

# Lecture 19: Scanning Confocal Microscopy (SCM)

- Rationale for SCM.
- Principles and major components of SCM.
- Advantages and major applications of SCM.

SCM can be considered as

- A simplified version of NSOM
- Or an advanced version of optical microscope

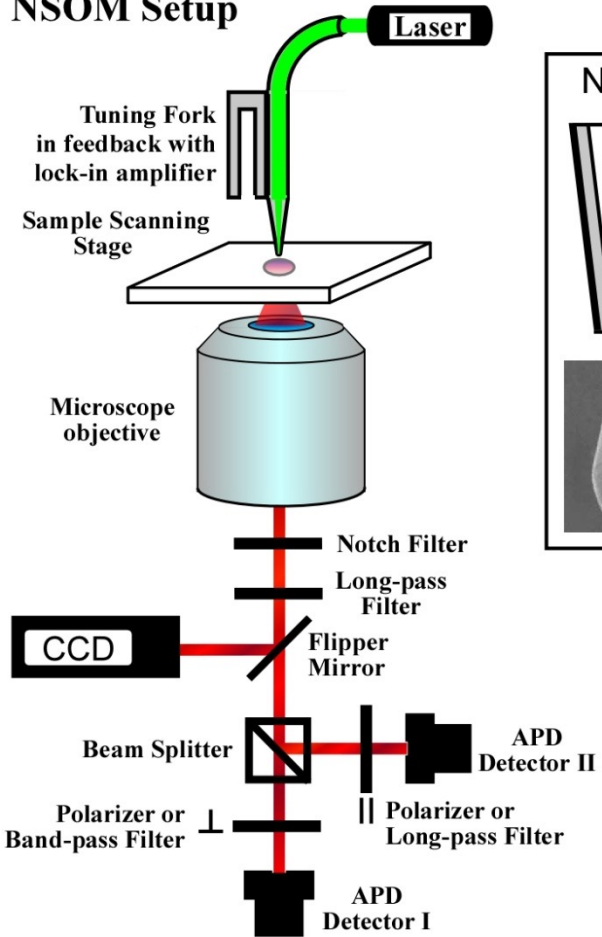
# Some limitations (disadvantages) of NSOM

## *A trade-off of high spatial resolution of NSOM*

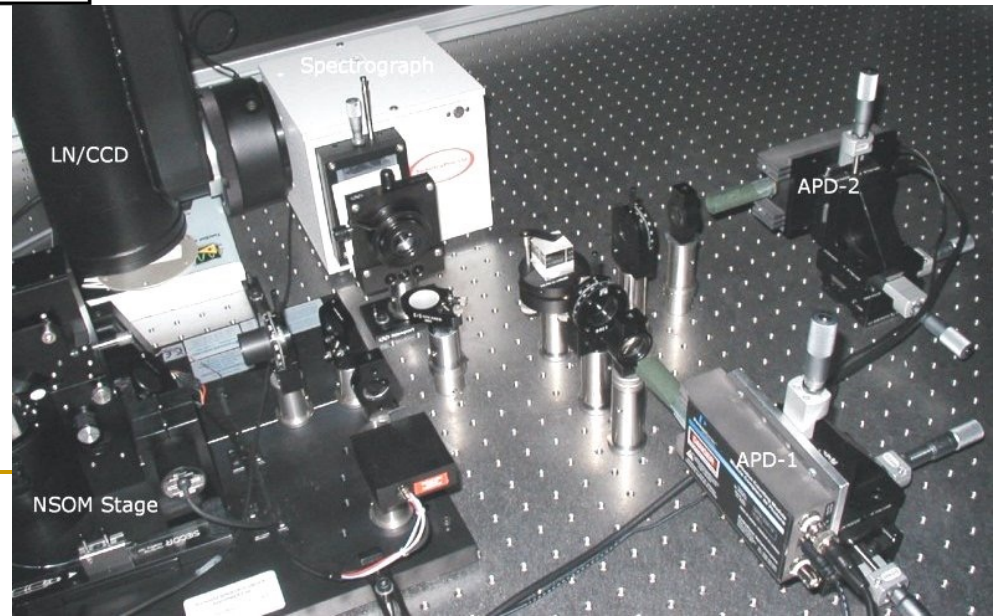
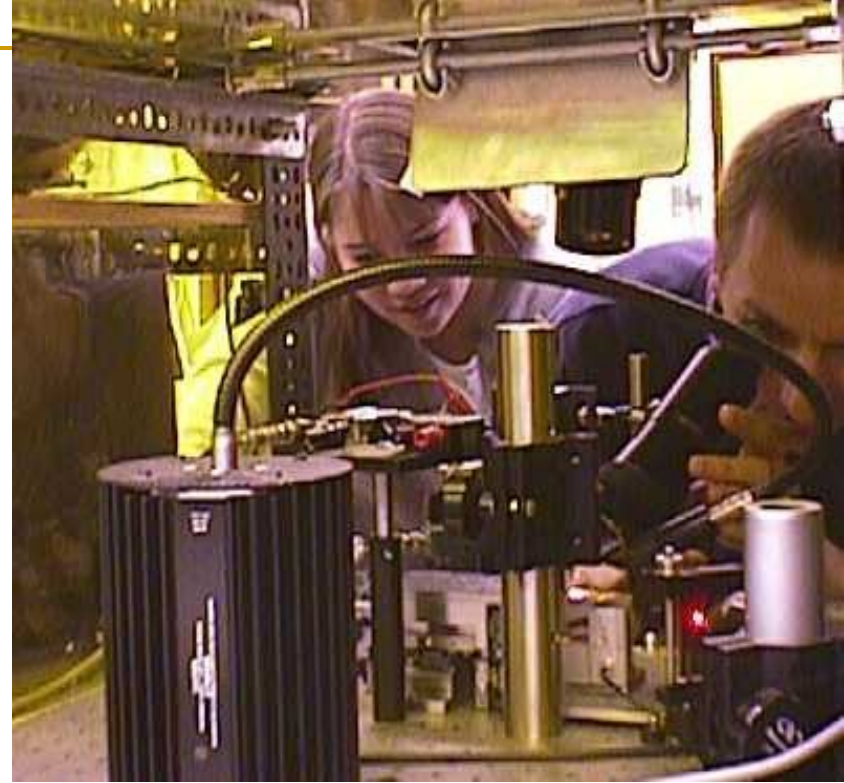
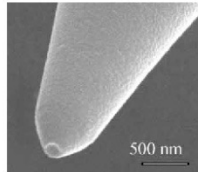
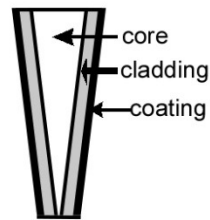
- Practically **zero working distance** (for objective) and an extremely **small depth of field** (for tip). Draw NSOM scheme on board.
- Very **low transmissivity** of apertures smaller than the incident light wavelength --- low intensity of incident light for excitation, a problem for weak fluorescent molecules (*challenging for molecules with fluorescence yield < 10%*).
- Extremely long scan times for high resolution images or large specimen areas.
- Only **surface features** can be imaged and studied --- limiting the application for sandwiched materials or devices (*double layer thin films, particles or molecules covered with insulating polymers*). Draw a scheme on board.
- Fiber optic probes are somewhat problematic for imaging soft materials due to their high spring constants, especially in shear-force mode.
- Feedback system is hard to maintain in liquid.
- Tips are expensive and hard to reproduce (*the aperture size and shape of the apex*) --- each time a new tip is used, the whole optical system (e.g. APD) has to be re-adjusted and optimized --- time consuming.

