LECTURE 9
Advanced Widefield Microscopy
Ilan Davis, March 2012

Image formation and airy rings

How deconvolution works

Design of a modern widefield digital acquisition system

OMX - fast simultaneous live and 3DSIM

Adaptive Optics Correcting Spherical aberration

Fluorescence revisited

http://www.olympusmicro.com/primer/techniques/fluorescence/fluorescenceintro.html

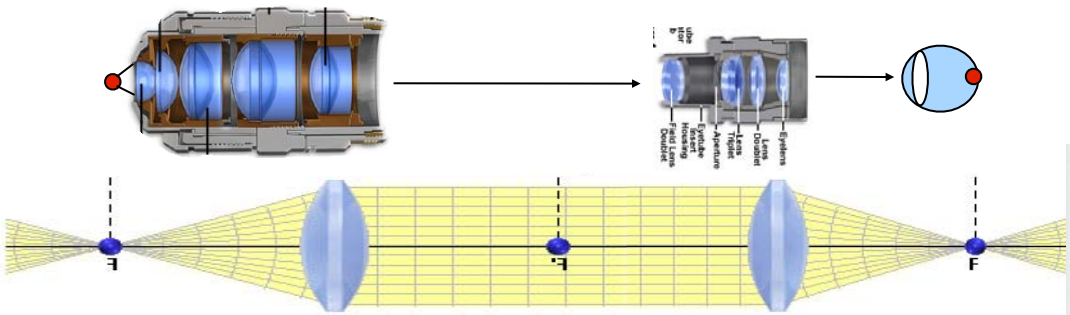
Three fundamental parameters commonly used in describing and comparing fluorophores are the extinction coefficient (**ε**), quantum yield (**Φ**), and fluorescence lifetime (**τ**).

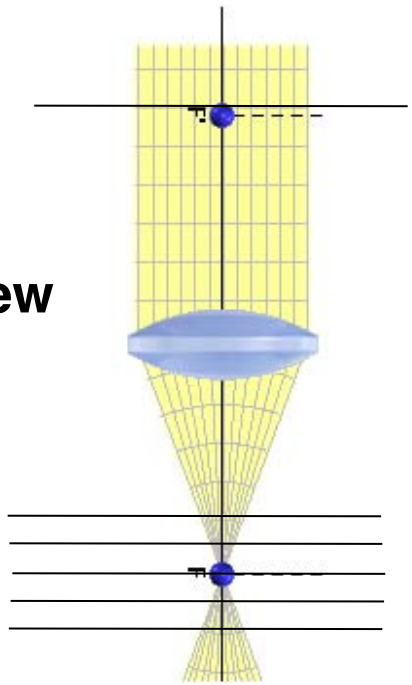
The extinction coefficient
The absorbance at a reference wavelength for **1M** in a cuvette having a **one-centimeter** path length. The reference wavelength is usually the wavelength of maximum absorption.

Quantum yield (sometimes incorrectly termed **quantum efficiency**)
Measure of the efficiency of fluorescence emission relative to all of the possible pathways for relaxation.
Expressed as the (dimensionless) ratio of photons emitted to the number of photons absorbed.
The probability that a given excited fluorochrome will produce an emitted photon (fluorescence).
0-1.

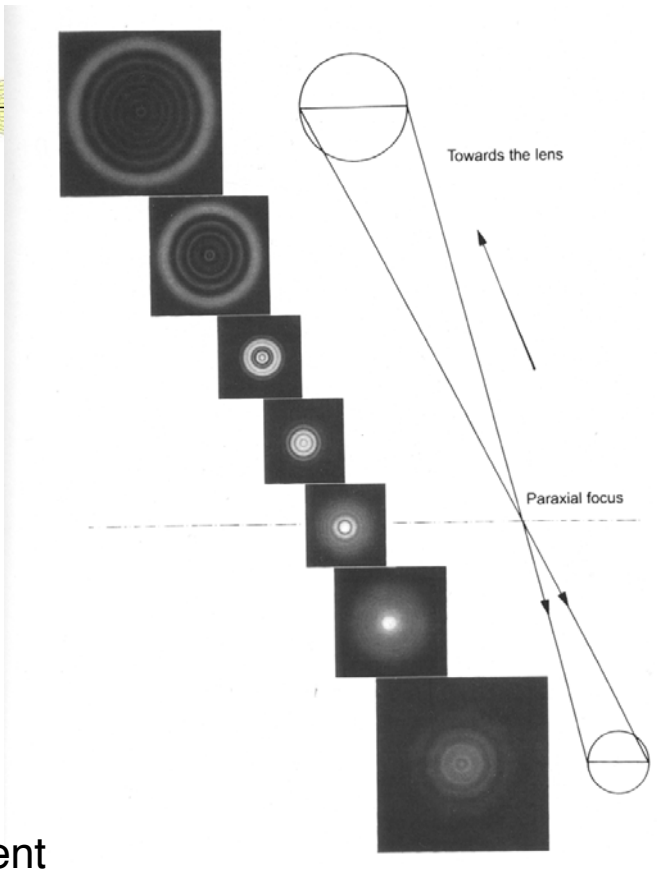
Doug Murphey et al

Image formation





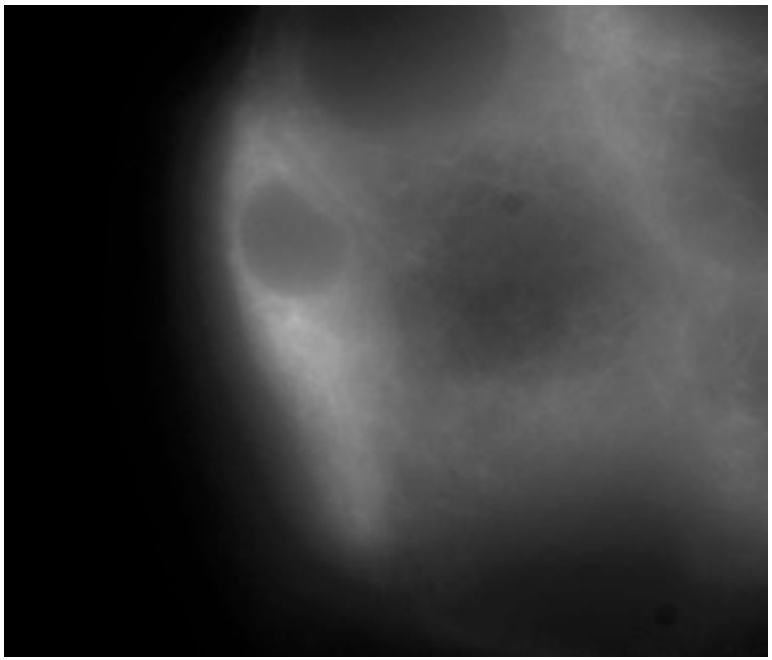
Different
Focal
plains



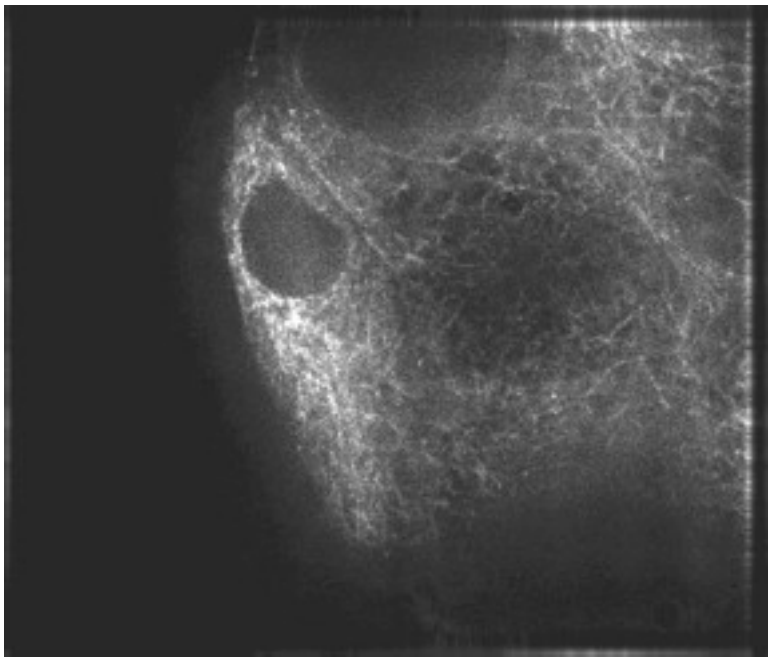
Out of focus light
-Airy rings in 3D view

How does Widefield Deconvolution Work
(restoring out of focus light to its point of origin)

