Microscopy Course 2013

Day 1: Monday 11 March - Fundamental Principles of Microscopy

Ilan		9:30	Welcome to the course
1	Richard	9.45-10.45	General introduction to light microscopy
2	Ian	10.45-12.00	Principles of microscopy and microscope anatomy
3	Ian	1.00-2.00	Contrast enhancement (phase contrast and DIC)
4	Eva	2.00-3.00	Basic Fluorescence Microscopy and sample prep
5	Richard	3.30-4.30	Basic image analysis
6	Mark	4.30-5.30	Fluorescent dyes and proteins

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Day 2: Tuesday 12 March - Imaging Molecules in Cells

7	Richard	9.00-10.00	Live cell imaging
8	Alan	10.00-11.00	Confocal, spinning discs and Multiphotons
9	Ilan	11.20-12.20	Advanced widefield microscopy and bespoke systems
10	James	1.30-2.30	Detectors for microscopy
11	Ian	2.30-3.30	F* techniques: FRET, FLIM, FCS, FRAP, FLIP
12	Chris E.	4.30-5.30	STED, FCS
13	Eva	5.00-5.20	Light-sheet microscopy of cellular dynamics

****

Day 3: Wednesday 13 March - New and emerging Imaging Approaches

14	Chris L.	9.00-10.00	Single molecule techniques
15	Rainer	10.00-11.00	Storm/PALM techniques.
16	Lothar	11.30-12.30	OMX
17	Errin	1.30-2.00	Electron Microscopy
18	Douglas	2.30-3.00	Image Management
19	Graeme	3.00-4.00	Applied Image analysis and Matlab

AND

****

Day 4: Thursday 14 March – Microscope demos and practical image analysis
9.00-12.00 and 14.00-17.00
DV live
Spinning disk live
OMX V2 dSTORM
OMX V3 SIM
Olympus scanning confocal
Olympus basic wide-field
Olympus TIRF
Image analysis

15 min demos + 5 min walking: 8 sessions in the morning, 8 sessions in the afternoon (4 people per group, 64 people in total)

Advanced Microscopy Course 2013
Introductory Lecture

Richard Parton - Richard.Parton@bioch.ox.ac.uk
Department of Biochemistry
University of Oxford

Goals of the lecture course

- The basic physics of optics and microscopes
- Explanation of how the light microscope works
- Make you aware of what is really important to good microscopy
- Describe the different techniques available and their application
- Explain of how to get the best from your microscope:
  - Specimen preparation and labeling
  - Contrast enhancement, phase and DIC
  - Fluorescence for detecting specific molecules
  - Digital acquisition and deblurring images

Why do we need to understand microscopy?

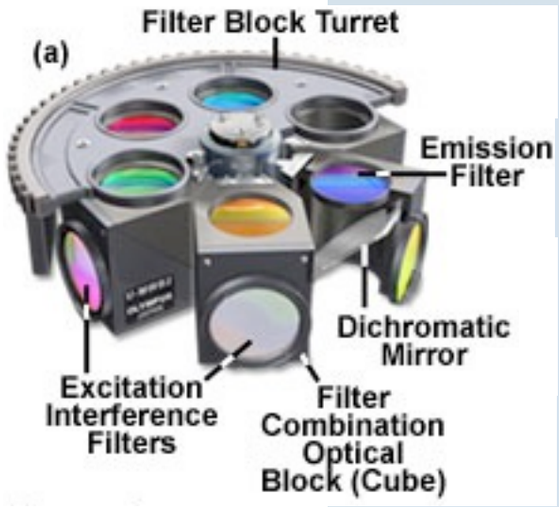
- Microscopes work purely to the laws of optical physics - there is no witchcraft!

- If you understand the principles involved then it is easy to understand how to get the best from your microscope.

- All the buttons and levers and knobs actually serve a purpose - don’t mess with them if you don’t understand!

Understanding what goes on in the Microscope

LECTURES 2-4



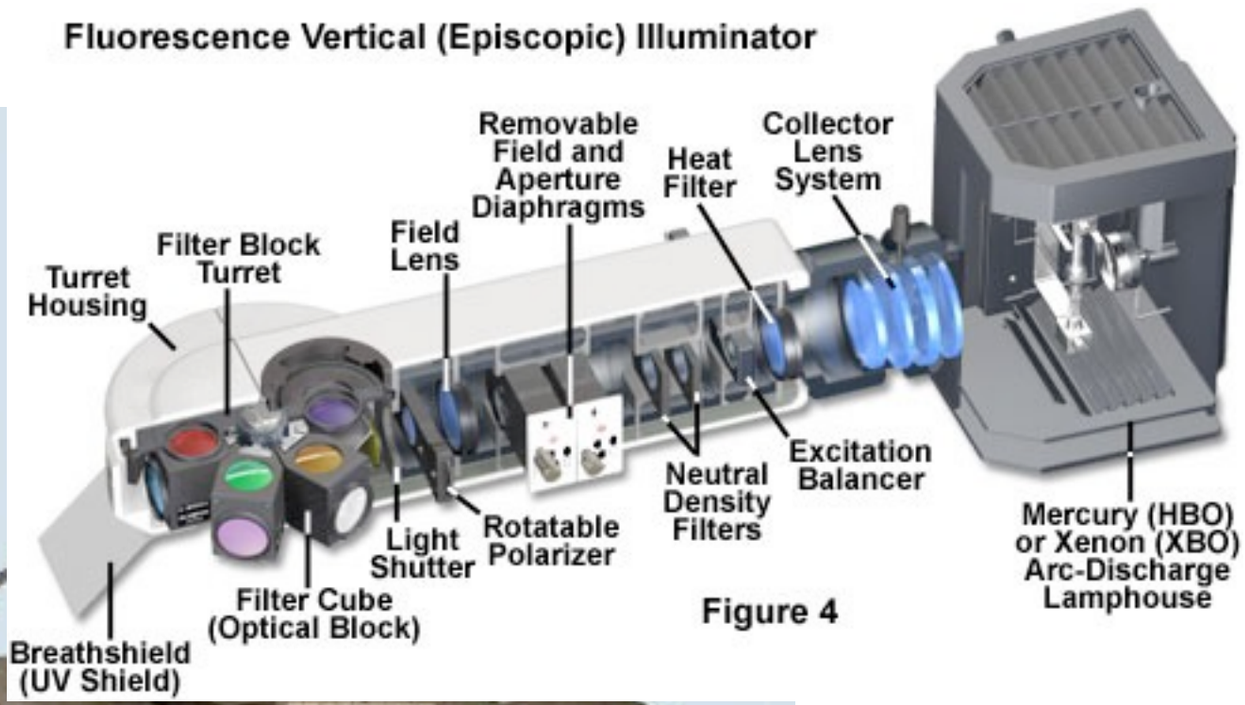


Figure 4

Figure 10

What can you do with a microscope?

100 years ago:

- Magnify small things to visualise more details

Now:

- Image specific molecules inside cells
- Determine how molecules interact with each other within cells
- Follow Changes in the distribution of molecules or the morphology of cells over time

Useful size range for light microscopy

We use the light microscope to image structures and substructures within the range: from about 300 μm down to about 0.3 μm (300 nm)

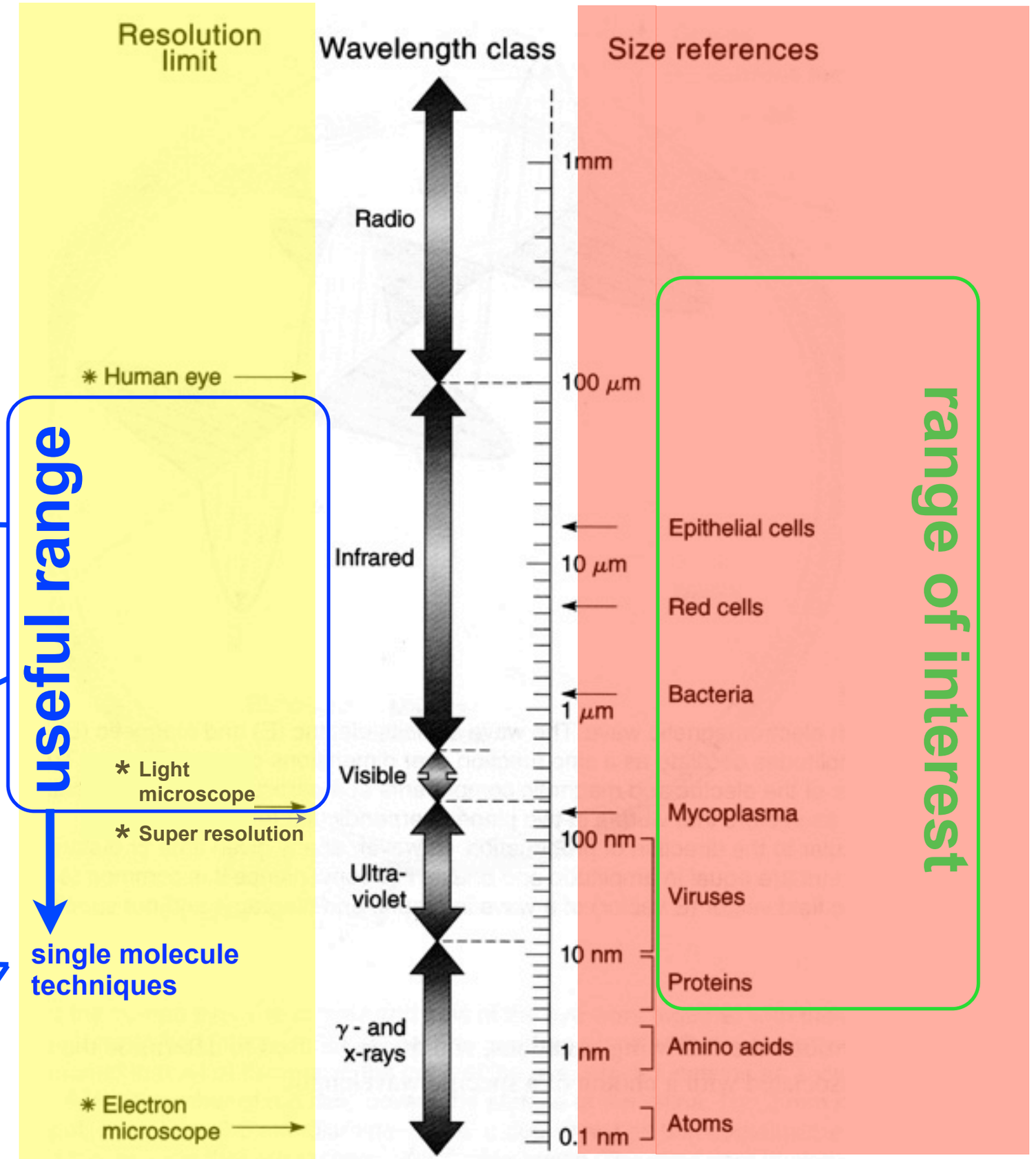
Eukaryote = 10 um
>50 um

Bacterium = 1 um

Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



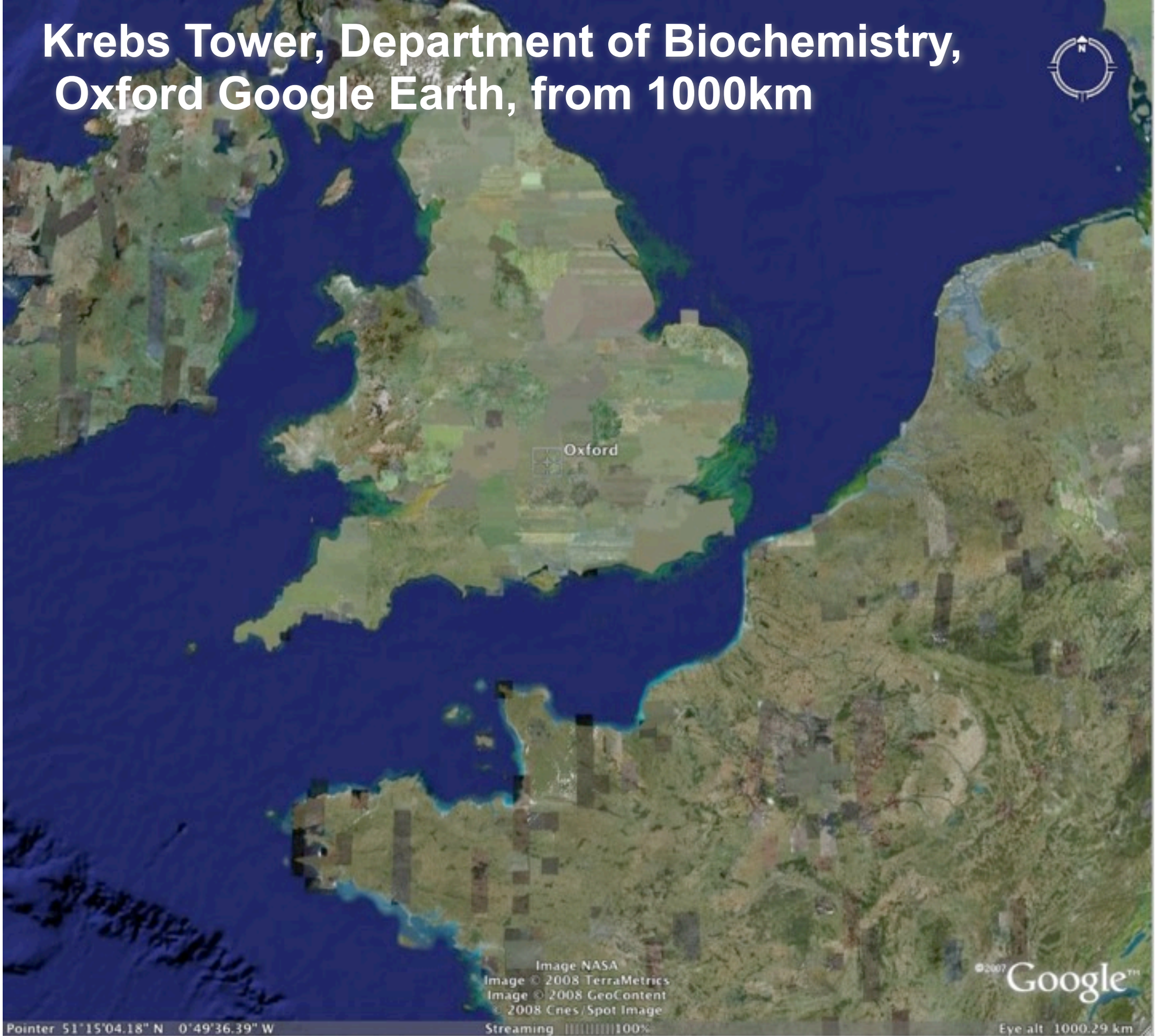
What is important in microscopy?

