FLIM, Confocal

- Last class
  - Single molecule imaging
  - Deconvolution
- This class
  - FLIM
  - Confocal

FLIM – Fluorescence Lifetime IMaging

FLIM – Fluorescence lifetime imaging

- Easier and more accurate quantitation
- Much harder to measure

For a population of fluorophores

$$B = QY * \varepsilon$$

$$F(t) = I_0(t) * N(t) * QY(t) * \varepsilon * \gamma(t)$$

γ(t) = photobleaching as a function of time
N(t) = number of fluorophores

$$\tau_d = \frac{1}{k_f + k_{nr}}$$

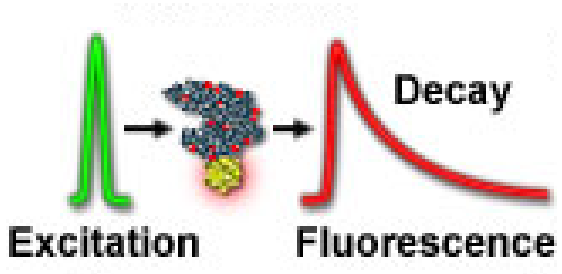
$$QY = \frac{k_f}{k_f + k_{nr}}$$

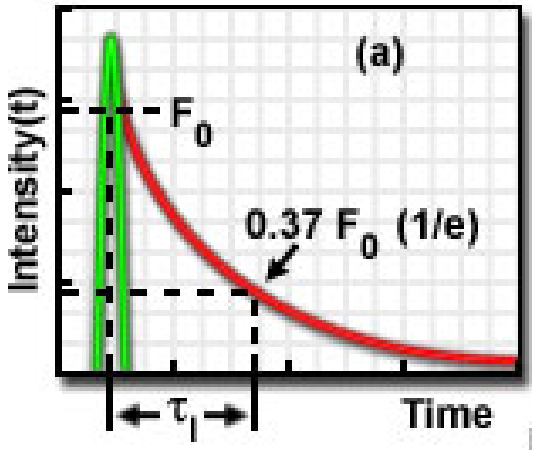
$$QY = \frac{k_f}{\tau_d}$$

k_f is mostly constant for a given molecule
k_f^{-1} is the radiation lifetime – how long it lives in the excited state before decay

Fluorescence Lifetime Imaging (FLIM)

- Examine physical properties of fluorophores
- Can avoid complications like photobleaching and concentration effects



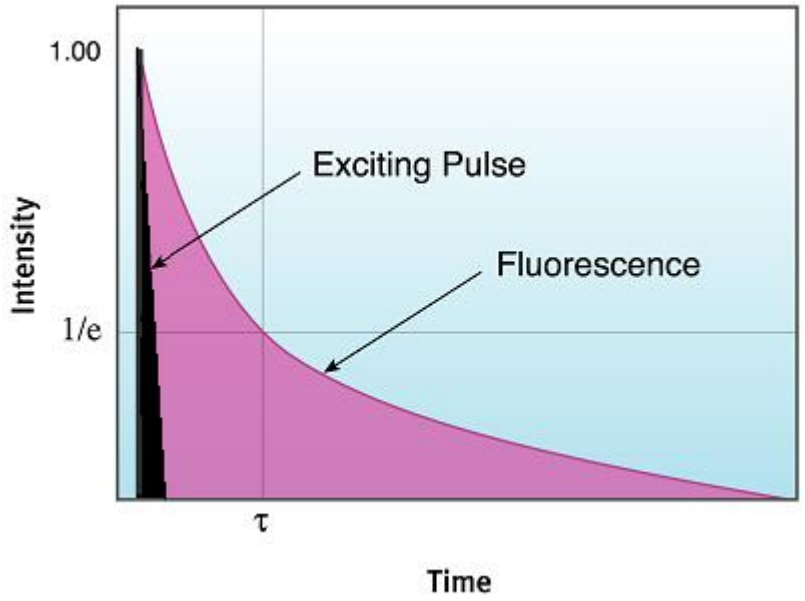


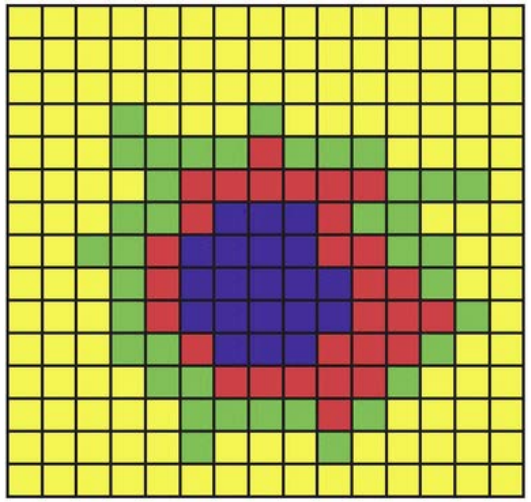
$$F(t) = F_0 e^{-t/\tau}$$

Fluorophore	Lifetime [ns]	Excitation Max [nm]	Emission Max [nm]	Solvent
ATTO 655	3.6	655	690	Water
Acridine Orange	2.0	500	530	PB pH 7.8
Alexa Fluor 488	4.1	494	519	PB pH 7.4
Alexa Fluor 647	1.0	651	672	Water
BODIPY FL	5.7	502	510	Methanol
Coumarin 6	2.5	460	505	Ethanol
CY3B	2.8	558	572	PBS
CY3	0.3	548	562	PBS
CY5	1.0	646	664	PBS
Fluorescein	4.0	495	517	PB pH 7.5
Oregon Green 488	4.1	493	520	PB pH 9
$Ru(bpy)_2(dcpby)[PF_6]_2$	375	458	650	Water
Pyrene	> 100	341	376	Water
Indocyanine Green	0.52	780	820	Water
Rhodamine B	1.68	562	583	PB 7.8

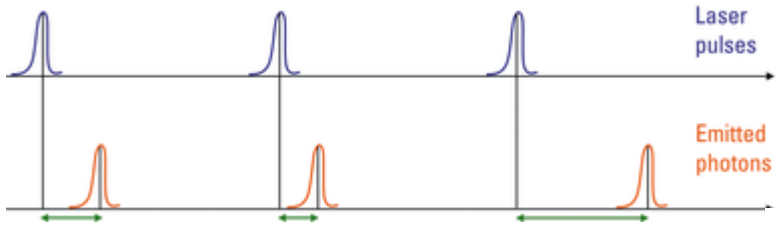
Measuring lifetime

- Need a very short excitation pulse
- Need to measure on very short time scales
- Collect image many times, at each delay to build up total image
- Takes a long time to image

