Practical light microscopy: an introduction

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Aim of today's talk:

- Explanation of the very (very) basics of how a light microscope works
- Illustration of the most important modifications that make the microscope more useful--phase contrast, DIC, fluorescence
- Outline the bacterial chemotaxis practical session

What can we do with a light microscope?

In a nutshell:

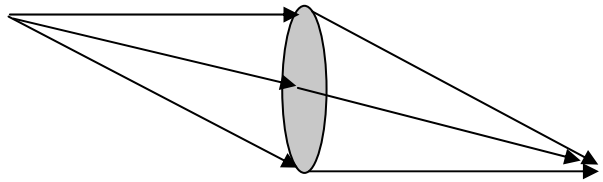
- Magnify things, resolve details not possible with the naked eye.

More specifically:

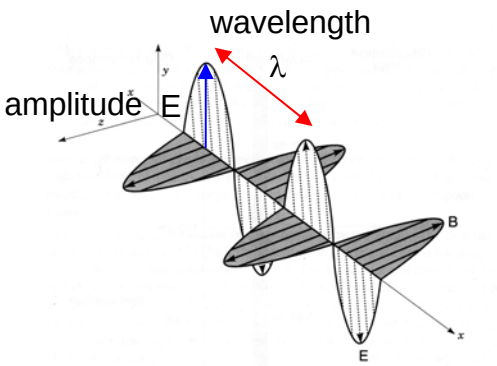
- Obtain information about the distribution of specific molecules inside cells, including sub-cellular structures
- Follow changes in cells or molecules over time.

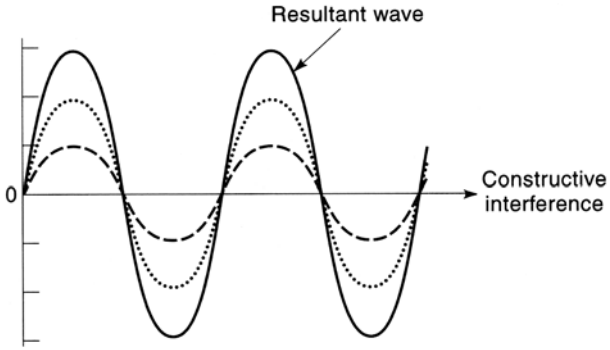
Light has both particle and wave properties

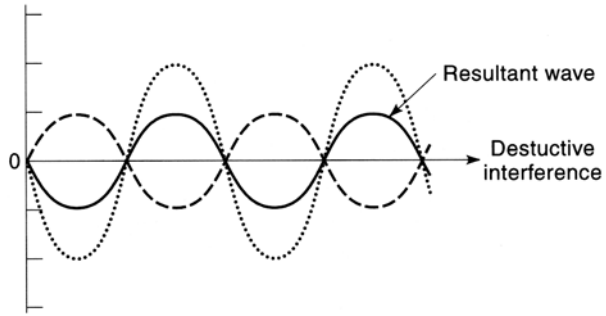
- Quantum mechanics -- too complicated for today!
- Light travels in a straight line (rays)--like a bullet



- Light also has "wave" properties, such as wavelength, and interference







Lenses utilize “refraction” of light

Interaction of light with matter can alter speed, and path, of light.

How? The electrons in the material interact with the vibrations of the electromagnetic field.

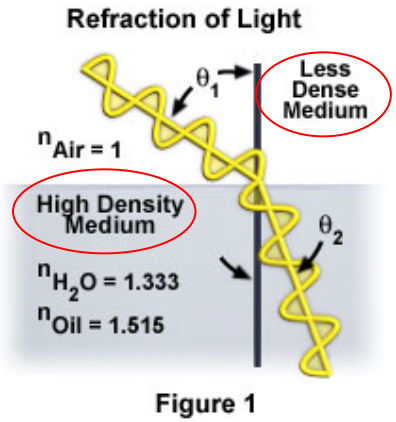
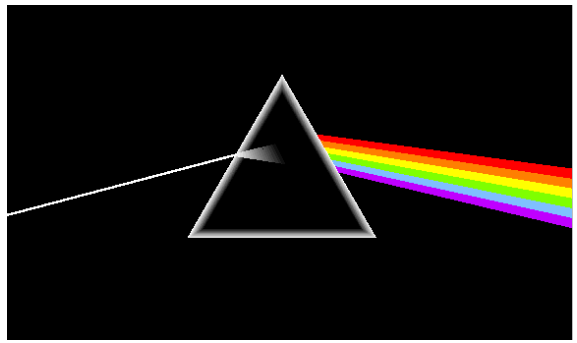
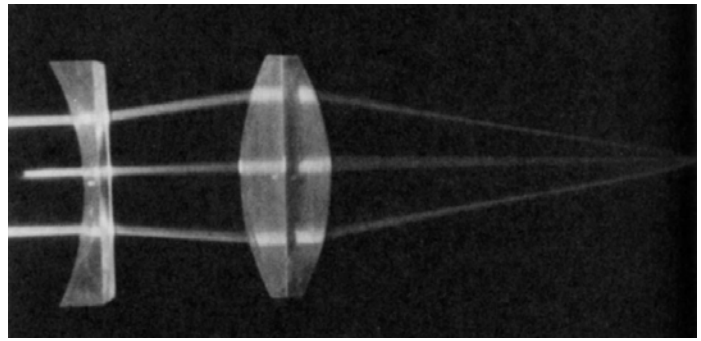


Figure 1

A prism: different wavelengths are refracted (bent) to different degrees



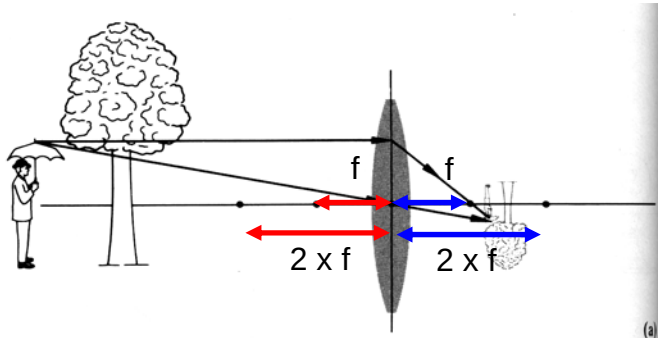
A lens: refraction at curved surfaces can cause the light rays to converge (or diverge)



How do lenses magnify?

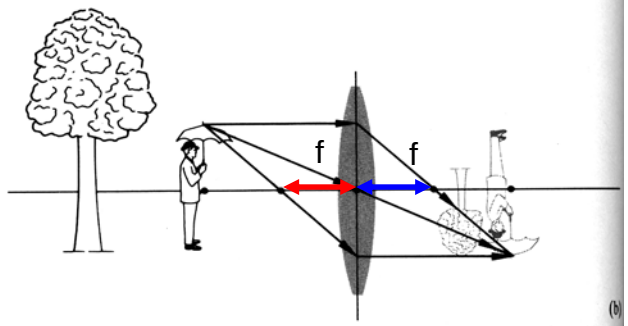
f = "focal length", or "focal distance", of lens.
Magnification depends on position of object relative to the lens.
This can be seen by "ray tracing" (black lines and arrows).

Object at distance greater than 2 x f from the lens



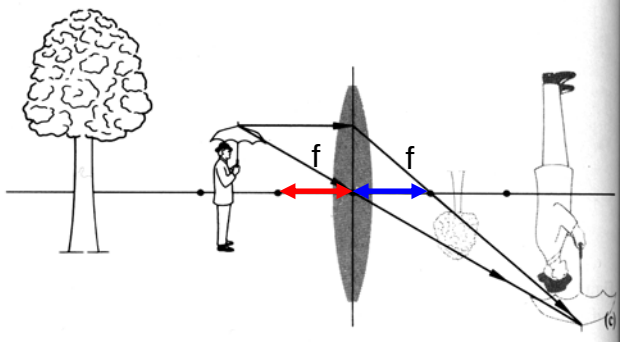
Miniature image formed, at distance between f and 2 x f

Object exactly at distance 2 x f from the lens



Same-size image formed, at distance 2 x f from the lens

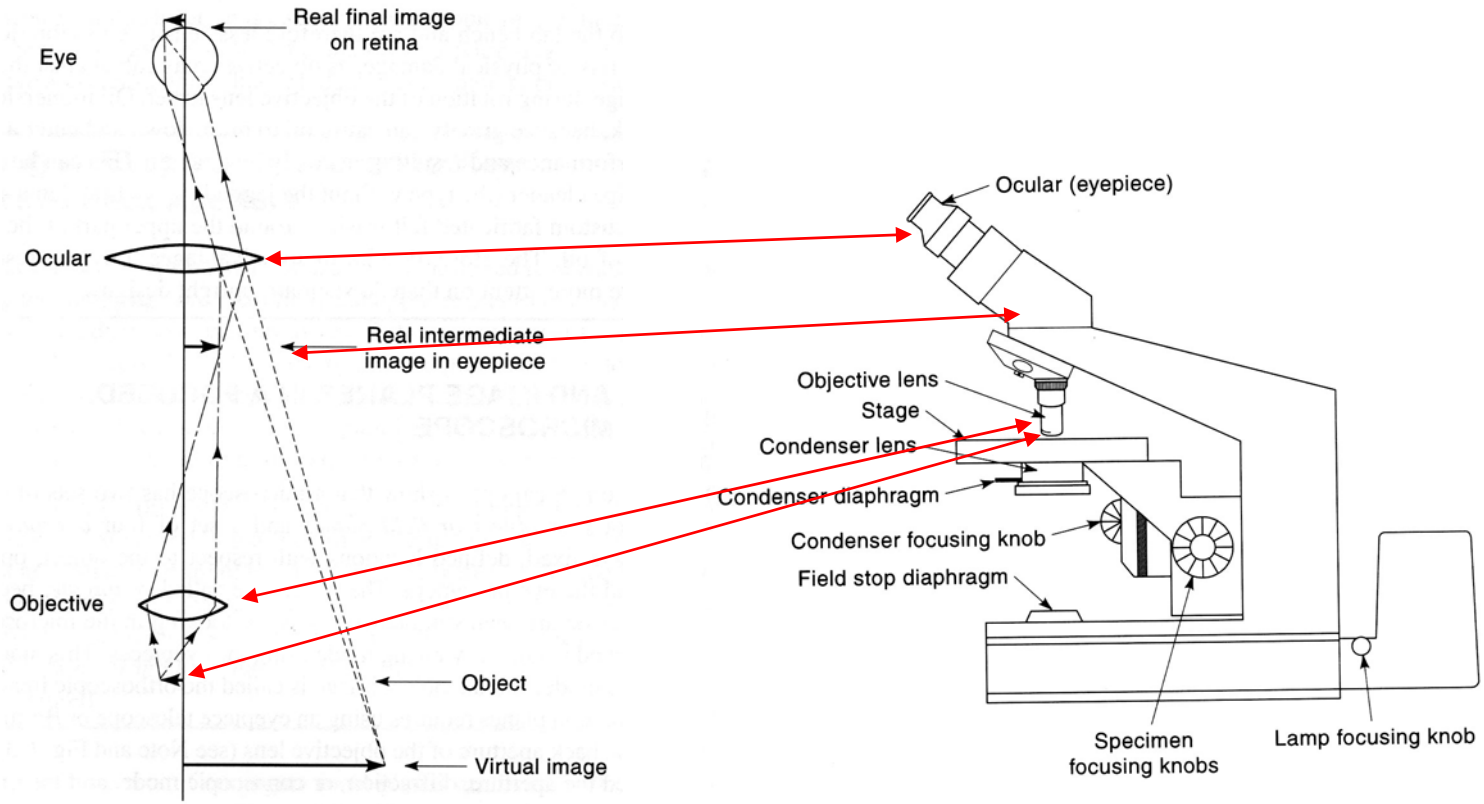
Object at distance between 2 x f and f from the lens



Magnified object formed, at distance than distance "f" the lens

The compound microscope

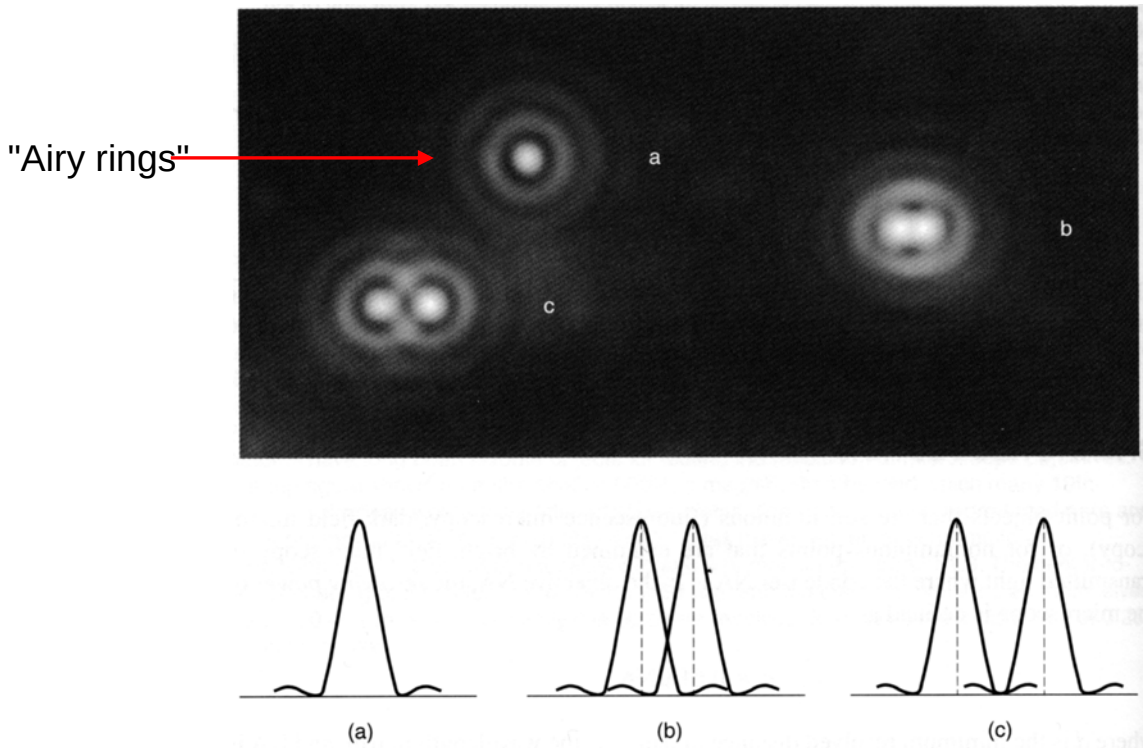
Conceptually, the compound microscope is not much different from a magnifying glass, but it has two different stages of magnification (hence "compound")--the objective and the eyepieces.
The objective creates an image inside the microscope, and this is further magnified by the ocular (eyepiece).
The eye is part of the "imaging system"!
In addition, everything is mounted on a stand, which makes it easy to focus, and there is a built-in illumination source.
The condenser lens (under the stage) directs the light correctly onto the sample.