1. Resolution
2. Sampling
3. Contrast
4. Noise

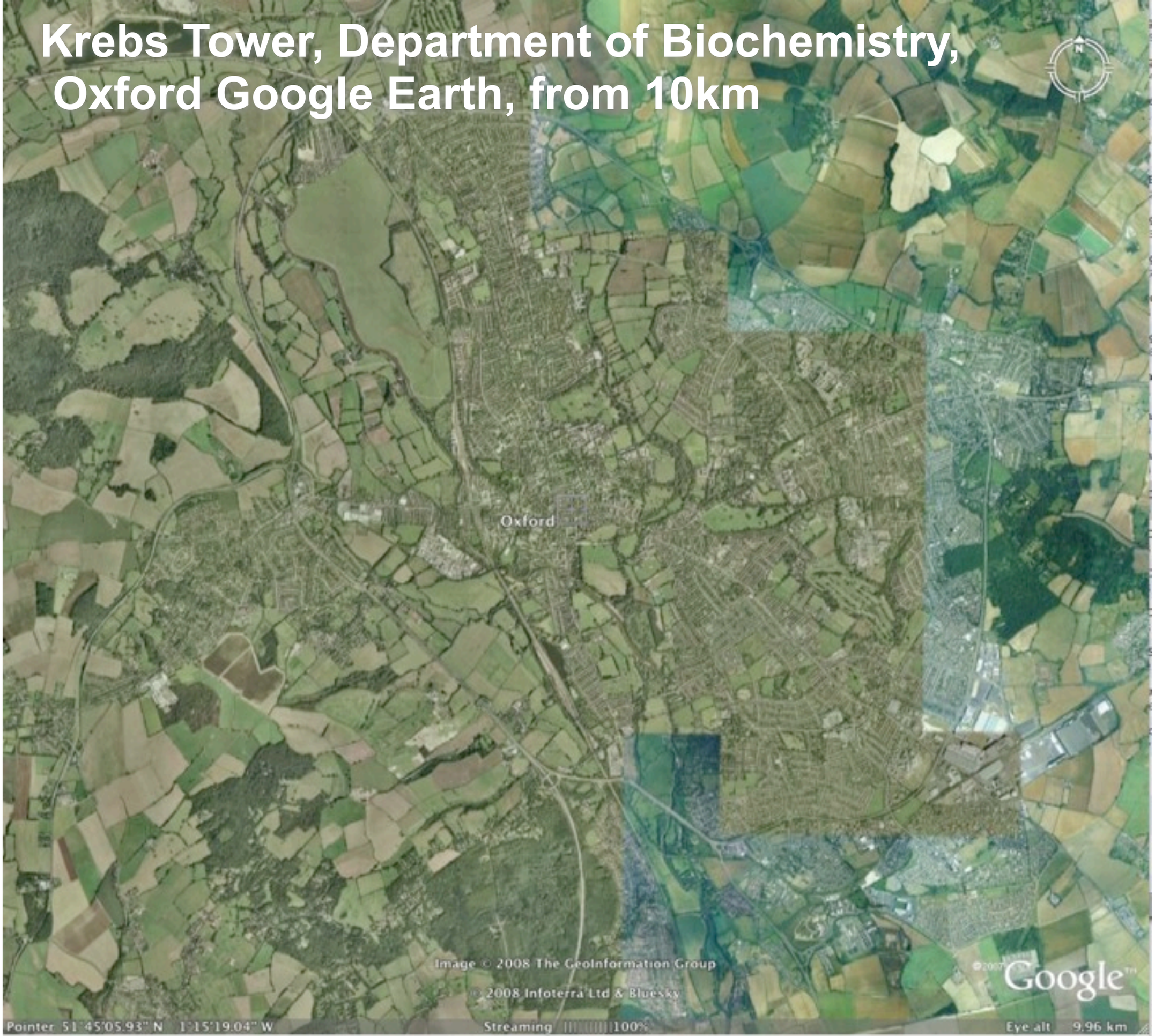
and nothing else!

Magnification is nothing without resolution!

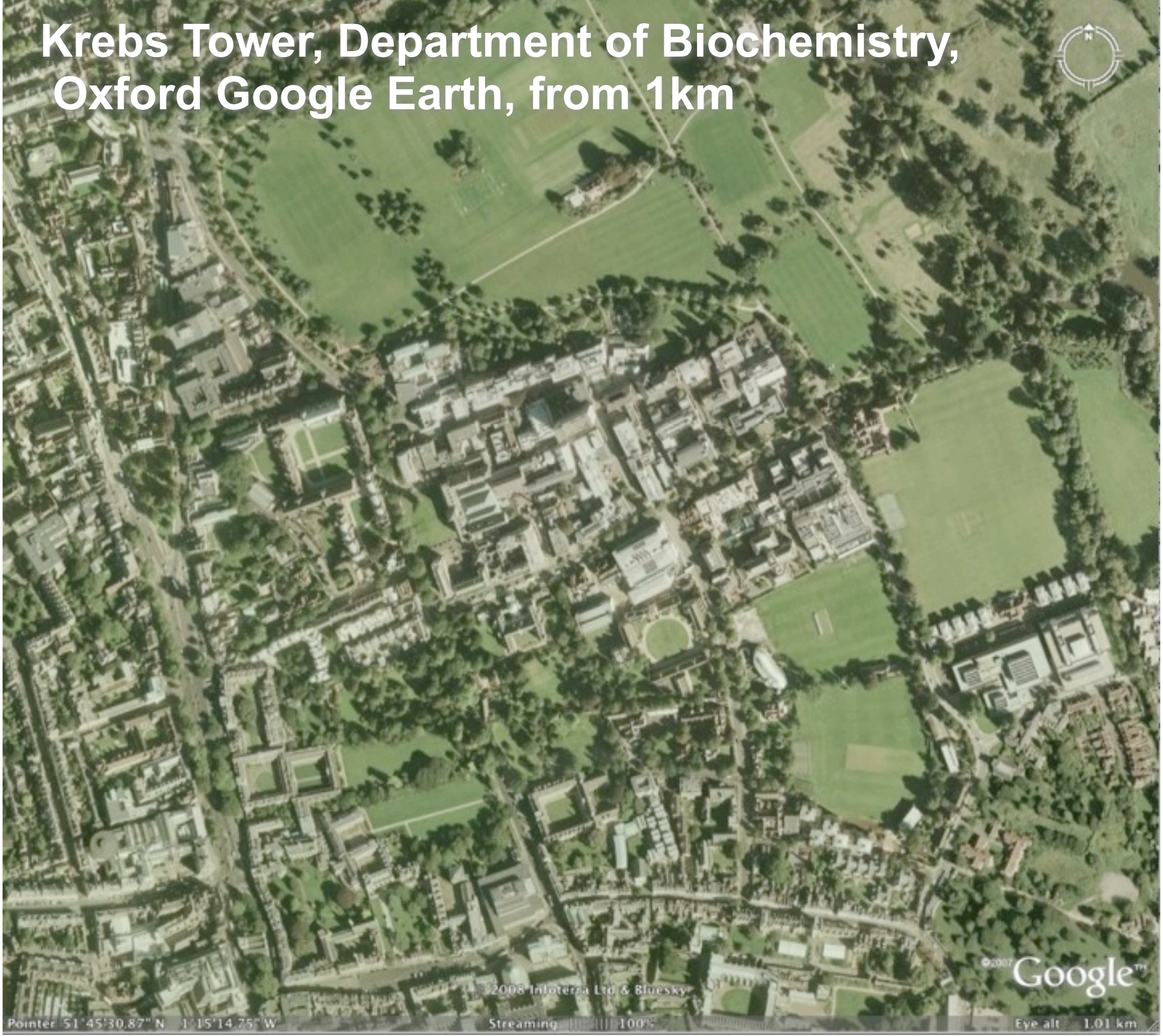
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 1000km



Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 10km



Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 1km



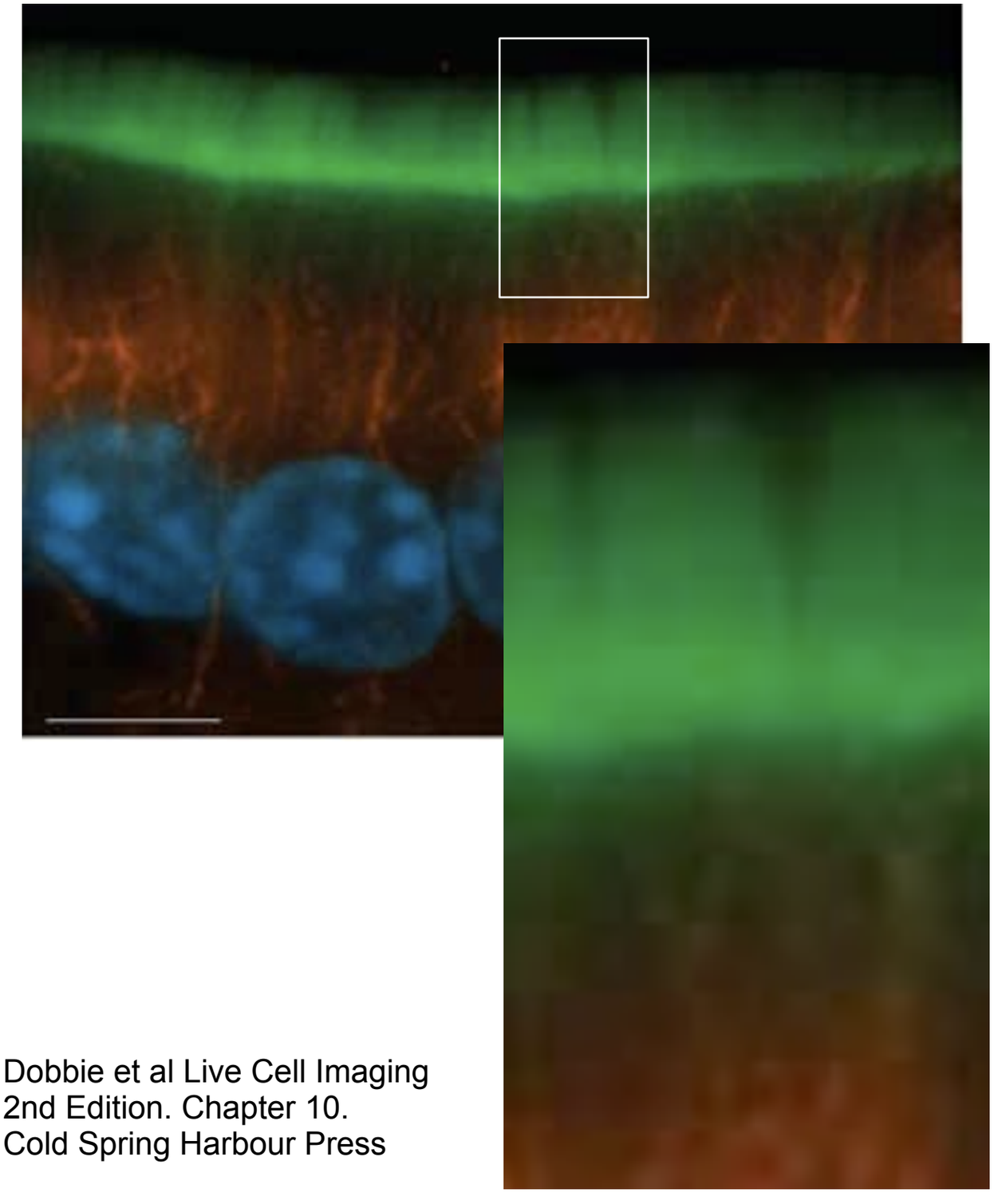
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 100m

Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 50m

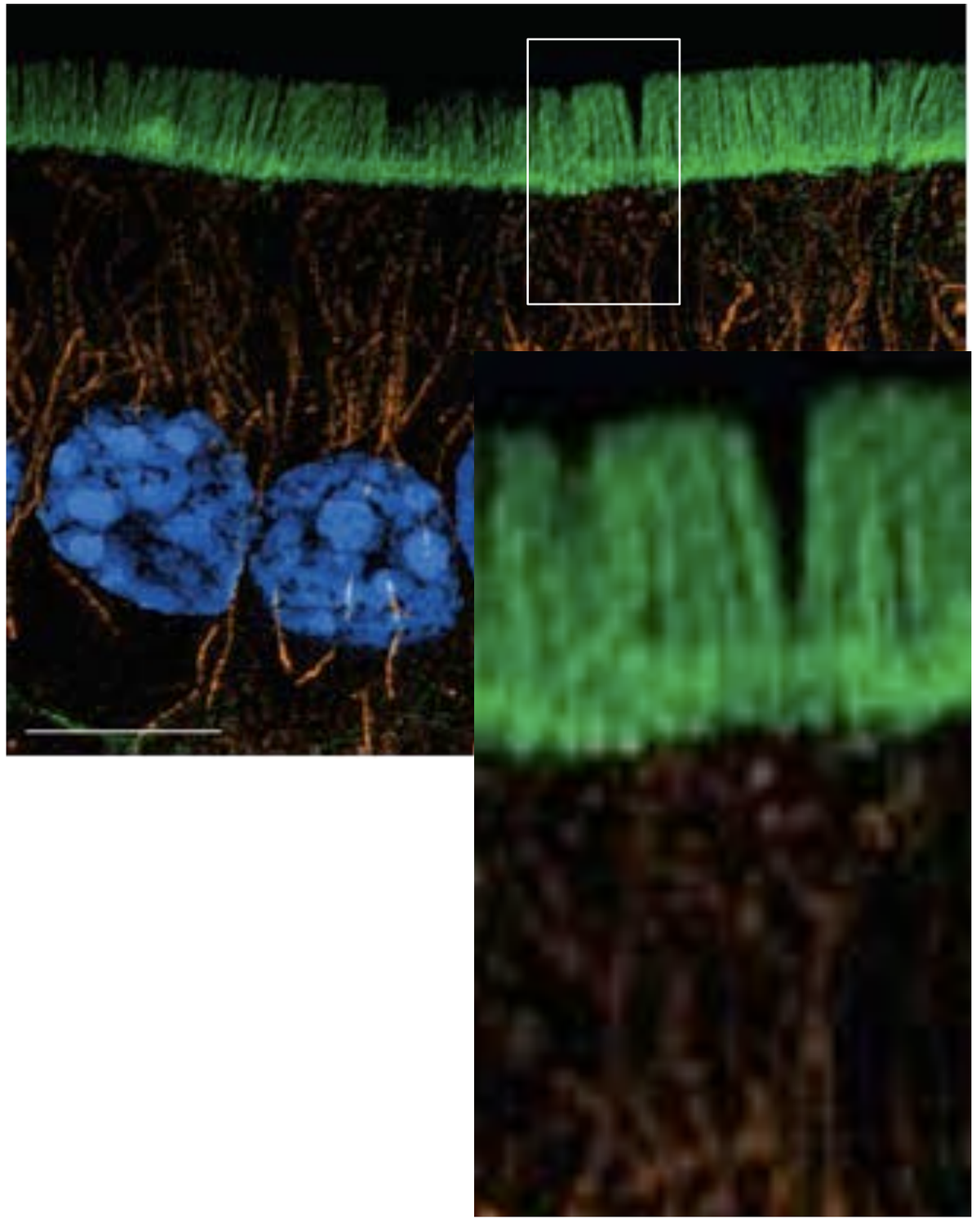

Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 10m
empty magnification!
© 2008 Infoterra Ltd & Bluesky
Google
Pointer 51°45'34.22" N 1°15'14.30" W
Streaming
Eye alt 10 m

RESOLUTION

Normal resolution



High resolution



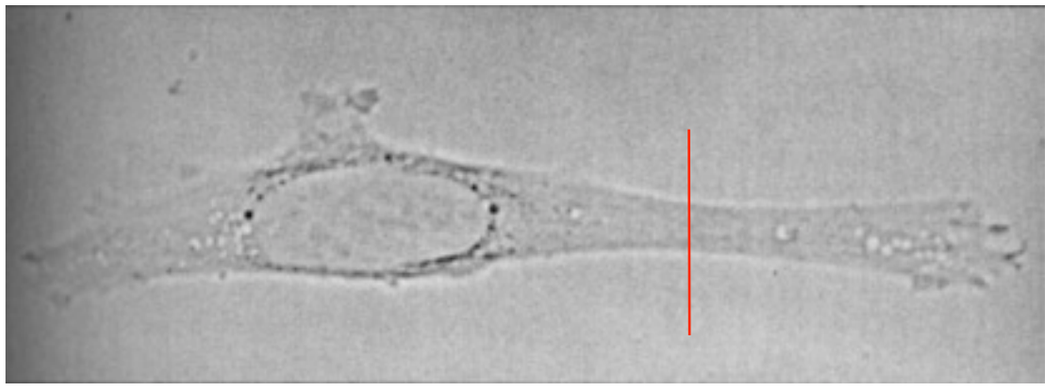
Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
Cold Spring Harbour Press

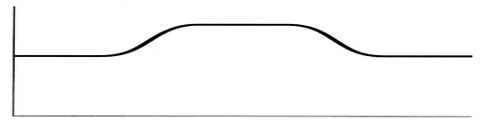
Resolution is nothing without contrast!

LECTURES 3-6

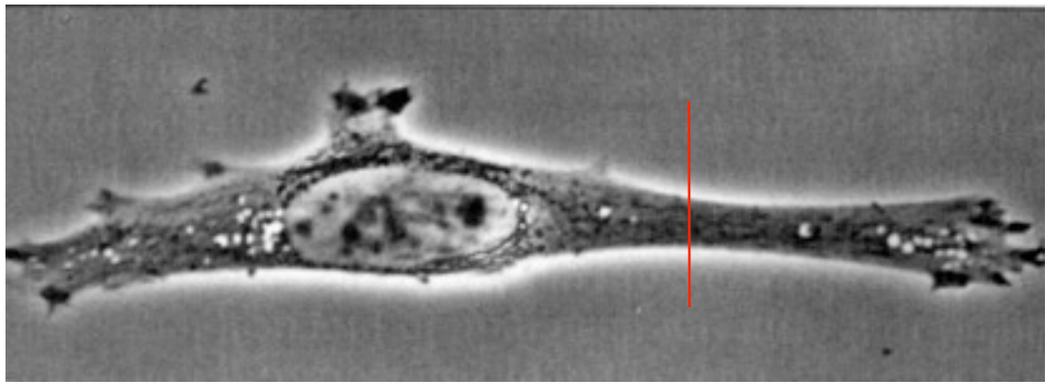
Contrast Enhancement:
Phase Contrast and DIC

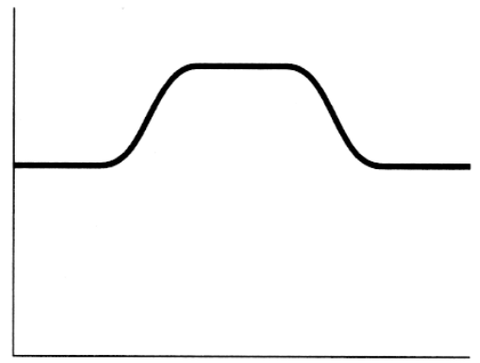
Normal contrast



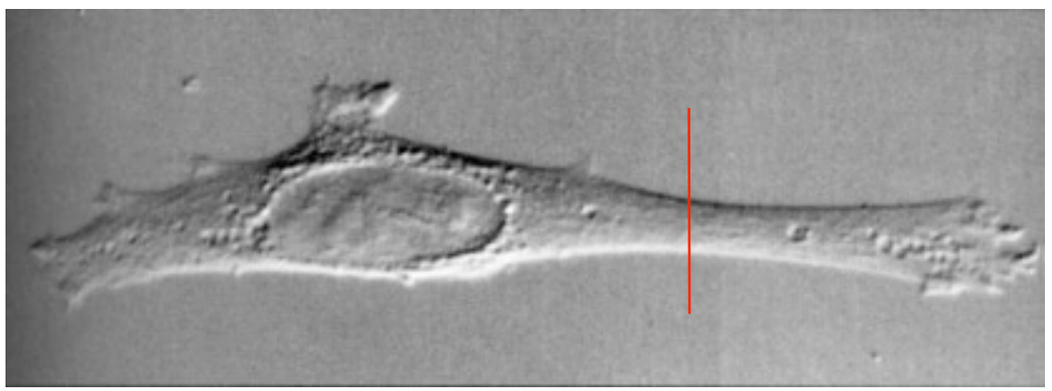


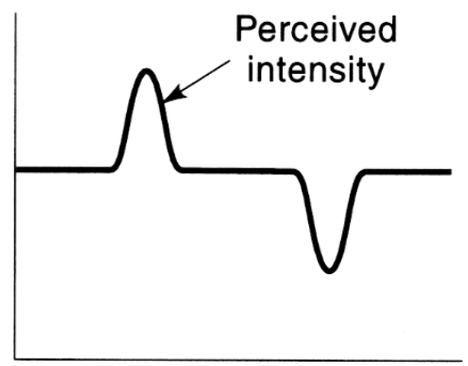
Phase contrast



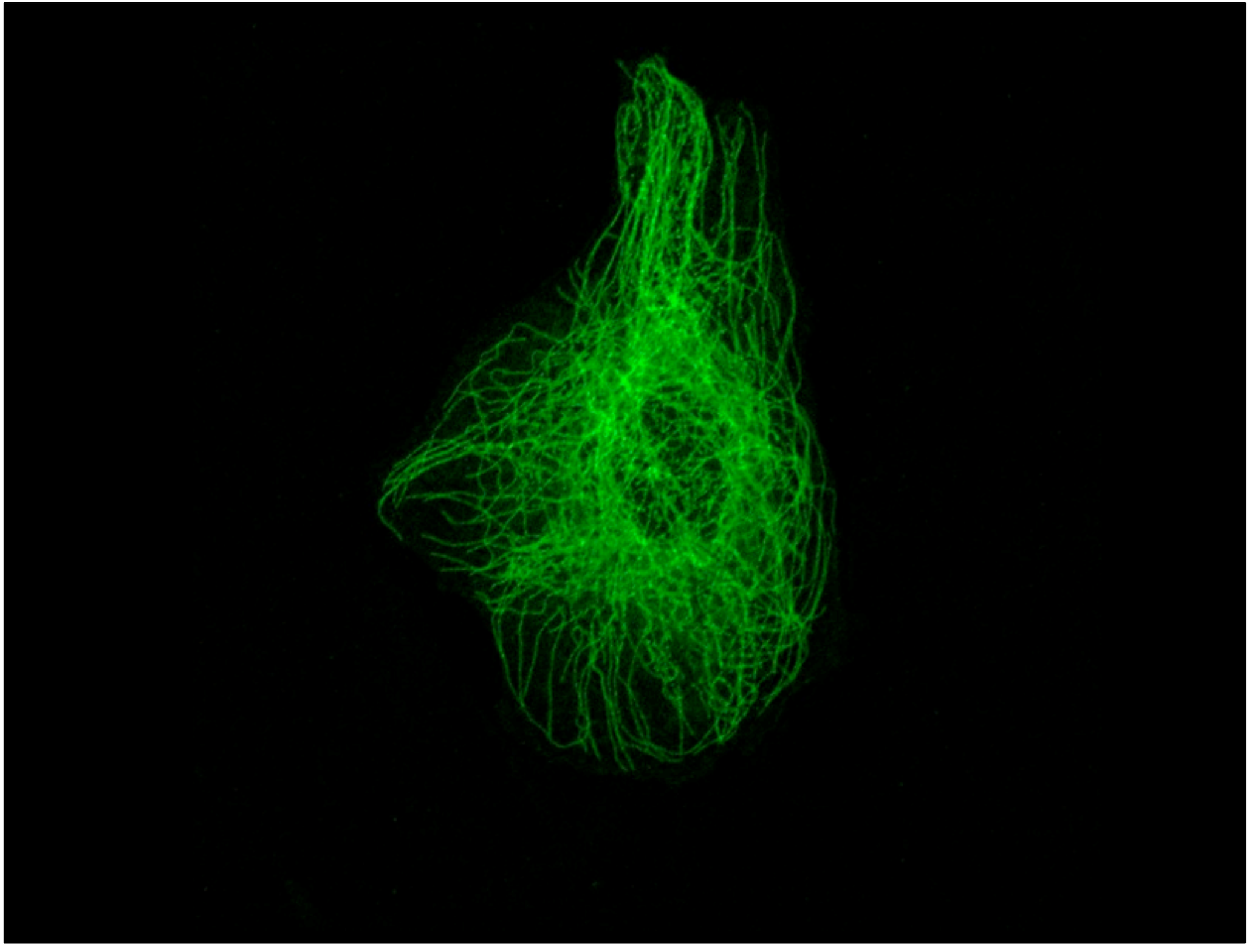


DIC





Contrast Enhancement:
Fluorescence



Understanding what limits Resolution

LECTURES 2,5,7

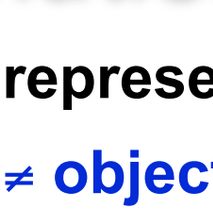
Convolution and the Point Spread Function

