Advanced Microscopy Course 2015
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

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

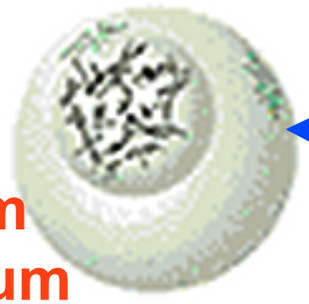
* Very sensitive, can follow distribution and interactions down to the molecular level

* Quantitative

* Allows experimental manipulation

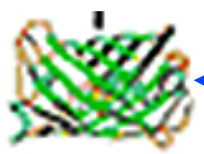
Useful size range for light microscopy

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

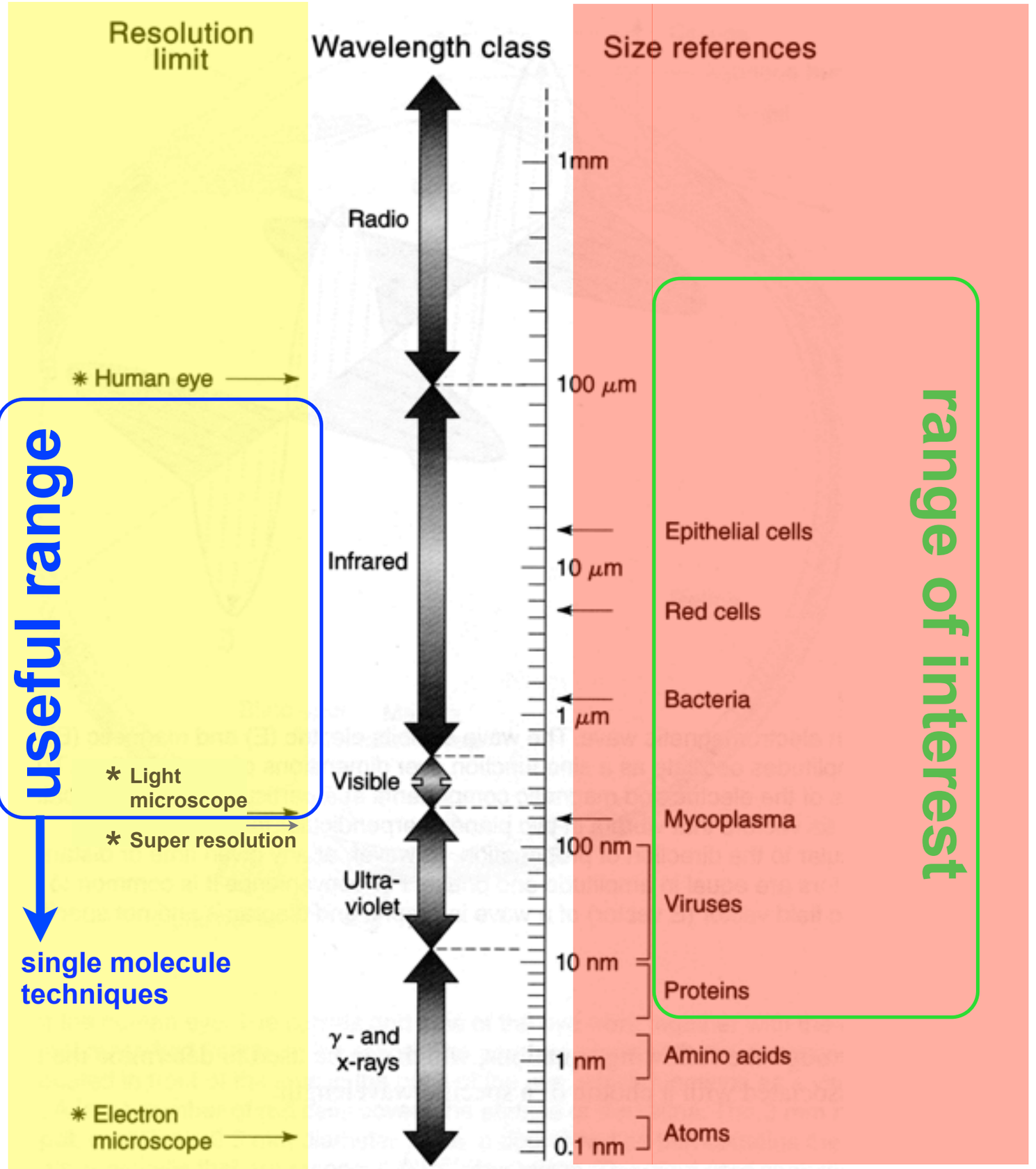
Bacterium = 1 um



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:

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!

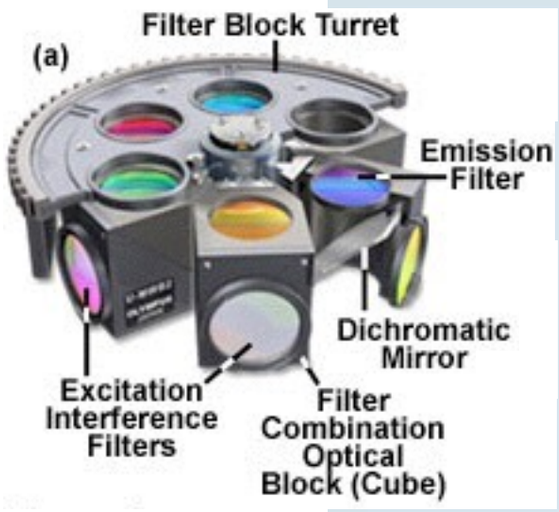
• If you understand the principles involved then it is easy to understand how to get the best from your microscope.

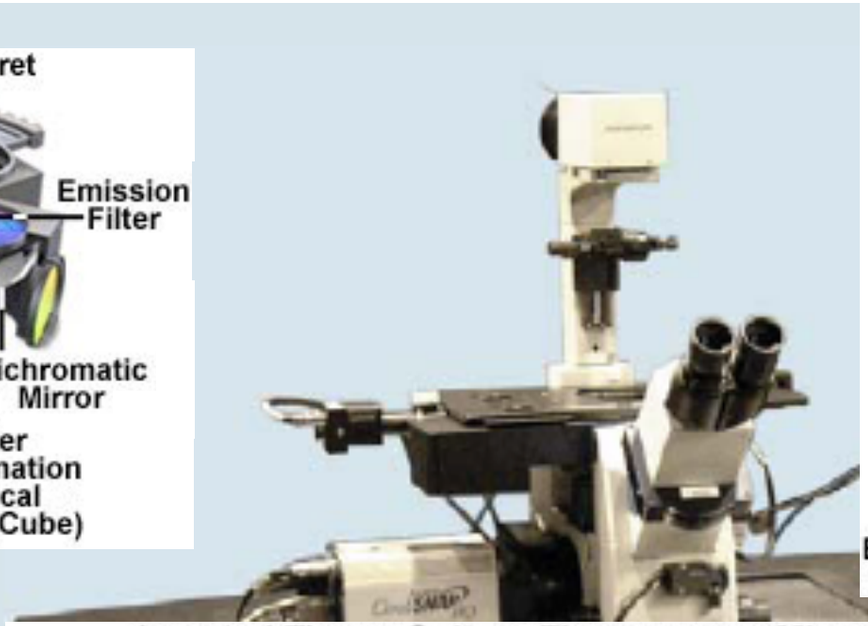
*** Microscopes are all basically the same**

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

Understanding what goes on in the Microscope

LECTURES 2-4 practicals 1 and 2





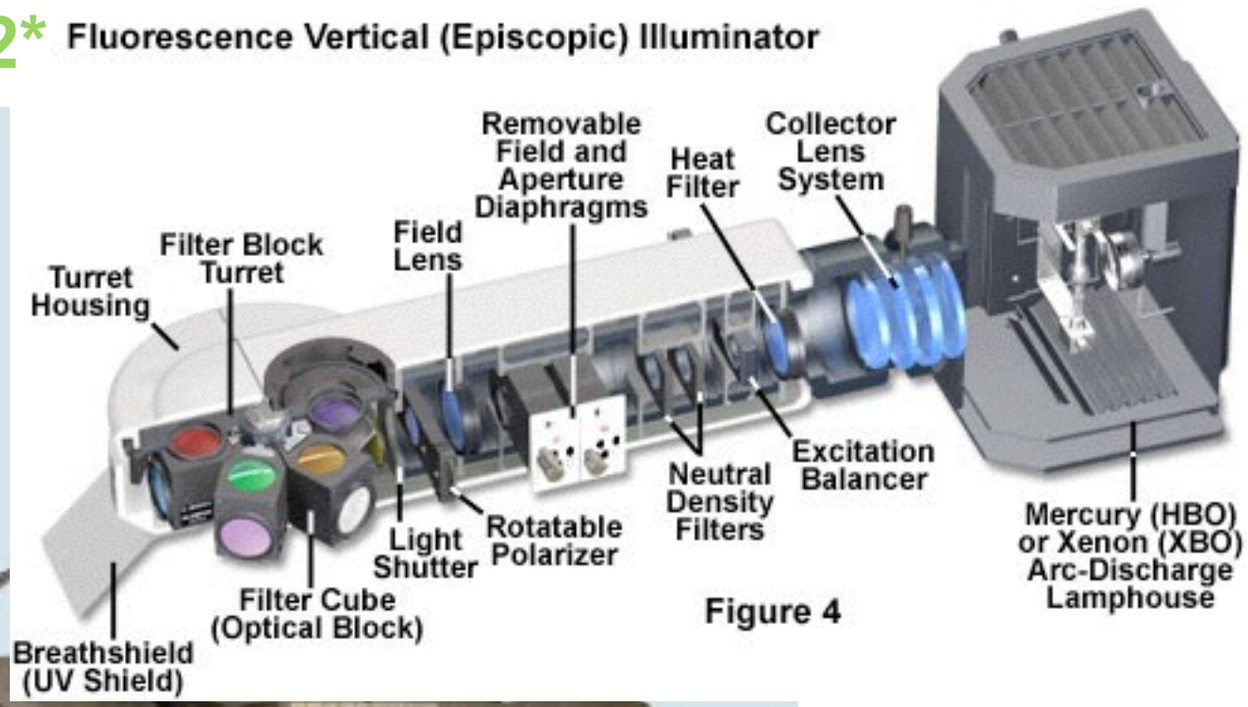


Figure 4

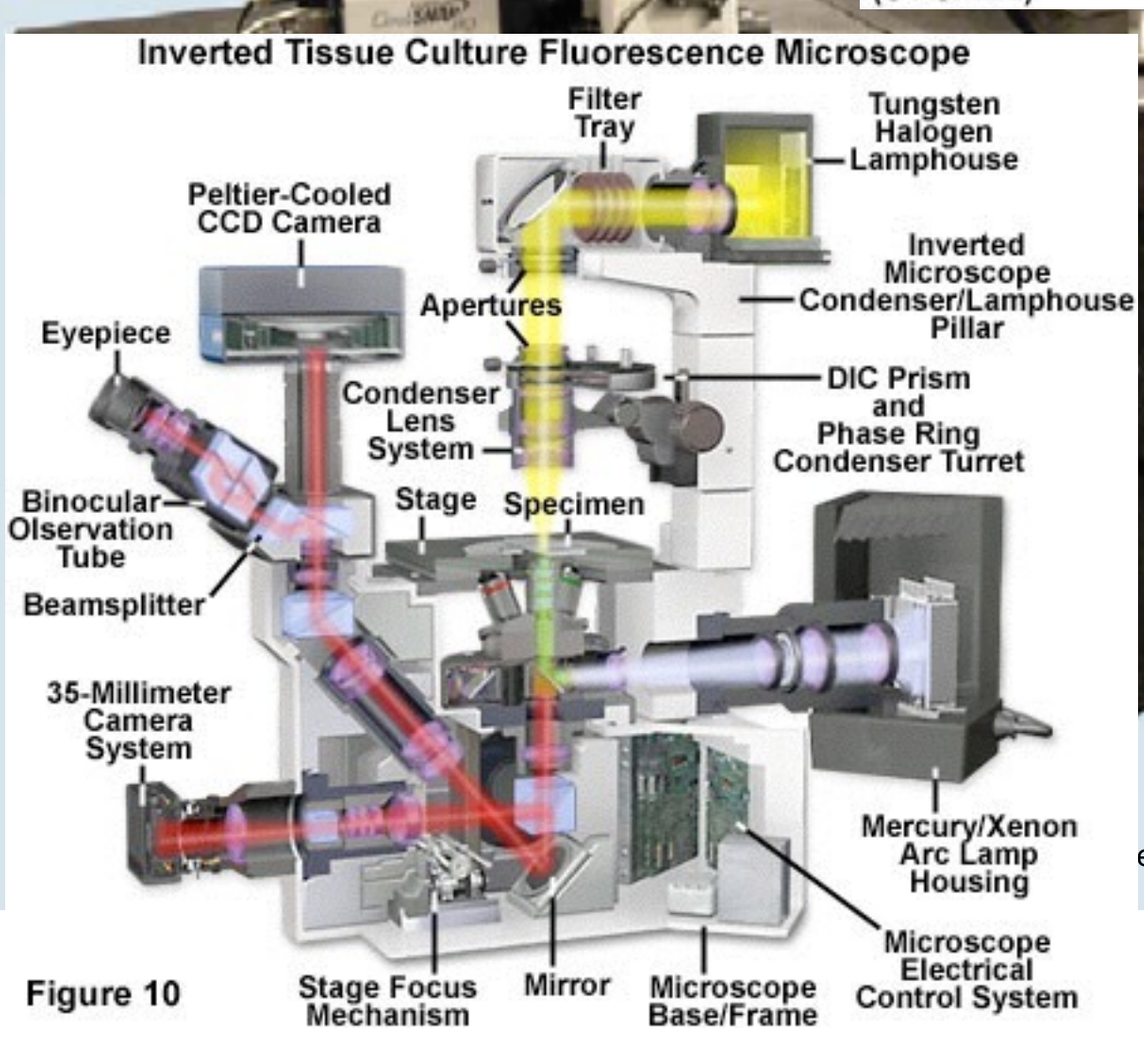


Figure 10

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

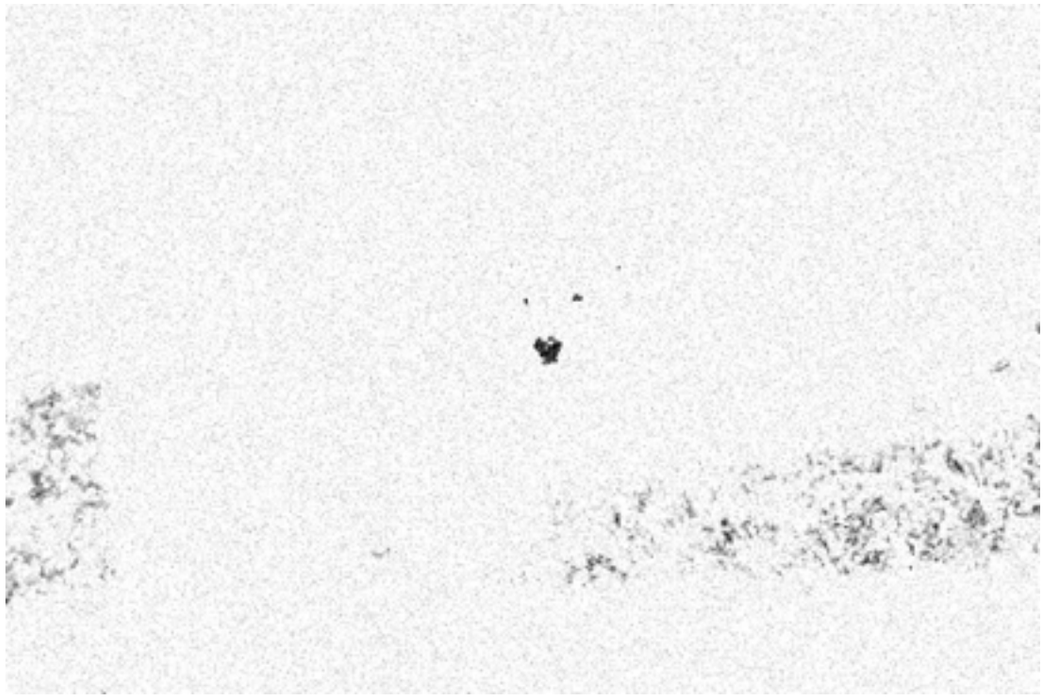
and nothing else!

