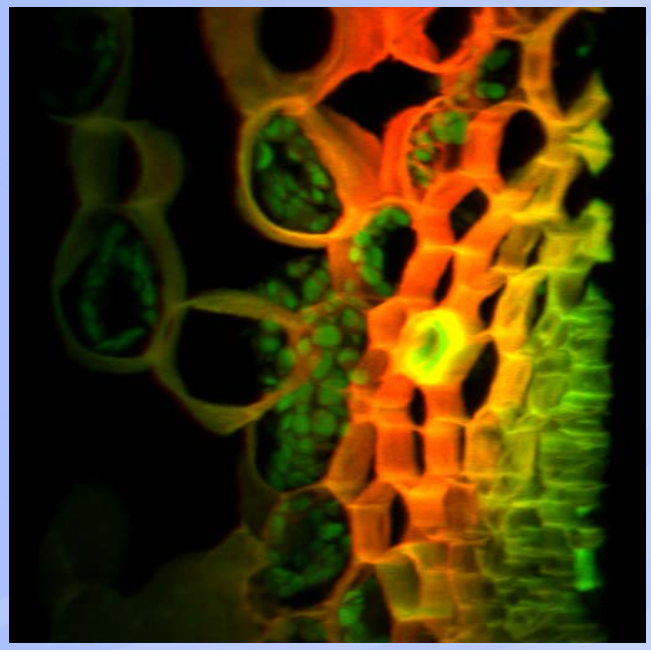
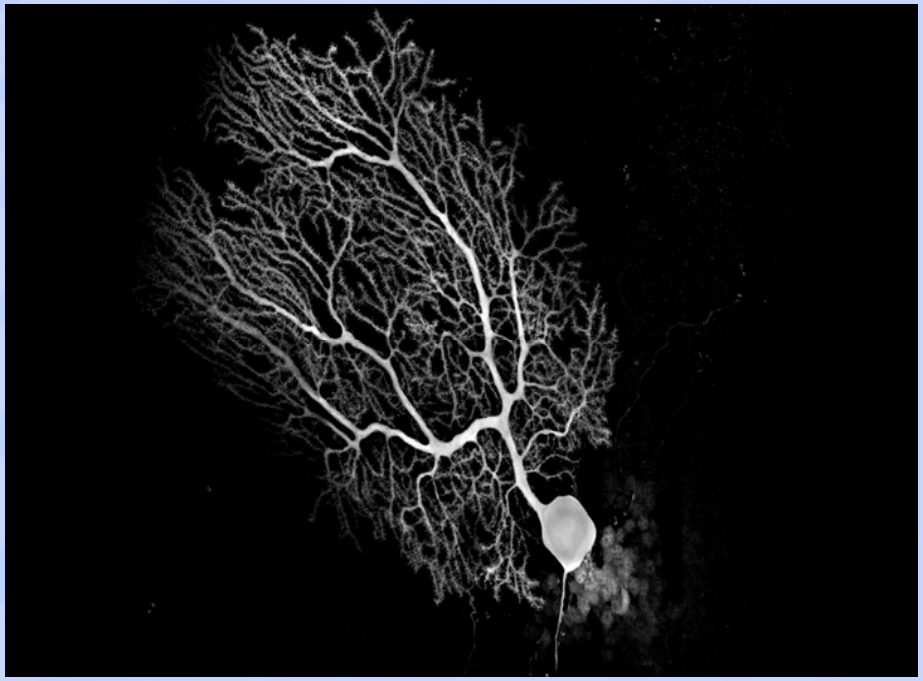
Microscopy Optical Sectioning





Stephen Ross Ph.D.
UCSF Principles and Practice
Of Light Microscopy
May 3rd, 2010

The Eyes of Science

Nikon

- **Confocal Microscopy In Principle**
- **Point Scanning Confocal**
- **Spinning Disk Confocal**
- **Swept Field Confocal**
- **Total Internal Reflection Fluorescence (TIRF)**

Broad definitions –An introduction to understanding the terminology

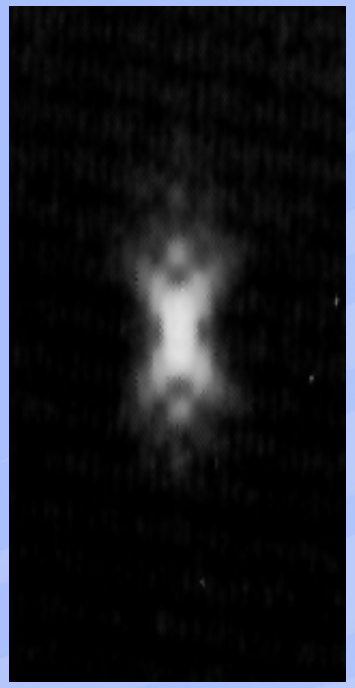
1. Define optical section in terms of the mathematics:
   - Lateral resolution: for diffraction limited techniques: where d is the smallest resolvable unit, λ is the wavelength of light, and NA is the numerical Aperture of the objective lens.

$$d_{xy} = \frac{1.22\lambda}{(2NA)}$$

Example: For a 1.4NA objective at 550nm, the resolution limit is 240nm.

Broad definitions –An introduction to understanding the terminology

1. Define optical section in terms of the mathematics:



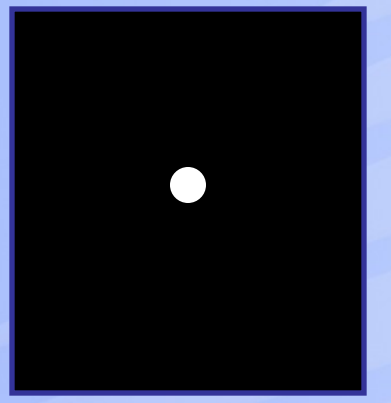
- Axial resolution (focal volume): Again, for diffraction limited techniques, the axial resolution is given as: where η is the refractive index of the media.

$$d_z = \frac{2\lambda \cdot \eta}{(NA_{obj})^2}$$

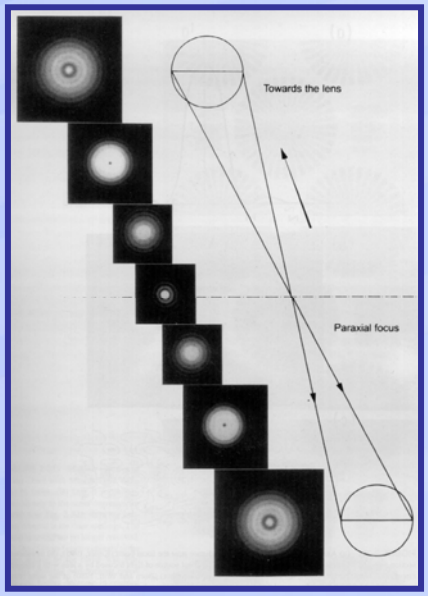
Example: For a 1.4NA objective in oil/oil, at 550nm, the resolution limit is 850nm.

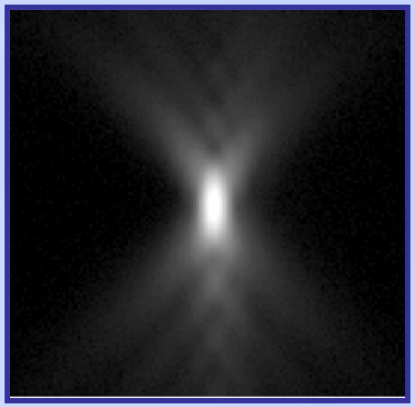
Point Spread Function

- **PSF defines the propagation of electromagnetic radiation or other imaging light rays from a point source or point object.**
- **The degree of spreading of the point object is a measure of the imaging system.**
- **The effect of the imaging system on a point object is termed the convolution of the optical system.**
- **Displayed by imaging a sub micron point in 3D (Z stack) and displaying as a volumetric airy disc. (X-Z projection)**



Diffraction limited Point Source





Optical Convolution of Point Source

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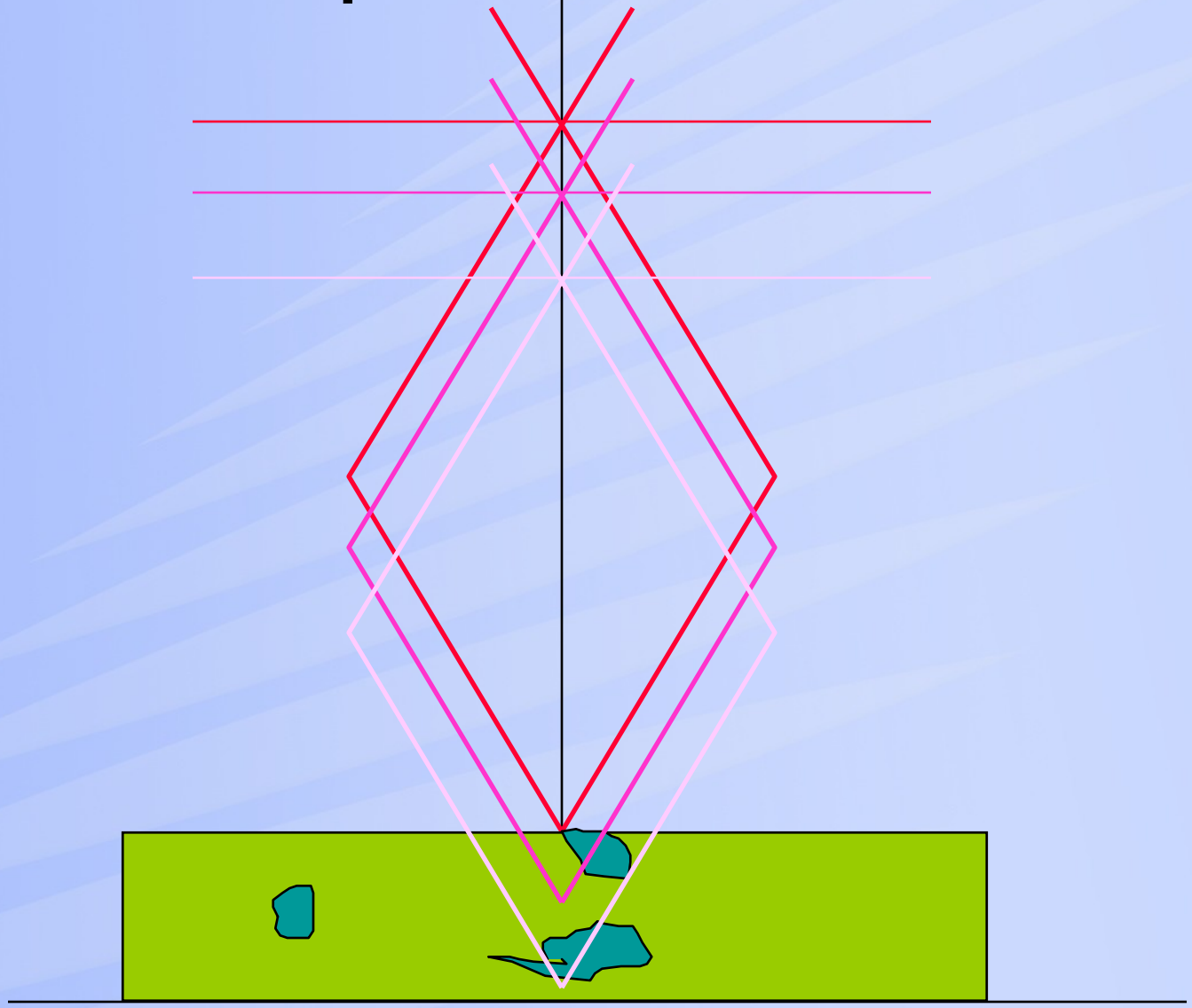
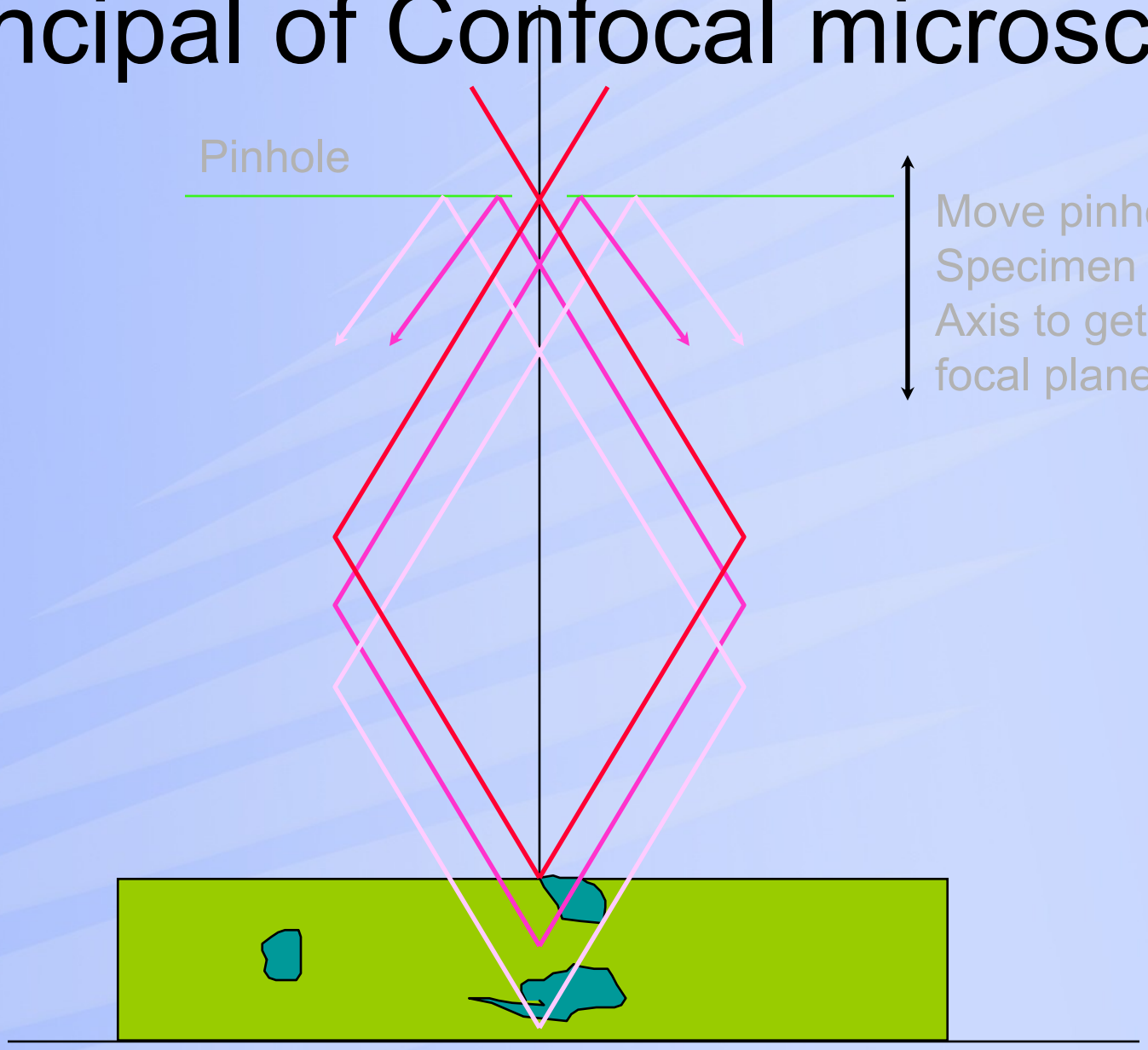
Multiple Focal Planes



Conventional light microscopy is limited by multiple focal planes in the same section, therefore:

- Physically cut thinner sections
  - Technically difficult
  - Highest resolution
  - Highest sensitivity
- Optical sectioning using confocal microscopy
  - Technically simple
  - Multiple section planes available
  - Complex computing

