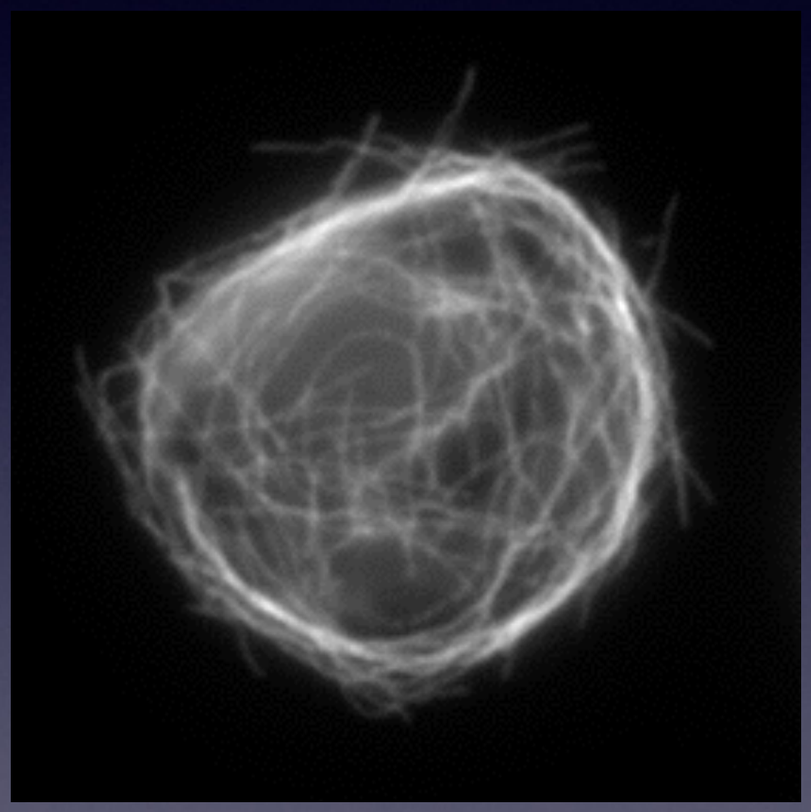
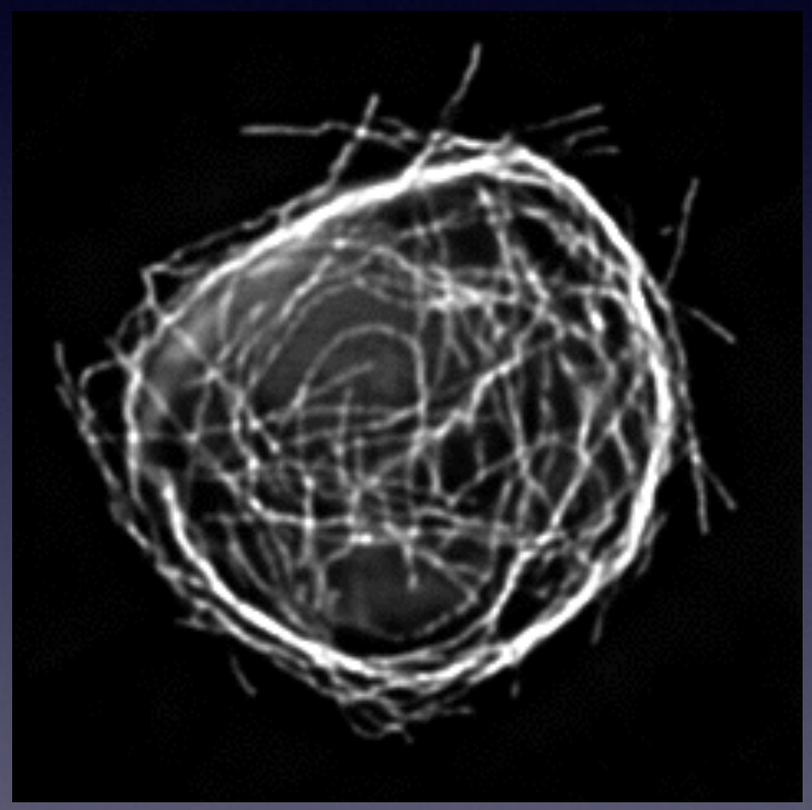
Lecture 9
Advanced Widefield Microscopy & Bespoke Microscopes



Widefield



Deconvolved Widefield

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Lecture 9
Advanced Widefield Microscopy & Bespoke Microscopes

Ian Dobbie
x13323

Overview

- Image formation and airy rings
- Beads and spherical aberration
- How deconvolution works
- Super fast acquisition
- Bespoke microscope design - pro's and cons

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What is a microscope image

- The microscope produces a magnified, but also distorted, image
- Record the light intensity on a camera.

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Microscopic imaging in mathematical terms.

- Take your sample
- Multiple it at every point by the imaging process in the microscope (convolve the PSF with the object).
- Produce the image.

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The most important things to think about.

Contrast :- What is the difference between what you want to see and everything else?

Resolution :- How small things can you see?

Nothing else

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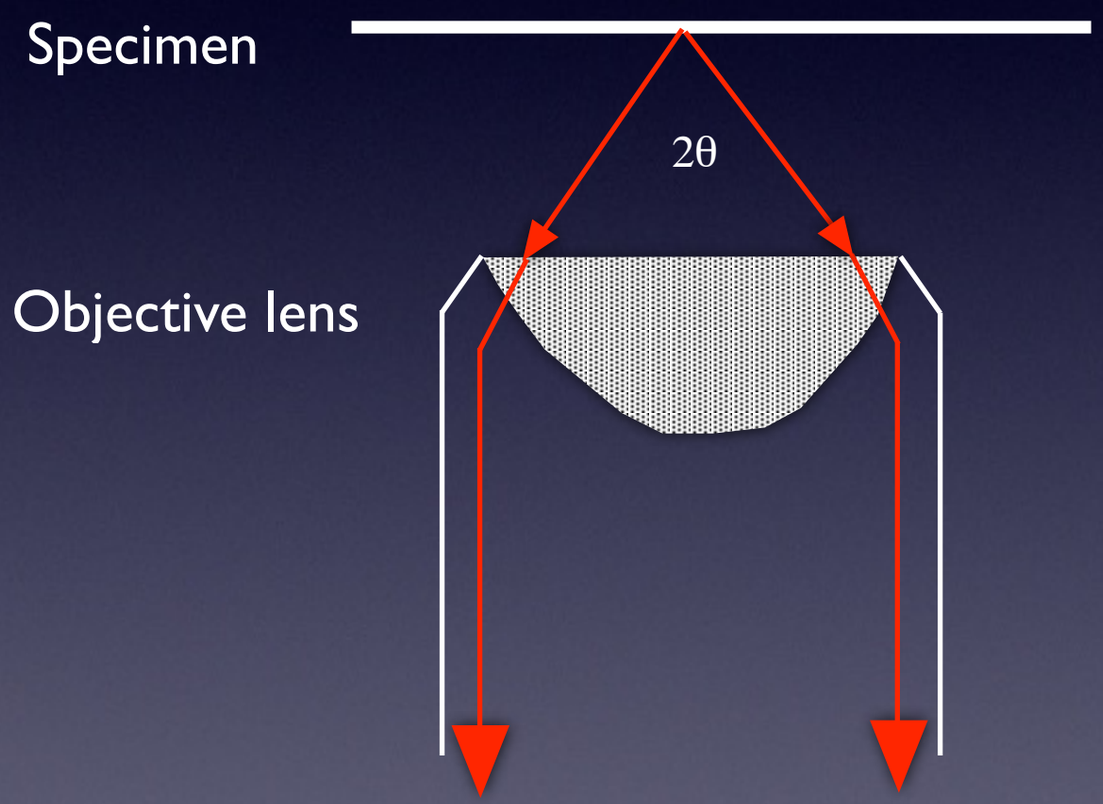
Microscope Resolution

- No lens has perfect resolution, even in theory
- Resolution depends on the angle (θ) of the cone of light that the objective can collect from the specimen.
- Rule of thumb: Resolution limit ~ $\lambda/2$



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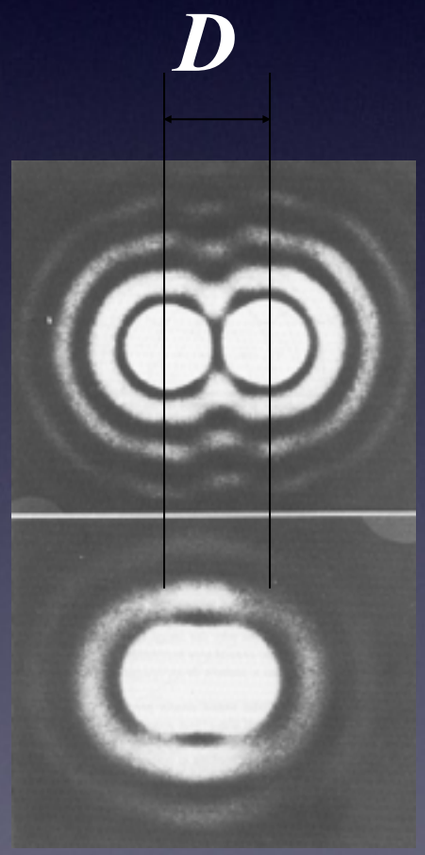
Resolution: A technical definition, the Rayleigh Criterion



D, the distance of two closest points that can be distinguished

$$D=1.22\ \lambda/(NA_{obj}+NA_{cond})$$

Epi-Fluorescence: $NA_{cond} = Na_{obj}$

so $D=1.22\lambda/2NA$

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The Point Spread Function - PSF

- The image of an infinitely small point.
- Limited by resolution
- 3D structure also very important.

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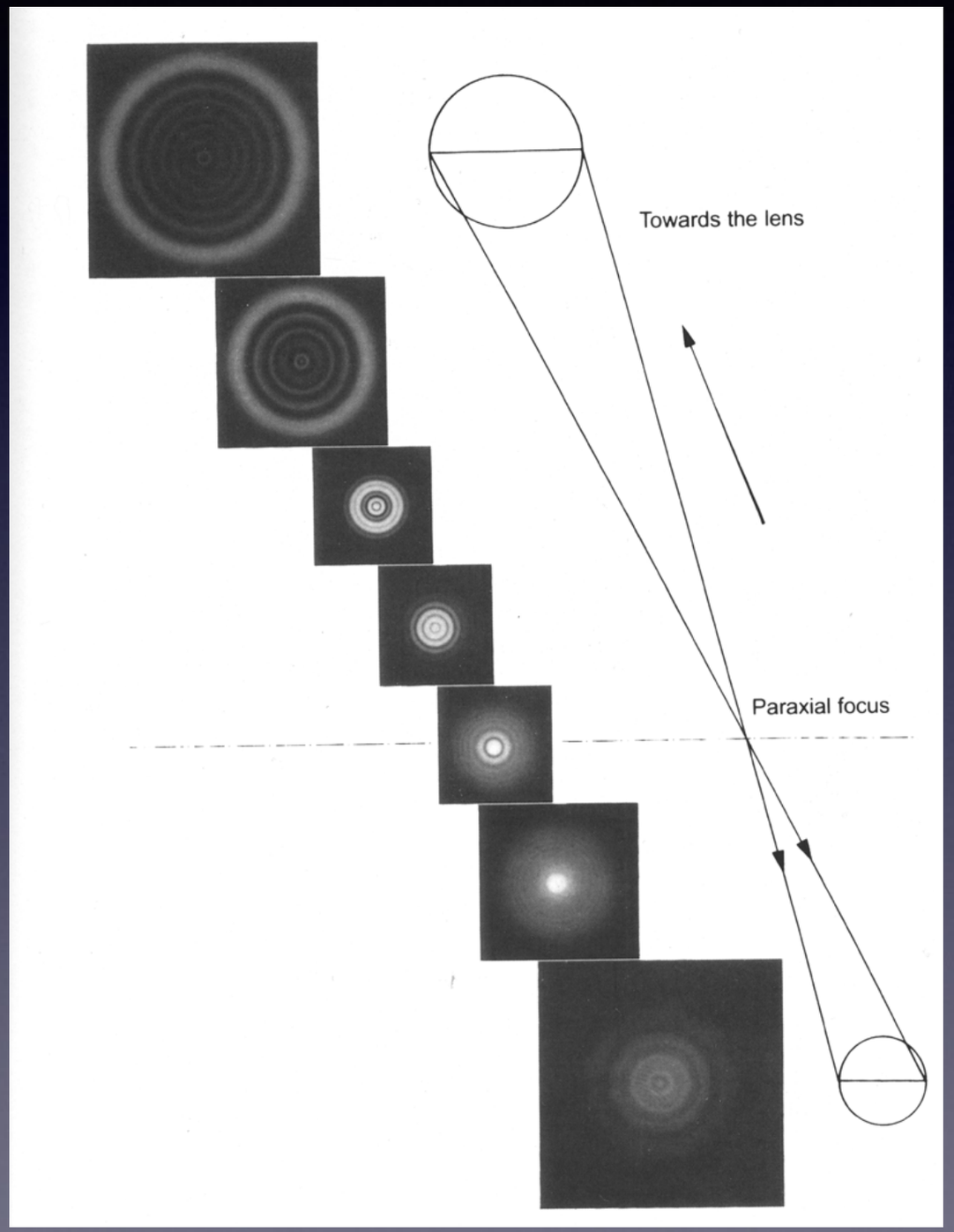
Image quality- the problem of "out-of-focus light" point spread function and airy rings

Sample object: a "sub-resolution" fluorescent bead



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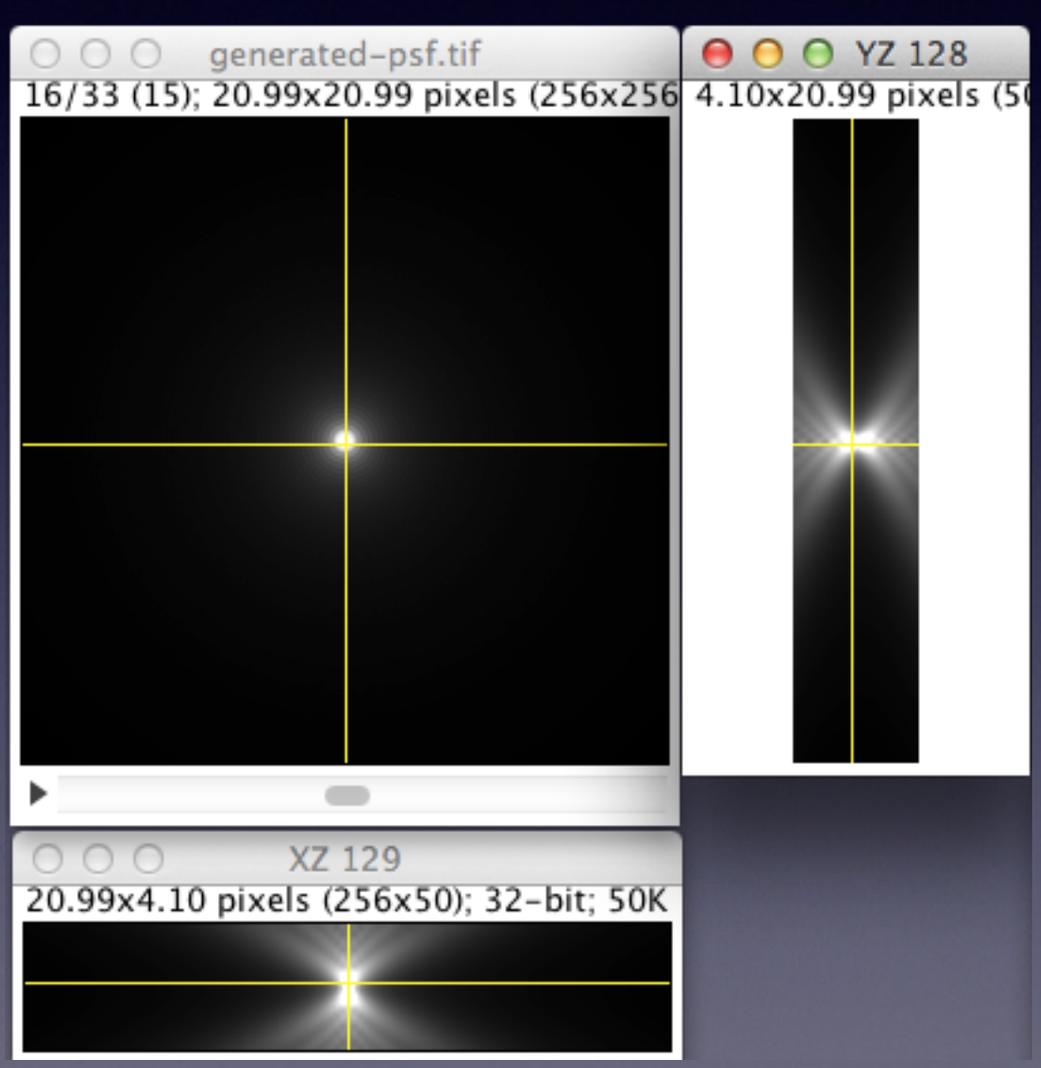
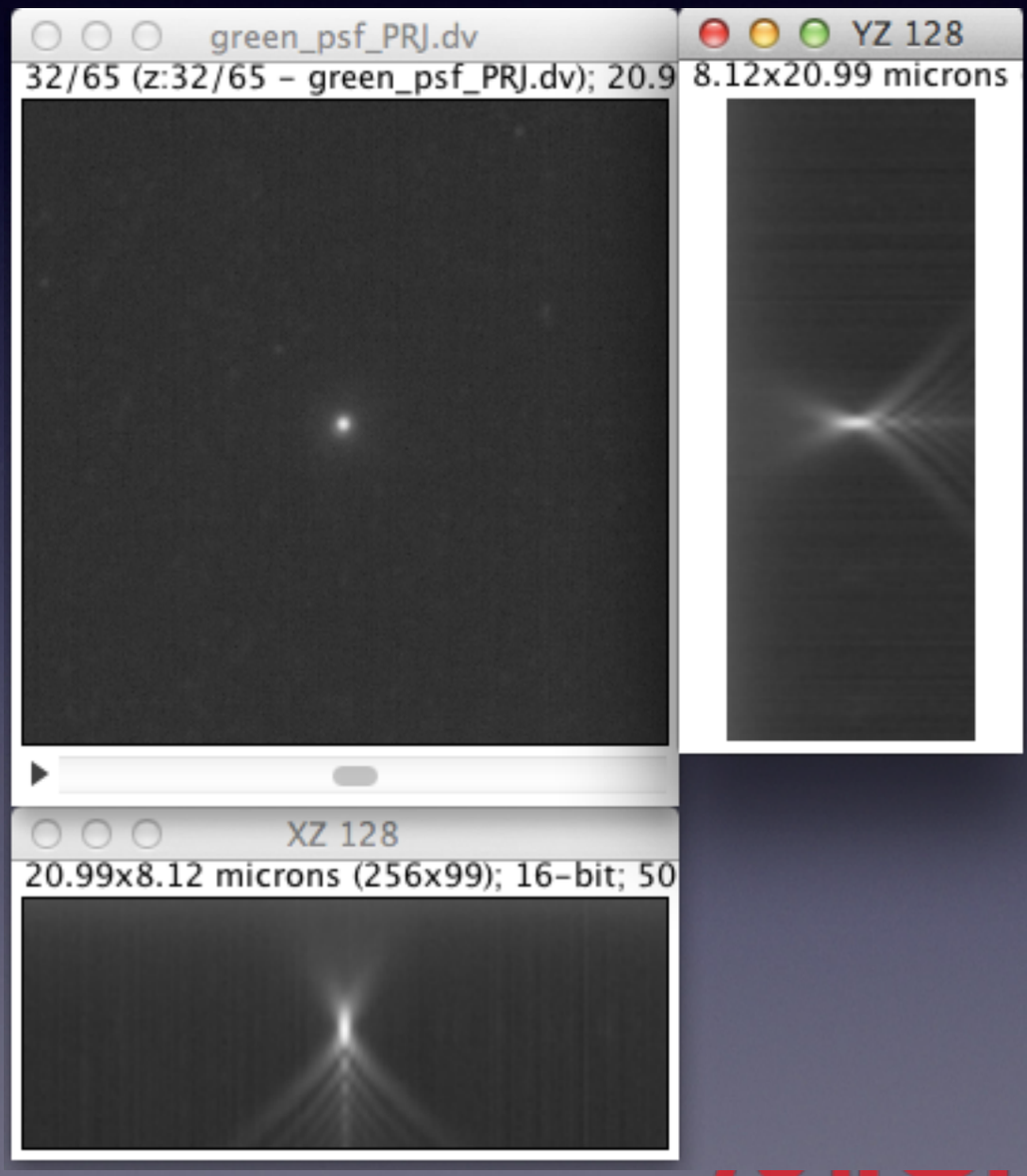
Theoretical and measured PSF Orthogonal views



