

# **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>

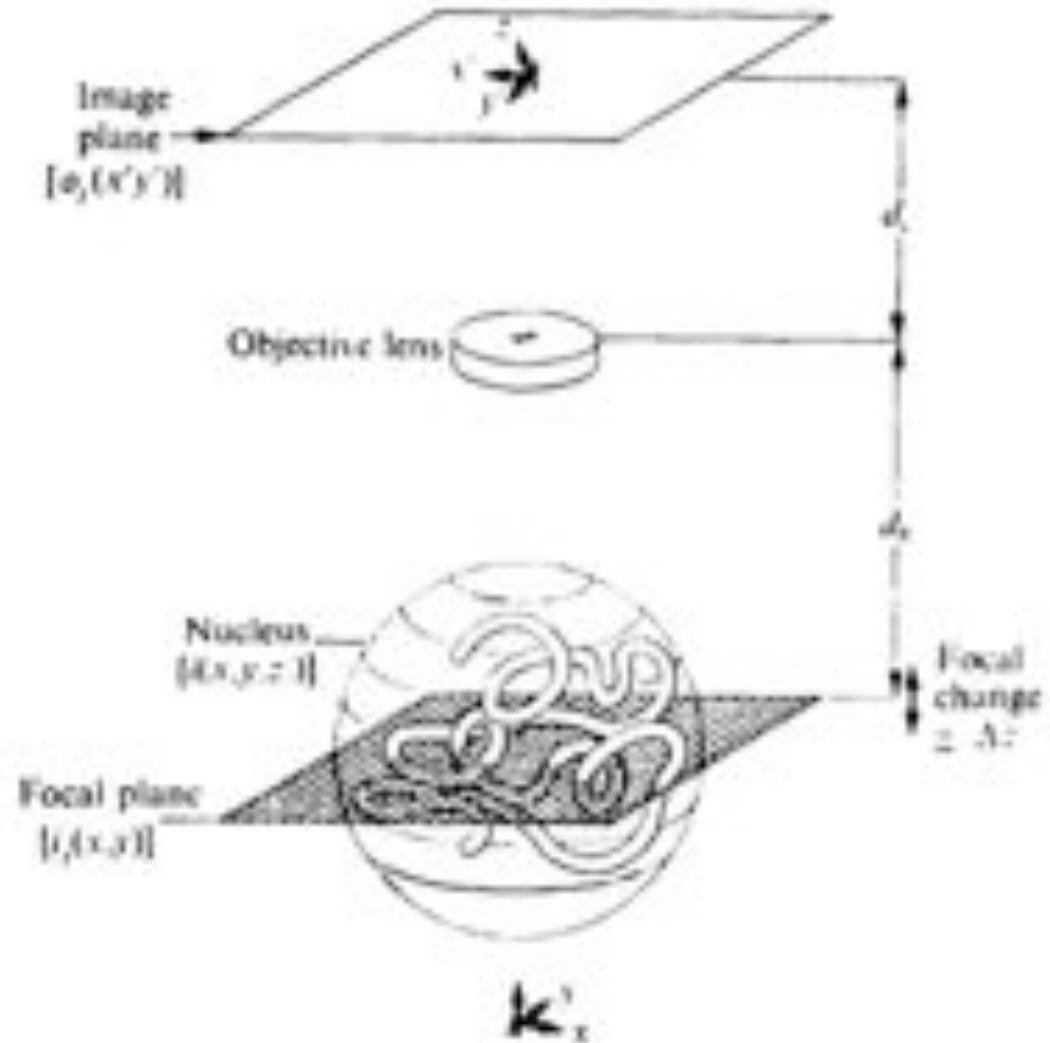
Reprinted from Science, Vol. 202, No. 4910, pp. 876-881, 21 April 1983  
© Macmillan Journals Ltd. 1983

## Three-dimensional architecture of a polytene nucleus

David A. Agard & John W. Sedat

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA

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 interwound coils and shows extensive interaction of the chromosomes with the nuclear envelope.

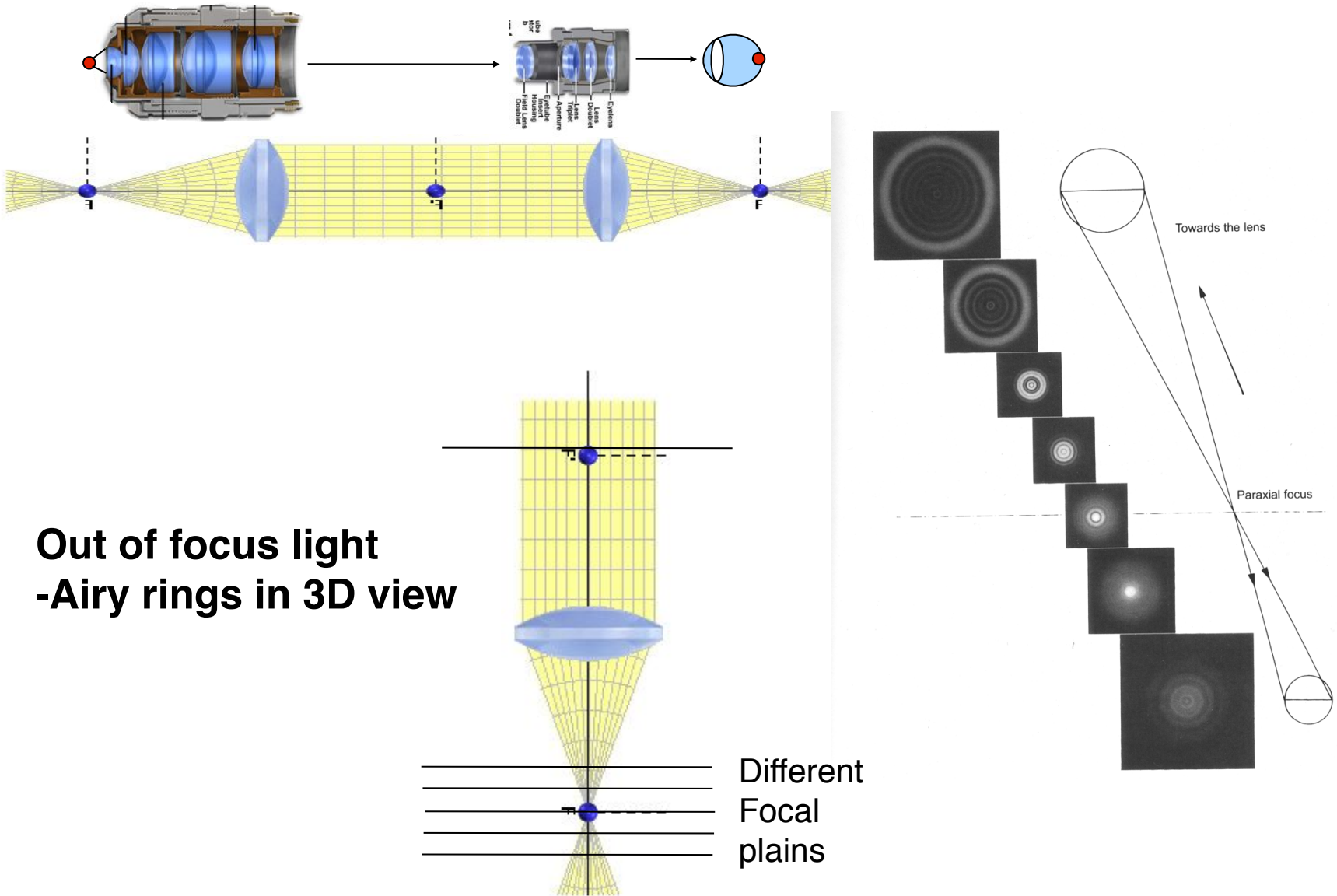


# Widefield Fluorescence microscopy (Olympus + Sedat/Agard DeltaVision)

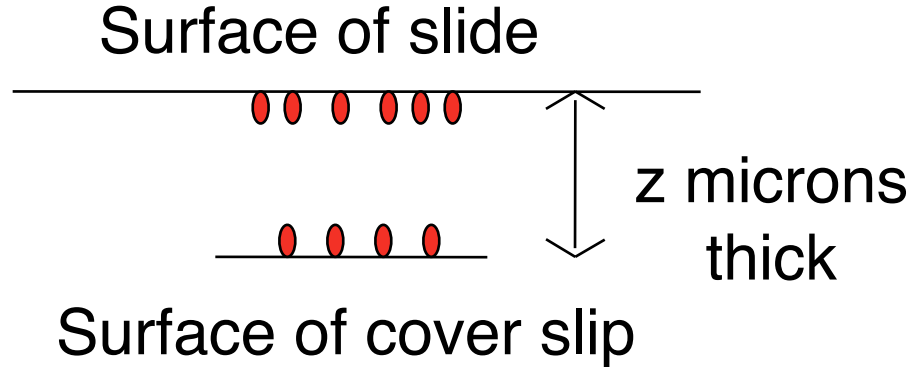


**Widefield deconvolution the most sensitive modality - but sometimes cannot be used**

# Image formation



# Bead slide

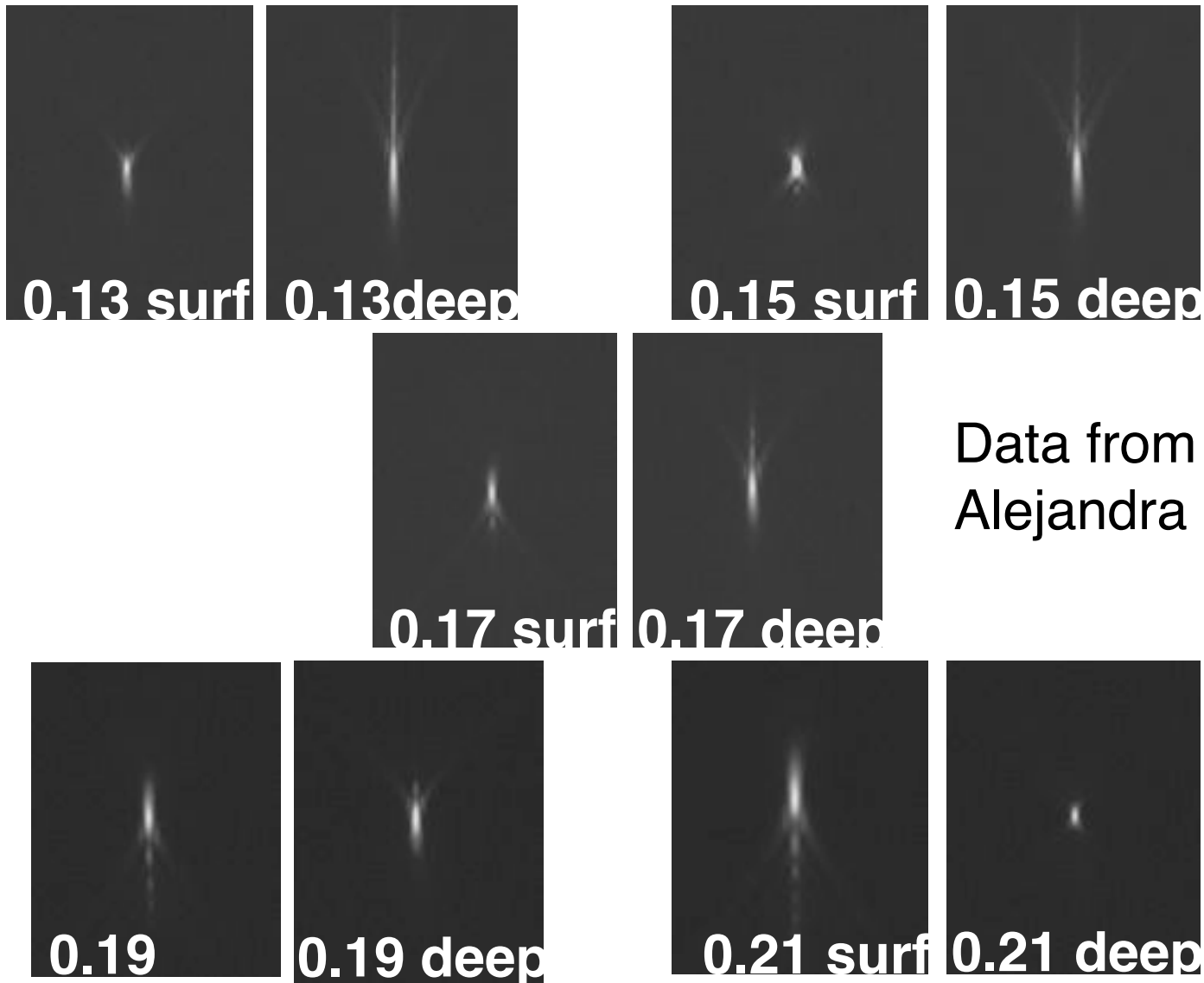


**Tetraspeck beads:** chromatic registration  
DAPI/FITC/Rhodamine/Cy5

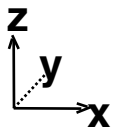
**Beads (PS Spec):** Single fluorochrome  
Brighter -better for generating  
point spread functions for deconvolution

**Inspeck Intensity beads:** Measure dynamic range

# Affects of deep imaging ( $90\mu\text{m}$ ) and collar settings on spherical aberration and psf of 60X/NA1.2<sub>w</sub>



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

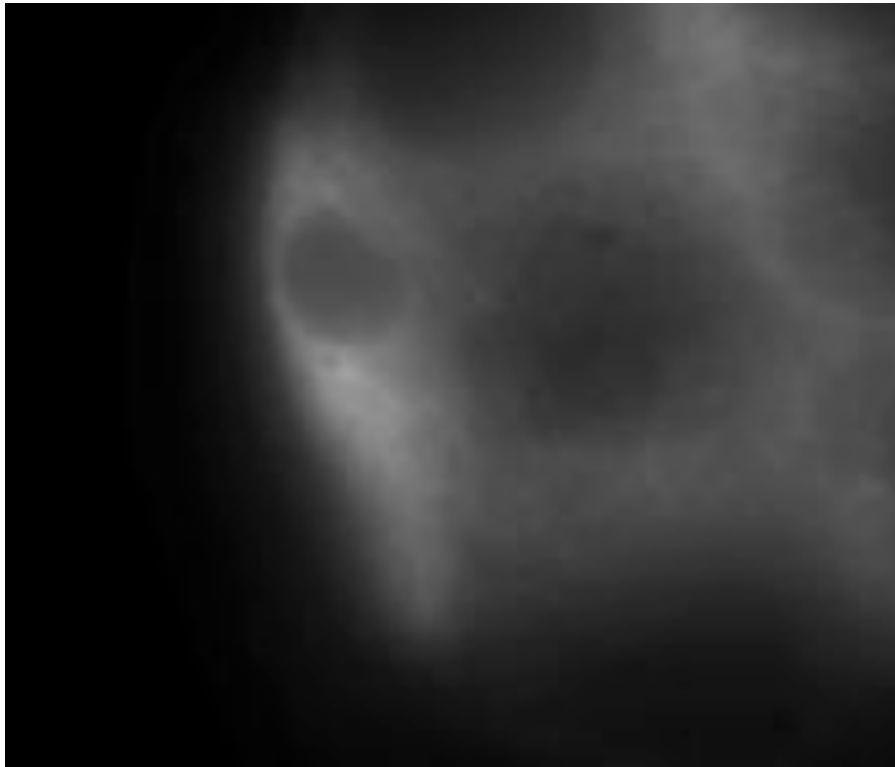


[http://www.olympusamerica.com/files/seg\\_bio/olympus\\_specialty\\_objectives.pdf](http://www.olympusamerica.com/files/seg_bio/olympus_specialty_objectives.pdf)

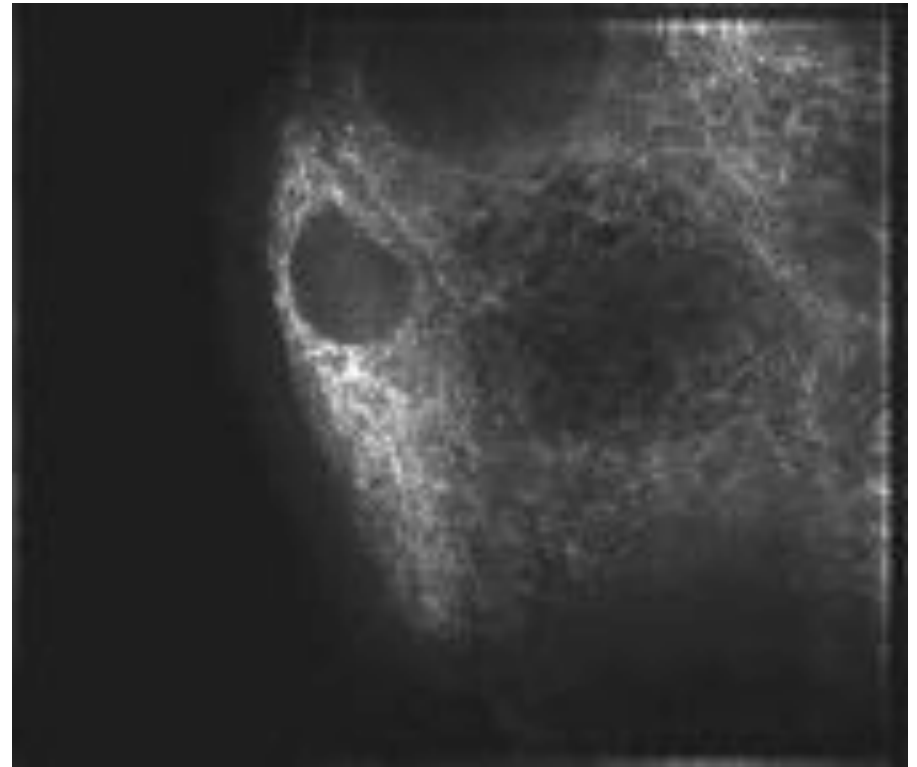
# How does Widefield Deconvolution Work?

