Deconvolution – Pros & Cons

3D microscopy

"Systematic errors can be measured and corrected for"

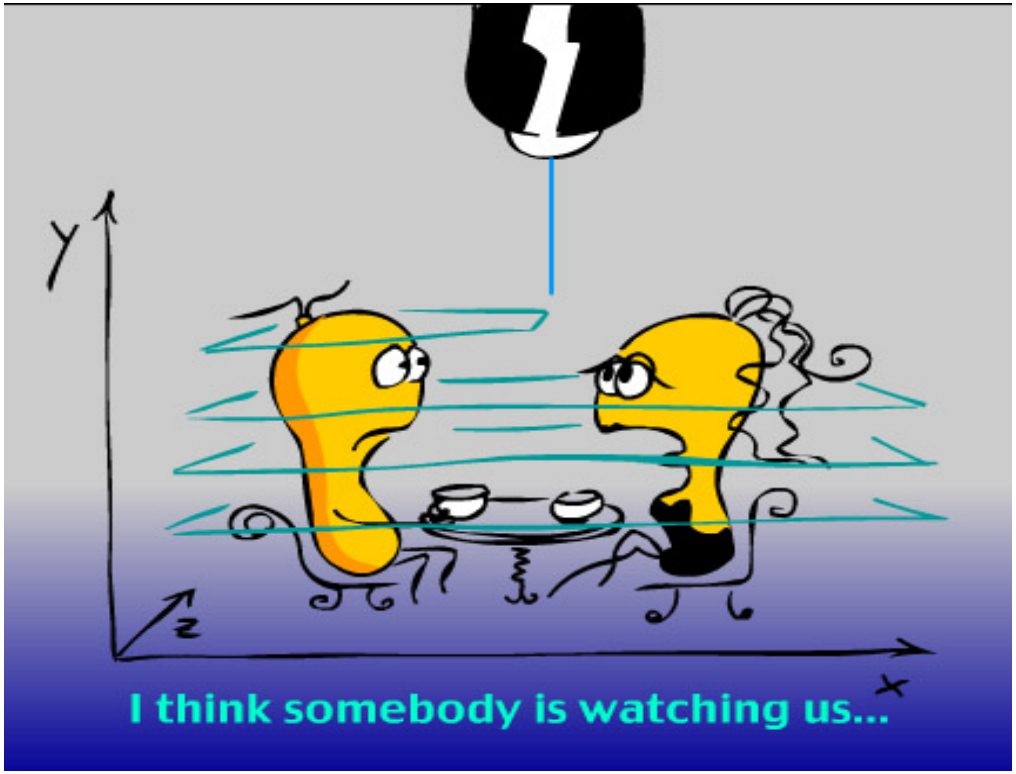
Or

"Enhancing contrast and removing noise helps see the resolution that's really there"

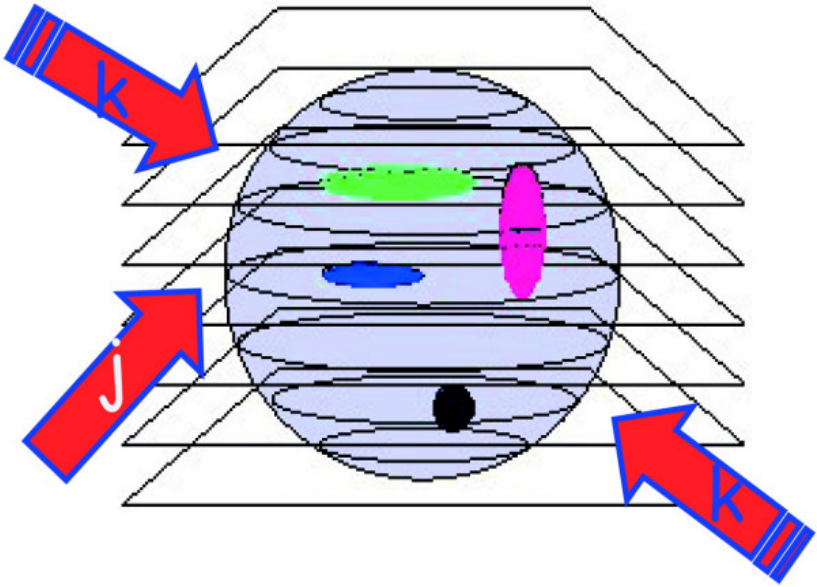
CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics

Dan White – Oct 2010

Optical sectioning – 3D imaging



Digital sampling of
3 spatial dimensions



X and Y are easy, right...?

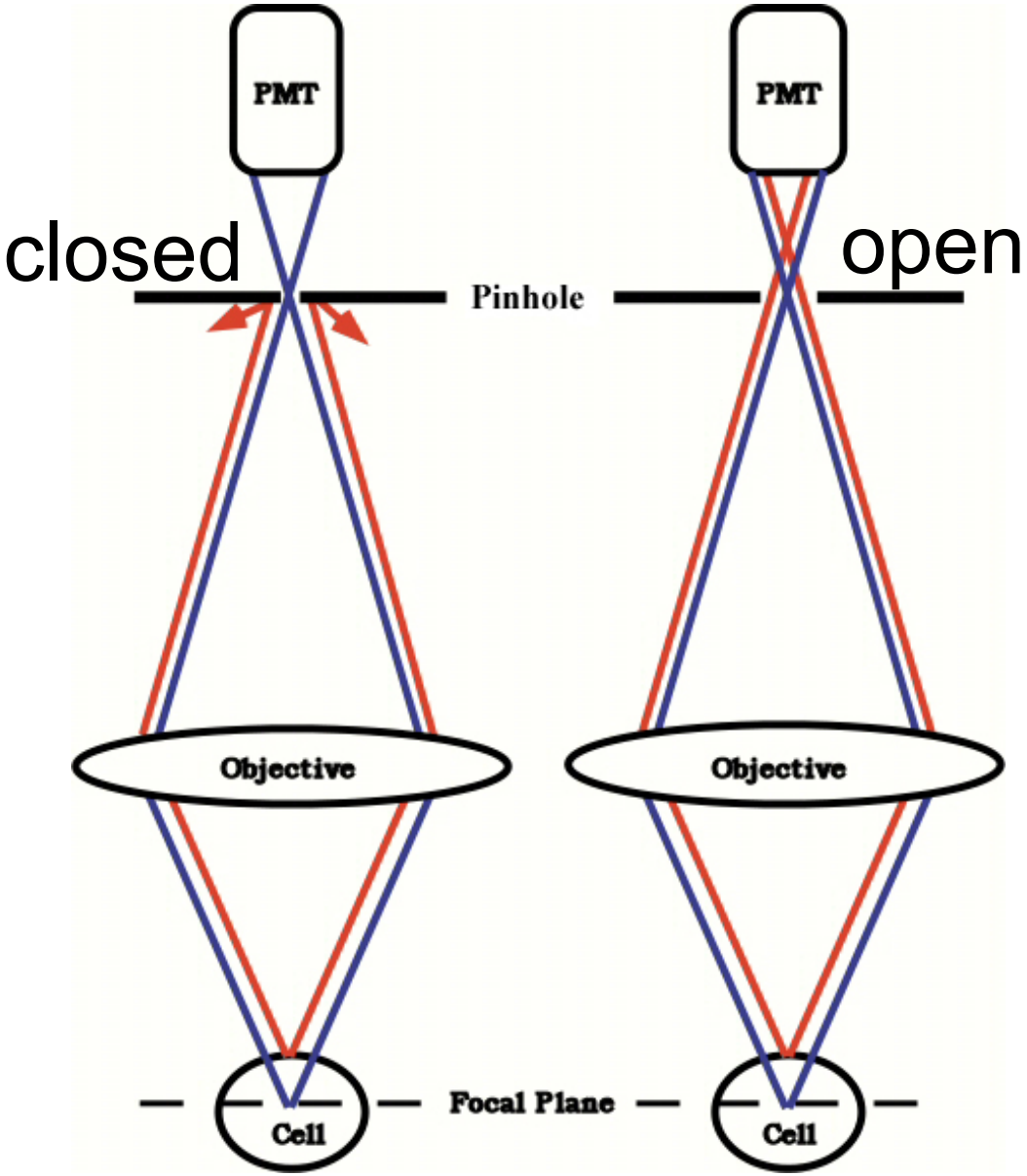
… but is Z special?

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

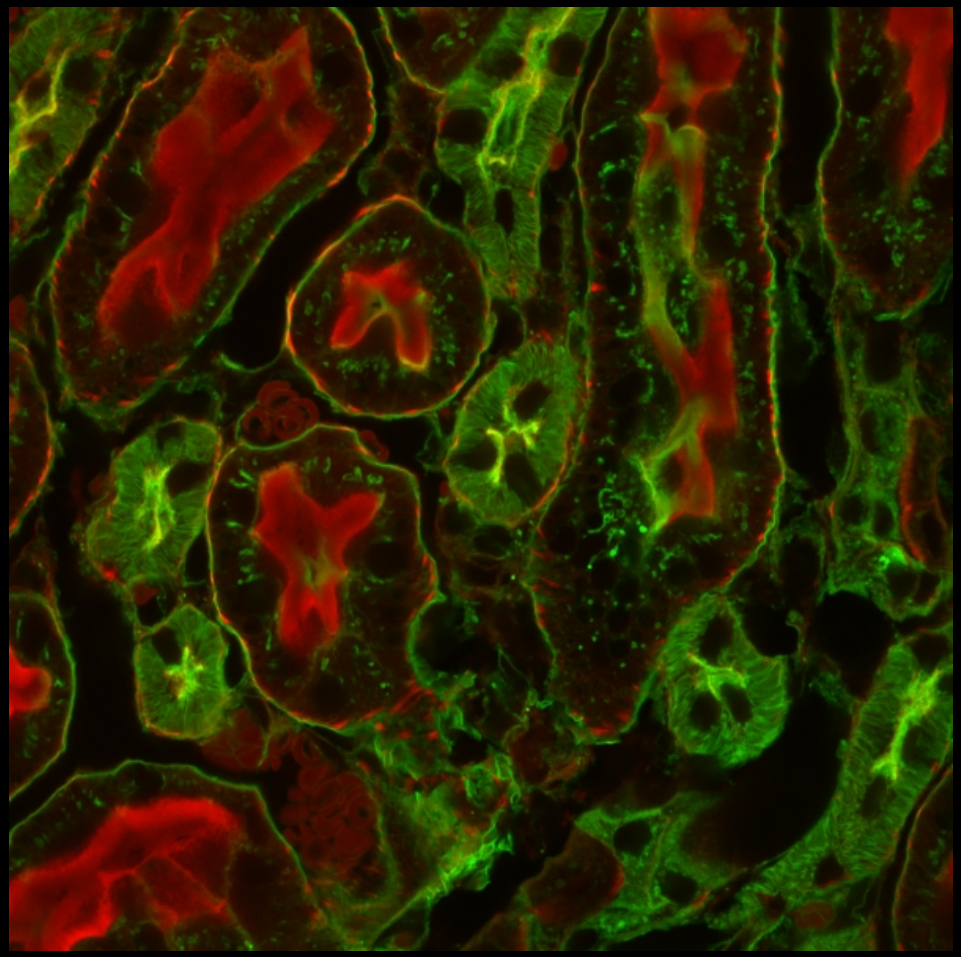
- structured illumination microscopy
- widefield microscopy + deconvolution

