Microscopy Course 2014 - lectures

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

****

Day 2: Tuesday 12 March - Imaging Cells and Tissues

7	Richard	9.00-10.00	Live cell imaging
8	Alan	10.00-11.00	Confocal, spinning discs and Multiphotons
9	Ian	11.20-12.20	Advanced widefield microscopy and bespoke systems
10	Matt	1.30-2.30	Cameras for microscopy
11	Sergi	2.30-3.30	F* techniques: FRET, FLIM, FCS, FRAP, FLIP
12	Chris L.	4.30-5.30	Single Molecule Techniques
13	Eva	5.00-5.30	Light-sheet microscopy

****

Day 3: Wednesday 13 March - Advanced imaging / Applied Image handling

14	Chris L.	9.00-10.00	STED, FCS
15	Rainer	10.00-11.00	Storm/PALM techniques.
16	Ian	11.30-12.30	OMX
17	Dominic	1.30-2.30	Applied Image analysis and Matlab
18	Douglas	2.30-3.00	Image Management
19	Errin	3.00-4.00	Electron Microscopy

****

AND

Day 4: Thursday 22 May – Microscope demos and image analysis
9.00-12.00 and 14.00-17.00

* DV live: Jupiter-YFP macrophages
* Spinning disk live: Jupiter-YFP macrophages
* OMX V2 dSTORM: Rainer's samples
* OMX V3 SIM: Golgi
* Olympus scanning confocal in the demo room: Golgi
* Olympus TIRF in prep area
* Lightsheet: Drosophila brain
* Image analysis

15 min demos (+ 5 min walking):
8 sessions in the morning,
8 sessions in the afternoon
(5 people per group, 80 people in total)

Microscopy Course 2014 - lectures and handout material may be downloaded from:

http://www.micron.ox.ac.uk/microngroup/2014_Lectures.php

Advanced Microscopy Course 2014
Introductory Lecture

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

Goals of the lecture course

- Explain why microscopy is so important
- Explain how the light microscope works:

  the basic physics of optics and microscopes
- 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
  - Dealing with images appropriately

Why is microscopy so important?

100 years ago:

Magnify small things to visualise more details

Now:

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) **

Why is microscopy so important?

* Spatial information at the cellular level not easily available from biochemistry

* Relatively non-invasive

* Very sensitive, can follow distribution and interactions down to the molecular level

* Quantitative

* Can be applied to live cells to follow sequences of events

* Allows experimental manipulation

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)

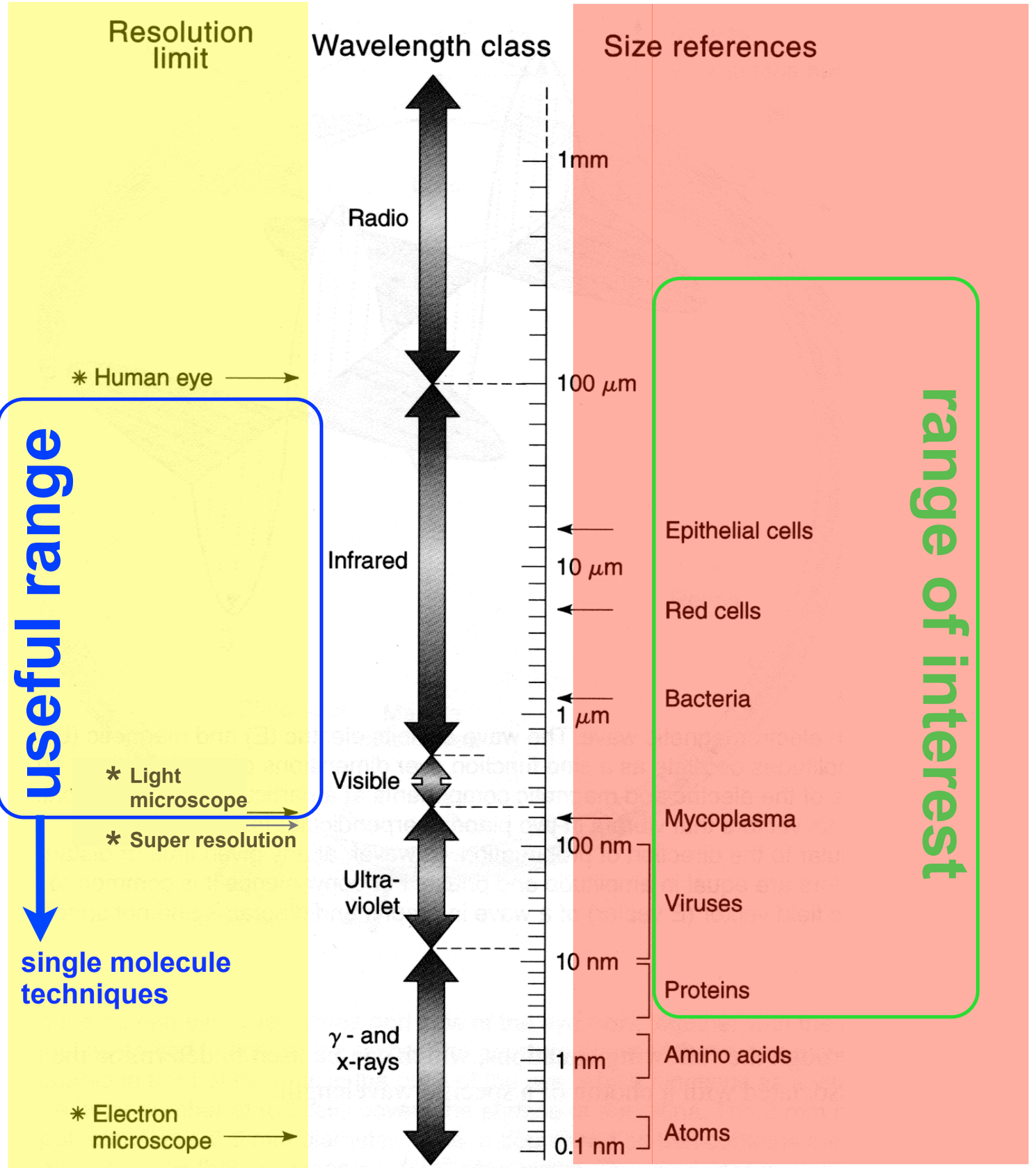
Eukaryote = 10 um
>50 um

Bacterium = 1 um

Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

Check out advanced imaging in the Biochemistry Dept:

Micron OXFORD

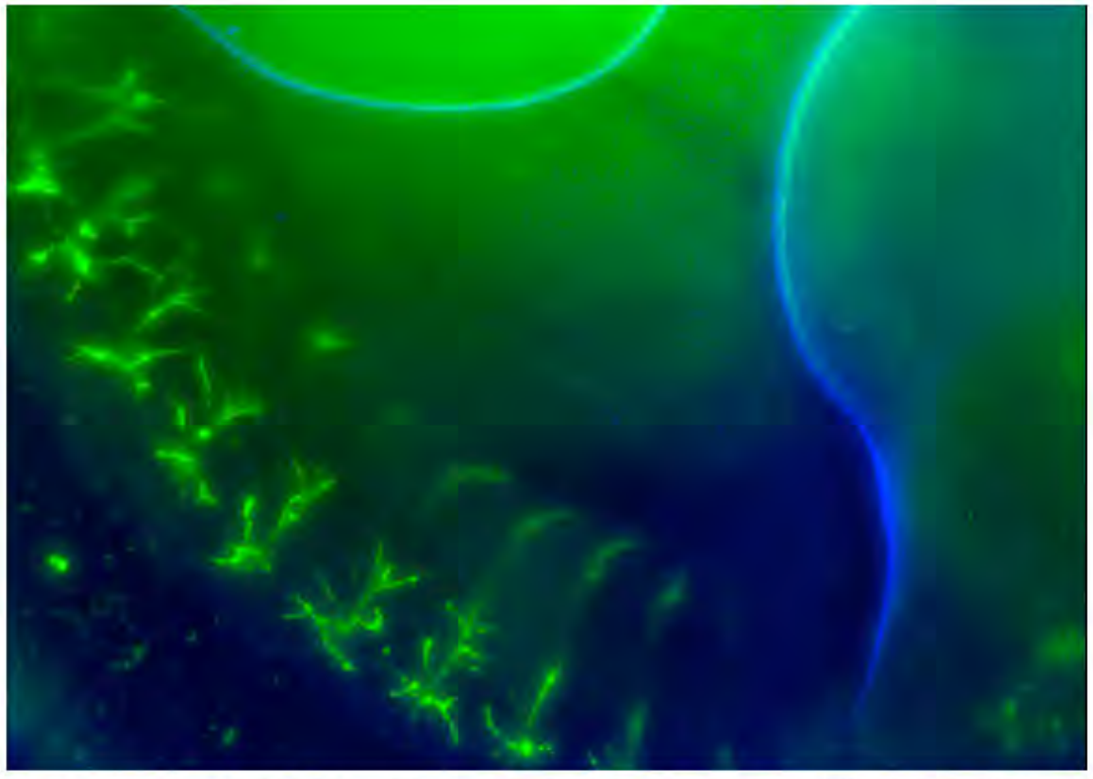
wellcome trust Strategic Award

Advanced Bioimaging Unit

UNIVERSITY OF OXFORD

Home | About Us | People | Research | Facilities | Resources | News & Media | Booking

Micron Oxford is located within the Department of Biochemistry and the Dunn School of Pathology in the South Parks Road science area at the University of Oxford, and is funded by a strategic award from the Wellcome Trust. We are a collaborative, multidisciplinary bioimaging unit working with biomedical researchers in the Oxford area and beyond to apply advanced cellular imaging techniques to address key questions in biology. We are focusing on the development and use of single molecule methods, light sheet and super-resolution microscopy.



This is a mouse embryo with Tuj-1 staining of Neuron-specific class III Beta-tubulin in green and DNA stained using DAPI in blue. (Anna Franz. Raff Lab).

Contact Us | more research images | view our Publications and our Research

SIMcheck | OME | Nano | Wiki

Micron Oxford @MicronOxford — 31 Jan

Congratulations to Lesterlin et al for their paper in @NatureMagazine with super-resolution images taken in micron! tinyurl.com/mgtu9ek

Micron Oxford @MicronOxford — 27 Jan

For more info on latest advances in #lightsheet #microscopy, check out collection of articles from @naturemethods tinyurl.com/lk9e2bv

Micron Oxford @MicronOxford — 24 Jan

exciting times in Micron as our new @ZEISS_Group #lightsheet Z.1 microscope arrives!

Expand

Bioimaging Facility — 22 Jan

Micron Oxford (2013). Department of Biochemistry / Dunn School of Pathology. Enquiries to micron; contact webmaster.

http://www.micron.ox.ac.uk/micron_home.php

Why do we need to understand microscopy?

*** Microscopes tend to be complicated and expensive**
Don't mess with what you don't understand

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

• All the buttons and levers and knobs actually serve a purpose - it is better if you understand what they do!

