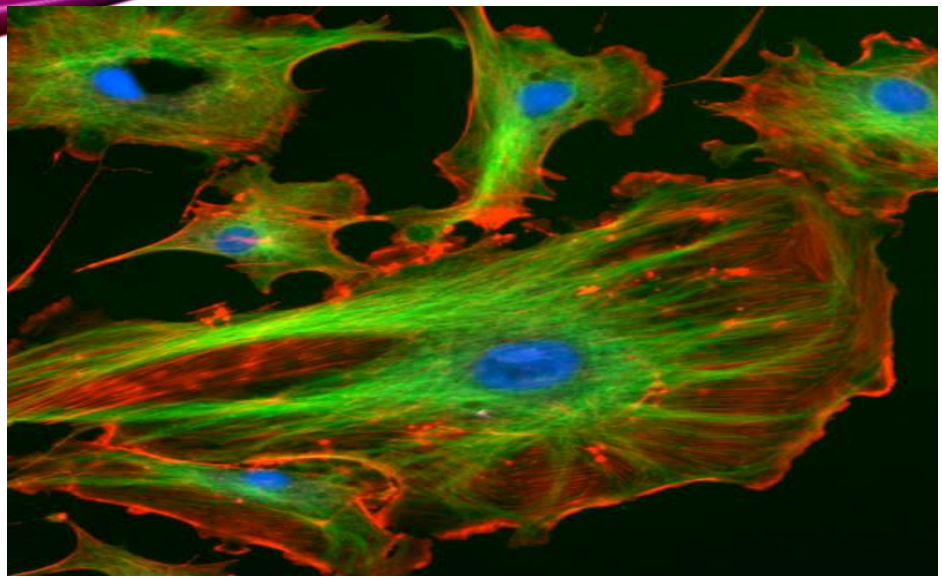


Cellular Stains

and Microscopy

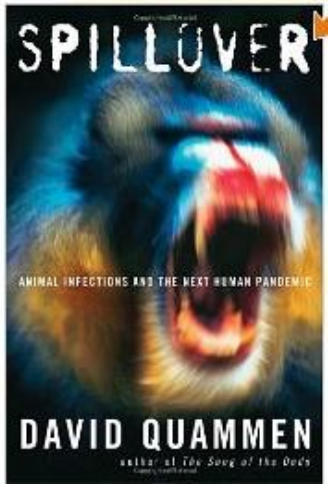
EXTRA CREDIT-1

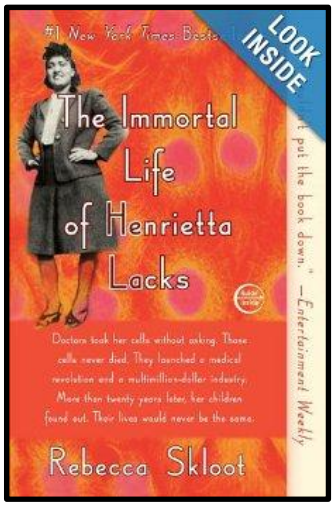
• Choose one of the books I have recommended, or one that I approve.

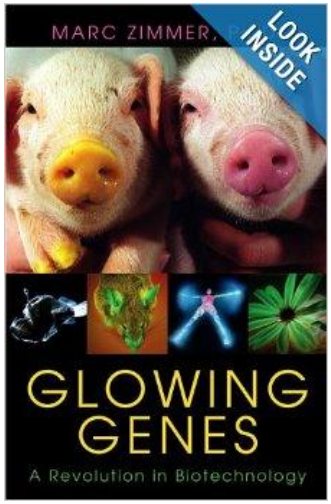
•500-750 word book **review**. This should NOT just be a summary of the text, but a critical review of the text as well. Generally, the ratio of summary to evaluation should be about 1:1.

• Total of 10 points possible.

• Due by Friday of week 13





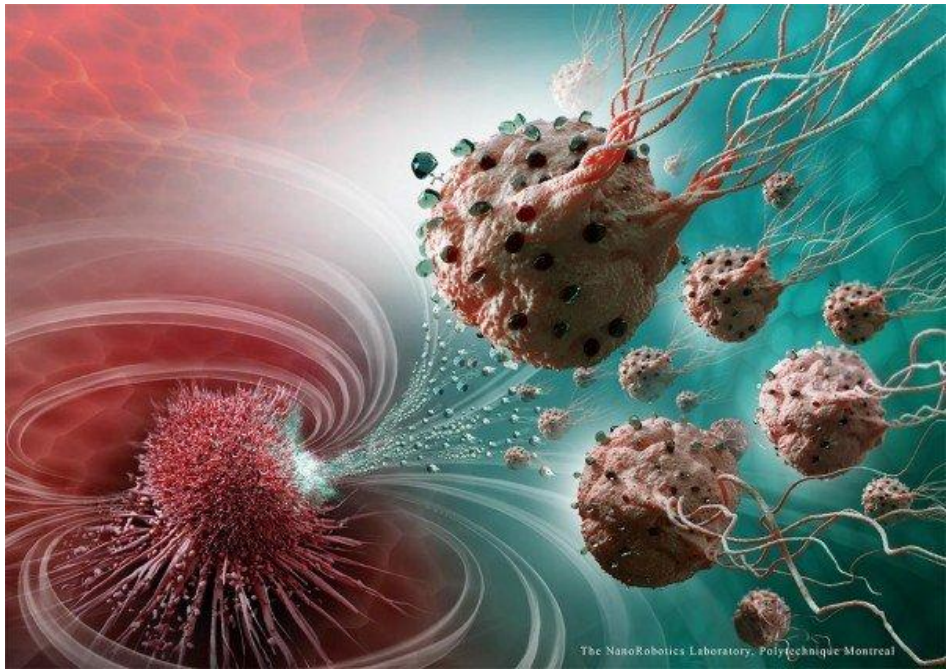


EXTRA CREDIT-2

• Prepare a 5 minute presentation on any current event in Cellular Biology.

•You will present your presentation to the class at the beginning of any Tue or Thur lecture of your choosing. Inform me at least 1 day in advance.

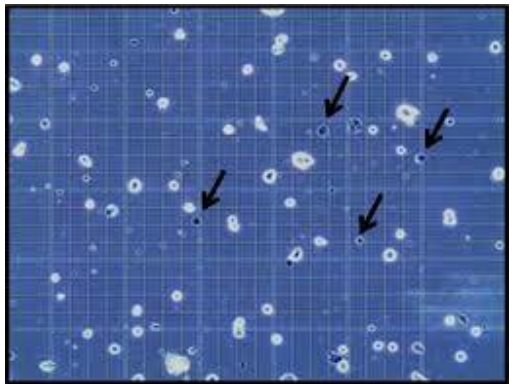
•Total of 10 points possible and must be completed by the end of week 13



Credit: NanoRobotics Laboratory, Polytechnique Montreal And Science Daily

CELLULAR STAINS AND DYES

What is cellular staining?
An auxiliary technique used in microscopy to enhance contrast in the specimen imaged. By using different stains, one can preferentially highlight certain cellular components (eg. nucleus, cytoskeleton, cell membrane, etc)

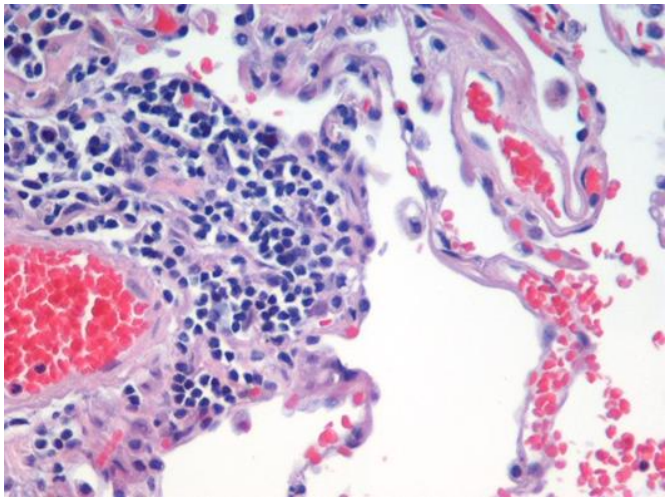


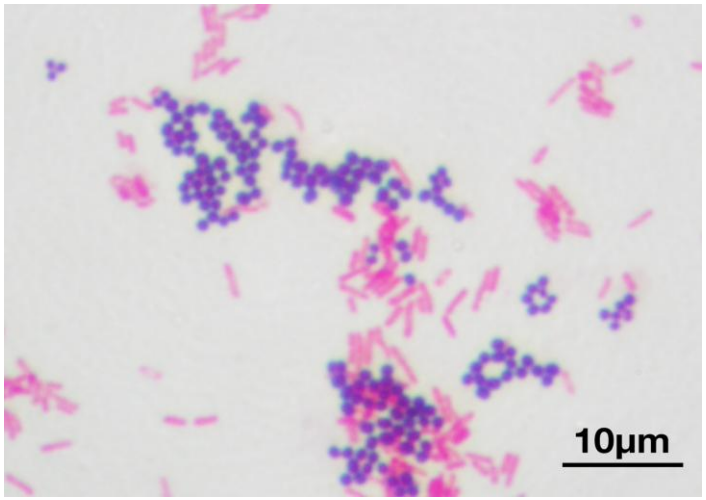
Vital Stains: stains which are taken up by dead cells but excluded by living cell

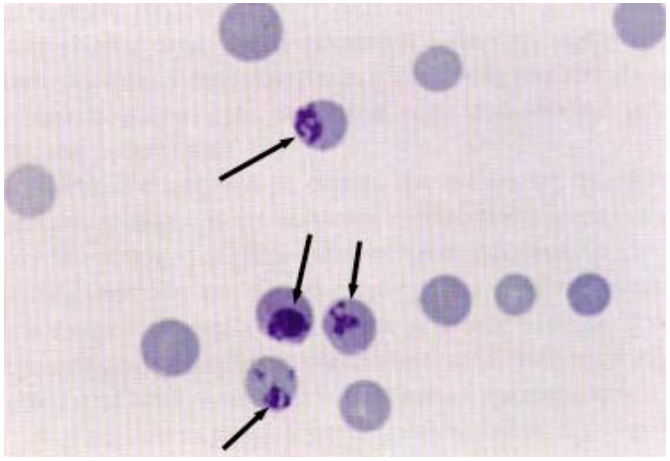
Supravital Stains: stains which enter and stain living cells. Most supravital stains are toxic if used in an organism.



