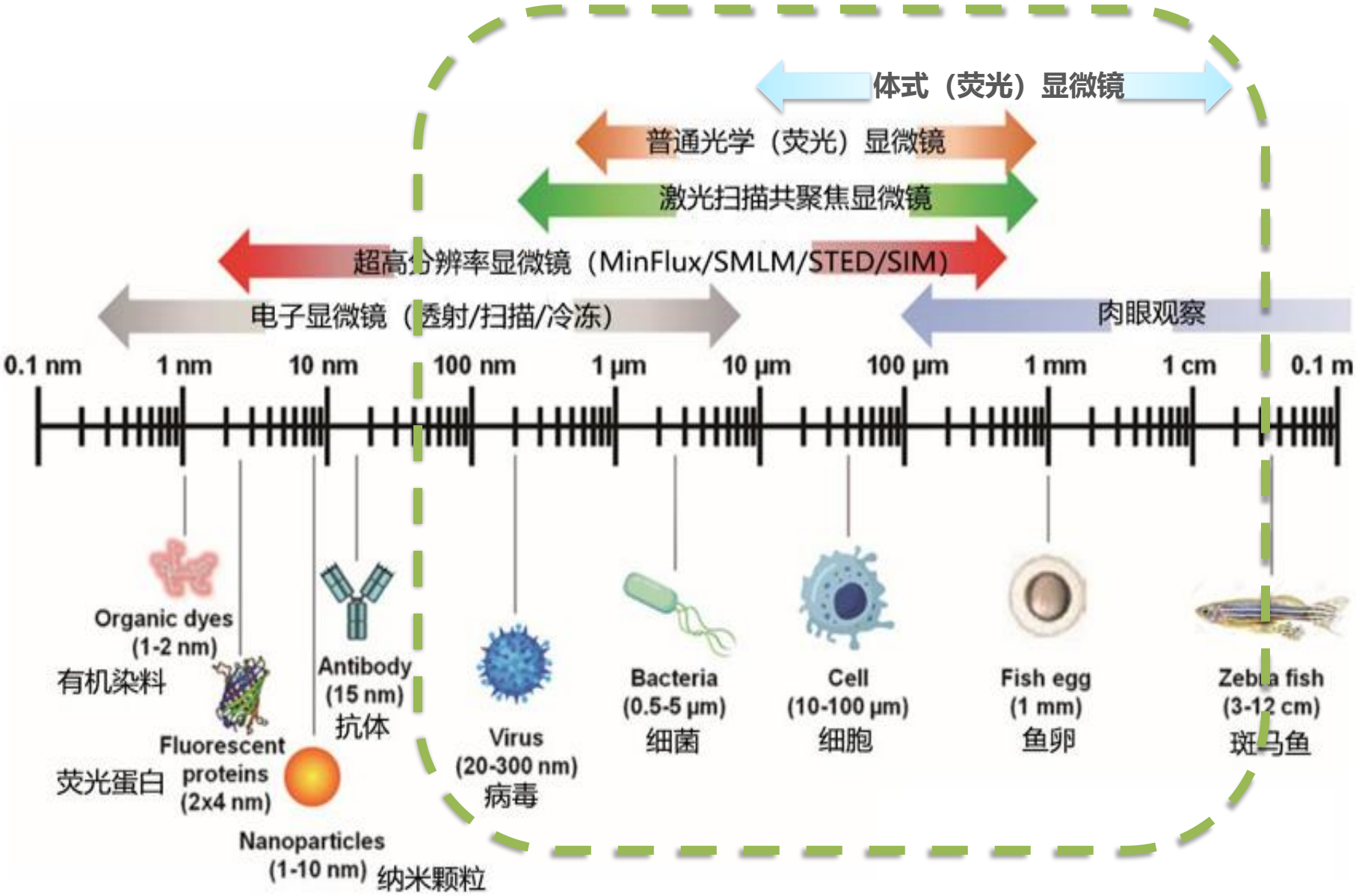
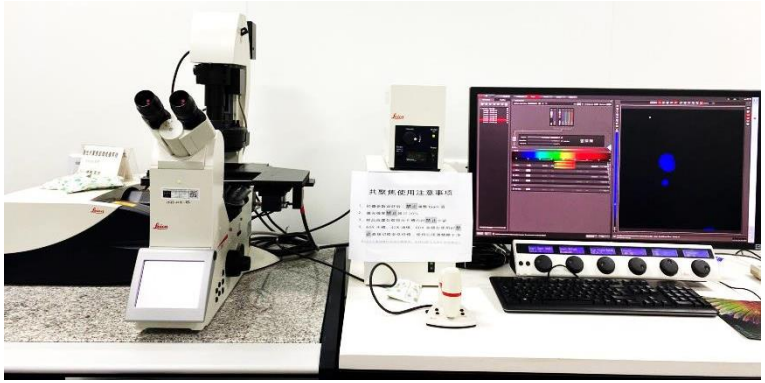
共聚焦显微成像技术及图像处理

水生所分测中心　　周芳

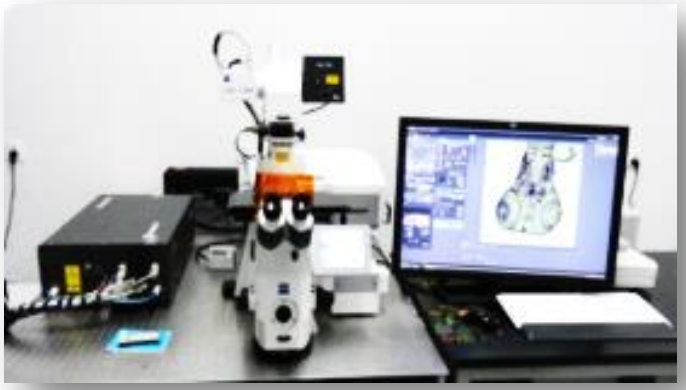
2020年8月7日


体式（荧光）显微镜
普通光学（荧光）显微镜
激光扫描共聚焦显微镜
超高分辨率显微镜（MinFlux/SMLM/STED/SIM）
电子显微镜（透射/扫描/冷冻）
肉眼观察
0.1 nm
1 nm
10 nm
100 nm
1 μm
10 μm
100 μm
1 mm
1 cm
0.1 m
Organic dyes
(1-2 nm)
有机染料
Fluorescent
proteins
(2x4 nm)
荧光蛋白
Nanoparticles
(1-10 nm)
纳米颗粒
Antibody
(15 nm)
抗体
Virus
(20-300 nm)
病毒
Bacteria
(0.5-5 μm)
细菌
Cell
(10-100 μm)
细胞
Fish egg
(1 mm)
鱼卵
Zebra fish
(3-12 cm)
斑马鱼

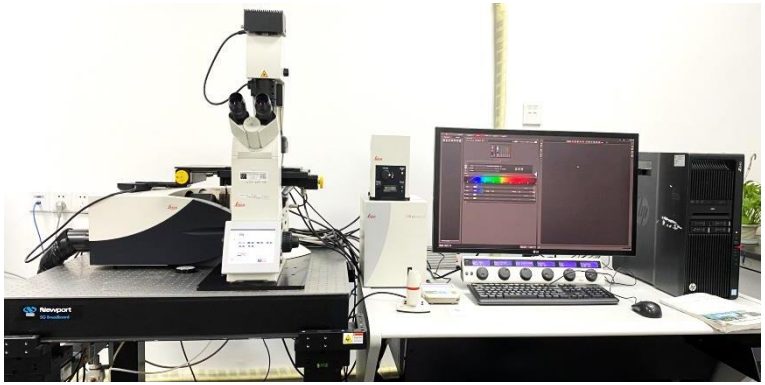
现有激光扫描共聚焦显微镜



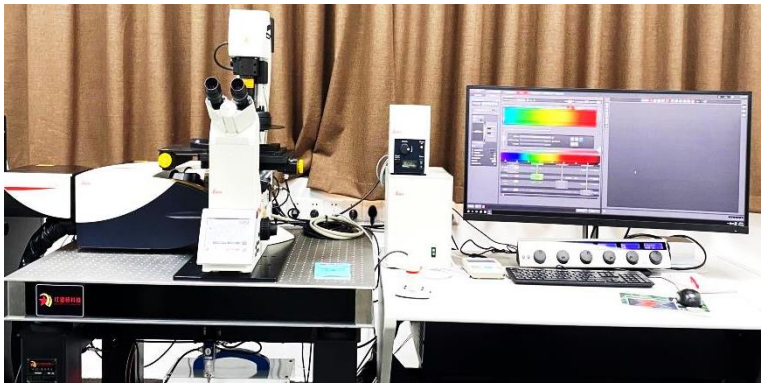
Leica SP8



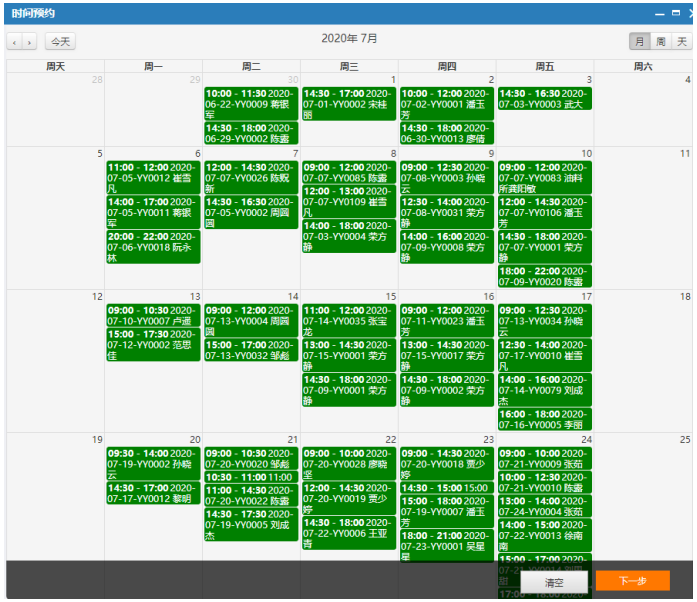
Zeiss LSM 710 NLO



Leica DLS



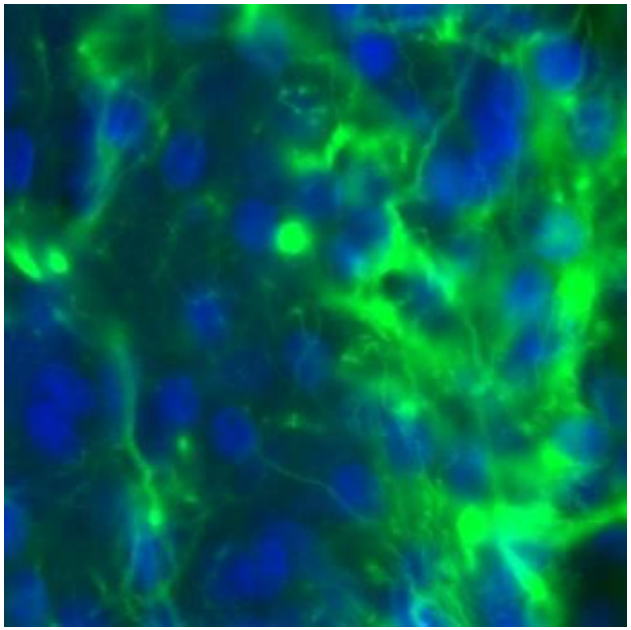
Leica STED



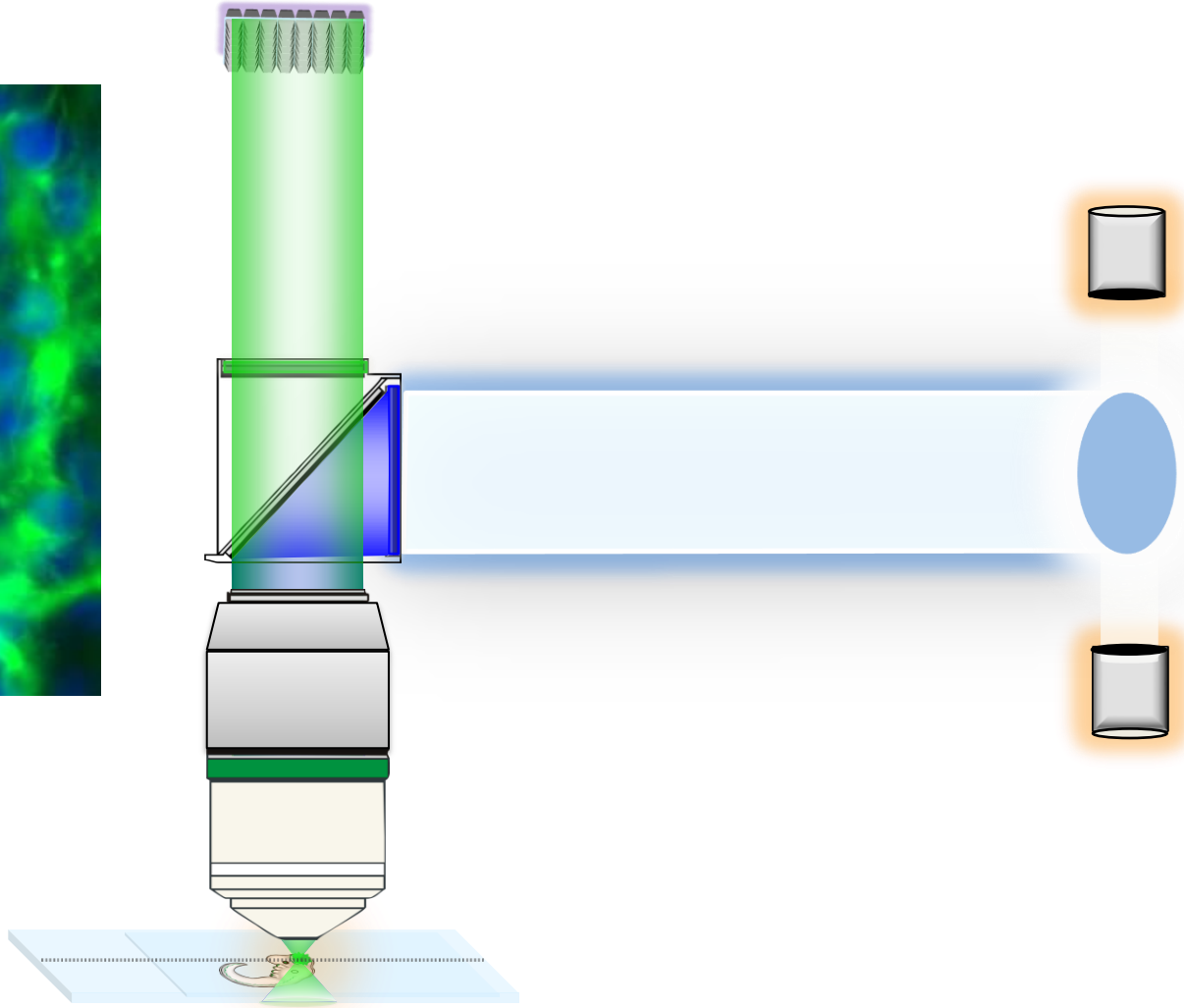
共聚焦——定义

以激光作为激发光源，采用光源针孔与检测针孔共轭聚焦技术，对样本进行断层扫描，以获得高分辨率光学切片的荧光显微系统。

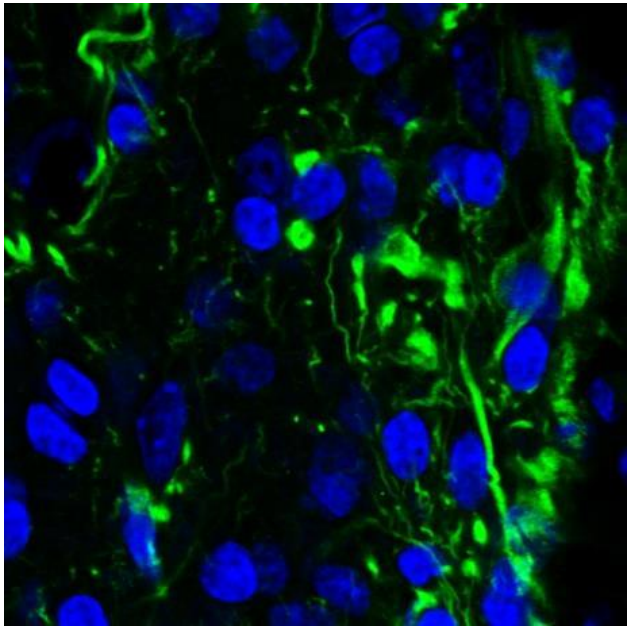
宽场荧光显微镜成像原理



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



共聚焦显微镜成像原理



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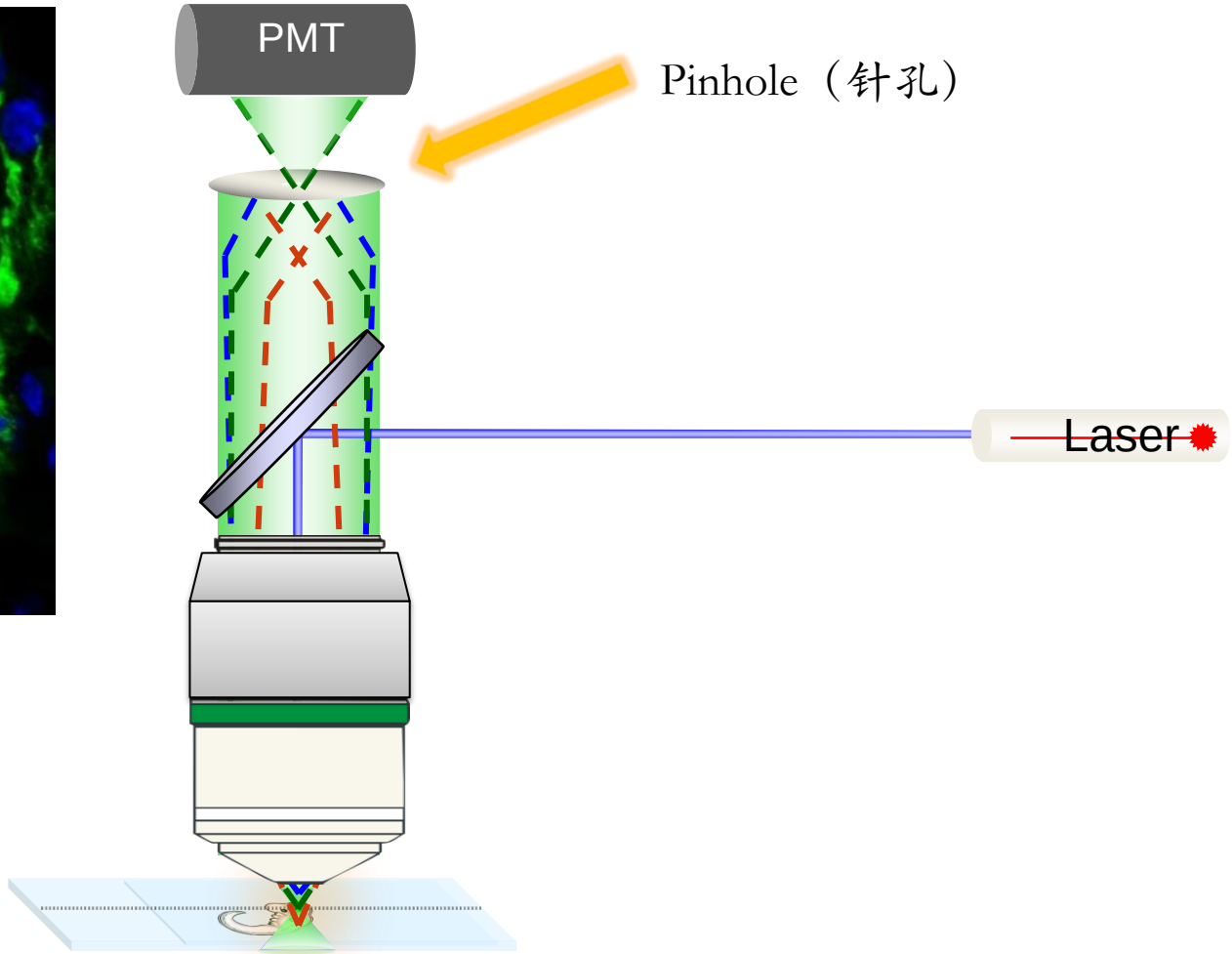
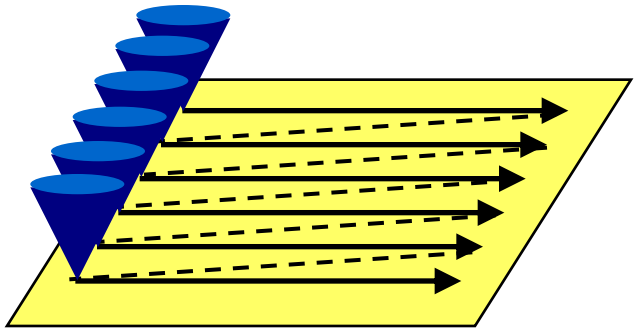
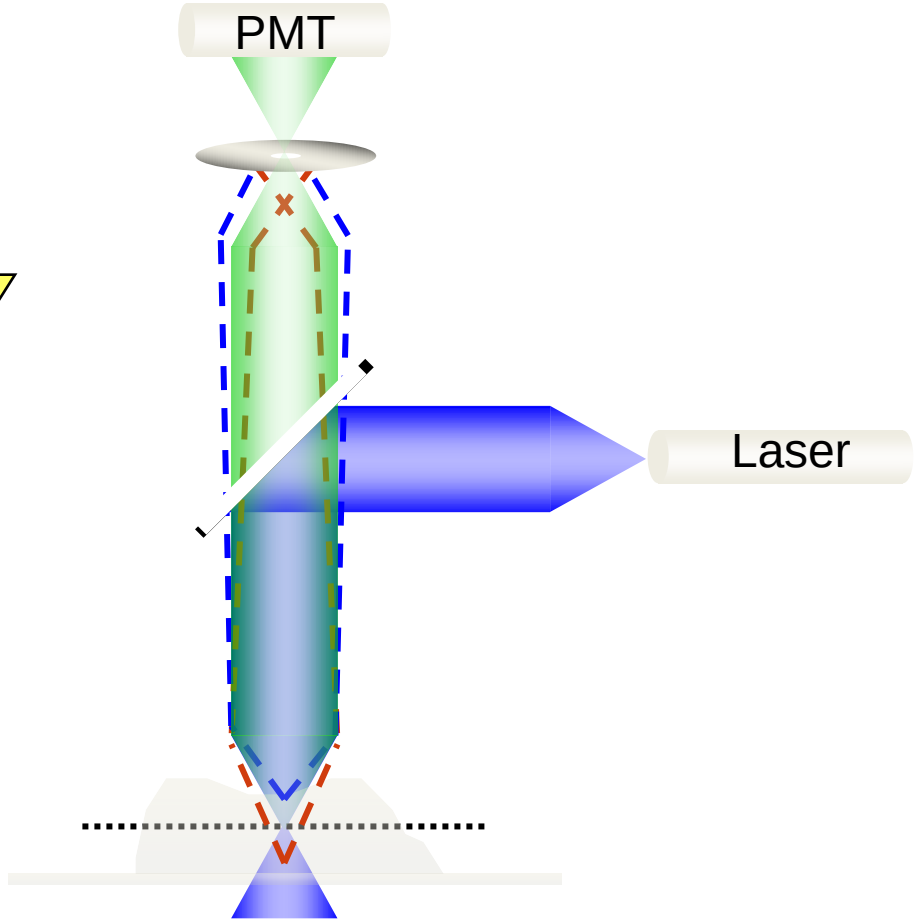


