纳米光学表征方法

分为三大类:

1. 扫描探针技术

2. 光谱技术

3. 成像技术

We are here!

分为三大类：

1. 扫描探针技术：
   - ◆ NSOM
   - ◆ AFM
   - ◆ STM
2. 光谱技术：
   - ◆ General introduction
   - ◆ Raman Spectrometry
3. 成像技术：
   - ◆ Confocal imaging
   - ◆ multi-photon imaging

We are here!

Imaging Technology

共聚焦显微镜的分类

1.单光子吸收共聚焦显微镜

2.背向散射光(backscattered light)共聚焦显微镜

3.双光子吸收共聚焦显微镜

内容

- 共聚焦激光扫描显微镜(CLSM)

  1. 光路

  2. 成像分辨率

  3. 采样速率(Nyquist Theorem)

- 反射 / 背向散射光成像

- 多光子成像技术

焦深

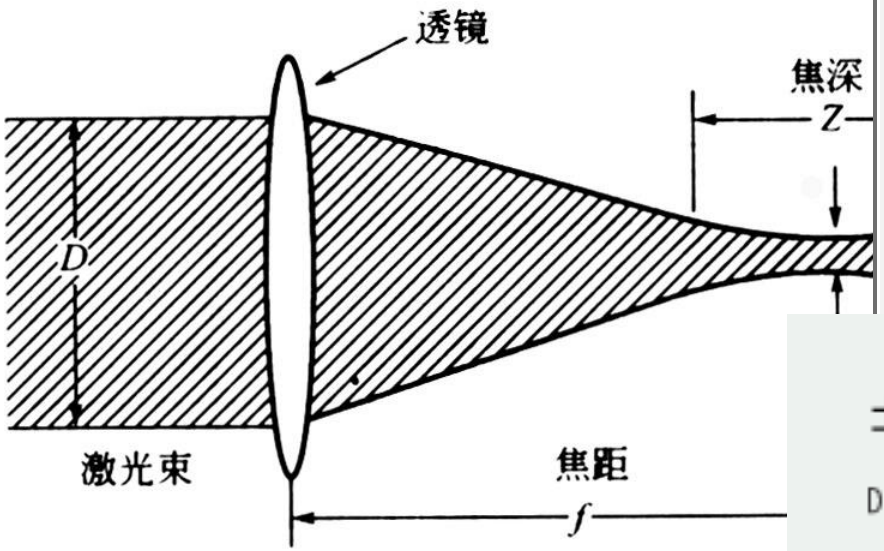
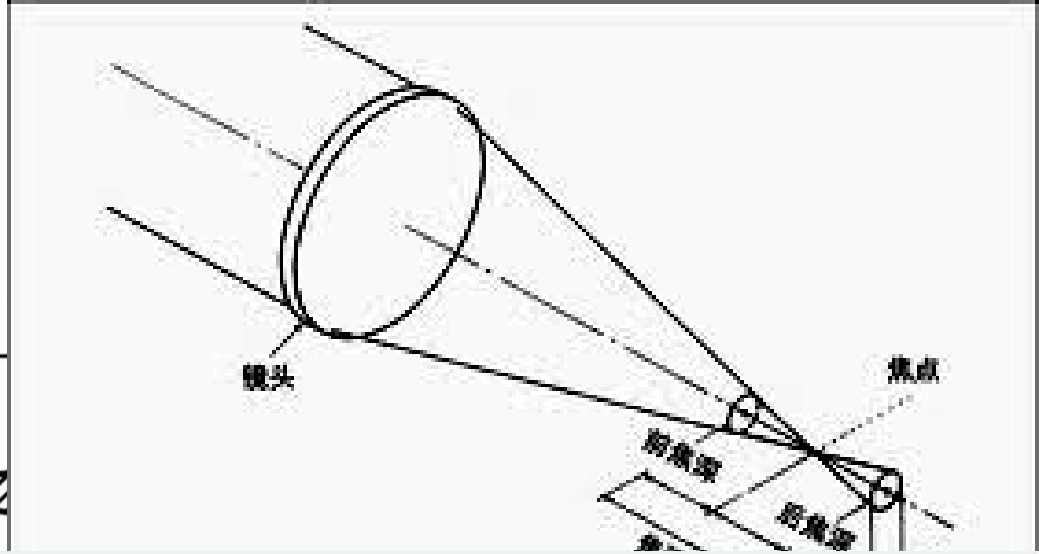


图 28　激光焦深示意



$$\pm D.O.F. = \frac{\omega \times 250{,}000}{N.A. \times M} + \frac{\lambda}{2(N.A.)^2} \ (\mu m)$$

D.O.F. : Depth Of Focus

ω : 眼睛的分辨能力0.0014（设眼睛的视角为5分）

M : 总倍率（物镜倍率×目镜倍率）

λ : 光的波长（可见光线 $\lambda = 0.55\mu m$）

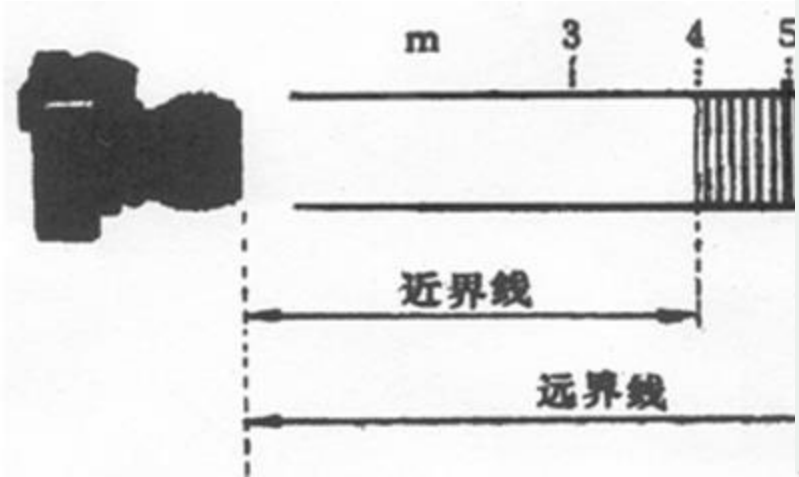
$$\rightarrow \quad \pm D.O.F. = \frac{350}{N.A. \times M} + \frac{0.275}{(N.A.)^2}$$

上式表明了N.A.越大焦深越小的特性。

例:MPLFLN100×，WHN10×时

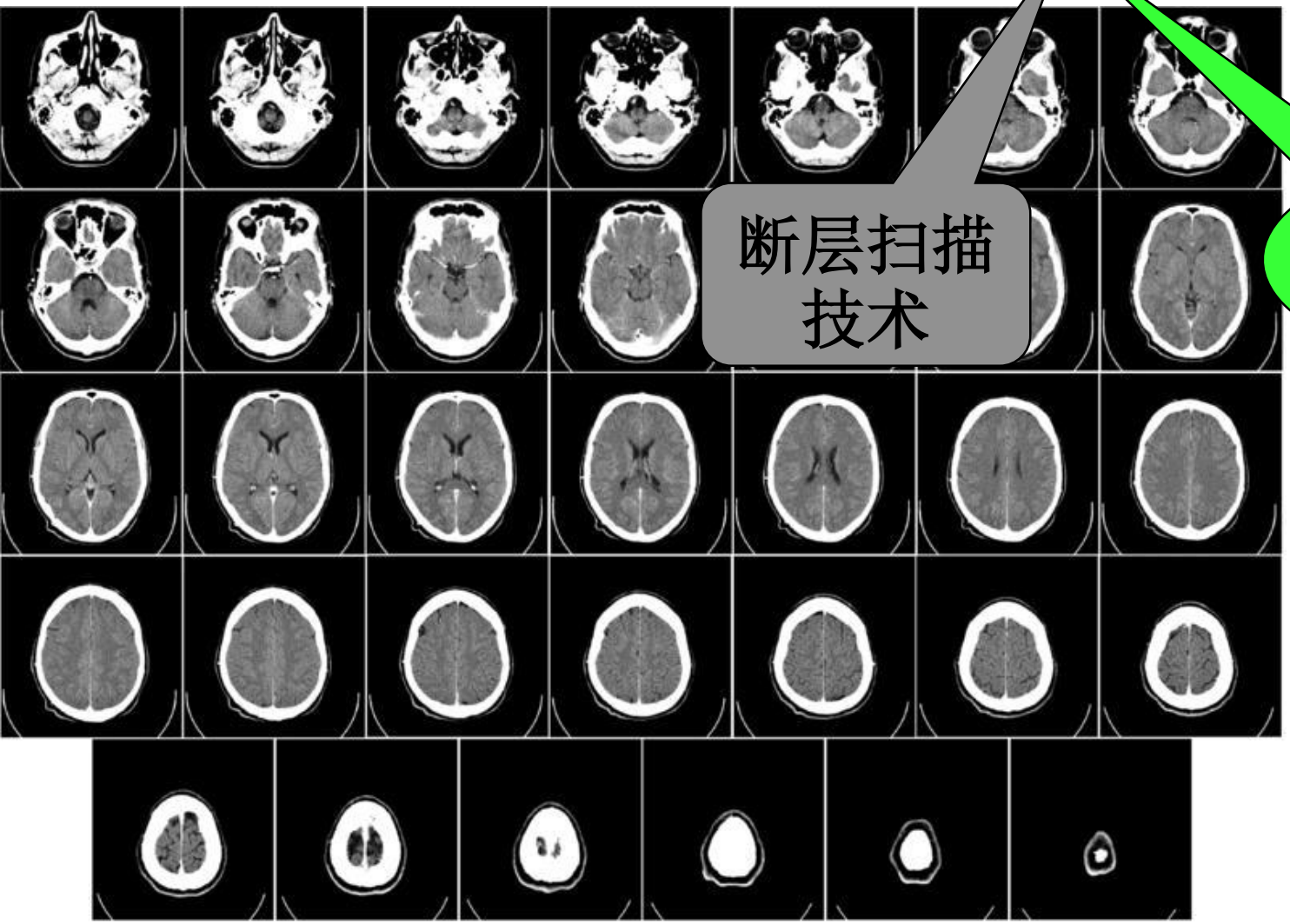
$$\pm D.O.F. = \frac{350}{0.90 \times 1000} + \frac{0.275}{0.81} = 0.39 + 0.34$$

$$= 0.73\mu m$$



Confocal laser scanning microscope

CT



断层扫描
技术

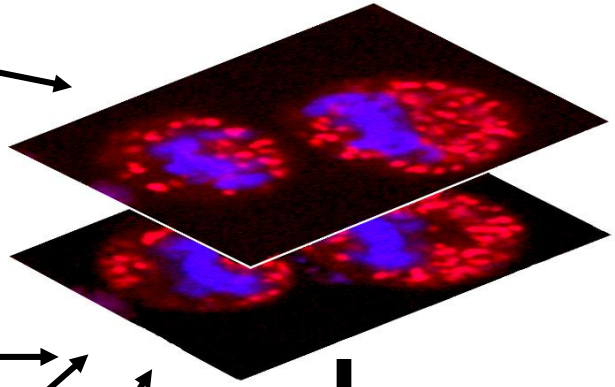
景深成
像技术

Optical Sectioning in Biological Microscopy

Conventional light microscopy doesn't work well on thick (>few microns) specimens.

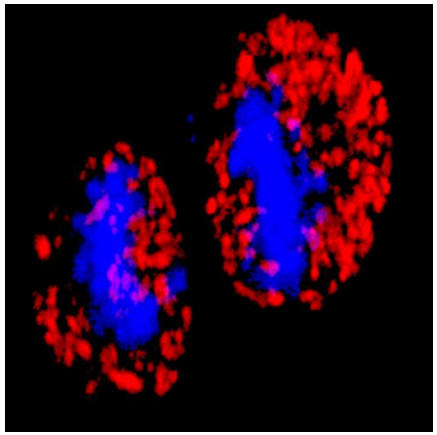
Fluorescently labeled sea urchin eggs

Widefield Fluorescence → Fixation and Physical Sectioning →



Live specimens

- Widefield Fluorescence → Deconvolution Methods (Computational) →
- **Confocal Microscopy** → **Confocal Aperture** →
- Multiphoton Microscopy → nonlinear processes →

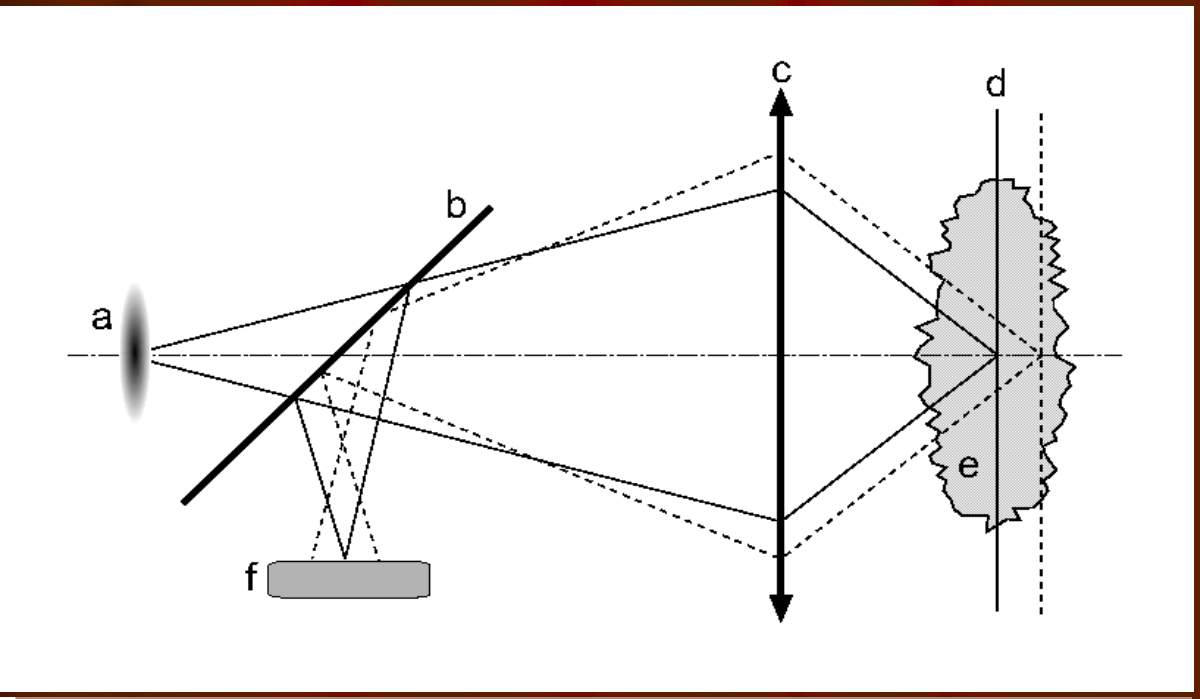


需掌握的要点：

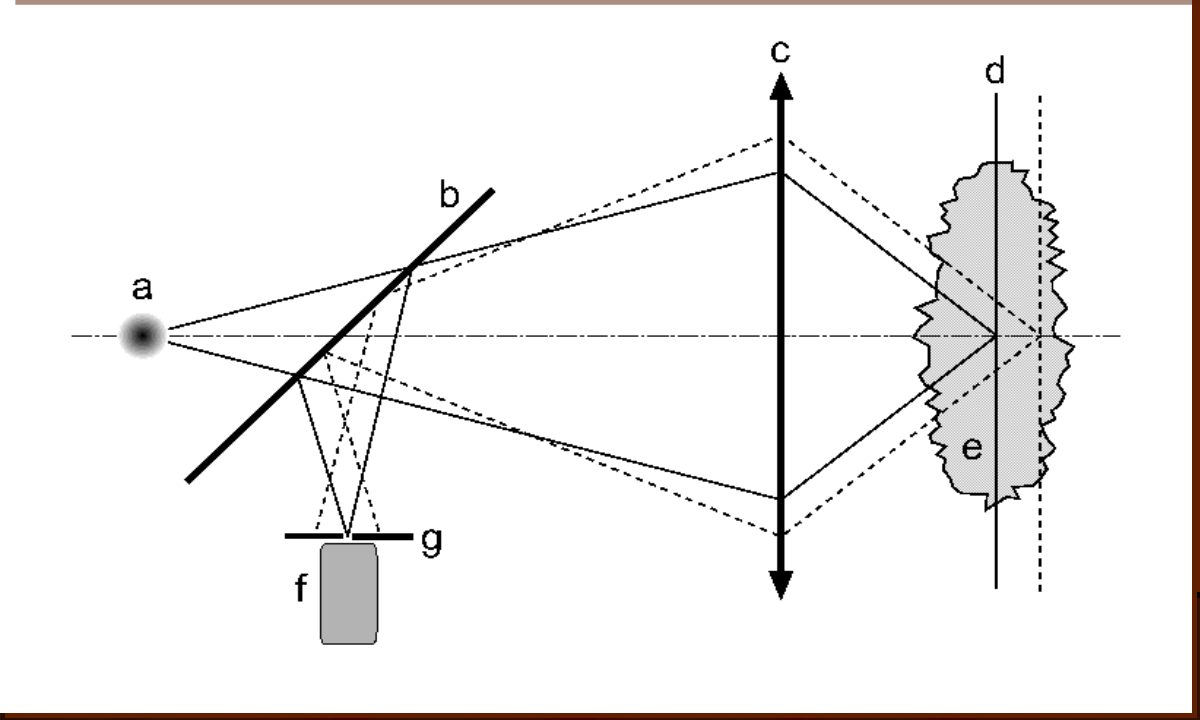
- 共聚焦扫描光学显微镜的组成
- 系统光路构成
- 光学分辨率- Airy disks
- 反射光/背向散射成像

共聚焦显微镜的优点

- 减少光散射造成的成像模糊
- 提高有效分辨率
- 提高信噪比
- 厚样品的清晰成像
- 垂直轴向扫描
- 大纵向成像深度
- 电子可调放大倍数



Wide-field microscopy

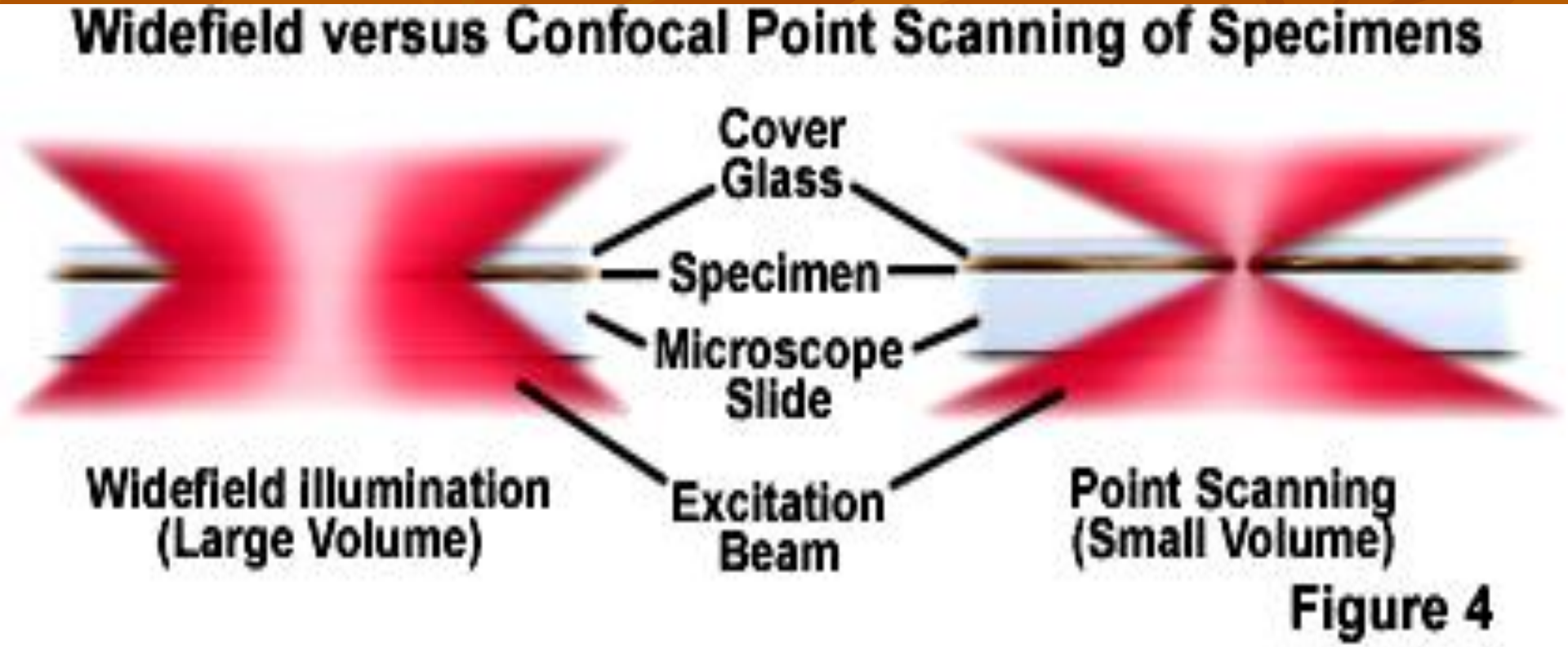


Confocal microscopy

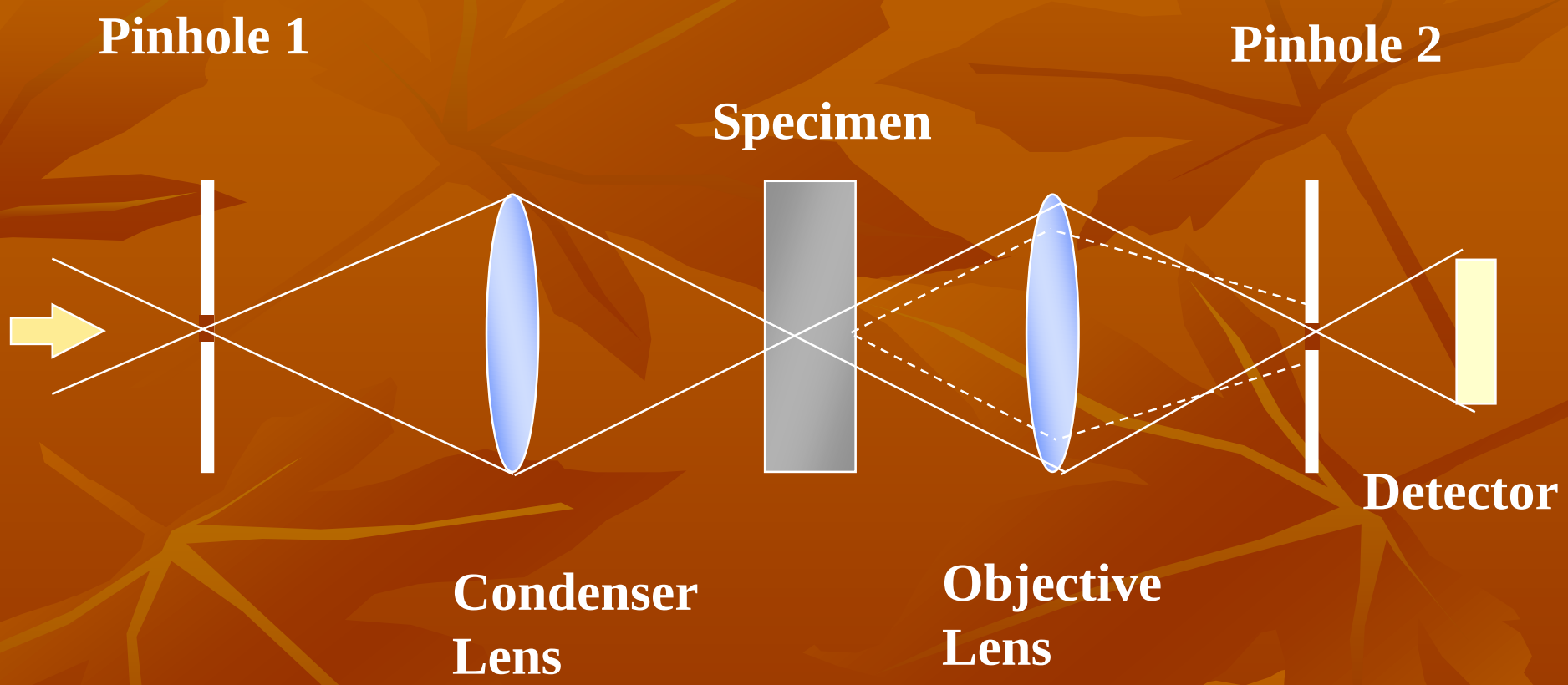
基本原理

传统的光学显微镜使用的是场光源，标本上每一点的图像都会受到邻近点的衍射或散射光的干扰；激光扫描共聚焦显微镜利用激光束经照明针孔形成点光源对标本内焦平面的每一点扫描，标本上的被照射点，在探测针孔处成像，由探测针孔后的光电倍增管（PMT）或致冷CCD逐点或逐线接收，迅速在计算机监视器屏幕上形成荧光图像。照明针孔与探测针孔相对于物镜焦平面是共轭的，焦平面上的点同时聚焦于照明针孔和发射针孔，焦平面以外的点不会在探测针孔处成像，这样得到的共聚焦图像是标本的光学横断面，克服了普通显微镜图像模糊的缺点。

