Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

CMB551 1A:

Microscopy and Image Analysis in Cell Biology

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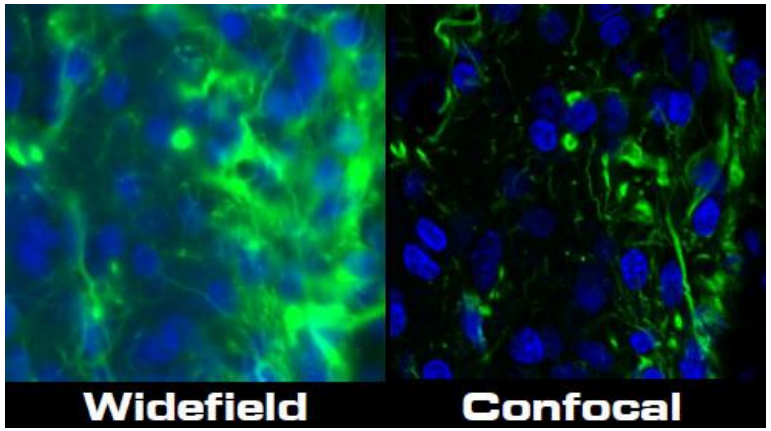
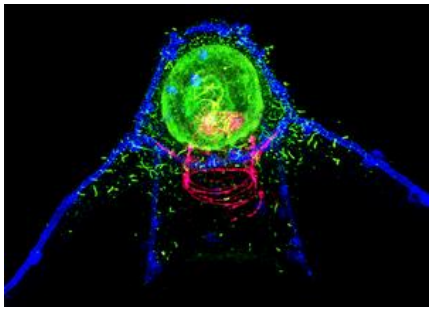
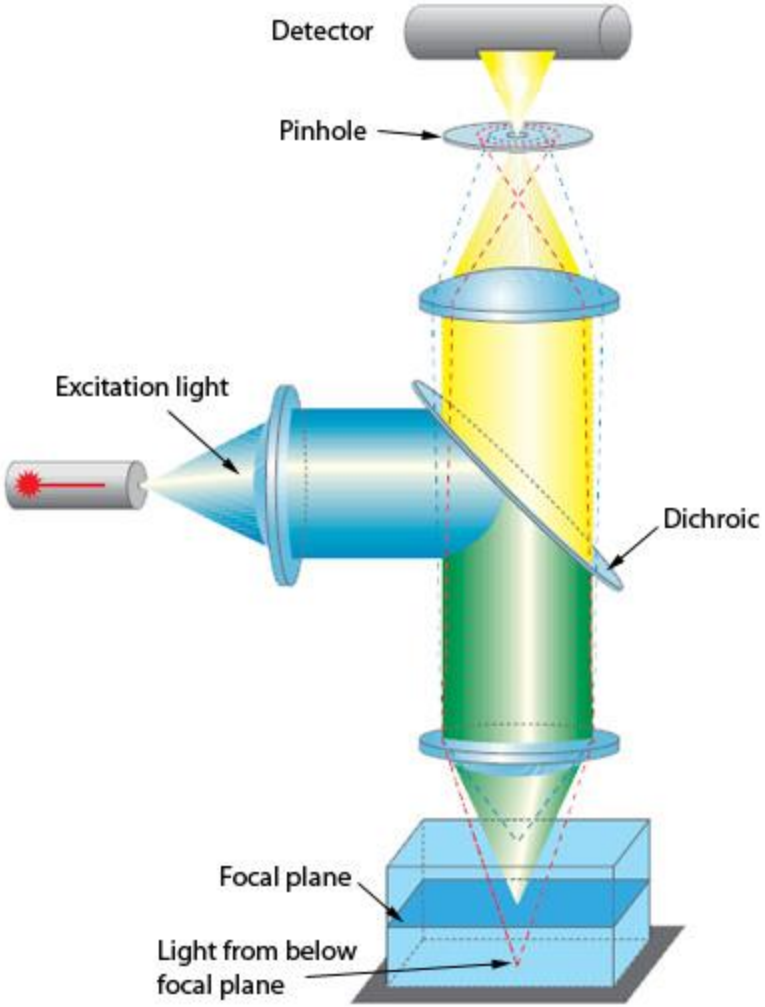
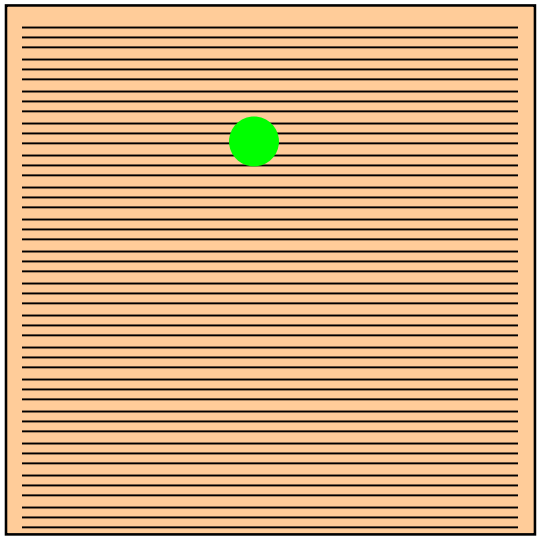
Other ways of doing something like this





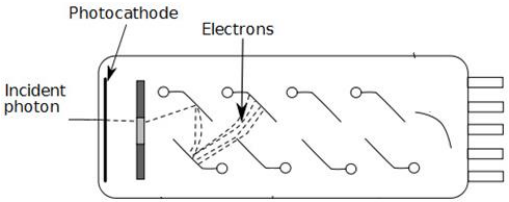


Without these problems . . .



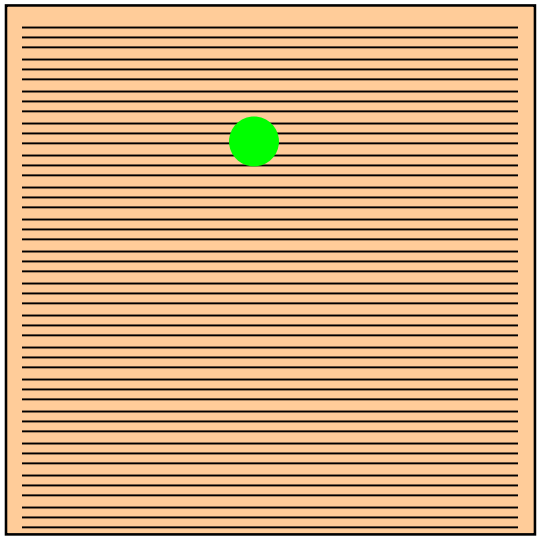
Point based consequences:

- Speed (eg 260,000 fold for 512 image)
- SNR (if quicker than 7 hr per image)
- Photodamage (bright spot to compensate)



Low detector QE, few photons

Without these problems . . .



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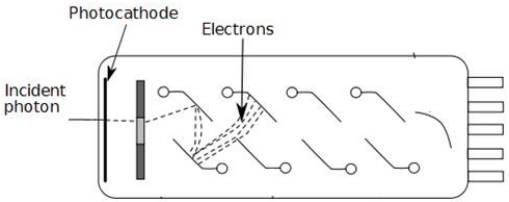
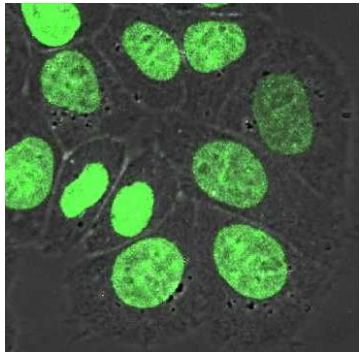
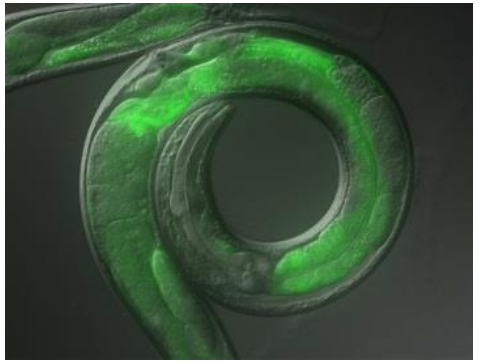


Low detector QE, few photons

Fixed

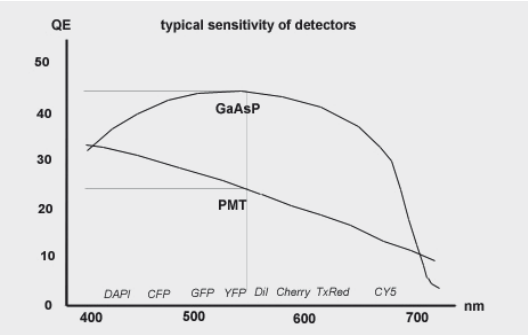
Live





What can we do about this?

Improve confocals



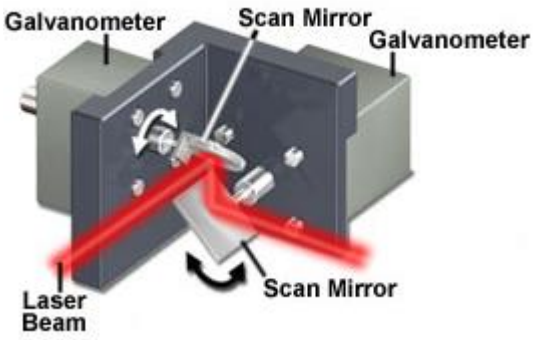
GaAsP detectors improve QE

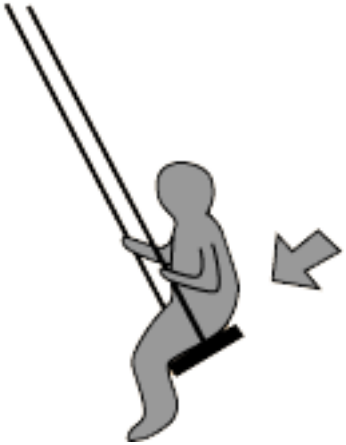
Drive confocals wisely

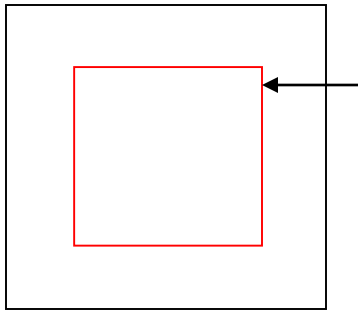
- Keep laser power to a minimum
- Open the pinhole a bit
- Under sample
- Get ok-ish images

- Use the best reagents
- Keep live samples otherwise as happy as possible

Resonant scanners







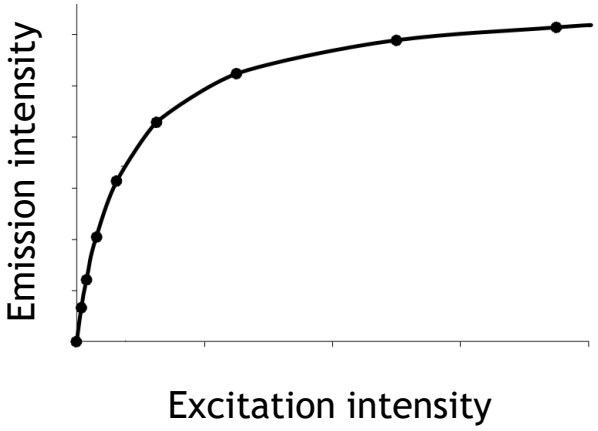
Smaller max amplitude so zoom is >1

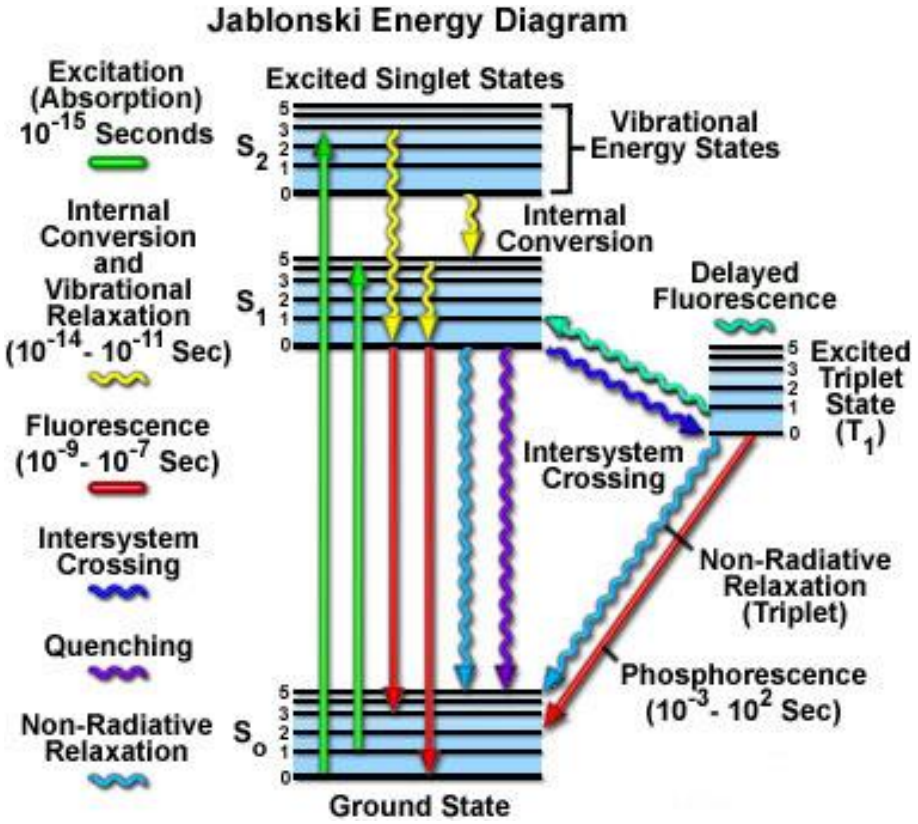
8000 Hz resonant scanner vs say 400 Hz standard scanner

Leica SP5 and SP8 have this feature

Everything else is the same except the scanner

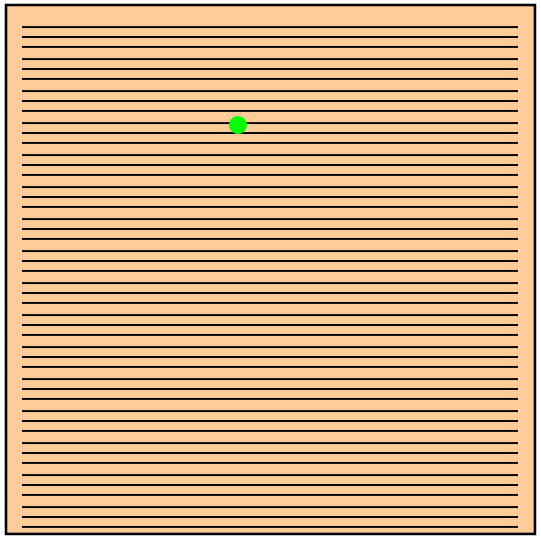
Consequences of scanning faster: reduced photobleaching

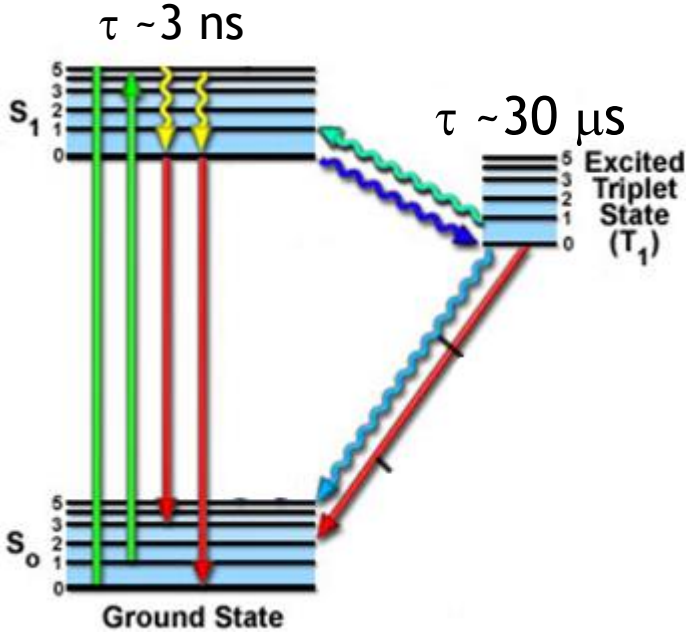




Faster scanning = lower fluorophore saturation
Phototoxicity and photobleaching are reduced

Consequences of scanning faster: reduced photobleaching





Example pixel dwell times: ~2 μSec standard, ~100 nSec resonant

The longer you illuminate, the greater the % of GFP accumulates in T

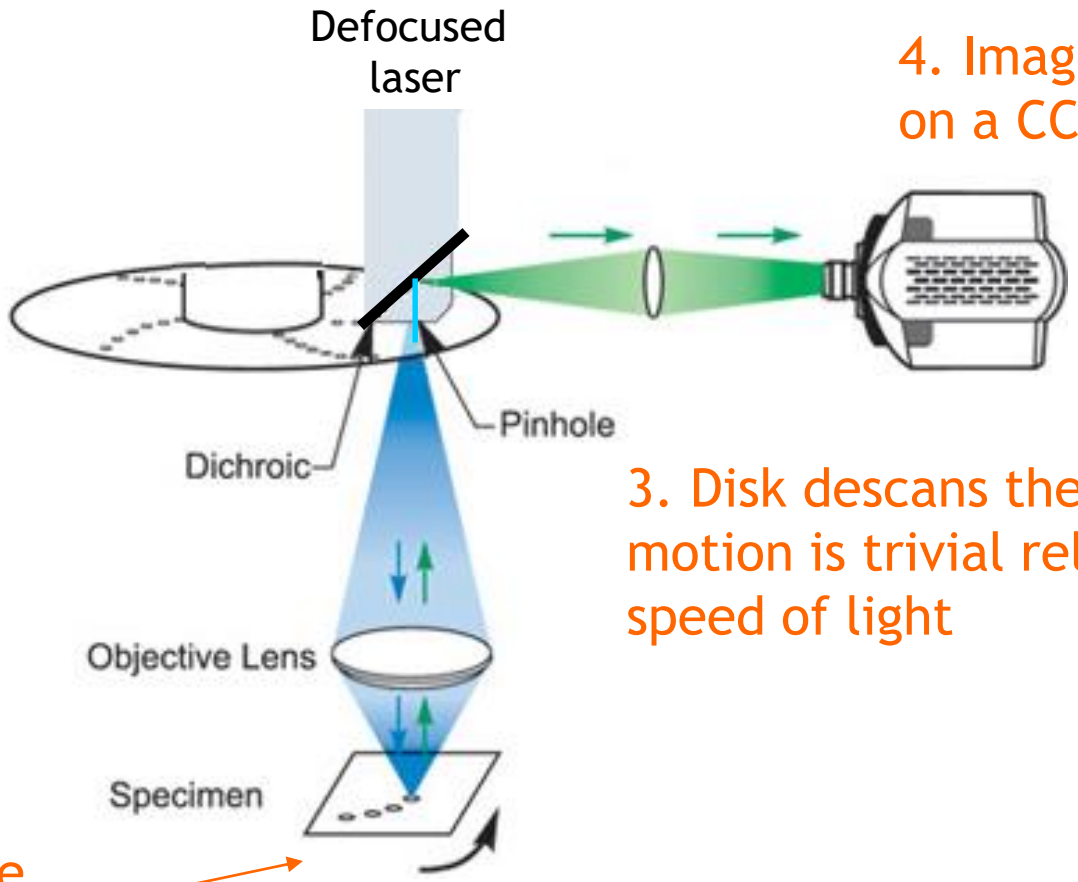
Triplet state is effectively permanent within the scale of pixel dwell time

