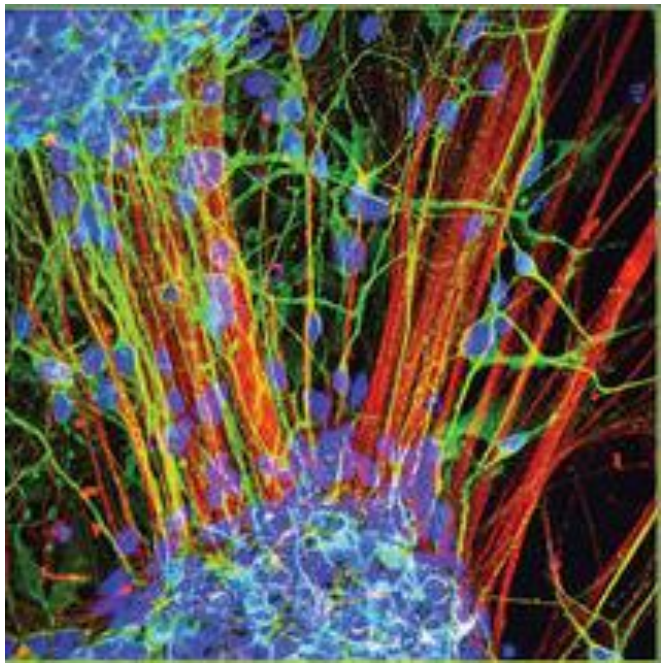
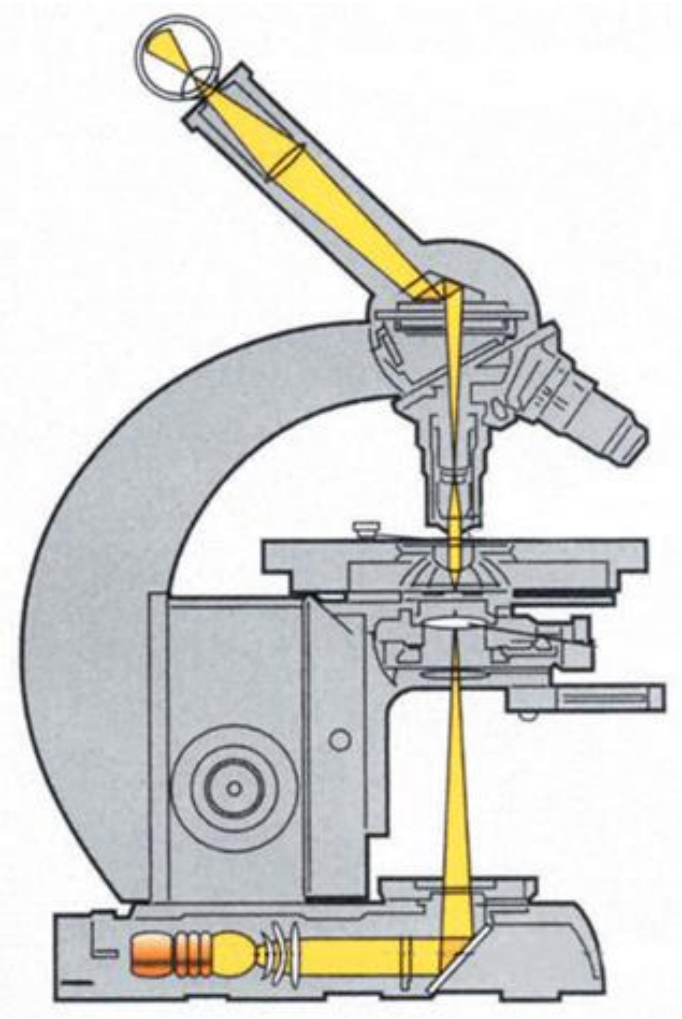


Biol 266 – Cell Biology

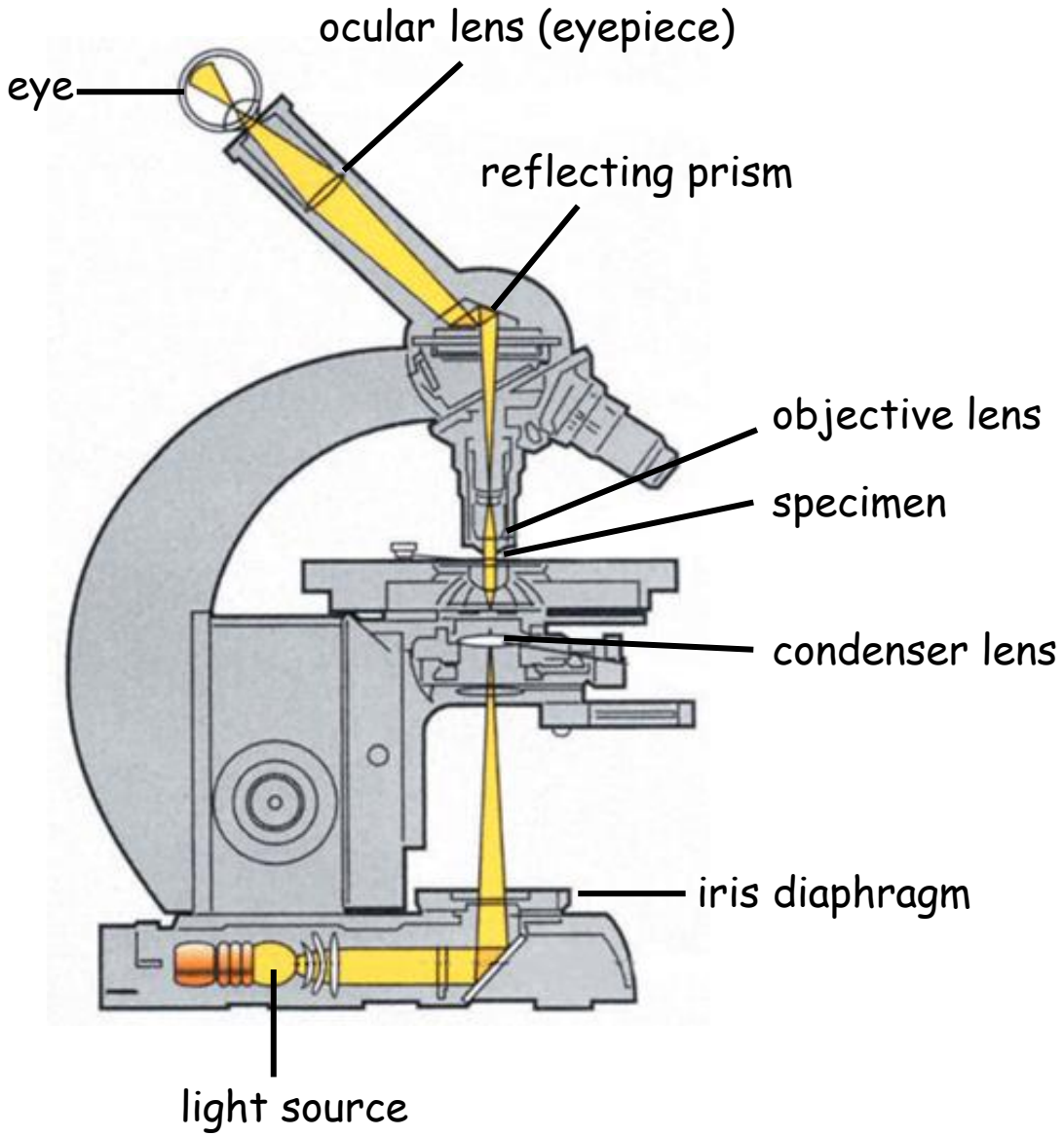
UNIT 2
"How cells are studied - I"