Working principle

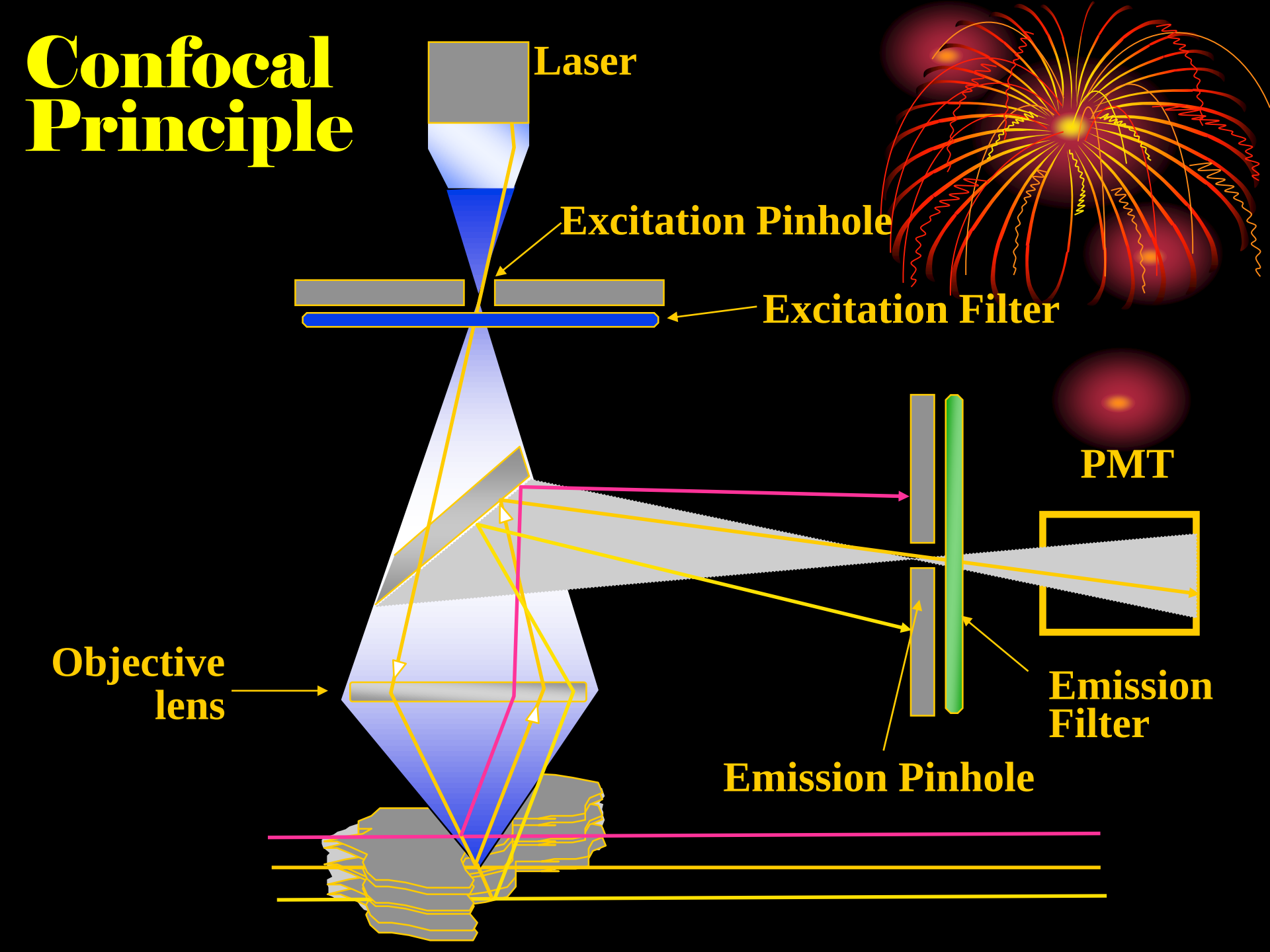


How a Confocal Image is Formed

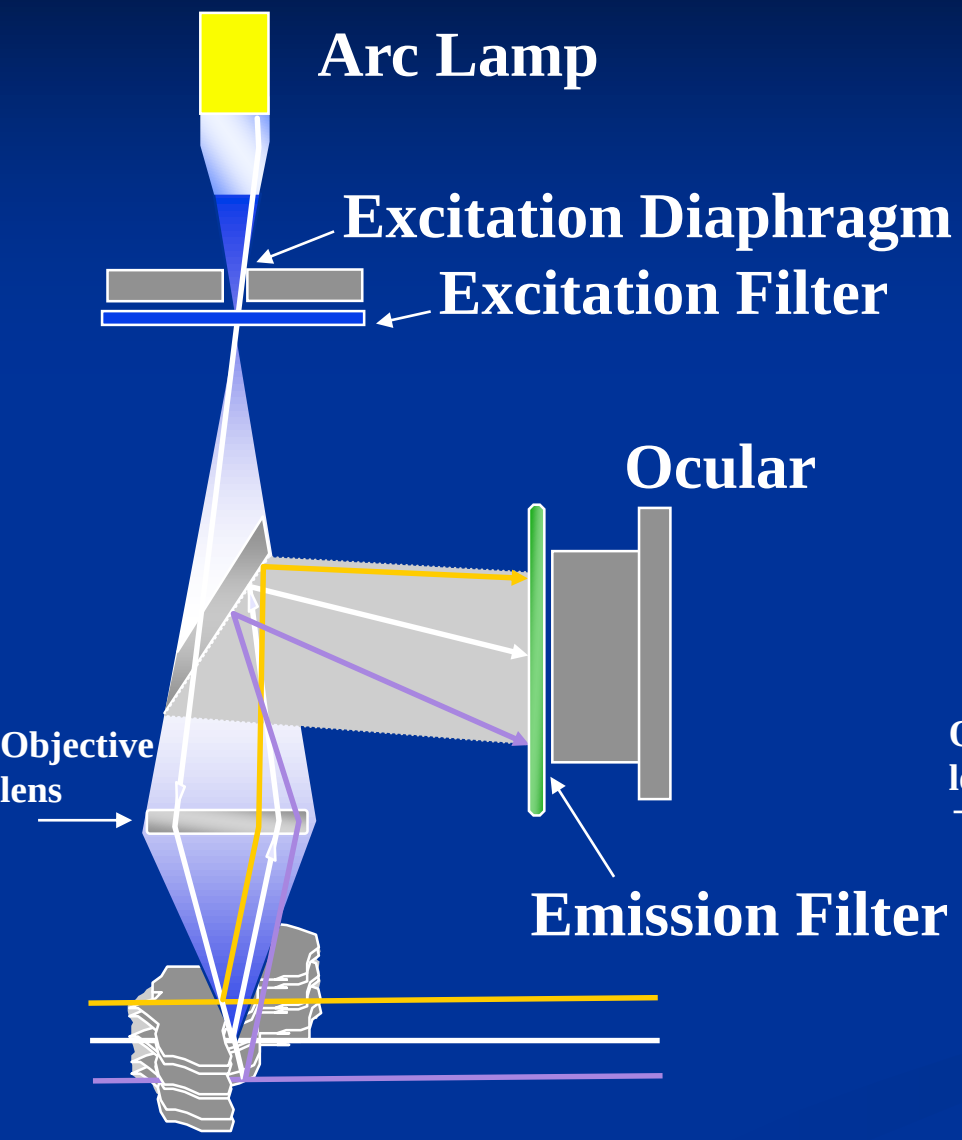


Modified from: Handbook of Biological Confocal Microscopy. J.B.Pawley, Plennum Press, 1989

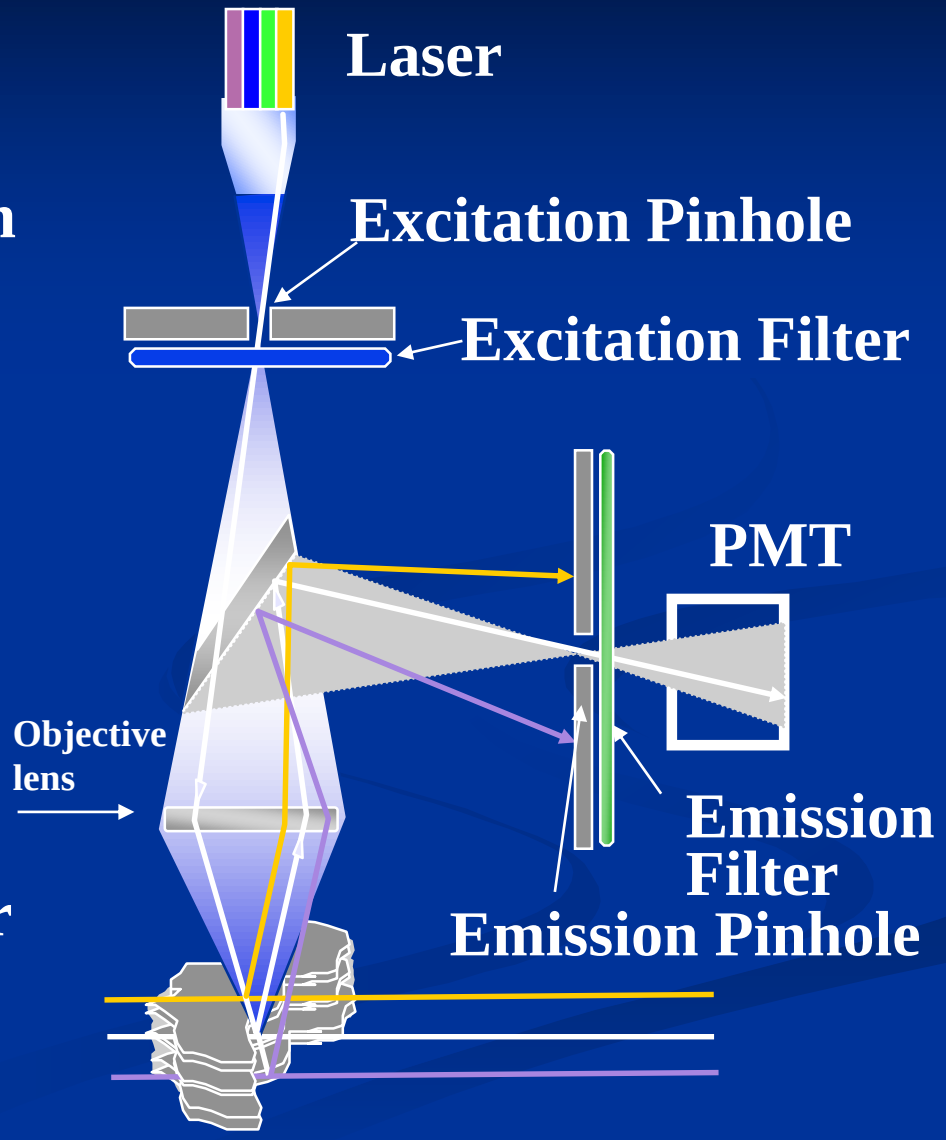
Confocal Principle



Fluorescent Microscope



Confocal Microscope



Confocal vs. widefield microscope

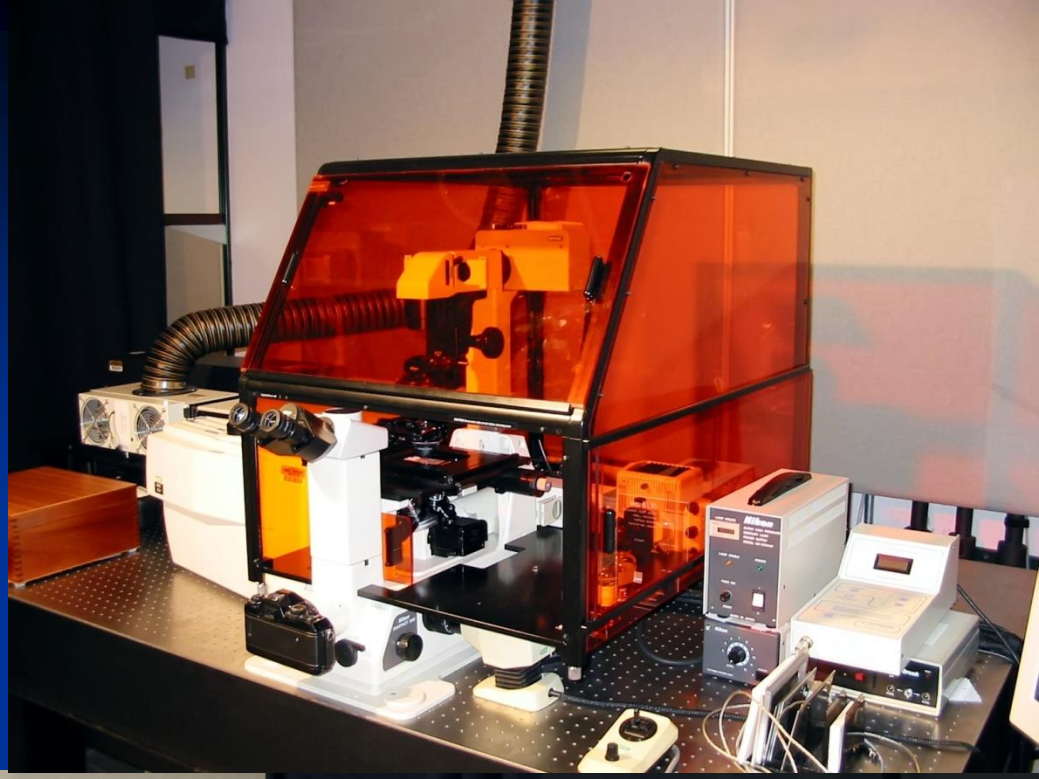
Widefield

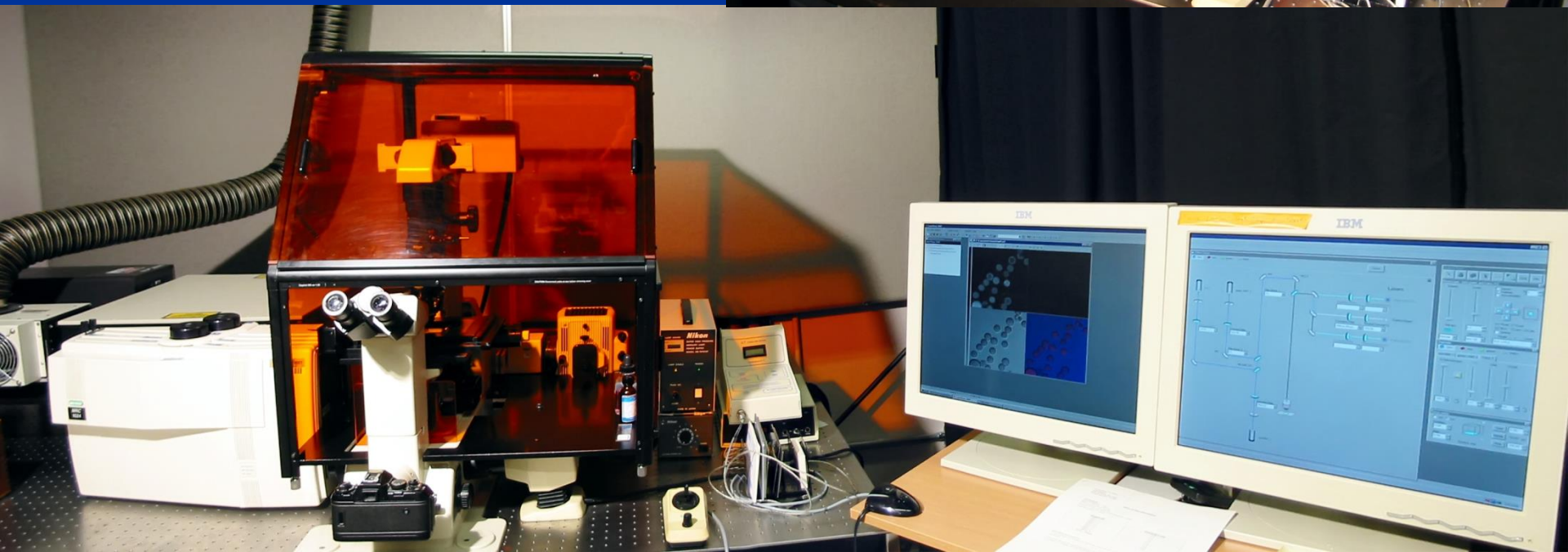
- 即时得到整个图像
  - 通过目镜成像
  - 成像快
  - 光损伤较大
- 背景噪声高 (也称为二次荧光)
- “价格便宜”

Confocal

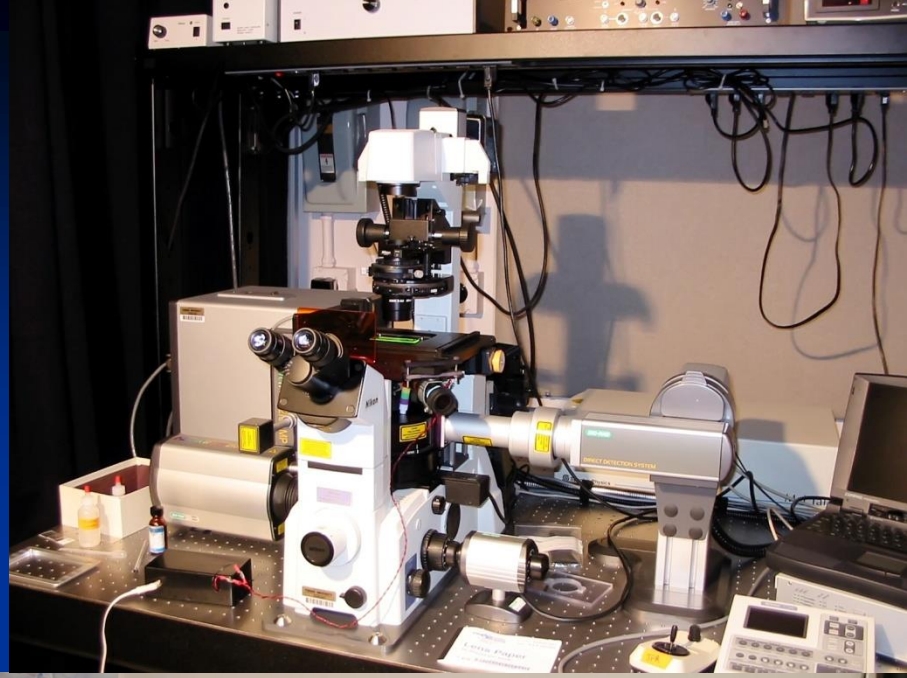
- 薄片成像(0.5-1.5μm)
- 最大厚度 ~50μm
- 图像对比度高
- 光损伤小
- 扫描成像方式
  - “成像慢”
  - 可数字化放大
- 有限的激光颜色数量
- 系统价格高