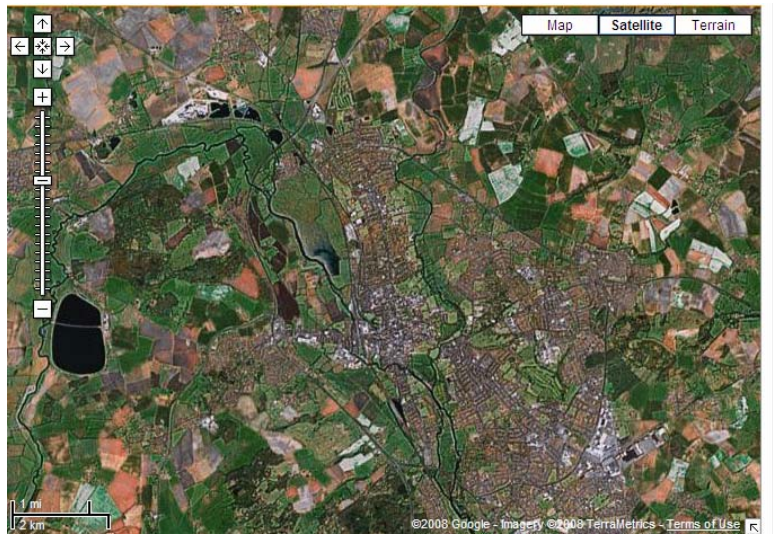


Magnification alone isn't sufficient

Resolution:

The ability to distinguish two points very close together

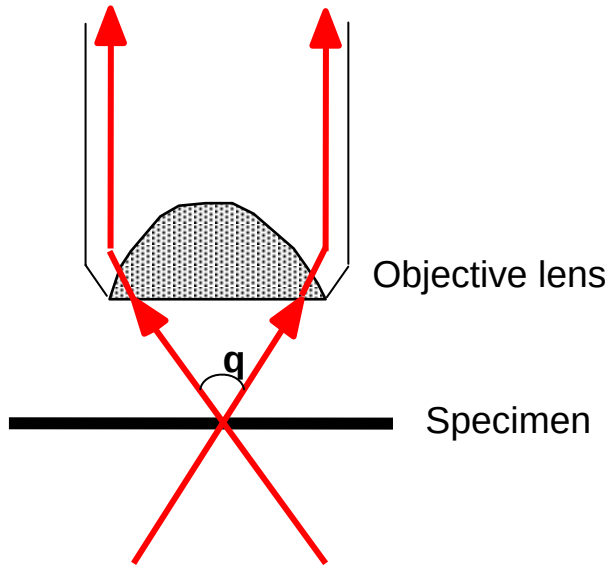


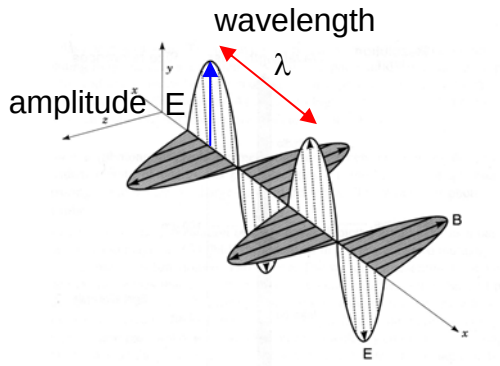


The Royal Oak

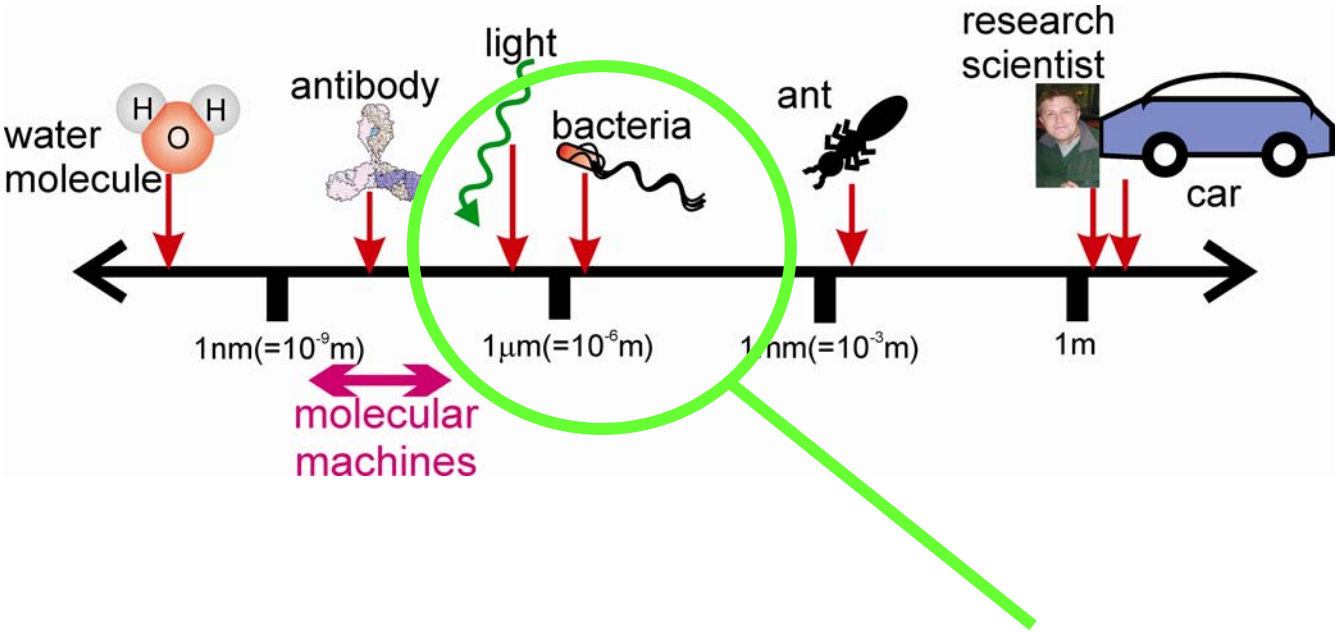
What determines the resolution limit?

- No lens has perfect resolution, even in theory
- Resolution depends on the angle (θ) of the cone of light that the objective can collect from the specimen.
- This angle, called Numerical Aperture, in turn depends on the lens diameter and on the distance from the specimen to the lens.
- For all practical purposes, improvements of Numerical Aperture in microscope design have reached their limits.
- The other factor affecting resolution is the wavelength of light itself.





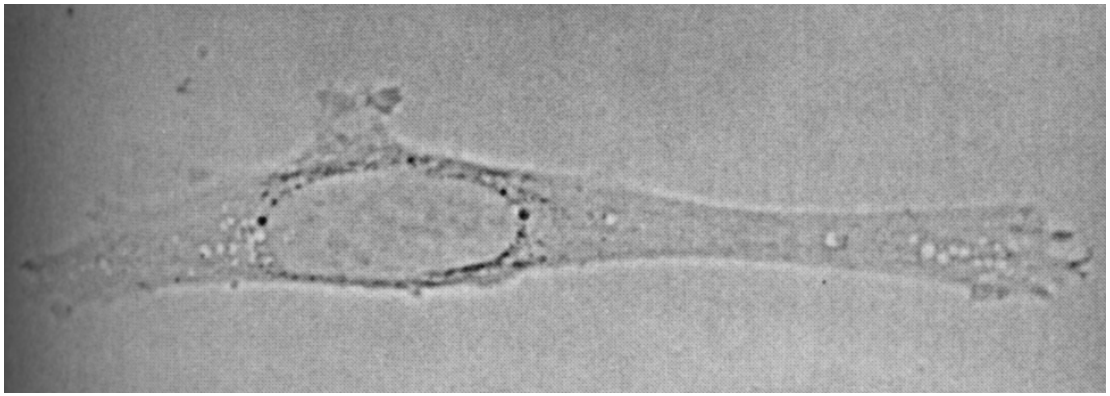
Useful size range for light microscopy



Using standard light microscopy most structures and substructures we observe are within a typical range from about 300 μm down to about 0.3 μm (300 nm), though we can detect single molecules under certain circumstances

By 1900 generic microscope designs were very similar to those of today, but…

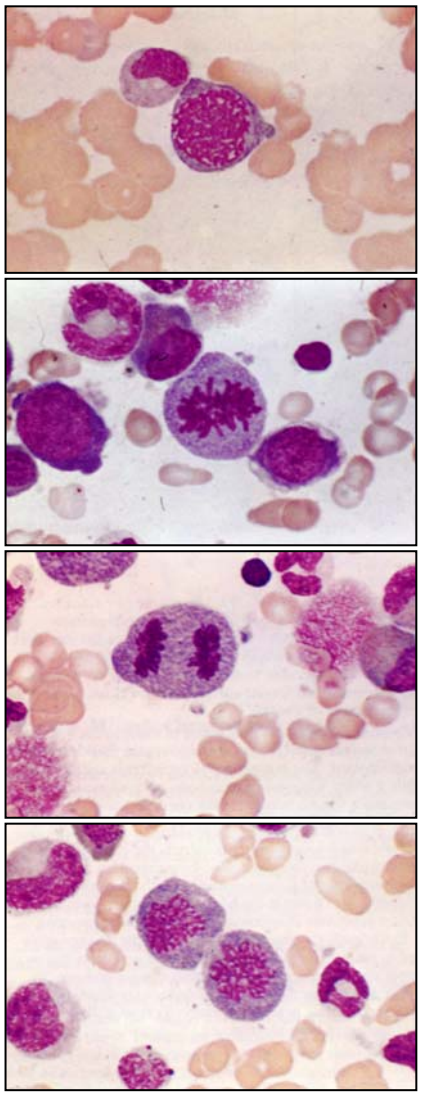
….cells are mostly water, and therefore mostly transparent!



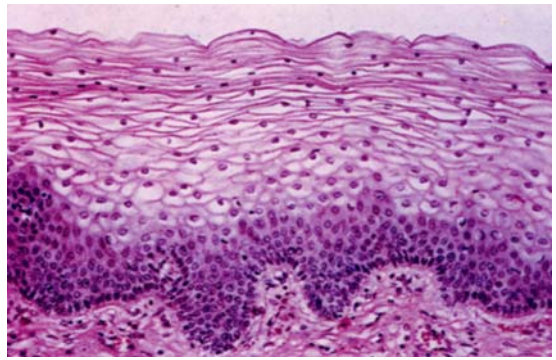
How can you generate <u>contrast</u> to "see" a transparent object?

Using chemical stains for cells and tissues

Mitosis in white blood cells--
Giemsa stain



Epithelial tissue--Haemotoxylin (basic dye) & Eosin (acidic dye) stain



Discoveries in biology emerged from cytochemistry:

Different types of granulocytes (white-blood cells) include :
Basophils: granules bound basic dyes (e.g. haemotoxylin)
Eosinophils: granules bound acid dyes (e.g. eosin)
Neutrophils: not stained with either acid- or basic-dyes

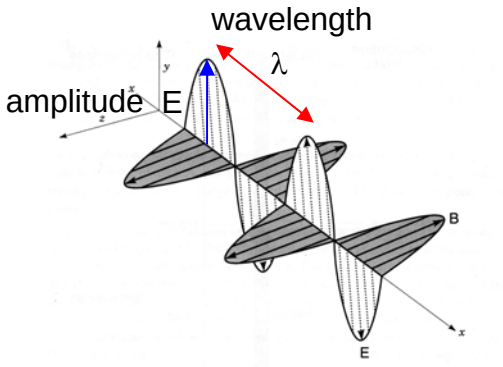
But these cells are FIXED

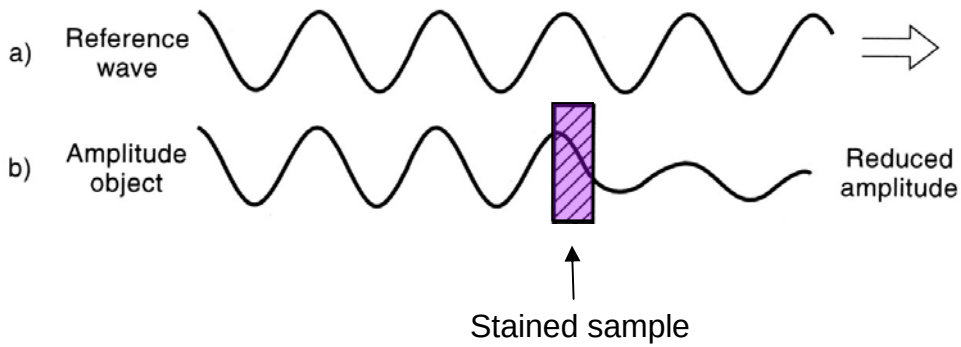
The big advances over previous 100 years:

- Imaging live cells (Phase contrast microscopy: 1930s, Differential contrast microscopy: 1950s)
- Imaging specific molecules inside cells (Immunofluorescence microscopy: 1960s and onwards)
- Imaging specific molecules inside live cells (Fluorescent labelled proteins:1980s, Green Fluorescent Protein: 1990s)

How to see non-fixed *living* cells?

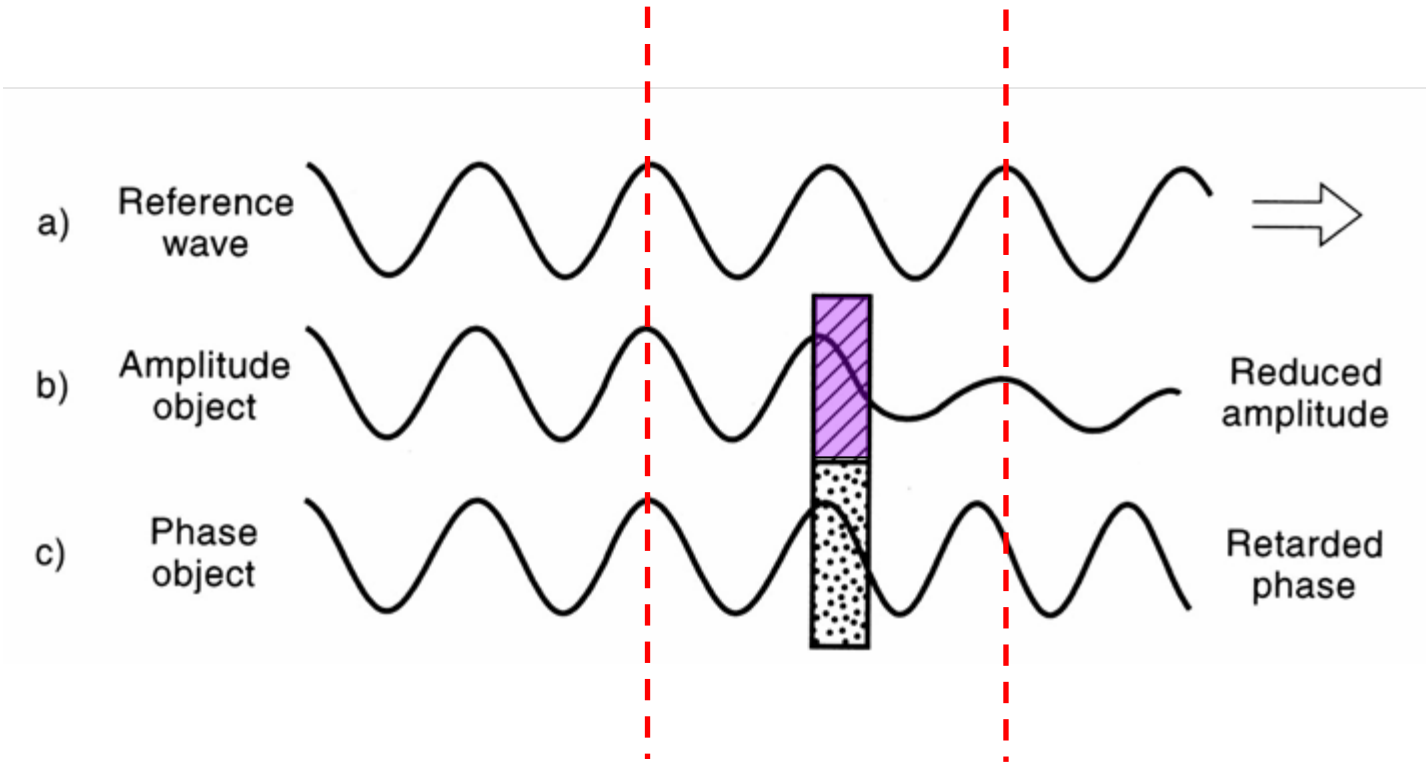
- How do stained samples generate contrast?
- Now need to think about light as electromagnetic radiation, i.e., waves.
- When stained samples absorb light, they reduce the <u>amplitude</u> of specific wavelengths





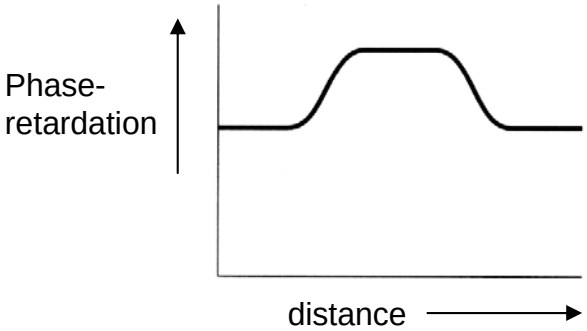
Transparent object do interact with light!

