Micron Advanced Light Microscopy Course 2016

Introductory lecture

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

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
- Day 5 - EM

- Additional Micron lectures on 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***
- Day 4 - Beyond conventional imaging ***LECTURES 9-11***
- Day 5 - EM

- Additional Micron lectures on imaging handling and analysis

Goals of the course:

Goals of the course:

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

  the basic physics of optics and microscopes

  designing and building bespoke microscope

Goals of the course:

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

  the basic physics of optics and microscopes

  designing and building bespoke microscope
- Make you aware of what is really important in good microscopy
- Describe the different techniques available and their application

Goals of the course:

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

  the basic physics of optics and microscopes

  designing and building bespoke microscope
- 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

Goals of the course:

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

  the basic physics of optics and microscopes

  designing and building bespoke microscope
- 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

Why is microscopy so important?

100 years ago:

Magnify small things to visualise more details

Why is microscopy so important?

100 years ago:

Magnify small things to visualise more details

Now:

Microscopy is fundamentally important to modern biology

Milestones in Microscopy

http://www.nature.com/milestones/milelight/index.html

1595	Invention of the microscope (Milestone 1)
1858	First histological stain (Milestone 2)
1871	Synthesis of fluorescein (Milestone 2)
1873	Diffraction limit theory (Milestone 3)
1911	First fluorescence microscope (Milestone 4)
1929	First epifluorescence microscope (Milestone 4)
1935	Phase contrast microscopy (Milestone 5)
1939	Polarization microscopy (Milestone 6)
1942	Immunofluorescence (Milestone 7)
1955	Differential interference contrast (Milestone 8)
1961	Concept of confocal microscopy (Milestone 9)
1967	The dichroic mirror (Milestone 4)
1972	Fluorescence correlation spectroscopy (Milestone 10)
1976	FRAP (Milestone 10)
	FRET (Milestone 11)
1980	Calcium probes (Milestone 12)
1981	Video-enhanced differential interference contrast (Milestone 8)
	TIRF microscopy (Milestone 13)
1983	Deconvolution microscopy (Milestone 14)
1987	Realization of confocal microscopy (Milestone 9)
1990	Two-photon microscopy (Milestone 15)
1993	Light sheet microscopy (Milestone 16)
	Single molecule microscopy (Milestone 17)
1994	GFP (Milestone 18)
1997	Fluorescent protein-based biosensors (Milestone 19)
1999	Red fluorescent proteins (Milestone 20)
2000	Breaking the diffraction limit: STED (Milestone 21)
2002	Photoactivatable fluorescent proteins (Milestone 20)
2006	Breaking the diffraction limit: PALM/STORM (Milestone 21)

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

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)

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

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)

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

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)

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

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)

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

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)

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

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) *

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

Why is microscopy so important?

*

*

*

*

*

*

Why is microscopy so important?

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

* Relatively non-invasive

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

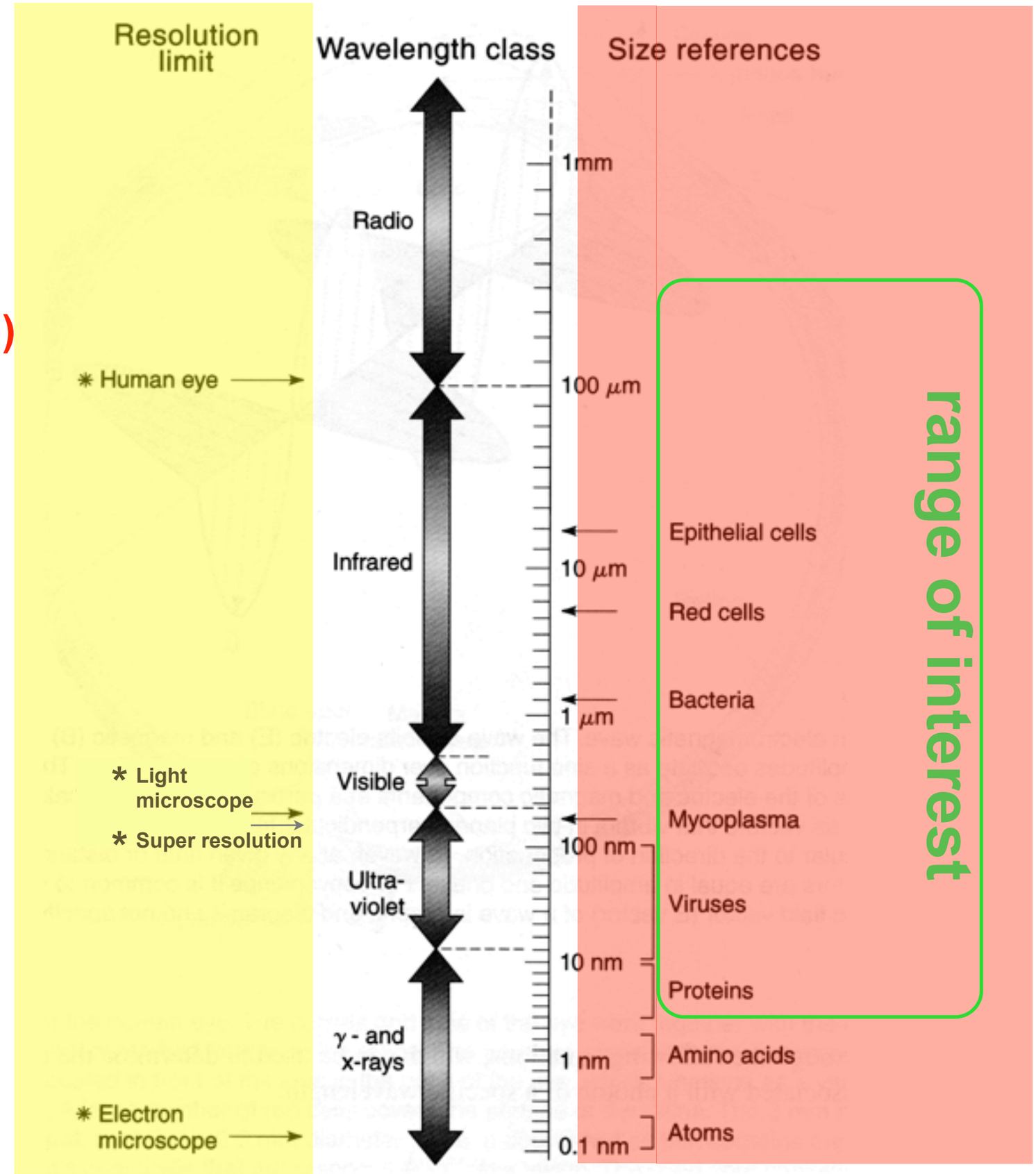
* Quantitative

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

* Allows experimental manipulation

Useful size range for light microscopy

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



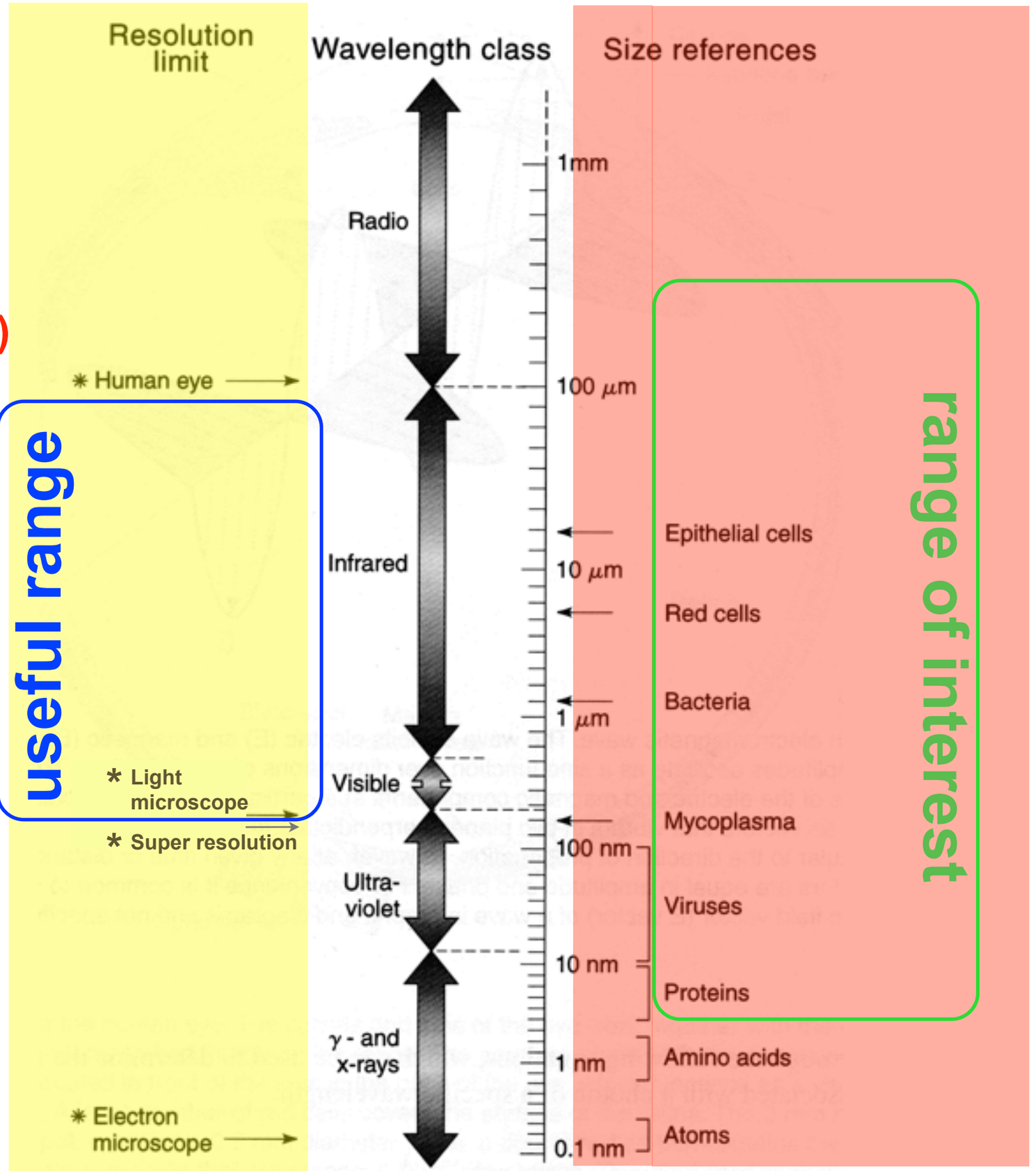
Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

Useful size range for light microscopy

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

Eukaryote = 10 um
>50 um

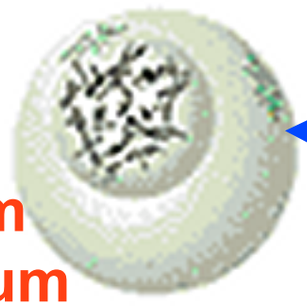
Bacterium = 1 um



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

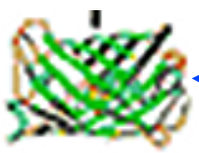
Useful size range for light microscopy

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



Eukaryote = 10 um
>50 um

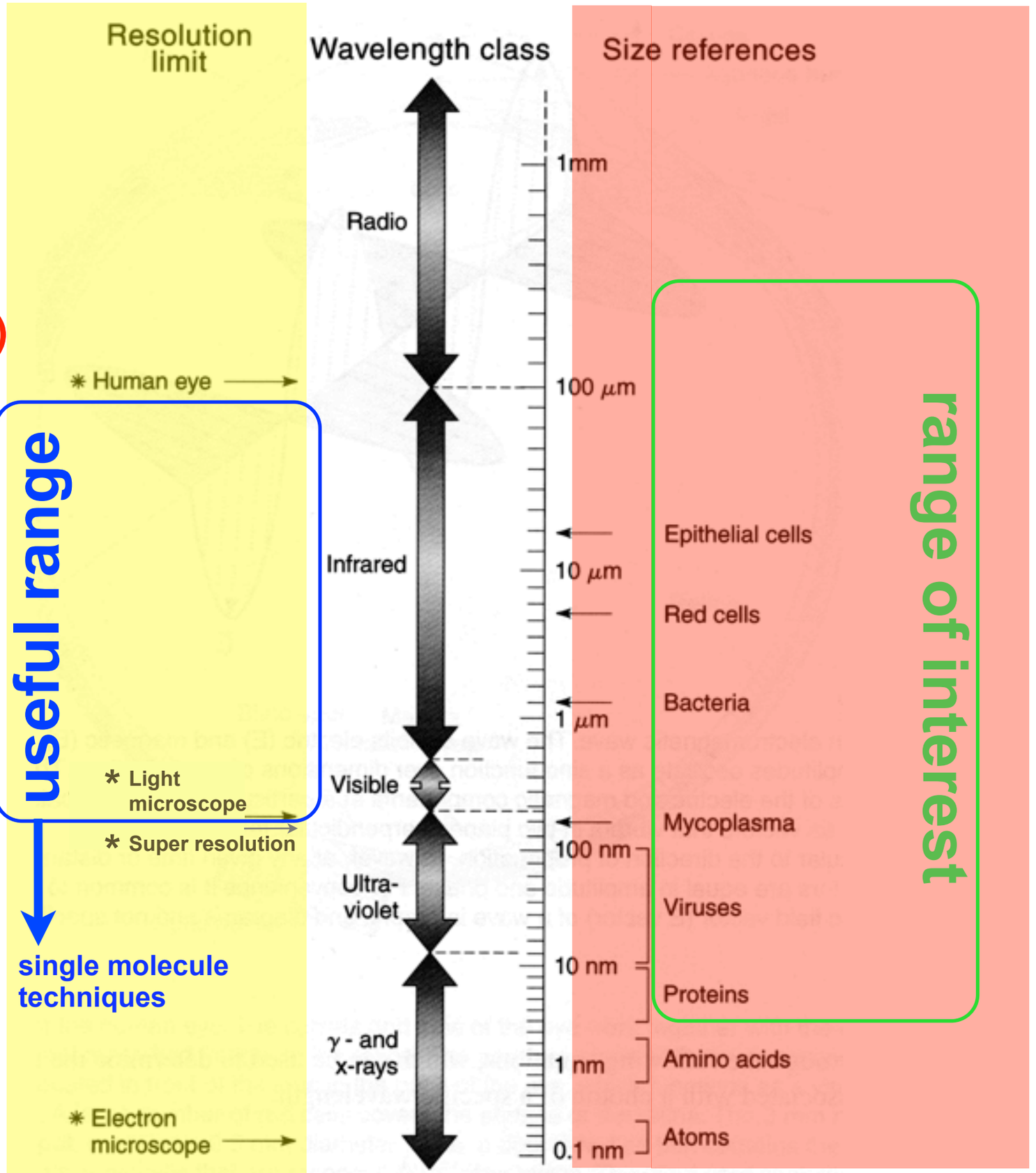
Bacterium = 1 um



Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



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

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!

Why do we need to understand microscopy?

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

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

Why do we need to understand microscopy?

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

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

*** Microscopes are all basically the same**

Why do we need to understand microscopy?

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

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

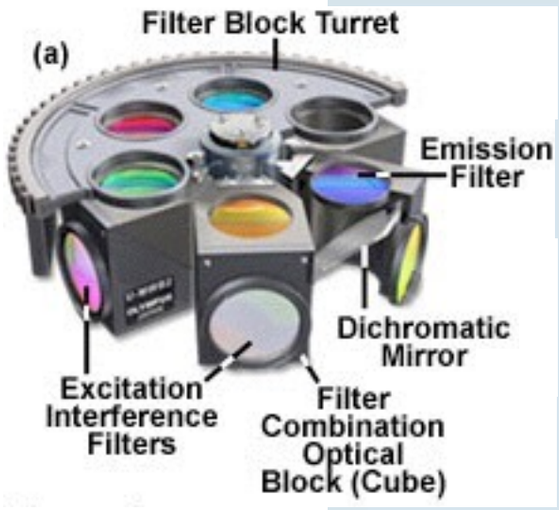
*** Microscopes are all basically the same**

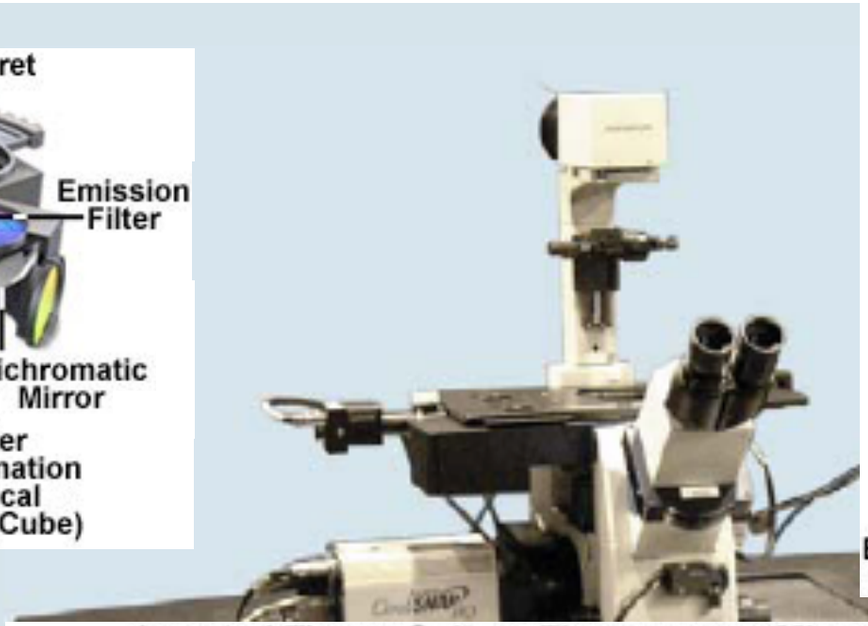
*** It is quite hard to break a microscope**