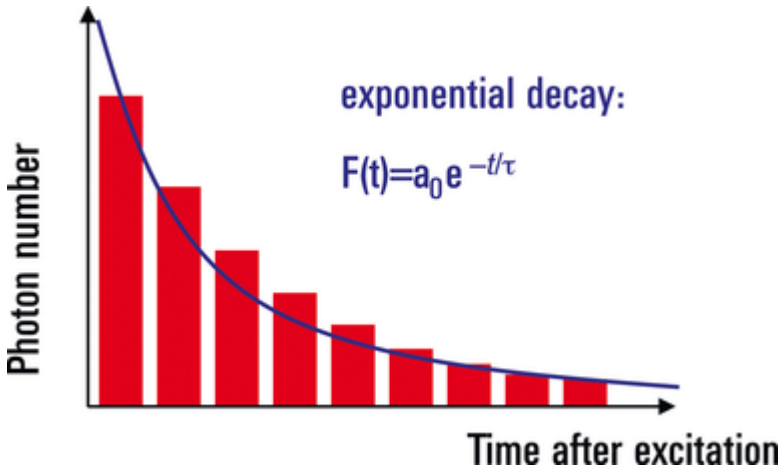


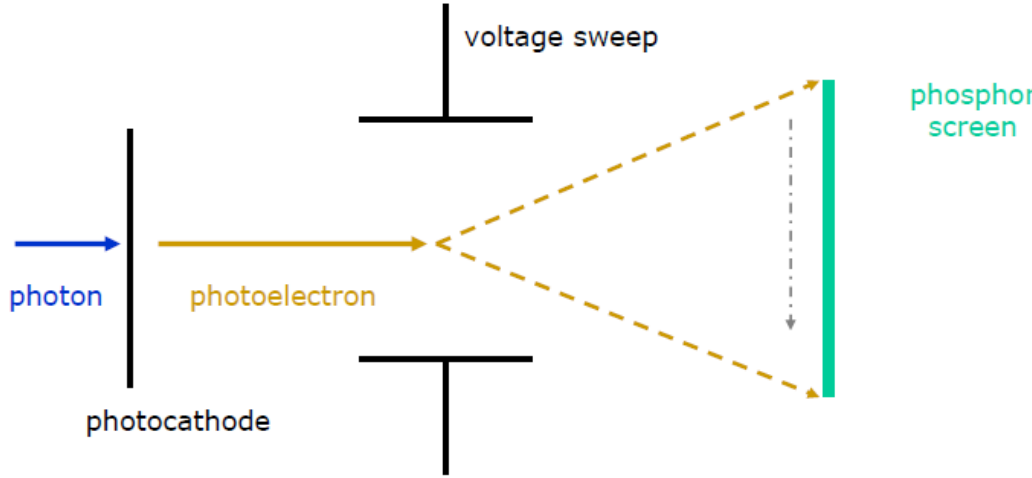


Fluorescence lifetime image

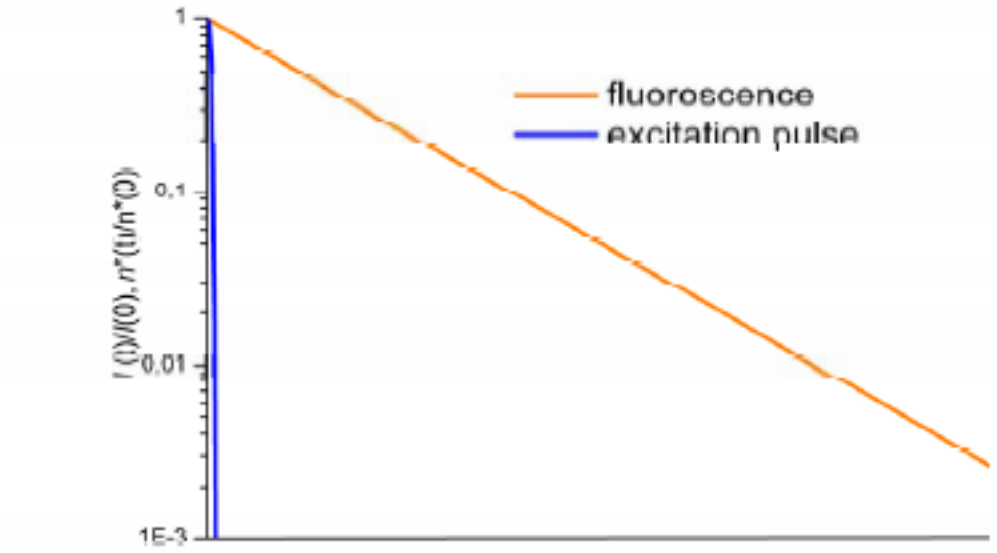


Wavelength-color look-up table



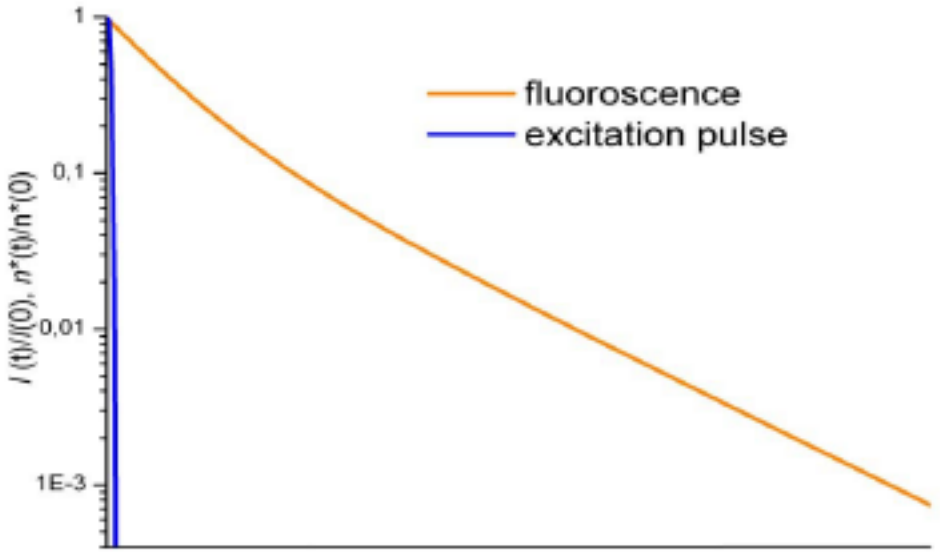


Fitting multiple time constants



single-exponential decay

$$I(t) = I(0)\exp(-t/\tau)$$



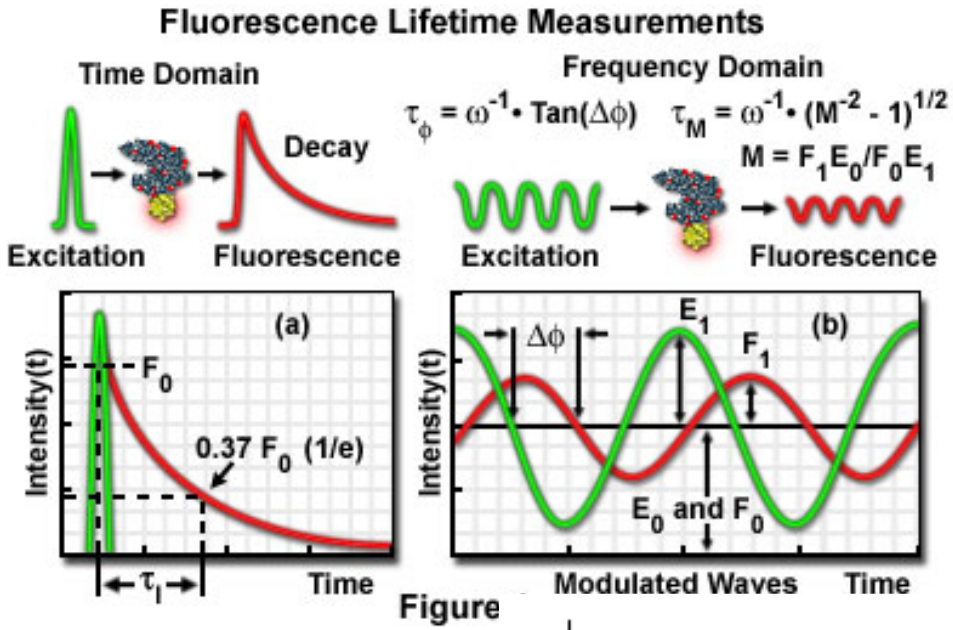
multi-exponential decay (at least two distinct lifetimes)

$$I(t) = I(0)\sum_i \alpha_i e^{-t/\tau_i}$$

Can only realistically fit up to 3 time constants. More than that will introduce artifacts

Frequency domain measurements

- Instead of watching decay, apply sinusoidal light
- Lifetime can be calculated from the phase delay of the fluorescence relative to the excitation
- Typically only works on one lifetime species
- If τ_{phase} is not equal to τ_m, then there is more than one lifetime

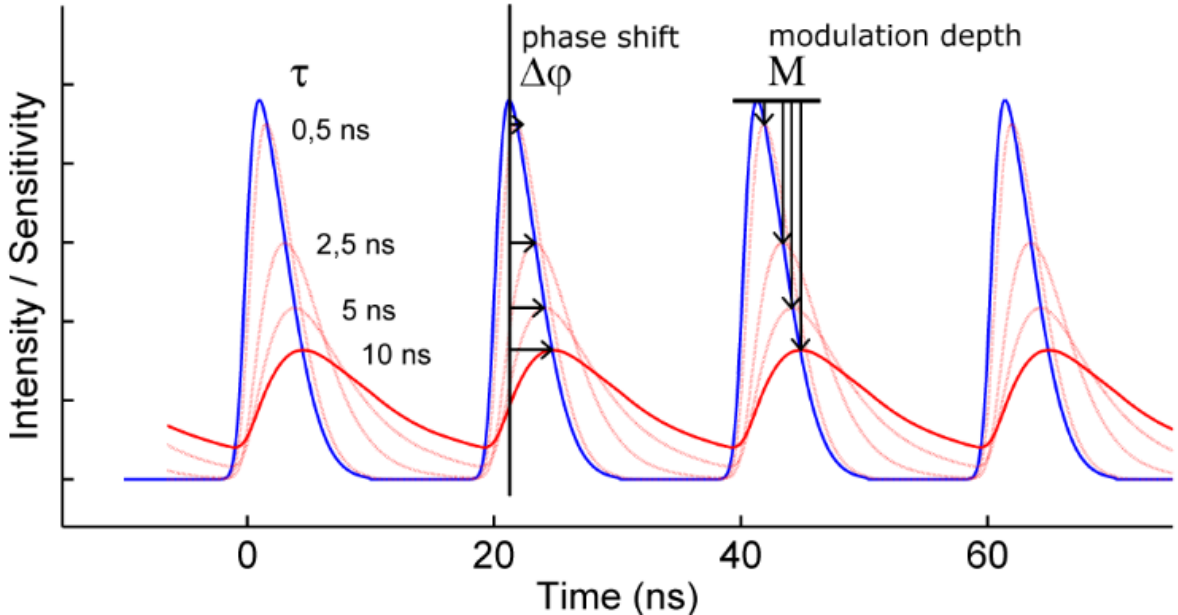


Figure

$$\Delta\varphi = \arctan(\omega_n \tau)$$

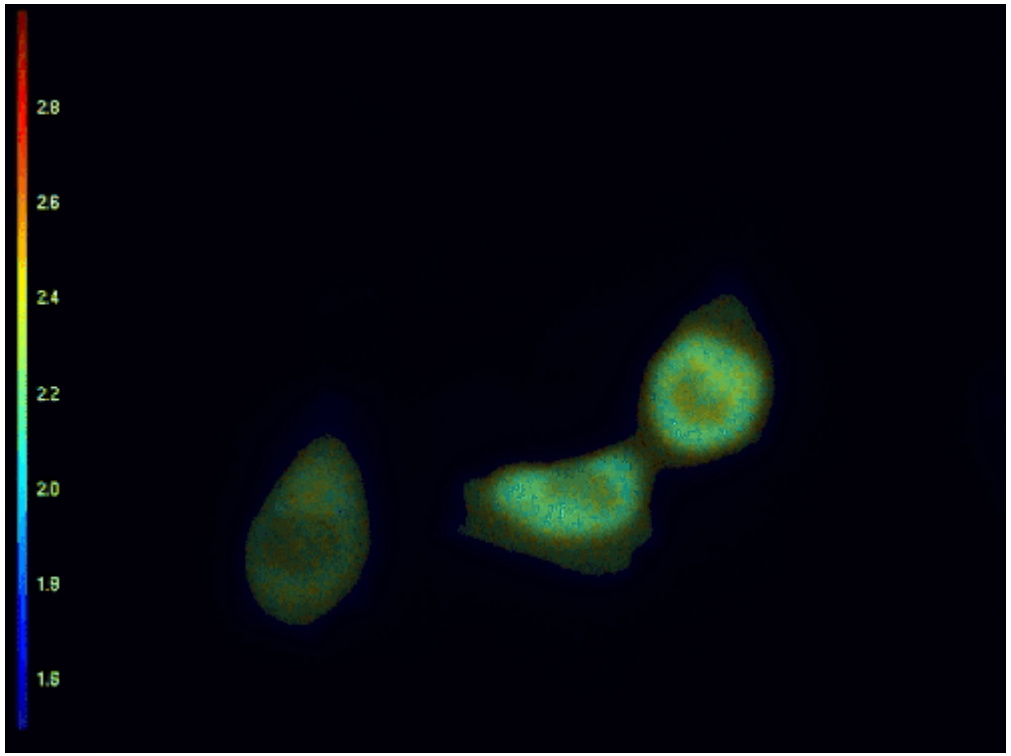
$$M = \frac{1}{\sqrt{1 + (\omega_n \tau)^2}}$$

