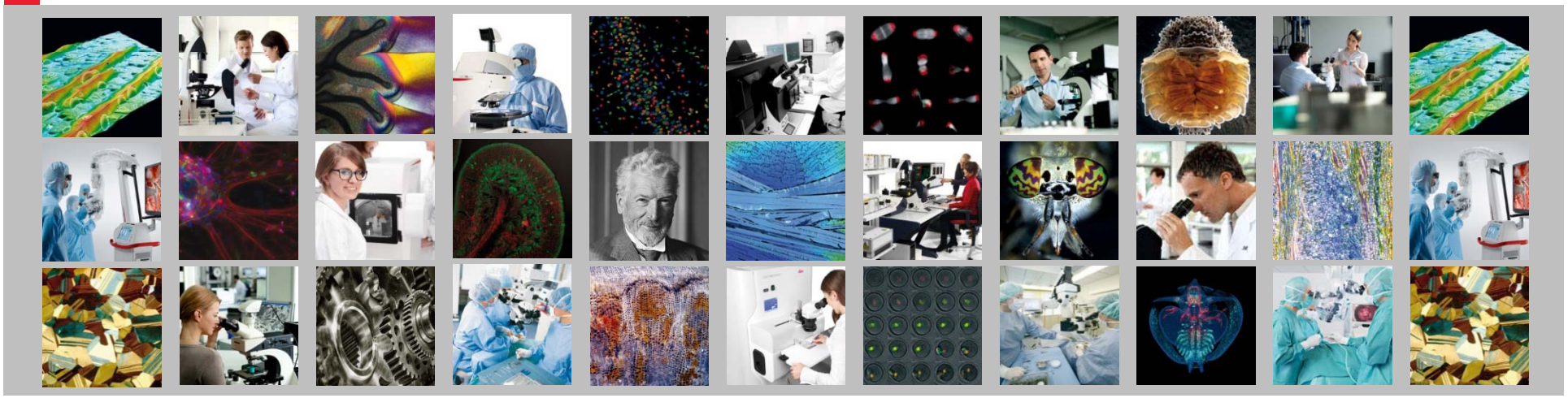
From Eye to Insight

Leica
MICROSYSTEMS

徕卡激光共聚焦显微镜原理、应用及操作



——期待您的发现

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Science Lab

显微镜成像原理：

把小细节看清楚的 3 个先决条件：

1. 放大：把小物像放大
2. 分辨率：能分辨两点之间的最小距离
3. 反差：辨认已经被放大和具有一定分辨率的细节

只有具备了上述 3 个条件，才尽可能真实地把物体信息转化为物体图像

	Magnification	Resolution
Human eye	1x	0.2 mm
Magnifier	10x	0.02 mm
Stereomicroscope	100x	2 µm
Compound microscope	1000x	0.2 µm
Electron microscope	1 Mio. x	0.2 nm

光学显微镜分类

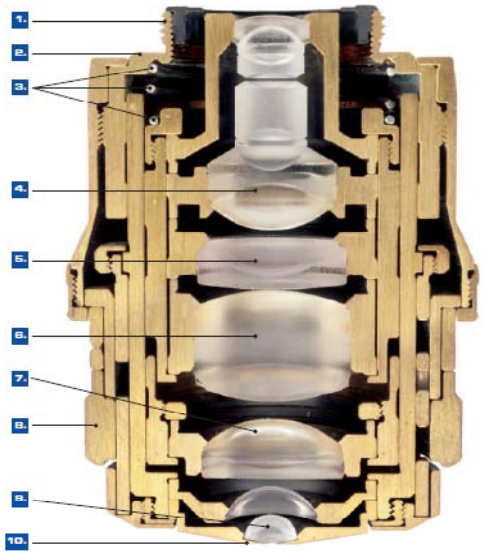
- 光学显微镜有**正置和倒置显微镜**（这两者也合称复合式 - Compound microscopes），以及**体视显微镜。**

- 光学显微镜分**宽视场显微镜**和**共聚焦**（激光扫描）显微镜两大类

正置显微镜

倒置显微镜

物镜的标识



PL APO
40x/0.85 CORR

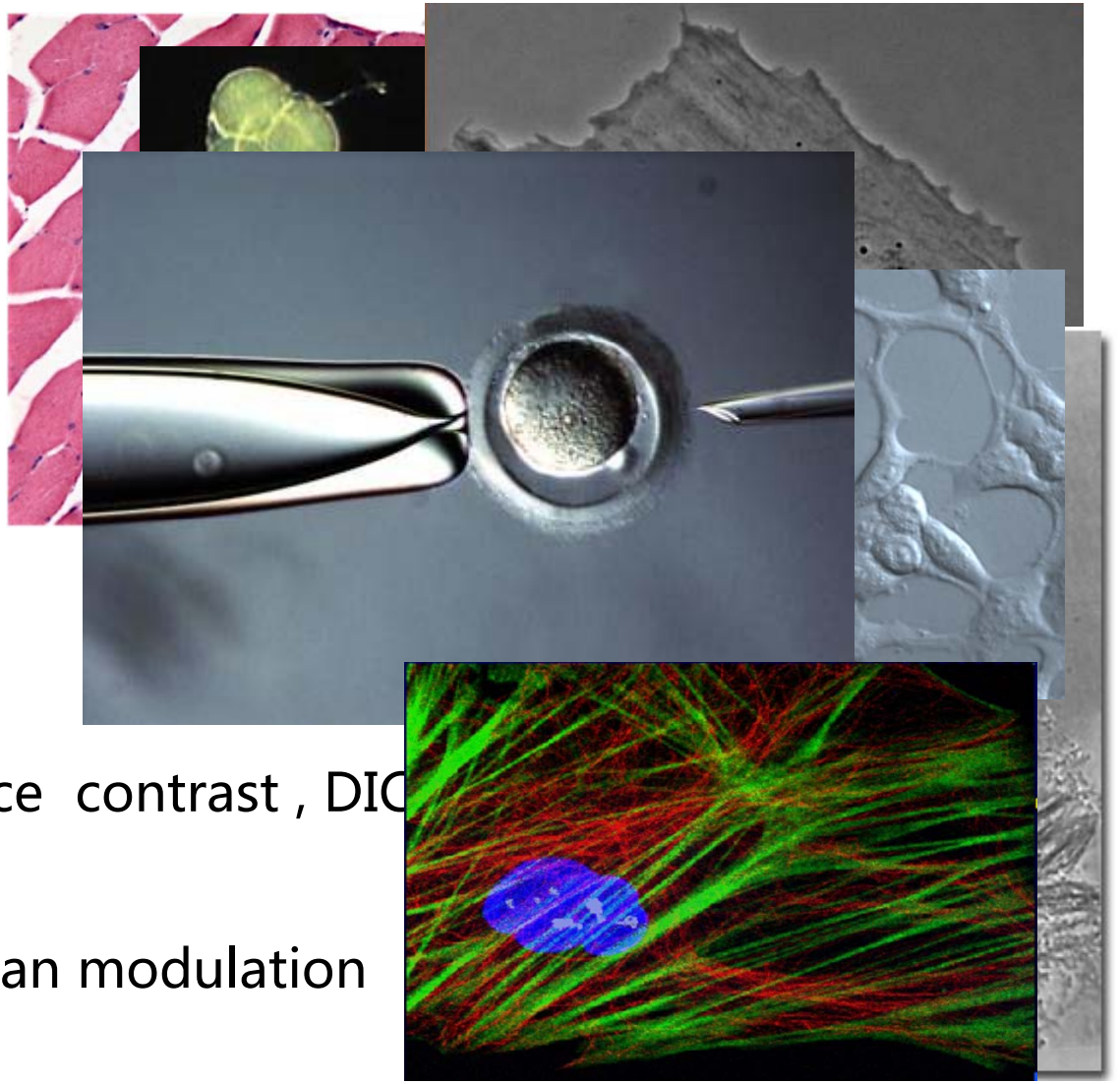
介质
Oil = 油镜，需镜油作为介质
W = 水镜
IMM = 浸镜，需水、甘油或油
DRY = 干镜

盖玻片规格

-	用于有或没有盖玻片
0	用于没有盖玻片
0.17	用于0.17mm厚度盖玻片
0-2	用于0-2mm厚度盖玻片
Corr	带有盖玻片厚度校正环

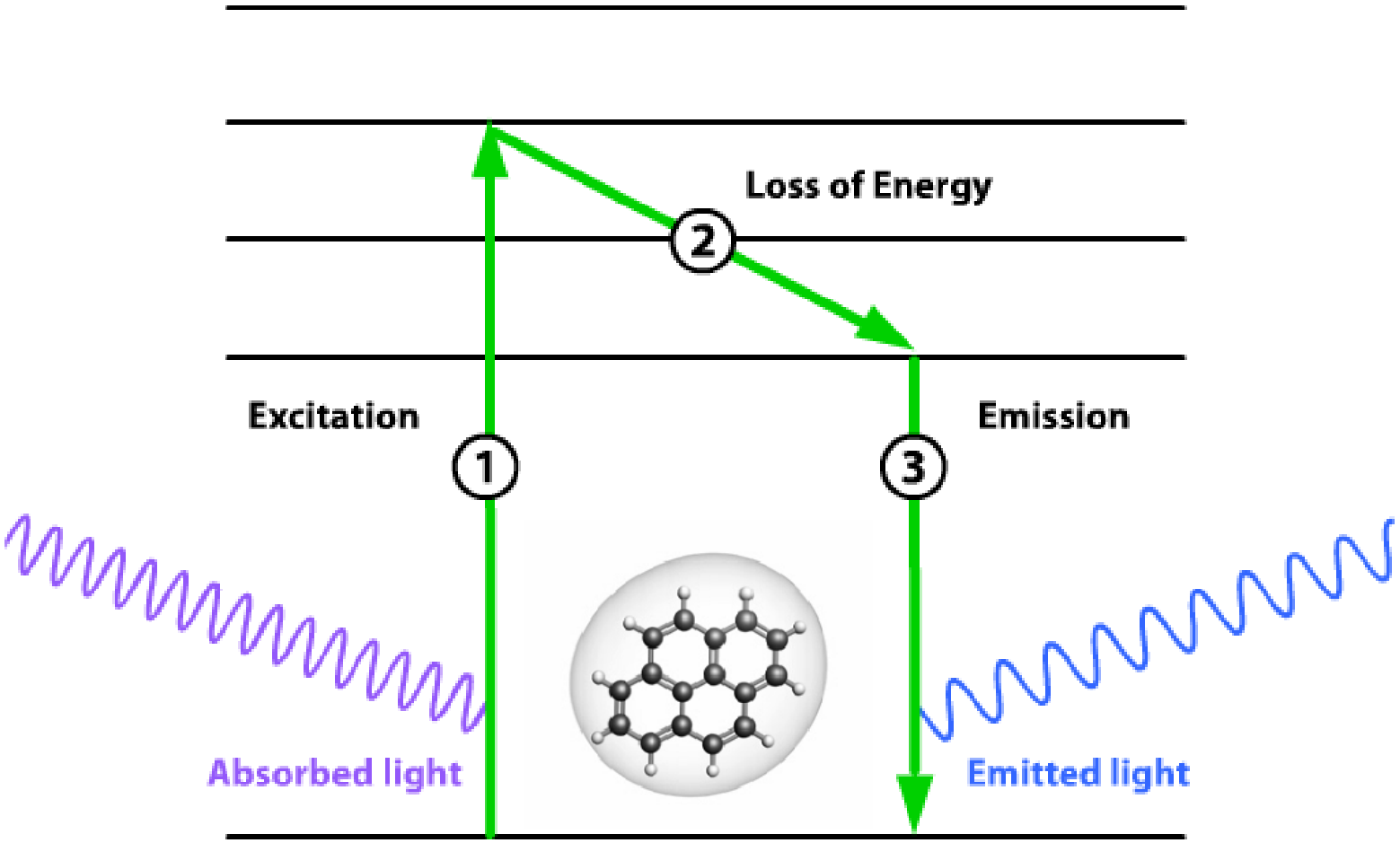
光学显微镜的不同观察方法

- 观察方法有：
  - 明场 (Bright Field)
  - 暗场 (Dark Field)
  - 相差 (Phase Contrast)
  - 偏光 (Polarized)
  - 微分干涉相差 (differential interference contrast，DIC)
  - 霍夫曼调制相差（Hoffman modulation contrast，HMC）
  - 荧光 (Fluorescence)



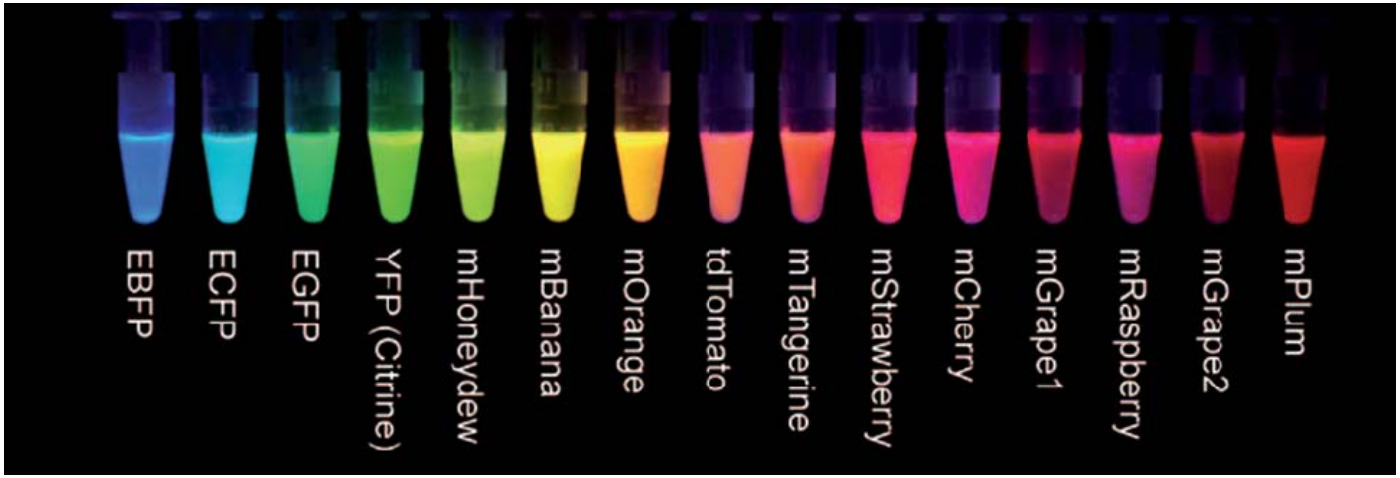
http://www.microscopy-uk.org.uk/mag/indexmag.html?http://www.microscopy-uk.org.uk/mag/artjul99/pollen.html
http://www.me.mtu.edu/~cchoi/practice.html
http://micro.magnet.fsu.edu/primer/techniques/polarized/biologicalpartone.html