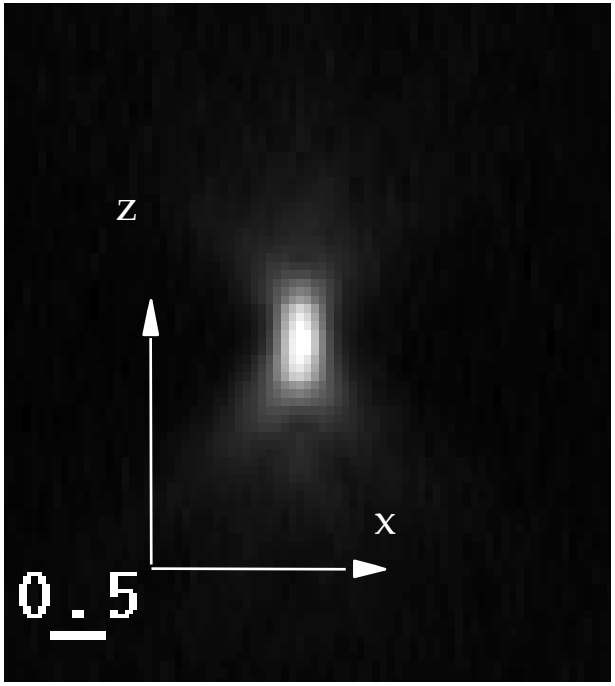
Before Deconvolution

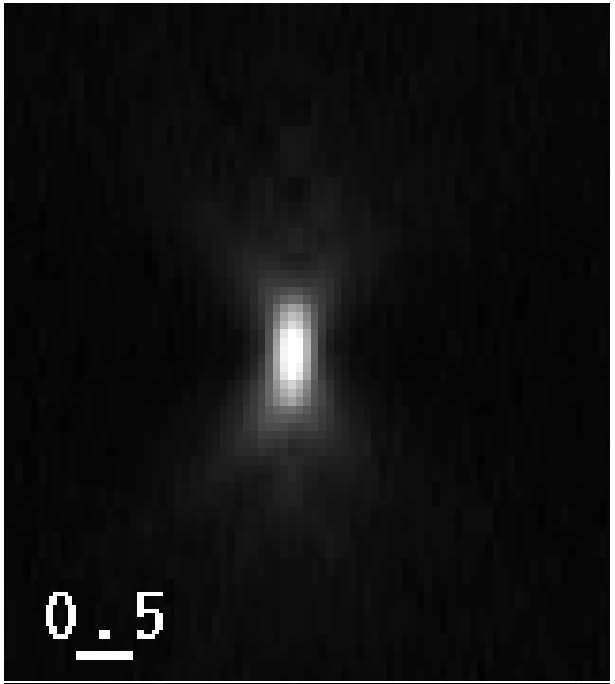


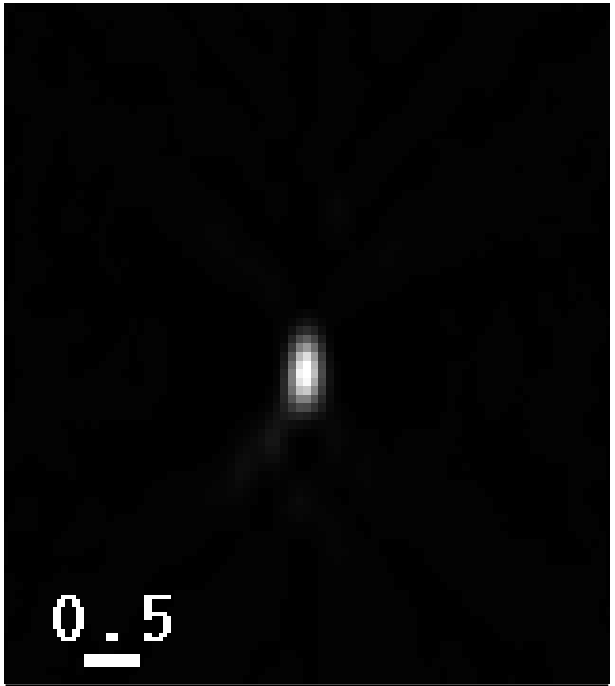
After Deconvolution

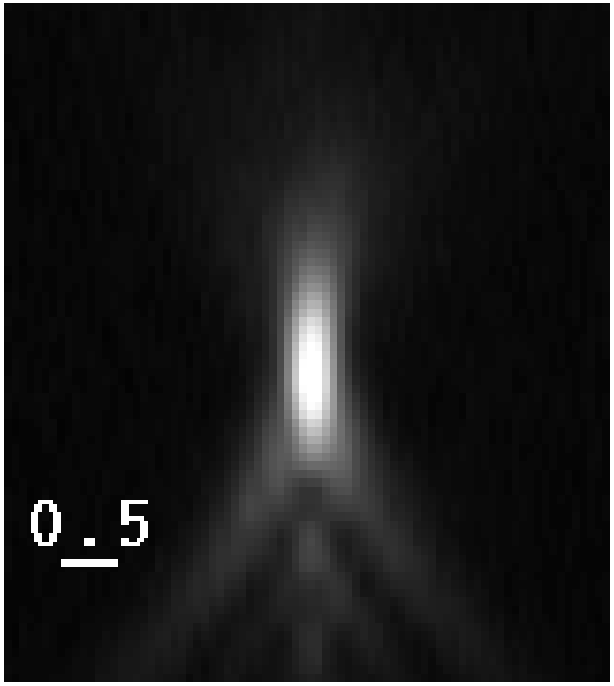


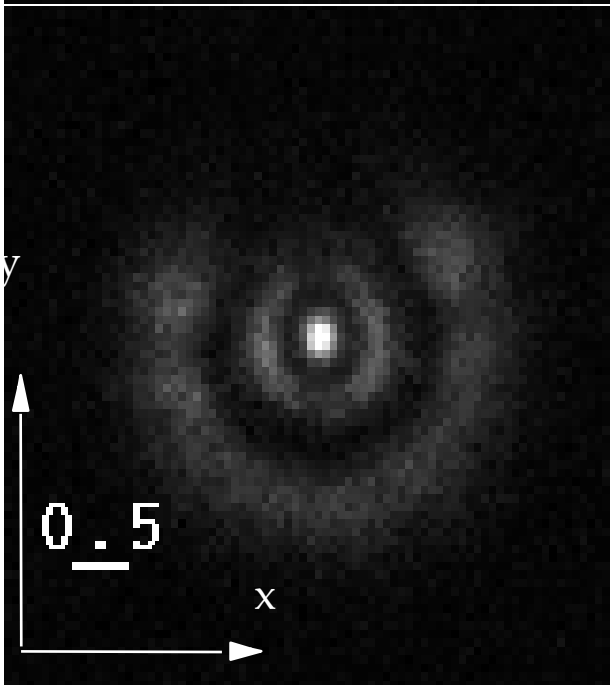
Richard Parton



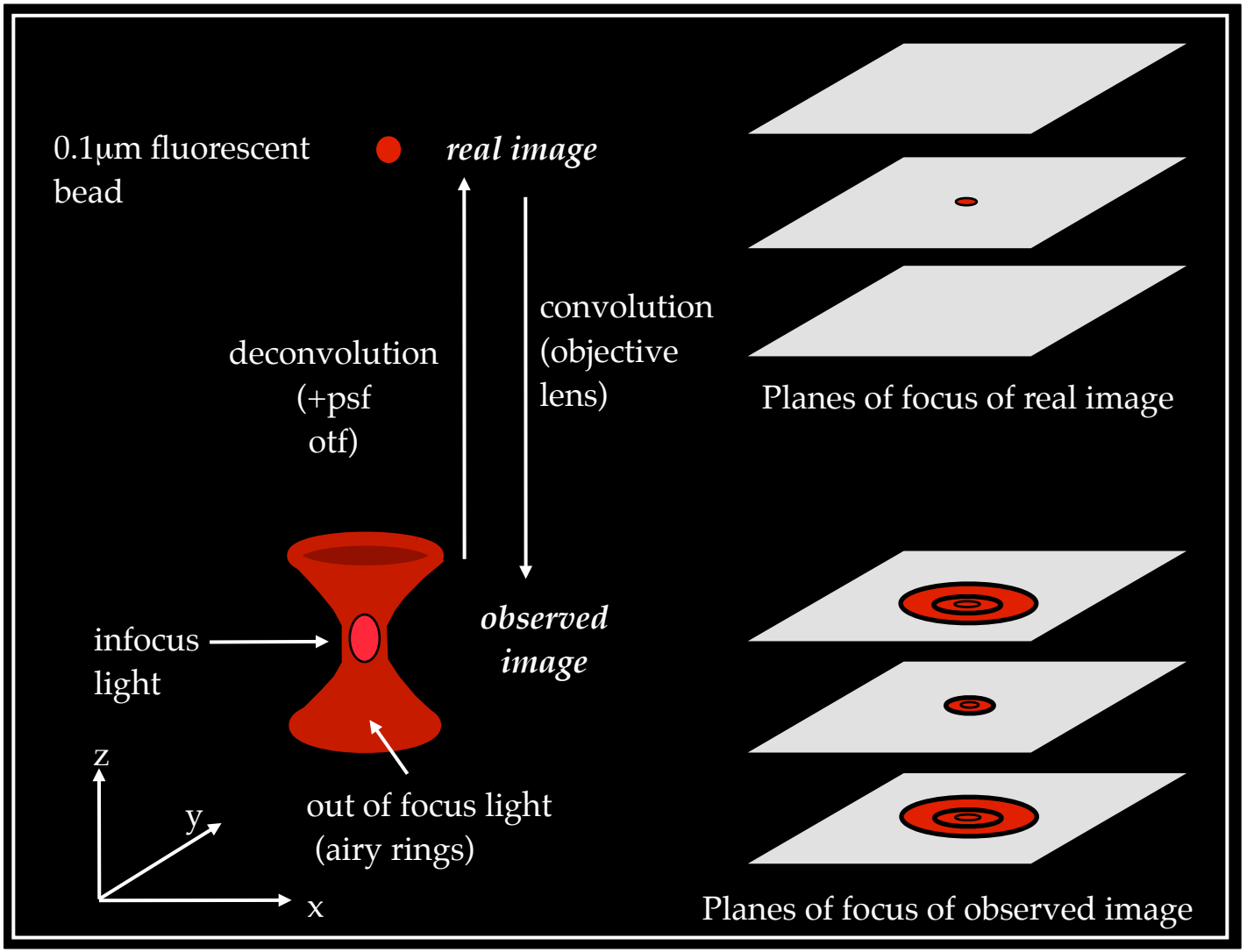








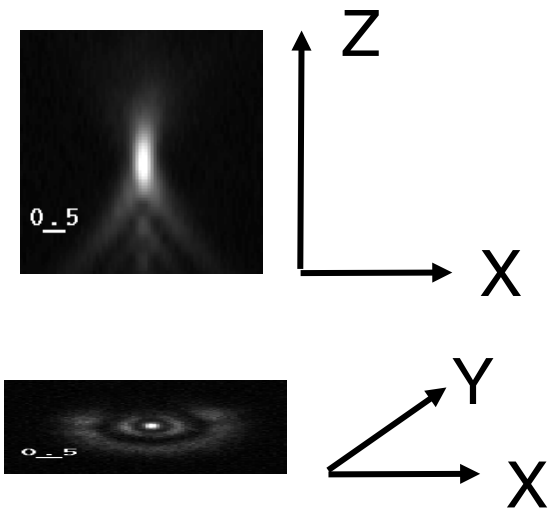
133	135	149	134	160	165	133	147	123
171	167	159	133	119	118	124	148	164
194	199	192	173	156	151	158	175	193
216	237	237	235	221	217	208	214	223
256	290	288	288	281	283	259	271	259
268	328	365	420	440	434	359	324	282
271	362	460	600	663	636	490	378	297
256	396	643	954	1140	1058	760	477	313
246	420	752	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
282	267	243	254	202	203	222	215	217
238	216	193	205	163	165	185	177	186


0.1µm fluorescent bead
real image
convolution (objective lens)
deconvolution (+psf otf)
Planes of focus of real image
infocus light
observed image
out of focus light (airy rings)
z
y
x
Planes of focus of observed image

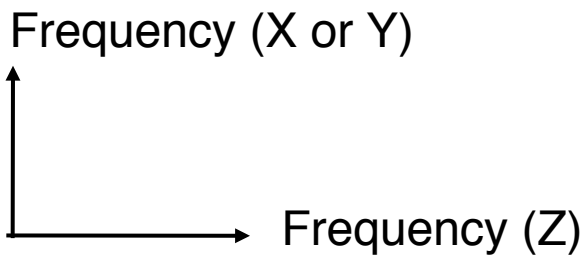
Deconvolution

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

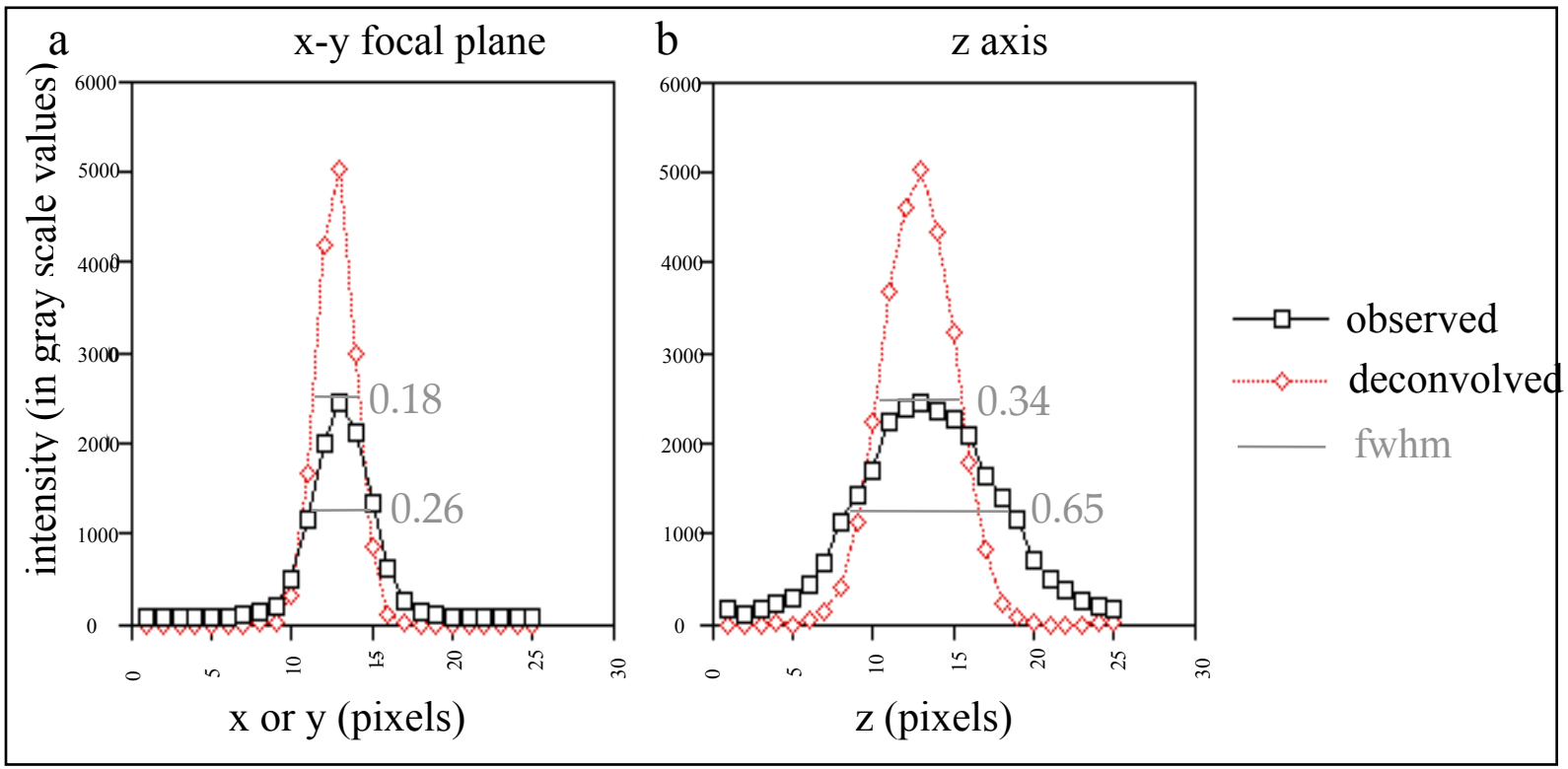
Point Spread Function
PSF (XYZ space)



