LECTURE 9
Advanced Widefield Microscopy
Ilan Davis, November 2014

- 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

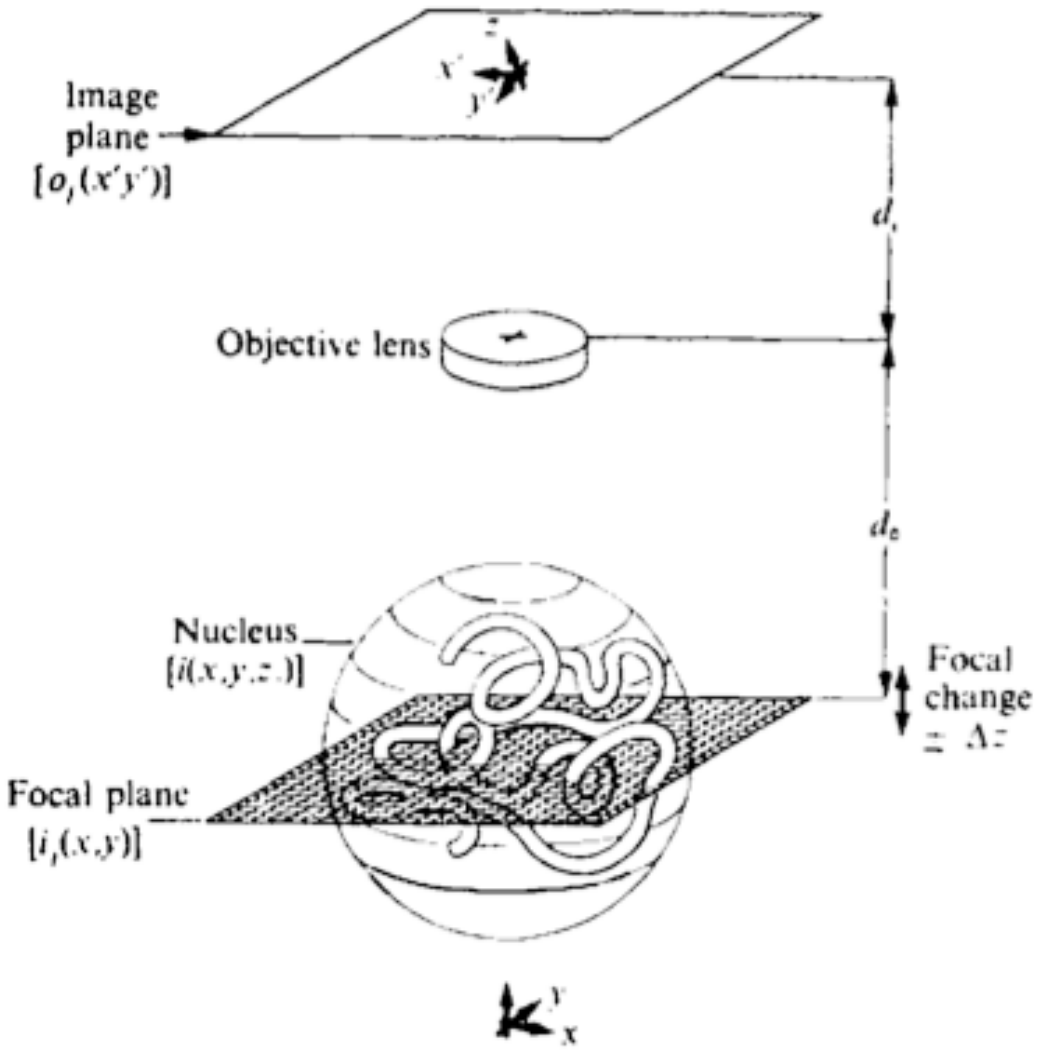
http://www.msg.ucsf.edu/agard/Publications/9-Agard-Nature-83.pdf

Reprinted from Nature, Vol. 302, No. 5910, pp. 676-681, 21 April 1983

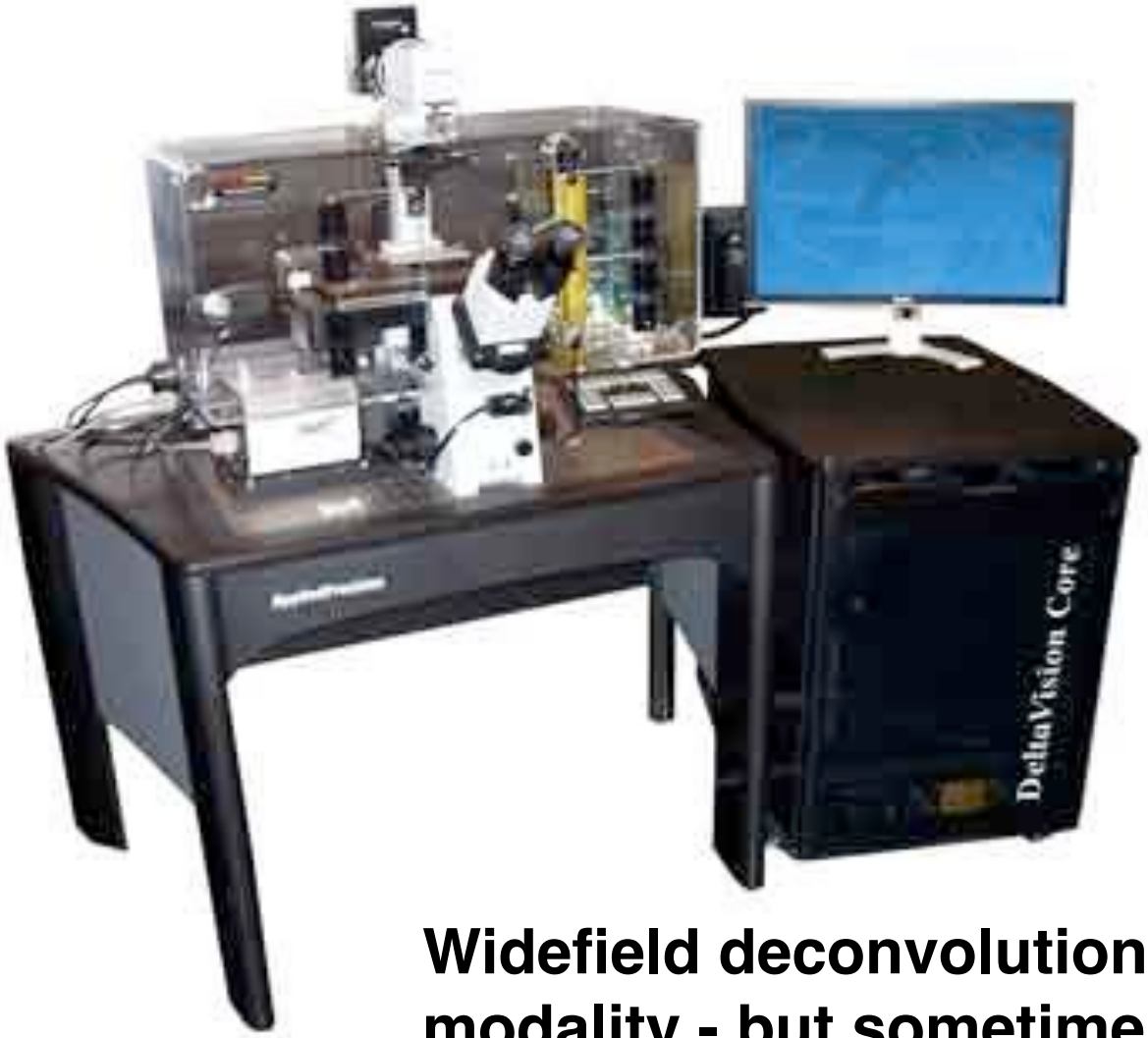
Three-dimensional architecture of a polytene nucleus

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

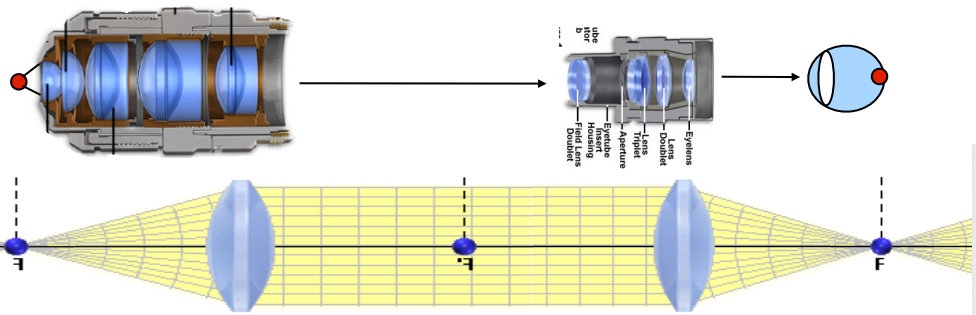


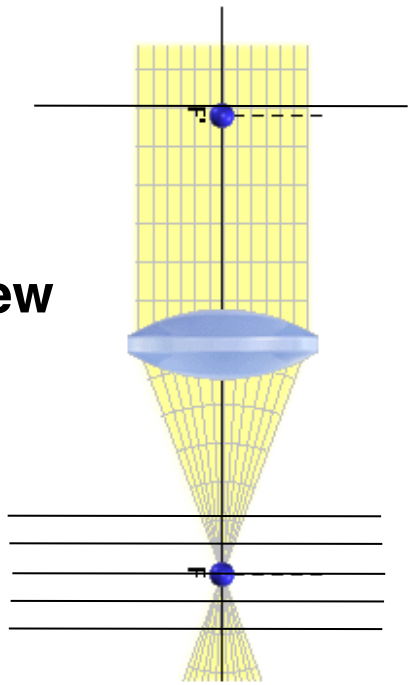
Widefield Fluorescence microscopy (Olympus + Sedat/Agard DeltaVision)



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

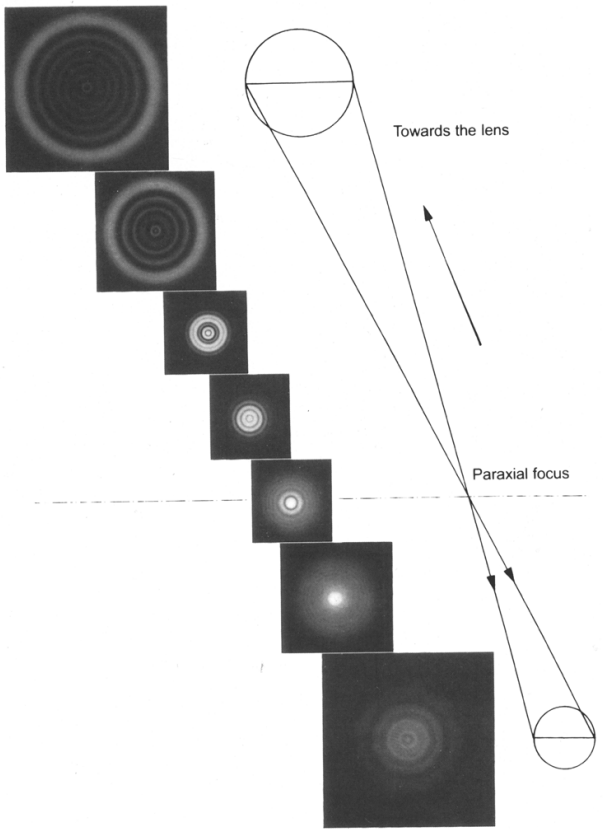
Image formation



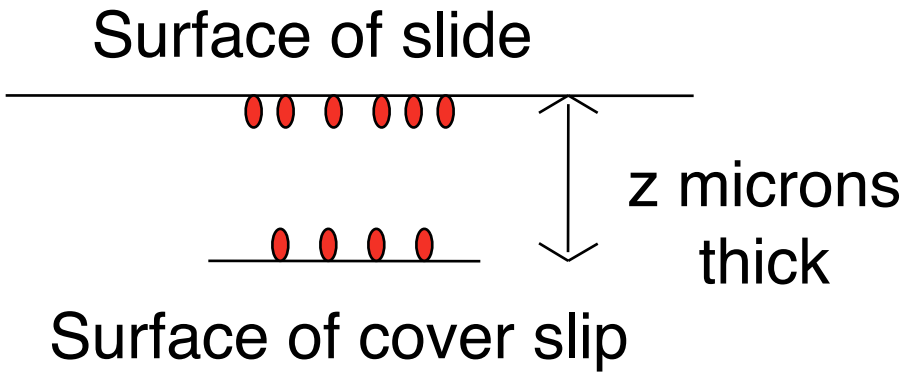


Out of focus light
-Airy rings in 3D view

Different Focal plains



Bead slide

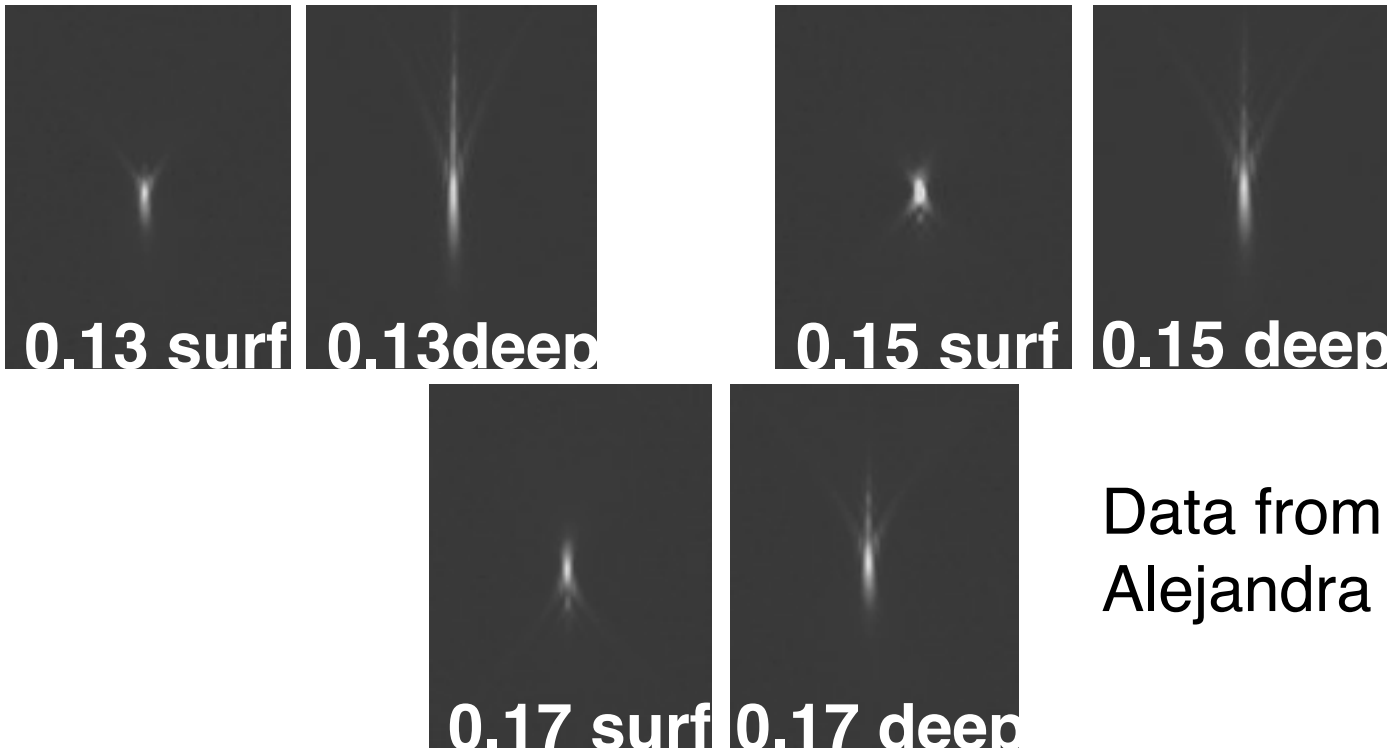


Tetraspeck beads: chromatic registration
DAPI/FITC/Rhodamine/Cy5

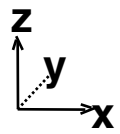
Beads (PS Spec): Single fluorochrome
Brighter -better for generating
point spread functions for deconvolution