Understanding what goes on in the Microscope

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

Understanding what goes on in the Microscope





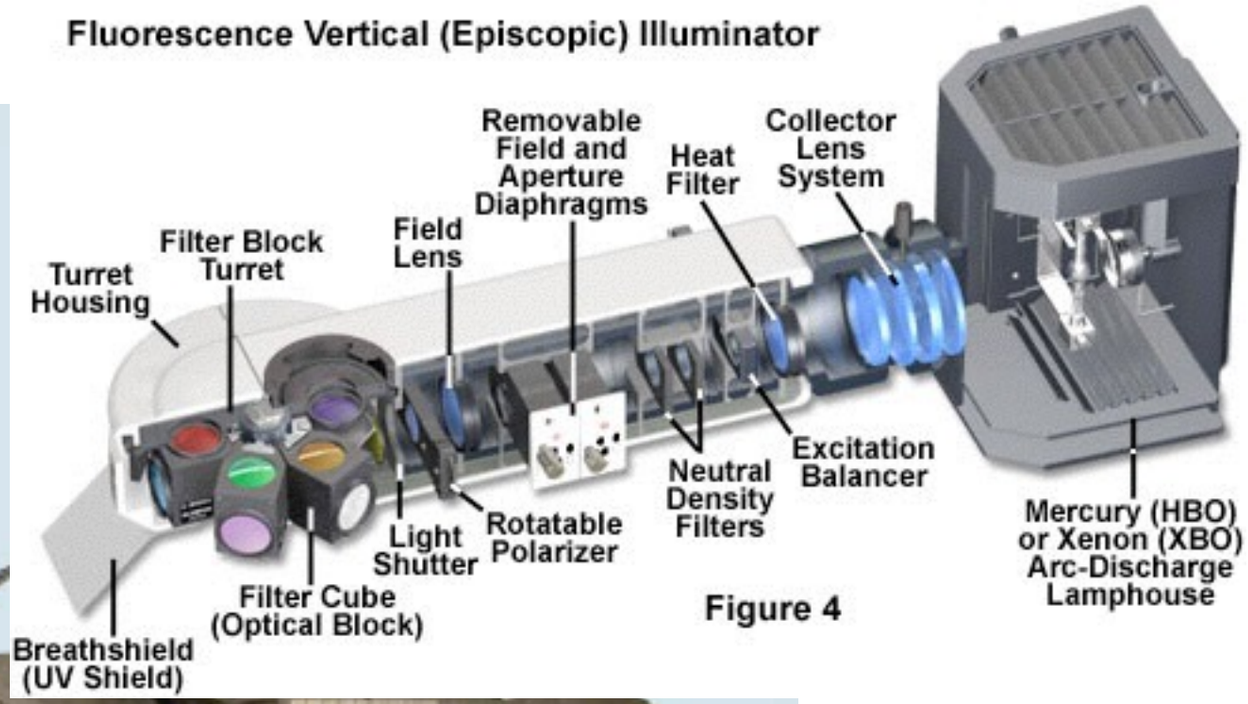


Figure 4

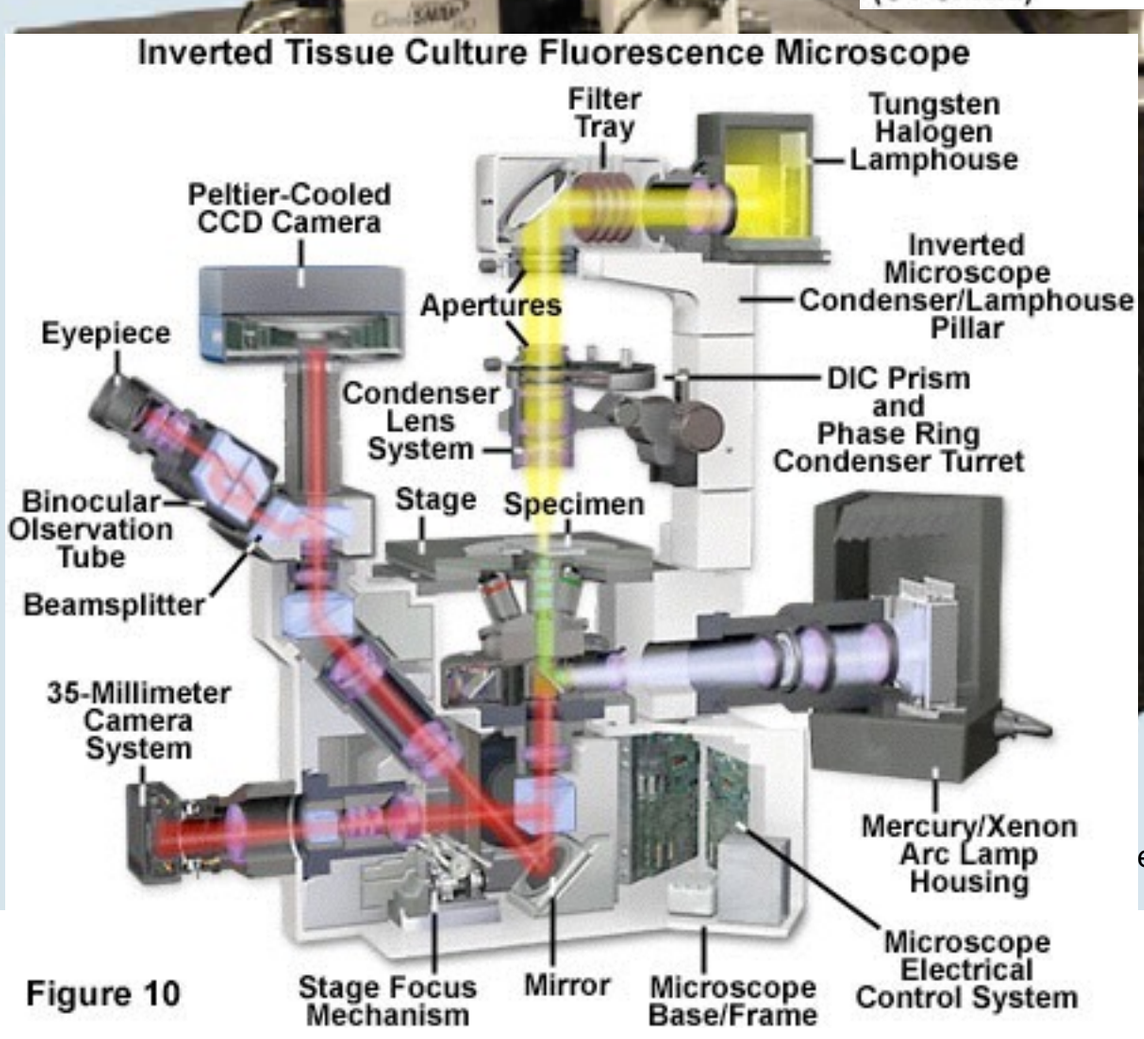


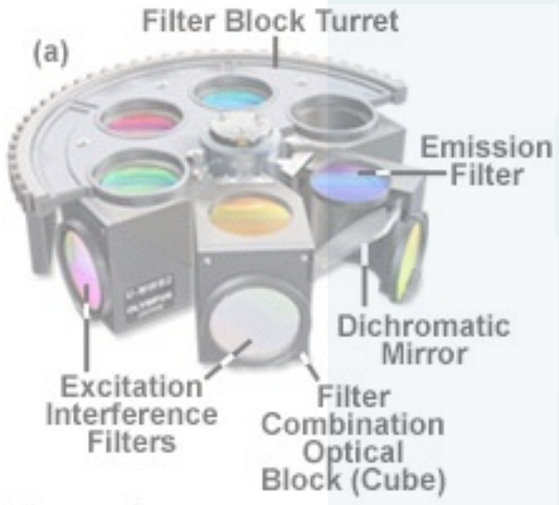
Figure 10

ehealthcare.com/api/deltavision.asp

http://www.olympusmicro.com/

Understanding what goes on in the Microscope

Lectures 2-4



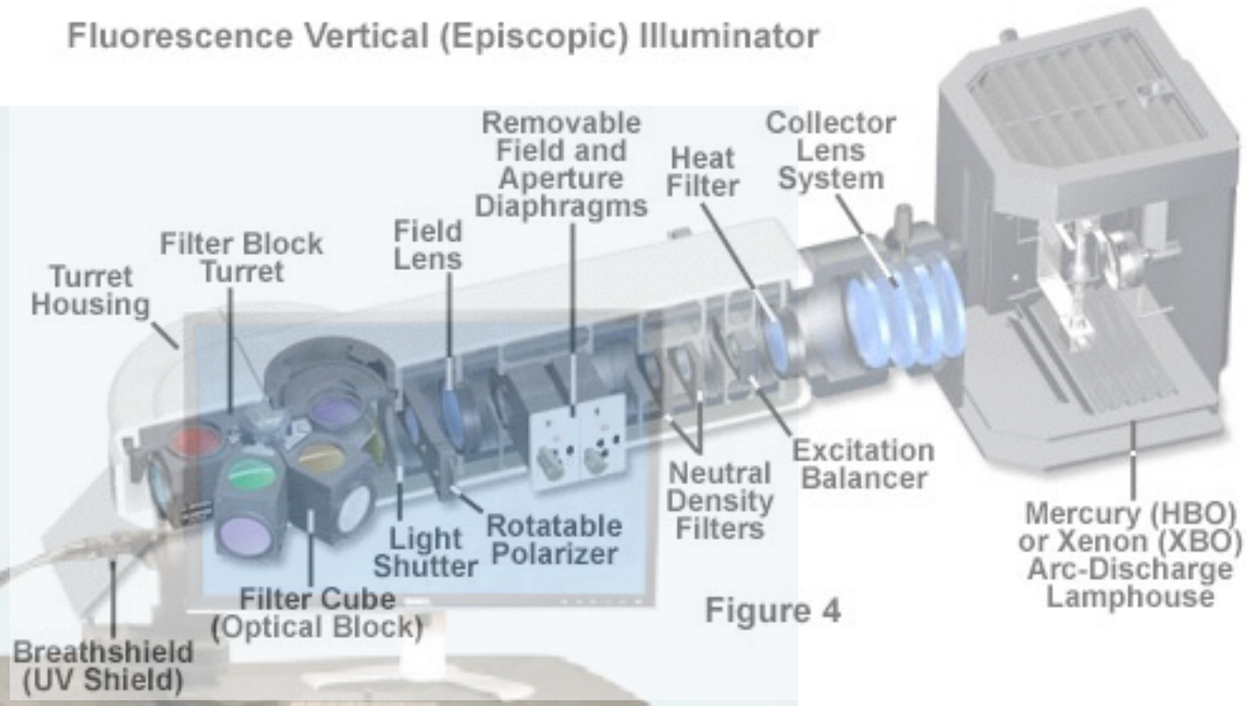


Figure 4

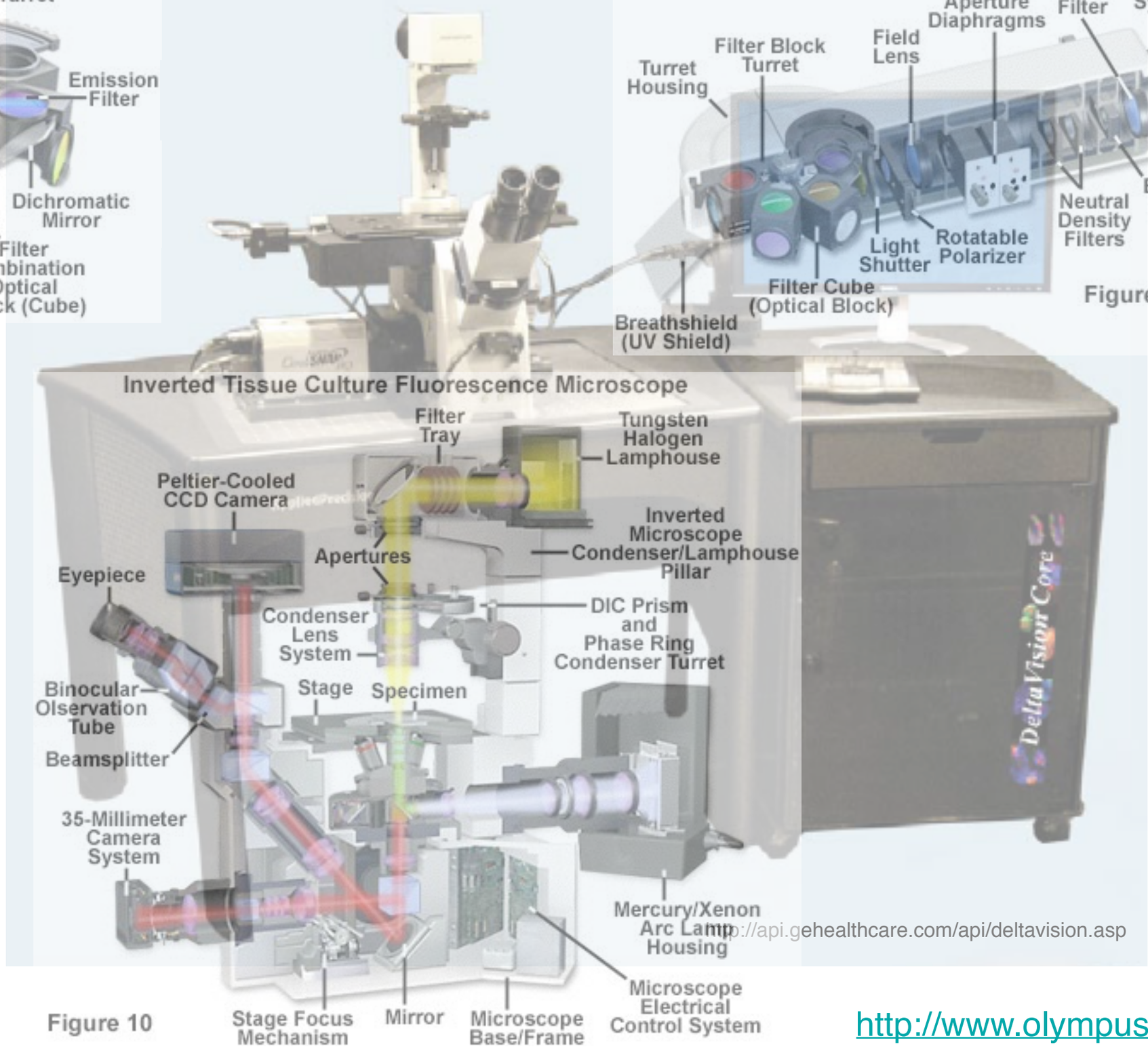


Figure 10

http://www.olympusmicro.com/

What is really important in microscopy?

…….the ability to see stuff

What is really important in microscopy?

…….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

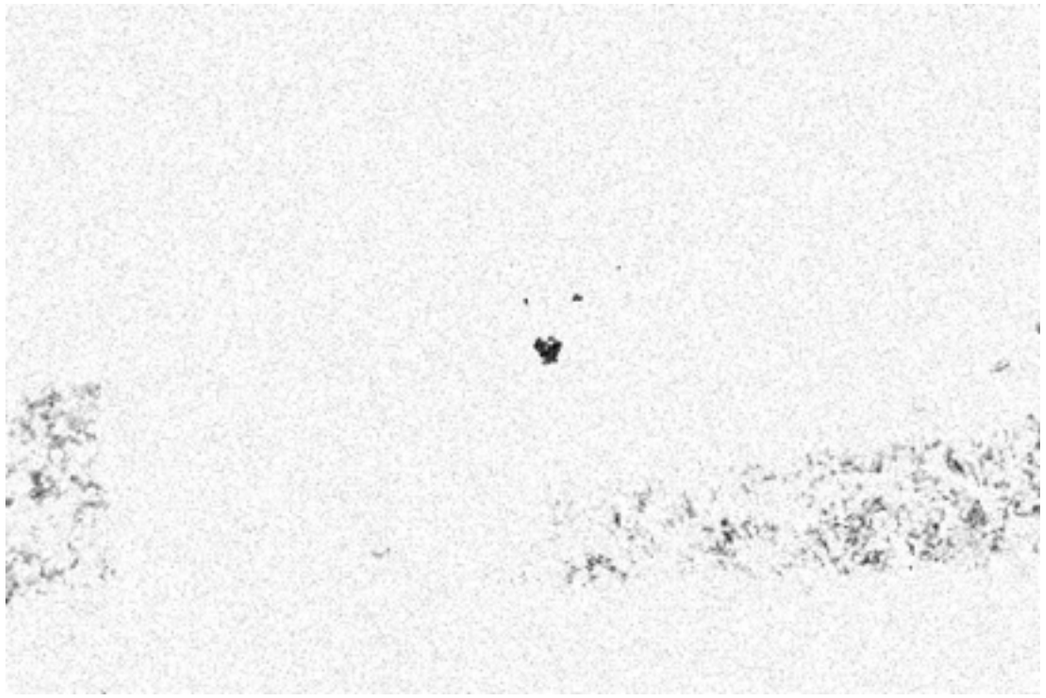
What is really important in microscopy?

Contrast .....the ability to distinguish stuff

What is really important in microscopy?

Contrast .....the ability to distinguish stuff

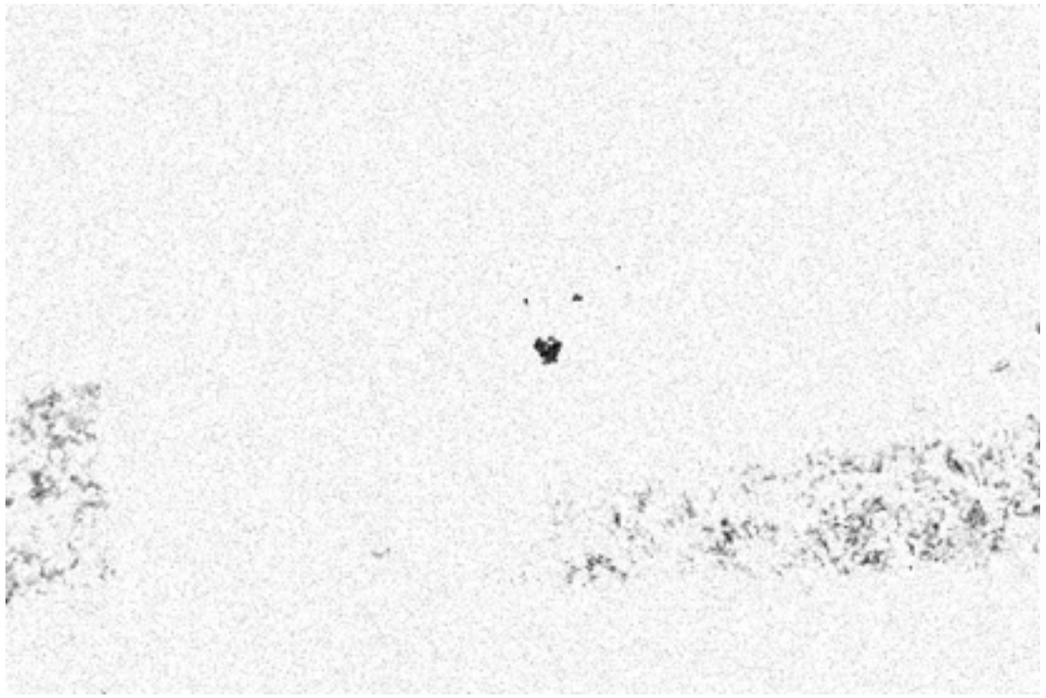
Biological specimens have low inherent contrast:



What is really important in microscopy?

Contrast .....the ability to distinguish stuff

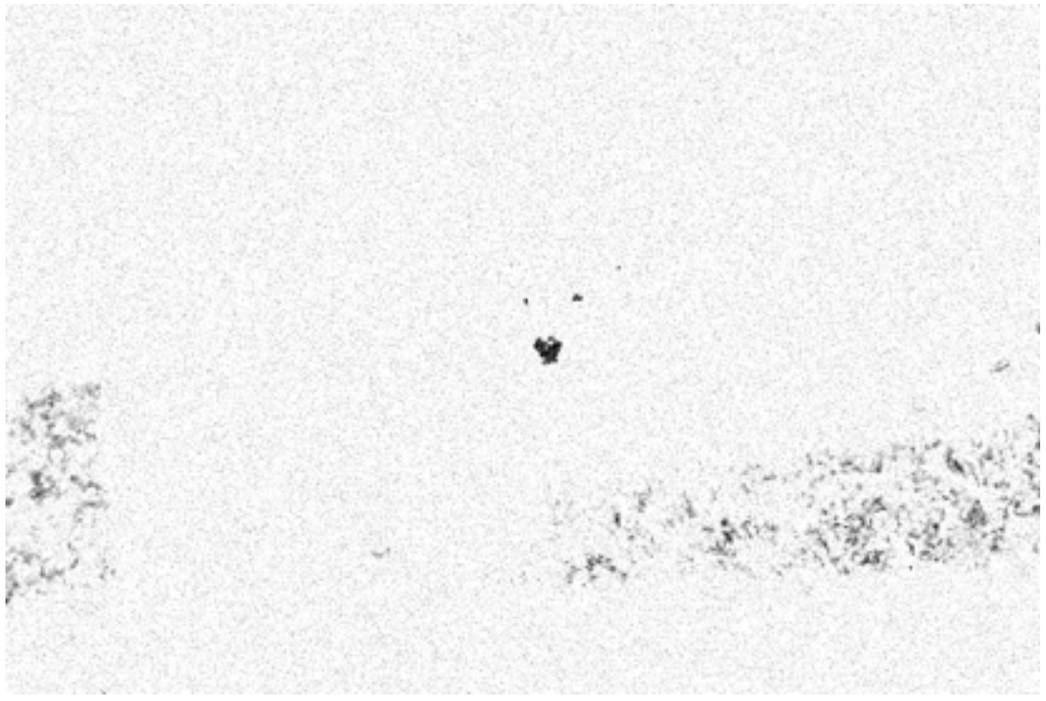
Biological specimens have low inherent contrast:

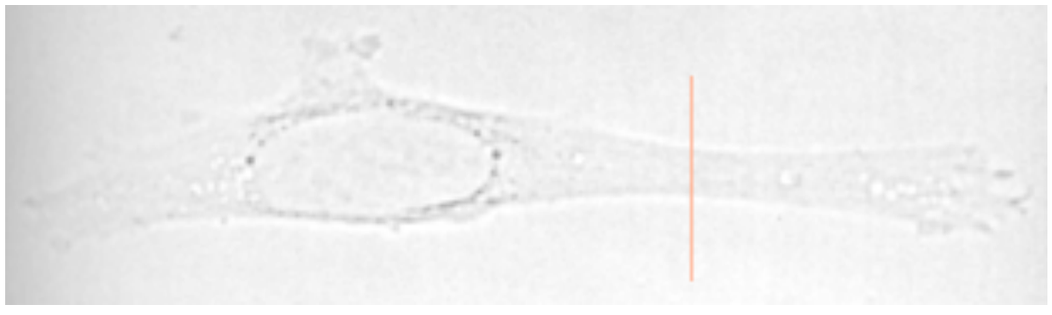


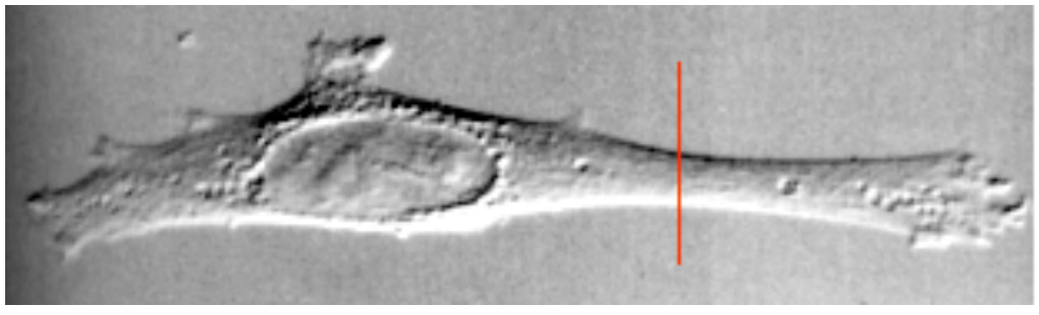
What is really important in microscopy?

