Advanced Microscopy Course 2012
Introductory Lecture

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

Program 2012:

Day 1: Mon 12 March - Fundamental Principles of Microscopy

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	13.00-14.00	Contrast enhancement (phase contrast and DIC)
4	Ilan	14.00-15.00	Basic Fluorescence Microscopy
5	Richard	15.30-16.30	Basic image analysis
6	Mark	16.30-17.30	Fluorescent dyes and proteins

Day 2: Tues 13 March - Imaging Molecules in Cells

7	Richard	9.00-10.00	Live cell imaging
8	Jordan	10.00-11.00	Confocal, spinning discs and Multiphotons
9	Ilan	11.20-12.20	Advanced widefield microscopy design (and OMX)
10	James	13.30-14.30	Detectors for microscopy
11	Ian	14.30-15.30	F* techniques: FRET, FLIM, FCS, FRAP, FLIP
12	Mark	16.00-17.00	Single molecule techniques

Day 3: Wed 14 March - New and emerging Imaging Approaches

13	Lothar	90.00-10.00	Breaking the limits: OMX, STED, SPIM
14	Rainer	10.00-11.00	STORM, PALM techniques
15	Ian	11.30-12.30	Building bespoke microscopes; image storage
16	Graeme	13.30-14.30	Applied Image analysis and Matlab
Ian (all)		14.30-15.30	Demonstration of self-taught practical exercises
ALL		16.00-17.00	Questions and Discussion
		6.00	Drinks & nibbles and informal discussions

AND

Day 4: Thursday 15 March - Demos of Micron microscopes

ALL	09.30-13.00	Capabilities and uses of different microscopes: Deltavision Elite wide-field fluorescence microscope, PerkinElmer spinning disk, Leica SP5 scanning confocal, OMX V3 super resolution microscope

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
- Explain of how to get the best from your microscope:

  - Specimen preparation and labelling
  - Contrast enhancement, phase and DIC
  - Fluorescence for detecting specific molecules
  - Digital acquisition and deblurring images

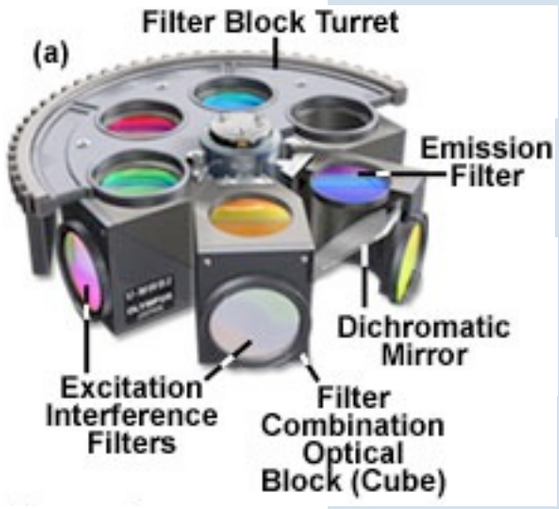
Why do we need to understand microscopy?

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

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

• 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



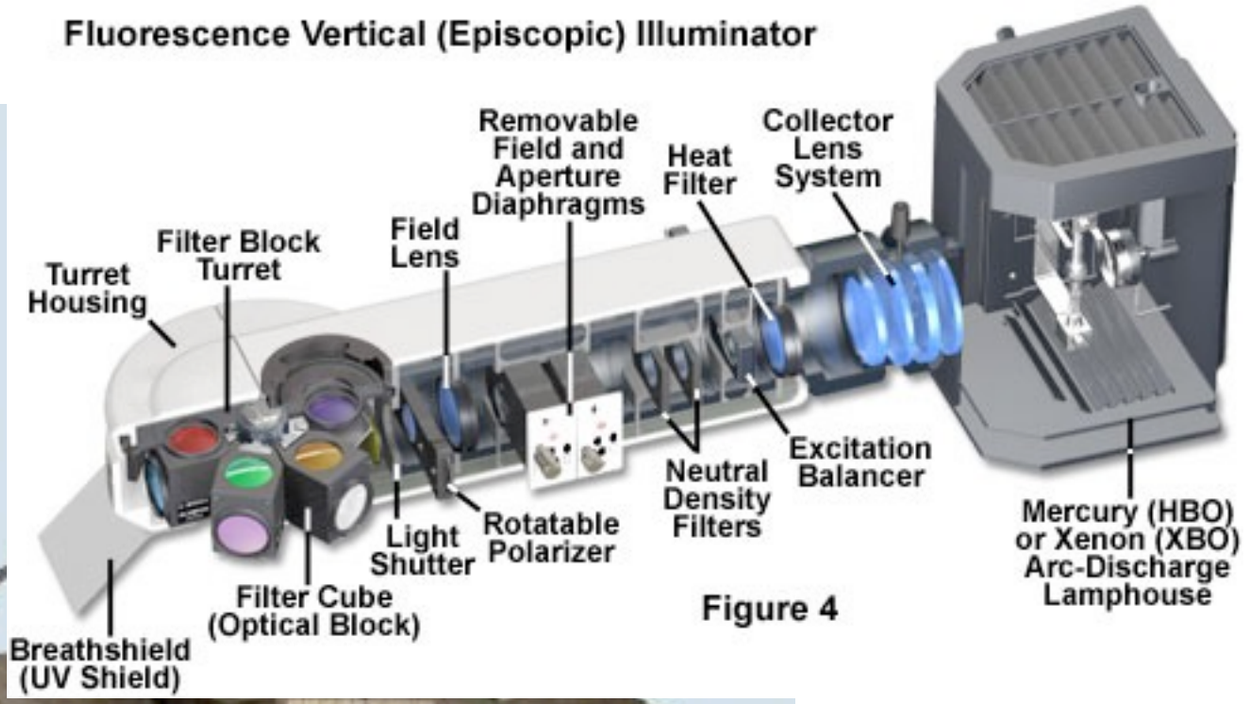


Figure 4

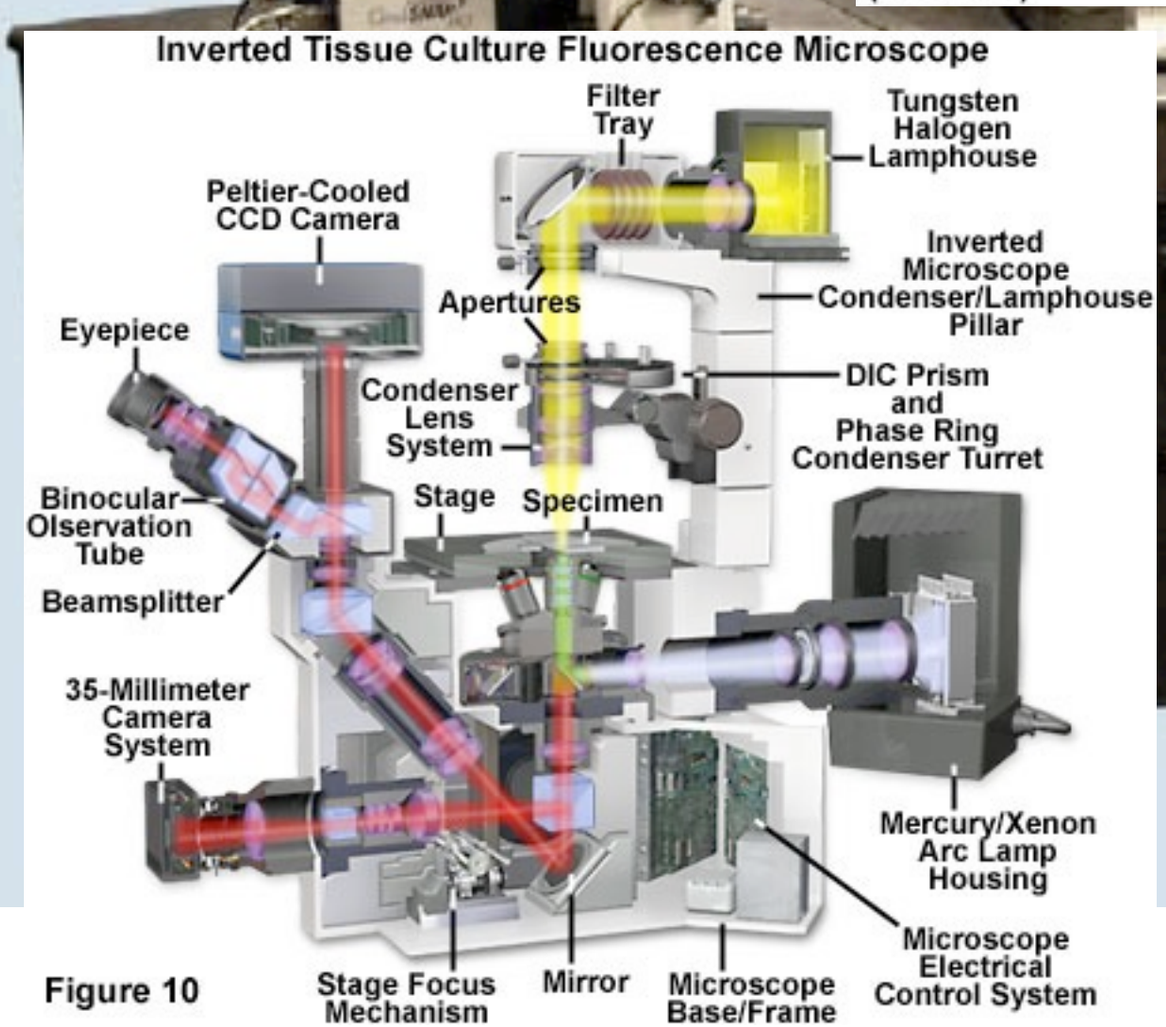


Figure 10

LECTURE 2-4

What can you do with a microscope?

100 years ago:

Magnify small things to visualise more details.

Now:

Image specific molecules inside cells.

Follow changes in the distribution of molecules or the morphology of cells over time.

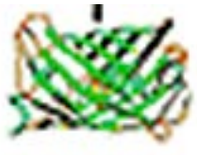
Determine how close molecules are to each other within cells

