Finish spinning disk, 2 photon, super resolution

- Last class
  - Confocal
  - Hyperspectral imaging
  - Spinning disk
- This class
  - Finish spinning disk
  - 2-photon imaging

Spinning disk – sample many confocal spots simultaneously

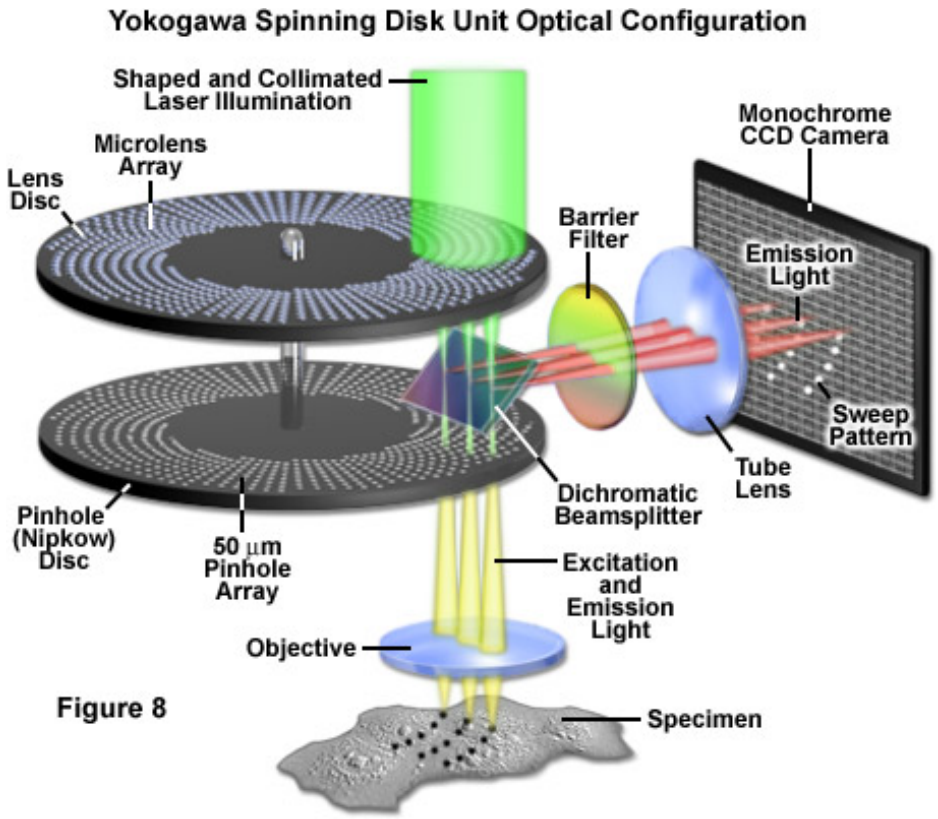
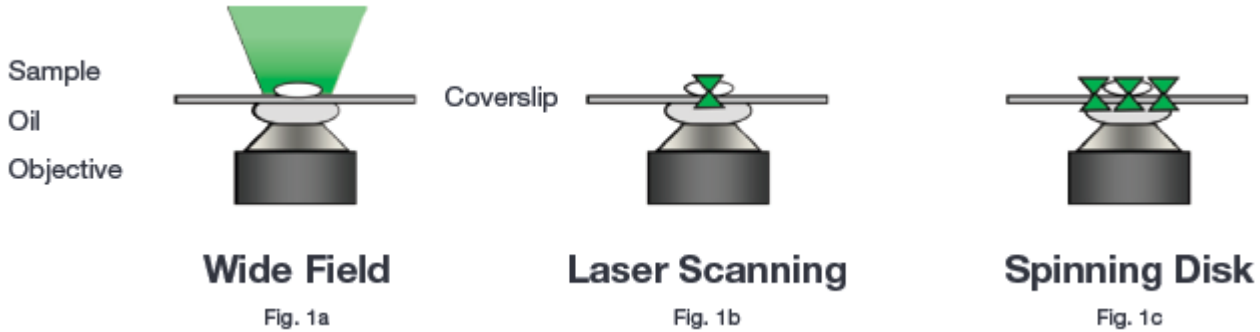
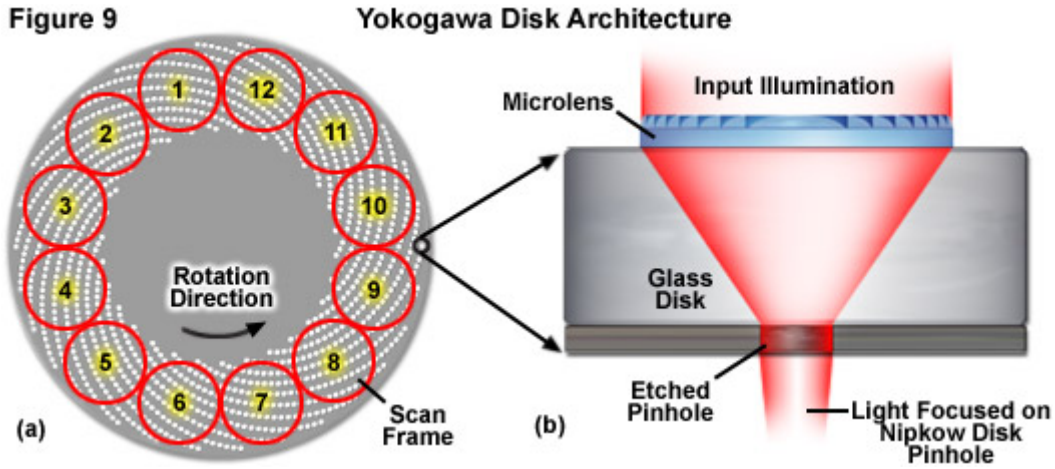


Figure 8





Possible artifacts

- Spin speed has to be synched with exposure time
- Mismatches will look like stripes in samples
- Camera readout can also add streaks
- No matter what frame rate you are running, you SHOULD be able to avoid streaks