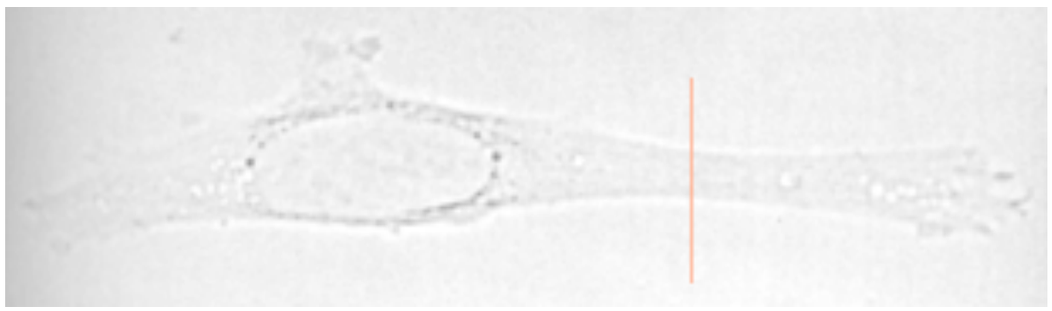
LECTURE 5

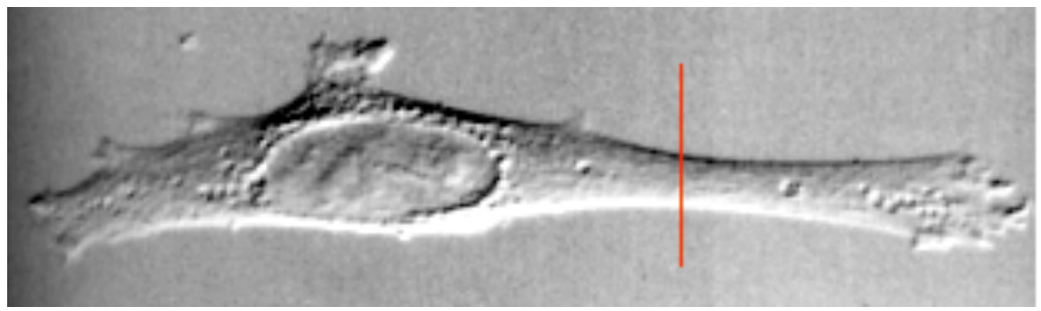
What is really important in microscopy?

Contrast .....the ability to distinguish stuff

Biological specimens have low inherent contrast:







.....can't resolve anything without contast

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

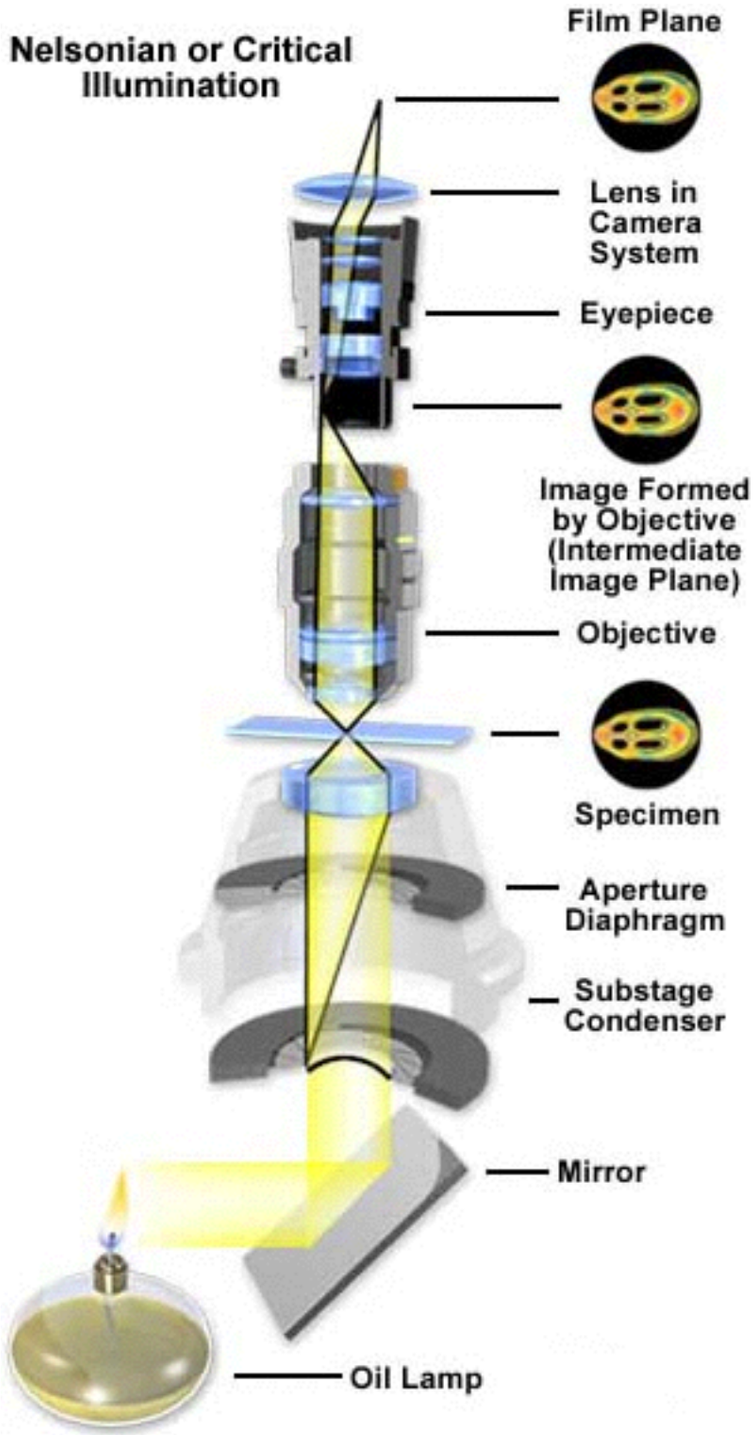
* Identifying the parts of you microscope, bright field

* Understanding the components: setting up Koehler illumination

* Adjusting the condenser and applying different contrast techniques

Illuminating the specimen: Critical vs Koehler

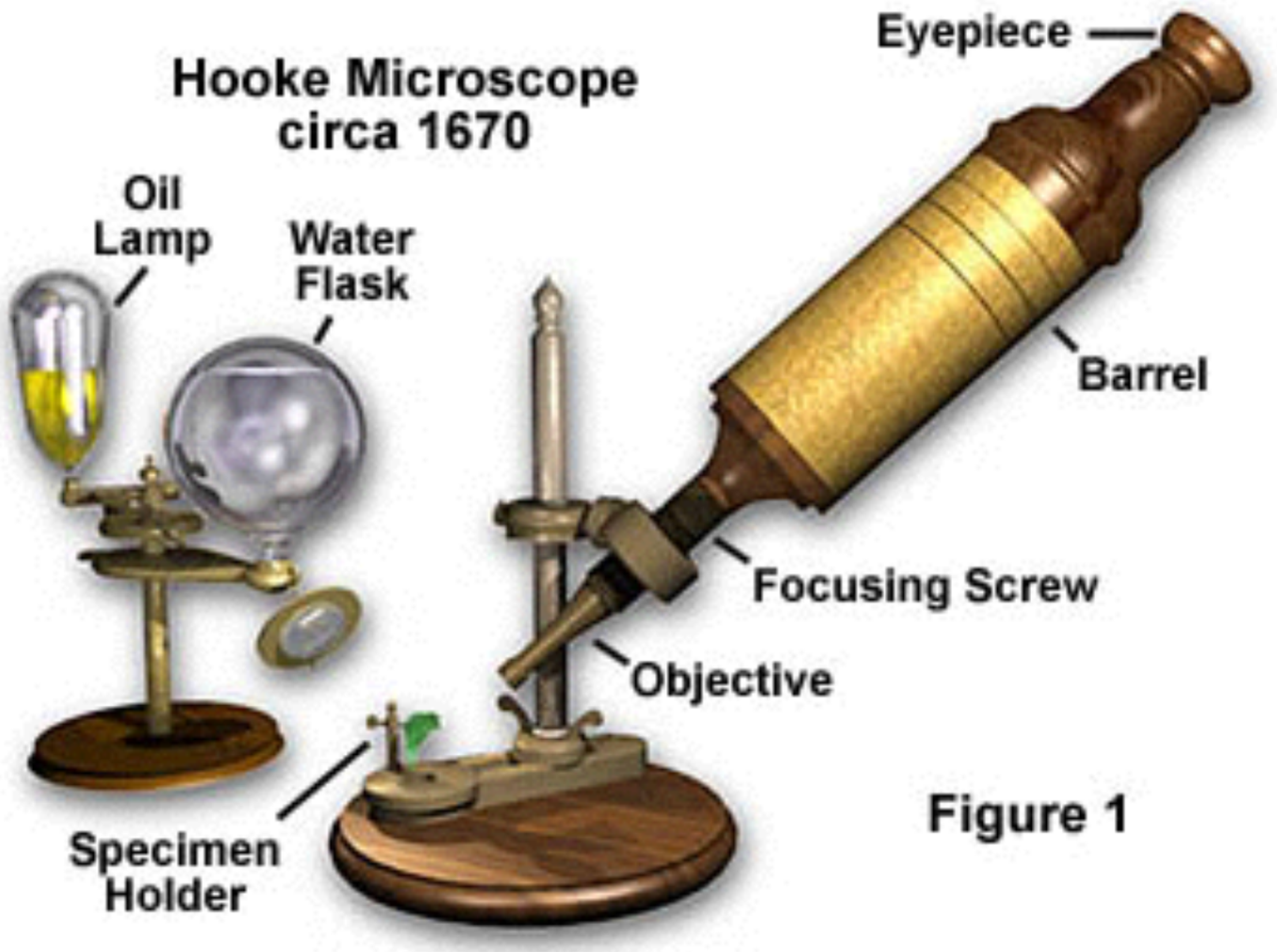
Critical = focusing an image of the illumination source on the specimen plane



CRITICAL

*** Bright illumination**

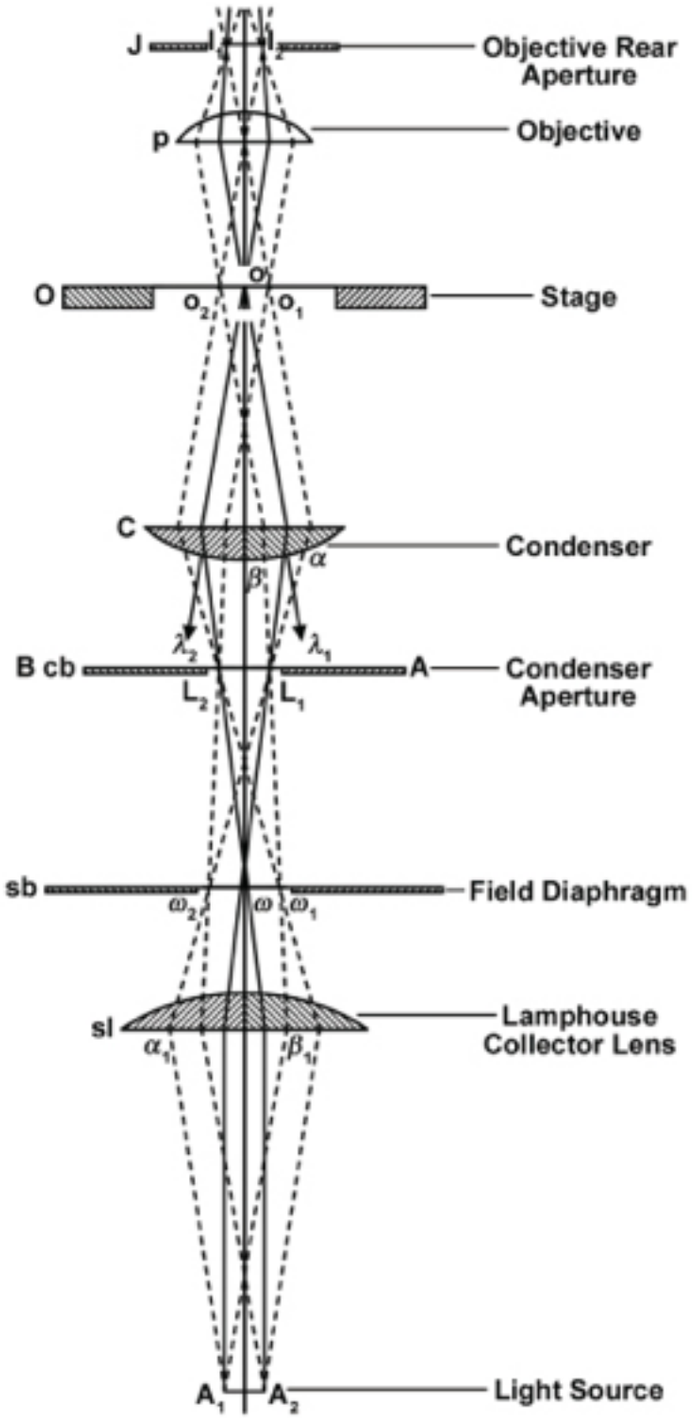
*** Not even illumination**



http://www.olympusmicro.com/primer/anatomy/introduction.html

Illuminating the specimen: Critical vs Koehler

Koehler = Illuminating the image plane with a maximally defocused image of the lamp
each point from the lamp contributes equally to illuminating the image plane