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

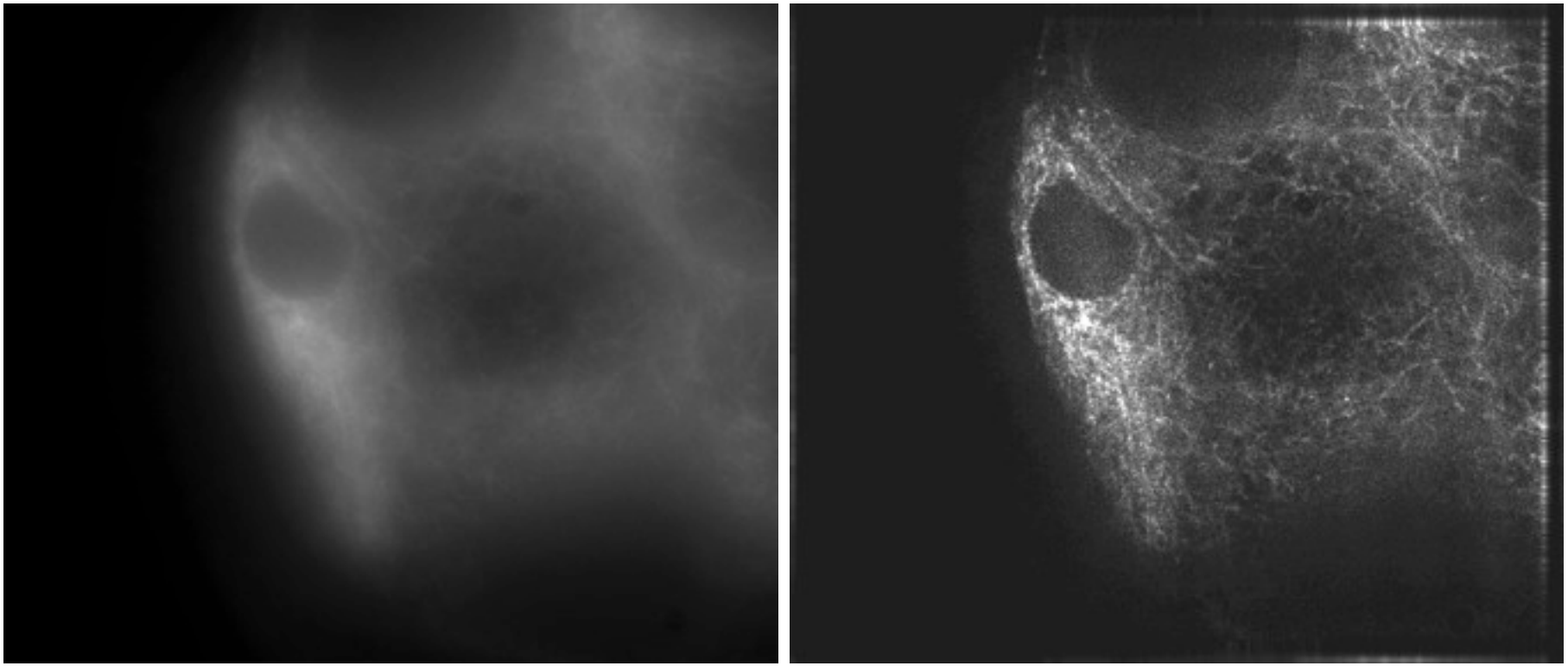
XLPN25XSVMP

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)

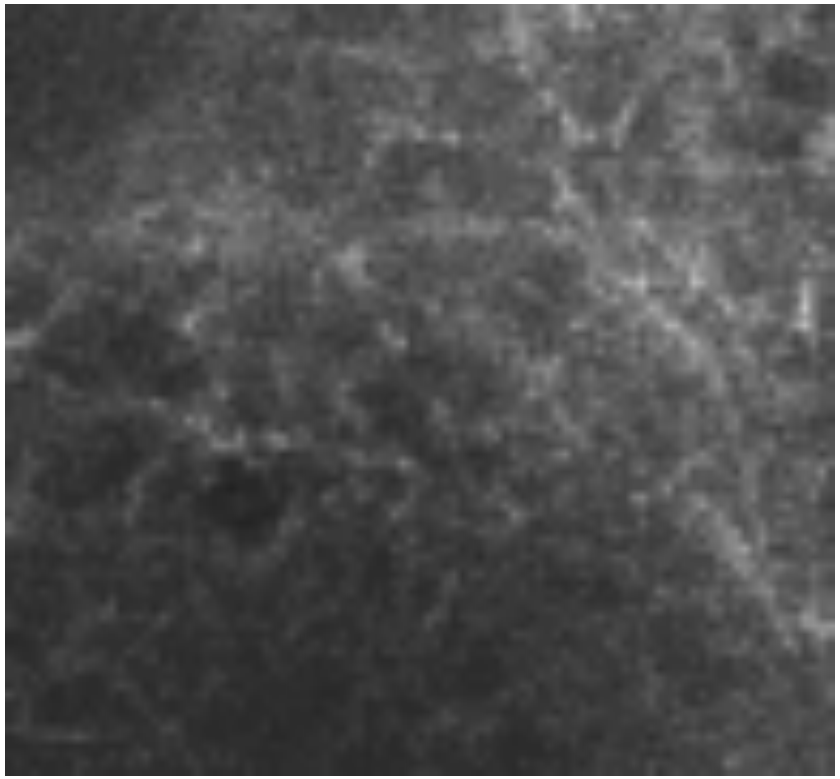
Before Deconvolution **After Deconvolution**

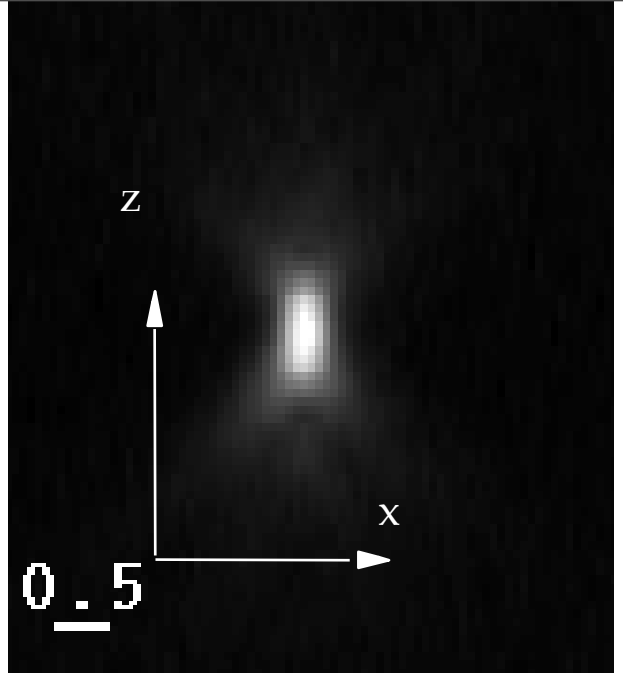


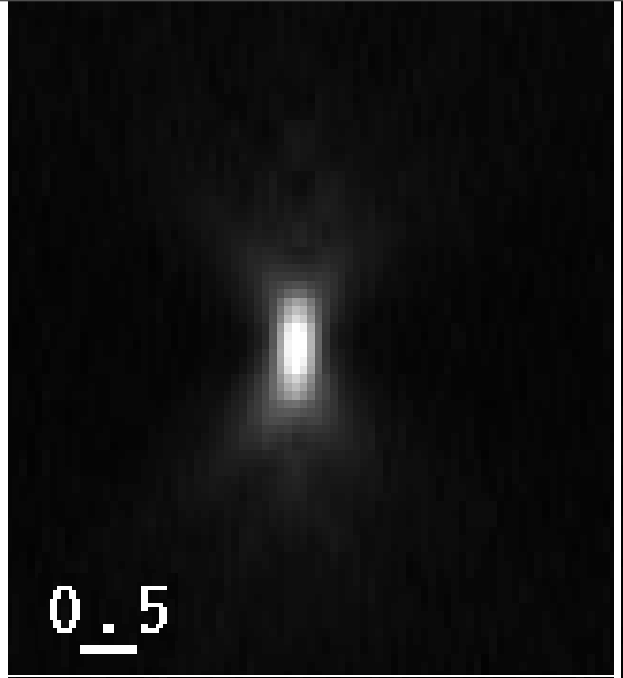
tauGFP (microtubules) in a *Drosophila* oocyte **Richard Parton**

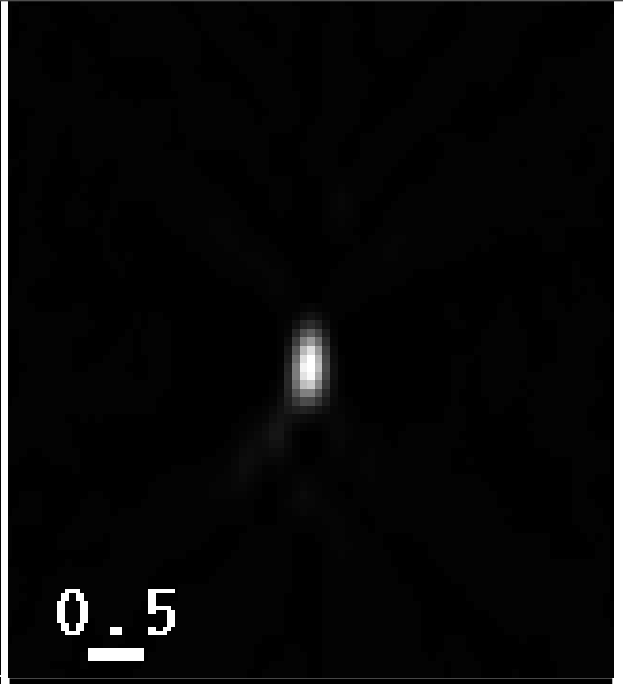
Before Deconvolution

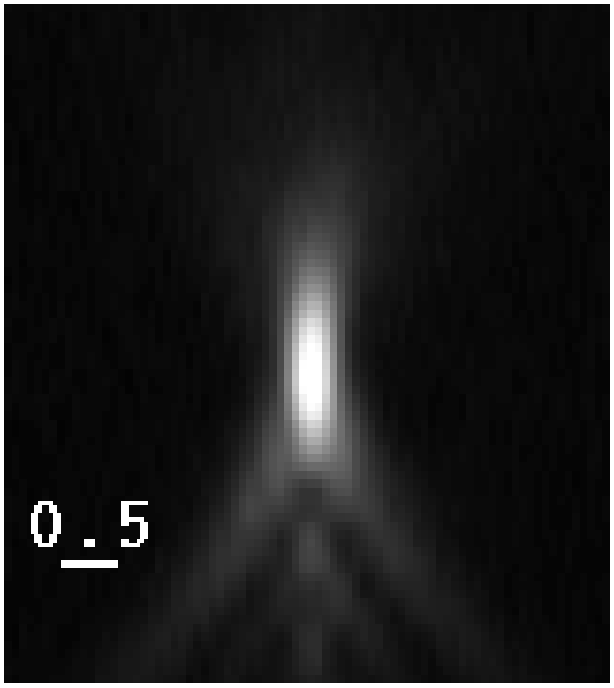
After Deconvolution

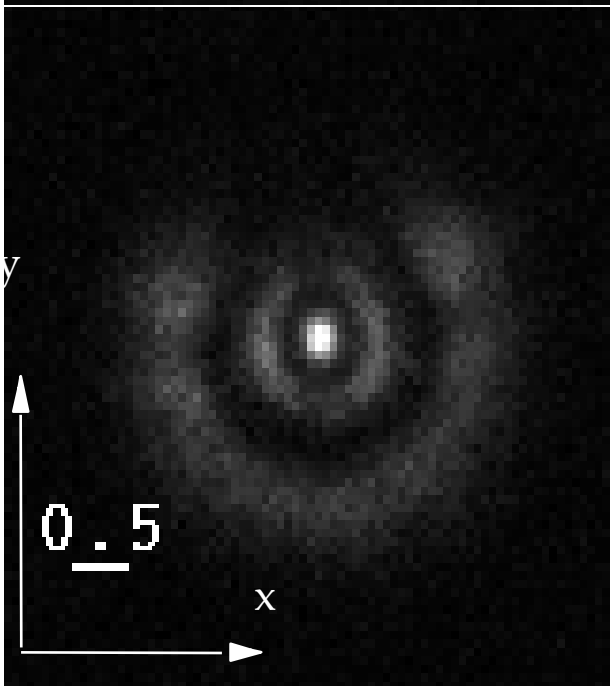












133	135	149	134	160	165	133	147	123
171	167	159	133	119	118	124	148	164
194	199	192	173	156	151	158	175	193
216	237	237	235	221	217	208	214	223
256	290	288	288	281	283	259	271	259
268	328	365	420	440	434	359	324	282
271	362	460	600	663	636	490	378	297
256	396	643	954	1140	1058	760	477	313
246	420	752	1171	1419	1296	903	520	310
233	439	857	1379	1691	1522	1027	551	296
197	483	1057	1801	2226	1937	1253	614	271
195	497	1126	1928	2386	2066	1311	622	257
208	510	1162	1982	2437	2111	1323	619	253
258	544	1193	1969	2370	2063	1301	613	263
286	562	1173	1893	2251	1959	1241	596	278
313	585	1129	1760	2073	1791	1156	577	300
365	622	1000	1407	1630	1378	930	514	357
386	629	916	1215	1388	1154	819	491	373
401	613	819	1026	1148	946	709	467	372
411	526	613	678	704	614	518	420	328
406	477	511	523	509	458	425	387	307
387	419	420	405	364	340	349	345	284
321	327	307	320	268	260	271	261	245
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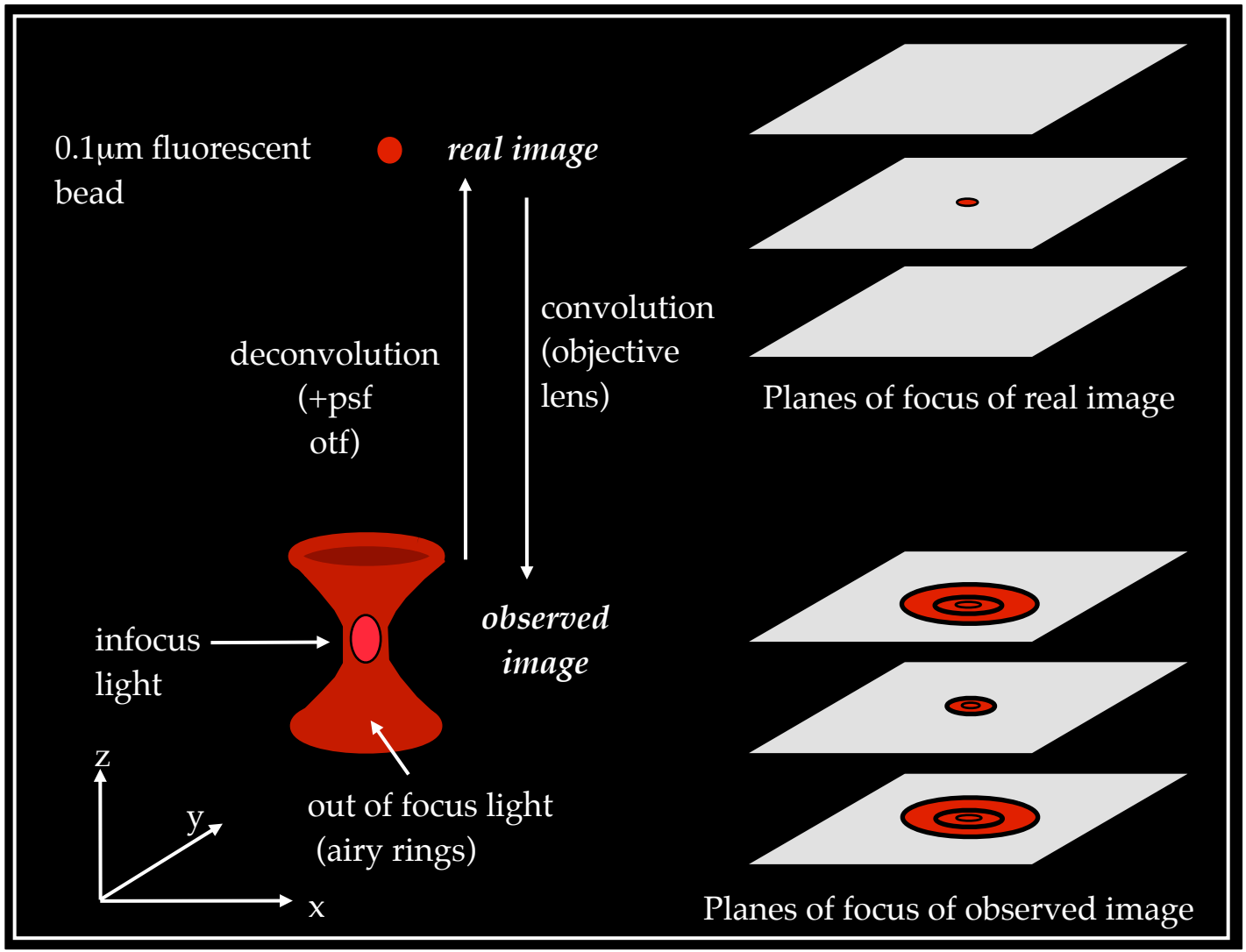
238	216	193	205	163	165	185	177	186

