Microscopy Course 2014 - lectures

Day 1: Monday 10th Nov. - Fundamental Principles of Microscopy

Ilan 11:30-12:00 Welcome to the course

1 Richard 12.00-12.45 General introduction to light microscopy

Lunch Break

2 Ian 1.45-2.30 Principles of microscopy and microscope anatomy

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Afternoon practical 2:30 – 5:30
(DTC/Micron prep area, 23 students Groups A and B change over 4:00)

Hands-on demos

- **Practical 1**: Learning to make specimens for bright-field microscopy (**Micron**: Students grp A, B. Richard, Eva, Ana).

- **Practical 2A**: Using microscopes - simple upright, invert (**DTC**: Students grp B, A. Ian, Chris L, Alan, [Ilan]).

ONBI Advanced Microscopy Course 2014

Week1 - Principles of light microscope design and fluorescence imaging

Introductory Lecture

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

Goals of the first week lectures:

- Explain why microscopy is so important
- Explain how the light microscope works:
  - the basic physics of optics and microscopes
  - designing and building bespoke microscopes
- Make you aware of what is really important in 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

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Why is microscopy so important?

100 years ago:

Magnify small things to visualise more details

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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)
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1942	Immunofluorescence (Milestone 7)
1955	Differential interference contrast (Milestone 8)
1961	Concept of confocal microscopy (Milestone 9)
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	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)

- 2014 Nobel Prize in Chemistry for Super Resolution: E. Betzig, S. Hell, W. Moerner

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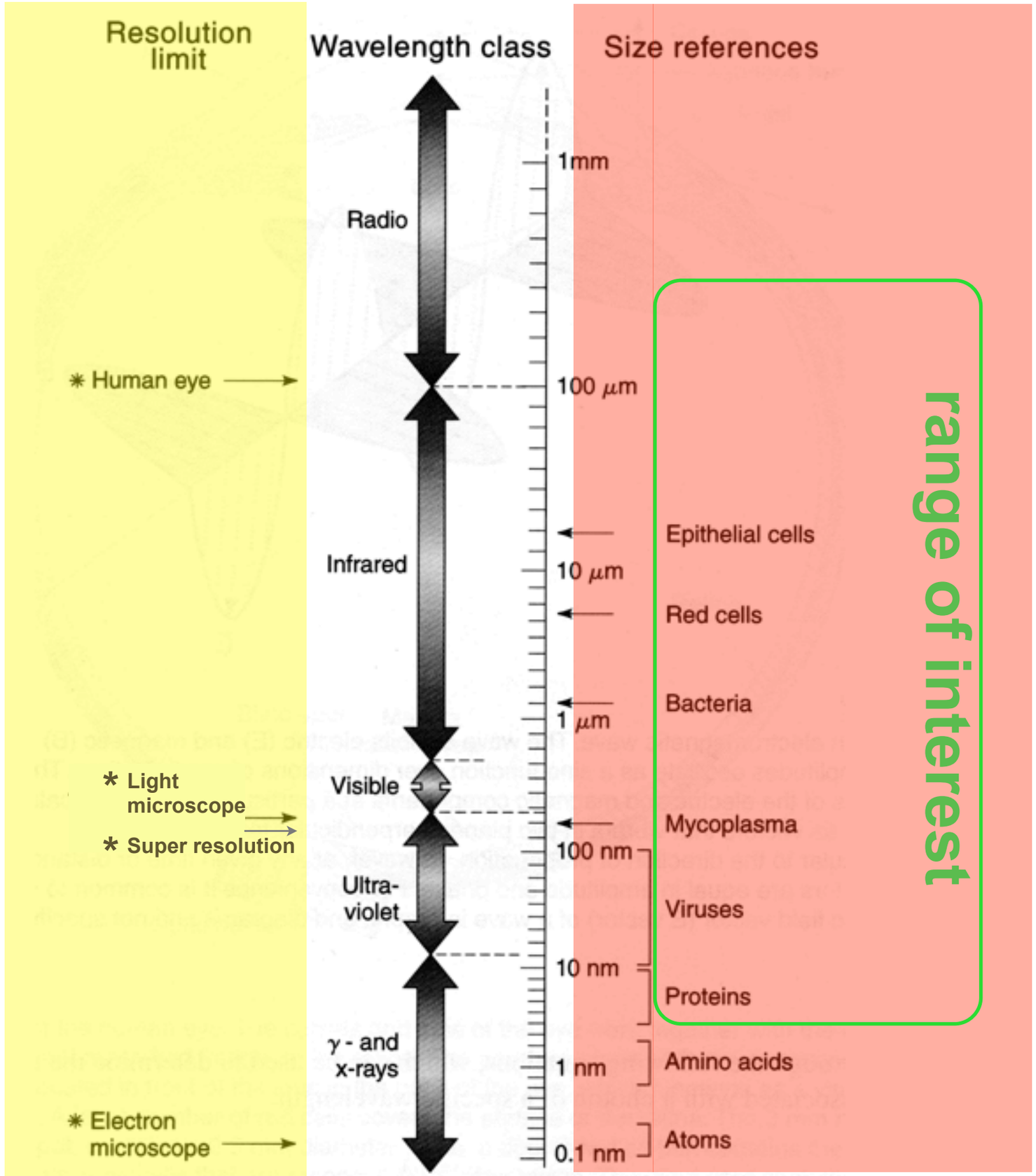
* Allows experimental manipulation

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)



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

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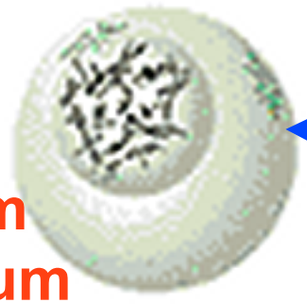
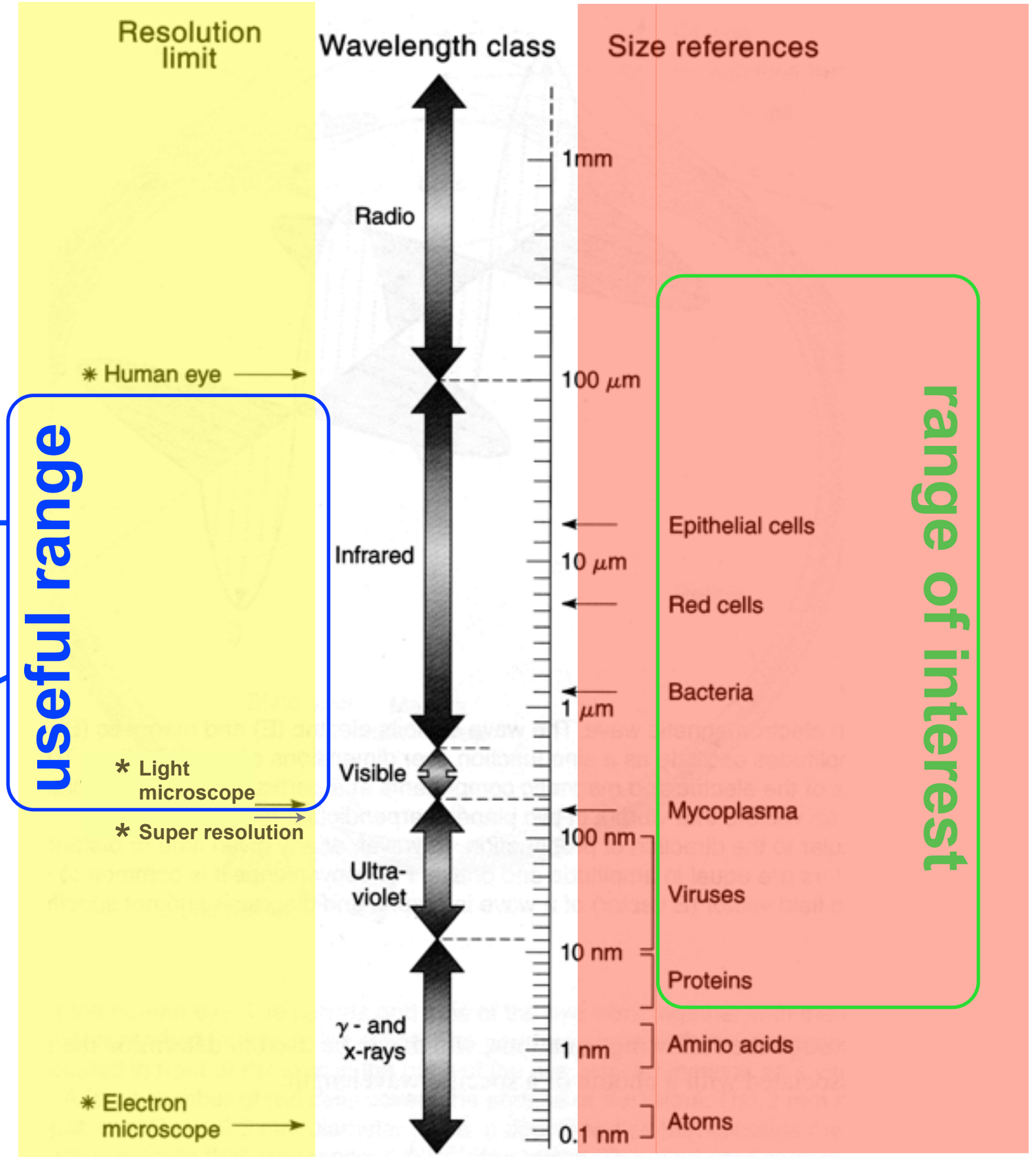


Eukaryote = 10 um
>50 um

Bacterium = 1 um



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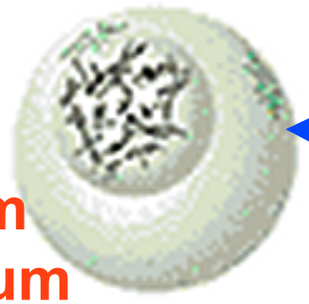


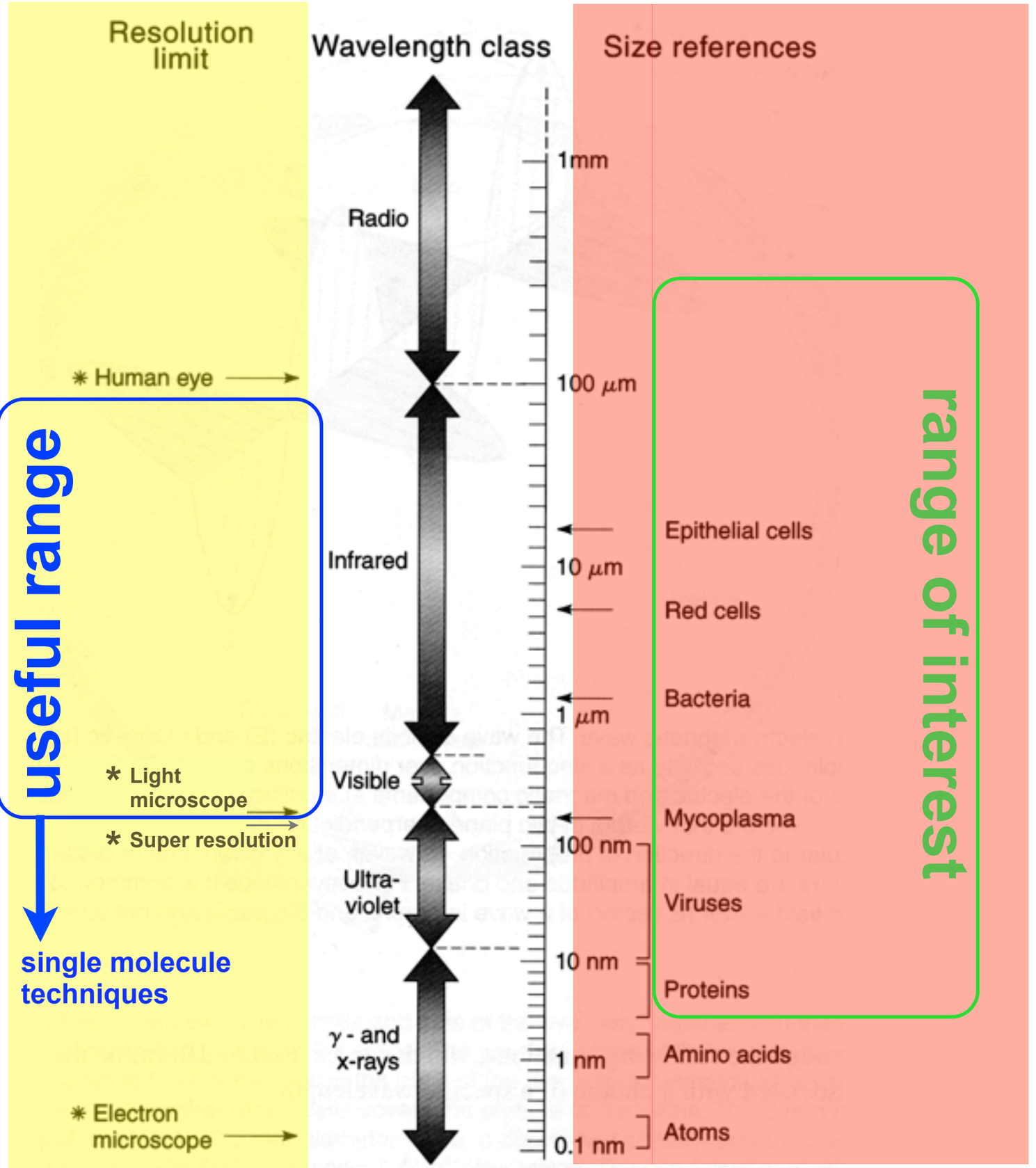
Eukaryote = 10 um
>50 um

Bacterium = 1 um

Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



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Why do we need to understand microscopy?

Why do we need to understand microscopy?

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

Why do we need to understand microscopy?

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

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*** Microscopes are all basically the same**

Why do we need to understand microscopy?

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

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*** Microscopes are all basically the same**

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

Understanding what goes on in the Microscope

http://api.gehealthcare.com/api/deltavision.asp

Understanding what goes on in the Microscope

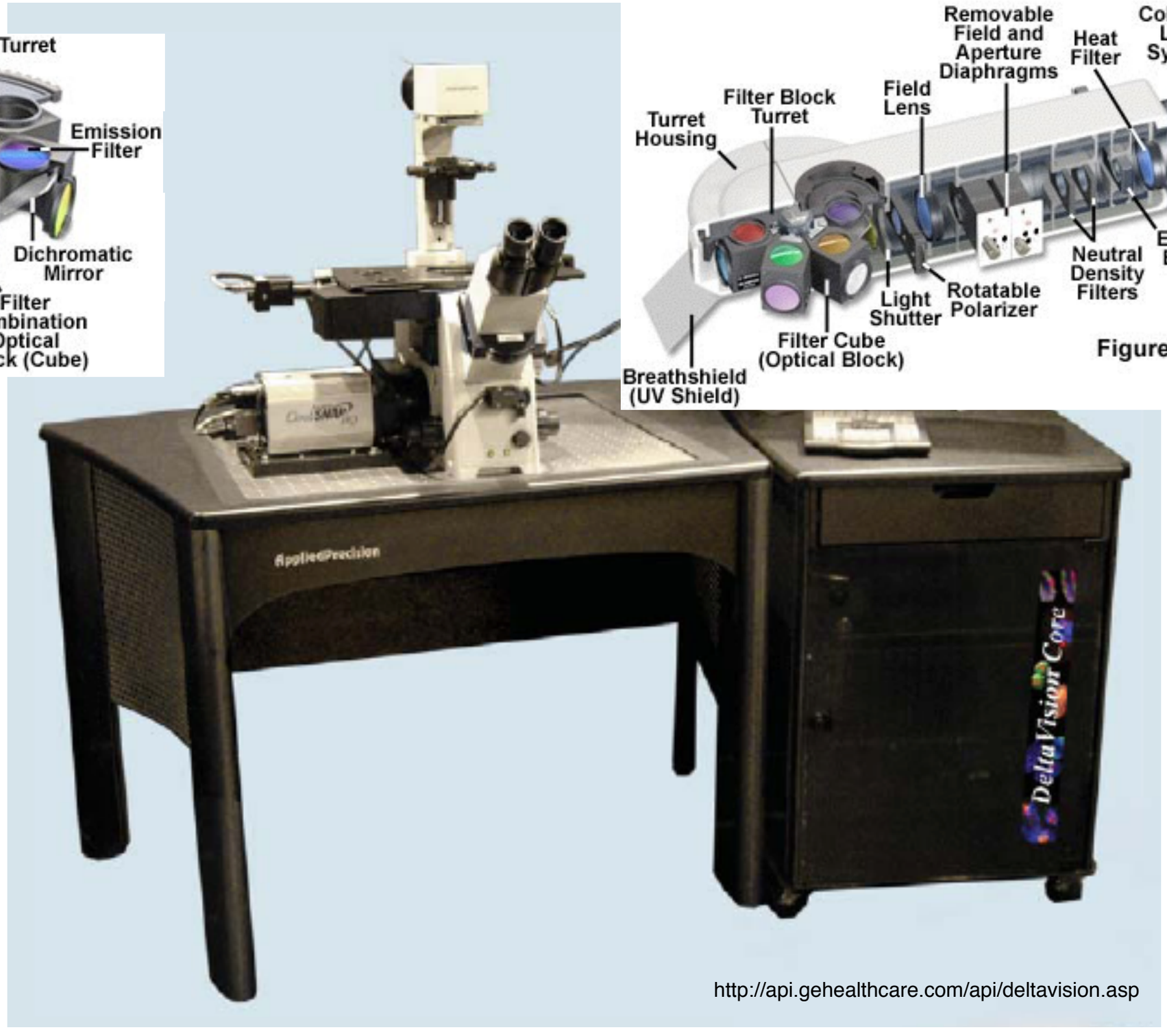

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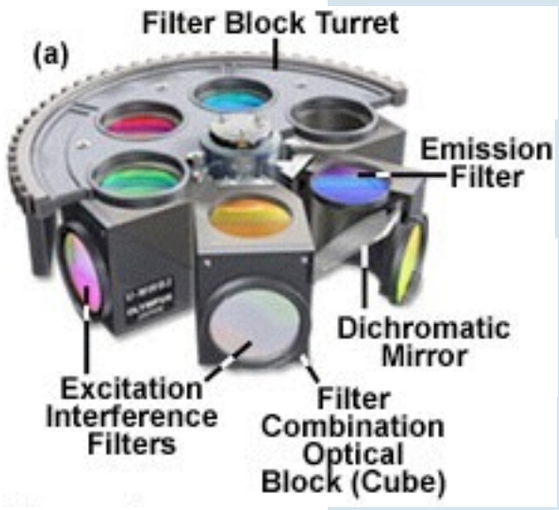
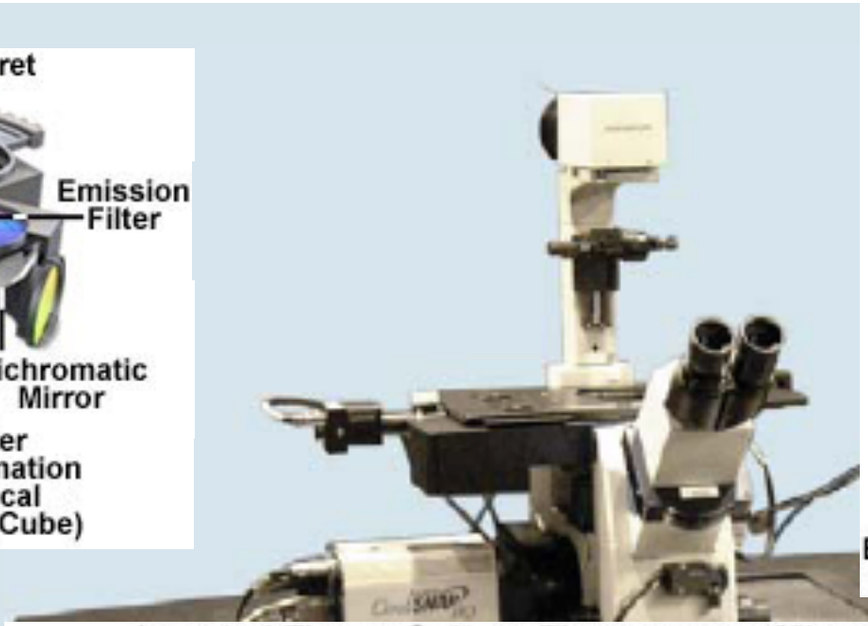
Figure 4

http://www.olympusmicro.com/

Understanding what goes on in the Microscope





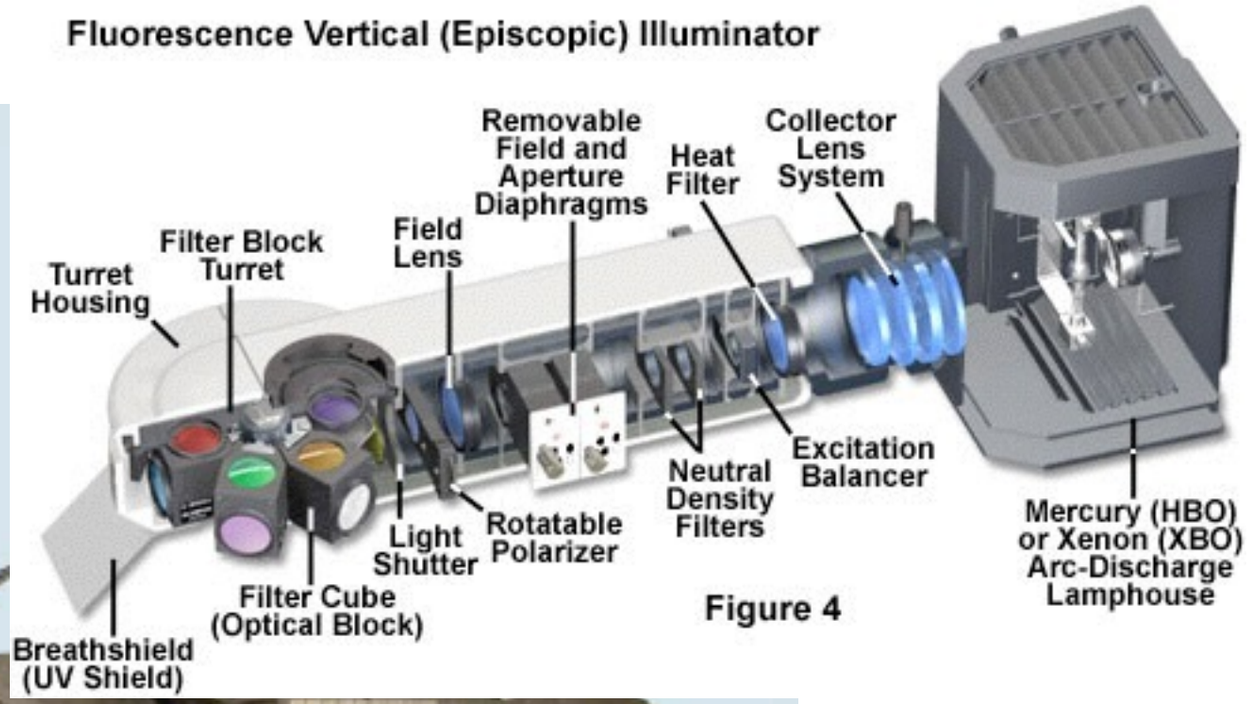


Figure 4

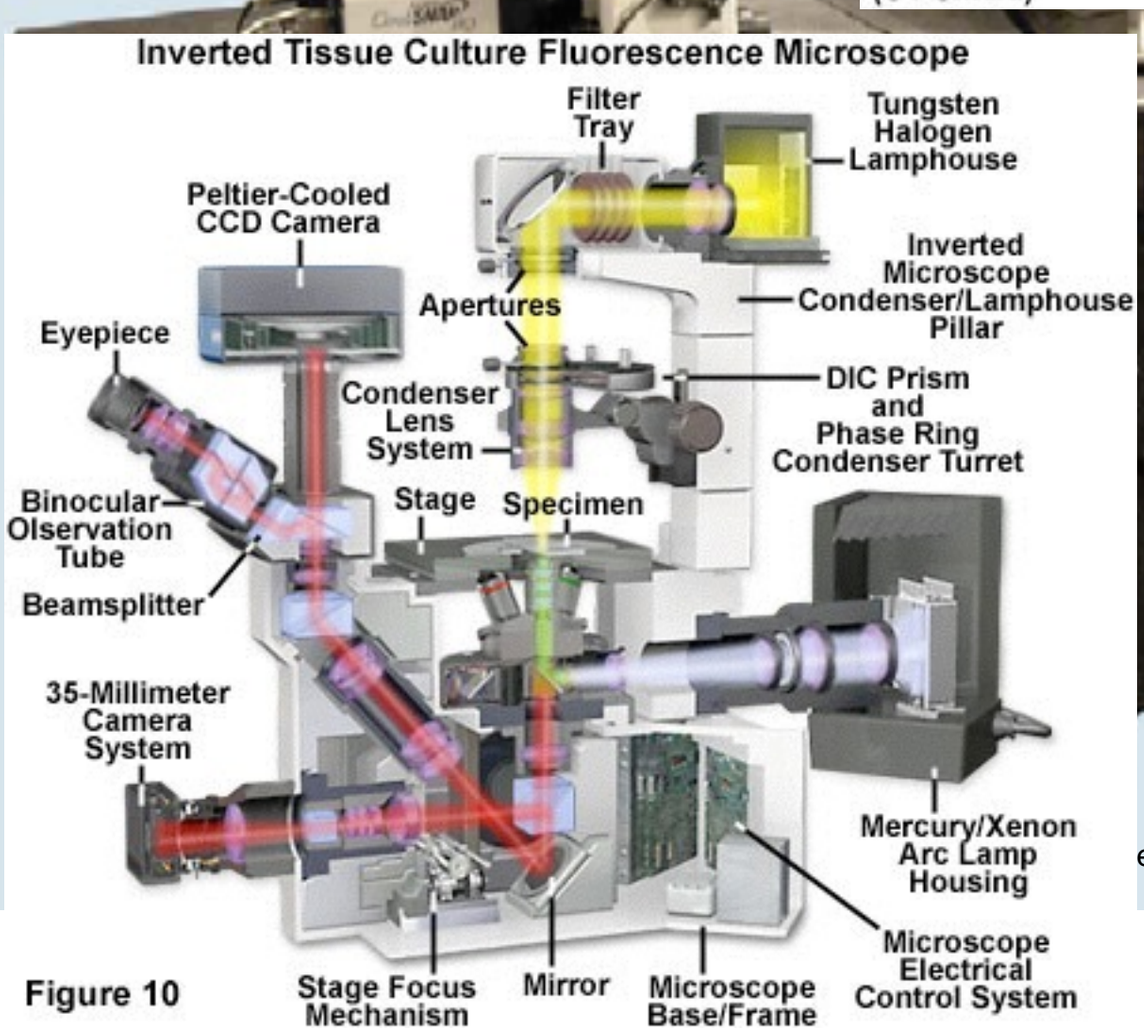


Figure 10

ehealthcare.com/api/deltavision.asp

http://www.olympusmicro.com/

What is really important in microscopy?

…….the ability to see stuff

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…….the ability to see stuff

1. Contrast

What is really important in microscopy?

…….the ability to see stuff

1. Contrast
2. Resolution

What is really important in microscopy?

…….the ability to see stuff

1. Contrast
2. Resolution
3. Sampling

What is really important in microscopy?

…….the ability to see stuff

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

What is really important in microscopy?

…….the ability to see stuff

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

and nothing else!

What is really important in microscopy?

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

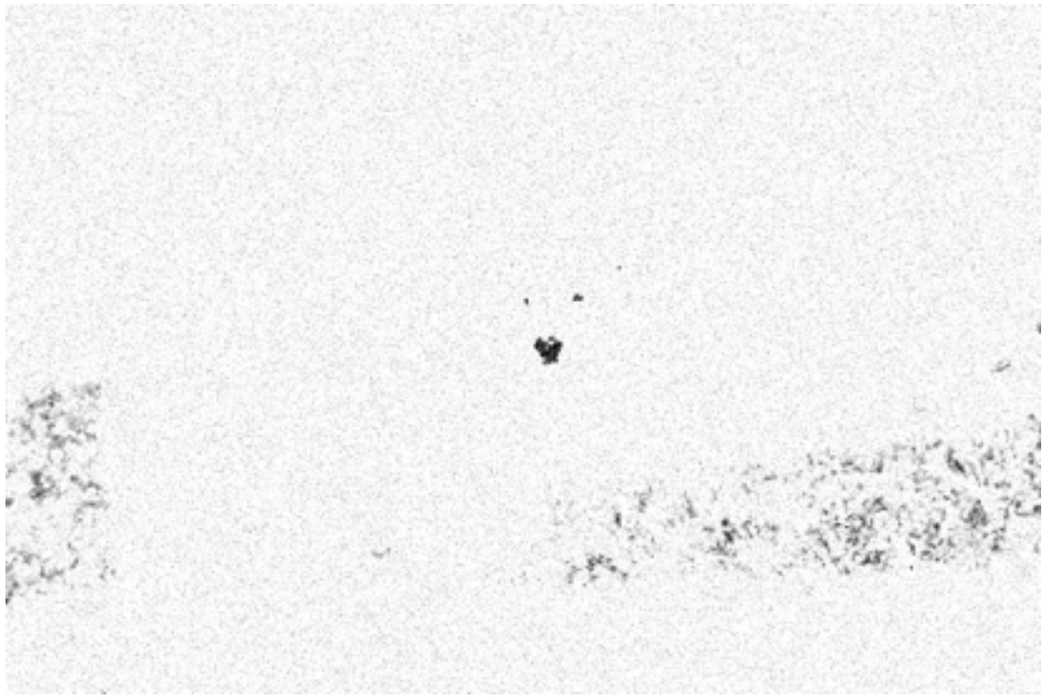
What is really important in microscopy?

Contrast .....the ability to distinguish stuff

What is really important in microscopy?