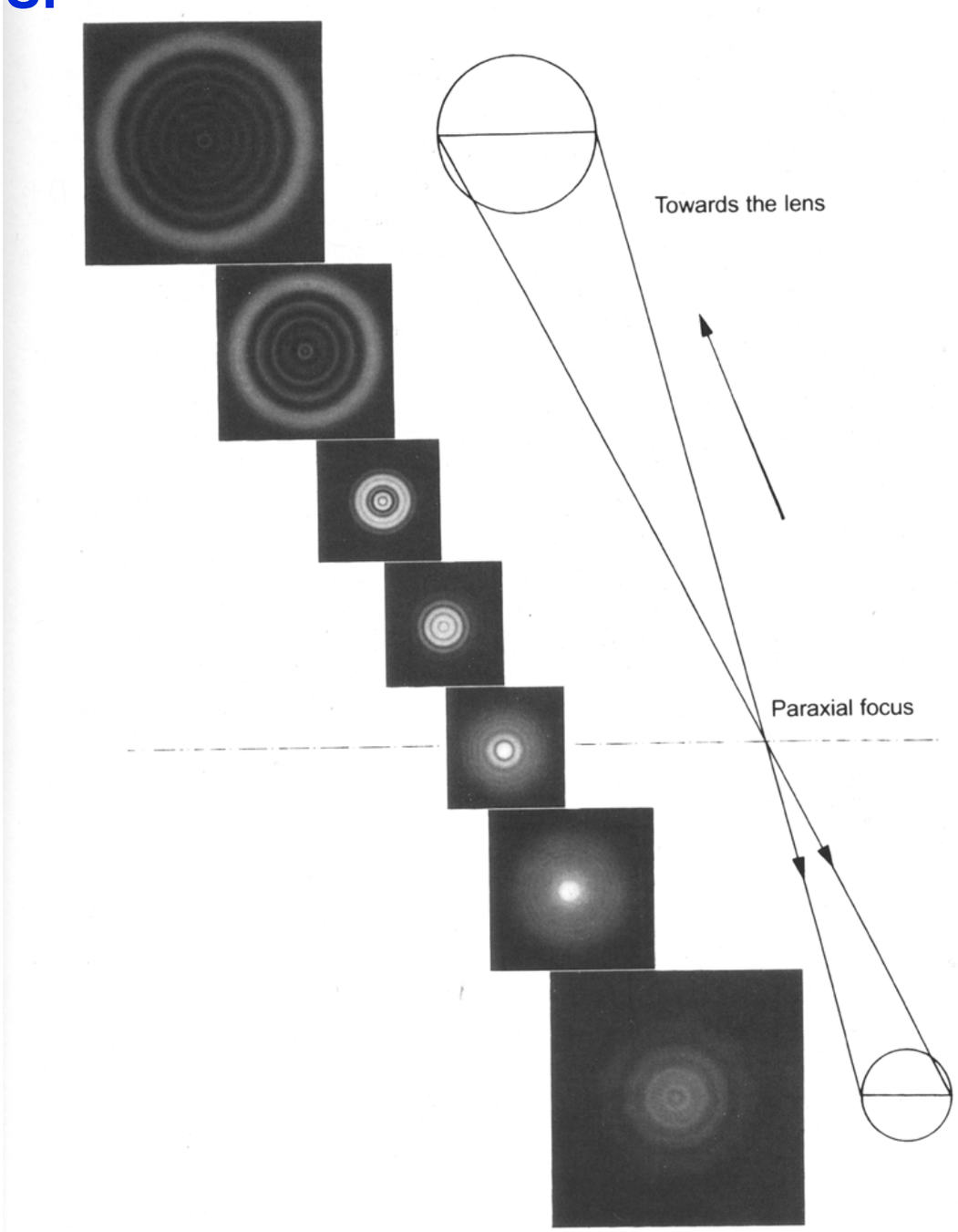
An image represents the output of the optics and detector of the imaging system

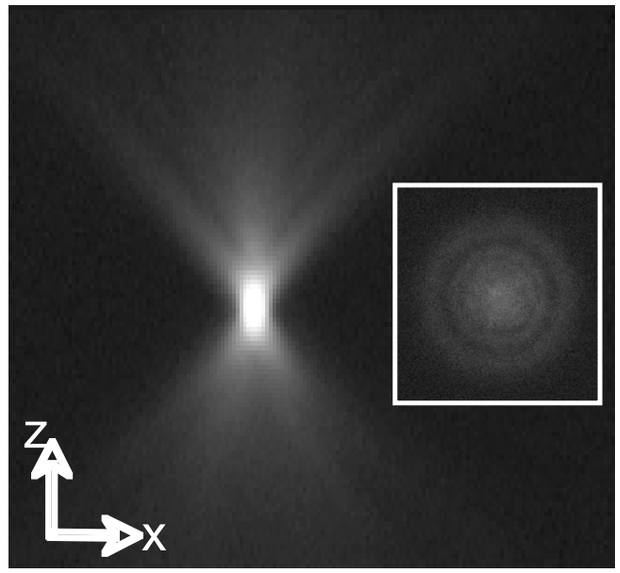
image ≠ object

image = object ⊗ PSF

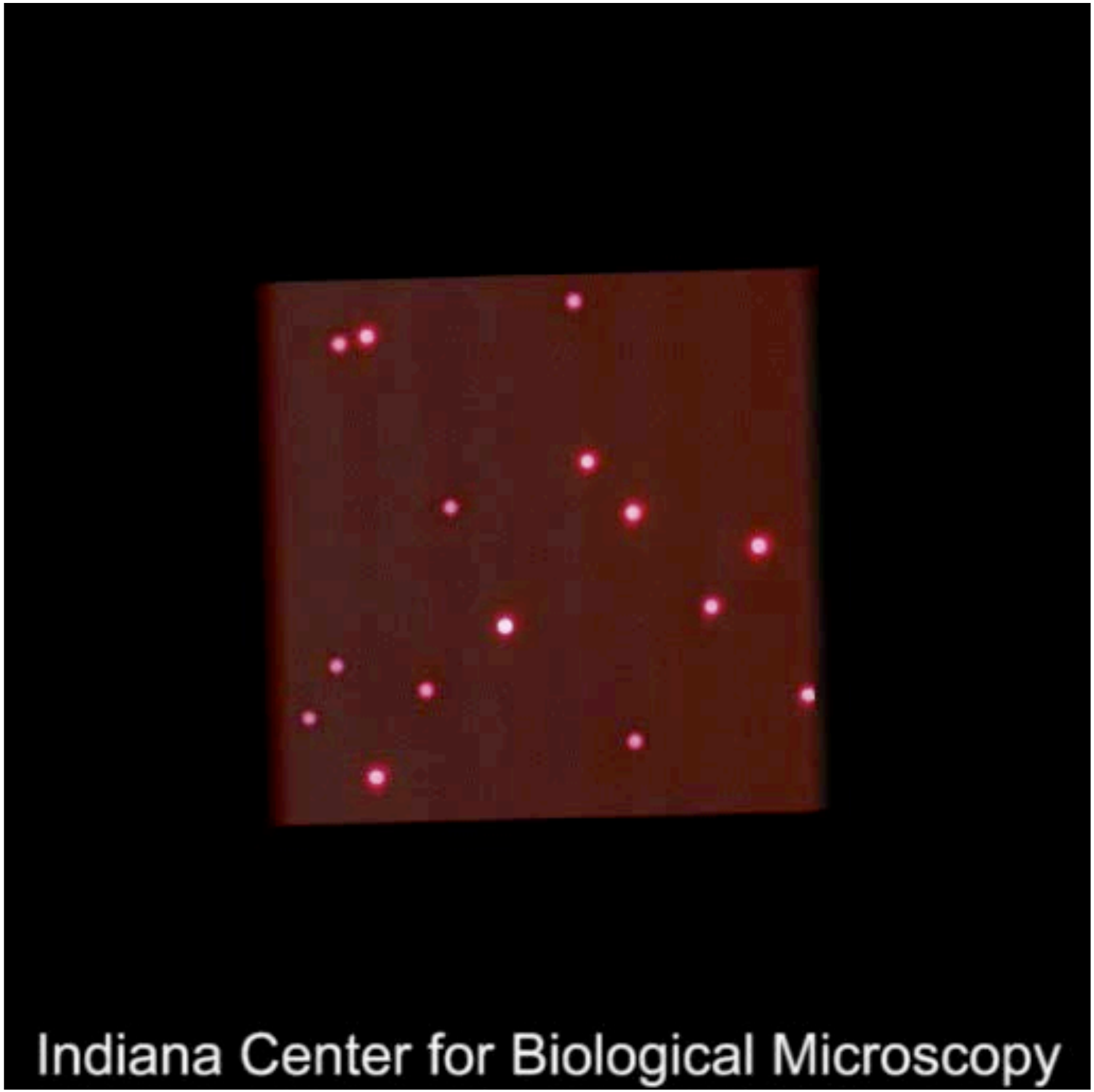
Sample object: a "sub-resolution" fluorescent bead



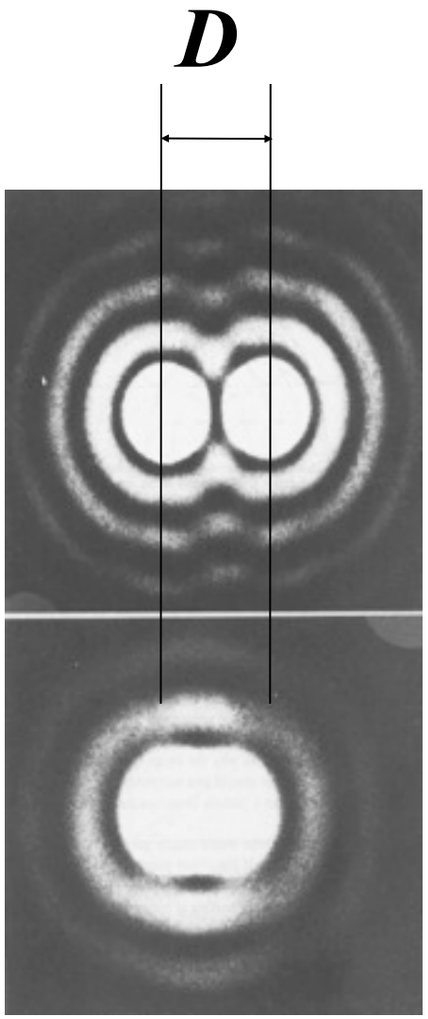




- "convolution" by the microscope optics = the PSF


Indiana Center for Biological Microscopy

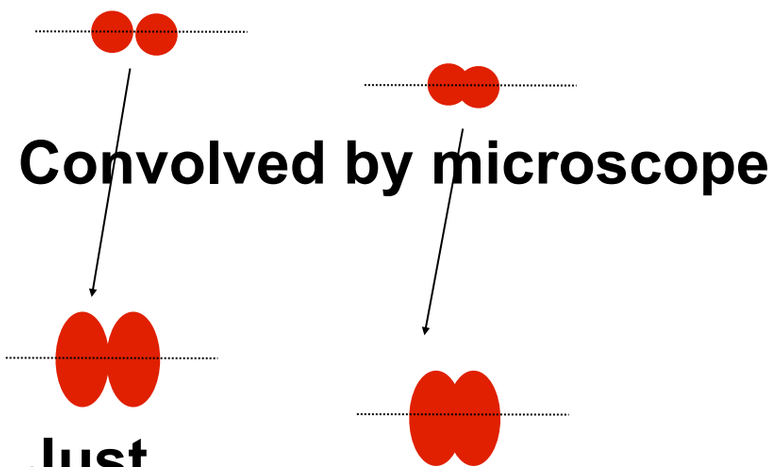
Calculating Lateral Resolution: The Rayleigh Criterion