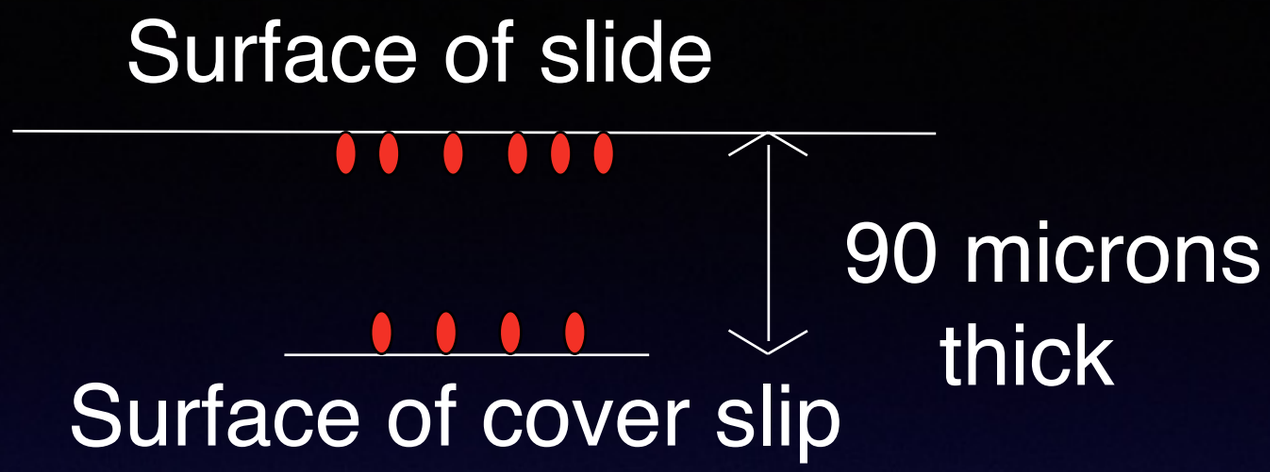
Generated PSF



Real PSF

OXFORD

Bead slide



Tetraspeck beads: chromatic registration
DAPI/FITC/Rhodamine/Cy5

Beads (PS Spec): Single fluorochrome
Brighter -better for generating
point spread functions for deconvolution

Inspec Intensity beads: Measure dynamic range

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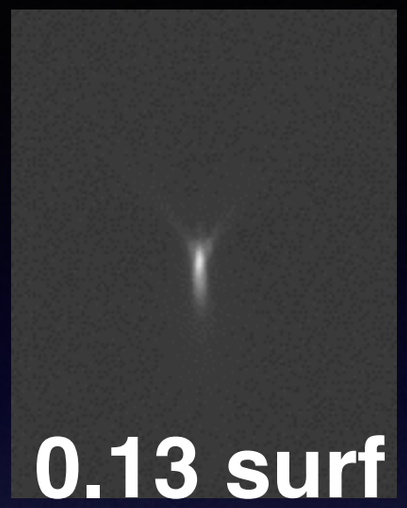
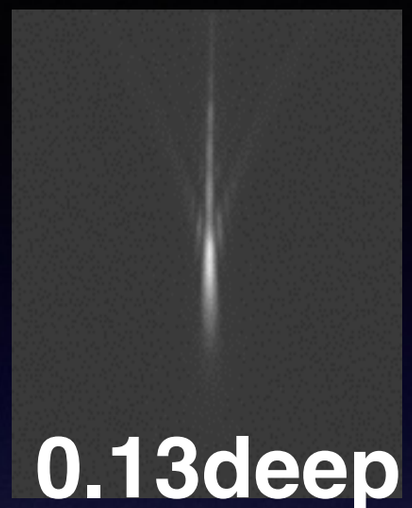
Affects of deep imaging (90μm) and collar settings on spherical aberration and psf of 60X/NA1.2w



0.13 surf



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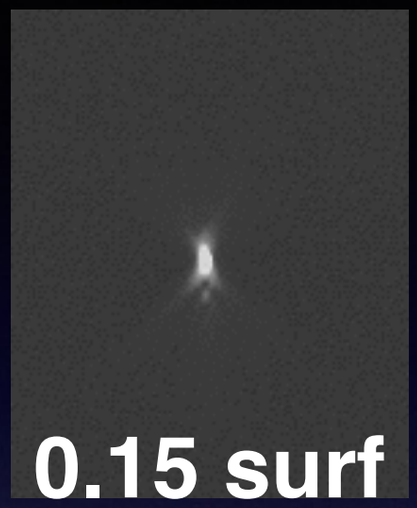

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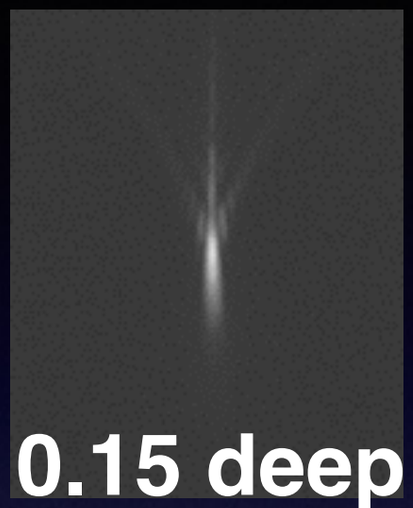
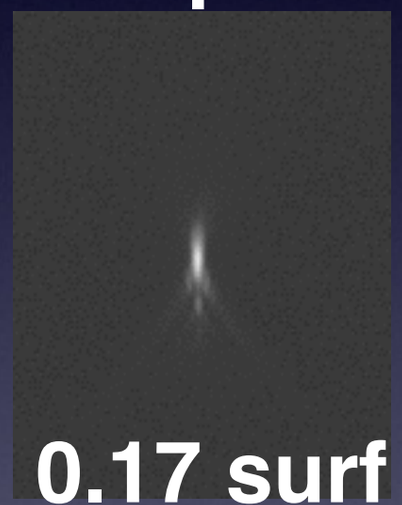


0.15 deep



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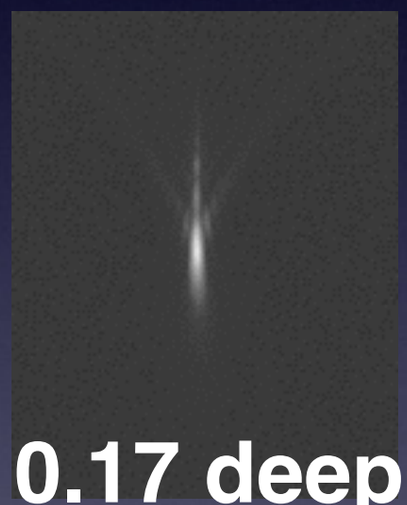
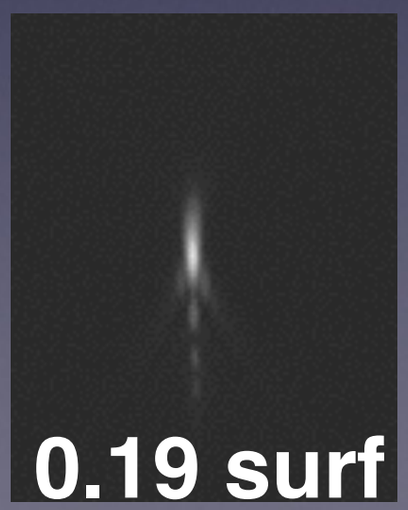
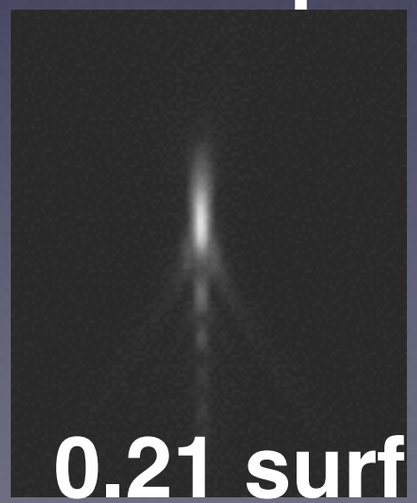


0.17 deep



0.19 surf

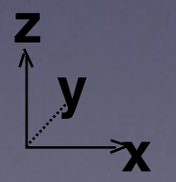
0.19 deep



0.21 surf

0.21 deep

Data from
Alejandra Clark



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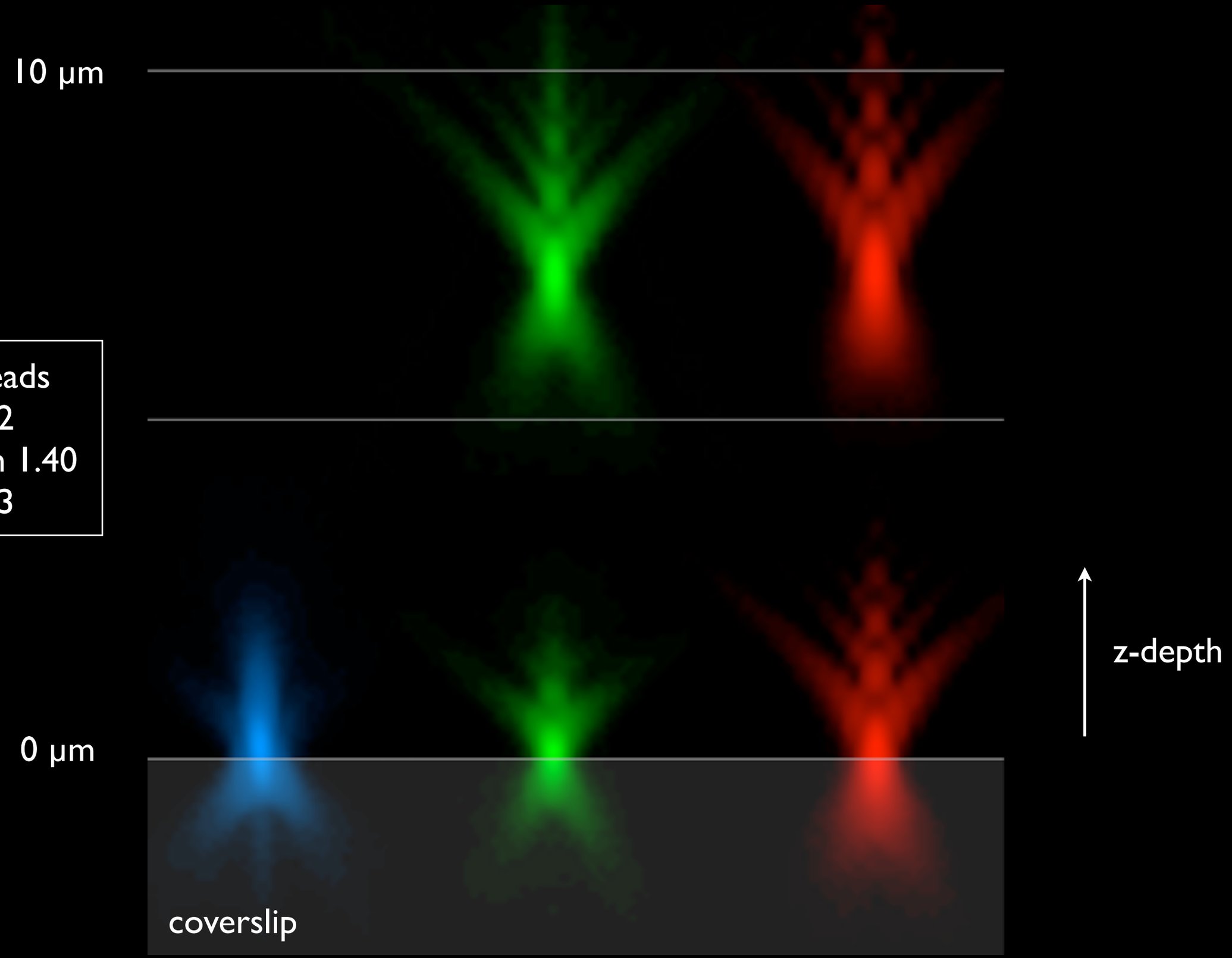
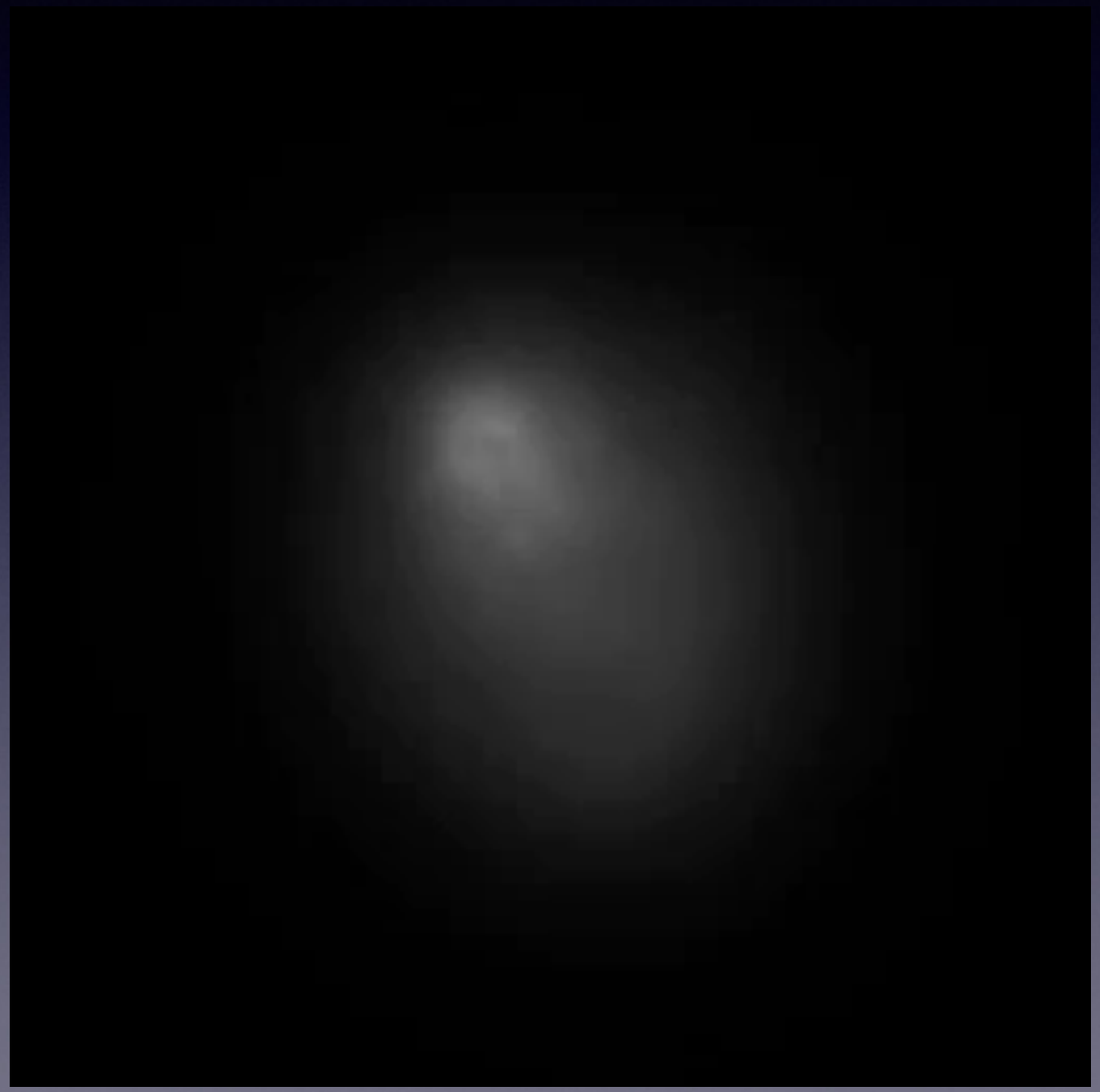
Spherical aberration dependent on wavelength, depth, RI