# NSOM vs. SCM

## NSOM Setup



## NSOM Tip



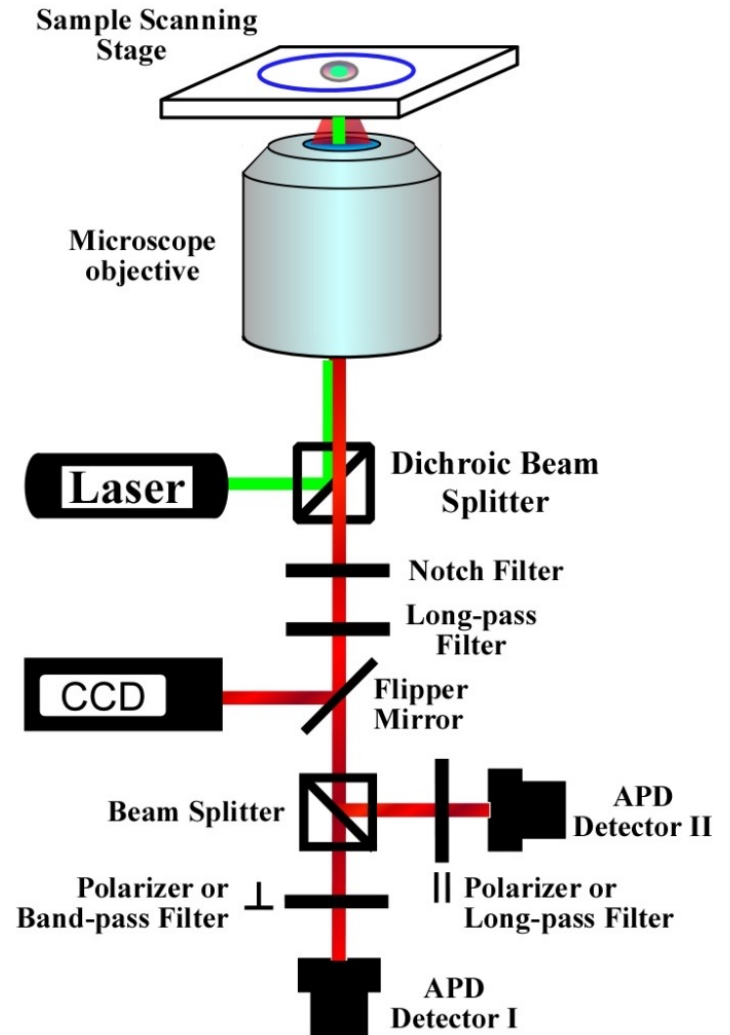
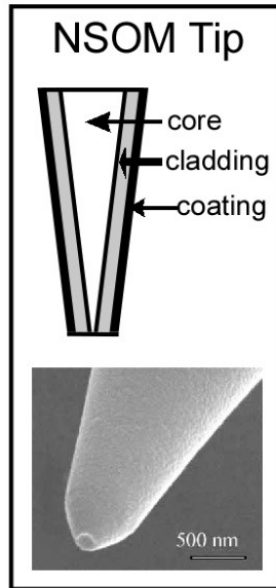
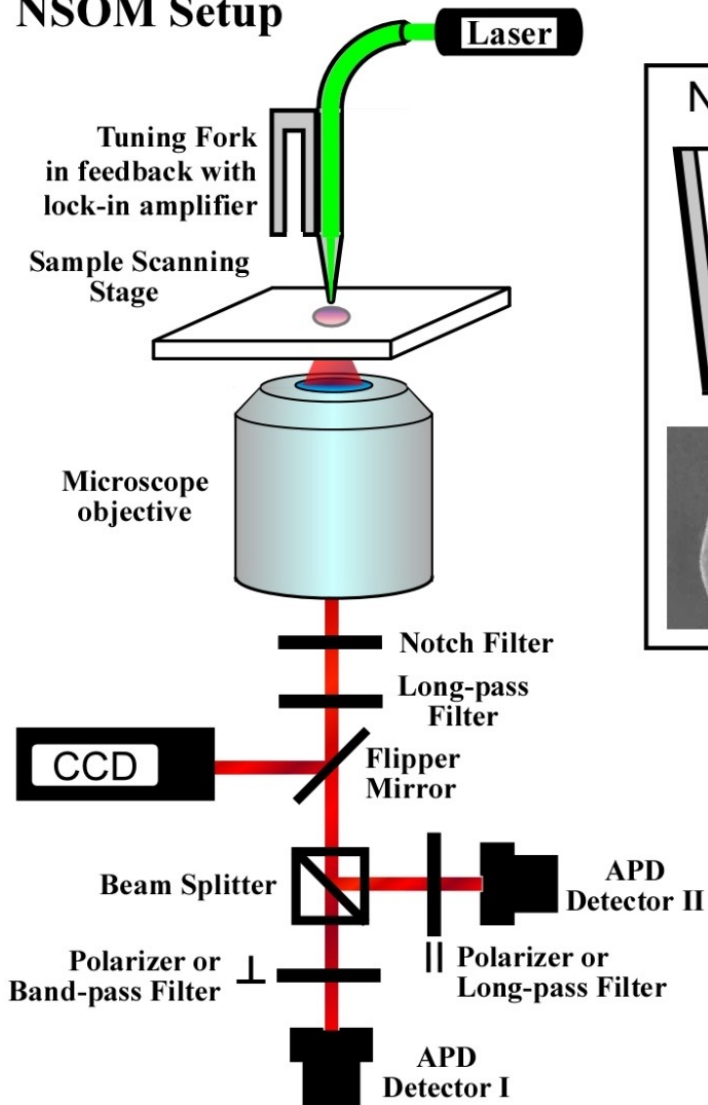
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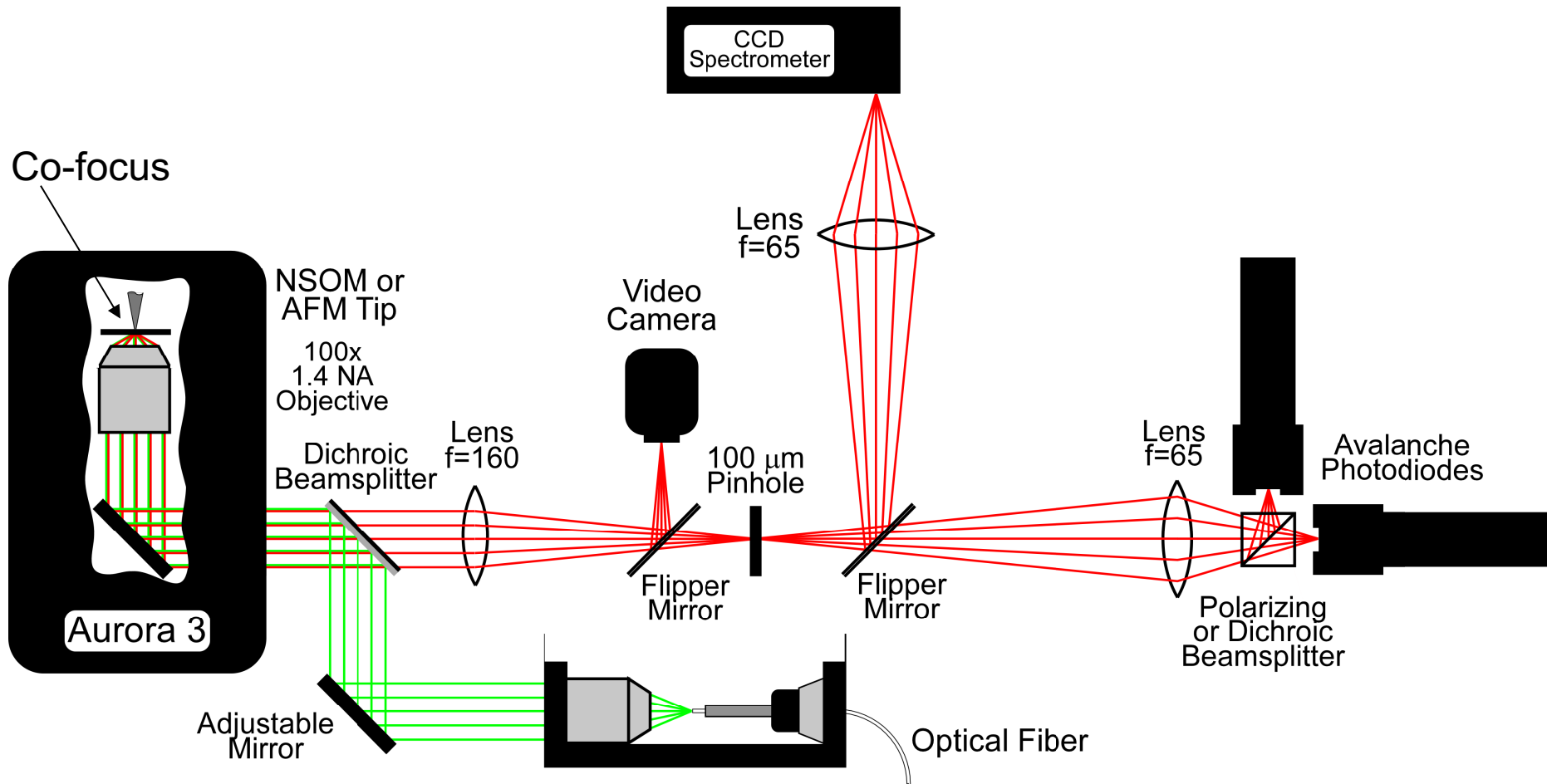
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# NSOM vs. SCM

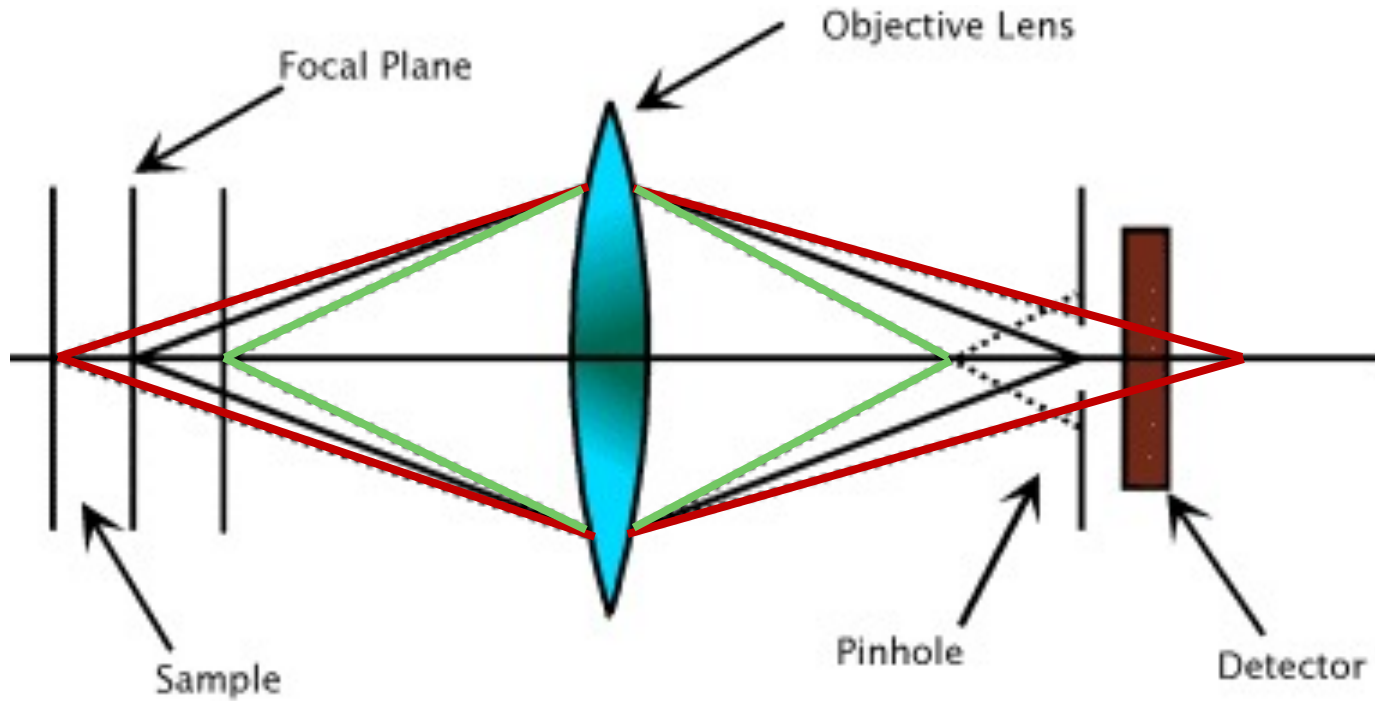
## NSOM Setup



# Optical paths of Scanning Confocal Microscope



# Confocal: *removing scattering light (noise)*



A good review on scanning confocal based single-molecule imaging

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**REVIEW ARTICLE**

Page 3597

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**Methods of single-molecule fluorescence spectroscopy and microscopy**

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# THE 2008 WOLF PRIZE IN CHEMISTRY

The Prize Committee for Chemistry has unanimously decided that the 2008 Wolf Prize be jointly awarded to



William E. Moerner  
Stanford University  
Stanford, California, USA



Allen J. Bard  
University of Texas  
Austin, Texas, USA

for the ingenious creation of a new field of science, **single molecule spectroscopy** and electrochemistry, with impact at the nanoscopic regime, from the molecular and cellular domain to complex material systems.



## The Nobel Prize in Chemistry 2014

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



Eric Betzig

A NSOM guy, now also focusing on Confocal

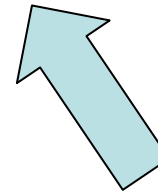
Eric Betzig, et al. *Science*, 262, 1422-1425 (1993).



Stefan W. Hell



William E. Moerner



Lecture 20:  
Scanning Confocal Microscopy

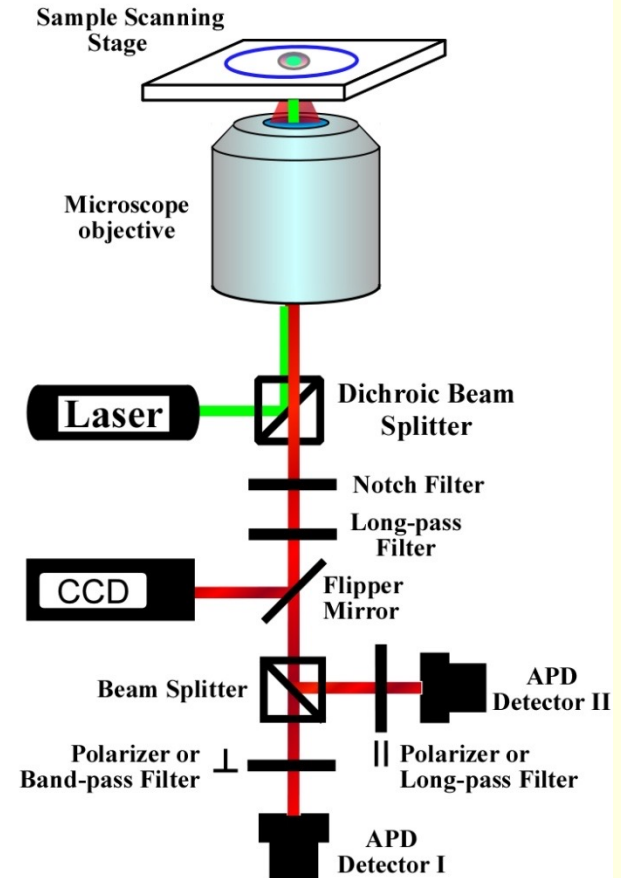
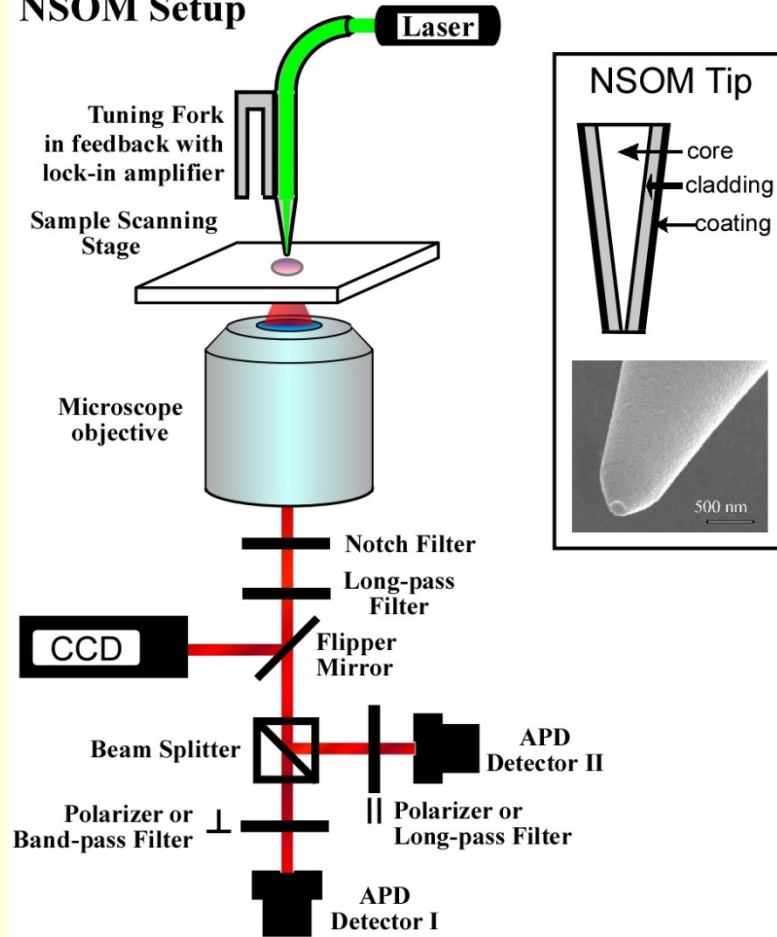
# Operation modes of SCM

