Micron Advanced Light Microscopy Course 2018

Introductory lecture

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Department of Biochemistry
University of Oxford

Introductory lecture:

- Organisation and goals of the course
- Why microscopy is important
- What is important for microscopy
- How to choose what technique to use

Organisation of the course:

- Day 1 - Principles of Microscopy
- Day 2 - Generating contrast
- Day 3 - Imaging approaches for molecules & cells
- Day 4 - Beyond conventional imaging

- Days 2-4 - Imaging handling and analysis

Organisation of the course:

- Day 1 - Principles of Microscopy ***LECTURES 1 & 2***
- Day 2 - Generating contrast ***LECTURES 3-5***
- Day 3 - Imaging approaches for molecules & cells ***LECTURES 6-8, M2***
- Day 4 - Beyond conventional imaging ***LECTURES 9-11***

- Days 2-4 - Imaging handling and analysis ***LECTURES M1,3,4,5***

Catering for a diverse intake: the knowledge base survey

*** To assess the starting knowledge**
*** To assess the diversity of experience**
*** To tailor the course material**

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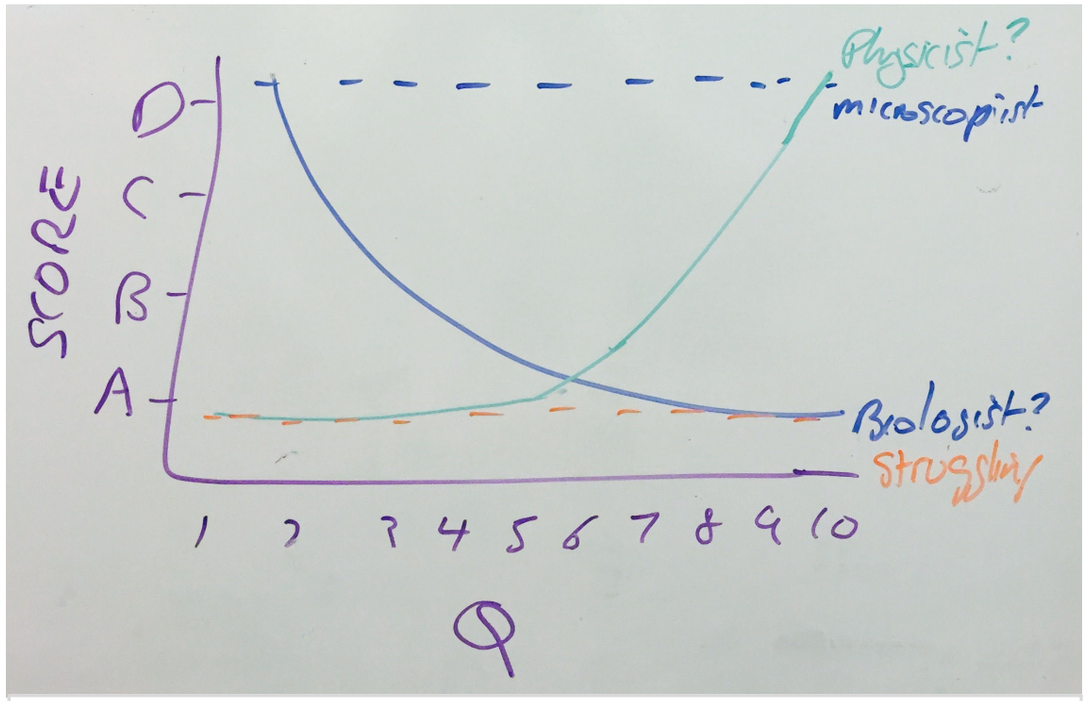
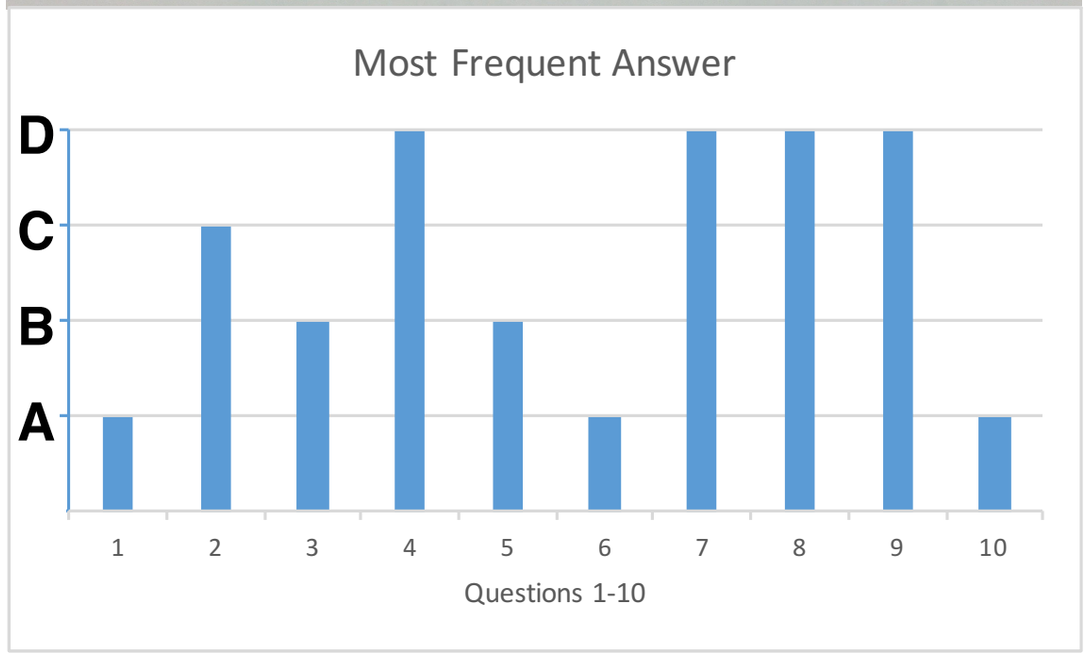
Simple answer scheme:
a) Have never heard of this
b) Have heard of this, but don't know what it means
c) Have a vague idea of what it means
d) Have a clear idea of what it means and can explain it



- Questions structured:
Q1-5 favour biologists
Q5-10 favour physicists / engineers
- questions increasing in difficulty



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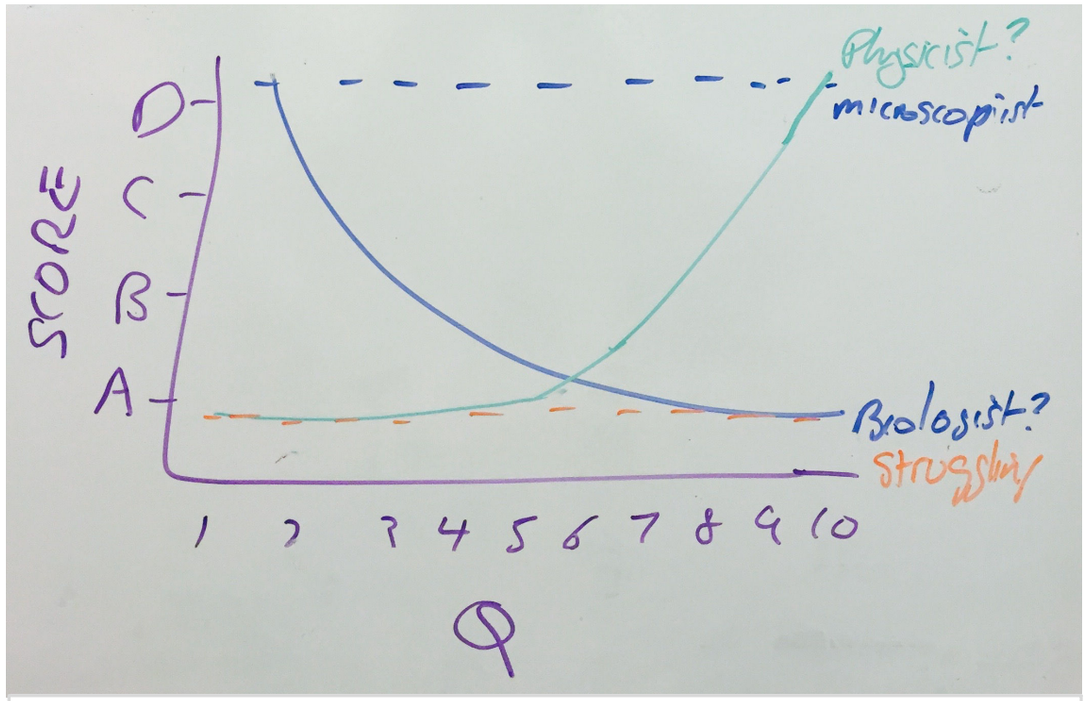

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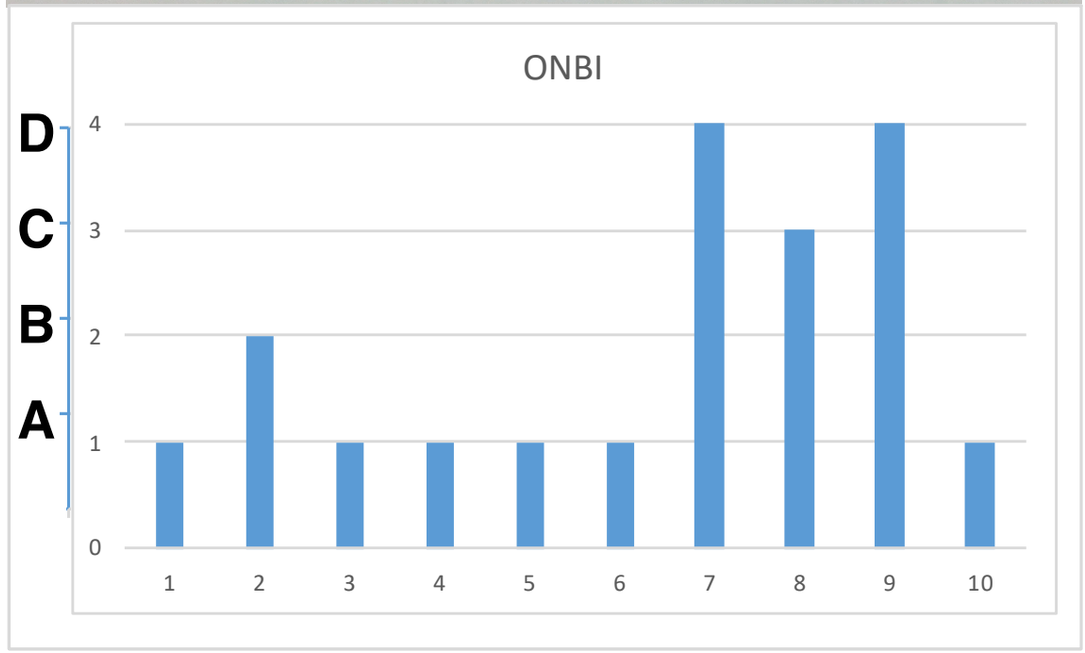

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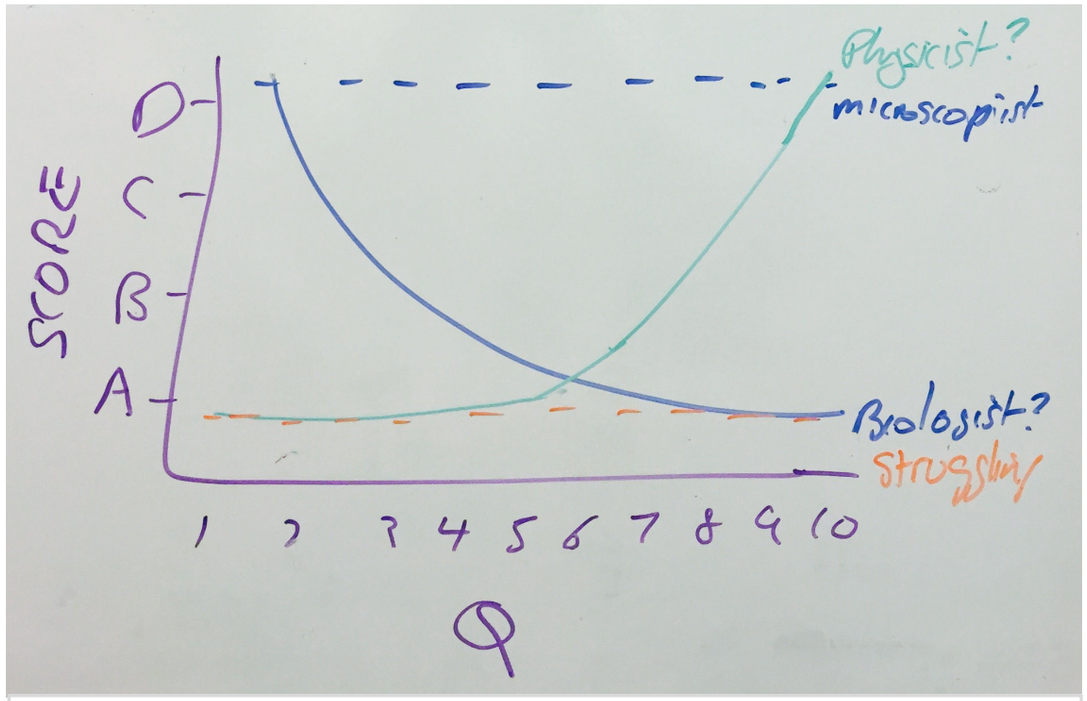

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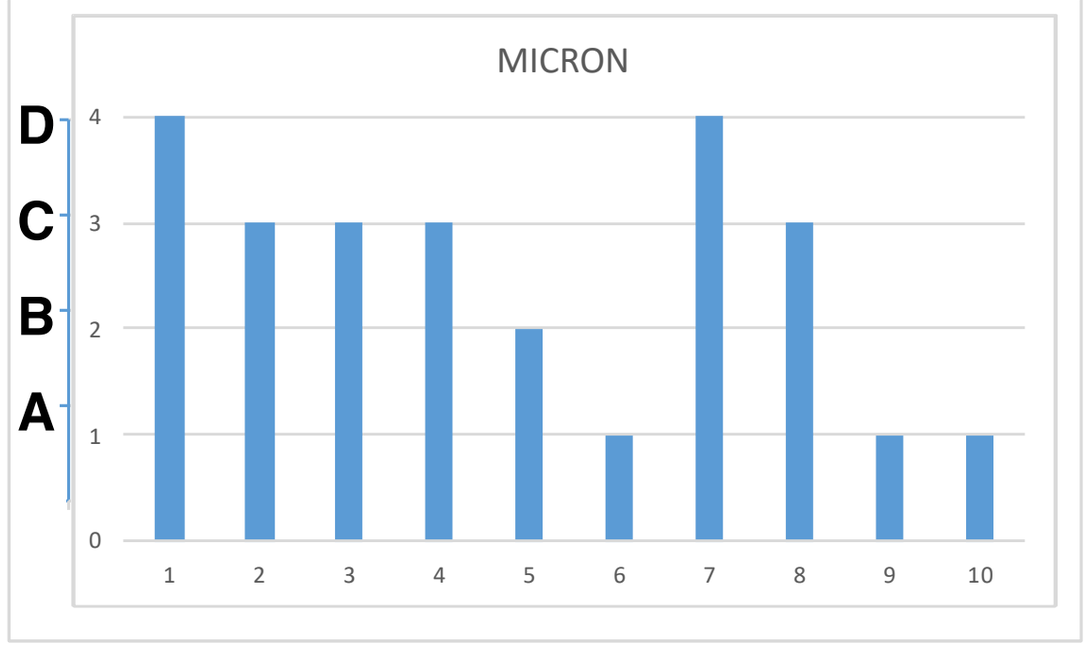

Why should you care about microscopy?

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Cell Journal 2018, Oct/Nov Research Articles:

>70% Include Some Form of Imaging

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  the basic physics of optics and microscopes

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  - Digital acquisition and deblurring images
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Why is microscopy 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)
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)

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- 2014 Nobel Prize in Chemistry for Super Resolution Betzig, Hell, Moerner * **

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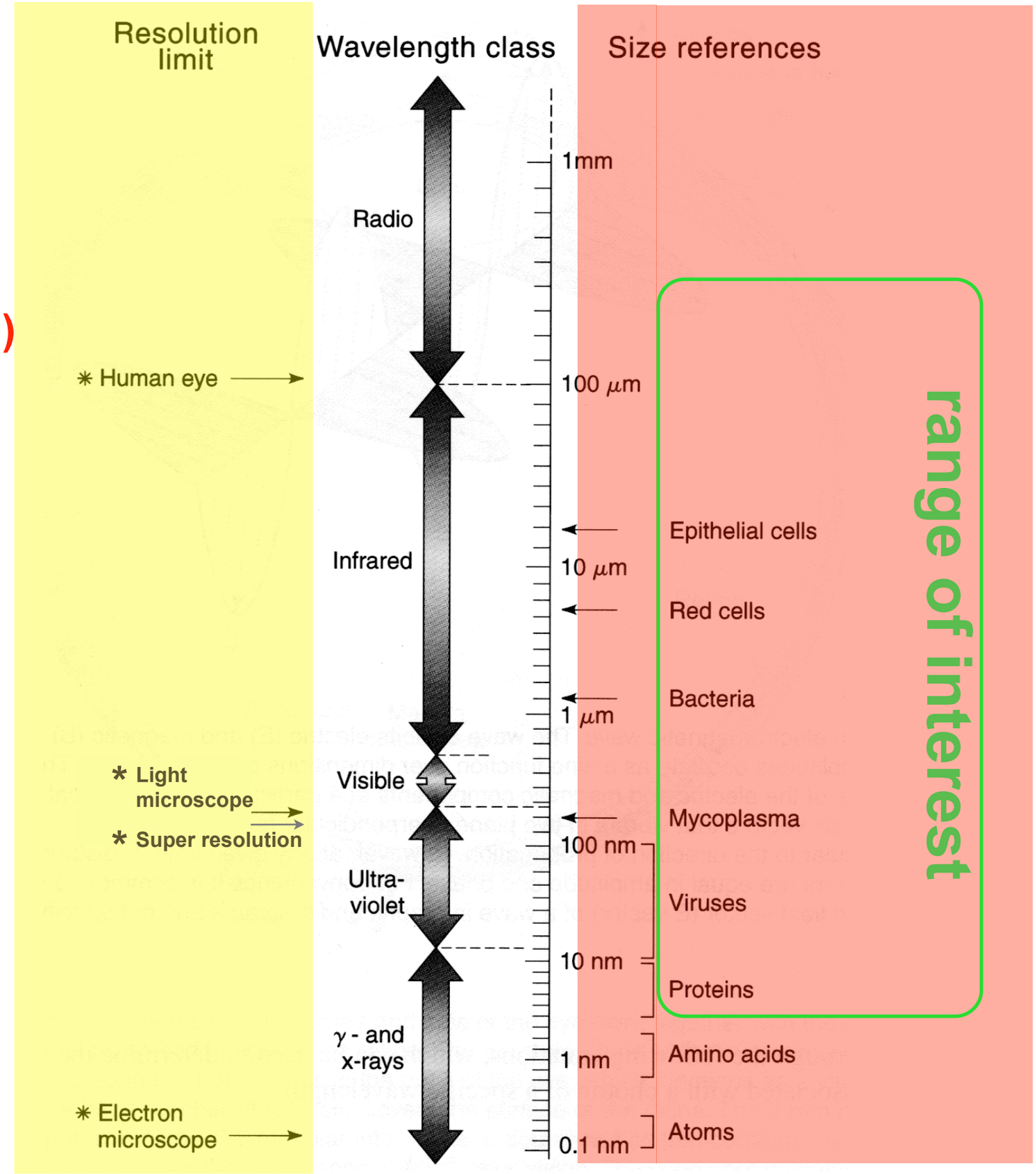
- **Can be applied to live cells to follow sequences of events**
- **Allows experimental manipulation**