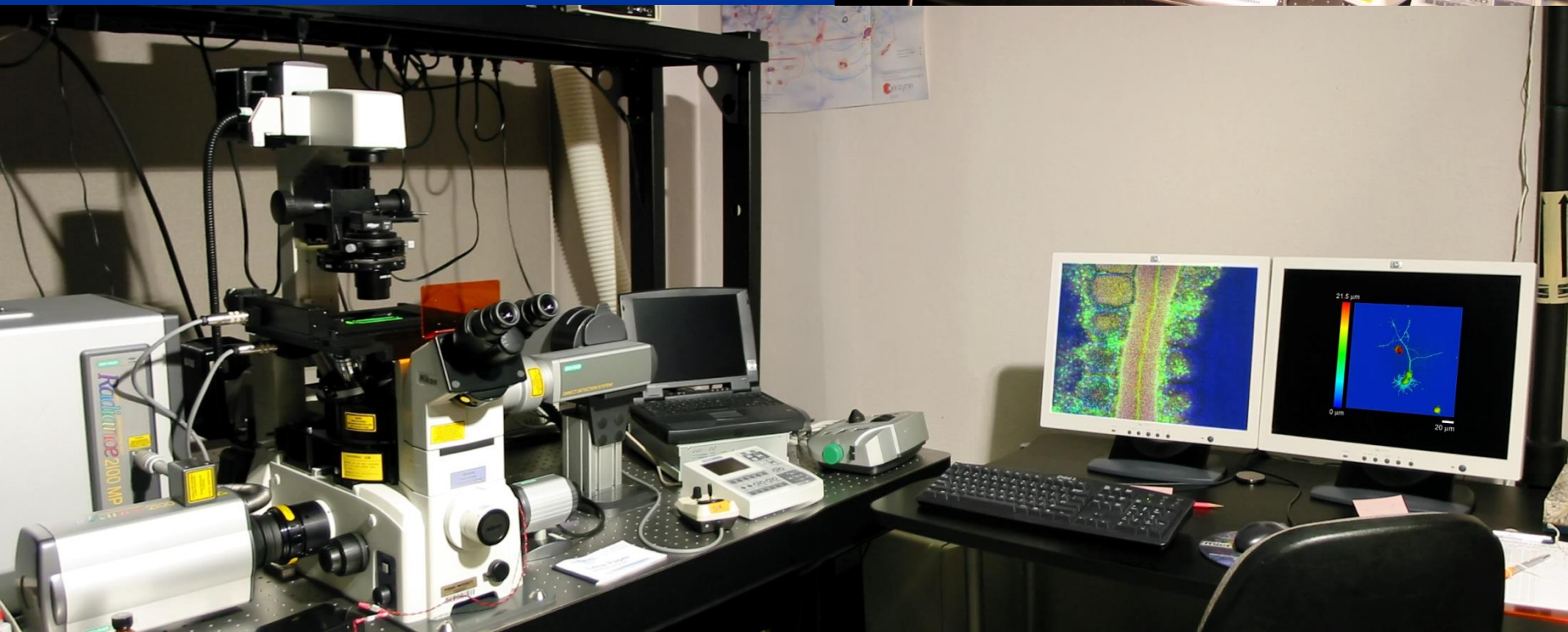
Bio-Rad MRC 1024 at Purdue



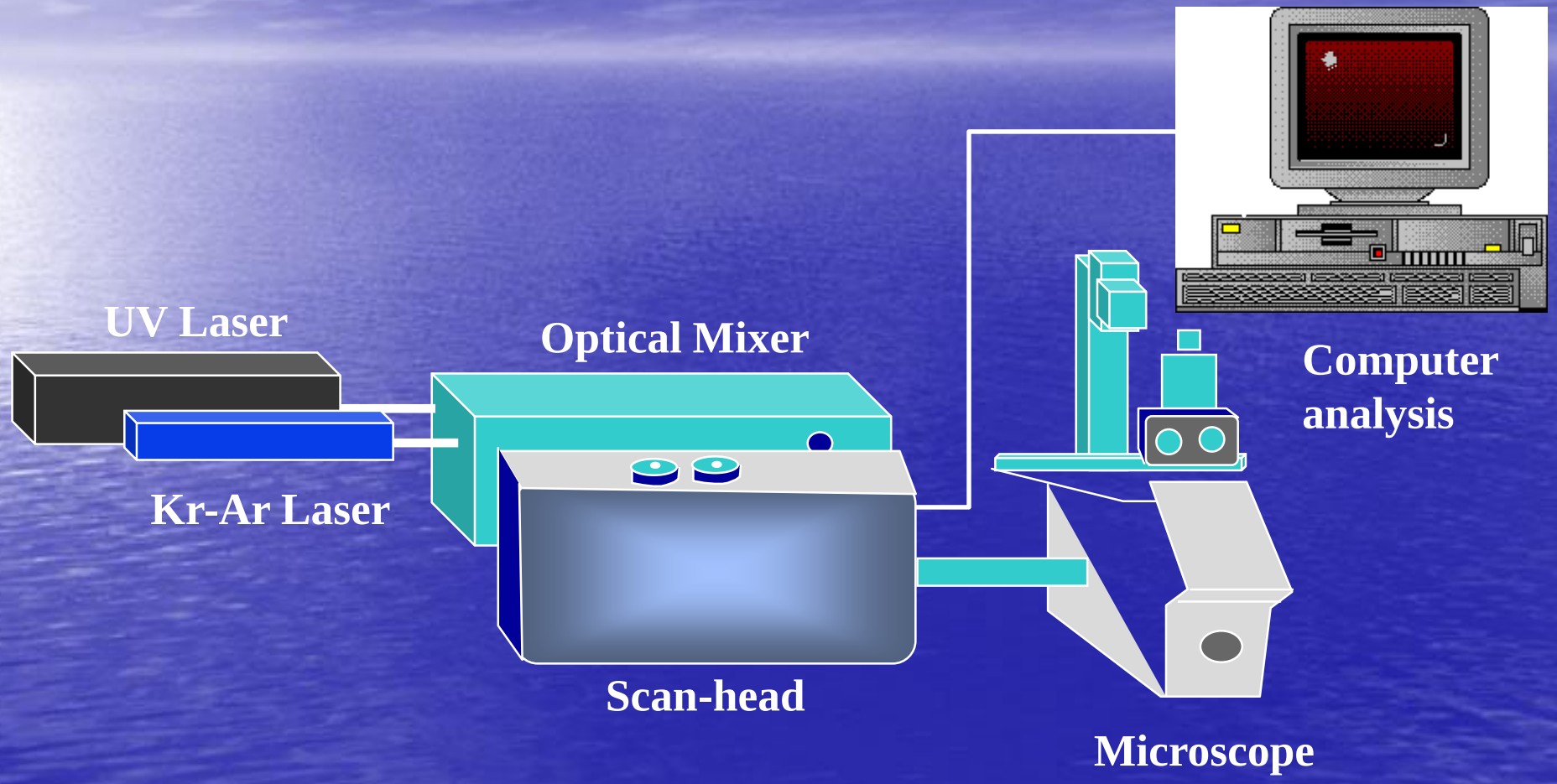


Radiance 2100MP at Purdue



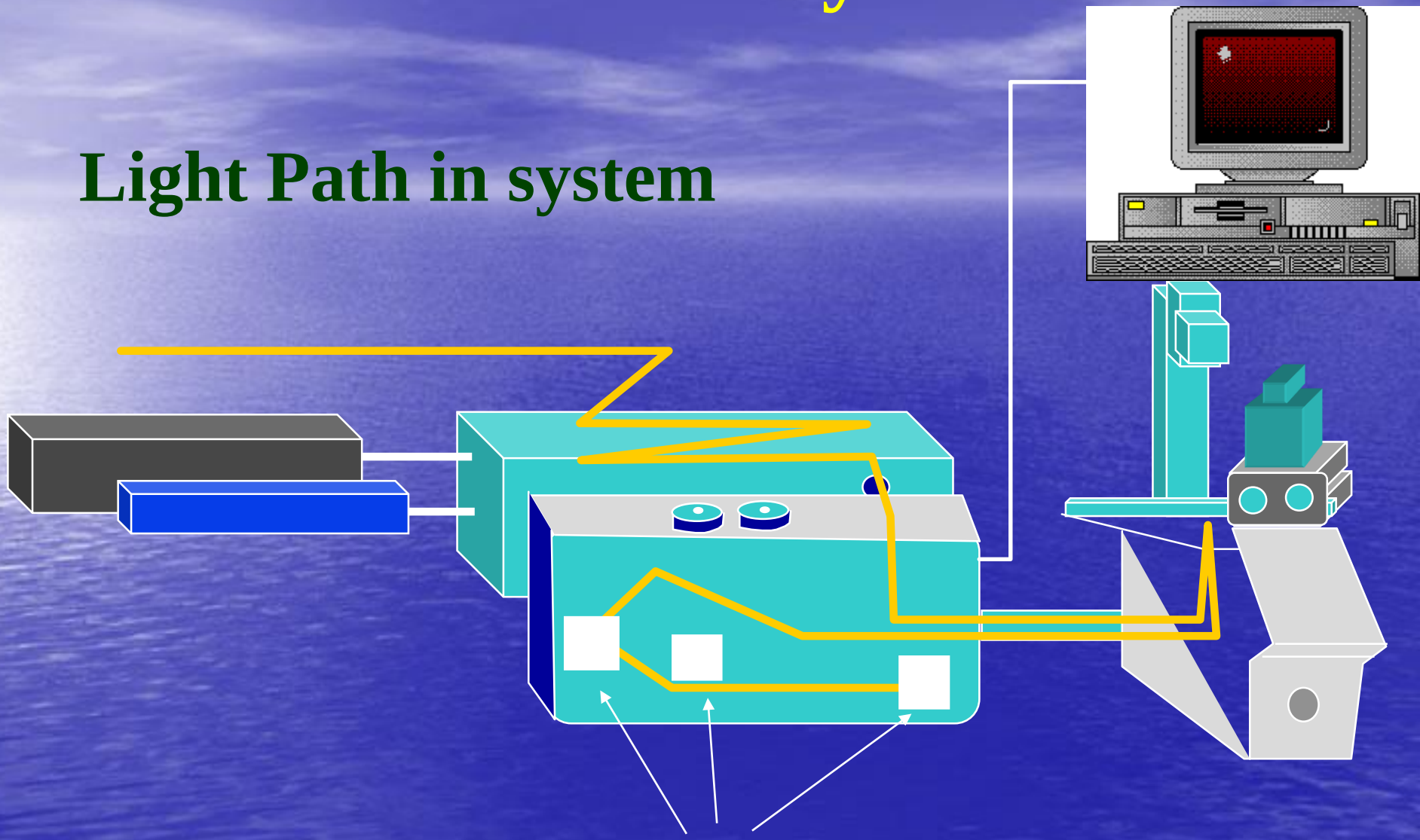


MRC 1024 System

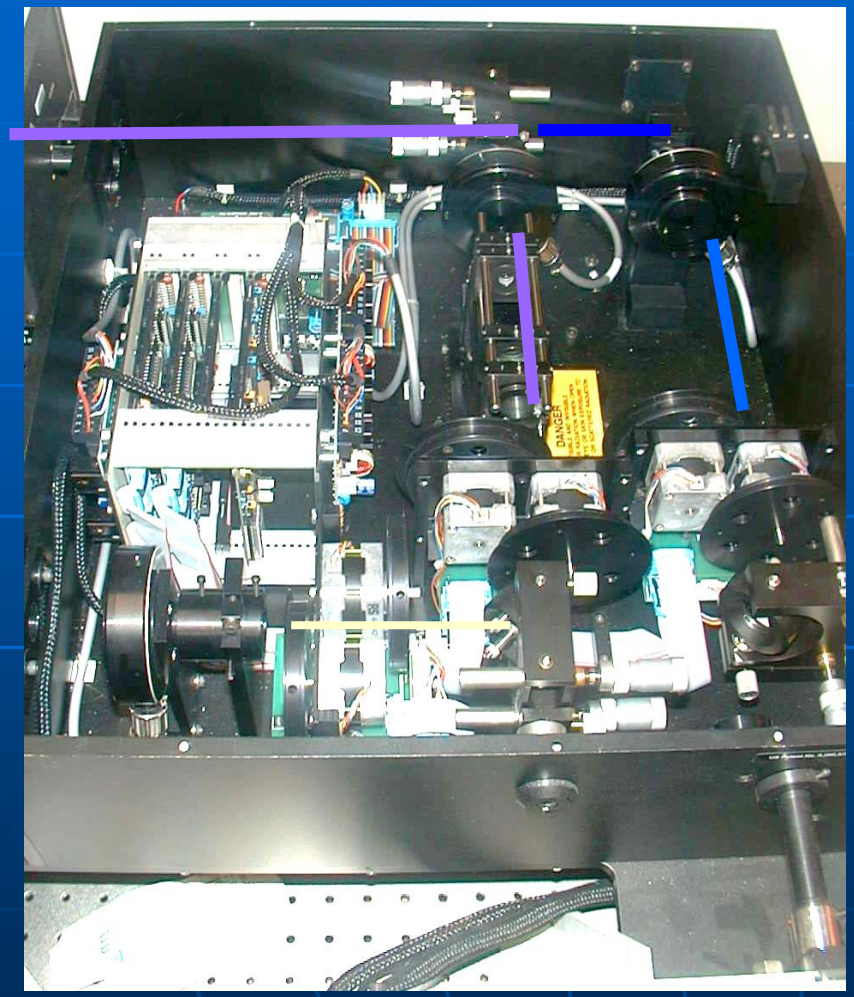


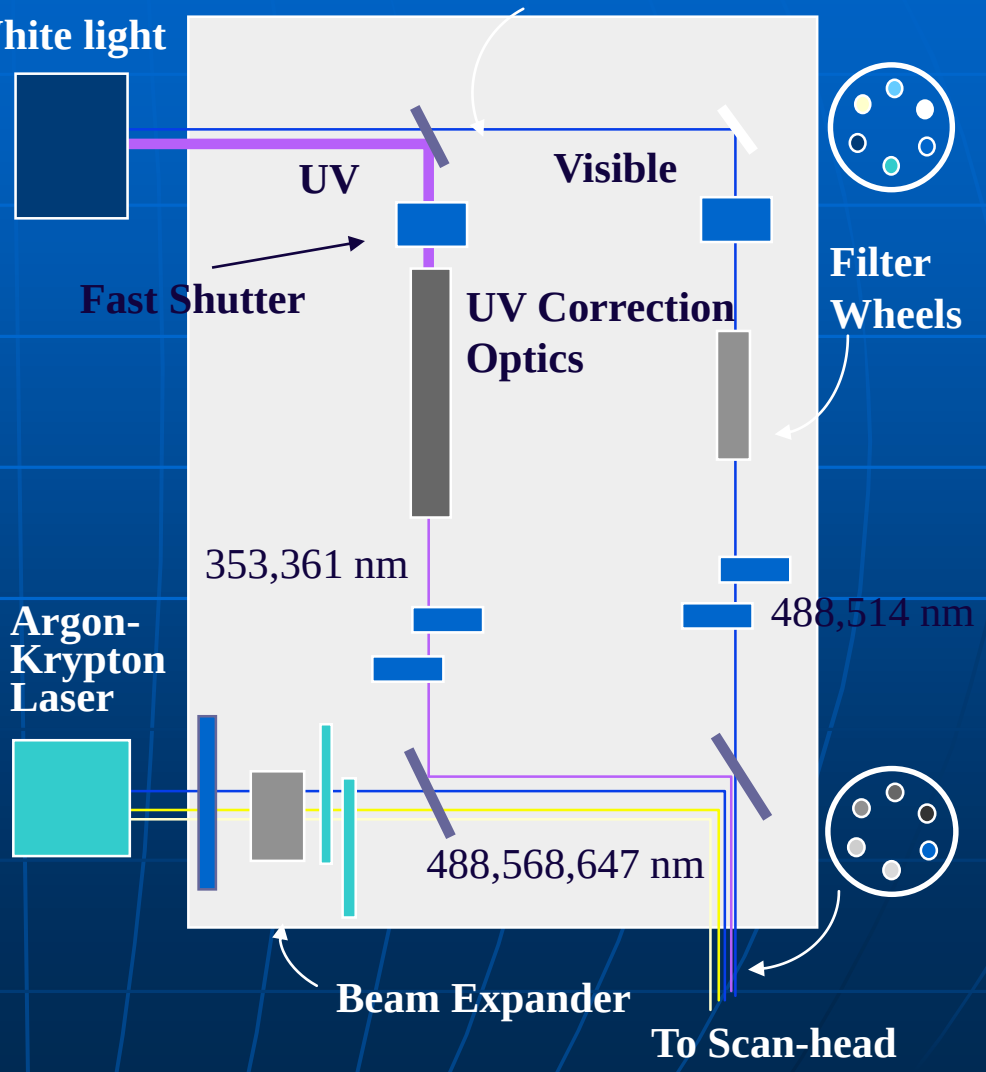
MRC 1024 System

Light Path in system

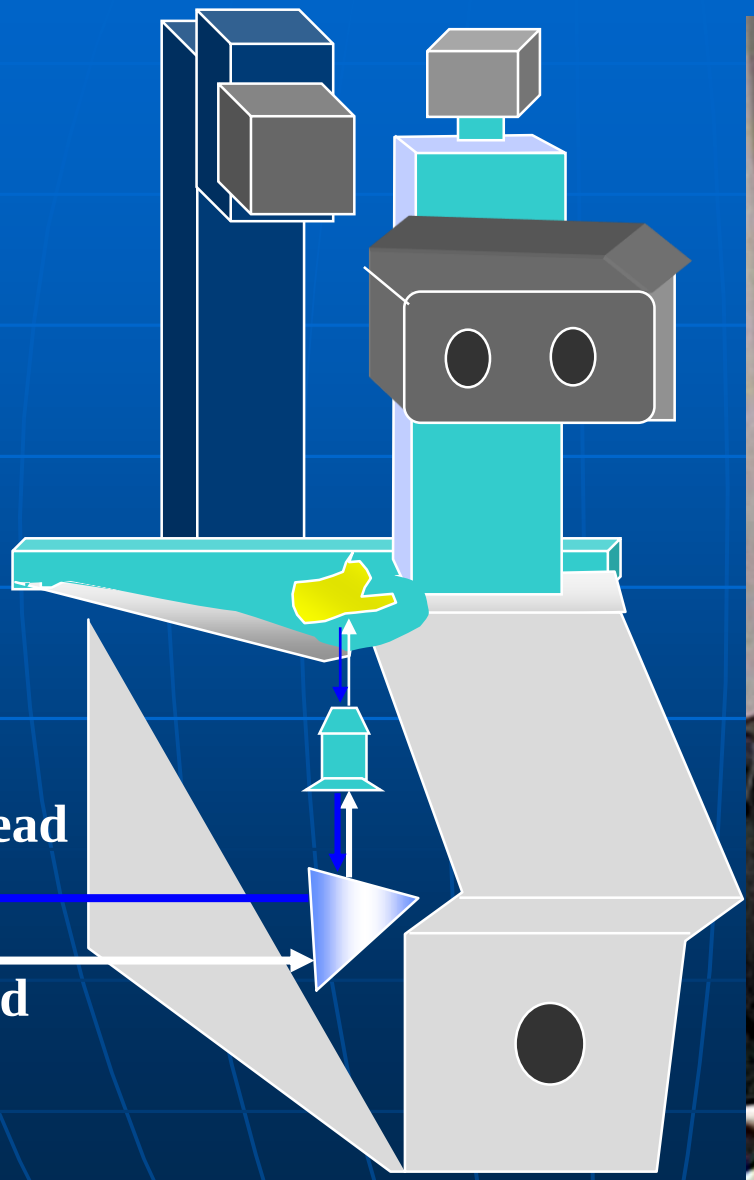


Optical Mixer - MRC 1024 UV

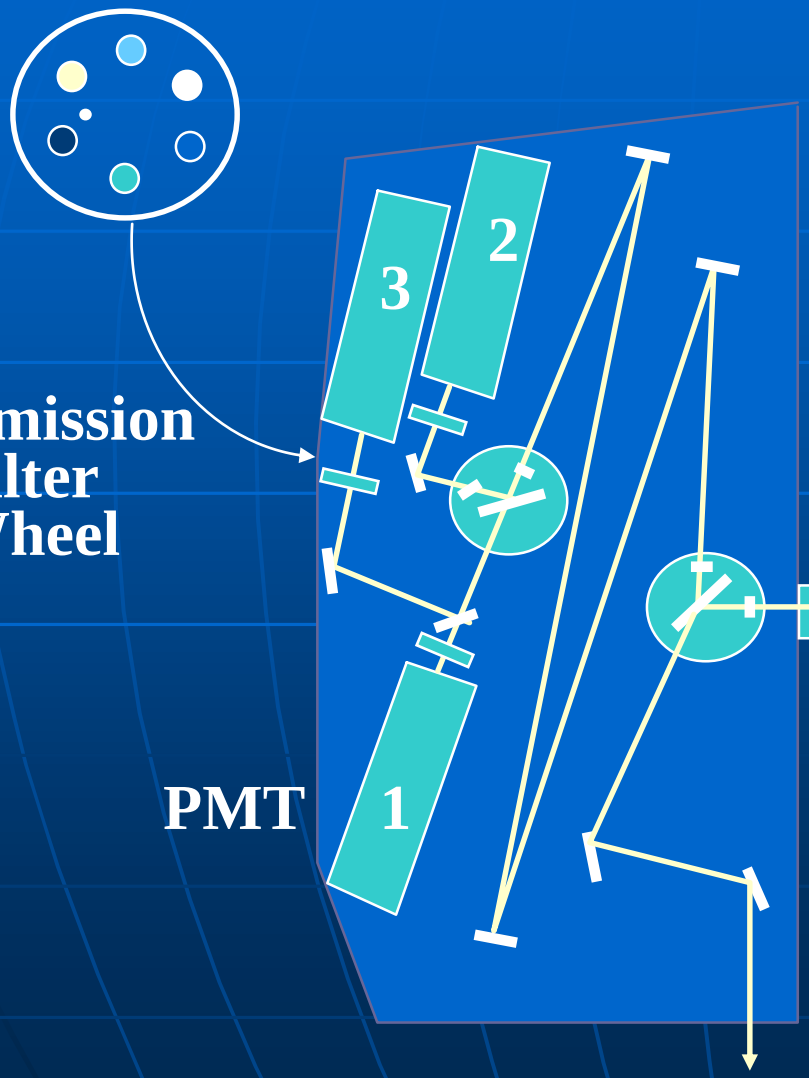




MRC 1024 Scan-head



MRC 1024 Scan-head



Emission Filter Wheel

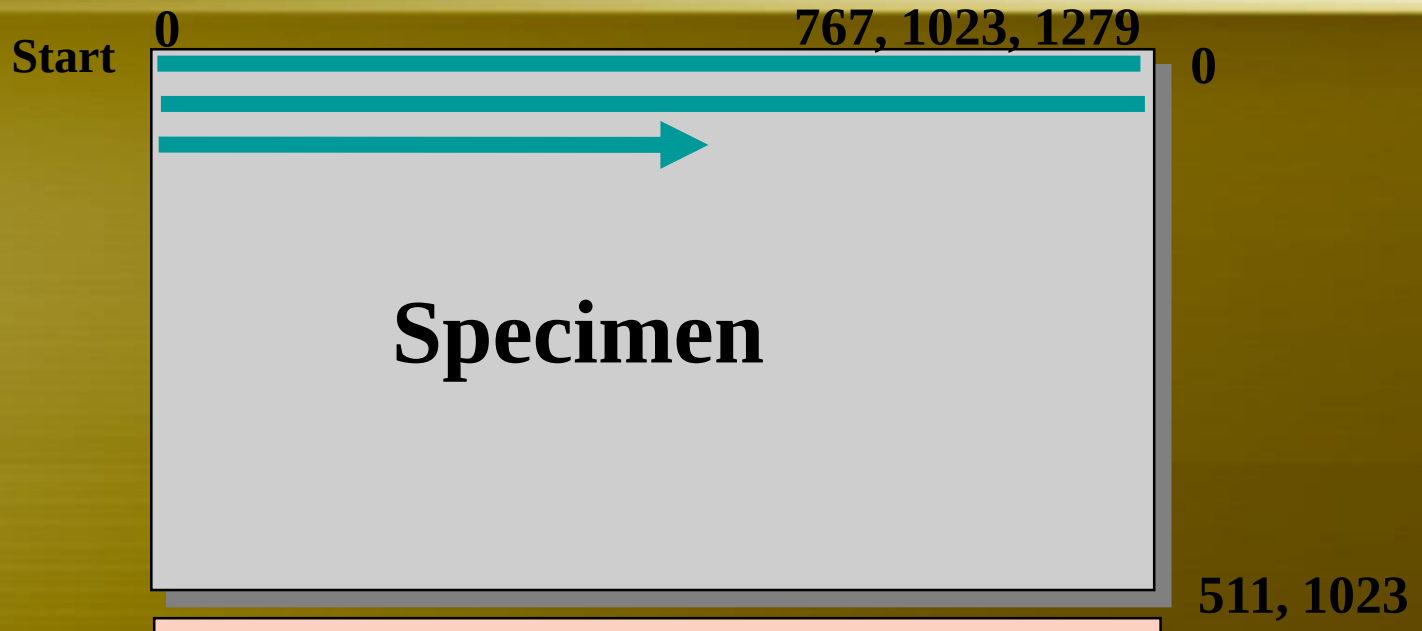
PMT

From Laser

Galvanometers

To and from Scan-head

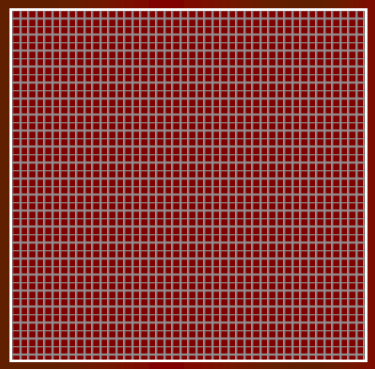
The Scan Path of the Laser Beam



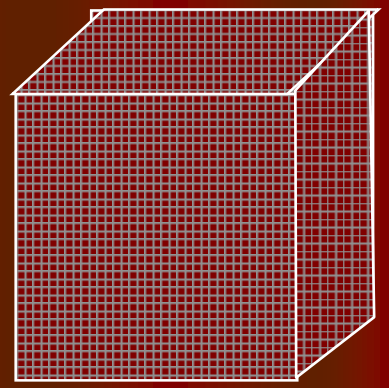
Frames/Sec	# Lines
1	512
2	256
4	128
8	64
16	32

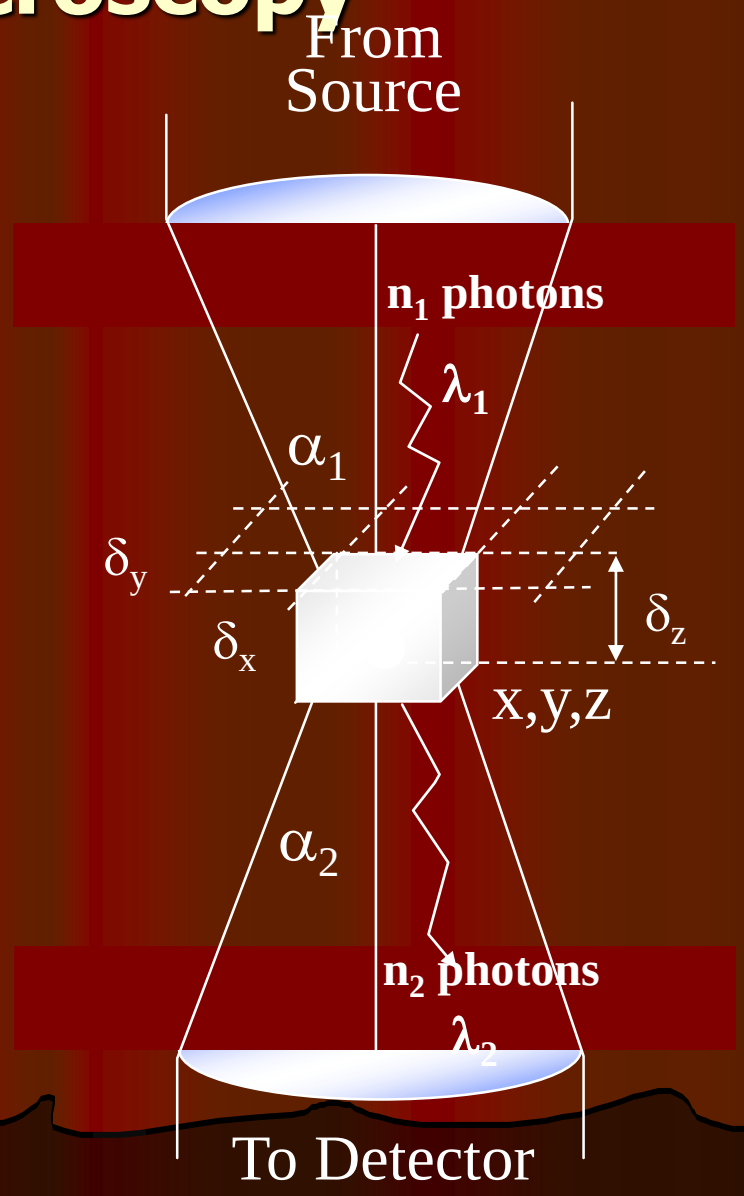
Fundamental Limitations of Confocal Microscopy

PIXEL
2D space



VOXEL
3D space





From: Handbook of Biological Confocal Microscopy. J.B.Pawley, Plennum Press, 1989

从基本原理上讲,共聚焦显微镜是一种现代化的光学显微镜,它对普通光学显微镜从技术上作了以下几点改进:

1.1 用激光做光源因为激光的单色性非常好,光源波束的波长相同,从根本上消除了色差。

1.2 采用共聚焦技术在物镜的焦平面上放置了一个当中带有小孔的挡板,将焦平面以外的杂散光挡住,消除了球差;并进一步消除了色差。

1.3 采用点扫描技术将样品分解成二维或三维空间上的无数点,用十分细小的激光束(点光源)逐点逐行扫描成像,再通过计算机组合成一个整体平面的或立体的像。而传统的光学显微镜是在场光源下一次成像的,标本上每一点的图像都会受到相邻点的衍射光和散射光的干扰。这两种图像的清晰度和精密度是无法相比的。

1.4 用计算机采集和处理光信号,并利用光电倍增管放大信号图在共聚焦显微镜中,计算机代替了人眼或照相机进行观察、摄像,得到的图像是数字化的,可以在电脑中进行处理,再一次提高图像的清晰度。而且利用了光电倍增管,可以将很微弱的信号放大,灵敏度大大提高。

由于综合利用了以上技术，可以说LSCM是显微镜制作技术、光电技术、计算机技术的完美结合,是现代技术发展的必然产物。

Gray Level & Pixelation

- **Analogous to intensity range**

  For computer images each pixel is assigned a value. If the image is 8 bit, there are 2^8 or 256 levels of intensity. If the image is 10 bit there are 1024 levels, 12 bit 4096 levels etc.

- The intensity analogue of a pixel is its grey level which shows up as brightness.

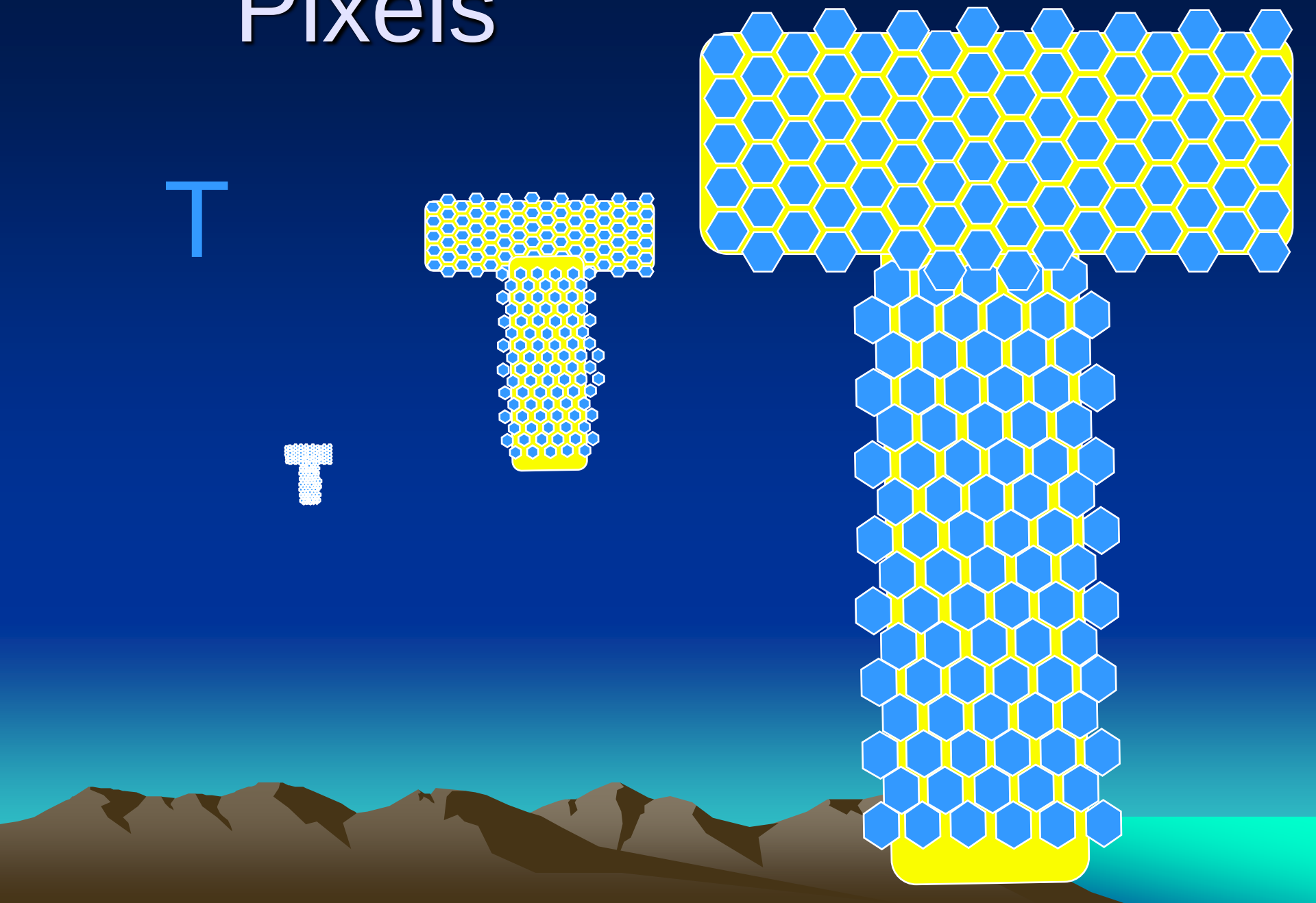
- The display will determine the possible resolution since on a TV screen, the image can only be displayed based upon the number of elements in the display. Of course, it is impossible to increase the resolution of an image by attributing more “pixels” to it than were collected in the original collection!

Pixels

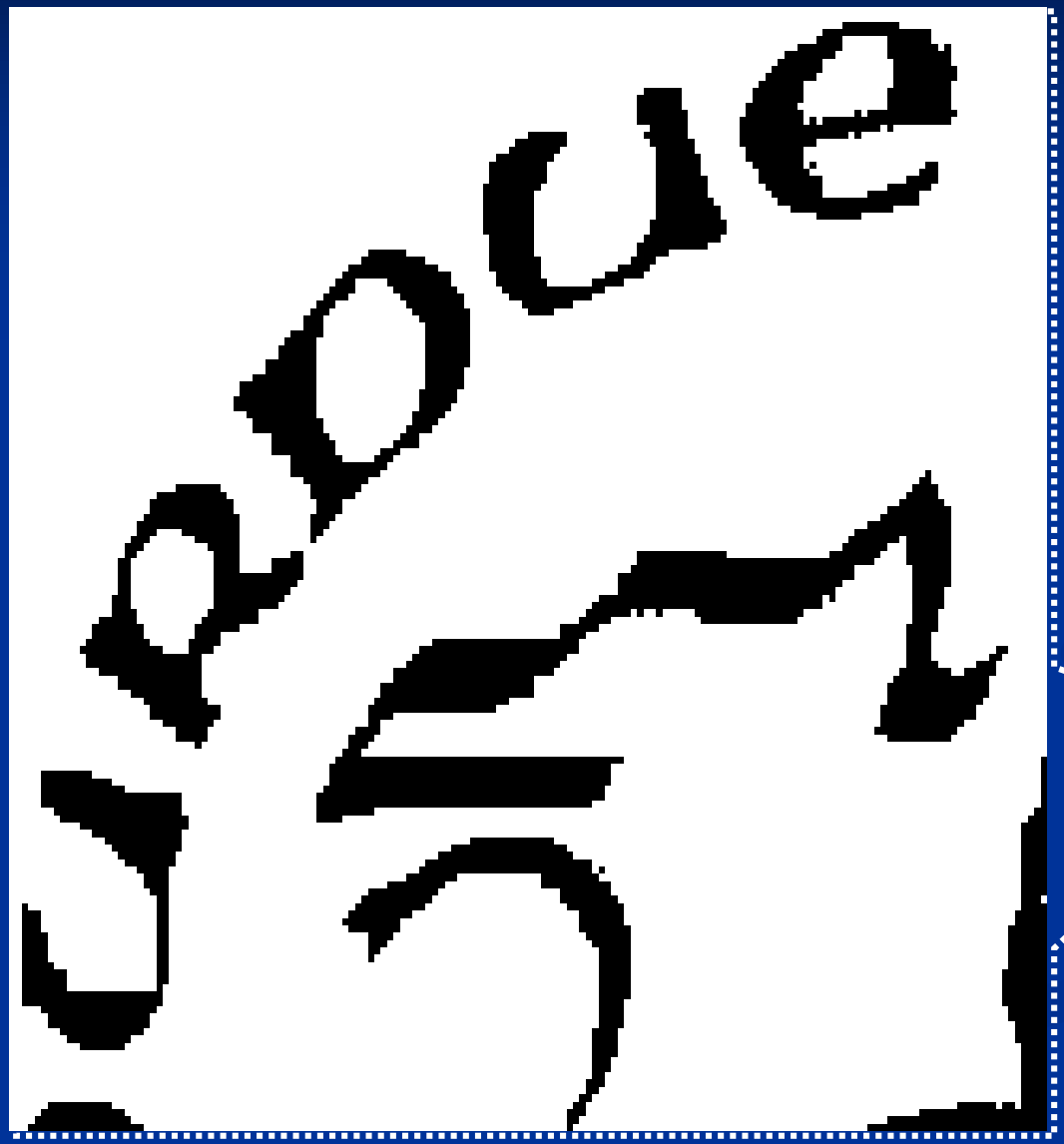
- **Pixels & image structure**

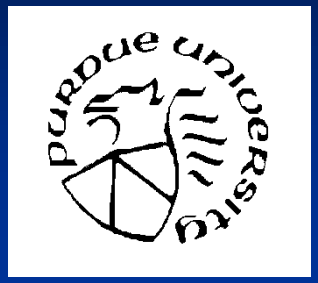
Hardcopy usually compromises pixel representation. With 20/20 vision you can distinguish dots 1 arc second apart (300m at 1m) so 300dps on a page is fine. So at 100m, you could use dots 300mm in size and get the same effect! Thus an image need only be parsimonius, *i.e.*, it only needs to show what is necessary to provide the expected image.