ω_n = Frequency of excitation light



Frequency domain

- Can take images fast
- Somewhat reasonable for live cell



FLIM advantages

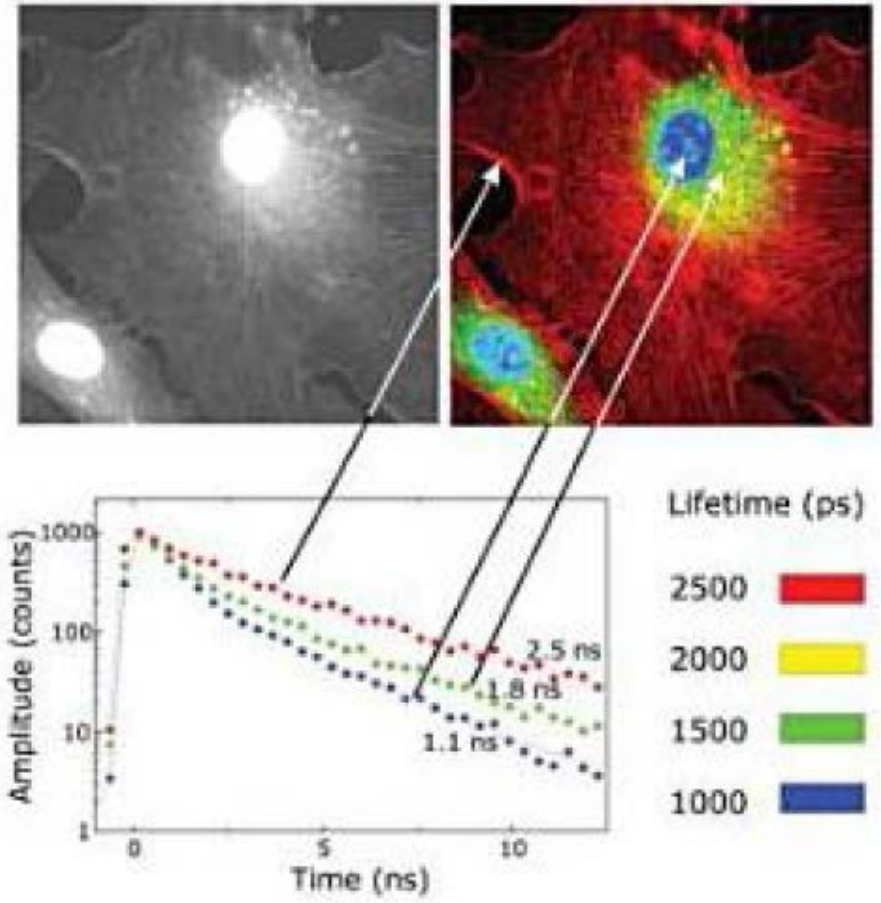
- Insensitive to concentration
- Insensitive to photobleaching
- Easily able to convert to absolute units
- In FRET, you only measure the donor lifetime, reduces quantification errors
- Much harder to analyze if there are multiple species of fluorophores

$$E = \frac{k_T}{k_T + k_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$

$$(\tau_{DA})^{-1} = k_T + k_D$$

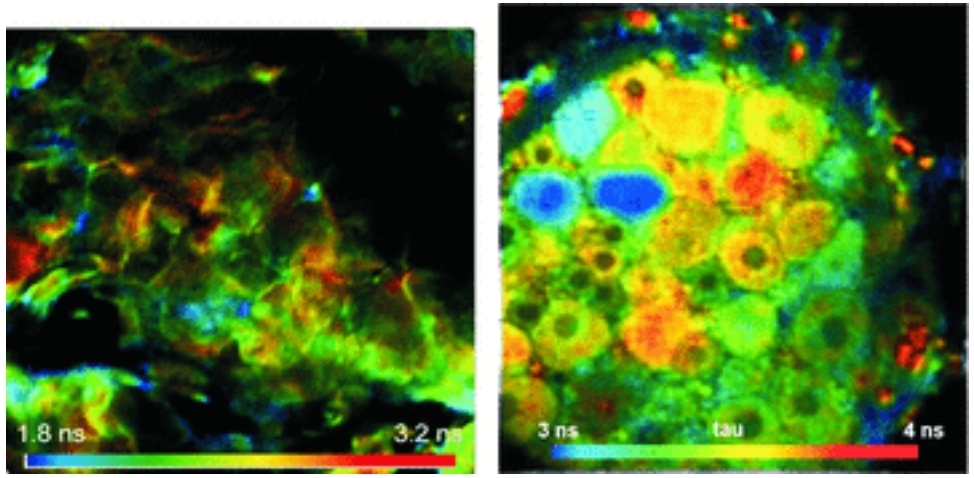
Donor lifetime in the presence of acceptor

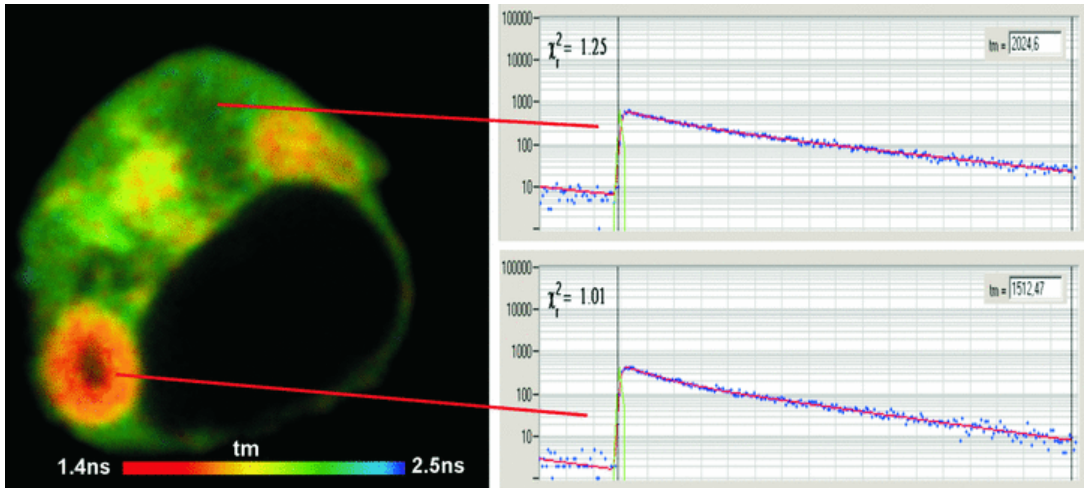
$$(\tau_D)^{-1} = k_D$$



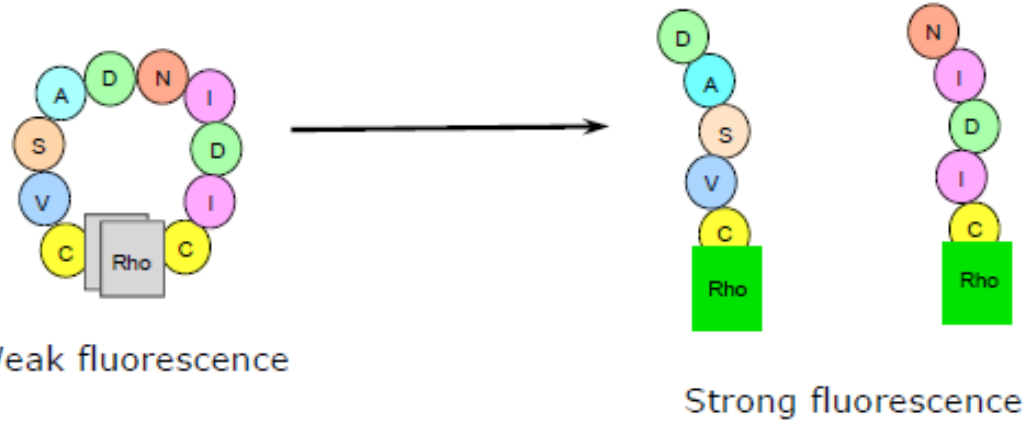
FLIM applications

- Measurements of pH – use dye that has a lifetime dependent on the local environment
- FRET interactions in cells





