光学显微成像分析

Light Microscopy

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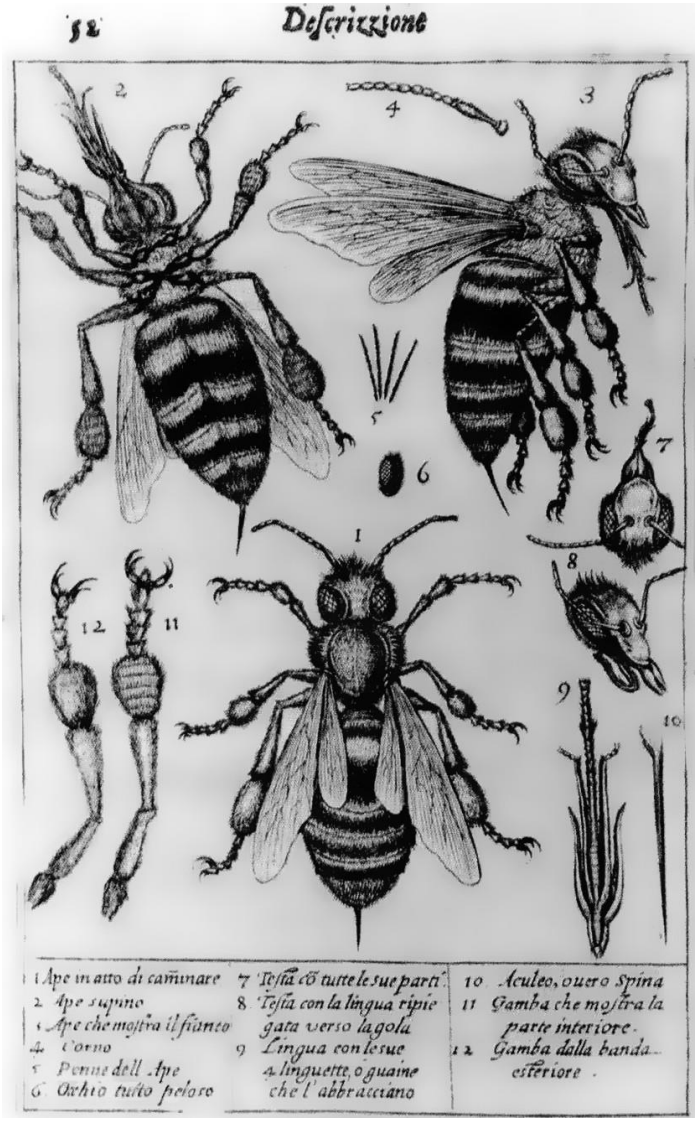
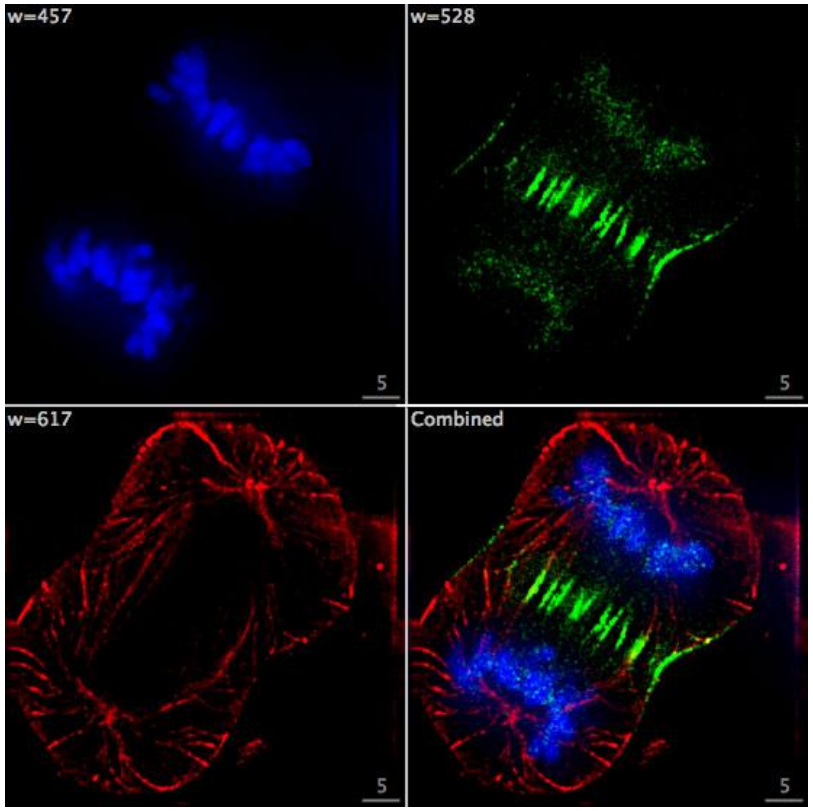
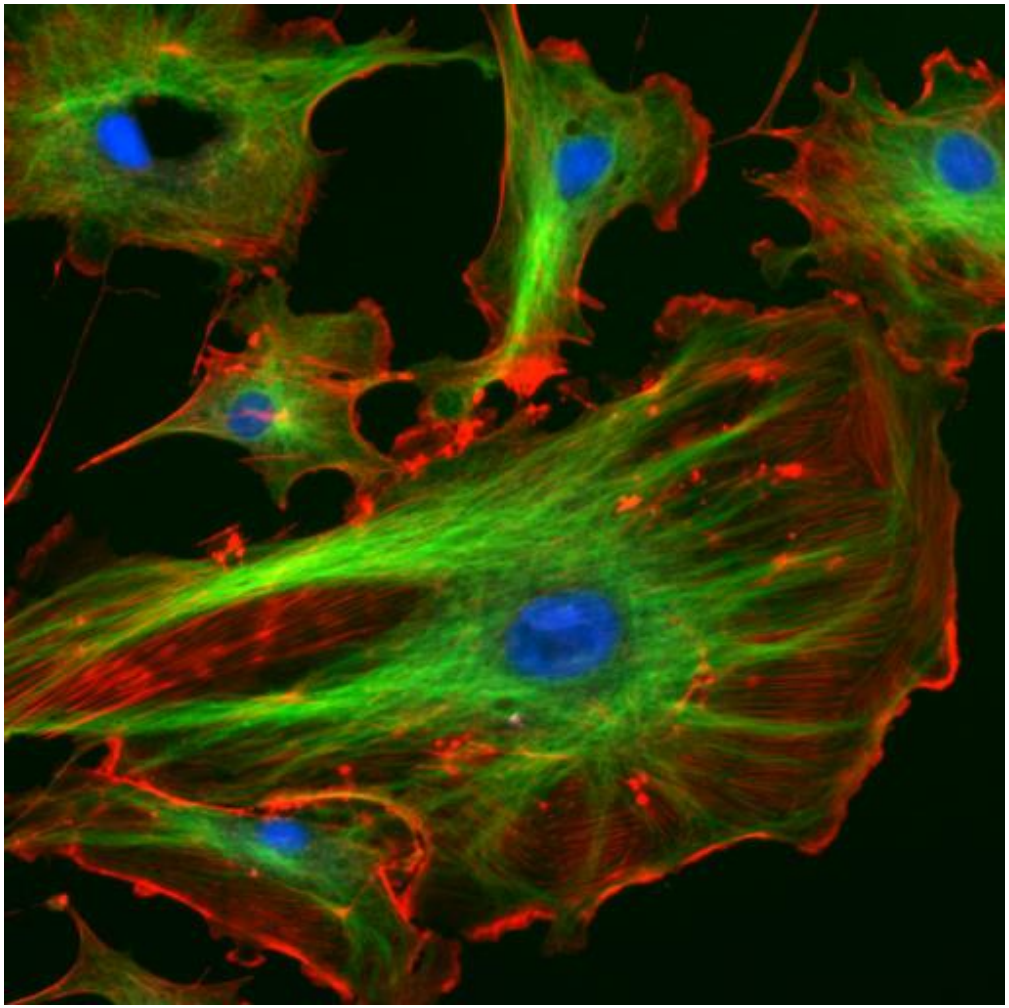


The oldest published image known to have been made with a microscope: bees by Francesco Stelluti, 1630

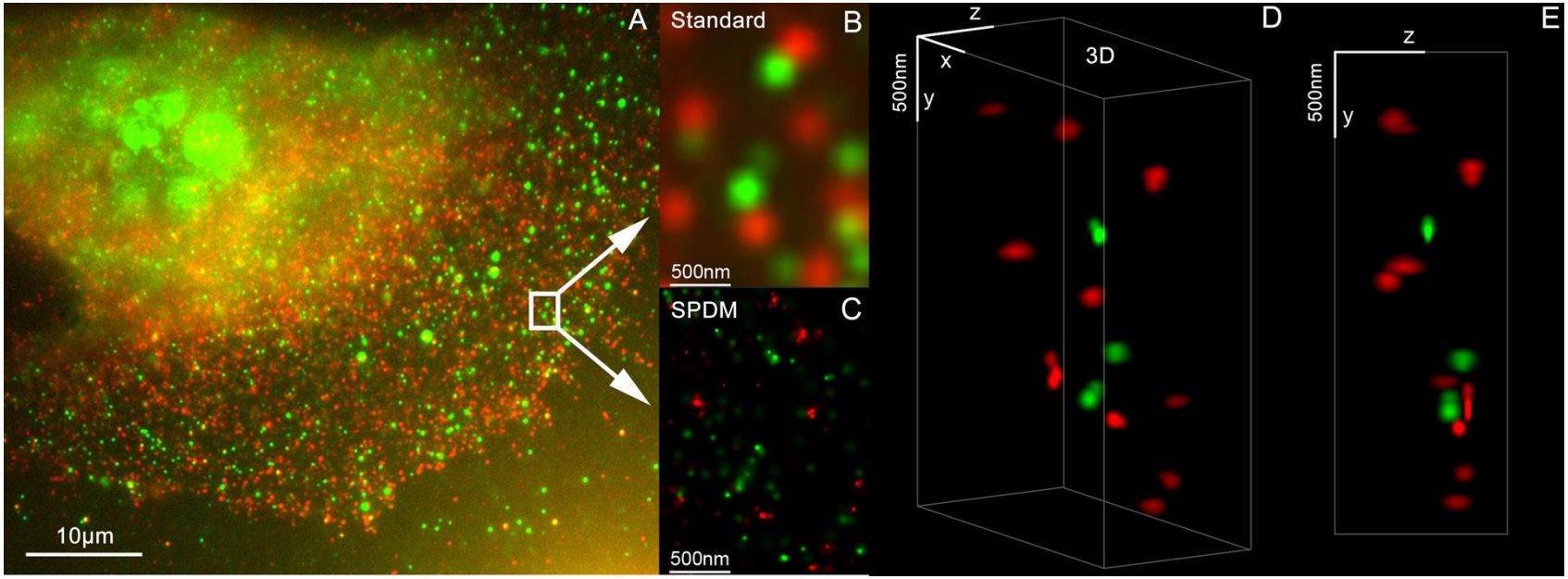
Gould (2000) The Lying Stones of Marrakech. Harmony Books. ISBN 0-609-60142-3.



Epifluorescent imaging of the three components in a dividing human cancer cell. DNA is stained blue, a protein called INCENP is green, and the microtubules are red. Each fluorophore is imaged separately using a different combination of excitation and emission filters, and the images are captured sequentially using a digital CCD camera, then overlaid to give a complete image.

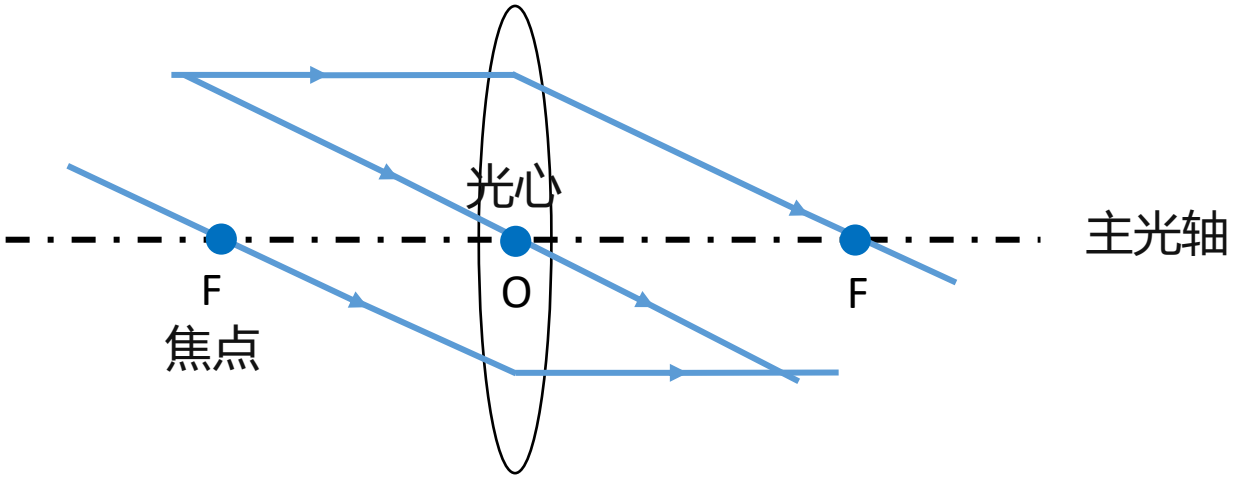


Endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to FITC and actin filaments are labeled red with phalloidin bound to TRITC. Bovine pulmonary artery endothelial (BPAE) cells



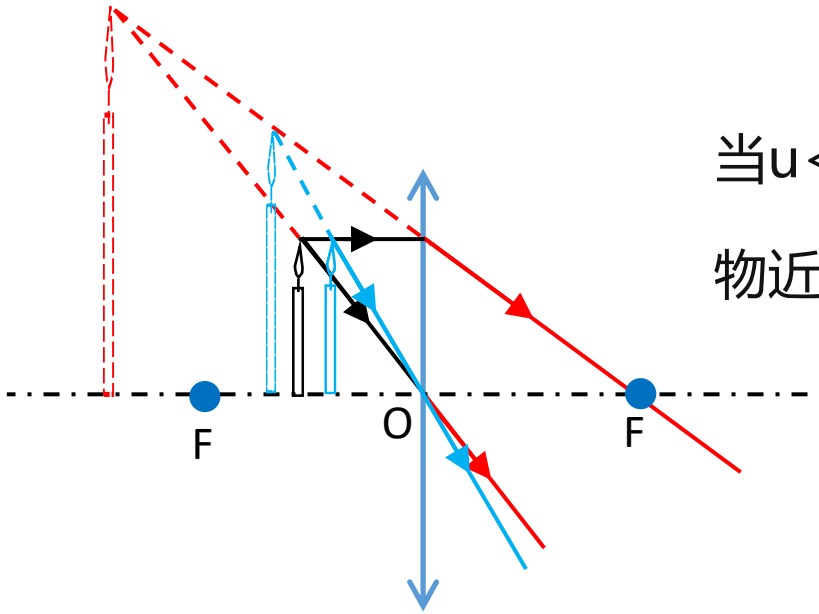
3D dual color super resolution microscopy with Her2 and Her3 in breast cells, standard dyes: Alexa 488, Alexa 568 LIMON

光学显微成像的基本原理—背景知识



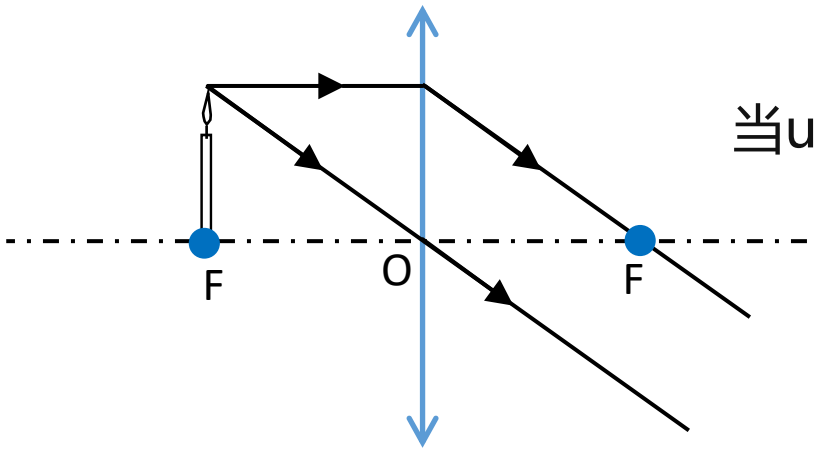
- 通过焦点的光线，平行于主光轴射出
- 通过光心的光线传播方向不改变
- 平行于主光轴上的光线汇聚到焦点