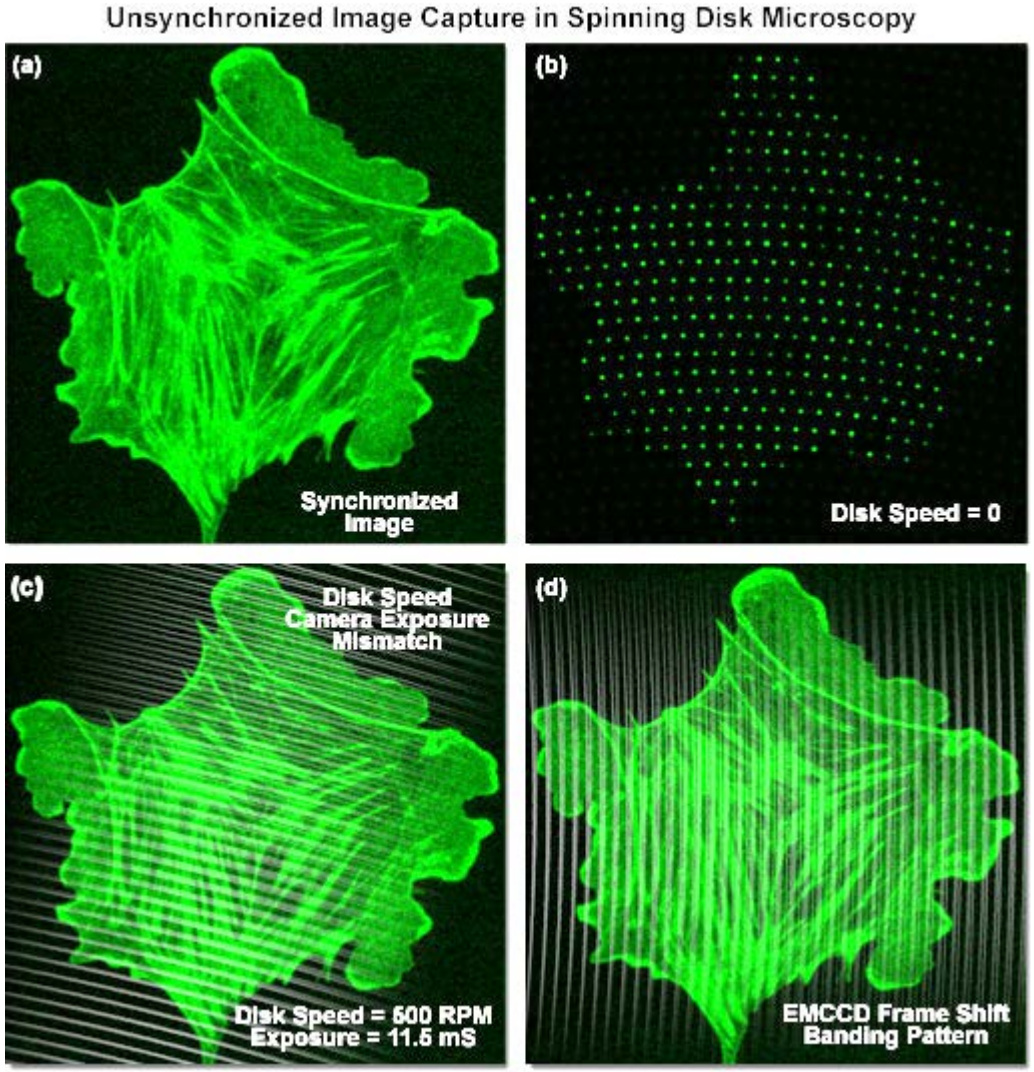
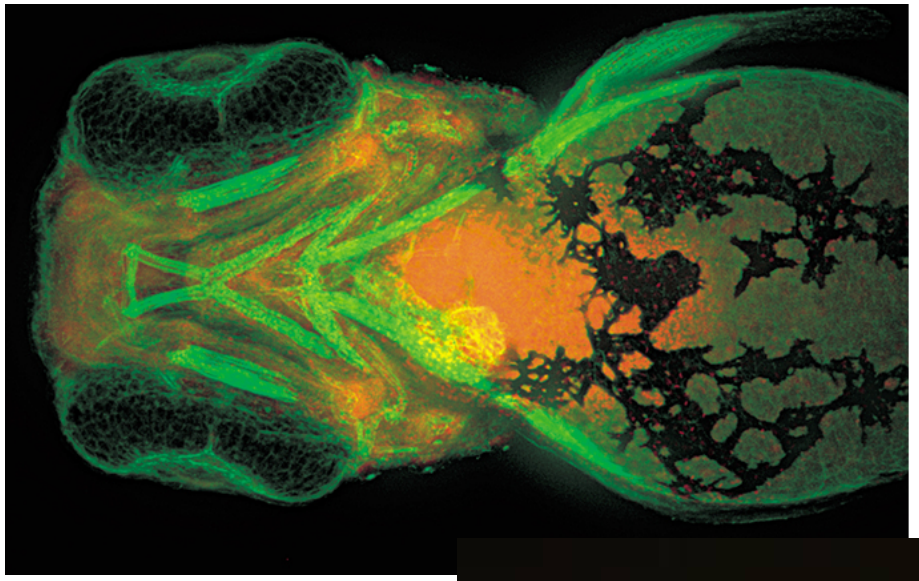
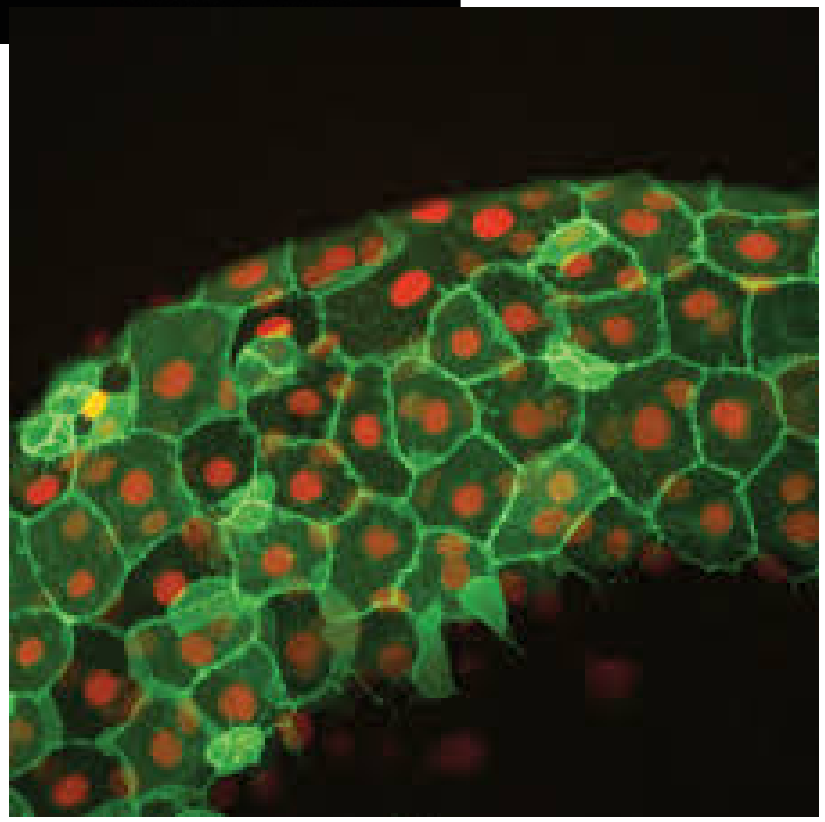
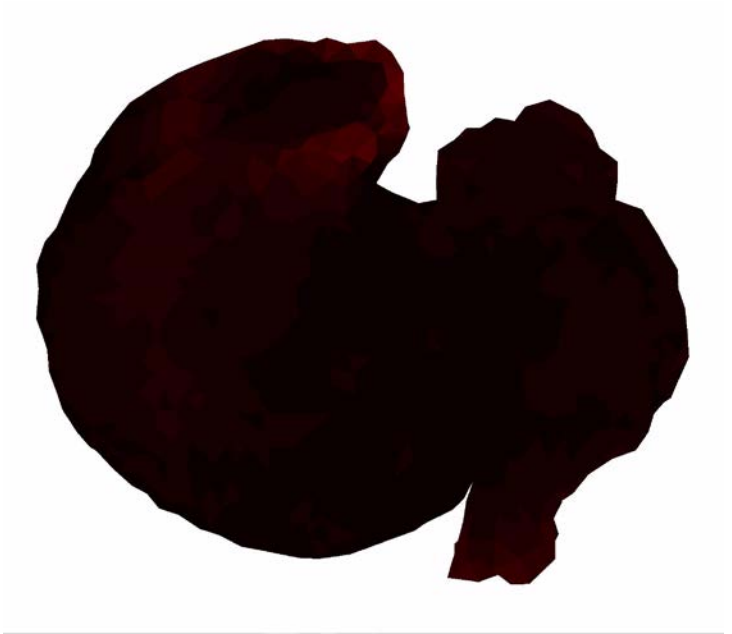