But the time between scans of the same spot is $>\tau_T$

http://www.leica-microsystems.com/science-lab/brighter-fluorescence-by-resonant-scanning/

The spinning disk principle

1. Pinholes are in an image plane so project many spots onto the sample



4. Image captured on a CCD

3. Disk descans the light – motion is trivial relative to speed of light

2. Spots scan the sample as the disk spins

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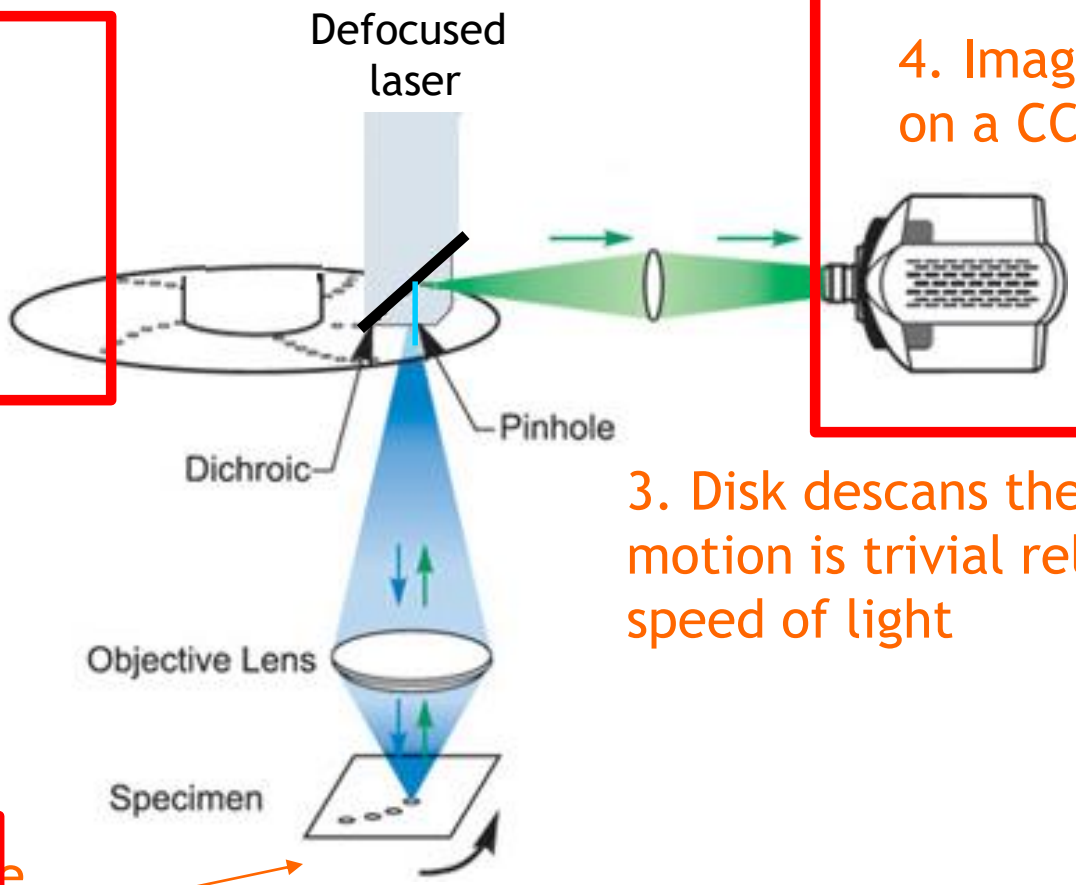

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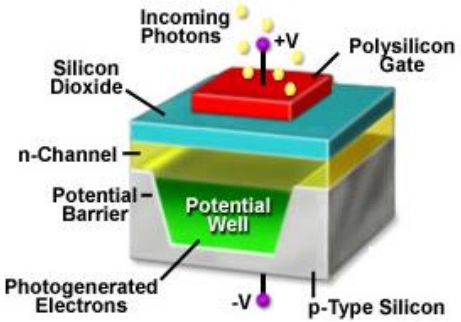
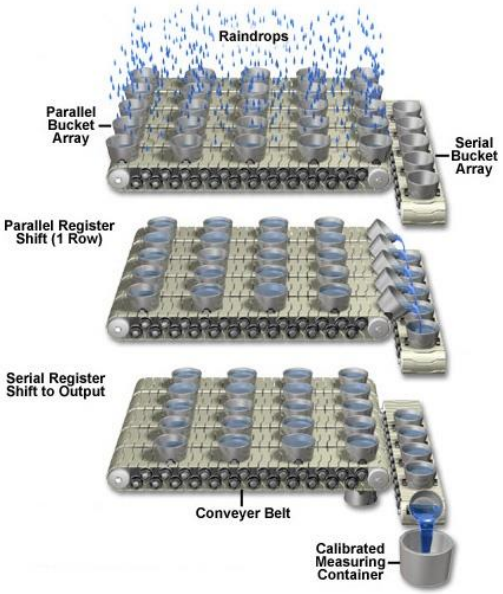
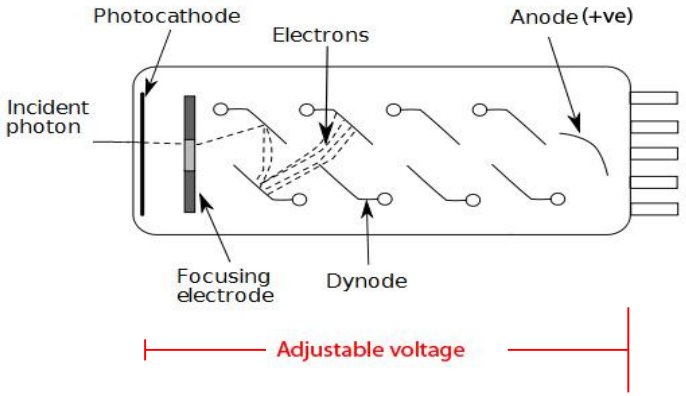
Detector comparison

CCD

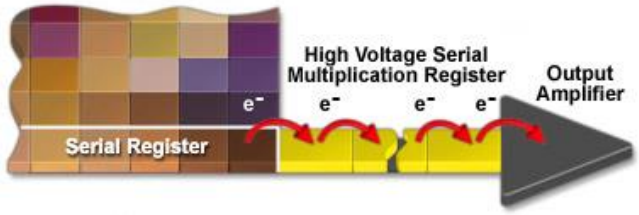




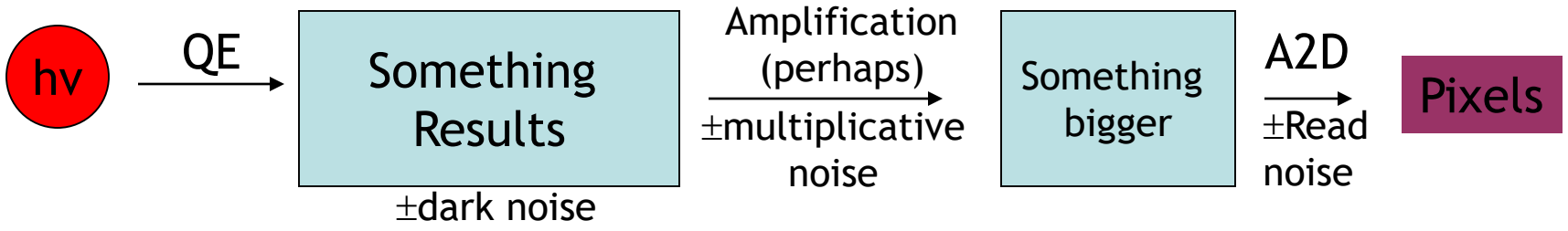
PMT



EMCCD



Photodetectors



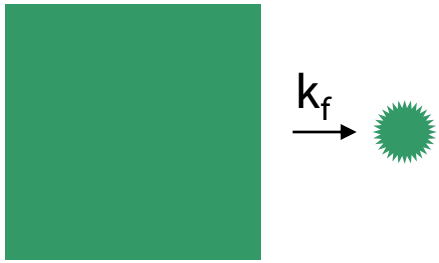
PMT	20%	An electron	Gain regulated cascade	More electrons
CCD	70%	Charge, e in well	No	Same
EMCCD	95%	Charge, e in well	em gain register	More electrons

wf 1,000-20,000 PSC 20-100 Disk 10-3,000

S:B over SNR

Shot Noise

Statistical noise in photon arrival, not from the detector

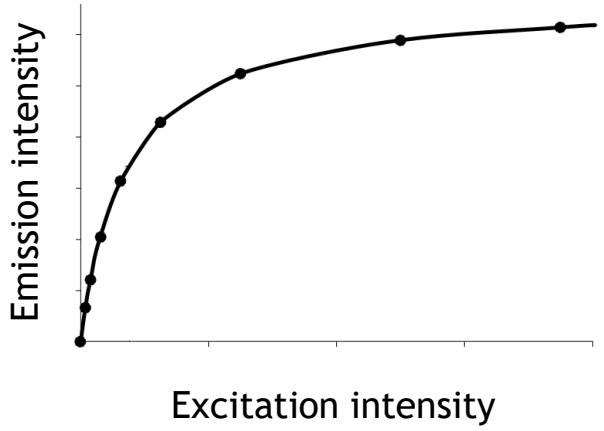


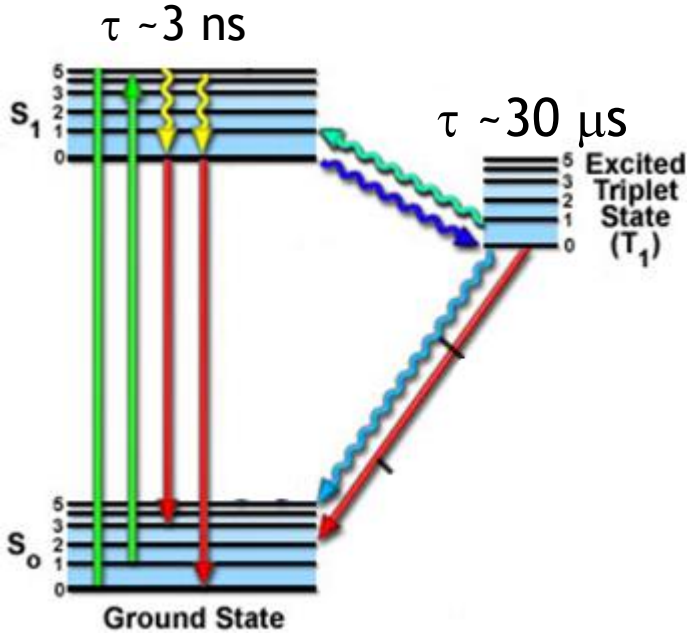
k_f	How many photons emitted in 1 second?
100,000 photons/sec	~100,000 (normal distribution)
2 photons/sec	Probably not 2, Poisson distribution

$$SNR = \frac{N}{\sqrt{N}} = \sqrt{N}$$

NSR: 5% error 400 with 1% error with 10,000 photons

Photobleaching advantages

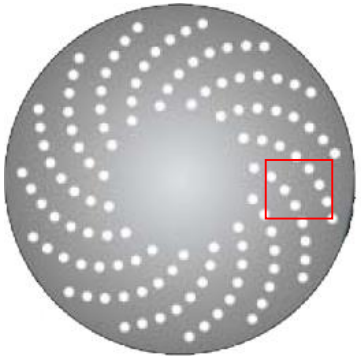




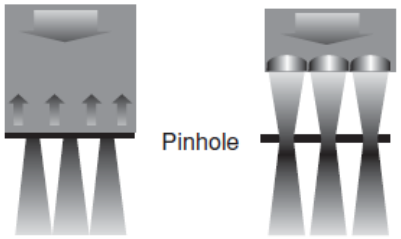
Each spot is less intense than in a point scanner

The stroboscopic avoidance of triplet

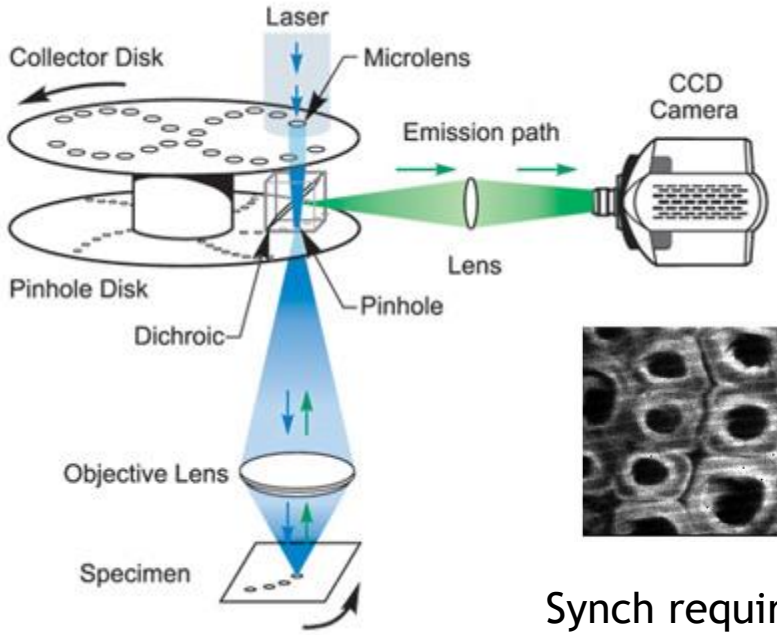
How does a spinning disk work?

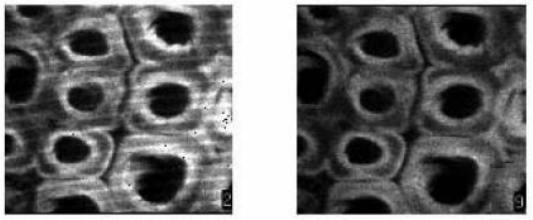


99% disk



~40% T

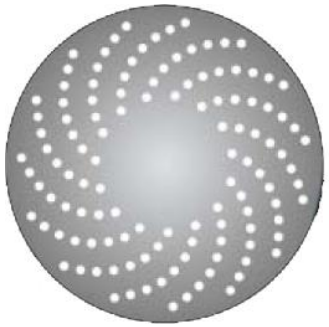




No zoom

Synch required above ~20 fps

Sectioning in spinning disks



Ideal pinhole diameter

= 0.5 λ M/NA

- 100x/1.4 = 20 μm
- 20x/0.5 = 11 μm

Trade-off for excitation intensity and emission confocality:
50-70 microns and fixed

The pinhole is > AU1 (often much more at low power lenses) so the sectioning and z-axis resolution will be less good than a point scanner

Sectioning in spinning disks

(Both these factors would be much worse without the microlens disk= increased transmission without having bigger pinholes)

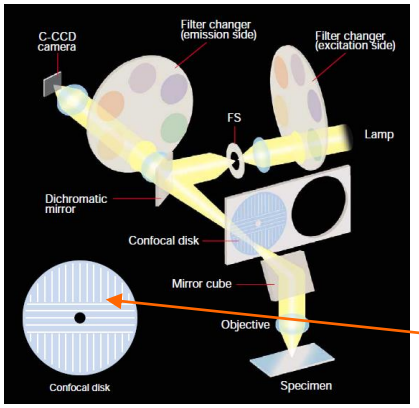
What works well on a spinning disk

Living things that need sectioning . . .

- Things which match the high magnification, high NA optimizations (eg subcellular imaging)
- Photosensitive samples
- Fast imaging

Not the dimmest samples
Not thick homogenous samples

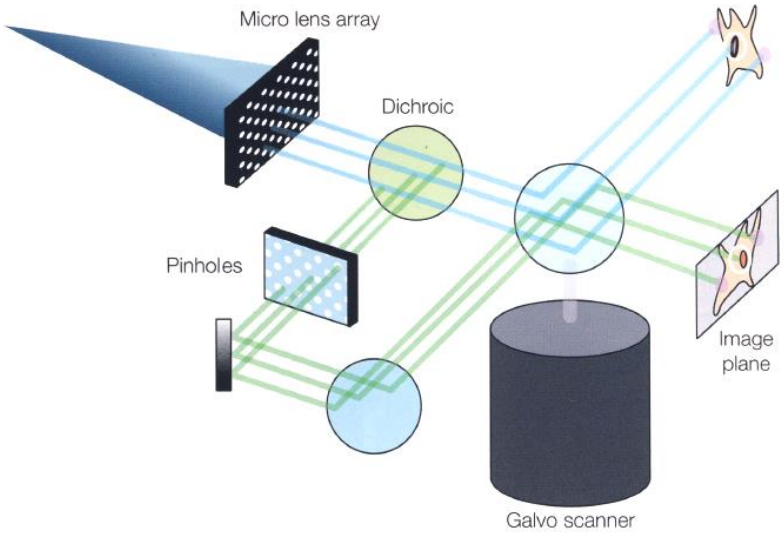
Things quite like a Yokogawa spinning disk . . .



