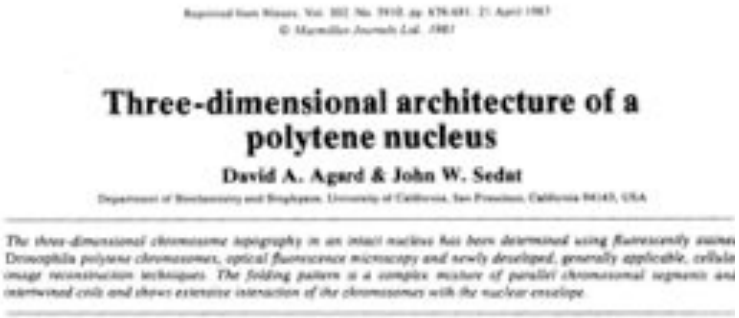
LECTURE 9
Advanced Widefield Microscopy
Ilan Davis, March 2013

- Image formation and airy rings
- Beads and spherical aberration
- How deconvolution works
- Design of a modern widefield digital microscope
- OMX - fast simultaneous live and 3DSIM
- Adaptive Optics Correcting Spherical aberration
- Bespoke microscope design - pros and cons
- Bespoke microscope principles and examples

Agard and Sedat, Nature 1983

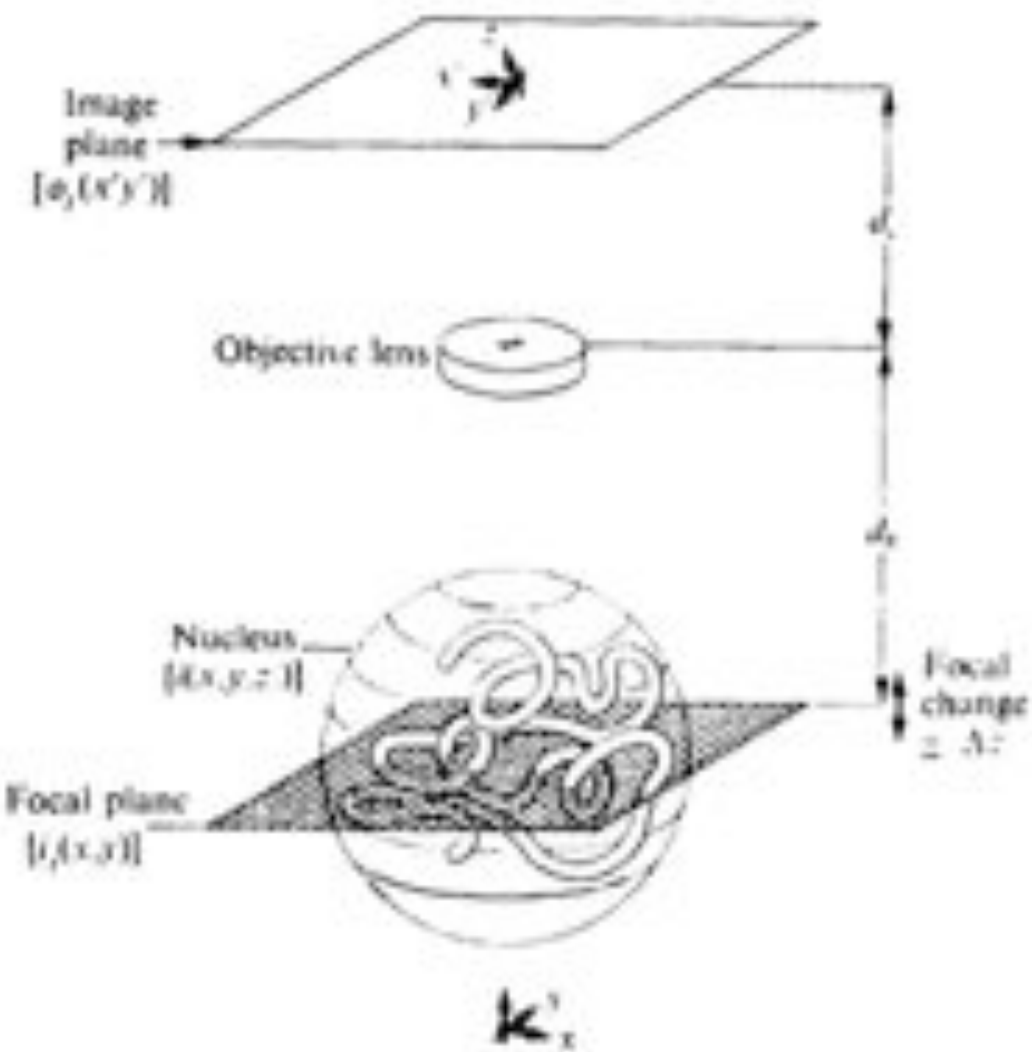
http://www.msg.ucsf.edu/agard/Publications/9-Agard-Nature-83.pdf



Three-dimensional architecture of a polytene nucleus

David A. Agard & John W. Sedat

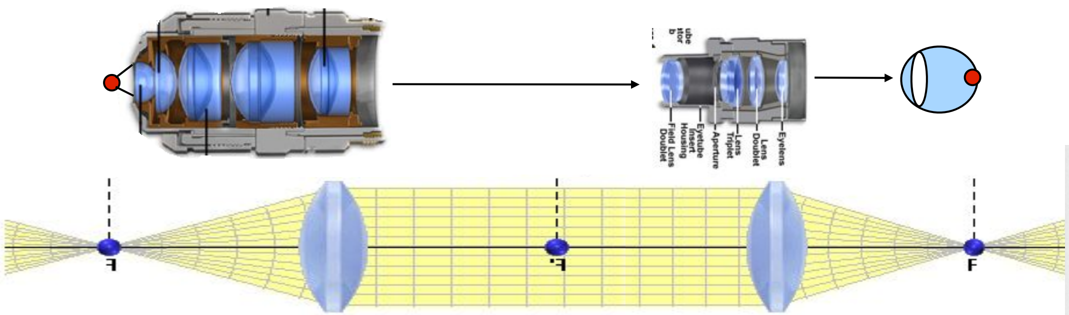
The three-dimensional chromosome topography in an intact nucleus has been determined using fluorescently stained Drosophila polytene chromosomes, optical fluorescence microscopy and newly developed, generally applicable, cellular image reconstruction techniques. The folding pattern is a complex mixture of parallel chromosomal segments and intertwined coils and shows extensive interaction of the chromosomes with the nuclear envelope.

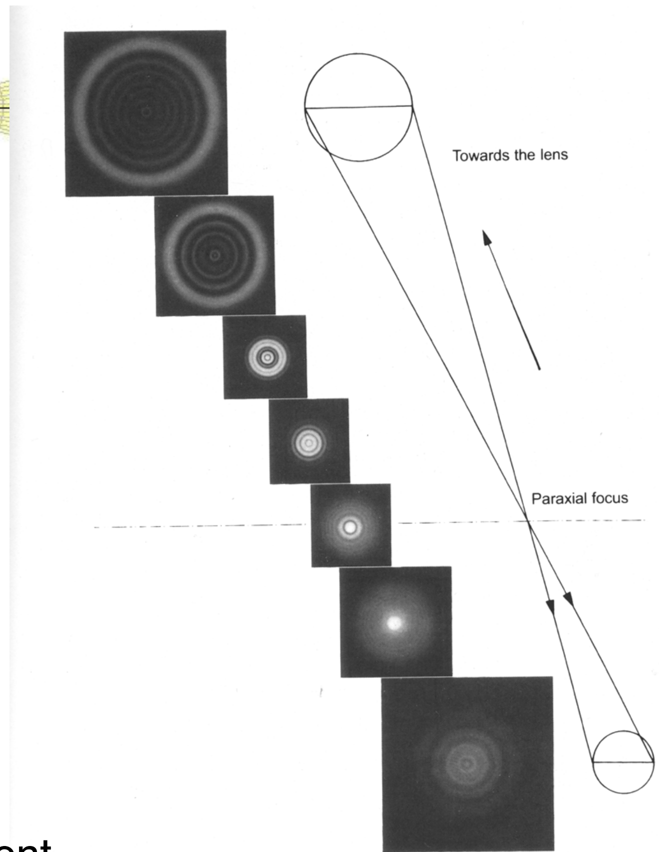


Widefield Fluorescence microscopy (Olympus + Sedat/Agard DeltaVision)

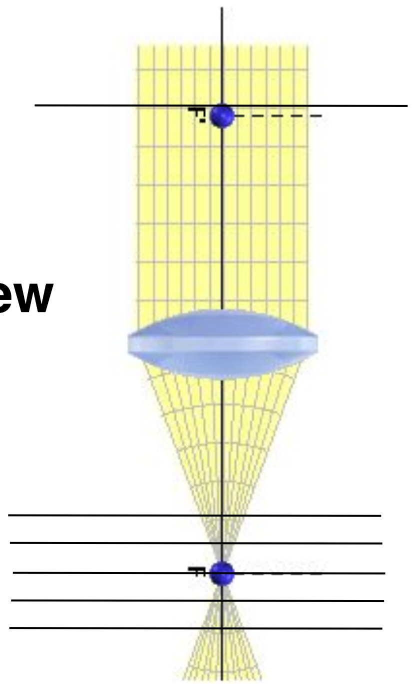
Widefield deconvolution the most sensitive modality - but sometimes cannot be used

Image formation



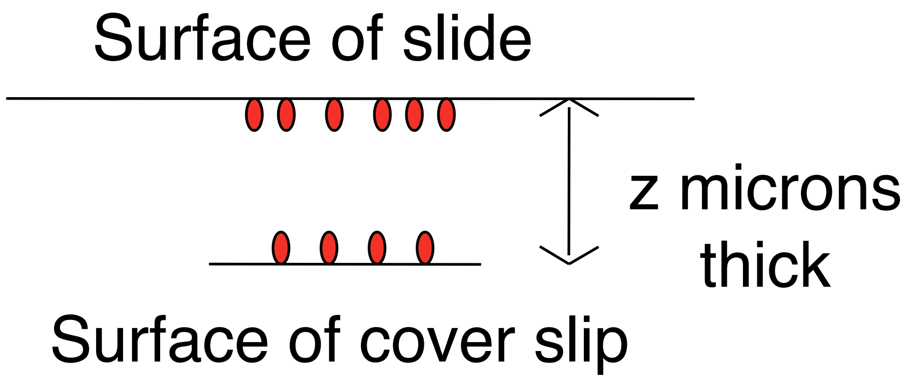


Out of focus light
-Airy rings in 3D view



Different
Focal
plains

Bead slide

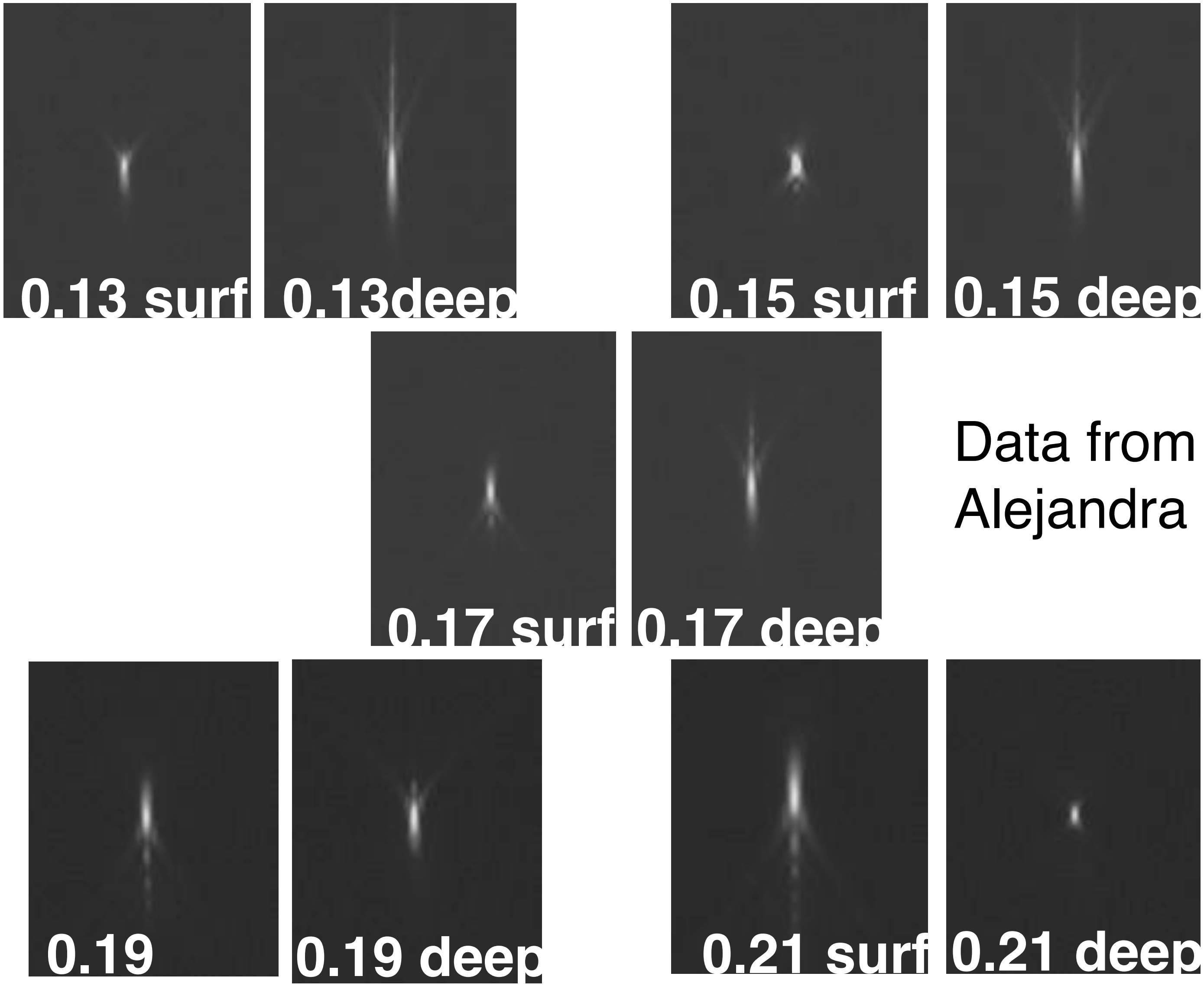


Tetraspeck beads: chromatic registration
DAPI/FITC/Rhodamine/Cy5

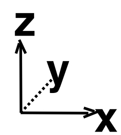
Beads (PS Spec): Single fluorochrome
Brighter -better for generating
point spread functions for deconvolution

Inspec Intensity beads: Measure dynamic range

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



Data from
Alejandra Clark



Special objectives from Olympus

- Water immersion x60psf NA1.2
- Silicon immersion objective x60SI NA1.3
- Multiphoton lens. Long working distance, highly corrected in IR light