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

*** Microscopes are all basically the same**

*** It is quite hard to break a microscope**

Understanding what goes on in the Microscope

LECTURES 2-4 practicals 1 and 2

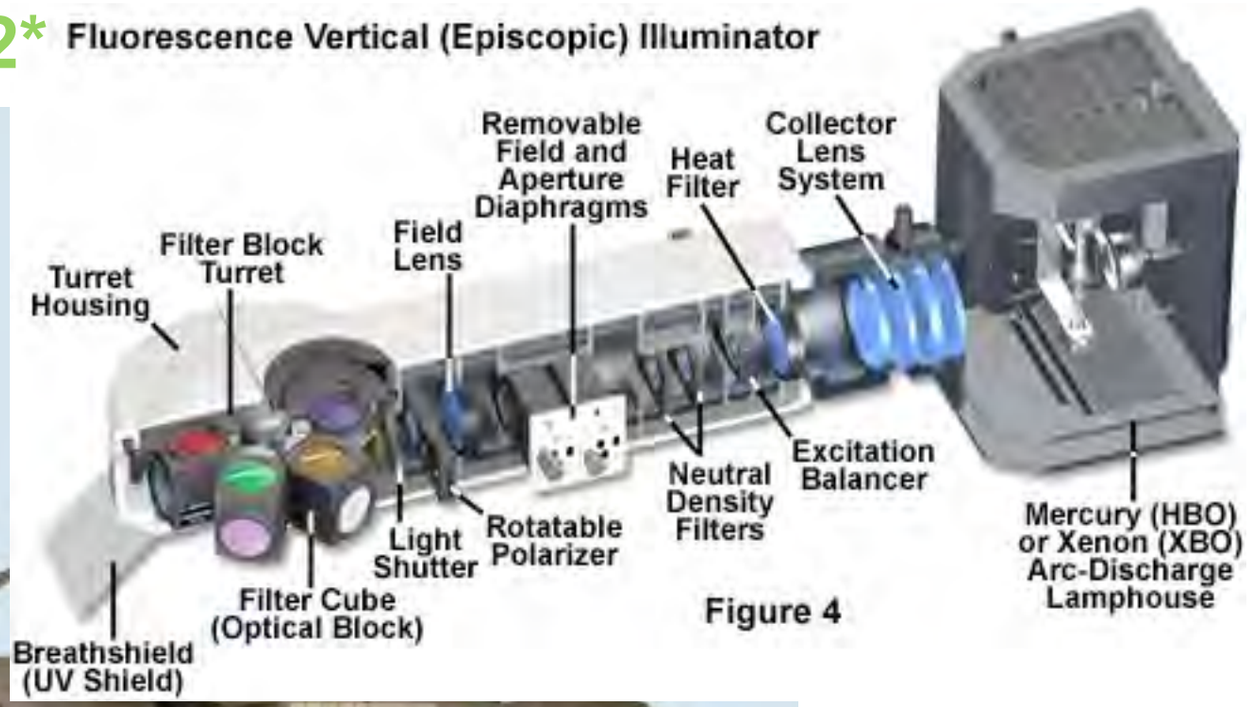


Figure 4

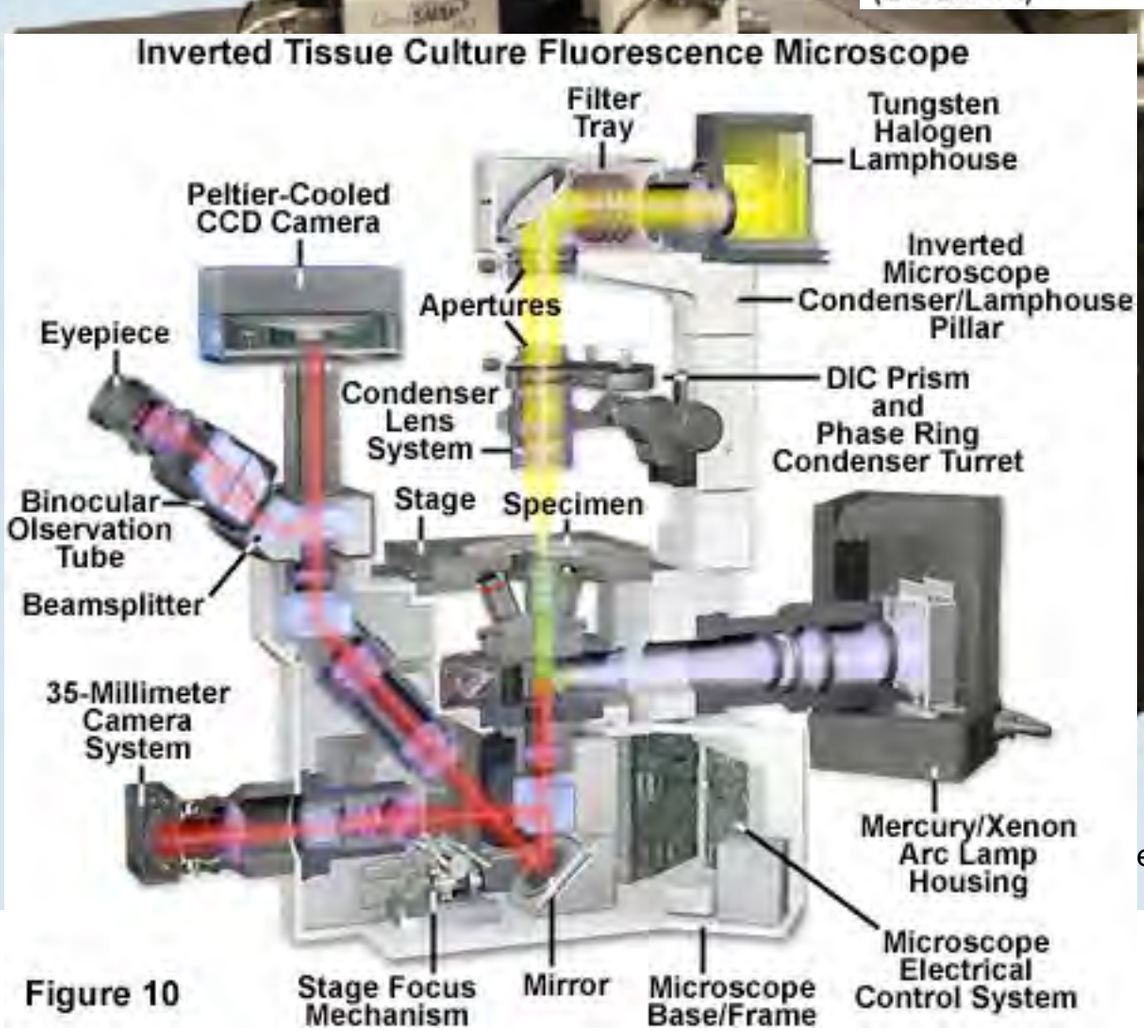


Figure 10

ehealthcare.com/api/deltavision.asp

http://www.olympusmicro.com/

What is really important in microscopy?

…….the ability to see stuff

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

and nothing else!

LECTURE 5

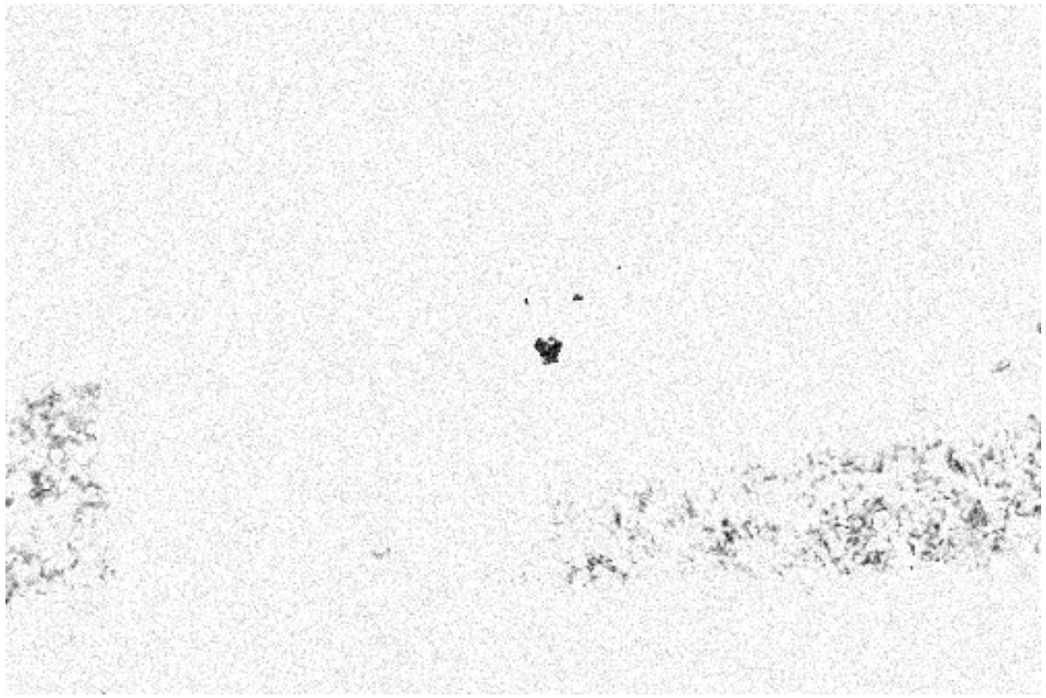
What is really important in microscopy?

1. Contrast
2. Resolution

What is really important in microscopy?

Contrast .....the ability to distinguish stuff

Biological specimens have low inherent contrast:



Bright Field Contrast Techniques

*LECTURE 3
Practical 1*

…….enhance features by transforming differences in the cell into differences in brightness

DIC

differential interference contrast

RMP: Onion epidermis bright field

Self Taught Practical Exercises - 1 Bright field

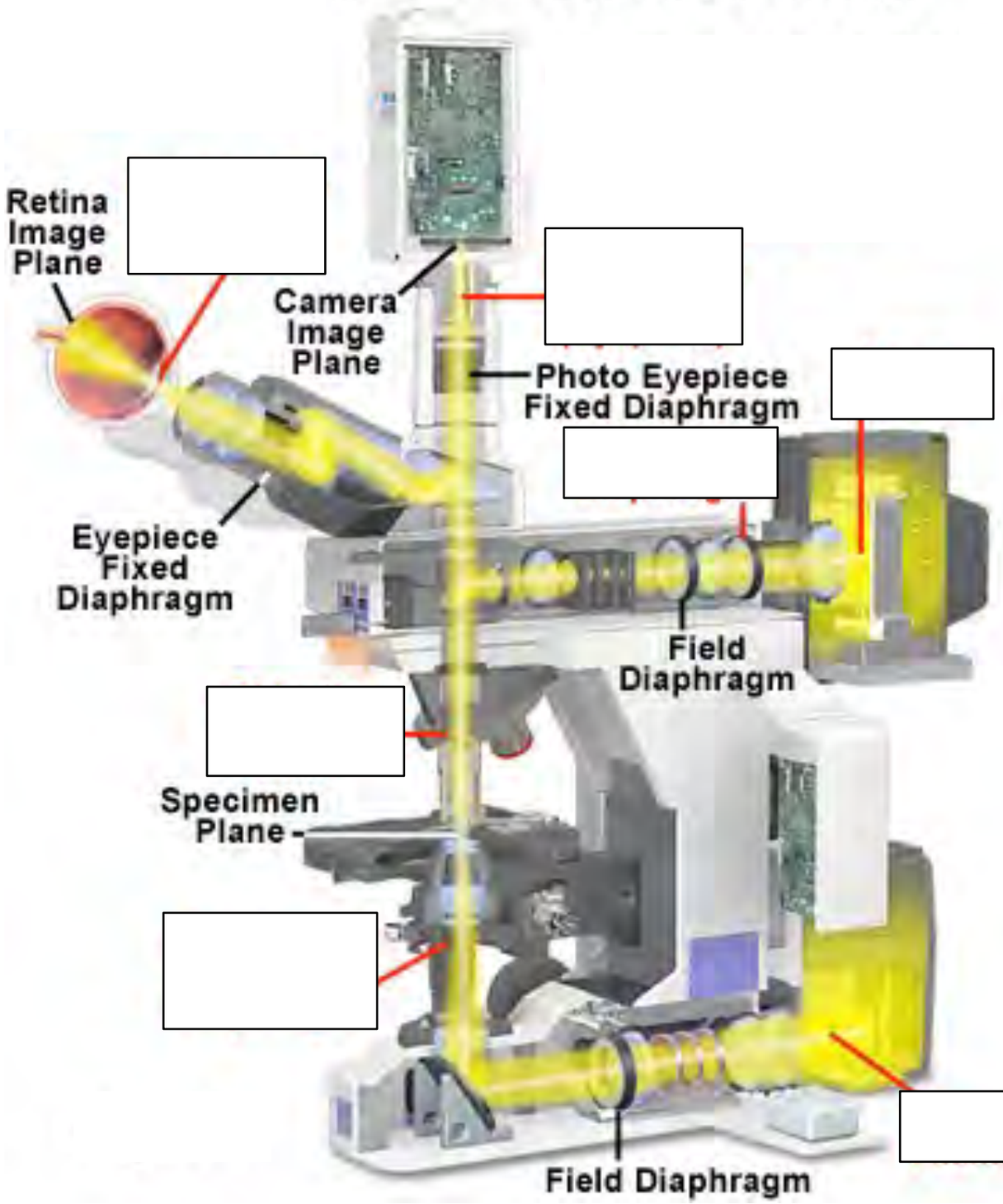
* Identifying the parts of the microscope, conjugate planes

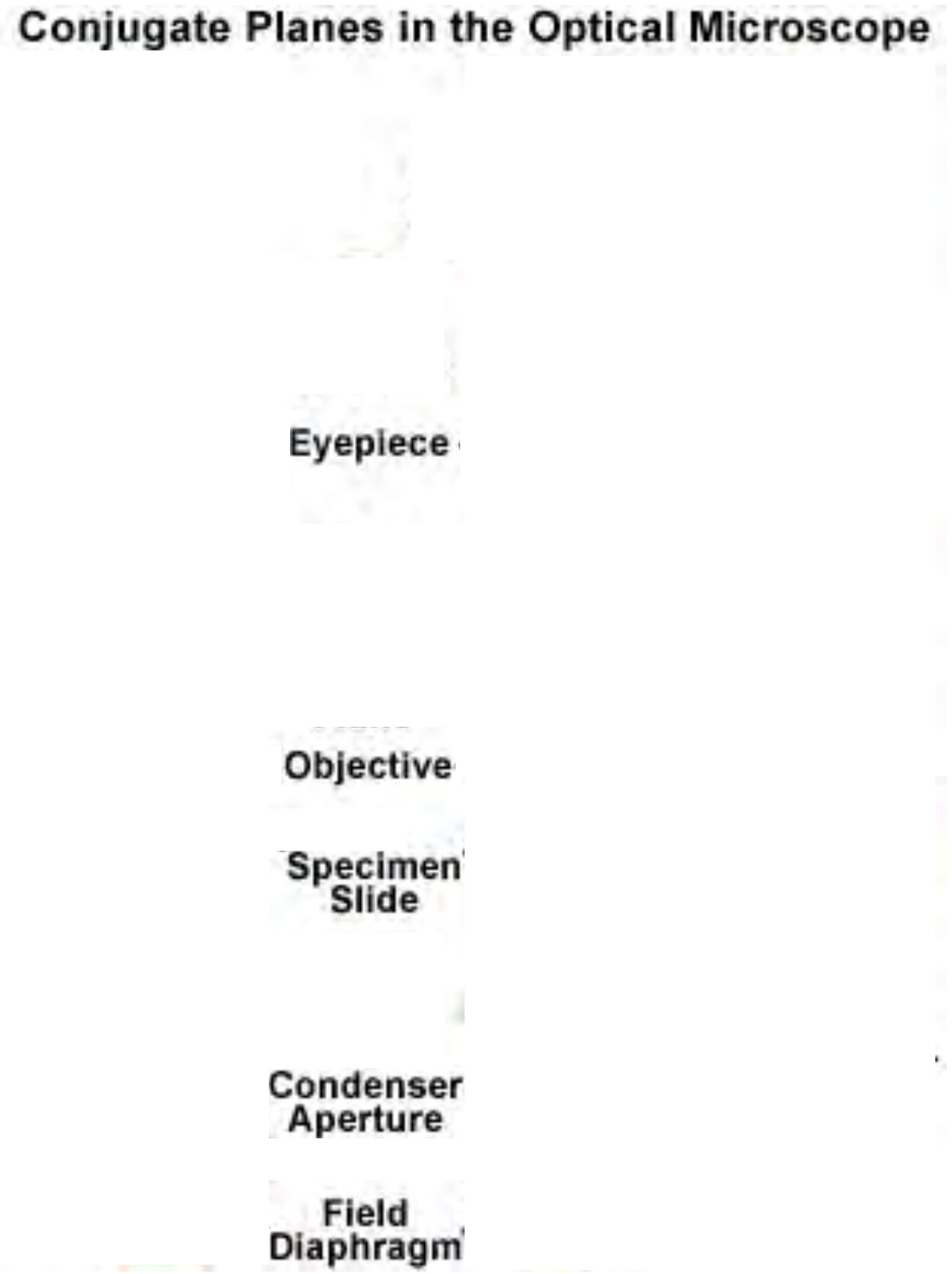
* Setting up Koehler illumination and adjusting the condenser