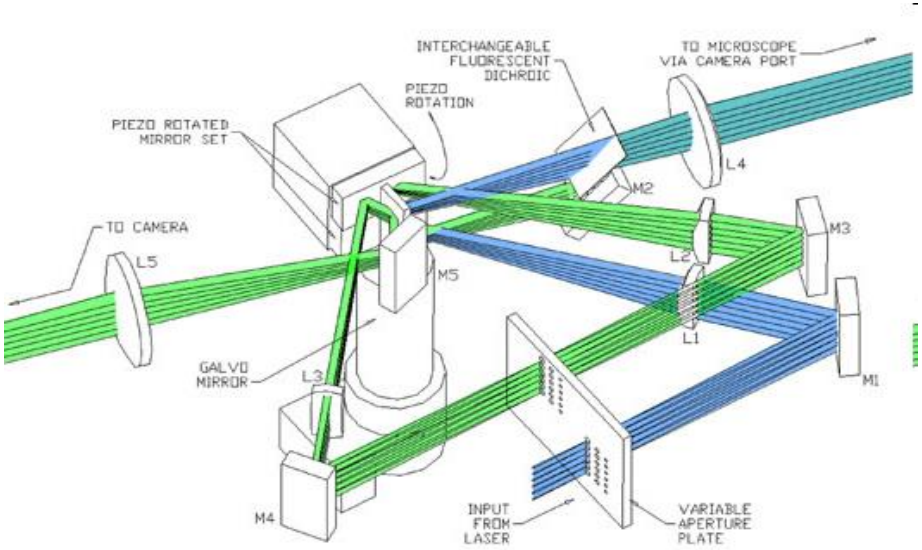
Slits not spots
Changeable

Swept field

Visitech Infinity



Prairie/Nikon



Similar to the microlens spinning disk in terms of strengths and weaknesses

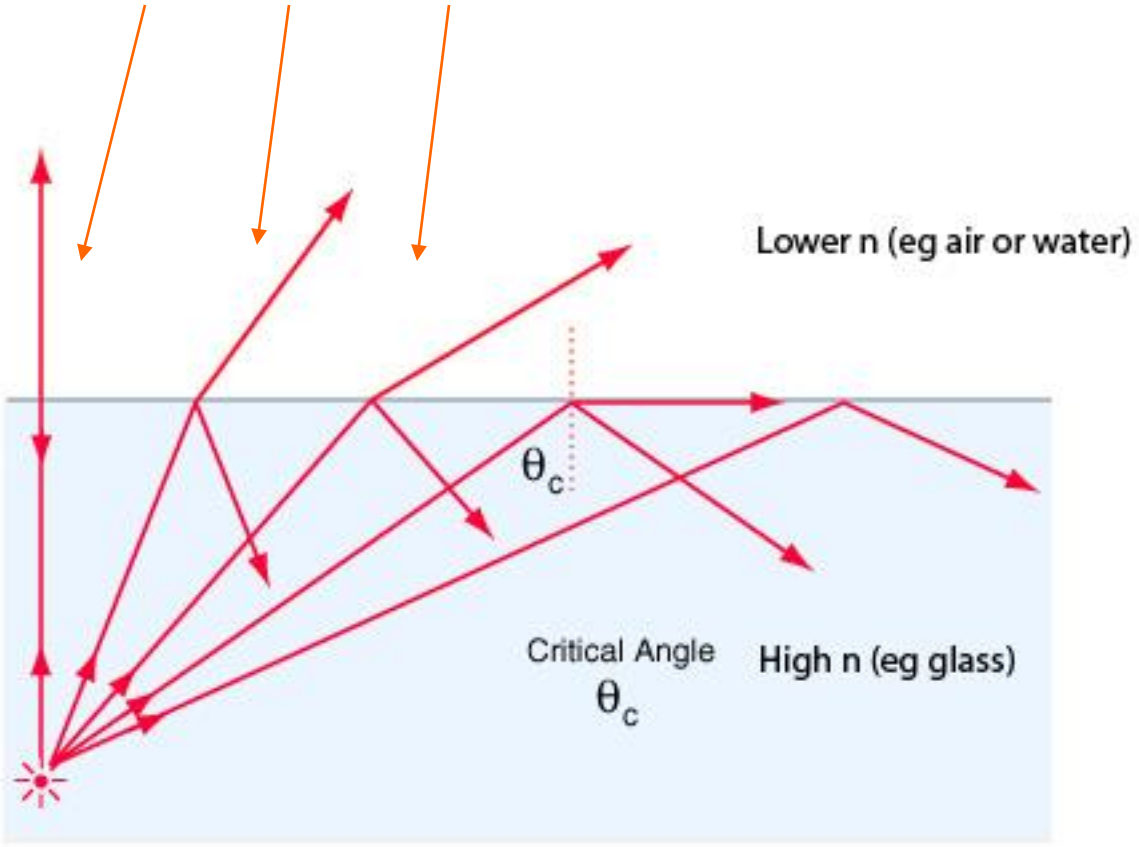
Sectioning by excitation

Excite a defined region of the z-axis and image pretty much all the fluorescence available

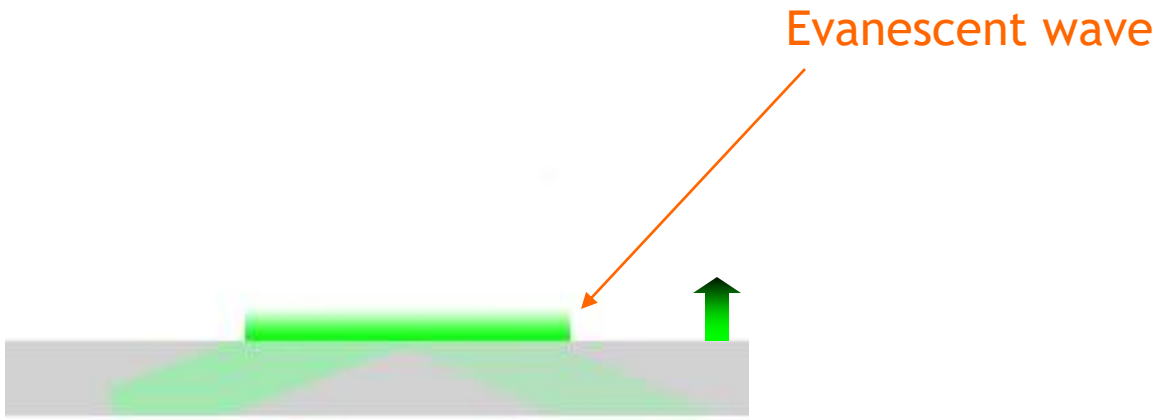
- TIRF
- Multi-photon excitation
- SPIM

Total internal reflection

Some reflected, some refracted



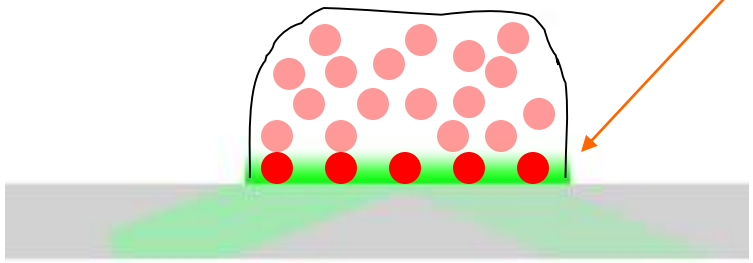
Evanescent wave



Exponential decay
of intensity

$$I_z = I_0 e^{-\beta z}$$

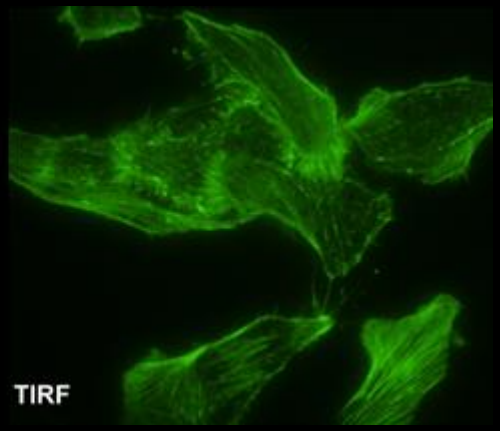
Evanescent wave



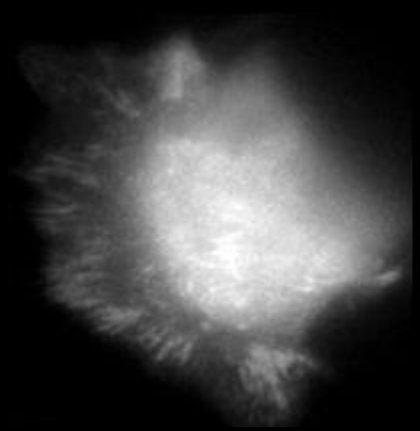
Plane of excitation
~100 nm thick

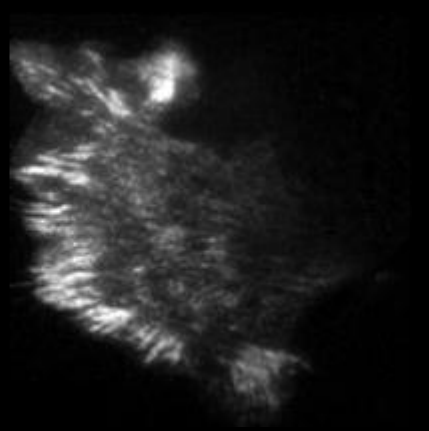
This is much thinner
than a confocal slice

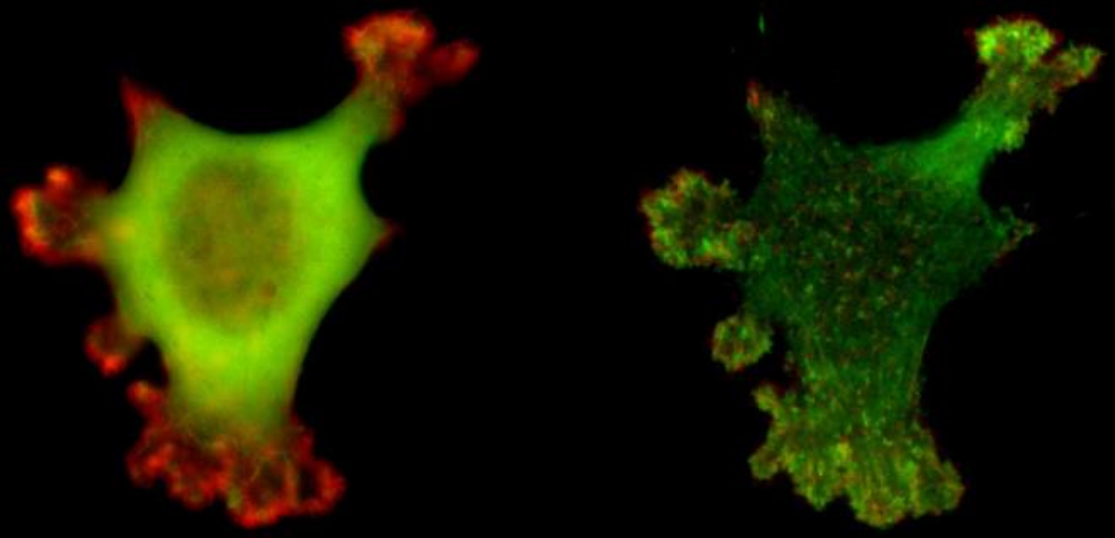
Widefield vs TIRF



Focal adhesions



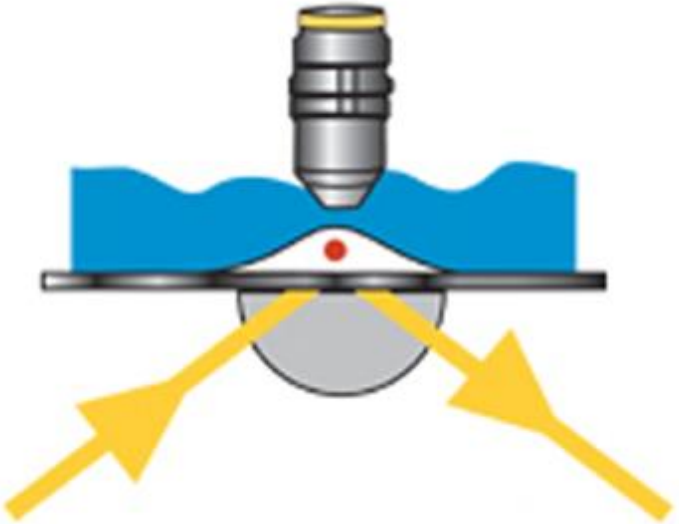




Myosin Actin

Two ways of generating and imaging TIRF

Prism-based



A bit more to align
Better SNR, lower background
Slight constraint on imaging objective
Sample access is difficult in some setups
Have to build your own

Objective-based

More convenient
Needs >1.45 NA objective
SNR still very good

Components of a TIRF system

Inverted microscope with a special TIRF objective

What is TIRF good for?

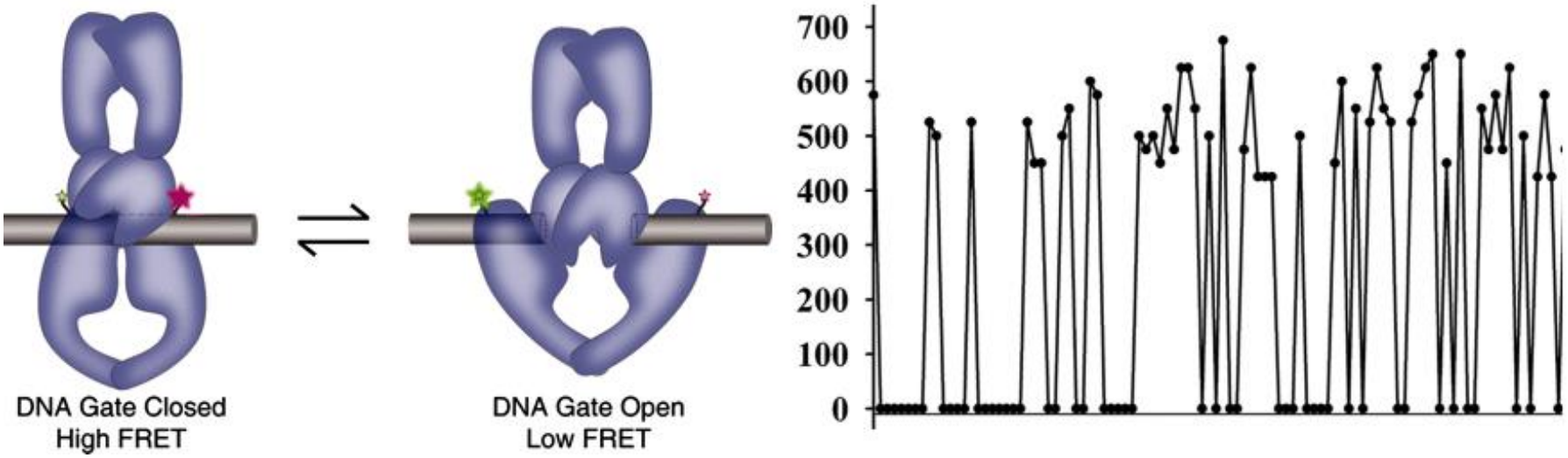
Anything at the edge of the cell/tissue

- Exocytosis/endocytosis
- Vesicle dynamics
- Cytoskeletal activity at the membrane – Focal adhesions
- Signalling in the membrane – translocation

Relatively distinct subset of samples gain from TIRF imaging

Single molecule imaging

Single molecule sensitivity allows single molecule biochemistry



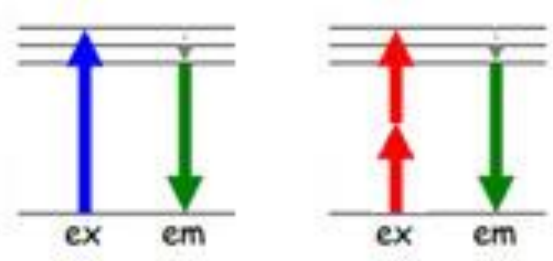
TIRF imaging is the highest SNR fluorescence imaging modality so good for single molecule studies, for which you need . . .

- A sensitive way of imaging
- A way of only having a few molecules in your imaging volume

- (also the basis of some super-resolution techniques . . .)

Two photon excitation

Single photon Two-photon



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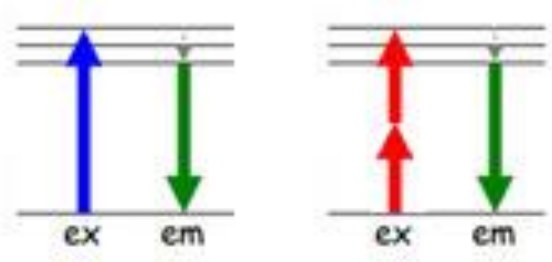
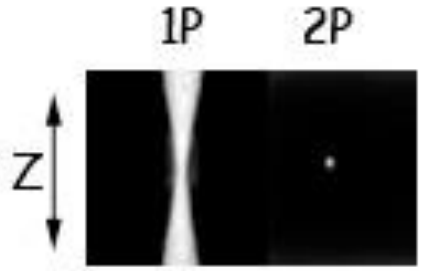
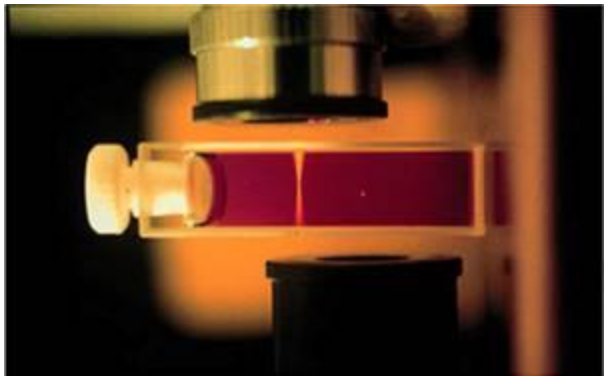
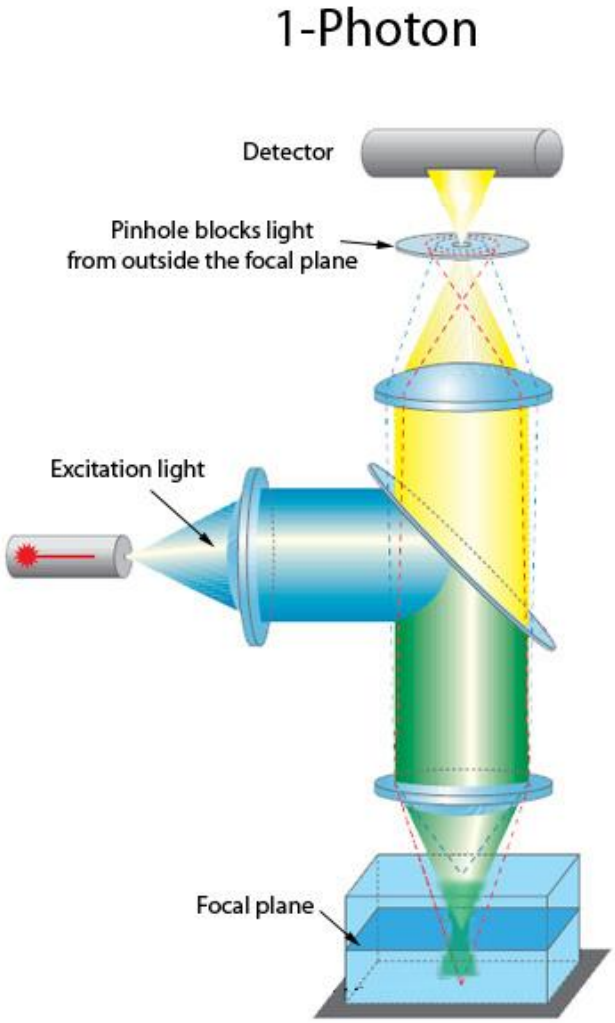