共聚焦扫描方式



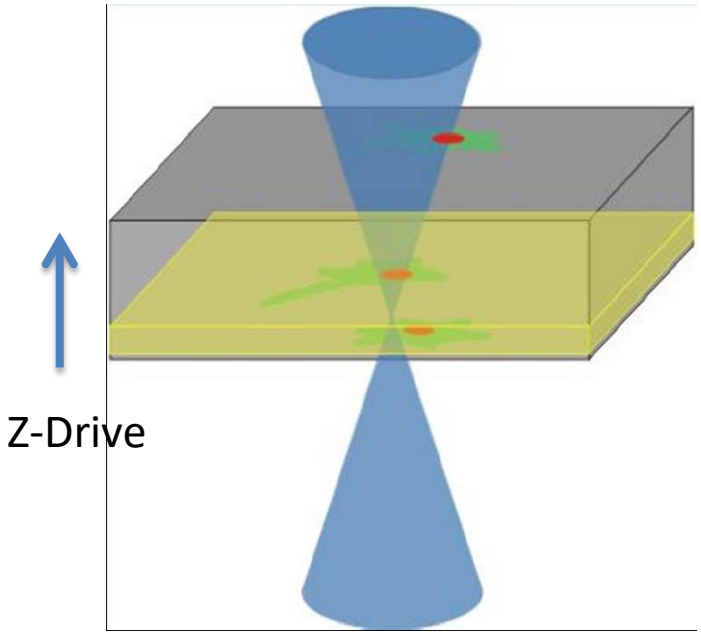
XY scanning

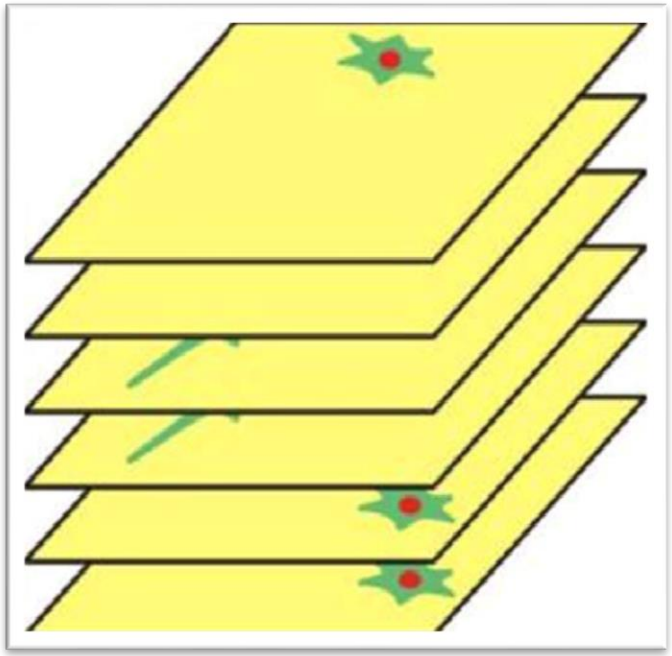


光学切面和三维信息采集

光学切片的厚度 d 取决于:

☑ 物镜NA ☑ 针孔大小 ☑ 发射波长





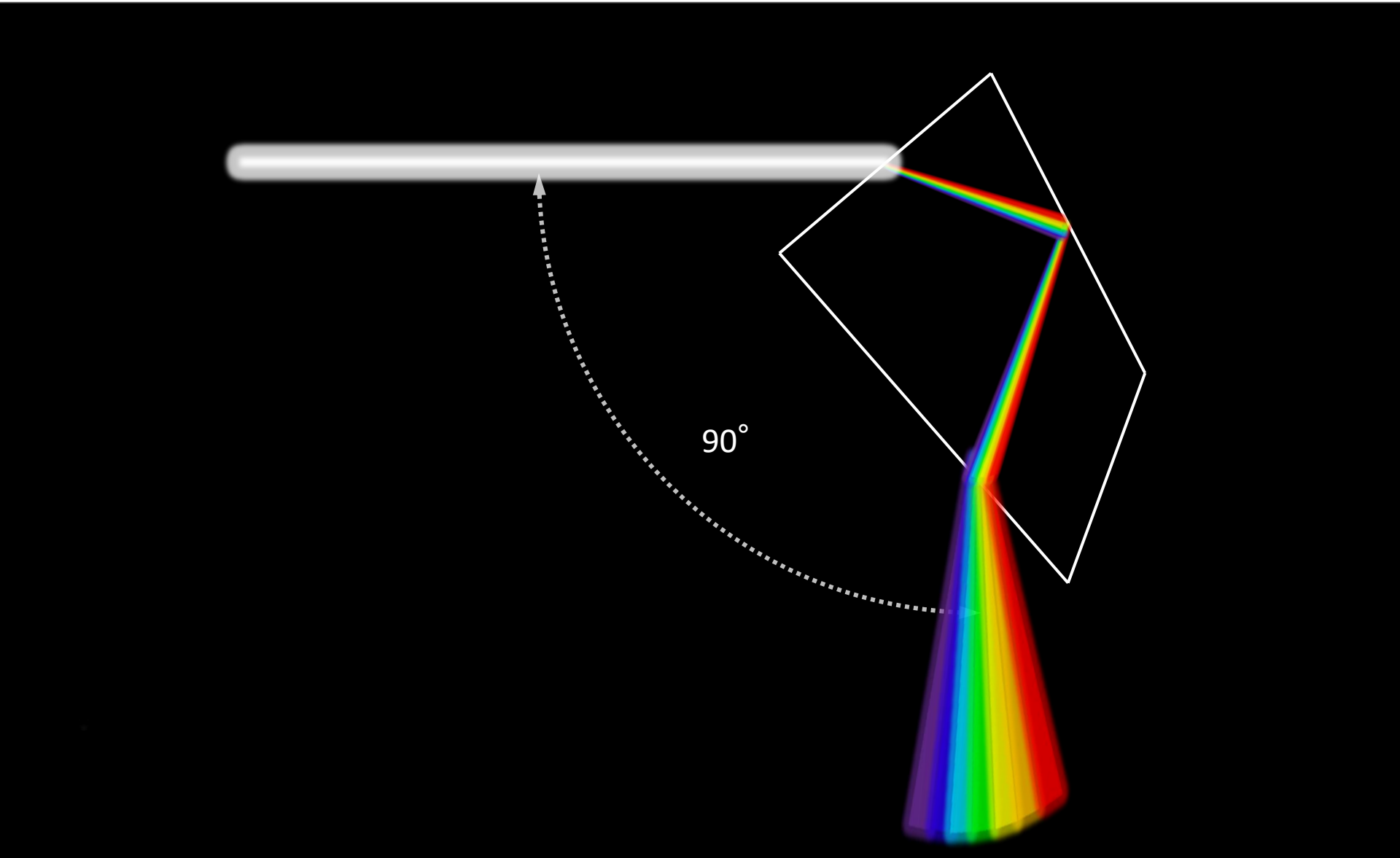
共聚焦基本结构

- 激光光源
- 扫描模块（包括光路通道、针孔、扫描镜、检测器）
- 倒置或者正置荧光显微镜
- 数字信号控制处理系统 ：计算机、数字信号处理器等
- 图像输出设备：显示器、存储器

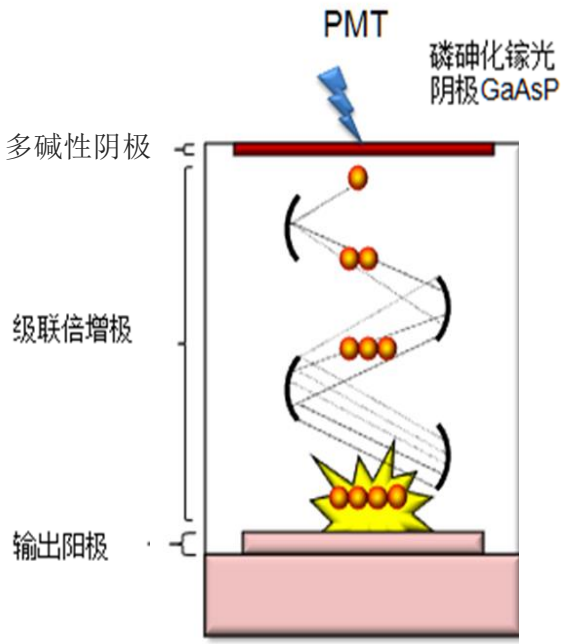
共聚焦光源

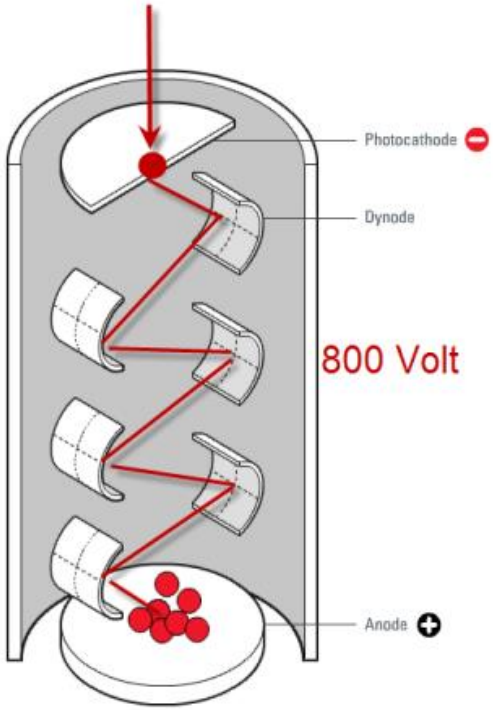
Laser	Laser	Laser	Laser
双光子690nm-1064nm	405nm	405nm	405nm
458nm/488nm/514nm	458nm/488nm/514nm	448nm	458nm/476nm/488nm/496nm/514nm
561nm	561nm	488nm	WIL 470nm-670nm
633nm	594nm	552nm	
	633nm	638nm	

共聚焦分光检测系统



共聚焦PMT检测器





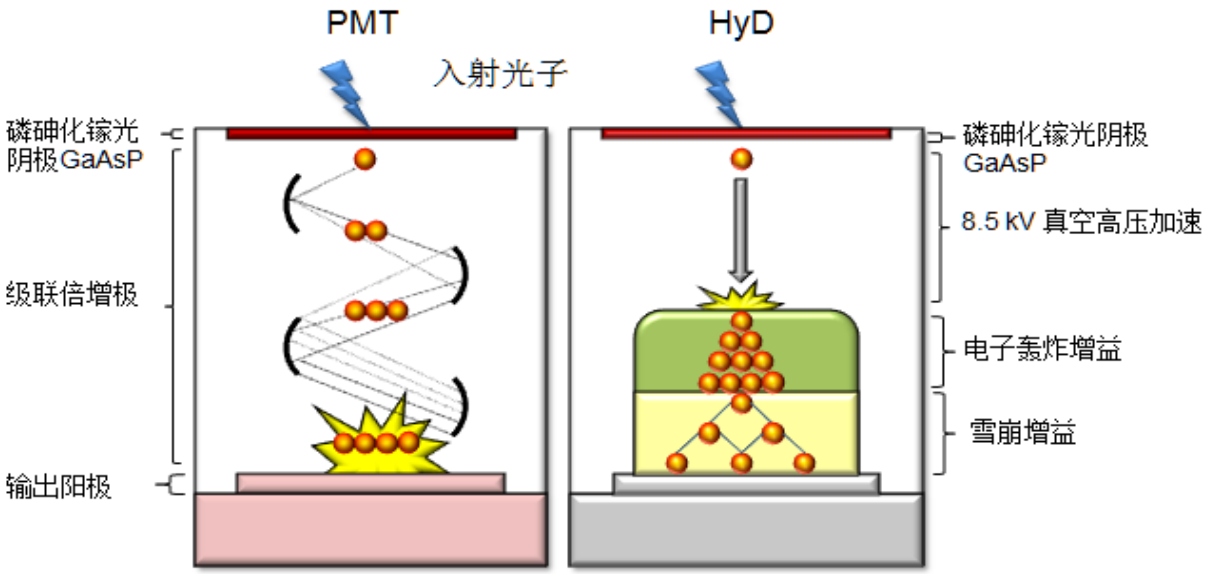
入射到PMT（光电倍增管）的光经过以下过程后才能输出信号：

（1）荧光到达光阴极面，发生光电转换，放出光电子（外光电效应）

（2）光电子经聚焦极汇集到第一倍增极，然后再相继经过各倍增极（发射二次电子），进行二次电子倍增

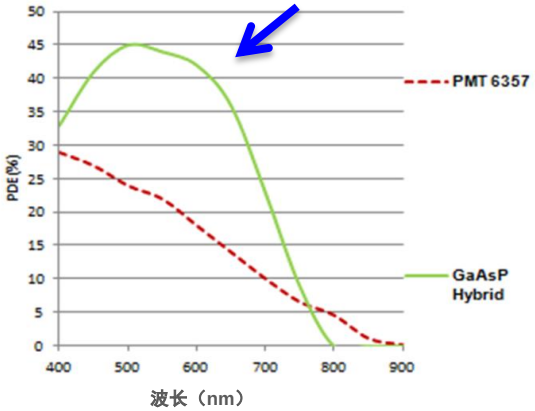
（3）最后由末极倍增极发射的二次电子通过阳极输出。

HyD™：超高灵敏检测器



- ✓ 超高灵敏
- ✓ 低噪声
- ✓ 大动态范围
- ✓ 快速响应

光子探测效率（PDE）比较



物镜

HC PL FLUOTAR	2.5x/0.07 DRY
HC PL APO CS2	10x/0.40 DRY
HC PL APO CS2	20x/0.75 DRY
HC PL APO CS2	40x/1.10 WATER
HC PL APO CS2	40x/1.30 OIL
HC PL APO CS2	63x/1.40 OIL

共聚焦的功能及优势

- 单张光学切片功能
- 多通道序列扫描
- 连续光学切片，三维图像重构
- 时间序列扫描
- 大视野拼图
- 多点标记扫描
- 光谱扫描
- 光操作

共聚焦的功能及优势

- 单张光学切片功能：高清晰度、高分辨率图像采集，Pinhole、去除杂散光，实现“光学切面”
- 多通道序列扫描
- 连续光学切片，三维图像重构
- 时间序列扫描
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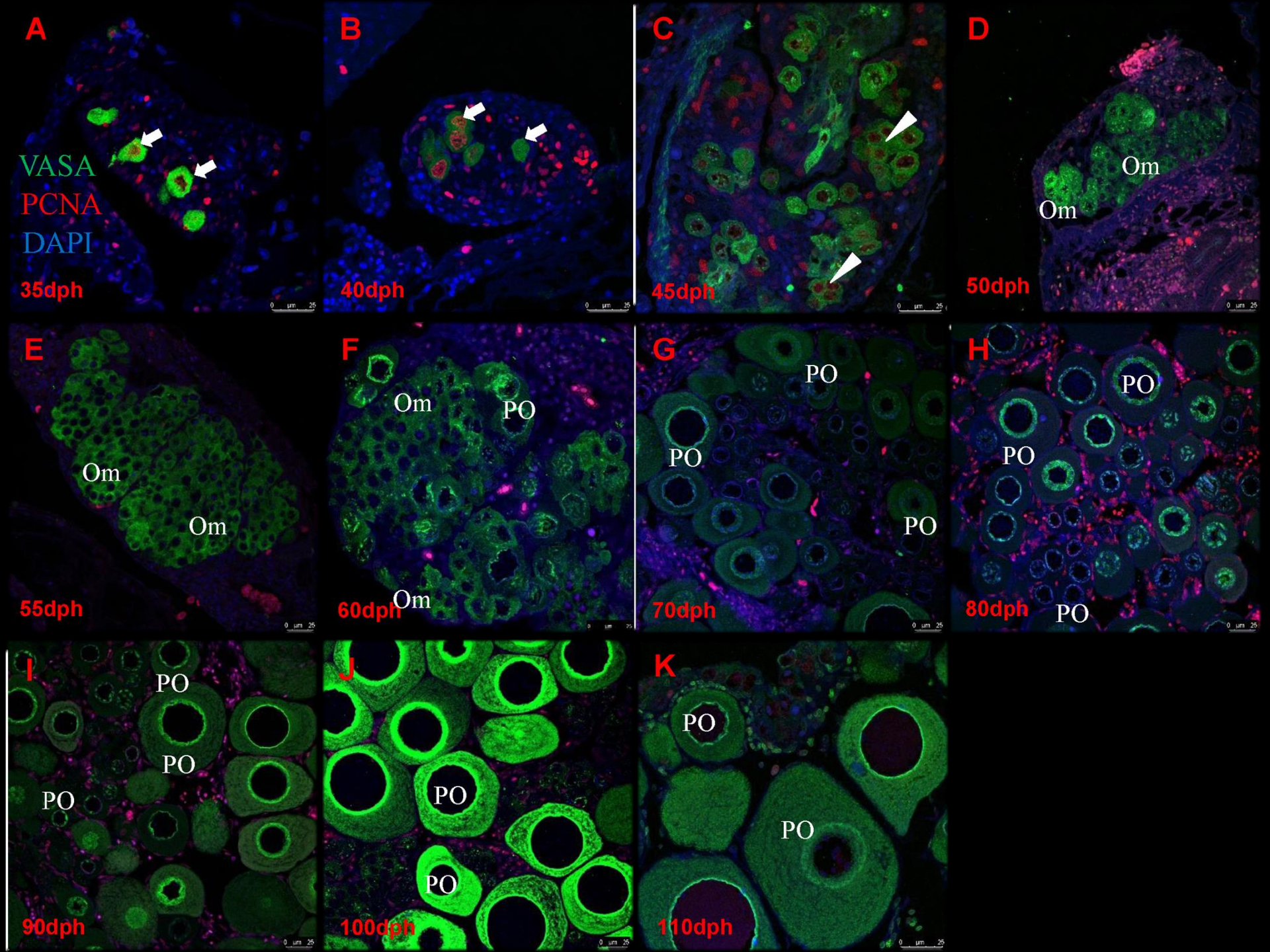
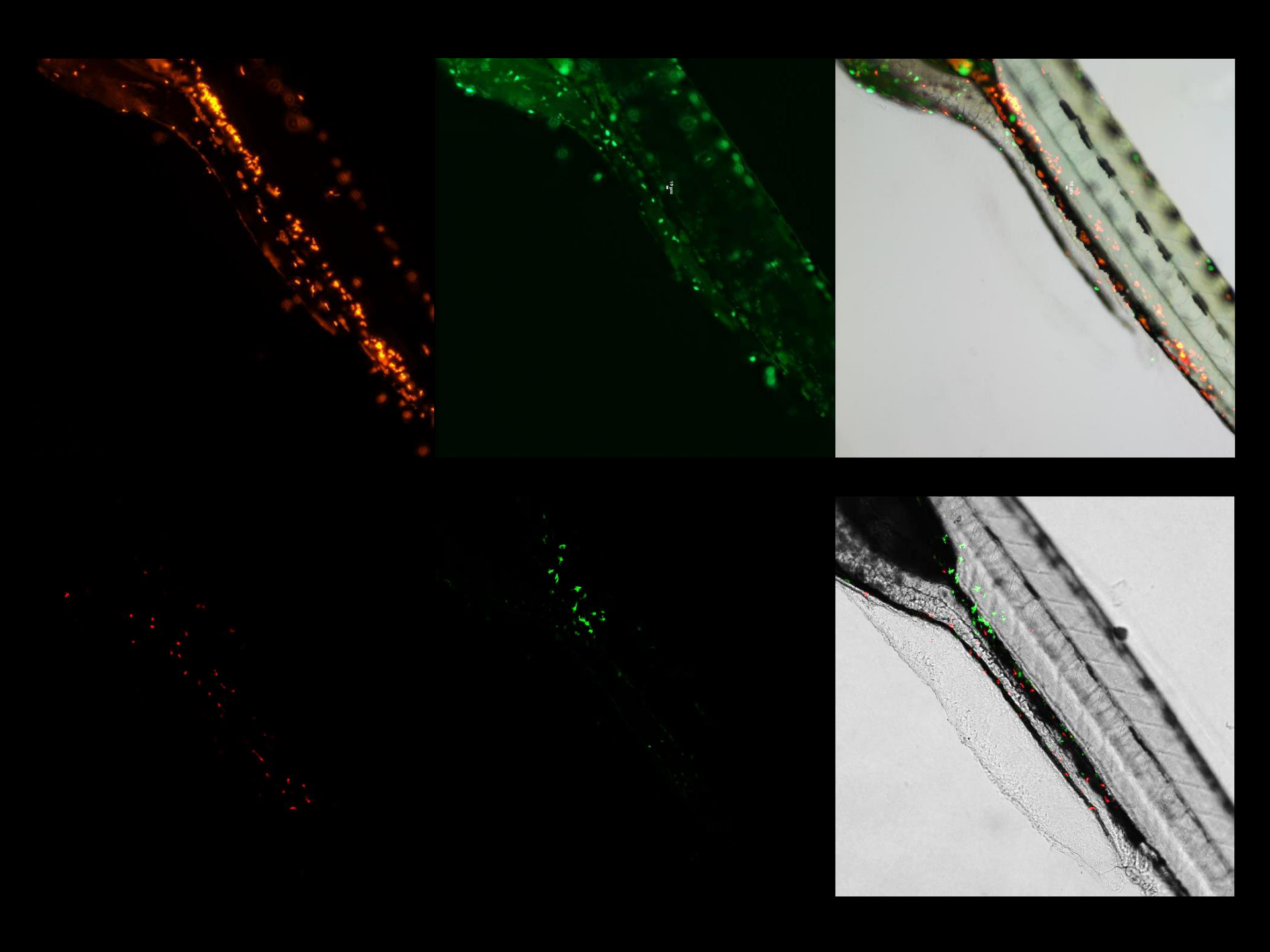

A
VASA
PCNA
DAPI
35dph
B
40dph
C
45dph
D
Om
Om
50dph
E
Om
Om
55dph
F
Om
PO
Om
60dph
G
PO
PO
PO
70dph
H
PO
PO
PO
80dph
I
PO
PO
PO
90dph
J
PO
PO
100dph
K
PO
PO
110dph