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

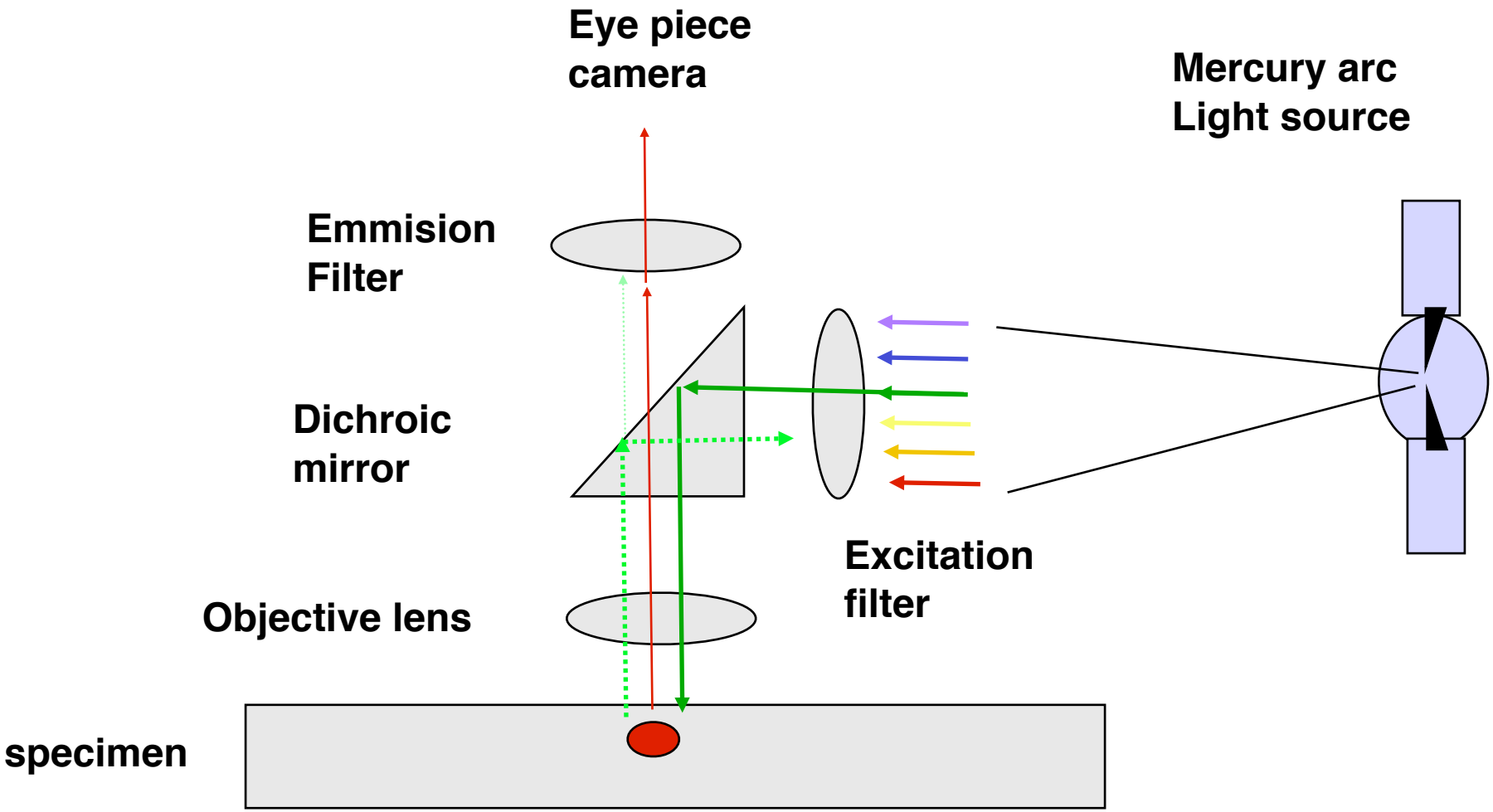
•Sedat/Agard ; Hoygens ; blind deconvolution

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

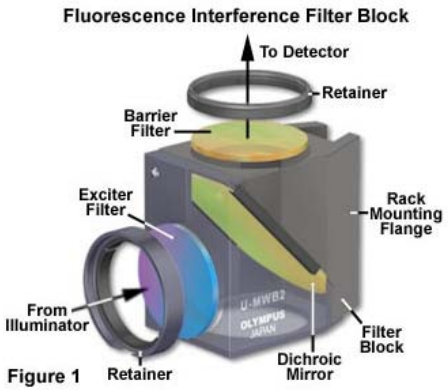
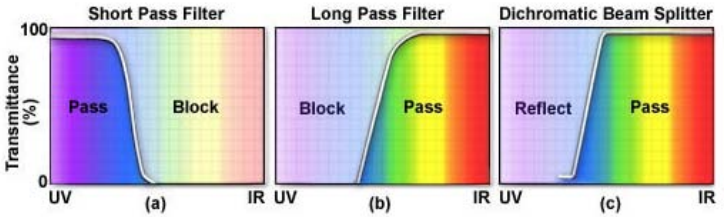


Figure 1



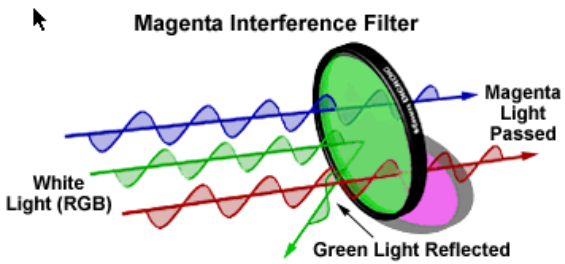
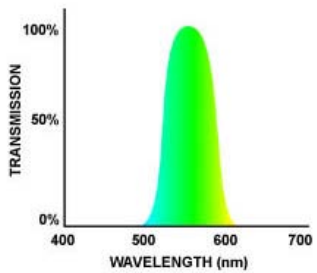


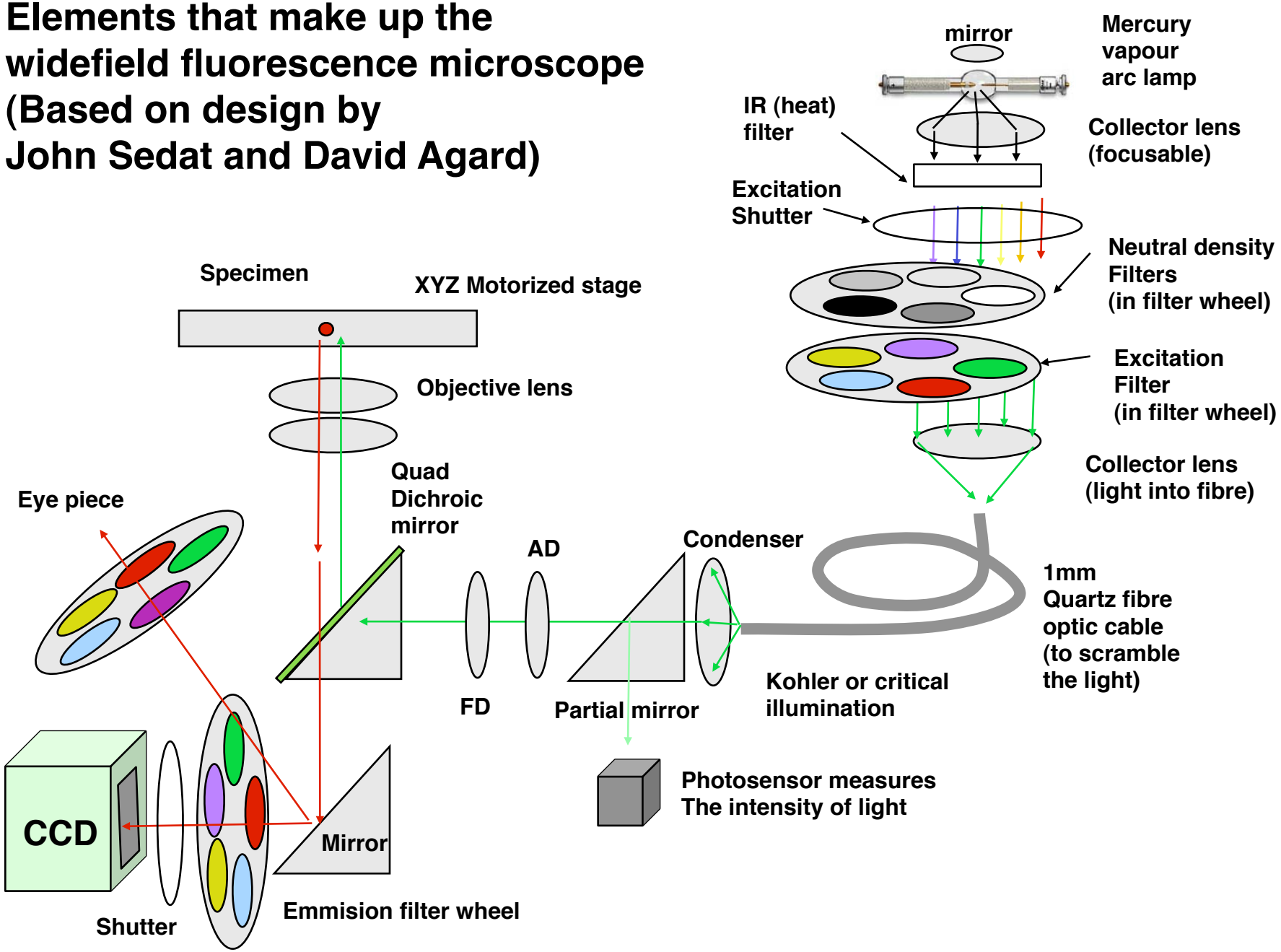
Figure 3



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

Widefield Fluorescence microscopy (Olympus + Sedat/Agard DeltaVision)

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

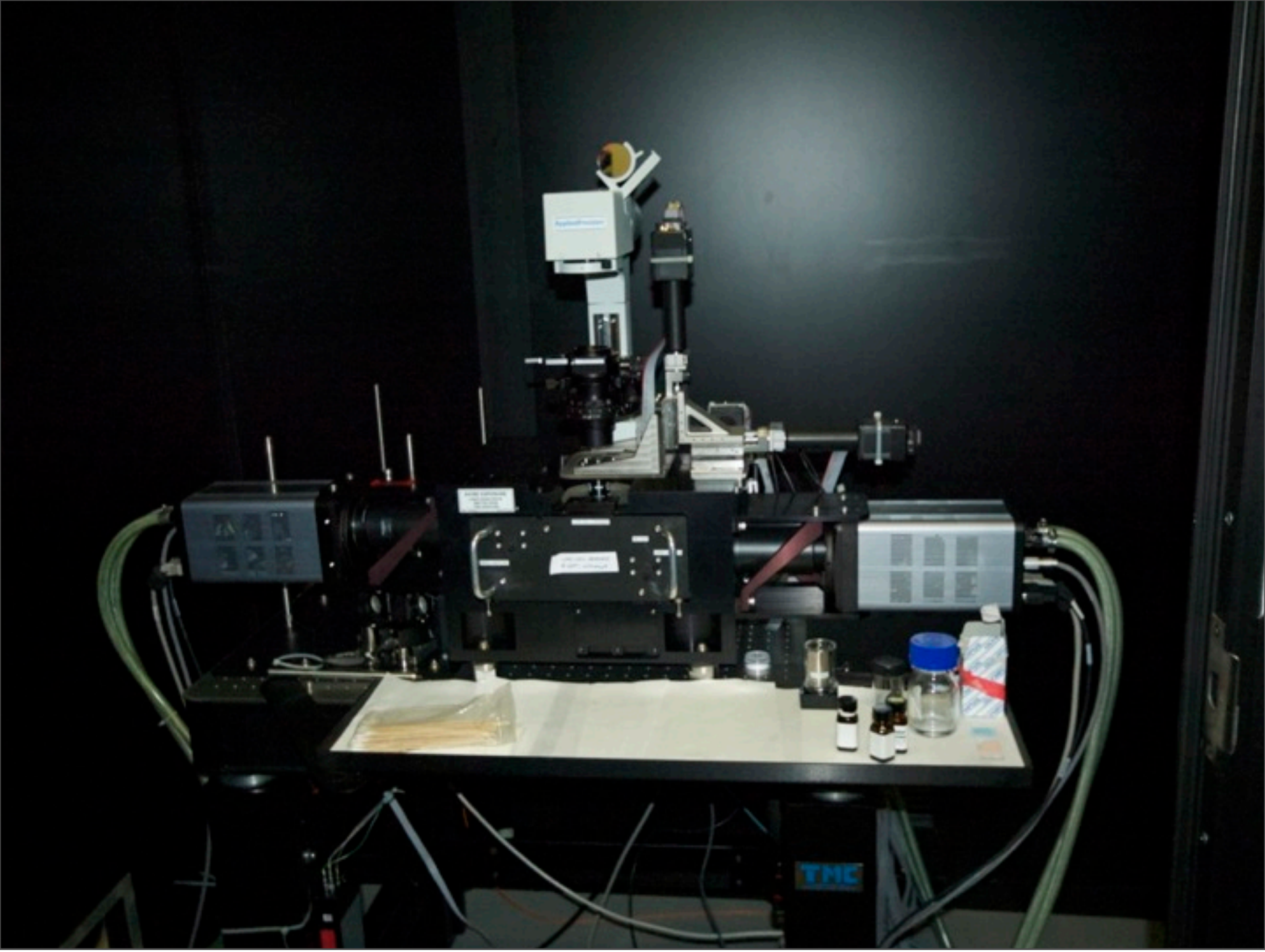


How can we improve the basic design of widefield microscopes ?