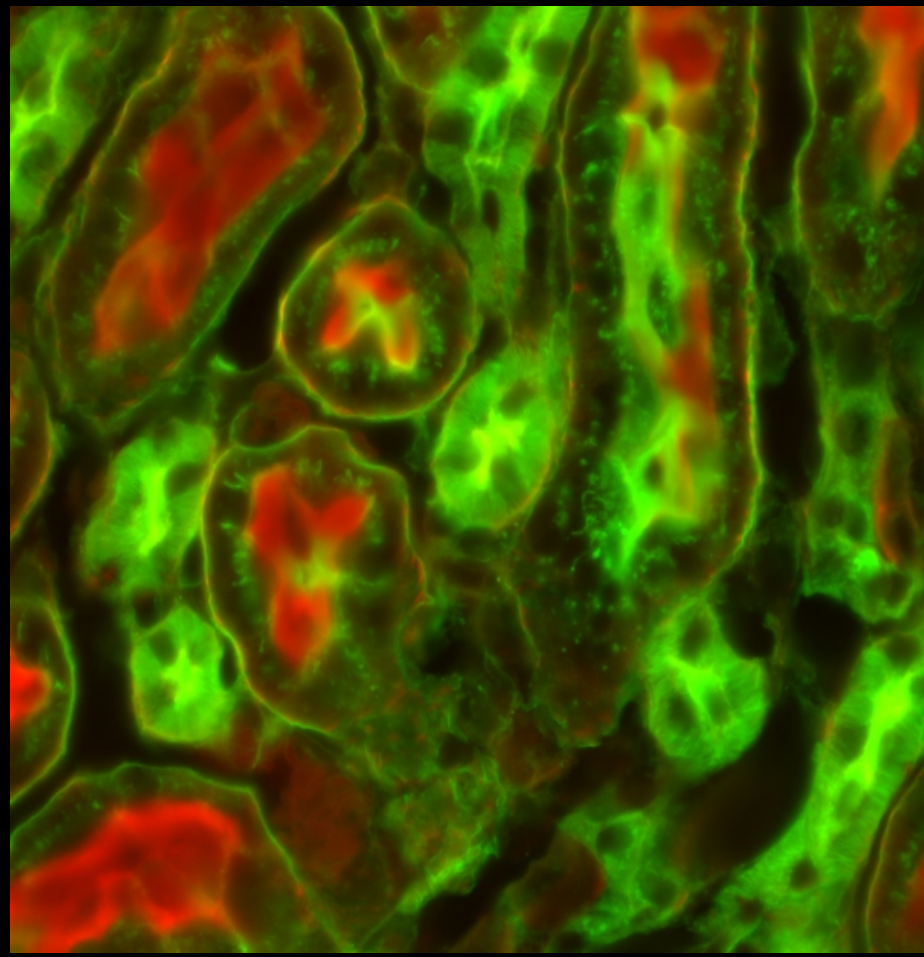


"confocal" pinhole rejects out of focus light from above and below the focal plane

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

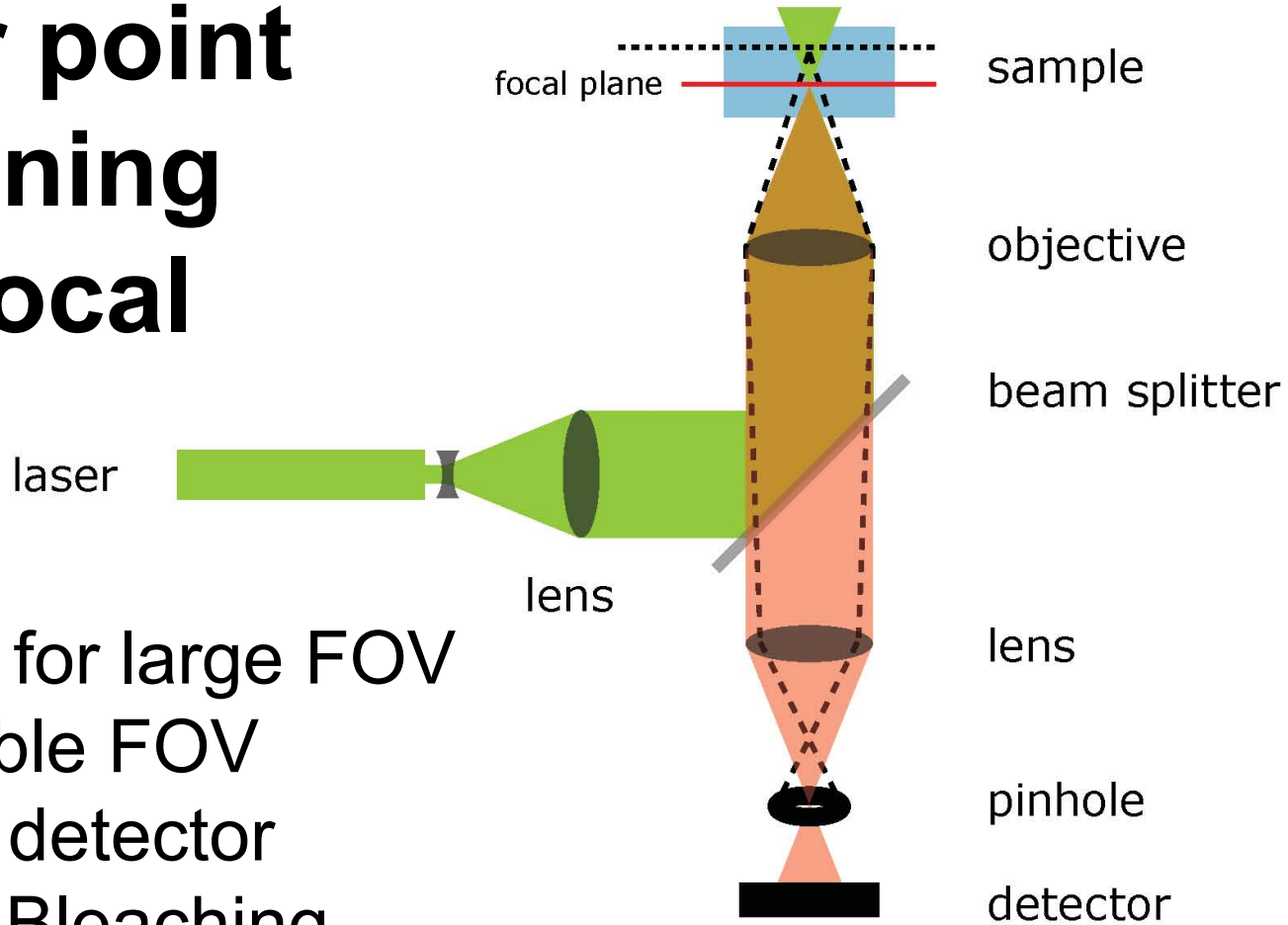
Alexa Fluor 568 phalloidin

For a closed pinhole:

PSF is much smaller in z, bit smaller in x,y

Michael Weber

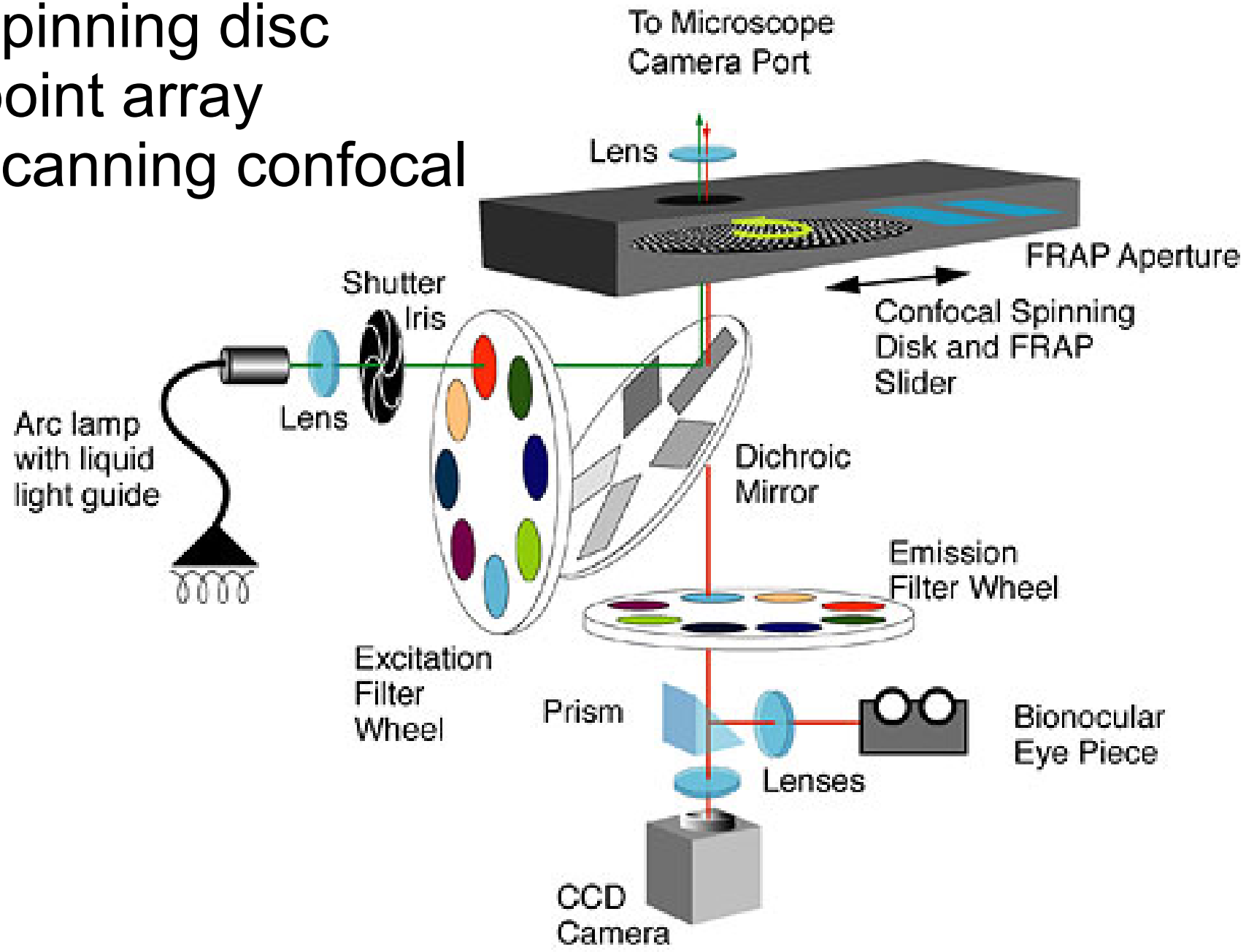
laser point scanning confocal



Michael Weber

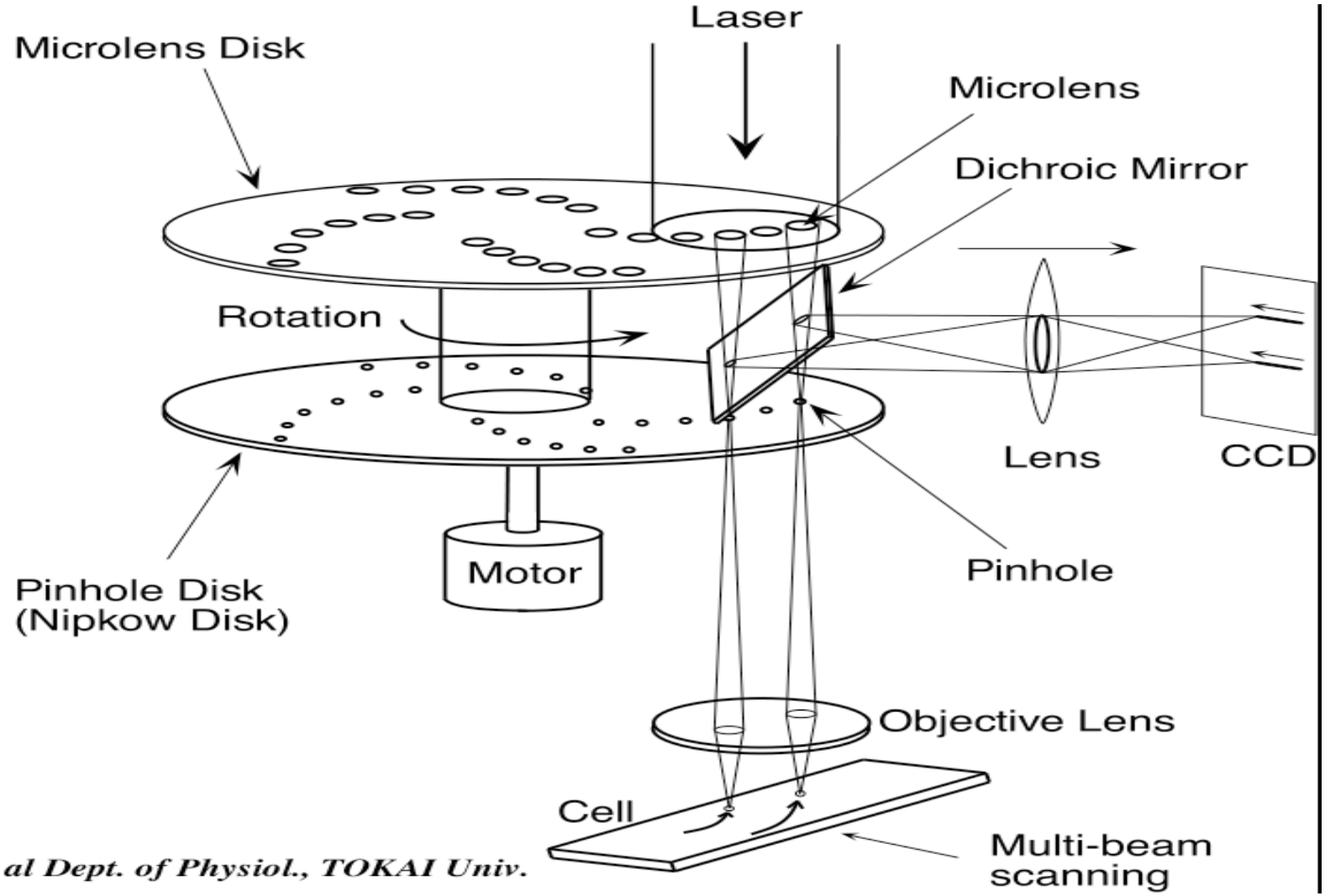
- Slow for large FOV
- Flexible FOV
- PMT detector
- High Bleaching
- “In Focus” signal = good contrast
- Good for fast images, smaller area at high resolution.

spinning disc
point array
scanning confocal



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

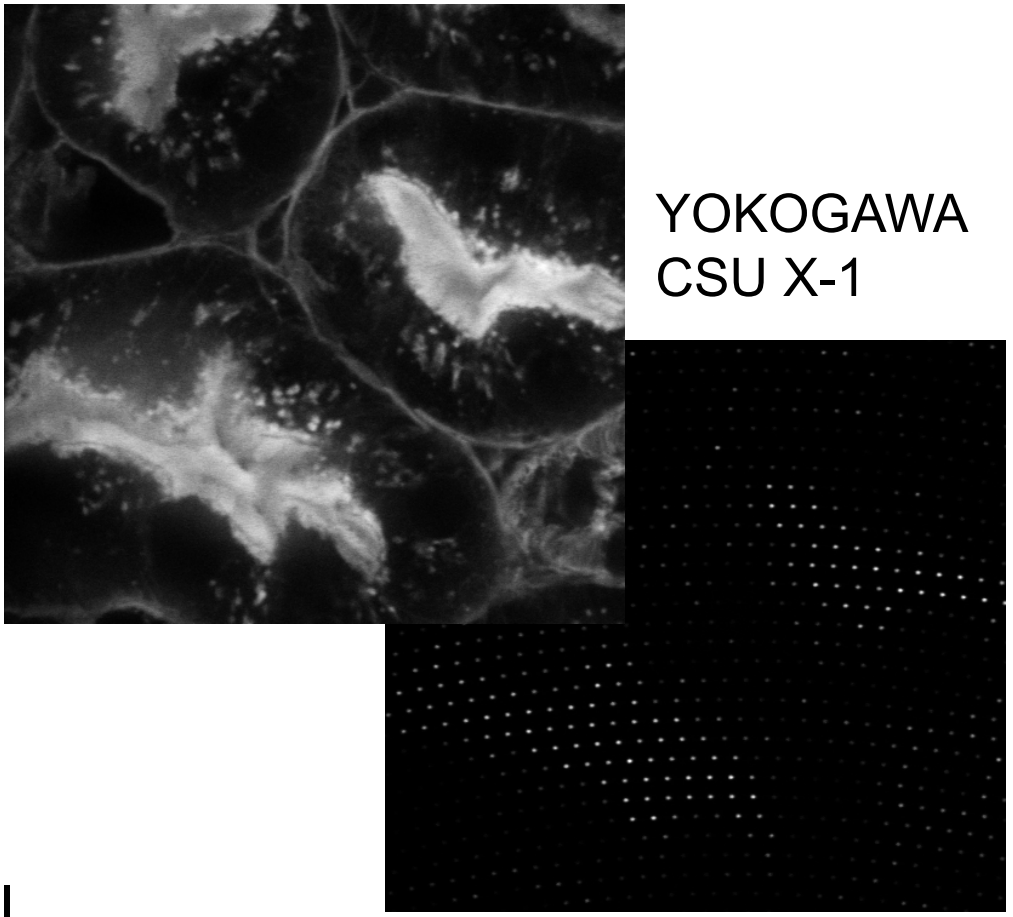
spinning disc confocal scanner head



H. Ishida et al Dept. of Physiol., TOKAI Univ.

Spinning Disk : Multi point scanning confocal

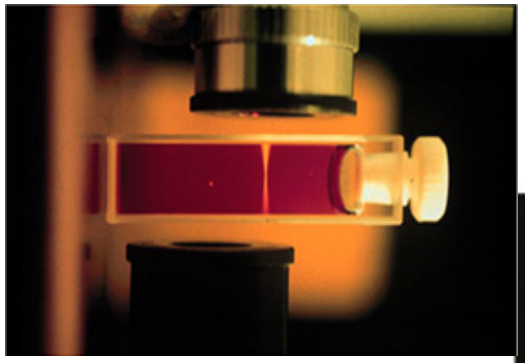
- “field” illumination method
- Detector : CCD / EM-CCD
- High speed!!!
  - Faster than point scan
  - But still noisy
- Lower photobleaching
- Good for live samples
- Lower Z resolution
  - compared to LSCM
  - more out of focus signal



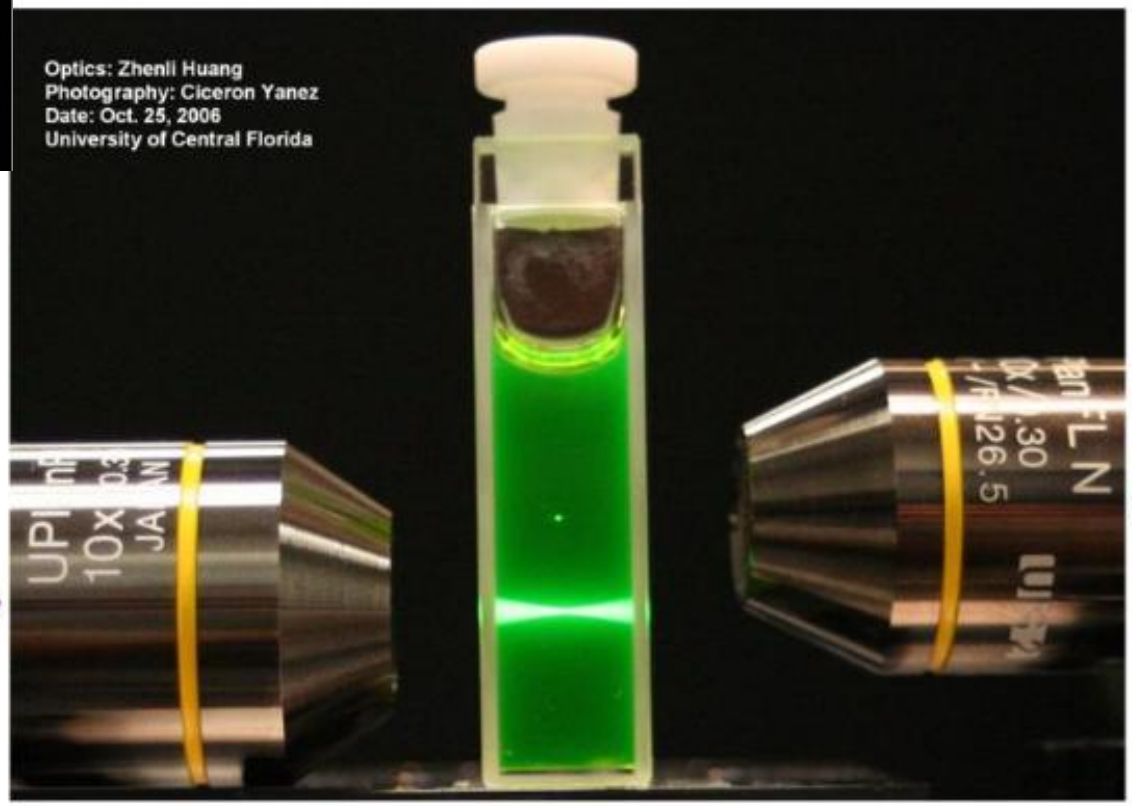
YOKOGAWA
CSU X-1

Britta Schroth-Diez

2 Photon Microscopy



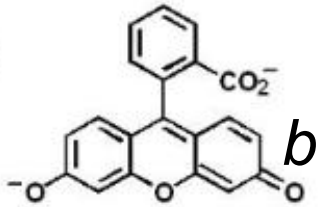
Brad Amos MRC



380nm,200fs

760nm,200fs

Fluorescein

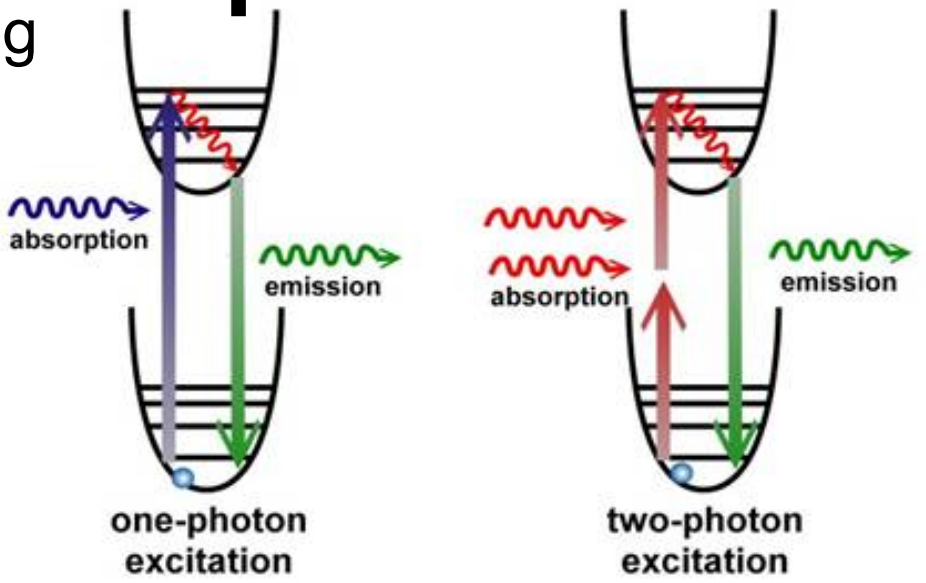


belfield.cos.ucf.edu

Two Photon Microscopy

- “point” illuminating technique
- detector : PMT
- Lower z resolution to LSCM (longer excitation)
- 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