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1. **Stage (sample) scanning** --- tunable from a NSOM set up. Highly desirable for manufacturers of NSOM to compete with SCM optical manufacturers. Installation of a NSOM means an option of SCM. **Major advantages: high resolution for scanning (0.1 nm).** *Slide.*
2. **Laser scanning** --- sample not moving, scanning the laser beam with mirrors. Major advantages: large area scanning and multiple laser excitation for biological systems. *Slide.*
3. **Nipkow disk** --- maintaining both the stage and light source stationary, scanning the specimen with an array of light points transmitted through apertures in a spinning. **Advantages: selective imaging, minimum damage to sample, no laser required.** It is not as common as the first two. *Slide.*

# NSOM vs. SCM

## NSOM Setup



# Operation modes of SCM

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# Mechanism of laser scanning confocal

*small tilt of laser beam leads to “out-of-focus”*

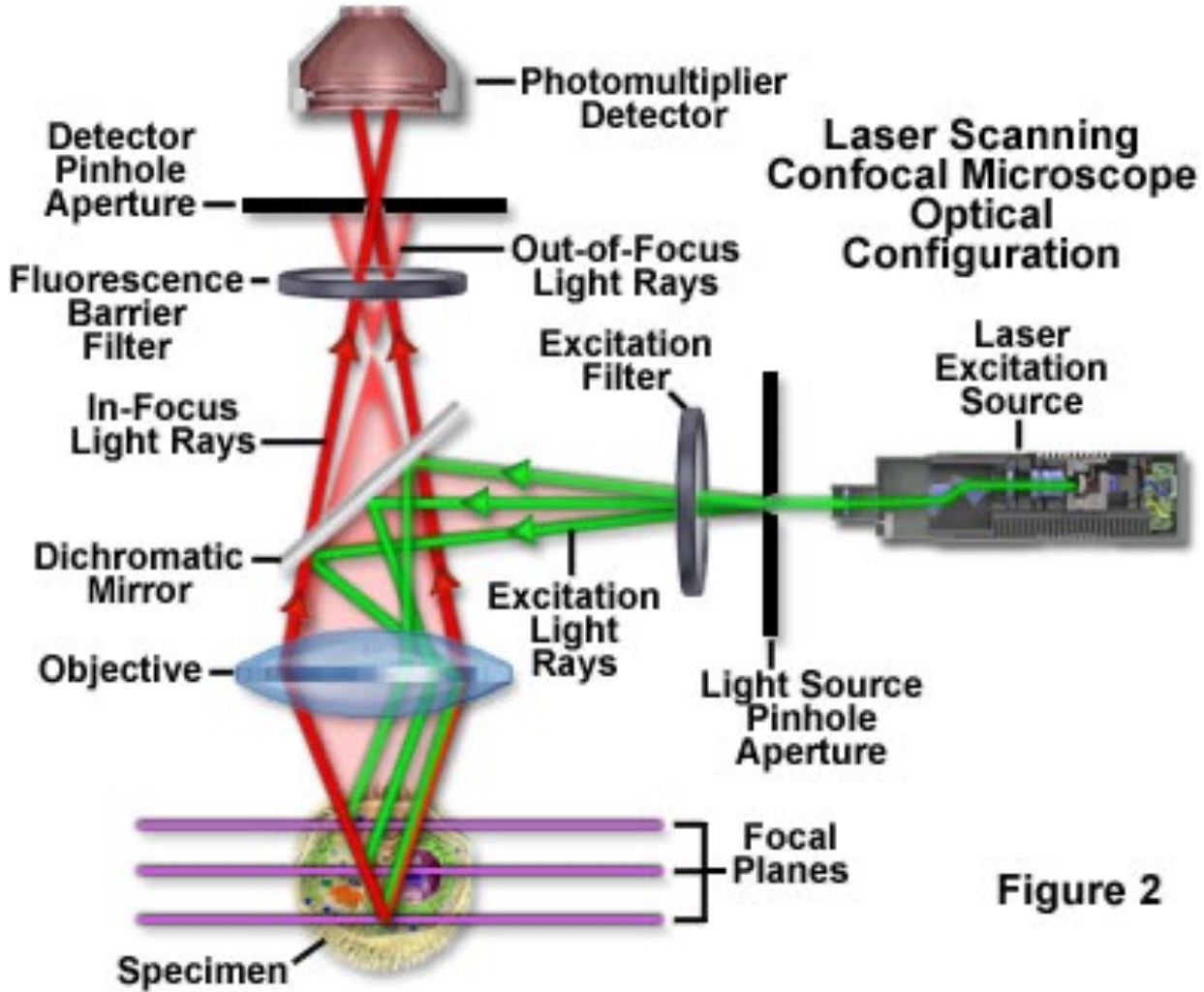


Figure 2

# Laser scanning control system

coincident moving of laser beam and optical accessories (e.g. pin holes)