D = the distance between the two closest points that can still be distinguished

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

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



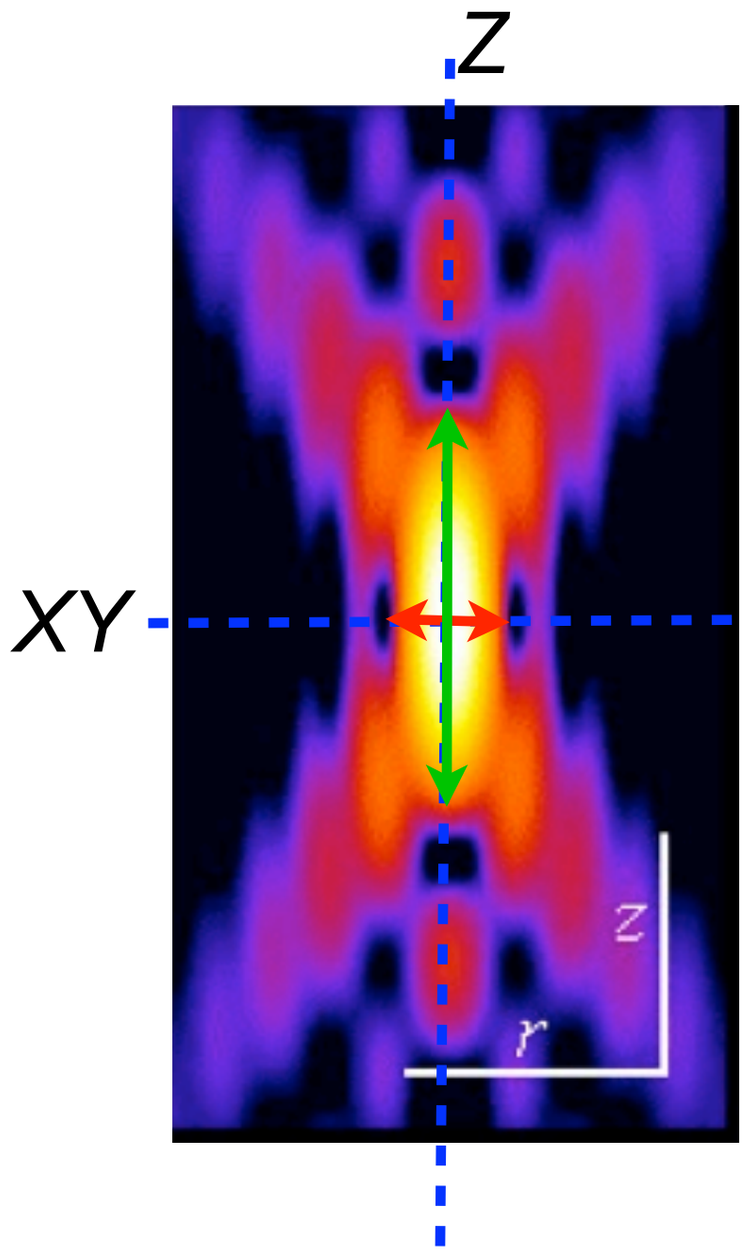
Limit of resolution $\approx \lambda_{em}/2$

Axial Resolution:

Resolution is worse in the axial dimension (along the optical axis, Z)

$$D_Z = 2\lambda\eta/(NA_{obj})^2 \ldots\ldots\ldots\ldots 705$$

(η = refractive index of the object medium)



Than it is in the lateral dimension (XY)

$$D_{XY} = 1.22\lambda/2NA_{obj} \ldots\ldots\ldots\ldots 227$$

FWHM

The relationship between the two is:

$$D_Z/Dxy = 3.28\eta/NA_{obj} \ldots\ldots \approx 3$$

Resolution: Down to the molecular scale?

resolution $\approx \lambda_{em}/2$ (Z resolution $\approx$ 2.5 times worse)

XY resolution ~ 230 nm
Z resolution ~ 700 nm

GFP is ~ 5 x5 x5 nm

Solution 1 - F* techniques

FRAP, FRET, FLIM etc

LECTURE 11

Solution 2 - Super resolution techniques

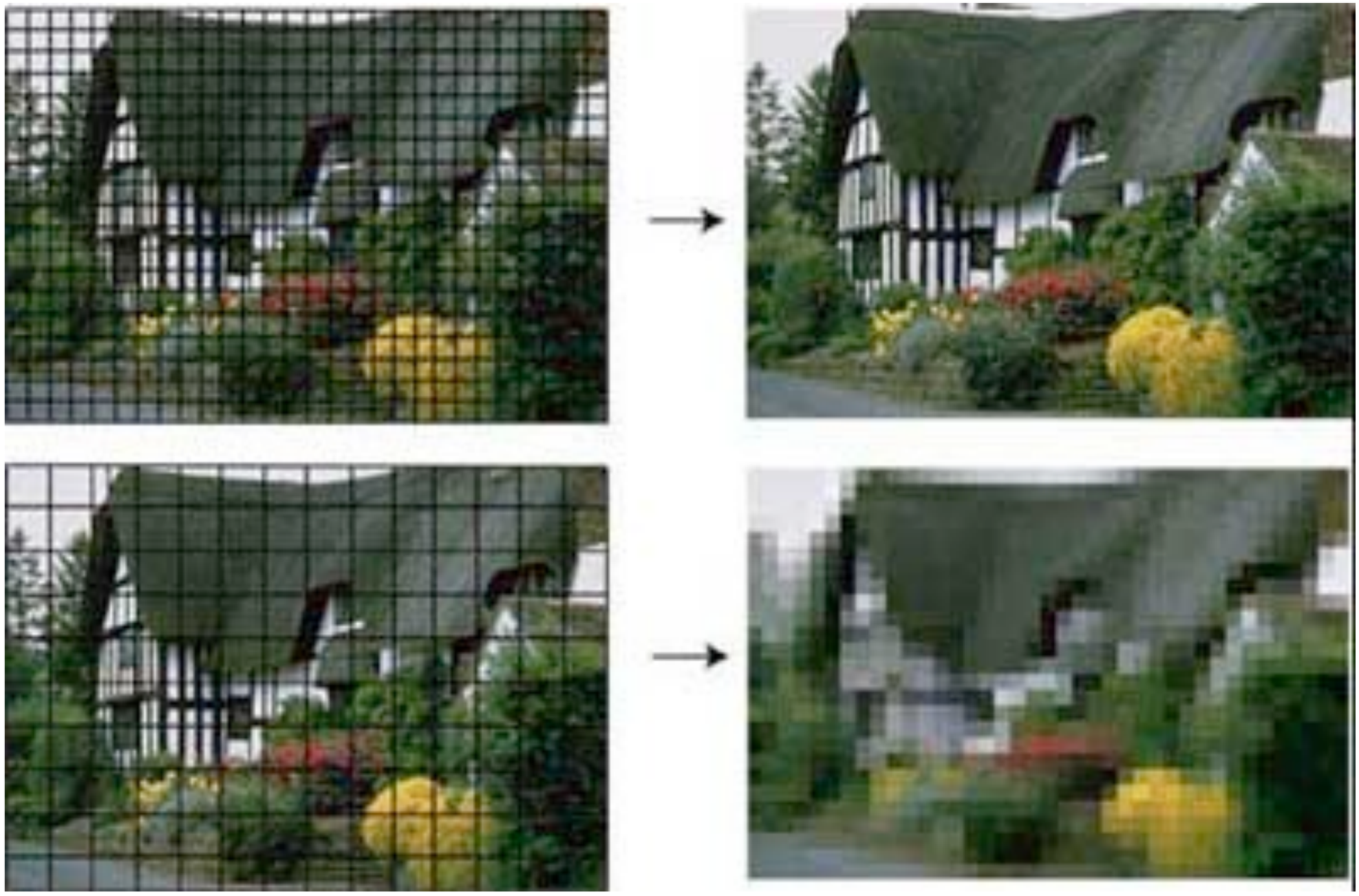
Localisation microscopy, Structured illumination, STED

LECTURES 12, 14-16

Resolution / Contrast / Sampling / Noise

LECTURE 5

Resolution and Sampling

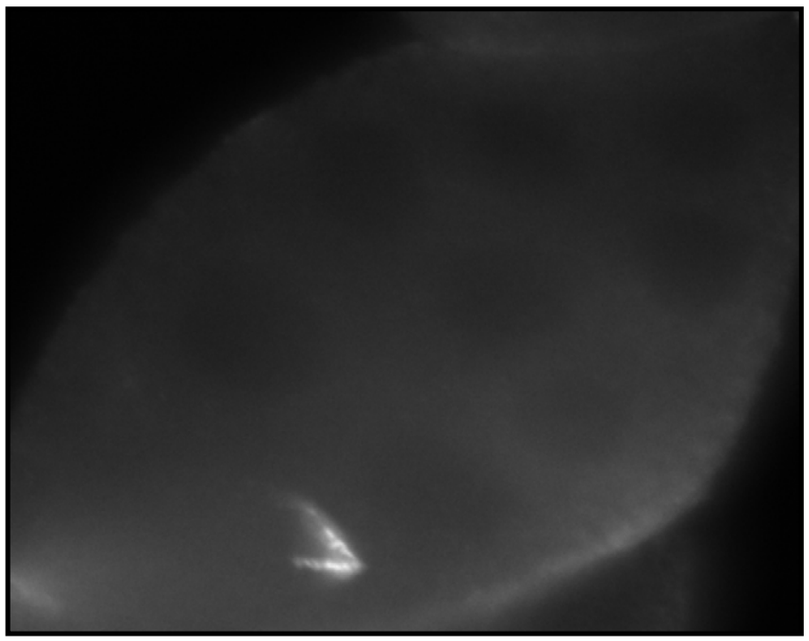


Undersampling limits the information in the image - mag and pixel size

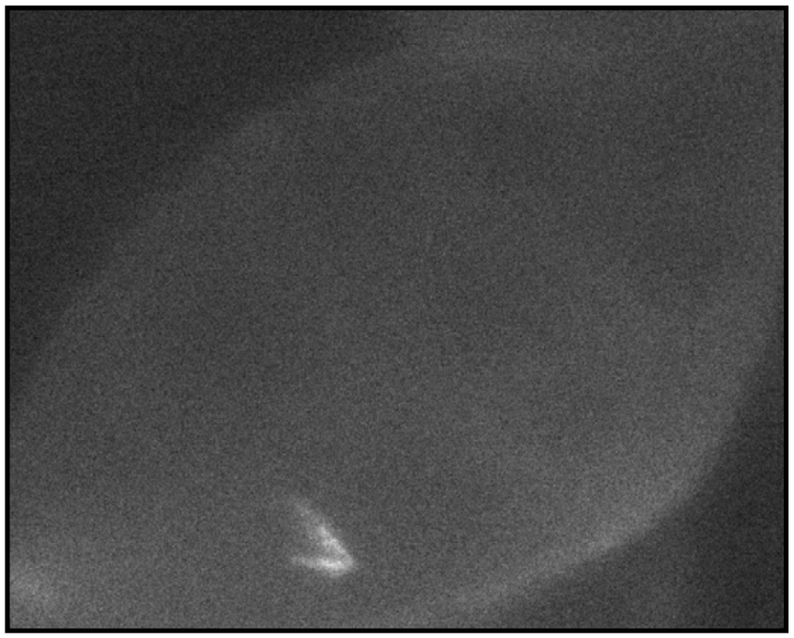
Resolution, Contrast and Noise (S/N ratio)

$$\text{S:N ratio} = \frac{\text{Signal}}{\text{Variation in the signal}}$$

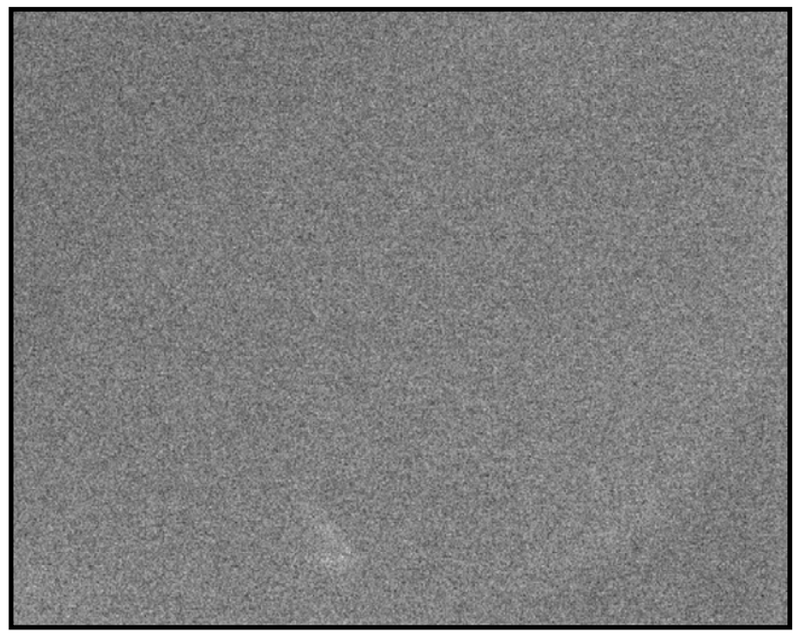
High signal / noise



Poor signal / noise



Very poor signal / noise



- Noise limits the contrast which limits the detail that can be resolved

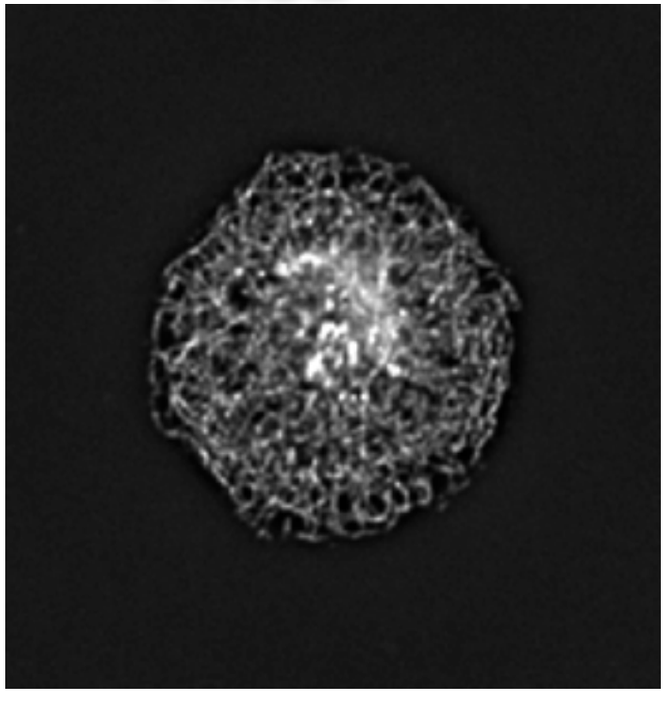
= Noise limits resolution

Which technique to use?