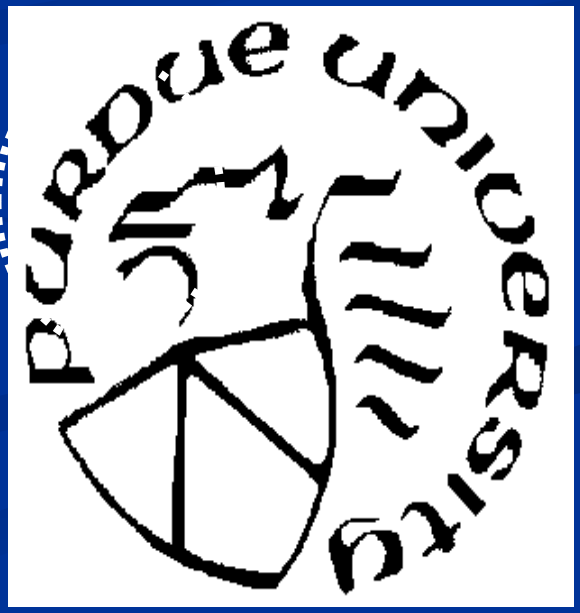
Pixels



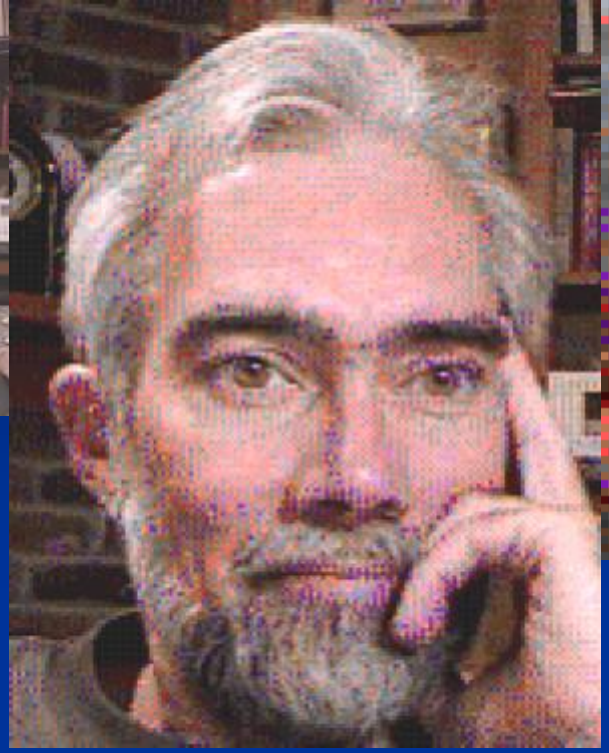
Magnification beyond acceptable limits



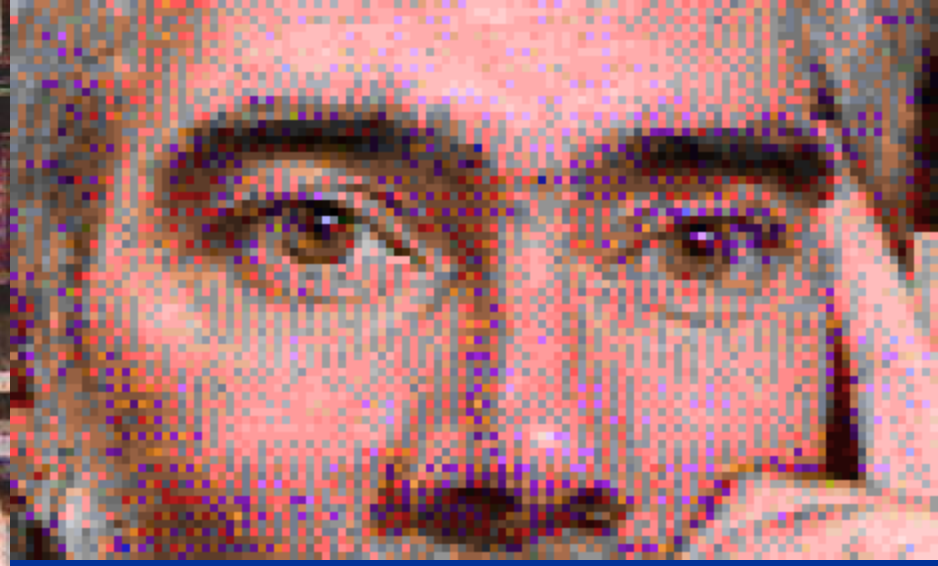




320x240 x 24



Zoom x 2



Zoom x 4

The final image appears to be very "boxy" this is known as "pixilation".

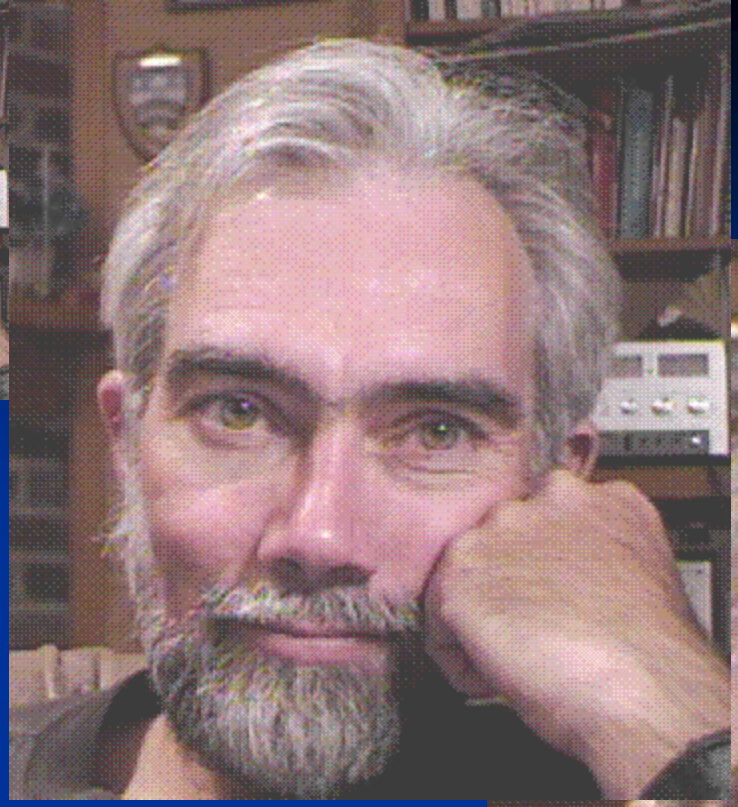
Magnifying with inadequate information. This is known as "empty magnification" because there are insufficient data points.

Zoom x 8

Socrates?….well perhaps not...

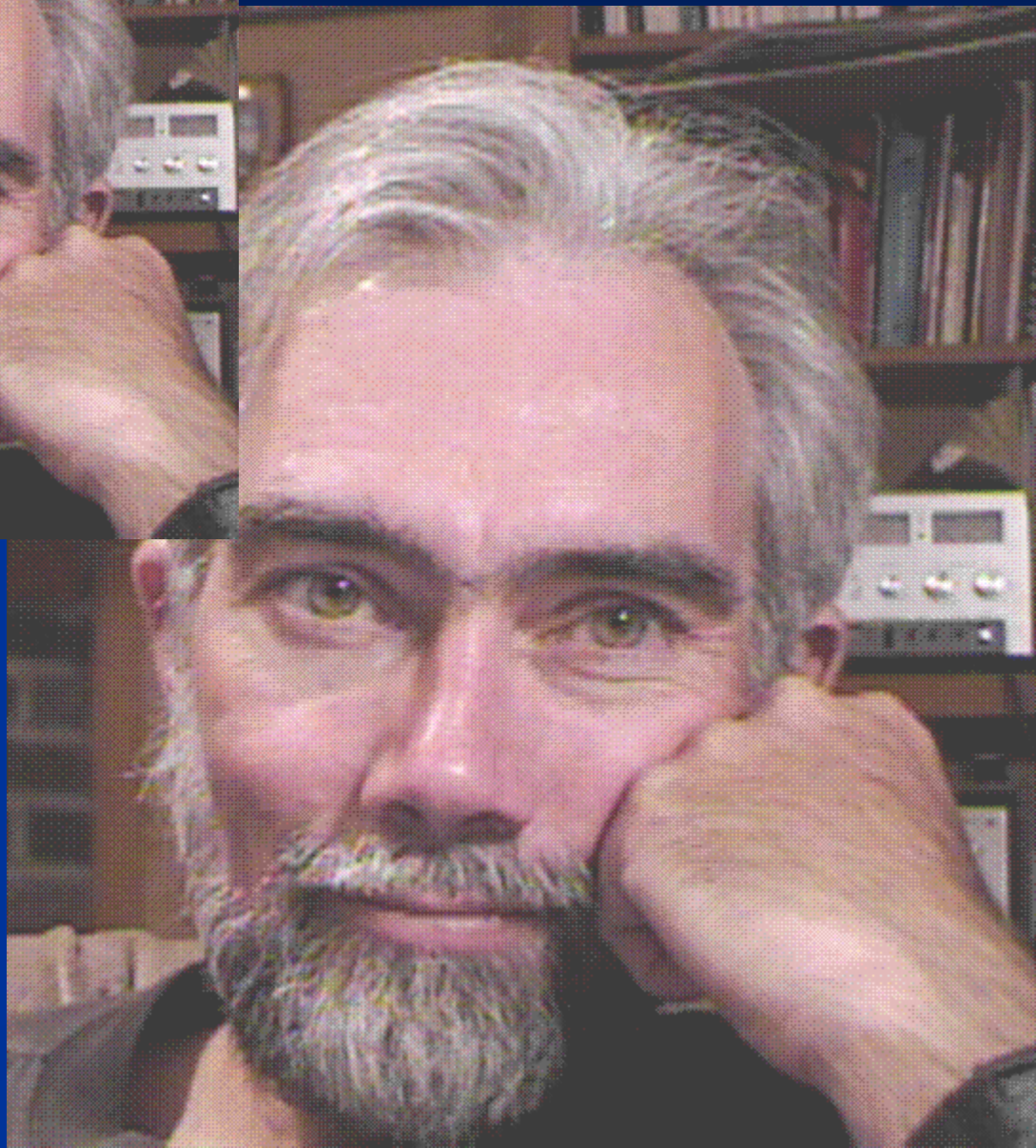
180x200x8

(288,000) 1X



361x400x8

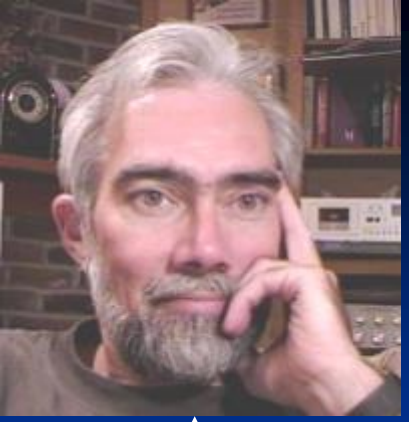
(1,155,200) 2x



541x600x8

(2,596,800) 1.5x)

Magnifying with adequate information. Here, the original image was collected with many more pixels - so the magnified image looks better!

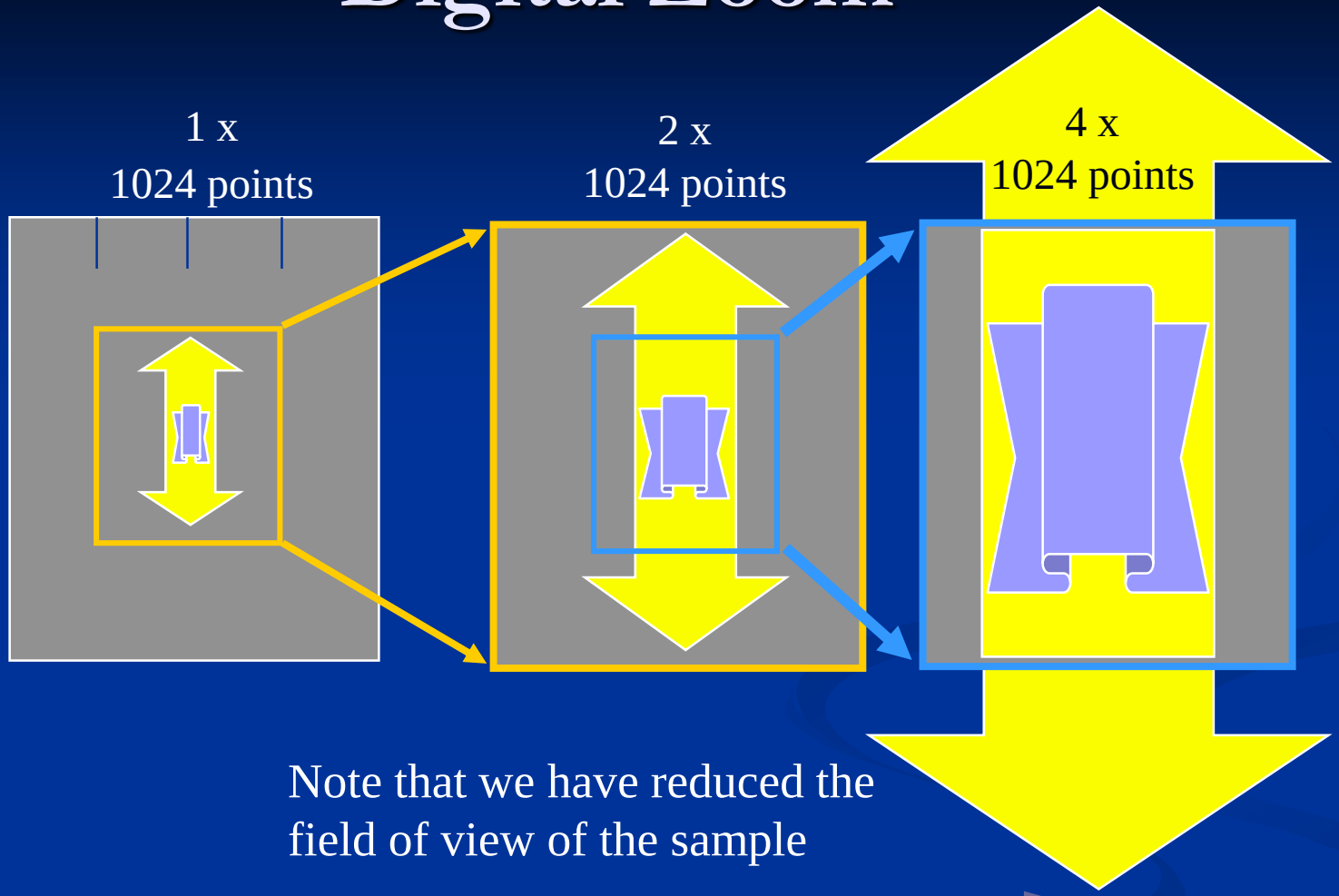


320 x 240 x 24

Originals collected at high resolution - compared to a low resolution image magnified

1500 x 1125 x 24

Digital Zoom



Note that we have reduced the field of view of the sample

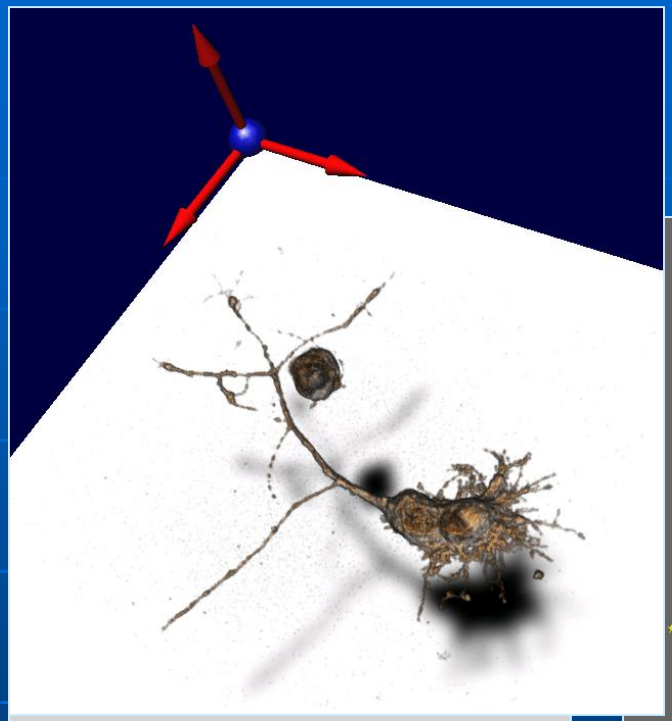
分辨率逐渐降低

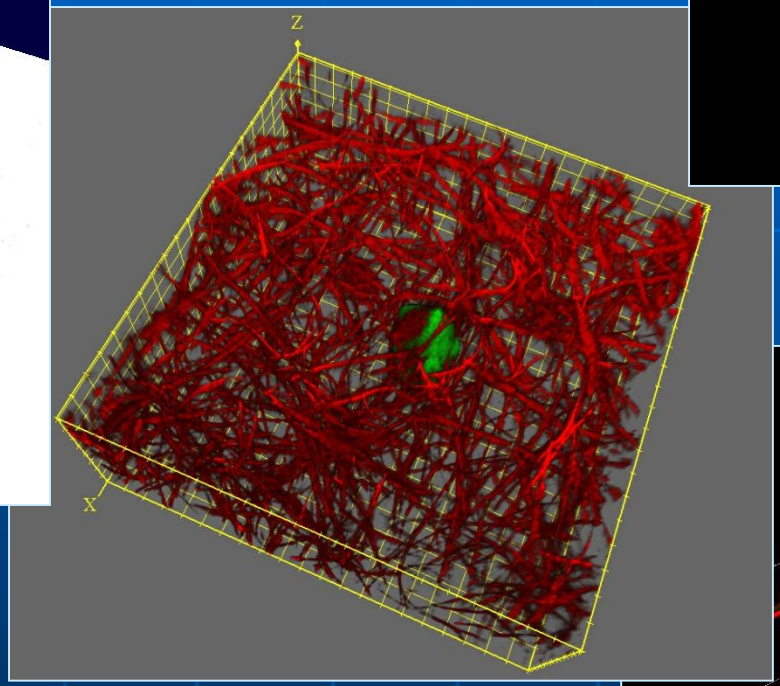
Note: There will only be a single zoom value where optimal resolution can be collected.

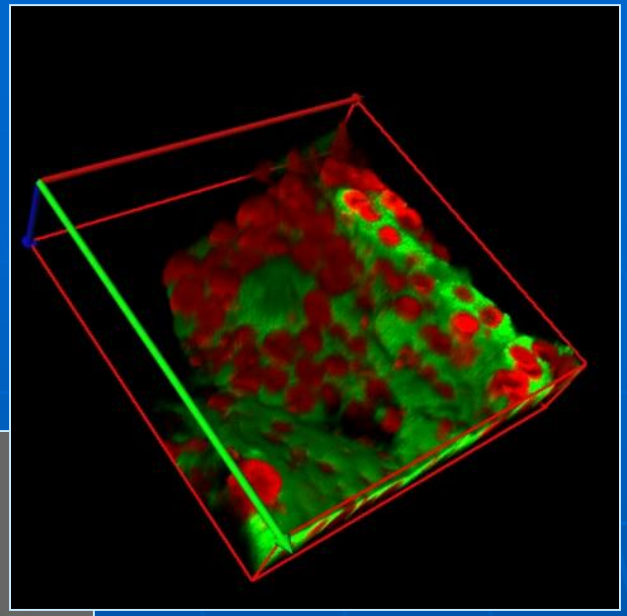
Sampling Theory

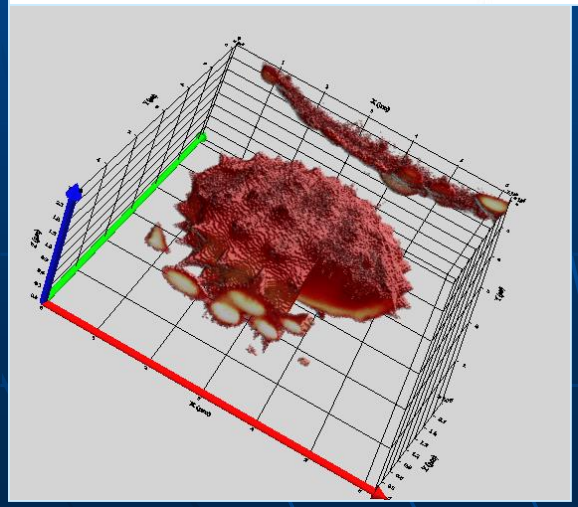
- **The Nyquist Theorem**
  - Nyquest theory describes the sampling frequency (f) required to represent the true identity of the sample.
  - *i.e.*, how many times must you sample an image to know that your sample truly represents the image?
  - In other words to capture the periodic components of frequency f in a signal we need to sample at least $2f$ times
- Nyquist claimed that the rate was $2f$.
- It has been determined that in reality the rate is $2.3f$ - in essence you must sample at least 2 times the highest frequency.
- For example in audio:
- To capture the 22 kHz in the digitized signal, we need to sample at least 44.1 kHz (unless of course you can't hear 22k Hz and then you don't need 44.1 kHz!)