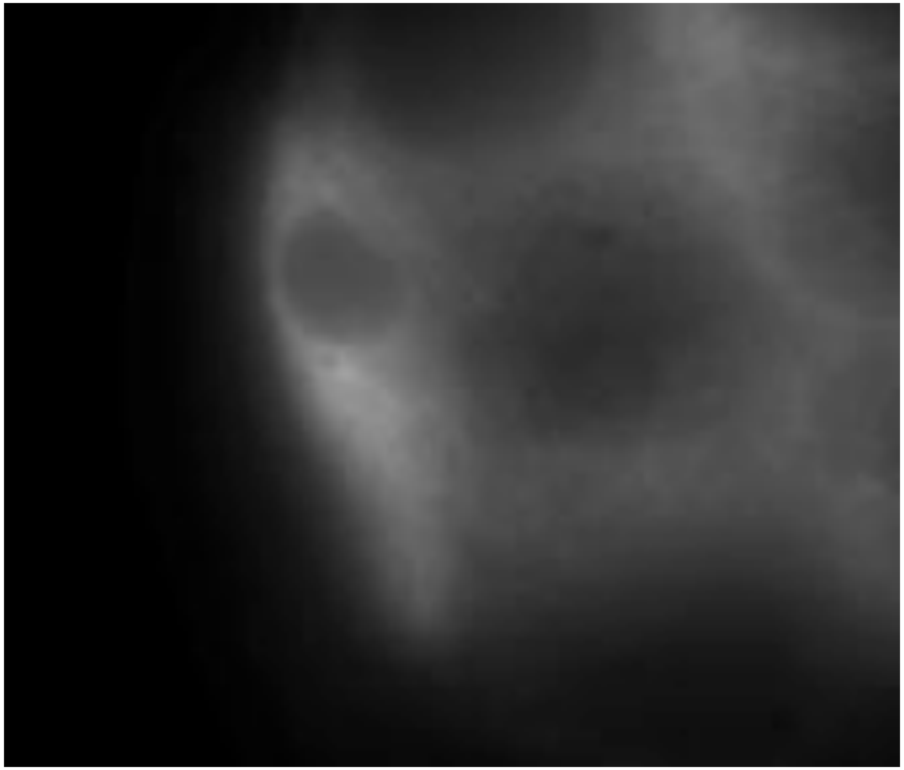
XLPN25XSVMP

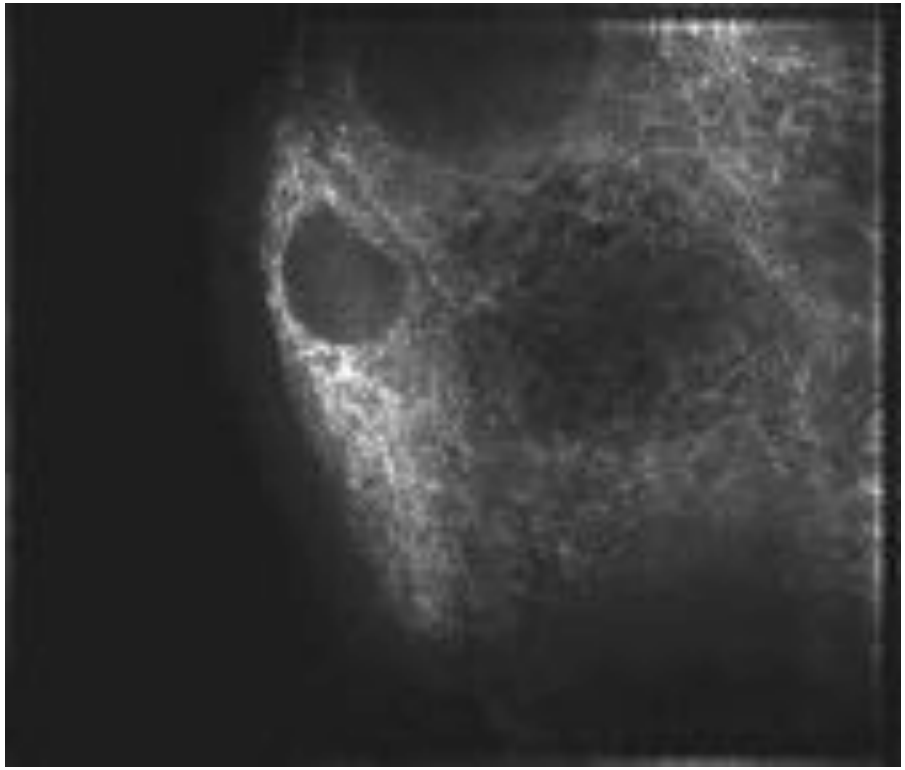
http://www.olympusamerica.com/files/seg_bio/olympus_specialty_objectives.pdf

How does Widefield Deconvolution Work?

(restoring out of focus light to its point of origin)

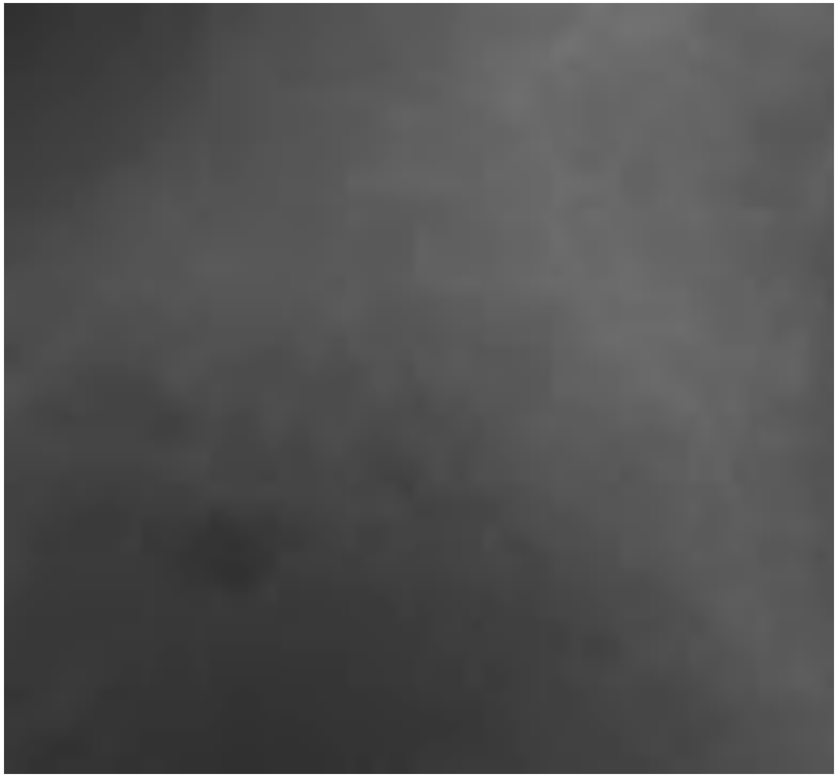
Before Deconvolution **After Deconvolution**

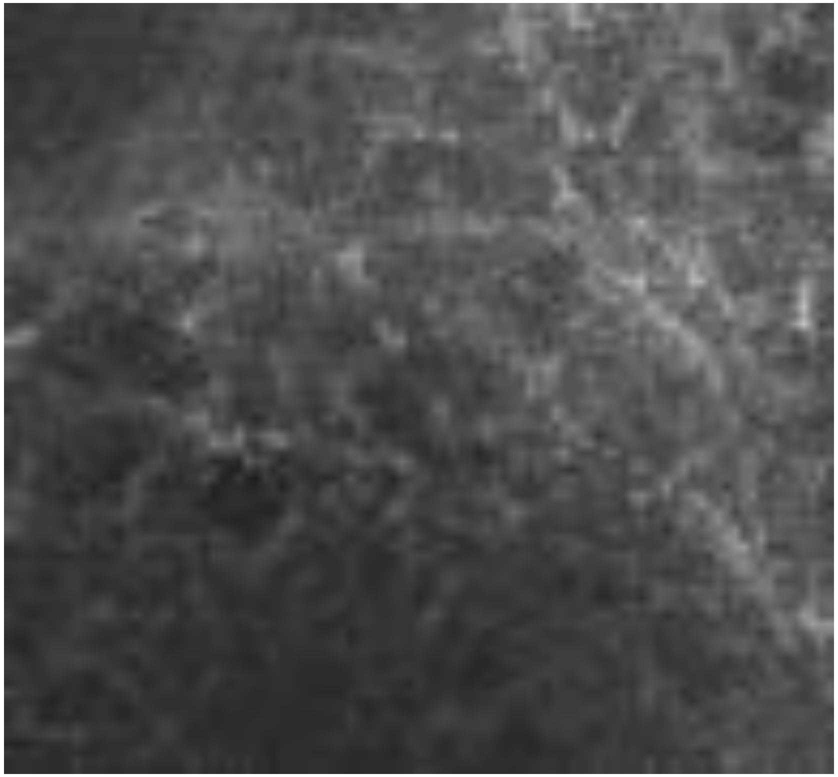


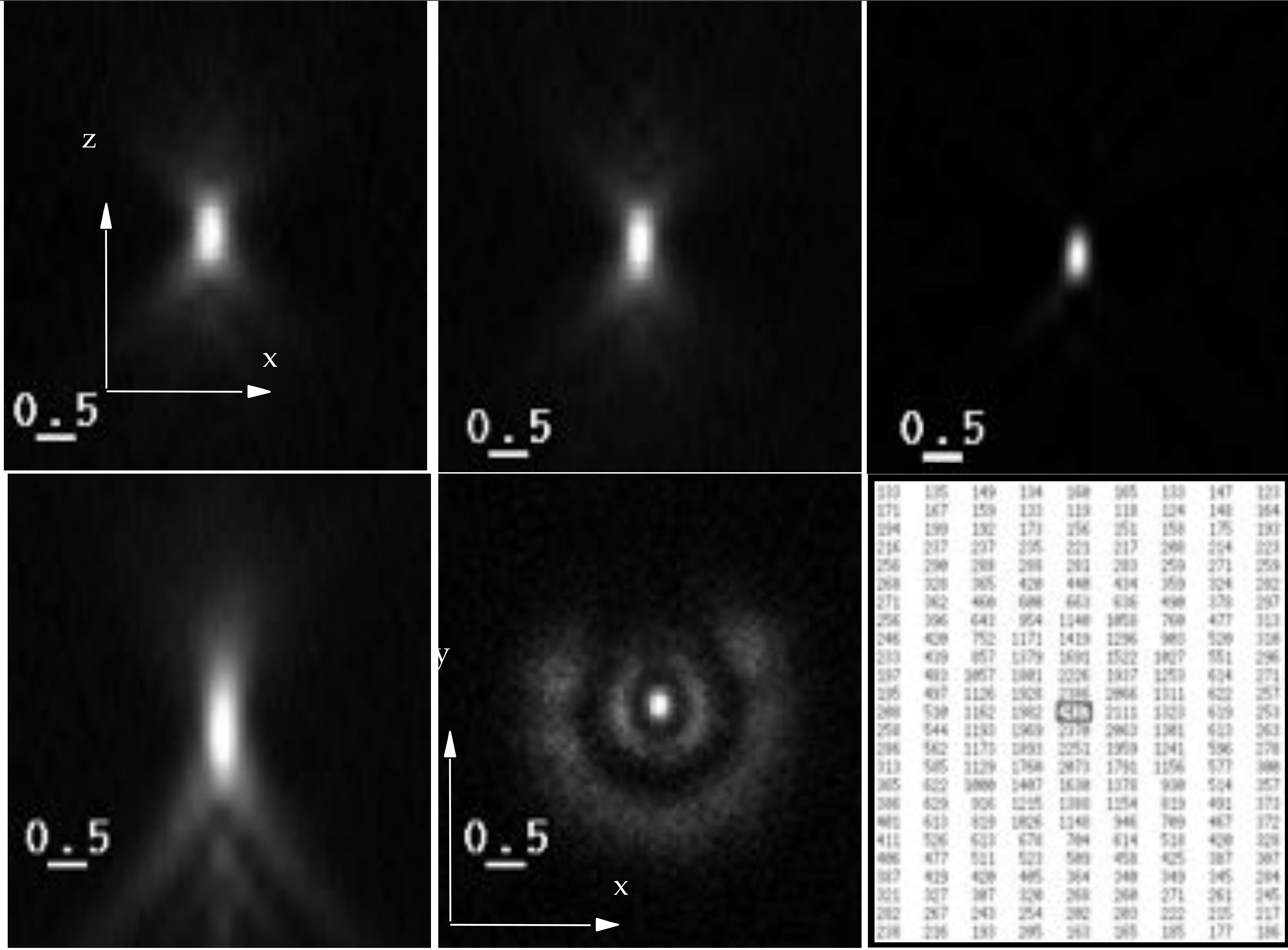


tauGFP (microtubules) in a *Drosophila* oocyte **Richard Parton**

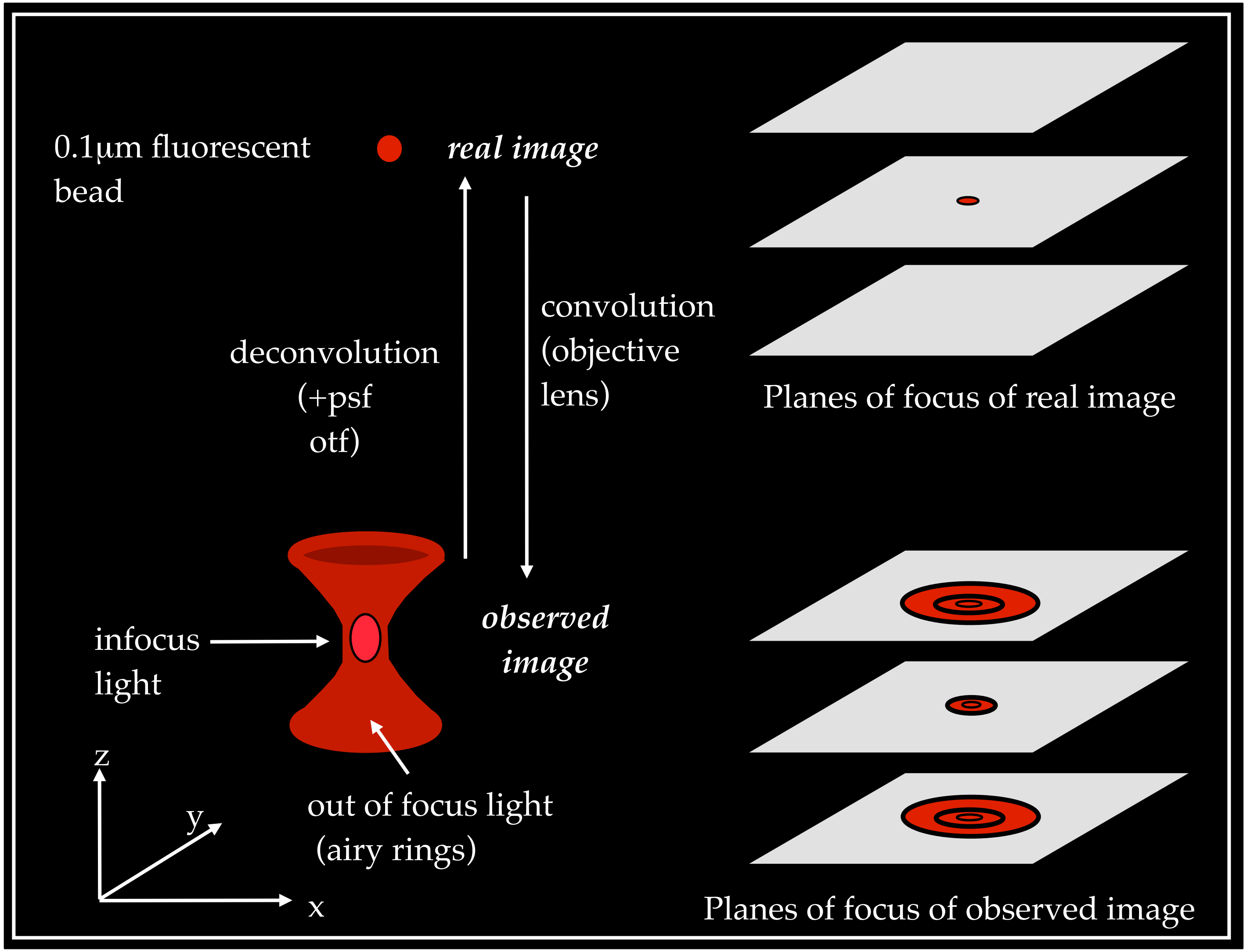
Before Deconvolution **After Deconvolution**