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

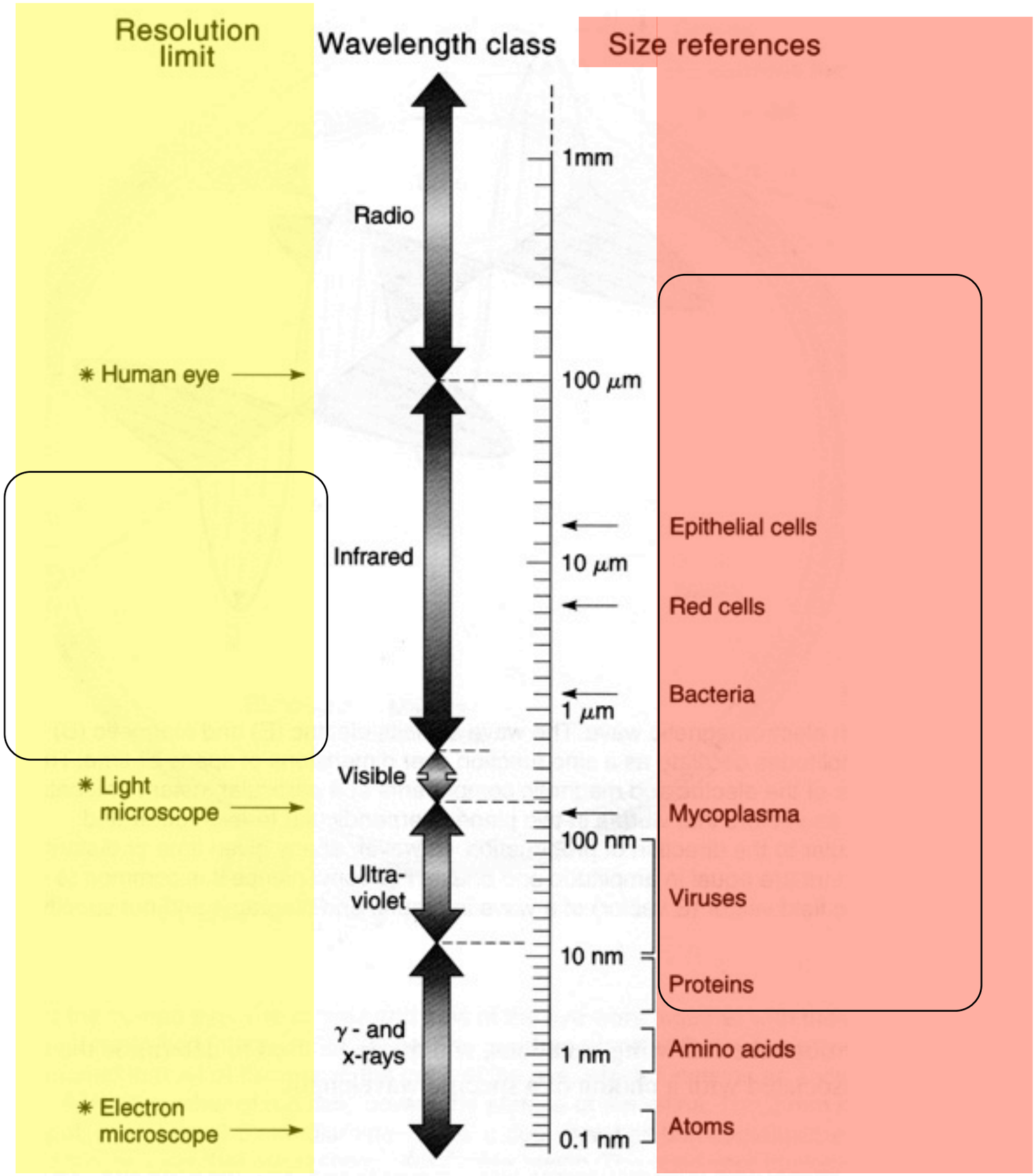
Bacterium = 1 um



Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



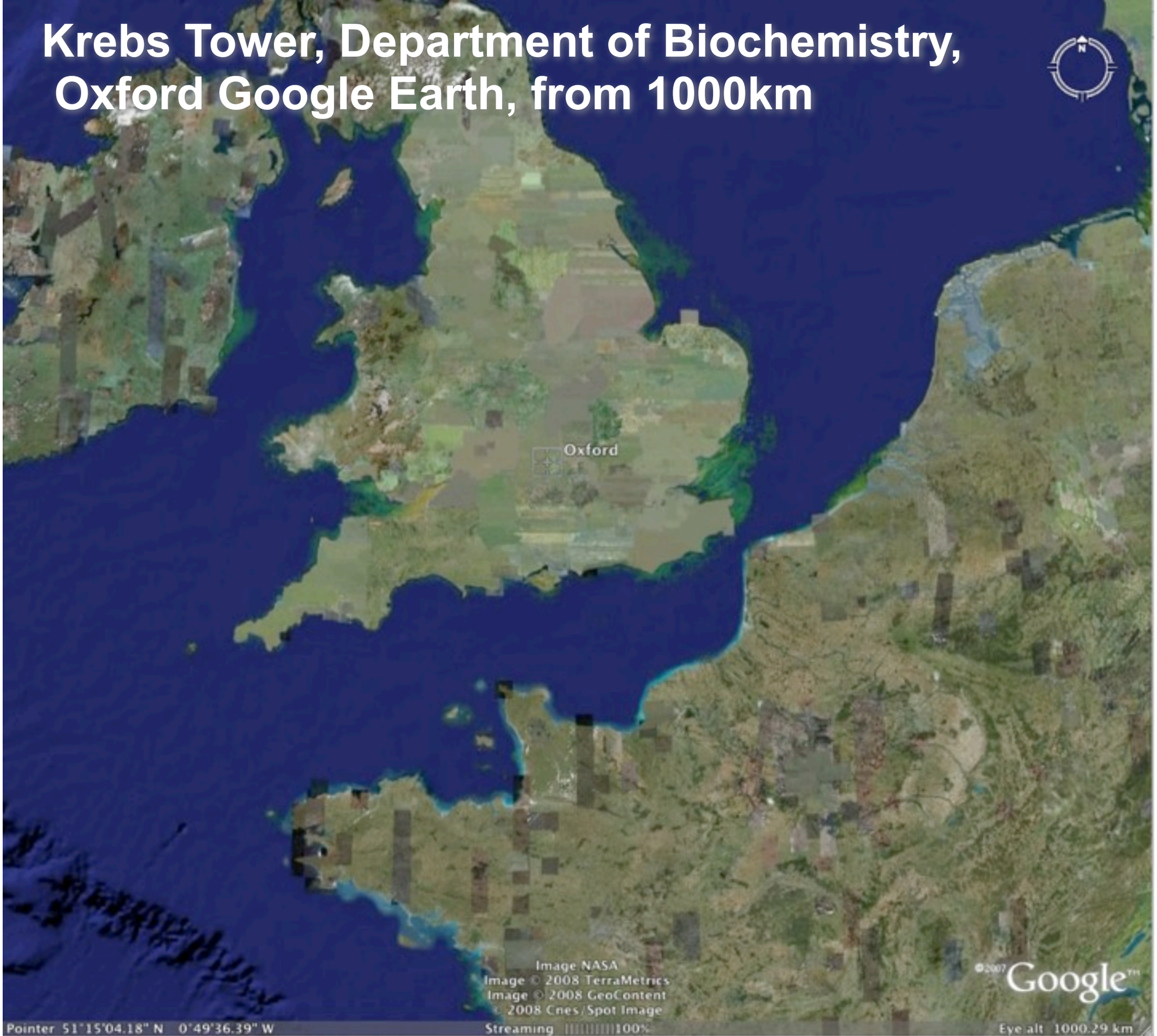
What is important in microscopy?

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

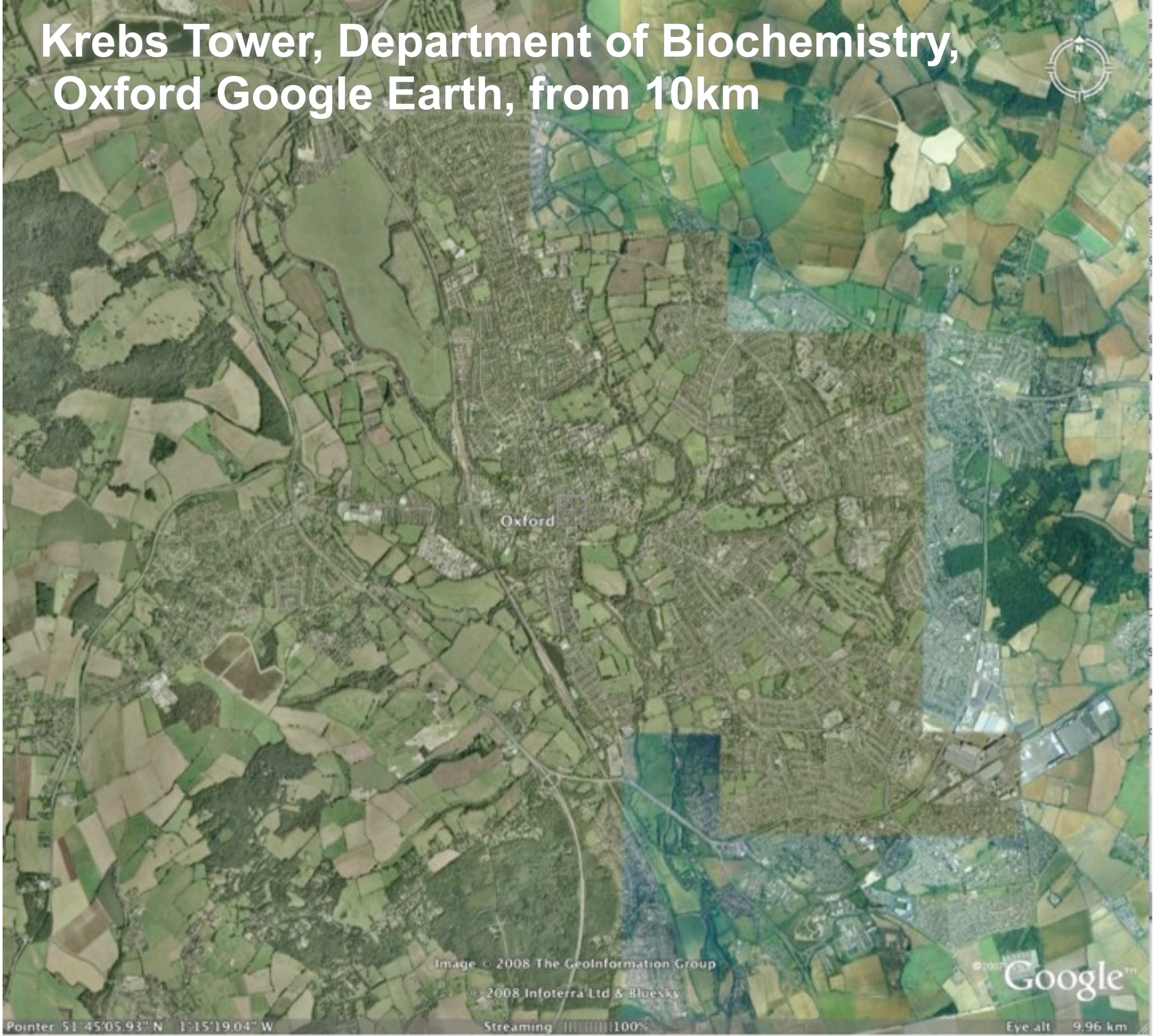
LECTURE 5

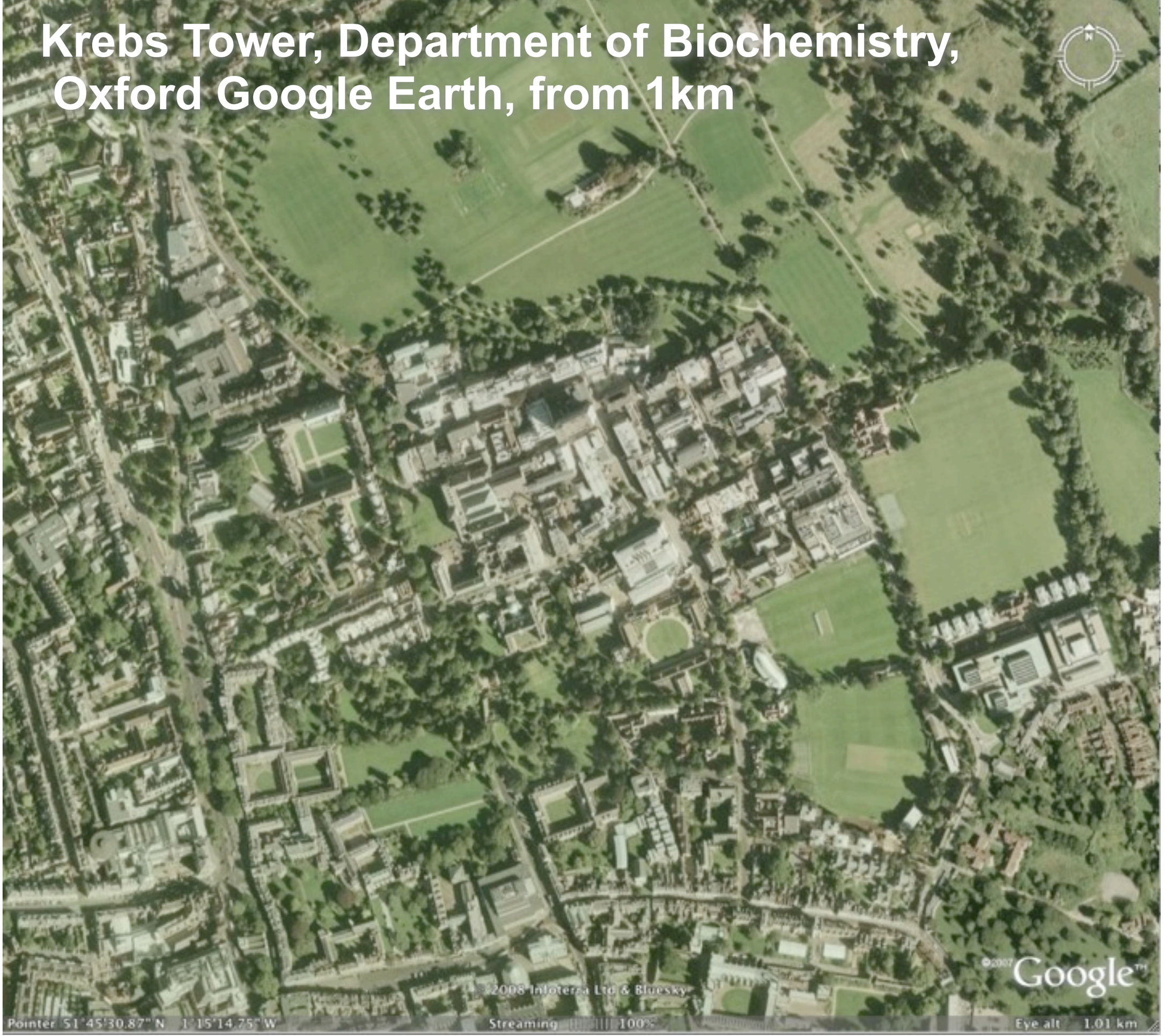
Magnification is nothing without resolution!

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



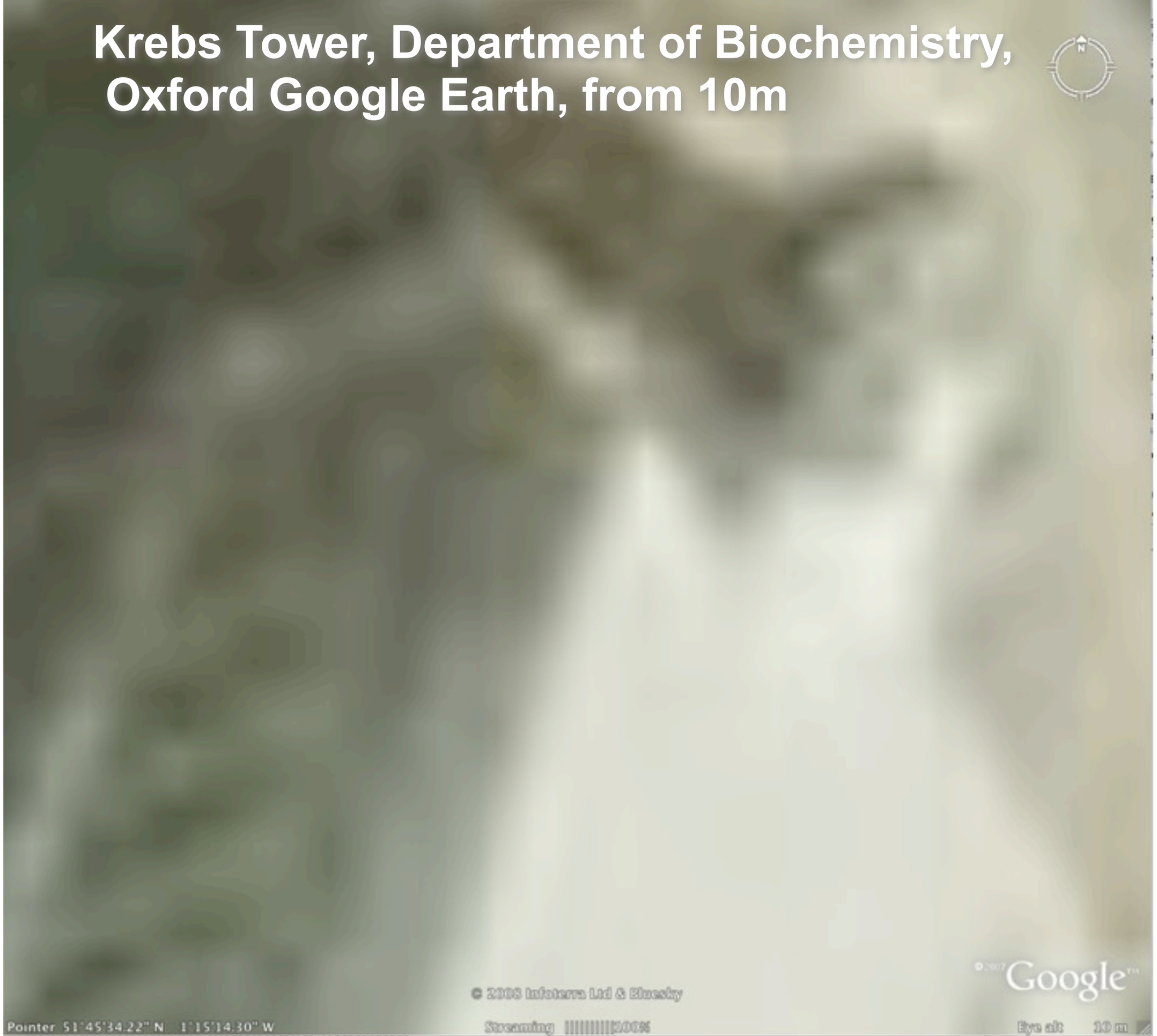
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 10km




Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 1km
©2008 Infoterra Ltd & Bluesky
©2007 Google™
Pointer 51°45'30.87" N 1°15'14.75" W
Streaming 100%
Eye alt 1.01 km

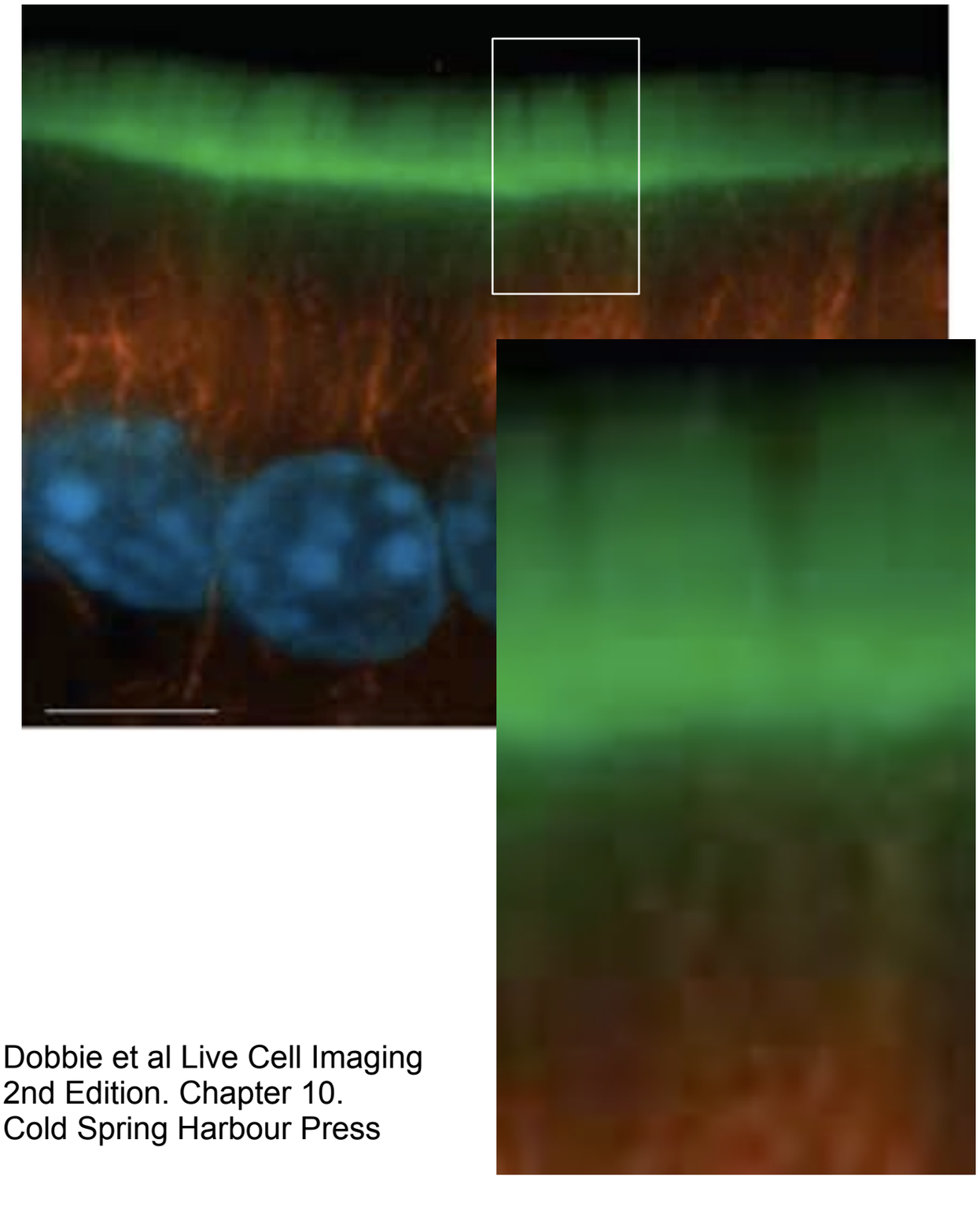

Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 100m
© 2008 Infoterra Ltd & Bluesky
©2007 Google™
Pointer 51°45'33.67" N 1°15'15.13" W
Streaming 100%
Eye alt 100 m

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

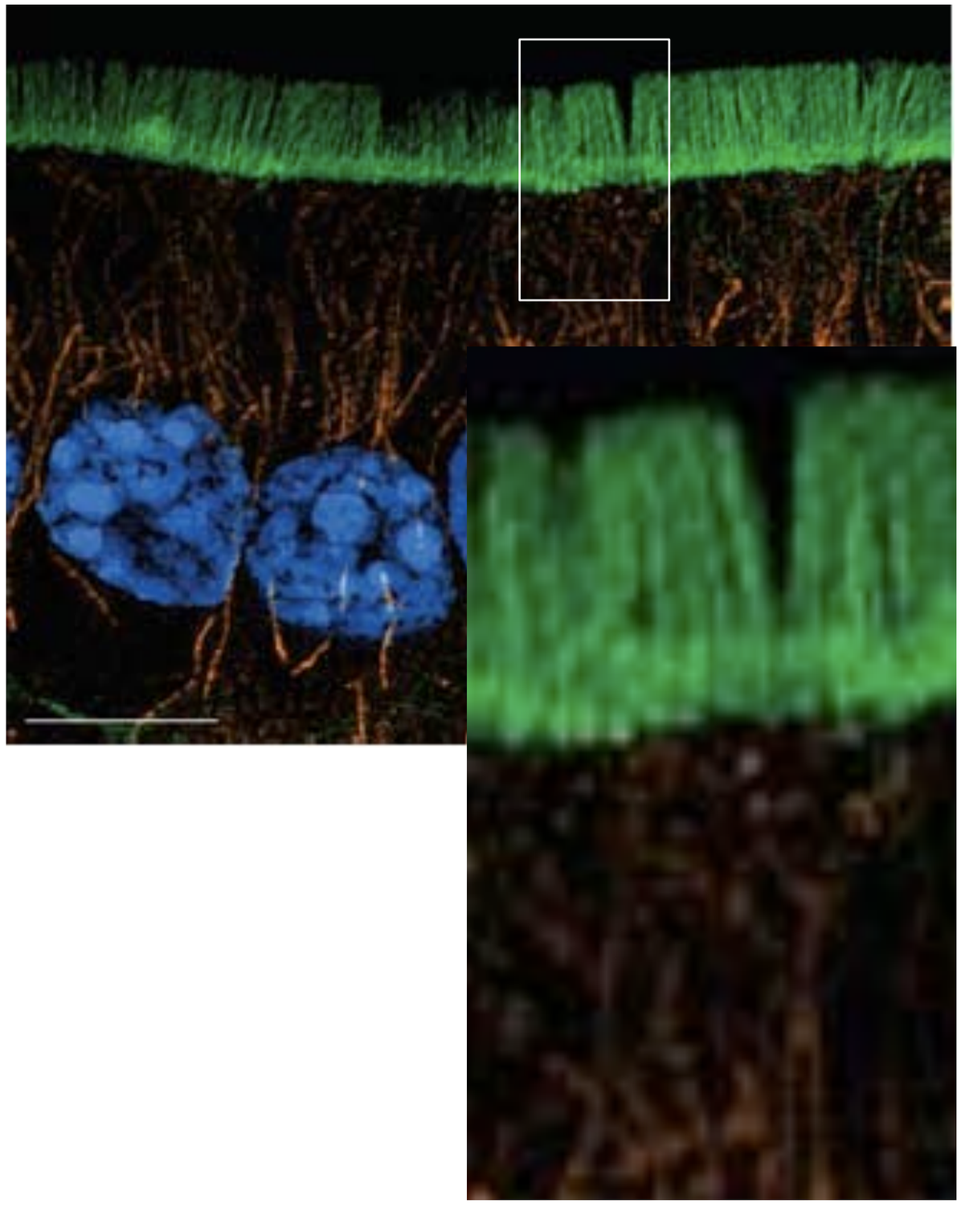

Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 10m
© 2008 Infoterra Ltd & Bluesky
Google
Pointer 51°45'34.22" N 1°15'14.30" W
Streaming 100%
Eye alt 10 m

RESOLUTION

Normal resolution



High resolution

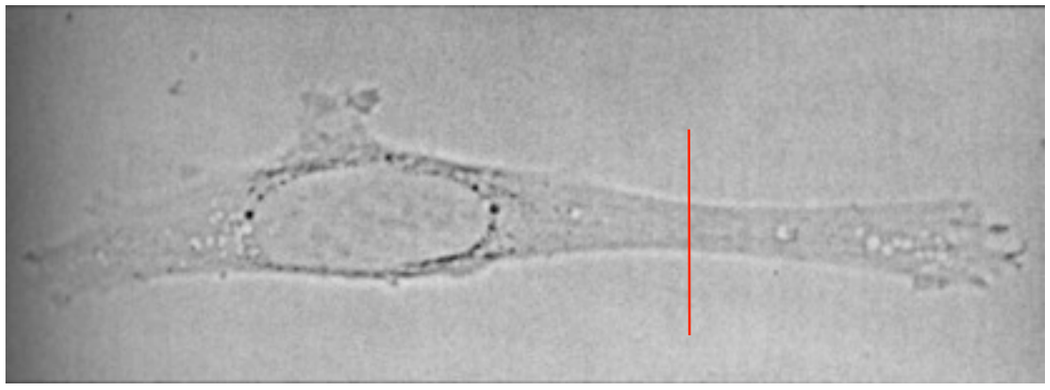


Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
Cold Spring Harbour Press

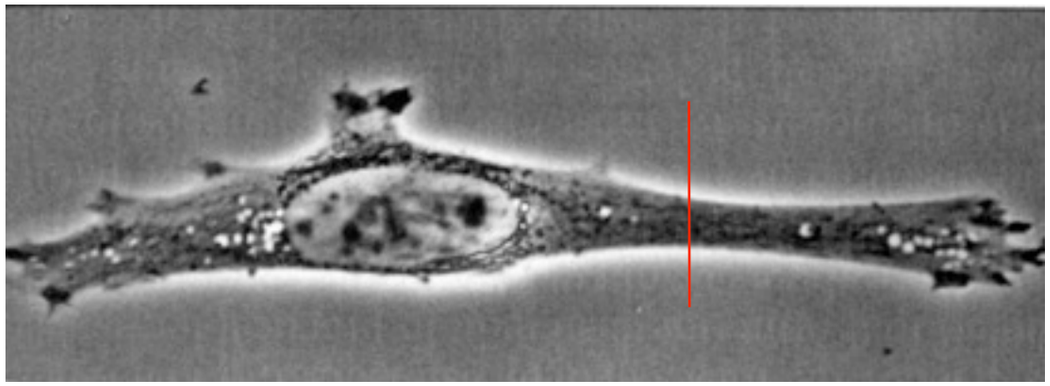
Resolution is nothing without contrast!

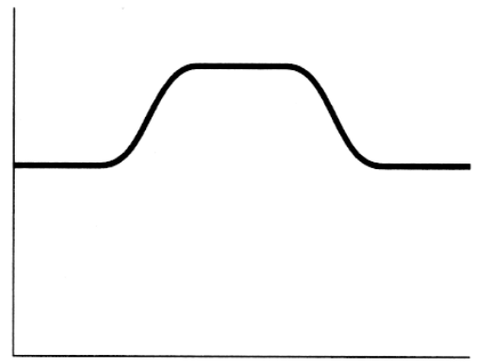
Contrast Enhancement:
Phase Contrast and DIC