LECTURES 3,4,5,6,7,8,9,10,11,12,13.14,14,16,17

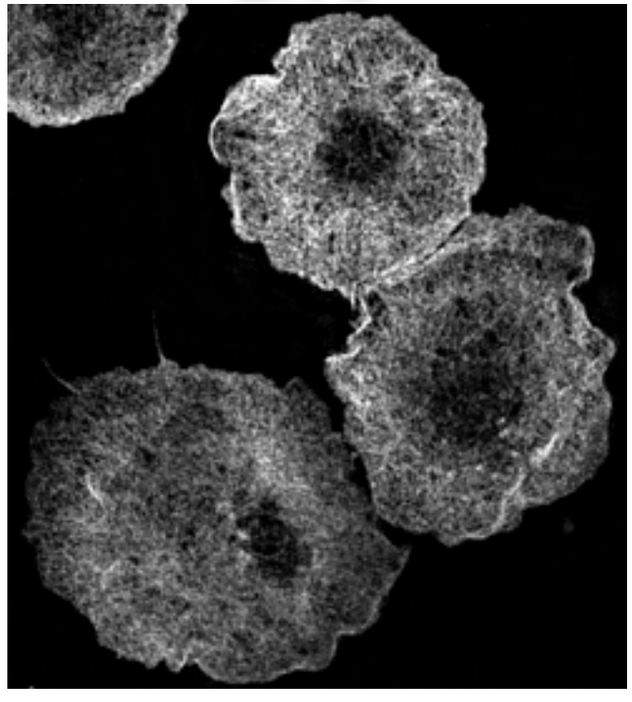
Which technique to use?

Fixed



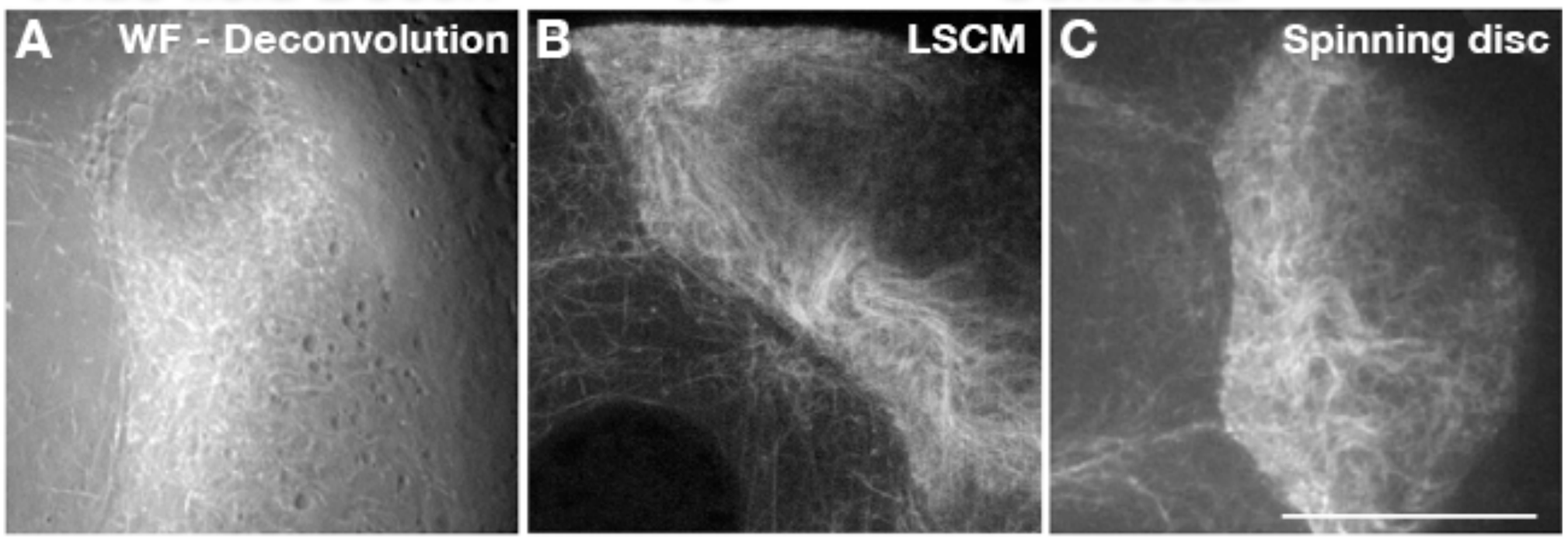
vs

Live



Drosophila macrophage: Phalloidin staining of fixed actin vs moesinGFP labelling of live actin

Wide field Decon vs Confocal



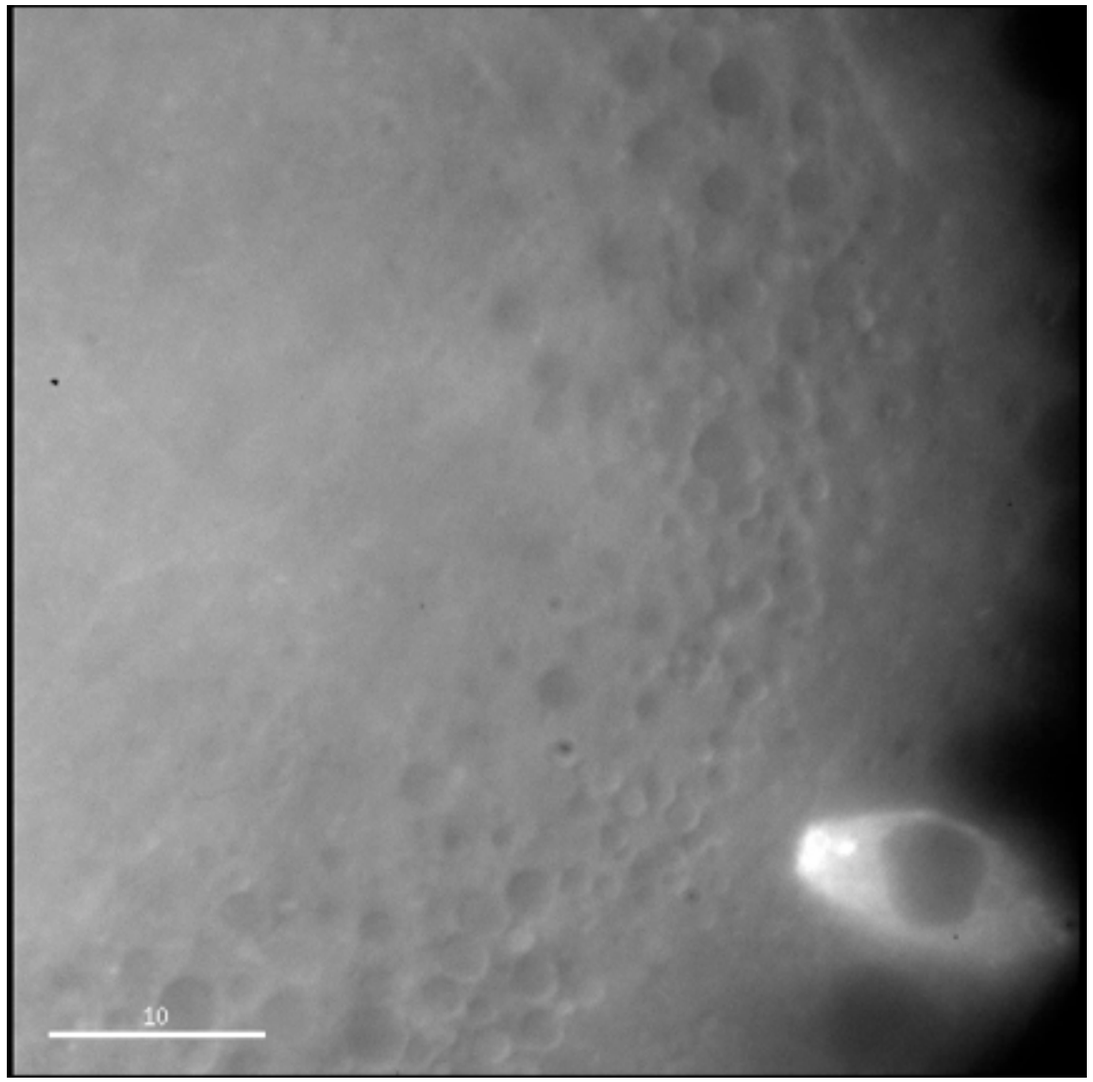
Live *Drosophila* oocyte: Tau-GFP labeling microtubules