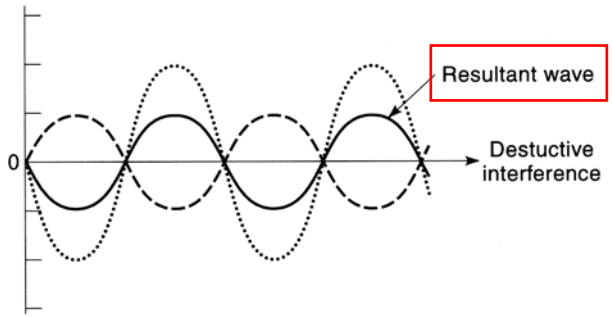
Light passing through any dense sample is slowed down (this is one aspect of refraction), which changes its phase relative to light not passing through the sample



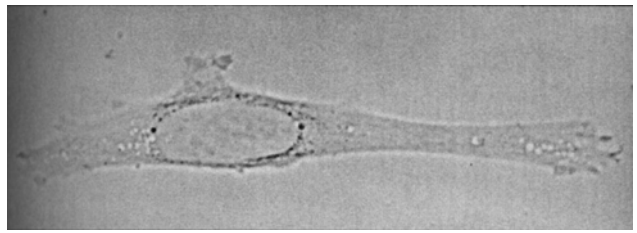
Phase-contrast microscopy:

- Invented by Zernike, a physicist, in the 1930s (Nobel Prize, 1953)
- Uses interference of light waves turn "invisible" phase differences into contrast. In essence is sensitive to spatial differences in refractive index

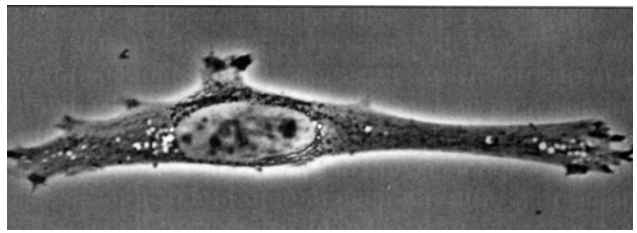




Normal contrast

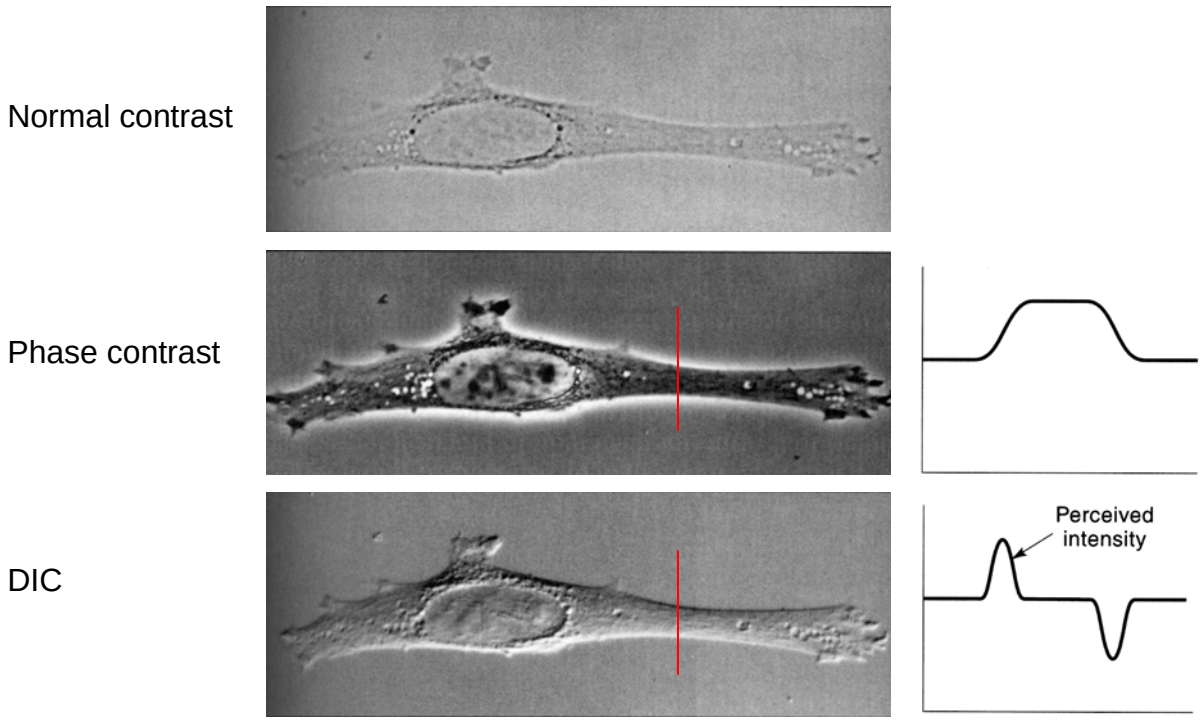


Phase contrast



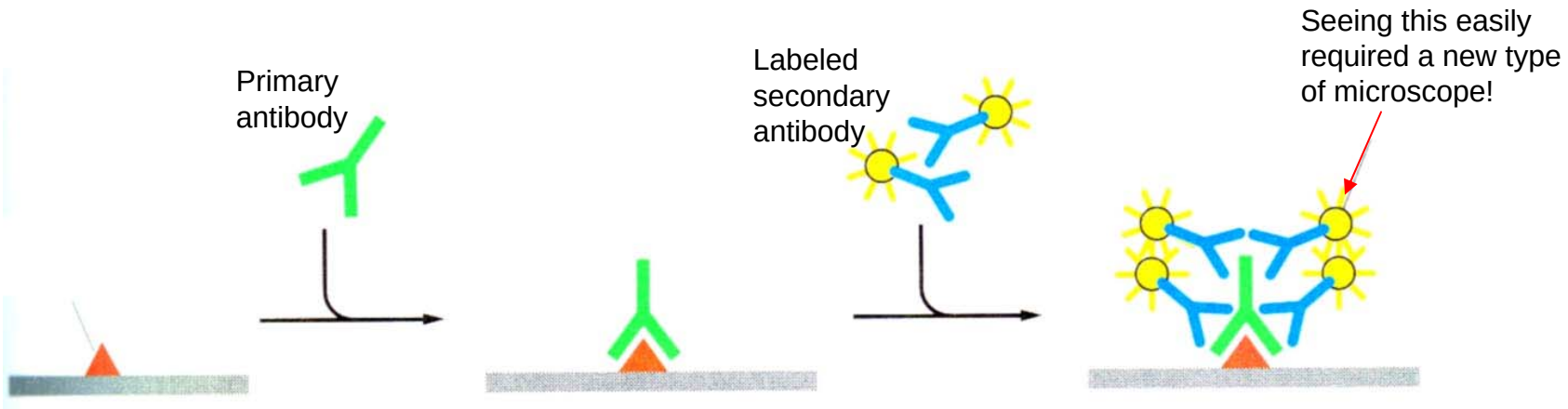
Differential interference contrast (DIC)

- Another phase-dependent method, more recent than phase contrast, and much more complicated technically.
- Also takes advantage of differences in phase, but measures relative phase difference (i.e. is sensitive to the spatial gradient of refractive index), not absolute phase difference.
- Differences are greatest at edges, giving 3-D contour effect



More specific stains:

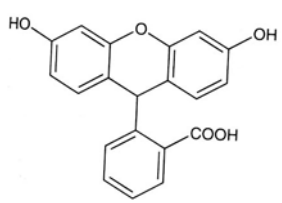
- Beginnings in late 1960s/early 1970s.
- Starts with chemically-fixed cells.
- Use antibodies to visualize specific components of cells
- Incubate with“primary" antibody that binds uniquely to a specific protein (e.g. "rabbit anti-actin")
- Then incubate with labeled “secondary" antibody that binds to the primary antibody (e.g. fluorescein-labelled "sheep anti-rabbit")



Fluorescence

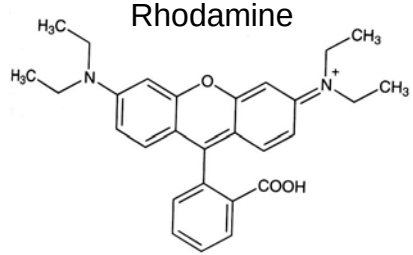
- Fluorophore absorbs light of a specific wavelength
- Rapidly emits light of longer wavelength (within nanoseconds)
- Can have multiple distinct fluorophore in the same experiment.
- Best results require modifications to the microscope design.

Fluorescein



Absorb: 490 nm (blue)
Emit: 520 nm (green)

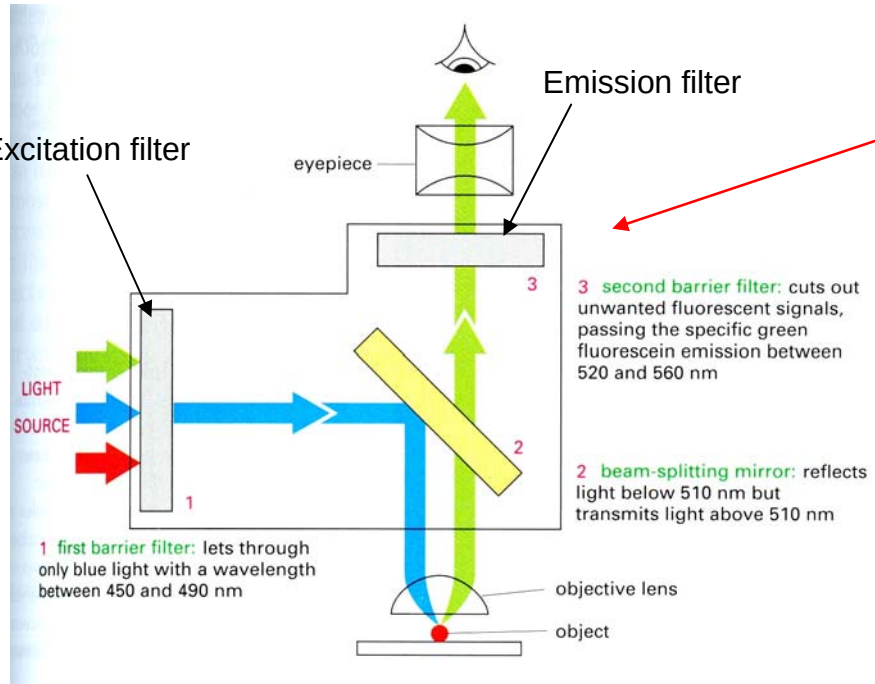
Rhodamine



Absorb: 550 nm (green-yellow)
Emit: 580 nm (orange-red)

“Epifluorescence” microscopes

Epifluorescence microscopy uses illumination from above ("epi-") and a special cube containing usually two colored filters plus a special beam-splitting ("dichroic") mirror



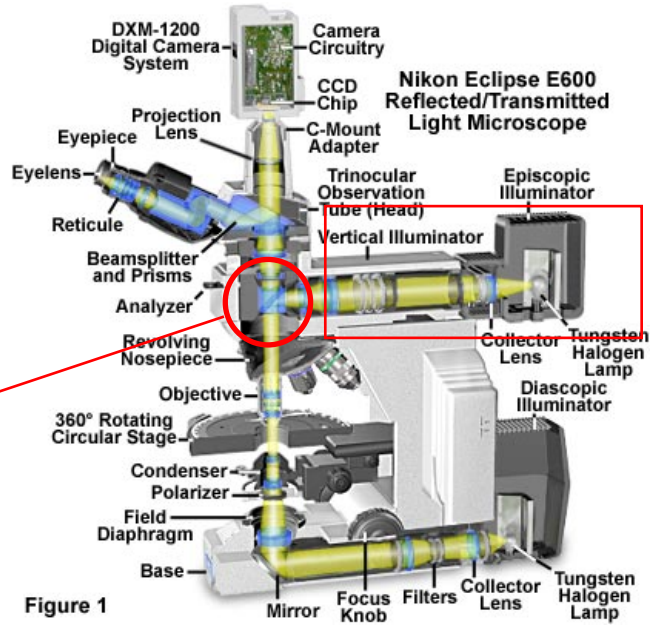
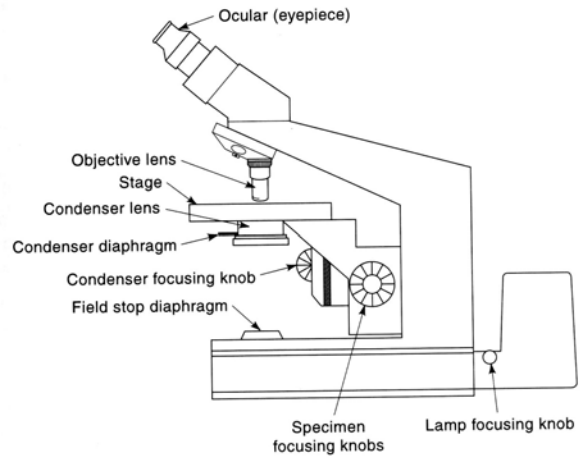
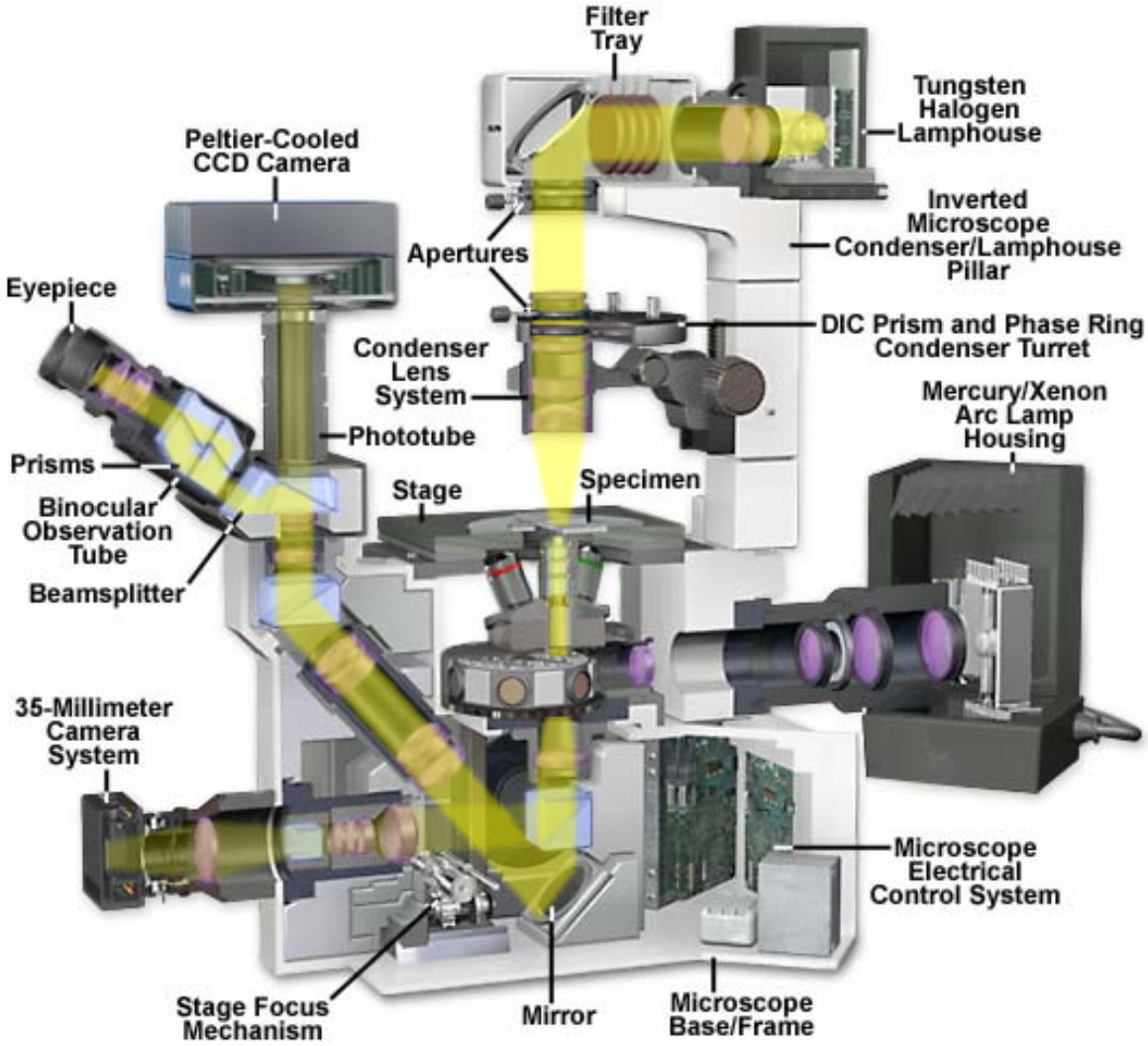


