Manawatu Microscopy & Imaging Centre

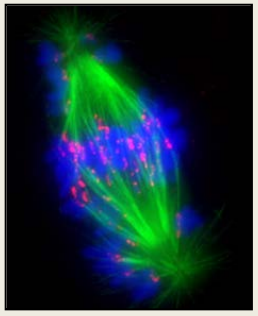
MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Light Microscopy and Digital Imaging Workshop

Matthew S. Savoian

M.S.Savoian@massey.ac.nz

Nov 14, 2014



Purpose:

Provide a primer on different light microscopy imaging and analysis techniques -and their limitations- using MMIC-based equipment as practical examples

Programme

Morning Session 10:00-12:00

Introduction to Light Microscopy

- Basic Concepts: Magnification, Resolution, Depth of Field
- Different Transmitted Light Modalities

Epi-Fluorescence Microscopy

- Mechanism of Fluorescence
- Widefield Epi-Fluorescence Microscope Components
- Fluorescent Probes/Stains (Fluorescent Proteins as Biosensors)
- Fundamentals of Digital Imaging
- Scanning Confocal Microscopy

Afternoon Session 13:00-15:00

ImageJ as a Tool for Digital Image Analysis

- ImageJ Basics
- Histograms, LUTs and Displays
- 2D and 3D Spatial Measurements
- Use of Linescans
- Quantitation of Fluorescence Intensity
- Quantifying Movement

Analysis of attendee data- as time permits

Principles of Microscopy

Microscopy allows us to view processes that would not be visible to the naked eye

- Object too small - we cannot see objects smaller than about 0.1mm or the thickness of a human hair)
- Object lacks contrast (Stains/Phase-Contrast/DIC)
- Process too slow (time-lapse) or not visible in nature (molecular dynamics or interactions-FRAP, FRET)

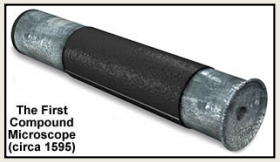
Every microscope has limits

Poor sample preparation is a recipe for disappointment and poor imaging

Milestones in Microscopy

100- Romans use crystals as “magnifying” and “burning” lenses

1595-Jensen makes first compound microscope



1665- Hooke publishes his “*Micrographia*” and coins the term “cell”

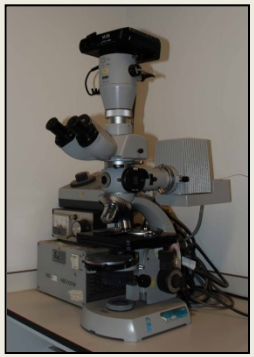
1676- Van Leeuwenhoek observes “animalcules” (bacteria)

1800s- Microscopes improved; theoretical limits of light microscopy determined

1931- Knoll and Ruska produce first Transmission Electron Microscope (TEM)

1945- Porter et al., use TEM to look at tissue culture cells

1965- First commercial Scanning Electron Microscope



1967- Modern Epi-fluorescence microscope invented

1980s- Macromolecular Reconstructions using TEM and tomography

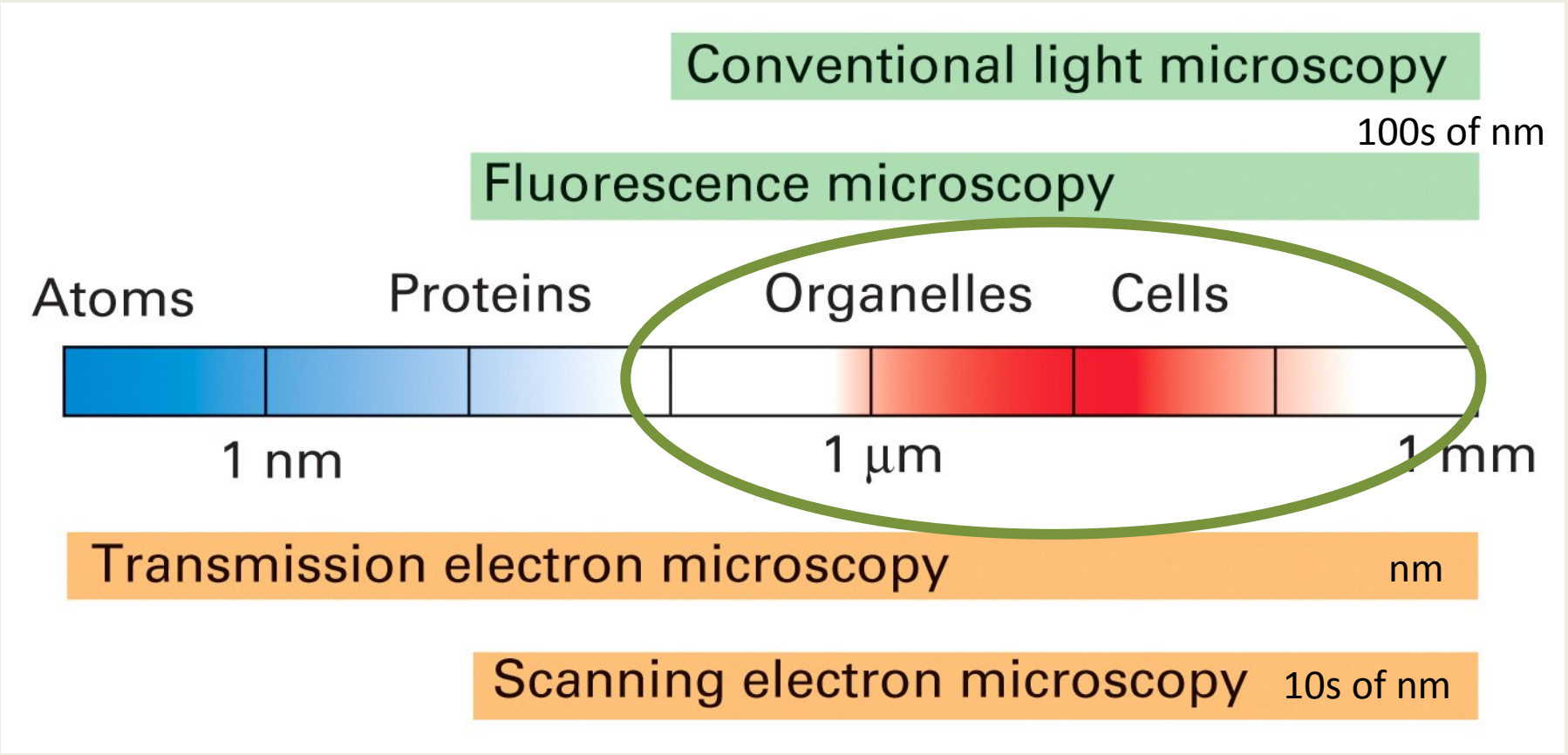
1987- Confocal microscope applied to cell biology

1994- Chalfie et al., use Green fluorescent protein (GFP) as an in vivo marker

2000s- super-resolution invented

?

Resolution of Different Microscopes



Common Light Microscope Imaging Methods

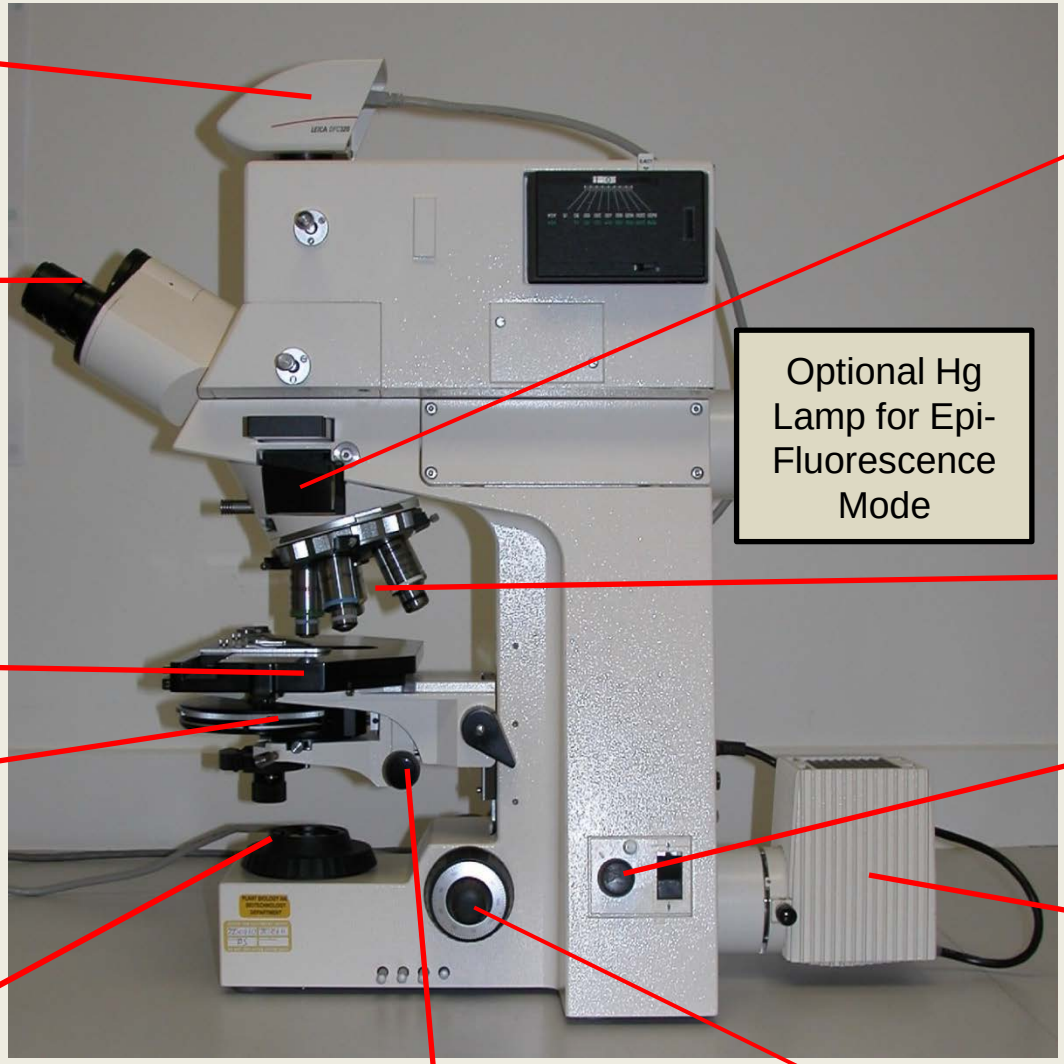
Transmitted Light Modalities (absorption/phase shift)

- Bright Field
- Phase-Contrast
- Differential Interference Contrast (DIC)

Epi-Fluorescence Light Modalities (emission)

- Widefield
- Scanning Confocal

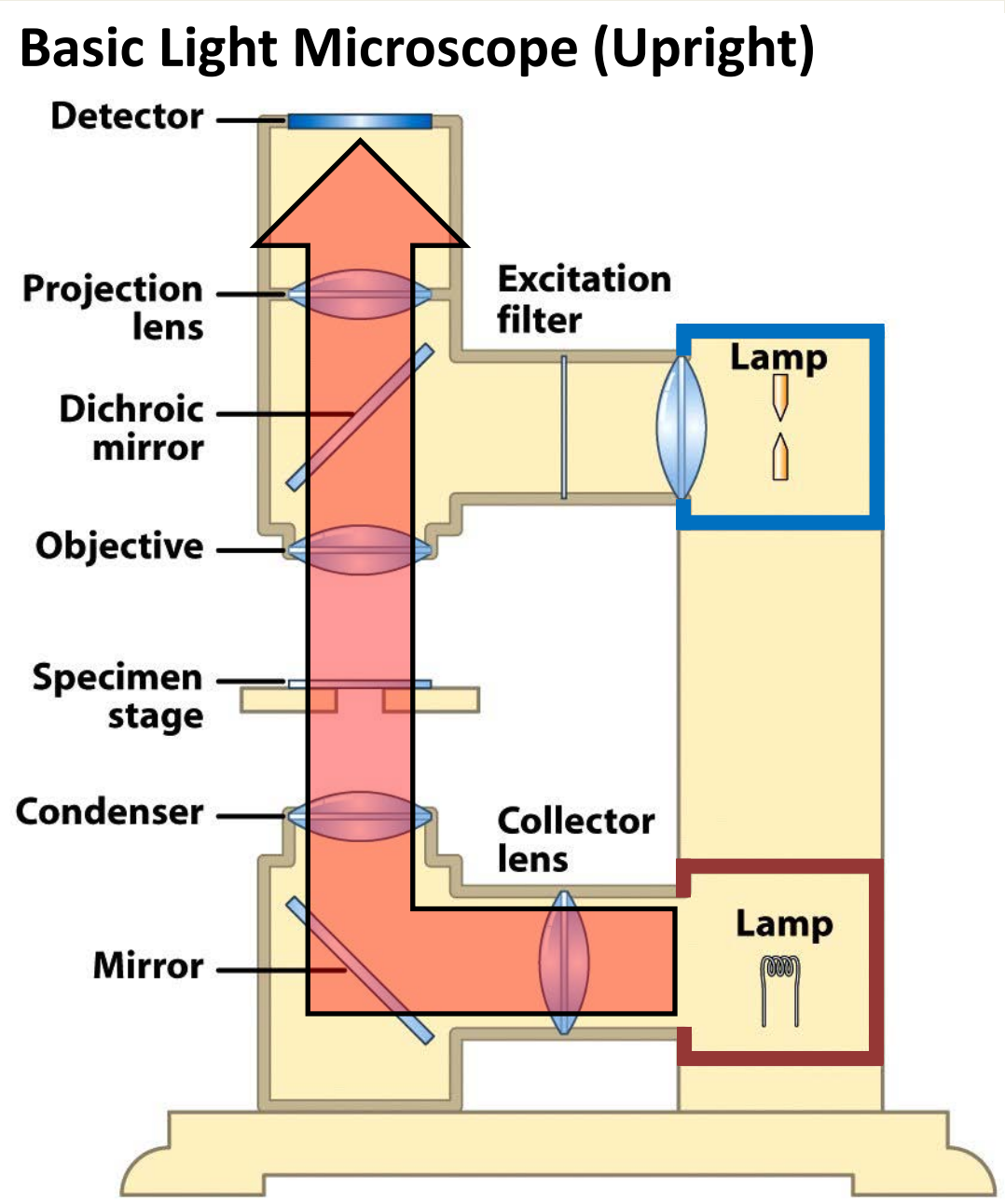
Upright Light Microscope Anatomy



Configured For:

Transmitted Light

- Brightfield
- Phase-Contrast
- Differential Interference Contrast



Modified from Lodish 6th Fig 9.10a

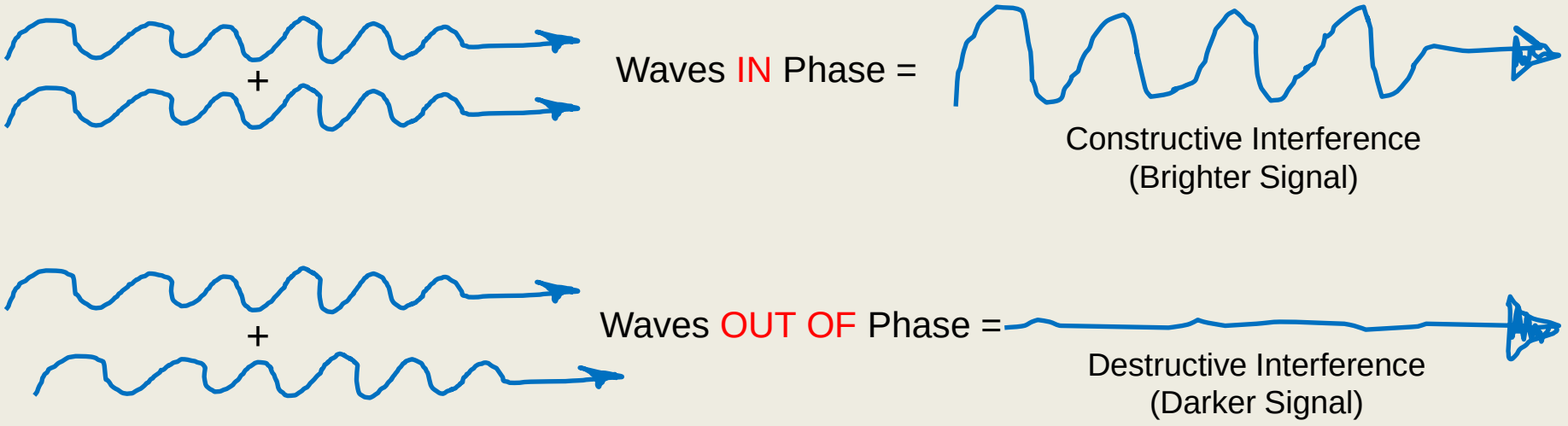
IMAGE FORMATION: Attributes of Microscopes

- Magnification
- Resolution

Light is a wave and a particle

Refraction: Bending of light as wave changes speed when travelling through different materials (e.g., a straw looking bent in a glass of water)

Diffraction: Bending of light as wave encounters an object or edge



These processes are the core of microscope image formation

Magnification

How big something appears

- Compound microscope used in conventional light microscopy utilises several lenses
- Objective lens (closest to specimen) – 2.5x-100x
- Projection lens (eyepiece/other) – 10x, etc.,
- Total magnification is the product of the magnification of the individual lenses
- Apparent Image Size can be misleading- size must be determined using calibration or scale bars

But magnification can be “empty”

Resolution

What is resolution?

Smallest distance apart at which two points on a specimen can still be seen separately

This is directly related to the light collecting capability of the optical system

---The Objective Lens---

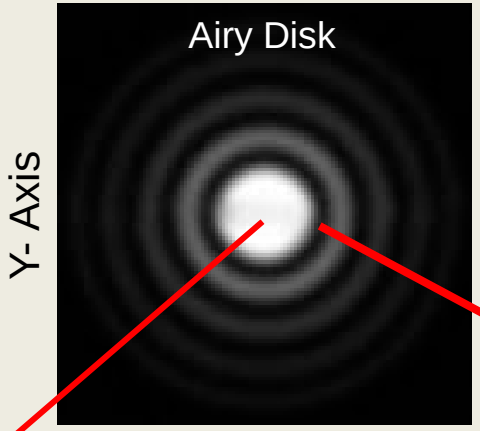
The Diffraction Pattern Defines the Image Characteristics

The Airy Disk (2D diffraction pattern)

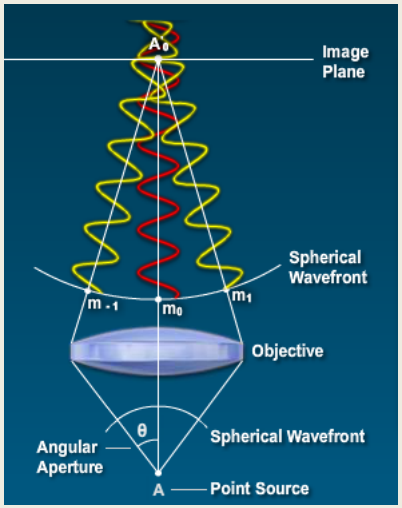
Using a self-luminous object as an example

Glowing Object (50nm)

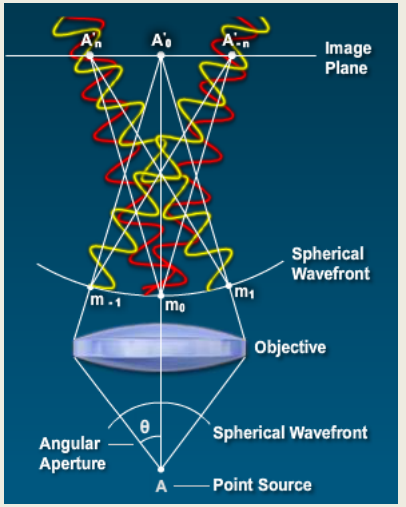
Diffraction Through Lens



zero order



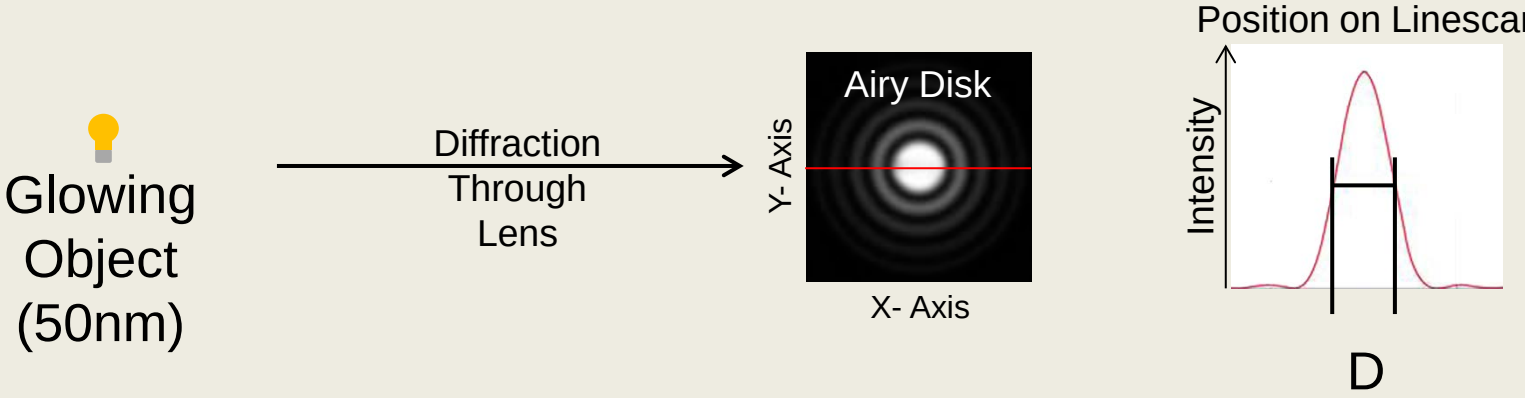
-1 order



Modified from http://zeiss-campus.magnet.fsu.edu

The Airy Disk (2D diffraction pattern) Dictates Object *Apparent Lateral Size*

Using a self-luminous object as an example



$D_{x,y}=0.61\lambda/N.A.$

λ=wavelength of emitted light

N.A.=Numerical Aperture of Objective Lens (light collecting power of lens)

For Example:

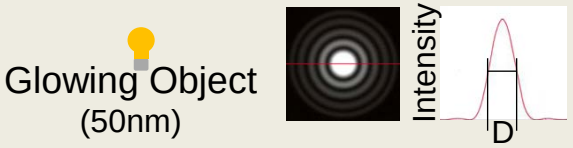
A 50nm bead imaged with a 100x oil Immersion Lens (NA 1.4) emitting 520nm (green) light

$D_{x,y}=0.61(520nm)/1.4$

$D_{x,y}=226nm$

The *minimum apparent* lateral size of *an* object viewed at 520nm light is 226nm

The Airy Disk Dictates Resolvable *Lateral Separation Distance*



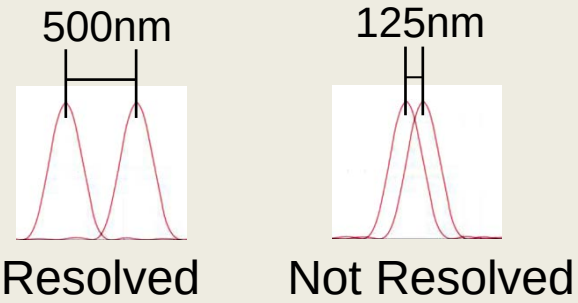
$D_{x,y}$= Lateral Resolution
$D_{x,y}=0.61\lambda/N.A.$

λ=wavelength of emitted light
N.A.=Numerical Aperture (light collecting power of lens)

For Example:
A 50nm bead imaged with a 100x oil Immersion Lens (N.A. 1.4) with 520nm (green) light

$D_{x,y}=0.61(520nm)/1.4$
$D_{x,y}=226nm$

Two objects spaced closer than 226nm appear as one

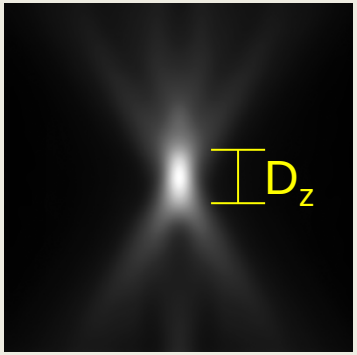


- Shorter wavelengths give higher resolution
- Higher N.A. gives higher resolution

Magnification has no impact on lateral resolution

The Point Spread Function is the 3D Diffraction pattern

Object
(50nm)



Axial Resolution $D_z = \lambda\eta/(N.A.)^2$

Emitted light
(520nm)

Lens Numerical
Aperture (1.4)

Refractive index of
mounting media (1.515)

$D_z = 520nm(1.515)/(1.4)^2$

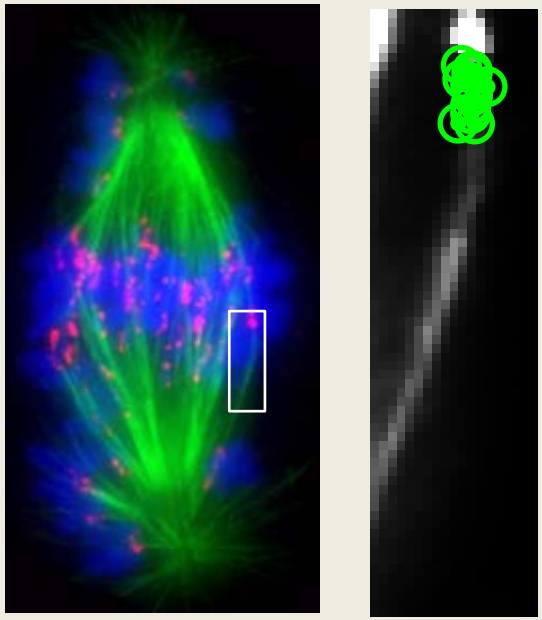
$D_z = 401nm$

The *minimum apparent axial size* and *separation distance* of an object emitting 520nm light is ~400nm

Axial (Z) resolution is ~ ½ of lateral (XY) resolution

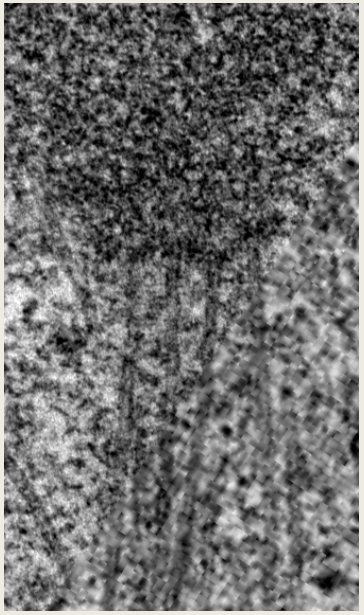
Magnification has no impact on axial resolution

Images are comprised of Airy Disks/PSFs



How do we exceed the diffraction limit?

TEM Image



http://pcwww.liv.ac.uk/~emunit/images/kinetochores.jpg

Alternative technologies

- Transmission Electron Microscopy (TEM)
  Resolution: ~5nm (Atomic!)

- "Super-resolution" Light Microscopy
  Resolution: ~70-150nm (depending on method)

Deciphering the Objective Lens

Magnification

Microscope Tube Focal Length (∞ or 160mm)

Optimal coverslip thickness

Corrected Aberrations
- U- Can transmit UV
- Plan- Entire field in focus
- Sapo/Apo- All colours focus in same plane

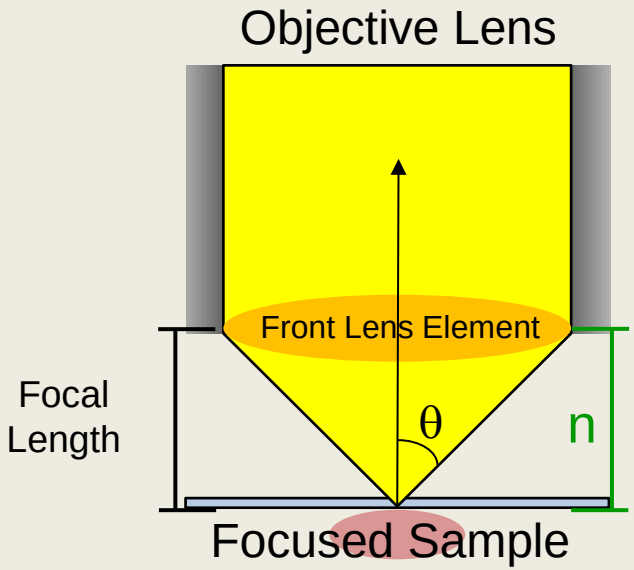
Numerical Aperture (N.A.)