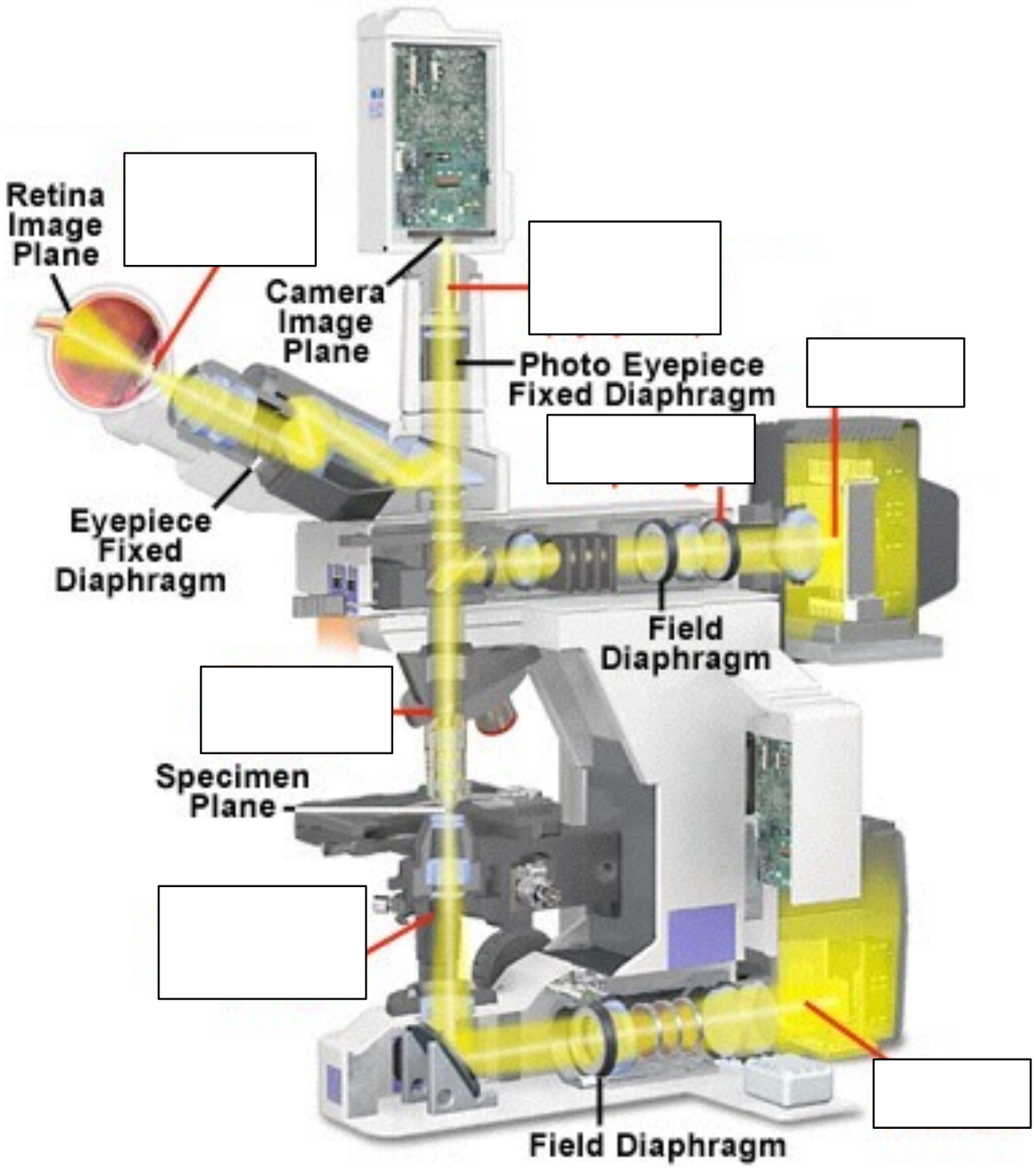
KOEHLER

*** Even illumination from an uneven source**

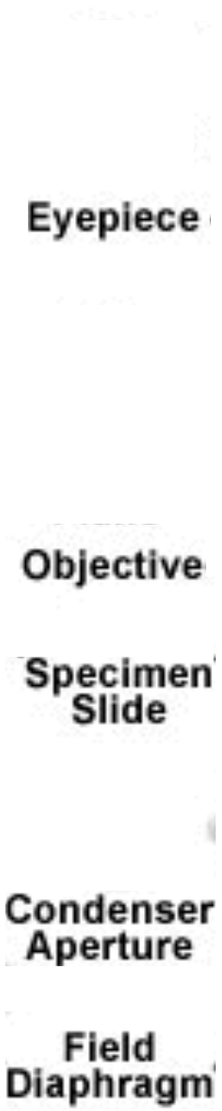
August Koehler
1866-1948

Koehler illumination and conjugate planes

Field or Image forming conjugate planes



Conjugate Planes in the Optical Microscope



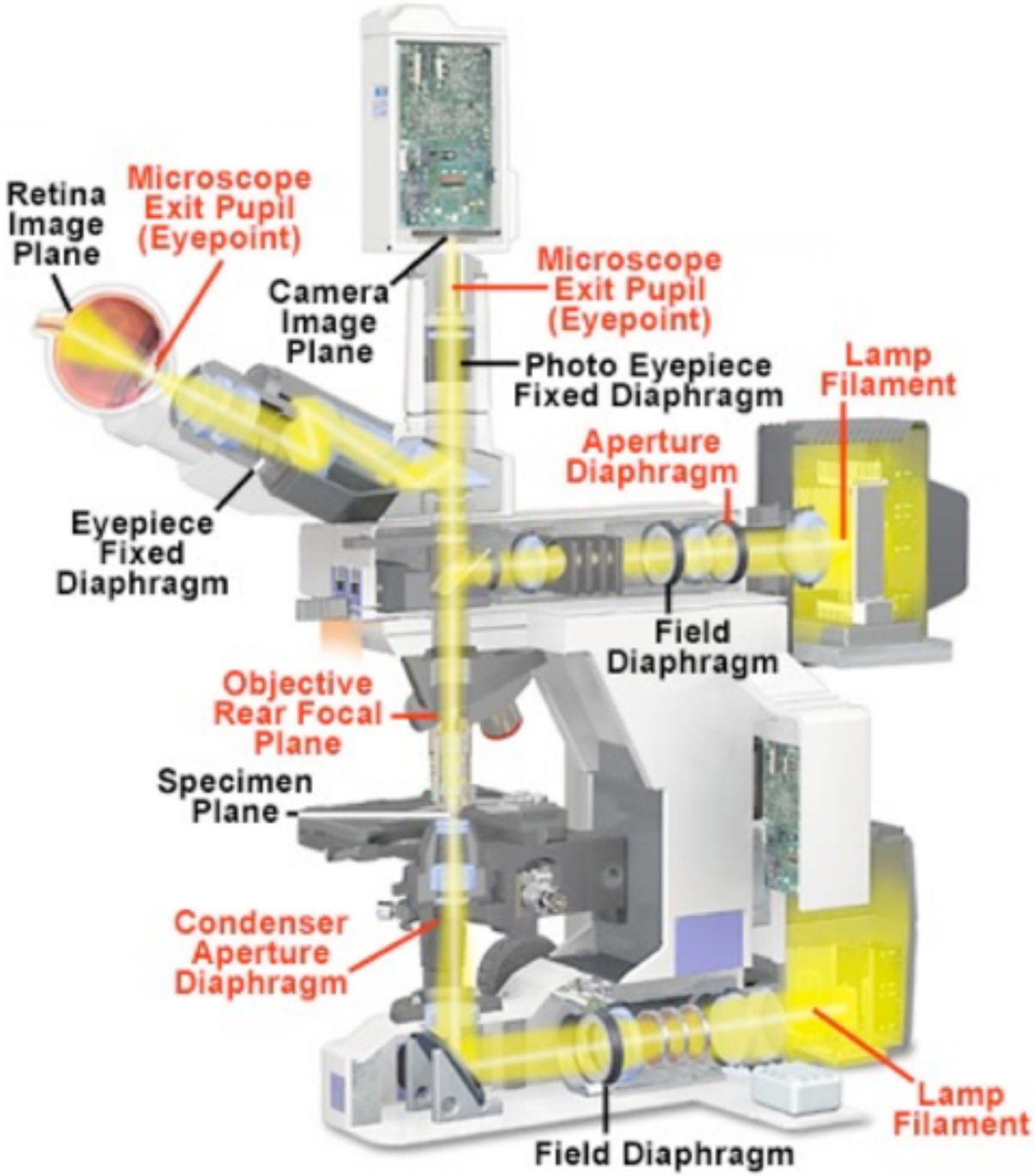
http://www.olympusmicro.com/

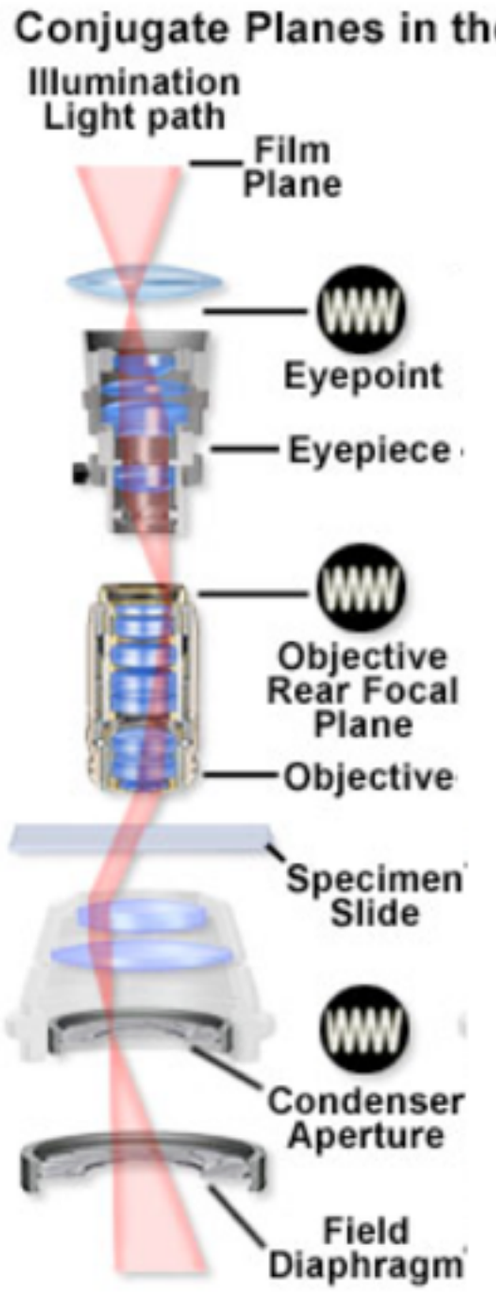
http://www.microscopyu.com/articles/formulas/formulasconjugate.html

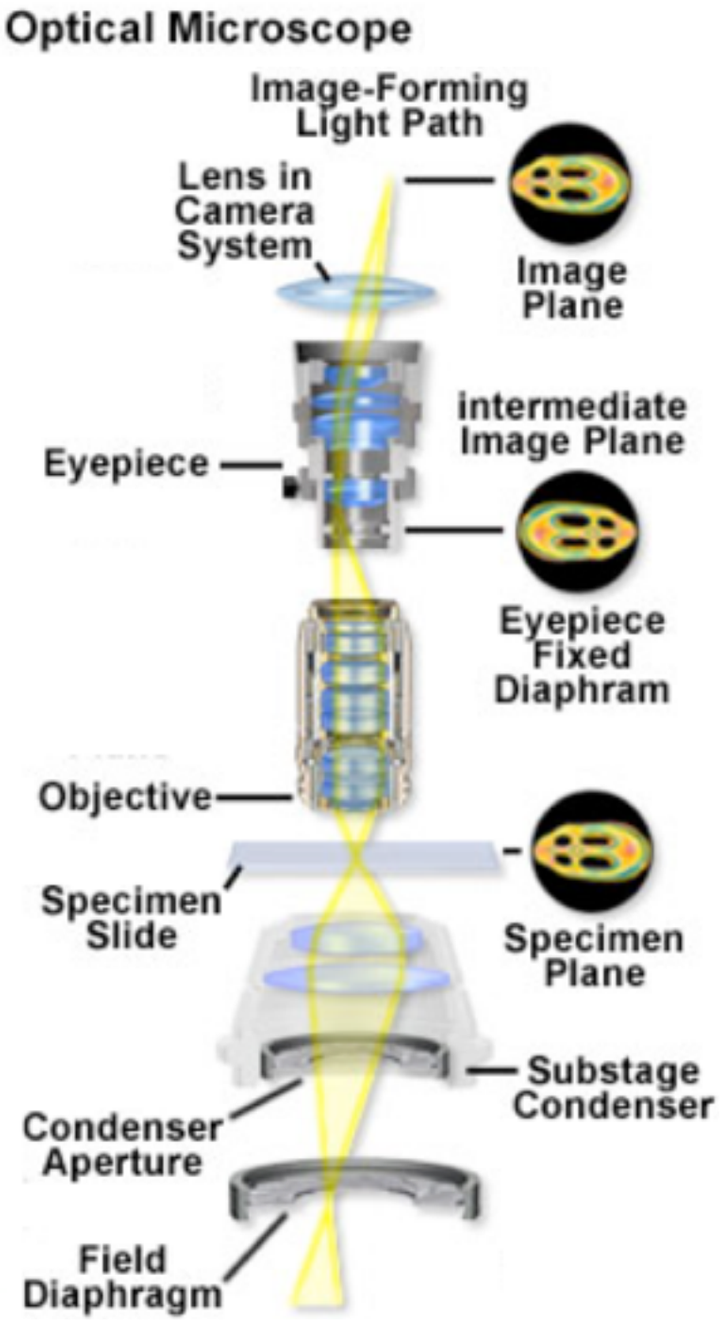
Koehler illumination and conjugate planes