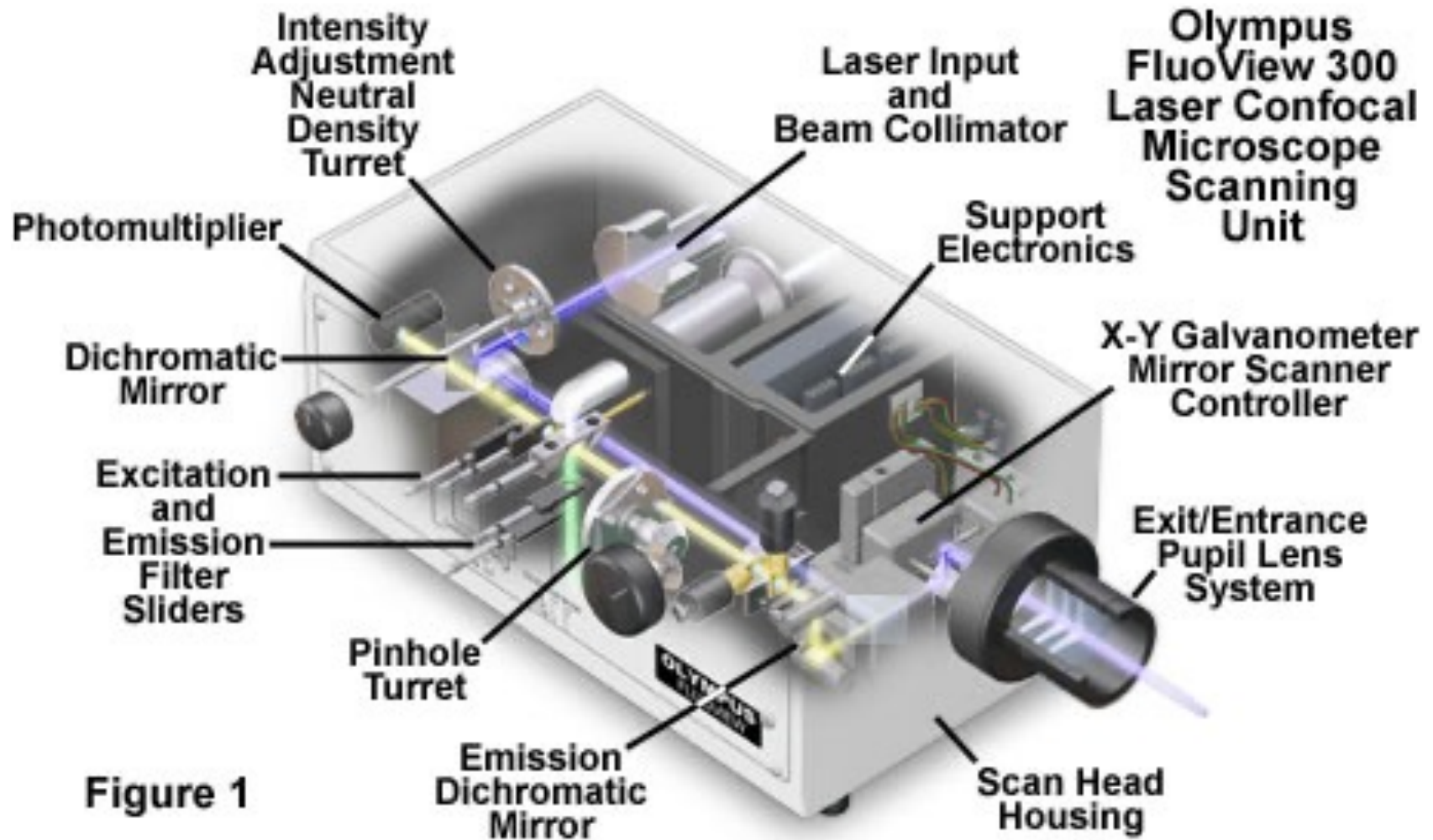


Figure 1

## Confocal Microscopy Information Flow Schematic Diagram

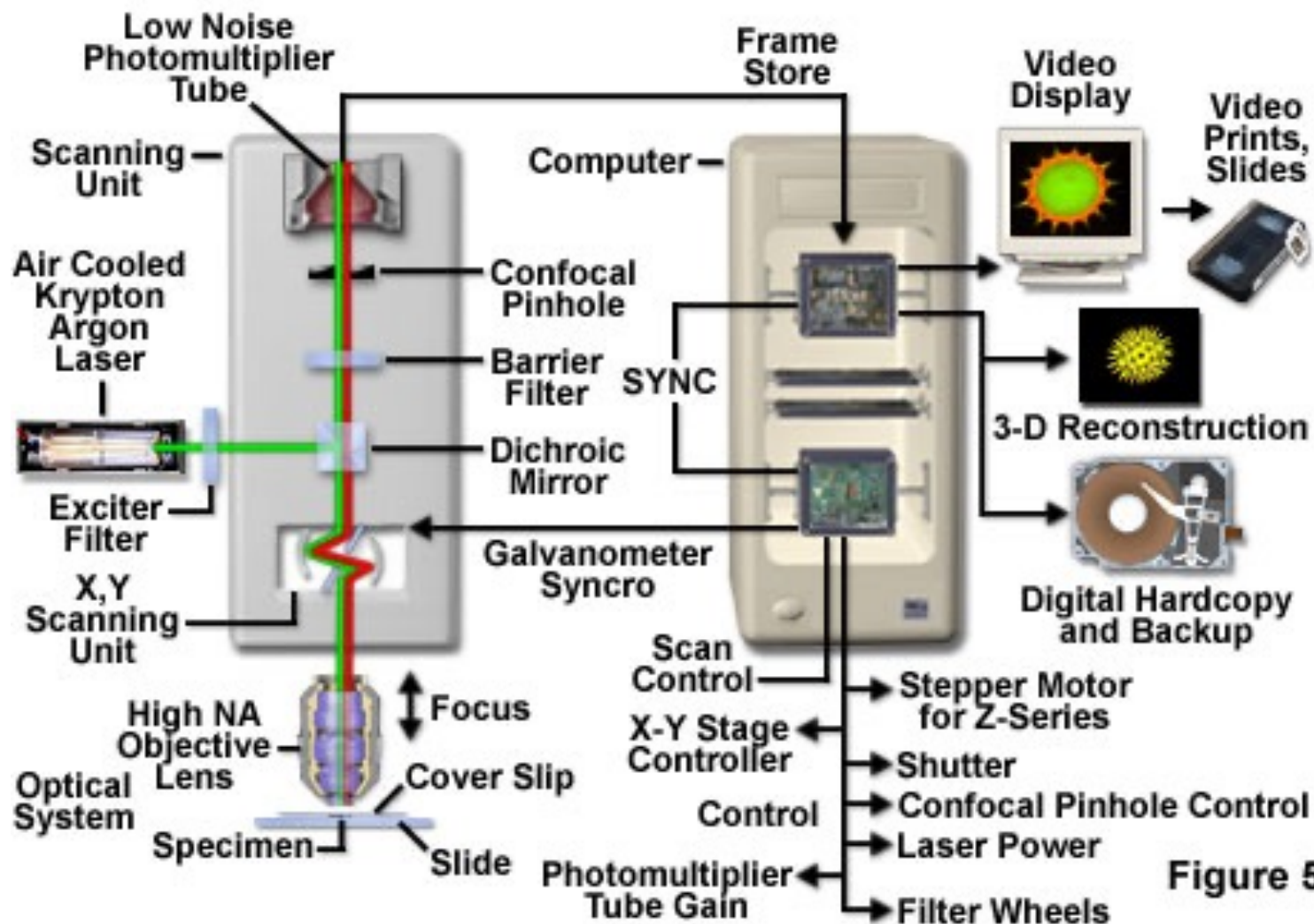


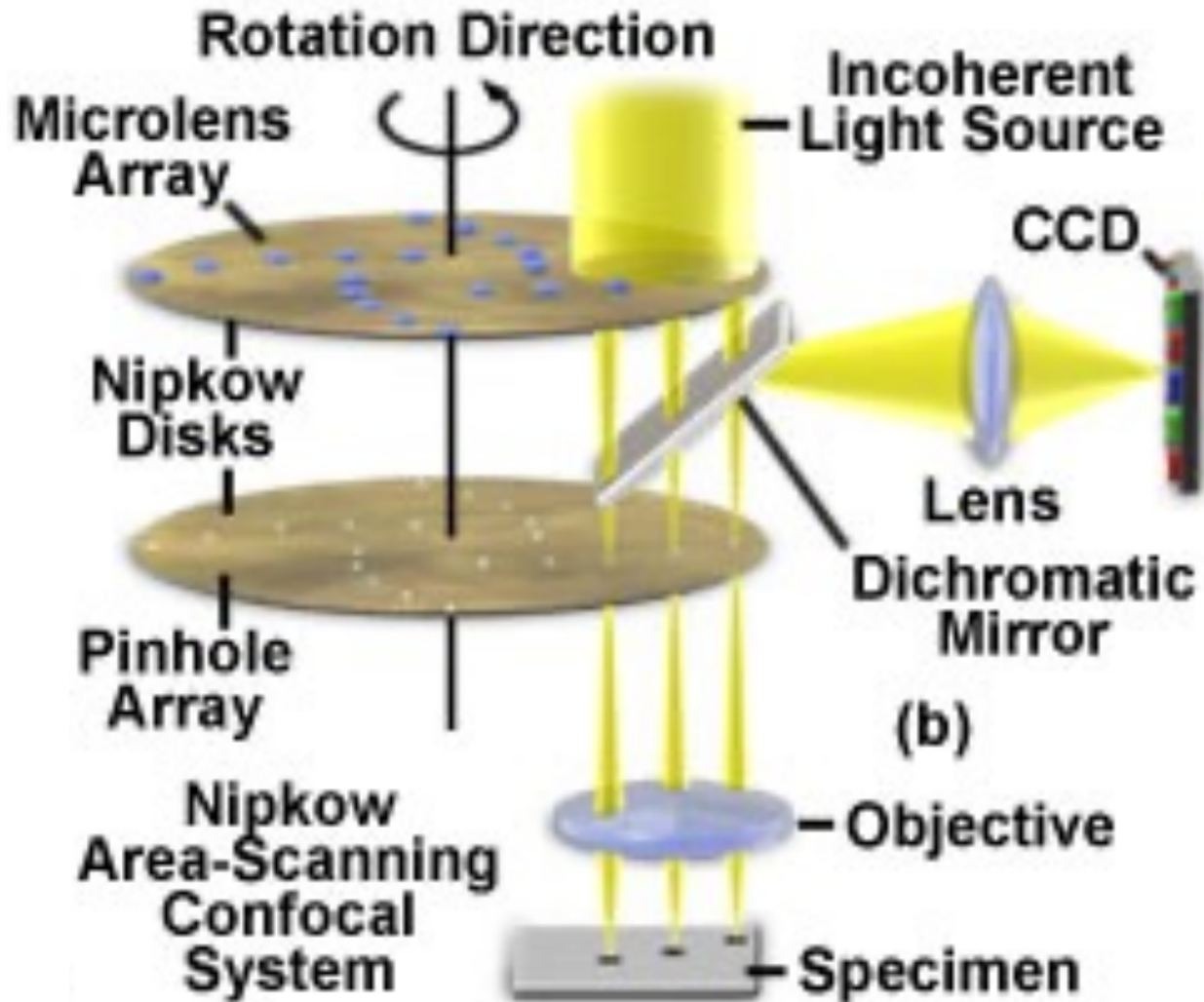
Figure 5



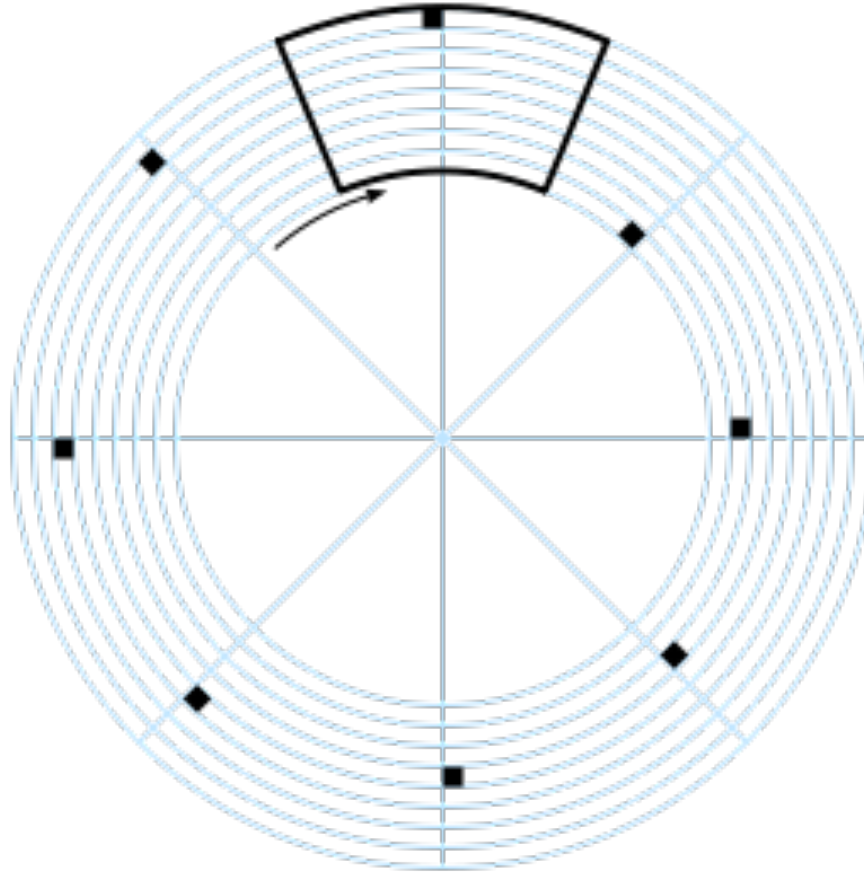
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# Laser scanning and Nipkow disk imaging