Immersion Oil Required
- Gly for glycerine
- Water for water

FN- Field Number (corresponds to diameter of ocular lens for best field of view)

Additional Details (e.g.)
- DIC/NIC-Differential Interference Contrast
- PH- Phase-Contrast

Objective Lens N.A. Determination



$N.A. = n \sin(\theta)$

n= Refractive Index between lens and sample
air=1.0
water=1.33
glass=1.518
oil=1.515

θ= angle between optical axis and widest ray captured by lens

Lower N.A. lenses collect less light; therefore images are less bright and *lower resolution*

Highest possible N.A. in air is ~0.95 0.95=1.0 (sin72)

Higher magnification lenses have a shorter focal length, greater θ and *commonly require oil to capture the light and achieve higher N.A.*

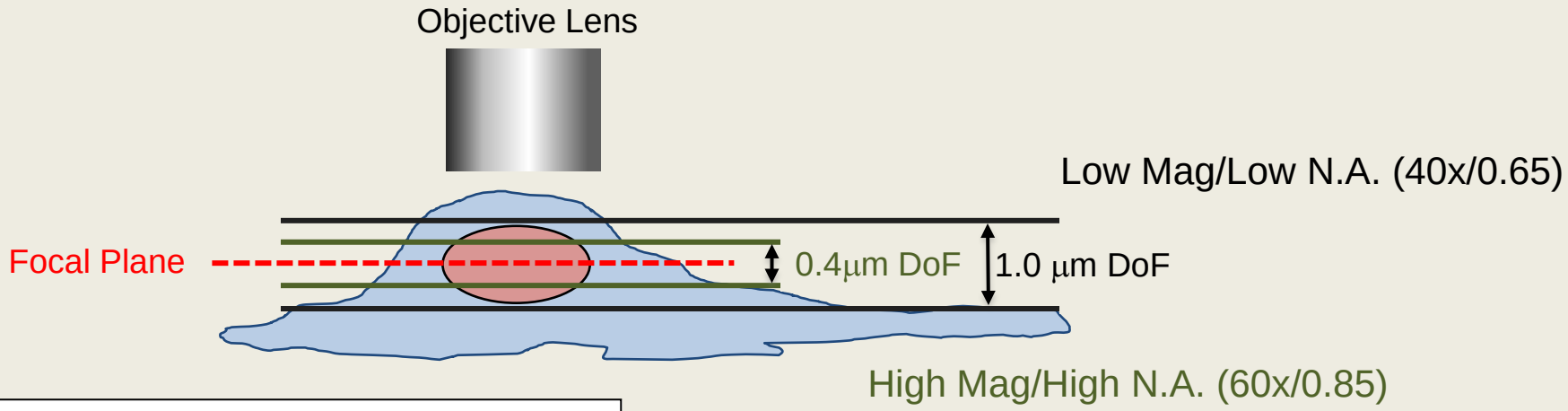
!!!oil should never contact a dry lens!!!

Addition of oil to a dry lens distorts light collecting pathway

Depth of Field

Amount of a specimen in focus at the same time

Depth of field (DoF) decreases with increased magnification and N.A.



Magnification	Numerical Aperture	Depth of Field (μm)
4x	0.10	15.5
10x	0.25	8.5
20x	0.40	5.8
40x	0.65	1.0
60x	0.85	0.40
100x	0.95	0.19

Table from www.olympusmicro.com/primer/anatomy/objectives.html

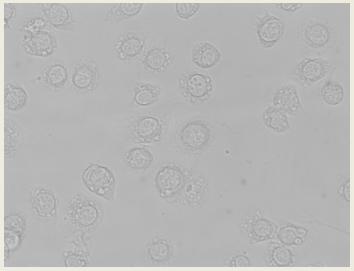
For the thinnest optical section use a high magnification and high N.A. lens

Contrast

or

Distinguishing detail relative to the background

Many samples have poor inherent contrast



Bright Field image of Insect Cells

Without contrast, magnification and resolution are irrelevant

In Transmitted Light Microscopy contrast can be generated by:

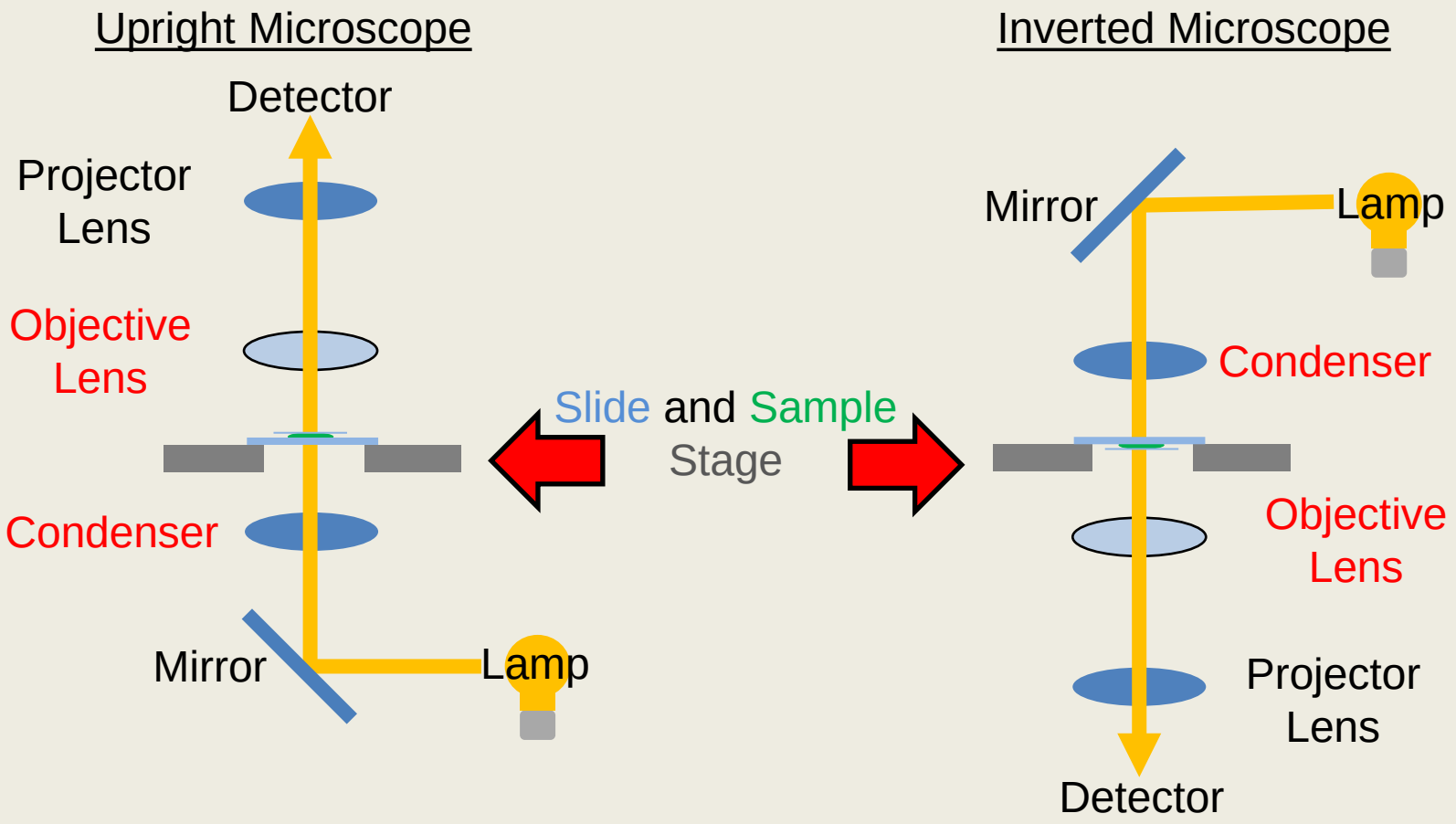
- Altering the light absorption of a sample (e.g., stains)
- Increasing the phase shift of light on a sample (special optics)

Transmitted Light Optical Contrasting Techniques

- Bright Field
- Phase-Contrast
- DIC/NIC (Differential Interference Contrast/Nomarski Interference Contrast)

Transmitted Light Microscopy

Light from tungsten lamp focused on specimen by condenser lens and travels *through* sample



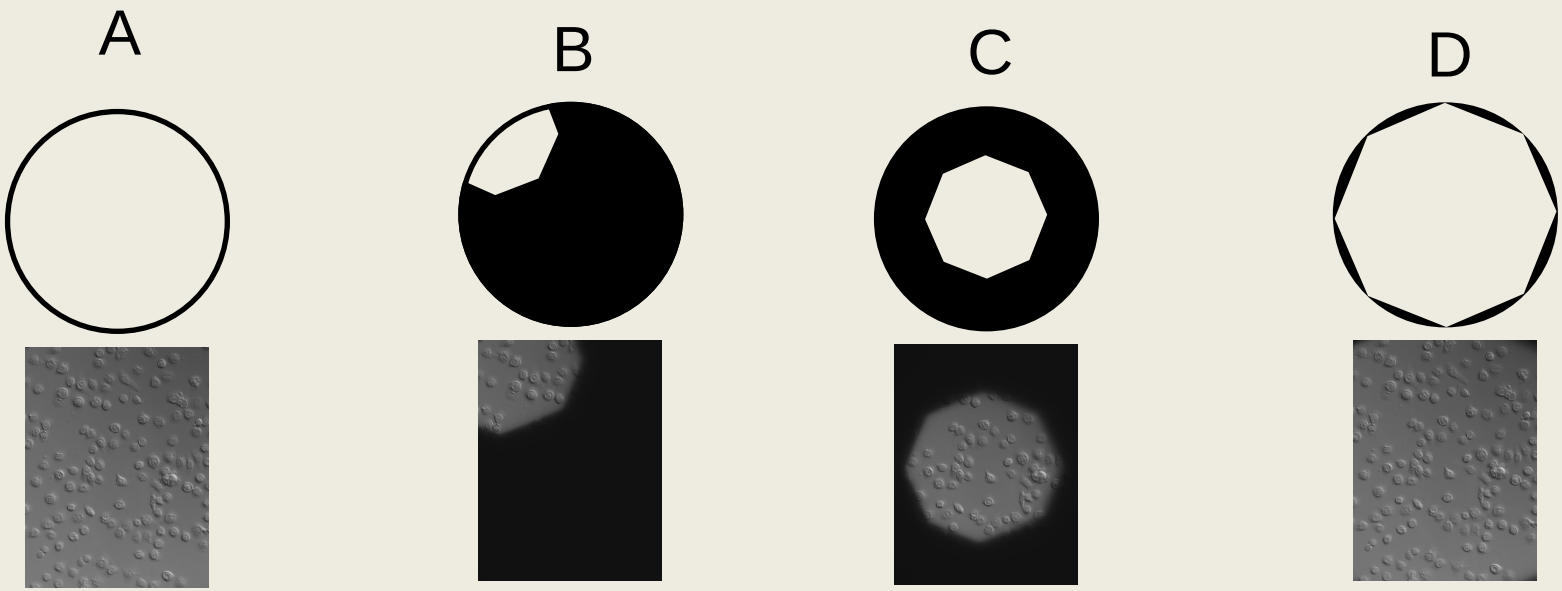
To achieve highest quality images it is essential that the sample is correctly illuminated

Köehler Illumination

- August Köehler, of the Zeiss corporation invented Köehler illumination in 1893
- Samples are uniformly illuminated
- Glare and unwanted stray light minimised
- Maximise resolution and contrast

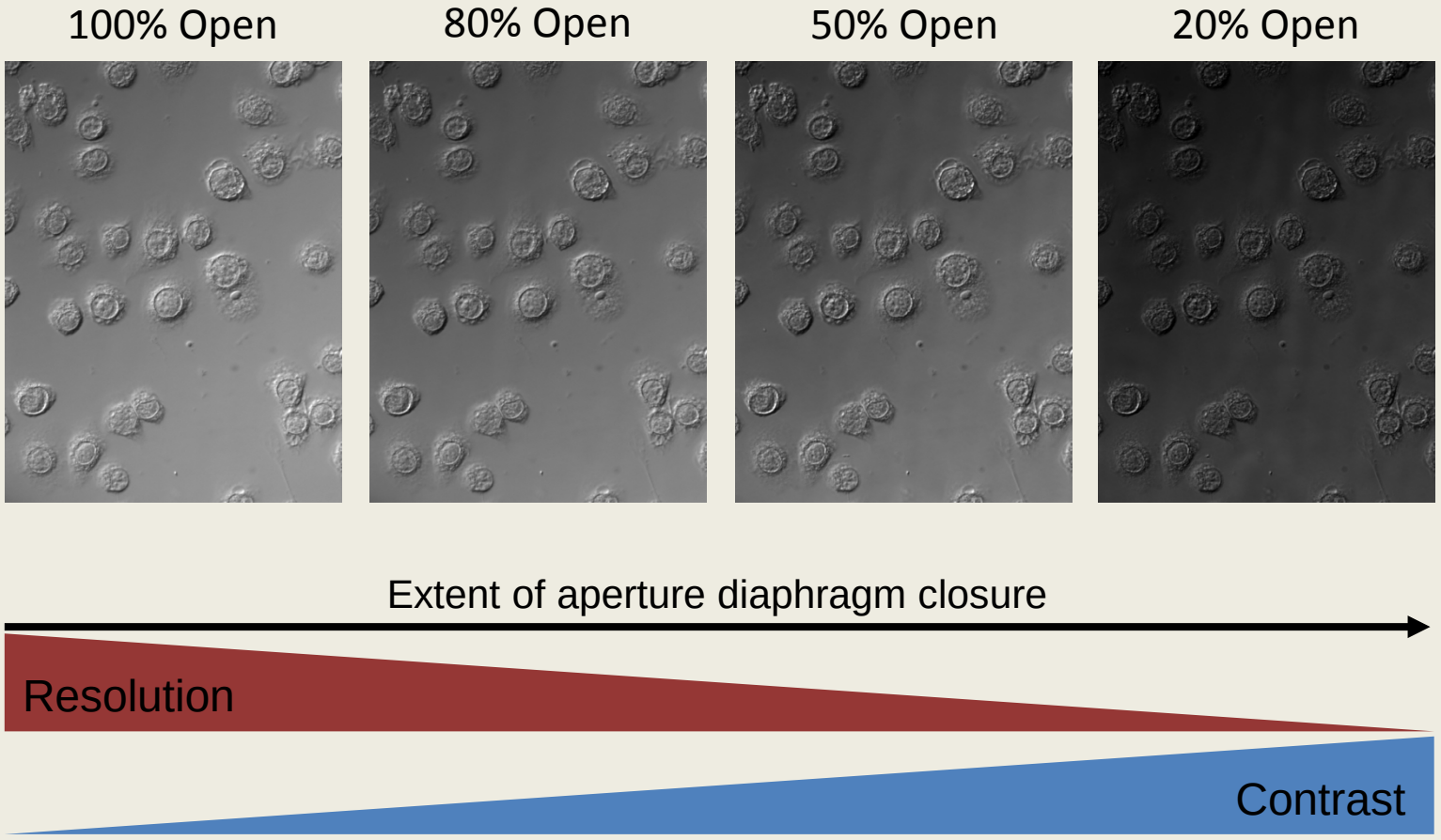
Setting Up Köehler Illumination

- A)Focus on sample with low power objective
- Close condenser field diaphragm
- Raise condenser up to highest position
- B)Lower condenser until diaphragm image (octagon) is in focus
- C)Centre using condenser centering screws
- D)Open field diaphragm until just filling field of view
- Adjust condenser aperture diaphragm



Transmitted Light Resolution $(D)_{x,y}=1.22\lambda$ /N.A.$_{objective}$+N.A.$_{condenser}$

The Condenser Diaphragm Balances System CONTRAST and RESOLUTION



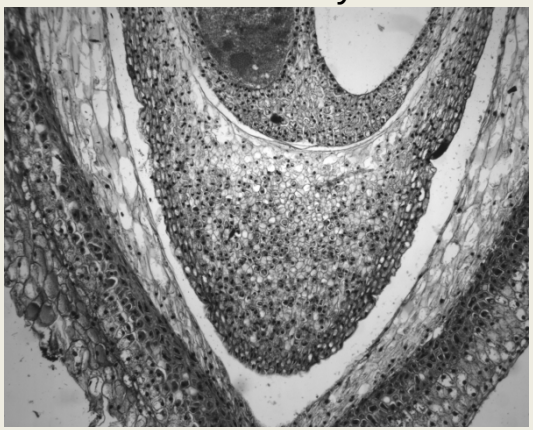
80% open is optimal for most applications

Bright Field Microscopy

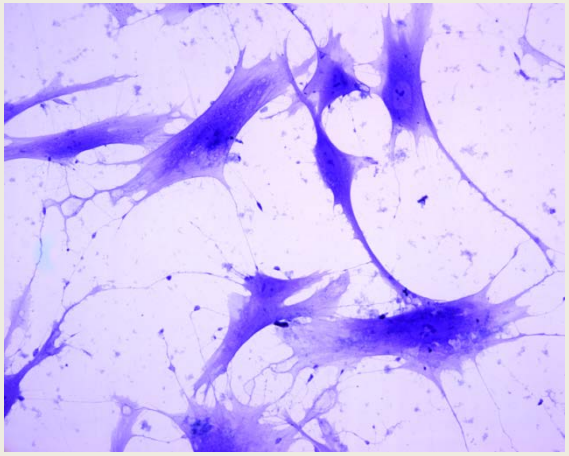
Image contrast produced by *absorption* of light

- Specimens commonly look coloured on white background
- May be due to natural pigments or introduced stains (e.g., histology)

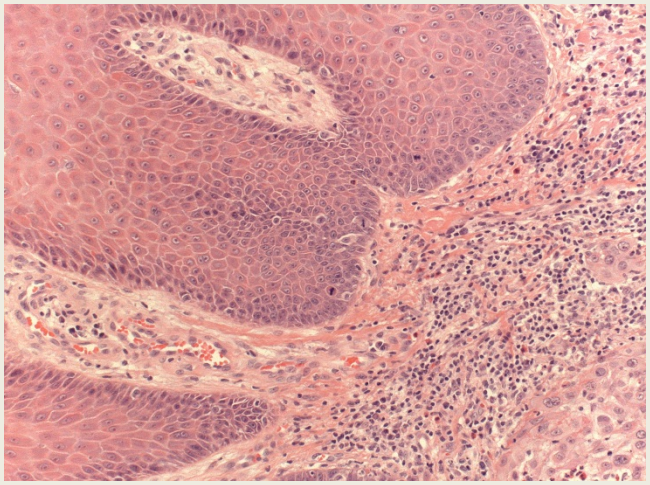
Plant embryo



Vertebrate Tissue Culture Cells



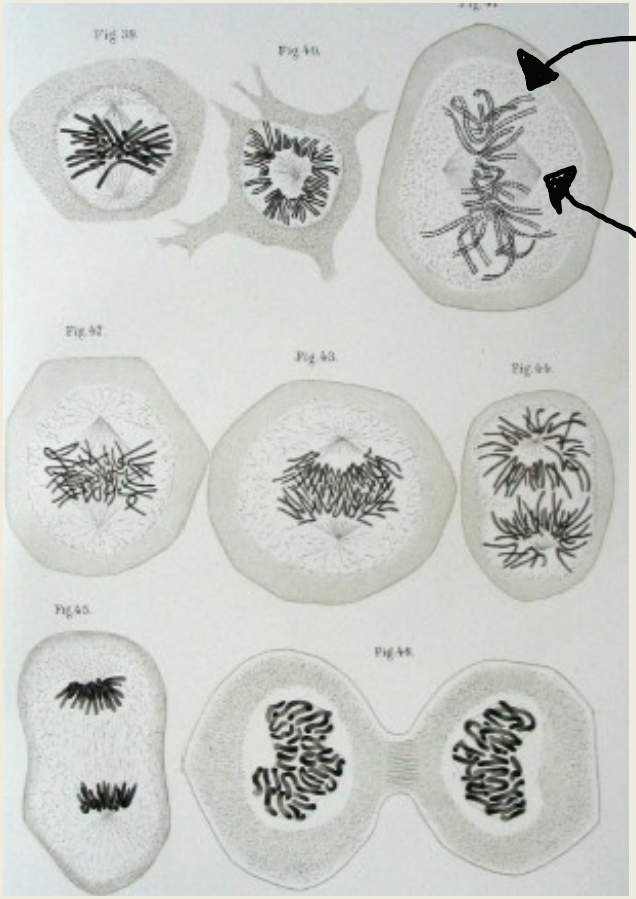
Human Tissue



Walther Flemming's 1882 illustrations of "MITOSIS" (Greek for "thread")

using *non-specific* aniline dyes

Salamander Gill Cells



But stained samples are DEAD!!!

Dynamics? Artefacts?

Phase-Contrast Microscopy

Human eyes detect differential absorption-

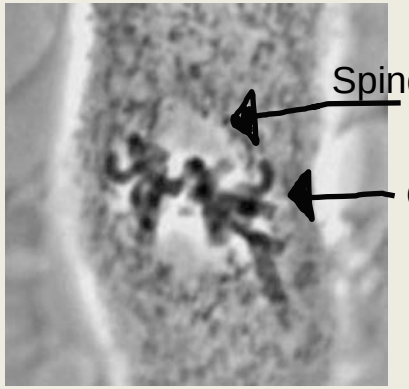
If light is not absorbed by a sample you cannot see it

Phase-Contrast Microscopy:

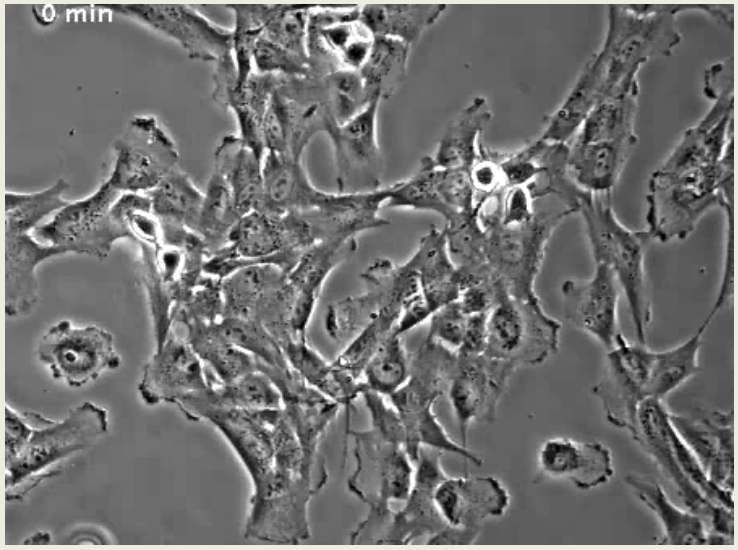
Small changes in the phase of light are converted into visible contrast changes

No staining is required

Vertebrate Mitotic Culture Cell



Vertebrate Culture Cells



Brito et al., 2008 JCB 182:623-629

. . . And that means you can study living samples!

Phase-Contrast Microscopy

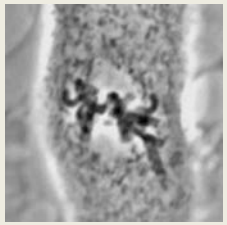
In Phase-Contrast microscopy the optical path of the microscope is modified so that it converts phase changes into an image

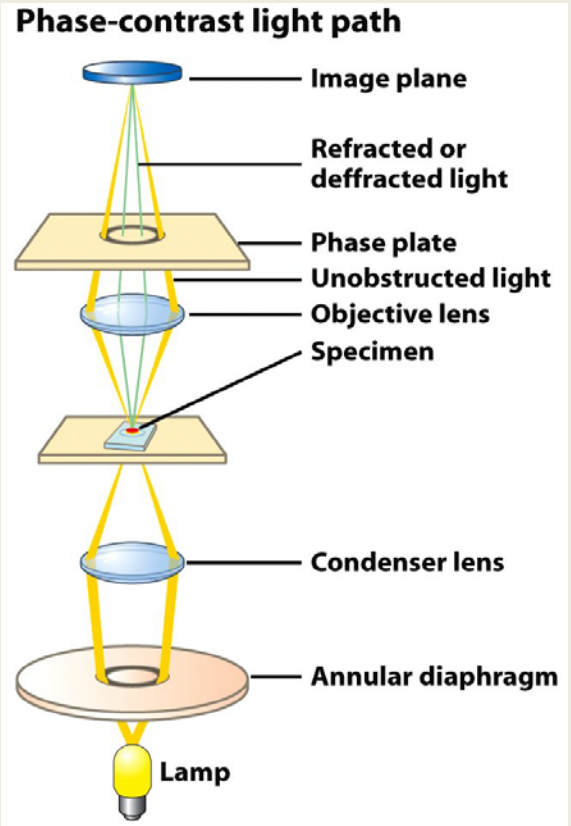
- Light from lamp emerges as a hollow cone

- Light is refracted by the sample
  But *not* the background

- A phase ring at the focal plane of the objective exaggerates phase differences between refracted and un-refracted light

These appear as intensity differences in recombined image





www.olympusmicro.com/primer/techniques/phasecontrast/phase.html

Differential Interference Contrast (DIC) Microscopy

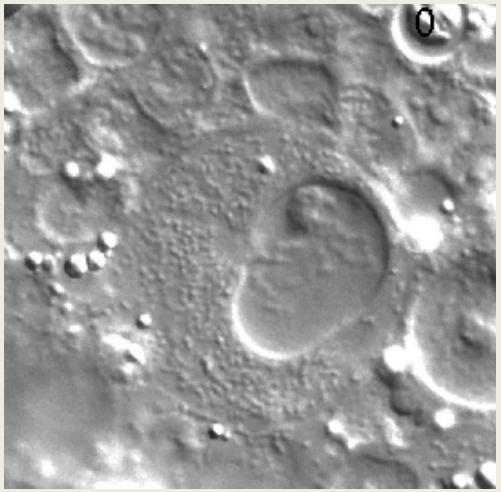
- Contrast based on exaggerating differences in Refractive Index of object and surrounding medium

- Objects have a 'relief' like appearance

DOES *NOT* PROVIDE TOPOLOGICAL INFORMATION

Surface analysis requires alternative techniques: e.g., Scanning Electron Microscopy (SEM)

Mitotically Dividing Neuroblast Stem Cell

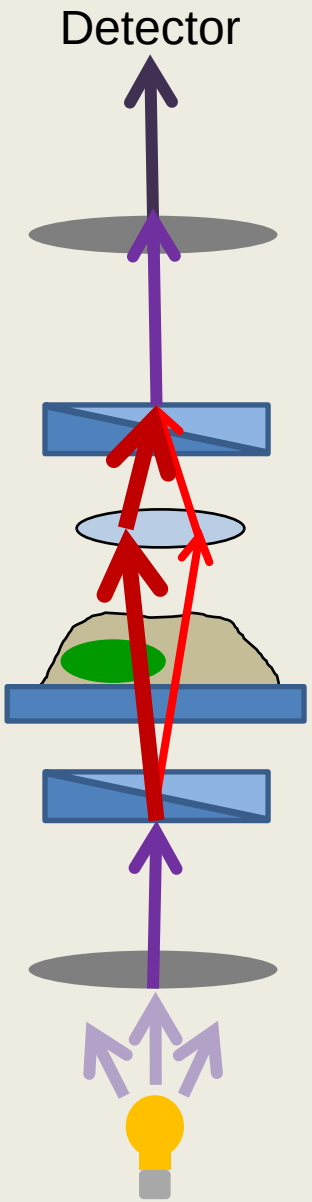


Generates the highest resolution image of any transmitted light method

Generates the thinnest optical section of any transmitted light method

Well suited for high resolution live cell studies

How Does DIC work?