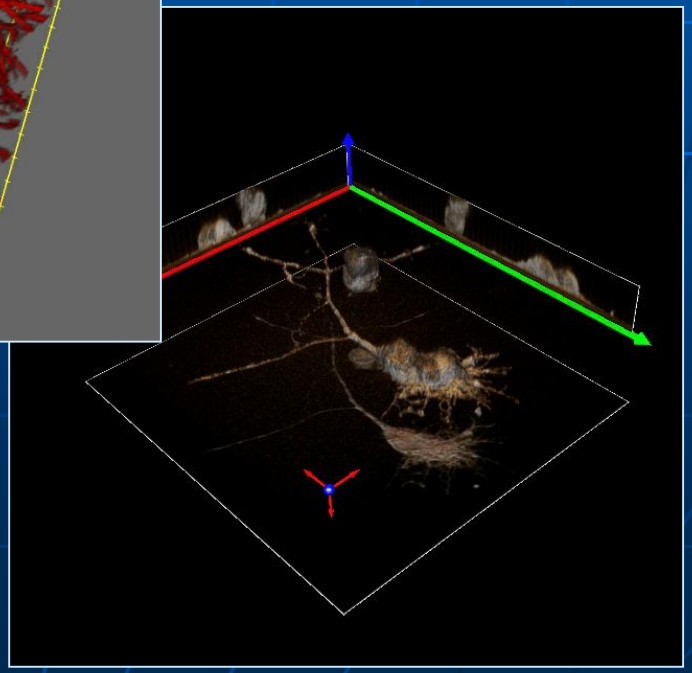
3D imaging using CLSM



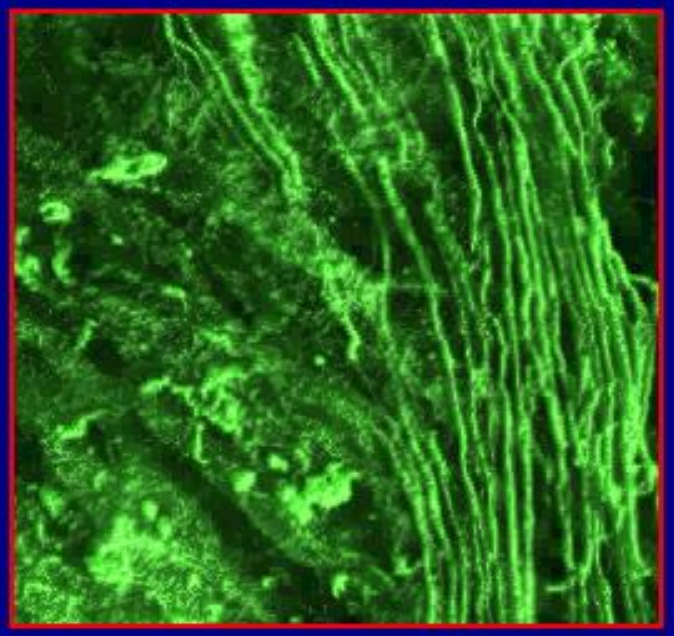




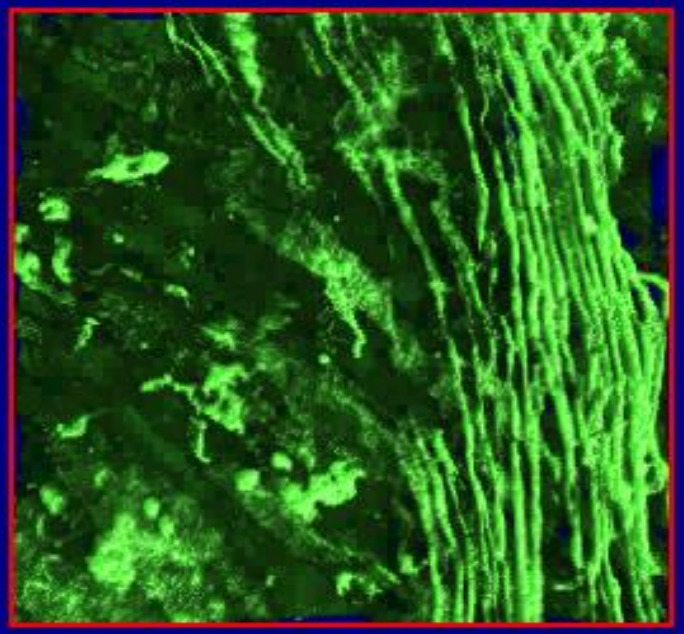




CLSM can employ autofluorescence! Example: topography of SIS



Raw image

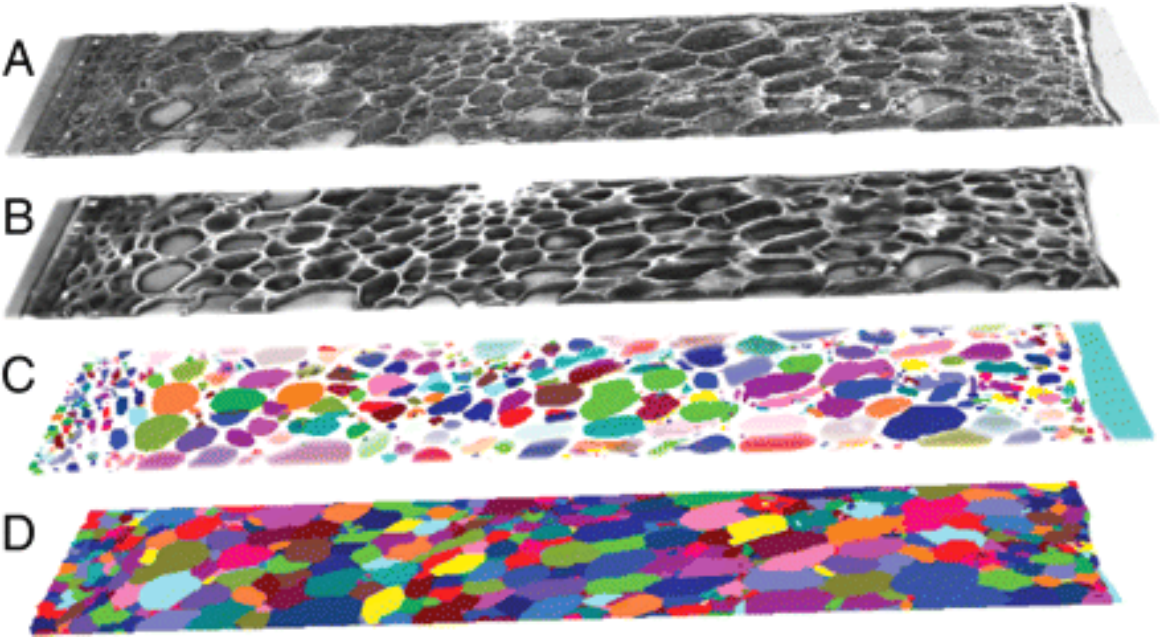


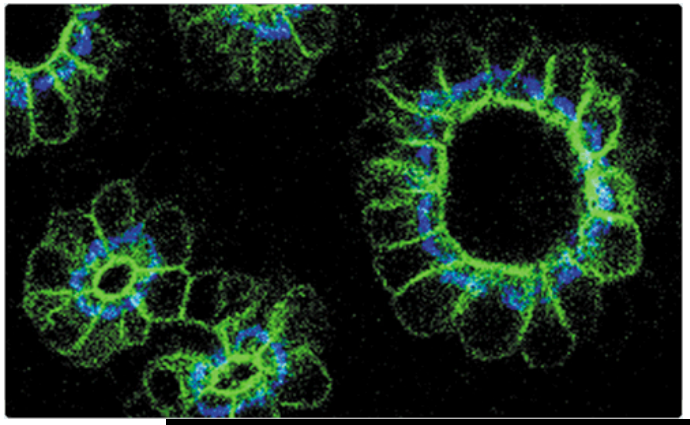
Deconvolved image

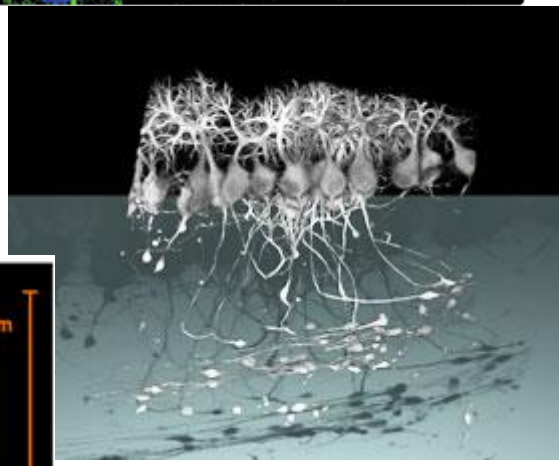
保证共聚焦成像需具备的几大因素

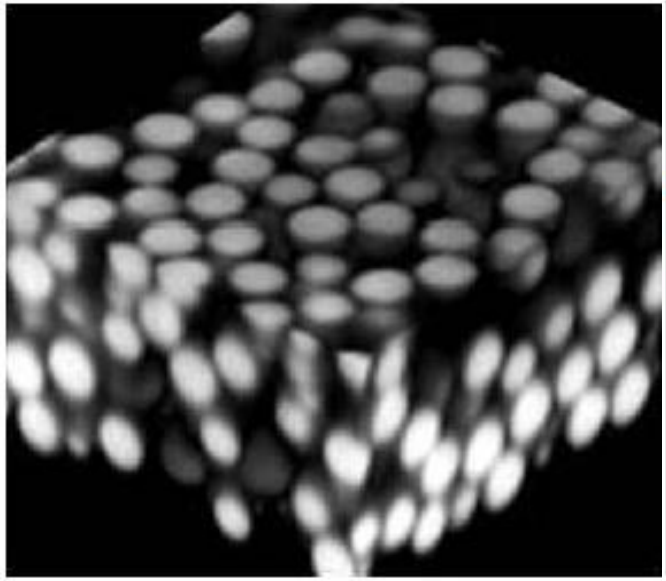
- 轴上分辨率
  - 决定了垂直切面上光强度的半高全宽值。
- 光场平面度 (小的畸变)
  - 必须能够得到样品的平面光场图像，否则Z方向的信息不准确。
- 消色差
  - 必须在全场测试以保证光出射是恒定的，由于色差的原因不能从镜像和切向采光。
- Z轴驱动的精度
  - 结果必须是可重复的

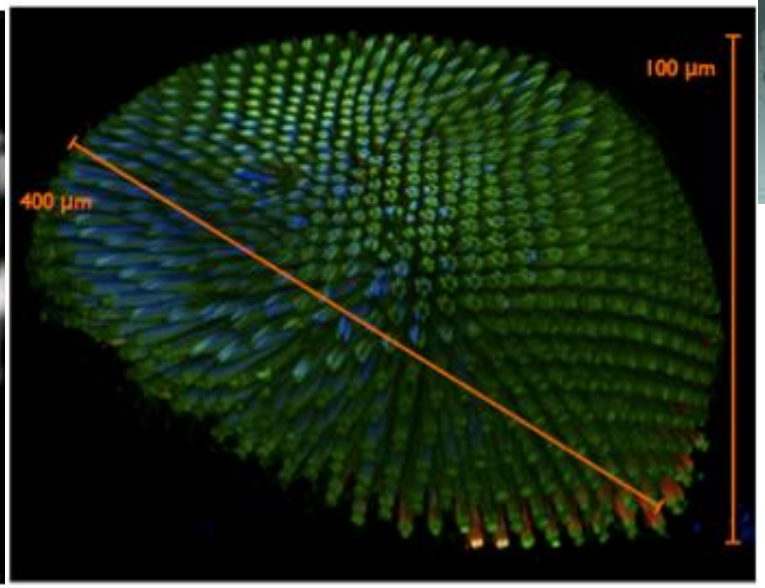
3D confocal imaging











共聚焦显微镜的分类

1.单光子吸收共聚焦显微镜

2.背向散射光(backscattered light)共聚焦显微镜

3.双光子吸收共聚焦显微镜

背向散射光共聚焦成像

背向散射可发生在以下不同的物理条件下，入射光波可基于不同的原理发生偏折:

- 来自大颗粒的扩散反射和米氏散射, 造成染山霞、对日照，可在气象雷达中看出;
- 电磁波和透射介质之间的非弹性碰撞，形成的Brillouin scattering和Raman scattering;
- 加速粒子和样品间的弹性碰撞形成Rutherford backscattering;
- 来自晶体的Bragg diffraction;
- 来自实验的因素(neutron backscattering, X-ray backscattering spectroscopy, XRD);
- Compton scattering, 来自Backscatter X-ray成像。

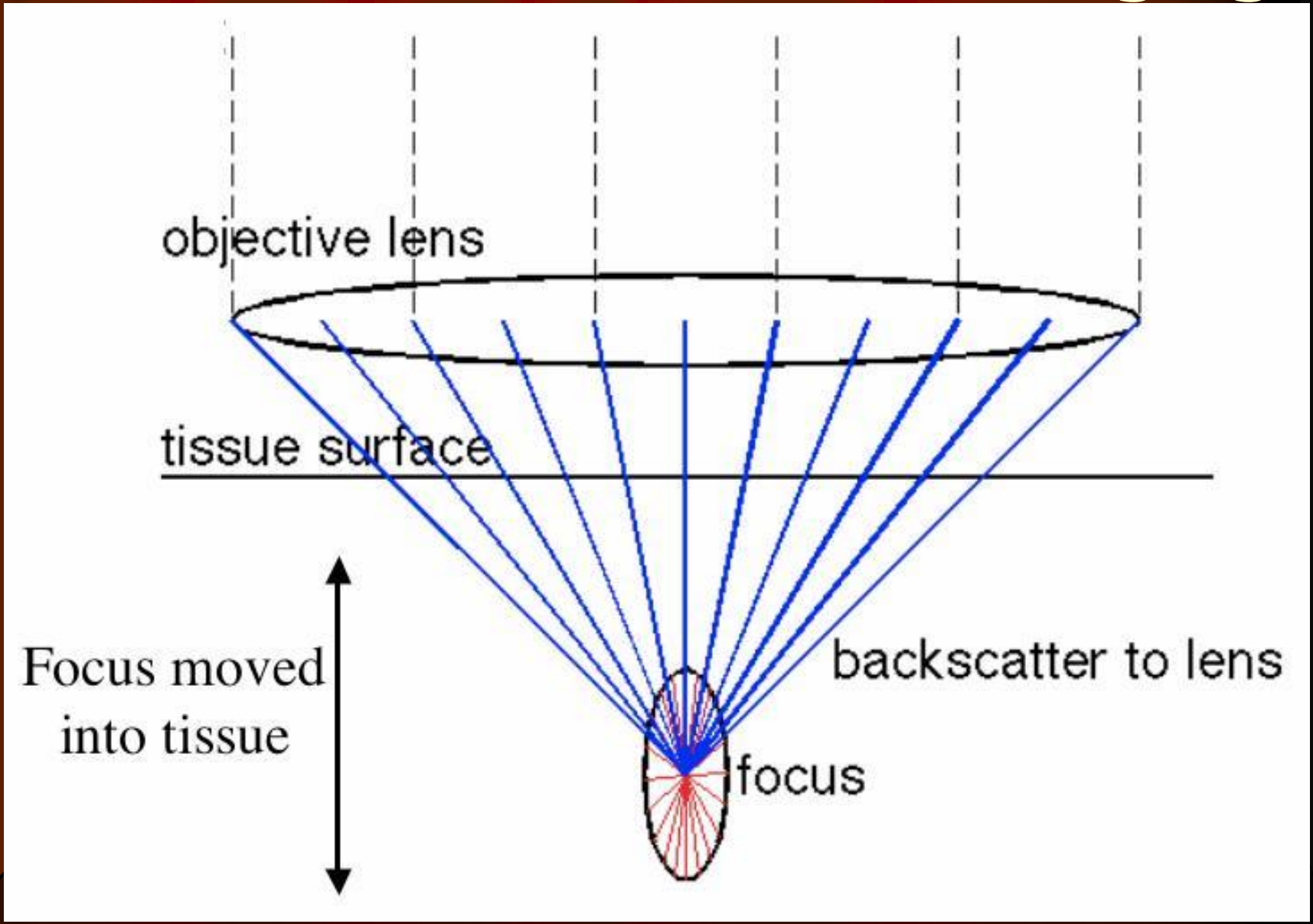
H, He

康普顿散射：高频率的X射线被轻元素中的电子散射后，波长随散射角的增加而增大。

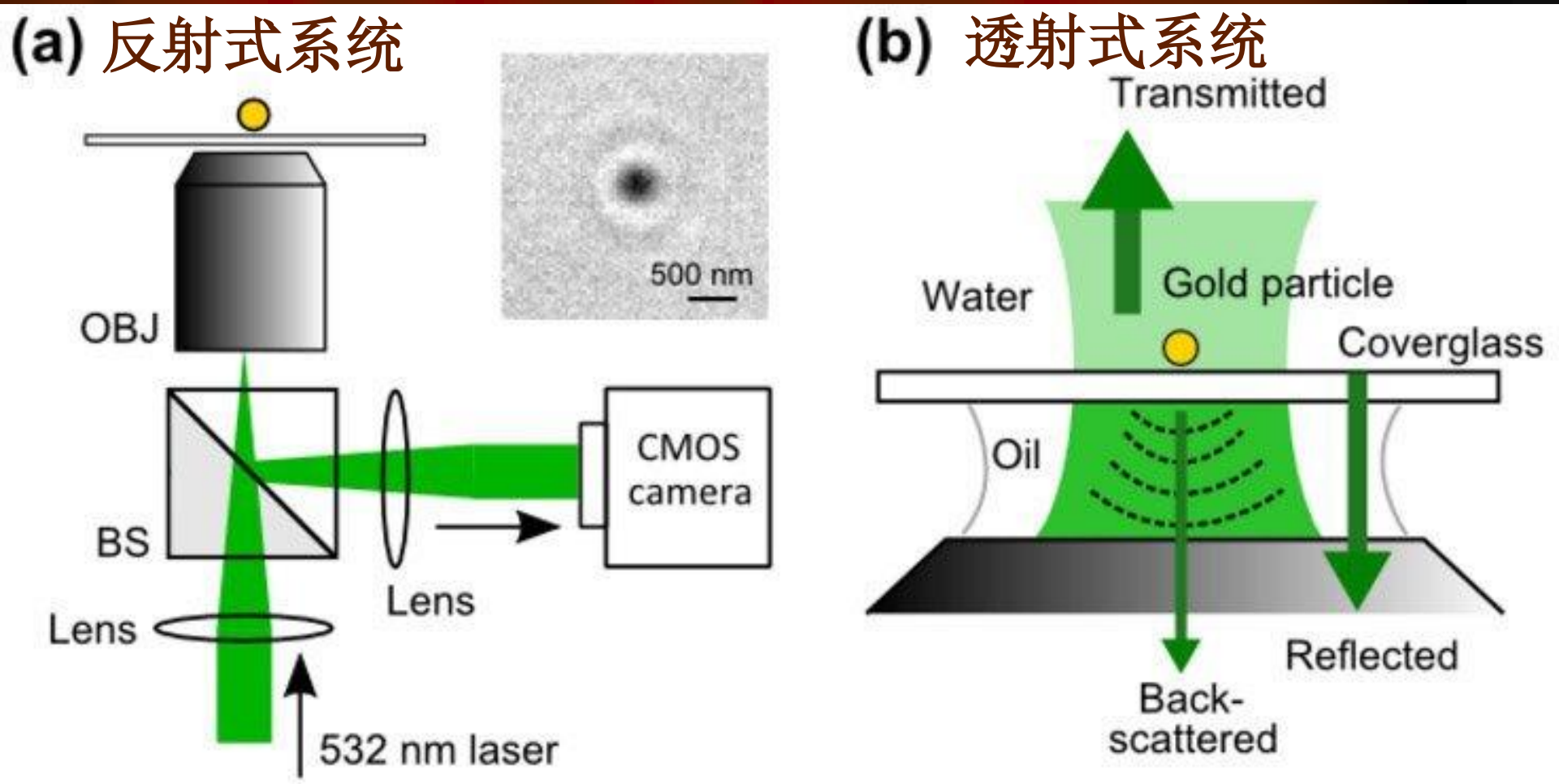
$$\Delta\lambda = \boxed{\frac{2h}{mc}} \sin^2\frac{\theta}{2}$$

康普顿波长

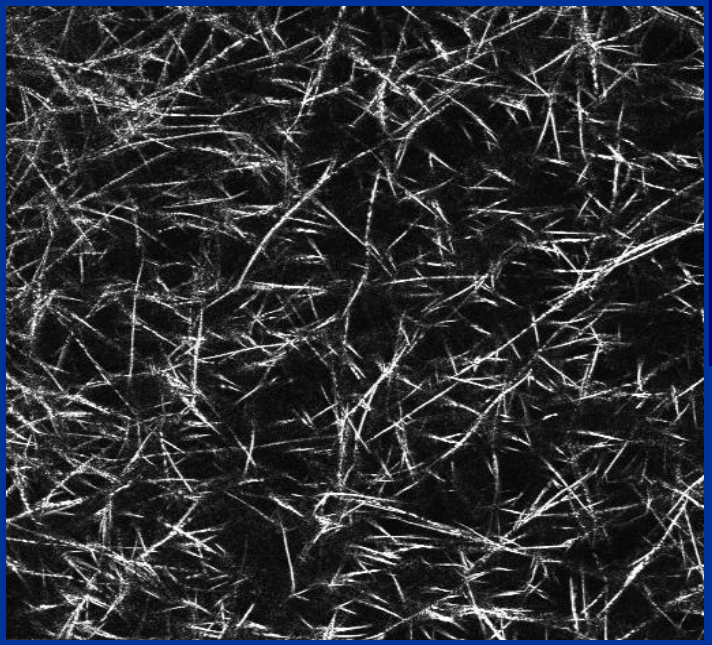
Backscattered/Reflection Imaging



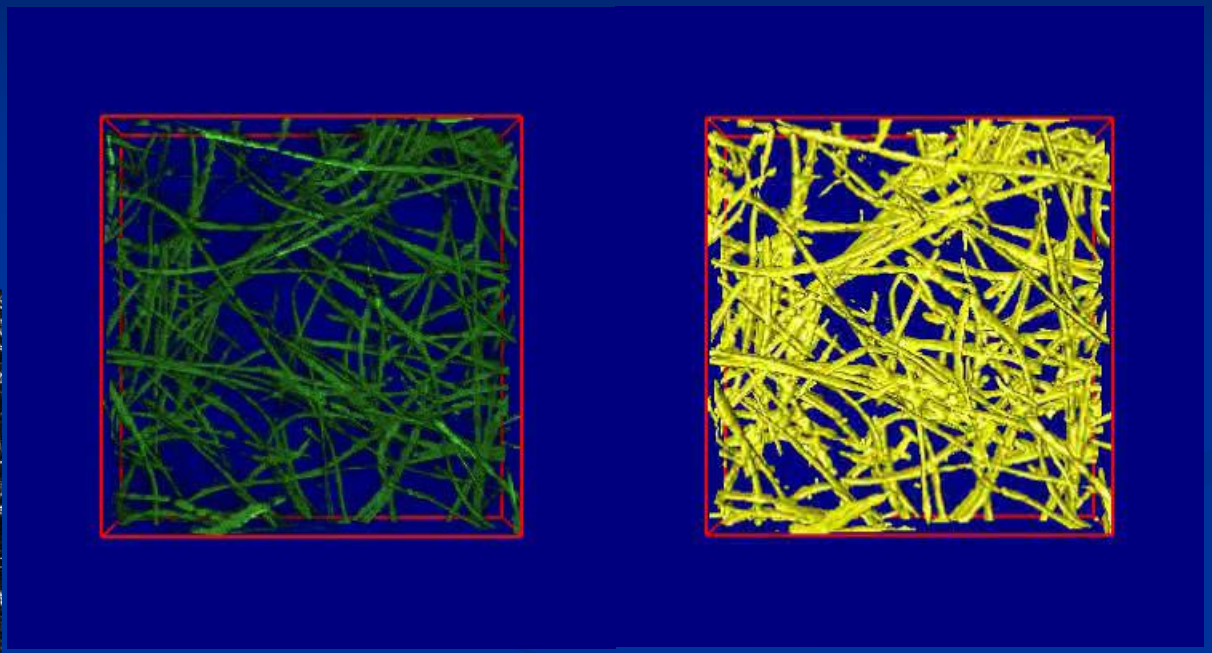
Backscattered/Reflection Imaging



背向散射光共聚焦成像

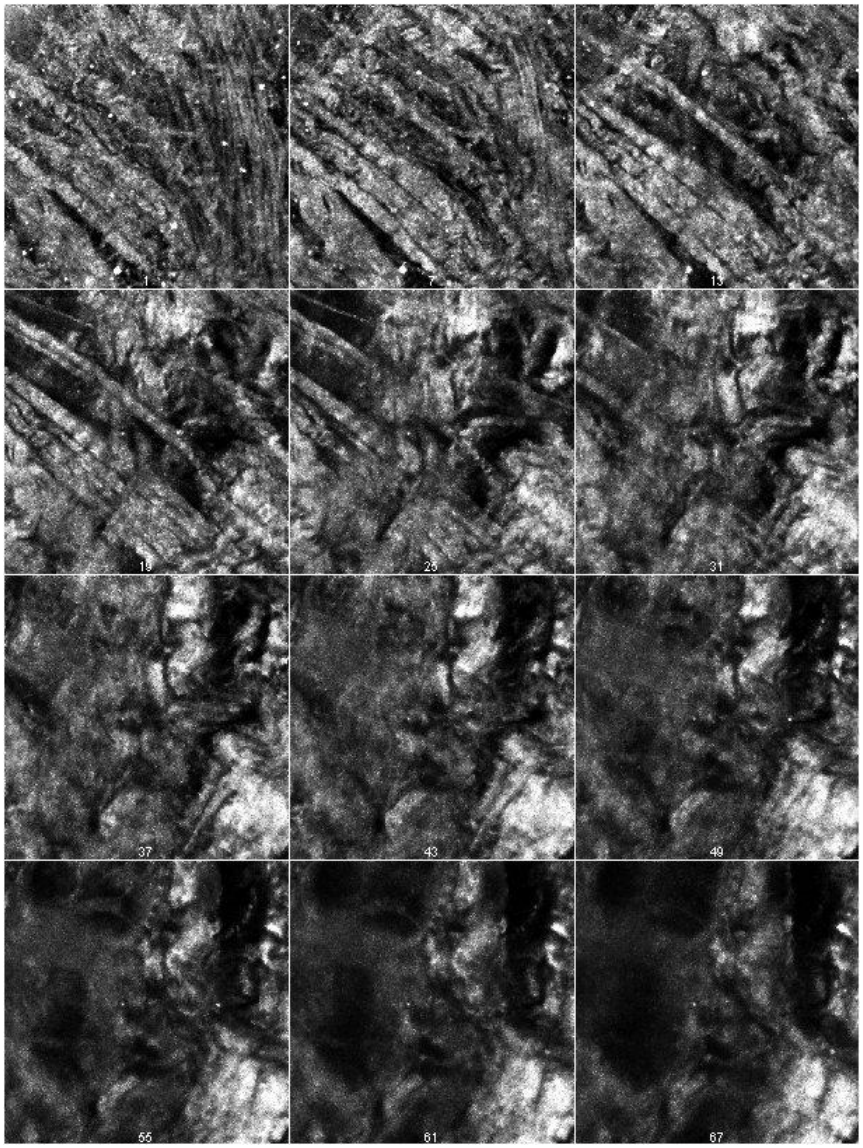


Collagen gel – an optical section

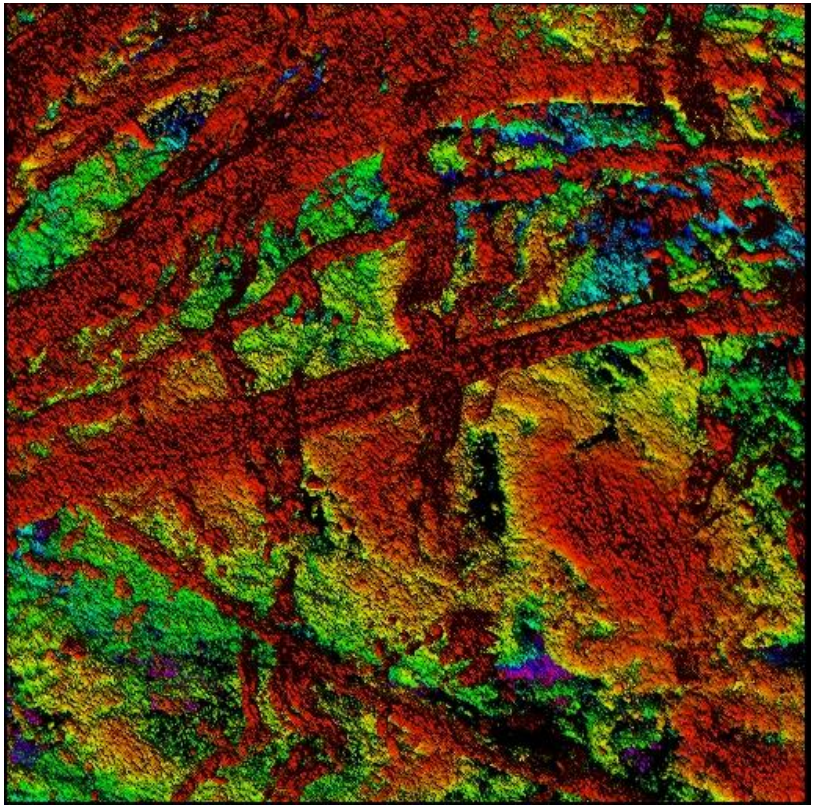


Collagen gel (胶原蛋白凝胶) visualized by BSL-confocal microscopy. Volume- (left) and surface-rendering (right) of a confocal dataset.