Peptide conformations



Weak fluorescence

Strong fluorescence

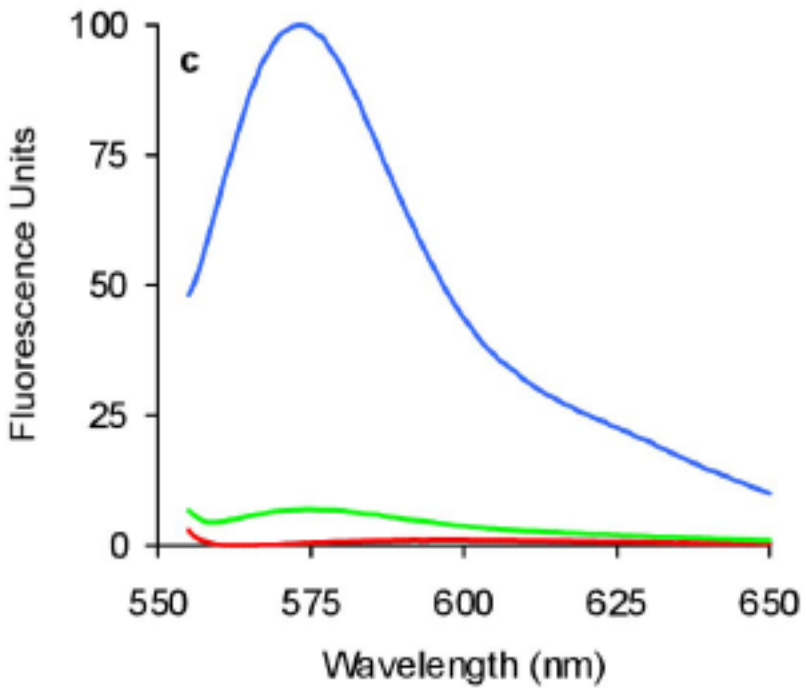


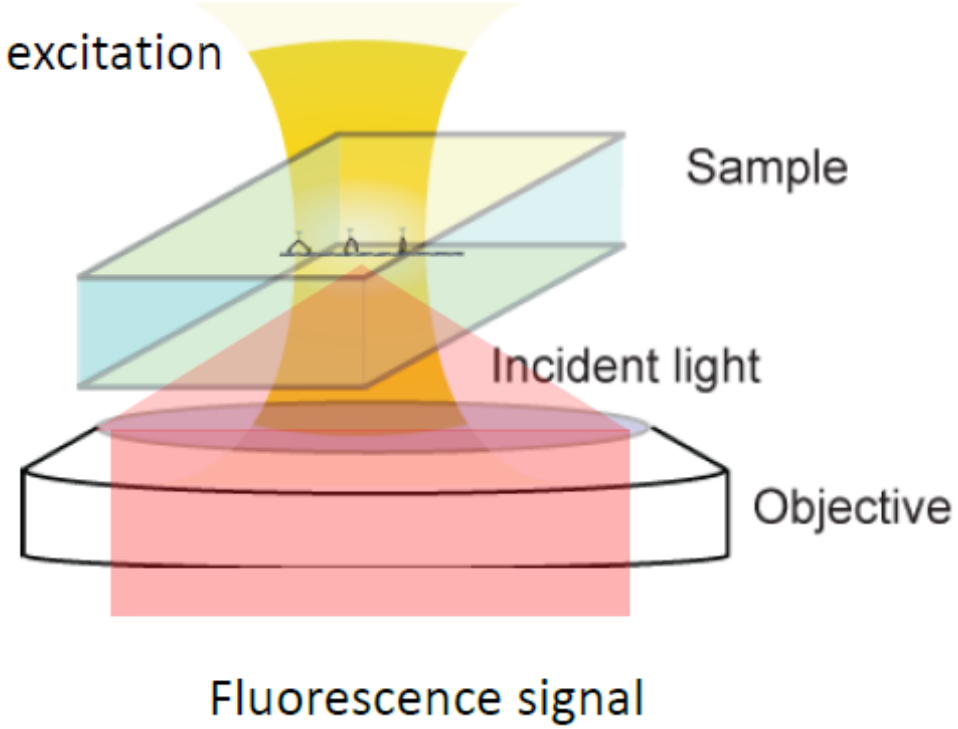
Table 3: Fluorescence Lifetime Parameters of Intact and Cleaved Labeled Peptides[a]

sample	τ_1 (ns)	f_1	α_1	τ_2 (ns)	f_2	α_2
pepF1-5R	2.44	0.95	0.52	0.14	0.05	0.48
pepF1-5R + Pronase	2.43	1.00				
pepF1-6R	2.50	0.95	0.37	0.076	0.05	0.63
pepF1-6R + Pronase	2.50	1.00				

Confocal microscopy

- 3D sectioning
- Increased resolution

Widefield fluorescence

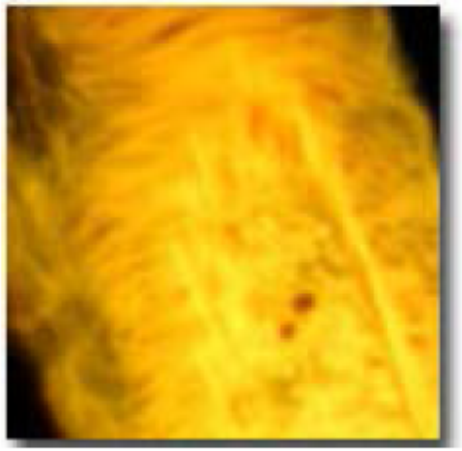


Bright light source illuminates the entire field of view at the same time

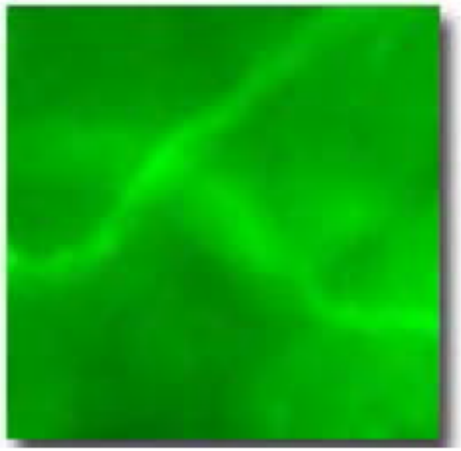
Fluorescence cube is placed between the objective and the camera

In epi-illumination, the objective acts as both the condenser and the objective – typically use high NA with low fluorescence glass