1) Light emitted from Lamp is polarised by Polariser 1

2) Polarised light passes through Wollaston Prism 1, is split into Ordinary (**O**) and Extraordinary (**E**) rays separated by diffraction limit

3) **O** and **E** differentially interact with sample- **O** (passes/refracts through nucleus)-pathway longer than **E**

4) Objective Lens focuses **O** and **E** into Wollaston Prism 2 for recombination

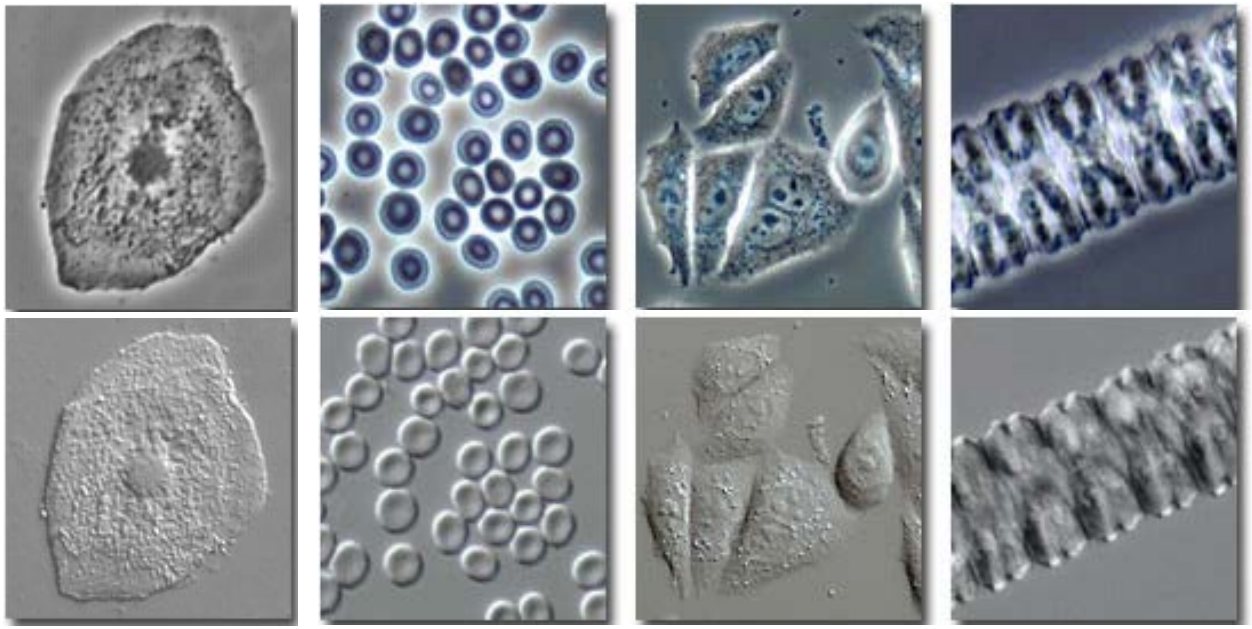
5) Combined ray passes through Polariser 2 and then into detector for viewing

Comparing Transmitted Light Optical Contrasting Techniques

Characteristic	Bright Field	Phase Contrast	DIC
Good for transparent samples?	No	Yes	Yes
Good for stained samples?	Yes	No	Yes
Halos?	No	Yes	No
3D effect?	No	No	Yes
Can use plastic slides/containers?	Yes	Yes	No
Brightness?	100%	1.3%	0.36-2.3%
Fluorescence light loss?	0%	28%	73%

Phase contrast

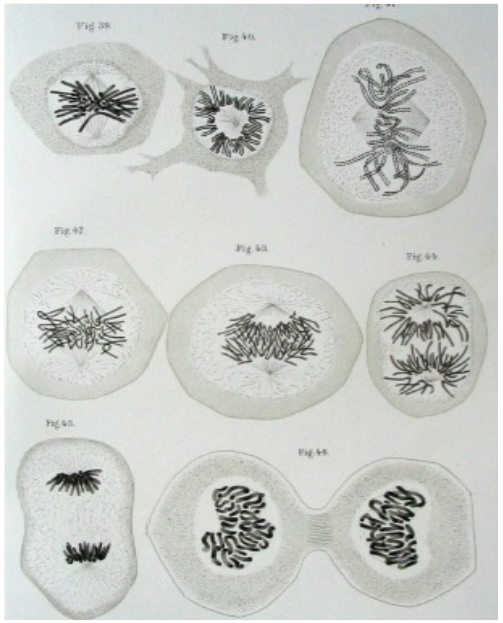
DIC



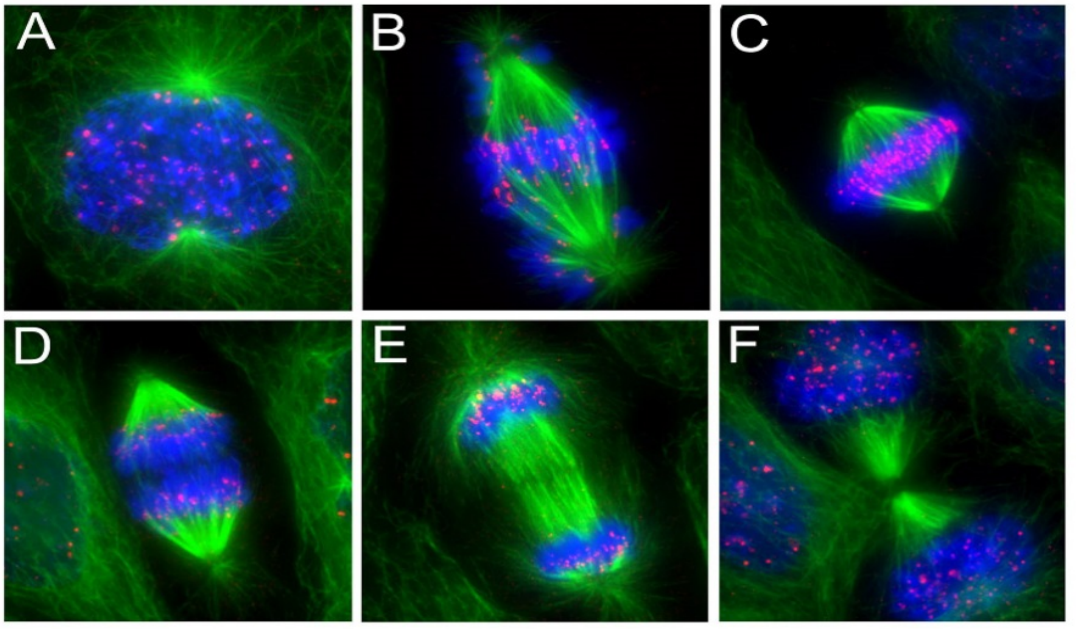
Modified from www.olympusmicro.com/primer/techniques/dic/dicphasecomparison.html

Epi-Fluorescence Microscopy:
A Tool for Molecule-Specific Imaging

Bright Field
(Dye Stained)



Indirect Immunofluorescence Staining
(Microtubules, Centromeres and DNA)

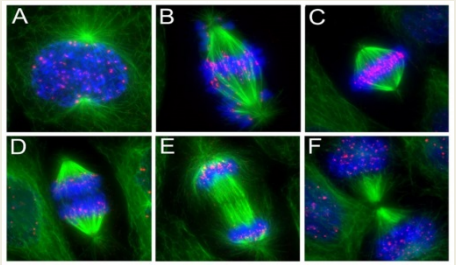


Dividing Vertebrate Cells (Salamander and Human)

Epi-Fluorescence Microscopy

Common Applications

- Co-localisation
- Dynamics
- Protein-Protein Interactions
- Protein Post-translational Modifications



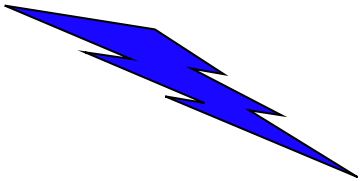
Epi-Fluorescence Microscope Configurations

- Widefield (classic fluorescence microscope)
- Scanning Confocal

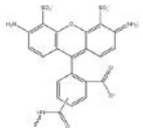
Fluorescence- The process whereby a molecule emits radiation following bombardment by incident radiation

What is Fluorescence and How Does it Work?

Excitation Light

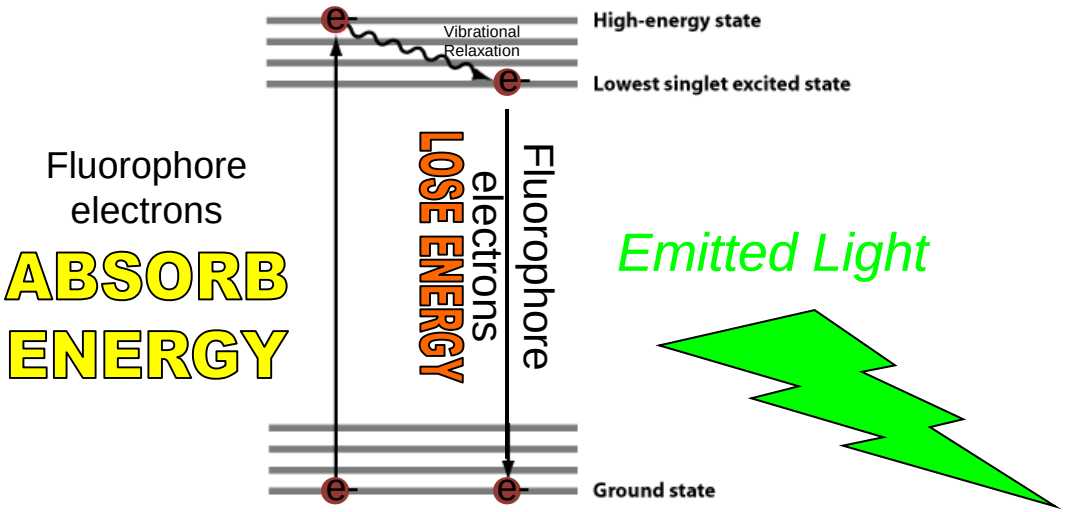


Fluorophore

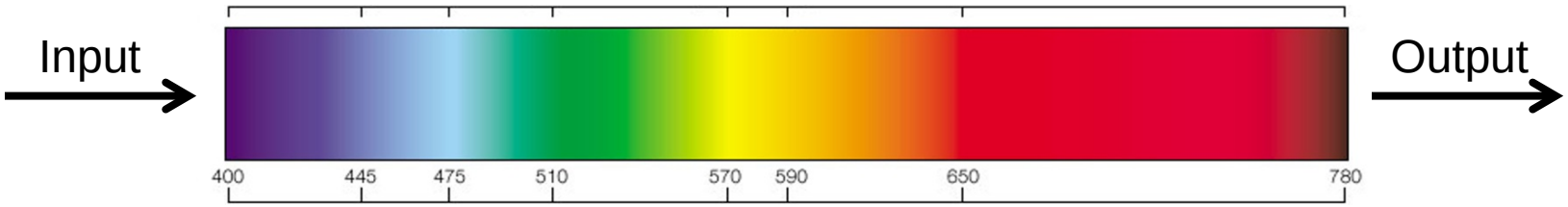


Alexa 488 Green Dye

Fluorescence energy diagram

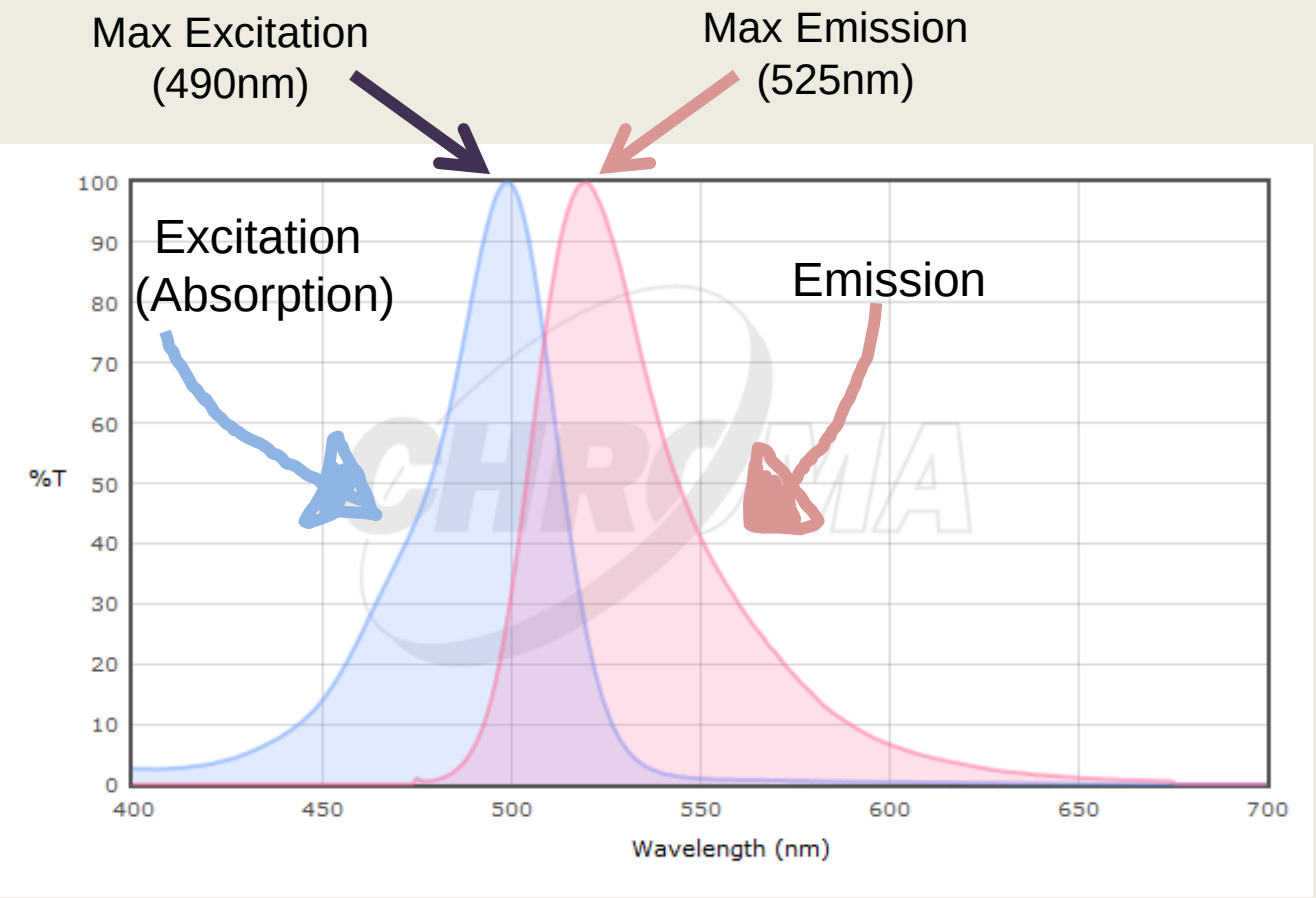


The emitted wavelength is *ALWAYS LONGER and Lower Energy* - Stoke's shift



Fluorophores Have Unique Fluorescence Spectra

Fluorescence Spectrum of Alexa 488

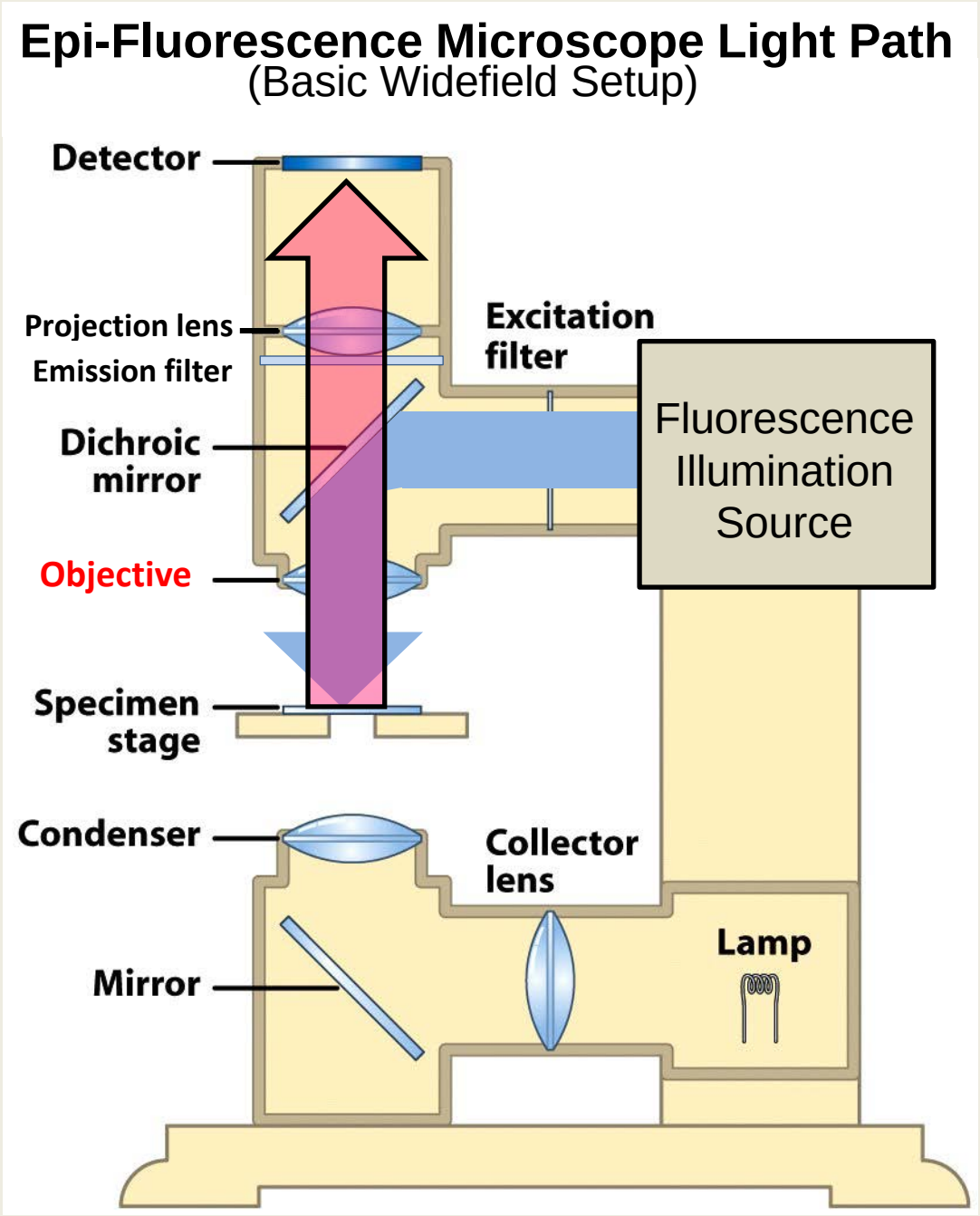


GAUSSIAN Absorption and Emission Profiles

Peak values listed by manufacturers

Prolonged excitation damages fluorophore and prevents emission
PHOTOBLEACHING

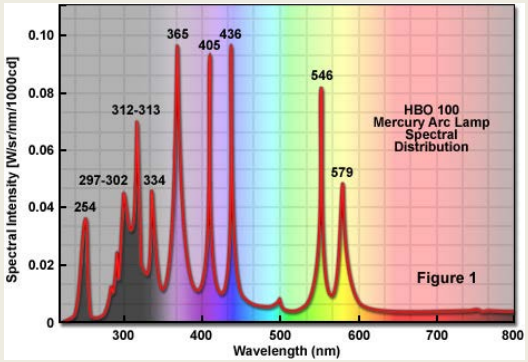
Epi-Fluorescence Microscope Light Path
(Basic Widefield Setup)



Modified from Lodish 6th Fig 9.10a

Illumination Sources

Hg Lamp- spectrum of excitation light wavelengths (350-600nm)



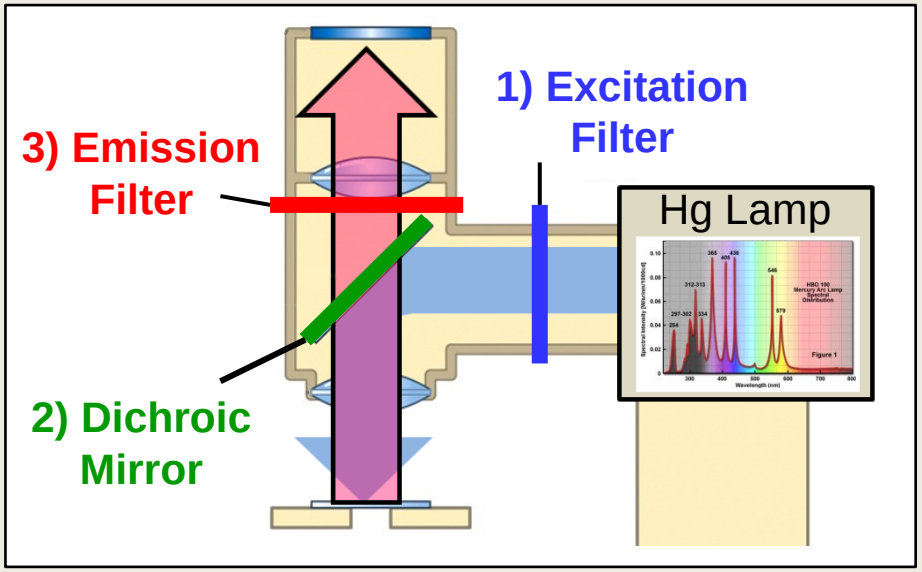
Lasers- Discreet wavelength per laser (e.g., 405nm, 488nm, 561nm, 633nm)

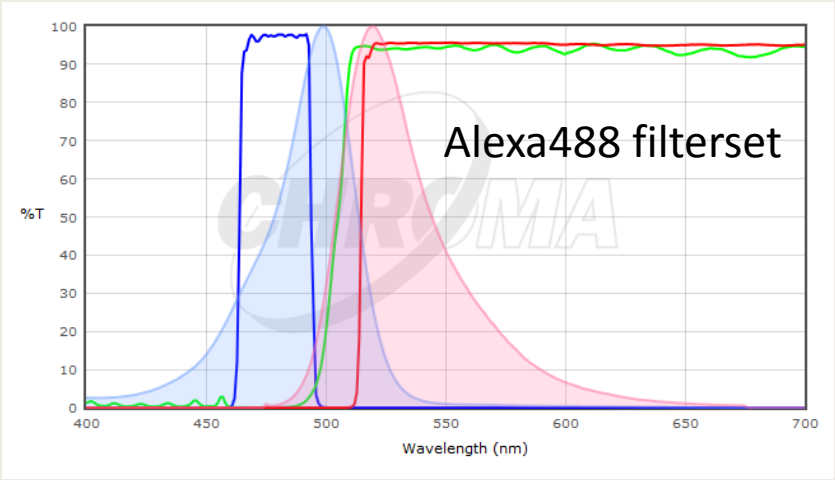
Alternatives:
Light Emitting Diodes (LEDs)- discreet wavelength per LED

Metal Halide Lamp (e.g., Xenon; broad spectrum of *visible* wavelengths

Epi-fluorescence Microscopes Require Filters

3 Component System





Bandpass Filter – blocks wavelengths outside of selected interval (e.g., AT480/30x; only 465-495nm transmitted)

Longpass Filter - blocks wavelength transmission below some value (e.g., AT515LP; ≥515nm transmitted)

Shortpass Filter - attenuates longer wavelengths and transmits (passes) shorter wavelengths

Dichroic mirror - reflects excitation beam and transmits emitted (e.g., AT505DC; ≥505nm transmitted)

3 Classes of Fluorescent Probes Provide Specific Labelling

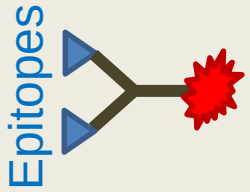
1) Dye-small organic molecule conjugates that directly bind their targets

Target Species	Probe Function	Example Probe
Various Ions	pH/Ion Concentration	pHRhodo/Fura-2
Lipids	Localisation	Nile Red
Proteins	Localisation	Fast Green
Actin	Localisation	Phallodin-alexa dye conjugate
Microtubules	Localisation	Taxol-alexa dye conjugate
Nucleic Acid	Localisation	Hoecsht33342, SYTO dyes
Mitochondria	Localisation	MitoTracker
ER	Localisation	ER-tracker
Lysosomes	Localisation	LysoTracker
Golgi	Localisation	Ceramide-BODIPY conjugate

All are cell membrane permeable and can be used on living samples

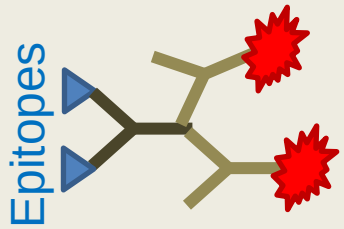
2) Dye-antibody conjugate labelling

Direct Immunofluorescence



- Antibody from host animal has fluorescent probe covalently attached
- Antibody-Probe binds to target epitope

Indirect Immunofluorescence



- Antibody from host animal 1 binds to target epitope
- Probe-conjugated antibody from animal 2 binds antibody 1

Pros:
Signal amplified

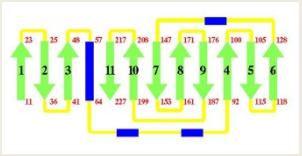
Cons:
Second antibody may non-specifically bind to sample resulting in “dirty” staining

Both require samples to be fixed and permeabilised with detergents

3) Dye-free genetically encoded labels

The Fluorescent Protein (FP) Revolution

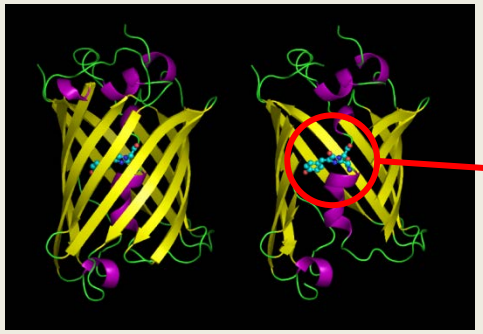
Green Fluorescent Protein (GFP)



1º Structure

11 β-sheets

4 α-helices



2º Structure

β-Barrel confers stability

Chromophore

(Ser65-Tyr66-Gly67)

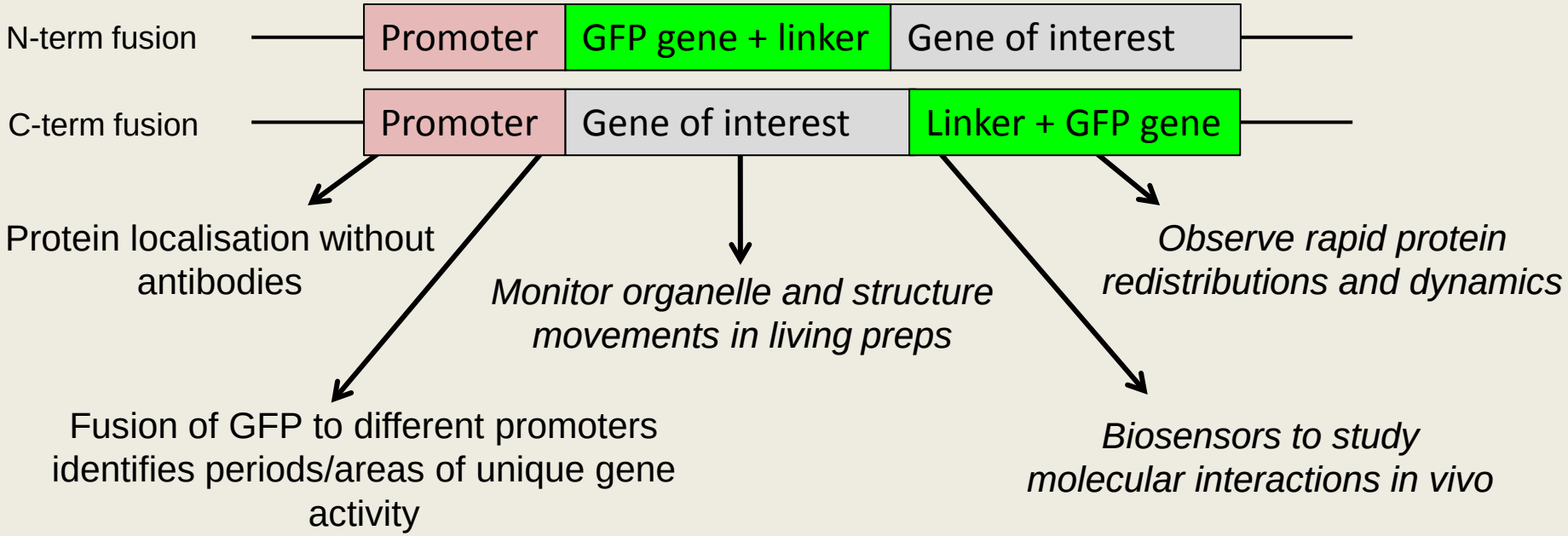
- Protein first isolated and studied in 1962 in “squeezates” by Shimomura
- Gene cloned in 1992 by Prasher et al.,
- Used as an in vivo marker by Chalfie and co-workers in 1994