Useful size range for light microscopy

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



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

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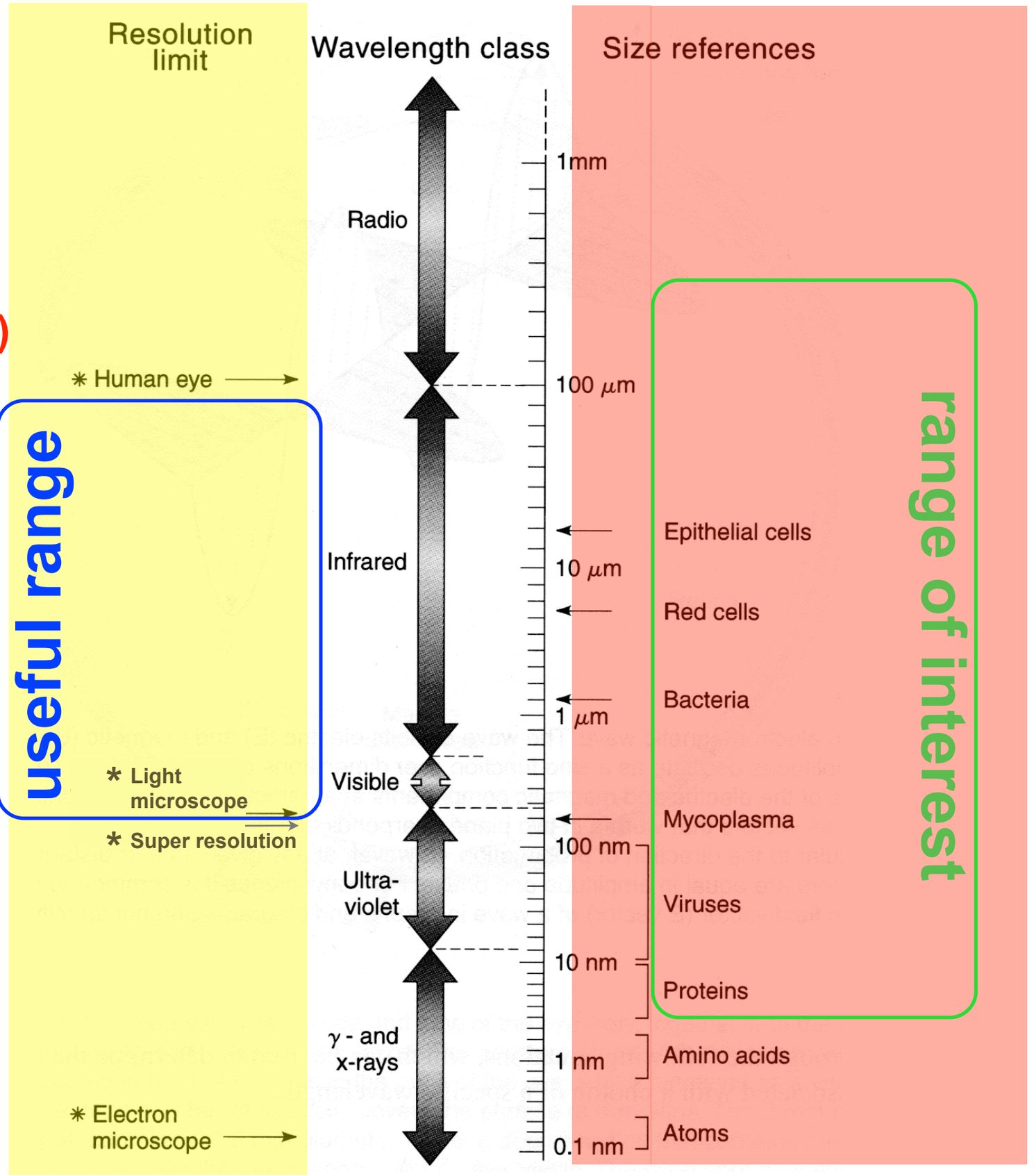
Eukaryote = 10 um
>50 um

Bacterium = 1 um



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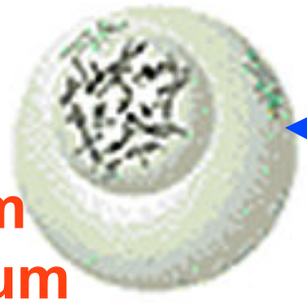
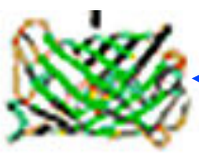


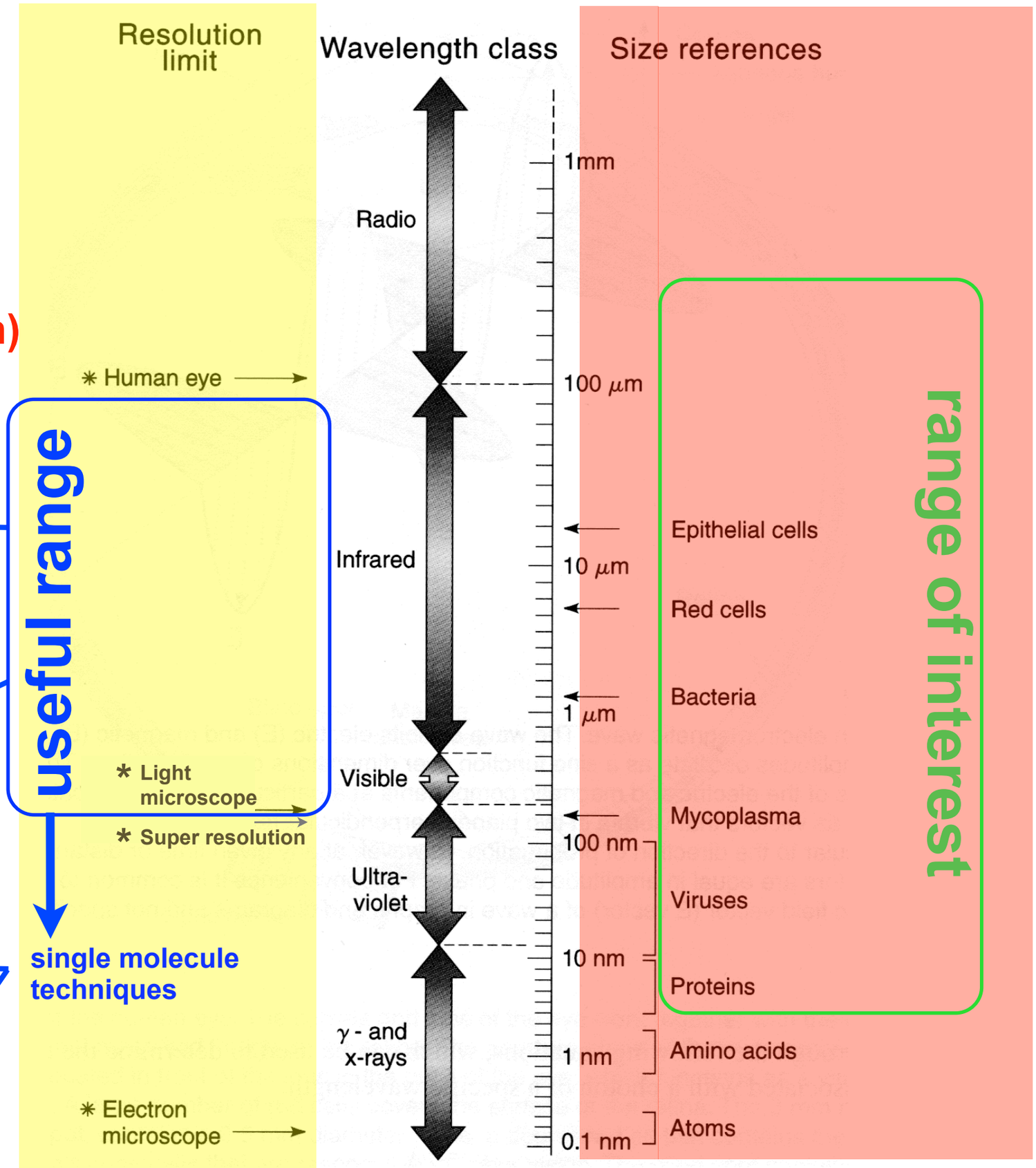
Bacterium = 1 um

Single GFP = 5 nm



Fluorescein = 1 nm

(1 nm = 10 Angstom)



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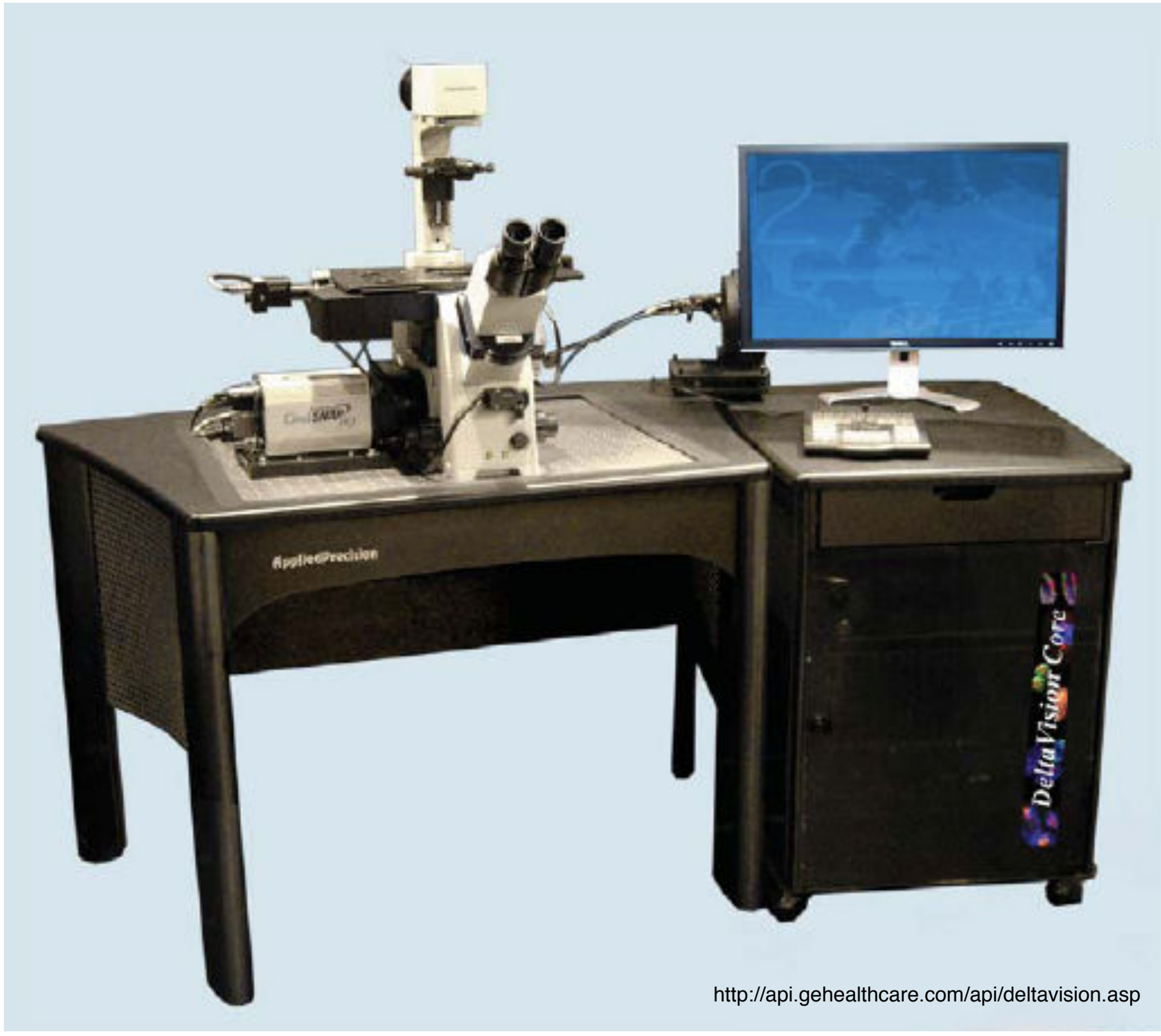
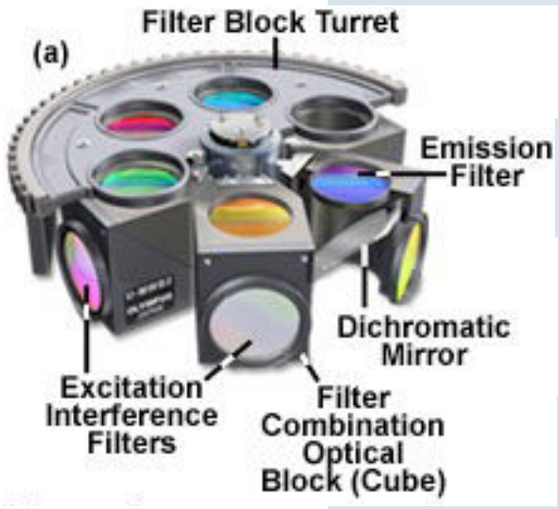
- **Microscopes are all basically the same**
- **It is quite hard to break a microscope**

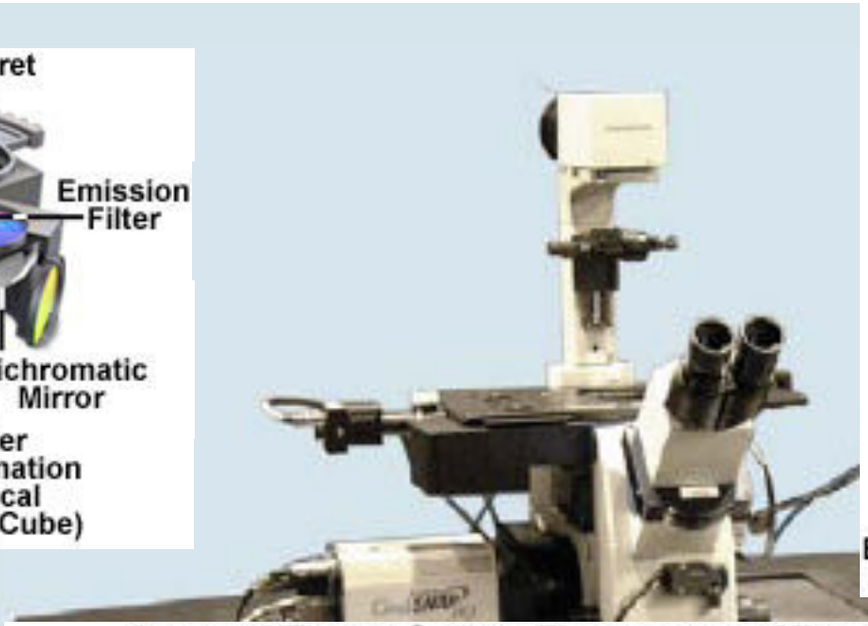
Understanding what goes on in the Microscope