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

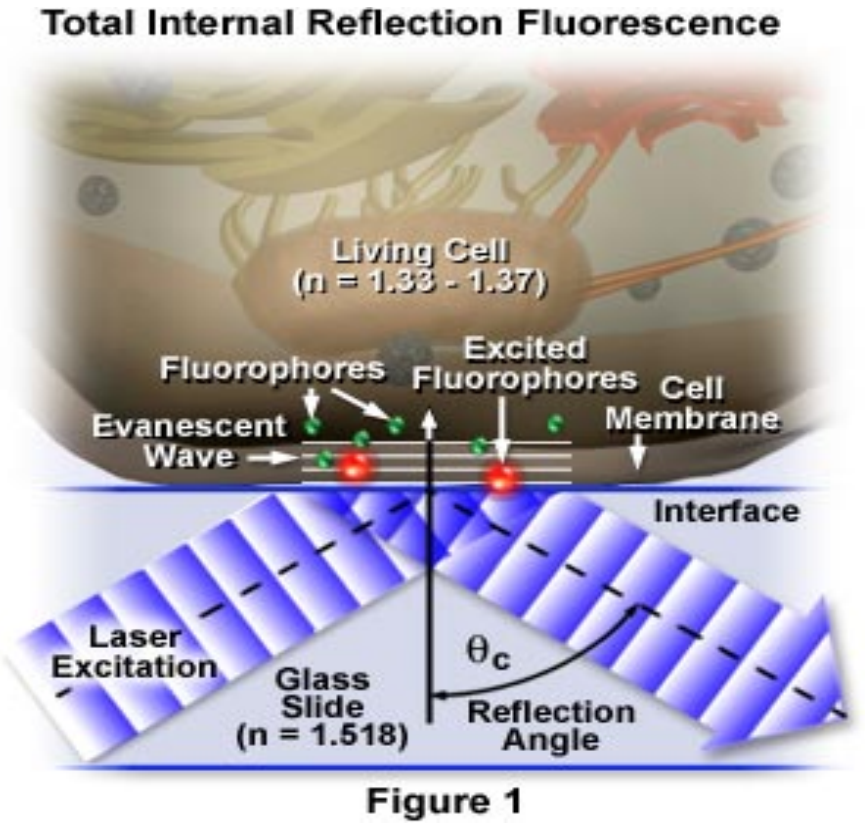
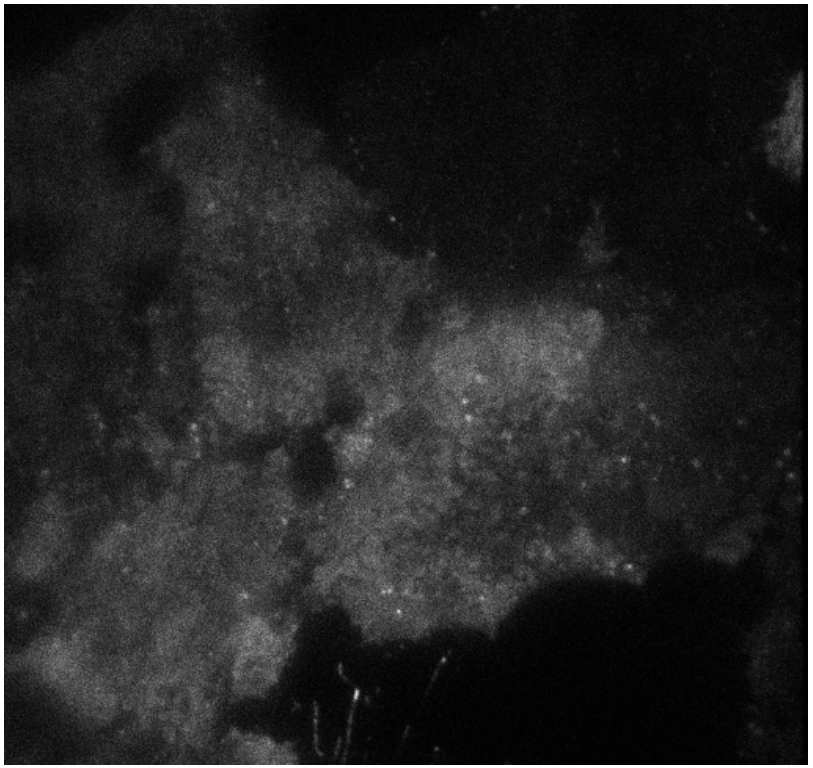


Figure 1

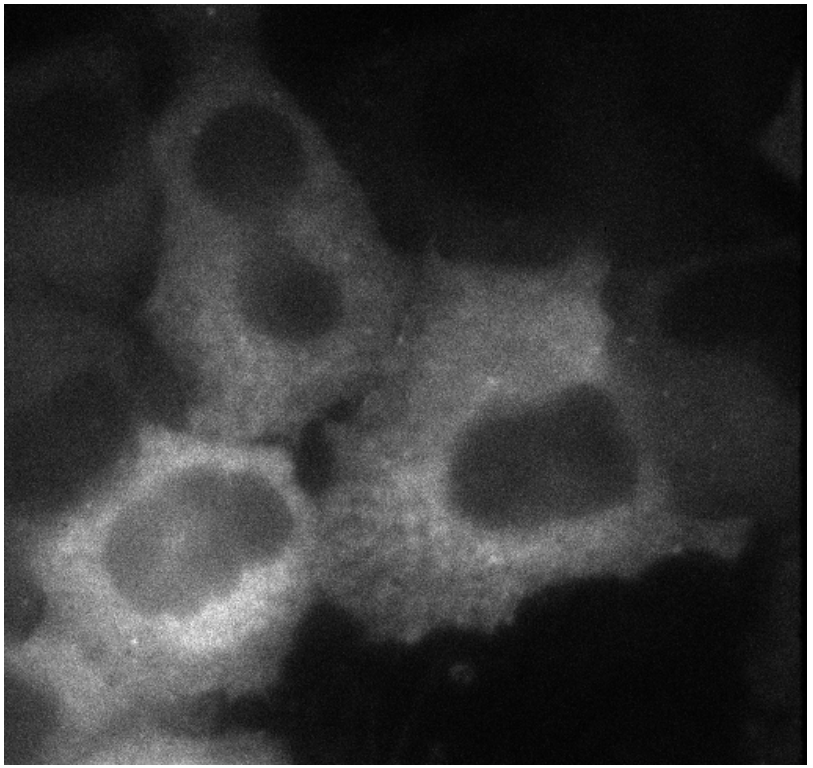
http://micro.magnet.fsu.edu/primer/java/tirf/reflect/index.html