The compound microscope:

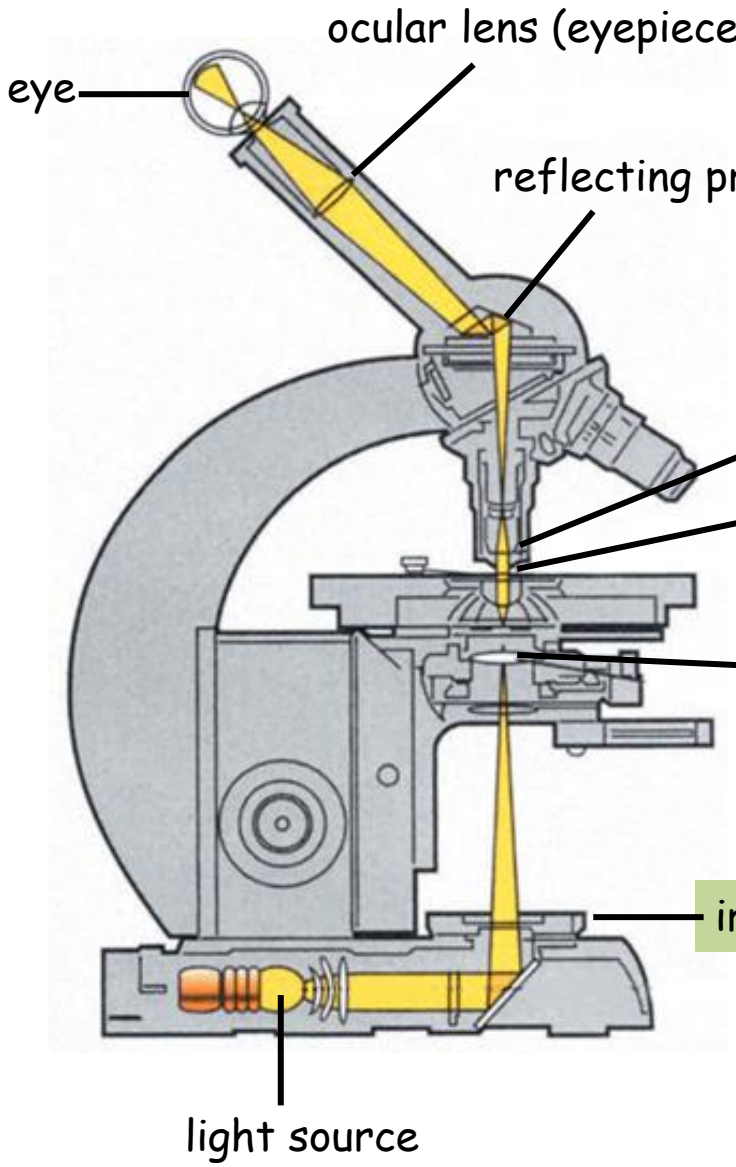
- most common microscope in use today
- contains several lenses that magnify the image of a specimen



The optical pathway in a modern compound optical microscope



The optical pathway in a modern compound optical microscope

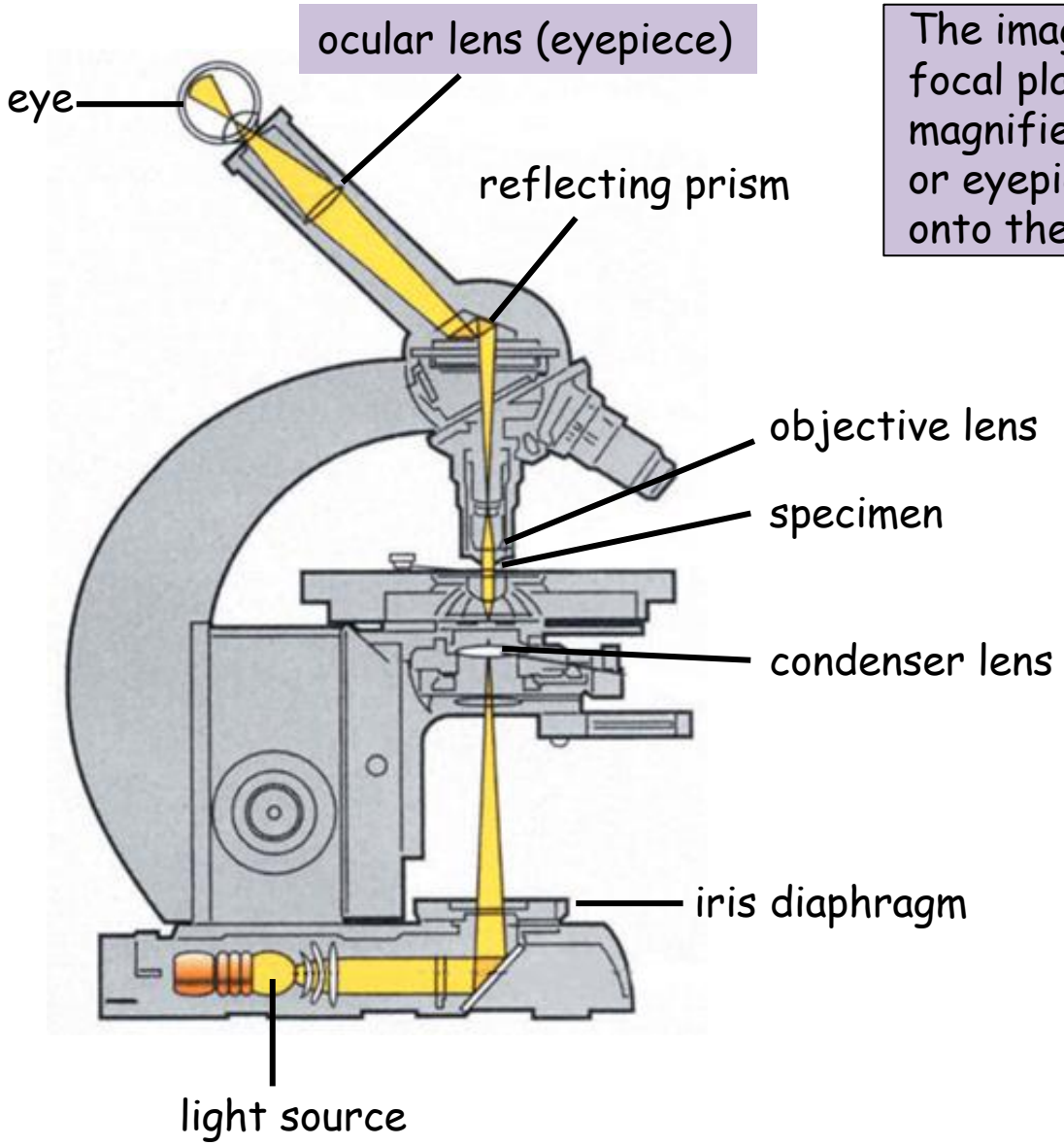


Picks up the light transmitted by the specimen and focus it on the focal plane of the objective lens, creating a magnified image