Ilan Davis, 2000



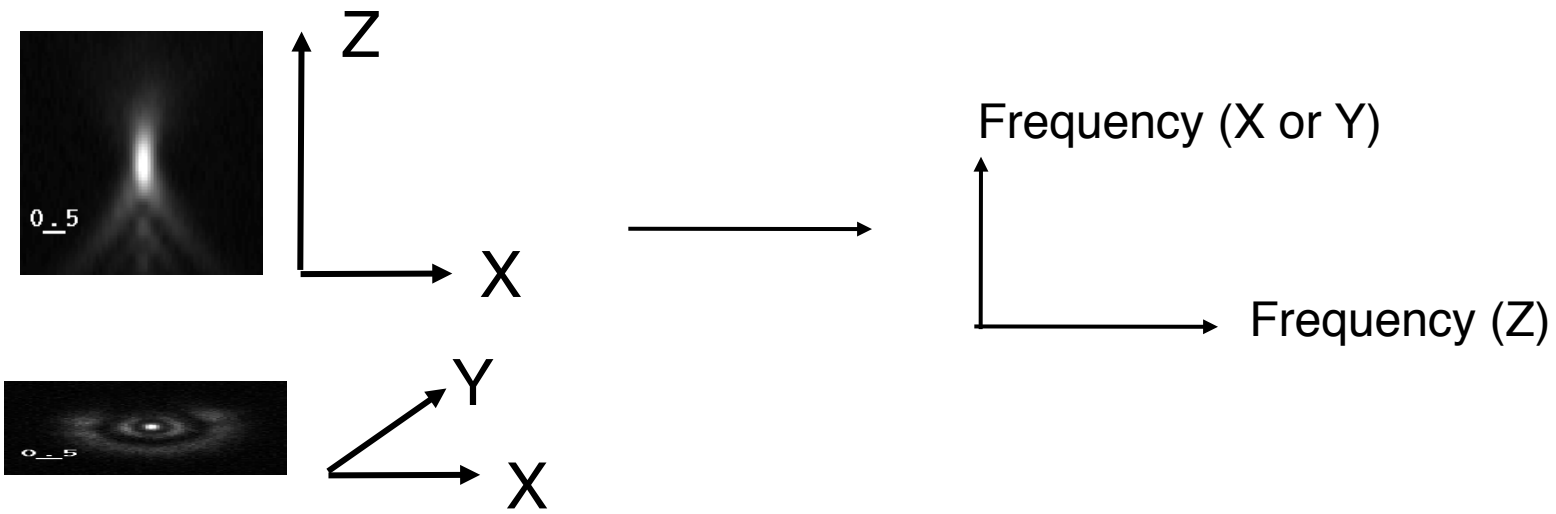
Ilan Davis, 2000

Deconvolution

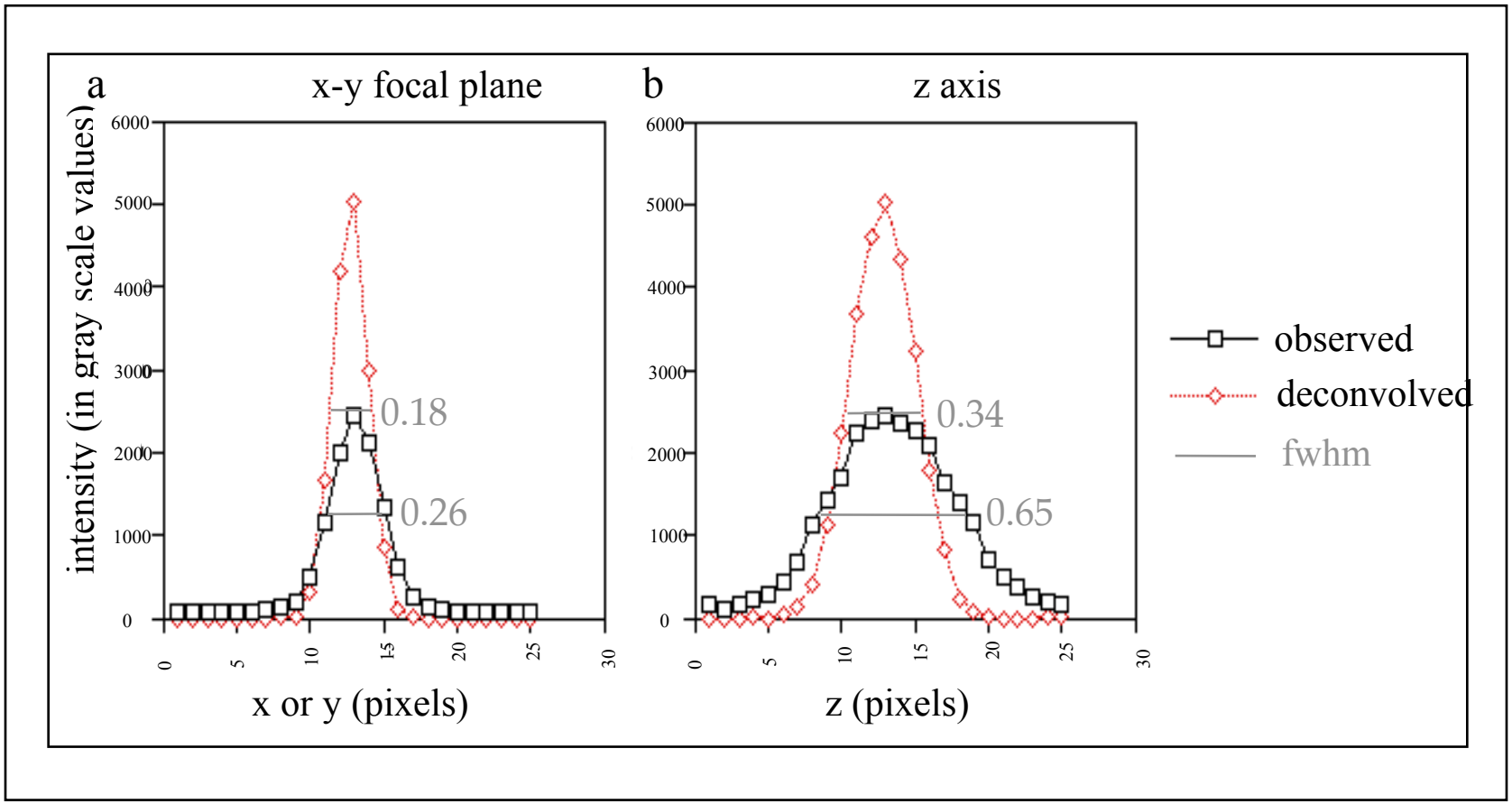
Calculations done in Fourier (frequency) space not XYZ space.
Uses Fast Fourier Transforms - much faster algorithm (developed in the 1960s)
Psf is converted to optical transfer function (only information in X and Z)
Several methods that vary in their implementation

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

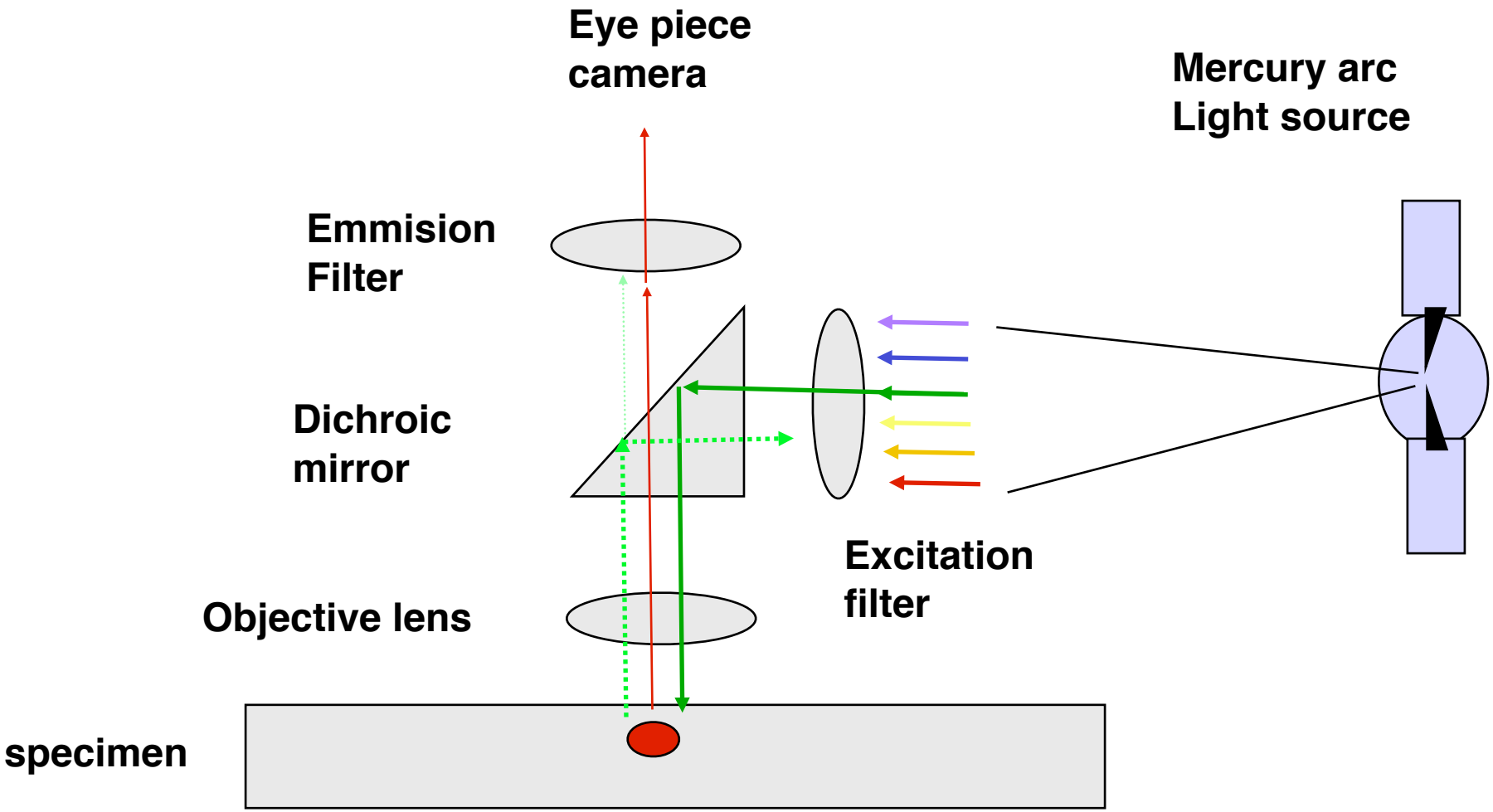
New methods (Sedat)

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

•Myopic deconvolution

Reminder

How do fluorescence microscopes work ?



Filter cubes

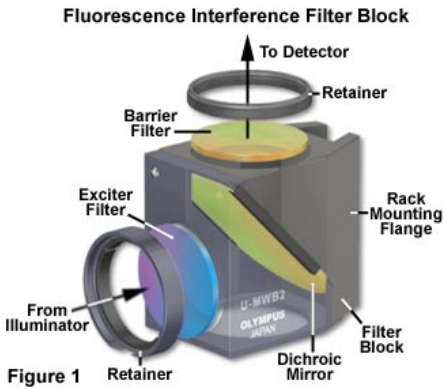
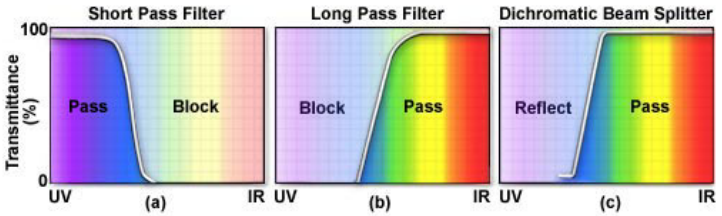


Figure 1



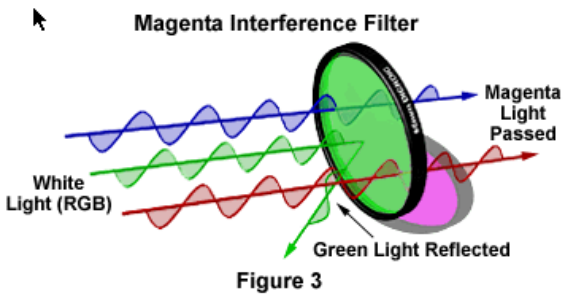
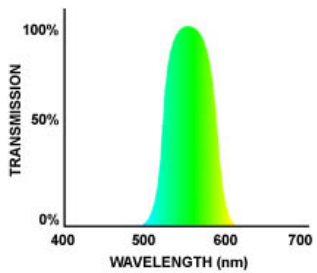
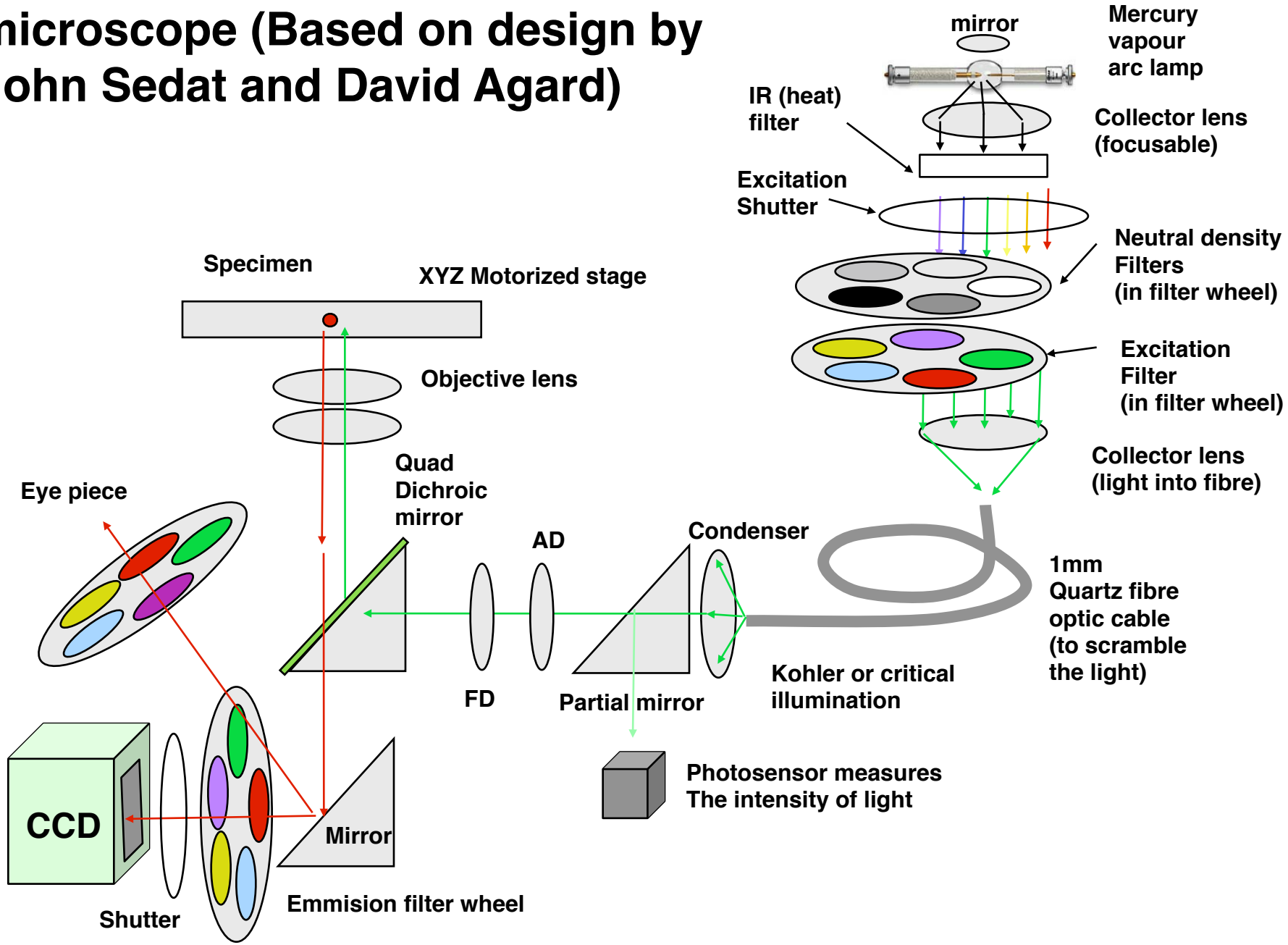


Figure 3

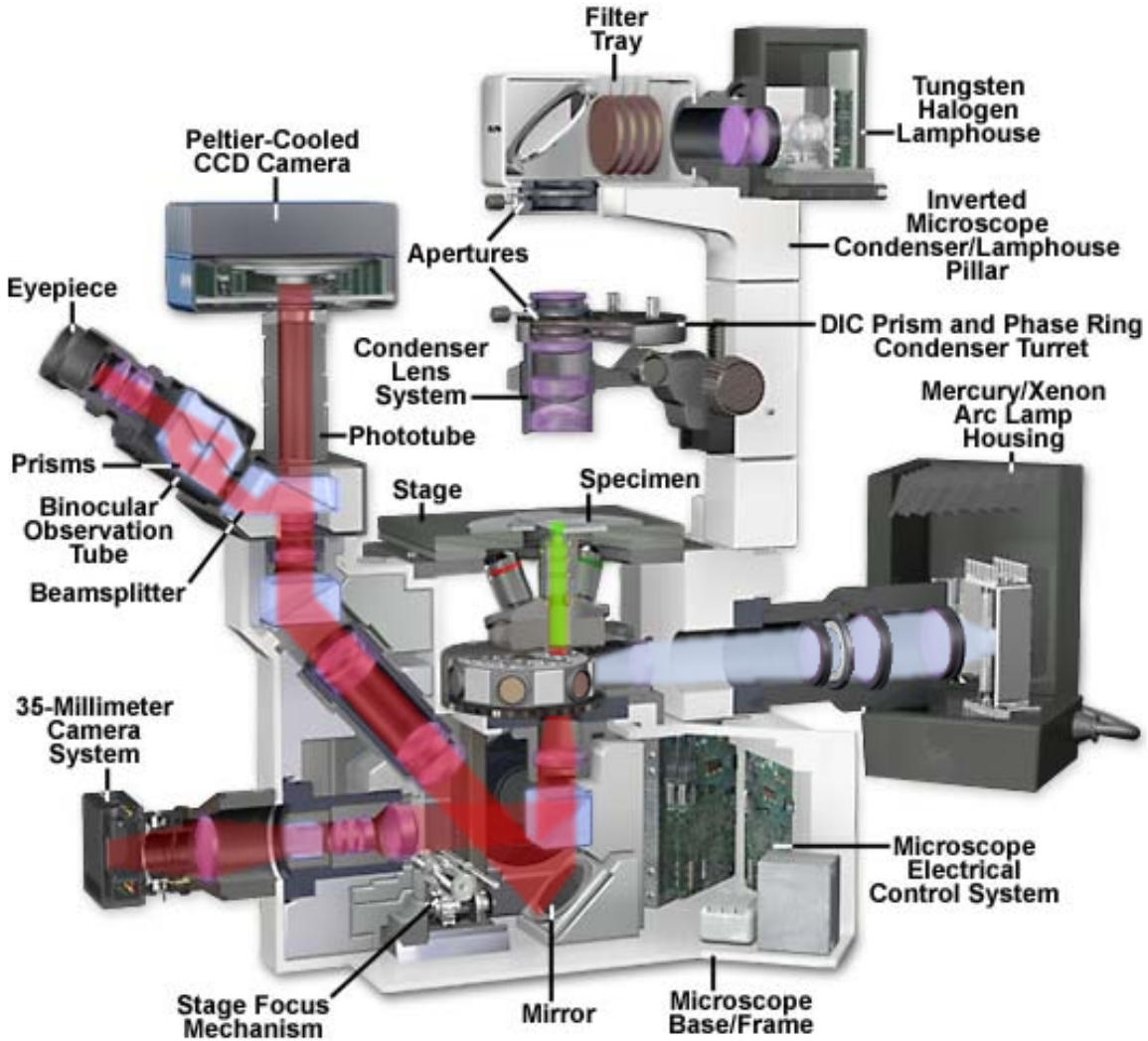


http://www.chroma.com/pdf/handbook4.pdf

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?