Figure 1



Olympus IX70 Inverted Microscope Light Pathways

Interactive Java Tutorial



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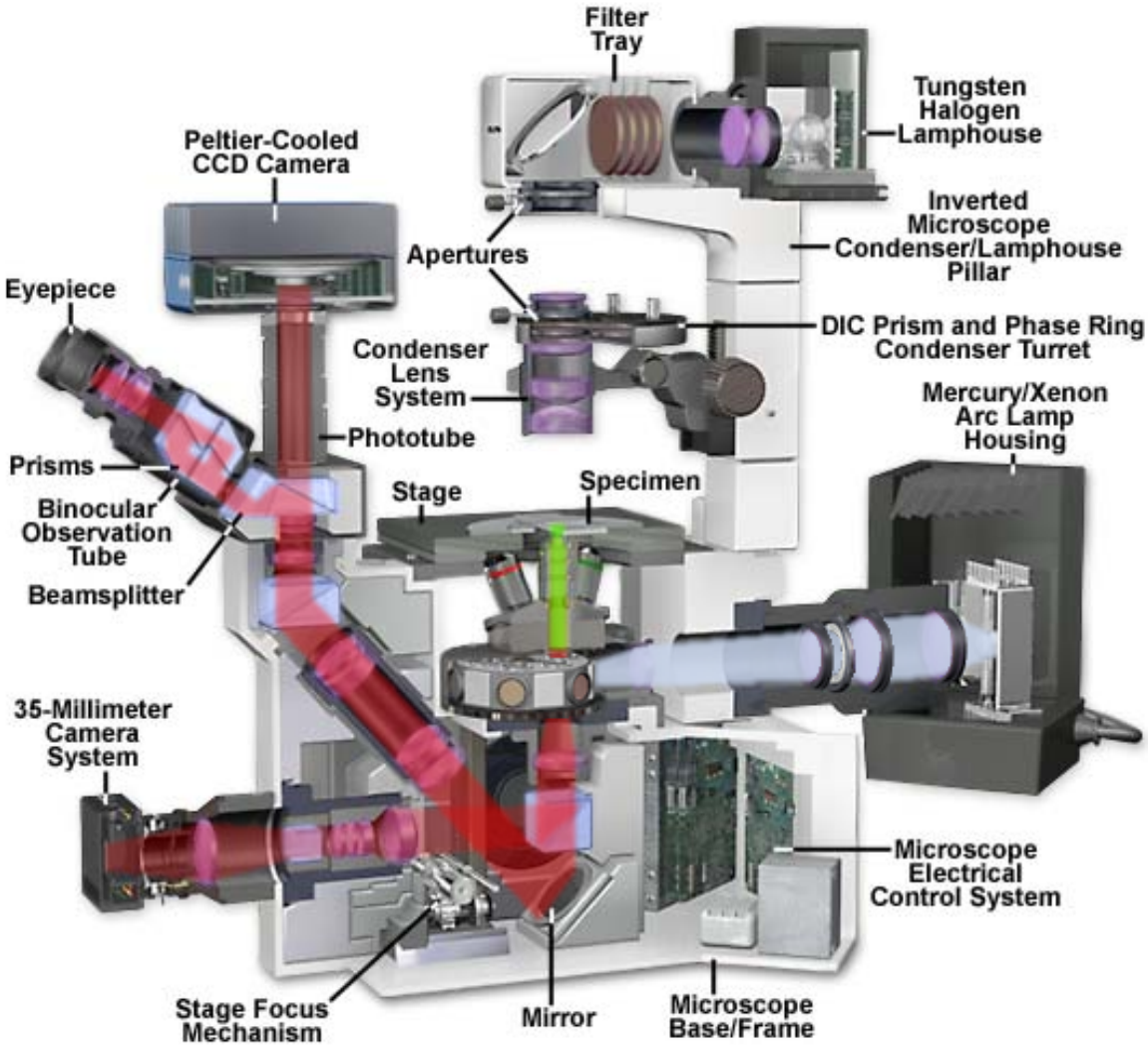


Seeing different molecules types in *living* cells?

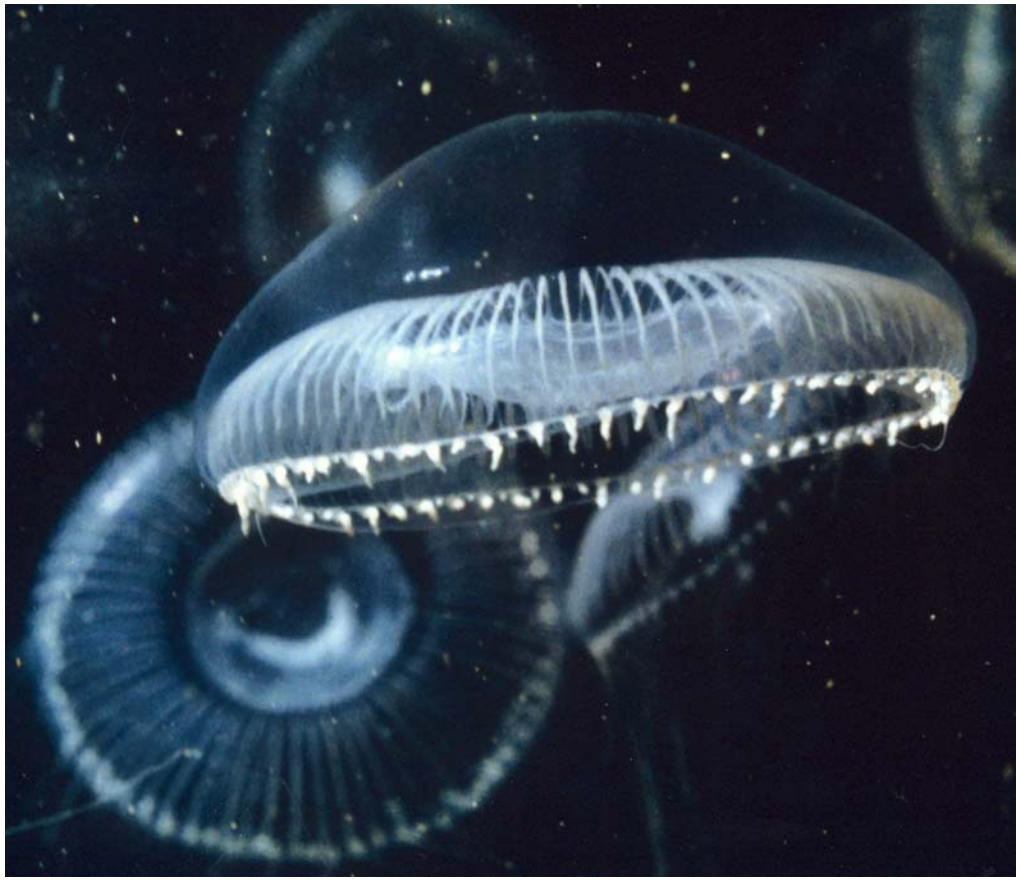
- Some fluorescent dyes bind to specific compartments or organelles
- Can also "microinject" labelled protein, or labelled antibody, directly into cells.

Difficulties:

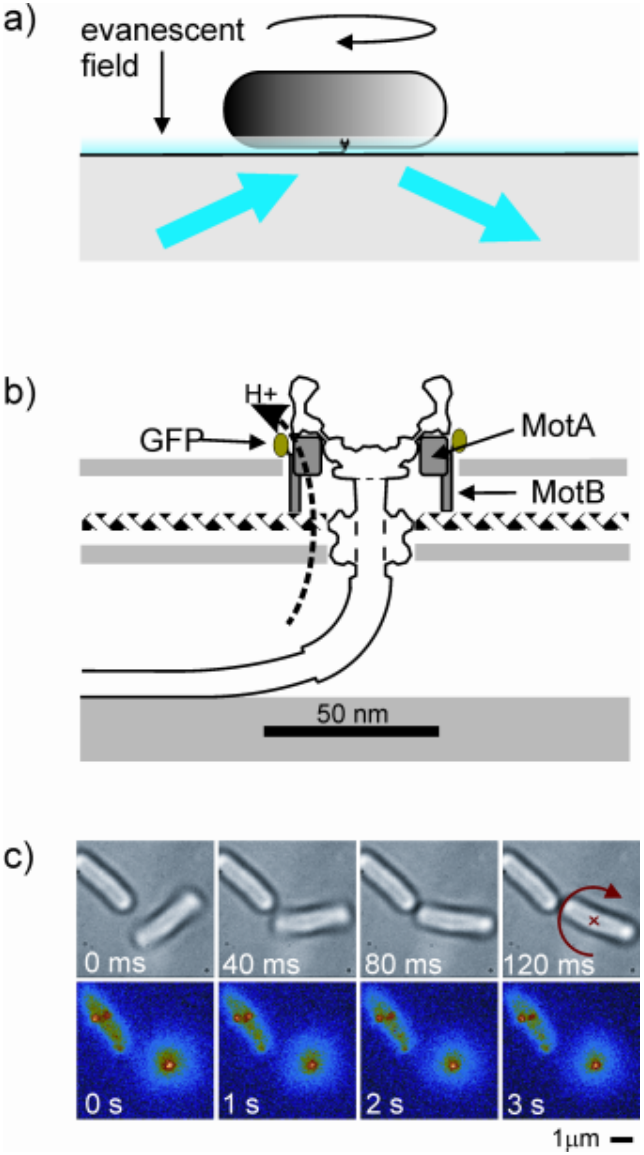
- Requires purified proteins or antibodies.
- These may perturb protein function.
- Many types of cells (cells in tissues, microorganisms, many plant cells) cannot be injected.

An answer: GFP

- Green Fluorescent Protein, a naturally fluorescent protein identified in the jellyfish *Aequorea victoria.*



Antibody-tethered cell-rotation assay:



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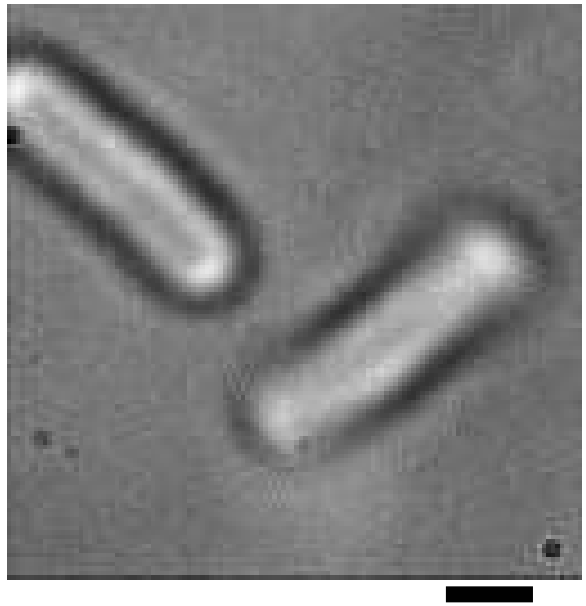

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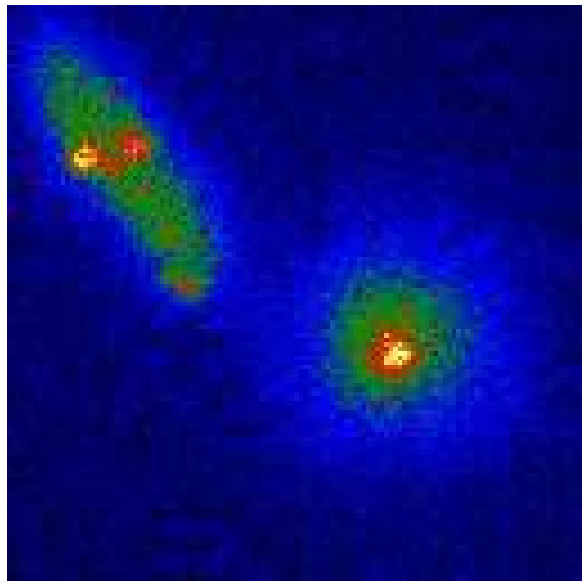
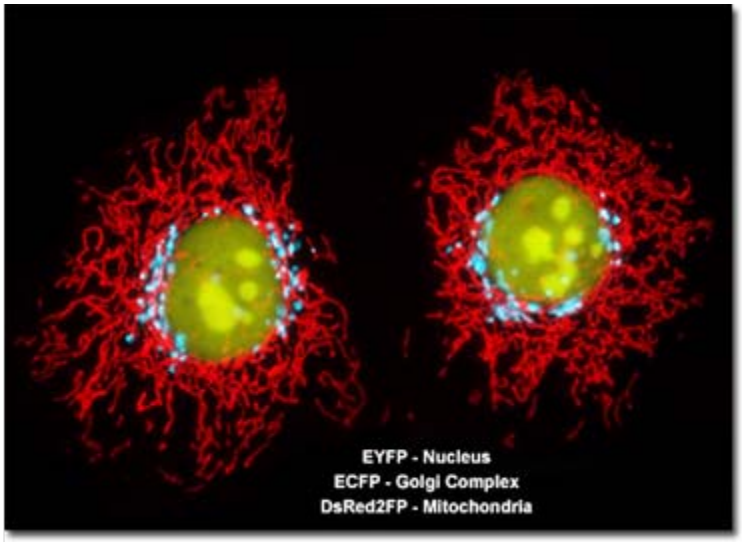