Normal contrast

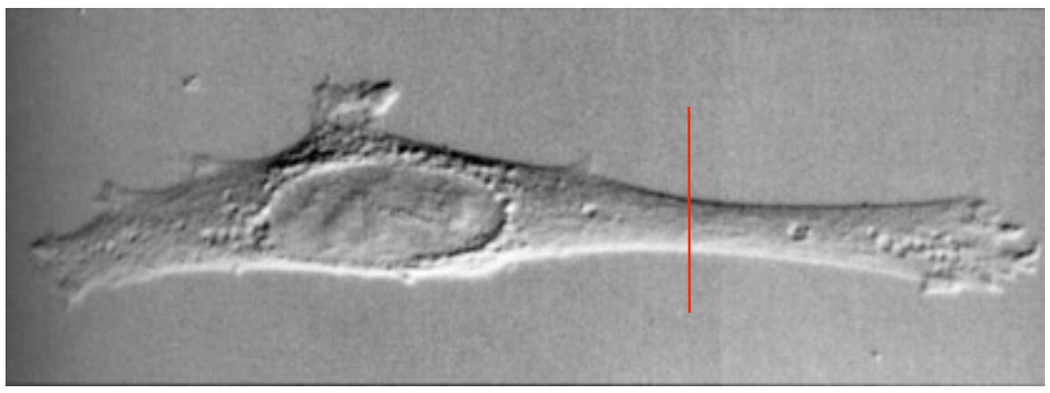


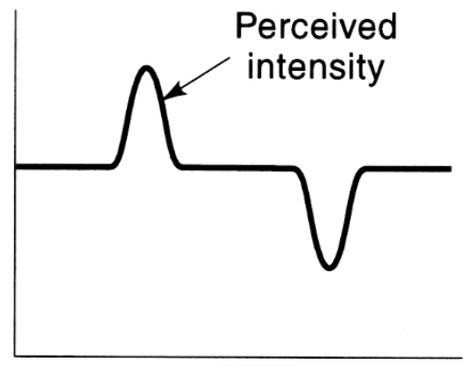
Phase contrast





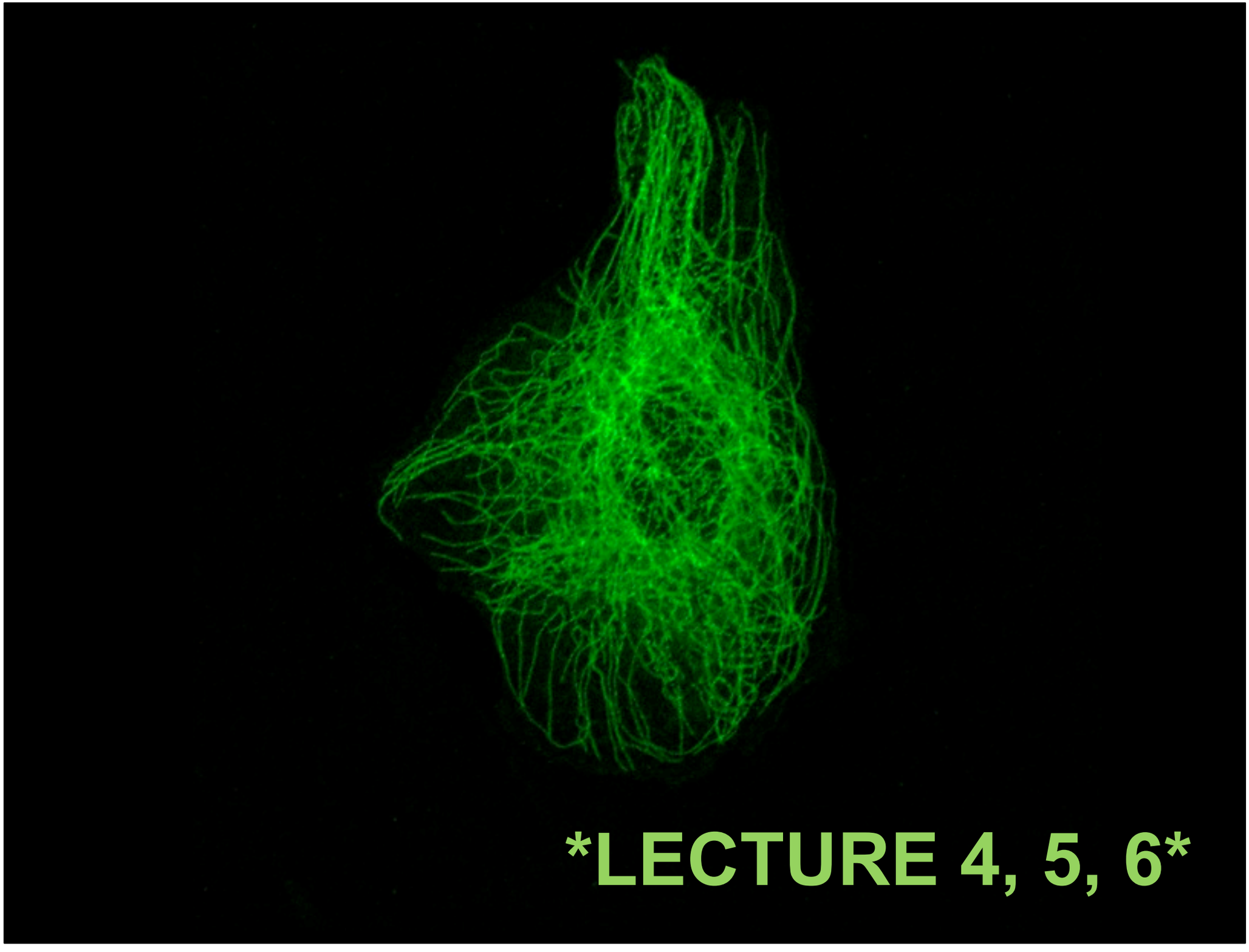
DIC





LECTURE 3

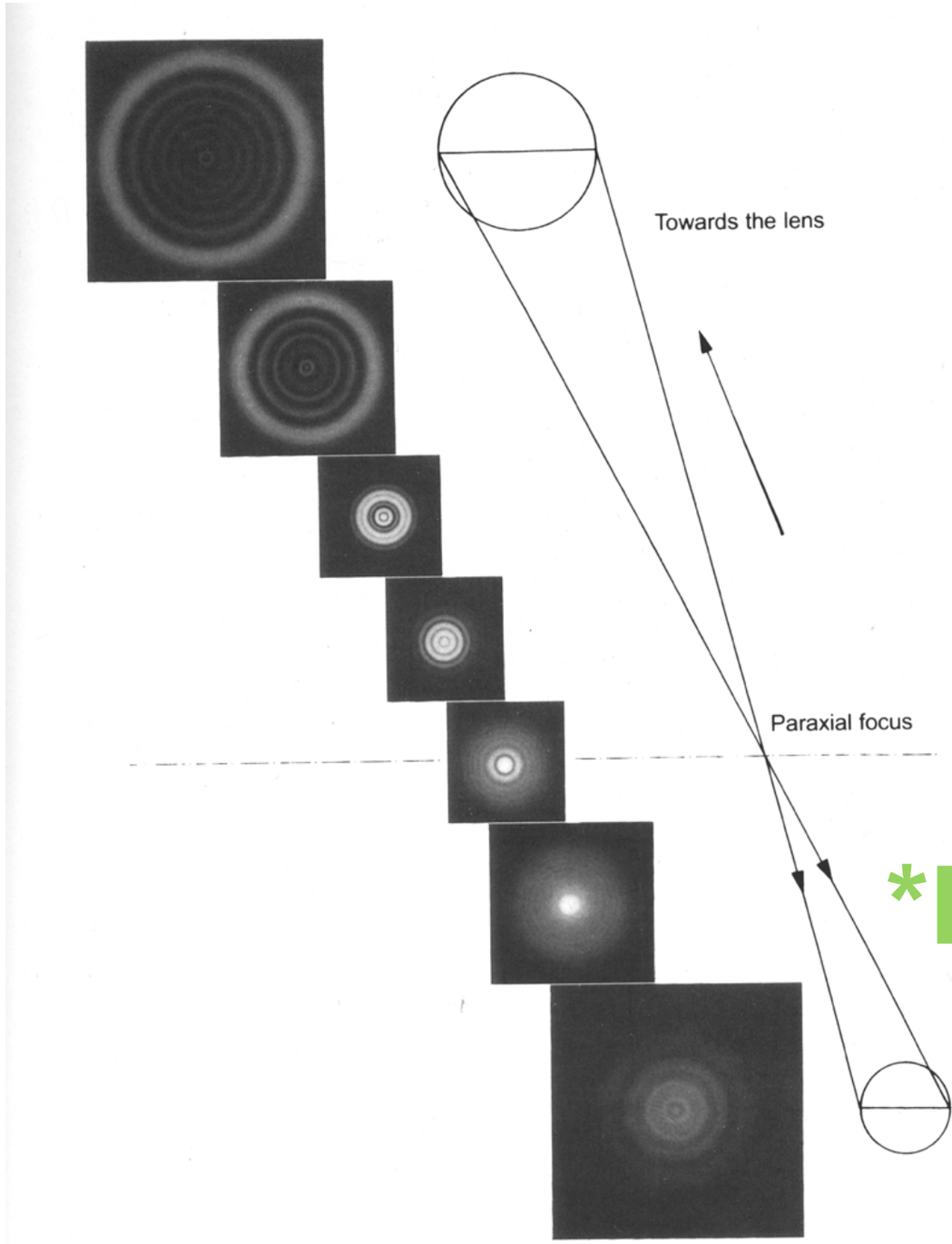
Contrast Enhancement:
Fluorescence

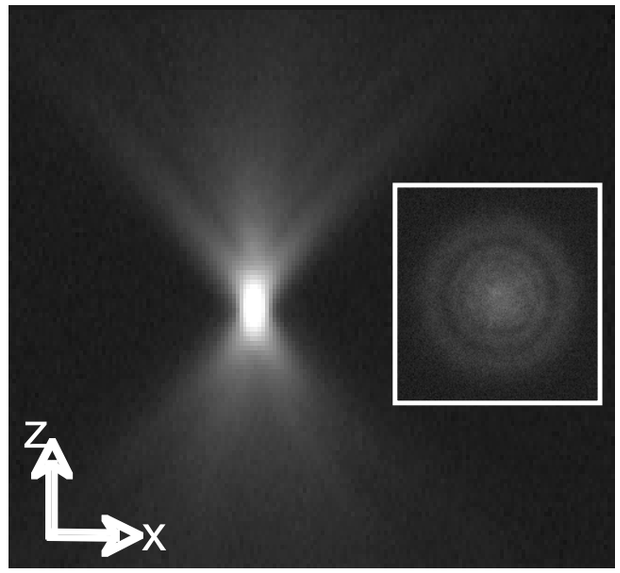


Understanding what limits Resolution

Convolution and the Point Spread Function

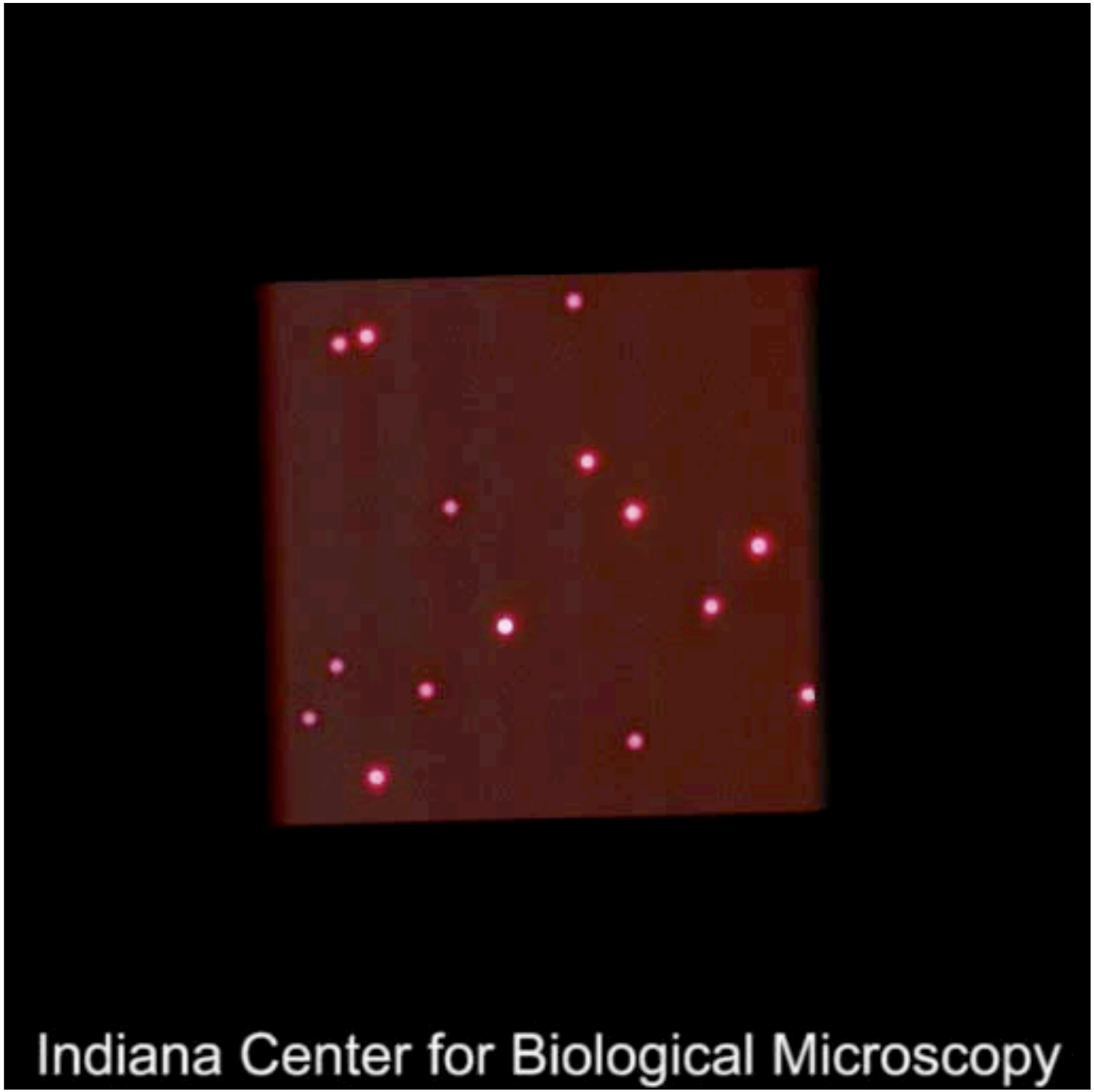
Sample object: a "sub-resolution" fluorescent bead