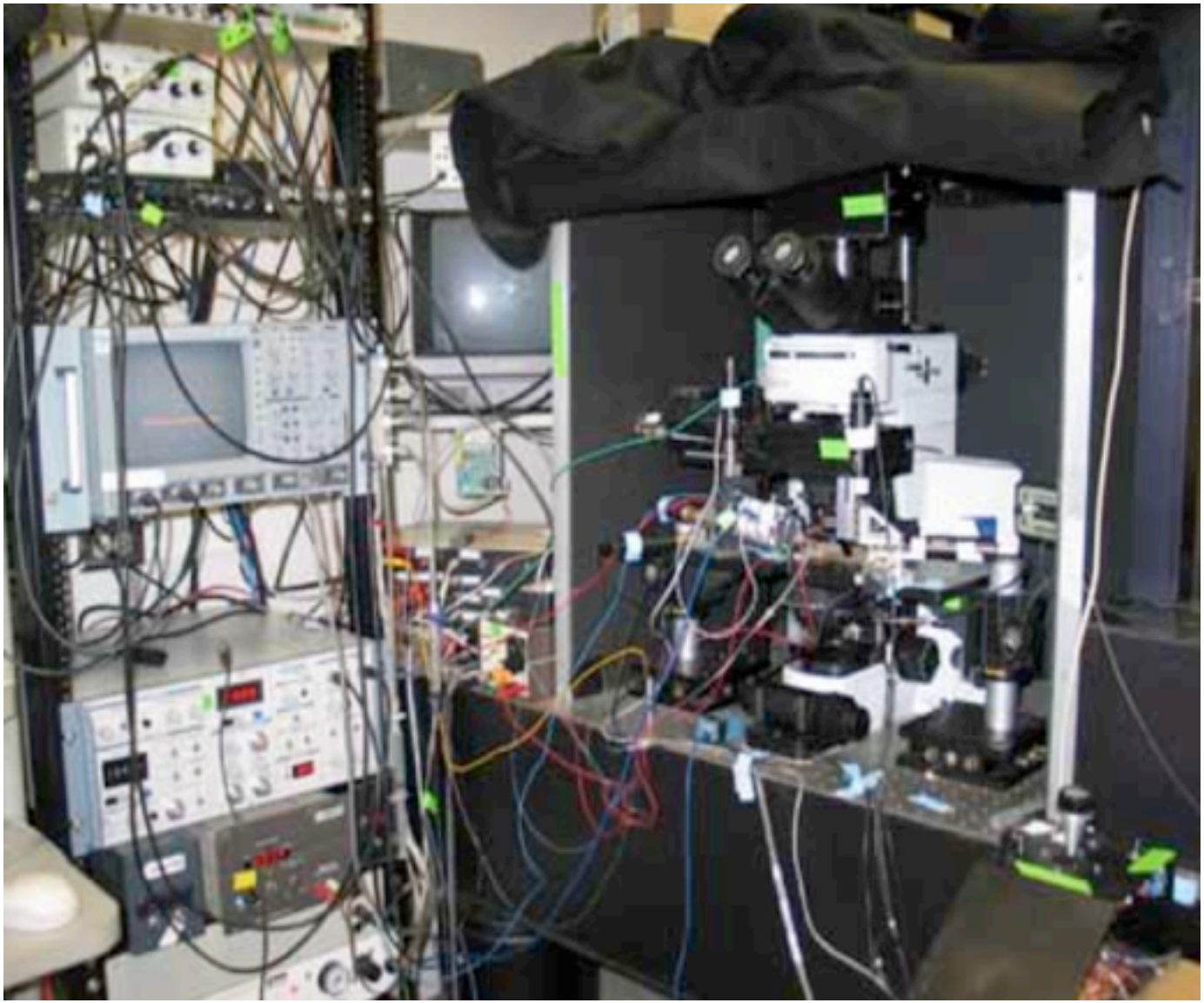
Specific applications -better than commercial microscopes

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

vibration isolation table

The basic ingredients

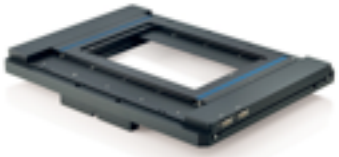
Objective lens and holder

vibration isolation table

The basic ingredients

Objective lens and holder

Stage



vibration isolation table

The basic ingredients

Objective lens and holder

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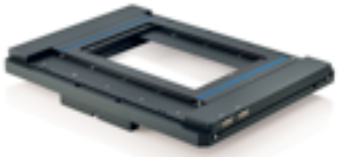

Cameras

vibration isolation table

The basic ingredients

Electronics

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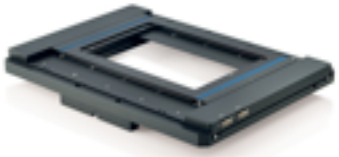

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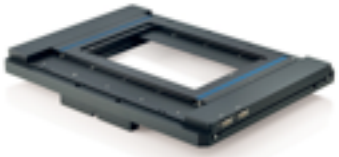


Cameras

vibration isolation table

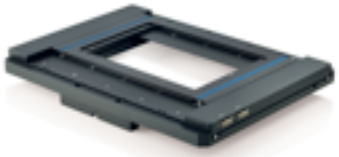
Electronics

Timing board TTL outputs

The basic ingredients

Objective lens and holder

Stage



Cameras

vibration isolation table

Electronics

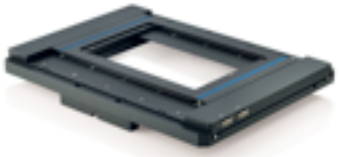
Timing board TTL outputs

Computer (normally a PC)

The basic ingredients

Objective lens and holder

Stage



Cameras

vibration isolation table

Electronics

Timing board TTL outputs

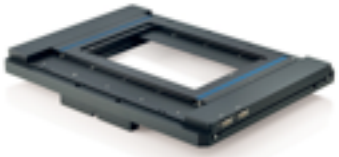
Computer (normally a PC)

Optics

The basic ingredients

Objective lens and holder

Stage



Cameras

vibration isolation table

Electronics

Timing board
TTL outputs

Computer (normally a PC)

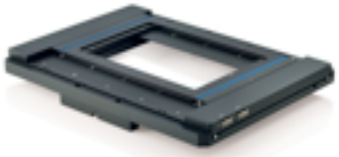
Optics

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

The basic ingredients

Objective lens and holder

Stage



Cameras

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Timing board
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Optics

Posts, flip mirrors, dichroics
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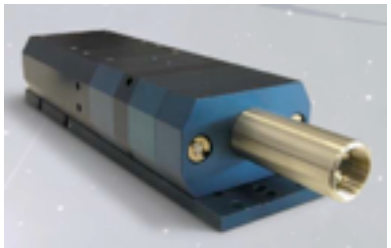
Lasers

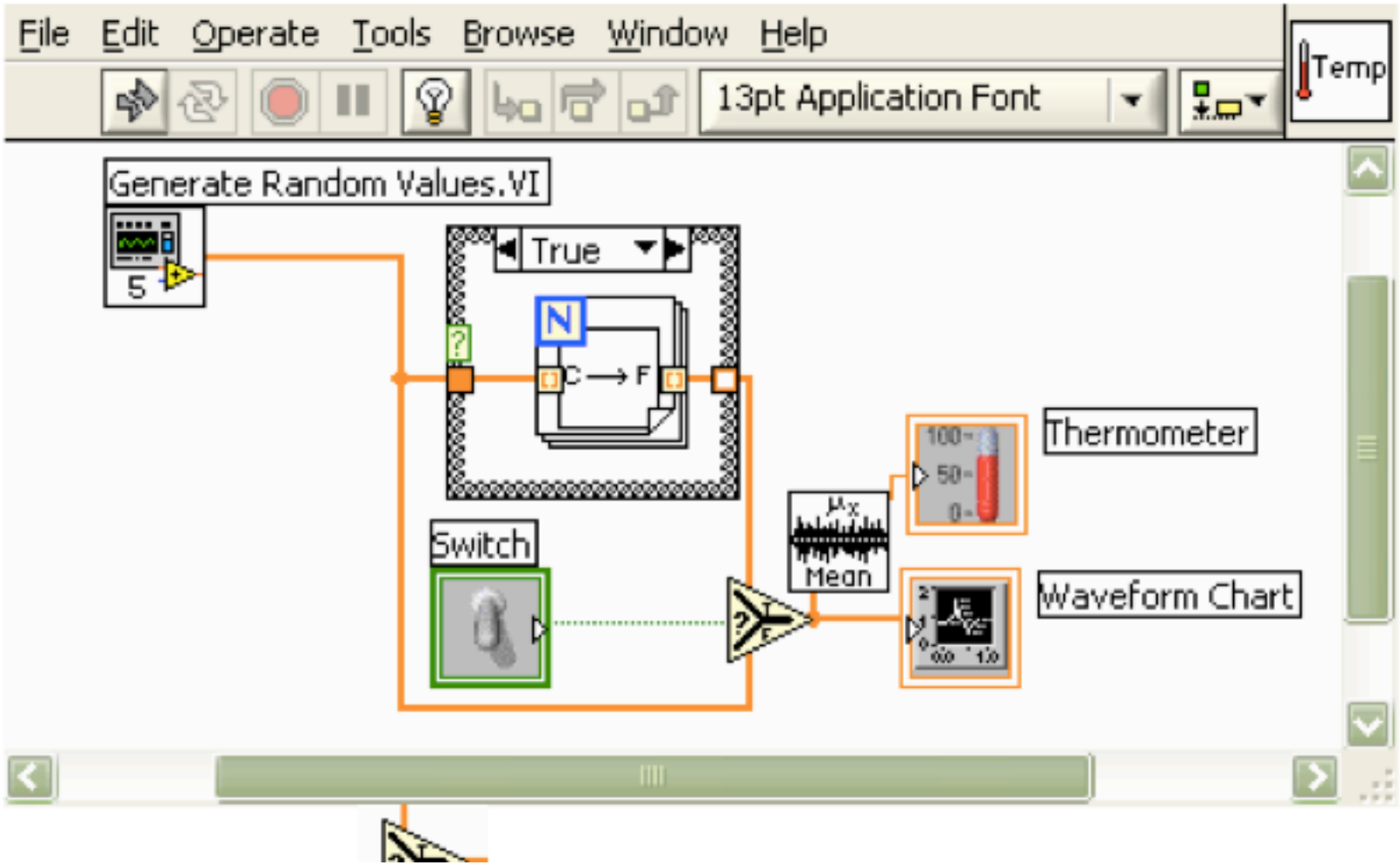


Software options

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

Lab view example

but we'll fix it.



Micromanager

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

µManager

THE OPEN SOURCE MICROSCOPY SOFTWARE

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

welcome to micro-manager!

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

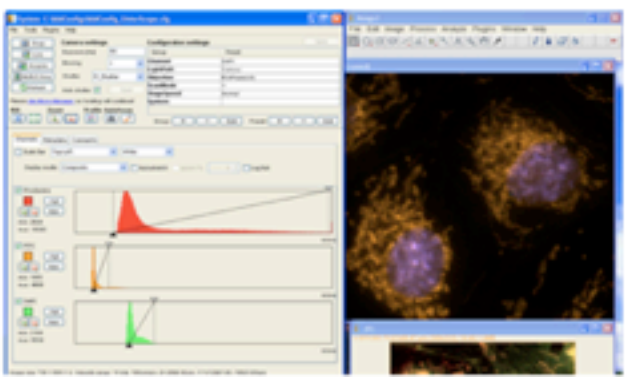
Micro-Manager Open Source Microscopy Software

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

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

How expensive is it?

Building costs

Hardware ~£100-250k

Salaries 1-3 years (~£50-£150)