Widefield images



human medulla

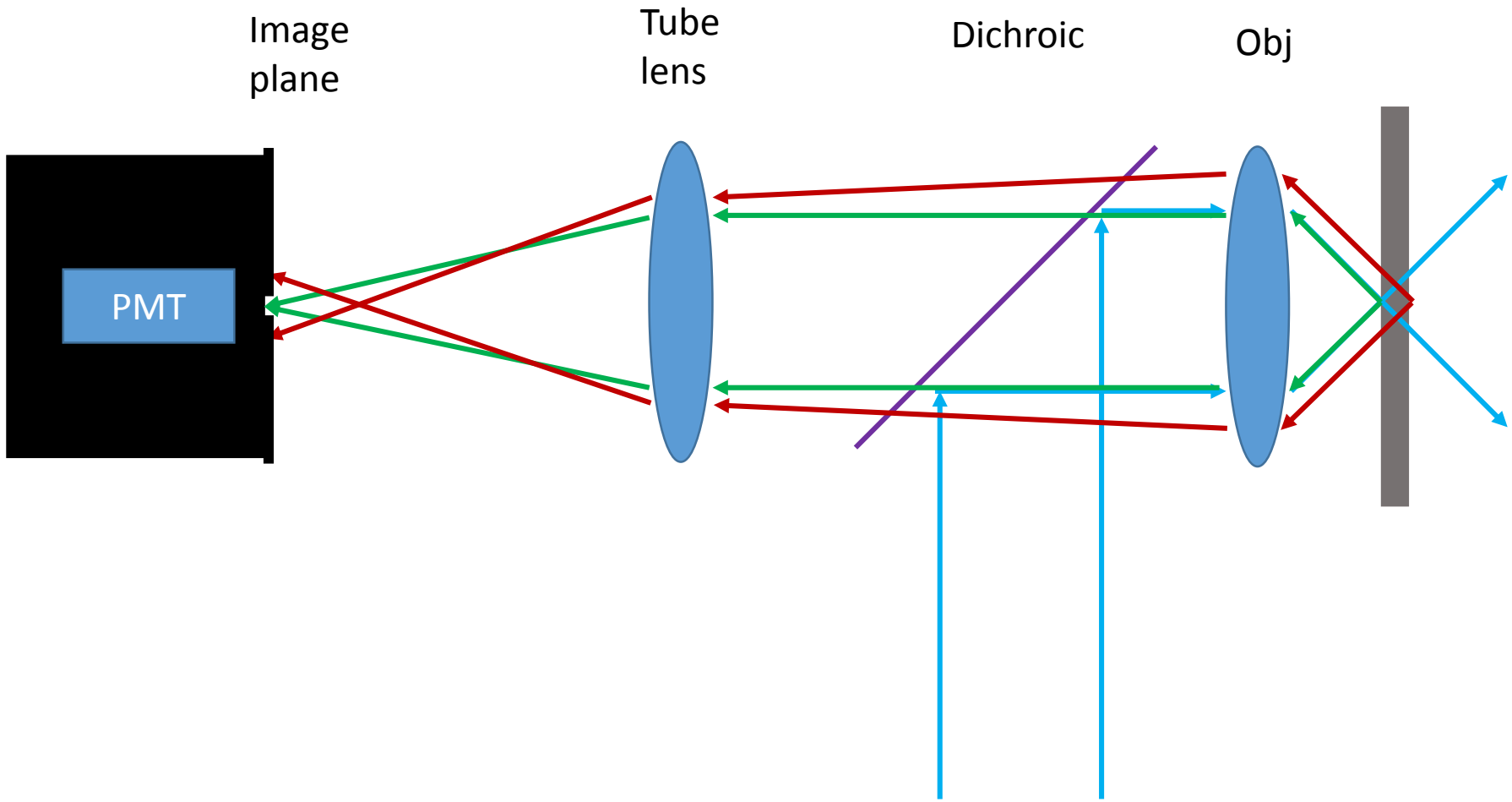


rabbit muscle fibers

sunflower pollen grain

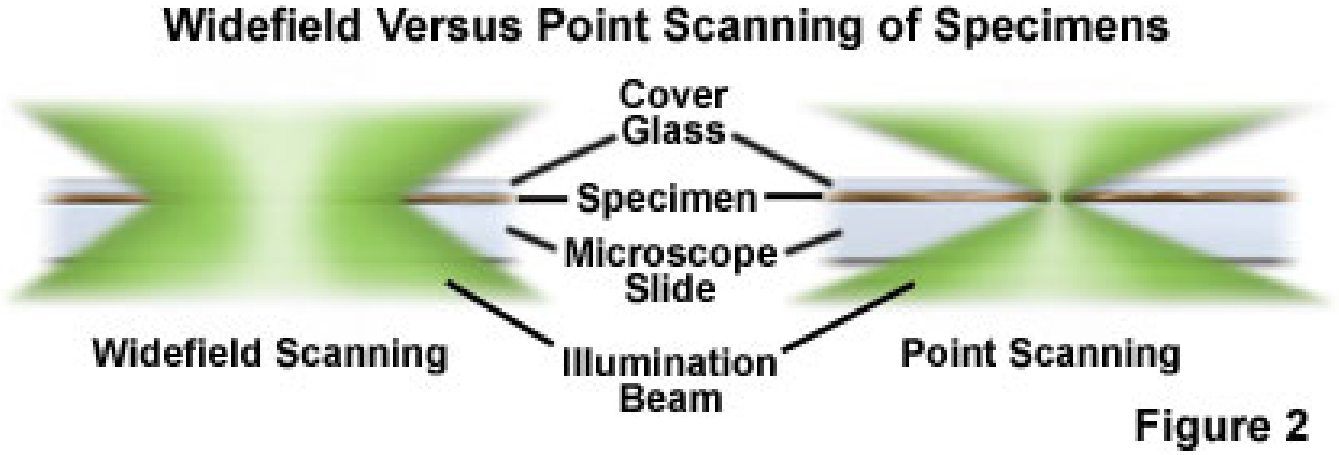
- Images are bright, but blurry and low contrast
- Epi-illuminator excites the whole thickness of the specimen, unable to control the depth of the field
- Bright fluorescent signal from out-of-focus objects give low-contrast
- Autofluorescence (fluorescence signal from unlabeled objects) of the cell increases the background

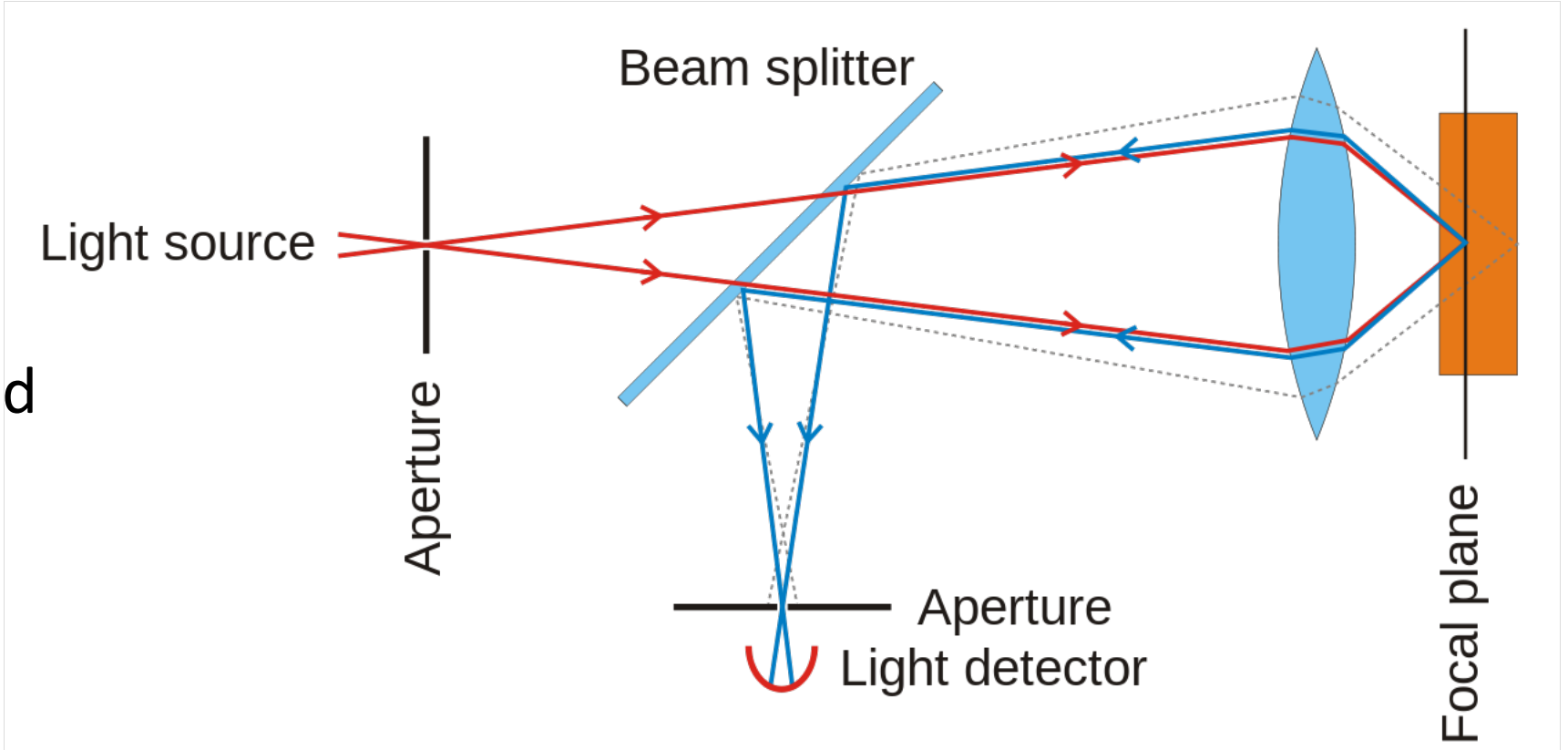
Confocal principle



Confocal principle

- Light is focused to a point in the sample
- Emitted photons imaged on to a plane
- Aperture is placed at the image plane
- Single element detector placed behind aperture



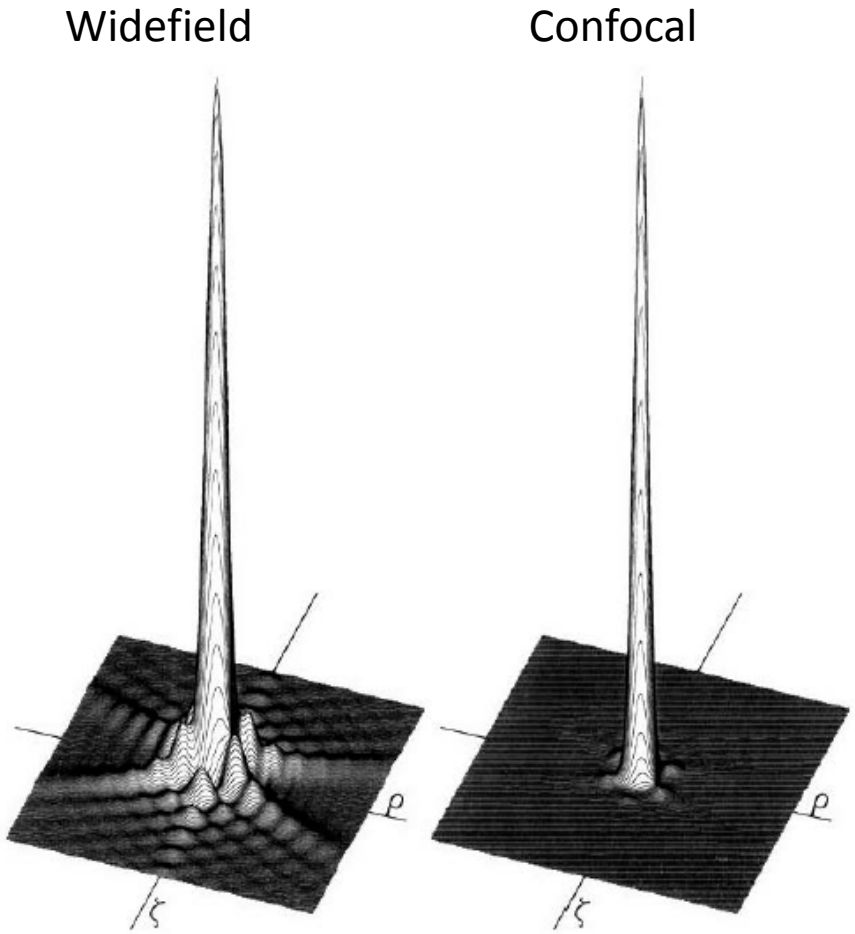


Improved resolution in confocal

- Resolution is now set both by excitation and emission light.
- New PSF is product of excitation (diffraction limited spot) and emission (diffraction limited, but with different wavelength).