Figure 10



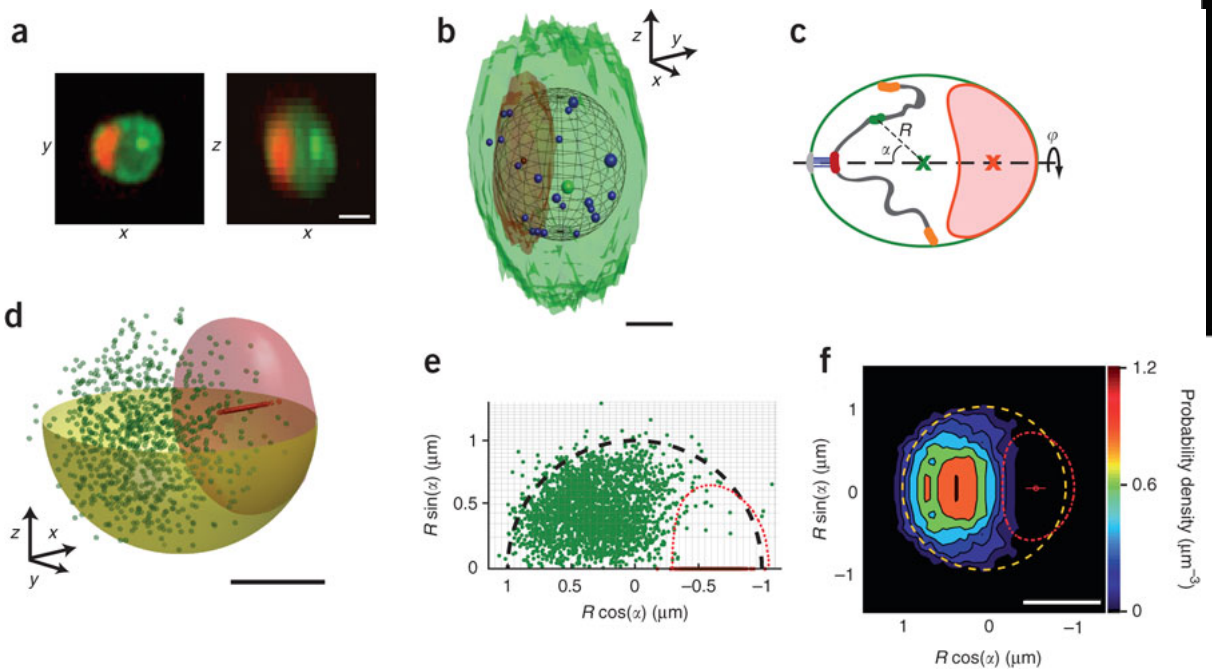


Voltage and calcium *in vivo*

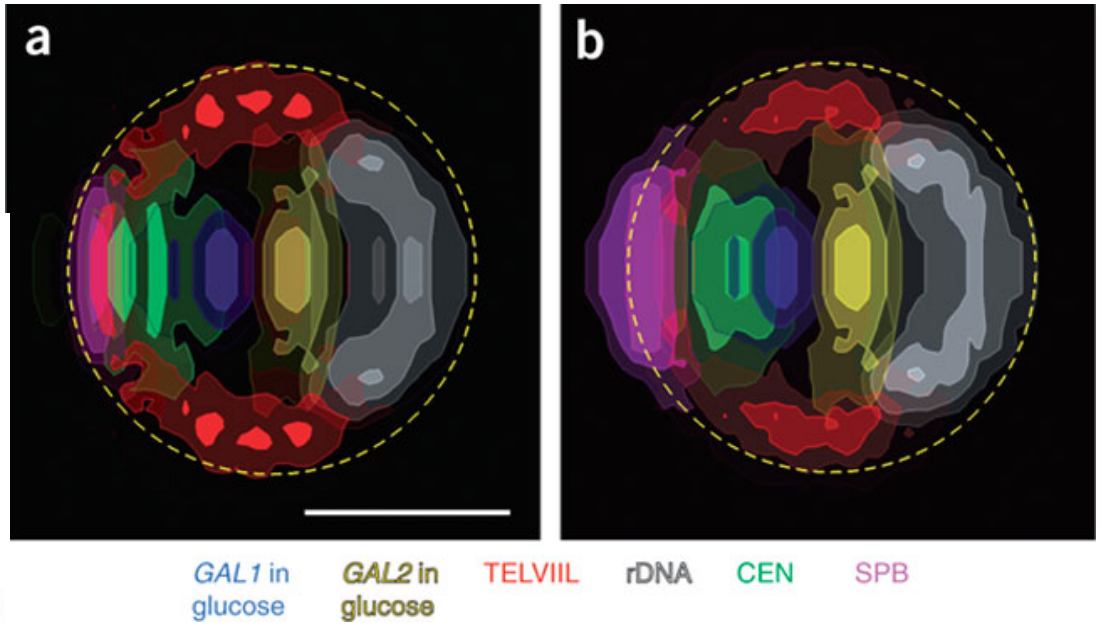


Applications of spinning disk

- Live cell, 3D imaging at fast rates



Gene territories in chromatin DNA
DNA diffuses quickly, high time resolution required