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

Understanding what goes on in the Microscope





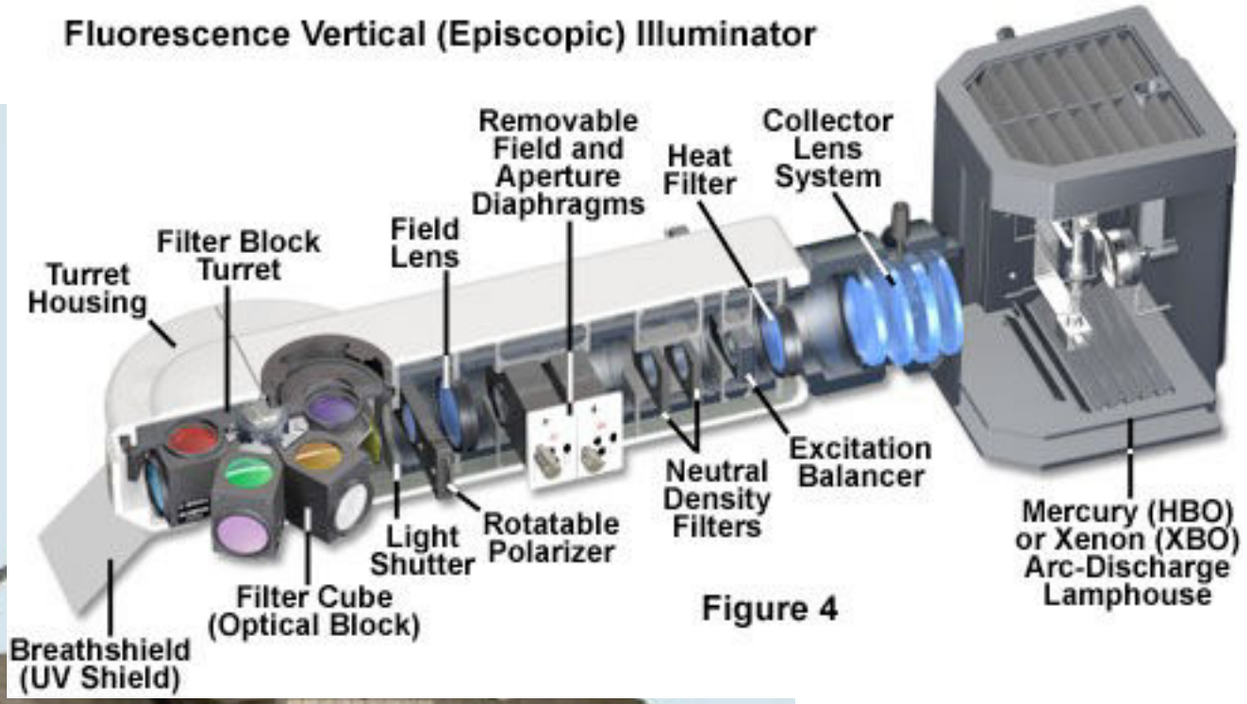


Figure 4

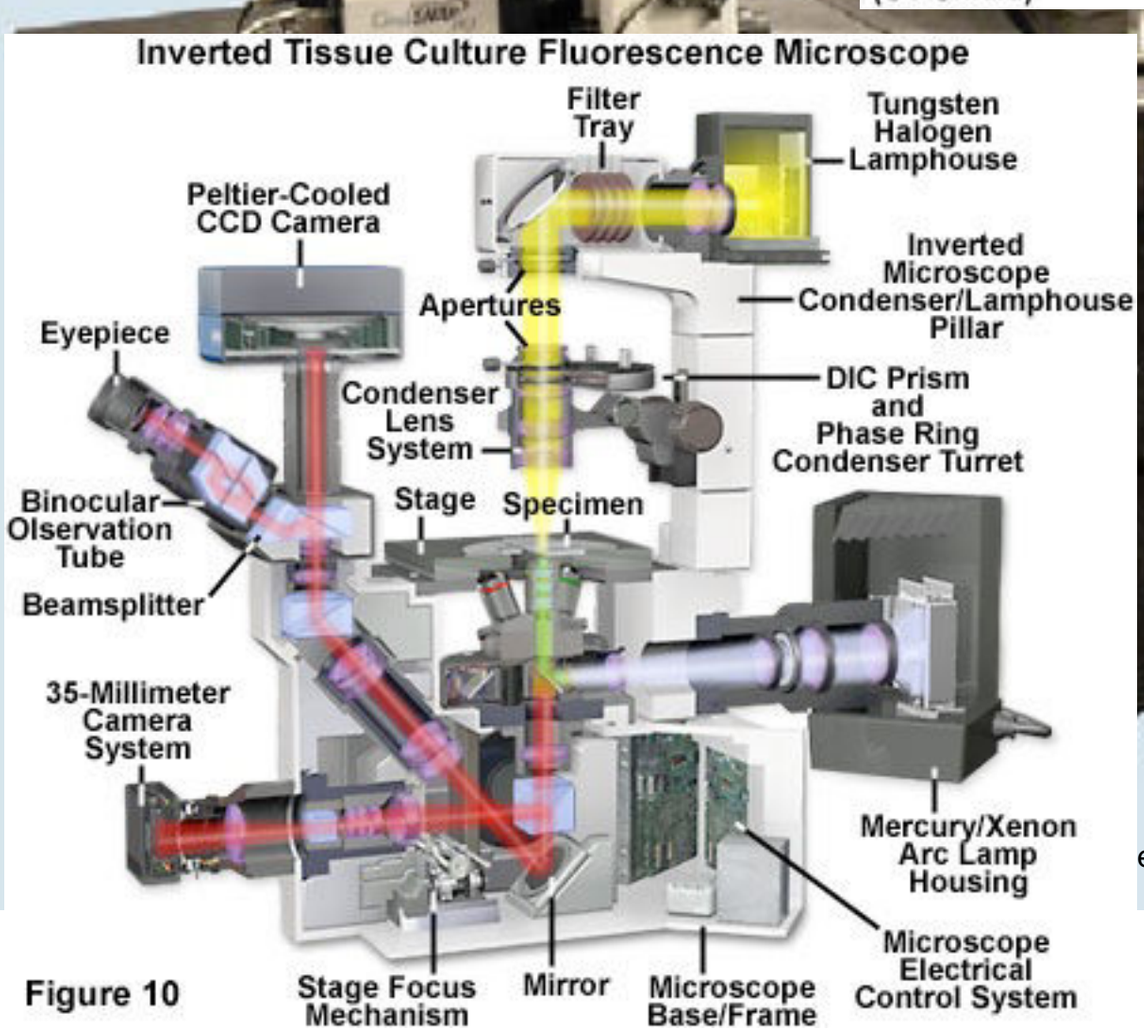


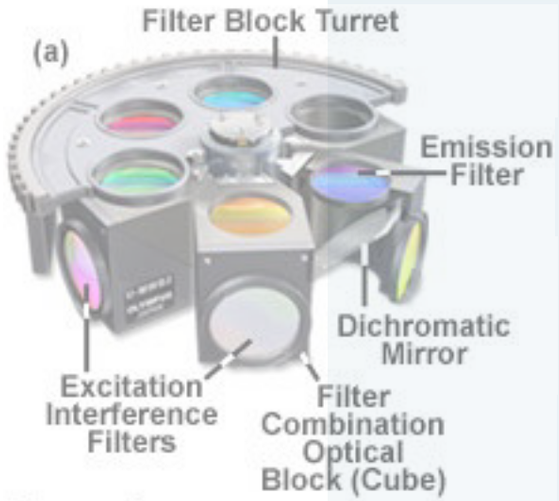
Figure 10

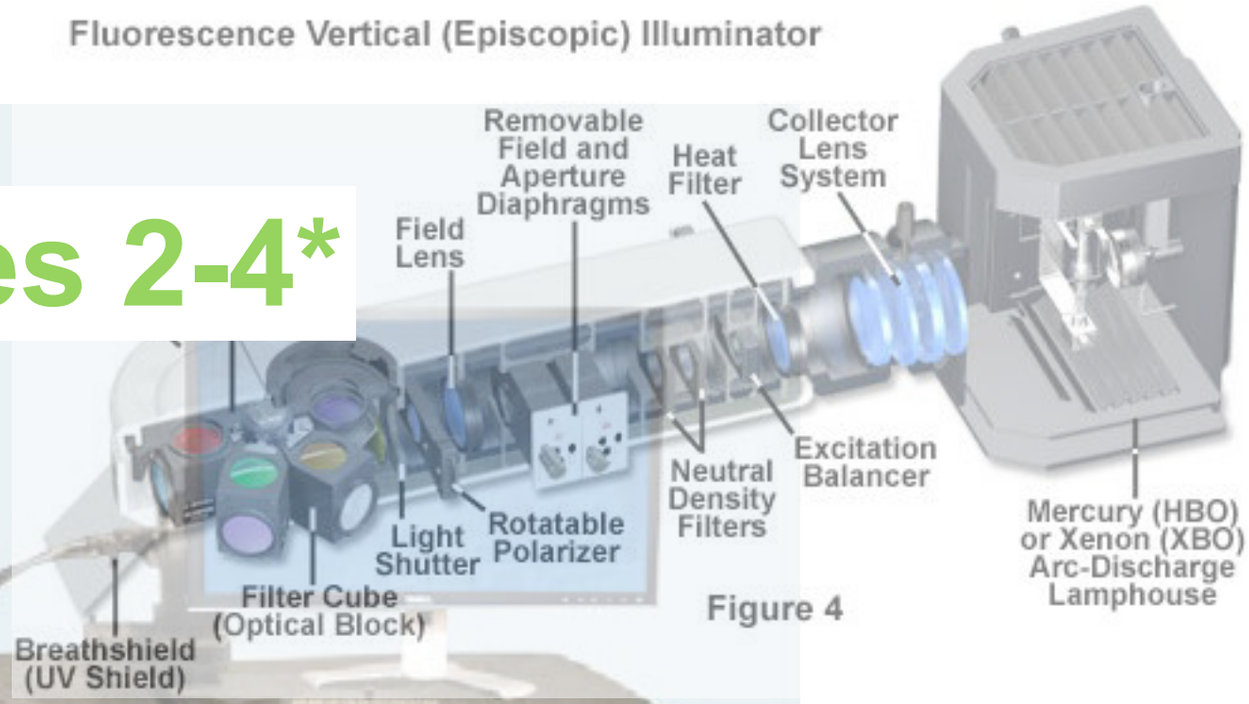
ehealthcare.com/api/deltavision.asp

http://www.olympusmicro.com/

Understanding what goes on in the Microscope

Lectures 2-4





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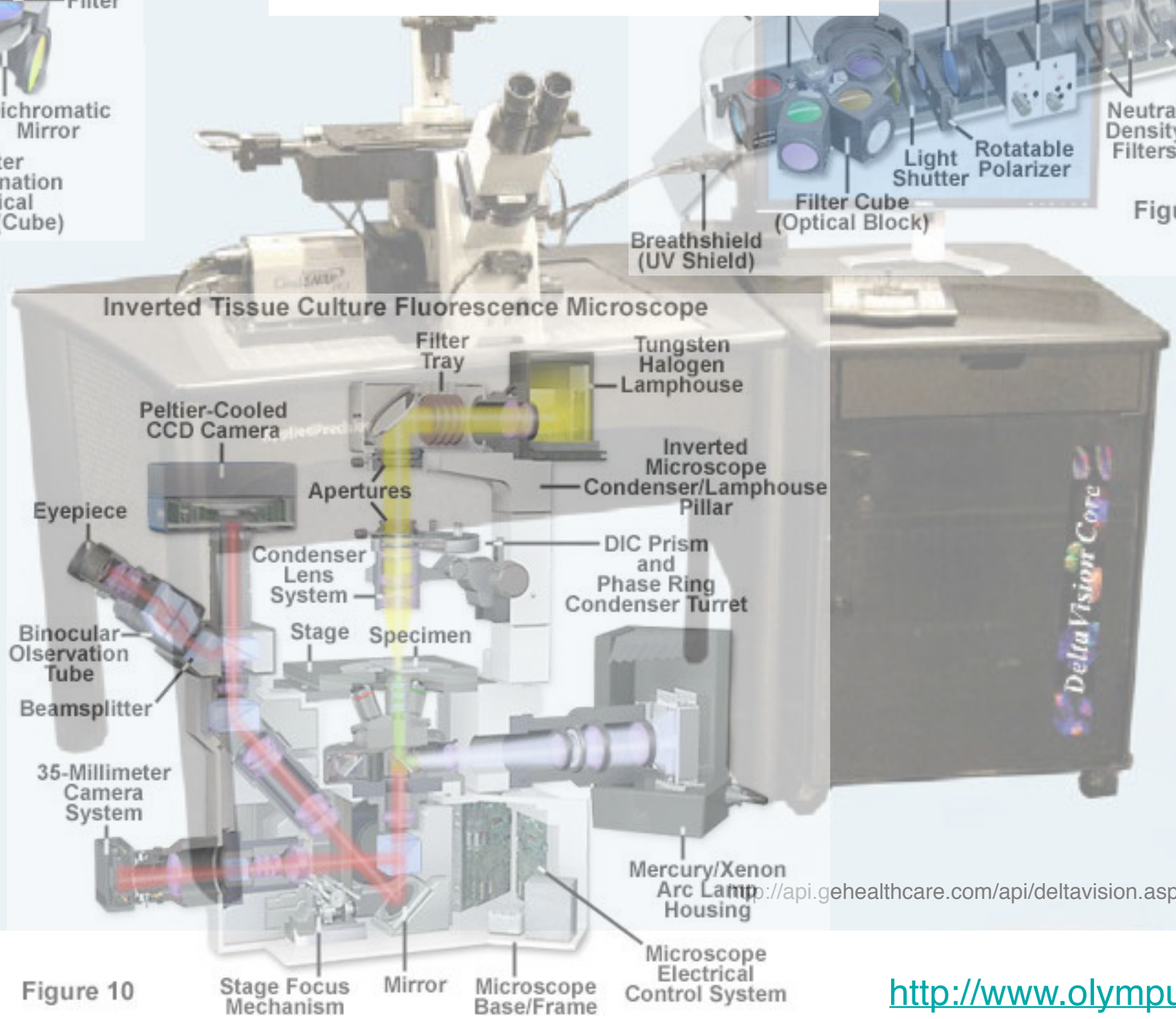

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What is really important in microscopy?

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…….the ability to see stuff

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1. Contrast

What is really important in microscopy?

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1. Contrast
2. Resolution

What is really important in microscopy?

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1. Contrast
2. Resolution
3. Sampling

What is really important in microscopy?

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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

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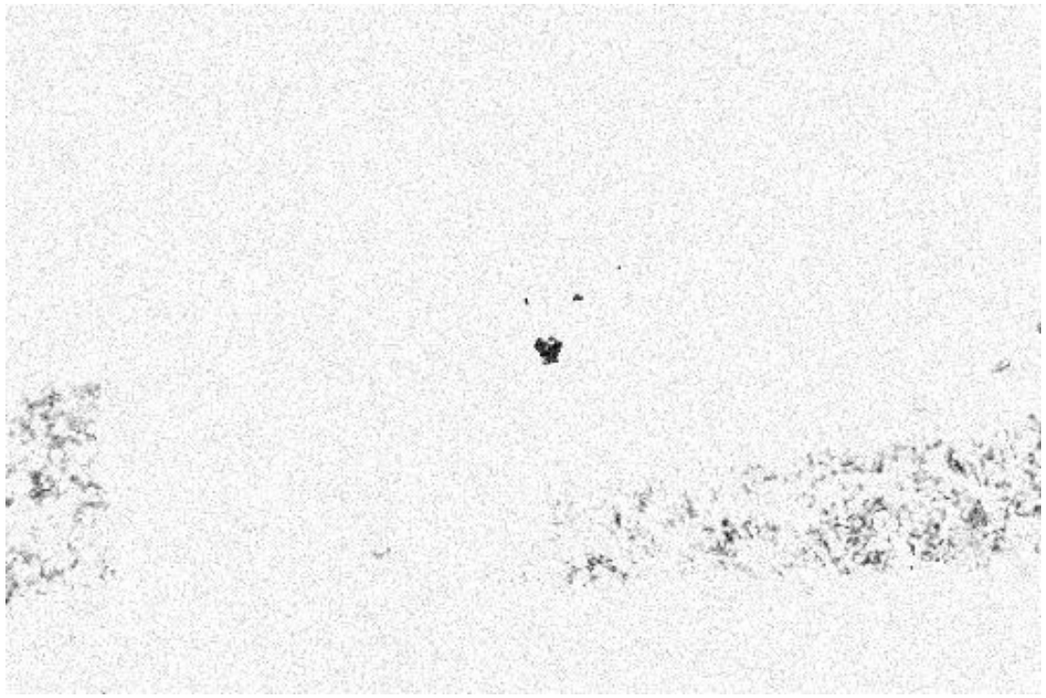
Contrast .....the ability to distinguish stuff

Biological specimens have low inherent contrast:



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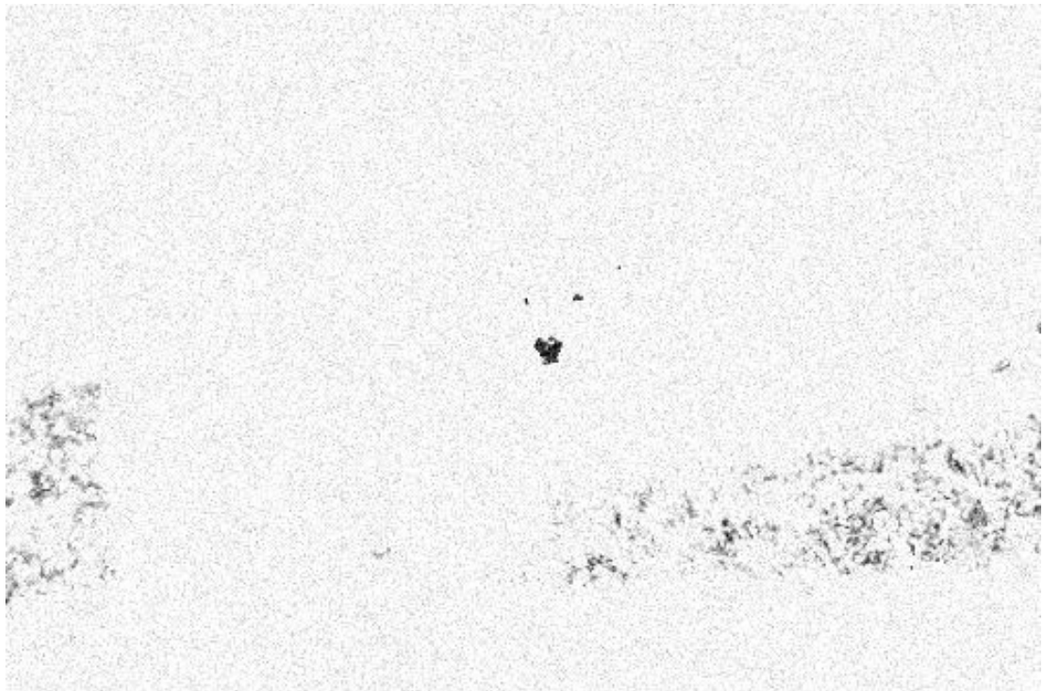

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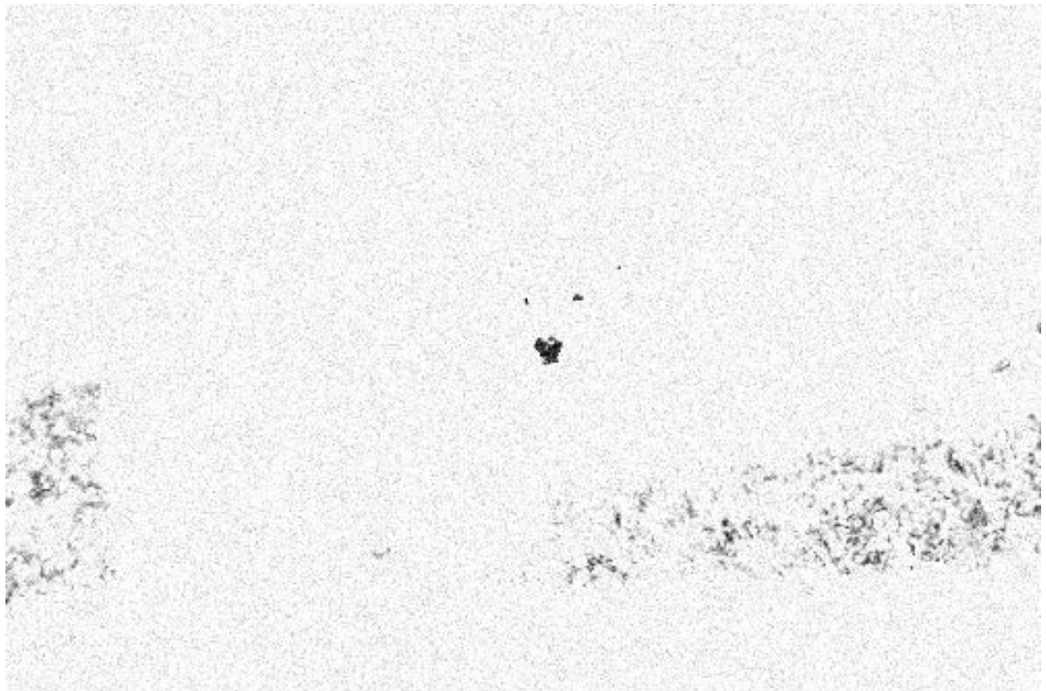

.....can’t resolve anything without contast

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

Bright Field Contrast Techniques

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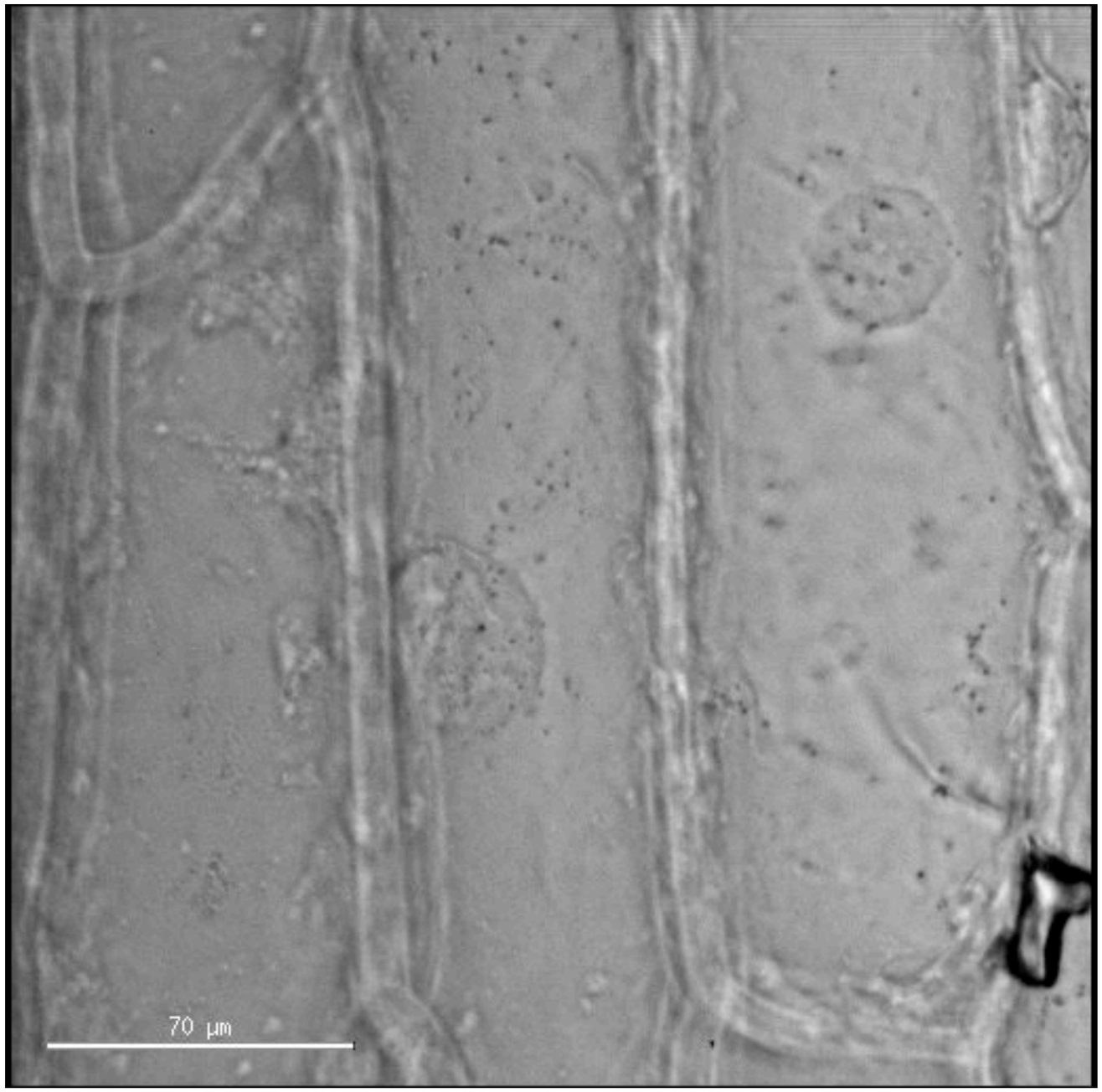
DIC differential interference contrast



RMP: Onion cytoplasmic streaming under DIC

Fluorescence Contrast Techniques

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

Fluorescence Contrast Techniques

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GFP
green
fluorescent
protein

Fluorescence Contrast Techniques

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

inorganic
fluorescent
labels

LECTURES 4, 5

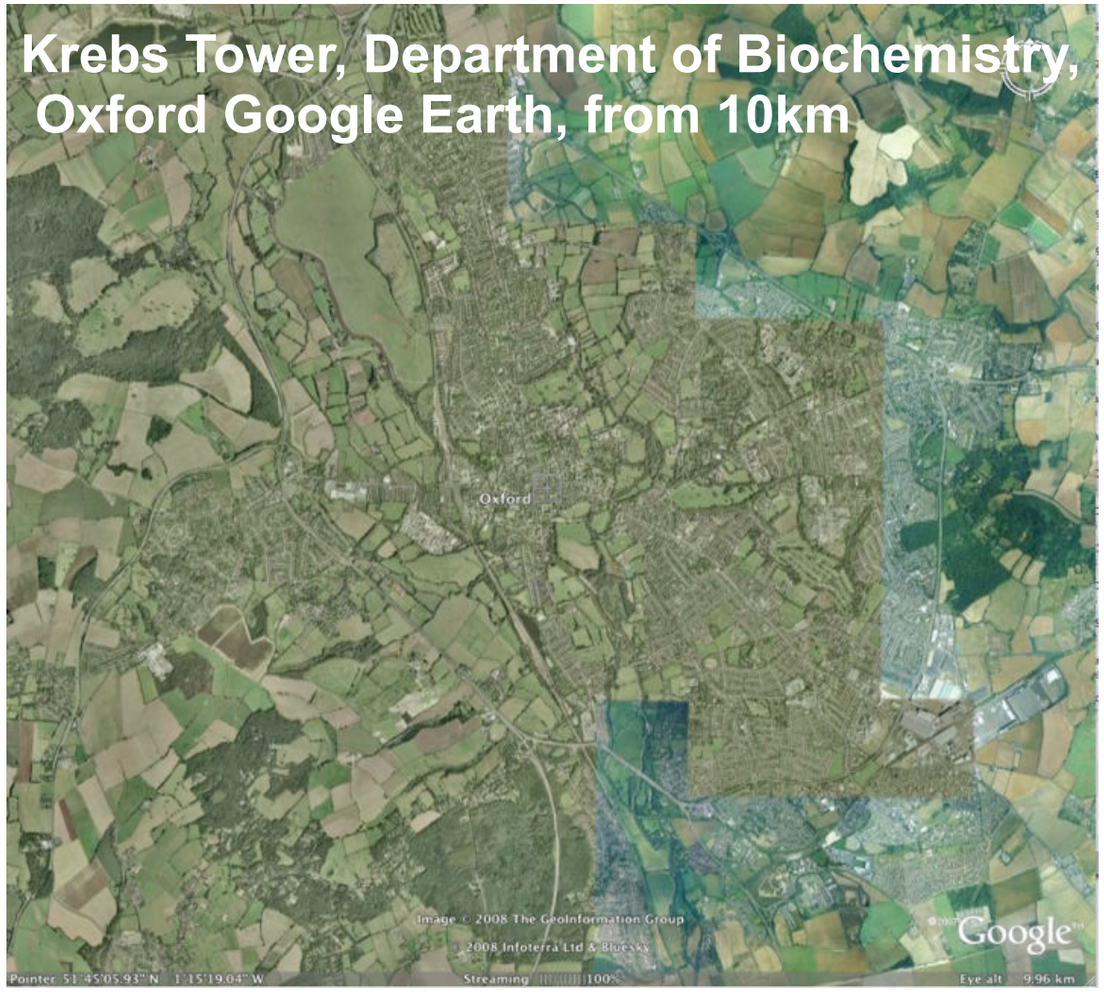
What is really important in microscopy?

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

What is really important in microscopy?