光学显微成像的基本原理—背景知识



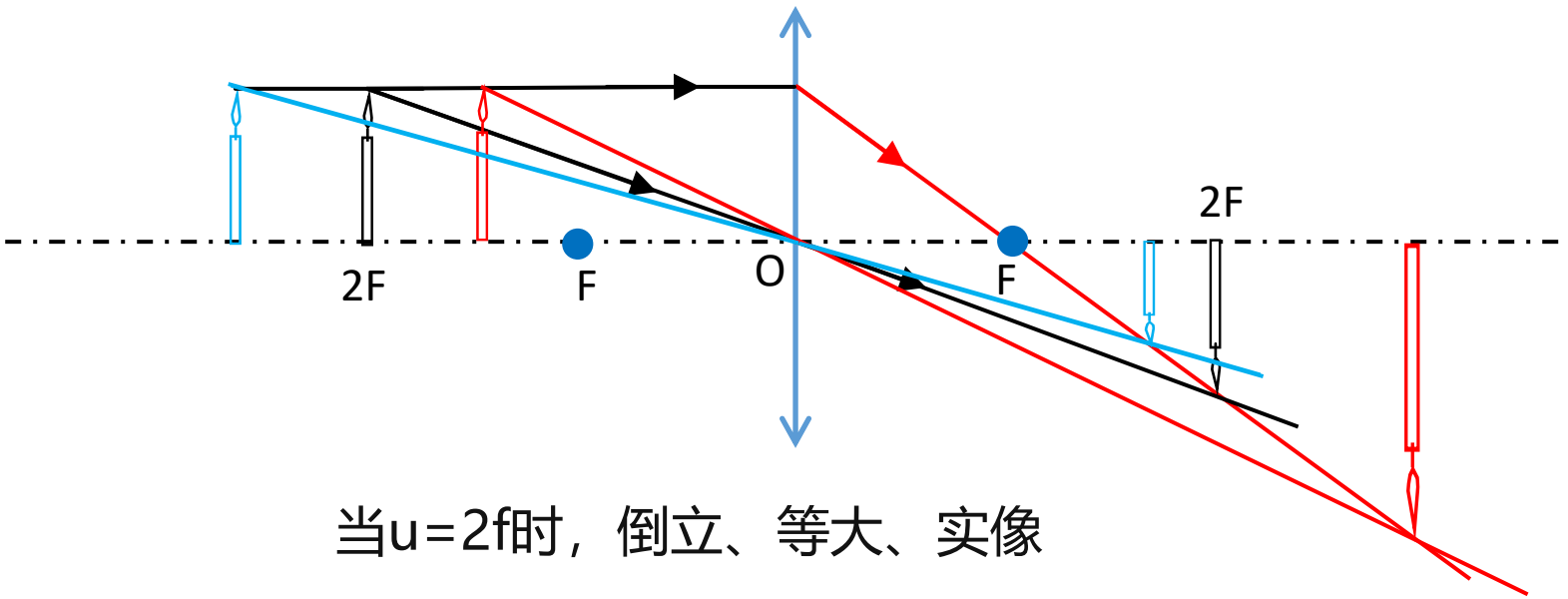
当u<f时，正立、放大虚像

物近像近像变小　　**放大镜**



当u=f时，平行光

光学显微成像的基本原理—背景知识



当u=2f时，倒立、等大、实像

当f<u<2f时，倒立、放大、实像

物近像远像变大

投影仪

当u>2f时，倒立、缩小、实像

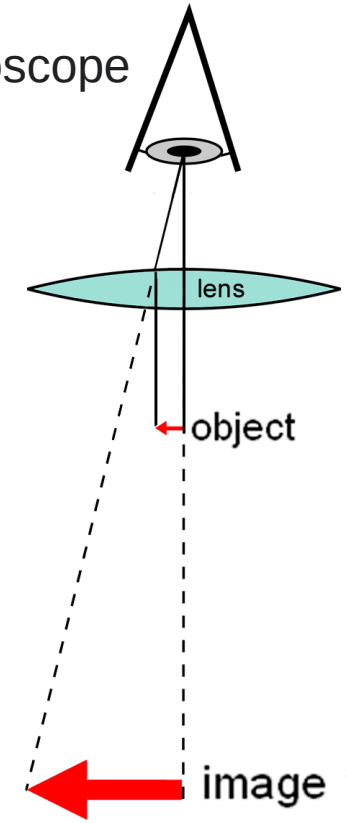
物近像远像变大

照相机

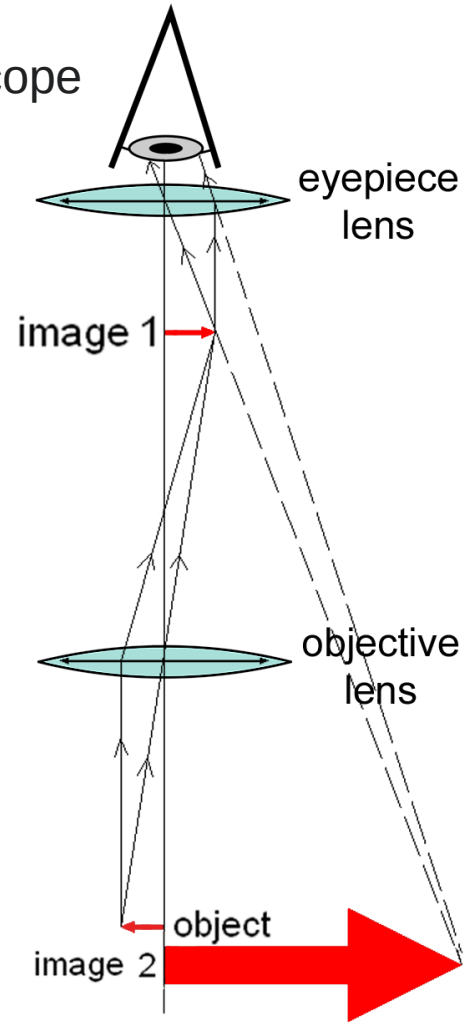
光学显微成像的基本原理

- 显微镜的放大原理：

simple microscope

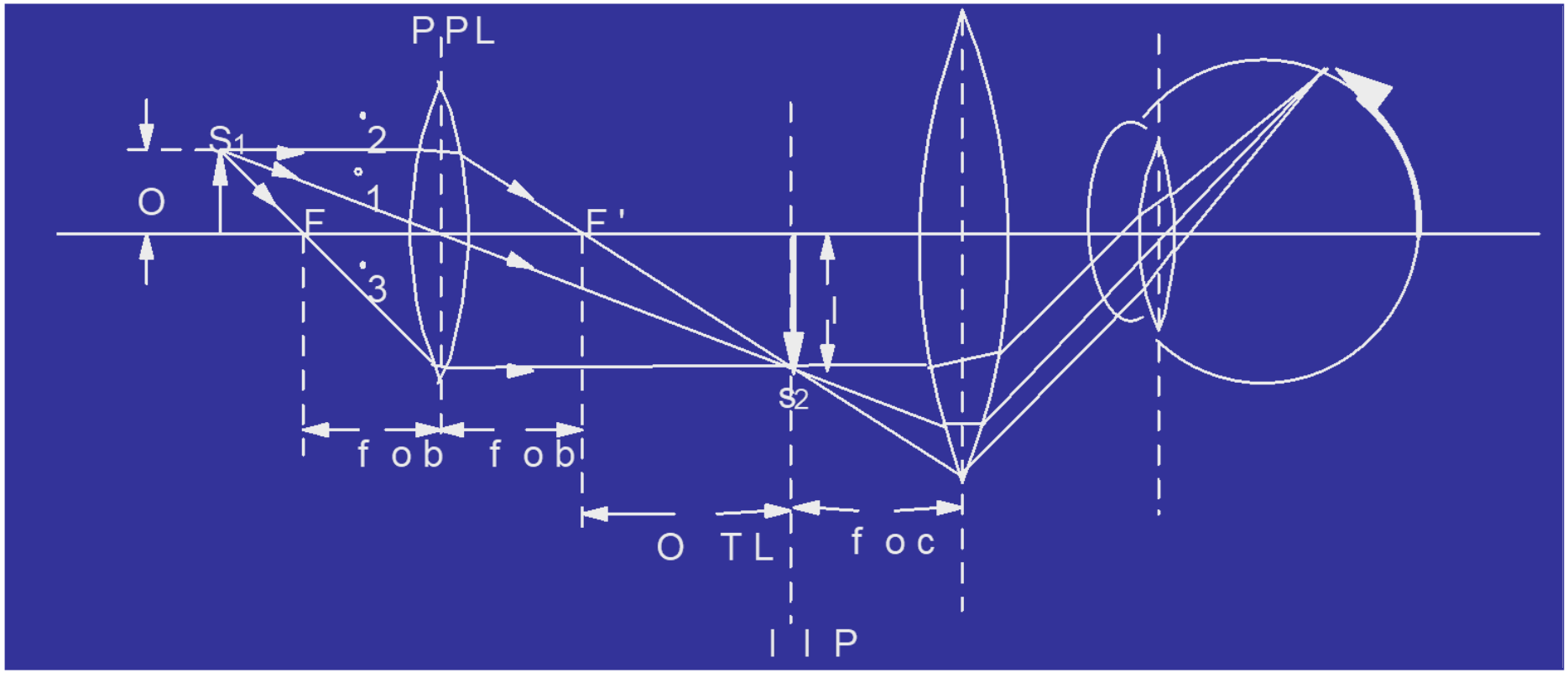


compound microscope



光学显微成像的基本原理

- 显微镜的放大原理：

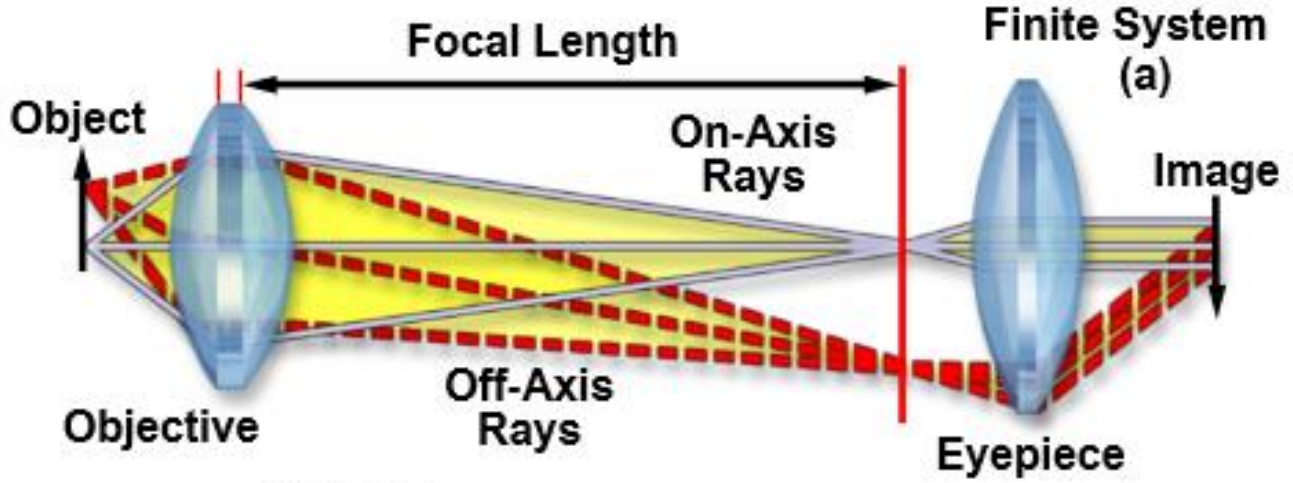


物体被放置在物镜的单聚焦点和双聚焦点之间。物镜产生的中间图像在目镜的前聚焦点和目镜之间聚焦。

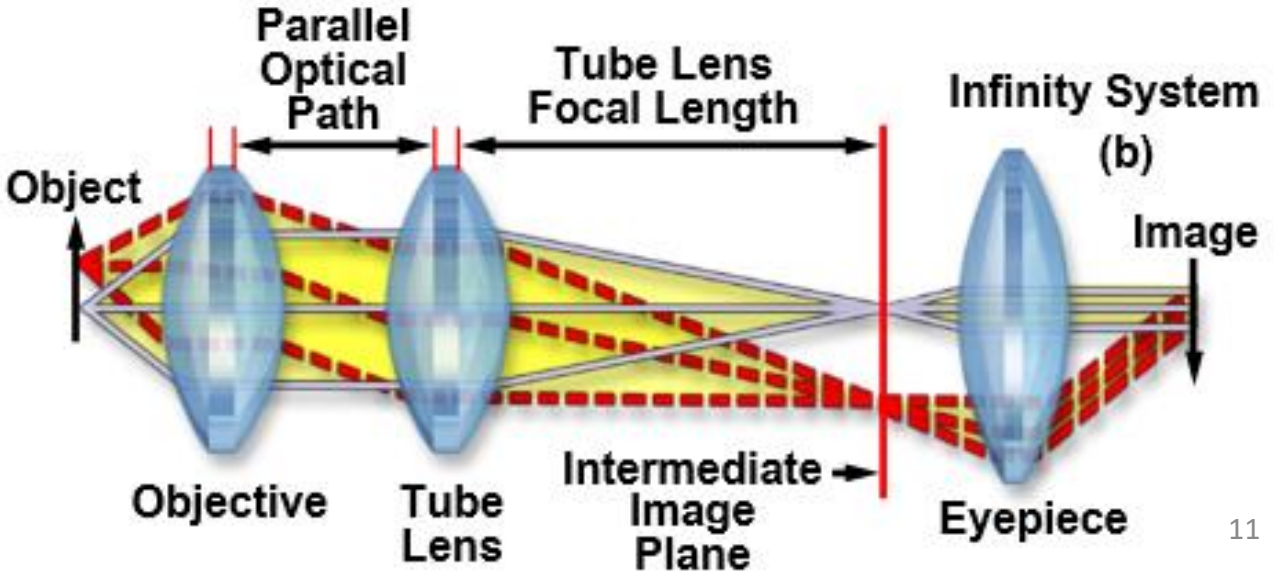
光学显微成像的基本原理

- 有限远光学系统与无限远光学系统

有限远
光学系统

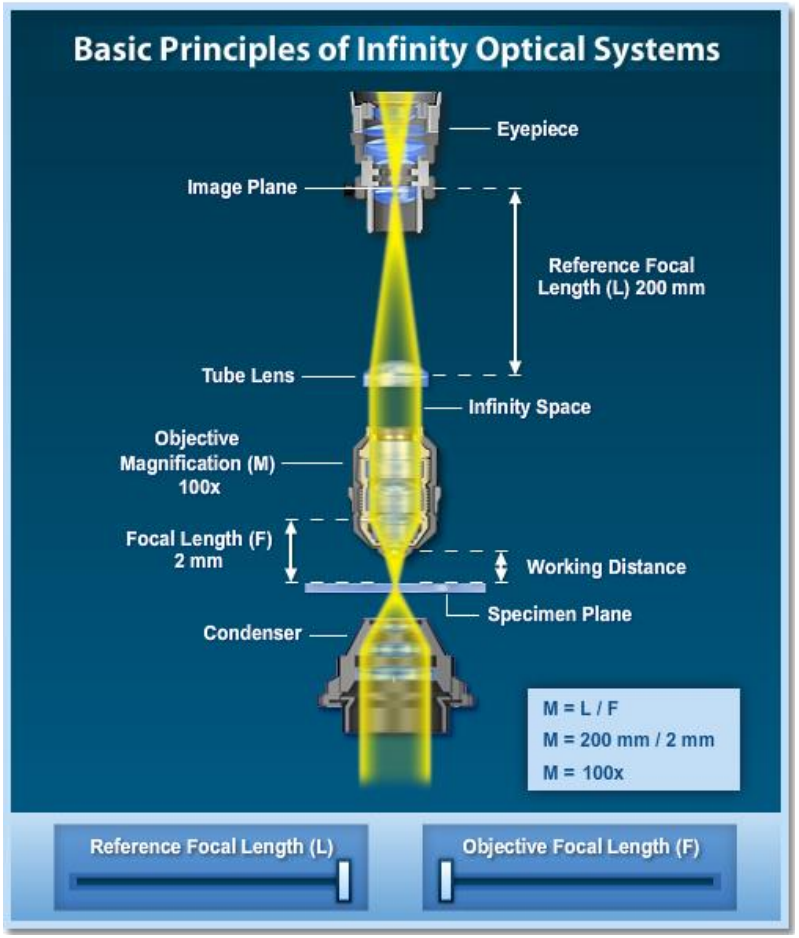


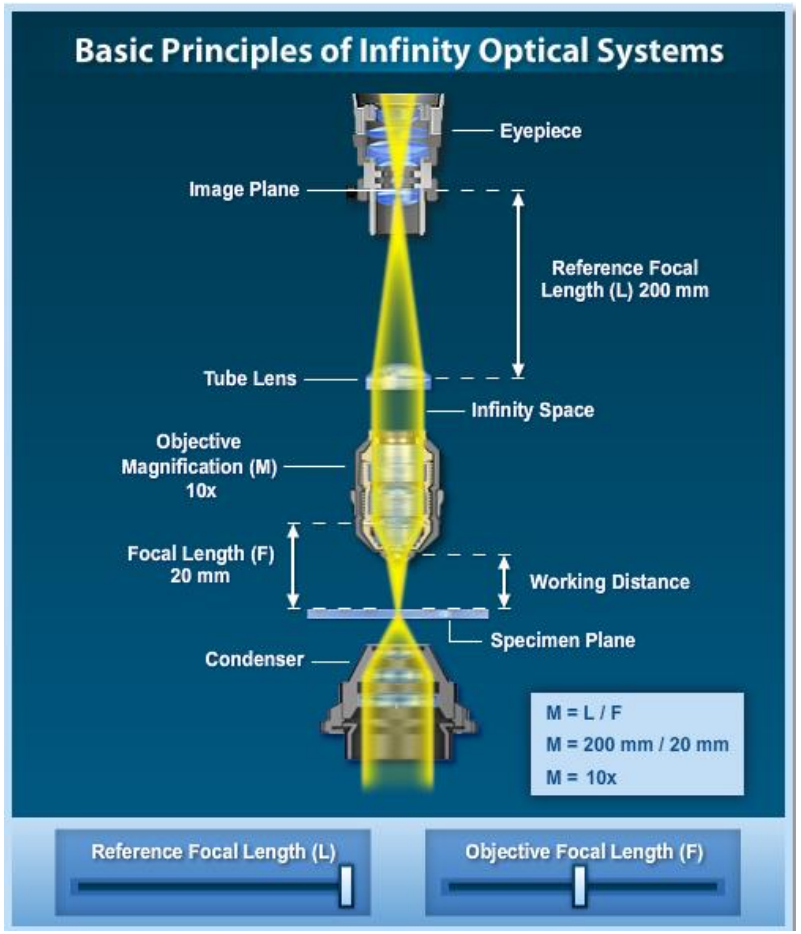
无限远
光学系统



光学显微成像的基本原理

- 无限远光学系统放大倍数

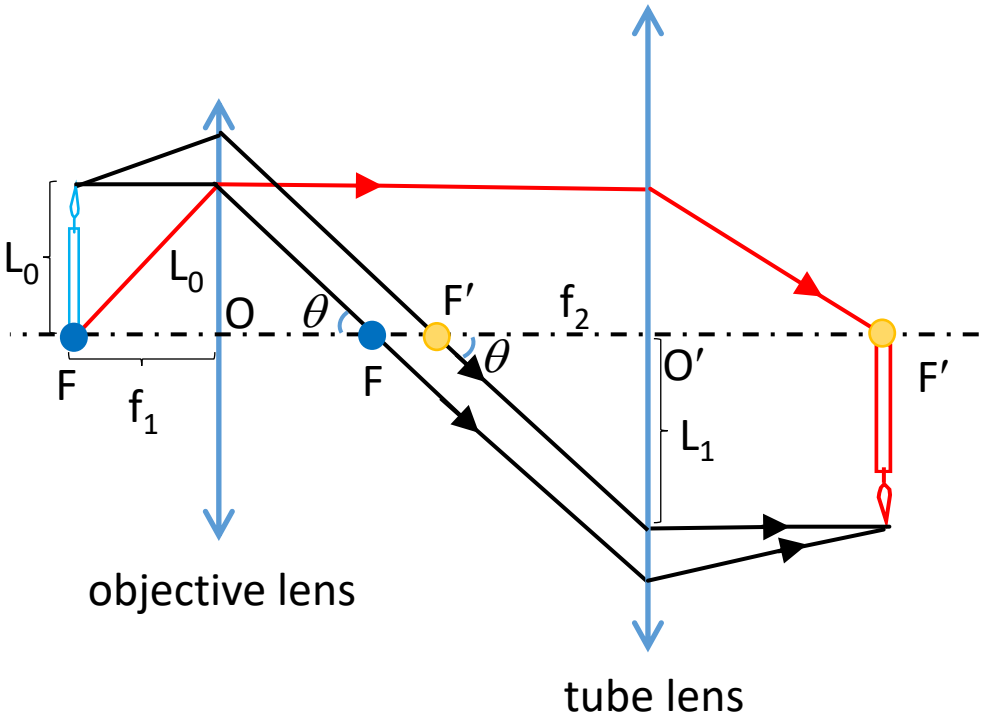




放大倍数由Tube Lens 的焦距Reference Focal Length 和物镜的焦距**Objective Focal Length**决定

光学显微成像的基本原理

- 无限远光学系统



$$\tan\theta = \frac{L_0}{f_1} = \frac{L_1}{f_2}$$

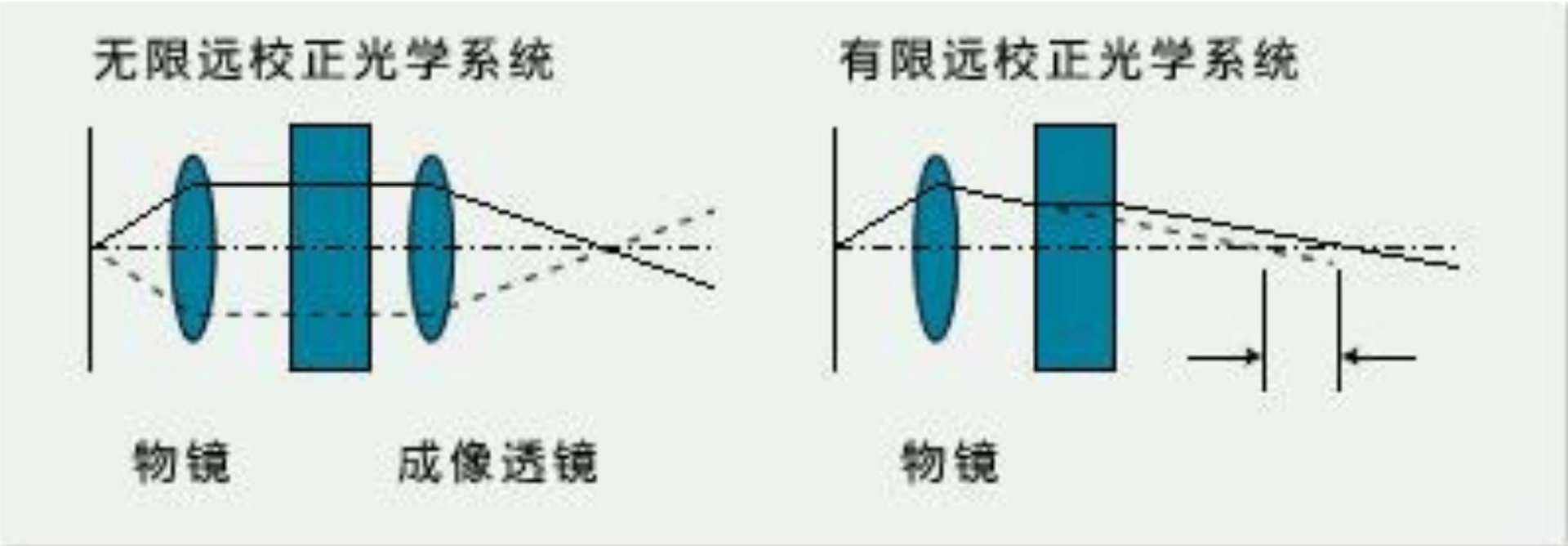
$$L_1 = \frac{f_2}{f_1} \times L_0$$

光学显微成像的基本原理

- 无限远光学系统的优势

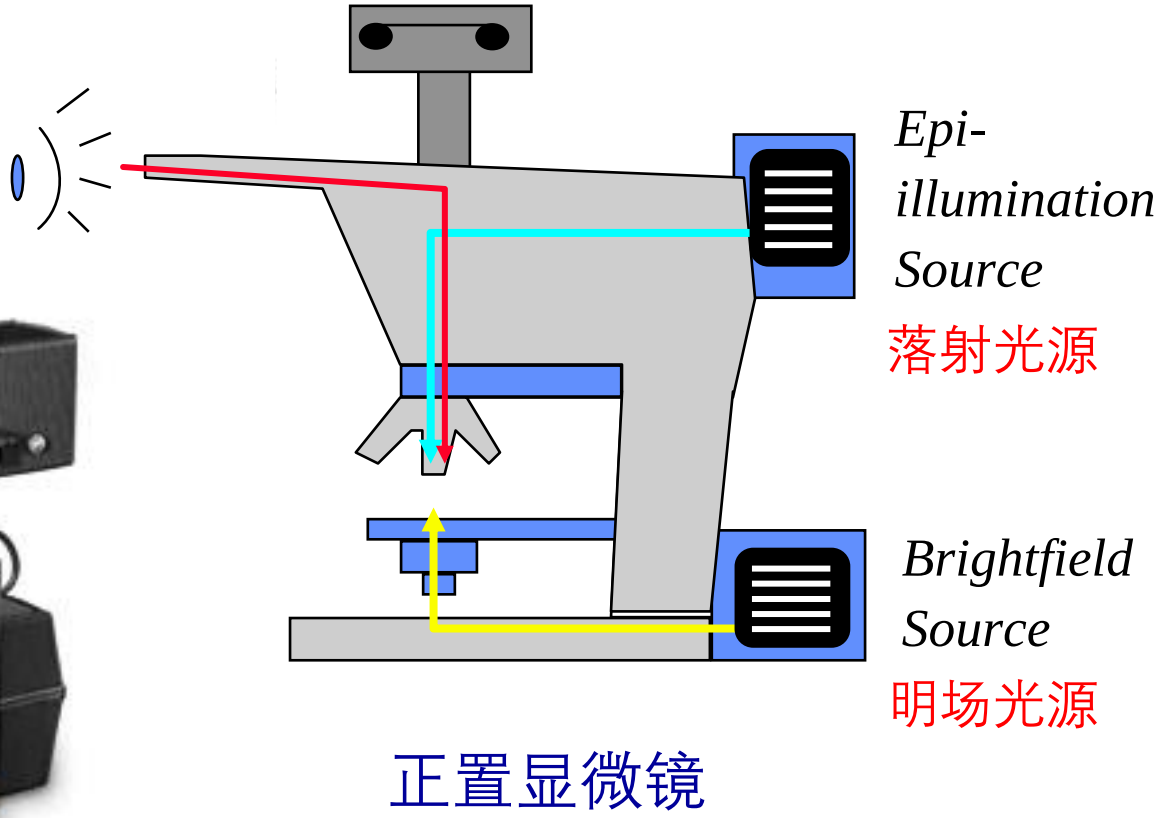
❖ 尽量减小物镜之后的光学器件对干扰光路的干扰;

❖ 移动物镜（而不是样品本身）来对焦。



光学显微成像的基本原理

- 正置与倒置显微镜的基本光路：