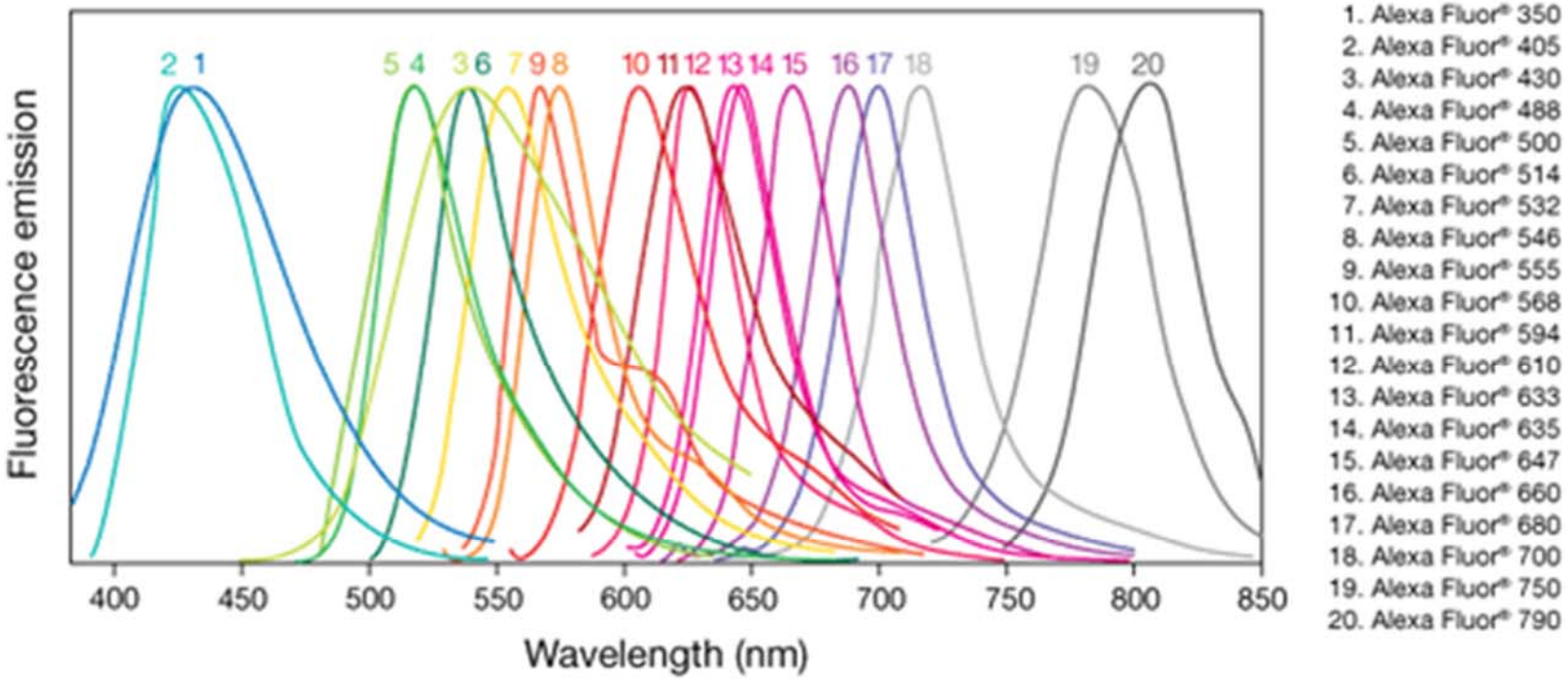
共聚焦最主要的观察对象：荧光信号



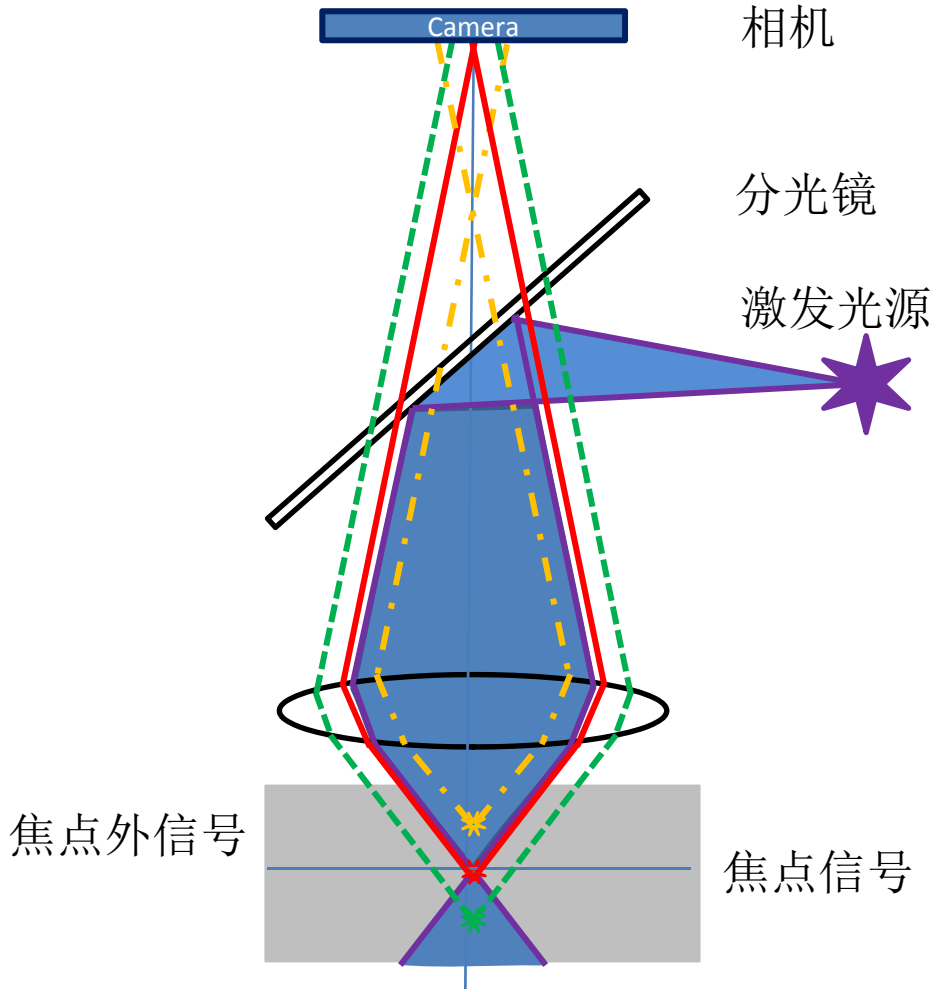
荧光蛋白或荧光染料

荧光蛋白与荧光染料

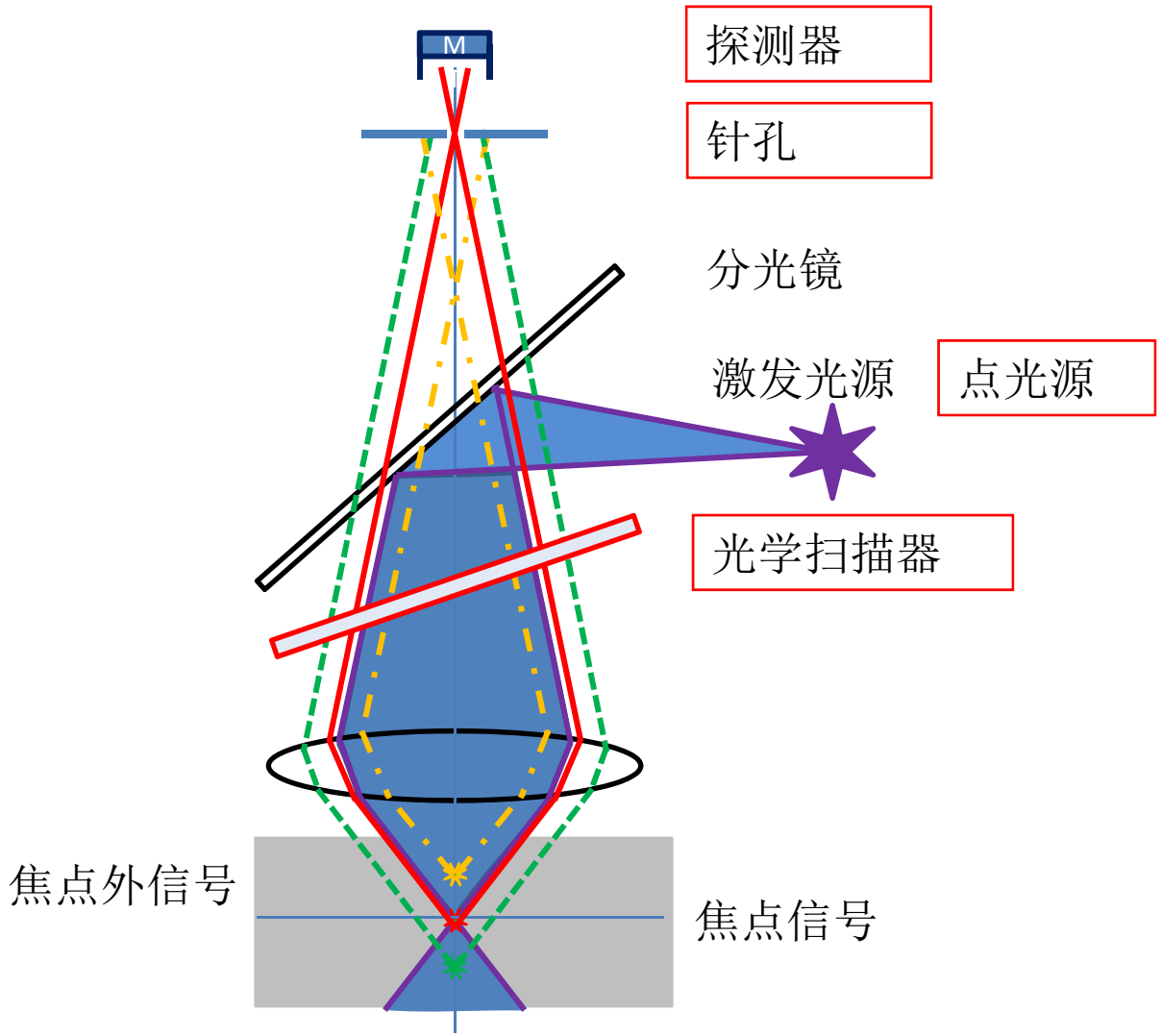

EBFP
ECFP
EGFP
YFP (Citrine)
mHoneydew
mBanana
mOrange
tdTomato
mTangerine
mStrawberry
mCherry
mGrape1
mRaspberry
mGrape2
mPlum