共聚焦的功能及优势

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- 多通道序列扫描：多色荧光标记成像，避免荧光串色
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- 大视野拼图
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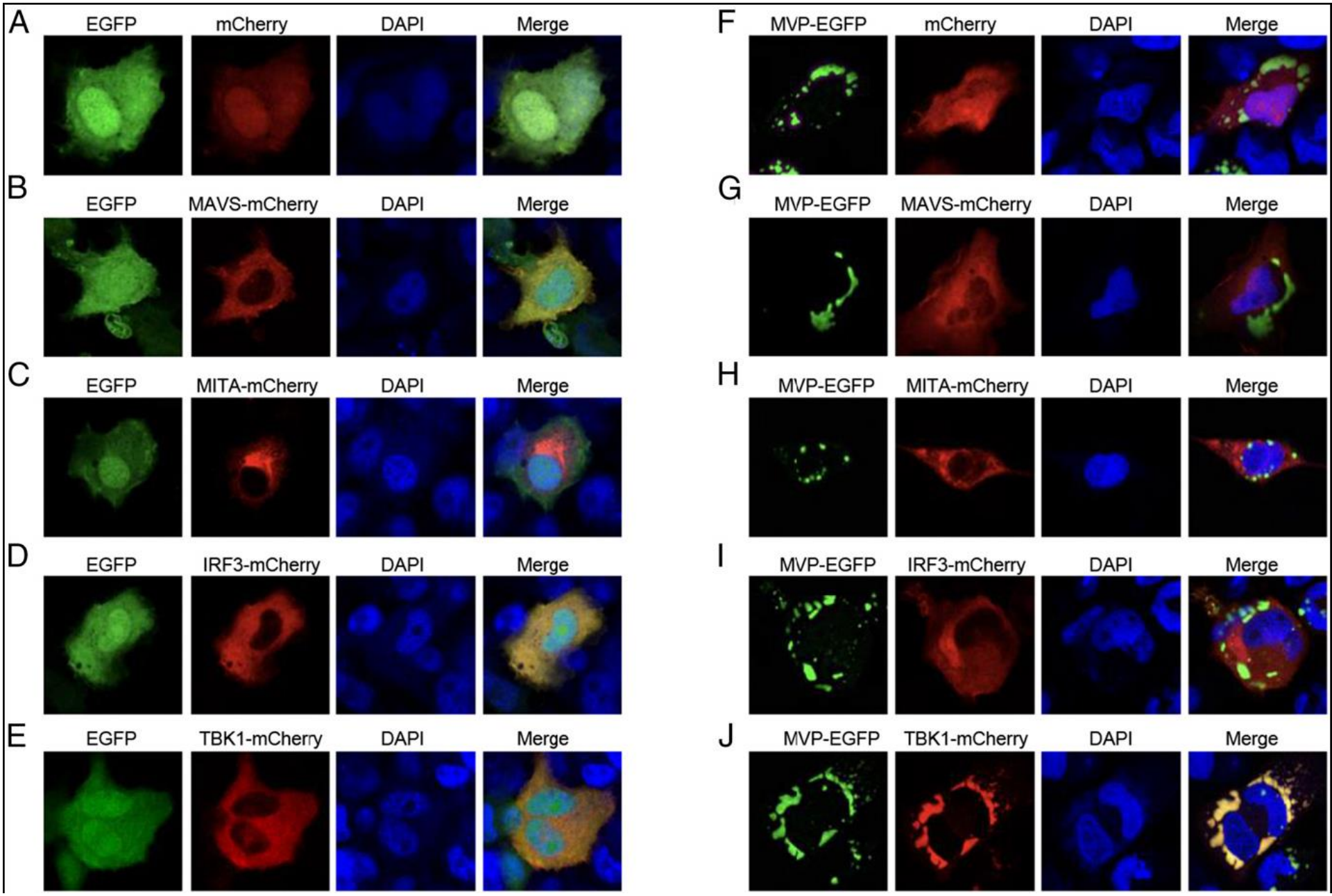
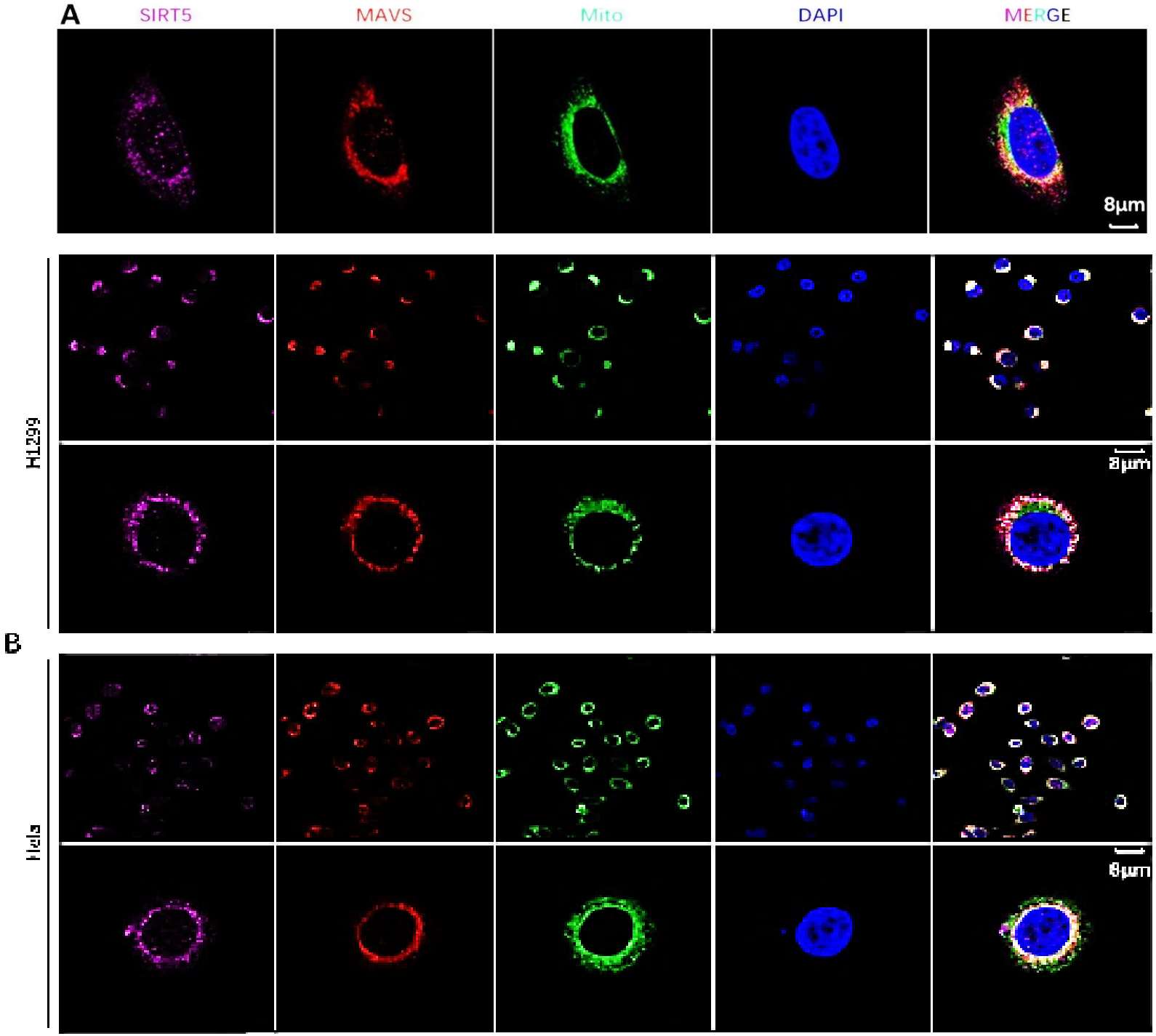
- 光谱扫描
- 光操作


A
EGFP
mCherry
DAPI
Merge
B
EGFP
MAVS-mCherry
DAPI
Merge
C
EGFP
MITA-mCherry
DAPI
Merge
D
EGFP
IRF3-mCherry
DAPI
Merge
E
EGFP
TBK1-mCherry
DAPI
Merge
F
MVP-EGFP
mCherry
DAPI
Merge
G
MVP-EGFP
MAVS-mCherry
DAPI
Merge
H
MVP-EGFP
MITA-mCherry
DAPI
Merge
I
MVP-EGFP
IRF3-mCherry
DAPI
Merge
J
MVP-EGFP
TBK1-mCherry
DAPI
Merge


A
SIRT5
MAVS
Mito
DAPI
MERGE
8μm
H1299
8μm
B
Hela
8μm

共聚焦的功能及优势

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- 多通道序列扫描：多色荧光标记成像，避免荧光串色
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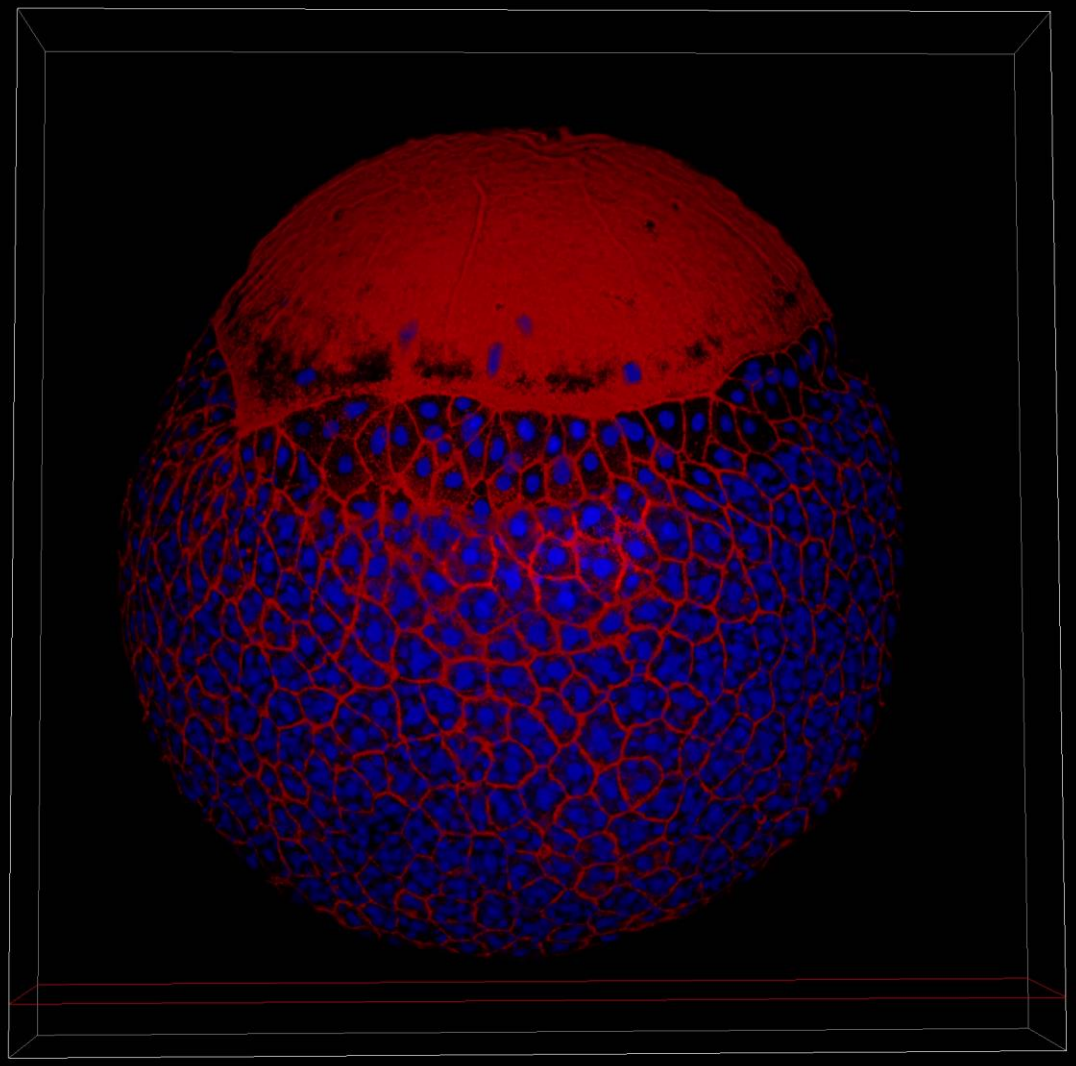

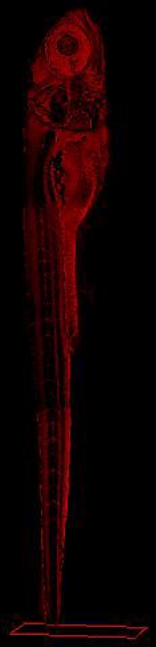

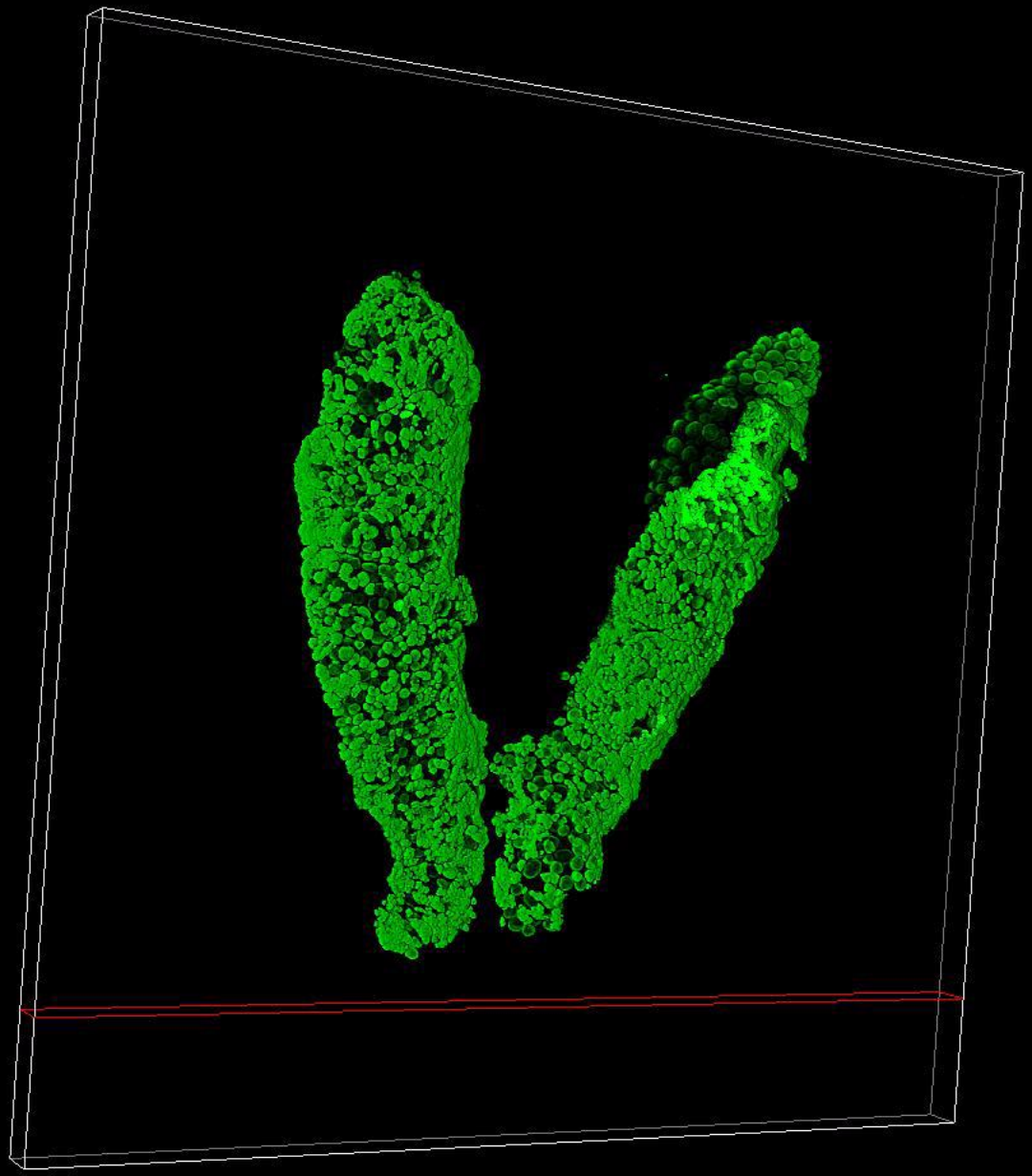

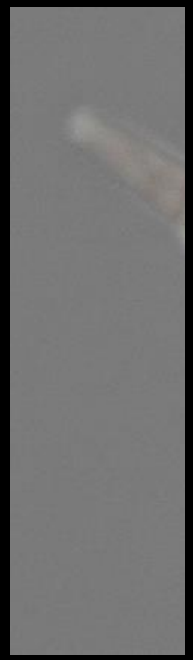

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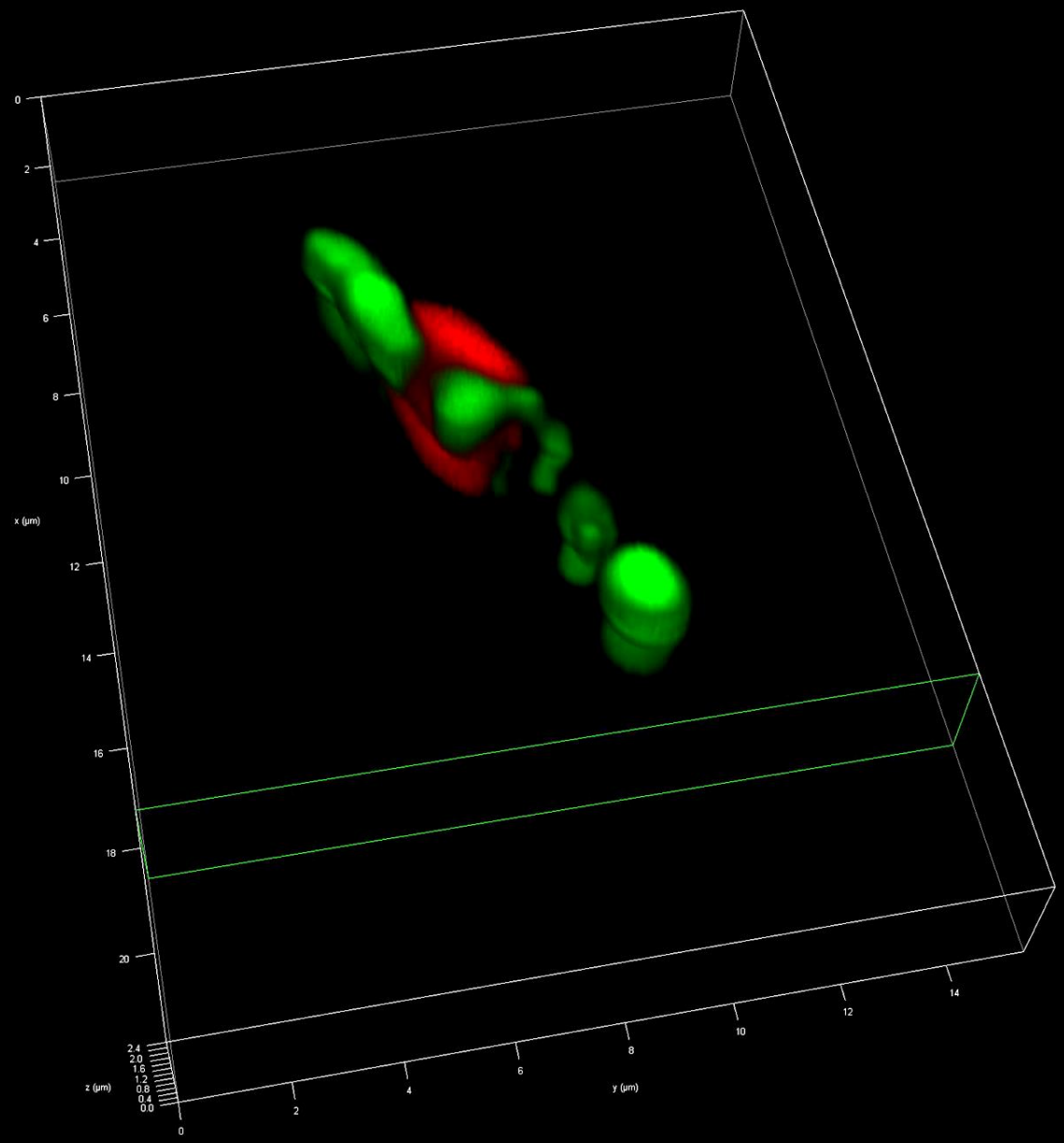

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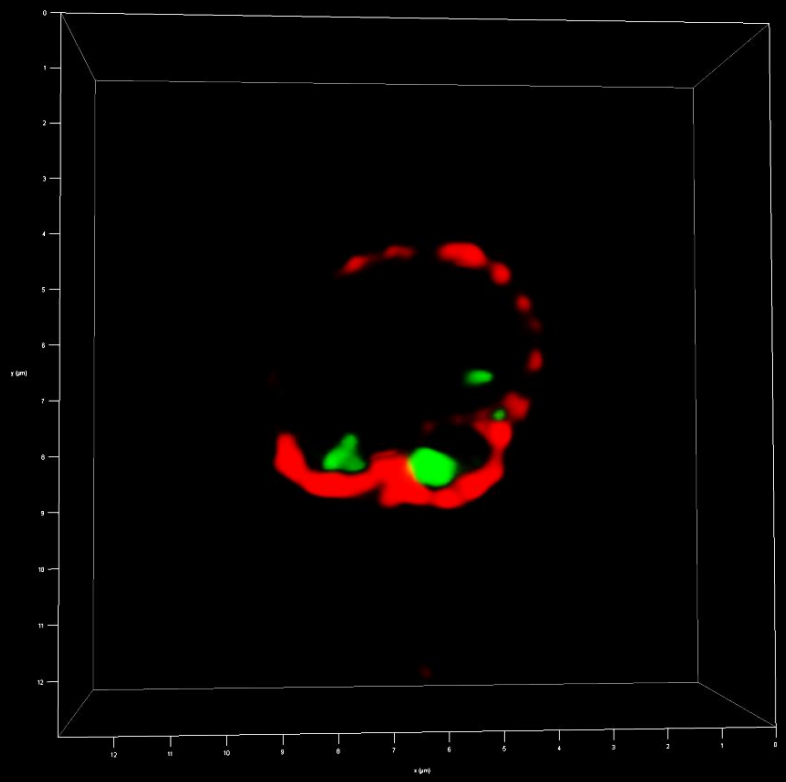
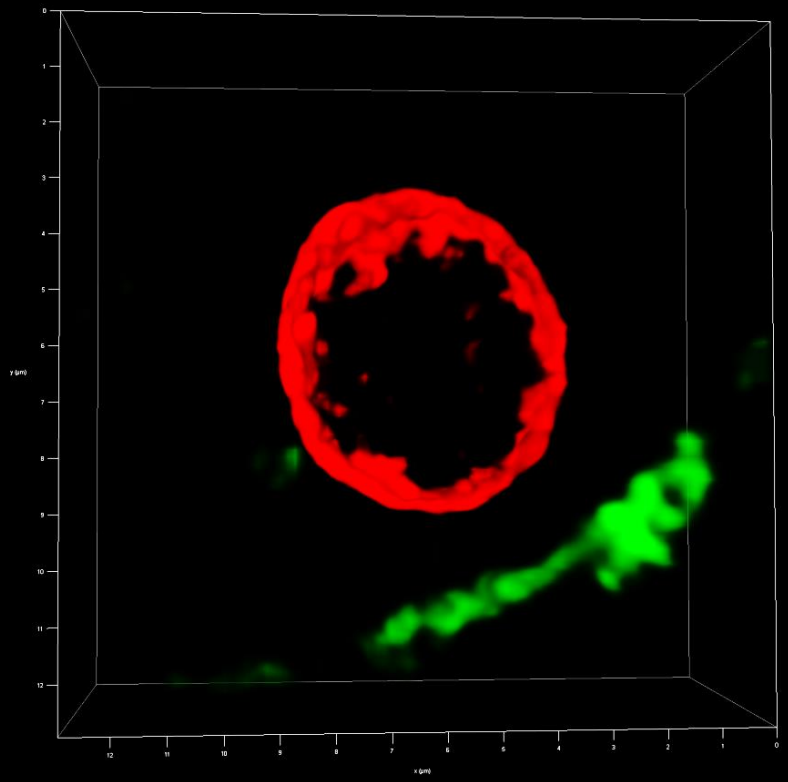