USES OF STAINS
Histology and Cytology





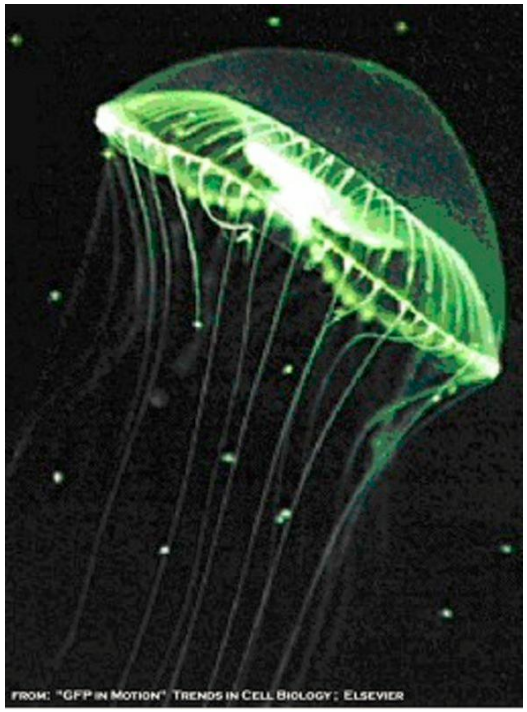


FLUORESCENCE

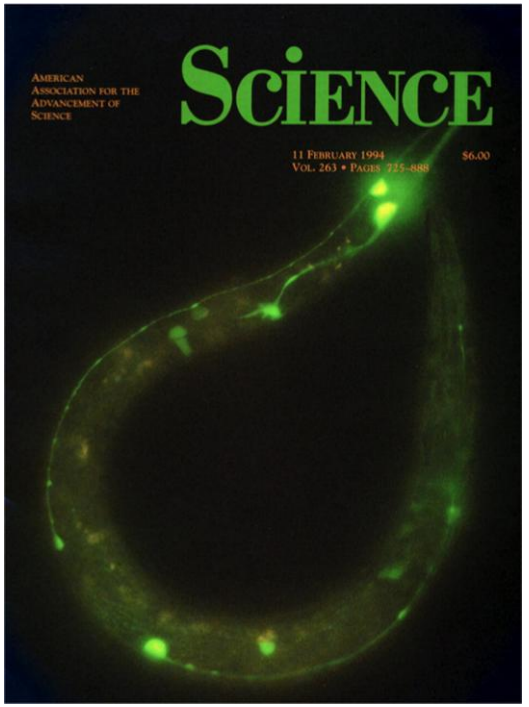
Form of microscopy where a fluorescent dye is used to label a molecule of interest. The fluorescent dye absorbs light at a high energy wavelength and emits it at a lower energy wavelength. Widely used technique due to its sensitivity and specificity for the intracellular molecule of interest.

Fluorophore = molecule the absorbs energy and converts it into light.

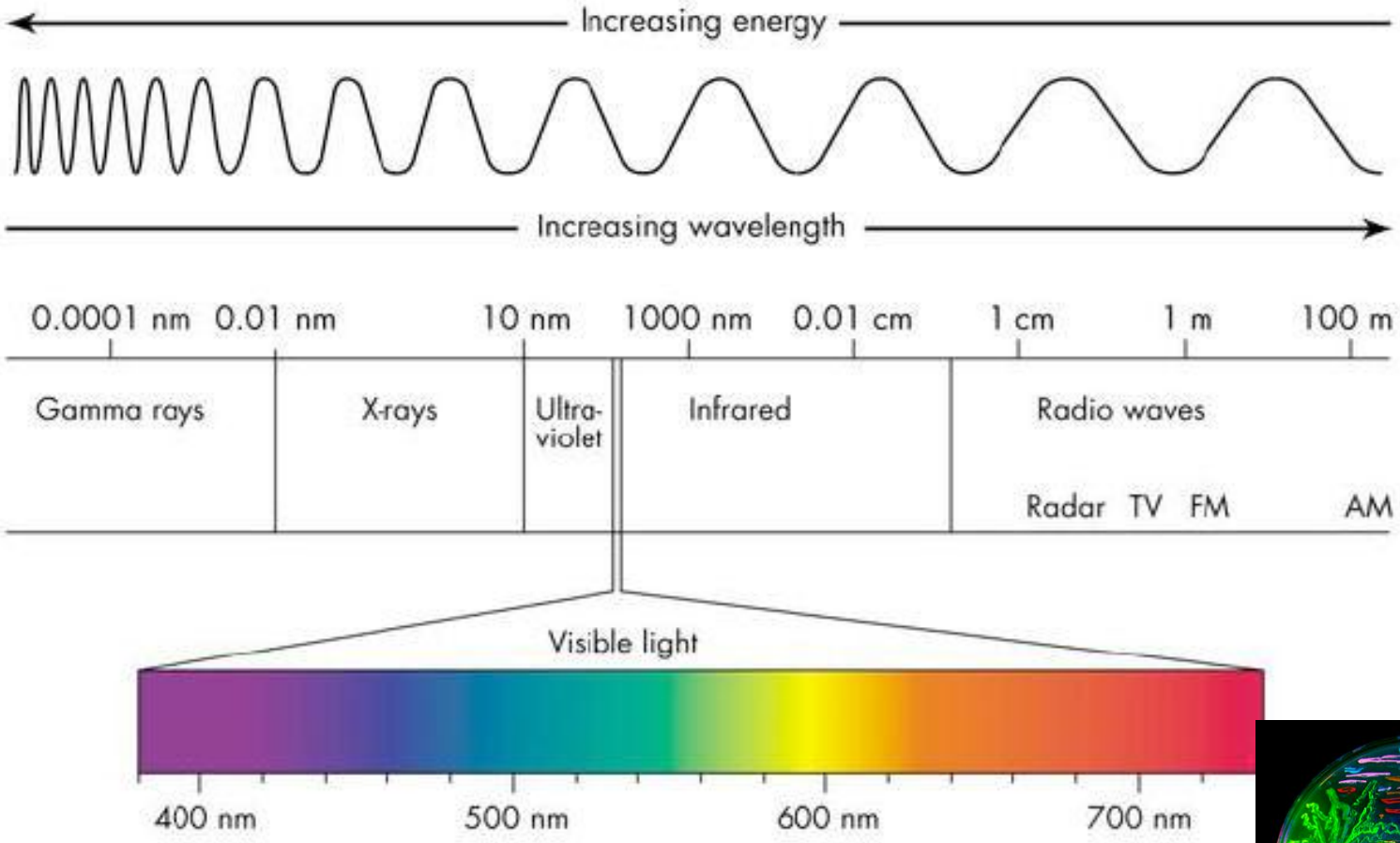
Absorbs light energy → excited state → emission of light energy