Contrast .....the ability to distinguish stuff

Biological specimens have low inherent contrast:



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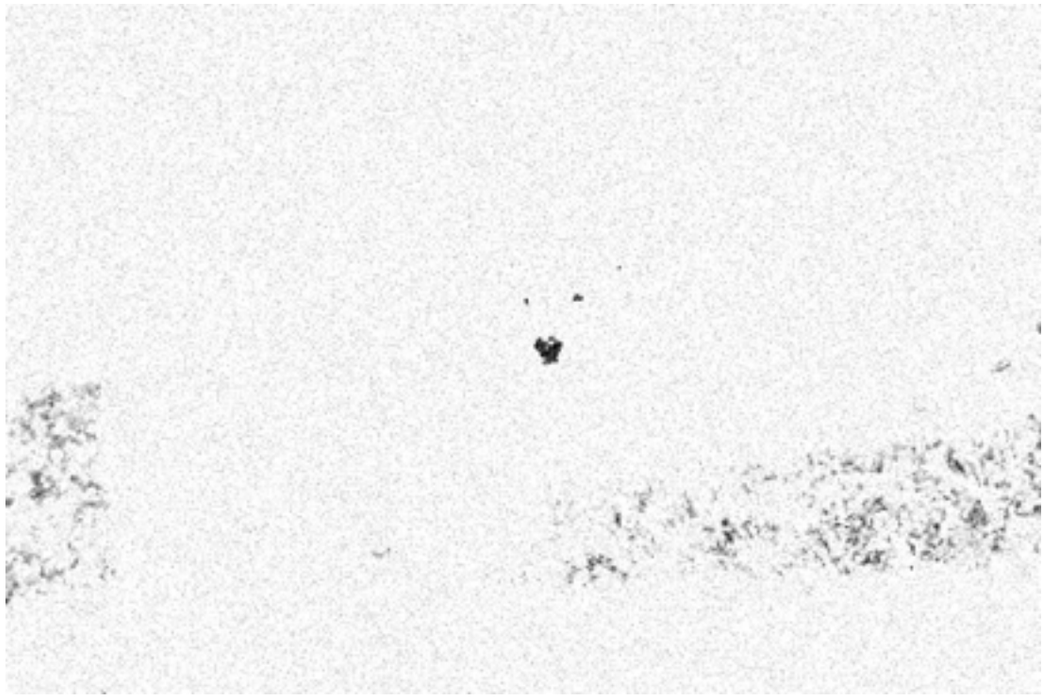

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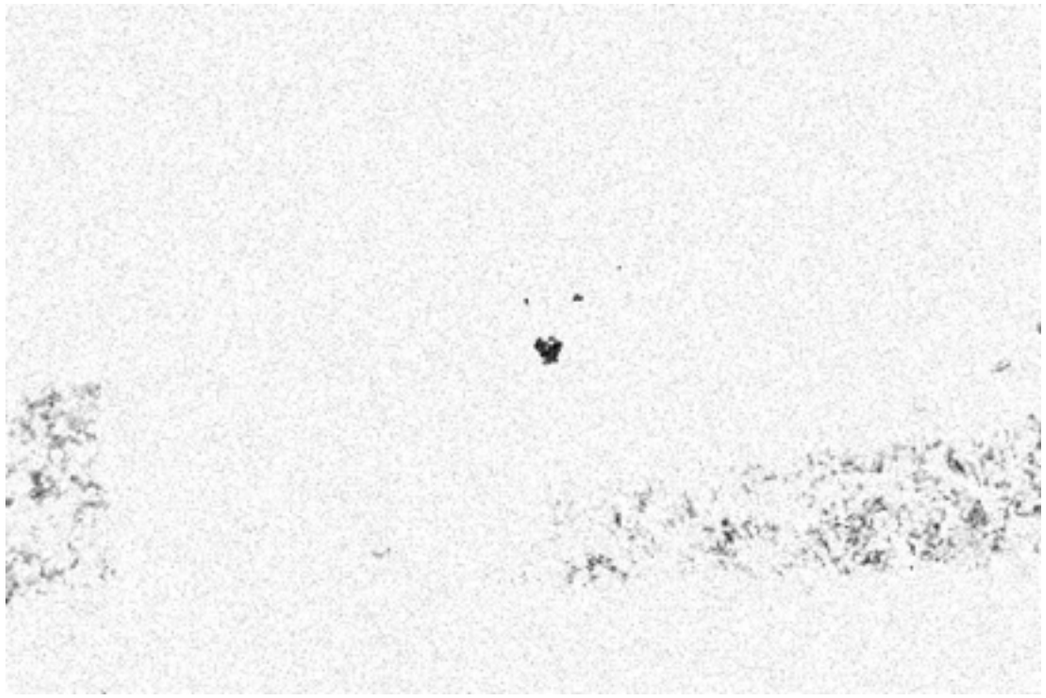

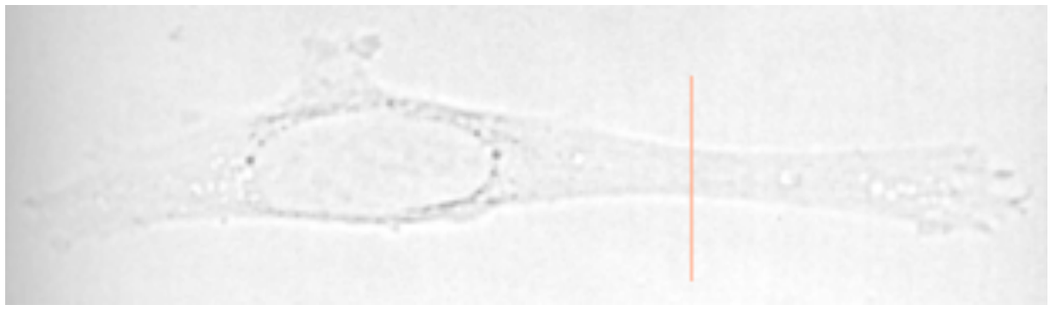

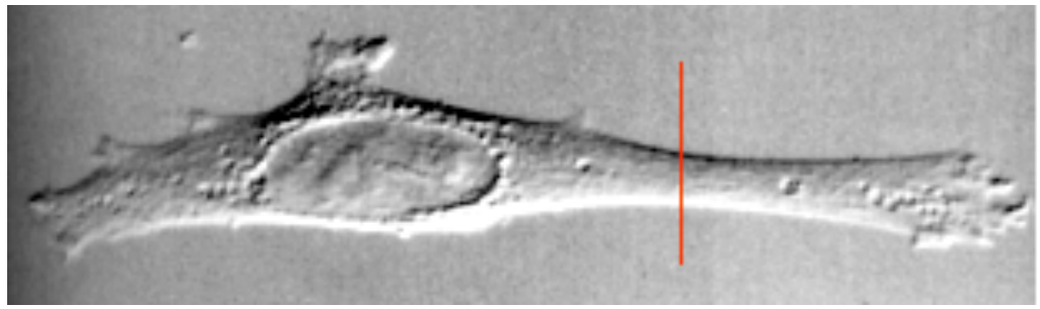

.....can’t resolve anything without contast

Bright Field Contrast Techniques

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

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RMP: Onion epidermis bright field

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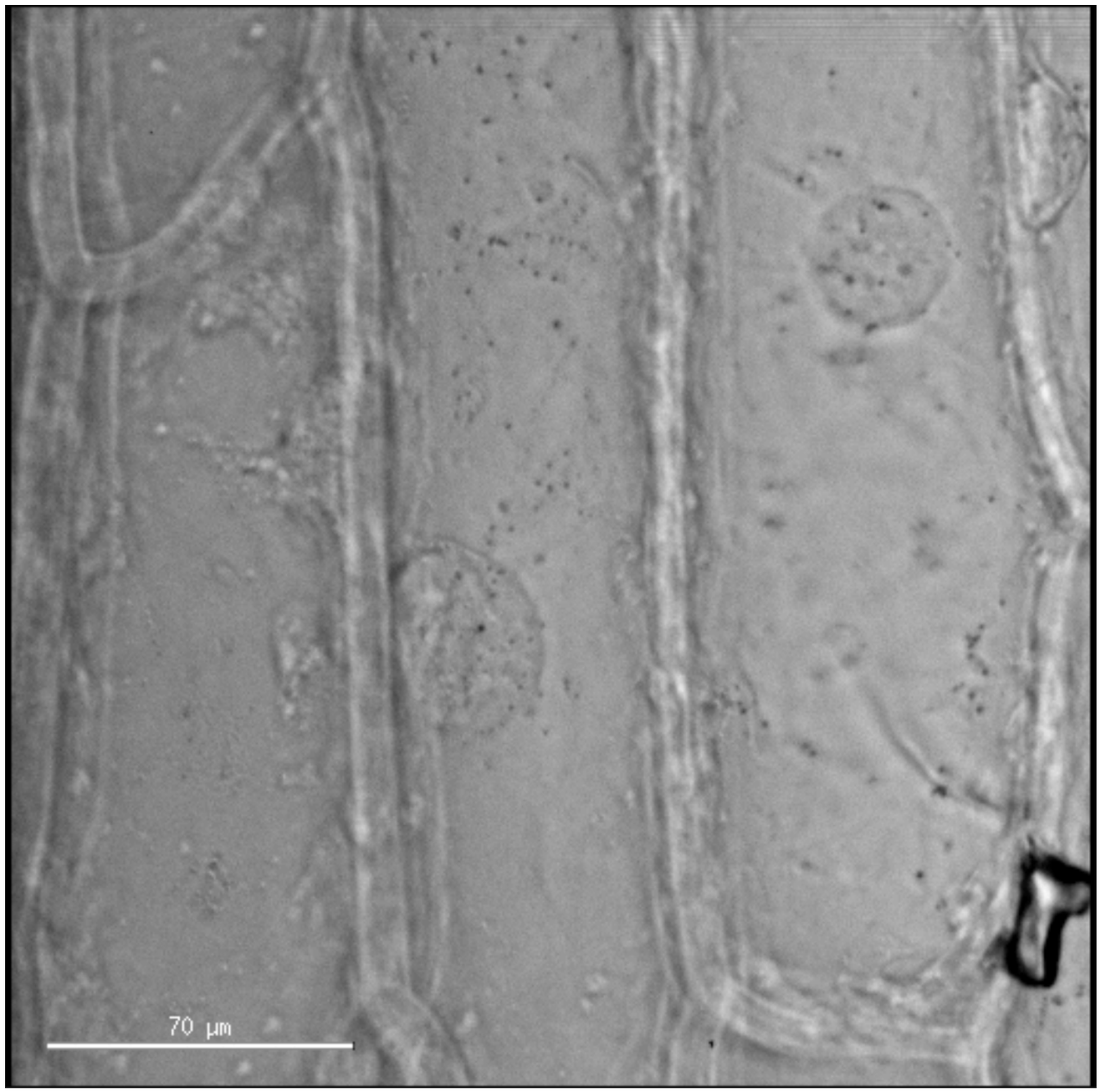
DIC differential interference contrast



RMP: Onion cytoplasmic streaming under DIC

Fluorescence Contrast Techniques

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

Fluorescence Contrast Techniques

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

GFP
green
fluorescent
protein

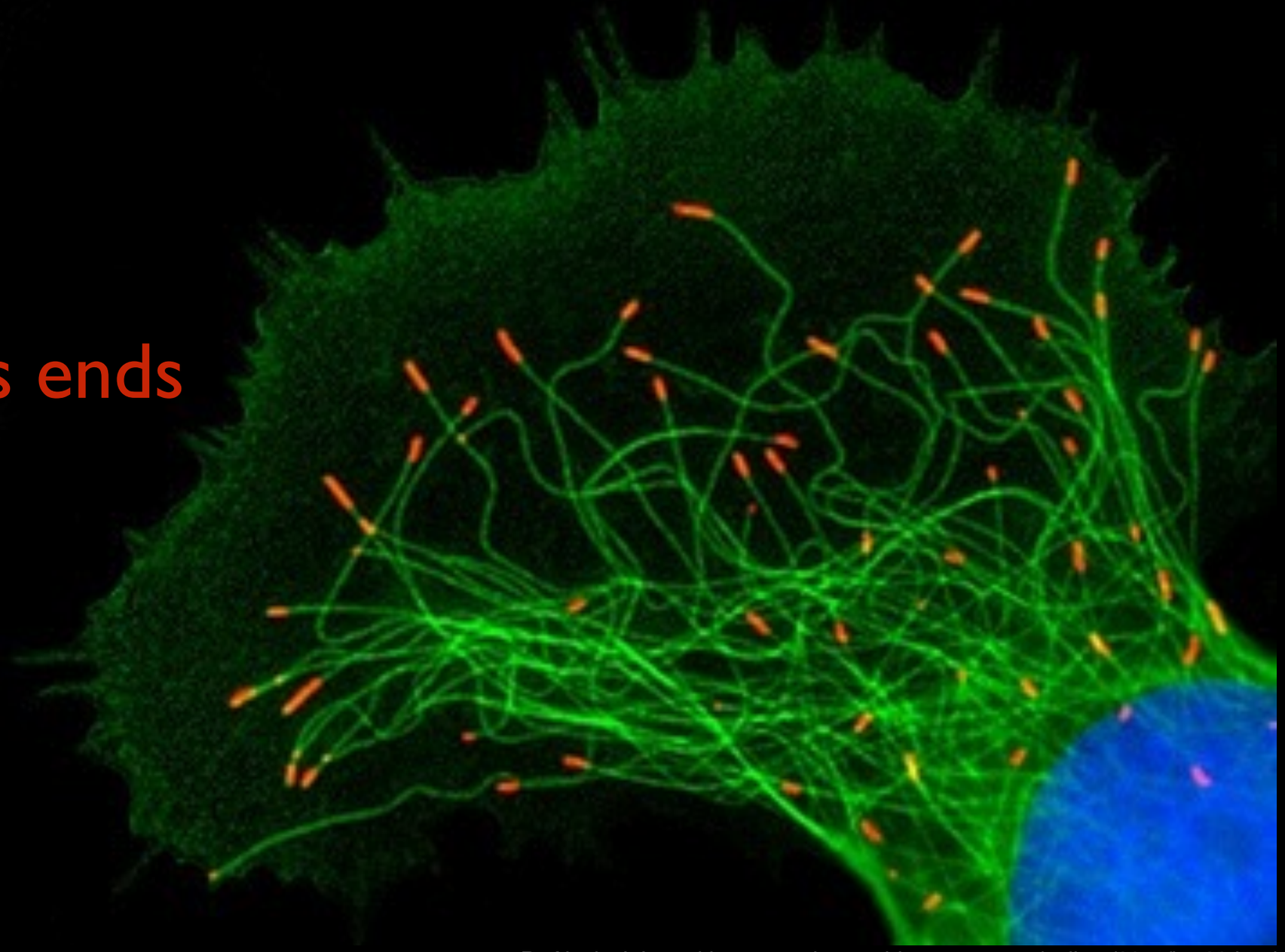
Fluorescence Contrast Techniques

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

Microtubules

Microtubule Plus ends

Nucleus



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

Practical - 1 Sample preparation

Practical - 2 Bright field contrast techniques

Practical - 3 Fluorescence

* Identifying the parts of the microscope, conjugate planes

* Setting up Koehler illumination and adjusting the condenser

* Different contrast techniques

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

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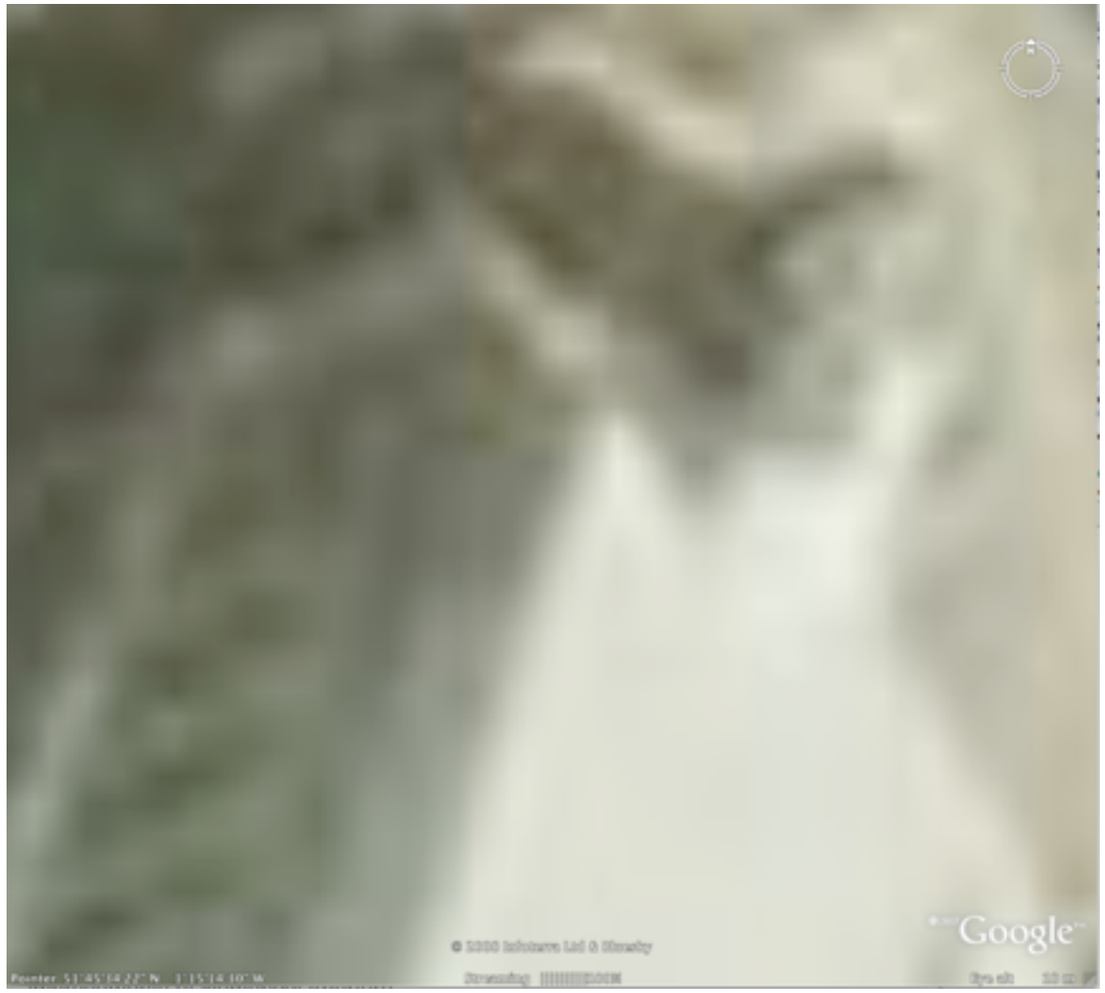
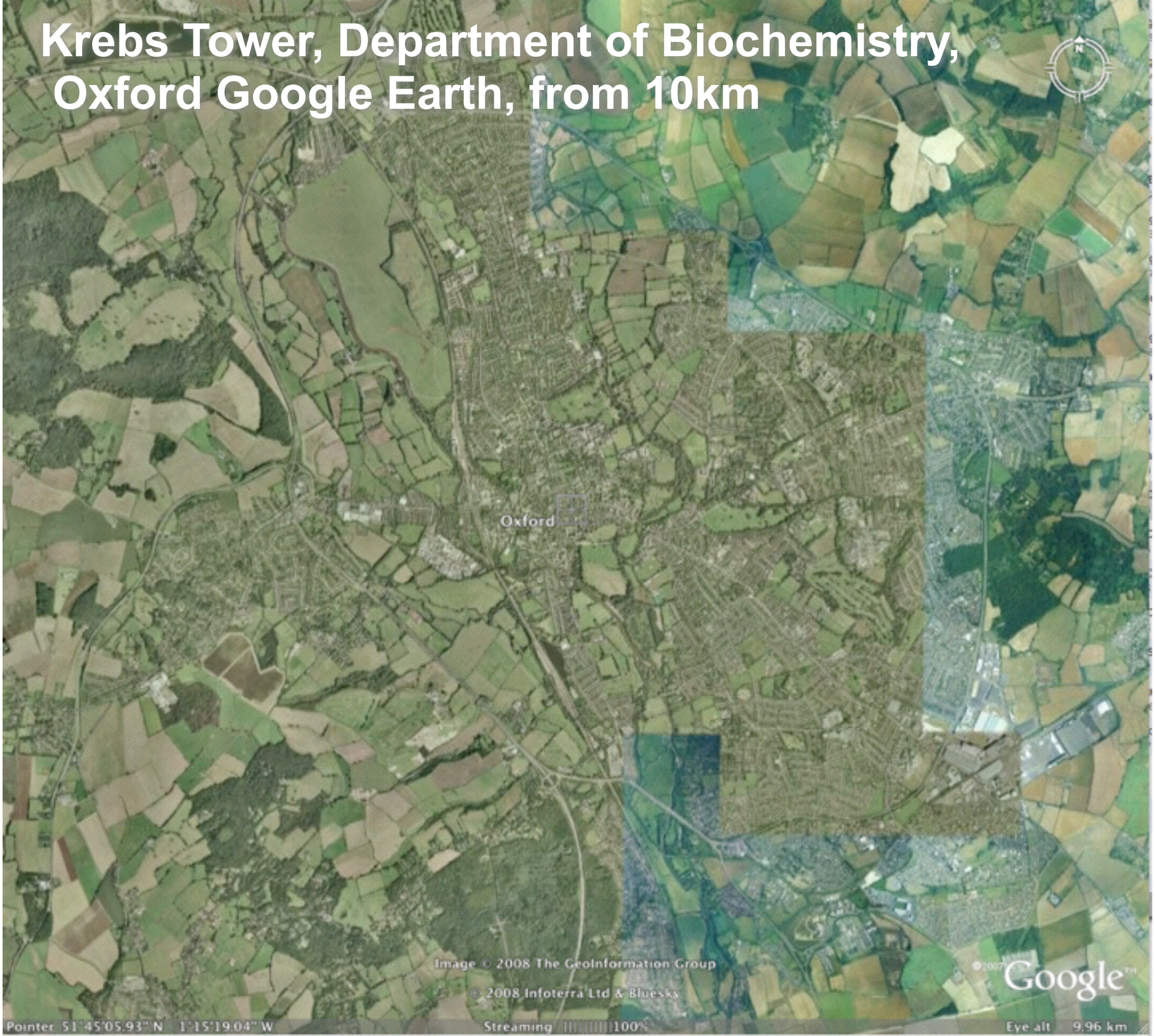
Magnifying is not enough:

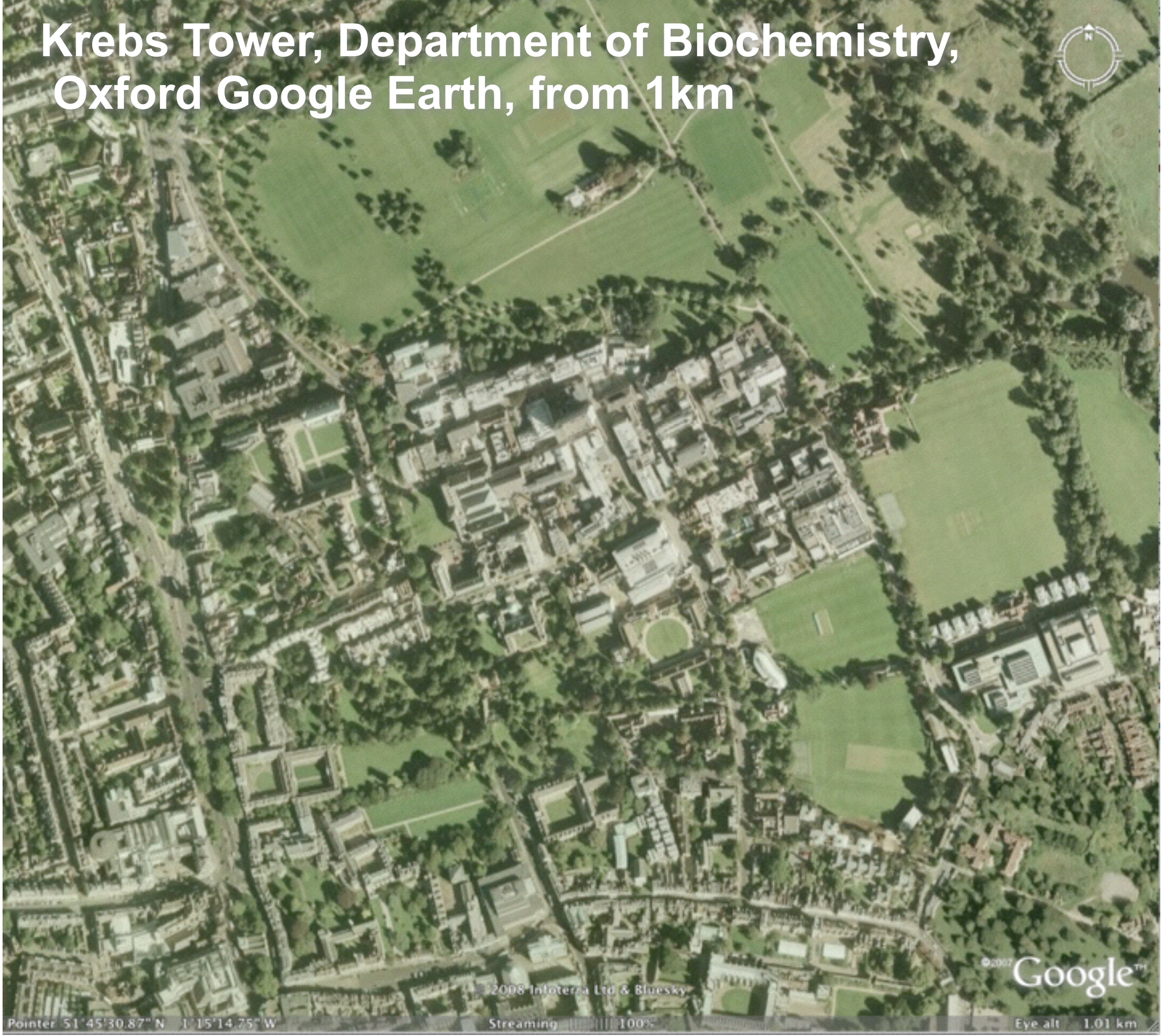


.....resolution is limited

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

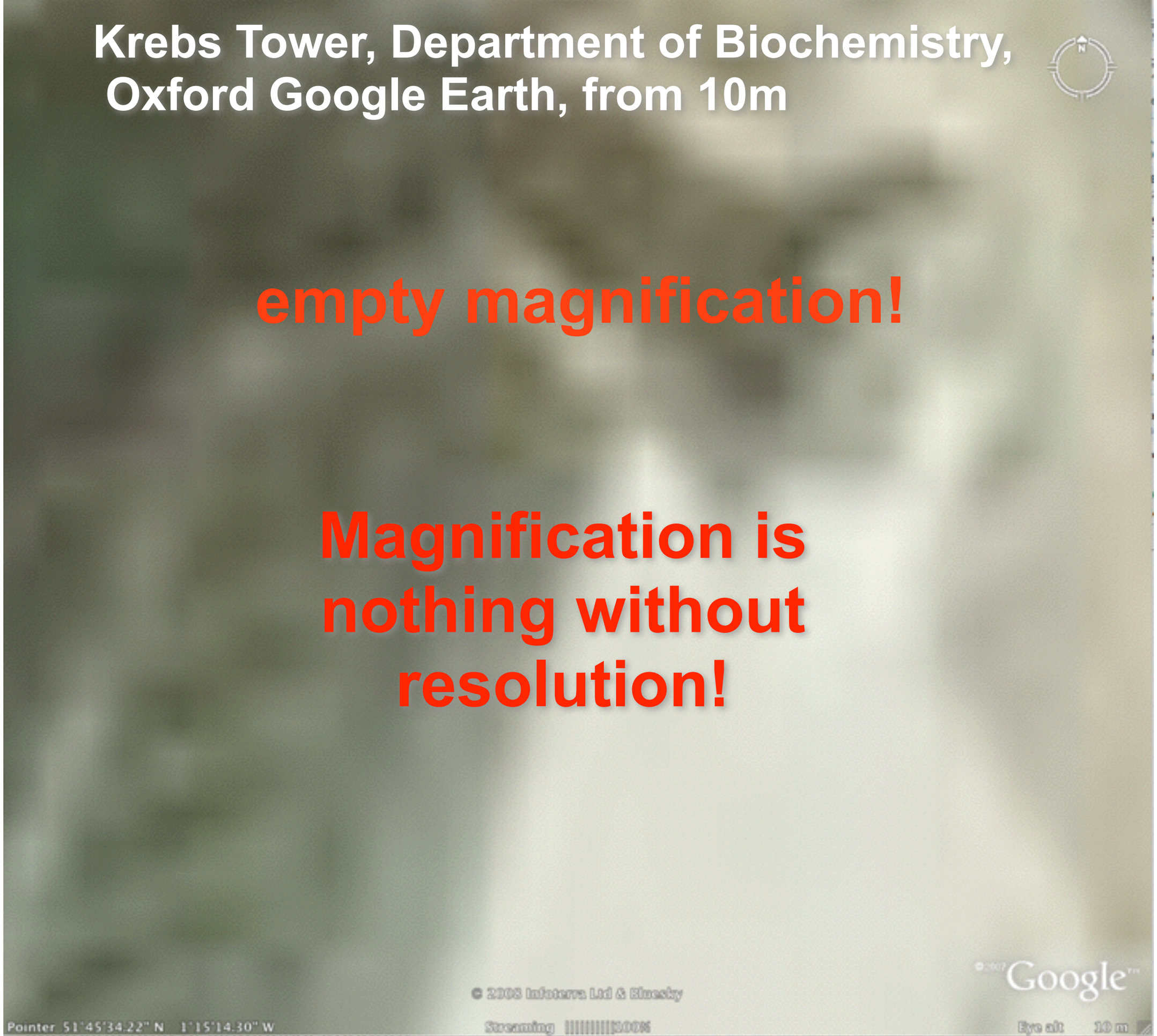


Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 1km



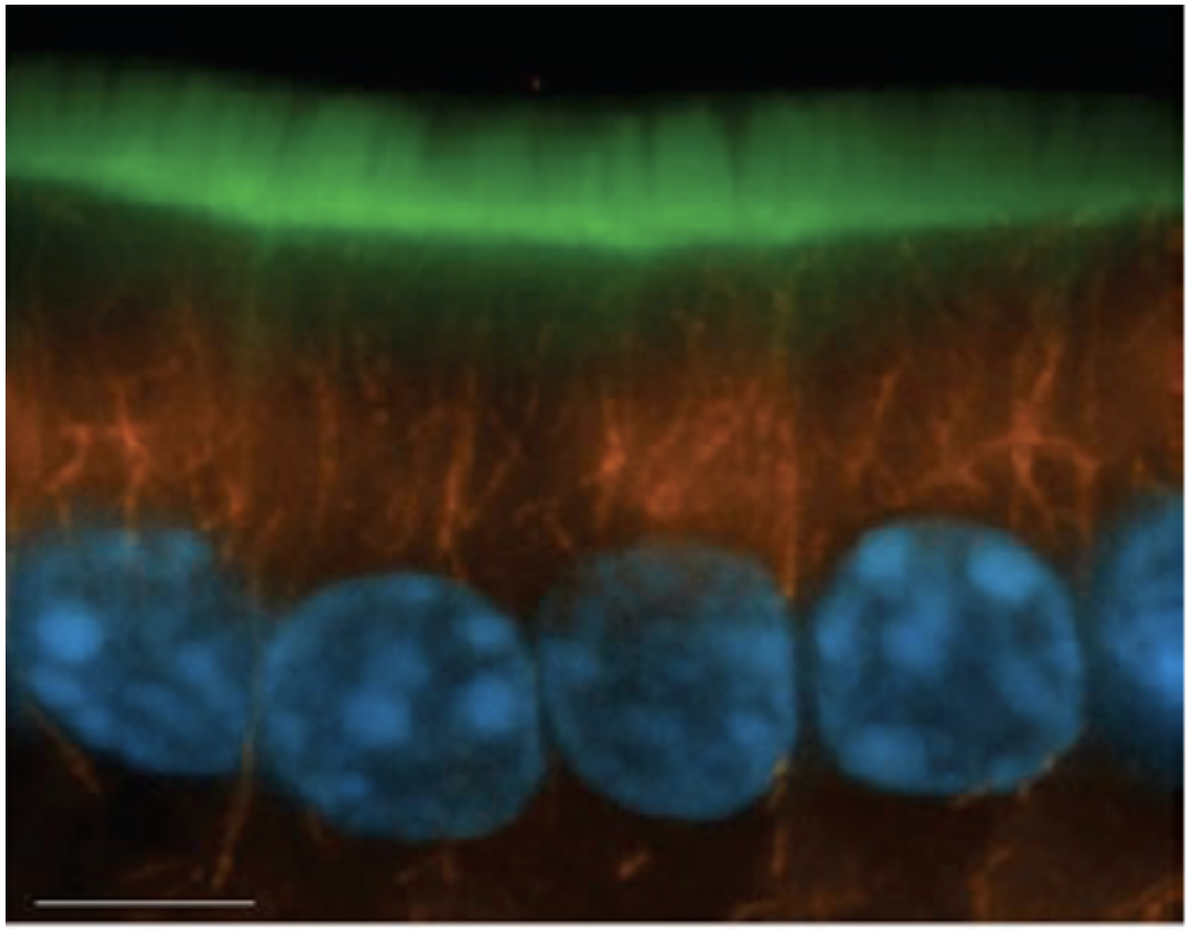
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 100m