Likelihood of gene sequence in nuclear space

Cell migration *in vivo*

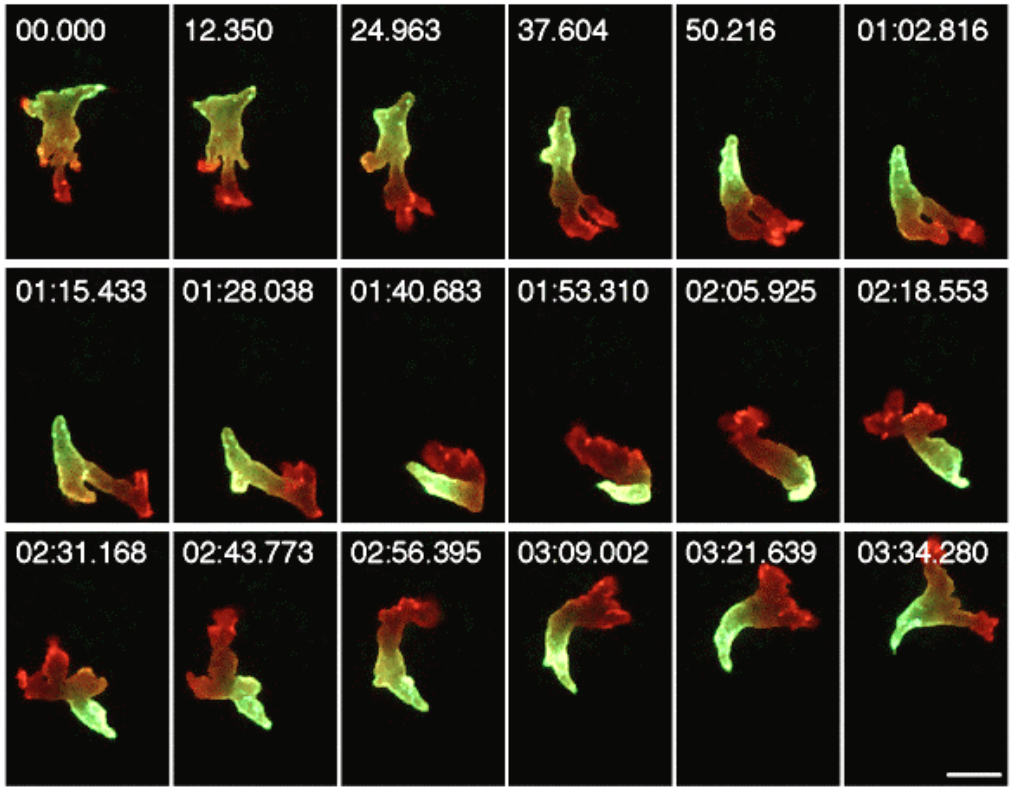


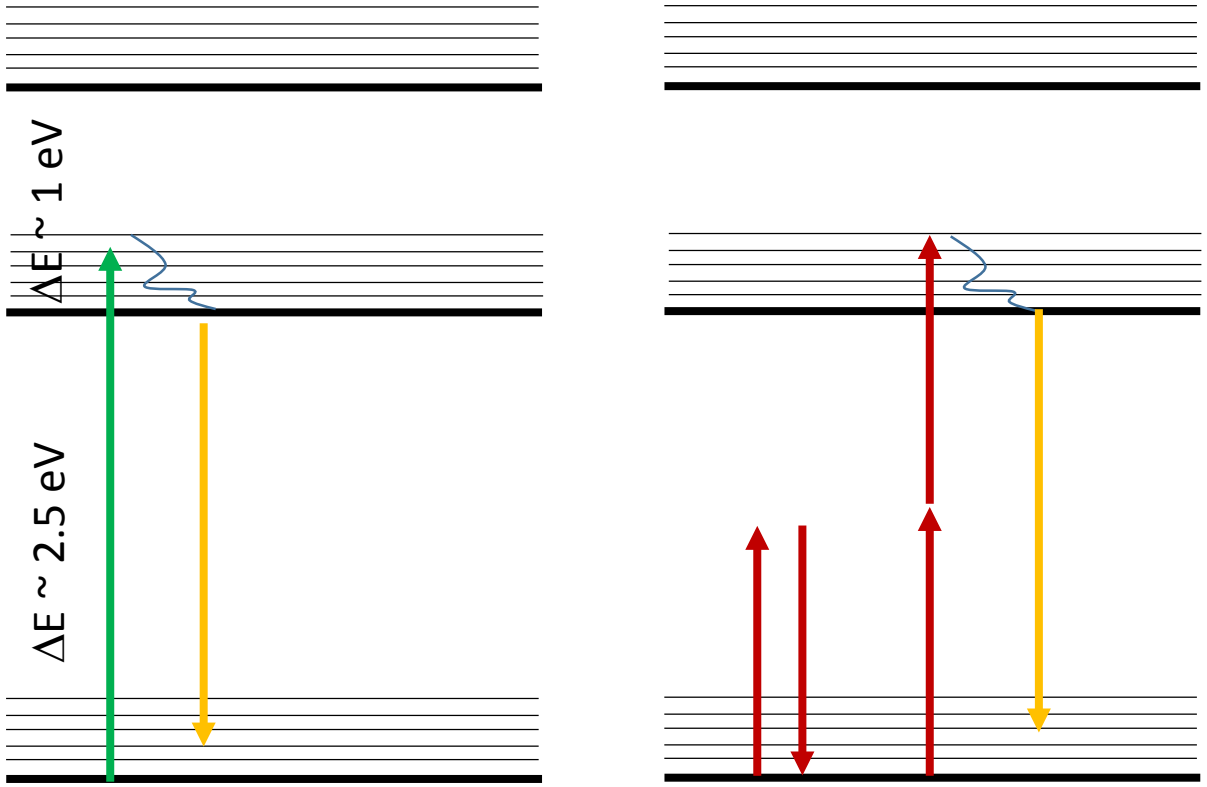
Fig. 3 Spinning disk confocal imaging of organelle subcellular localization. Time-lapse maximum intensity projection image of a neutrophil expressing γtubulin-GFP (*green*) and a nuclear probe, mCherry-histone H2B (*red*). The Microtubule Organizing Center (MTOC-*arrow*) is localized in front of the nucleus during neutrophil random motility in vivo. Scale bar: 10 μm

Two photon imaging

- Because 2 photons are better than 1
- Very high axial resolution

Moving from ground state to excited state

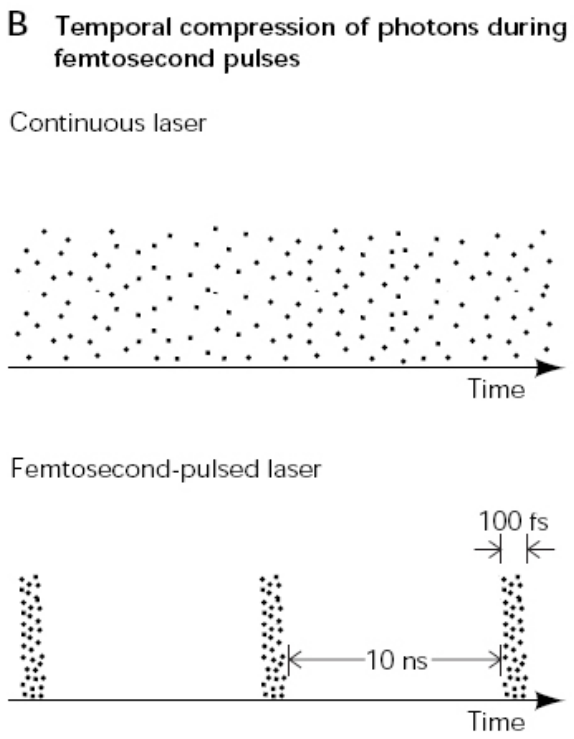
- The energy between the ground state and the excited state is a void
- However, if you send in a photon, the electron will live in the void for a VERY short time
- If the atom gets hit by a second photon during that VERY short time, the electron can be promoted completely into the excited state



$$E = \frac{hc}{\lambda} = \frac{1240\ eVnm}{\lambda}$$

2 Photon characteristics

- In order to stimulate the same fluorophore twice during the lifetime of the virtual state, the intensity has to be VERY high
- To achieve the high intensities, we have to use pulsed lasers
- Pulses are very short, so during that time they have a high intensity