GFP and Fluorescent Protein Technology have provided *unparalleled* insights into biological processes

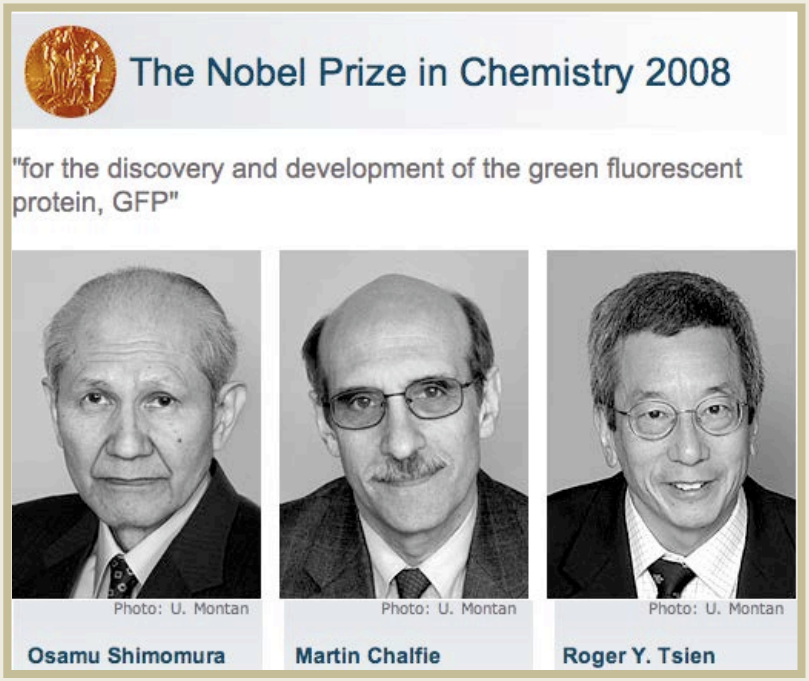
GFP Glows WITHOUT Additional Cofactors or Agents

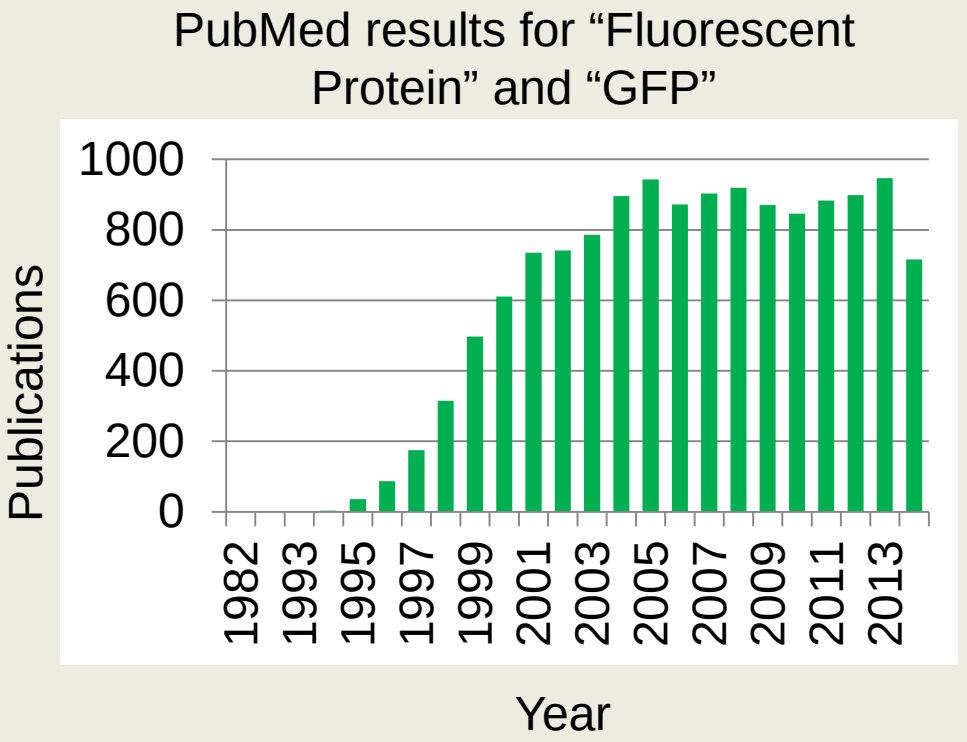
- 238 a.a. long
  - ~27 kDa
- Stable at physiological range of Temperatures and pHs
  - Rapid folding (and glowing)

GFP is NON-TOXIC, uses conserved codons and can be fused to genes of interest from any organism



The Fluorescent Protein Revolution





The Fluorescent Protein (FP) Palette

FPs engineered/isolated from other organisms with variants covering the spectrum

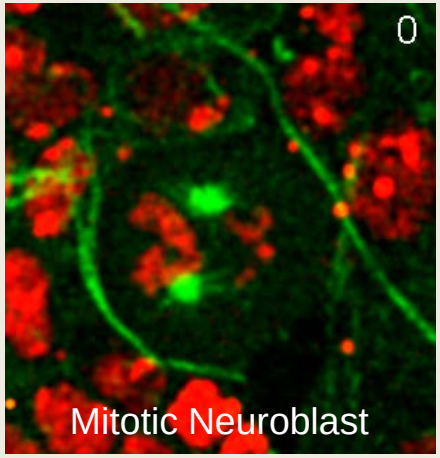
Chromophore differs but all have β-Barrel

Protein*	Color of spectral class	Excitation peak (nm)	Emission peak (nm)	Association state‡
EBFP2	Blue	383	448	Weak dimer
ECFP‡‡	Cyan	433/445	475/503	Weak dimer
mCerulean	Cyan	433/445	475/503	Monomer
mTFP1	Cyan-green	462	492	Monomer
mEGFP	Green	488	507	Monomer
mEmerald	Green	487	509	Monomer
sfGFP	Green	485	510	Weak dimer
EYFP‡‡	Yellow	514	527	Weak dimer
mVenus	Yellow	515	528	Monomer
mCitrine	Yellow	516	529	Monomer
YPet	Yellow	517	530	Weak dimer
mKO	Orange	548	559	Monomer
tdTomato	Orange	554	581	T-dimer
TagRFP	Orange	555	584	Monomer
mRFP1‡‡	Red	584	607	Monomer
mCherry	Red	587	610	Monomer
mKate	Far-red	588	635	Monomer
mPlum	Far-red	590	649	Monomer

Modified from Shaner et al., 2007

In vivo Molecular Specificity

Tubulin::EGFP Histone:mCherry



Many suffer from forming dimers/tetramers– can lead to artefacts

The Fluorescent Protein (FP) Palette

Protein*	Color of spectral class	Excitation peak (nm)	Emission peak (nm)	Association state‡
EBFP2	Blue	383	448	Weak dimer
ECFP‡‡	Cyan	433/445	475/503	Weak dimer
mCerulean	Cyan	433/445	475/503	Monomer
mTFP1	Cyan-green	462	492	Monomer
mEGFP	Green	488	507	Monomer
mEmerald	Green	487	509	Monomer
sfGFP	Green	485	510	Weak dimer
EYFP‡‡	Yellow	514	527	Weak dimer
mVenus	Yellow	515	528	Monomer
mCitrine	Yellow	516	529	Monomer
YPet	Yellow	517	530	Weak dimer
mKO	Orange	548	559	Monomer
tdTomato	Orange	554	581	T-dimer
TagRFP	Orange	555	584	Monomer
mRFP1‡‡	Red	584	607	Monomer
mCherry	Red	587	610	Monomer
mKate	Far-red	588	635	Monomer
mPlum	Far-red	590	649	Monomer

FP experiment considerations:

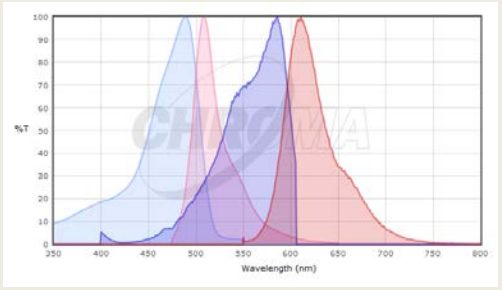
1) Does FP interfere with protein function?

- Is placement better on N or C term?
  - Does tag form multimers?

2) Is FP bright and photostable enough for experiment?

3) Are FPs spectrally distinct?

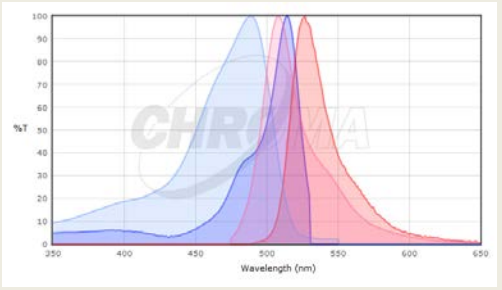
EGFP and mCherry



Well defined

Vs.

EGFP and EYFP



Extreme overlap-hard to resolve

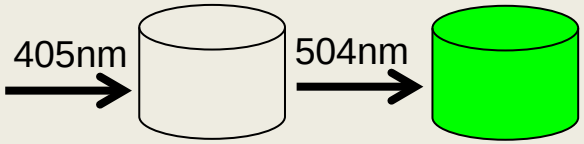
Fluorescent Proteins as Optical Highliters

Fluorescent Proteins as Highliters

Some Fluorescent Proteins can be differentially controlled by light

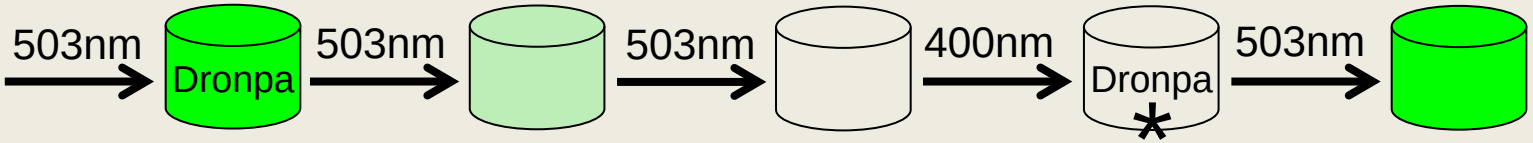
Photoactivatable (on with UV light)

- PA-GFP (ex. 504nm; em. green)
- PA-mCherry1 (ex. 564nm; em. red)



Photoswitchable (on/off)

		Excite (nm)	Inactivate (nm)	Activate (nm)
Dronpa	(em. green)	503	503	400
rsEGFP2	(em. green)	478	503	408
Dreiklang	(em. green/yellow)	511	405	365
rsCherry	(em. red)	572	450	550



Fluorescent Proteins as Highliters

Photoconvertible

		Conversion Wavelength (nm)
▪ PS-CFP2	cyan-to-green	405
▪ Dendra2	green-to-red	480
▪ PCDronpa2	green-to-red	405
▪ mEOS2	green-to-red	405
▪ Kaede	green-to-red	405
▪ psmOrange2	orange-to-far red	489

Fluorescent Proteins can serve as timers

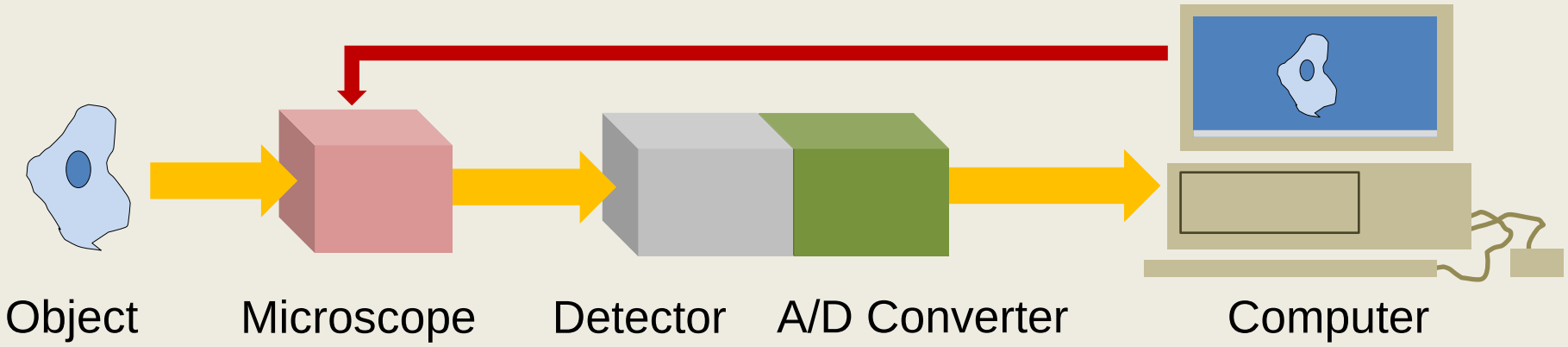
mCherry Derivatives

	Blue-to-Red Fluorescence Conversion Time (Hours)
▪ Fast-FT	~4
▪ Medium-FT	~7
▪ Slow-FT	~28

DsRed derivatives- all tetrameric

DSRed-E5 green-to-red ~18 hours

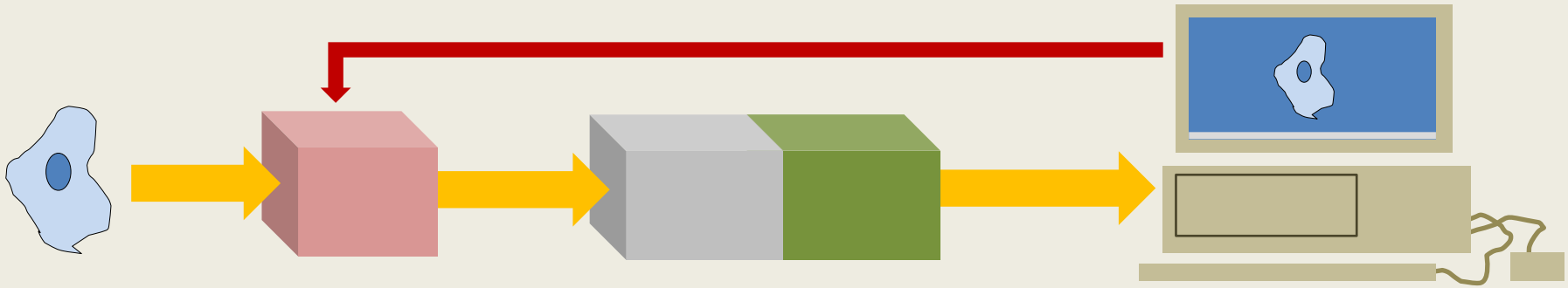
Image Acquisition: Digital Imaging



Digital Imaging

- Easy work flow from microscope to presentation (seminars, publications, etc.,)
- Software allows data manipulation and analysis at your desk
- Storage footprint and expense minimal

The Pathway of Digital Image Formation



Object

Emits *Photons*

Microscope

Transmits *Photons*

Detector

Captures *Photons* And Turns them into *VOLTs*

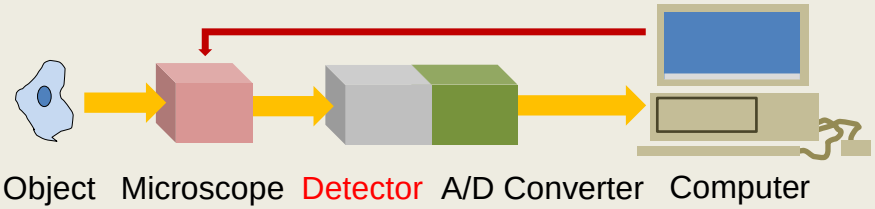
A/D Converter

Turns *Volts* into *Pixels* (x,y and grey value data)

Computer

Controls Acquisition and allows Visualisation/Analysis of Photons in Quantitative Way

The Pathway of Digital Image Formation



Detectors

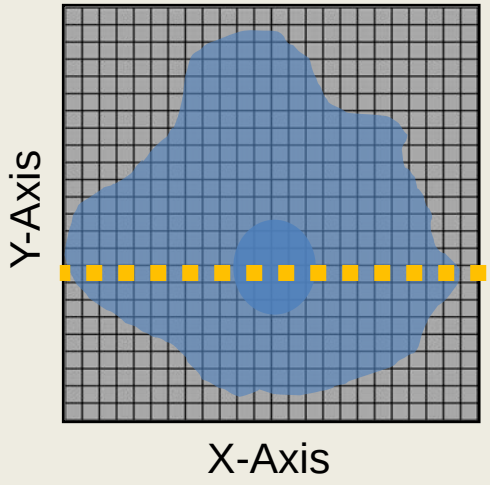
Photosensitive devices that transduce incoming photons into PROPORTIONATE AND SPATIALLY ORGANISED voltage distributions

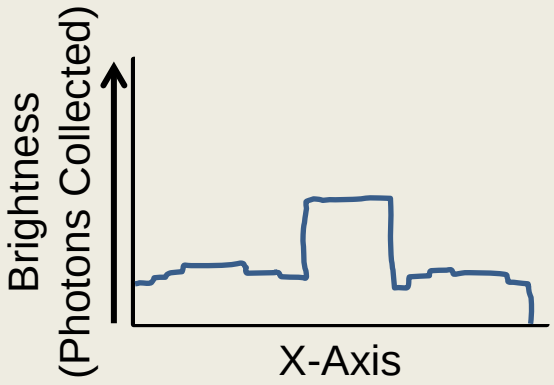
In other words. . .

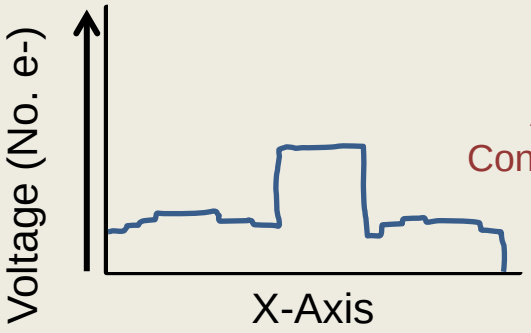
The Pathway of Digital Image Formation

It makes a map!

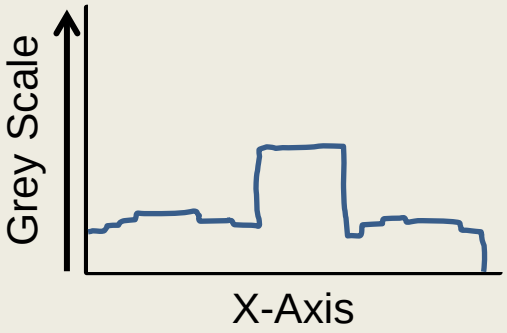
Each map unit is a pixel:
x,y information and brightness information







A/D
Conversion

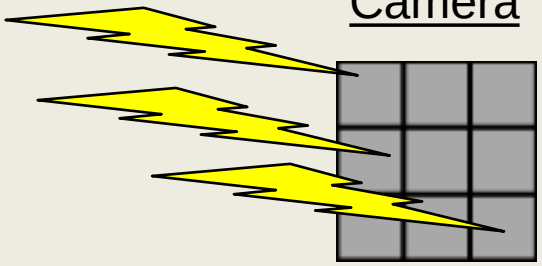


The Pathway of Digital Image Formation: Detectors

Digital Camera
- Charge Coupled Device (CCD)
- Complementary Metal-Oxide Superconductor (CMOS)

Photomultiplier Tube (PMT)

Camera



Entire image formed simultaneously from arrays of *physically subdivided* detectors (pixels)

PMT

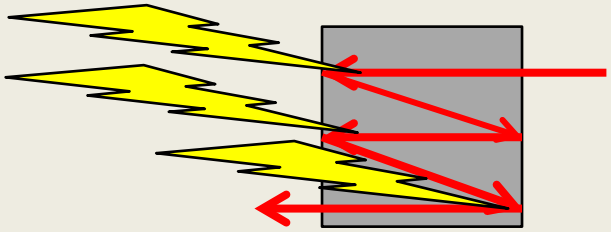


Image formed spot by spot (raster scanning)

The Pathway of Digital Image Formation: Detector Characteristics

Physical Pixel Size: Not so important- apparent size is (see next)

Pixel Number: Not so important– most CCDs <2MPx (1400x1080)

Dynamic Range: Total range of shades

8bit= 2^8=256

12bit= 2^{12}=4095

16bit= 2^{16}=65,535

Quantum Efficiency: Efficiency of electron production per photon collision

CCD/CMOS 60-90%

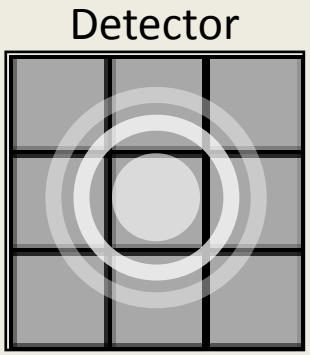
PMT ~15%

Noise: Non-signal-based contributors to the image

- Shot/Photon Noise- Random emission of photons from sample
- Thermal Noise- random e- due to thermal fluctuation in detector
- Electronic Noise- when signal transmitted from detector to A/D converter

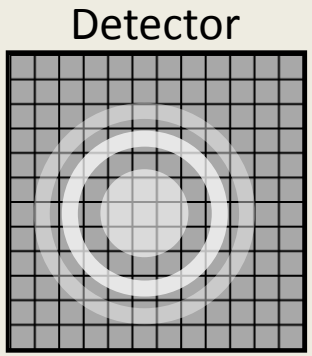
Detector Characteristics: Pixel Size (Spatial Information)

Pixel size should be matched to system resolution

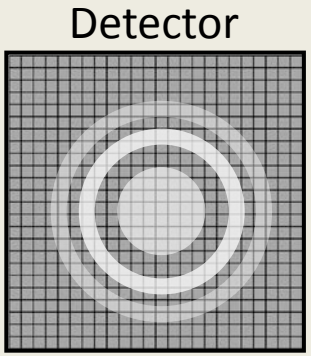


"Undersampled"

Detail Lost



Optimal



"Oversampled"

- Empty Magnification
- Signal Intensity Lost

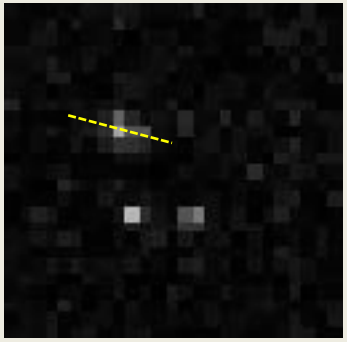
Each pixel should *appear* 1/3 to 1/2 the size of the Airy Disk

Detector Characteristics: Pixel Size

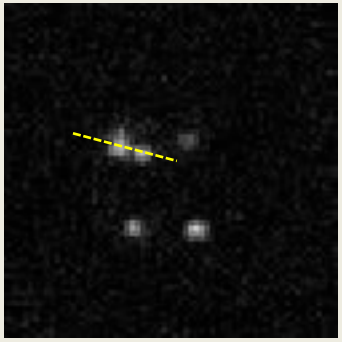
Pixel Size Limits Image Information

0.5µm beads imaged using different pixel sizes

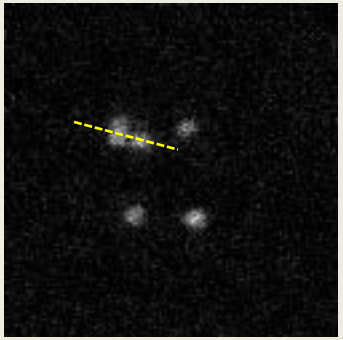
240nm pixel



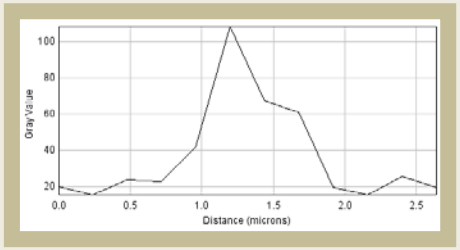
96nm pixel

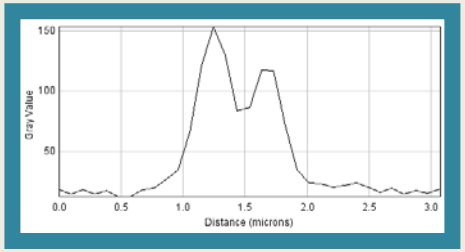


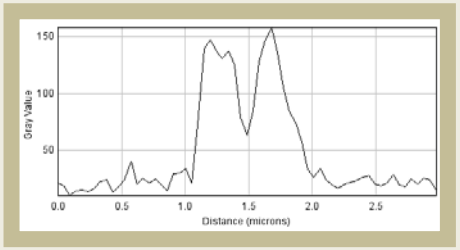
48nm pixel



Linescans across area denoted by arrow







“Undersampled”

Optimal

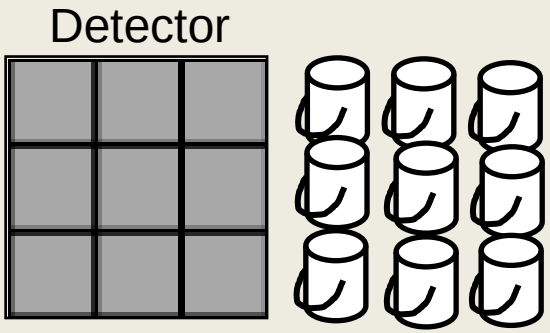
“Oversampled”

Oversampling offers little spatial improvement but may decrease image brightness or increase scan time

Detector Characteristics: Dynamic Range
(Intensity Information)

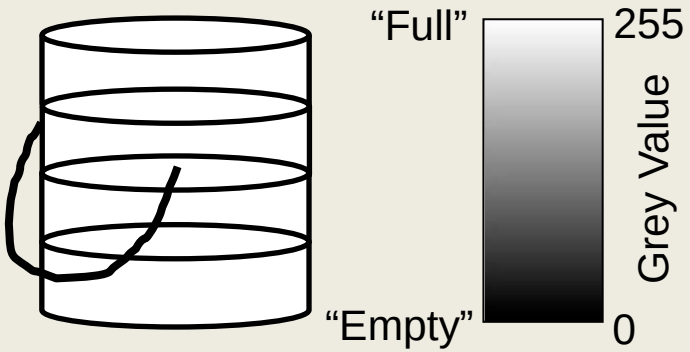
Most monochrome images are 8 bit (2^8 =256 shades)
Displayed as a pseudo-coloured LOOK UP TABLE (LUT)

RGB colour images are 24 bit (Red8bit+Green8bit+Blue8bit data)