Focuses the light onto the specimen – no magnification

Restricts the amount of light entering the lens

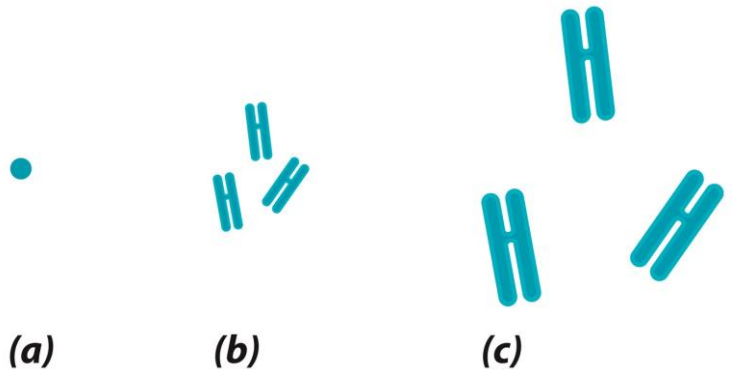
The optical pathway in a modern compound optical microscope



The image on the objective focal plane is further magnified by the ocular lens, or eyepiece, and projects it onto the human eye

The total magnification is the product of the magnification of the individual lenses.
If the objective lens magnifies 100-fold (a 100X lens) and the ocular lens magnifies 10-fold (a 10X lens), the final magnification will be 100 x 10 = 1000-fold

However, the most important property of the microscope is not its magnification but its resolving power (resolution).

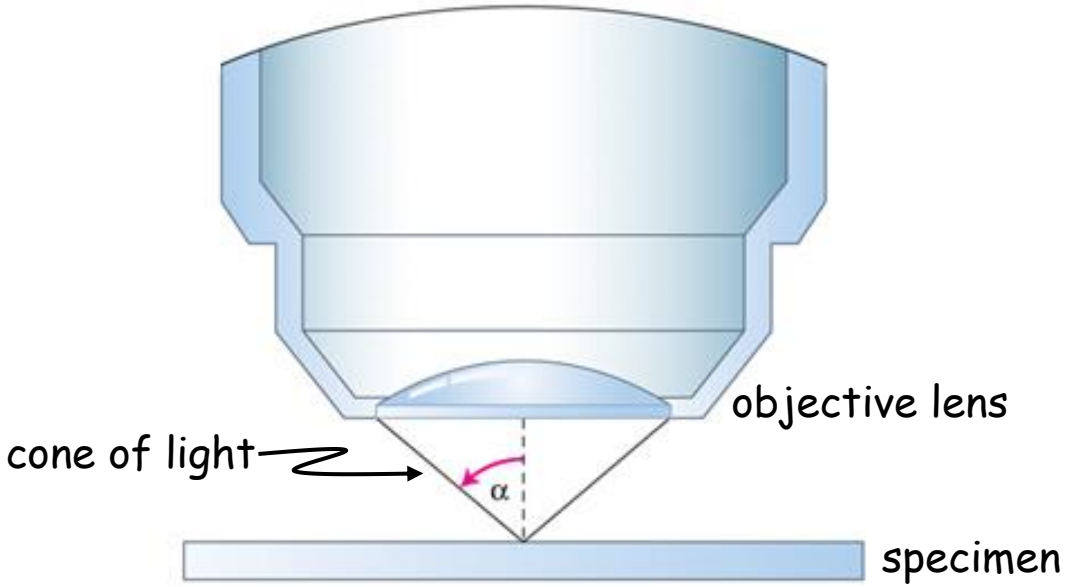


Magnification versus resolution: (a) to (b) has increased magnification and resolution, but (b) to (c) only increased magnification

Resolution (D) is the ability to see two nearby points as distinct images. The smaller the value of D, the better the resolution.

Resolution 1 is better than resolution 2

Resolution is determined by the objective lens and its ability to gather the "cone of light" coming from the specimen. The light comes into the objective lens as a cone due to diffraction by the specimen.



α represents half the angle of the cone of light

$$D = \frac{0.61\,\lambda}{n \sin\alpha}$$

- λ is the wavelength of incident light in nm
- n is the refractive index of the medium between the specimen and the objective

$$D = \frac{0.61\,\lambda}{n \sin\alpha}$$

The lower the wavelength, the better the resolution.

The shortest wavelength visible light is 450 nm (blue).

In contrast, an electron in an electron microscope with an accelerating voltage of 100,000 V has a wavelength of 0.004.

In theory, the resolution of such an electron microscope is ~100,000 times greater than that of the light microscope.

A fundamental limitation on all microscopes: a given type of radiation cannot be used to probe details smaller than its own wavelength (λ).

We can partially circumvent this limitation by increasing α, which will decrease D. The best objectives have an α value of 70° (e.g. sin90 = 1, sin70 = 0.94, sin45 = 0.71. Thus as α increases, the denominator increases, lowering the value of D).

$$D = \frac{0.61\,\lambda}{n \sin\alpha}$$

A fundamental limitation on all microscopes: a given type of radiation cannot be used to probe details smaller than its own wavelength (λ).