Total cost ~£150-350k

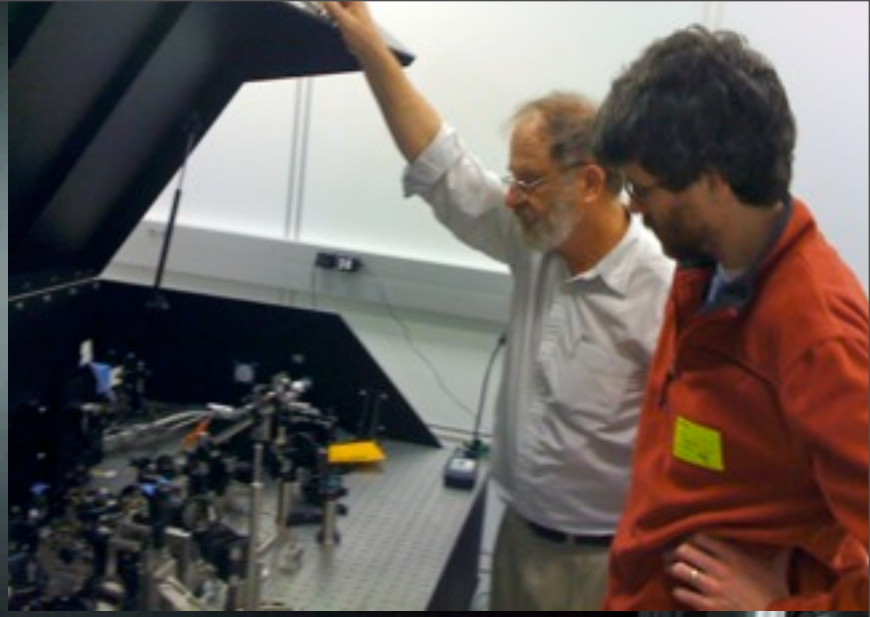
Commercial OMX system ~£750k

OMX

David Agard

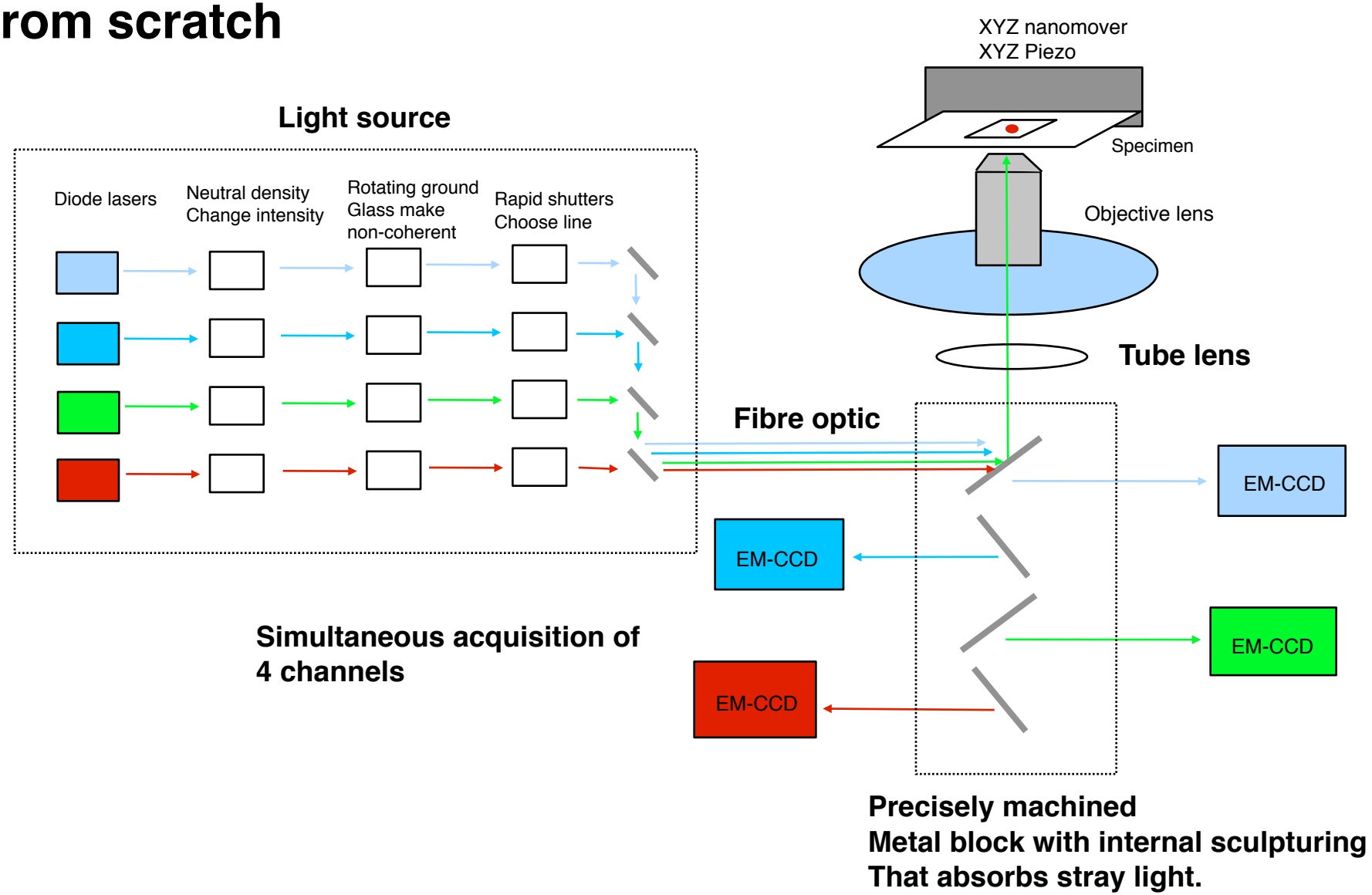
Richard Parton

Lothar Schermelleh

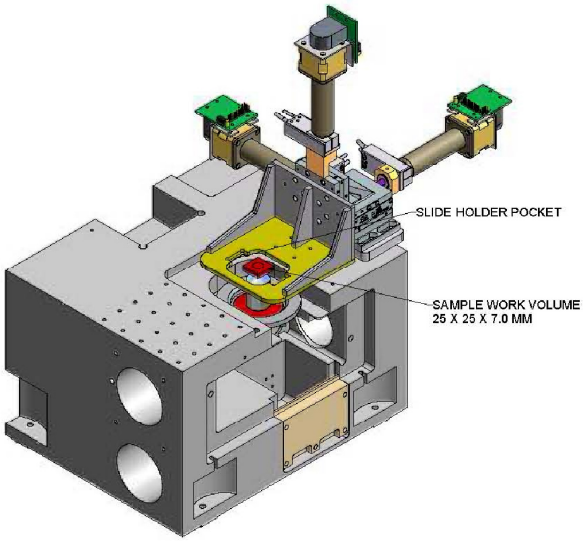


John Sedat and Ian Dobbie

OMX - Redesigning widefield microscopy from scratch

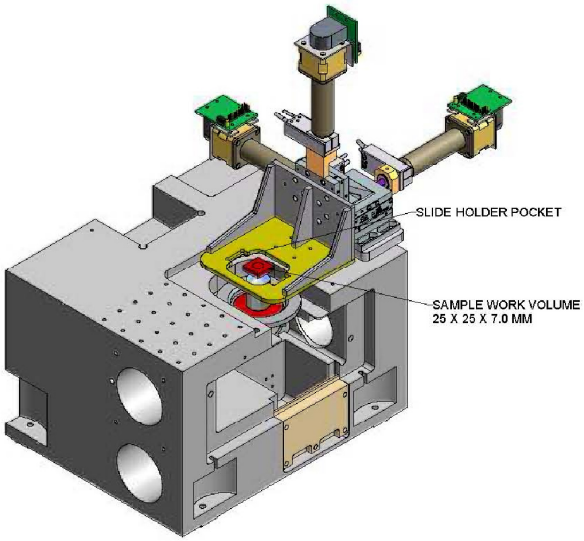


OMX (John Sedat, David Agard and Mats Gustafsson)

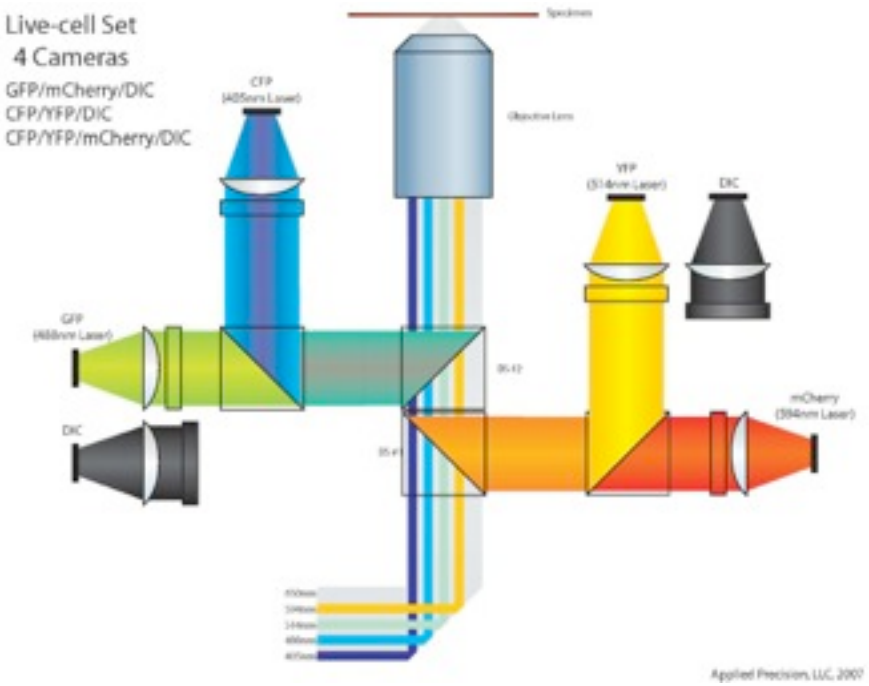


Precisely machined
Metal block with internal sculpturing
That absorbs stray light
Maximized emission light efficiency

OMX (John Sedat, David Agard and Mats Gustafsson)

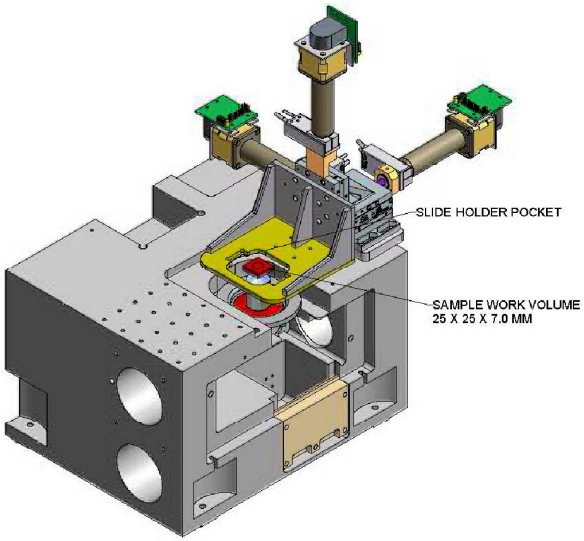


Precisely machined
Metal block with internal sculpturing
That absorbs stray light
Maximized emission light efficiency



4 laser excitation lines
4 simultaneous acquisition lines CCDs

OMX (John Sedat, David Agard and Mats Gustafsson)



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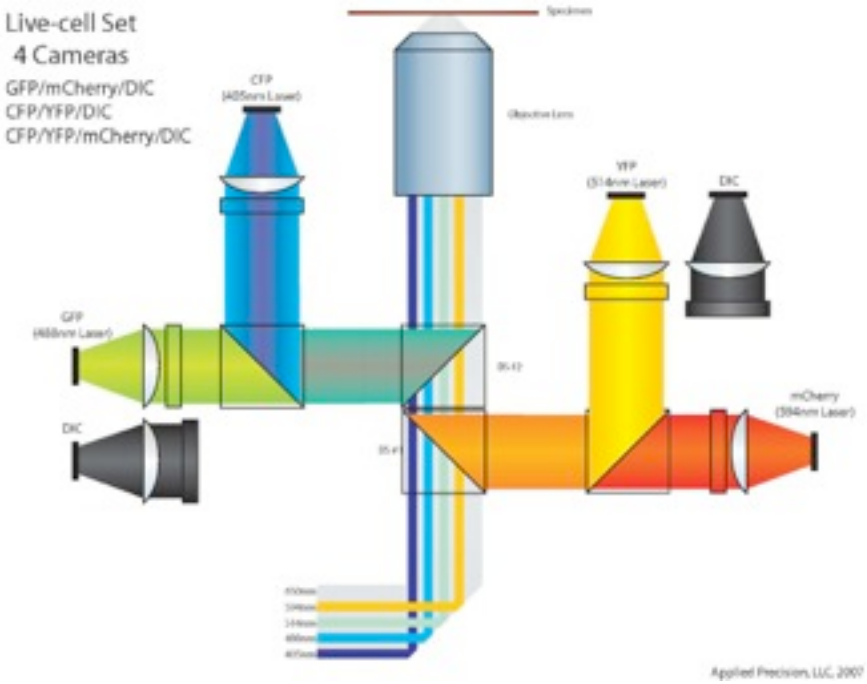
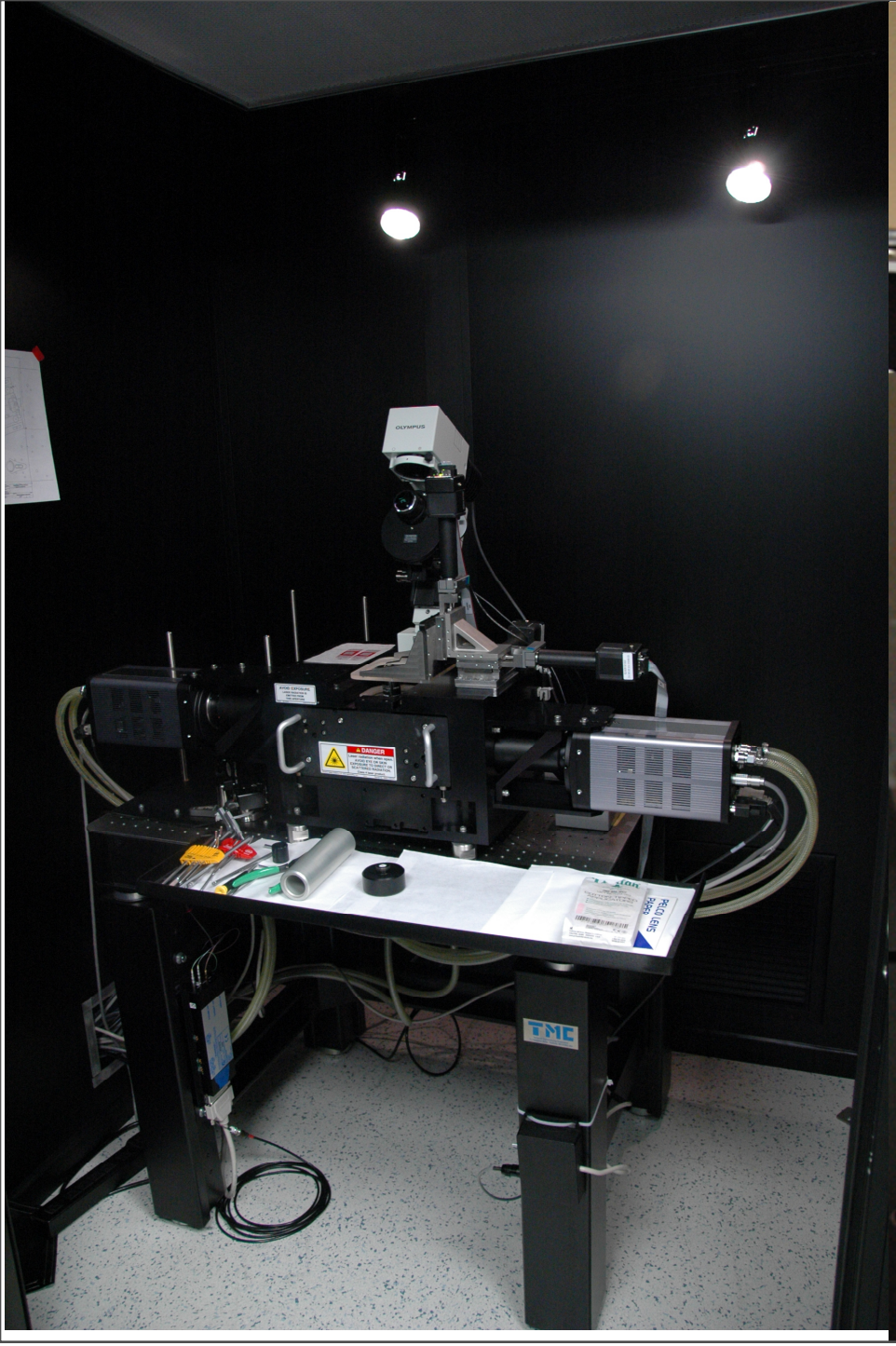
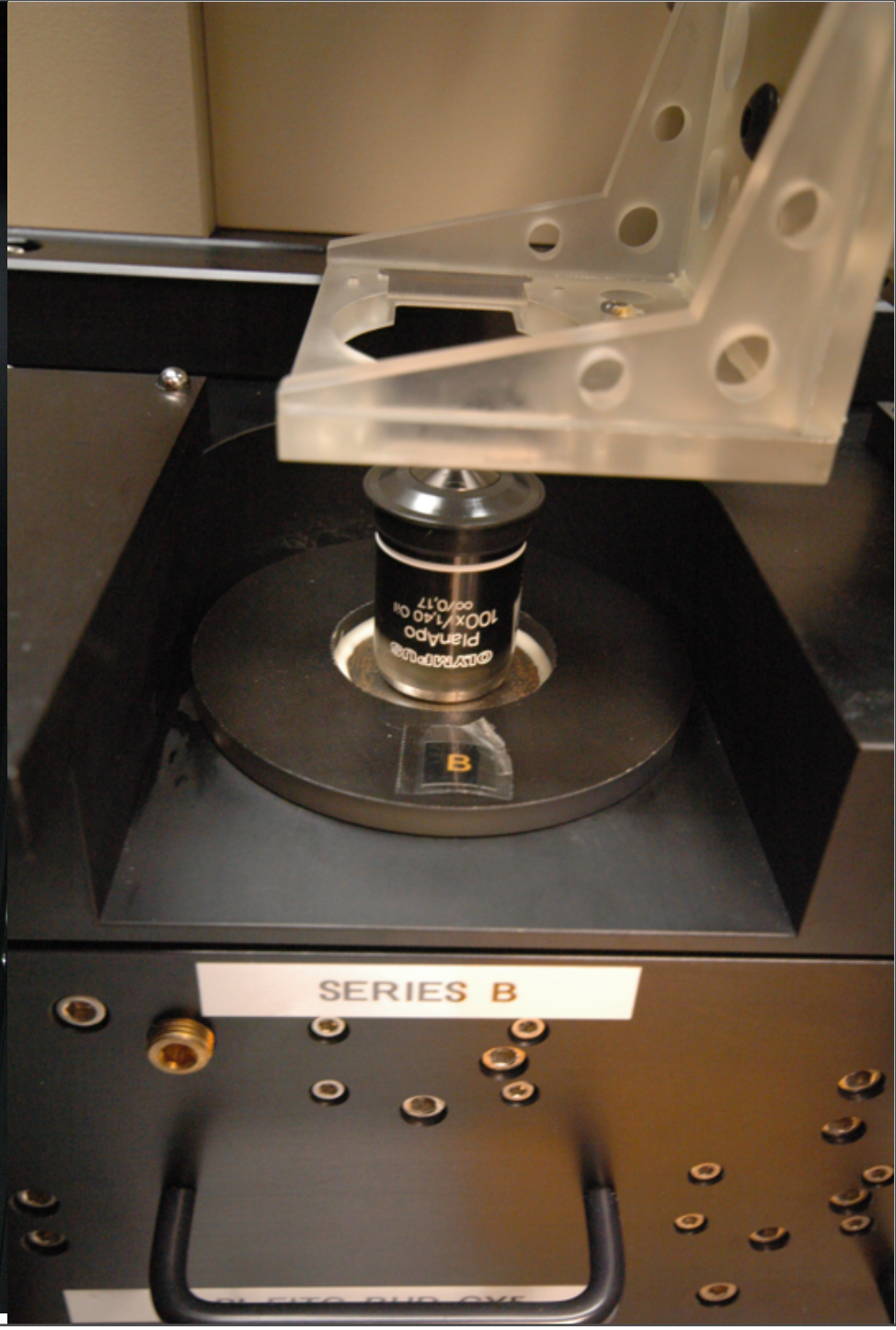