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

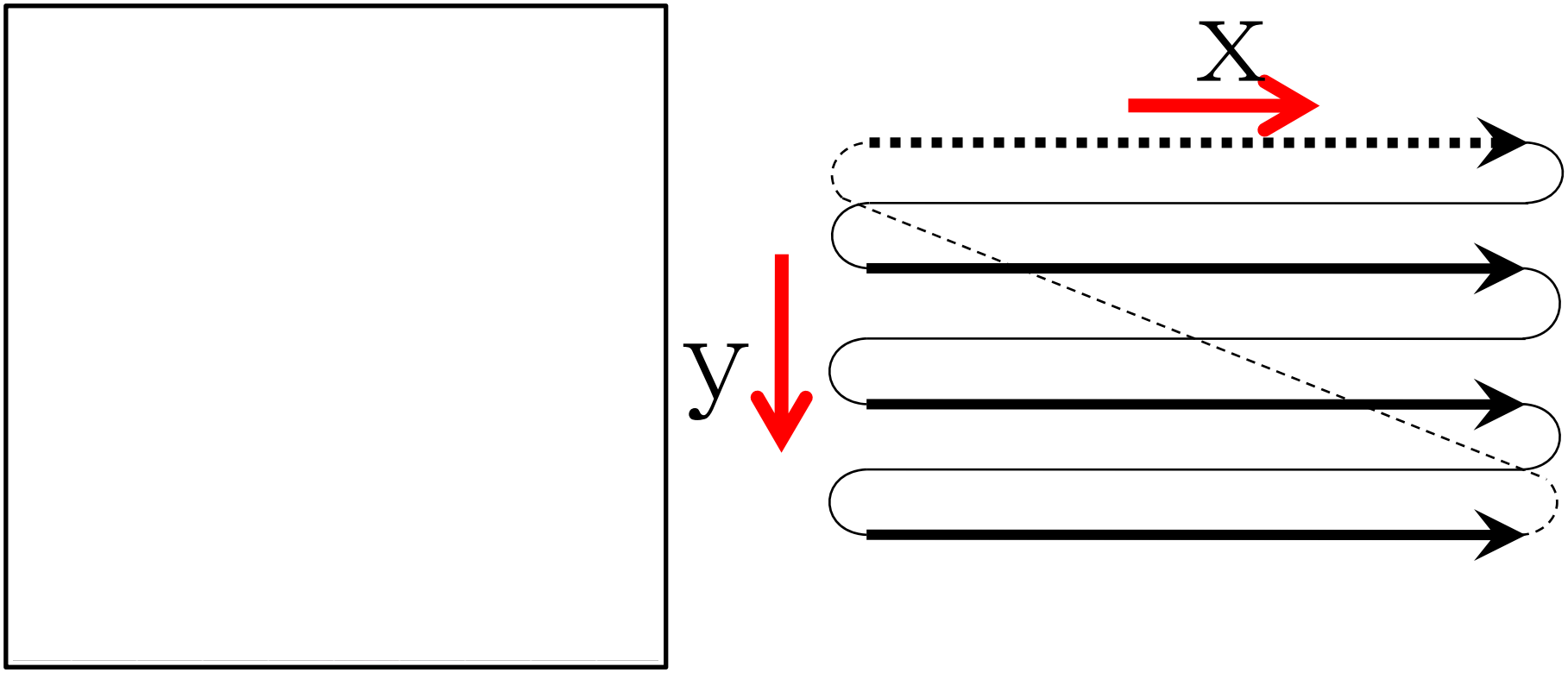
宽场荧光显微镜的局限



共聚焦显微镜原理

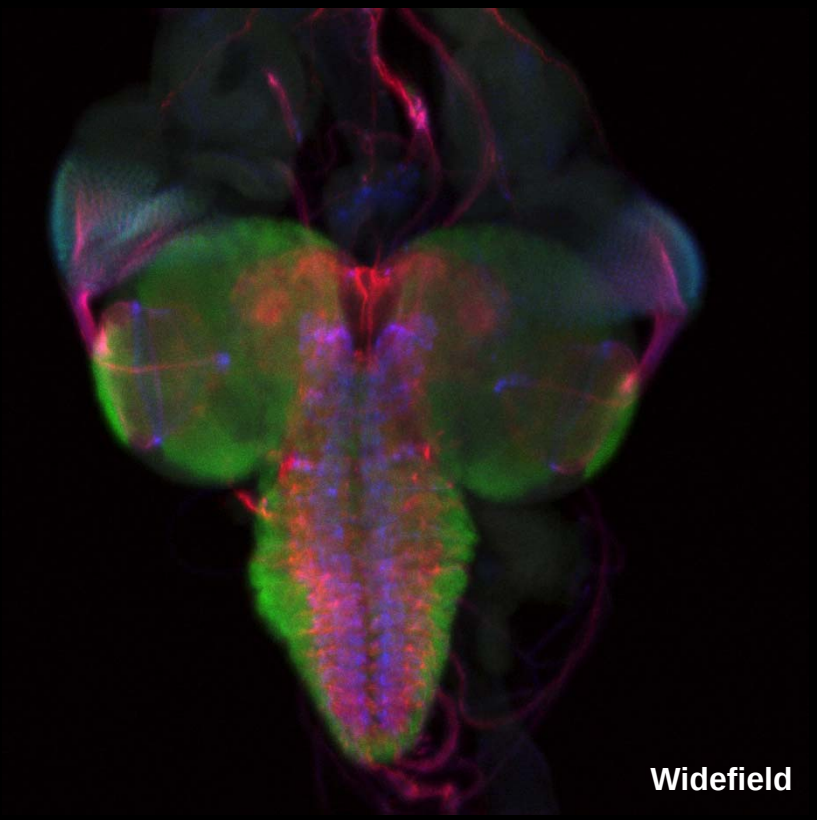


扫描过程

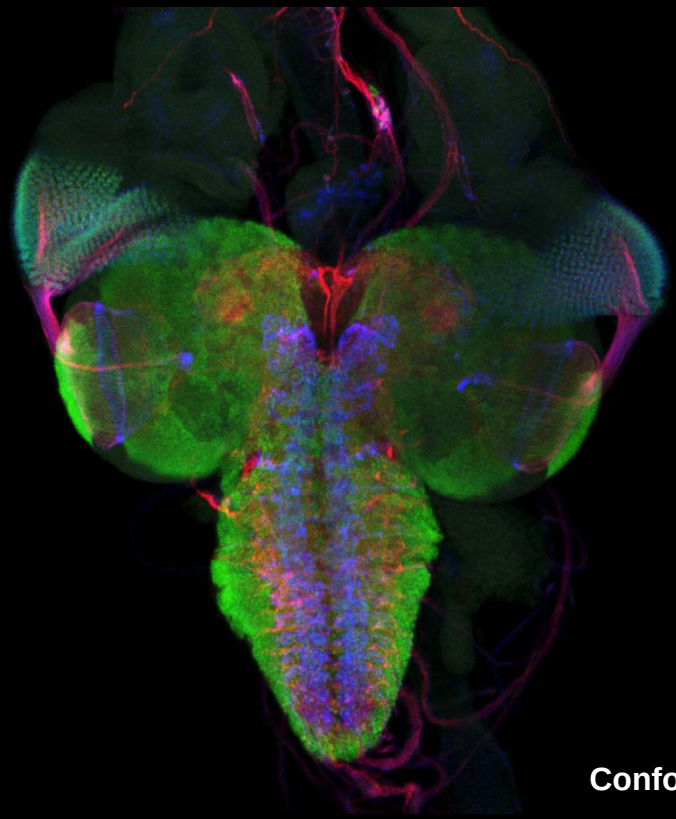


由于使用点光源和探测针孔，共聚焦显微镜每次只能探测一个特定的点的信息。为了能够得到一个二维的图像，必须要在x-和 y-方向移动采样点。（“扫描”）

宽场 vs. 共聚焦荧光成像



Widefield

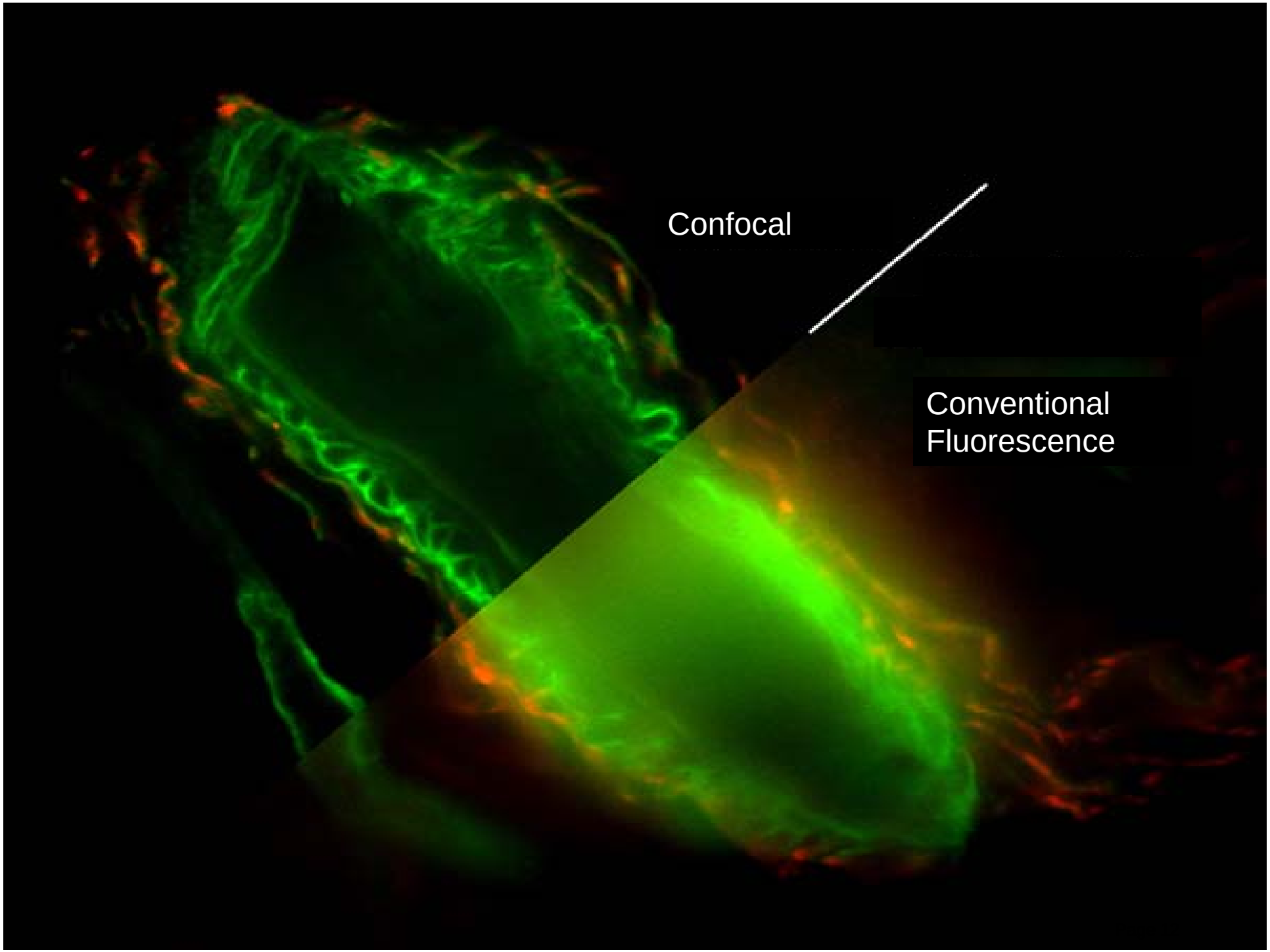


Confocal

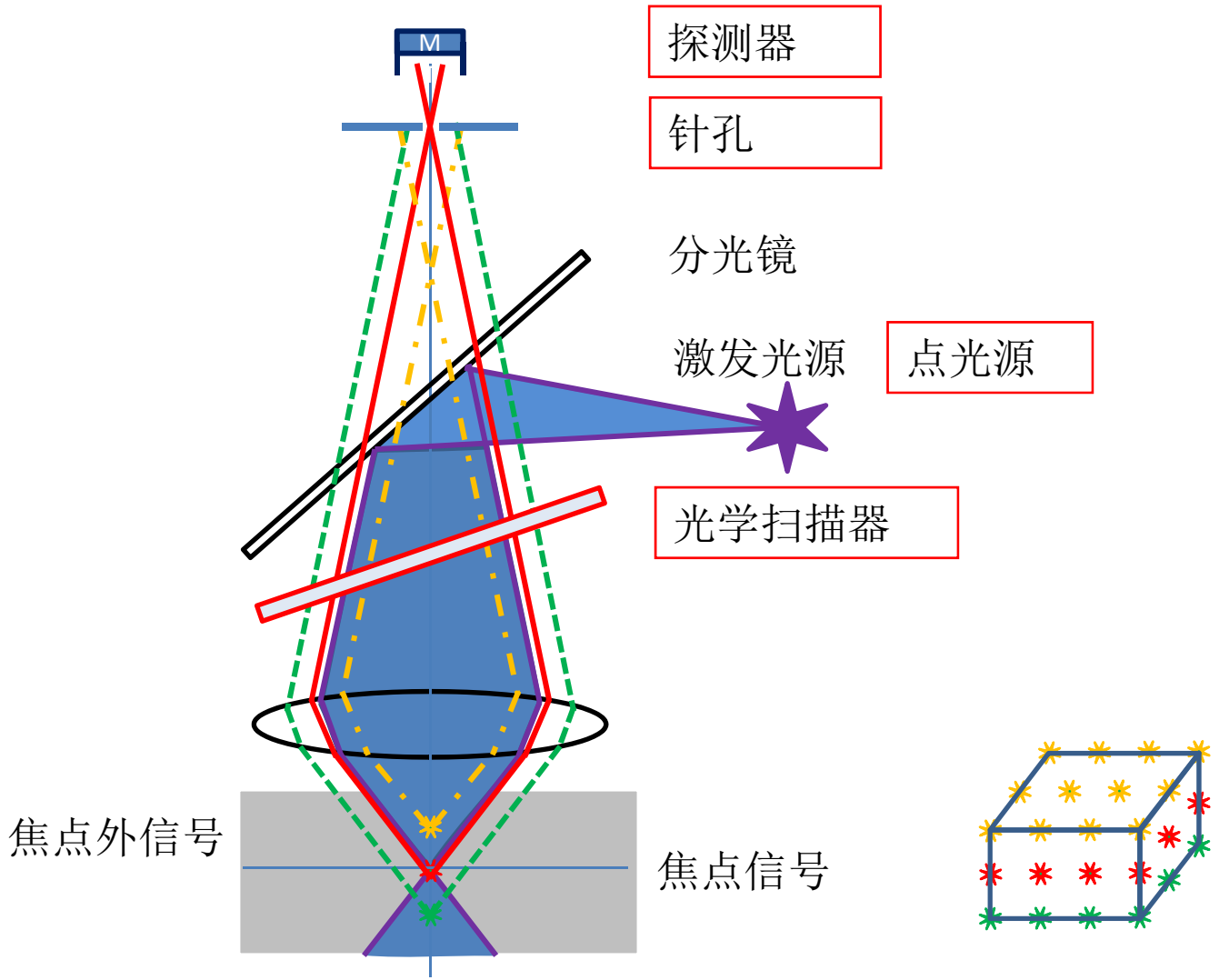
Drosophila melanogaster (larvae)

Green: Nuclei (RNA binding protein), Alexa 488; Red: Axons, Cy 3; Blue: Axon endings (of MJ94-positive neurons), Cy 5

Courtesy: Dr. Christoph Melcher, Research Center Karlsruhe


Confocal
Conventional
Fluorescence

共聚焦显微镜原理



激光共聚焦显微镜应用

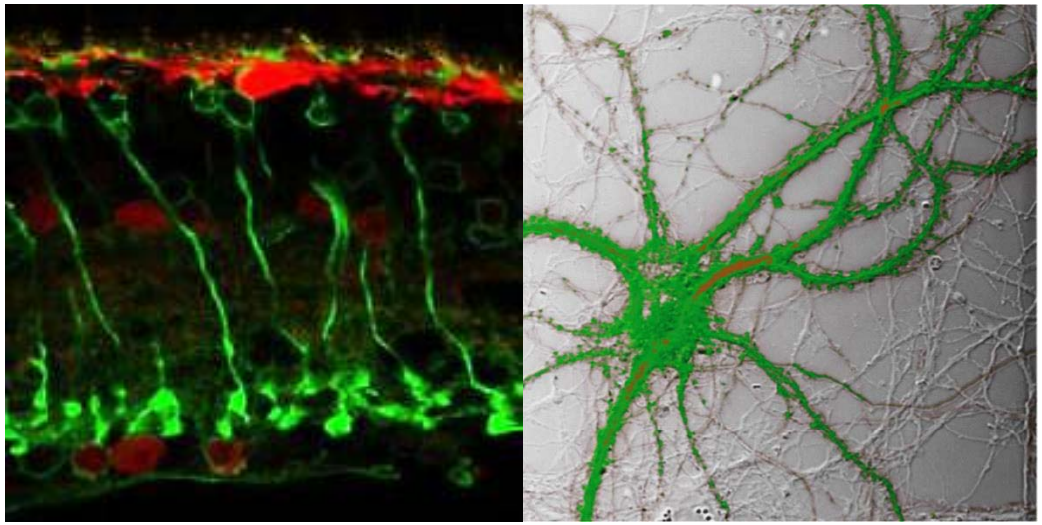
Leica MICROSYSTEMS

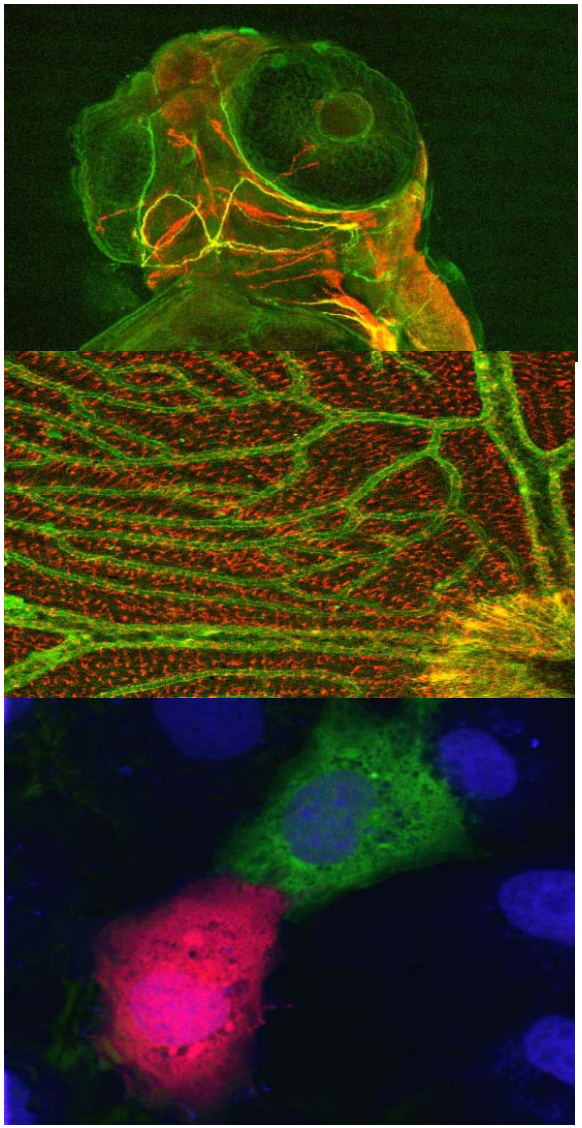
1. 共聚焦图像

原位鉴定细胞或组织里的生物大分子；
观察细胞或亚细胞形态结构

2. 活细胞

活体细胞或者组织功能的实时动态监测

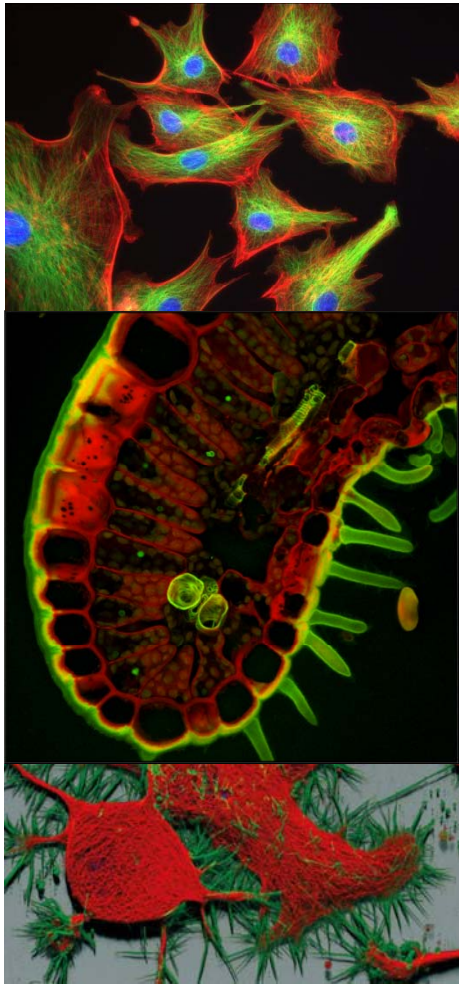




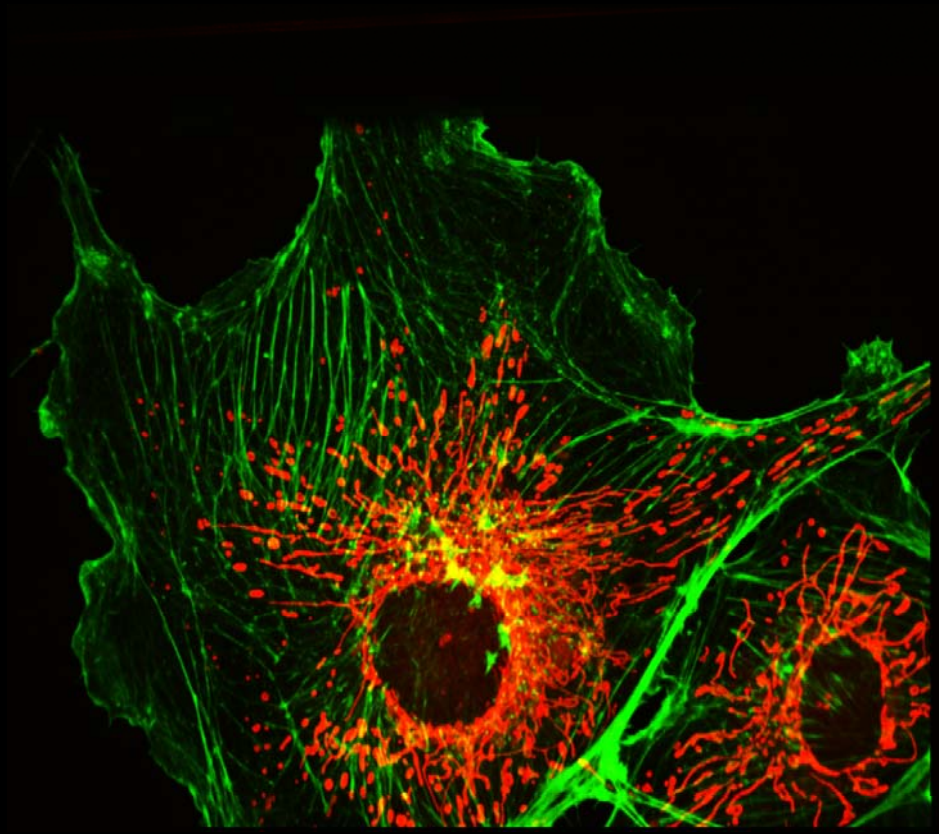
共聚焦显微镜应用

原位鉴定细胞或组织里的生物大分子、观察细胞或亚细胞形态结构

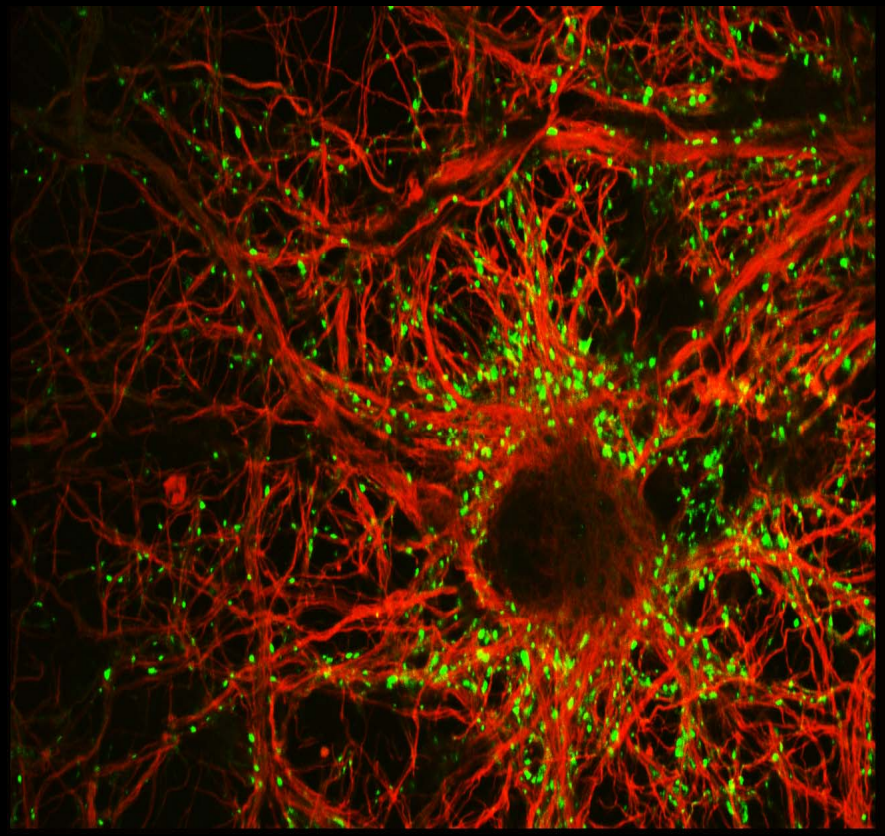
原位检测核酸；
检测蛋白质及其他大分子；
检测蛋白质共定位；
细胞器的观察和测定（线粒体、溶酶体、内质网和高尔基体）；
大图拼接、多点扫描；
……



1. 多色标记荧光



上皮细 胞 MitoTracker Red (线粒体) 、phallacidin (微丝)