Another way is to increase the refractive index of the medium between the specimen and the objective lens (n).
(e.g. n = 1.0 for air, n = 1.5 for oil. Thus, using oil increases resolution by 33%)

$$D = \frac{0.61\,\lambda}{n \sin\alpha}$$

The limit of resolution of a light microscope:

With the visible light of shortest wavelength (blue, λ = 450 nm), an immersion oil objective (n = 1.5) and the best objective lens (α = 70°, sin α = 0.94):

$$D = \frac{0.61 \times 450 \text{ nm}}{1.5 \times 0.94} = 194 \text{ nm } (\sim 0.2 \ \mu\text{m})$$

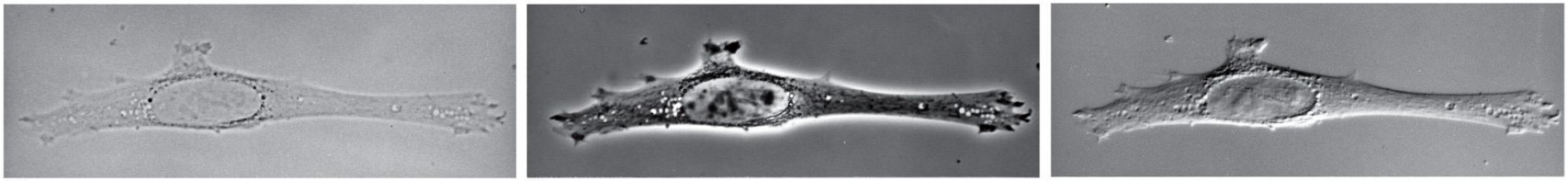
No matter how many times the image is magnified, the light microscope can never resolve objects that are less than ~ 0.2 μm in size

Three common types of light microscopy

A. Brightfield – no contrast other than natural is provided, image is projected on a background of the cone of light that enters the objective lens.

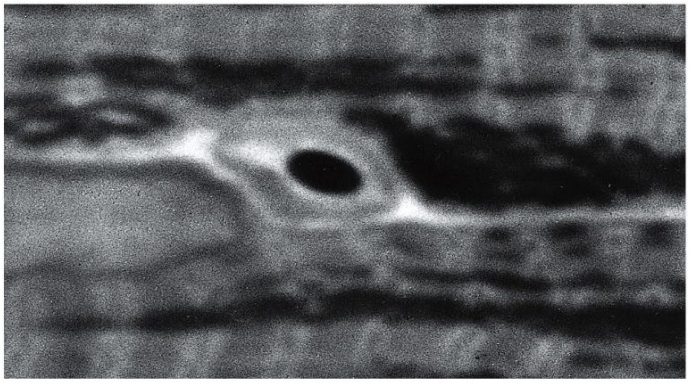
B. Phase-contrast – as light passes through a sample it is slowed in a medium of higher refractive index. The refracted and unrefracted light are recombined to form the image. If light is out of phase it will be less bright, in phase it will be more bright. Requires a phase plate to be inserted into the microscope after light passes through the objective lens.

C. Differential interference contrast (DIC) or Nomarski interference – based on interference between polarized light and the medium (refractive index) through which it travels. Method of choice for thicker samples.

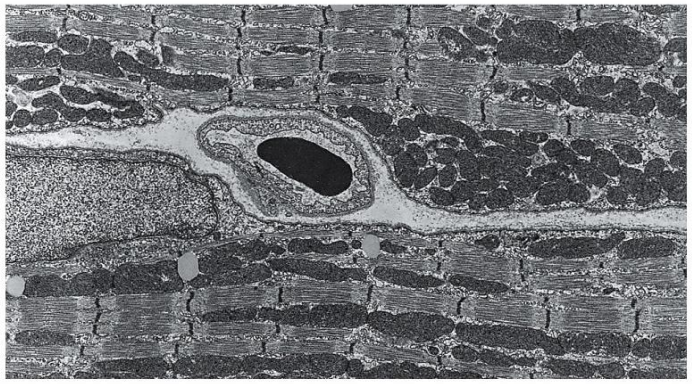


Transmission electron microscope (TEM)

Transmission electron microscopes (TEMs) use electrons instead of light to form images.



(a)

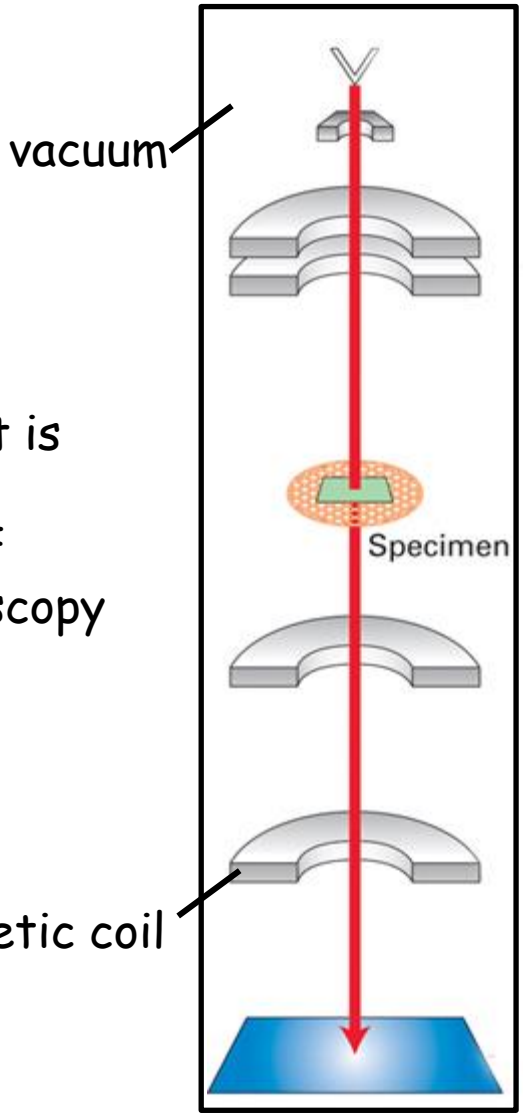


(b)

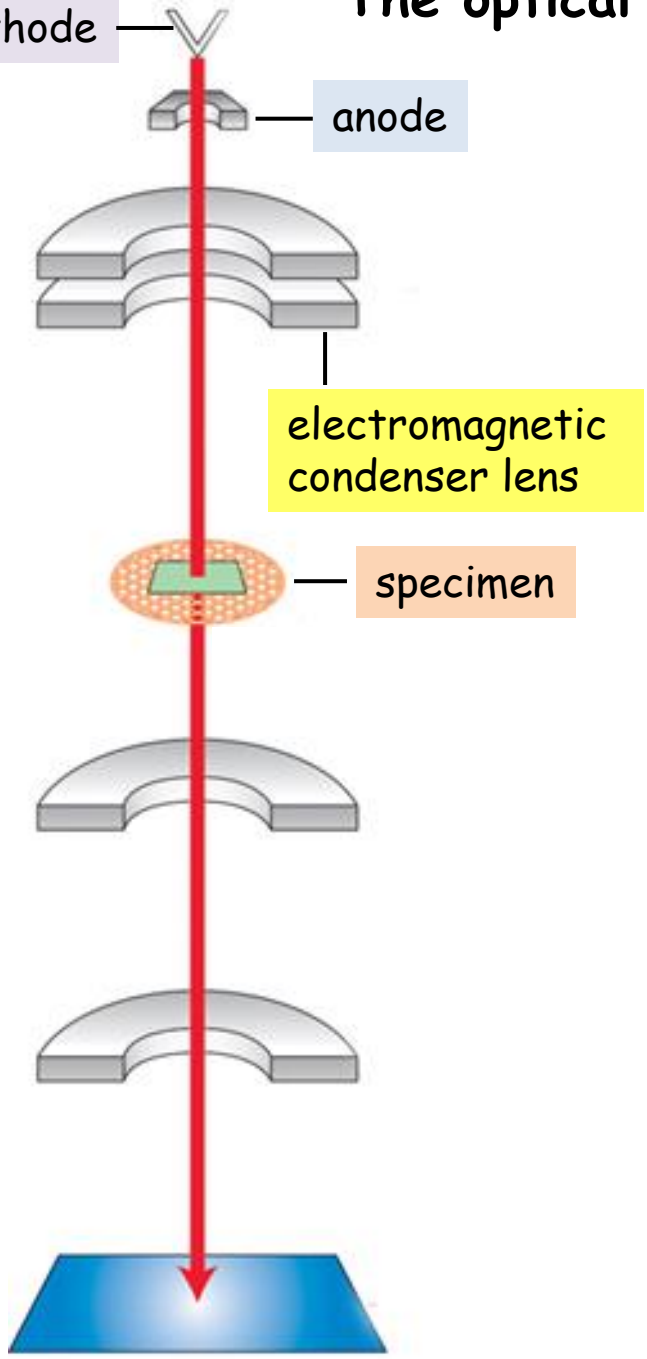
Comparison of skeletal muscle images from a light (top) and electron (bottom) microscope at a comparable magnification of 4500 times actual size.