Principal of Confocal microscopy



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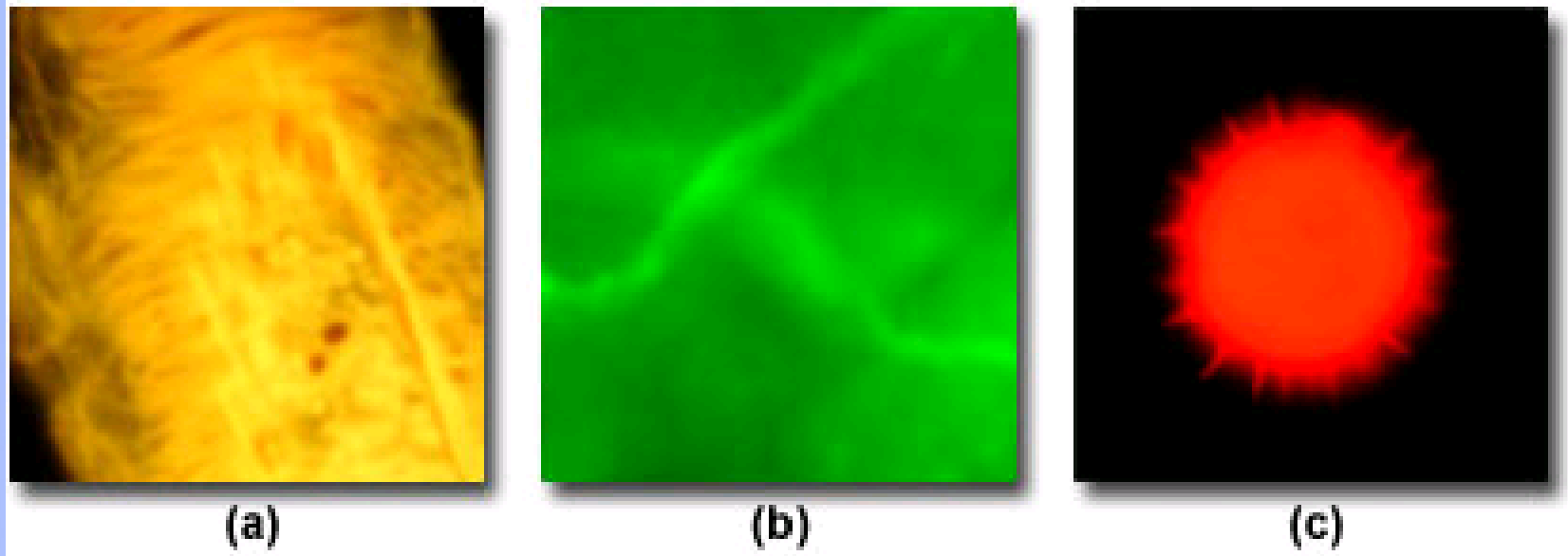
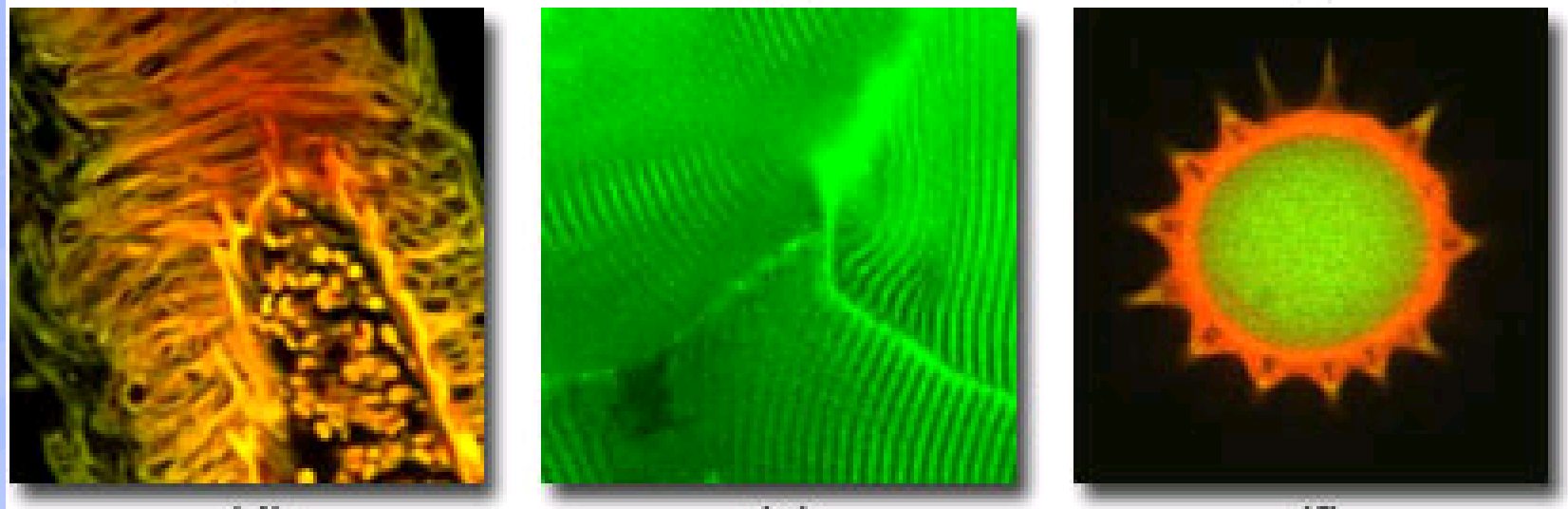
Pinhole size and resolution

- Pinhole size should be related to width of point spread function
- Width of point spread function = resolution of lens × magnification of lens = 1 Airy unit
  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 22 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 9.4 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 8.1 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 6.8 μm

Confocal and Widefield Fluorescence Microscopy

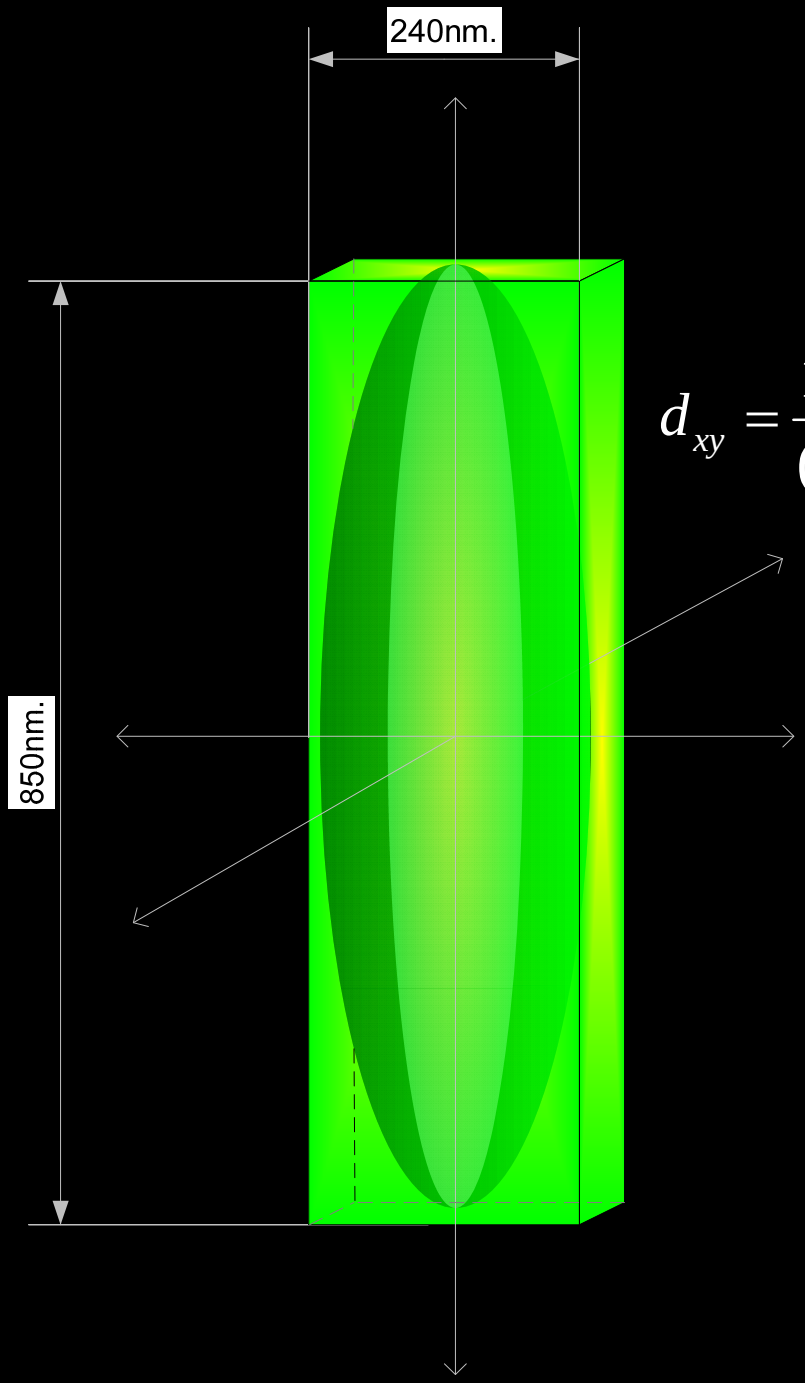


(a) (b) (c)



(d) (e) (f)

Figure 1



Diffraction limited volume :

NA = 1.4

η (Refractive Index) = 1.515

λ (Wavelength) = 550 nm

$$d_{xy} = \frac{1.22\lambda}{(2NA)} = \frac{1.22 \cdot 0.55\ \mu m}{(2 \cdot 1.4)} = 0.24\ \mu m = 240\ nm$$

$$d_z = \frac{2\lambda \cdot \eta}{(NA_{obj})^2}$$

$$= \frac{2 \cdot (0.55\ \mu m) \cdot 1.515}{(1.4)^2} = 0.85\ \mu m = 850\ nm$$

Components of a confocal

Light source
Generally laser based

Detector
PMT or CCD

Controller and
image acquisition
computer

Microscope

Scan Head
w/pinholes

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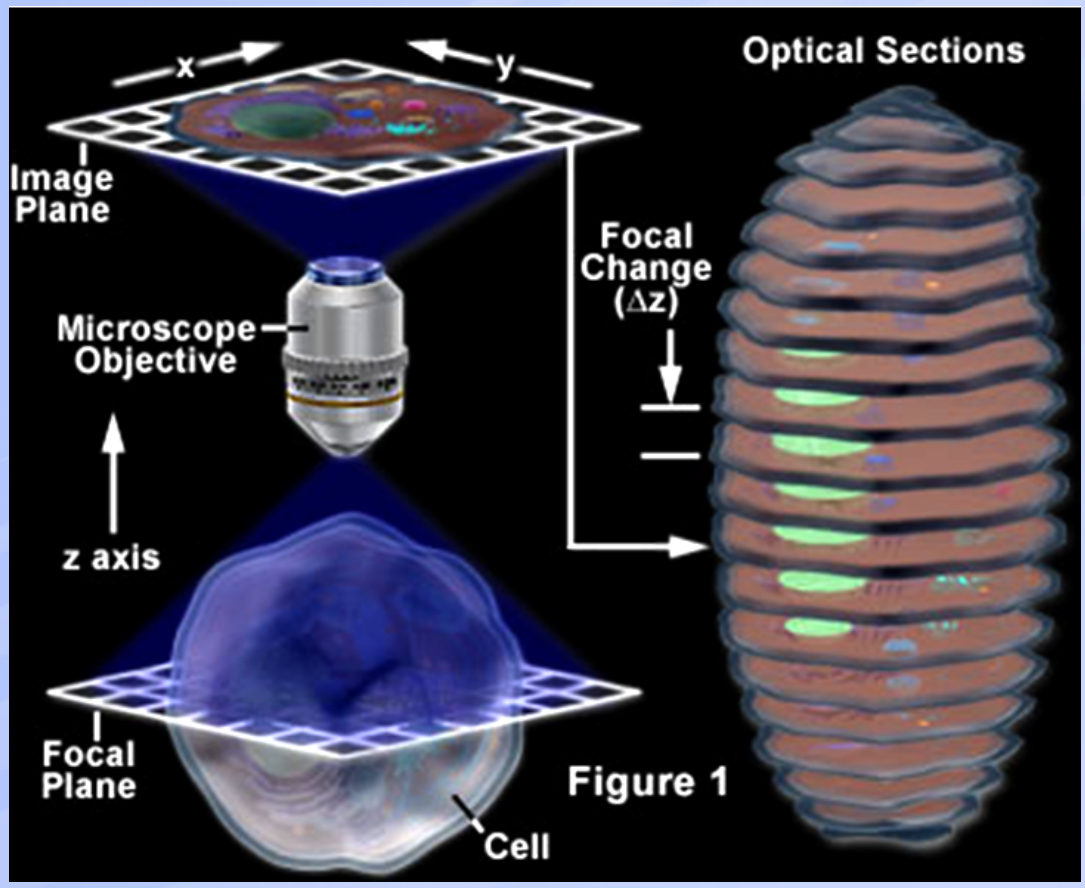
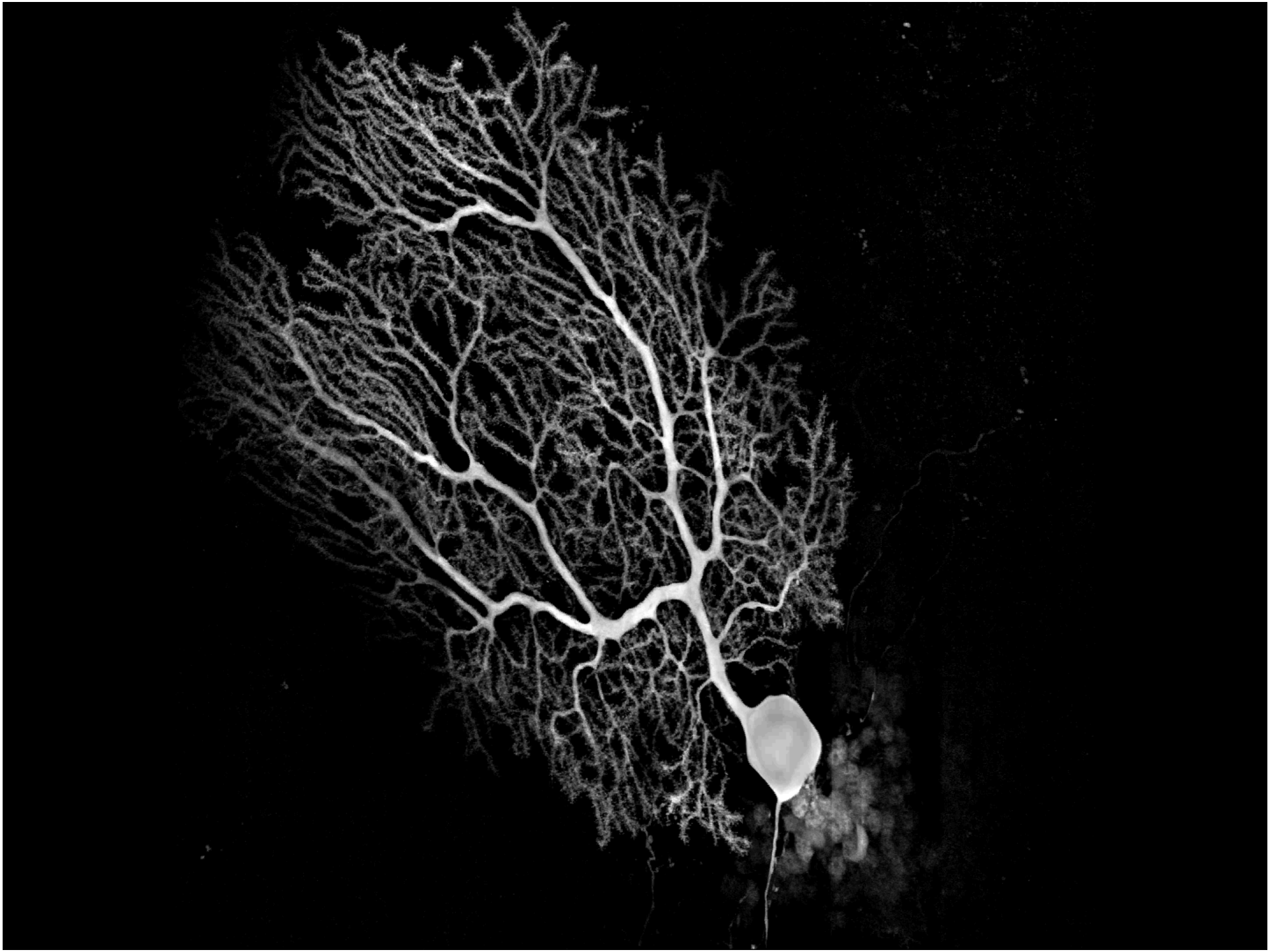
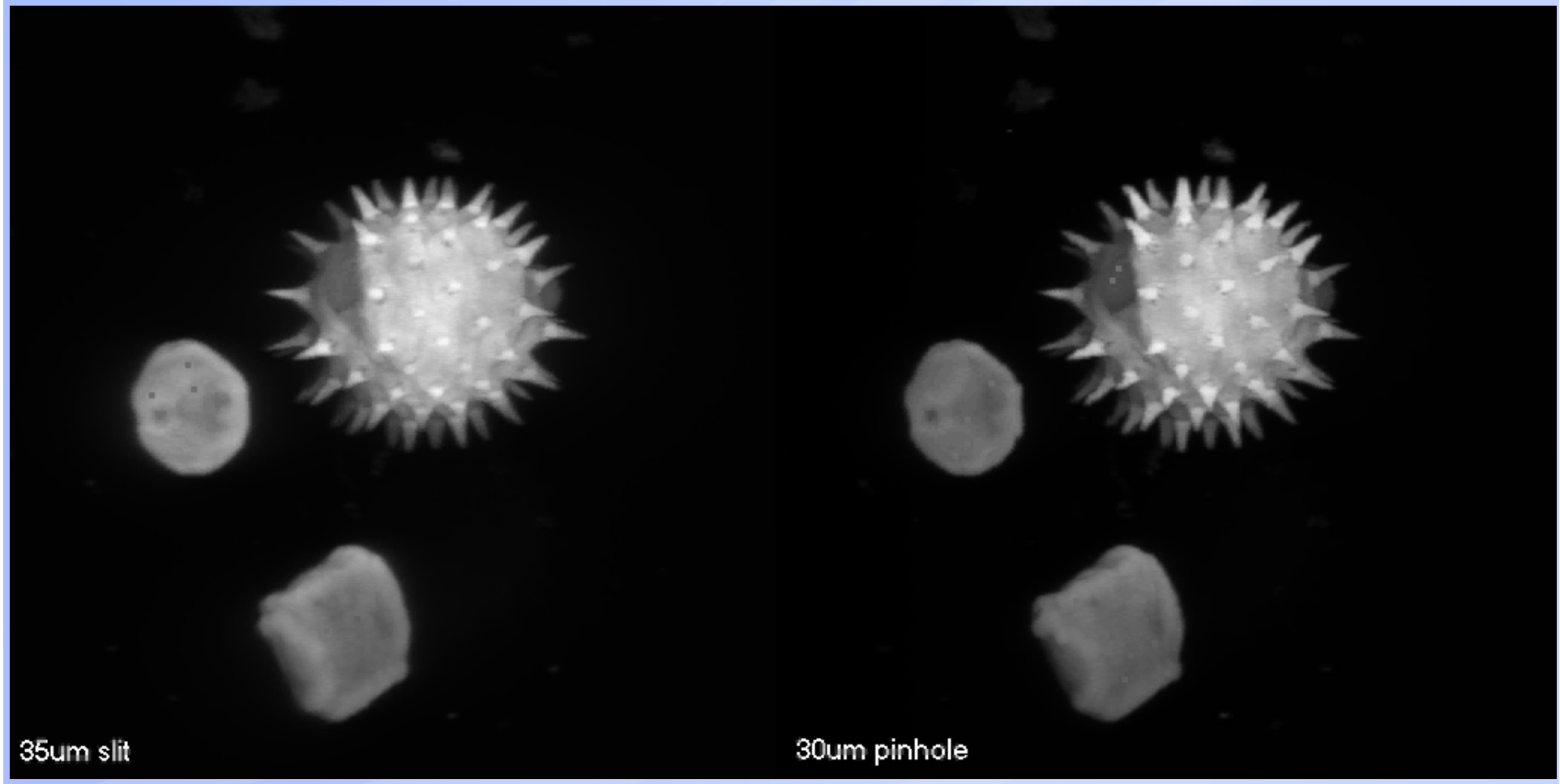
3D reconstruction



Once the “Z-stack” has been collected and sent to the computer, Sections of the stack can be reconstructed into a 3D volume.