y (µm)
x (µm)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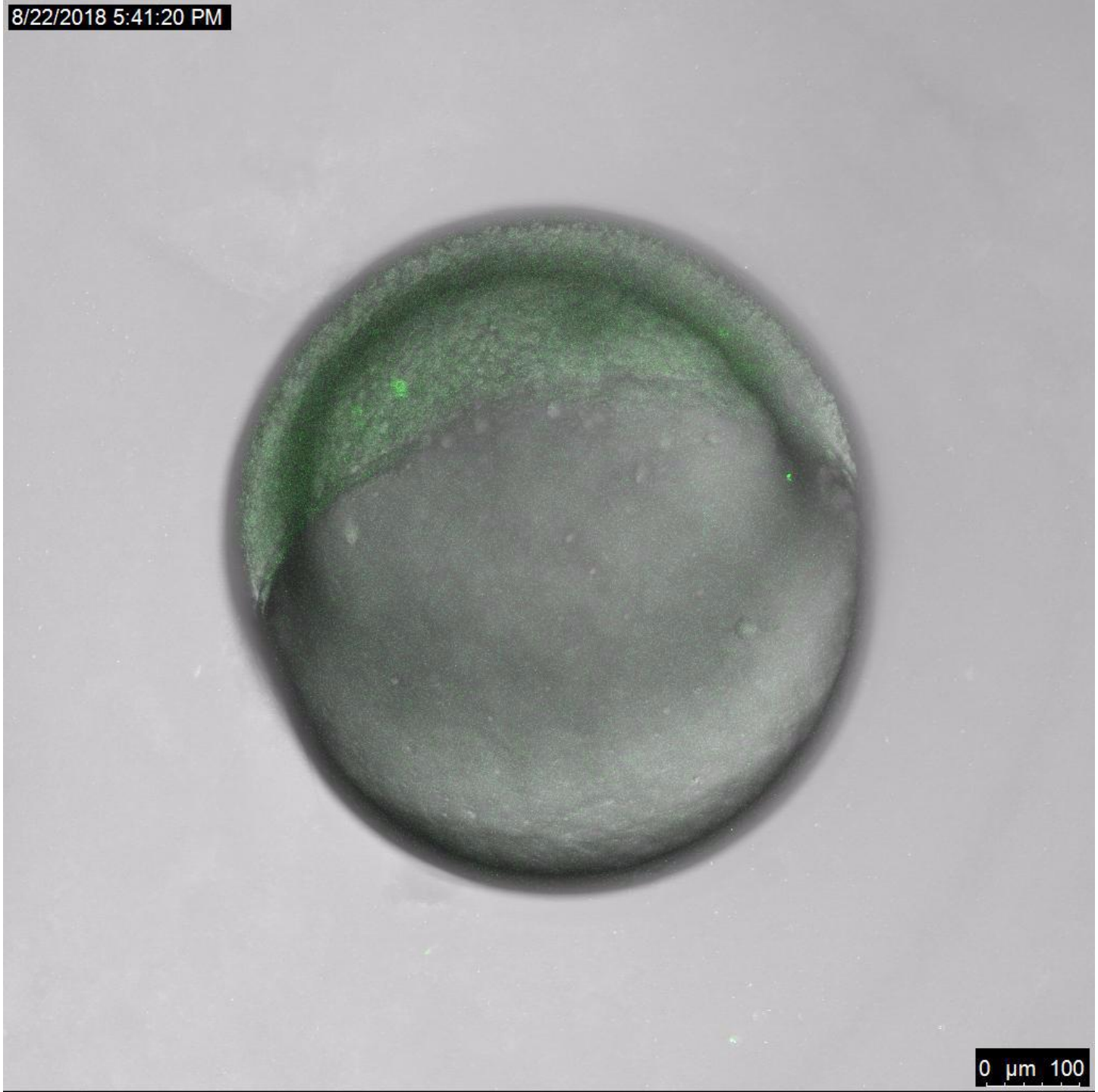
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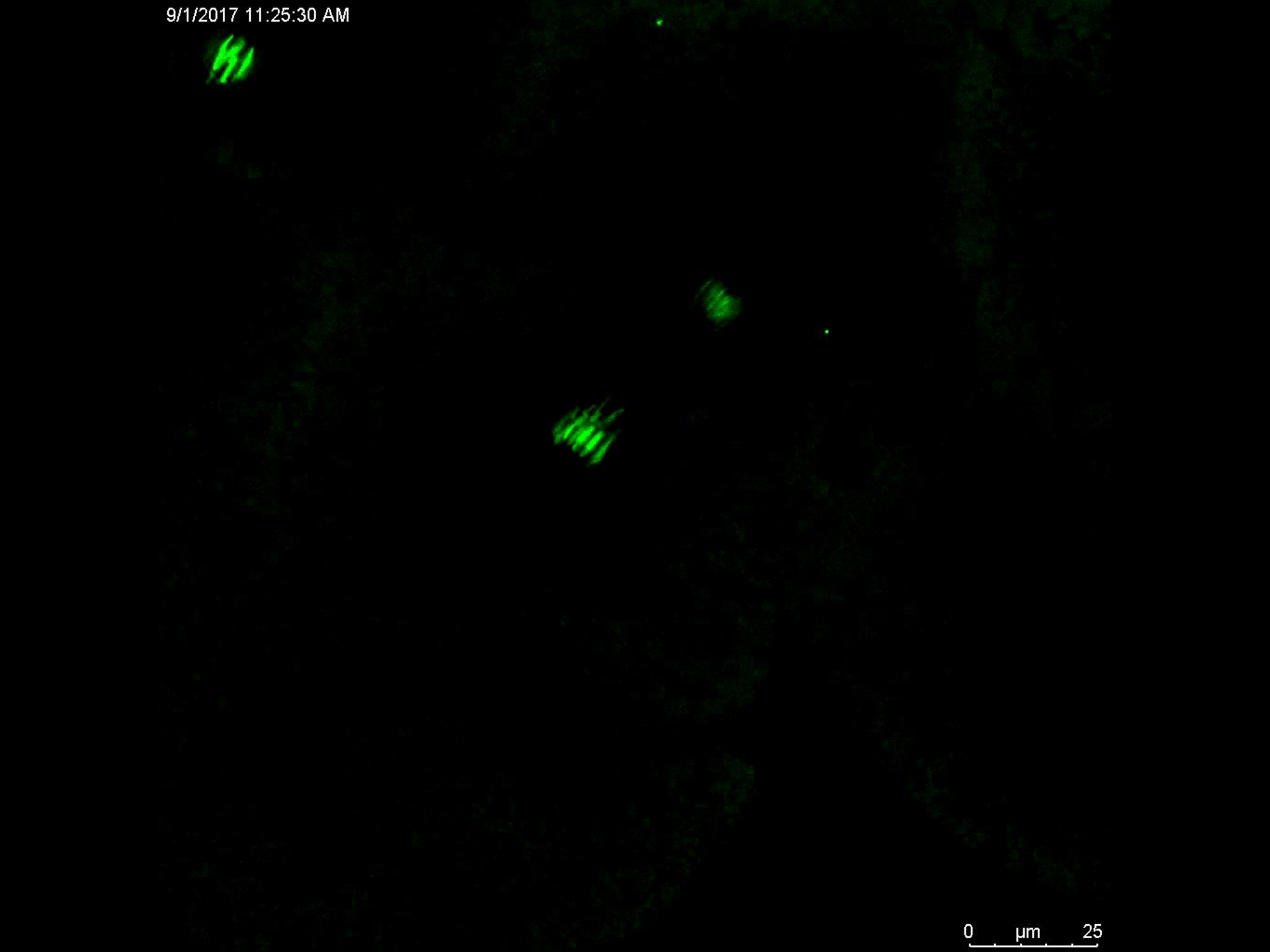
0 µm 100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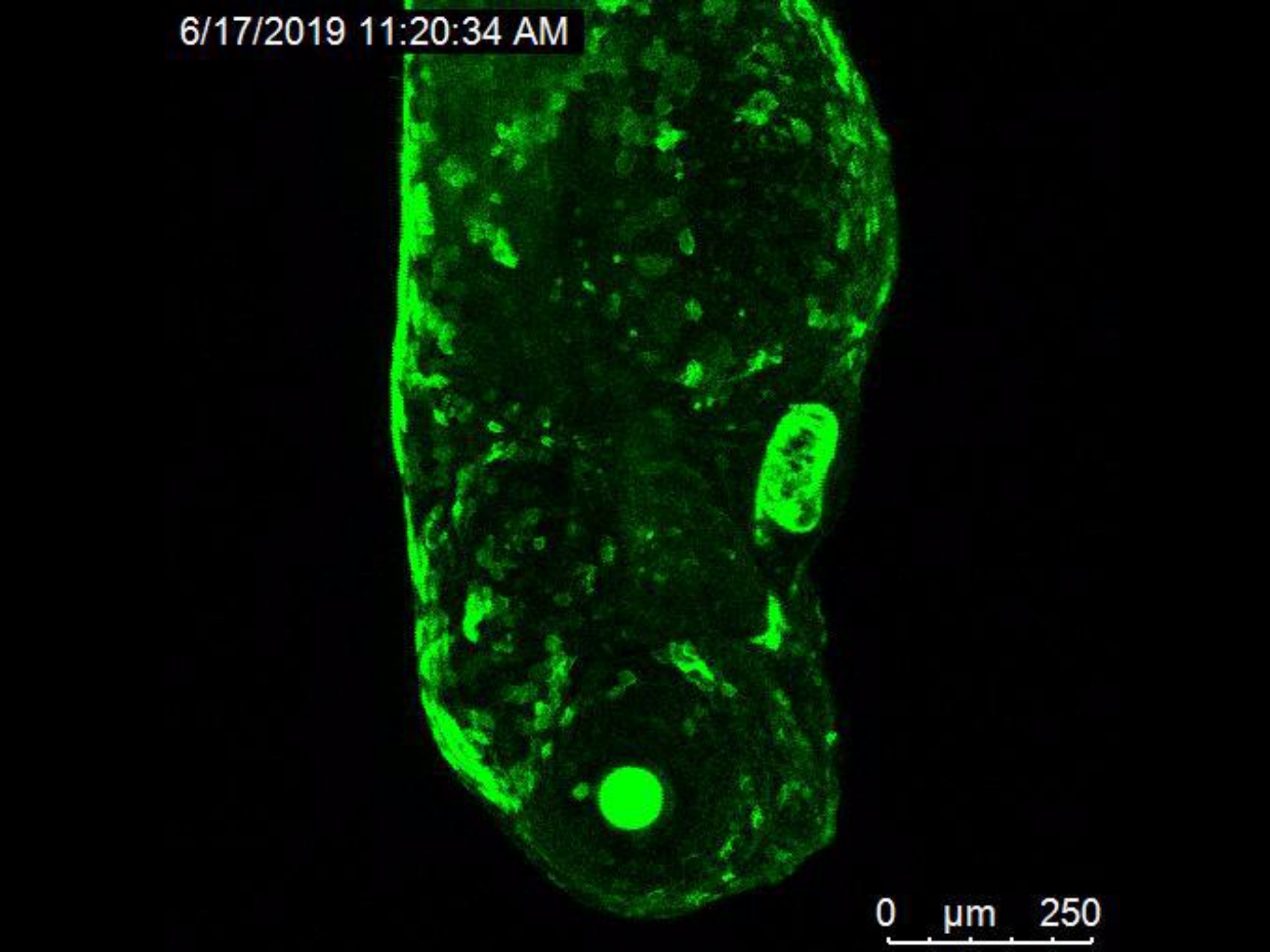
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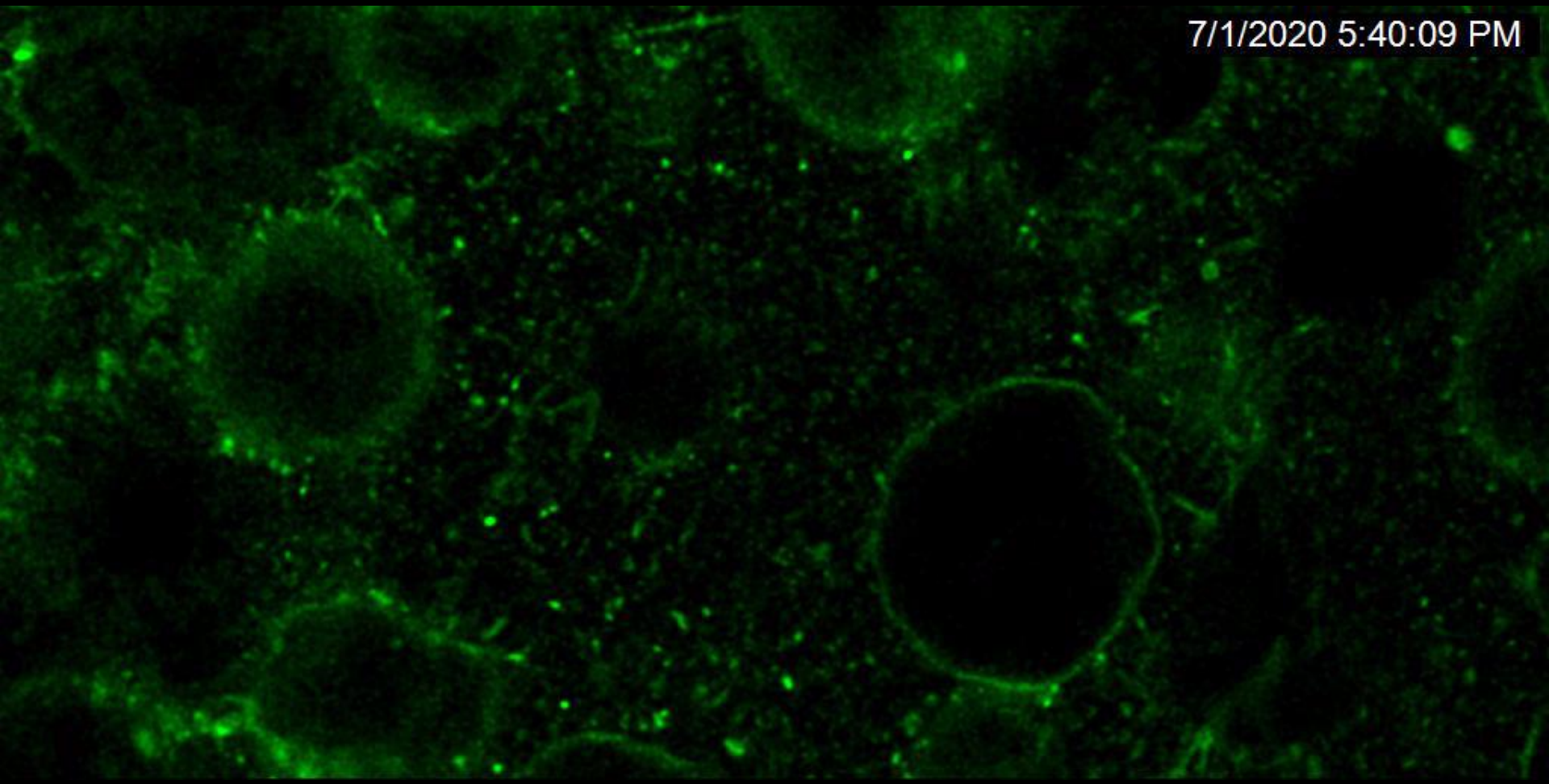
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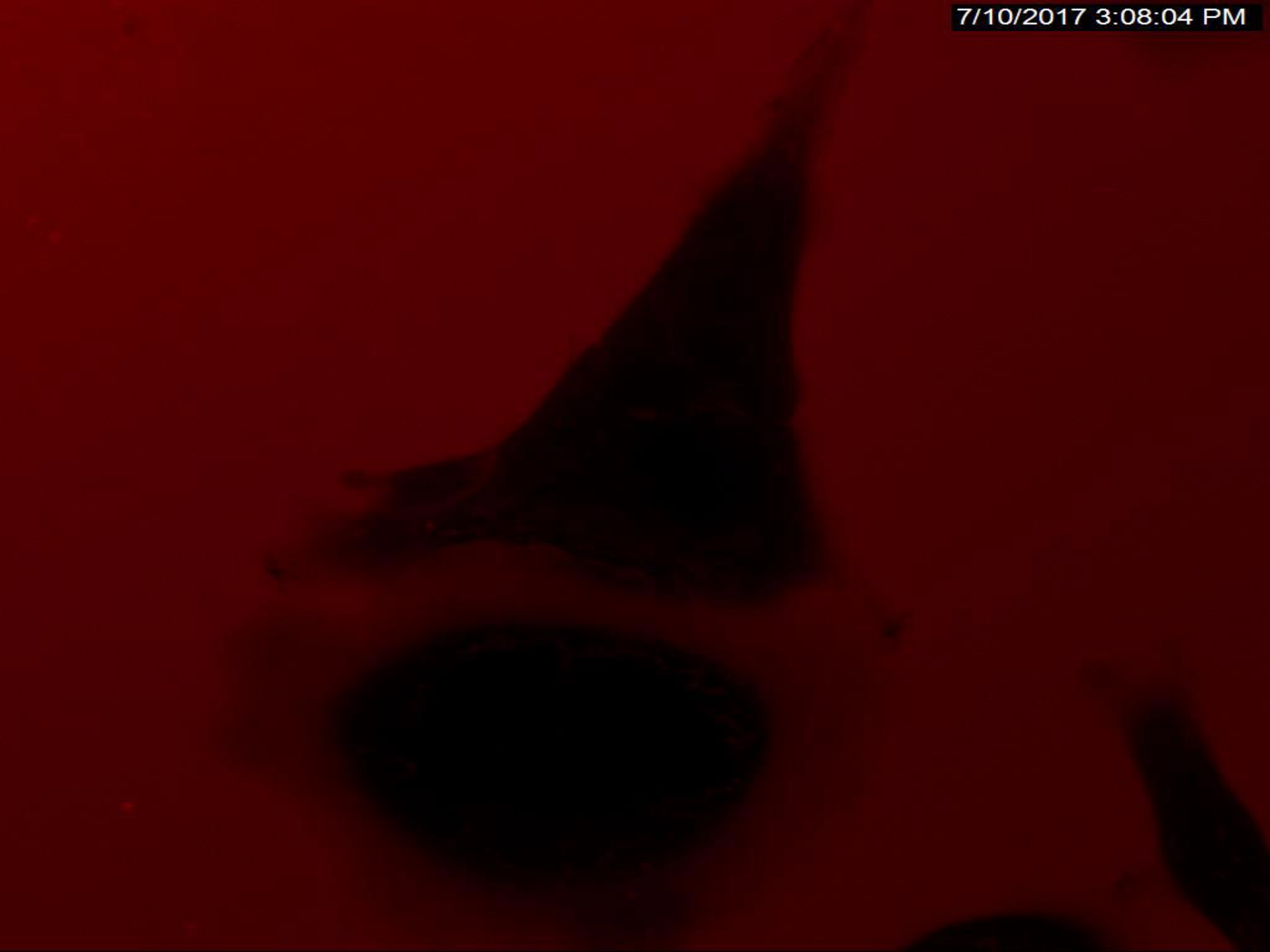
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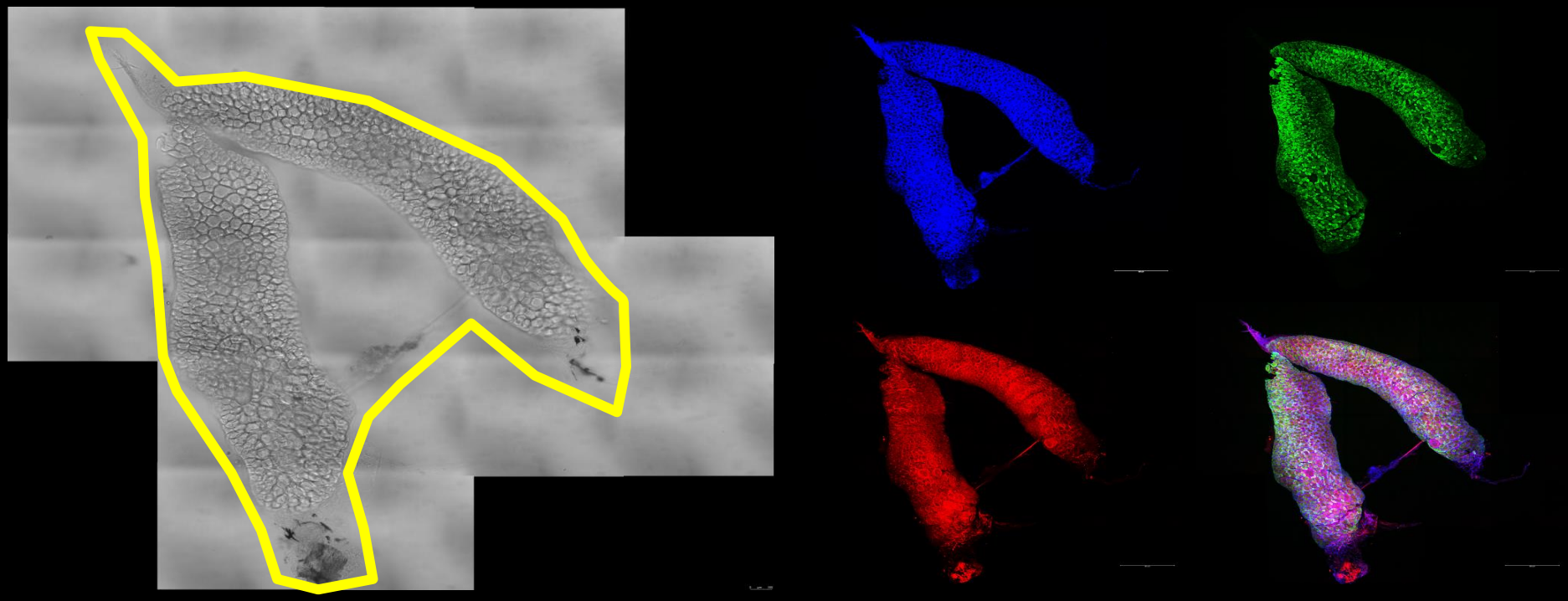
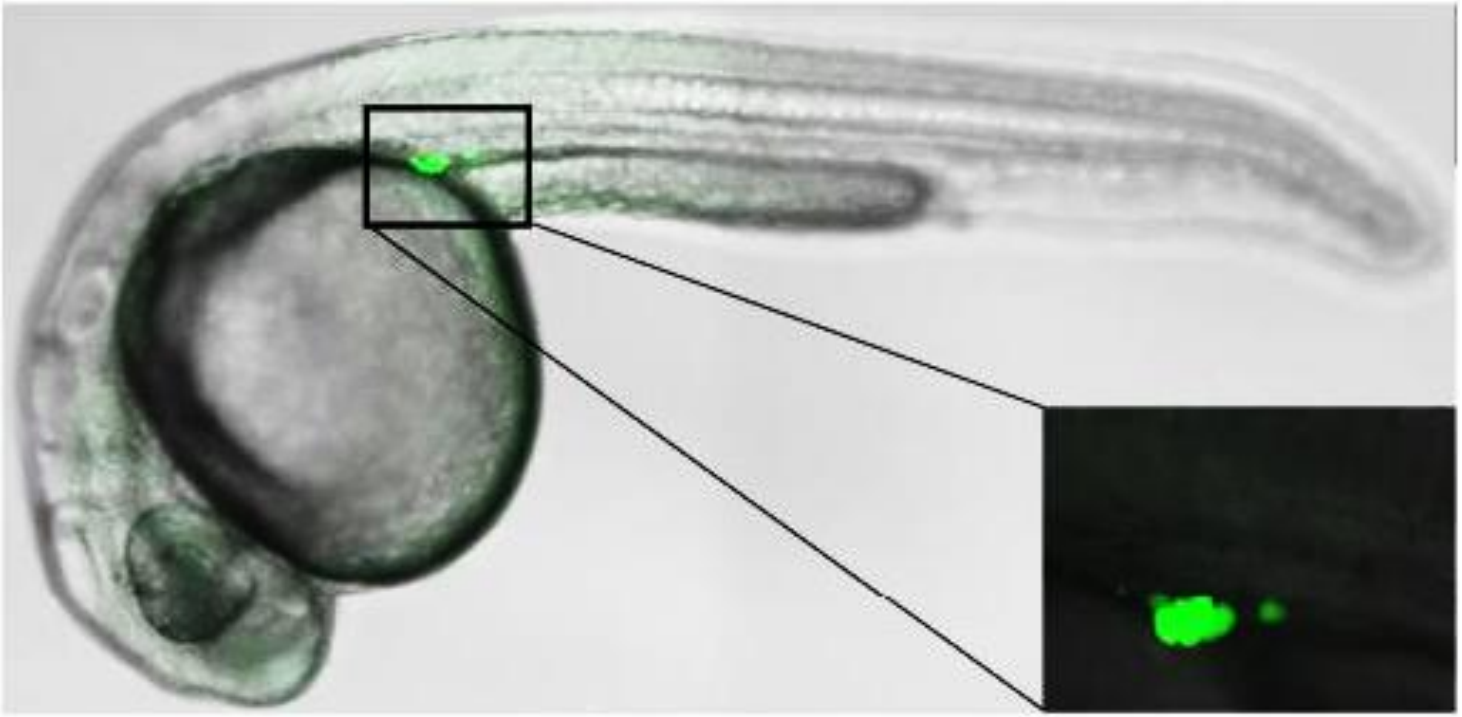
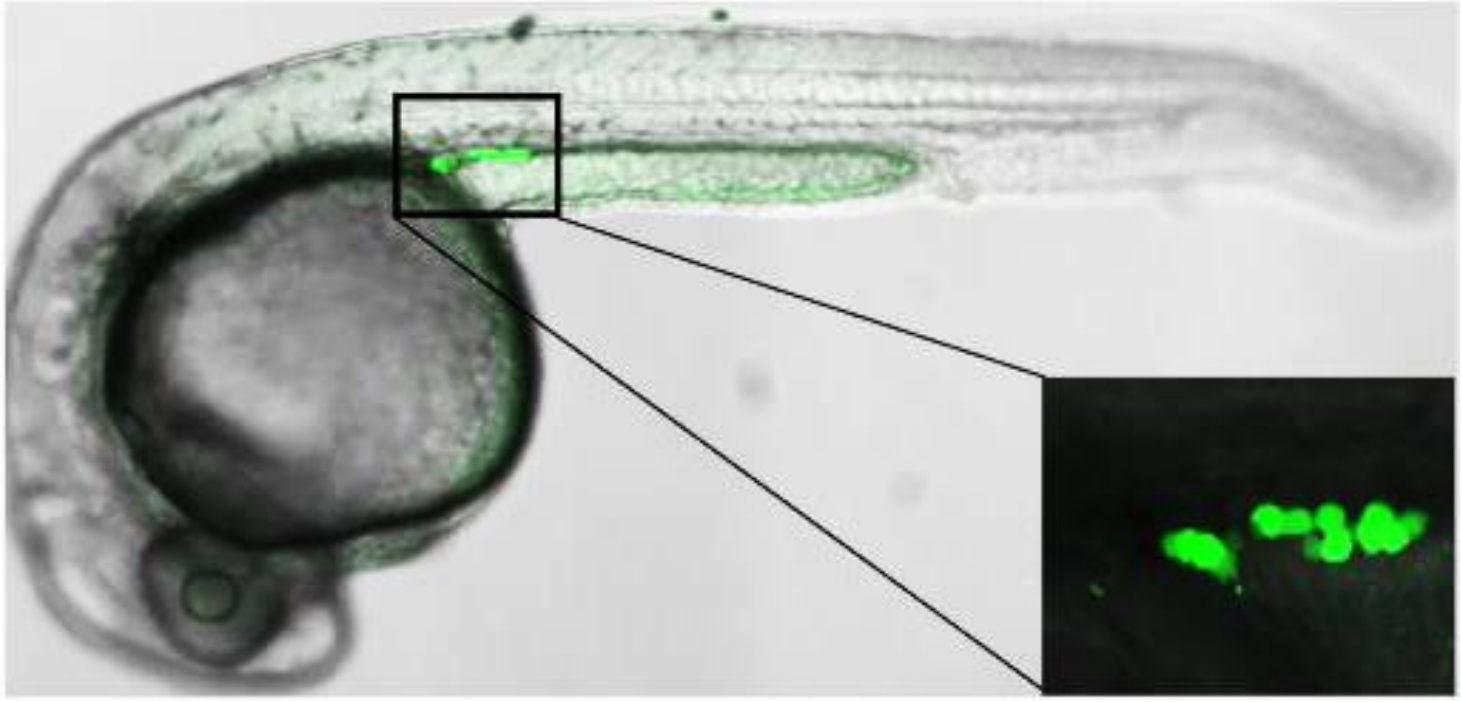
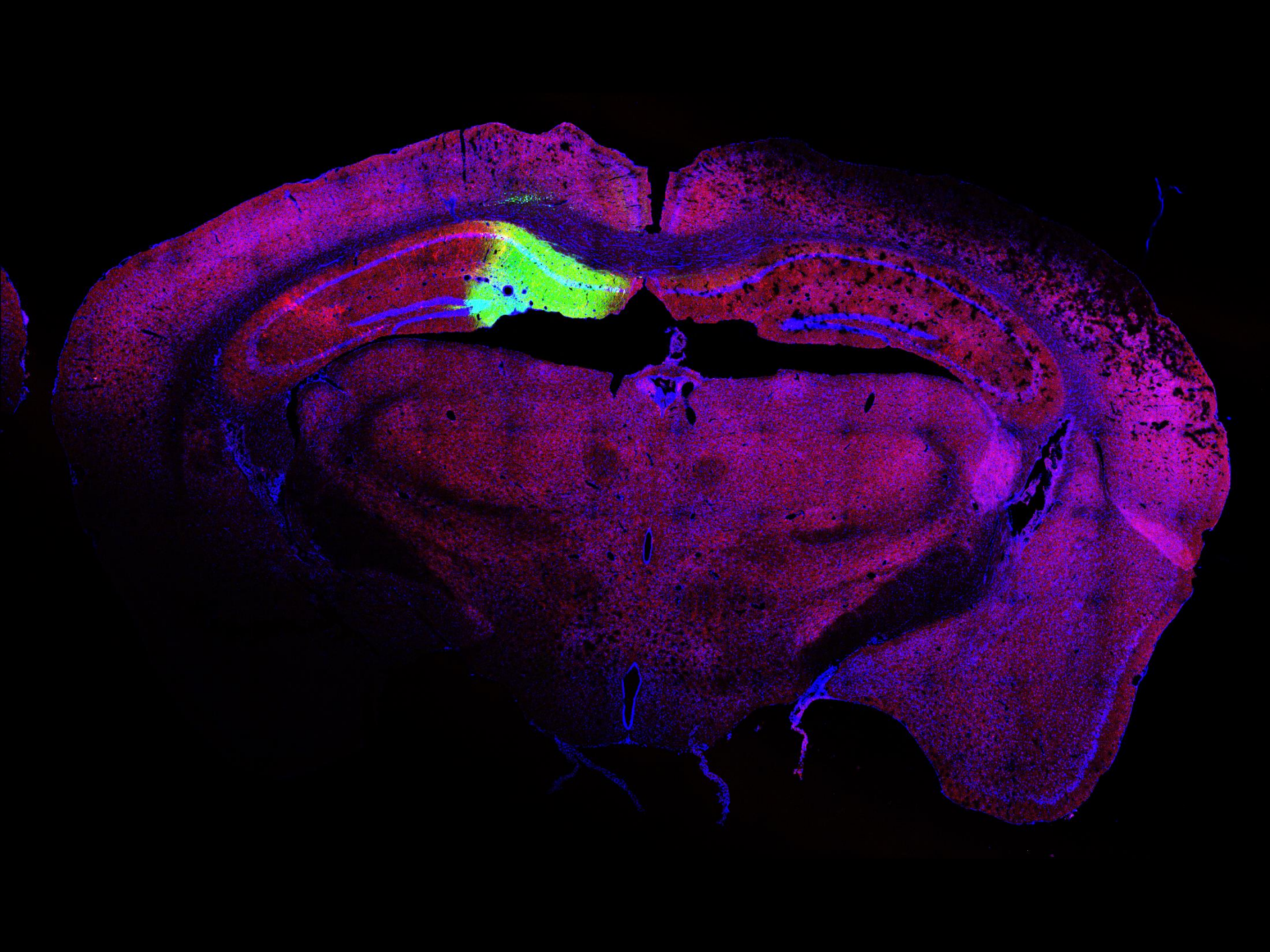
- 多点标记扫描
- 光谱扫描
- 光操作