Conventional Epi-Fluorescence Image



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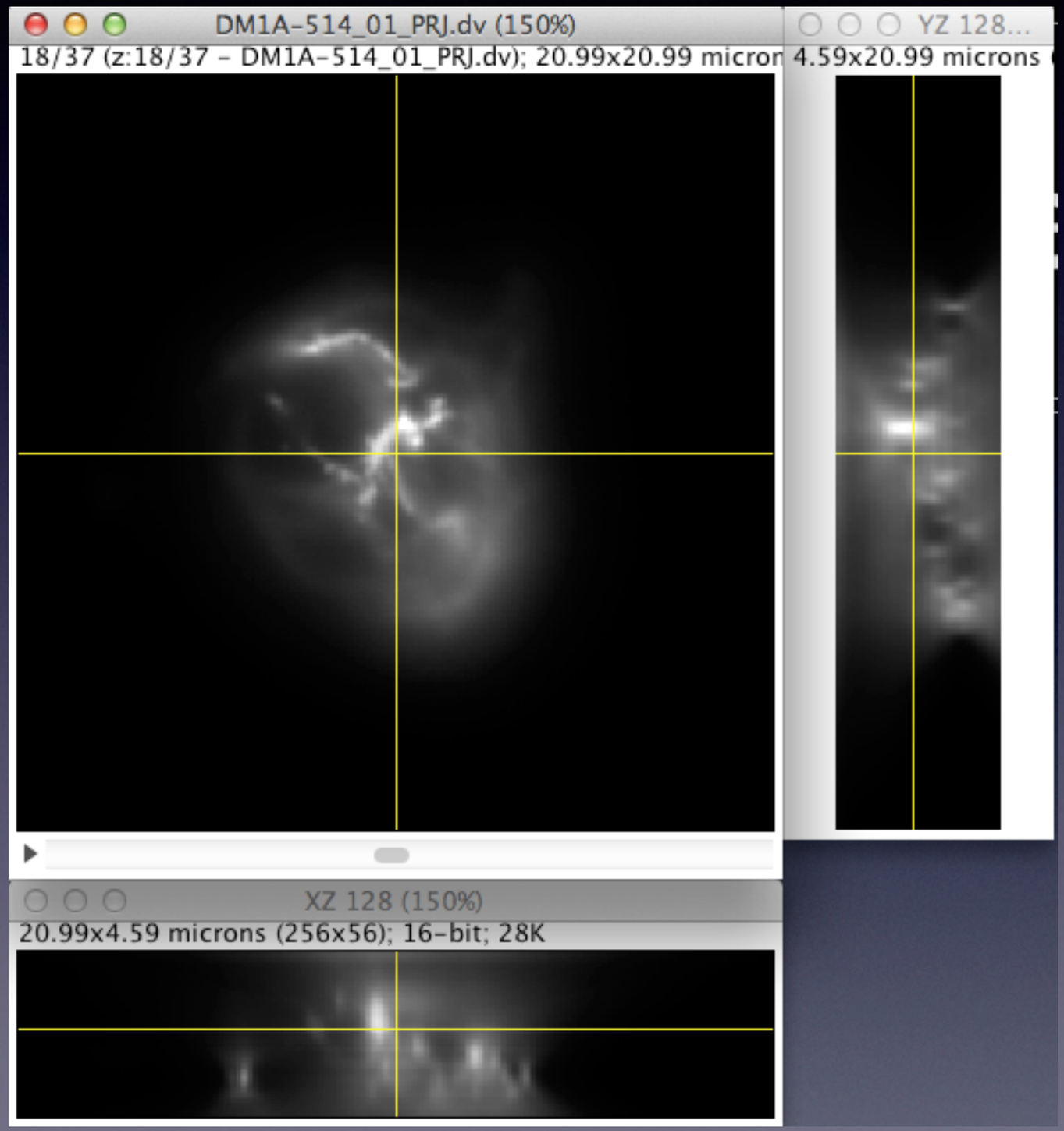
Orthogonal views



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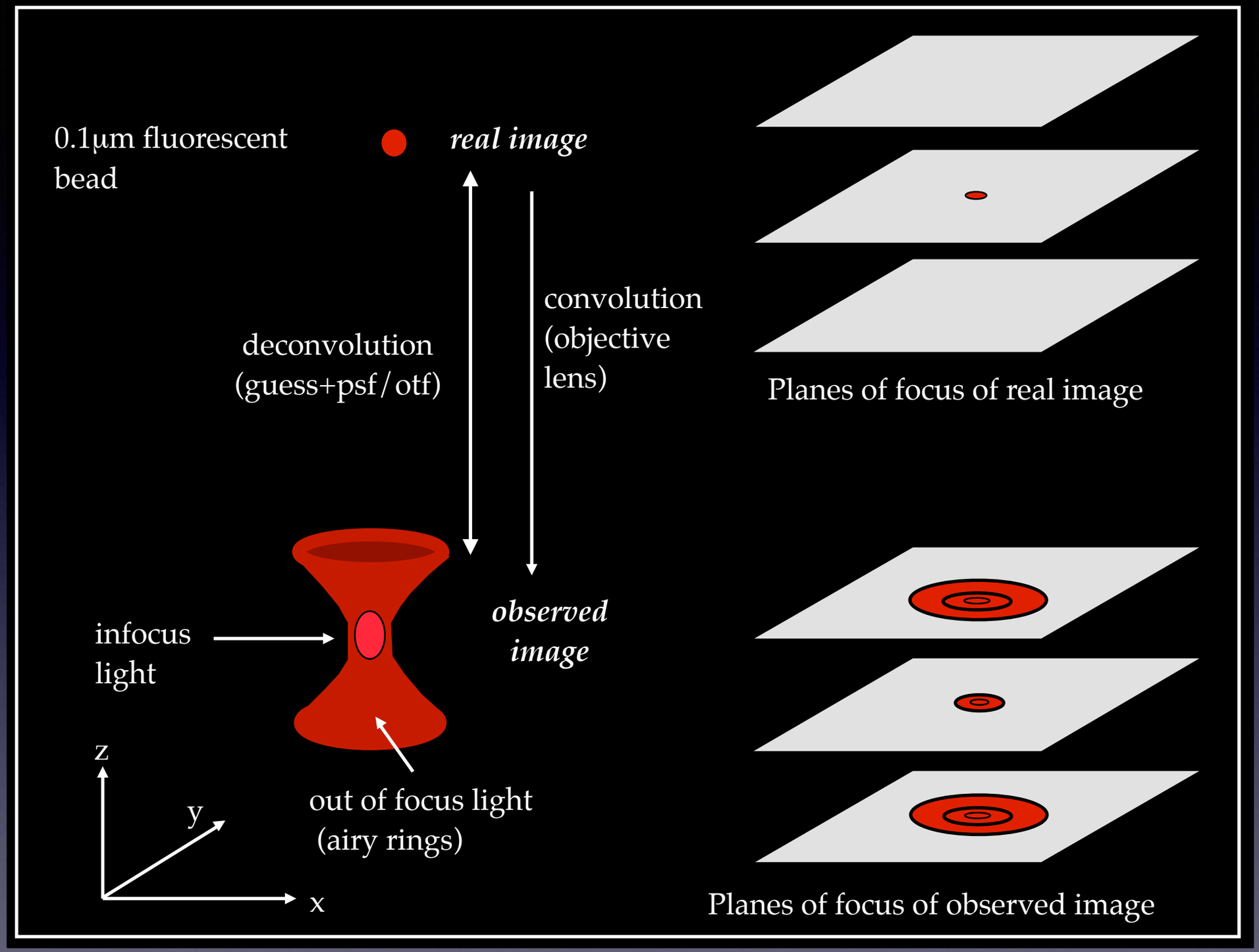

Ilan Davis, 2000

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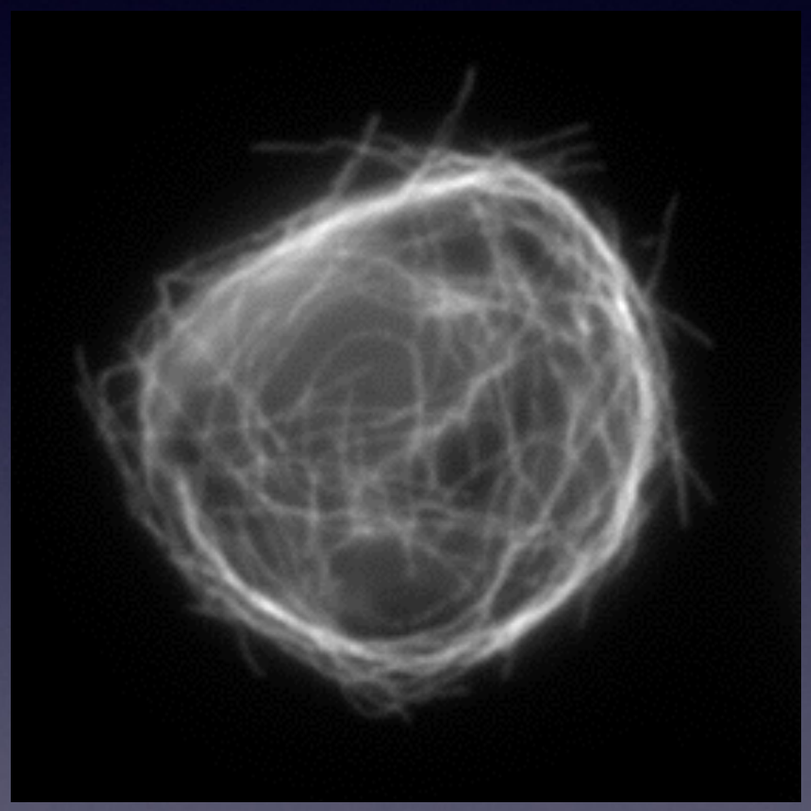
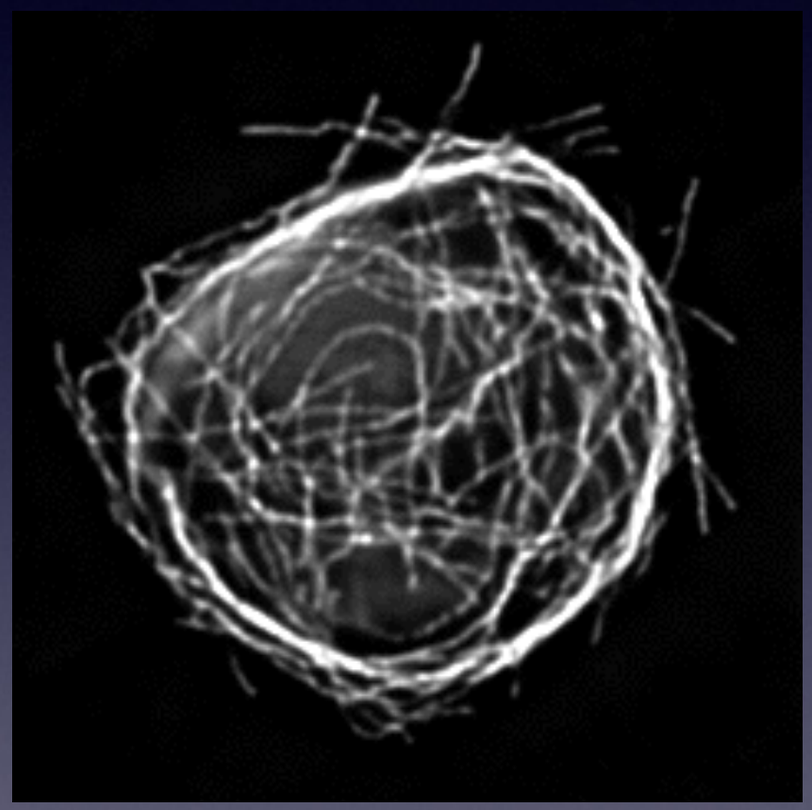
Original Image versus Deconvolved image.



Widefield



Deconvolved Widefield

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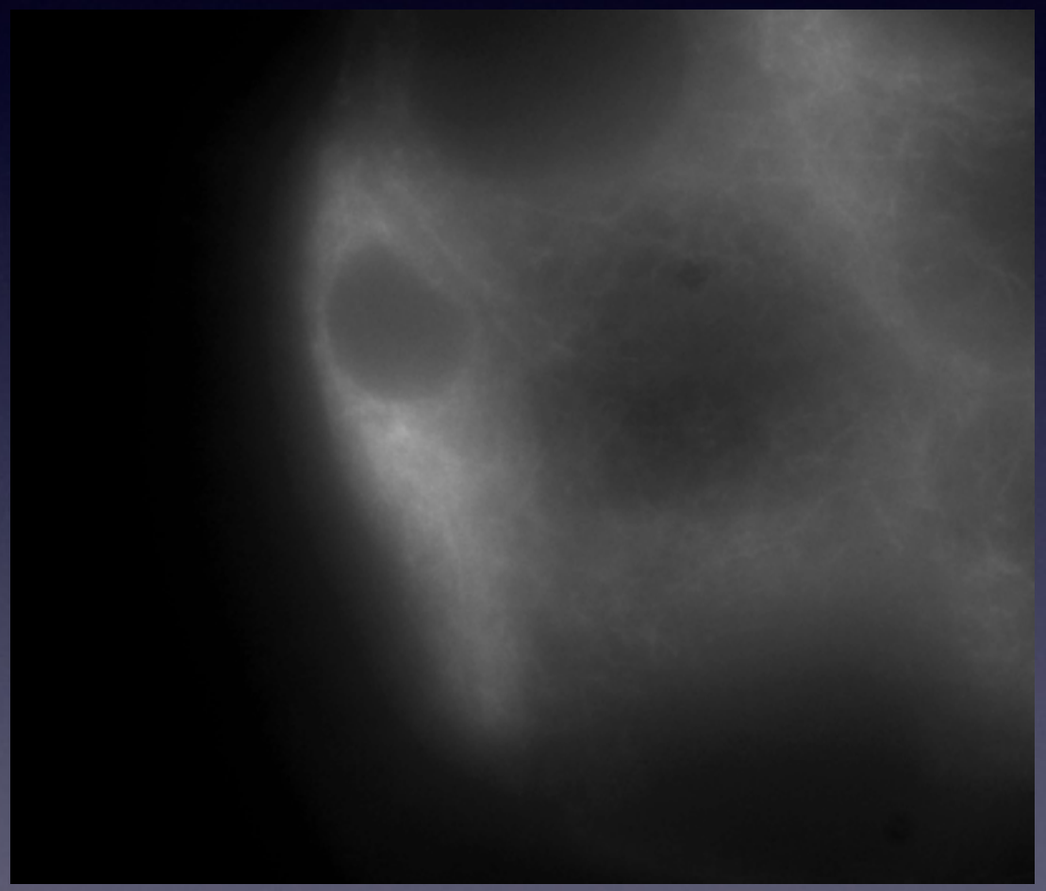
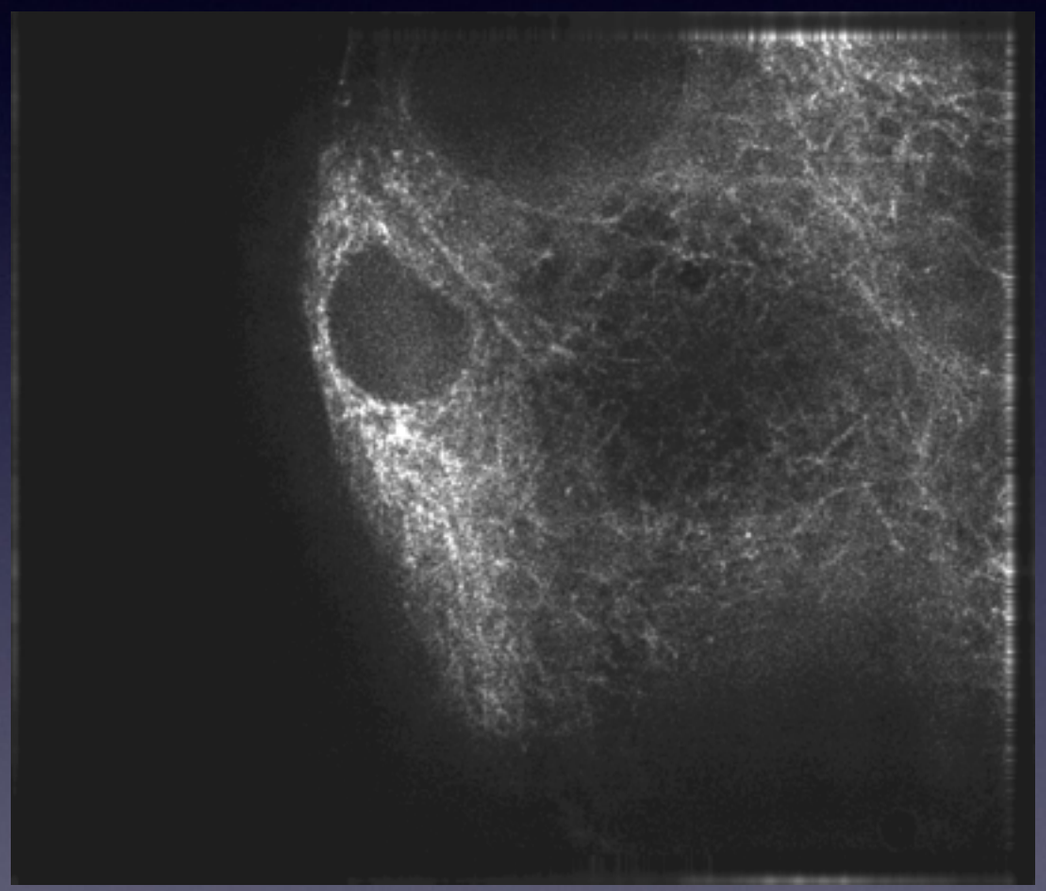
A real example of deconvolution

Before deconvolution



After deconvolution



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Improvements in Deconvolution

- Increases contrast as out of focus background is removed
- Reduces signal spread, hence increases resolution