TIRF

only the objects *on* the coverslip

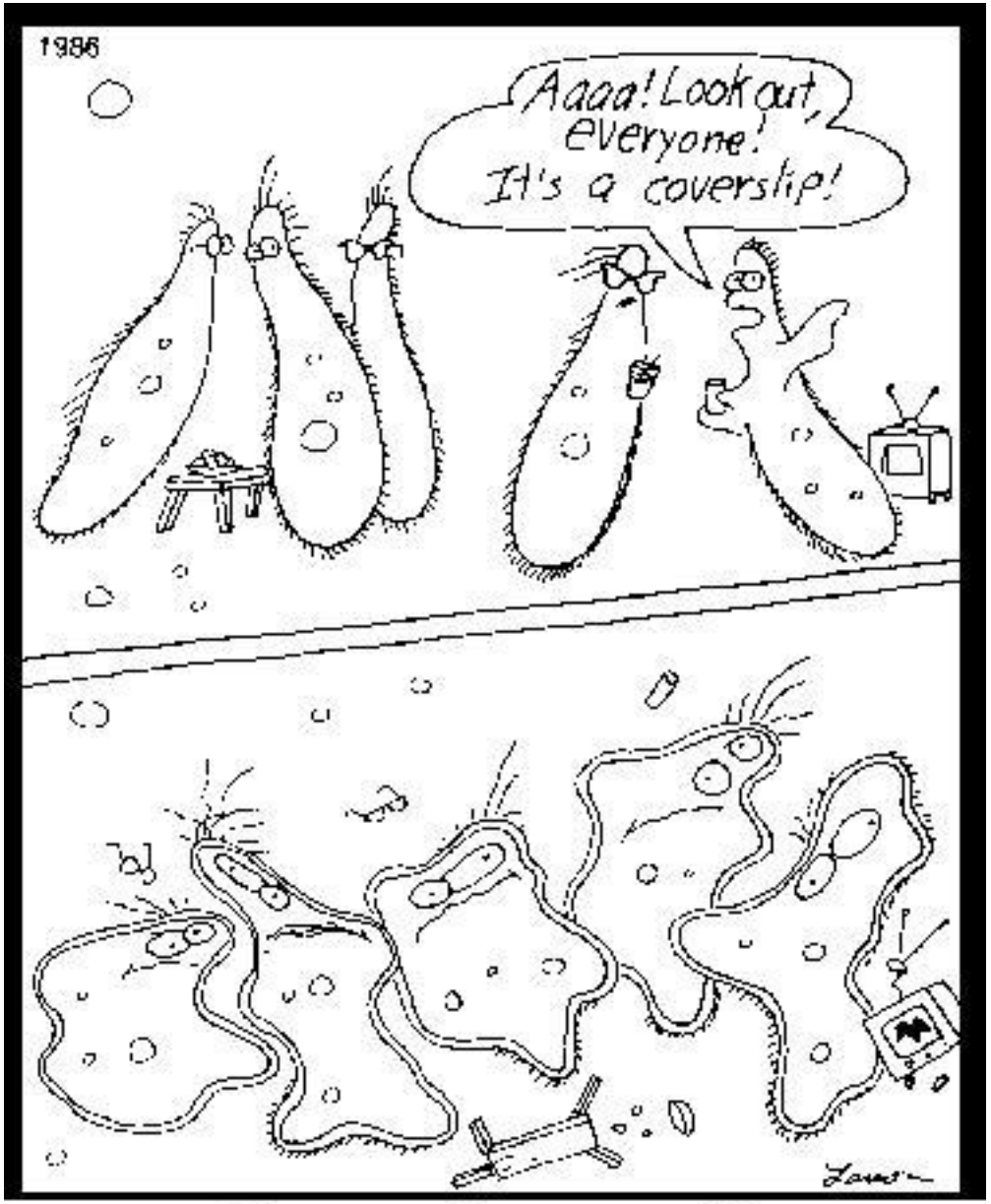


TIRF



Wide field

Britta S-D, Silke G

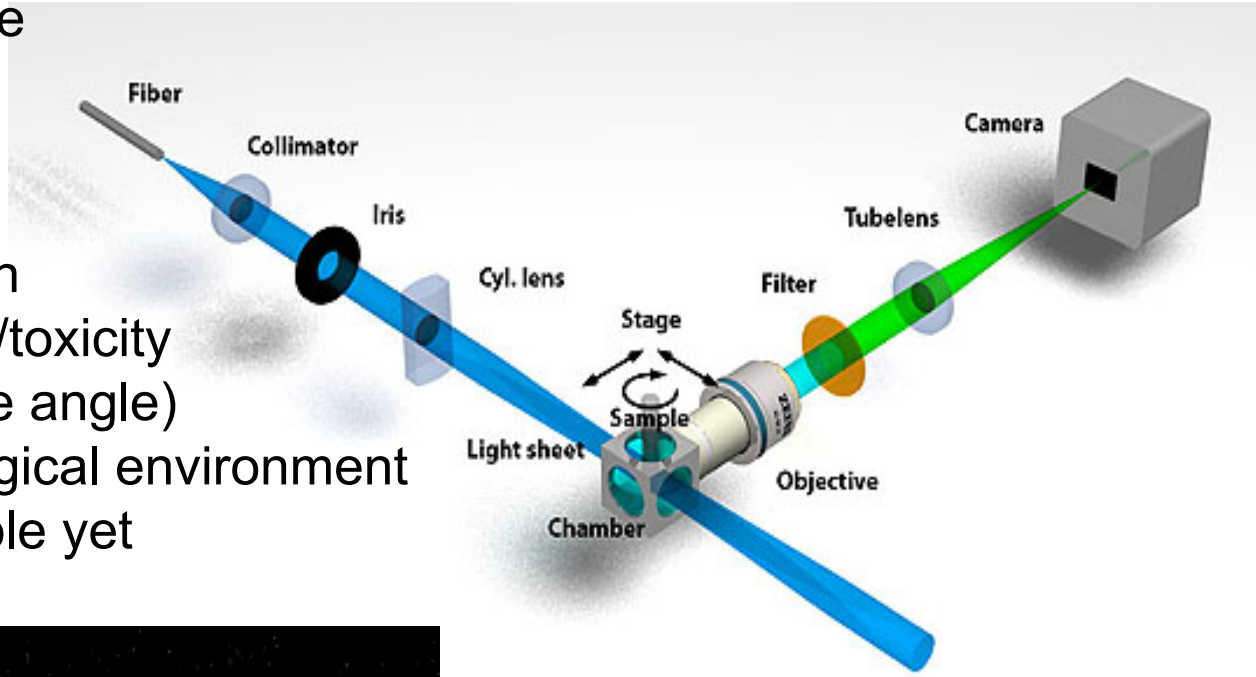


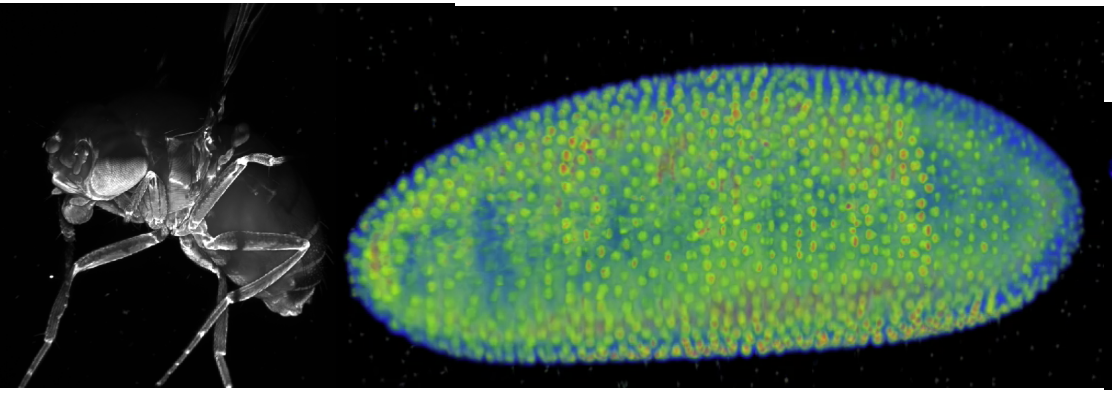
Life on a microscope slide

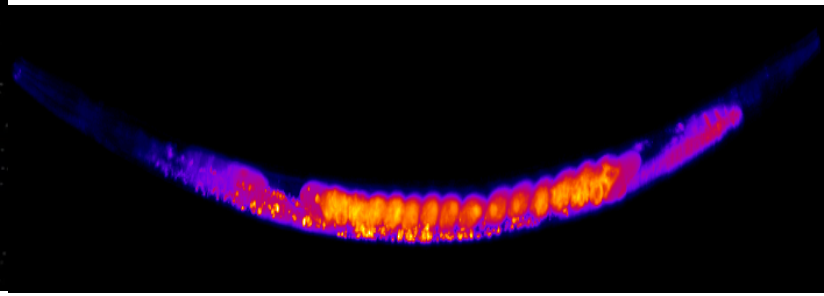
Sticking biology to flat glass is not very physiological...

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

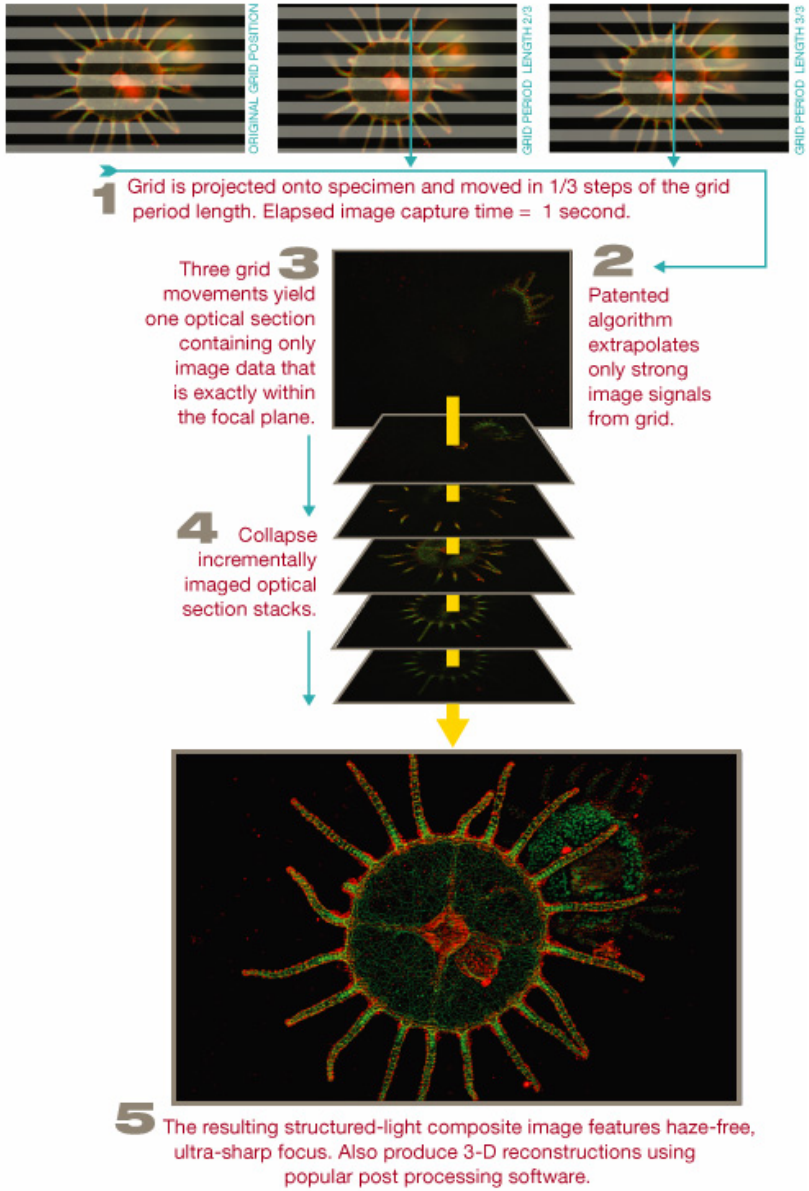






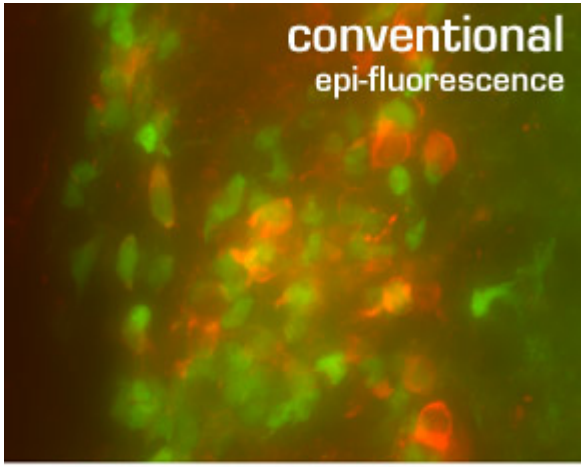
White, Preibisch, Saalfeld, Ejsmont, Sarov, Tomancak *http://www.huisken.org/jan/spim.html*

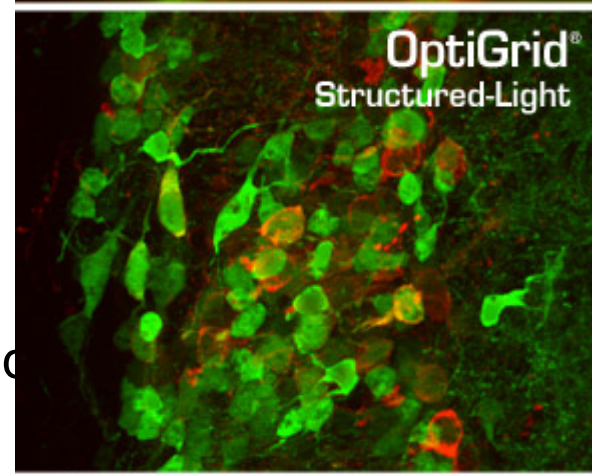
Structured illumination

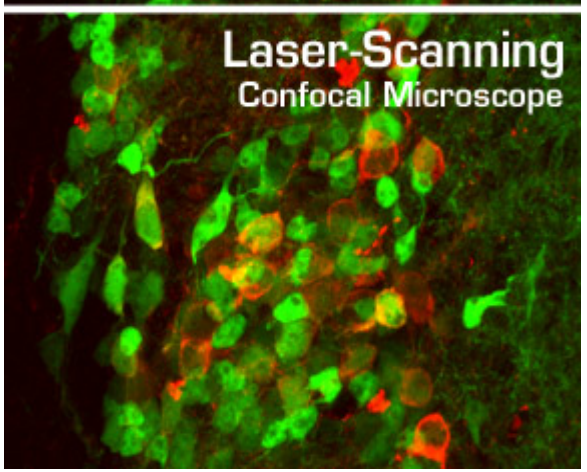


Optigrid, ApoTome and OMX

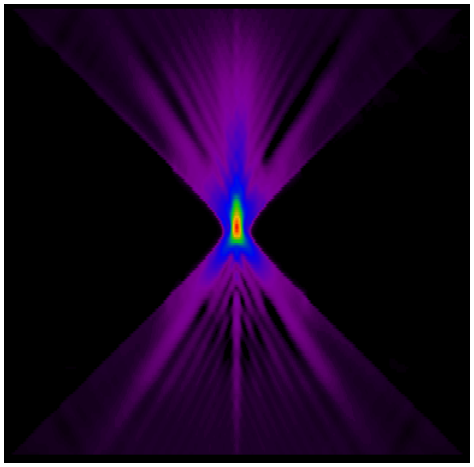
- "field" illumination
- detector : CCD
- slow/medium speed
- high flexibility
- image processing required (fast)
- Good for fixed thinner samples, instead of confocal