Field or Image forming conjugate planes

Aperture or illuminating conjugate planes



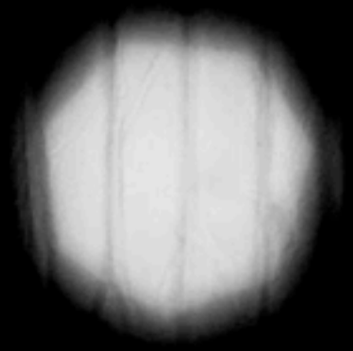




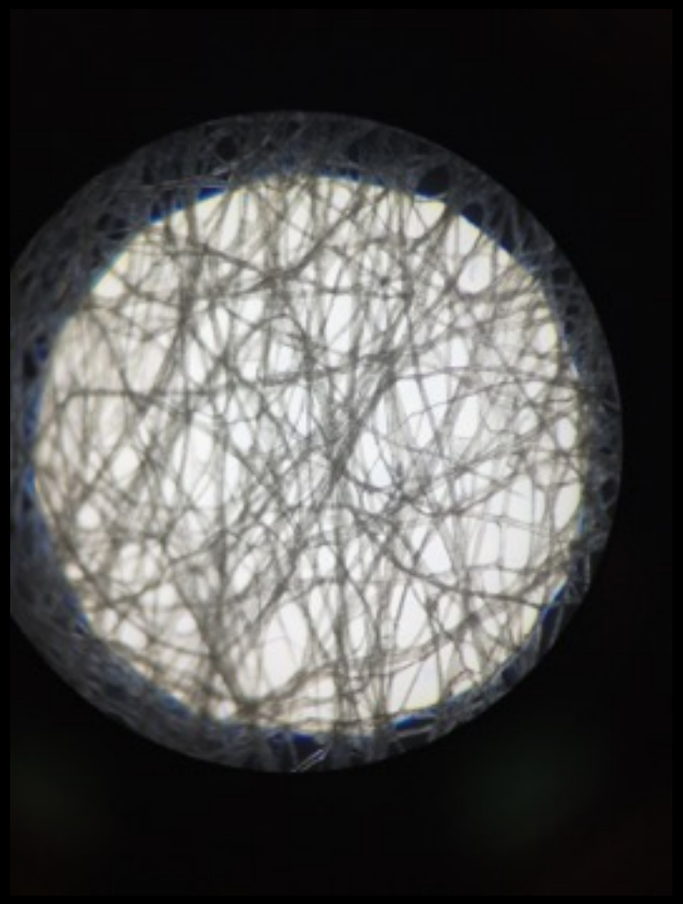
http://www.olympusmicro.com/

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

Bright-field Koehler setup (field diaphragm)



Lens tissue sample



Bright Field
low contrast

Aperture Iris
Adjusted

Dark Field
high contrast

Fluorescence contrast techniques

....use selective fluorescent probes to label features

GFP
green
fluorescent
protein

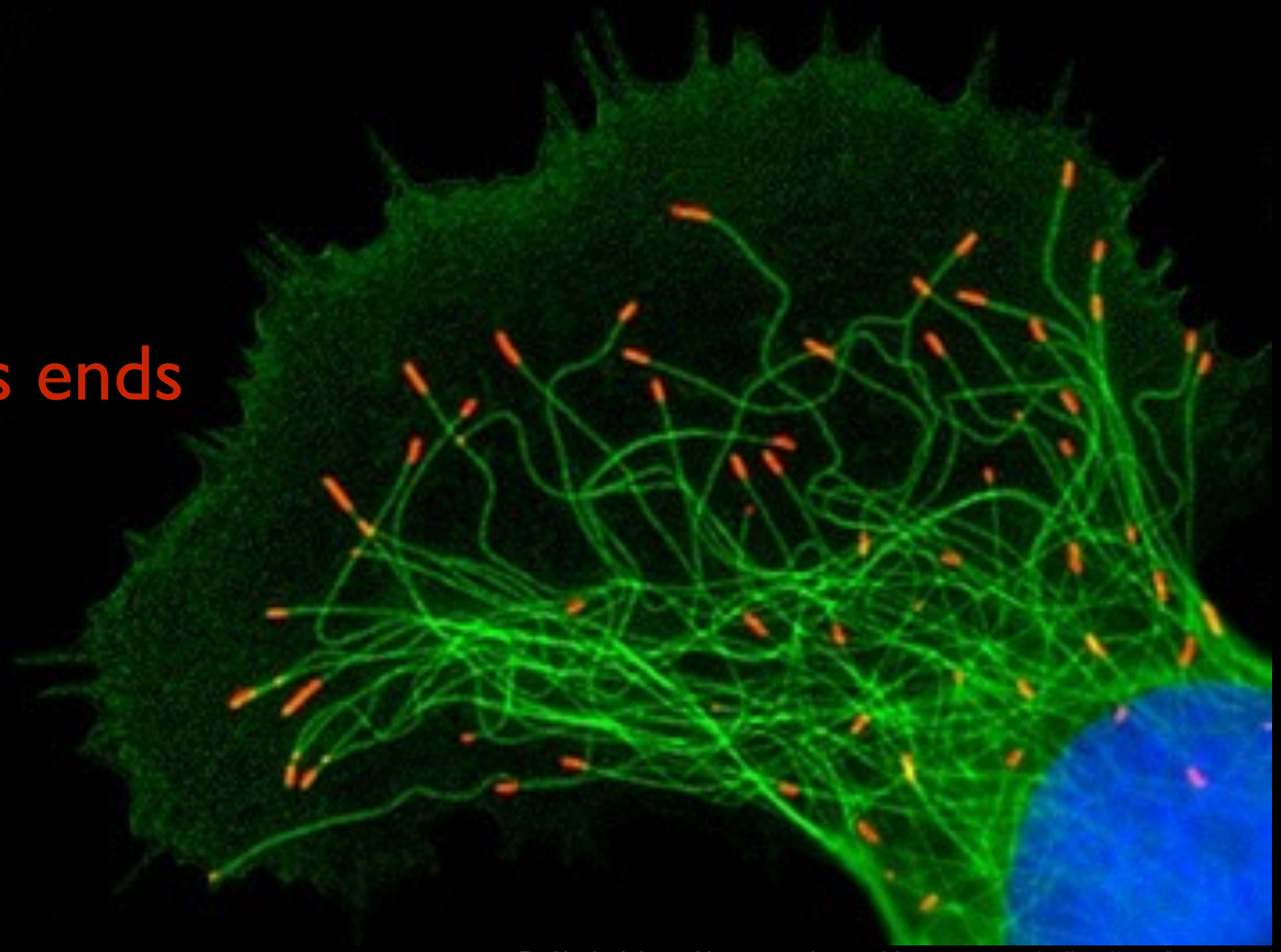
Fluorescence Contrast Techniques

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

Microtubules

Microtubule Plus ends

Nucleus



***LECTURES 4, 6**

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

Self Taught Practical Exercises - 2 Fluorescence

* 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 (bead slides)

* How to leave the microscope for the next user

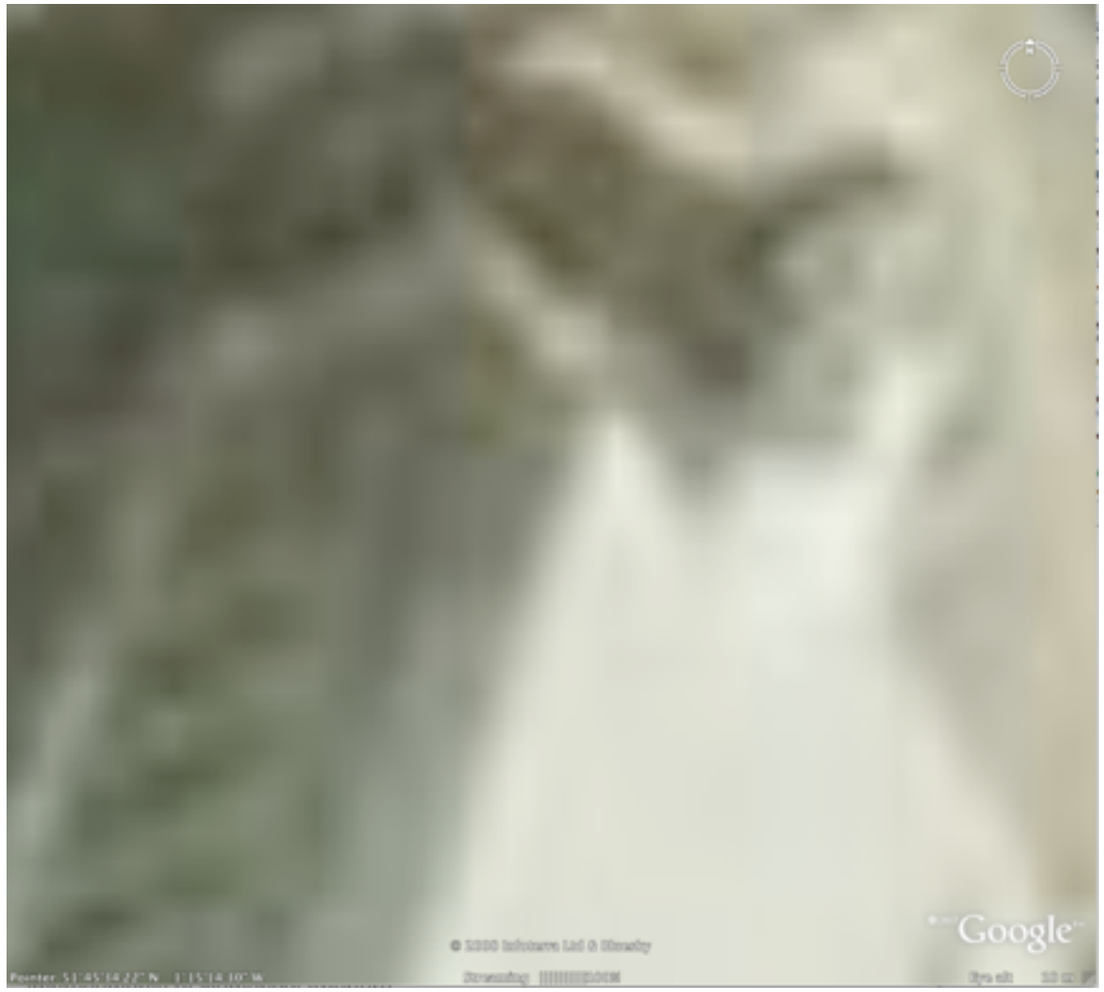
What is really important in microscopy?

1. Contrast
2. Resolution

What is really important in microscopy?

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

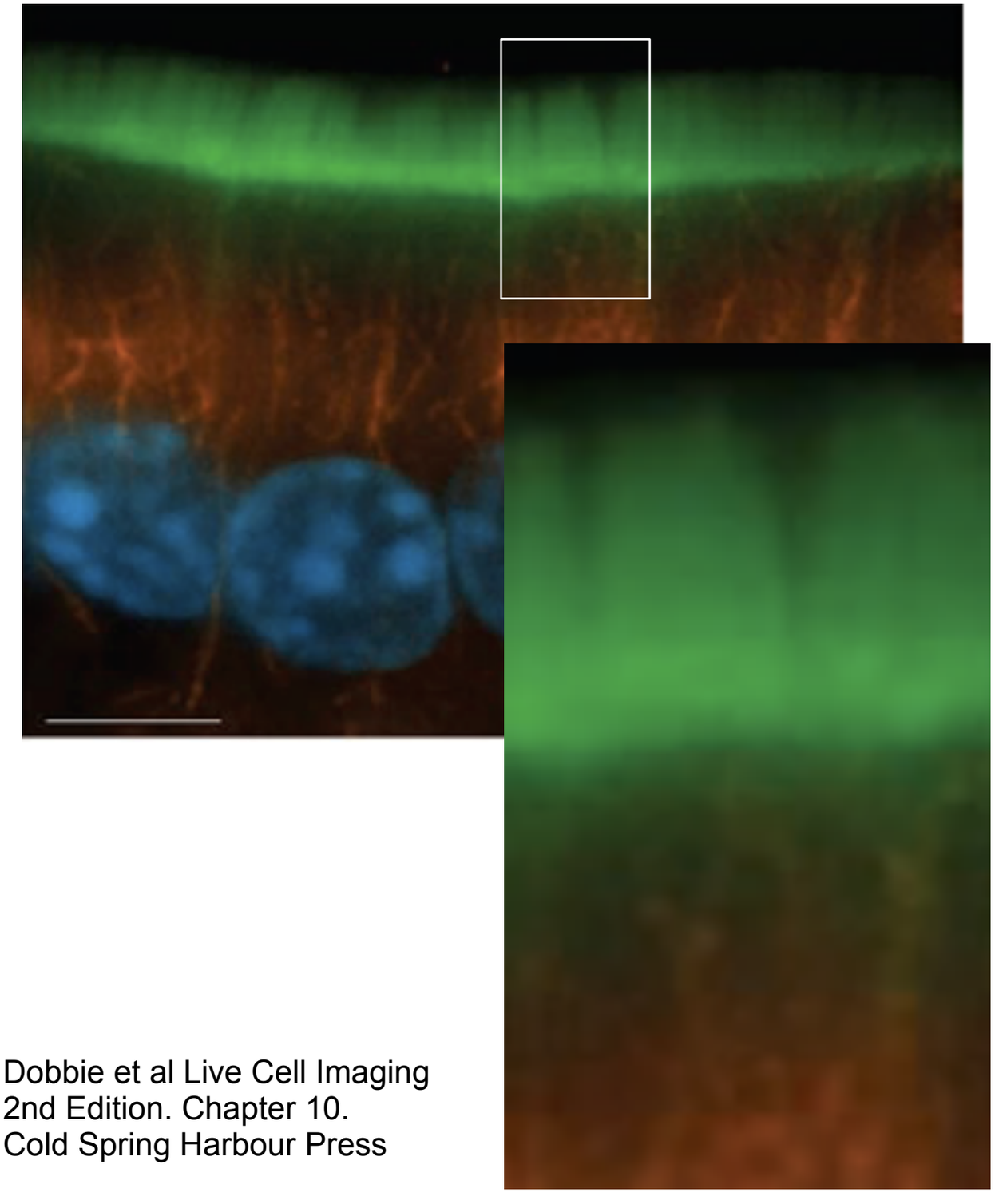
Magnifying is not enough:



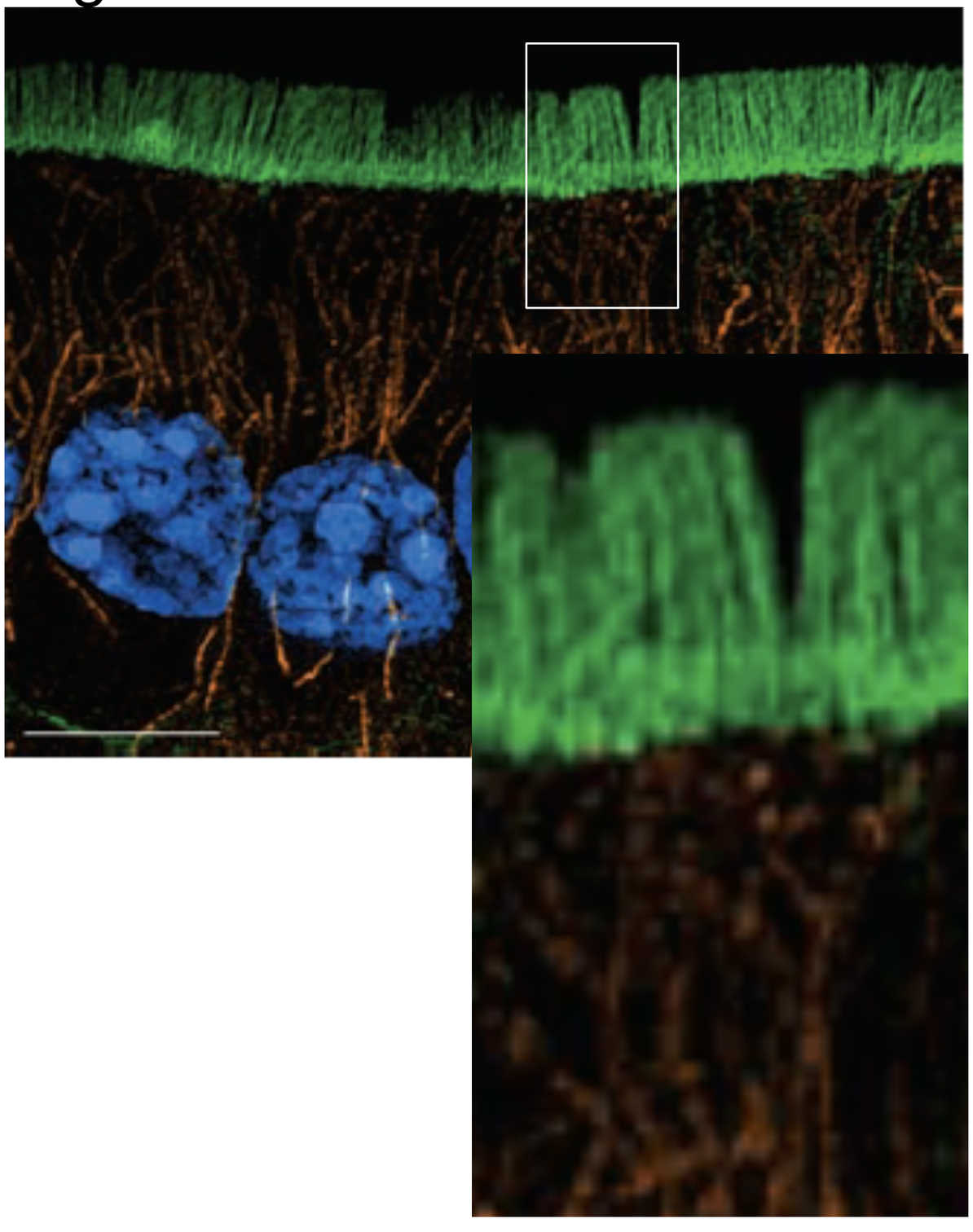
.....resolution is limited

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)

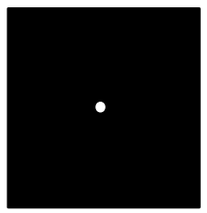
Convolution and the Point Spread Function

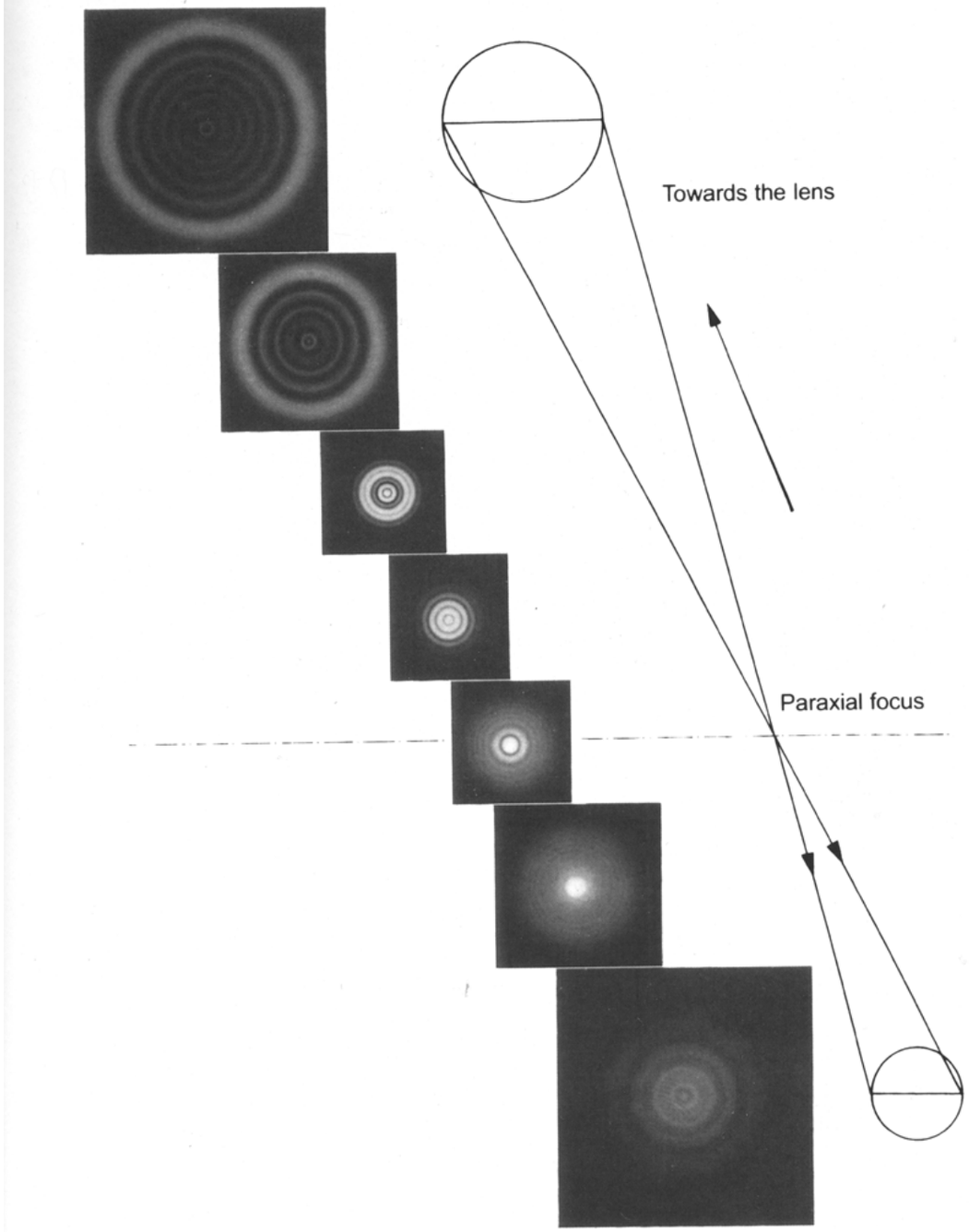
An image represents the output of the optics and detector of the imaging system

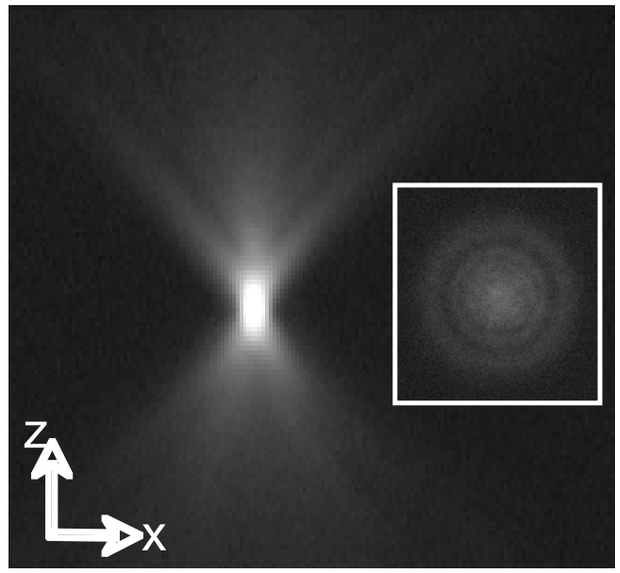
image ≠ object

image = object ⊗ PSF

Sample object: a "sub-resolution" fluorescent bead





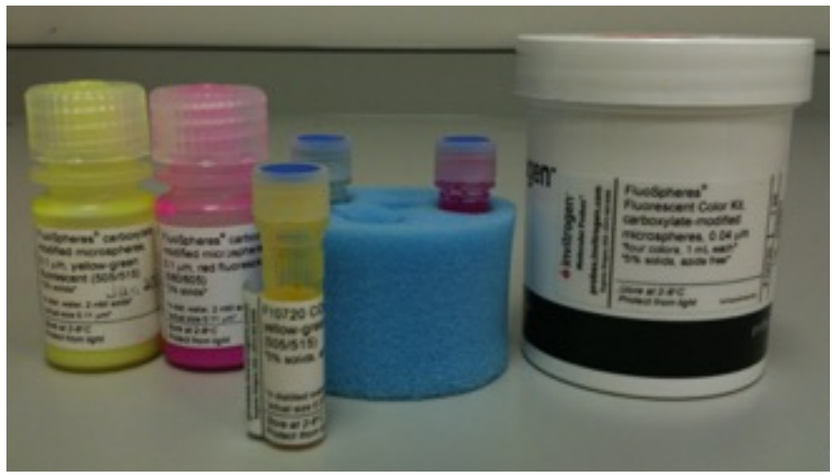


Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

- "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

Preparing a bead slide:

* Self Taught Practical 2 *

For PSF slides:

1. Dilute fluoresce **lute enough that a single bead**

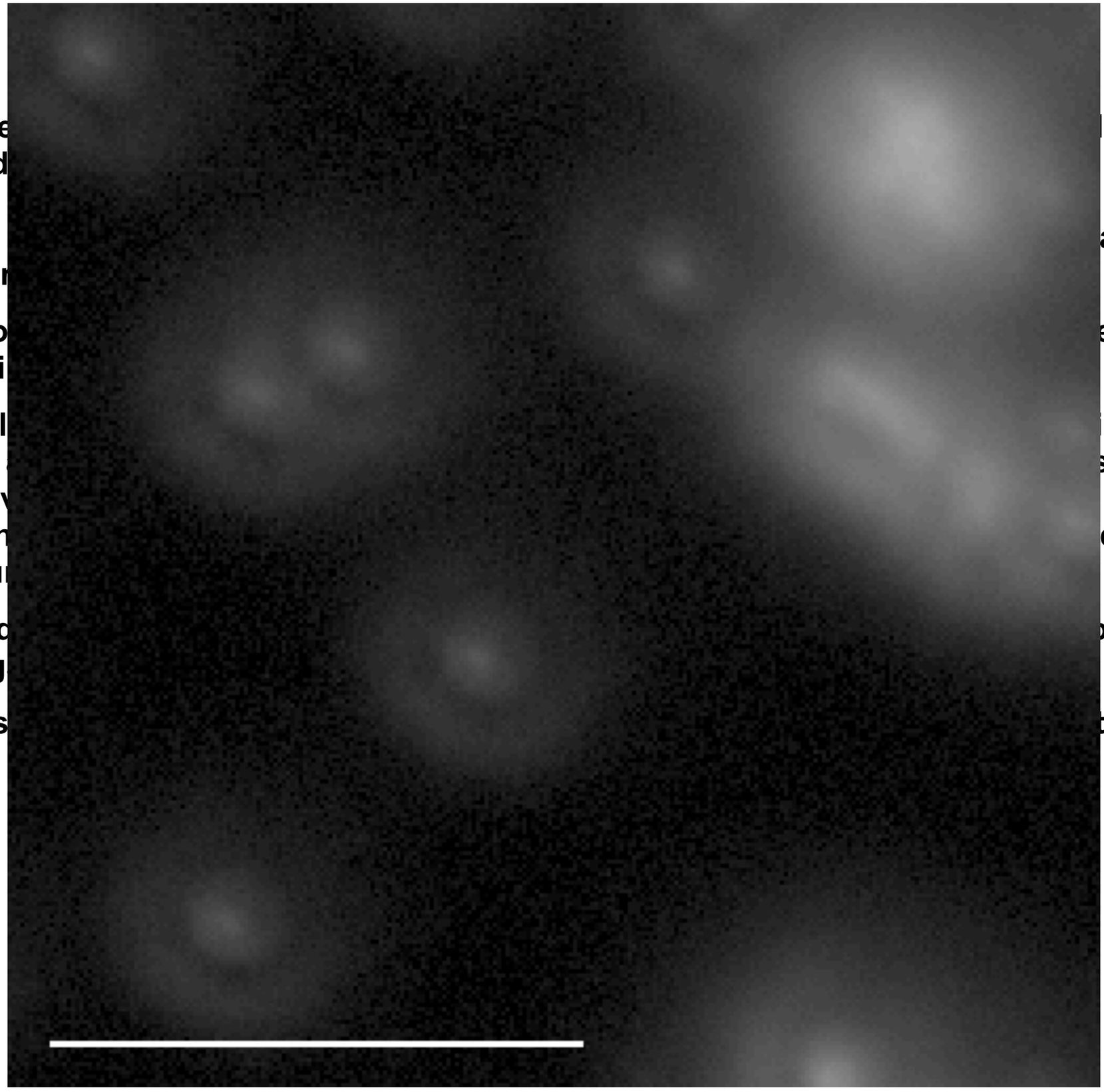
2. Apply 10-20 µl **a pipette tip, and allow to air dr**