Ilan Davis, 2000



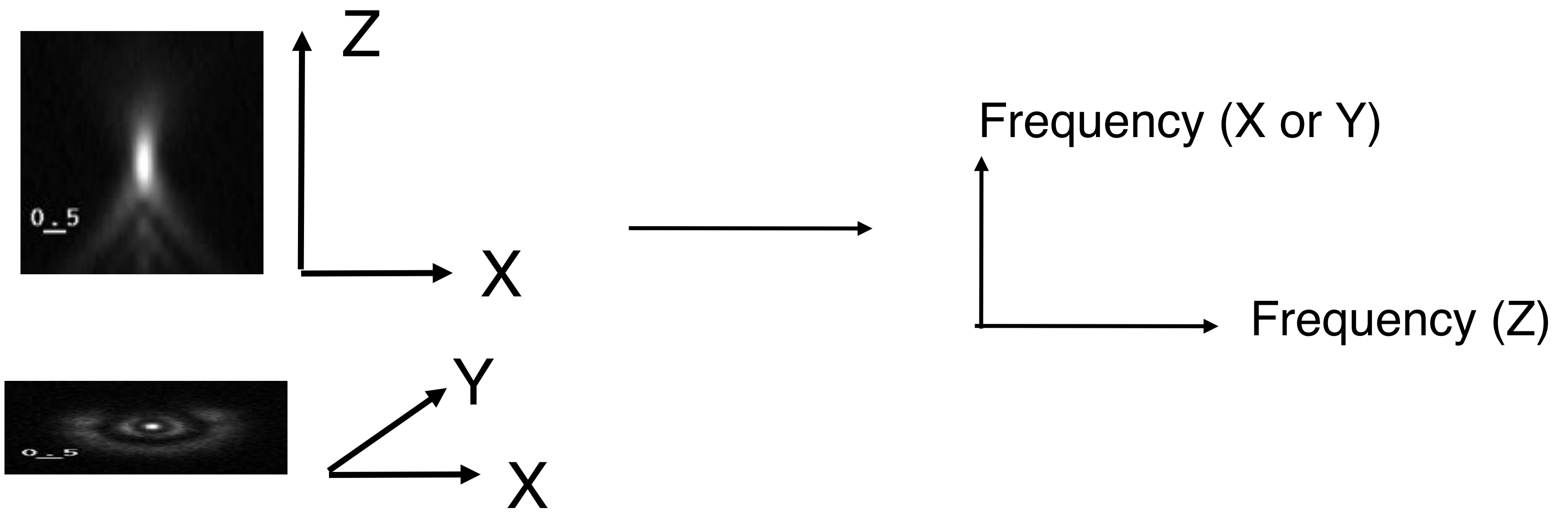
Ilan Davis, 2000

Deconvolution

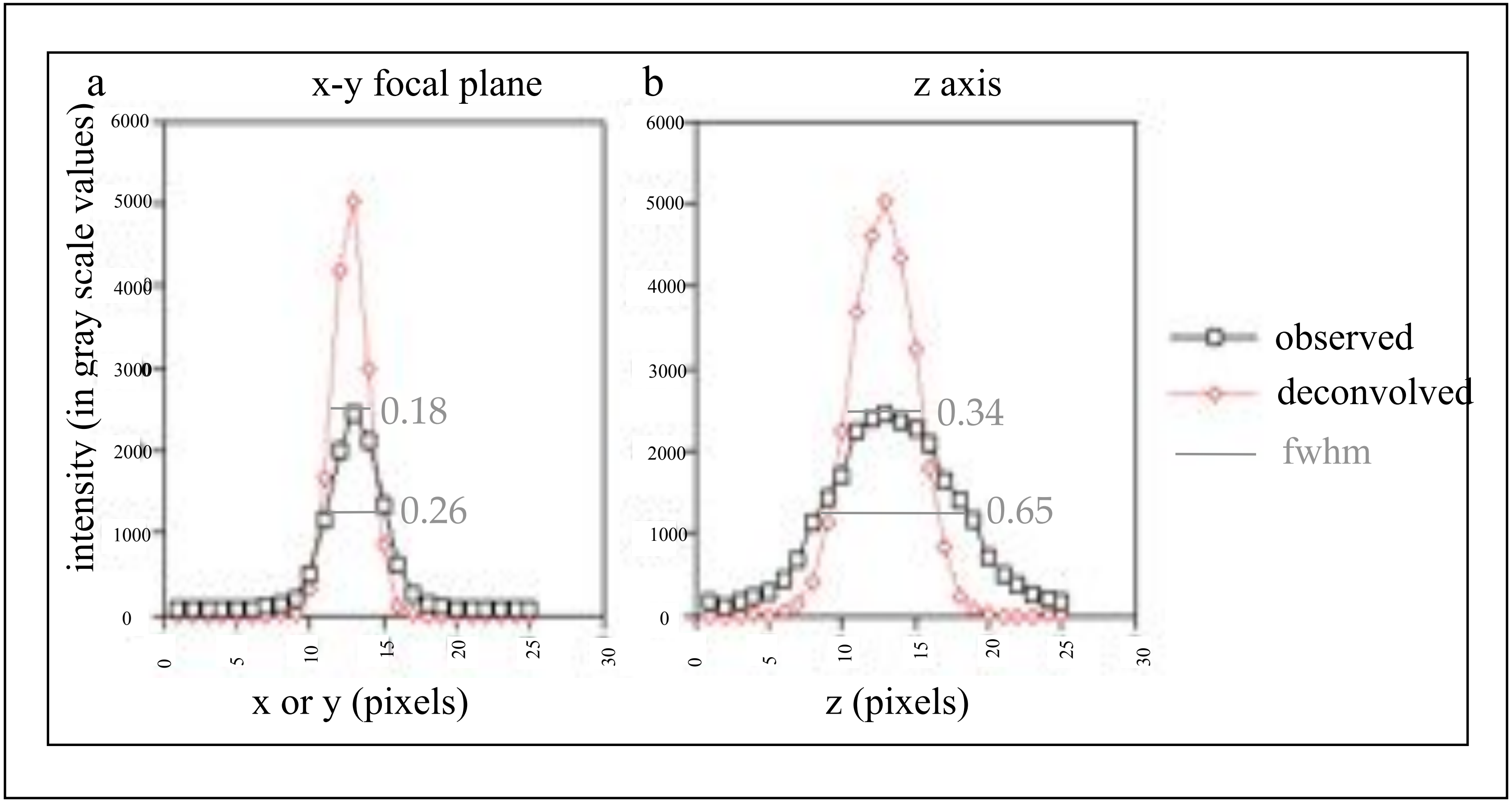
Calculations done in Fourier (frequency) space not XYZ space.
Uses Fast Fourier Transforms - much faster algorithm (developed in the 1960s)
Psf is converted to optical transfer function (only information in X and Z)
Several methods that vary in their implementation

Point Spread Function
PSF (XYZ space)

Optical Transfer Function
OTF (XZ frequency space)



Increase in resolution (XY and Z) after deconvolution



Types of Deconvolution

•No neighbour, nearest neighbour - poor substitute

•2D deconvolution - Not as good

•3D constrained iterative approaches

•Sedat/Agard ; Hoygens ; blind deconvolution

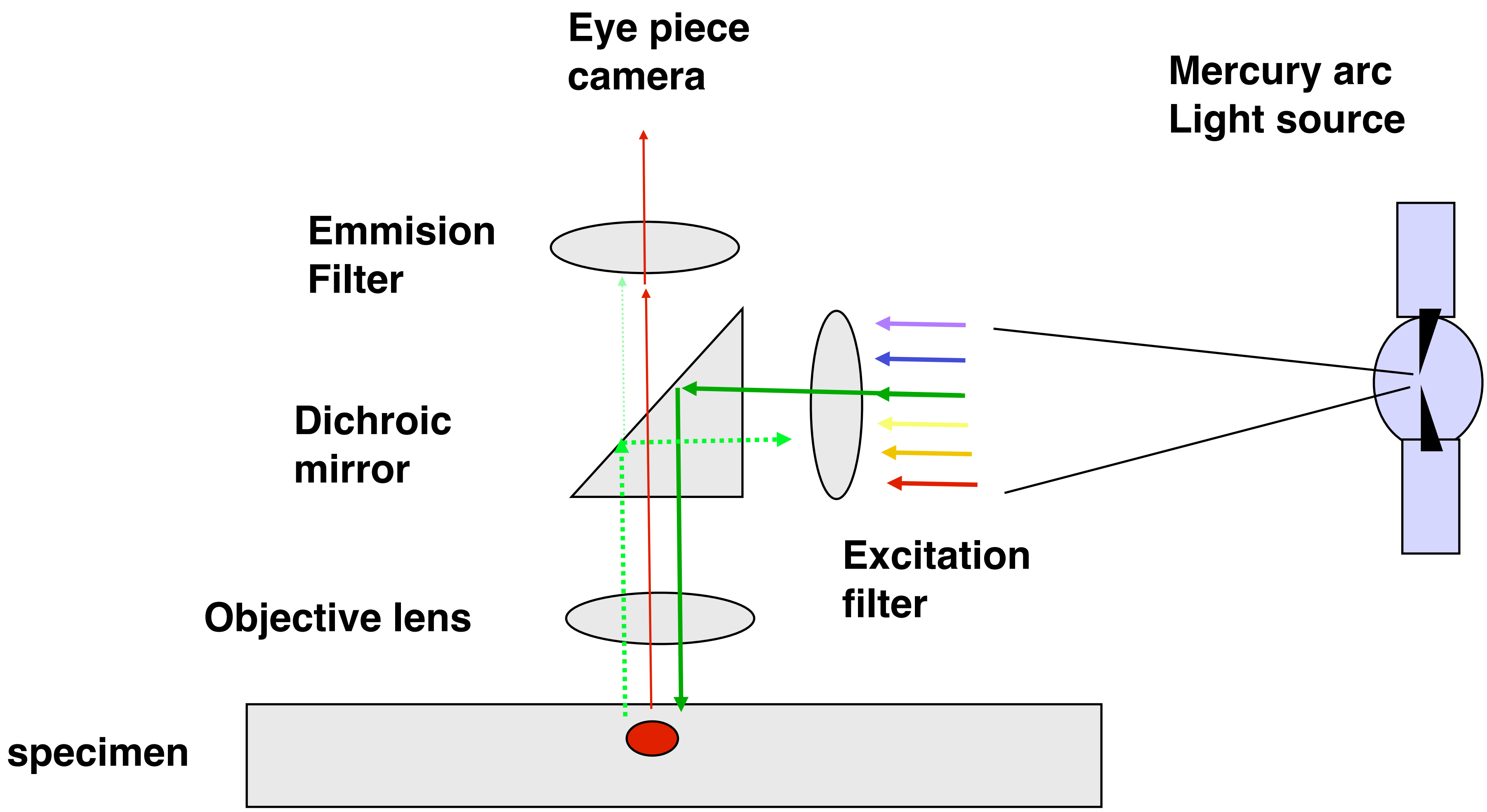
New methods (Sedat)

•Pupal functions (used to sharpen Hubble telescope) include information in otf in X, Y and Z and phase. Phase retrieval

•Myopic deconvolution

Reminder

How do fluorescence microscopes work ?



Filter cubes

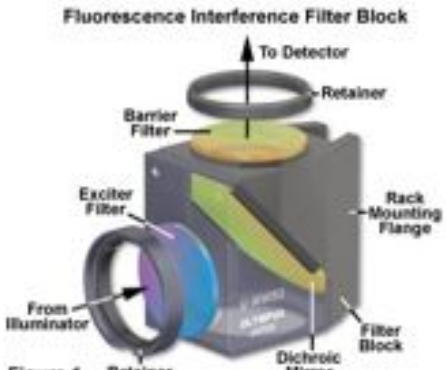
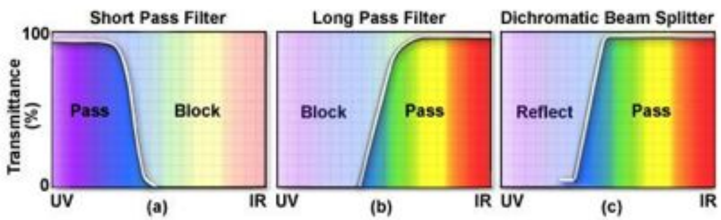


Figure 1



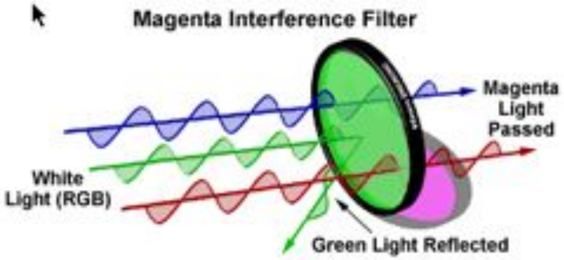
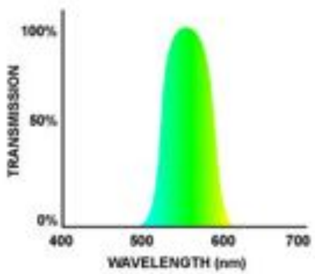
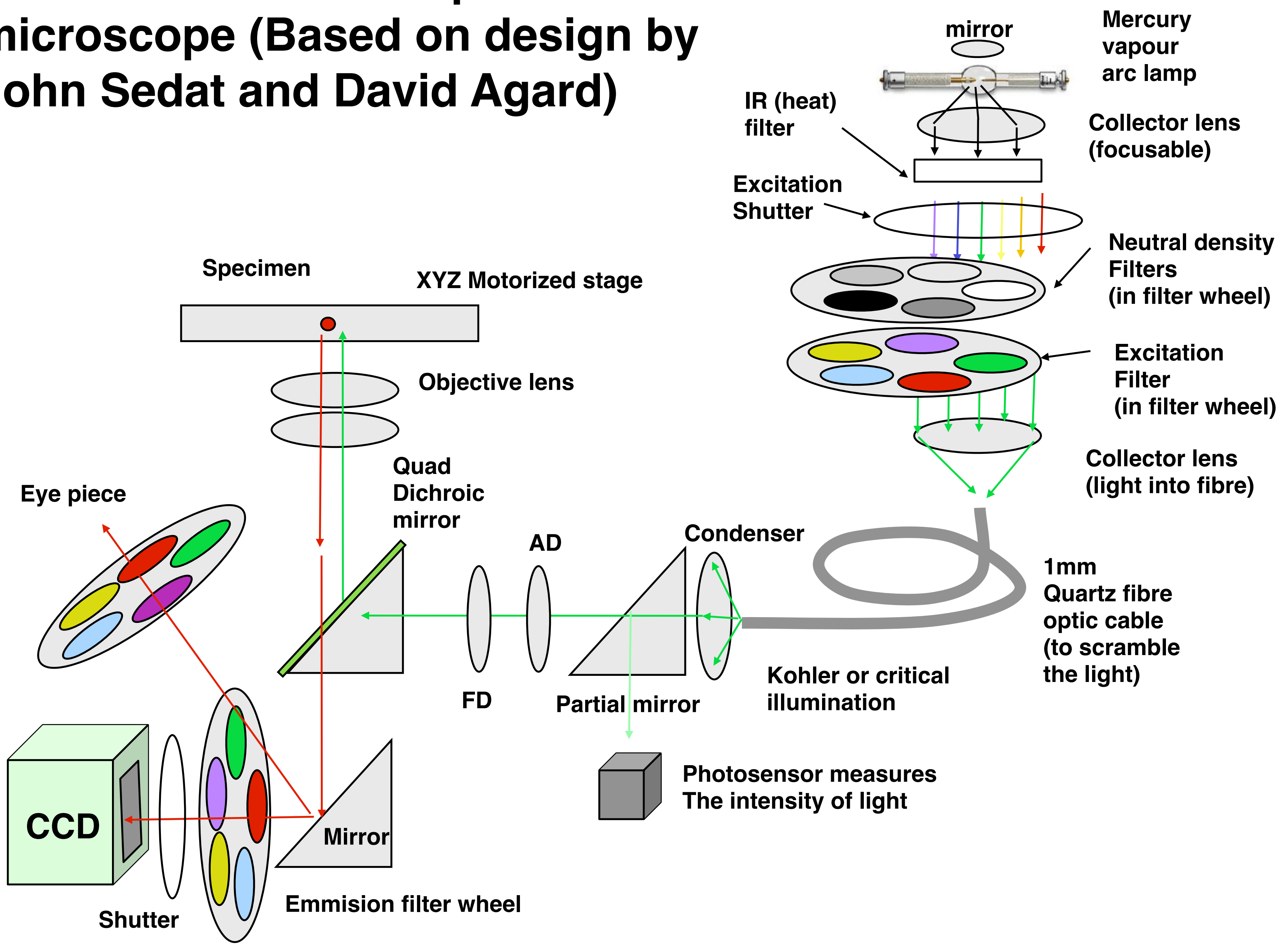