Image Processing

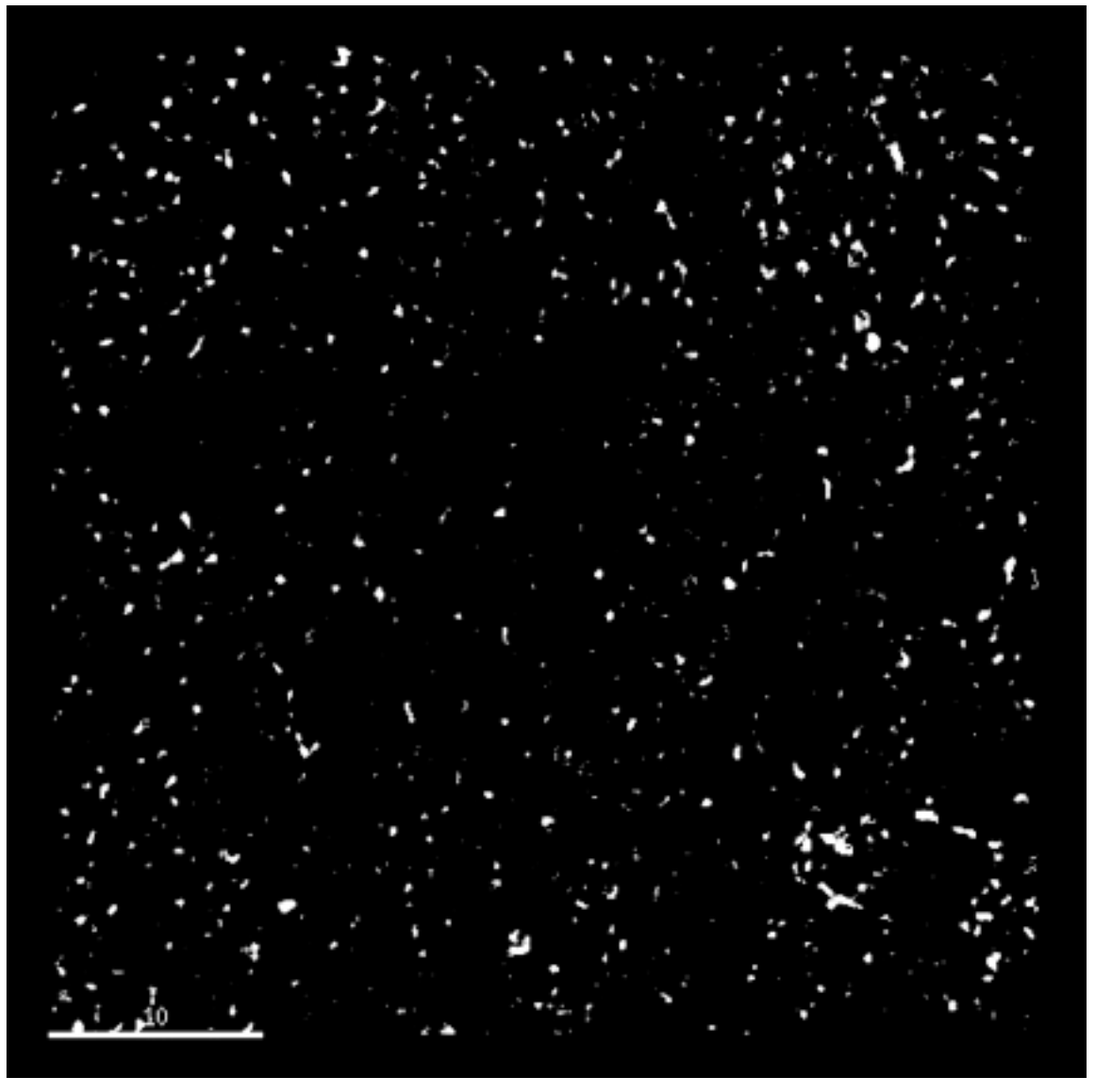
LECTURES 5, 19

Computational enhancement / automation

Raw - Widefield

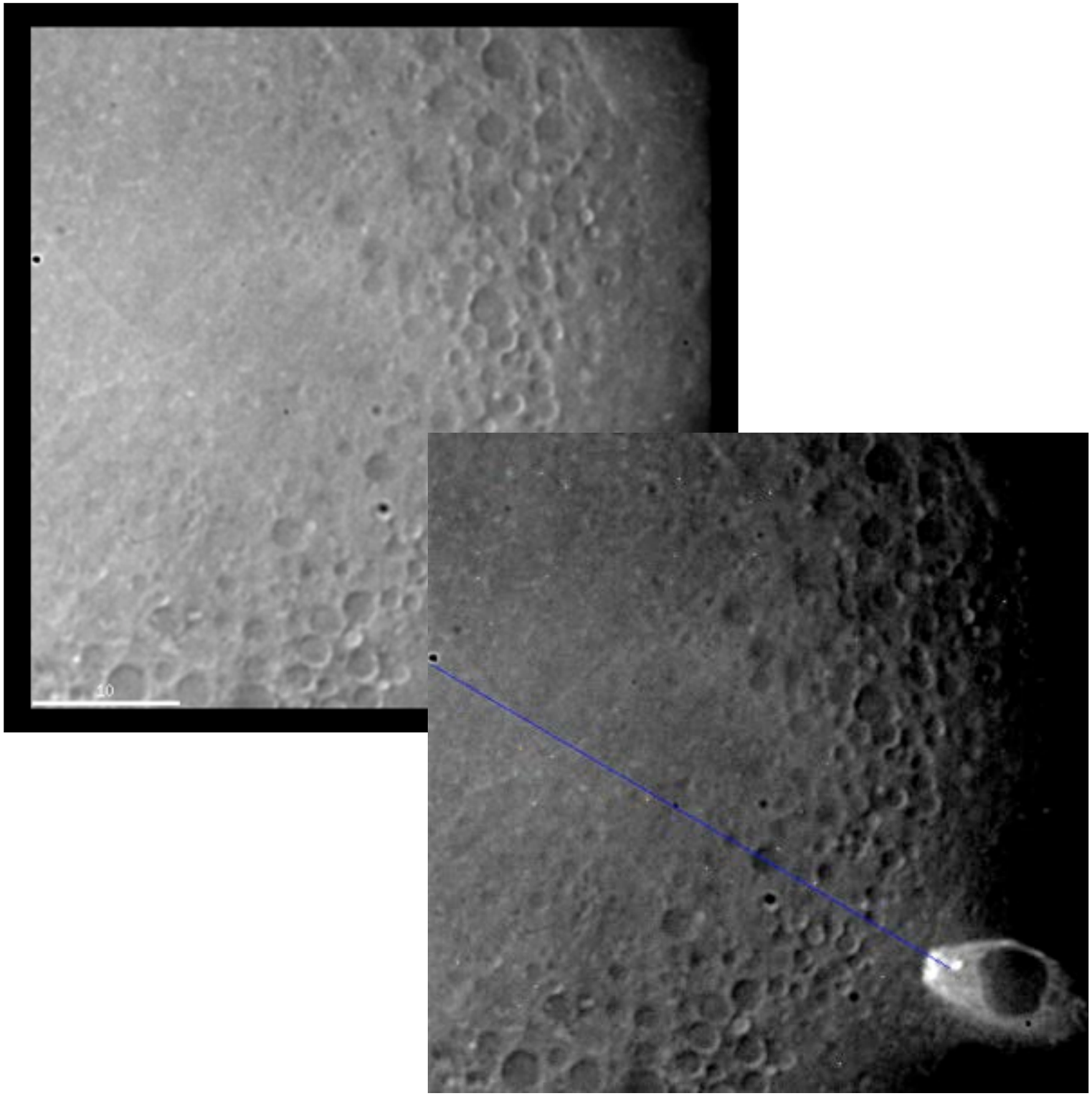


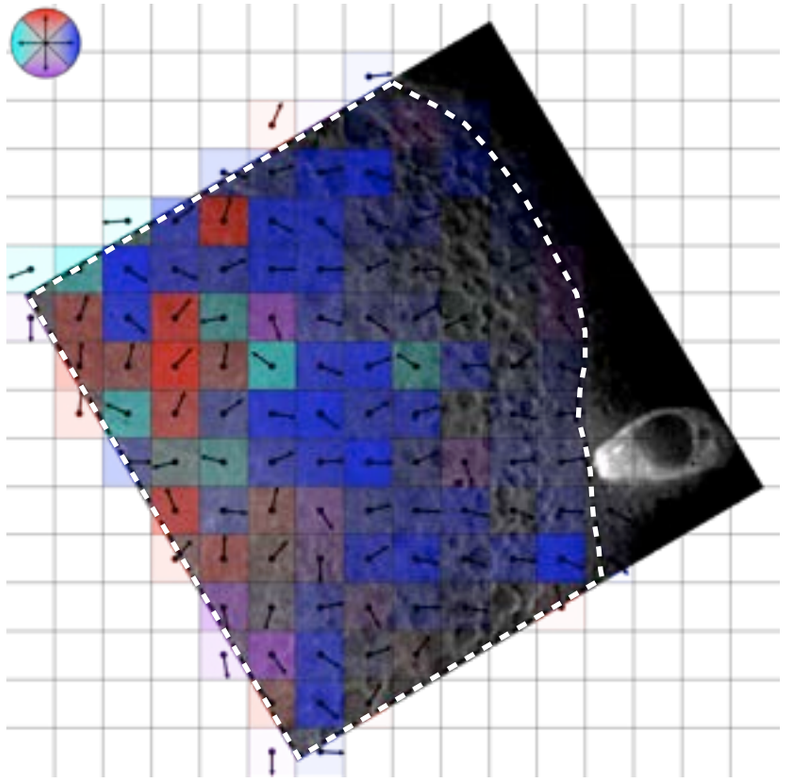
Processed

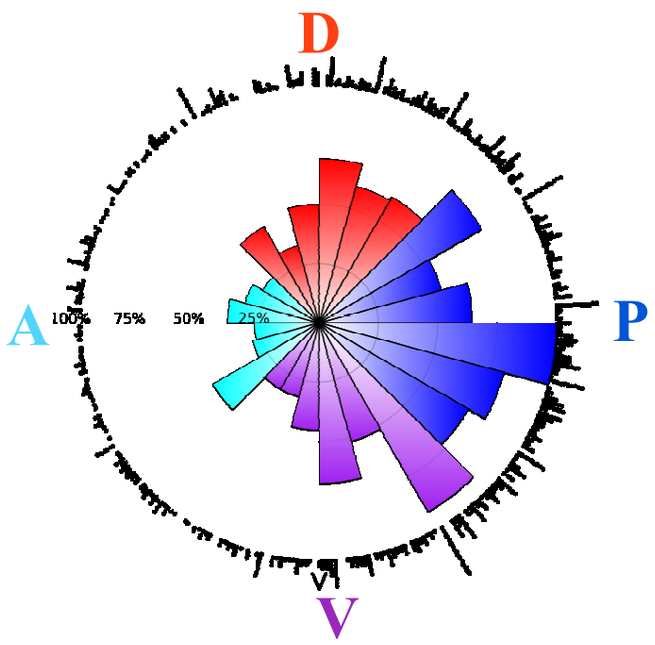


Parton, R. M., Hamilton, R. S., Ball, G., et al. (2011). JCB 194(1), 121–135. doi:10.1083/jcb.201103160

Live imaging - not just a pretty picture







Parton, R. M., Hamilton, R. S., Ball, G., et al. (2011). JCB 194(1), 121–135.
doi:10.1083/jcb.201103160

(Russell Hamilton, Particle Stats: http://www.particlestats.com)

Microscopy is fundamentally important to modern biology

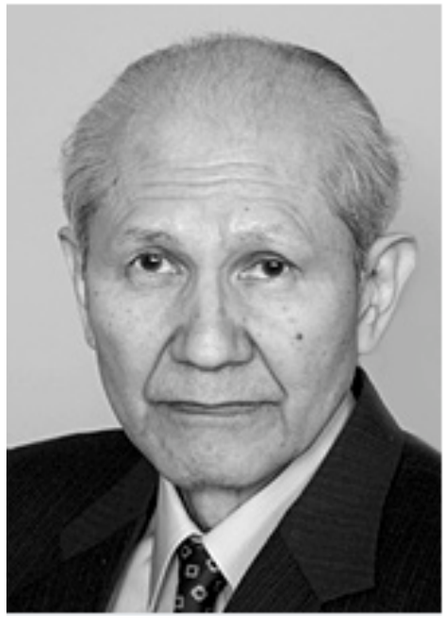
Milestones in Microscopy

http://www.nature.com/milestones/milelight/index.html

1595	Invention of the microscope (Milestone 1)
1858	First histological stain (Milestone 2)
1871	Synthesis of fluorescein (Milestone 2)
1873	Diffraction limit theory (Milestone 3)
1911	First fluorescence microscope (Milestone 4)
1929	First epifluorescence microscope (Milestone 4)
1935	Phase contrast microscopy (Milestone 5)
1939	Polarization microscopy (Milestone 6)
1942	Immunofluorescence (Milestone 7)
1955	Differential interference contrast (Milestone 8)
1961	Concept of confocal microscopy (Milestone 9)
1967	The dichroic mirror (Milestone 4)
1972	Fluorescence correlation spectroscopy (Milestone 10)
1976	FRAP (Milestone 10)
	FRET (Milestone 11)
1980	Calcium probes (Milestone 12)
1981	Video-enhanced differential interference contrast (Milestone 8)
	TIRF microscopy (Milestone 13)
1983	Deconvolution microscopy (Milestone 14)
1987	Realization of confocal microscopy (Milestone 9)
1990	Two-photon microscopy (Milestone 15)
1993	Light sheet microscopy (Milestone 16)
	Single molecule microscopy (Milestone 17)
* 1994 *	GFP (Milestone 18)
1997	Fluorescent protein-based biosensors (Milestone 19)
1999	Red fluorescent proteins (Milestone 20)
* 2000 *	Breaking the diffraction limit: STED (Milestone 21)
2002	Photoactivatable fluorescent proteins (Milestone 20)
* 2006 *	Breaking the diffraction limit: PALM/STORM (Milestone 21)

The Nobel Prize in Chemistry 2008

"for the discovery and development of Green Fluorescent Protein"