Contrast .....the ability to distinguish stuff

Biological specimens have low inherent contrast:







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

Bright Field Contrast Techniques

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

Bright Field Contrast Techniques

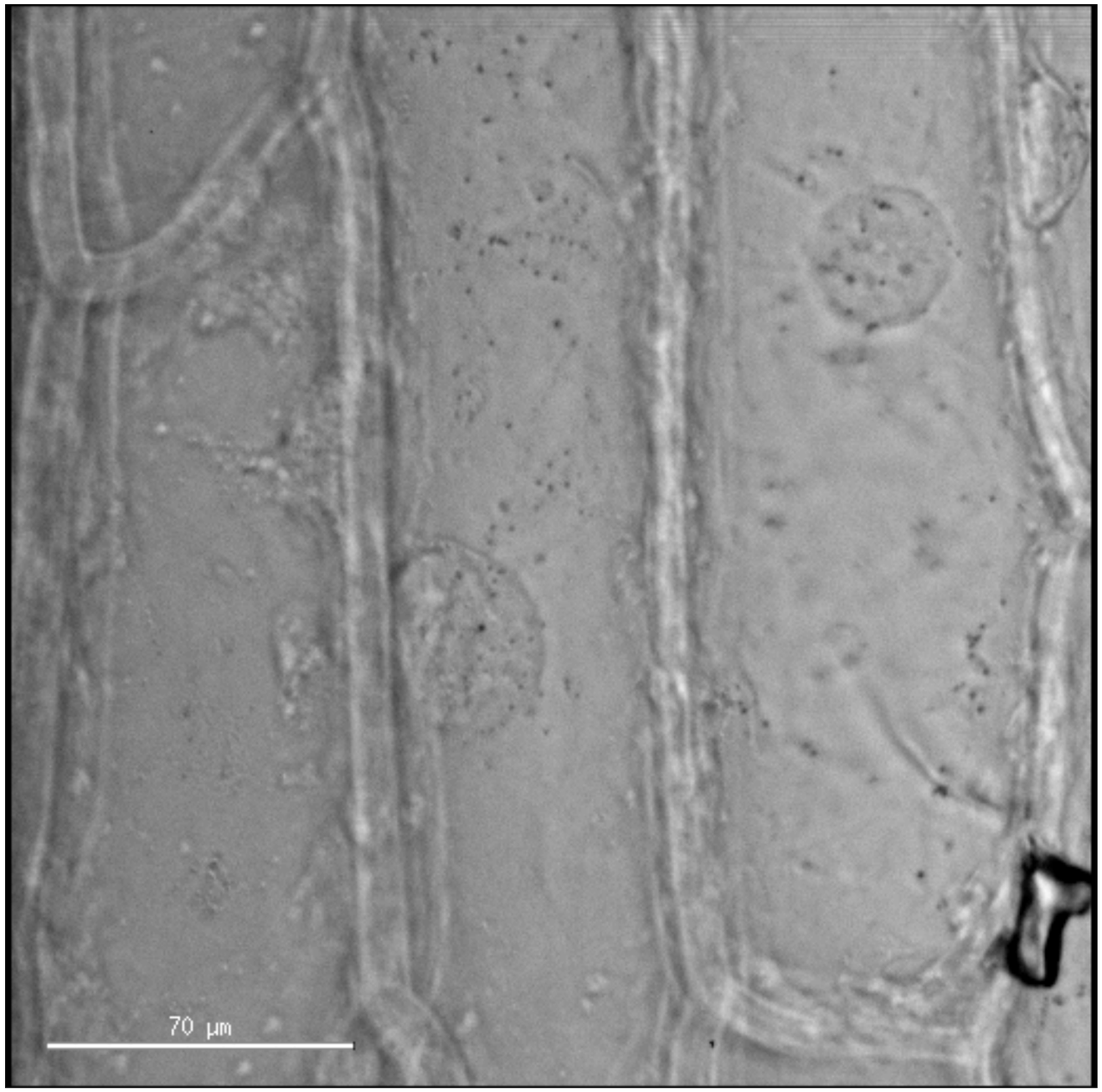
…….enhance features by transforming differences in the cell into differences in brightness

RMP: Onion epidermis bright field

Bright Field Contrast Techniques

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

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

inorganic
fluorescent
labels

LECTURES 4, 5

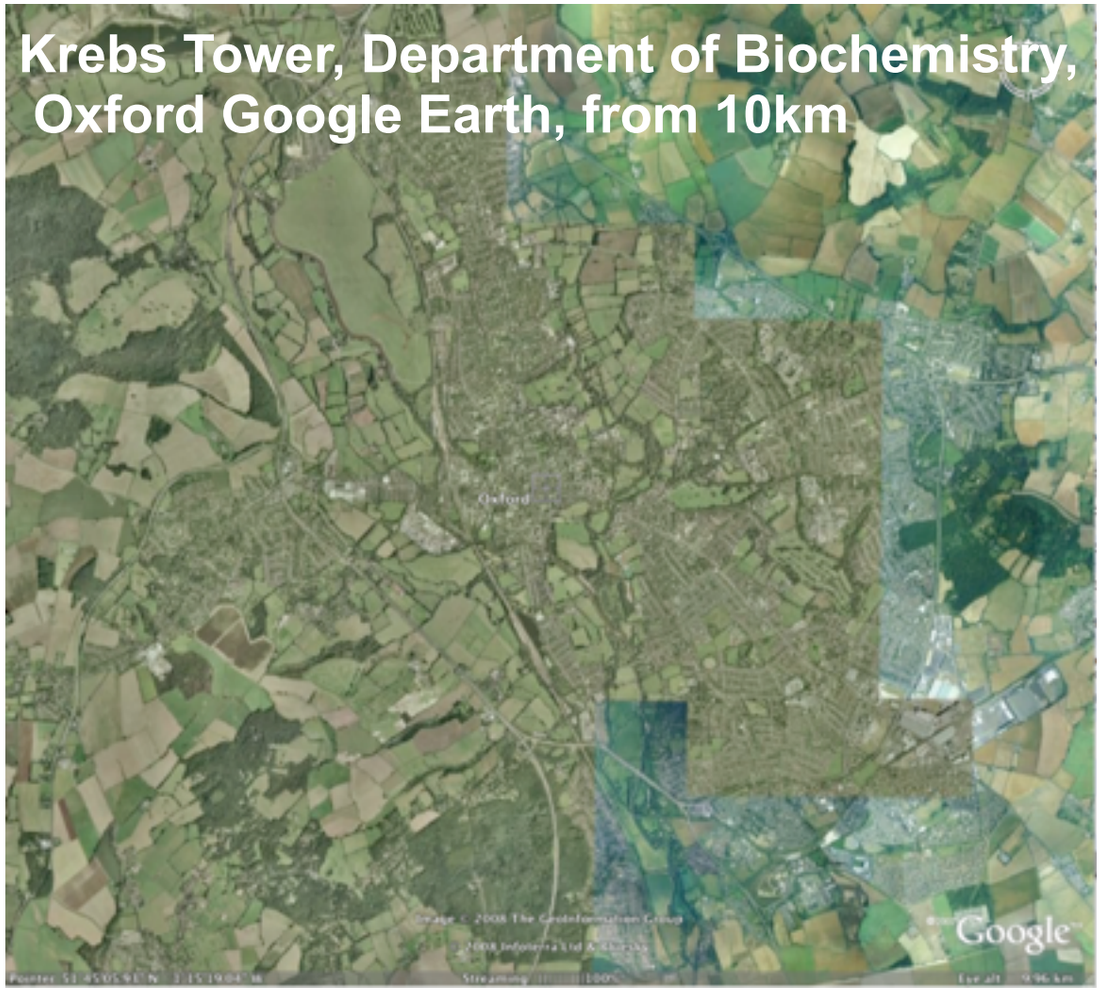
What is really important in microscopy?

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

What is really important in microscopy?

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

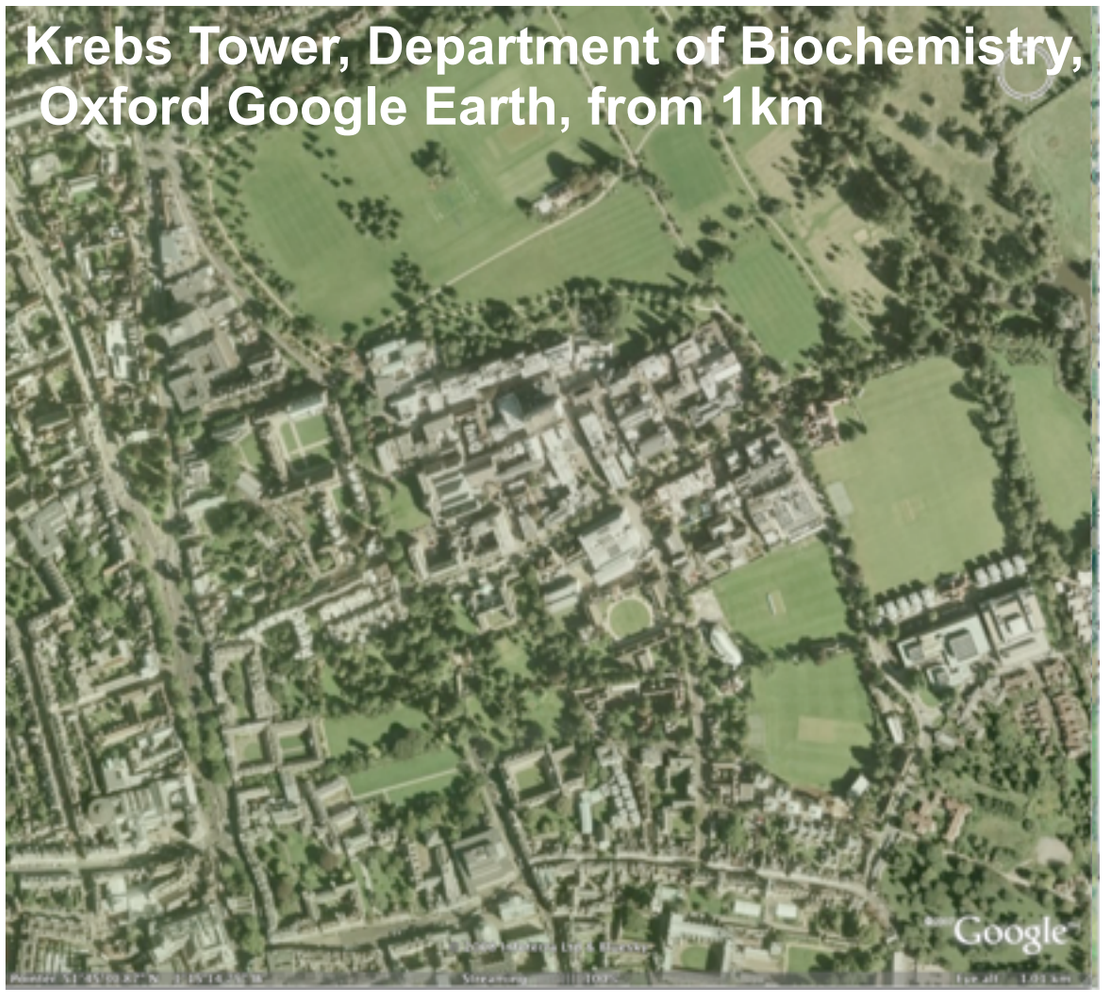
Magnifying is not enough:



What is really important in microscopy?

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

Magnifying is not enough:



What is really important in microscopy?

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

Magnifying is not enough:

What is really important in microscopy?

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

Magnifying is not enough:

What is really important in microscopy?

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

Magnifying is not enough:

What is really important in microscopy?

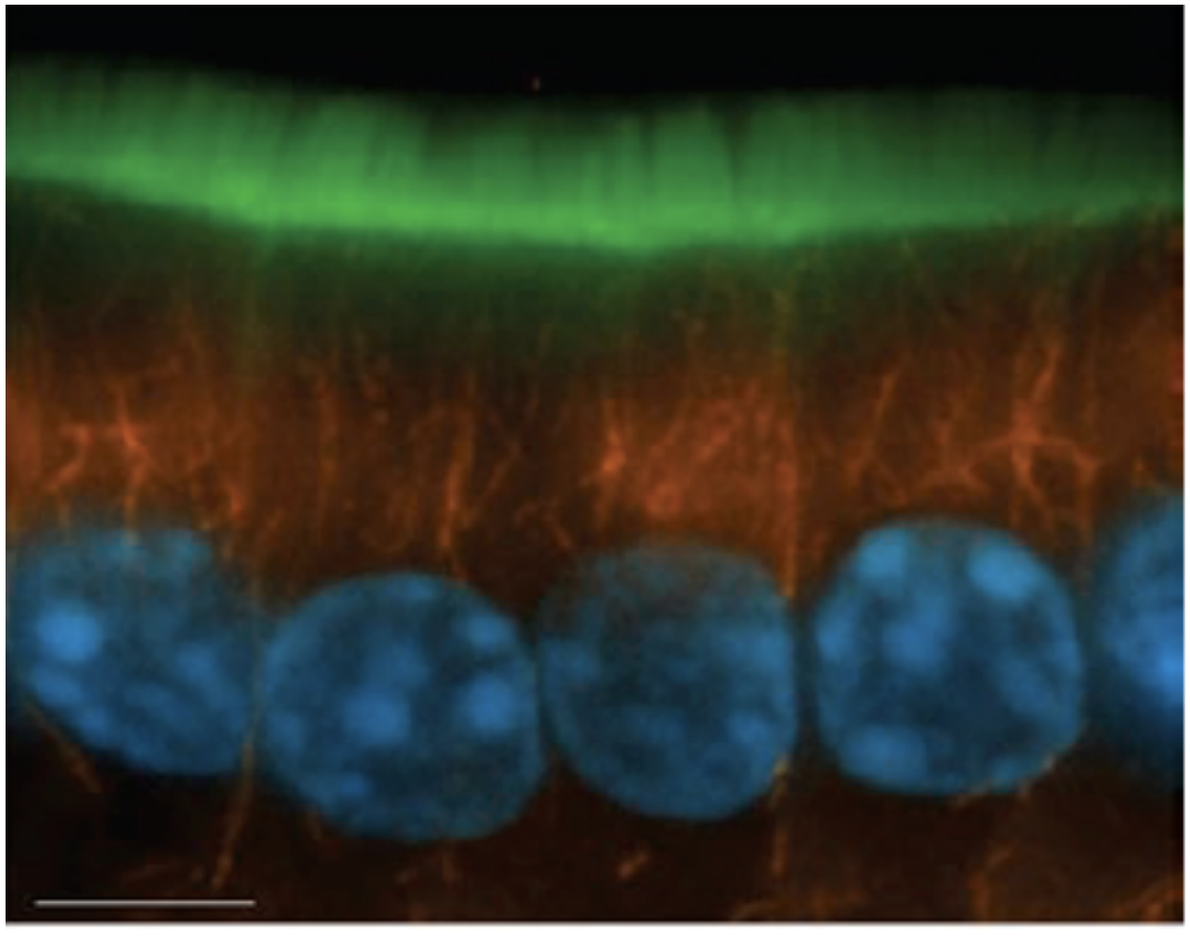
Resolution .....the ability to see small stuff

Magnifying is not enough:

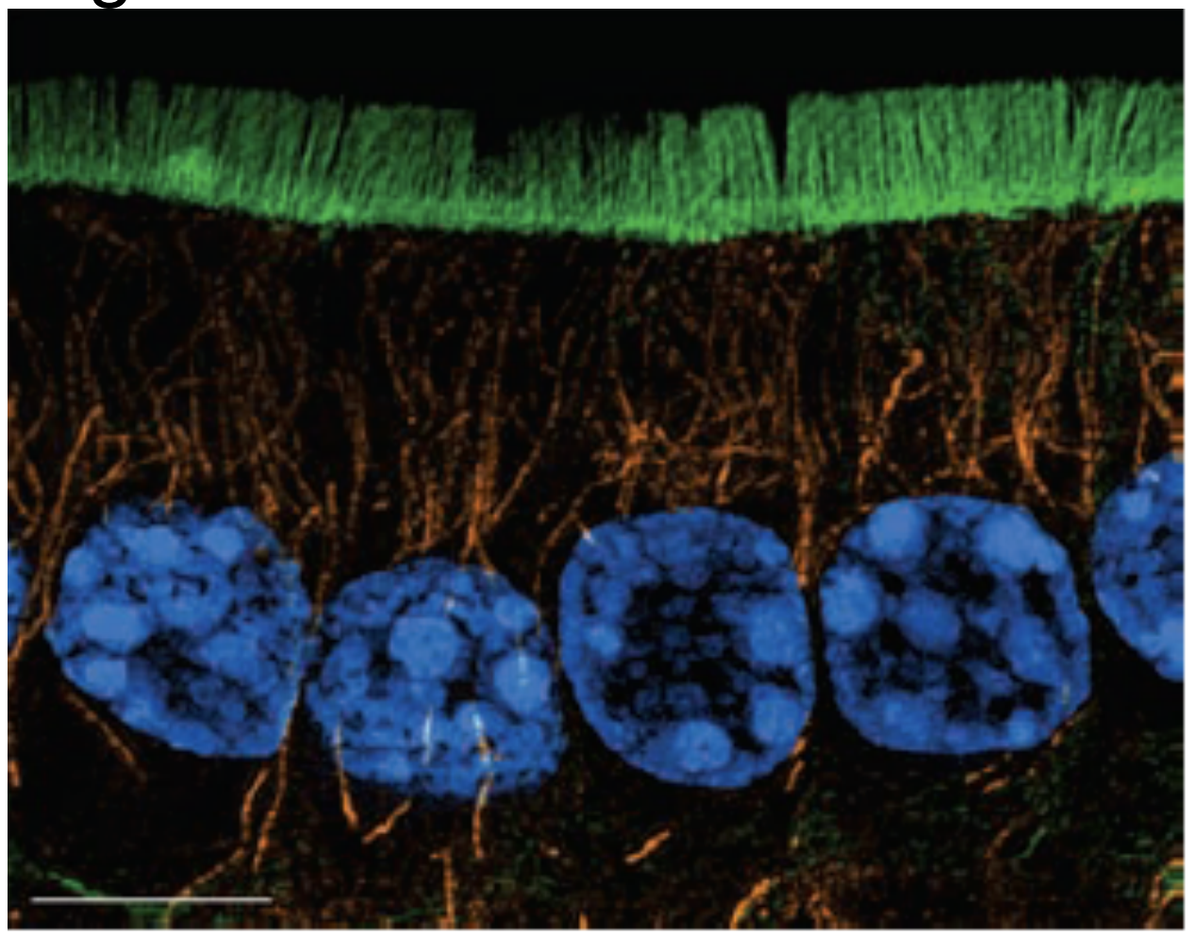
.....resolution is limited

RESOLUTION

Normal resolution



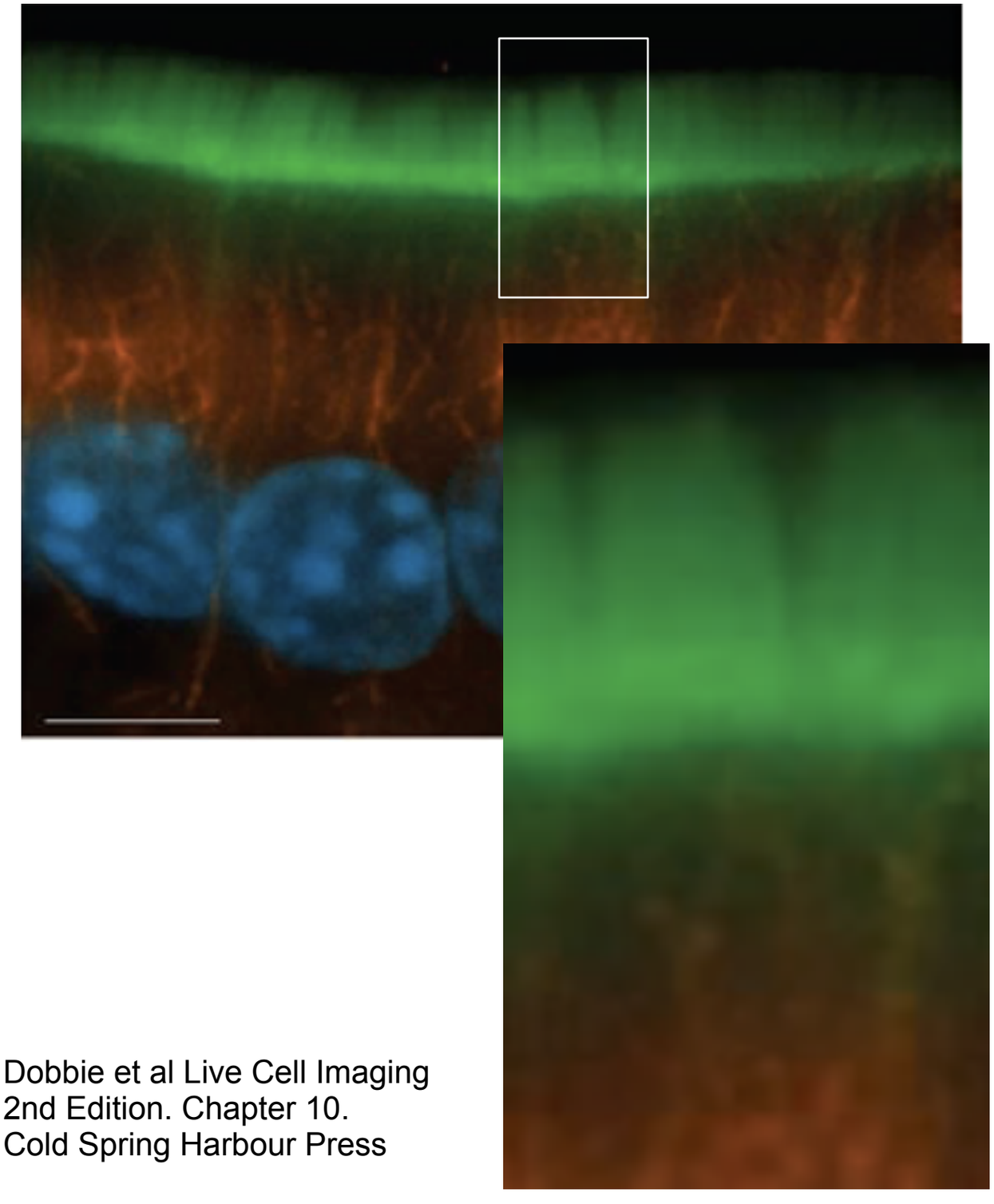
High resolution



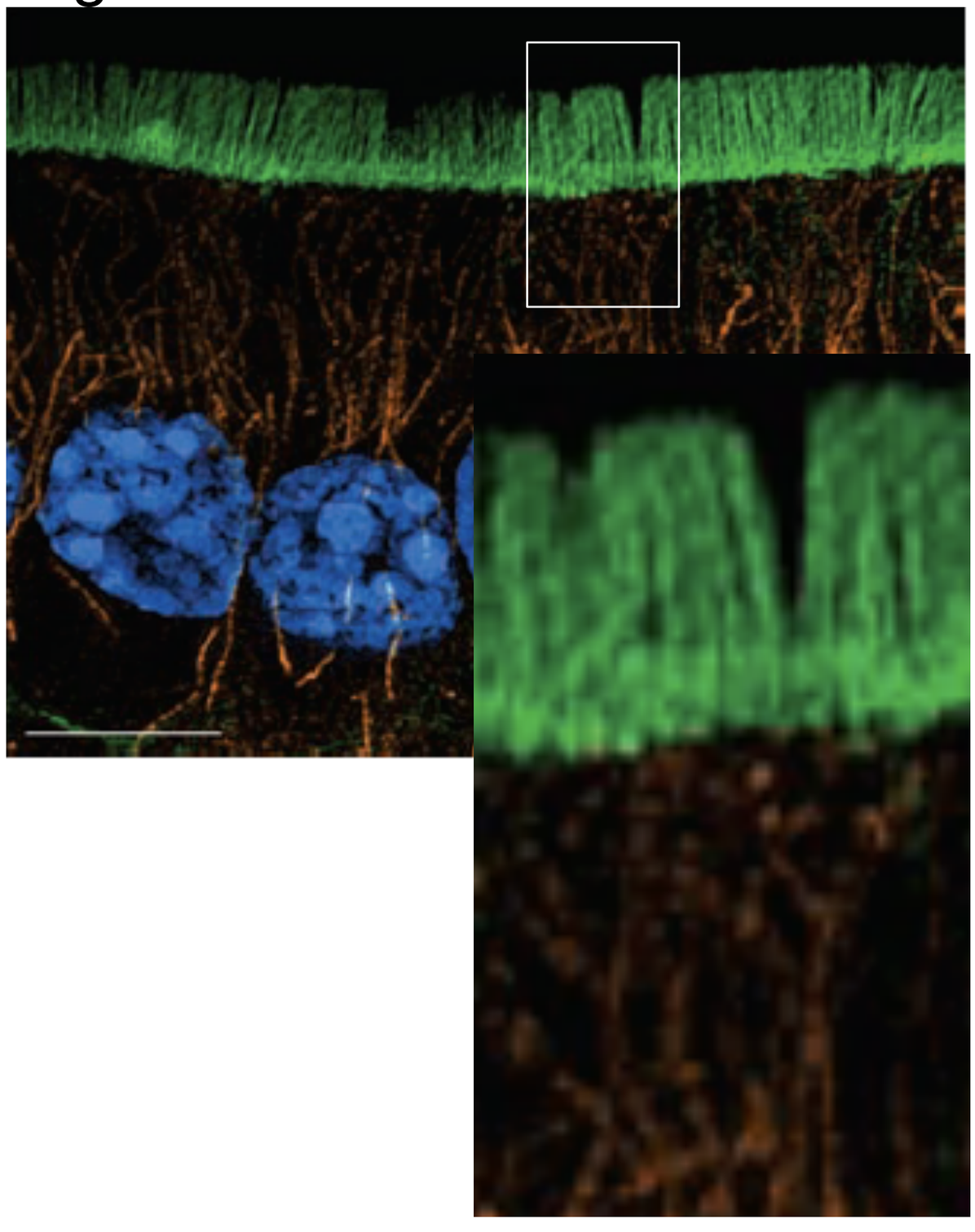
Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
Cold Spring Harbour Press

RESOLUTION

Normal resolution



High resolution



Dobbie et al Live Cell Imaging
2nd Edition. Chapter 10.
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

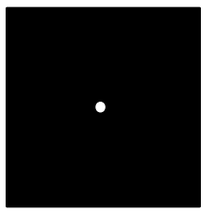
Convolution and the Point Spread Function

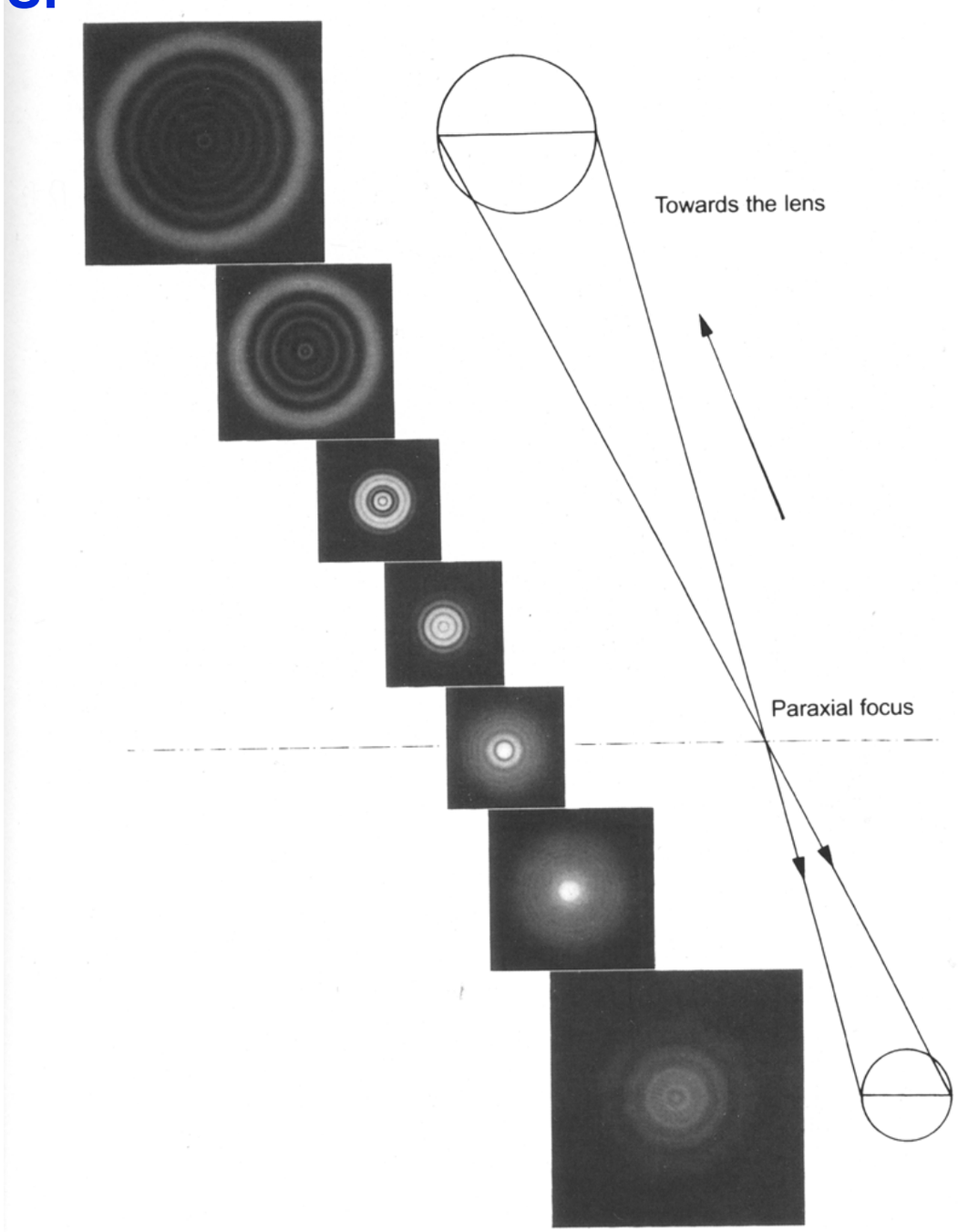
An image represents the output of the optics and detector of the imaging system

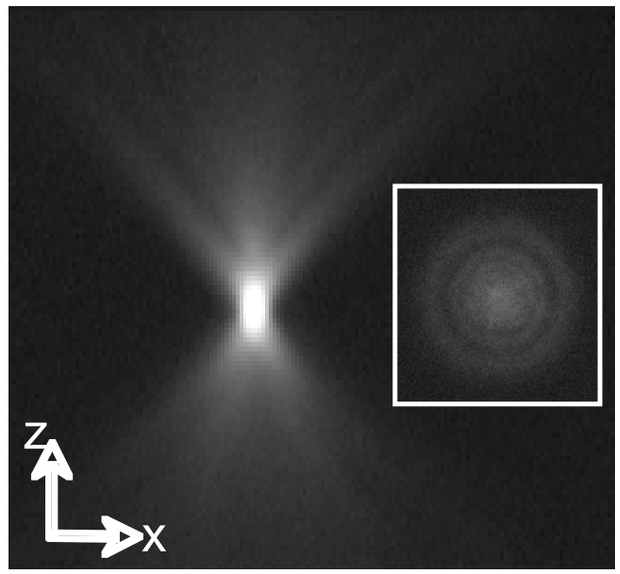
image ≠ object

image = object ⊗ PSF

Sample object: a "sub-resolution" fluorescent bead







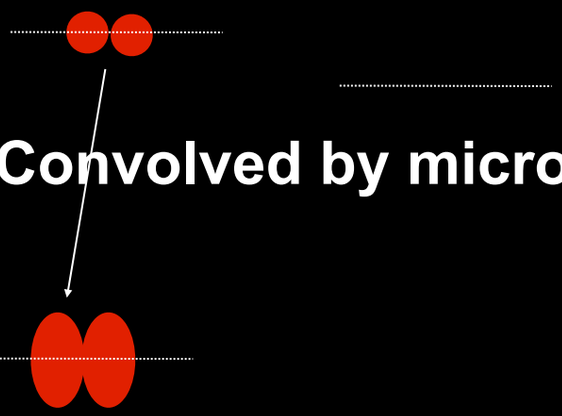
Fundamentals of light microscope and electronic imaging. Douglas B. Murphy. Wiley-Liss 2001

LECTURE 4

- "convolution" by the microscope optics = the PSF

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects

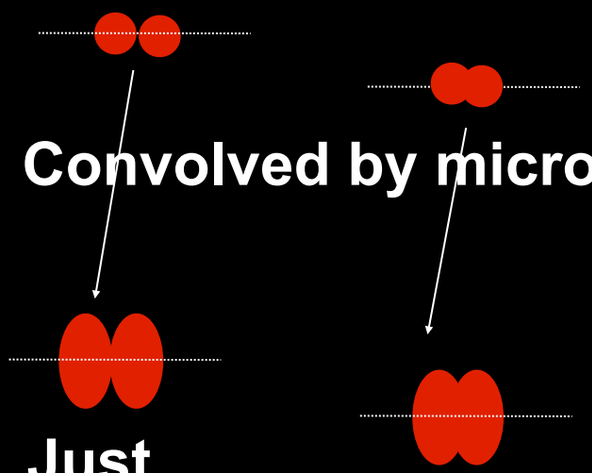


Convolved by microscope

Just resolved

Optical resolution: The Rayleigh Criterion (D_R)

Two small objects



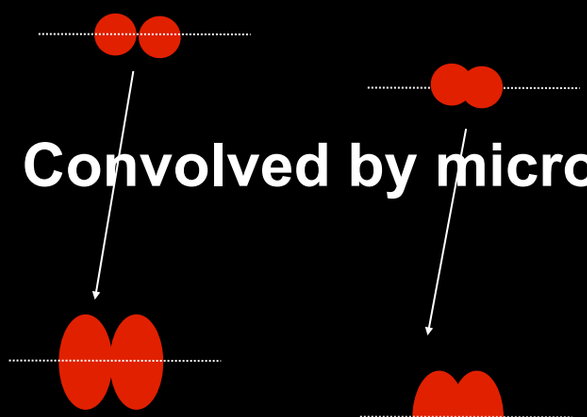
Convolved by microscope

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

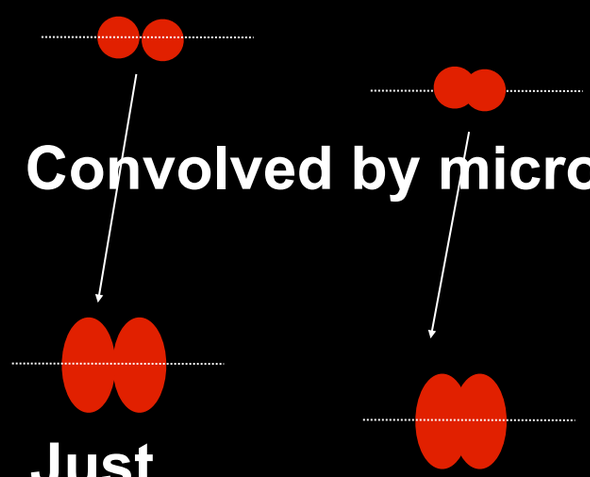
Just resolved

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

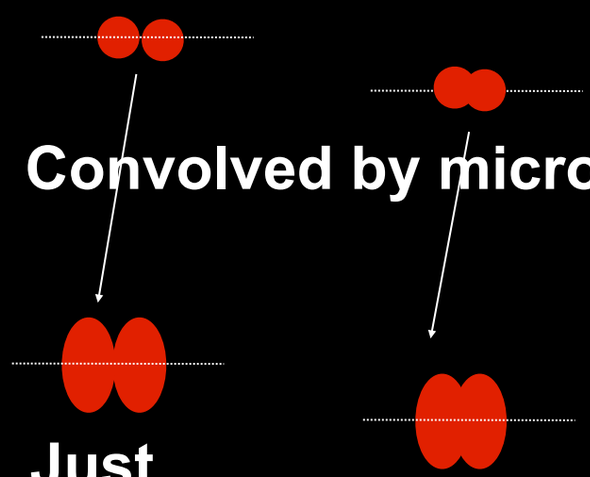
Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

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

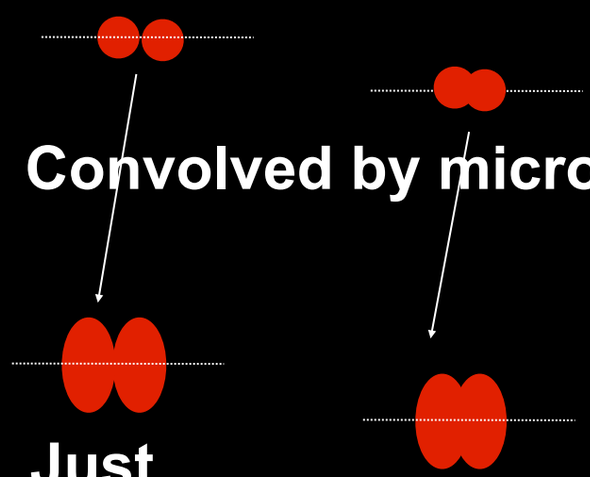
Resolution (D_R) depends upon the objective and wavelength of light:

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

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:

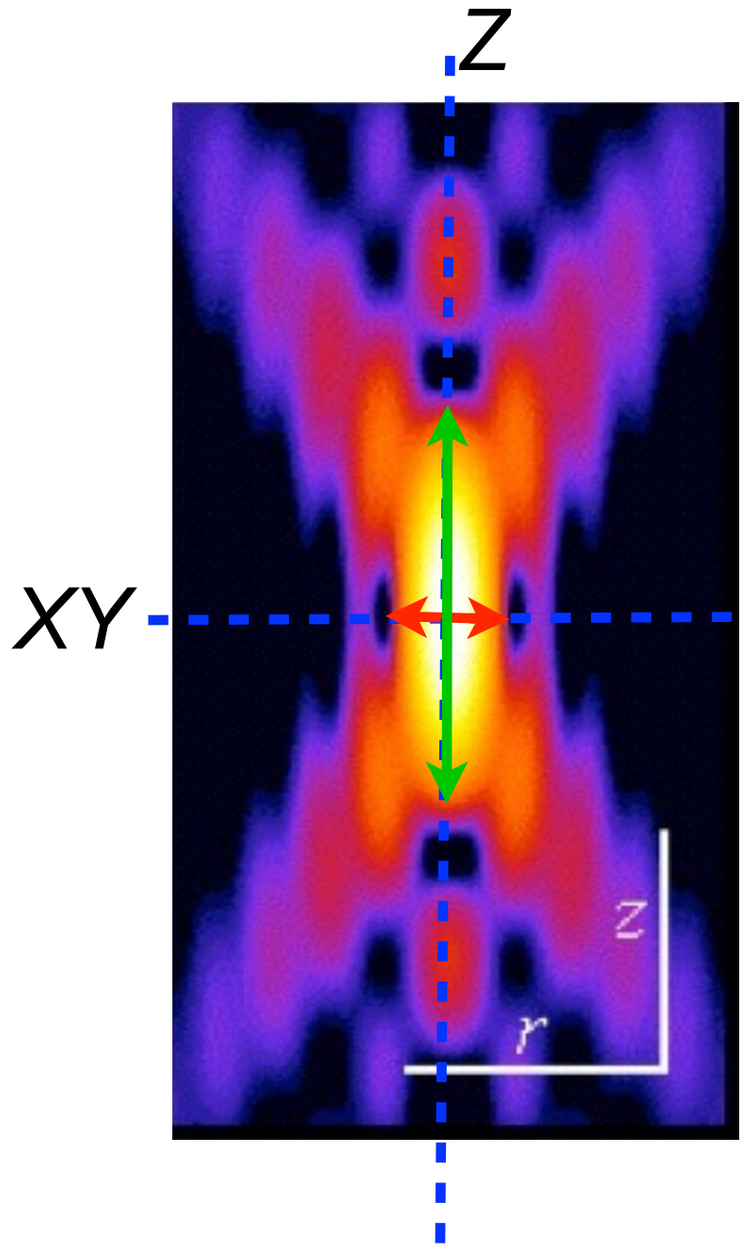
$$D_R = 1.22 \times \lambda / Na_{obj} + Na_{cond}$$

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:

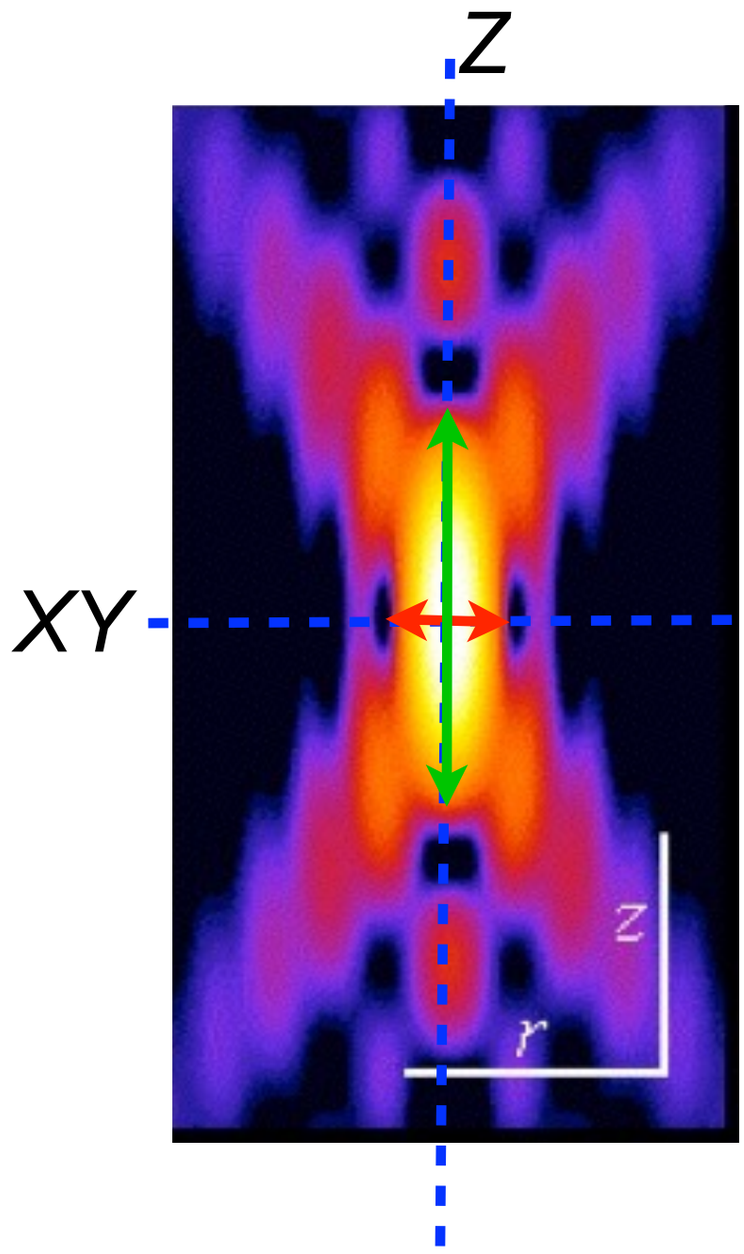


Axial Resolution:

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)

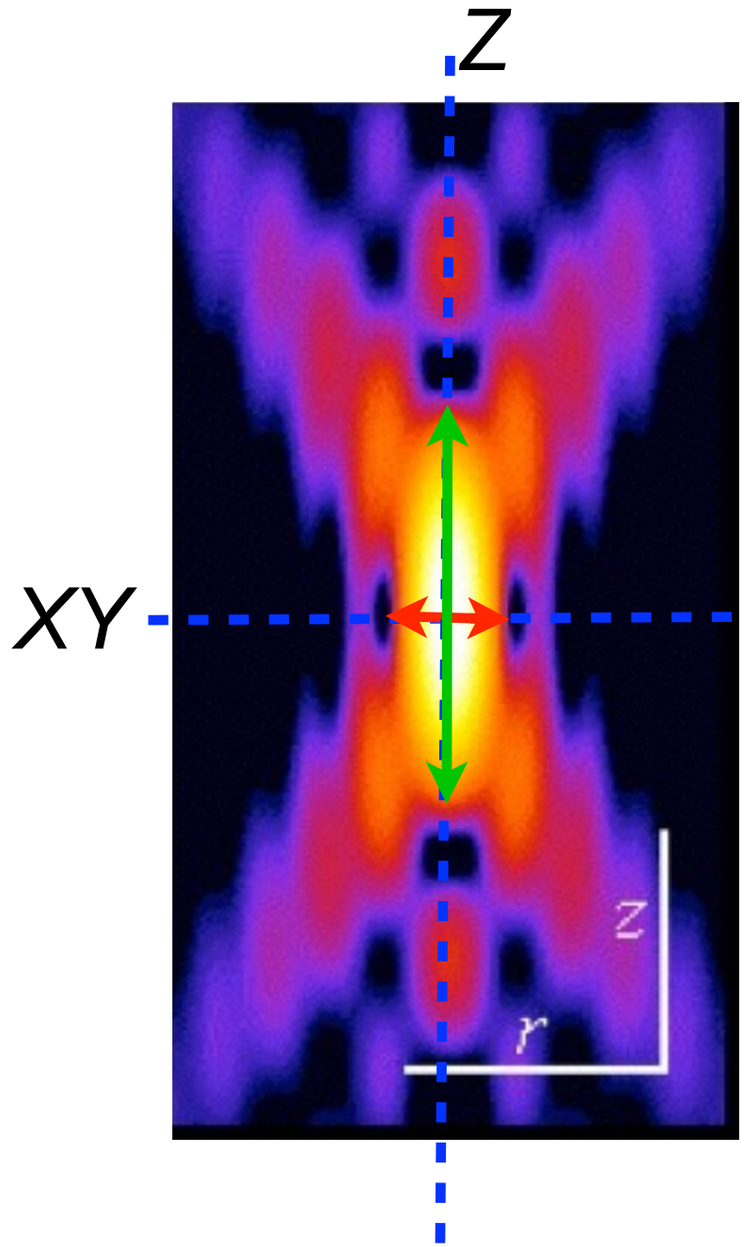
$$D_Z = 2\,\lambda\eta/(NA_{obj})^2 \ldots\ldots\ldots\ldots 705$$

(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

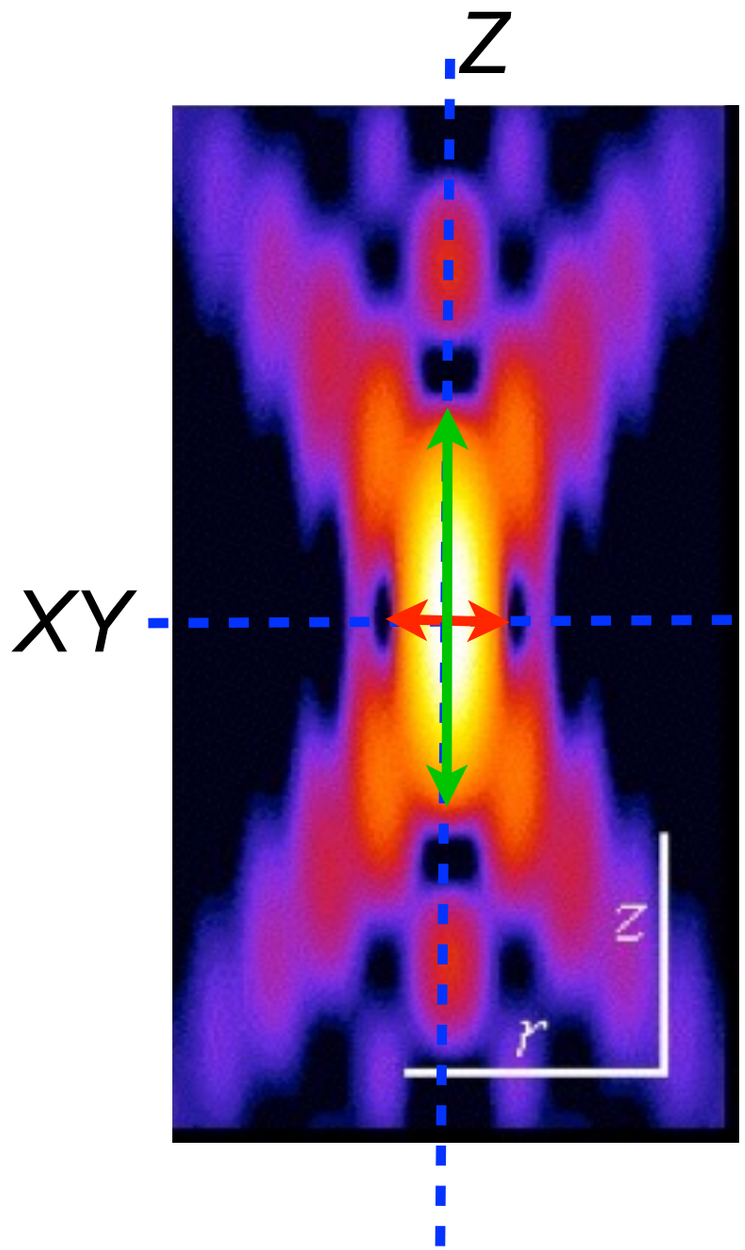


Axial Resolution:

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

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

(η = refractive index of the object medium)



Than it is in the lateral dimension (XY)

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

FWHM

The relationship between the two is:

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

Resolution: Down to the molecular scale?

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

XY resolution ~ 230 nm
Z resolution ~ 700 nm

GFP is ~ 5 x5 x5 nm 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 $\approx$ 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 8

Solution 2 - Super resolution techniques

Localisation microscopy, Structured illumination, STED

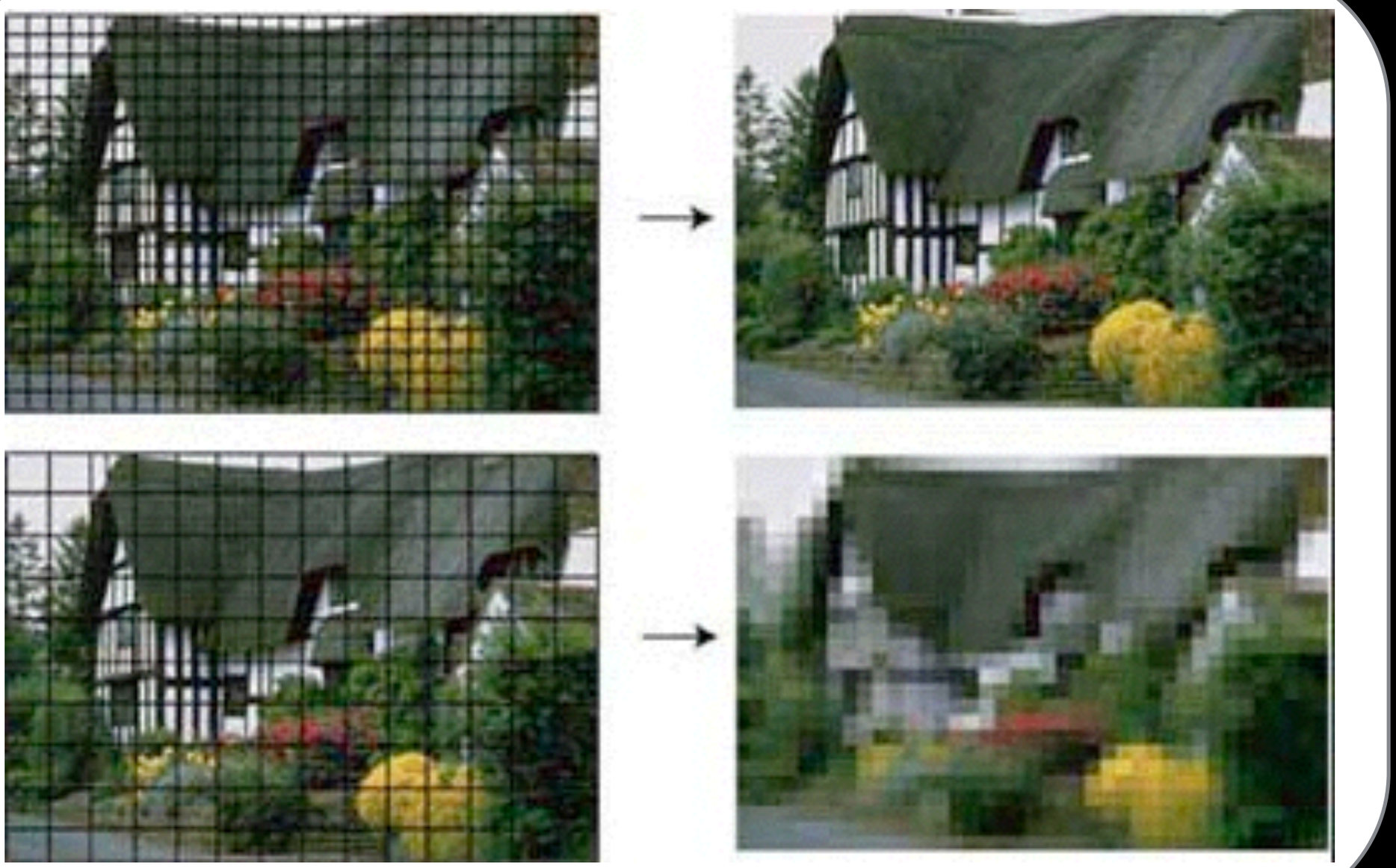
LECTURES 9-11

What is really important in microscopy?

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

Sampling

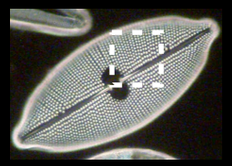
……..correctly reading the available information



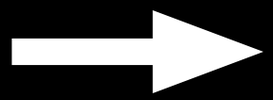
……..poor sampling limits the resolution achieved

Resolution .....Magnification and Sampling

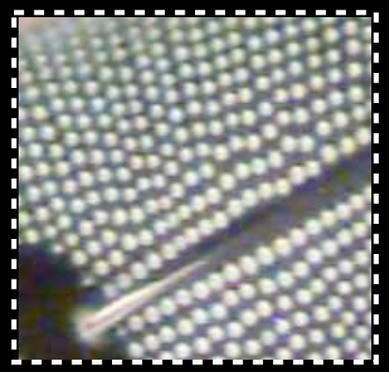
Specimen
Fine Detail



Detail imaged
by microscope

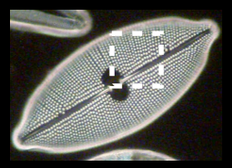


*magnification
*optical resolution



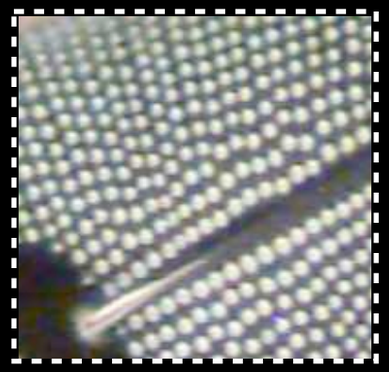
Resolution .....Magnification and Sampling

Specimen
Fine Detail

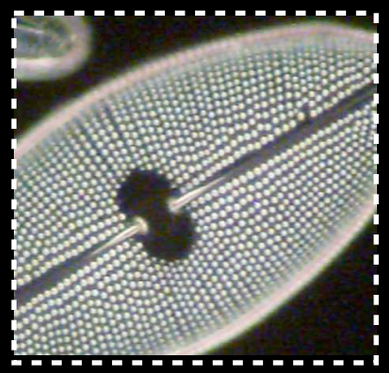


Detail imaged
by microscope

*magnification
*optical resolution

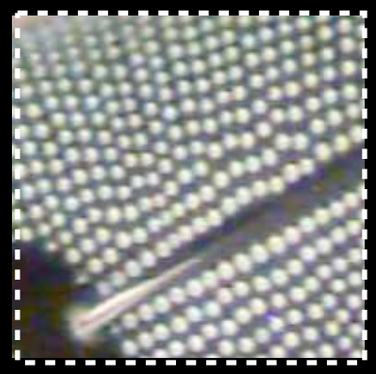


not magnified enough



Undersampling
all detail not resolved
Large field of view

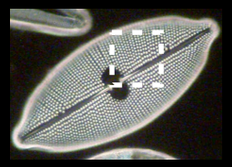
auxiliary
magnification
to match image
to detector



All resolvable
detail recorded

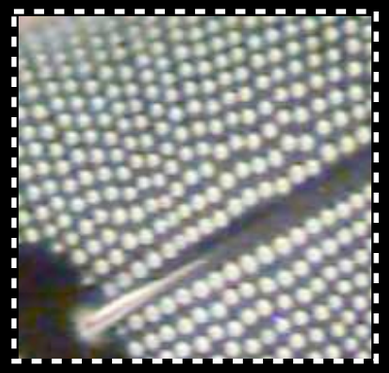
Resolution .....Magnification and Sampling