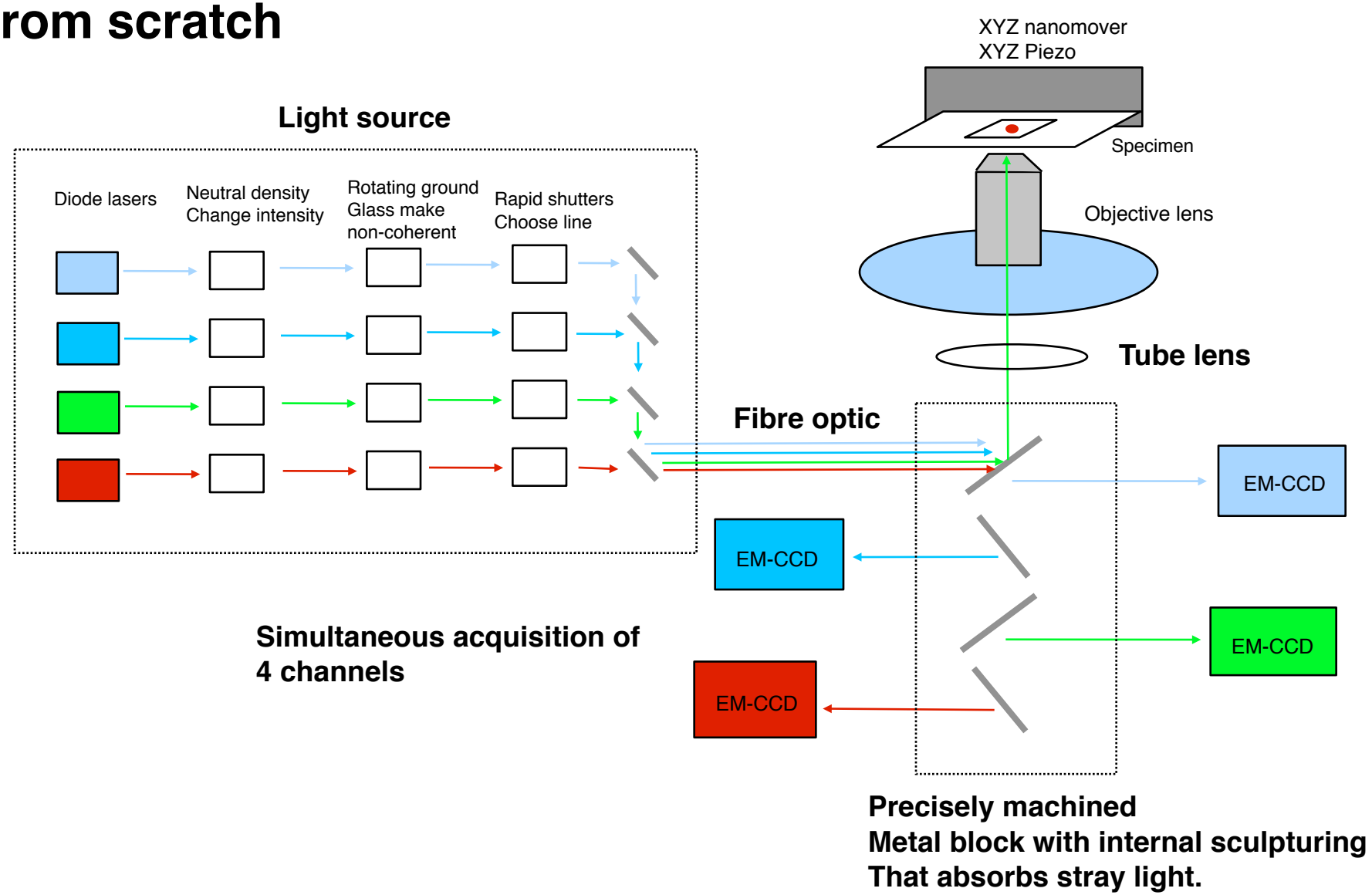
OMX

Richard Parton

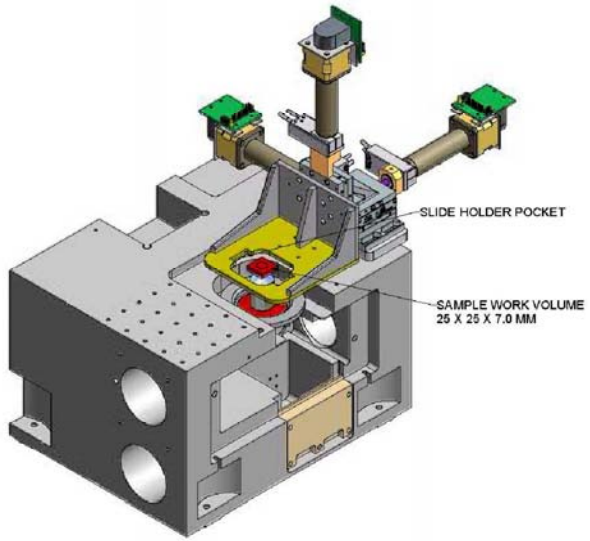
John Sedat and Ian Dobbie



OMX - Redesigning widefield microscopy from scratch

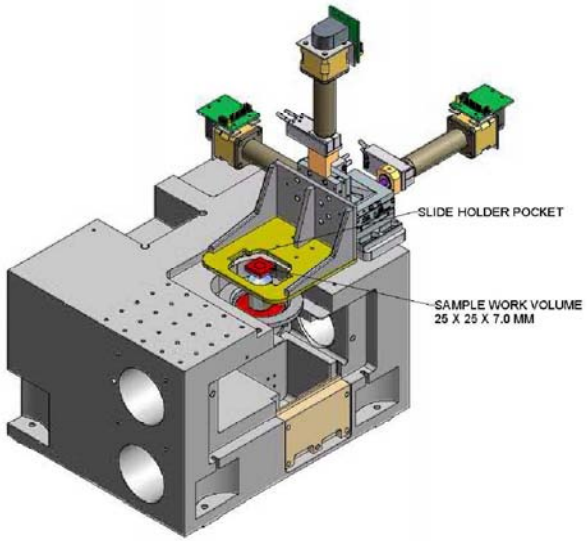


OMX (John Sedat, David Agard and Mats Gustafsson)



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

OMX (John Sedat, David Agard and Mats Gustafsson)



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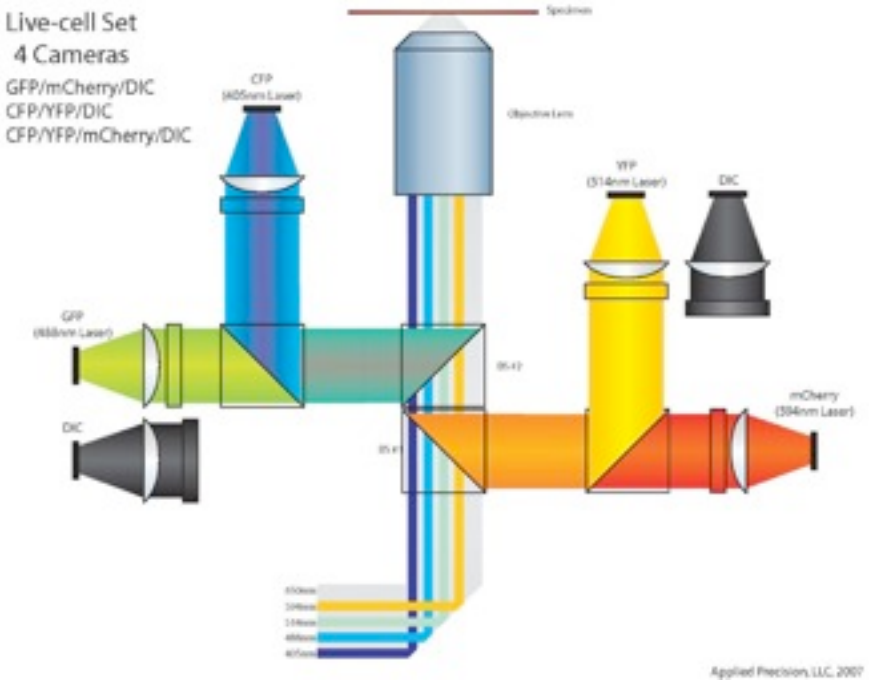
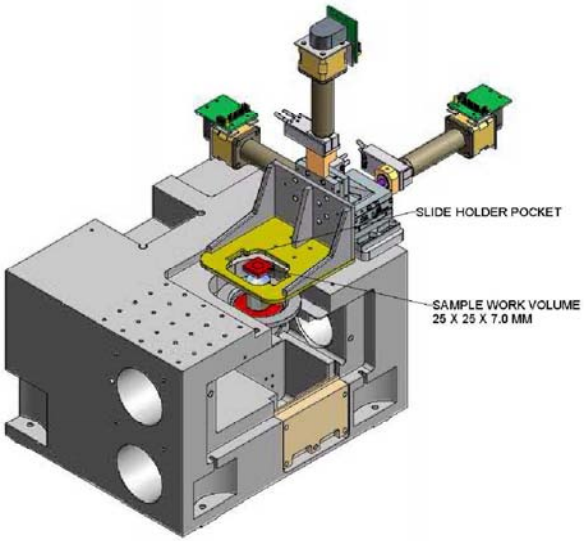


4 laser excitation lines
4 simultaneous acquisition lines CCDs

OMX (John Sedat, David Agard and Mats Gustafsson)