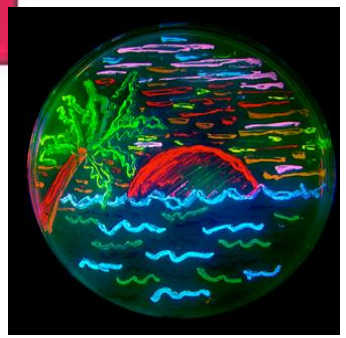




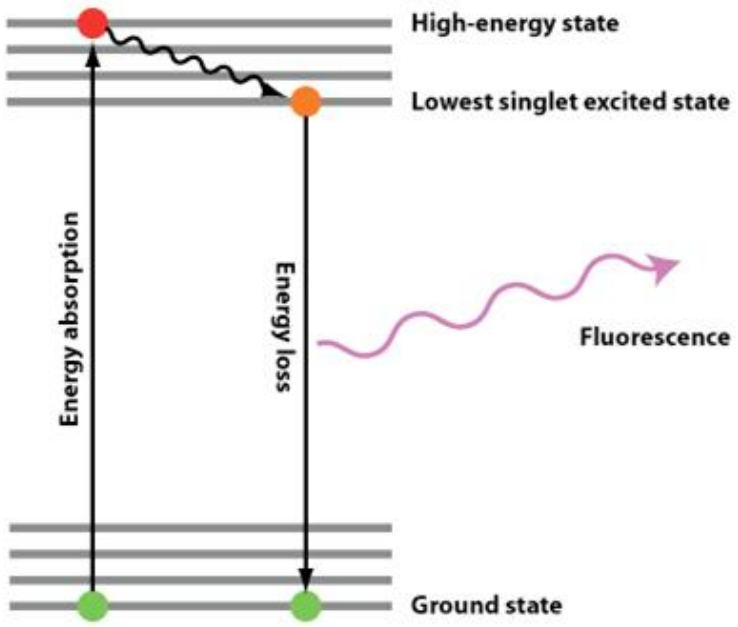


LIGHT ENERGY

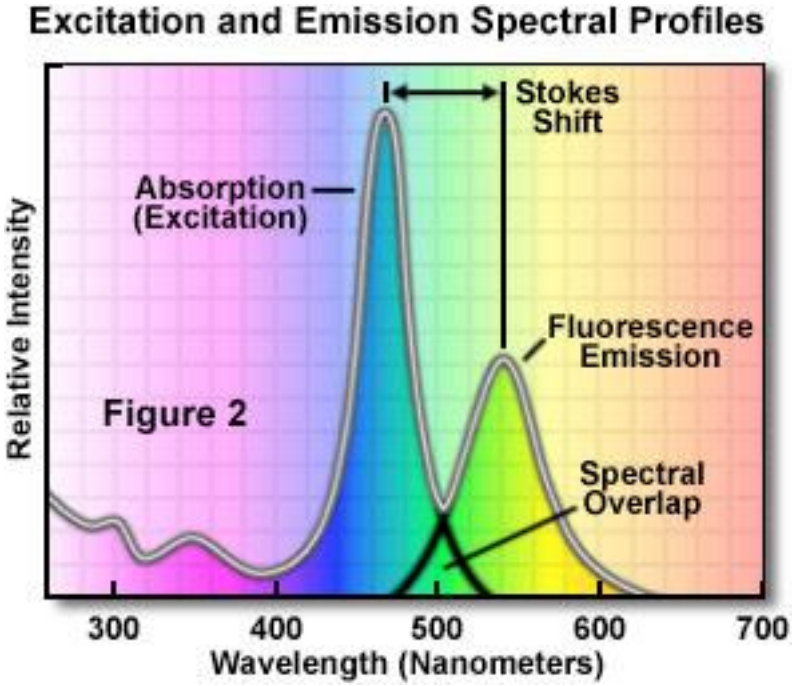




TWO PART PROCESS OF FLUORESCENCE



http://www.scienceinyoureyes.com/index.php?id=79



http://www.olympusmicro.com/primer/lightandcolor/fluoroexcitation.html

Fluorescence excitation spectrum = the range of wavelengths at which the fluorophore absorbs light energy

Fluorescence emission spectrum = the range of wavelengths at which the fluorophore emits light energy

EXCITATION AND EMISSION

Excitation and emission spectrums of some popular fluorescent markers:

Green Fluorescent Protein

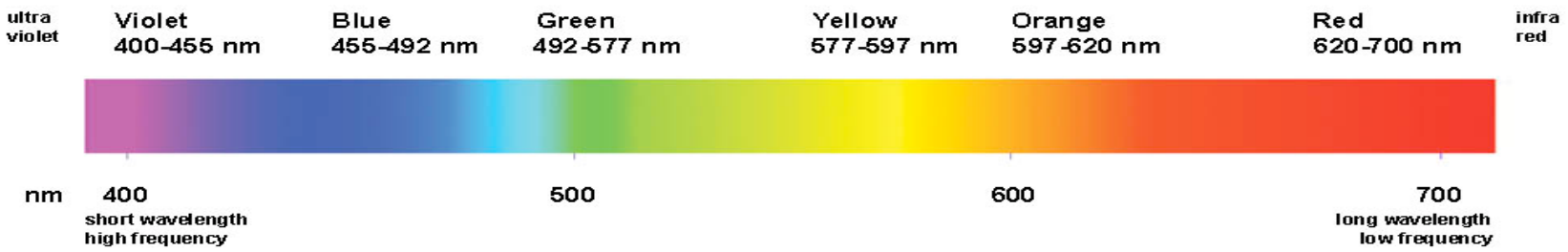
excitation maximum 395nm
emission maximum 509nm → green

DAPI stain

excitation maximum 355nm
emission maximum 462nm → blue

Texas Red stain

excitation maximum 591nm
emission maximum 608nm → red



APPLICATIONS FOR FLUORESCENCE IN CELLULAR BIOLOGY

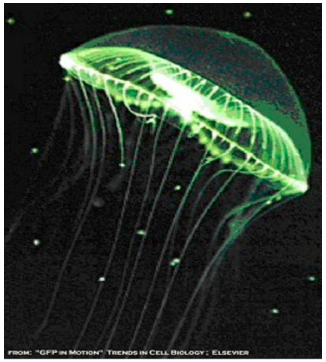
TAGGING PROTEINS WITH GFP

Protein first isolated from the jellyfish *Aequorea victoria* which is also a fluorophore – absorbs energy at 395nm and emits energy at 509nm (green)

The GFP gene is often used in cellular biology as a reporter of expression. A GFP-tagged protein of interest is transfected into a cell. Expression of the protein of interest will result in expression of the GFP protein as well (same promoter) – leading to fluorescence.

Can be used to tag essentially any protein. Essentially non-toxic to living cells and organisms and can be studied over time.

Many fluorescent protein derivatives have now been designed of different fluorescent properties.

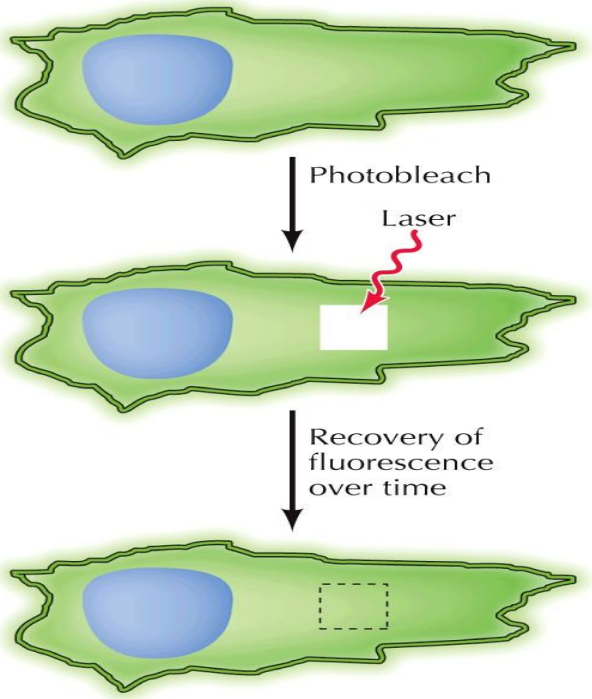






BLEACHING AND FRAP

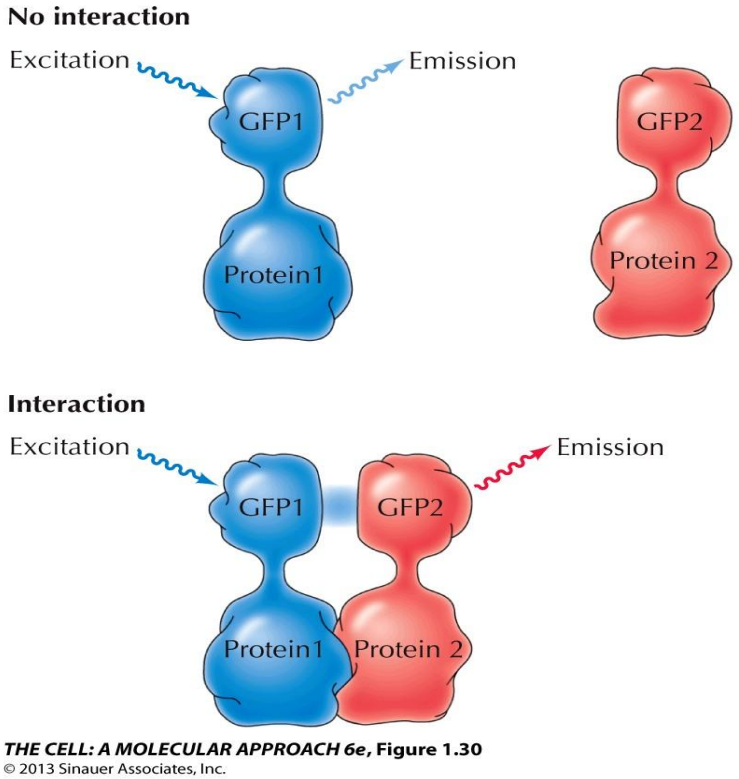
Fluorescence Recovery after Photobleaching (FRAP)
Technique to study the movement of proteins in a cell. A region of fluorescence in a cell is bleached by over exposure to light. Fluorescence recovers over time due to the movement of unbleached GFP-labeled molecules into the region.



***THE CELL: A MOLECULAR APPROACH 6e*, Figure 1.29**
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FRET AND PROTEIN INTERACTION

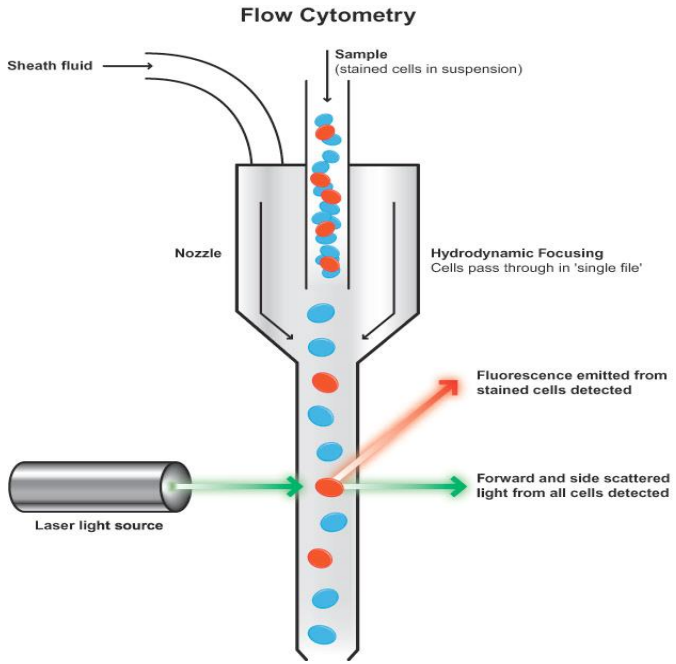
Fluorescence Resonance Energy Transfer (FRET)
Technique to study the interactions of two proteins with one another. The proteins are coupled to two different fluorescent dyes. Light is emitted to excite the first protein, which excites the second protein if they interact, resulting in emission.