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

**Before Deconvolution**



**After Deconvolution**



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

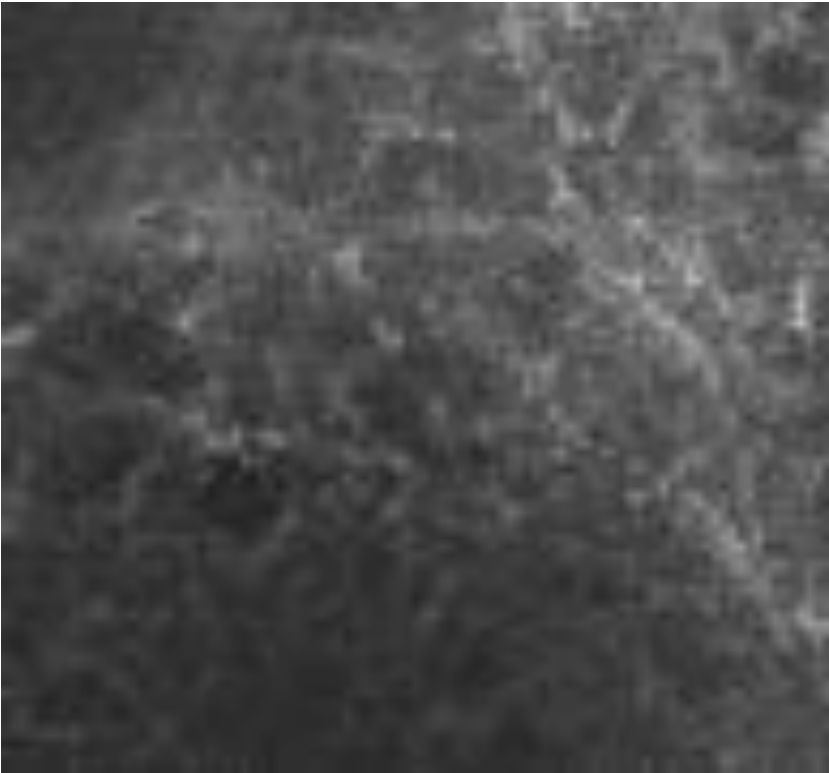
**Richard Parton**

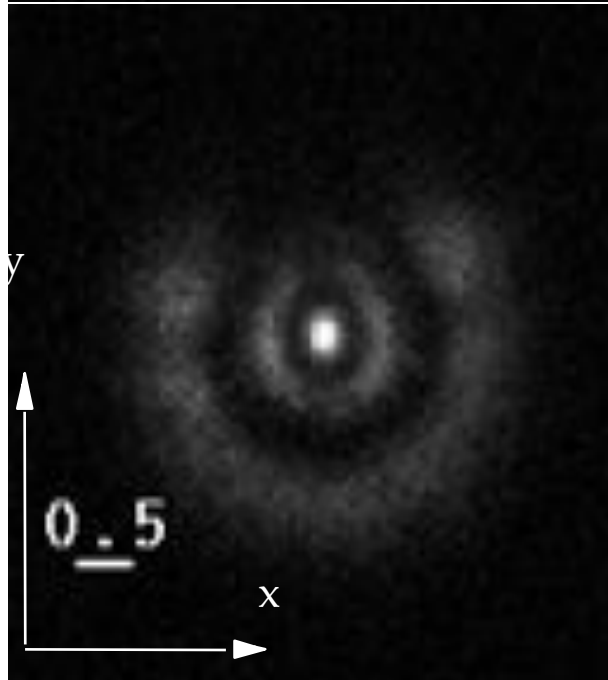
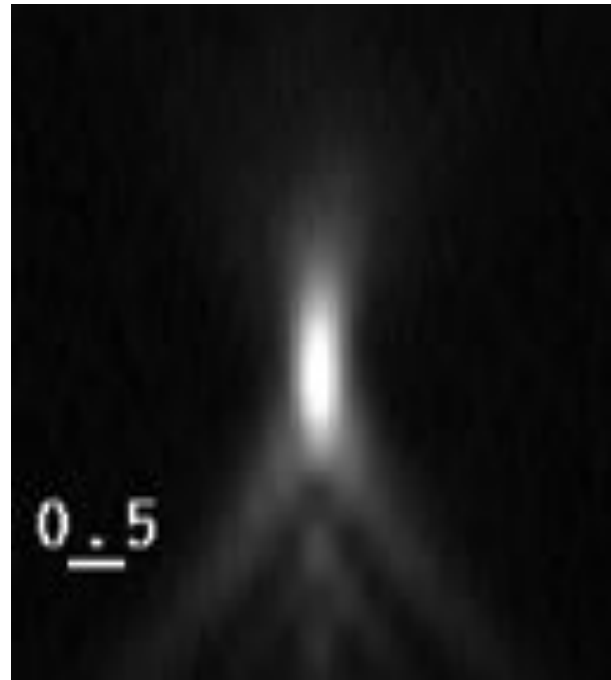
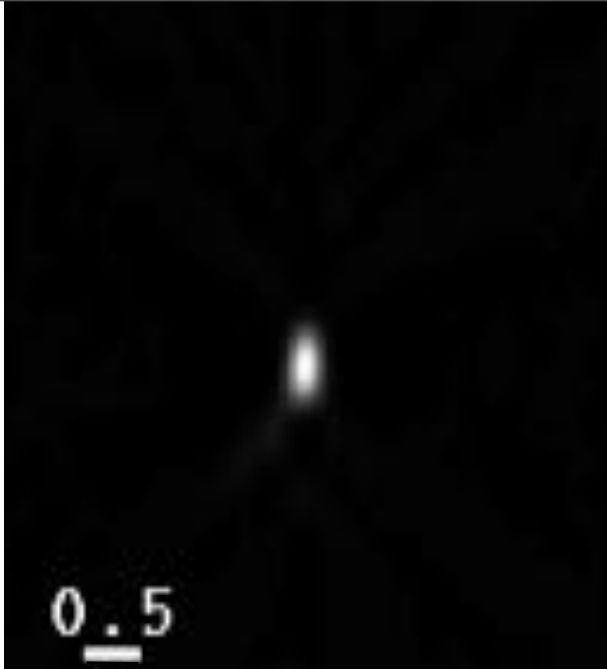
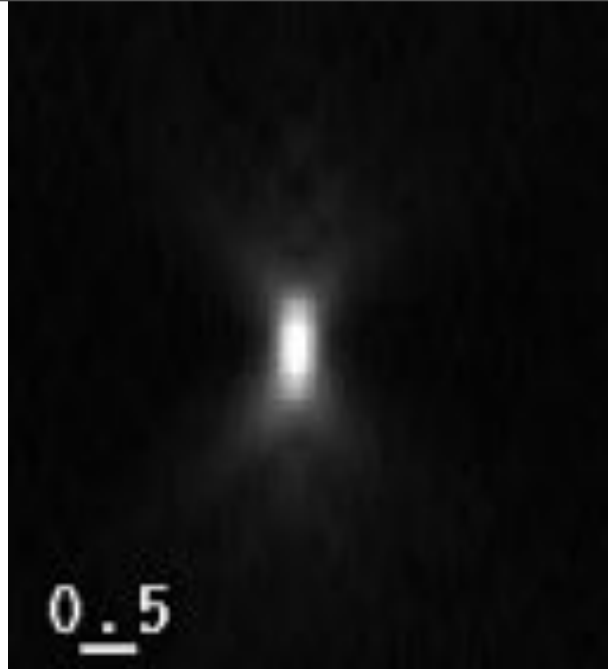
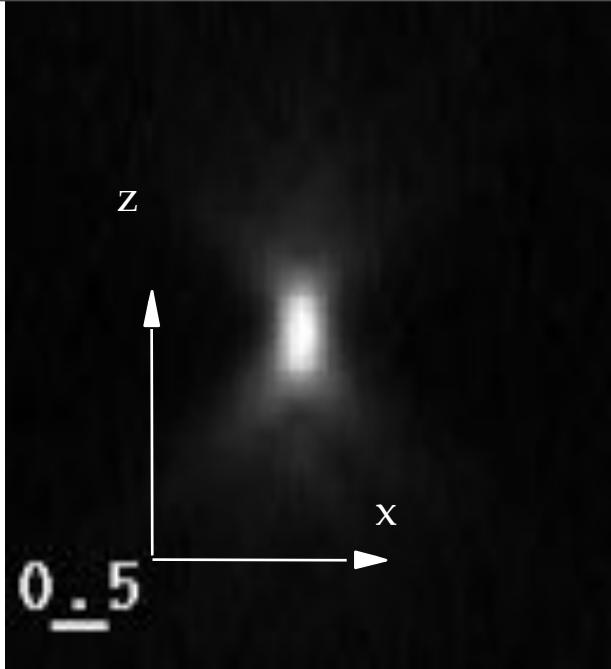


**Before Deconvolution**



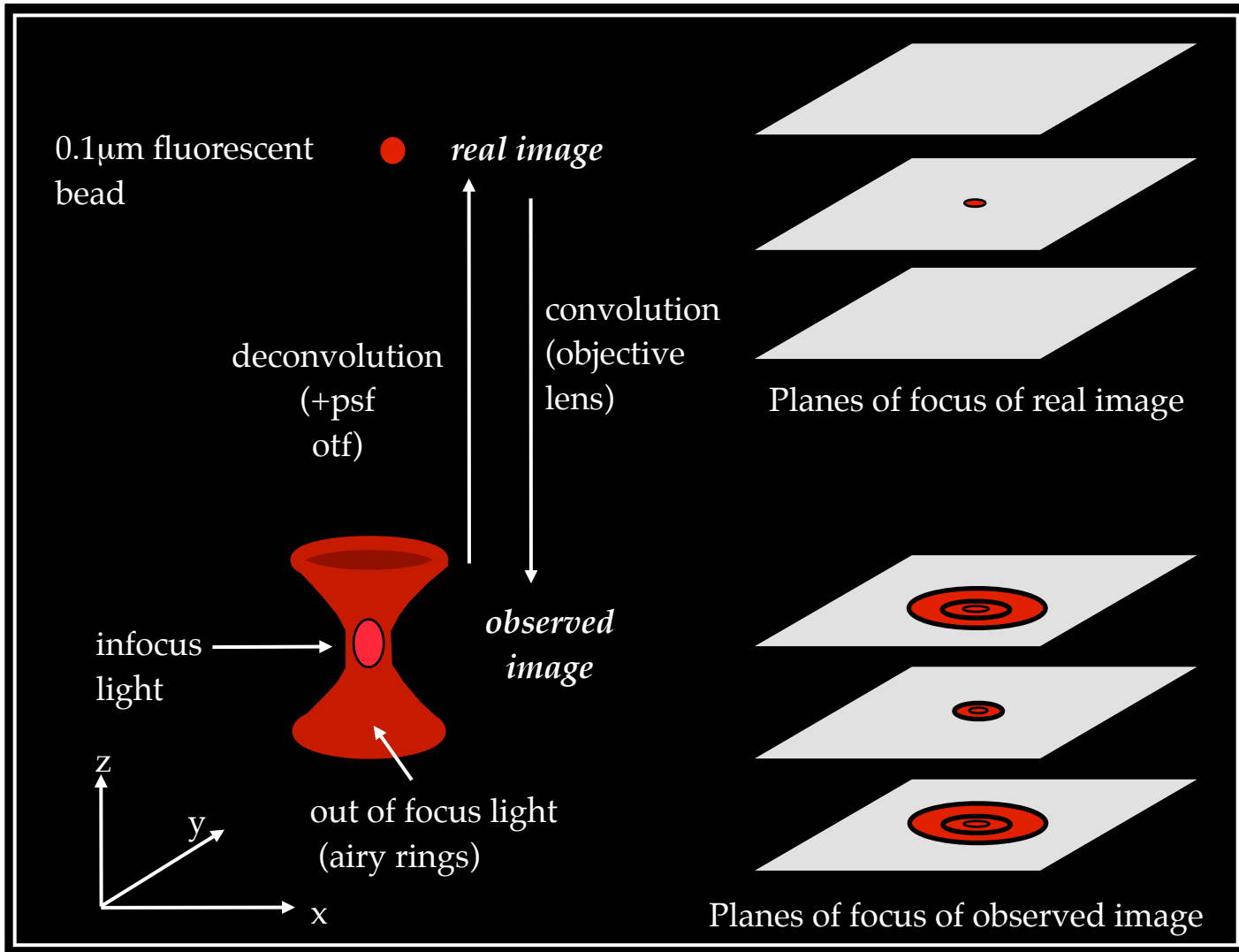
**After Deconvolution**





110	135	149	134	100	105	133	147	123
171	167	159	133	119	118	124	148	164
194	199	192	171	156	151	159	175	193
214	217	217	205	213	217	200	214	223
256	290	288	268	281	283	259	271	259
268	320	305	428	448	434	359	324	282
271	362	468	688	663	636	498	378	297
256	396	641	954	1148	1018	768	477	313
246	428	752	1171	1419	1296	988	528	318
213	439	857	1379	1691	1522	1027	551	296
197	481	1057	1881	2226	1917	1253	614	271
195	497	1126	1928	2386	2068	1311	622	257
288	538	1182	1982	2378	2111	1323	619	253
258	544	1193	1989	2378	2083	1381	613	263
288	582	1173	1893	2251	1958	1241	596	278
313	585	1129	1798	2873	1791	1156	577	308
385	622	1088	1487	1638	1378	938	514	357
386	629	906	1215	1388	1154	819	491	371
481	613	819	1828	1248	946	788	467	372
411	526	613	678	784	614	518	428	328
486	477	511	523	588	458	425	387	387
587	419	428	485	354	248	348	345	284
321	327	387	328	268	268	271	261	245
282	287	243	254	282	283	222	215	217
238	236	193	205	263	265	185	177	186

Ilan Davis, 2000



Ilan Davis, 2000

# Deconvolution

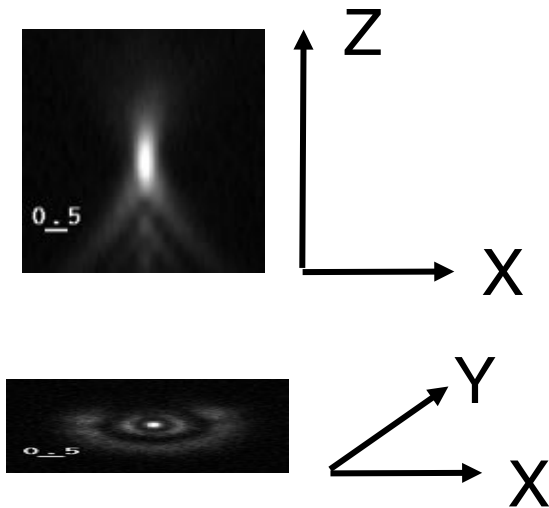
Calculations done in Fourier (frequency) space not XYZ space.