正置显微镜

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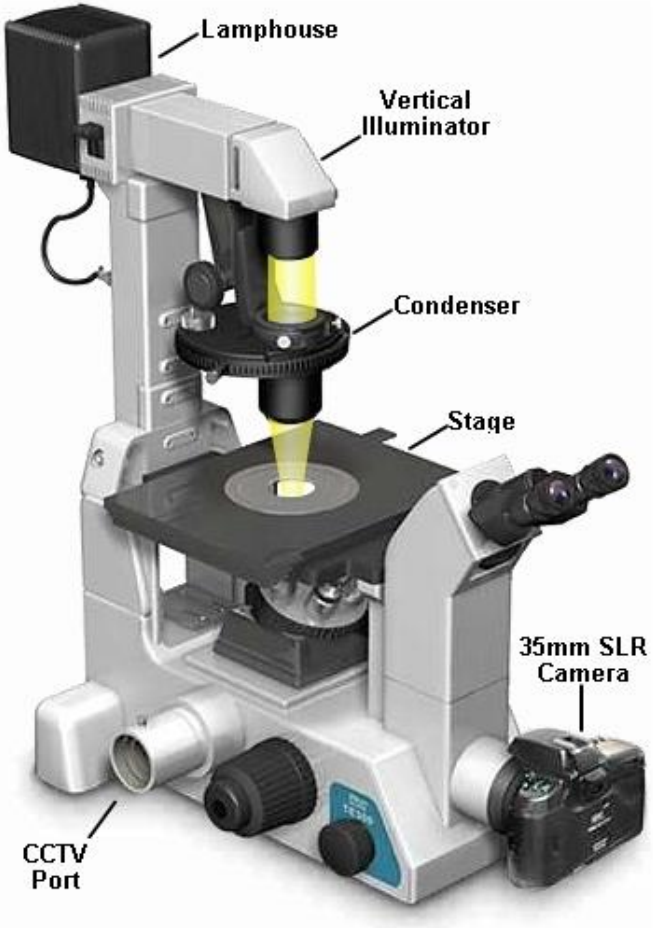
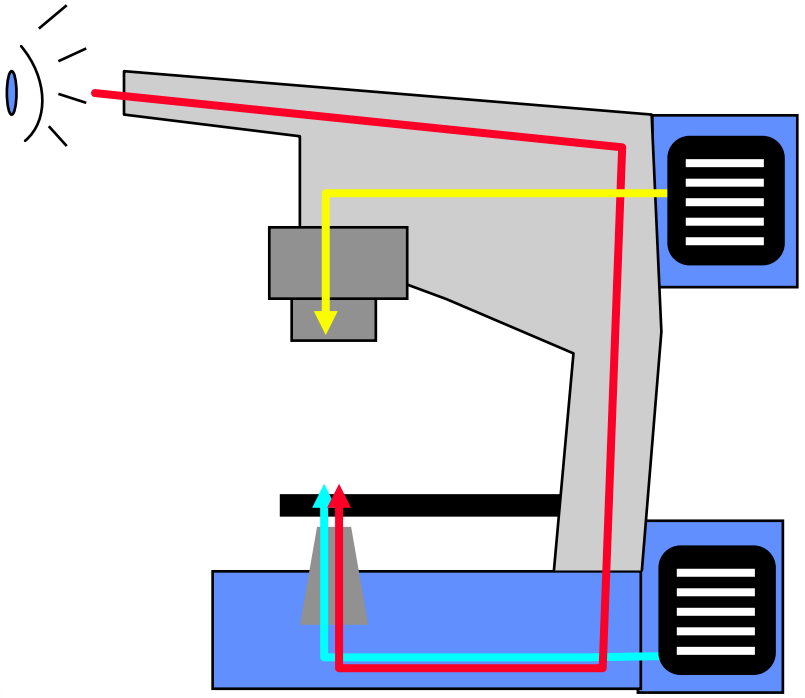
- 正置与倒置显微镜的基本光路：





Brightfield Source
明场光源

Epi-illumination Source
落射光源

倒置显微镜

光学显微成像的基本原理

- 物镜（Objectives）：

Q：怎么理解Tube length是∞？

Q：Numerical Aperture数值孔径是什么？

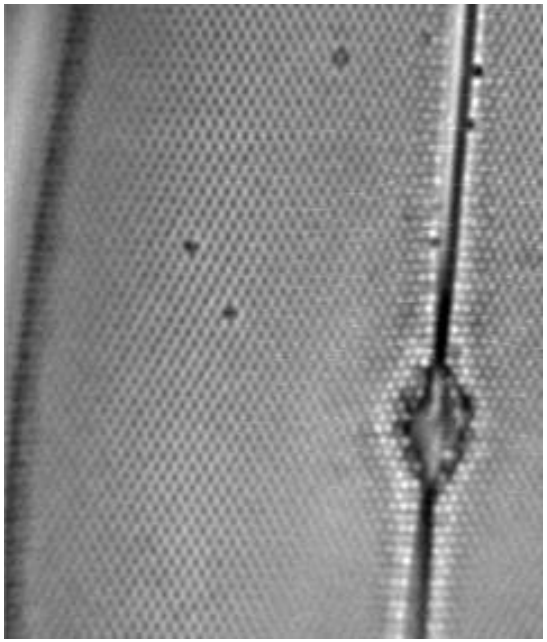
光学显微成像的基本原理

- 显微镜的分辨率：

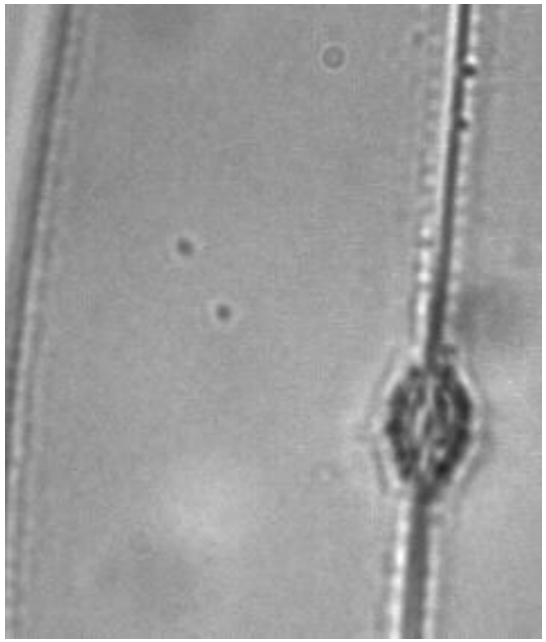
❖ 显微镜的分辨率取决于物镜的数值孔径（NA）：

$$d = \frac{0.61\lambda}{\mathrm{NA}} = \frac{0.61\lambda}{n\sin\theta}$$

λ = wavelength of light
d = minimal distance to distinguish between two close objects
$n \sin \theta$ = numerical aperture



High NA



Low NA

光学显微成像的基本原理

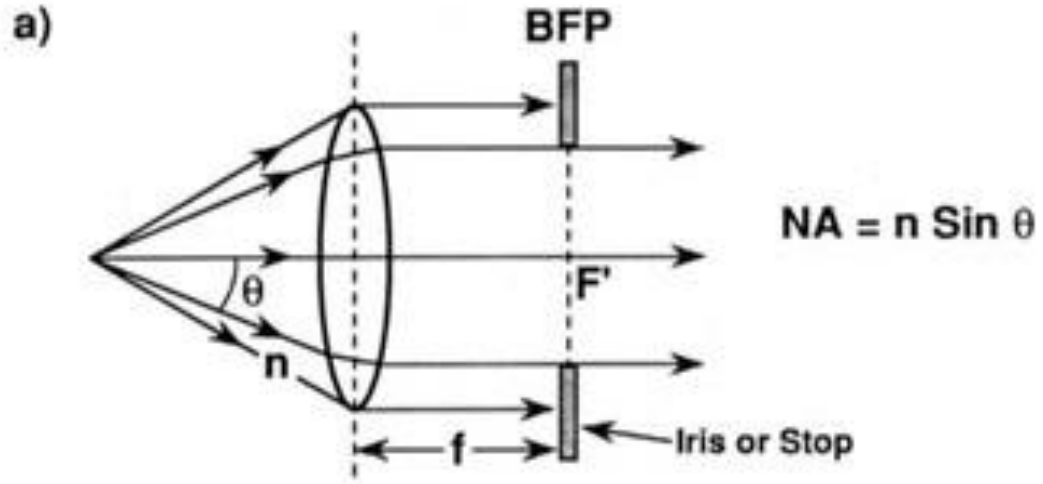
- 显微镜的分辨率：

❖ 数值孔径（Numerical Aperture，NA）的定义：

$$\mathrm{NA} = n\sin\theta$$

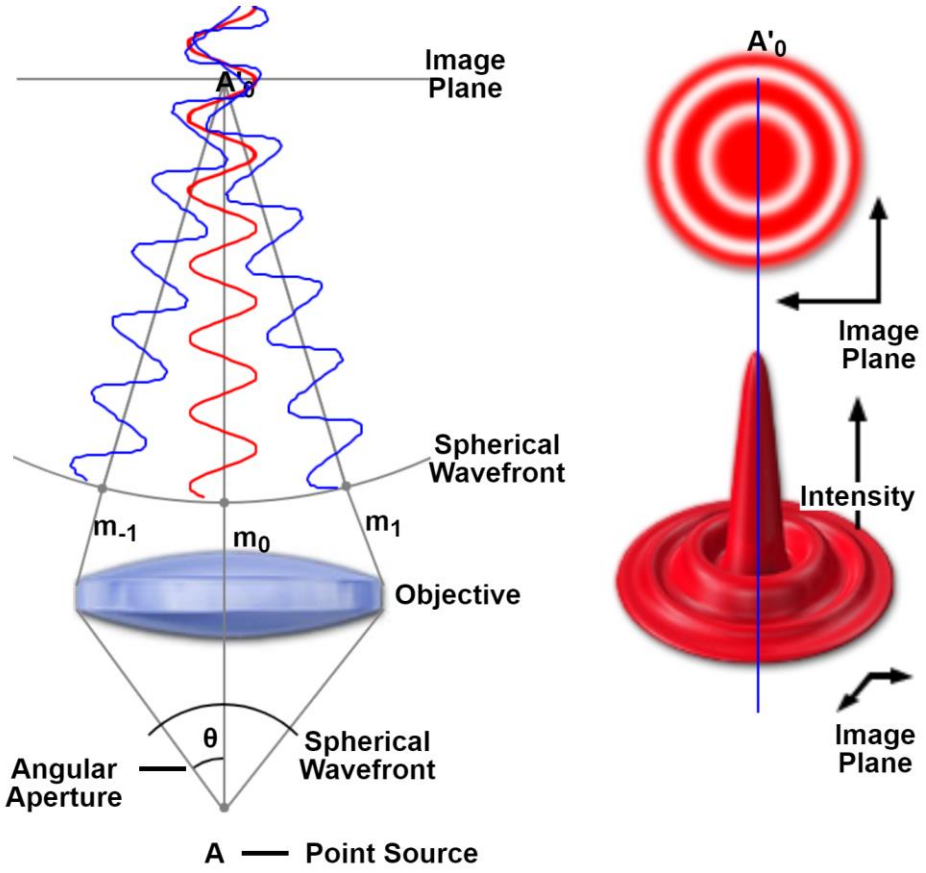
→ n：样品与第一个透镜之间的介质的折射率

→ θ：透镜的孔径角（如下图所示）



光学显微成像的基本原理

- 显微镜的理论分辨率由Abbe极限确定：
  - ❖ 光波的干涉所形成的物理极限；
  - ❖ 可由Airy disk的半径计算得到。



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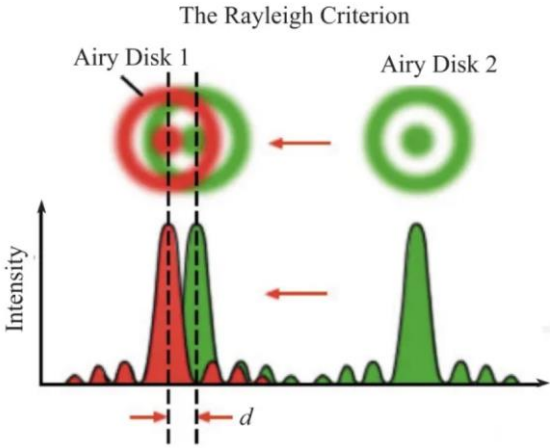
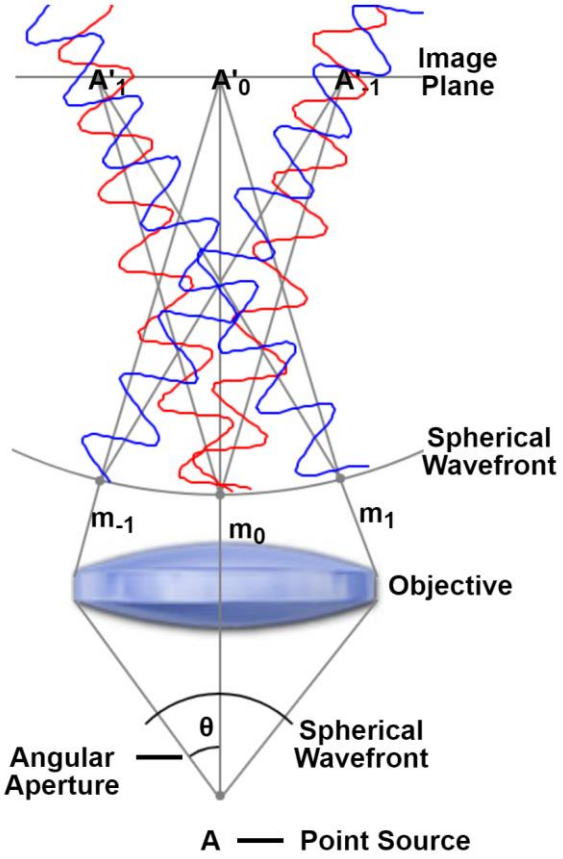
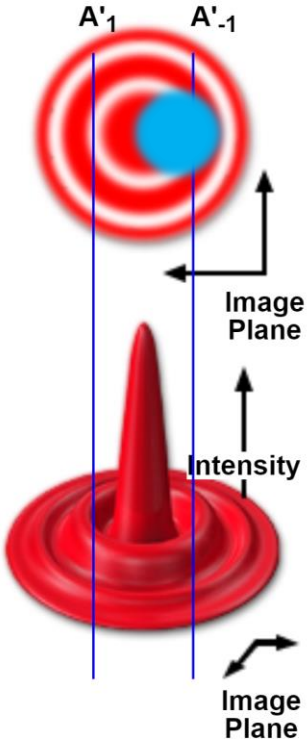