* Different contrast techniques

Koehler illumination and conjugate planes

Field or Image forming conjugate planes





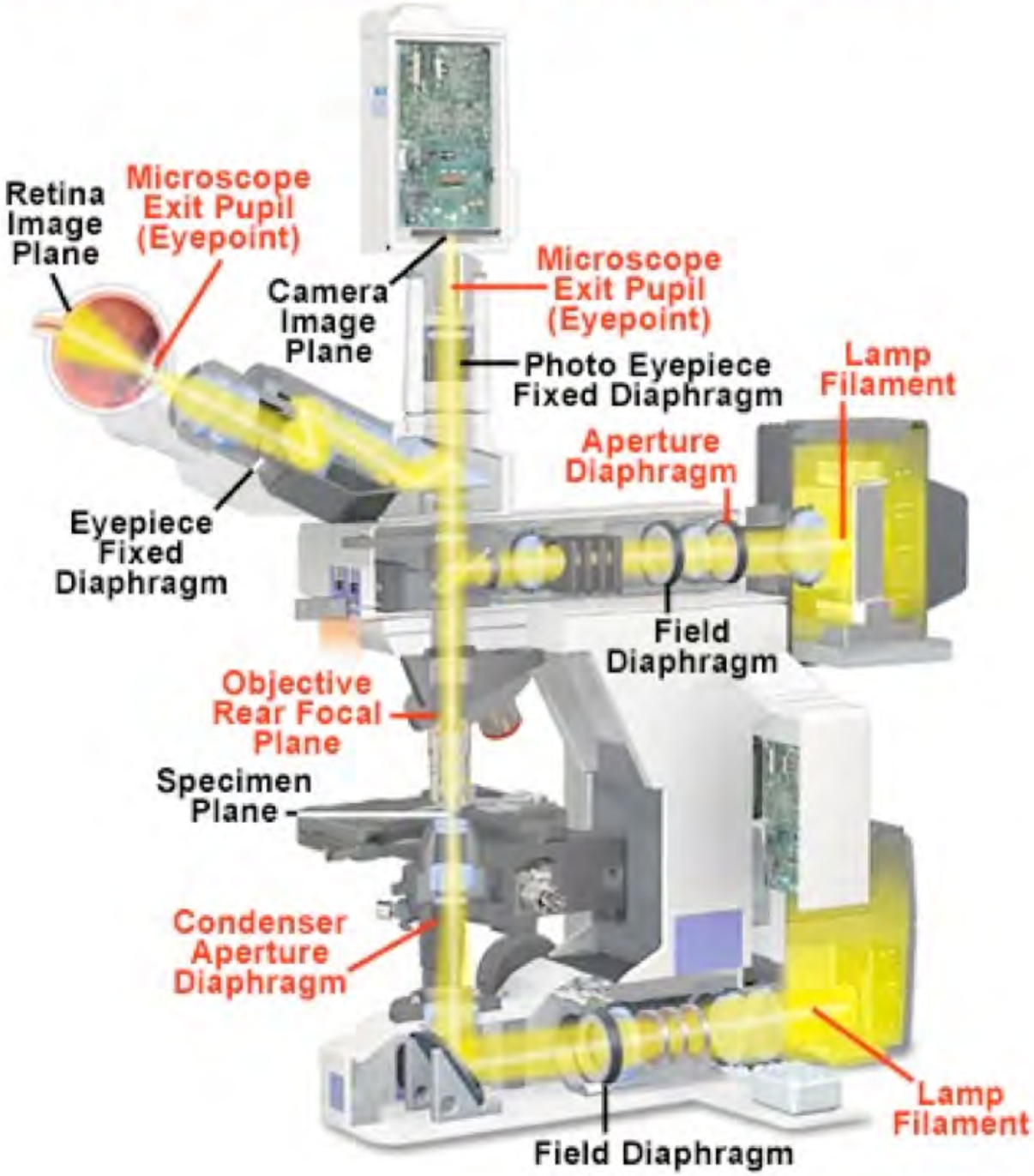
http://www.olympusmicro.com/

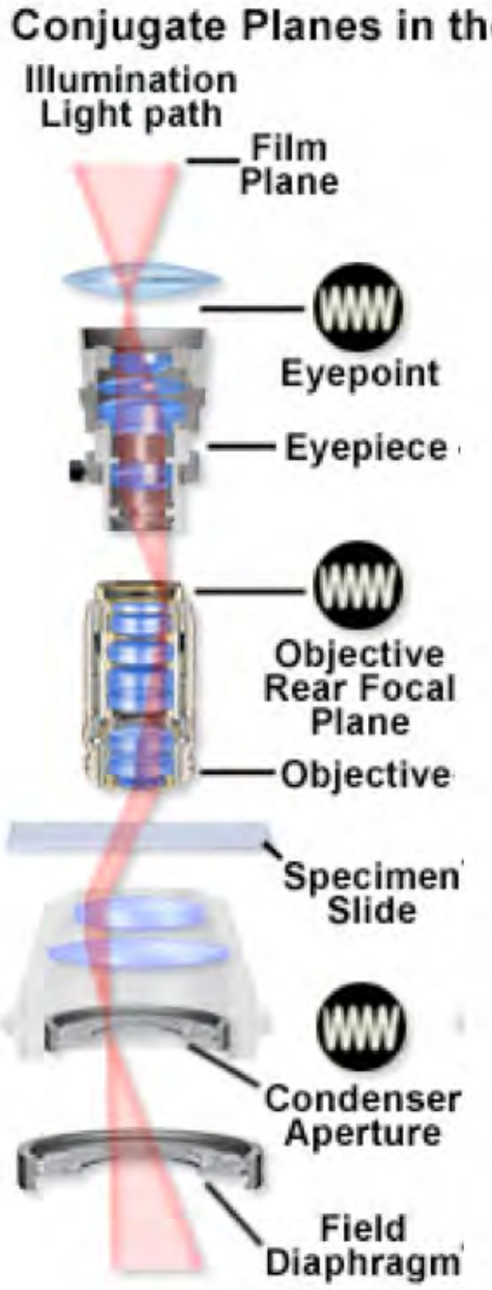
http://www.microscopyu.com/articles/formulas/formulasconjugate.html

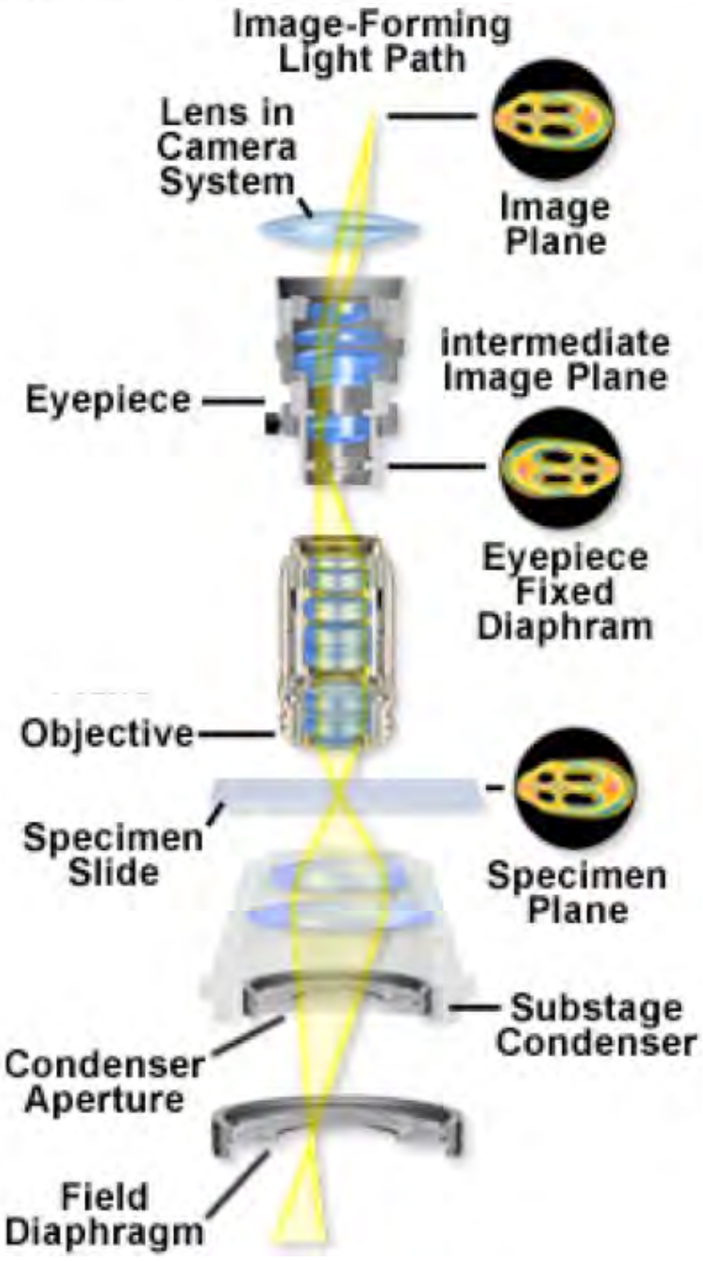
Koehler illumination and conjugate planes

Field or Image forming conjugate planes

Aperture or illuminating conjugate planes



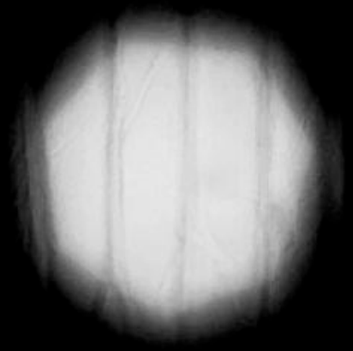




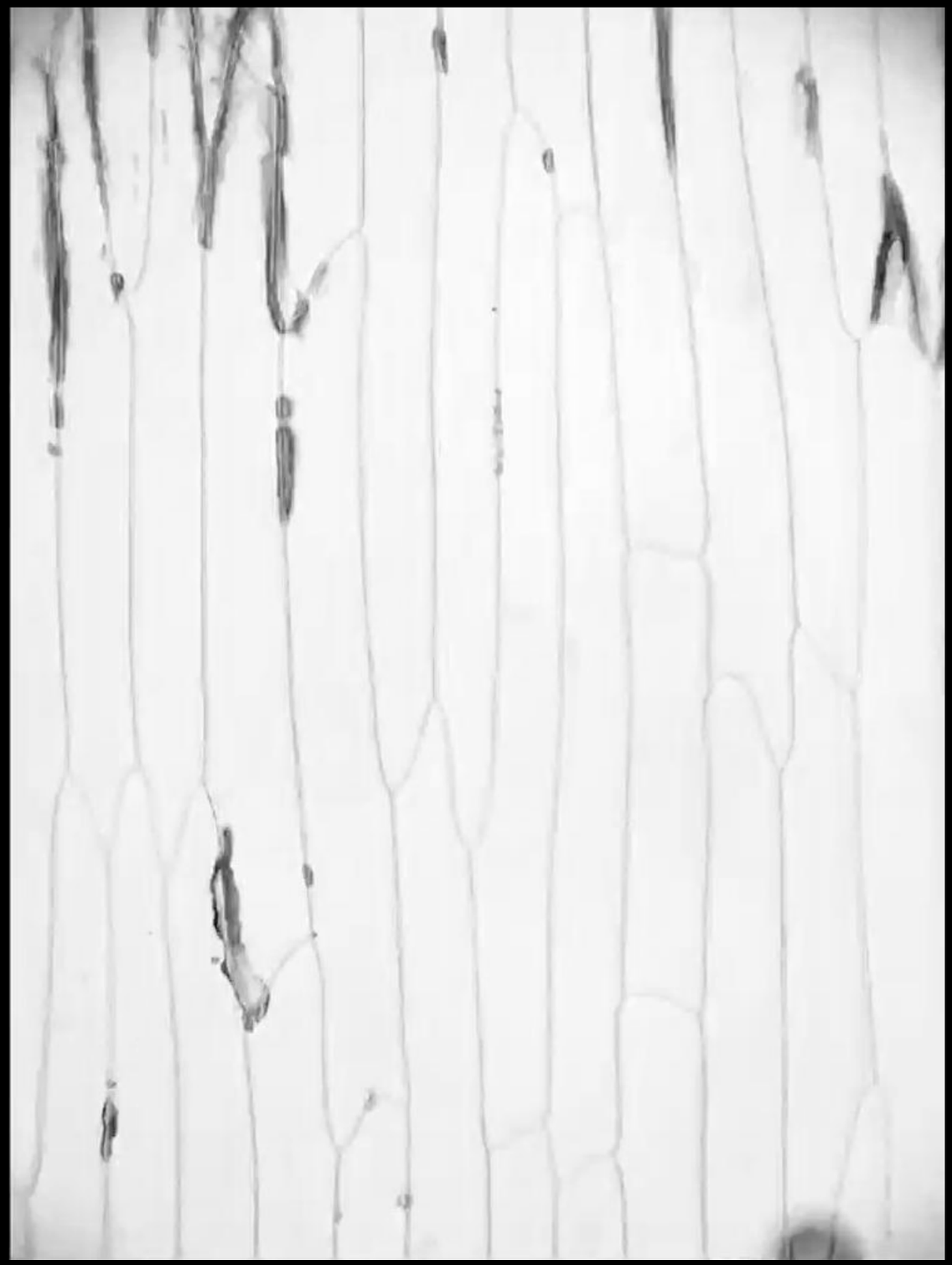
http://www.olympusmicro.com/

http://www.microscopyu.com/articles/formulas/formulasconjugate.html

Bright-field Koehler setup (field diaphragm)