Uses Fast Fourier Transforms - much faster algorithm (developed in the 1960s)

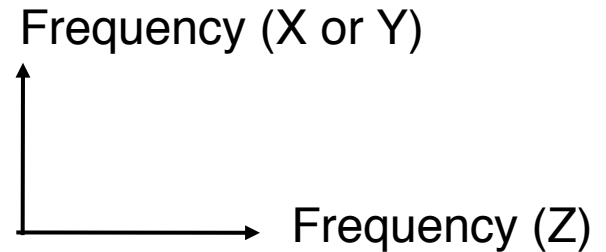
Psf is converted to optical transfer function (only information in X and Z)

Several methods that vary in their implementation

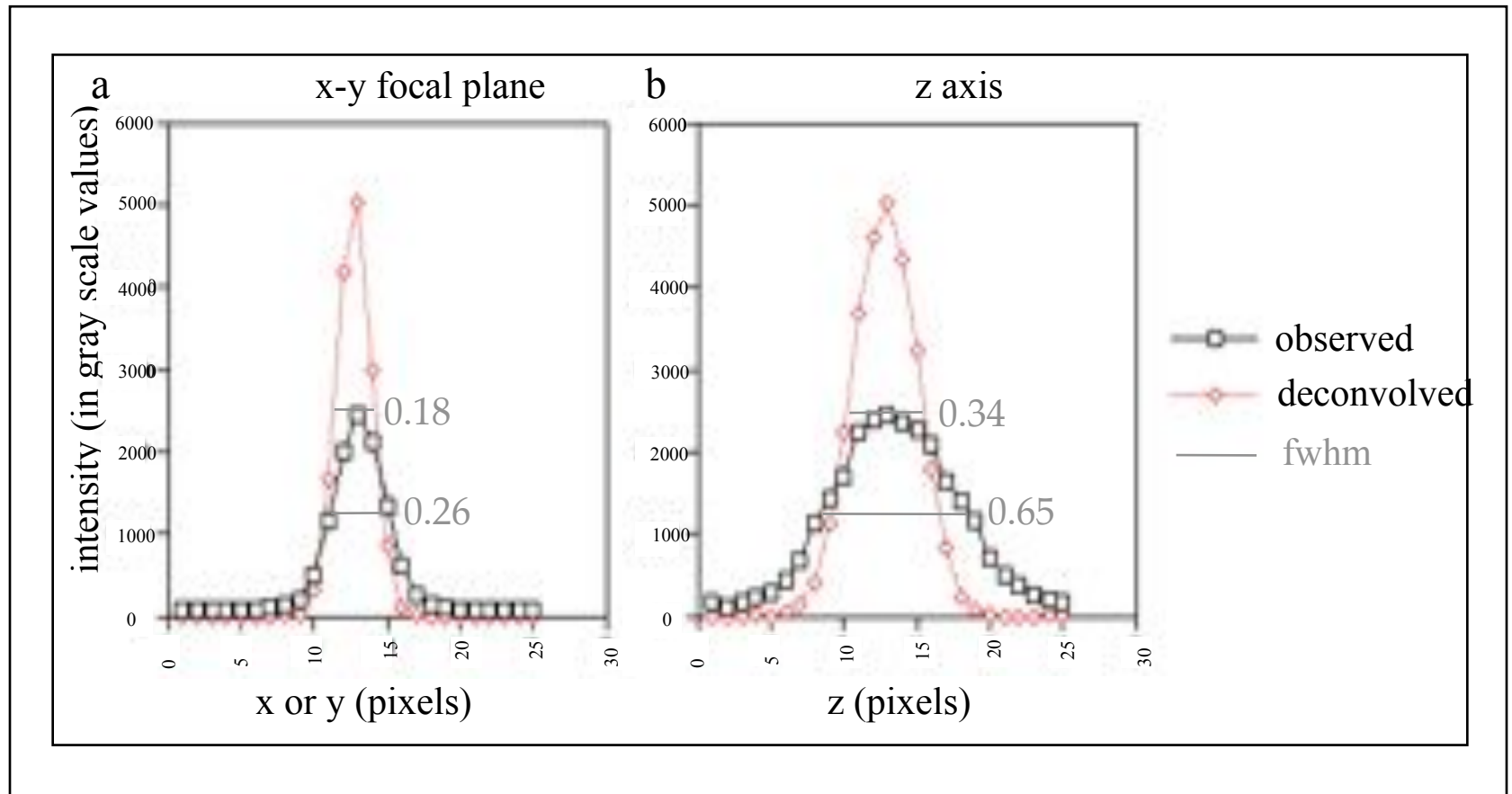
**Point Spread Function  
PSF (XYZ space)**



**Optical Transfer Function  
OTF (XZ frequency space)**



# Increase in resolution (XY and Z) after deconvolution



# Types of Deconvolution

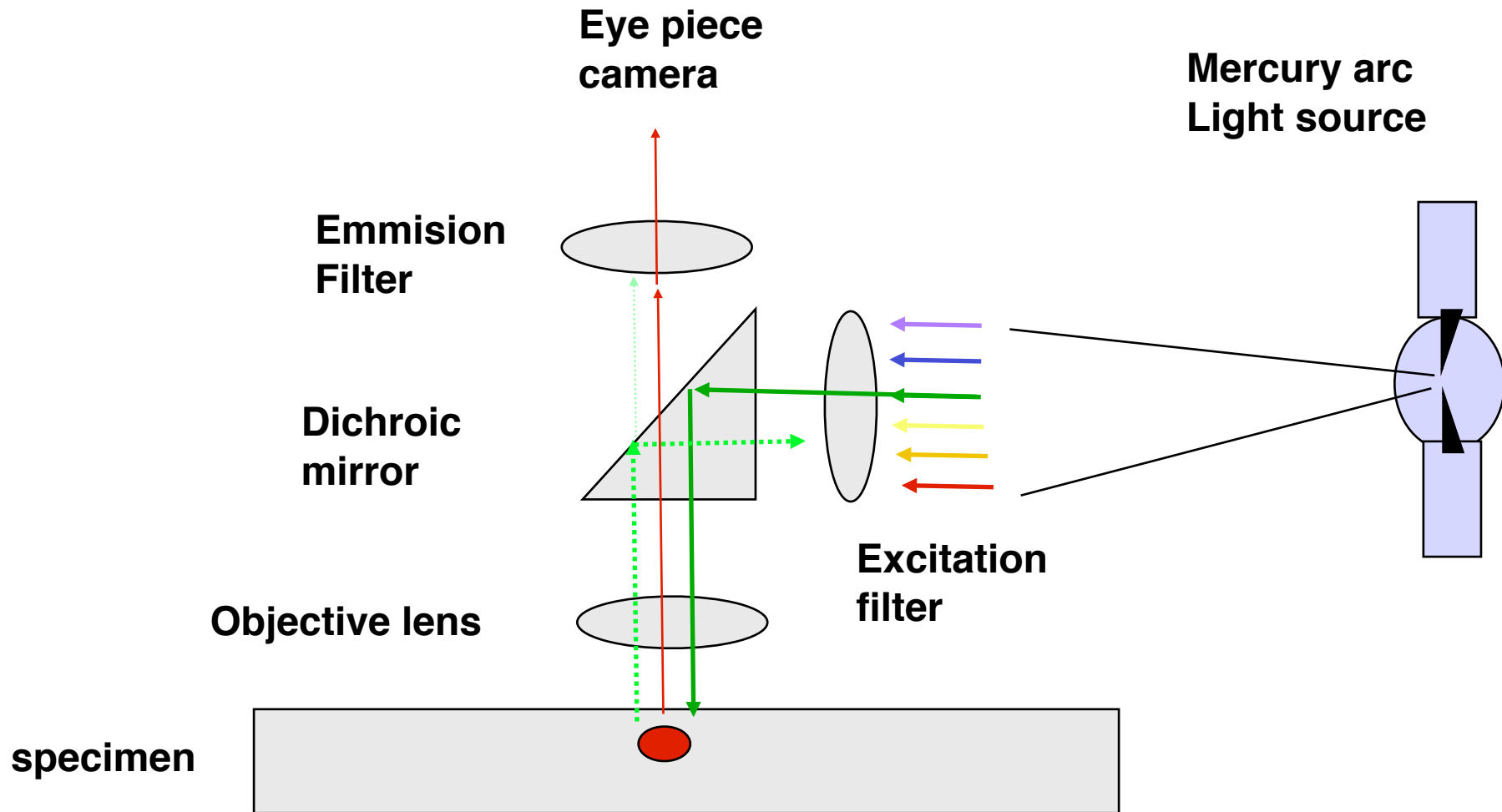
- No neighbour, nearest neighbour - poor substitute
- 2D deconvolution - Not as good
- 3D constrained iterative approaches
- Sedat/Agard ; Hoygens ; blind deconvolution

## **New methods (Sedat)**

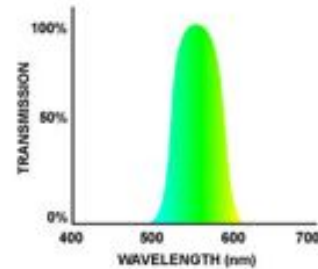
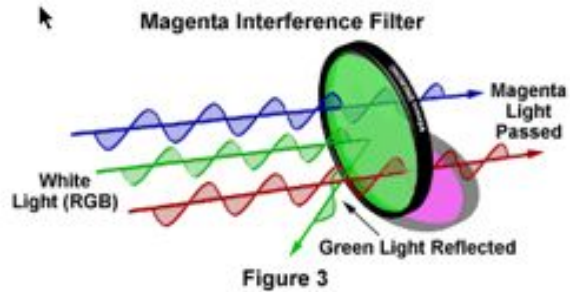
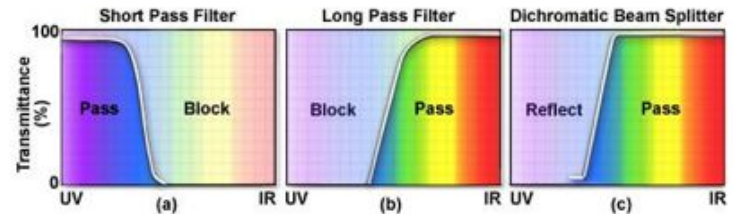
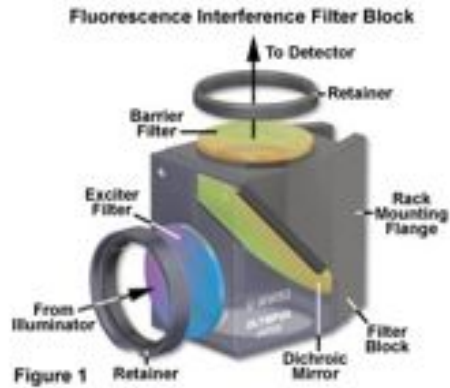
- Pupal functions (used to sharpen Hubble telescope) include information in  $o$ tf in X, Y and Z and phase. Phase retrieval
- Myopic deconvolution

# Reminder

## How do fluorescence microscopes work ?



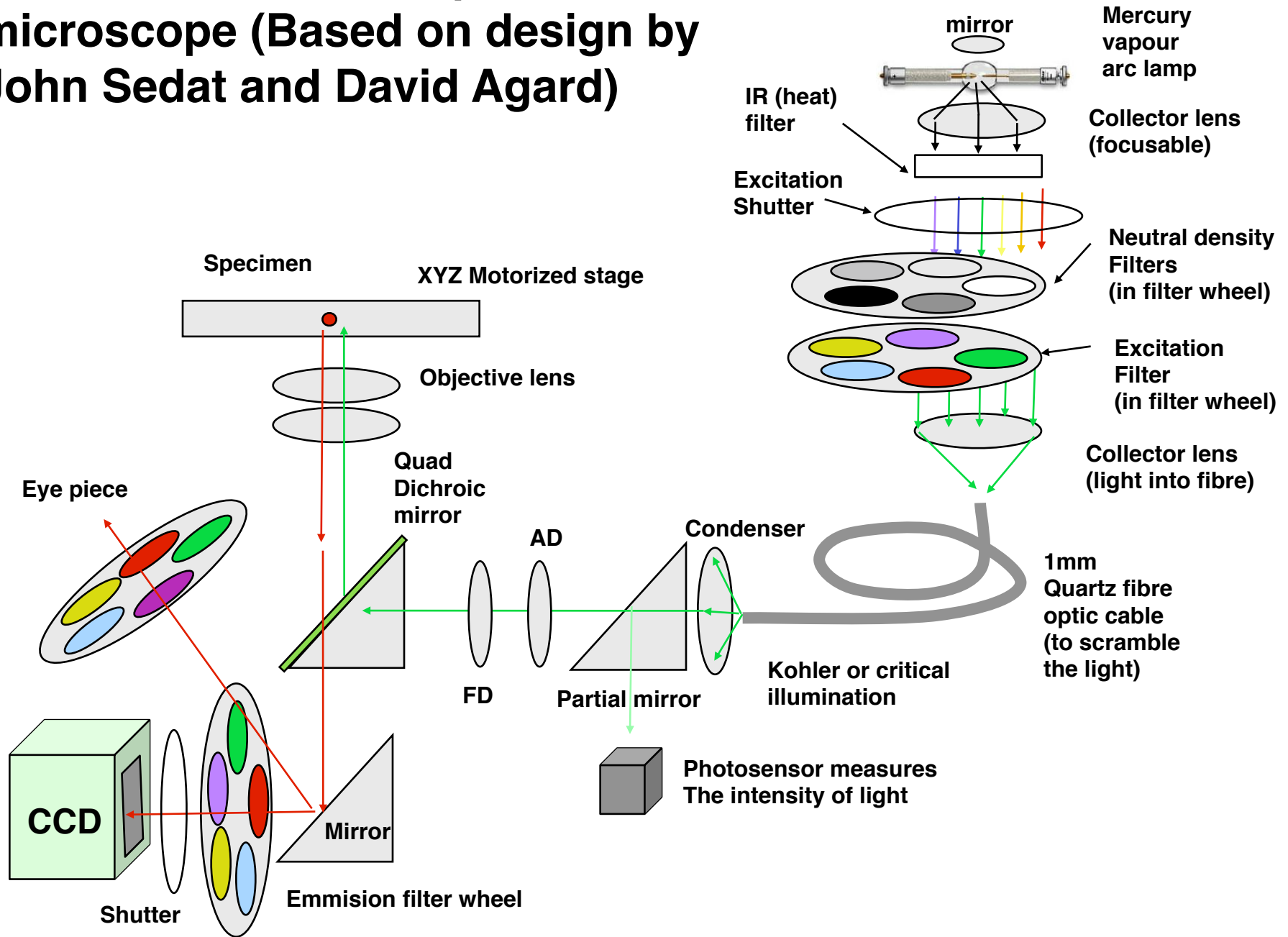
# Filter cubes



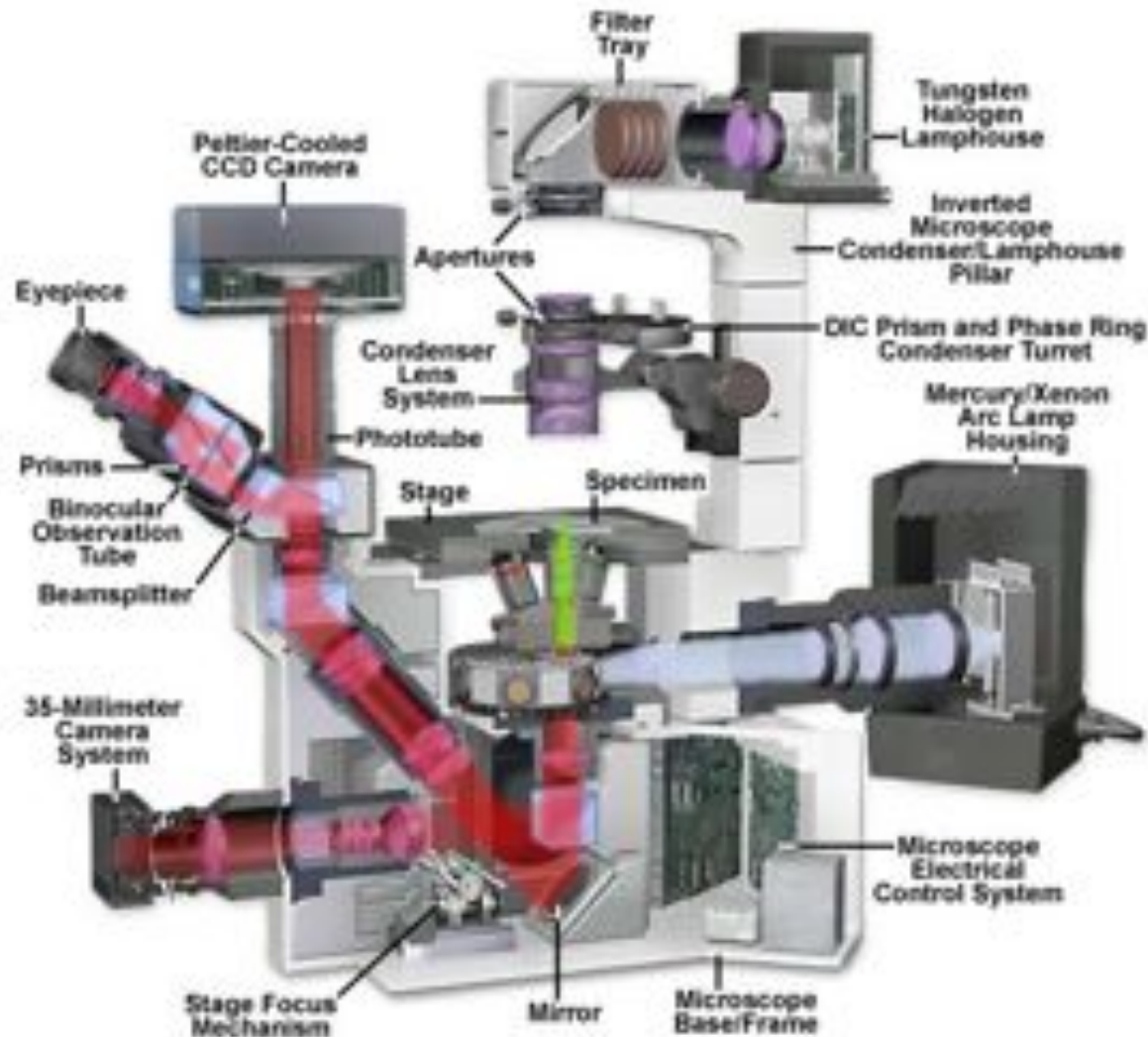
<http://www.chroma.com/pdf/handbook4.pdf>