Comparison between TEM and light microscope

1. TEM is larger (>2m) compared to a light microscope (~30cm)
2. TEM uses electrons instead of light.
3. Beam of electrons is projected downwards while light is projected upwards
4. TEM uses electromagnetic coils to focus the beam of electrons and to magnify the image while light microscopy uses glass lenses
5. The TEM is maintained in a vacuum while the light microscope operates in air



The optical path in a transmission electron microscope



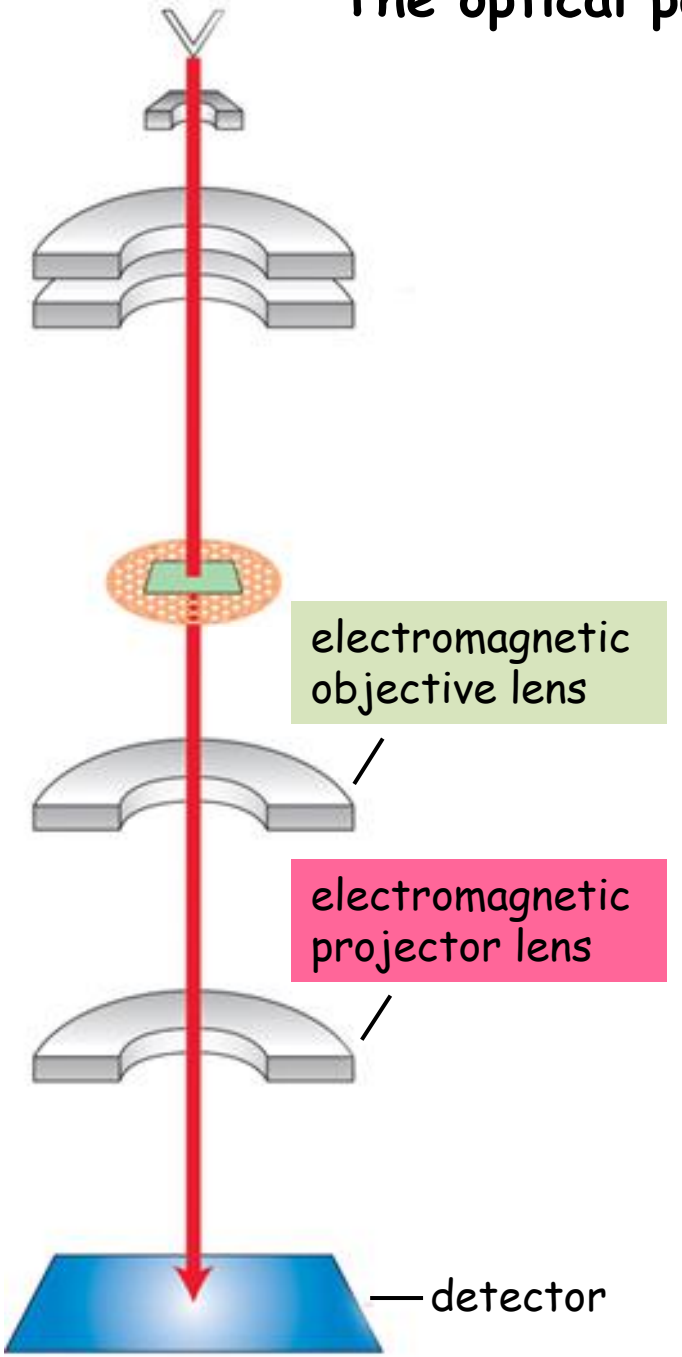
Electrons are emitted by a cathode when it is electrically heated. The electric potential of the cathode is kept at 50,000 – 100,000 volts

The electric potential of the anode is zero. The drop in voltage causes the electrons to accelerate as they move toward the anode.

A beam of electrons is focused onto the specimen plane by the electromagnetic condenser. Like the light microscope, the condensor does not create a magnified image of the specimen.

Specimen is extremely thin (50 – 100 nm), cut with a special instrument called an ultramicrotome.

The optical path in a transmission electron microscope



The electromagnetic objective lens picks up the electrons that have passed through the specimen and **magnifies** the image in the focal plane of the objective lens.

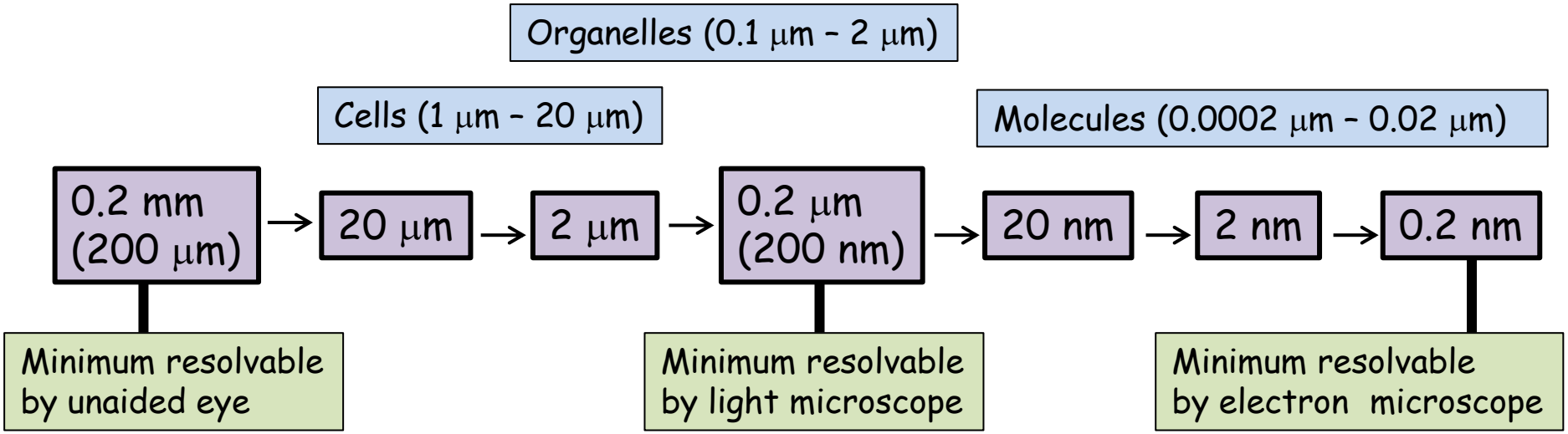
The electromagnetic projector lens (equivalent to the ocular lens in the light microscope) picks up the electrons from the focal plane of the objective lens and both focuses and **magnifies** them onto the specimen detector.

