Optical Sectioning – Pros & Cons

3D microscopy

"The 1,000,000 euro confocal is not always the right microscope for the job."

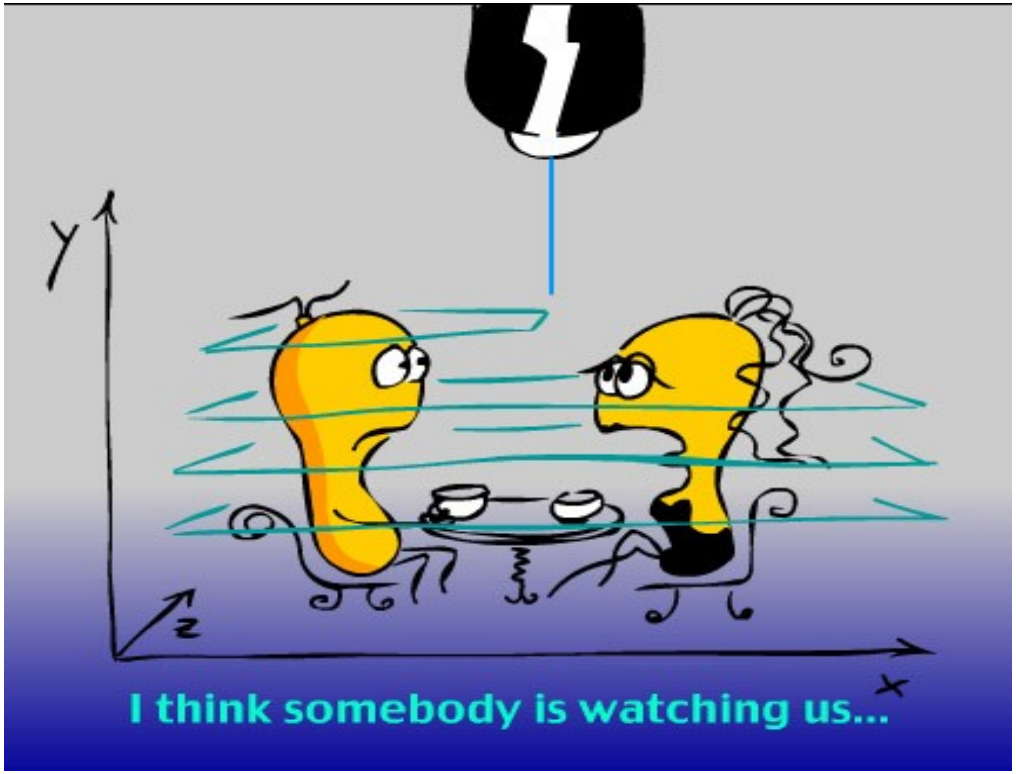
Or

"Driving a Ferrari over a muddy field is not very sensible, especially when you have a nice tractor available."

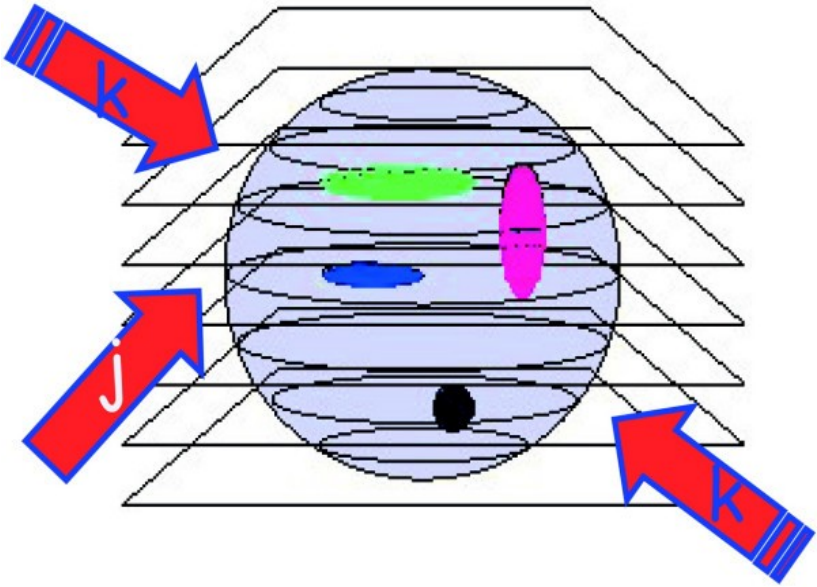
CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics

Dan White – Oct 2009

Optical sectioning – 3D imaging



Digital sampling of
3 spatial dimensions



X and Y are easy, right...?

… but is Z special?

www.biomedical-engineering-online.com

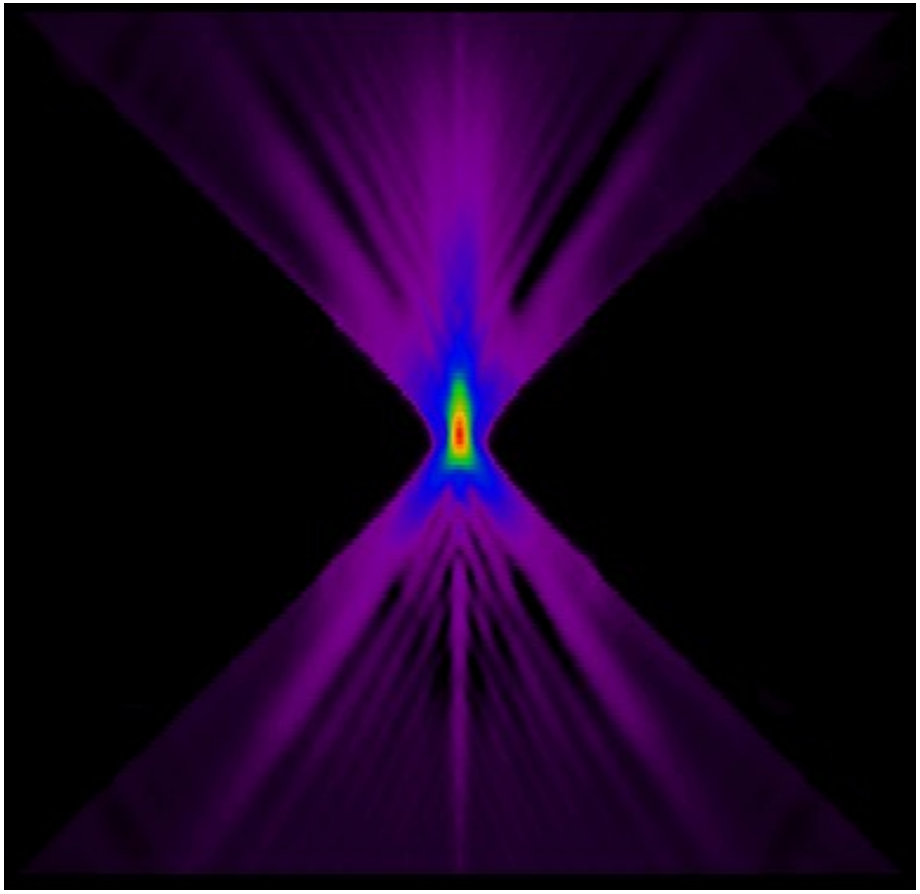
Optical sectioning - methods:

- WM widefield microscopy
- CLSM confocal laser single point scanning
- SPD confocal multi point scanning
- 2P two photon
- TIRF total internal reflection fluorescence
- SPIM single plane illumination

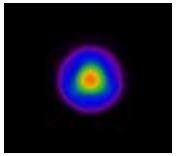
- structured illumination microscopy
- widefield microscopy + deconvolution

Image of a point is not a point!

The image of a point is convolved by the point spread function – PSF.



PSF size:
WF>SD>SPIM>C



Widefield microscope PSF
1.4 NA Oil lens
Watery sample

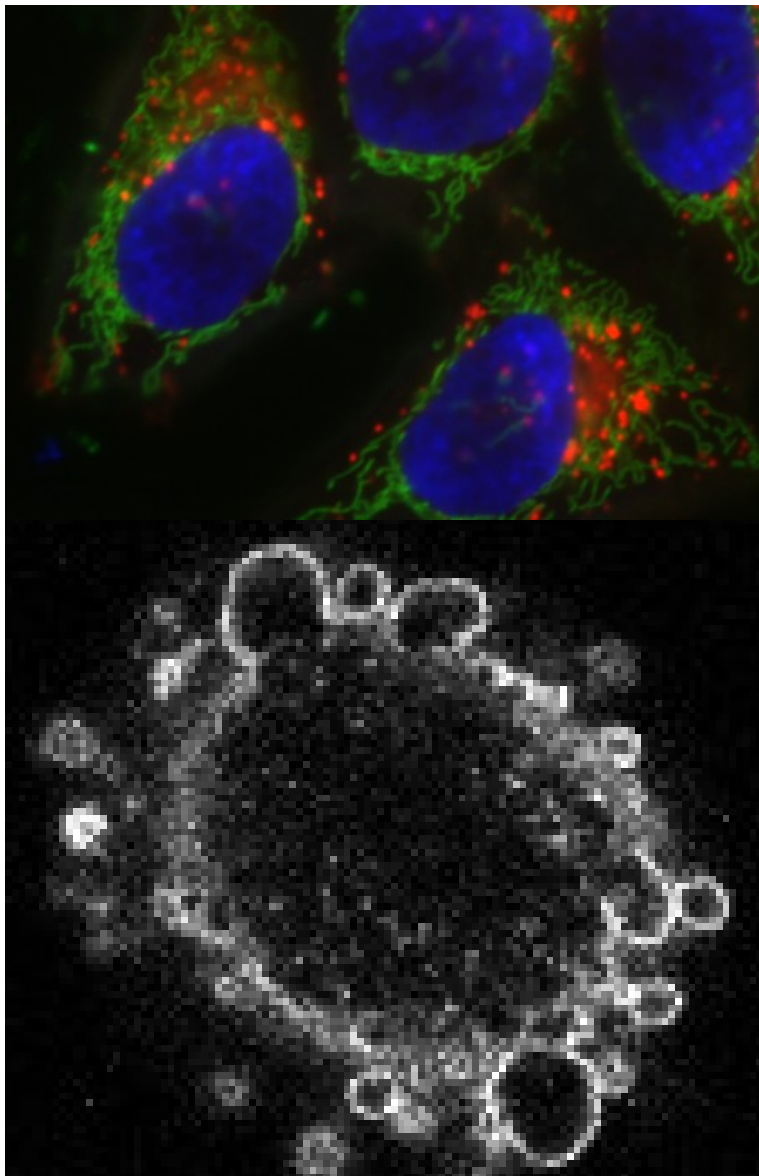
PSF is very much bigger in z
than in x and y
Why?

Higher NA =
better resolution,
especially in Z!