Photon flux for CW or ultrafast laser:

CW: 1 W @ 488 nm
-> 2.5 x 10^{18} photons/sec

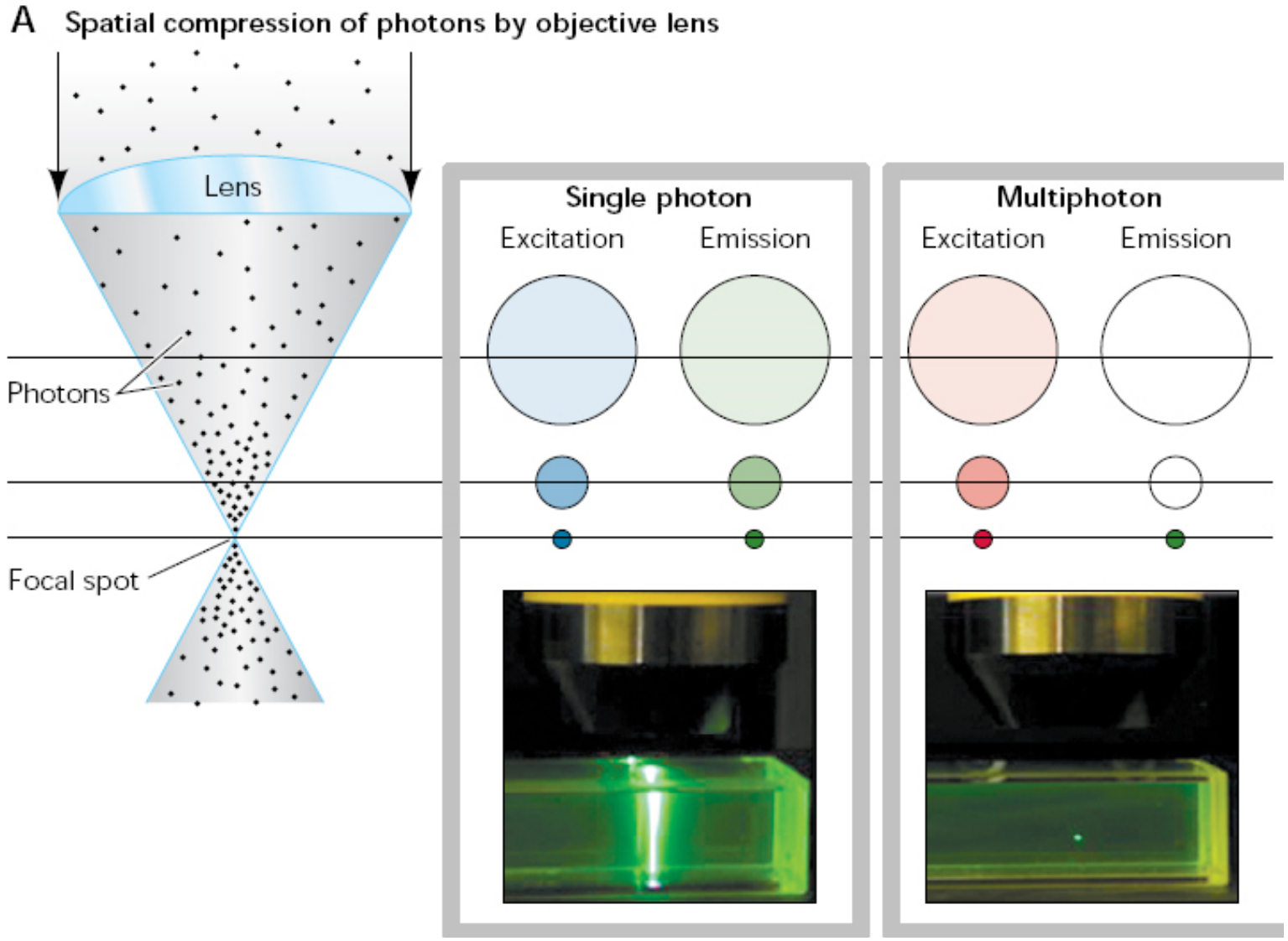
MaiTai:
1 W @ 920 nm
100 fs pulse
80 MHz rep rate

->125,000 W/sec during pulse
= 5.8 x 10^{23} photons/sec

More power, or shorter pulse, lead to higher intensities

2 Photon absorption α Intensity^2

- Absorption needs 2 photons, so absorption is proportional to I^2
- Doubling absorption requires quadrupling intensity
- Excitation ONLY occurs in focused region where intensity is high enough
- Pulsed light is (relatively) harmless to cells, so that's good.



Localized excitation in 2 Photon

- Since we control the only point where excitation occurs, we don't need the pinhole
- We don't photobleach the out of plane sample
- Allows for scattered photons to be collected
- Move detector as close to objective as possible. We're not imaging