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- Confocal Microscopy In Principle
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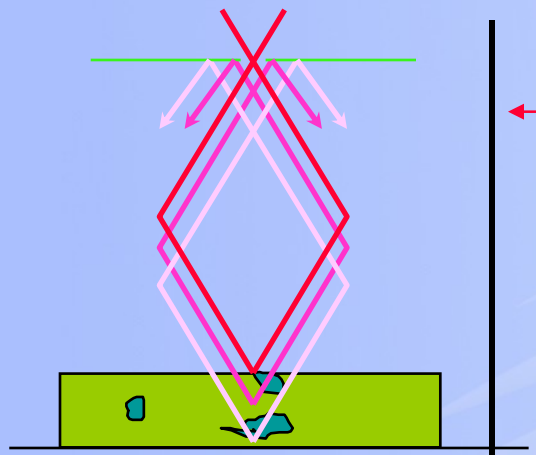
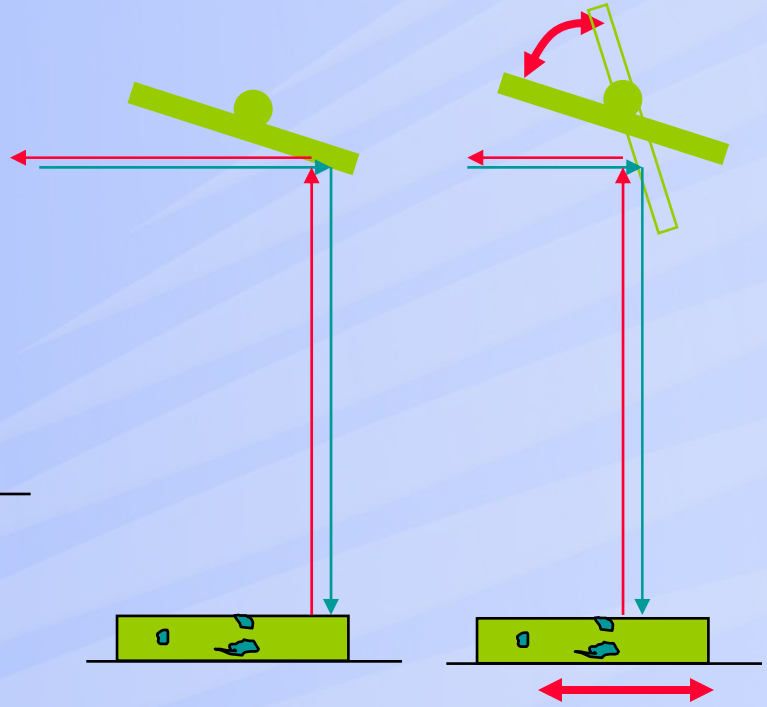
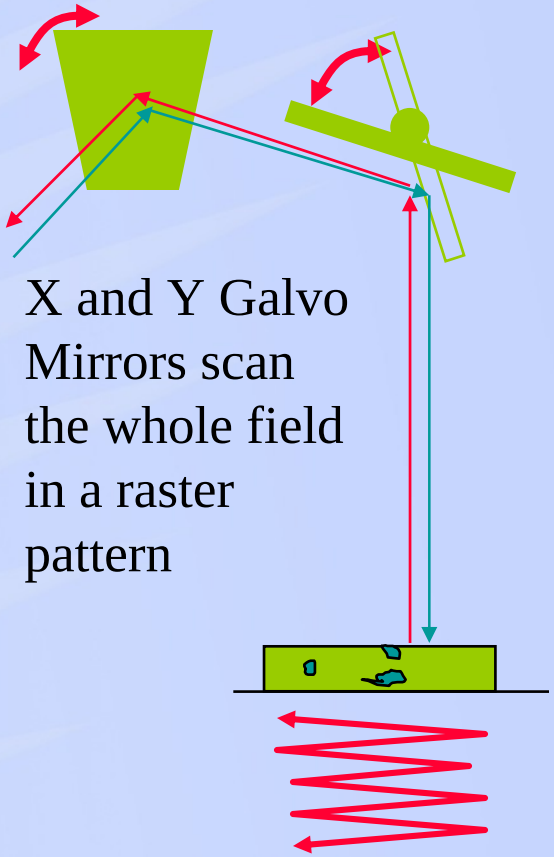
How a Point Scanner Works



The pinhole will only provide information about a single point in space. Therefore move specimen or move the exciting light





X and Y Galvo Mirrors scan the whole field in a raster pattern

- Note that the mirrors also descan the returning light
- Many systems have been developed to control the scan mirrors
- Also motorize Z-axis to sample points in vertical axis

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Commonly used Lasers

• Argon	Ar	457, 488, 514 nm
• Krypton-Ar	Kr-Ar	488, 568, 647 nm
• Helium-Neon	He-Ne	lots single line sources 543, 594, 633 nm
• He-Cadmium	He-Cd	325 - 441 nm
• Diodes	350, 405, 442, 473, 488, 491,514, 532, 561,593, 638, more coming!	

Photomultipliers (PMTs)

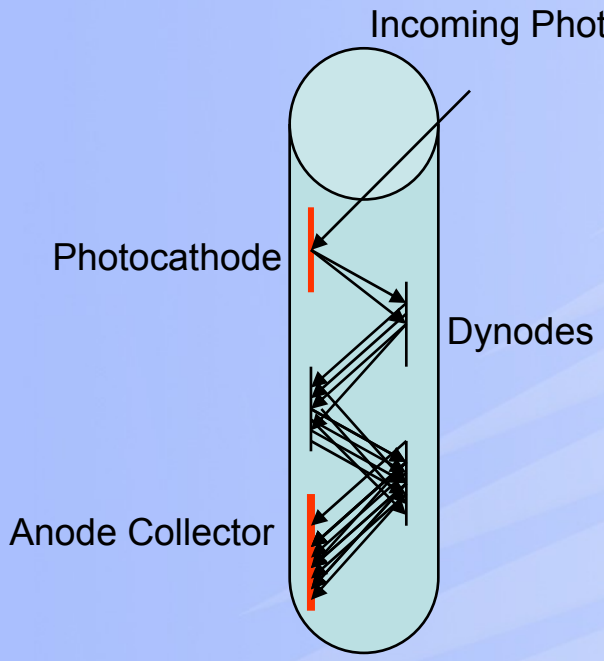
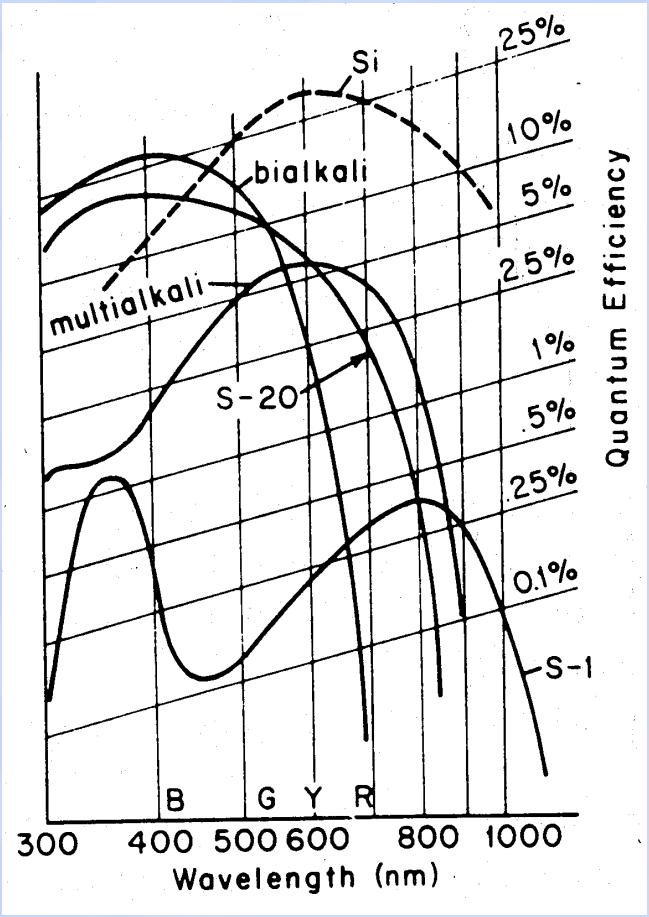


Photo Multiplier Tube

•Maximize the light budget returning from the scan head by intensification.

•Subject to electrical noise to much gain will degrade image and demand excessive averaging



PMTs must be matched with detection wavelength

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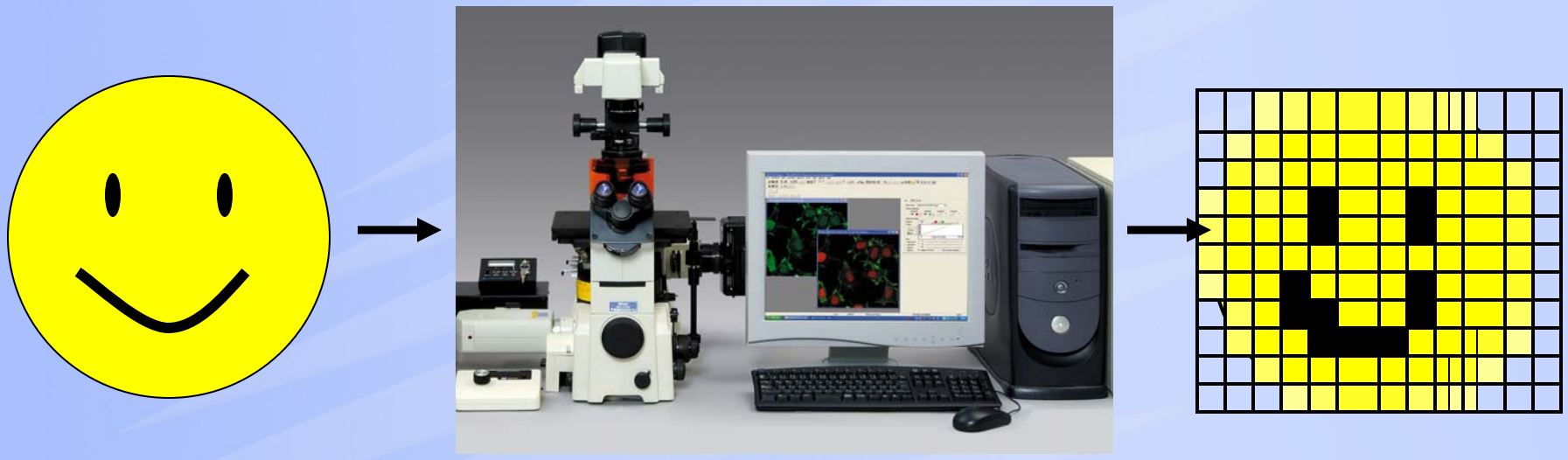
Computer builds digital image from scanned data



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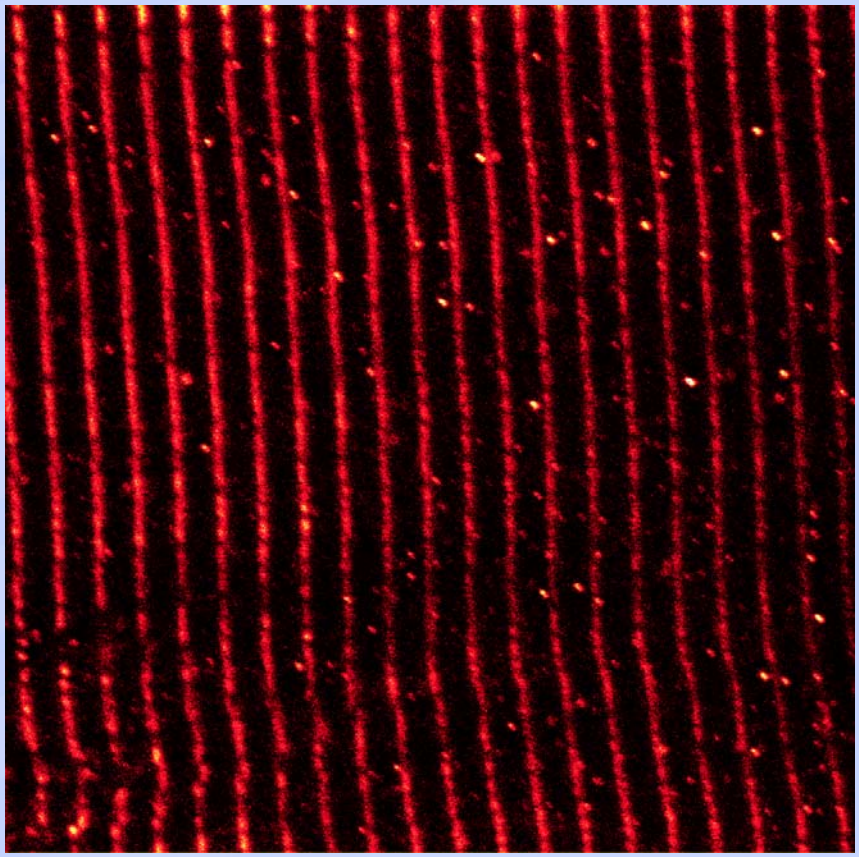
Due to PMT Noise, Averaging is commonly used to enhance S/N

1 frame



8 frames

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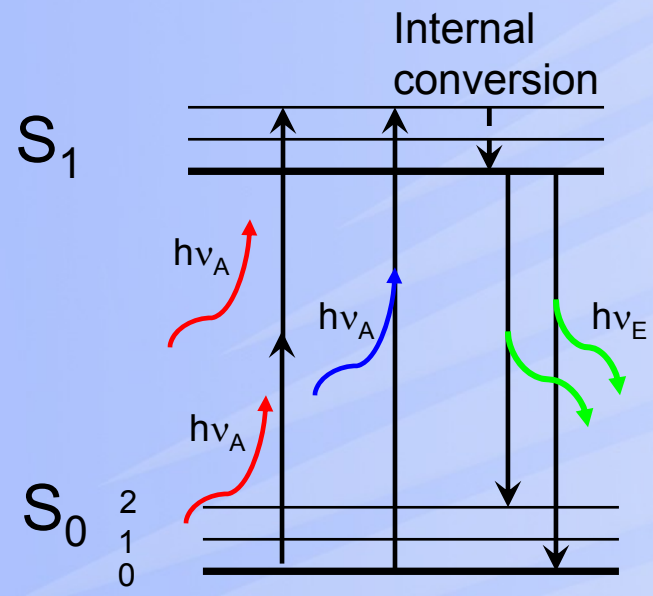
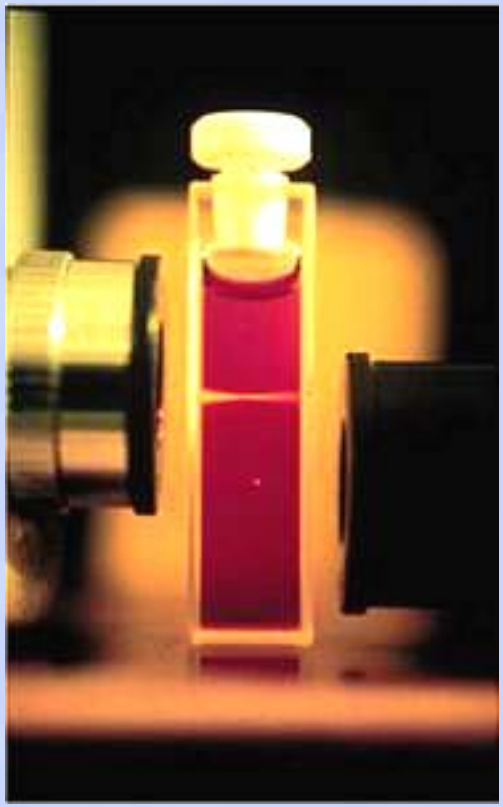
Multi-photon excitation





Brad Amos, MRC, Cambridge

Multi-photon excitation does not excite out-of-focus light, so you can get rid of pinhole

- Confocal Microscopy In Principle
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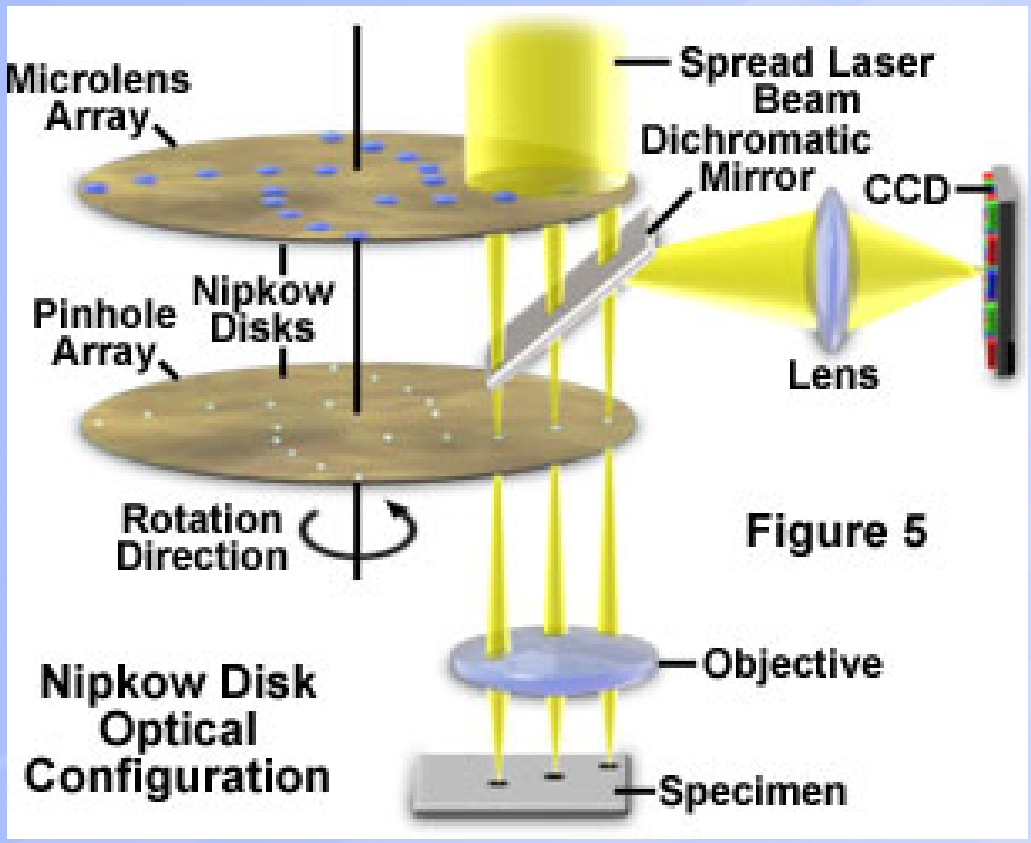
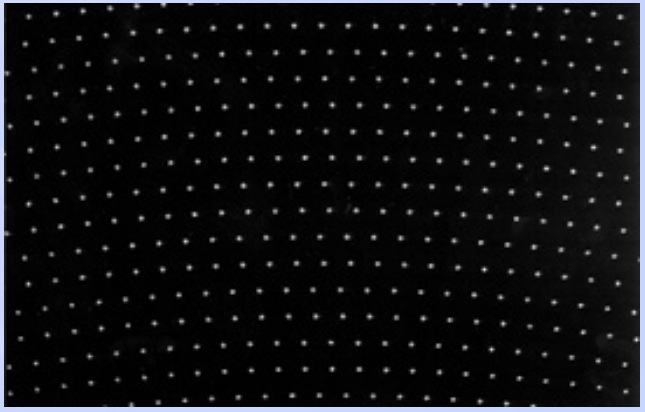
Spinning Disc Confocal





