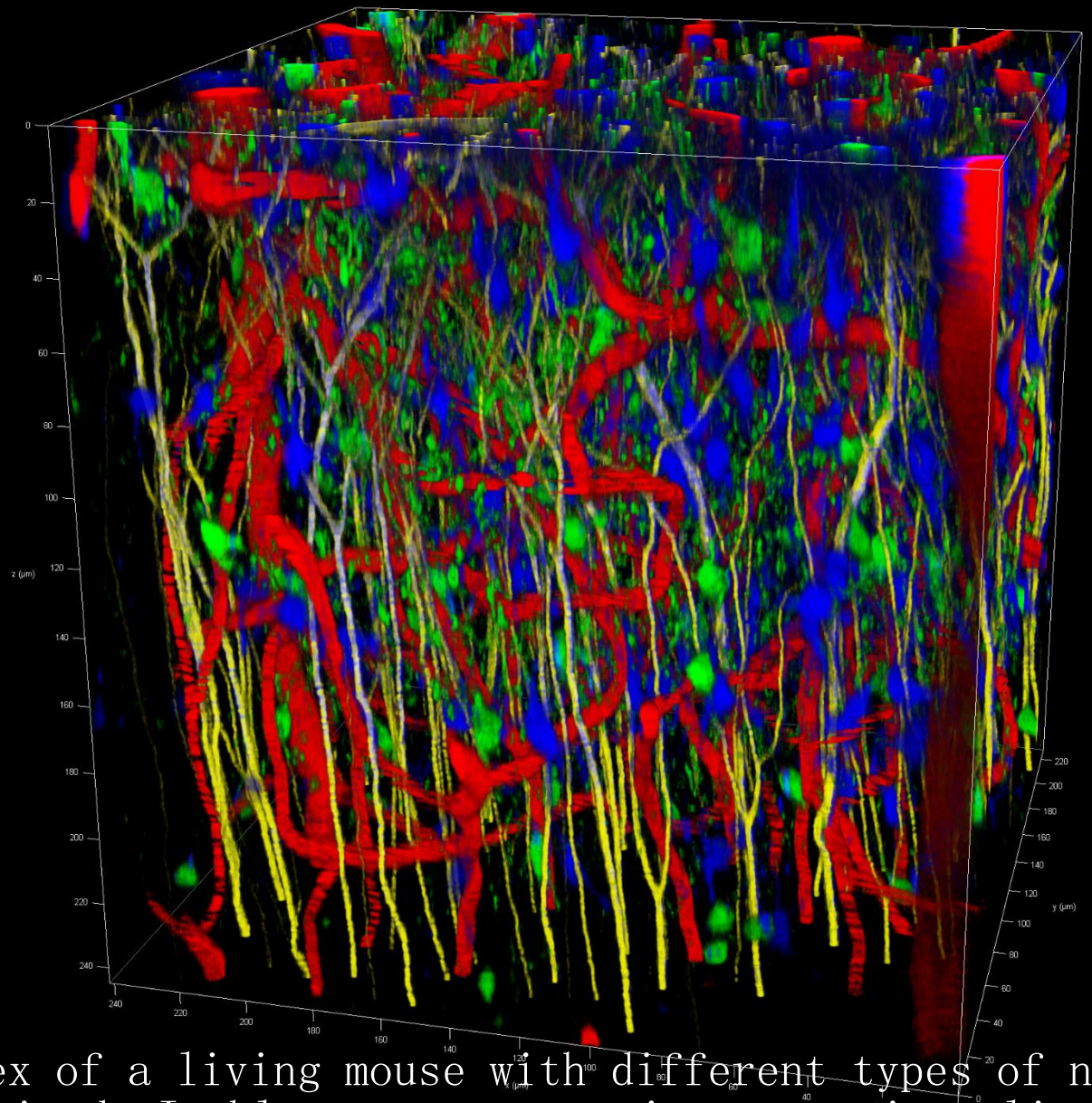
Over 1mm penetration depth with two photon. The sample was a six month old narcotized mouse expressing neuronal YFP.