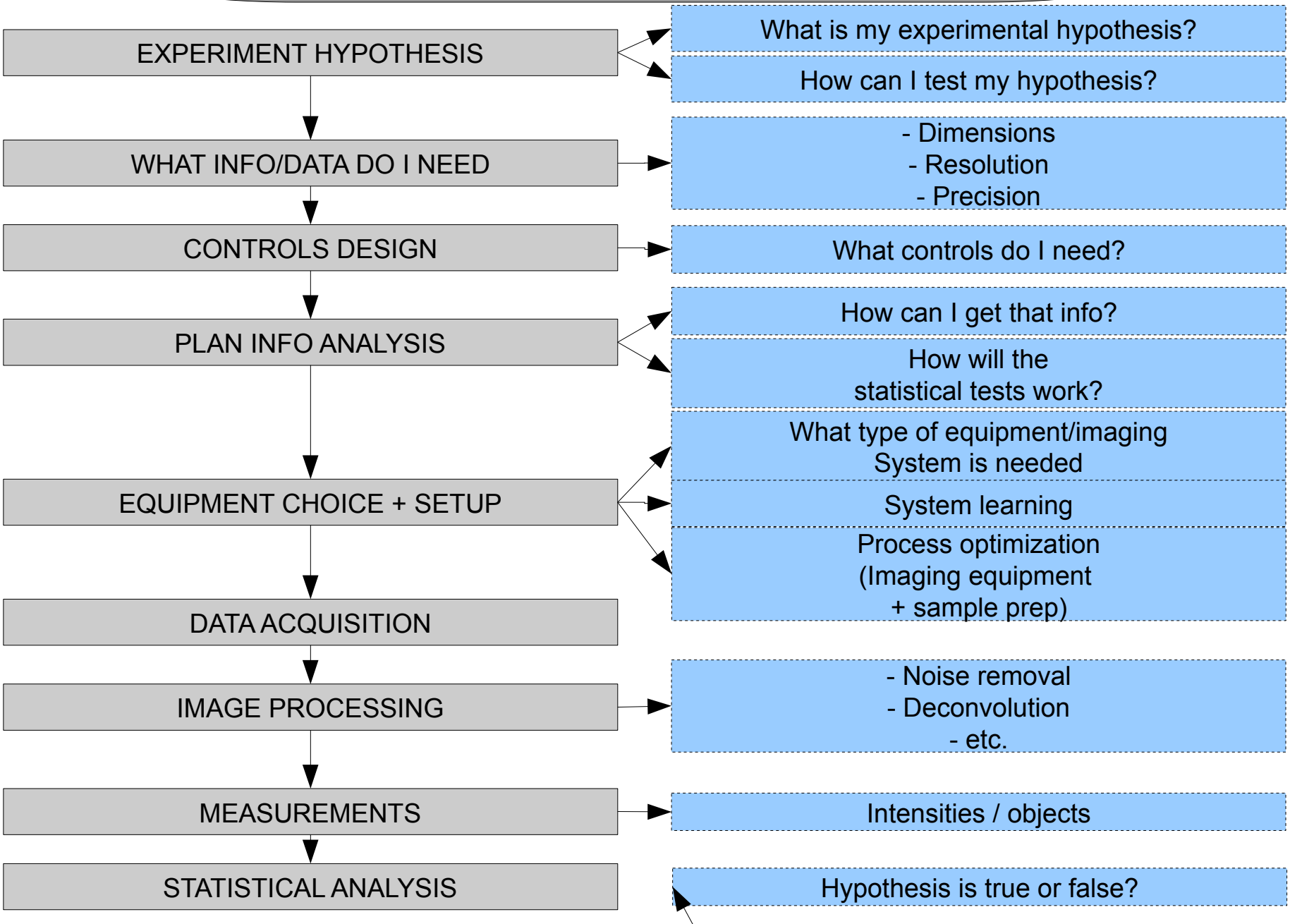
Jesse Lipp, Dan White

Imaging Experiment Planning:

- What **BIOLOGY** an I trying to **measure**?
  - What is the hypothesis under test?
- Do I need 3D, 4D, xD information
  - Resolution? Sampling: Space, Time, Intensity
- Choose appropriate microscope
  - Don't use Confocal LSM just because its the newest or most expensive or because that what others in your lab use
- Optimise microscope system
  - get best data from your sample
- Do the right controls!
- Measure Something!
  - Statistics to test hypothesis
  - how many data points/images/cells?



Imaging Experiment Work Flow



Pixel Size / Resolution

Remember !!!

Spatial sampling:

~1/3 x smallest feature.

Remember !!!

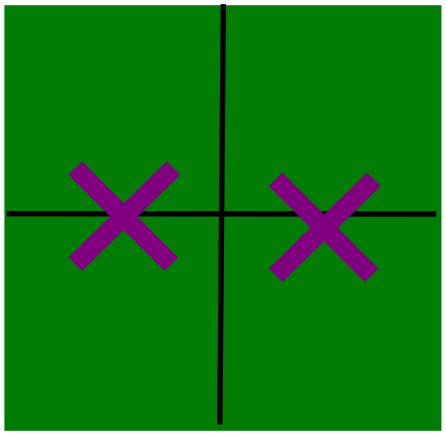
A pixel is NOT a little square...

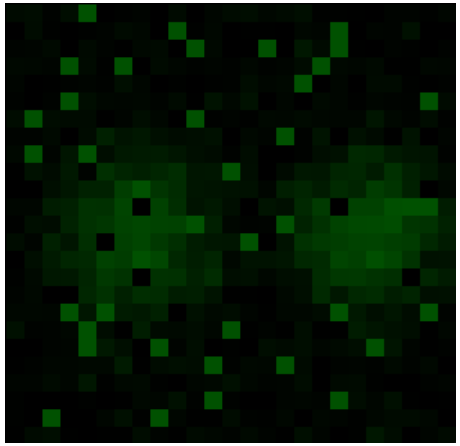
it is a spatial sample at some location x,y,z in space and time

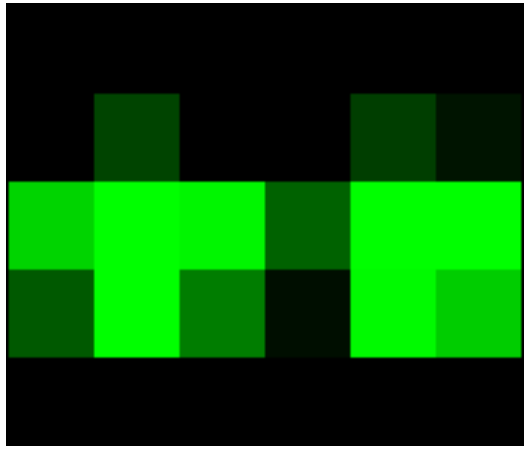
Remember !!! Think like a spectroscopist, not a photographer!