$$D = \frac{0.61\,\lambda}{n \sin\alpha}$$

For a TEM:
λ of electron is ~0.004 nm
N.A. = 0.01
So, D = 0.2 nm

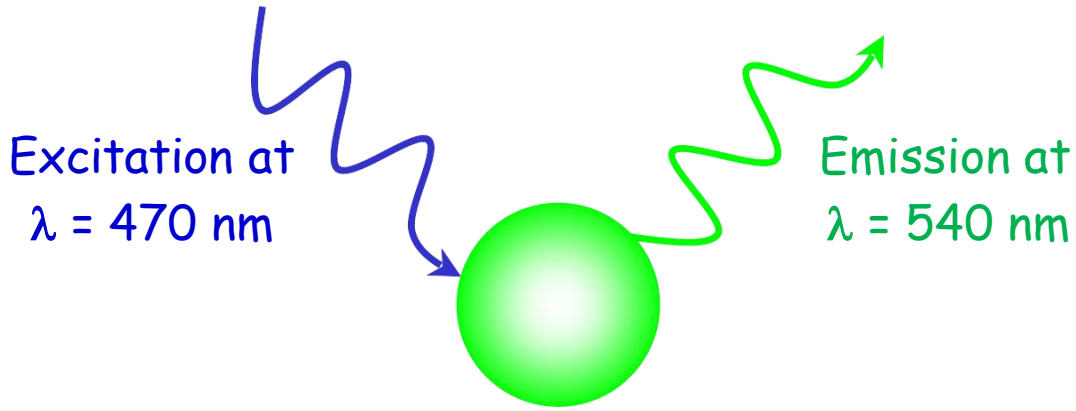
For a light microscope, D = 200 nm.
Therefore, a TEM has 1000-fold more resolution compared to a light microscope

The limits of resolution, the sizes of cells and of their component parts, and the units in which they are measured



Fluorescence microscopy

- Fluorescent molecules absorb light at one wavelength (the excitation wavelength) and emit light (fluoresce) at another, longer wavelength (the emission wavelength)



- If such a compound is illuminated at its excitation wavelength and viewed through a filter that allows only light of the emitted wavelength to pass, it is seen to glow against a dark background.

$$E = \frac{hc}{\lambda}$$

E – the energy of a photon of light **[photon is the basic component (unit) of light]**