(d) Brightfield and (e) TIRF images of GFP-MotB *E. coli* mutant. Black bar=1μm

Extending the palette:

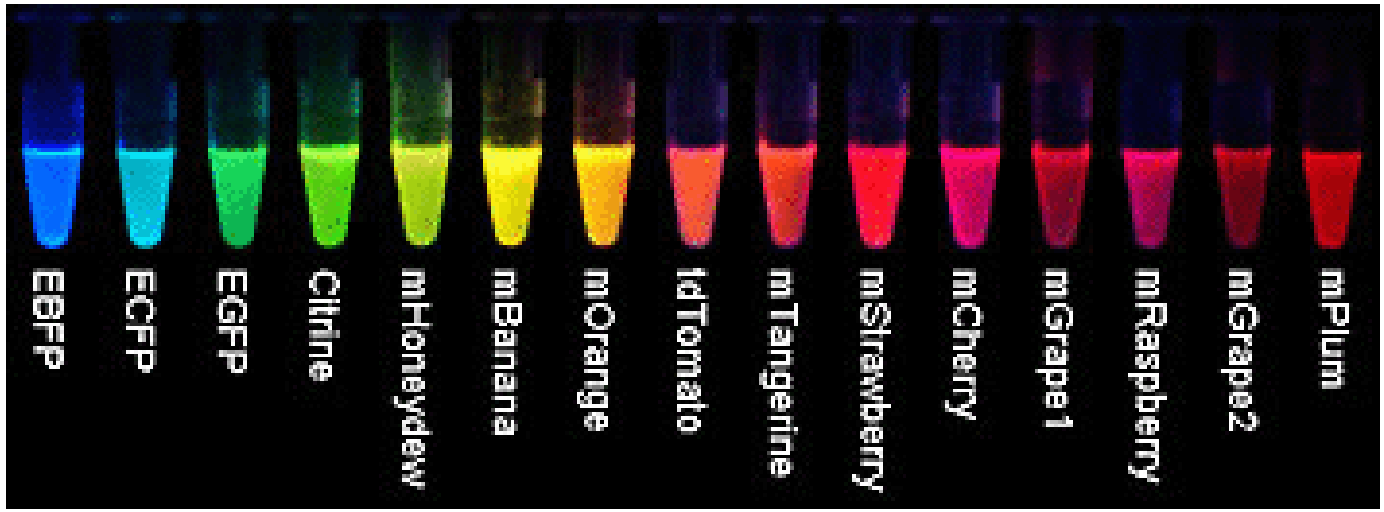


EYFP = enhanced Yellow Fluorescent Protein (GFP derivative)

ECFP = enhanced Cyan Fluorescent Protein (GFP derivative)

DsRed2FP = Red Fluorescent Protein (coral protein, unrelated to GFP, and not monomeric)

Changing the properties of GFP and RFP by genetic engineering



Advanced methods for fluorescence microscopy:

How to improve the quality of fluorescent imaging?

•Deconvolution microscopy: using computation to put "out-of-focus" light back where it belongs

•Confocal microscopy: using tricks of geometric optics to remove "out-of-focus" light before it hits the detector

•TIRF: delimiting the excitation volume to improve image contrast

How to measure protein interactions and dynamics?

•FRET (Foerster Resonance Energy Transfer)

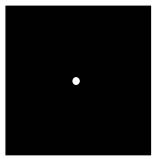
•FLIM (Fluorescence Lifetime Imaging)

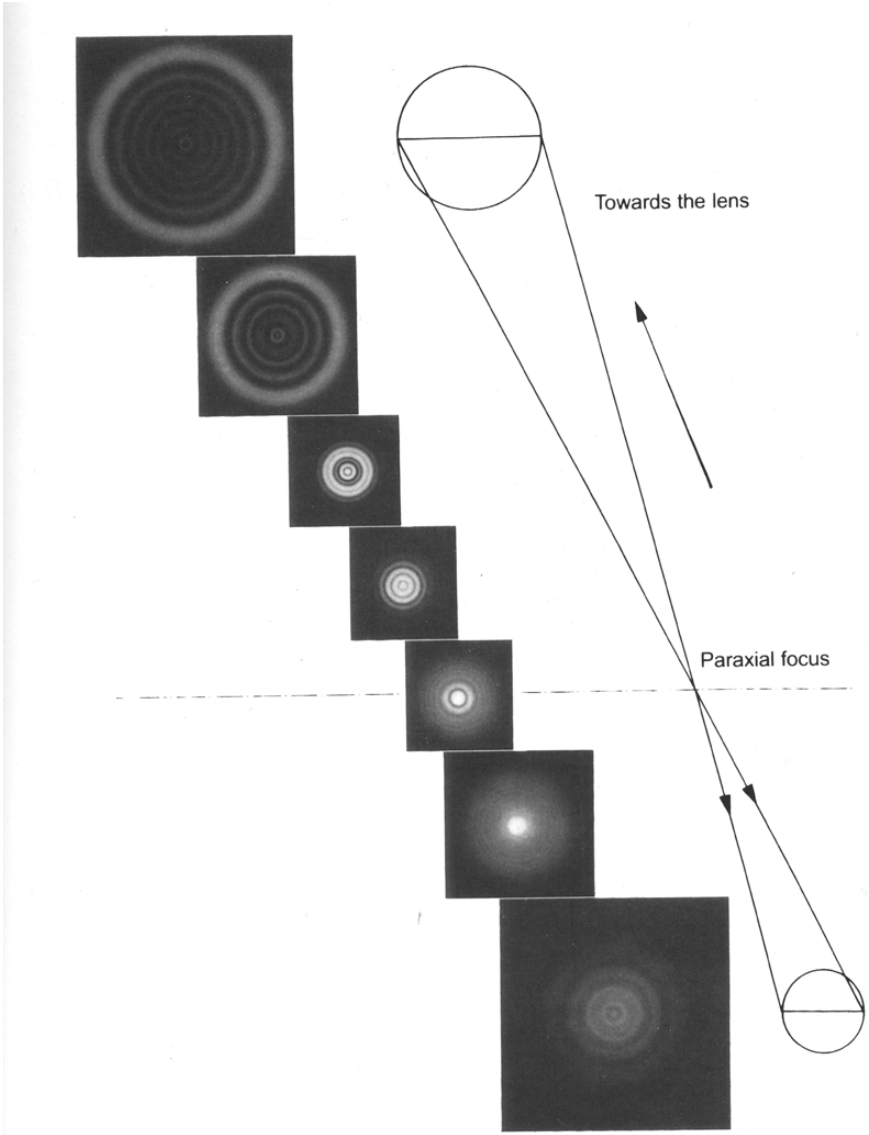
•FRAP/FLP (Fluorescence Recovery After/Loss In Photobleaching)

•FCS (Fluorescence Correlation Spectroscopy)

“off-focus” problems

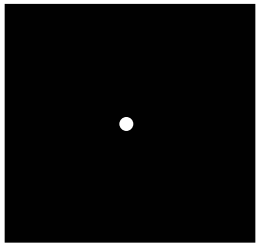
Sample object: a "sub-resolution" fluorescent bead



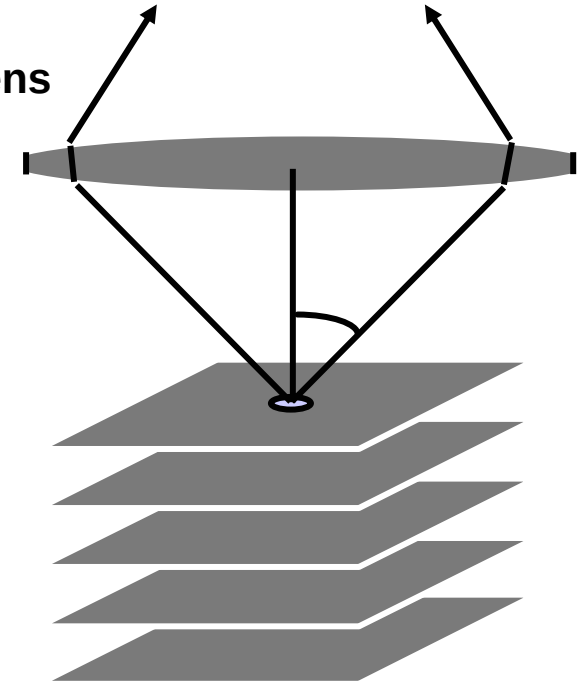


Deconvolution of off-focus light

X-Z view of sample object

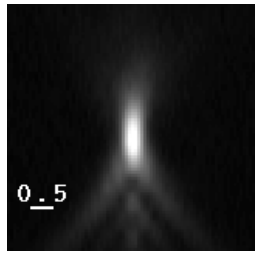


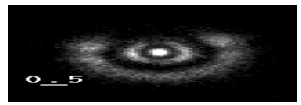
Objective lens



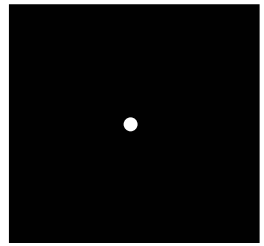
Planes of focus (z stack)