Bright-field Koehler setup - aperture diaphragm



Fluorescence Contrast Techniques

.....use selective fluorescent probes to label features

GFP
green
fluorescent
protein

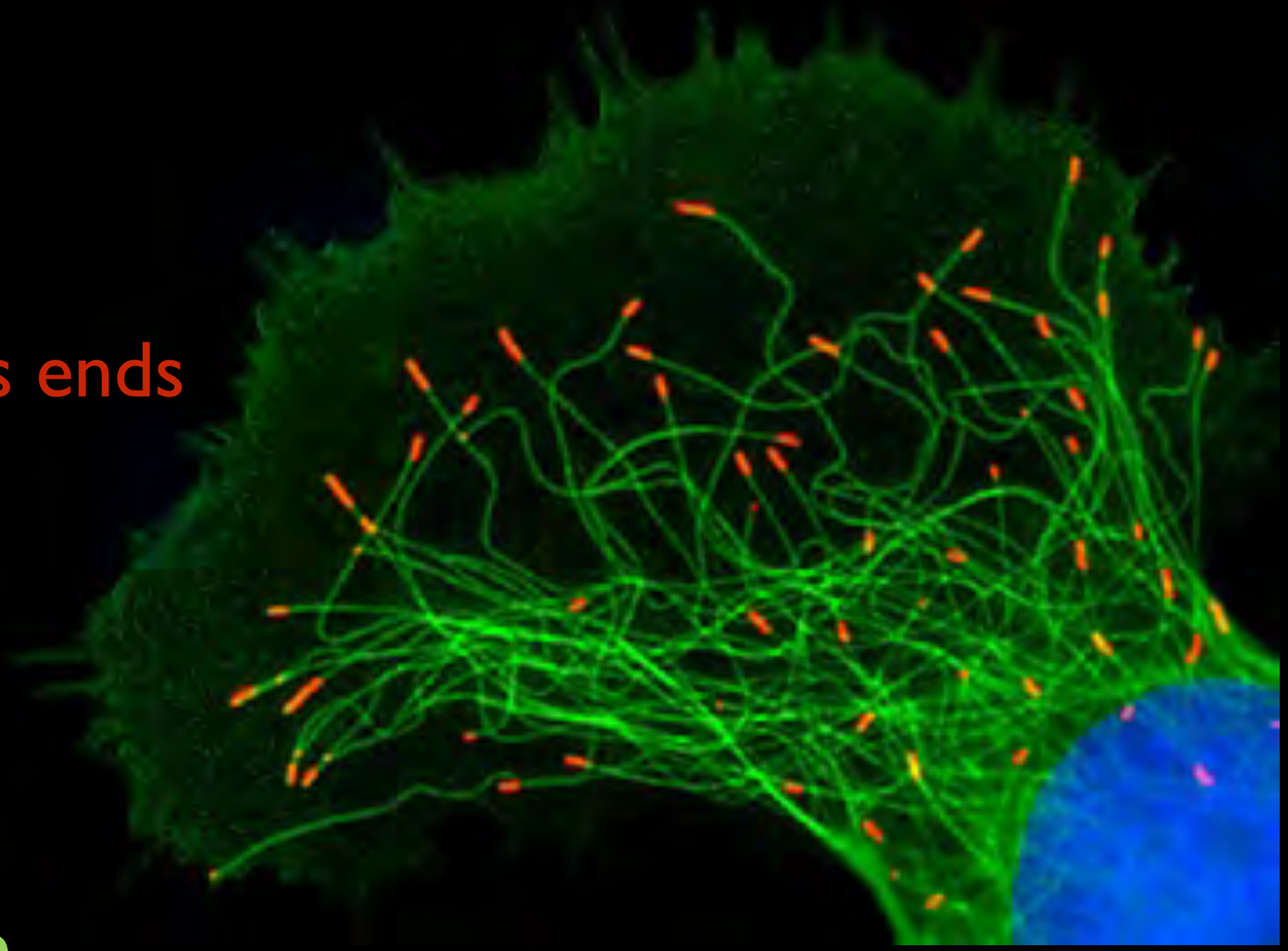
Fluorescence Contrast Techniques

.....use selective fluorescent probes to label features

Microtubules

Microtubule Plus ends

Nucleus



***LECTURES 4, 6-9**

Dr Alexis J. Lomakin www.olympusbioscapes.com/gallery/2011/hm29.html

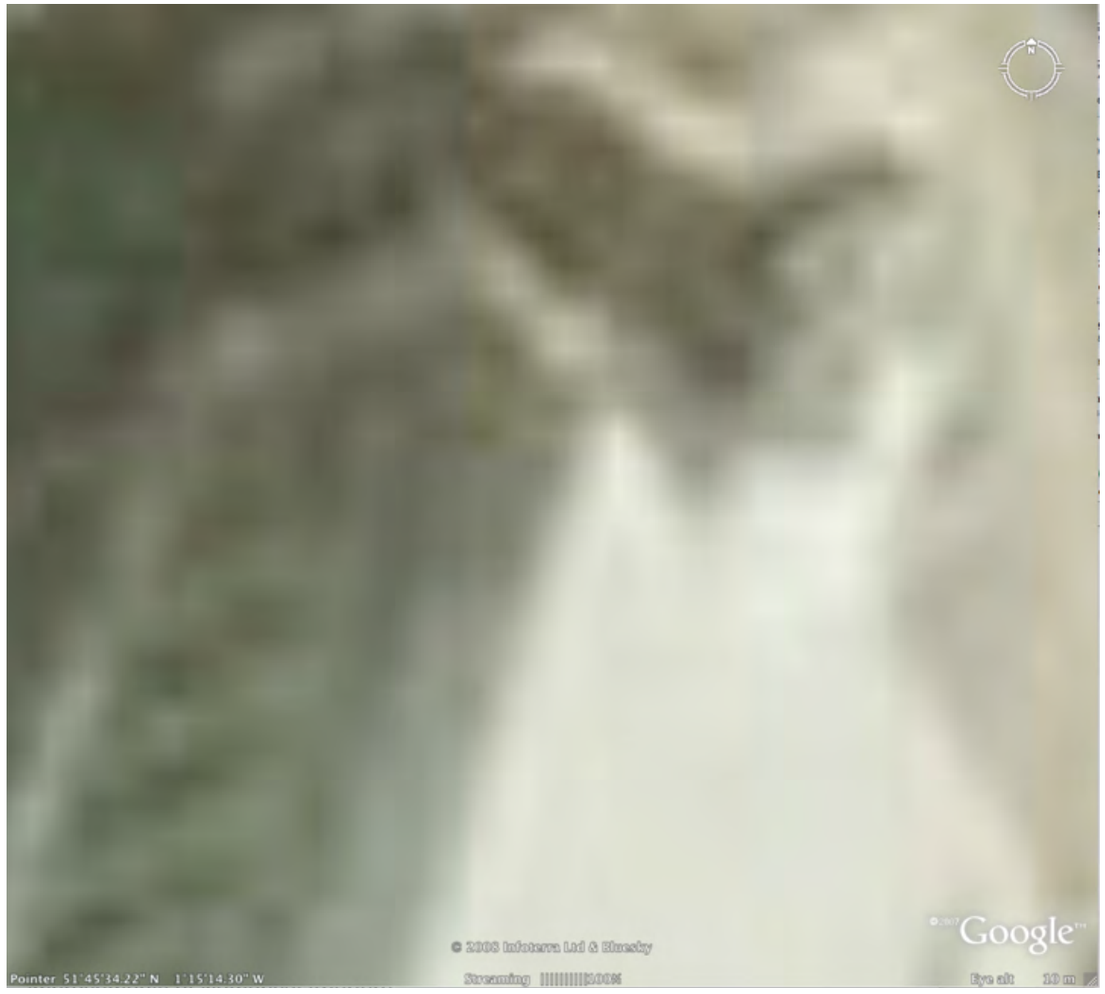
What is really important in microscopy?

1. Contrast
2. Resolution

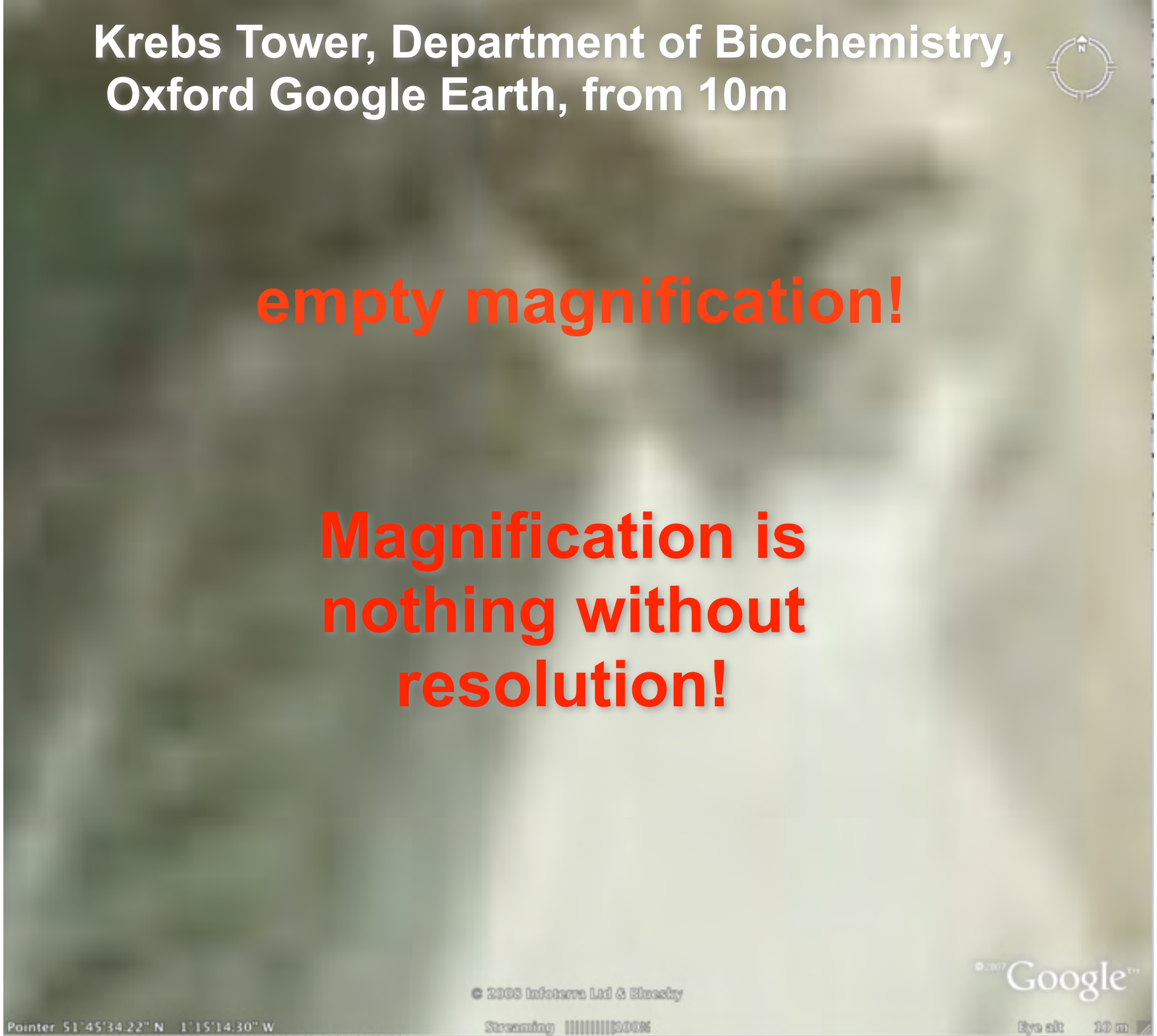
What is really important in microscopy?