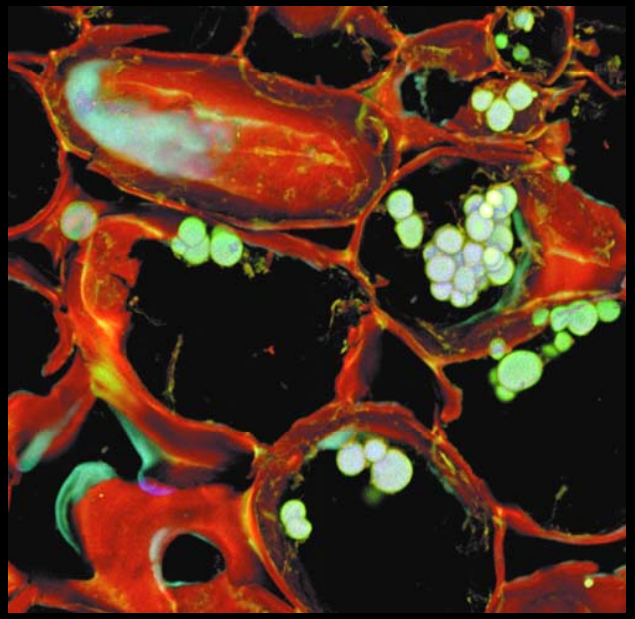


神经元 tubulin和synapsin的定位

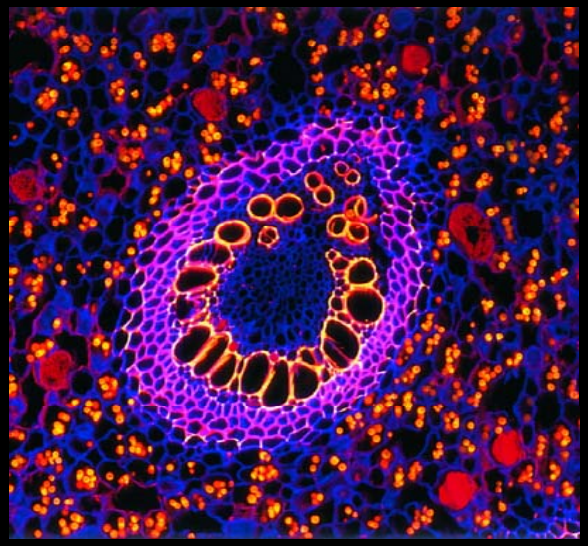
石南属植物茎横切面

灯芯草

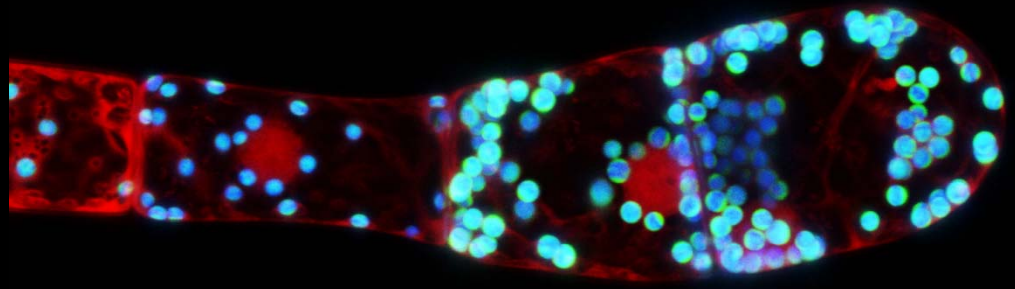
青色：淀粉粒
红色：细胞壁



植物根尖横断面
自发荧光

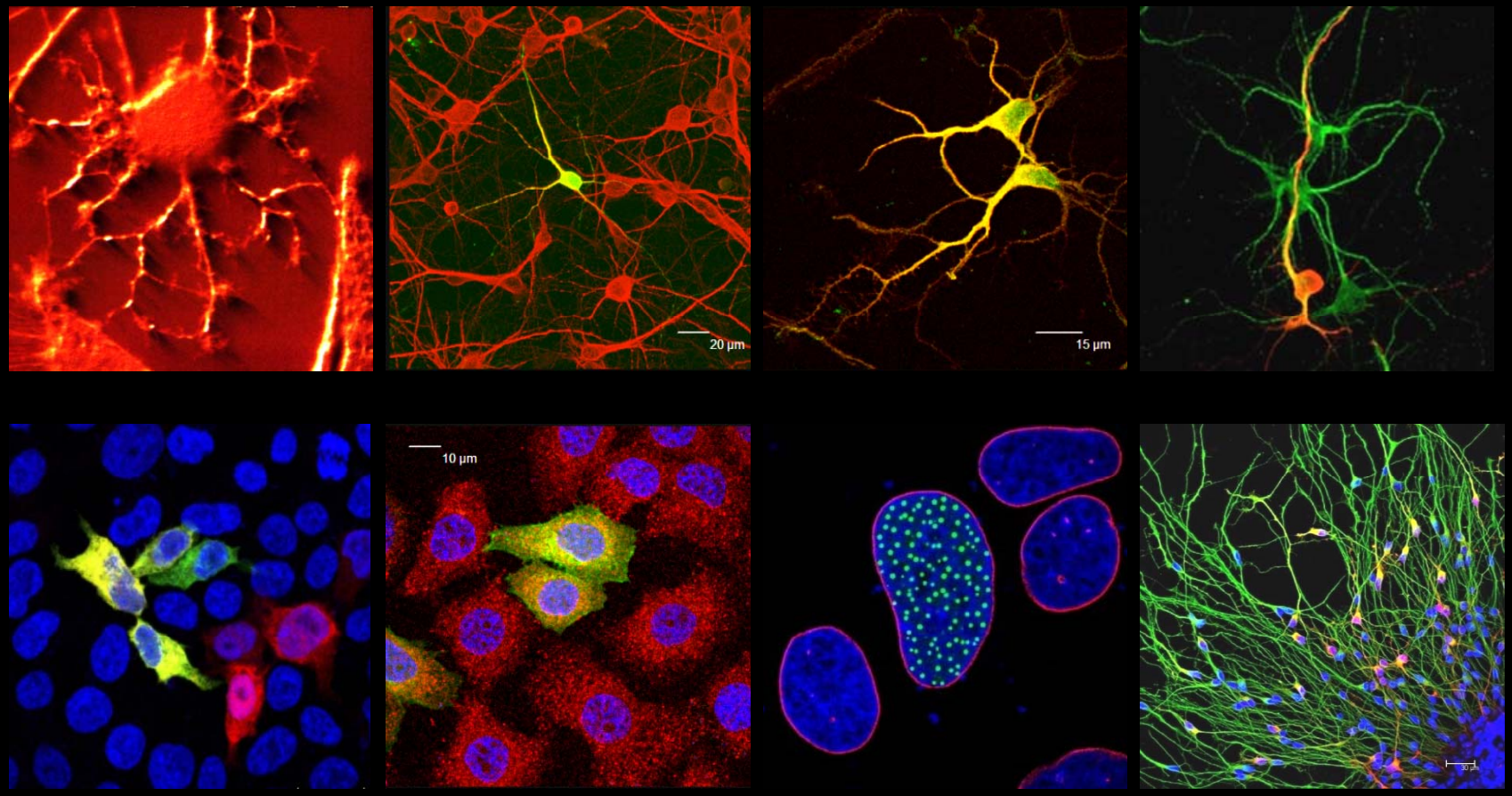


烟叶。
绿色: GFP, 红色:YFP
蓝色: 叶绿素自发荧光



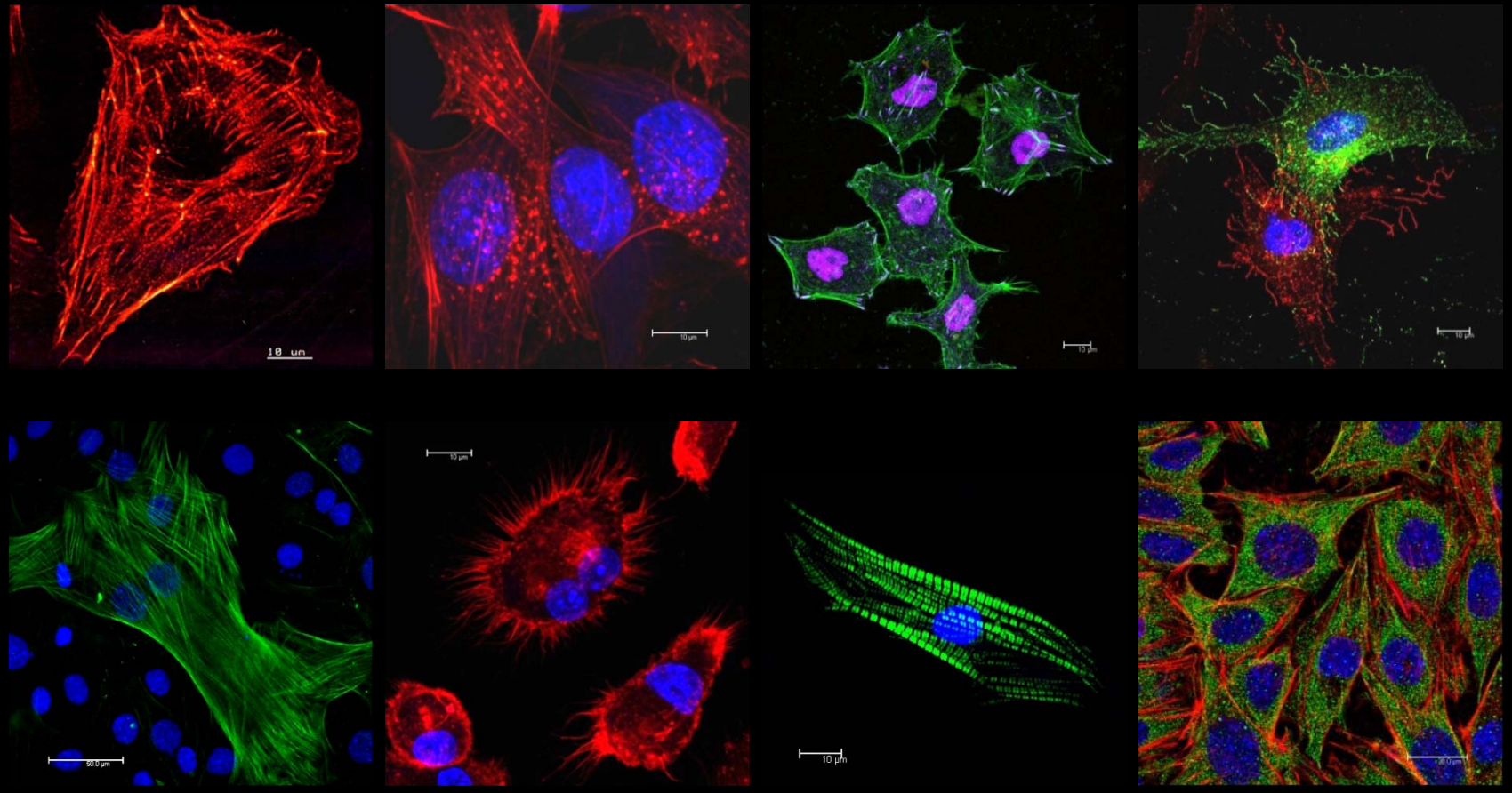
❖ *Confocal 应用*

---细胞

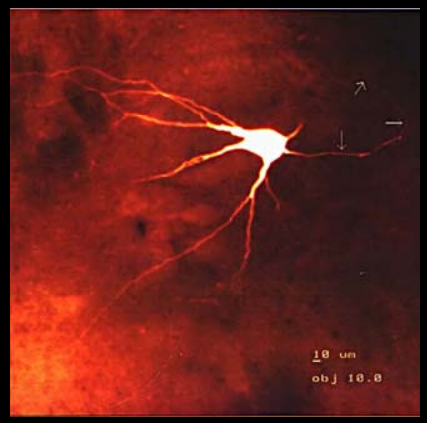


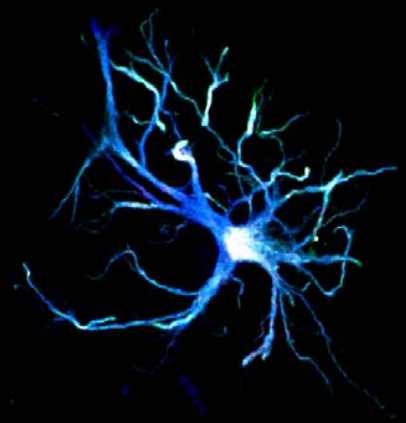
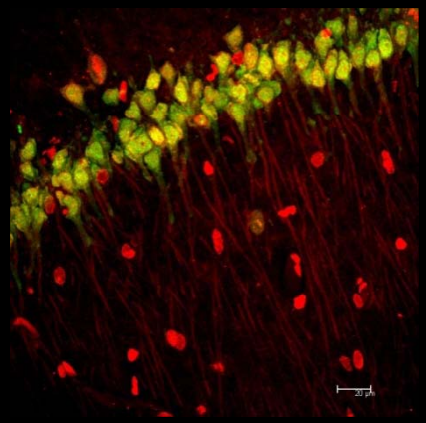
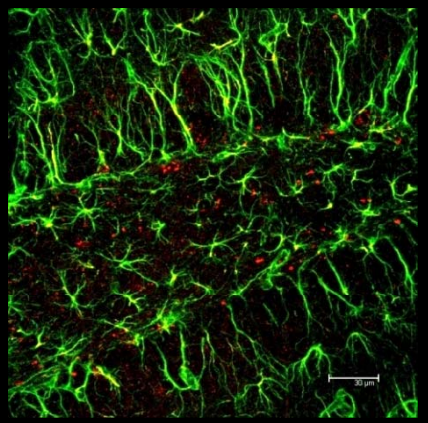
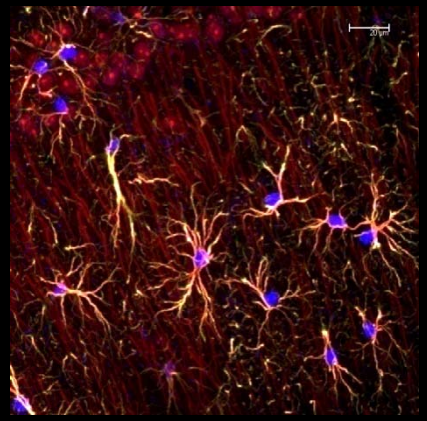
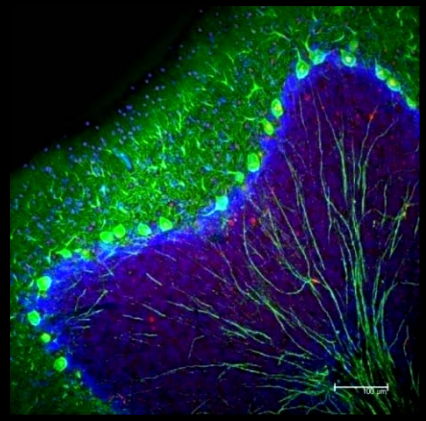
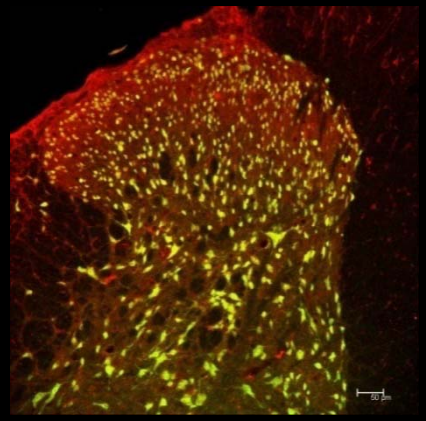
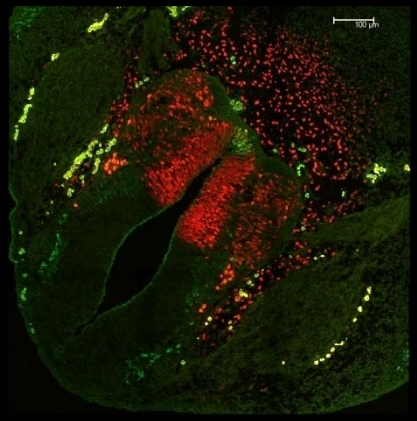
致谢复旦大学黄娅琳教授

---细胞

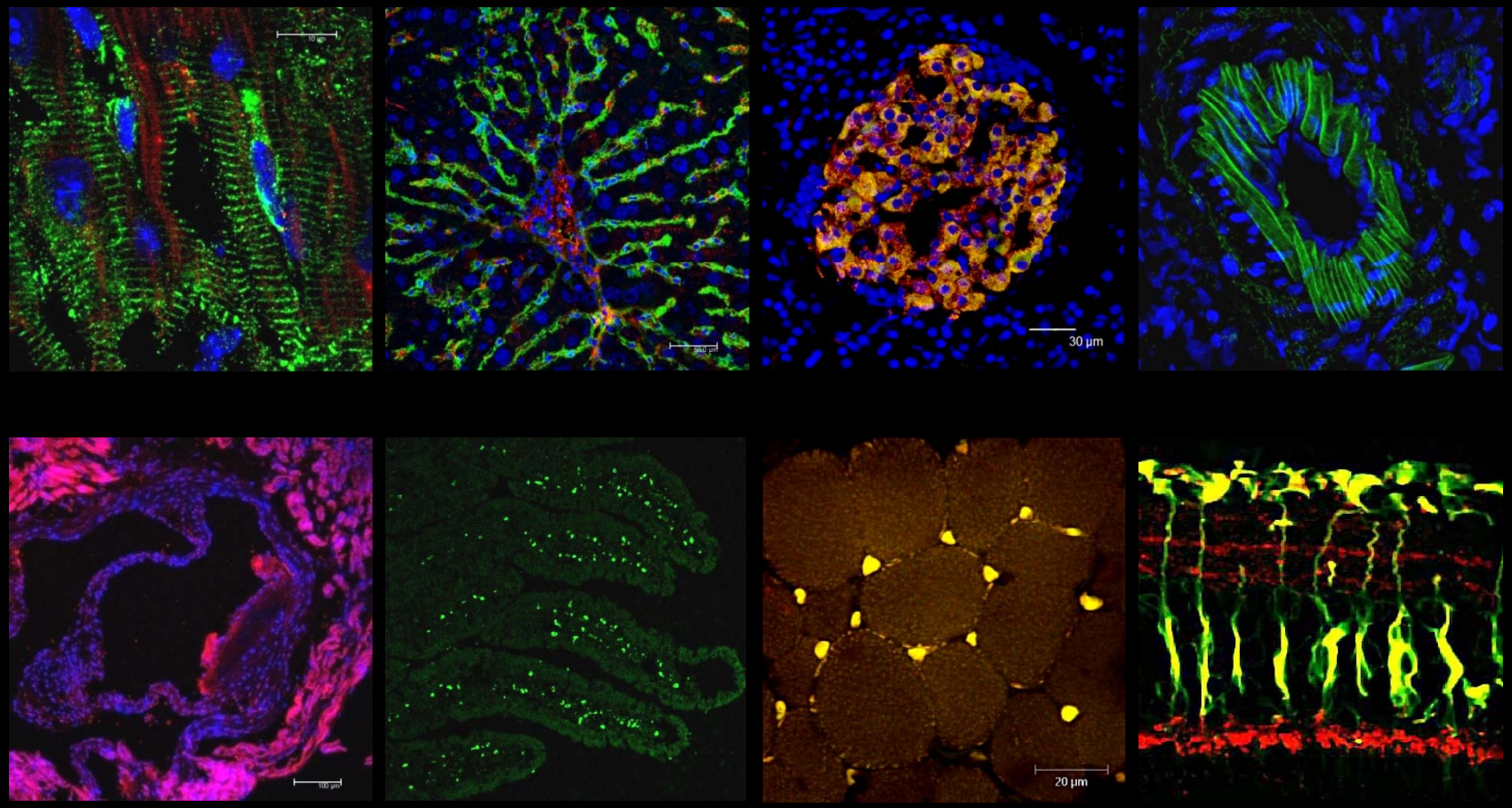


---组织



---组织



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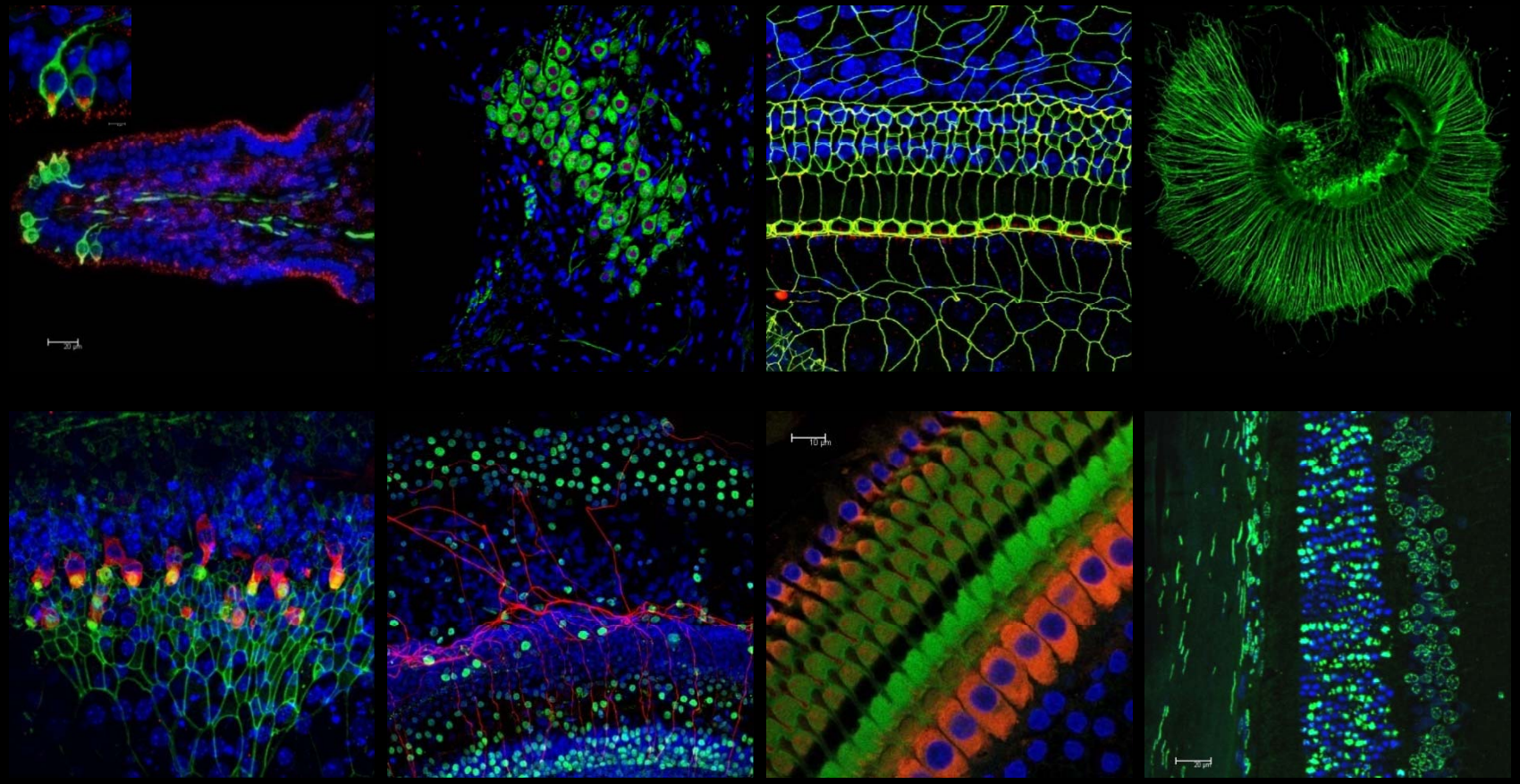
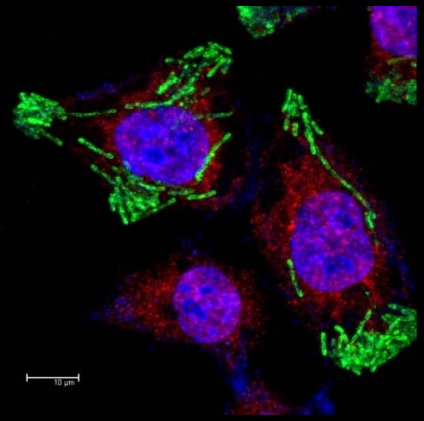
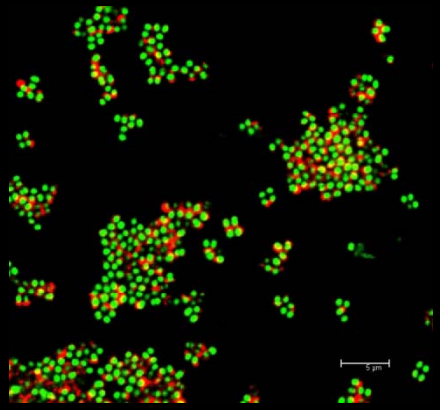
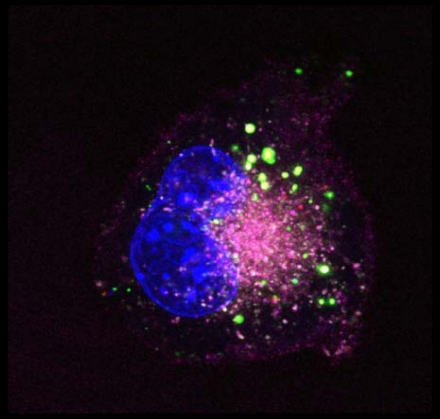
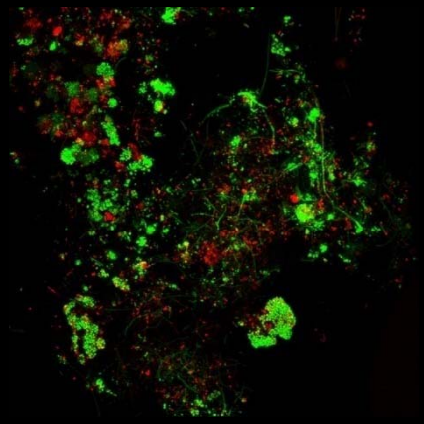