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

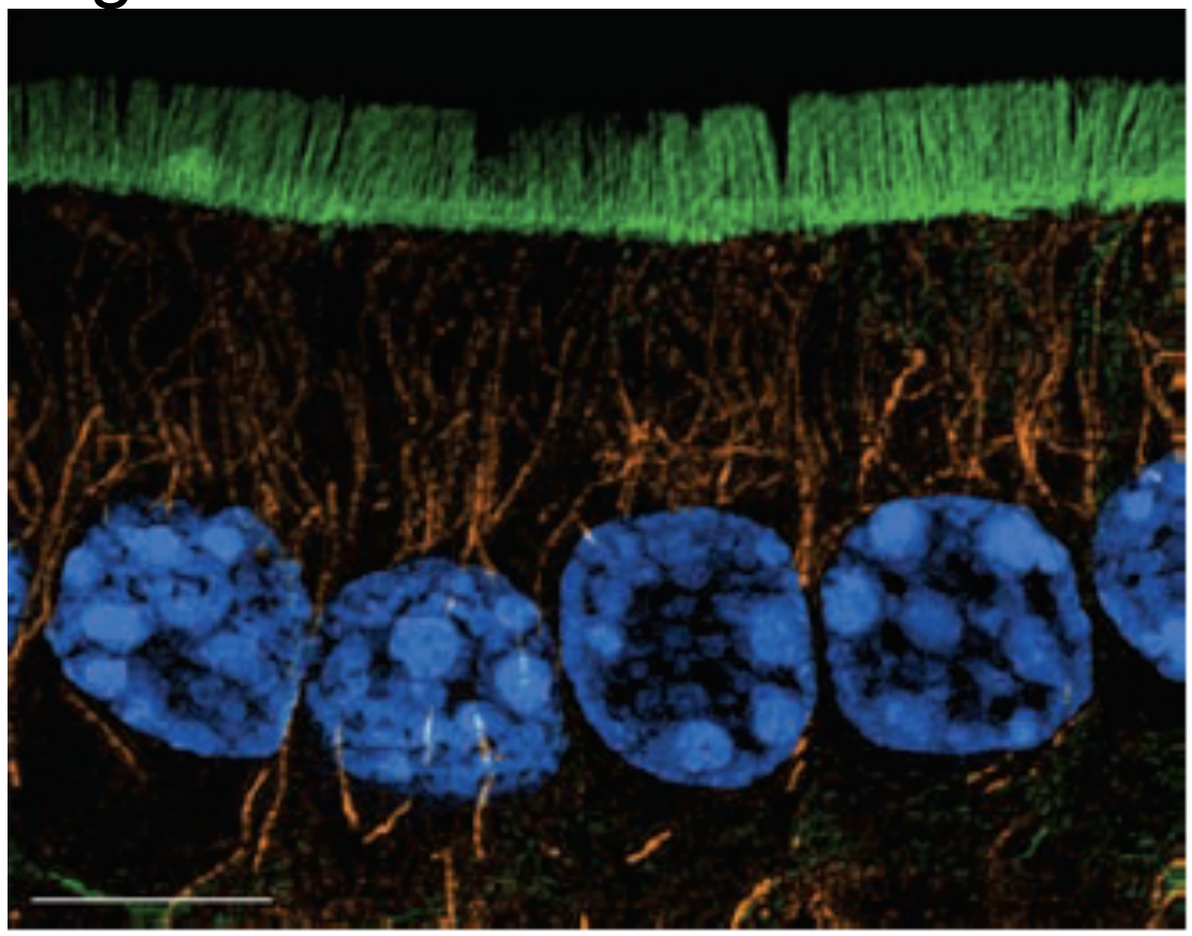

Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 10m
empty magnification!
Magnification is nothing without resolution!
© 2008 Infoterra Ltd & Bluesky
Google
Pointer 51°45'34.22" N 1°15'14.30" W
Eye alt 10 m

RESOLUTION

Normal resolution



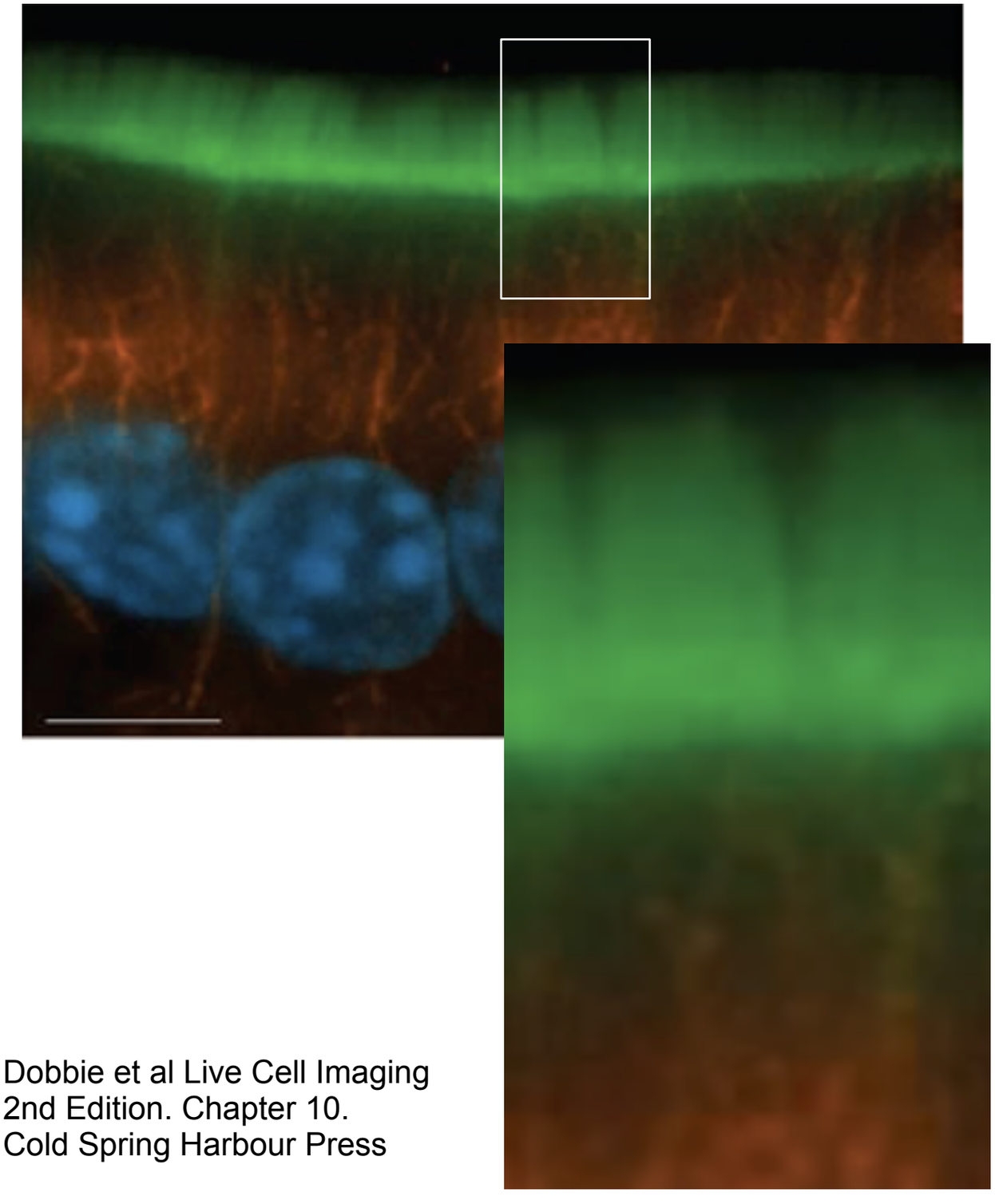
High resolution



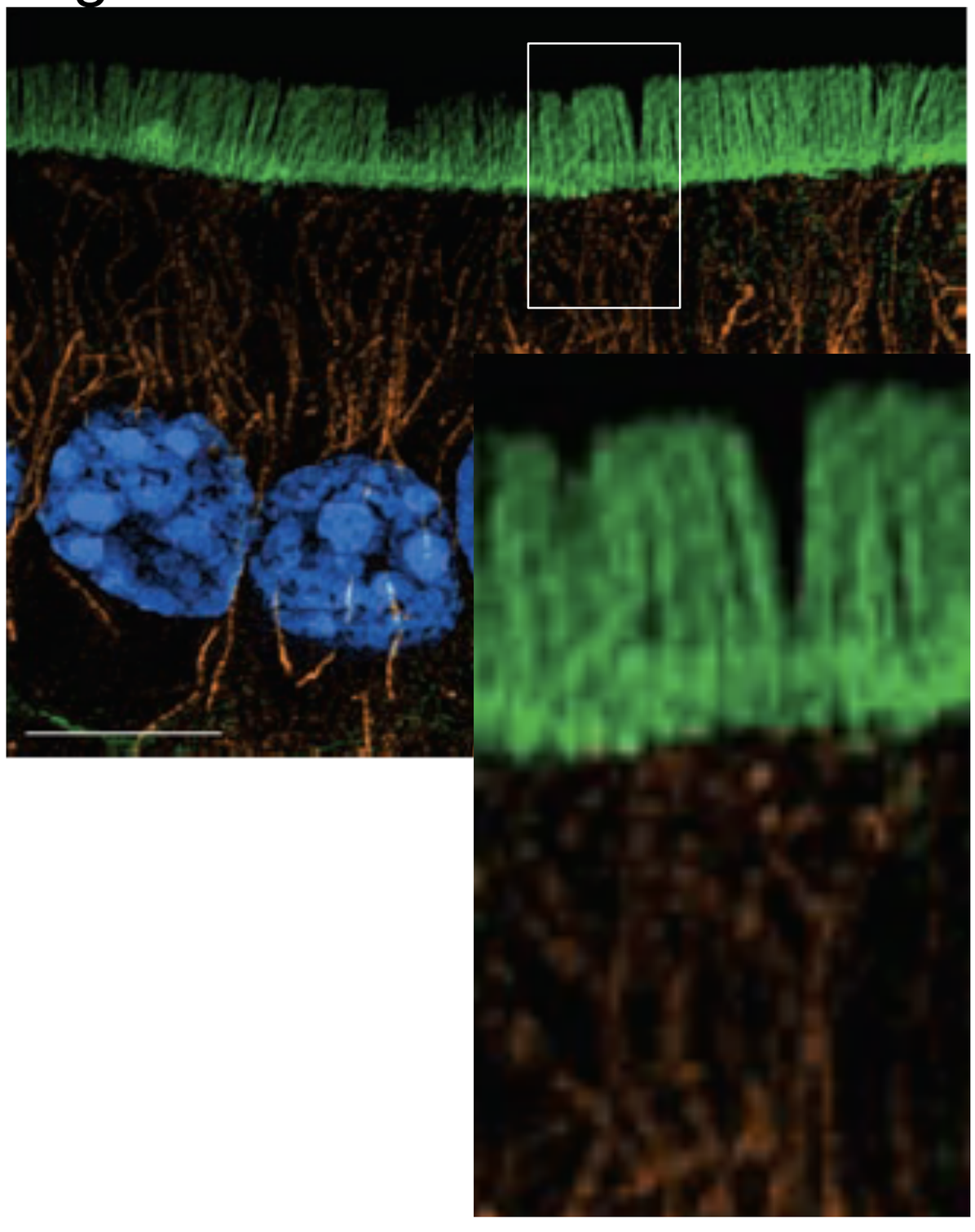
Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
Cold Spring Harbour Press

RESOLUTION

Normal resolution



High resolution



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Cold Spring Harbour Press

Understanding what limits Resolution

* 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

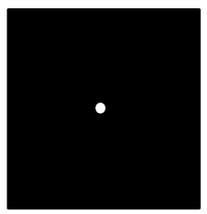
Convolution and the Point Spread Function

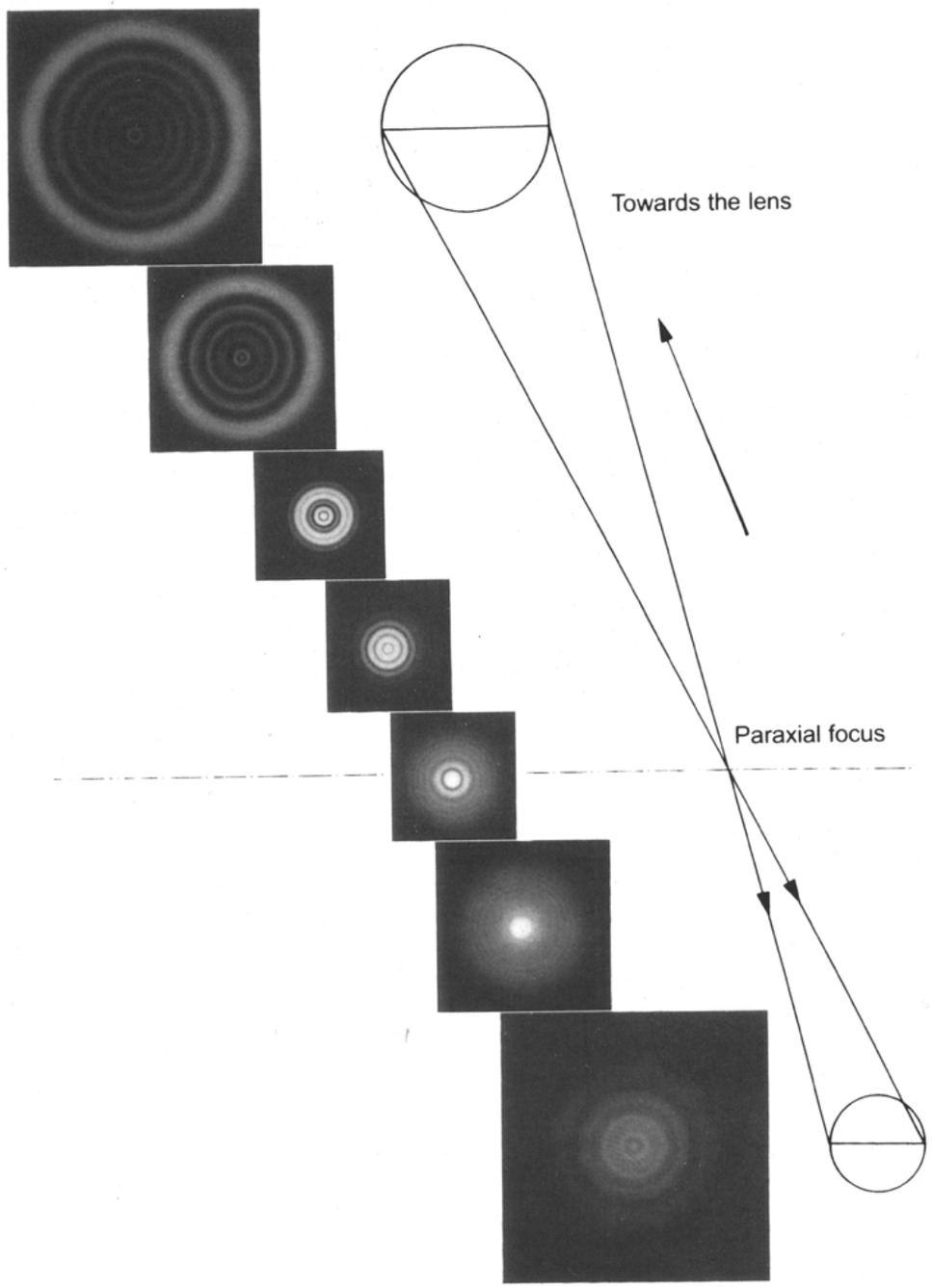
An image represents the output of the optics and detector of the imaging system

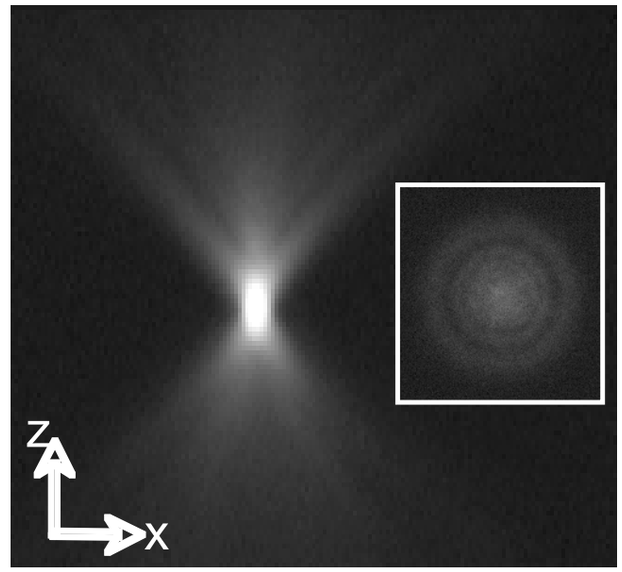
image ≠ object

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

Practicals 4-5 Fluorescence and the PSF

* 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

* Using the PSF to assess optical performance and aberrations

Bead slides - Practical 4:

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)

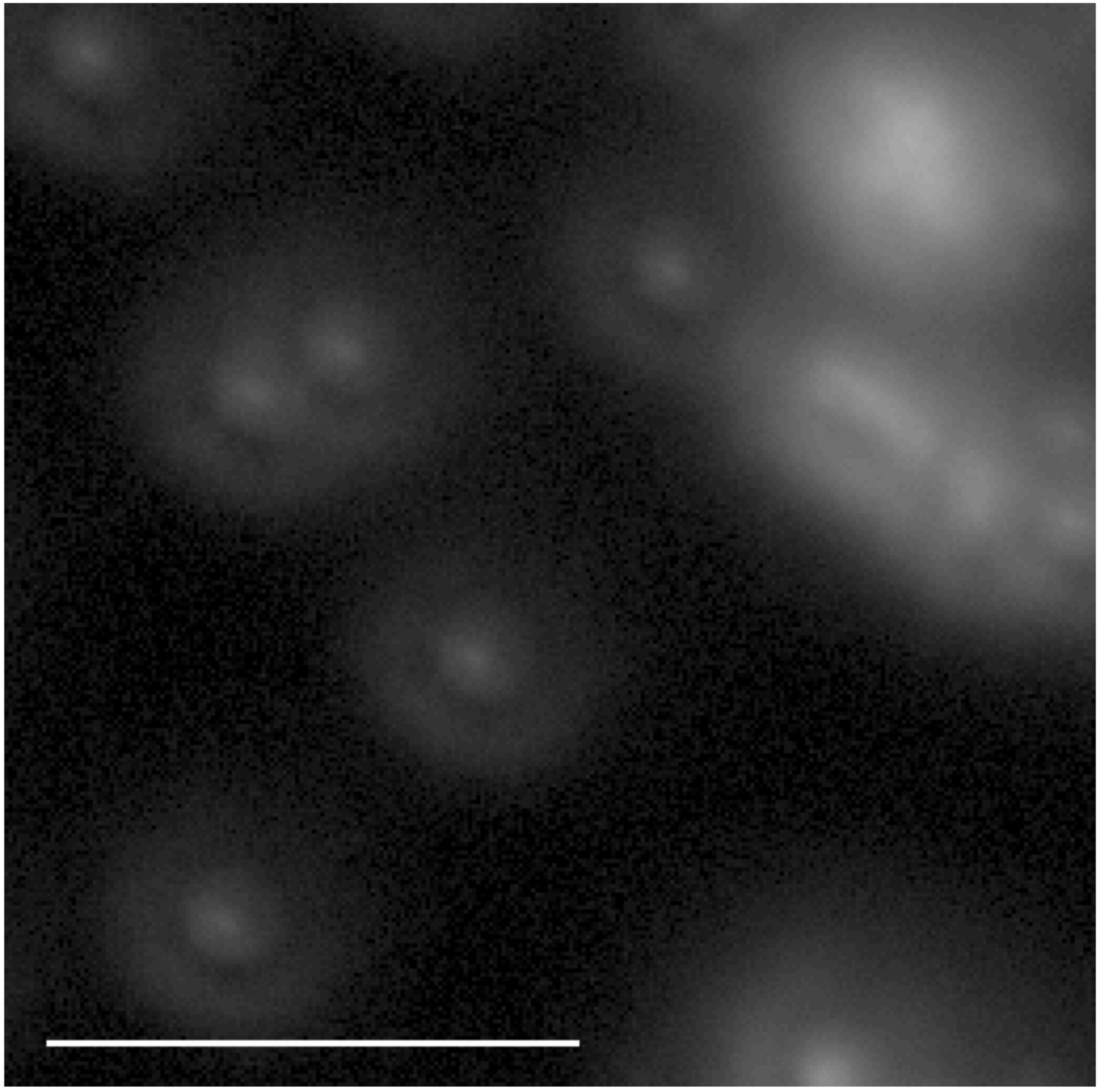
Diameter

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

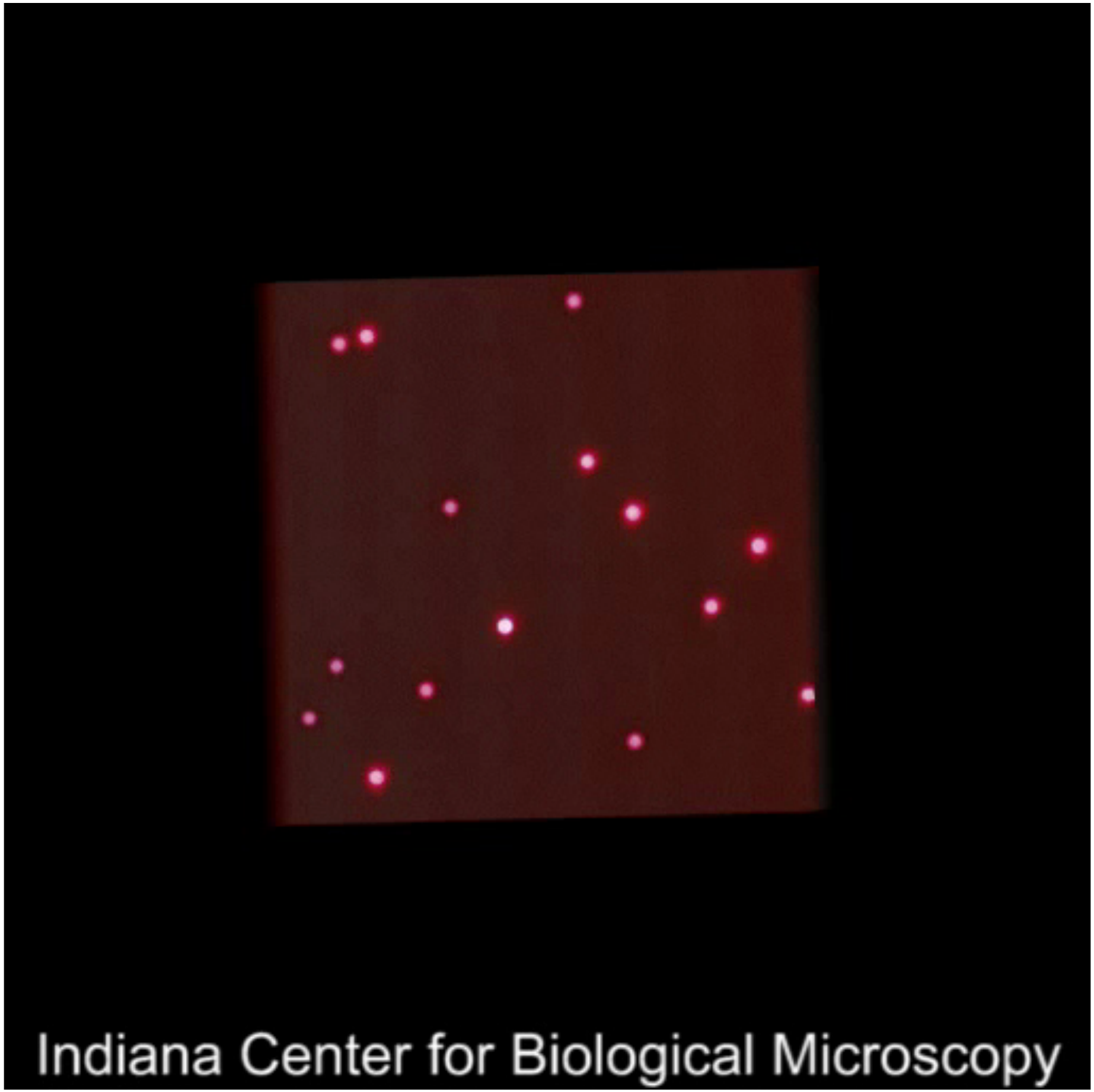
Product Name	SKU #	Calibration Type	Product Size	List Price GBP
Constellation™ Microspheres for Imaging, mixture of assorted sizes & colors	C-14837	Fluorescence Microscope Calibration	3 mL	68.50
FocalCheck™ Fluorescence Microscope Test Slide #1, for alignment, intensity, & calibration	F36909	Confocal 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

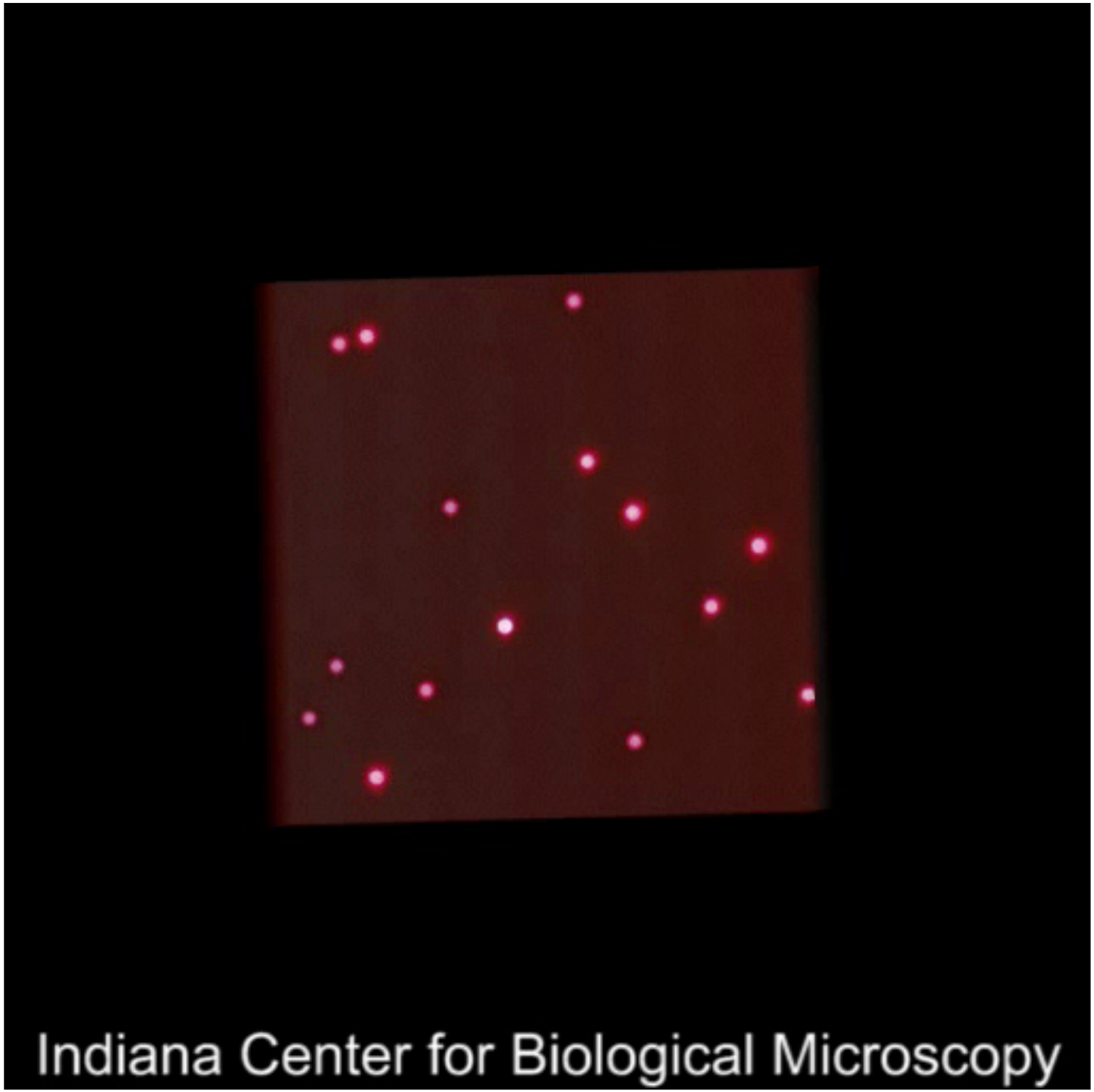
http://www.lifetechnologies.com/uk/en/home/references/molecular-probes-the-handbook/tools-for-fluorescence-applications-including-reference-standards-and-optical-filters/fluorescence-microscopy-reference-standards-and-antifade-reagents.html#head3

Bead slides - Practical 4:



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

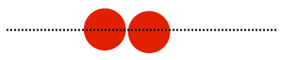

Indiana Center for Biological Microscopy


Indiana Center for Biological Microscopy

Calculating Lateral Resolution: The Rayleigh Criterion

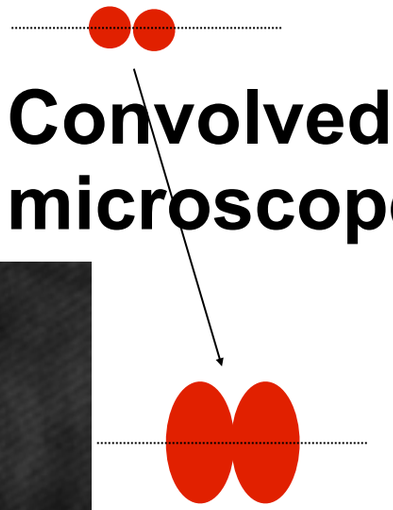
Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects

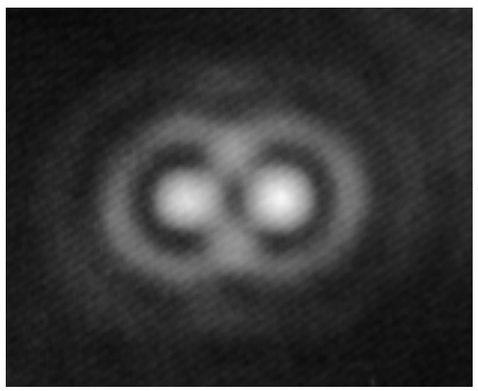


Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects



Convolved by microscope



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

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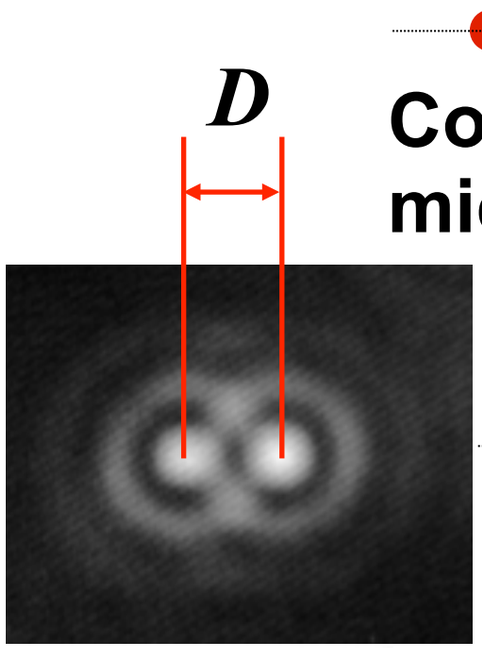
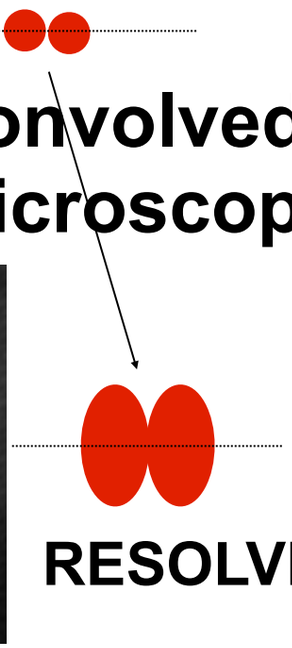
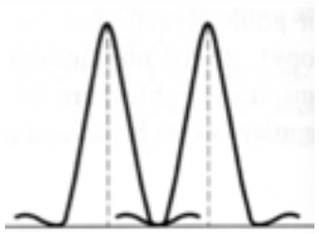