2 points X and X – resolution limit apart, d

Pixel size = d/3







under sampled

Can't see 2 objects

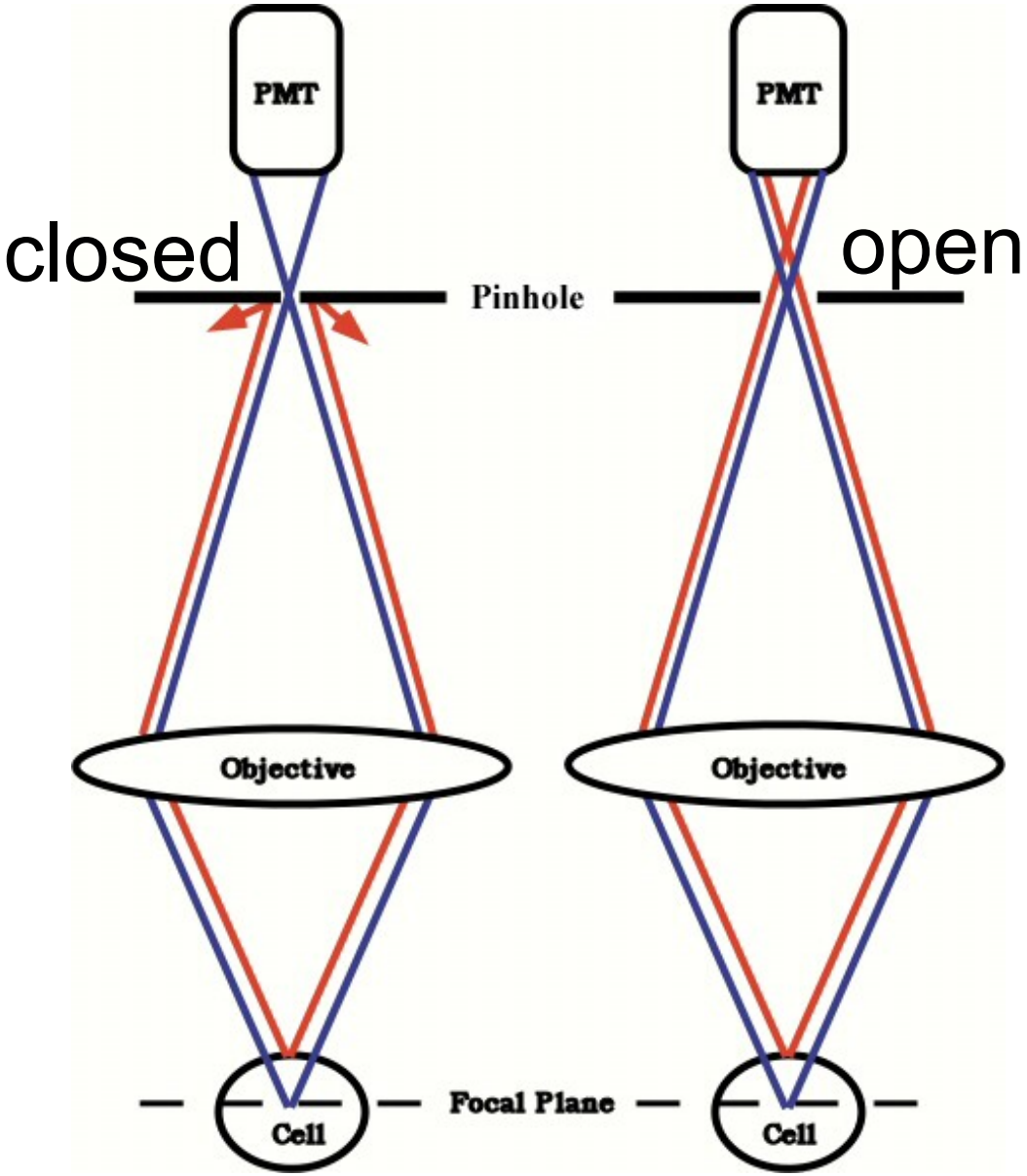
over sampled

Noisy, low contrast

correct sampling

Good contrast

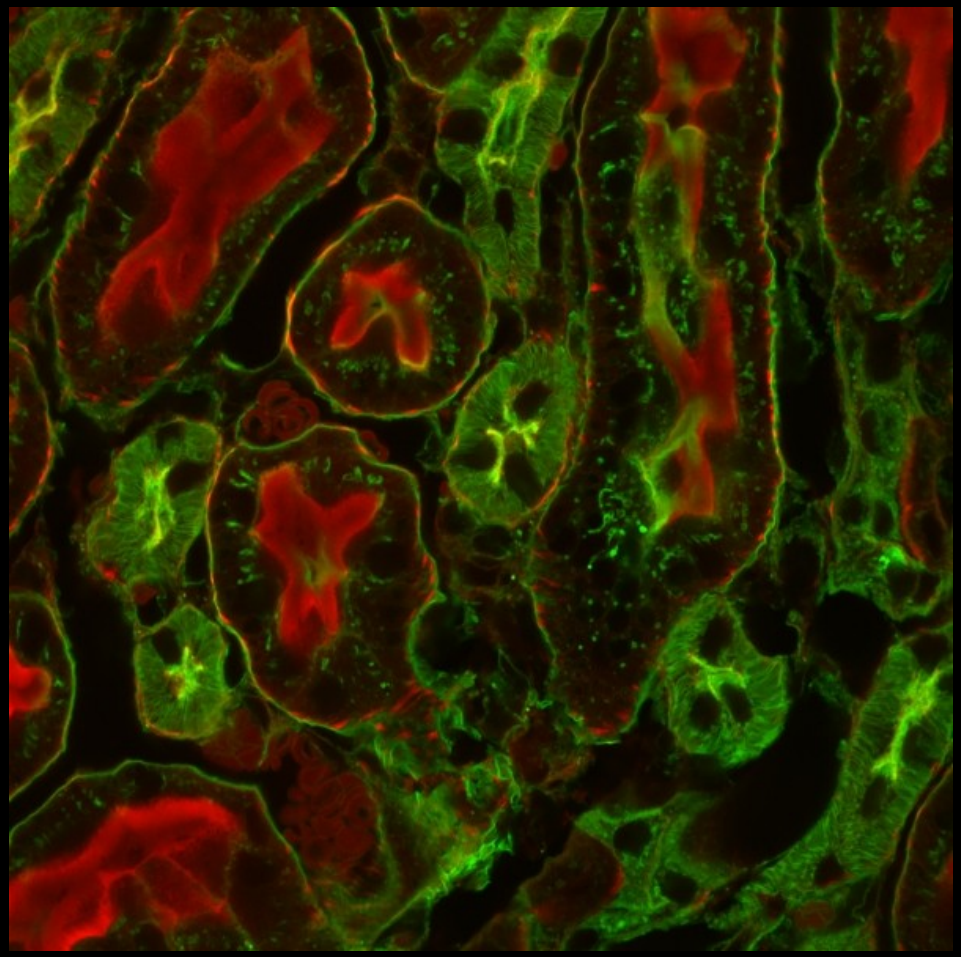
See 2 objects

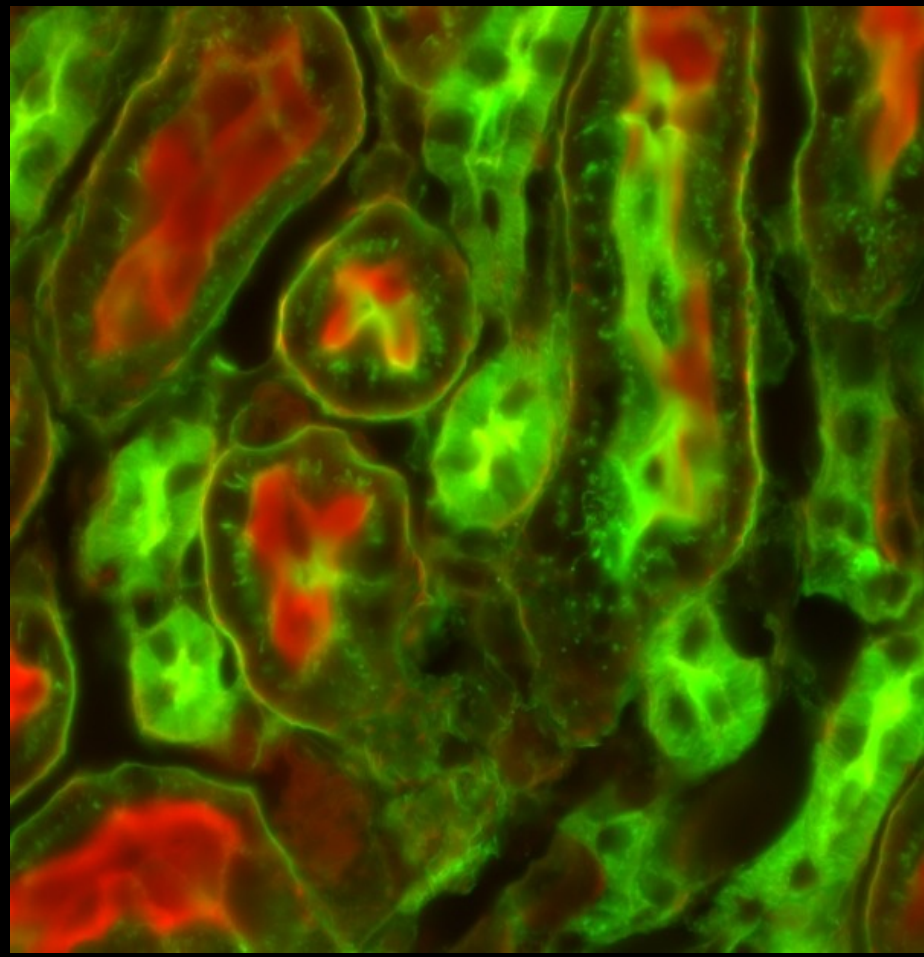


"confocal" pinhole rejects out of focus light from above and below the focal plane