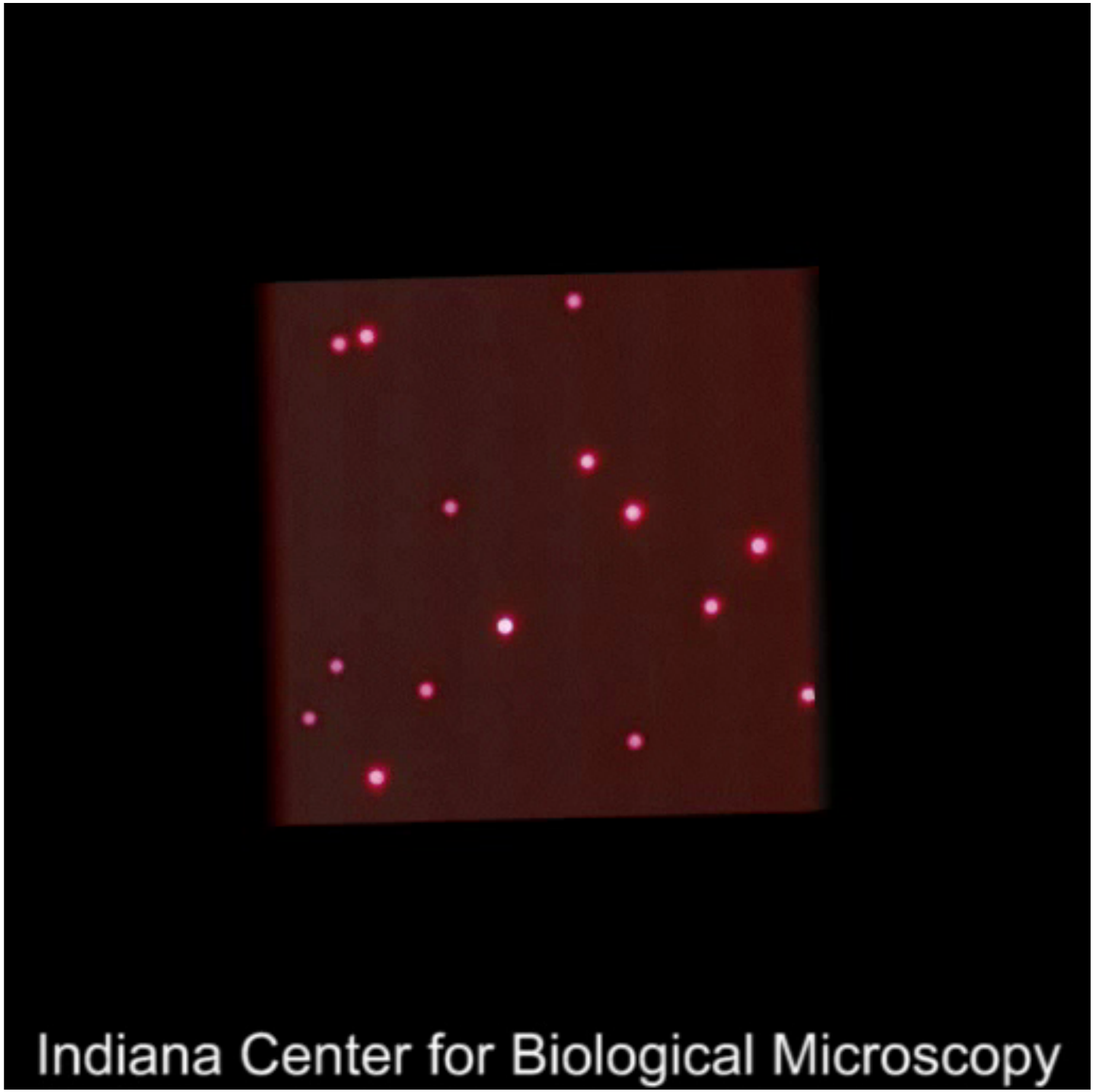
3. Apply 10-20µl o **e previously prepared coversli**

4. While most cal **res to image structures** **s several µm away from the cov** **in parallel with depositing th** **djusted by varying the amou**

5. Calibration slic **old, Vectashield or Ag**

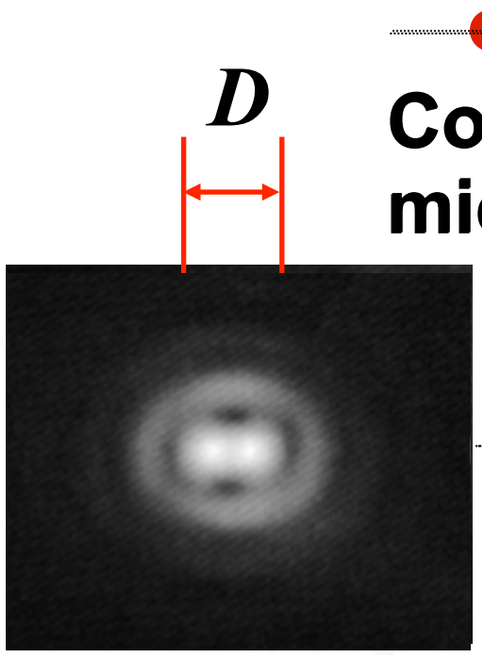
6. Seal the covers **t 4 degrees.**




Indiana Center for Biological Microscopy

Calculating Lateral Resolution: The Rayleigh Criterion

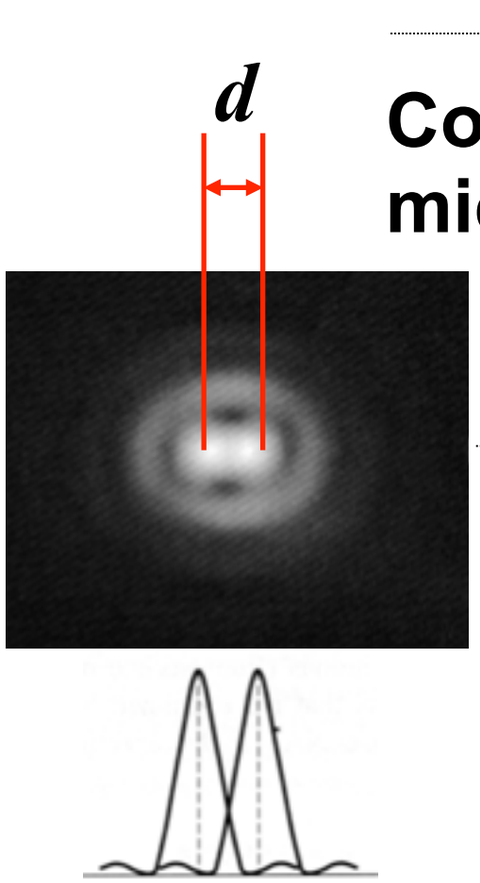
Two small objects



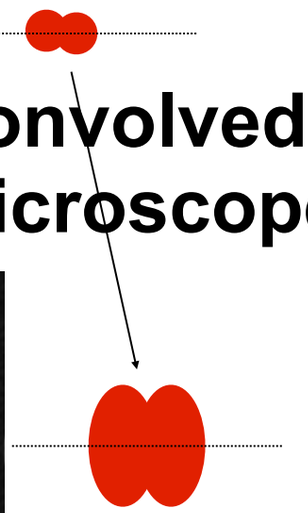
D = the distance between the two closest points that can still be distinguished

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:

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

$D = 227\ nm$

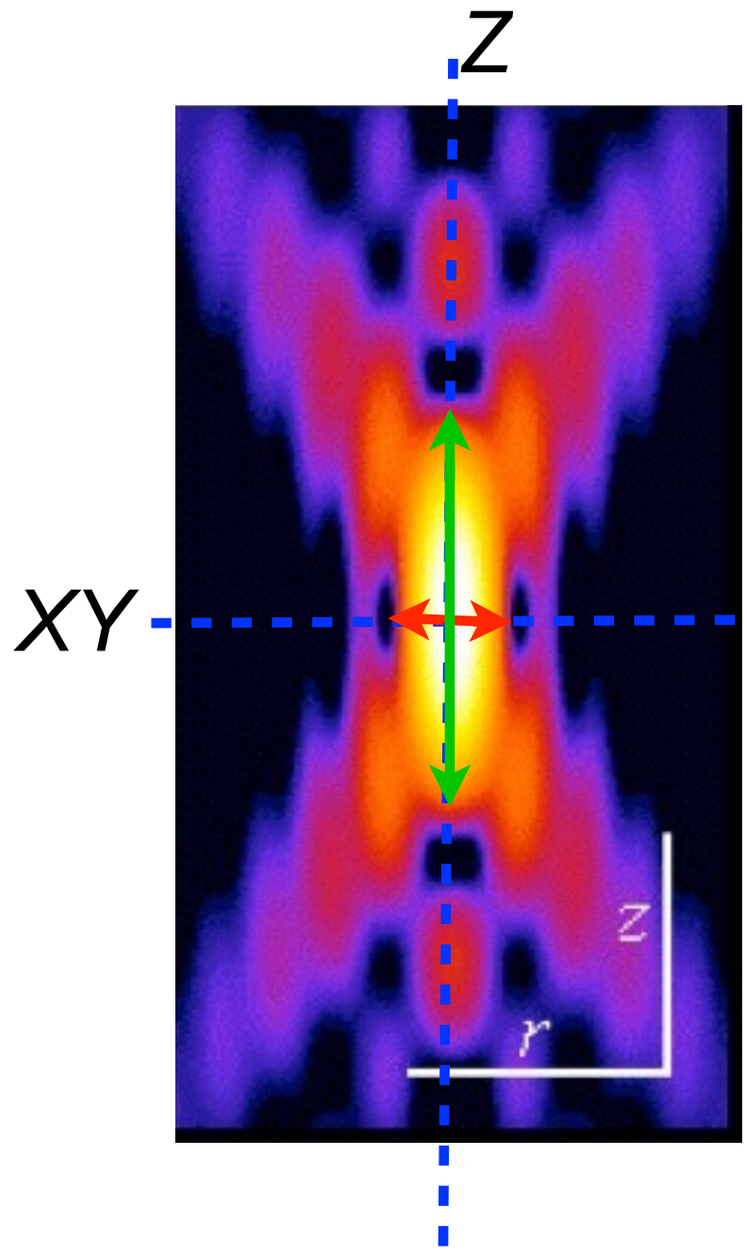
Limit of resolution $\approx \lambda_{em}/2$

Axial Resolution:

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

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

(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 ~ 230 nm
Z resolution ~ 700 nm

GFP is ~ 5 x5 x5 nm

Solution 1 - F* techniques

FRAP, FRET, FLIM etc

LECTURES 11, 14

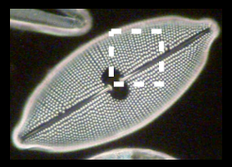
Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

LECTURES 12, 15-16, 19

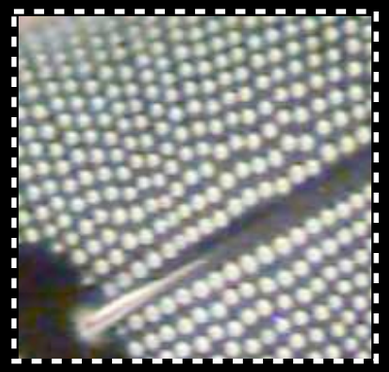
Resolution .....Magnification and Sampling

Specimen
Fine Detail

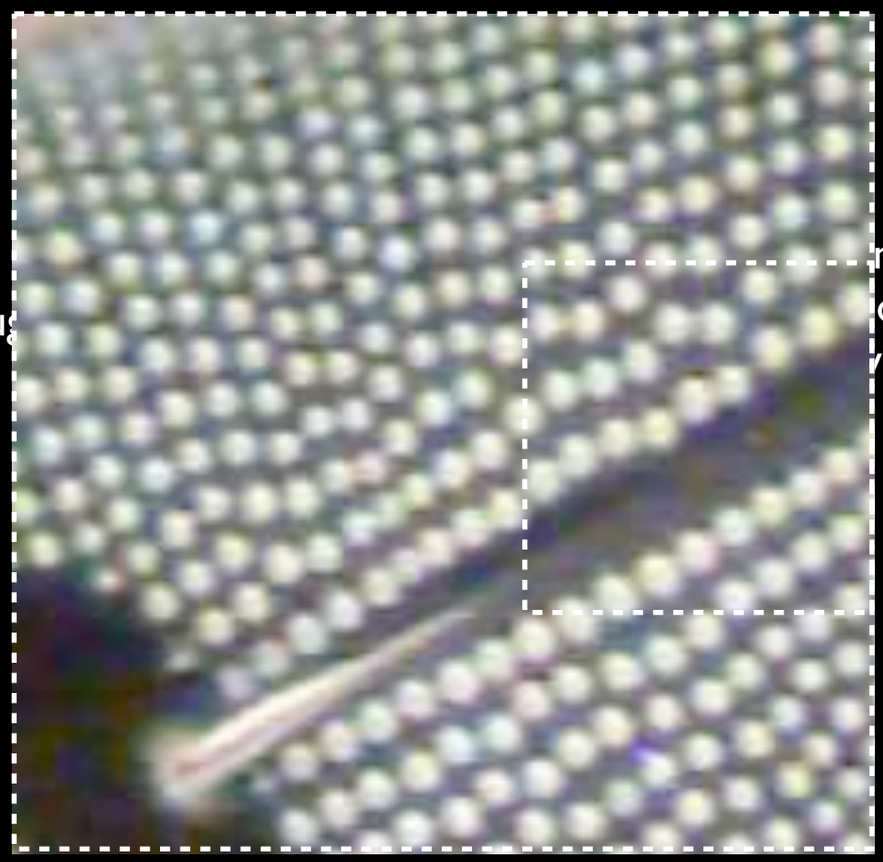


Detail imaged
by microscope

*magnification
*optical resolution



too magnified enough



ng
olved
iew

crop
auxiliary
magnification
to detector

to match image
to detector

Oversampling
Empty
magnification

All resolvable
detail recorded

Blurred image

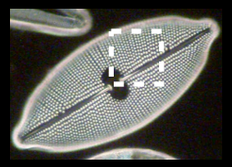
Limited field of
view

What is the optimum magnification.....?

For optimal imaging: magnification must match the resolution to the detector

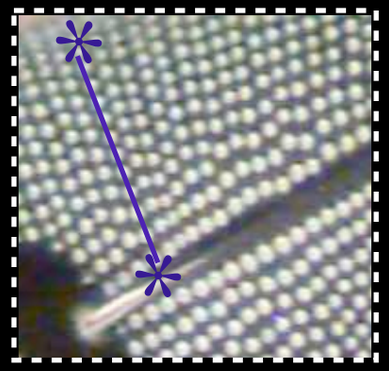
Resolution .....Magnification and Sampling