Each pixel is like a bucket

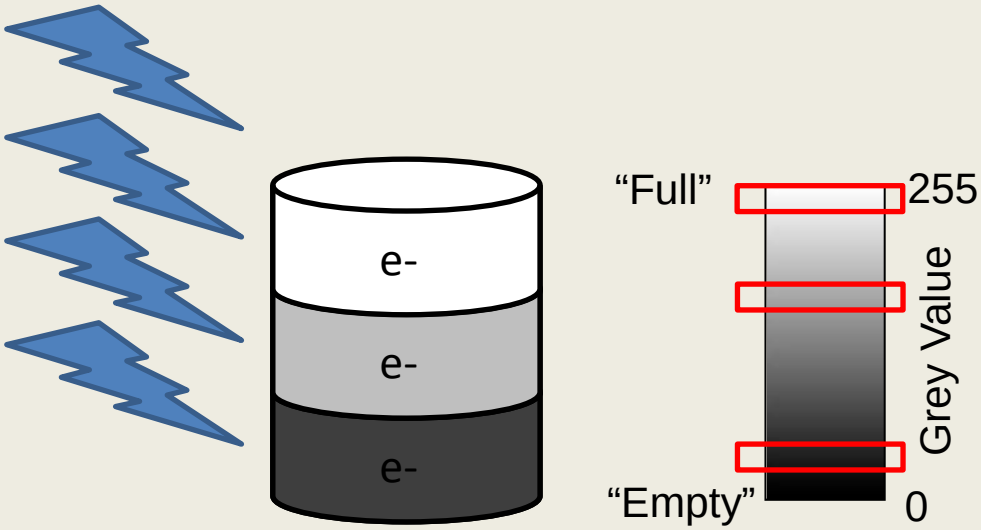
As photons strike detector, electric charge builds (fills the bucket)



The bucket’s depth defines dynamic range

Dynamic Range (Intensity Information)

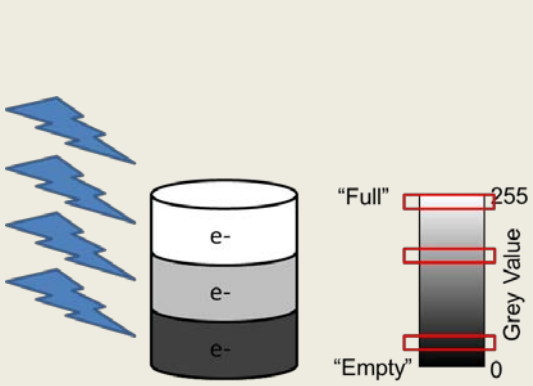
As photons strike, electric charge *PROPORTIONATELY* accumulates (fills the bucket)

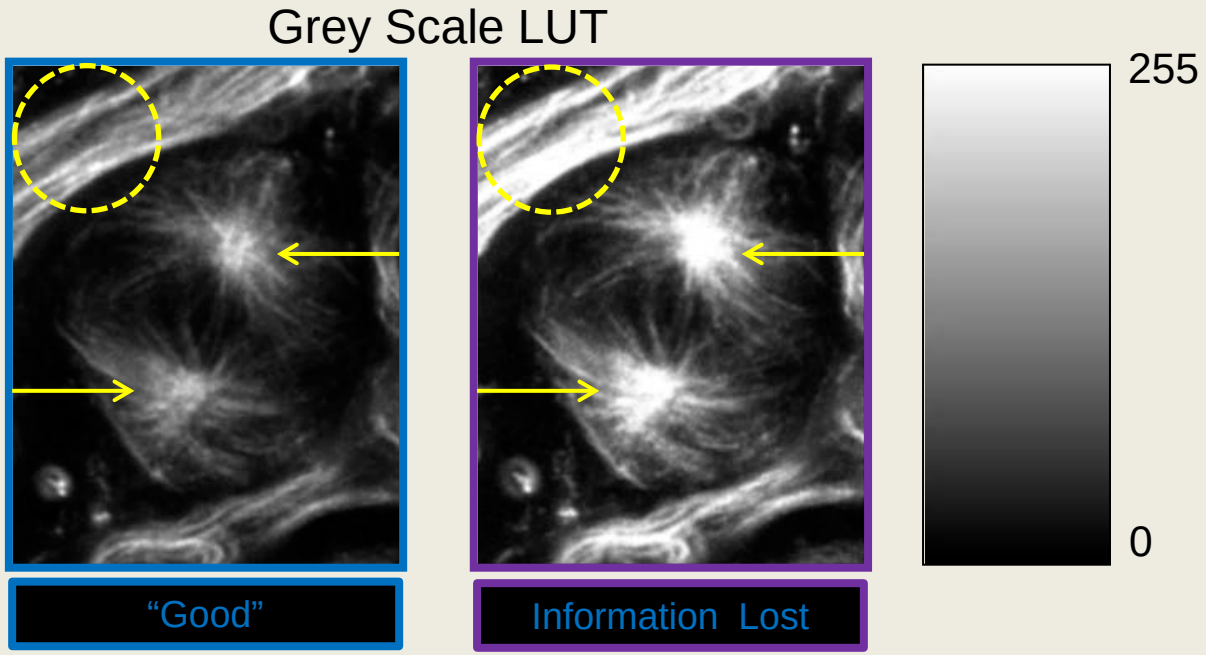


"bucket full"
Detector SATURATED

Additional photons not displayed

Dynamic Range (Intensity Information)



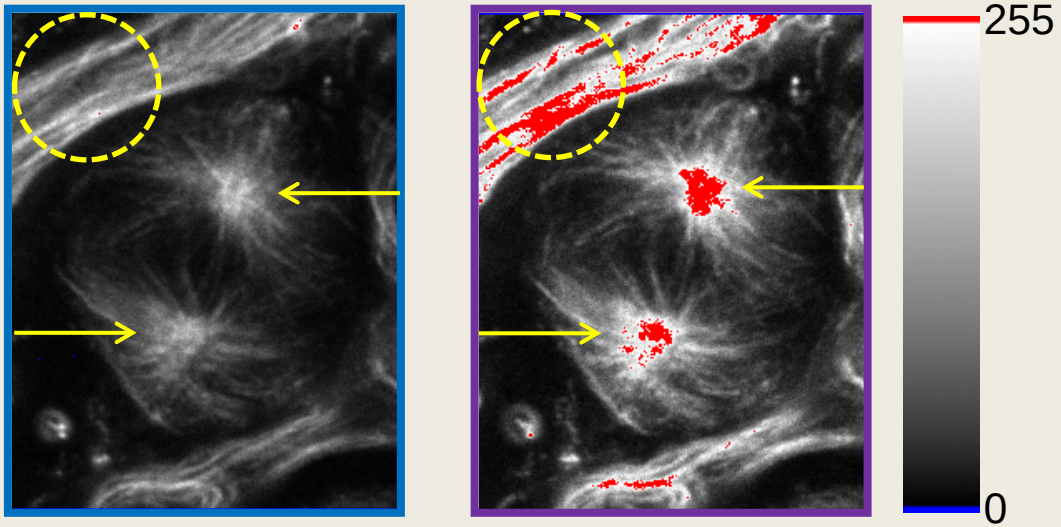


Excessive "white" areas– spatial and intensity detail not visible

- *Loss of information due to saturation?*
- *No data lost- monitor screen too bright?*

Dynamic Range (Intensity Information)

Look Up Tables can reveal saturation/underexposure



"HiLo" LUT

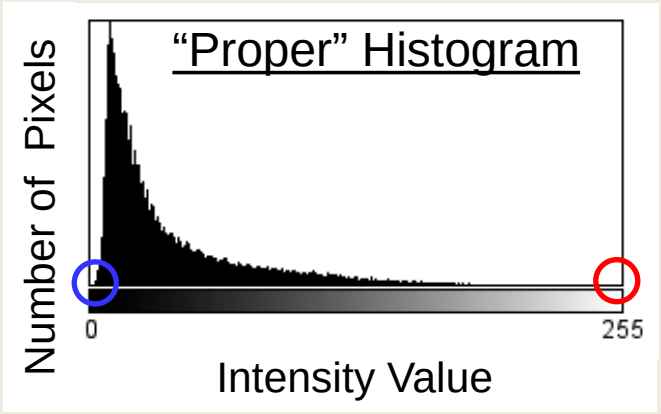
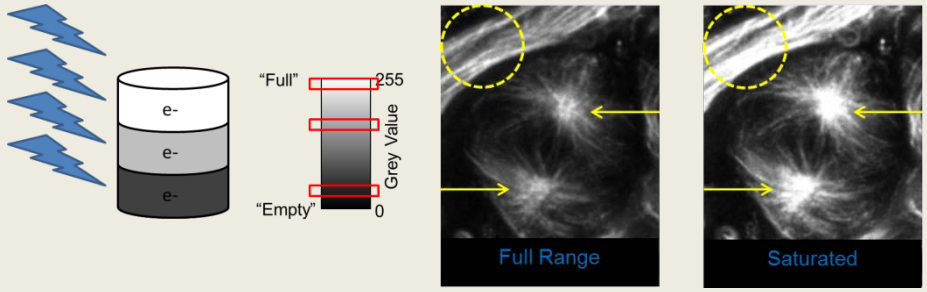


Image Saturated

INFORMATION PERMANETLY LOST

Dynamic Range (Intensity Information)

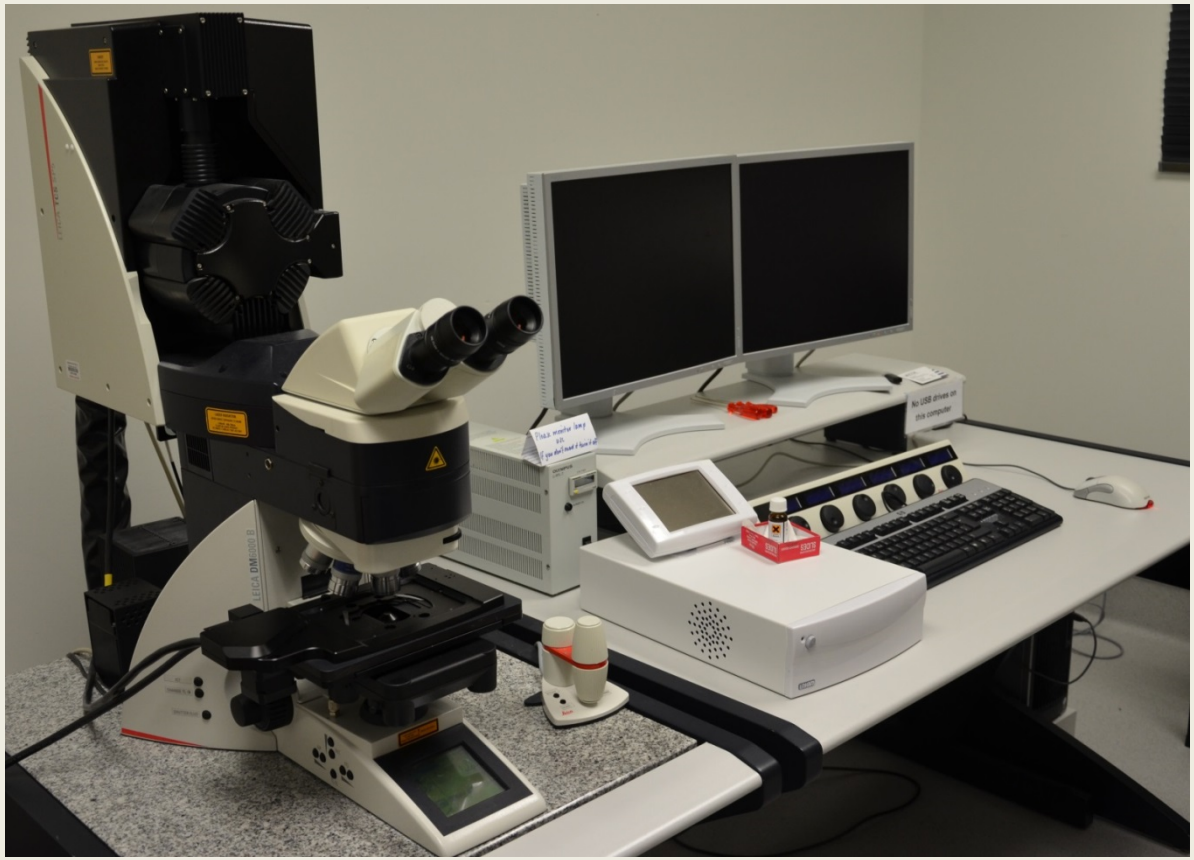


As photons strike, electric charge *PROPORTIONATELY* accumulates (fills the bucket)

Below saturation, fluorescence intensity is proportional to collected photons and can be quantified as a metric of molecular concentrations

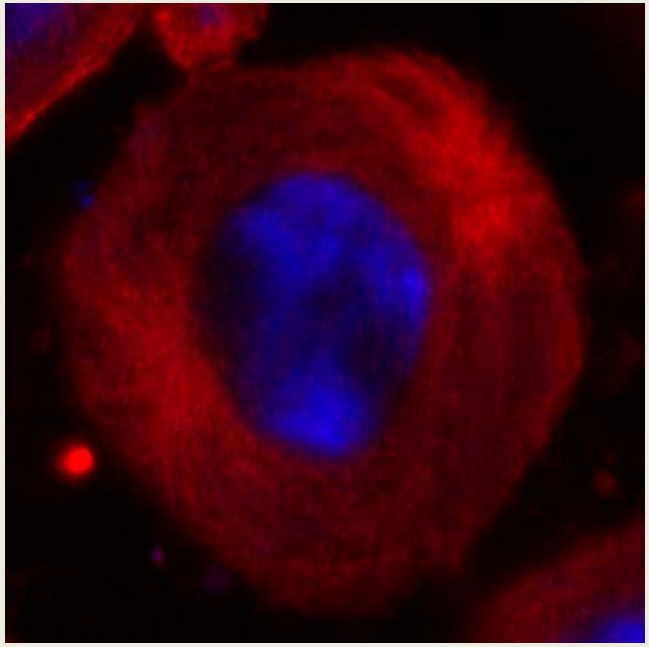
(Which we will explore later)

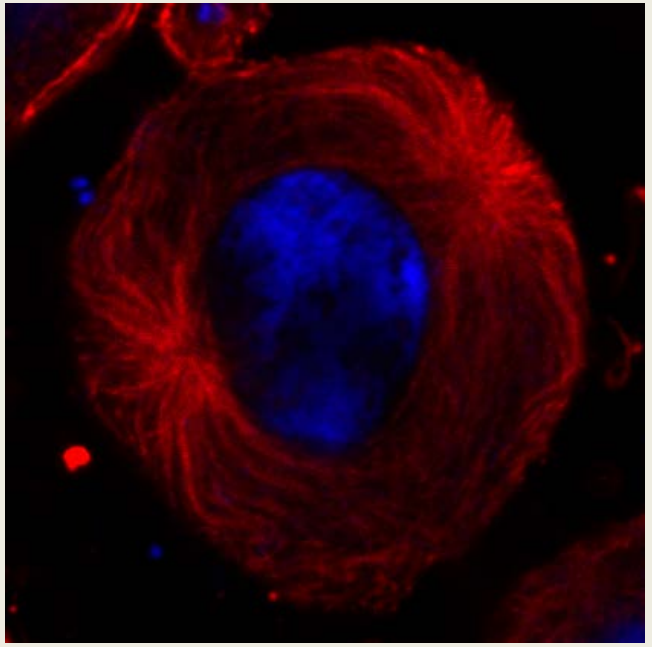
Scanning Confocal Microscopy (SCM)



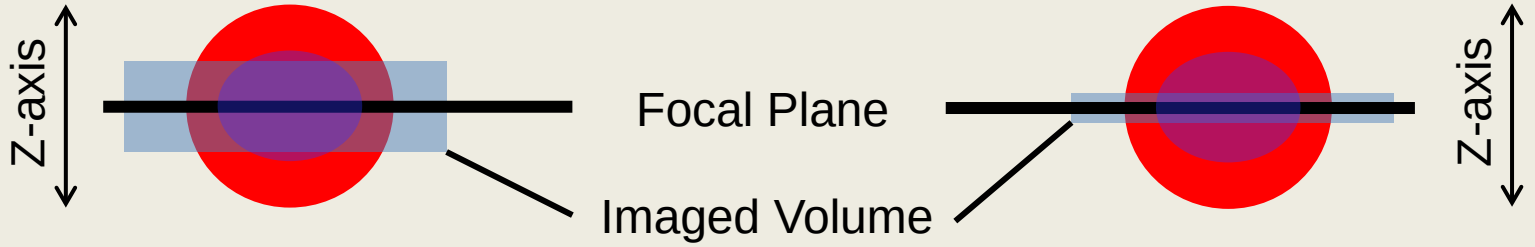
A Hardware Approach to Improving Epi-Fluorescence Image Quality

Scanning Confocal Microscopy Provides Thin Optical Sections





Drosophila cells stained for Microtubules and DNA



Background fluorescence is collected from above and below focal plane

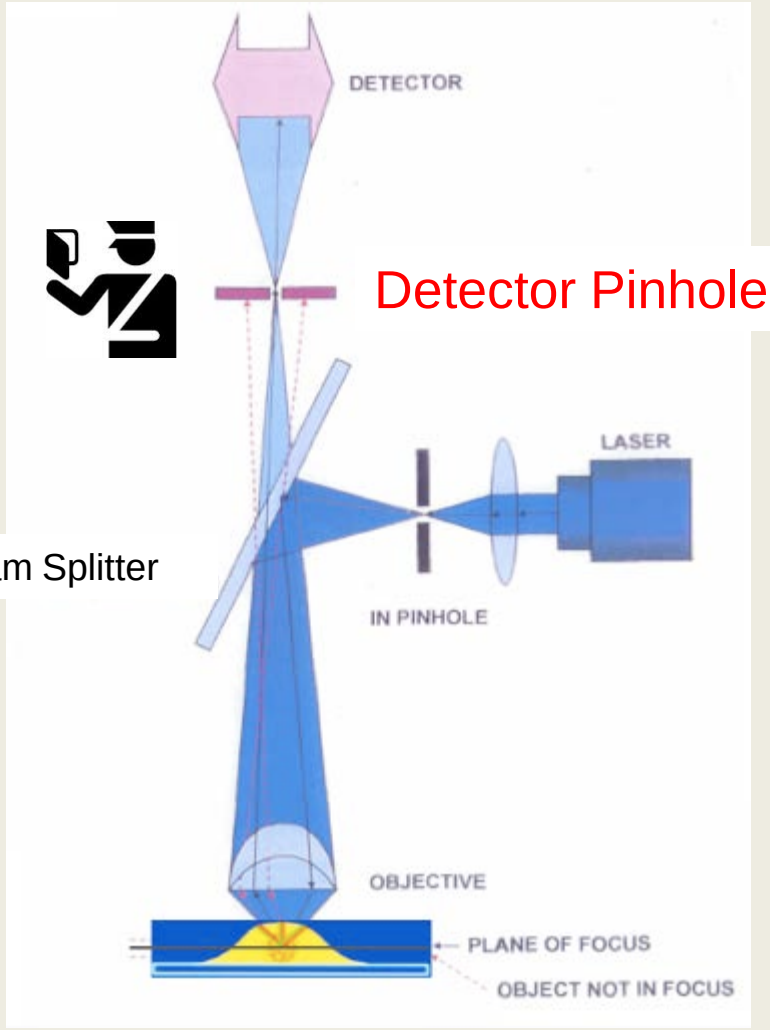
Collected fluorescence limited to focal plane

SCM: The Confocal Principle

The sharpened image is due to the “pinhole”

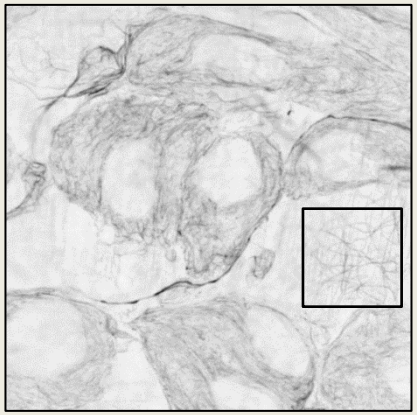
An excitation laser is scanned across the sample

Pinhole located in front of detector blocks emitted light not originating from the focal plane

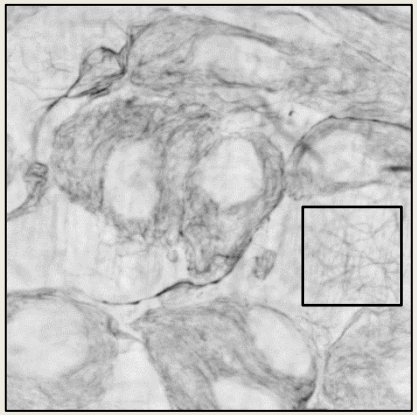


SCM: The Pinhole Dictates Optical Section thickness

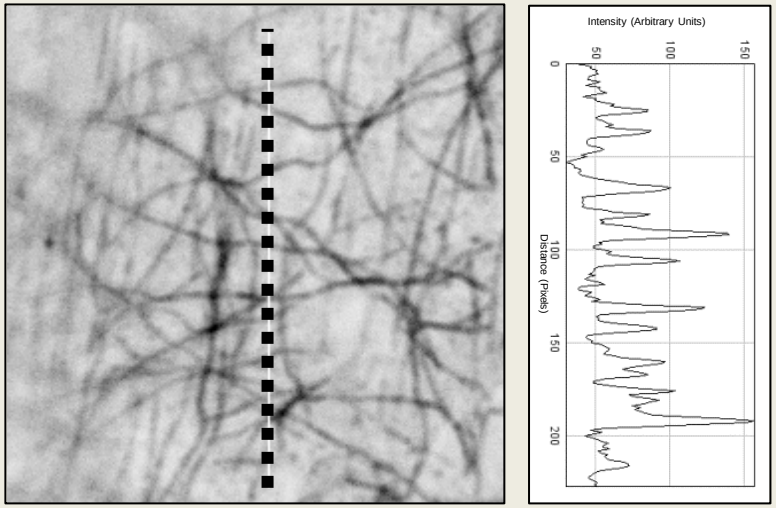
Pinhole size 1.0 Airy Units (Default)

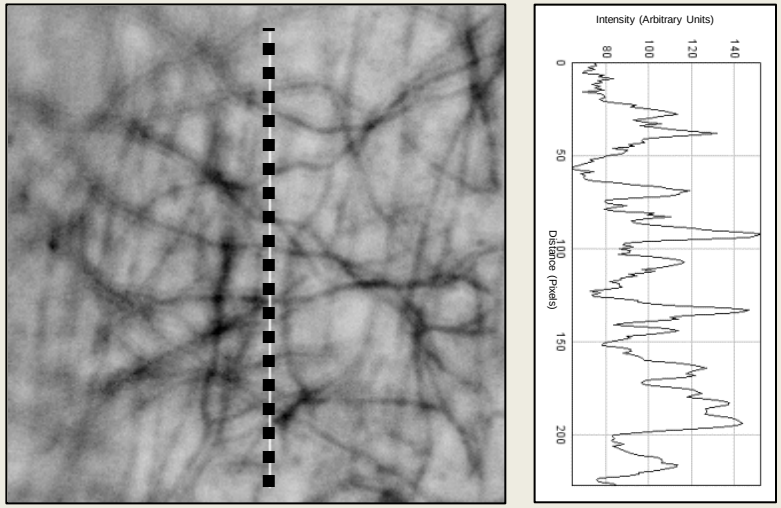


Pinhole size 2.0 Airy Units



Images of Microtubules in *Drosophila* cells



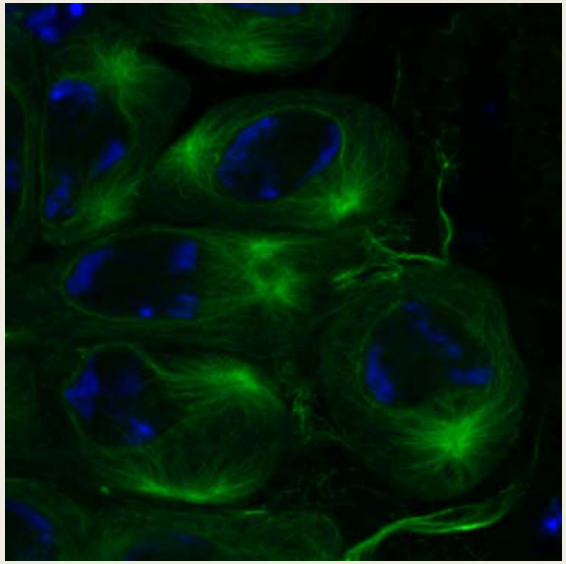


Opening the pinhole increases image blur

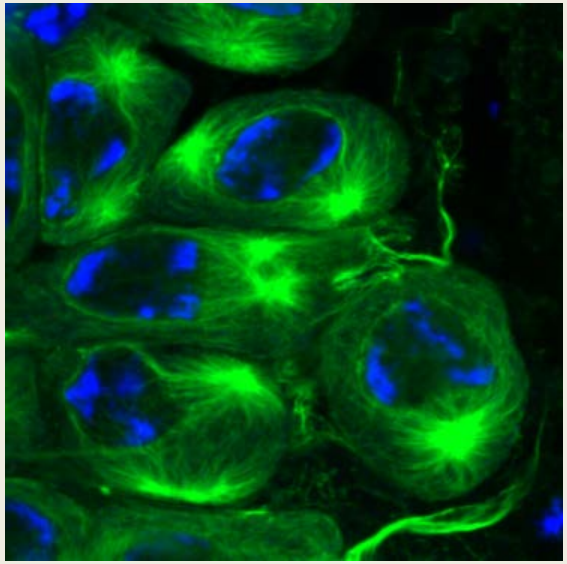
SCM: The Pinhole Size Determines Image Brightness

Images of *Drosophila* cells imaged with identical settings *EXCEPT* for the pinhole diameter
(Microtubules DNA)

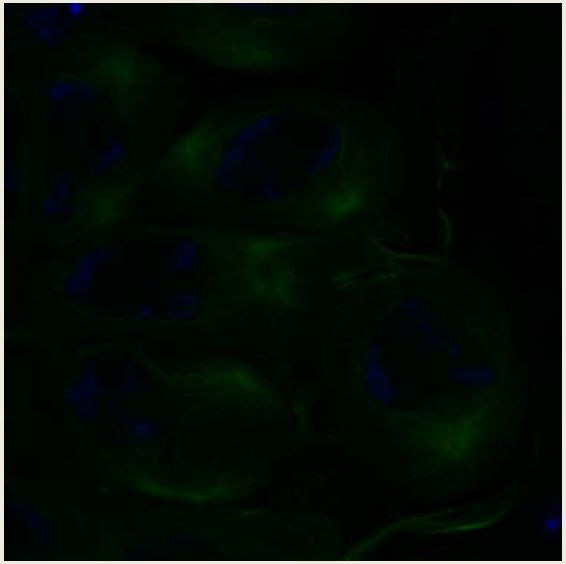
1.0 Airy Units (Default)



2.0 Airy Units



0.5 Airy Units



A larger pinhole creates a thicker optical section and allows more light to be captured

Pinholes < 1 Airy Unit reduce signal intensity but DO NOT significantly improve image quality

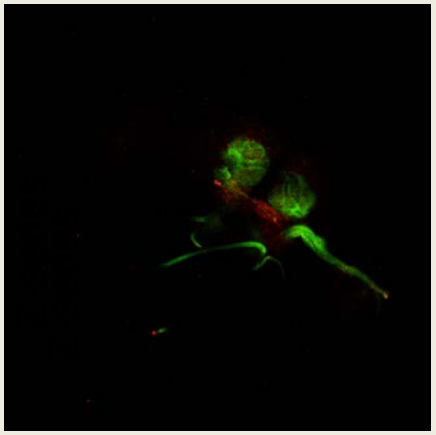
SCM: 3D Reconstructions

Any automated epi-fluorescence microscope can collect optical sections

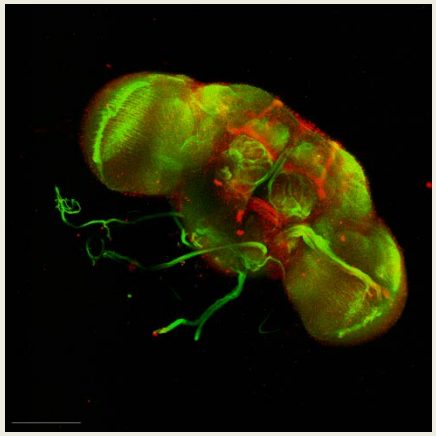
Scanning Confocal Microscopy EXCELS with THICK specimens

Fruit fly Brain (52 sections, 2µm steps)

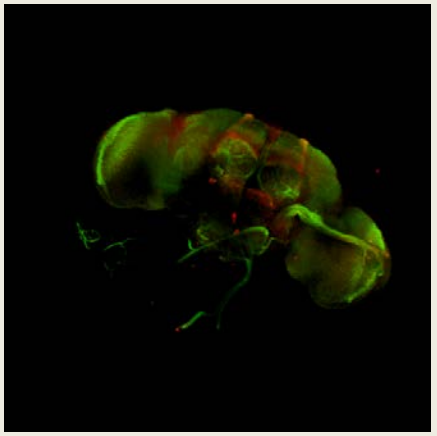
Z-series



Max. Intensity Proj.