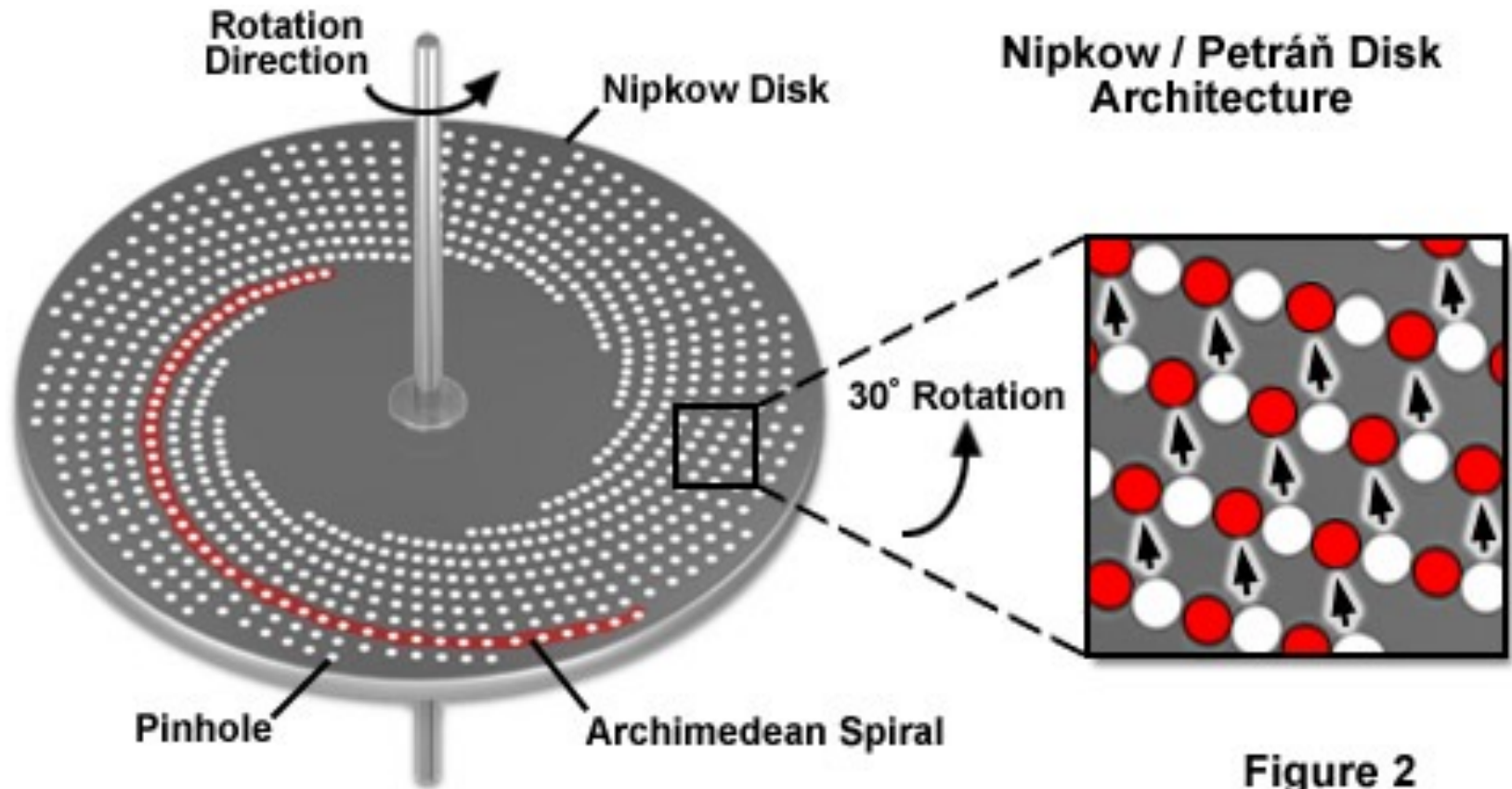


# Nipkow disk



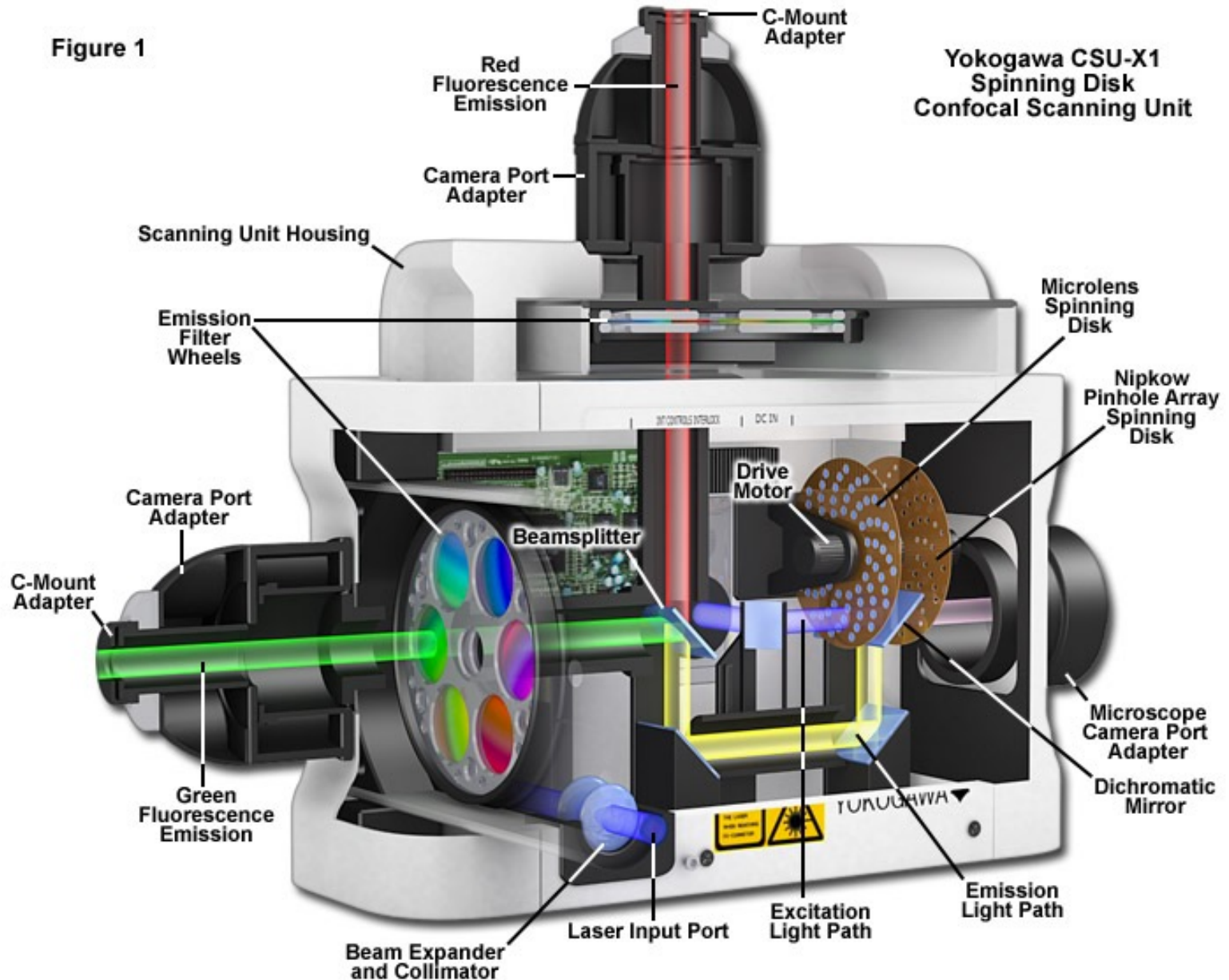
A Nipkow disk (patented in 1884), also known as scanning disk, is a mechanical, geometrically operating image scanning device, invented by Paul Gottlieb Nipkow.

# Nipkow disk

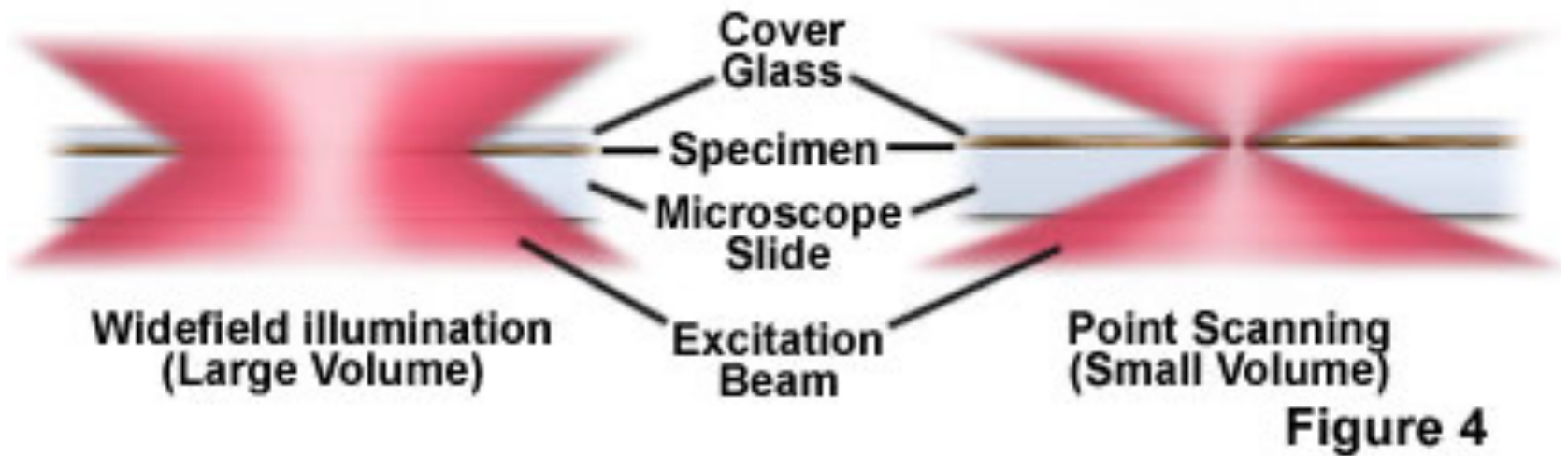


# Spinning (Nipkow) disk confocal microscopy

Figure 1

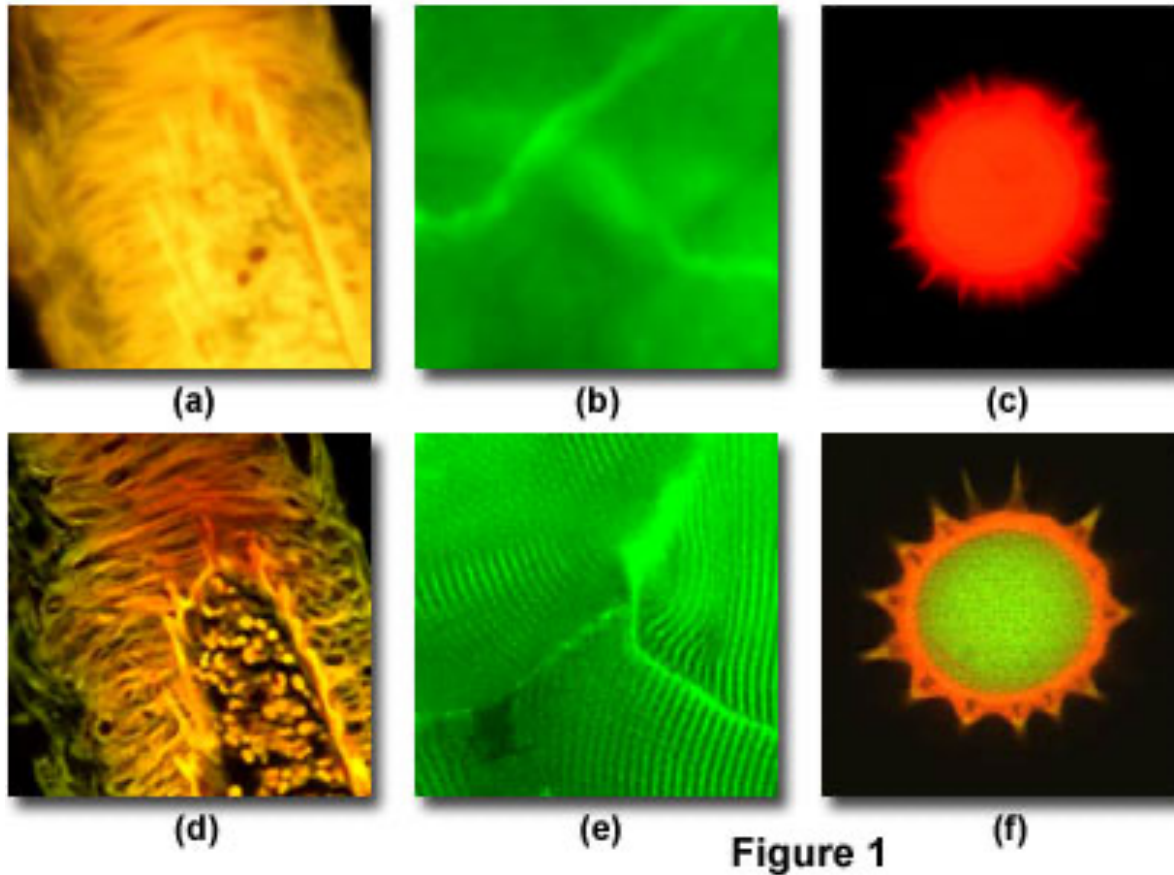


## Widefield versus Confocal Point Scanning of Specimens



Small illumination volume provides higher spatial resolution.