# 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?**

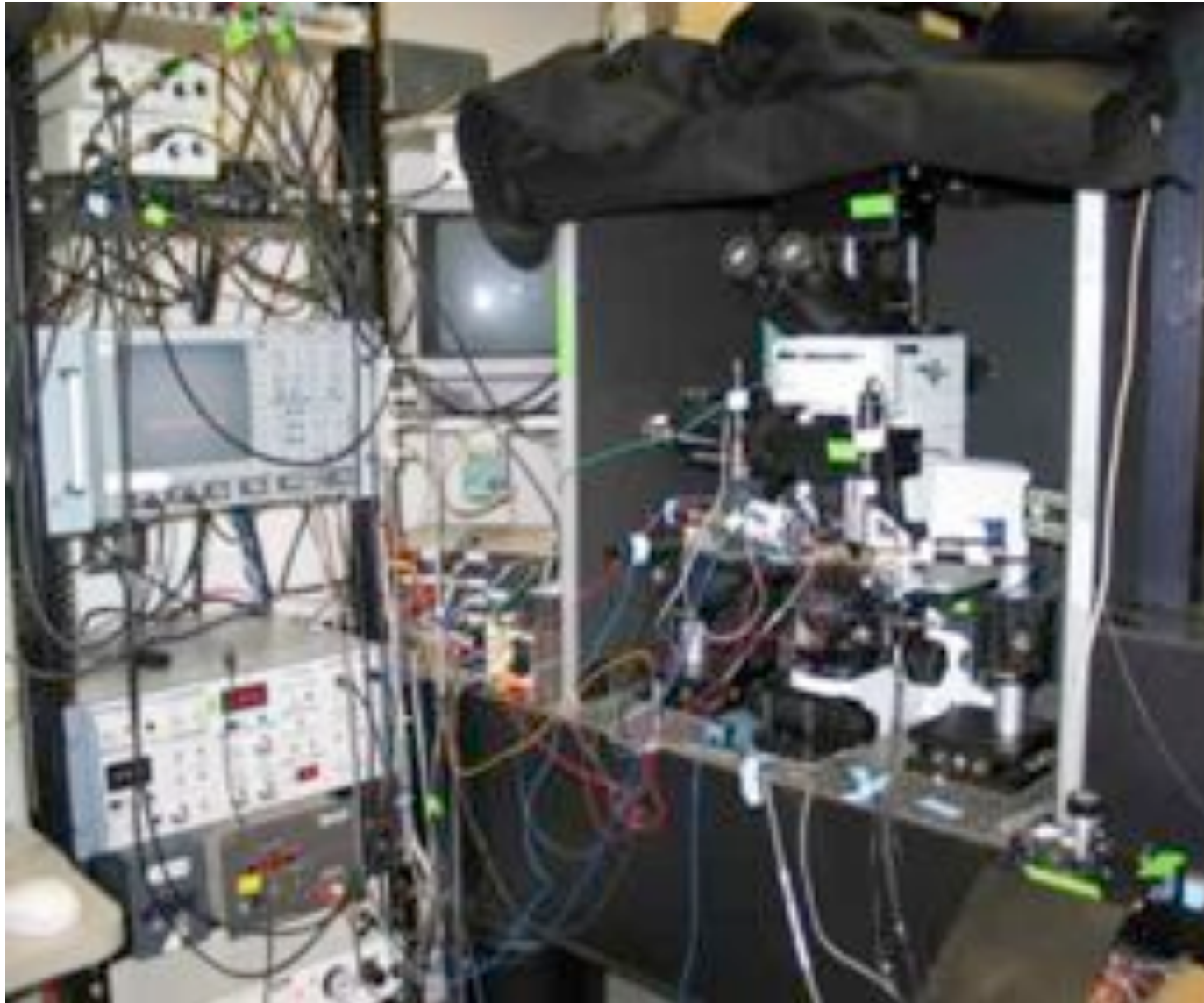
**Specific applications -better than  
commercial microscopes**

**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



**vibration  
isolation  
table**

# The basic ingredients

**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



Stage



Cameras



photometrics  
evolvo  
BY DELTA



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**



**Stage**



**Cameras**



**vibration  
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**Electronics**

**Timing board  
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**Computer (normally a PC)**

# The basic ingredients

**Objective lens  
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**Computer (normally a PC)**

**Optics**

# The basic ingredients

**Objective lens  
and holder**



**Stage**



**Cameras**



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**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**

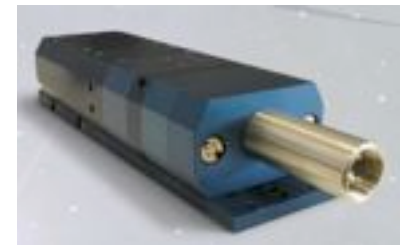


**Computer (normally a PC)**

**Optics**

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

**Lasers**

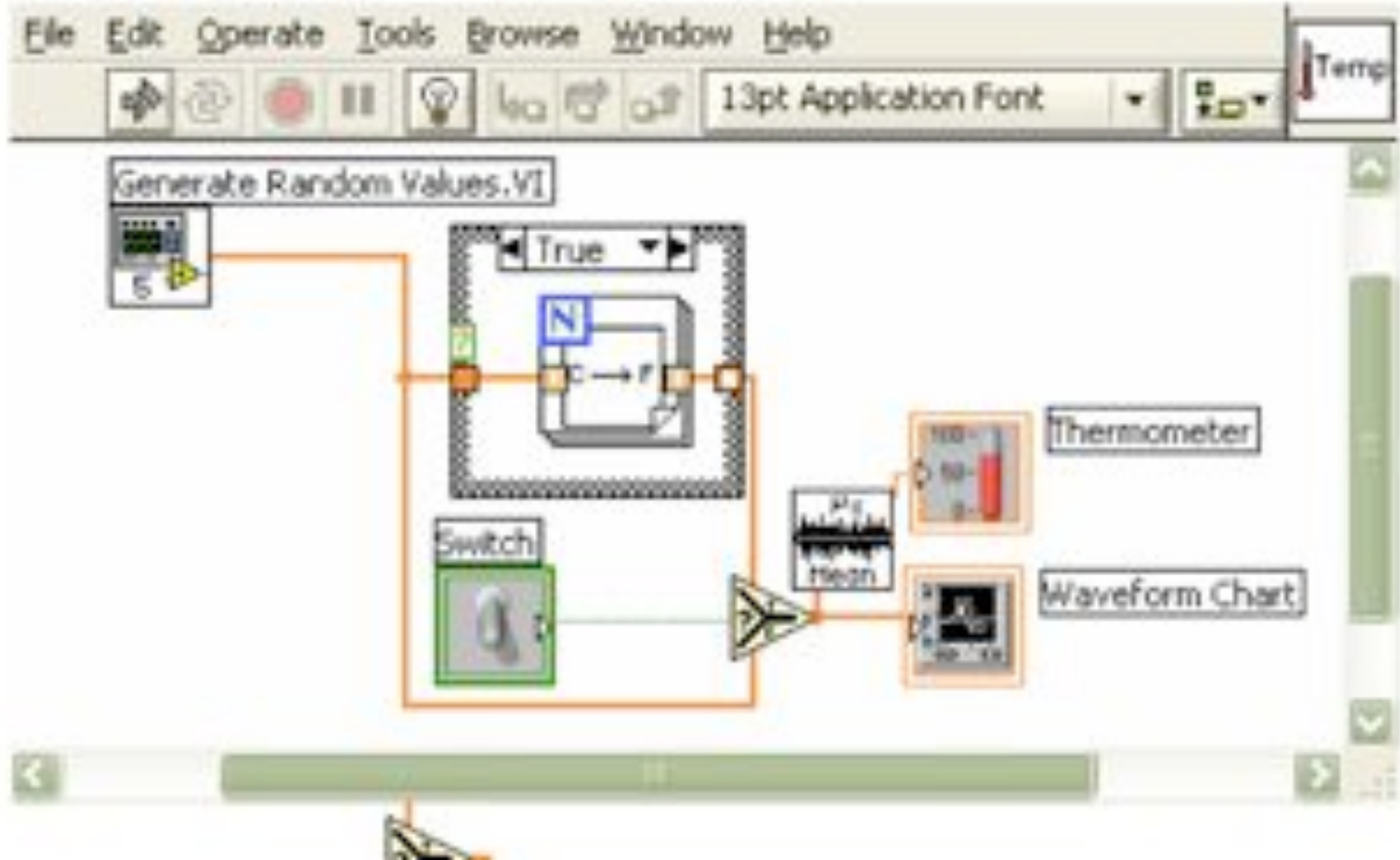


# Software options

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

# Lab view example

but we'll fix it.



# Micromanager

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



The screenshot shows the homepage of the Micromanager website. At the top left, the logo "µManager" is displayed above the tagline "THE OPEN SOURCE MICROSCOPY SOFTWARE". A navigation bar contains links for "OVERVIEW", "DOWNLOAD", "DOCUMENTATION", "SERVICES", "PROGRAMMING", "SUPPORT", "EVENTS", "CREDITS", and "LOG IN". A sidebar on the left features a "welcome to micro-manager!" message with a small image of eyes, a "News" section with a list of recent updates, and a "Support for Nikon and" link. The main content area has a large background image of a microscope. Below this, the heading "Micro-Manager Open Source Microscopy Software" is followed by the µManager logo and a descriptive paragraph. A call to action encourages downloading the latest version (1.4) and watching a ScreenCast. At the bottom, a screenshot of the software interface shows a control panel and a live microscopy image of cells.

µManager

THE OPEN SOURCE MICROSCOPY SOFTWARE

OVERVIEW - DOWNLOAD - DOCUMENTATION - SERVICES - PROGRAMMING - SUPPORT - EVENTS - CREDITS - LOG IN

welcome to micro-manager!



## Micro-Manager 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



# 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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- Often necessary for specific specialised problems.
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- Can provide extremely flexible systems