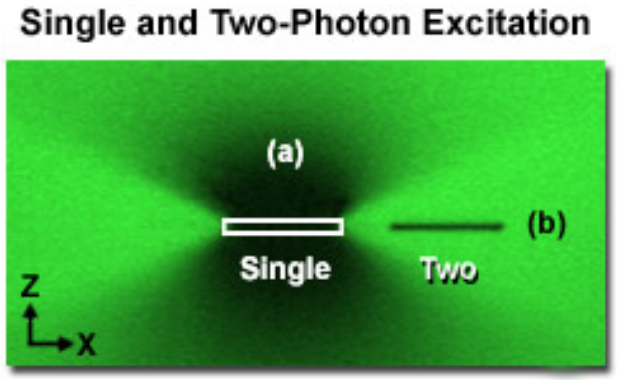


Figure 3

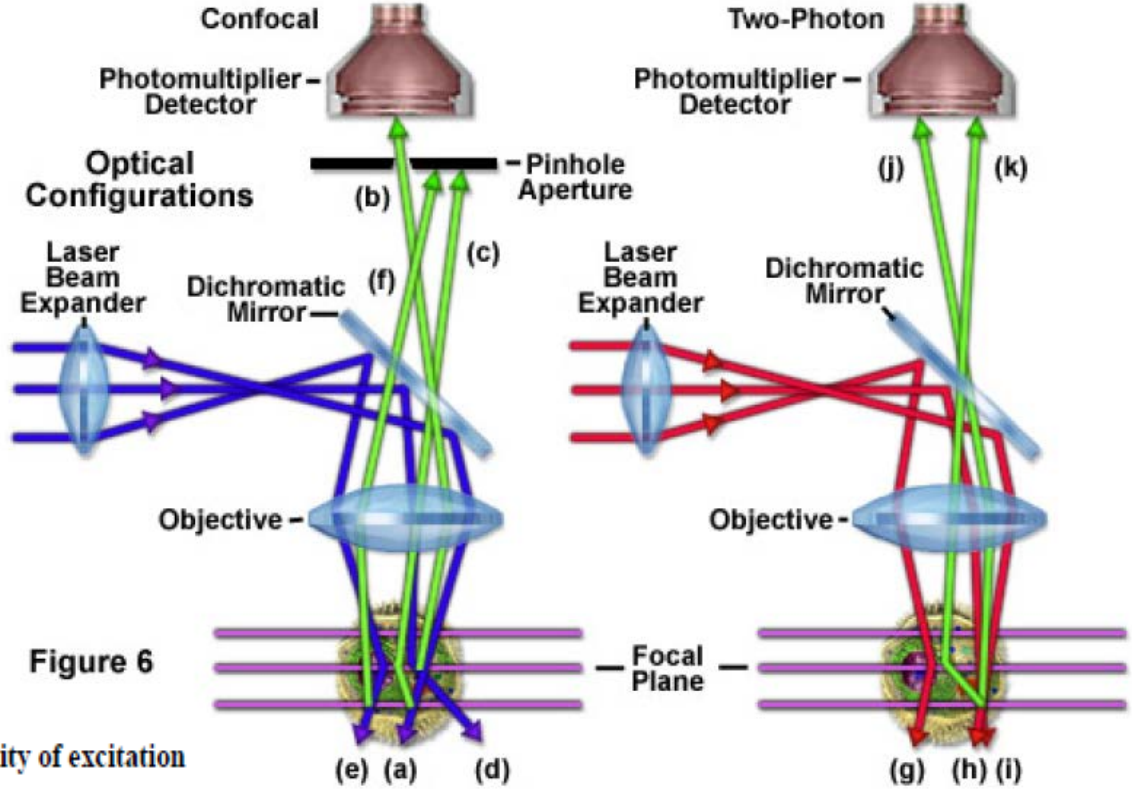


Figure 6

$$n_a \propto \delta\left(\frac{P_{avg}^2}{\tau f^2}\right)\left(\pi\frac{NA^2}{hc\lambda}\right)^2$$

n_a: probability of excitation
δ: excitation cross section
P_{avg}: average power incident light (peak power)
τ: pulsewidth
f: repetition rate
NA: Numerical aperture
h: Planck's constant
c: Speed of light
λ: Wavelength

Resolution in 2 photon

- The x,y resolution is the product of the excitation and emission PSF
- Since we're exciting with IR light (large λ) resolution is worse than confocal of similar dyes
- Better than widefield, but occurs only at a single z plane

Widefield

$$r_{x,y} = \frac{0.61\,\lambda}{NA}$$

$$r_z = \frac{1.4\,n\,\lambda}{NA^2}$$

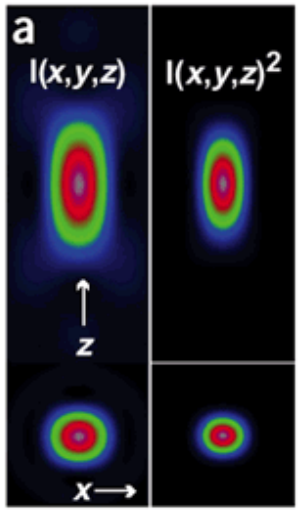
Confocal

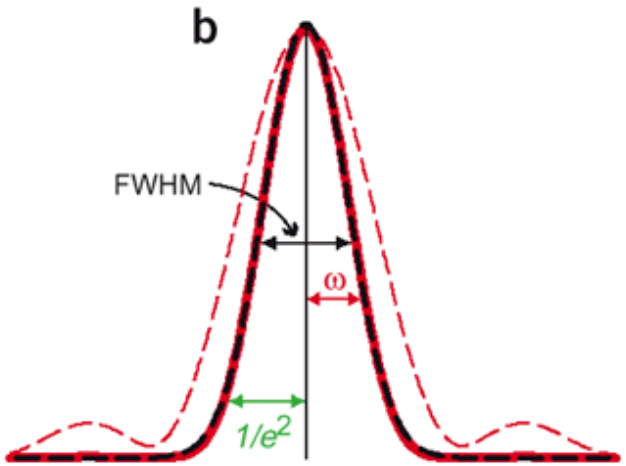
$$r_{x,y} = \frac{0.46\,\lambda}{NA}$$

2 Photon

$$r_{x,y} = \frac{.325\,\lambda}{\sqrt{2}NA^{.91}}$$

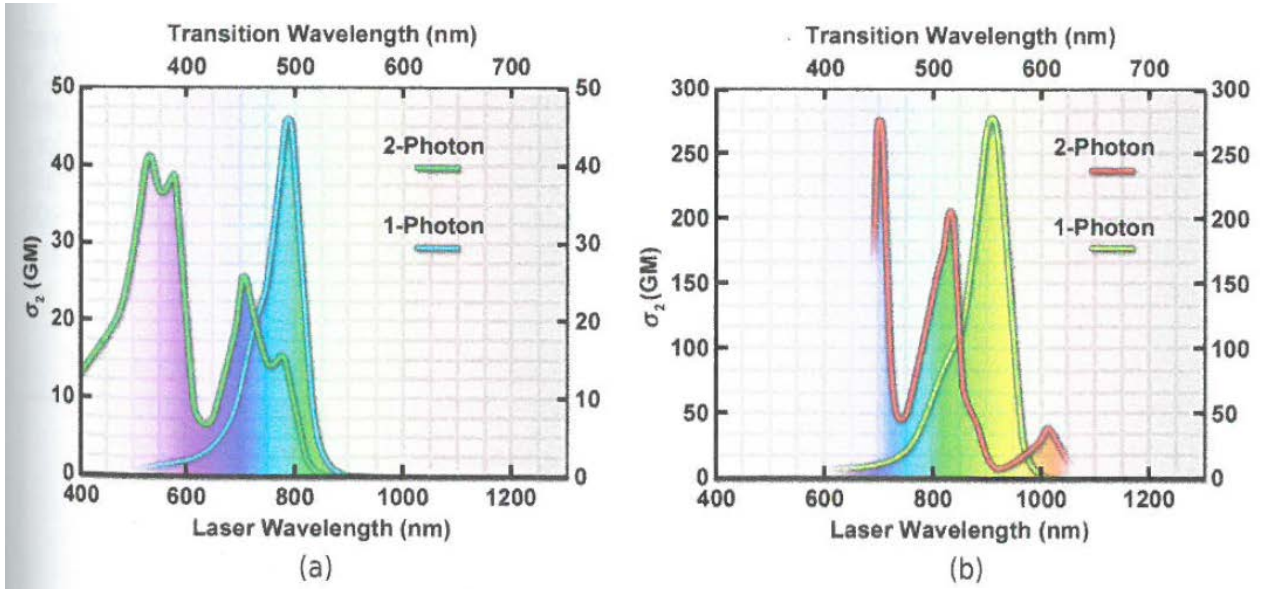
$$r_z = \frac{0.532\,\lambda}{\sqrt{2}} * \frac{1}{n - \sqrt{n^2 - NA^2}}$$