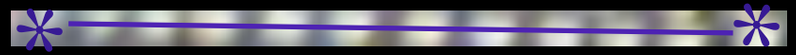
Specimen
Fine Detail



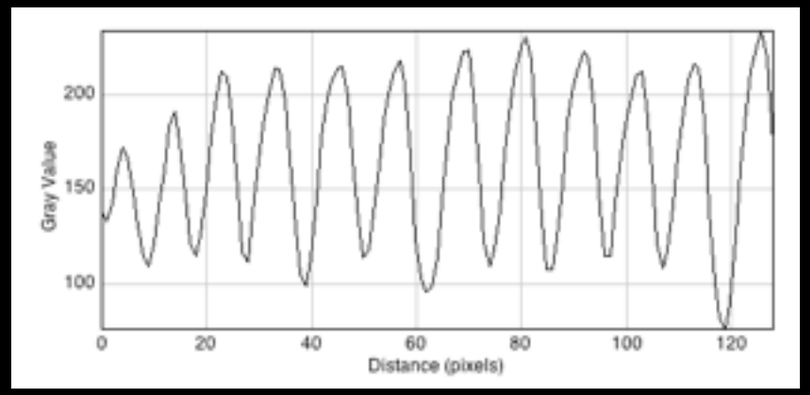
Detail imaged
by microscope

*magnification
*optical resolution





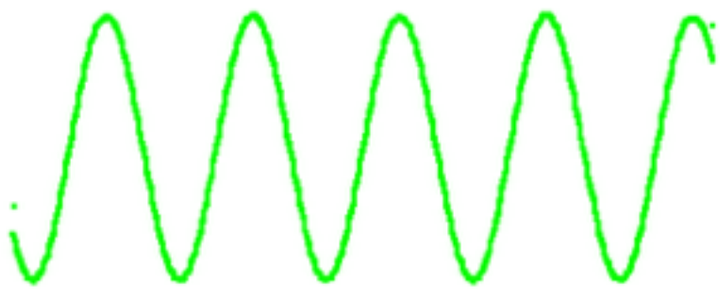
intensity profile ~ a sine wave



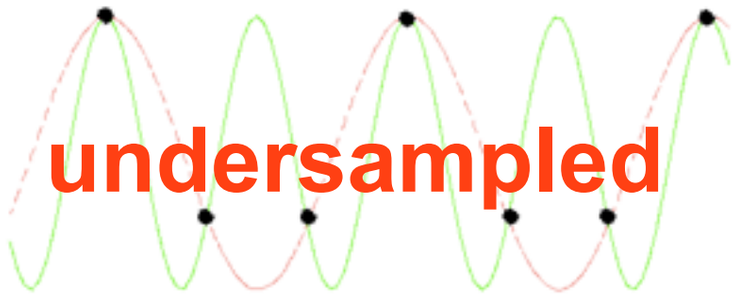
Optimum = 2.3 times per cycle

= Nyquist sampling

sampling a sine wave

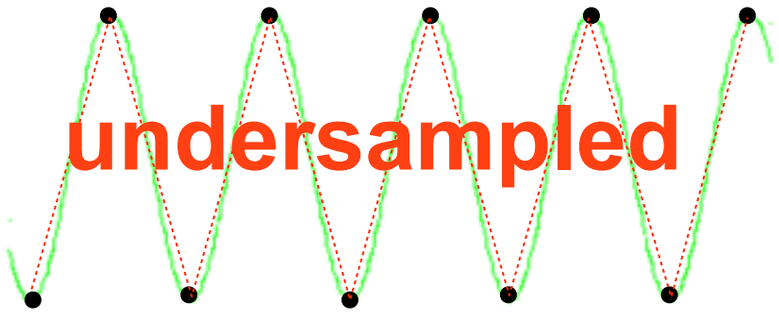


Sampling 1.5 times per cycle



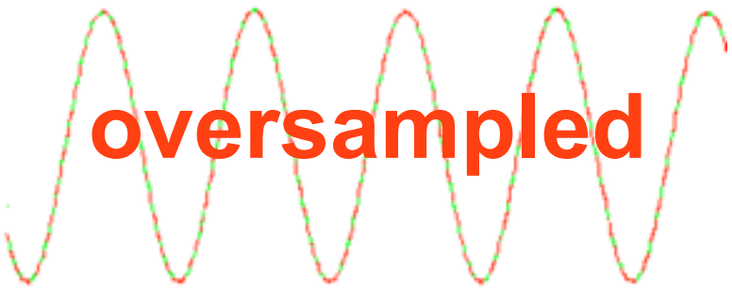
undersampled

Sampling 2.0 times per cycle



undersampled

Sampling many times per cycle



oversampled

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)

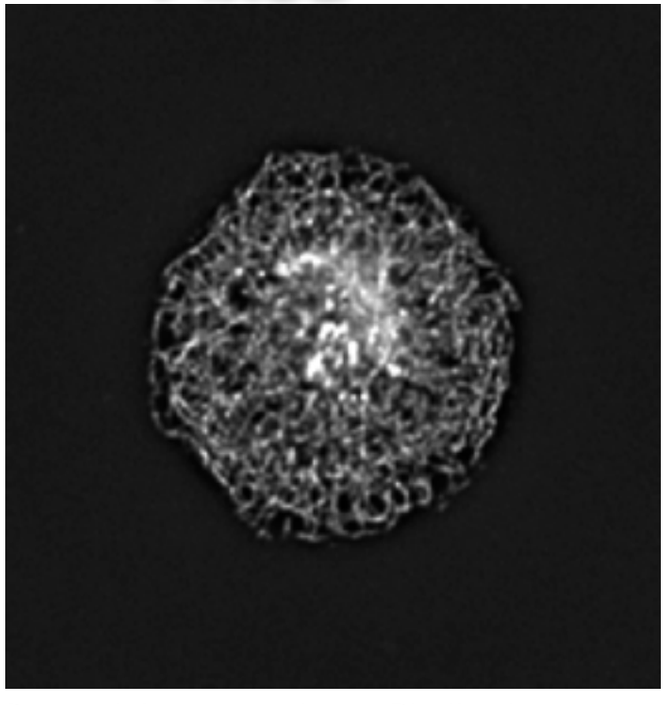
Super-resolution techniques (optical and post aquistion)

Techniques for molecular dynamics and interactions

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

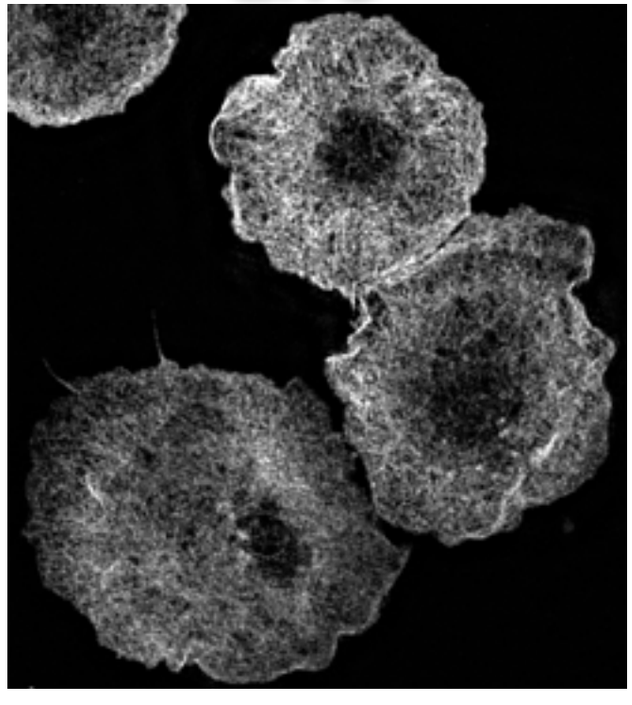
Which technique to use?

Fixed



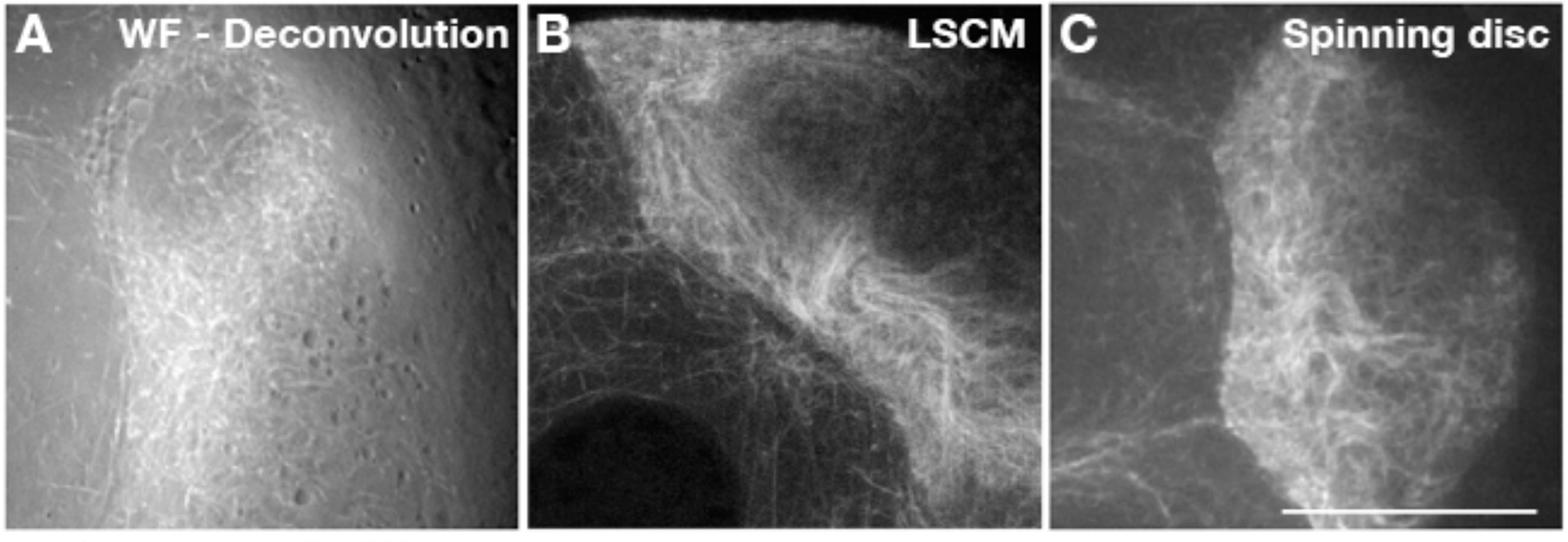
vs

Live



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

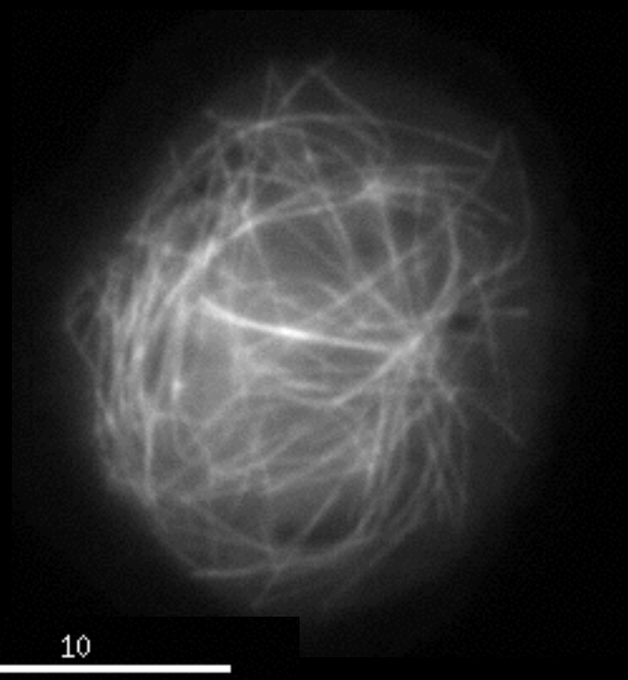
Wide field Decon vs Confocal



Live *Drosophila* oocyte: Tau-GFP labeling microtubules

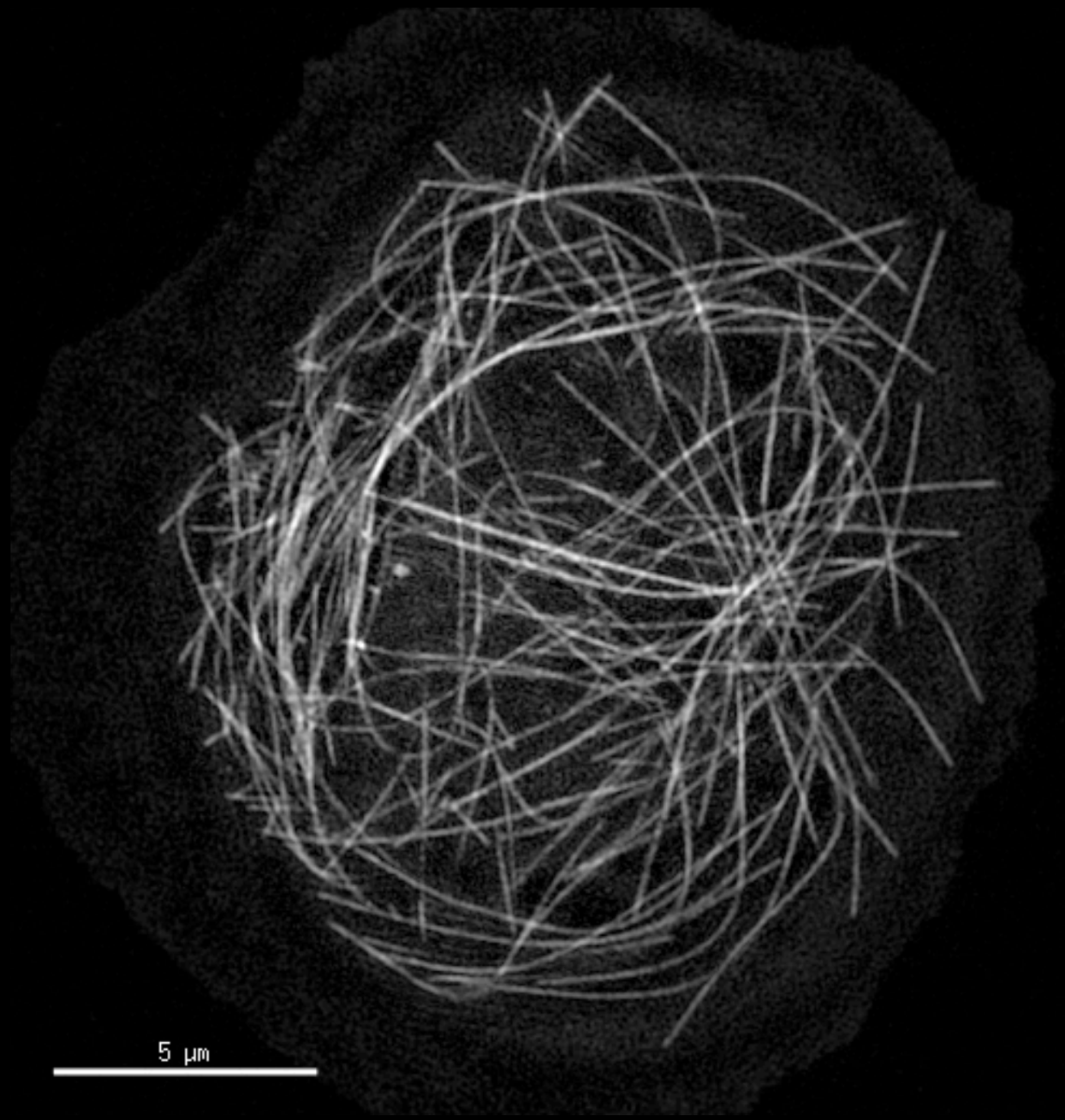
Live-cell super-resolution!