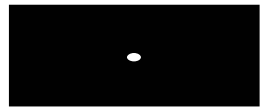
X-Z view of raw image stack





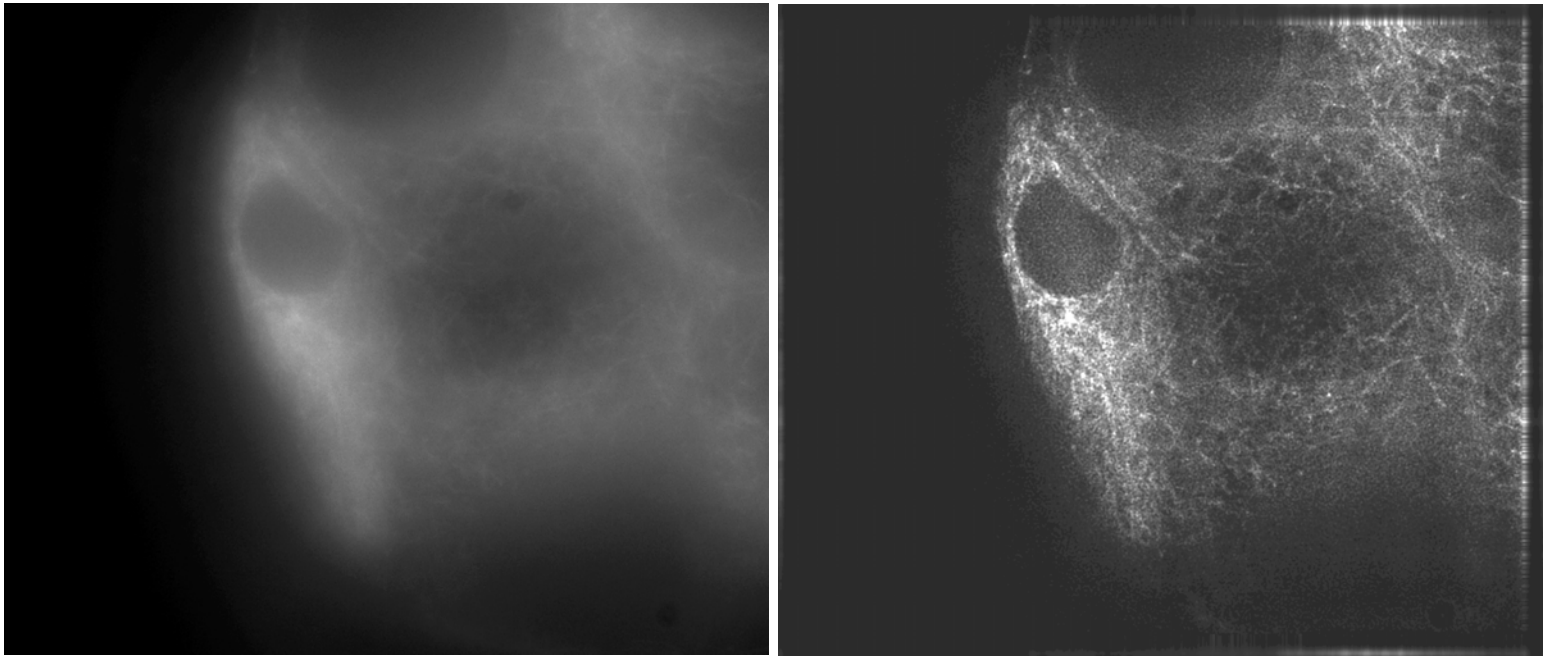
X-Z view of deconvolved image stack





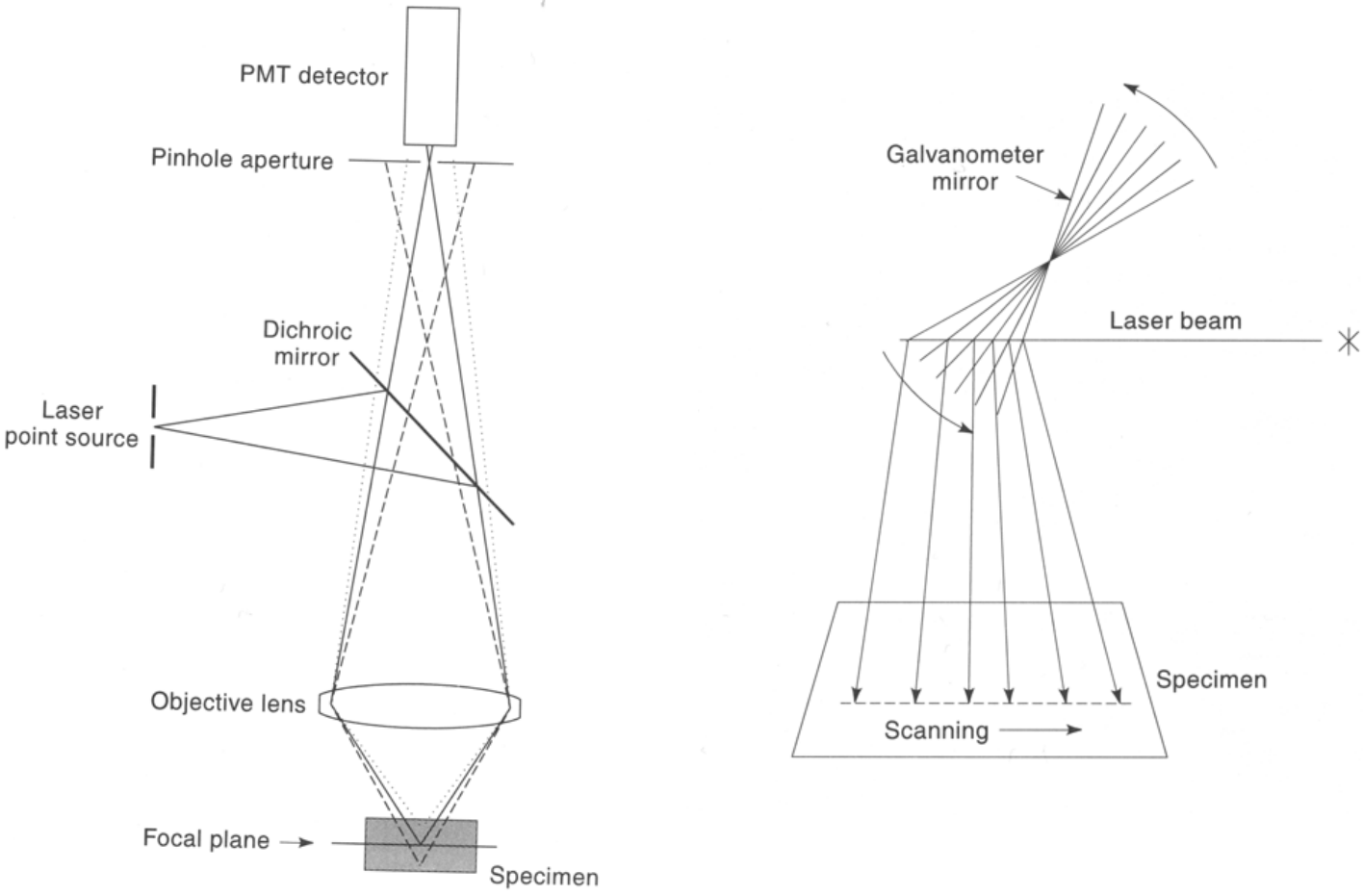
Before deconvolution

After deconvolution



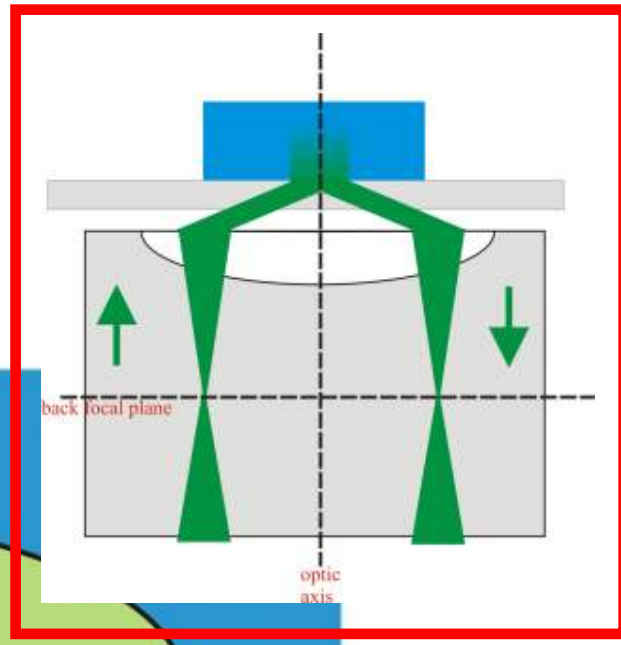
GFP tag in Drosophila embryo

Confocal microscope (laser scanning)



At the pinhole aperture, in-focus light from the specimen is again "in focus", and all of it goes through the pinhole, but out-of-focus light from the specimen is now "out-of-focus" and spread out, contributing little to the total signal received by the photomultiplier

Total-internal-reflection-fluorescence (TIRF):



$n_1=1.3$

$n_2=1.5$

$\alpha_{critical}$

$\alpha_{critical}=\sin^{-1}(n_1/n_2)$

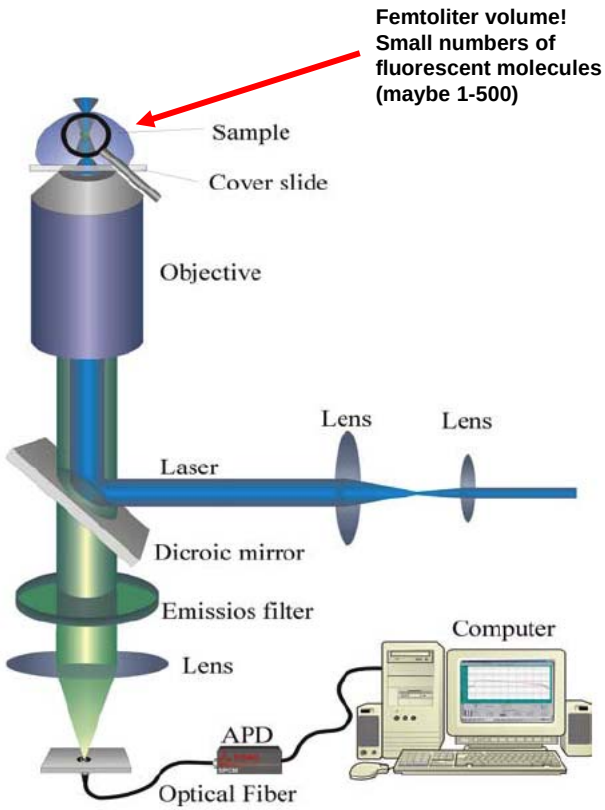
Measuring molecular interactions and dynamics

- Fluorescence correlation spectroscopy (FCS)
- Fluorescence recovery after/loss in photobleaching (FRAP/FLIP)
- Foerster resonance energy transfer (FRET) and multi-colour imaging
- Fluorescence lifetime imaging (FLIM)

Fluorescence correlation spectroscopy (FCS)

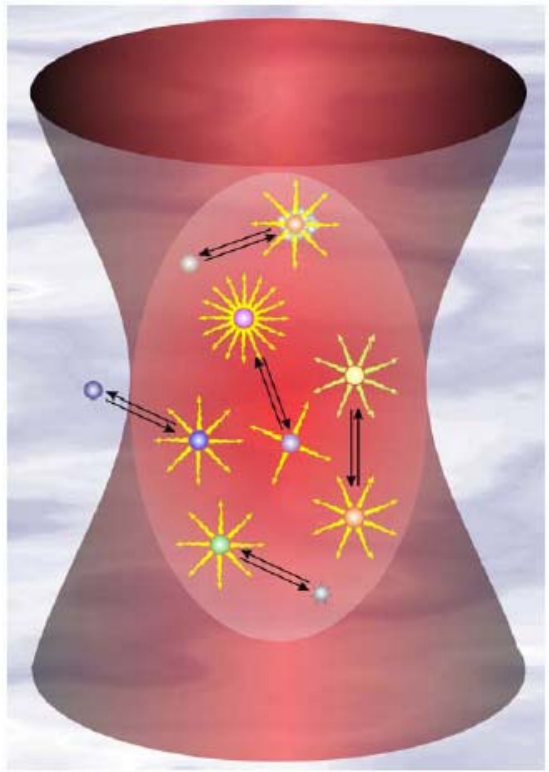
Good for measuring concentrations, diffusion coefficients and turnover

Experimental set-up

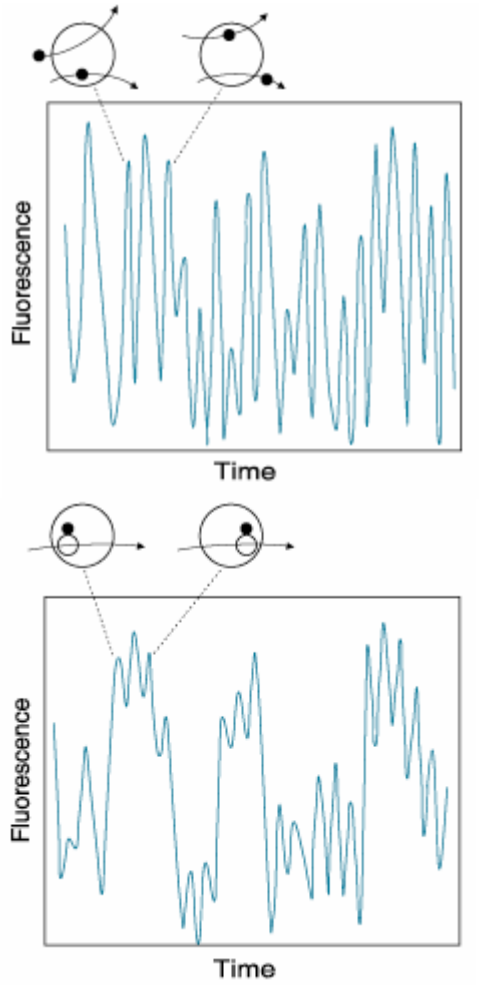


P. Schwille and E. Haustein, MPI Goettingen

Reasons for fluctuations in fluorescence



Different diffusion coefficients give rise to different fluctuations



Invitrogen website

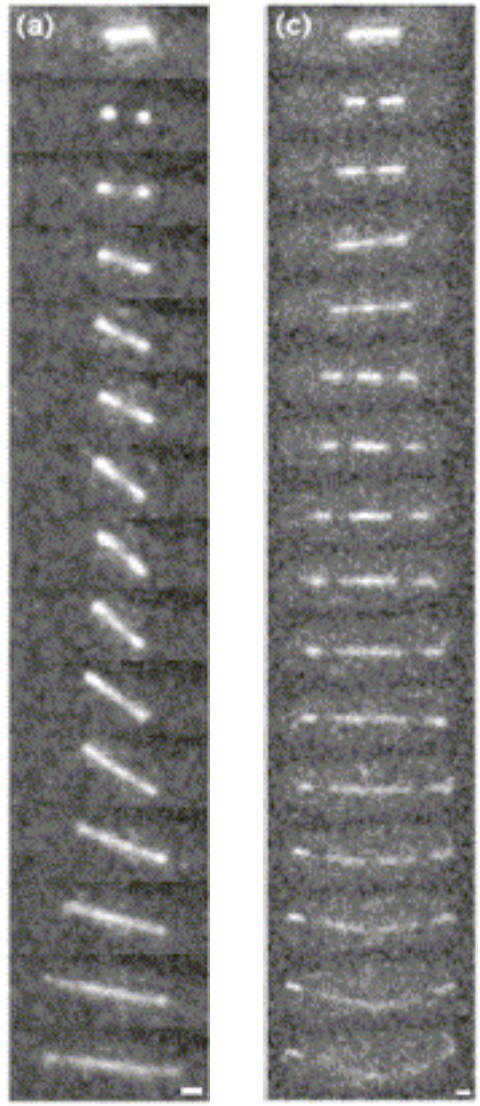
Fluorescence recovery after photobleaching (FRAP)

Start with structure of interest uniformly labeled with fluorophore (fluorescent dye or GFP-fusion protein).

Then, photobleach a region, and follow recovery in space and time

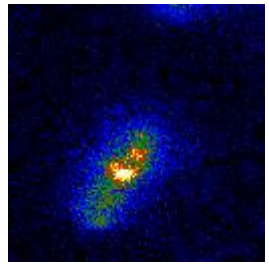
Fission yeast mitotic spindles

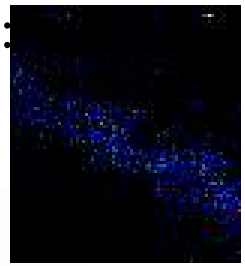
Pre-anaphase Anaphase

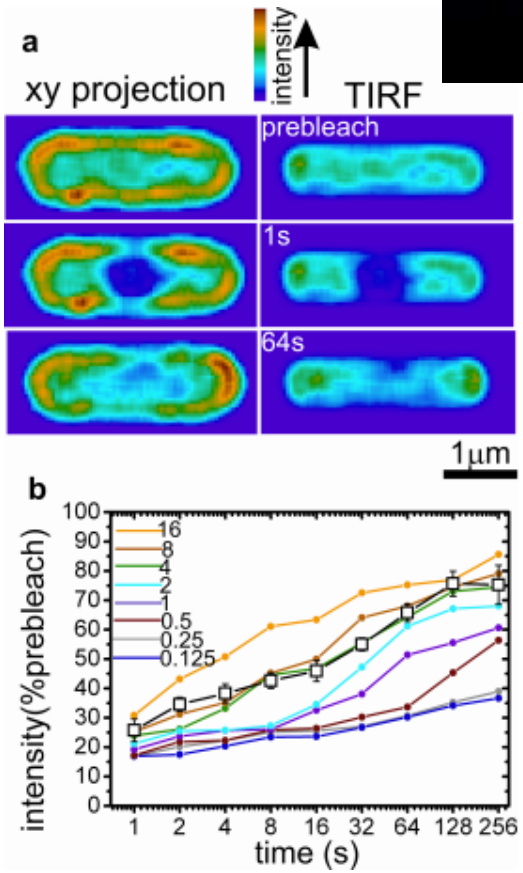


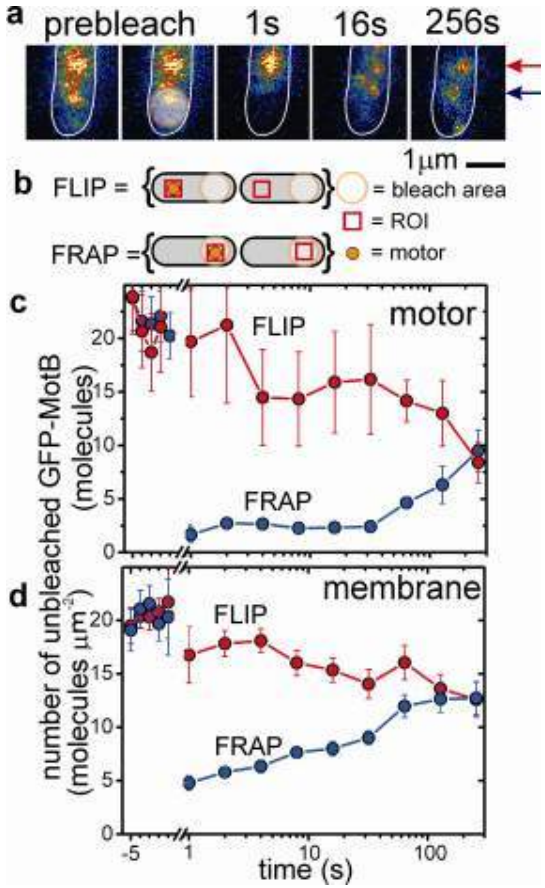
Focused Laser FRAP/FLIP (…*Loss In* Photobleaching):

Either track individual particles directly, or apply Monte-Carlo 2D simulations to estimate diffusion coefficient:



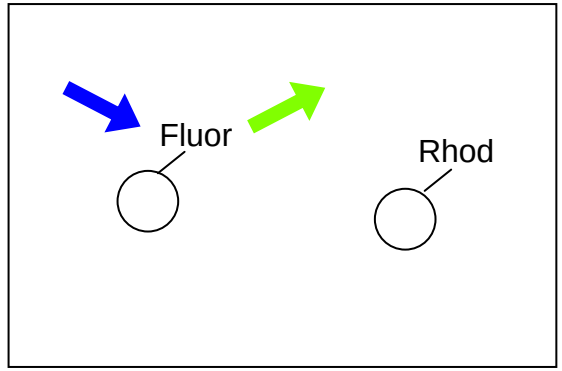




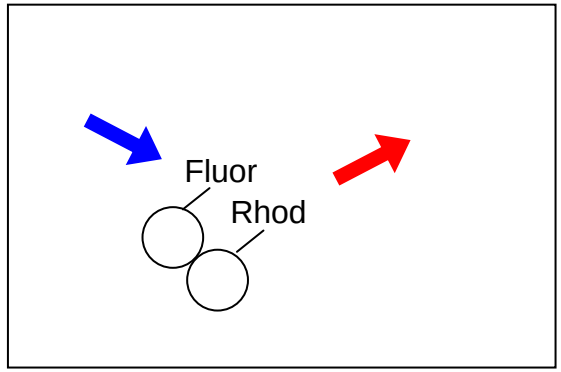


Foerster resonance energy transfer (FRET)