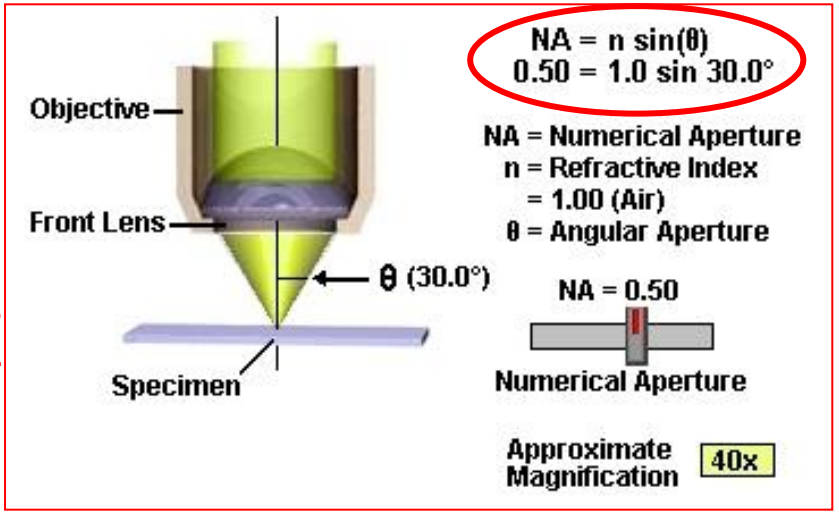
光学显微成像的基本原理

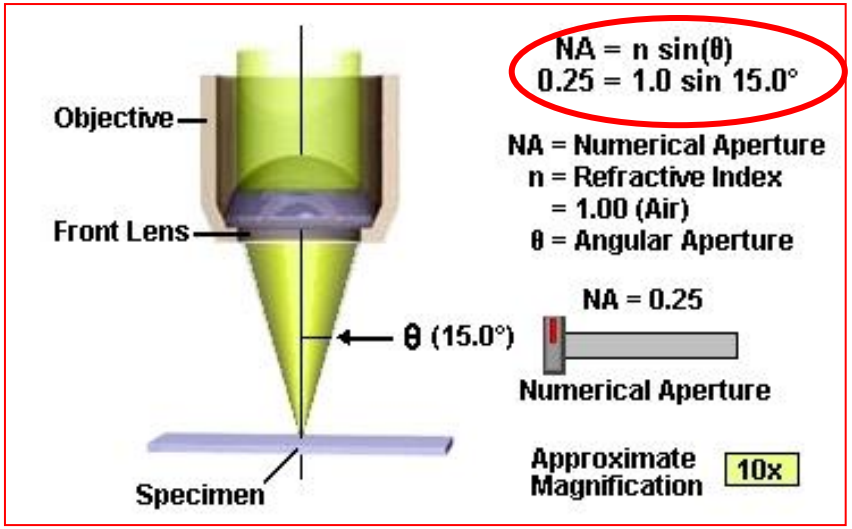
- 显微镜的分辨率取决于物镜的数值孔径：

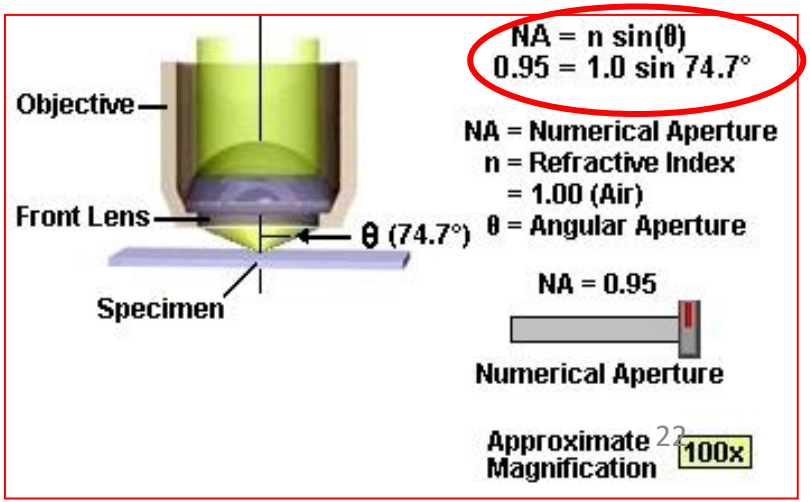
$$d = \frac{0.61\lambda}{\mathrm{NA}} = \frac{0.61\lambda}{n\sin\theta}$$

→镜头接收光线的角度越大，分辨率越大；

→NA值越大的镜头，工作距离越短







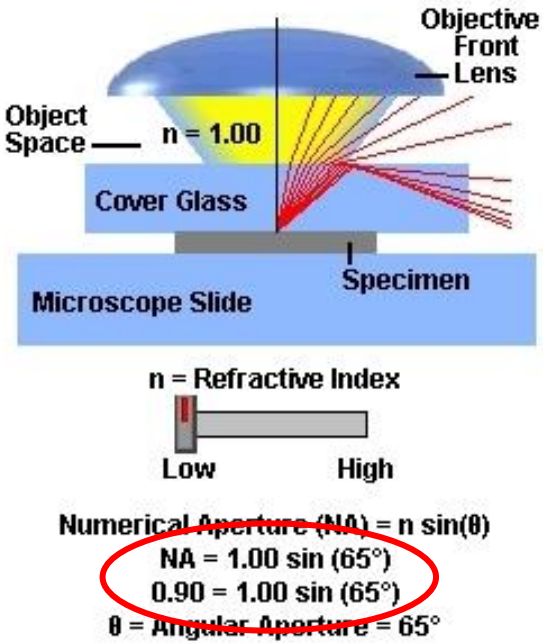
光学显微成像的基本原理

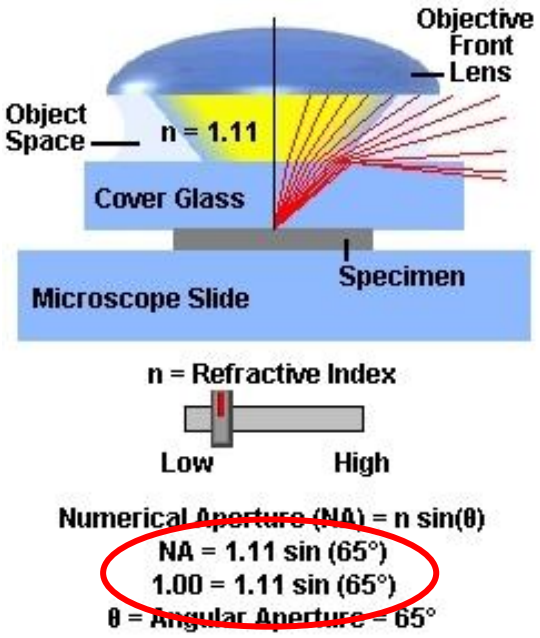
- 显微镜的分辨率取决于物镜的数值孔径：

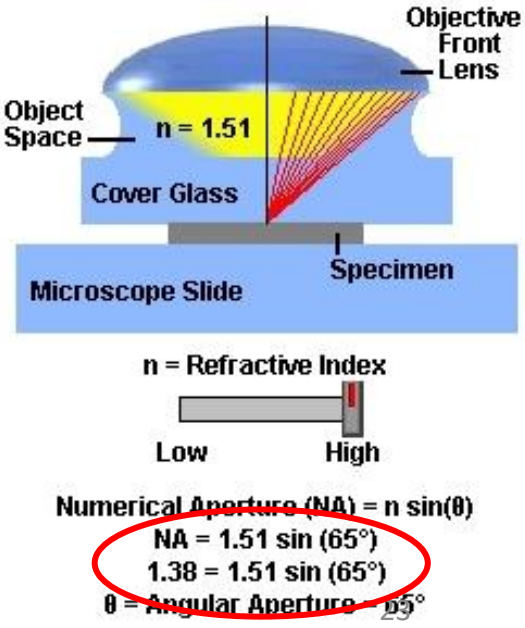
→ 通过改变样品与镜头之间的介质折射率，可以提高镜头的分辨率。

$$d = \frac{0.61\lambda}{\mathrm{NA}} = \frac{0.61\lambda}{n\sin\theta}$$

$$n_1 \bullet \sin\theta_1 = n_2 \bullet \sin\theta_2$$

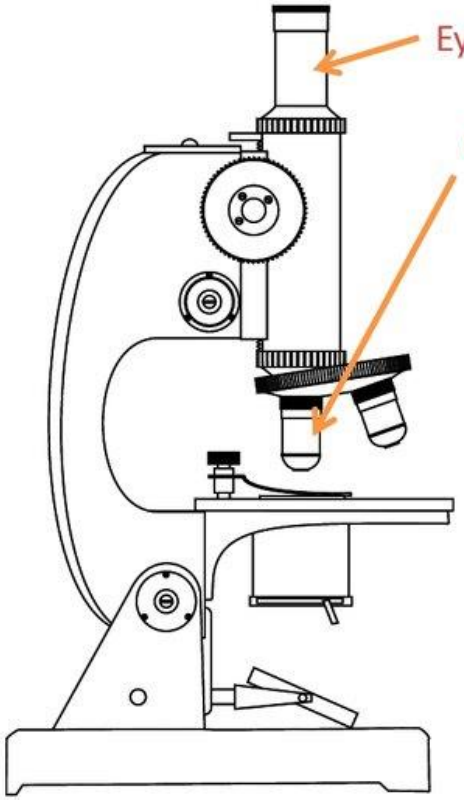






光学显微成像的基本原理

How do we find the overall magnification of a light microscope?



Eyepiece Magnification	Objective Magnification	Overall Magnification
X10	X4	40
X10	X10	100
X10	X40	400
X10	X100	1000

光学显微成像的基本原理

- 显微镜的对比度（contrast）：

❖ 单有分辨率而无对比度无法得到可用的图像:

$$CONTRAST = (I_p - I_{bg}) / I_{bg}$$

1 2 3

光学显微成像的基本原理

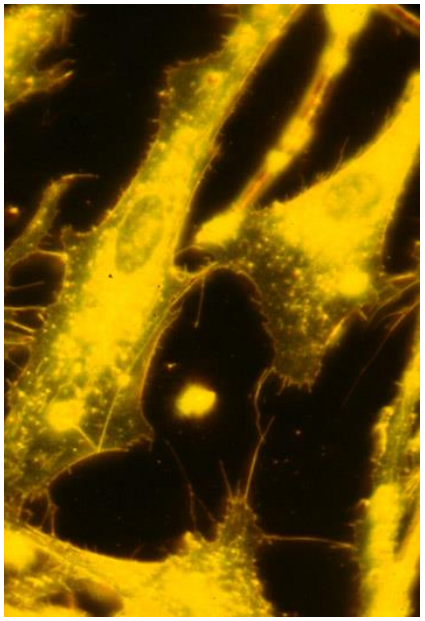
- 光学显微镜提高对比度的不同模式：

CONTRAST MODES OF LIGHT MICROSCOPY

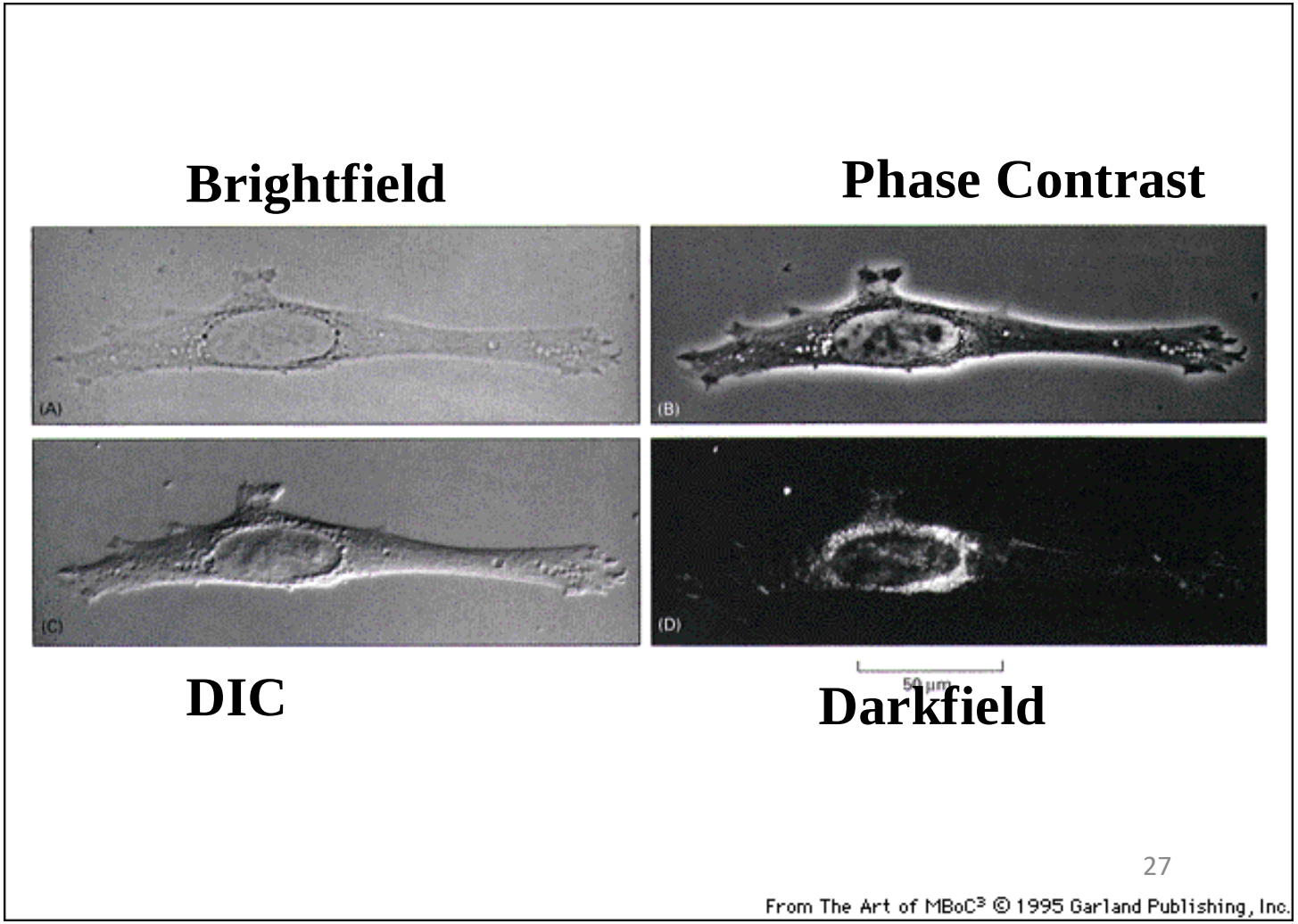
MODE	MECHANISM OF CONTRAST
Brightfield	Absorption of light
Phase contrast	Optical path length (index, density)
DIC	Rate of change of optical path
Widefield fluorescence	Absorption of light, quantum yield of fluorophore
Confocal fluorescence	same as fluorescence
Darkfield	light scattering by edges in specimen
Interference reflection contrast	interference between reflections from ventral cell surface and substratum
Polarization	Extinction between crossed polars caused by specimen birefringence