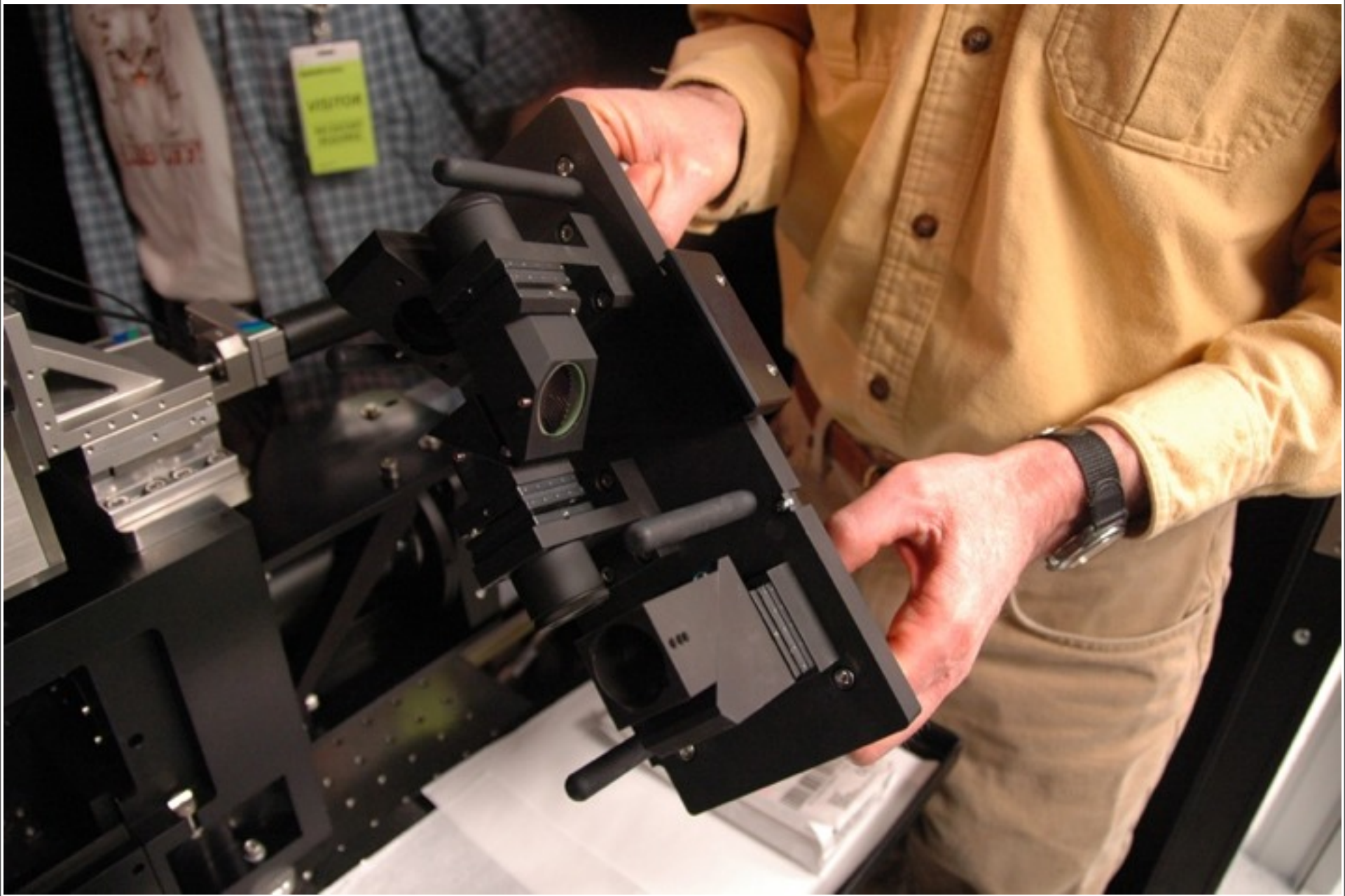
4 laser excitation lines
4 simultaneous acquisition lines CCDs

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

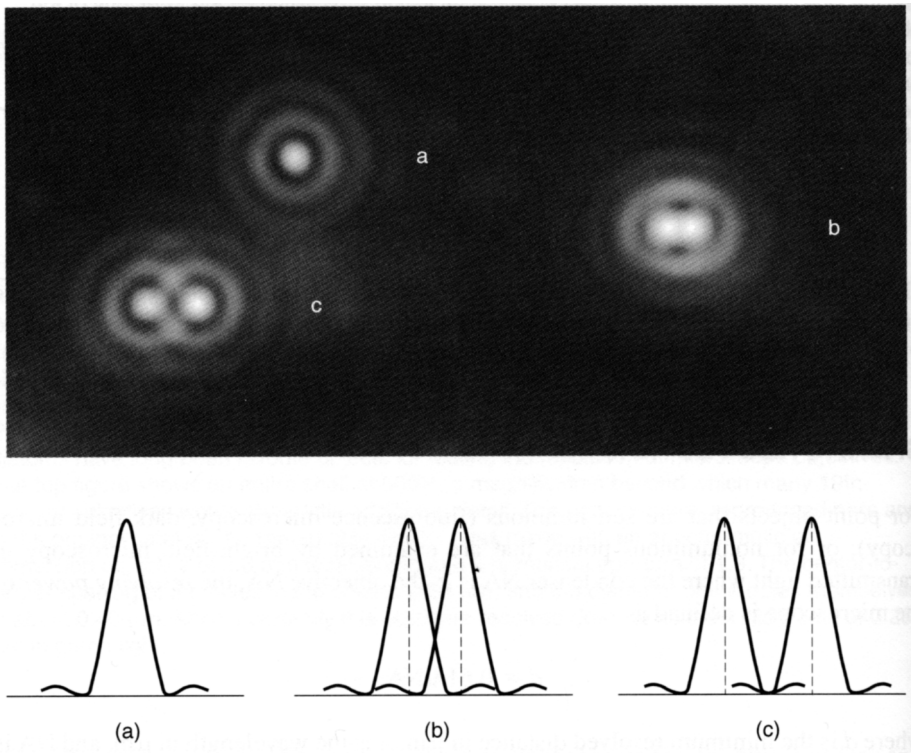

DANGER
Vantus

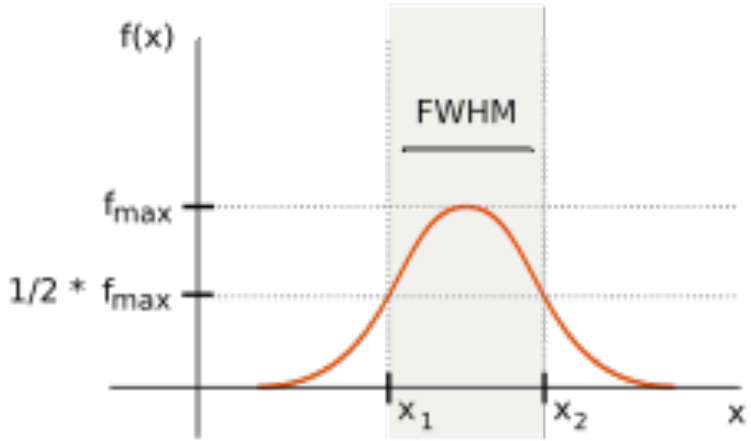

OLYMPUS
DANGER
TMC


PlanApo
100x/1.40 Oil
B
SERIES B



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





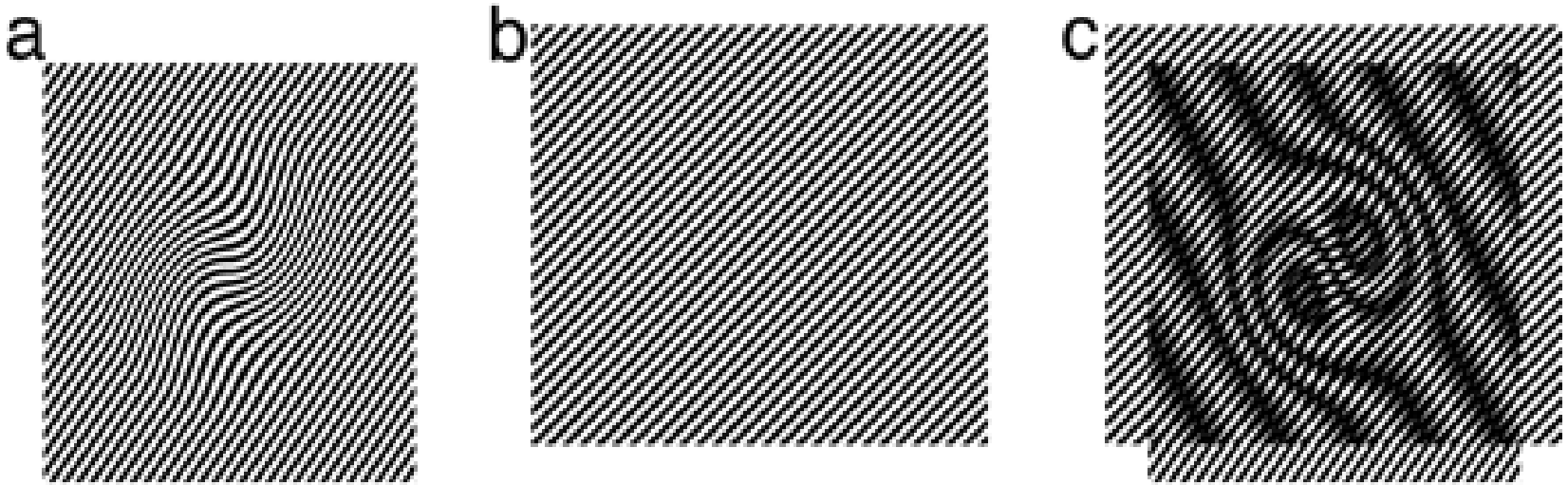
How can we overcome this limit long standing limit?

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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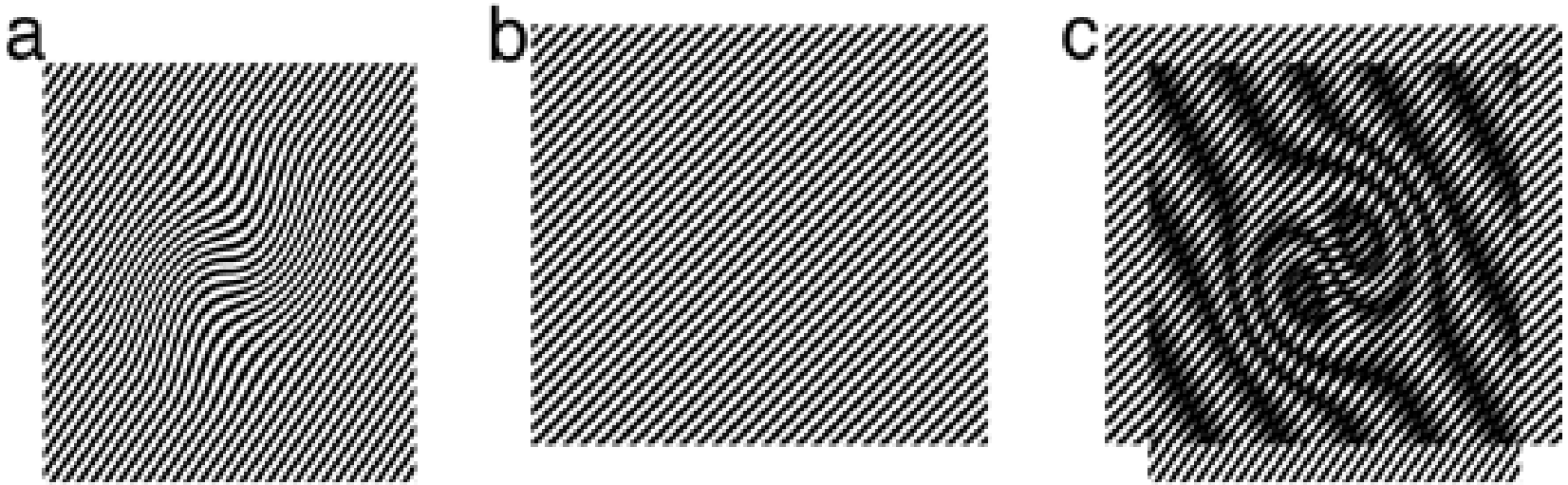


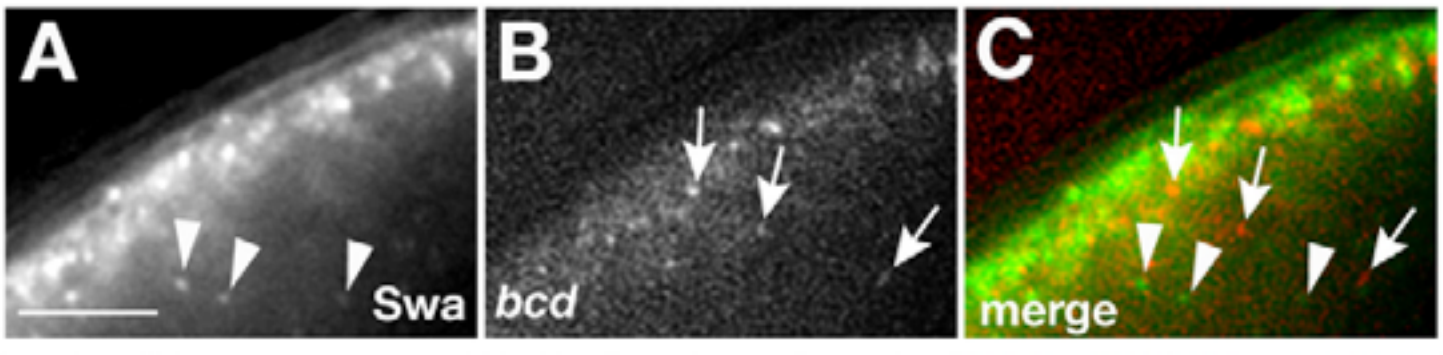
More tomorrow from Lothar

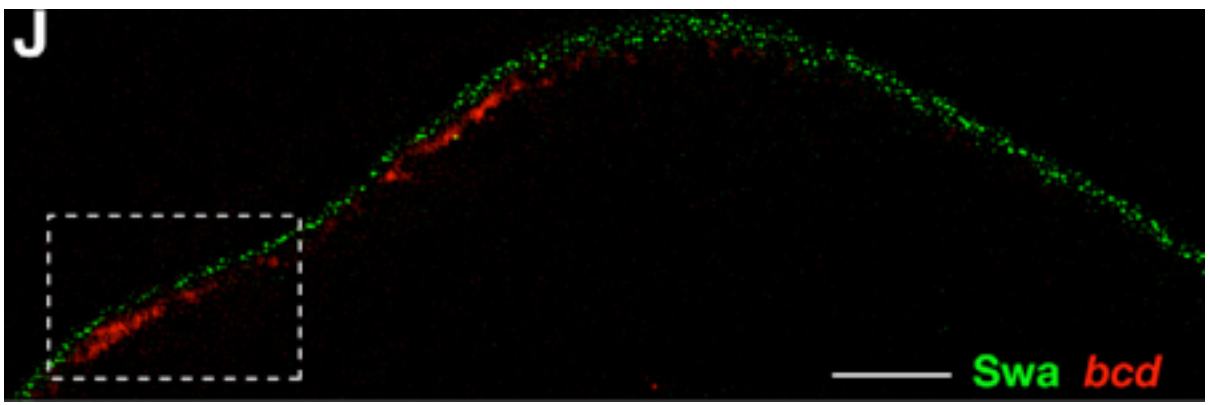
Live multidimensional imaging on OMX

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

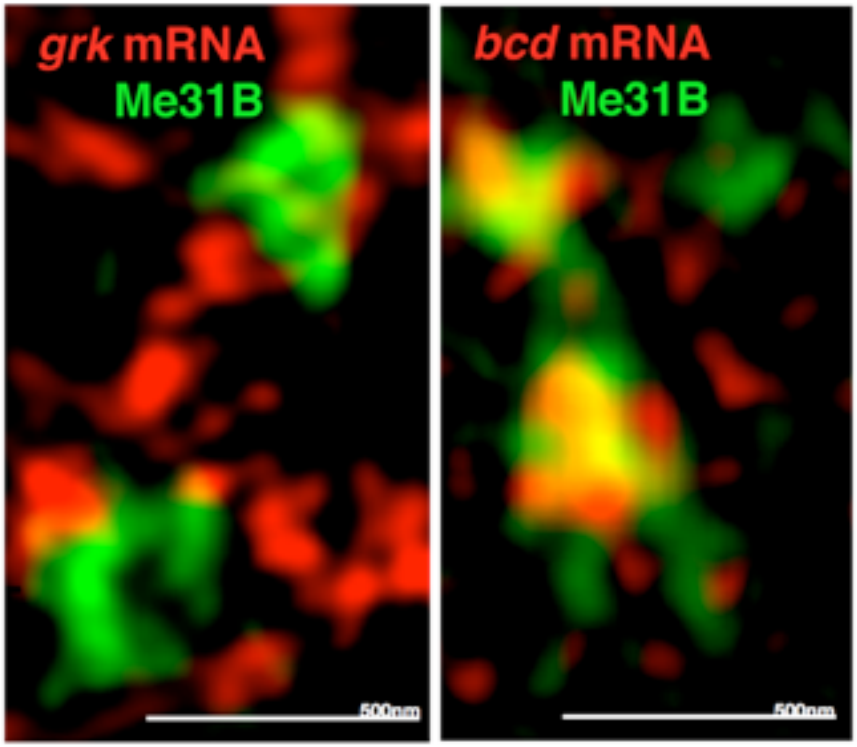
nature cell biology

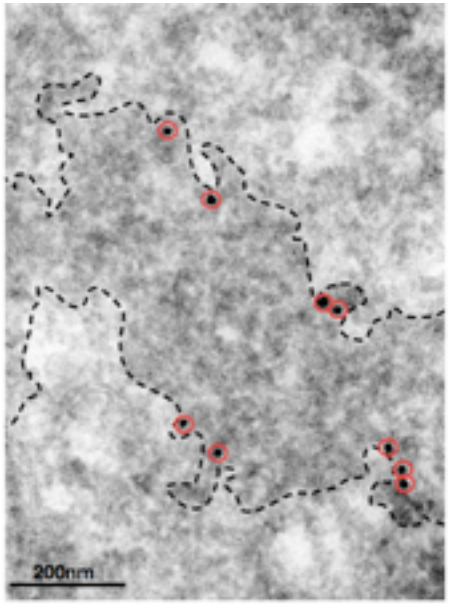
Drosophila patterning is established by differential association of mRNAs with P bodies