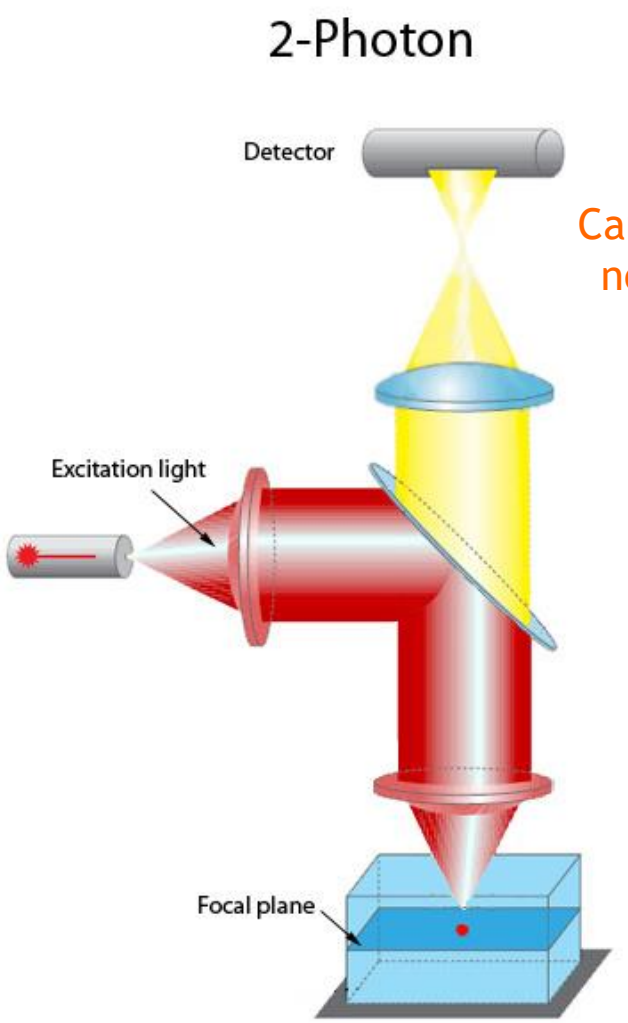




Excitation is limited to a small focal volume where photons are most concentrated

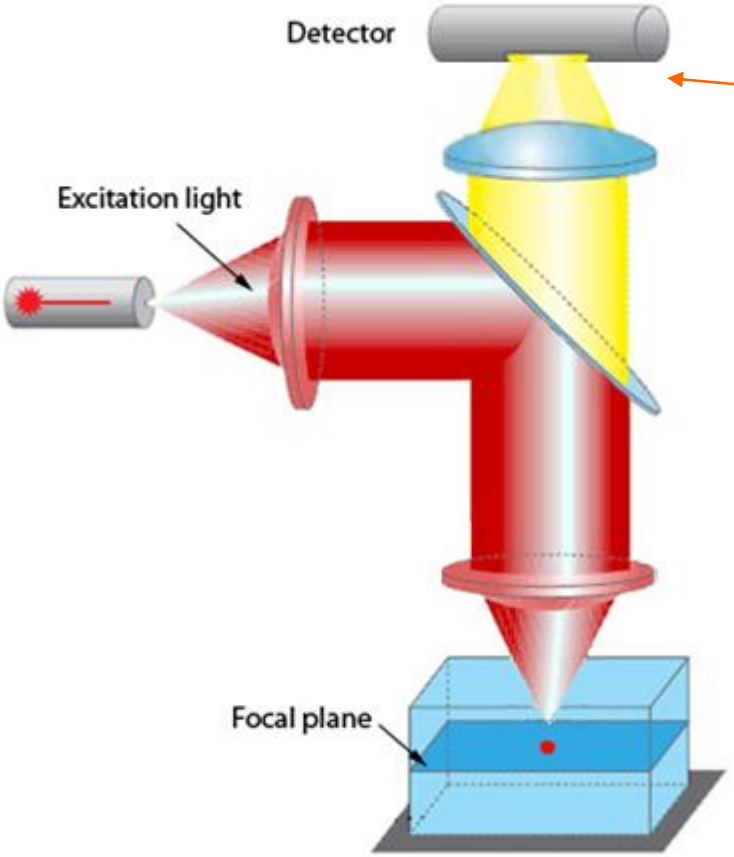
Differences to a single photon confocal





Can use all the light,
no pinhole needed

Non-descanned detector



Since we don't need to go through a pinhole, we don't even need to descan and the PMT can be close to the objective and efficient

Is it a confocal?

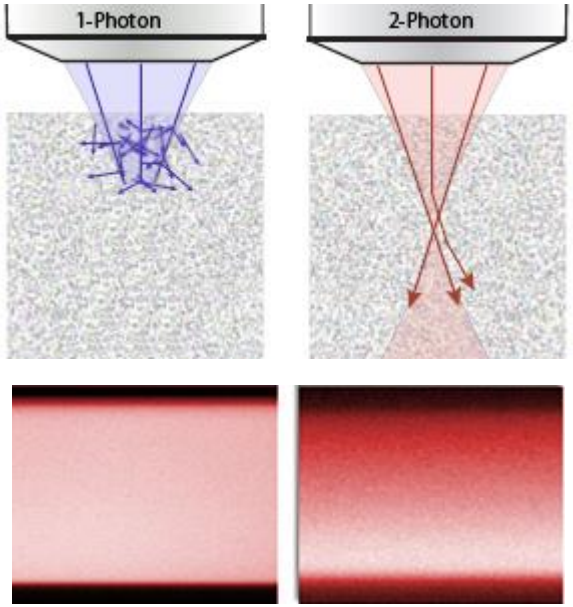
(this arrangement makes it very sensitive to room light)

2-photon advantages

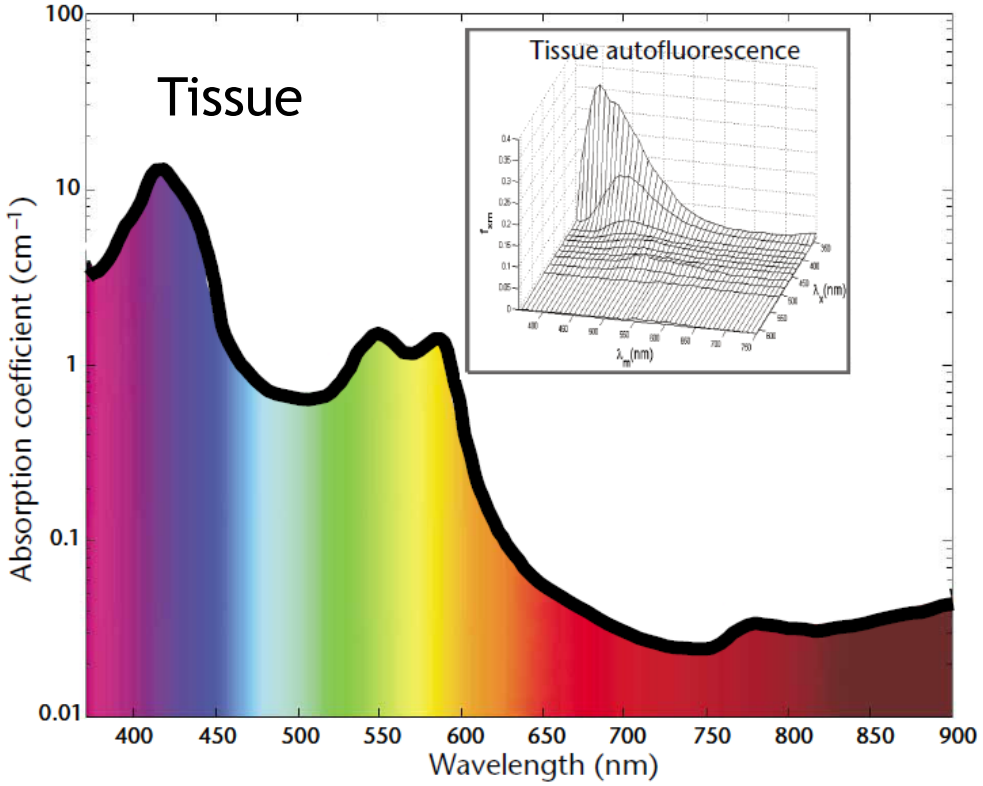
Main advantage: Imaging thicker specimens

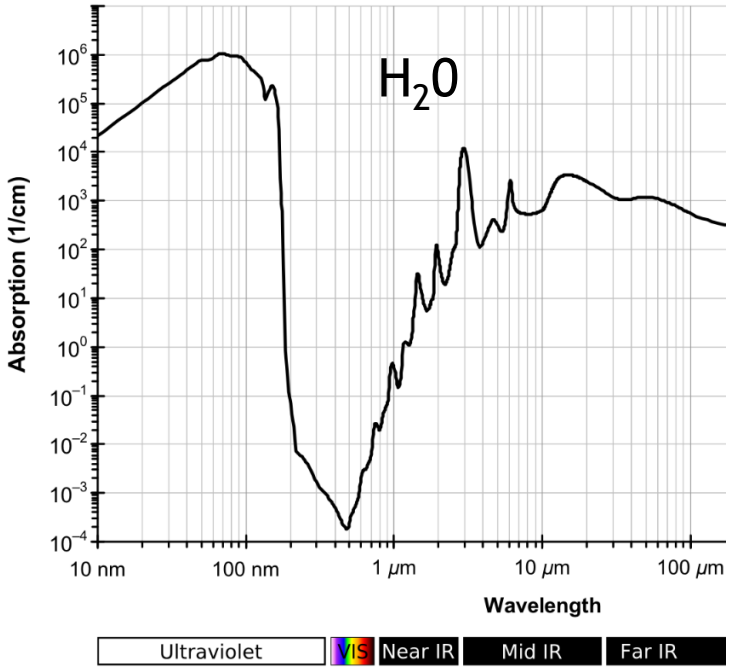
The longer wavelength excitation penetrates further into the sample. The scattered excitation light doesn't cause background fluorescence.

The excitation is also not attenuated by fluorophore absorption above the plane of focus



Tissue absorption of light of λ . . .

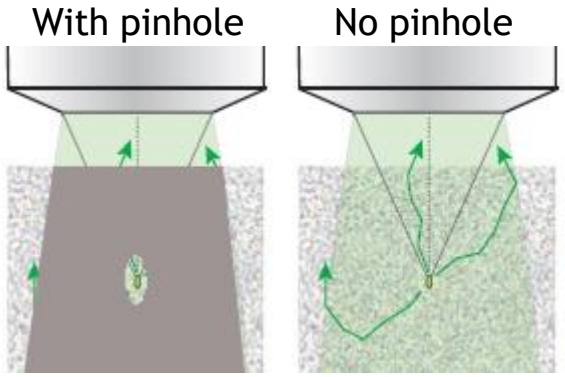




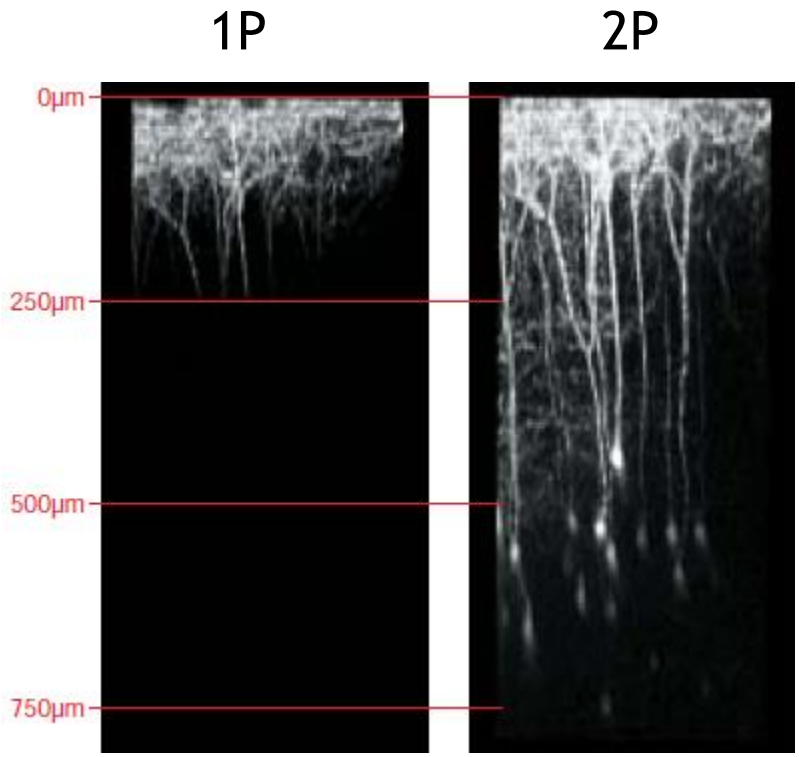
700-1200 nm is a good window between absorption/scatter by tissue and absorption by water

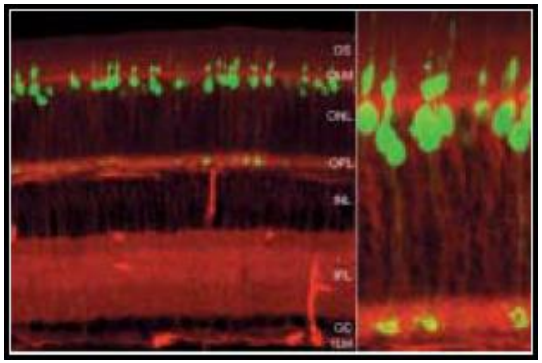
Emission advantage

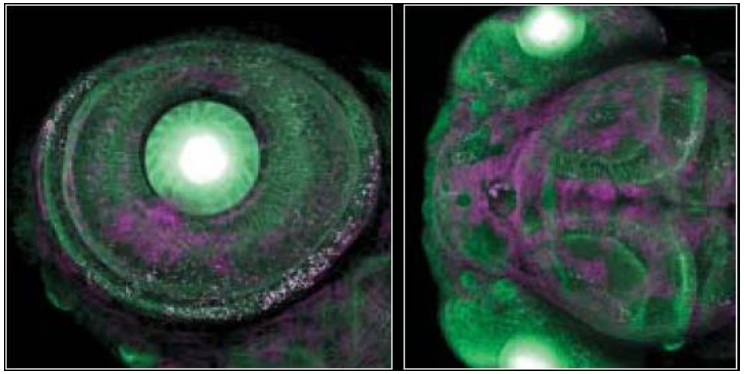
Because we are able to image all the light (no pinhole) this is less affected by scatter

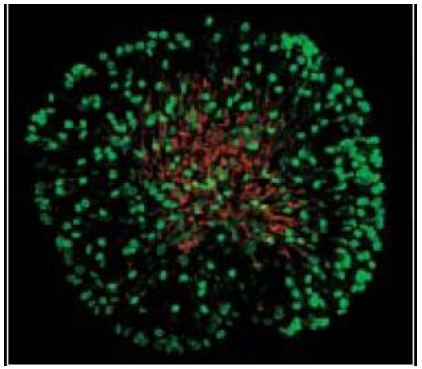


The NDD is closer and more efficient for a scattered beam path (which is hard to move efficiently through several lenses)



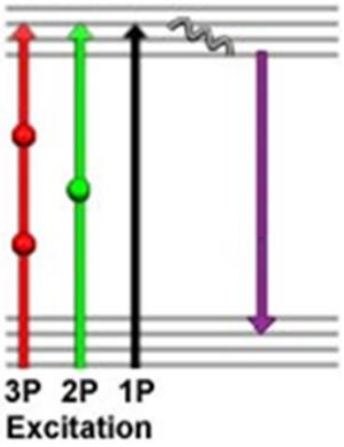




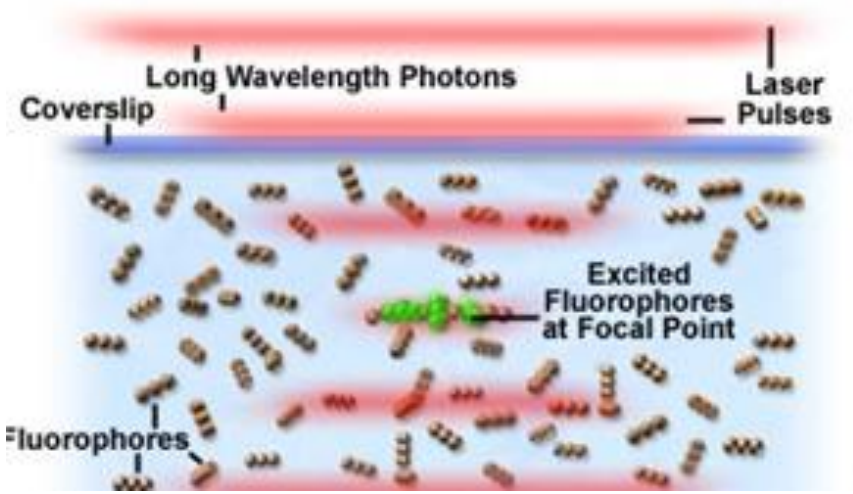


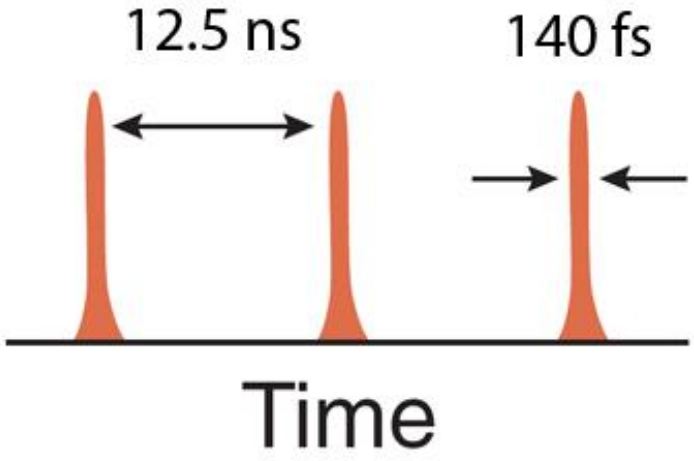
Pretty pictures from the Olympus FV1000 MPE brochure

A Pulsed laser is required for MPE



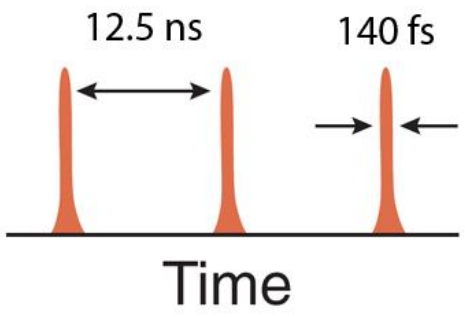
For efficient MPE we need photon concentration in space and time . . .