Figure 3

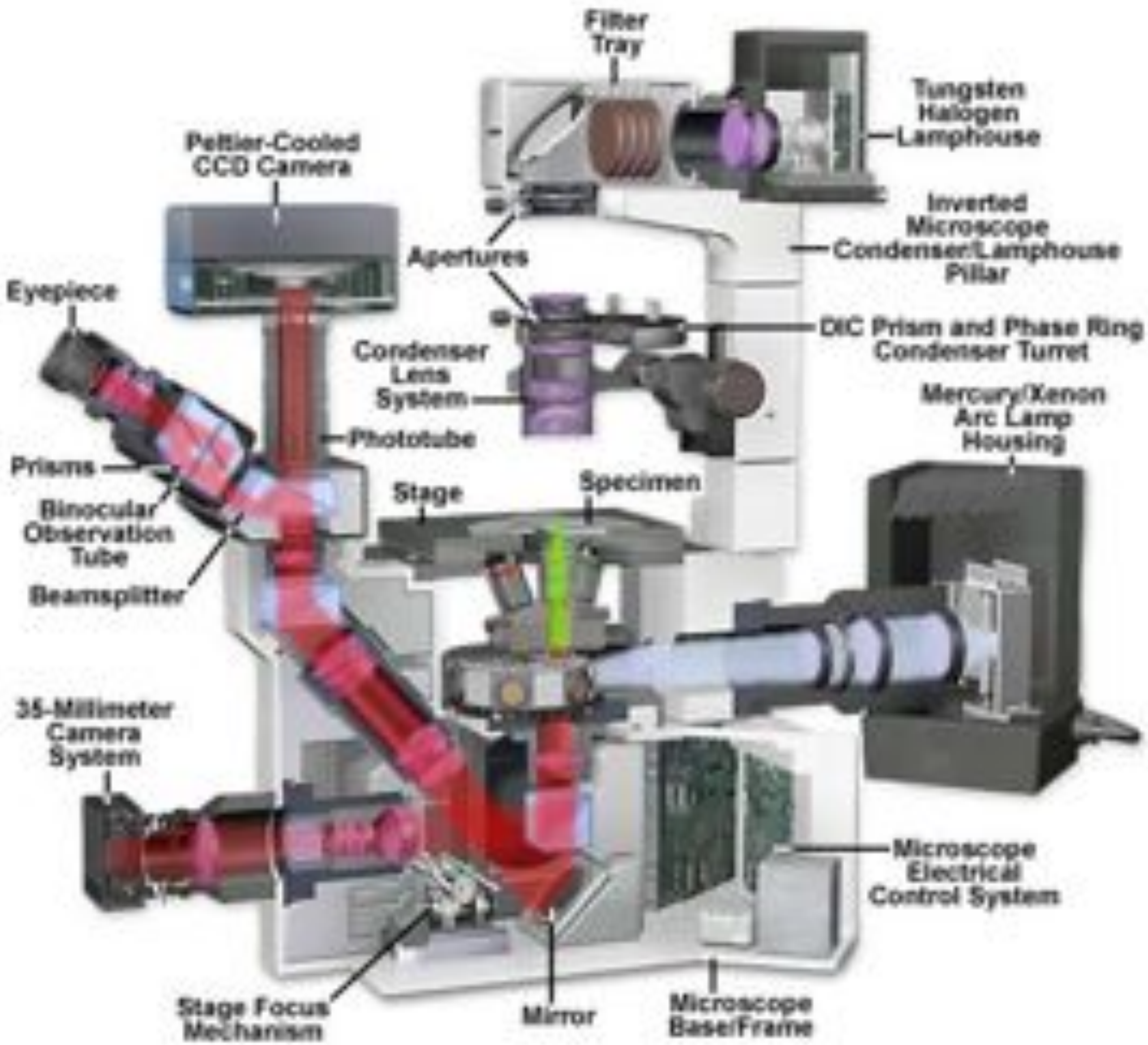


http://www.chroma.com/pdf/handbook4.pdf

Elements that make up the widefield fluorescence microscope (Based on design by John Sedat and David Agard)



Problem: the design of all conventional microscope stands



How can we improve the basic design of widefield microscopes?

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

The solution -build your own bespoke microscope

Mark Leake's Slimfield TIRF microscope (Biophysics prize)

Bespoke Microscopes

Why bother?

Bespoke Microscopes

Why bother?

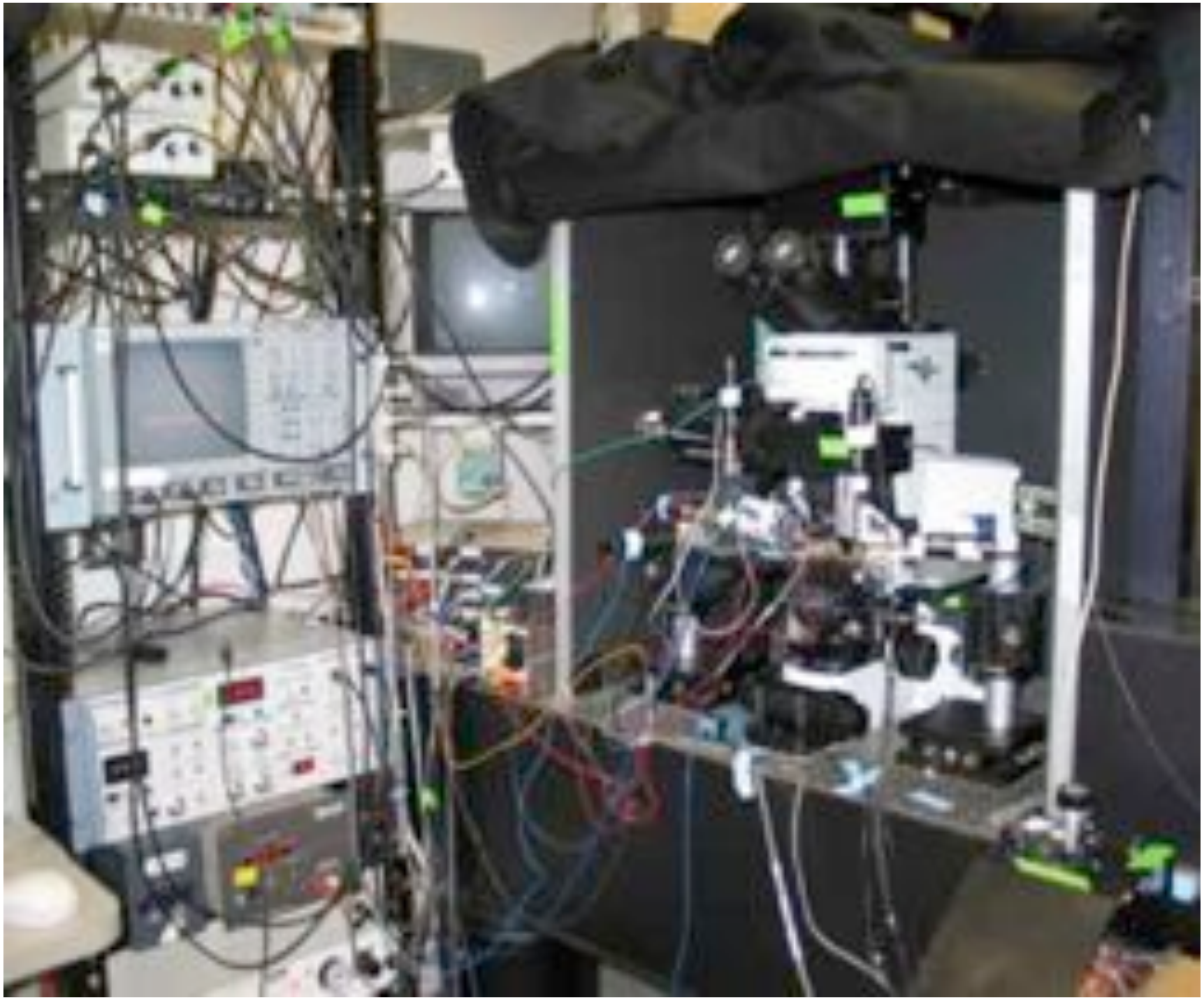
Specific applications -better than commercial microscopes

Flexibility

Cost

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**

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

The basic ingredients

The basic ingredients

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Objective lens and holder

vibration isolation table

The basic ingredients

Objective lens and holder

Stage

vibration isolation table

The basic ingredients

Objective lens and holder

Stage

Cameras

vibration isolation table

The basic ingredients

Electronics

Objective lens and holder

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Cameras

vibration isolation table

The basic ingredients

Objective lens and holder

Stage

Cameras

vibration isolation table

Electronics

Timing board TTL outputs

The basic ingredients

Objective lens and holder

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Cameras

vibration isolation table

Electronics

Timing board TTL outputs

Computer (normally a PC)

The basic ingredients

Objective lens and holder

Stage

Cameras

vibration isolation table

Electronics

Timing board TTL outputs

Computer (normally a PC)

Optics

The basic ingredients

Objective lens and holder

Stage

Cameras

vibration isolation table

Electronics

Timing board TTL outputs

Computer (normally a PC)

Optics

Posts, flip mirrors, dichroics lenses, filter wheels, shutters, fibres, AOTF

The basic ingredients

Objective lens and holder

Stage

Cameras

vibration isolation table

Electronics

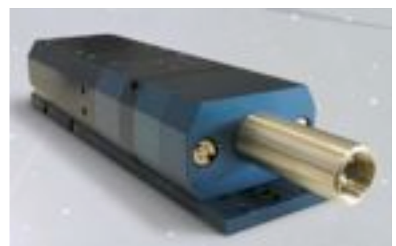
Timing board TTL outputs

Computer (normally a PC)

Optics

Posts, flip mirrors, dichroics lenses, filter wheels, shutters, fibres, AOTF

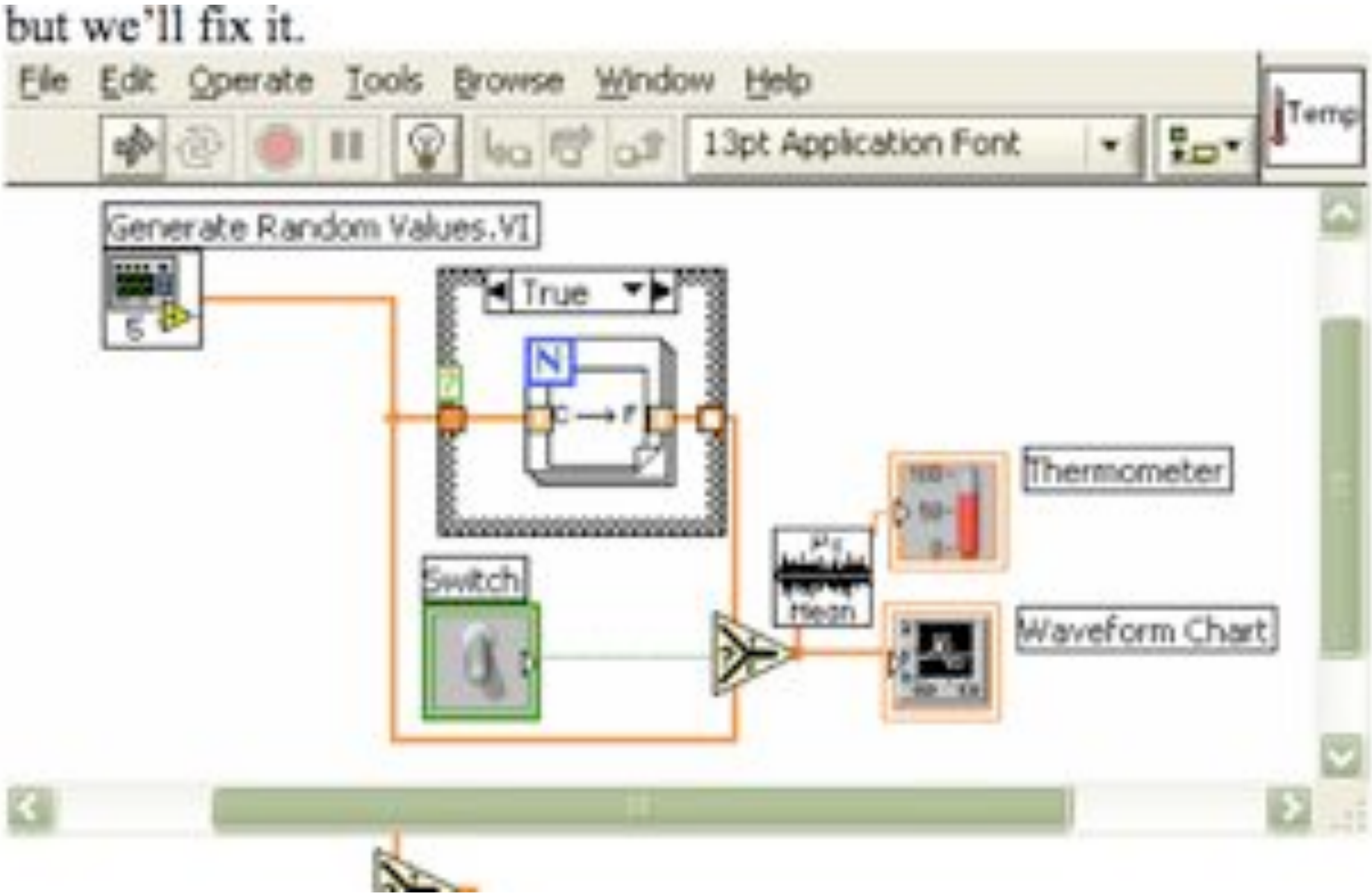
Lasers



Software options

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

Lab view example



Micromanager

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

Some rules of thumb

- Clean and dust free environment
- Oscilloscope and soldering iron
- 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

Justification for Bespoke Systems

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

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

How expensive is it?