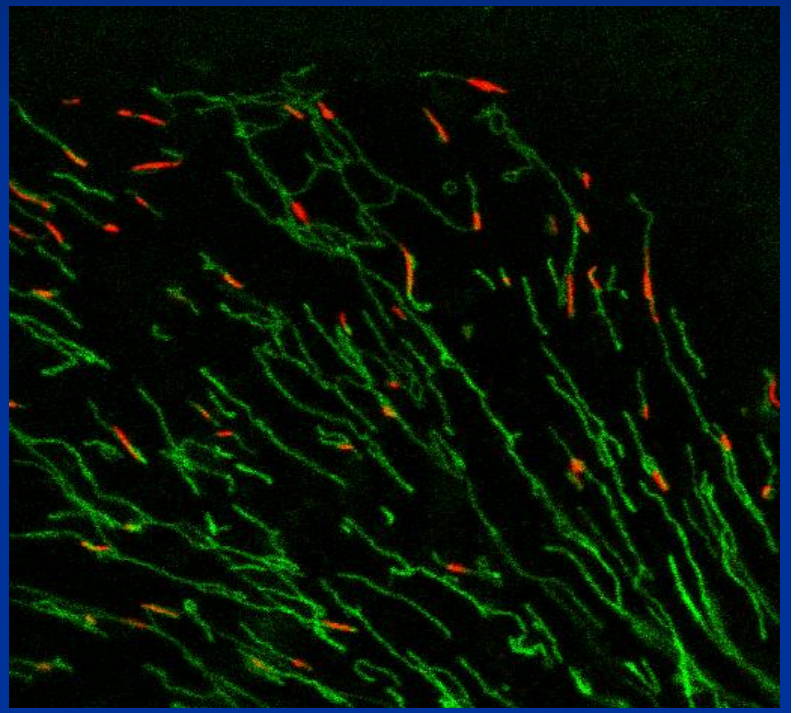
Backscattered light: tissue imaging



Left: Optical sections of SIS visualized with BSL-confocal technique. Bottom: color-coded height map revealing the topography of height map of SIS

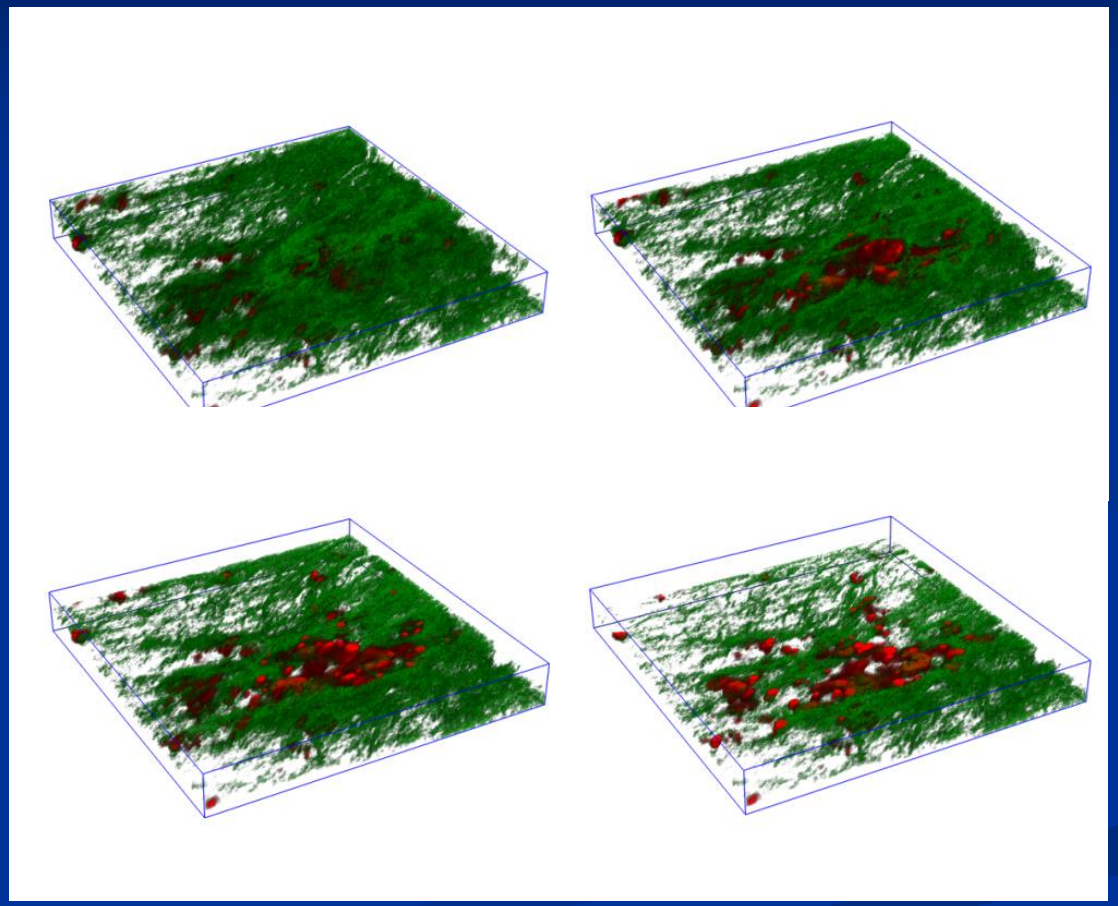


Example: BSL and AF signals combined



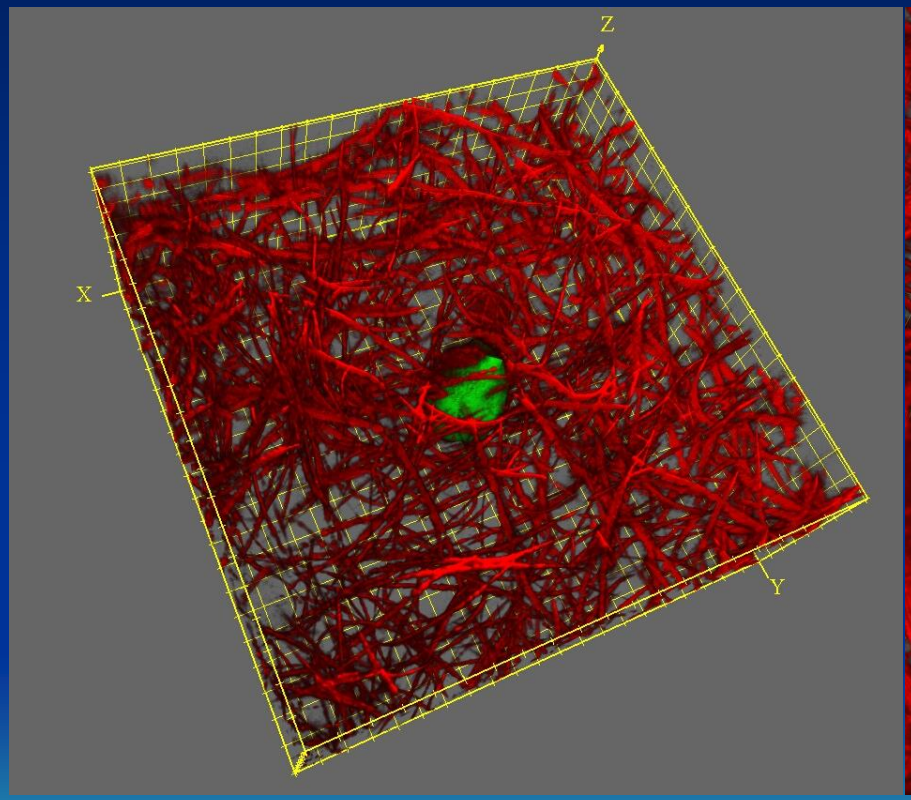
$HepG_2$ cells grow embedded within a collagen matrix

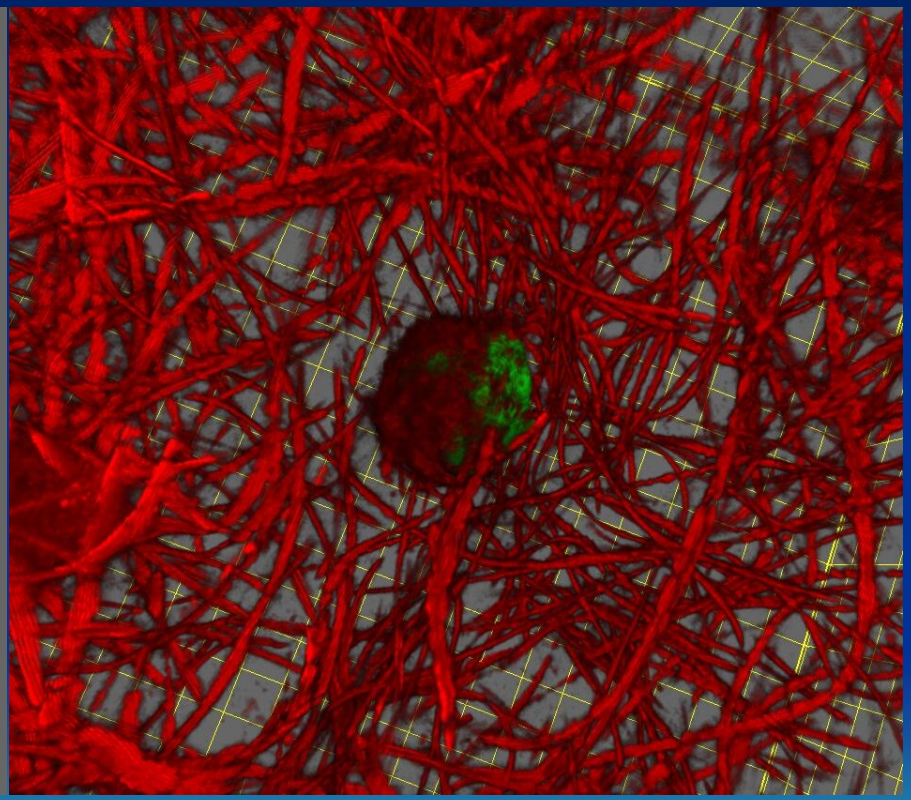
2D BSL imaging



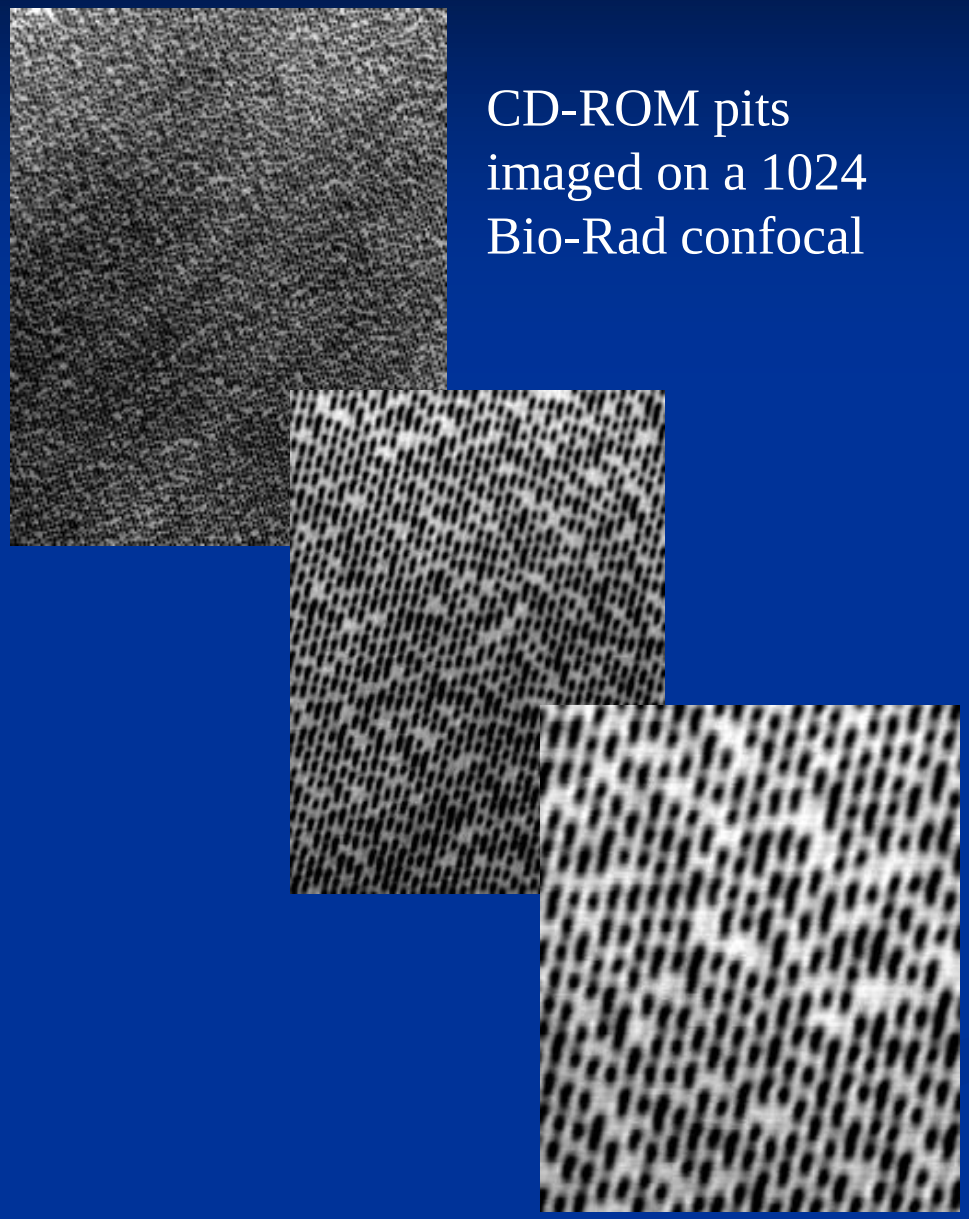
AF: autofluorescence

Backscattered light and autofluorescence signals combined: collagen gel & $HepG_2$ cells



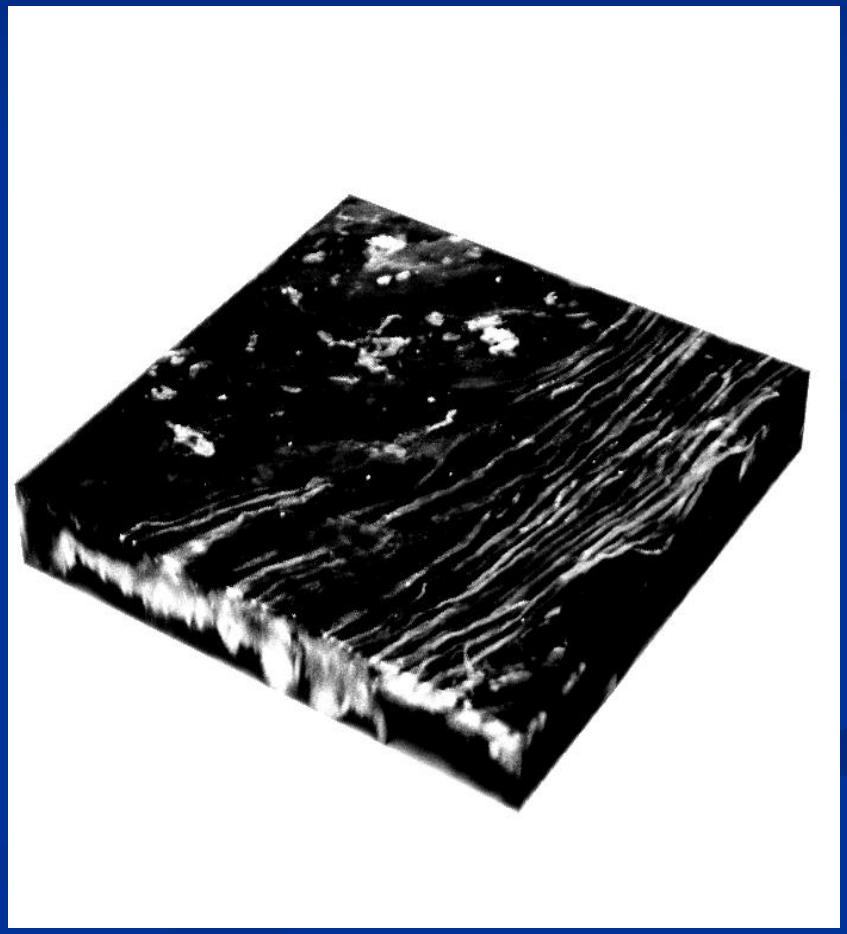


背向散射共聚焦成像



CD-ROM pits imaged on a 1024 Bio-Rad confocal

Collagen imaged by a 1024 confocal image



背向散射共聚焦成像

背向散射光共聚焦成像

Advantages：由于没有使用光探针，故无光子漂白现象。

Problems：有来自于显微镜组件的反射光干扰。

共聚焦显微镜的分类

1.单光子吸收共聚焦显微镜

2.背向散射光(backscattered light)共聚焦显微镜

3.双光子吸收共聚焦显微镜

双光子成像共聚焦显微镜

光吸收

带间
光吸收

自由载流
子吸收

双光子/多
光子吸收

带间光吸收：设入射光的能量为 $h\nu$，半导体材料的禁带宽度为 E_g，如果 $h\nu$ 大于 E_g，那么半导体材料价带中的电子就有可能吸收光子能量而跃迁至导带中，这一过程称为带间光吸收。假设入射光的能量正好等于材料的禁带宽度，则有：

$$\begin{aligned} E_g &= h\nu \\ &= hc/\lambda \end{aligned} \qquad \ldots(1.1)$$

其中 c 为真空中的光速。根据上式，硅材料的禁带宽度为 1.12 eV，则其对应的入射光截至波长约为 1100 nm。大于此波长的入射光由于光子能量低于硅材料的带隙宽度，因此将不能在硅材料中发生带间光吸收。在入射光能量大于材料带隙宽度时，入射光光强与其在材料中的传播距离满足以下关系：

$$I(x) = I_0 \mathrm{e}^{-\alpha x} \qquad \ldots(1.2)$$

其中 x 为传播距离，I_0 为 x=0 时的初始光强，α为吸收系数。

自由载流子光吸收：在入射光能量小于材料带隙宽度时，带间光吸收是无法发生的。但由于光场的存在，材料导带和价带中的电子会产生运动，吸收一部分入射光的能量，这就是自由载流子光吸收。自由载流子光吸收的吸收系数为：

$$\alpha=\frac{e^5\lambda^2}{4\pi^2c^2n\varepsilon_0}\frac{N}{m_e\mu_e} \qquad \ldots(1.3)$$

其中 n 为材料的折射率，N 为材料中的自由载流子浓度，ε_0 为介电常数，μ_e 和 m_e 分别为电子的迁移率和有效质量。显然，自由载流子光吸收系数与自由载流子浓度成正比关系，浓度越高则吸收越强。同时，吸收系数也正比于波长的二次方，这说明自由载流子光吸收在长波情况下将更为明显。

双光子吸收：通常情况下，原子必须吸收特定大小的能量才能由低能级跃迁至高能级。对光吸收过程来讲，光子能量必须恰好等于高低能级之差时才可以被吸收。但是在强激光作用下，原子也有可能同时吸收两个光子来完成跃迁，这两个光子的能量之和达到一定的数值即可。这种吸收即称为双光子吸收。双光子吸收属于一种三阶非线性效应，具有良好的空间选择性，并对样品的损伤较小。

同理，还有三光子吸收甚至多光子吸收。

形成双光子吸收的条件：

1.红外脉冲光源；

2.数值孔径NA尽量大；

3.光子密度高，即：红外激光器功率密度高。

4.脉冲重复频率低；

5.荧光团浓度高；

6.生物样品的折射率η尽量高

Two-Photon Microscopy

双光子吸收/激发技术的原理：

在强激光激发下，荧光分子同时吸收两个光子，从基态跃迁到两倍光子能量的激发态的过程，在经过一个很短的所谓激发态寿命的时间后，荧光分子发射出一个波长较短的光子。两个光子可以是相同波长的，也可以是不同波长的，但必须是同时吸收（两个光子到达被激发分子的时间间隔小于1fs）。也可以这样理解，先吸收一个光子的能量跃迁到一个虚拟的中间态，然后再吸收一个光子的能量跃迁到激发态。

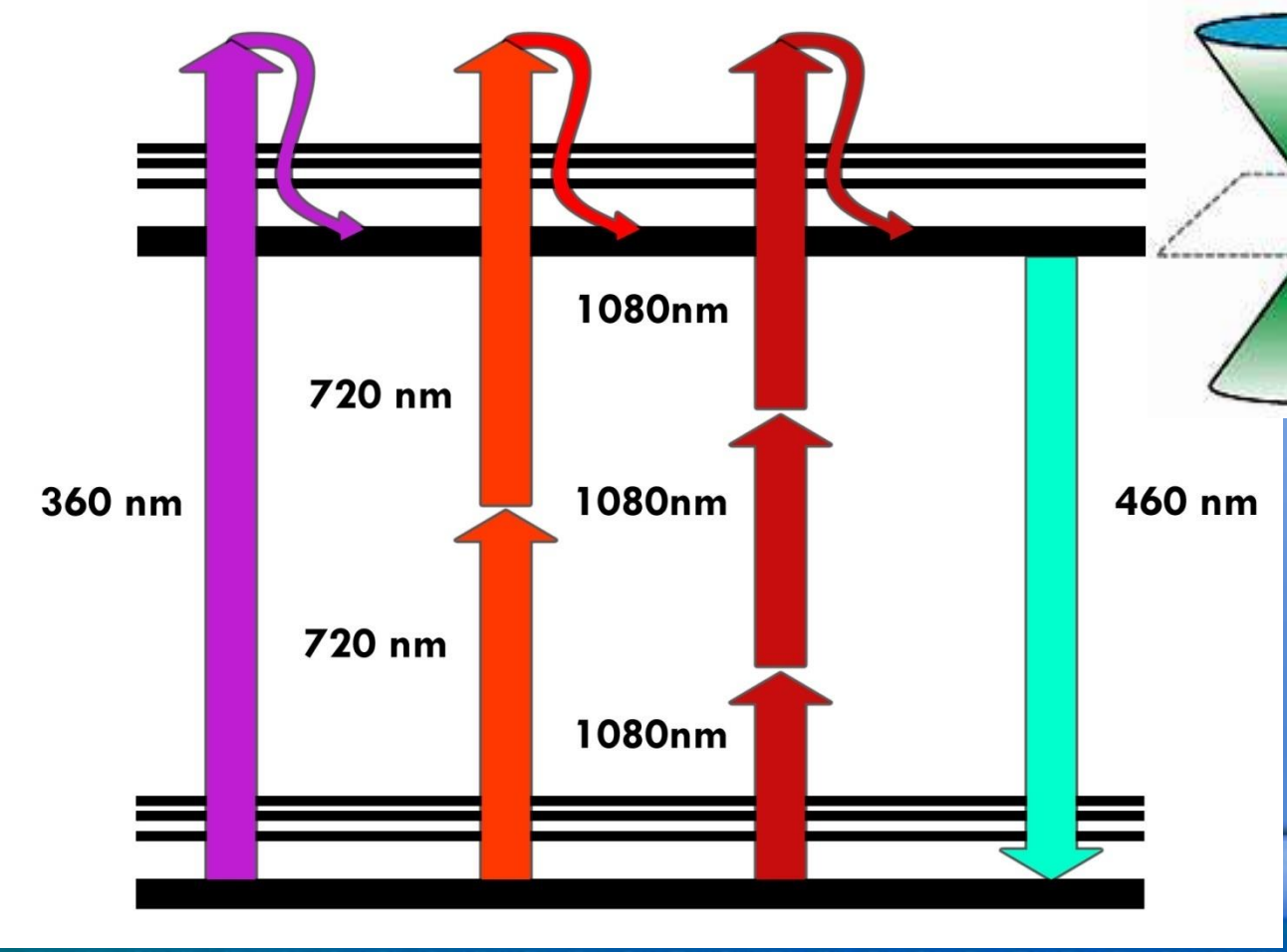
Two-Photon Microscopy

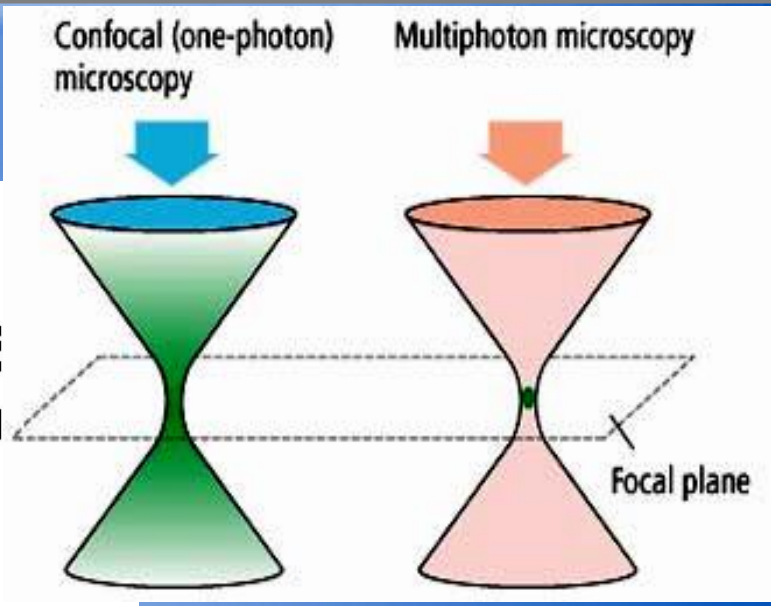
双光子激发需要很高的光子密度，为了不损伤细胞，双光子显微镜使用高能量锁模脉冲激光器。在使用高数值孔径的物镜将脉冲激光的光子聚焦时，物镜的焦点处的光子密度是最高的，双光子激发只发生在物镜的焦点上，所以双光子显微镜不需要共聚焦针孔，提高了荧光检测效率。

成像过程：仍然需要借助于共聚焦的点对点扫描实现二维成像。

Multi-Photon Microscopy

Resolution is improved (in comparison to the same excitation wavelength)

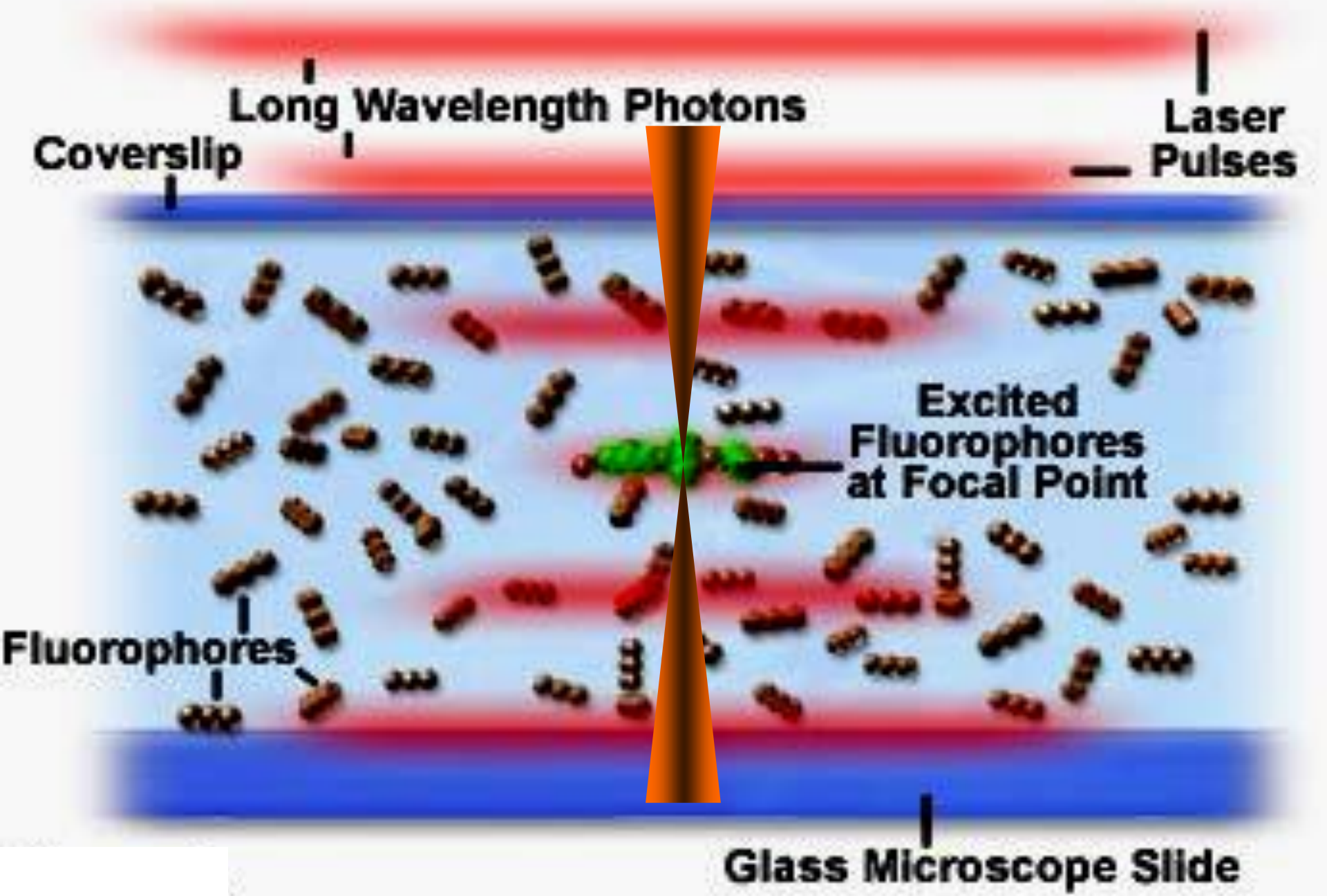




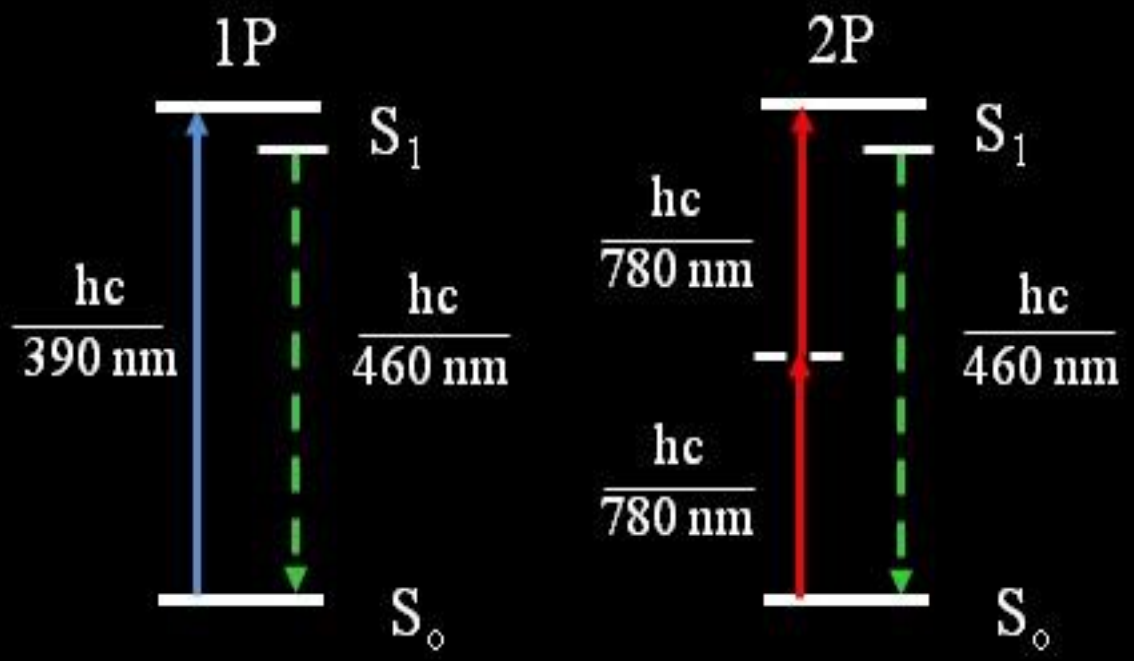
Power issue !!!