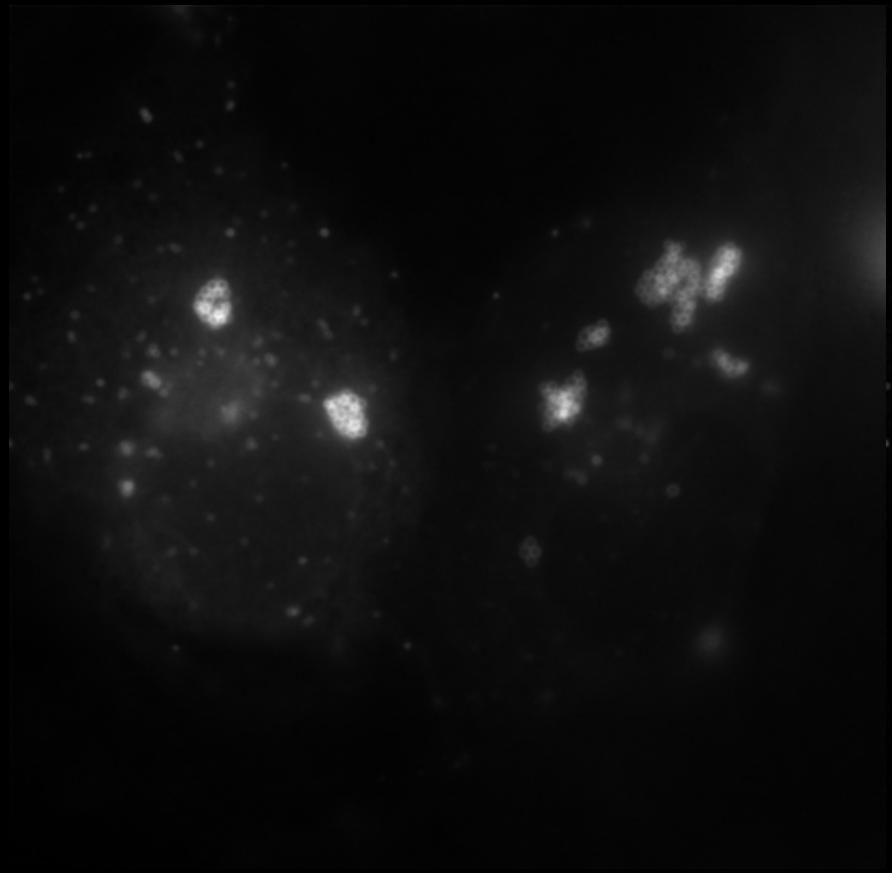


Widefield Microscopy + Deconvolution

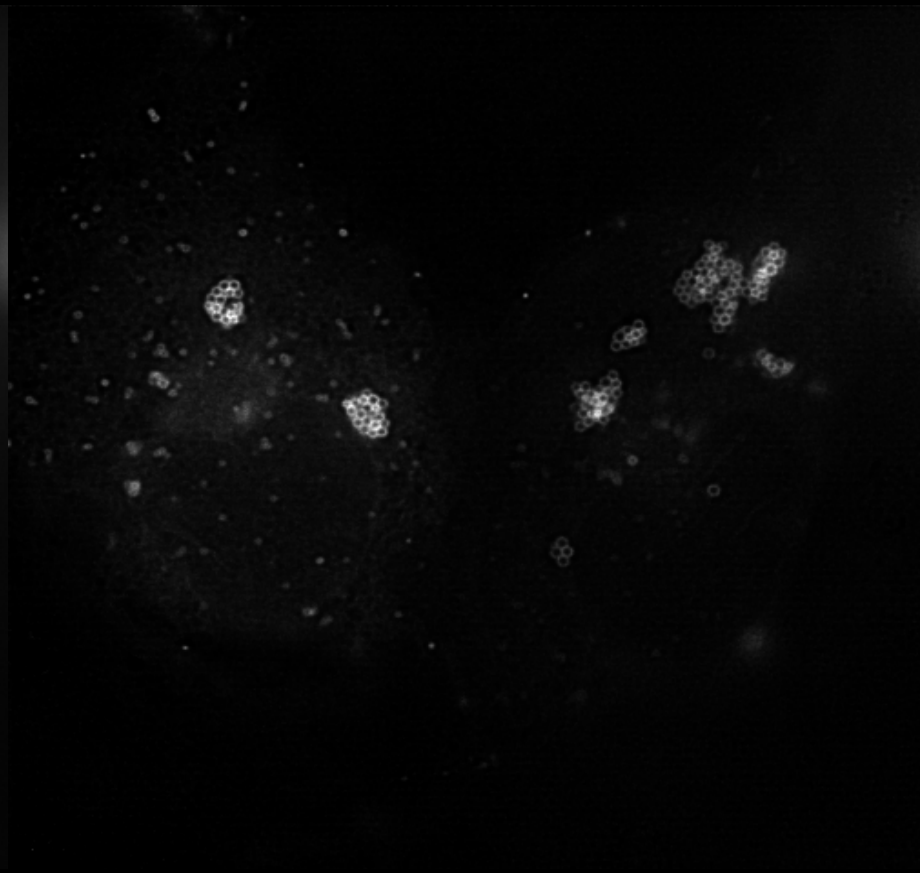


- “field” illuminating technique
- Detector : CCD
- High temporal resolution (high speed)
- High flexibility
- Low bleaching / photo toxicity
- Very high signal:noise (low speckle)
- Image processing required (automated)
- Good for fixed and living samples.