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- Confocal Microscopy In Principle
- Point Scanning Confocal
- Spinning Disk Confocal
- Swept Field Confocal
- Total Internal Reflection Fluorescence (TIRF)

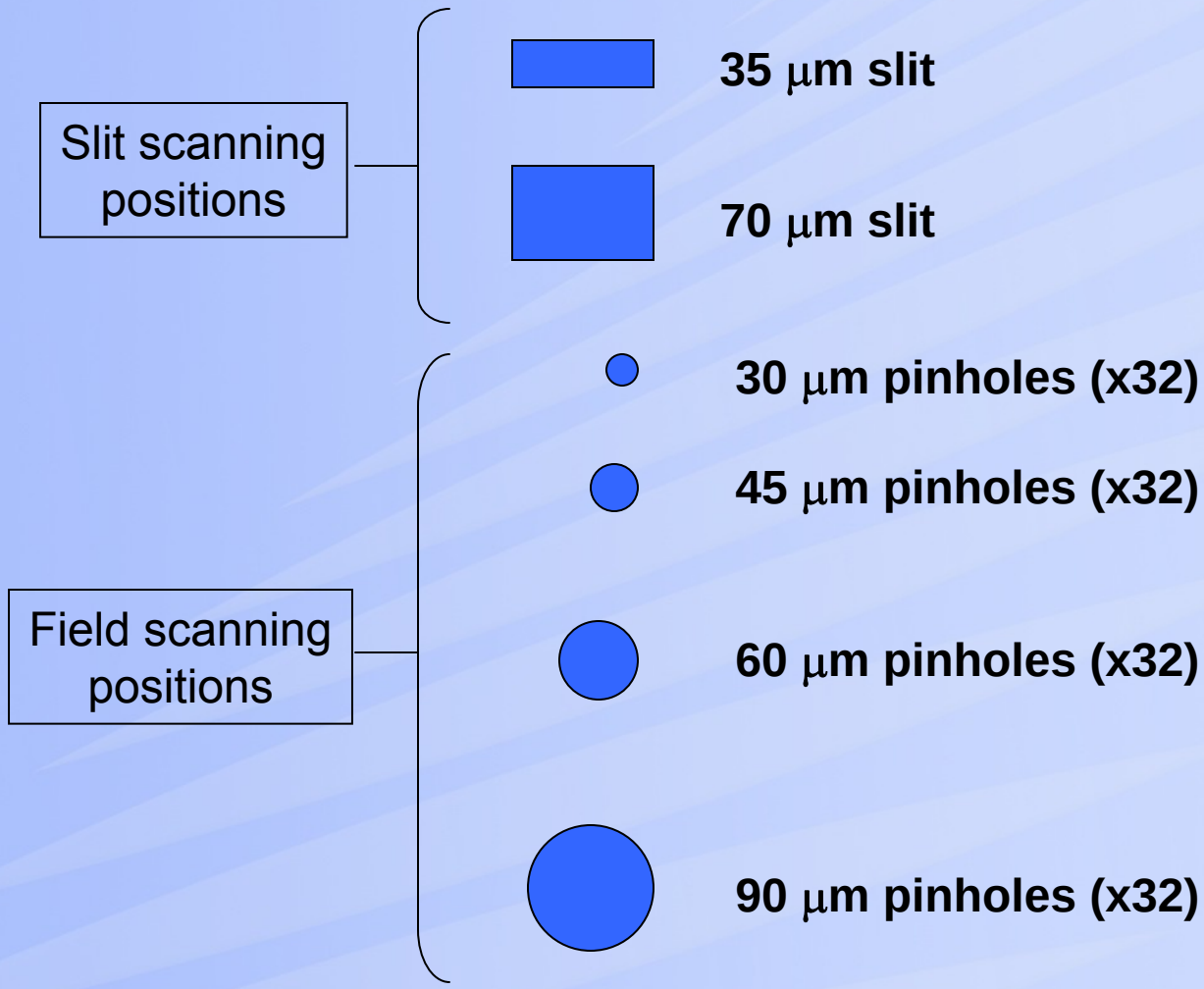
SFC Design Overview

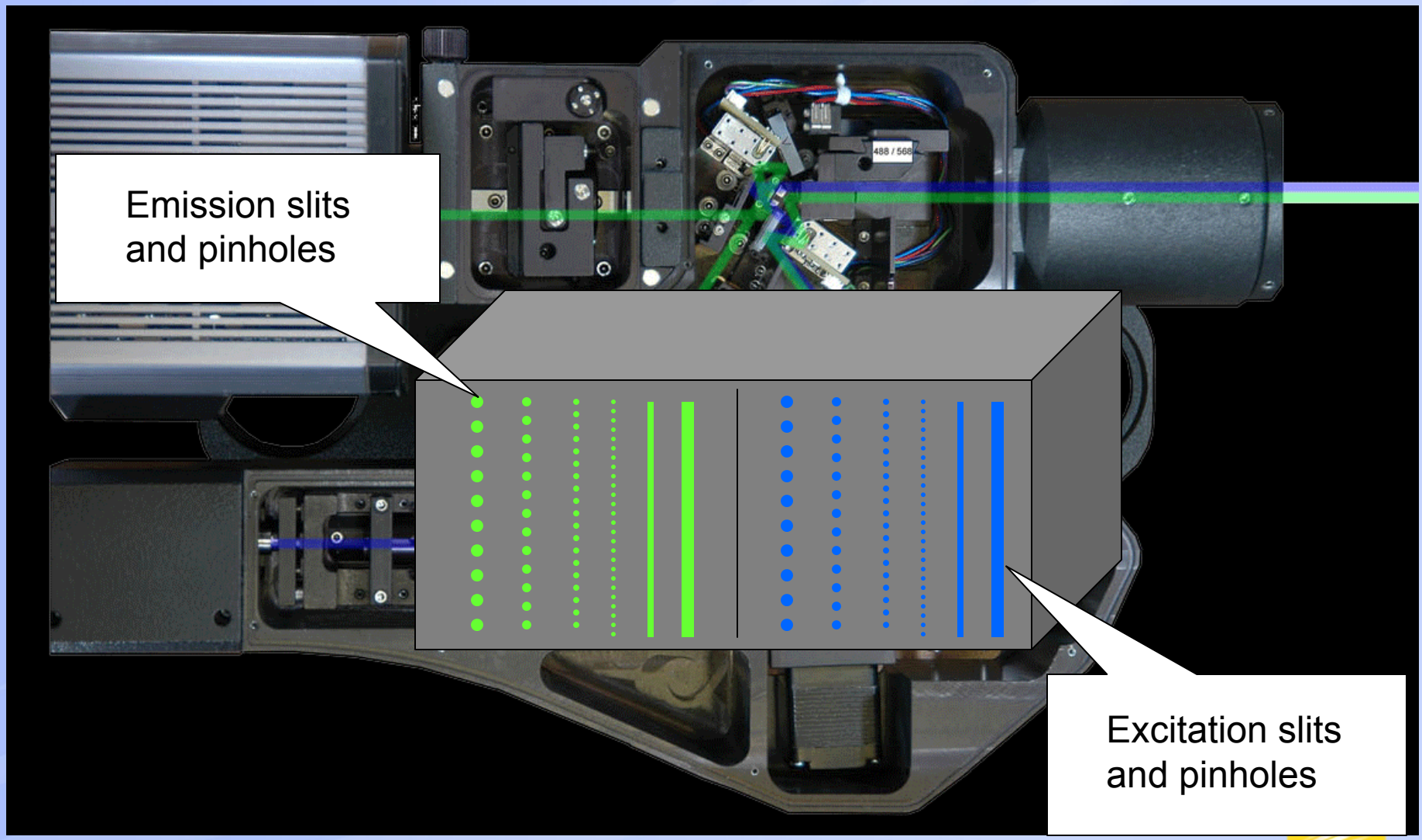
- Supravideo rate, high frequency slit scanner.
- Variable pinhole, linear array field scanner.
- CCD camera-based detector.
- Linear scanning at high frequency minimizes phototoxicity.

SFC Design Overview, cont'd.

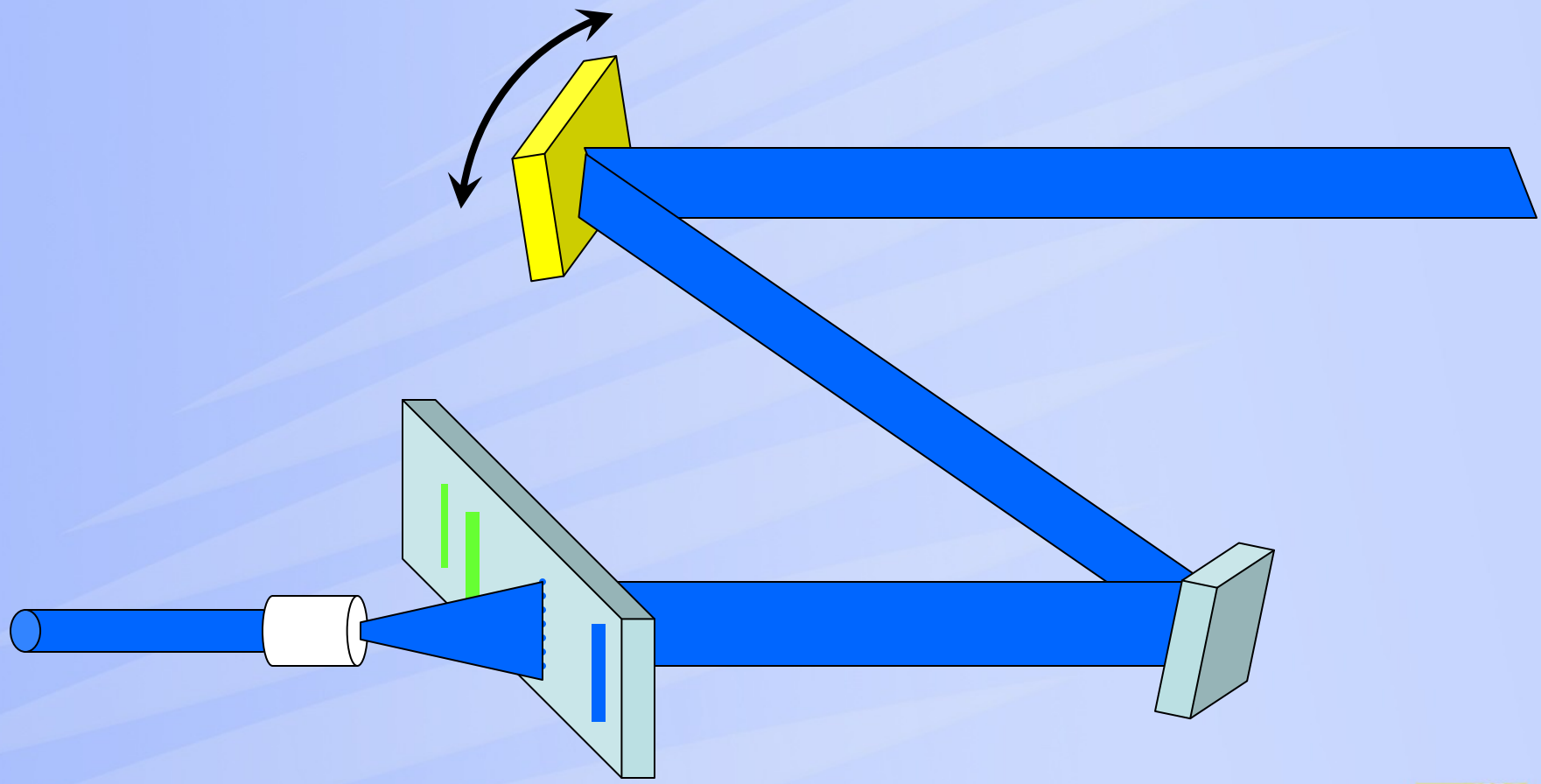
- Variable pinhole sizes match various objective lenses.
- Linear pinhole array (1-D) and separate excitation and emission light path minimize emission crosstalk.

SFC Aperture Positions




Emission slits
and pinholes
488 / 568
Excitation slits
and pinholes

Laser beam is shaped into a line, then scanned



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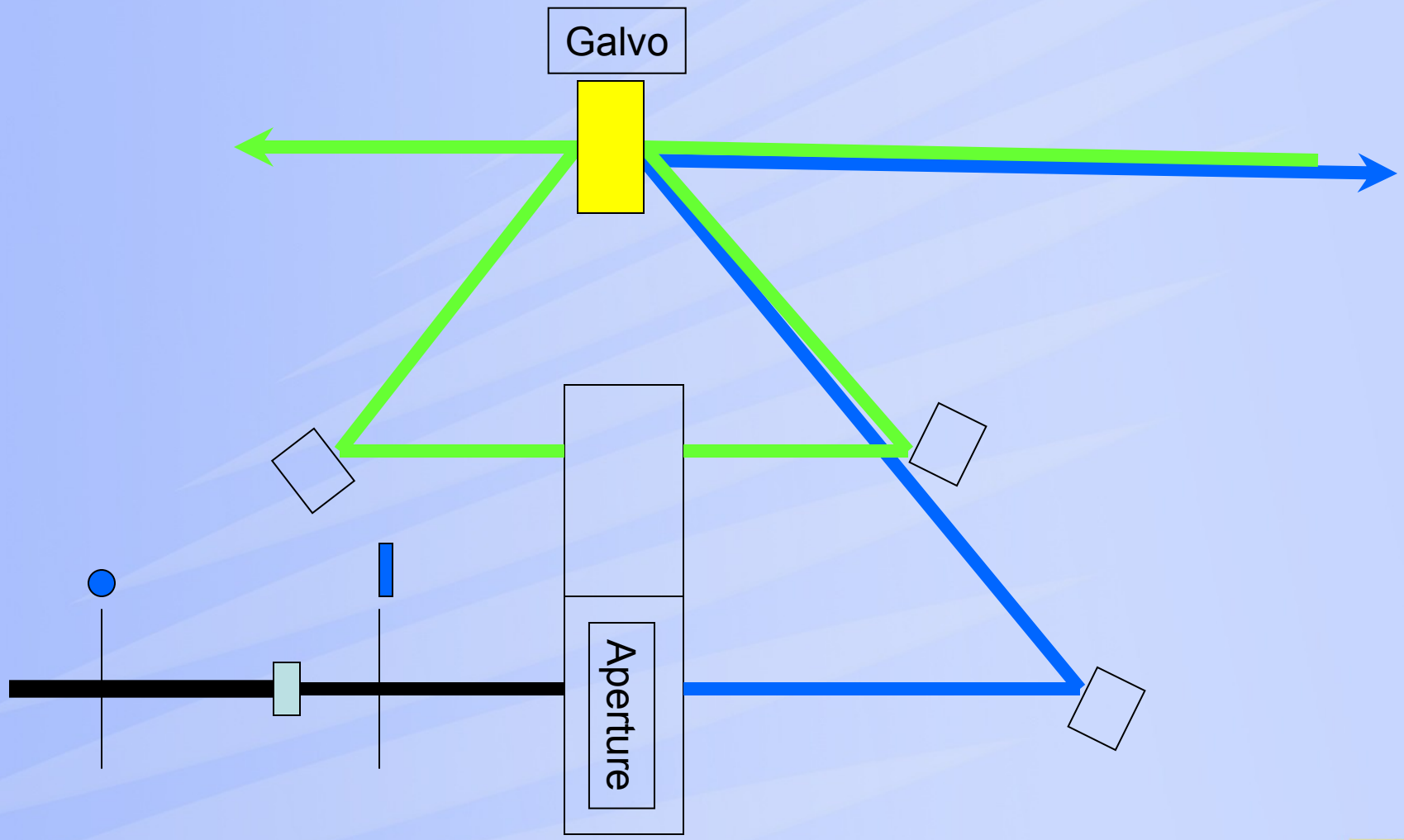
Slit scanner lightpath (simplified)



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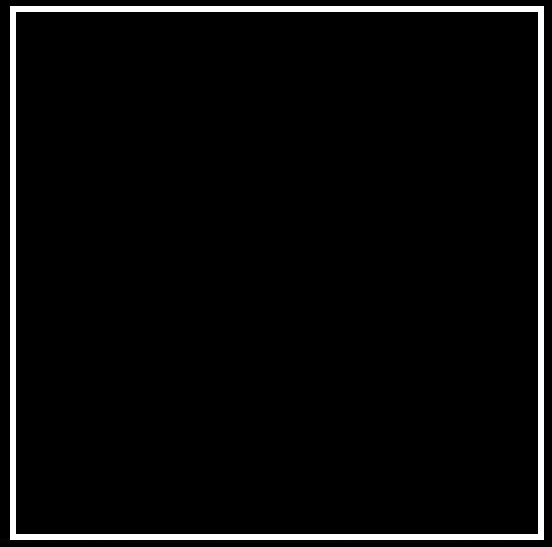
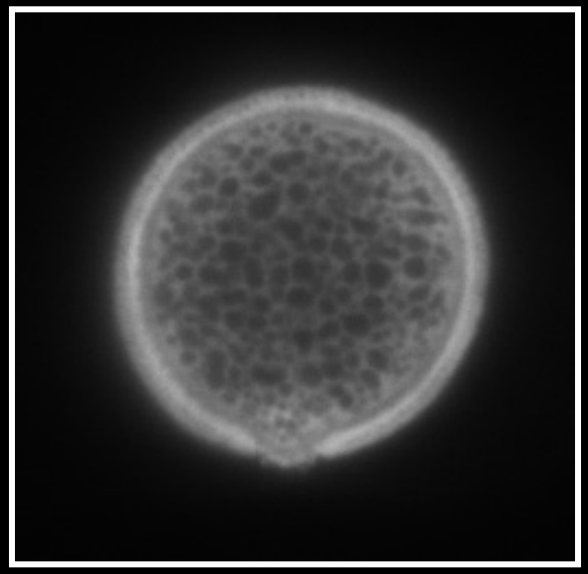
Galvo “Sweep”