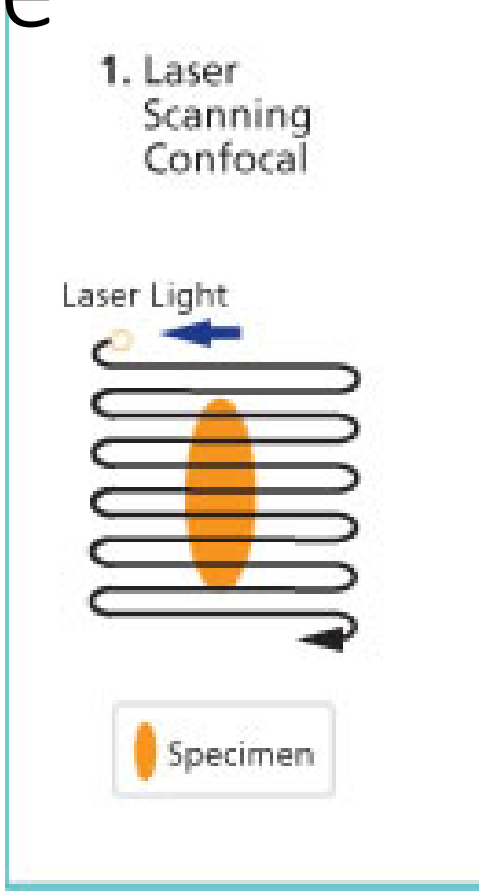
$$d_{x,y} = \frac{0.4\lambda}{NA}$$

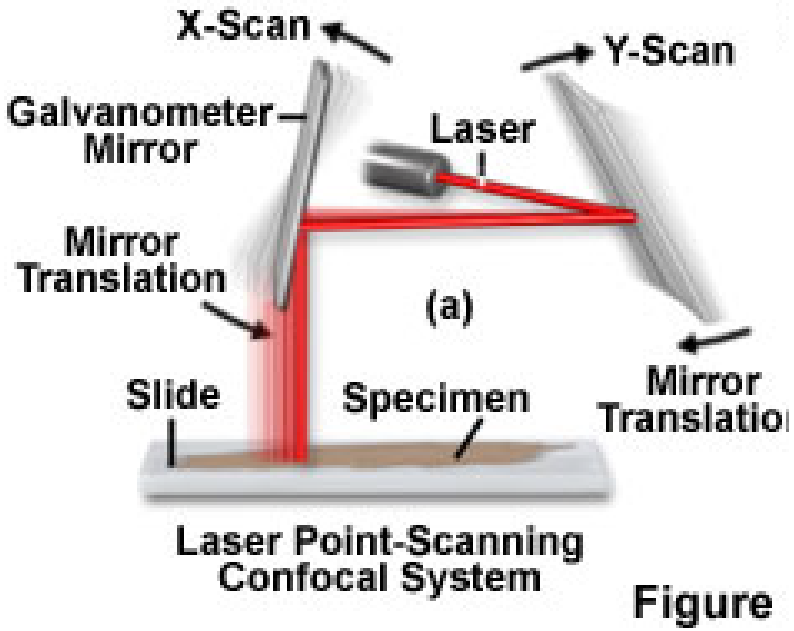
Optimal diffraction



Scan to form an image

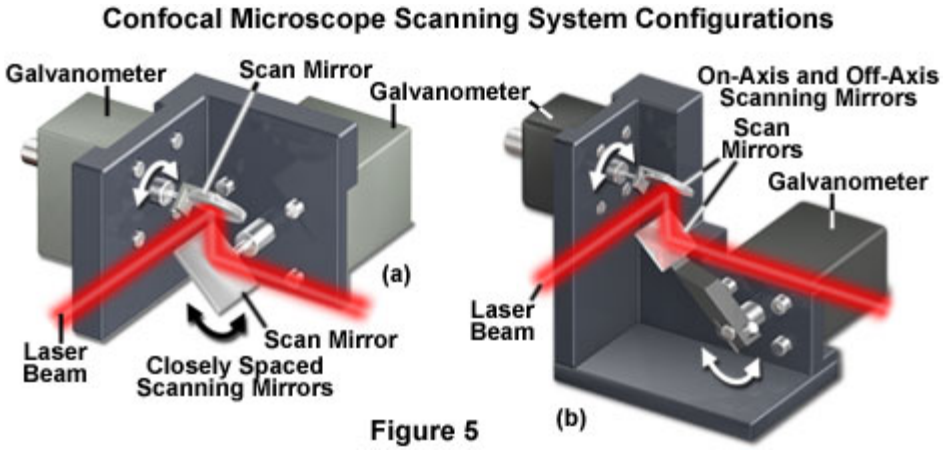
- Confocal only illuminates a single point
- Have to scan the point around the sample to build up spatial information
- Use fast moving galvo mirrors to rapidly move the laser across the sample
- Typically move in a raster pattern
- Tradeoff between time on pixel and frame rate