- Uses a pair of distinct but compatible fluorophores (e.g. fluorescein and rhodamine, or CFP and YFP), each attached to a different protein
- If proteins are close together (6-8 nm), the energy emitted by the shorter-wavelength fluorophores can be "immediately" absorbed by the longer-wavelength fluorophore
- To be "compatible", the emission spectrum of the shorter-wavelength fluorophore must overlap considerably with the excitation spectrum of the longer-wavelength fluorophore
- Since FRET occurs only when two proteins are very close together, it can be used to judge whether two proteins are present in the same complex in vivo (far superior to "co-localization at the light level")



No FRET



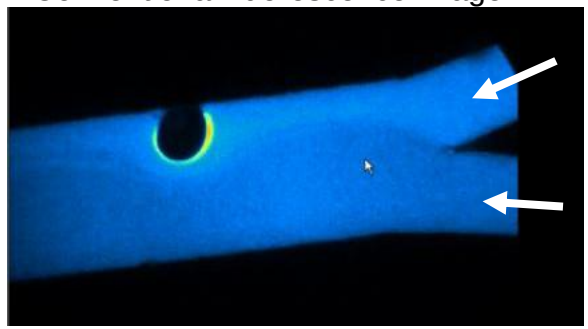
FRET

Fluorescence lifetime imaging (FLIM)

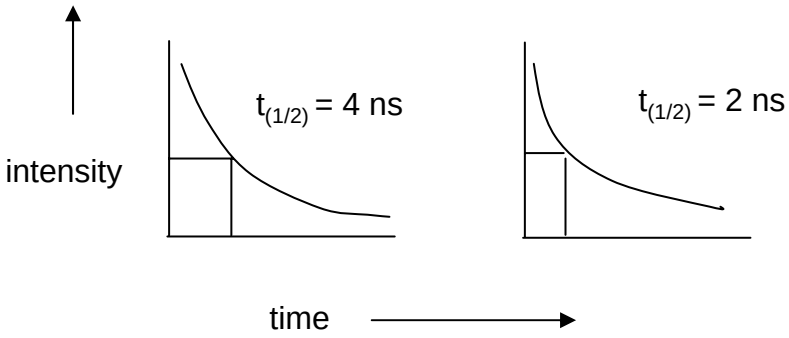
- Changes in the molecular environment of a fluorochrome (including interactions with FRET partners) can alter the fluorescence lifetime
- This can be used to assay protein-protein interactions, among other things

Example: the merging of two flowing microchannels of fluorescent dye

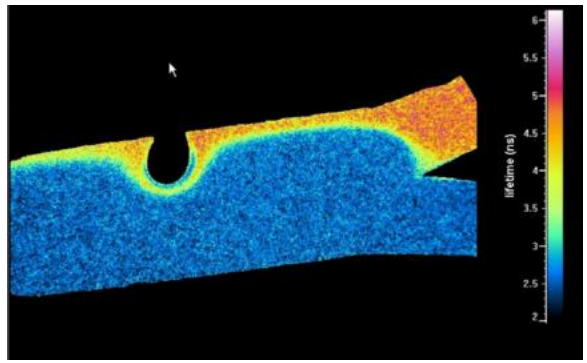
Conventional fluorescence image



Decay of fluorescence after excitation:

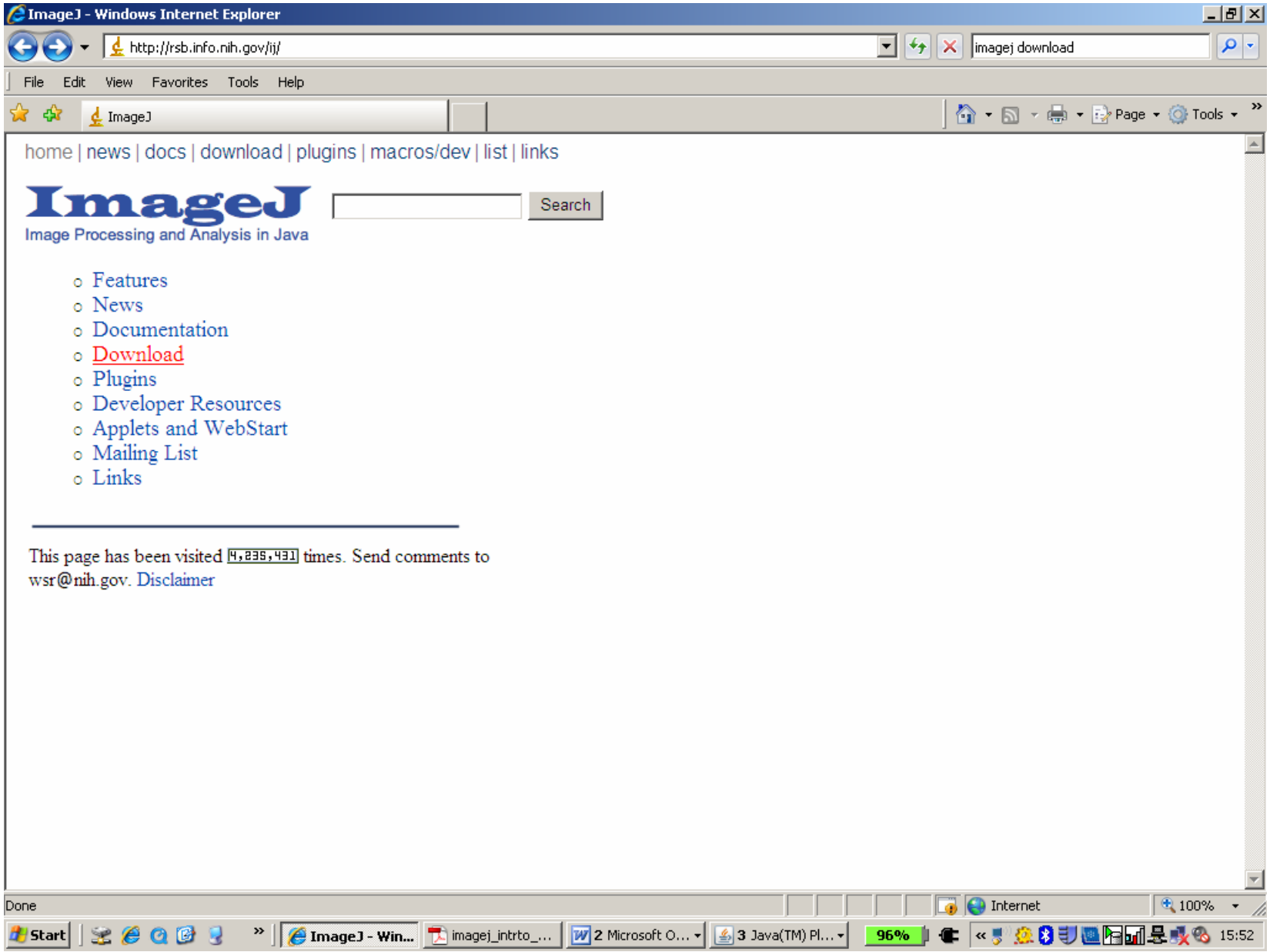


Fluorescence lifetime image



http://www.cheng.cam.ac.uk/news/2004/flim.html

The bacterial chemotaxis practical…



http://rsb.info.nih.gov/ij/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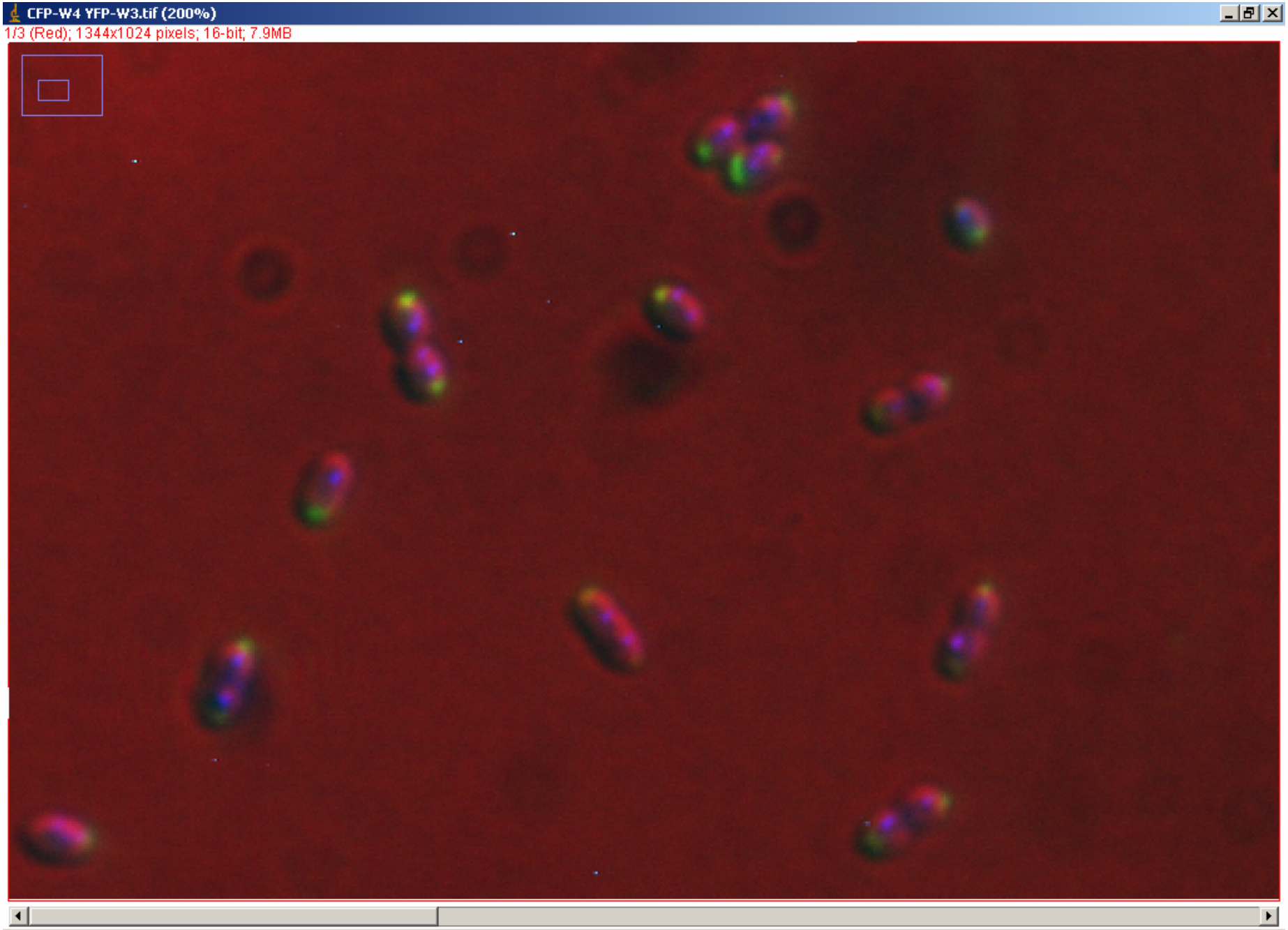
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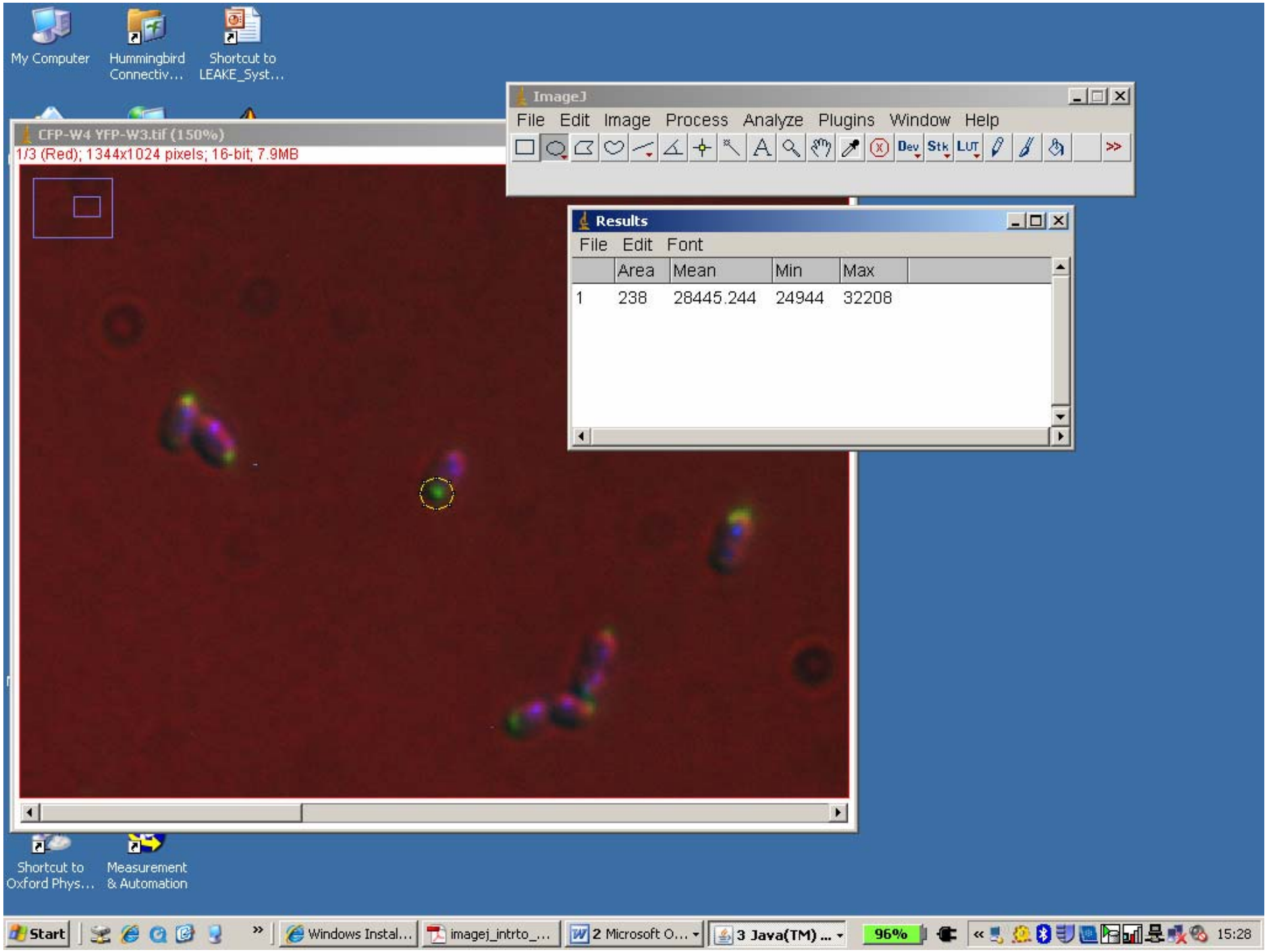
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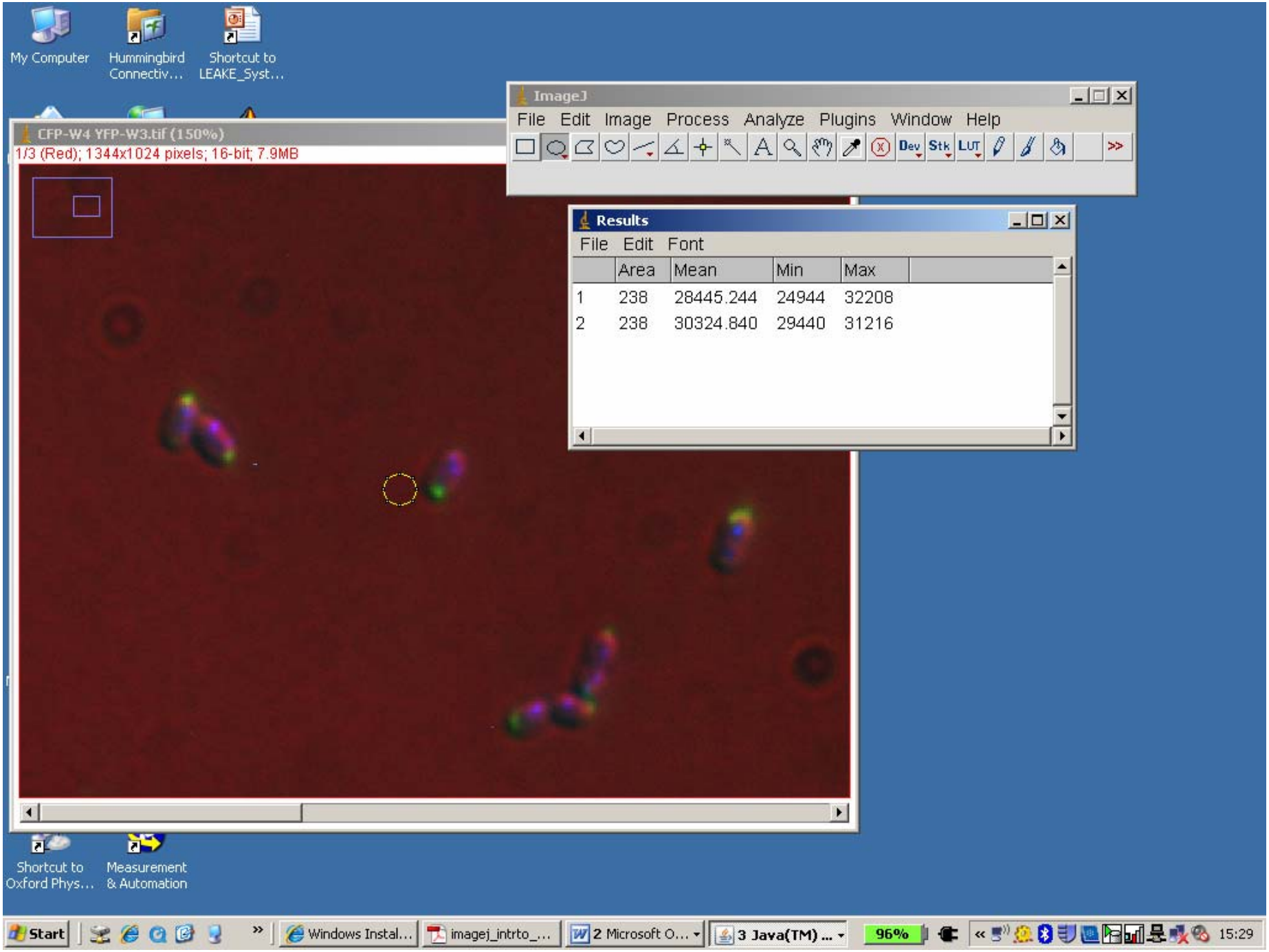
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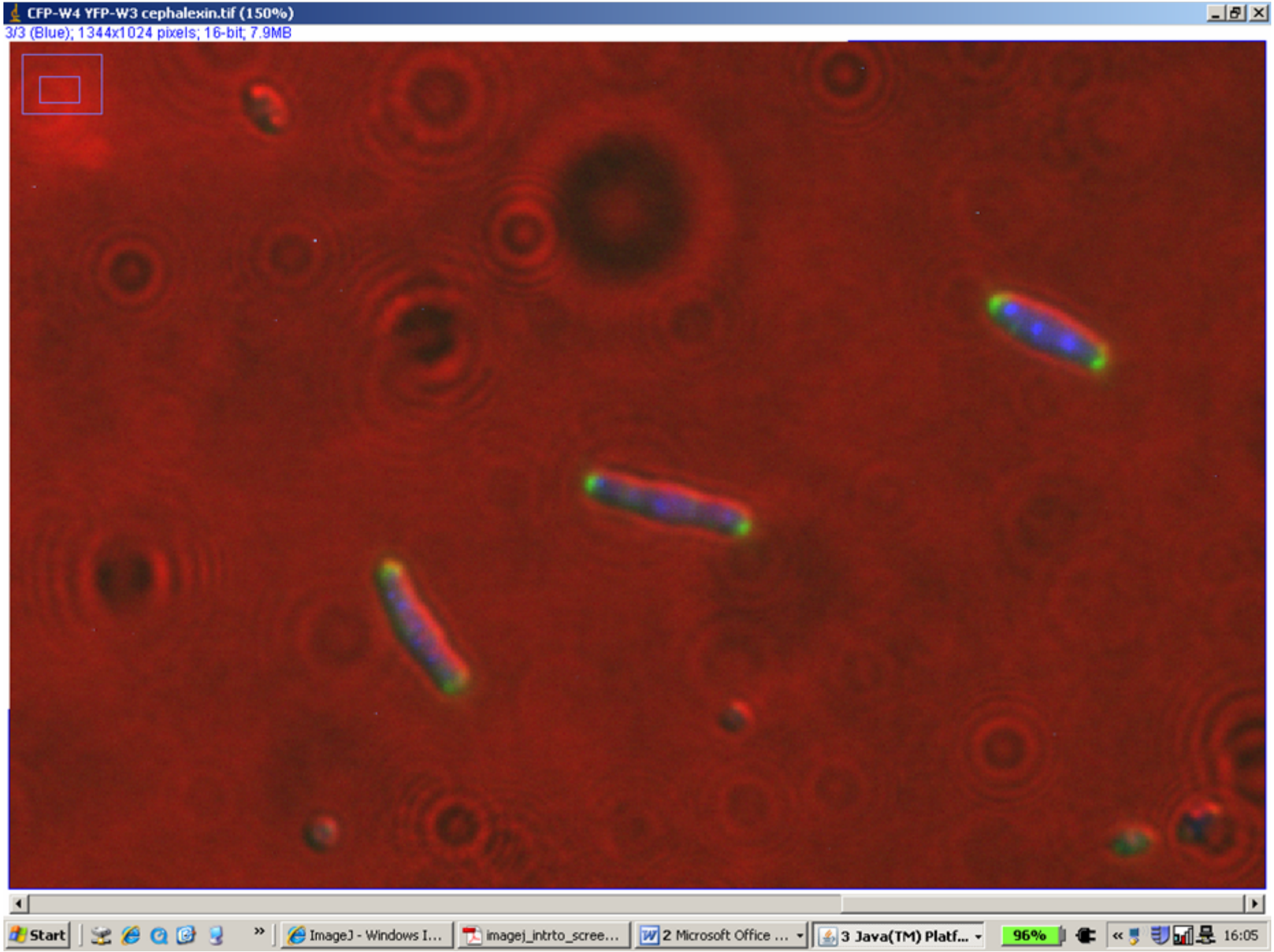
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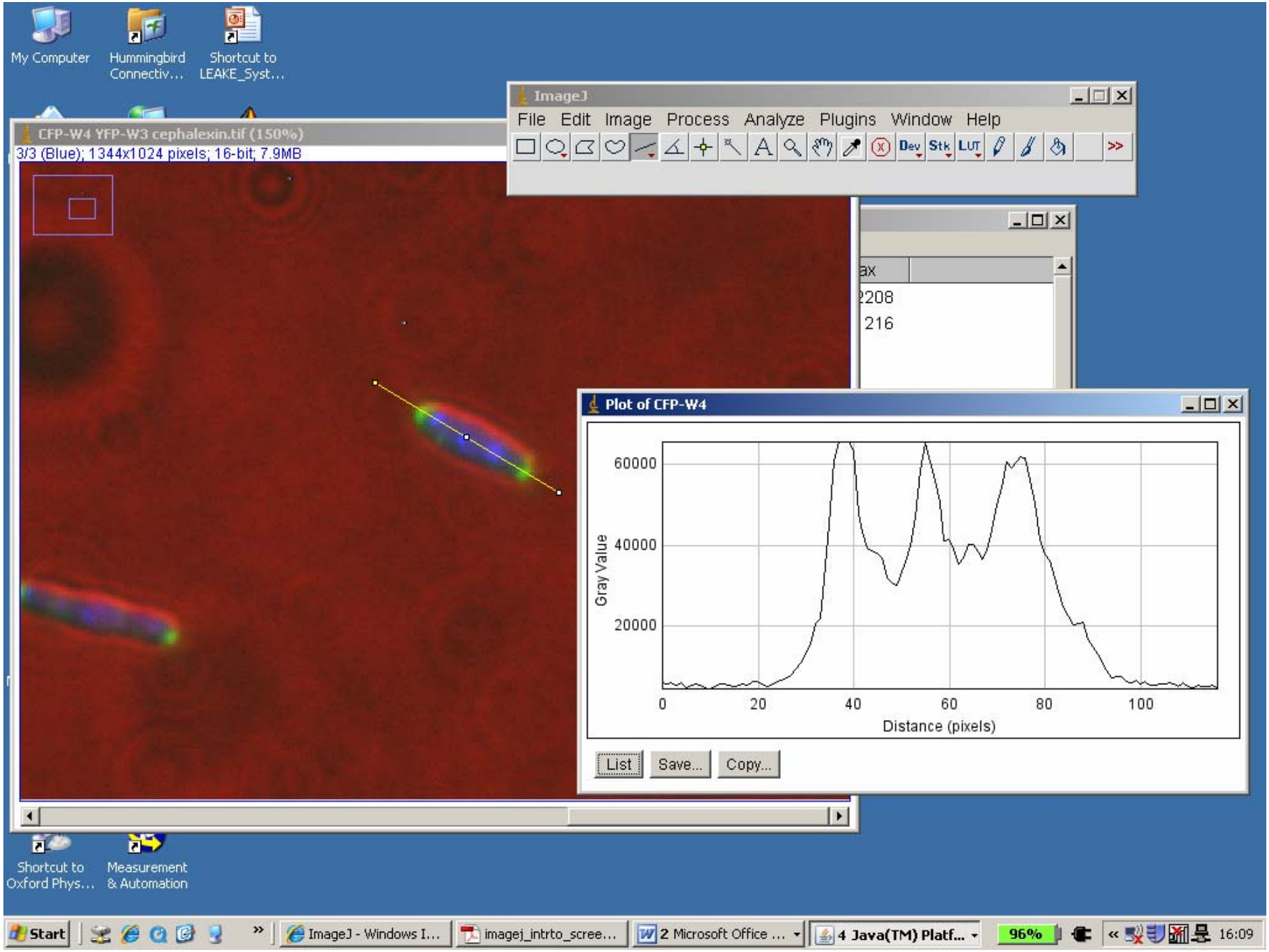
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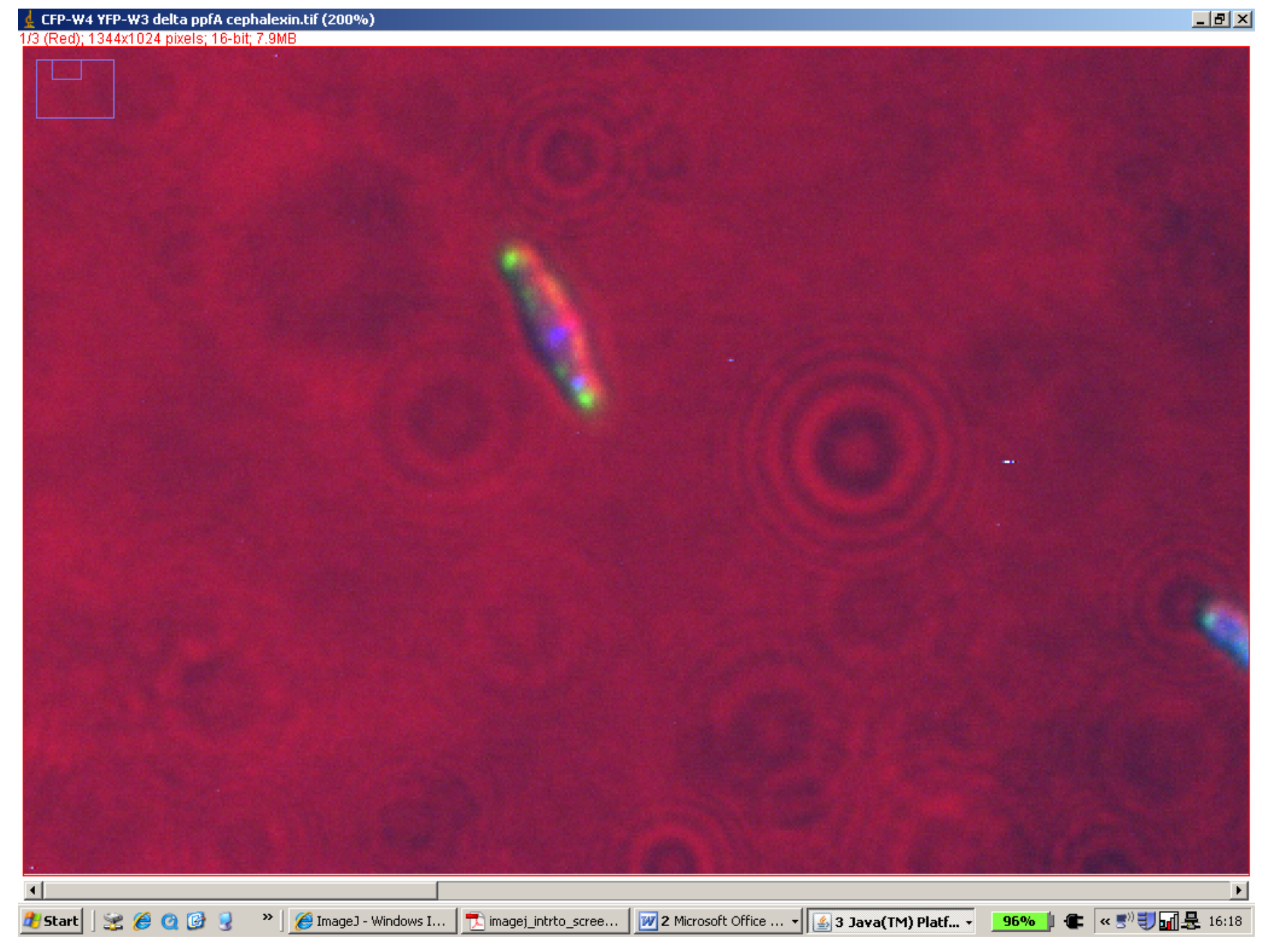
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Bibliography

Useful articles:

•Axelrod D, Burghardt TP & Thompson NL (1984)
Total internal reflection fluorescence. *Annu. Rev. Biophys. Bioeng.* 13, 247–268.

•Leake MC. et al (2006) Stoichiometry and turnover in single, functioning membrane protein complexes. *Nature* 443, 355-8.

•Thompson S, Wadhams GH & Armitage JP. (2006) The positioning of cytoplasmic protein clusters in bacteria. *PNAS* 103, 8209-14.

•Tsien R. (1998) The green fluorescent protein. *Annu. Rev. Bioch.* 67, 509-44.

•Wadhams GH et al (2003) Targeting of two signal transduction pathways to different region of the bacterial cell. *Mol. Microbiol.* 50, 763-70.

• Wadhams GH & Armitage JP. (2004) making sense of it all: bacterial chemotaxis. *Nat. Rev. Mol. Cell Biol.* 5, 1024-37.

Useful websites:

•http://www.olympusmicro.com/primer/techniques/fluorescence/.html

•http://www.physics.ox.ac.uk/users/leake

•http://rsb.info.nih.gov/ij/