---微生物





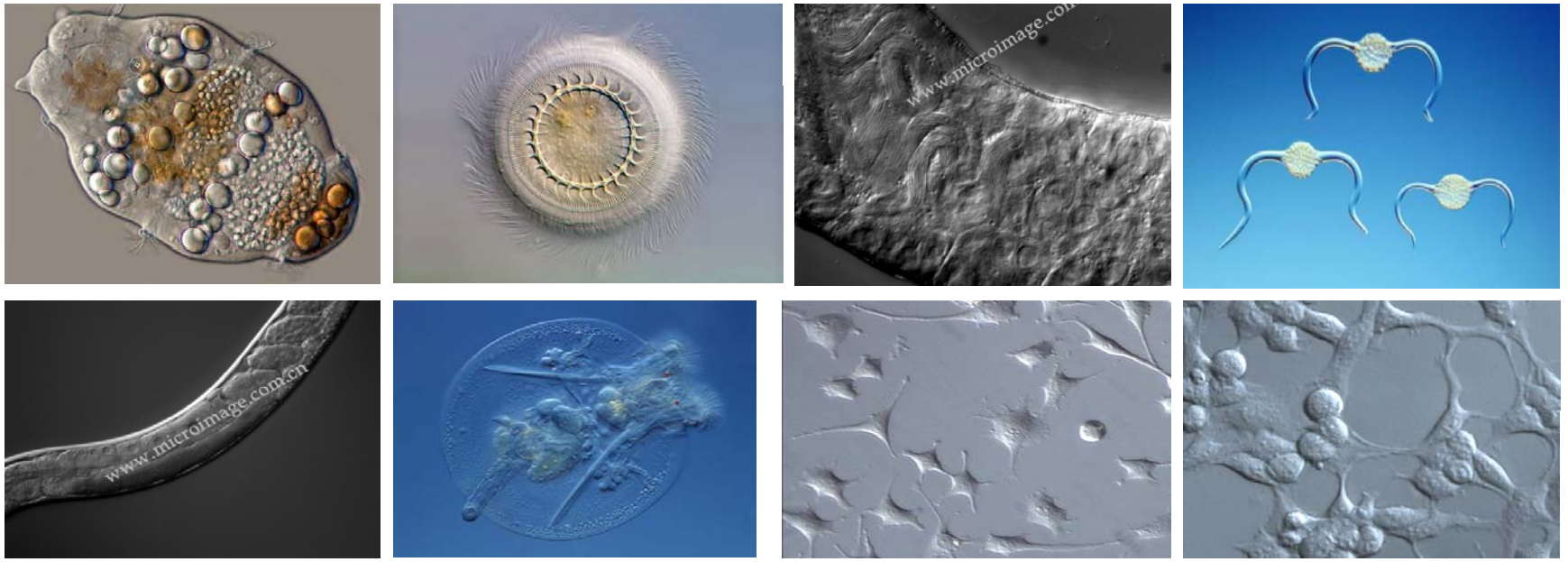




2. 透射光扫描（DIC）

除了明场透射光成像也可配置全自动DIC成像

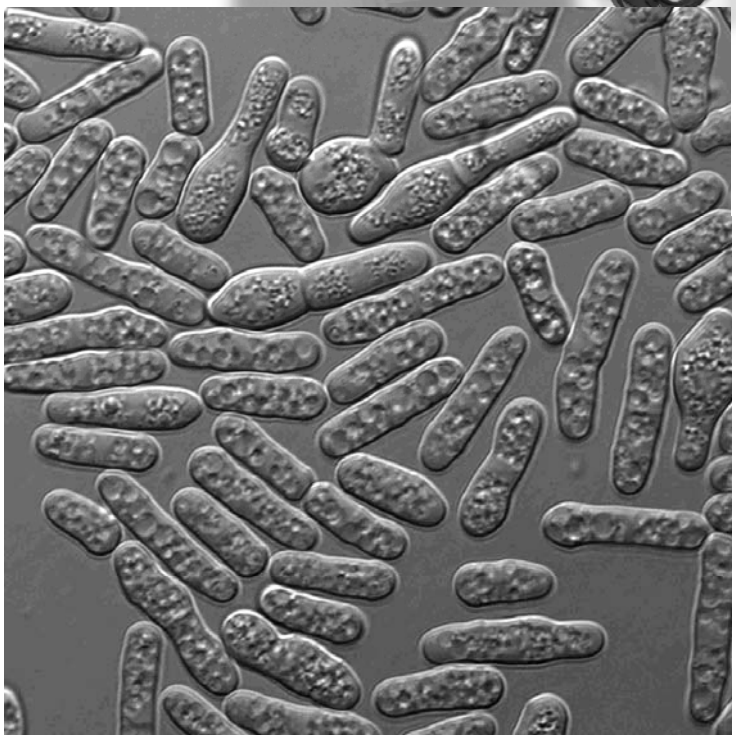
利用激光透射扫描成像，可获得清晰的具有立体感的图片。

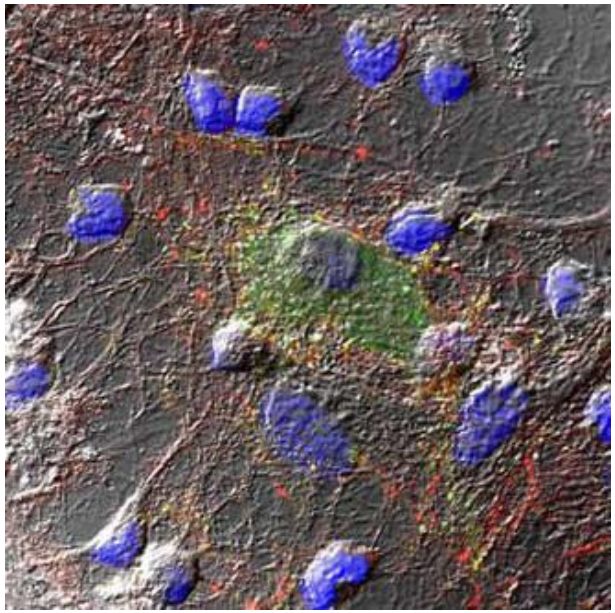


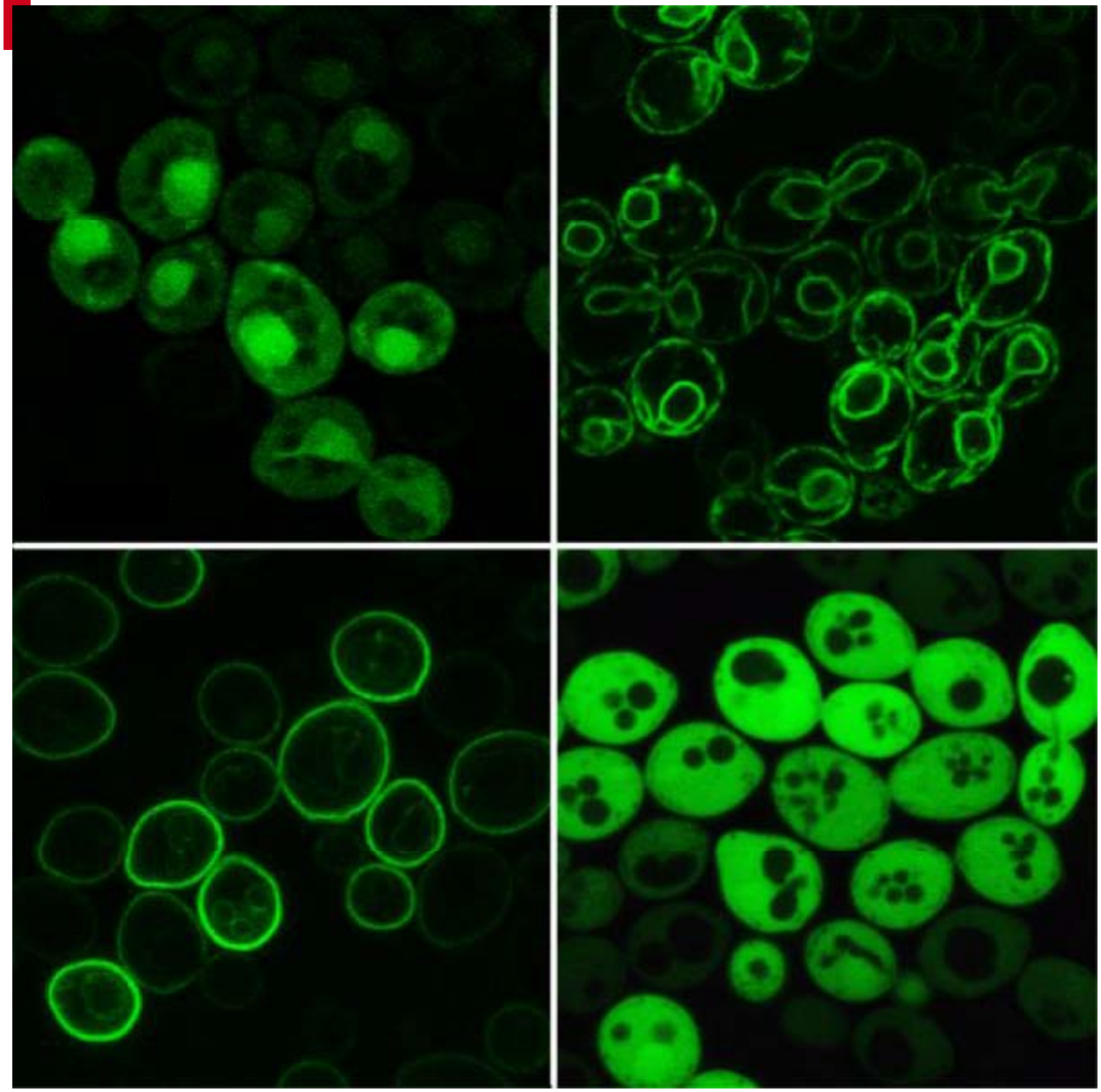
徕卡激光共聚焦 - 全自动软件控制DIC

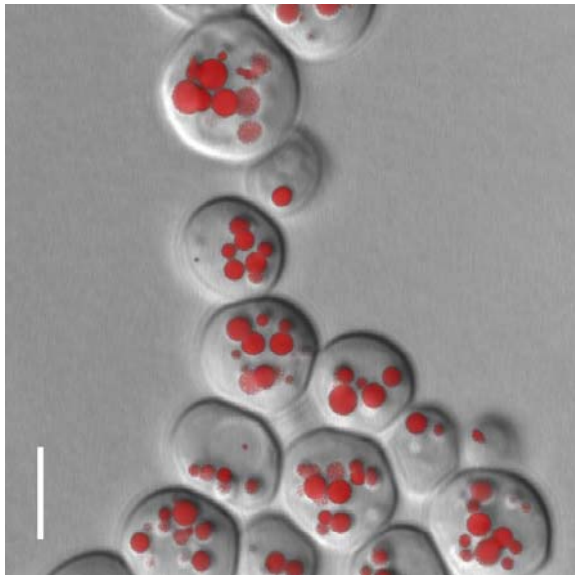
✓ 全自动DIC（微分干涉）

- 自动切换荧光和DIC两种观察方式
- 荧光观察时，DIC棱镜自动移出光路，保证足够的荧光通透量
- 避免DIC棱镜造成的荧光颜色叠加误差





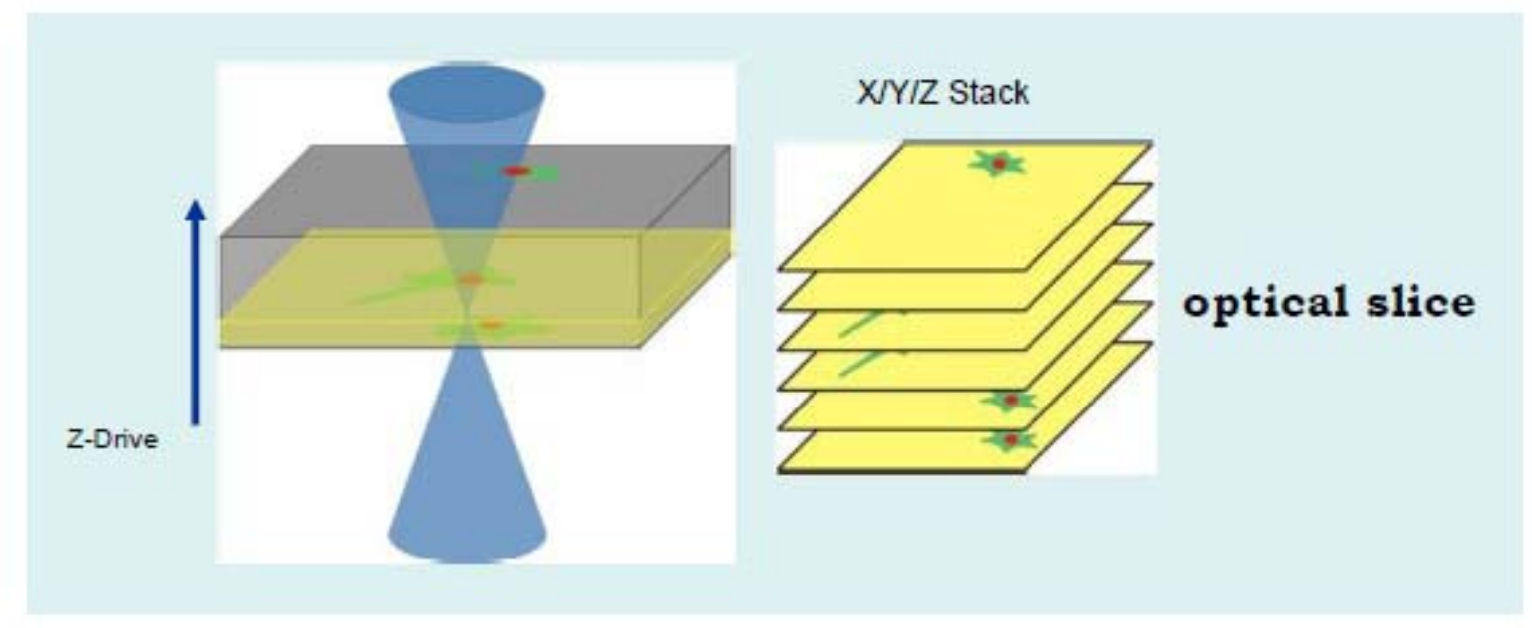




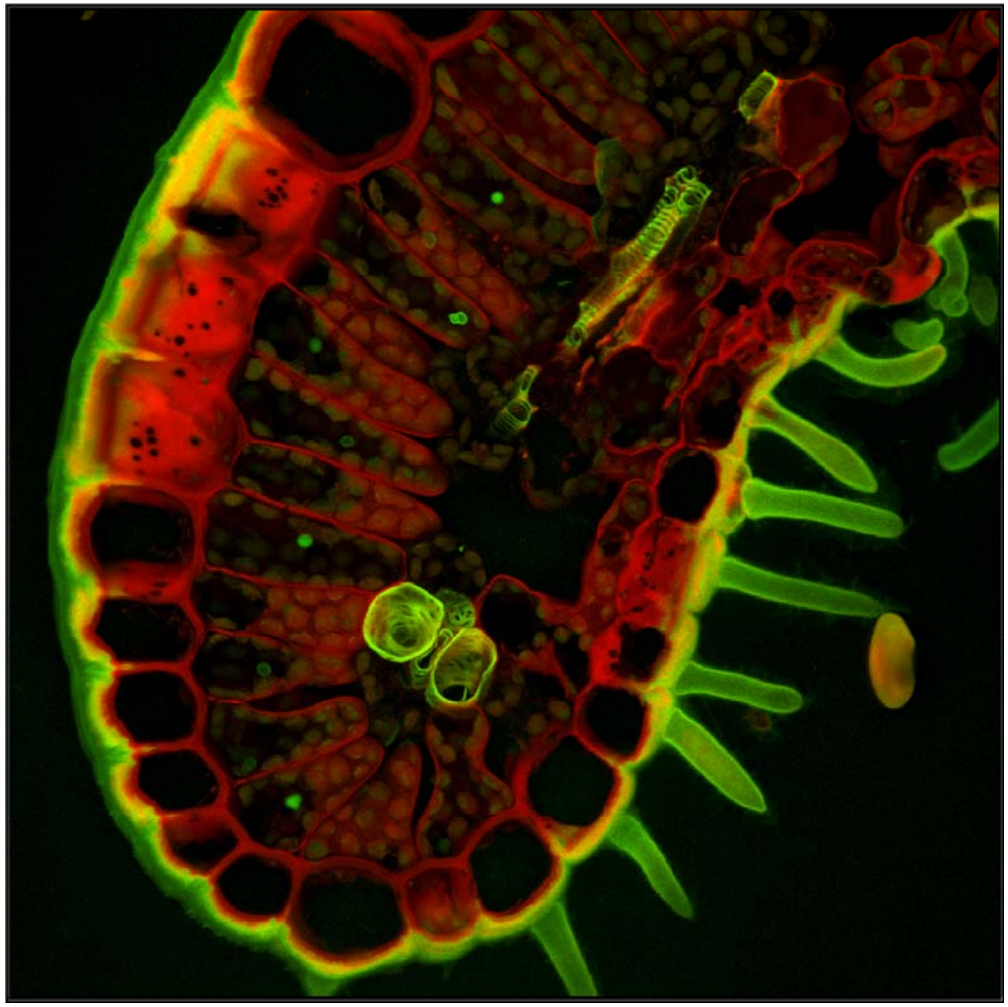
3. 三维构建

分层扫描（切片扫描）

通过调节针孔的大小，可控制样品扫描层面的厚度。即样品中相当薄的一层被探测到，而其他层面不影响成像。

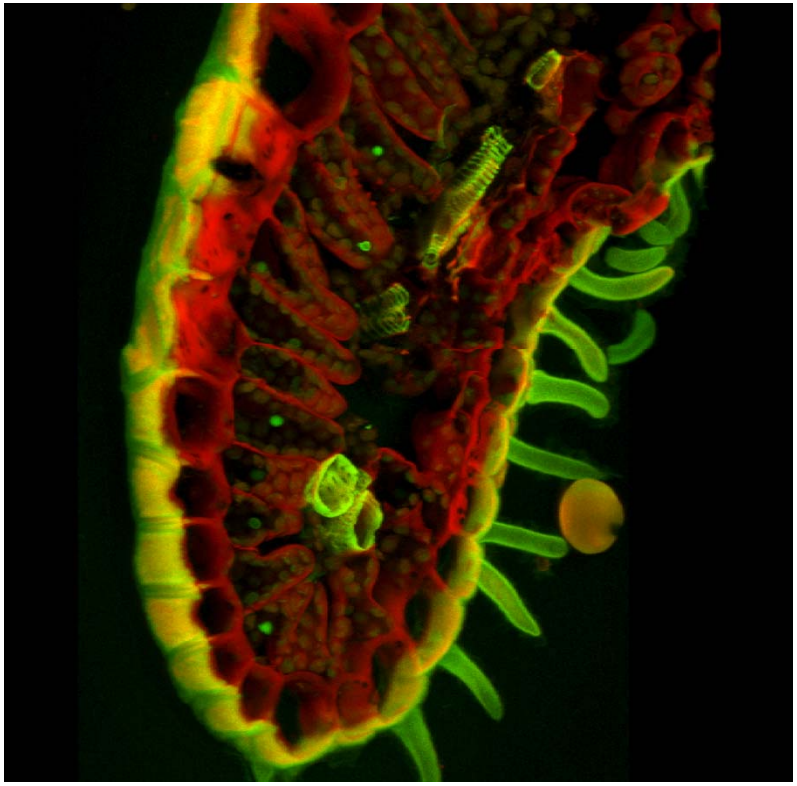


共聚焦成像– 光学切片



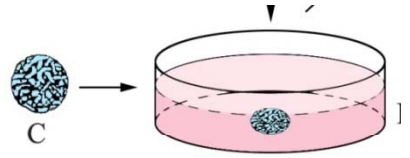
Sample: Erica, leaf-section, Stack: 40µm, 80 sections, 0,5µm distance
Objective: 40x/1,25 Color: 488nm/ 500-553; 603-789
Rec: 1024x1024 pix, 210nm pixel size

- 焦点外的信号不会到达探测器
- 可以对样本进行光学切片

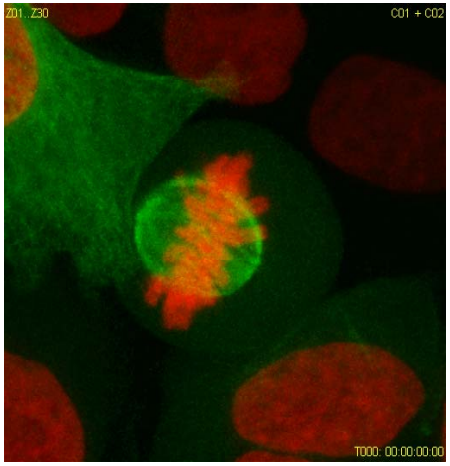


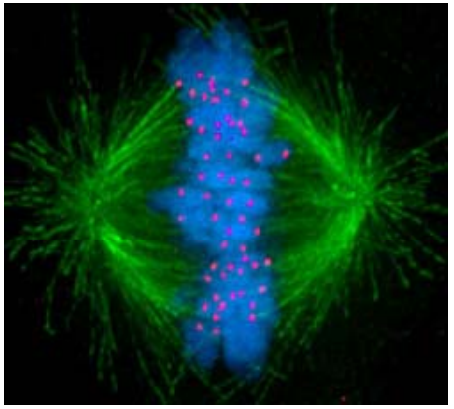
共聚焦显微镜应用

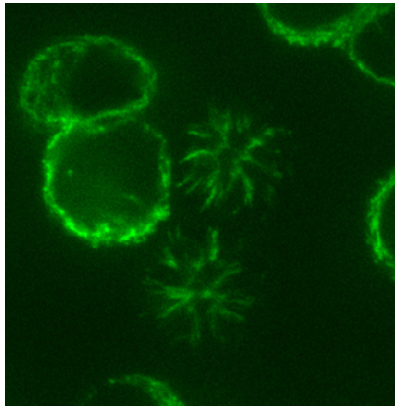
- 活体细胞或者组织功能的实时动态监测



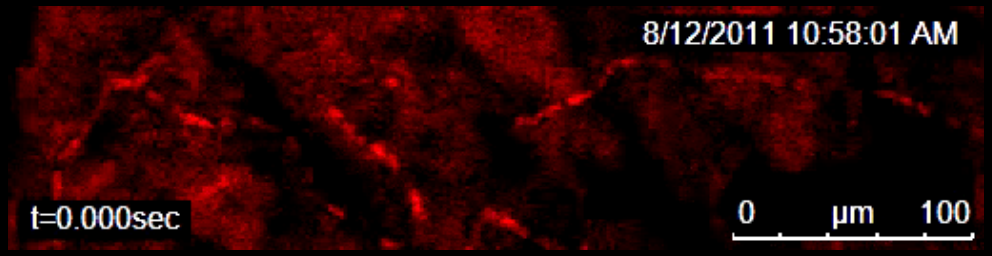
细胞的运动：迁移、变形、分裂
细胞内的运动：骨架变化、分子、细胞器、微粒的运输
分子的分布及量的变化：钙离子、氢离子、活性氧
荧光共振能量转移（FRET）；
荧光漂白恢复（FRAP）；
……