Building costs

Hardware ~£100-250k

Salaries 1-3 years (~£50-£150)

Total cost ~£150-350k

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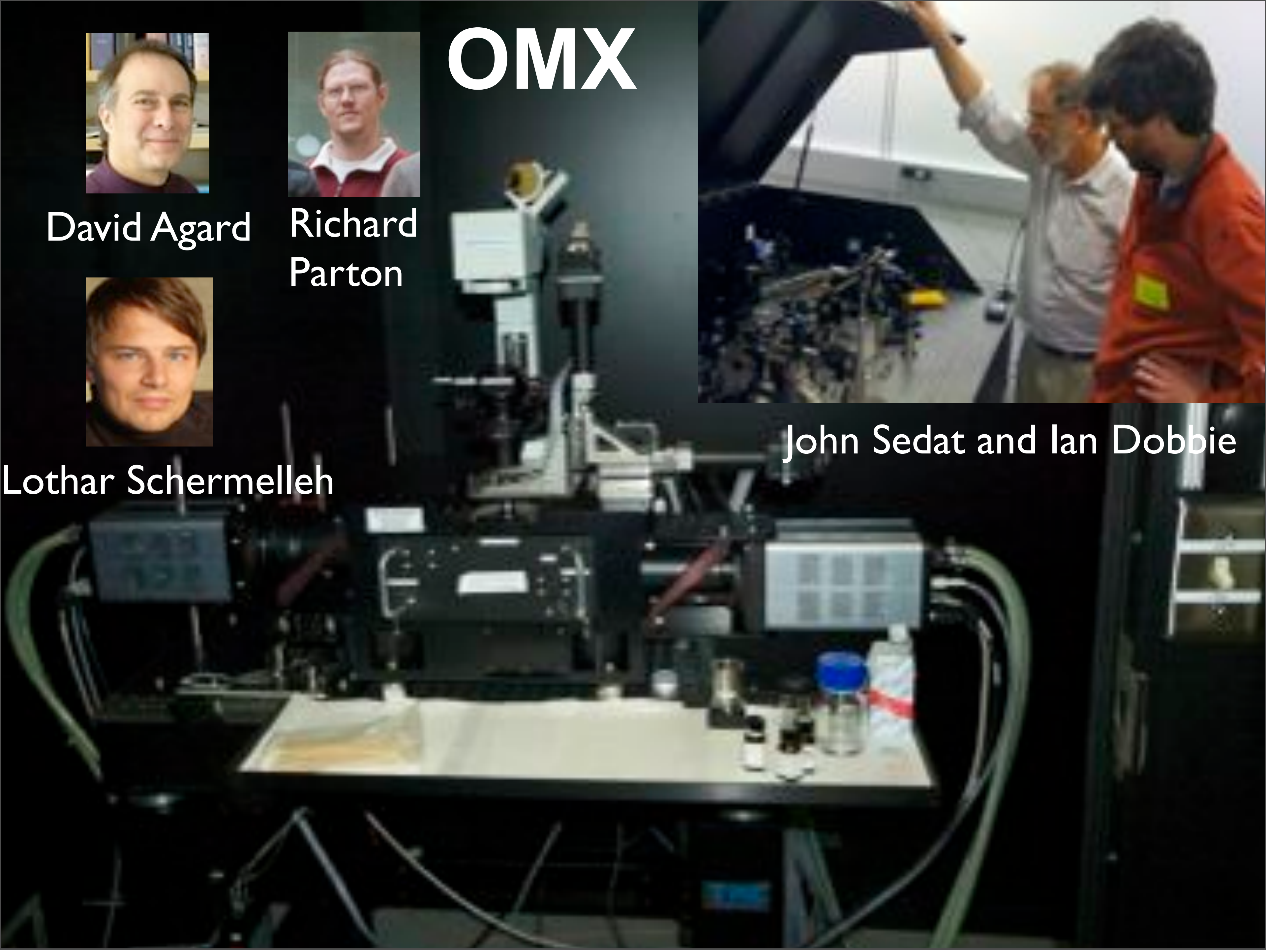
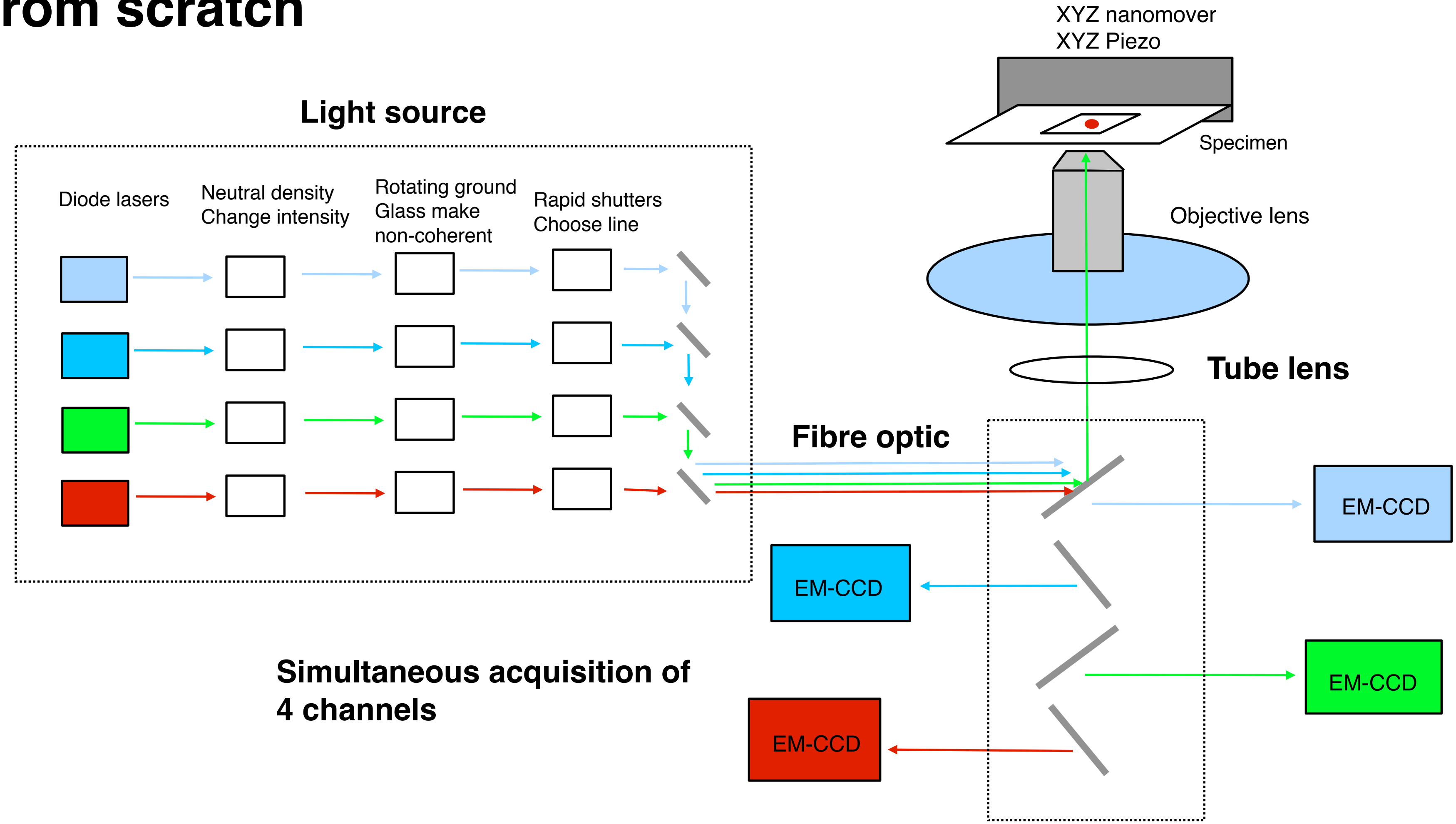
Commercial OMX system ~£750k


OMX
David Agard
Richard Parton
Lothar Schermelleh
John Sedat and Ian Dobbie

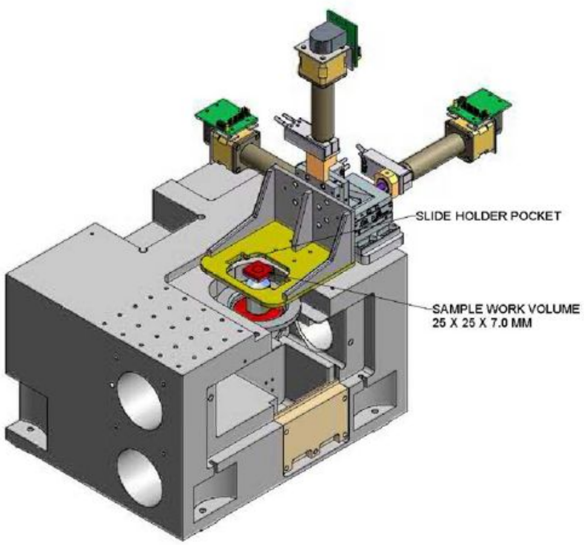
OMX - Redesigning widefield microscopy from scratch



Simultaneous acquisition of 4 channels

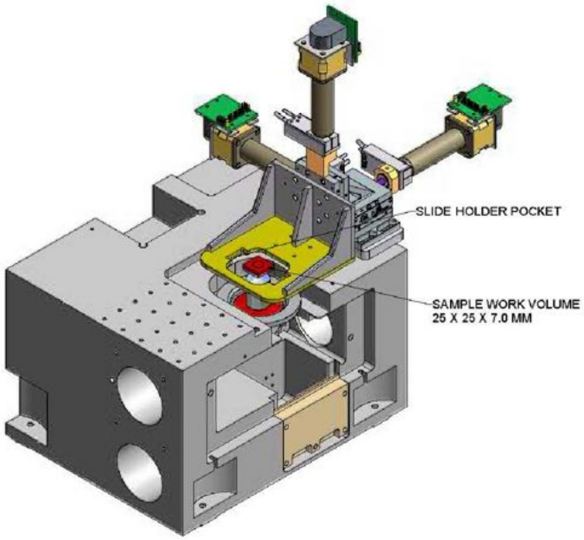
**Precisely machined
Metal block with internal sculpturing
That absorbs stray light.**

OMX (John Sedat, David Agard and Mats Gustafsson)



Precisely machined
Metal block with internal sculpturing
That absorbs stray light
Maximized emission light efficiency

OMX (John Sedat, David Agard and Mats Gustafsson)



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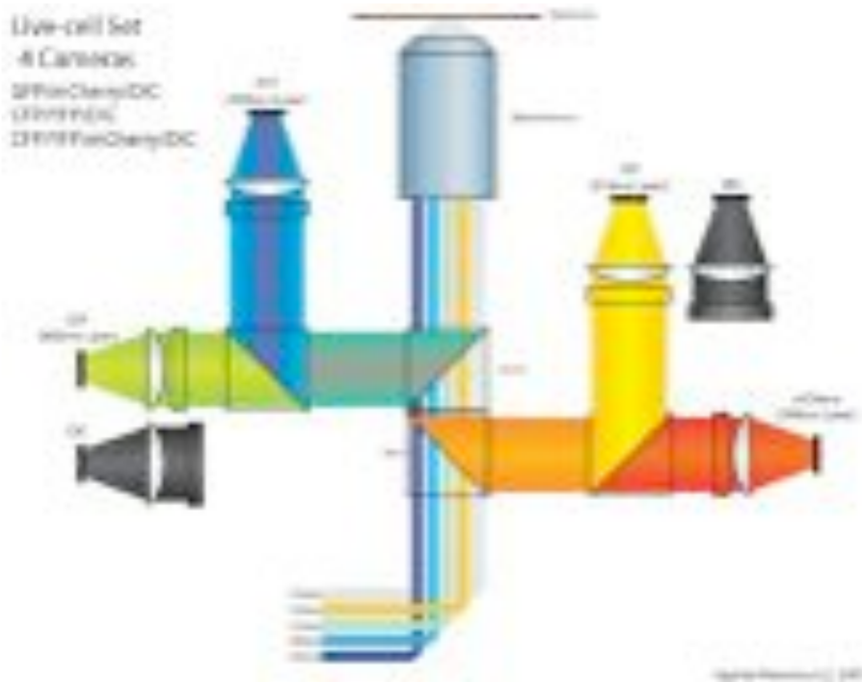
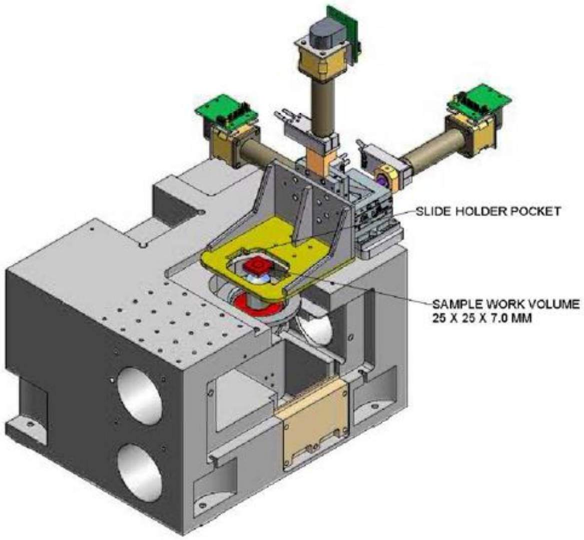


4 laser excitation lines
4 simultaneous acquisition lines CCDs

OMX (John Sedat, David Agard and Mats Gustafsson)



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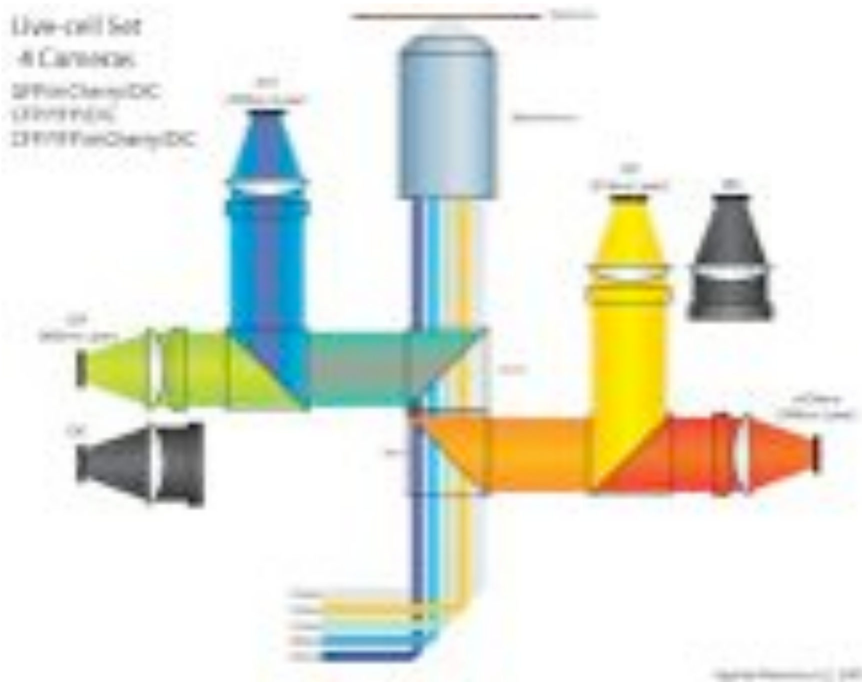
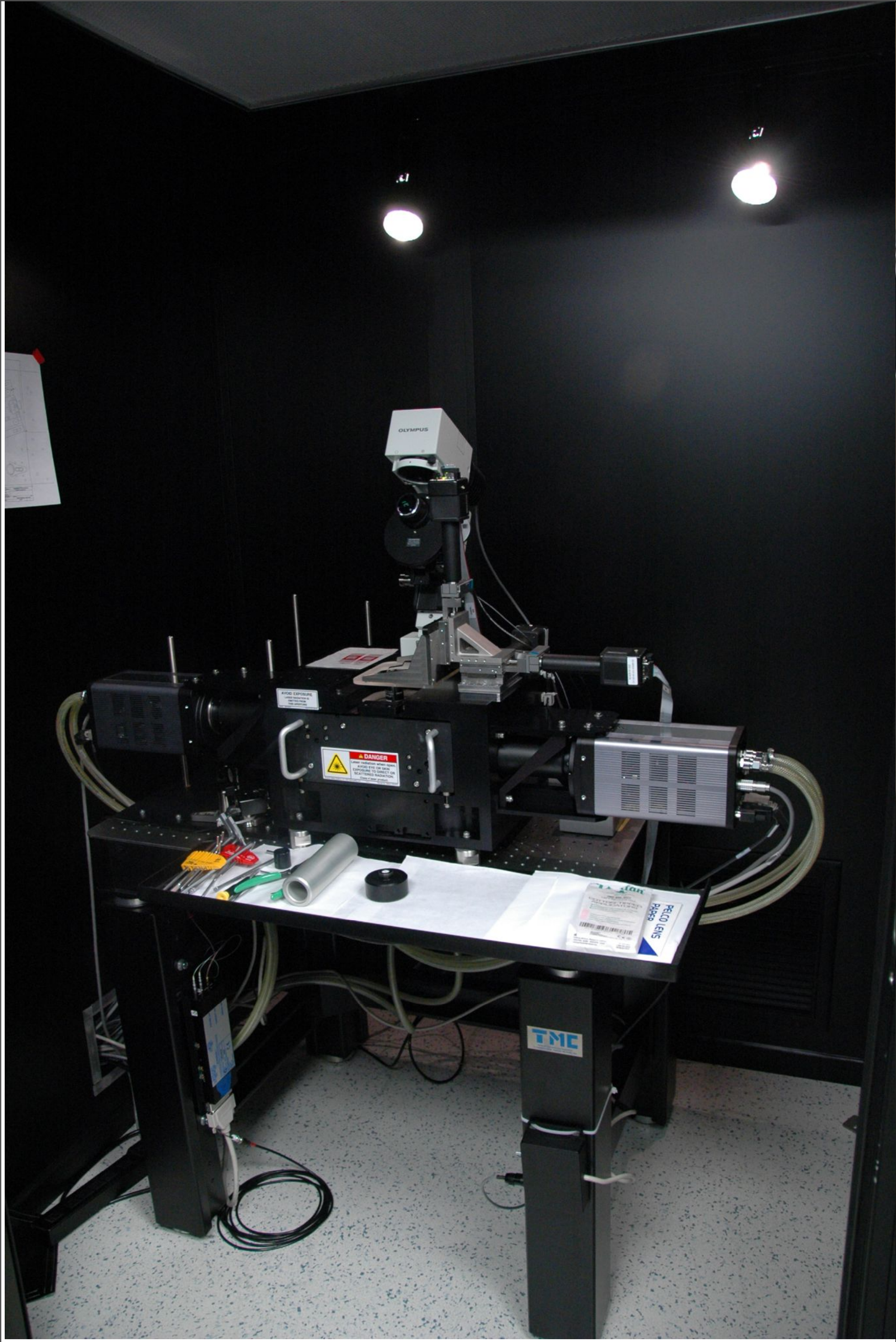