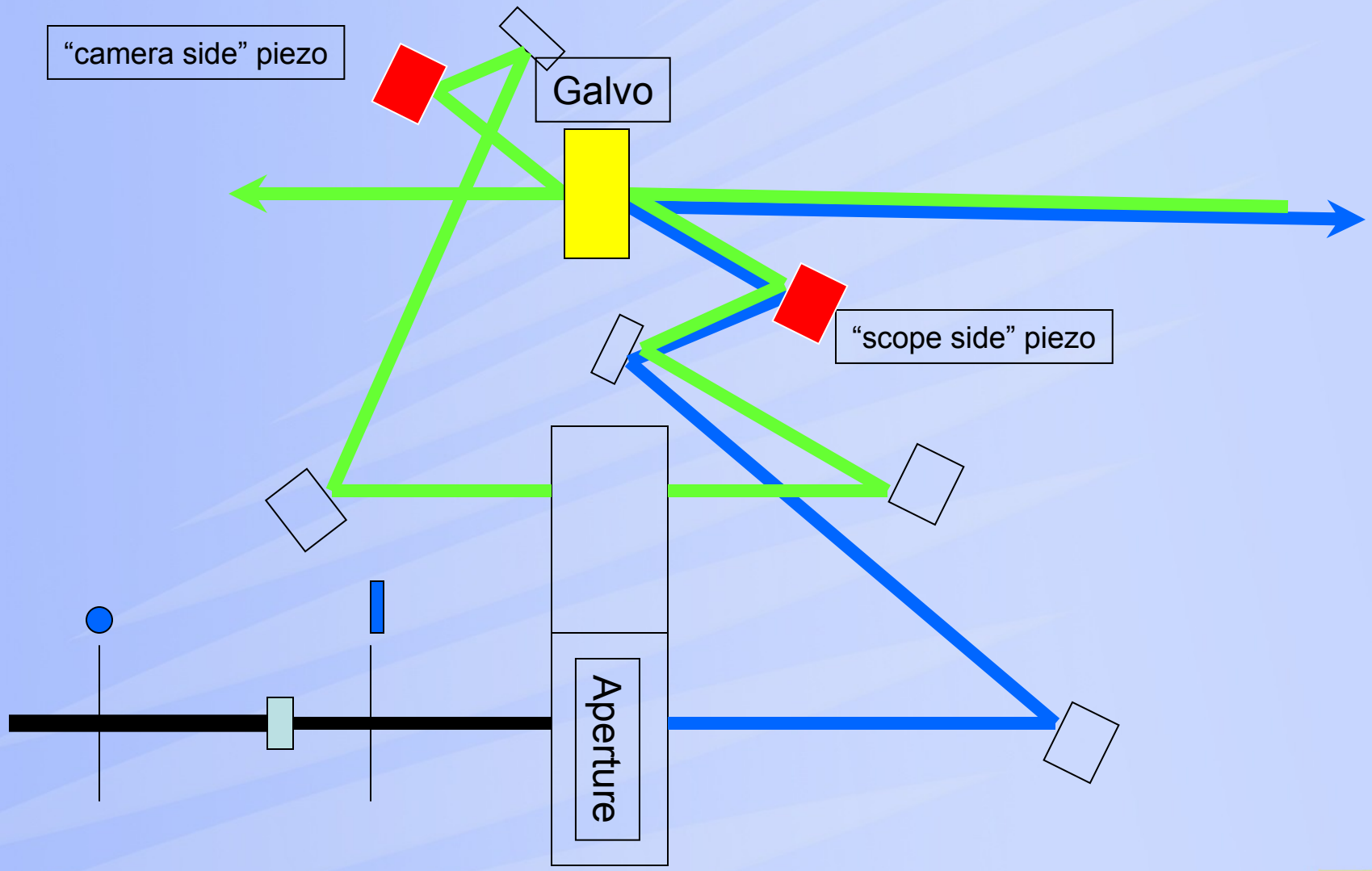


Low frequency
galvo sweep



High frequency
galvo sweep

Field Scanner lightpath (simplified)



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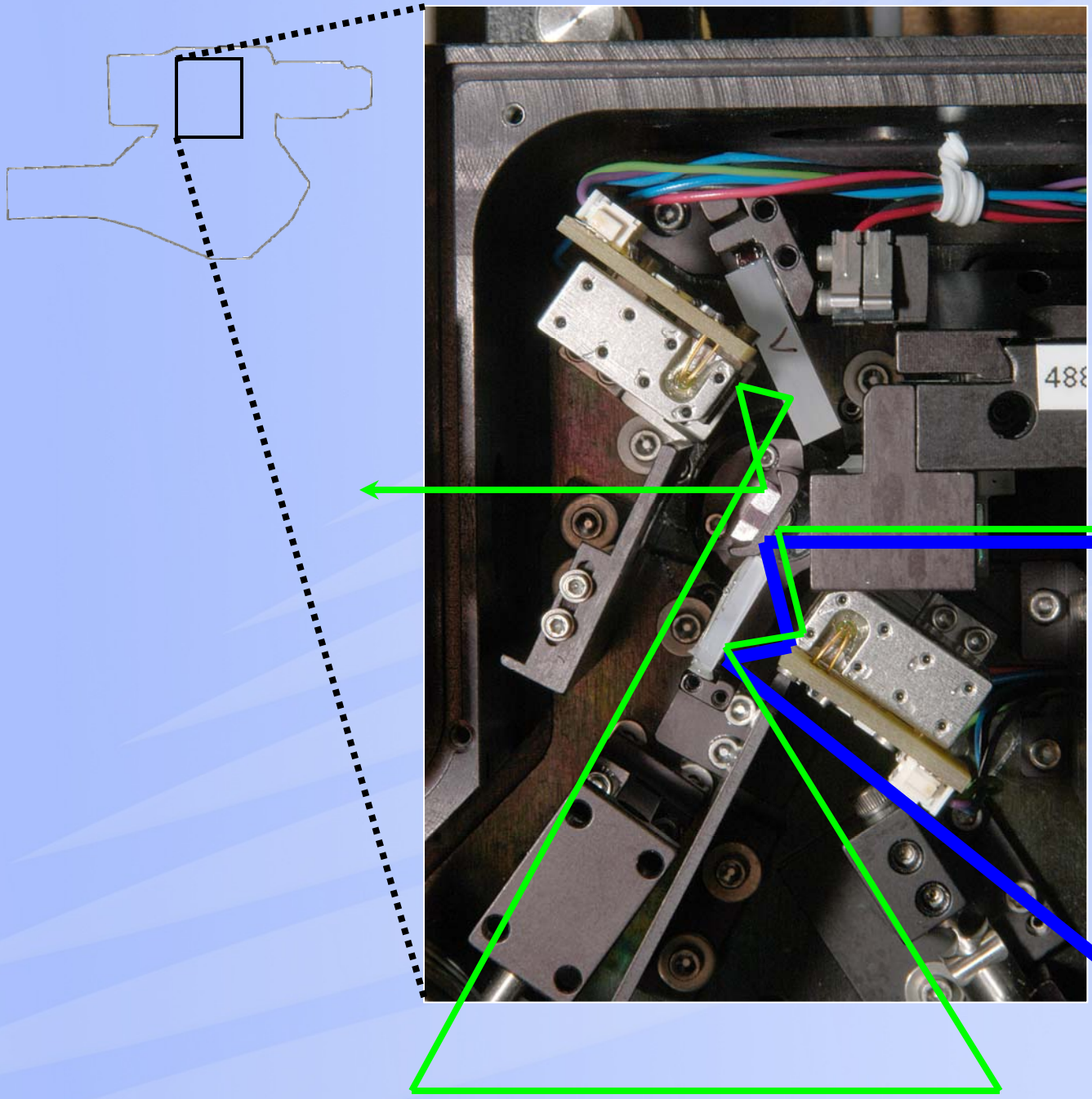

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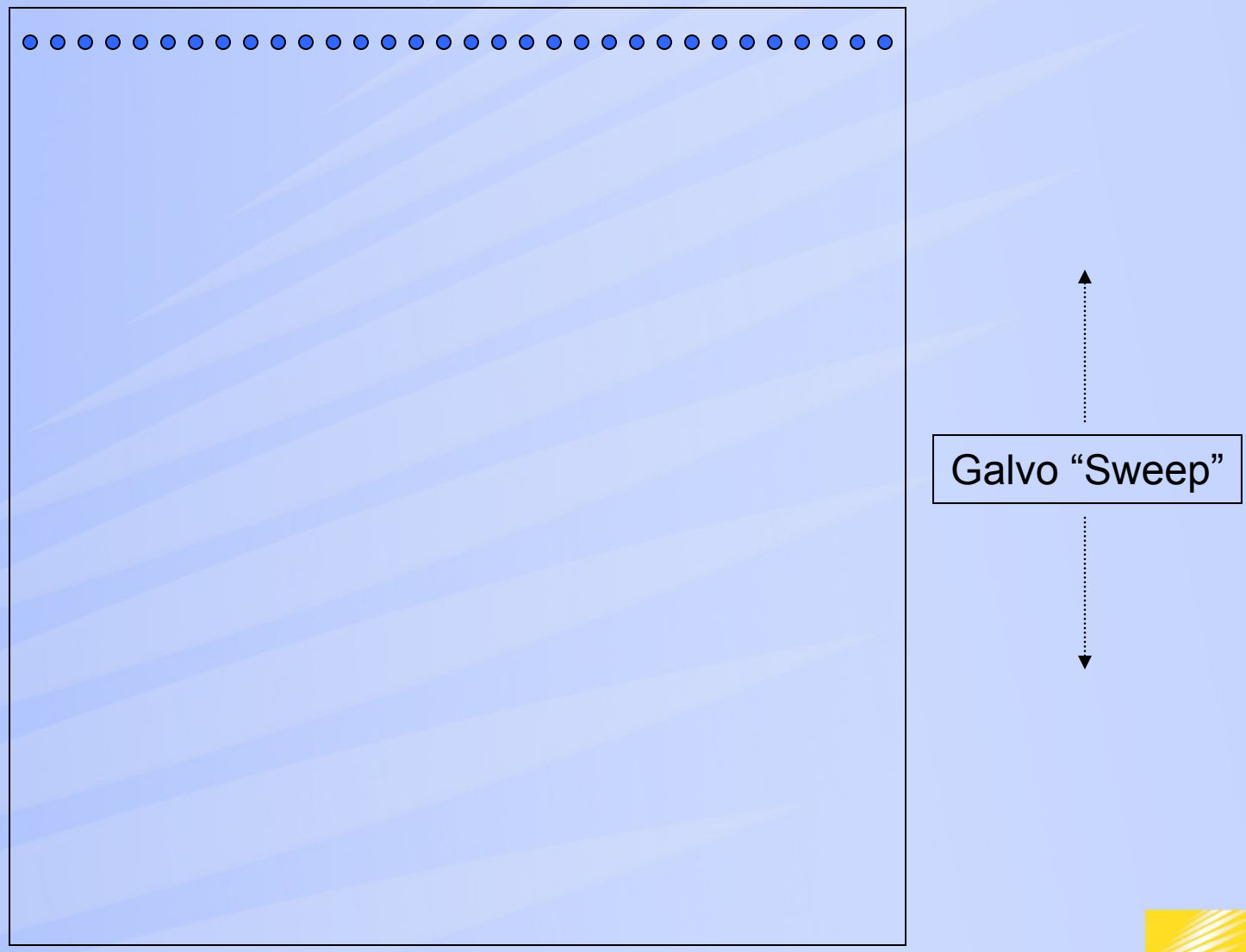
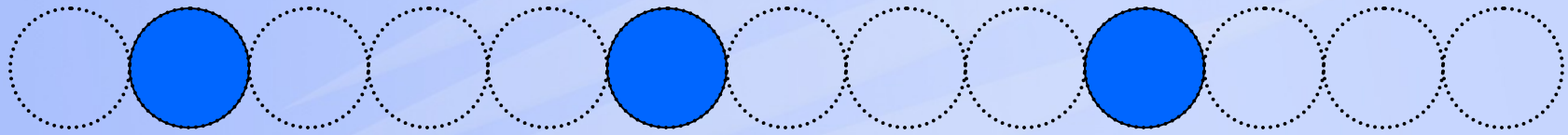
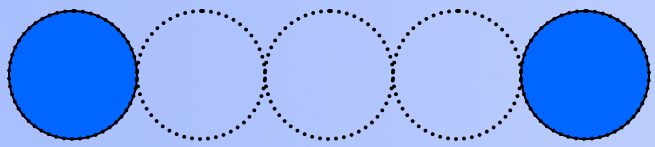

Galvo “Sweep”

Piezo movement of Pinhole image

CCD Frame #1 (or odd #s)



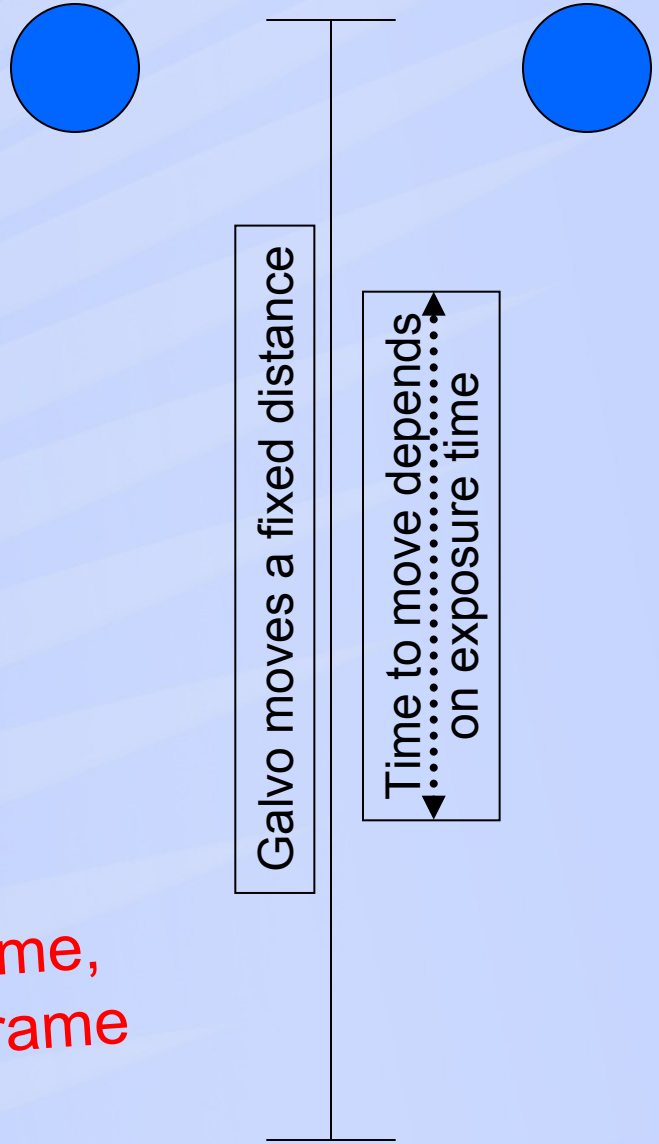
CCD Frame #2 (or even #s)



Piezos move a fixed distance

Time to move depends on exposure time

It takes 2 frames for one complete “piezo sweep”

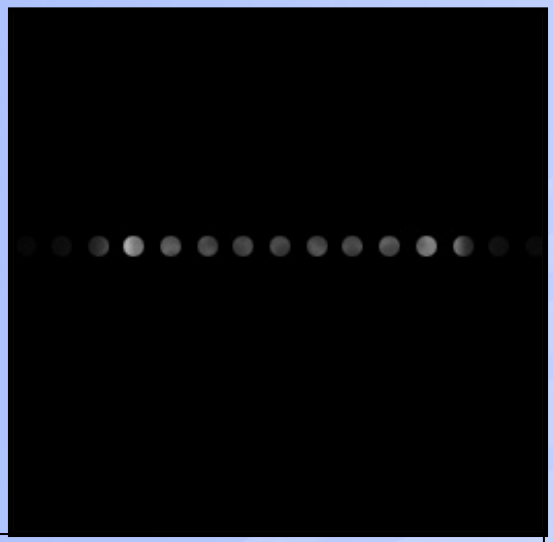


Galvo sweeps **multiple** cycles/frame, and piezos sweep only **once** per frame

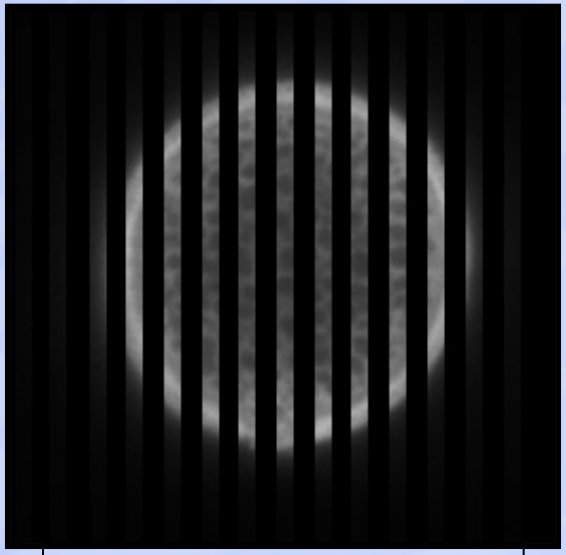
Galvo cycles multiple times / frame

Field-scanning combines multiple galvo sweeps
with bidirectional piezo movement

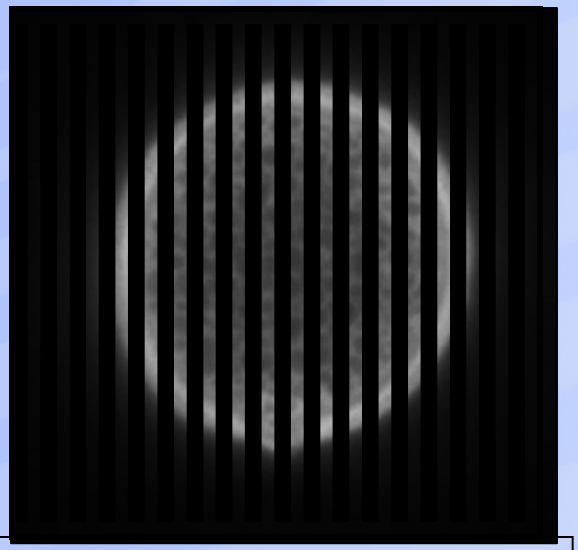

Piezo step
Galvo “Sweep”