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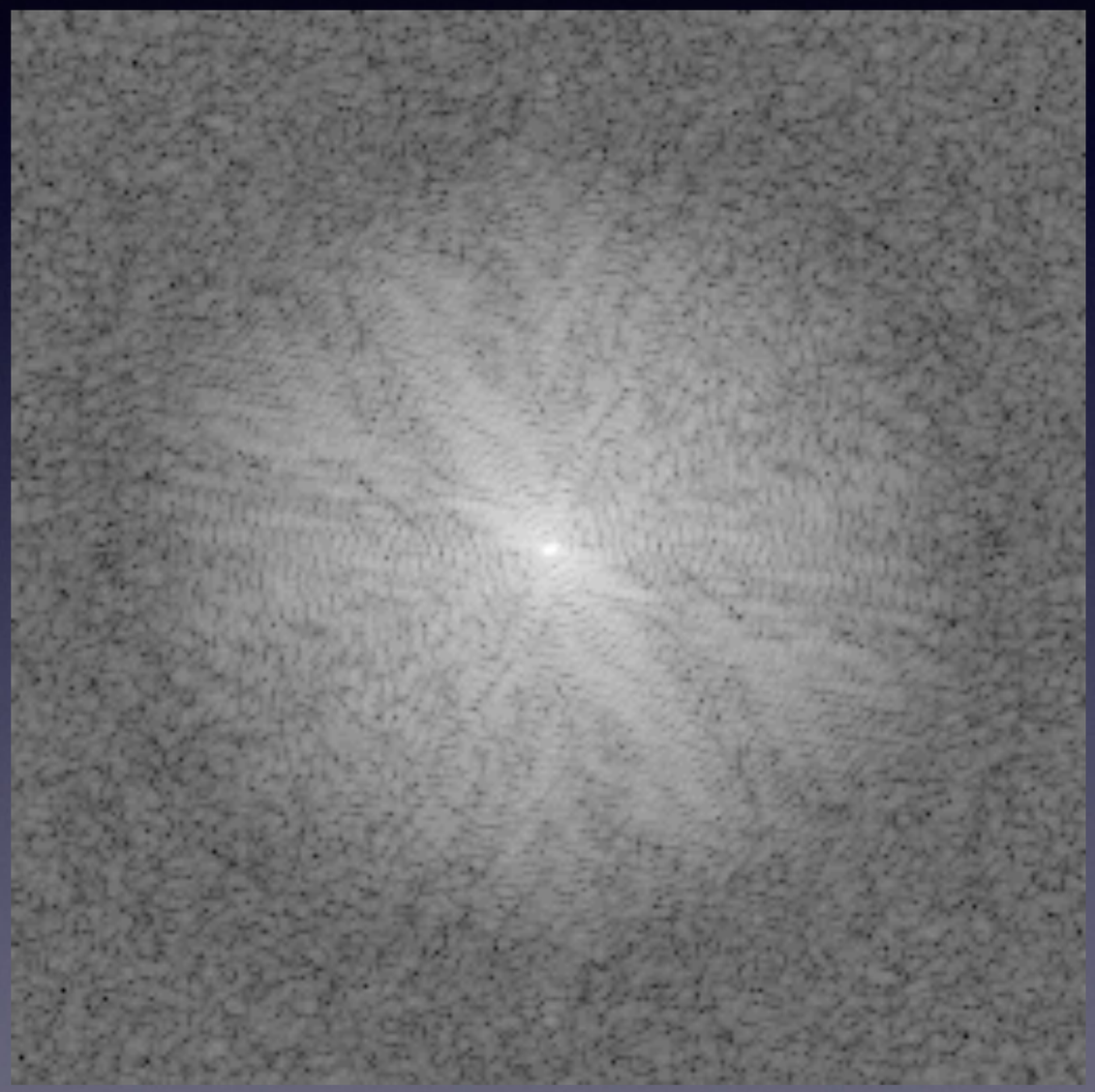
Fourier Transform



FFT of a single slice (Z plane) of image stack

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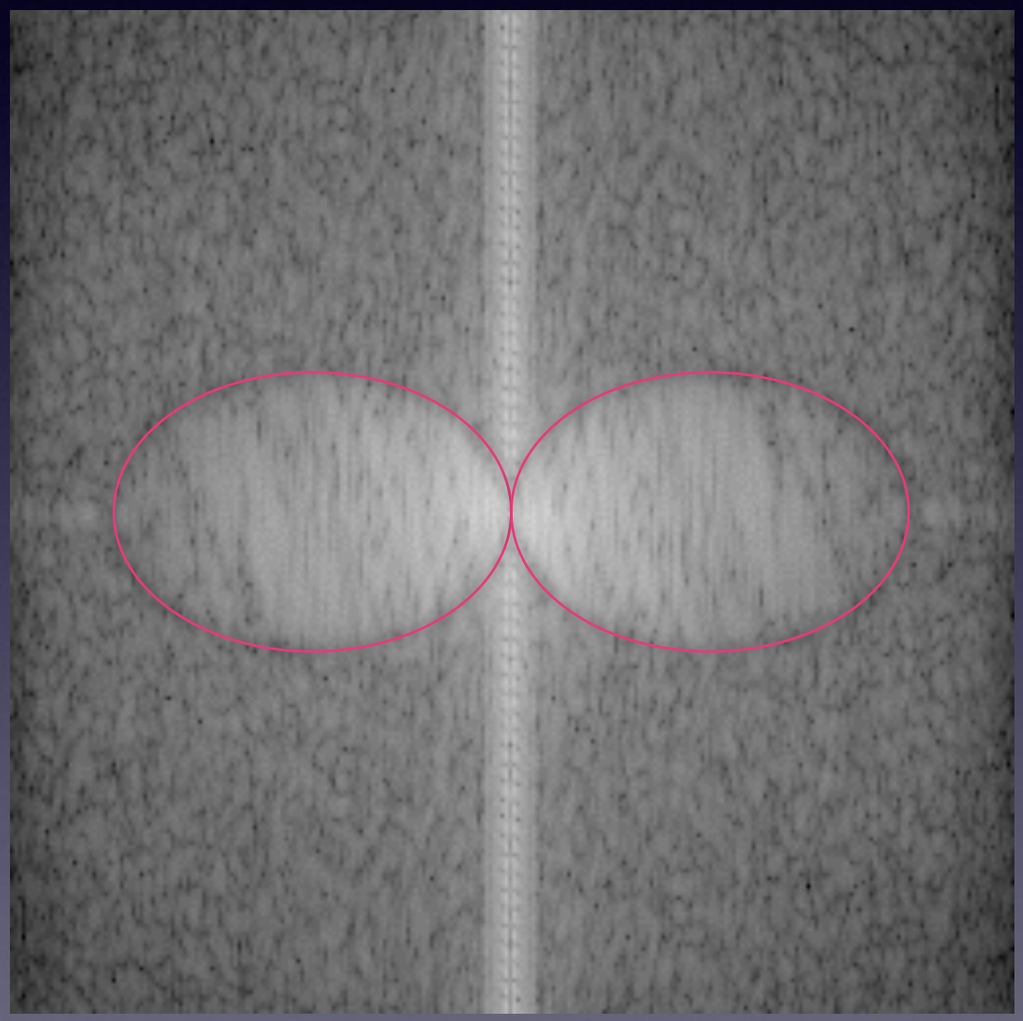
Fourier Transforms in XZ plane



FFT of an orthogonal slice of image stack

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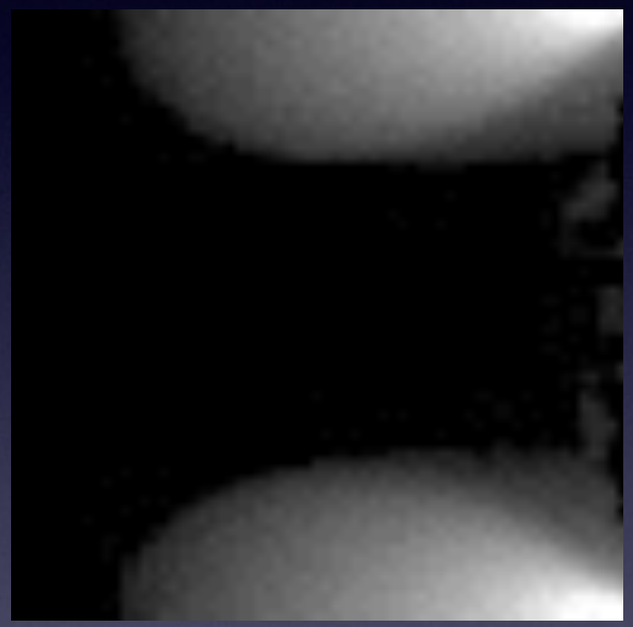
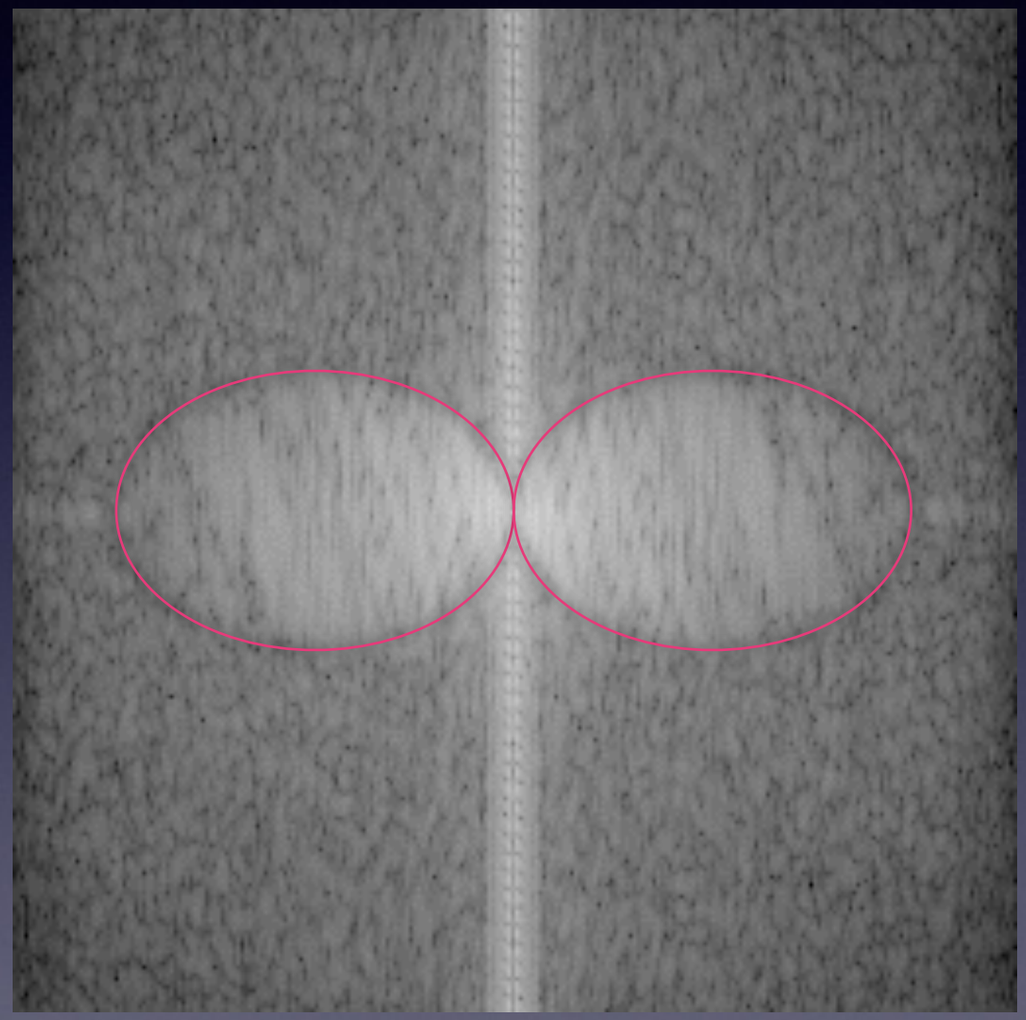
Applying the PSF in practice



Optical Transfer Function
OTF - FFT of the PSF



FFT of an XZ section of
the real image

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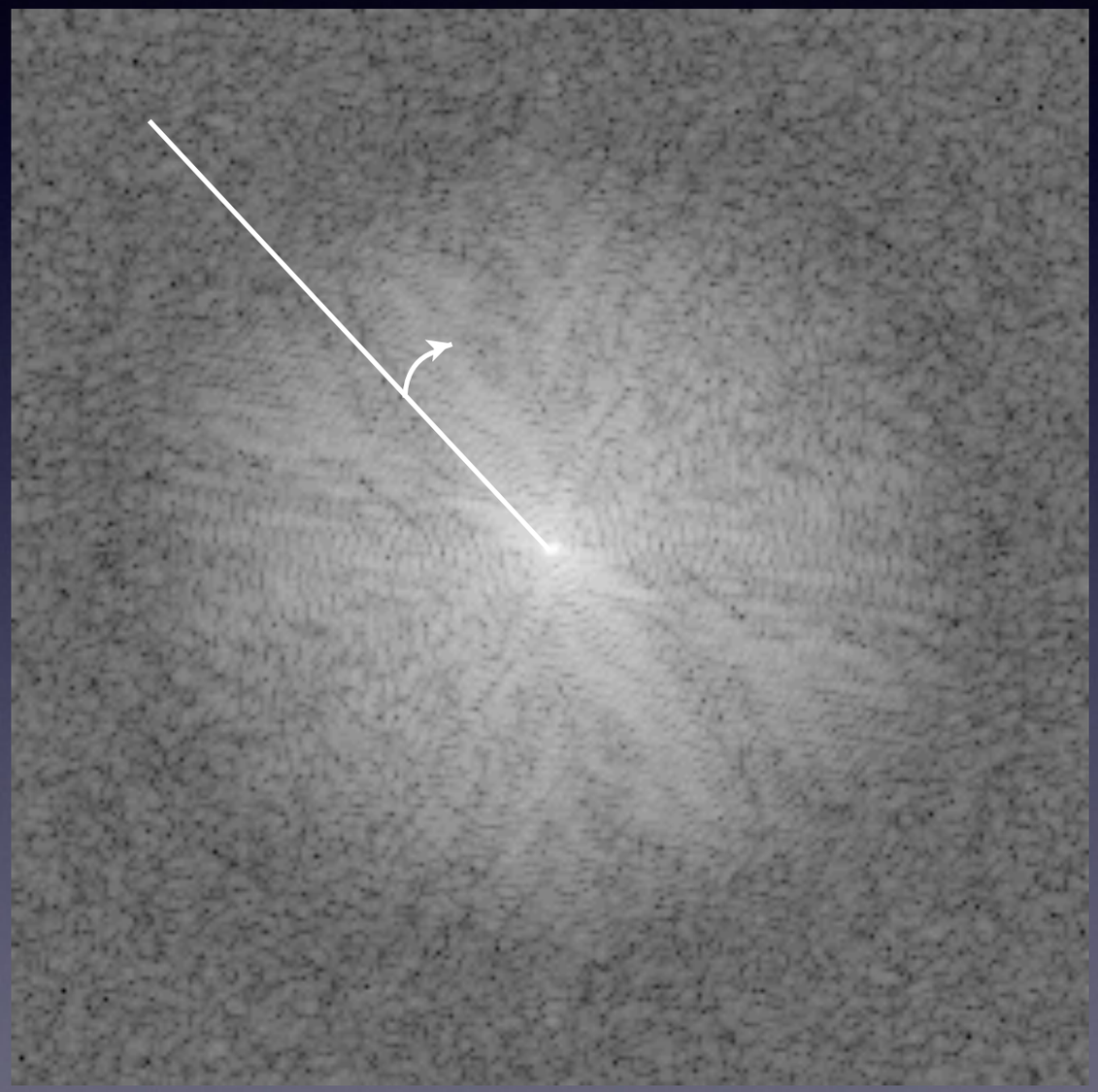
Fourier Transforms to Assess Resolution



FFT of a single slice (Z plane) of image stack

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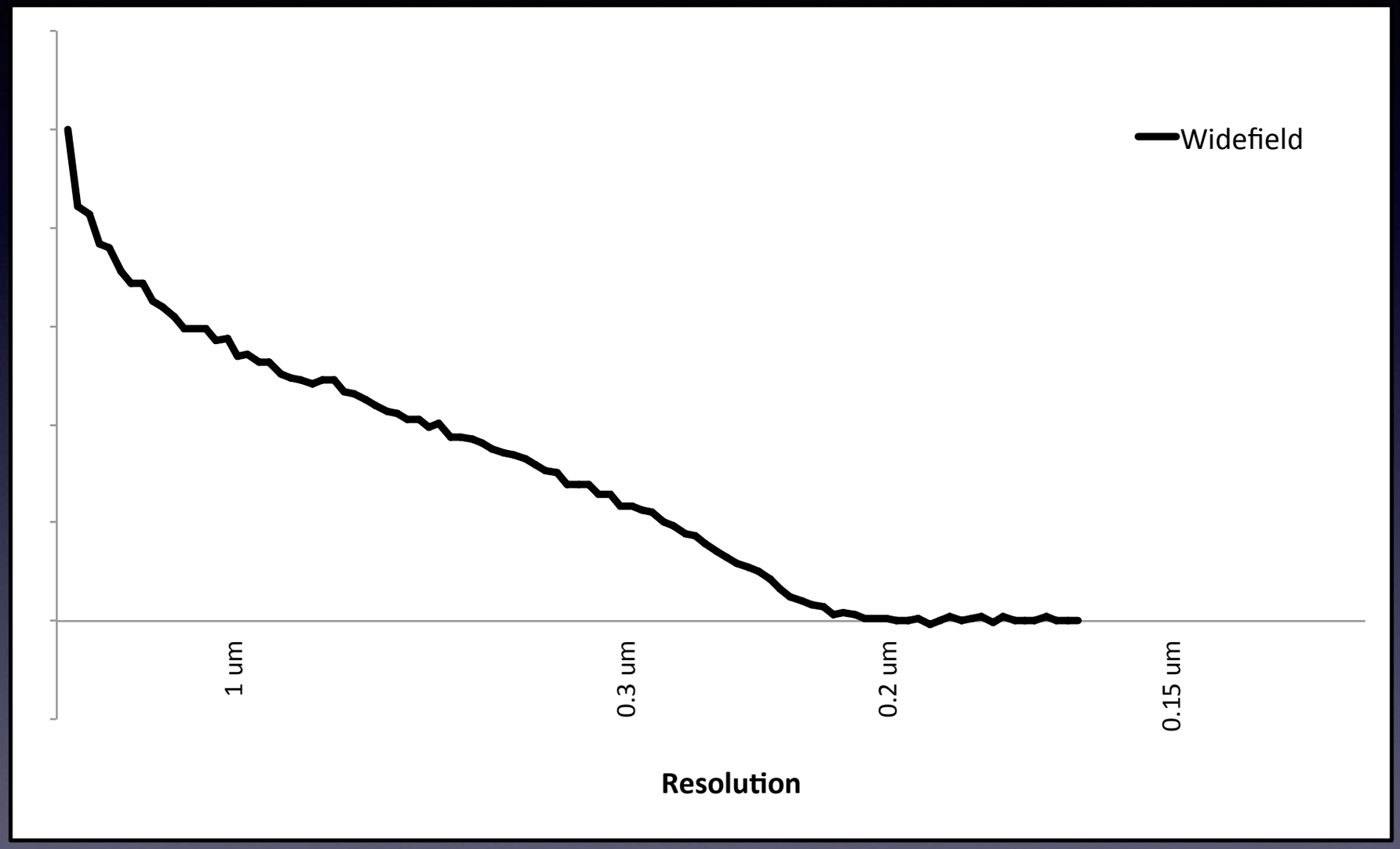
Radial Integrals of FTs