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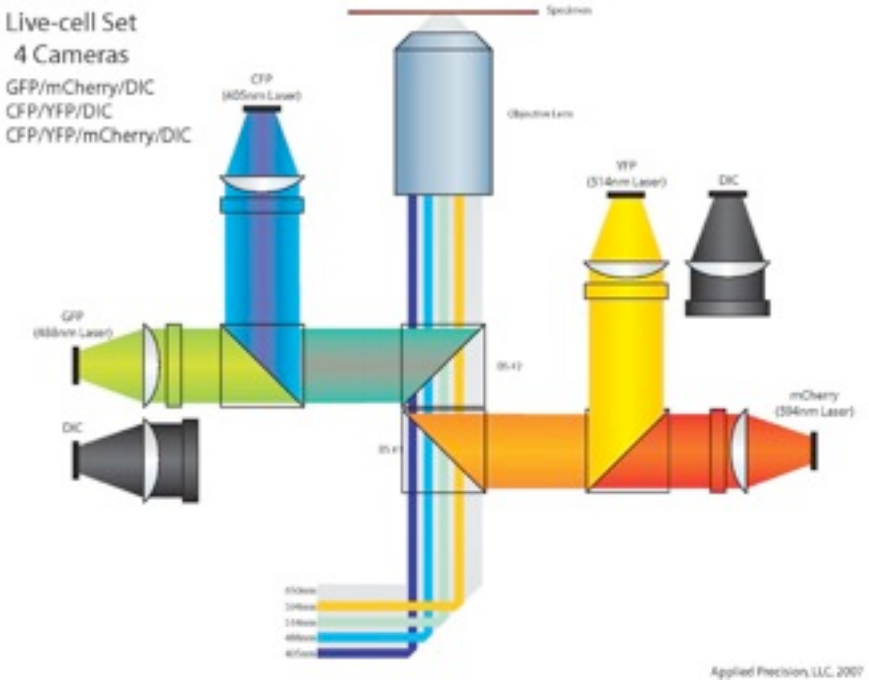
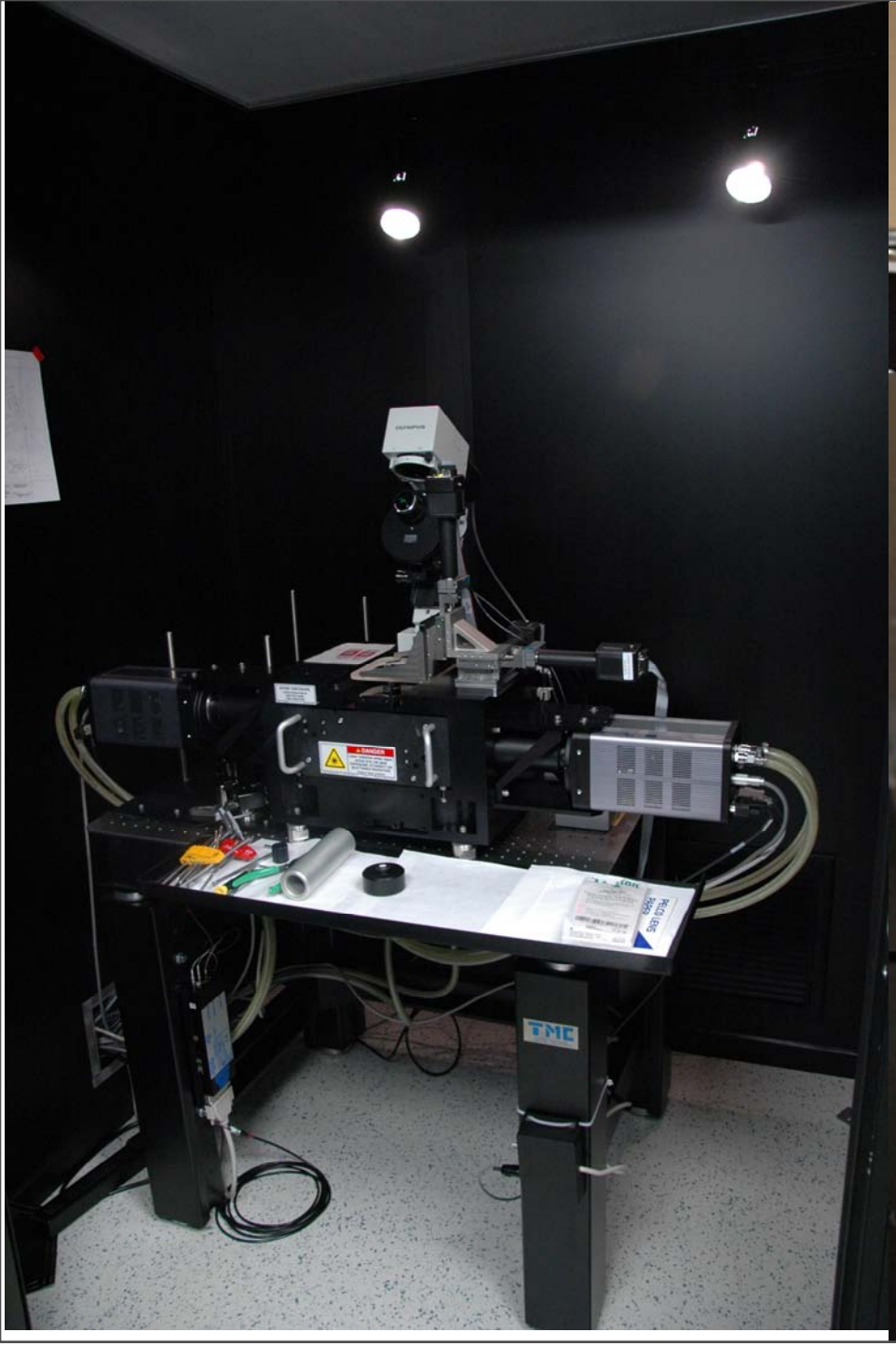
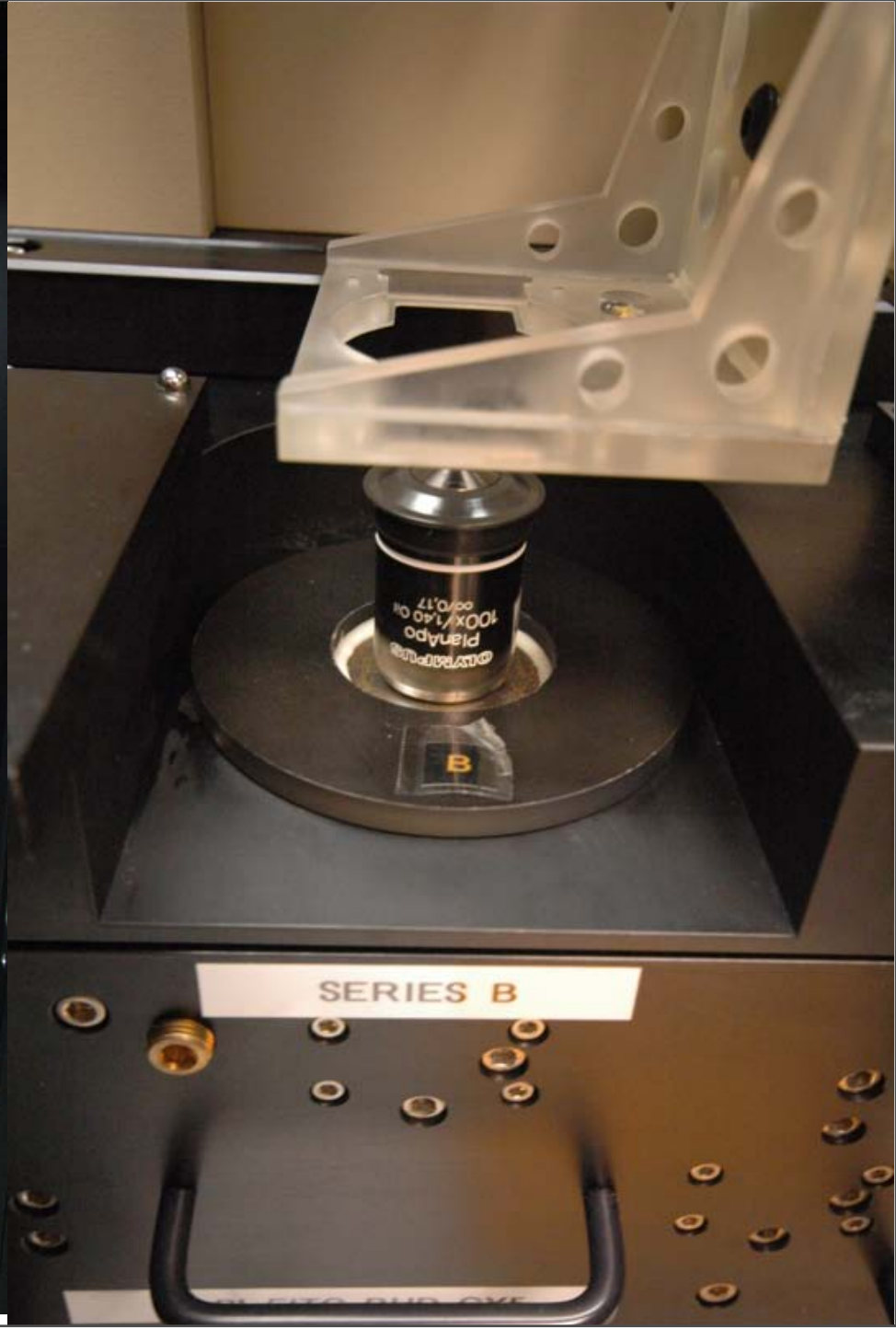


4 laser excitation lines
4 simultaneous acquisition lines CCDs

We have the second replica of the prototype instrument - 7 manufactured so far worlwide.


DANGER
VENTUS


TMC


SERIES B
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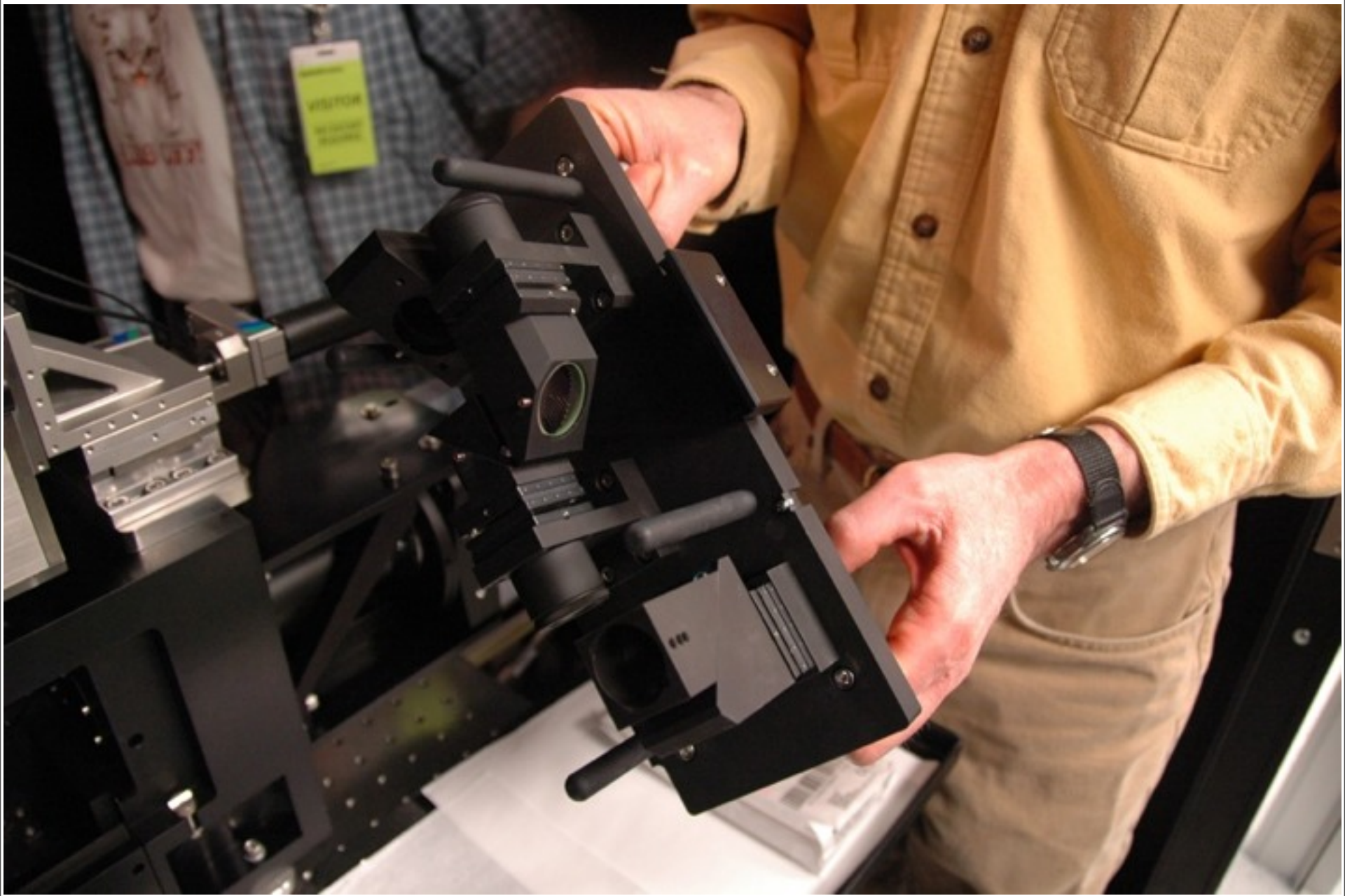
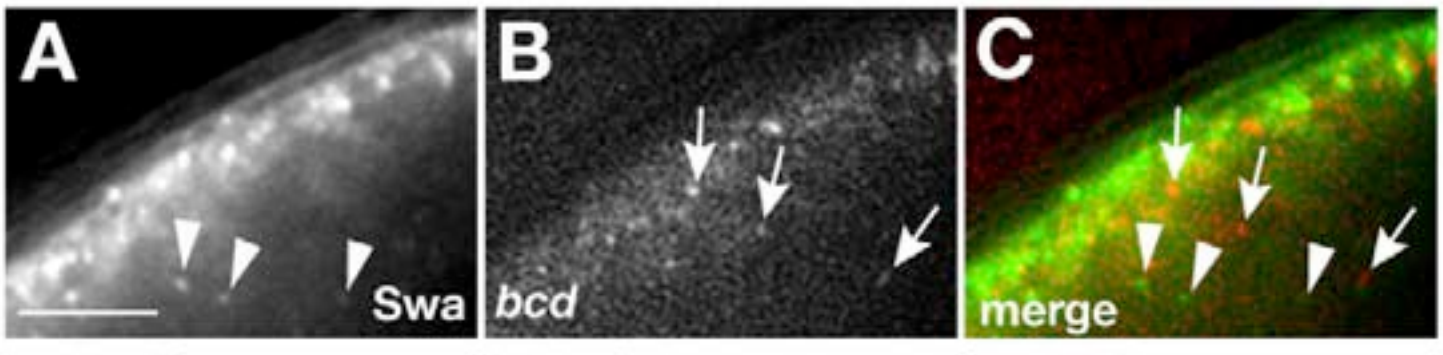


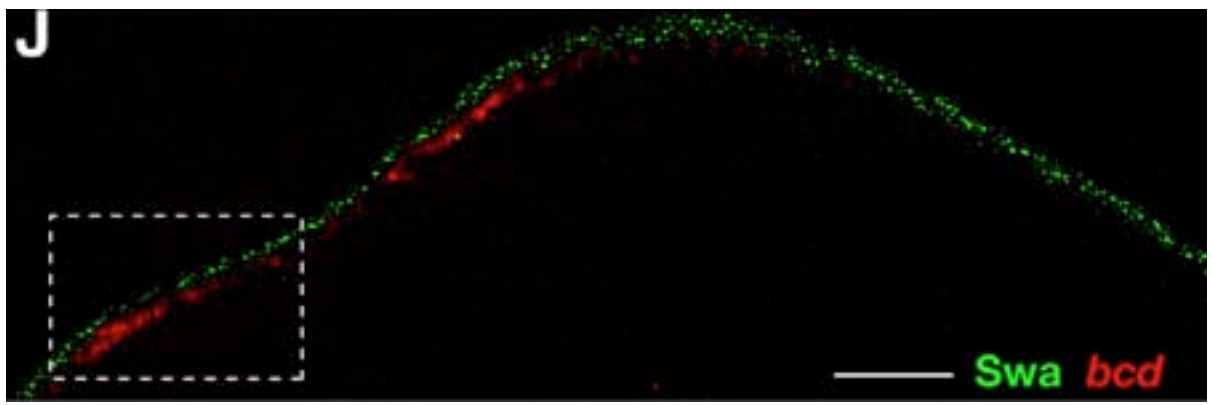
Live multidimensional imaging on OMX

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

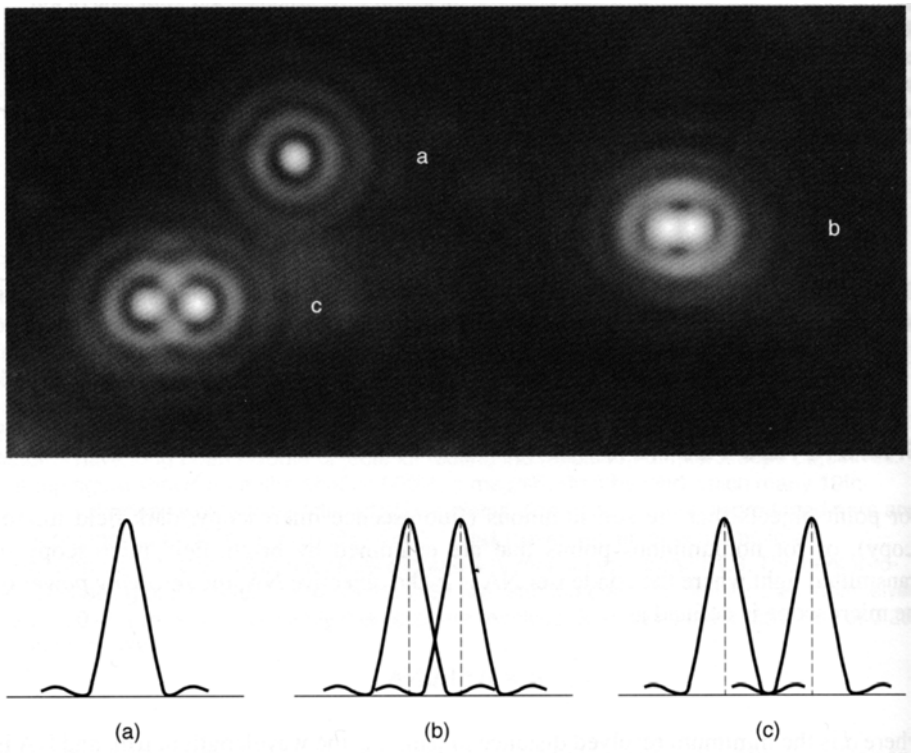
Distinguishing direct from indirect roles for *bicoid* mRNA localization factors