Resolution .....the ability to see small stuff

Magnifying is not enough:

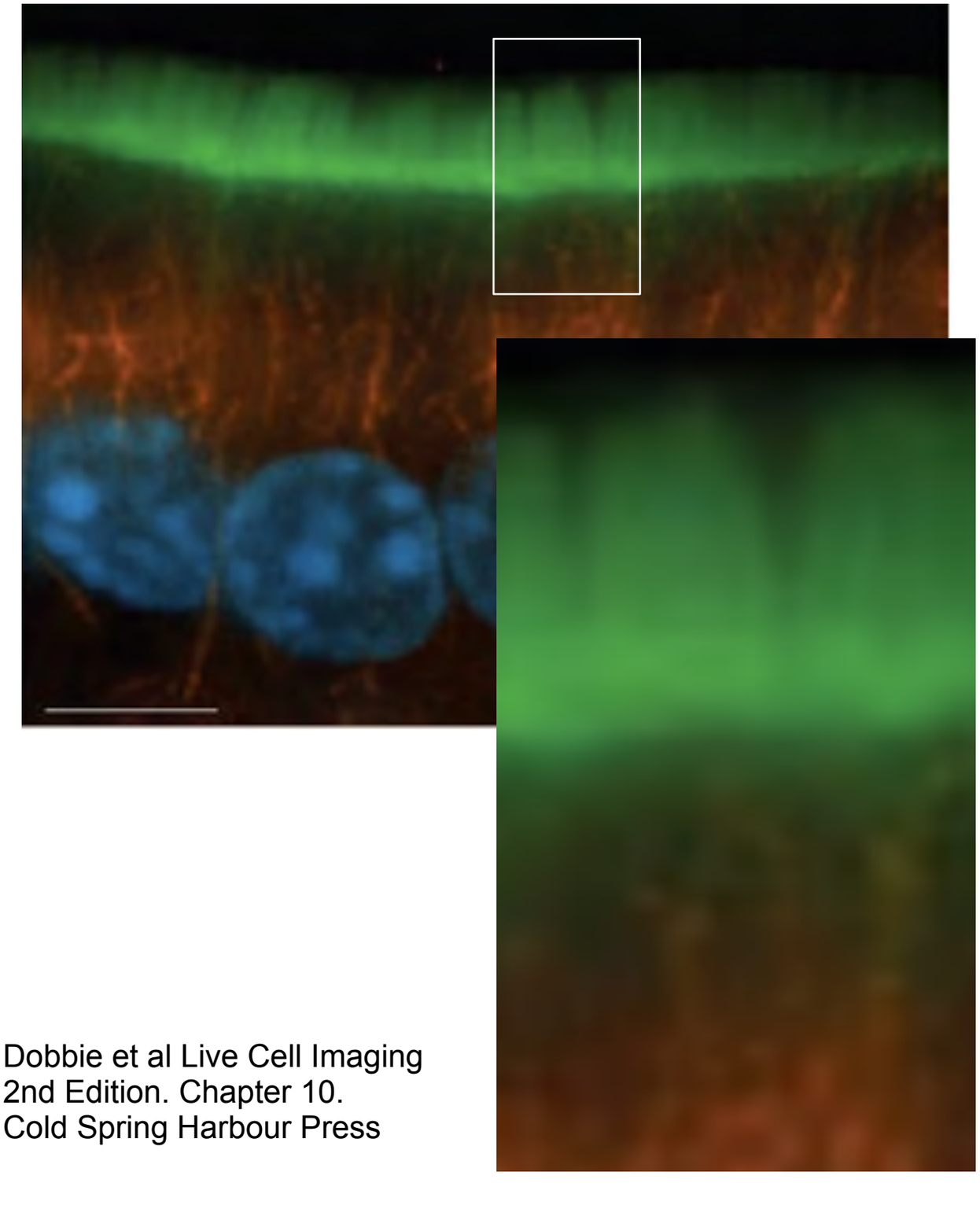


.....resolution is limited

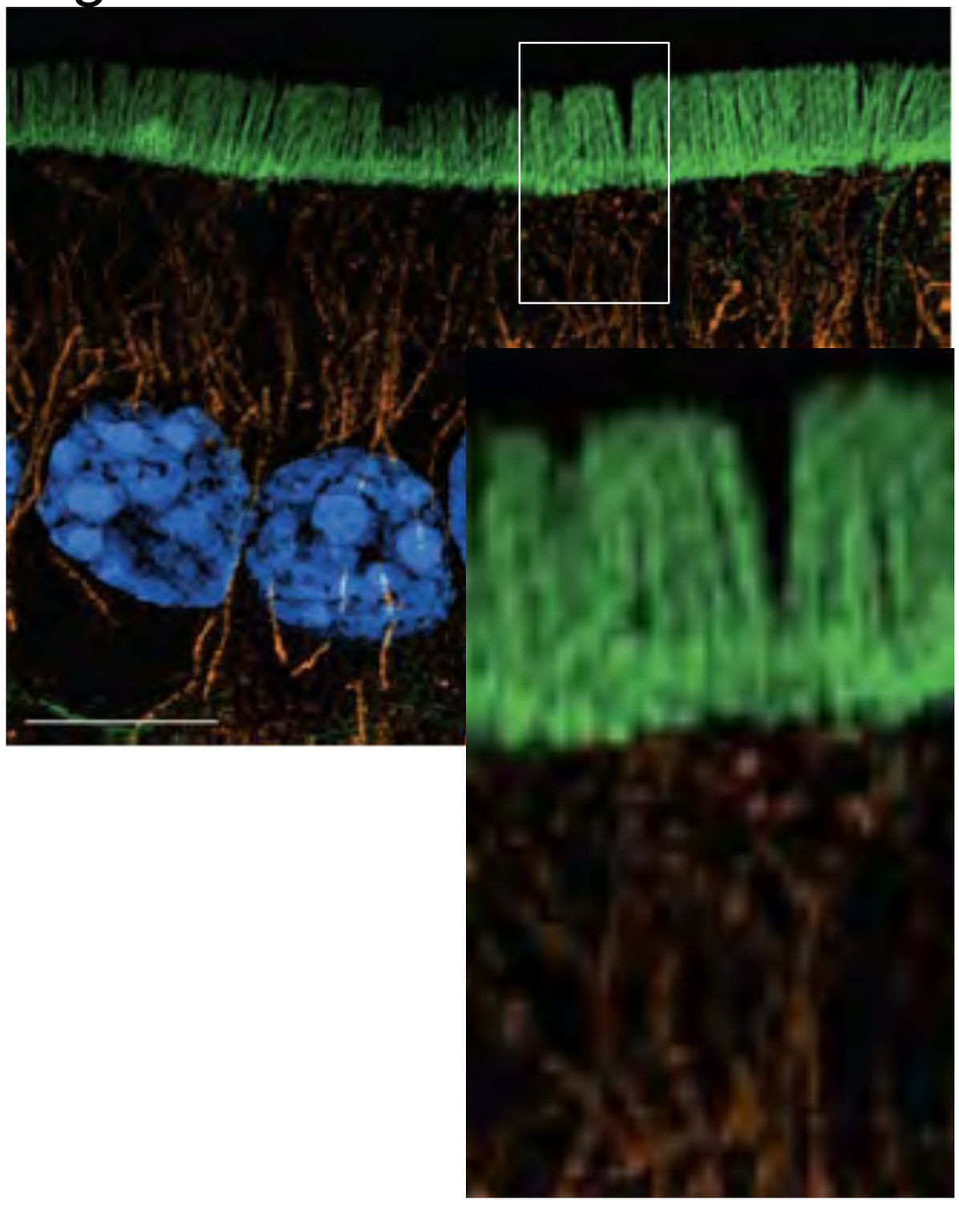

Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 10m
empty magnification!
Magnification is nothing without resolution!
© 2008 Infoterra Ltd & Bluesky
Google
Pointer 51°45'34.22" N 1°15'14.30" W
Eye alt 10 m

RESOLUTION

Normal resolution



High resolution



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

Understanding what limits Resolution

RESOLUTION *LECTURE 2*

* Convolution and the Point Spread Function

* The Rayleigh Criterion (D)

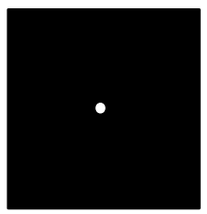
Convolution and the Point Spread Function

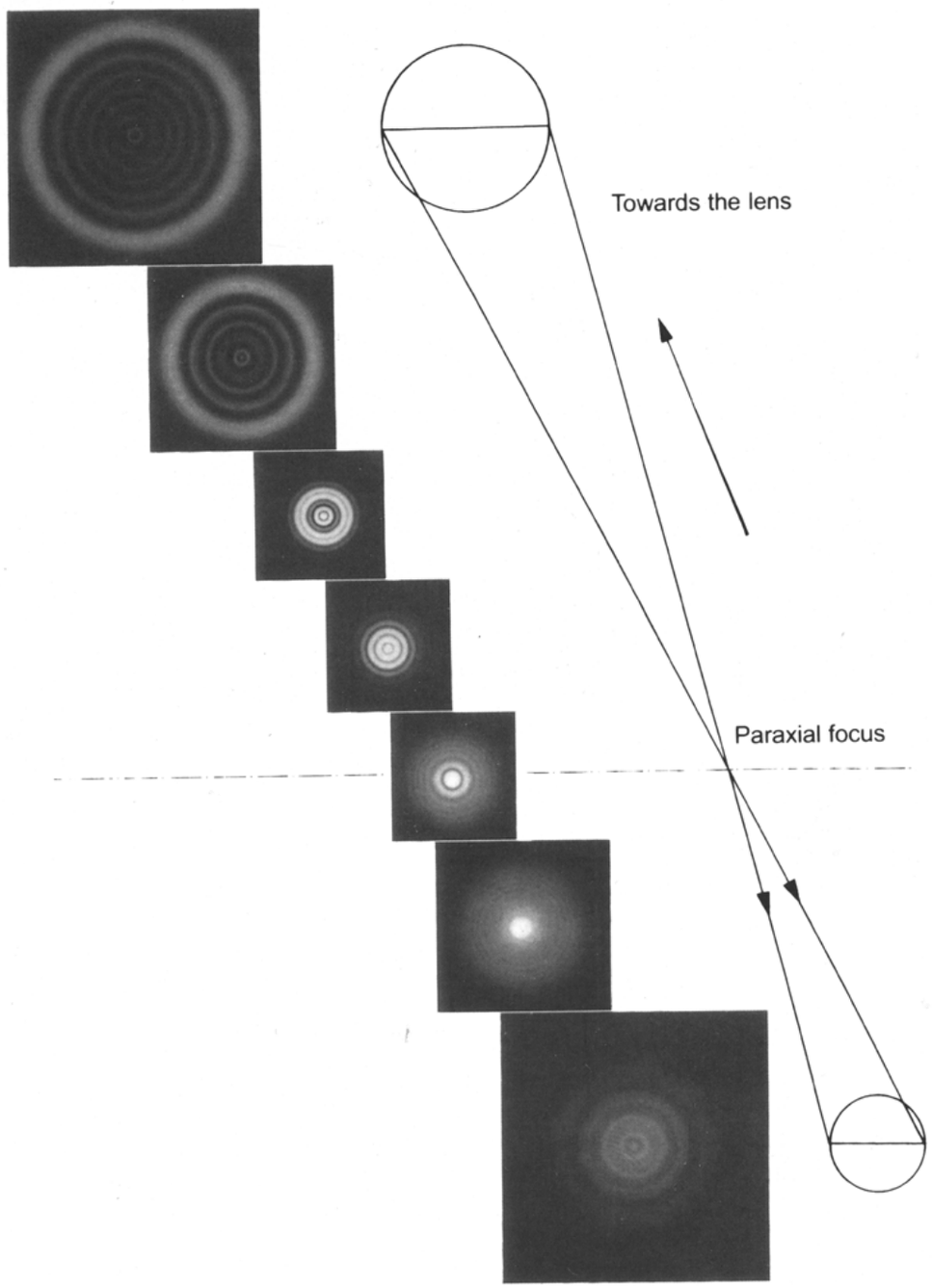
An image represents the output of the optics and detector of the imaging system

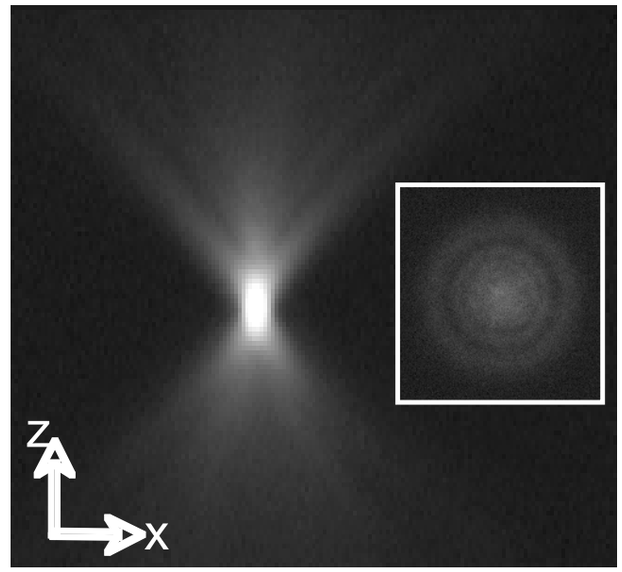
image ≠ object

image = object ⊗ PSF

Sample object: a "sub-resolution" fluorescent bead







Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

- "convolution" by the microscope optics = the PSF

Preparing a bead slide:

* Self Taught Practical 2 *

Brand

- Constellation™ (1)
- FocalCheck™ (3)
- MultiSpeck™ (1)
- PS-Speck™ (1)
- TetraSpeck™ (7)

Color

- Assorted Colors (4)
- Blue (9)
- Dark Red (7)
- Deep Red (1)
- Green (9)
- Orange (8)
- Red (1)

Diameter

- 0.1 µm (3)
- 0.2 µm (2)
- 0.5 µm (3)
- 1 µm (2)
- 4 µm (4)
- 6 µm (1)