共聚焦的功能及优势

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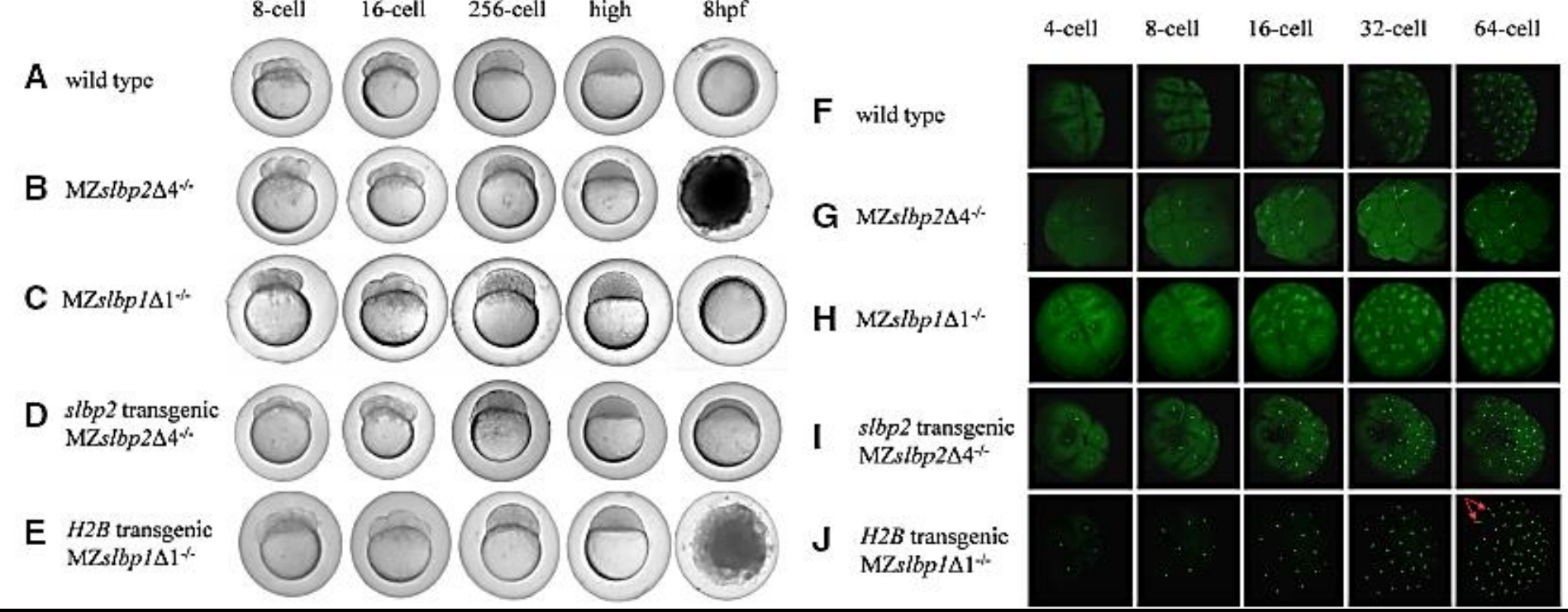
- 光操作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16-cell
256-cell
high
8hpf
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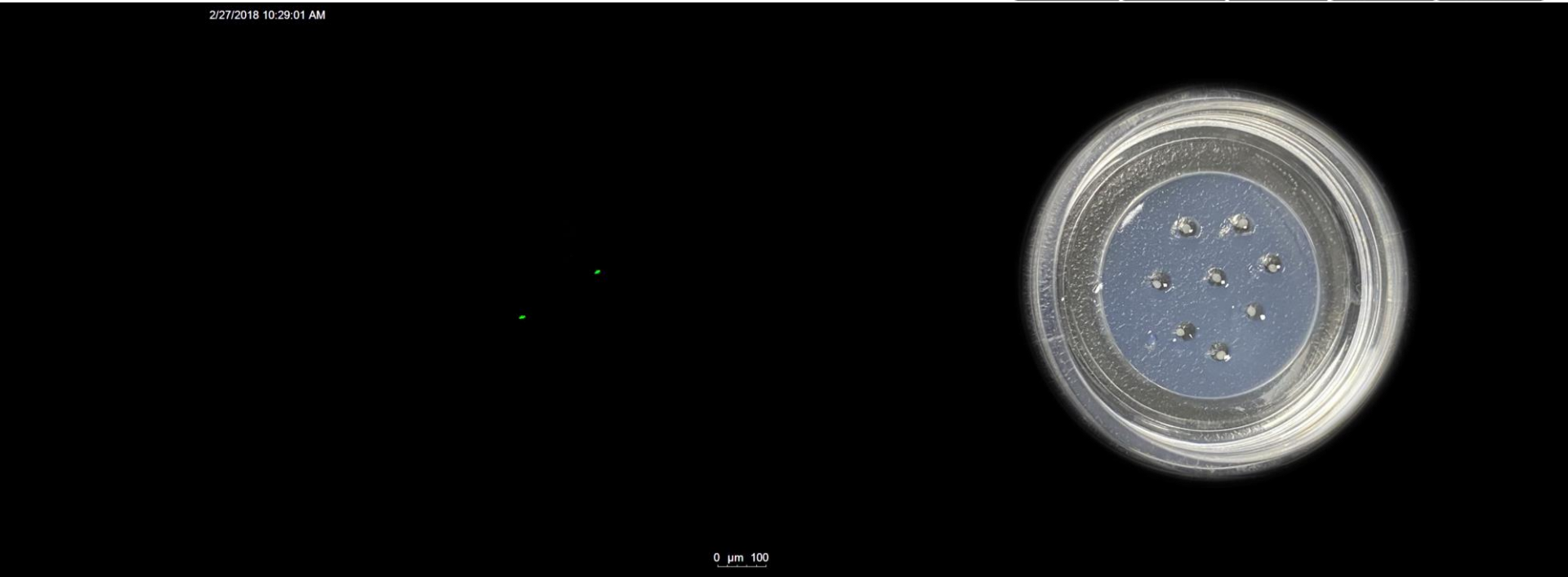
B MZslbp2Δ4-/-
C MZslbp1Δ1-/-
D slbp2 transgenic MZslbp2Δ4-/-
E H2B transgenic MZslbp1Δ1-/-
4-cell
8-cell
16-cell
32-cell
64-cell
F wild type
G MZslbp2Δ4-/-
H MZslbp1Δ1-/-
I slbp2 transgenic MZslbp2Δ4-/-
J H2B transgenic MZslbp1Δ1-/-


2/27/2018 10:29:01 AM
0 µm 100

共聚焦的功能及优势

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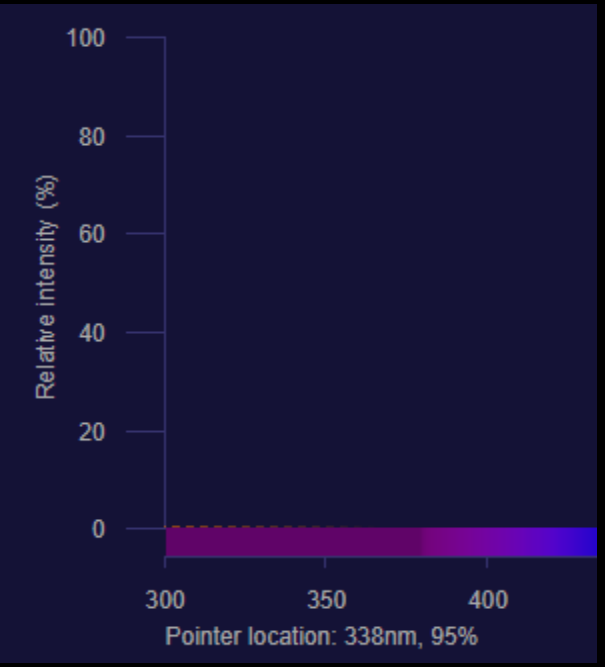
- 光谱扫描：自发荧光、串色荧光的准确分离
- 光操作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80
60
40
20
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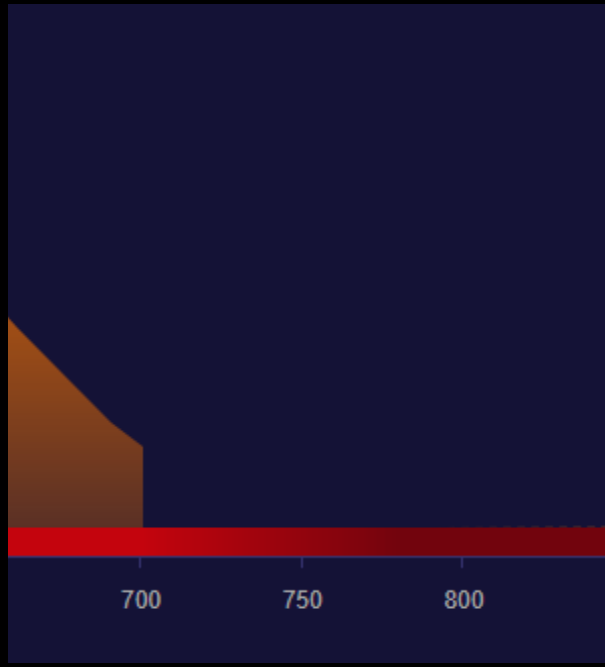
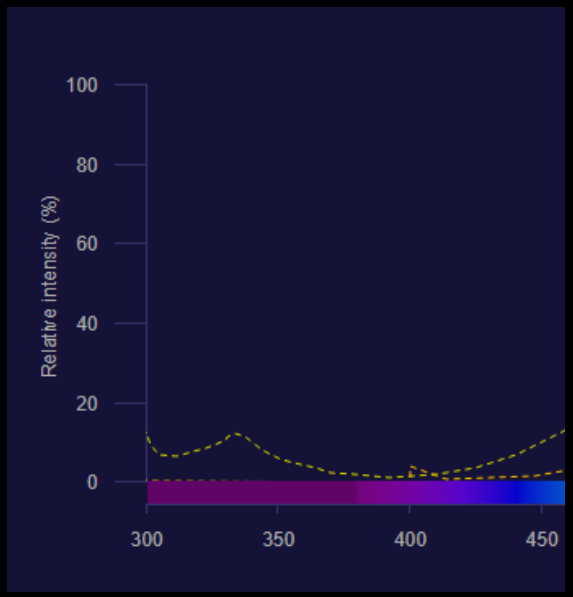
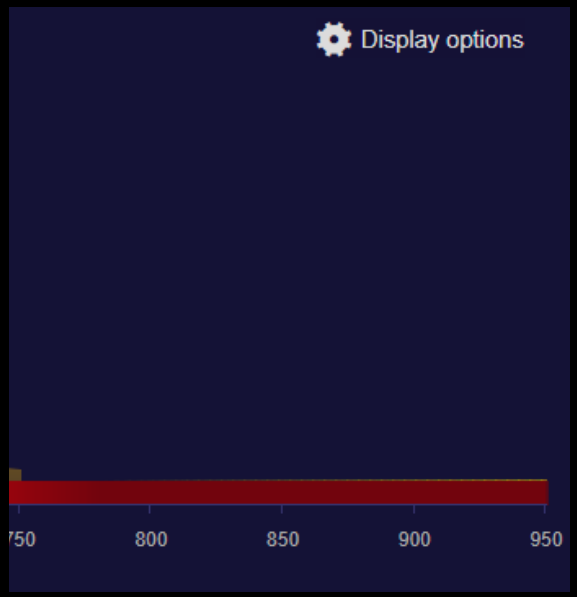
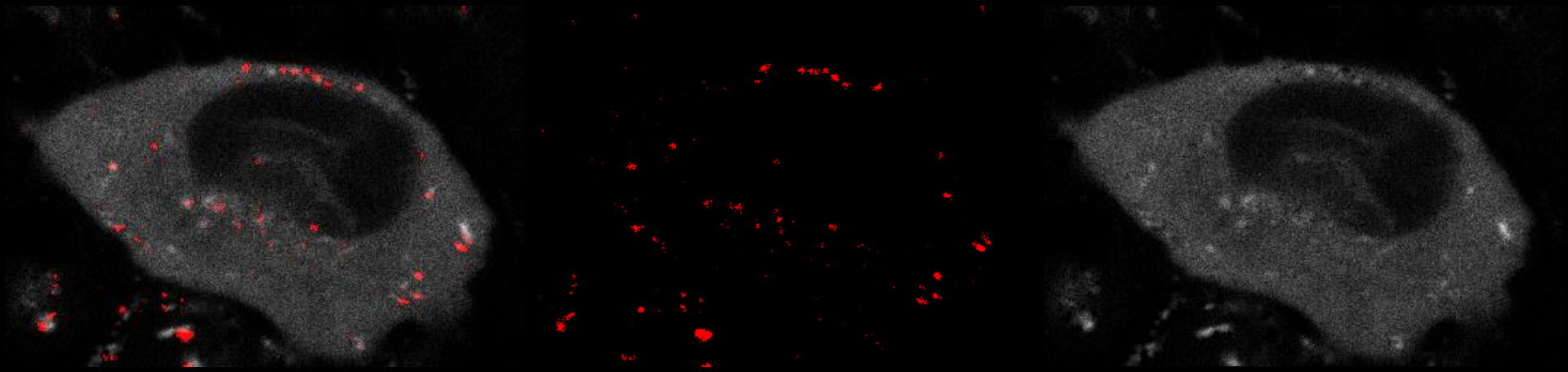
Relative intensity (%)
300
350
400
Pointer location: 338nm, 95%


700
750
800


100
80
60
40
20
0
Relative intensity (%)
300
350
400
450


Display options
750
800
850
900
950

λ=560nm



共聚焦的功能及优势

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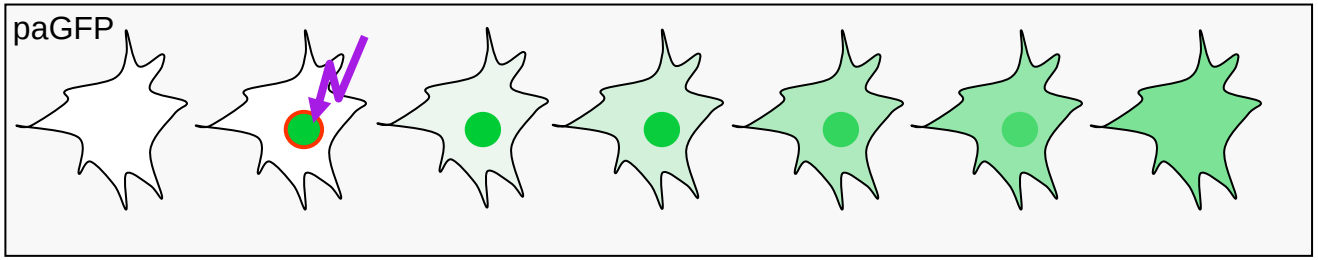
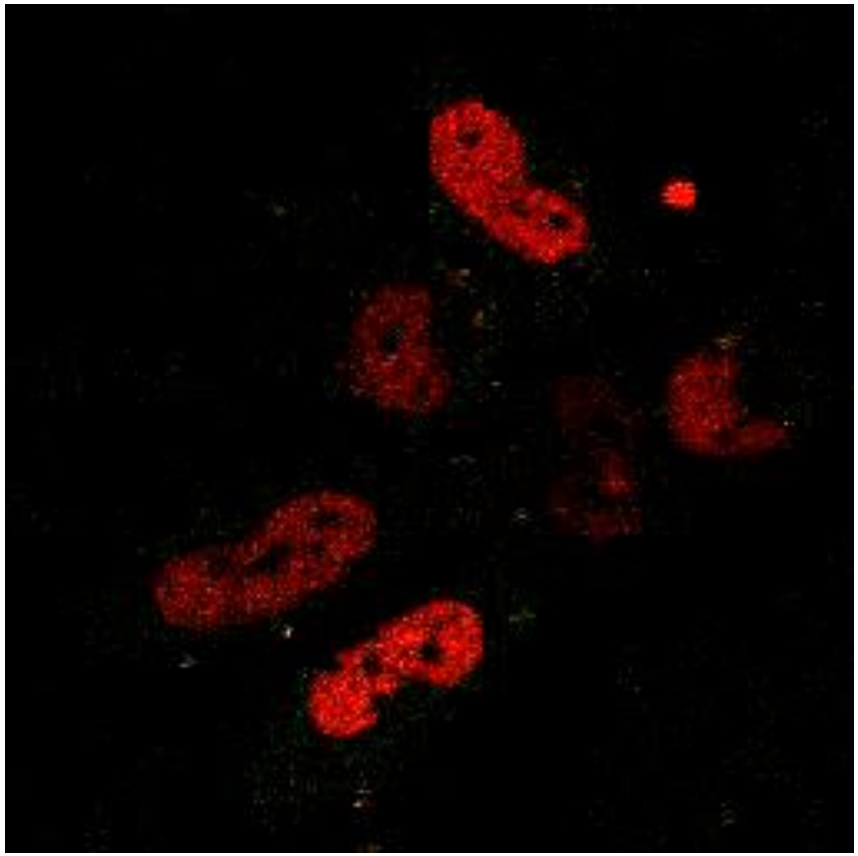
- 光操作：光激活、光转换、光损伤、光漂白等实验