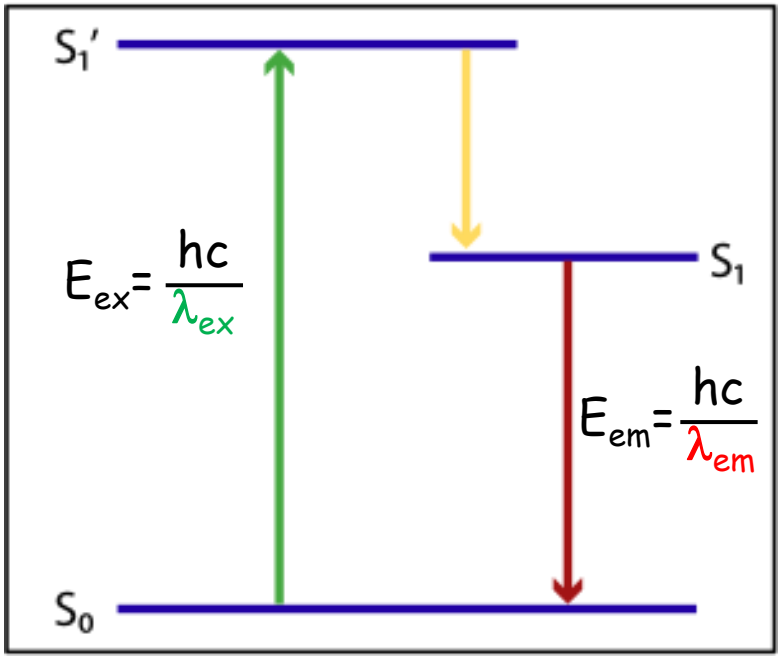
h – Planck's constant

c-speed of light

L – wavelength of a photon

The energy (E) of a photon of light is inversely proportional to its wavelength

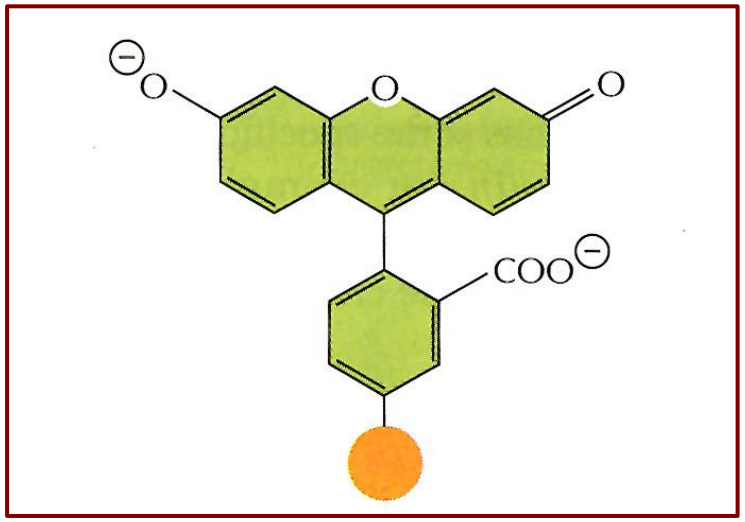
Fluorescence is a three-stage process



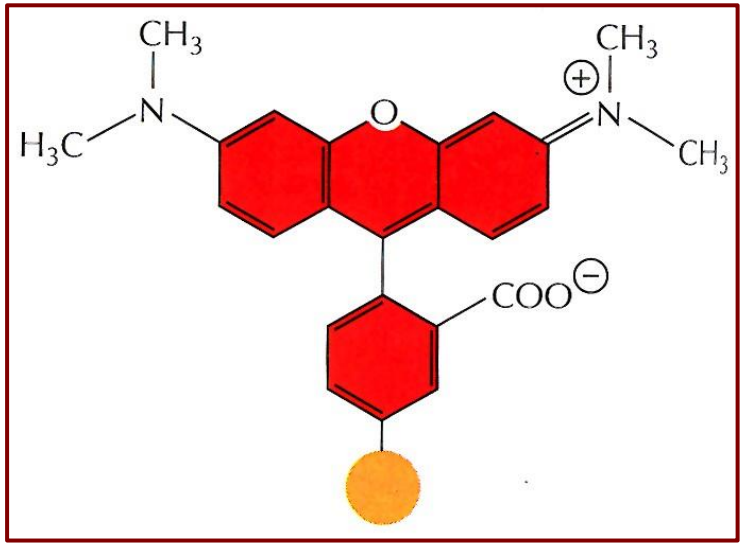
Excitation: A photon of energy is supplied by an external source (e.g. a laser) and absorbed by a fluorescent molecule creating an excited state (S_1')

Energy dissipation: The energy of S_1' is partially dissipated, yielding a relaxed state (S_1)

Fluorescence emission: A photon of energy is emitted, returning the fluorescent molecule to its ground state (S_0)

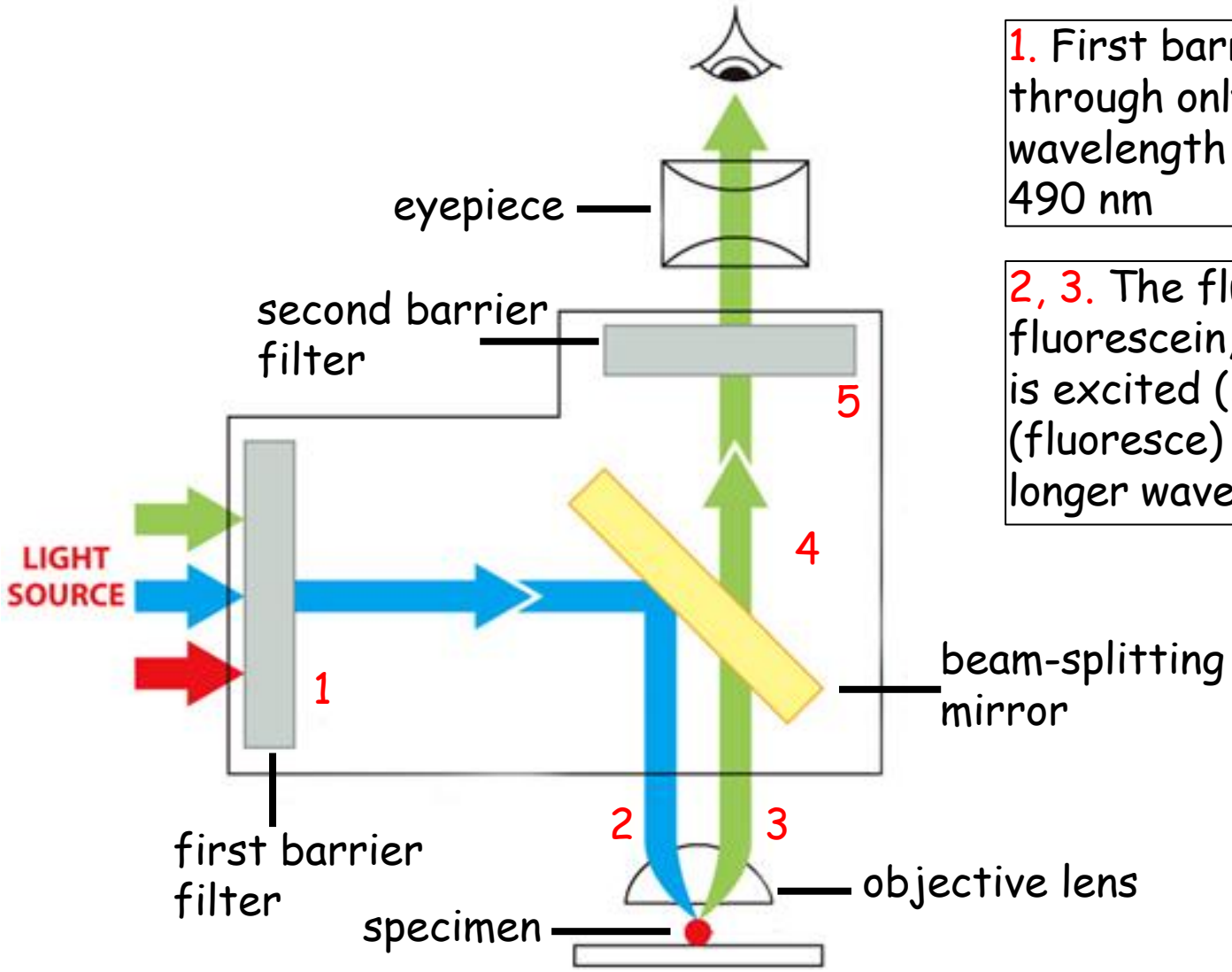


Fluorescein emits green light when activated by light of the appropriate wavelength



Tetramethylrhodamine emits red light when activated by light of the appropriate wavelength