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



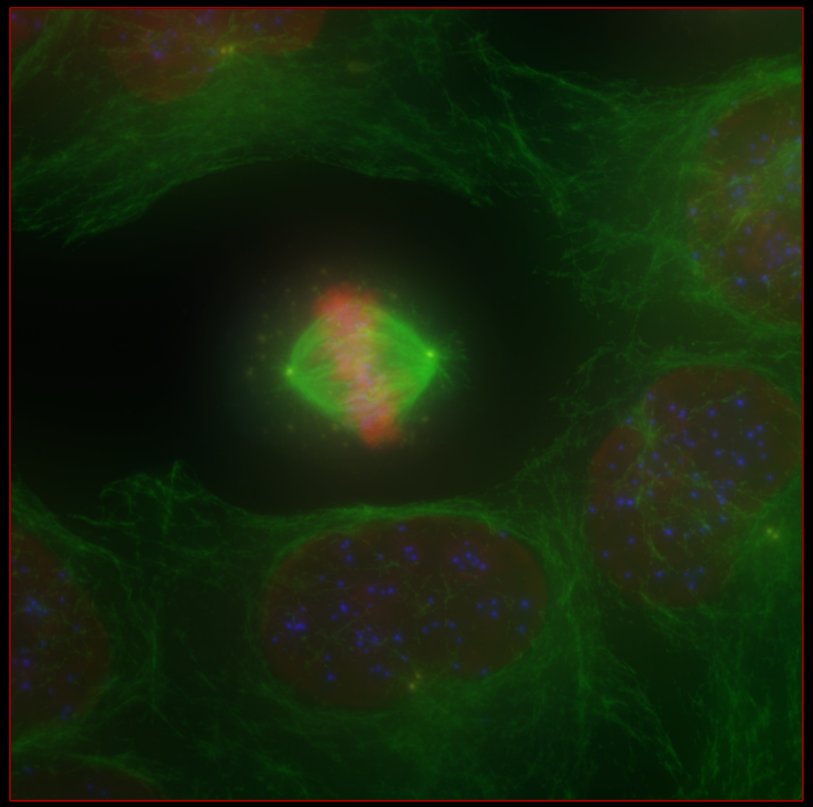
raw image



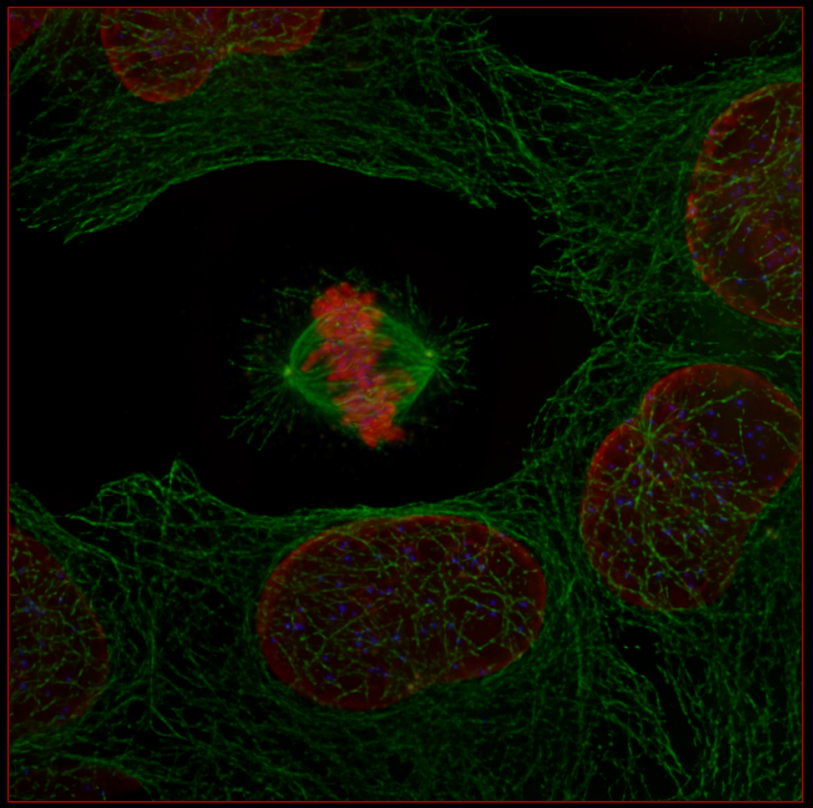
deconvolved image

Johanna Spandl

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



raw image
max Z projection

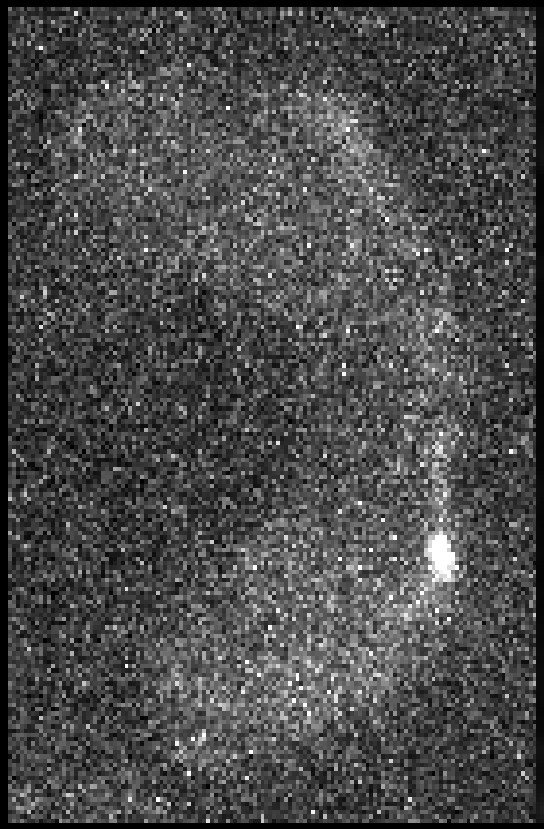


deconvolved image
max Z projection

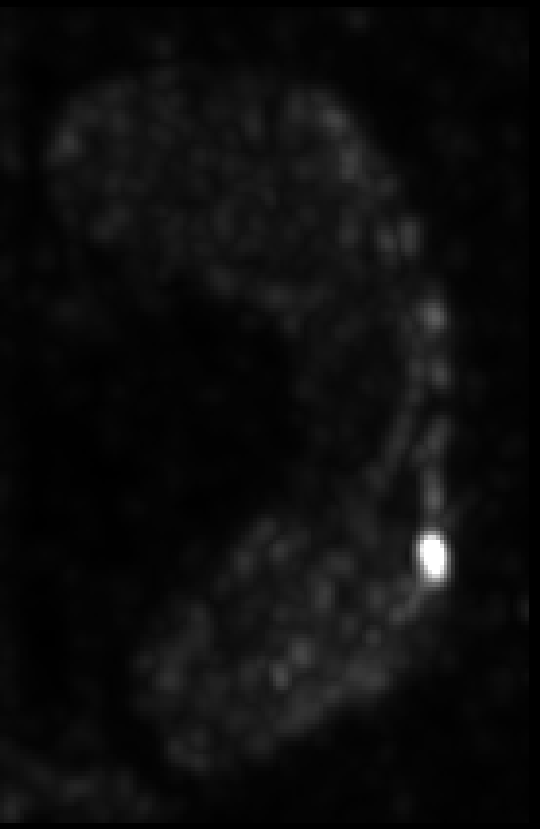
Alex Bird / Dan White

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

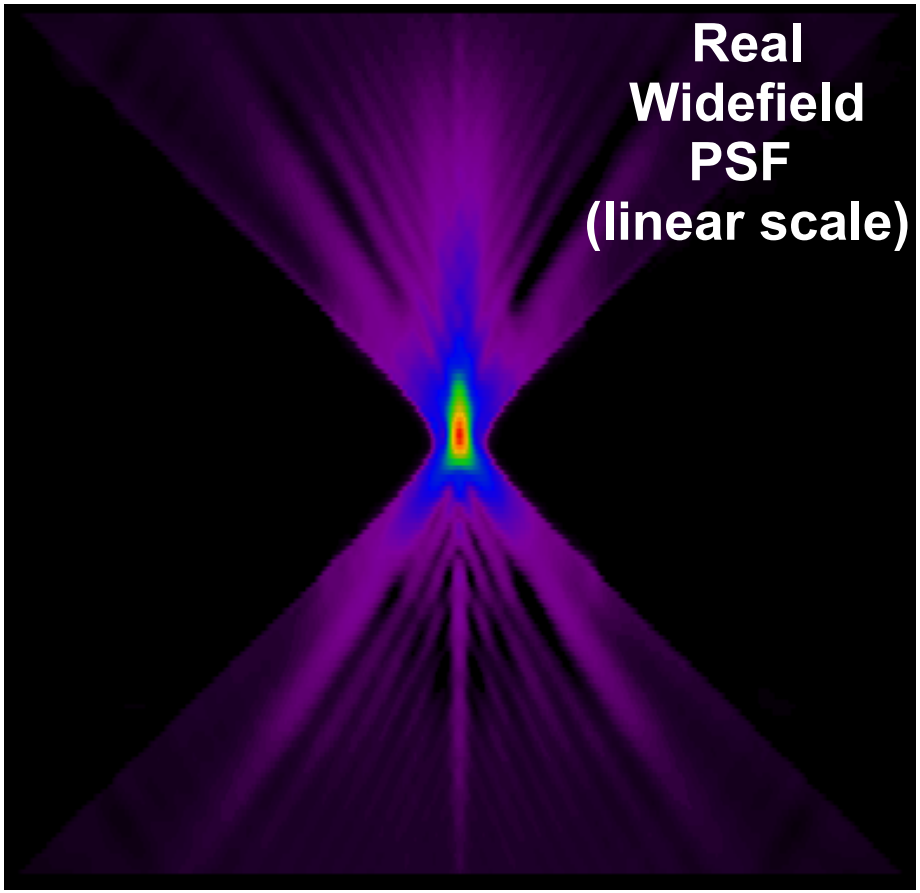


deconvolved image
max Z projection

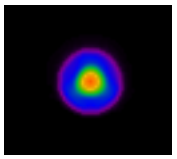
Davide Accardi / Dan White

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
Measured PSF
1.4 NA Oil lens
Watery sample
=
Spherical Aberation!

Jesse Lipp, Dan White

Pixel Size / Resolution

Remember !!!

Spatial sampling:

~1/3 x smallest feature.

Remember !!!

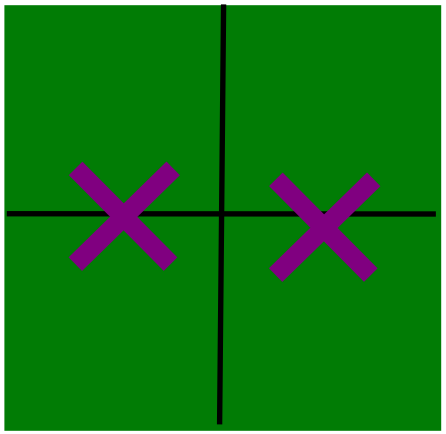
A pixel is NOT a little square...

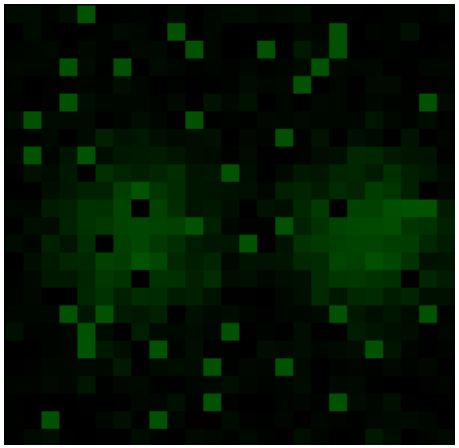
it is a spatial sample at some location x,y,z in space and time

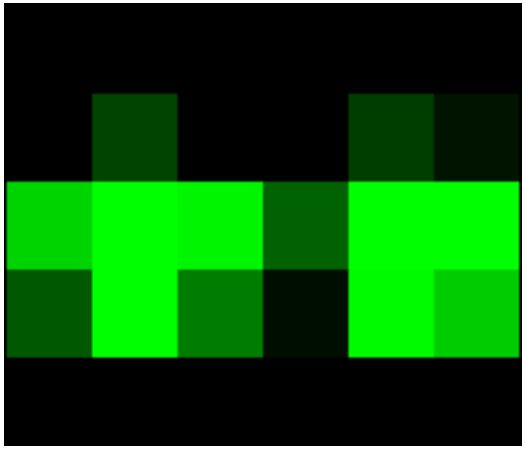
Remember !!! Think like a spectroscopist, not a photographer!

2 points X and X – resolution limit apart, d

Pixel size = d/3







under sampled

Can't see 2 objects

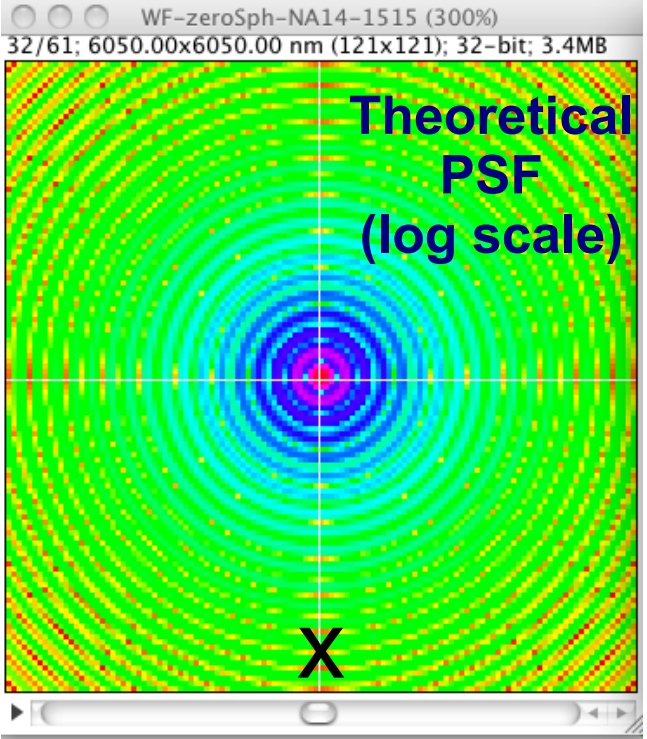
over sampled

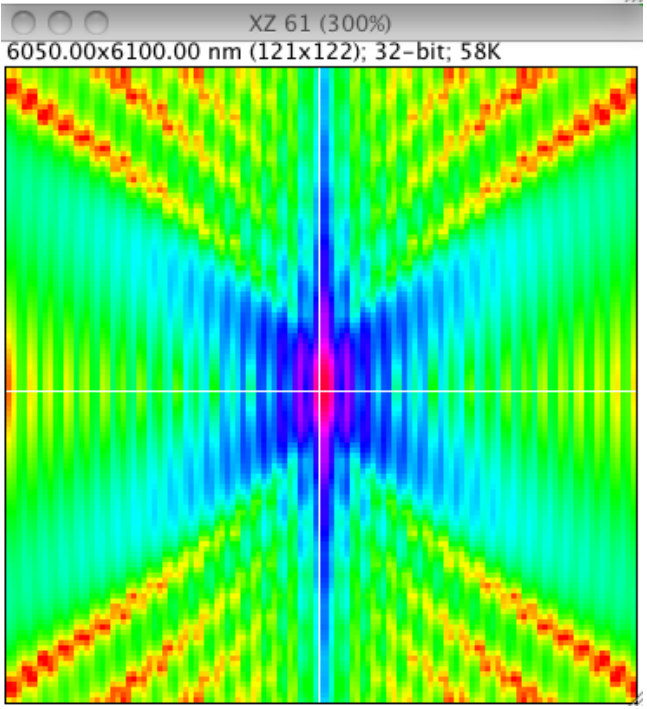
Noisy, low contrast

correct sampling

Good contrast

See 2 objects





Z

Image of a point is not a point!

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

Widefield microscope PSF
1.4 NA Oil lens 1.515
Glassy sample 1.515

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

PSF size:
WF>SD>SPIM>C

Higher NA =
better resolution,
especially in Z!

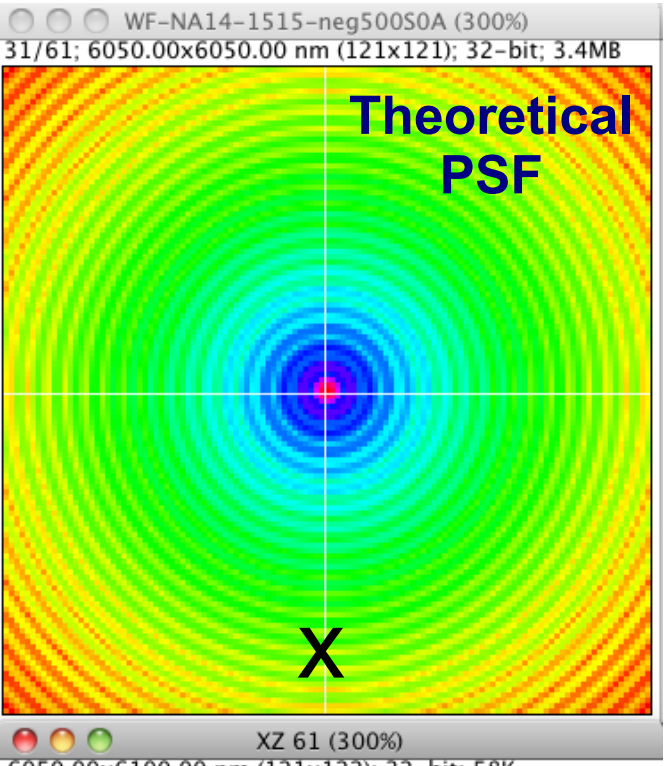
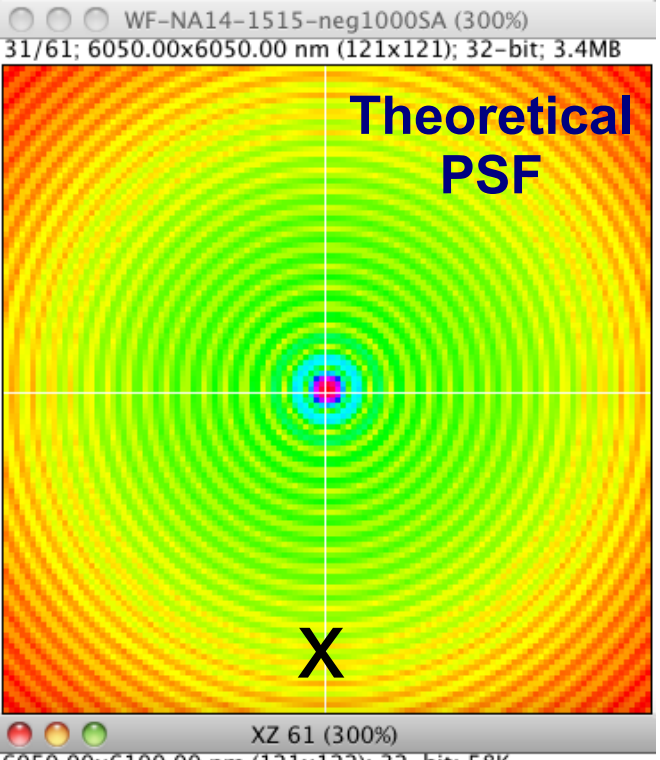


Image of a point is not a point!

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

Widefield microscope PSF
1.4 NA Oil lens 1.515
Watery Sample
1 Wave spherical aberration
(eg approx. 10 um of water)

PSF is very asymmetric in Z
and also shifted in Z



Z

Image of a point is not a point!