http://depts.washington.edu/keck/intro.htm

Make the PSF smaller? Confocal Sectioning





closed pinhole

open pinhole

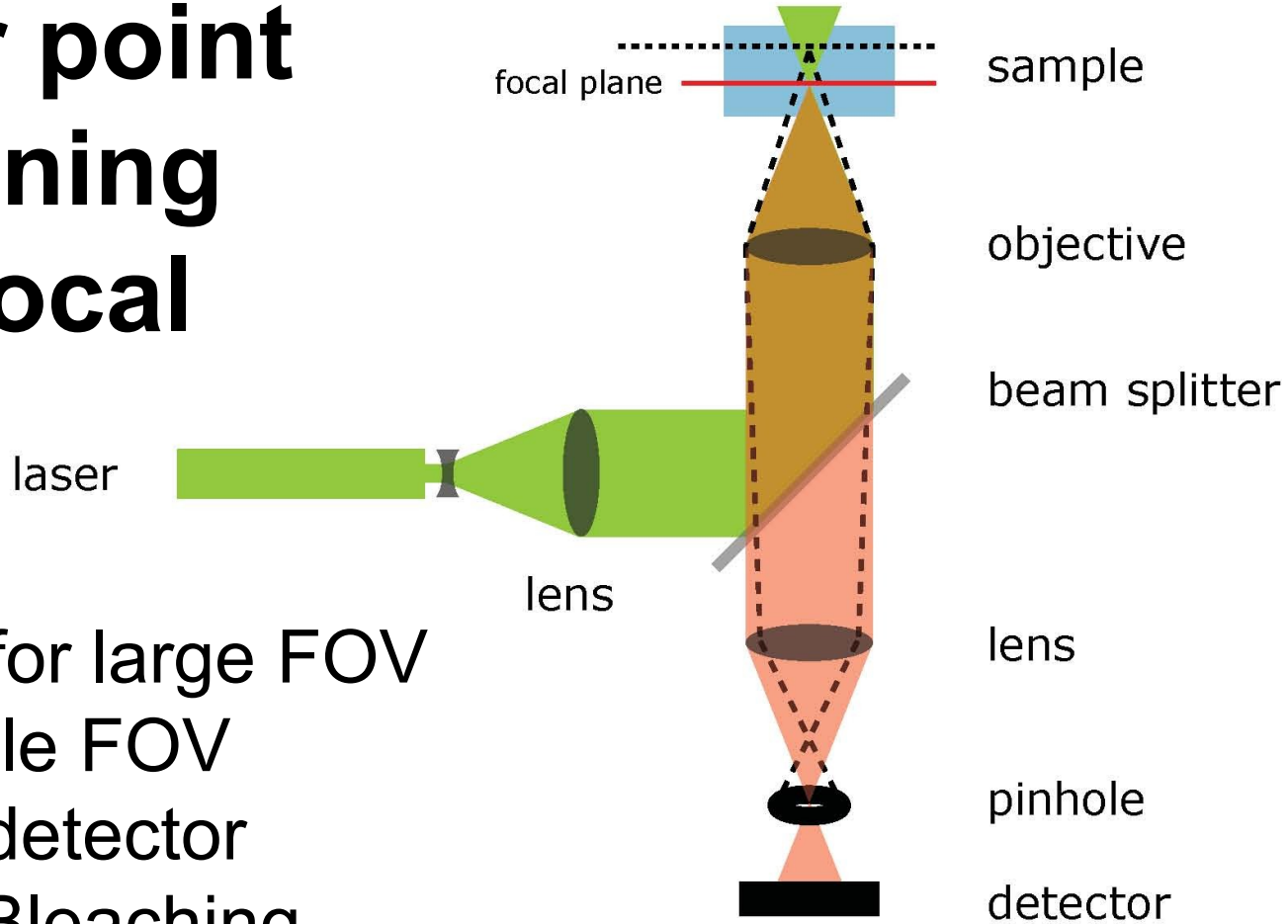
50 μm

Mouse kidney section
Alexa Fluor 488 WGA
Alexa Fluor 568 phalloidin

For a closed pinhole:
PSF is much smaller in z, bit smaller in x,y

Michael Weber

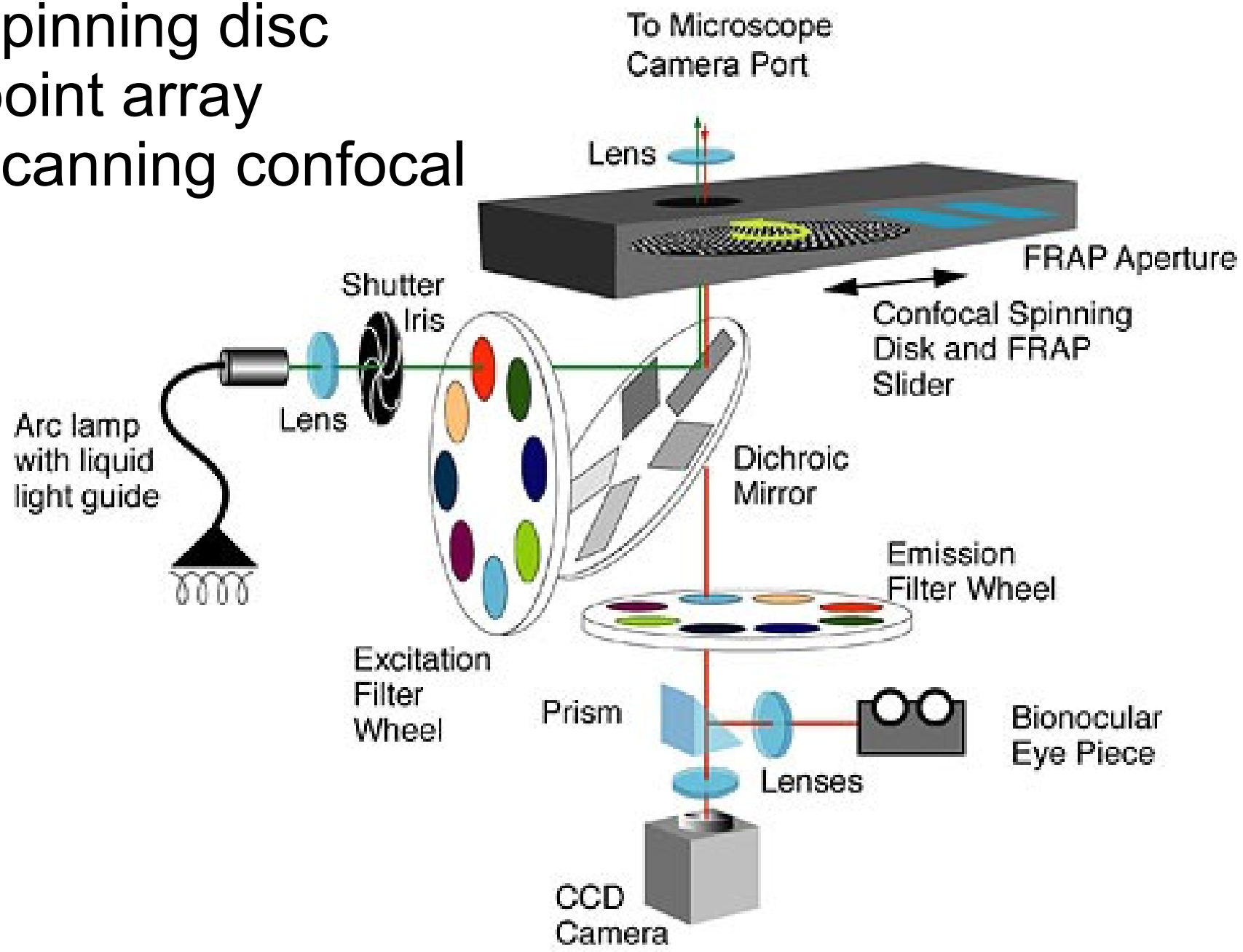
laser point scanning confocal



Michael Weber

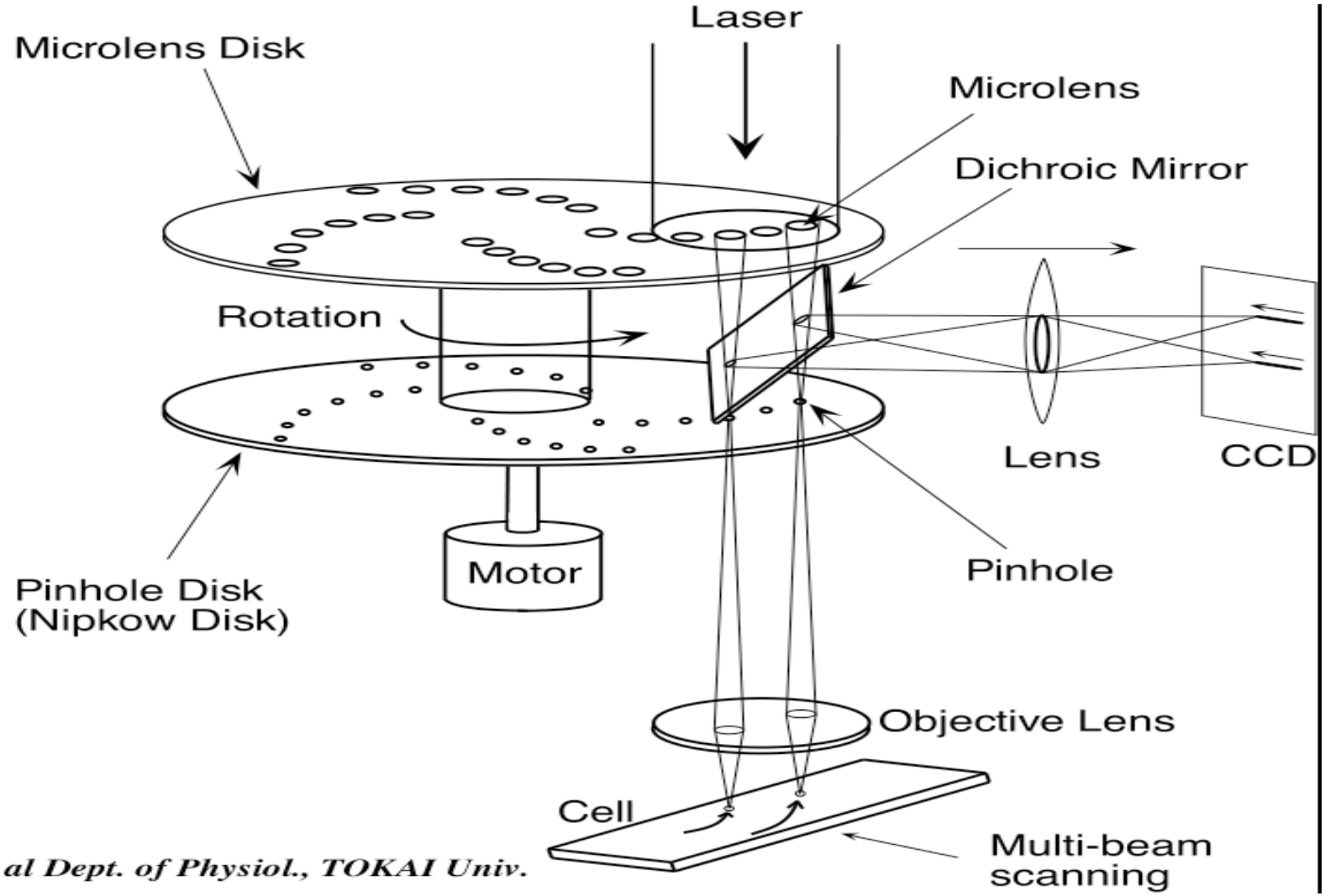
- Slow for large FOV
- Flexible FOV
- PMT detector
- High Bleaching
- “In Focus” signal = good contrast
- Good for fast images, smaller area at high resolution.

spinning disc
point array
scanning confocal



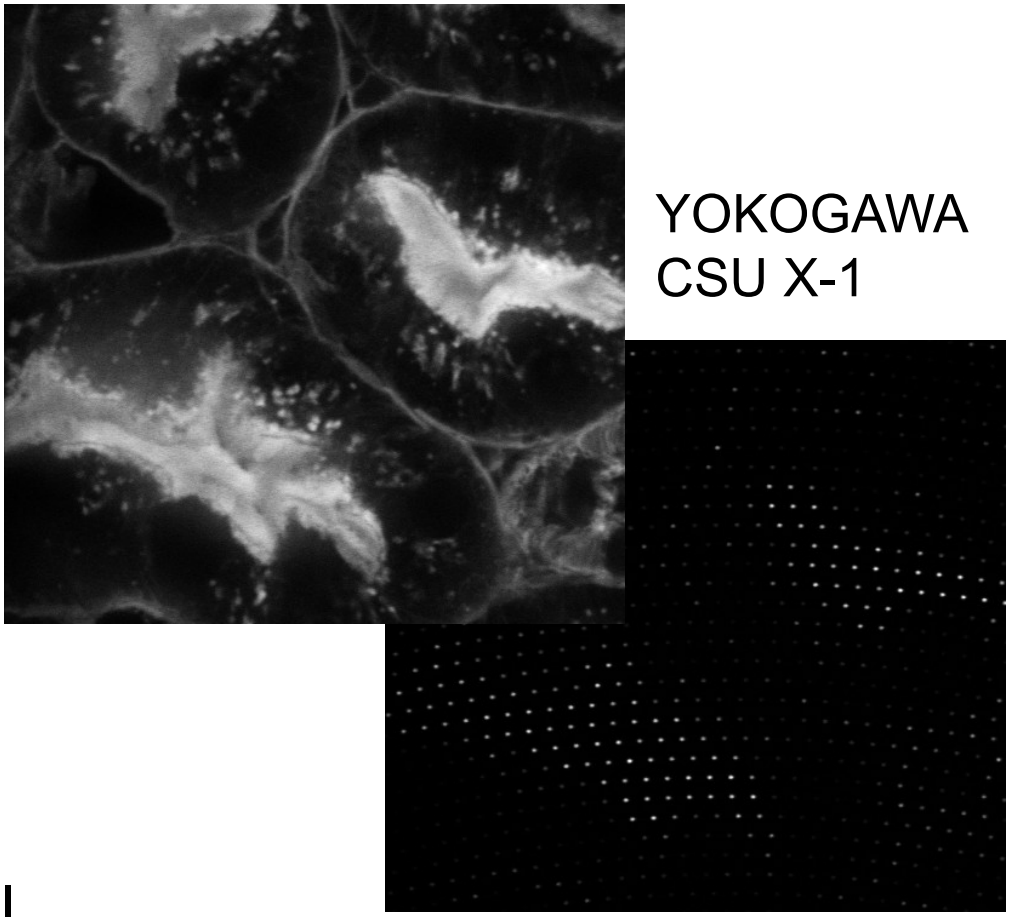
http://www.atto.com/technologies/imaging.shtml

spinning disc confocal scanner head



Spinning Disk : Multi point scanning confocal

- “field” illumination method
- Detector : CCD / EM-CCD
- High speed!!!
  - Faster than point scan
  - But still noisy
- Lower photobleaching
- Good for live samples
- Lower Z resolution
  - compared to LSCM
  - more out of focus signal