Convolved by microscope



RESOLVED

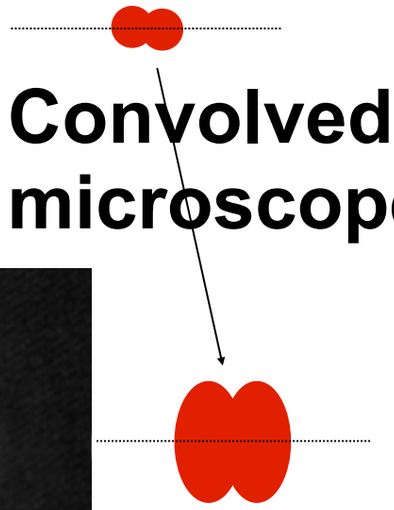


D = the distance between the two closest points that can still be distinguished

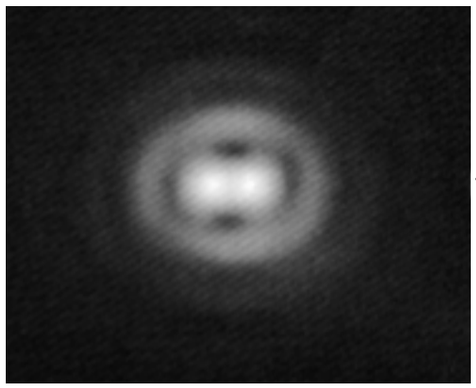
Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects



Convolved by microscope



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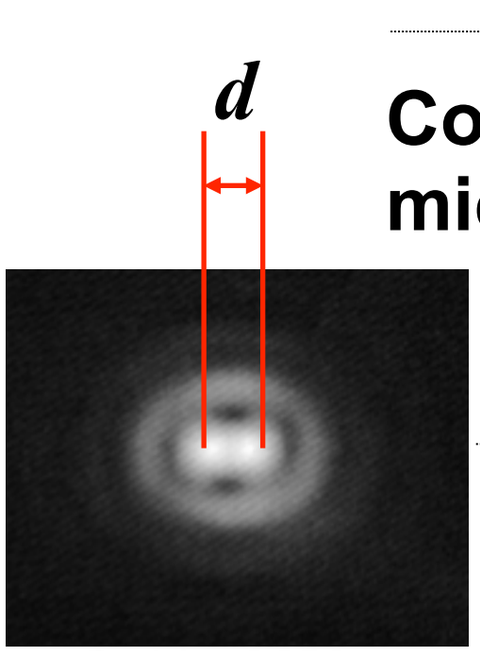

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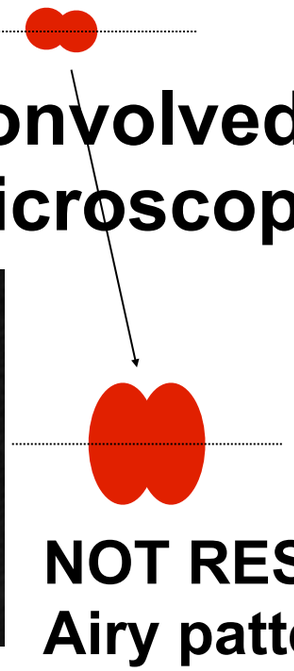
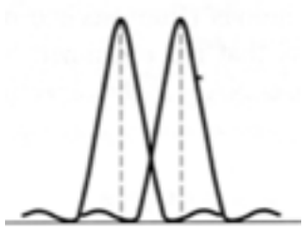


NOT RESOLVED
Airy patterns must Overlap by less Than 42% of the Maximal intensities



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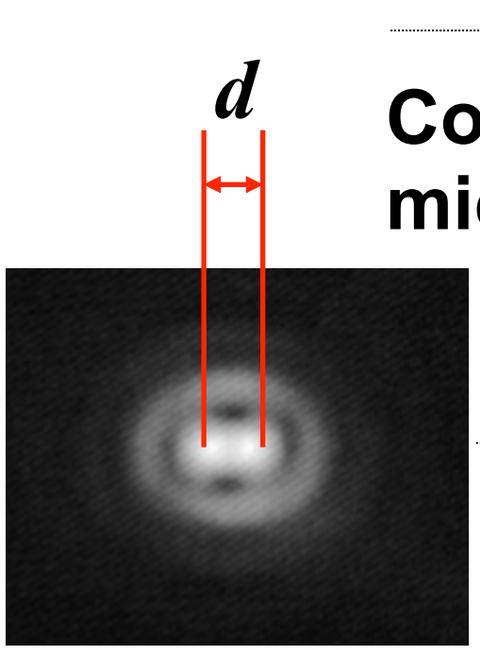
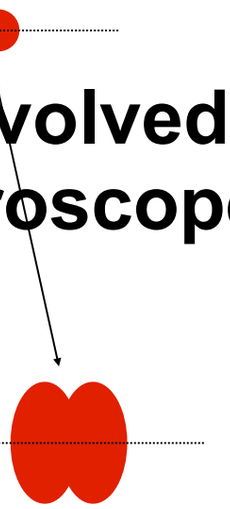
Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects



Convolved by microscope



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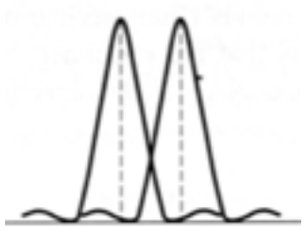


D = the distance between the two closest points that can still be distinguished

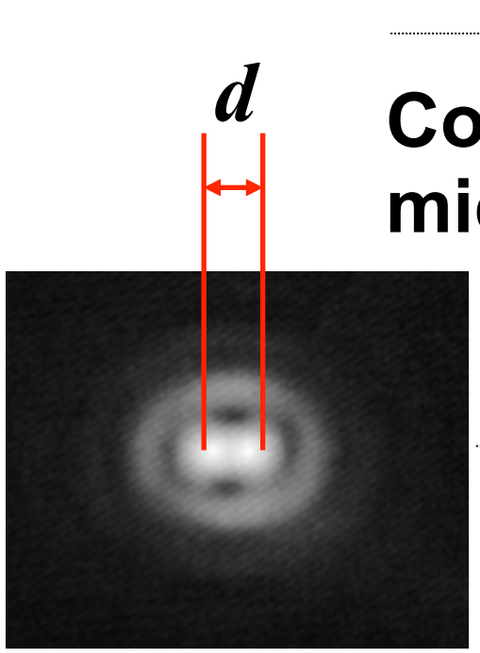
$$D=1.22\ \lambda/(NA_{obj}+NA_{cond})$$

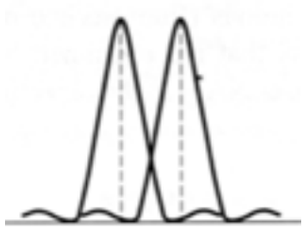
Epi-Fluorescence: $NA_{cond} = Na_{obj}$

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

Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects





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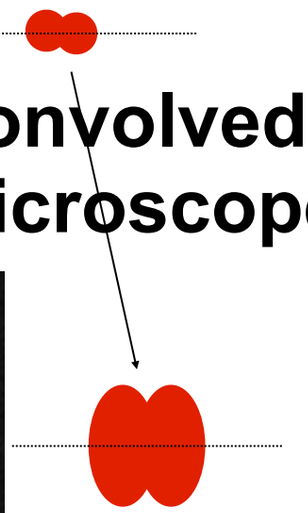

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D = the distance between the two closest points that can still be distinguished

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

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

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

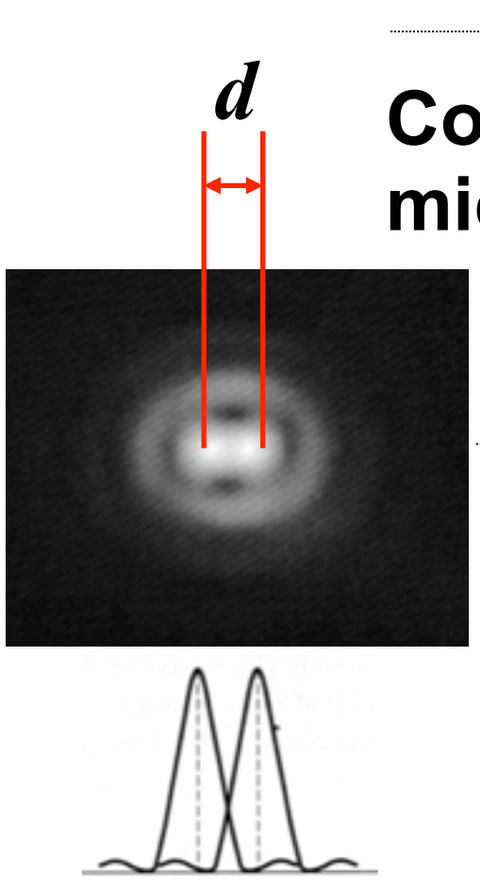
$$D = 1.22 \times 520_{nm} / 2 \times 1.4$$

$$D = 227\ nm$$

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

Calculating Lateral Resolution: The Rayleigh Criterion

Two small objects



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

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

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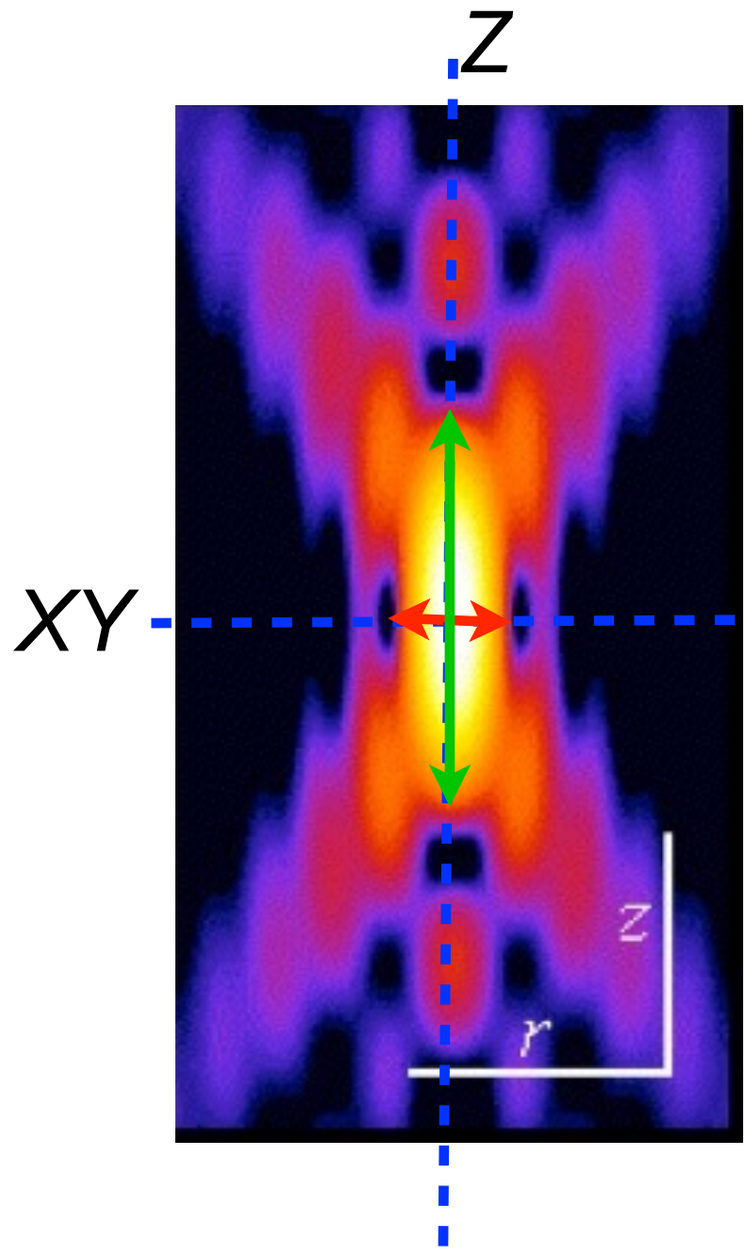
Axial Resolution:

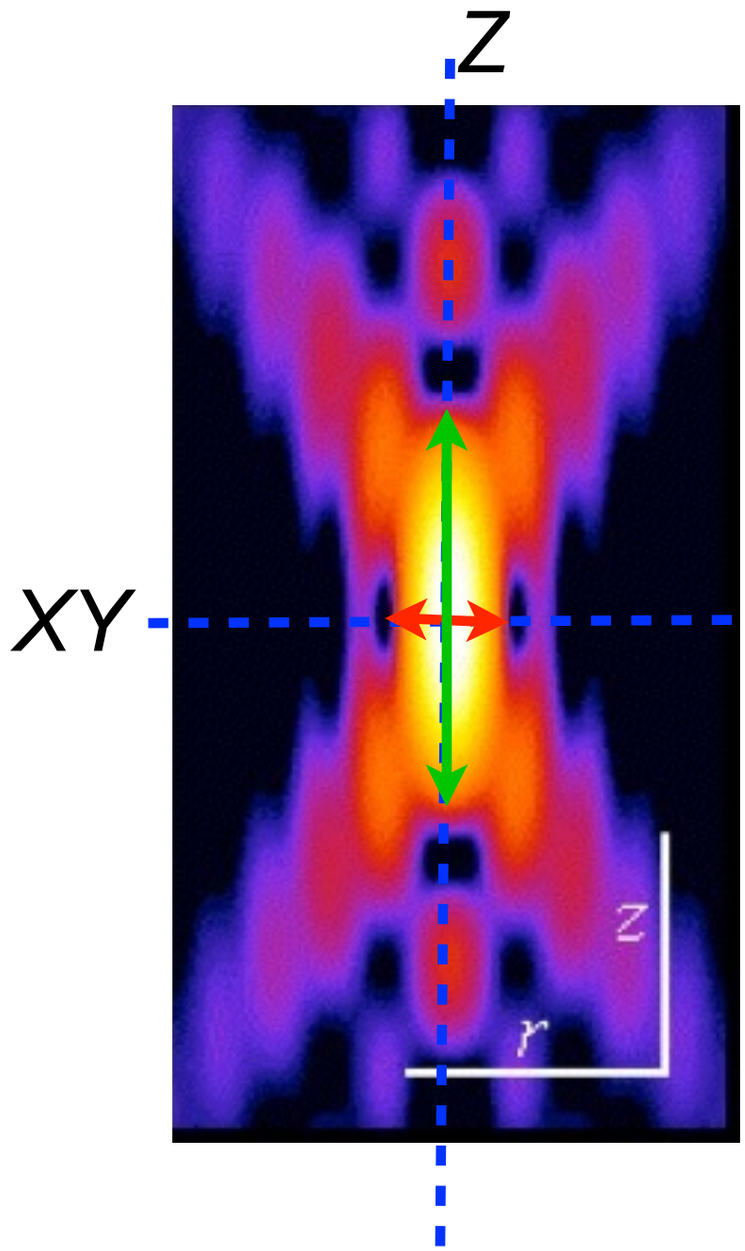


Axial Resolution:

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

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

(η = refractive index of the object medium)



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

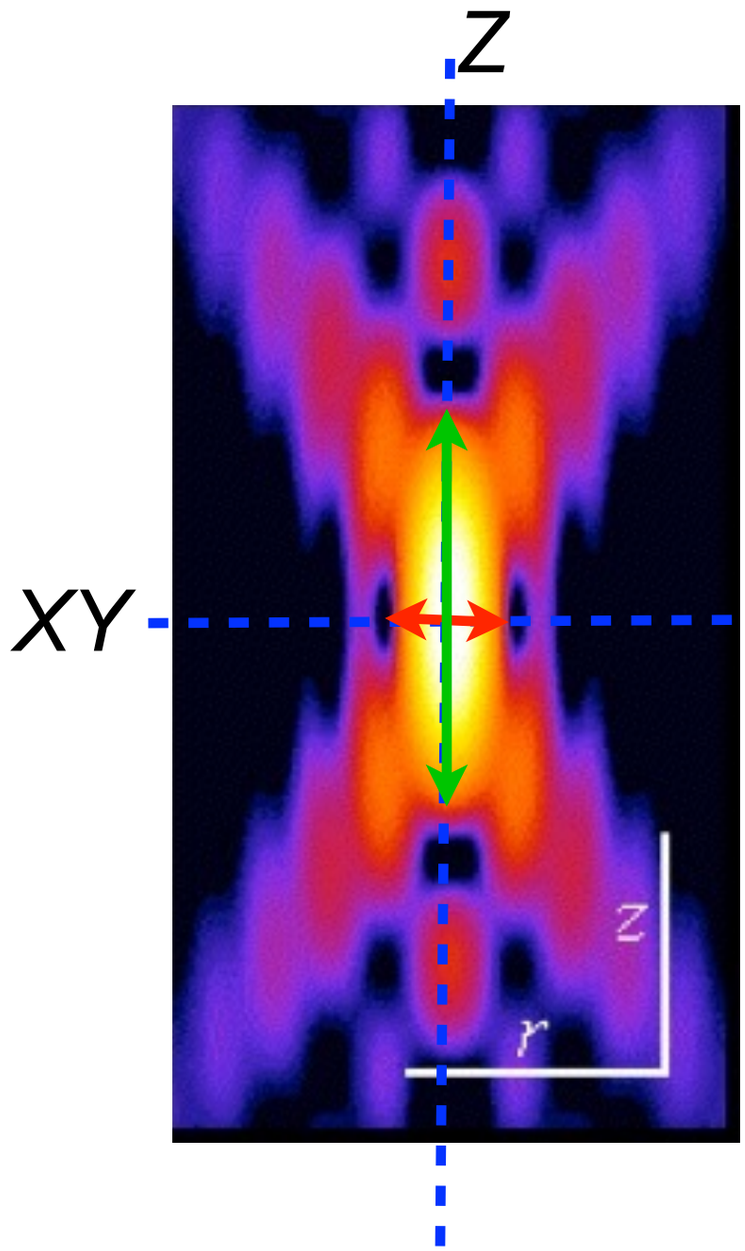
$$D_Z = 2\lambda\eta/(NA_{obj})^2 \quad \ldots\ldots\ldots\ldots 705$$

(η = refractive index of the object medium)

Than it is in the lateral dimension (XY)

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

FWHM



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

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

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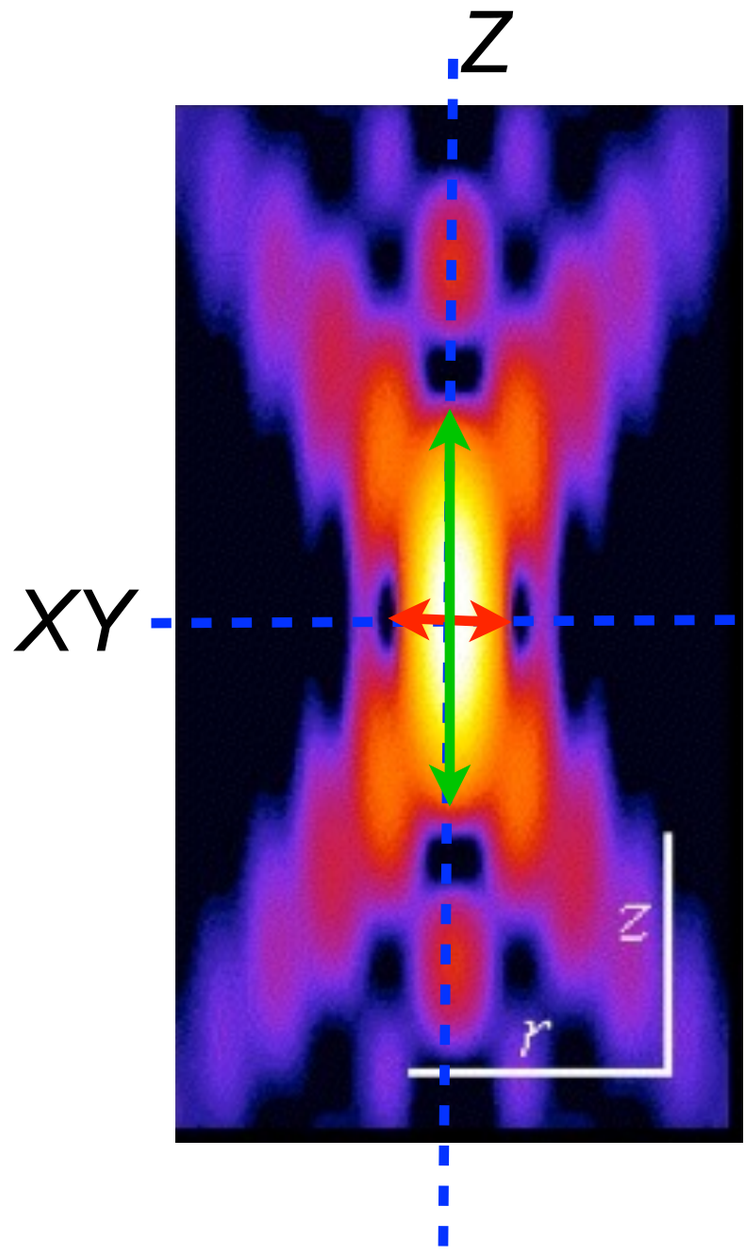


Than it is in the lateral dimension (XY)

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

FWHM

The relationship between the two is:

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

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 MT is ~ 25 nm diameter

Resolution: Down to the molecular scale?

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

XY resolution ~ 230 nm
Z resolution ~ 700 nm

GFP is ~ 5 x5 x5 nm MT is ~ 25 nm diameter

Solution 1 - F* techniques

FRAP, FRET, FLIM etc

LECTURE 12

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 MT is ~ 25 nm diameter

Solution 1 - F* techniques

FRAP, FRET, FLIM etc

LECTURE 12

Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

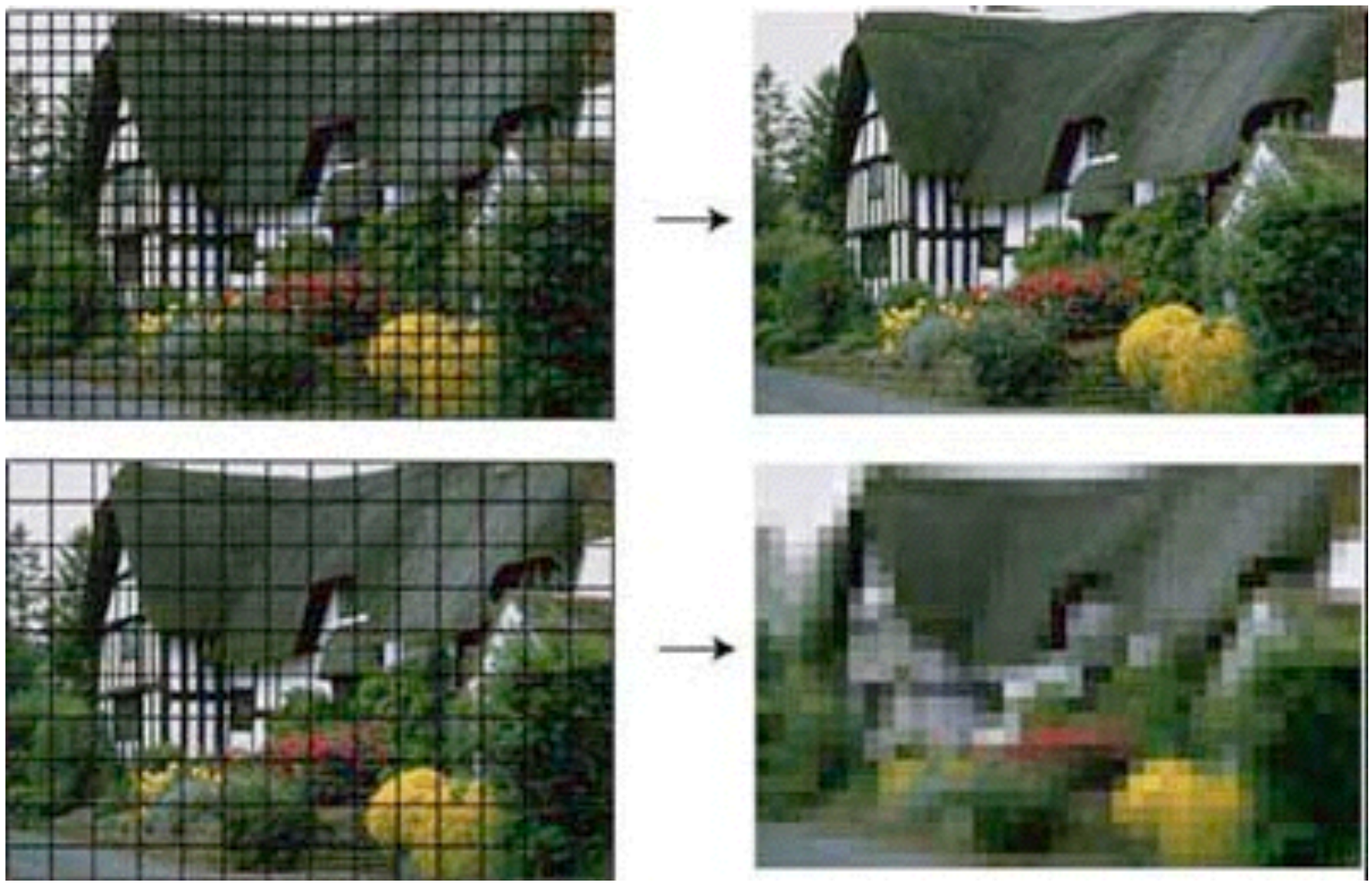
LECTURES 13-15

What is really important in microscopy?

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

Sampling

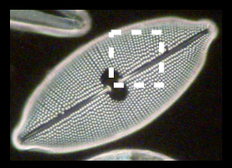
……..correctly reading the available information



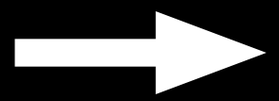
……..poor sampling limits the resolution achieved

Sampling.....appropriate magnification for the detector

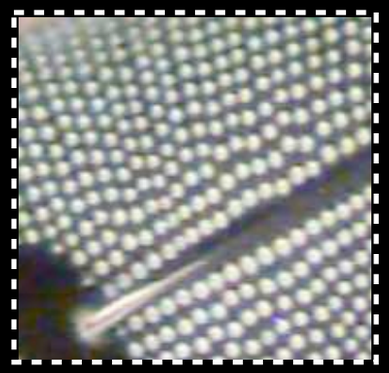
Specimen
Fine Detail



Detail imaged
by microscope



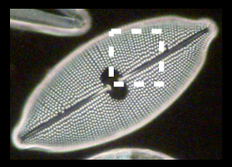
*magnification
*optical resolution



Diatom image: http://www.micromagnus.net

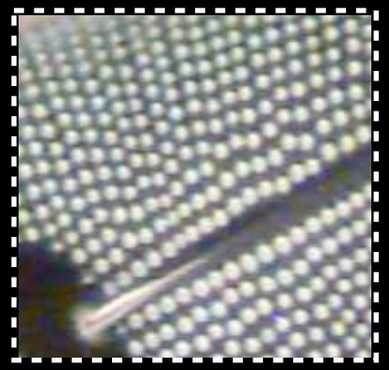
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

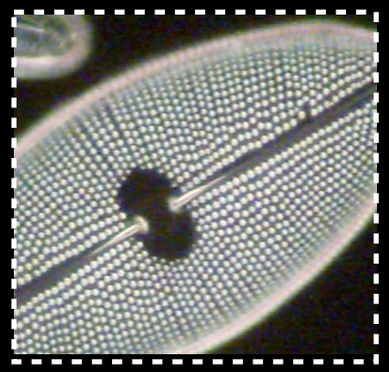


Detail imaged
by microscope

*magnification
*optical resolution



not magnified enough

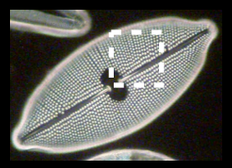


Undersampling
all detail not resolved
Large field of view

Diatom image: http://www.micromagnus.net

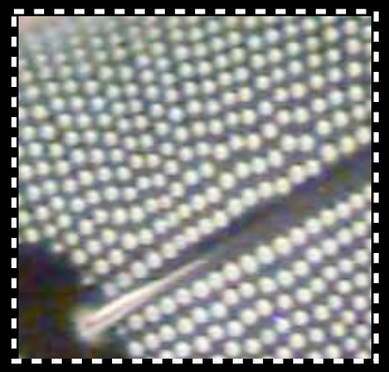
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

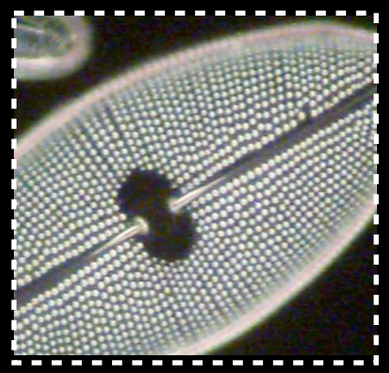


Detail imaged
by microscope

*magnification
*optical resolution



not magnified enough



Undersampling
all detail not resolved
Large field of view

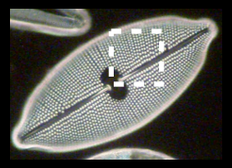
auxiliary
magnification
to match image
to detector

All resolvable
detail recorded

Diatom image: http://www.micromagnus.net

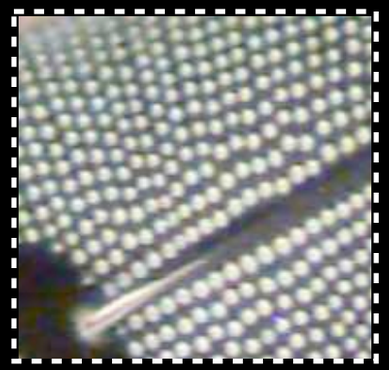
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

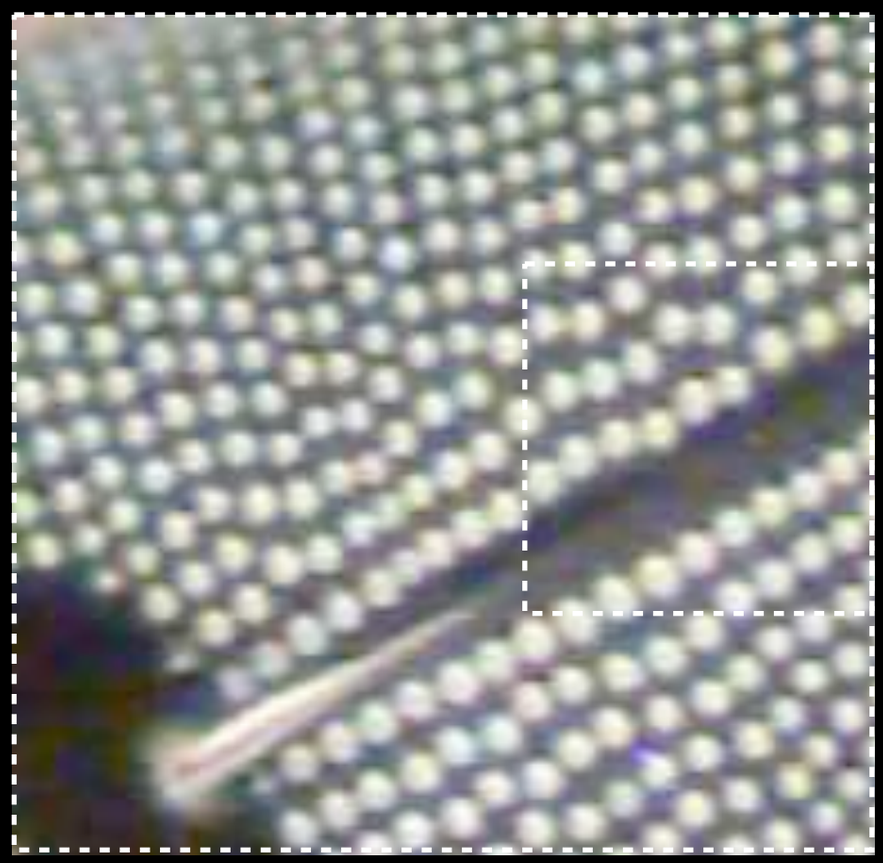


Detail imaged
by microscope

*magnification
*optical resolution



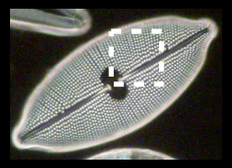
too magnified



Diatom image: http://www.micromagnus.net

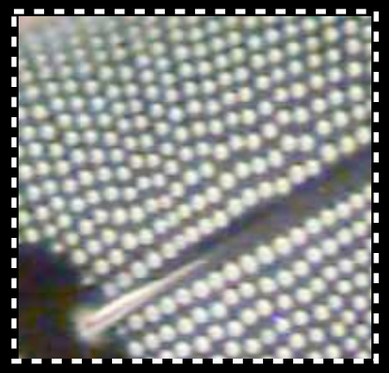
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