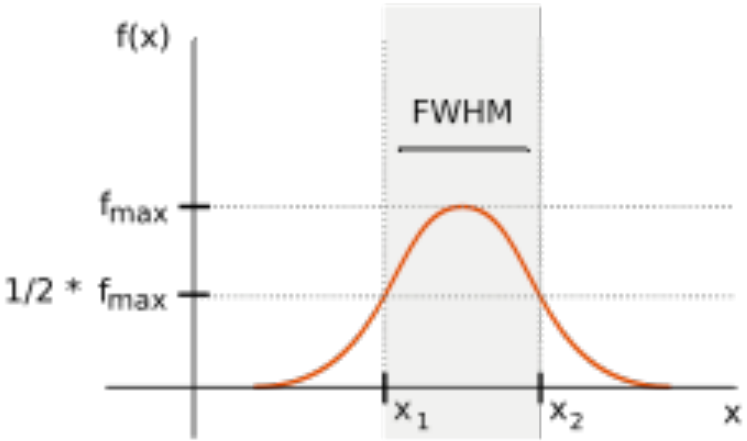
Timothy T. Weil[1,2,3], Despina Xanthakis[1], Richard Parton[3], Ian Dobbie[3], Catherine Rabouille[1], Elizabeth R. Gavis[2,*] and Ilan Davis[3]





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





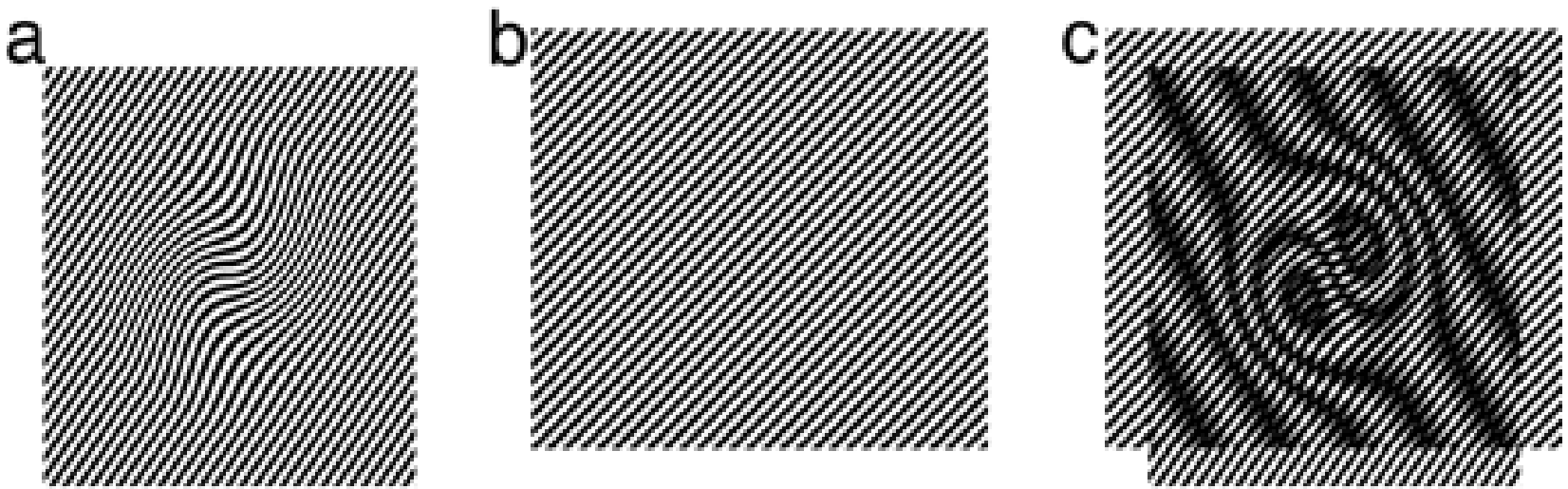
How can we overcome this limit long standing limit?

Structured Illumination

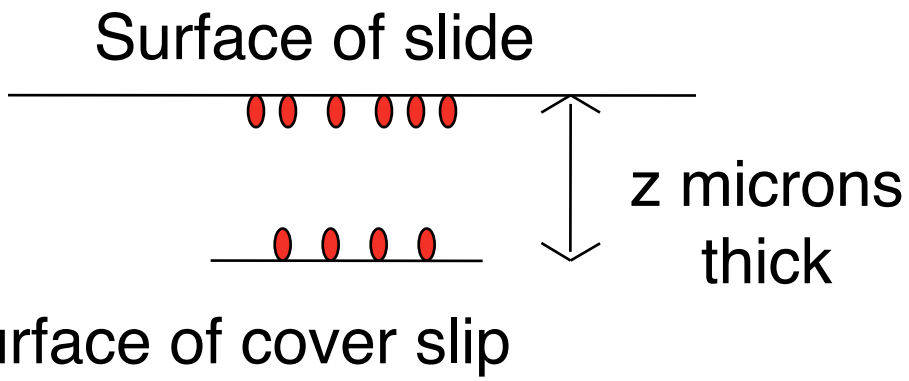
Surpassing the lateral resolution limit by a factor of two using structured illumination. Journal of microscopy Gustafsson, G.L., (2000) 198, 82.

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

Resolution extension through Moire effect



Bead slide: 0.1micron and 0.5 micron

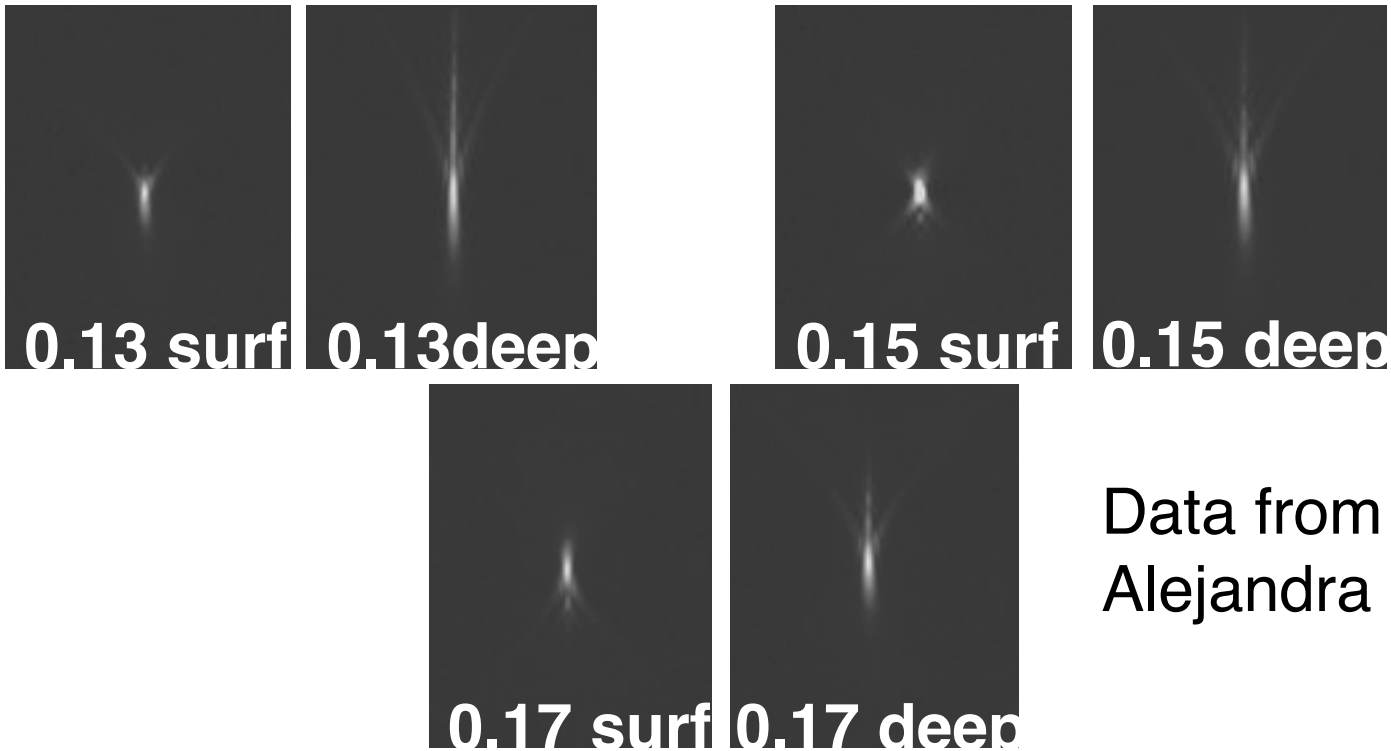


Tetraspeck beads: chromatic registration
DAPI/FITC/Rhodamine/Cy5

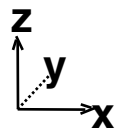
Beads (PS Spec): Single fluorochrome
Brighter -better for generating
point spread functions for deconvolution