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

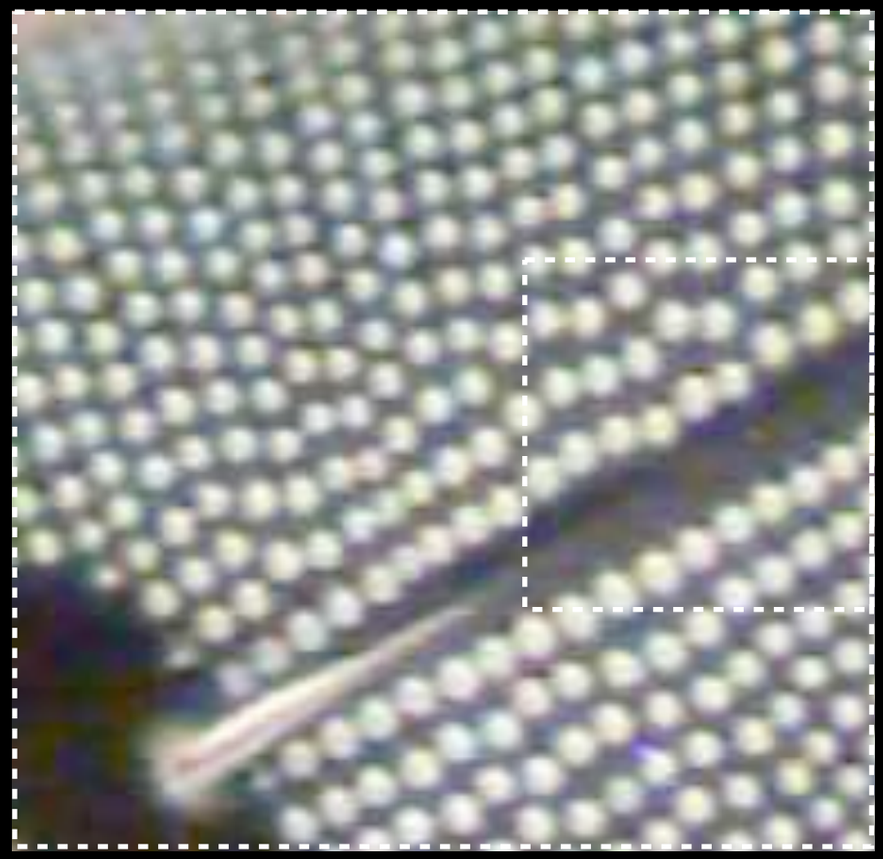
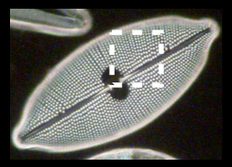


image
on detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

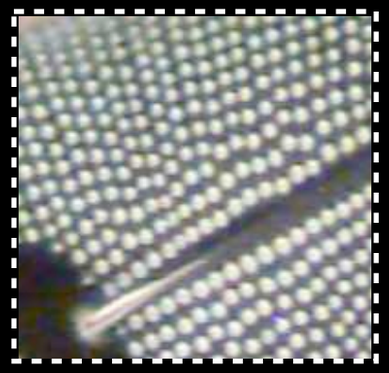
Resolution .....Magnification and Sampling

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

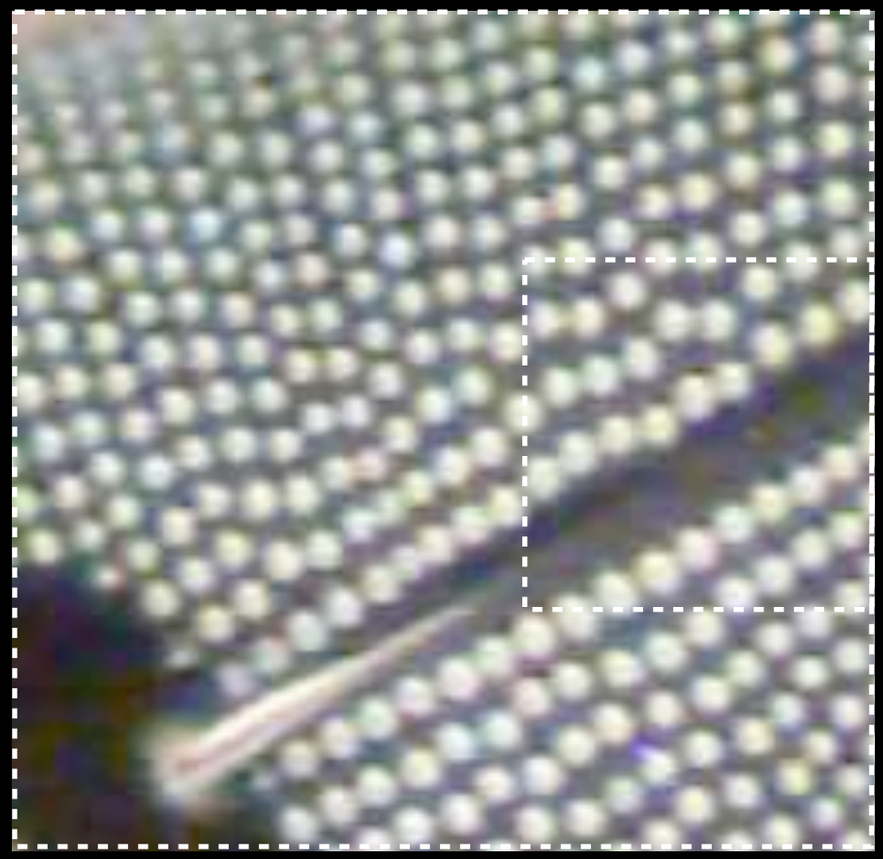


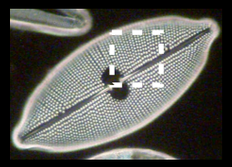
image
on detector

Oversampling
Empty
magnification
Blurred image
Limited field of
view

What is the optimum magnification.....?

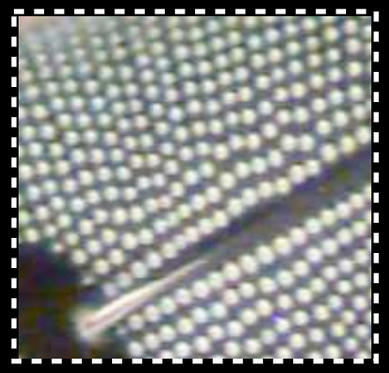
Resolution .....Magnification and Sampling

Specimen
Fine Detail



Detail imaged
by microscope

*magnification
*optical resolution



too magnified

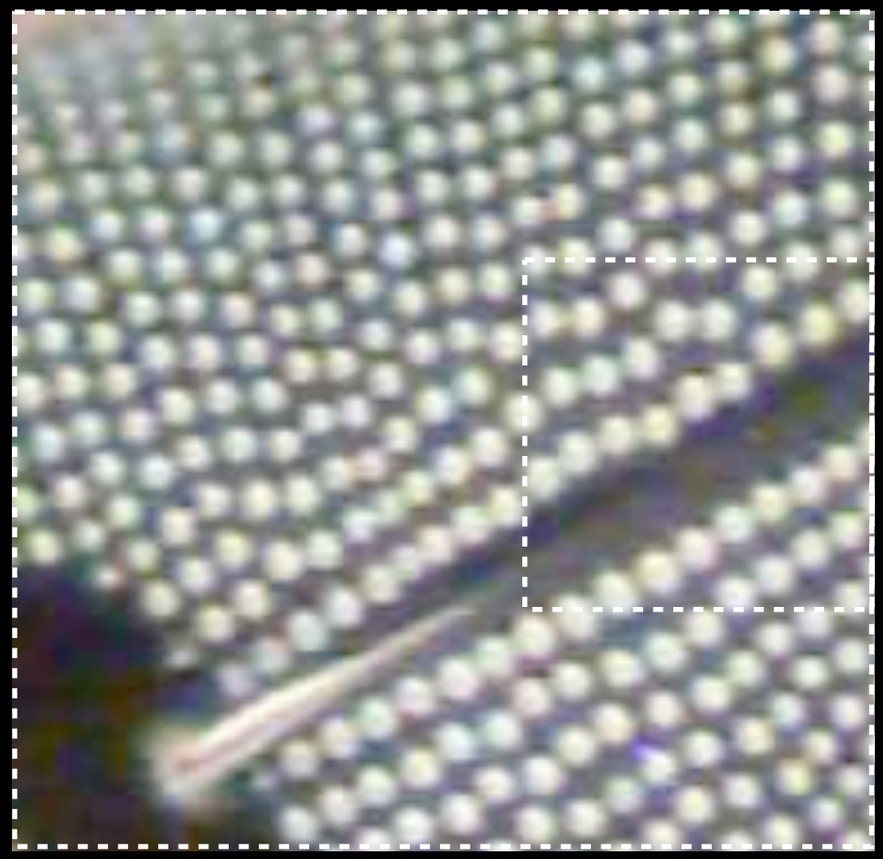


image
on detector

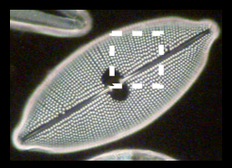
Oversampling
Empty
magnification
Blurred image
Limited field of
view

What is the optimum magnification.....?

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

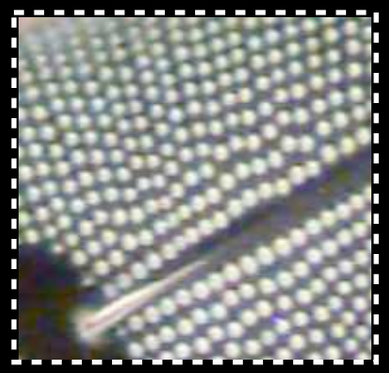
Resolution .....Magnification and Sampling

Specimen
Fine Detail



Detail imaged
by microscope

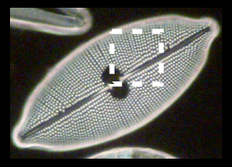
*magnification
*optical resolution



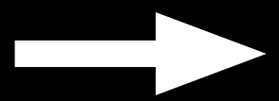
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

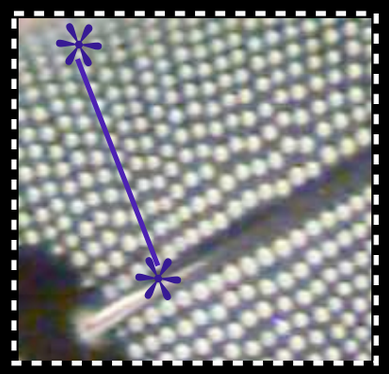
Specimen
Fine Detail

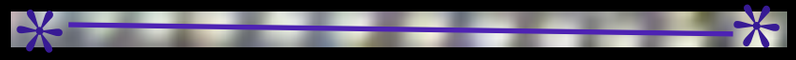


Detail imaged
by microscope

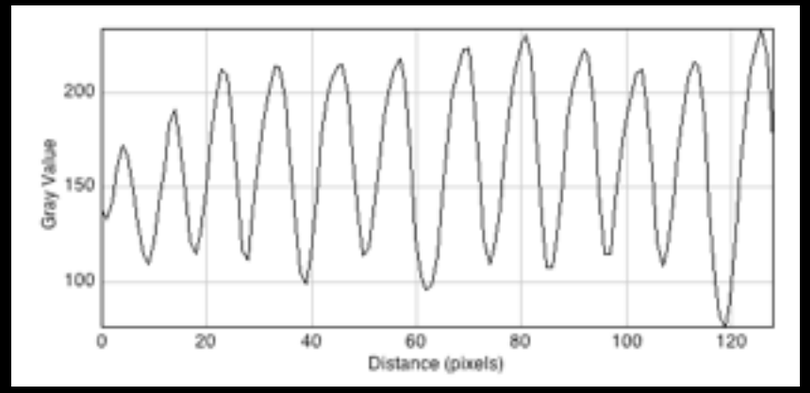


*magnification
*optical resolution





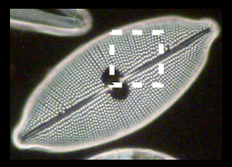
intensity profile ~ a sine wave



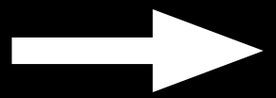
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

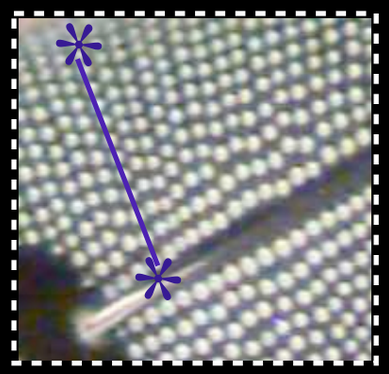
Specimen
Fine Detail

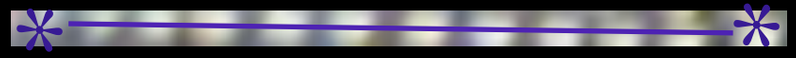


Detail imaged
by microscope

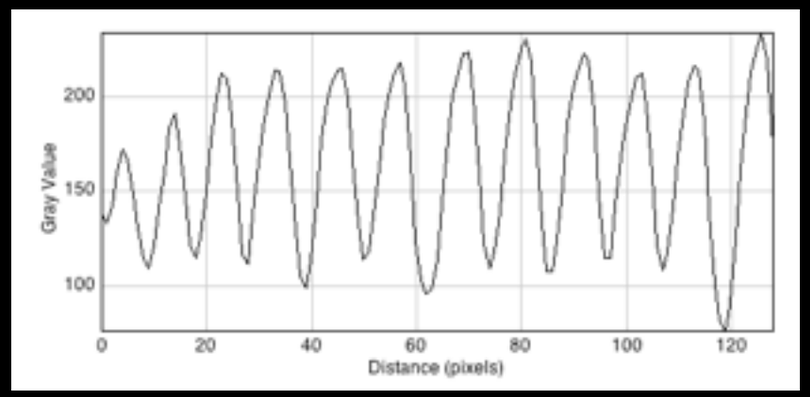


*magnification
*optical resolution

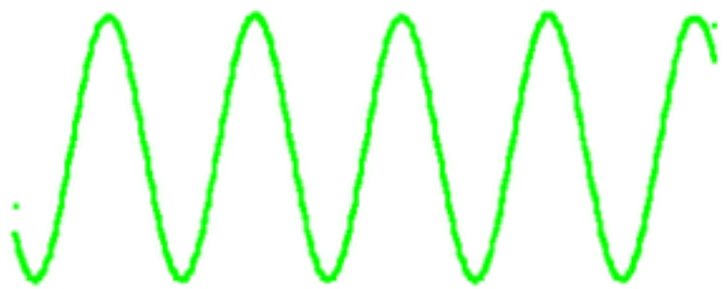




intensity profile ~ a sine wave



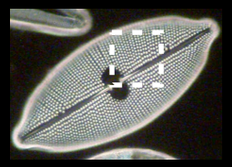
sampling a sine wave



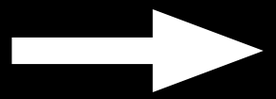
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

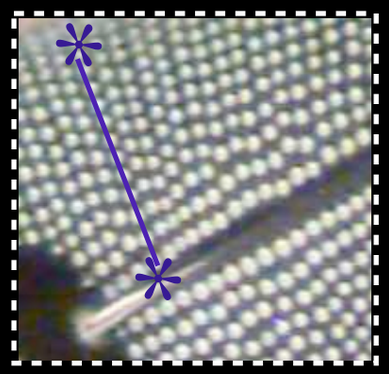
Specimen
Fine Detail

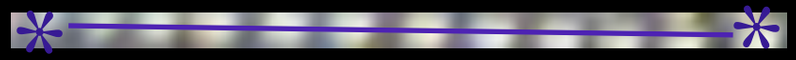


Detail imaged
by microscope

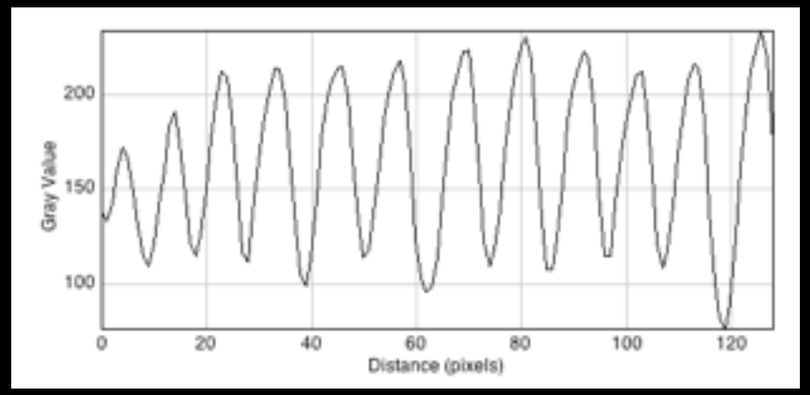


*magnification
*optical resolution

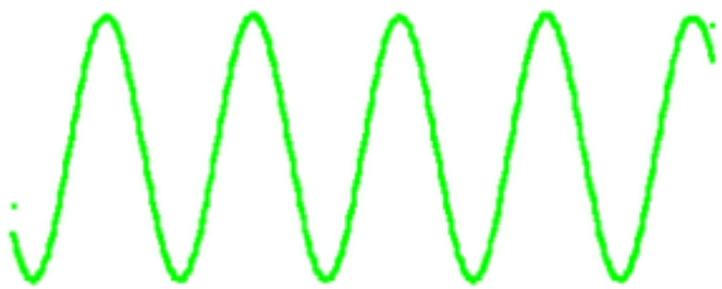




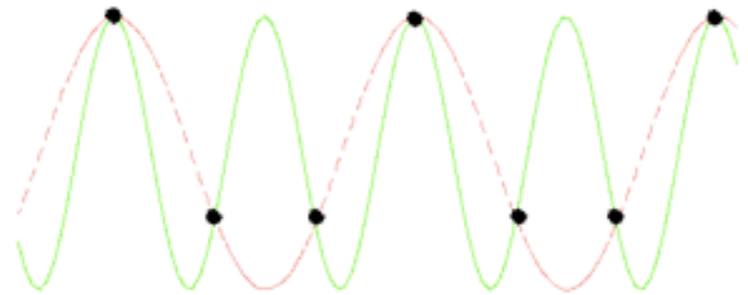
intensity profile ~ a sine wave



sampling a sine wave



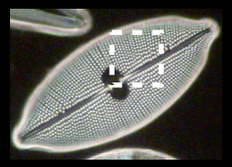
Sampling 1.5 times per cycle



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

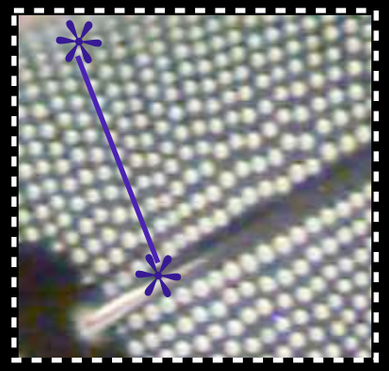
Resolution .....Magnification and Sampling