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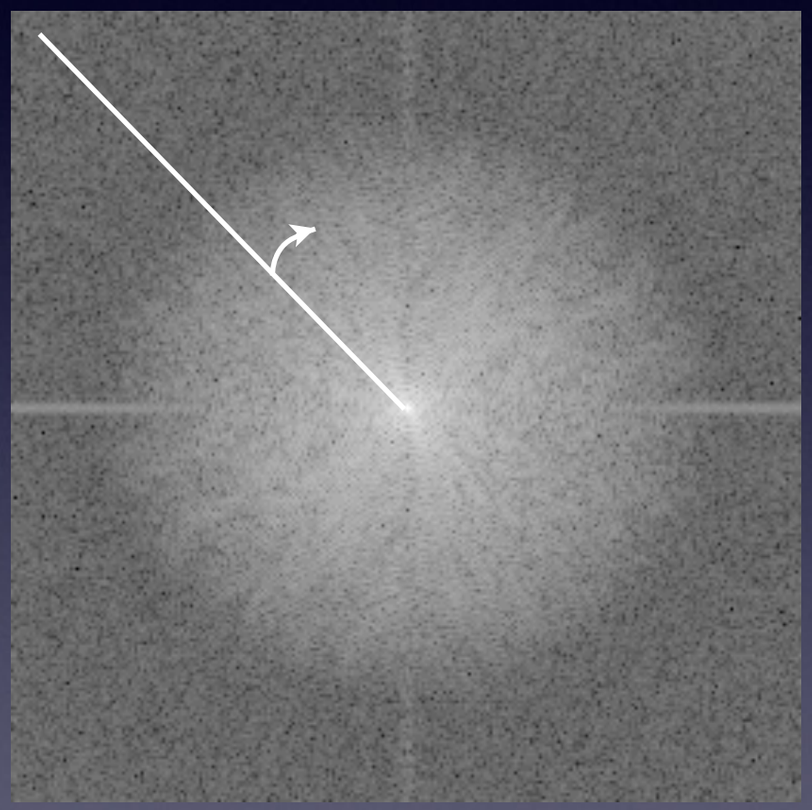
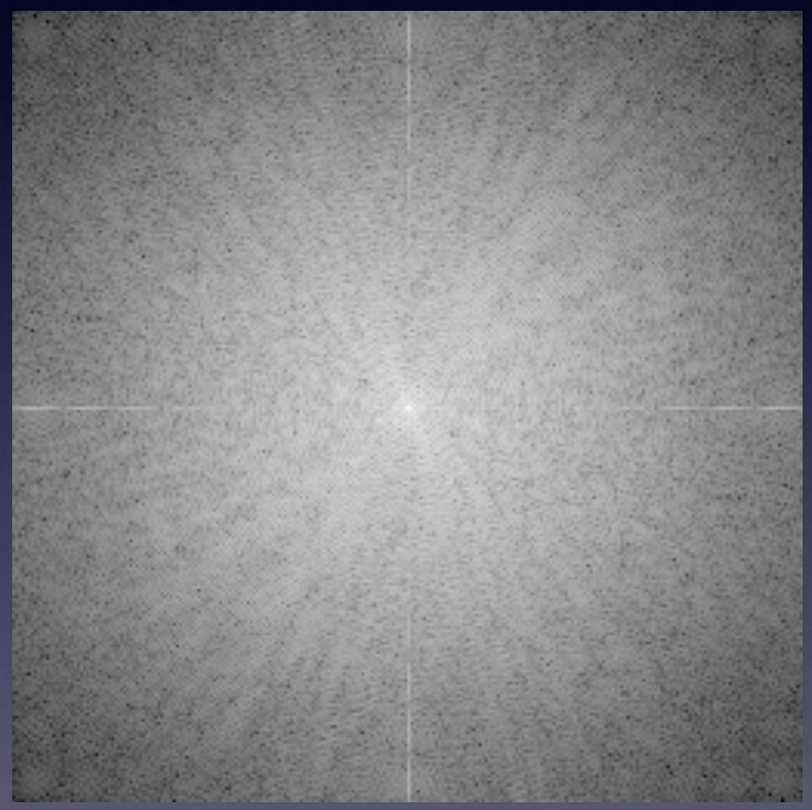
Fourier Transforms to Assess Resolution



FFT of Widefield



FFT of deconvolved Widefield

FTs of Microtubule images at equivalent scale

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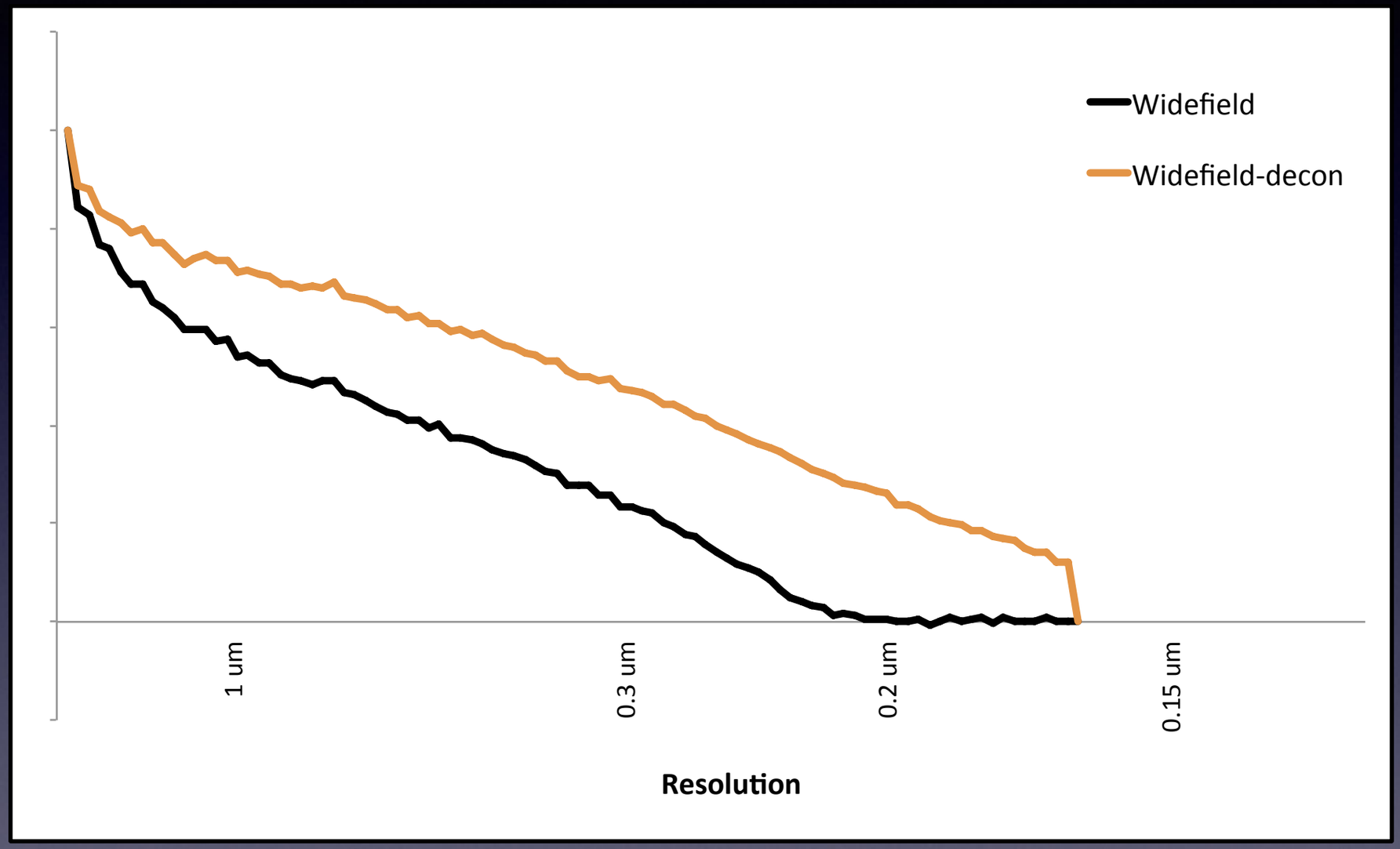
Radial Integrals of FTs



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How a DIC prism effects fluorescence imaging

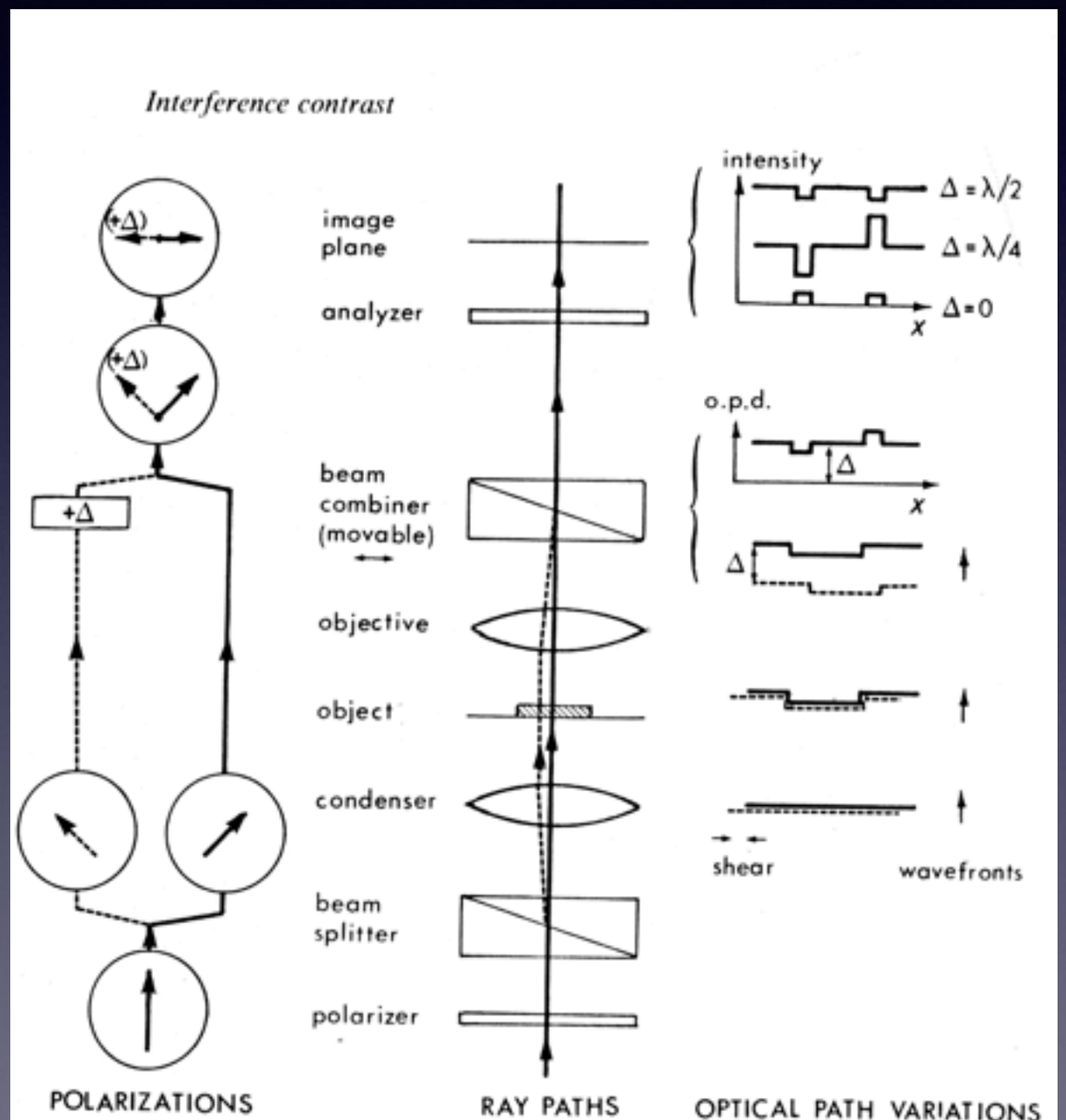

Fig. 38. The optical system for interference contrast.

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With/without DIC prism

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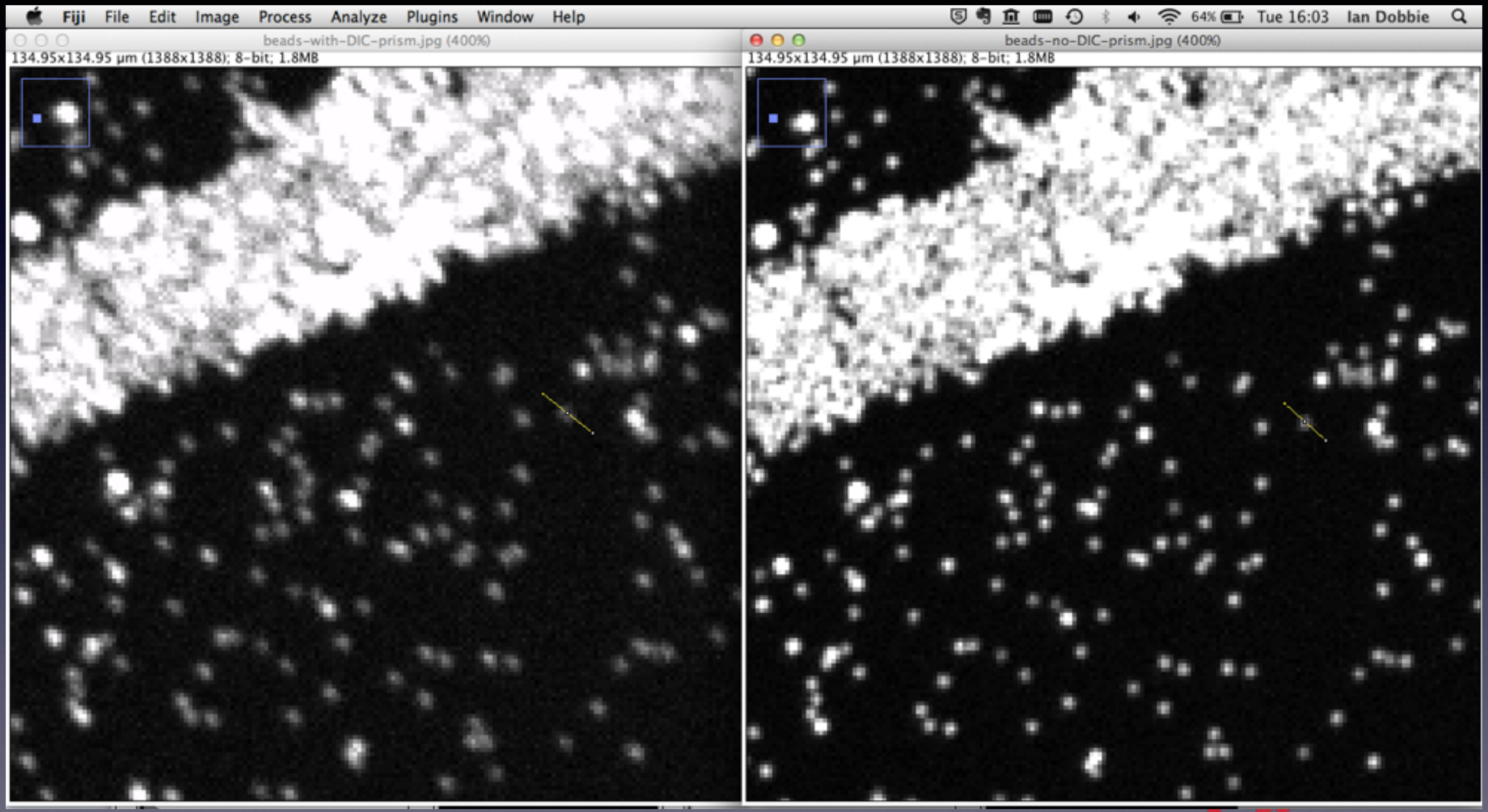
With/without DIC prism