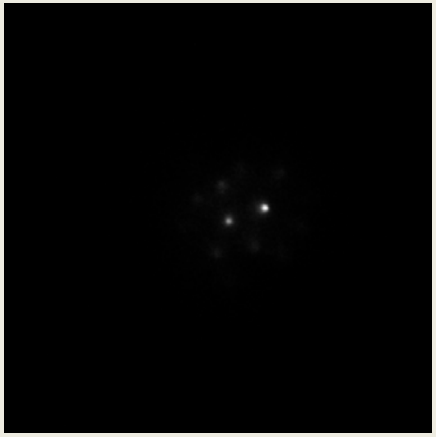


Volume

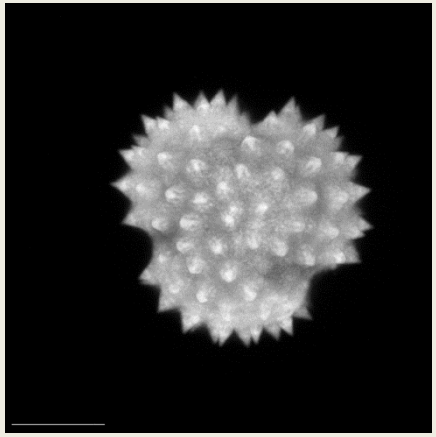


Pollen Grain (52 sections, 0.4µm steps)

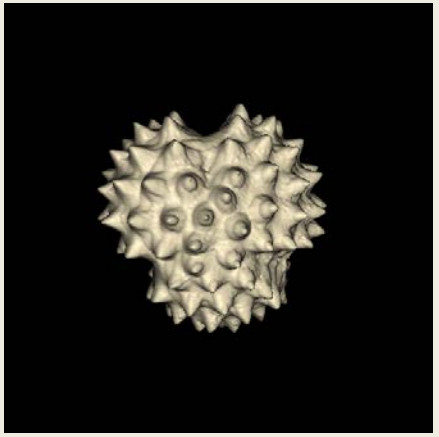
Z-series



Max. Intensity Proj.



Surface Rendering



Scanning Confocal Microscopy vs. Widefield Epi-Fluorescence Microscopy

Pros:

- Thinner optical section
- Superior signal:background
  3D reconstructions from optical slices
- Better for imaging into thick specimens (5μm vs 50μm)

- Ability to bleach/activate in fixed area of virtually any shape (FRAP/FRET)
- The ability to magnify without loss of intensity

Cons:

- Substantial loss of emitted sample signal (<90%)
- Excitation lasers may rapidly photobleach sample
- SLOW scan speed so not ideal for studying living/fast events

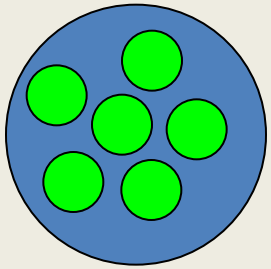
In other words, experimental needs dictate the technique

More than “pretty pictures”: Light Microscopy As A Quantitative Tool

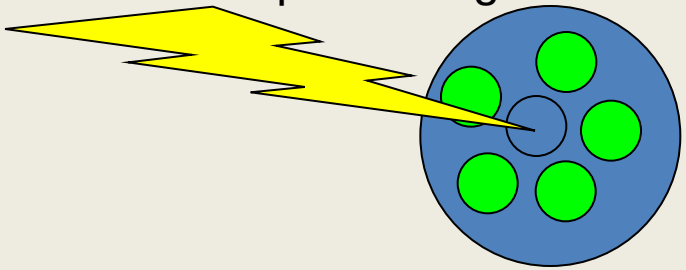
Measuring Protein Dynamics:

Fluorescence Recovery After Photobleaching (FRAP)

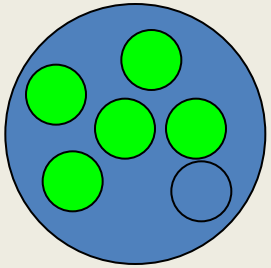
1) Pre-bleach: GFP-tagged molecules dynamically associate with structure



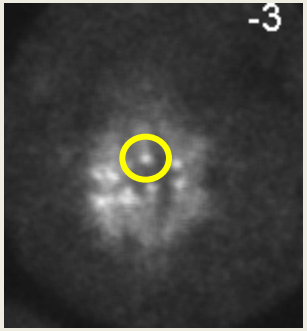
2) Bleach: HIGH ENERGY LIGHT IRREVERSIBLY damages targeted chromophores preventing further fluorescence



3) Recovery: Fluorescence returns to the structure as unbleached molecules exchange with and “dilute out” bleached ones



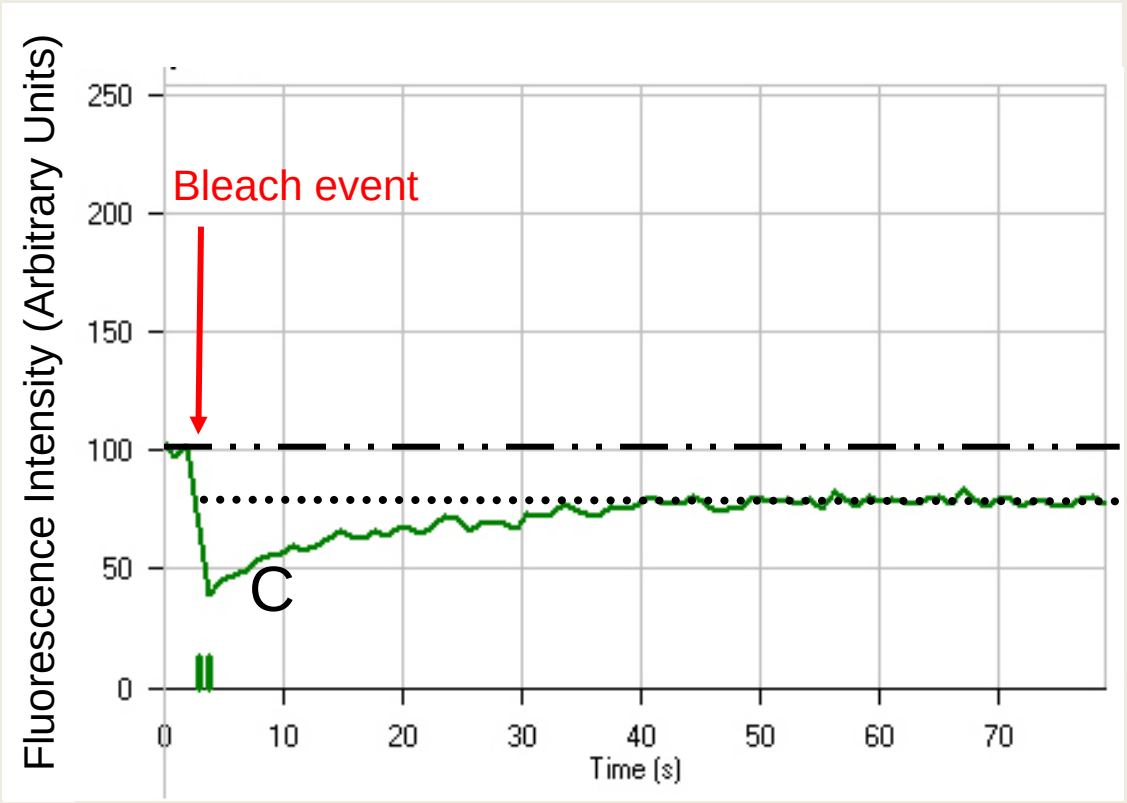
FRAP at work: Kinetochore Protein Dynamics



Drosophila mitotic cell expressing GFP tagged Klp67A

FRAP reveals:

- % of protein pool that is dynamically exchanging
- Rate of mobility



A Pre-bleach fluorescence intensity

B Post-bleach intensity plateau

Difference between A-B reveals non-dynamic population

C Slope identifies mobility rate

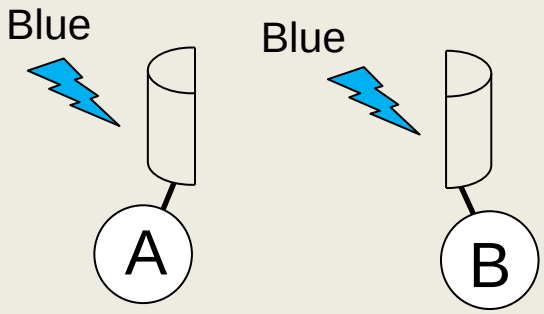
Steeper is more rapid

$T_{1/2}$ ~6 sec

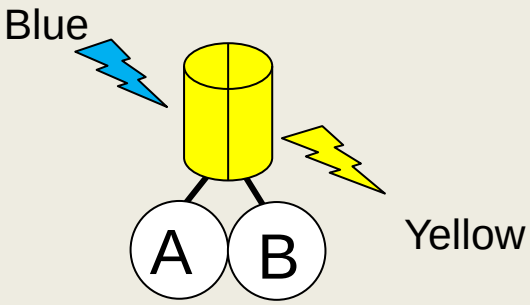
Studying Protein-Protein Interactions: Bimolecular Fluorescence Complementation (BiFC)

- Fluorescent Protein cloned as two separate halves

(e.g., YFP; N-term a.a. 1-154 + C-term 155-238) fused to candidate interactors (A, B)



- Neither fragment glows

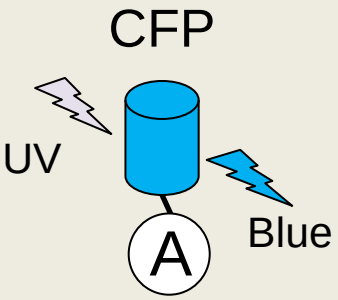


- A-B interact and YFP halves come together; YFP fluoresces

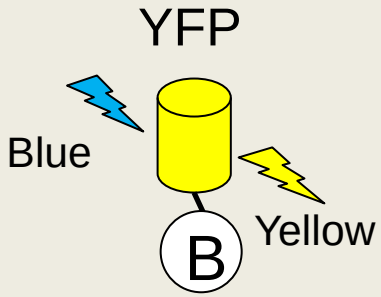
Quantify fluorescence intensity of each to reveal efficiency of binding

- A and B need to be within ~10nm
- Binding irreversible- not good for dissociation kinetics

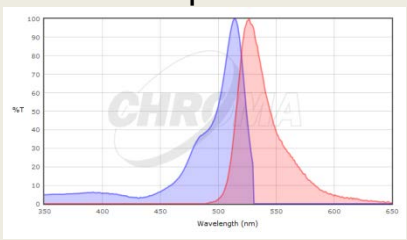
Studying Protein-Protein Interactions: Förster Resonance Energy Transfer (FRET)



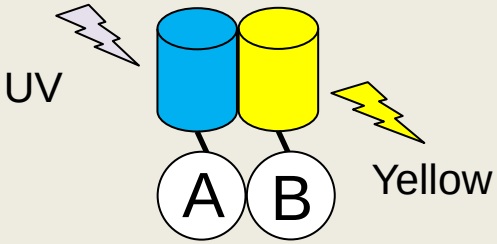
CFP Spectrum



YFP Spectrum

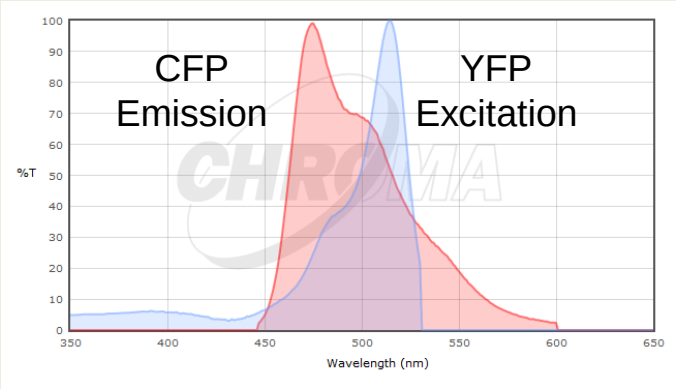


DONOR-ACCEPTOR



Proteins A and B interact

CFP/YFP Spectrum

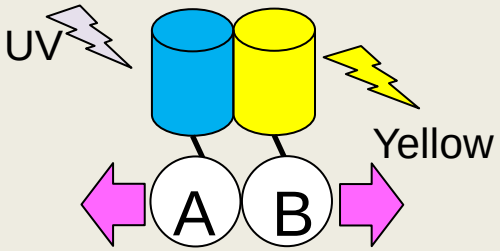


Measure fluorescence intensity to reveal efficiency of binding

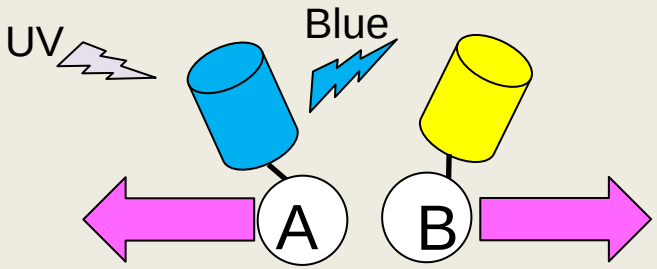
- Donor Emission must OVERLAP Acceptor Excitation
- Chromophores are ≤10nm apart

FRET as a Quantitative Biosensor

Sites and durations of Mechanical Tension

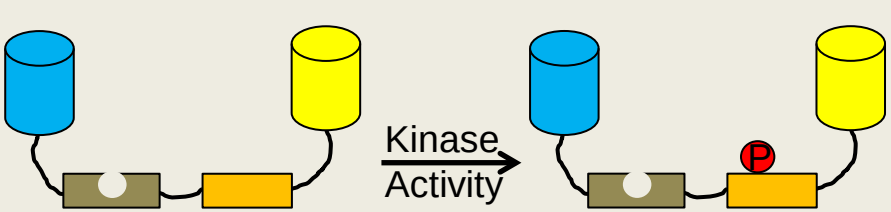


Tension LOW:
A contacts B;
FRET



Tension HIGH:
A and B separated
FRET LOST

Protein Modifications e.g., Local kinase activity

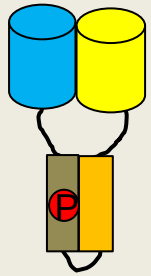


1. Default State

NO FRET

2. Phosphorylation of Substrate

NO FRET



3. Intramolecular binding P-Substrate Binds PBD

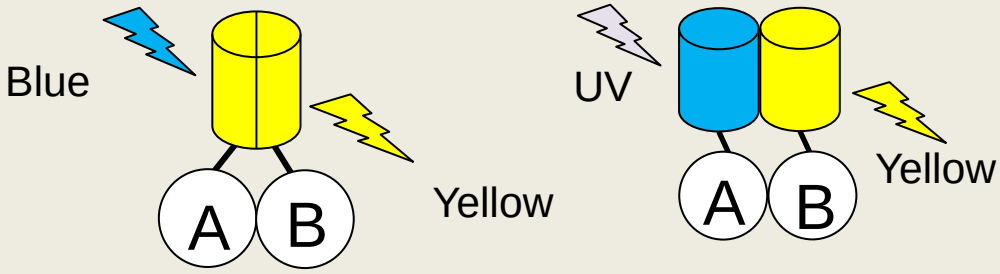
FRET

Phospho-amino acid Binding Domain (PBD)

Kinase Substrate

Kinase Substrate (Phosphorylated)

BiFC and FRET: Further Considerations



Chromophore interaction is a function of DISTANCE and ORIENTATION

N-terminal fragment fused at the N-terminal protein A + C-terminal fragment fused at the N-terminal protein B
N-terminal fragment fused at the N-terminal protein A + C-terminal fragment fused at the C-terminal protein B
N-terminal fragment fused at the C-terminal protein A + C-terminal fragment fused at the N-terminal protein B
N-terminal fragment fused at the C-terminal protein A + C-terminal fragment fused at the C-terminal protein B
C-terminal fragment fused at the N-terminal protein A + N-terminal fragment fused at the N-terminal protein B
C-terminal fragment fused at the N-terminal protein A + N-terminal fragment fused at the C-terminal protein B
C-terminal fragment fused at the C-terminal protein A + N-terminal fragment fused at the N-terminal protein B
C-terminal fragment fused at the C-terminal protein A + N-terminal fragment fused at the C-terminal protein B

And don’t forget, the linker needs to be long and flexible enough to permit interactions as well!

It's Alive!!!!!!!

Dealing with Living Material

- What is physiological temperature?
- How metabolically active is it? Do waste products induce immediate insult? Is gas required?

RADIATION

Excitation light induces photobleaching and phototoxicity

- Shorter λ → higher energy → higher resolution → more phototoxic
- Longer λ →less phototoxic but poorer resolution

- Limit exposure time/laser excitation power → but this means a weaker signal
- Limit z-series → but this means less spatial information
- Limit sampling (framing) rate → but this means poorer temporal resolution

Compromise based on EMPIRICAL DETERMINATION BALANCING *WANTS* vs *NEEDS*

Useful Online References and Primers:

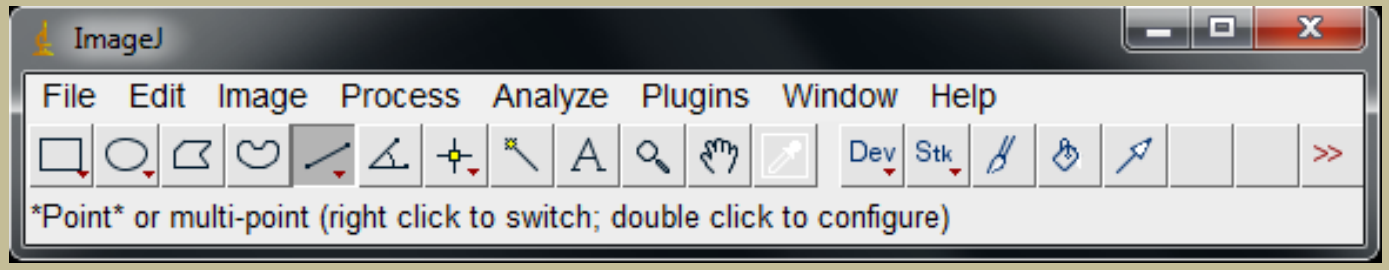
http://www.microscopyu.com/
http://zeiss-campus.magnet.fsu.edu/index.html
http://www.olympusmicro.com/index.html

Online spectra comparison
http://www.chroma.com/spectra-viewer

Questions?

LUNCH TIME!

ImageJ: A Free to Use Image Analysis Programme



By

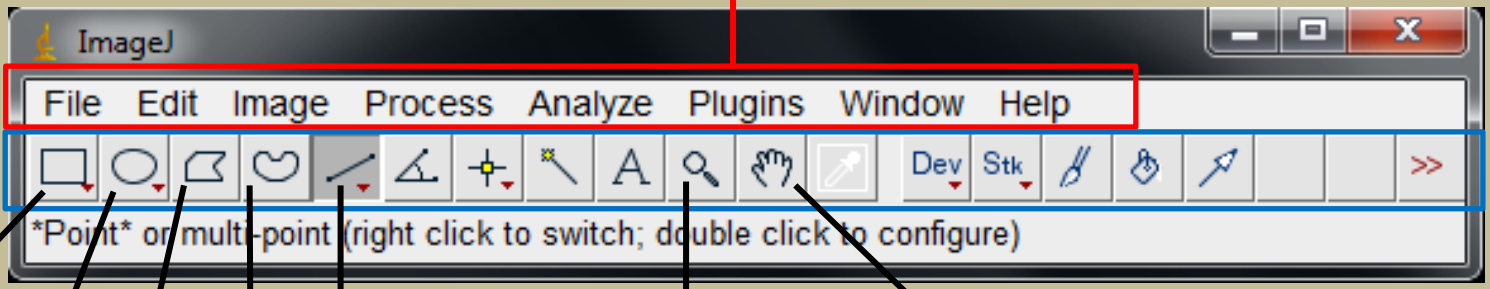
Wayne Rasband

http://imagej.nih.gov/ij/

There are multiple routes to answering any experimental challenge

If you have questions. . . ASK!

Getting Around ImageJ: Layout



Function-specific "sub-programmes"

MENUS

OPTIONS

Rectangle Tool

Circle Tool

Polygon Tool

Freeform Shape Tool

Line Tool

Zoom In/Out (shift +/-)

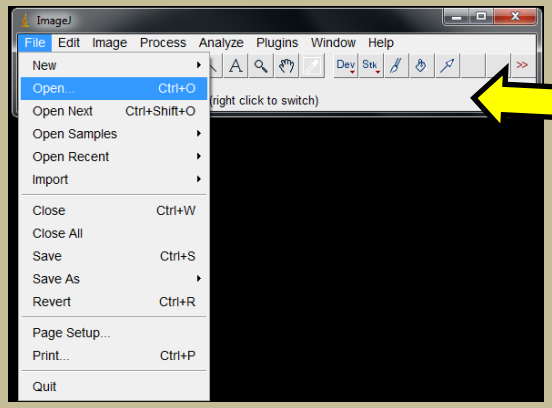
Move Image within window (when zoomed)

Tools for Defining
Region of Interest (ROI)

Getting Around ImageJ: Loading Data Sets

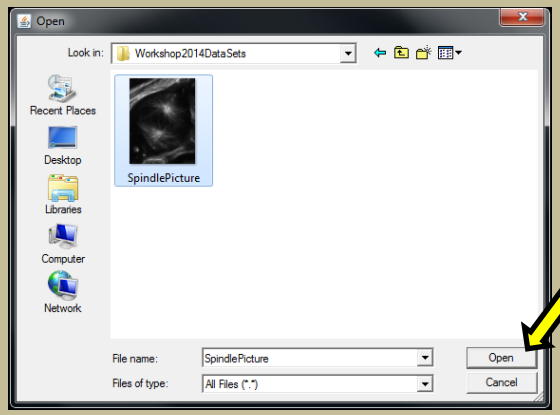
ImageJ can open just about any data format. . .
(e.g., .Lif, .avi, .tif)

➢ Open “SpindlePicture” image from “Workshop2014DataSets” folder



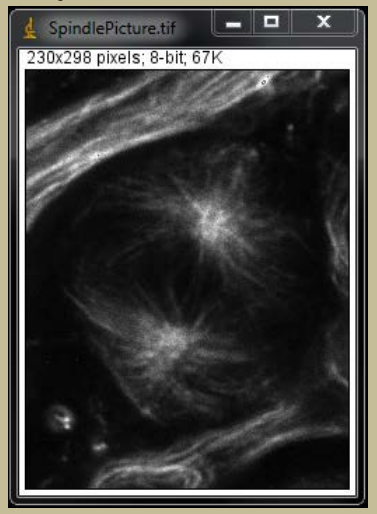
“Drag and Drop” Data Set onto ImageJ Programme Bar

OR



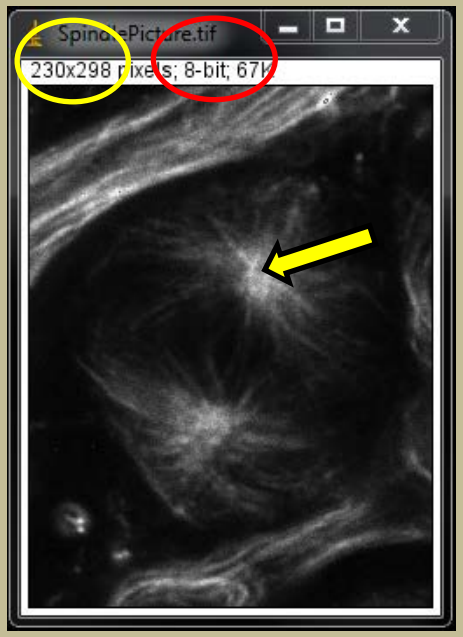
Click “Open”

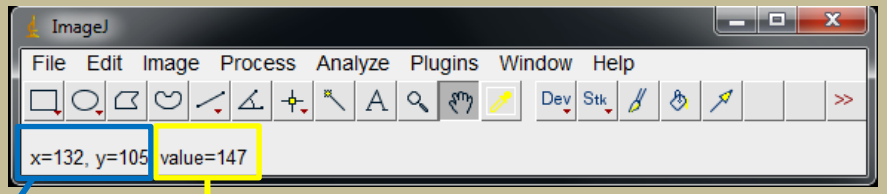
SpindlePicture.tif



Getting Around ImageJ: Histograms, LUTs & Displays

Image Size Bit Depth= # Shades

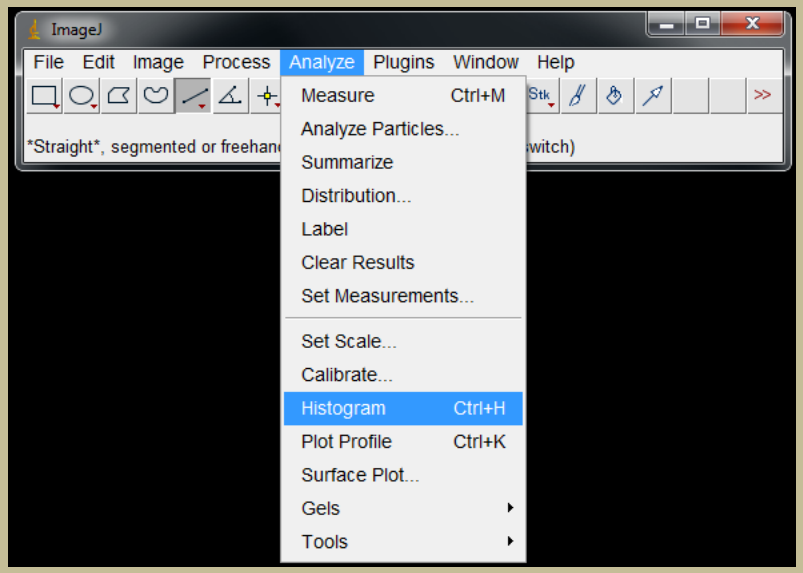


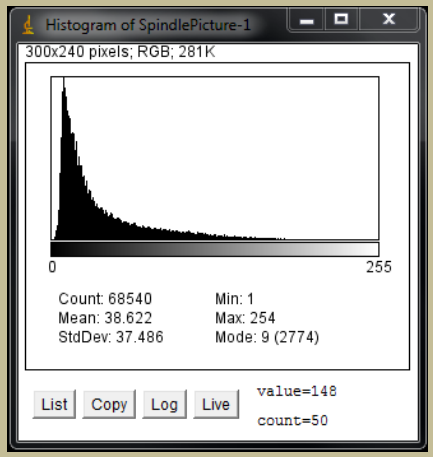


Cursor Coordinates

Pixel Intensity at Cursor

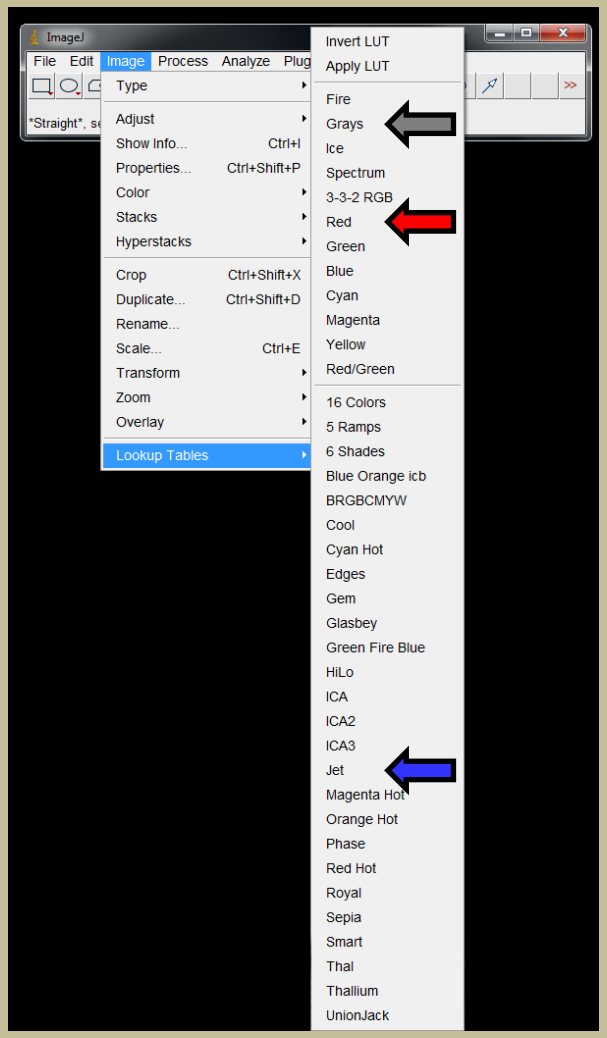
Histogram: Distribution of Shades in an Image