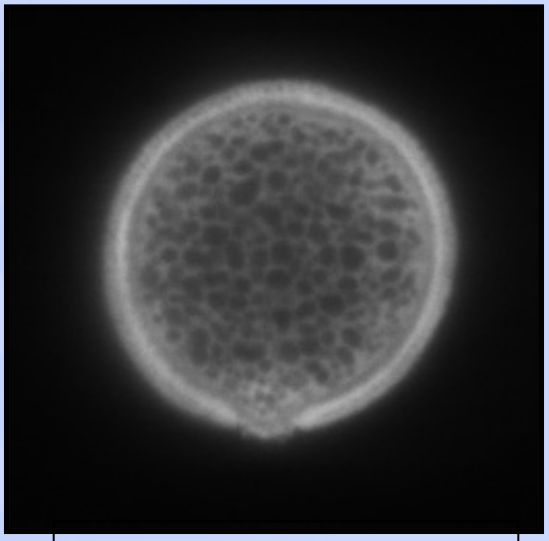
Galvo, Piezos stationary



Sweeping galvo only



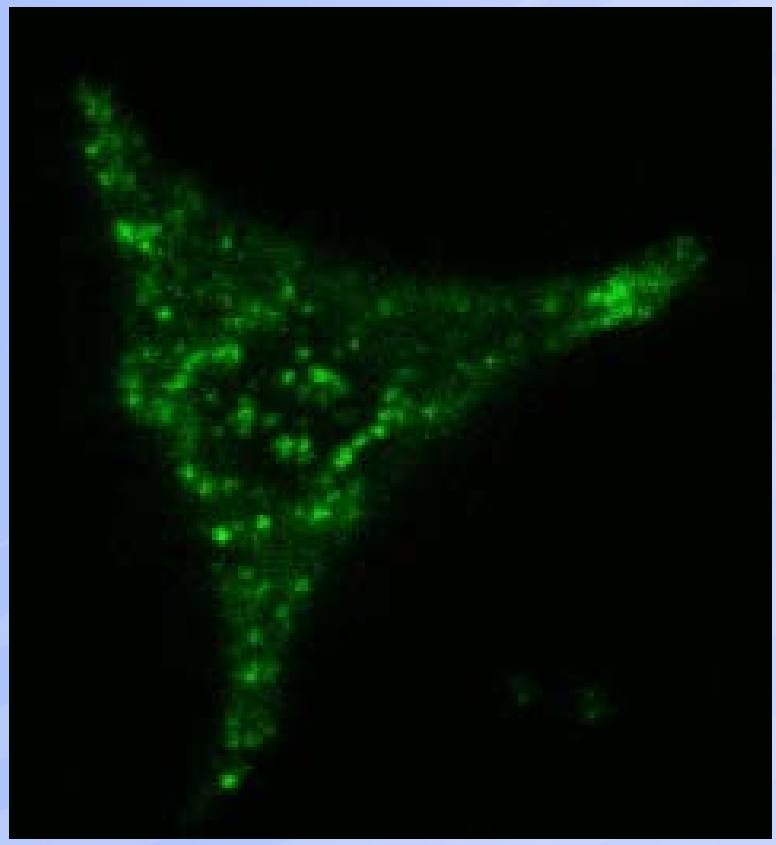
Sweeping galvo & piezos



Piezo sweep synch with frame rate

Performance

- Slit scan imaging to 1000Hz with appropriate detector.
- Pinhole scan imaging to 100Hz.
- Axial resolution from ~ 0.4μm to 1.2μm over 6 scanning modes.



10 minute timelapse (35um slit) Beta cell with eGFP labeled insulin.

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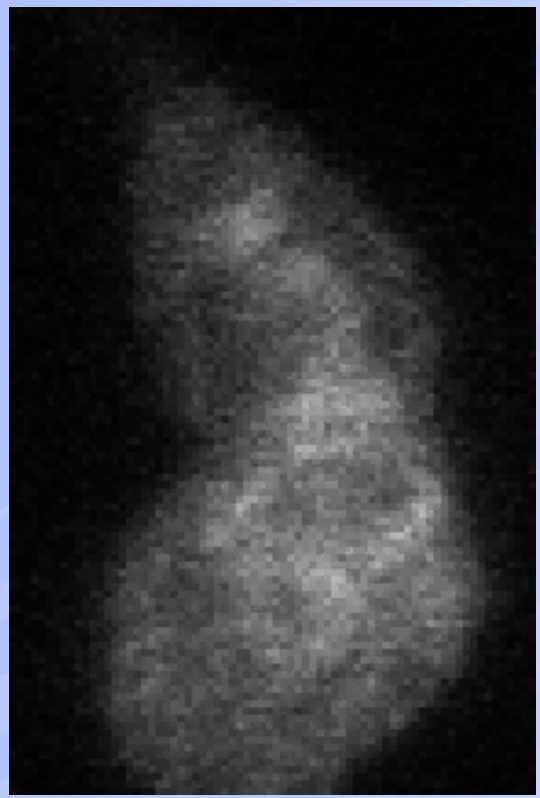
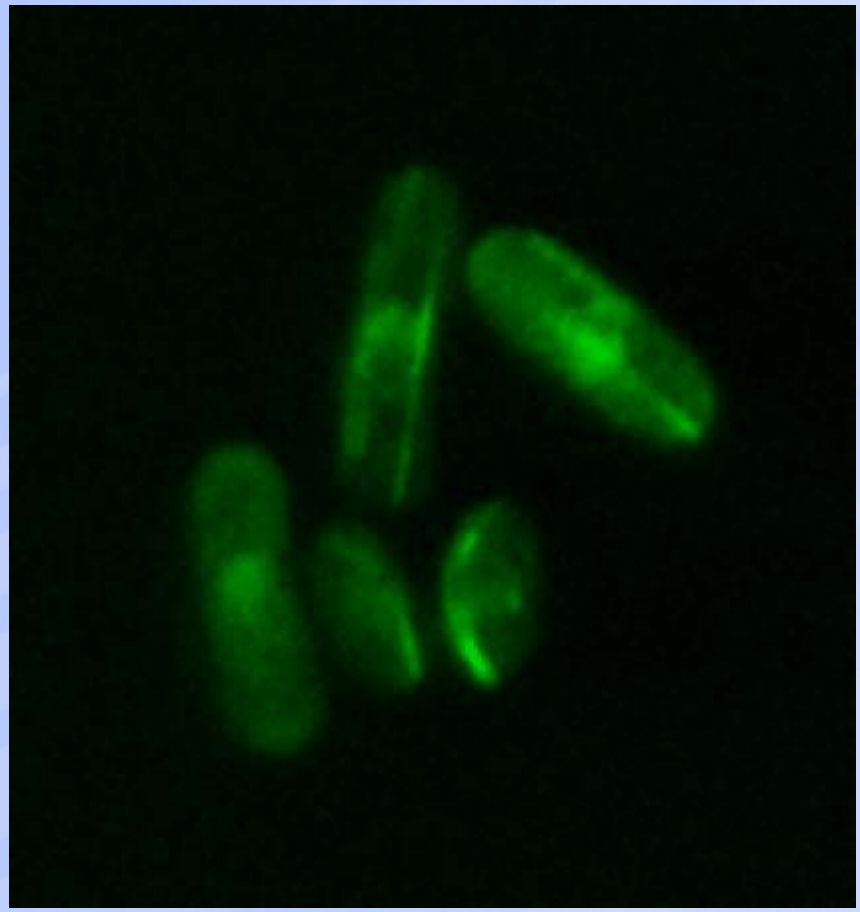


Calcium sparks at 80 FPS in smooth muscle cells (Fluo4)



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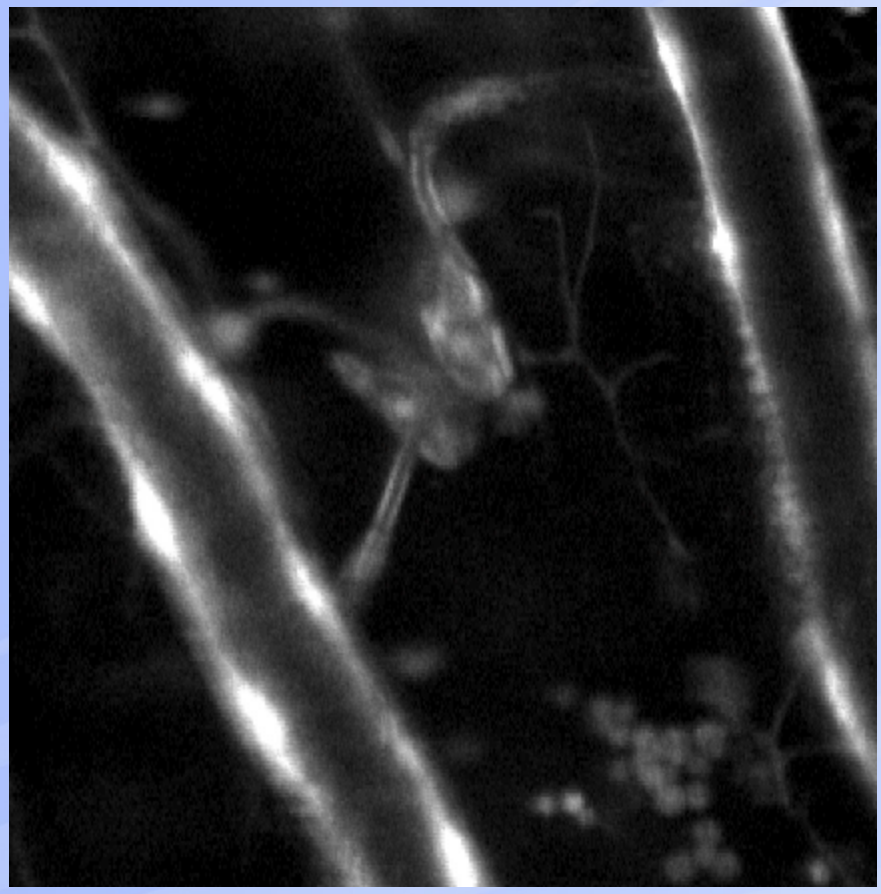


Drosophila Embryo Heart Beating 50 FPS, GFP expressed in smooth muscle.

- **Confocal Microscopy In Principle**
- **Point Scanning Confocal**
- **Spinning Disk Confocal**
- **Swept Field Confocal**
- **Total Internal Reflection Fluorescence (TIRF)**

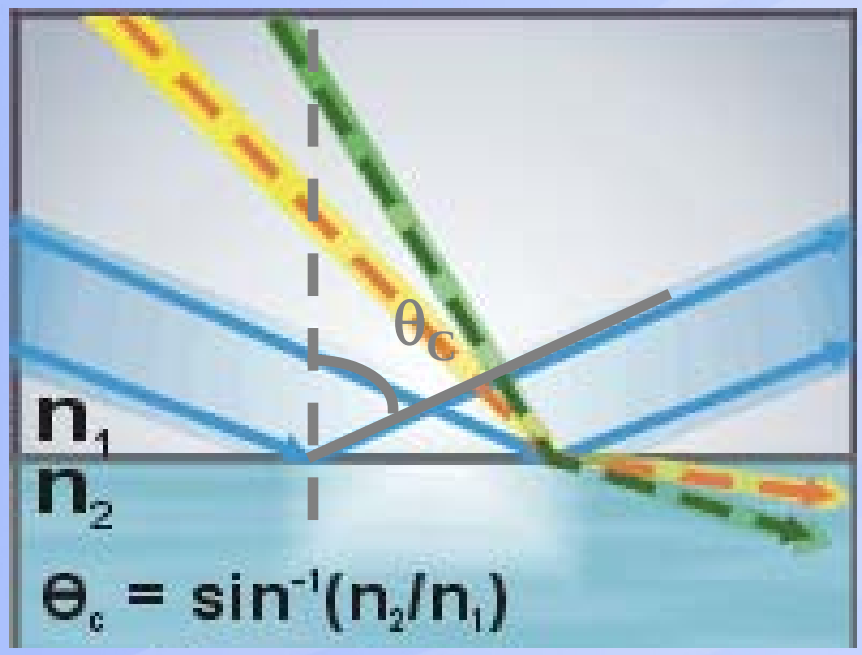
What is Evanescent Wave or Total Internal Reflection Fluorescence (TIRF) Microscopy?

- **An optical phenomenon that can occur when light strikes the interface between two media of different refractive indices**

- **When light is totally internally reflected it does not propagate through the sample, but causes an electromagnetic field (termed the evanescent wave) that extends in the z-direction**

- **The evanescent wave intensity decreases exponentially, and extends only a few hundred nanometers into the second media**

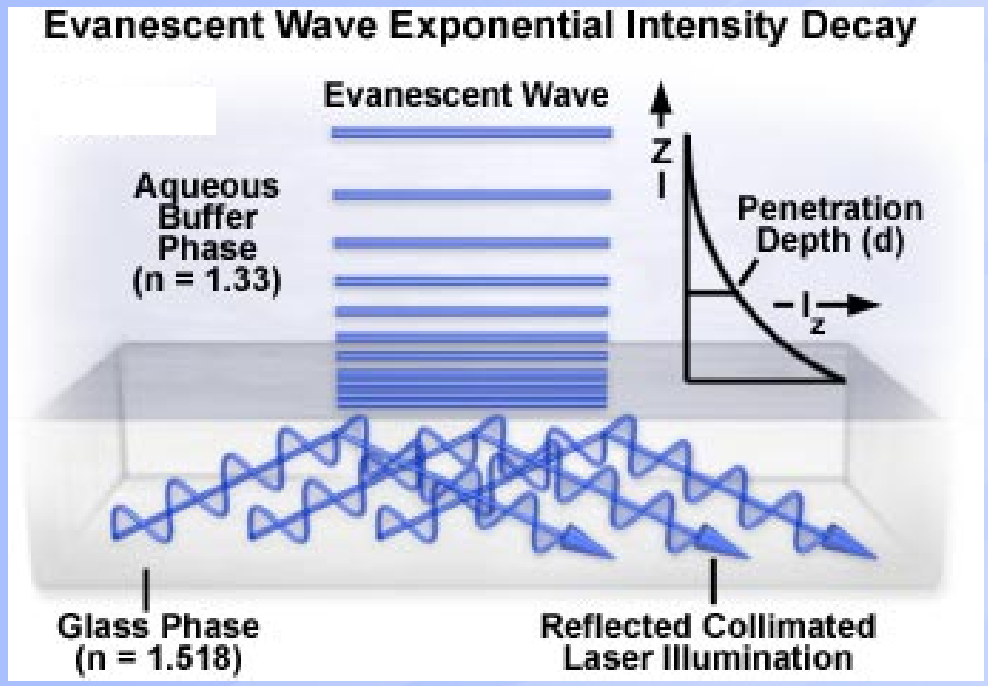
Glass (n=1.515)



Live cells (n=1.33-1.38)

Criterion for TIR

- $N_1 > N_2$
- **The angle of incidence must be ≥ the critical angle (θ_C)**



$$E_z = E_0 \exp(-z/d_p)$$

$$d_p = \frac{\lambda_i}{2\pi n_1 \sqrt{\sin^2\theta_i - (n_2/n_1)^2}}$$

- The energy of the evanescent wave at any point in z is a function of location in the field and the penetration depth

- The penetration depth of the evanescent wave is a function of the illumination wavelength.

- only 50nm to 100nm is typically useful illumination

Why is this useful for fluorescence microscopy?

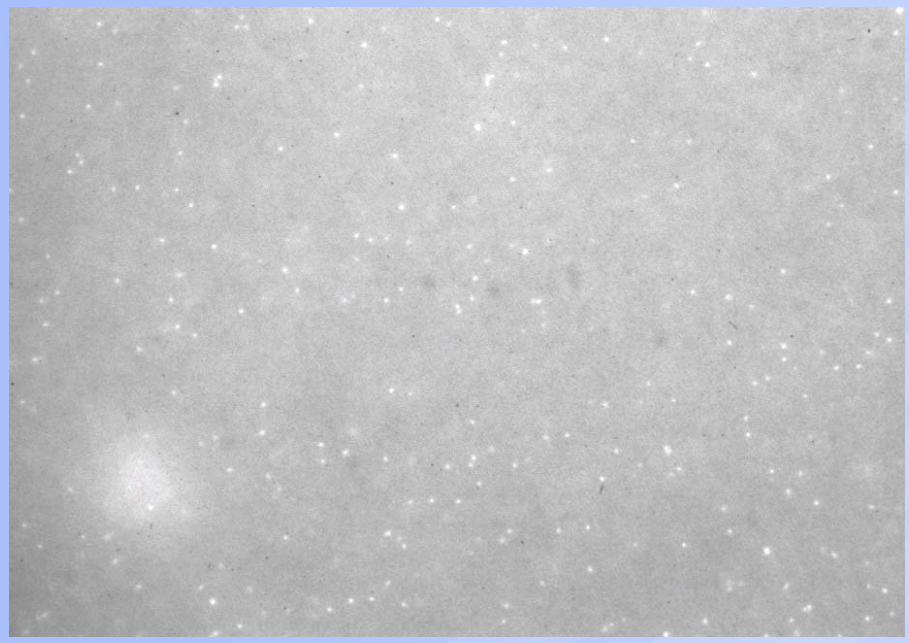
- **The evanescent wave will typically only excite fluorophores within 50nm-80nm of the cover slip.**

- **Excitation of this small proportion of fluorophores in the thin optical section results in an extremely large increase in the S/N**

- **As a result of the increase in S/N very small signals, such as single molecule fluorescence, can be resolved above the noise floor**

TIRF is all about

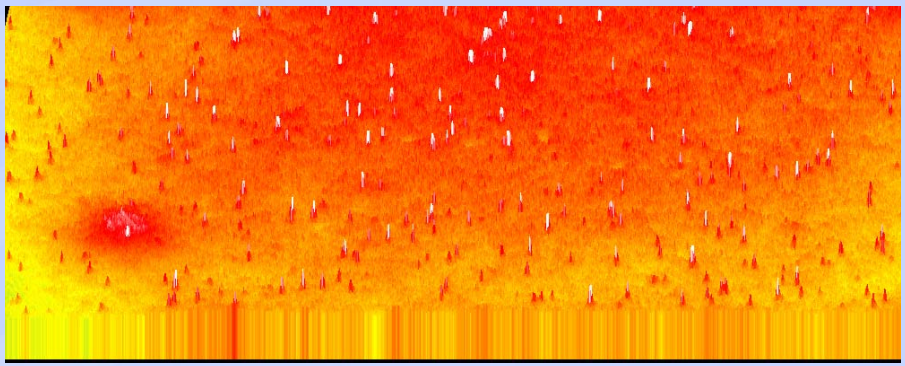
SIGNAL to NOISE!