Inspec Intensity beads: Measure dynamic range

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

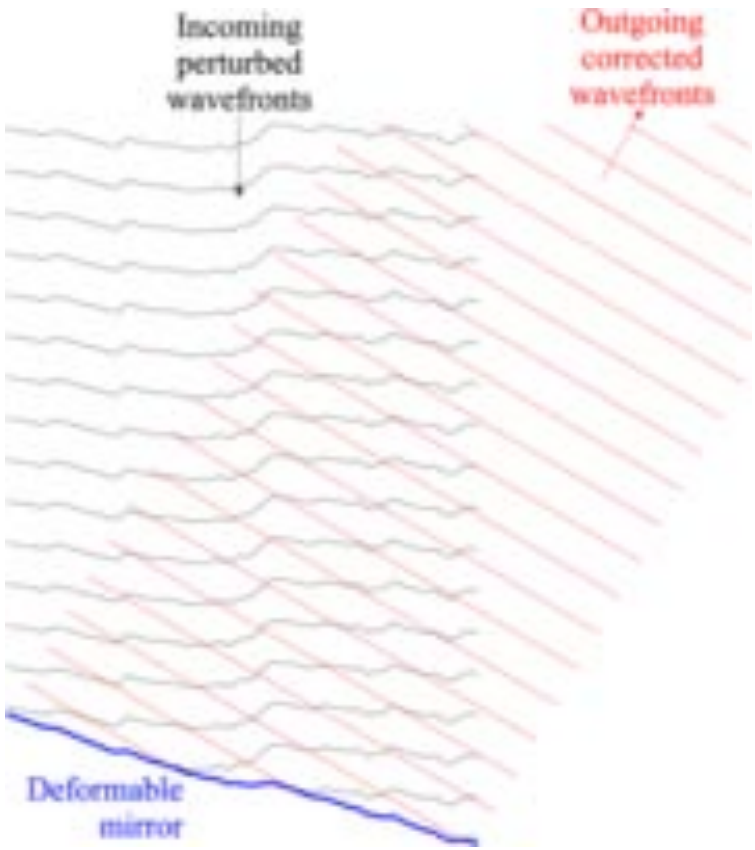


Data from
Alejandra Clark

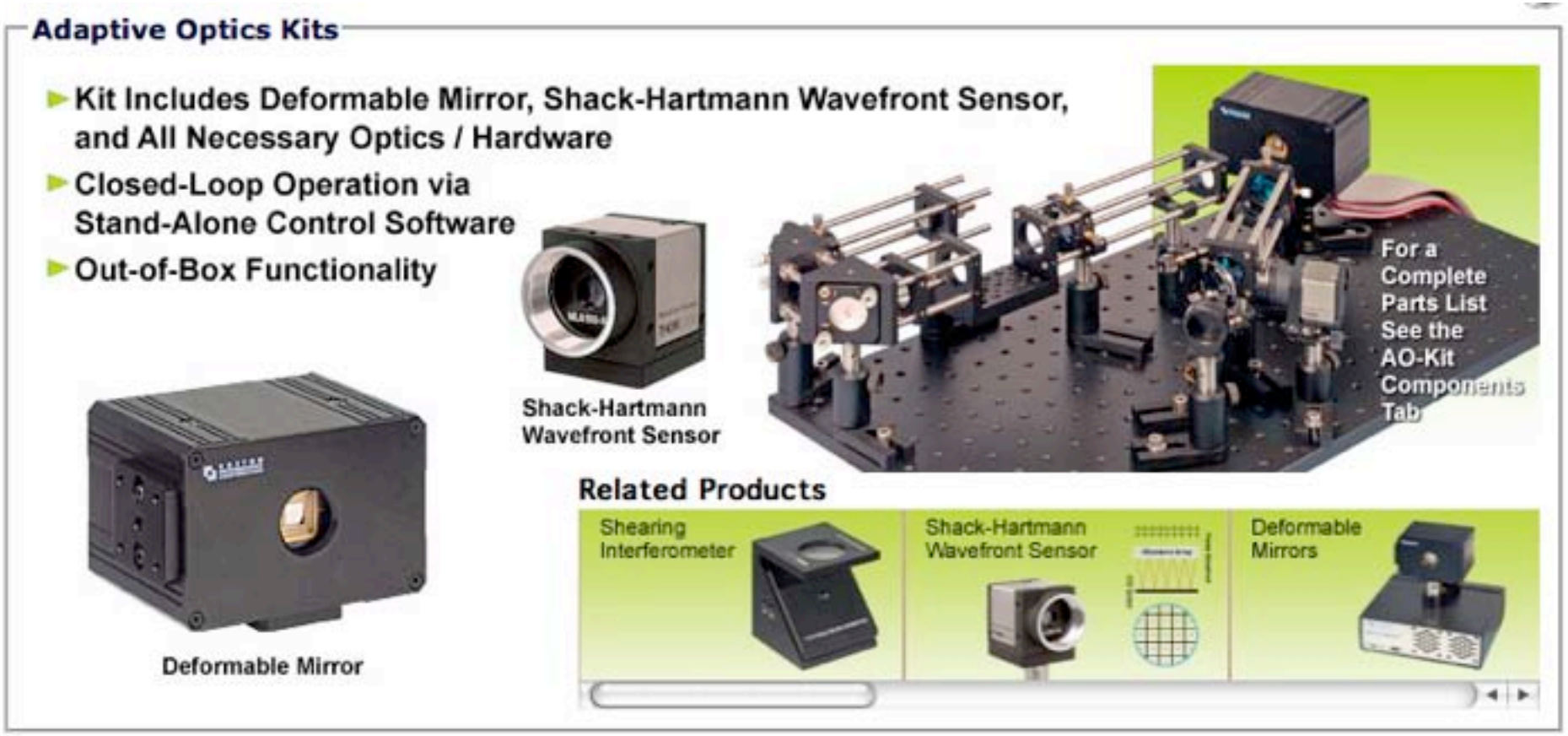


Adaptive Optics

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.

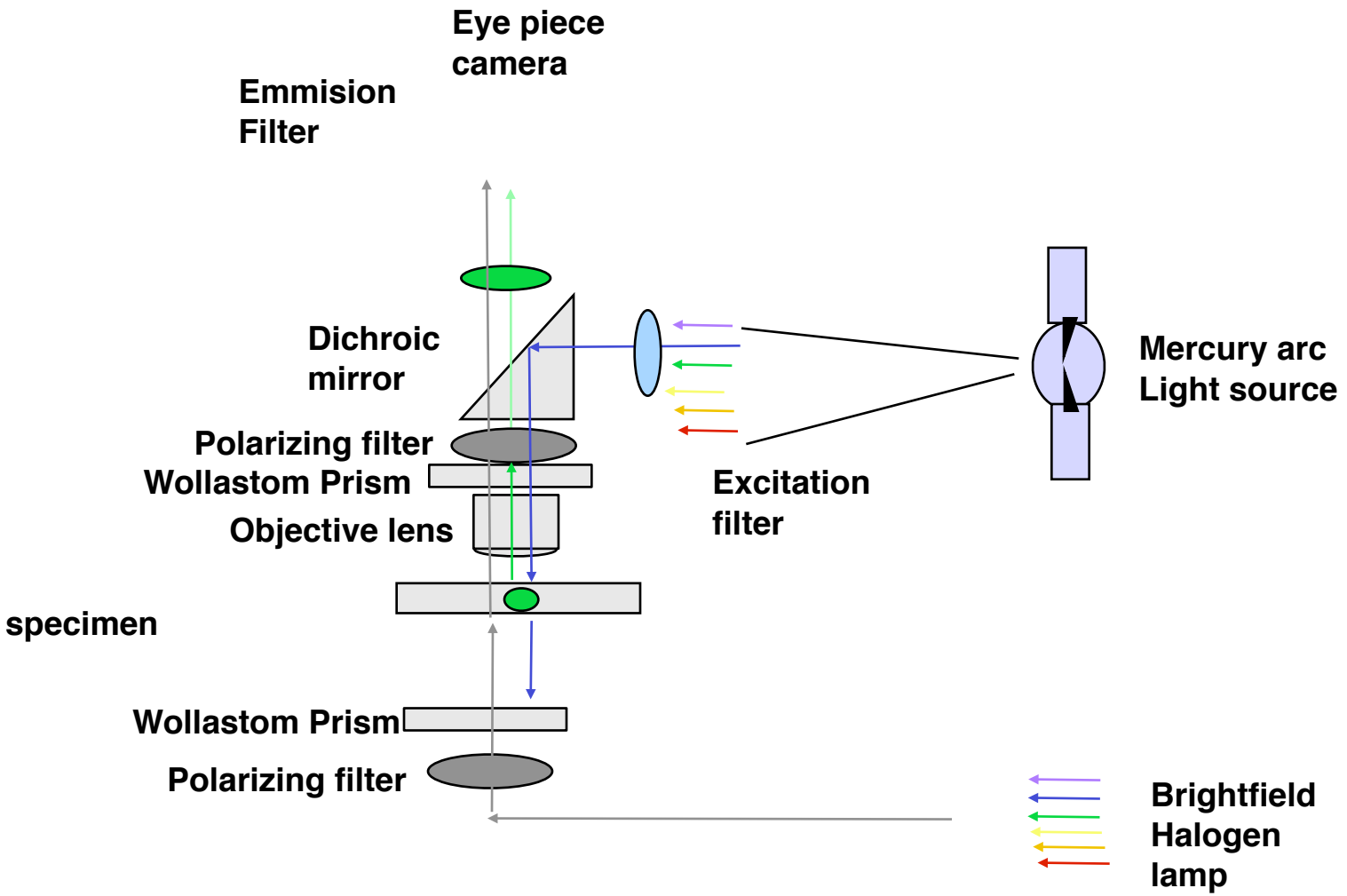


From Thorlabs



The hard part - algorithms for shaping the deformable mirror

How to use DIC and Fluorescence without loss of signal by polarizing filter?



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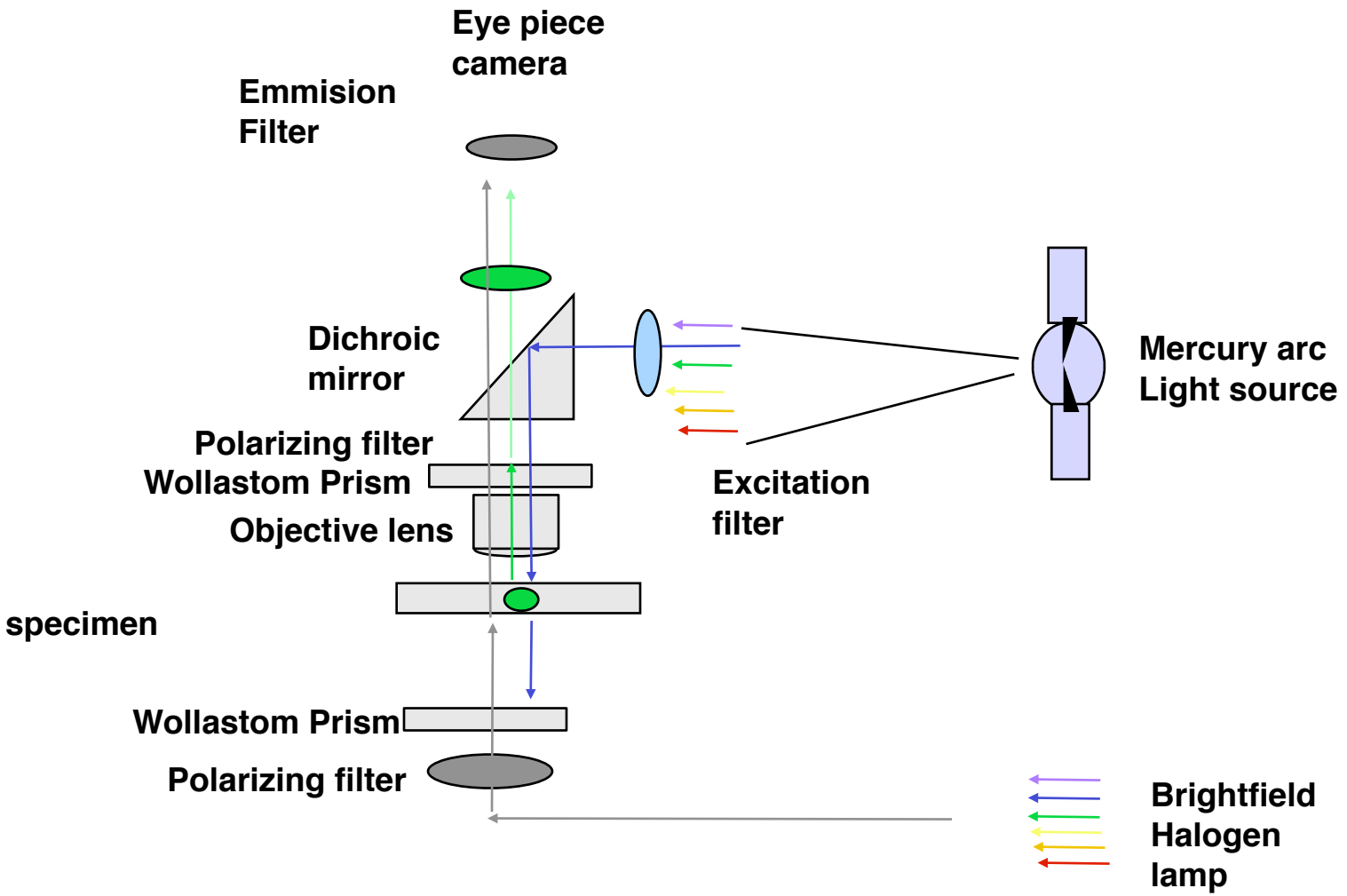

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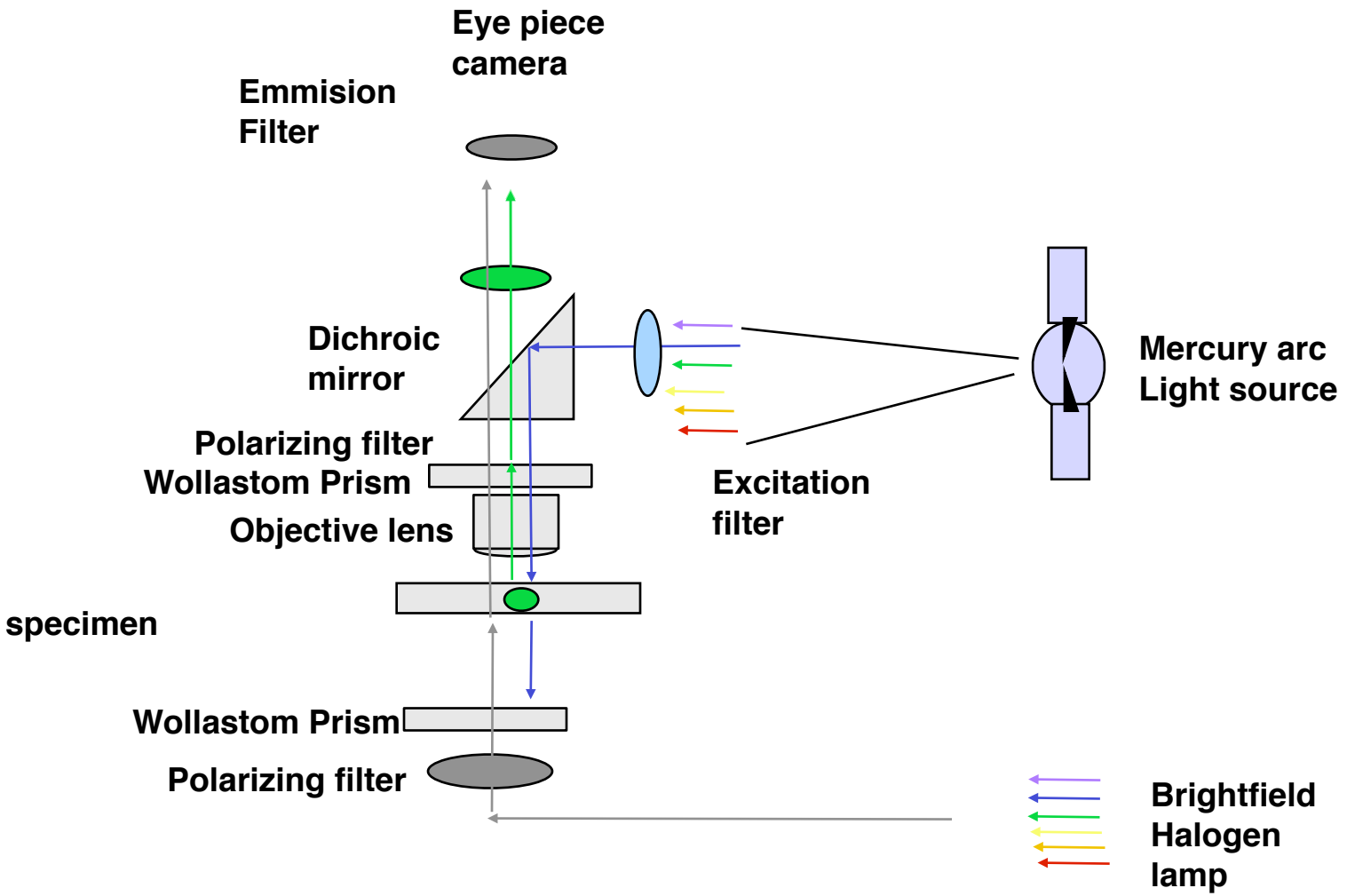