光学显微成像的基本原理

- 光学显微镜提高对比度的不同模式：

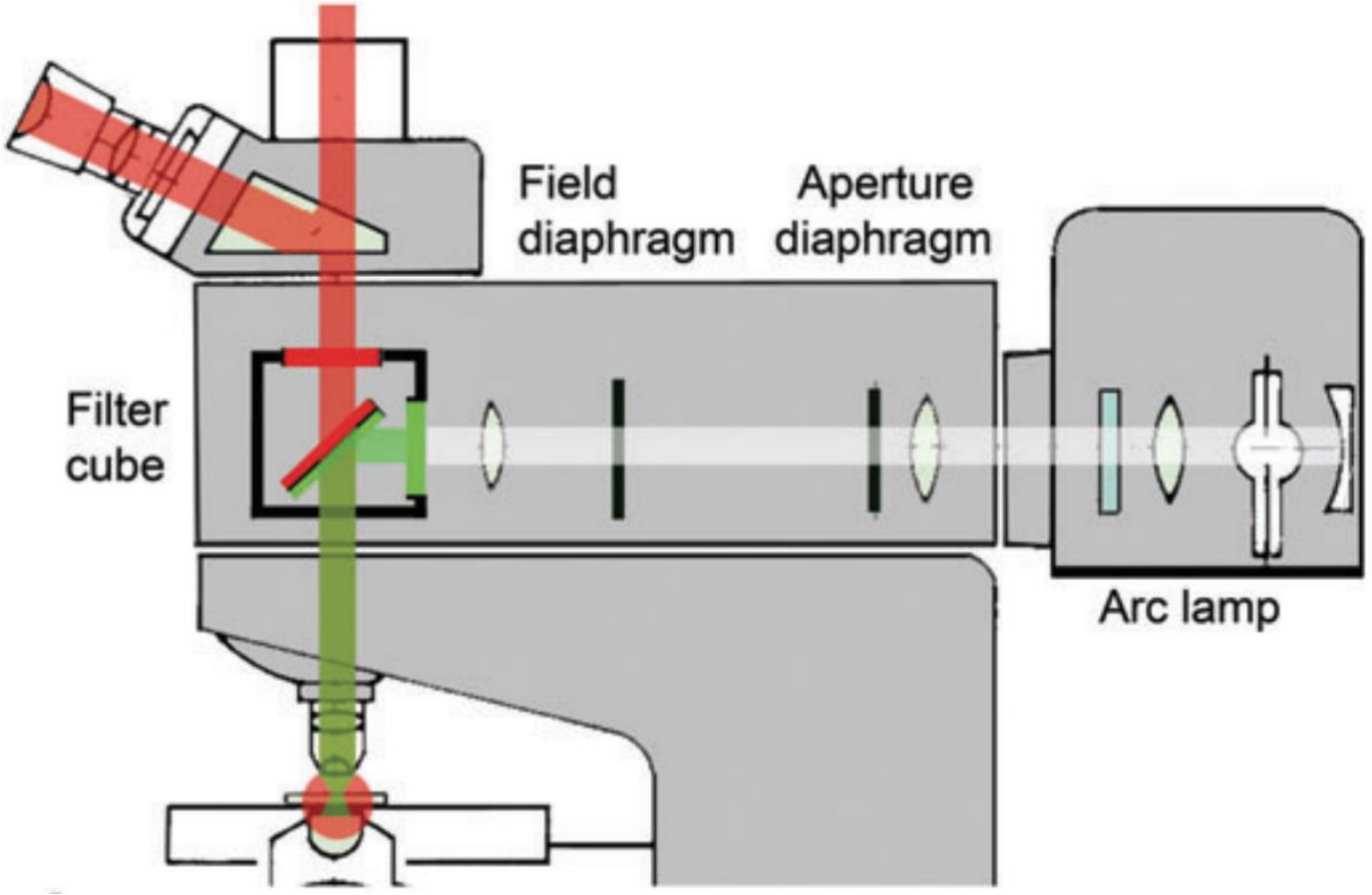


Fluorescence



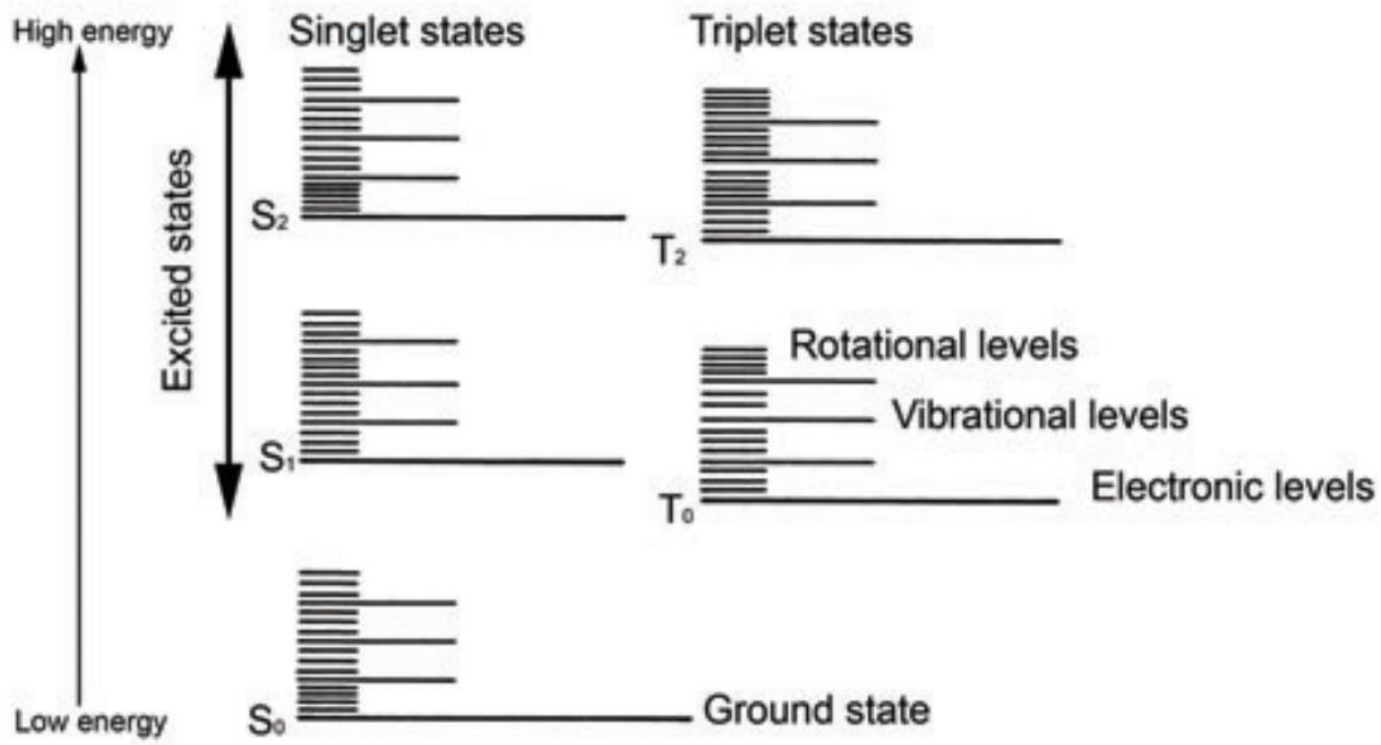
荧光显微成像

- 光路构造：



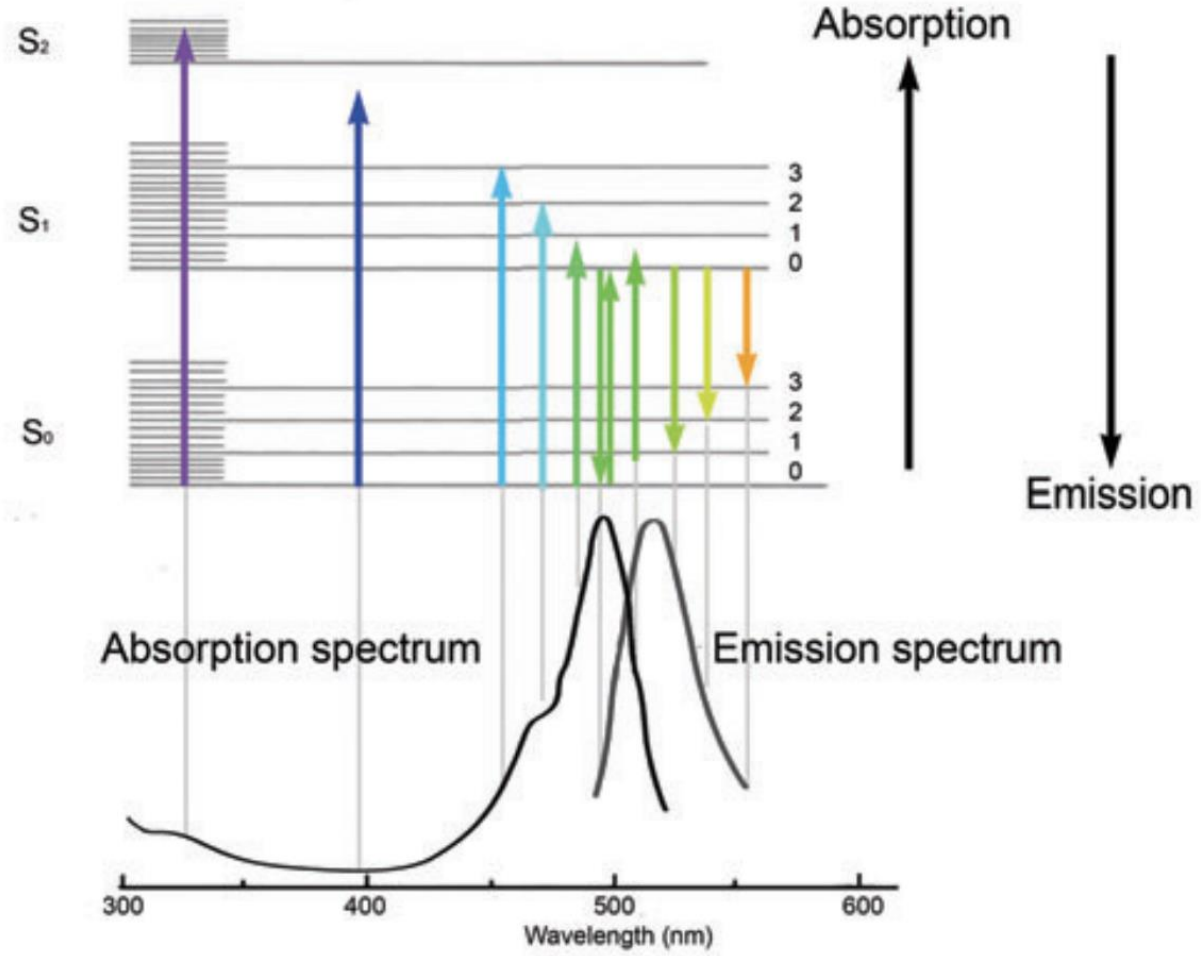
Nature Methods, 2, 910–919 (2005)

荧光显微成像



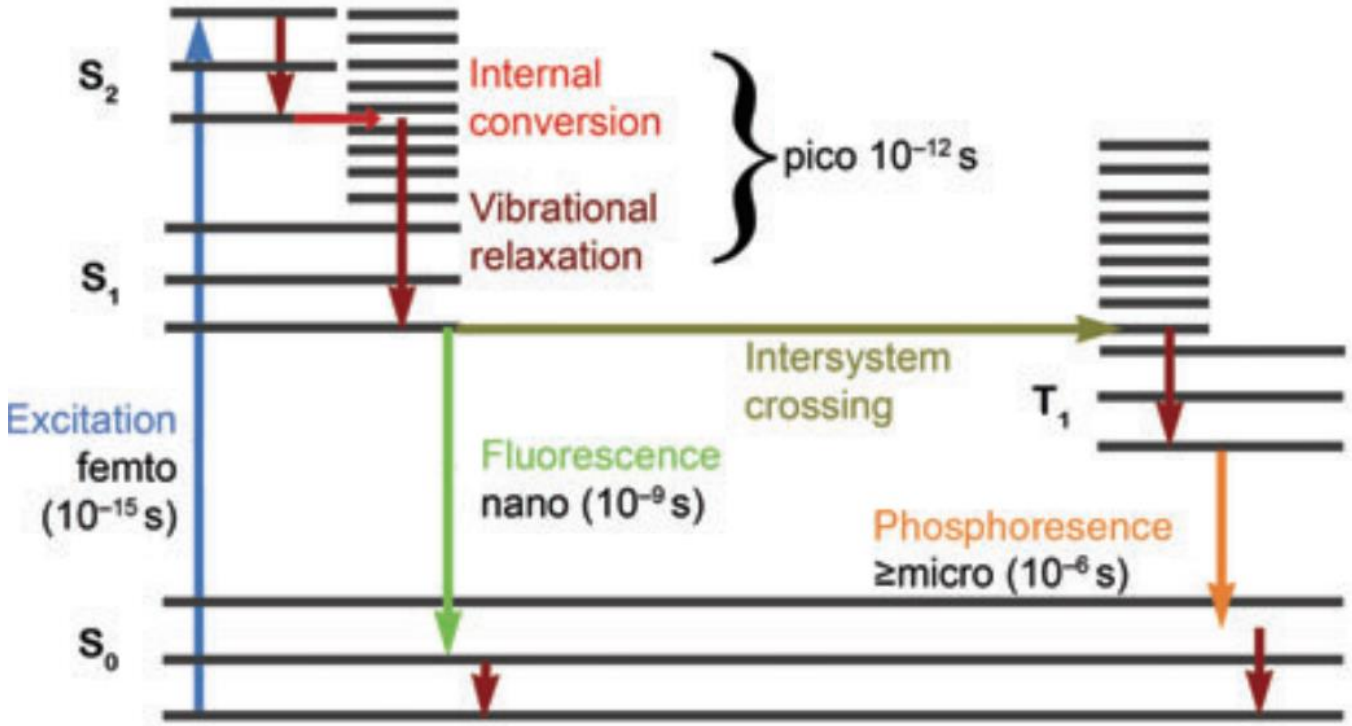
Nature Methods, 2, 910–919 (2005)

荧光显微成像



Nature Methods, 2, 910–919 (2005)

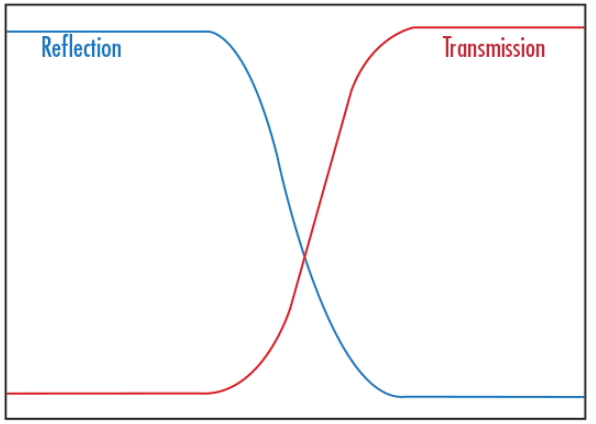
荧光显微成像



Nature Methods, 2, 910–919 (2005)

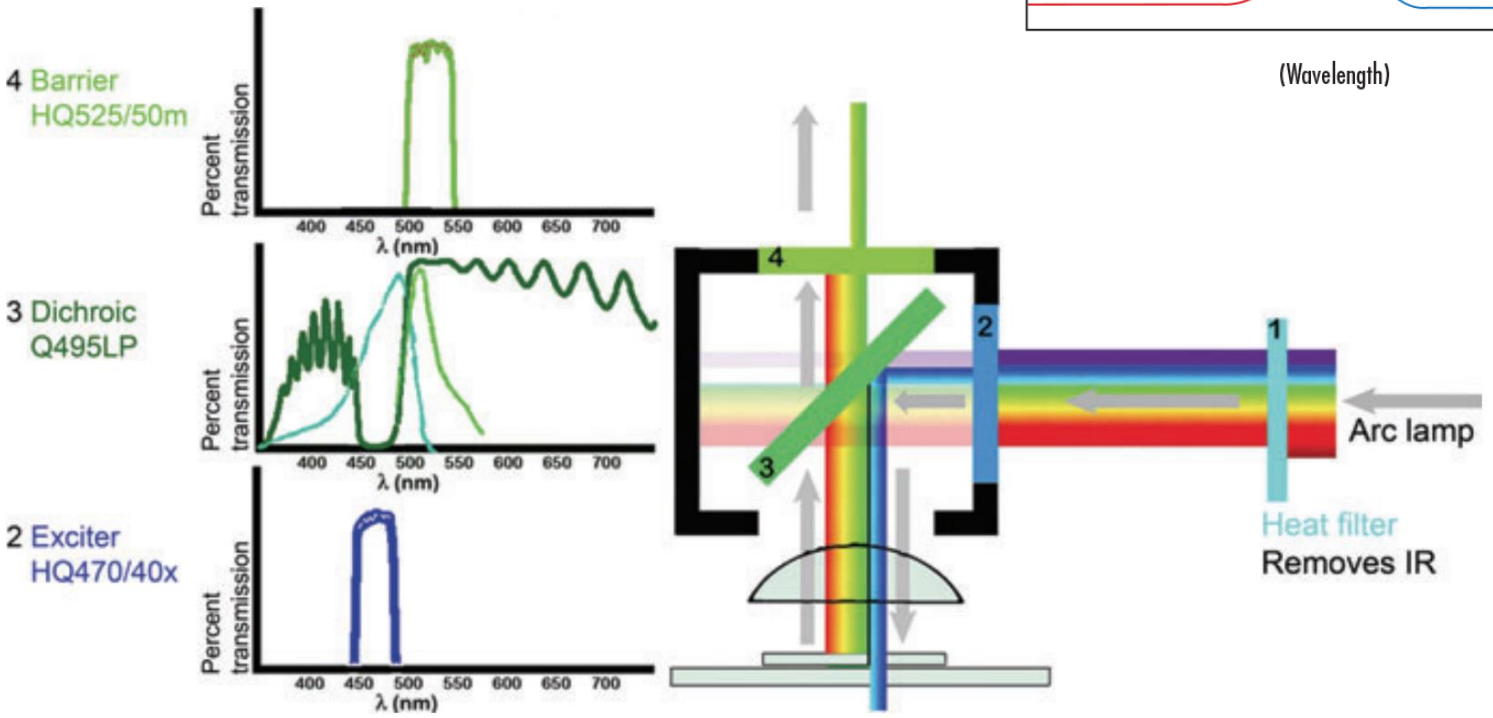
荧光显微成像

- 双色分光镜（Dichromatic mirror）：



→ 全反射短波长的激发光

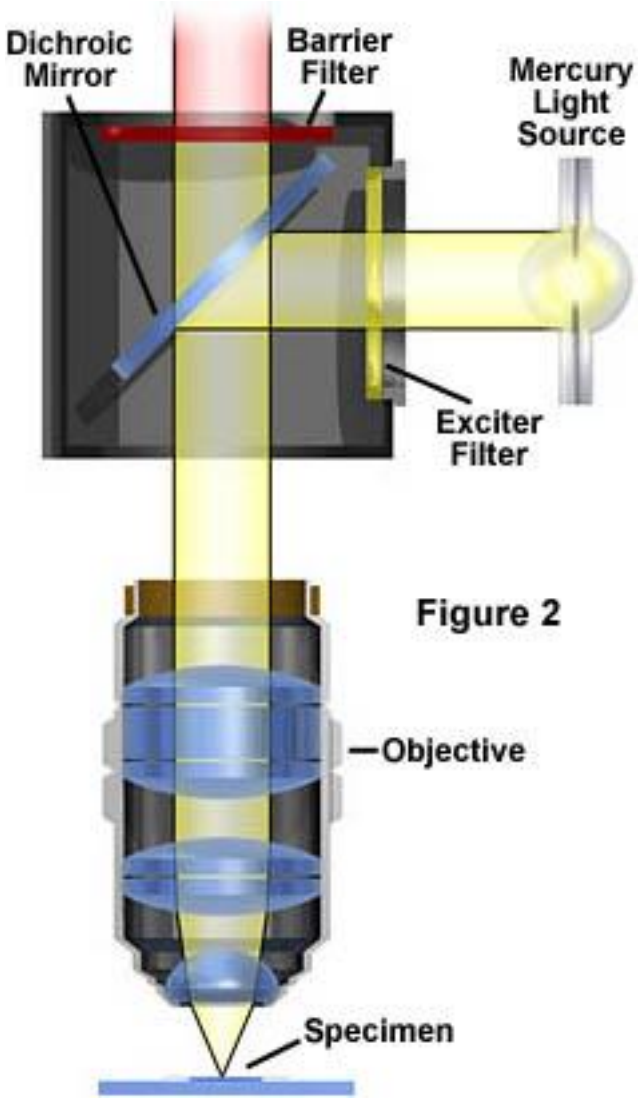
→ 透射长波长的荧光信号



Nature Methods, 2, 910–919 (2005)