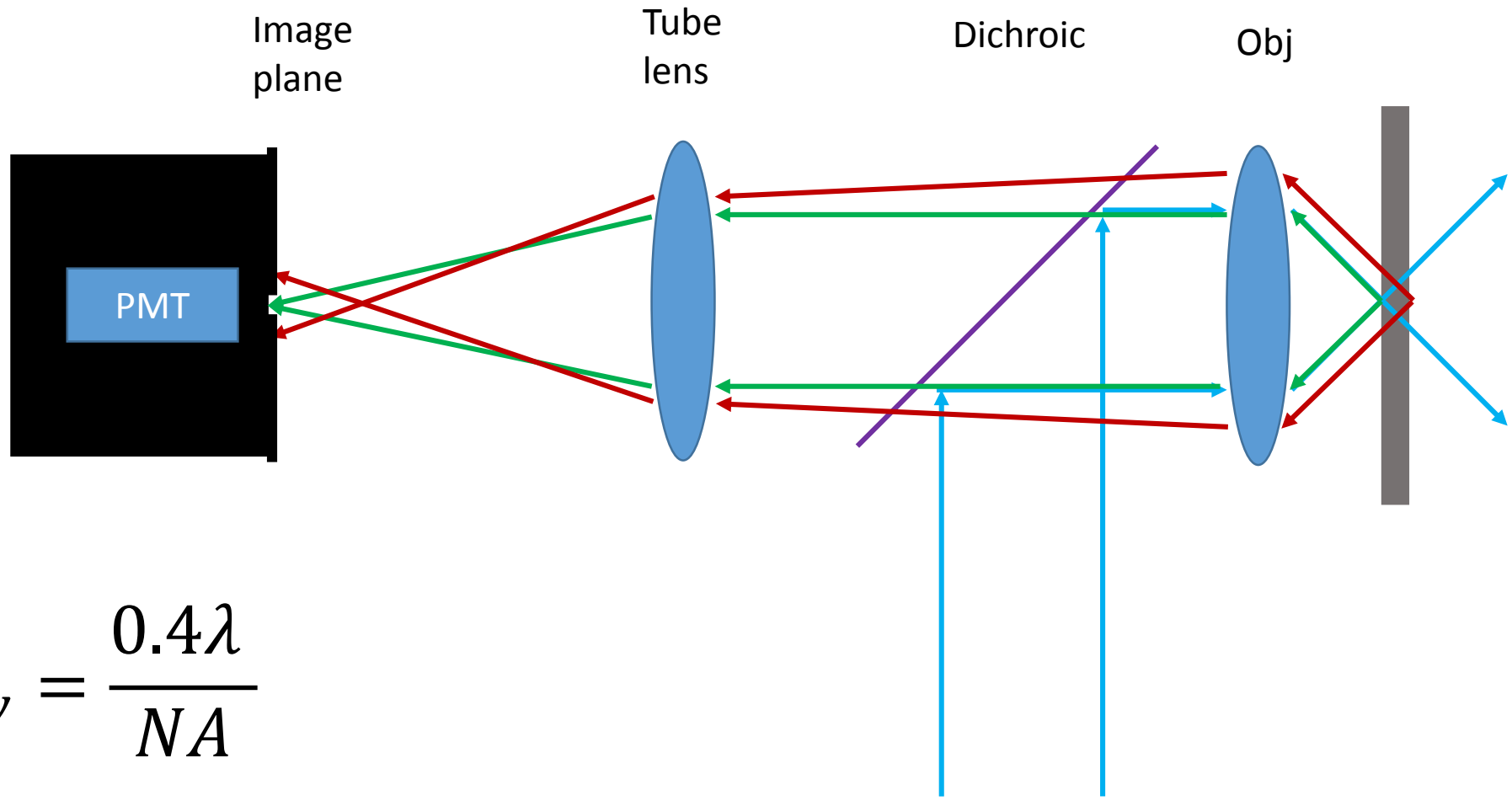


Fast galvo can scan around 1 kHz

On a 512 x 512 pixel image -> 2 µs per pixel, 500 ms total exposure



Confocal principle



$$d_{x,y} = \frac{0.4\lambda}{NA}$$

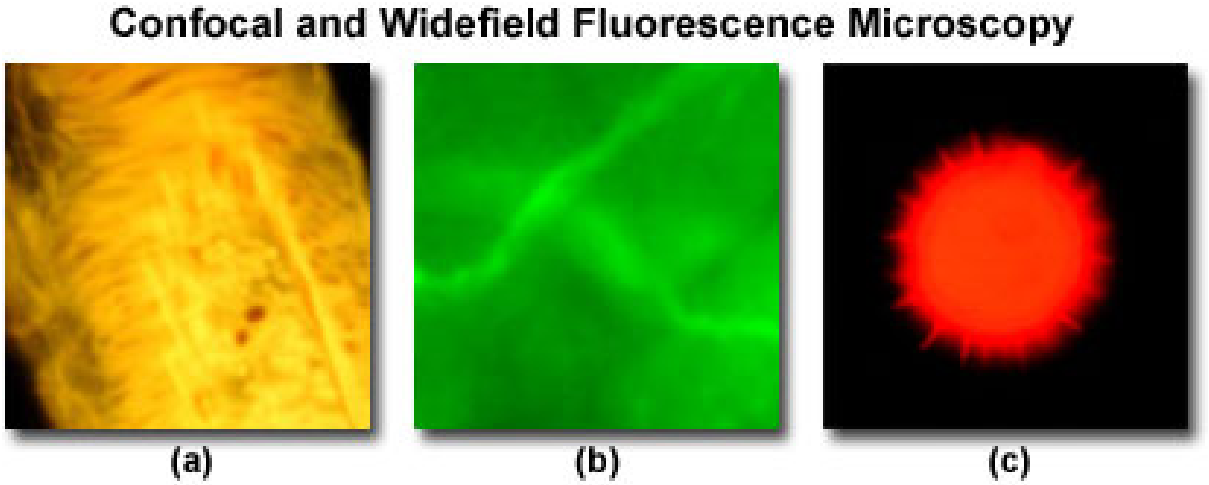
Confocal images

Confocal advantages:

-Blocks out of focus light rays, increasing resolution and contrast

-Higher flexibility in image size and acquisition

-Allows 3D sectioning

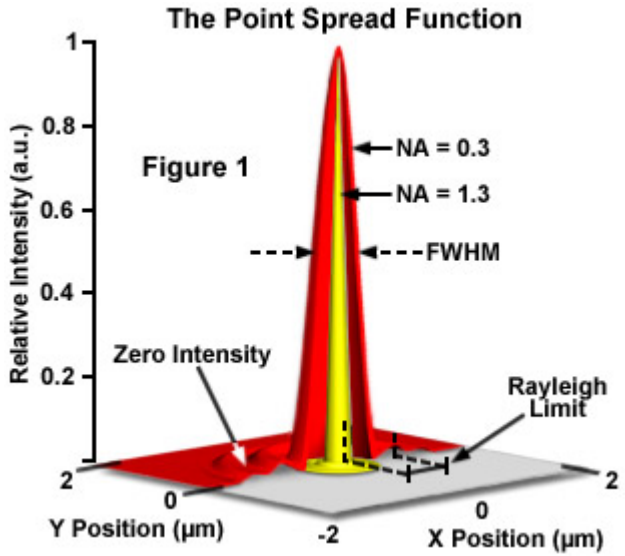


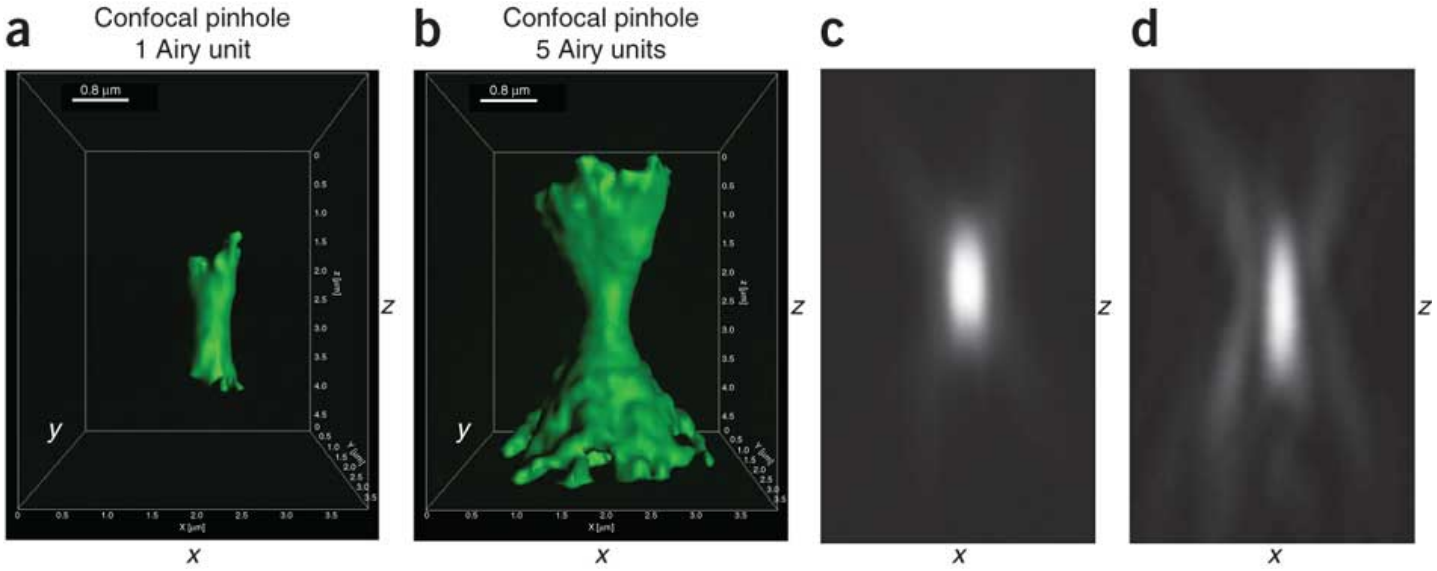
$$d_{x,y} = \frac{0.4\lambda}{NA}$$

3D PSF

- PSF has a width in x and y, we've seen this before
- In confocal, now we can think about a z- detection PSF as well, set by the pinhole
- Narrower the pinhole, the better z resolution

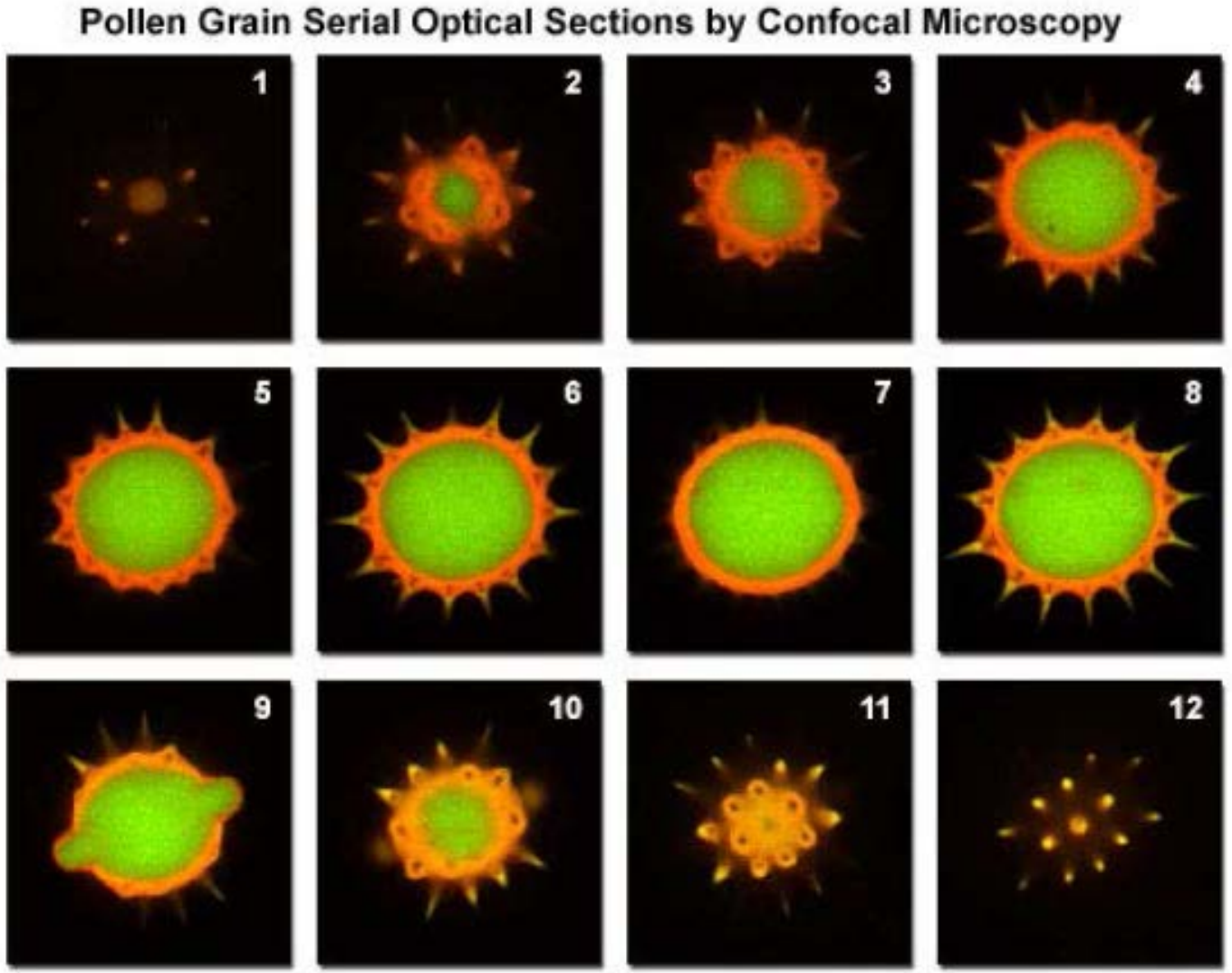
$$d_z = \frac{1.4\lambda \mathrm{n}}{NA^2}$$