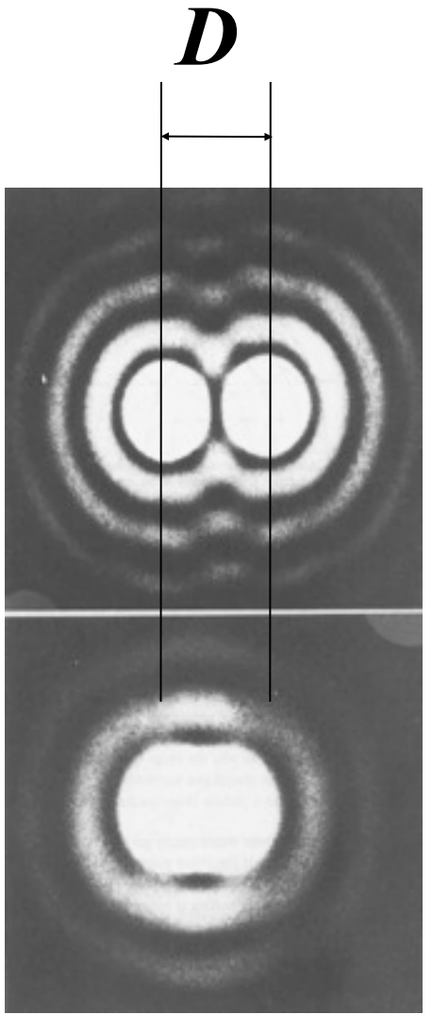


LECTURE 4-5

- "convolution" by the microscope optics = the PSF


Indiana Center for Biological Microscopy

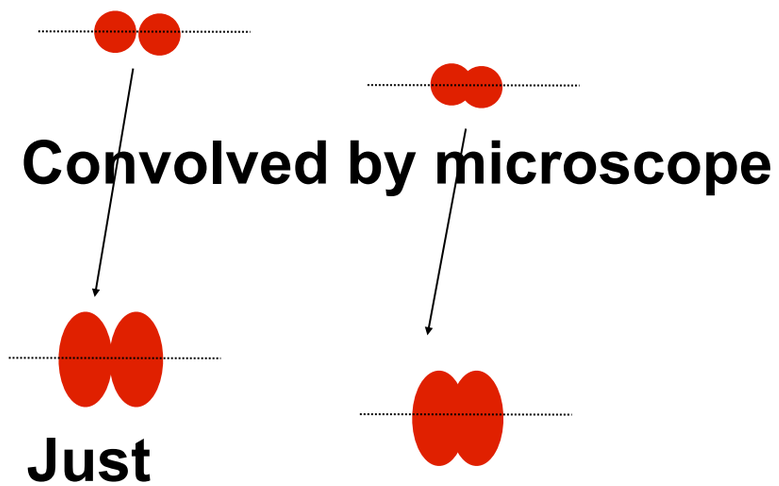
Lateral Resolution: The Rayleigh Criterion



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

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

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



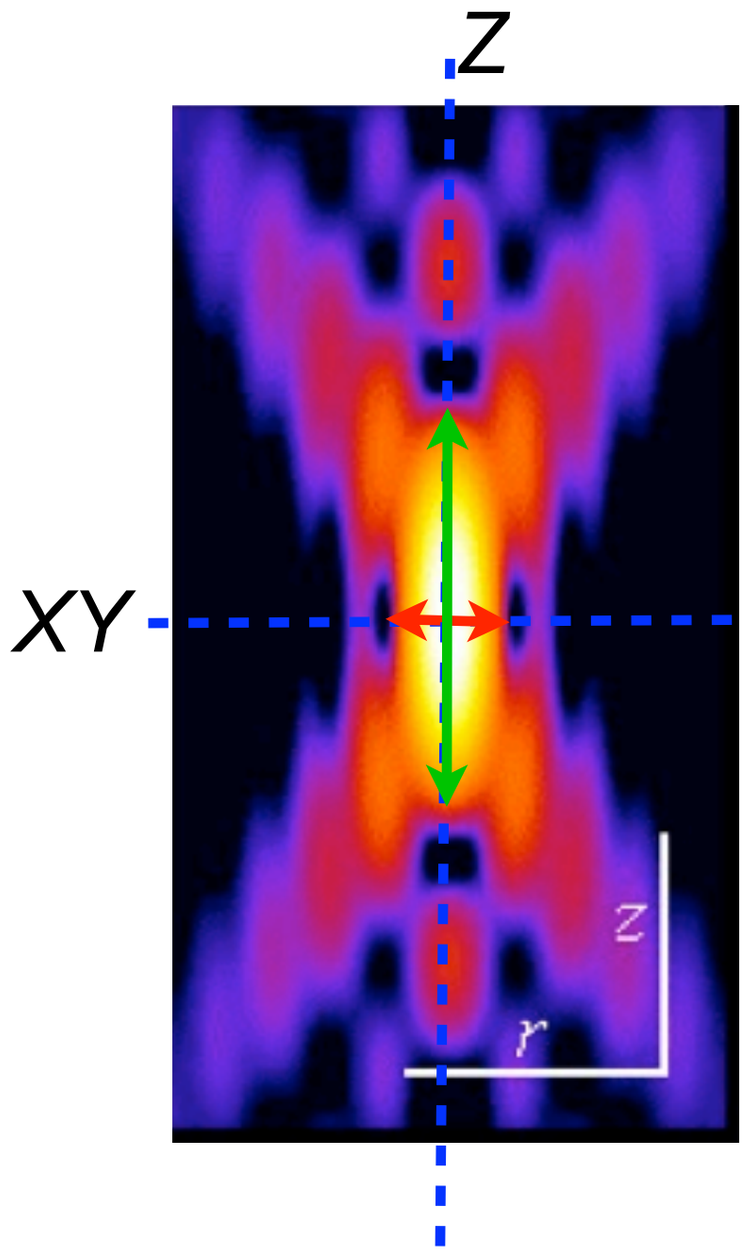
Limit of resolution $\approx \lambda_{em}/2$

Axial Resolution:

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

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

(η = refractive index of the object medium)



Than it is in the lateral dimension (XY)

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

FWHM

The relationship between the two is:

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

Resolution: Down to the molecular scale

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

XY resolution ~ 200 nm
Z resolution ~ 500 nm

GFP is ~ 5 x5 x5 nm

Solution 1 - F* techniques

FRAP, FRET, FLIM etc

LECTURES 11 and 12

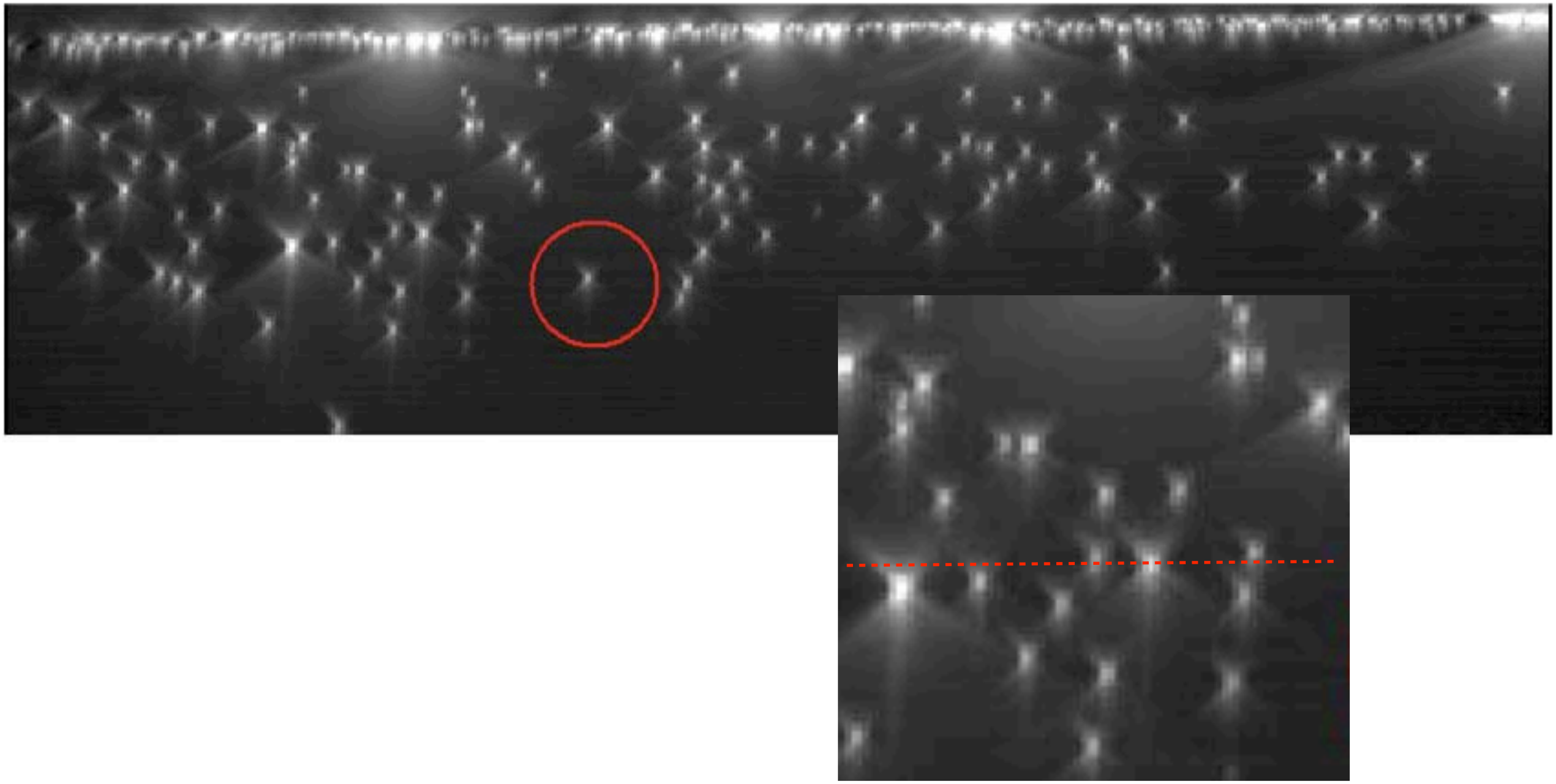
Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

LECTURES 13 and 14

Contrast and “out of focus” information

“out of focus” information



Techniques for improved fluorescence imaging

- Fluorescence deconvolution

  Wide-field vs Deconvolution

  The PSF and “out of focus” information

  Convolution and deconvolution

LECTURE 4,9

- Confocal microscopy

  The confocal principle and optical sectioning

  Point scanner and spinning disc

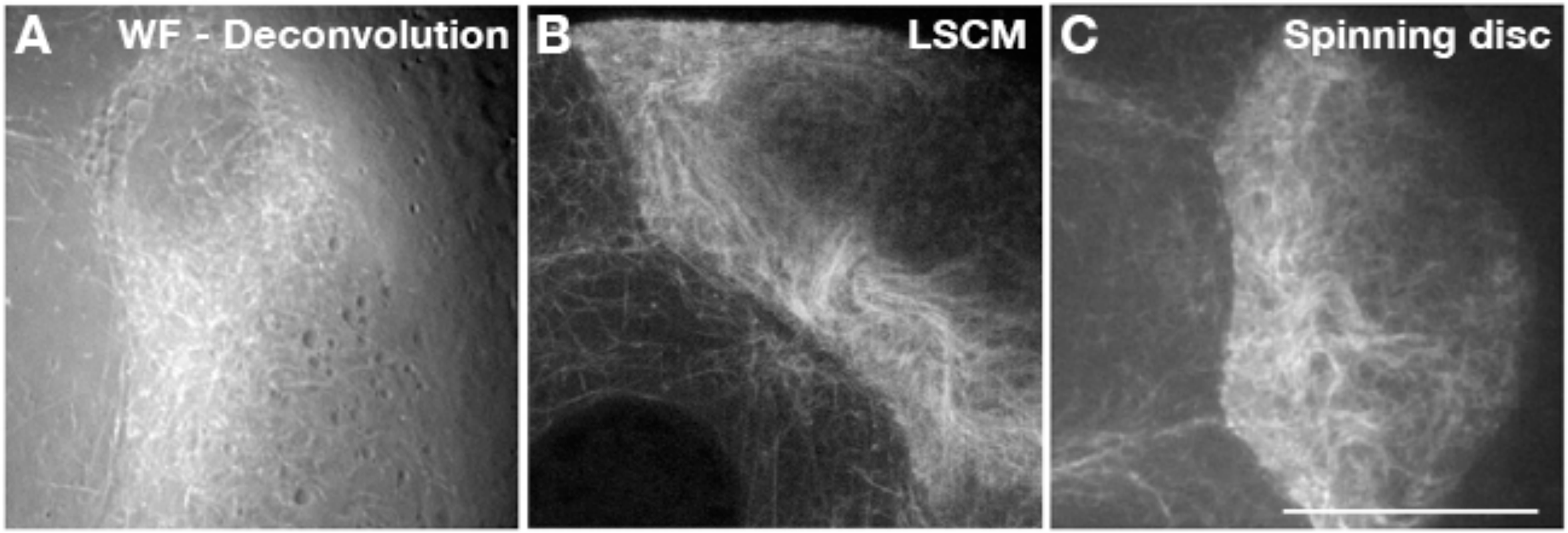
LECTURE 7,8

- Super-resolution and Total Internal Reflection Microscopy

  Evanescent wave excitation ***LECTURE 13,14***

Wide field Decon

Confocal PSF

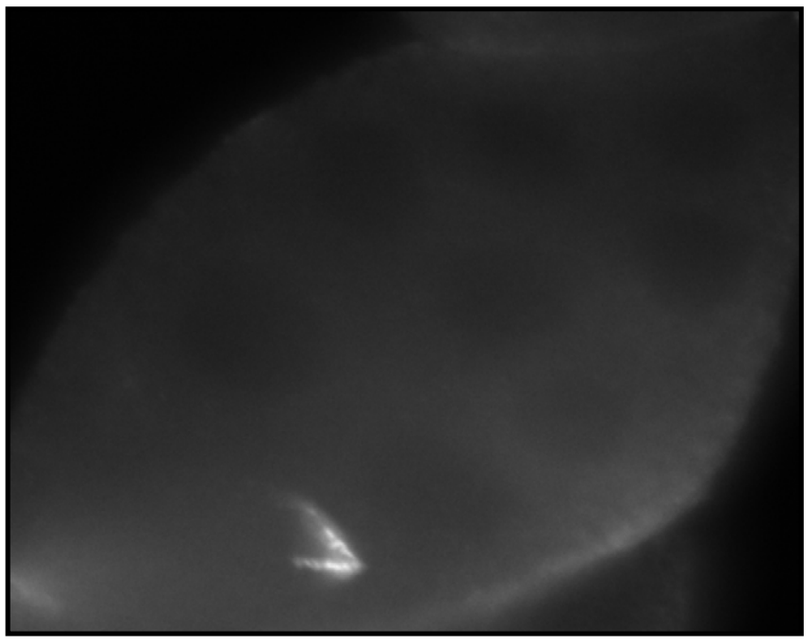


Resolution / Sampling / Noise

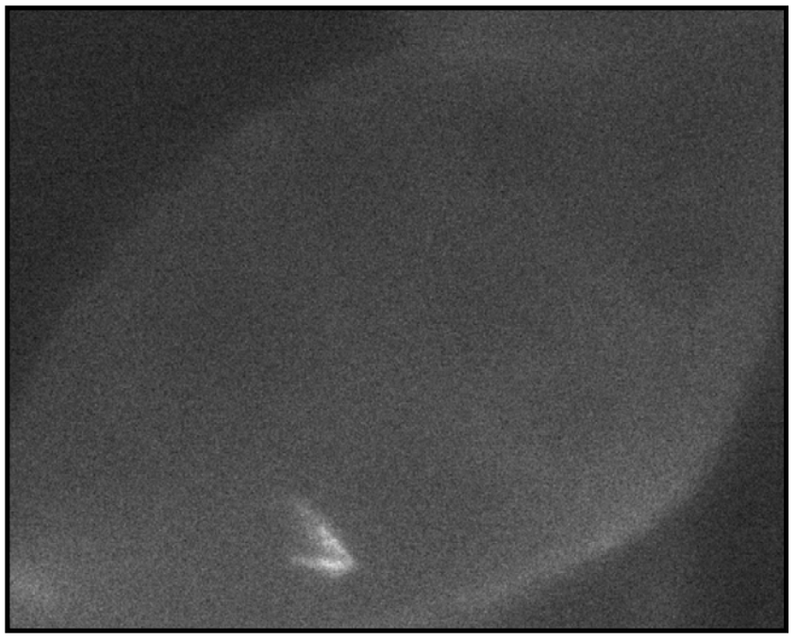
Resolution Contrast and noise (S/N ratio)