Product Name	SKU #	Calibration Type	Product Size	List Price GBP
Constellation™ Microspheres for Imaging, mixture of assorted sizes & colors	C-14837	Fluorescence Microscope Calibration	3 mL	68.50
FocalCheck™ Fluorescence Microscope Test Slide #1, for alignment, intensity, & calibration	F36909	Confocal Microscope Calibration	1 each	200.00
FocalCheck™ Fluorescence Microscope Test Slide #2, for spectral imaging systems	F36913	Confocal Microscope Calibration	1 each	206.00
FocalCheck™ Fluorescence Microscope Test Slide #3, 5 colors, high & low intensities	F36914	Confocal Microscope Calibration	1 each	191.00
MultiSpeck™ Multispectral Fluorescence Microscopy Standards Kit (in suspension)	M-7901	Fluorescence Microscope Calibration	1 kit	216.00
PS-Speck™ Microscope Point Source Kit (blue, green, orange & deep-red fluorescent beads)	P-7220	Fluorescence Microscope Calibration	1 kit	168.00
TetraSpeck™ Fluorescent Microspheres Sampler Kit	T-7284	Confocal Microscope Calibration	1 kit	199.00
TetraSpeck™ Fluorescent Microspheres Size Kit (mounted on slide)	T14792	Confocal Microscope Calibration	1 kit	252.00
TetraSpeck™ Microspheres, 0.1 µm, fluorescent blue/green/orange/dark red	T-7279	Confocal Microscope Calibration	0.5 mL	205.00
TetraSpeck™ Microspheres, 0.2 µm, fluorescent blue/green/orange/dark red	T-7280	Confocal Microscope Calibration	0.5 mL	205.00
TetraSpeck™ Microspheres, 0.5 µm, fluorescent blue/green/orange/dark red	T-7281	Confocal Microscope Calibration	0.5 mL	205.00
TetraSpeck™ Microspheres, 1.0 µm, fluorescent blue/green/orange/dark red	T-7282	Confocal Microscope Calibration	0.5 mL	205.00
TetraSpeck™ Microspheres, 4.0 µm, fluorescent blue/green/orange/dark red	T-7283	Confocal Microscope Calibration	0.5 mL	195.00

http://www.lifetechnologies.com/uk/en/home/references/molecular-probes-the-handbook/tools-for-fluorescence-applications-including-reference-standards-and-optical-filters/fluorescence-microscopy-reference-standards-and-antifade-reagents.html#head3

Self Taught Practical Exercises - 2 Bright field

* Familiarise yourself with the components of the fluorescence path

* Be aware of correct alignment and use of different components

* Observe airy rings and point spread function

* How to leave the microscope for the next user

Preparing a bead slide:

* Self Taught Practical 2 *

For PSF slides:

1. Dilute fluorescent beads 1:10^3-10^7 in ddH_2O after thorough vortexing. They should be dilute enough that a single beads can be found without Airy discs from neighboring beads encroaching.

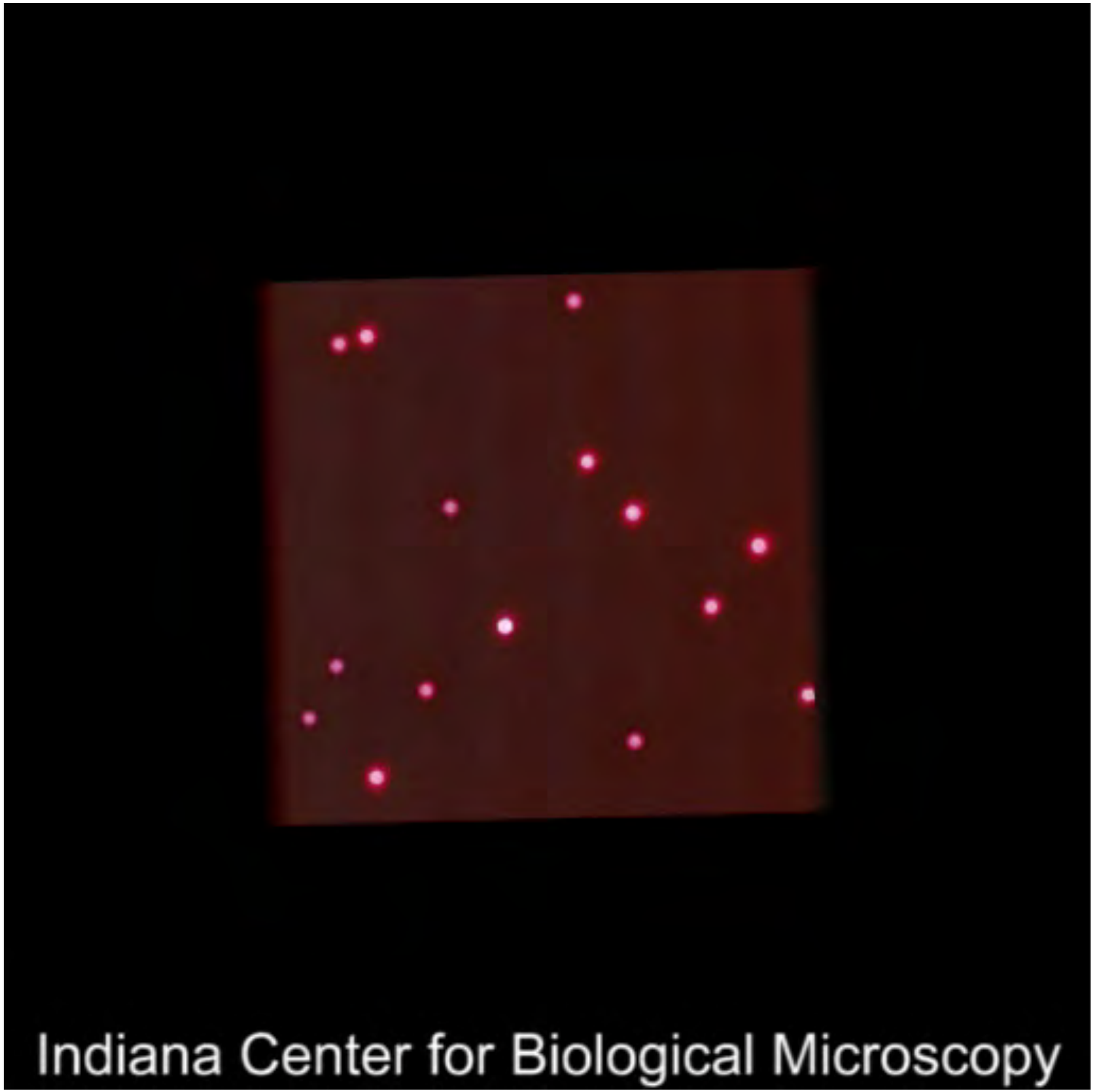
2. Apply 10-20 µl bead suspension to a clean coverslip (usually No 1; 22x22), spread with a pipette tip, and allow to air dry.

3. Apply 10-20µl of 90% glycerol solution to a pre-cleaned microscope slide, and mount the previously prepared coverslip by gently lowering it onto the glycerol drop to avoid air pockets.

4. While most calibrations will be performed using beads on the coverslip, if the user desires to image structures at >10µm depth into the sample, calibrations may be performed on beads several µm away from the coverslip. In this case, PSF beads may also be deposited on the slide itself, in parallel with depositing them on the coverslip, The distance between slide and coverslip may be adjusted by varying the amount of glycerol used to mount the coverslip.

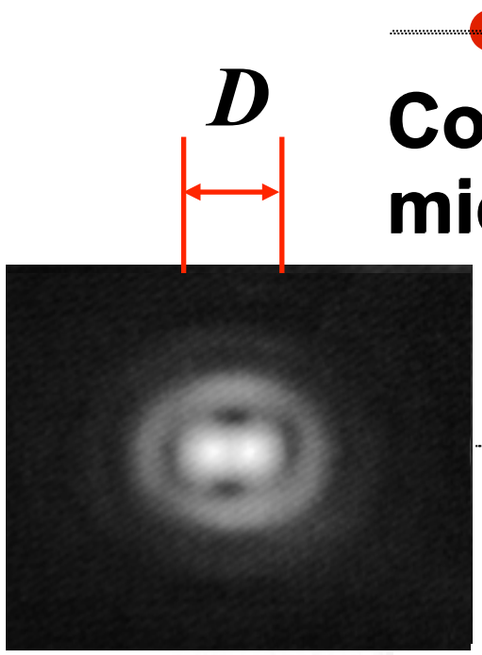
5. Calibration slides may be produced for various mounting media,: e.g. using Prolong Gold, Vectashield or Agar instead of glycerol.

6. Seal the coverslip with clear nail varnish, wait for it to dry and repeat. Store slides flat at 4 degrees.


Indiana Center for Biological Microscopy

Calculating Lateral Resolution: The Rayleigh Criterion

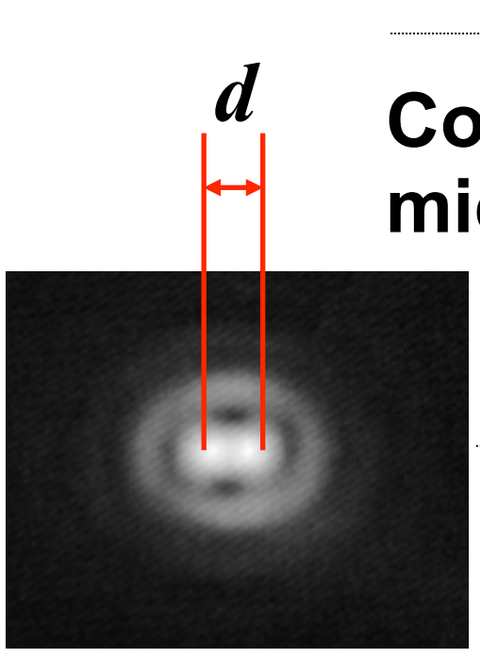
Two small objects



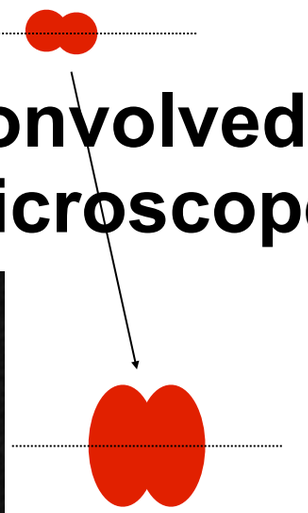
D = the distance between the two closest points that can still be distinguished

Calculating Lateral Resolution: The Rayleigh Criterion

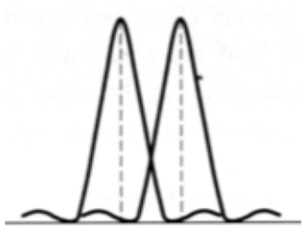
Two small objects



Convolved by microscope



NOT RESOLVED
Airy patterns must Overlap by less Than 42% of the Maximal intensities



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

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

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

Considering x100 objective, Na 1.4, fluorescence emission 520 nm:

$D = 1.22 \times 520_{nm} / 2 \times 1.4$

$D = 227\ nm$

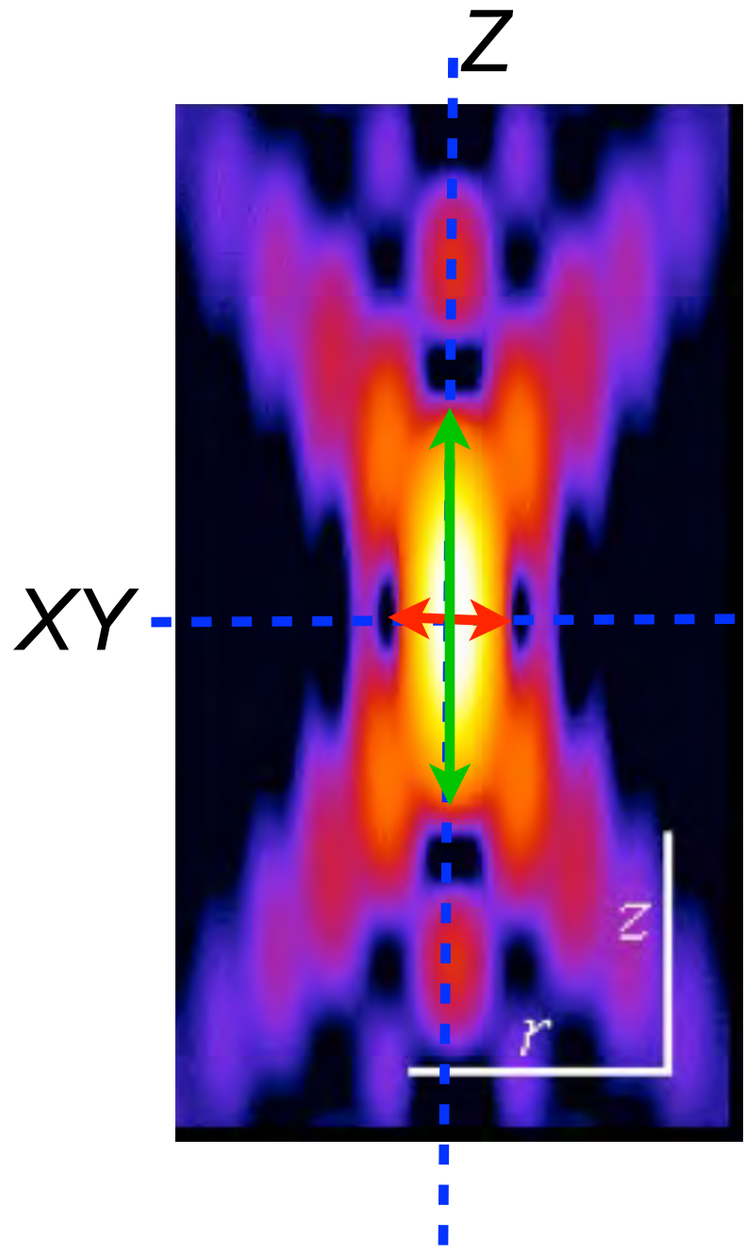
Limit of resolution $\approx \lambda_{em}/2$

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

LECTURES 11, 12

Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

LECTURES 12, 14-16

Which technique do I use?