THE CELL: A MOLECULAR APPROACH 6e, Figure 1.30
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FLOW CYTOMETRY

Flow Cytometry
def. A laser-based biophysical technology employed in cell counting, cell sorting (FACS), and biomarker detection. Allows for simultaneous multiparametric analysis of the physical and chemical characteristics of a sample of cells.

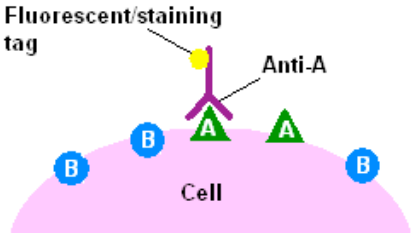


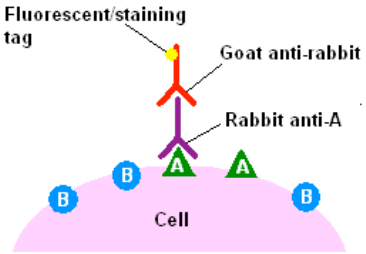
IMMUNOFLUORESCENCE

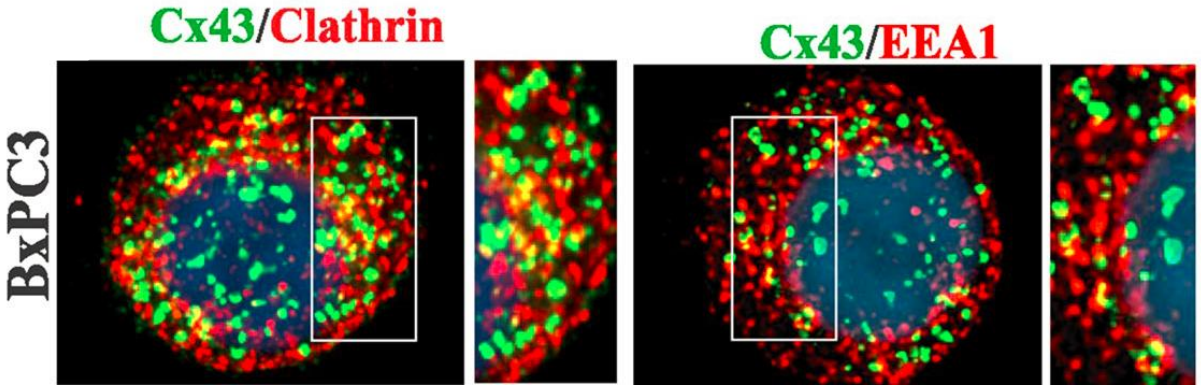
Immunostaining

def. The use of any antibody-based method to detect a specific protein (antigen) in a sample. (Immunofluorescence-uses tagged antibodies)



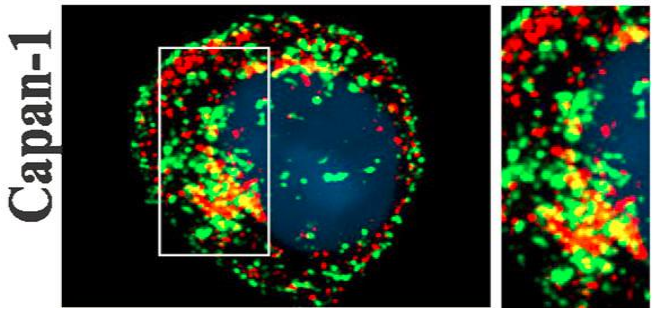


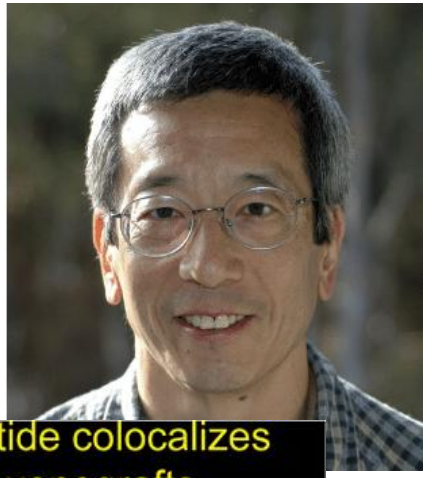


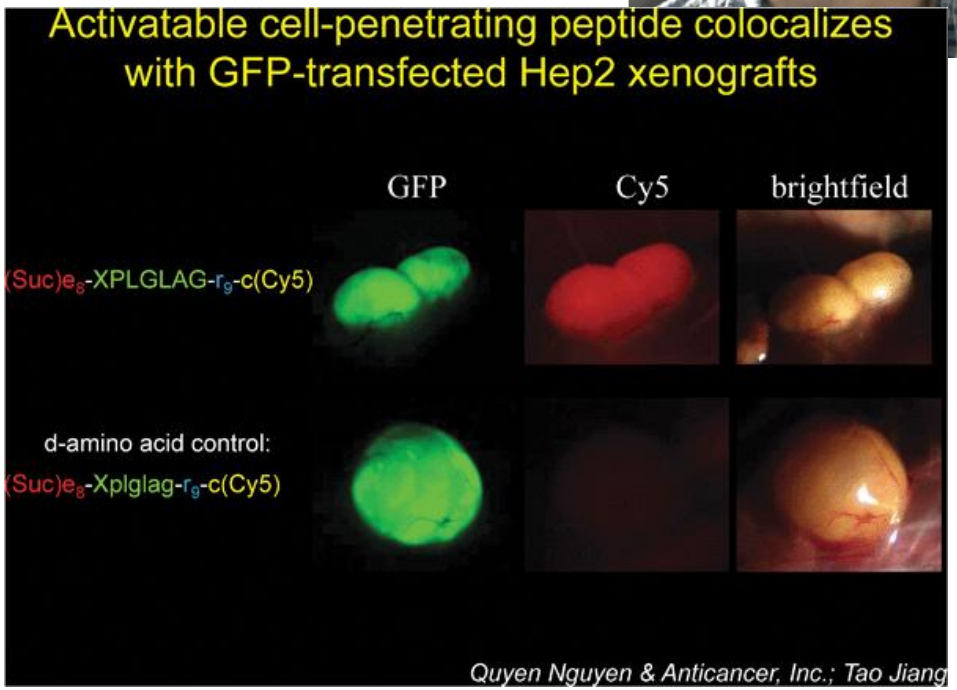


FLUORESCENCE AND: Location, location, location....









DAPI

DAPI stands for 4',6-diamidino-2-phenylindole

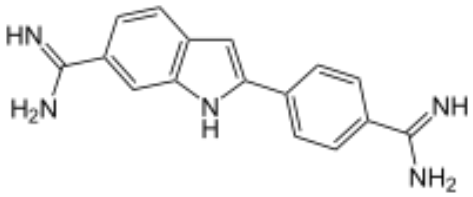
Passes through intact or damaged cell membranes, which means it can be used in both live or fixed cells.

Binds strongly to A-T rich regions in DNA

Absorbs maximally at 355nm (UV) and emits maximally at 462nm (blue)

Also used to identify Mycoplasma contamination

Most commonly used for fluorescence imaging, but may also be used as a marker in flow cytometry.



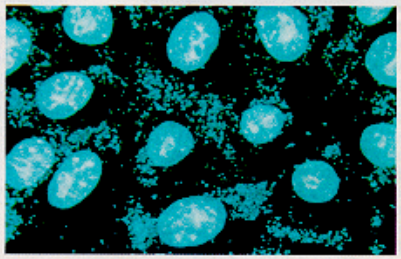


Figure 3 *DNA-fluorochrome staining, VERO cells, positive for mycoplasma (heavily contaminated).*

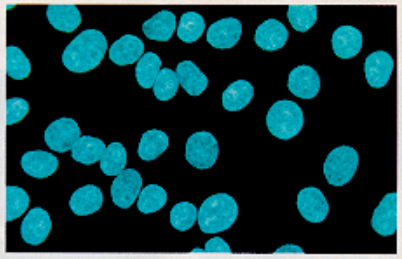


Figure 4 *DNA-fluorochrome staining, VERO cells, negative for mycoplasma.*

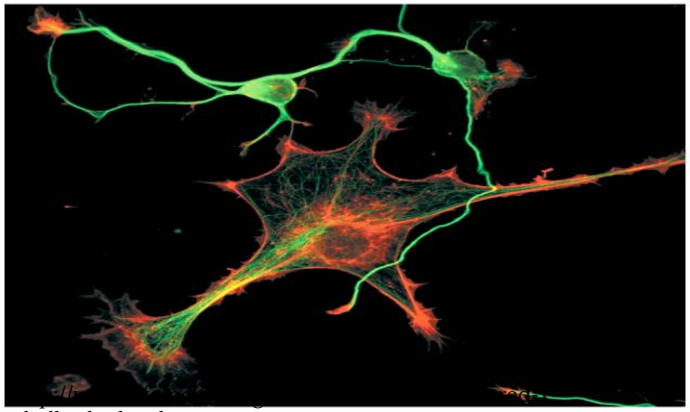
Texas Red - Phalloidin

Texas Red is a fluorophore that excites at 591nm and emits at 608nm (yellow)

Texas Red is fairly easily conjugated to proteins, such that the conjugated protein effectively becomes a fluorophore. This is the case with Texas Red – Phalloidin conjugate stain.