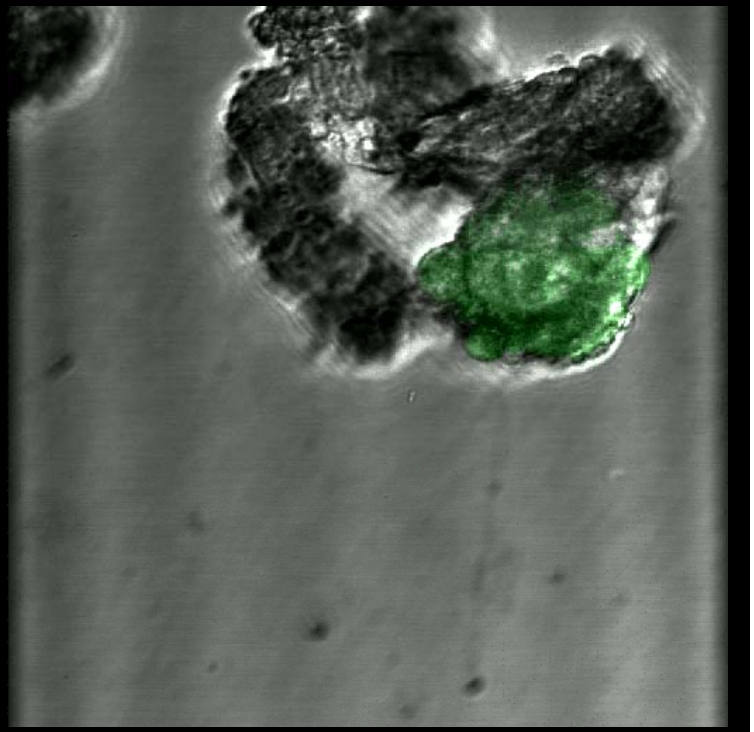


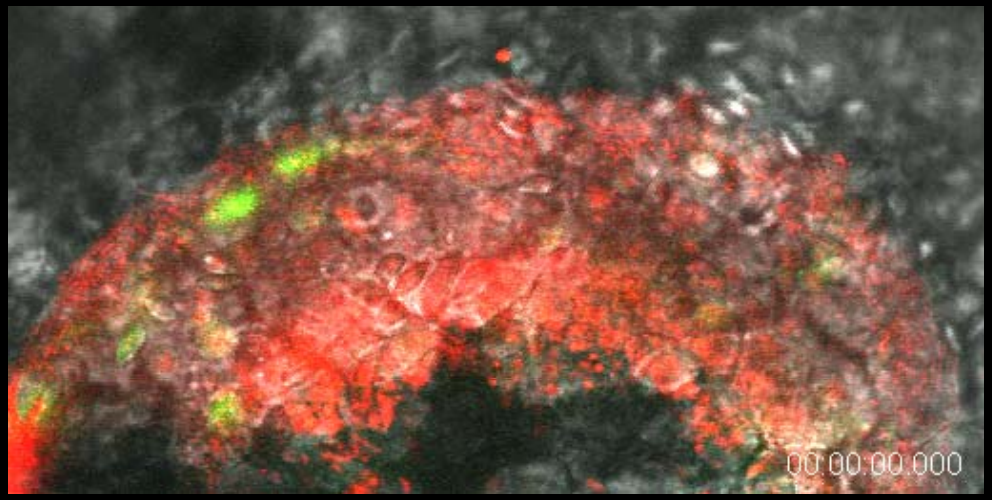




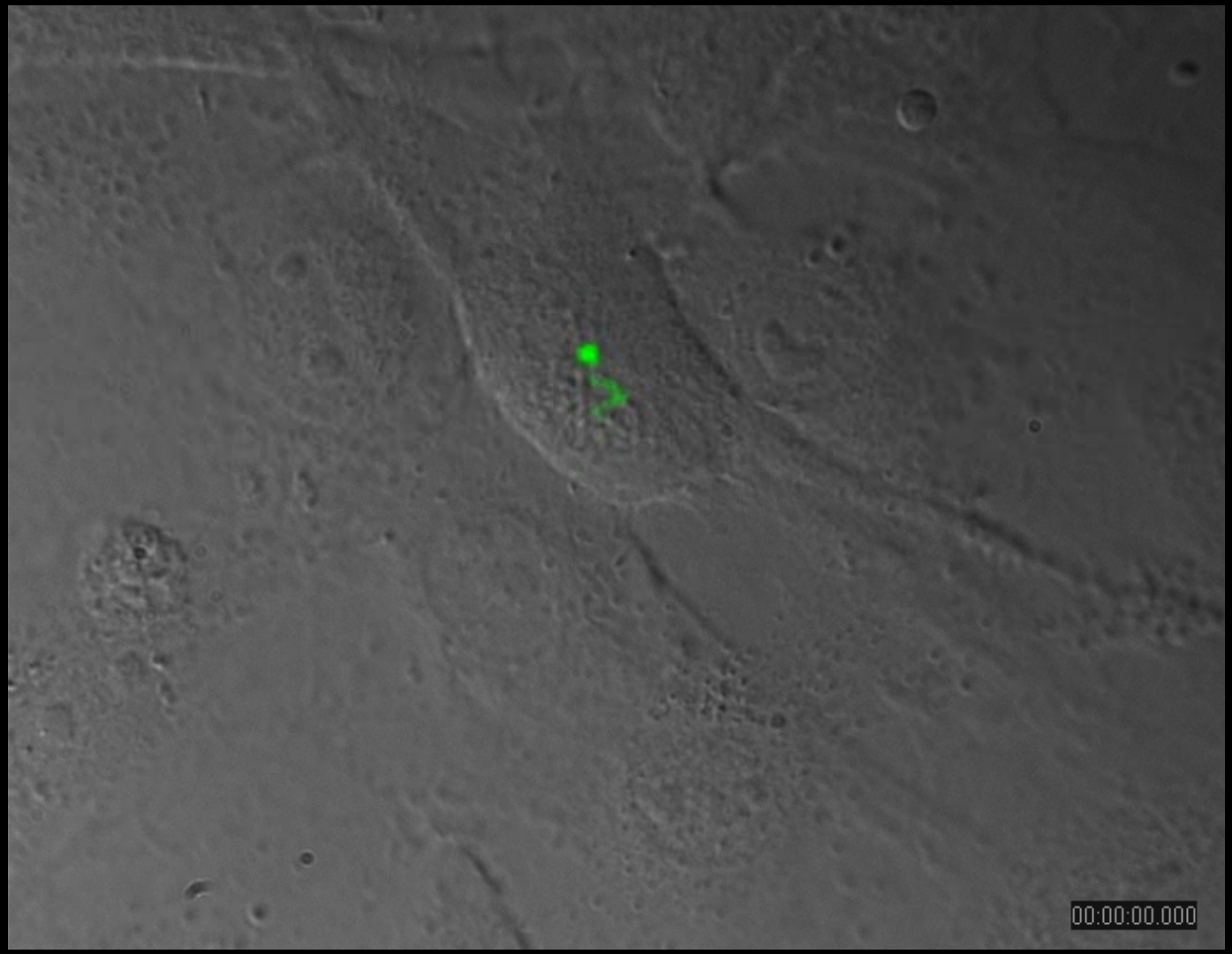
细胞的运动



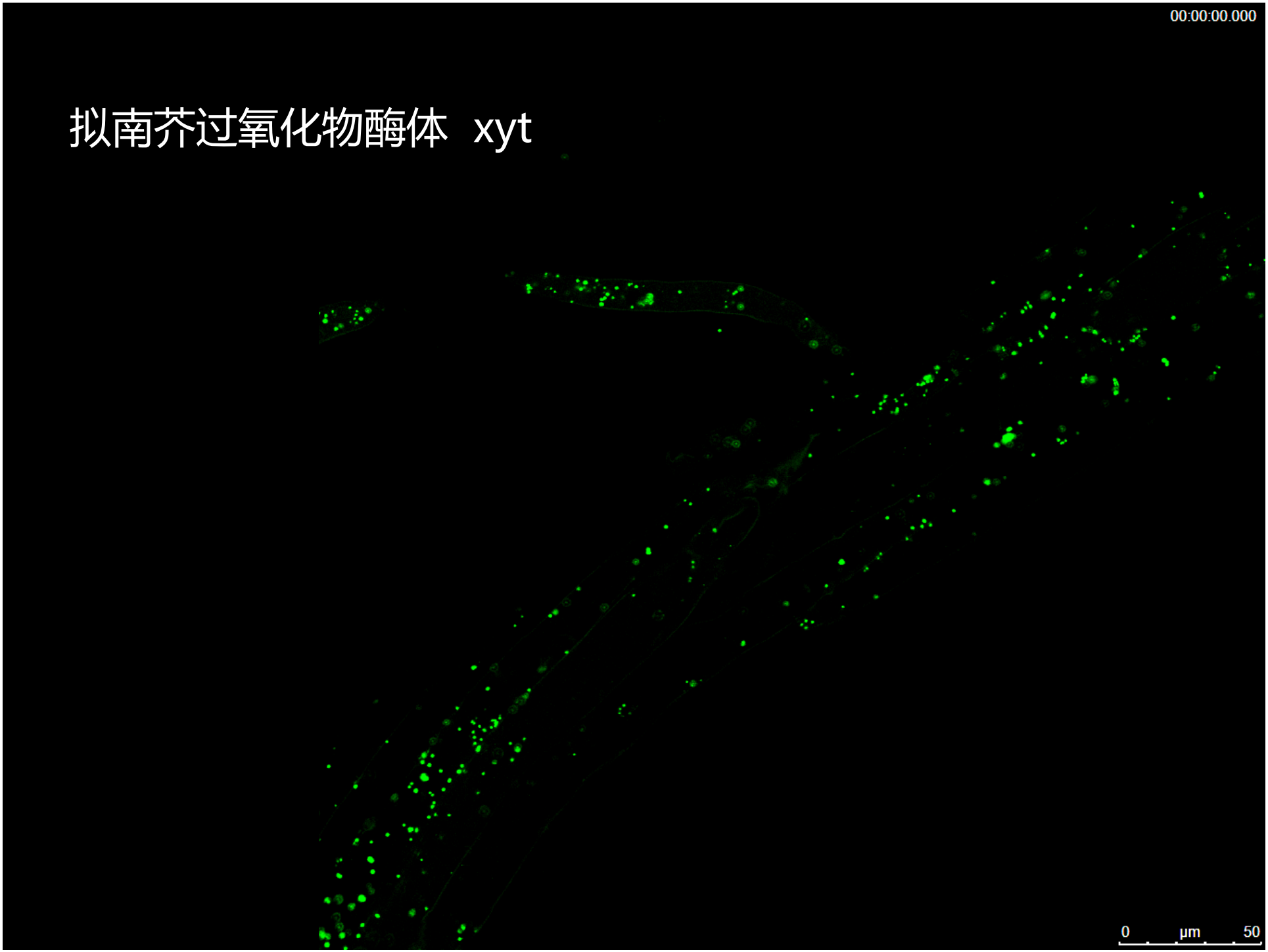




细胞连续增殖培养



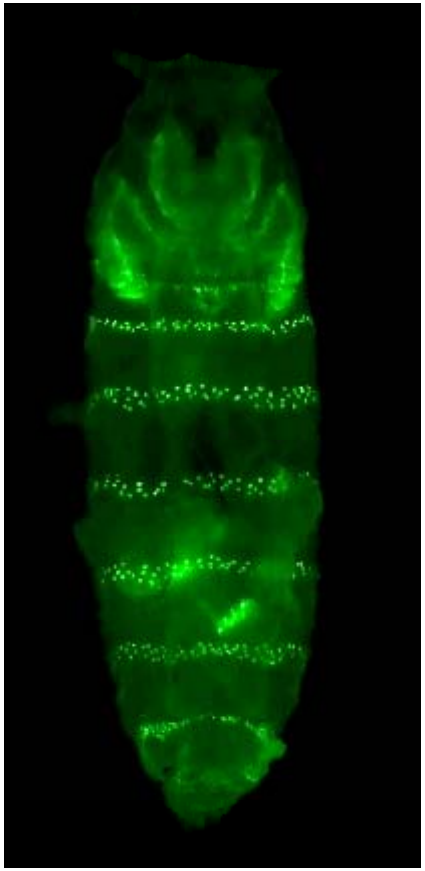
细胞有丝分裂


00:00:00.000
拟南芥过氧化物酶体 xyt
0 μm 50

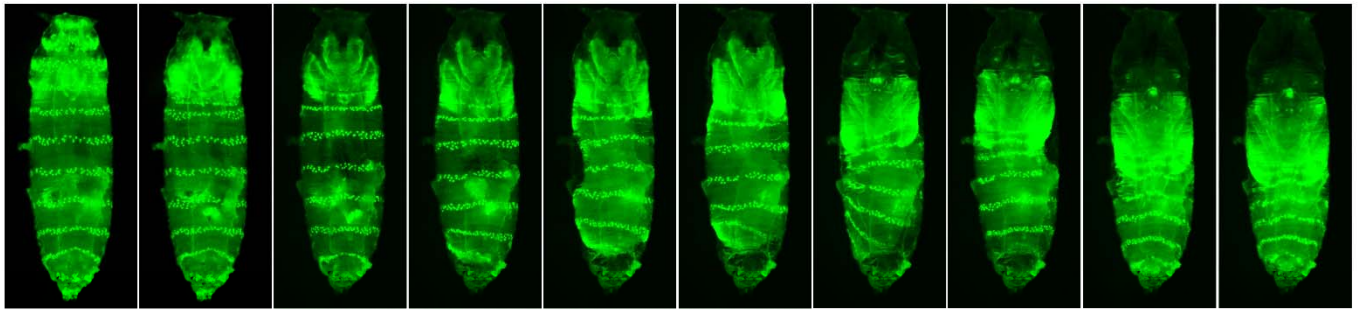
多维全景图像的采集及拼接

Macro Time-Lapse

X-Y-Z-T / 15 h

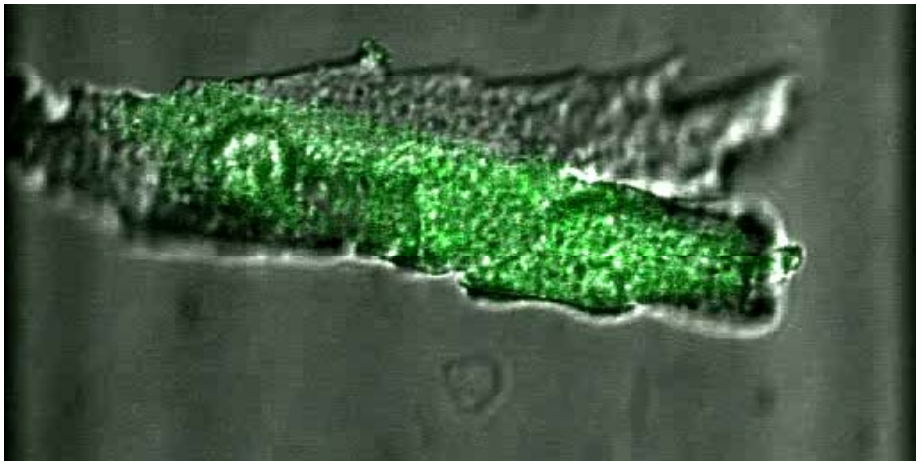


果蝇幼虫蛹化过程体节变化

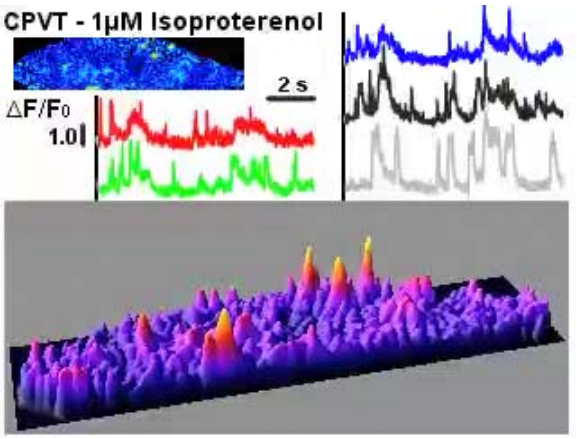


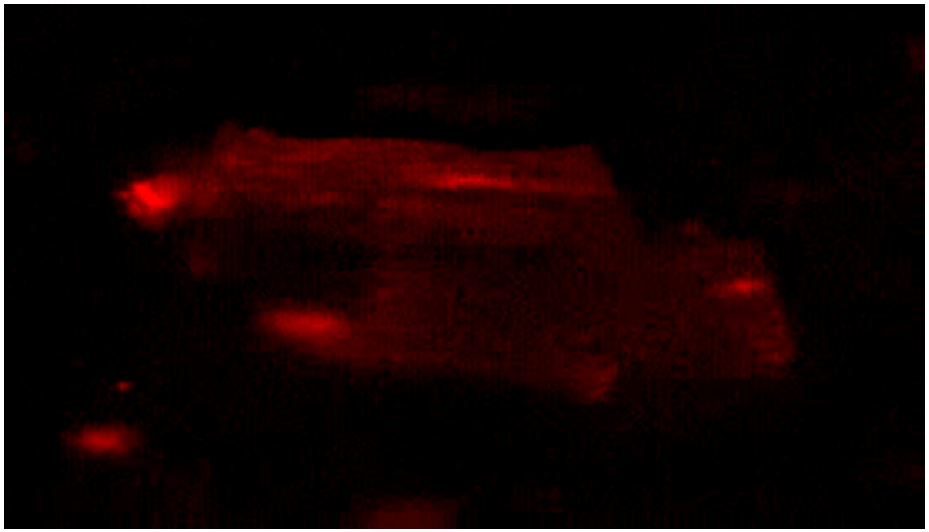
Sample: 15 hours time-lapse imaging of Drosophila after pupation. A fluorescence protein (Venus) was expressed in posterior compartment of each segment and the signal was detected clearly even under the presence of puparium.

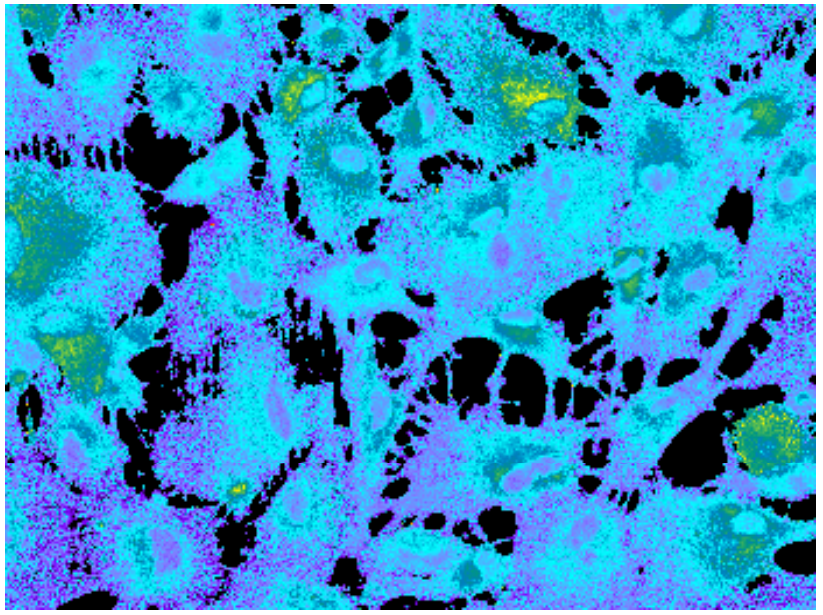
实时定量测定细胞内Ca^{2+}的变化