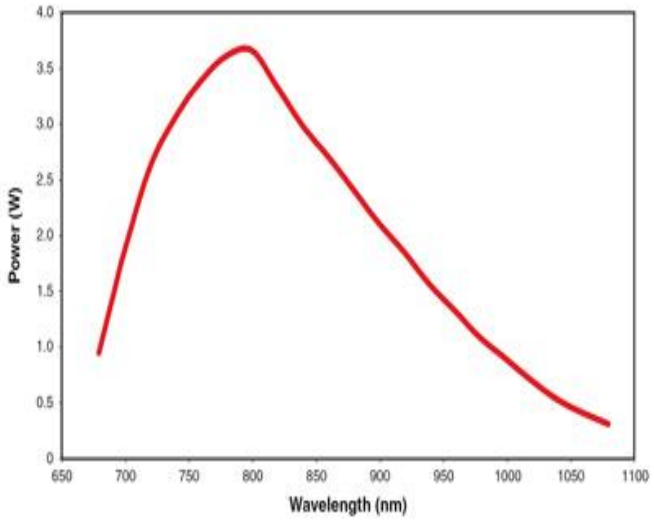


http://www.olympusmicro.com/primer/techniques/fluorescence/multiphoton/multiphotonintro.html

A pulsed laser



Chameleon Ultra II
Femtosecond pulsed laser

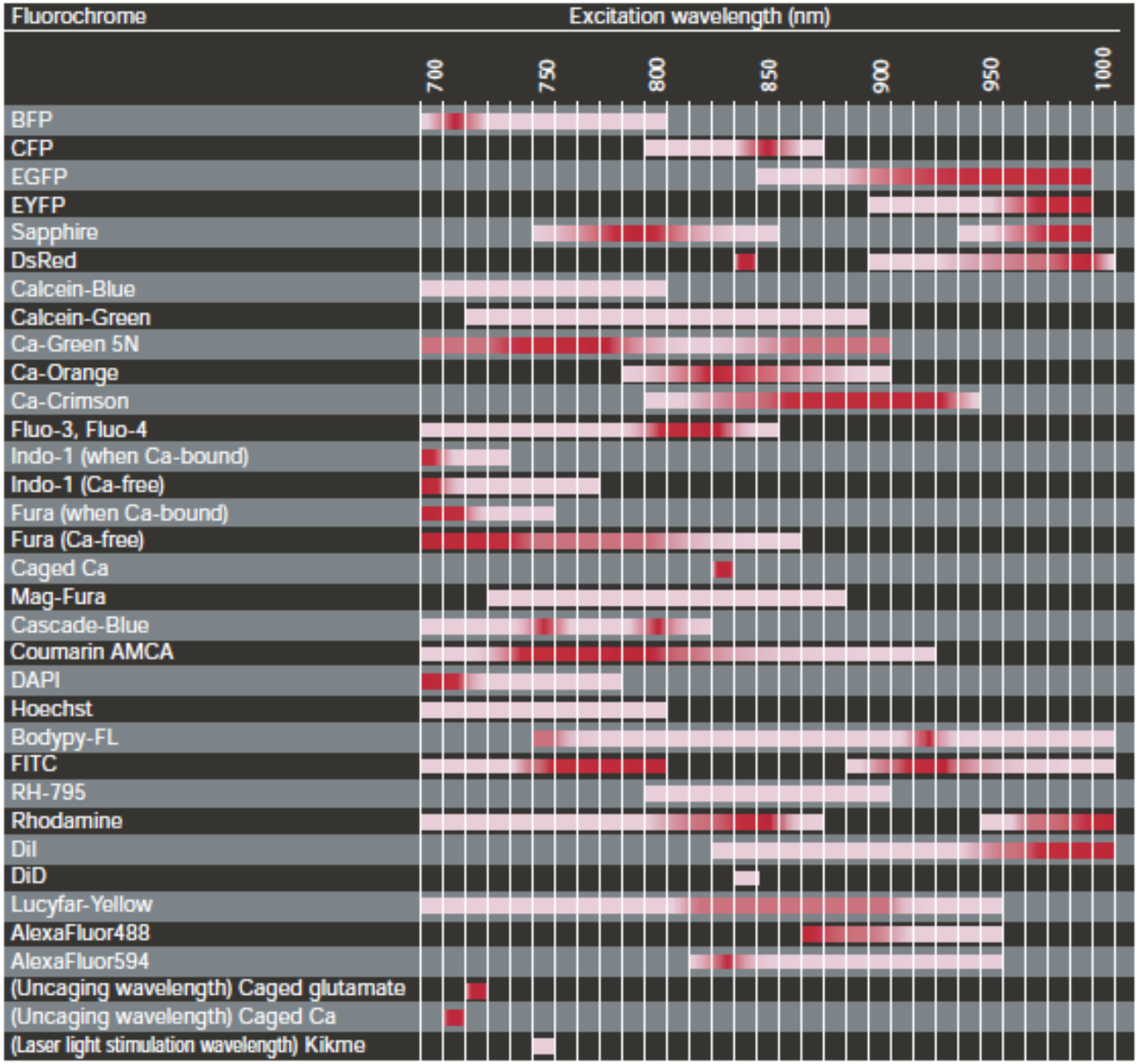


3-4 W of power at peak

Tunable 680-1080 nm @40 nm/sec

This gives flexibility, since these laser cost about $200,000 each
its not normally practical to have several per machine like with 1 photon

Excitation spectra



Most good 1P fluor's are ok for 2P (weak ones are normally even worse in 2P)

Not exactly double 1-photon

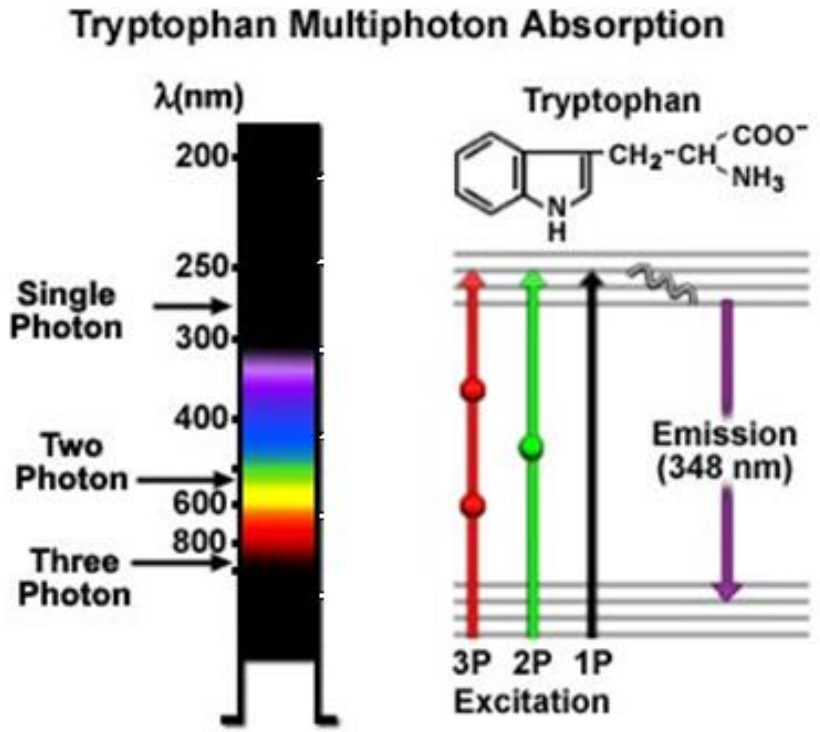
Tends to be broader and blue shifted

This makes multi-channel imaging easier in some ways and more difficult in others

Photobleaching and toxicity

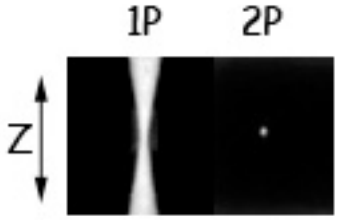
Good luck!
Extremely toxic λ
Won't go through glass

Much less phototoxicity associated with these λ



Multi-photon excitation provides a means of exciting UV and blue fluorophores with less phototoxity

Photobleaching and toxicity



Photodamage in 2P is confined to the thin layer being imaged

	Power ($\propto$photons/μm^2
Wide-field	X
1 Photon	10^5X
2 Photon (average)	10^6X
2 Photon (peak)	10^{11}X

But the damage in that area can be worse

In general, for thick samples 2P has an advantage over 1P
For thin samples, 2P is often worse than 1P

There may be strange forms of damage due to very high field strength – ROS, DNA breaks, tweezing

2-photon vs. 1-photon

- **Improved SNR with thick samples**

- The IR 2PE is less phototoxic in many cases, especially for UV dyes.

- Photobleaching/damage restricted to plane being imaged. Photobleaching or uncaging is possible with fine z-axis resolution

- Resolution is slightly less good

- Multi-channel acquisition is harder (excitation cross-sections are normally much broader) and limited in excitation λ

- No control of optical section thickness

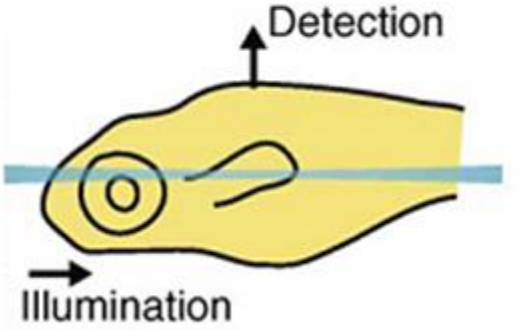
- Lasers are expensive and require exact alignment and can produce heating and other damage

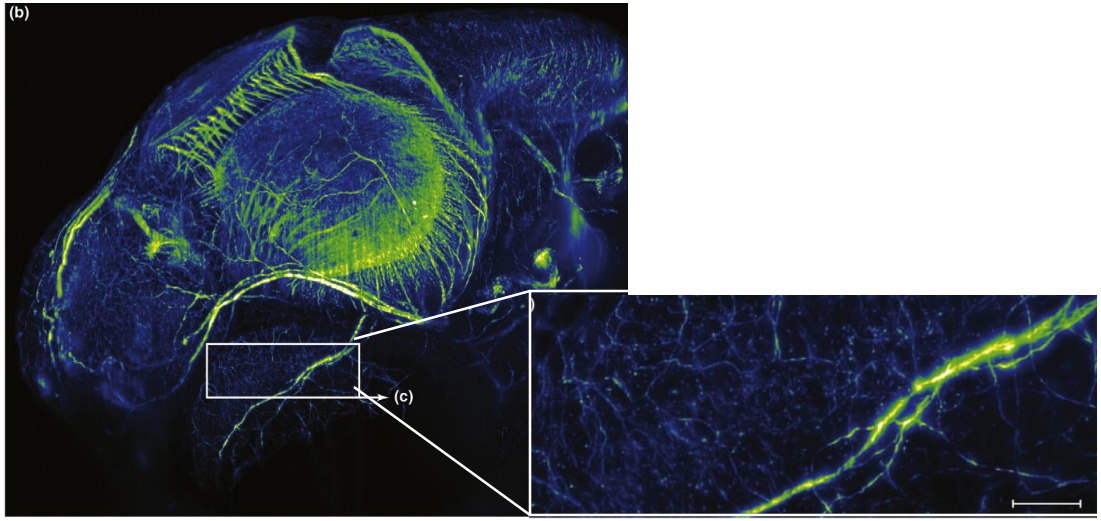
Don’t use a 2-photon system unless you need the advantages

Selective Plane Illumination Microscopy (SPIM)
Light sheet fluorescence microscopy

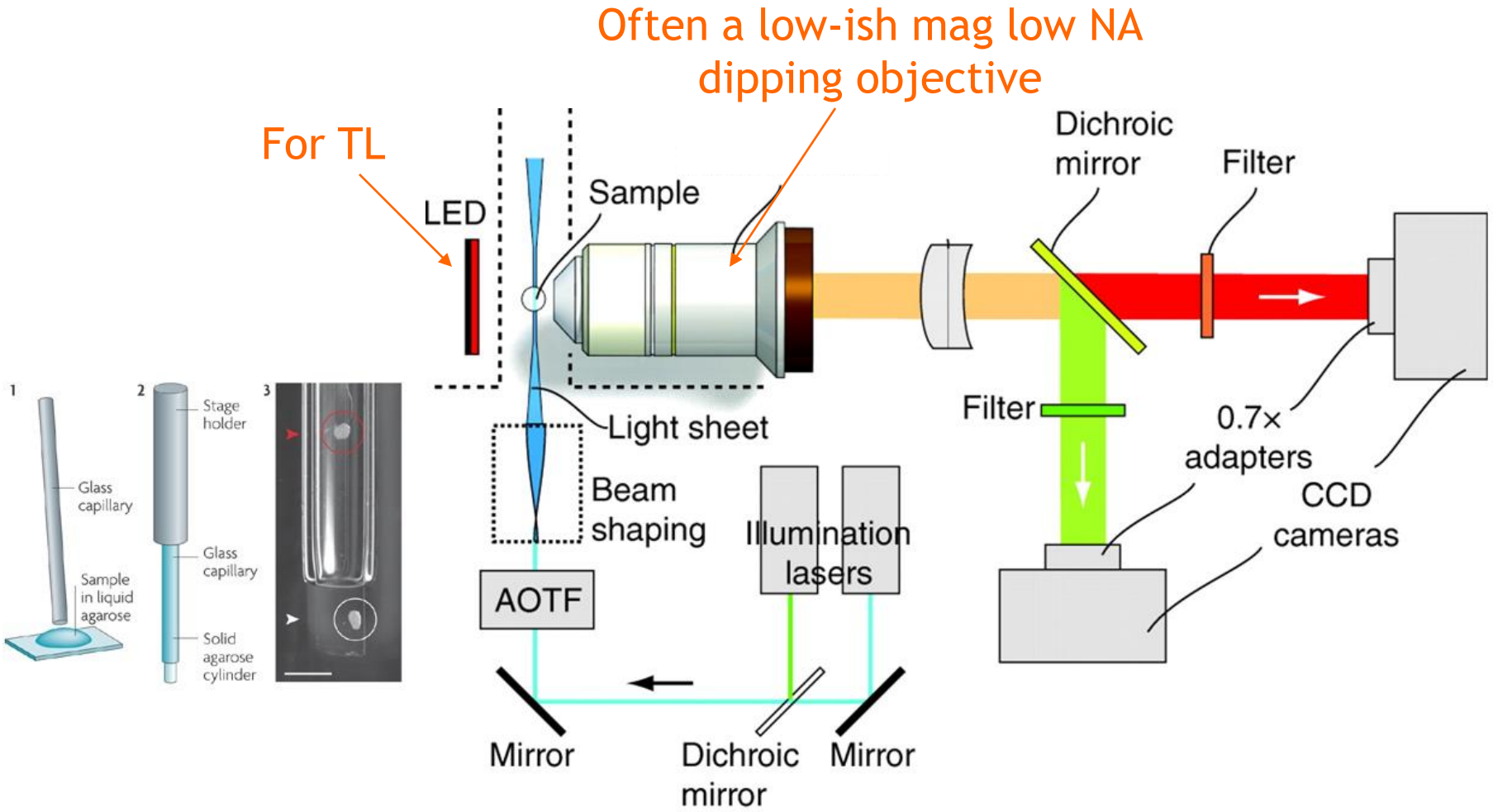
Widefield CCD detection

Sheet illumination
from the side

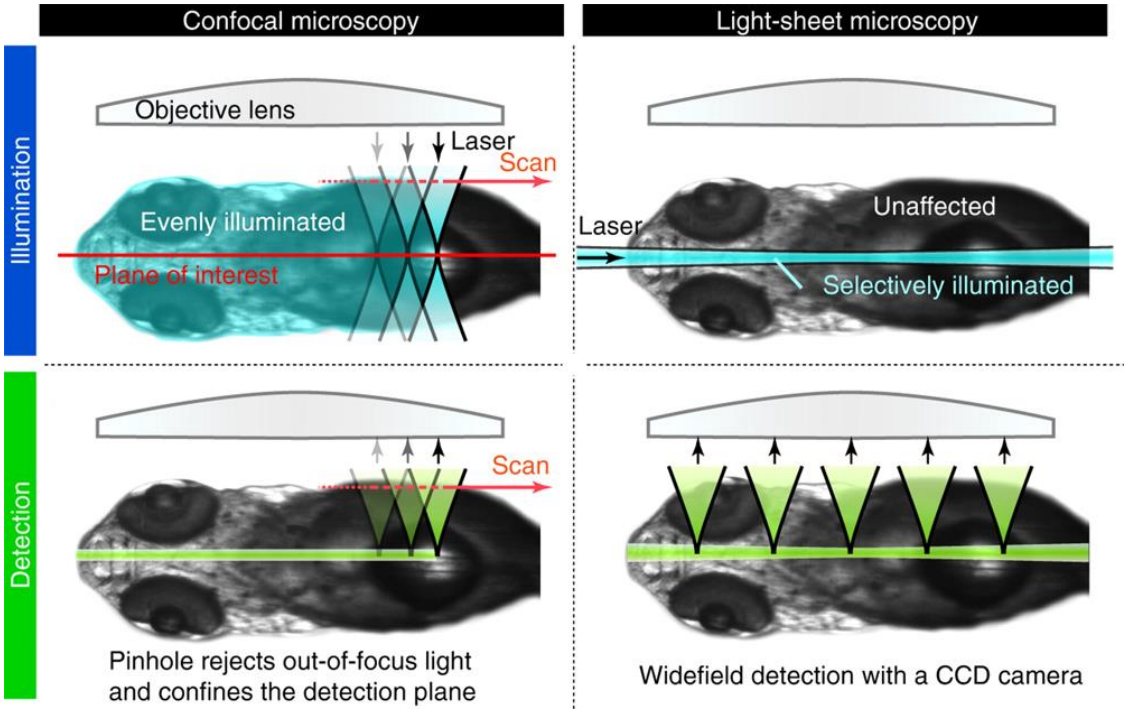




Components of an example SPIM system



Plane illumination photodamage advantage



Reduced region of illumination and photodamage

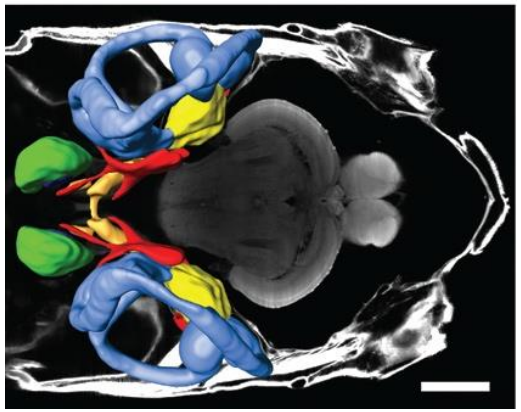
n times better for a z-stack of n slices