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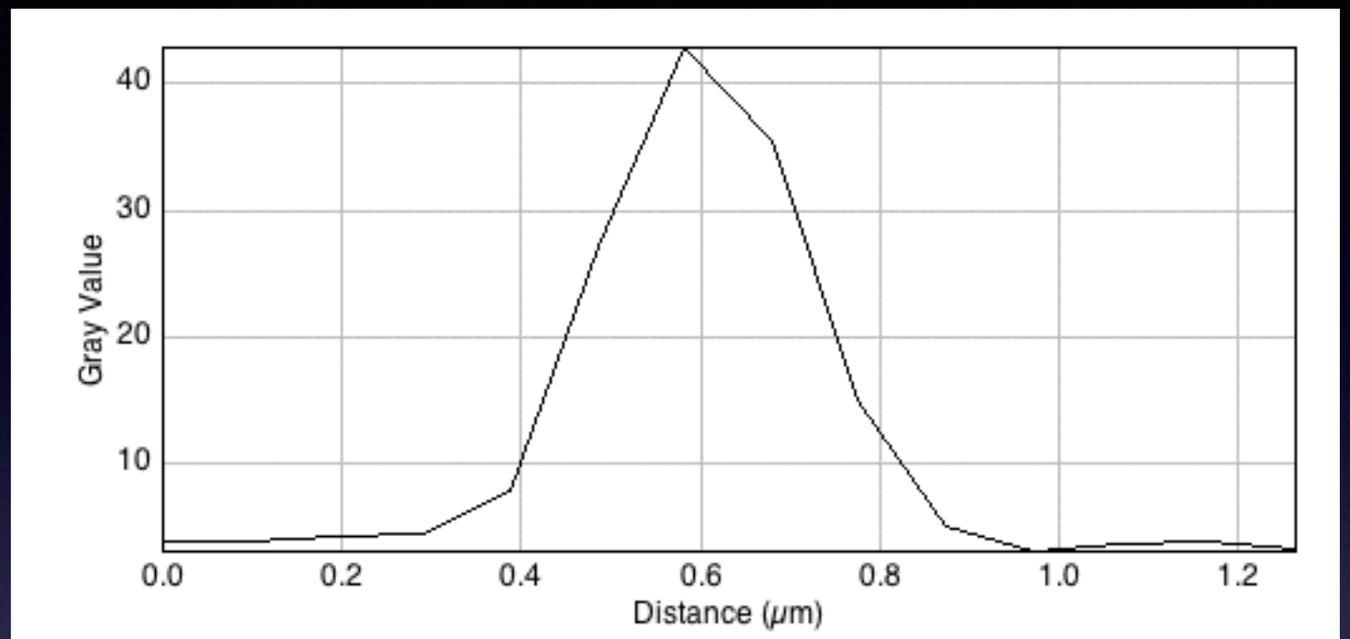
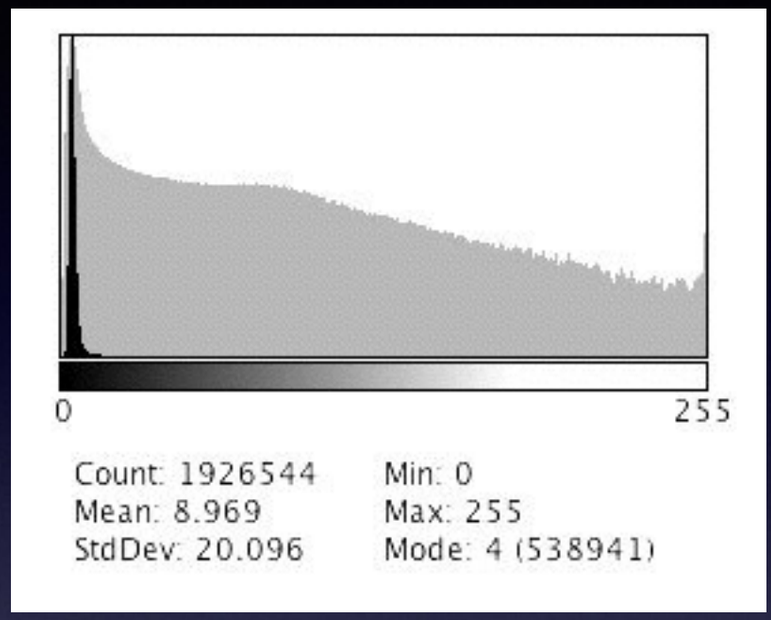
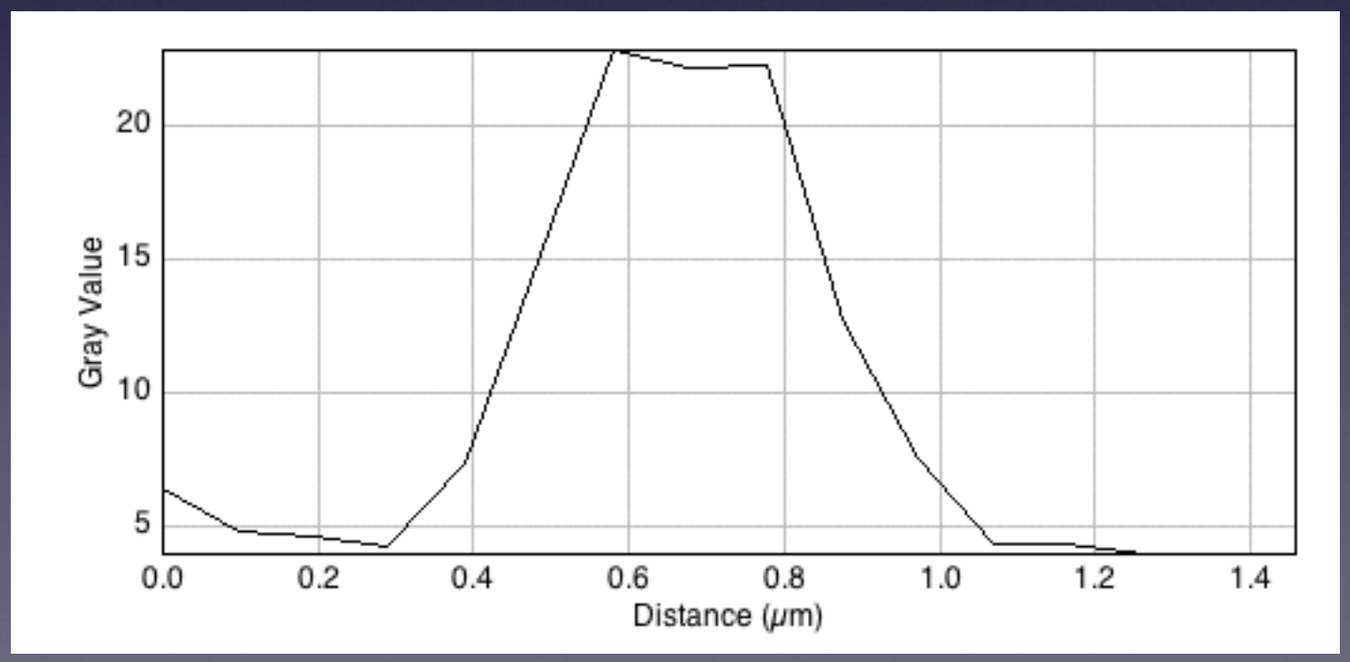
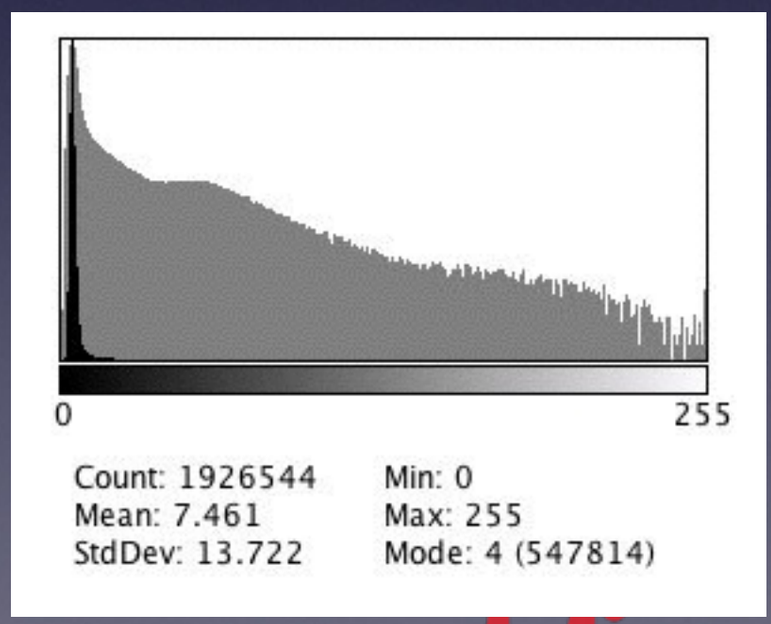
Line scans and histograms









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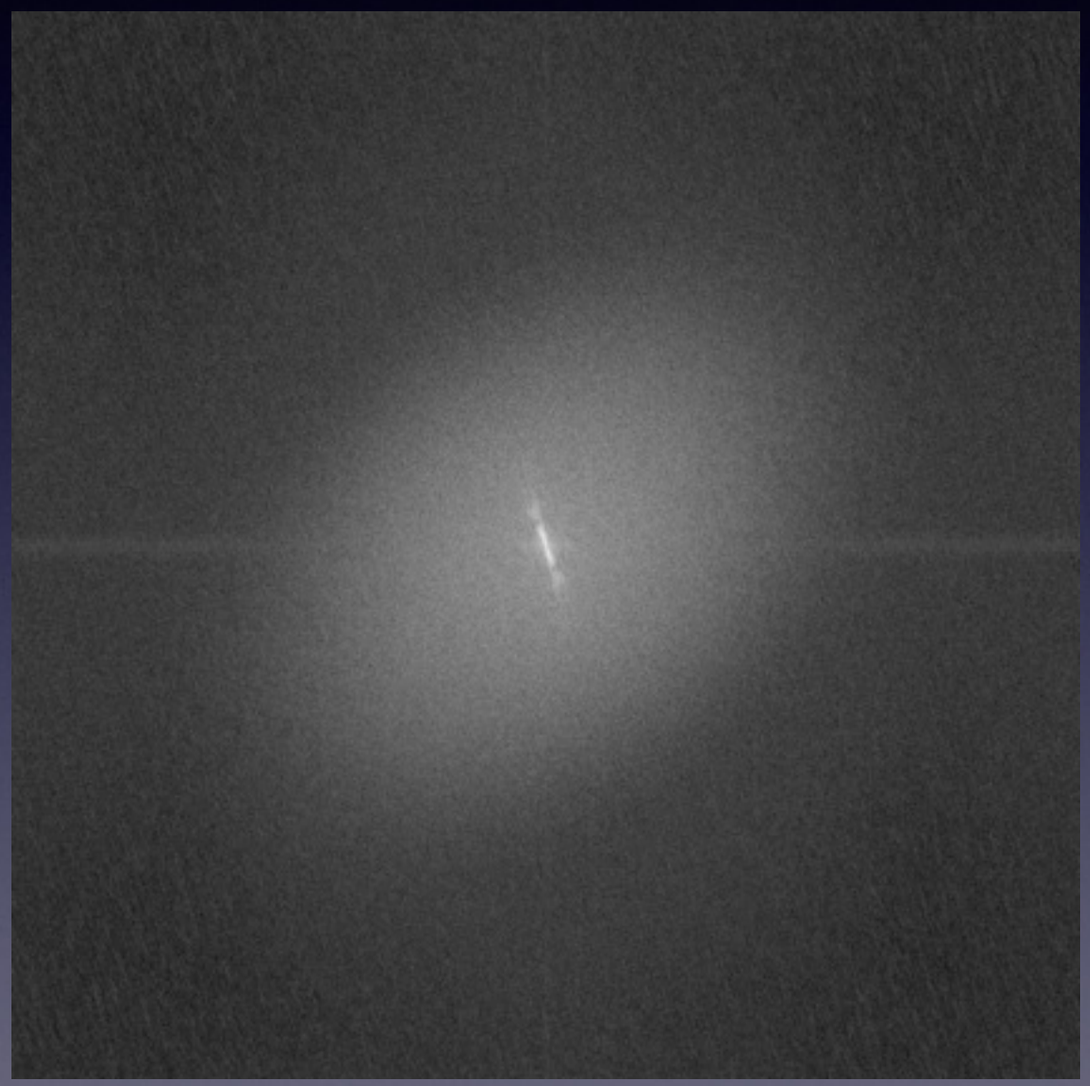
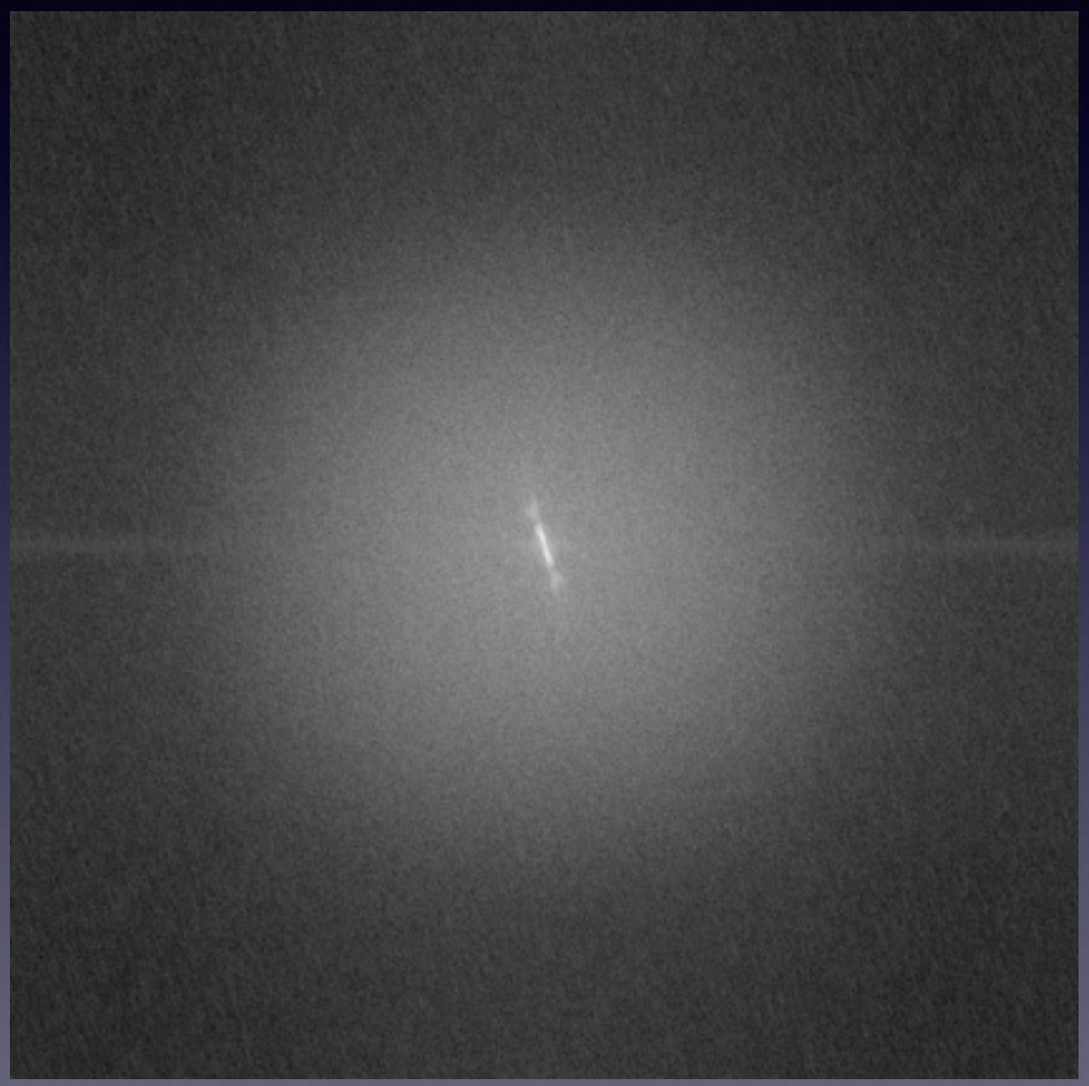
FFTs with/without DIC prism





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Super Fast Acquisition (FastZ)

- Ramp the Z position instead of stepping it
- Take images as fast as possible during ramp
- Delay between stacks to allow stage to return to initial position

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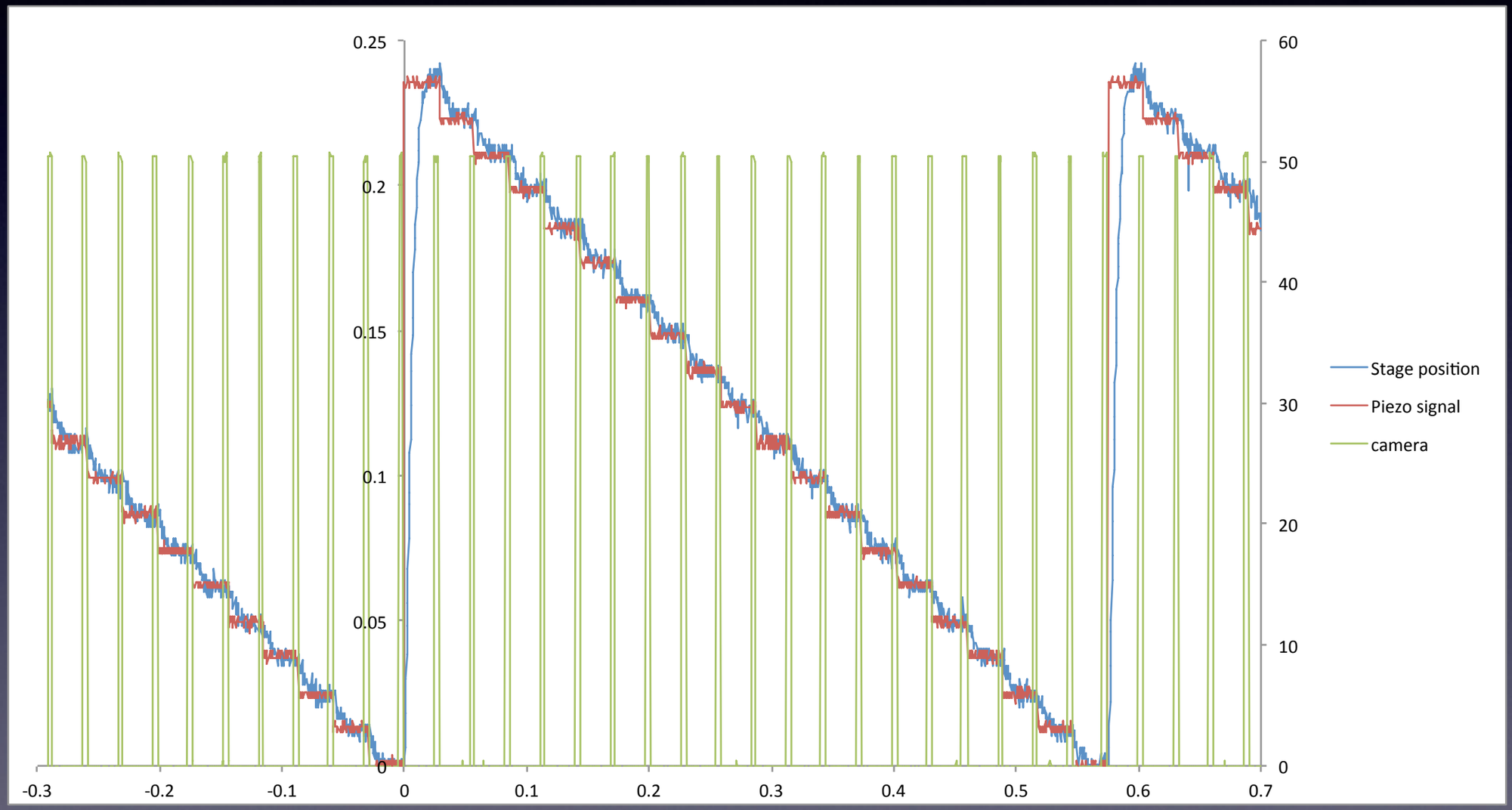
Covential widefield Z stack