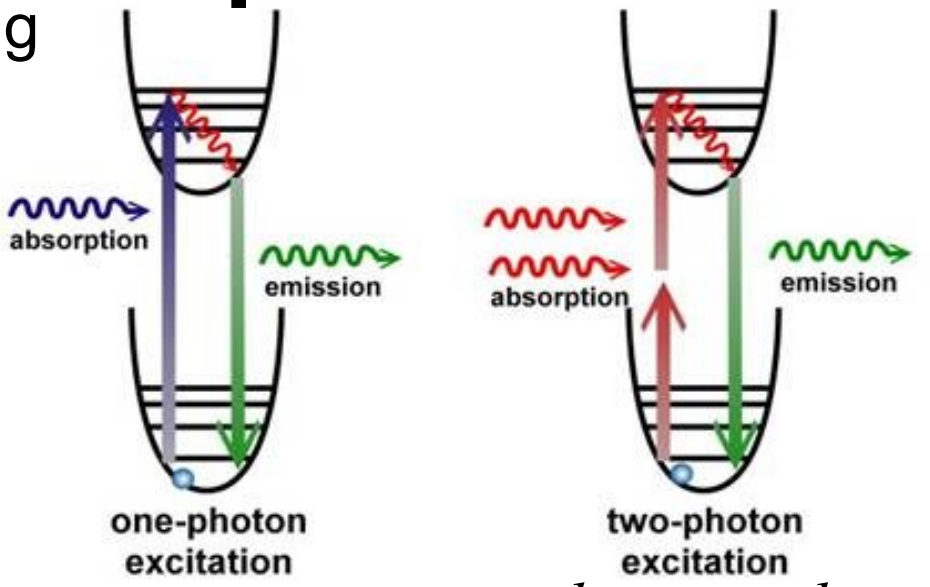
YOKOGAWA
CSU X-1

Britta Schroth-Diez

Two Photon Microscopy

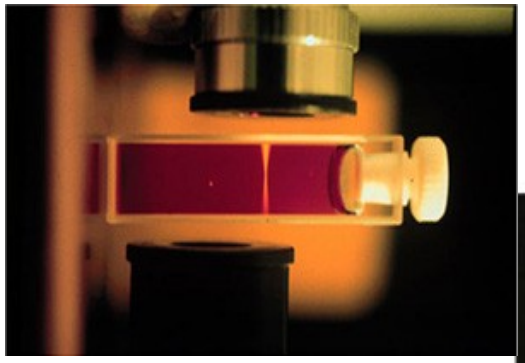
- “point” illuminating technique
- detector : PMT
- even higher Z resolution compared to LSCM (in theory)
- high penetration depth - up to 500 um
- low temporal resolution (slow speed)
- low photobleaching
- low illumination light scattering
- high price (expensive laser)
- 1 or maybe 2 “colours” only
- Good for
  - Thick samples
  - Low light scattering
  - Living Samples

2 photon effect

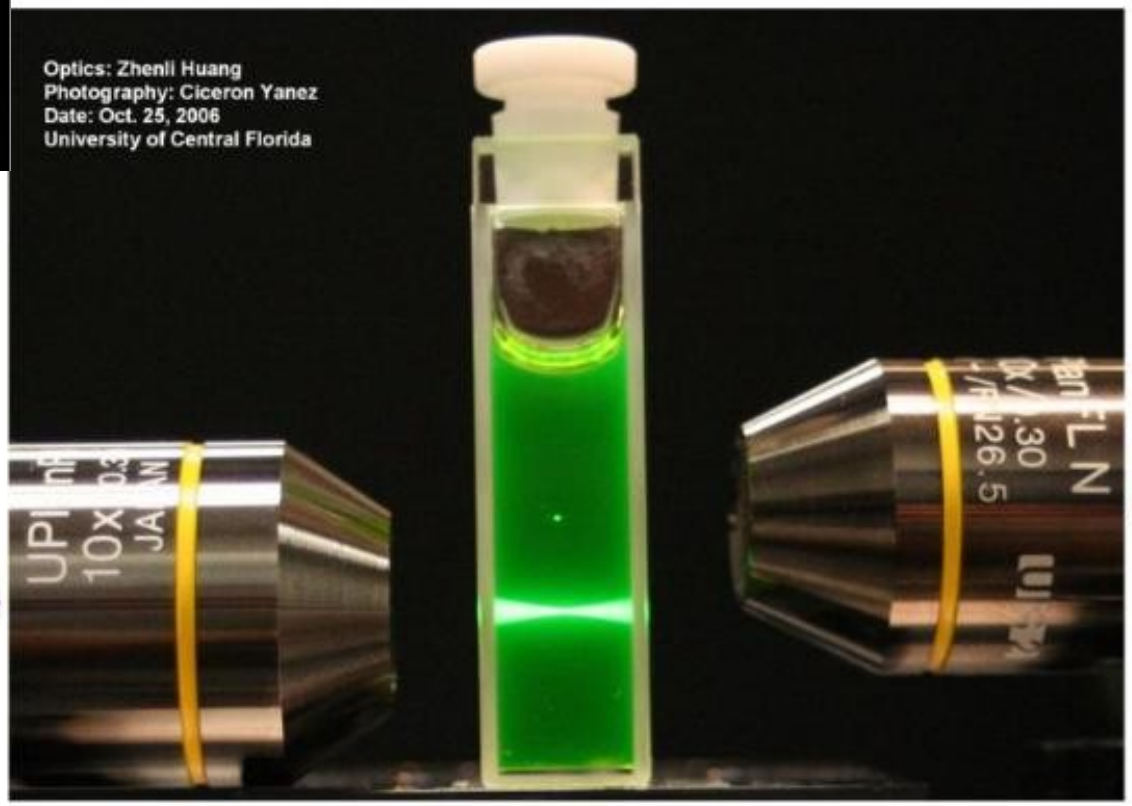


www.phys.ntu.edu.tw

2 Photon Microscopy



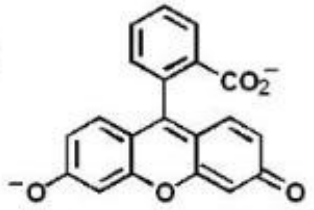
Brad Amos MRC



380nm,200fs

760nm,200fs

Fluorescein



belfield.cos.ucf.edu

Total Internal Reflection Fluorescence

- field illuminating technique
- detector : CCD
- fast
- very high Z resolution
  - ~150 nm
- one optical section only at plane close to cover glass
- Living sample
  - Fast dynamics

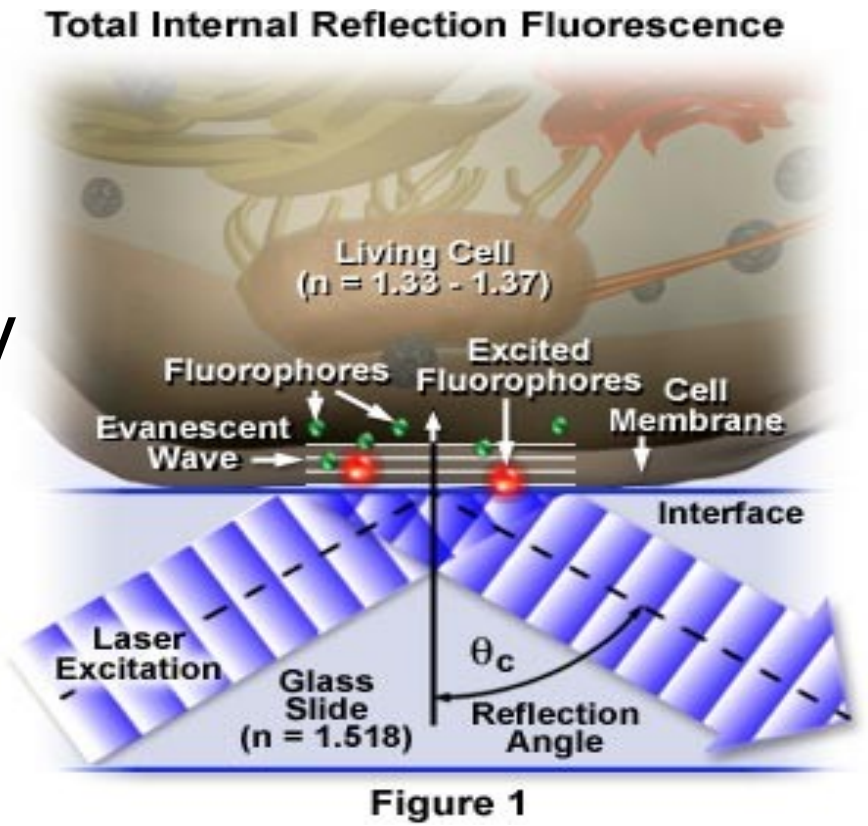
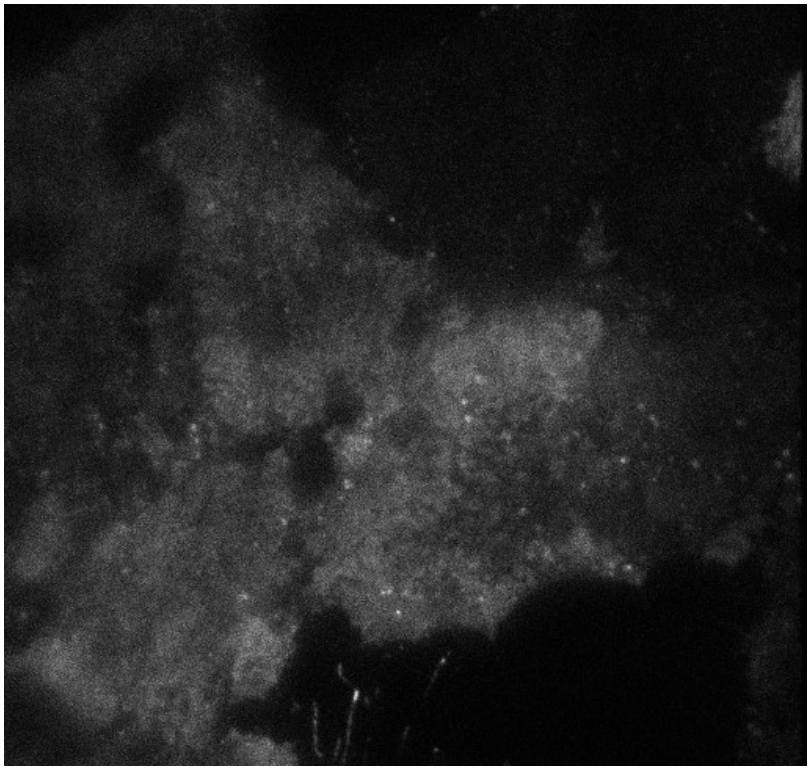