The excitation intensities are overall much lower than confocal

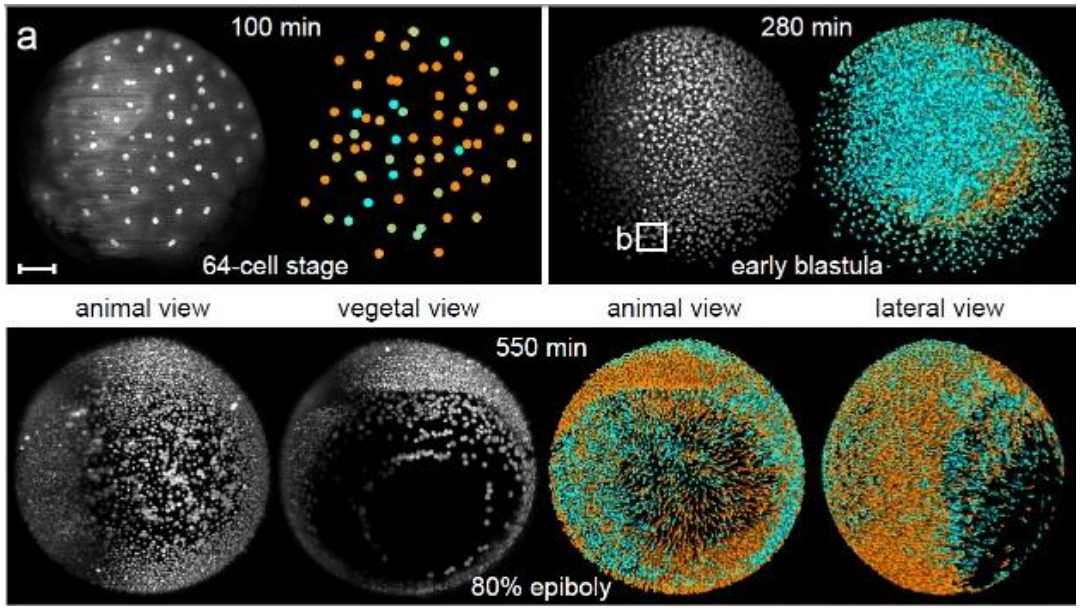
Selective plane illumination microscopy techniques in developmental biology Jan Huisken and Didier Y. R. Stainier Development 2009

Samples good for SPIM etc

The sectioning, speed, efficiency and low photodamage make it ideal for study of living samples up to a few mm across



Thin-sheet laser imaging microscopy for optical sectioning of thick tissues, Santi et al 2009



Reconstruction of zebrafish early embryonic development by Scanned Light Sheet Microscopy Keller et al 2009

SPIM systems are good for a range of samples imaged poorly by other techniques

Optical clearing: SCALE reagent

- 4 M urea
- 10% (wt/vol) Glycerol
- 0.1% (wt/vol) Triton X-100
- pH of 7.7

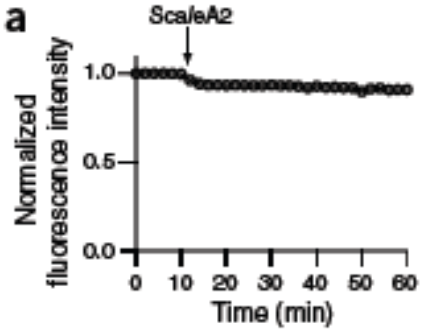
Refractive index of 1.387 and 486 nm

Soak your sample in it for a couple of weeks . . .

http://www.nature.com/neuro/journal/v14/n11/full/nn.2928.html

It clears fixed samples by removing refractive index changes

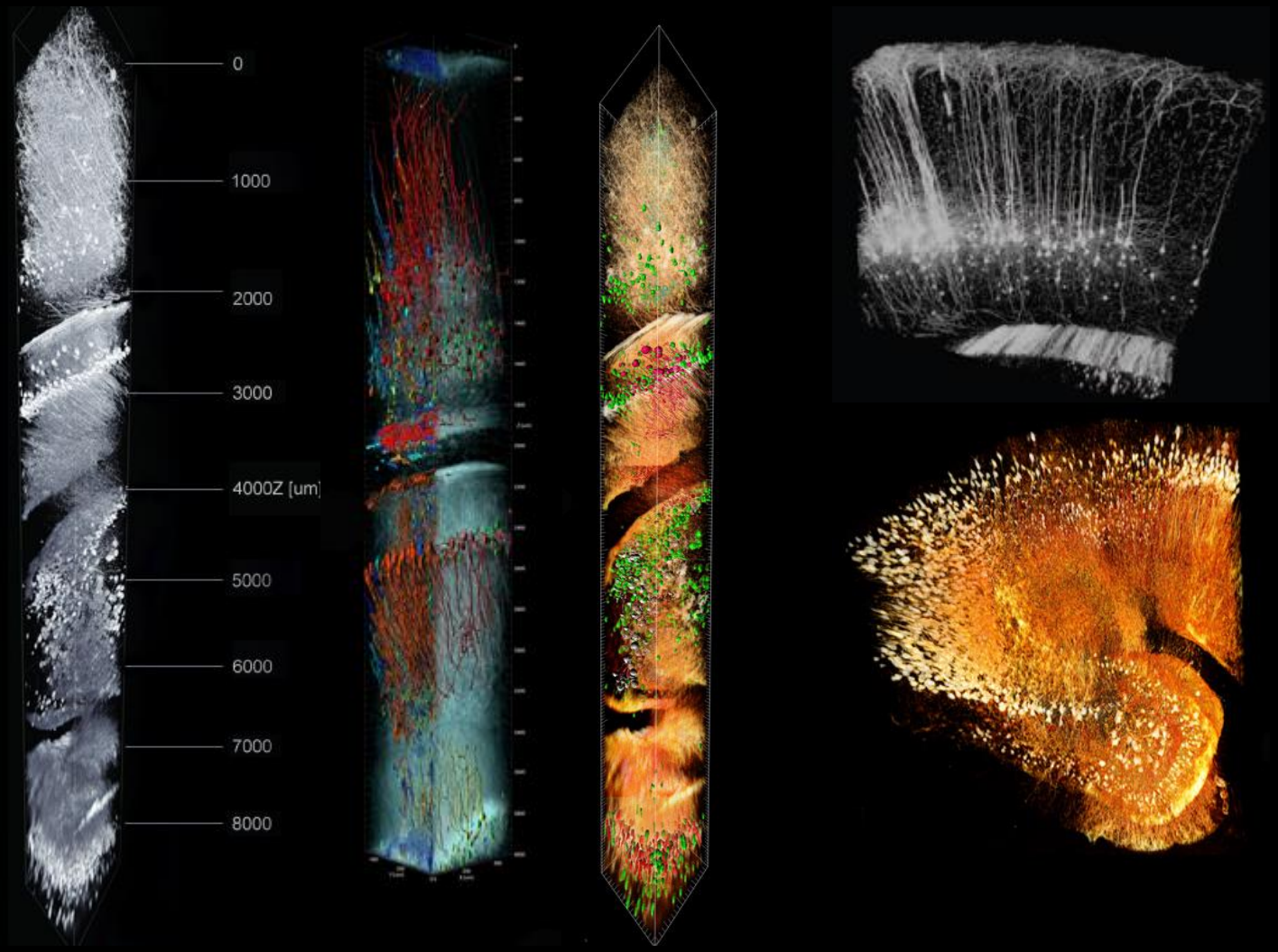
Doesn’t destroy fluorescence

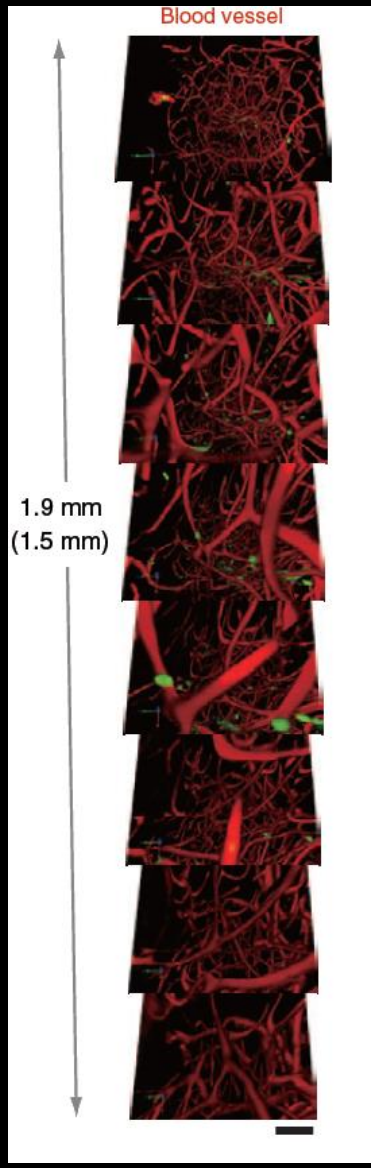


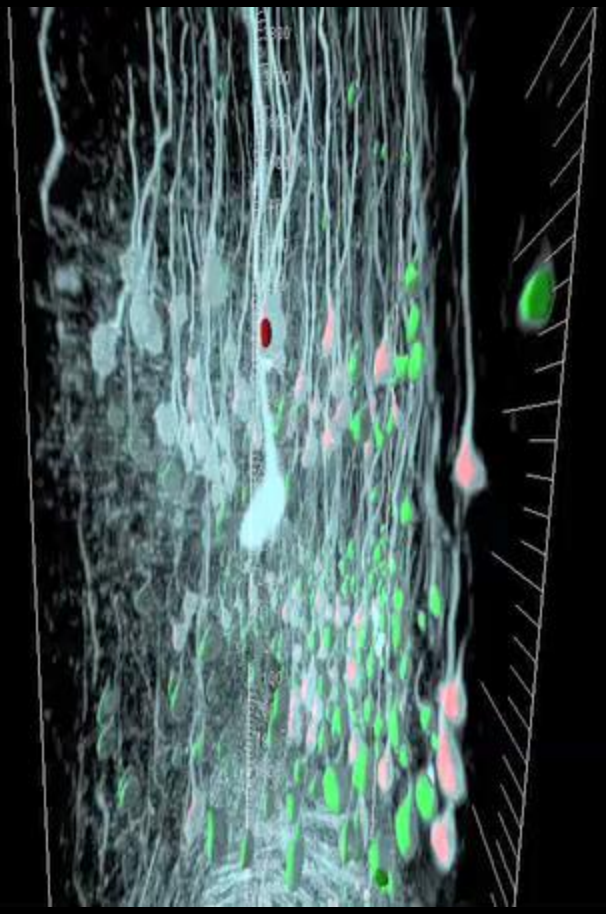
Preserves tissue structure

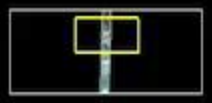
And allows . . .

SCALE reagent









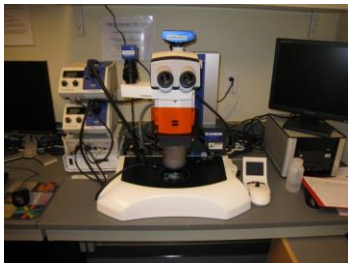
Movies of data

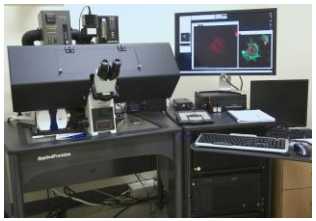
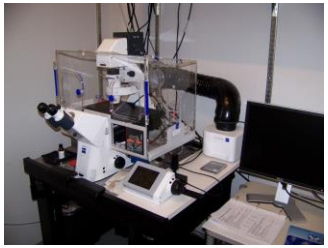
Tools available for you

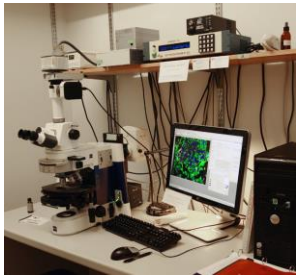
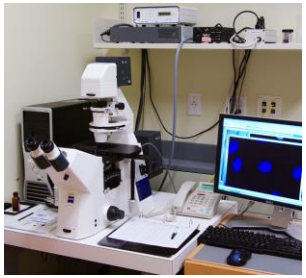
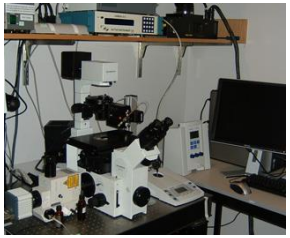
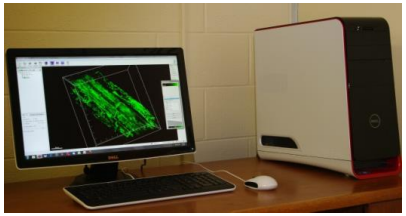
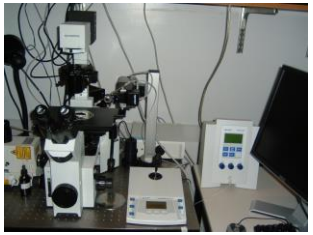
Light Microscopy Core Facility

DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

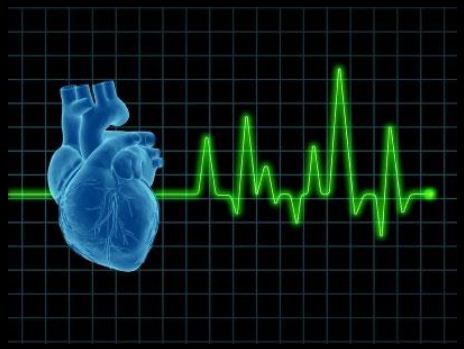


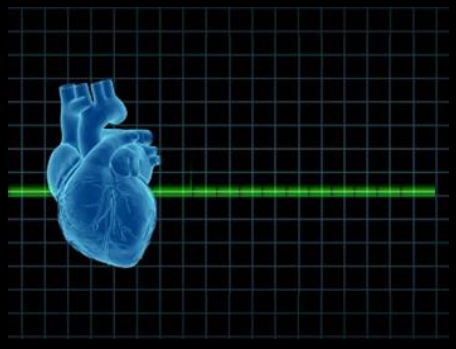



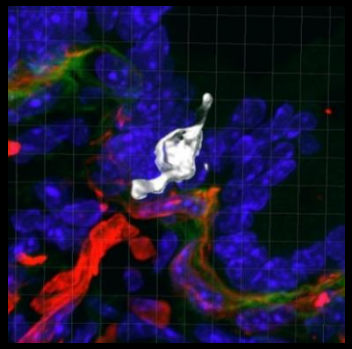




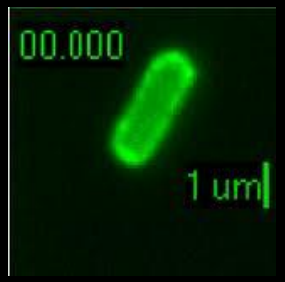






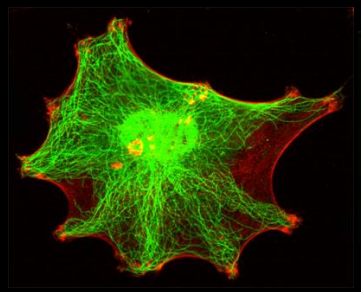
Choose wisely

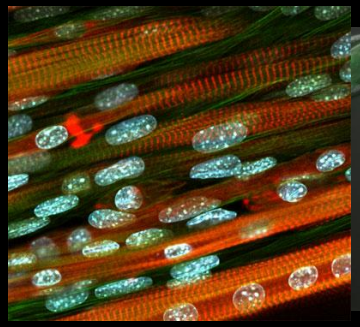


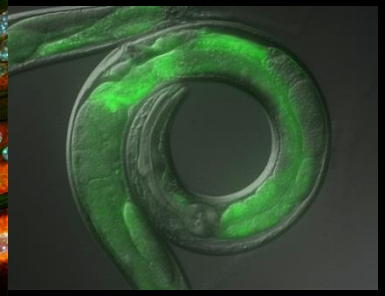


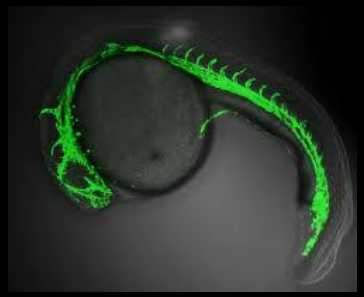


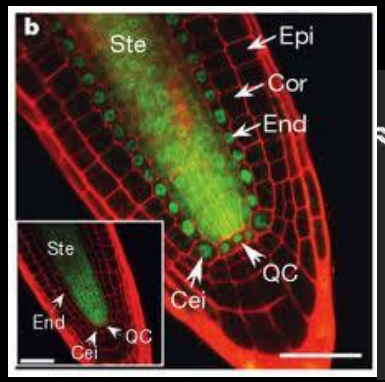


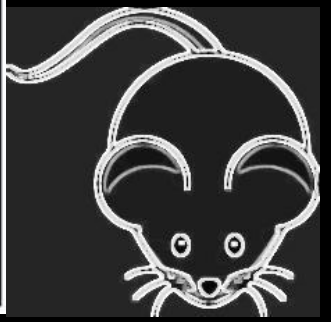












Modality comparison

Modality	Sensitivity	Speed	Photo-damage	Sectioning	Samples
WF Fluorescence	Good	Camera fast	Not bad	No	Cells, thin sections
TIRF	Good	Camera fast	Not bad	“100 nm”	Things close to the membrane, single molecules
Confocal point scanning	Poor	Slow as point based	A concern	Adjustable >500 nm	Nearly anything, only helps with thick samples/sectioning
Resonant PS confocal	Poor	Point-based still	Better than normal confocal	Adjustable >500 nm	Living samples that need high-speed/ low photodamage
Spinning Disk	Good (but goes through a disk)	Camera fast	Good	Fixed by disk, depends on objective	Live samples, cells to embryos
2 Photon	Poor	Slow as point based	It depends	By excitation	Things too thick for 1P

But testing a few is often a good idea

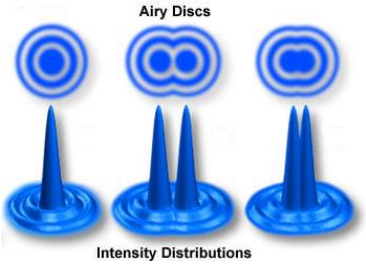
Commercial choices: the big four

The best microscopes are definitely from [redacted]

(Redacted by the LMCF legal department)

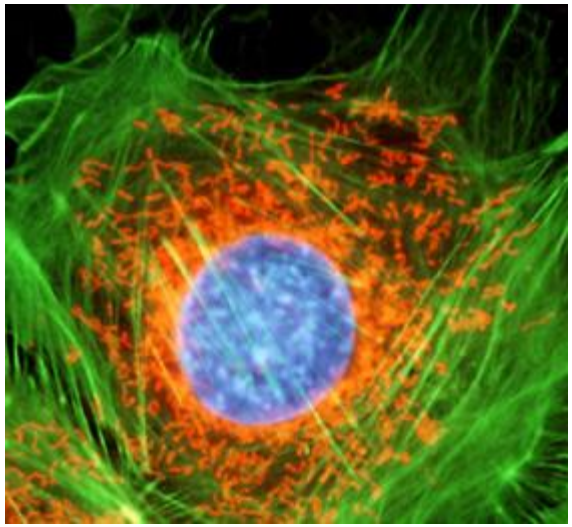
Super resolution

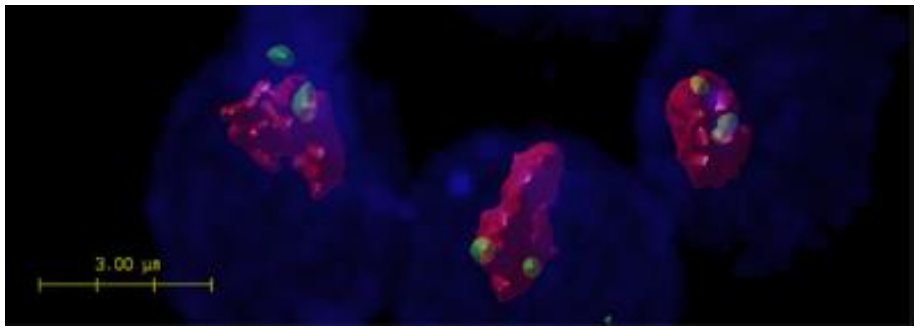
How to improve the resolution of fluorescence microscopy