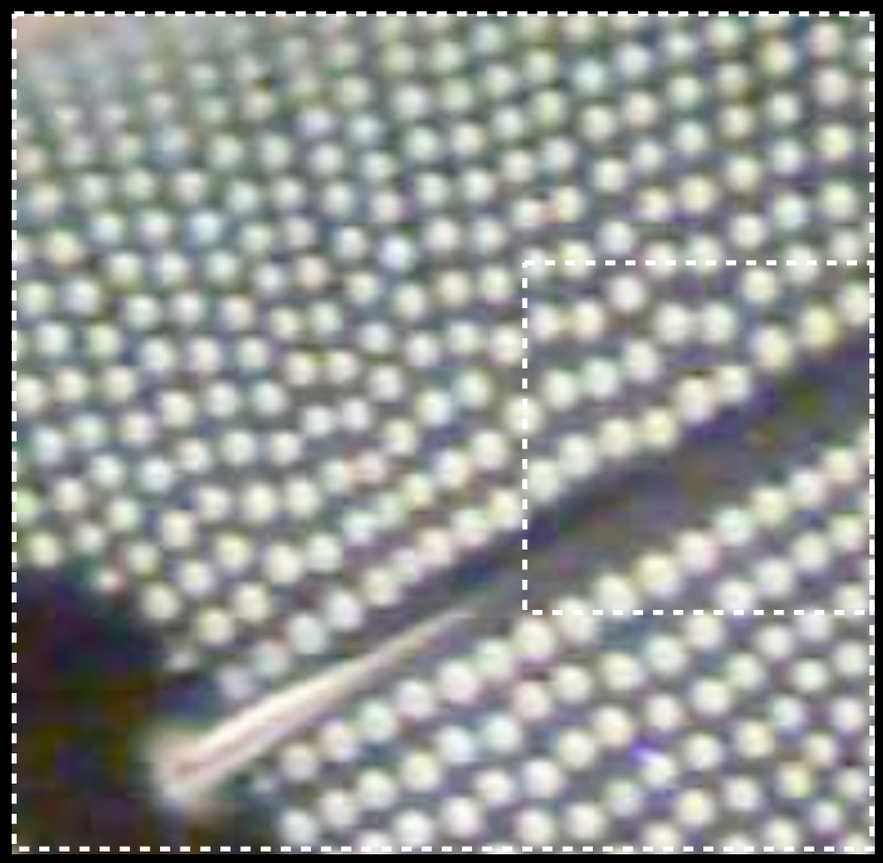


Detail imaged
by microscope

*magnification
*optical resolution



too magnified



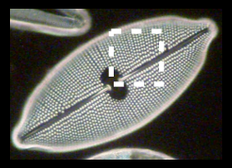
crop
to match image
to detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

Diatom image: http://www.micromagnus.net

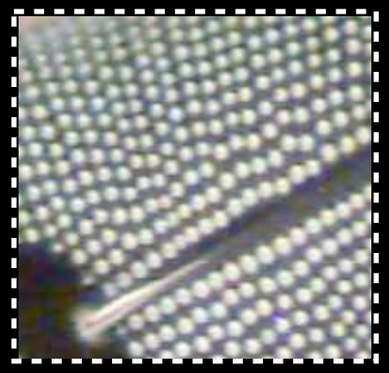
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

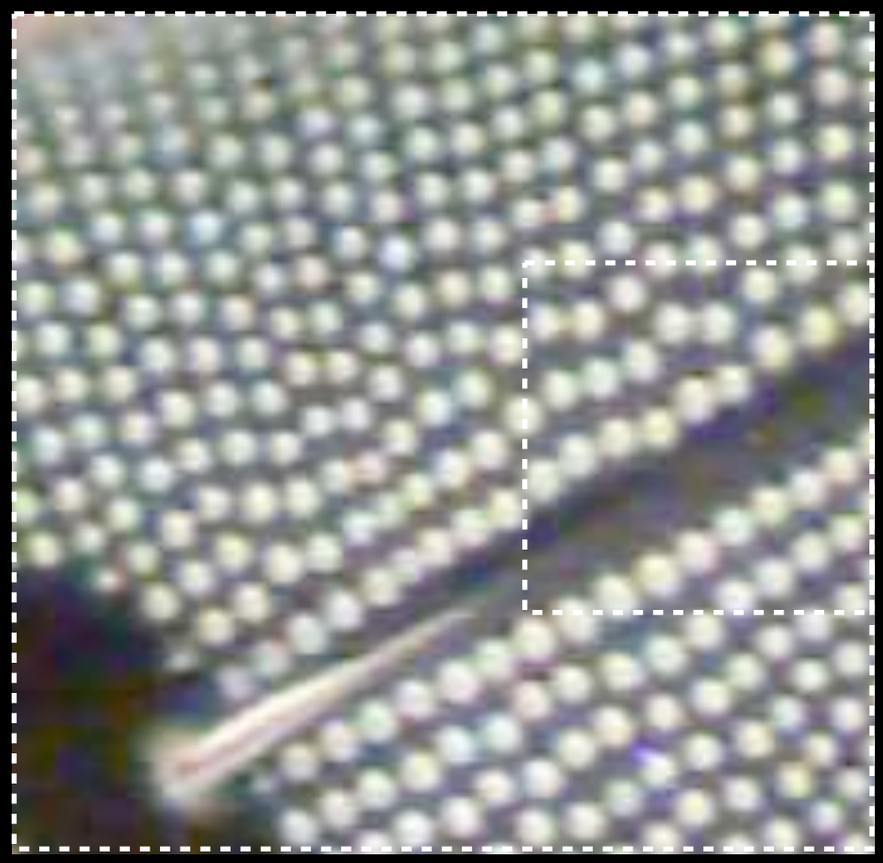


Detail imaged
by microscope

*magnification
*optical resolution



too magnified



crop
to match image
to detector

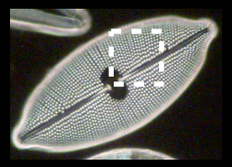
Oversampling
Empty
magnification
Blurred image
Limited field of
view

What is the optimum magnification.....?

Diatom image: http://www.micromagnus.net

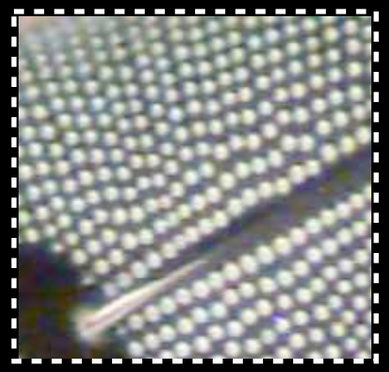
Sampling.....appropriate magnification for the detector

Specimen
Fine Detail

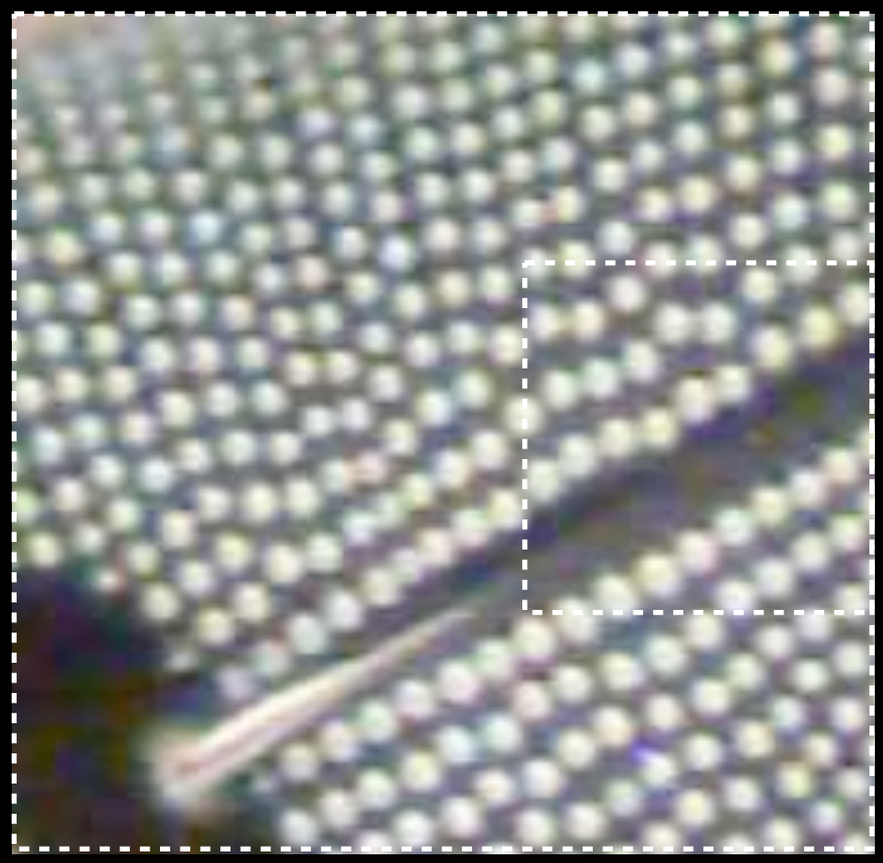


Detail imaged
by microscope

*magnification
*optical resolution



too magnified



crop
to match image
to detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

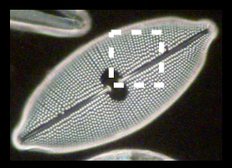
What is the optimum magnification.....?

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

Diatom image: http://www.micromagnus.net

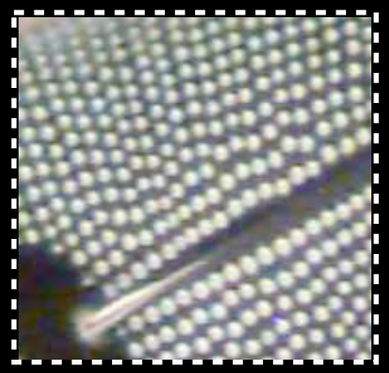
Resolution .....Magnification and Sampling

Specimen
Fine Detail



Detail imaged
by microscope

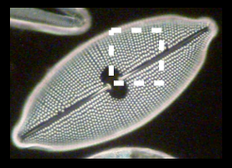
*magnification
*optical resolution



Diatom image: http://www.micromagnus.net/index_micro.html

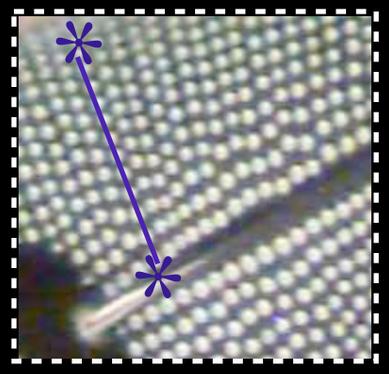
Resolution .....Magnification and Sampling

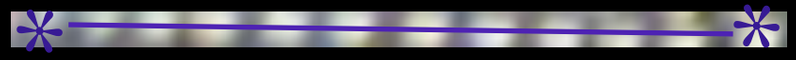
Specimen
Fine Detail



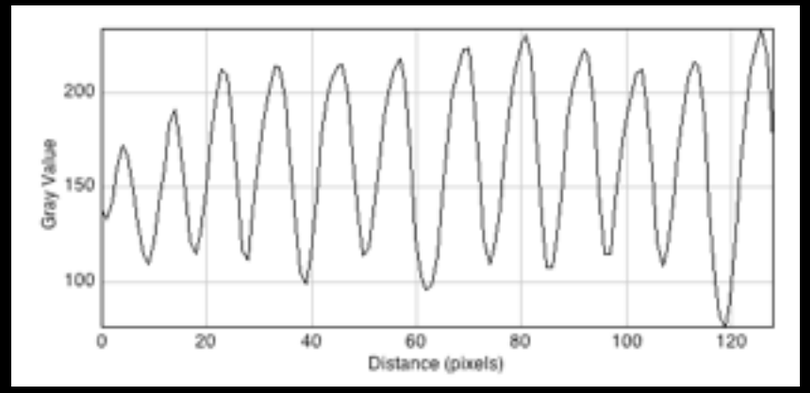
Detail imaged
by microscope

*magnification
*optical resolution





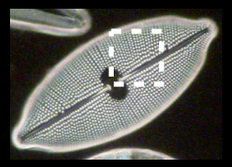
intensity profile ~ a sine wave



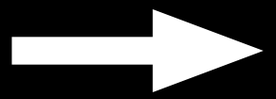
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

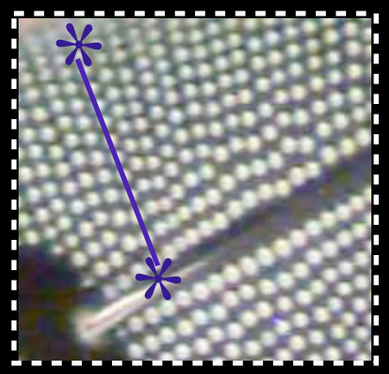
Specimen
Fine Detail

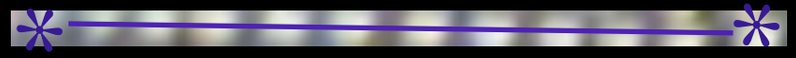


Detail imaged
by microscope

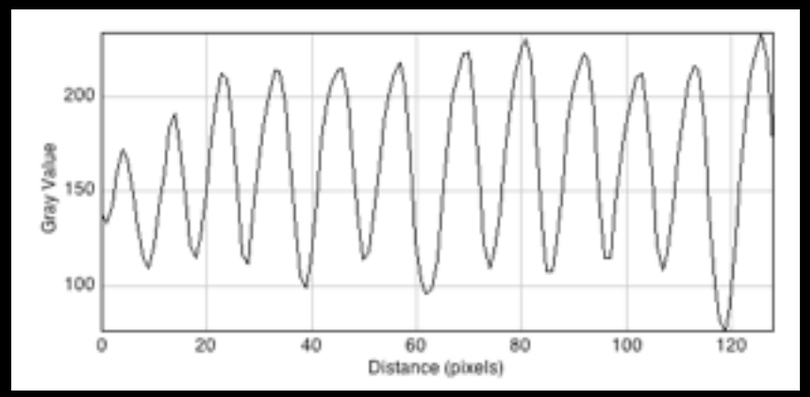


*magnification
*optical resolution

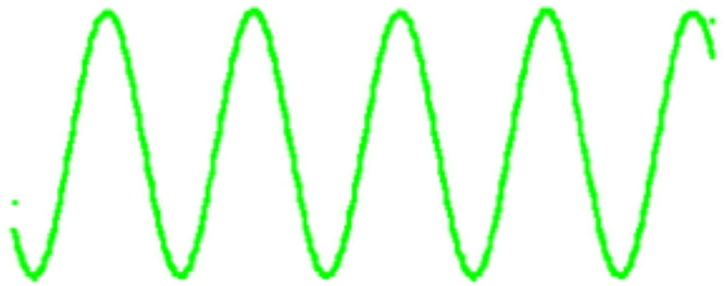




intensity profile ~ a sine wave



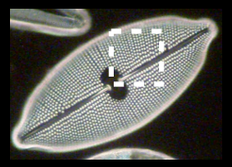
sampling a sine wave



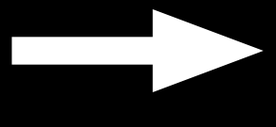
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

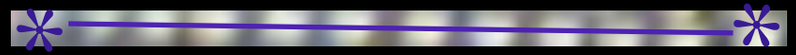
Specimen
Fine Detail



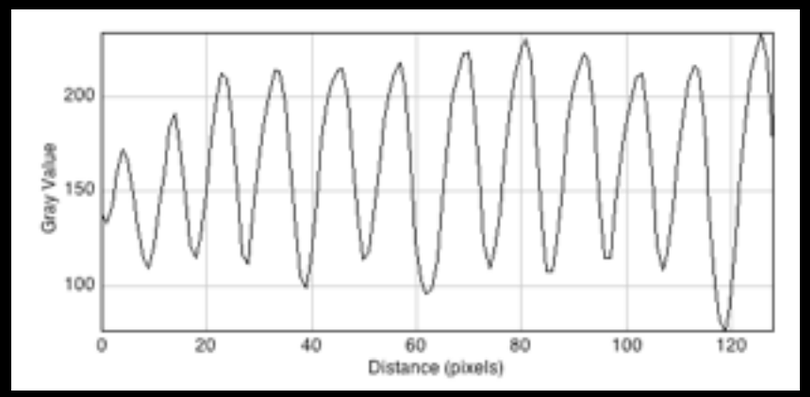
Detail imaged
by microscope



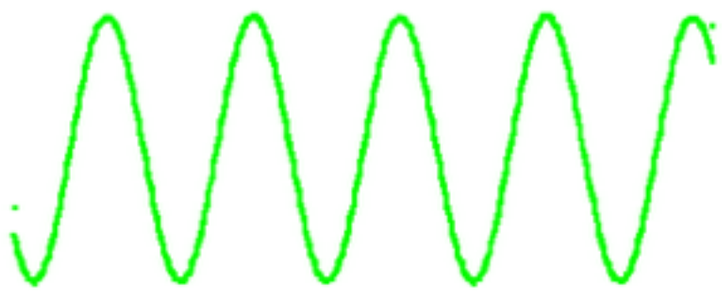
*magnification
*optical resolution



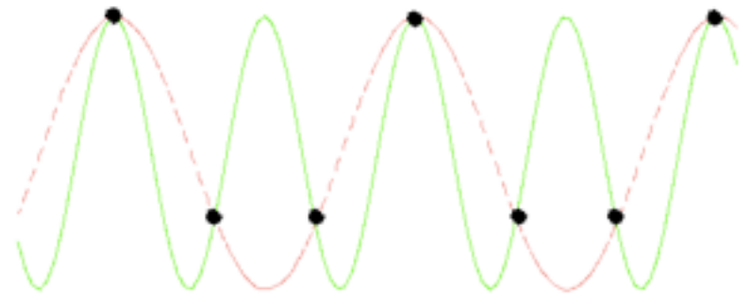
intensity profile ~ a sine wave



sampling a sine wave



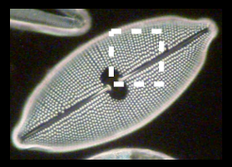
Sampling 1.5 times per cycle



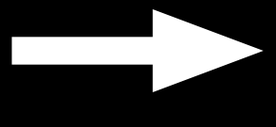
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

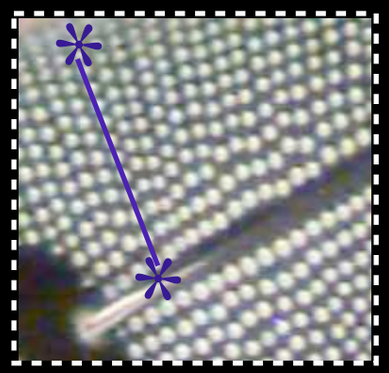
Specimen
Fine Detail

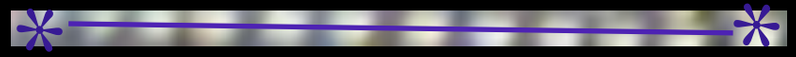


Detail imaged
by microscope

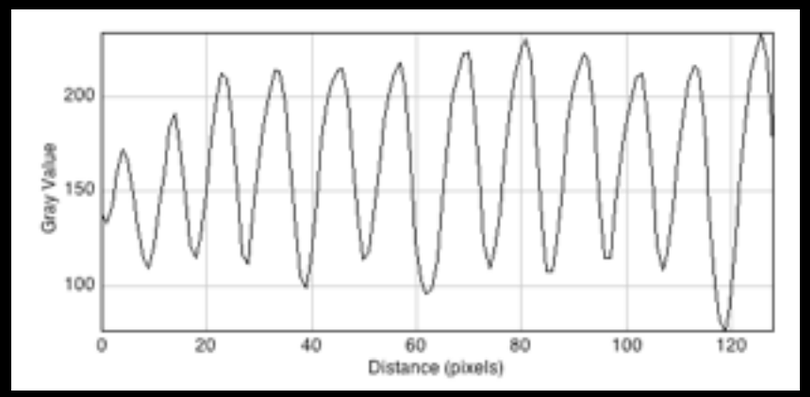


*magnification
*optical resolution

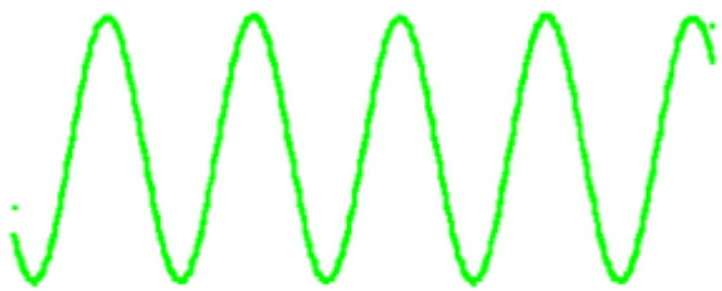




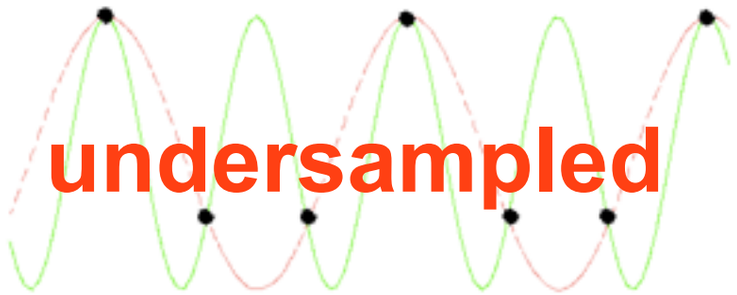
intensity profile ~ a sine wave



sampling a sine wave



Sampling 1.5 times per cycle

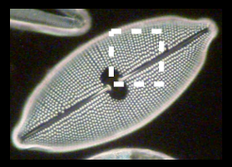


undersampled

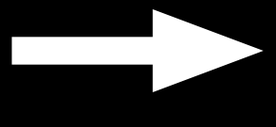
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

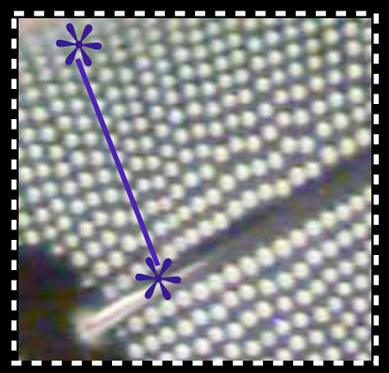
Specimen
Fine Detail



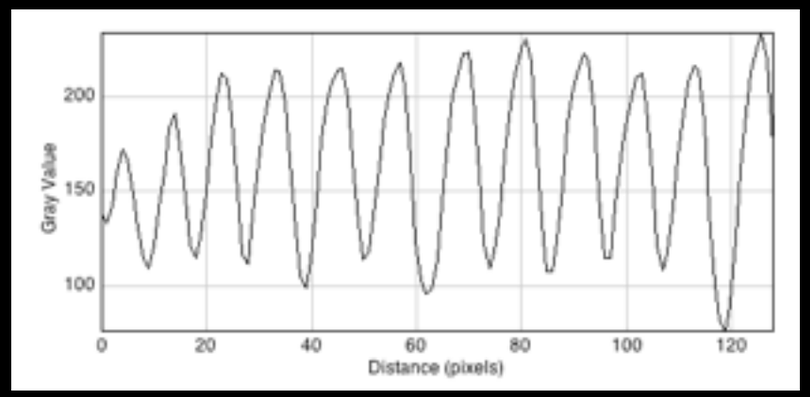
Detail imaged
by microscope



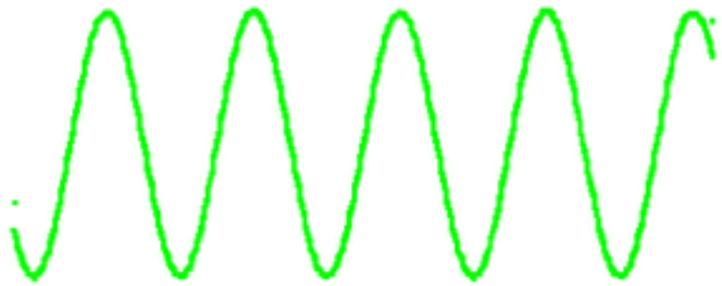
*magnification
*optical resolution



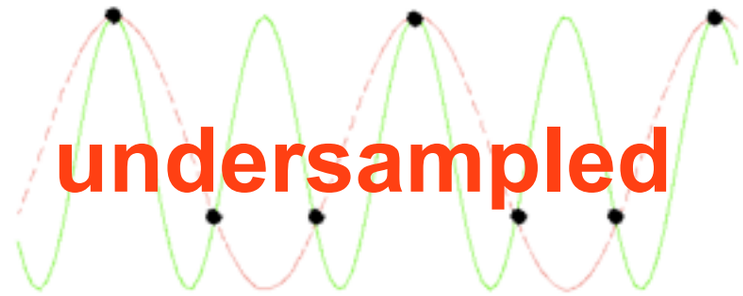
intensity profile ~ a sine wave



sampling a sine wave

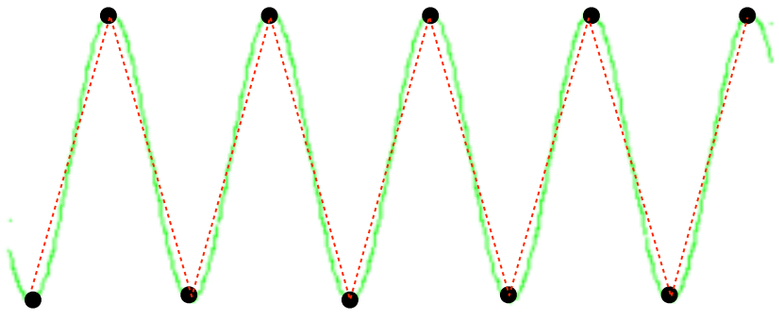


Sampling 1.5 times per cycle



undersampled

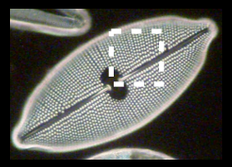
Sampling 2.0 times per cycle



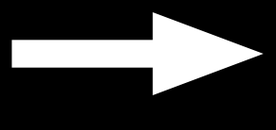
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

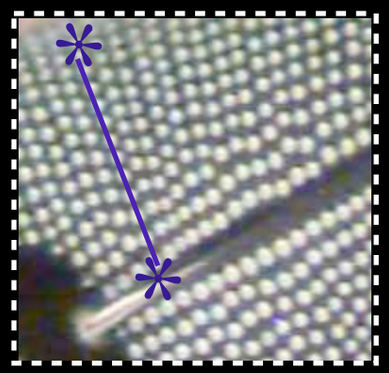
Specimen
Fine Detail

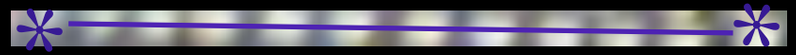


Detail imaged
by microscope

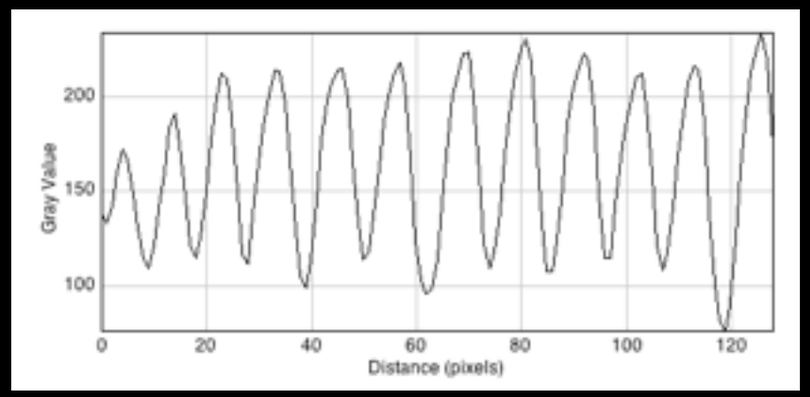


*magnification
*optical resolution

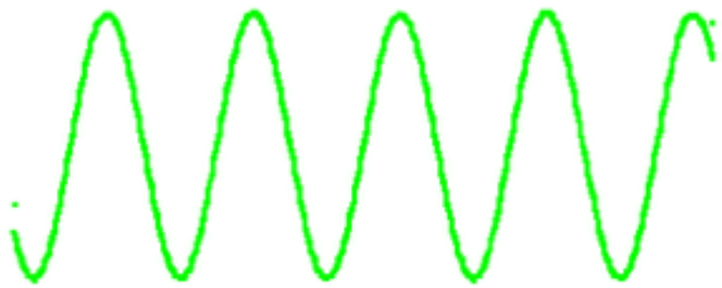




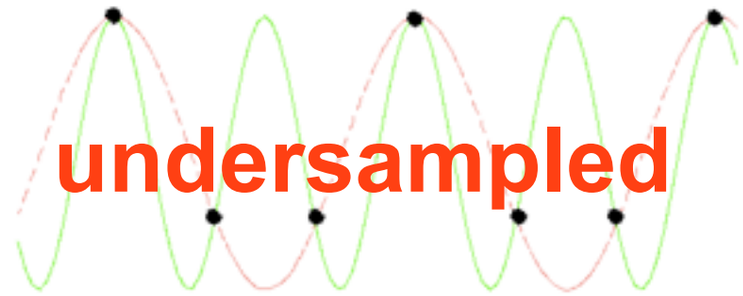
intensity profile ~ a sine wave



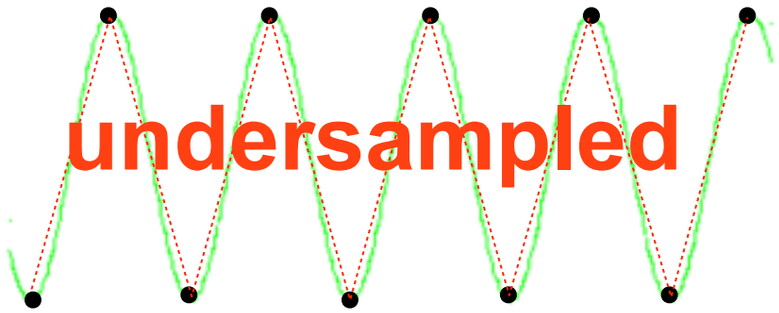
sampling a sine wave



Sampling 1.5 times per cycle



Sampling 2.0 times per cycle



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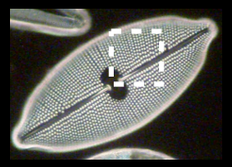

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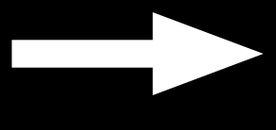
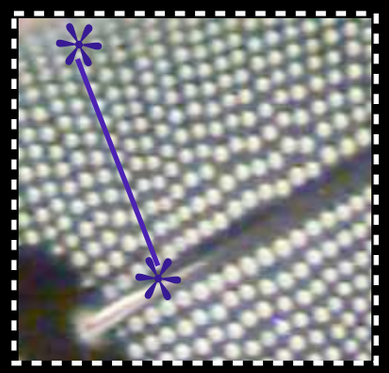