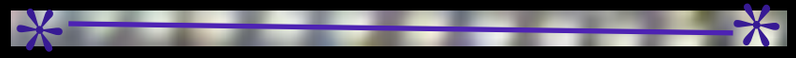
Specimen
Fine Detail



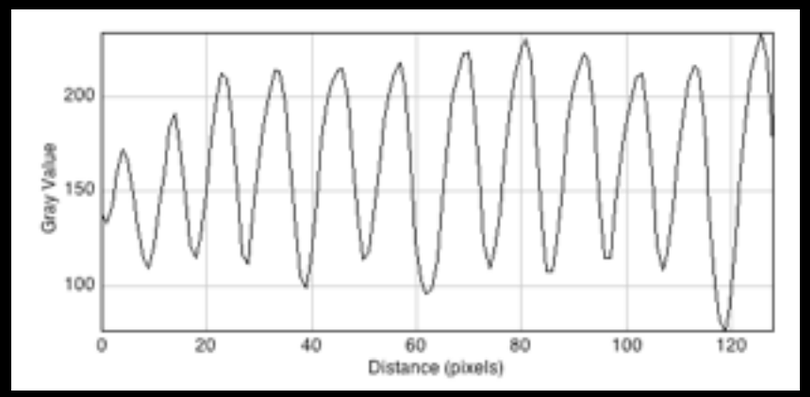
Detail imaged
by microscope

*magnification
*optical resolution

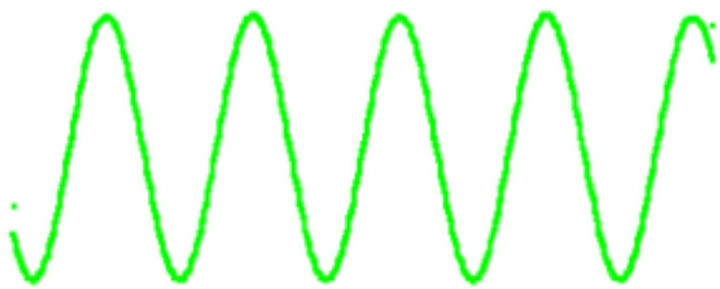




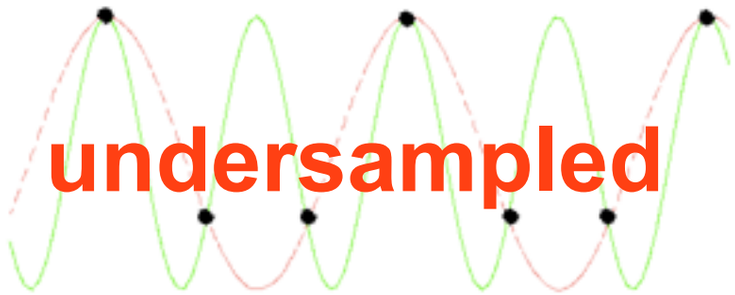
intensity profile ~ a sine wave



sampling a sine wave



Sampling 1.5 times per cycle

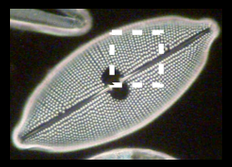


undersampled

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

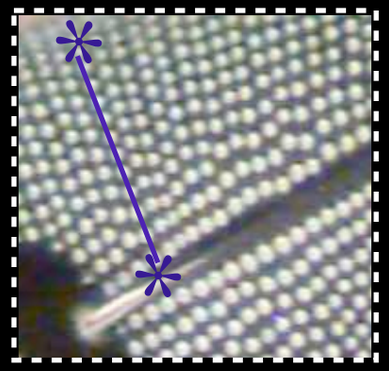
Resolution .....Magnification and Sampling

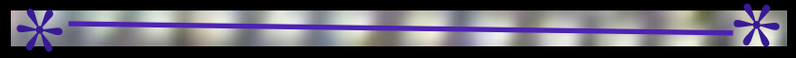
Specimen
Fine Detail



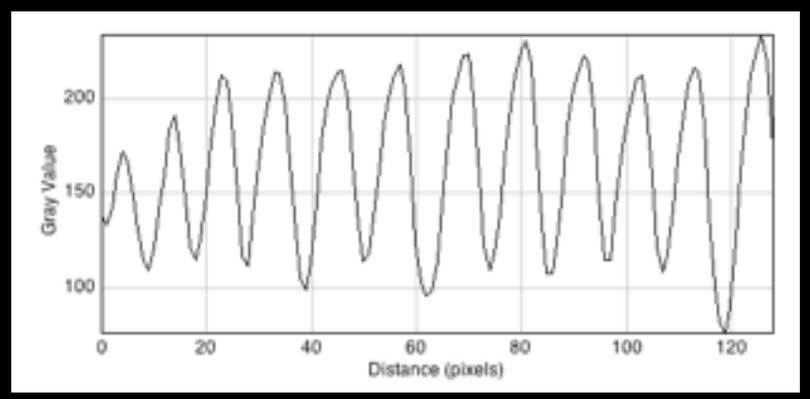
Detail imaged
by microscope

*magnification
*optical resolution

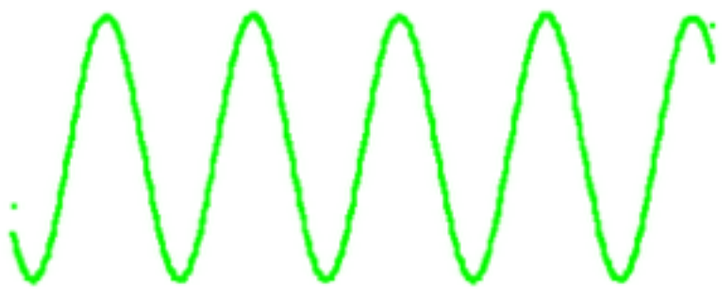




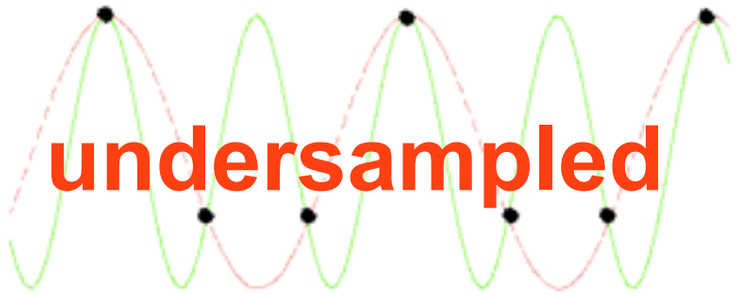
intensity profile ~ a sine wave



sampling a sine wave

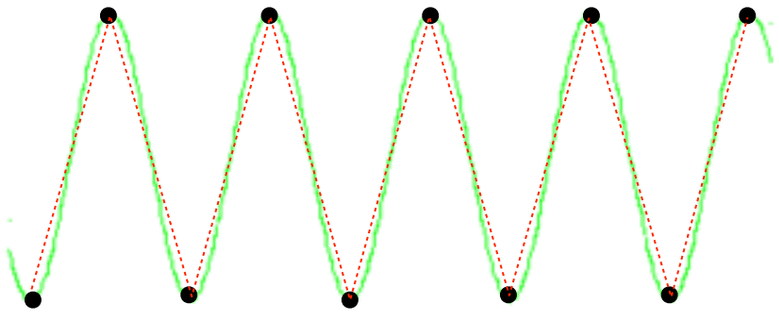


Sampling 1.5 times per cycle



undersampled

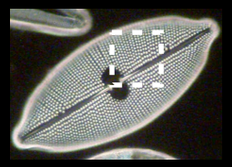
Sampling 2.0 times per cycle



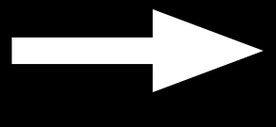
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

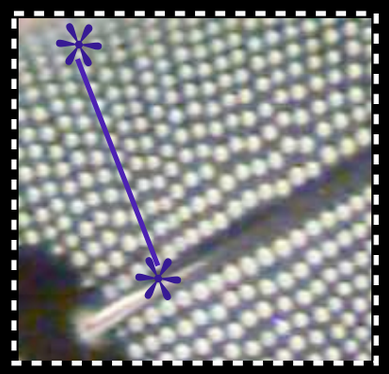
Specimen
Fine Detail

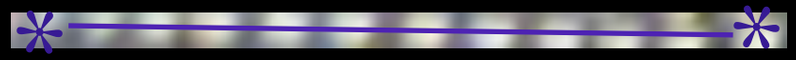


Detail imaged
by microscope

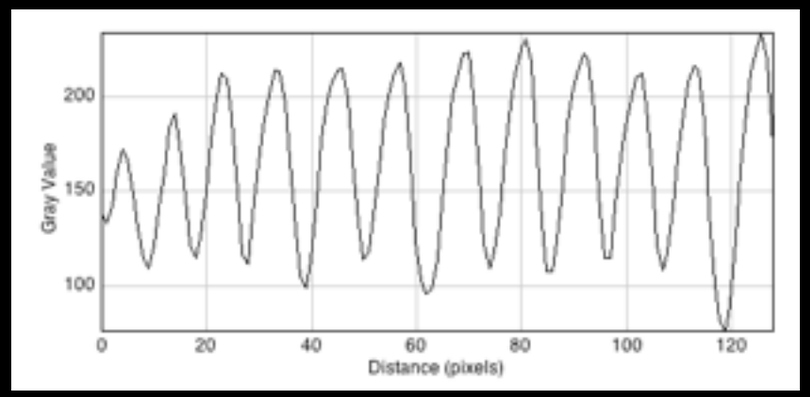


*magnification
*optical resolution

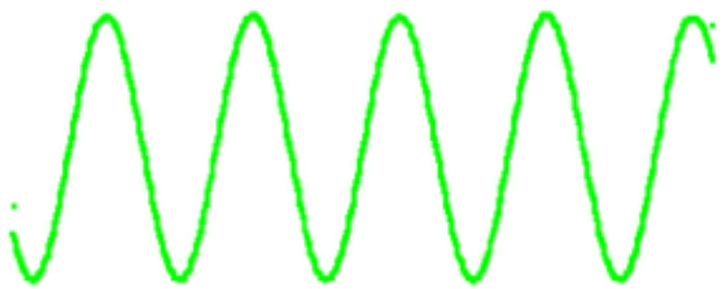




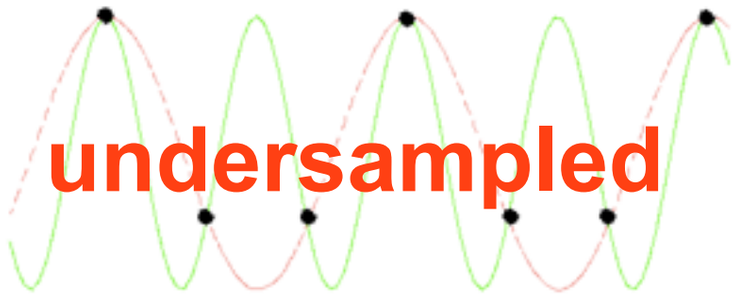
intensity profile ~ a sine wave



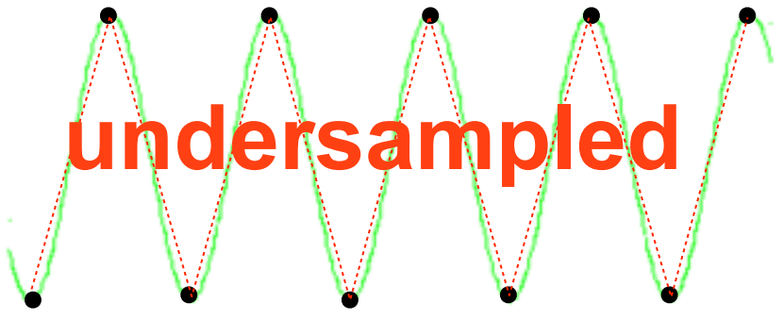
sampling a sine wave



Sampling 1.5 times per cycle



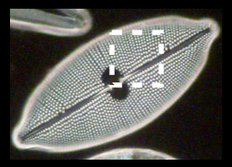
Sampling 2.0 times per cycle



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

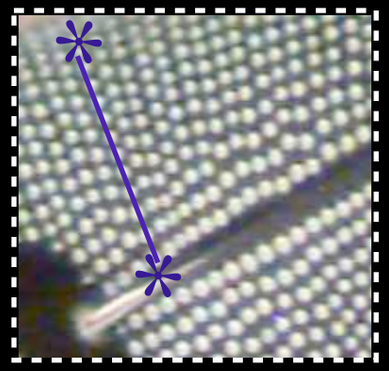
Resolution .....Magnification and Sampling

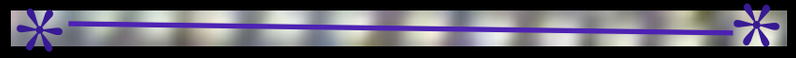
Specimen
Fine Detail



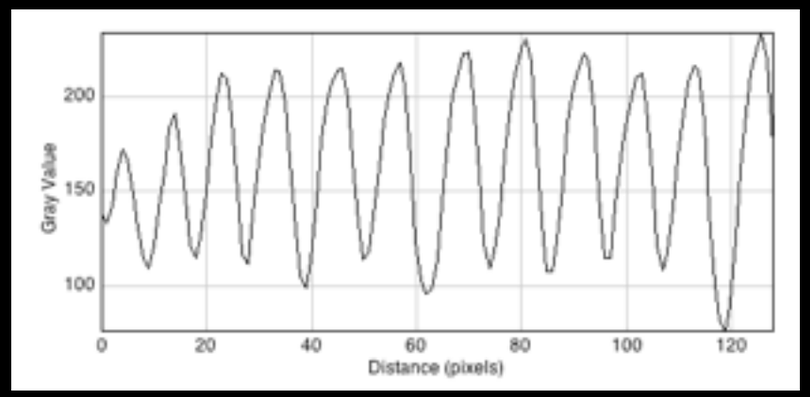
Detail imaged
by microscope

*magnification
*optical resolution

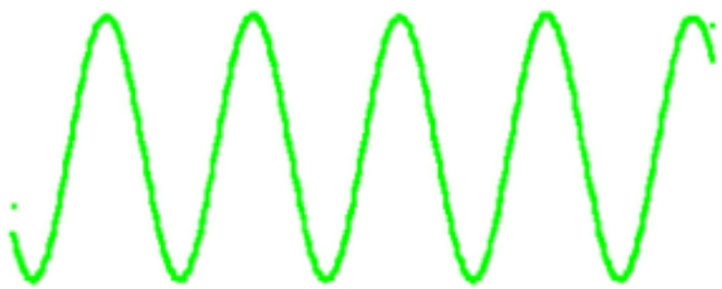




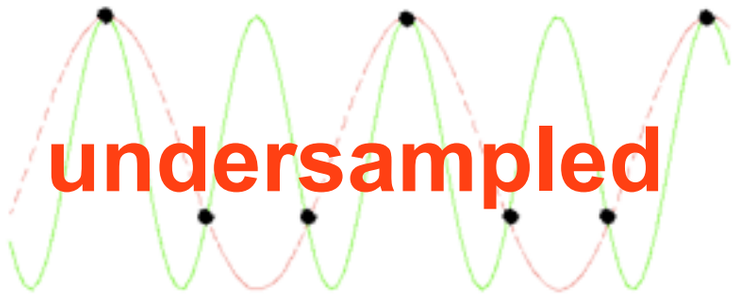
intensity profile ~ a sine wave



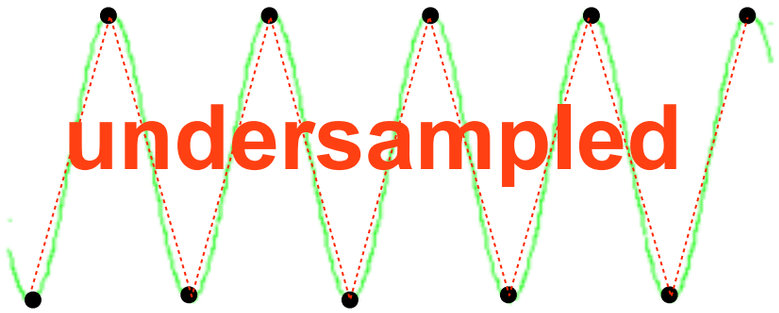
sampling a sine wave



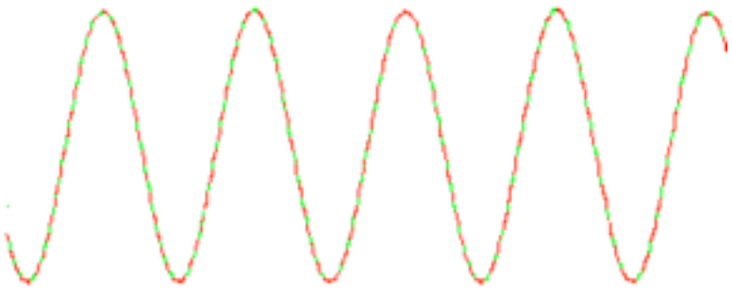
Sampling 1.5 times per cycle



Sampling 2.0 times per cycle



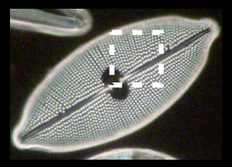
Sampling many times per cycle



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

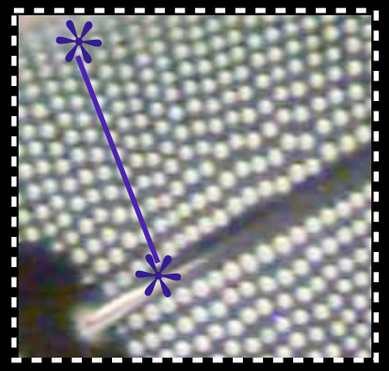
Resolution .....Magnification and Sampling

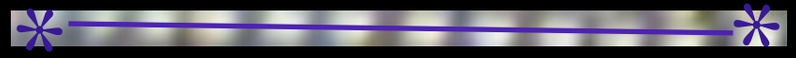
Specimen
Fine Detail



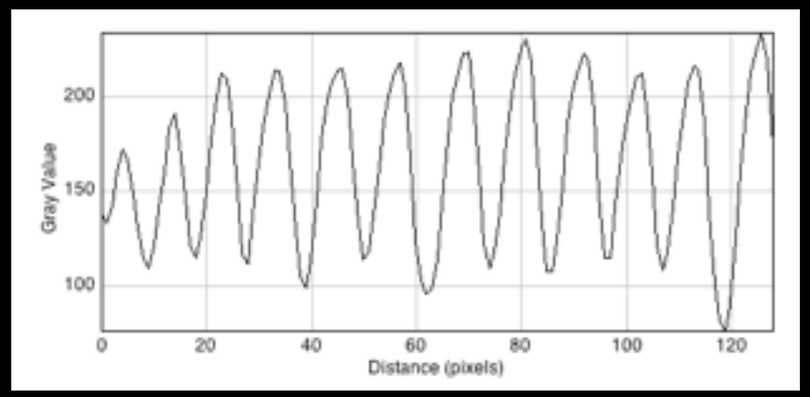
Detail imaged
by microscope

*magnification
*optical resolution

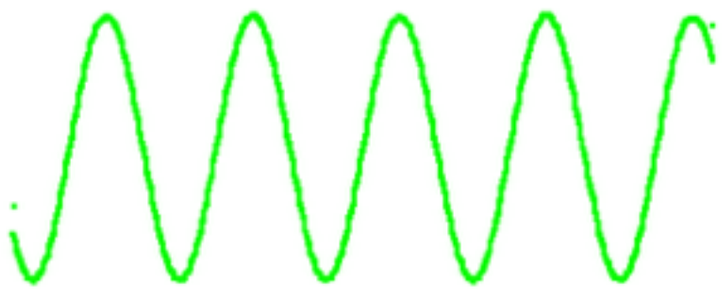




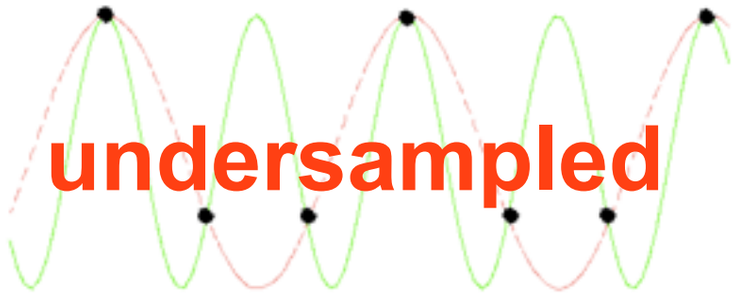
intensity profile ~ a sine wave



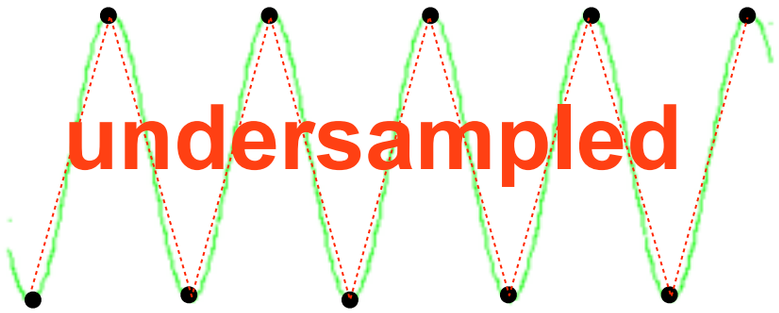
sampling a sine wave



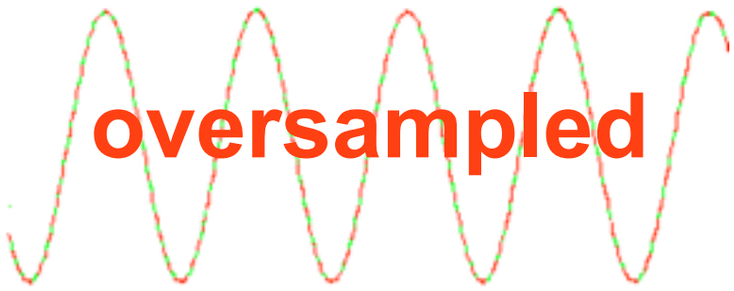
Sampling 1.5 times per cycle



Sampling 2.0 times per cycle



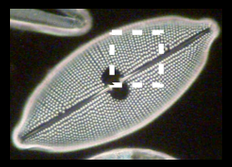
Sampling many times per cycle



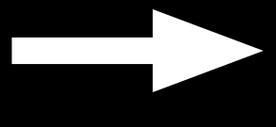
Diatom image: http://www.micromagnus.net/index_micro.html