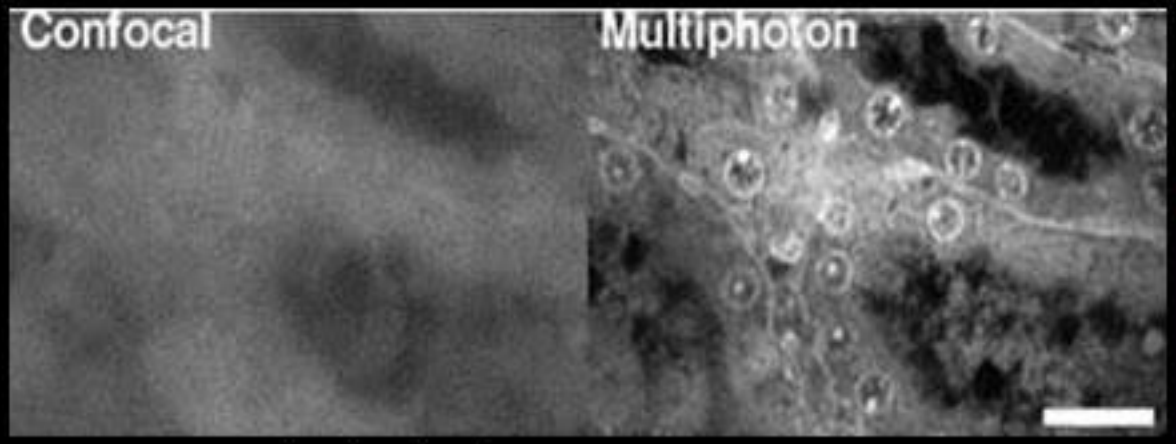
激发态与基态之间的能量差相当于两个光子的能量和

Fluorophore Excitation in Multiphoton Microscopy

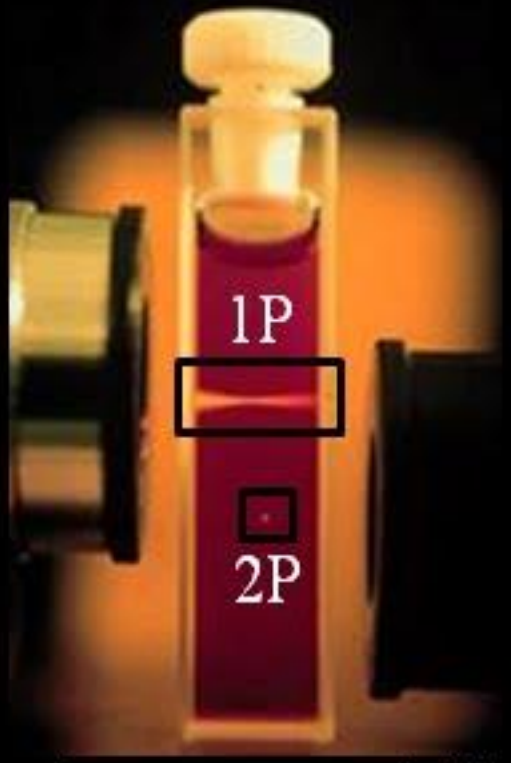


Multi-Photon Microscopy





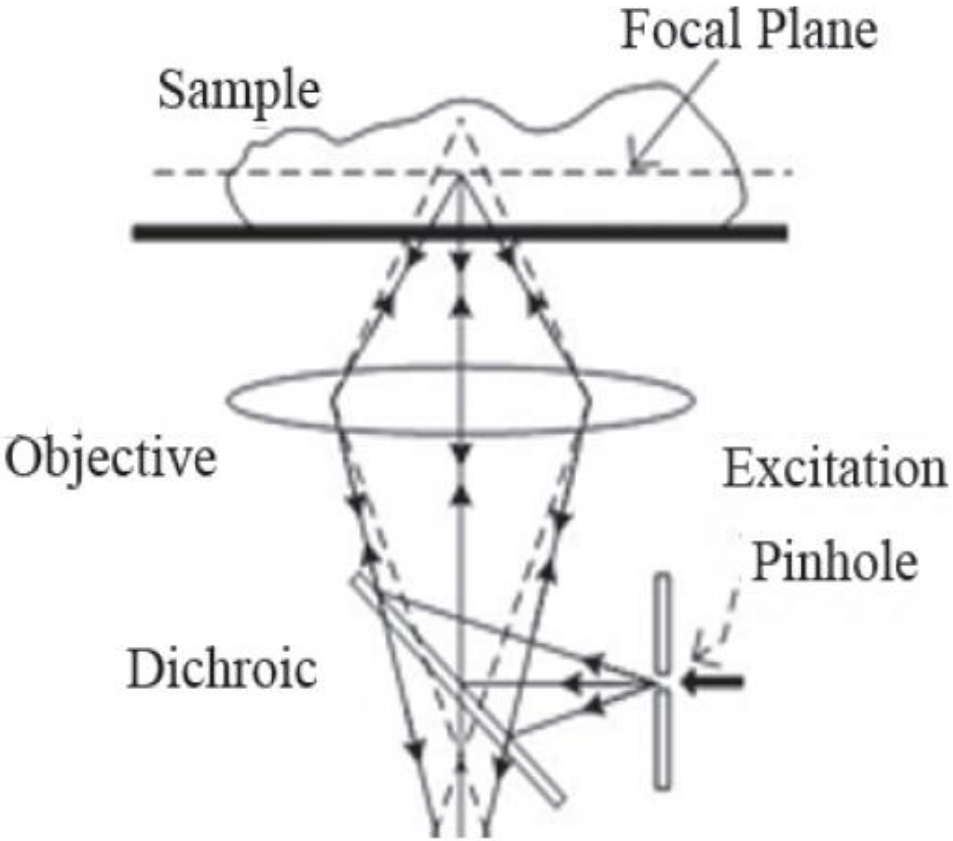
Centonze, Biophysical J. 1998



Brad Amos MRC, Cambridge

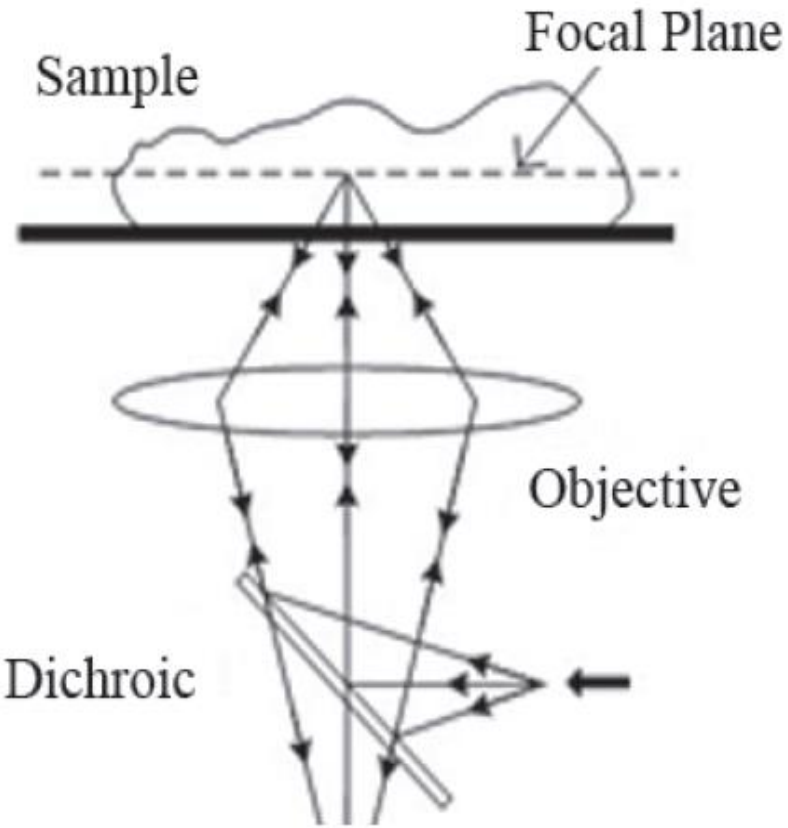
↑ Viability

↑ Penetration depth



(a) LSCM原理示意图

a) Schematic diagram of laser scan confocal microscope

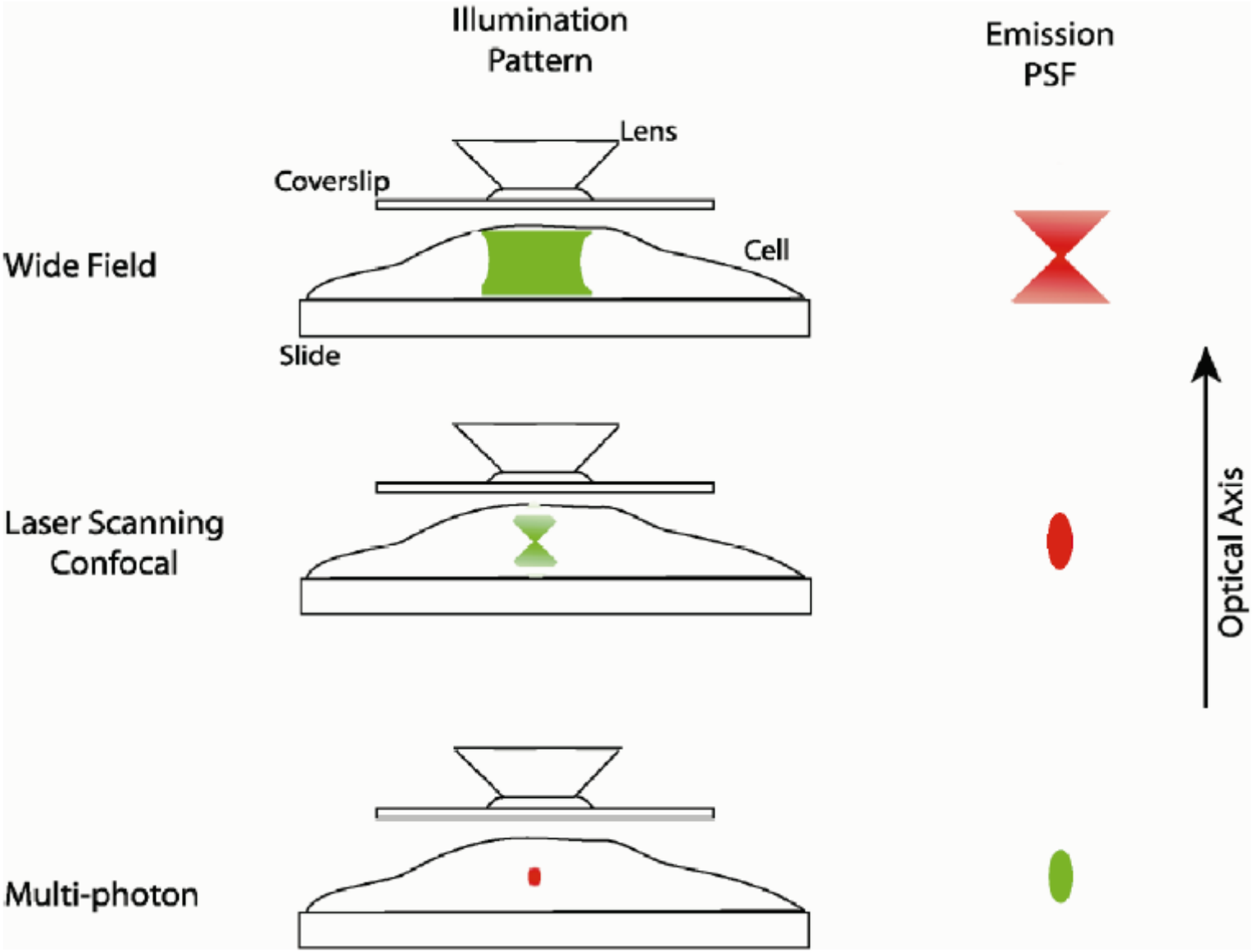


(b) TPELSM原理示意图

(a) Schematic diagram of two-photon exciation laser scan microscope

多光子激发只有在焦点附近的极小区域内才能发生，无需共聚焦针孔便可实现三维高分辨成像。

Wide-field vs confocal vs 2-p



Multi-Photon Microscopy: IR Lasers

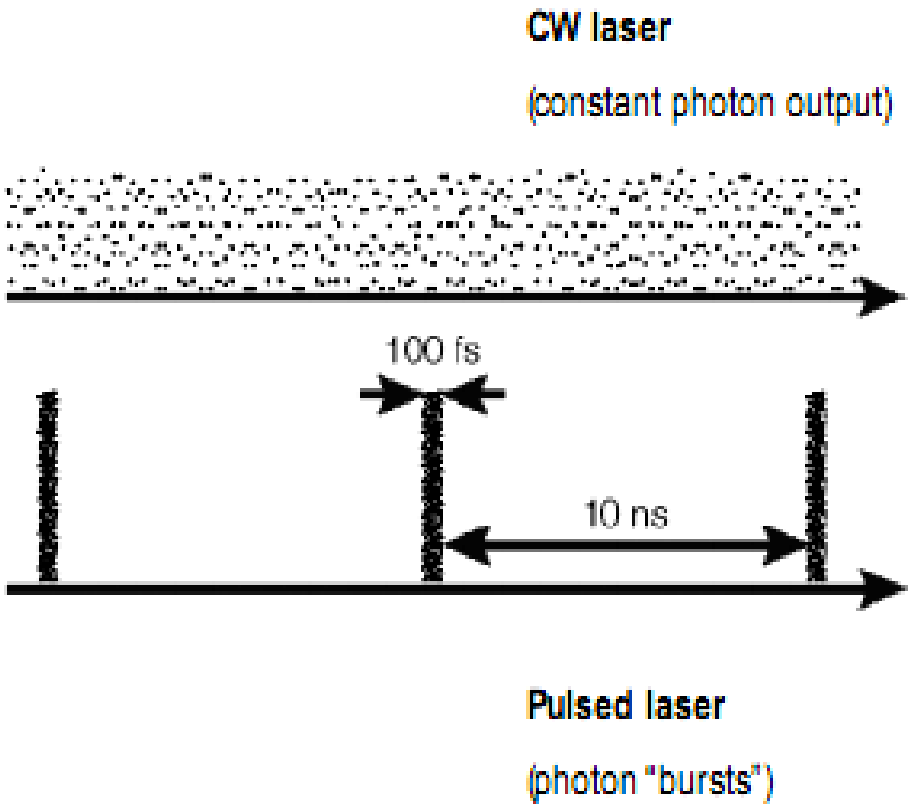
Fluorochrome excitation width pulsed IR lasers

CW laser (continuous wave) for LSM

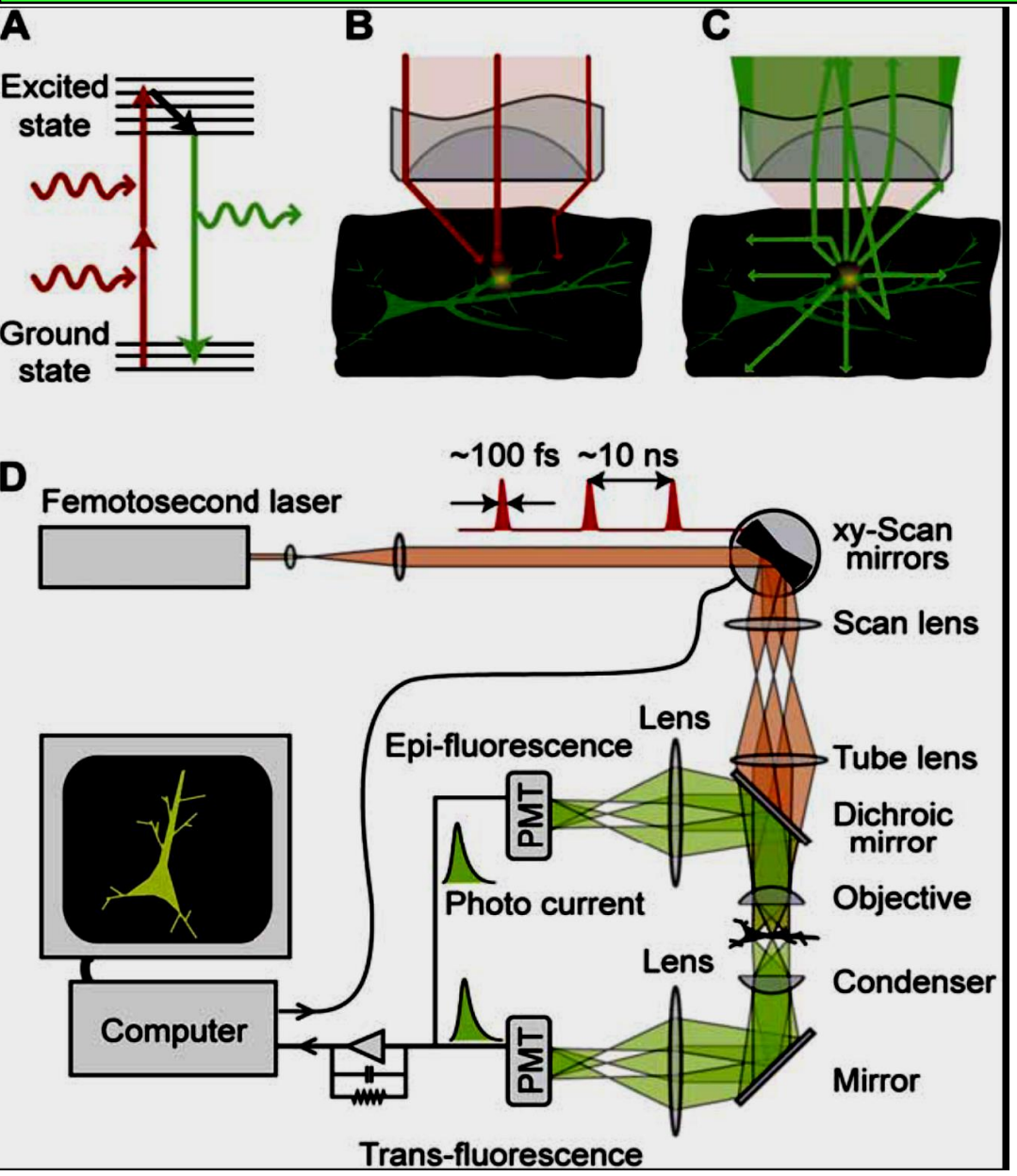
Continuous emission with low average power (e.g. 30mW Argon laser, 1mW HeNe543)

Pulsed laser

Ultra short pulses with a very high peak power (0,5 W average power with 100 fs pulses correspond to a peak power of 65.8 kW).



Two-photon microscopy



焦点处的吸收概率

$$P_a = \langle P \rangle^2 F_p \xi \left(\frac{N_A}{2\pi c} \right)^2$$

C –荧光团浓度

δ – 双光子吸收截面

F_p – 重复频率

ξ – 双光子优势因子(由脉冲形状决定)

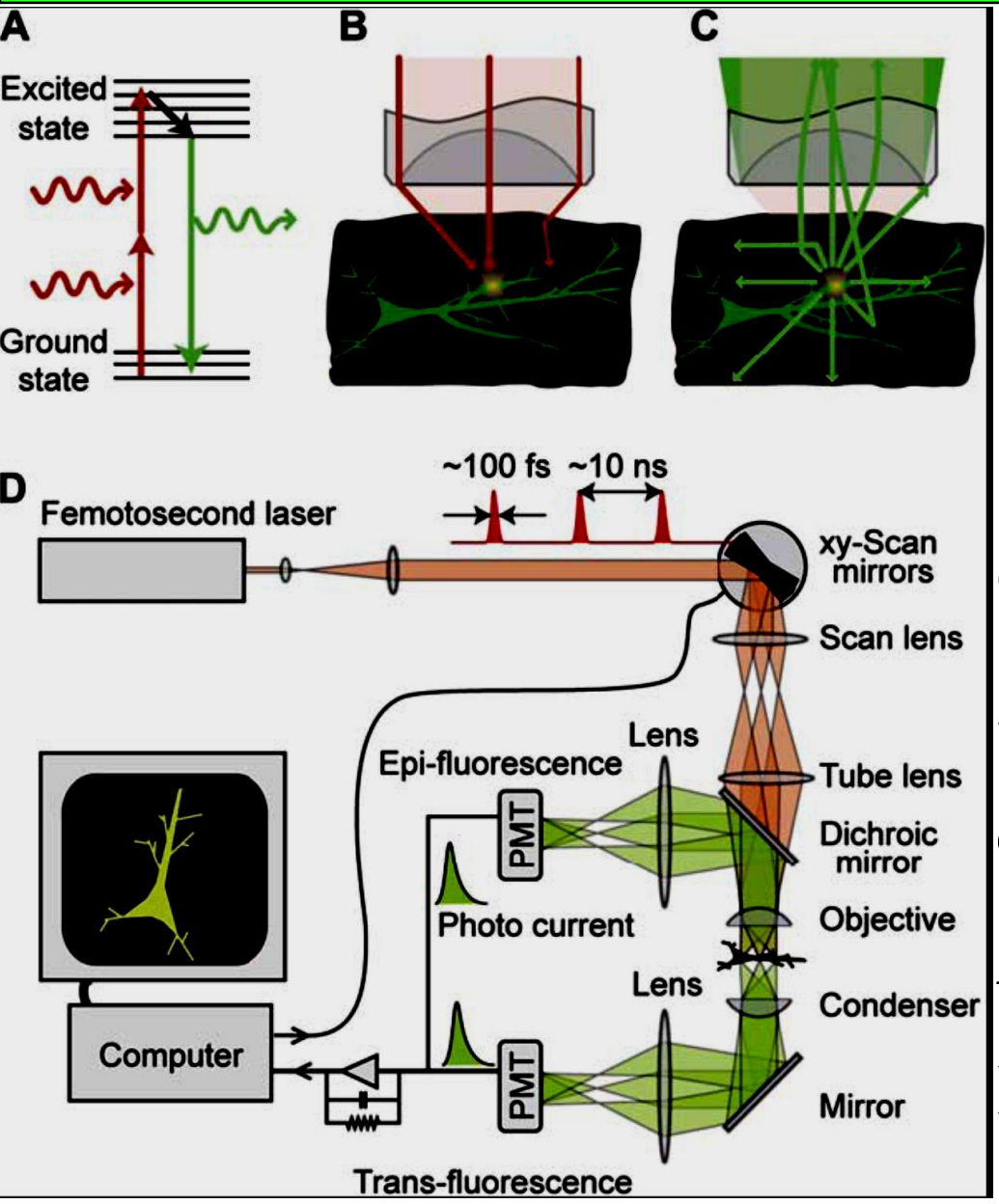
η – 折射率

$\langle P \rangle^2$ – 平均光子密度

总吸收概率

$$P_a = \frac{\delta C \langle P \rangle^2 \eta \xi}{2\pi c}$$

Two-photon microscopy



焦点处的能量吸收概率

$$\frac{dW}{dt} = \frac{8\pi^2\omega}{n^2c^2} I^2 \operatorname{Im}(\chi^{(3)})$$

W–代表能量吸收概率

ω – 入射光频率

n–光敏树脂的折射率

c–真空中的光速

I–激光强度

$\operatorname{Im}(\chi^{(3)})$ –三阶磁化率的虚部

双光子吸收的横截面比单光子吸收的横截面要小的多，需能量密度为TW/cm^{2}量级的高强度超短脉冲激光器。此外，由于树脂中存在淬灭剂，自由基浓度超过一定阈值时，聚合才会开始。即：当辐照时间为常数时，存在一个激光强度阈值；当激光强度为常数时，存在一个辐照时间阈值。