*magnification
*optical resolution



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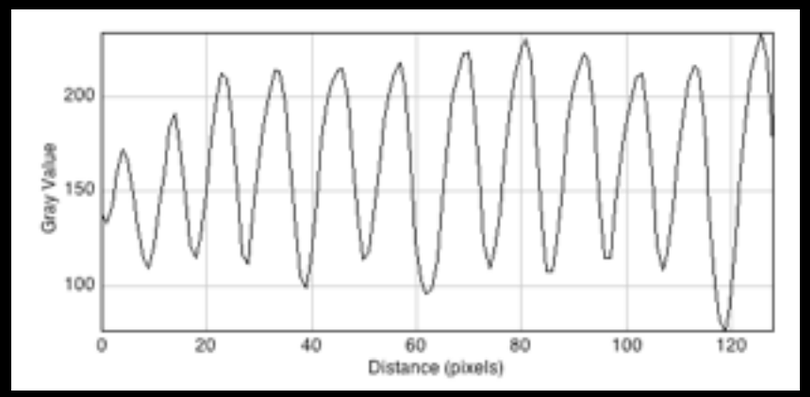
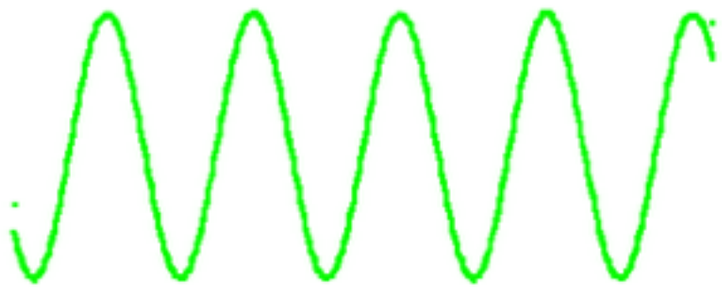


sampling a sine wave



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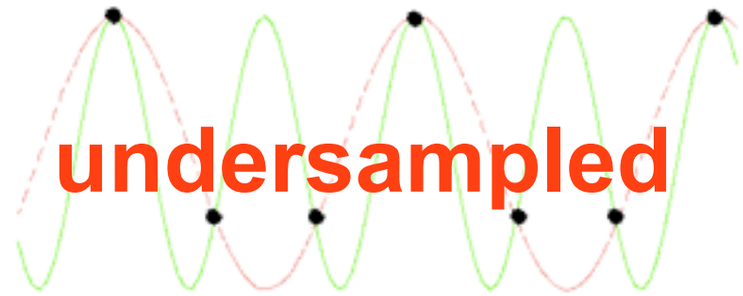
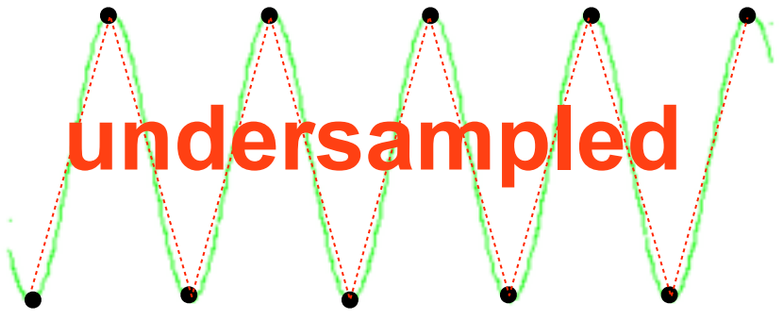
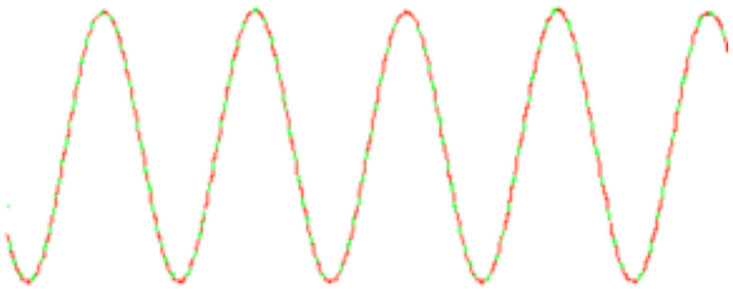


Sampling 2.0 times per cycle



Sampling many times per cycle



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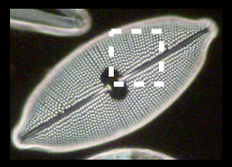

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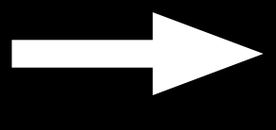

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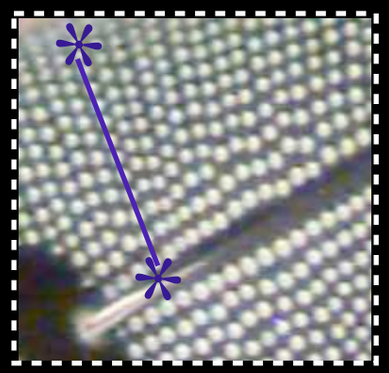

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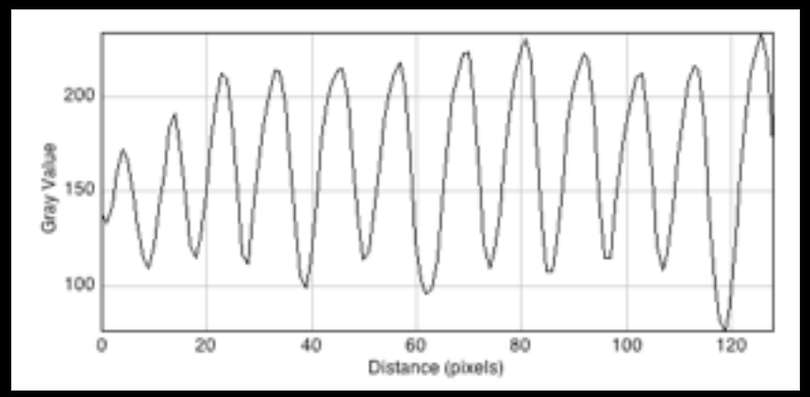
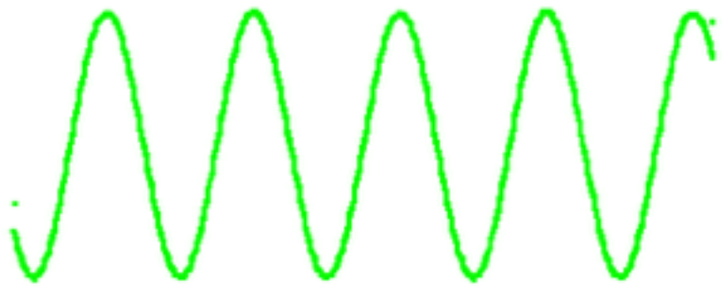
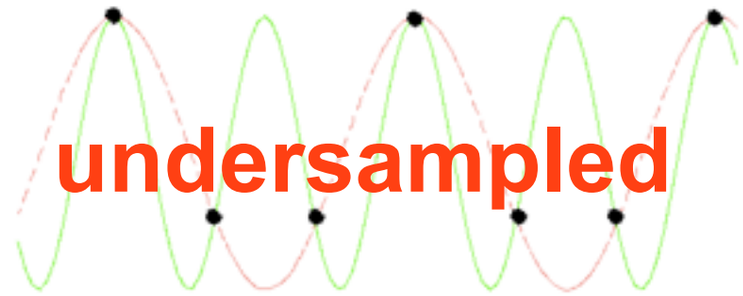


sampling a sine wave



Sampling 1.5 times per cycle



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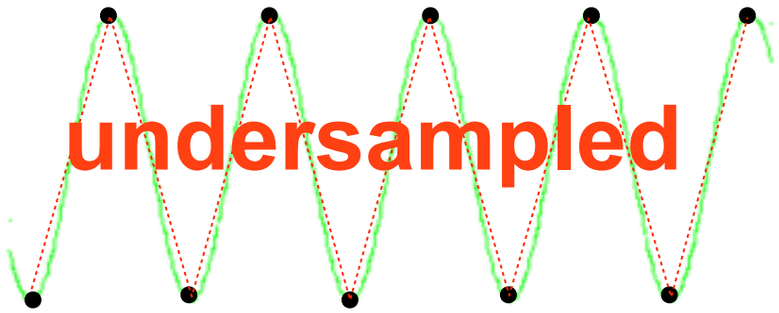

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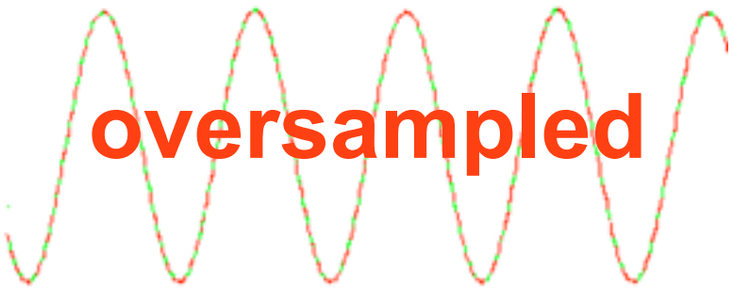

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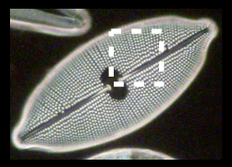

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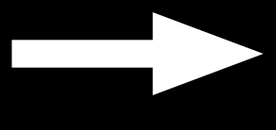

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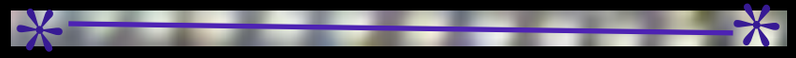

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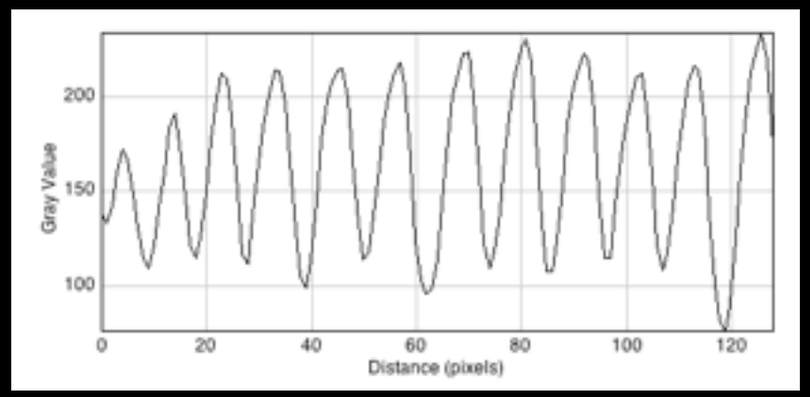
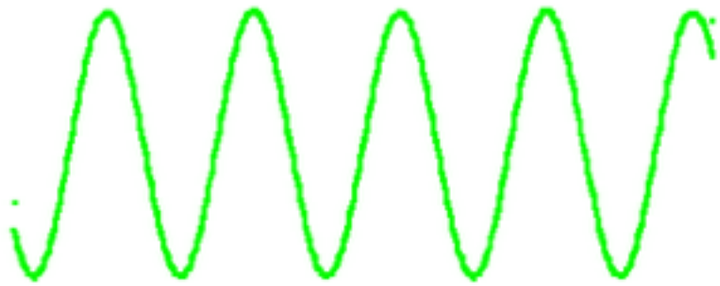


Optimum = 2.3 times per cycle

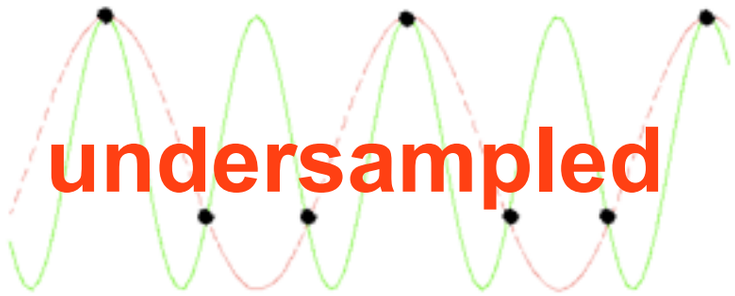
= Nyquist sampling

Diatom image: http://www.micromagnus.net/index_micro.html

sampling a sine wave

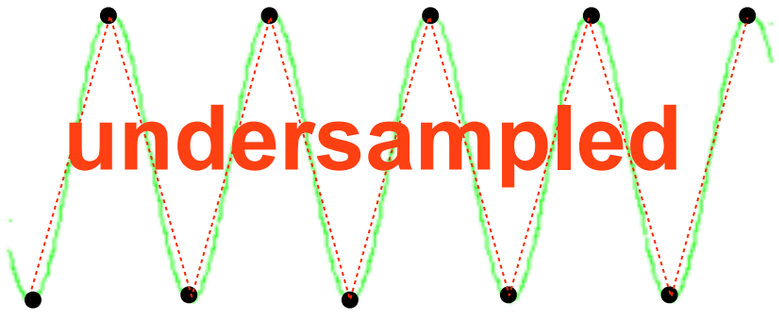


Sampling 1.5 times per cycle



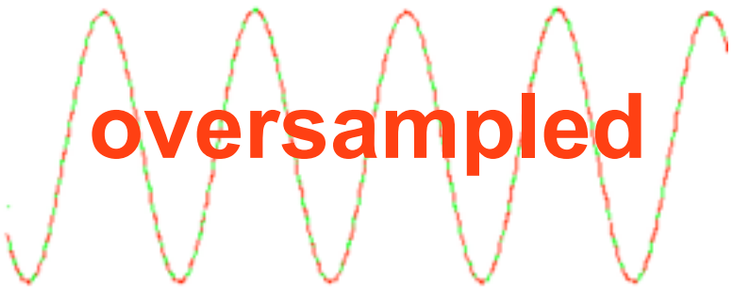
undersampled

Sampling 2.0 times per cycle



undersampled

Sampling many times per cycle



oversampled

Resolution .....Magnification and Sampling

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

optimal total mag X resolvable distance = detector element size

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$$\text{optimal total mag} = \text{Na} \times \frac{2 \times \text{Detector Element}}{1.22 \times 520\ \text{nm}}$$

≈ ideal pixel size ~ 80 nm

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Rearranging to find the optimum magnification ....

$$\text{optimal total mag} = \text{Na} \times \frac{2 \times \text{Detector Element}}{1.22 \times 520\ \text{nm}} \approx \text{ideal pixel size} \sim 80\ \text{nm}$$

$$1.4 \times \frac{6600\ \text{nm} \times 2 \times 3}{1.22 \times 520} \approx \mathbf{87 \times mag}$$

x100 obj = GOOD SAMPLING

What is really important in microscopy?

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

Noise / Signal to Noise (S/N)

Noise / Signal to Noise (S/N)

http://rogewu.comyr.com/brad-pitt-meet-joe-black-wiki.php

Noise / Signal to Noise (S/N)

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Brigitte Bardot, Jours de France Magazine Cover

Signal to Noise - definitions:

- **One of the most important limitations to image quality and image processing**

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

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Signal to Noise - definitions:

- One of the most important limitations to image quality and image processing

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

- Noise is NOT background, auto-fluorescence or dark signal
- Good image data has a high S:N ratio (>4)
- Fundamental limit = Poisson distributed statistics of photon detection (shot noise)

Poisson distributed variation

$$\text{S:N ratio} = \frac{n}{\sqrt{n}}$$

- Statistics of photon counting dictate the minimum useful signal

Average signal = 9, S:N ratio = 3
Average signal = 100, S:N ratio = 10
Average signal = 10,000, S:N ratio = 100

A meaningful difference in intensity needs to be at least three times the noise level

- Additional sources of noise from digitisation, detector readout, thermal noise.

Resolution, contrast, noise

- **Noise limits the contrast which limits the details that can be resolved**
  = Noise limits resolution

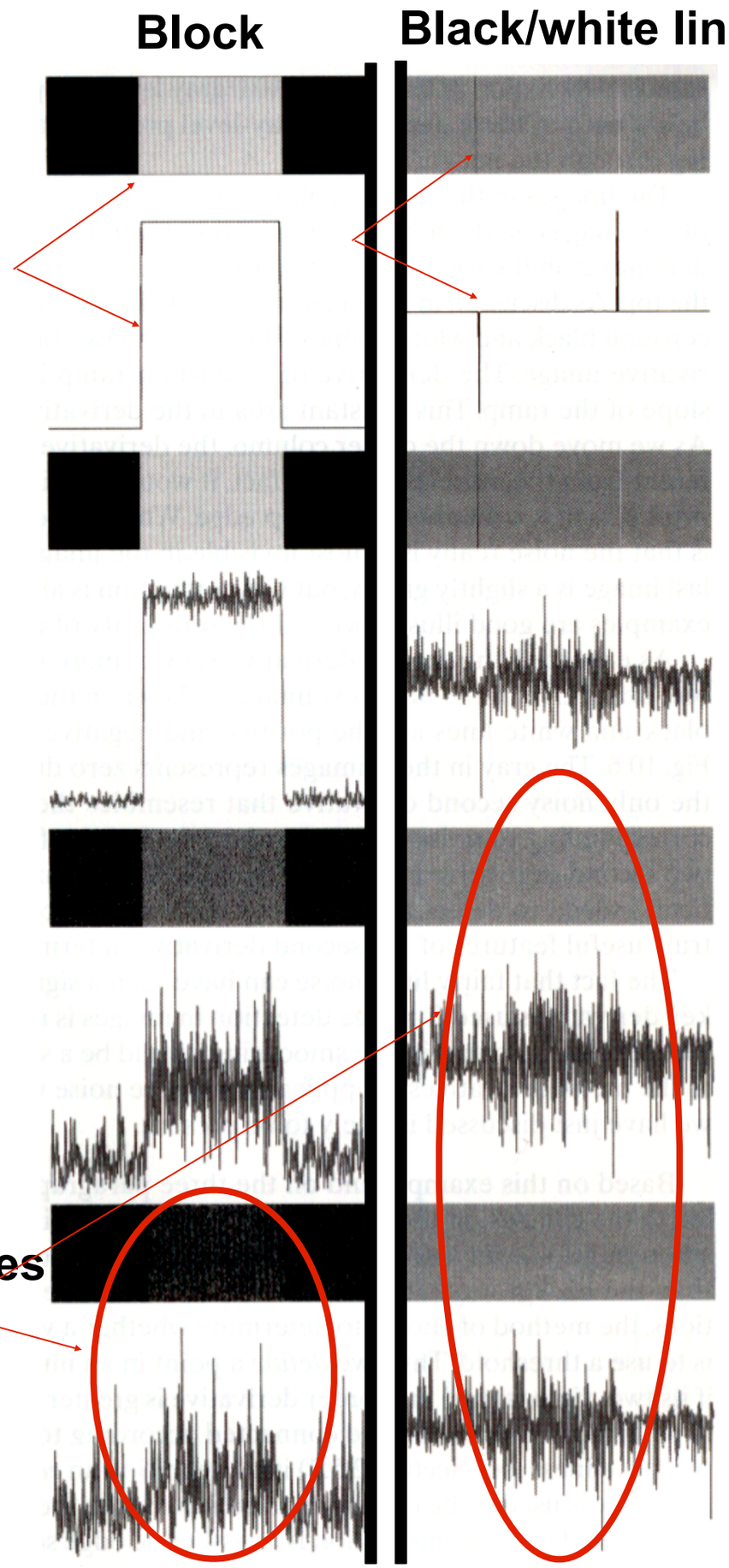
Resolution, contrast, noise

Boundaries and lines easily resolved in the absence of noise

Increasing levels of Gaussian noise

Decreasing S/N

Boundaries and lines no longer resolved



Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

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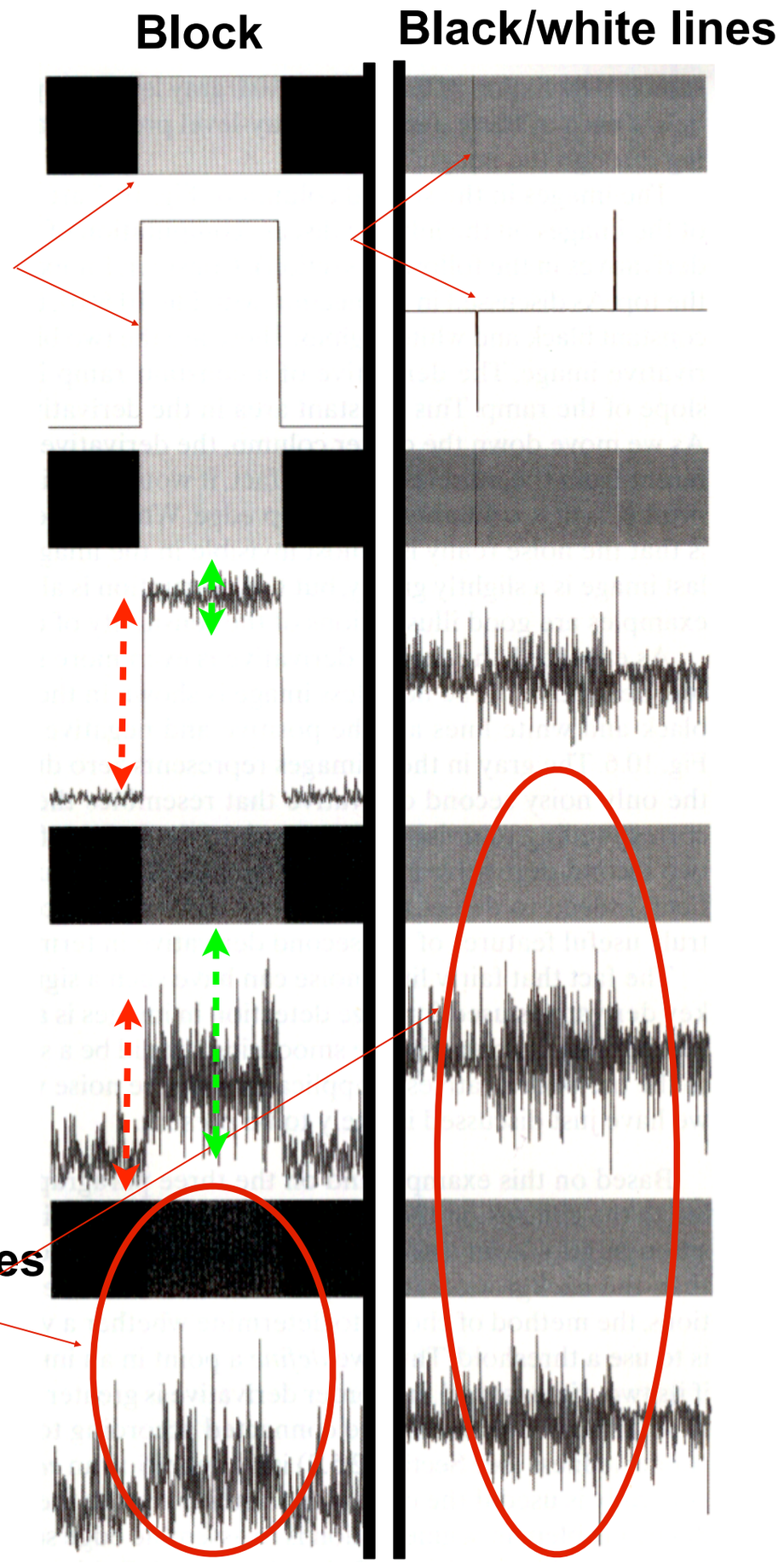


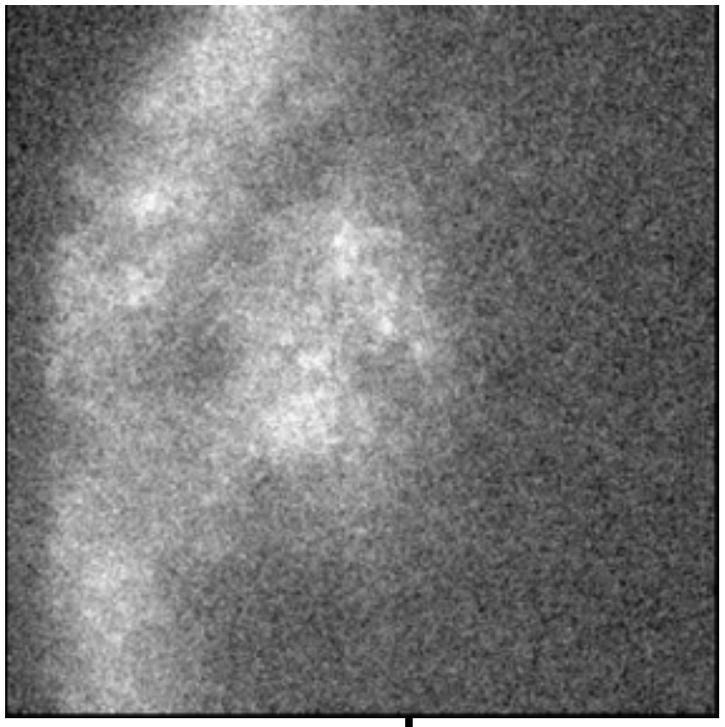
The **difference between signal and background** must be at least 3X the **noise** to be detectable

Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

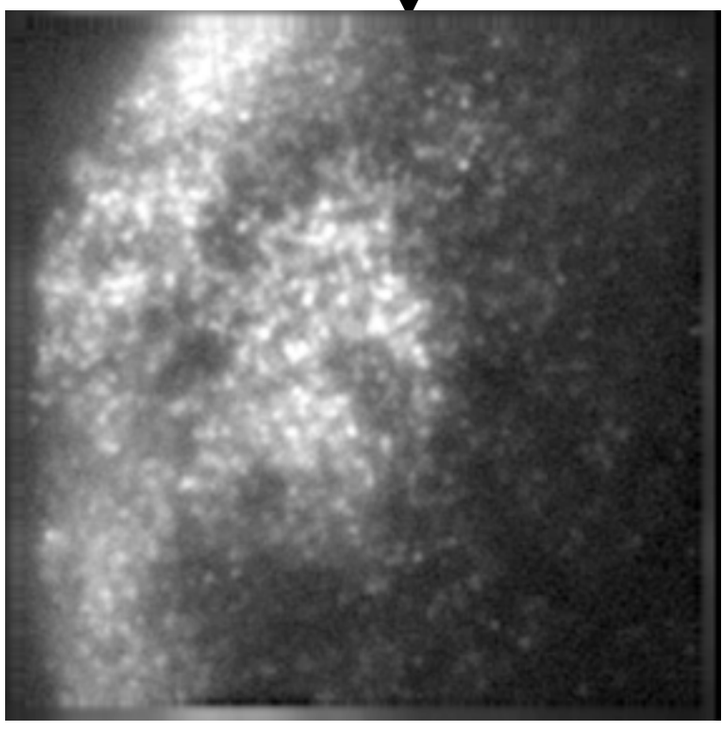
Improving signal to noise

increased signal increases S/N = improved contrast

noisy image (scaled)



5x integration time



increased number of photons counted

improved S/N

ImageJ

Avoid propagating noise

- **Noise is additive:**
  SO subtracting one noisy image from another propagates noise

  THEREFORE
  Subtract an AVERAGE signal to avoid noise propagation
  Or
  Where the signal is non-uniform across the field subtract a 4x AVERAGED image to avoid noise propagation

http://www.dpcorner.com/topics/

Which technique do I use?

Practical 7: Comparison of techniques

Optimising your imaging

Optimising your imaging

* Asking the right questions

Optimising your imaging

* Asking the right questions

* Picking the right technique

Optimising your imaging

* Asking the right questions
* Picking the right technique
* Applying the technique well

Optimising your imaging

* Asking the right questions

* Picking the right technique

* Applying the technique well

* Analysing / interpreting the data properly

Be clear what you want from your experiment

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Qualitative data
Quantitative data
Dynamics

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
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Be aware of the different techniques

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Dynamics

Be aware of the different techniques

VISIT A FACILITY

Their strengths
Their weaknesses
Their availability

Understand the limitations of your material

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
Dynamics

Be aware of the different techniques

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Their strengths
Their weaknesses
Their availability

Understand the limitations of your material

Viability
Thickness
Brightness

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?

LECTURES 8-16

Which technique do I use?

Bright field and fluorescence (contrast generation)

LECTURES 8-16

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Bright field and fluorescence (contrast generation)

Live cell imaging
Fixed material imaging

(dynamics vs detail)

LECTURES 8-16

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Bright field and fluorescence (contrast generation)

Live cell imaging
Fixed material imaging (dynamics vs detail)

Confocal techniques (scanning, optical sectioning)

Wide field techniques (Speed, sensitivity)

LECTURES 8-16

Which technique do I use?

Bright field and fluorescence (contrast generation)

Live cell imaging
Fixed material imaging (dynamics vs detail)

Confocal techniques (scanning, optical sectioning)

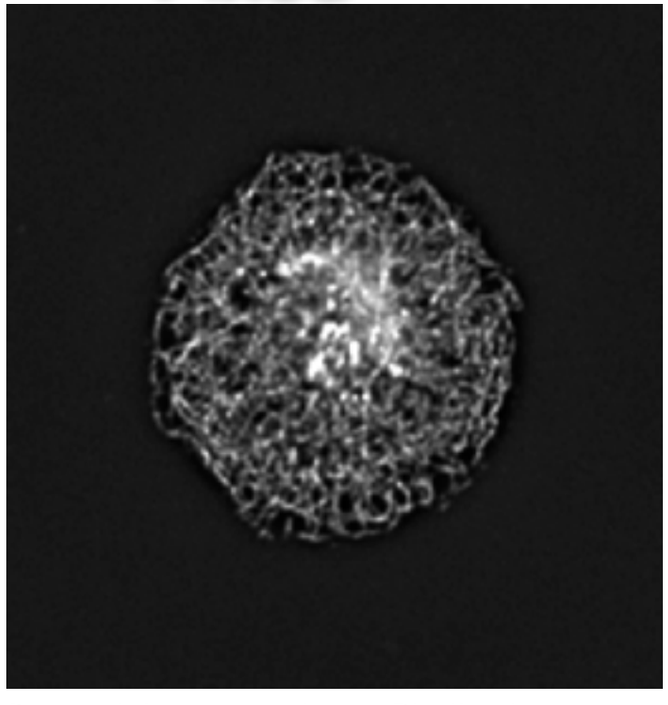
Wide field techniques (Speed, sensitivity)

Super-resolution techniques
Techniques for molecular scale dynamics / interactions

LECTURES 8-16

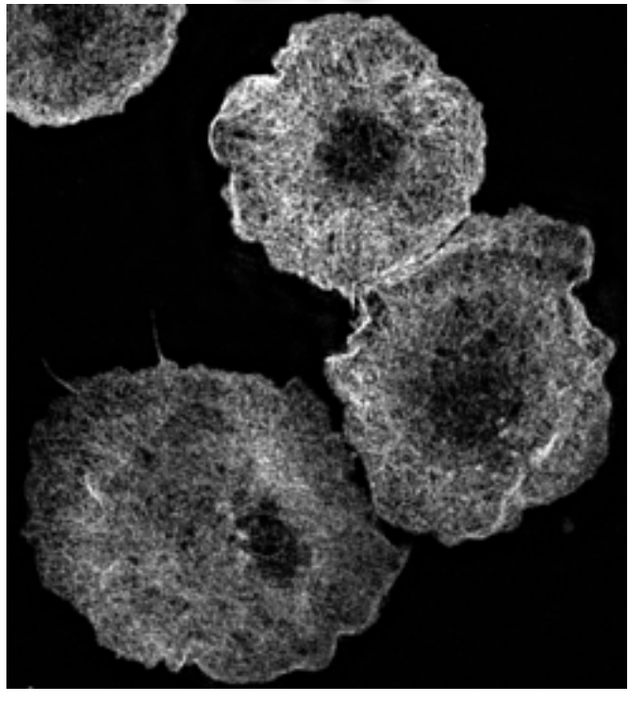
Which technique to use?