Figure 1

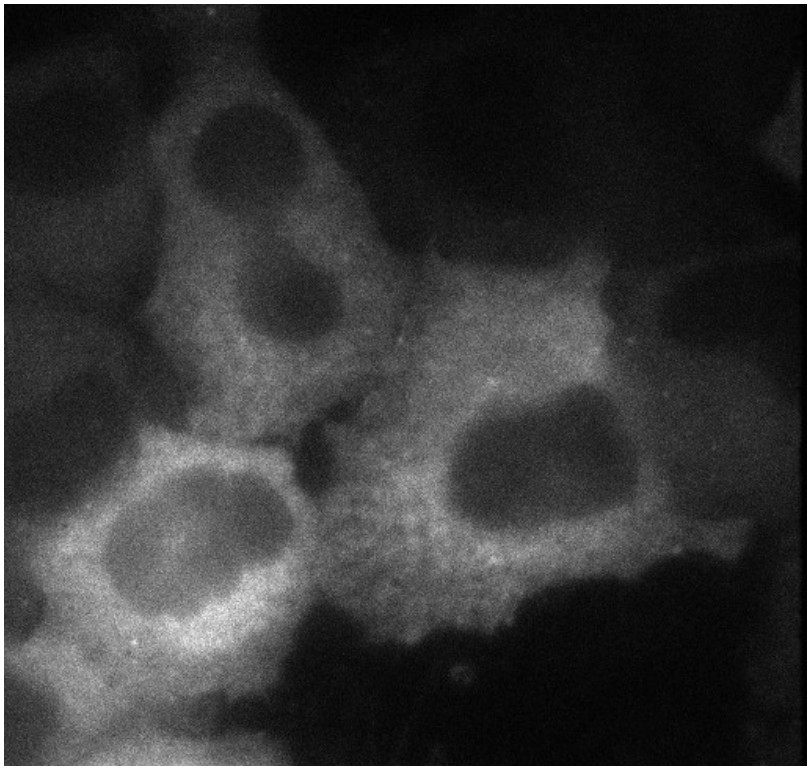
http://micro.magnet.fsu.edu/primer/java/tirf/reflect/index.html

TIRF

only the objects *on* the coverslip

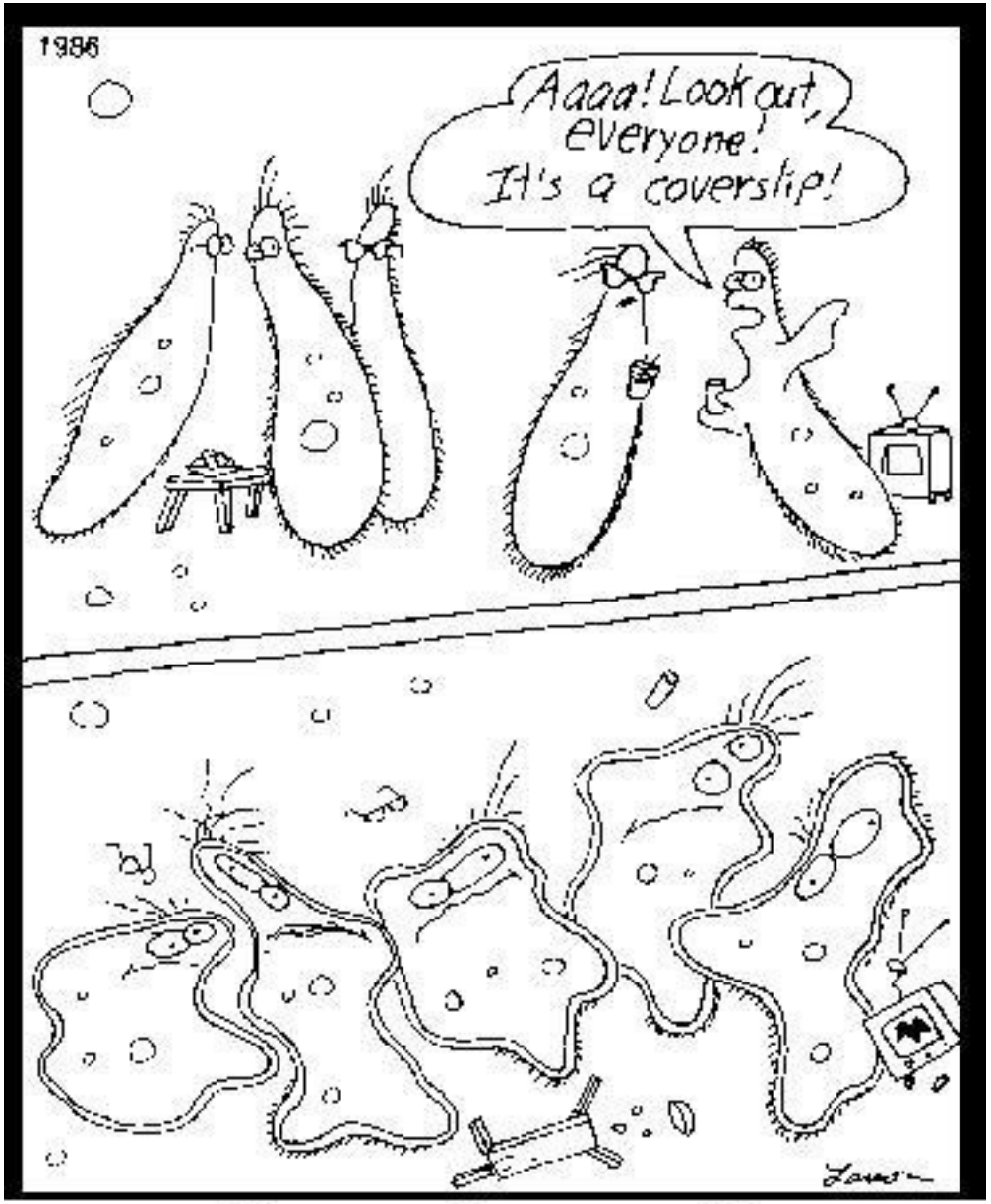


TIRF



Wide field

Britta S-D, Silke G

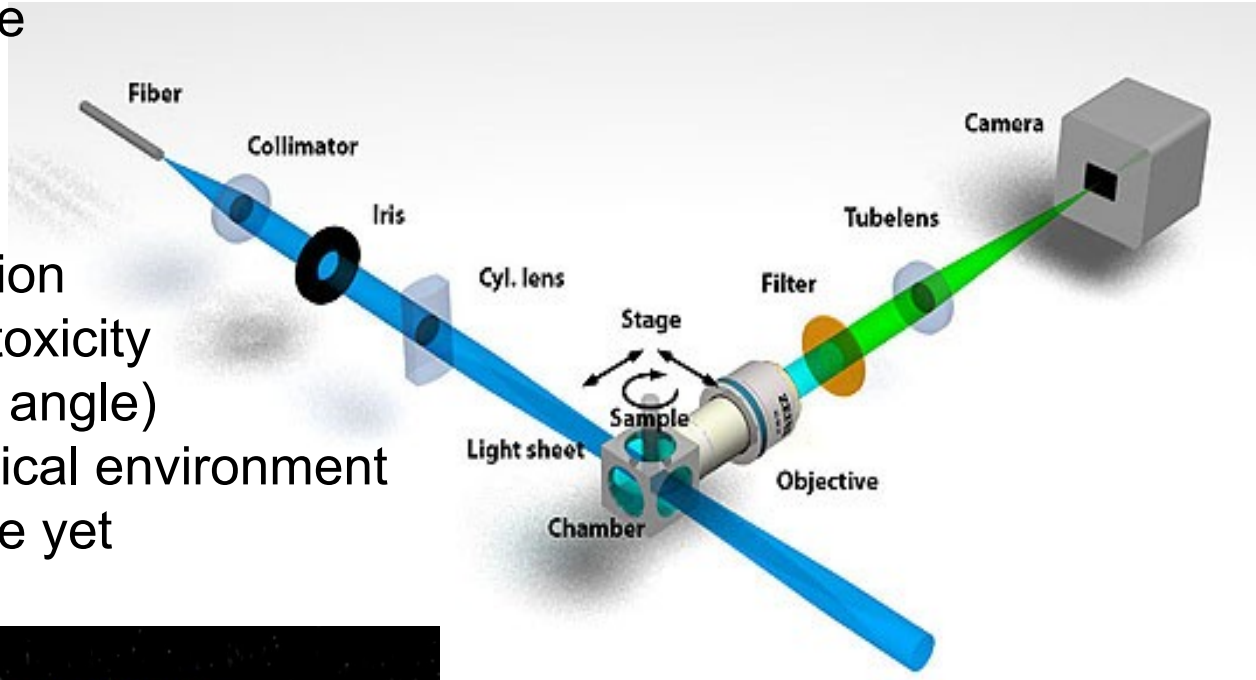


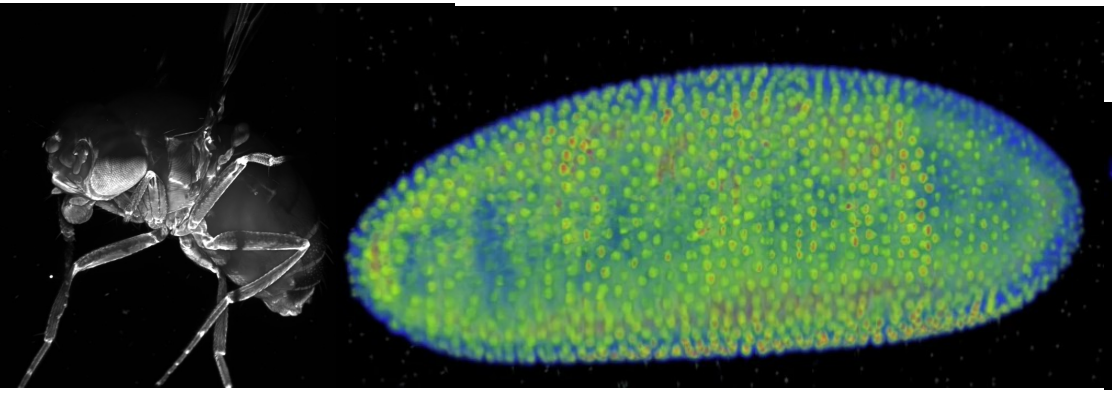
Life on a microscope slide

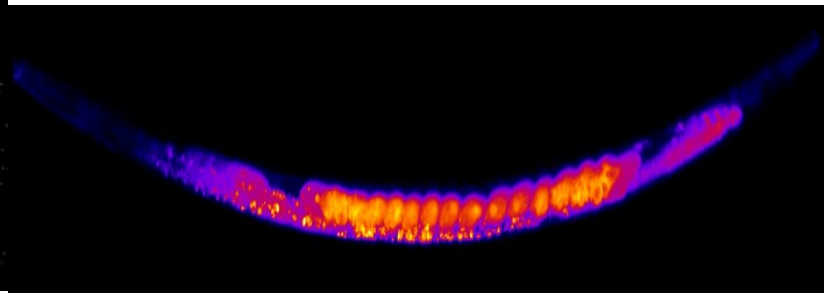
Sticking biology to flat glass is not very physiological...