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

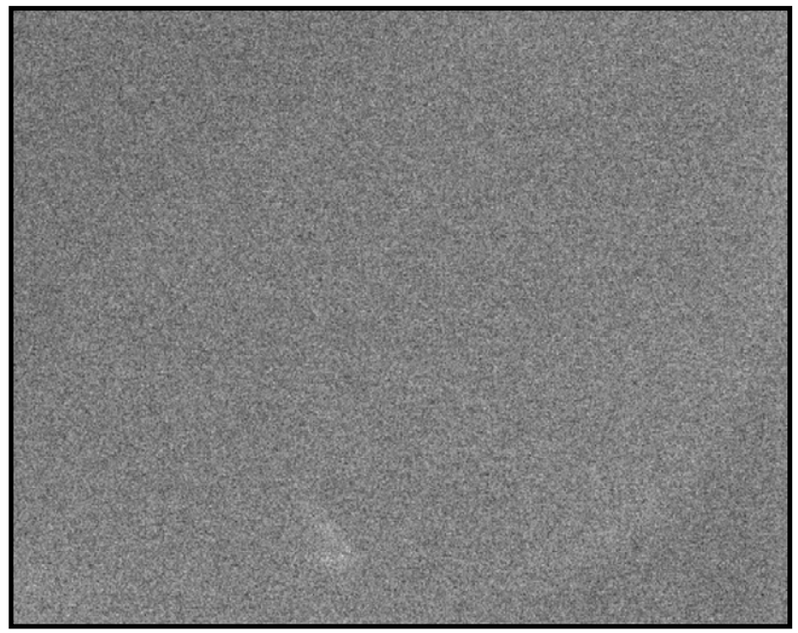
High signal / noise



Poor signal / noise



Very poor signal / noise



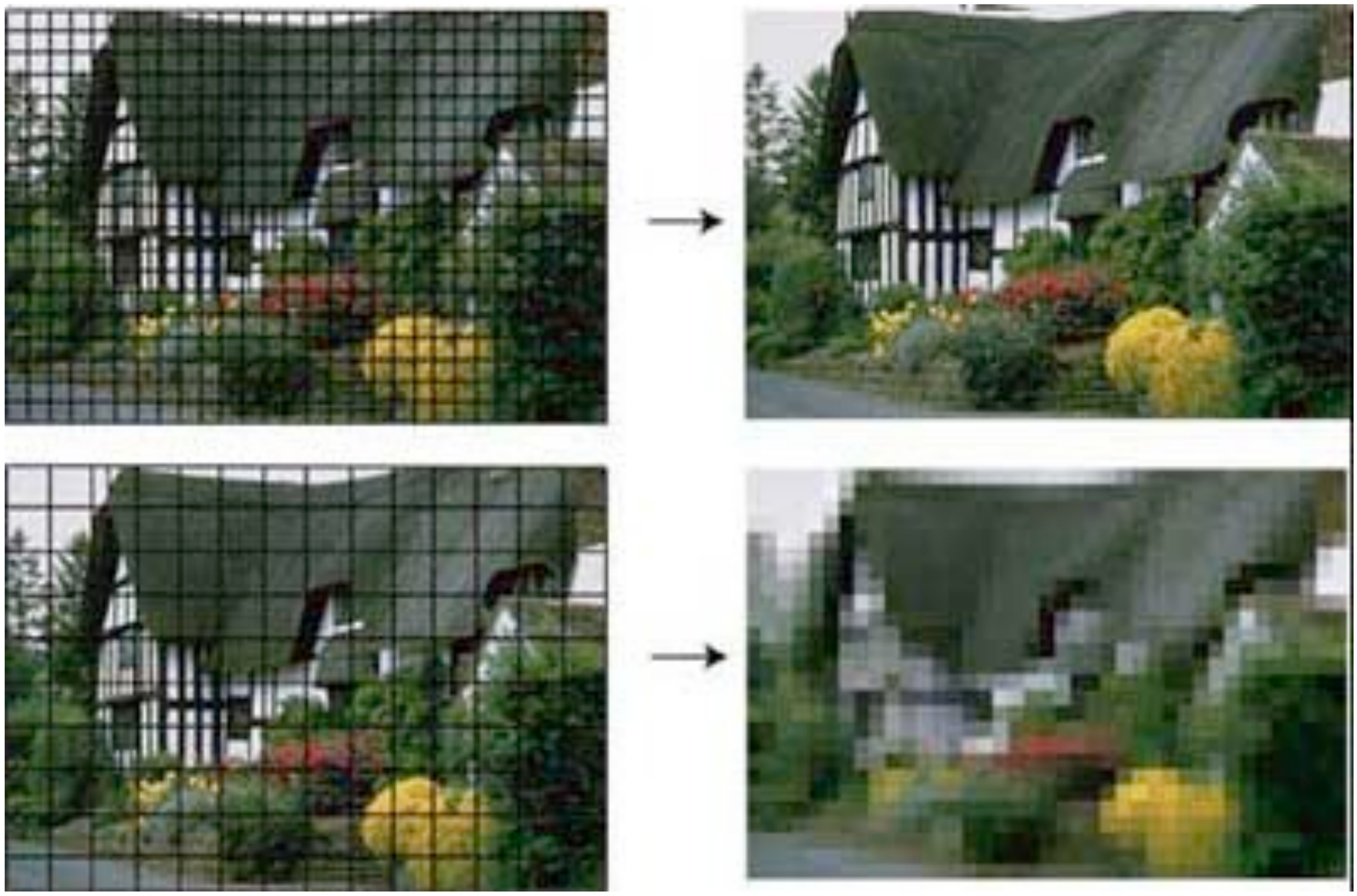
- Noise limits the contrast which limits the detail that can be resolved