**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**

# How expensive is it?

**Building costs**

**Hardware ~£100-250k**

**Salaries 1-3 years (~£50-£150)**

**Total cost ~£150-350k**

**Commercial OMX system ~£750k**

# OMX



David Agard



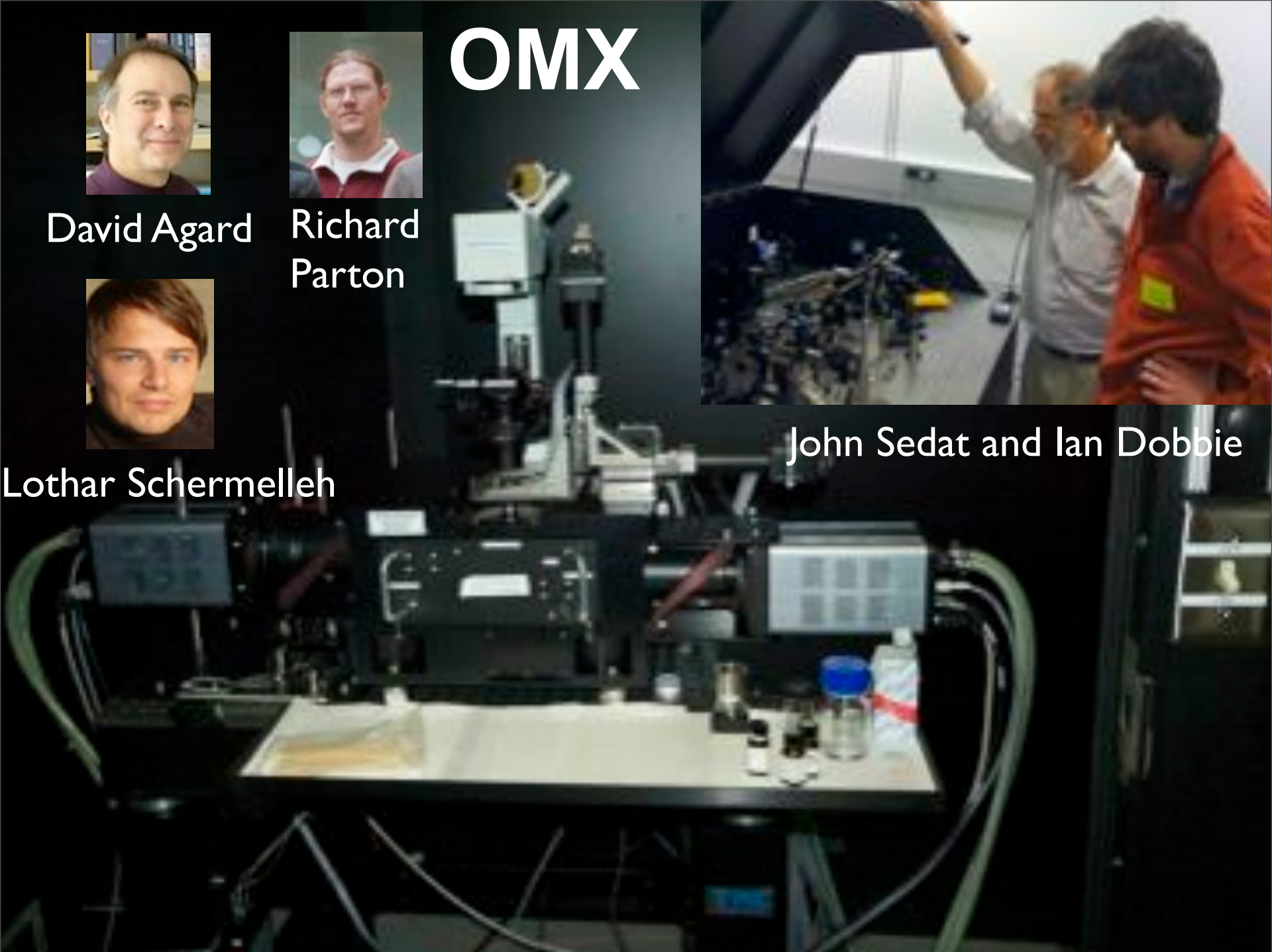
Richard Parton



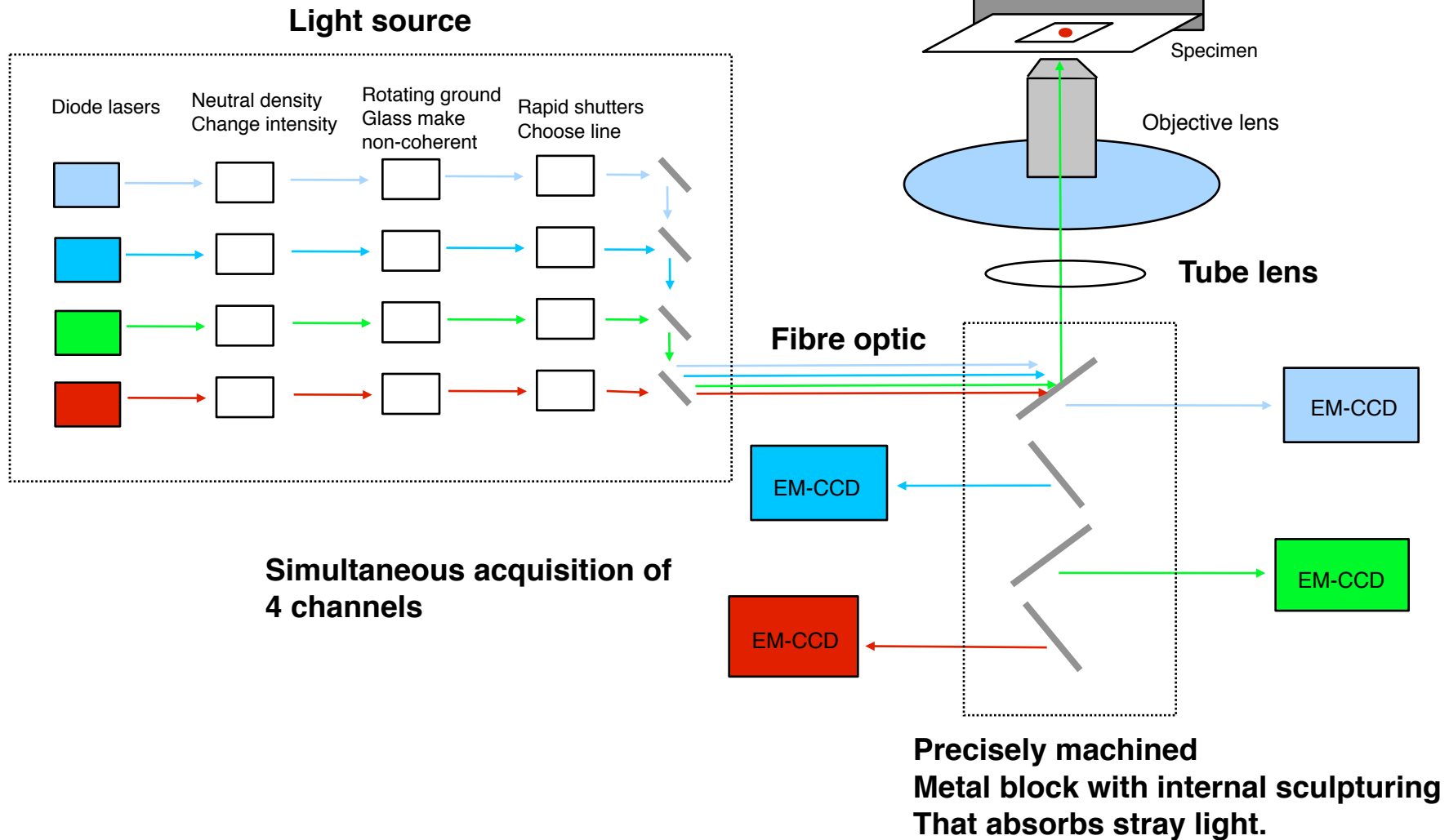
Lothar Schermelleh



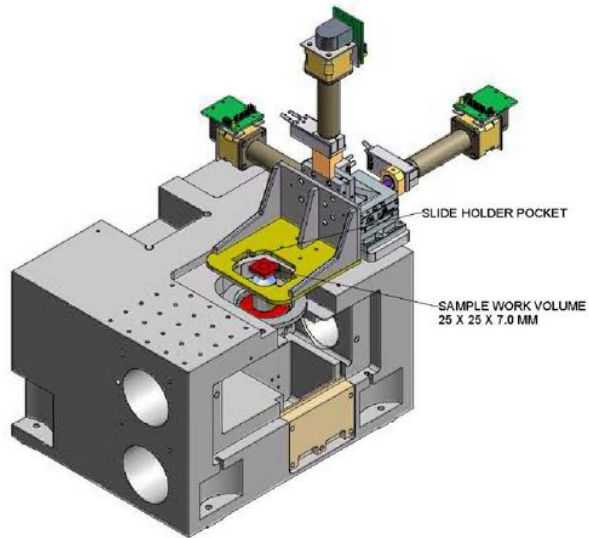
John Sedat and Ian Dobbie



# OMX - Redesigning widefield microscopy from scratch



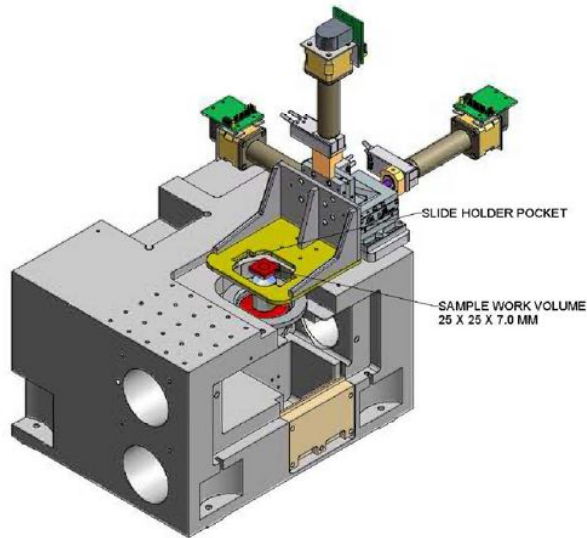
# OMX (John Sedat, David Agard and Mats Gustafsson)



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

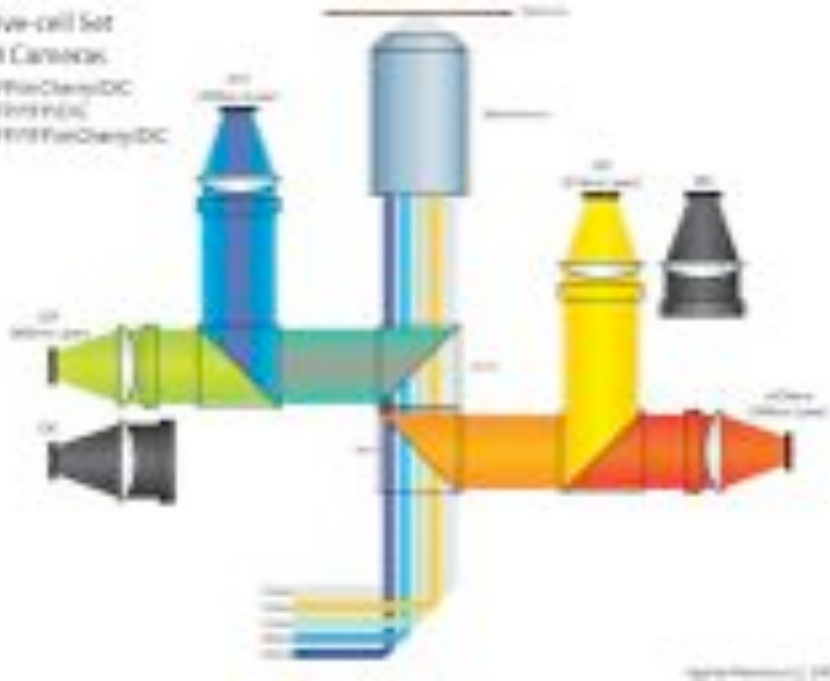


# OMX (John Sedat, David Agard and Mats Gustafsson)



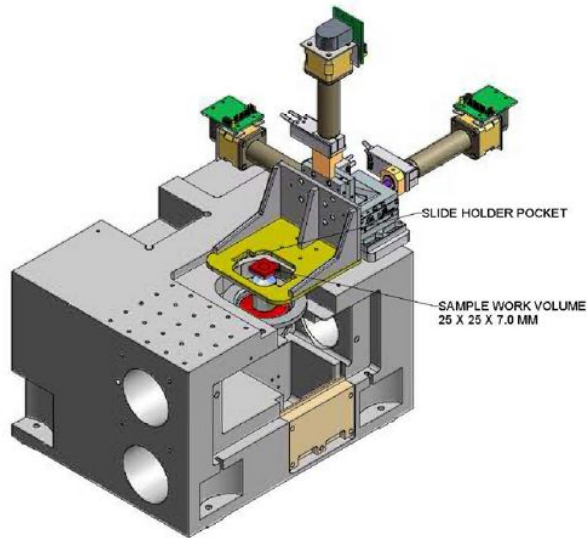
**Precisely machined  
Metal block with internal sculpturing  
That absorbs stray light  
Maximized emission light efficiency**

Live-cell set  
- 4 Channels  
SPFowCherryDC  
C2P1P1P1DC  
C2P1P1P1DC



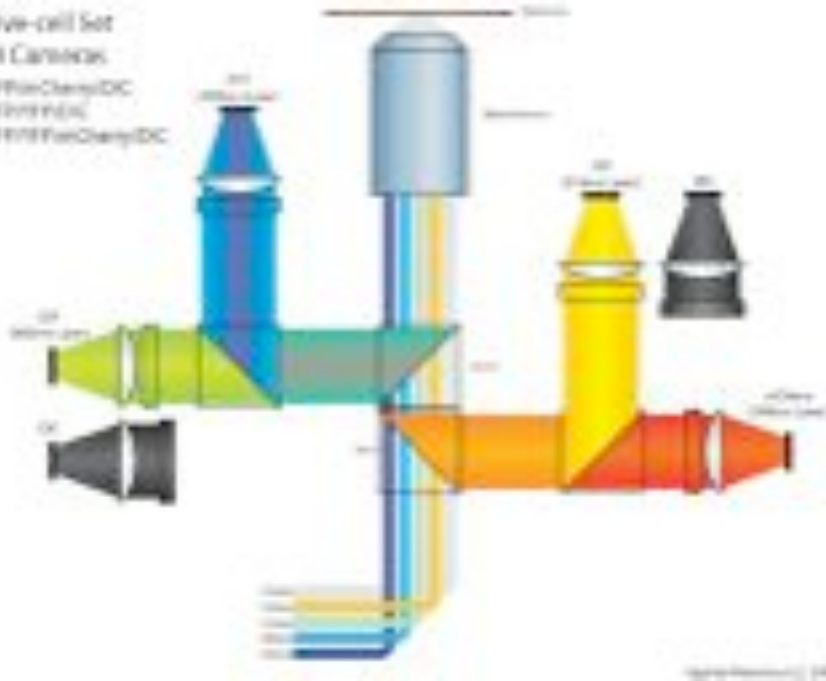
**4 laser excitation lines  
4 simultaneous acquisition lines CCDs**

# OMX (John Sedat, David Agard and Mats Gustafsson)



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

Live-cell set  
- 4 Channels  
SPFowCherryDC  
CSPFowCherryDC  
DFFowCherryDC

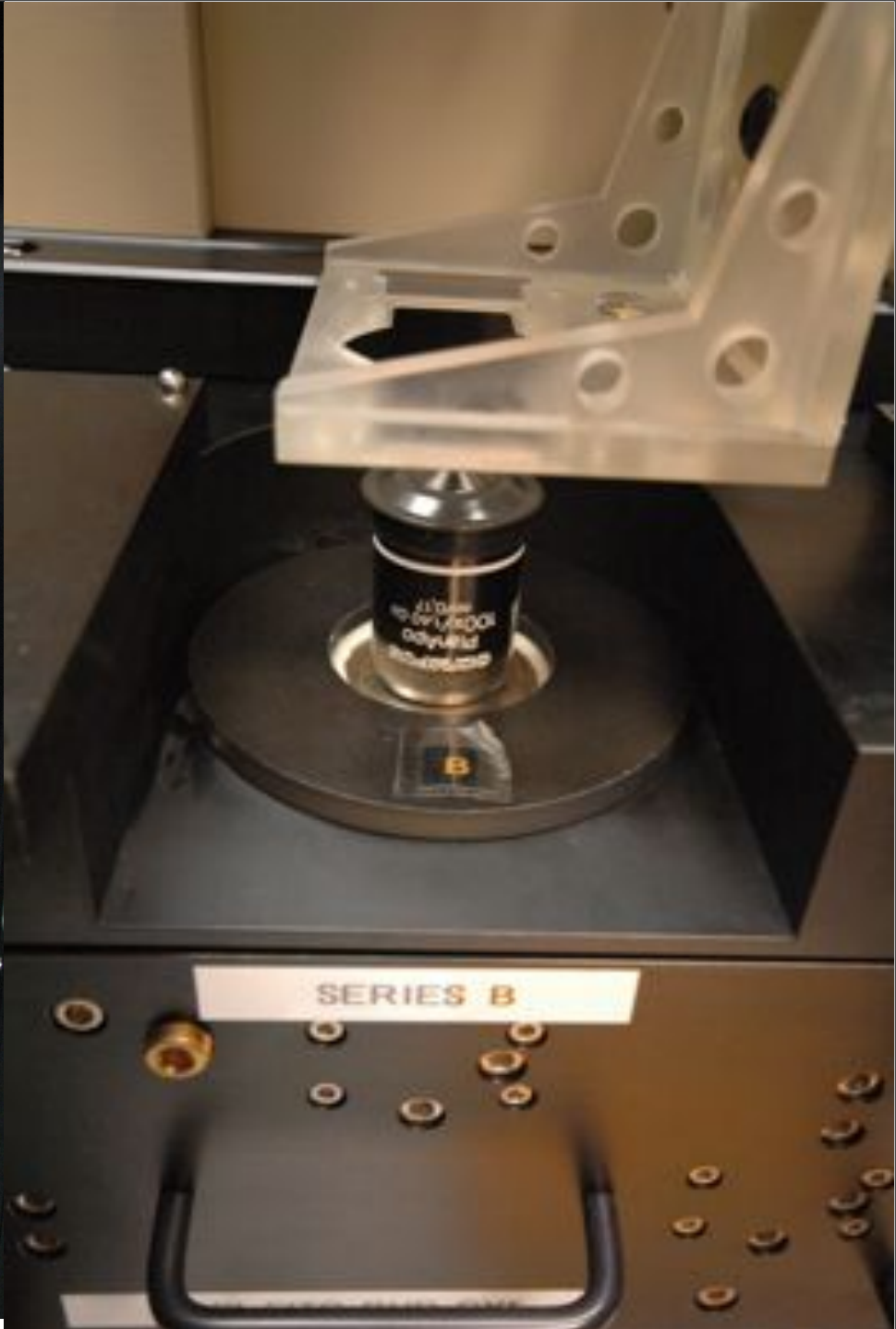


**4 laser excitation lines  
4 simultaneous acquisition lines CCDs**

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



Monday, 11 March 13

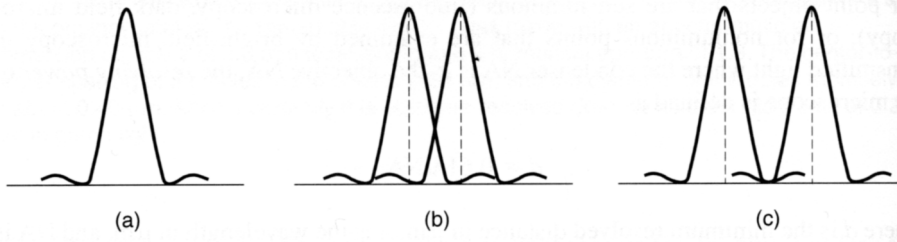
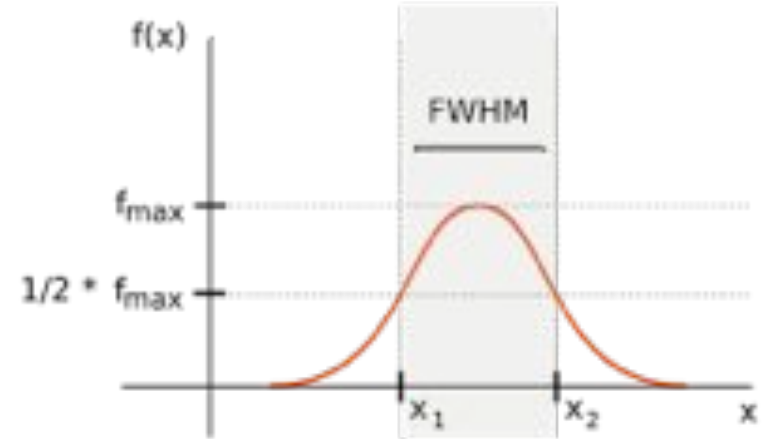
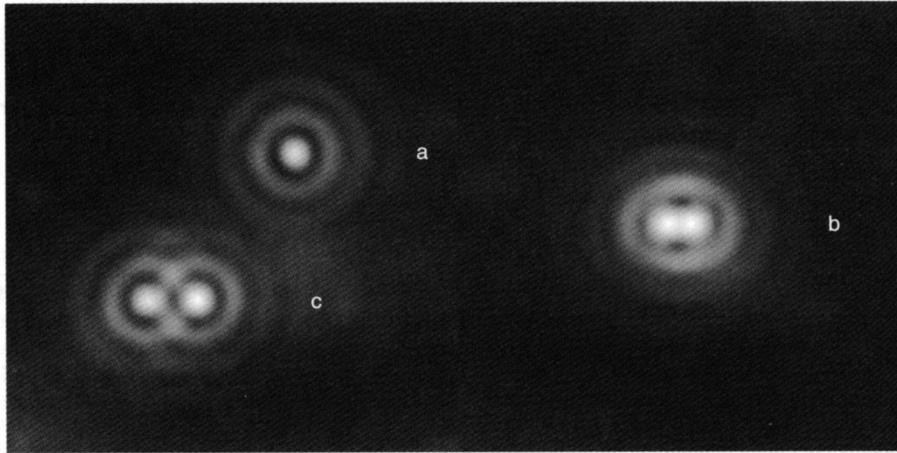