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

Magnifying is not enough:



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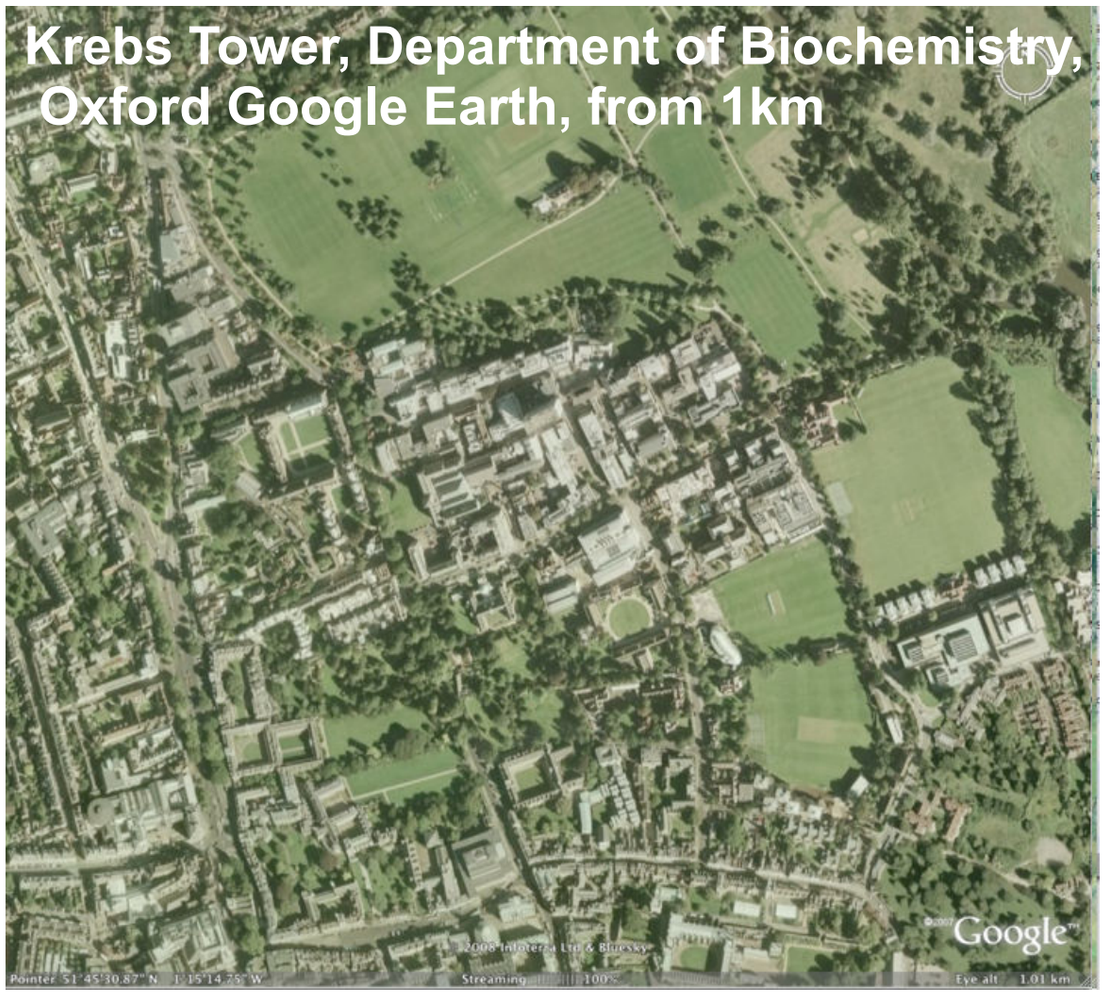

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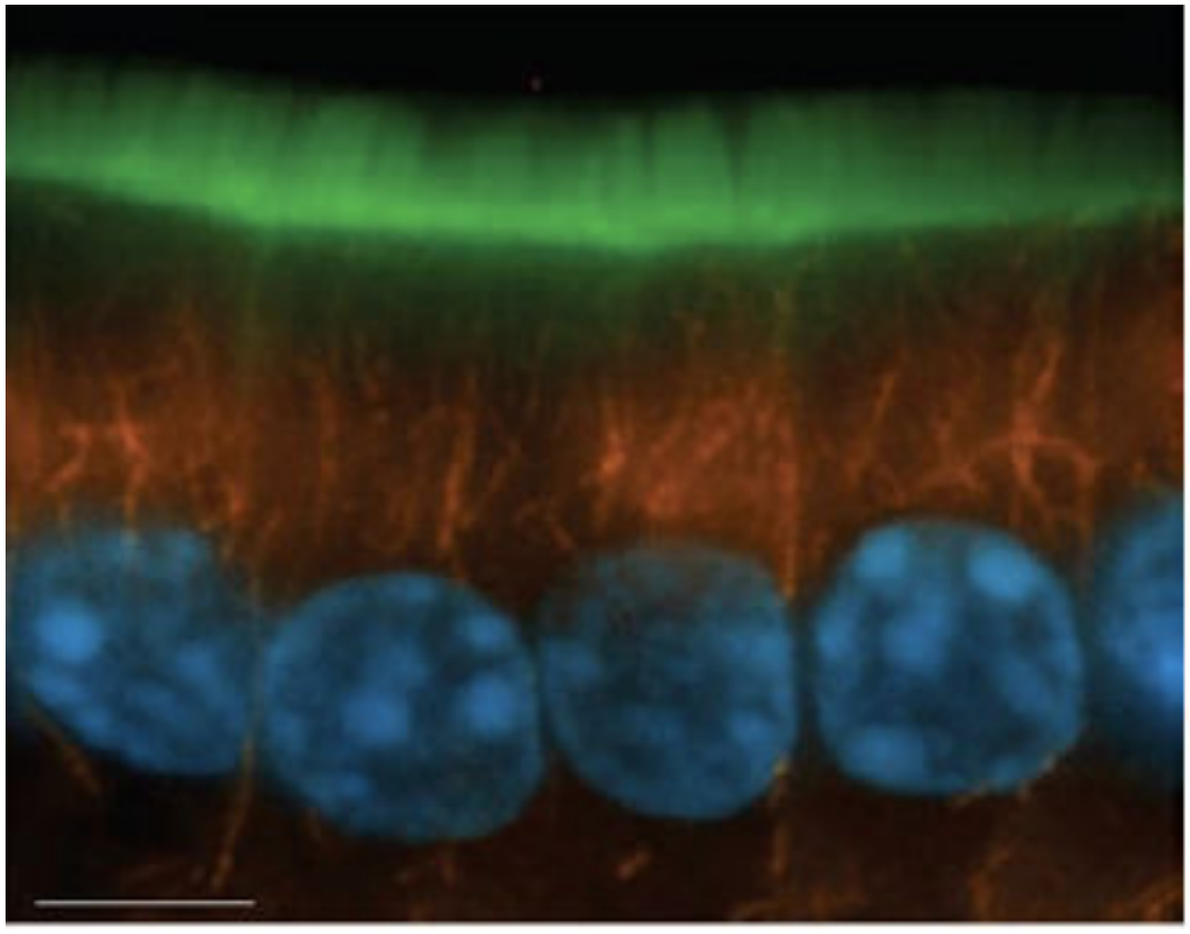
Resolution .....the ability to see small stuff

Magnifying is not enough:

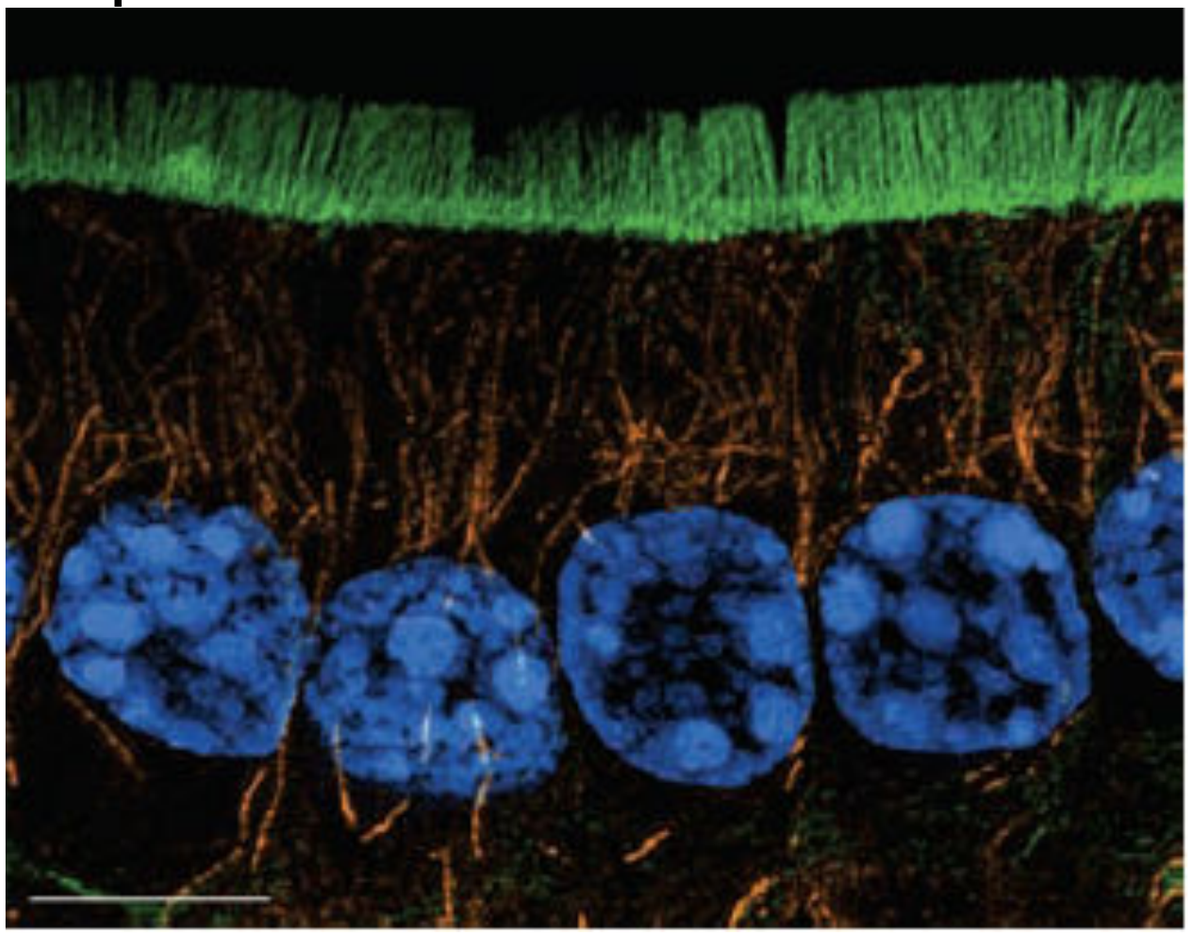
.....resolution is limited

RESOLUTION

Normal resolution



Super resolution



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

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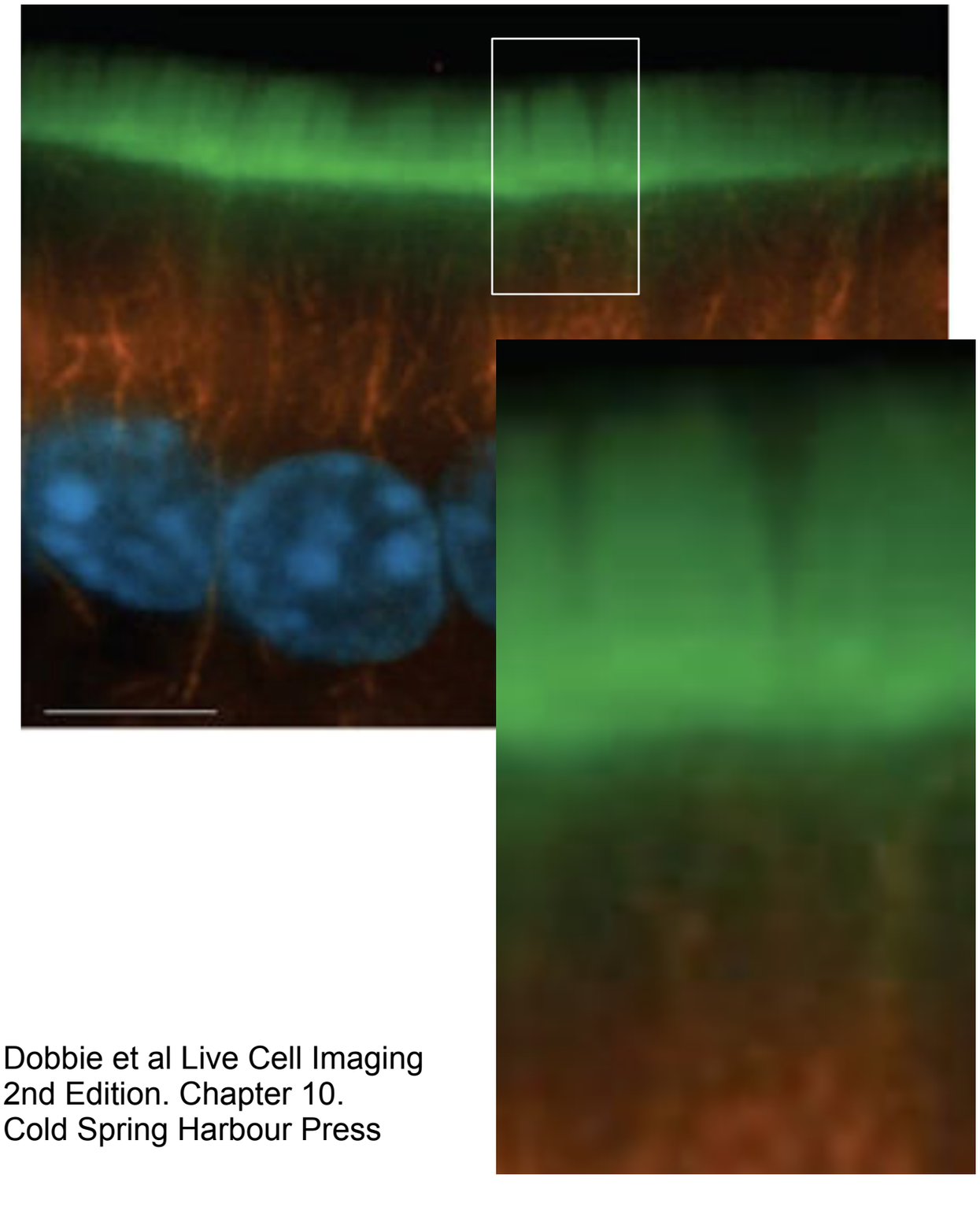

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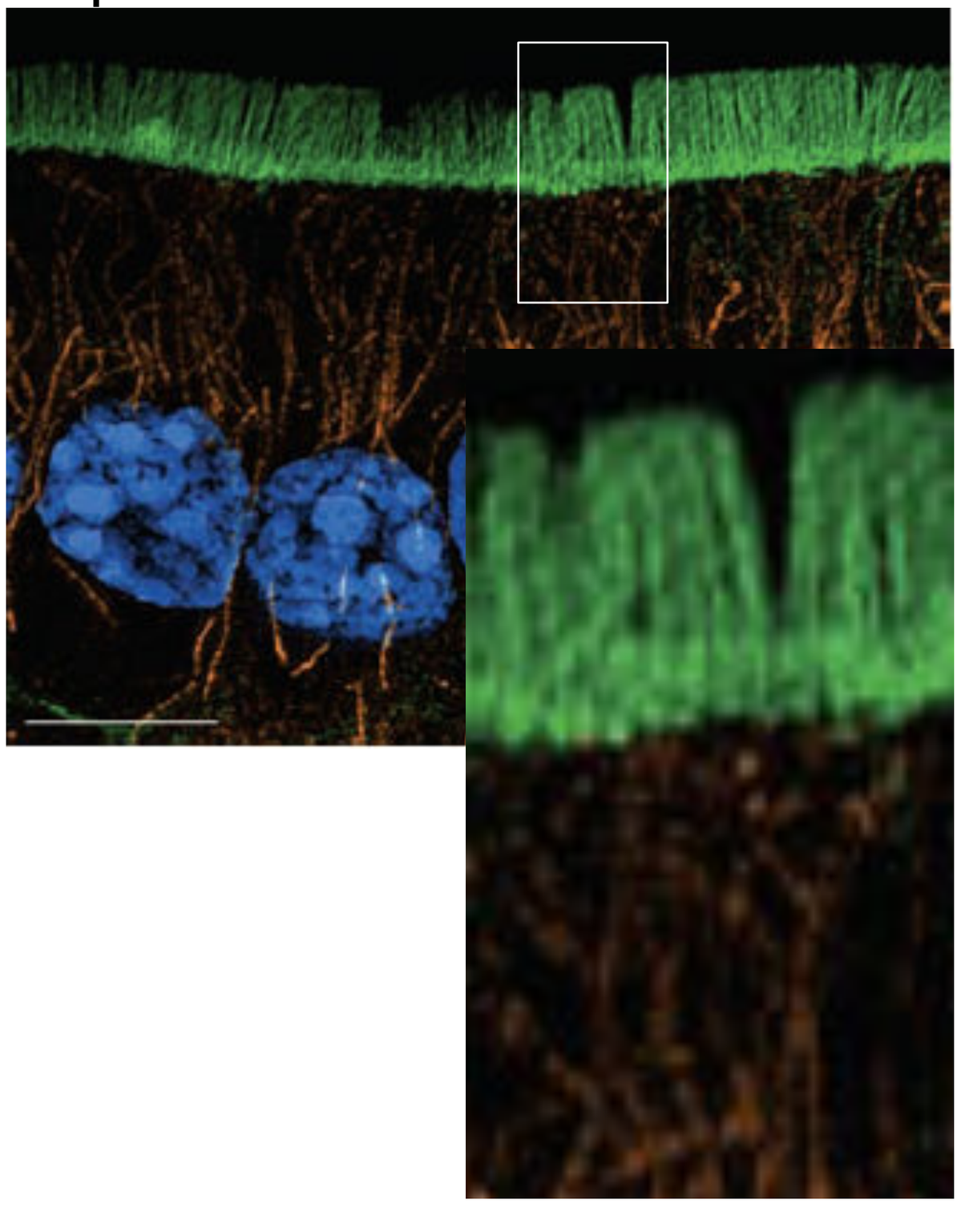

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Understanding what limits Resolution

* 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

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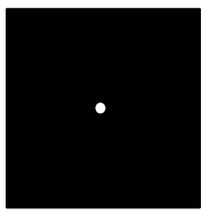
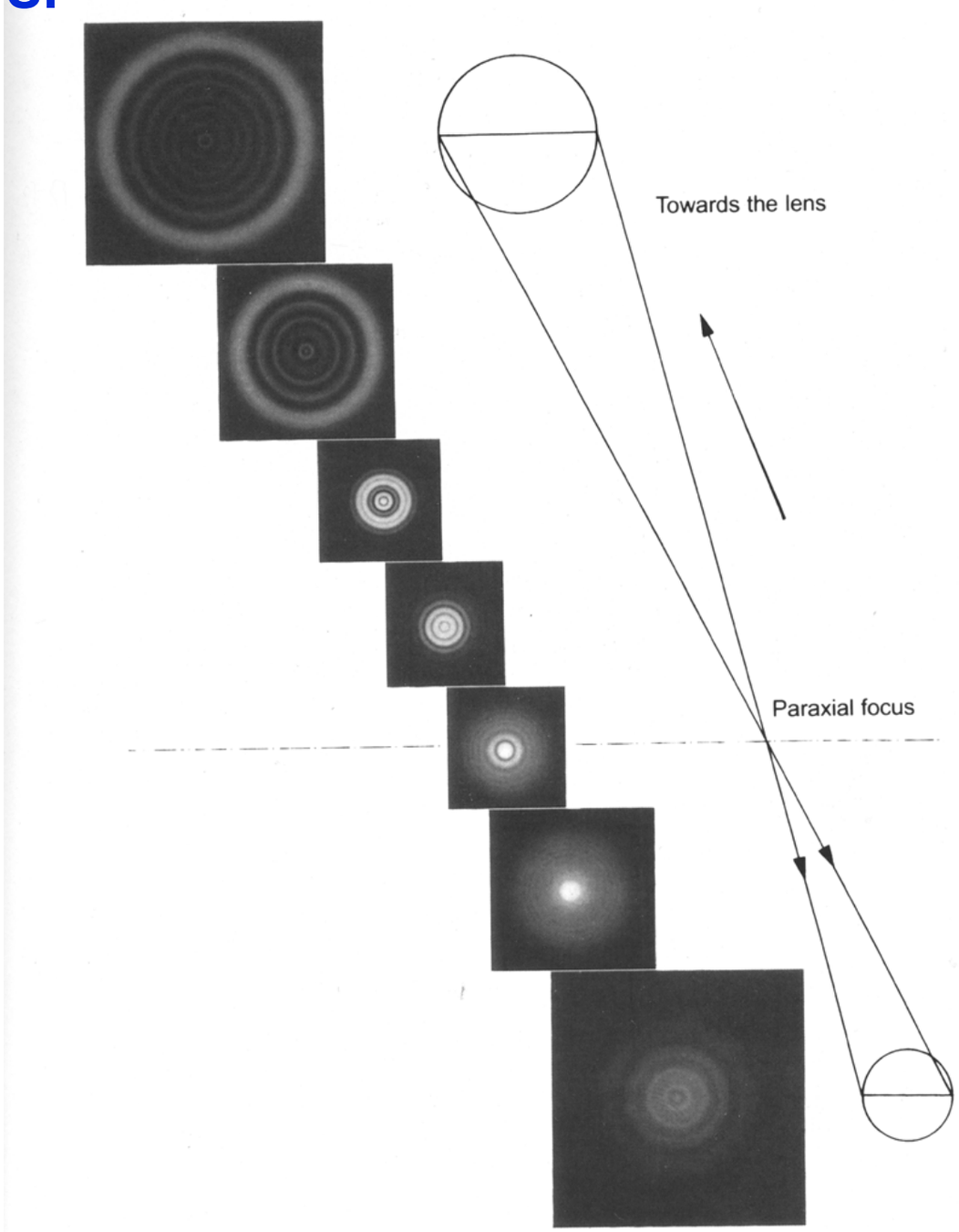
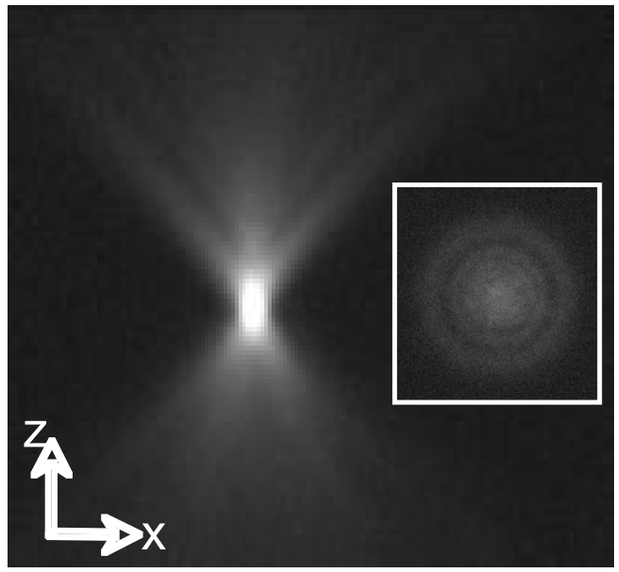
Sample object: a "sub-resolution" fluorescent bead