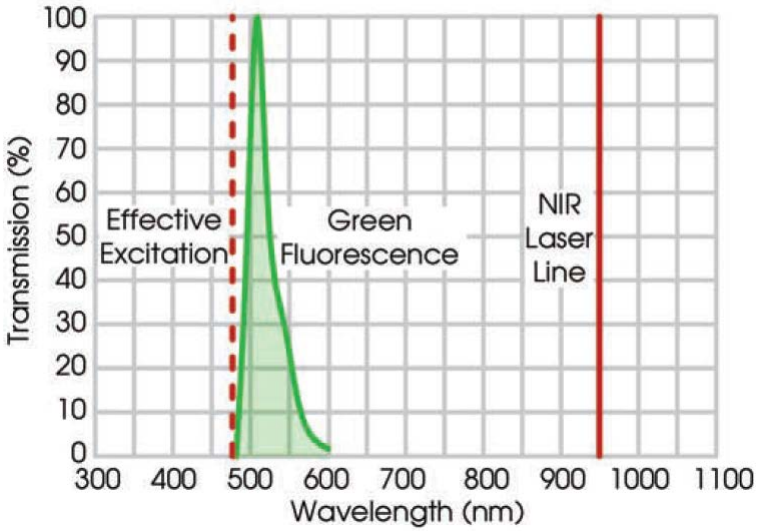


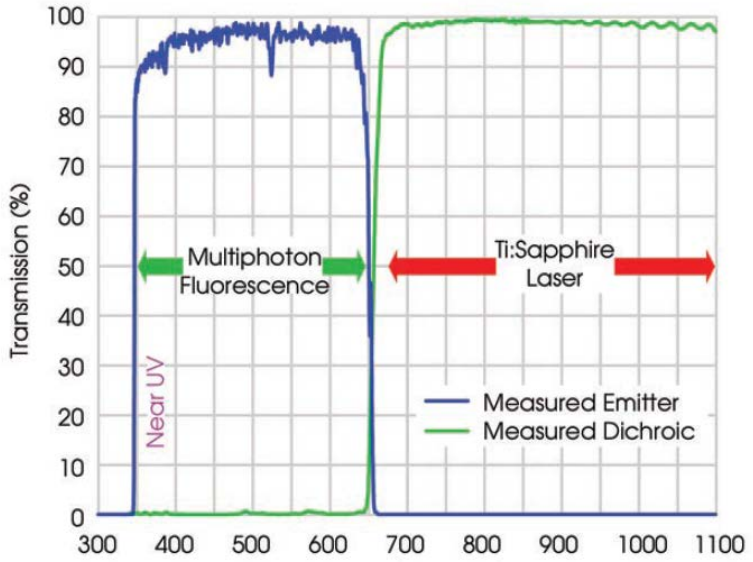


Two photon spectra

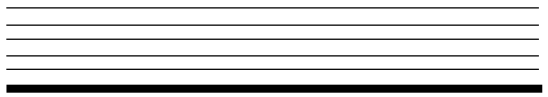
- For still mostly unknown reasons, the 2 photon spectrum is not just 2 times longer than the single photon
- Unpredictable, but it's almost always blue shifted to what you expect
- Fluorescence emission is exactly the same as 1 photon
- Excitation and emission filters unique to 2 photon modality

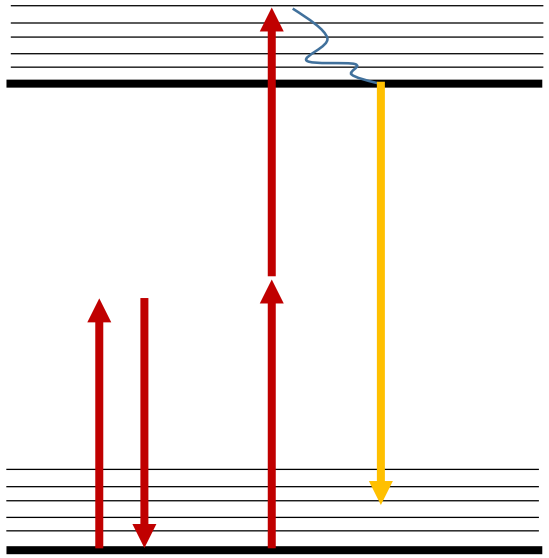






2 Photon



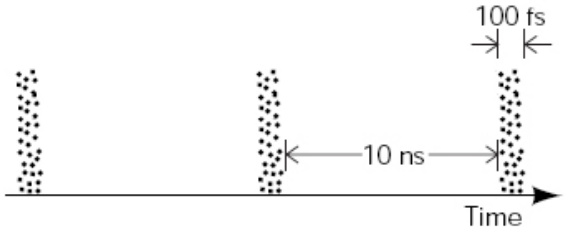


$$E = \frac{hc}{\lambda} = \frac{1240\ eVnm}{\lambda}$$

B Temporal compression of photons during femtosecond pulses

Continuous laser

Femtosecond-pulsed laser



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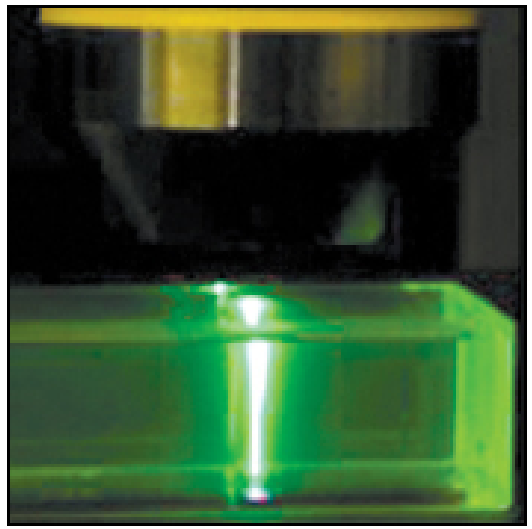
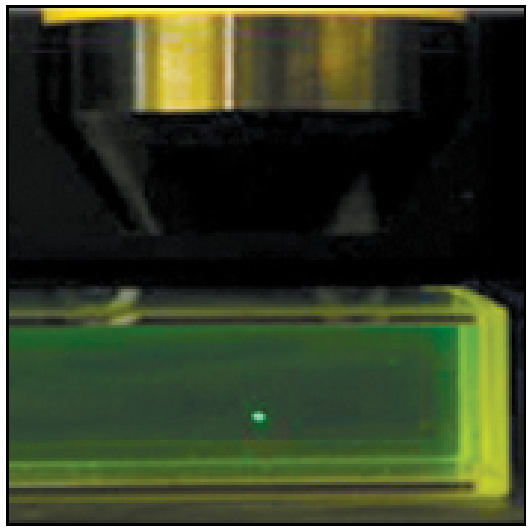




Common dyes and FPs for 2 photon

- In general, very hard to predict 2 photon absorption spectrum
- If it's a good 1 photon dye, it's likely it's a good 2 photon dye
- Best to use something that has been reported before
- 2 photon cross section often given in GM (Goeppert Mayer) – Nobel winner for predicting 2 photon in the 30s