Getting Around ImageJ: Histograms, LUTs & Displays

LOOK UP TABLES (LUTs) change image displays but *not* their intensity values



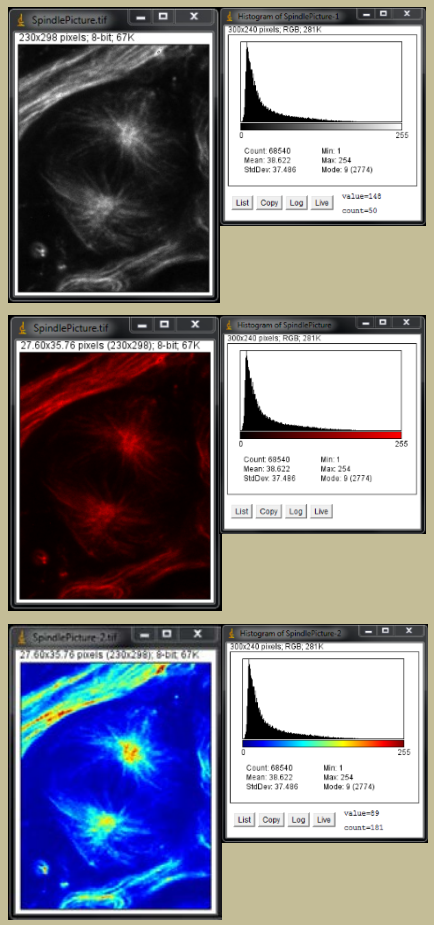
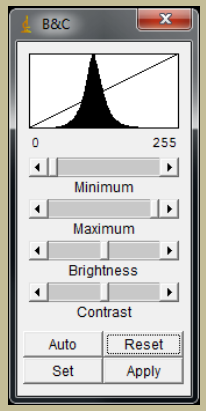
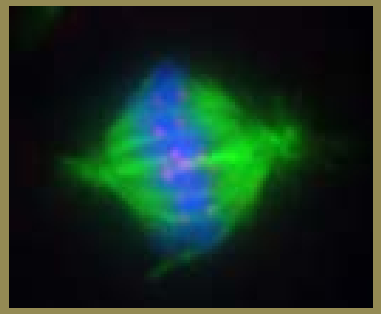


Image->Adjust->Brightness/Contrast: changes display but not image data



Getting Around ImageJ: Histograms, LUTs & Displays

An RGB colour image is 3 intensity channels with 3 different LUTs

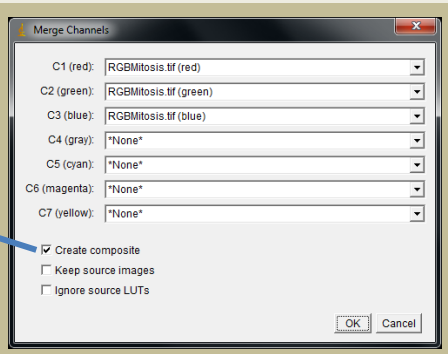


Channel1=Red=Kinetochores
Channel2=Green=Microtubules
Channel3=Blue=DNA

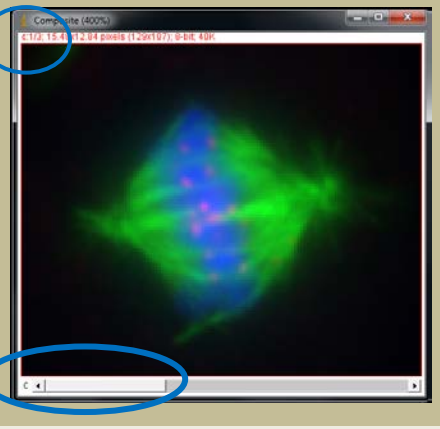
- Open “RGBMitosis” image from “Workshop2014DataSets” folder
- Look at Values with cursor, Try to alter LUT
- Image->Color->Split Channels
- Image->Color->Merge Channels

Make a Composite Image

Composite=Colour Image with Separate LUTs



Note: Channel #



- Manipulate LUTs and Brightness/Contrast for each Channel

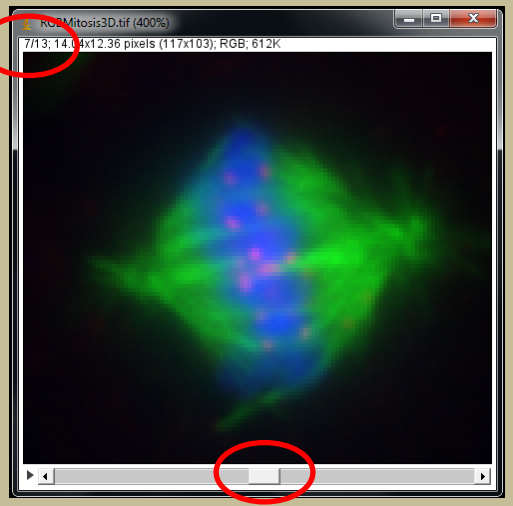
Save altered LUT choices as RGB image

- Image->Color->Type->RGB Color
- File->Save As->Tiff

Getting Around ImageJ: Histograms, LUTs & Displays

- Open “RGBMitosis3D” image from “Workshop2014DataSets” folder

z-plane information



z-plane slider

3D data sets are called “Stacks”

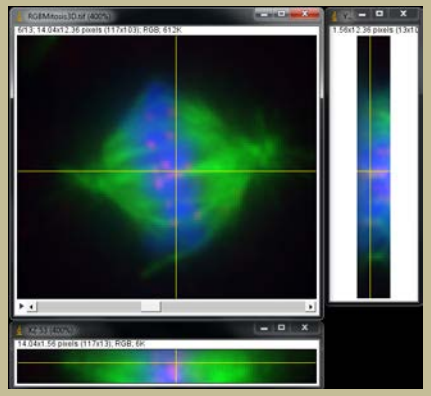
- Move through the volume- different information lay in different sections

Stacks can be manipulated

- Image->Stacks

To further view the 3D Information:

- Image->Stacks->Orthogonal Views



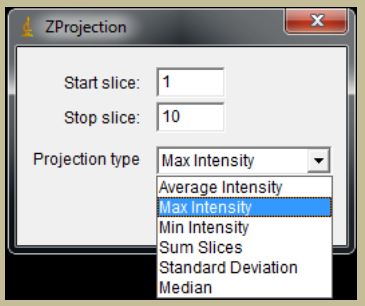
- Move through the volume by dragging the crosshair

- *ANY* image can be saved by selecting it and going to:
  - File->Save As->Tiff->. . .

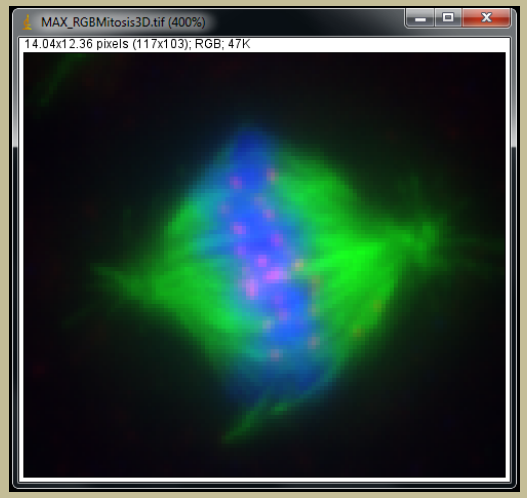
Getting Around ImageJ: Histograms, LUTs & Displays

To collapse the volume into a single 2D projection:

- Image->Stacks->Z Project



- Set top and bottom limits (exclude “empty” sections)
- Choose “Max Intensity”



Is having more sections better? Worse?

Getting Around ImageJ: Measurements

Spatial Analyses Require Image Calibration

- Image->Properties. . .

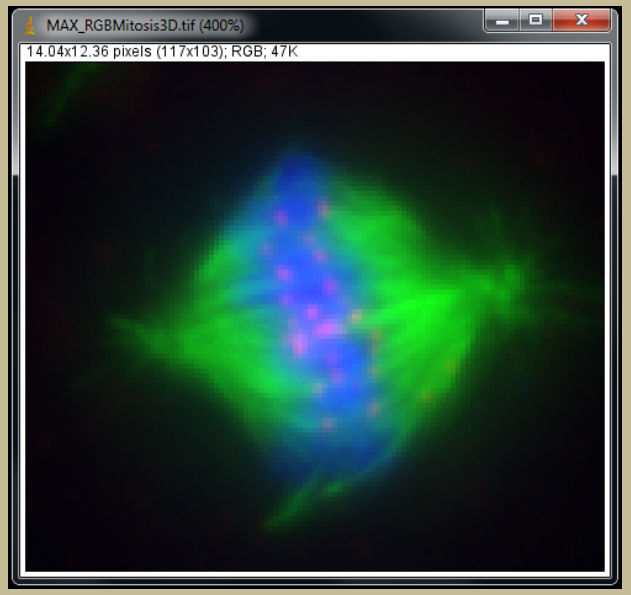
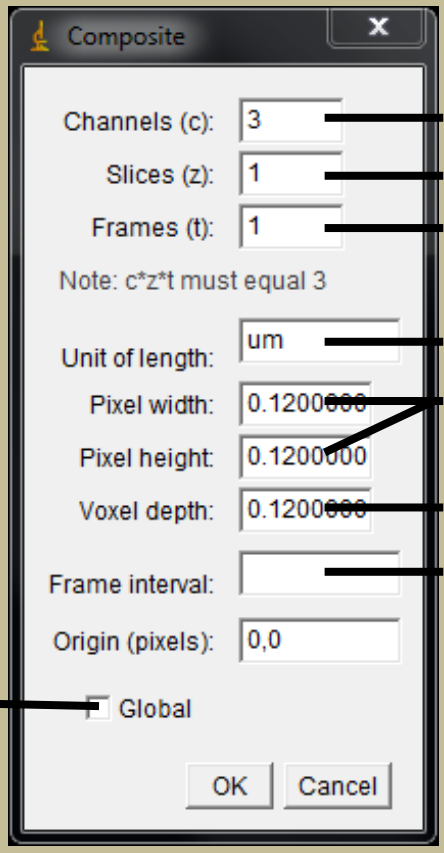


Image Properties
(commonly in file header)



channels

z-steps

time points

length units

apparent pixel dimensions

z-step size

Time between frames

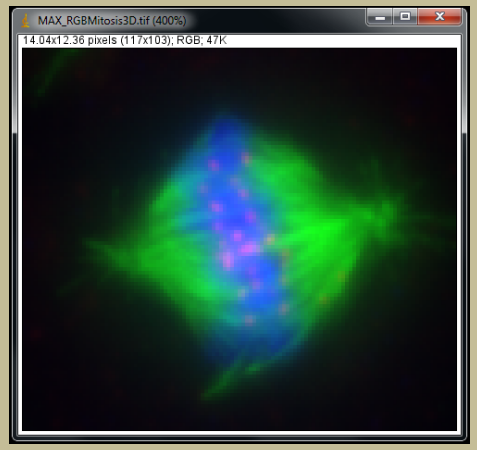
Apply properties values to all open images

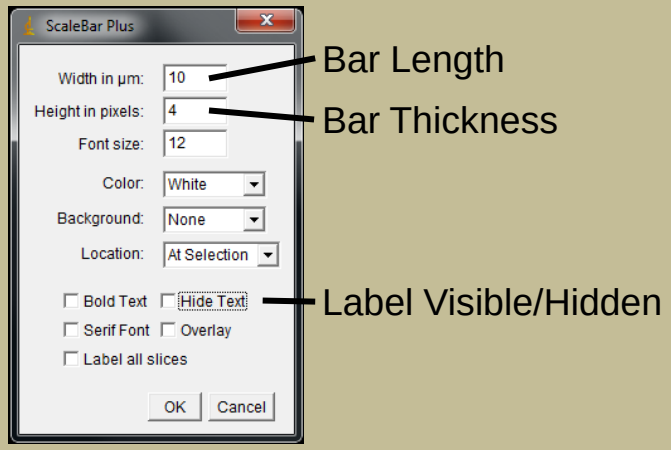
If not in the file header ask/determine empirically

Getting Around ImageJ: Measurements

To add a Scale Bar

- Analyze->Tools->Scale Bar. . .

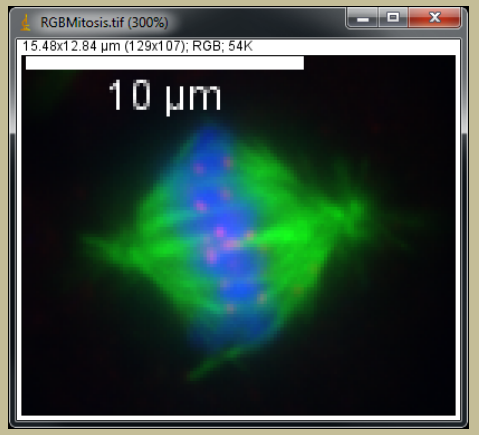




Bar Length

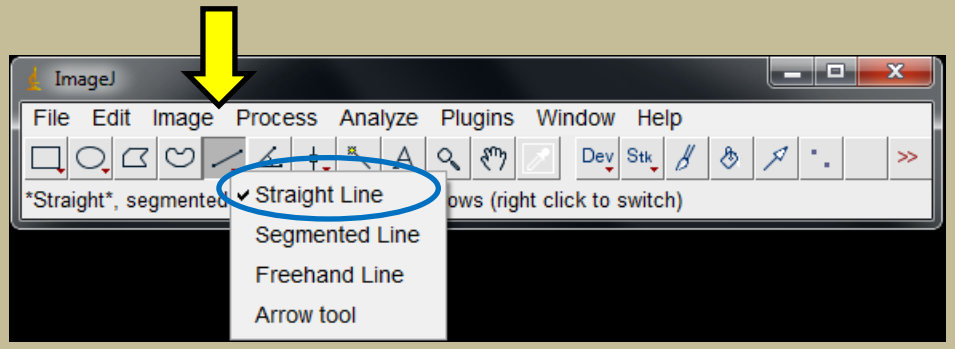
Bar Thickness

Label Visible/Hidden



Getting Around ImageJ: 2D Distance Measurements

- Open “3DMeasureRGB” from “Workshop2014DataSets” folder
- Collapse to Max. Int. Proj
- Use Line Tool to draw line between centrosomes



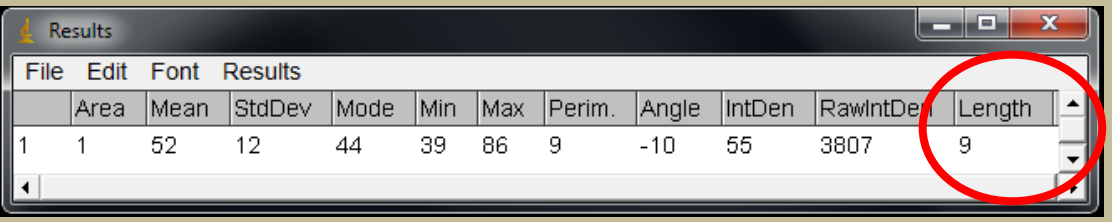
Different line options are accessed by Right Click

Measure Line By:

- Analyze->Measure

OR

- Ctrl + M

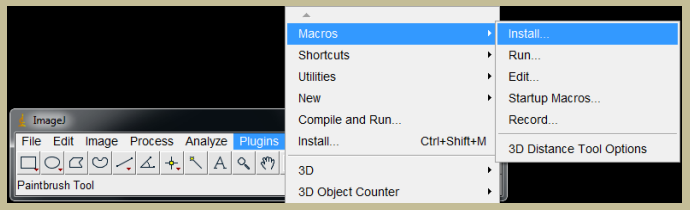


- Copy and Paste Results in Spreadsheet (i.e., Excel)

Getting Around ImageJ: 3D Distance Measurements

- Open “3DMeasureRGB” from “Workshop2014DataSets” folder

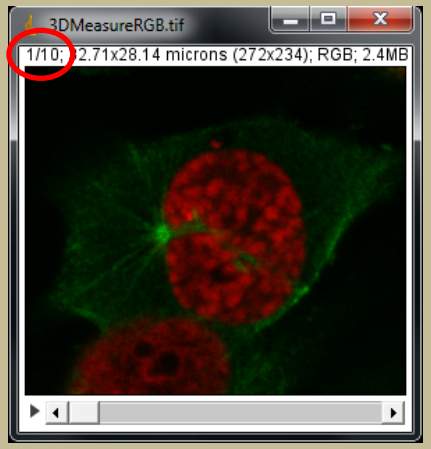
- Install Macro “3D-Distance-Tool”



OR

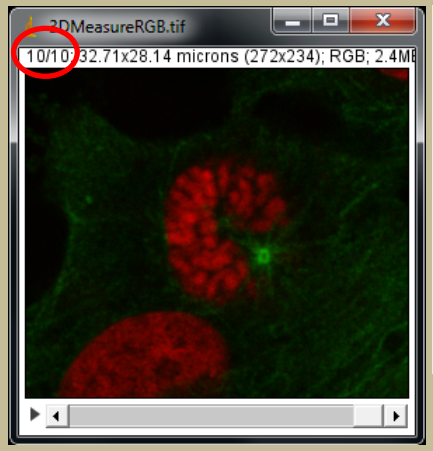
- Drag and drop “3D-Distance-Tool” on Toolbar

- Plugins->Macros-> “3D-Distance-Tool Options”



Run Macro

- Left click to position first marker



- Alt + Left click to position second marker in different z-plane

- Distance Listed

Separation distance in x,y,z is 12.58 µm vs. 9 µm in x,y

2D projections may be misrepresentations of separations and distances

Getting Around ImageJ: Object Counting/Analysis

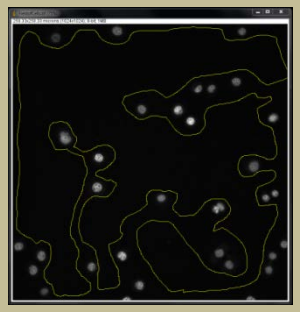
- Open “FieldofCells” image from “Workshop2014DataSets” folder

How many nuclei are in the field and how large are they?

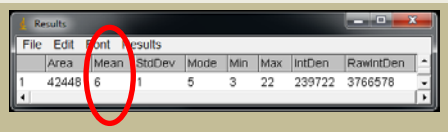
We could manually count and measure or have the computer do the labour

Remember:
Object Signal Intensity = Signal of Interest + Background

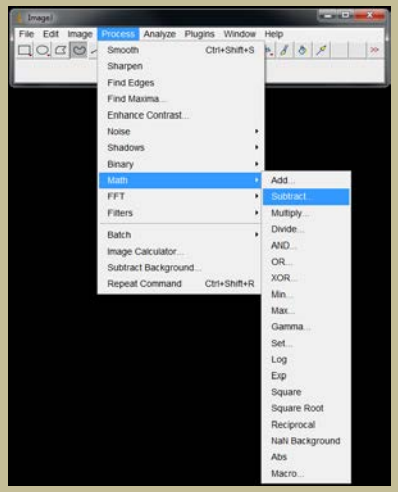
1) Determine Background



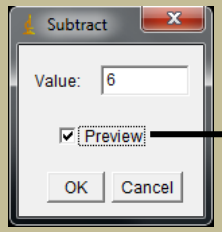
- Use Freeform tool to define background (more area is better)
- Measure and Determine Mean Intensity



2) Subtract Background



- Process->Math->Subtract



Preview Result

Corrected Resultant Image

Background=0

Getting Around ImageJ: Object Counting/Analysis

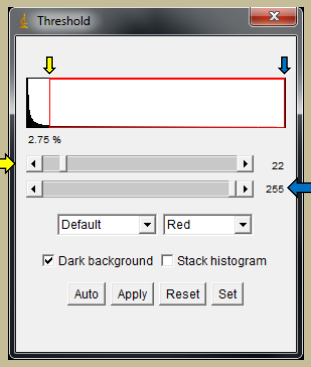
Thresholding and Automated Analysis

Segmentation: Defining objects of interest from the background and one another

- Image->Adjust->Threshold

Corrected Resultant Image

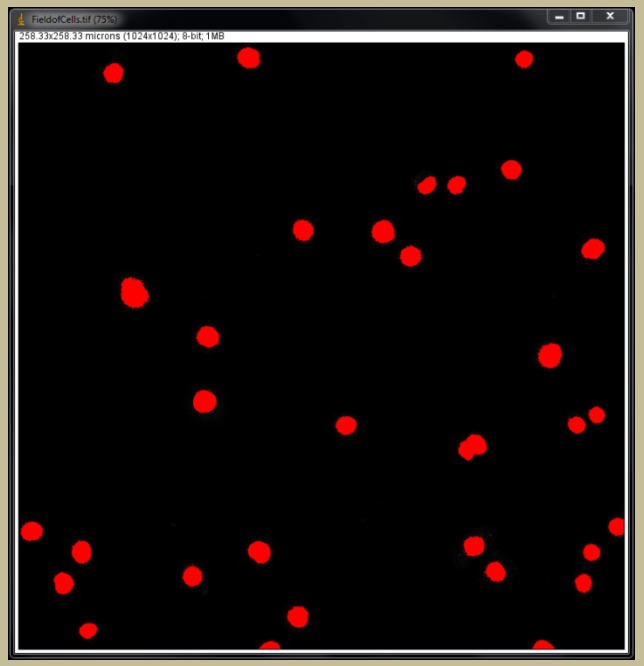
Thresholding includes/excludes intensity ranges



Set lower limit

Set upper limit

Only intensities between 22-255 will be registered



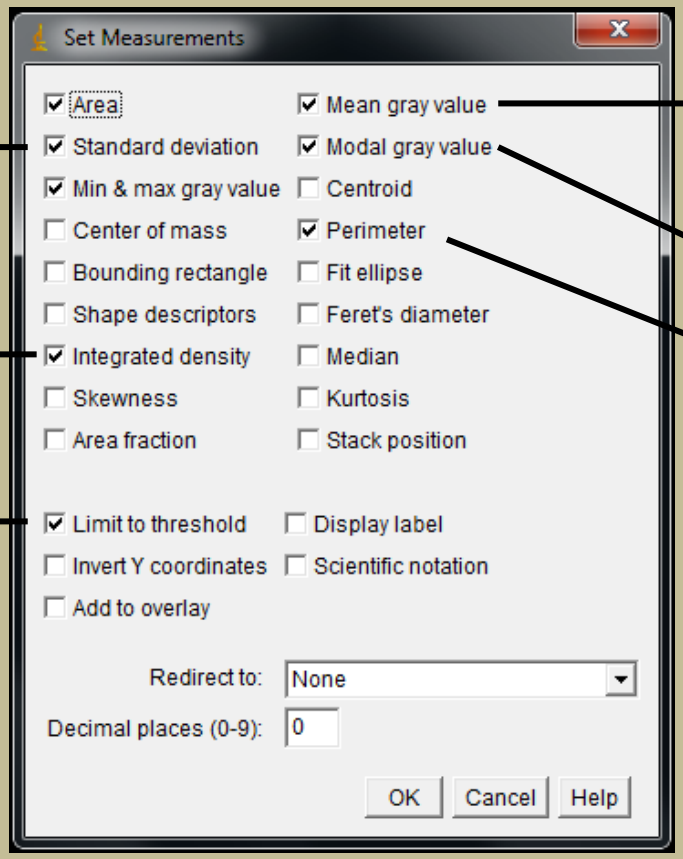
8 bit image reduced to 2 bit (red signal on black background)

Getting Around ImageJ: Object Counting/Analysis

Thresholding and Automated Analysis

- Analyze->Set Measurements

[Define Parameters to be Measured]



Area, Deviation and Intensity Boundaries

Summation of intensity values

Only thresholded objects analysed

Summation of all intensity values/total # of pixels

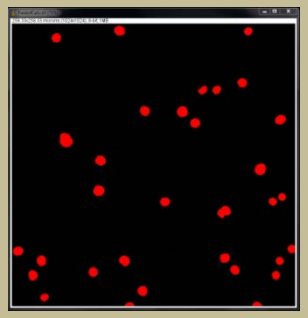
Most frequent intensity value

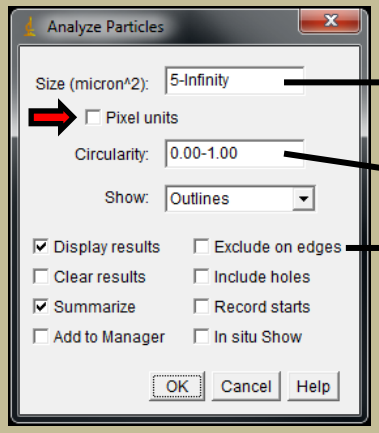
Perimeter

Getting Around ImageJ: Object Counting/Analysis

Thresholding and Automated Analysis

- Analyze->Analyze Particles





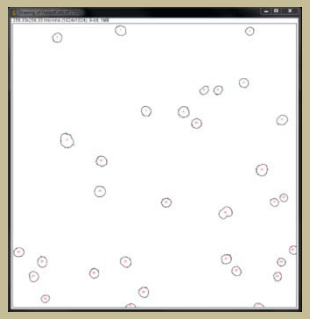
Particle size range (real units or *pixels*)

Circle=1.00

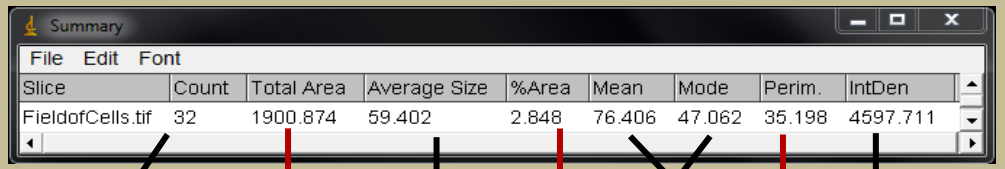
Do not analyse particles touching edge of screen

OUTPUT

Outlines of Thresholded/ Analysed Particles



Summary of Results Table



Total Particle #

Total Area (um)

Avg Area (um^2)

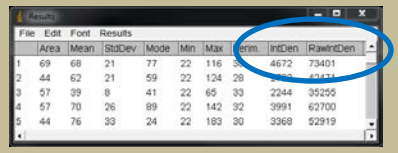
% image area thresholded

Intensity Data

Avg. Perim (um)

Avg. Int. Den (Mean Int. *Area)

Individual Results Table



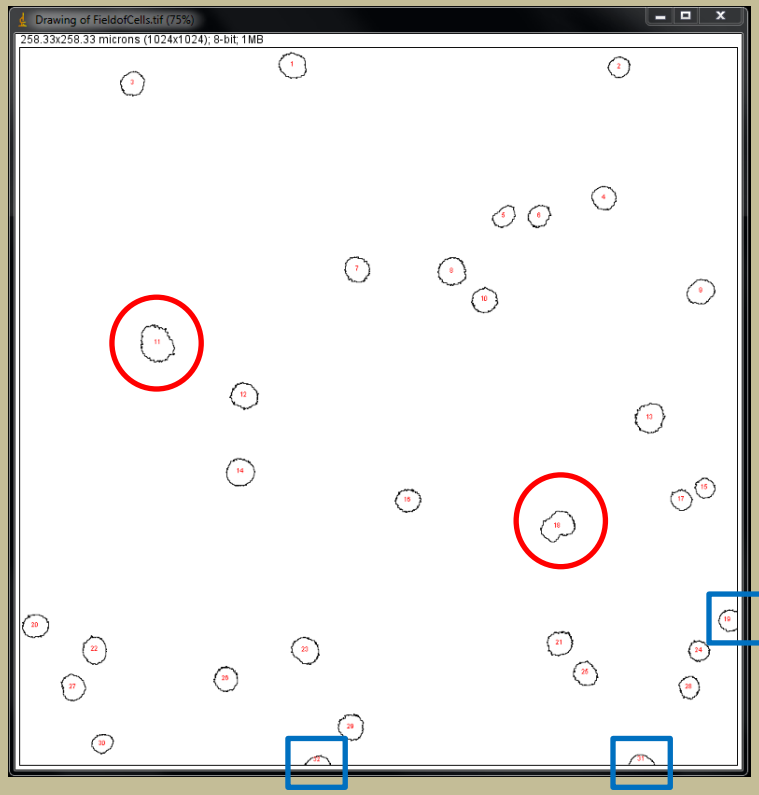
Intensity in two forms:

*Mean Int.*Area* *Sum of Int.*

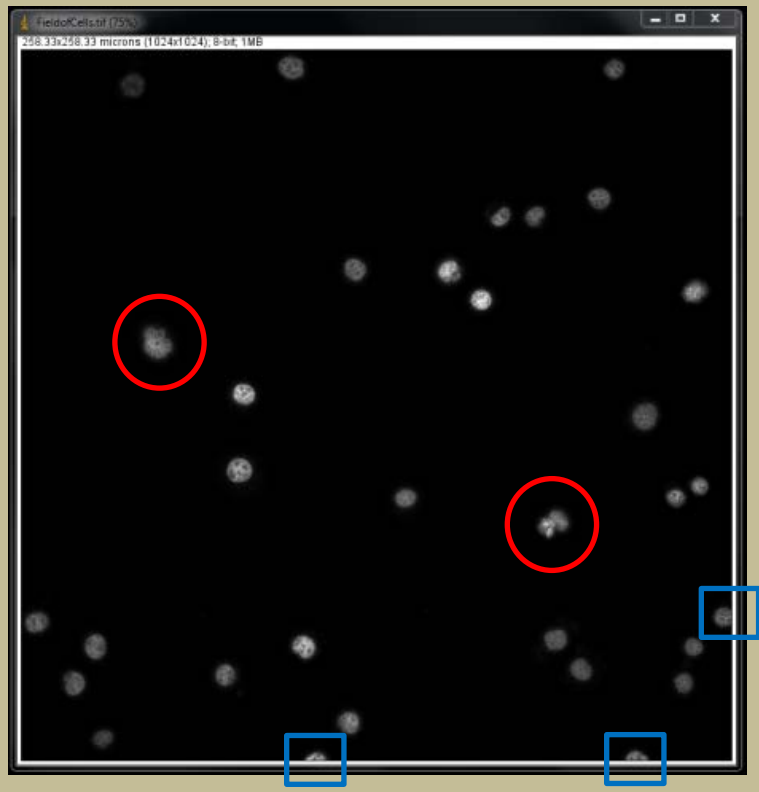
Getting Around ImageJ: Object Counting

Thresholding and Automated Analysis

BUT COMPUTERS ARE IMPERFECT!