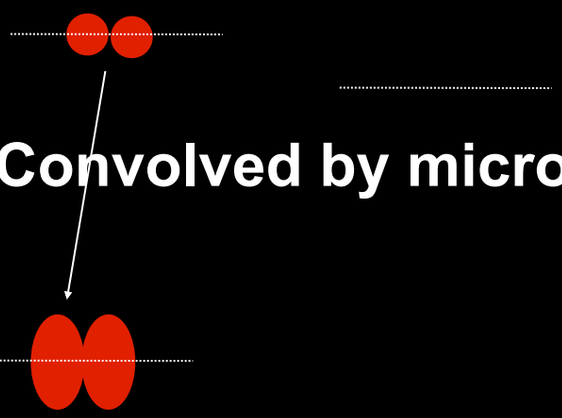
Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

LECTURE 4

- "convolution" by the microscope optics = the PSF

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects

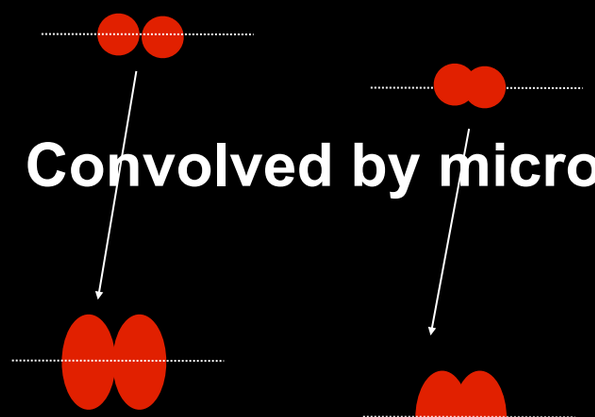


Convolved by microscope

Just resolved

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



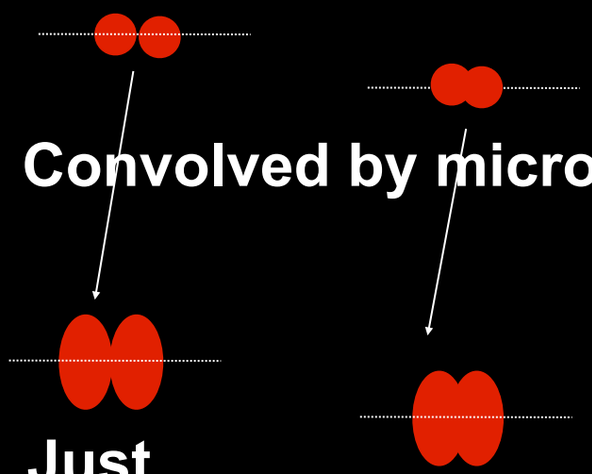
Convolved by microscope

Just resolved

Not resolved Airy patterns must Overlap by less Than 42% of the Maximal intensities

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



Convolved by microscope

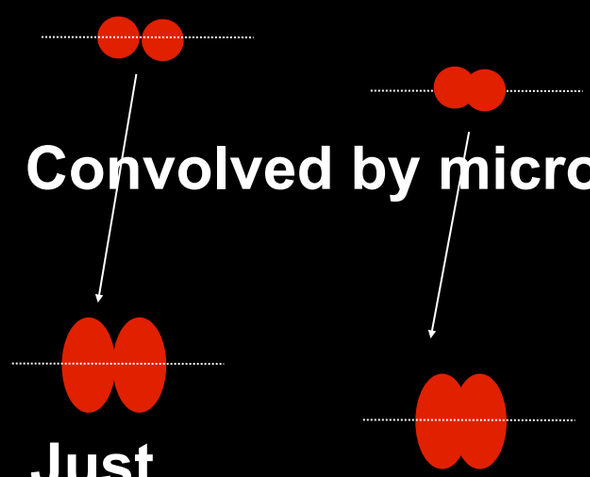
Just resolved

Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

Resolution (D_R) depends upon the objective and wavelength of light:

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



Convolved by microscope

Just resolved

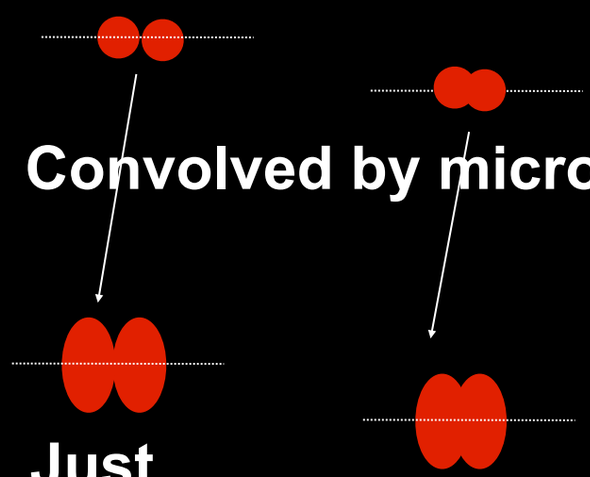
Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

Resolution (D_R) depends upon the objective and wavelength of light:

$$D_R = 1.22 \times \lambda / Na_{obj} + Na_{cond}$$

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



Convolved by microscope

Just resolved

Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

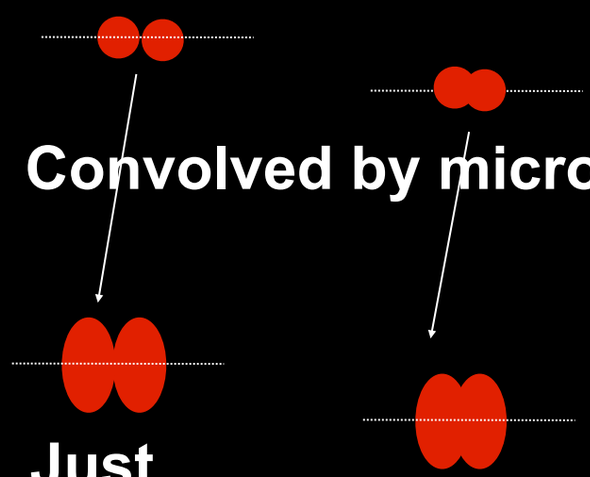
Resolution (D_R) depends upon the objective and wavelength of light:

$$D_R = 1.22 \times \lambda / Na_{obj} + Na_{cond}$$

Considering x10 objective, Na 0.25, fluorescence emission 520 nm:

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



Convolved by microscope

Just resolved

Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

Resolution (D_R) depends upon the objective and wavelength of light:

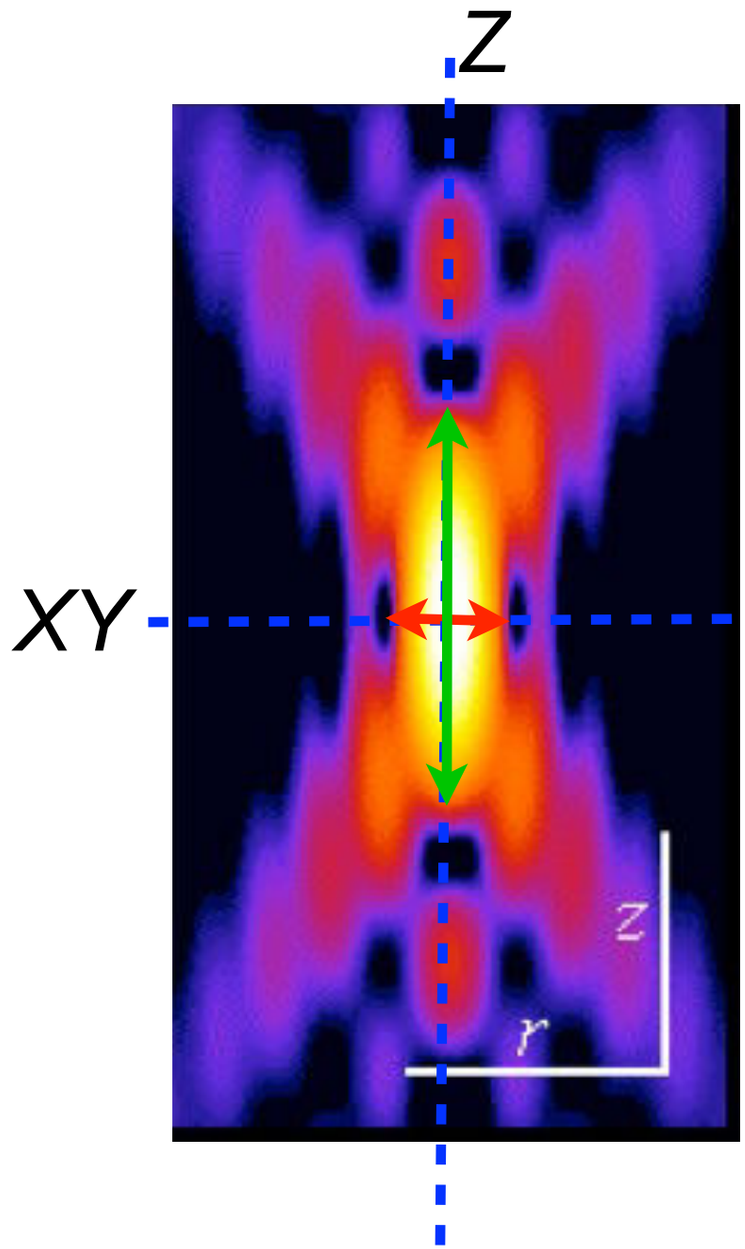
$$D_R = 1.22 \times \lambda / Na_{obj} + Na_{cond}$$

Considering x10 objective, Na 0.25, fluorescence emission 520 nm:

$$D_R = 1.22 \times 520_{nm} / 2 \times 0.25$$

$$D_R = 1.269 \text{ um}$$

Axial Resolution:

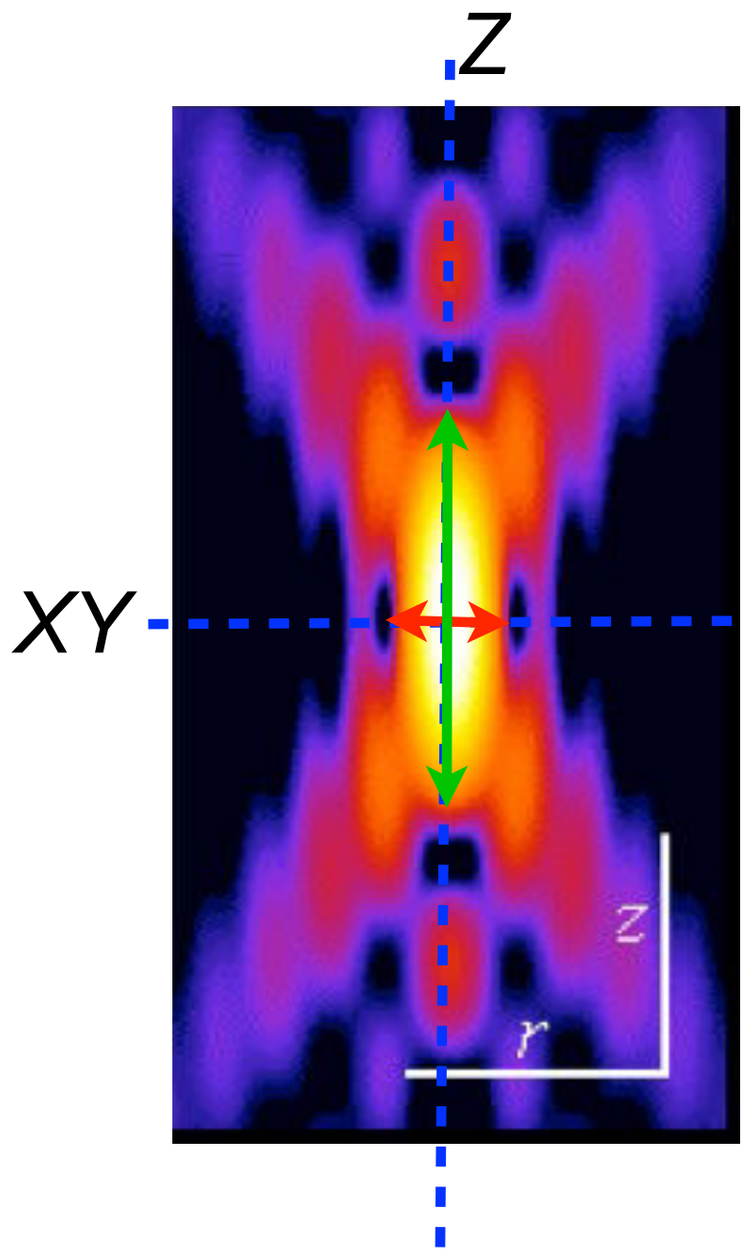


Axial Resolution:

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)



Axial Resolution:

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

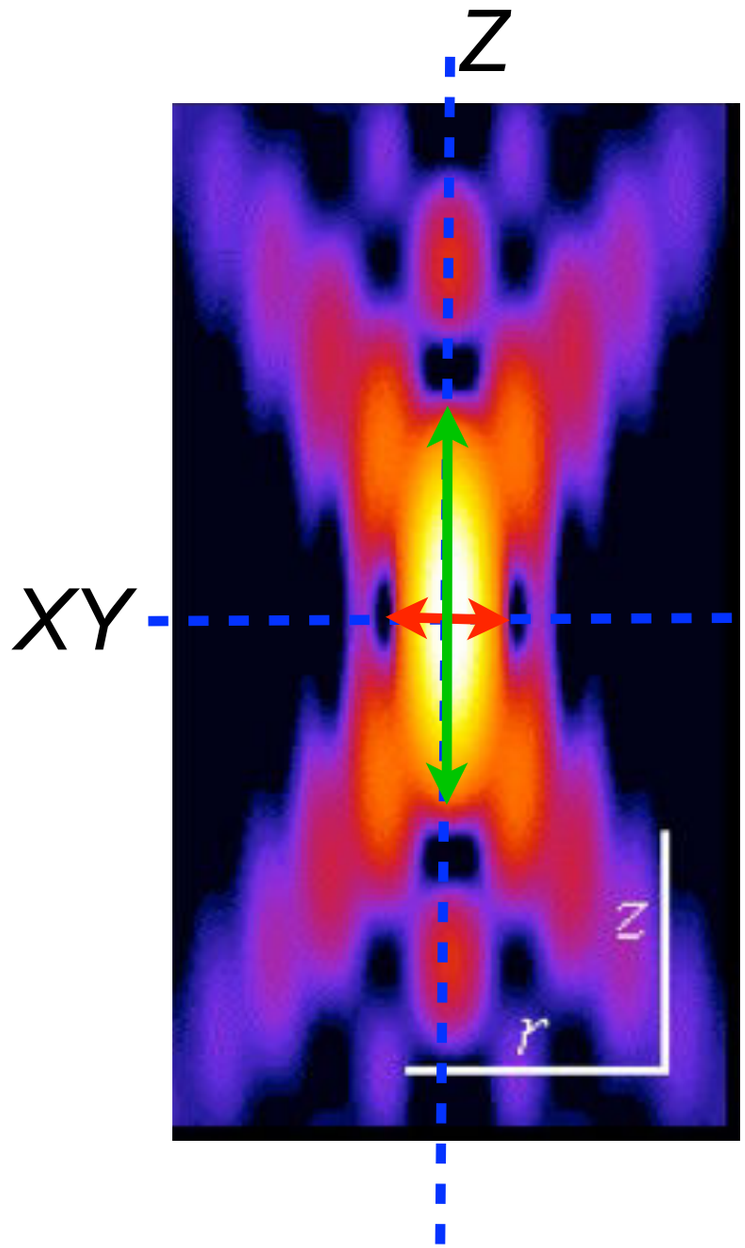
$$D_Z = 2\,\lambda\eta/(NA_{obj})^2 \quad \ldots\ldots\ldots\ldots\ 705$$

(η = refractive index of the object medium)

Than it is in the lateral dimension (XY)

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

FWHM

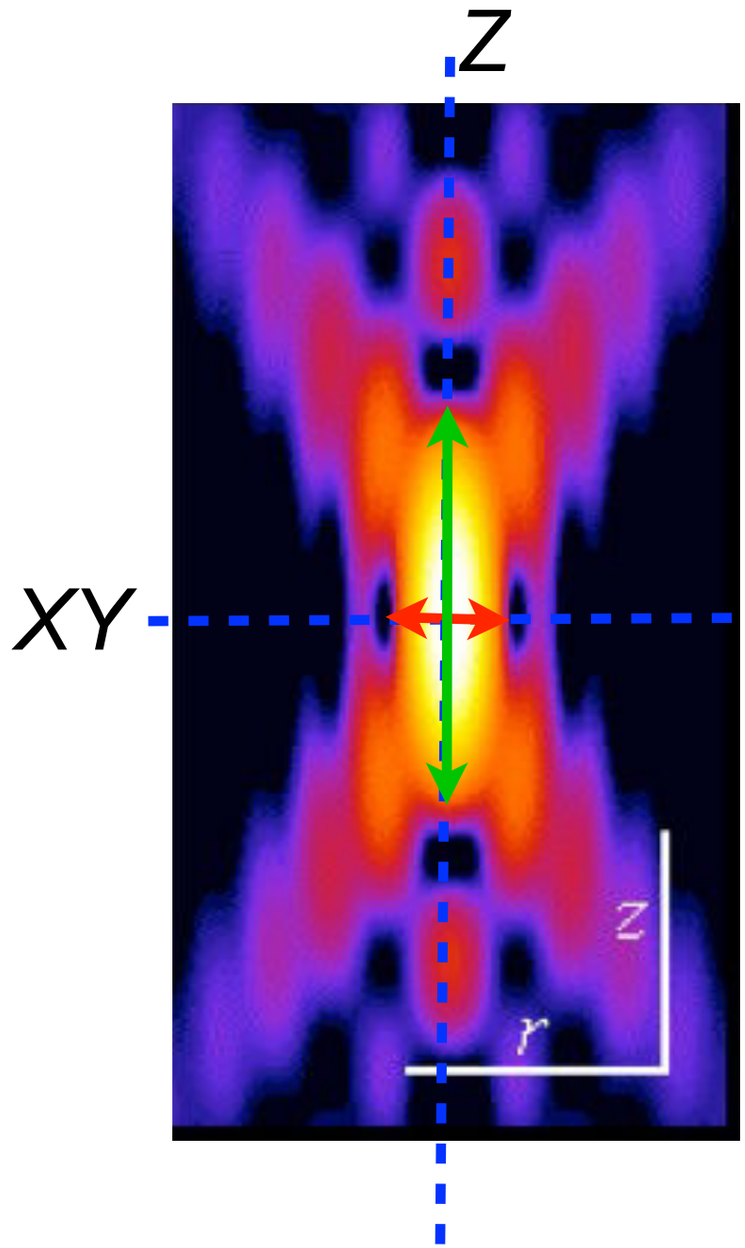


Axial Resolution:

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

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

(η = refractive index of the object medium)



Than it is in the lateral dimension (XY)

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

FWHM

The relationship between the two is:

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

Resolution: Down to the molecular scale?