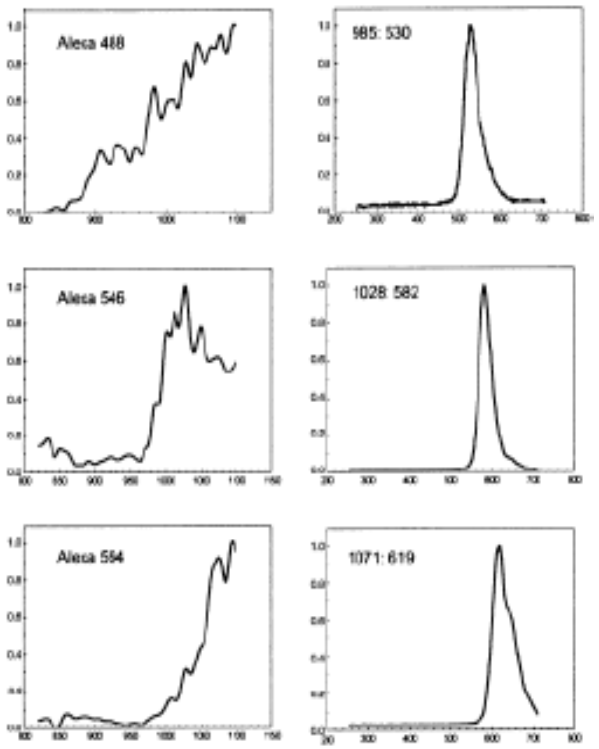
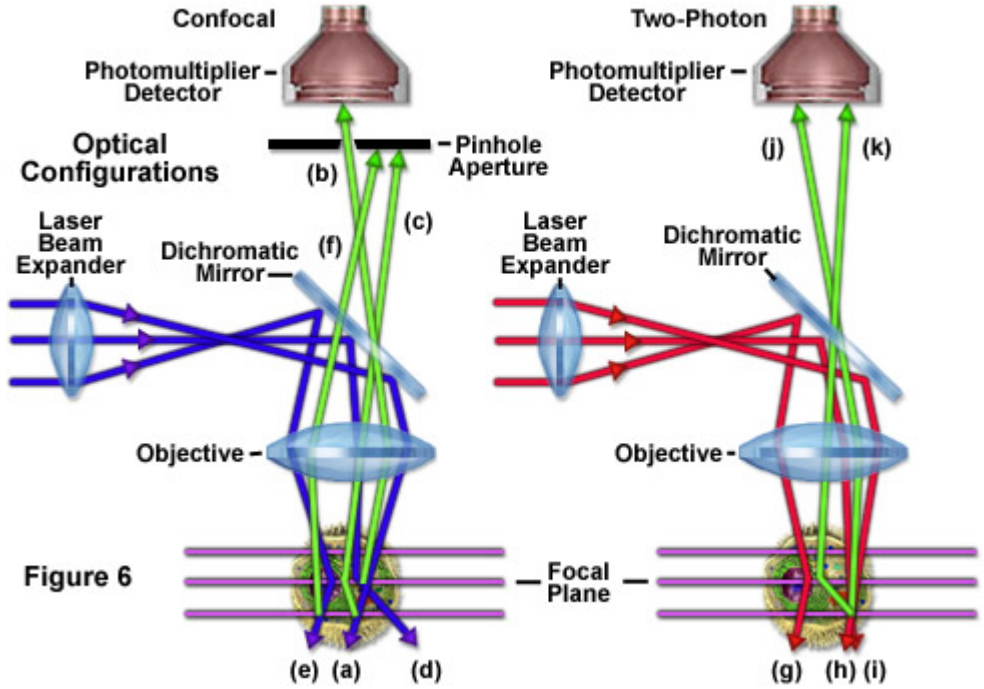


Table 1. Fluorophores and Chromophores for Two-Photon Excitation

Fluorophores/Choromophores	Φ[a] (GM)	2PE[b] (nm)	Em. (nm)	Note	References
Calcium indicators					
Fluo -3, -4, -5F, 4FF et al.		810[c]	520–530		(Yasuda et al., 2004)
Oregon Green BAPTA -1, -2 et al.		810[c]	520		(Yasuda et al., 2004)
Calcium green-1 + Ca^{2+}; Calcium green-1 – Ca^{2+}	30, 2	820	530		(Xu and Webb, 1996; Xu et al., 1996)
Fura-2 + Ca^{2+}; Fura-2 – Ca^{2+}	6, 0.2	800	505		(Wokosin et al., 2004)
Indo-1 + Ca^{2+}; Indo-1 – Ca^{2+}	3.5, 1.5	700	400		(Xu and Webb, 1996; Xu et al., 1996)
Quantum dots					
Quantum dots	up to 47,000	broad	variable		(Larson et al., 2003)
Fluorescent proteins					
eCFP	100–200	800–900	505		(Zipfel et al., 2003)
eGFP	100–200	900–1000	510		(Zipfel et al., 2003)
eYFP	100–200	930–1000	530		(Zipfel et al., 2003)
mRFP, mCherry		1030[c]	610	Ytterbium-doped laser	(Campbell et al., 2002; Shaner et al., 2004)
Photoswitchable fluorescent proteins (see also Lukyanov et al., 2005)					
paGFP		750[g]	515		(Patterson and Lippincott-Schwartz, 2002; Schneider et al., 2005)
Kaede		730[d]	520→580	green to red; tetramer	(Ando et al., 2002)
KFP1		1120[d]	600	tetramer	(Chudakov et al., 2003)
Dronpa		780[d,e], 1010[d,f]	520	reversible	(Ando et al., 2004; Habuchi et al., 2005)
psCFP		800[d]	470→510	cyan to green	(Chudakov et al., 2004)
PA-mRFP		760[d]	605		(Verkhusha and Sorkin, 2005)
KikGR		760[c]	520→590	green to red; tetramer	(Tsutsui et al., 2005)
Dendra		960[d]	505→575	green to red	(Gurskaya et al., 2006)
mEosFP		780[d]	520→580	green to red	(Wiedenmann et al., 2004)
Caged glutamate					
MNI-glutamate	0.06	730			(Matsuzaki et al., 2001)
Caged calcium					
DM-nitrophen	0.013	730		K_d: 2 nM[h], 1.5 mM[i]	(Brown et al., 1999; Momotake et al., 2006)
Azid-1	1.4	700		K_d: 230 nM[h], 0.12 mM[i]	(Brown et al., 1999; Momotake et al., 2006)
NDBF-EGTA	0.6	710		K_d: 14 nM[h], 1 mM[i]	(Momotake et al., 2006)

2 Photon enables deep tissue imaging

- Tissue scatters, making confocal impossible
- Two photon can collect the scattered photons because we do not need the pinhole
- The excitation light can travel further because it is in the IR
- Imaging deep in tissue is primary reason to use 2 photon



$$\sigma_s = \frac{2\pi^5}{3}\frac{d^6}{\lambda^4}\left(\frac{n^2-1}{n^2+2}\right)^2$$

On to Matlab…