Fixed



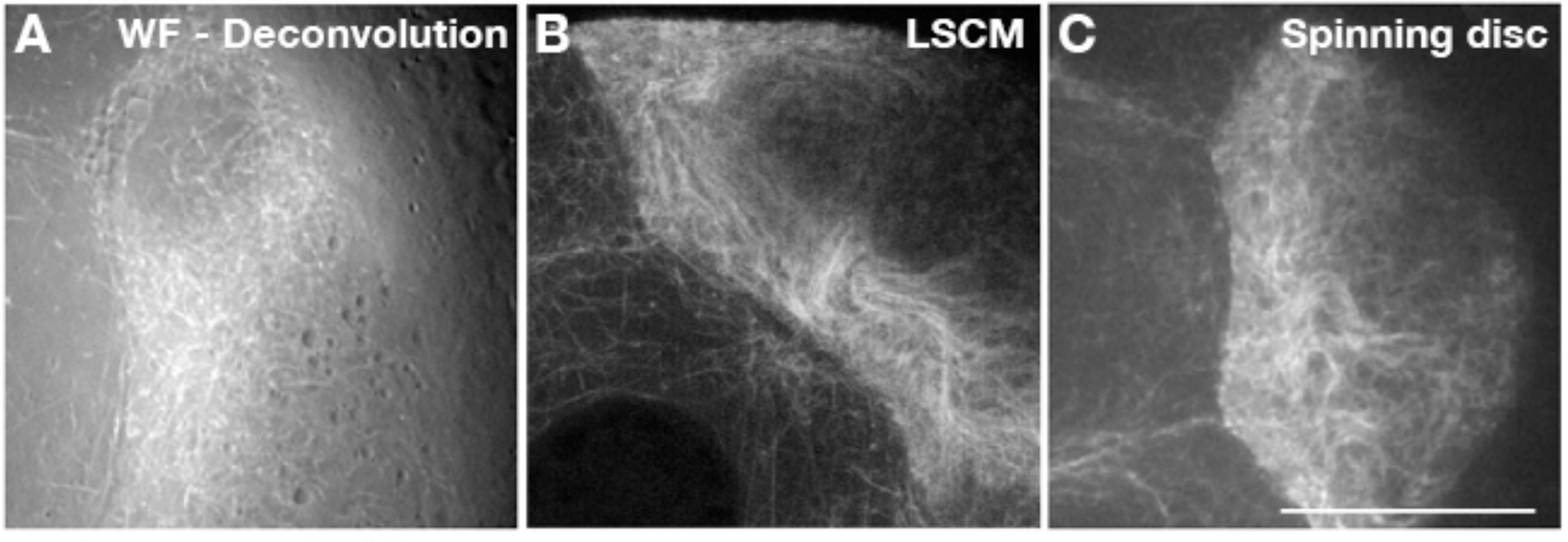
vs

Live



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

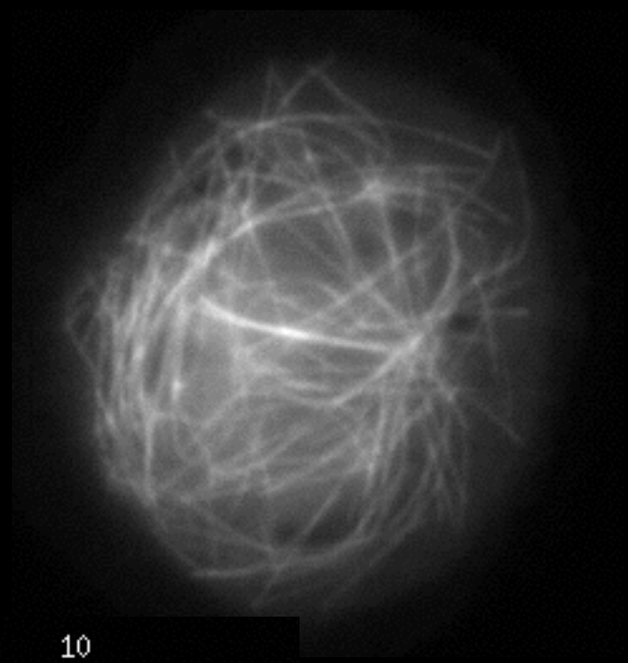
Wide field Decon vs Confocal



Live *Drosophila* oocyte: Tau-GFP labeling microtubules

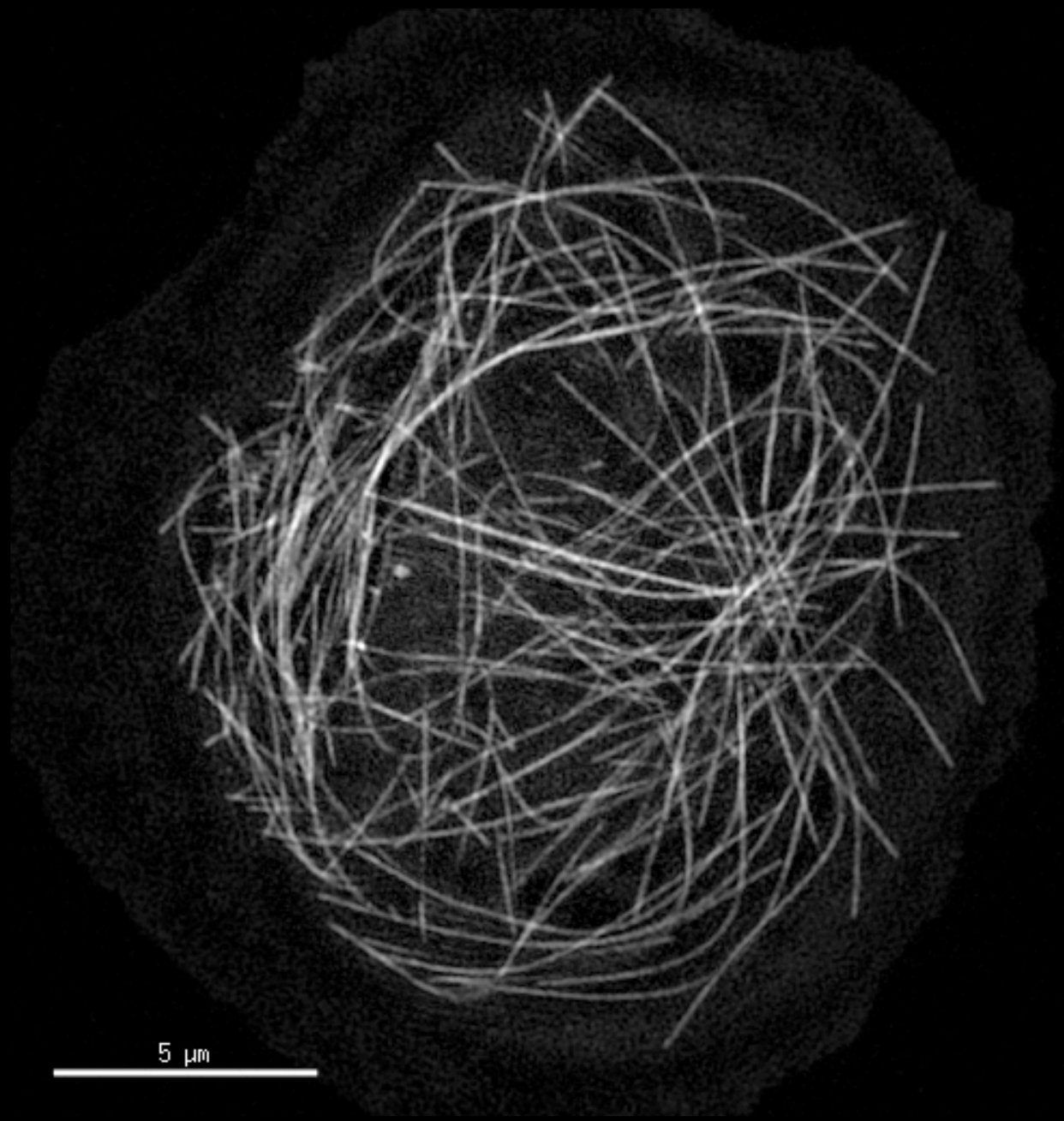
Live-cell super-resolution!

CONVENTIONAL



XY resolution ~250 nm

3D-SIM OMX-BLAZE

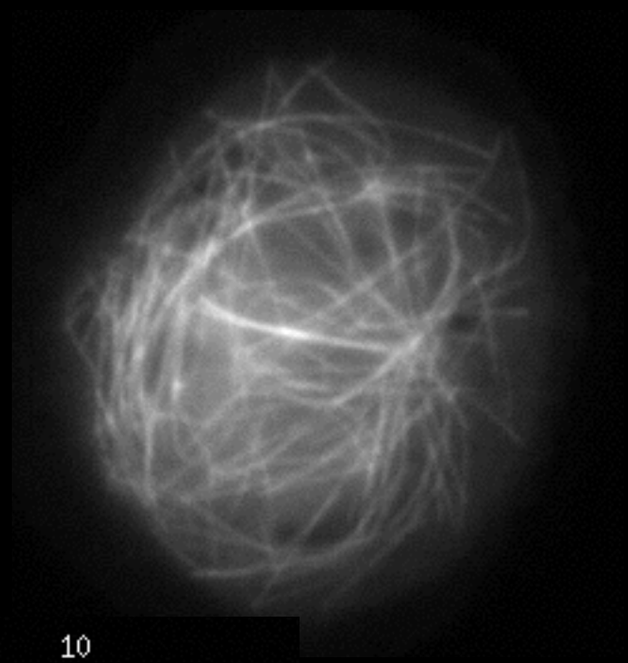


XY resolution ~130 nm

Jupiter-GFP tagged MT dynamics in a living Macrophage

Live-cell super-resolution!

CONVENTIONAL



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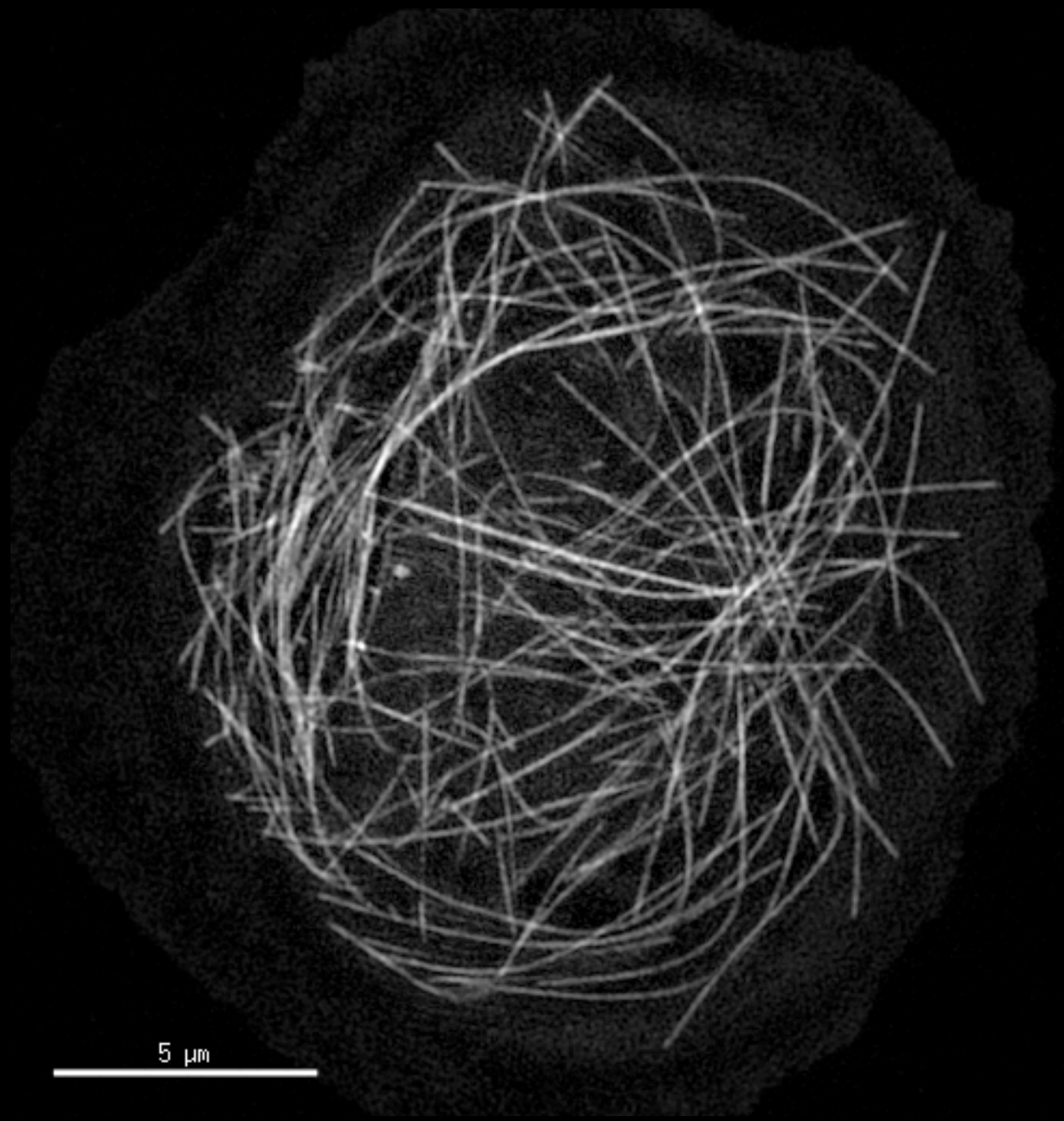

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http://www.theguardian.com/science/2011/sep/11/genetically-modified-glowing-cats

Introductory Image Analysis Course:

Foundations of Image Analysis for Biomedical and Life-Science Research

Dominic Waithe

Image handling

Enhancement

Analysis

Reference Material

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

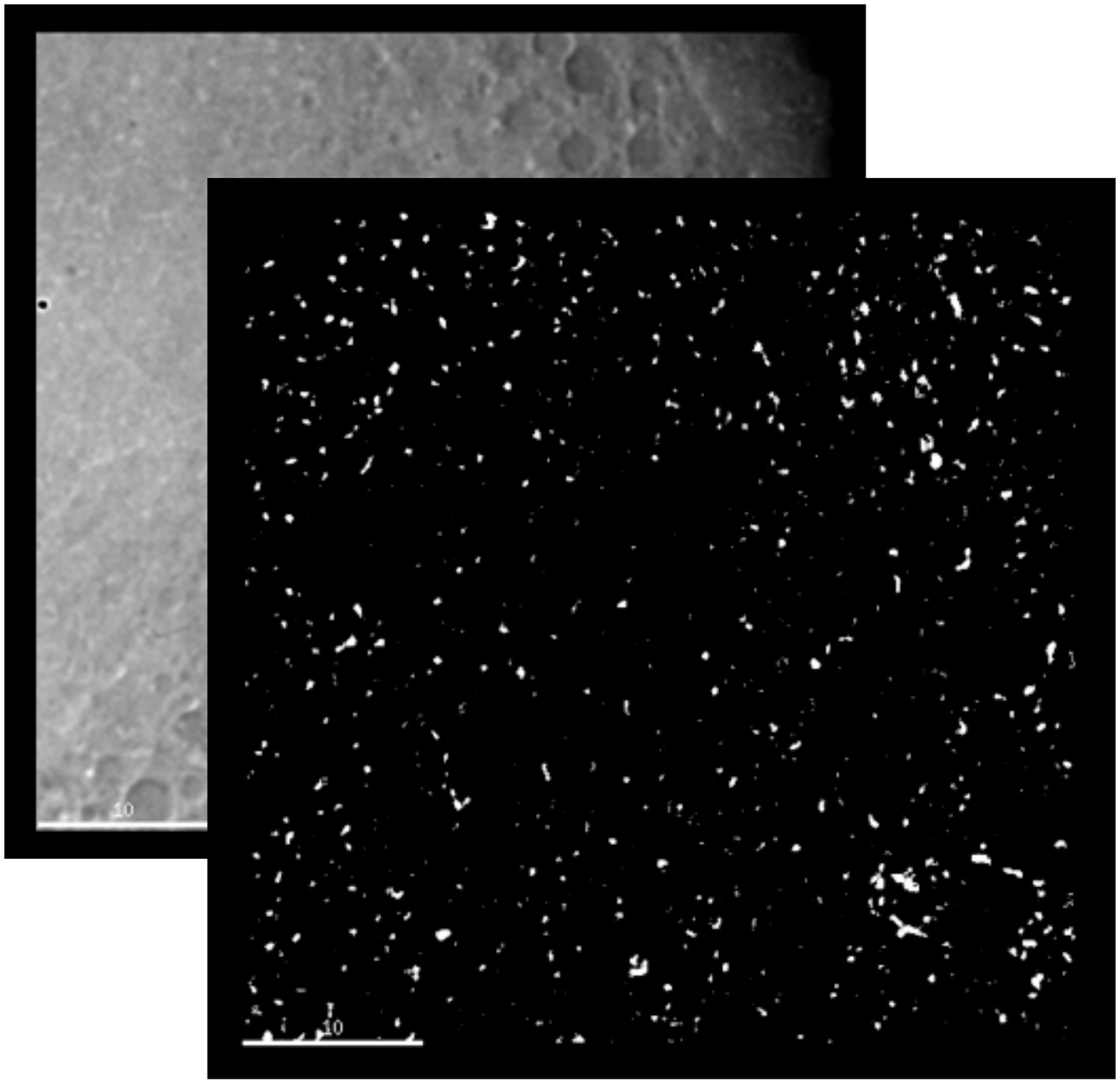
http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html
Very comprehensive

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

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

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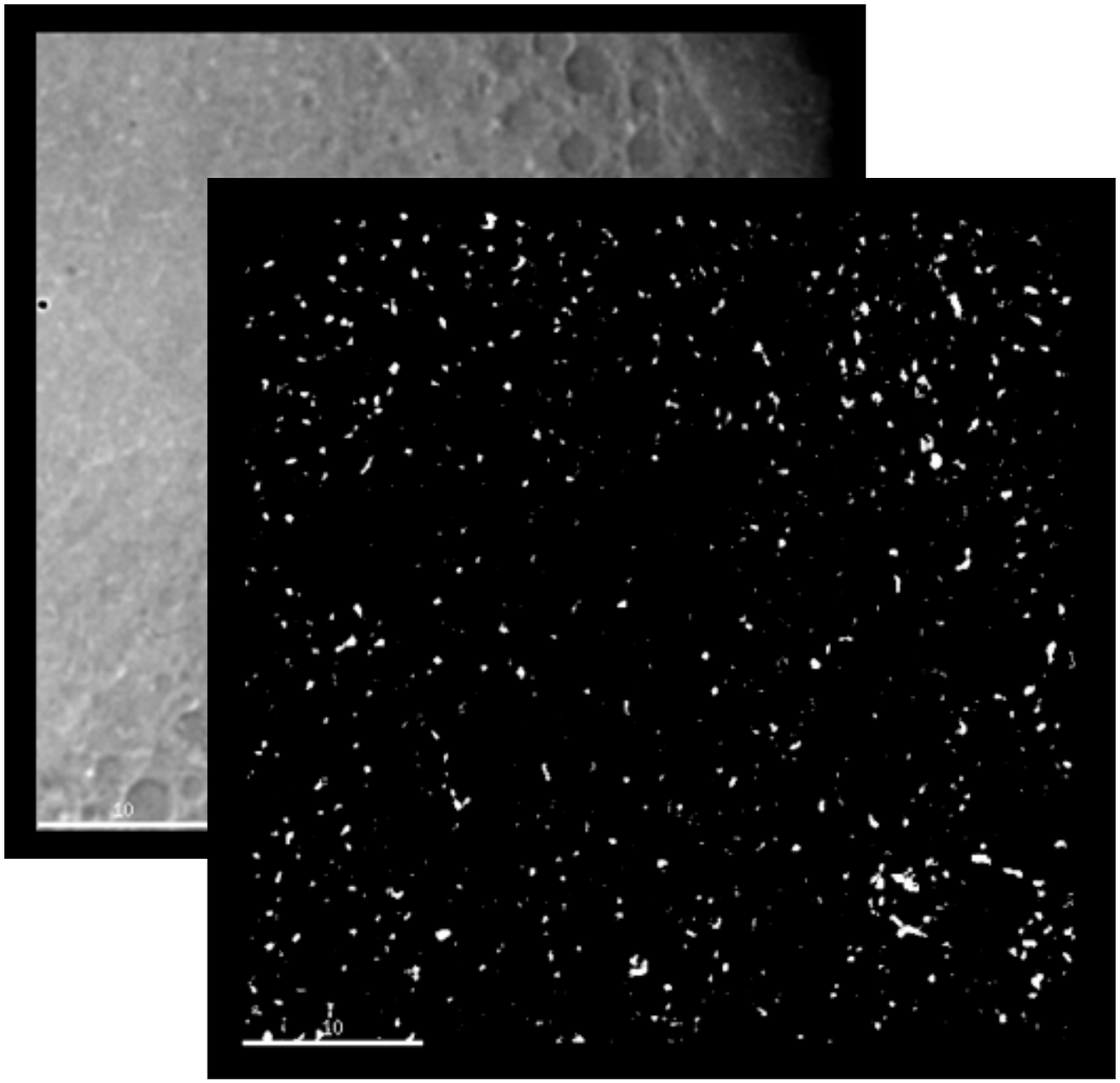

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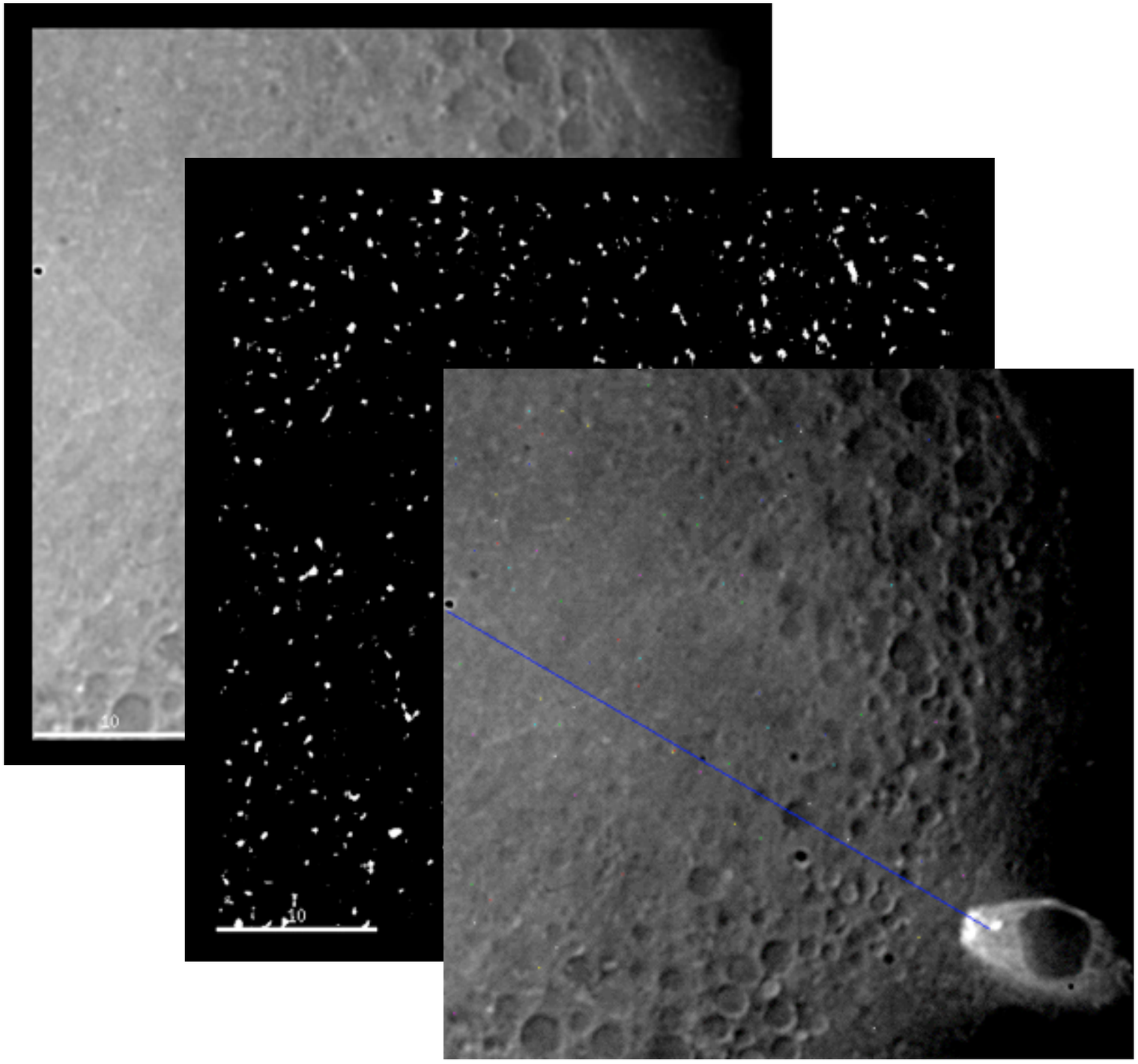

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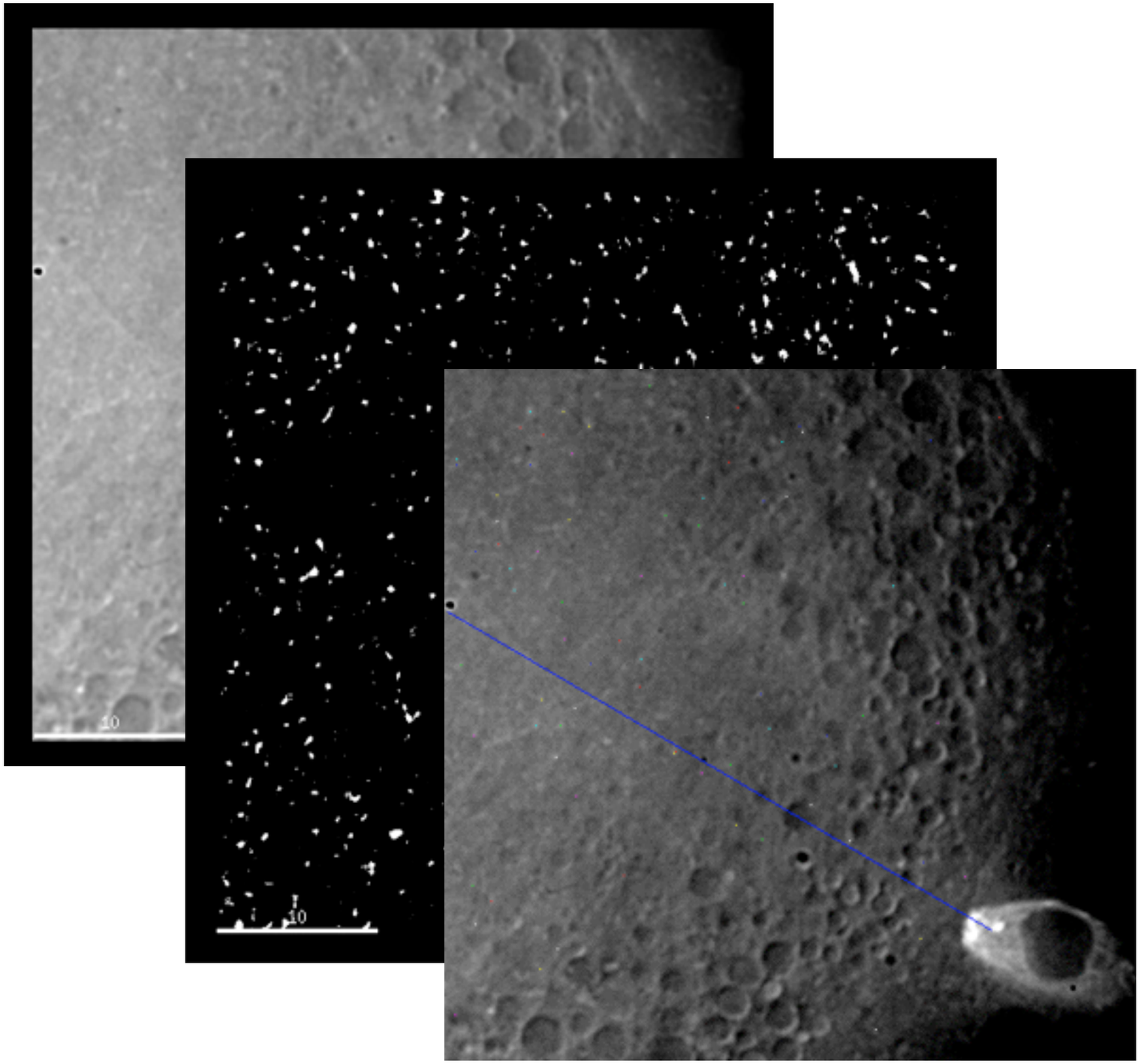