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

XY resolution ~ 230 nm
Z resolution ~ 700 nm

GFP is ~ 5 x5 x5 nm 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 8

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 8

Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

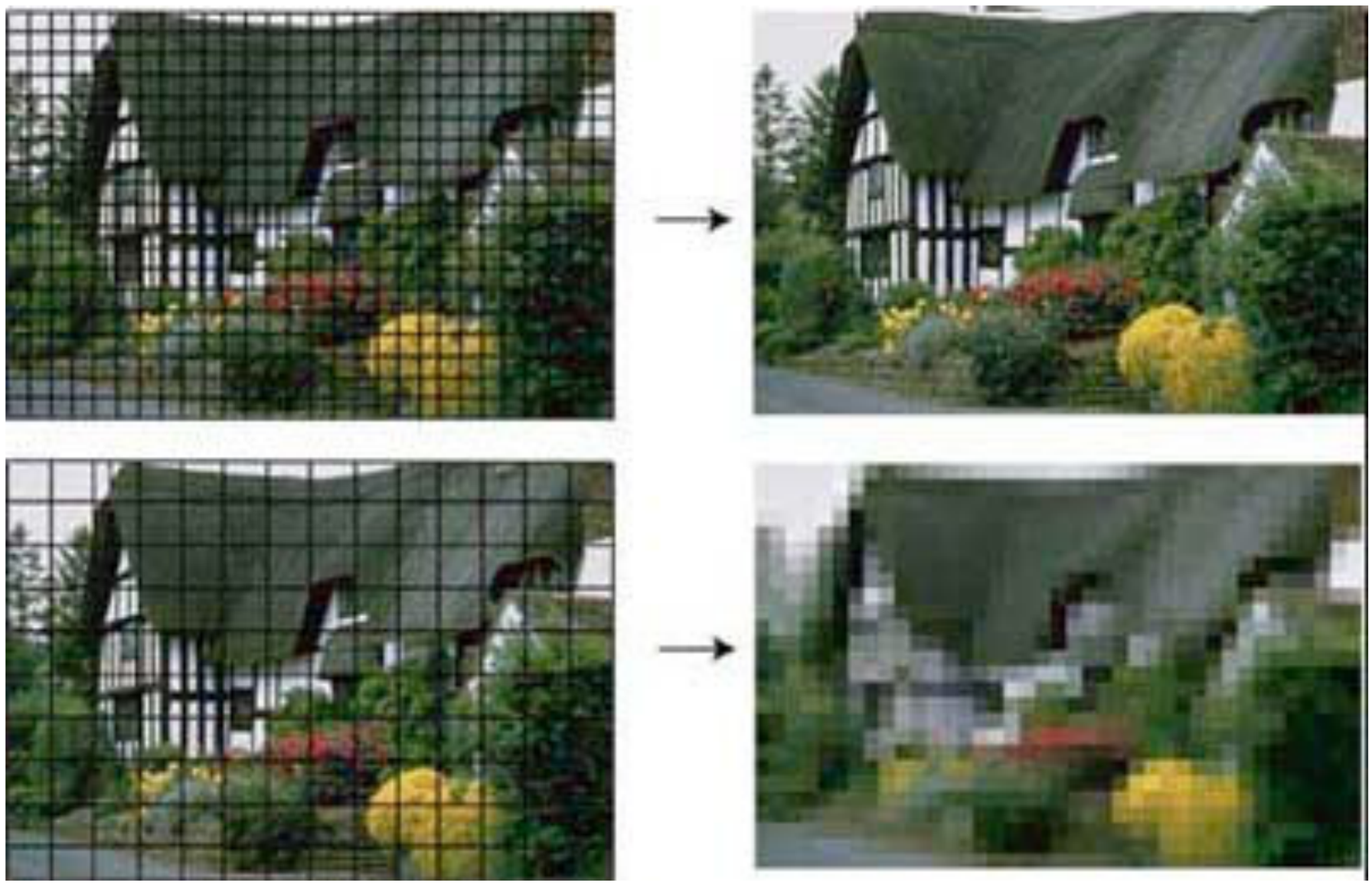
LECTURES 9-11

What is really important in microscopy?

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

Sampling

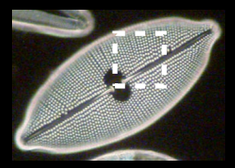
……..correctly reading the available information



……..poor sampling limits the resolution achieved

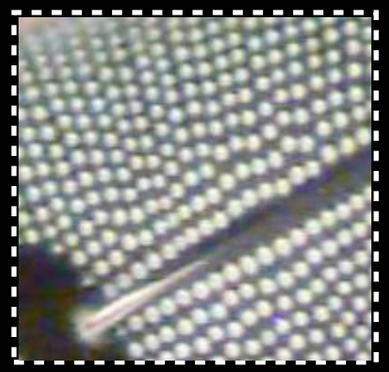
Resolution/sampling .....& Magnification

Specimen
Fine Detail



Detail imaged
by microscope

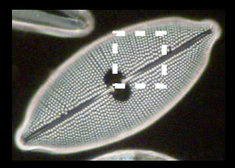
*magnification
*optical resolution



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

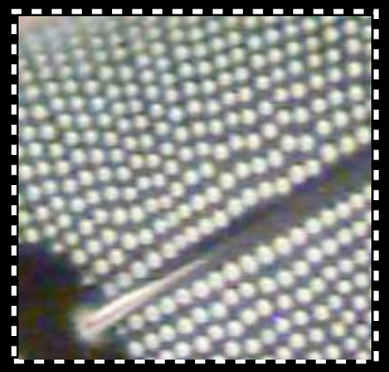
Resolution/sampling .....& Magnification

Specimen
Fine Detail

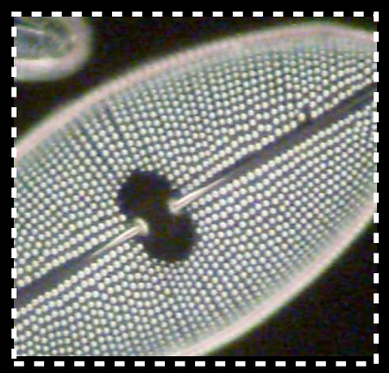


Detail imaged
by microscope

*magnification
*optical resolution

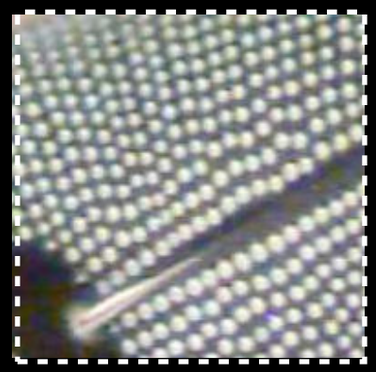


not magnified enough



Undersampling
all detail not resolved
Large field of view

auxiliary
magnification
to match image
to detector

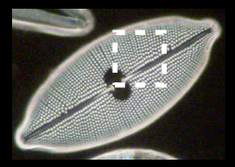


All resolvable
detail recorded

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

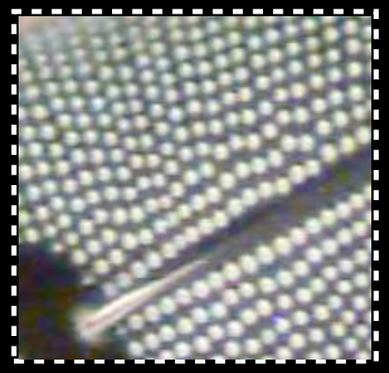
Resolution/sampling .....& Magnification

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

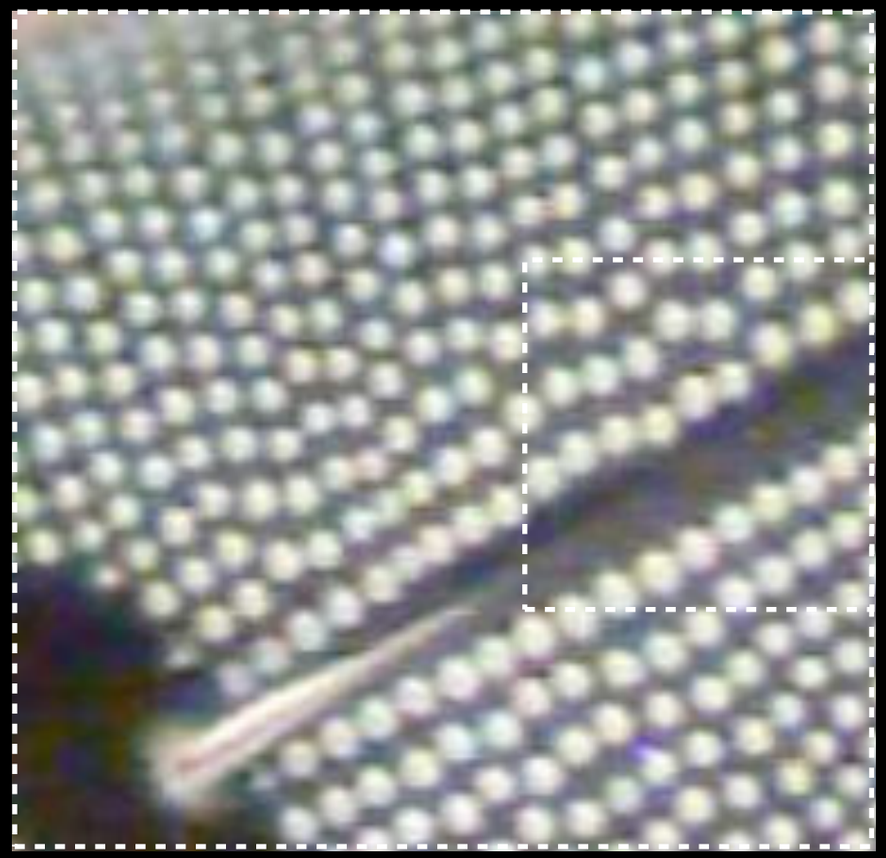


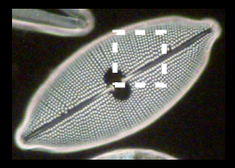
image
on detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

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

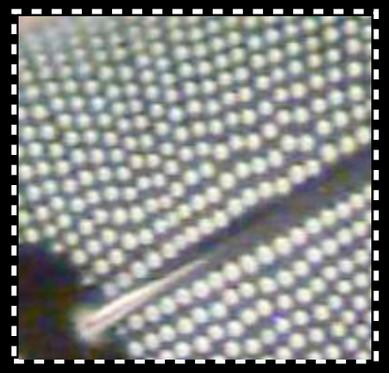
Resolution/sampling .....& Magnification

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

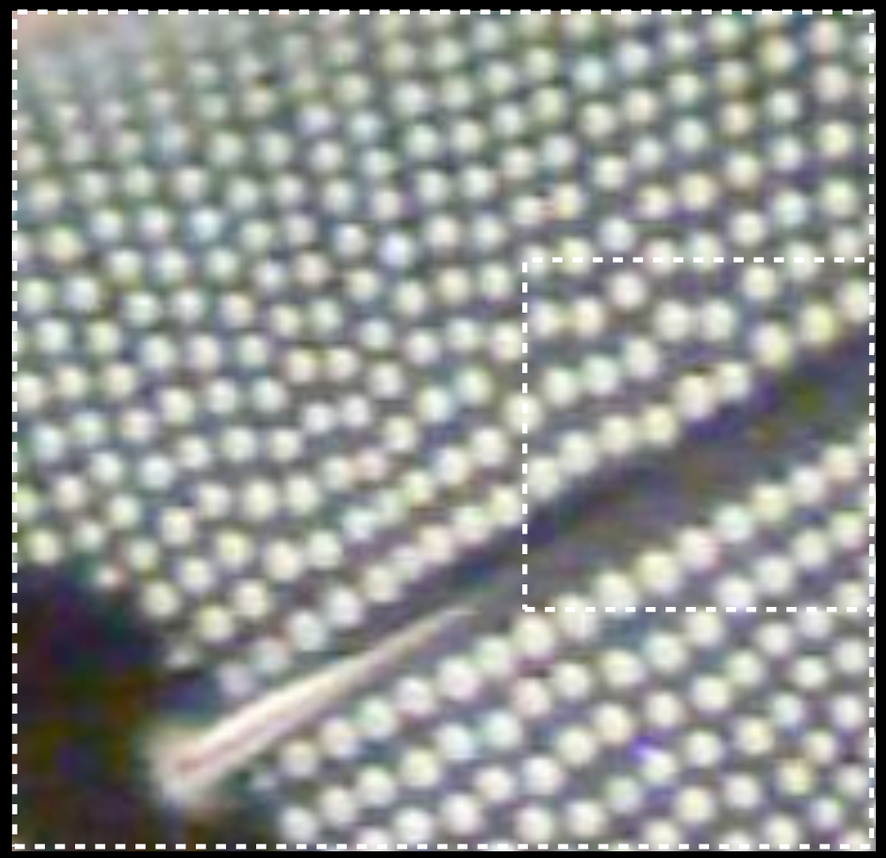


image
on detector

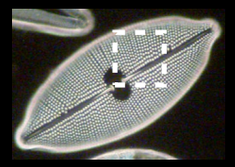
Oversampling
Empty
magnification
Blurred image
Limited field of
view

What is the optimum magnification.....?

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

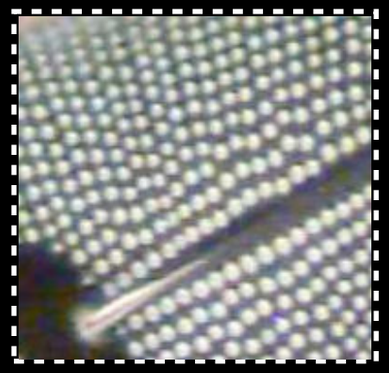
Resolution/sampling .....& Magnification

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

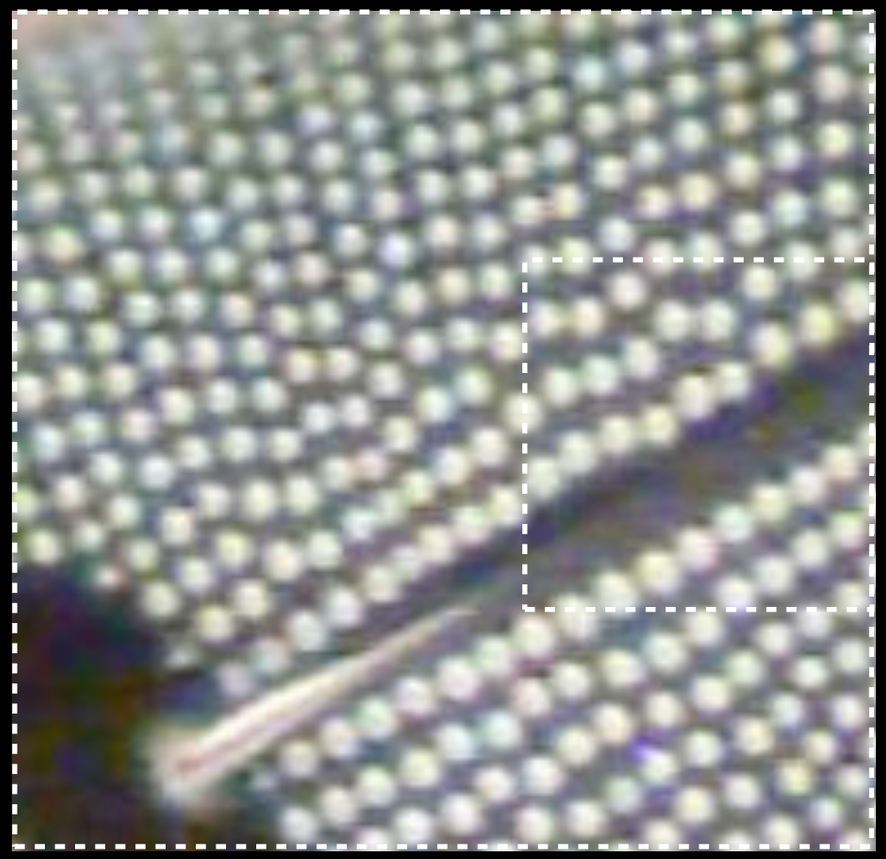


image
on detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

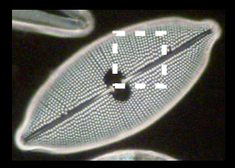
What is the optimum magnification.....?

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

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

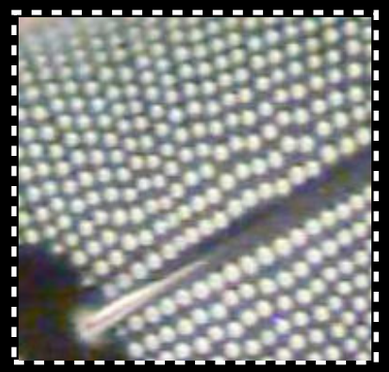
Resolution/Sampling .....& Magnification

Specimen
Fine Detail



Detail imaged
by microscope

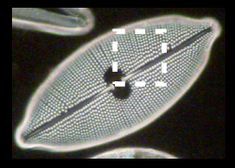
*magnification
*optical resolution



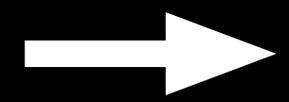
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution/Sampling .....& Magnification