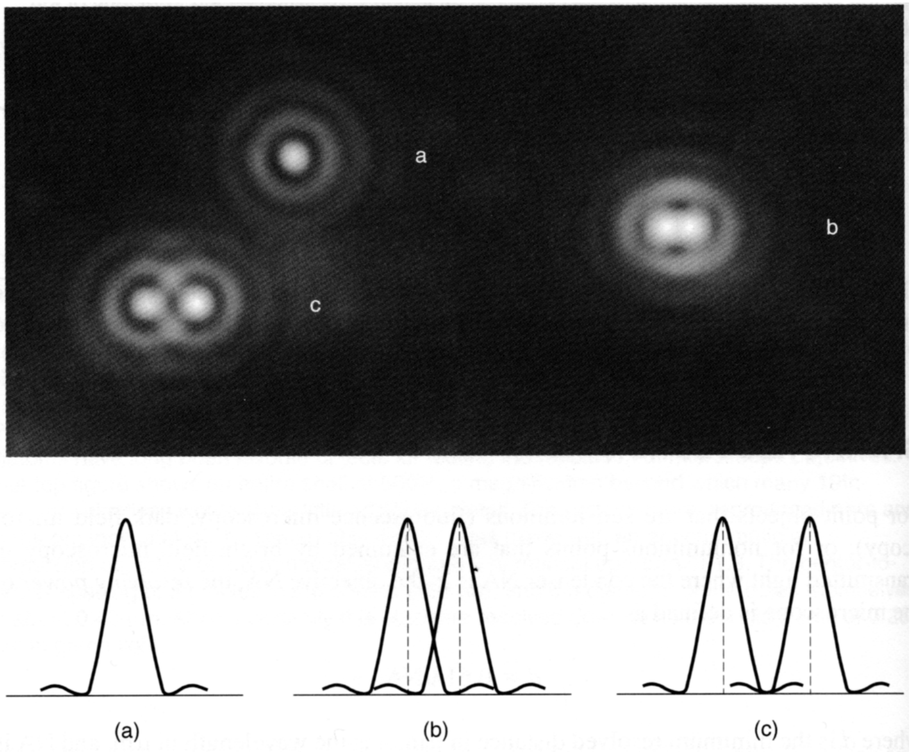
4 laser excitation lines
4 simultaneous acquisition lines CCDs

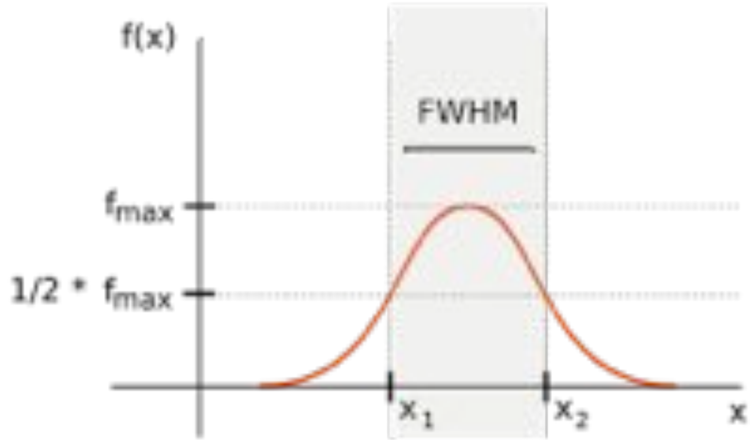
We have the second replica of the prototype instrument - 30 manufactured so far worlwide.


DANGER
TMC

SERIES B

Resolution limit -500nm light is approx 250nm in XY and 750nm in Z





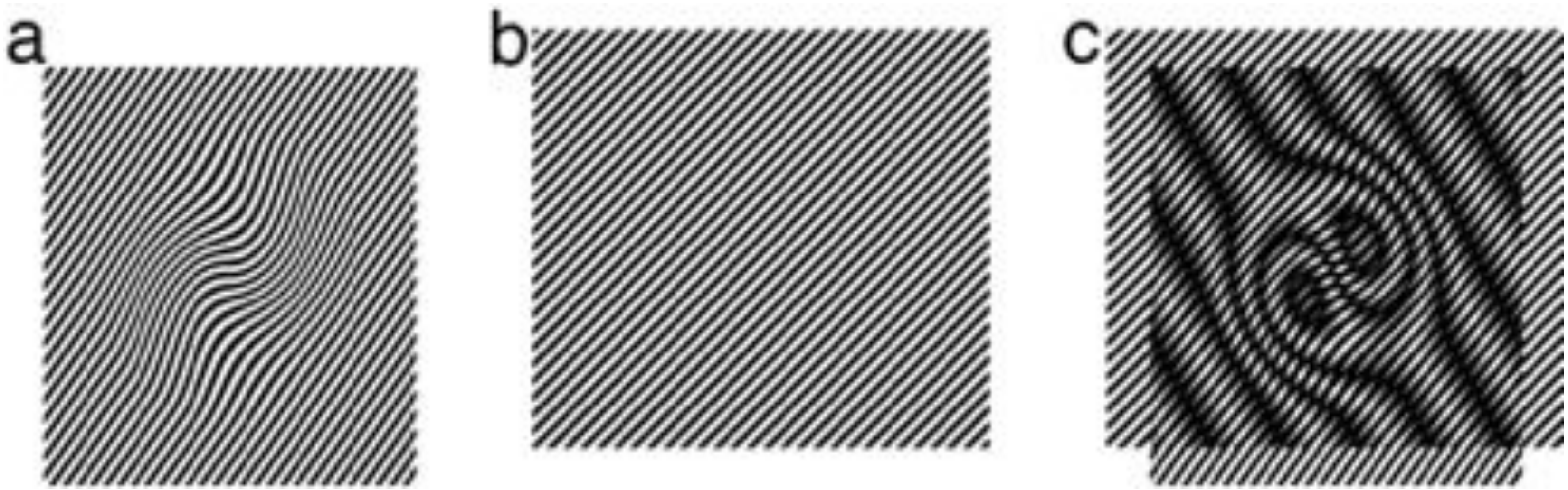
How can we overcome this limit long standing limit?

Structured Illumination

Surpassing the lateral resolution limit by a factor of two using structured illumination. Journal of microscopy Gustafsson, G.L., (2000) 198, 82.

http://www.blackwell-synergy.com/links/doi/10.1046/j.1365-2818.2000.00710.x

Resolution extension through Moire effect



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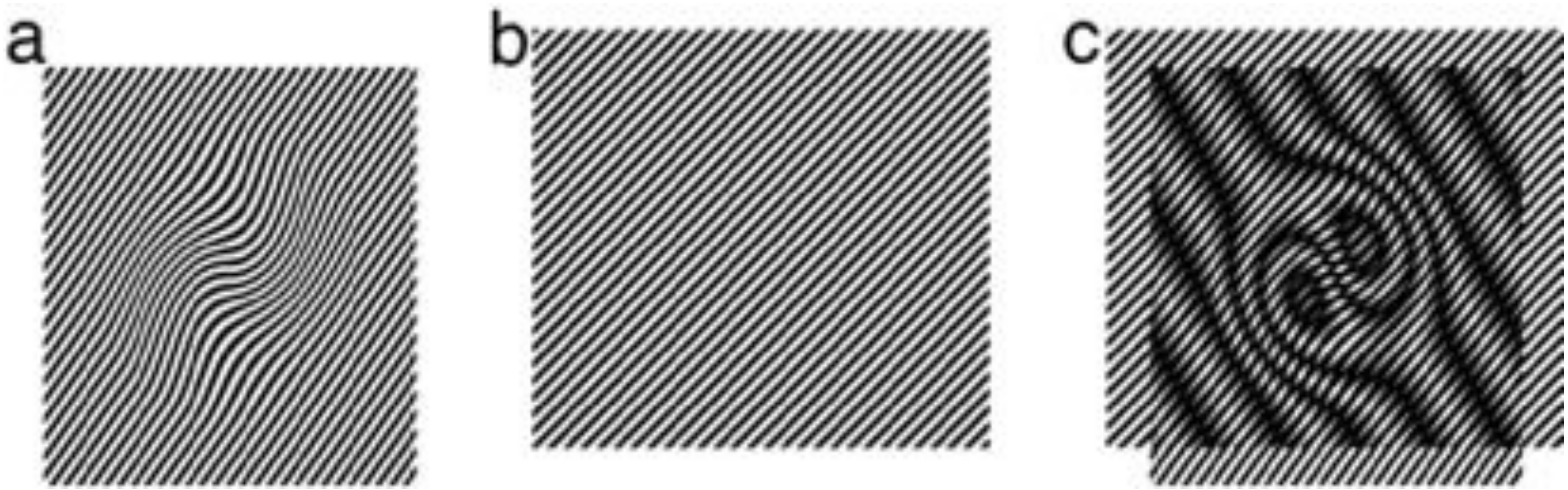


More tomorrow from Lothar

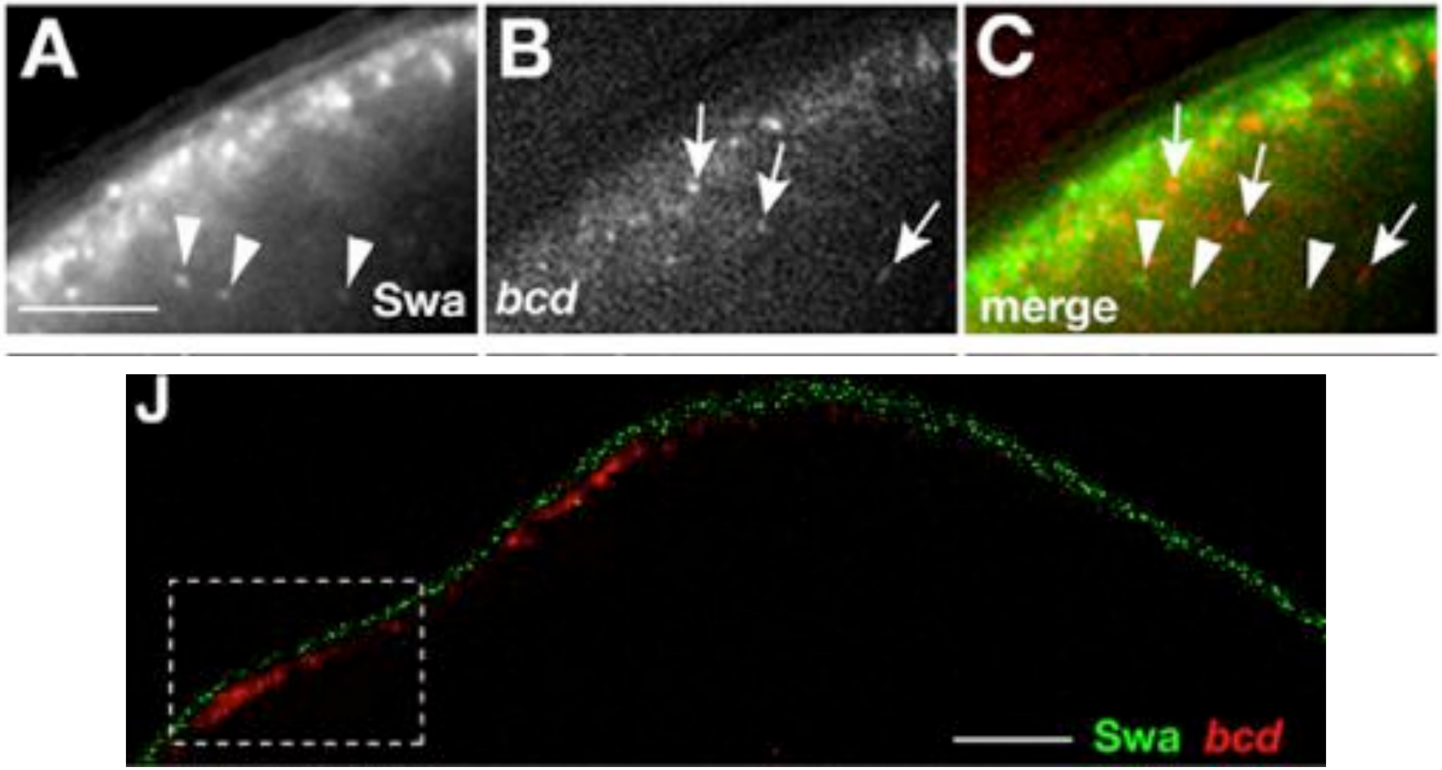
Live multidimensional imaging on OMX

RESEARCH ARTICLE 169

Development 137, 169-176 (2010) doi:10.1242/dev.044867

Distinguishing direct from indirect roles for *bicoid* mRNA localization factors

Timothy T. Weil[1,2,3], Despina Xanthakis[1], Richard Parton[3], Ian Dobbie[3], Catherine Rabouille[1], Elizabeth R. Gavis[2,*] and Ilan Davis[3]



LETTERS

nature cell biology

Drosophila patterning is established by differential association of mRNAs with P bodies

Timothy T. Weil, Richard M. Parton, Bram Herpers, Jan Soetaert, Tineke Veenendaal, Despina Xanthakis, Ian M. Dobbie, James M. Halstead, Rippei Hayashi, Catherine Rabouille and Ilan Davis

NATURE CELL BIOLOGY VOLUME 14 | NUMBER 12 | DECEMBER 2012

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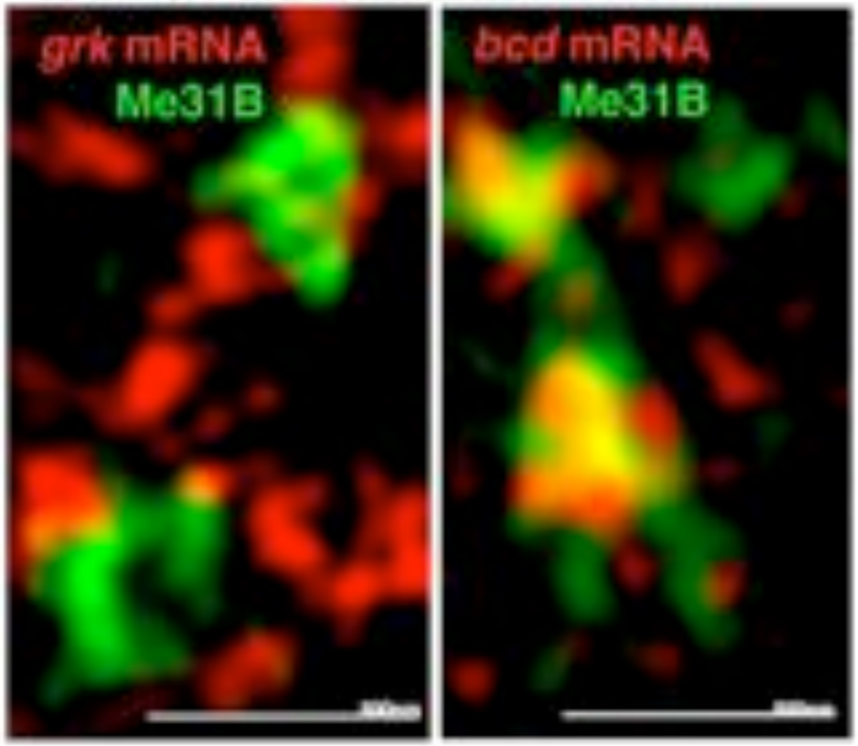
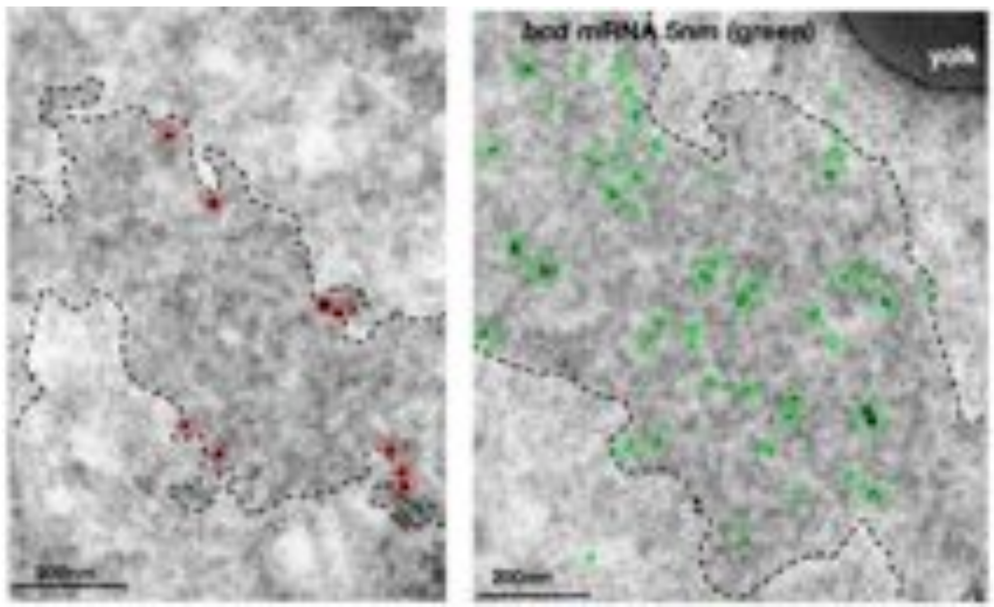
3D-SIM of P bodies assocated with RNA