SIGNAL to NOISE
Epi-fluorescence
=1.3
For this specimen

3D Intensity Histogram of image on the left

Epi-Fl Image
5mm deep solution of 200nm fluorophor coated polystyrene micro-spheres.



Low Intensity (black) → High Intensity (white)

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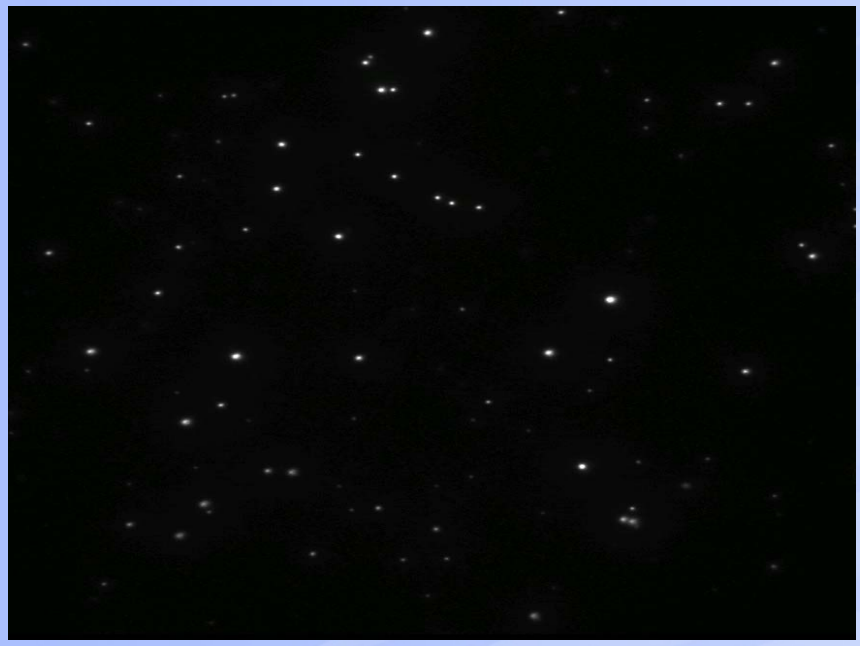
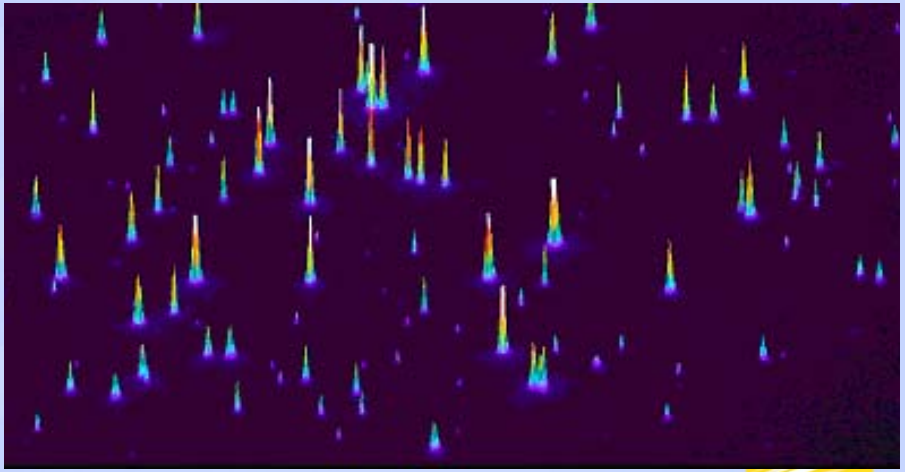


SIGNAL to NOISE
TIRF
=35
For the same specimen

3D Intensity Histogram of image on the left



Low Intensity (black) ➡ High Intensity (white)

TIRF Image

5mm deep solution of 200nm fluorophor coated polystyrene micro-spheres.

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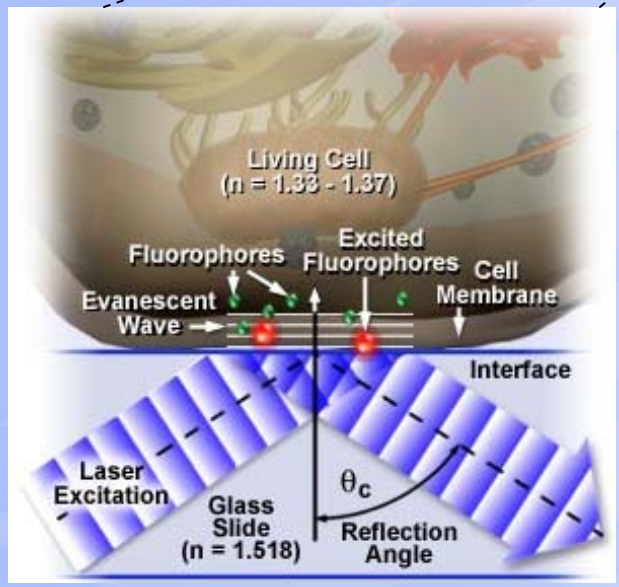
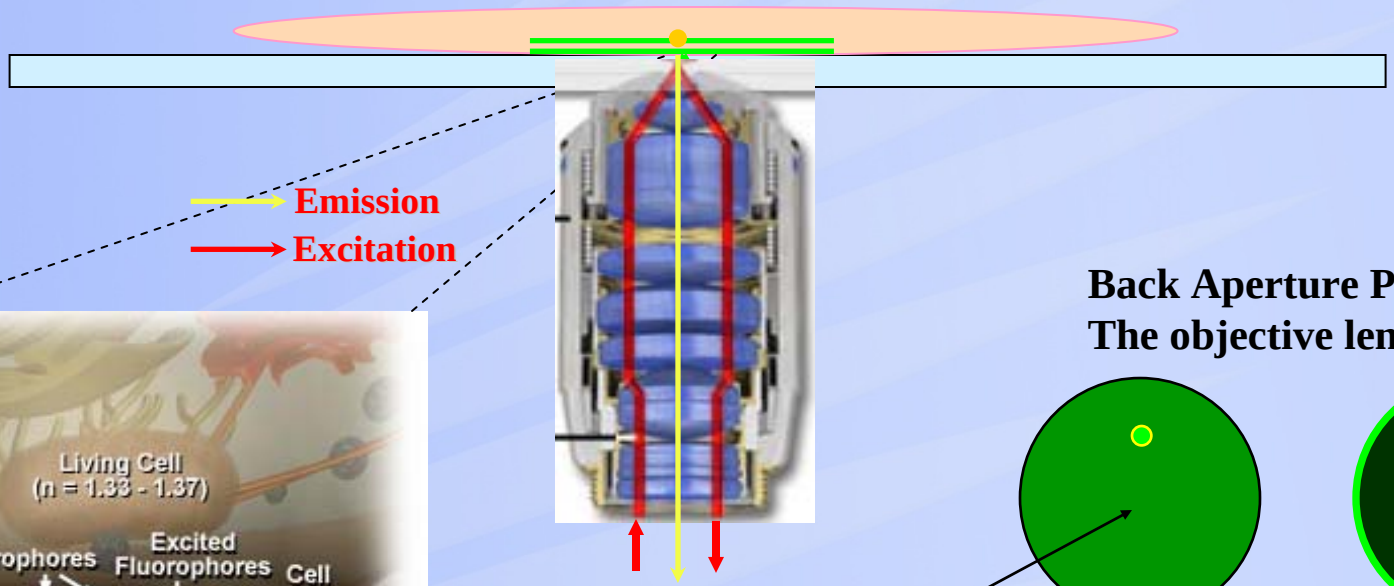
What are some of the applications of TIRF ?

- Endocytosis and Exocytosis
- Dynamics of membrane associated proteins
- Protein arrangement at membranes
- Focal adhesions
- Growth cone migration
- Receptor/Ligand interactions
- Biophysical studies of single molecule behavior

- ???? For you to figure out

Objective lens TIRF Method





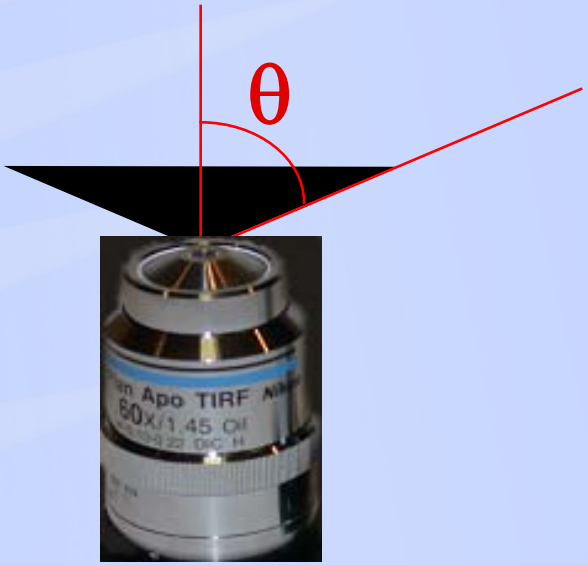
Back Aperture Plane of The objective lens

When a laser is focused at the back aperture plane of the objective in the low NA regions (left) it causes epi-illumination, however when the laser focus is moved to the high NA regions, beyond the n of the specimen., the light is totally internally reflected (right). Consequently, the aperture plane darkens and reveals an "Eclipse" of light at the perimeter.

High NA objective lenses are necessary for Total Internal Reflection, utilizing the lens method, so that light can come out of the lens at angles at or beyond the critical angle for TIR

$$NA = n \sin \theta$$

- **Numerical Aperture**
- **Mounting Media Refractive Index**
- **Angle of the Cone of illumination**



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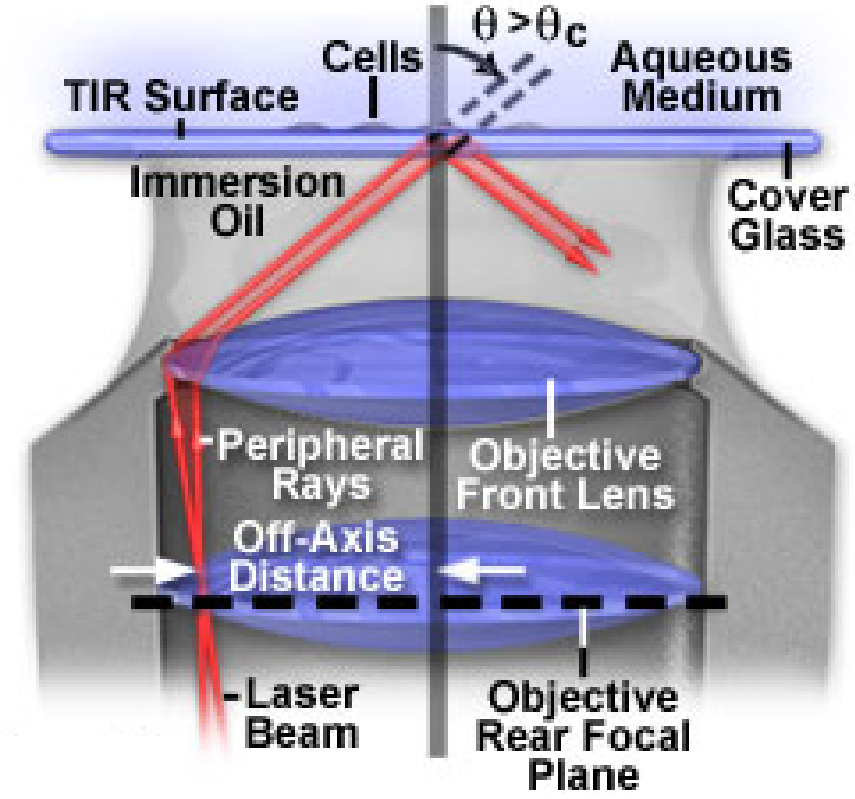
High Numerical Aperture Objective TIRF



Special objective lenses provide the NA Necessary to achieve Total Internal Reflection Through the objective lens.

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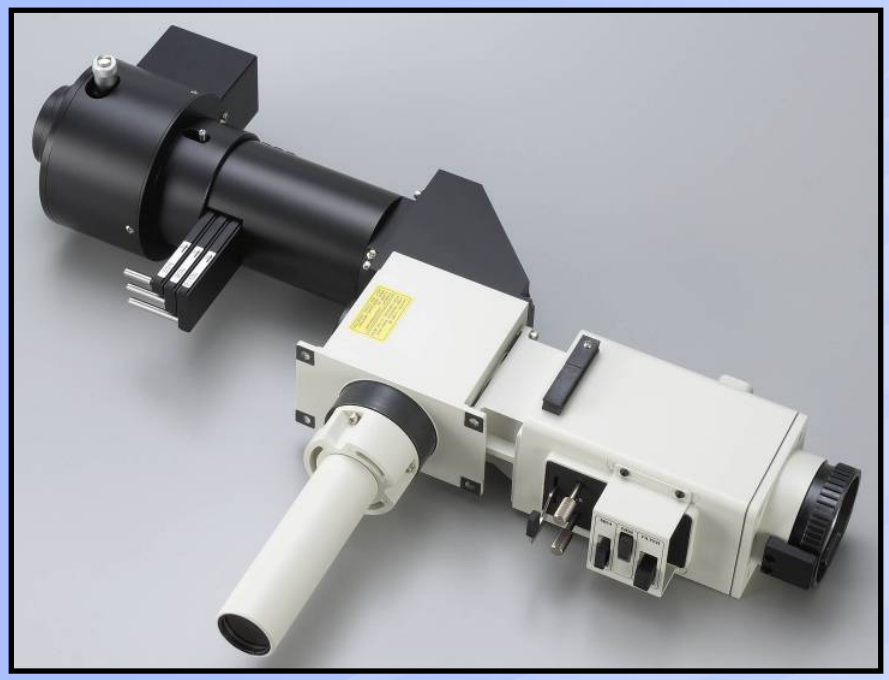