## Confocal and Widefield Fluorescence Microscopy



Fluorescently  
stained human  
medulla

Fluorescently  
stained rabbit  
muscle fibers

sunflower pollen  
grain

# Resolution and contrast of SCM imaging

## Airy Disks and Diffraction Pattern Intensity Profiles

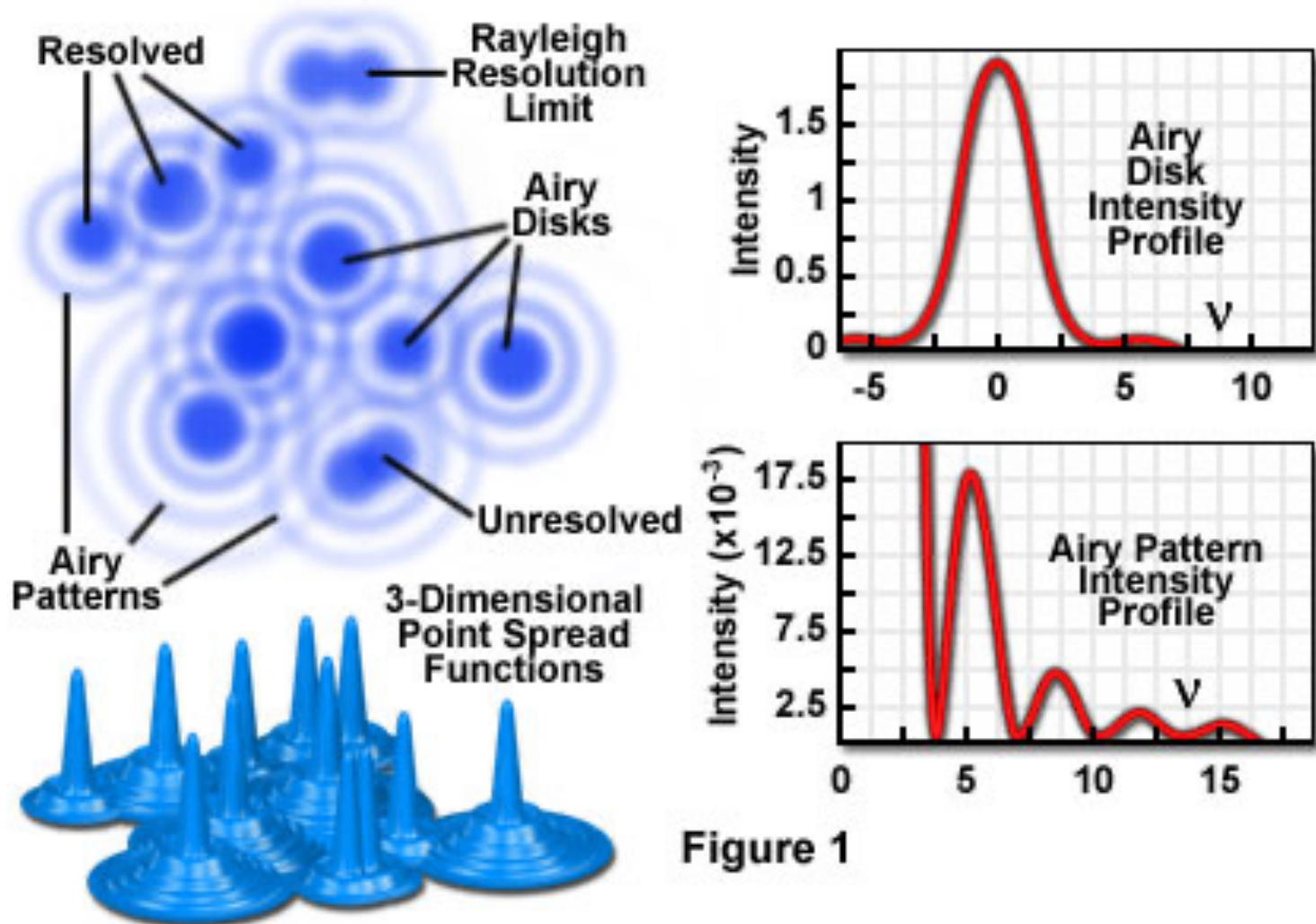
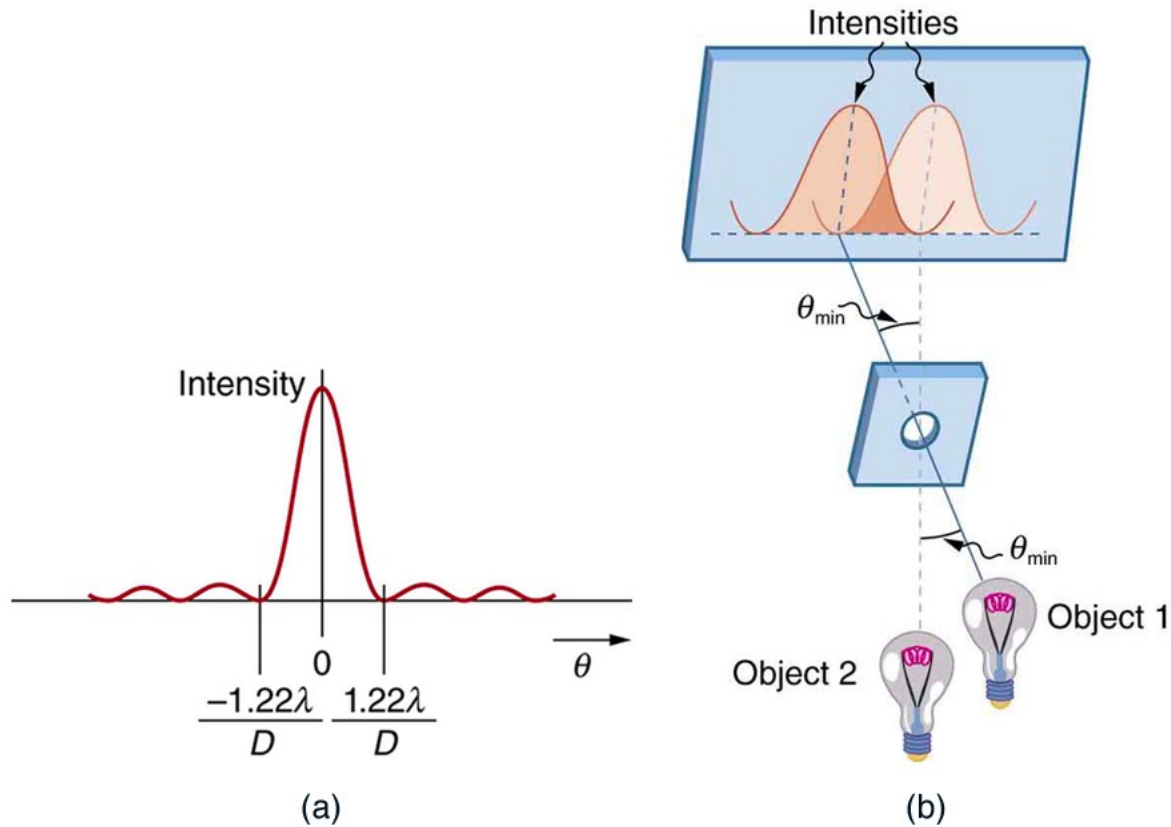


Figure 1





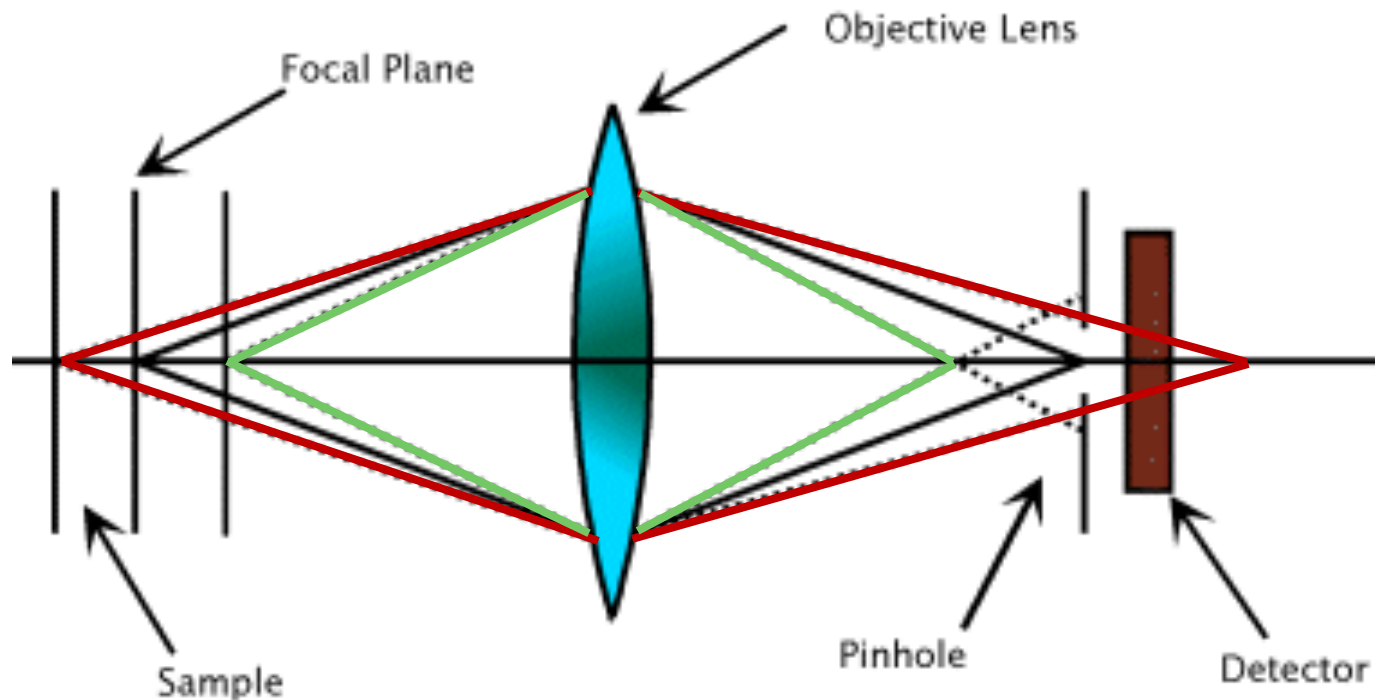
The Rayleigh criterion for the diffraction limit to resolution states that *two images are just resolvable when the center of the diffraction pattern of one is directly over the first minimum of the diffraction pattern of the other*. The first minimum is at an angle of  $\theta = 1.22 \lambda/D$ , so that two point objects are just resolvable if they are separated by the angle

$$\theta = 1.22 \lambda/D$$

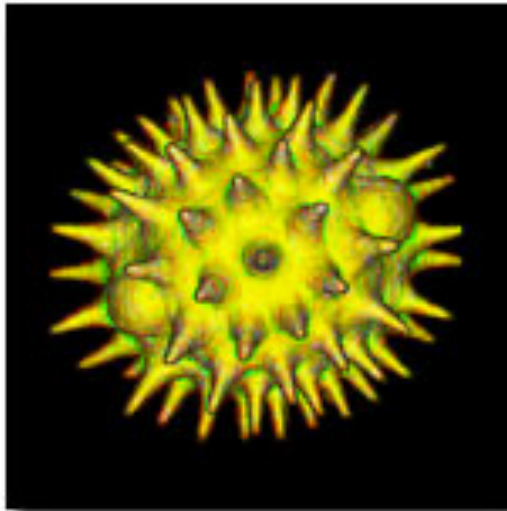
where  $\lambda$  is the wavelength of light (or other electromagnetic radiation) and  $D$  is the diameter of the aperture, lens, mirror, etc., with which the two objects are observed. In this expression,  $\theta$  has units of radians.

## 3-D imaging by Laser Scanning Confocal Microscopy

- Only the emission from the focal plane is detected through a pinhole.
- Synchronized scanning control of the laser beam enables the specimen to be **optically sectioned** along the **z** axis.
- Thus, 3-D imaging is allowable --- ***layer-by-layer scanning***.

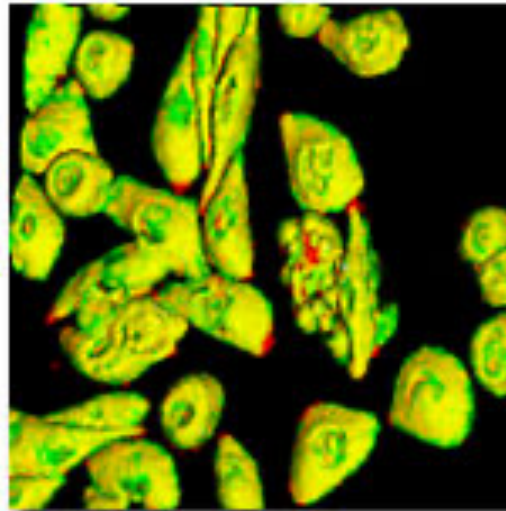


## Three-Dimensional Volume Renders from Confocal Optical Sections



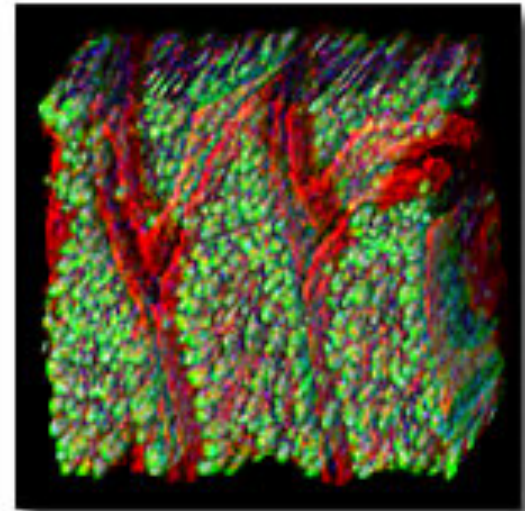
(a)

Sunflower pollen grain



(b)