Review Original image as 8bit "Grays" LUT



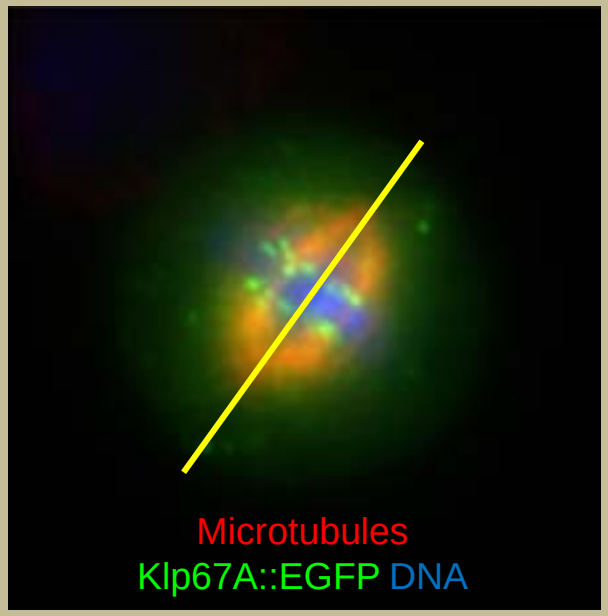
Nuclei missed/# underestimated

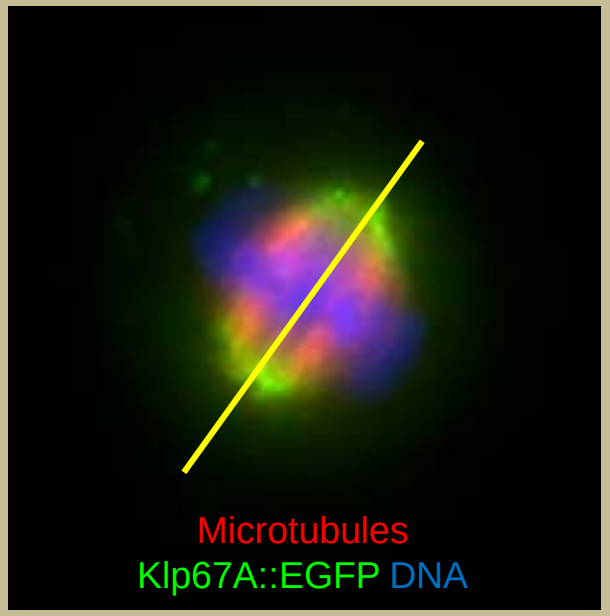
Partial nuclei counted/# overestimated, area underestimated

(can remove via "Exclude on edges")

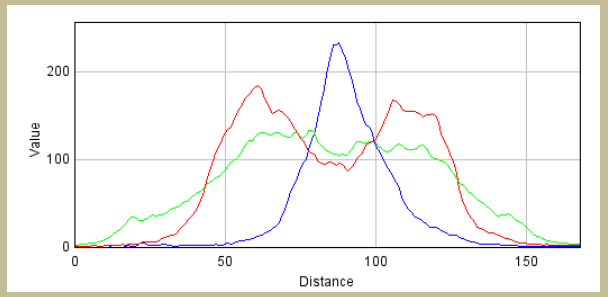
Getting Around ImageJ: Comparing and Quantifying Fluorescence

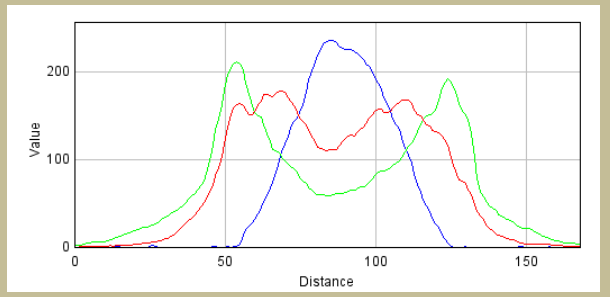
Linescans reveal intensity *distributions*





How does the distribution of Klp67A vary?

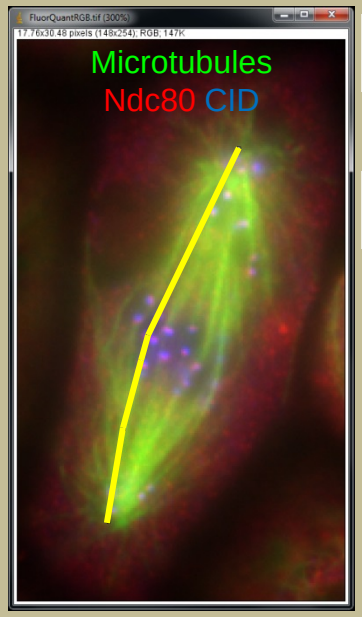




Getting Around ImageJ: Comparing and Quantifying Fluorescence

Linescans compare intensity *distributions*

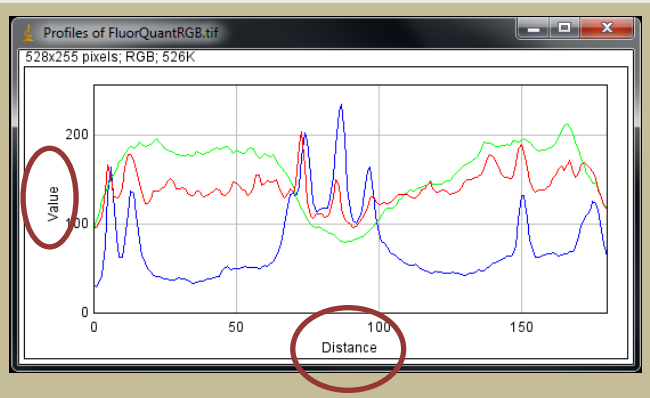
- Open “FluorQuantRGB” image from “Workshop2014DataSets” folder



- Use line tool to draw line ROI across structures/features of interest

Use multi-segment line since object is not straight

- Plugins->Colour Functions->RGB Profiler

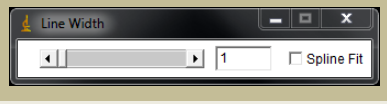


Distance in PIXELS

Intensity in Arbitrary Units

Changing line width or orientation affects profile

- On Line Tool->Double left click

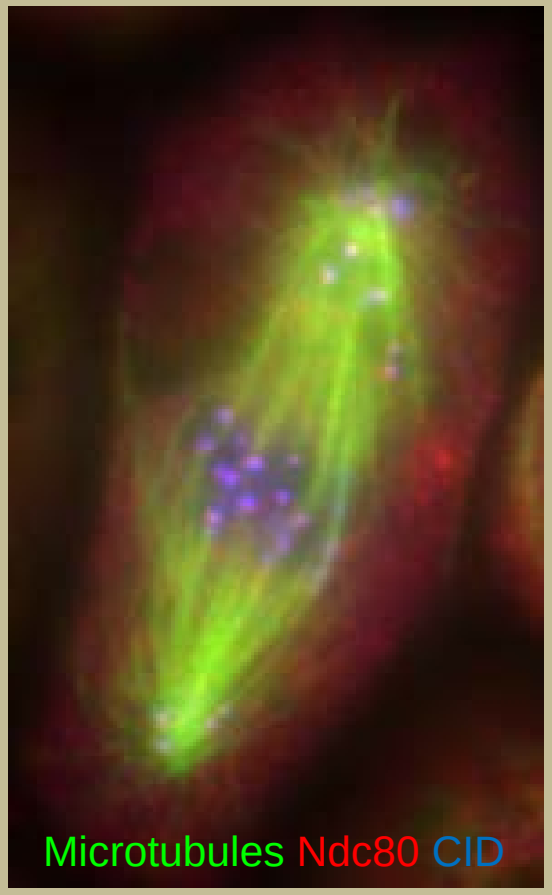


To save plot:

- File->Save As->Tiff

Getting Around ImageJ: Comparing and Quantifying Fluorescence

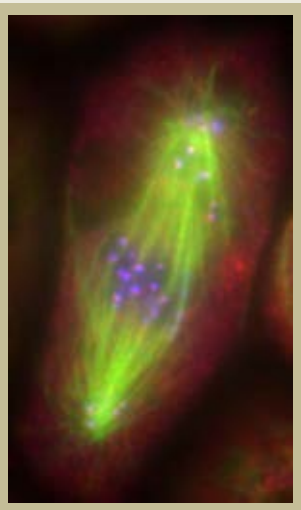
Quantifying Subcellular Intensities



How do we quantify the discreet accumulations of the protein shown in RED?

Getting Around ImageJ: Comparing and Quantifying Fluorescence

- Open “FluorQuantRGB” image from “Workshop2014DataSets” folder



But any intensity data is R+G+B

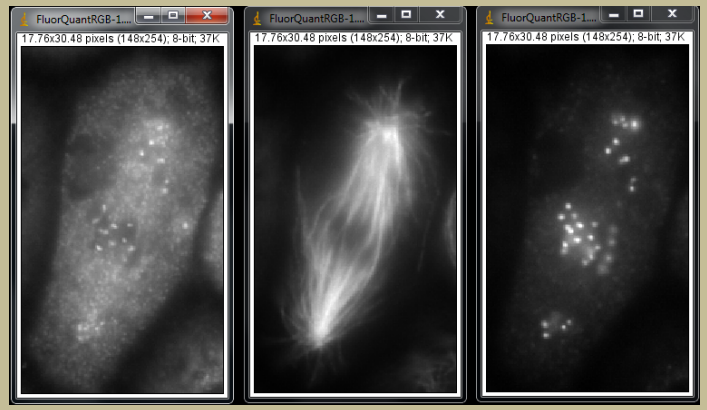
We want Red Channel Intensity only

Need to isolate red channel

- Image->Color->Split Channels

Three individual channels

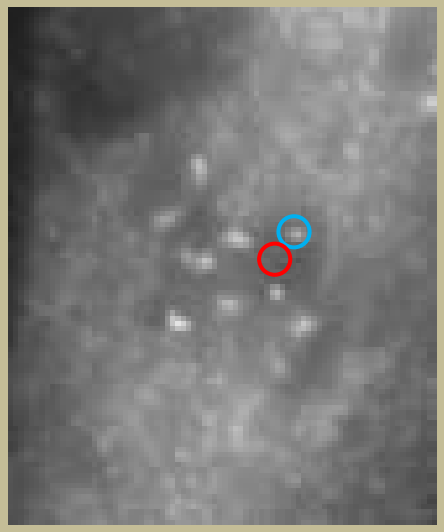
Red Green Blue



Getting Around ImageJ: Comparing and Quantifying Fluorescence

Remember: Signal Intensity = Signal of Interest + Background
This varies within the image so can't globally subtract it

Red Channel



- Draw ROI encompassing *Object*
- Measure Intensity (Ctrl + M)
- Move ROI to *appropriate BACKGROUND*
- Measure Intensity (Ctrl + M)

Signal
Background

Results

File Edit Font Results

	Area	Mean	StdDev	Mode	Min	Max	Perim.	IntDen	RawIntDen
1	1	114	17	98	93	165	3	86	5947
2	1	100	4	103	93	107	3	75	5213

- Copy and Paste Results in Spreadsheet (i.e., Excel)
- Use Equation:

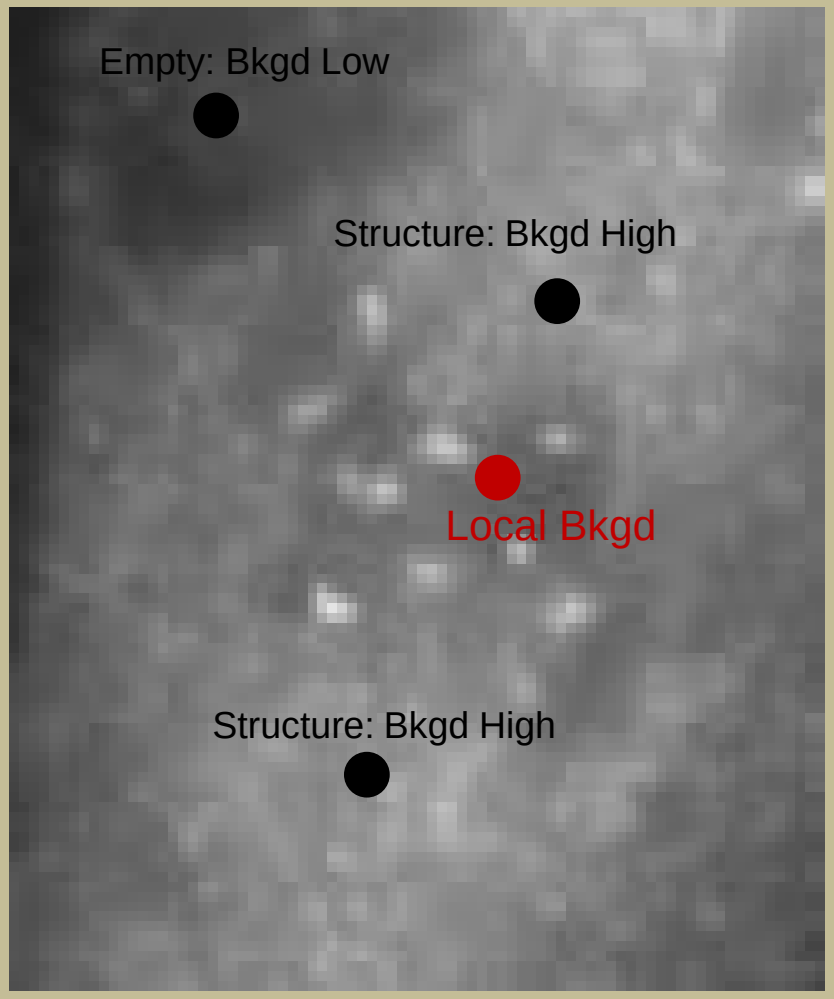
$$Intensity_{Corrected} = (Intensity_{Signal} - Intensity_{Background})/Intensity_{Background}$$

$$Intensity_{Corrected} = (5947-5213)/5213$$

0.14 *Arbitrary Units*

Getting Around ImageJ: Comparing and Quantifying Fluorescence

What is “appropriate” Background and why does if matter?



Background *MUST* reflect measured object’s local environment

$Intensity_{Corrected} = (Int._{Signal} - Int._{Background})/Int._{Background}$

Background too high=$Intensity_{Corrected}$ too low
Background too low= $Intensity_{Corrected}$ too high

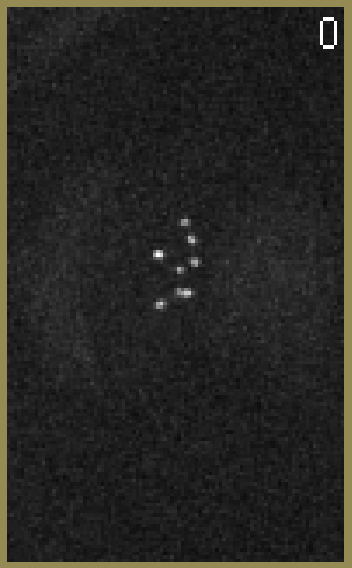
To compare data between samples/slides, *imaging conditions should be constant*

This means that exposure/laser power/gain/etc., must be determined for brightest sample first (to avoid saturation)

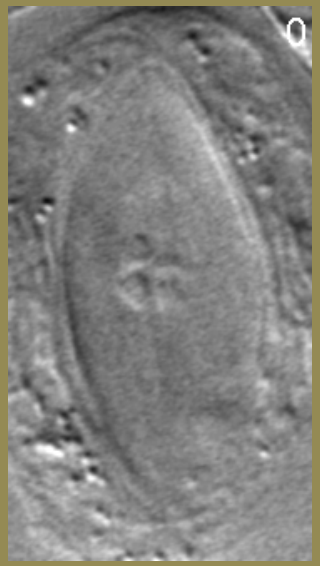
Getting Around ImageJ: Quantifying Movement

(Demonstration Only)

Centromeres labelled with EGFP



DIC



Dividing fly cells

How fast do the chromosomes move during division?

Fluorescence and Transmitted Light data can be tracked

Useful data requires adequate SPATIAL and Temporal resolution (~3 pixels movement per time point)

Getting Around ImageJ: Quantifying Movement

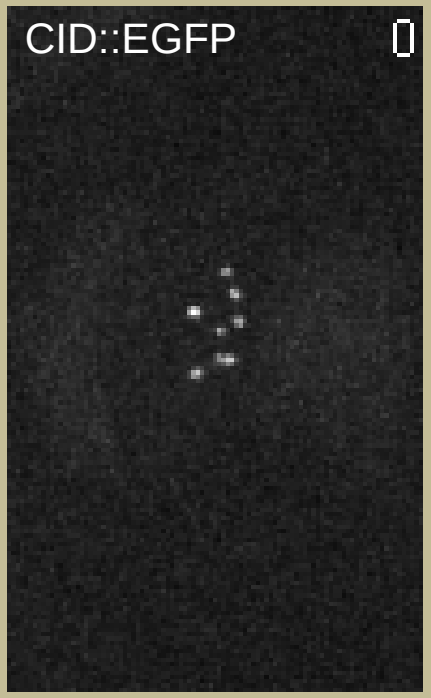
Object “automatic tracking” plugins for ImageJ:

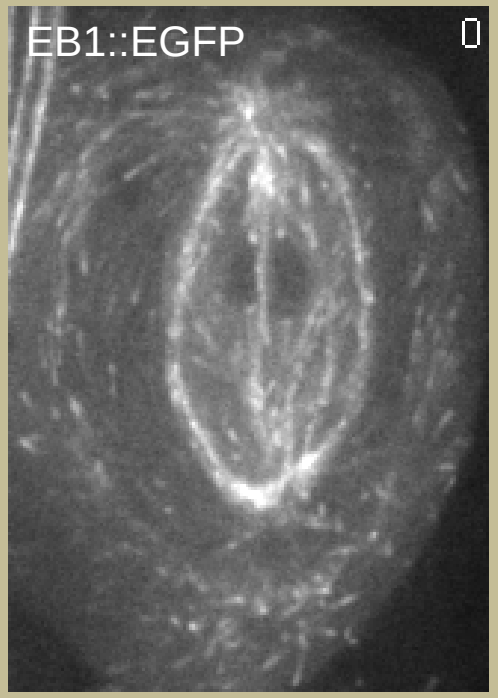
- Difference Tracker
- MTrackJ2
- MultiTracker
- ObjectTracker
- SpeckleTrackerJ
- SpotTracker
- TrackMate

All based on segmentation

Requires:

- Thresholding
  (defining object vs. background)
- Defining object/particle size
- Objects MUST remain *distinct*
  to be followed with confidence



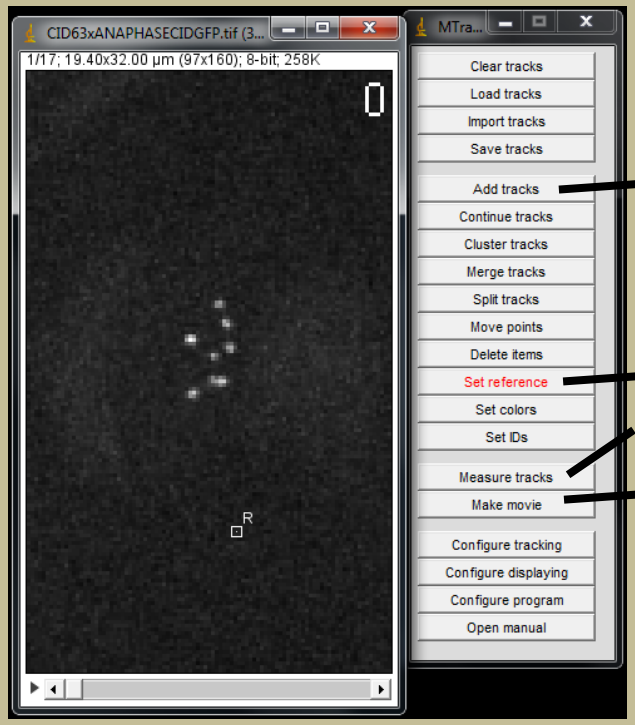


Getting Around ImageJ: Quantifying Movement

Semi-Automated Tracking

MTrackJ By Erik Meijering

http://www.imagescience.org/meijering/software/mtrackj/

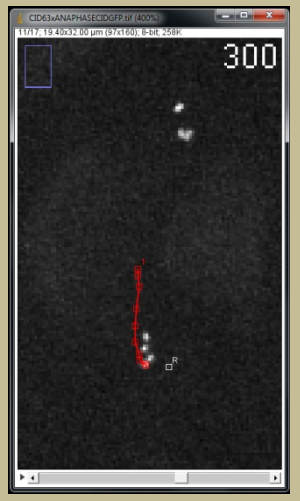


Initiate new set of measurements

Define reference (R) for movements

Calculate displacement and velocity

Overlay user defined path on data



Each mouse click positions data point and advances to next frame

Copy/export data for further analysis

Getting Around ImageJ: Quantifying Movement

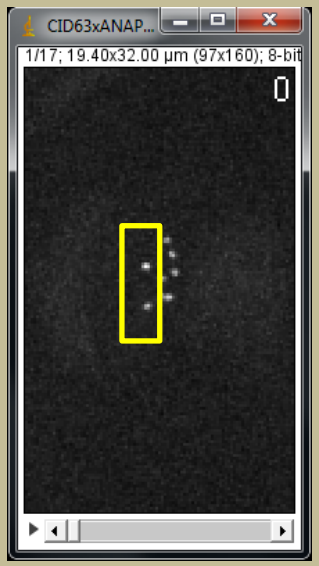
Kymographs: Time/Space Plots

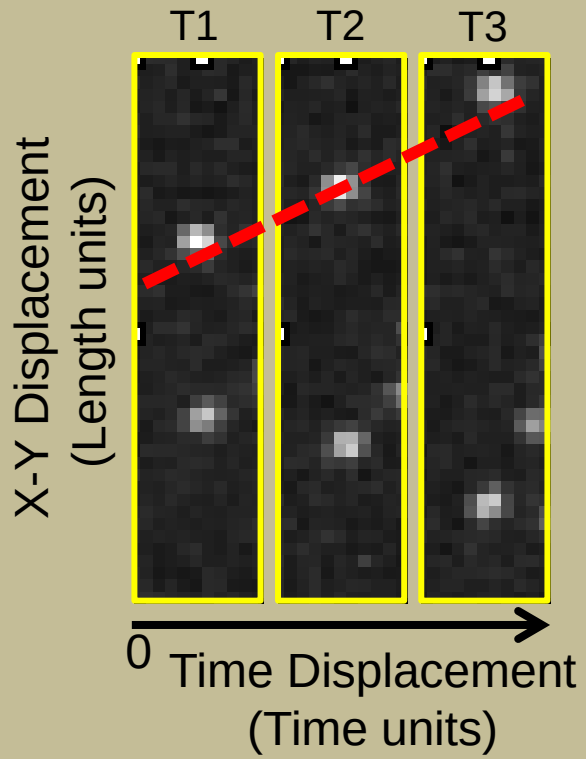
e.g., Kbi Kymograph, Kymograph, MultipleKymograph

Kbi Kymograph (Kbi Tools Plugins) By Natsumaro Kutsuna

http://hasezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ImageJKbiPlugins

What is a kymograph?





Because pixels are calibrated in space and time

SLOPE=VELOCITY

Getting Around ImageJ: Quantifying Movement

Kymographs: Time/Space Plots

Basic procedure illustrated with Kbi Kymograph

Open data set

Make Max. Int. projection to reveal object movement pathway

Draw line along object pathway

Duplicate line on original data set

- Edit->Selection->Restore Selection

Make kymograph

- Plugins->Kbi_Kymograph

Analyse kymograph to get slope/velocity

- Draw line along object edge
- Plugins->Kbi_KymoMeasure
- Calibrate
- Copy/Export velocity

Acknowledgements

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

MMIC Manawatu Microscopy & Imaging Centre

Jordan Taylor (TEM)
J.W.Taylor@massey.ac.nz

Niki Murray (SEM)
N.A.Murray@massey.ac.nz

Remember, MMIC is now free for Massey Work!