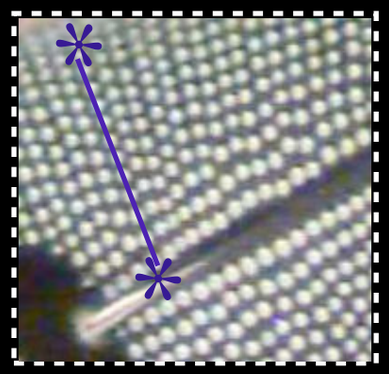
Specimen
Fine Detail

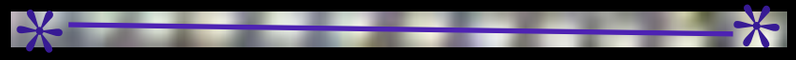


Detail imaged
by microscope

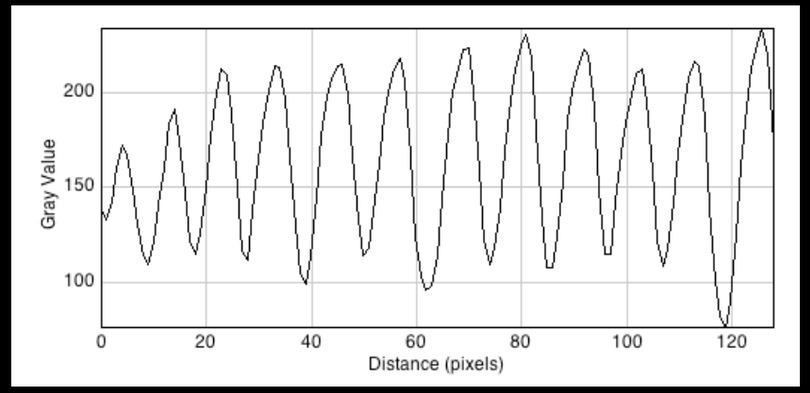


*magnification
*optical resolution





intensity profile ~ a sine wave

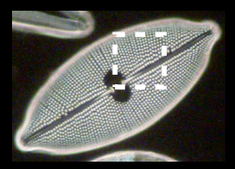


Diatom image: http://www.micromagnus.net/index_micro.html

Resolution/Sampling

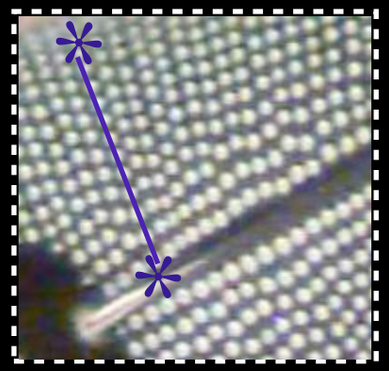
……..& Magnification

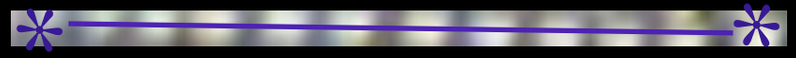
Specimen
Fine Detail



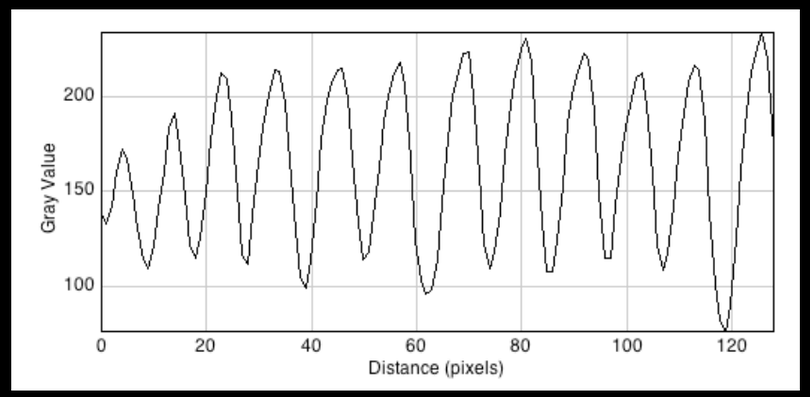
Detail imaged
by microscope

*magnification
*optical resolution

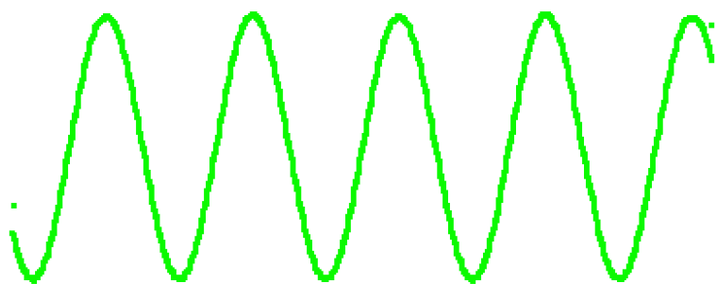




intensity profile ~ a sine wave



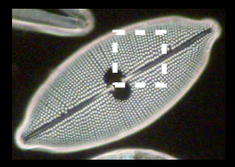
sampling a sine wave



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

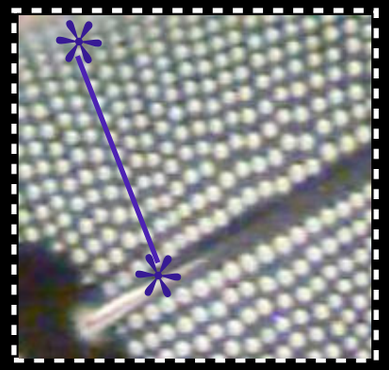
Resolution/Sampling

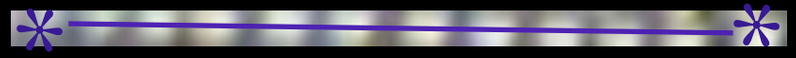
Specimen
Fine Detail



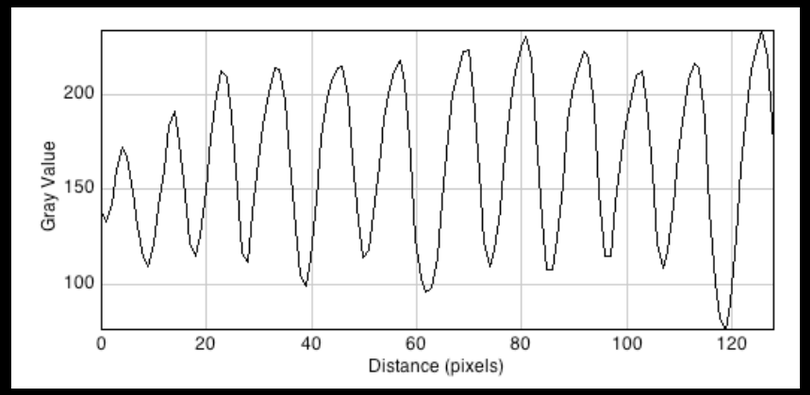
Detail imaged
by microscope

*magnification
*optical resolution



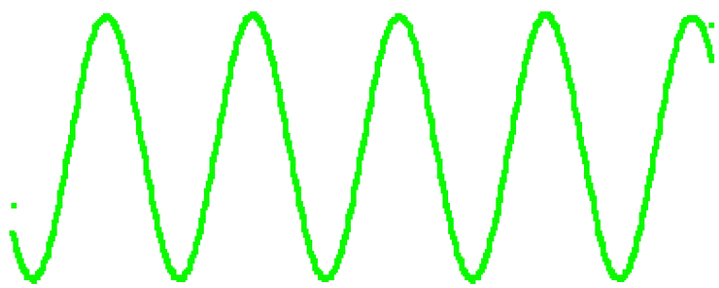


intensity profile ~ a sine wave

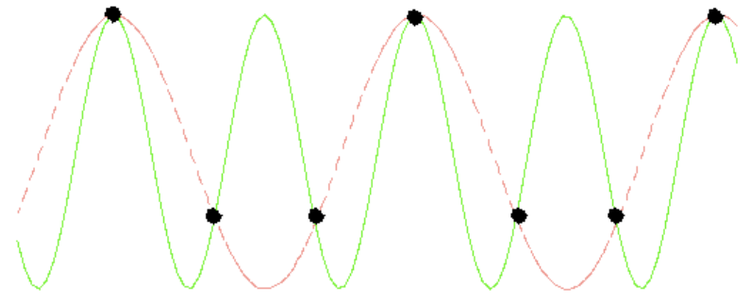


.....& Magnification

sampling a sine wave



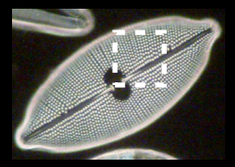
Sampling 1.5 times per cycle



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

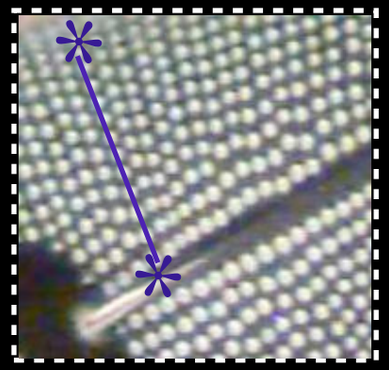
Resolution/Sampling

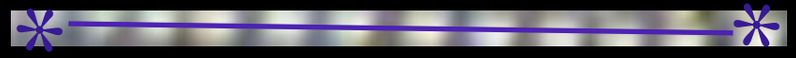
Specimen
Fine Detail



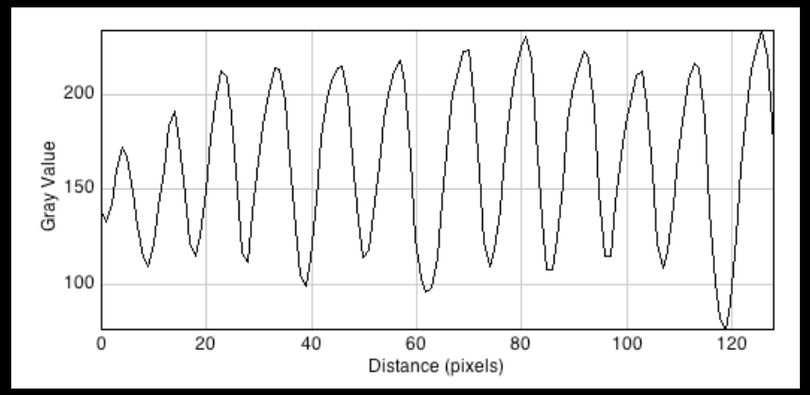
Detail imaged
by microscope

*magnification
*optical resolution



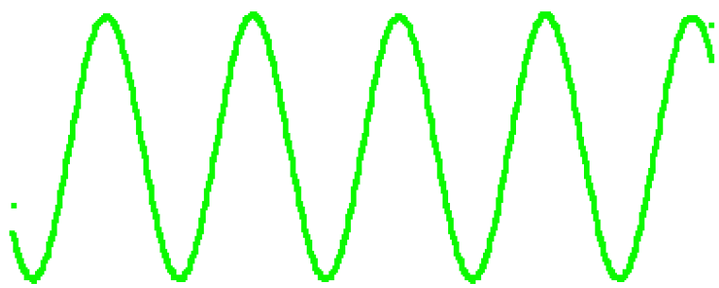


intensity profile ~ a sine wave

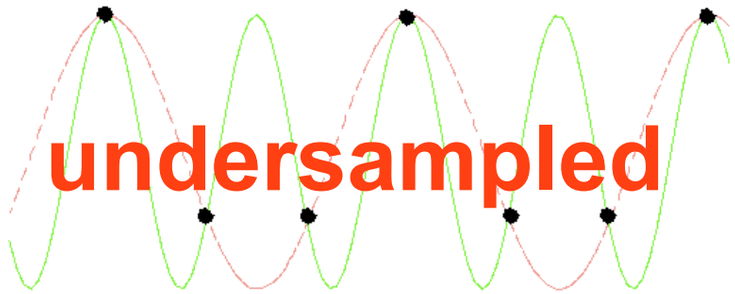


.....& Magnification

sampling a sine wave



Sampling 1.5 times per cycle



undersampled

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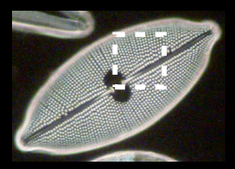
Resolution/Sampling

Specimen
Fine Detail



Detail imaged
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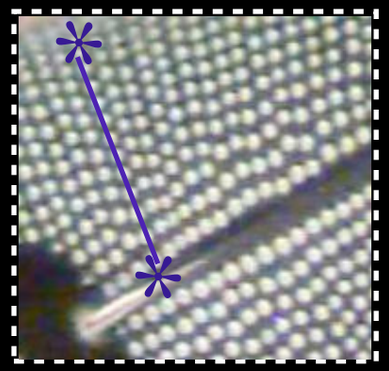
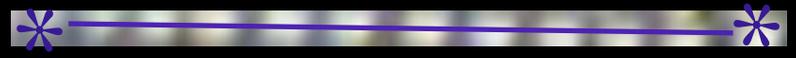
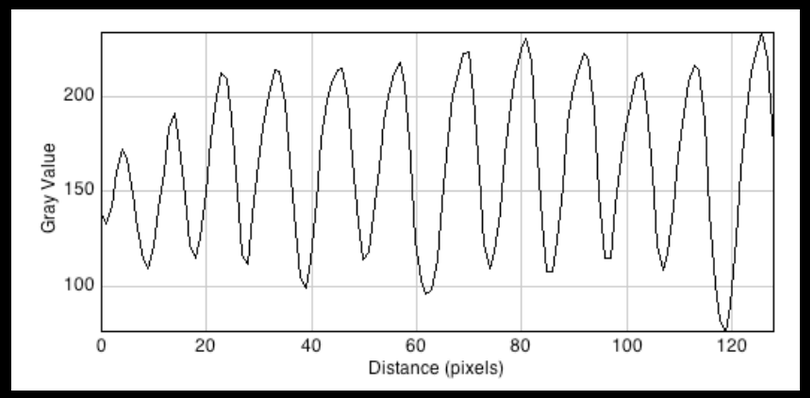
*optical resolution





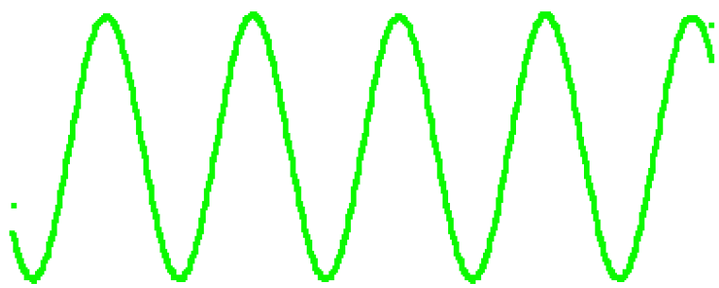
intensity profile ~ a sine wave



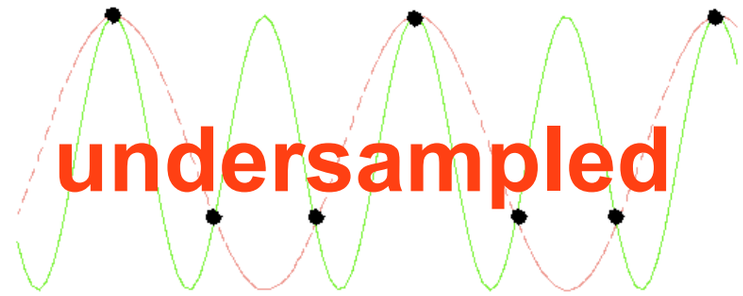
Diatom image: http://www.micromagnus.net/index_micro.html