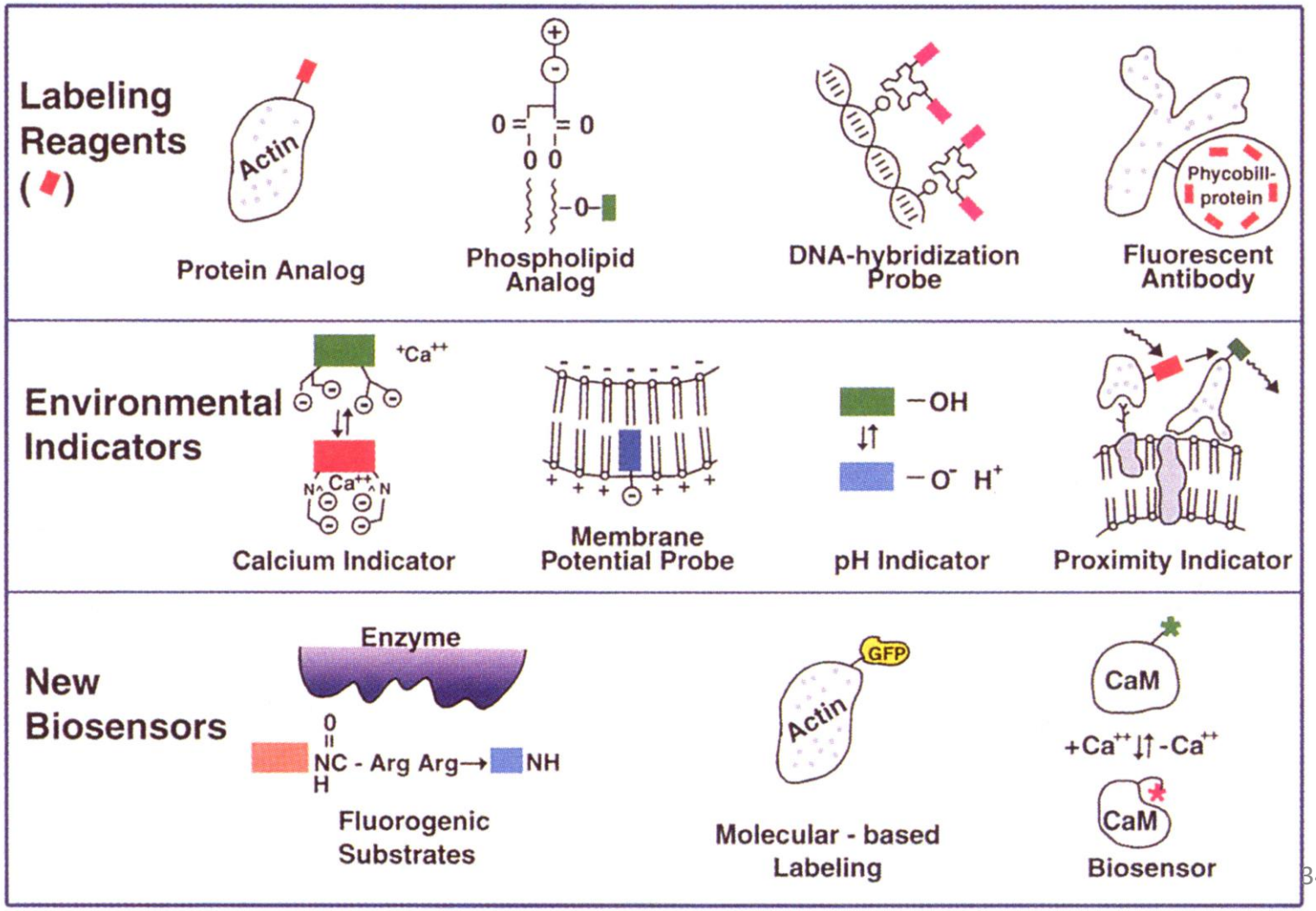
荧光显微成像

- 双色分光镜（Dichromatic mirror）：



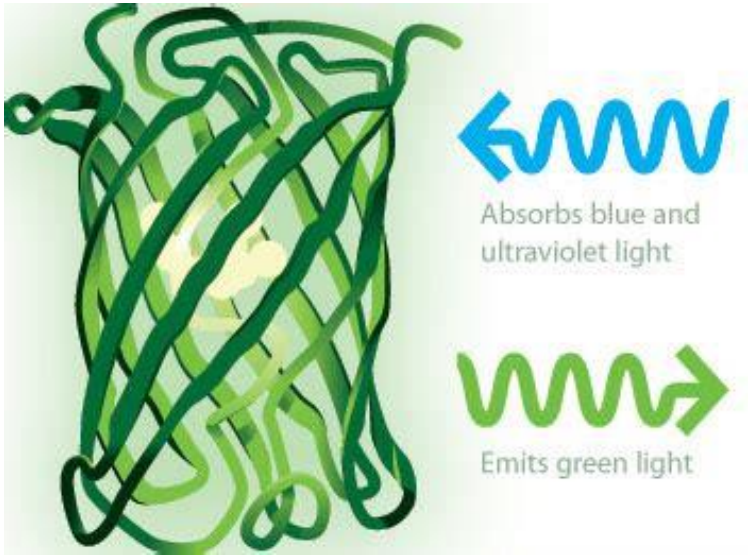
荧光显微成像

- 荧光基团的引入：



荧光显微成像

- 绿色荧光蛋白（GFP）的发明是科学史上的突破性进展。



2008 Nobel Prize in Chemistry

荧光显微成像

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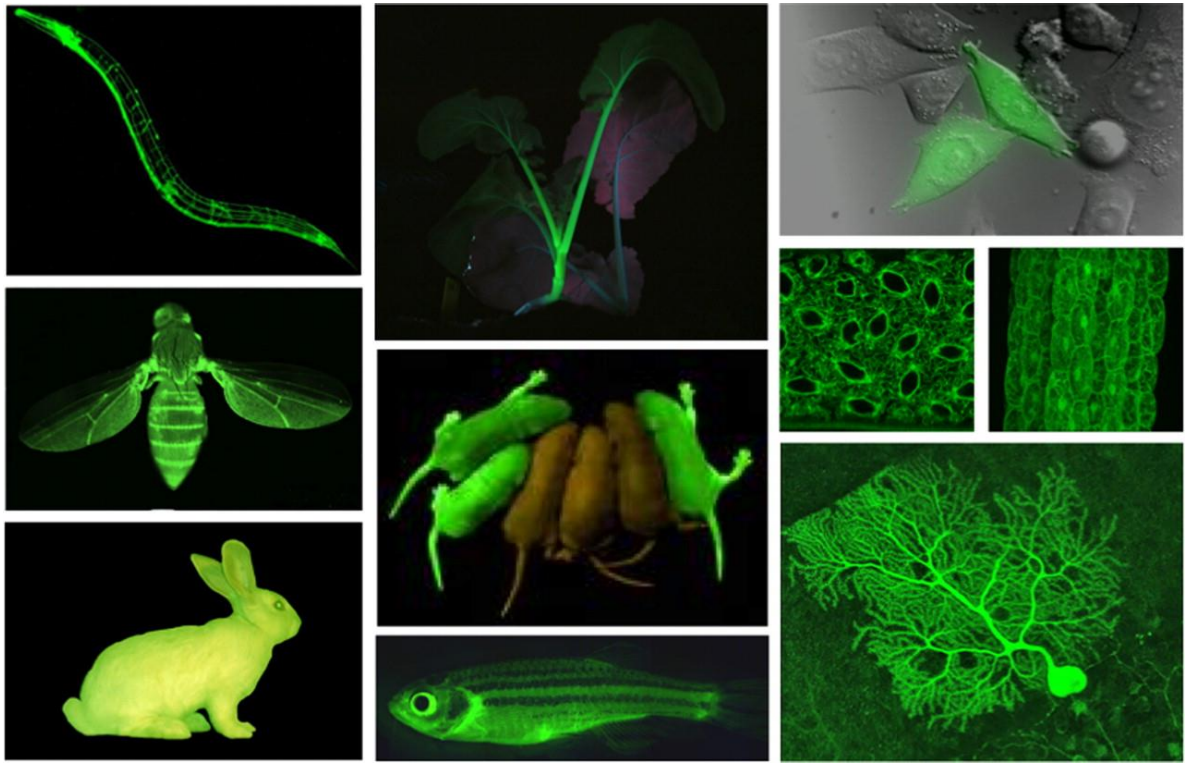
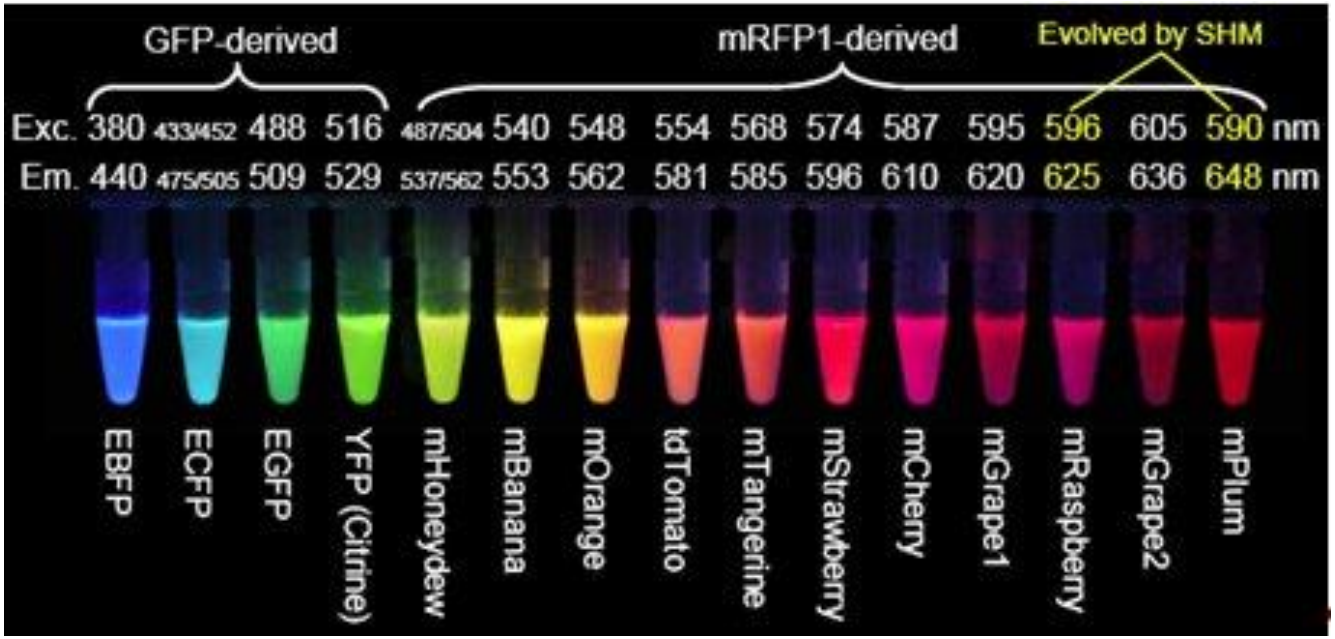


2008 Nobel Prize in Chemistry

荧光显微成像

- GFP及其相关荧光蛋白可覆盖整个可见光谱

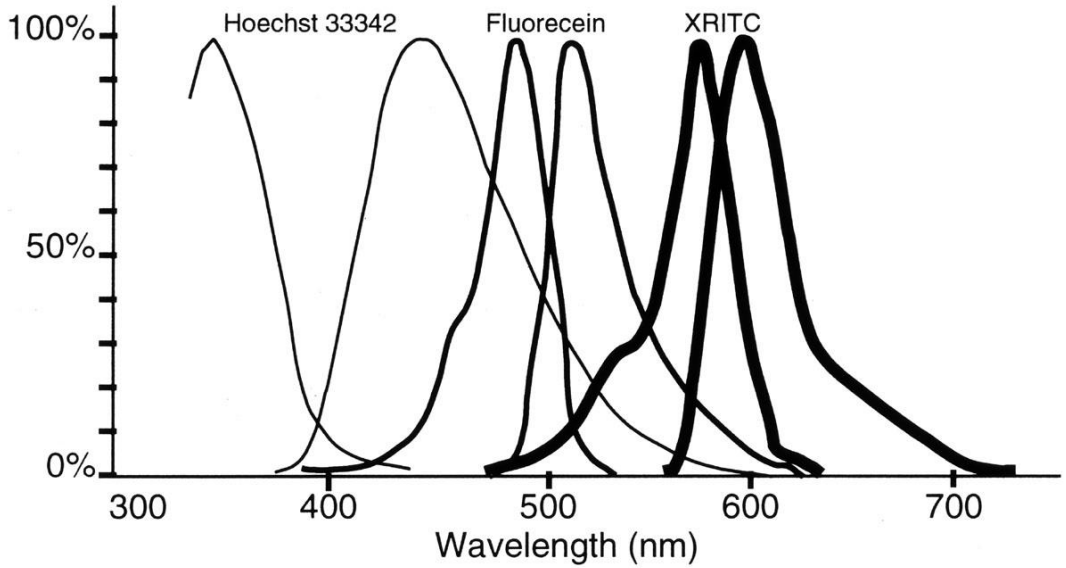


2008 Nobel Prize in Chemistry

荧光显微成像

- 多色荧光成像：

❖ 通过选择不同激发/发射波长的荧光标记，可以对不同的蛋白或细胞器件进行同时荧光成像。



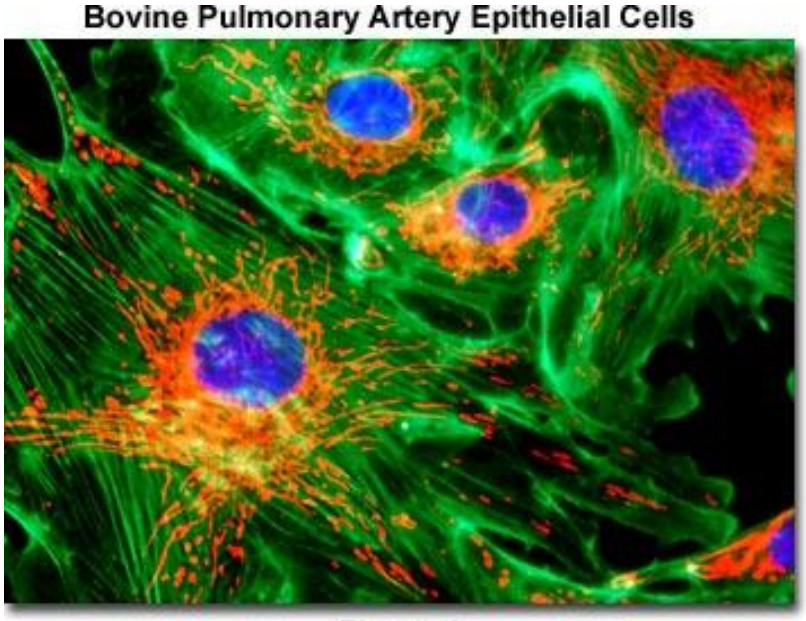
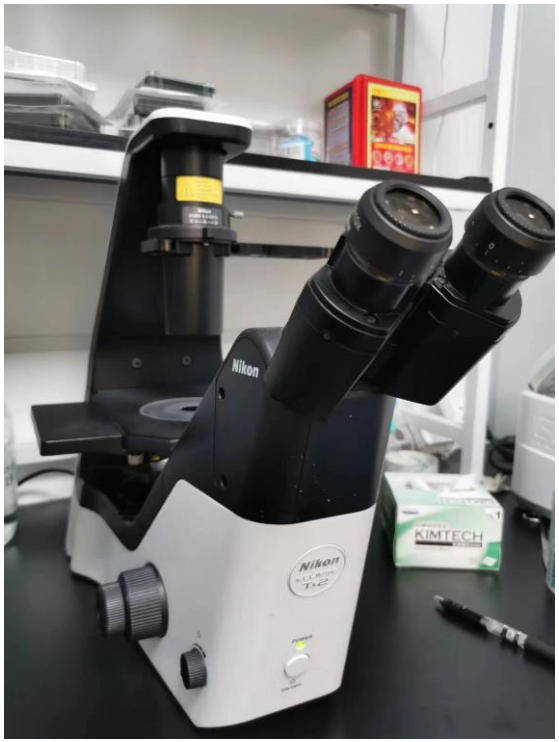
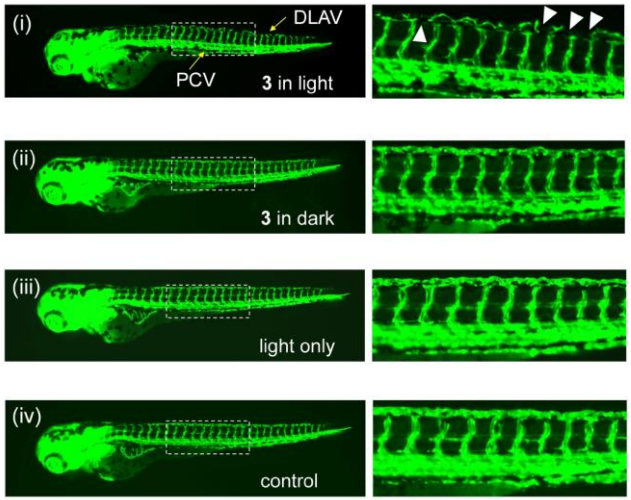


Figure 1

ECLIPSE Ti2系列 | 倒置显微镜



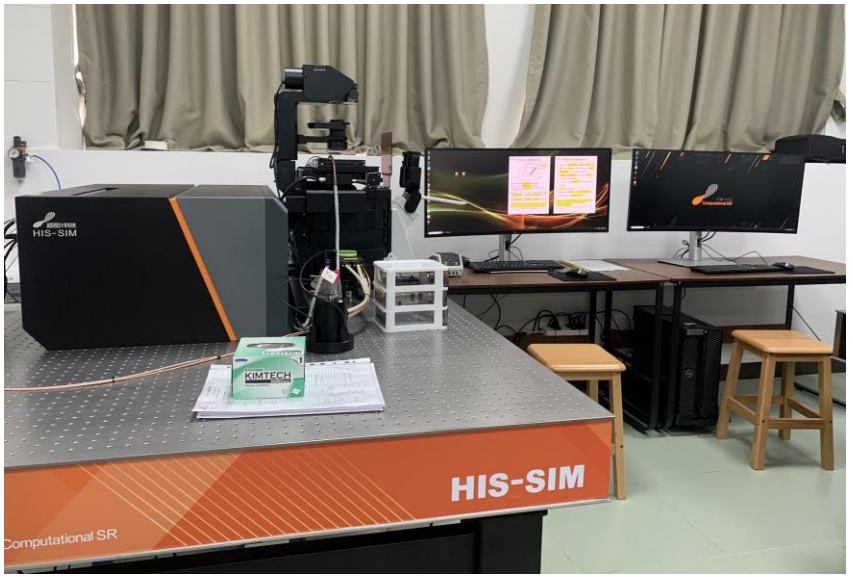
ECLIPSE Ti2 尼康倒置荧光显微镜

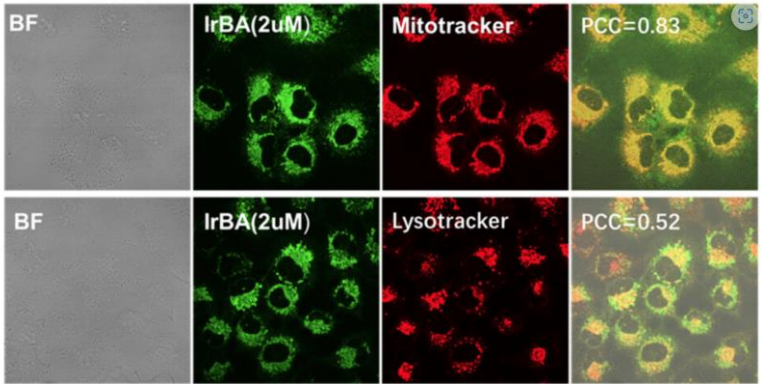


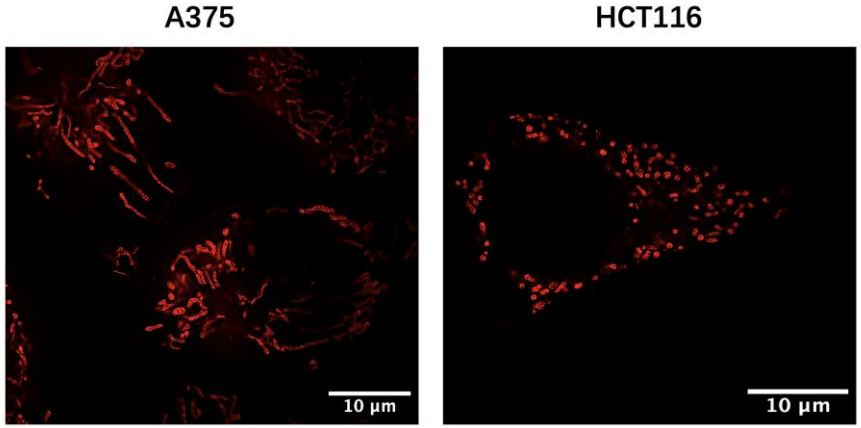
Angew. Chem. Int. Ed. **2022**, *61*, e202201103.