Phalloidin is a toxin produced by the death cap (*Amanita phalloides*). It binds F-actin (filamentous) in cells and prevents depolymerization.

Toxic to living cells. Does not easily cross intact cell membranes.



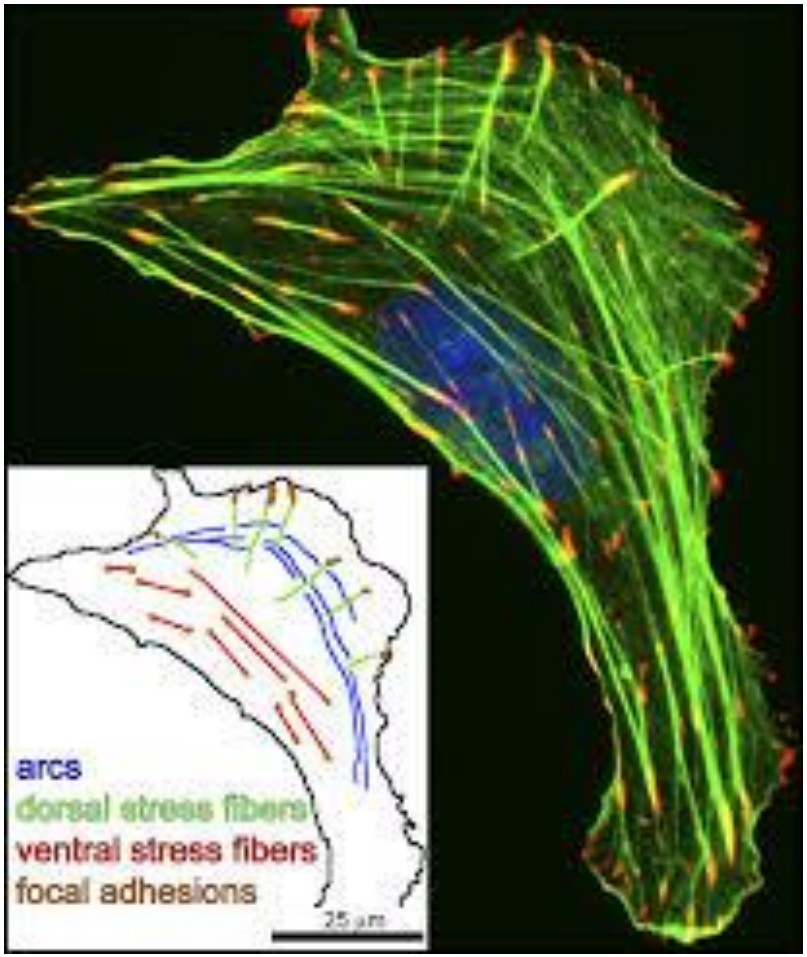
phalloidin.html

ORGANIZATION OF F-ACTIN

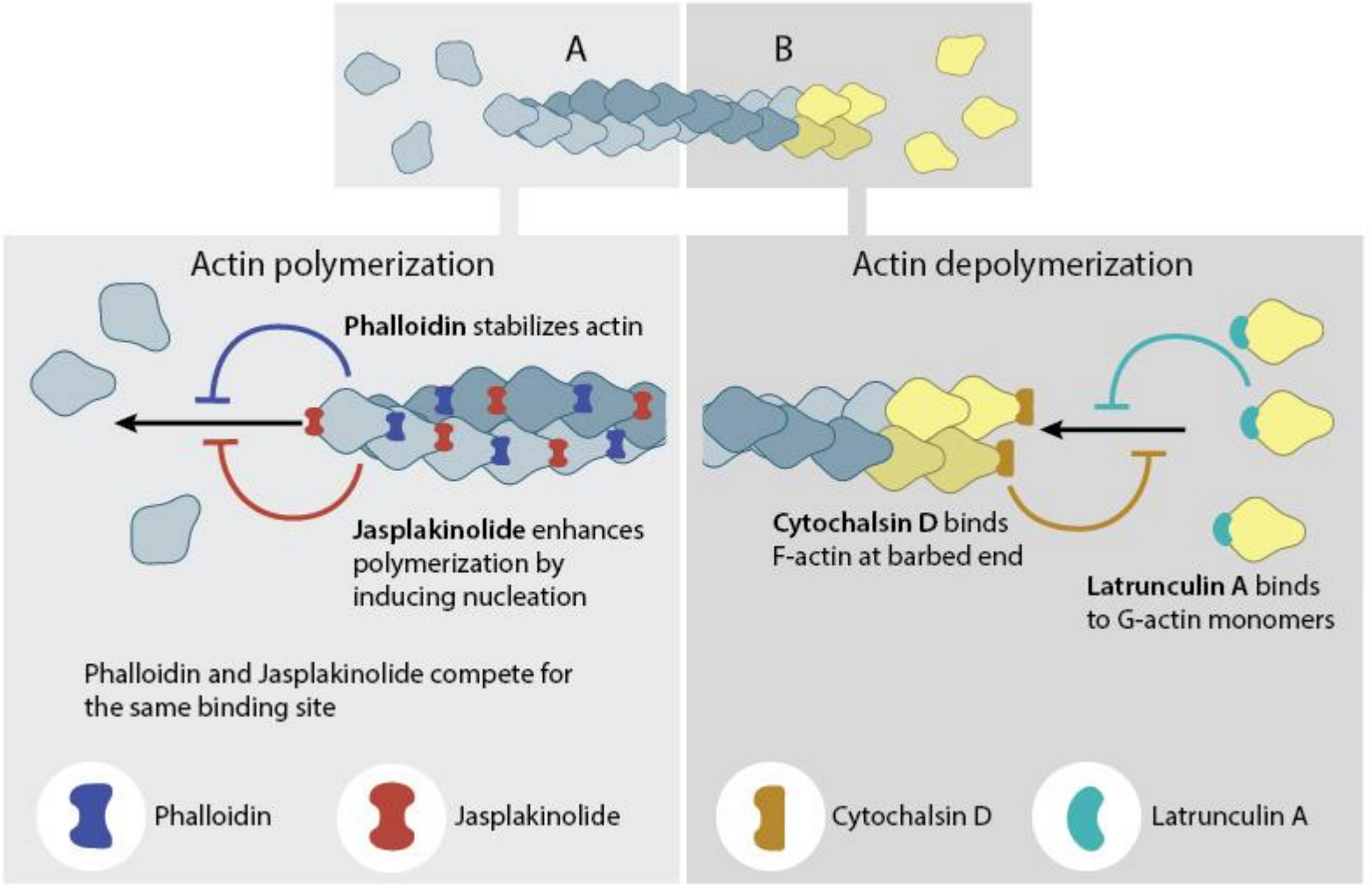
We will correlate the presence of well-organized filamentous actin with cell health and ability to establish an adherent culture.

Focal Adhesions: Assembly of actin filaments and proteins responsible for attachement.

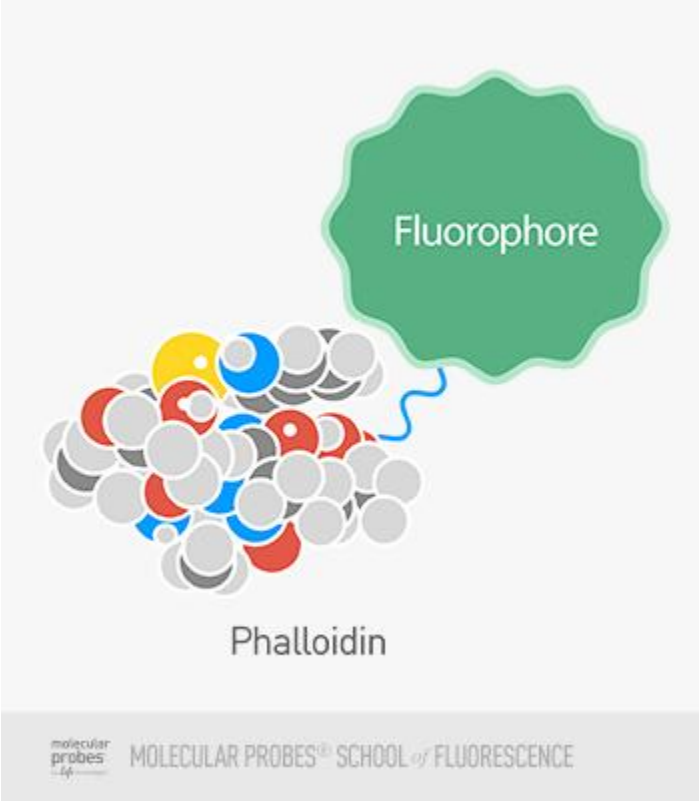
Stress Fibers: Contractile actomyosin bundles important for cell adhesion and morphogenesis



SMALL MOLECULE MODULATION OF F-ACTIN



FLUORESCENT STAINING



Fluorophores can be conjugated to proteins that will bind specific structures in the cells.