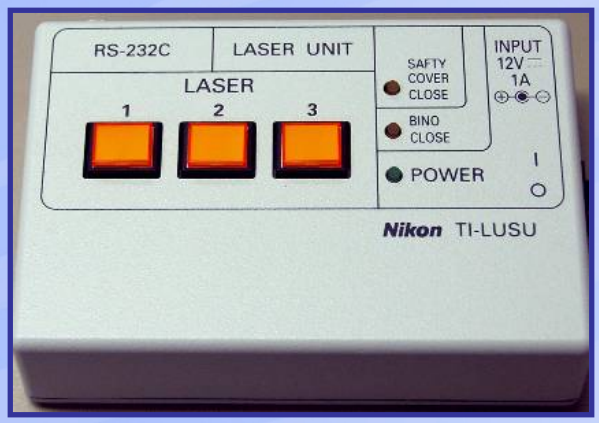

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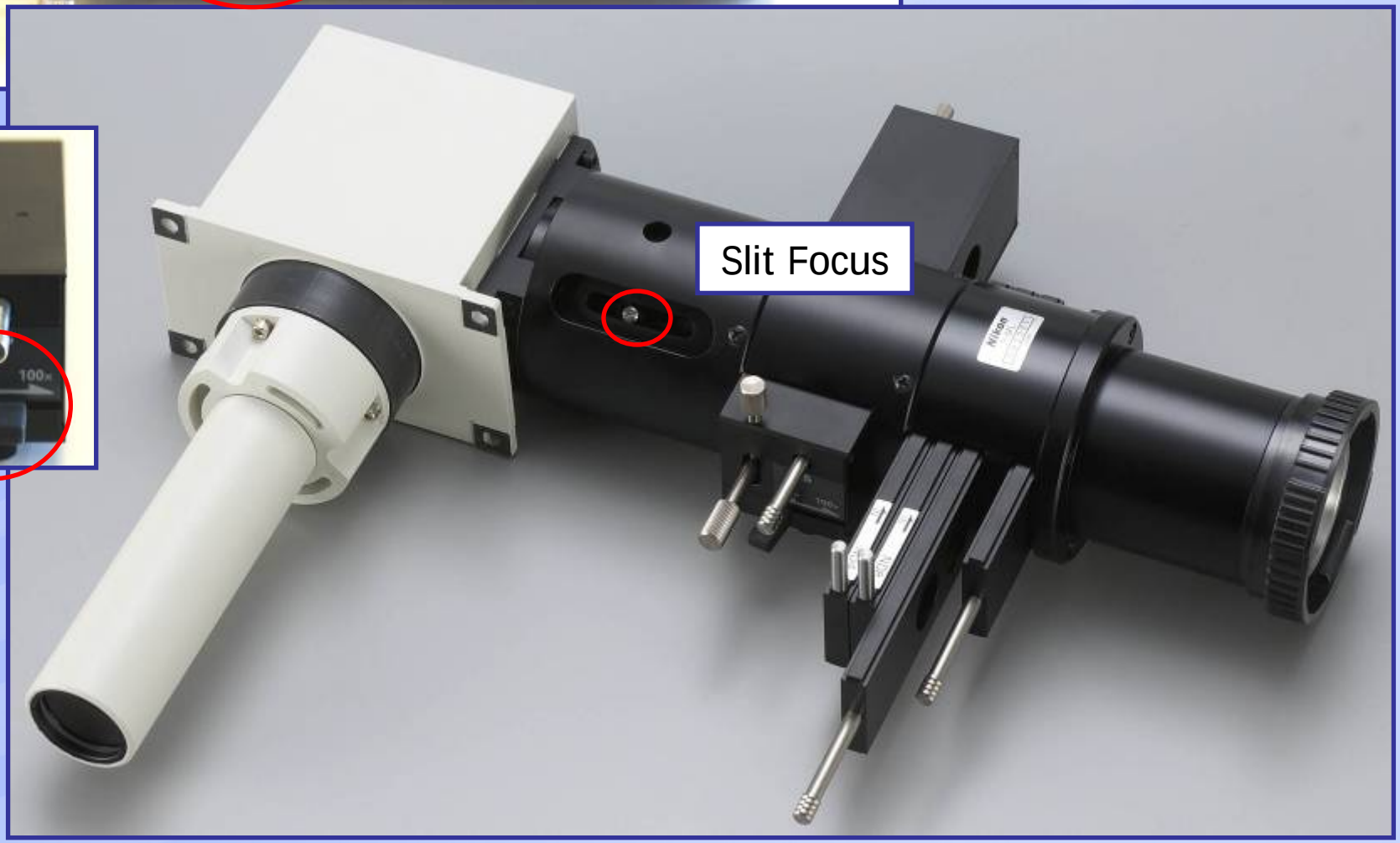
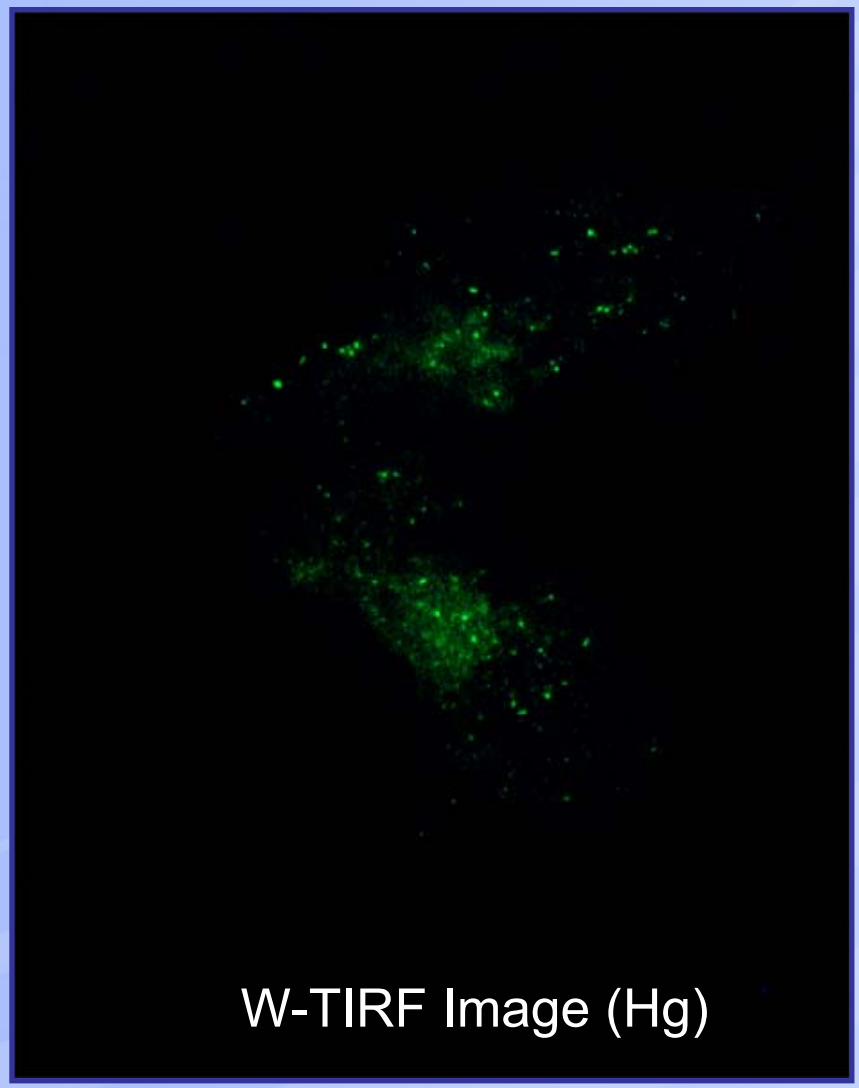
Ti-SFL

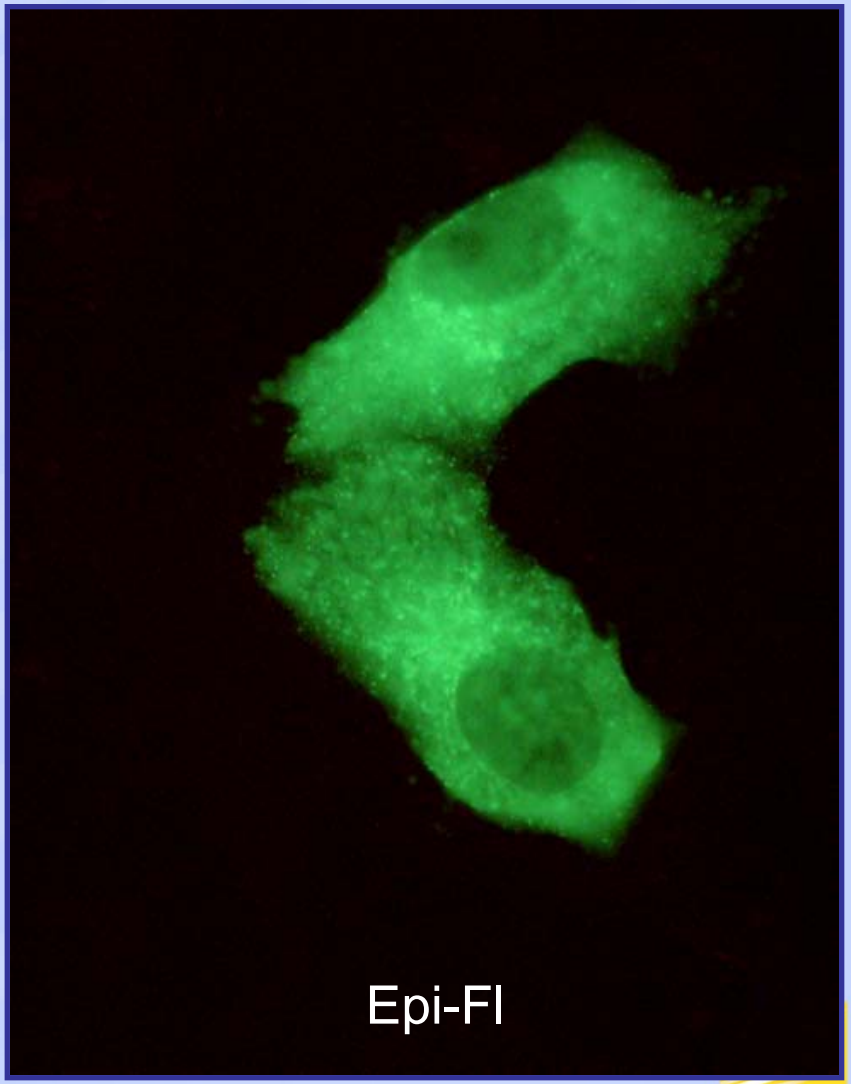


COS Cell Expressing GFP Clathrin

(Taken with “white light” TIRF)



W-TIRF Image (Hg)



Epi-Fl

Images by: Prof. Dan Axelrod, Univ. of Michigan

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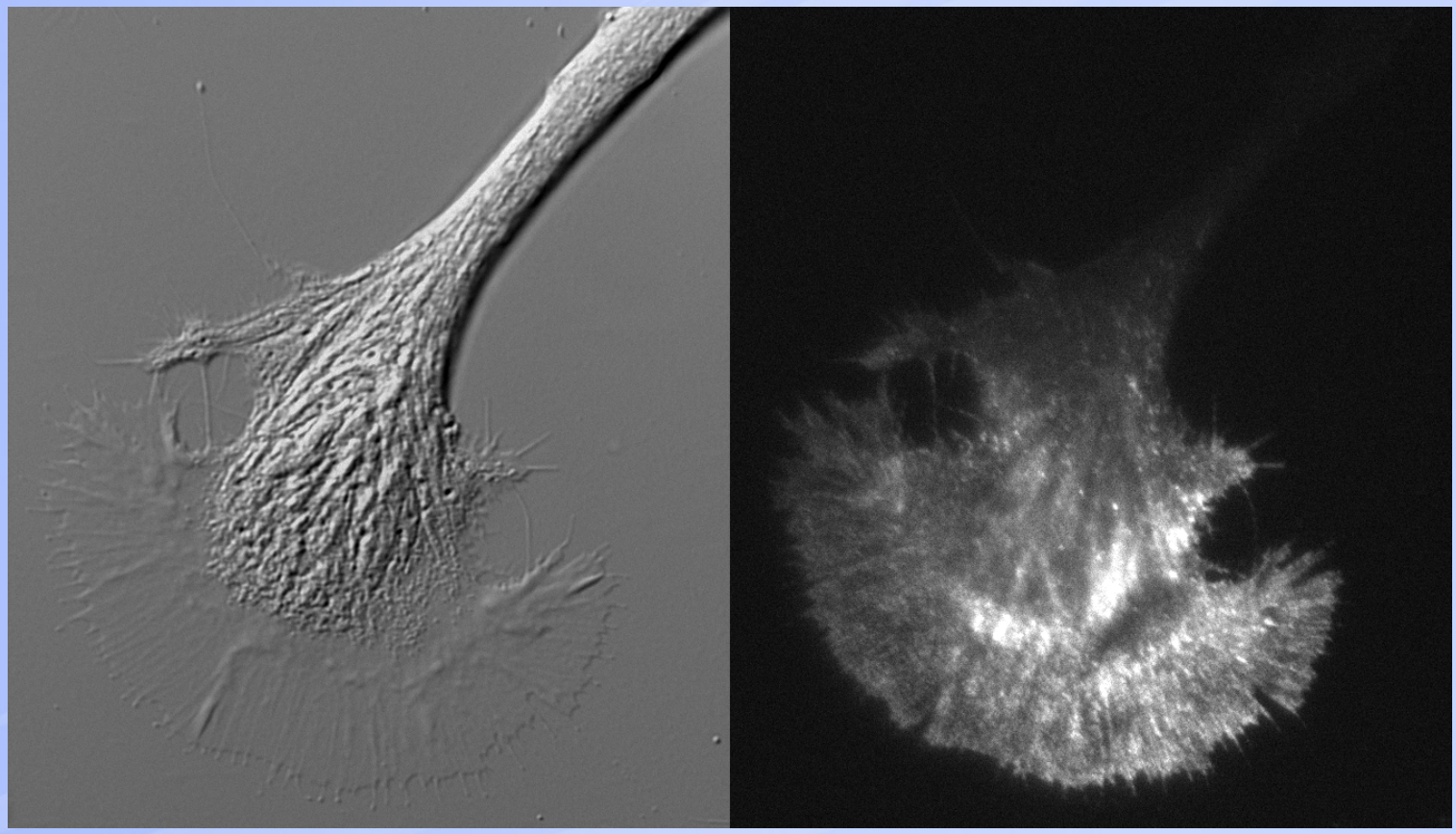
Aplysia Growth Cone, primary culture

Alexa 488 Actin

Camera: Roper Coolsnap HQ, 0.1Hz



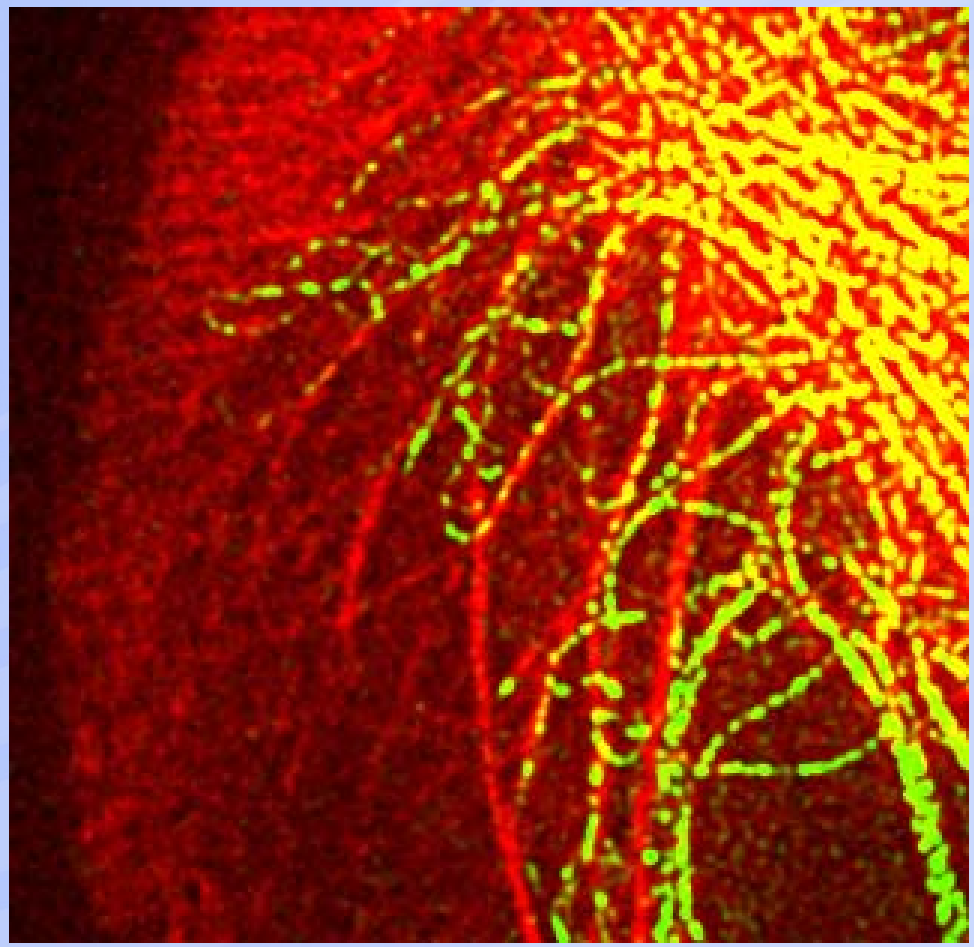
Imaged by Andy Schaefer, Paul Forscher Lab. , Yale University

Aplysia Growth Cone, primary culture

Alexa 488 Actin

Alexa 568 Tubulin

Camera: Roper Coolsnap HQ, 0.1Hz



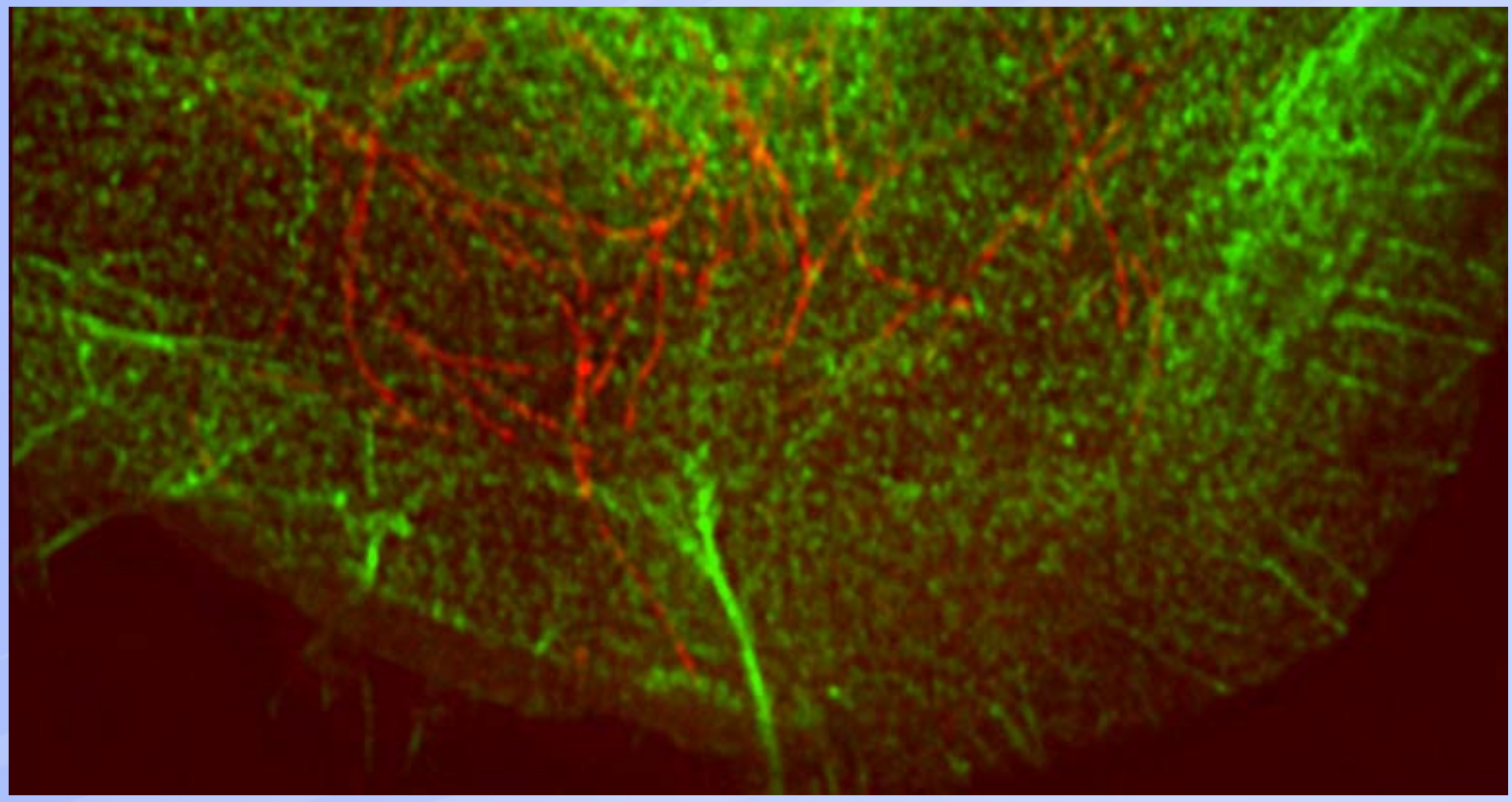
Imaged by Andy Schaefer, Paul Forscher Lab. , Yale University

Aplysia Growth Cone, primary culture

Alexa 488 Actin (speckled concentration)

Alexa 568 Tubulin

Camera: Roper Coolsnap HQ, 0.1Hz



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Thank You

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