= Noise limits resolution

LECTURE 5

Resolution and Sampling

LECTURE 5,9

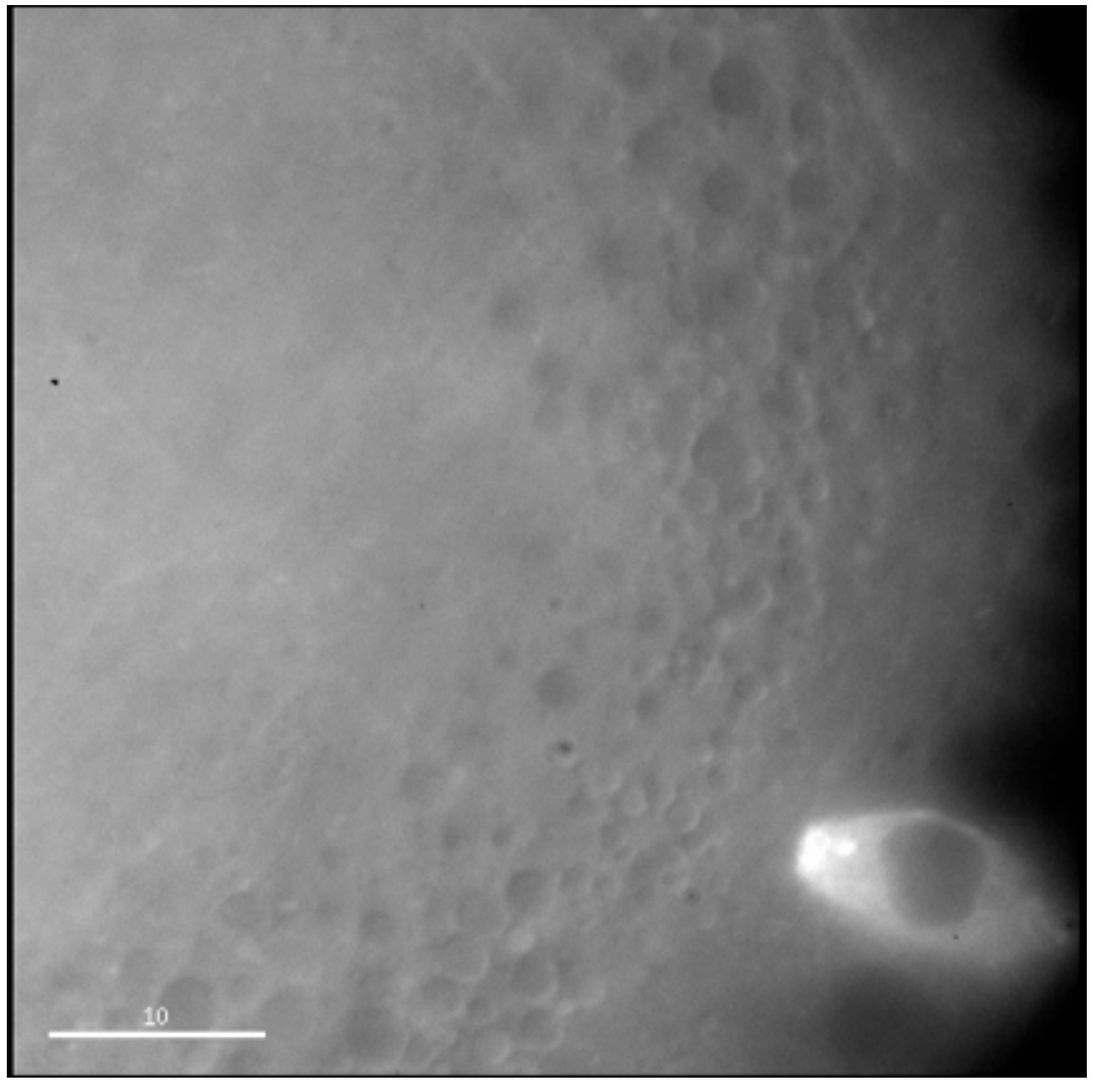


Undersampling limits the information in the image - mag and pixel size

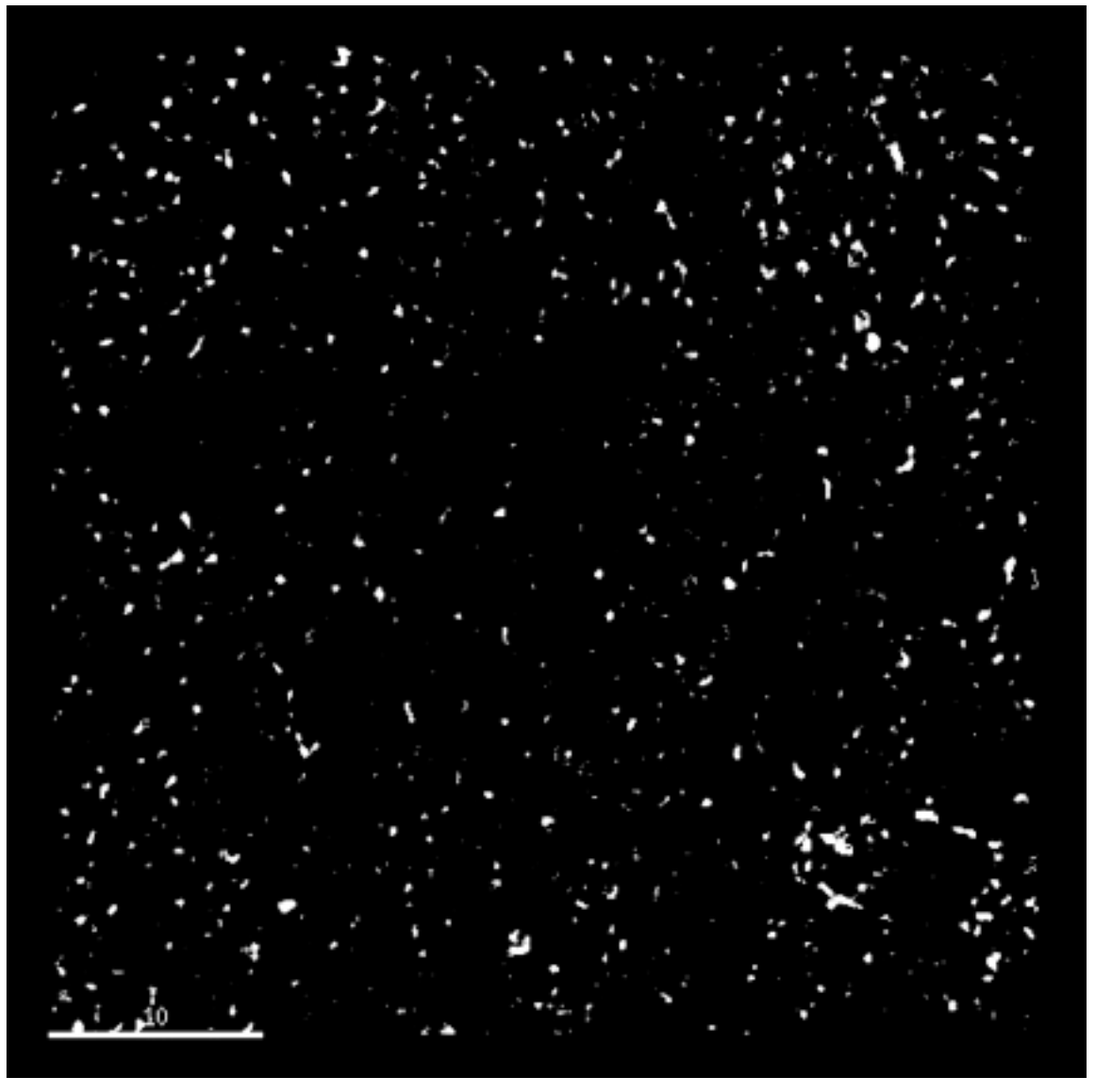
Image Processing

Computational enhancement / automation

Raw - Widefield



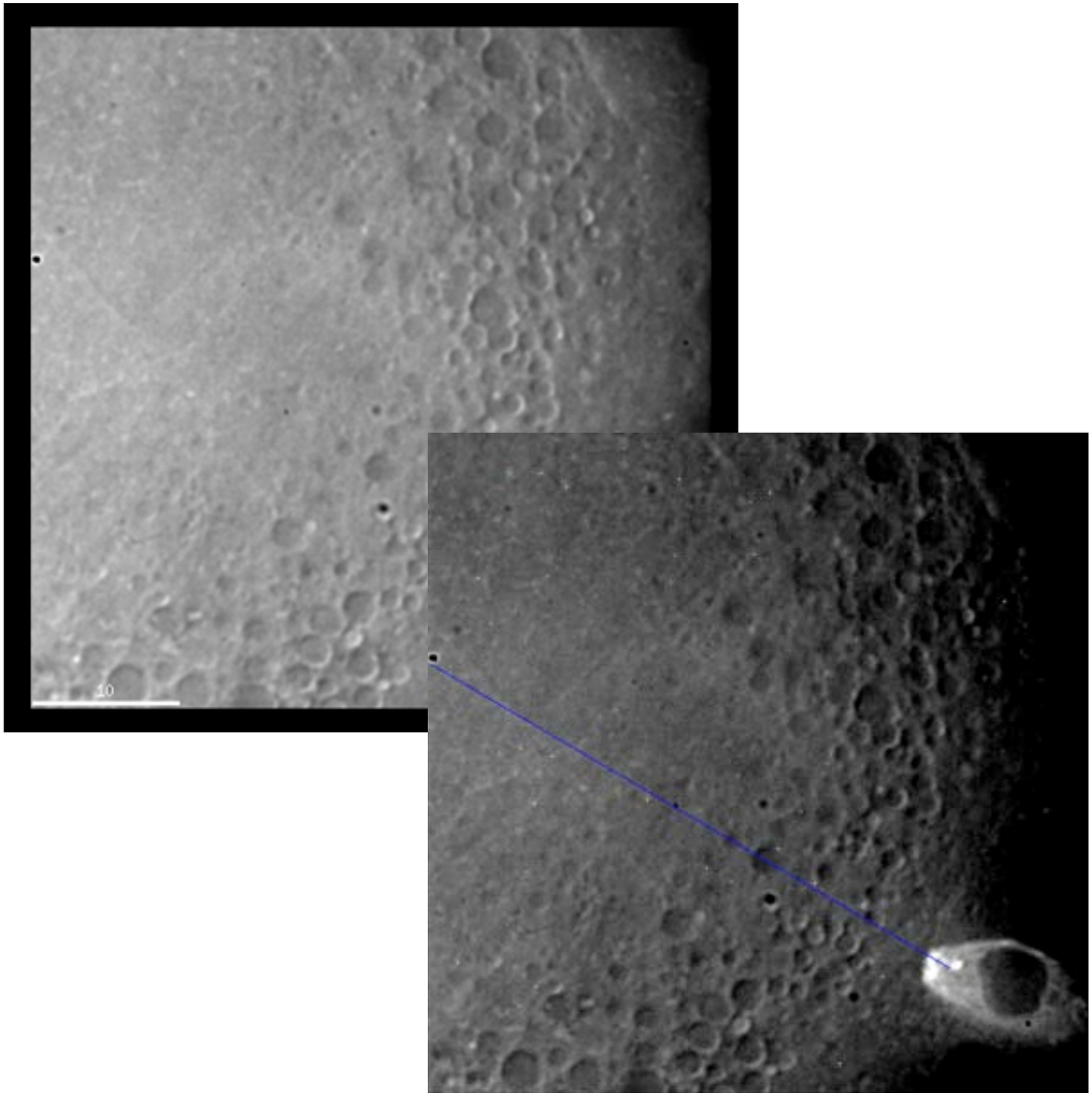
Processed

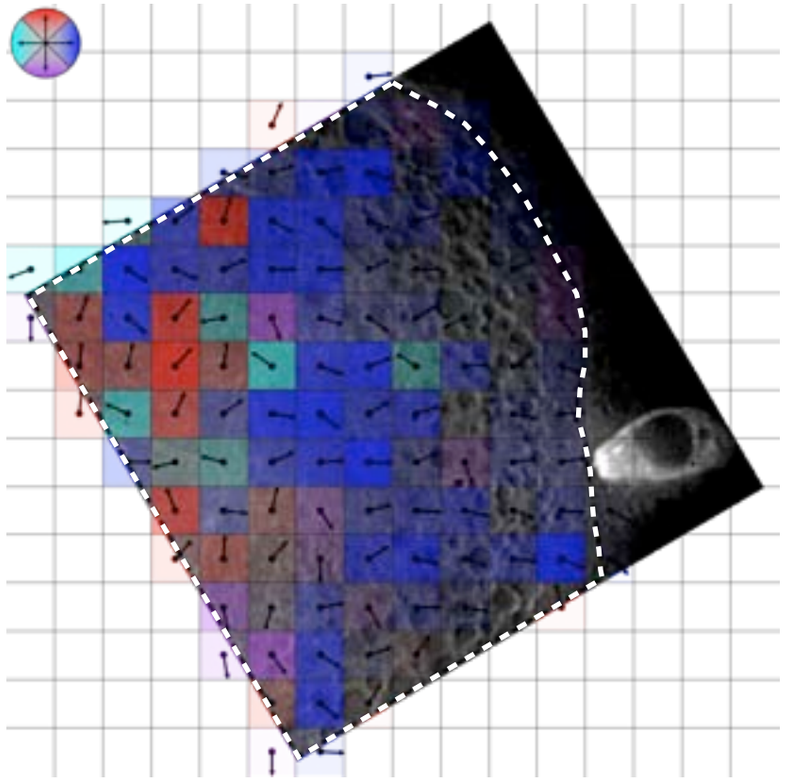


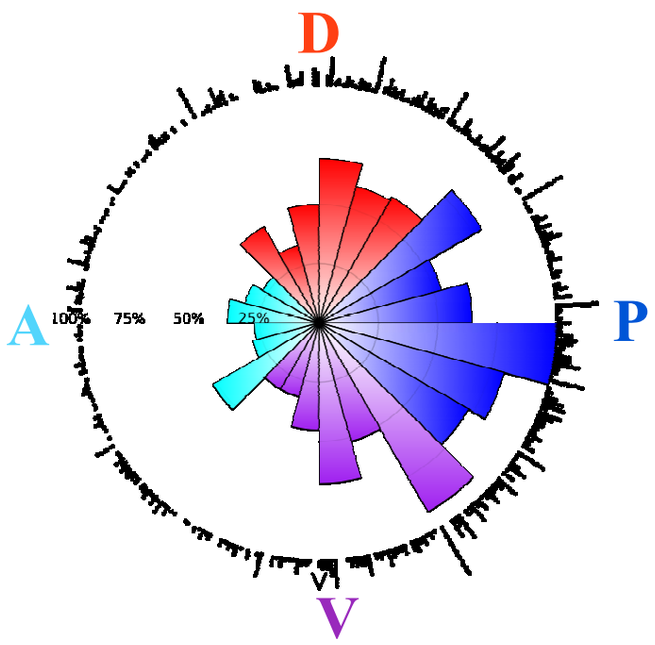
LECTURE 5, 16

(Richard Parton; Graeme Ball)

Live imaging - not just a pretty picture







(Richard Parton, Graeme Ball)