- Phalloidin-A Mushroom Toxin that binds F-Actin
- DAPI-Binds DNA
- Antibodies can be raised against specific proteins and conjugated with a fluorophore

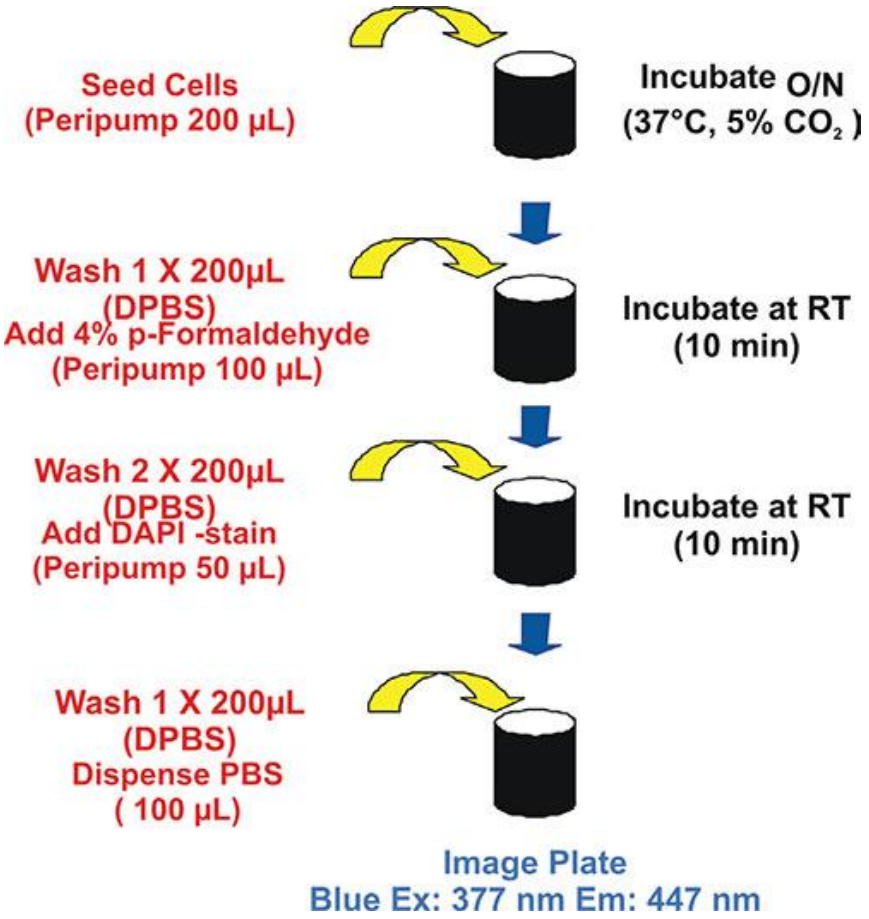
CELL PREPARATION FOR STAINING

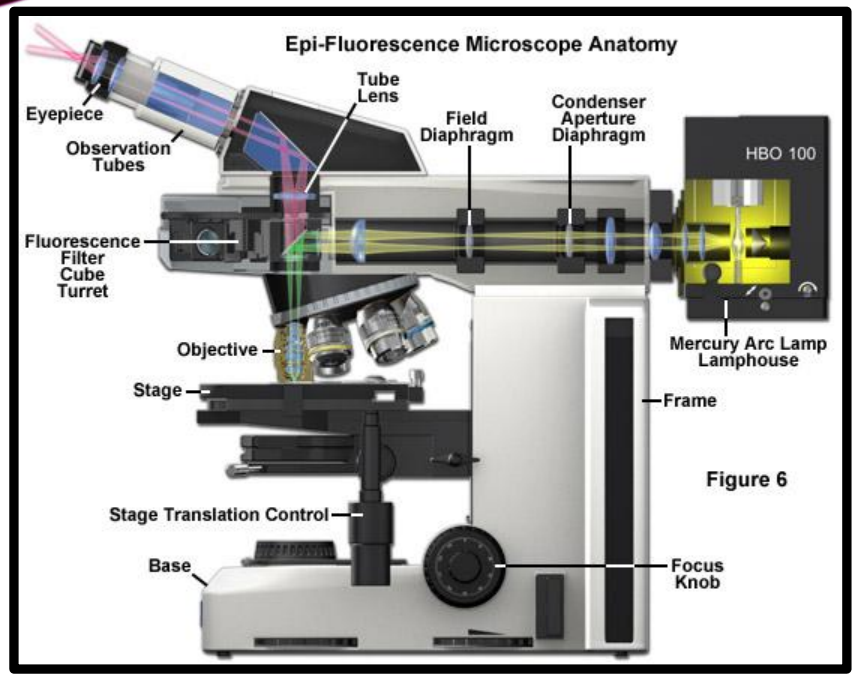
Fixation
def. Use of chemicals to kill a sample while preserving biological materials as close to their natural state as possible. Avoids apoptosis and postmortem decay (autolysis, putrification)
Ex. Paraformaldehyde: fixes tissues by cross-linking the proteins.
CAUTION

Quenching
def. Solution used to decrease the fluorescence of a substance. Used to decrease inherent cellular fluorescence prior to staining
Ex. Tris-Cl

Permeabilization
def. Treatment of cells with a mild surfactant which dissolves cell membranes, allowing larger molecules to enter inside the cell
Ex. Triton X-100

FLUORESCENT STAINING

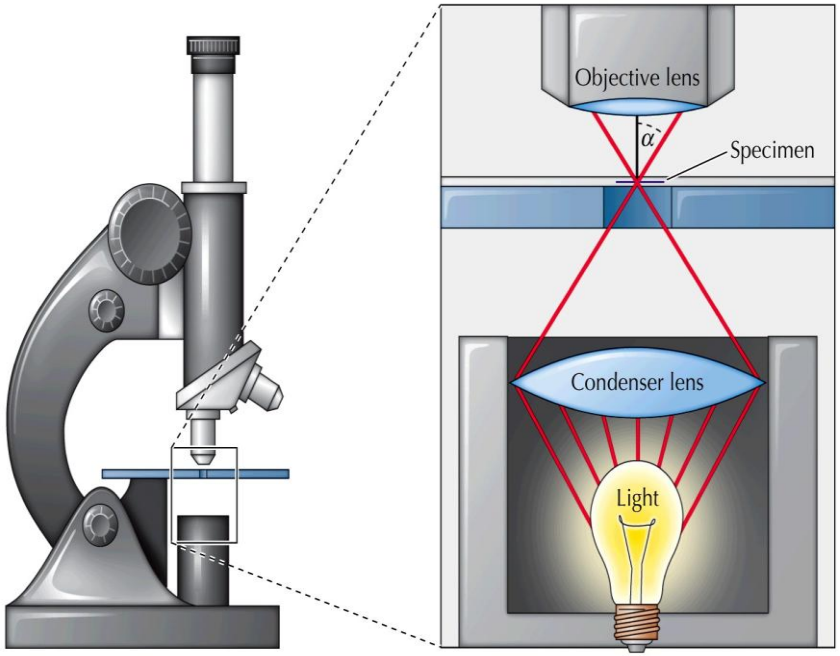




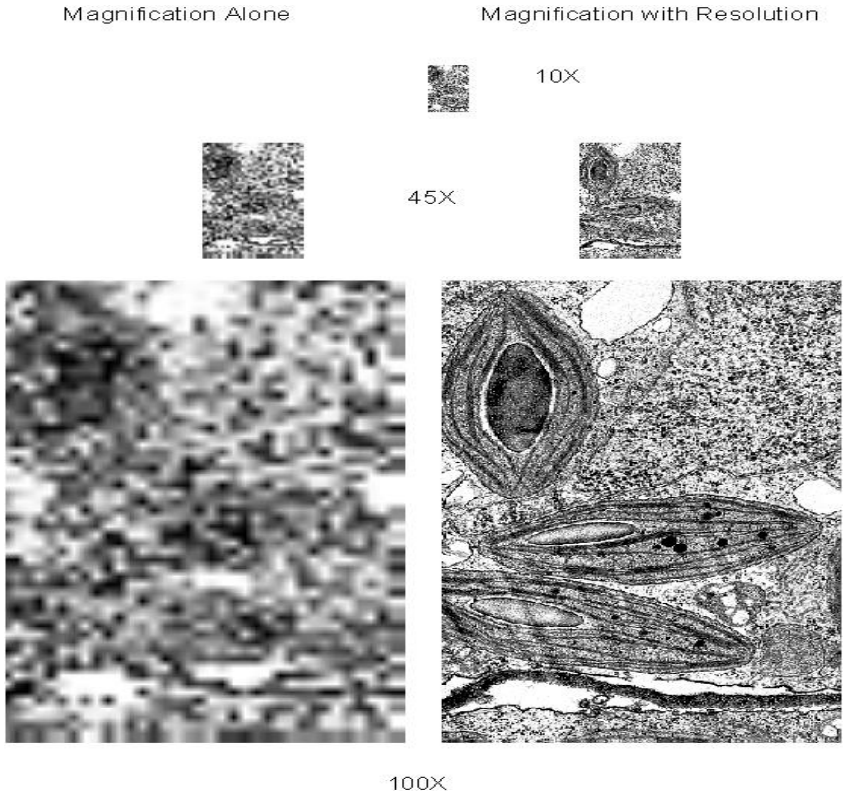
FLUORESCENCE MICROSCOPY

LIGHT MICROSCOPY

• Able to **magnify** objects up to 1000x. Sufficient for objects such as cells and large organelles (nuclei, mitochondria). The **resolution** power, however, of the light microscope limits its ability to observe the fine details of cell structures. **Limit of resolution for light microscopy is 0.22um**