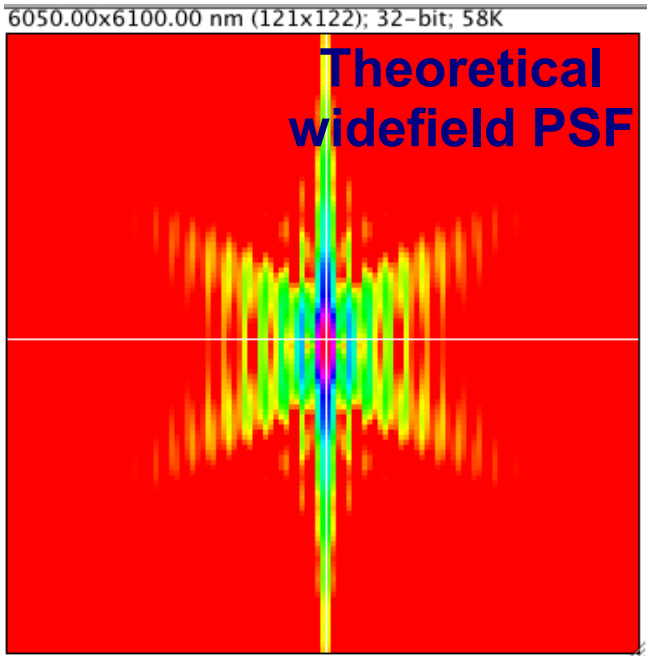
The image of a point is convolved by the point spread function – PSF.

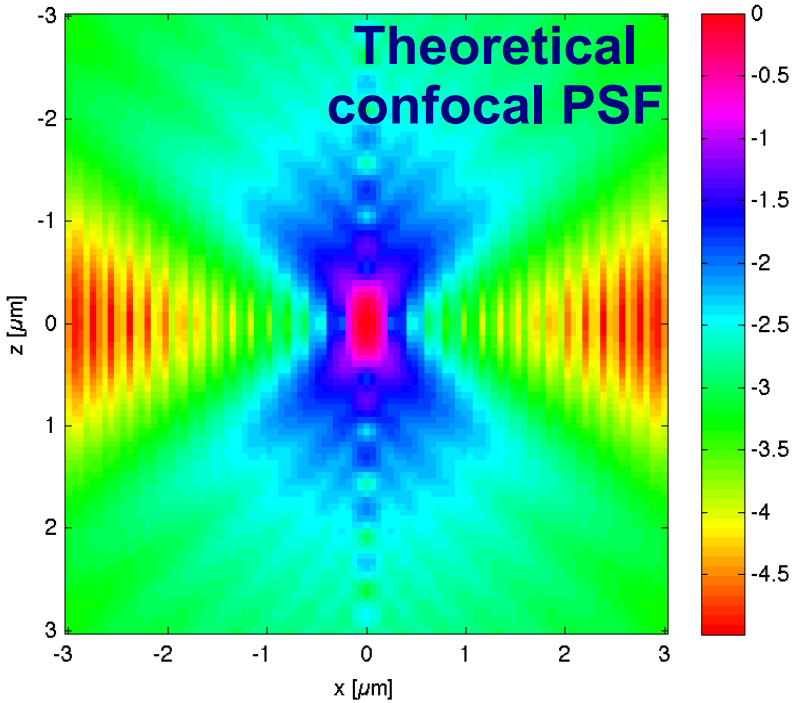
Widefield microscope PSF
1.4 NA Oil lens 1.515
2 Waves spherical aberration

PSF is very very very asymmetric in Z and also shifted very badly in Z

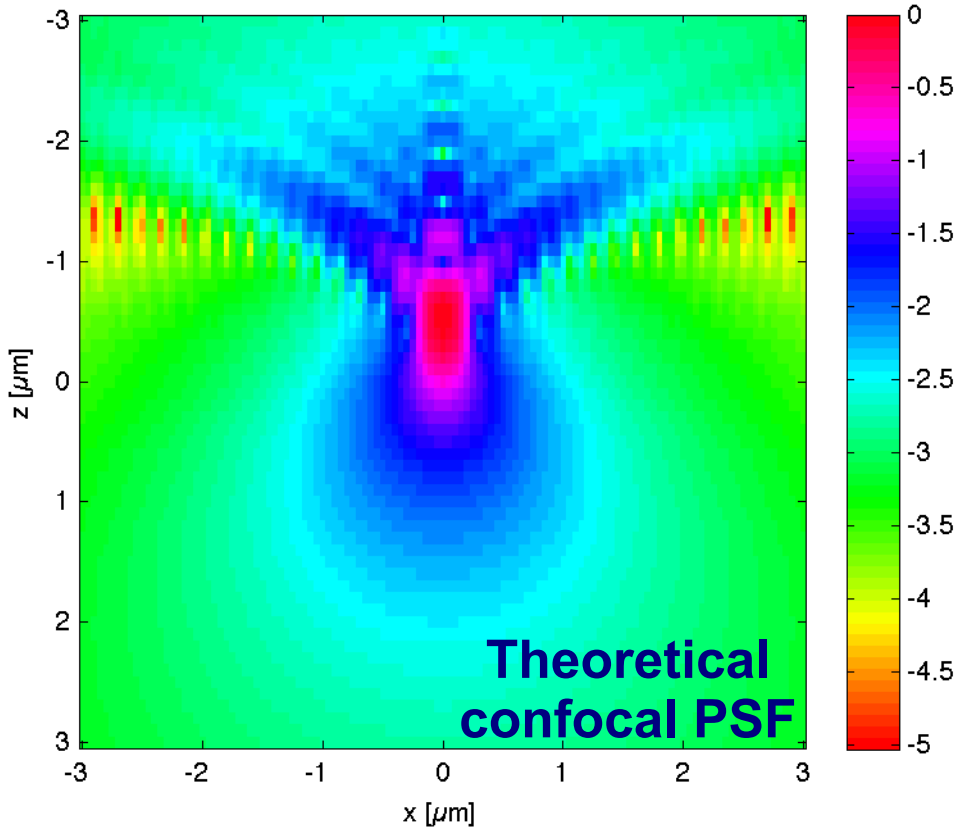
Confocal PSF is smaller than WF, but still significant in size.

PSFs with
1.4 NA Oil lens 1.515
Glassy sample 1.515



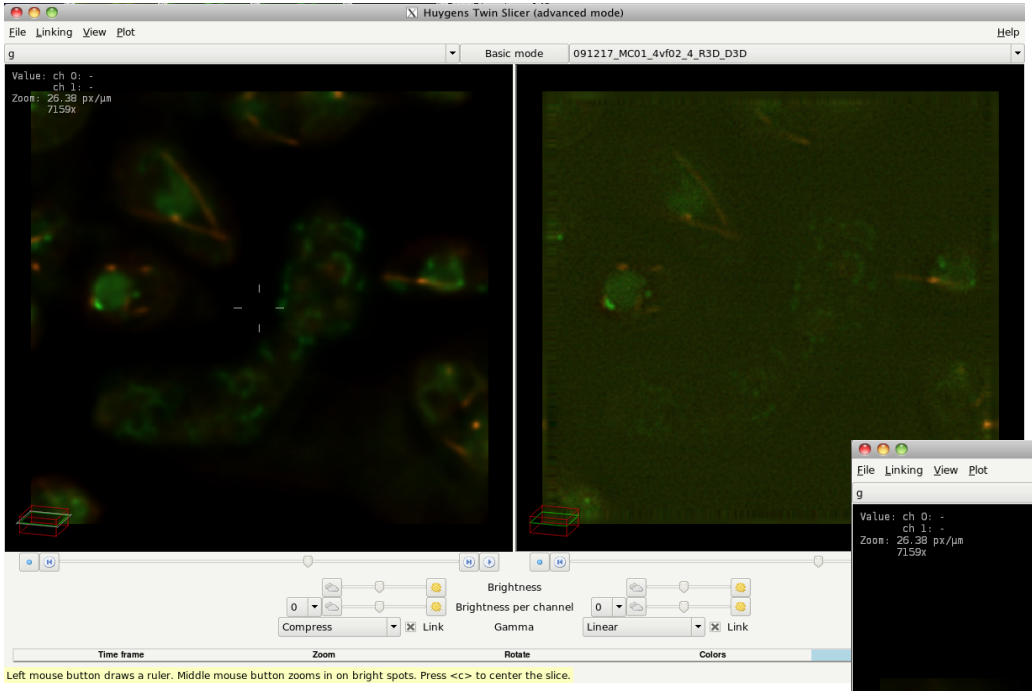


Spherical Aberation Kills Confocal Imaging



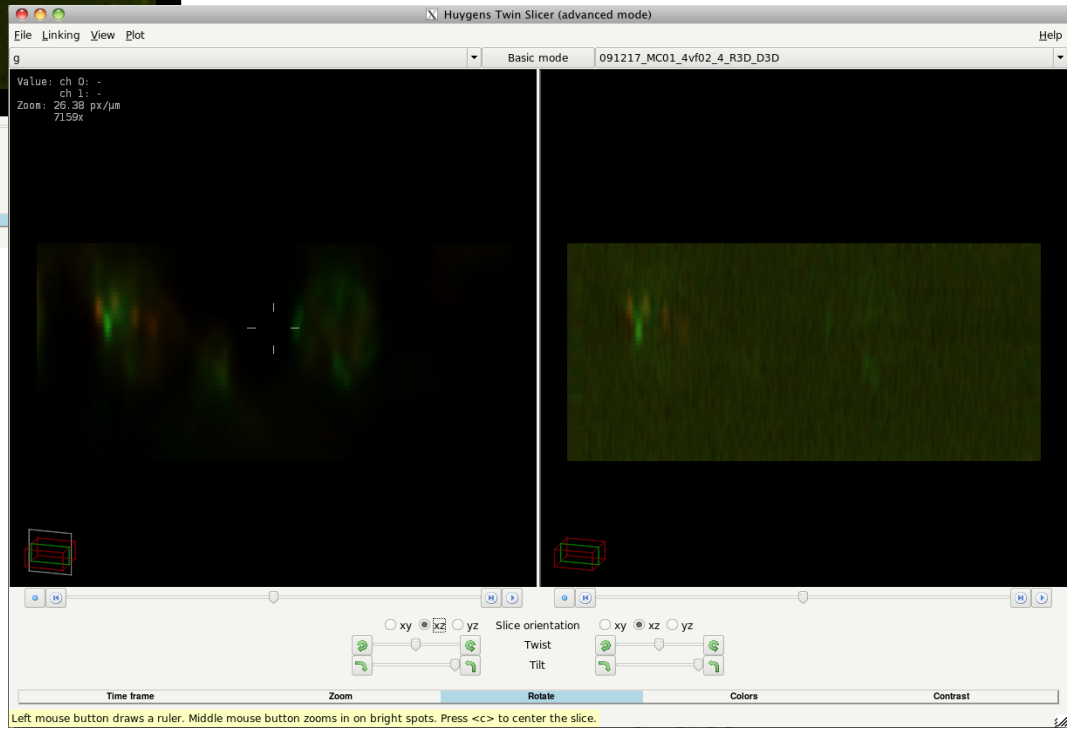
PSF with
1.4 NA Oil lens
1.515
Watery sample 1.33
10 um away from
0.17 mm coverglass

Quick and Dirty vs. Slow and Careful



Default "High Noise" settings on DeltaVision don't handle very noisy images or CCD Offset well

More careful deconvolution using Huygens does a better job of dealing with noise and CCD offset



Tips to get it right:

1) Measure the PSF, or
    Correct aberrations to get close to perfect PSF
        Coverslip, immersion and mounting media.

2) Test different noise parameters until you get a reasonable result
    Don't enhance noise or smooth detail.
    The is a happy optimum

3) Look for “left over” intensity – wrong PSF

4) Look for “new” false objects, not in raw image.

5) Correct spatial sampling – noisy data OK.