20 Z planes as fast as possible

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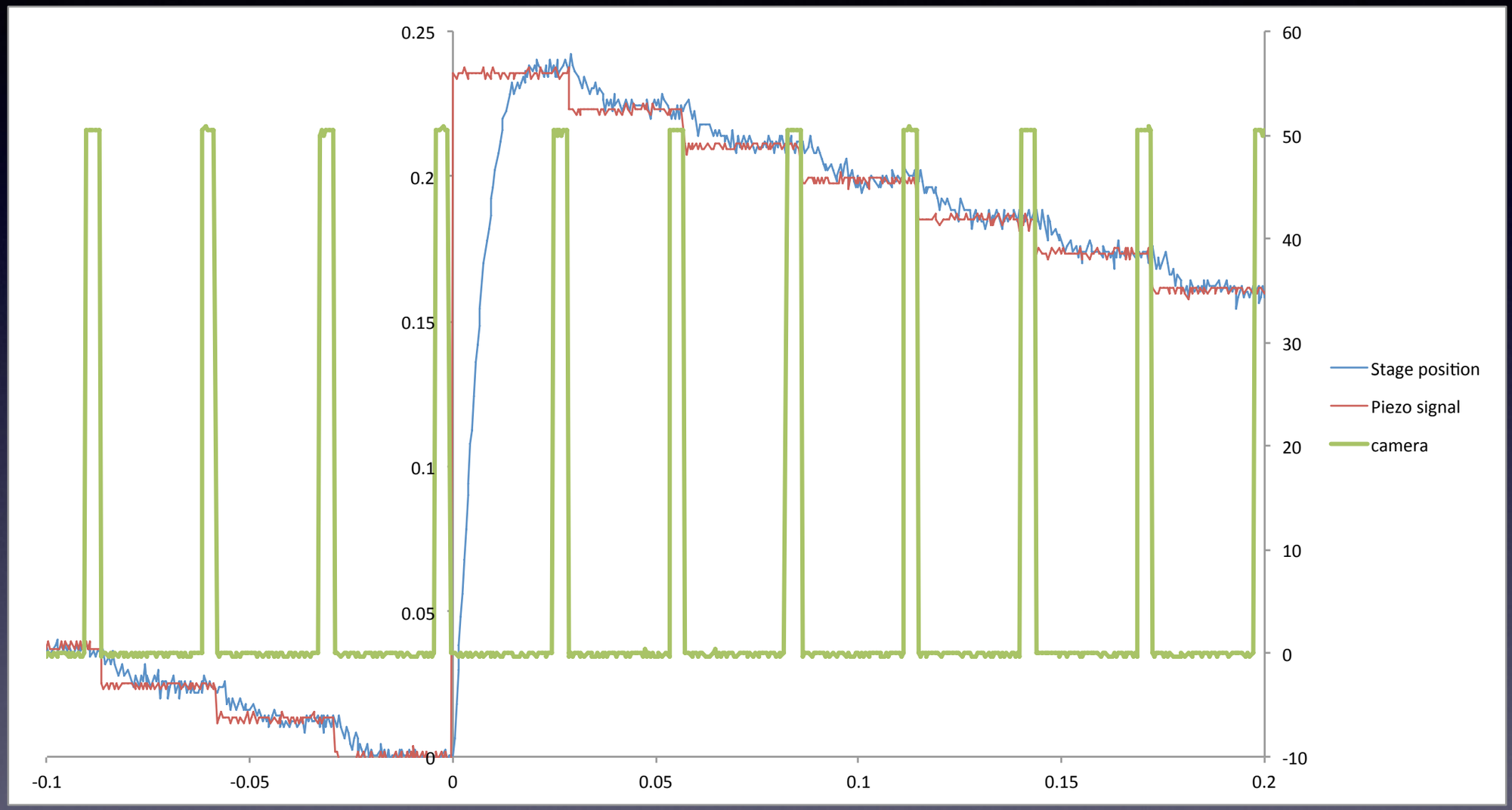
Covential Z stack



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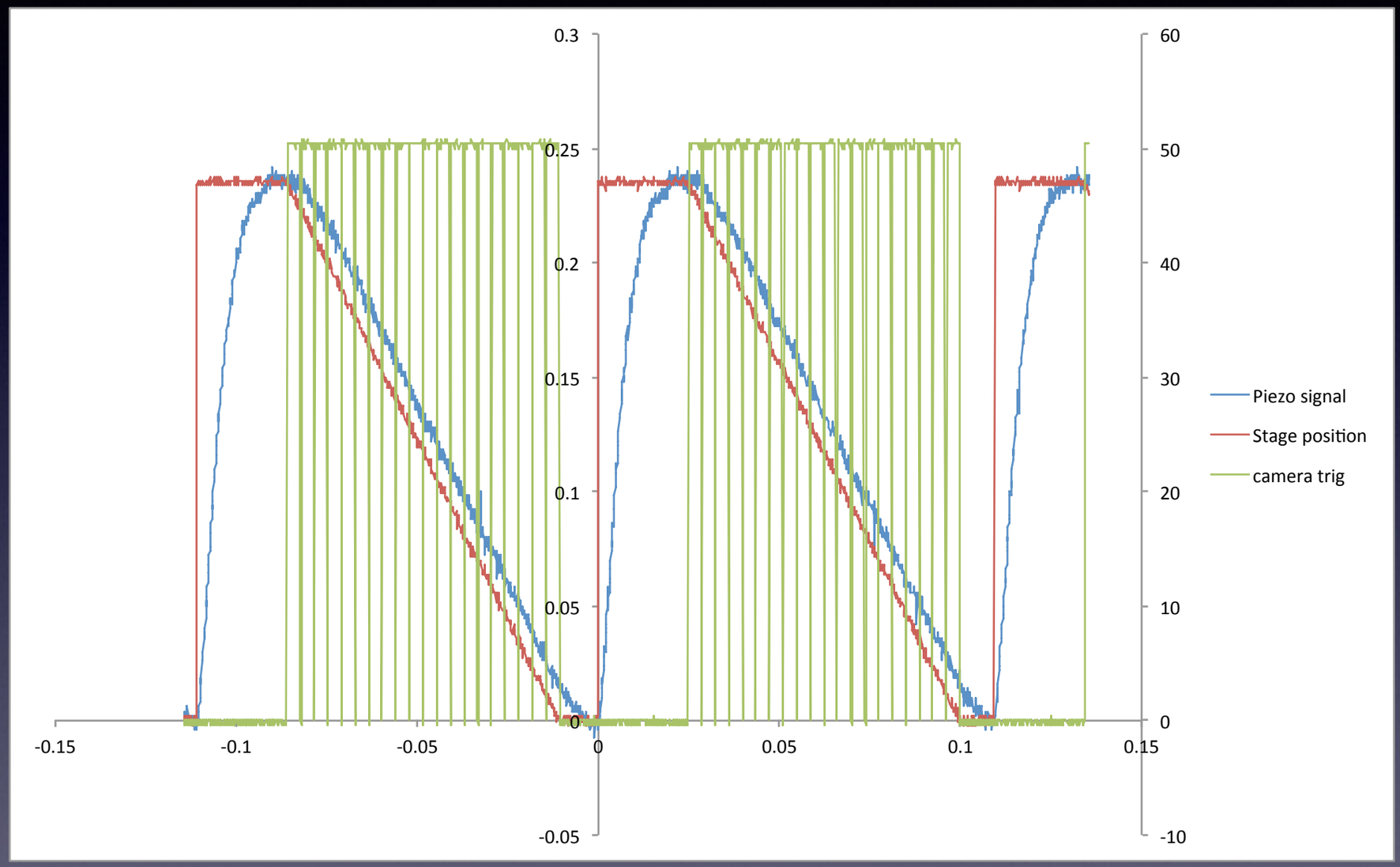
Ramp Z stack



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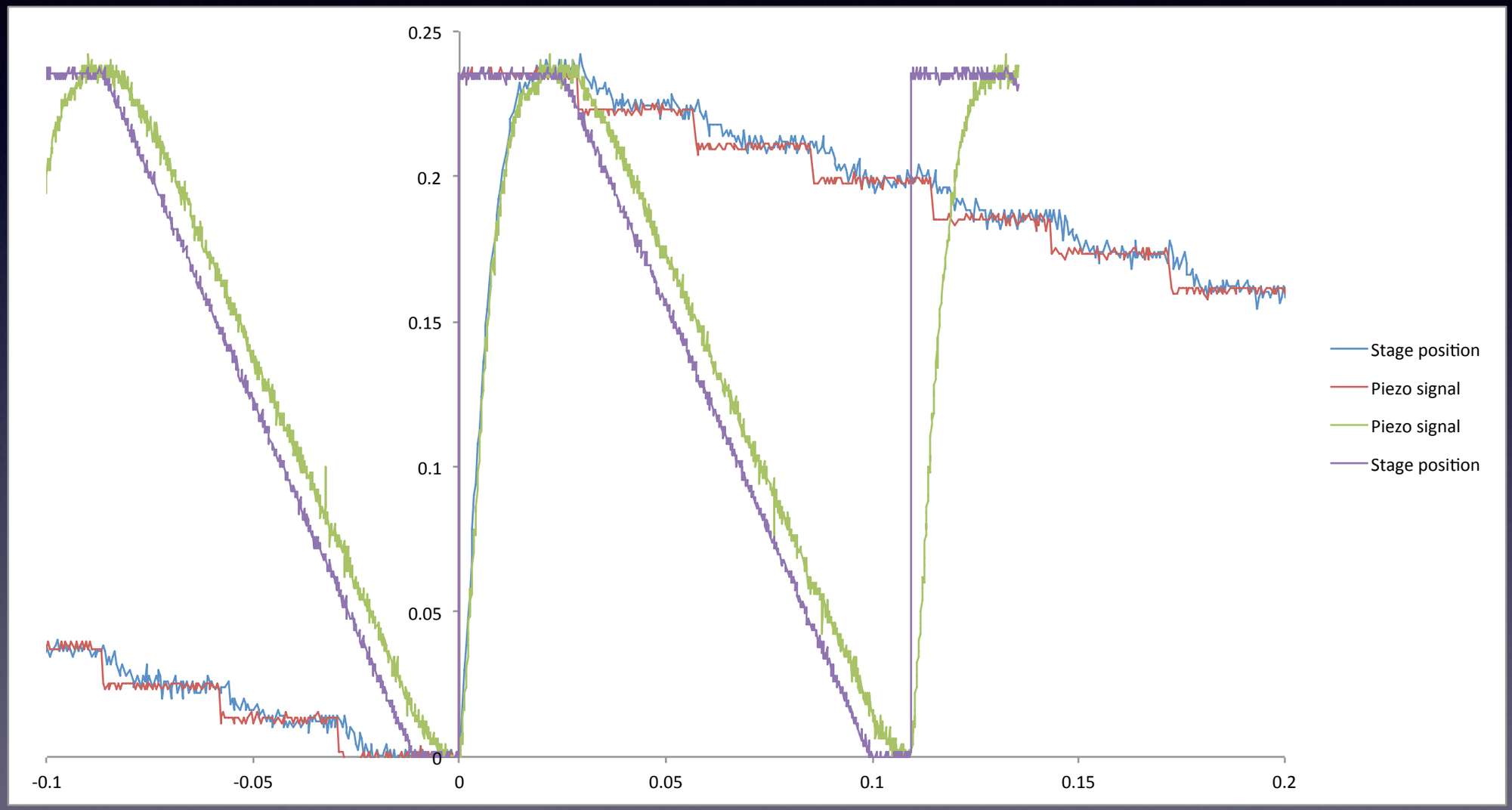
Comparison: FastZ to normal



Speed increases

- Depends on stack height, image size, exposure time.
- Test sample, 512x512 pixel images, 1 ms exposure 20 Z slices of 200 nm.
- Conventional cycle time = 575 ms
- FastZ cycle time = 109 ms

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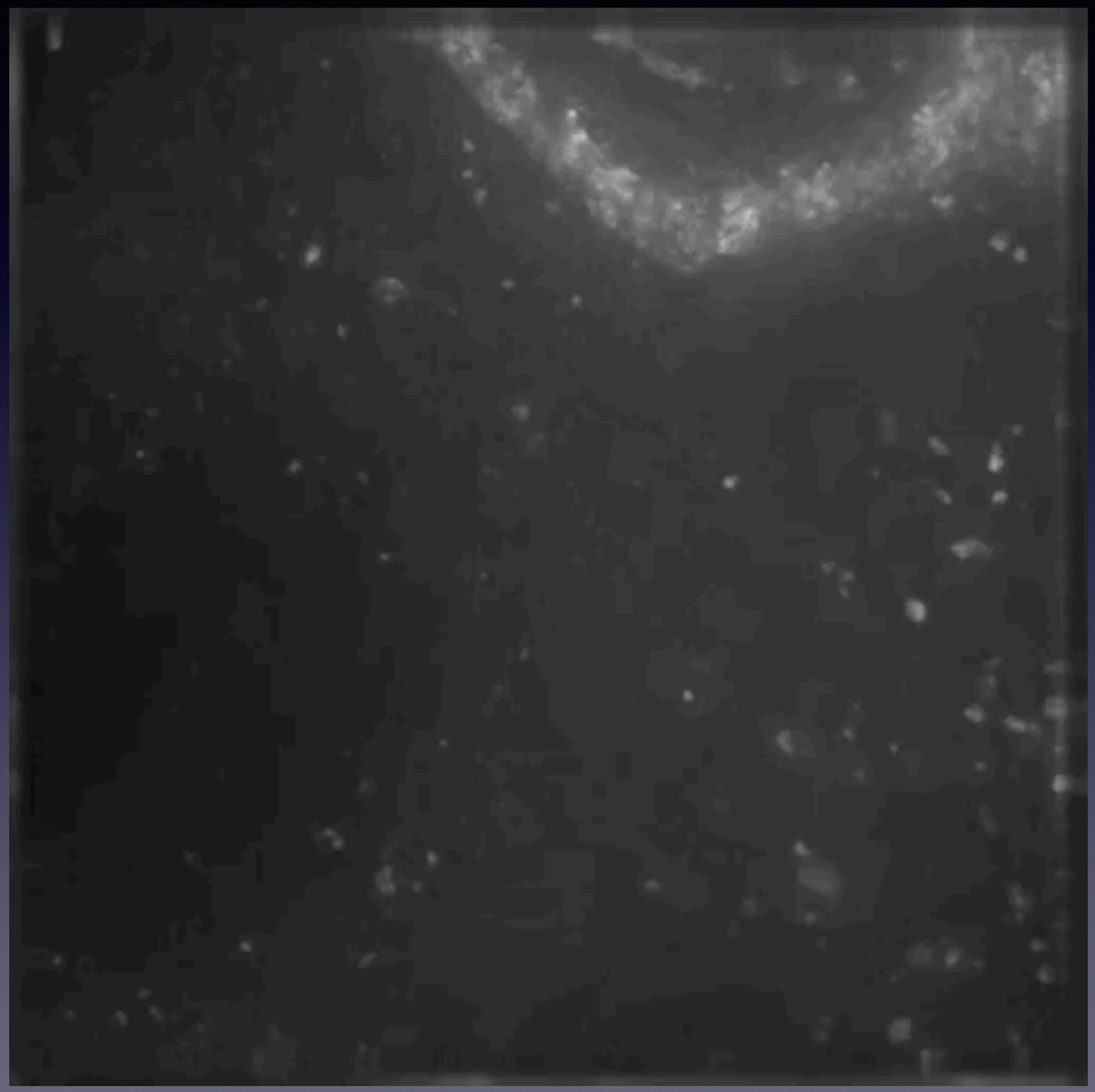
FastZ - Results



Me31B-GFP Drosophila oocyte
25-slices, 8 stacks/s - 200 frames/s

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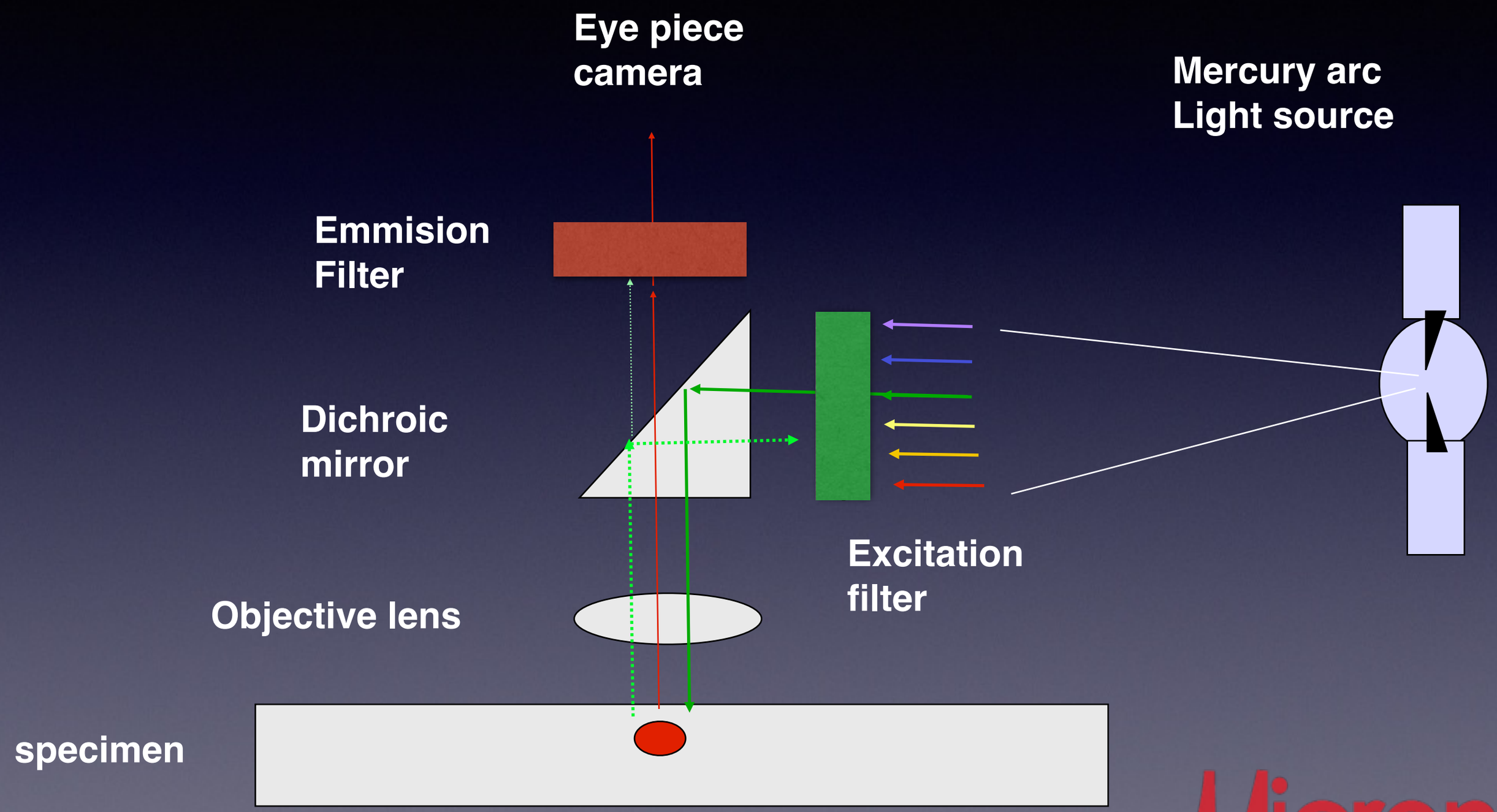
Reminder

How do fluorescence microscopes work ?