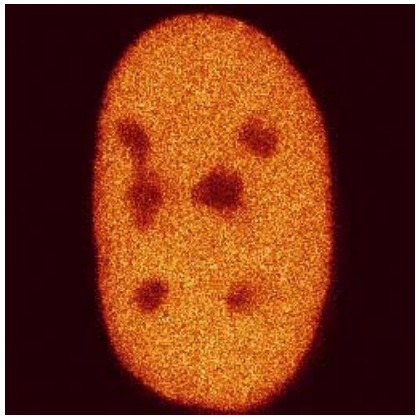
心肌细胞

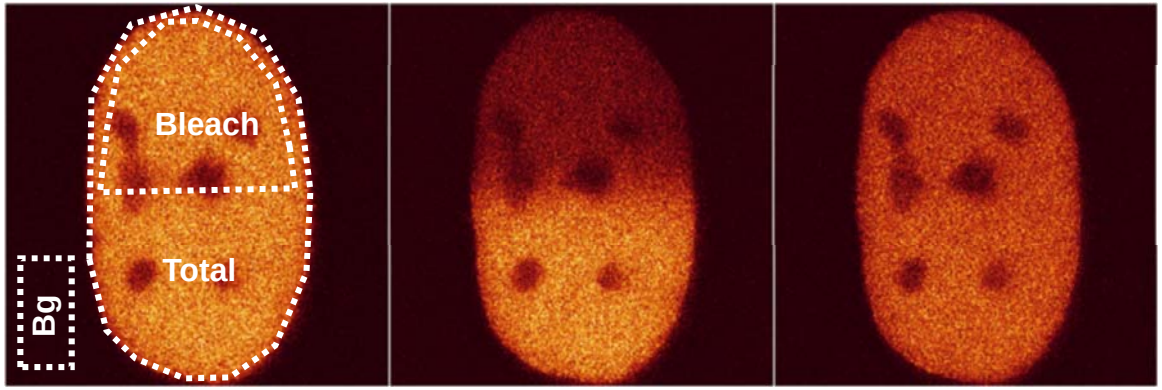






FRAP

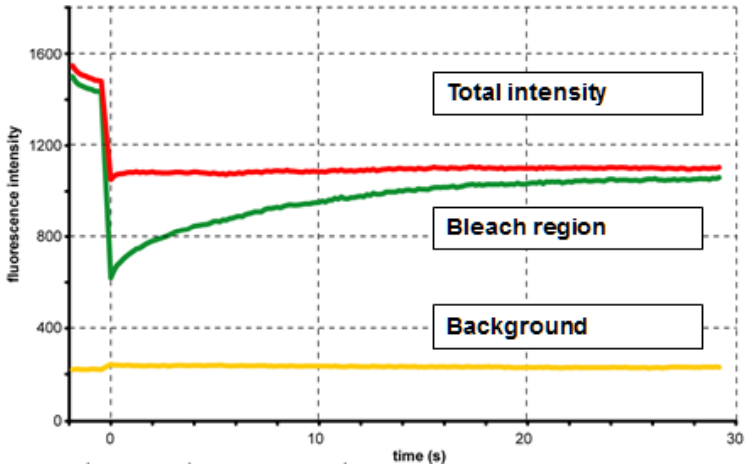


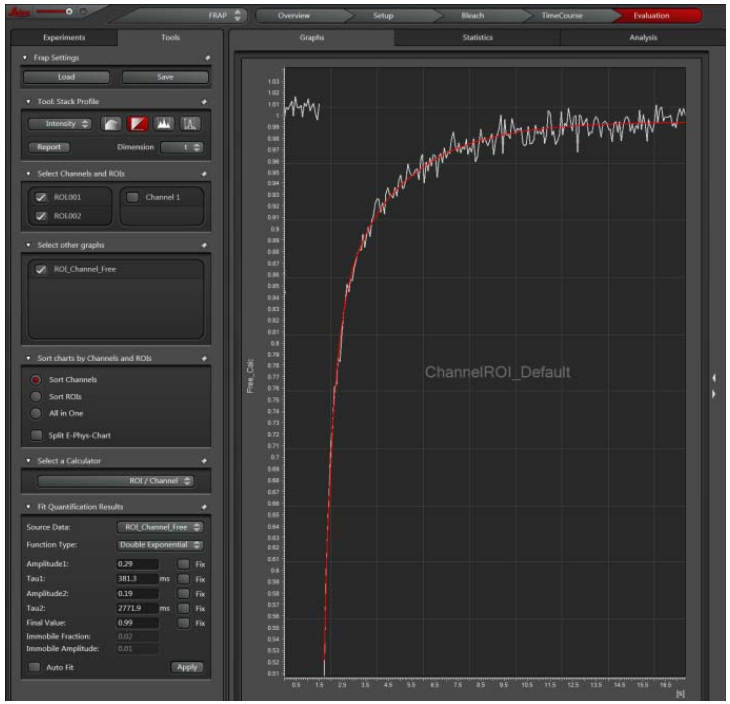


Quantitation

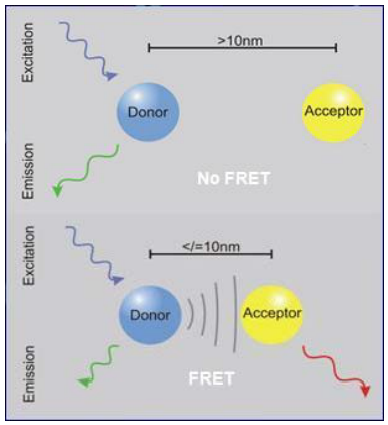
Measure average fluorescence intensities in regions of interests.

Courtesy of Peter Lenart, EMBL, Heidelberg



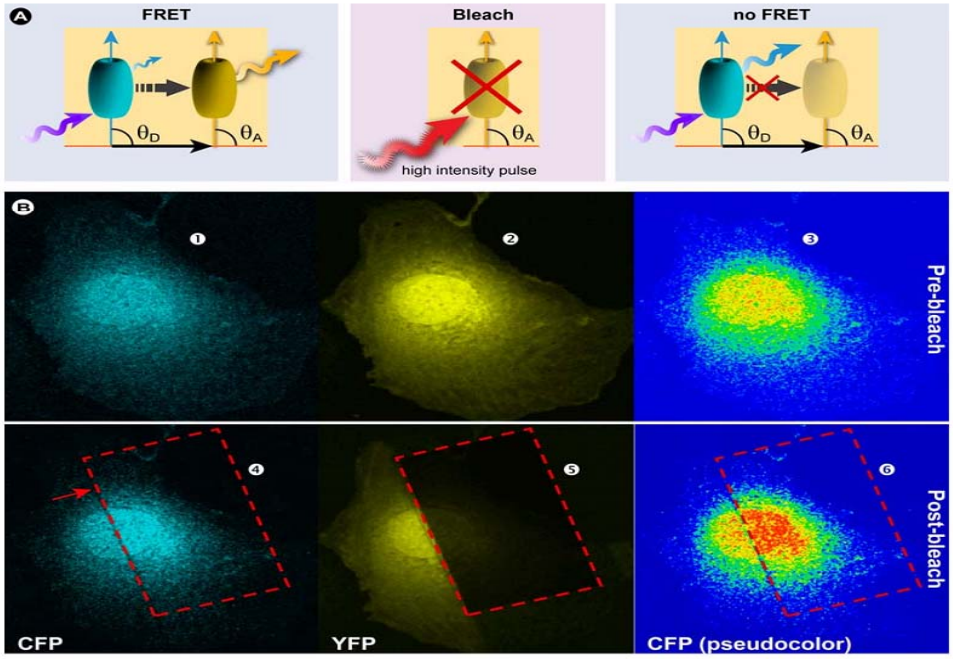


FRET



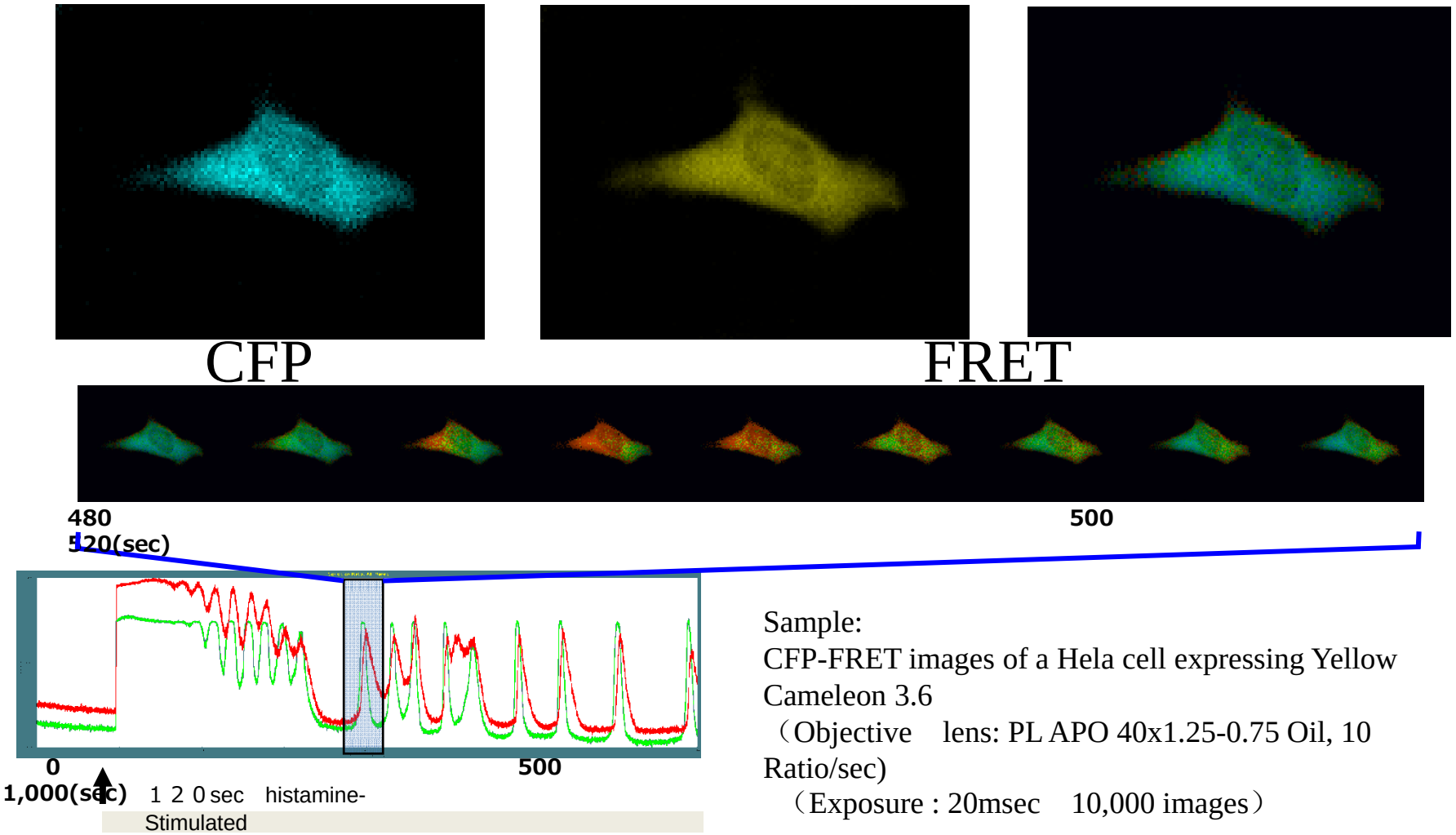
- **Acceptor Photobleaching-----FRET AB**

$$FRET_{eff} = (D_{post} - D_{pre}) / D_{post}$$



FRET —荧光共振能量转移

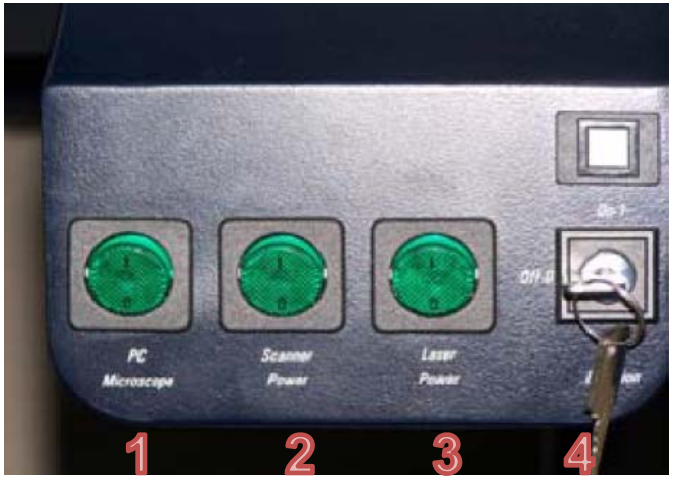
➢ **Sensitized Emission----FRET SE**



Sample:
CFP-FRET images of a Hela cell expressing Yellow Cameleon 3.6
（Objective lens: PL APO 40x1.25-0.75 Oil, 10 Ratio/sec)
（Exposure : 20msec 10,000 images）