SPIM
Single Plane Illumination Microscopy

- field illumination technique
- detector: CCD
- high speed
- Isotropic Resolution xyz
  Multi angle reconstruction
- very low photobleaching/toxicity
- lower Z resolution (single angle)
- 3D mounting in physiological environment
- not commercially available yet

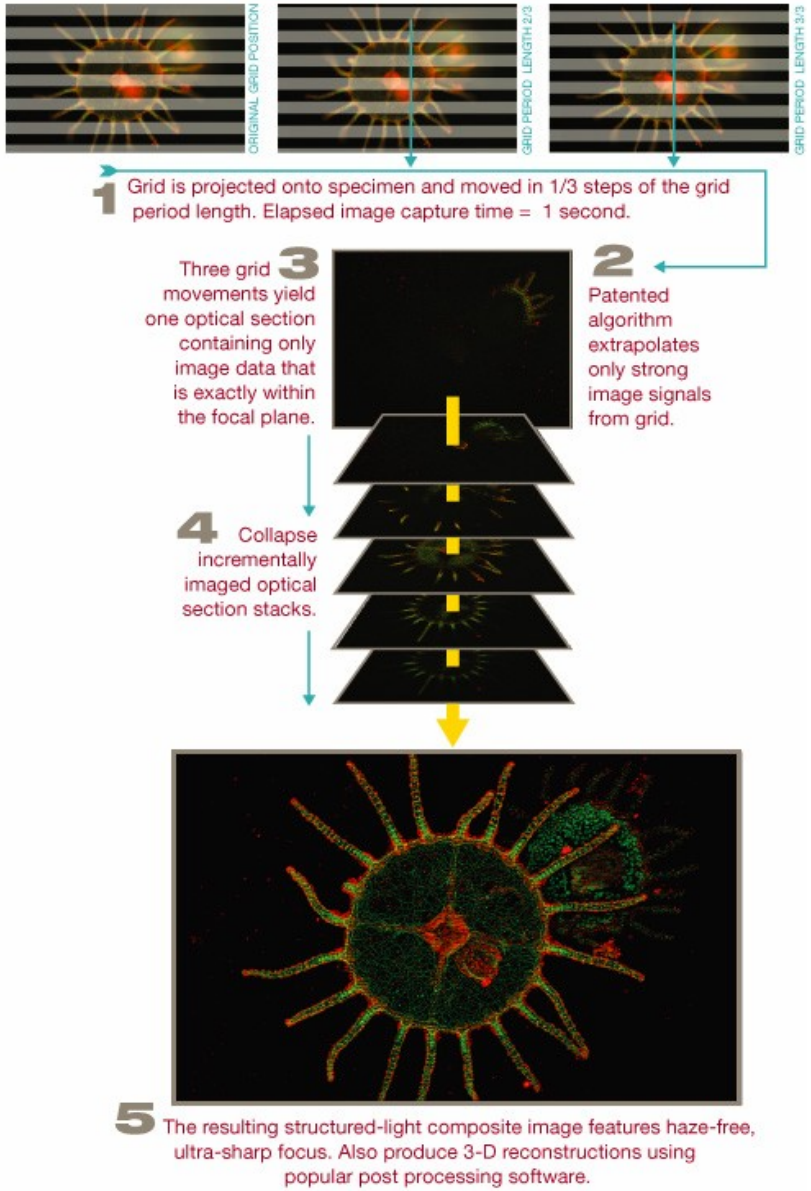






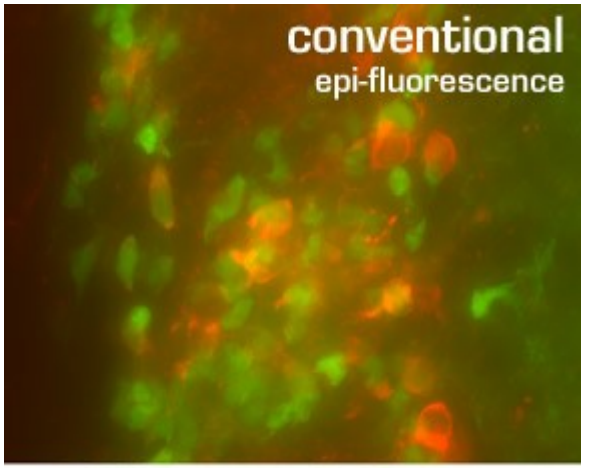
White, Preibisch, Saalfeld, Ejsmont, Sarov, Tomancak *http://www.huisken.org/jan/spim.html*

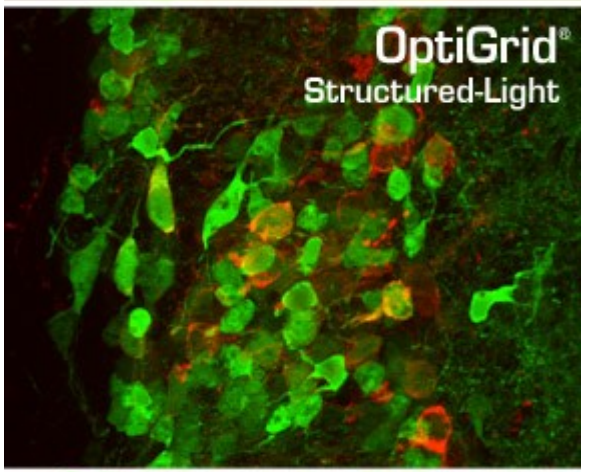
Structured illumination

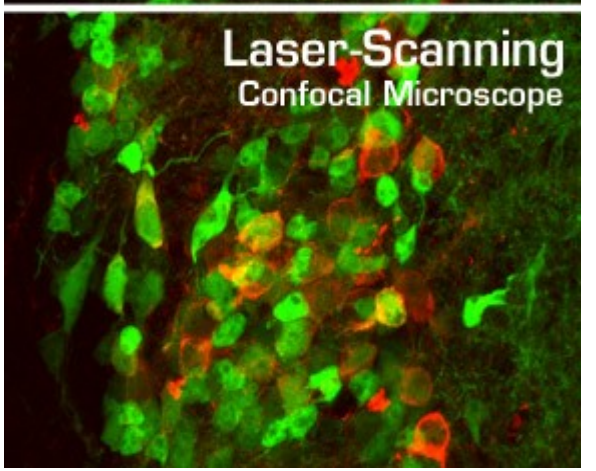


Optigrid, ApoTome and OMX

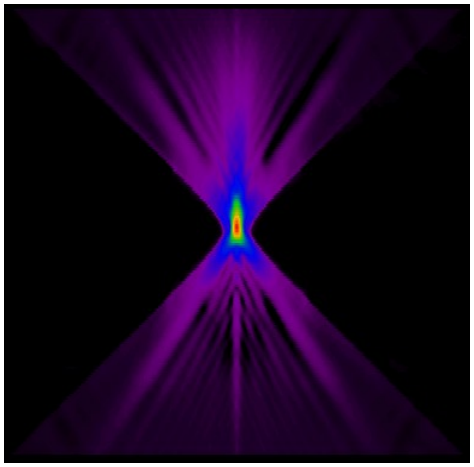
- "field" illumination
- detector : CCD
- slow/medium speed
- high flexibility
- image processing required (fast)
- Good for fixed thinner samples, instead of confocal