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, 17, 18

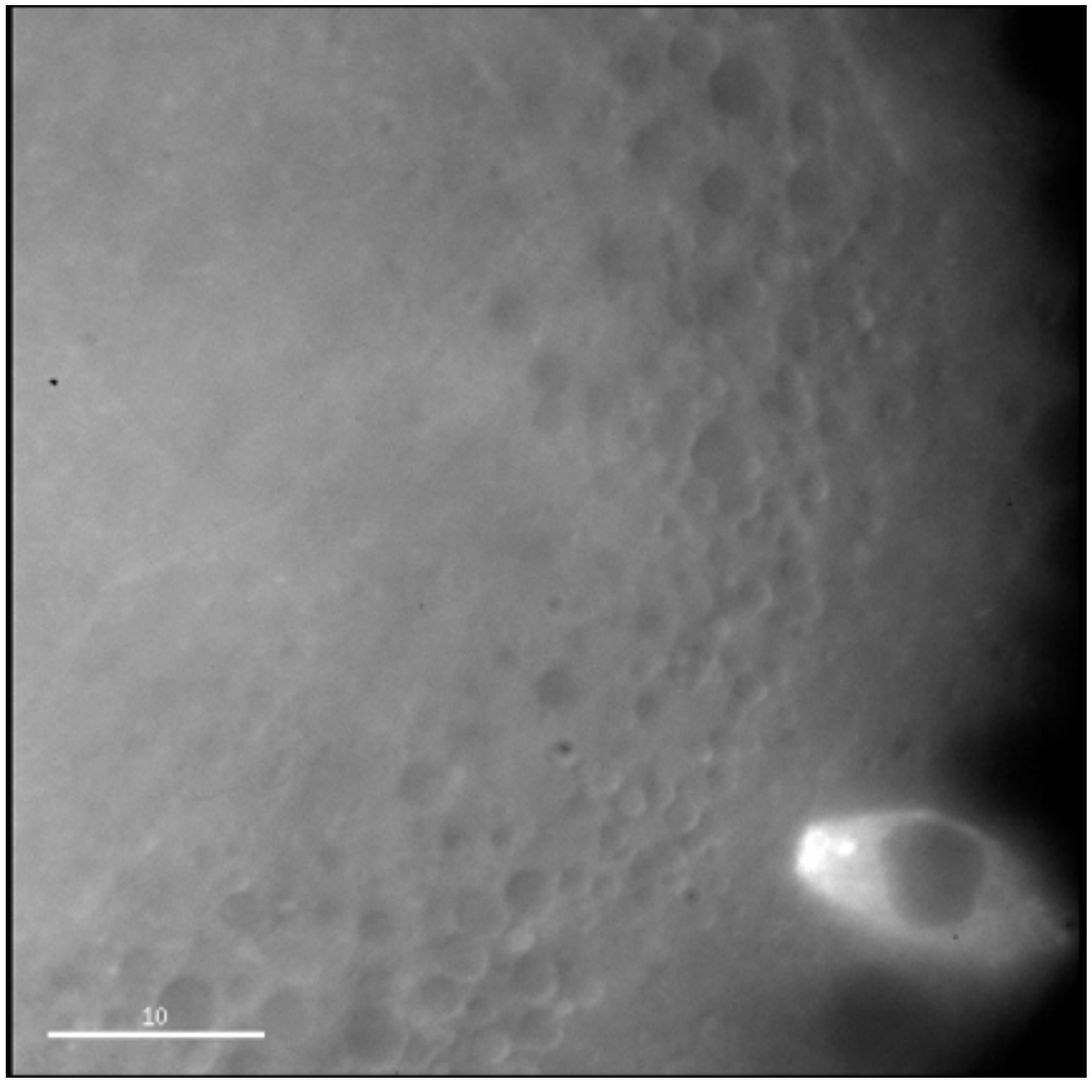
Image handling

Enhancement

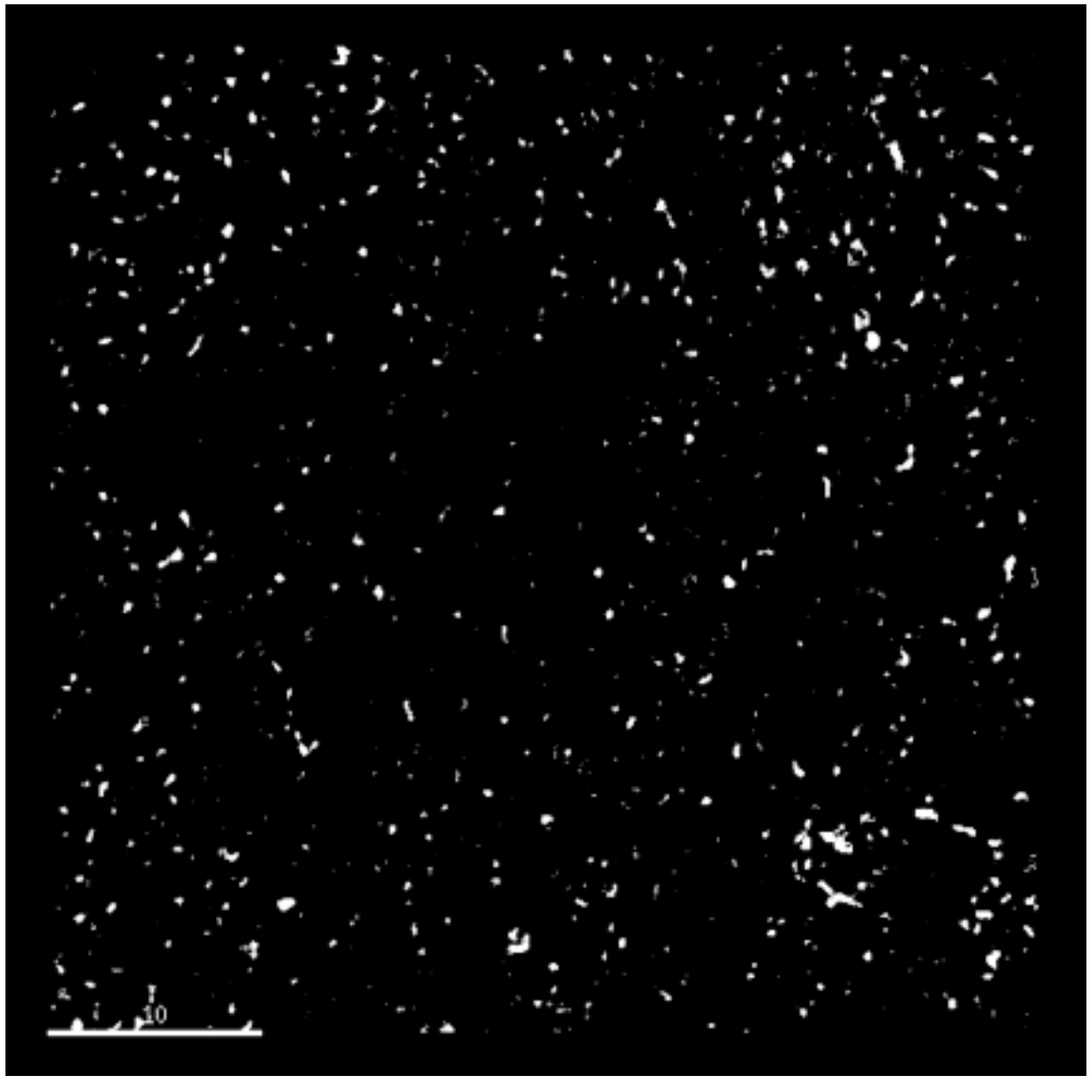
Analysis

Computational enhancement and processing

Raw - Widefield

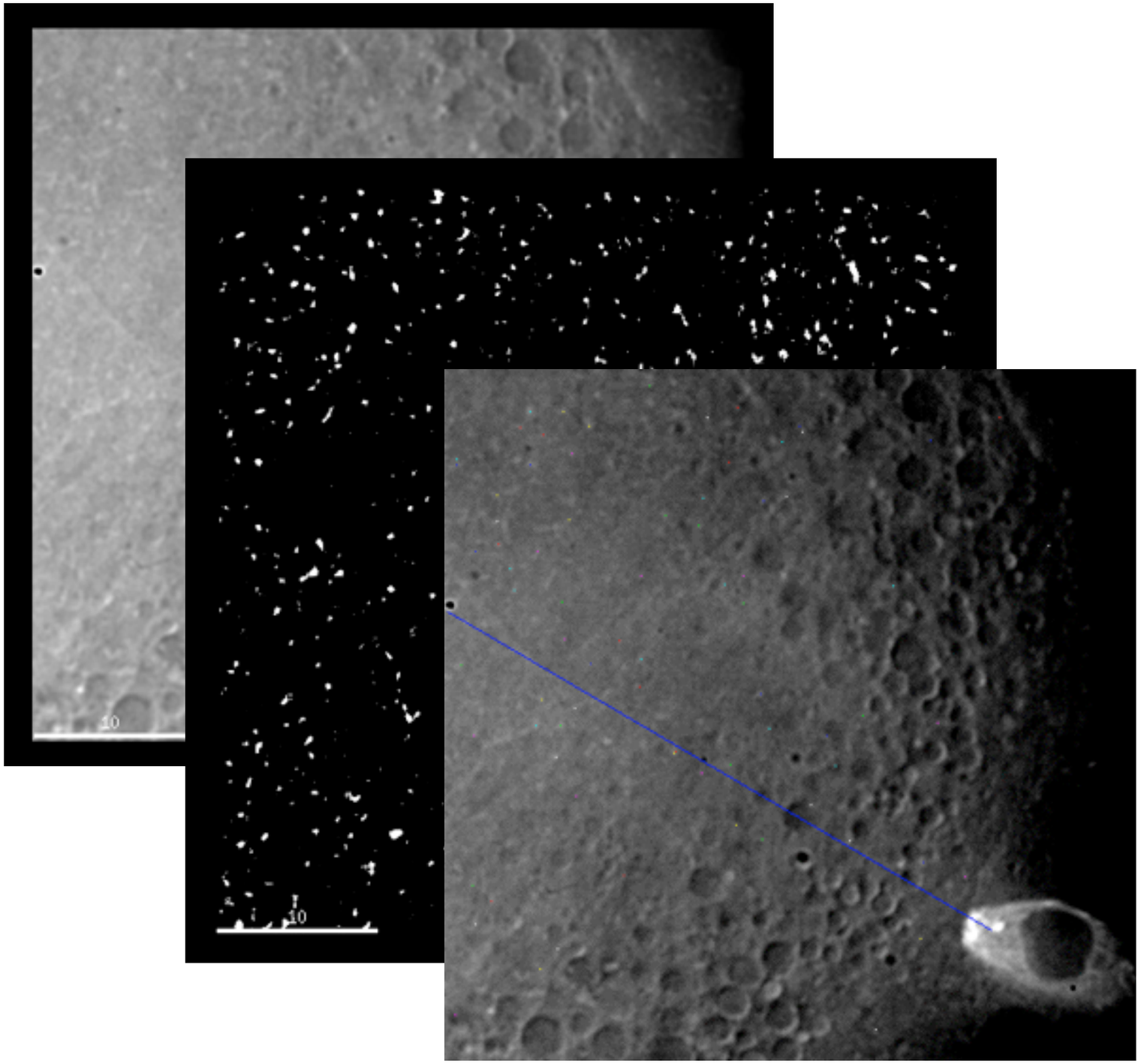


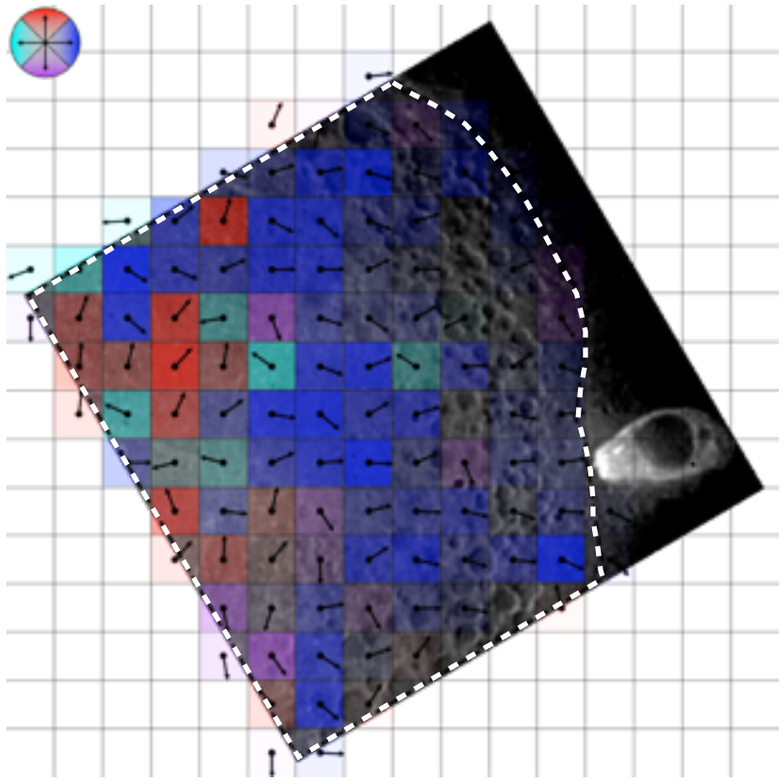
Processed

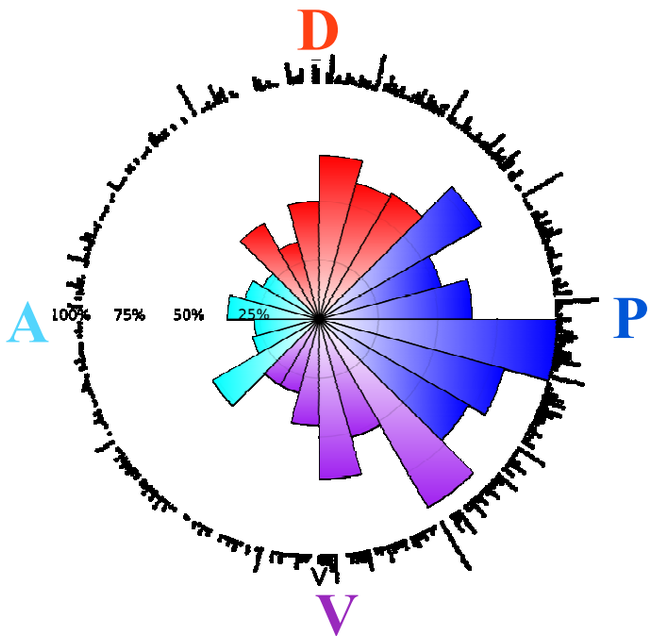


Parton, R. M., Hamilton, R. S., Ball, G., et al. (2011). JCB 194(1), 121–135. doi:10.1083/jcb.201103160

Live imaging - not just a pretty picture







LECTURE 17 - David

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)

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

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

Resolution .....Magnification and Sampling

For optimal imaging the magnification must match the resolution to the detector (eye or camera).....

optimal total mag X resolvable distance = detector element size

Considering Fluorescence imaging x10 objective; 0.25 Na; 520 nm emission, eyepiece x10.....

total mag	resolvable distance	Detector Element
x10 objective X x10 eyepiece	1.22 x **λ520**/ 2Na	eye is 0.15 mm (taking into account Nyquist)

Rearranging to find the optimum magnification ....

optimal total mag = Na X (2 x Detector Element /1.22 x 520 nm) ≈ **120 x**

x10 eyepiece
x10 objective

= 100x

undersampled

optimal total mag ≥ Na X 500
optimal total mag ≤ Na X 1000

undersampled<125x

oversampled>250x