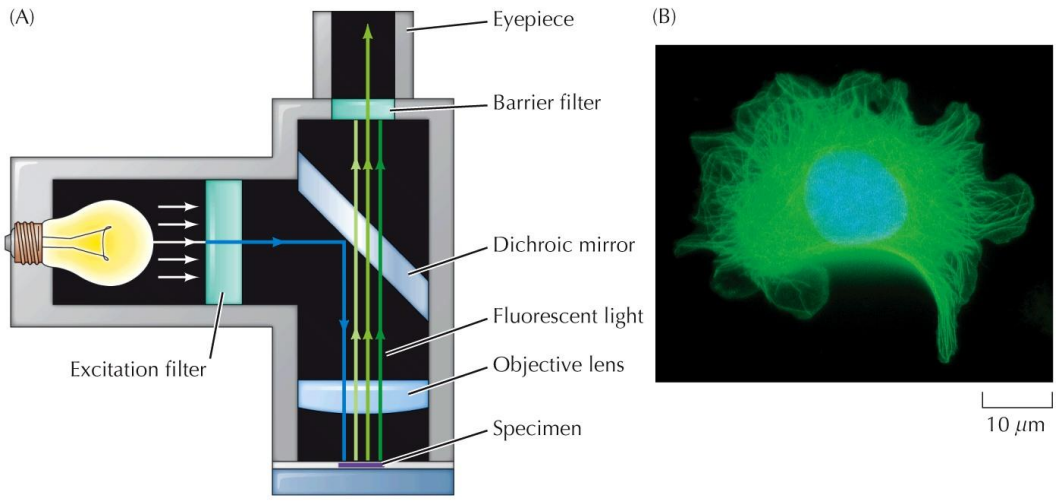


THE CELL: A MOLECULAR APPROACH 6e, Figure 1.23
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LIGHT MICROSCOPY

Fluorescence microscopy: use of a fluorescent dye to label molecules of interest within a cell. The dye absorbs light at one wavelength and emits at a second. The specimen can then be illuminated by the "excitation" wavelength and filters use to detect the "emission" wavelength.



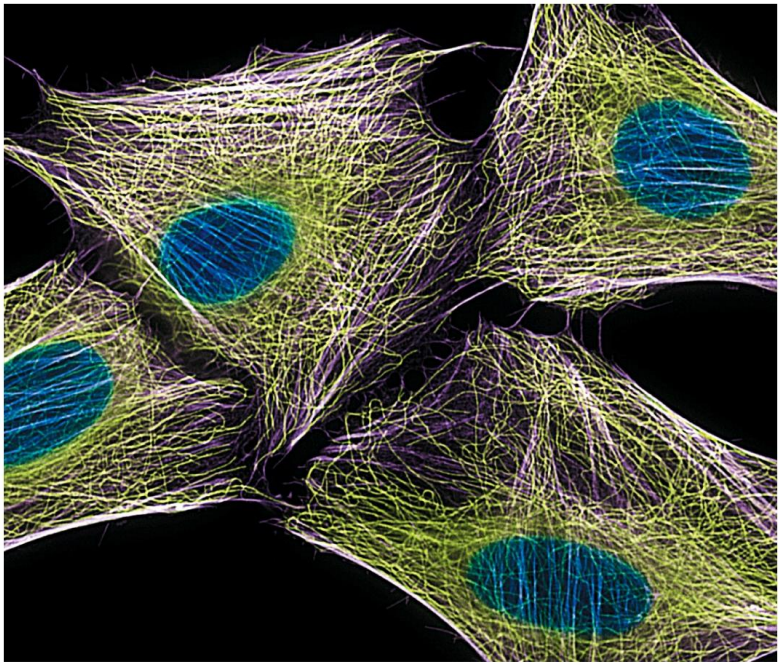
THE CELL: A MOLECULAR APPROACH 6e, Figure 1.27
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Green fluorescent protein (GFP)

Protein expressed by the jellyfish *Aequorea victoria* and first used as a fluorescent marker in the 1990s. GFP can be used as a reporter of expression of a protein of interest using recombinant DNA techniques.

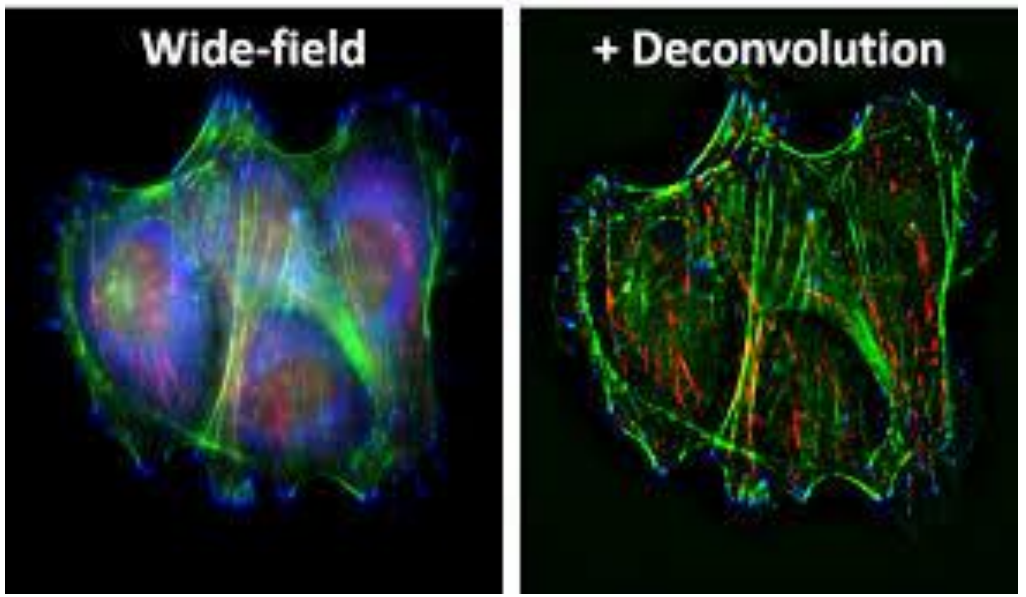
Wide Field vs Confocal Fluorescence Microscopy

Confocal microscopy allows images of increased contrast and detail to be obtained by analyzing fluorescence from only a single point in the specimen. Produces a much sharper image than standard, **widefield microscopy**, which analyzes a larger region resulting in noise from light scatter.



THE CELL: A MOLECULAR APPROACH 6e, Figure 1.32

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HOW SELECTIVE IS YOUR FILTER?



Figure 6

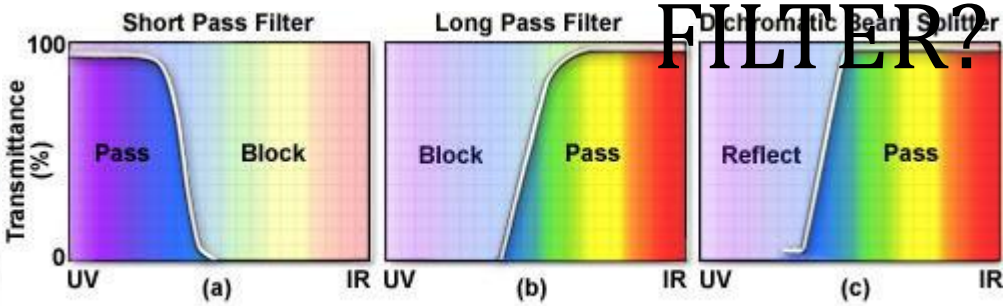
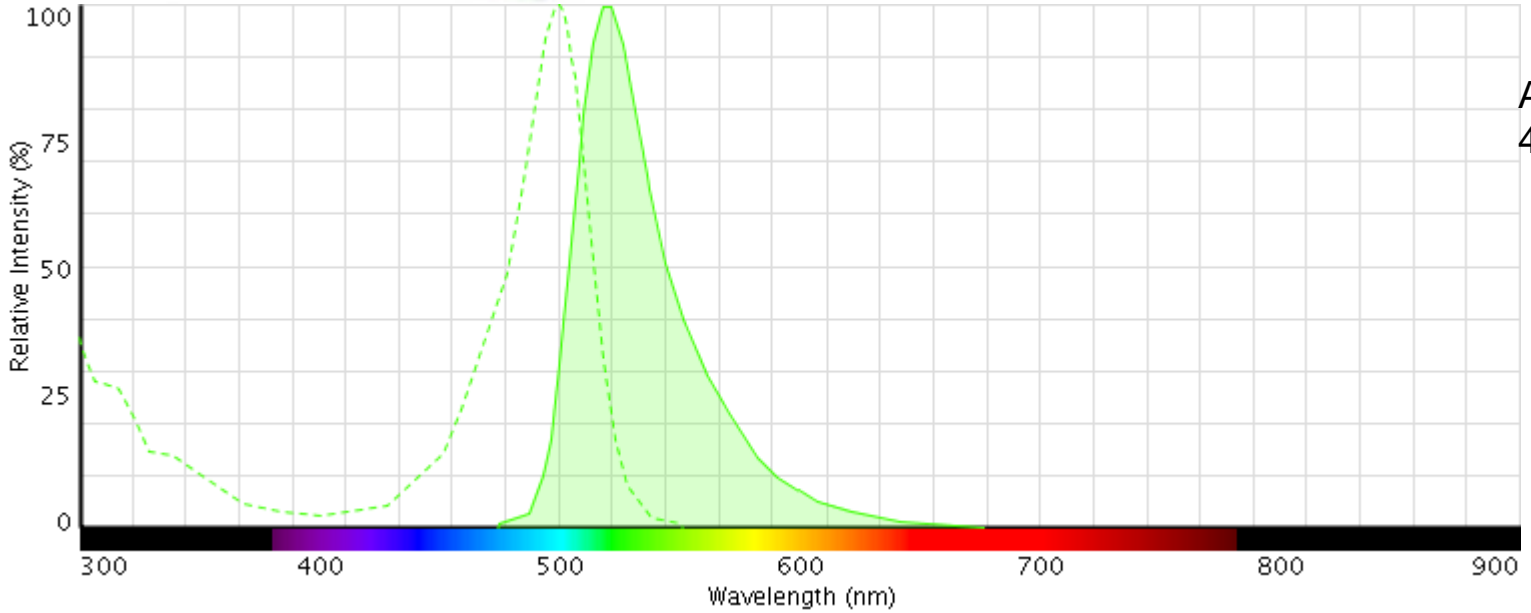