Chinese hamster ovary (CHO) line mutated with GFP and HIV protein

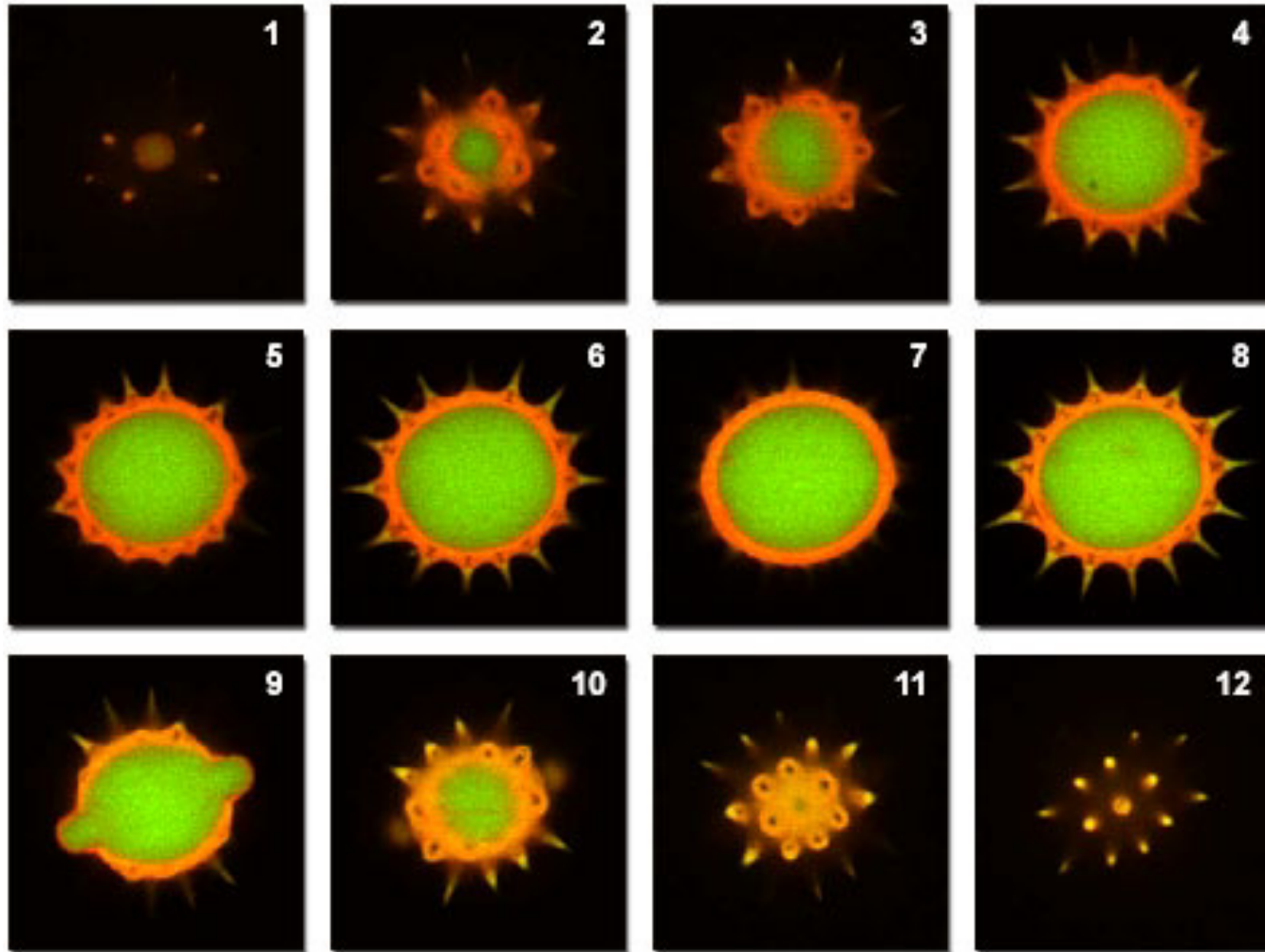


(c)

Mouse intestine section labeled with several fluorophores, created from stacking of 45 optical sections.

Figure 7

**Pollen Grain Serial Optical Sections by Confocal Microscopy**

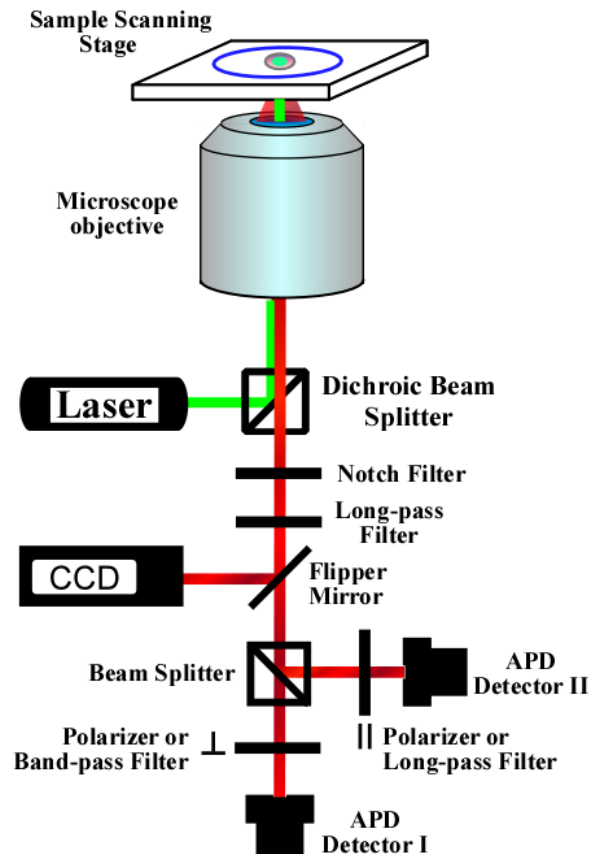


**Figure 6**

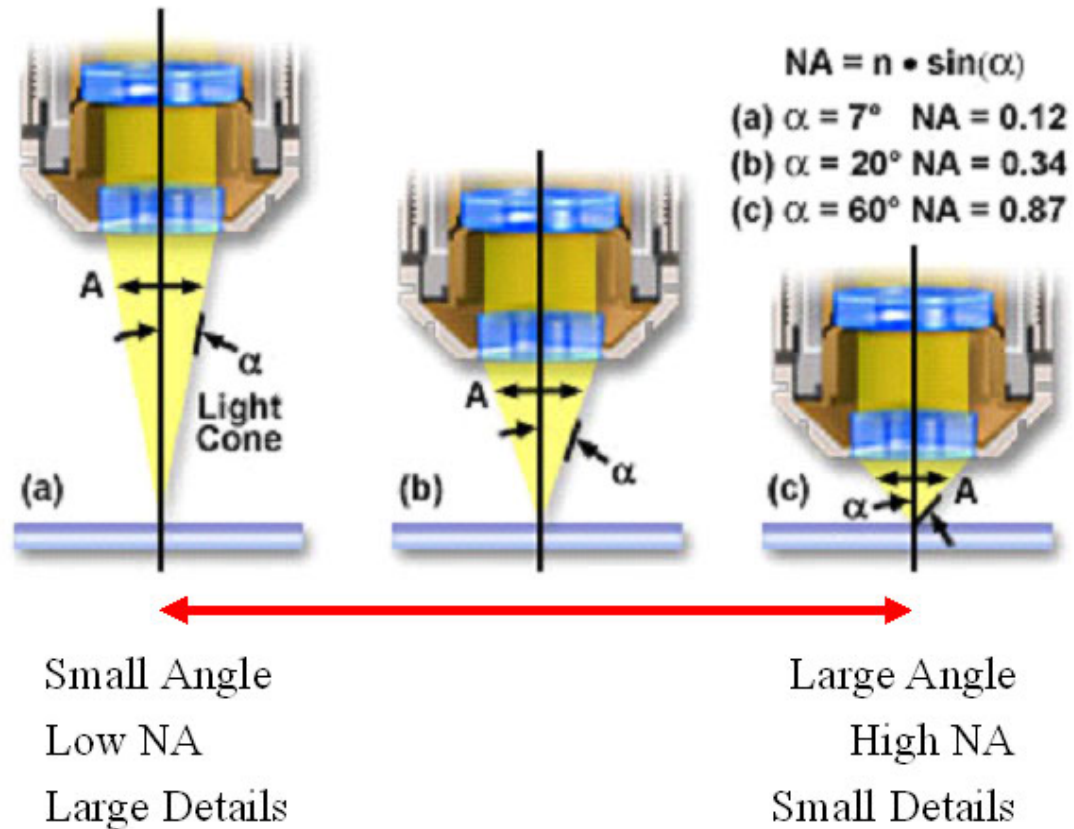
# Major optical components of SCM

- **Major instrument component:** scanner (stage), laser source (CW, pulsed), detector (PMT, APD, CCD (spectra, color image)). *See a slide.*
- **Major optical components:**
  1. **Objective** --- focusing both excitation and emission. Large NA (numerical aperture) objectives (NA > 1.2) are normally used for efficient collection of emission.
  2. **Notch filter** --- extremely narrow blocking of a specific wavelength (good for lasers).
  3. **Bandpass Filter** --- A filter that transmits a defined region (or band) of wavelengths.
  4. **Shortpass (SP) Filter** --- An optical interference or colored glass filter that attenuates longer wavelengths and transmits (passes) shorter wavelengths.
  5. **Longpass (LP) Filter** --- An optical interference or colored glass filter that attenuates shorter wavelengths and transmits (passes) longer wavelengths.
  6. **Beamsplitter** --- A common optical device used for separating an incident beam of light into two or more components that are subsequently projected in different directions. Beamsplitters are available in a variety of configurations to suit particular requirements. **A)** Prism beamsplitters, **B)** polarizing beamsplitters are composed of a crystalline birefringent material to produce linearly polarized light, **C)** dichromatic (often termed dichroic) mirrors act as beamsplitters to reflect excitation wavelengths back into the source while transmitting longer wavelength secondary fluorescence emission to the eyepieces or detector.
  7. **Neutral Density (ND) Filter** - Extensively utilized for a variety of applications in optical microscopy, neutral density filters are neutral gray in color (resembling **smoked** glass) and **are designed to reduce transmitted light intensity** evenly across either a small number of wavelengths or the entire wavelength spectrum without altering the spectral profile of illumination.
  8. **Pinhole** - placed near the light source and detector, enabling the microscope to **produce thin optical sections of focal planes in the specimen.**

# A scheme of SCM

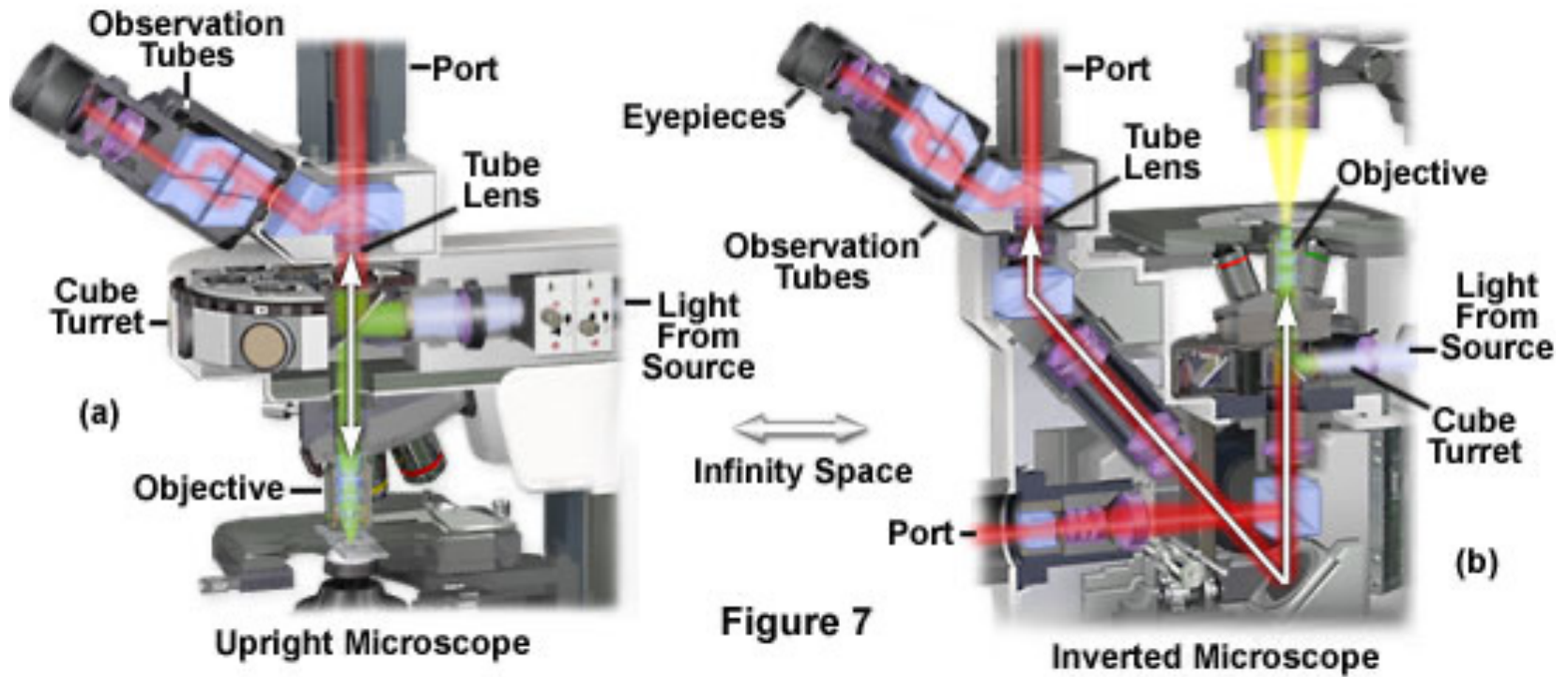


Numerical Aperture (NA) of an objective and the effect on optical resolution and light collection