Resolution = 0.61 λ / NA

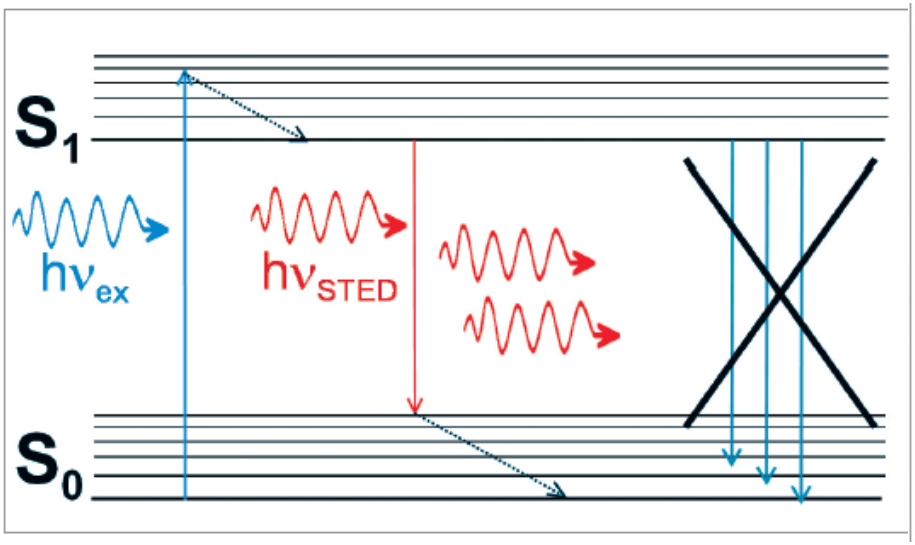
It should be said that it's really quite good already ~200 nm

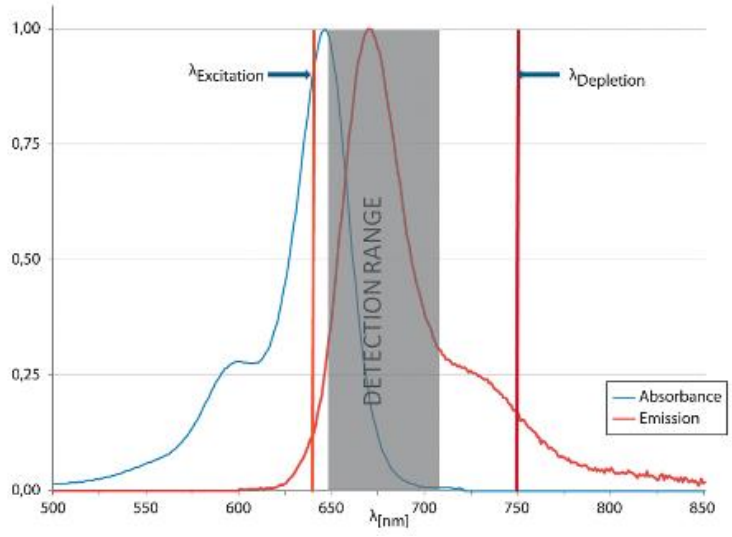




1. STED: STimulated Emission Depletion

More strange things about the quantum mechanics of fluorophores

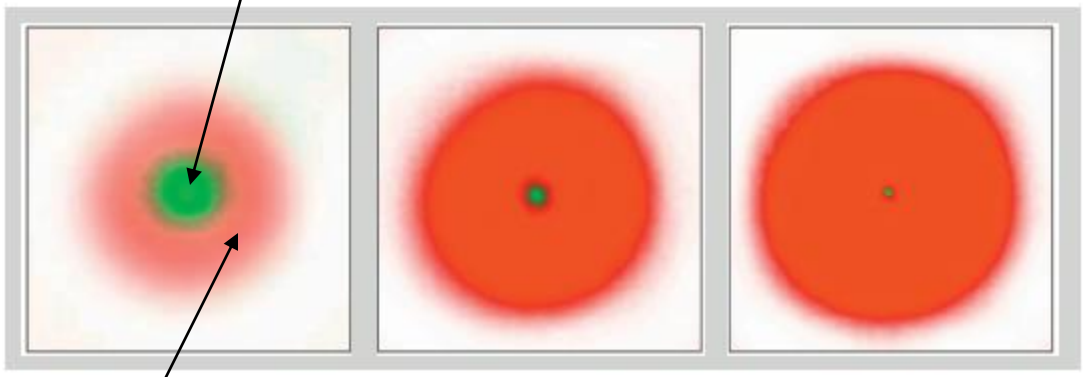




A long wavelength photon can deplete the fluorescence

How STED can get us better resolution

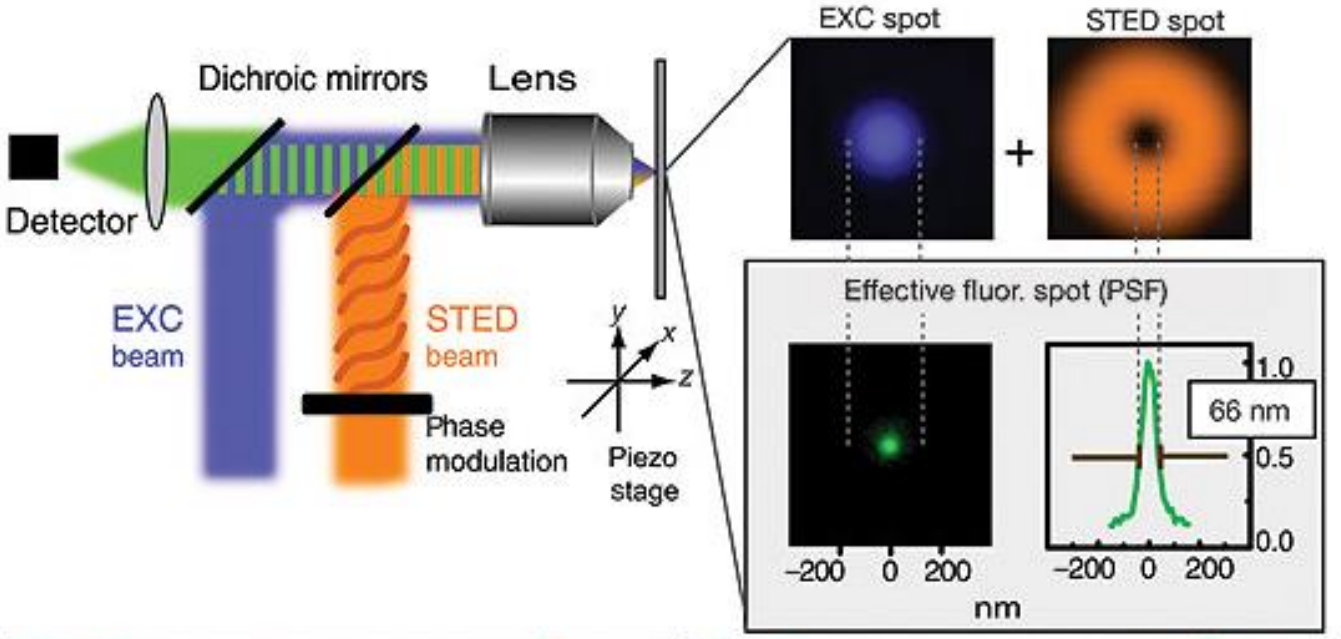
Normal 1P excitation spot

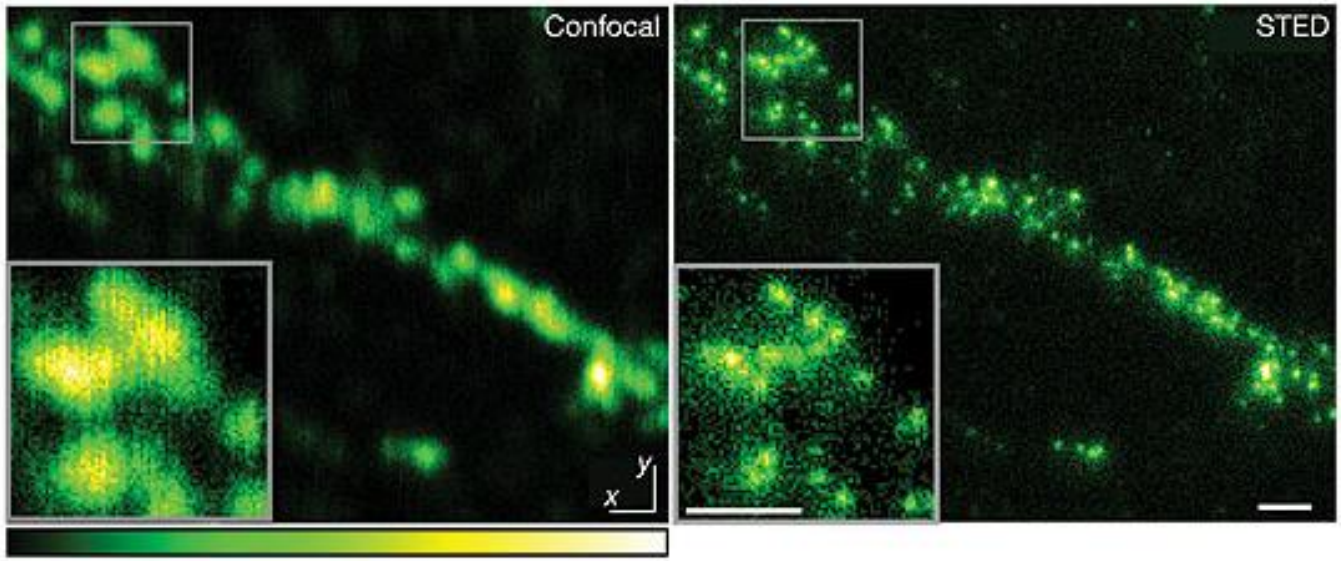


Doughnut of depletion

Bigger doughnut, smaller spot

STED system


Dichroic mirrors
Lens
Detector
EXC
beam
STED
beam
Phase
modulation
Piezo
stage
y
x
z
EXC spot
STED spot
+
Effective fluor. spot (PSF)
1.0
66 nm
0.5
0.0
−200
0
200
−200
0
200
nm


Confocal
STED
y
x

Strengths and weaknesses of STED

- ✓ Point scanning confocal with improved XY resolution ~3X
- ✓ Fast – pretty much as a standard confocal

- Fluorophore limitations, multiple fluors difficult

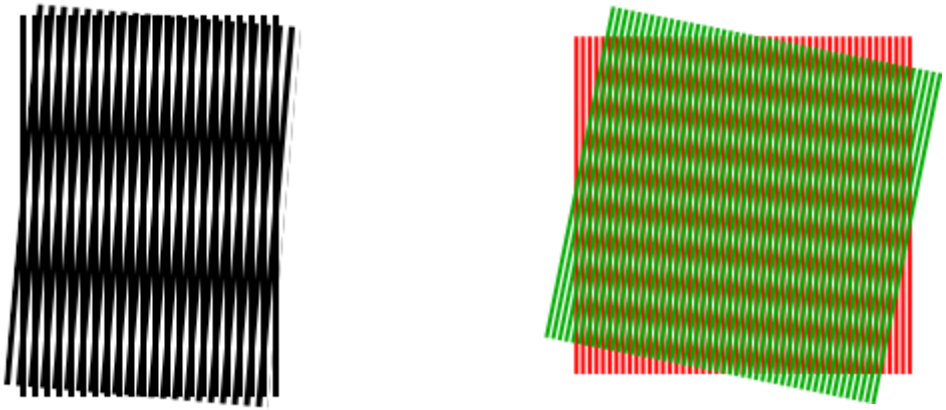
- Requires precise alignment

- The power required for depletion is not ideal for living cells

- Corruption with depth

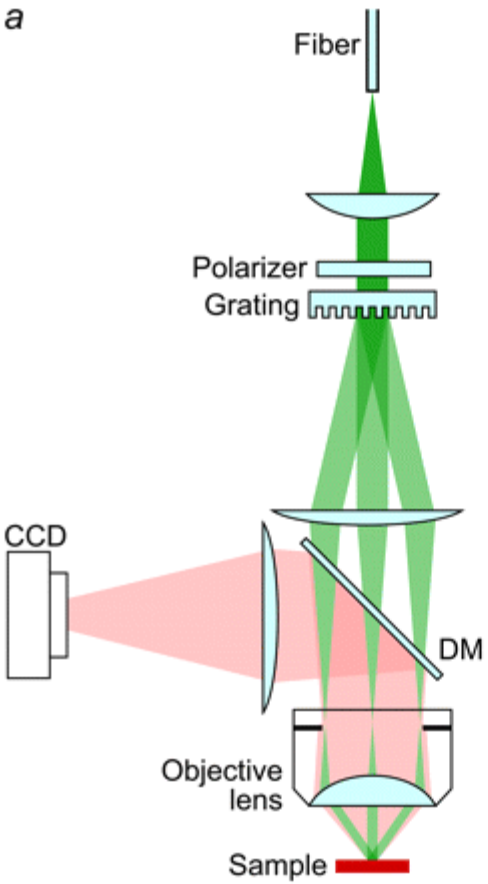
- Z-resolution not improved

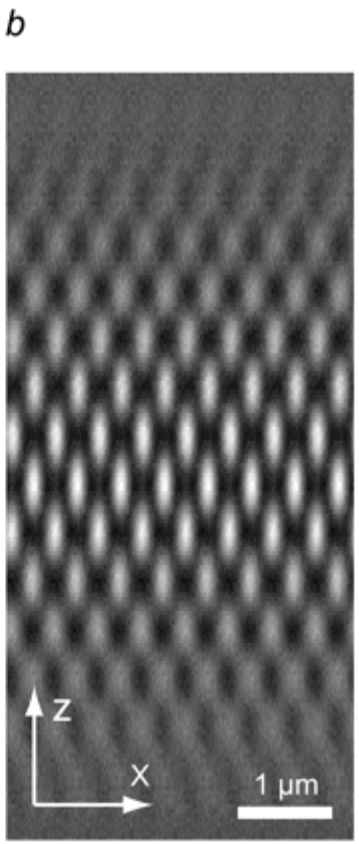
2. Structured illumination extends the passband

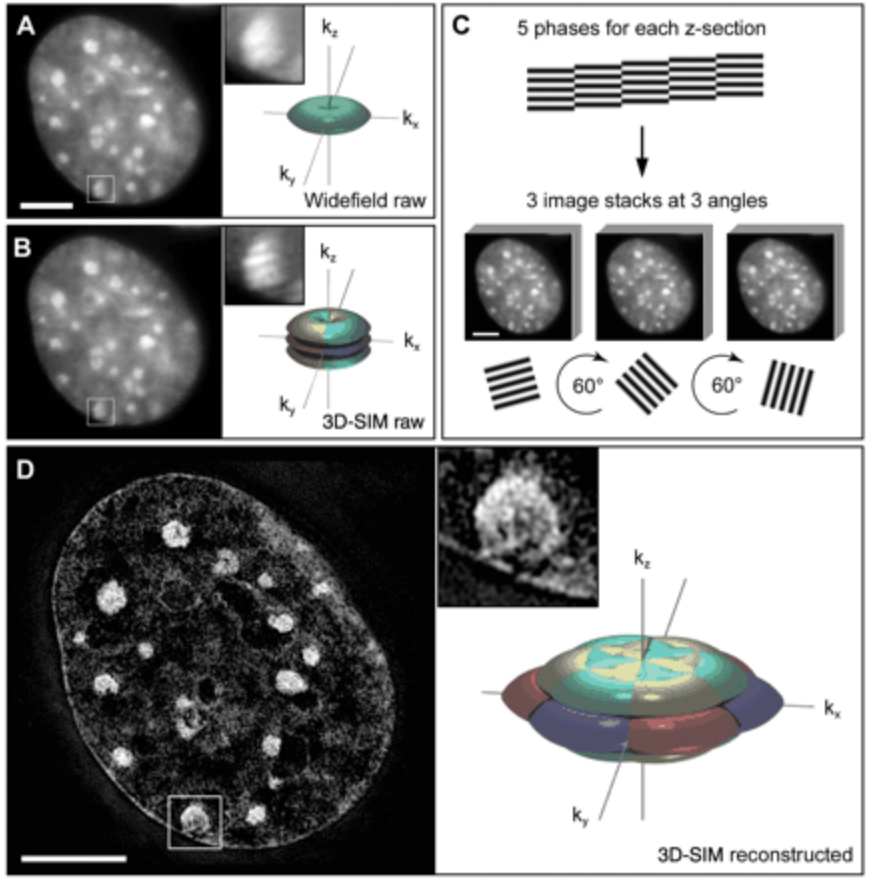


We can image the moire fringes and with knowledge of the illumination structure we can capture the object in finer detail than ordinarily possible