DIVE光谱型多光子系统



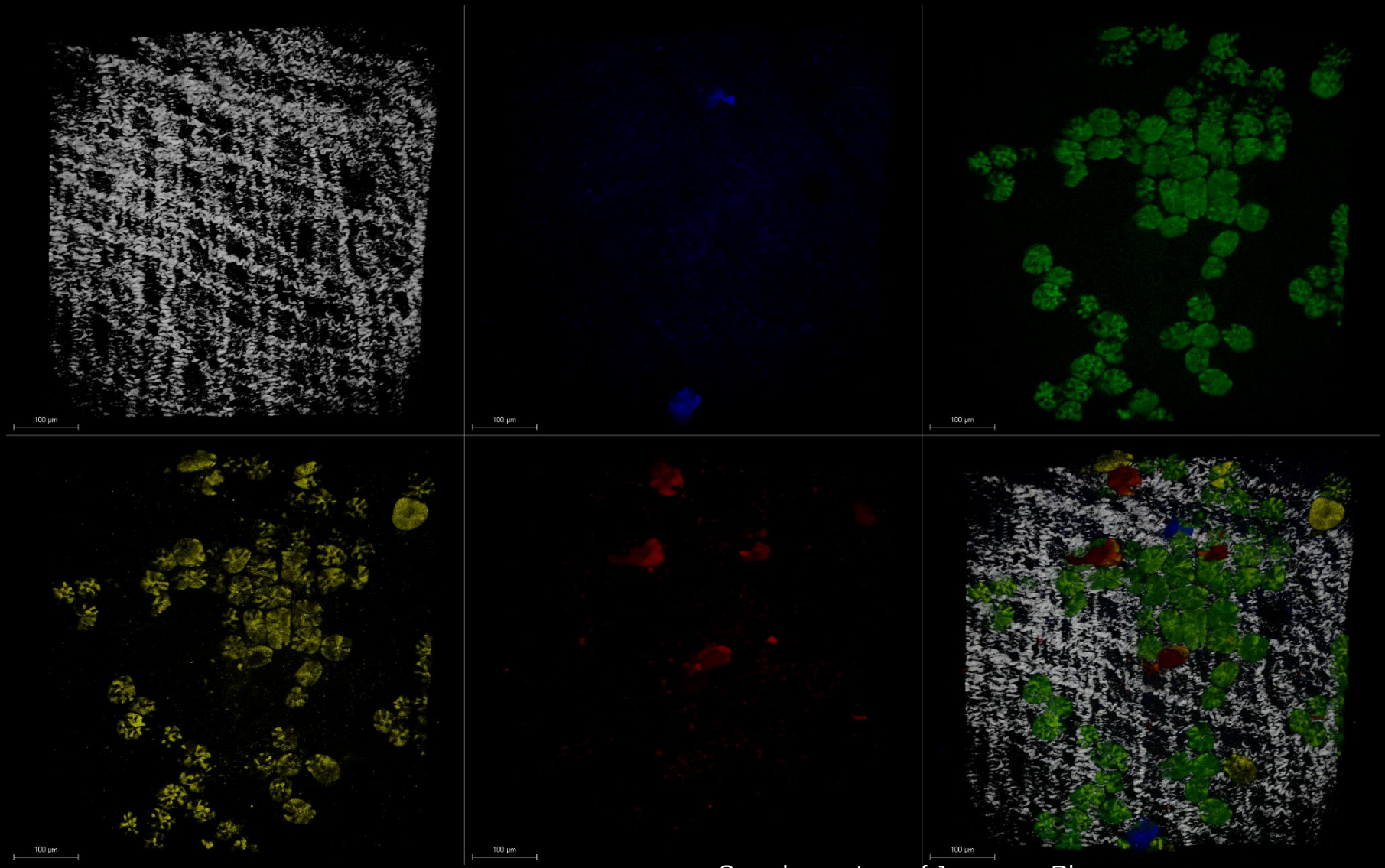
4Tune光谱检测单元

World‘s only true spectral NDD



The cortex of a living mouse with different types of nerve cells stained: In blue astrocytes, in green microglia, in yellow neurons and in red the blood system. The experiment was done by our beta-tester site at the DZNE in Bonn, Germany.

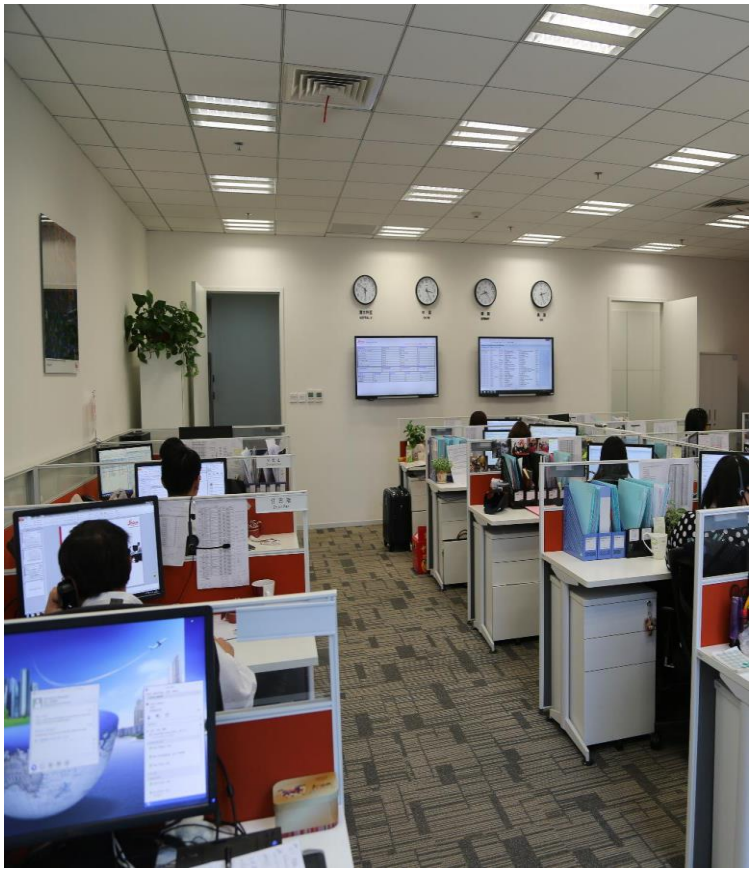
DIVE provides Spectral Freedom



Confetti Mouse, small intestine – gray SHG collagen,
green stem cells,
red, blue, yellow progeny of stem cells

Sample courtesy of Jacco van Rheenen.
University of Utrecht, NL

徕卡用心，服务贴心



徕卡自进入中国以来，以其杰出的产品质量和卓越的产品性能得到了广大中国用户的广泛青睐，并取得的巨大发展。

自2012年以来，徕卡客户服务将“成为客户首选服务供应商和服务业务及品质的行业标杆”定为新的发展战略，以“徕卡用心，服务贴心”为其服务指导，在过去的时间里，徕卡在客户服务中心，在线技术支持，现场服务及物流等多方面强化服务流程，提升服务响应，改善服务态度，使其服务焕然一新，成为徕卡品质内涵的重要组成部分！

400-650-6632

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