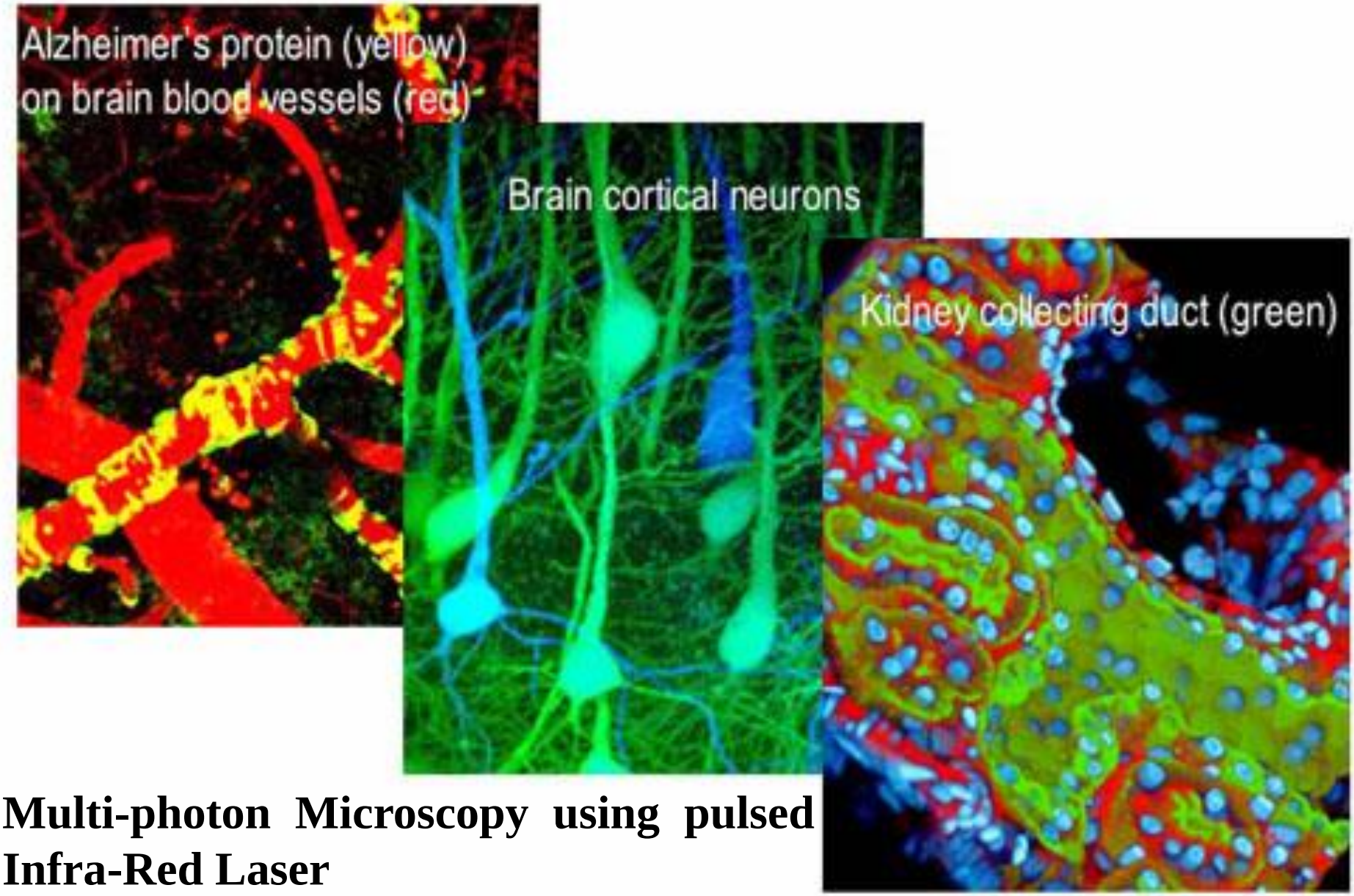
光学非线性特性和阈值特性都有助于分辨率超越衍射极限。

小结

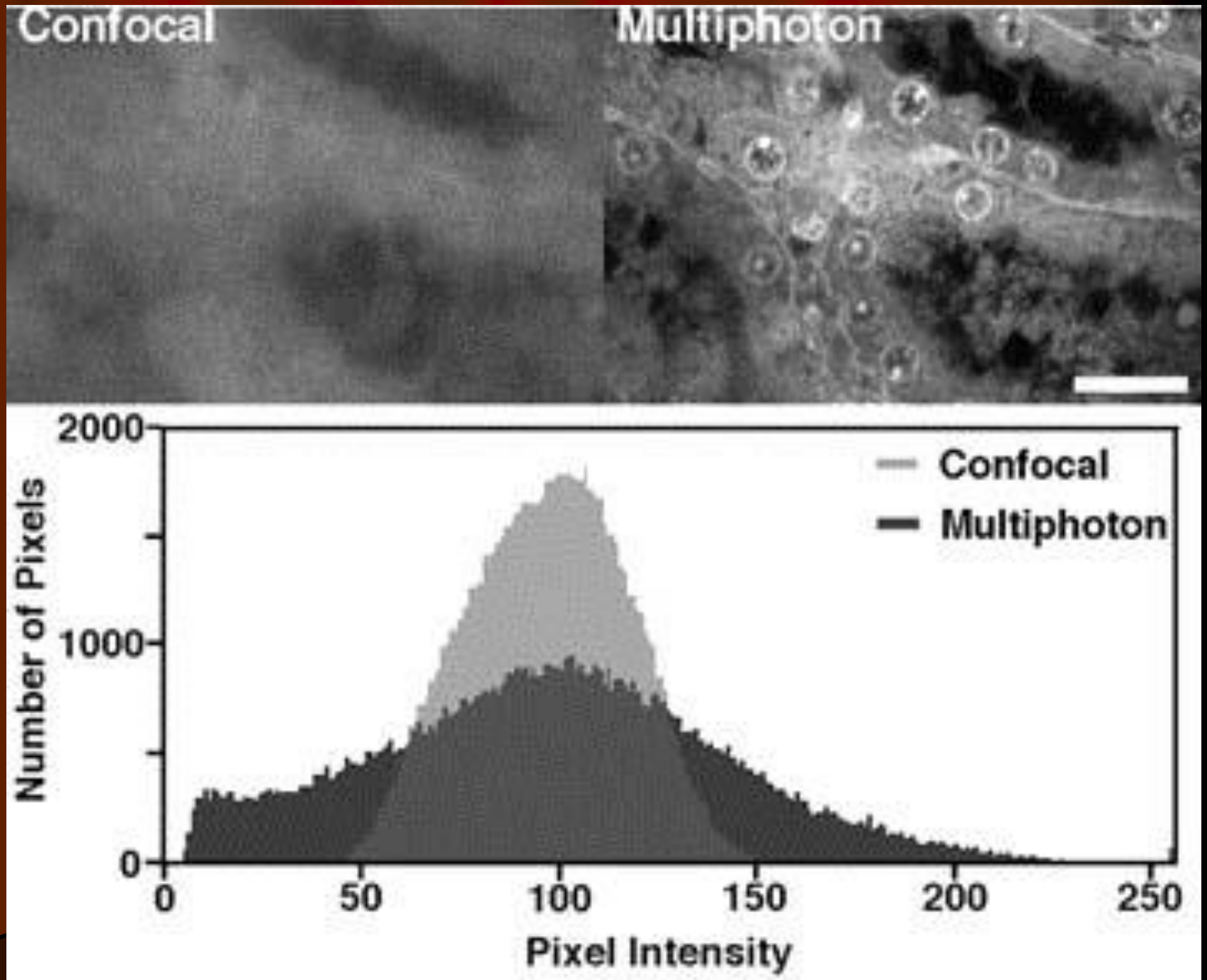
- 焦点处的吸收概率对估计光损伤和光子漂白效应很重要。
- 对于不同的生物样品和荧光剂，选用的脉冲宽度不同。

Multiphoton microscopy

Multiphoton microscopy



Multi-photon Microscopy using pulsed Infra-Red Laser


Confocal
Multiphoton
Confocal
Multiphoton
Number of Pixels
2000
1000
0
0
50
100
150
200
250
Pixel Intensity

对于某些具有非中心对称结构的细胞或组织，如胶原蛋白、细胞外间质的筋膜和眼角膜等，可以利用双光子结合二次谐波成像技术（SHG）实现成像。二次谐波技术和双光子显微镜在装置上具有通用性，SHG信号收集不受前向发射性质的限制，可以背向收集信号(backscattering)，因此很容易与双光子显微成像联合使用，进行成像比较，实现信号互补。

多光子显微术：强有力的生物成像工具

真正具有单分子灵敏度的“**分子成像**”

尤其是对以下特定生物目标的成像：

- 常规的荧光染色剂
- GFPs
- 内部产生荧光的生物体

◆ 具有亚微米的成像分辨率

◆ 可对较厚或混浊介质样品的剖面成像

◆ 宽广的生物和诊断应用：

✓ 基因表达

✓ 蛋白质相互作用

✓ 钙浓度分析

✓ 神经活动

✓ 疾病诊断

✓ 光学-生物探测

多光子激发的优点：

除了可限制成像面的光漂白和光损伤，多光子激发还有如下优点：

◆对于很多生物样品而言，近红外散射子少于蓝光

◆更有效的光收集功能

- 由于使用红外激光，具有更深的穿透功能
- 可调的激发波长—多色激发；
- 非线性效应成像（SHG）；
- 激发波长和发射波长更容易分开。

◆能够激发荧光剂在紫外波段的吸收

- 可使用UV荧光剂；
- 可多色成像。

减小光毒效应

- **荧光在组织体内成像时会产生光毒效应。**

  荧光团对基态的非辐射转换会引起较强的反应。然而，不同于共焦和全场显微镜的荧光成像，多光子成像仅仅位于光扫描面的荧光团，没有体样品的荧光团激发，因此大大降低了光毒效应的产生。

- 原理上，所有从多光子激发的发射光子均可参与成像，没有共焦孔径的阻碍。

- 紫外荧光团通过没有在紫外波段消像差的透镜照明也可被激发，因为这些激发波长不必通过该透镜接收。

多光子激发成像的限制

- ✓ 与共焦显微镜相比，对荧光团的分比率略低。这种分辨率损失可采用共焦孔径加以排除，但代价是信号强度损失增加。

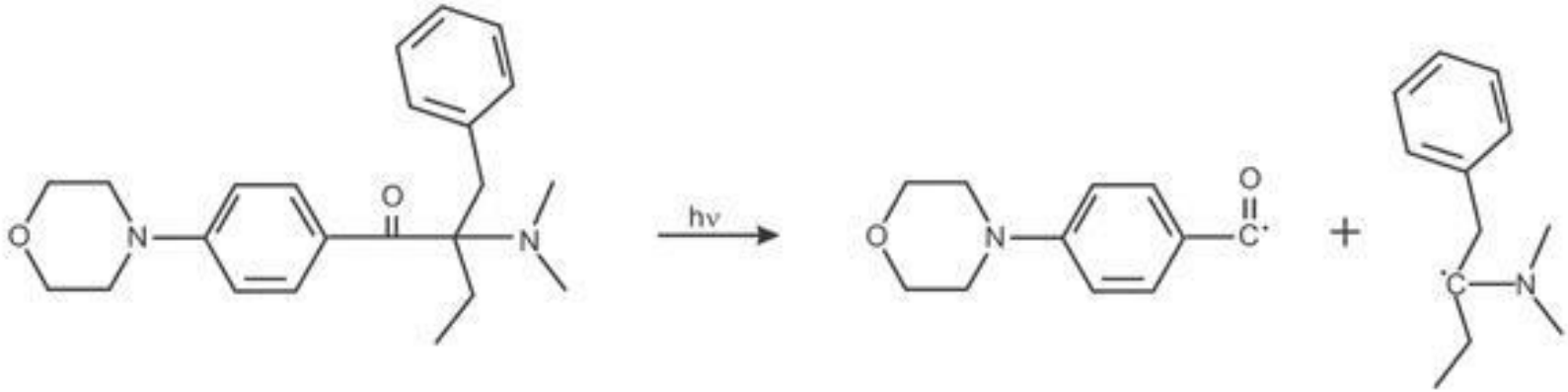
- ✓ 如果样品中含有吸收激发波长的载色体，如黑色素染料，会发生热损伤。

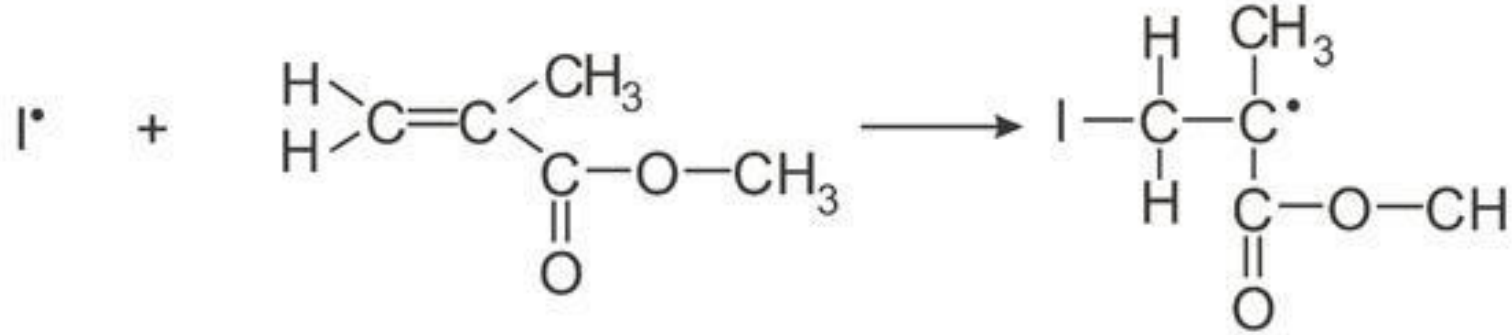
- ✓ 仅用于荧光成像

- ✓ 使用成本很高

Two-photon nanofabrication

Photo-chemical reaction



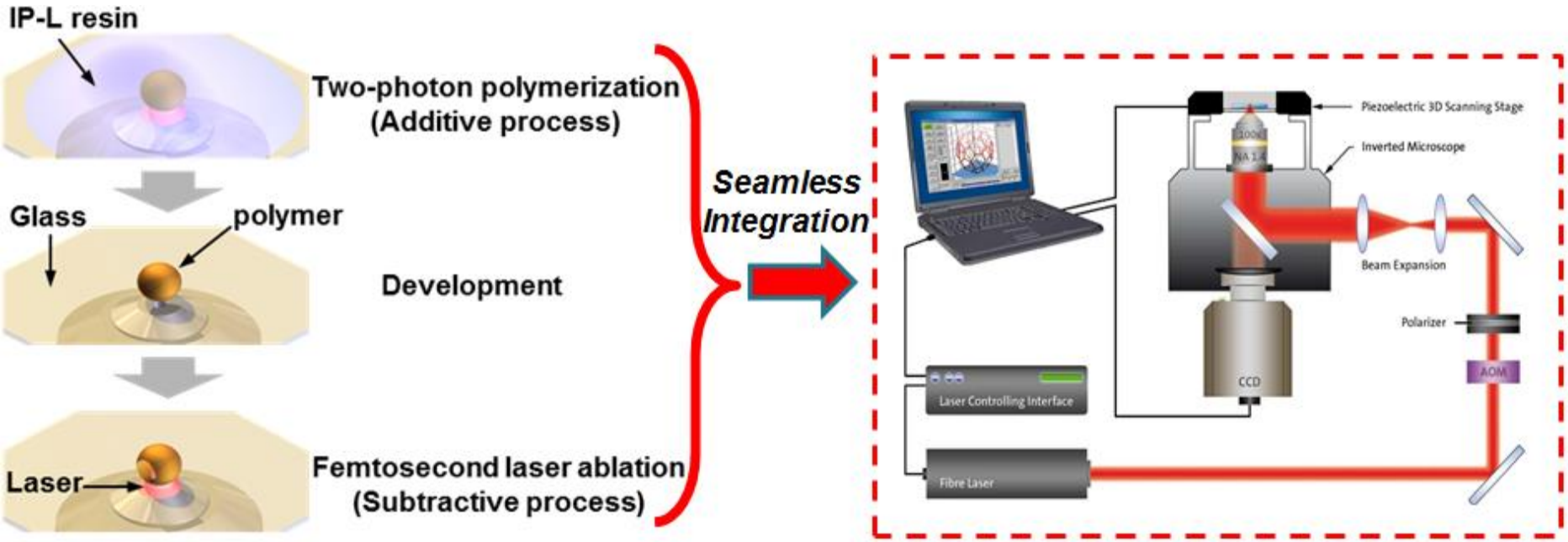


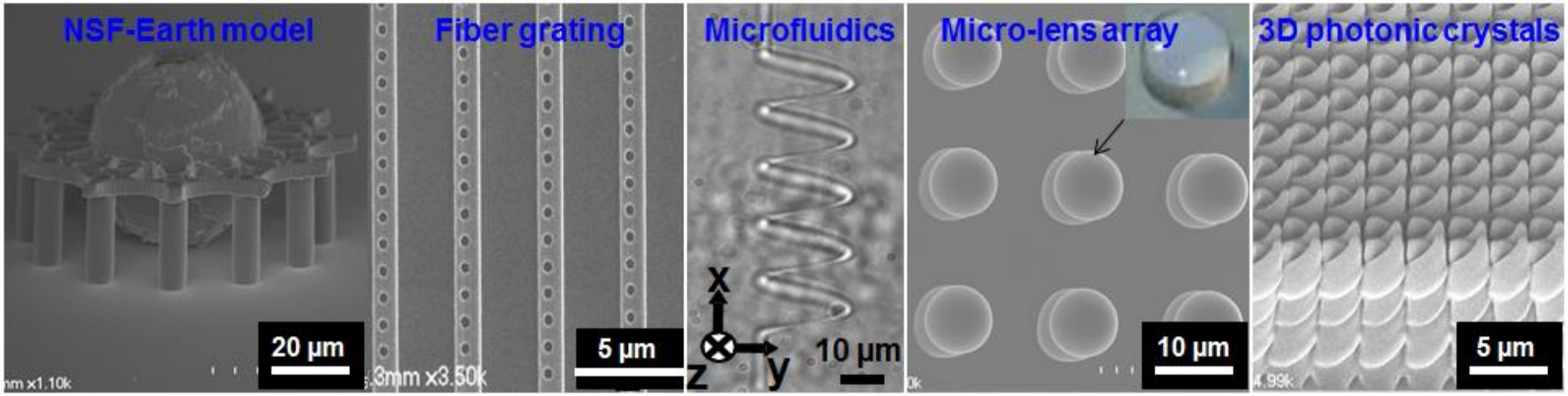
radicalized initiator

monomer

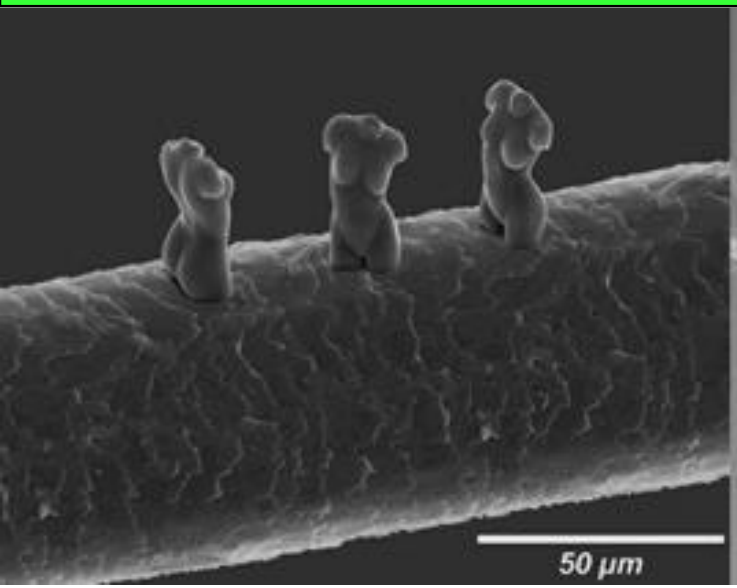
radicalized monomer

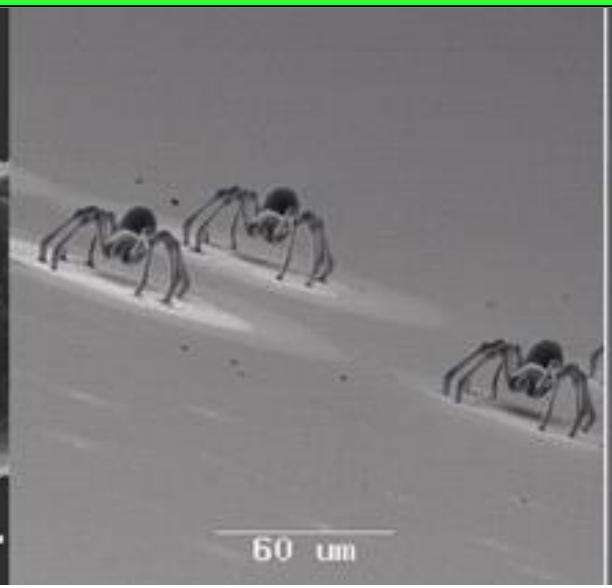
Two-photon nanofabrication

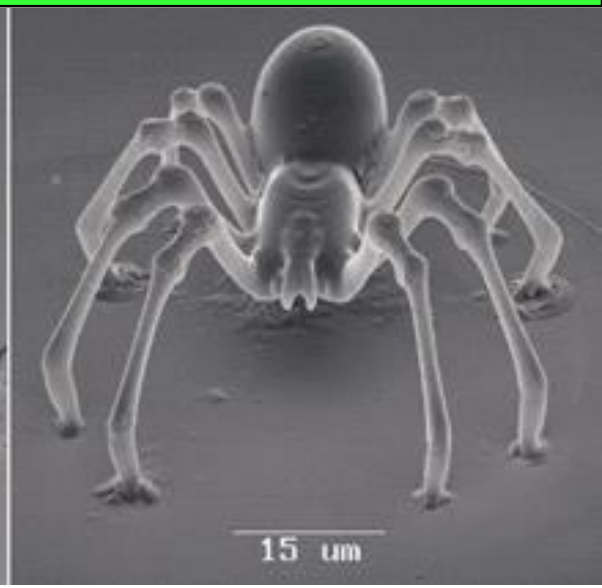


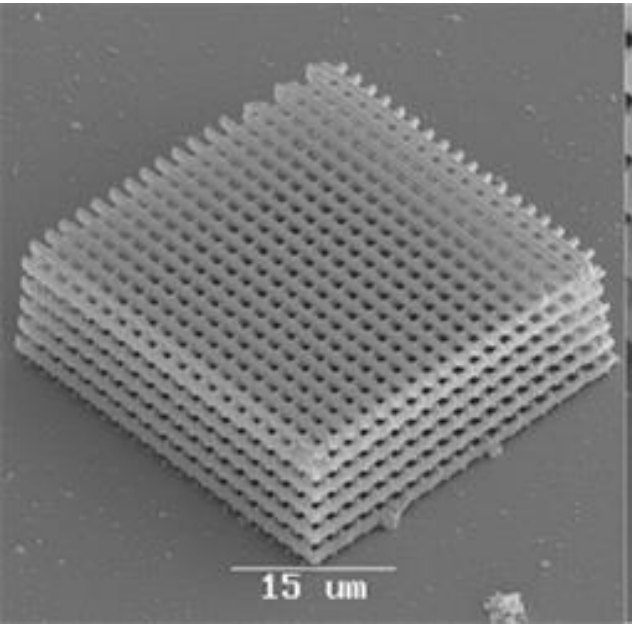


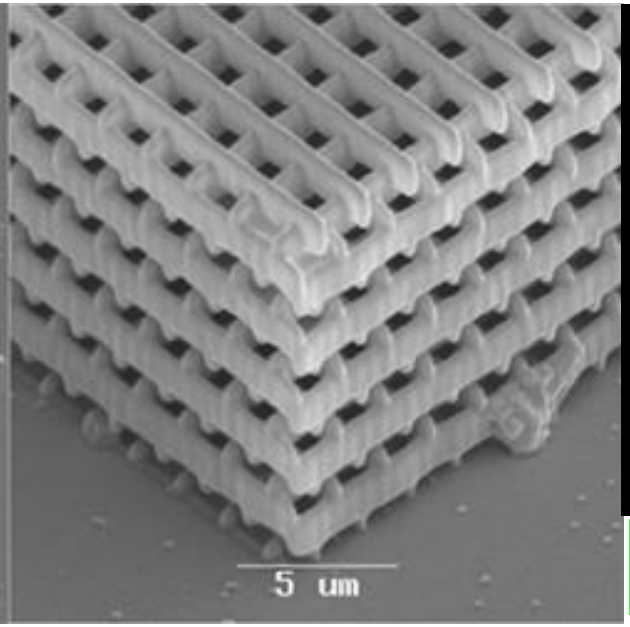
Nanofabrication: Two-photon polymerization

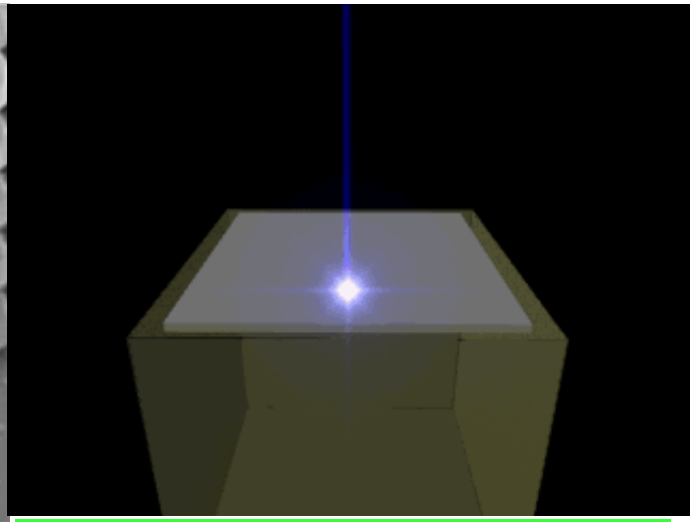






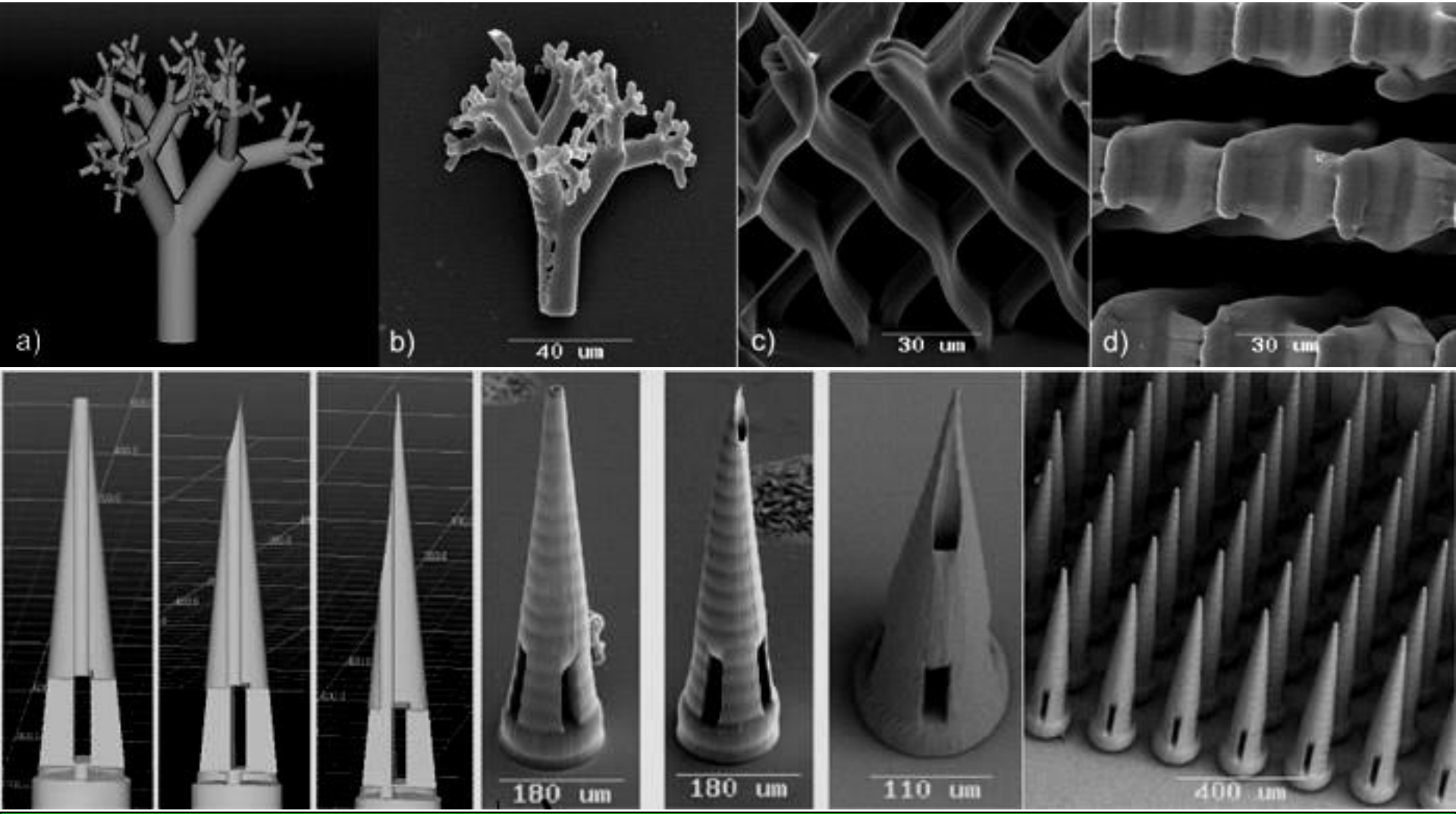




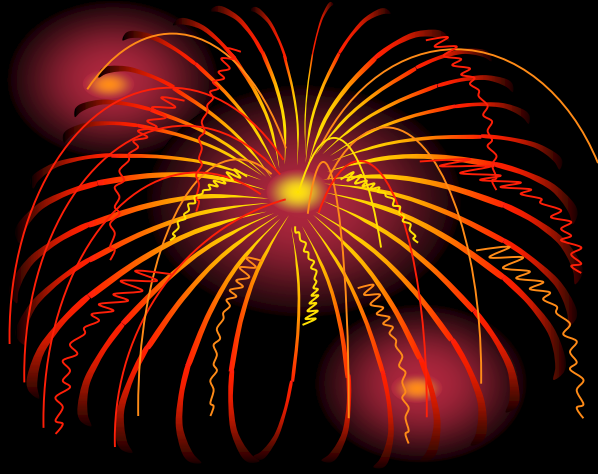


Laser sterolithography

Nanofabrication: Two-photon polymerization



http://asdn.net/asdn/nanotools/two-photon_polymerization.shtml



THE END