3D Structured illumination



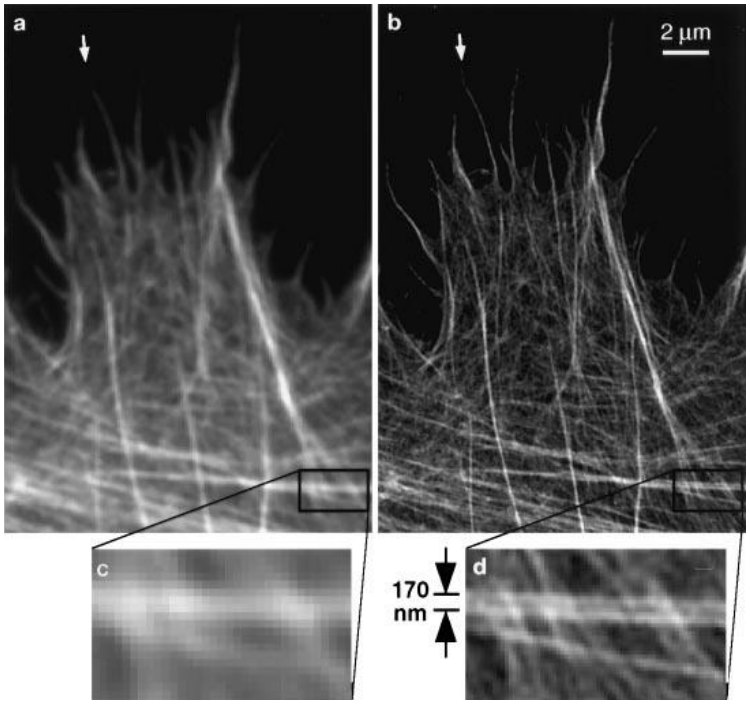


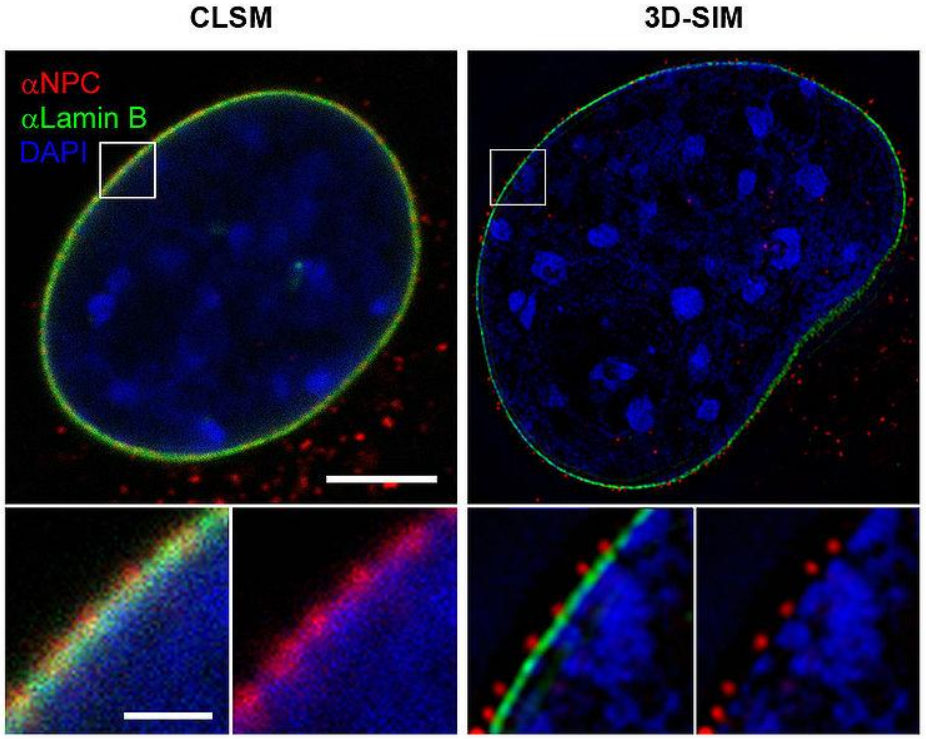


Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy

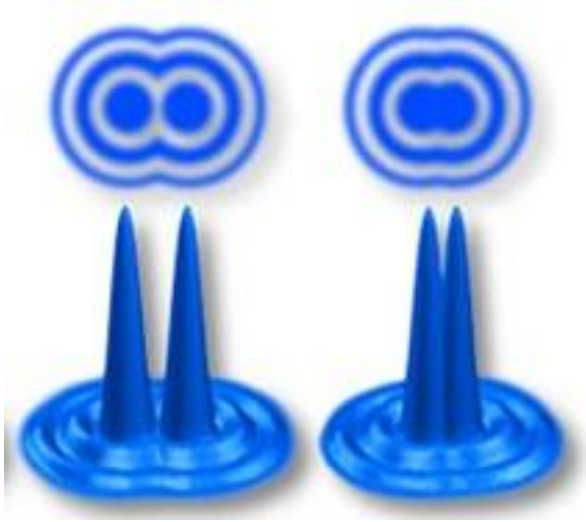
SIM in practice

- 100 nm XY by 200-300 nm Z resolution
- Essentially a widefield technique
- Normal dyes
- Two fold resolution improvement still pretty useful



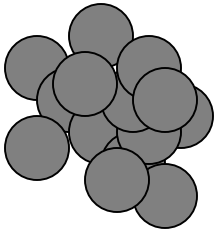


3. Core principle of the next approach



We can't separate two objects beyond our diffraction limited resolution but . . .

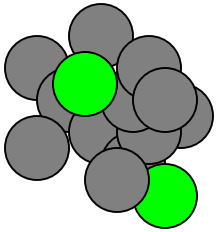
. . . If you only have one object, you can position the centroid with very high accuracy, say 1 to a few nm



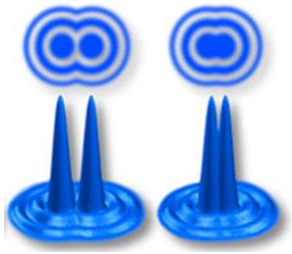
Dark sample with PA-fluorophore

Weak photoactivation to produce a few isolated fluorphores

Image and bleach individual fluorophores and map their position

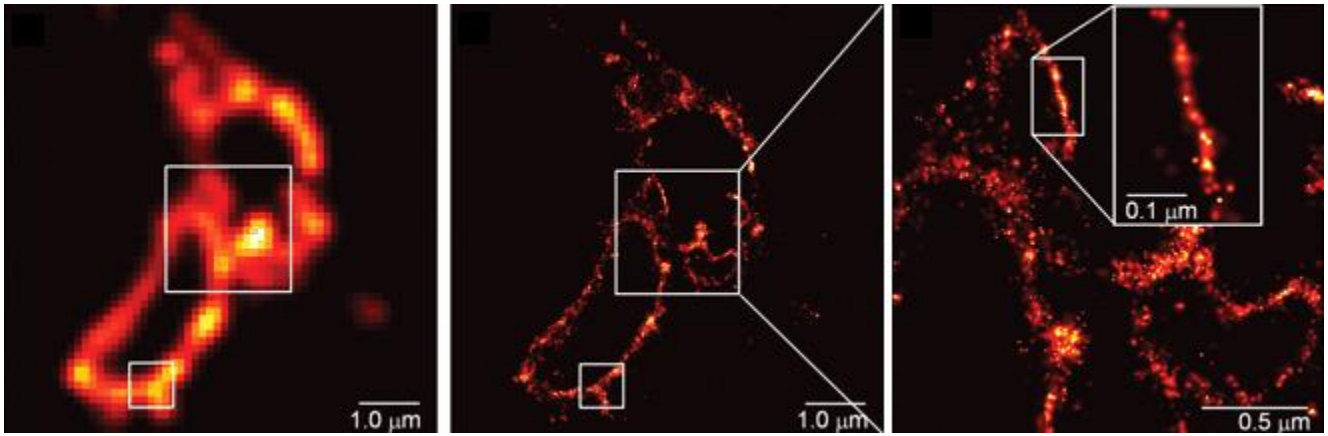


Many iterations to produce super-resolution map/image



TIRF image

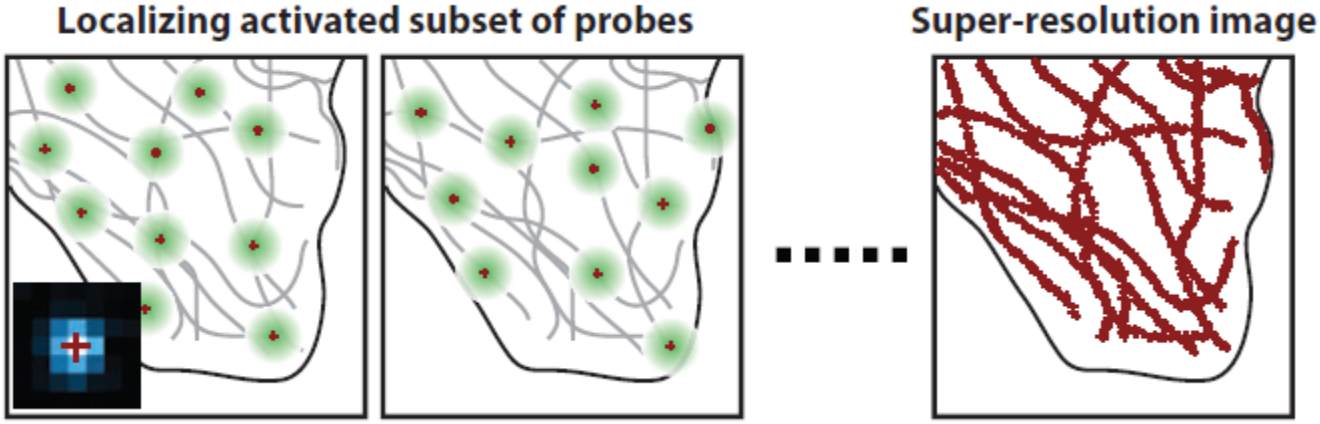
PALM Super-resolution



Resolution can be improved more than 10 fold

Imaging Intracellular Fluorescent Proteins at Nanometer Resolution

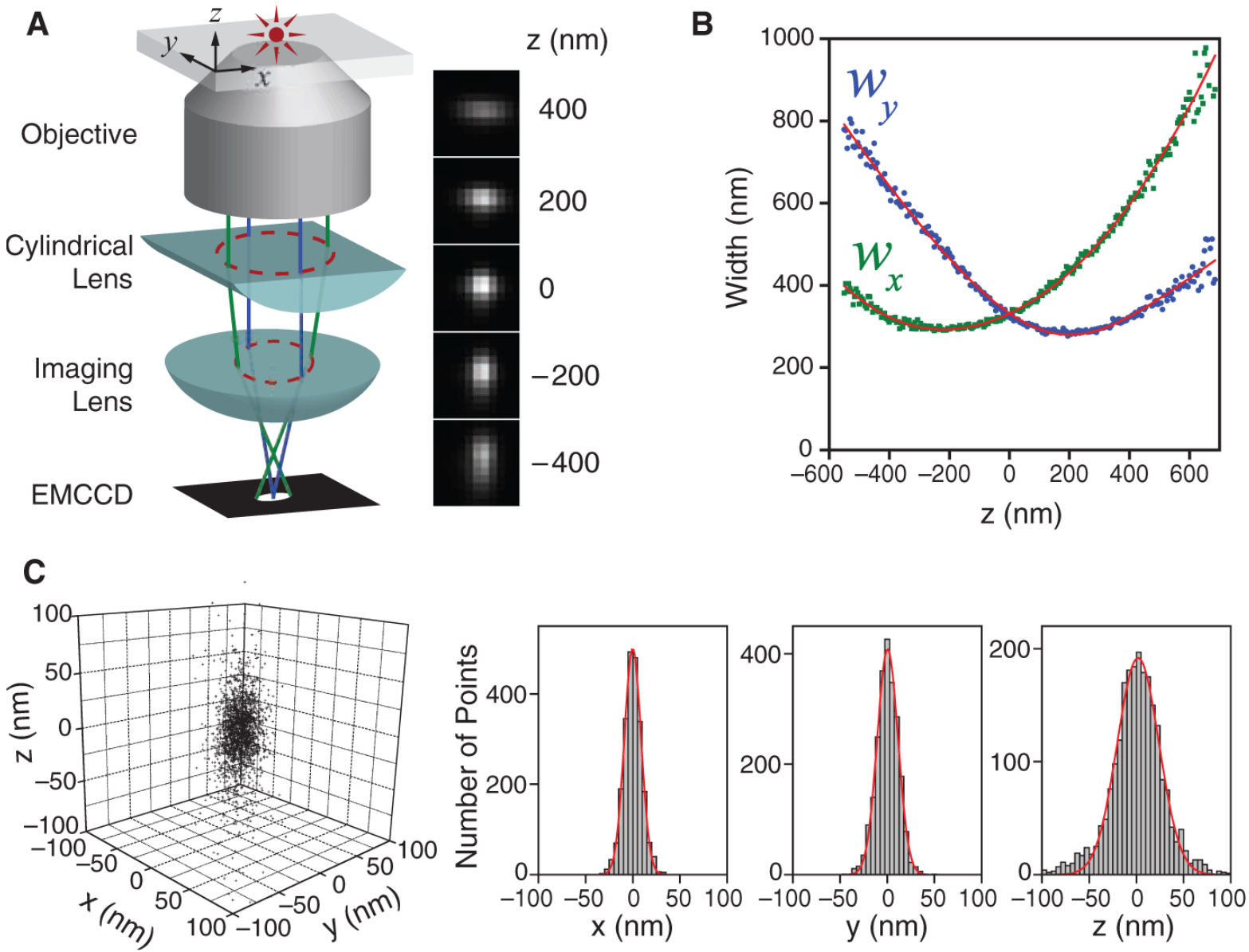
Processing the images



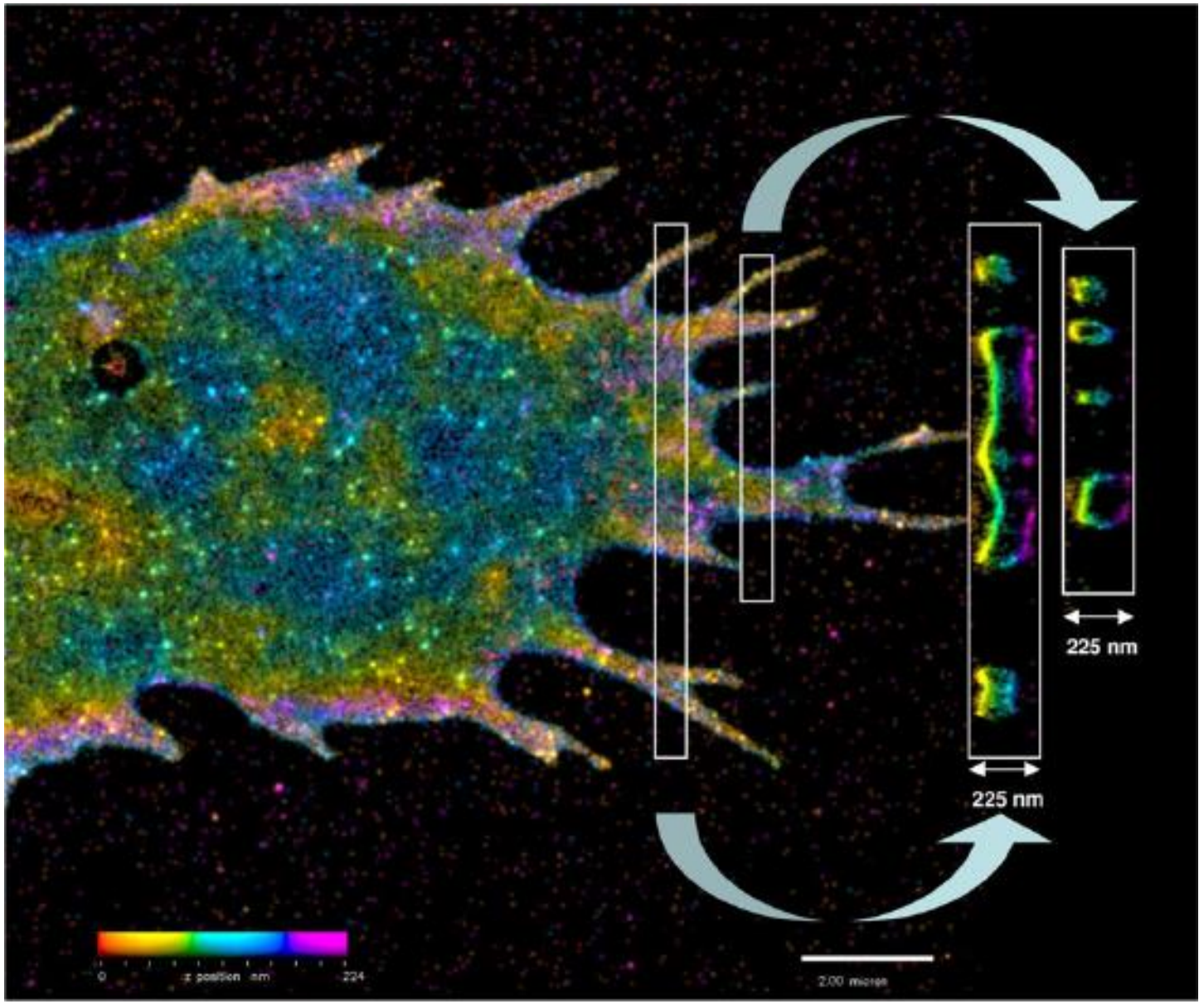
- 10,000 images, ~10 min, few GB
- Fiducials help correct drift

- Individual molecules must be separated by >resolution
- But sequential so >10^5 molecules per μm^2 possible
- Accuracy of centroid fitting is $\lambda/2NA \sqrt{\#photons}$
  about 50 photons per point (STORM more than FPs)

Astigmatism: 3D STORM



iPALM



Which super resolution approach is best?

Well they do pretty different things . . .

STED– Confocal like
Limited fluorophores, damage, no z-axis improvement

Structured illumination
Smallest improvement in resolution, but 3D improvement
Most versatile and like “normal” microscopy
Probably live cell compatible

PALM/STORM family
Best resolution
Temporal constraint
How easy are the 3D versions?
Unlike “normal” imaging -

http://www.nature.com/nmeth/collections/superresmicroscopy/index.html
http://www.annualreviews.org/doi/full/10.1146/annurev-cellbio-100109-104048

What should I understand?

- How fluorescence works
- How all those spectra, filters, lamps, objectives add up to a photon efficient imaging combination. How to choose a filter for a particular fluorophore.
- Resolution (in fluorescence terms) – what it means, what it doesn't
- What factors into a wise choice of an objective
- NA and its consequences
- How CCD cameras work and are used in microscopy. When to use an EMCCD.
- What all the components in the TL path do and HOW TO KOHLER A SCOPE (maybe the conjugate planes explanation)
- Contrast – principles of brightfield, Phase contrast, DIC
- The confocal principle, advantages and disadvantages
- How a confocal works, what the components do and how to adjust them
- Resolution and sampling in 2D and 3D
- Advantages and disadvantages of Spinning disk
- TIRF and the type of samples it works for
- Advantages and disadvantages of multiphoton

More information

This is a fairly comprehensive collection of review articles and interactive tutorials about the optics involved in microscopes http://www.olympusmicro.com/primer/anatomy/anatomy.html

This book has a very good for transmitted light and optical basics: Fundamentals of Light Microscopy and Electronic Imaging – Douglas B Murphy (Duke has an eBook)

Optical Microscopy by Davidson and Abramowitz is a 40 page review article you can download here http://www.olympusmicro.com/primer/opticalmicroscopy.html

Review articles about fluorescence microscopy
http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html

Spinning disks http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html

TIRF http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html

SPIM http://dev.biologists.org/content/136/12/1963

Multiphoton articles/tutorials
http://micro.magnet.fsu.edu/primer/techniques/fluorescence/multiphoton/multiphotonhome.html

Reviews on multiphoton imaging
http://zeiss-campus.magnet.fsu.edu/referencelibrary/multiphoton.html