Monday, 11 March 13

THE FUTURE: 3D SIM live on V3-blaze

3D Structured Illumination

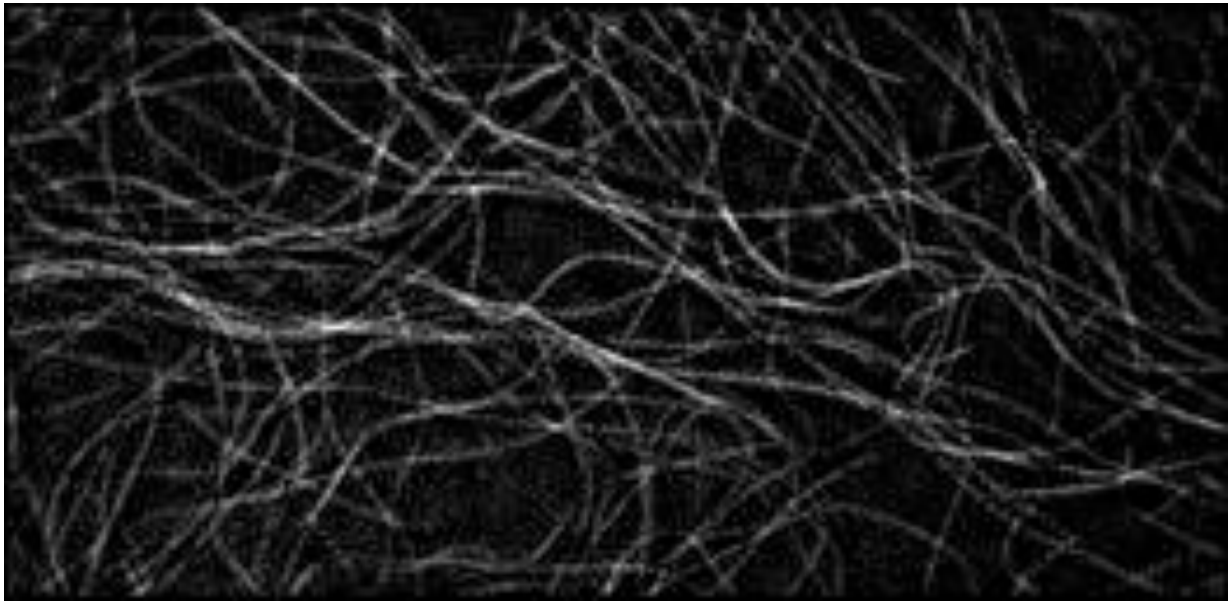
Conventional Widefield deconvolution

MT: Jupiter-GFP - captured at API.
(1fps, 30 time points)

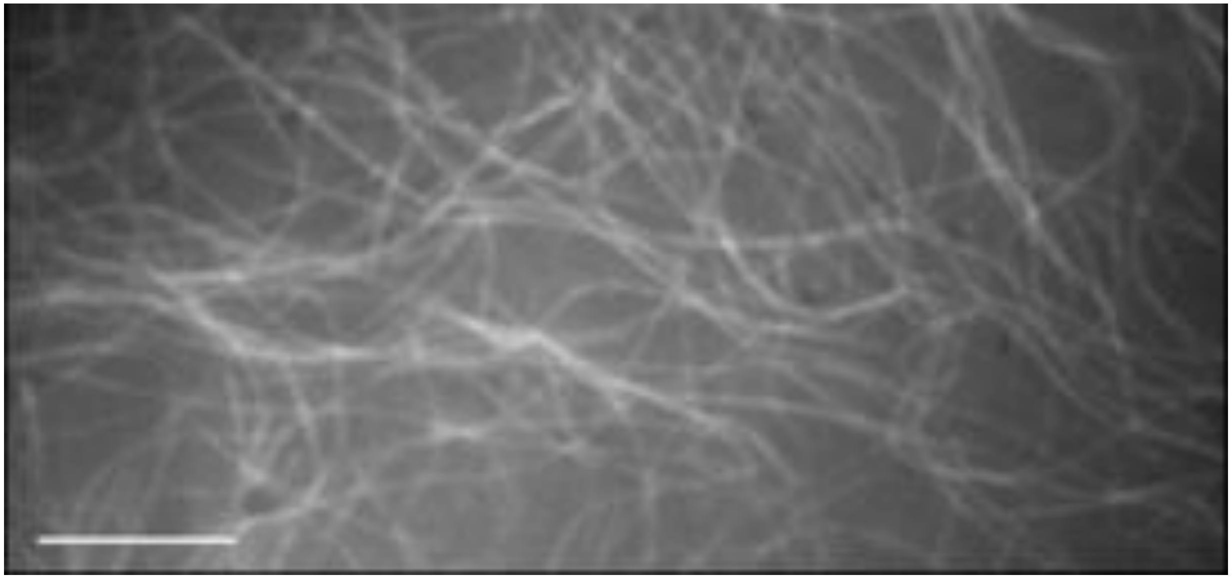
Parton, Goodwin, Atkins

THE FUTURE: 3D SIM live on V3-blaze

3D Structured Illumination



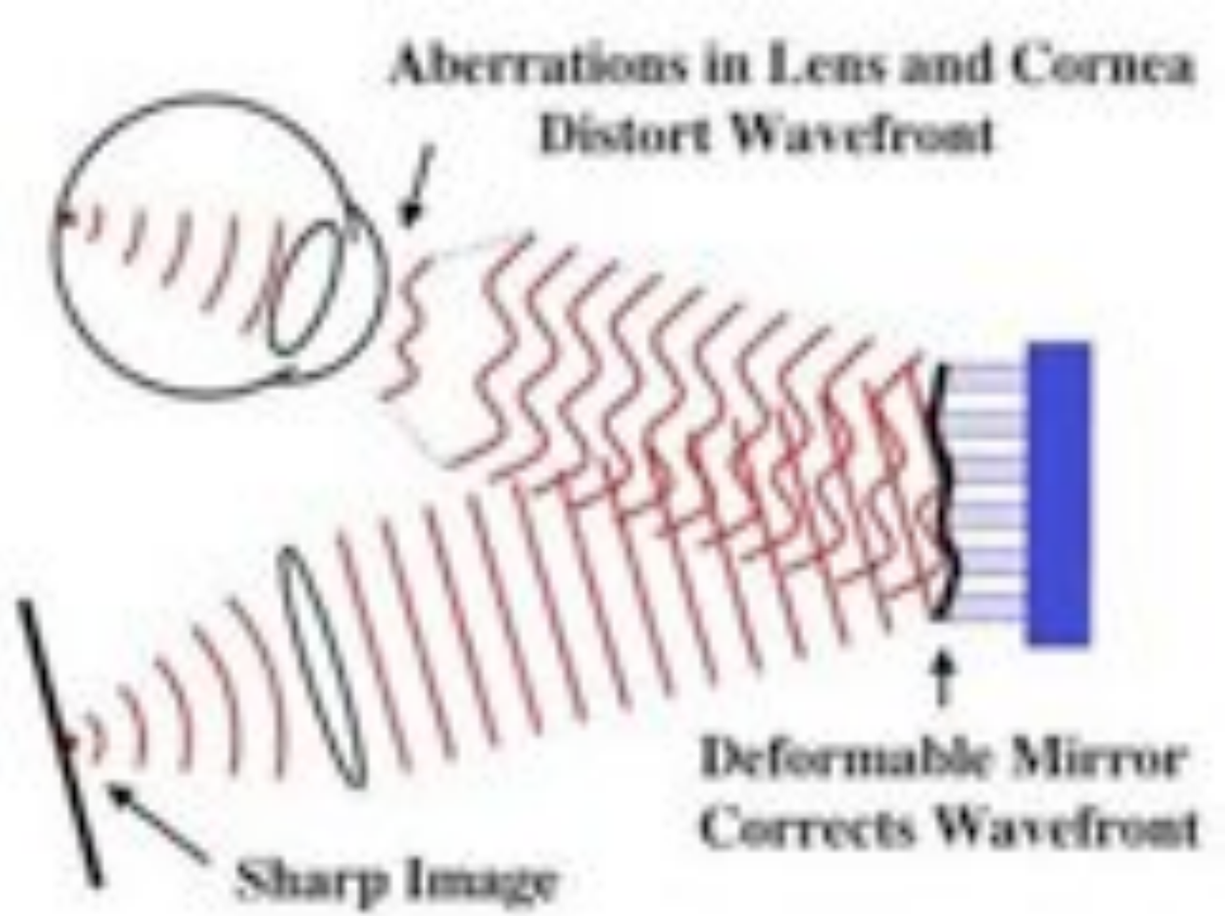
Conventional Widefield deconvolution



MT: Jupiter-GFP - captured at API.
(1fps, 30 time points)

Parton, Goodwin, Atkins

Adaptive Optics
Zam K, Hanser B, Gustafsson MGL, Agard DA, Sedat JW. Computational adaptive optics for live three-dimensional biological imaging. Proc. Natl. Acad. Sci. USA 98: 3790-3795, 2000.



From Thorlabs

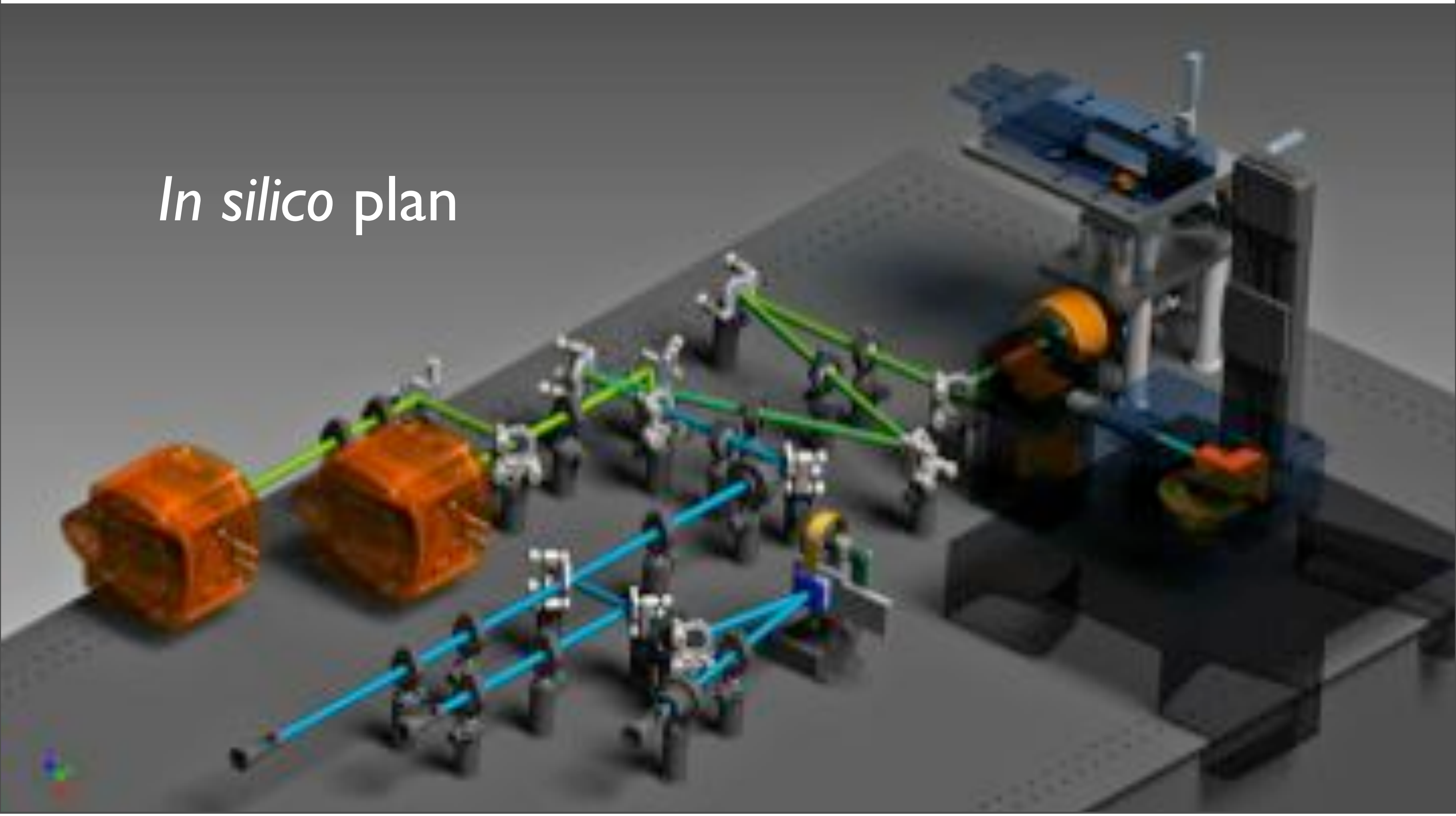
The hard part - algorithms for shaping the deformable mirror