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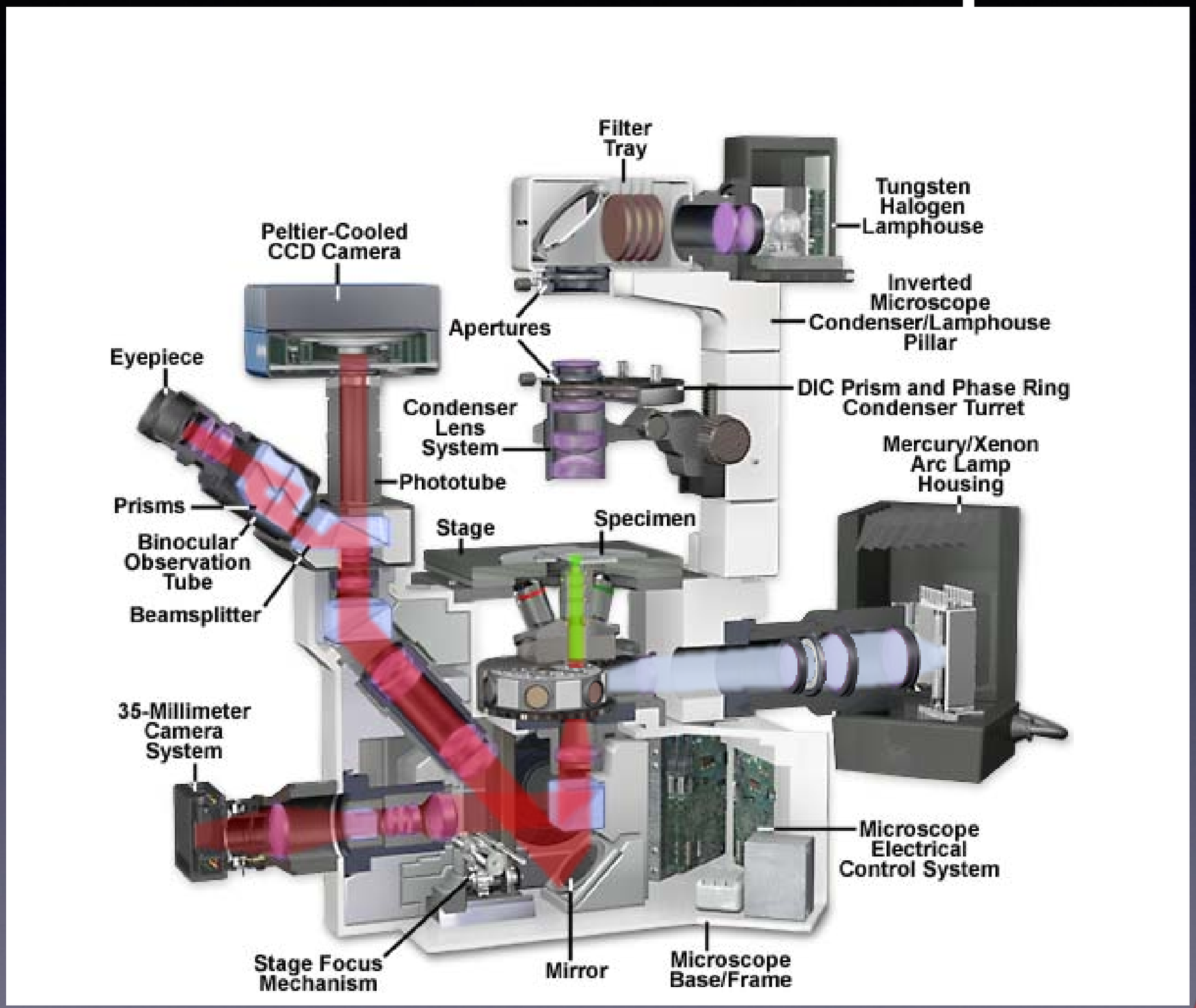
Problem: the design of all conventional microscope stands



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How can we improve the basic design of widefield microscopes?

By dispensing with the normal microscope stand and building your own microscope from optical components on a breadboard

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The solution -build your own bespoke microscope

Mark Leake with the Slimfield TIRF microscope (Biophysics prize)

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Bespoke Microscopes

Why bother?

Specific applications -better than commercial microscopes

Flexibility

Cost

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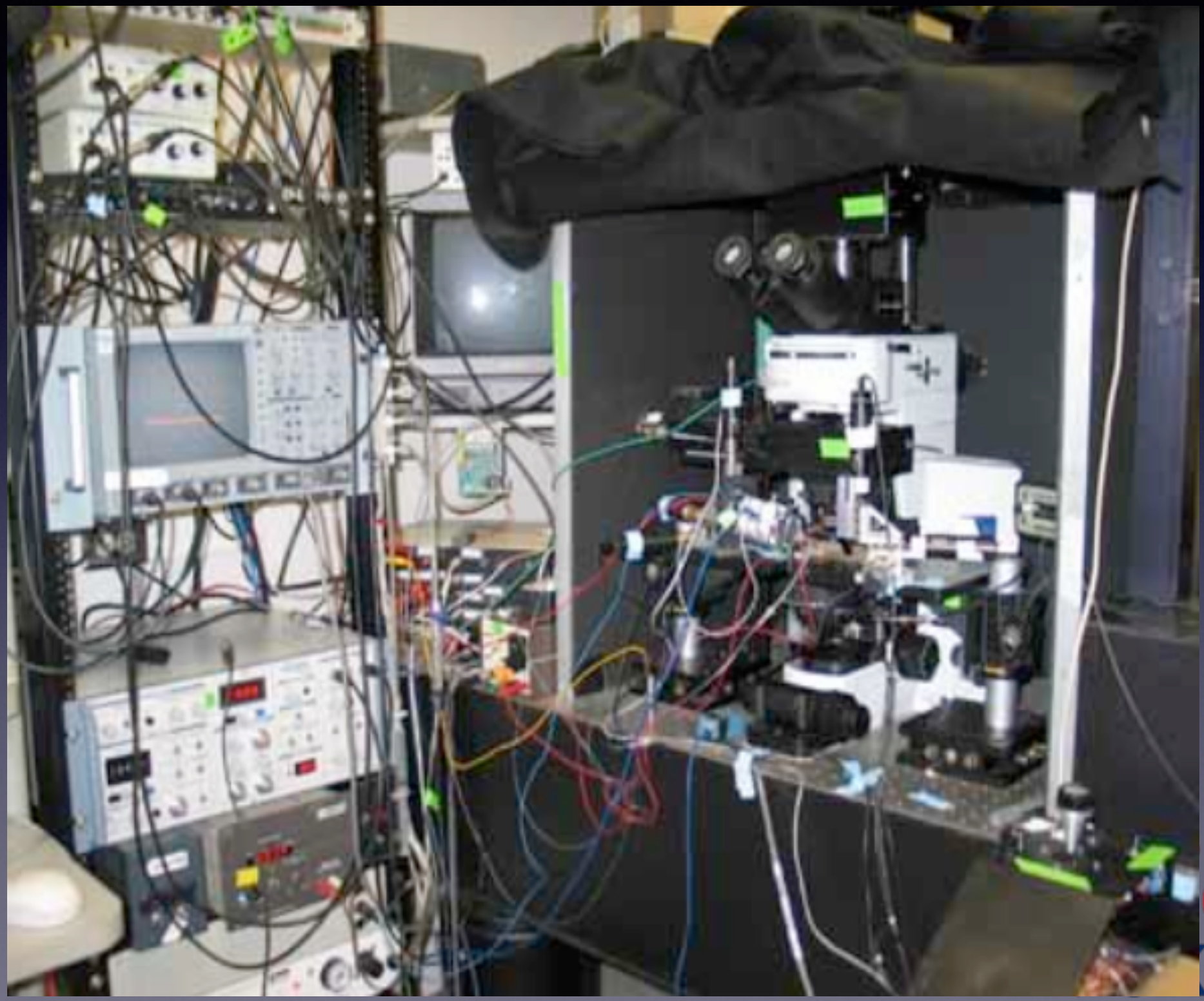
Popular bespoke microscope

Multiphoton for neuroscience work



Bespoke Microscopes

Why NOT to bother?

- Salary of physicist/engineer required
- Long building time required (it's hard)
- Not supported by a company
  (repairs are costly and lengthy)
- Not always easy to use by biologists

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Example of Bespoke Microscopes

OMX-T microscope
Designed and built by John Sedat and Dave Agard, UCSF

Live PALM microscope
Designed and built by Stephan Uphoff and Achillefs Kapanidis, Micron Oxford

WOSM
Designed and built by Nick Carter and Rob Cross, Warwick University

Openspim
Designed and built by Pavel Tamacek and his team at Dresden MPI

Holographic microscope
Irwin Said and Richard Berry, Micron Oxford

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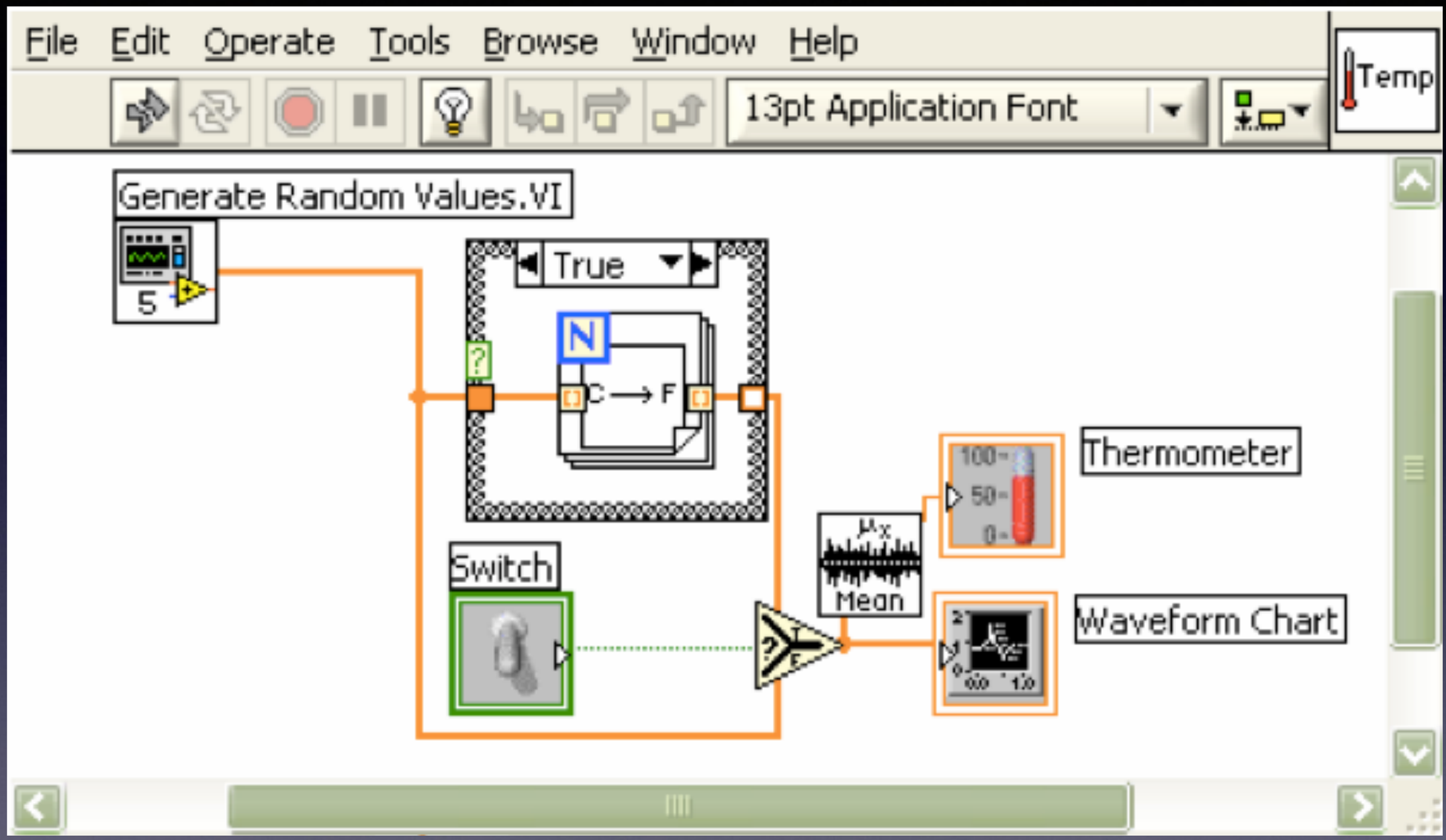
Software options

- Lab view
- Micromanager
- DIY: SDKs - C++, Python, Visual basic

Lab view example



Micromanager

http://valelab.ucsf.edu/~MM/MMwiki/

µManager

THE OPEN SOURCE MICROSCOPY SOFTWARE

OVERVIEW · DOWNLOADS · DOCUMENTATION · DEVICES · PROGRAMMING · SUPPORT · EVENTS · CREDITS · LOG IN

welcome to micro-manager!

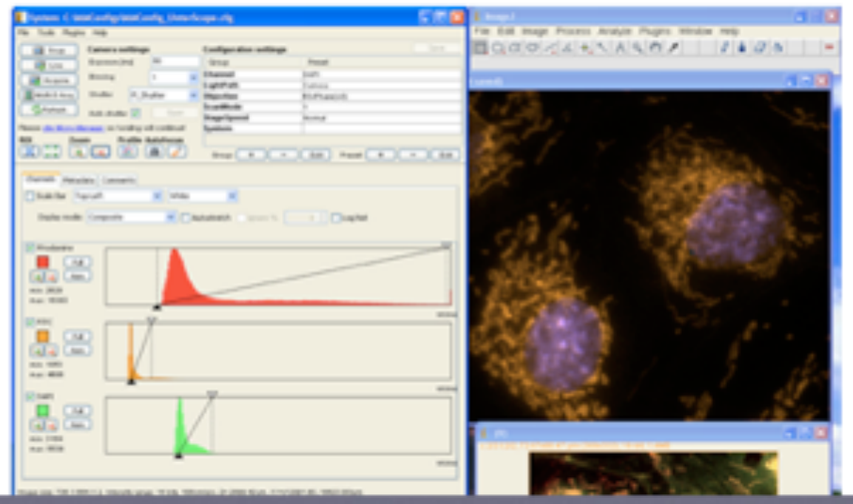
Micro-Manager Open Source Microscopy Software

µ-Manager

The Open Source Microscopy Software

µManager is a software package for control of automated microscopes. Together with the image processing application ImageJ, µManager provides a comprehensive, freely available, imaging solution.

Download the most recent version (1.4) from our website. Also check out our **ScreenCast** for a quick tour on getting started.



µManager has a simple and clean user interface, through which it lets you execute common microscope image acquisition strategies such as time-lapses, multi-channel imaging, z-stacks, and combinations thereof. µManager works with microscopes from all four major manufacturers (Leica, Nikon, Olympus and Zeiss), most scientific-grade cameras and

News

- Micro-Manager Programmer Job Opening!
- ImageJ Conference
- [Open SPIM]
- Micro-Manager 1.4 Released
- Recap of Micro-Manager at 2011 ASCB meeting
- New Getting Started ScreenCast
- [Watch Micro-Manager in Action]
- Support for Nikon and

Some rules of thumb

- Clean and dust free environment
- Oscilloscope and soldering iron - you will need them!
- Good tools and spare parts
- Important to think about user interface
- Important to think about continuity of the project and workflow of experiments
- Important to think about data analysis

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Justification for Bespoke Systems

- Often necessary for specific specialised problems.
- Easily optimised for several parameters, speed, sensitivity etc...
- Can provide extremely flexible systems

BUT think hard as it is likely to be harder, longer and more expensive than at first thought.

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How expensive is it?

Building costs

Hardware ~£100-250k

Salaries 1-3 years (~£50-£150)

Total cost ~£150-350k

Commercial OMX system ~£750k

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Summary

- Recap on image formation
- Fluorescent beads showing aberrations
- How deconvolution works
- Bespoke microscope building projects pro's and cons.

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