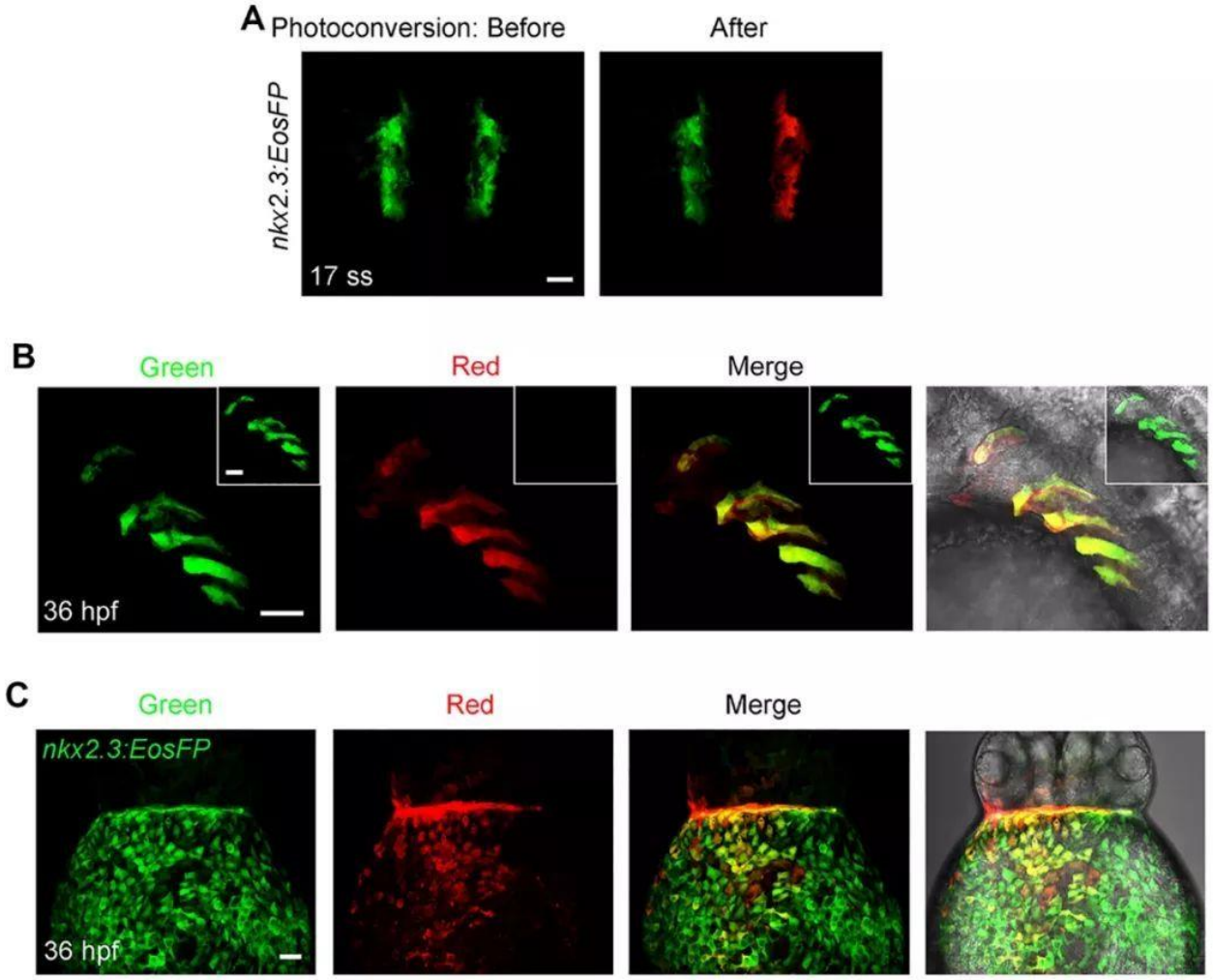
光激活





PA-GFP and HcRed cotransfected NRK cells

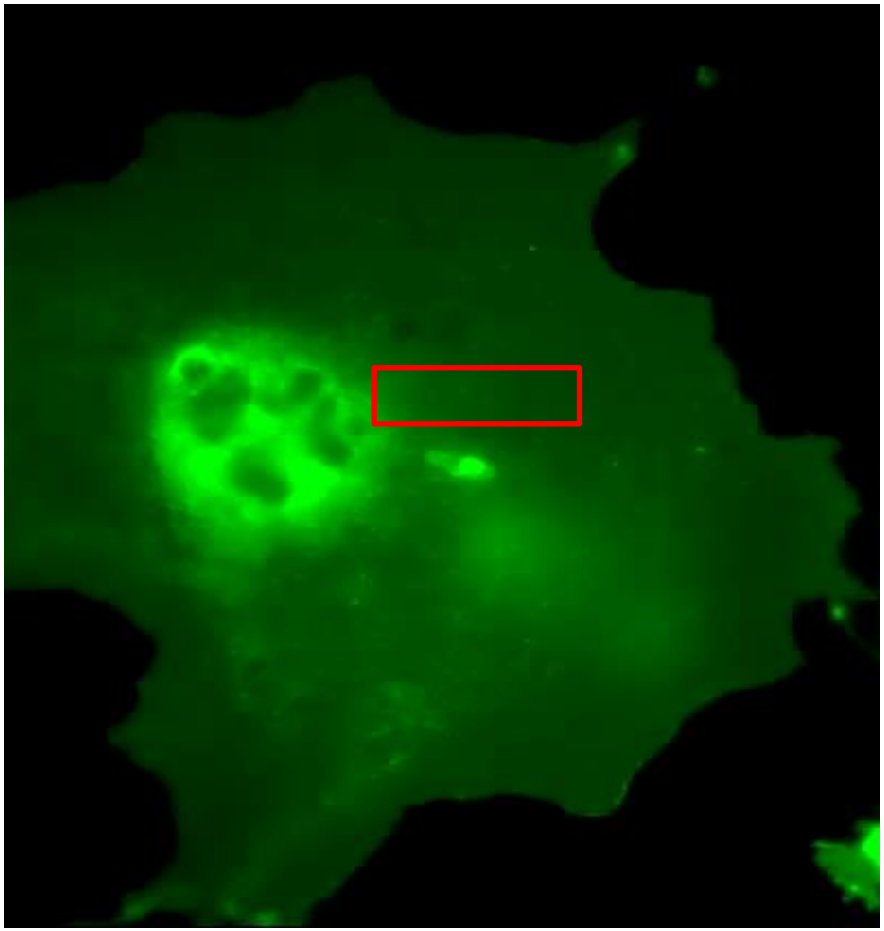
光转换

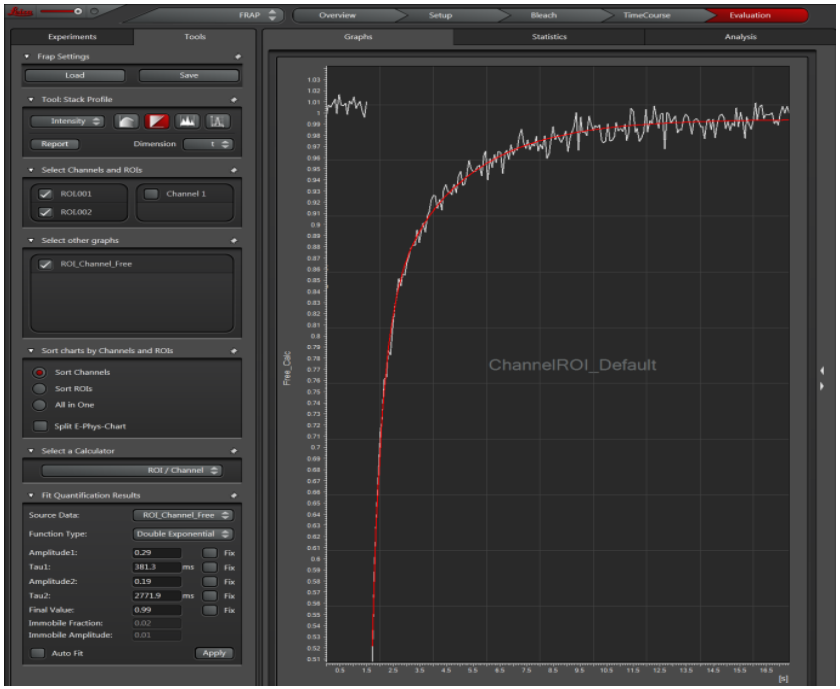


nkx2.3-EosFP

BMP signaling is required for nkx2.3-positive pharyngeal pouch progenitor specification in zebrafish

光漂白



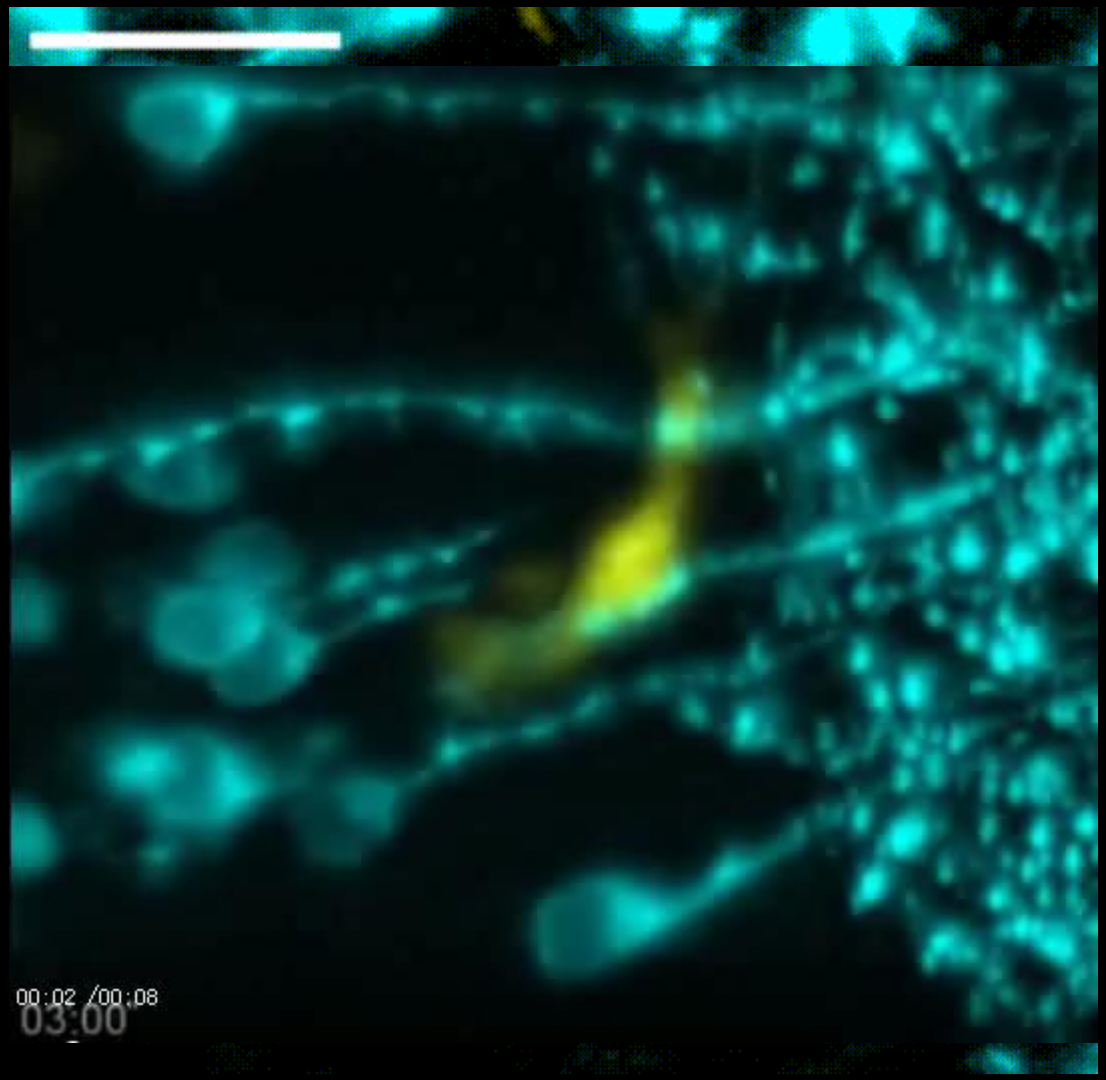


- 测量质膜或胞质不同组分的侧向扩散；
- 研究不同条件下(如细胞缺氧性损伤)膜受体和脂质的迁移；
- 研究细胞骨架动力学；

COS cells expressing p75-GFP
Courtesy Prof. Dr. Ralf Jacob, University Marburg

光损伤

1025nm
脉冲激光



2020，EMBL，Matteo Rauzi and Lars Hufnagel，Scientific Reports

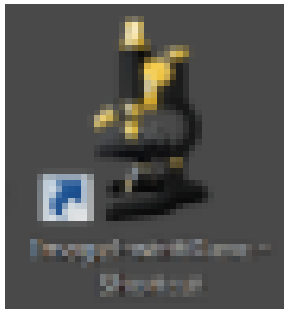
图像处理及数据分析

图像处理:

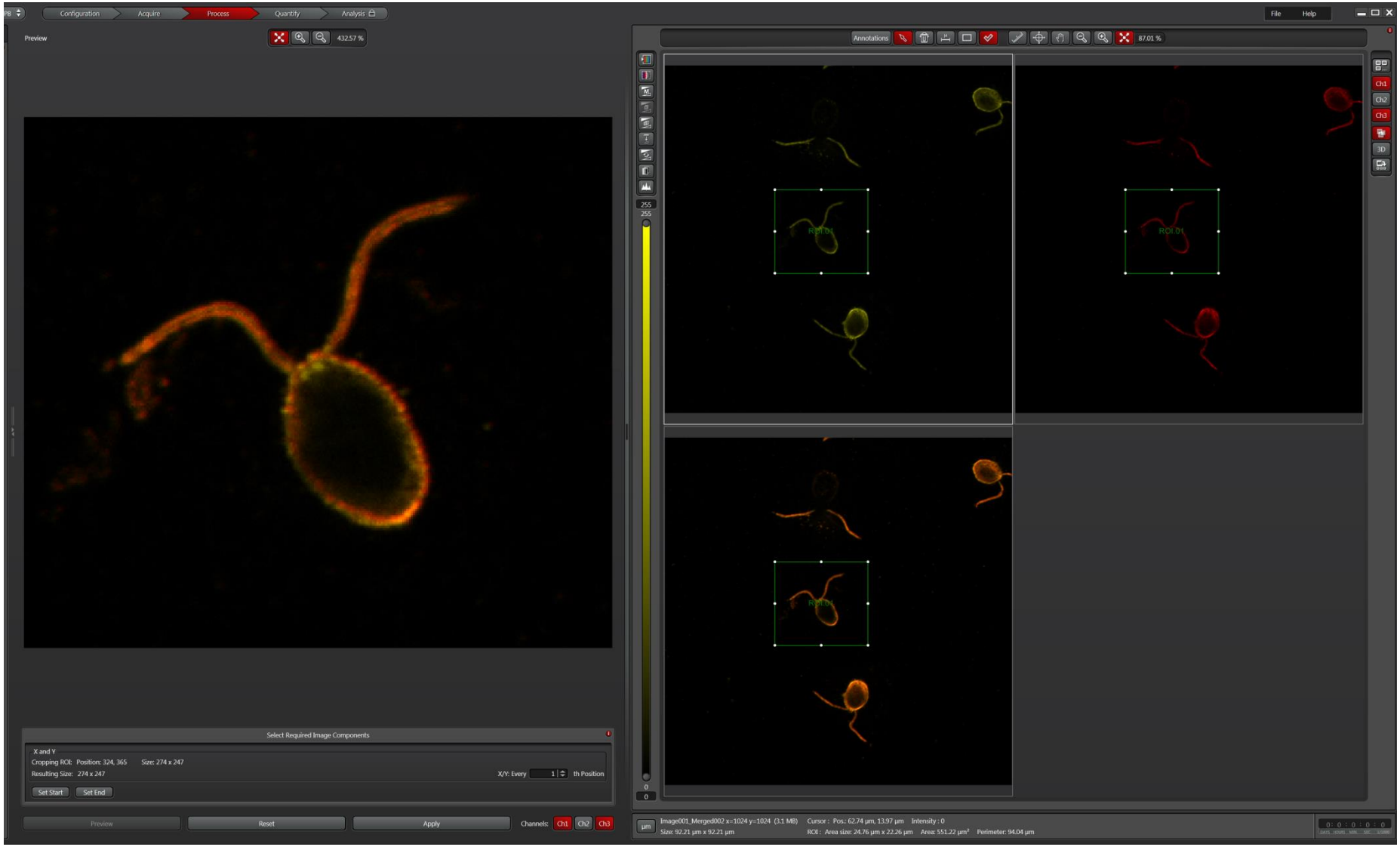
- 编辑：Crop、Merge、Project
- 调整：去背景、对比度
- 去噪：滤镜、椒盐噪声
- 去卷积：Huygens、Lightning
- 3D重构

图像分析:

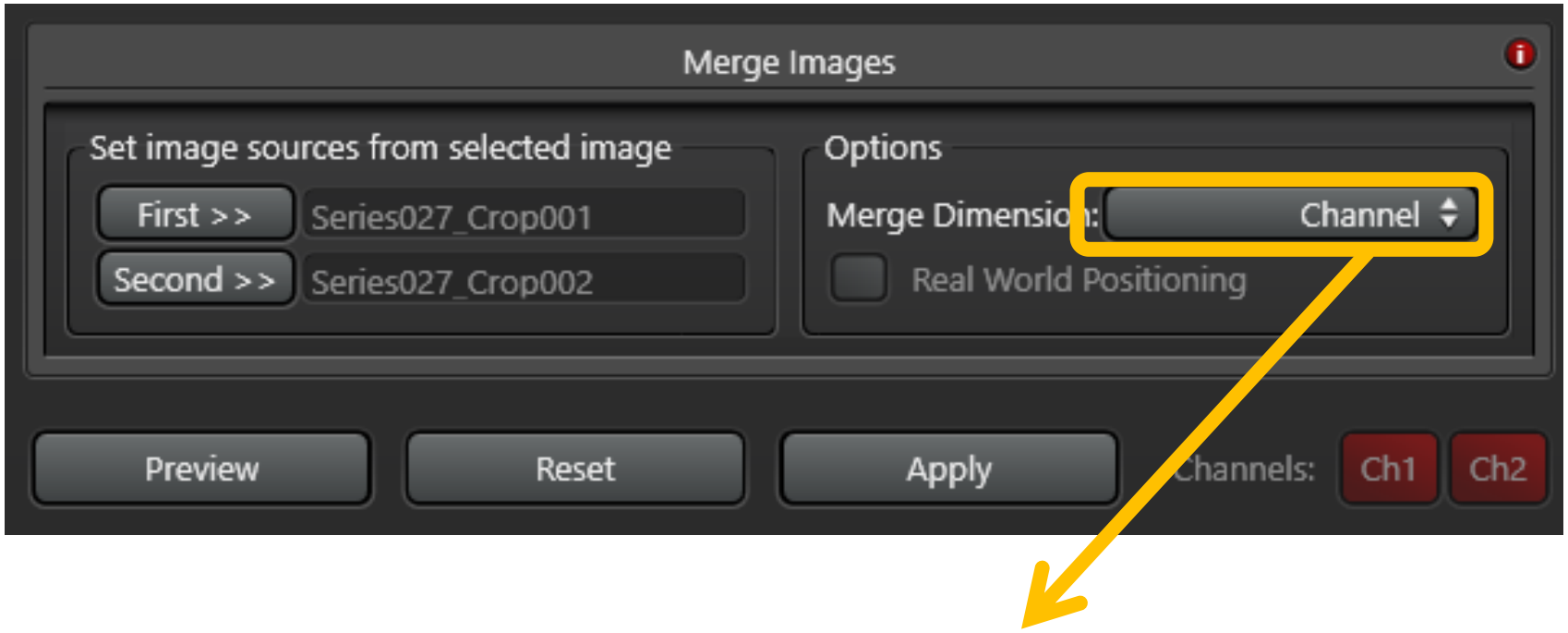
- 细胞计数
- 荧光强度
- 共定位分析



- 图像处理-Crop


Configuration
Acquire
Process
Quantify
Analysis
File
Help
Preview
432.57 %
Annotations
87.01 %
Ch1
Ch2
Ch3
3D
255
255
0
0
ROI:01
Select Required Image Components
X and Y
Cropping ROI: Position: 324, 365 Size: 274 x 247
Resulting Size: 274 x 247
X/Y: Every 1 th Position
Set Start
Set End
Preview
Reset
Apply
Channels: Ch1 Ch2 Ch3
µm
Image001_Merged002 x=1024 y=1024 (3.1 MB)
Size: 92.21 µm x 92.21 µm
Cursor : Pos.: 62.74 µm, 13.97 µm Intensity : 0
ROI : Area size: 24.76 µm x 22.26 µm Area: 551.22 µm² Perimeter: 94.04 µm

• 图像处理-Merge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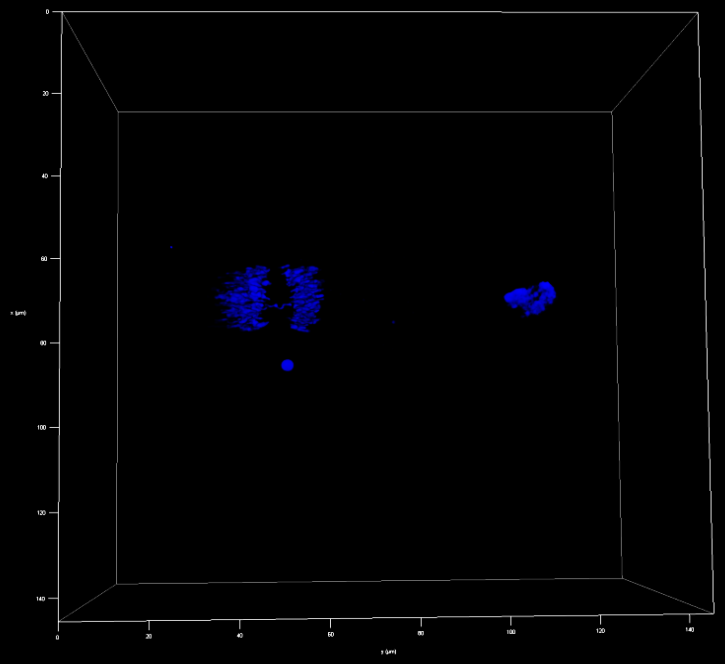
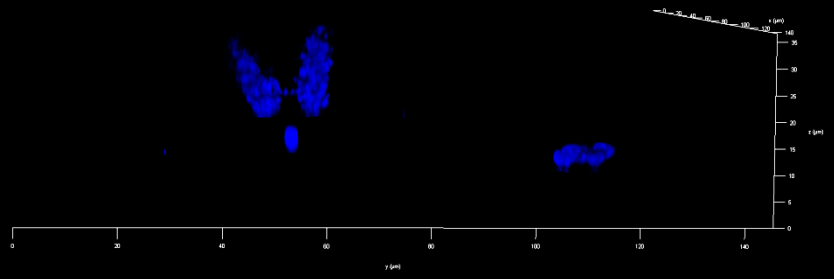
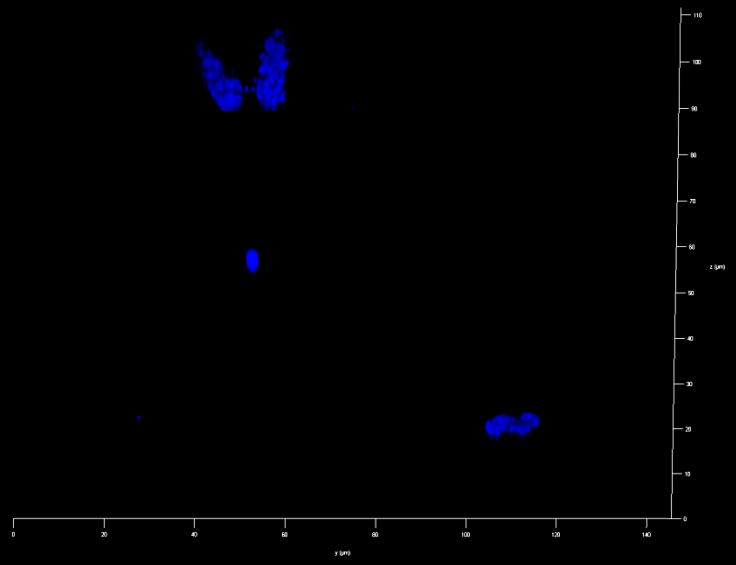
Set image sources from selected image
First >>
Series027_Crop001
Second >>
Series027_Crop002
Options
Merge Dimension:
Channel
Real World Positioning
Preview
Reset
Apply
Channels:
Ch1
Ch2