激光共聚焦显微镜--1 (FV3000)

智能高速超分辨显微镜 (HIS-SIM)







JACS **2023**, *145*, 10082-10091.

特种荧光显微成像系统

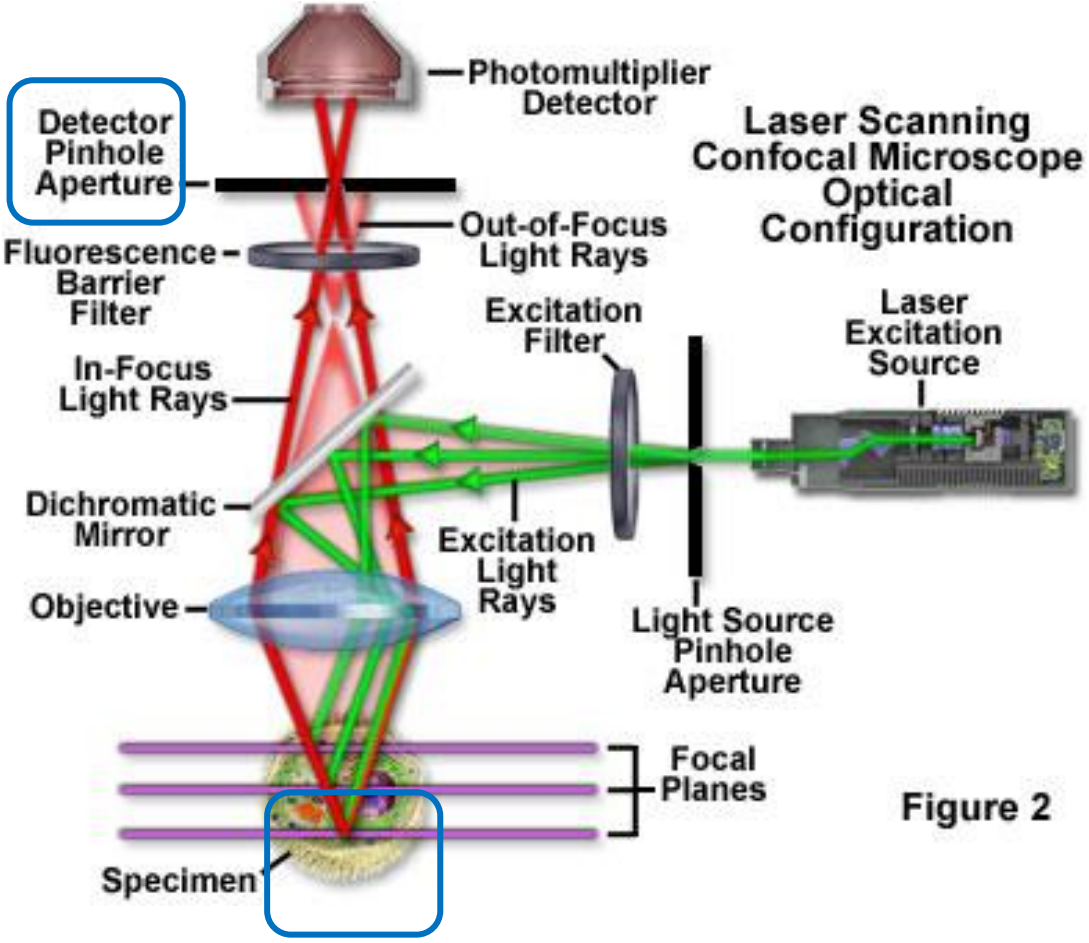
- 激光共聚焦显微成像（Laser Scanning Confocal Microscopy）：