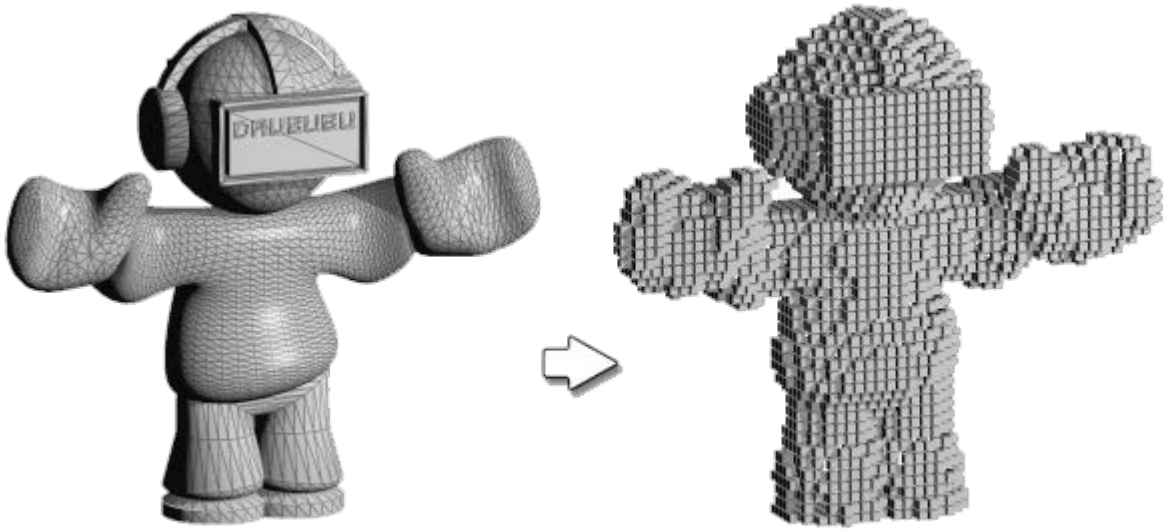
3D imaging

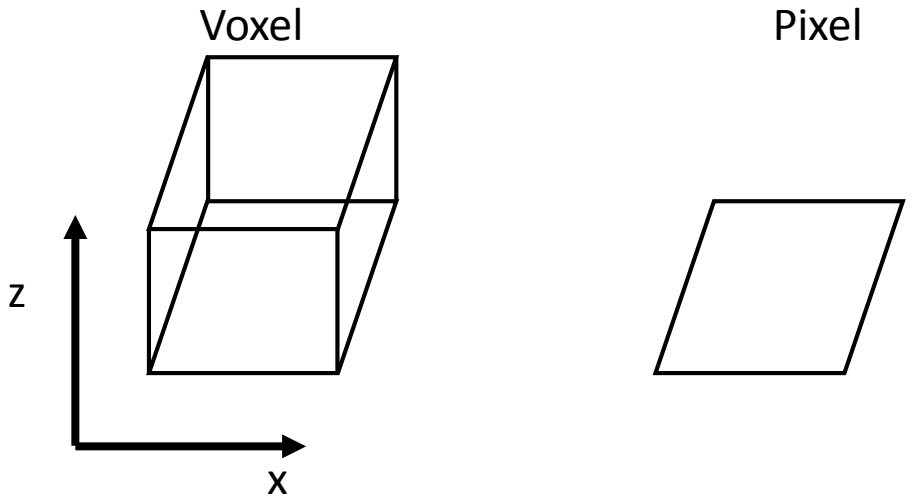
- Use galvo mirrors to take X-Y image
- Motorized stage to move sample in Z
- Take another X-Y image
- Repeat



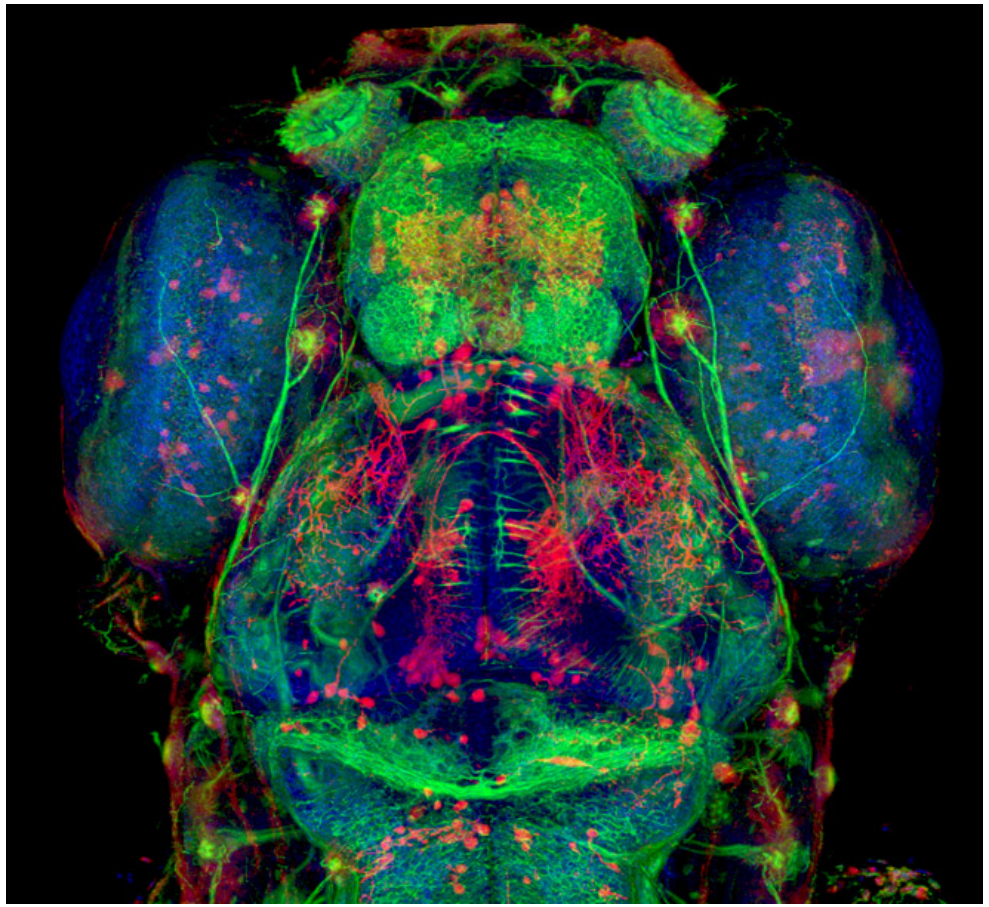
Voxels

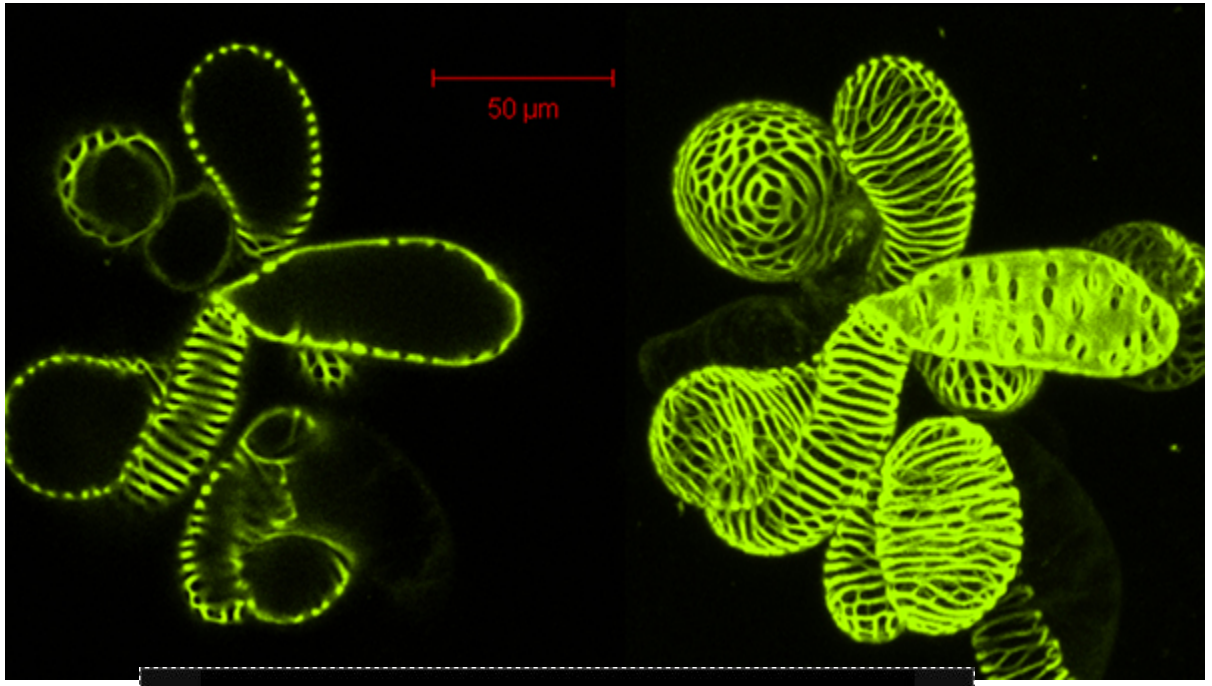
- Regular grid in 3D space
- Has dimensions in x, y, and z
- Z dimensions set by confocal system
- Each voxel is assigned an intensity value in each color
- Z resolution is lower than x,y resolution
- Sampling at least at the Nyquist frequency (1/2 size of smallest feature)





3D reconstruction





And on to Matlab