Optimising your imaging

* Asking the right questions

* Picking the right technique

* Applying the technique well

* Analysing / interpreting the data properly

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

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
Dynamics

Be aware of the different techniques

VISIT A FACILITY

Their strengths
Their weaknesses
Their availability

Understand the limitations of your material

DISCUSS YOUR APPLICATION

Viability
Thickness
Brightness

Which technique do I use?

Bright field and fluorescence (contrast generation)

Live cell imaging
Fixed material imaging (sample prep)

Confocal techniques (scanning, optical sectioning)

Wide field techniques (Speed, sensitivity)

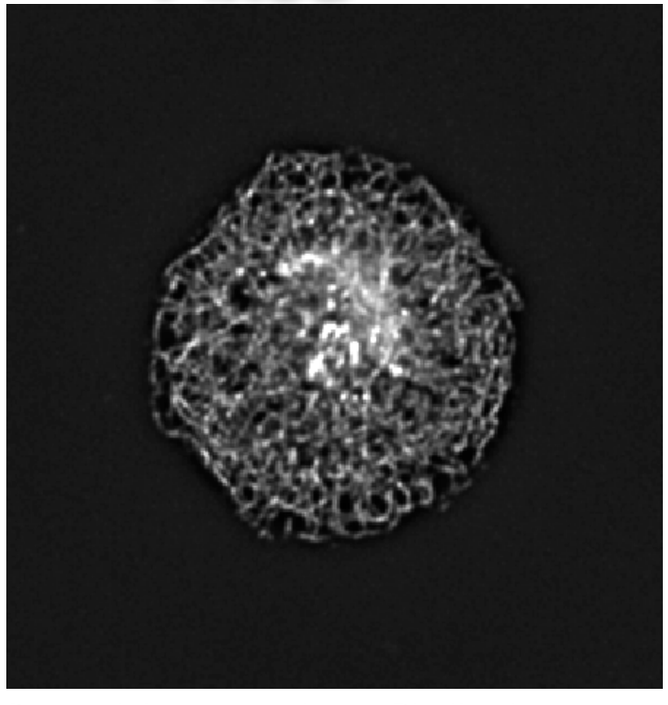
Super-resolution techniques (optical and post aquistion)

Techniques for molecular dynamics and interactions

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

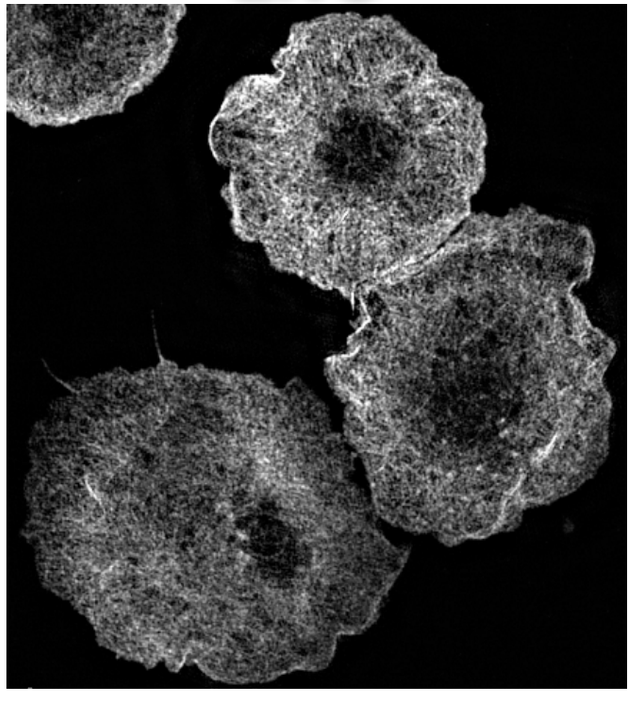
Which technique to use?

Fixed



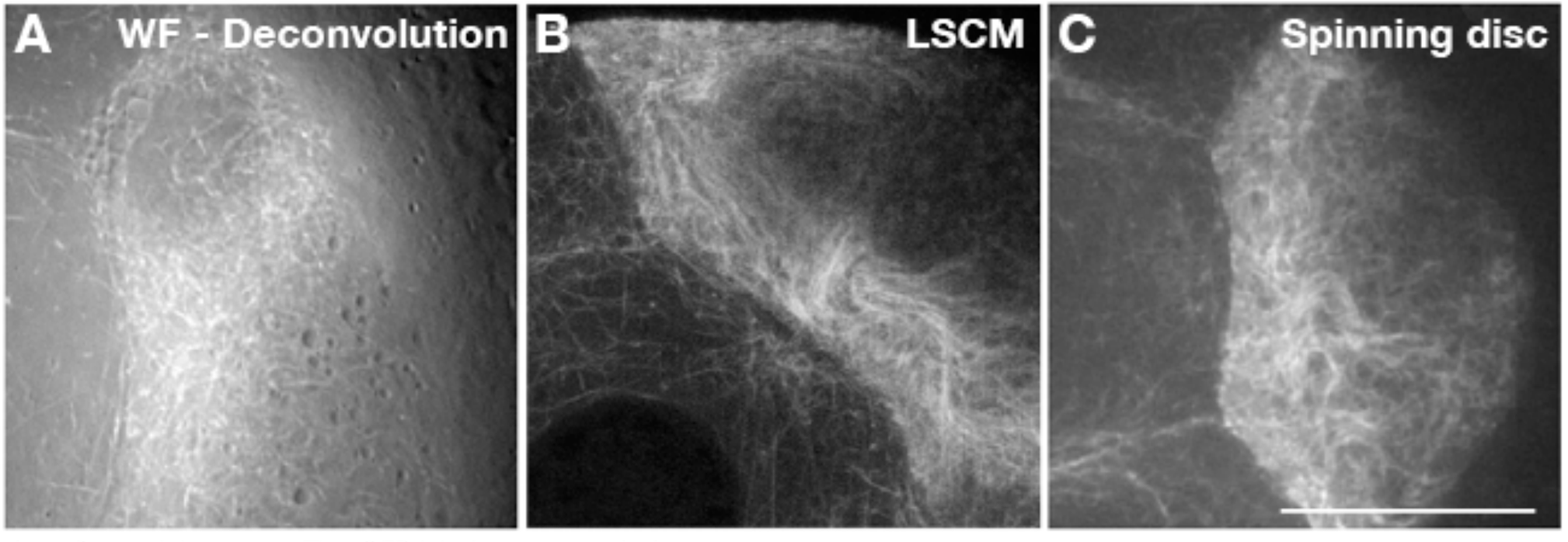
vs

Live



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

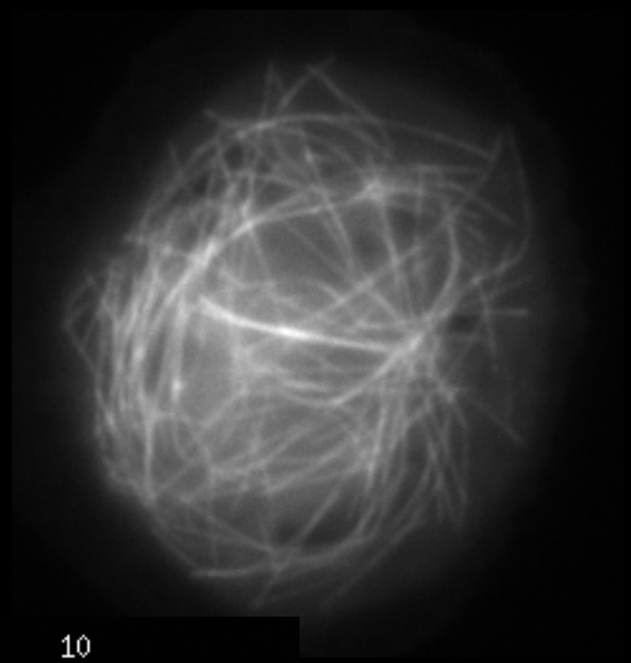
Wide field Decon vs Confocal



Live *Drosophila* oocyte: Tau-GFP labeling microtubules

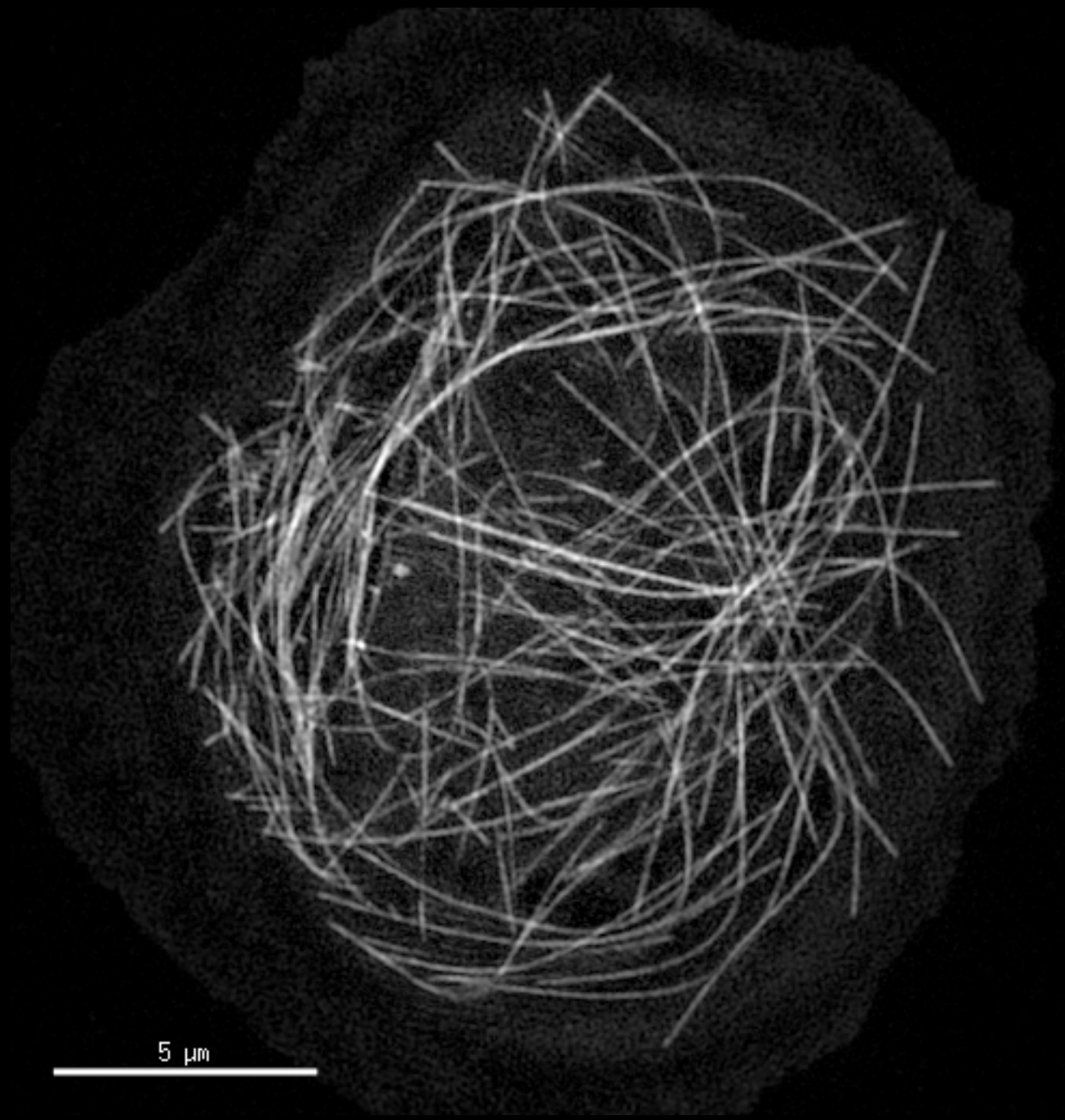
Live-cell super-resolution!