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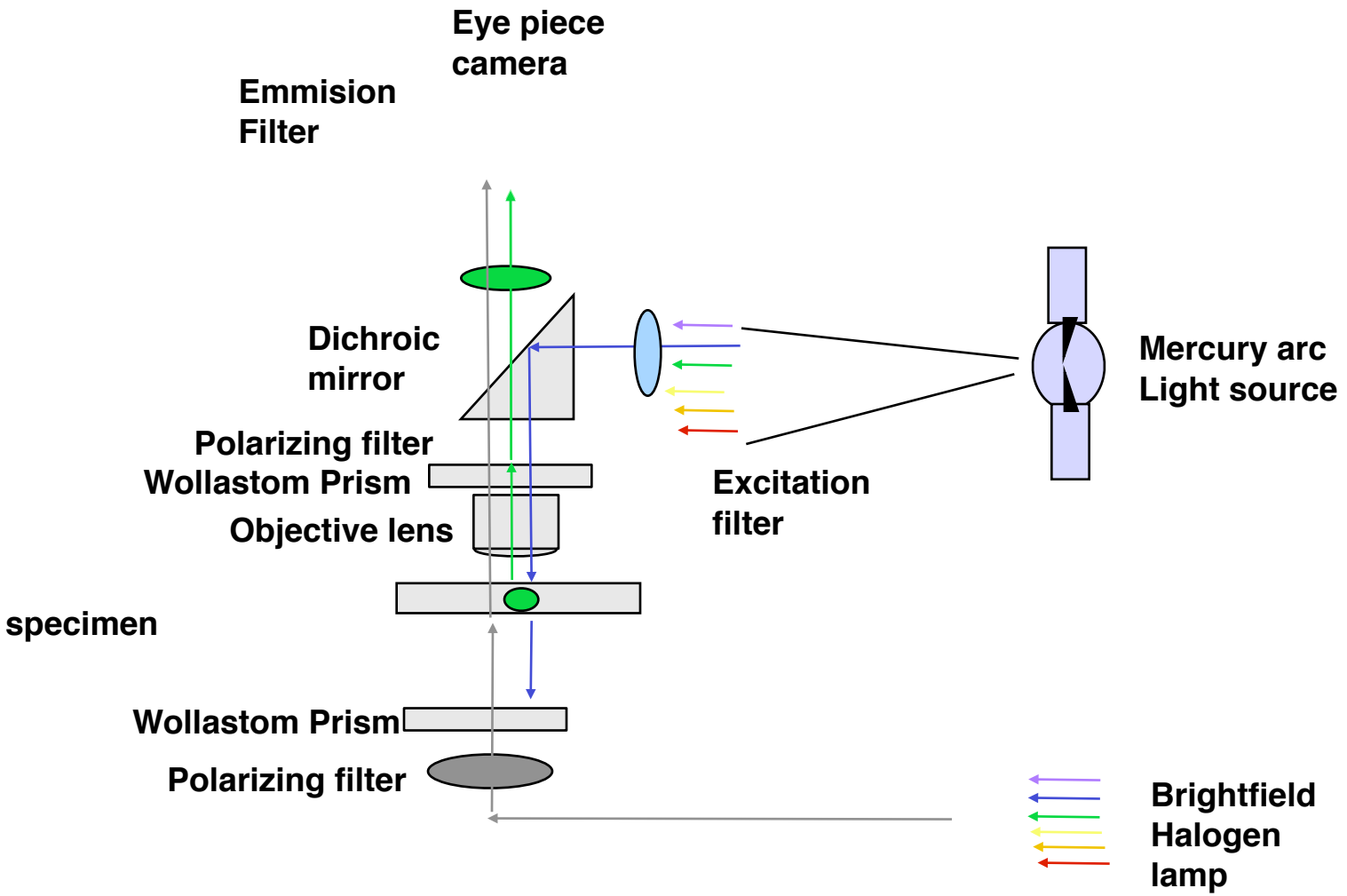

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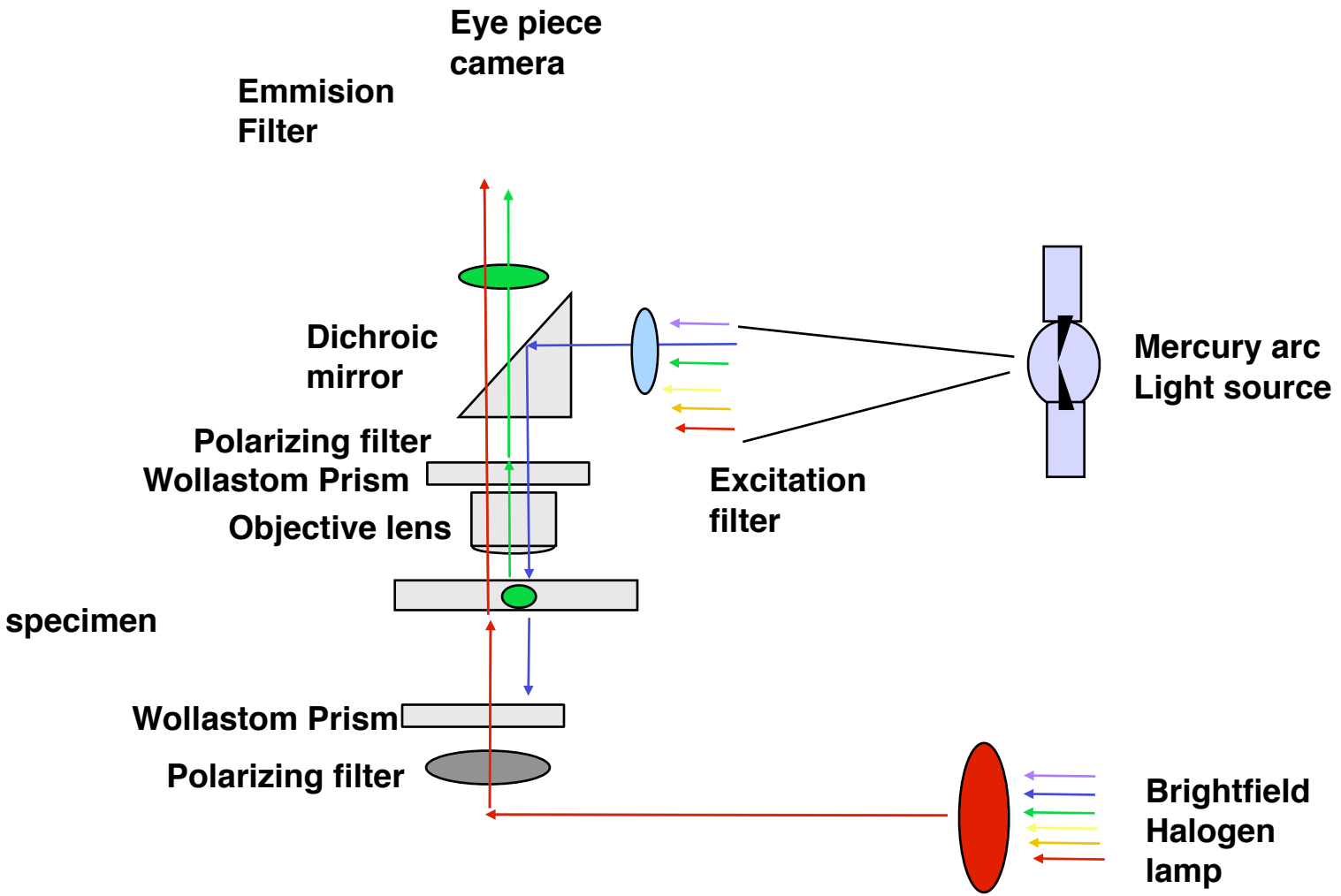

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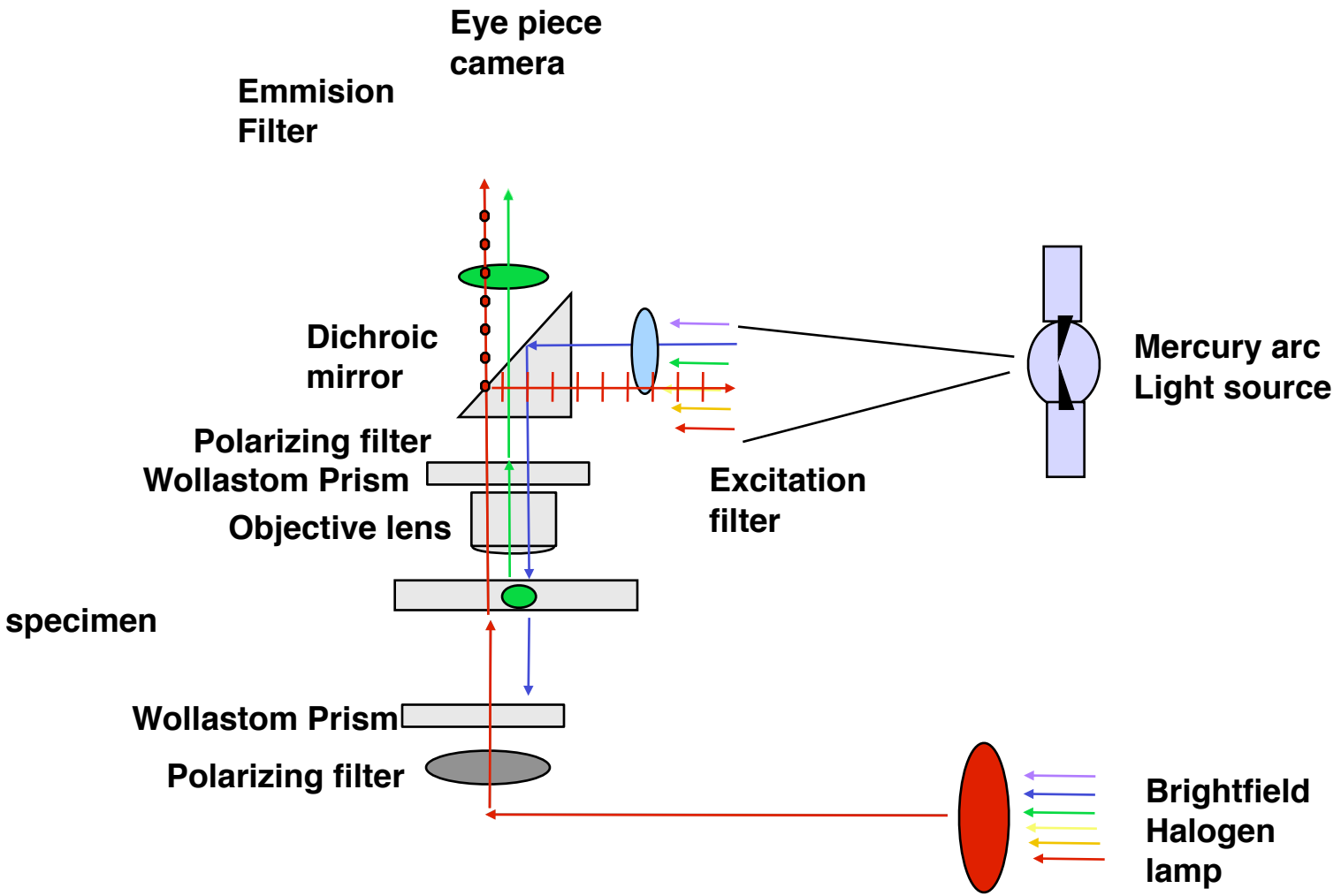

Simultaneous DIC and fluorescence (avoid loss of fluorescence intensity)

DIC/FITC cube where dichroic mirror acts as polariser only in red light instead of the analyser. Available for FITC/ rhodamine/DIC and other flavours.

Analyser removed to emission filter wheel.

The End