Options
Merge Dimension:
Channel
Channel
t
x
y
z
Apply

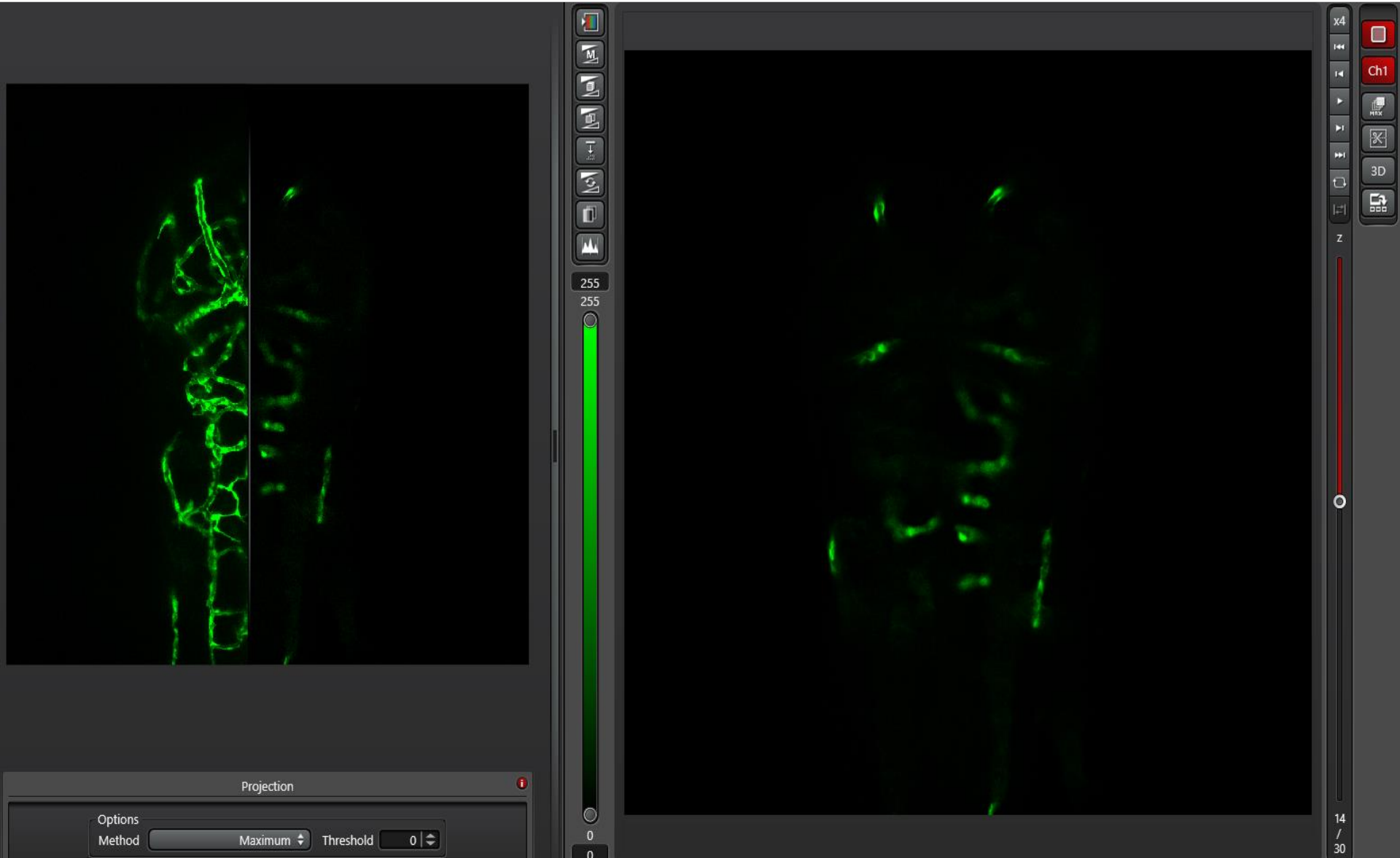
Real World Positioning



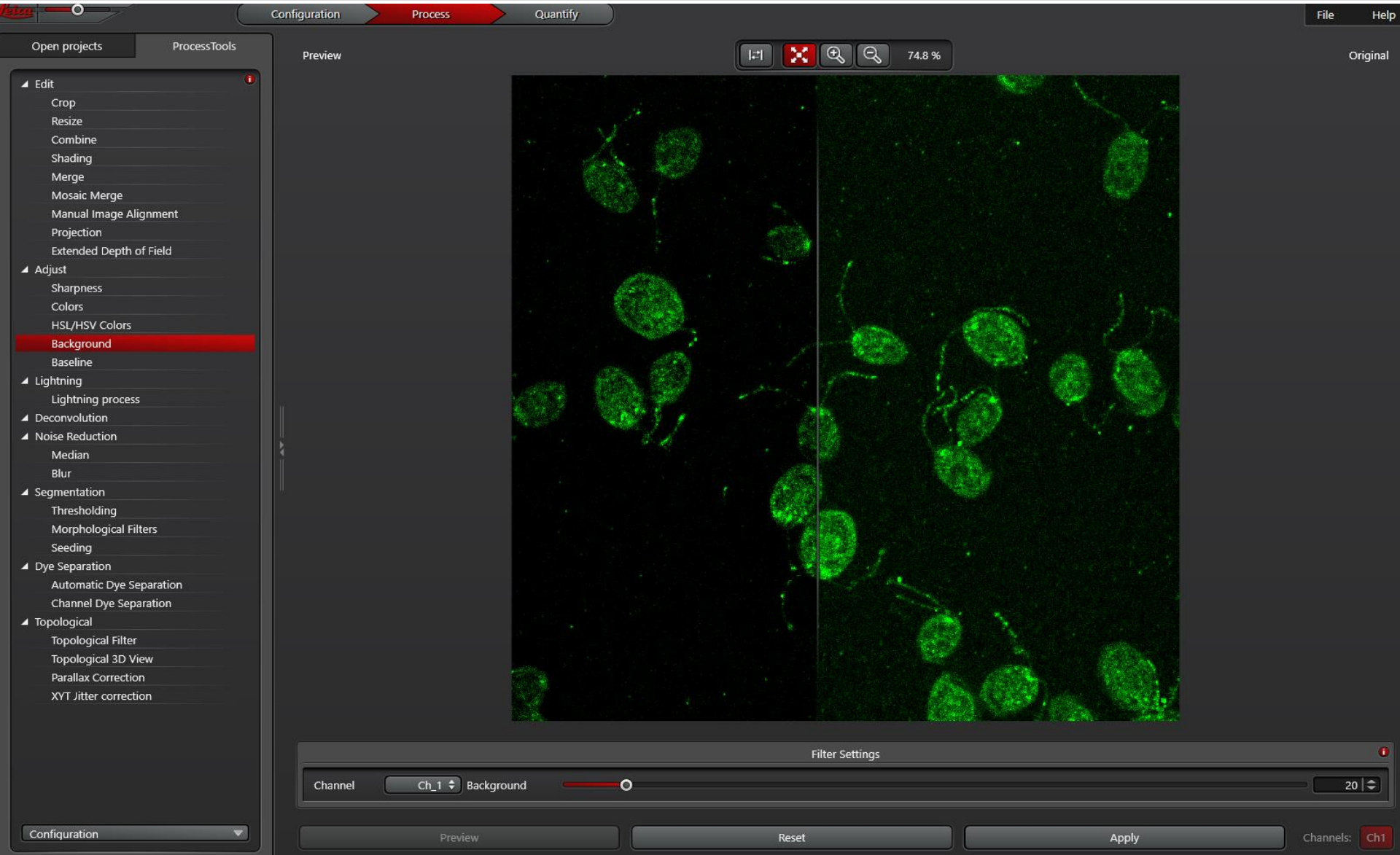




- 图像处理-Projection

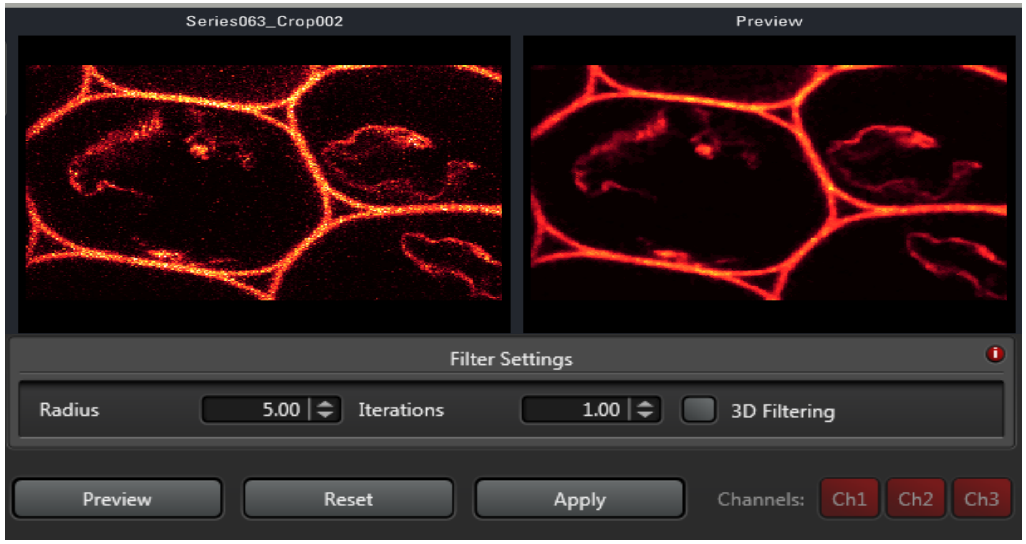


• 图像处理-对比度、去背景

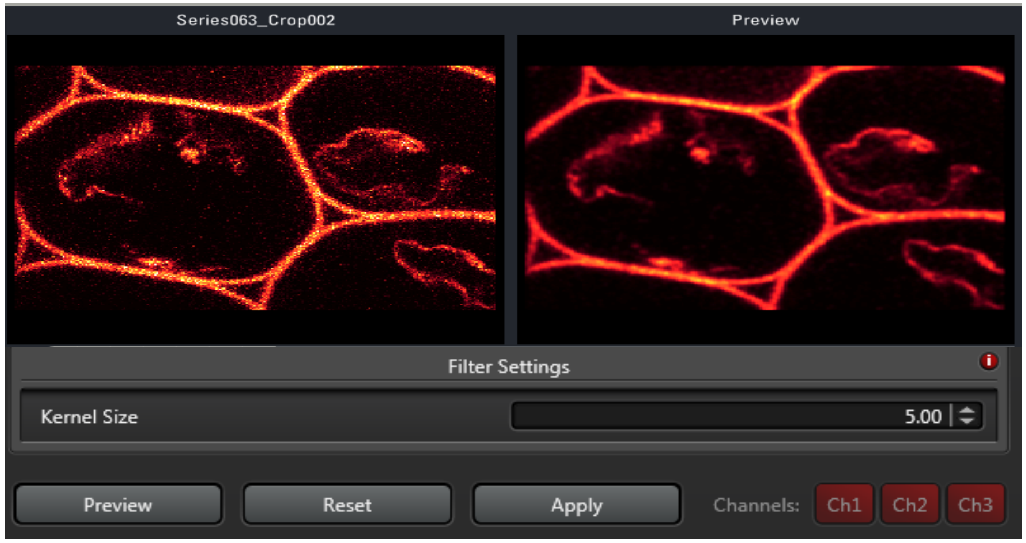

Configuration
Process
Quantify
File
Help
Open projects
ProcessTools
Preview
74.8 %
Original
Edit
Crop
Resize
Combine
Shading
Merge
Mosaic Merge
Manual Image Alignment
Projection
Extended Depth of Field
Adjust
Sharpness
Colors
HSL/HSV Colors
Background
Baseline
Lightning
Lightning process
Deconvolution
Noise Reduction
Median
Blur
Segmentation
Thresholding
Morphological Filters
Seeding
Dye Separation
Automatic Dye Separation
Channel Dye Separation
Topological
Topological Filter
Topological 3D View
Parallax Correction
XYT Jitter correction
Configuration
Filter Settings
Channel
Ch_1
Background
20
Preview
Reset
Apply
Channels:
Ch1

• 图像处理–常用滤镜

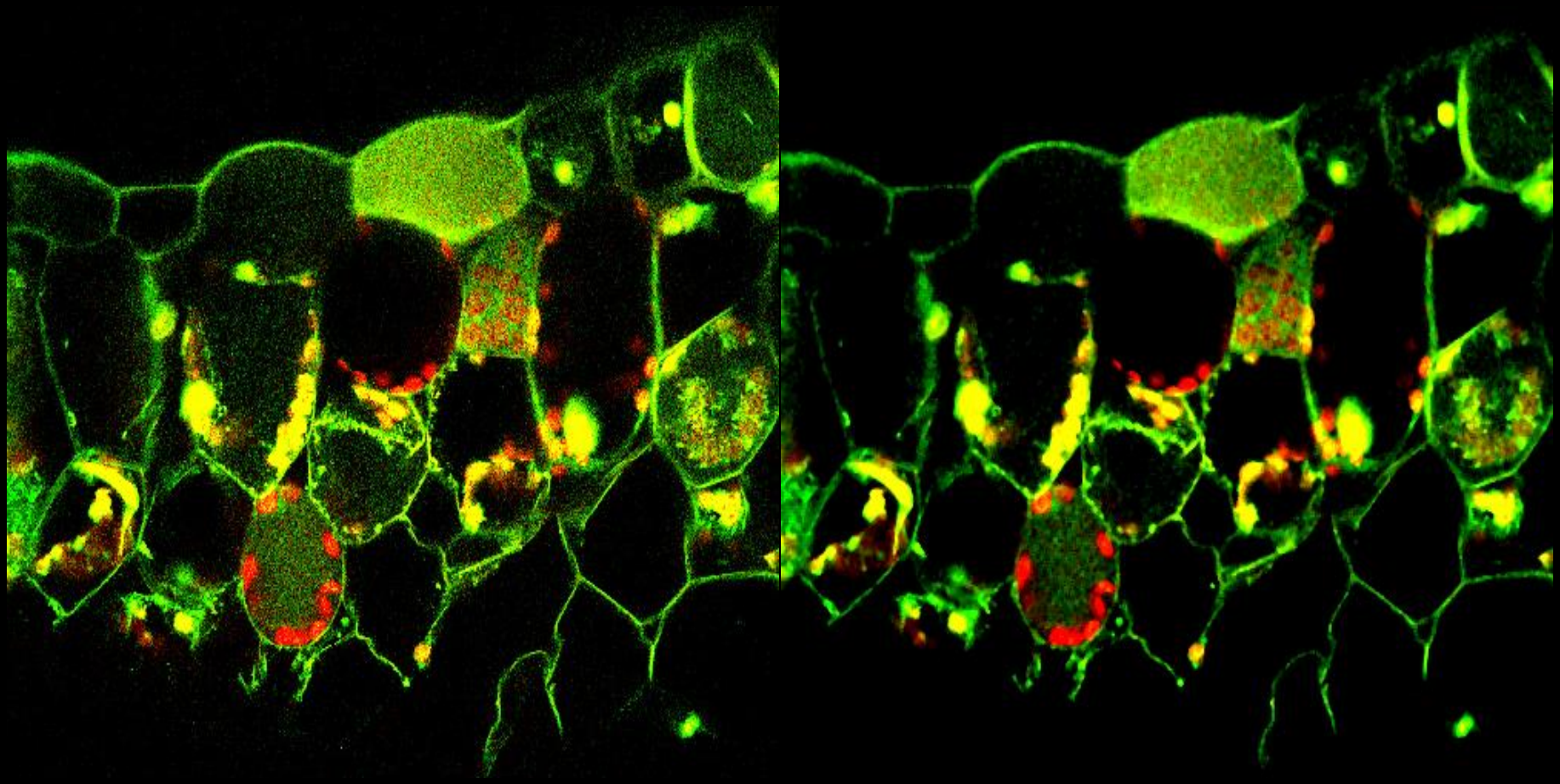
Median滤镜



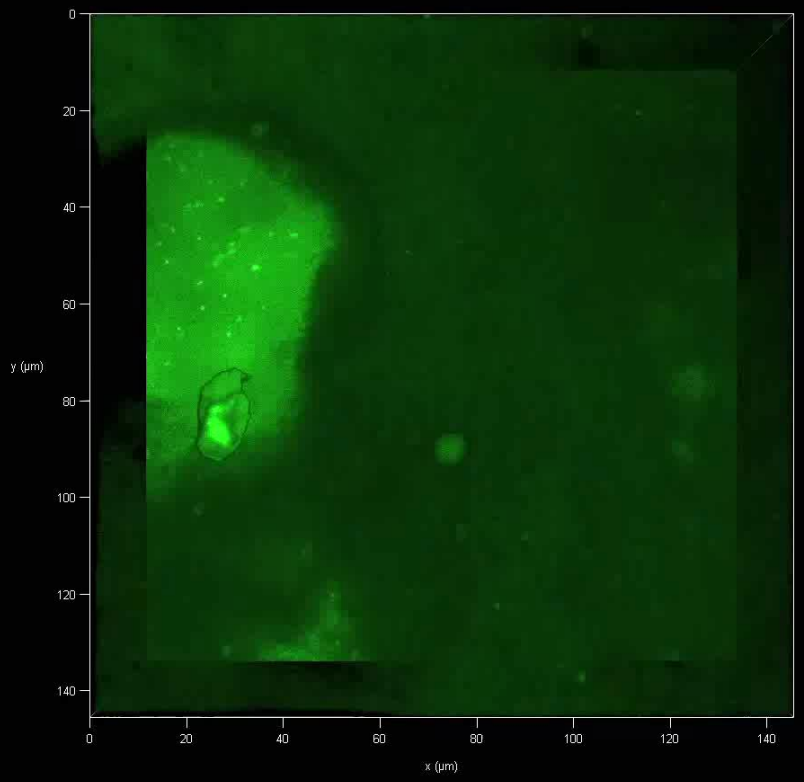
Gaussian Blur滤镜

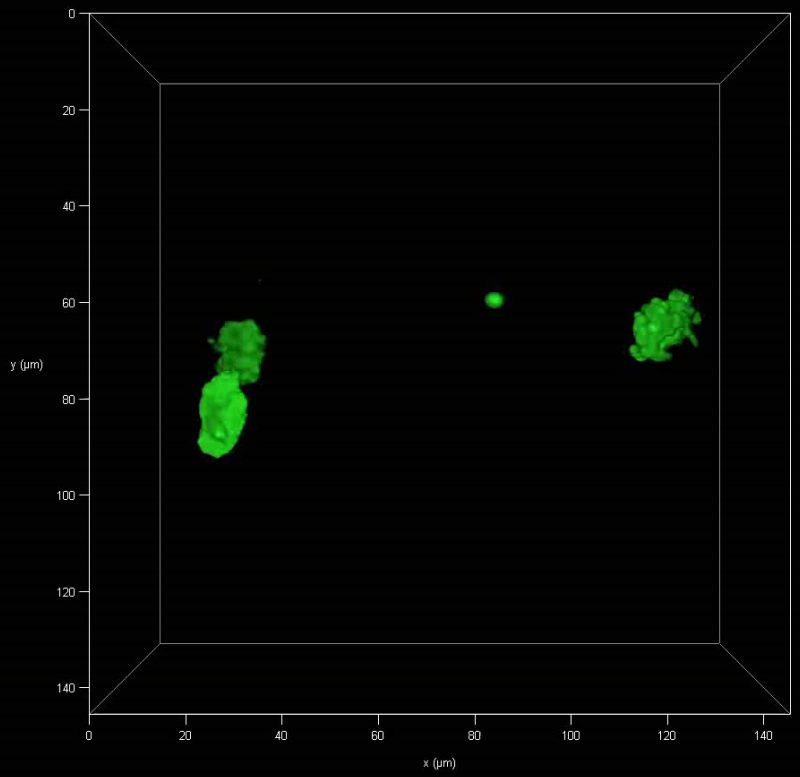


- 图像处理–Despeckle去椒盐噪声



• 图像处理-Huygens





- 图像处理-3D重构

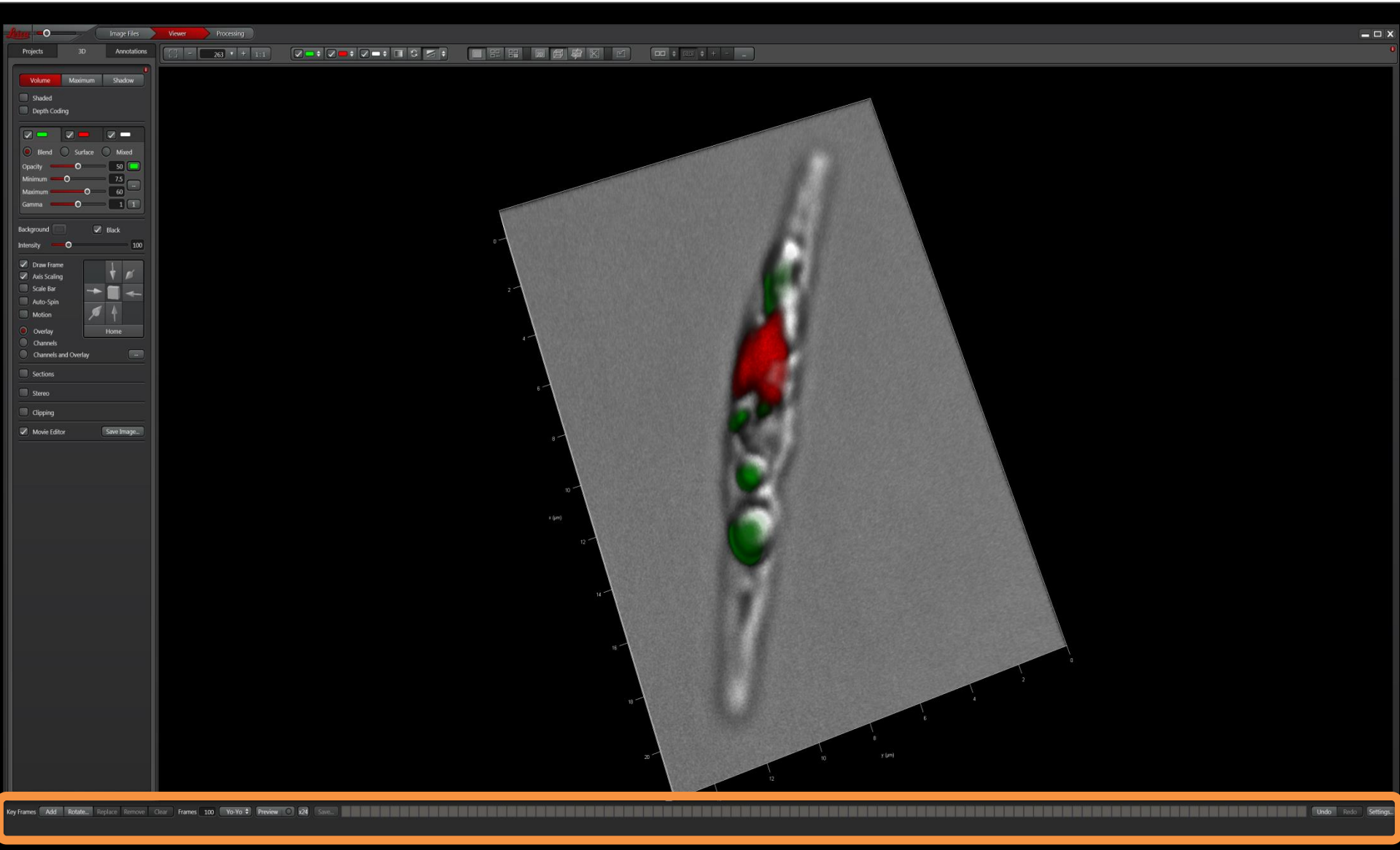


Image Files
Viewer
Processing
Projects
3D
Annotations
Volume
Maximum
Shadow
Shaded
Depth Coding
Blend
Surface
Mixed
Opacity
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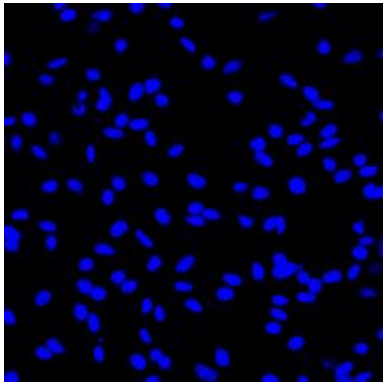
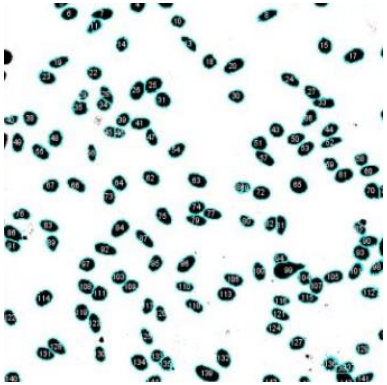
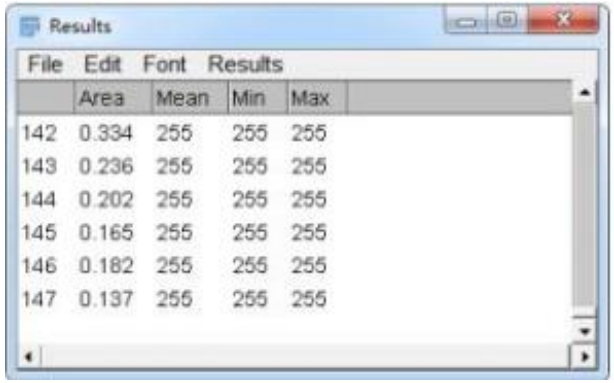
Gamma
Background
Black
Intensity
Draw Frame
Axis Scaling
Scale Bar
Auto-Spin
Motion
Home
Overlay
Channels
Channels and Overlay
Sections
Stereo
Clipping
Movie Editor
Save Image...
Key Frames
Add
Rotate...
Replace
Remove
Clear
Frames
Yo-Yo
Preview
Save...
Undo
Redo
Settings...