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

Microscopy is fundamentally important to modern biology

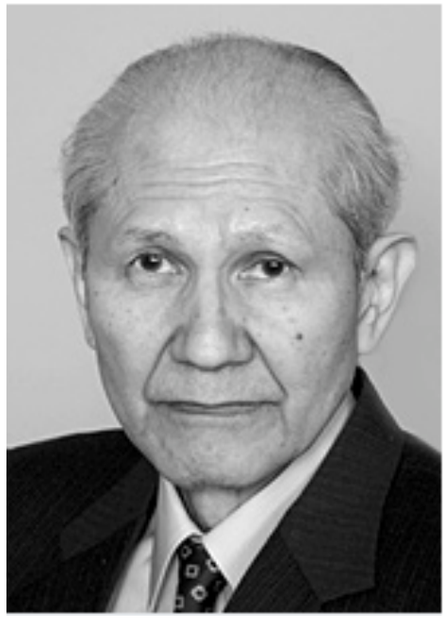
Milestones in Microscopy

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

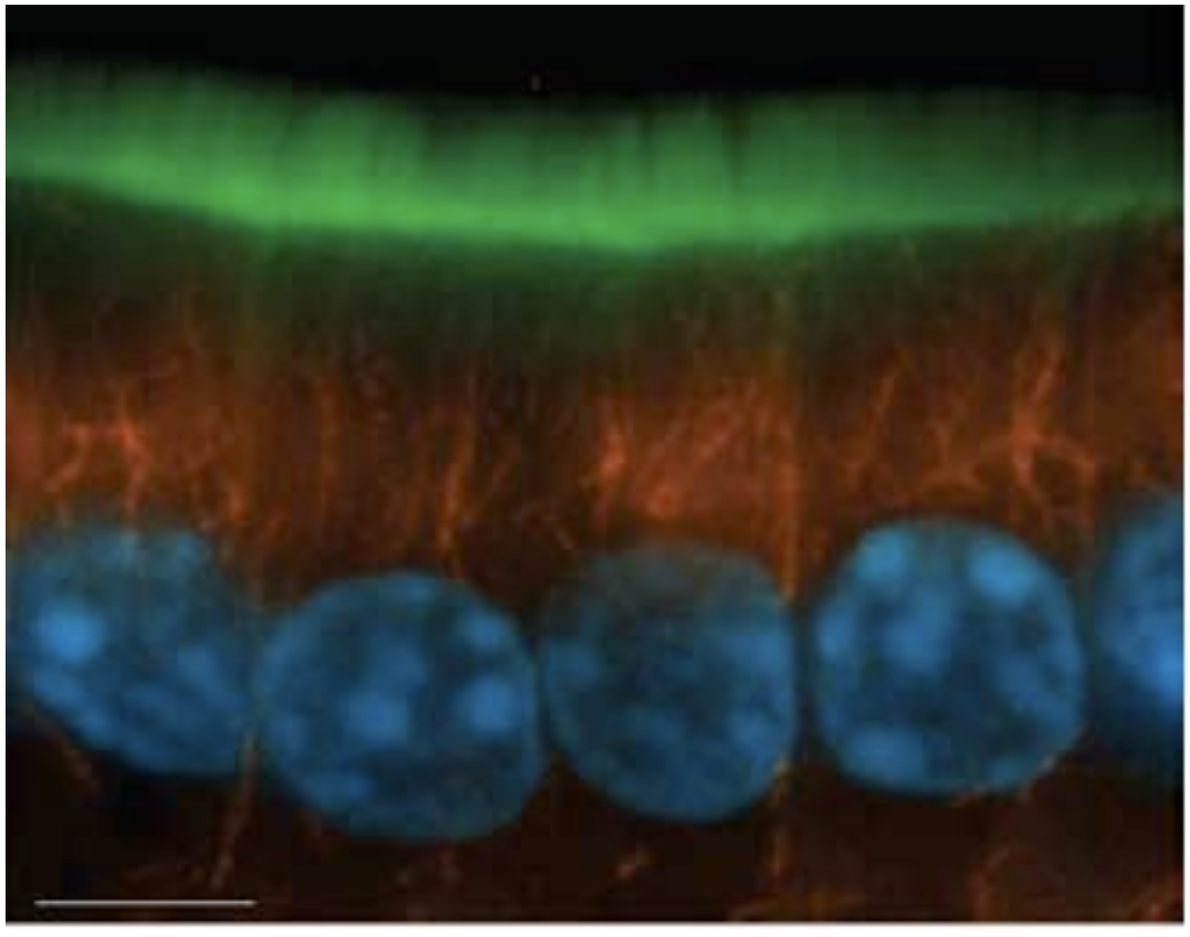
Manipulating the colour of GFP 1999

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

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

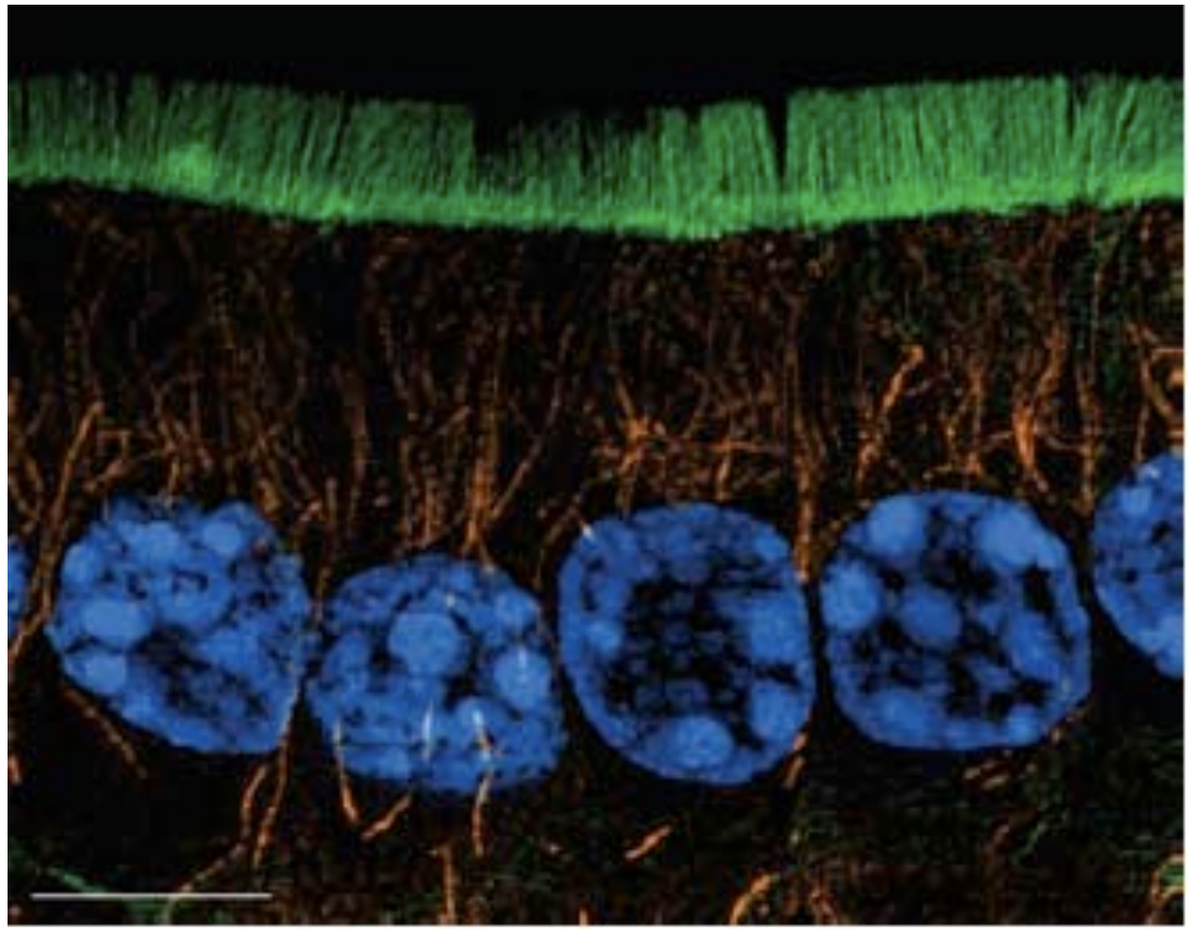
Super-resolution: beyond the diffraction limit

CONVENTIONAL



XY resolution ~250 nm

3D SIM



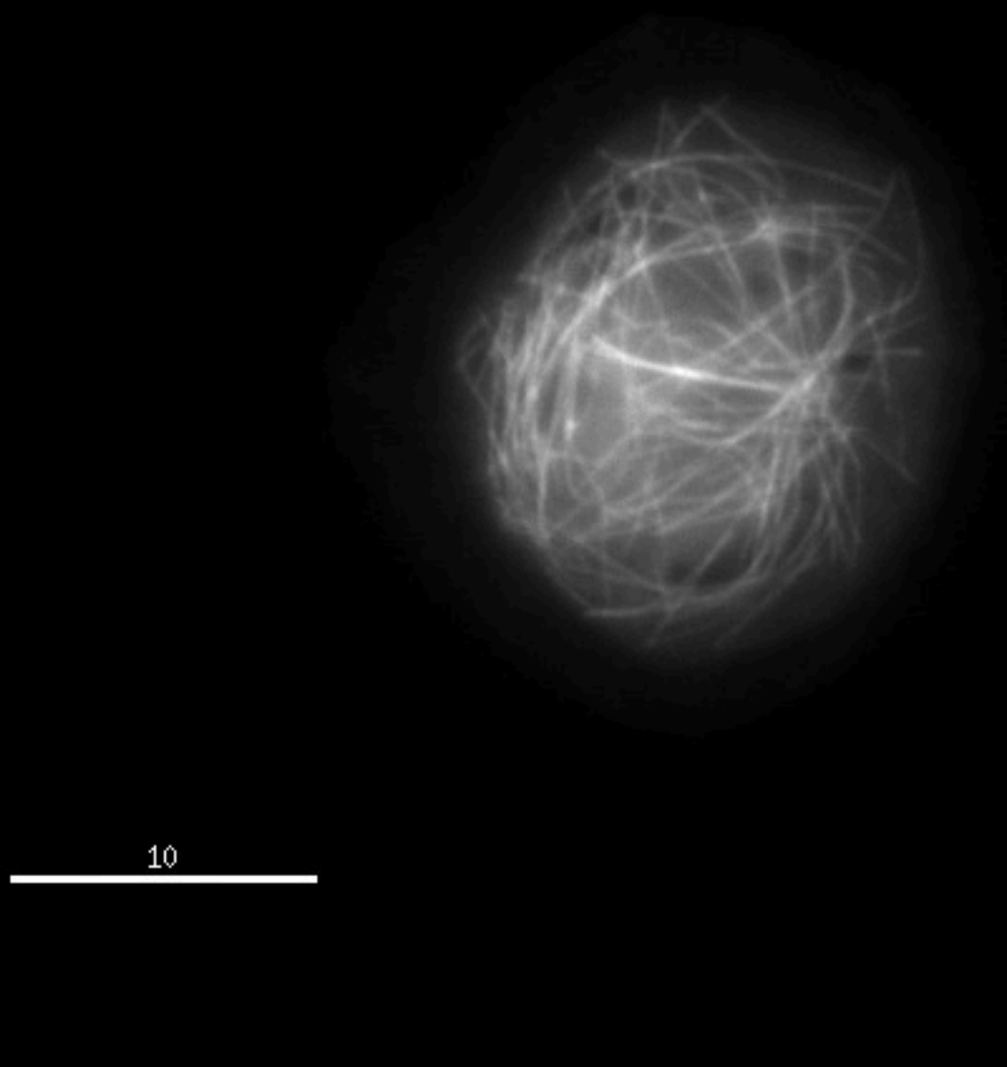
XY resolution ~130 nm

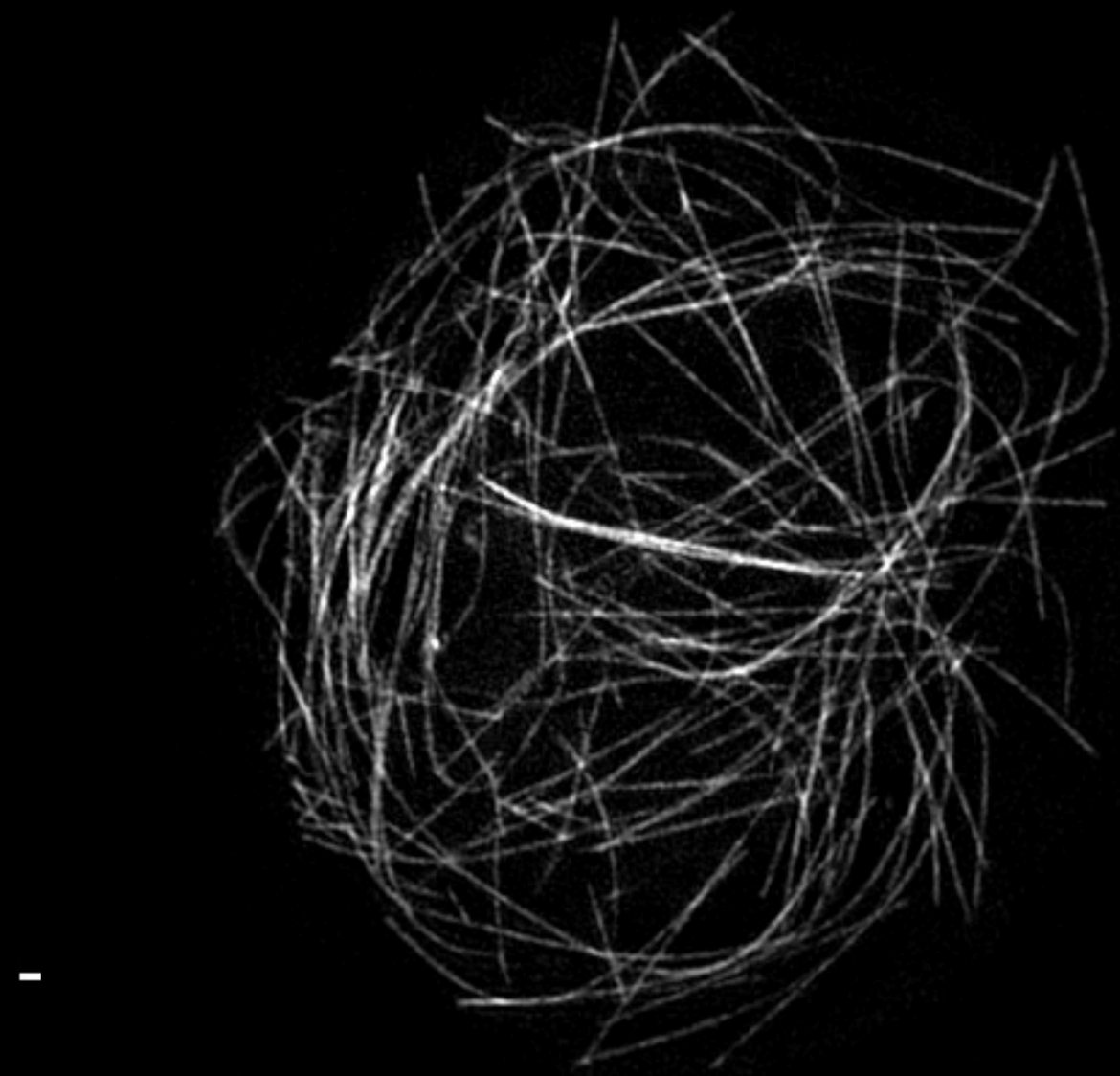
Image From Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
Cold Spring Harbour Press

LECTURE 13,14

Super-resolution: beyond the diffraction limit

CONVENTIONAL



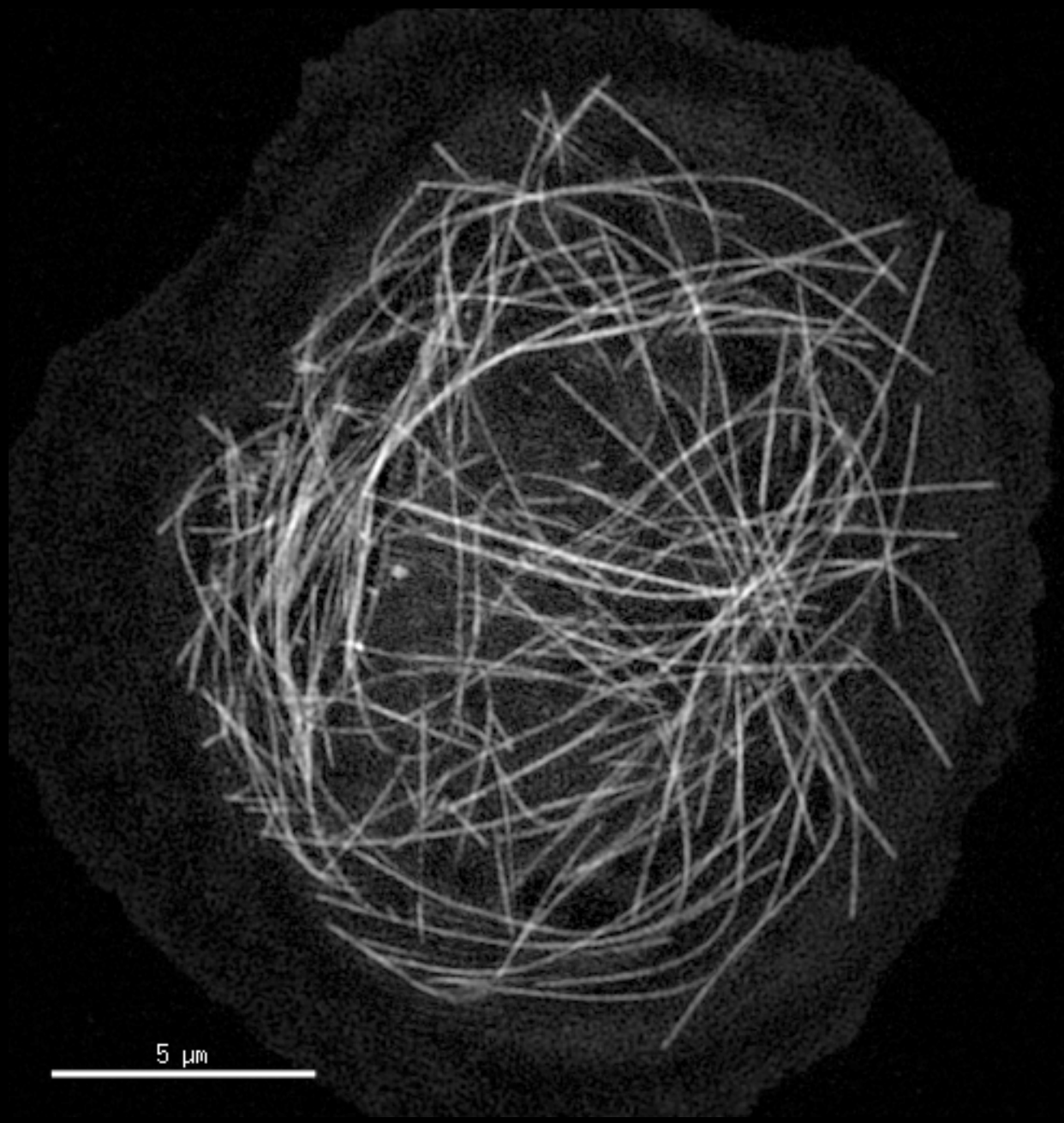


XY resolution ~250 nm

XY resolution ~130 nm

LECTURE 13,14

Live-cell super-resolution!



Jupiter-GFP tagged MT dynamics in a living Macrophage

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

Molecular Biology of the Cell, fifth edition.
Alberts et al. Chapter 9: Visualizing cells, page 579-616

END