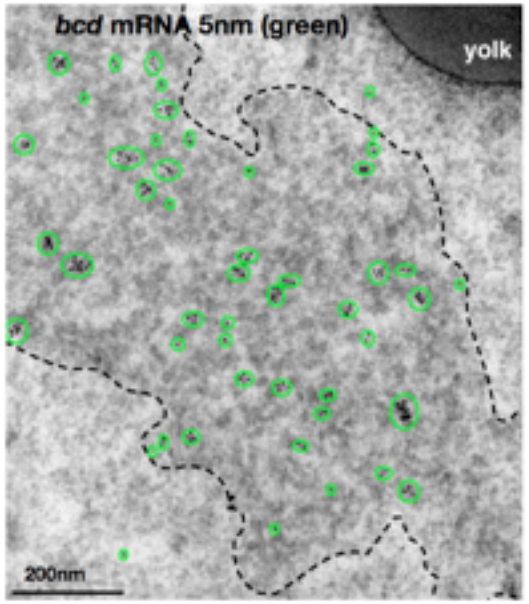
Timothy T. Weil[1,5], Richard M. Parton[1,5], Bram Herpers[2,6], Jan Soetaert[1,6], Tineke Veenendaal[3], Despina Xanthakis[2,3], Ian M. Dobbie[1], James M. Halstead[1], Rippei Hayashi[4], Catherine Rabouille[2,3,7] and Ilan Davis[1,7]

NATURE CELL BIOLOGY VOLUME 14 | NUMBER 12 | DECEMBER 2012

3D-SIM of P bodies assocated with RNA







THE FUTURE: 3D SIM live on V3-blaze

3D Structured Illumination

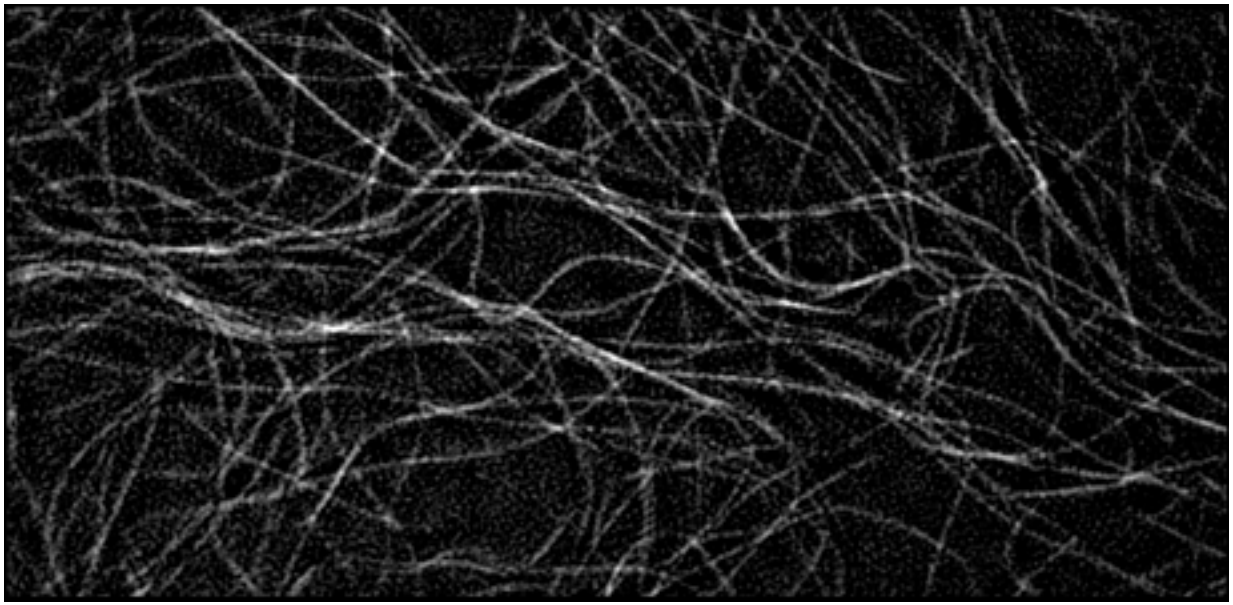
Conventional Widefield deconvolution

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

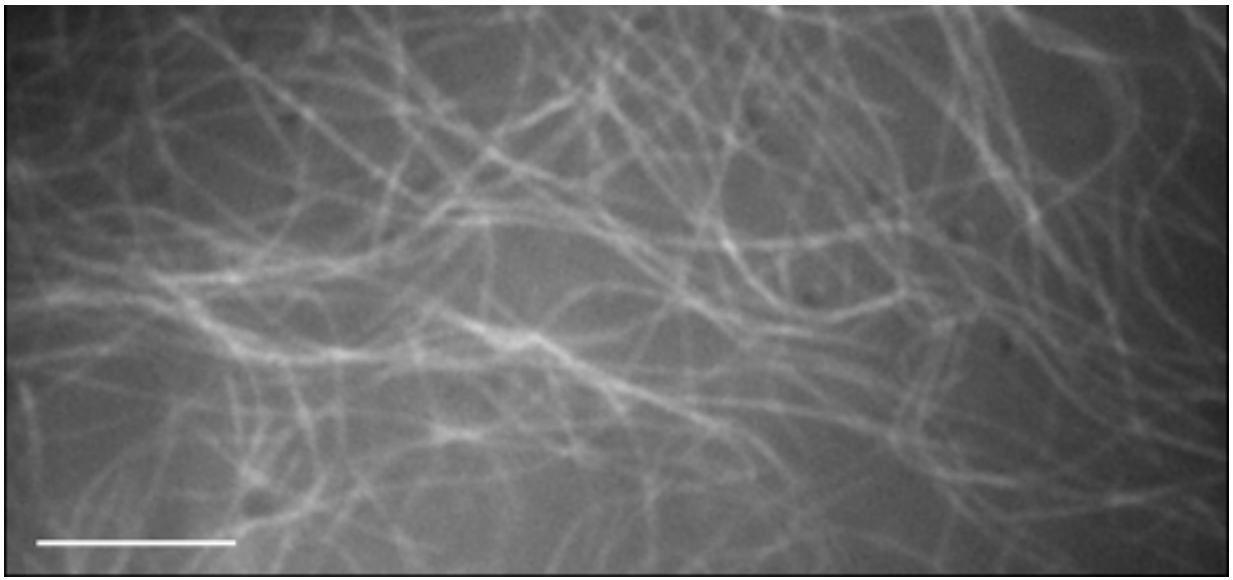
Parton, Goodwin, Atkins

THE FUTURE: 3D SIM live on V3-blaze

3D Structured Illumination



Conventional Widefield deconvolution

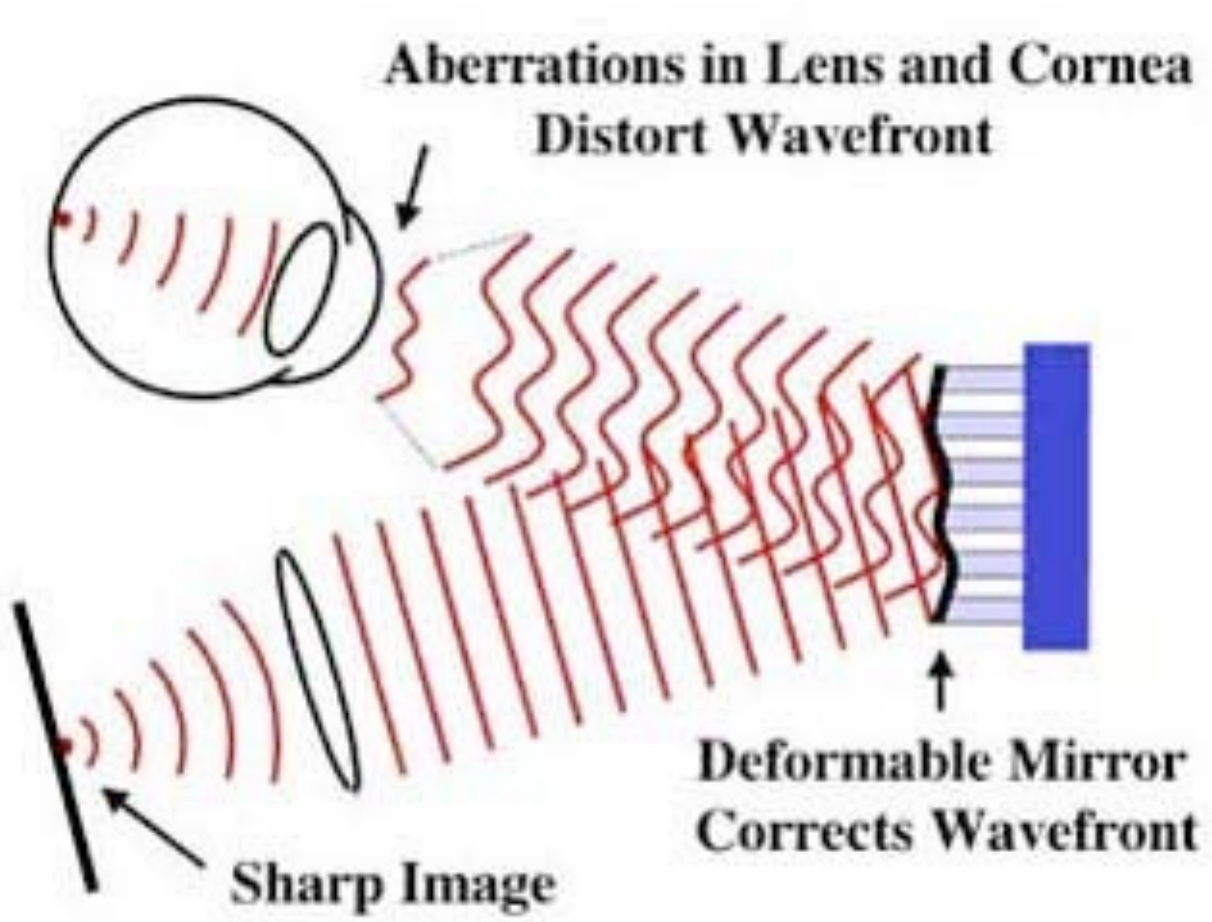


MT: Jupiter-GFP - captured at API.
(1fps, 30 time points)

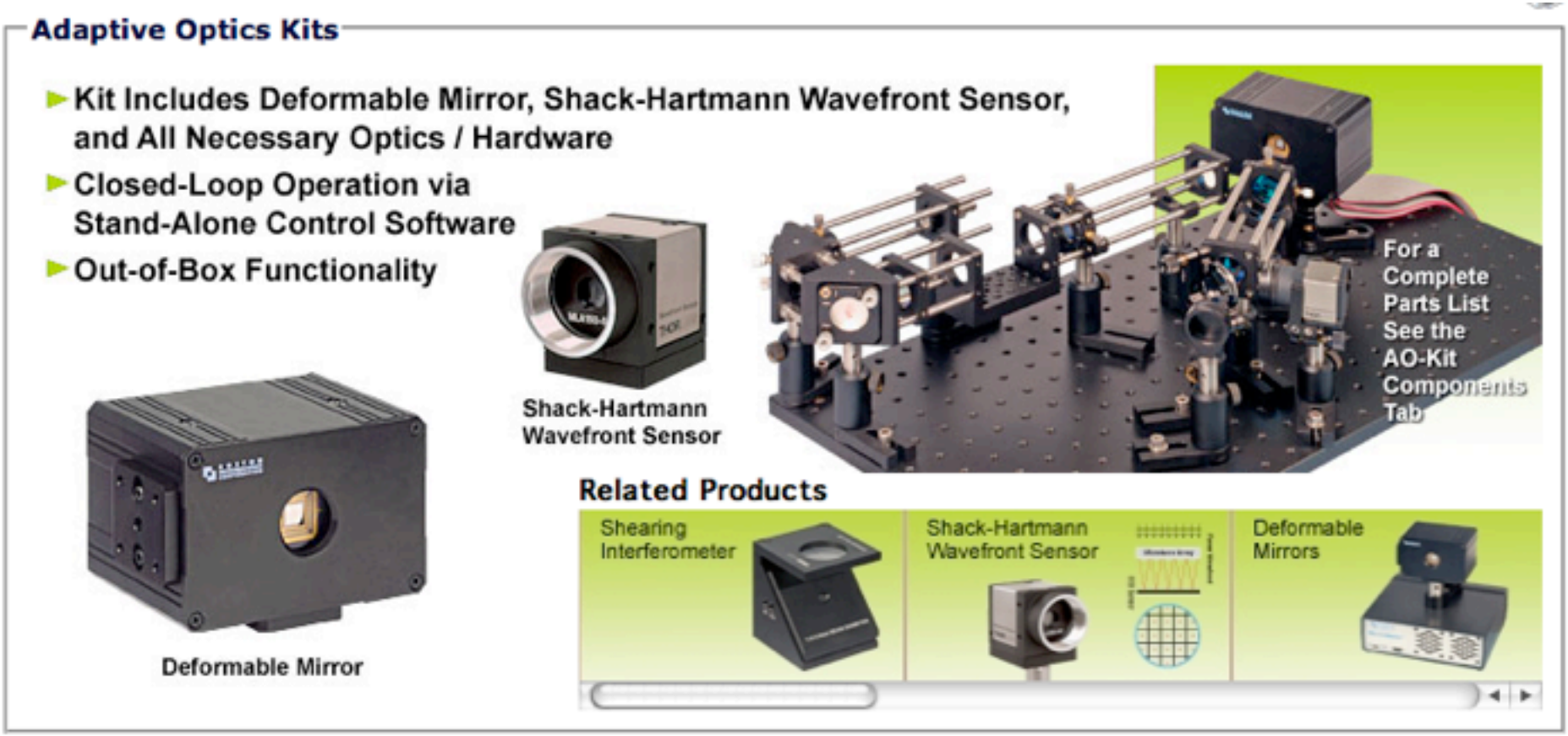
Parton, Goodwin, Atkins

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



The hard part - algorithms for shaping the deformable mirror

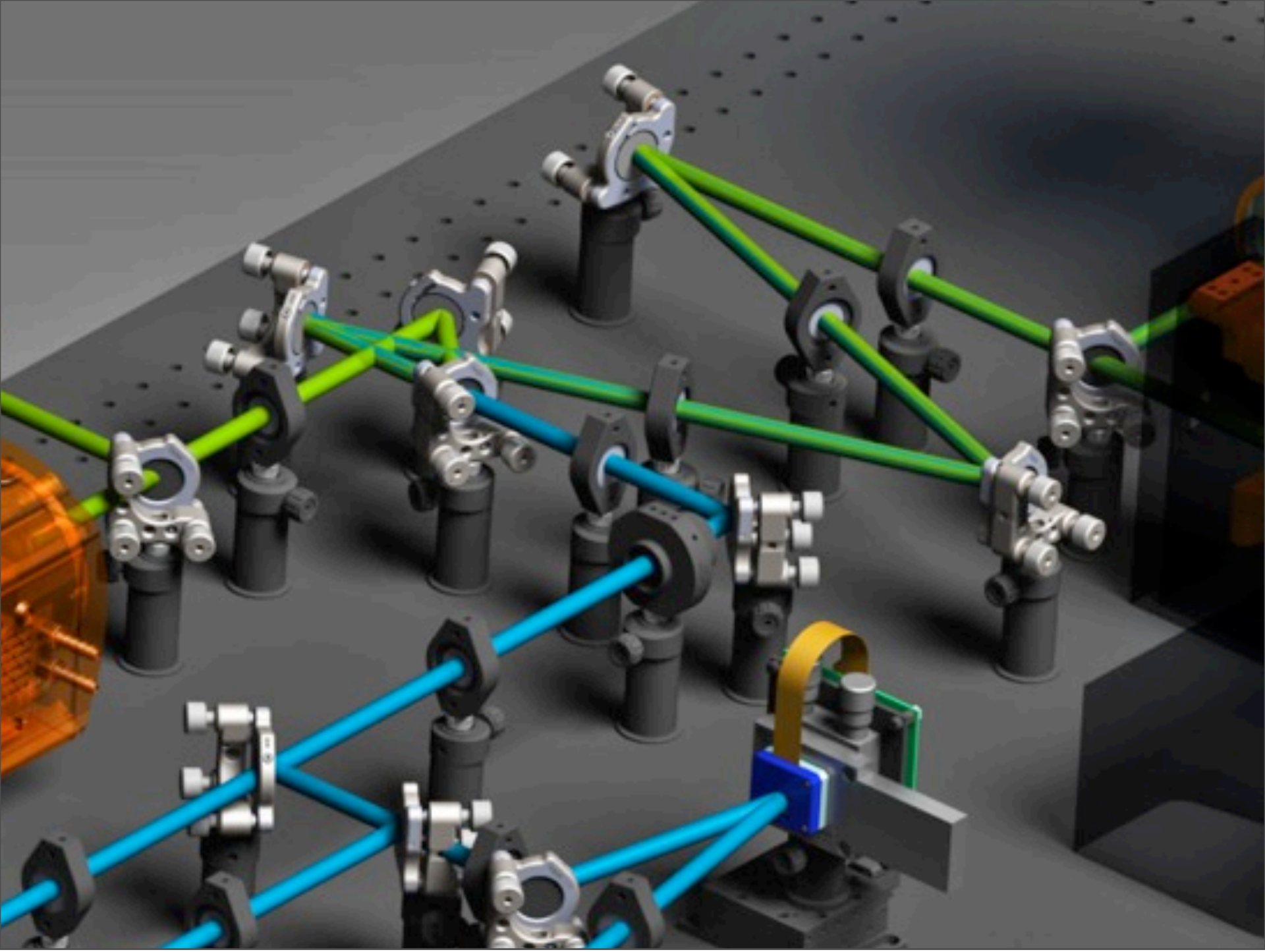
Martin Booth - Engineering / CNSB, Oxford