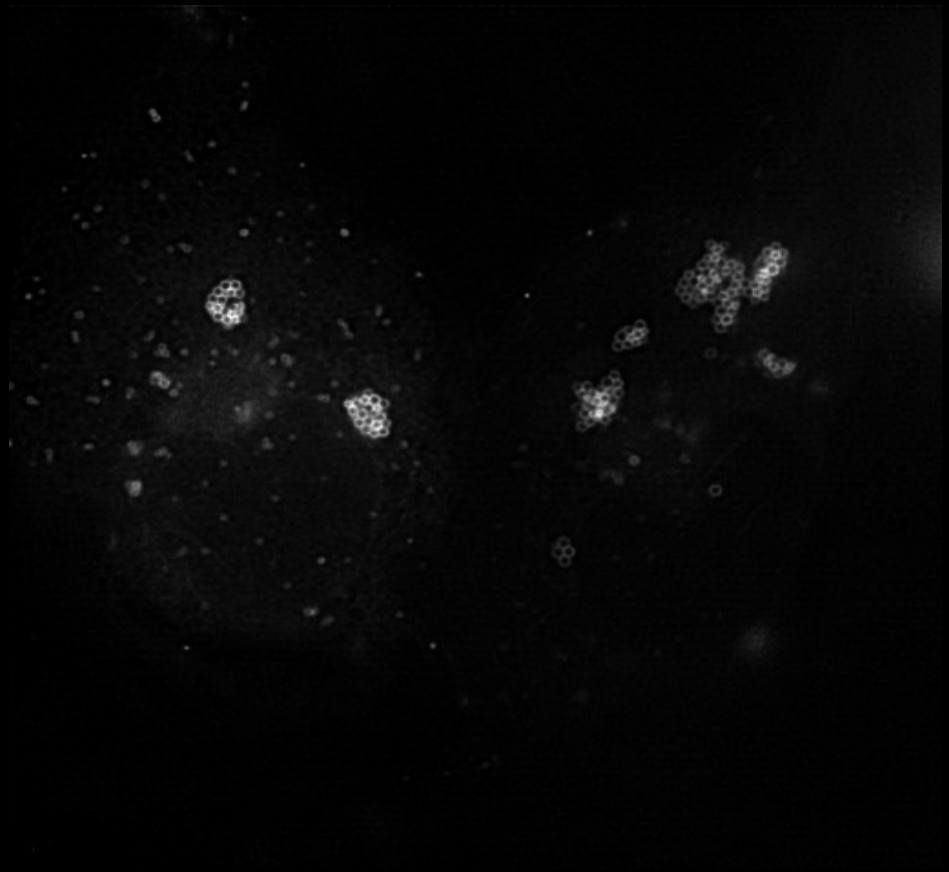


Widefield Microscopy + Deconvolution

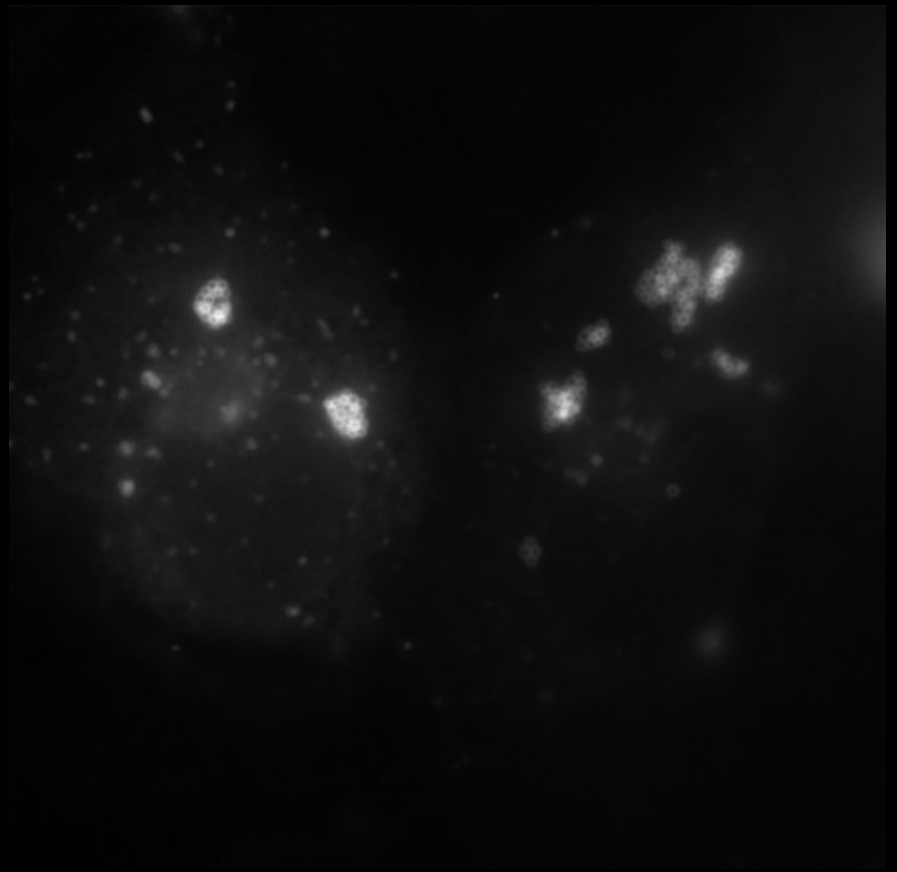


- “field” illuminating technique
- Detector : CCD
- High temporal resolution (high speed)
- High flexibility
- Low bleaching / photo toxicity
- Very high signal:noise (low speckle)
- Image processing required (automated)
- Good for fixed and living samples.

Wide-field fluorescence + deconvolution
Single plane – 2D blind deconv.



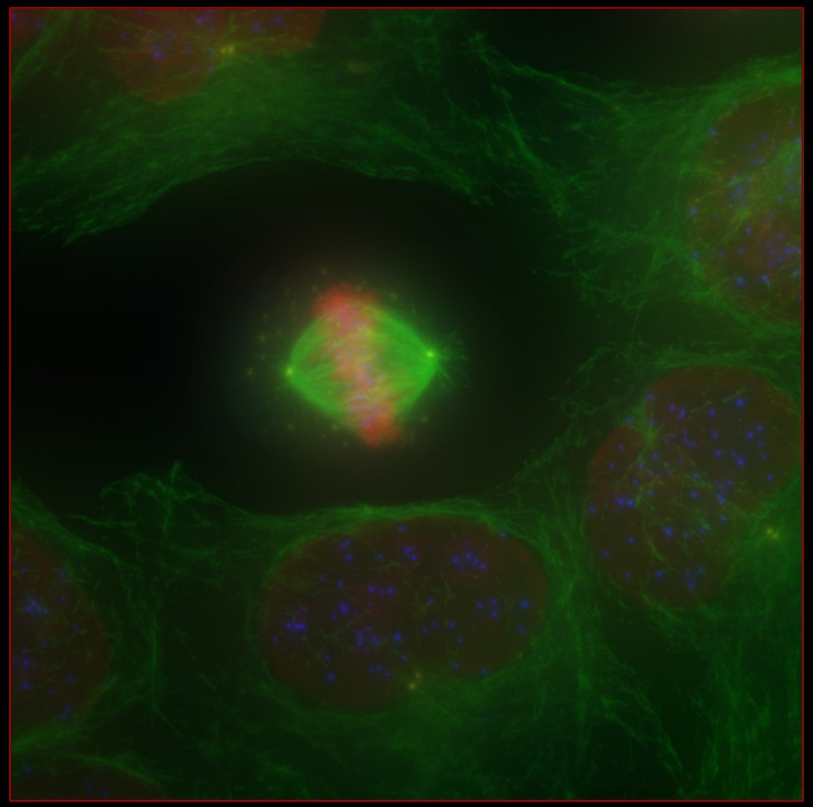
raw image



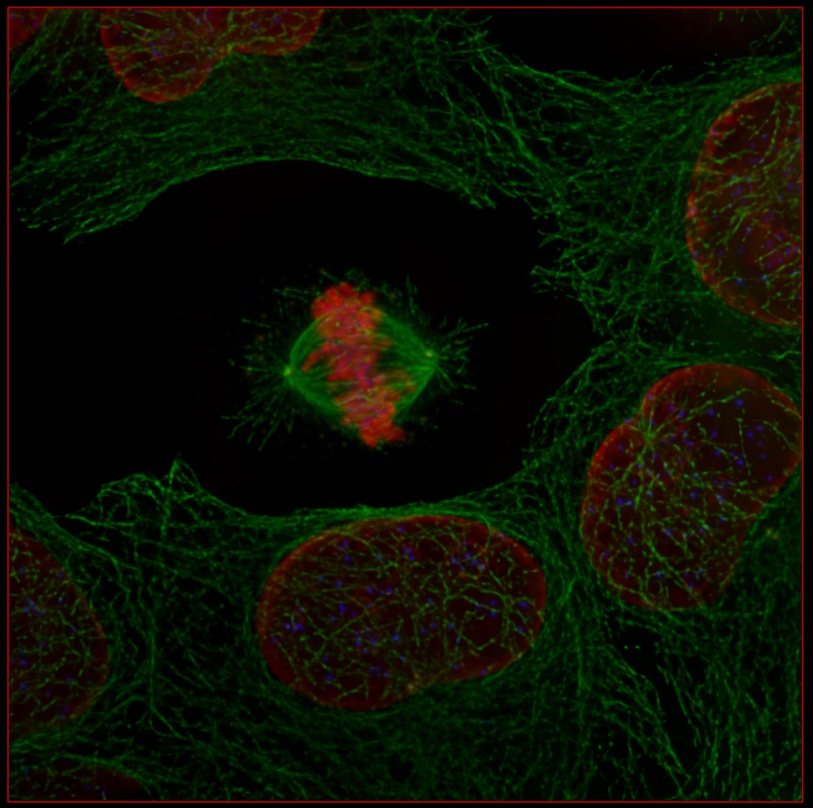
deconvolved image

Johanna Spandl

Wide-field fluorescence + deconvolution
z-stack mono layer cultured cells on glass



raw image
max Z projection

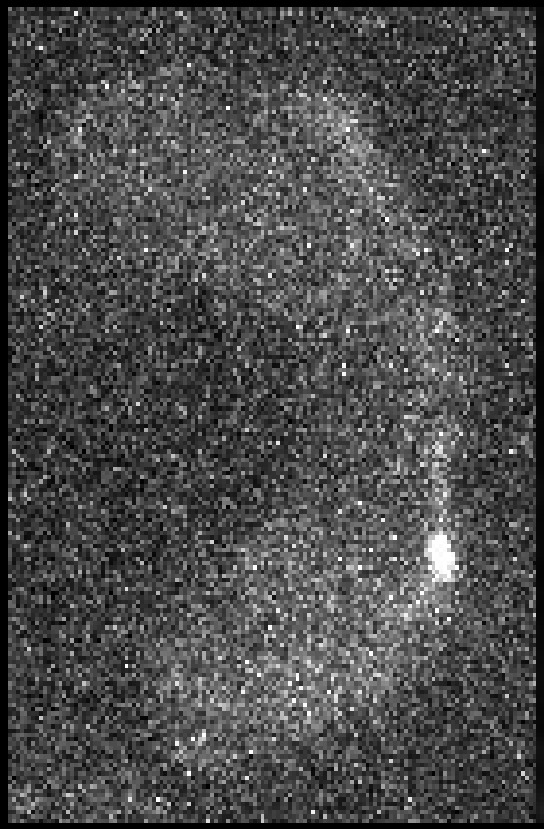


deconvolved image
max Z projection

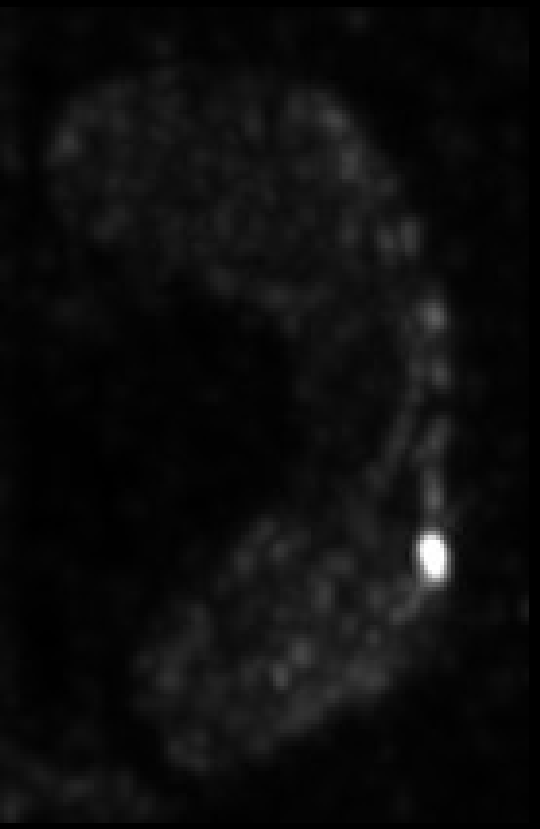
Alex Bird / Dan White

Low signal:noise yeast live 3D imaging
Spinning Disk Confocal

Deconvolution is also good at noise removal, revealing details and improving contrast in all kinds of microscopy images.



raw image
max Z projection



deconvolved image
max Z projection

Davide Accardi / Dan White

Take Home Messages

1) Don't use laser scanning confocal just because it most expensive.
Choose the **right microscopy tool for the job**.

2) Know the thickness of your **cover-slip**: accuracy – reproducibility.
Don't put expensive samples on cheap glass!

3) Know **refractive indices** of your sample mounting medium and lens immersion medium.
Contrast – Penetration – lens with correction collar?.

4) Know your detector: CCD, PMT, APD, eye
sensitivity, spectral response. **Never, ever, saturate it (= lost info)!**

5) Know your x,y,z,t,i scanning / sampling system. It determines the **information quality** in the images.

6) Know and clean your **objectives**: N.A, transmission efficiency, chromatic correction, field flatness.
Measure Beads:PSF. Pixel/Voxel size must be matched to the **resolution** you need to see, Nyquist says **~3x smaller than object, xyzt.**

7) Keep your raw data in original format, on the file-server.
Preserve Meta Data (don't export as TIFF)! Work on copies.

8) Optimise detector settings / scan speed to get the **signal:noise (image quality)** needed for analysis

9) Think **Spectroscopy, not Photography**.
Pretty pictures are nice, but scientists measure things. **Controls + Statistics**.

10) **Deconvolution** is nearly always a good idea.