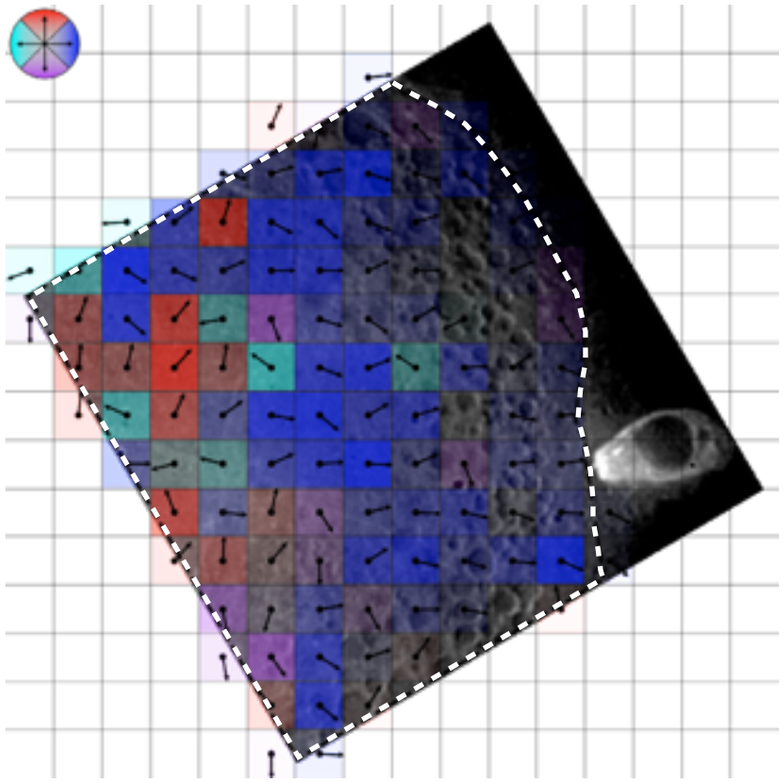

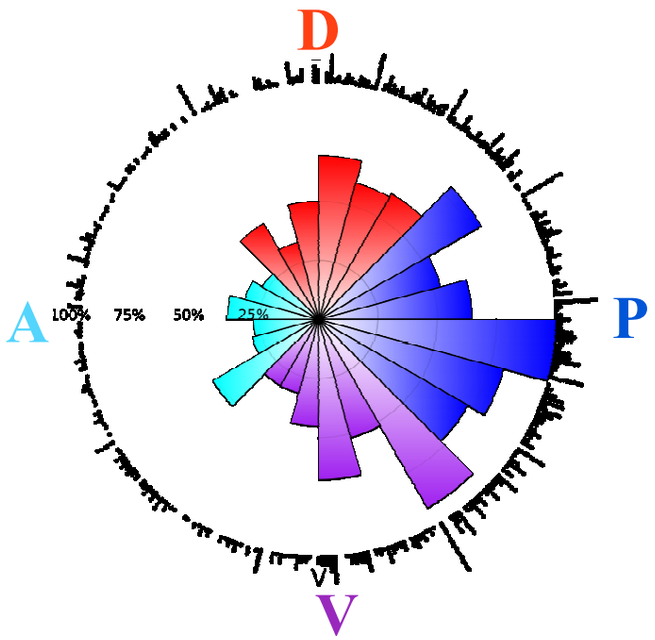

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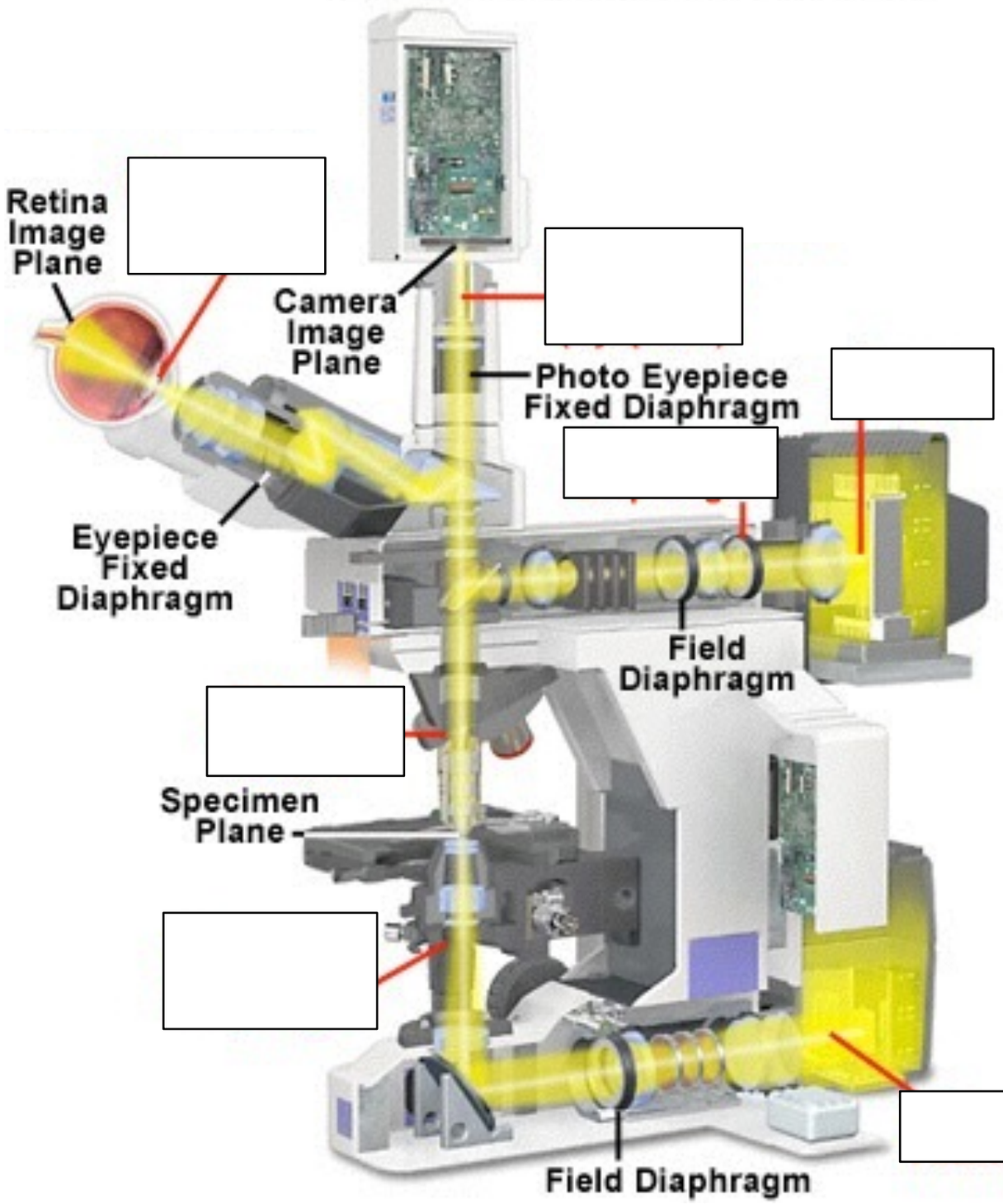
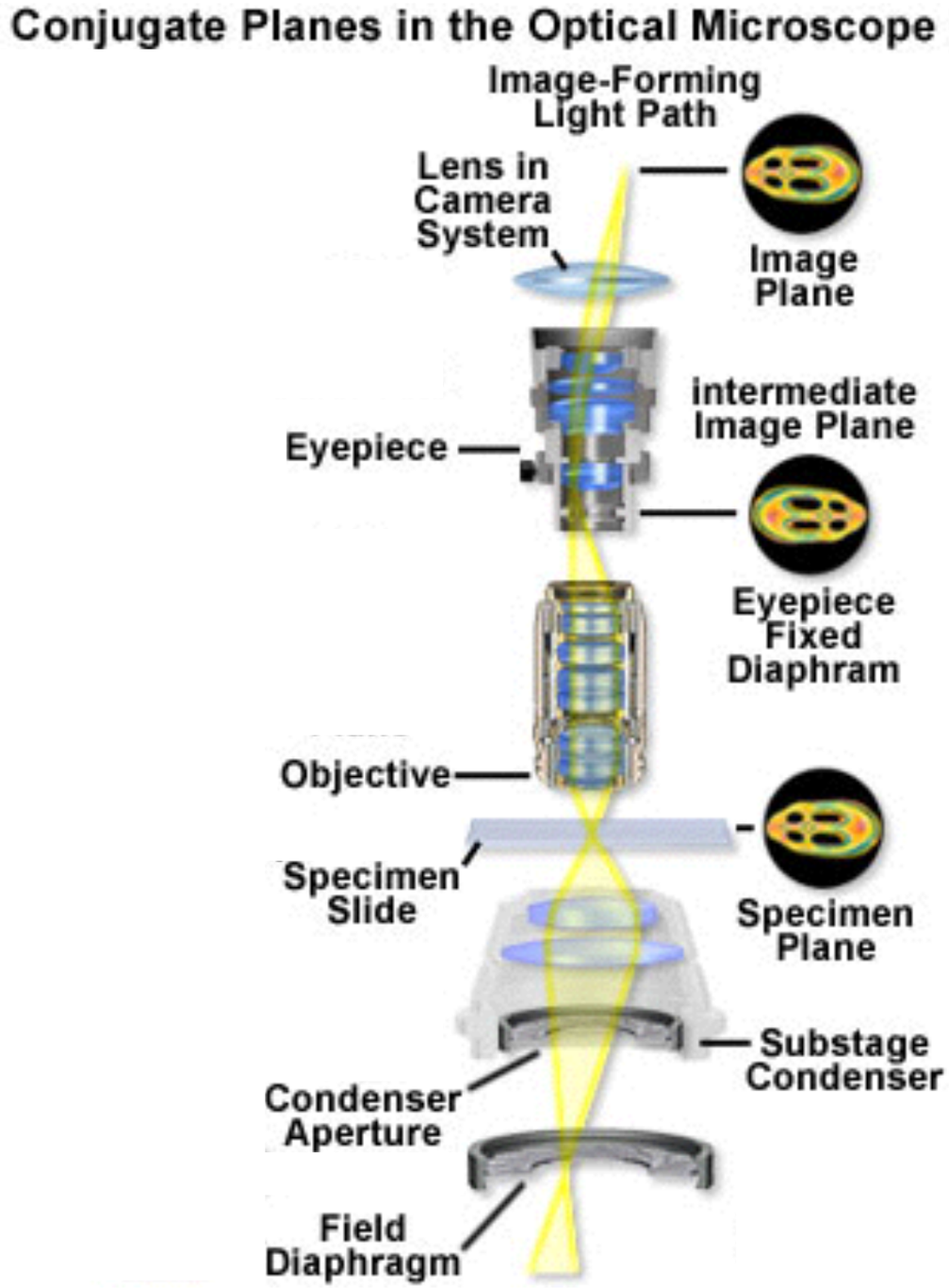
(Russell Hamilton, Particle Stats: http://www.particlestats.com)

Koehler illumination and conjugate planes

Field or Image forming conjugate planes





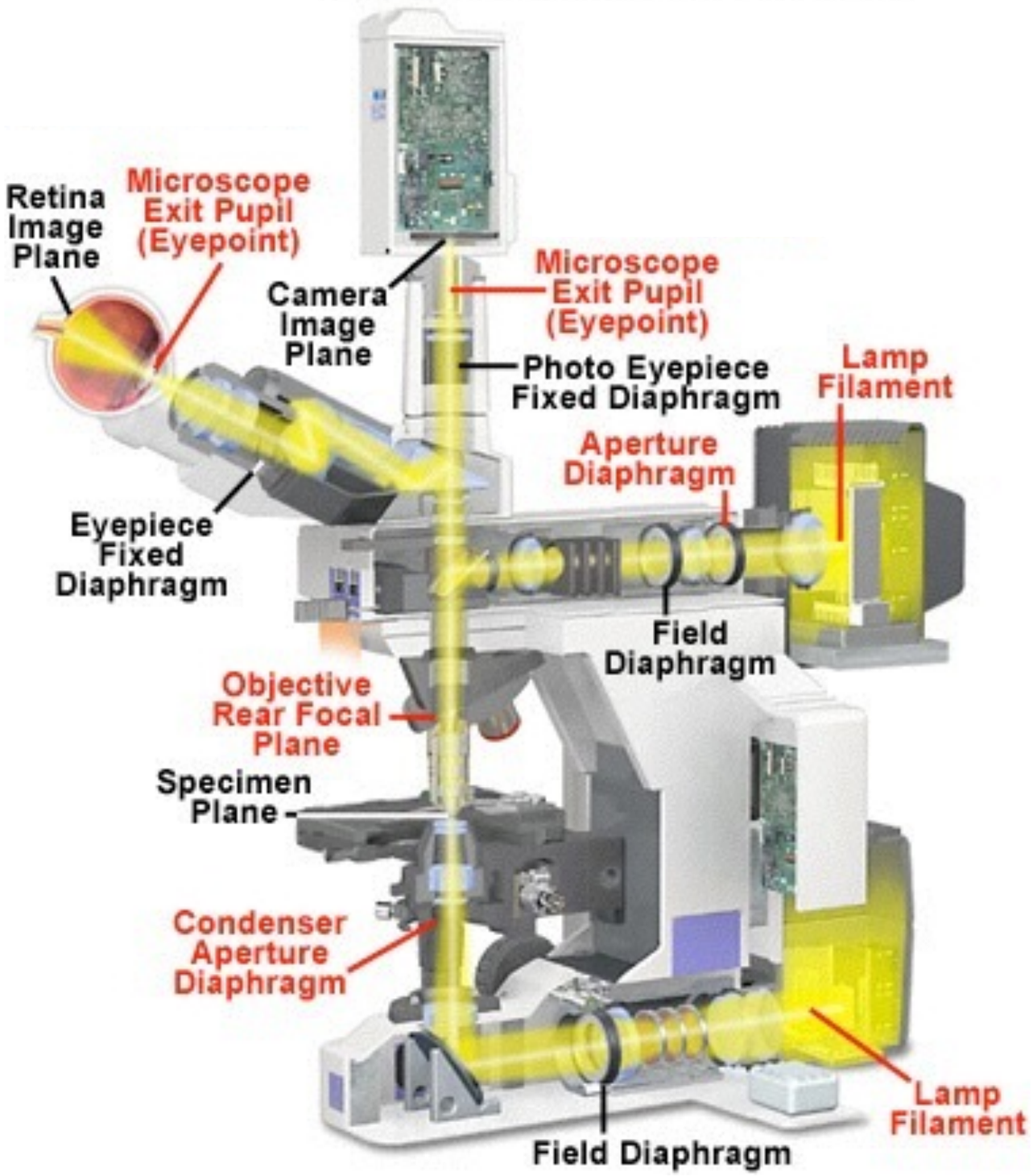
http://www.olympusmicro.com/

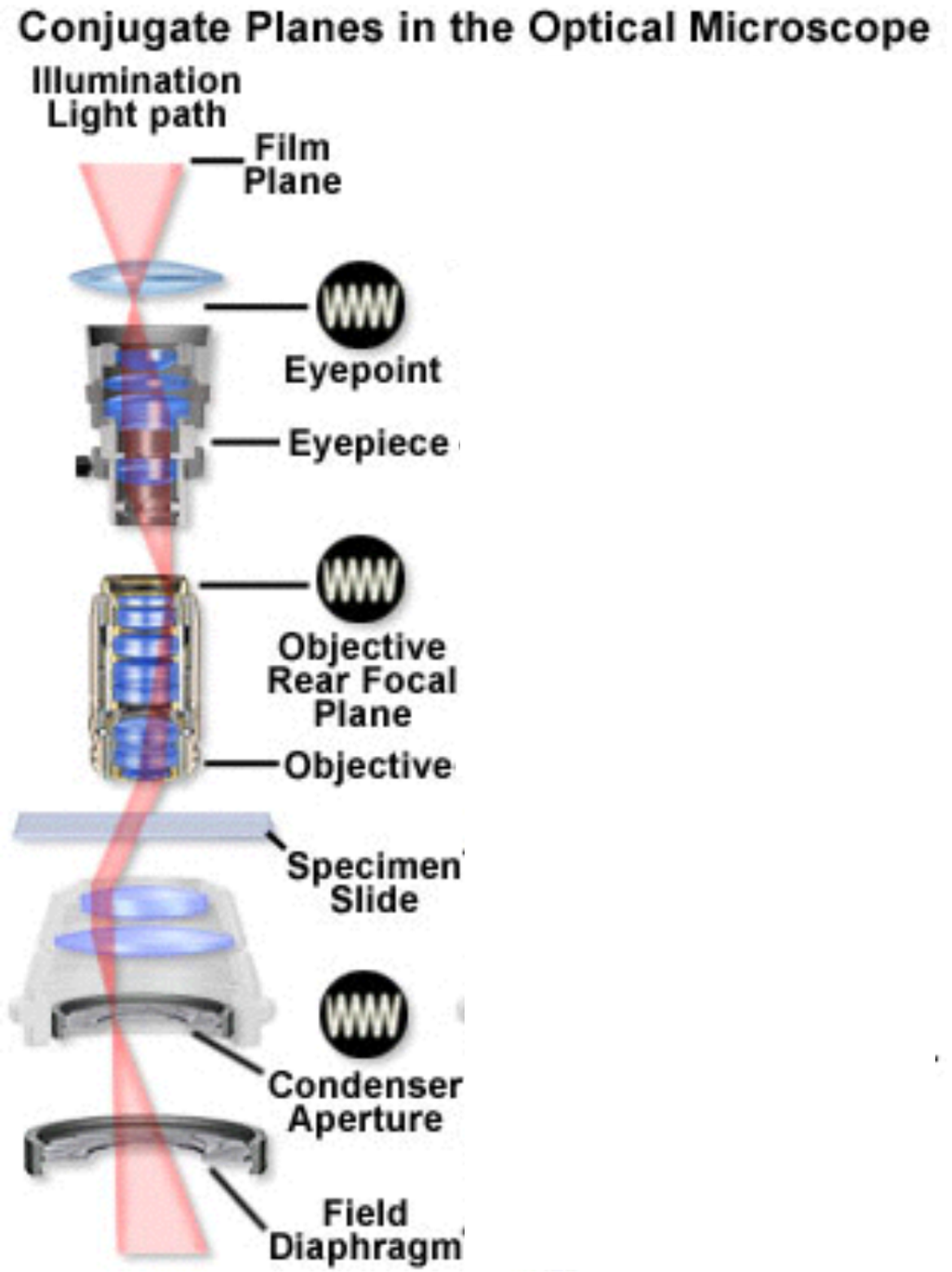
http://www.microscopyu.com/articles/formulas/formulasconjugate.html

Koehler illumination and conjugate planes

Field or Image forming conjugate planes

Aperture or illuminating conjugate planes





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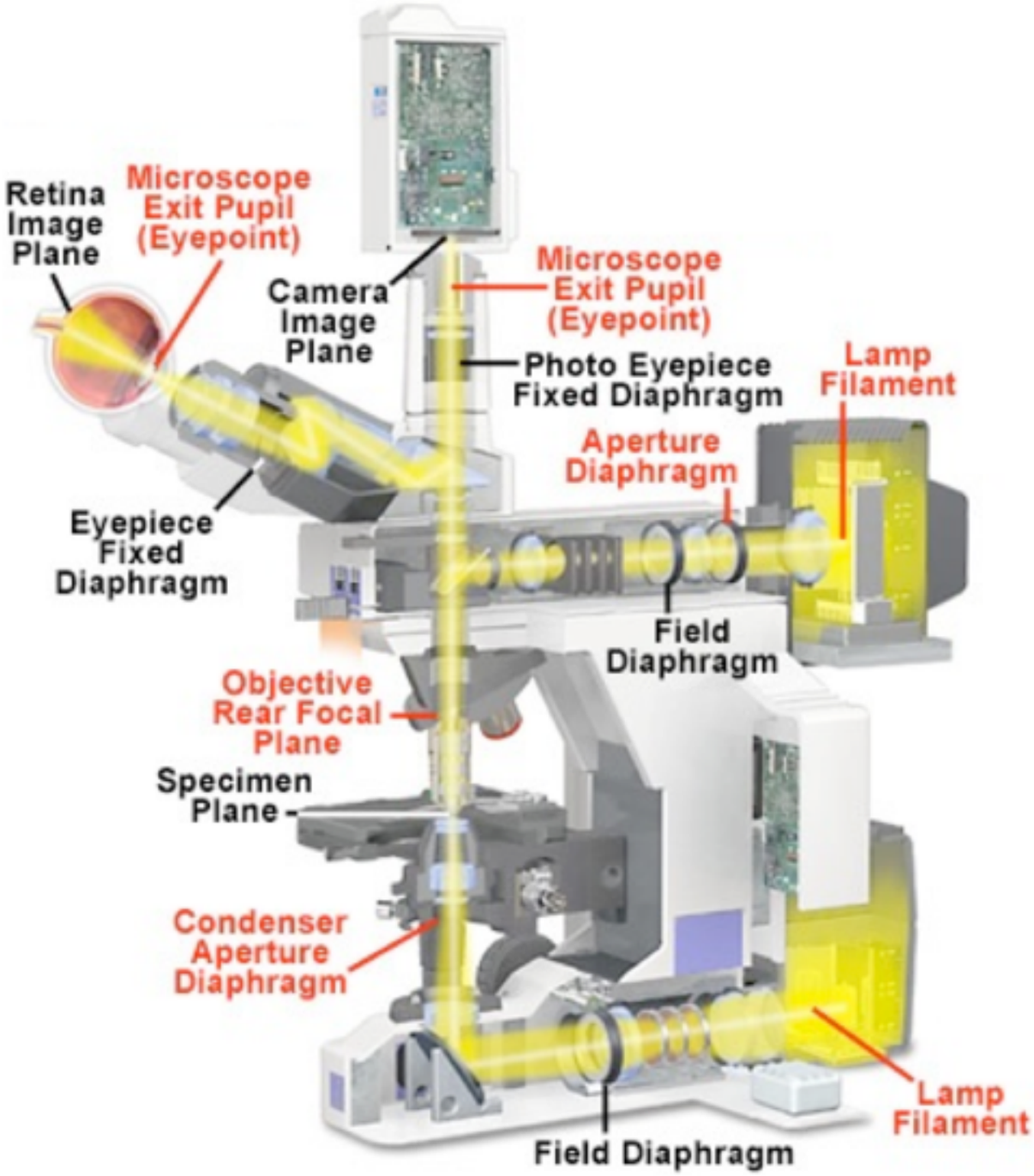

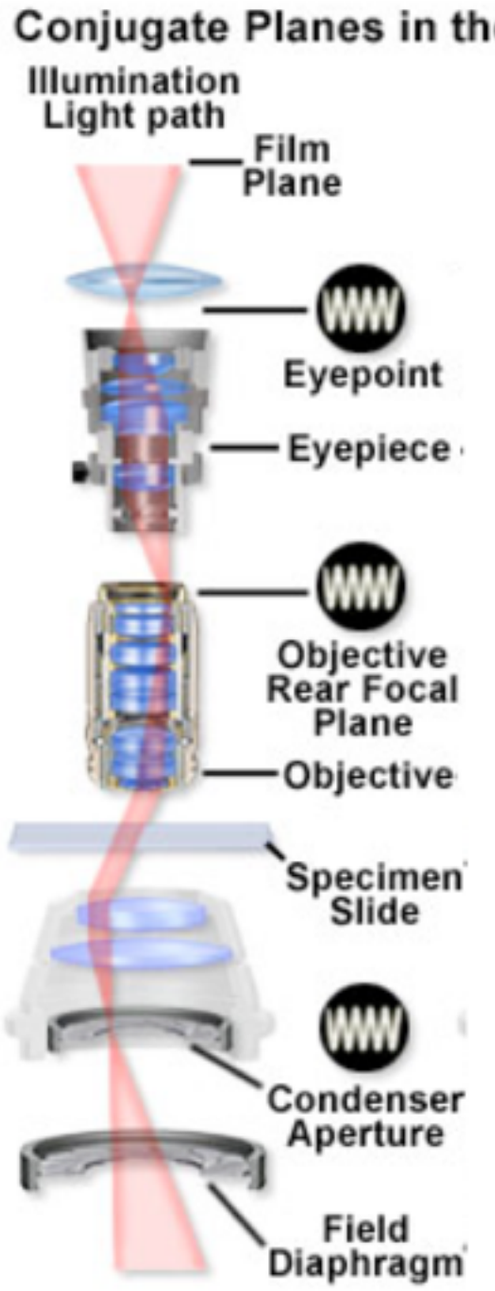

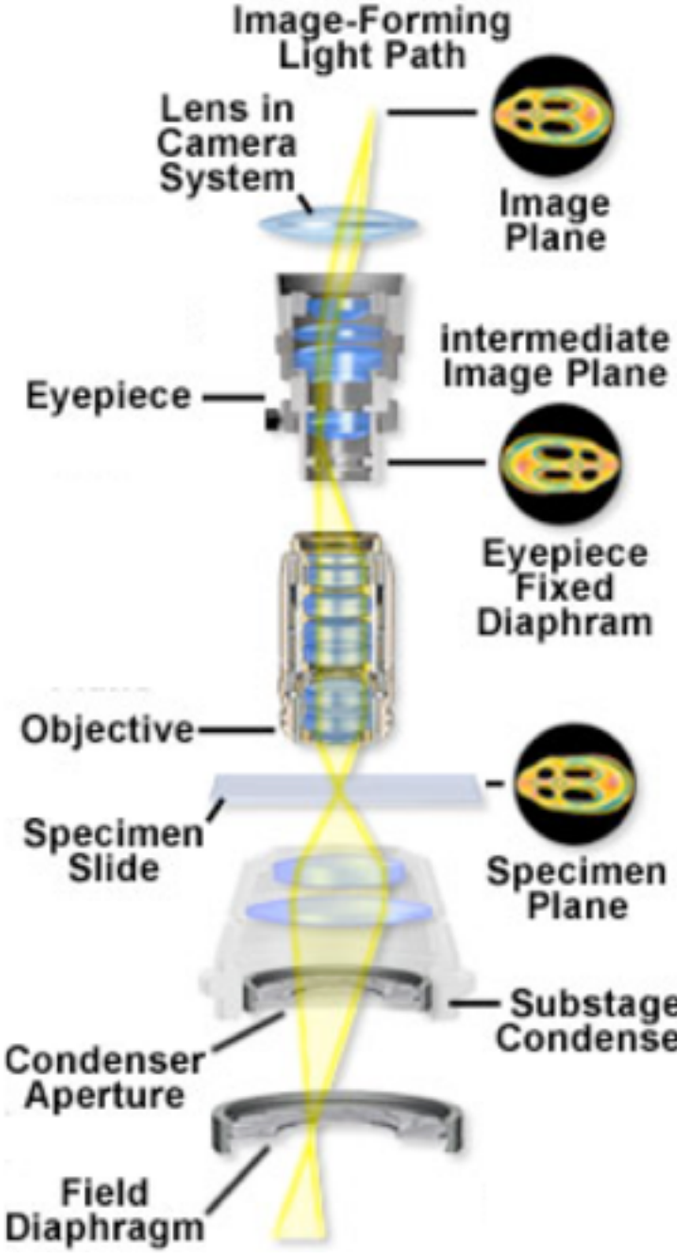

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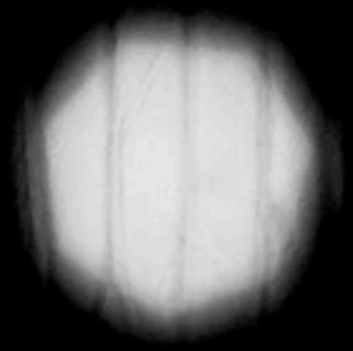
Bright-field Koehler setup (field diaphragm)



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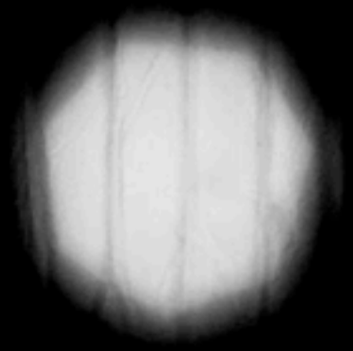
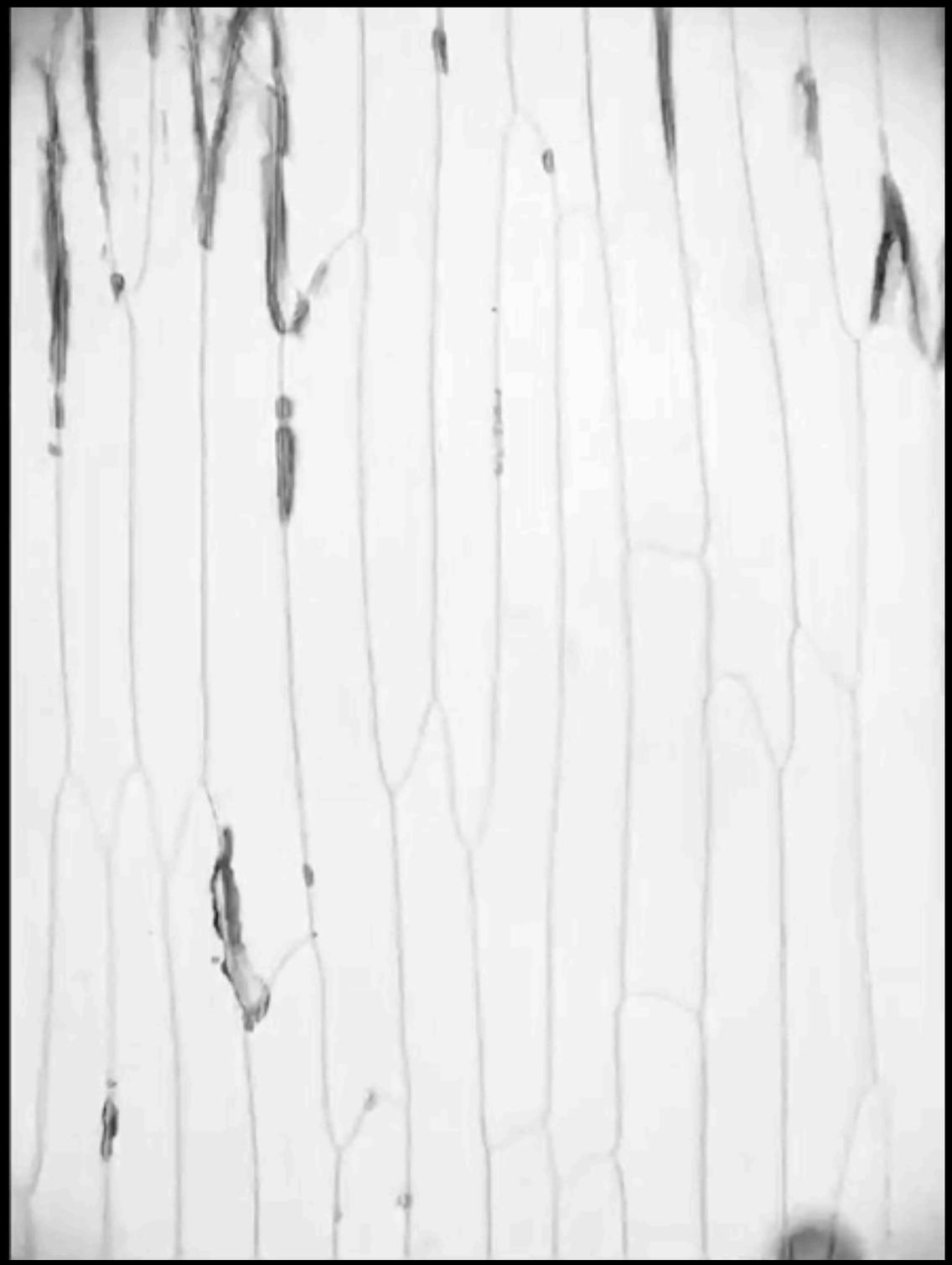


Bright-field Koehler setup - aperture diaphragm



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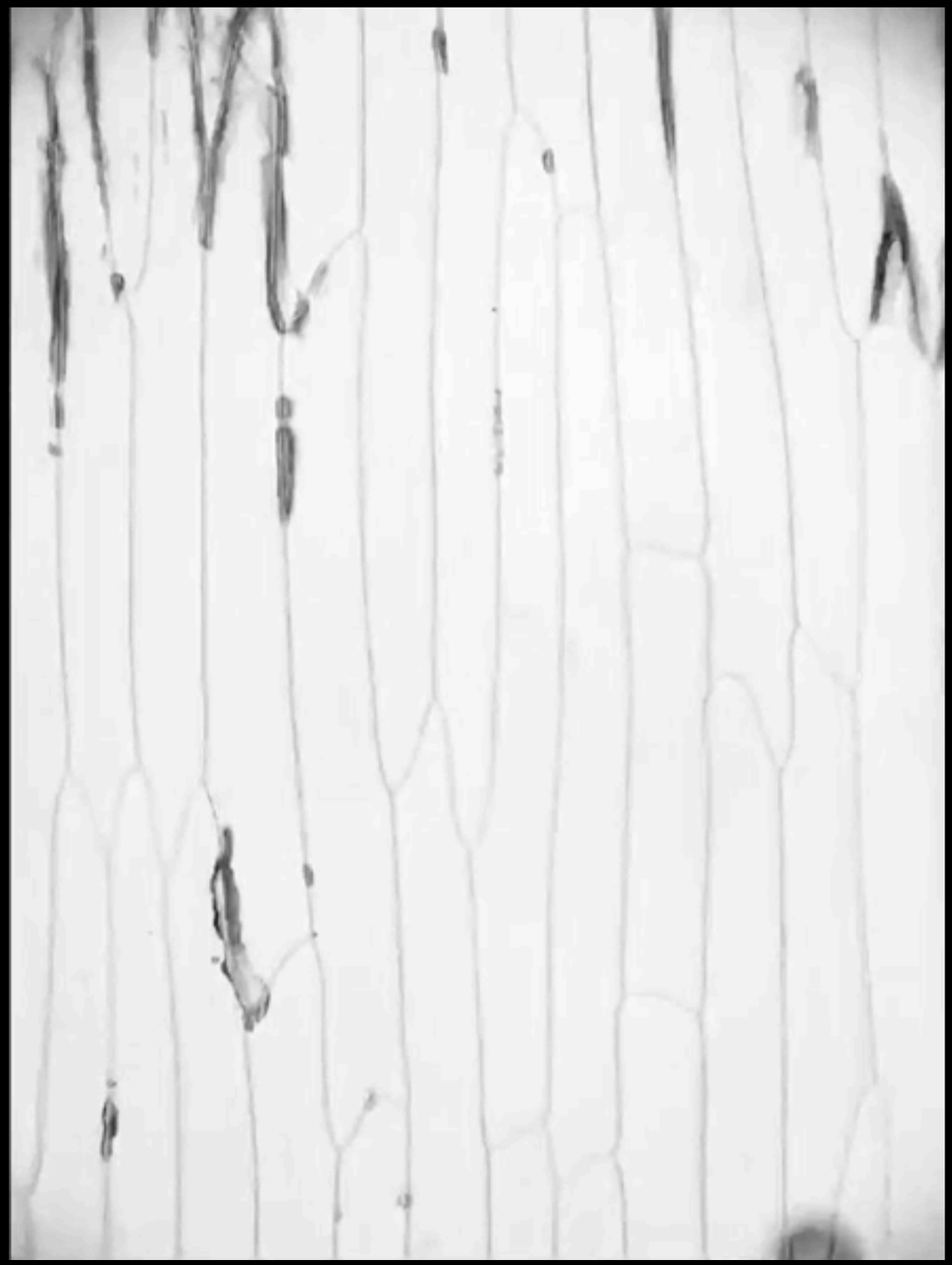

Preparing a bead slide:

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.