Figure 2



Alexa 488

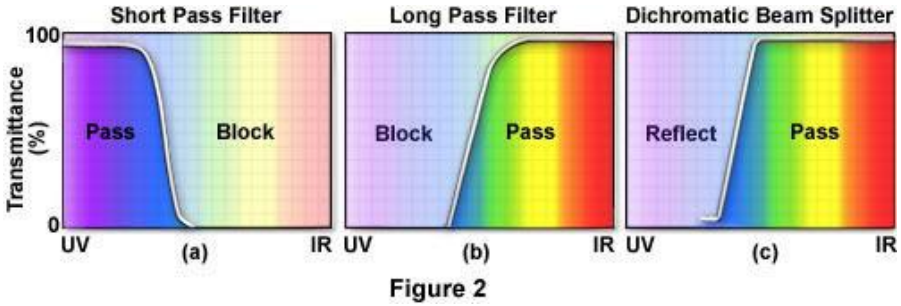


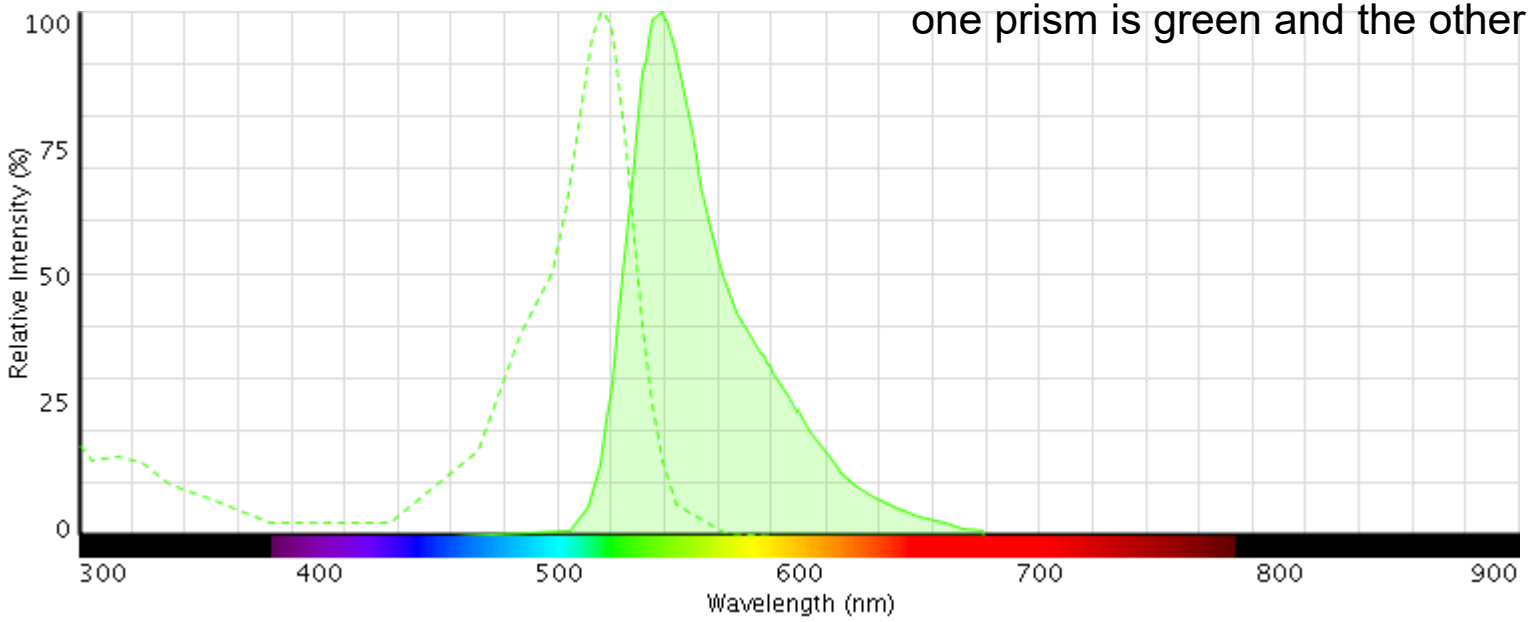
Figure 2

Our green fluorophore would be collected by our short pass filter.

So would our orange fluorophore.

This is why you often see green and red and filters band widths are carefully chosen.

Need to trust that what you collect with one prism is green and the other is red.



Alexa 555 Orange

USE AND CARE OF THE MICROSCOPE
inverted, phase-contrast

- Remember: magnification is the objective lens (4x, 10x, 20x, 40x) multiplied by the eye-piece (10x)

- Always turn off the light when done and replace the dust cover

- Use the appropriate phase shift ring as indicated on the objective lens (4x = Ph0 10x, 20x, 40x = Ph1 20x, 40x = Ph2)

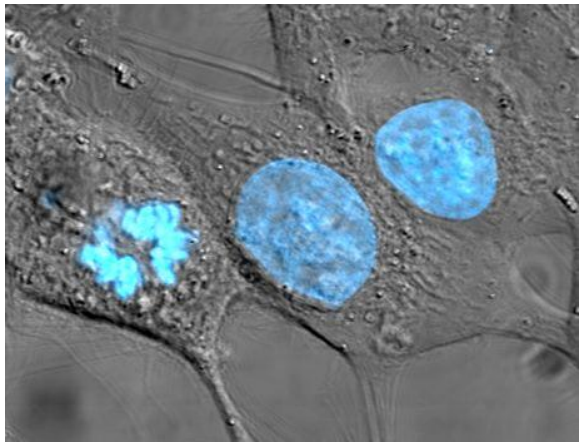


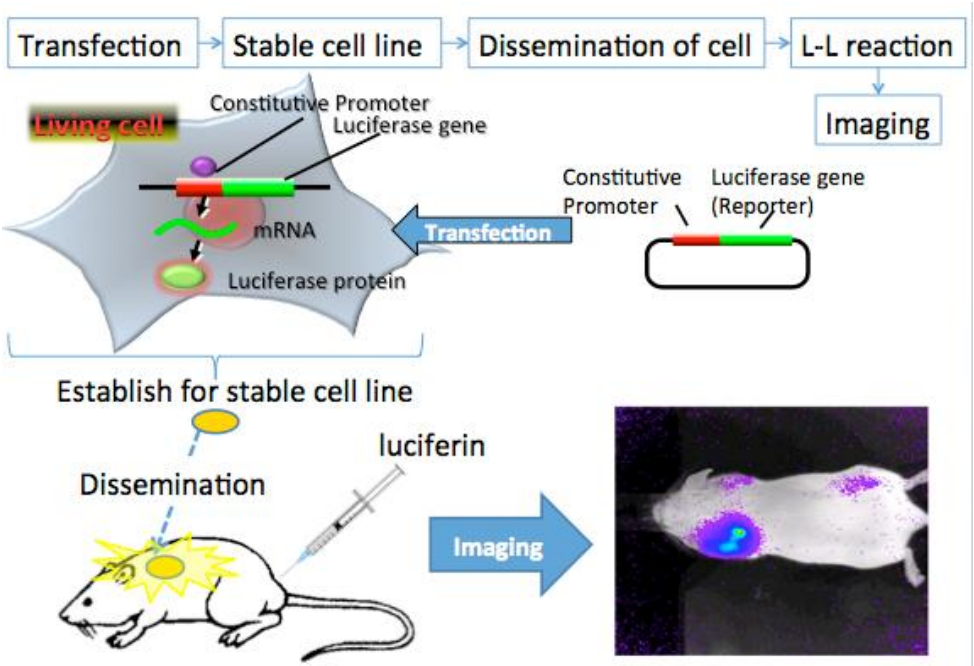
USE AND CARE OF THE MICROSCOPE
EVOS

• Digital inverted microscope for use in bright-field, phase-contrast and fluorescence microscopy.
• Be sure that the phase annuli selector is in the correct position that corresponds to the selected objective and contrast method.
• Use the light shield box to reduce ambient light and improve image quality.
• Bring a USB flash drive if you'd like to save images for lab reports.
• Always remember to turn off the microscope and replace the dust cover when done.



A Final Word...




Transfection
Stable cell line
Dissemination of cell
L-L reaction
Imaging
Living cell
Constitutive Promoter
Luciferase gene
mRNA
Luciferase protein
Transfection
Constitutive Promoter
Luciferase gene (Reporter)
Establish for stable cell line
Dissemination
luciferin
Imaging



STRUCTURE AND ACTIVITY OF CELLS

CELLS alive!

An online overview of prokaryotic and eukaryotic plant and animal cells

Inner Life of the Cell (Full Version - Narrated) (7:57)

Inner Life of the Cell (Excerpt – Musical Score Only) (3:12)