Further development: OMX-T

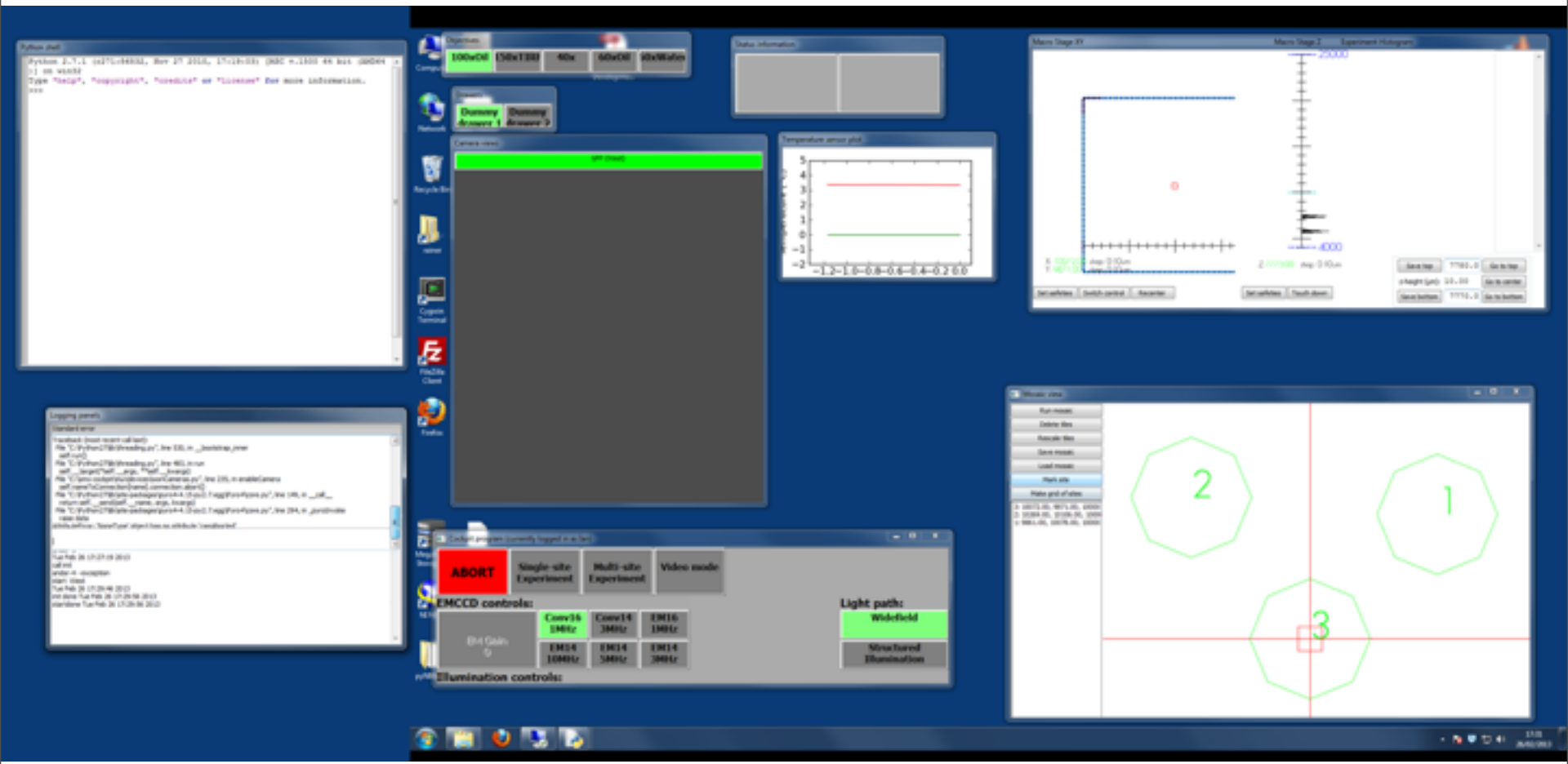
Rainer Kauffman and Ian Dobbie

Based on John Sedat's design

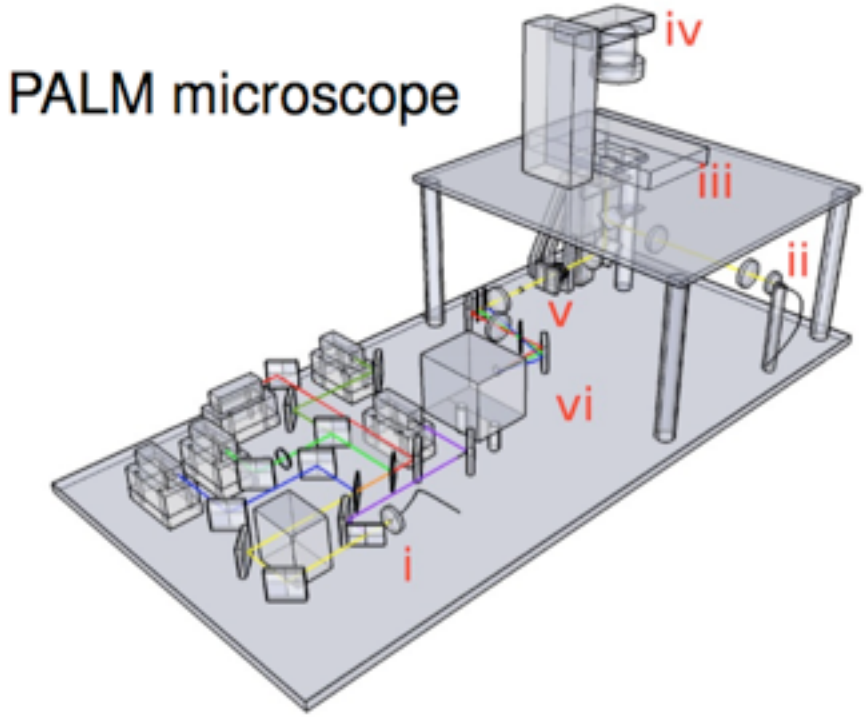
In silico plan

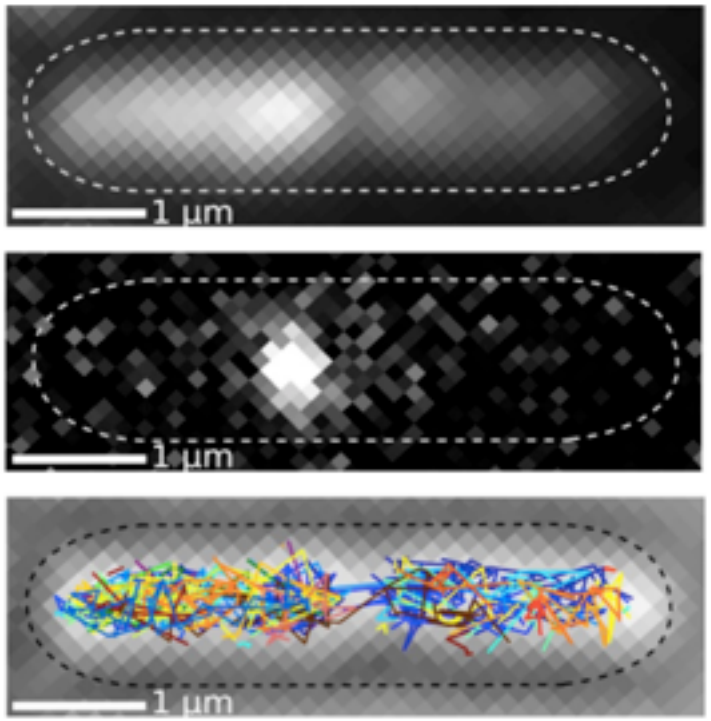


Cockpit - from John Sedat



Home built PALM / DSTORM



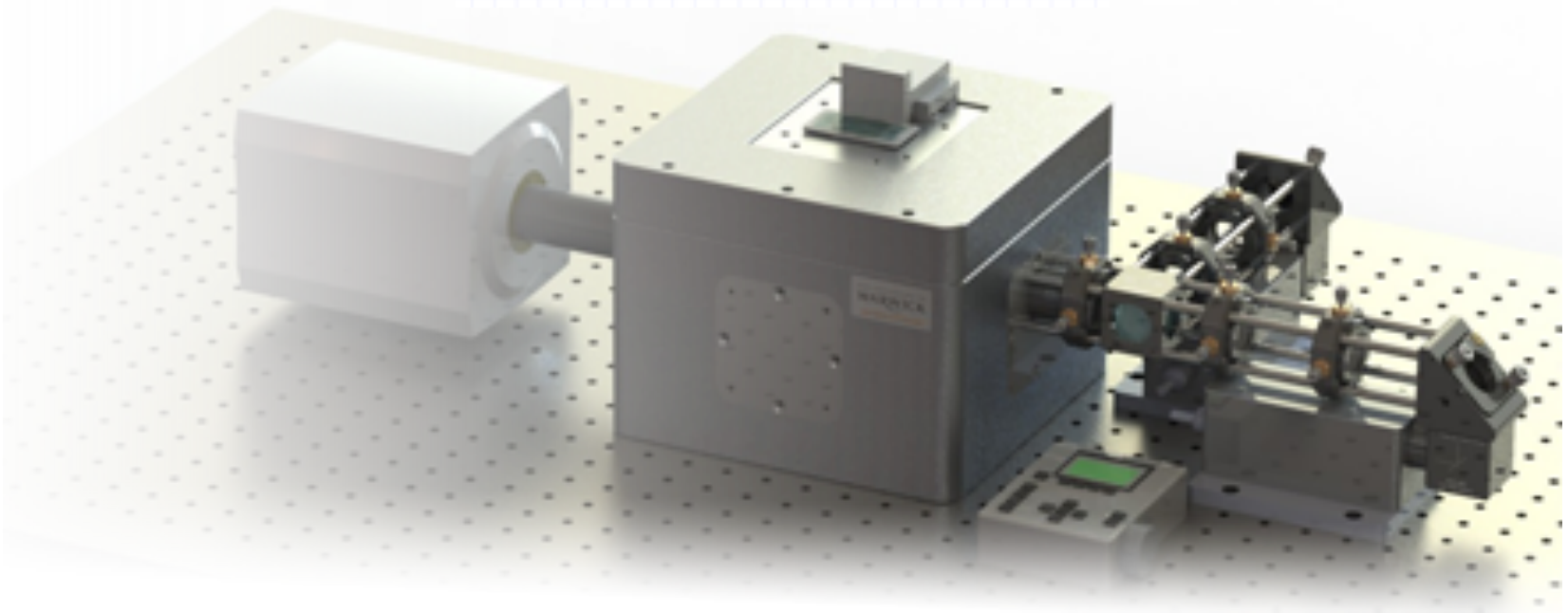


Stephan Uphoff and Achillefs Kapanidis

Half way houses

Warwick Open Source Microscope

WOSM



http://wosmic.org

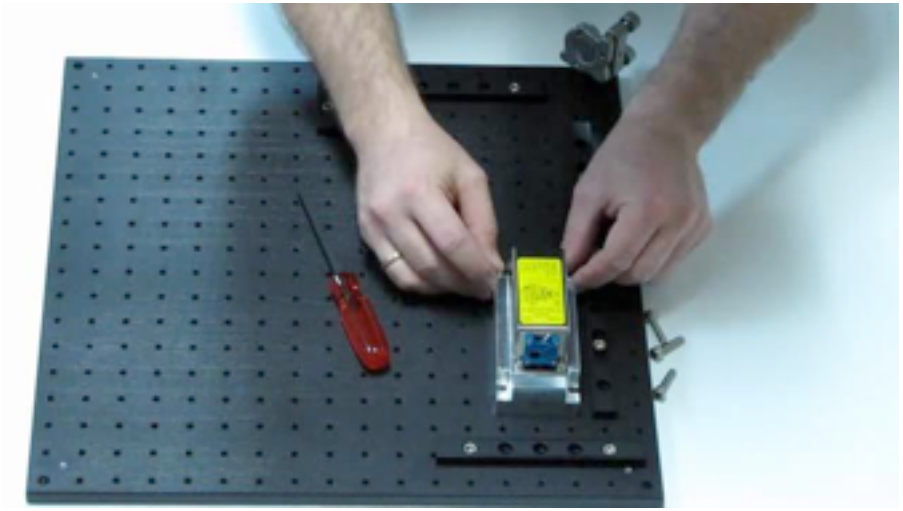
Nick Carter and Rob Cross

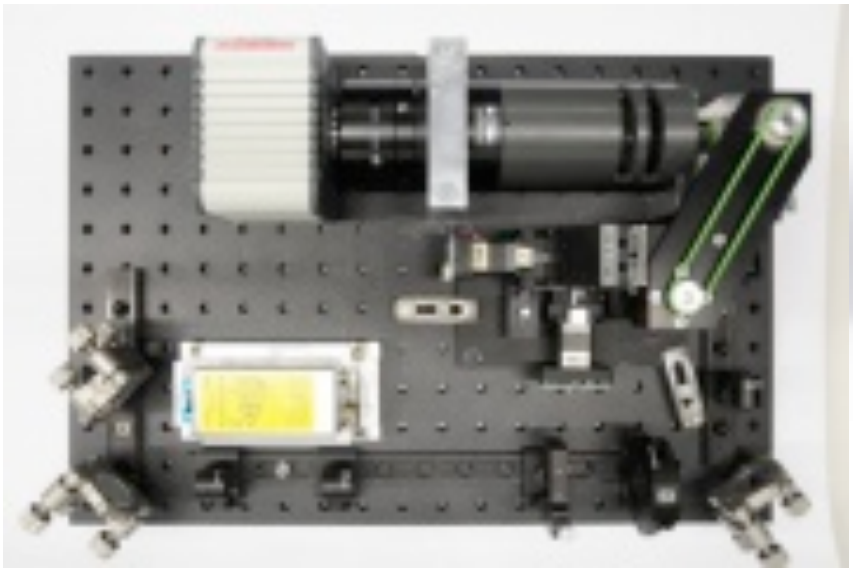
3D printer

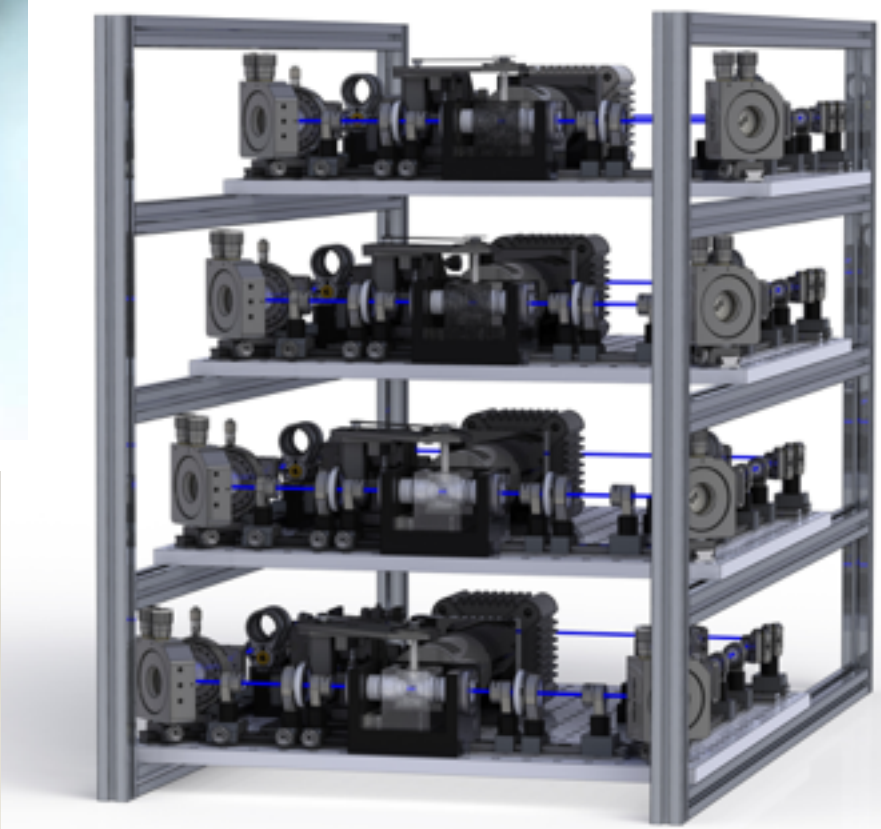
http://airwolf3d.com/about-us/

Openspim

http://openspim.org/Welcome_to_the_OpenSPIM_Wiki







SPIM Farm

openspim

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People

Pavel Tomancak Research Group Leader at the MPI-CBG in Dresden. Provides ideas, concepts, research questions and funding.

Jan Huisken Research Group Leader at the MPI-CBG in Dresden.

Peter Gabriel Pitrone Microscopy technician in the Tomancak lab. Designed and built the OpenSPIM.

Kevin Eliceiri Director of LOCI University of Madison. Collaborator and provides live cell imaging expertise on Open SPIM project.

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

Luke Stuyvenberg Student Programmer at LOCI University of Madison. Works on the µManager OpenSPIM steering interface.

Thanks