❖ 相干激光由物镜聚焦于样品上的扫描点；

❖ 荧光在检测器前面的小孔再次聚焦；

❖ 背景荧光在再次聚焦的时候失焦；

❖ 降低背景，提高实际分辨率。



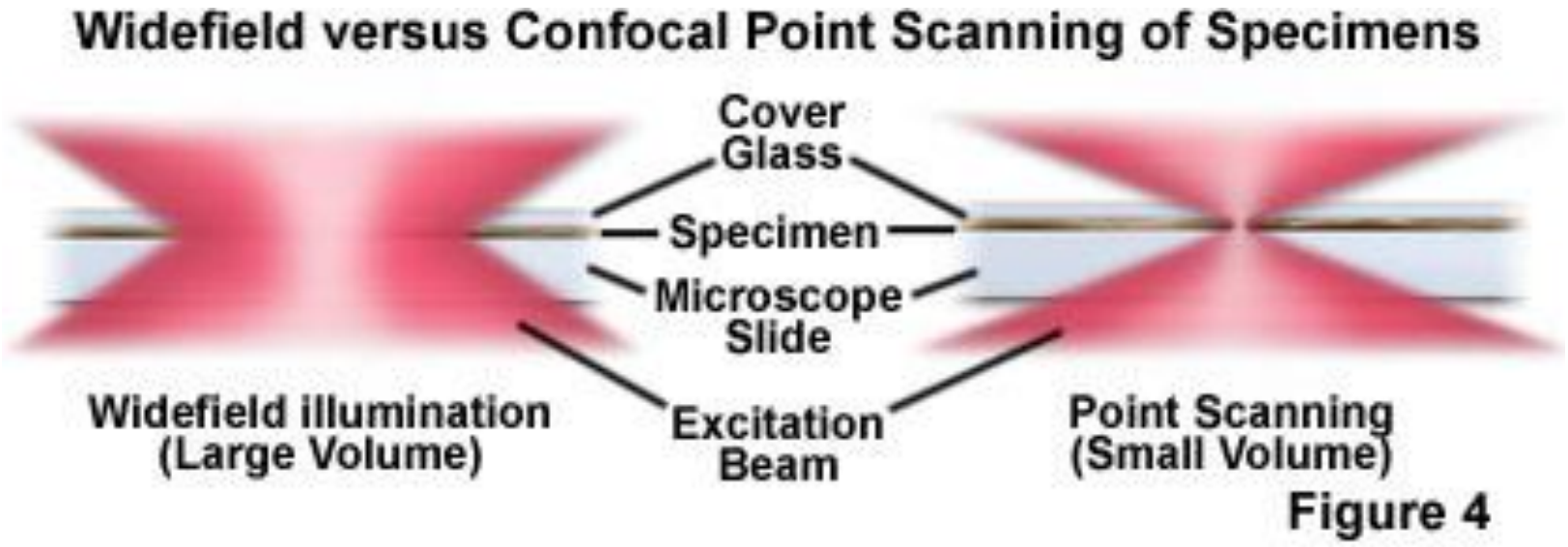
FSU, microscopy primer

特种荧光显微成像系统

- 激光共聚焦（Laser Scanning Confocal Microscopy）：

❖ 共聚焦的扫描点比普通荧光显微镜的照射体积小很多；

❖ 因此图像需要扫描后合成。



特种荧光显微成像系统

- 共聚焦与宽场荧光成像效果的对比

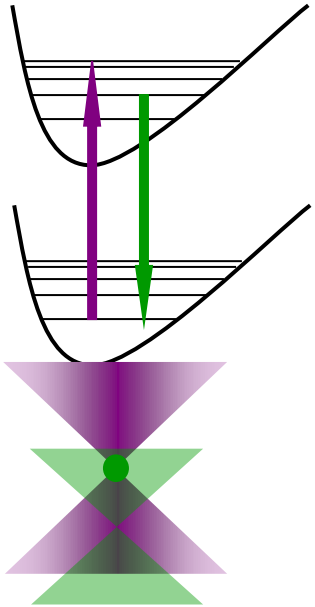
ISU Advanced
Bioimaging Facility

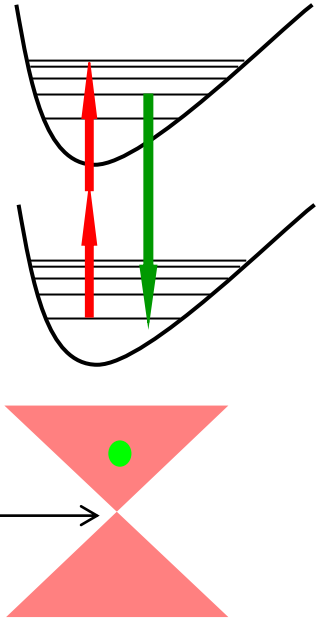
特种荧光显微成像系统

- 双光子显微成像（Two-photon excitation microscopy）：

❖ 两个光子同时（10 × e^{-18}秒以内）到达荧光分子 → 激光

❖ 只有在光子浓度很高的焦点附近才能达到双光子激发条件



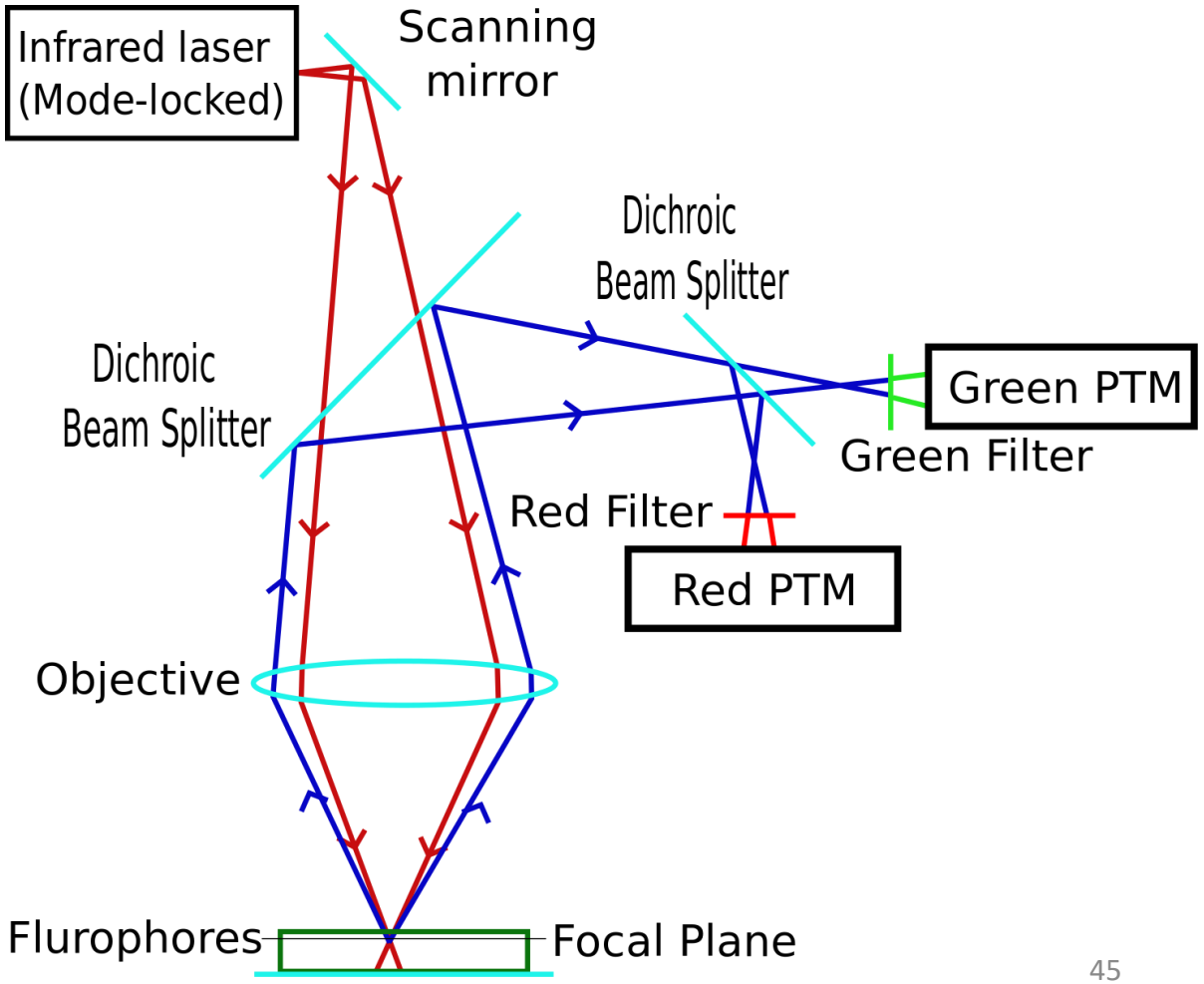


1 - Photon

2 - Photon

特种荧光显微成像系统

- 双光子显微镜的基本构造与激光共聚焦类似：



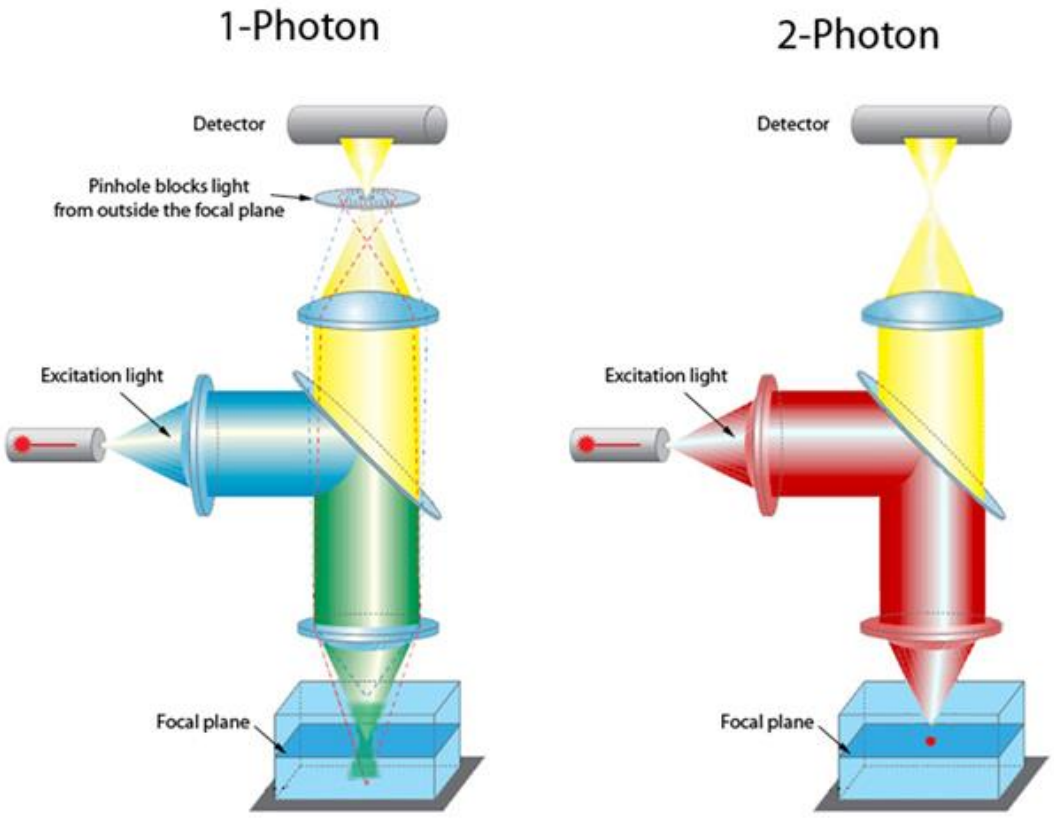
特种荧光显微成像系统

- 双光子显微成像与激光共聚焦的对比：

❖ 激发波长不同

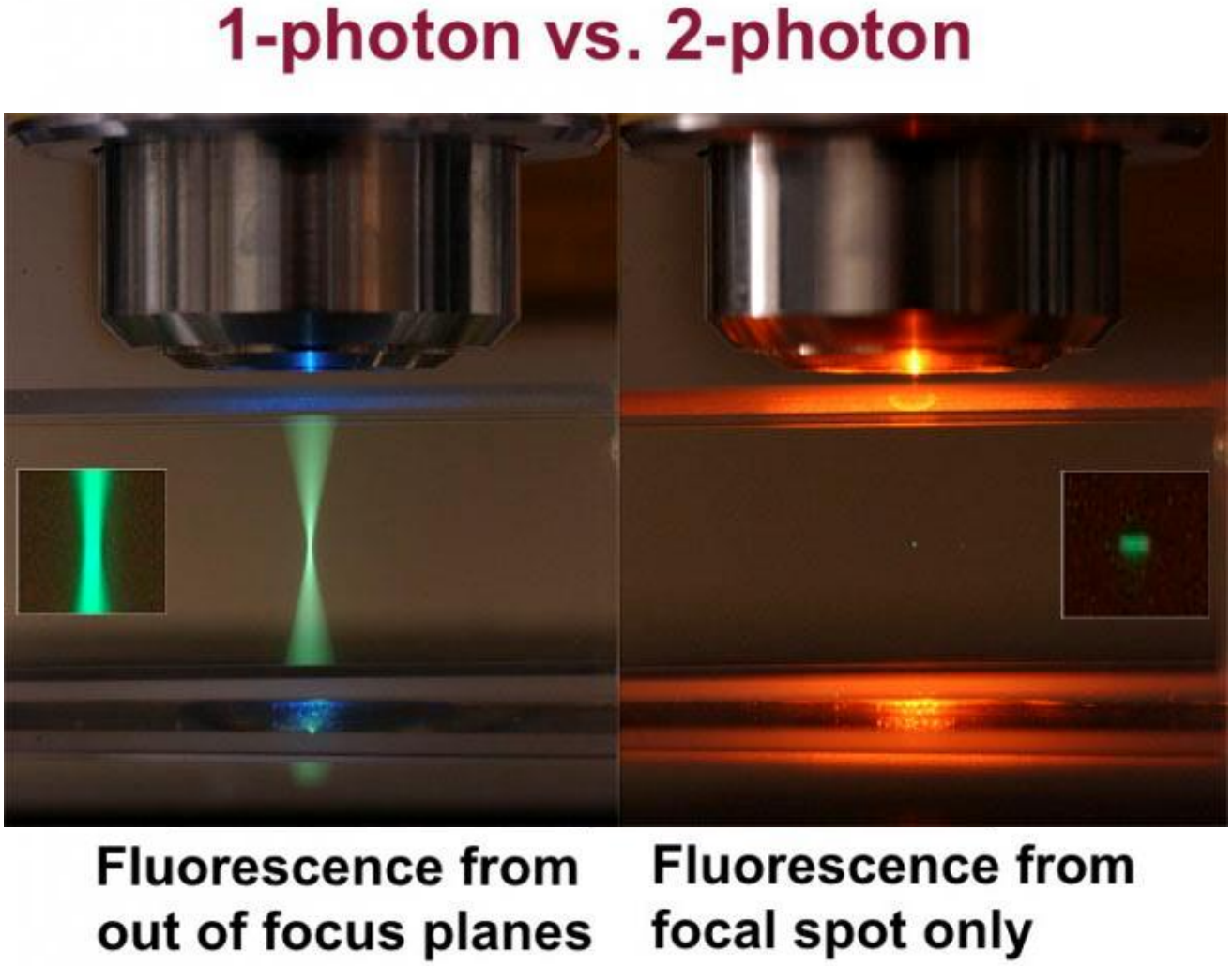
❖ 双光子无需滤光小孔

双光子激发荧光需要有一个强度的阈值，低于这个阈值后，两个光子同时到达的概率几乎为零，因此也就不会有荧光产生。焦点以外的区域基本没有希望达到这个阈值。即便是在焦点处，达到这个阈值也不容易，需要采用瞬时功率高达15万W的飞秒激光。因此，不需要共聚焦显微镜中的小孔来隔离非焦点处的荧光。



特种荧光显微成像系统

- 双光子显微成像与激光共聚焦的对比：



特种荧光显微成像系统

- 双光子显微成像的应用优势：

→ 3-D sectioning effect

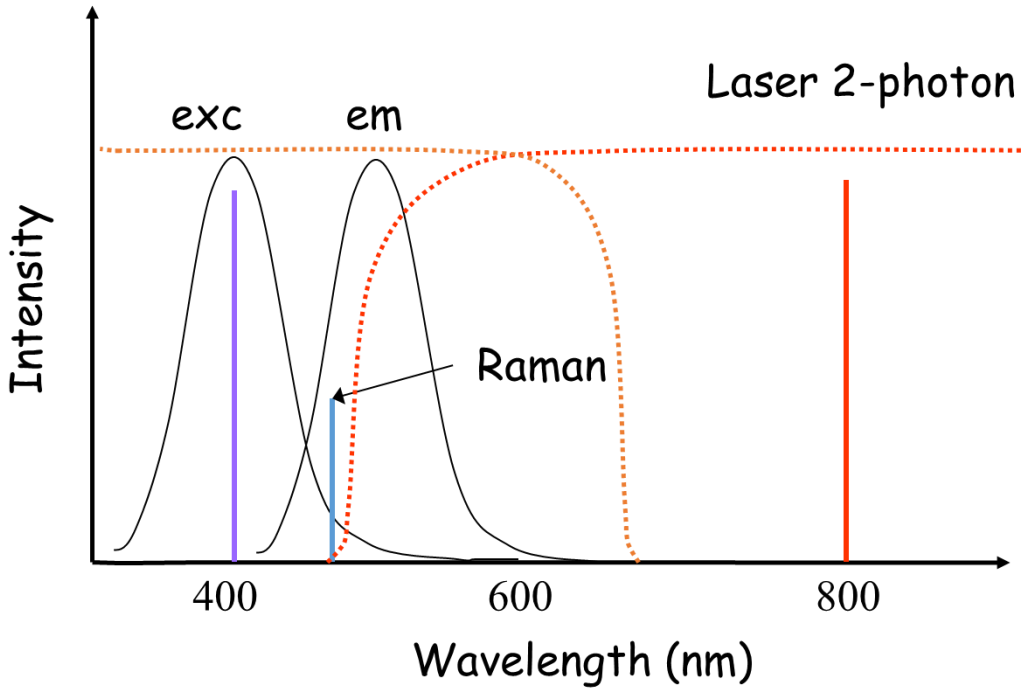
→ Absence of photo bleaching in out of focus regions

→ Large separation of excitation and emission

→ No Raman from the solvent

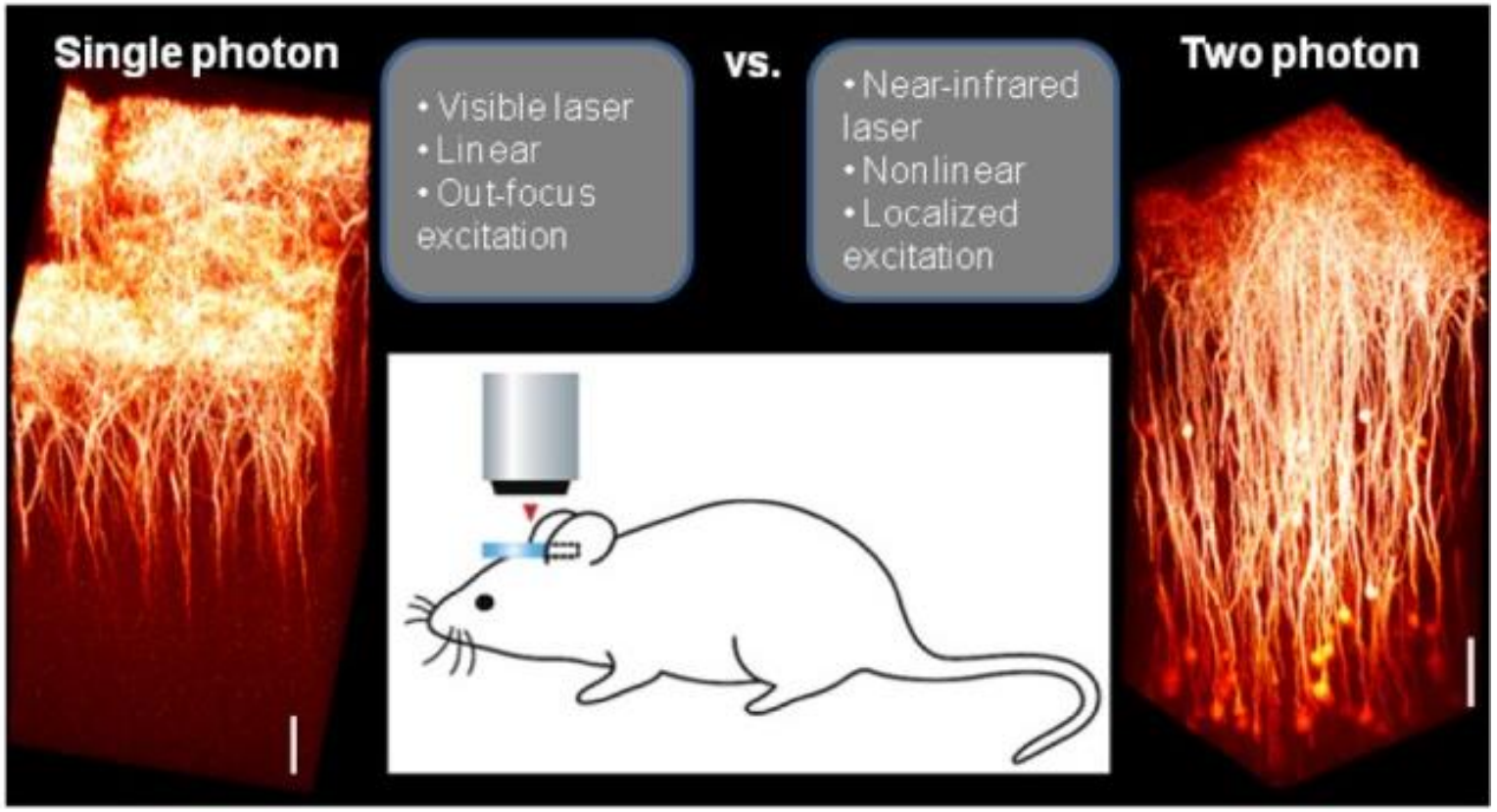
→ Deep penetration in tissues

→ Single wavelength of excitation for many dyes



特种荧光显微成像系统

- 双光子显微成像在组织穿透方面的显著优势：



比较单光子与双光子荧光显微镜对小鼠大脑皮层的成像（Korean J Physiol Pharmacol 2016; 20(1): 1-8）

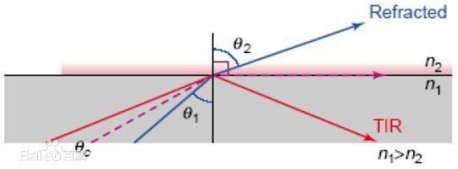
特种荧光显微成像系统

Total internal reflection fluorescence microscopy (TIRFM):

❖ 全反射（TIR）与隐失波（evanescent wave）：

→ 当入射光在折射界面上发射全反射（TIR）的时候，沿着界面平行方向会产生隐失波（Evanescent wave）；

→ 隐失波的波长与入射光一致，进入介质中30~300nm。



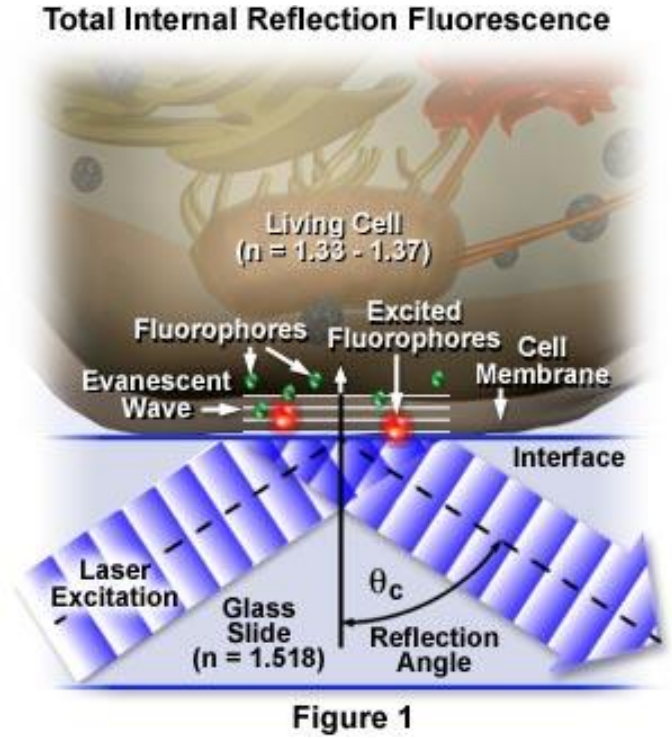
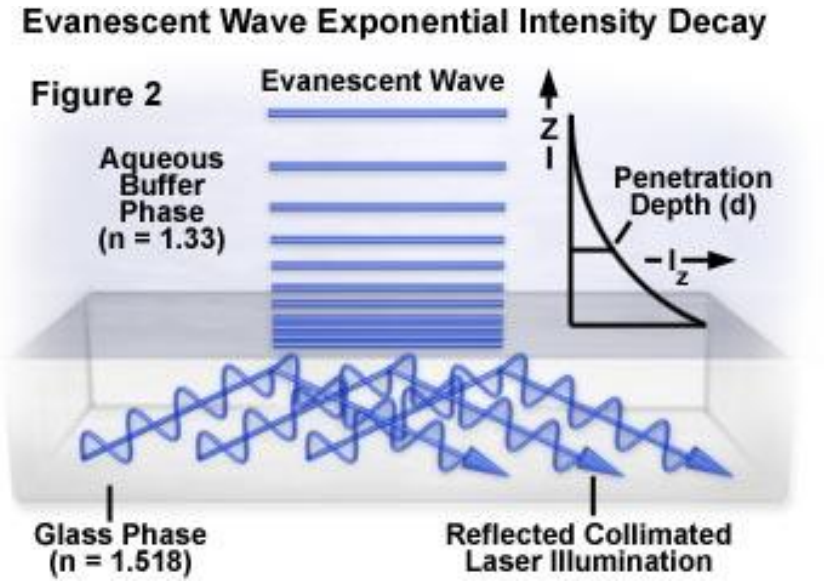


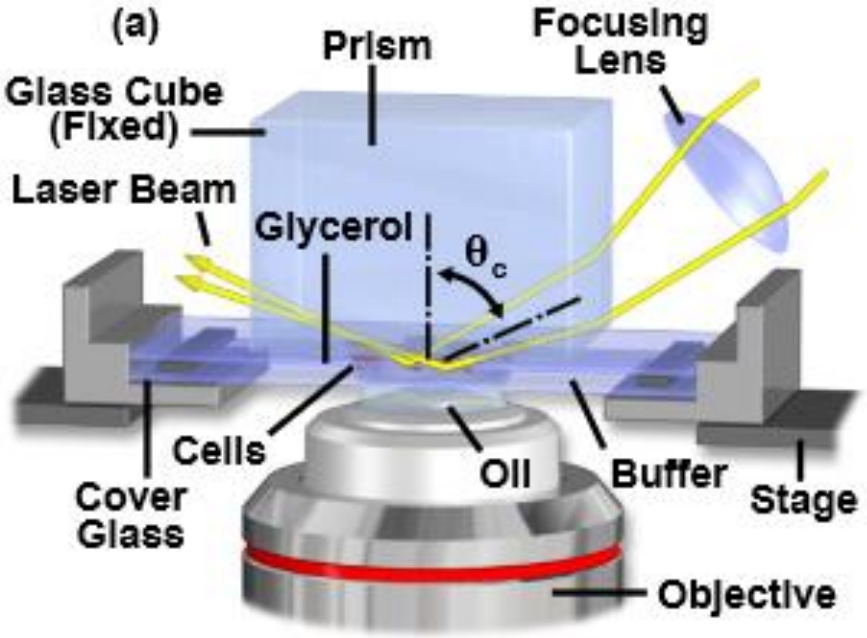
Figure 1



特种荧光显微成像系统

Total internal reflection fluorescence microscopy (TIRFM):

❖ 仪器构造:



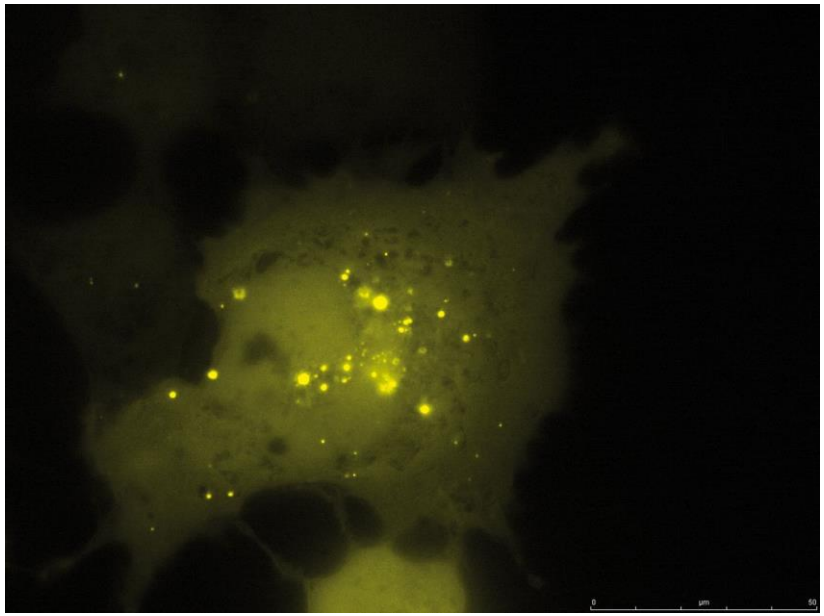
特种荧光显微成像系统

Total internal reflection fluorescence microscopy (TIRFM):

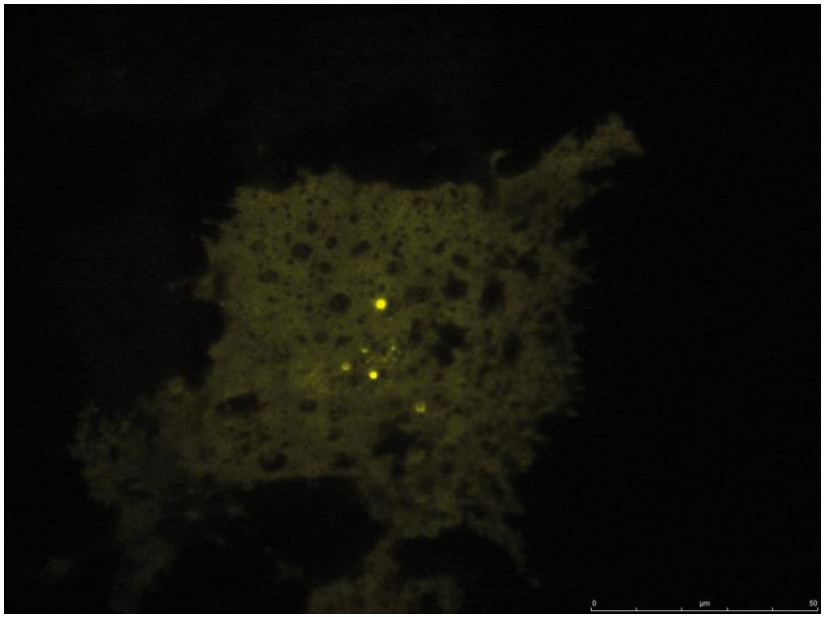
❖ 应用:

→Super-resolution Microscopy

→可用于单分子荧光研究。



Widefield(Tubulin expressing CFP)



TIRF(Tubulin expressing CFP)

leica-microsystems