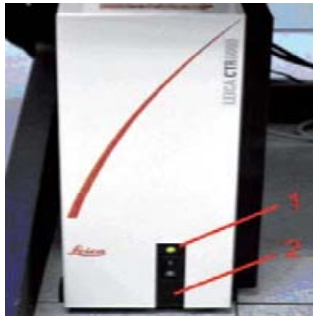
共聚焦基本操作流程：

开机：



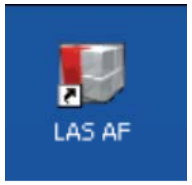
依次打开“PC Microscope”、“Scanner Power”及“Laser Power”三个圆形按钮，然后将“Laser Power”按钮右侧的激光开关钥匙（Laser Emission）顺时针旋转 90 度至“On-1”位置

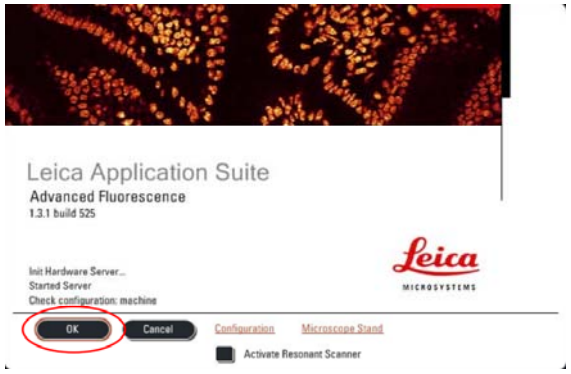
打开荧光电源



启动徕卡共聚焦软件

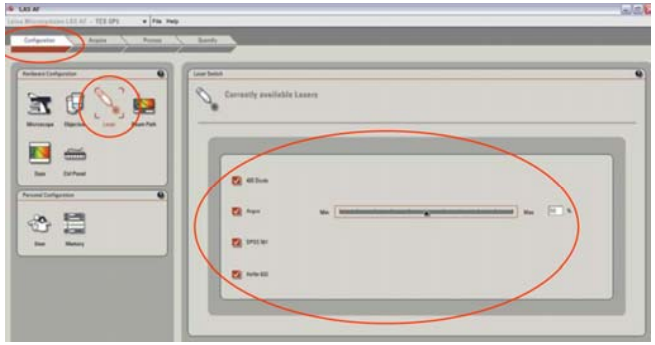
- 双击电脑显示屏桌面上的“LAS AF”图标启动徕卡共聚焦软件并点击OK按钮



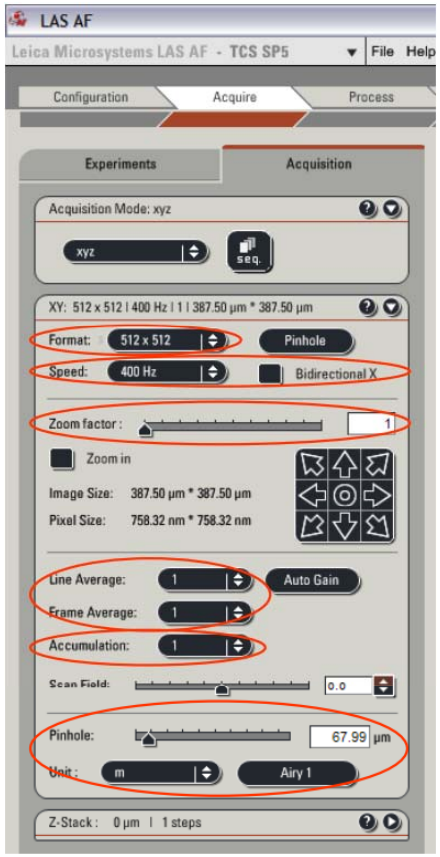


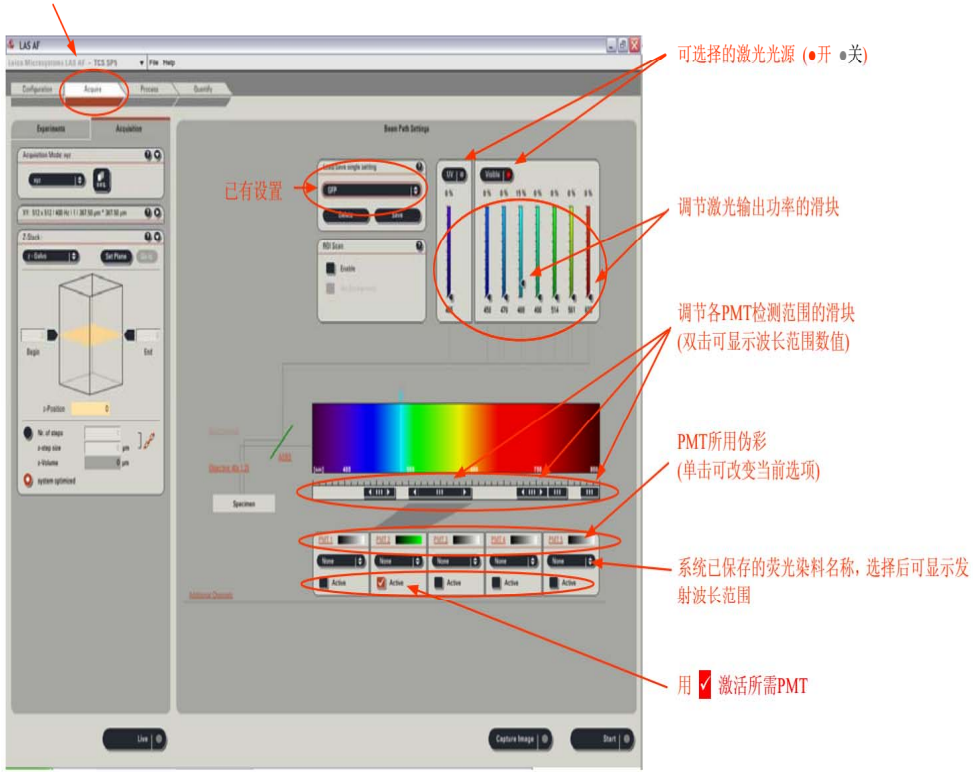
图像采集

- 激光打开



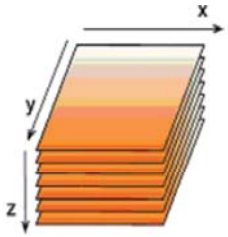
- 光路设置



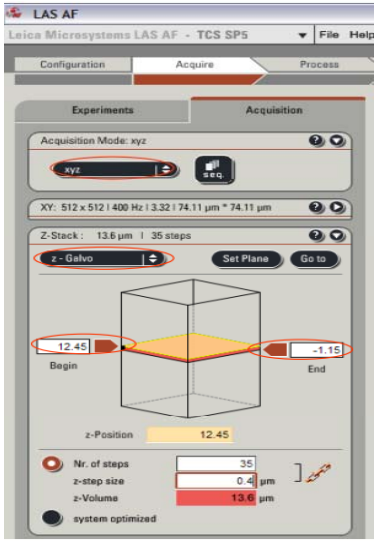


图像采集

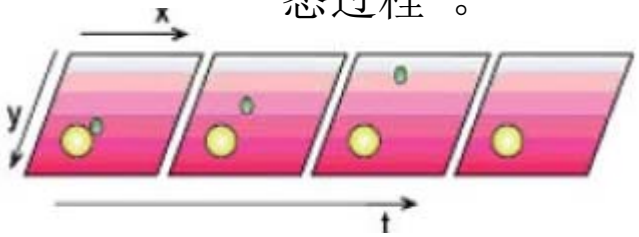
- XYZ图像采集

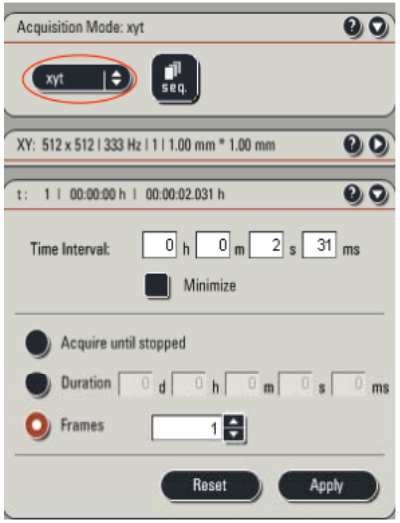


Z轴层切用于观察样品中目标的空间分布



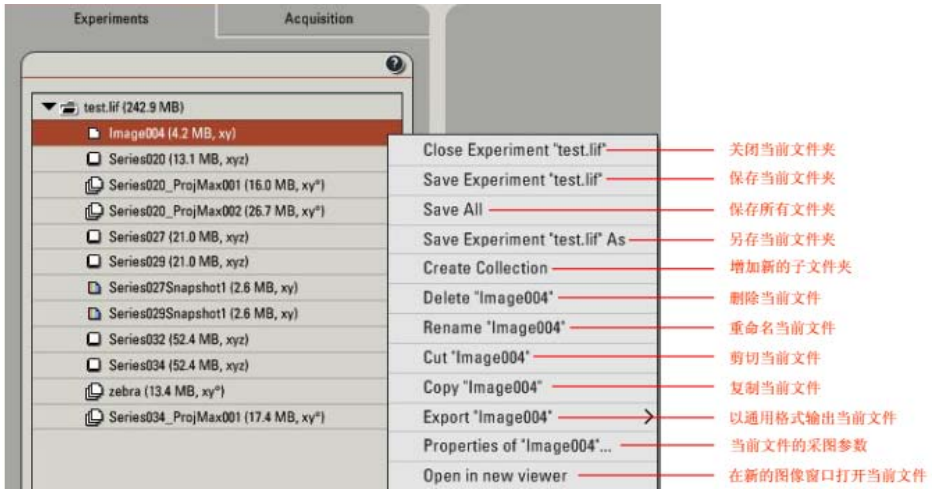
- XYT图像采集 时间序列扫描多用于活细胞成像，记录动态过程 。



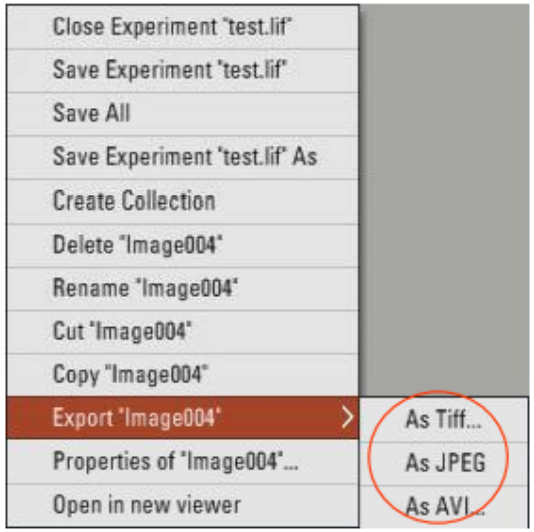


文件保存和输出

- 文件保存



- 文件的导出



关机

3 2 4 1

1. 关闭显微镜荧光电源。
2. 若使用过油镜，需用无水乙醚与无水乙醇混合液(体积比 7： 3)或无水乙醇清洁镜头。
3. 关闭 LAS AF 软件。
4. 将电脑桌右侧“Laser Power”按钮右侧的激光开关钥匙(Laser Emission)逆时针旋转 90 度至“On-0”位置。
5. 关闭“Scanner Power”按钮。
6. 在电脑上进行图像数据的输出。注意：使用空白光盘刻录，不得使用任何其他形式的移动存储介质。
7. 关闭电脑后，关闭“PC Microscope”按钮。
8. 风扇停止后(关闭激光开关钥匙约 5 分钟后)，关闭“Laser Power”按钮。记录关机时间、仪器状况等信息。

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- Surface Metrology
- Surgical Microscopy
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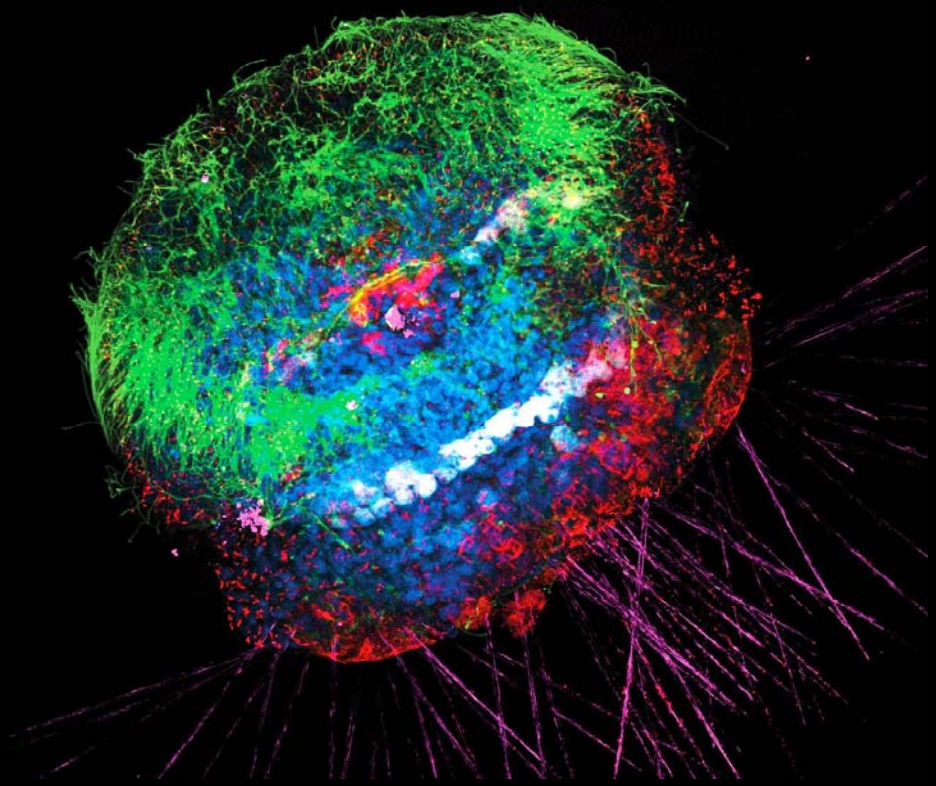

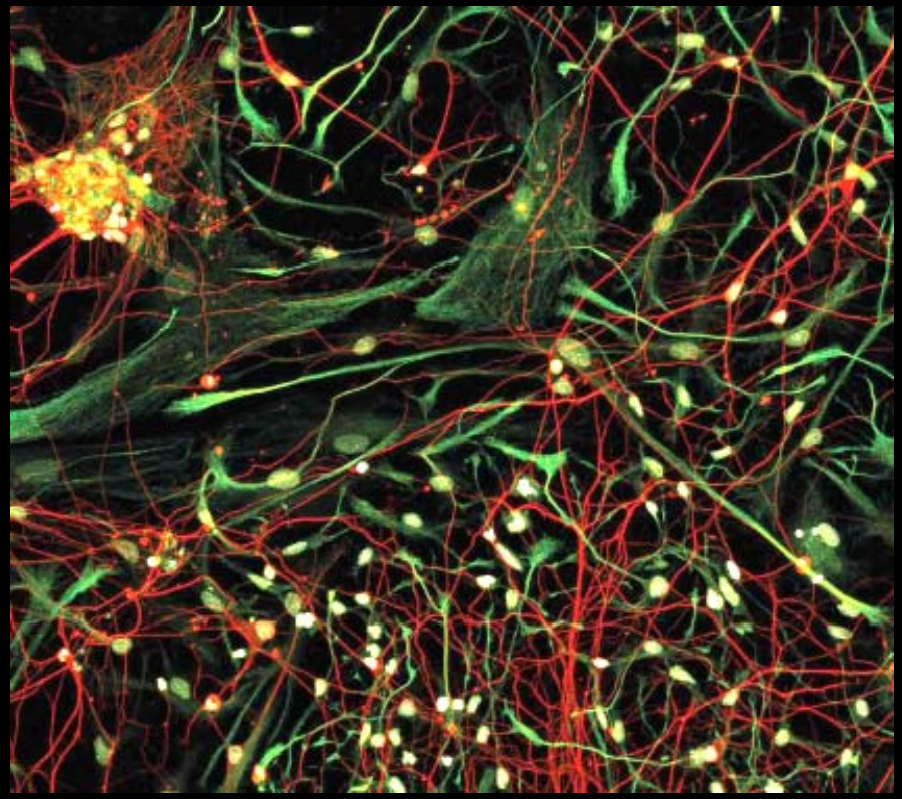

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THANX

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