Resolution .....Magnification and Sampling

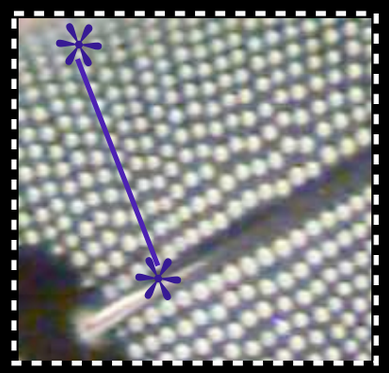
Specimen
Fine Detail

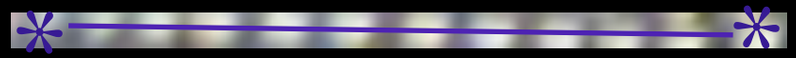


Detail imaged
by microscope

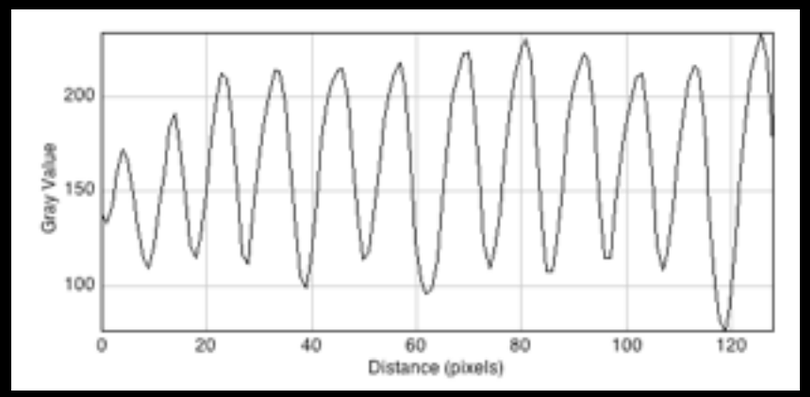


*magnification
*optical resolution





intensity profile ~ a sine wave

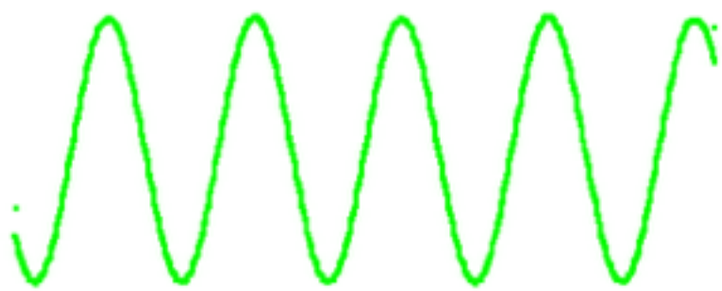


Optimum = 2.3 times per cycle

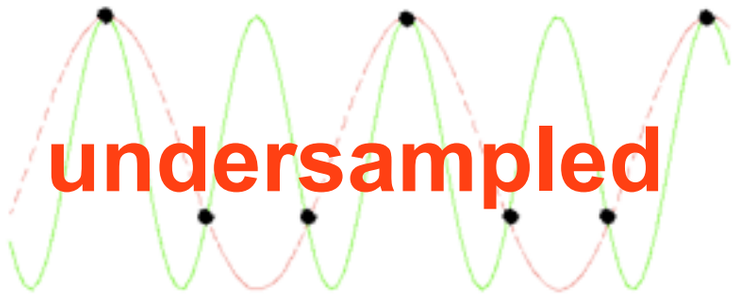
= Nyquist sampling

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

sampling a sine wave

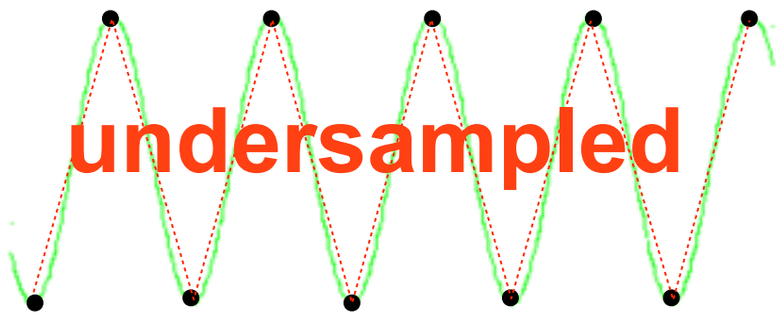


Sampling 1.5 times per cycle



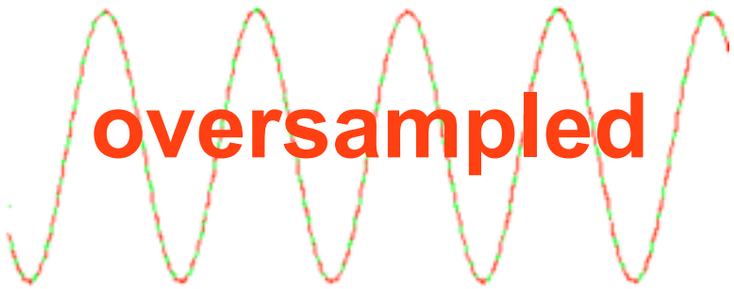
undersampled

Sampling 2.0 times per cycle



undersampled

Sampling many times per cycle



oversampled

Resolution ……..Optimum Magnification and Sampling

Resolution ……..Optimum Magnification and Sampling

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

Resolution ……..Optimum Magnification and Sampling

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,.....

Resolution ……..Optimum Magnification and Sampling

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)

Resolution .....Optimum Magnification and Sampling

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

Resolution ……..Optimum Magnification and Sampling

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

…….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)

Noise / Signal to Noise (S/N)

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

Noise / Signal to Noise (S/N)

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

Brigitte Bardot, Jours de France Magazine Cover

Signal to Noise - definitions:

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

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

Signal to Noise - definitions:

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

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

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

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)

$$\text{Poisson distributed variation} \quad \text{S:N ratio} = \frac{n}{\sqrt{n}}$$

- 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

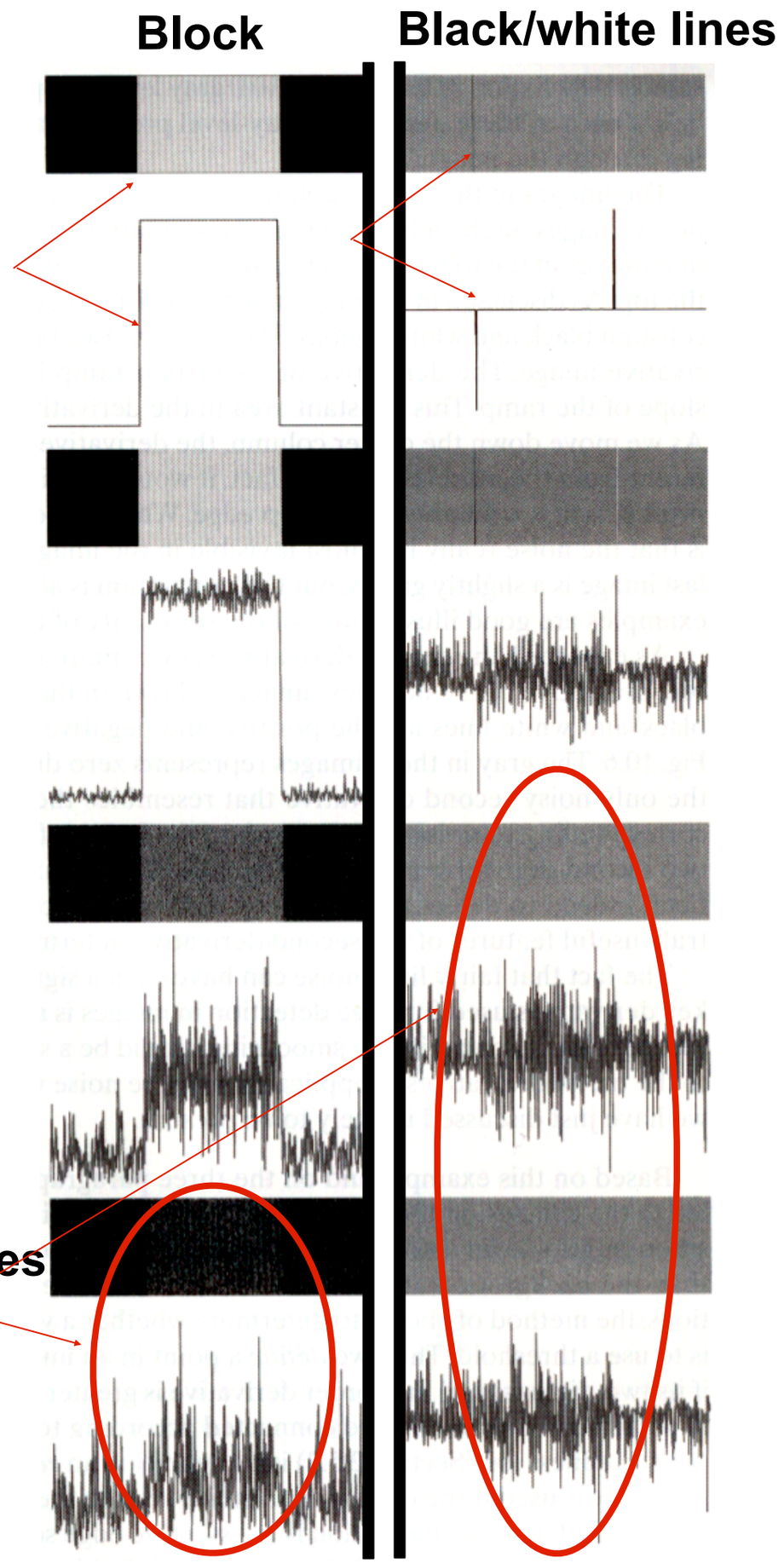
Resolution, contrast, noise

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.

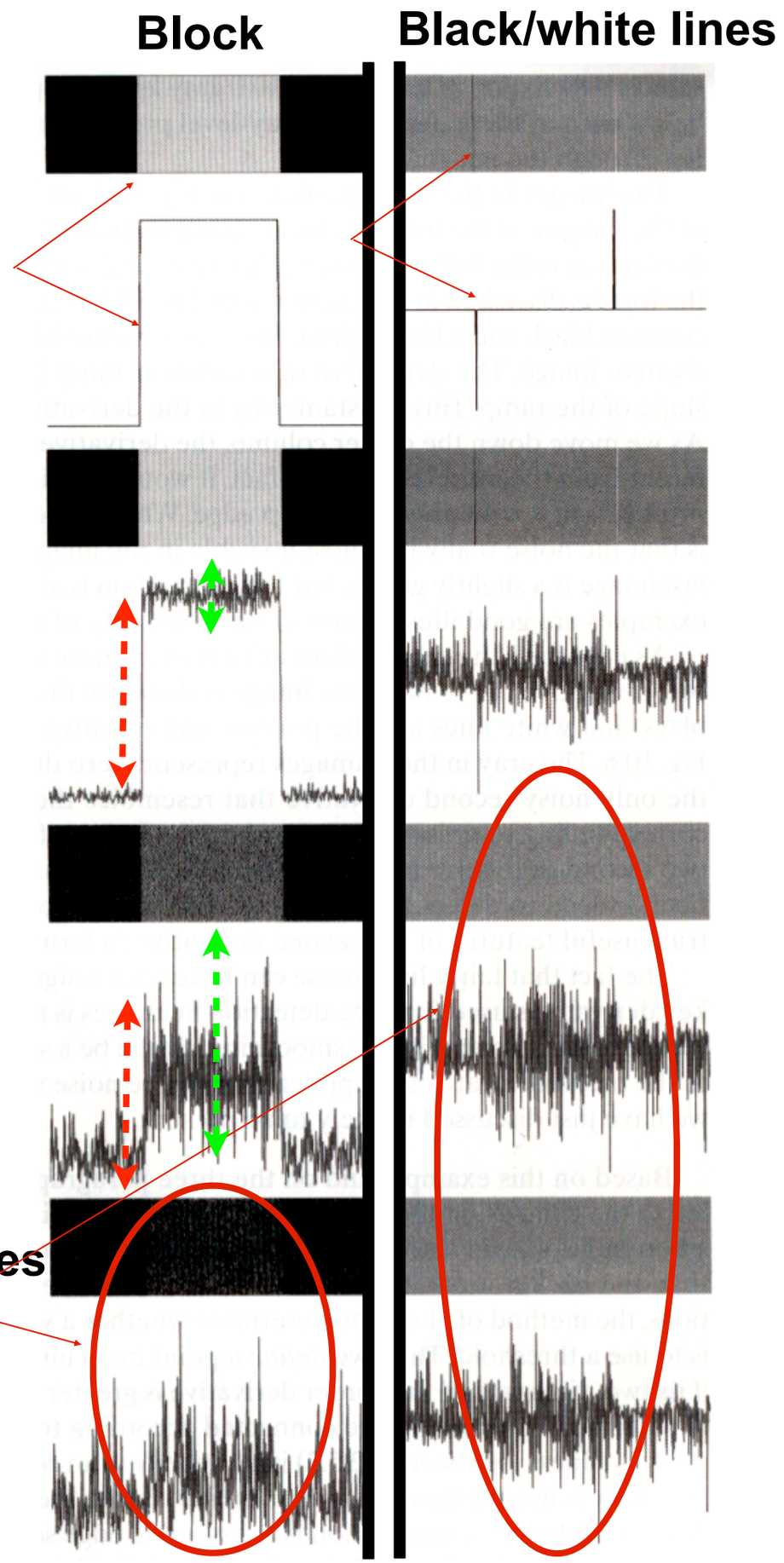
Resolution, contrast, noise

Boundaries and lines easily resolved in the absence of noise

Increasing levels of Gaussian noise

Decreasing S/N

Boundaries and lines no longer resolved



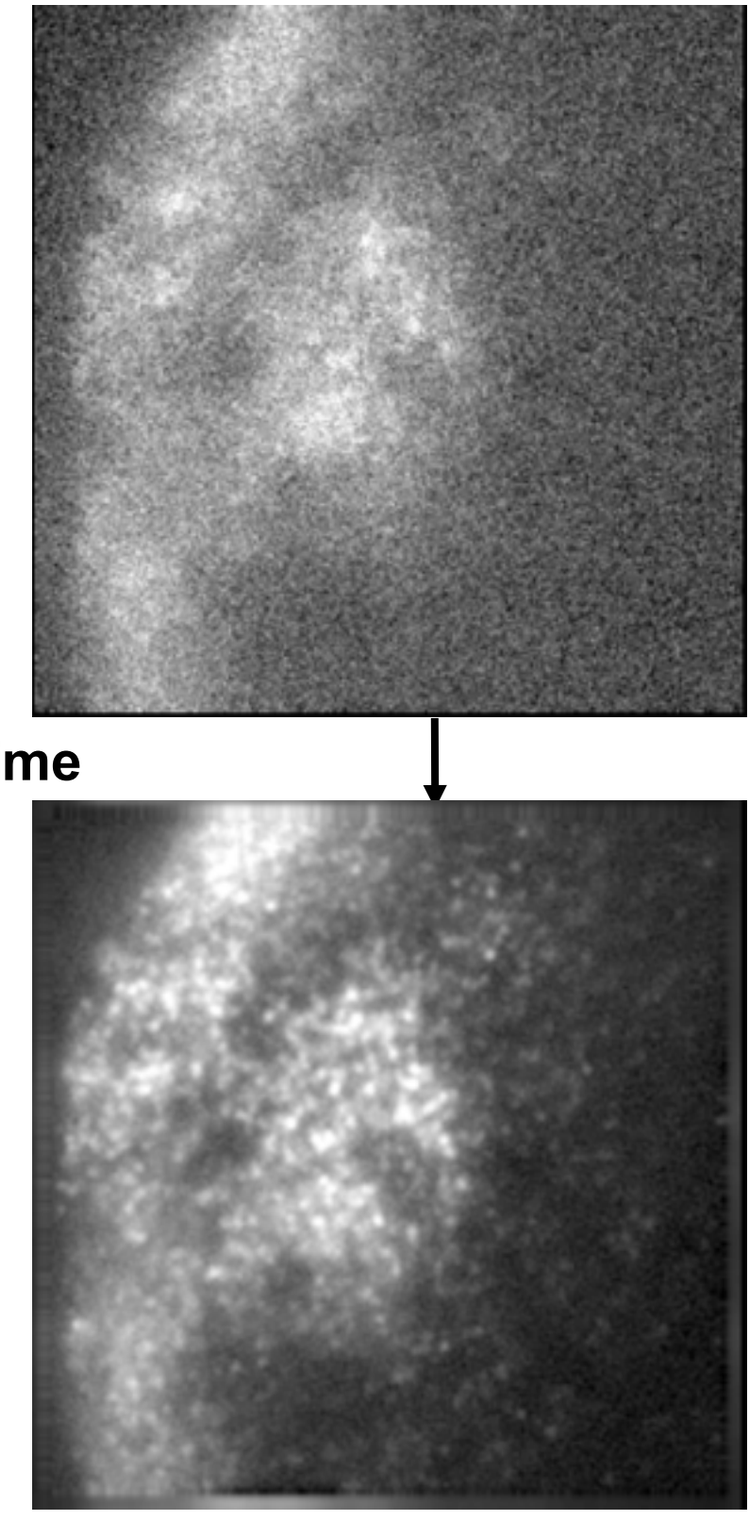
The **difference between signal and background** must be at least 3X the **noise** to be detectable

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

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

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

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

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

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?

Which technique do I use?

Bright field / fluorescence (contrast generation)

Which technique do I use?

Bright field / fluorescence (contrast generation)

Live cell imaging
Fixed material imaging

(dynamics vs detail)

Which technique do I use?

Bright field / fluorescence (contrast generation)

Live cell imaging
Fixed material imaging

(dynamics vs detail)

Confocal techniques (scanning, optical sectioning)

Wide field techniques (Speed, sensitivity)

Lectures 6 & 7

Which technique do I use?

Bright field / fluorescence (contrast generation)

Live cell imaging
Fixed material imaging

(dynamics vs detail)

Lectures 4 & 6

Confocal techniques (scanning, optical sectioning)

Wide field techniques (Speed, sensitivity)

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