.....& Magnification

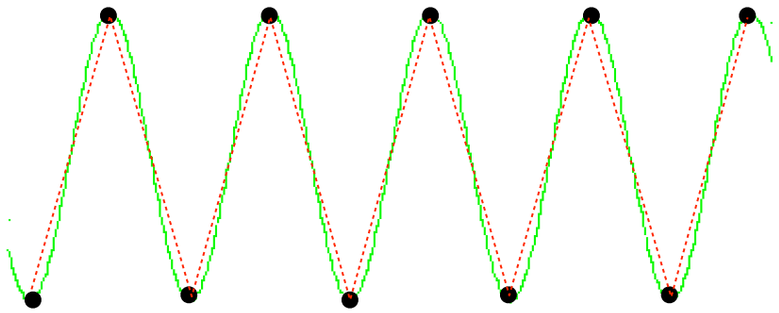
sampling a sine wave



Sampling 1.5 times per cycle

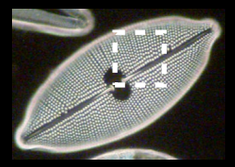


Sampling 2.0 times per cycle



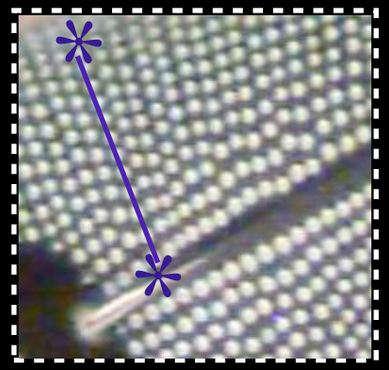
Resolution/Sampling

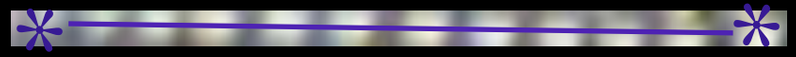
Specimen
Fine Detail



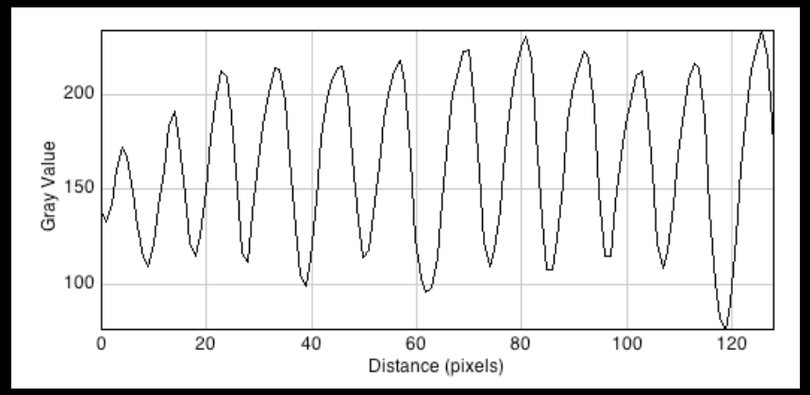
Detail imaged
by microscope

*magnification
*optical resolution



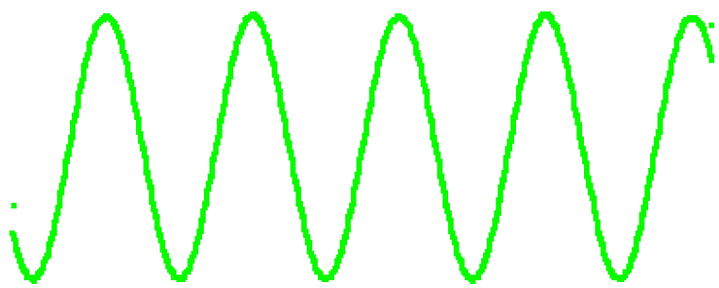


intensity profile ~ a sine wave

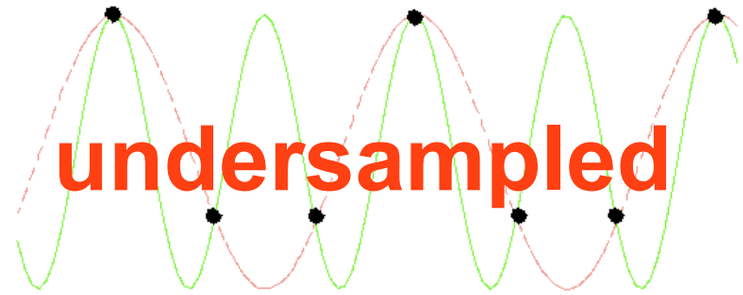


.....& Magnification

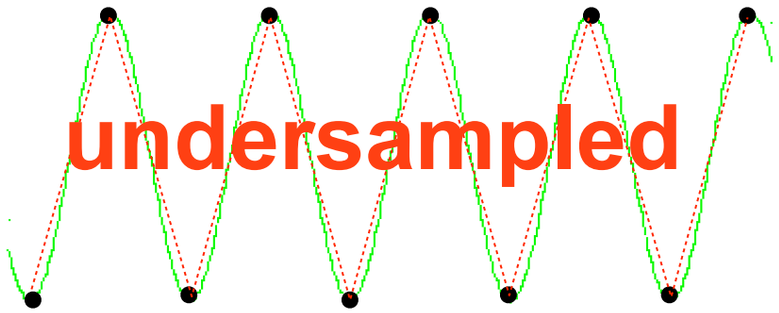
sampling a sine wave



Sampling 1.5 times per cycle



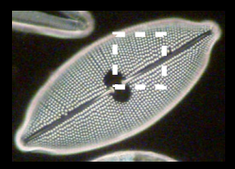
Sampling 2.0 times per cycle



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

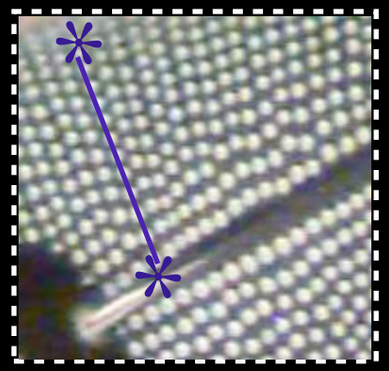
Resolution/Sampling

Specimen
Fine Detail

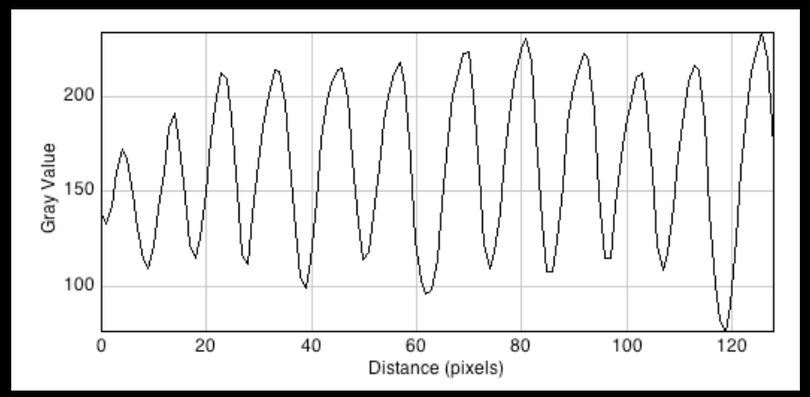


Detail imaged
by microscope

*magnification
*optical resolution

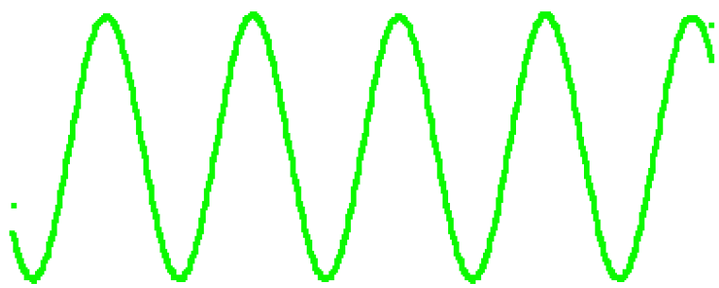


intensity profile ~ a sine wave

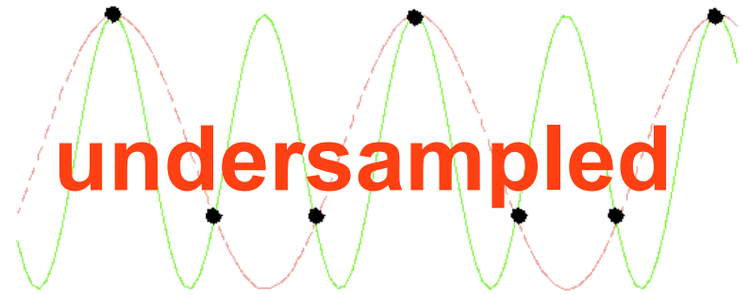


.....& Magnification

sampling a sine wave

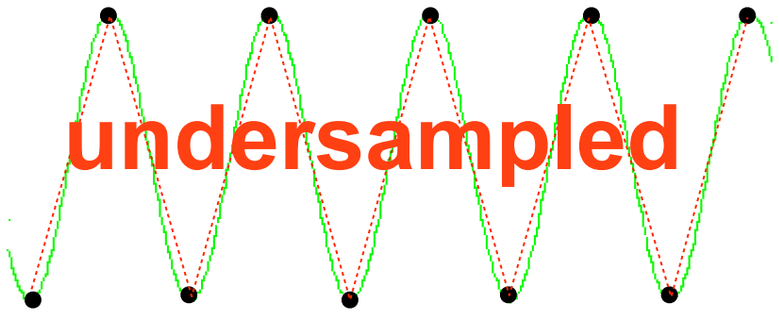


Sampling 1.5 times per cycle



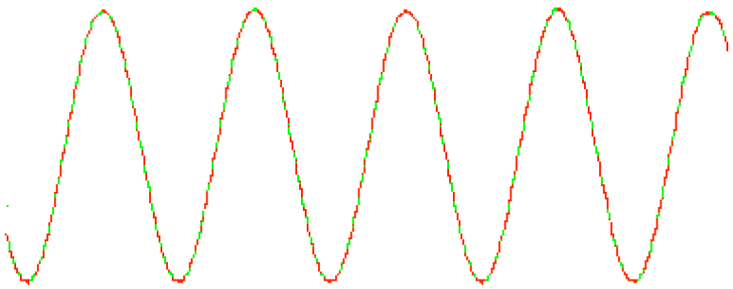
undersampled

Sampling 2.0 times per cycle



undersampled

Sampling many times per cycle

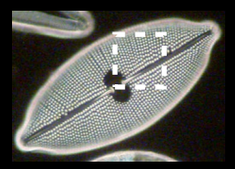


Diatom image: http://www.micromagnus.net/index_micro.html

Resolution/Sampling

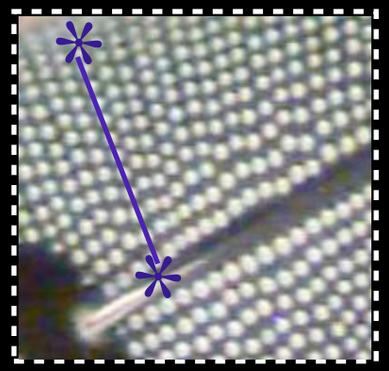
……..& Magnification

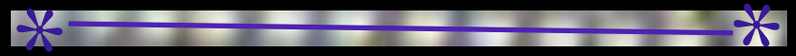
Specimen
Fine Detail



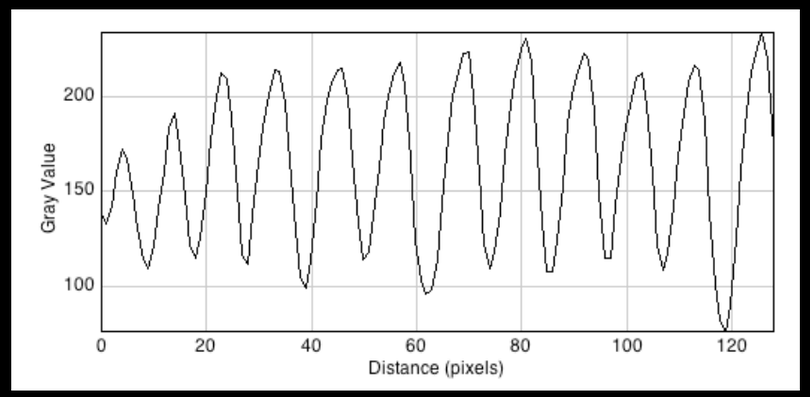
Detail imaged
by microscope

*magnification
*optical resolution

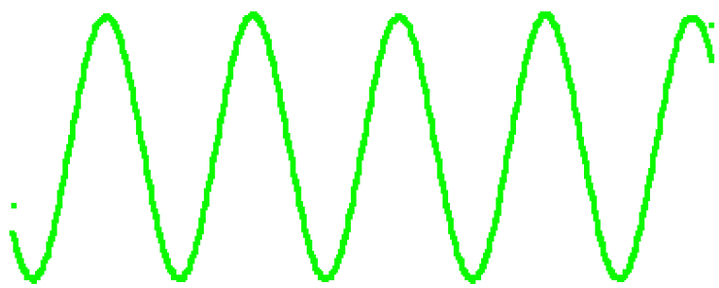




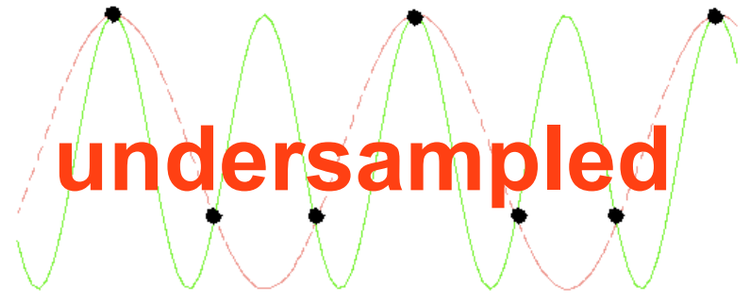
intensity profile ~ a sine wave



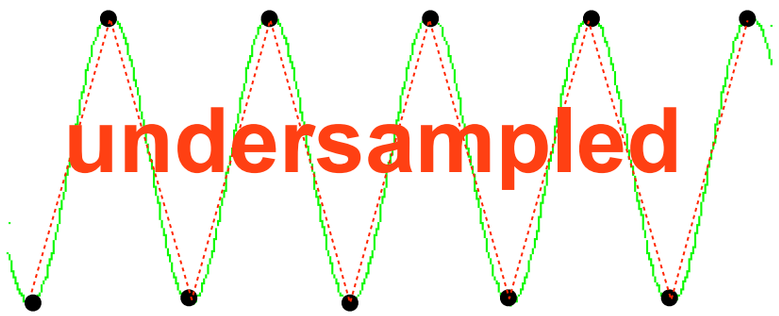
sampling a sine wave



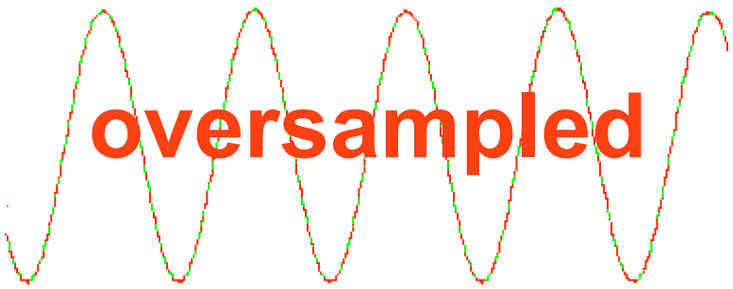
Sampling 1.5 times per cycle



Sampling 2.0 times per cycle



Sampling many times per cycle



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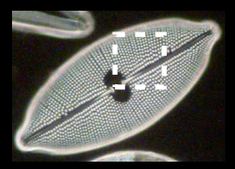
Resolution/Sampling

……..& Magnification

Specimen
Fine Detail



Detail imaged
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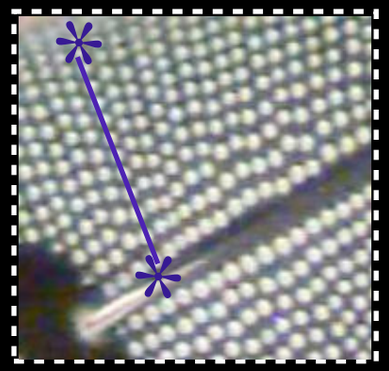
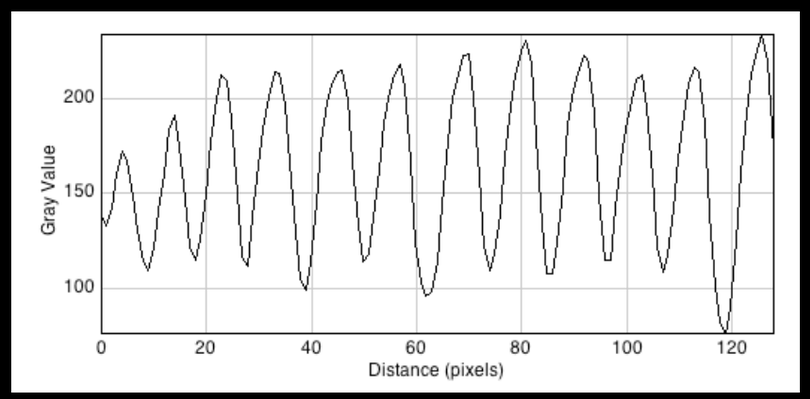


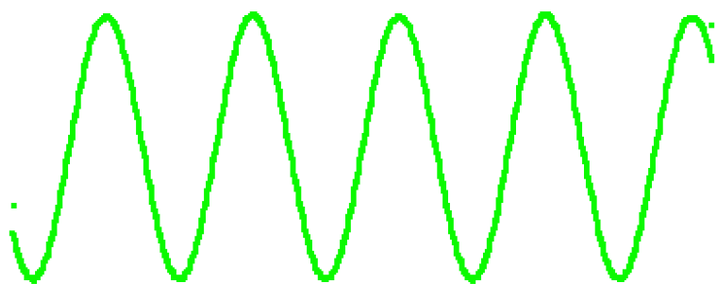
intensity profile ~ a sine wave



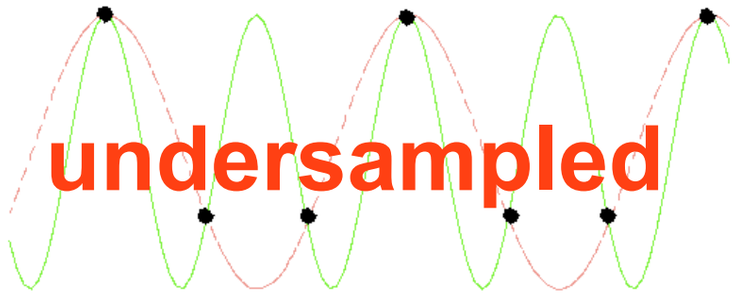
Optimum = 2.3 times per cycle

= Nyquist sampling

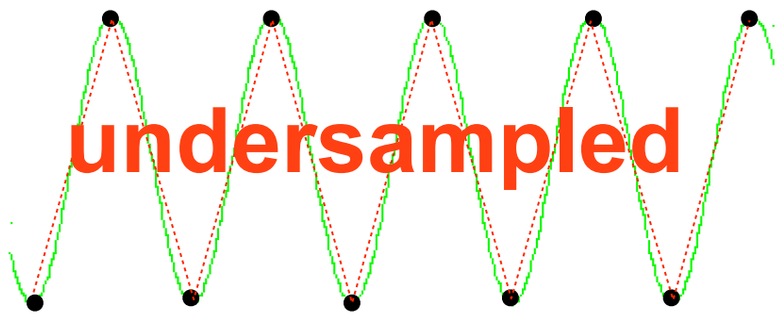
sampling a sine wave



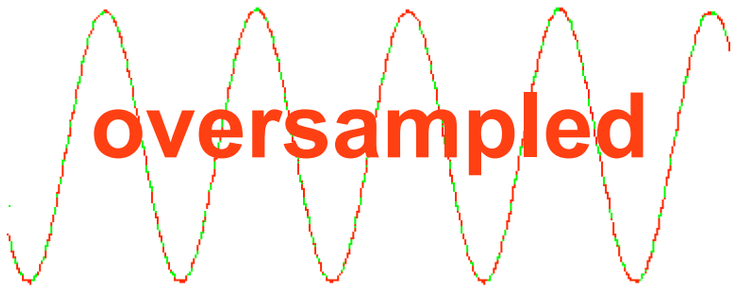
Sampling 1.5 times per cycle



Sampling 2.0 times per cycle



Sampling many times per cycle



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

Resolution/Sampling & Optimum magnification

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

optimal total mag X resolvable distance = 3x detector element size

Considering Fluorescence imaging x100 objective; 1.4 Na; 520 nm emission,.....

total mag	**resolvable distance**	**Detector Element**
x100 objective X x1.0 Aux mag	1.22 x **λ520**/ 2Na	Camera pixel element = 6.6 um (x3 taking into account Nyquist)

optimal total mag = (2Na) 3 x Detector Element /1.22 x 520 nm

≈ 87 times magnification

x100 obj = GOOD SAMPLING

.....OR

pixel size must be ~1/3 of the resolution

What is really important in microscopy?

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

Noise / Signal to Noise (S/N)

https://www.forbes.com/2001/01/26/0126movers.html#12f309d936c5

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Noise / Signal to Noise (S/N)

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Signal to Noise - definitions:

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

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

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

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$$\frac{\text{mean}}{\text{S.D.}}$$

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

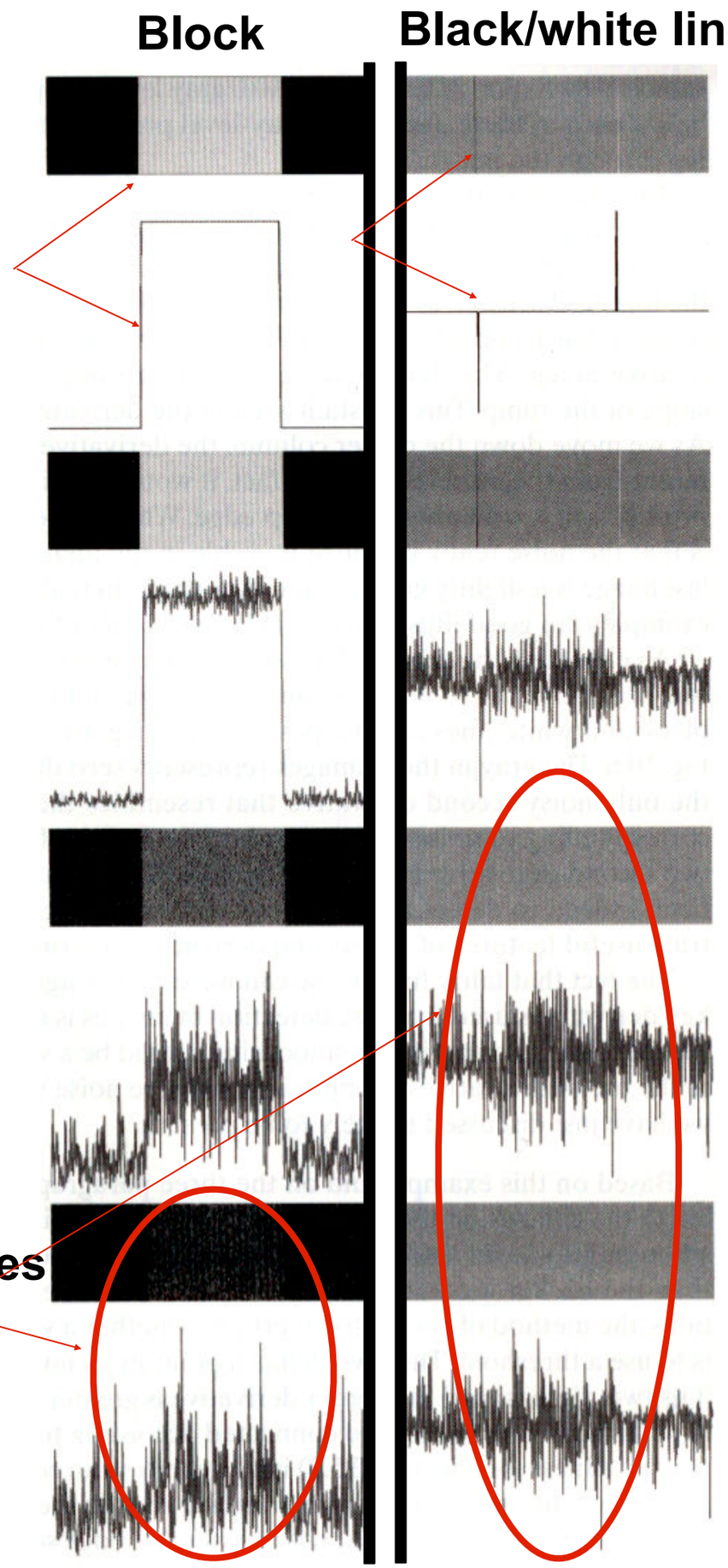
- Additional sources of noise from digitisation, detector readout, thermal noise.

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.

Resolution, contrast, noise

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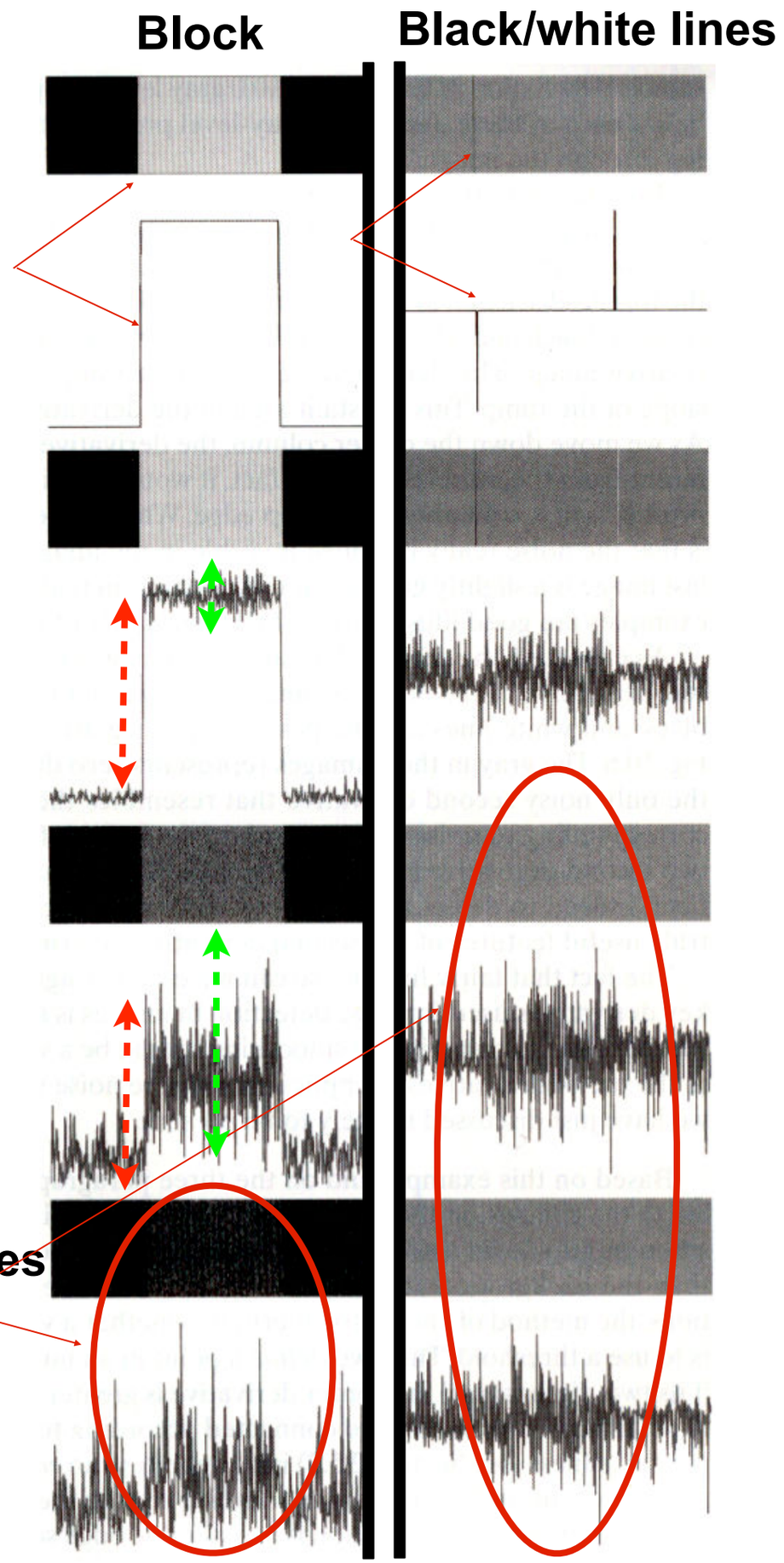


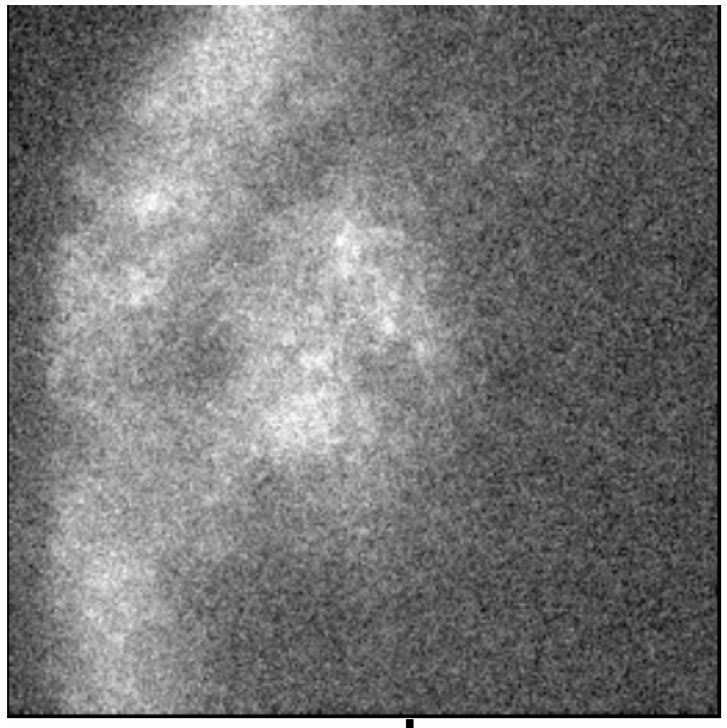
The **difference between signal and background** must be at least 3X the **noise** to be detectable

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

Improving signal to noise

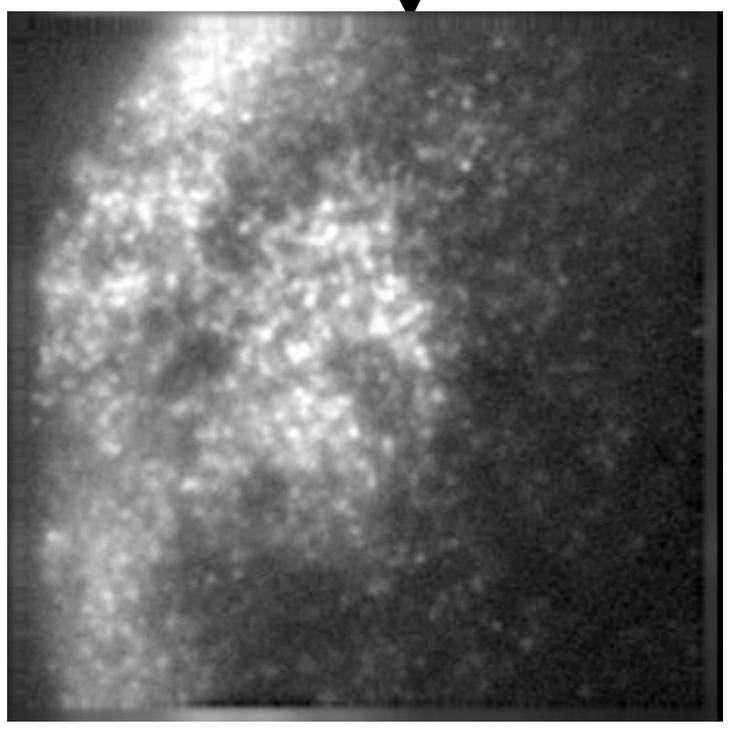
increased signal increases S/N = improved contrast

noisy image (scaled)



increased number of photons counted

5x integration time



improved S/N

ImageJ

Which technique do I use?

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

Be clear what you want from your experiment

Qualitative data

Quantitative data

Dynamics

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
Dynamics

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
Dynamics

Be aware of the different techniques

Be clear what you want from your experiment

UP TO YOU

Qualitative data
Quantitative data
Dynamics

Be aware of the different techniques

Their strengths
Their weaknesses
Their availability

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VISIT A FACILITY

http://www.micron.ox.ac.uk/microngroup/about.php

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Viability
Thickness
Brightness

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DISCUSS YOUR APPLICATION

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Thickness
Brightness

Which technique do I use?

http://www.micron.ox.ac.uk/microngroup/facilities.php

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Bright field / fluorescence (contrast generation)

Lectures 1,4 & 6

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Live cell imaging
Fixed material imaging

(dynamics vs detail)

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Lectures 6 & 7

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Lectures 6 & 7

Super-resolution techniques
Techniques for molecular scale dynamics / interactions

Lectures 8-11

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