- Osamu Shimomura

Bioluminescence, Aquorin and Green fluorescent protein 1962

- Martin Chalfie

Expression of Green fluorescent protein in other organisms 1994

- Roger Tsien

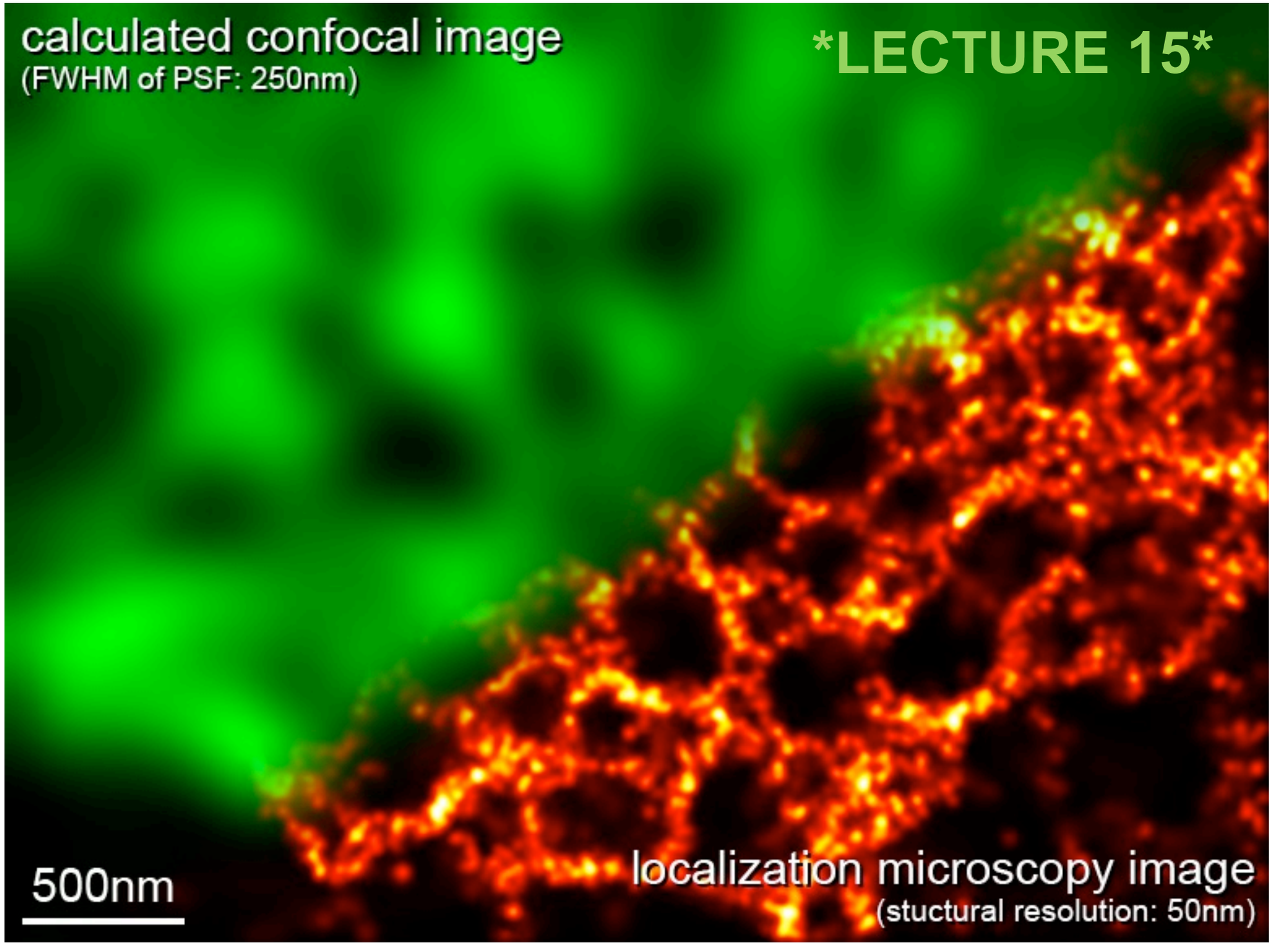
Manipulating the colour of GFP 1999

http://www.nature.com/milestones/milelight/full/milelight18.html

http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/

Super-precision: beyond the diffraction limit

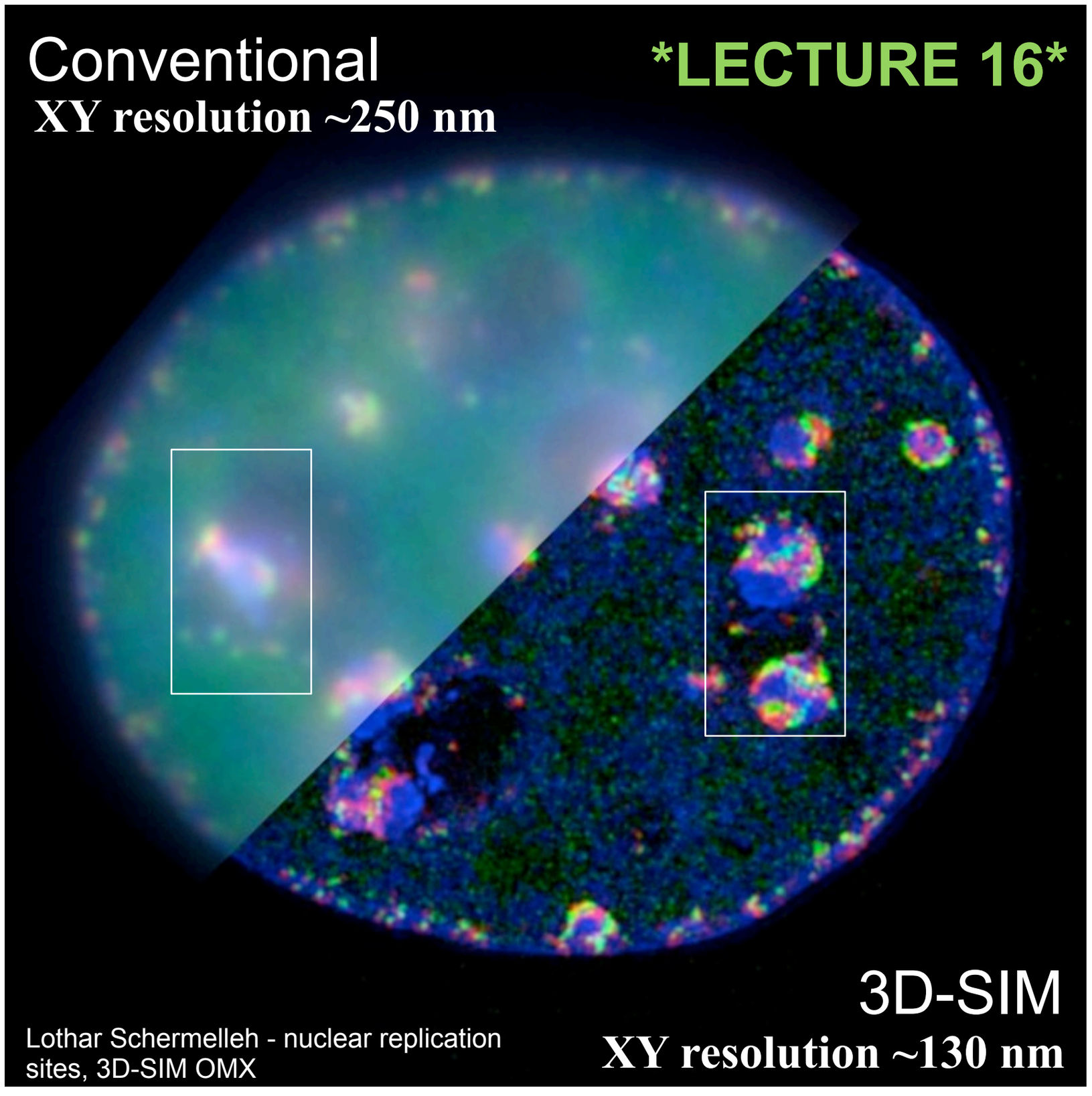
Localisation Microscopy

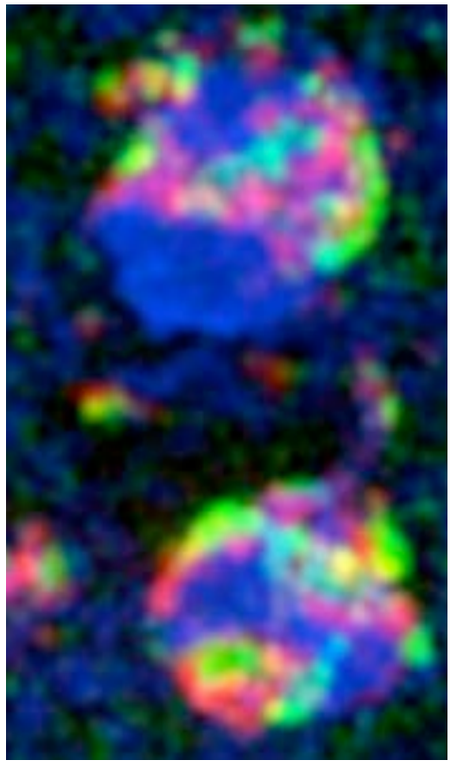


Rainer - MT fixed tissue culture cell, comparison of confocal and DSTORM

Super-resolution: beyond the diffraction limit

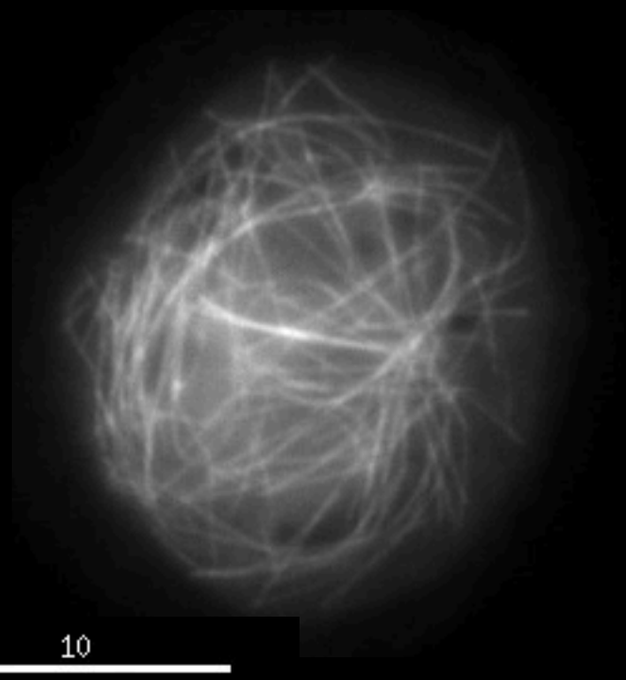
3D-Structured Illumination Microscopy





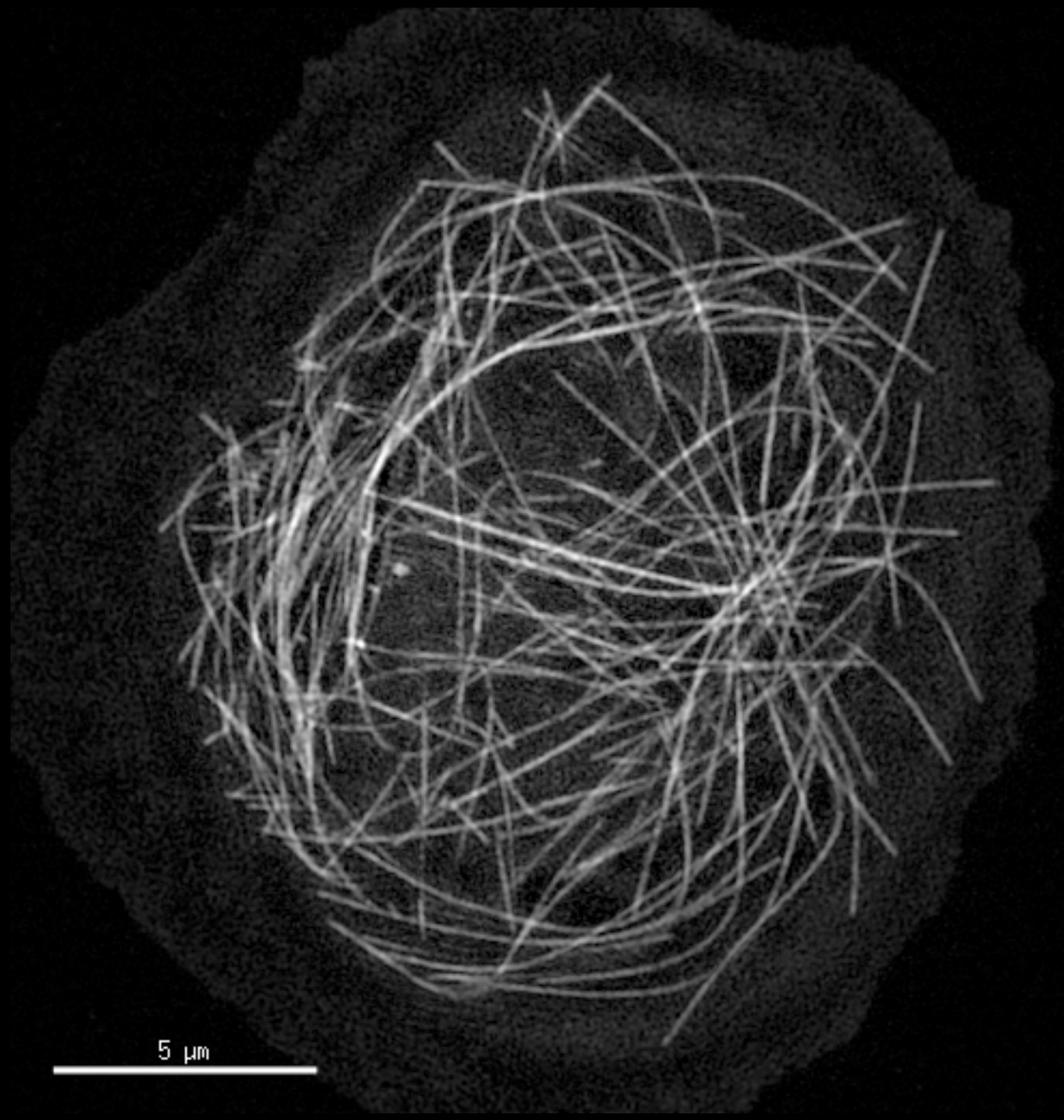
Live-cell super-resolution!

CONVENTIONAL



XY resolution ~250 nm

3D-SIM OMX-BLAZE



XY resolution ~130 nm

Jupiter-GFP tagged MT dynamics in a living Macrophage

END

Reference Material

http://www.olympusmicro.com/
Very comprehensive and well written

http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html
Very comprehensive

Fundamentals of light microscope and electronic imaging
Douglas B. Murphy. Wiley-Liss 2001
ISBN 0-471-25391-X

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