Monday, 11 March 13



Monday, 11 March 13

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



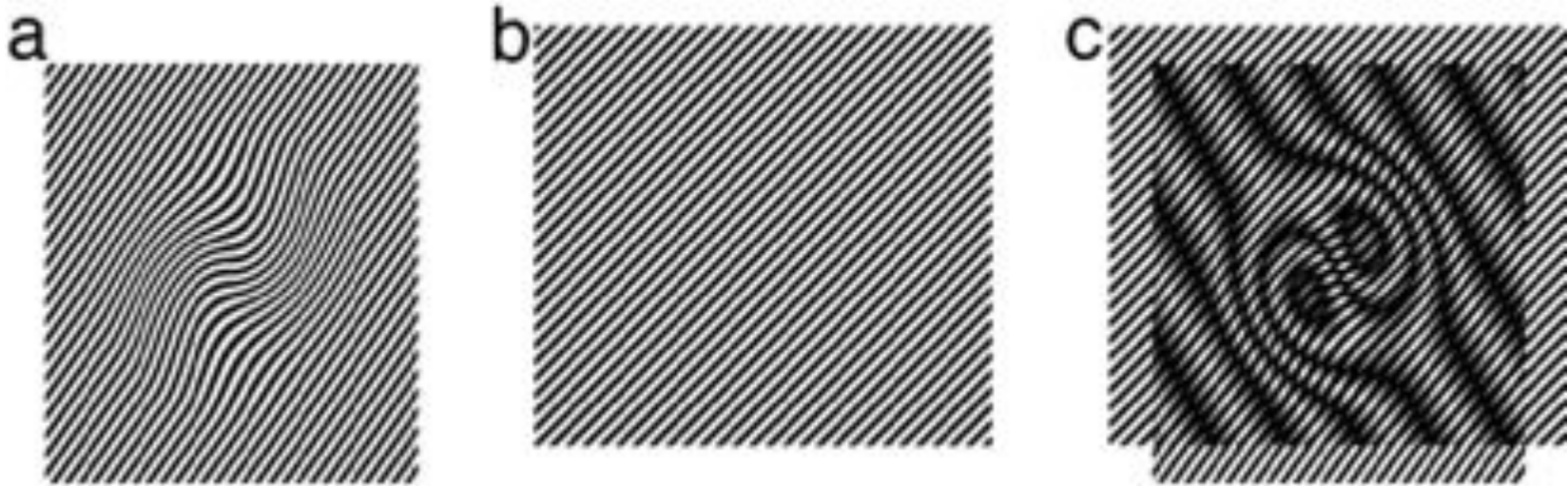
**How can we overcome this limit  
long standing limit?**

# 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

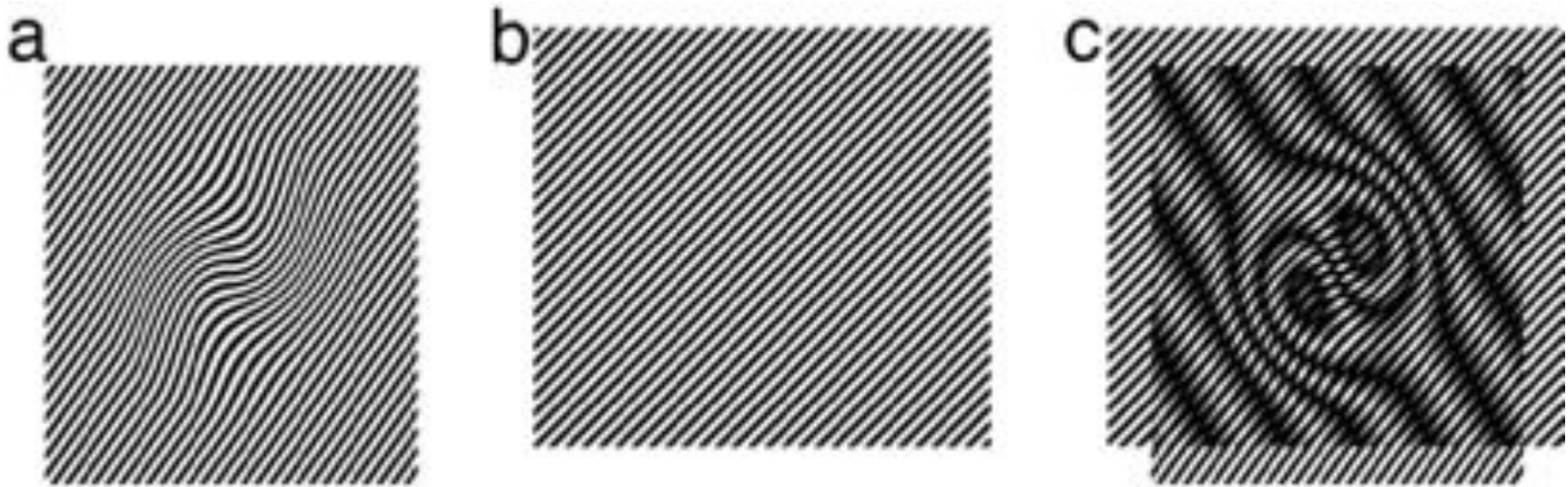


# Structured Illumination

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

## Resolution extension through Moire effect



# More tomorrow from Lothar



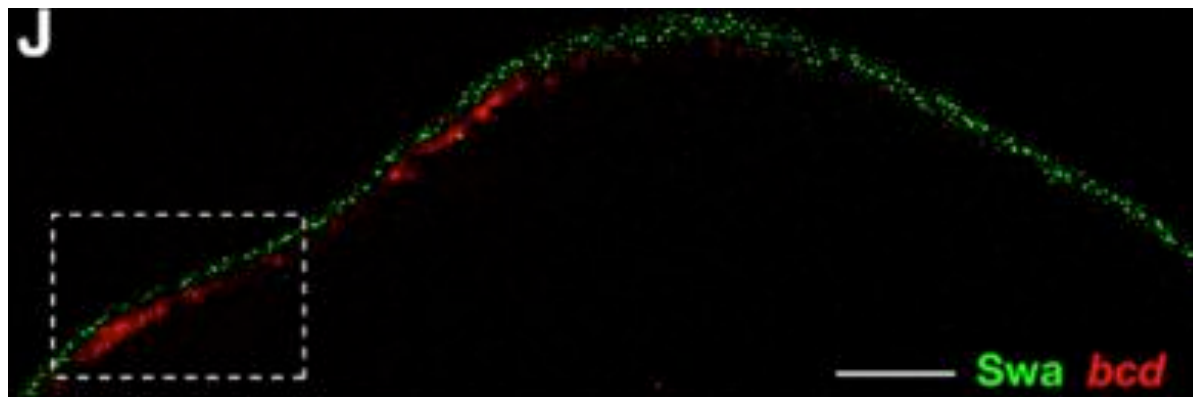
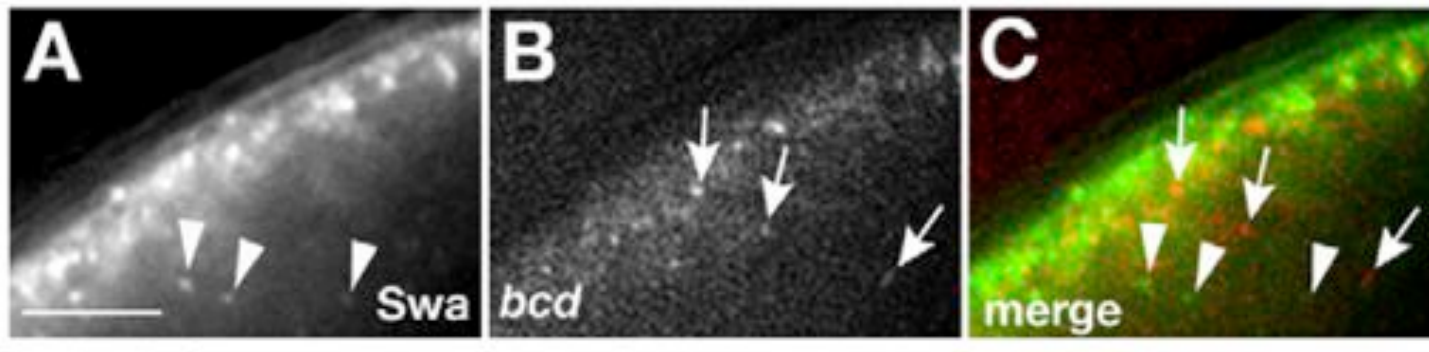
# Live multidimensional imaging on OMX

RESEARCH ARTICLE 169

Development 137, 169–176 (2010) doi:10.1242/dev.044967

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

Timothy T. Weill<sup>1,2,3</sup>, Despina Xanthakis<sup>1</sup>, Richard Parton<sup>3</sup>, Ian Dobbie<sup>3</sup>, Catherine Rabouille<sup>1</sup>, Elizabeth R. Gavis<sup>2,\*</sup> and Ilan Davis<sup>3</sup>



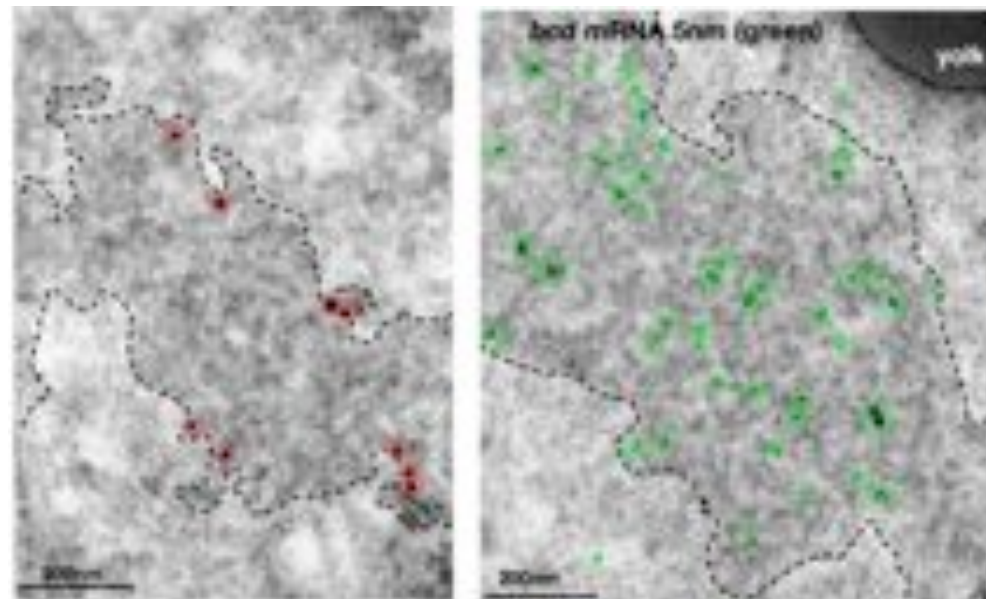
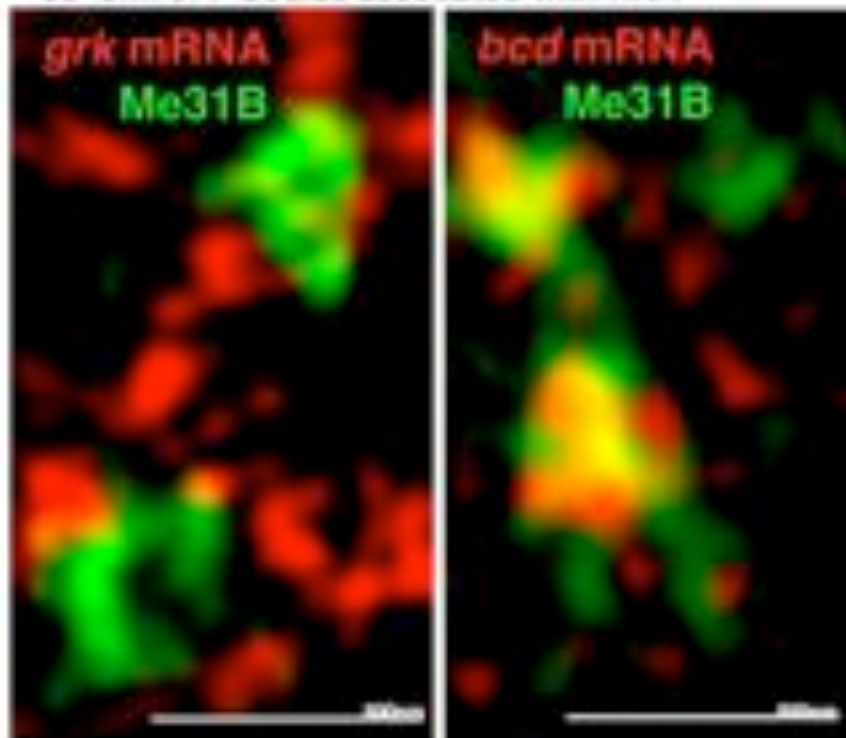
## *Drosophila* patterning is established by differential association of mRNAs with P bodies

Timothy T. Weil<sup>1,2</sup>, Richard M. Parton<sup>1,2</sup>, Bram Hejpers<sup>1,2</sup>, Jan Soutaert<sup>1,2</sup>, Elinke Vennema<sup>1</sup>, Despina Karthakis<sup>1,2</sup>, Ian M. Dobbie<sup>1</sup>, James M. Halstead<sup>1</sup>, Rippel Hayashi<sup>1</sup>, Catherine Rabouille<sup>1,2</sup> and Ian Davis<sup>1,2</sup>

NATURE CELL BIOLOGY VOLUME 14 | NUMBER 12 | DECEMBER 2012

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### 3D-SIM of P bodies associated with RNA