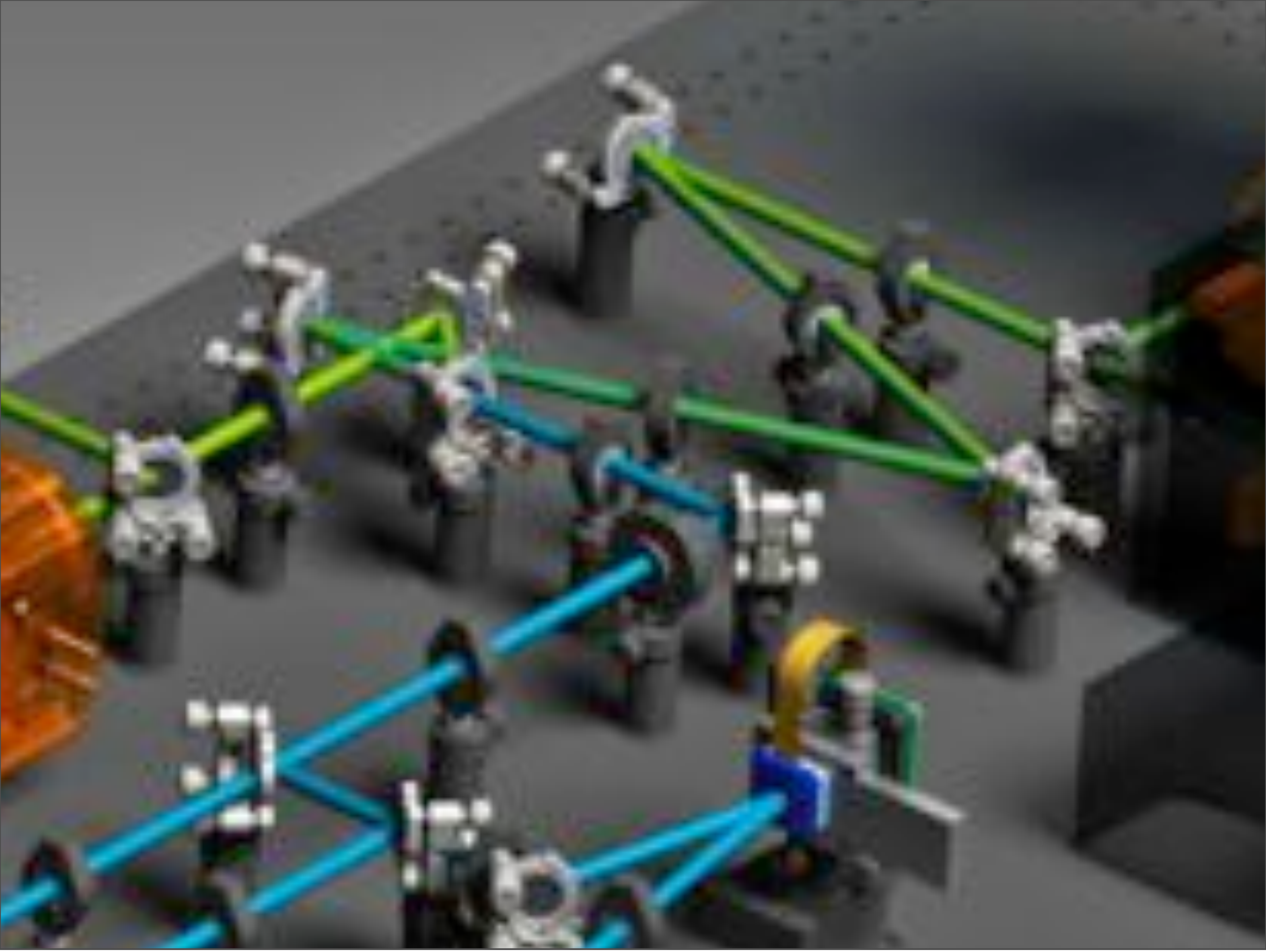
Martin Booth - Engineering / CNSB, Oxford

Further development: OMX-T

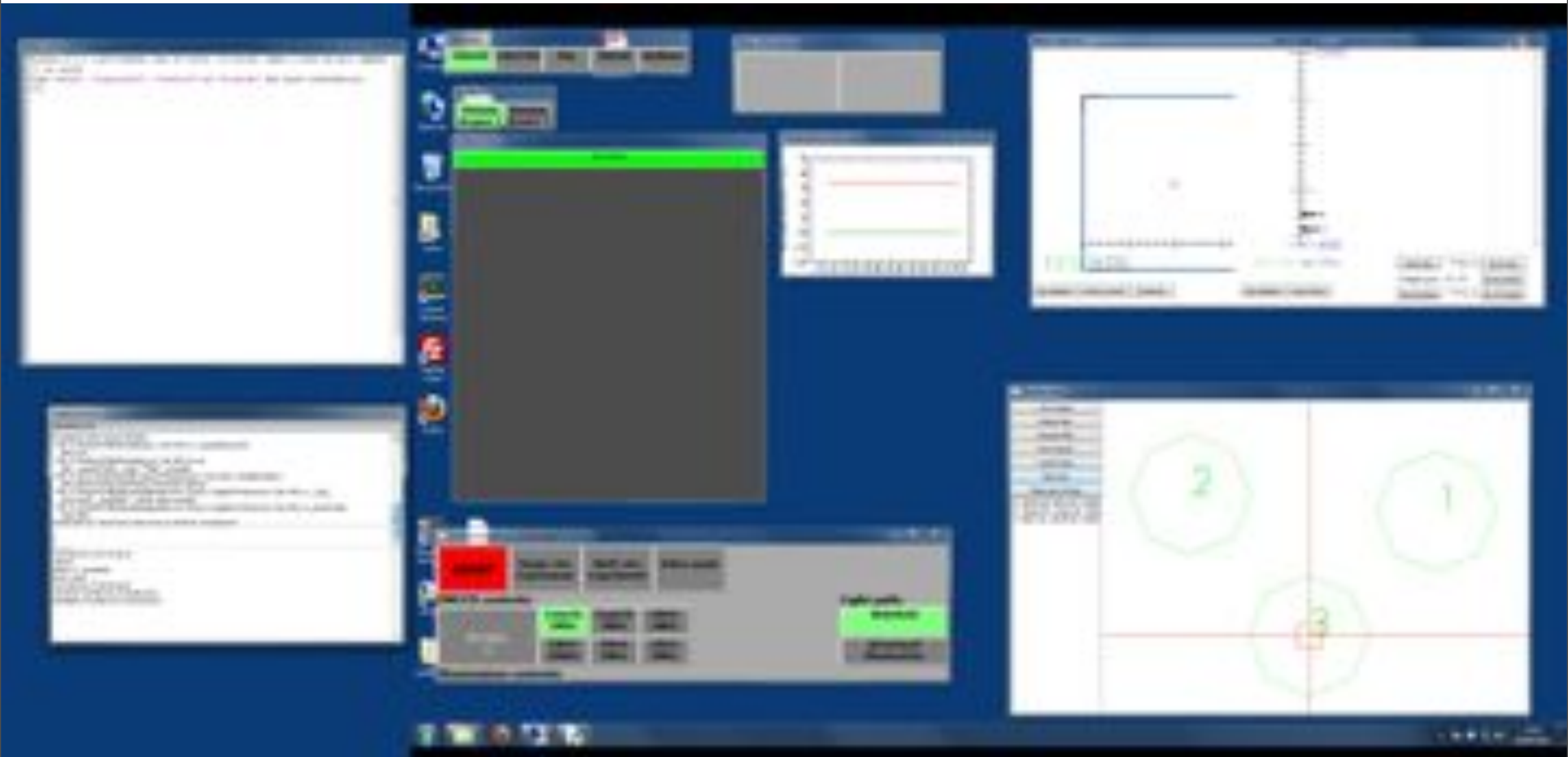
Rainer Kauffman and Ian Dobbie

Based on John Sedat's design

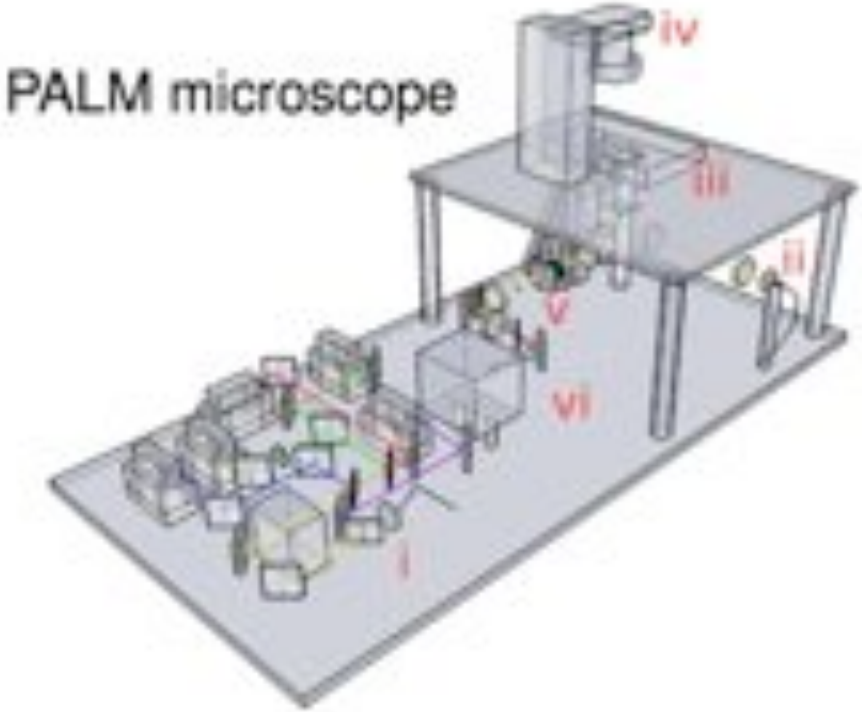


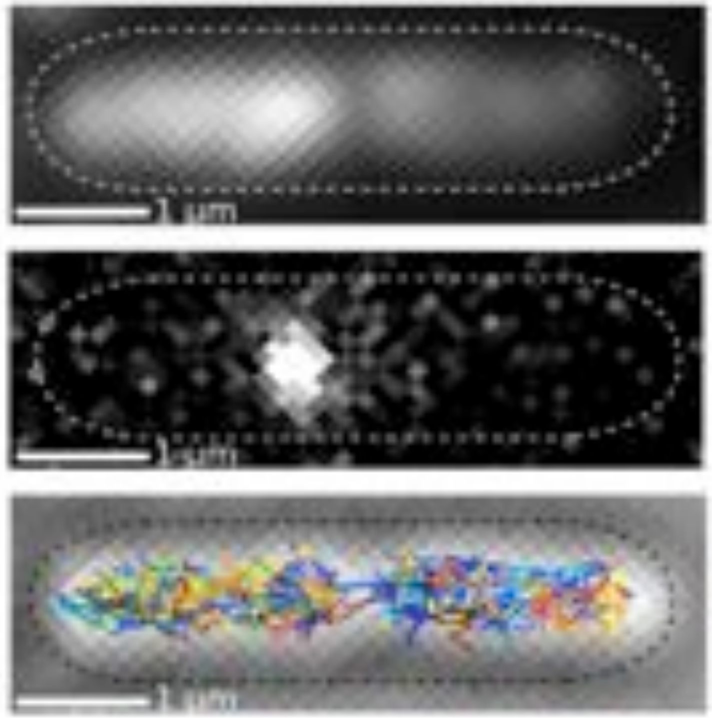


Cockpit - from John Sedat



Home built PALM / DSTORM





Stephan Uphoff and Achillefs Kapanidis

Half way houses

Warwick Open Source Microscope

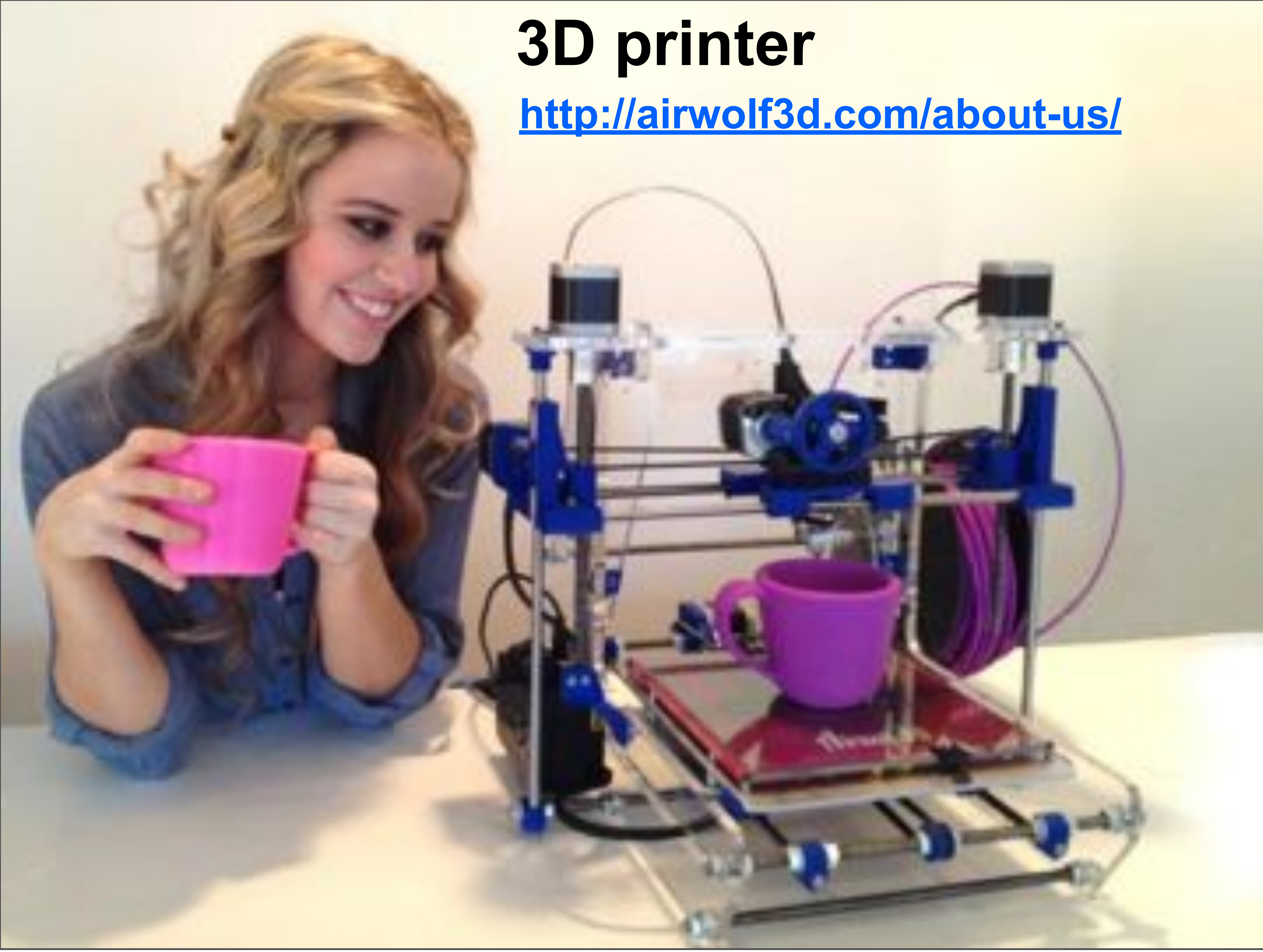
WOSM

http://wosmic.org

Nick Carter and Rob Cross

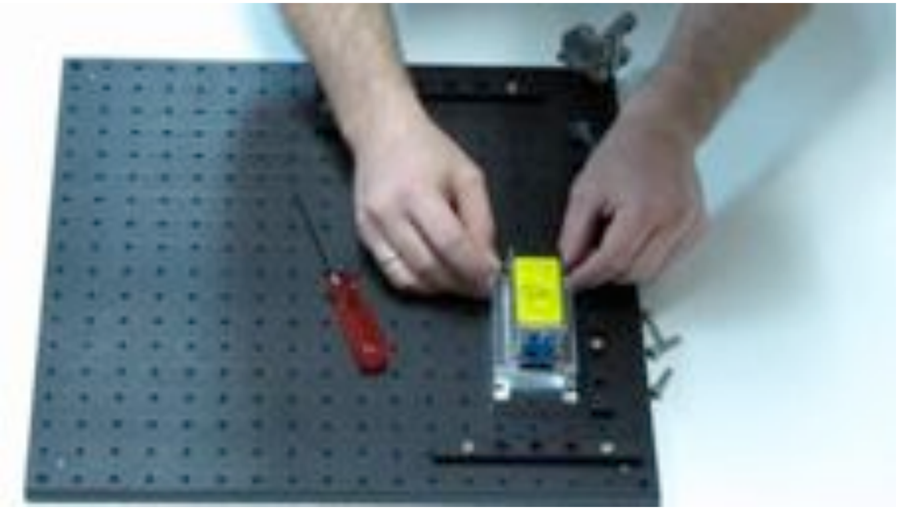
3D printer

http://airwolf3d.com/about-us/



Openspim

http://openspim.org/Welcome_to_the_OpenSPIM_Wiki



SPIM Farm


openspim
People
Pavel Tomancak
Jan Huisken
Peter Gabriel Pitrone
Kevin Eliceiri
Johannes Schindelin
Luke Stuyvenberg

The End