# Two styles of SCM setup

Parallel Infinity Space in Upright and Inverted Fluorescence Microscopes



# Olympus FV 1000

**FluoView™ FV1000  
Laser Scanning Confocal  
Microscope**



**BX61 Automated Research  
Microscope with CCD  
Camera Systems**



# Zeiss LSM 510

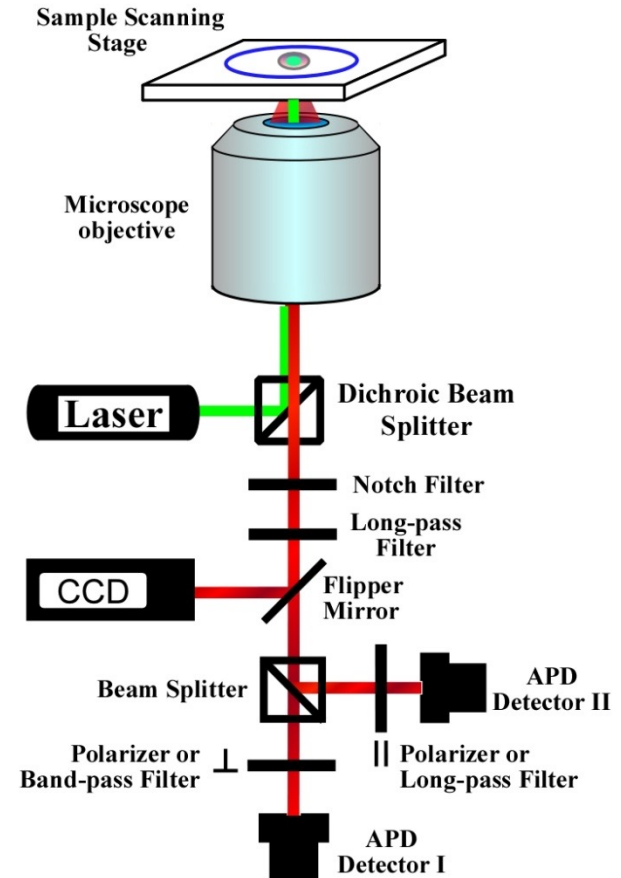
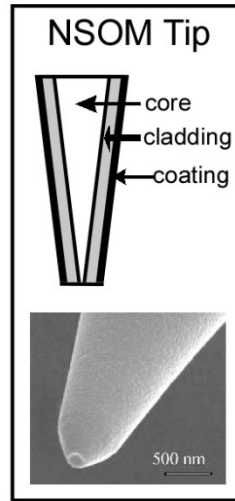
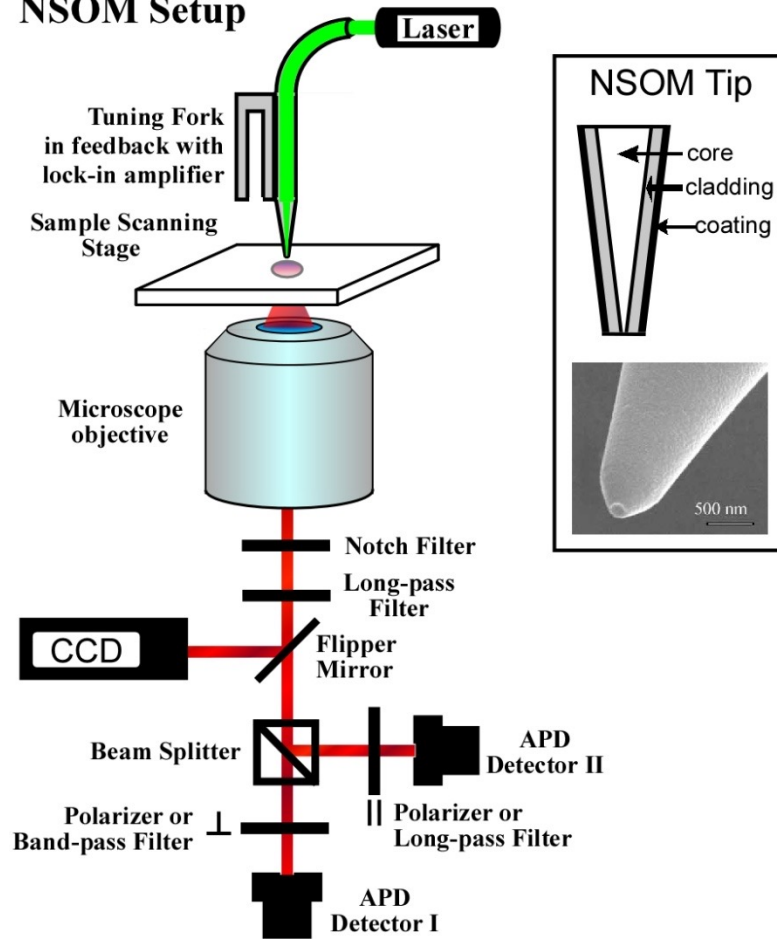


# Advantages of SCM over NSOM

- High **spatial resolution** of NSOM --- critical for studying nanostructured materials, but not necessary for single-molecule imaging, because the molecules can be dispersed such that they are **far enough apart** (> 300 nm) that diffraction limited optics can resolve individual ones.
  - Historically, detection of the fluorescence from single molecules on a surface at room temperature was originally achieved with NSOM.
  - Soon after this discovery, confocal microscopy had emerged as a more popular technique to detect single molecules. Although confocal microscopy is not capable of **sub-wavelength resolution** imaging, it has a number of advantages over NSOM. *One representative person is Morner, slide.*
1. Very **high optical excitation powers** are possible, whereas with NSOM, the upper limit of the excitation power is severely limited. The higher fluorescence intensities that accompany higher excitation powers raises the S/N and reduces the **time integration** needed for any given experiment. This is important for **faster imaging rates** and for looking at fluorescence signals which fluctuate on short (millisecond) time scales.
  2. SCM is relatively easy to set up in comparison to NSOM, since no feedback system to be maintained. Setting up and optimizing shear force **distance regulation** is the most time consuming step in NSOM.
  3. For NSOM, the position of the APD needs to be adjusted each time a new tip is used, and should also be optimized, or at least checked for optimization, each time a new region of the sample is imaged (i.e. when the course position of the sample is adjusted). This is because the tip, each time replaced or moved slightly, is not aligned confocal with the detector. In contrast to this, the focal spot of SCM objective does not change. Multiple samples can be imaged **over days**, and probably **weeks**, without any drifts substantial enough to require adjusting the position of the APD --- **high throughput screening**.
  4. The excitation power of SCM is more easily measured. SCM is capable of **power dependence** studies, which simply cannot be done with NSOM because the high power regime is inaccessible. For NSOM, the differences in the throughput of each tip and differences in the efficiency of coupling junctions (typically, one which couples light from a free space laser beam to the single mode fiber, and another fiber-to-fiber splice to couple into the NSOM probe) prevent simply measuring the power of the input beam.

# NSOM vs. SCM

## NSOM Setup



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# Major applications of SCM

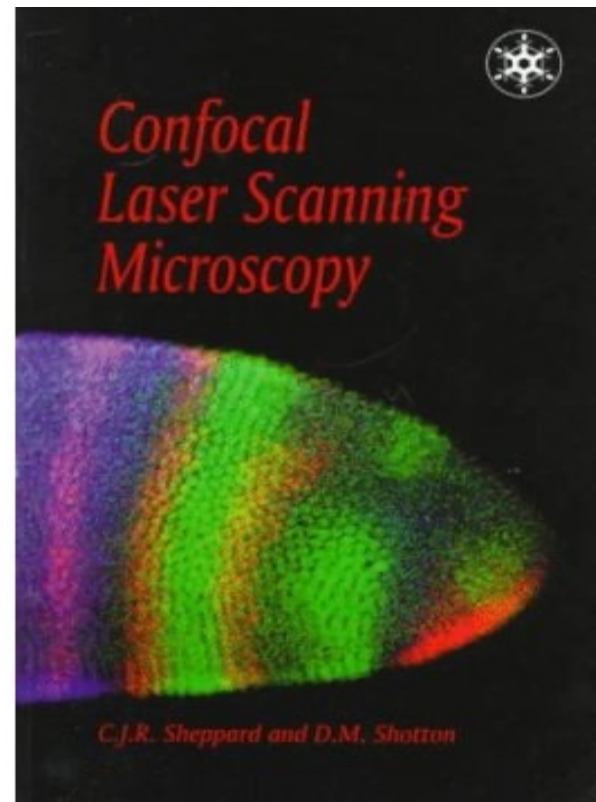
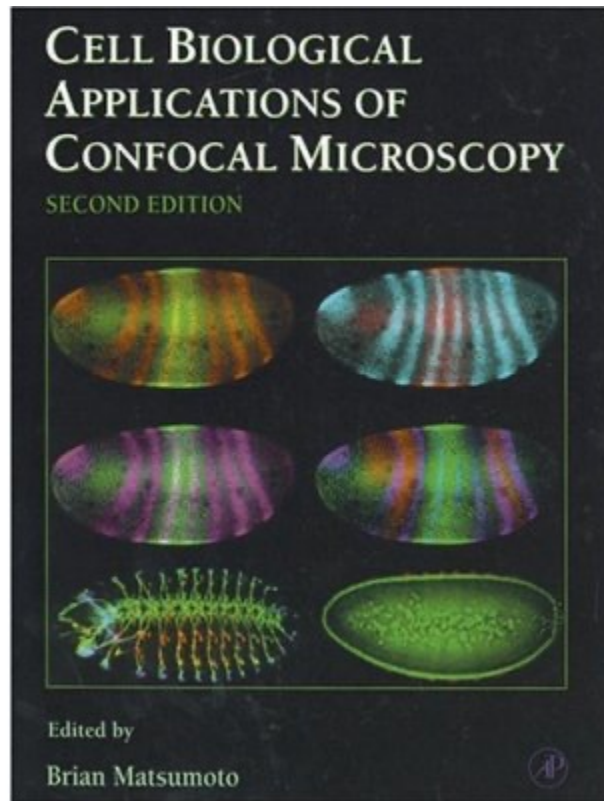
1. Single-molecule imaging and spectroscopy (high throughput), particularly in solutions, *where NSOM is hardly to be applied, and no spatial resolution required.*
  2. Single-molecule rotation and diffusion.
  3. Protein folding and dynamic conformations.
  4. In situ single-cell imaging of virus attack.
  5. Nanocrystal optoelectronics.
  6. Single-molecule Raman spectrometry.
-

SCM allows for detecting at different depths into sample



Protection from oxygen and moisture is often required.

Many books and readings available



And **Google**: “scanning confocal” + your topic