• 图像分析-细胞计数

➢ 细胞形状规则，可以和背景分离





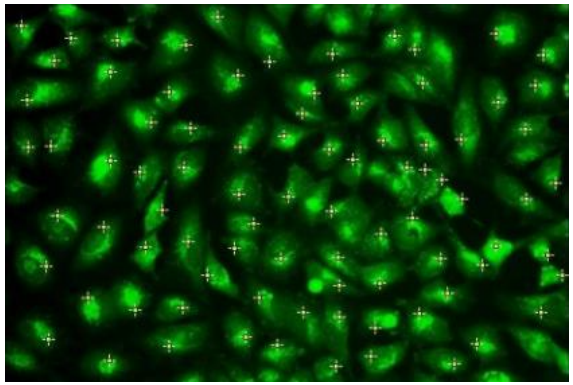


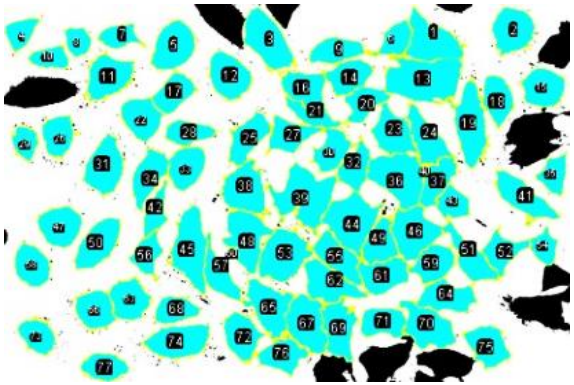
Results

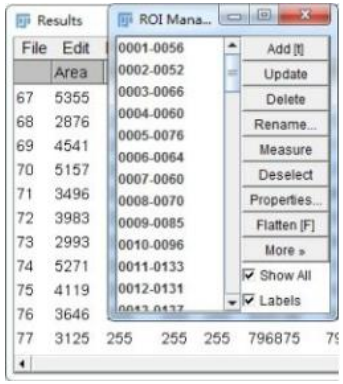
File Edit Font Results

	Area	Mean	Min	Max
142	0.334	255	255	255
143	0.236	255	255	255
144	0.202	255	255	255
145	0.165	255	255	255
146	0.182	255	255	255
147	0.137	255	255	255

➢ 细胞形状不规则，且细胞重叠







Results

File Edit

	Area
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68	2876
69	4541
70	5157
71	3496
72	3983
73	2993
74	5271
75	4119
76	3646
77	3125 255 255 255 796875

ROI Mana...

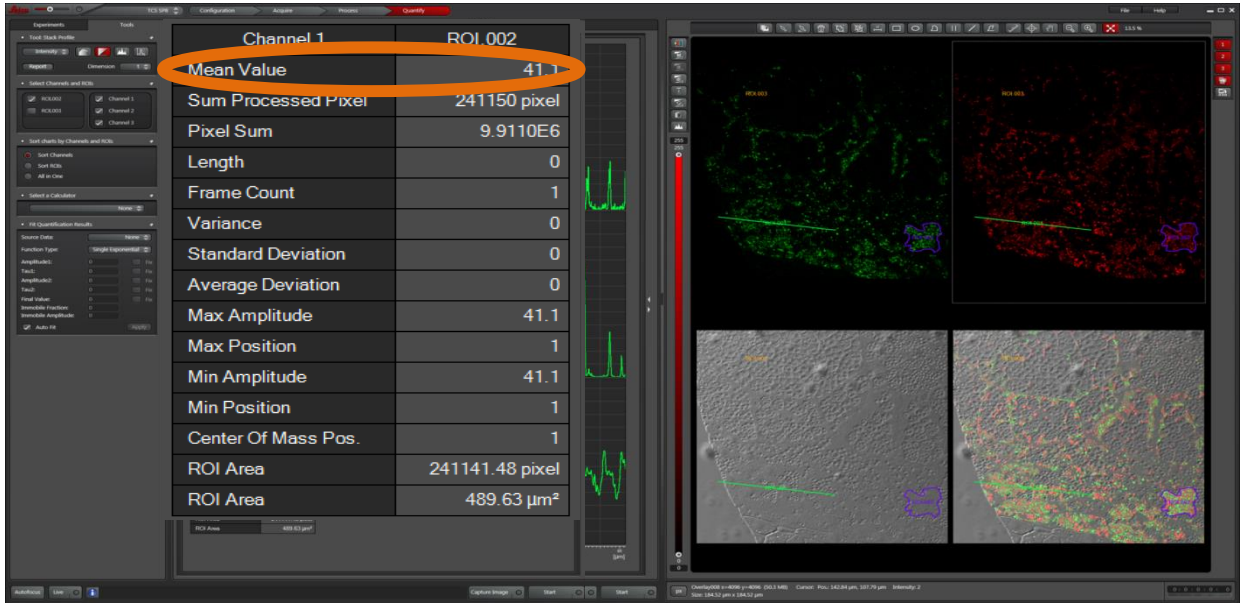
0001-0056
0002-0052
0003-0066
0004-0060
0005-0076
0006-0064
0007-0060
0008-0070
0009-0085
0010-0096
0011-0133
0012-0131

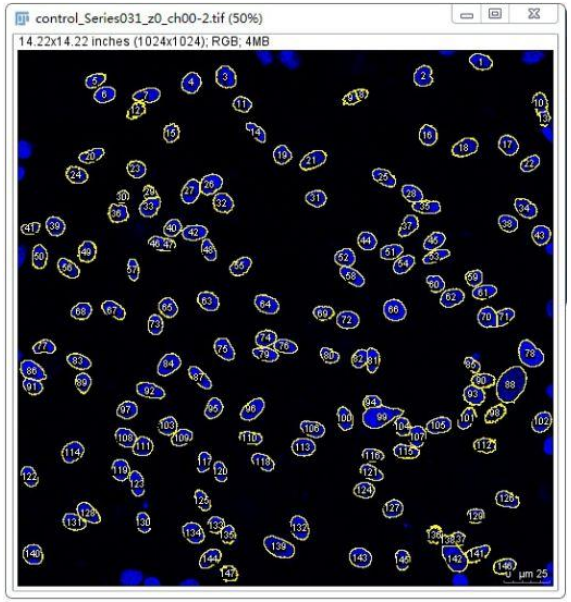
Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
Show All
Labels

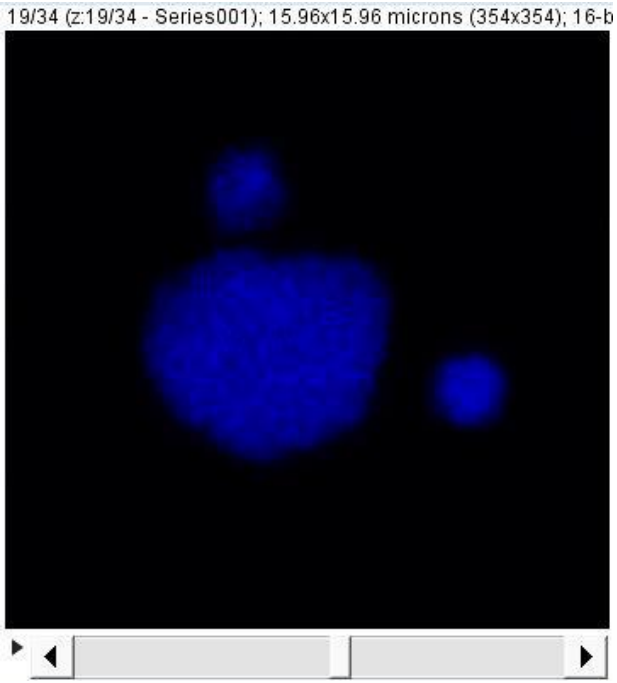
• 图像分析-荧光定量

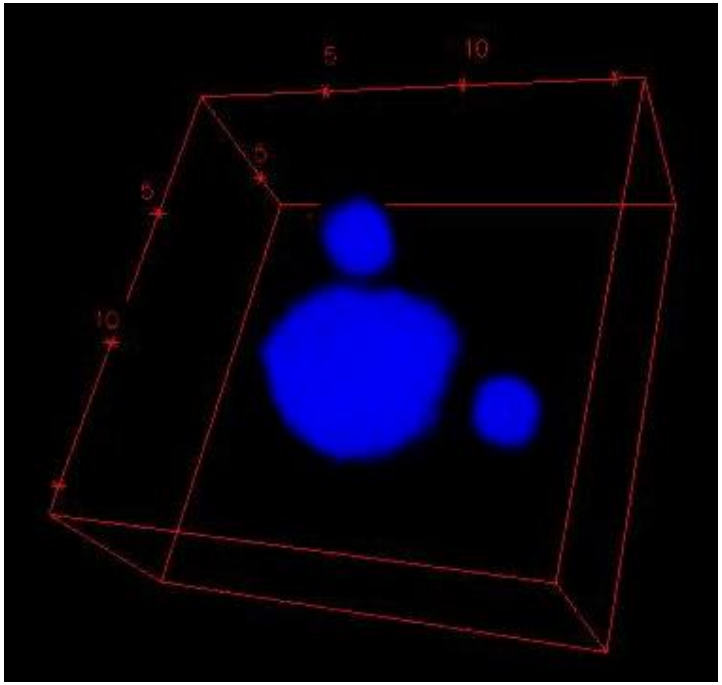
半定量分析

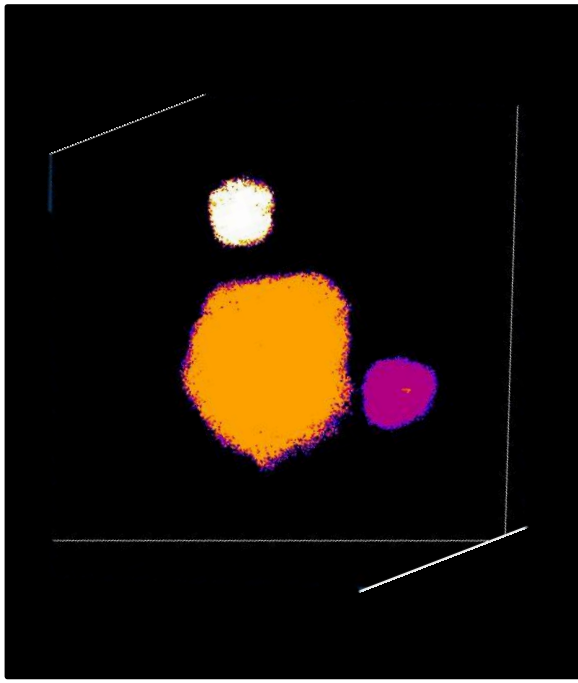
- 平均荧光强度
- ROI荧光强度
- 单细胞荧光强度
- 3D荧光强度





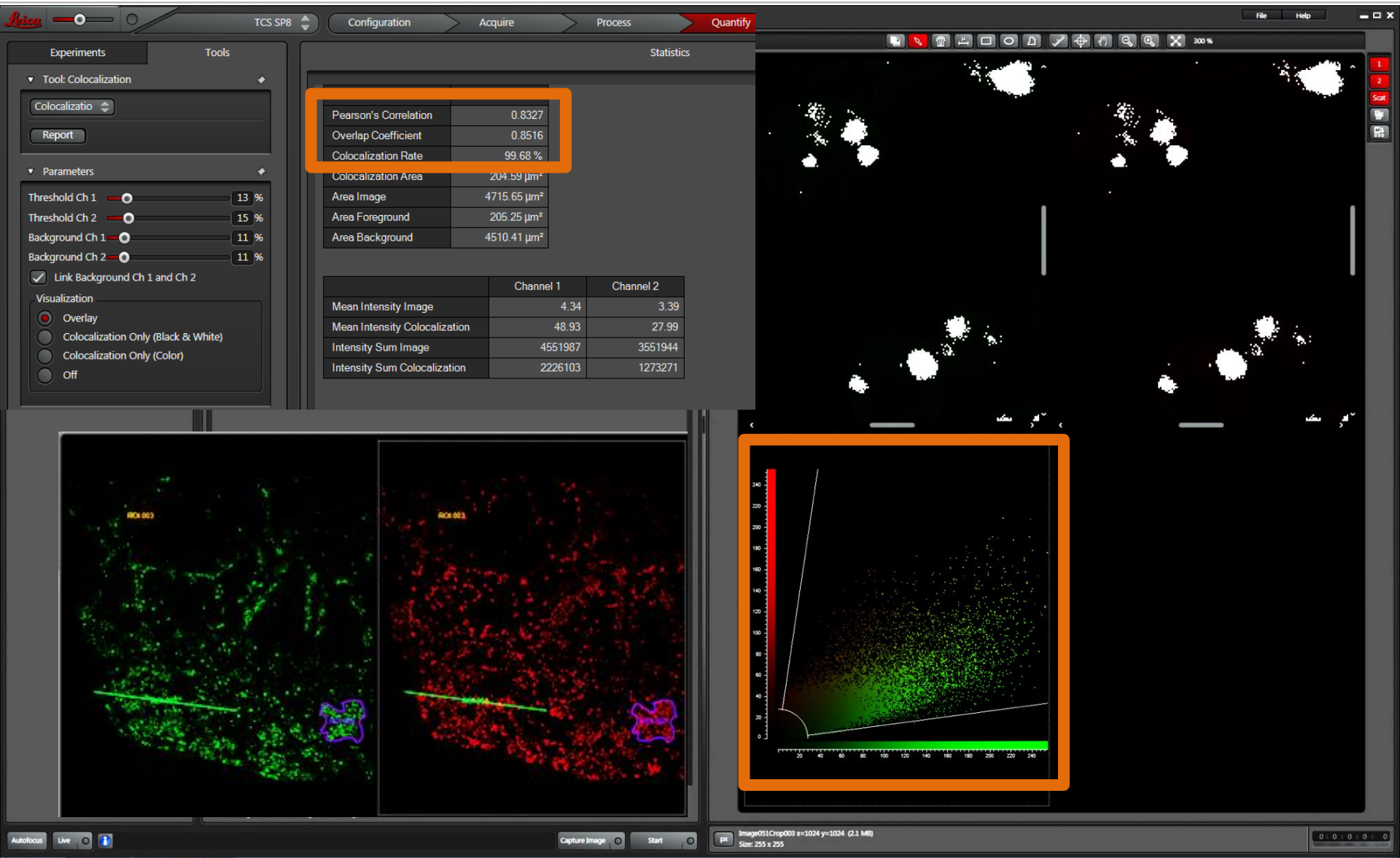






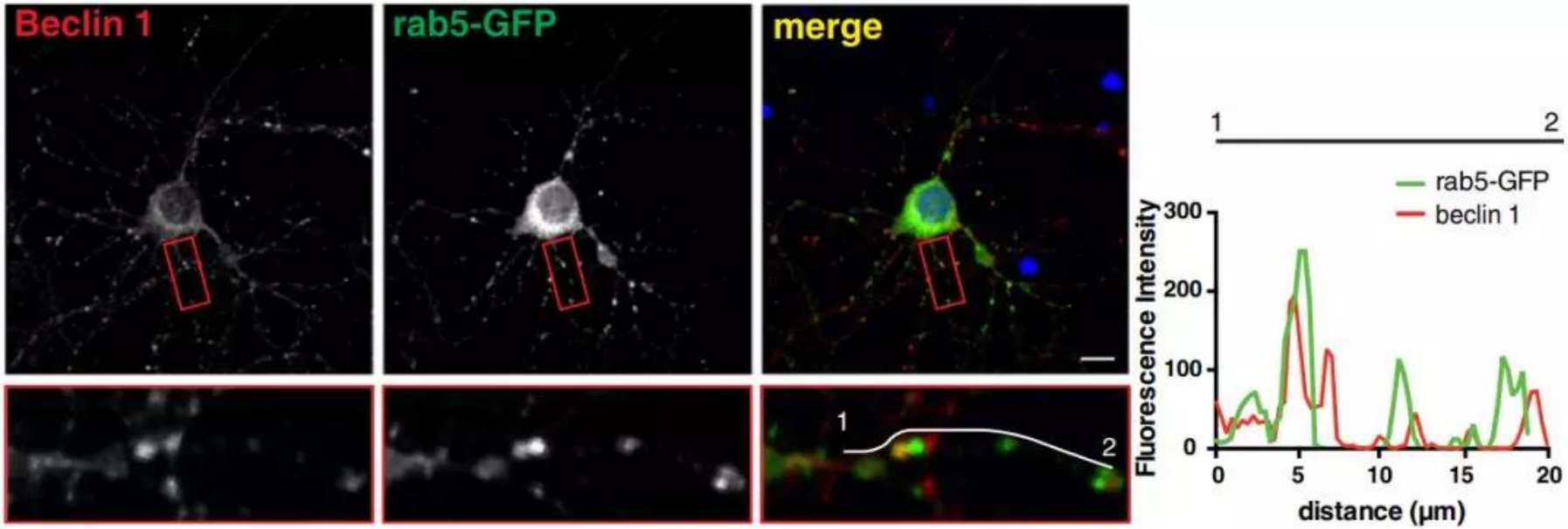
Volume (micron^3)	Surface (micron^2)	Nb of obj. voxels	Nb of surf. voxels	IntDen	Mean
12.940	168.663	30407	10006	20430756	671.910
115.184	1185.341	270671	73422	178369584	658.990
8.643	128.653	20311	7631	13134186	646.654

• 图像分析–共定位分析


TCS SP8
Configuration
Acquire
Process
Quantify
Experiments
Tools
Tool: Colocalization
Colocalizatio
Report
Parameters
Threshold Ch 1 13 %
Threshold Ch 2 15 %
Background Ch 1 11 %
Background Ch 2 11 %
Link Background Ch 1 and Ch 2
Visualization
Overlay
Colocalization Only (Black & White)
Colocalization Only (Color)
Off
Statistics
Pearson's Correlation 0.8327
Overlap Coefficient 0.8516
Colocalization Rate 99.68 %
Colocalization Area 204.59 µm²
Area Image 4715.65 µm²
Area Foreground 205.25 µm²
Area Background 4510.41 µm²
Channel 1
Channel 2
Mean Intensity Image 4.34 3.39
Mean Intensity Colocalization 48.93 27.99
Intensity Sum Image 4551987 3551944
Intensity Sum Colocalization 2226103 1273271
File
Help
300 %
Autofocus
Live
Capture Image
Start

- Colocalization Rate
- Scatter Plot散点图
- Plot profile图

Pearson's Correlation	描述两荧光通道的灰度值分布相关的系数，数值范围为－1～＋1，－1表明完全负相关，如红色通道荧光值下降，则绿色通道上升，即呈负相关；0表明没有显著的共定位；1表明完全正相关，即红色通道变亮，绿色通道也变亮。
Manders' Colocalization Coefficients (MCC) Overlap Coefficient	描述两荧光通道重叠程度，数值范围是0～1，共定位越显著数值越接近1。
Colocalization Rate	描述两荧光通道共定位百分比%: Colocalization Rate =Colocalization Area/Area Foreground



谢谢！

IHB光学成像交流群

该二维码7天内(8月14日前)有效，重新进入将更新

周芳

TEL：180 4056 1678

MAIL：

zhoufang@ihb.ac.cn

王鑫

TEL：136 3865 1076

MAIL：

wangxin@ihb.ac.cn

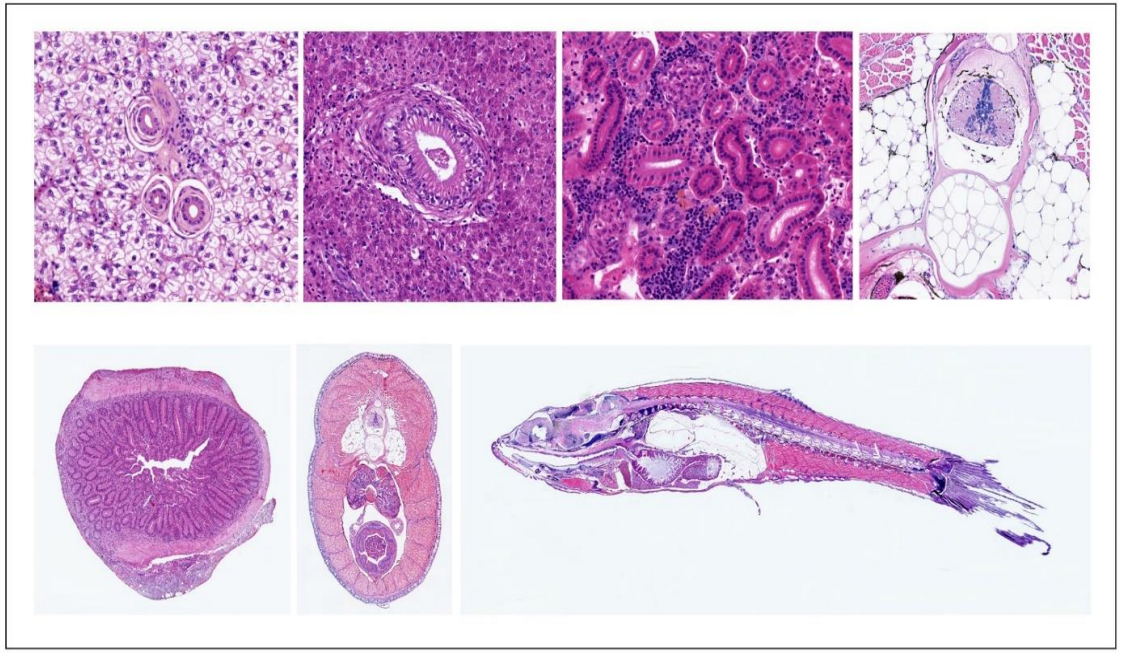
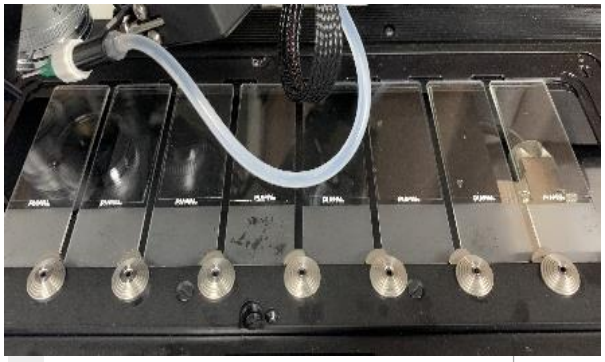
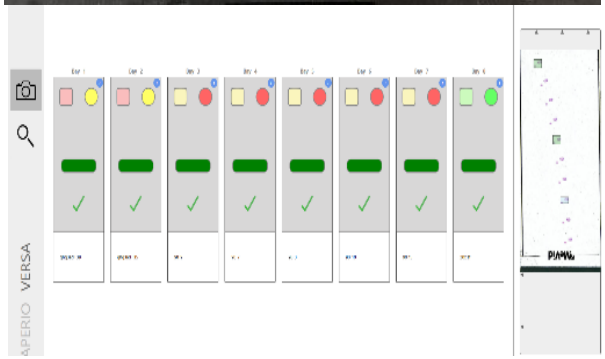
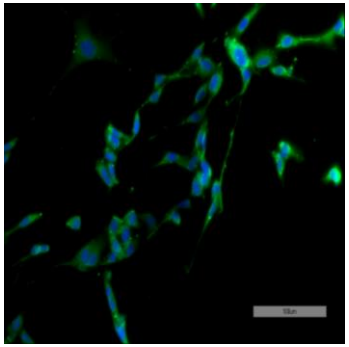
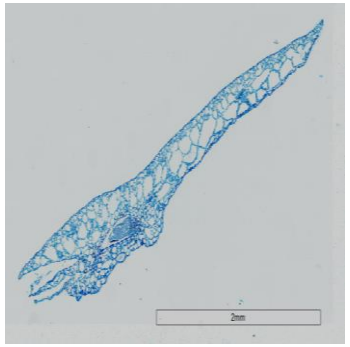
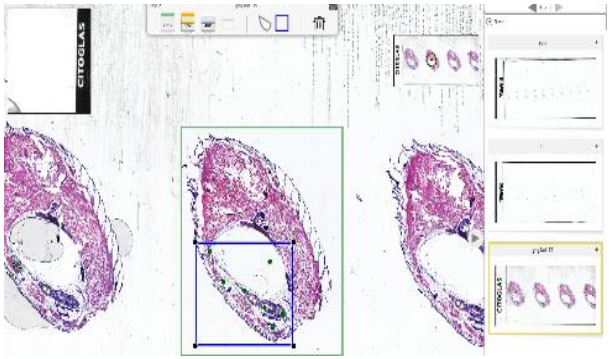
王光欣

TEL：135 4506 8558

MAIL：

wanggx@ihb.ac.cn

光学成像技术平台已熟练开展的检测项目

- **普通共聚焦成像及FRAP、FRET、光转化、光激活等特殊成像**
  用于测定荧光强度、共定位、三维重构和蛋白动力学数据和蛋白相互作用等结果
- **双光子共聚焦成像**
  利用双光子激发化学合成小分子纳米材料标记目标蛋白，或进行厚样品的深度观察
- **超分辨STED成像**
  获得分辨率XY≤120nm的全光谱超高分辨率成像
- **特定单细胞或区域制备**
  在显微镜下精确从各种各样的组织样本中分离出特定的单细胞或整个区域的组织
- **病理玻片扫描**
  批量、高清、快速、多通道扫描

组织切片制样及切片扫描