CONVENTIONAL



XY resolution ~250 nm

3D-SIM OMX-BLAZE



XY resolution ~130 nm

Jupiter-GFP tagged MT dynamics in a living Macrophage

Image Processing

LECTURES 5, 18, 19

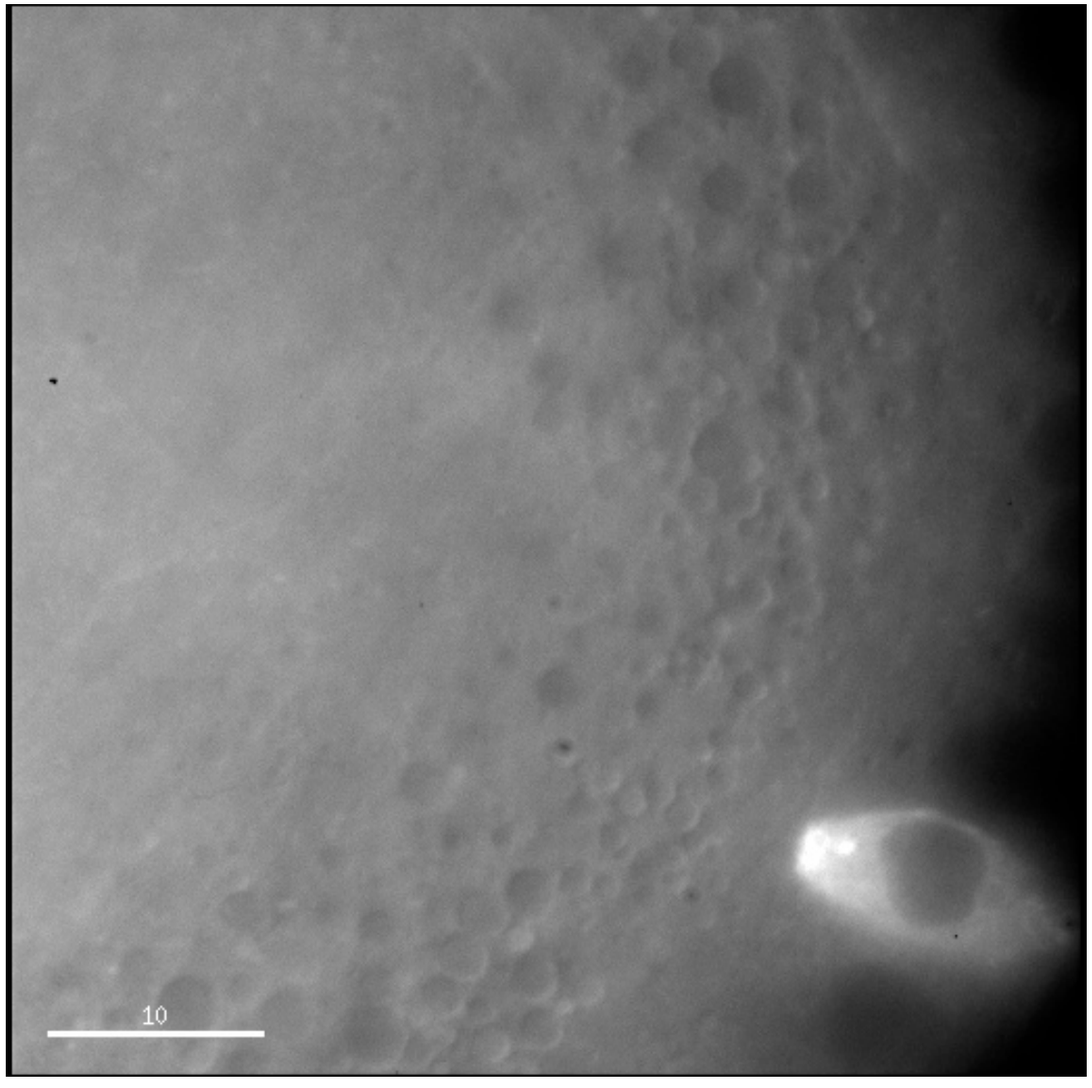
Image handling

Enhancement

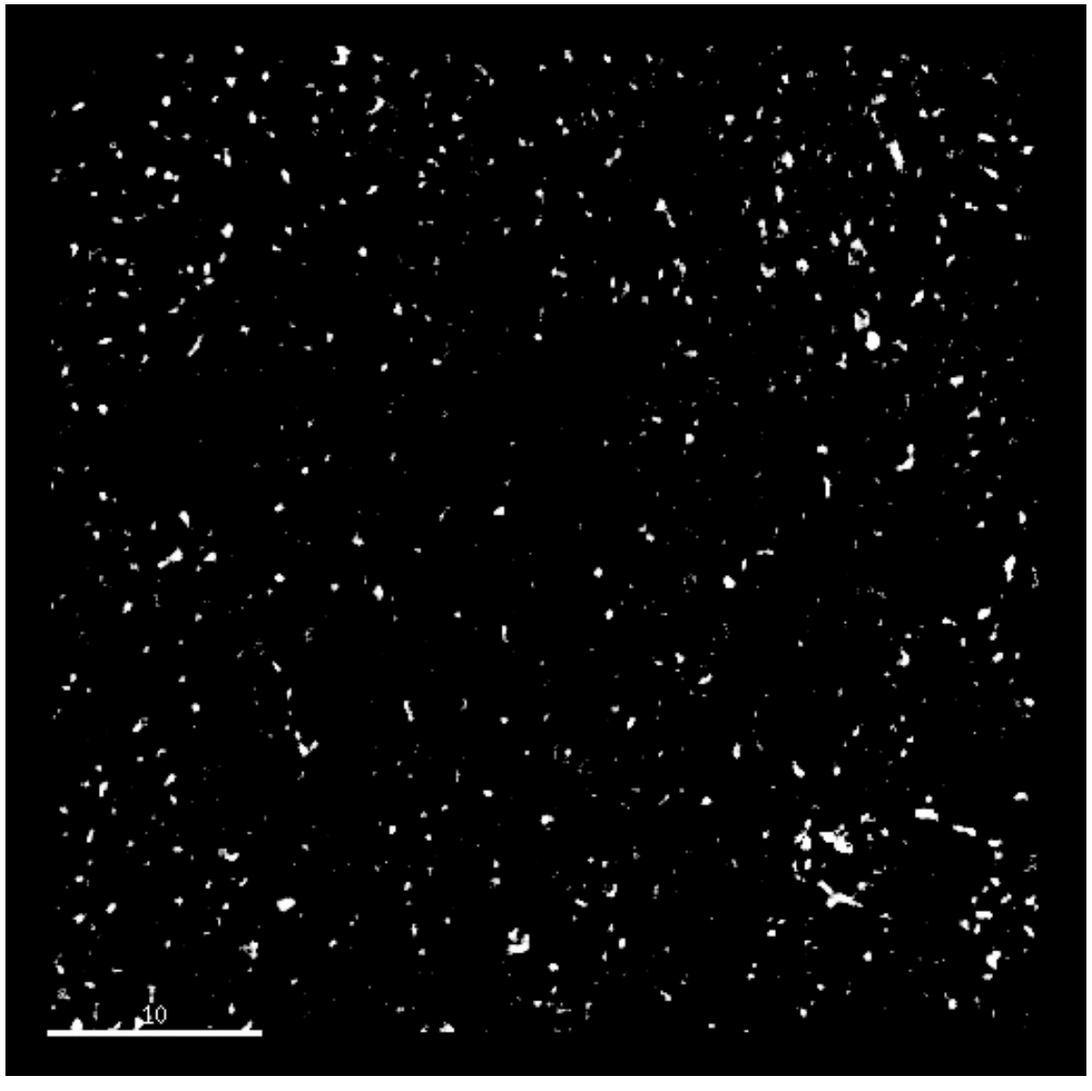
Analysis

Computational enhancement and processing

Raw - Widefield

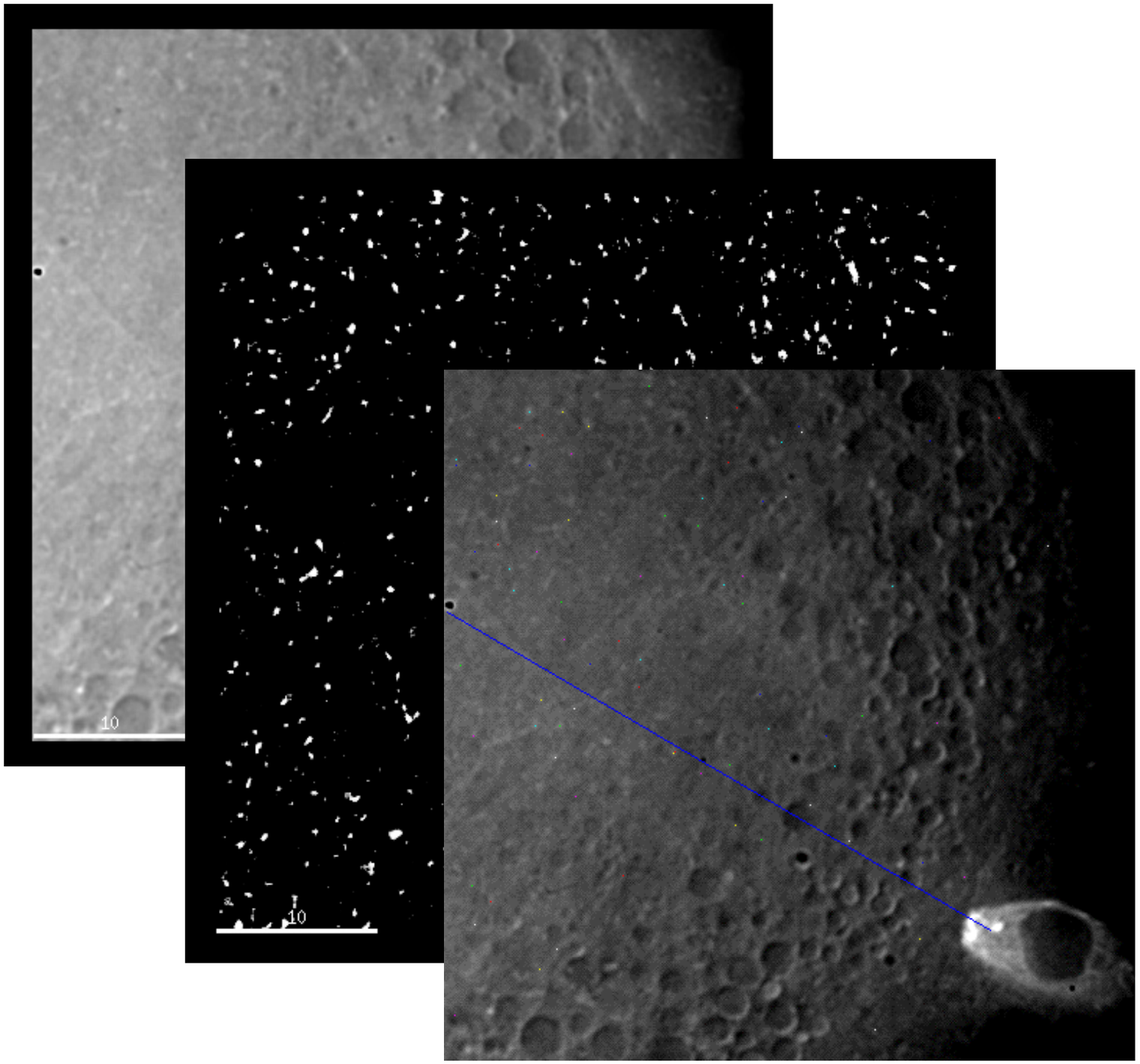


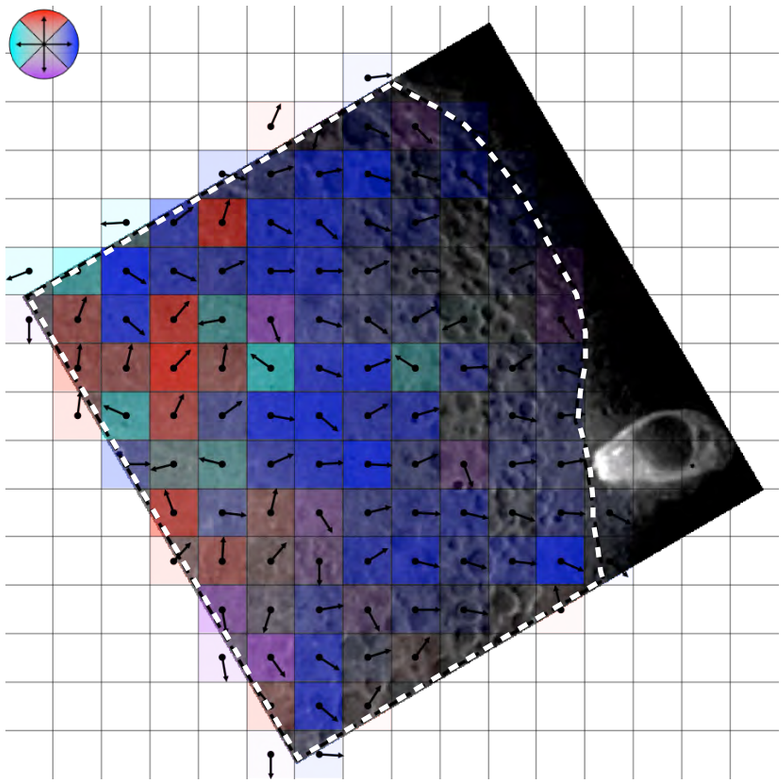
Processed

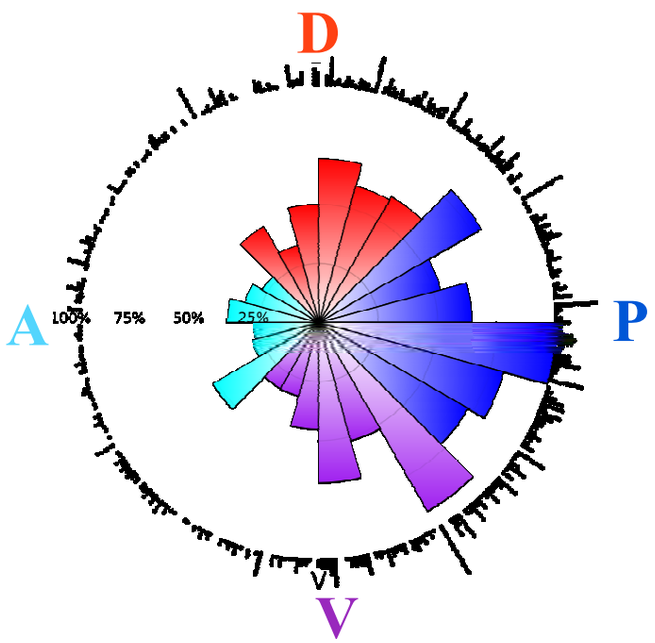


Parton, R. M., Hamilton, R. S., Ball, G., et al. (2011). JCB 194(1), 121–135. doi:10.1083/jcb.201103160

Live imaging - not just a pretty picture







LECTURE 17 - Dominic

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)

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

http://www.biology.uoc.gr/courses/BIOL493/documents/book.pdf

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

http://www.theguardian.com/science/2011/sep/11/genetically-modified-glowing-cats