# **THE FUTURE: 3D SIM live on V3-blaze**

**3D Structured  
Illumination**

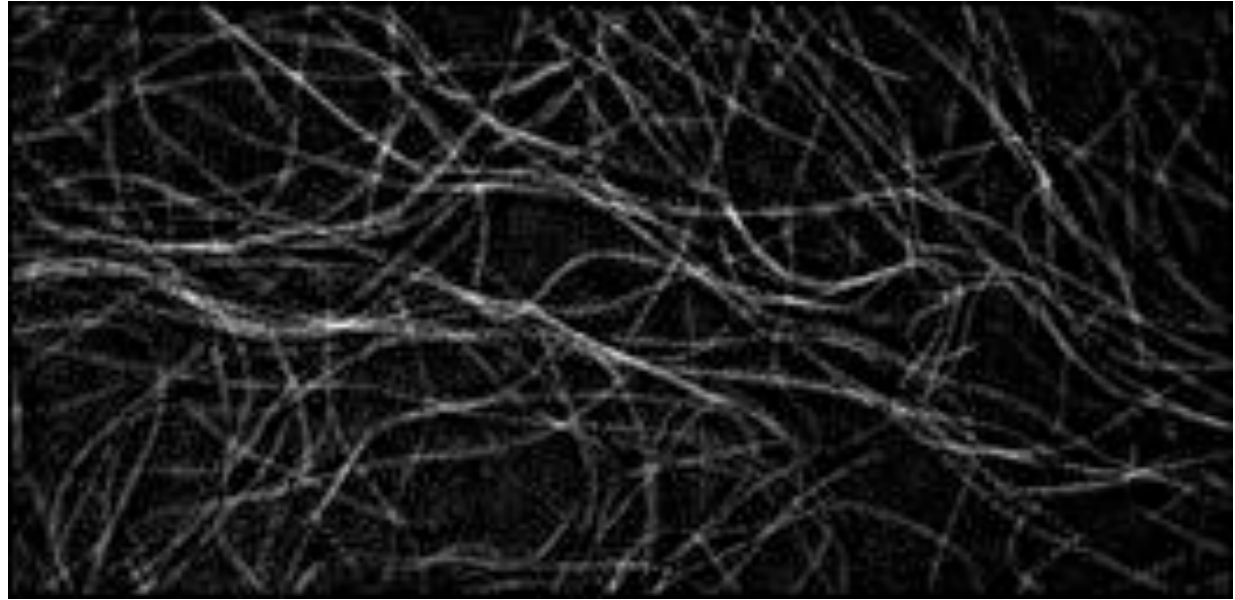
**Conventional  
Widefield  
deconvolution**

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

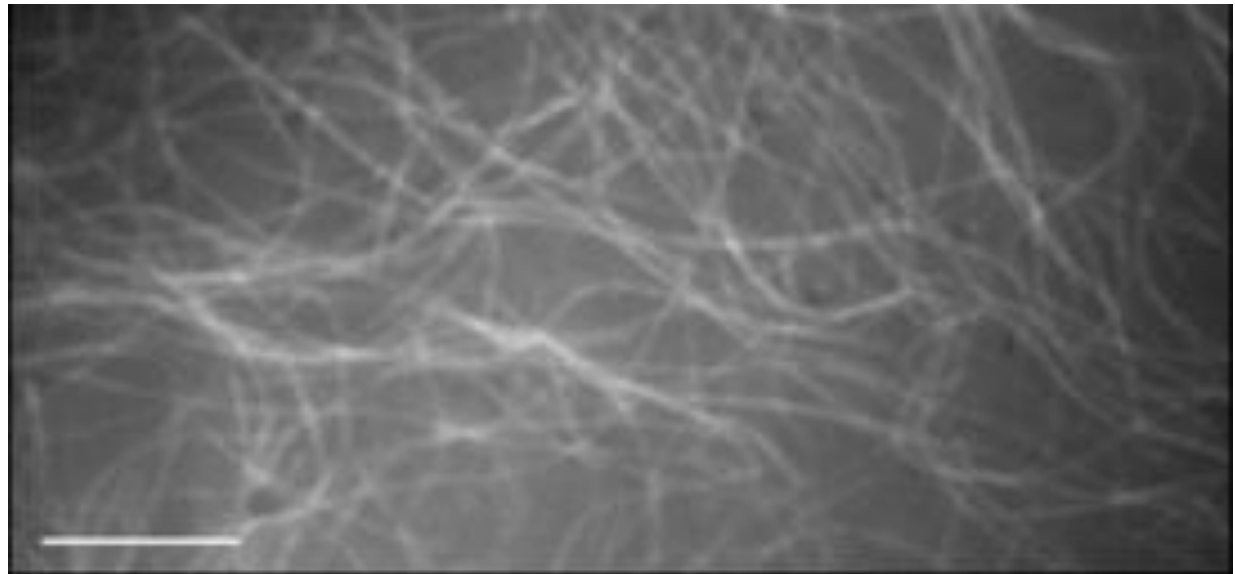
**Parton, Goodwin, Atkins**

# THE FUTURE: 3D SIM live on V3-blaze

**3D Structured  
Illumination**



**Conventional  
Widefield  
deconvolution**



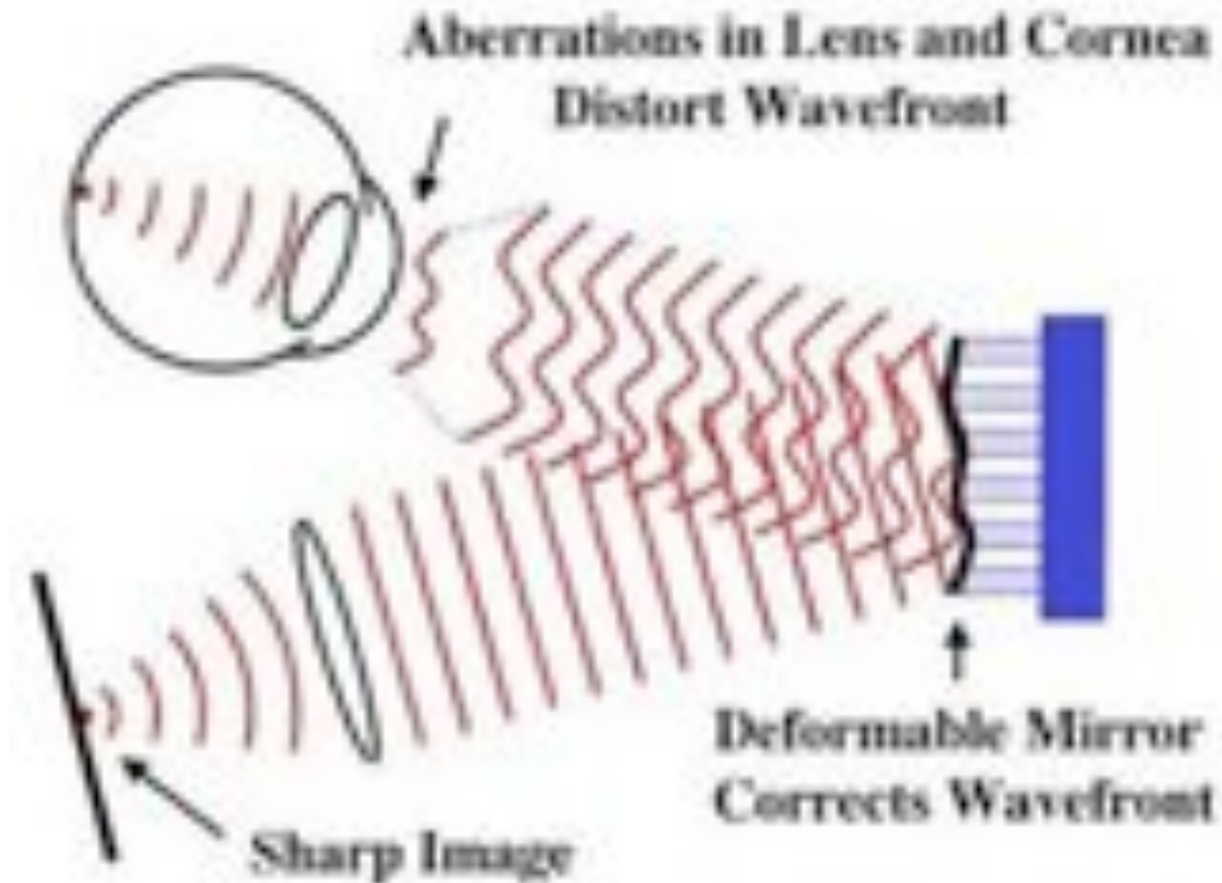
**MT: Jupiter-GFP - captured at API.  
(1fps, 30 time points)**

**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

## Adaptive Optics Kits

- ▶ Kit Includes Deformable Mirror, Shack-Hartmann Wavefront Sensor, and All Necessary Optics / Hardware
- ▶ Closed-Loop Operation via Stand-Alone Control Software
- ▶ Out-of-Box Functionality



Deformable Mirror



Shack-Hartmann Wavefront Sensor



## Related Products



**The hard part - algorithms for shaping the deformable mirror**

**Martin Booth - Engineering / CNSB, Oxford**

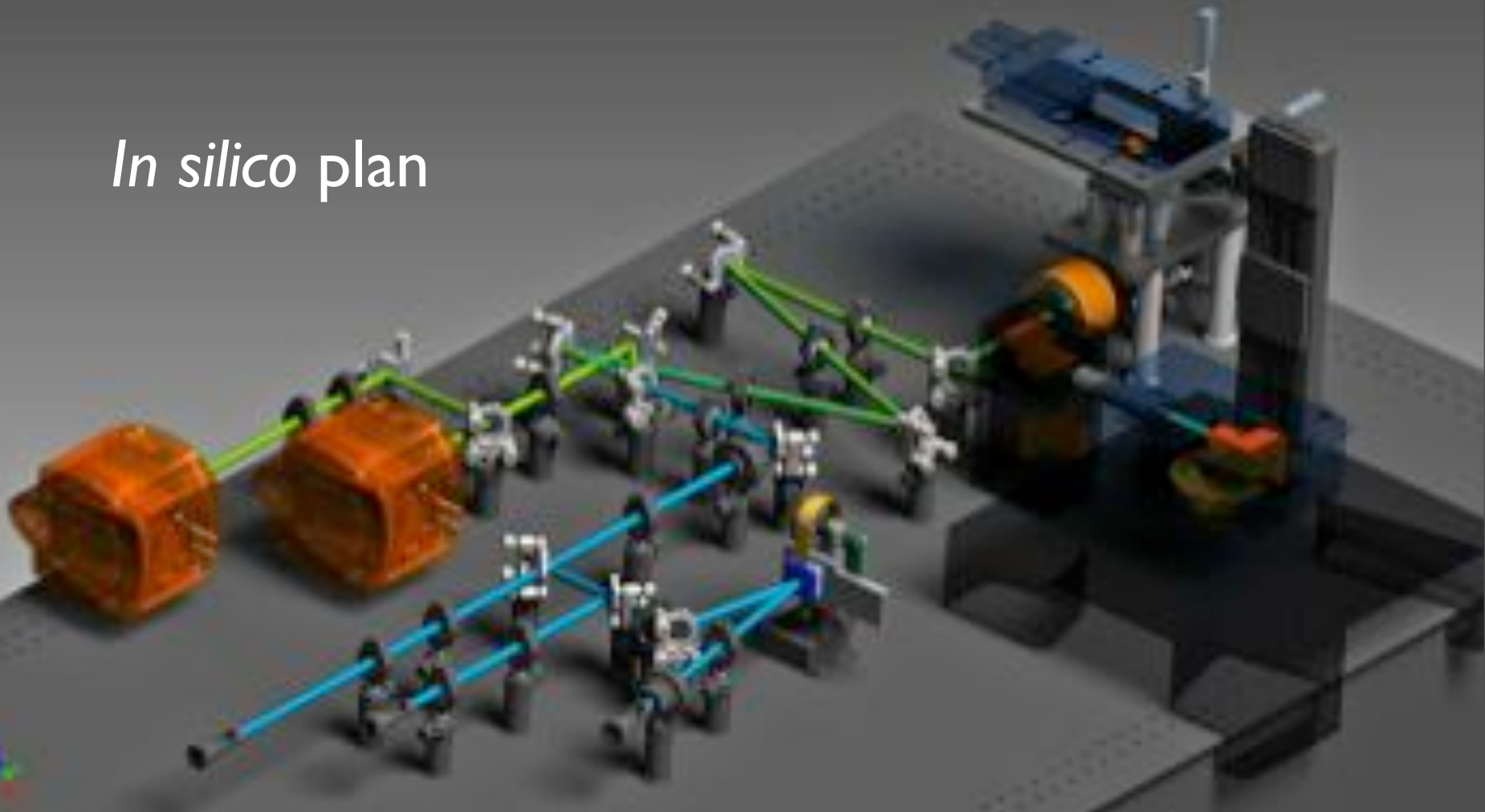


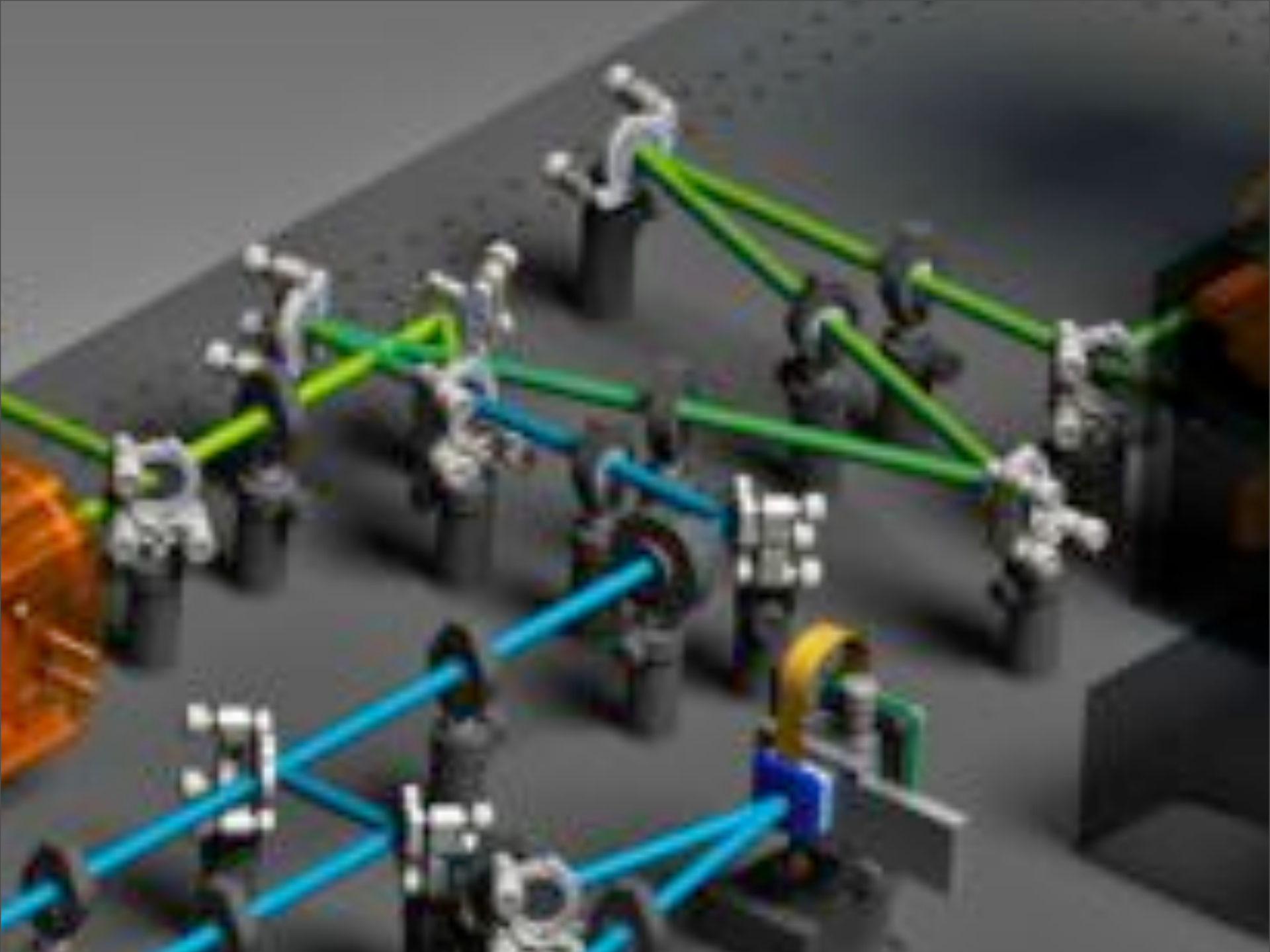
# Further development: OMX-T

Rainer Kauffman  
and Ian Dobbie

Based on John Sedat's  
design

*In silico* plan

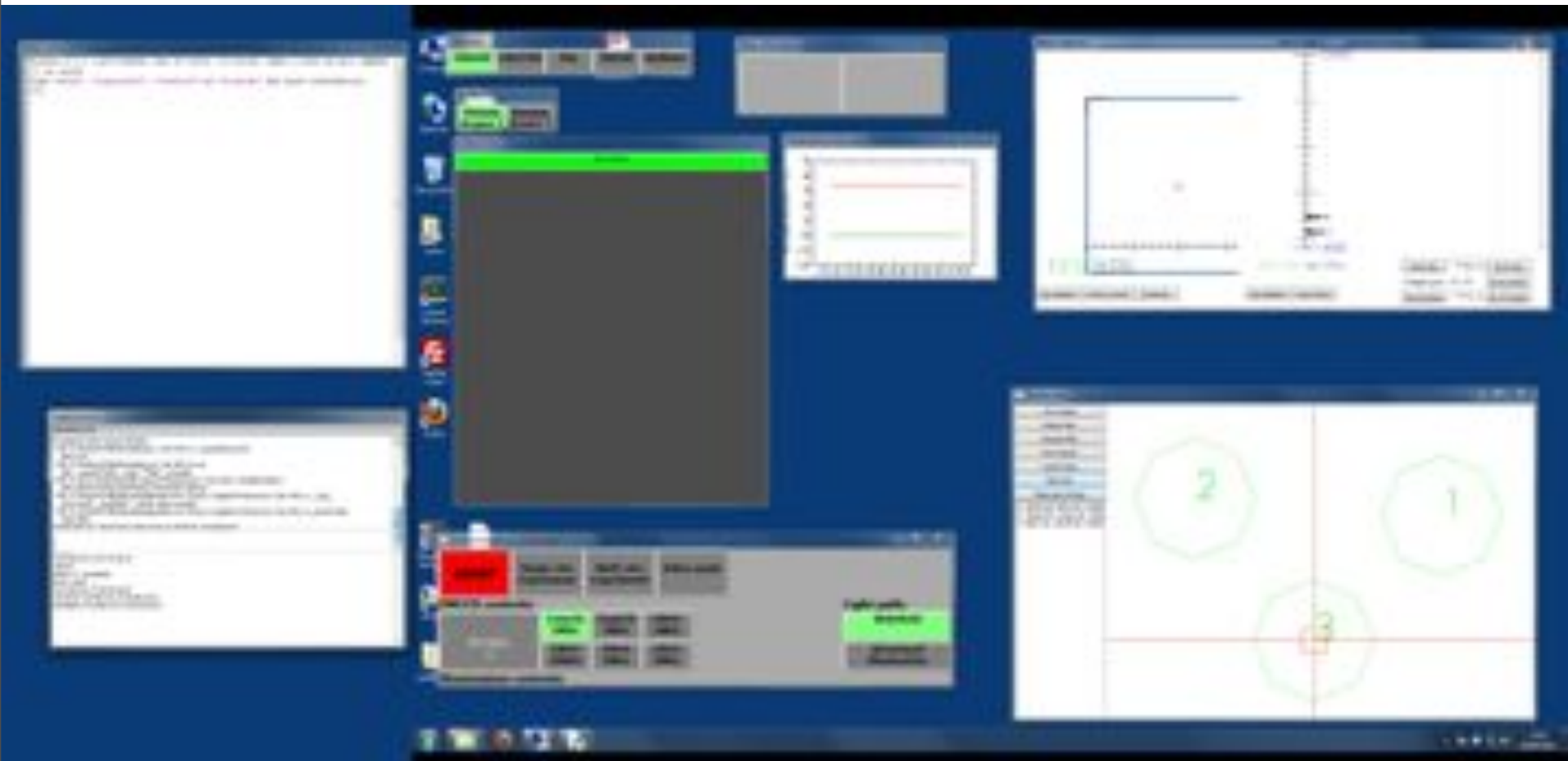




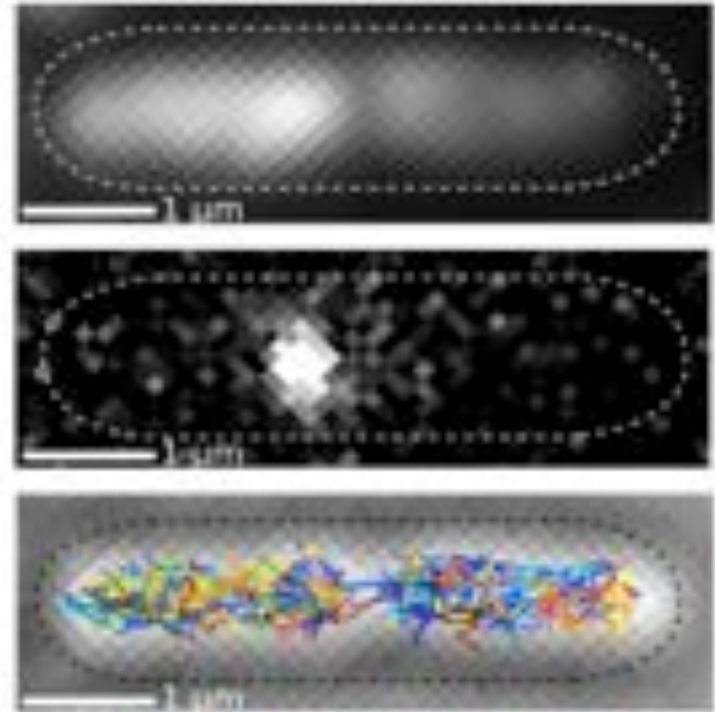
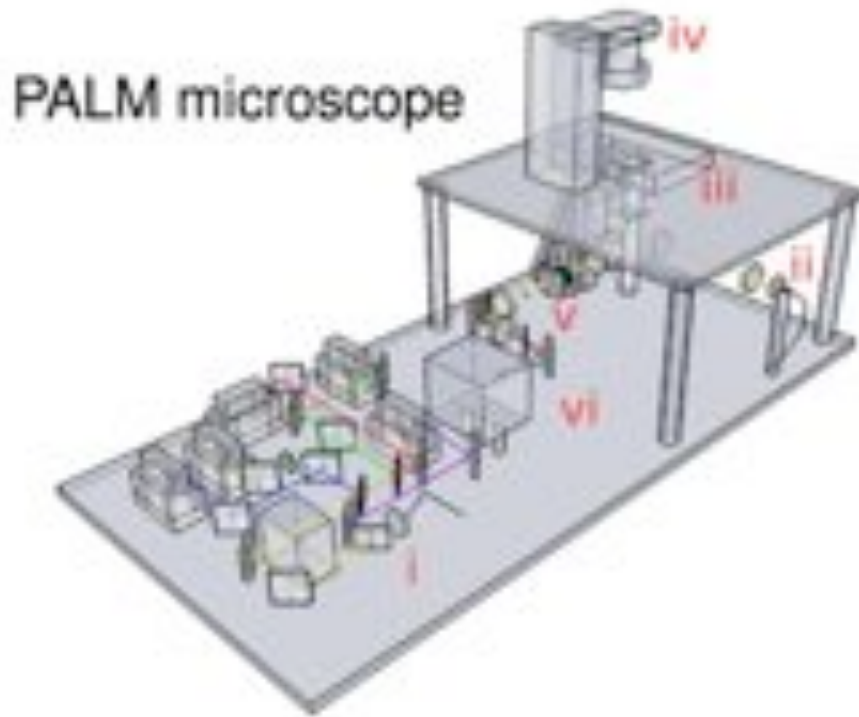
Monday, 11 March 13



# Cockpit - from John Sedat



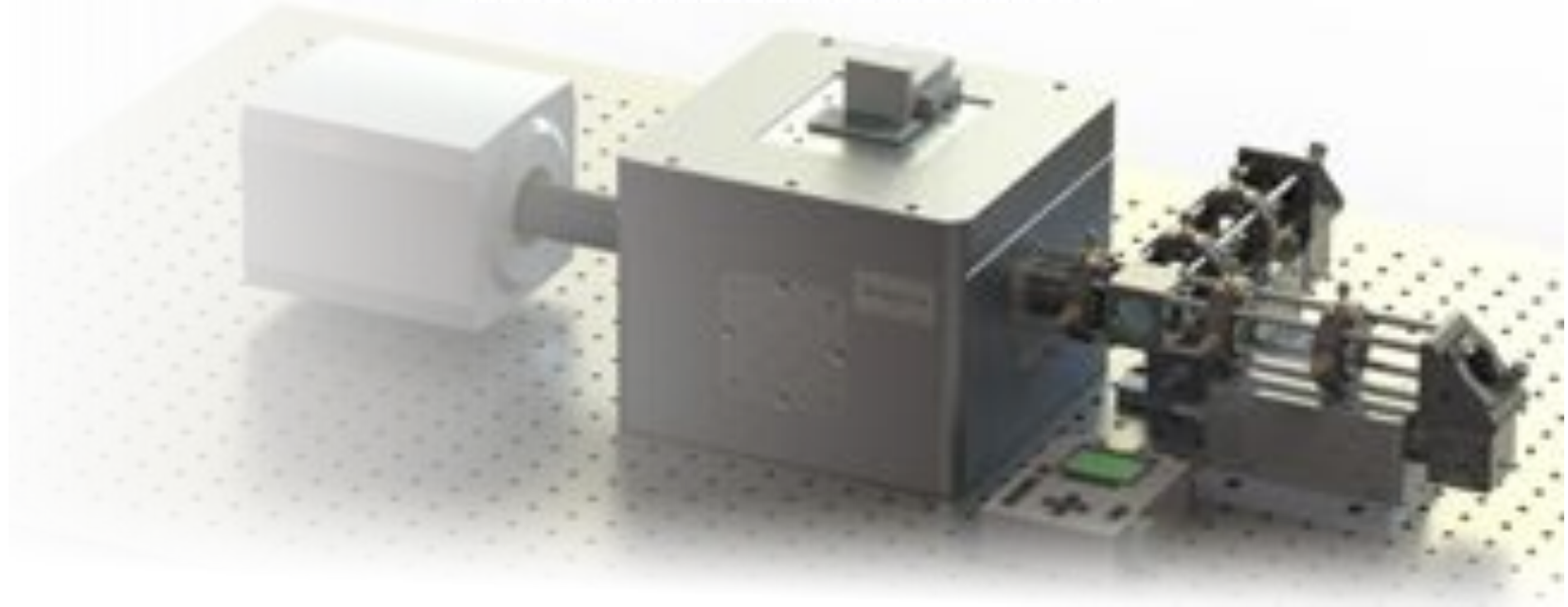
# Home built PALM / DSTORM



**Stephan Uphoff and Achillefs Kapanidis**

# Half way houses

Warwick Open Source Microscope  
WOSM

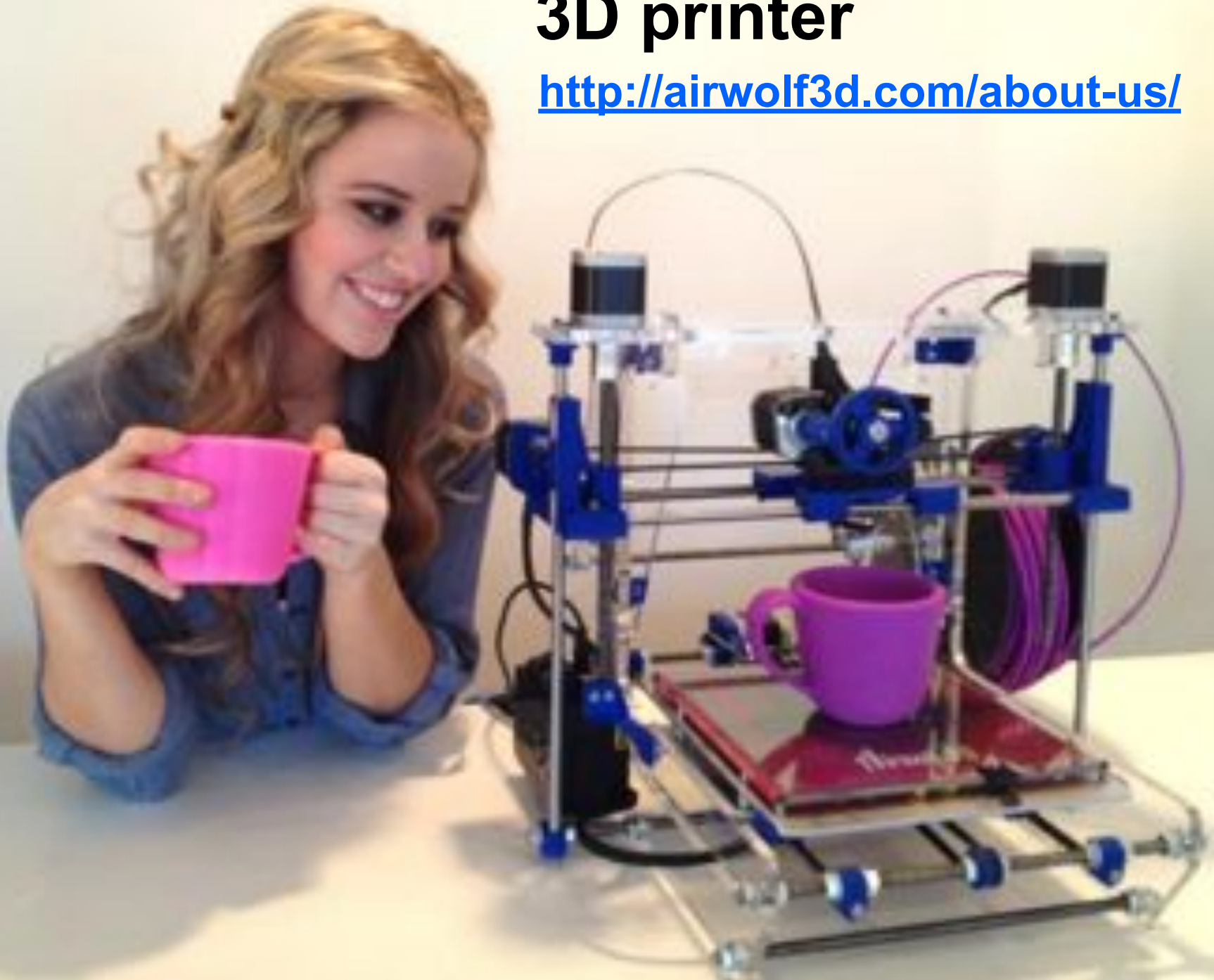


<http://wosmic.org>

Nick Carter and Rob Cross

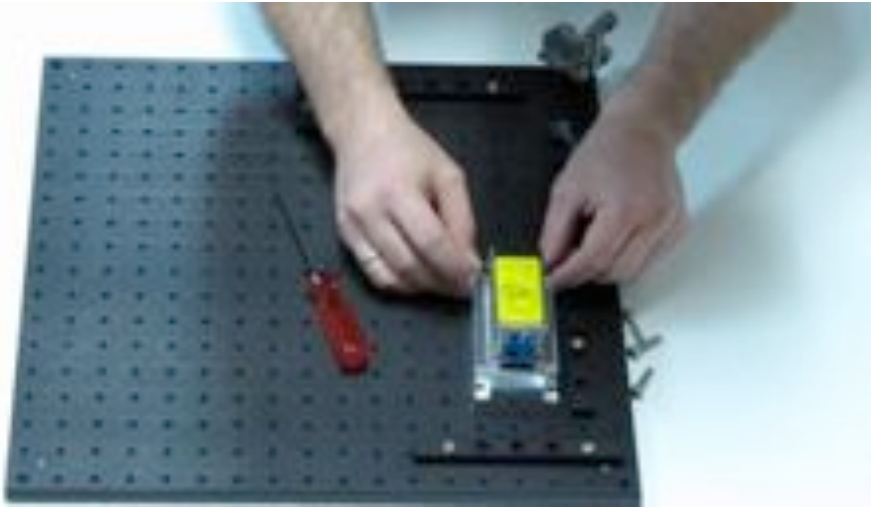
# 3D printer

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



# Openspim

[http://openspim.org/Welcome\\_to\\_the\\_OpenSPIM\\_Wiki](http://openspim.org/Welcome_to_the_OpenSPIM_Wiki)



**SPIM Farm**

- Home
- Help list
- Security
- Operation
- Frequently asked Questions
- Getting
- News
- Downloads
- Recent changes
- Twitter
- What links here
- Special changes
- Special pages
- Privacy policy
- Disclaimers

## People



**Pavel Tomancak**

Research Group leader of the MPI-CBG in Dresden. Provides ideas, concepts, research questions and funding.



**Jan Hulskan**

Research Group leader of the MPI-CBG in Dresden.



**Peter Gabriel Fibene**

Microscopy technician in the Tomancak world. Designed and built the OpenSPIM.



**Kevin Elwell**

Director of LOCI at University of Madison. Collaborator and provides free cell imaging expertise on Open SPIM project.



**Johannes Schindelin**

Senior Programmer at LOCI at University of Madison. Wrote the original OpenSPIM steering interface. Develops and maintains Fiji.



**Luke Stuyvenberg**

Student Programmer at LOCI at University of Madison. Works on the original OpenSPIM steering interface.

**The End**