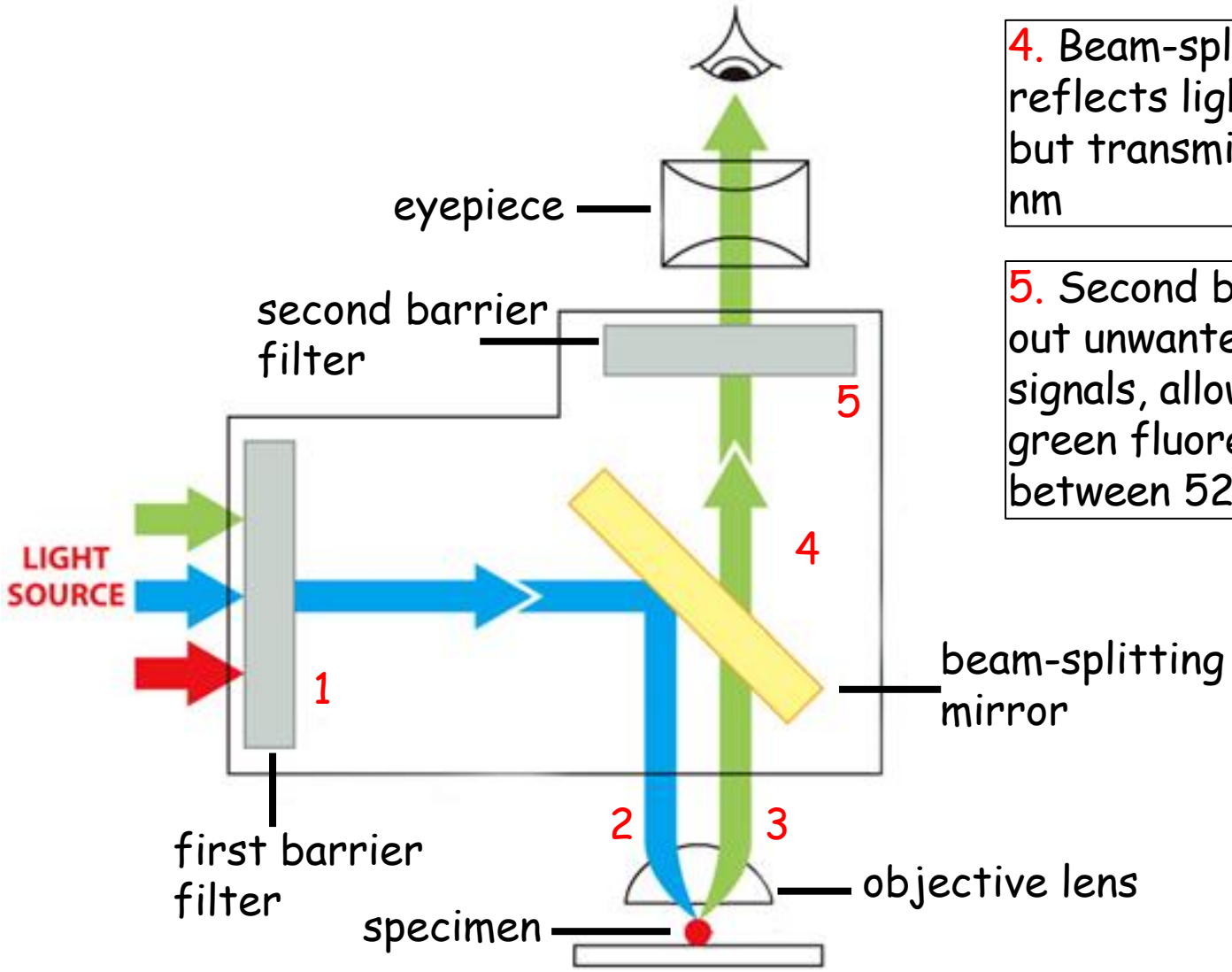
The optical path in a fluorescence microscope



1. First barrier filter: lets through only blue light with a wavelength between 450 and 490 nm

2, 3. The fluorescent dye, fluorescein, in the specimen is excited (2) to emit light (fluoresce) at a specific and longer wavelength (3)

The optical path in a fluorescence microscope



4. Beam-splitting mirror reflects light below 510 nm but transmits light above 510 nm

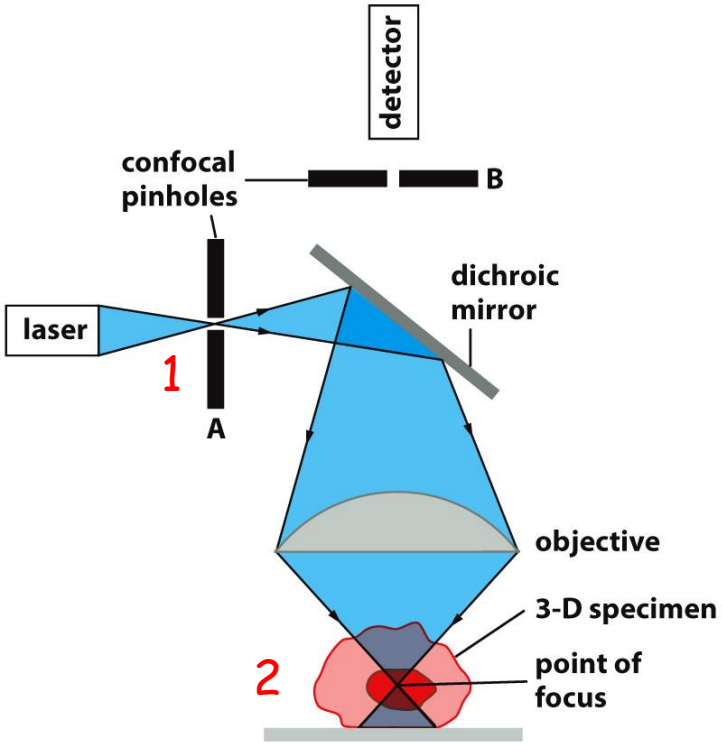
5. Second barrier filter cuts out unwanted fluorescent signals, allowing through the green fluorescein emission between 520 and 560 nm

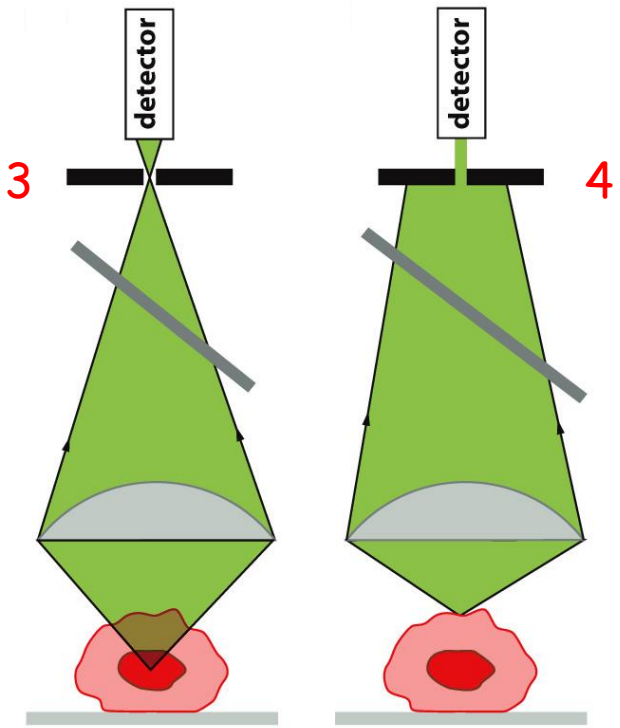
Confocal microscopy improves the image of a conventional florescence microscope

Conventional fluorescence microscopy generates a blurry image because light from above and below the plane of focus are also collected.

Confocal microscopy focuses a single point of light at a specific depth in the specimen.

The optical path in a confocal fluorescence microscope





1. Light from a laser passes through a pinhole.

2. A dichroic mirror and an objective lens then focus the light at a specific depth in the specimen.

3. Emitted light from the specimen is focused at a second (confocal) pinhole and reaches the detector.

4. Emitted light from elsewhere in the specimen is largely excluded from the second pinhole.

(a) Conventional fluorescence microscopy

(b) Confocal fluorescence microscopy

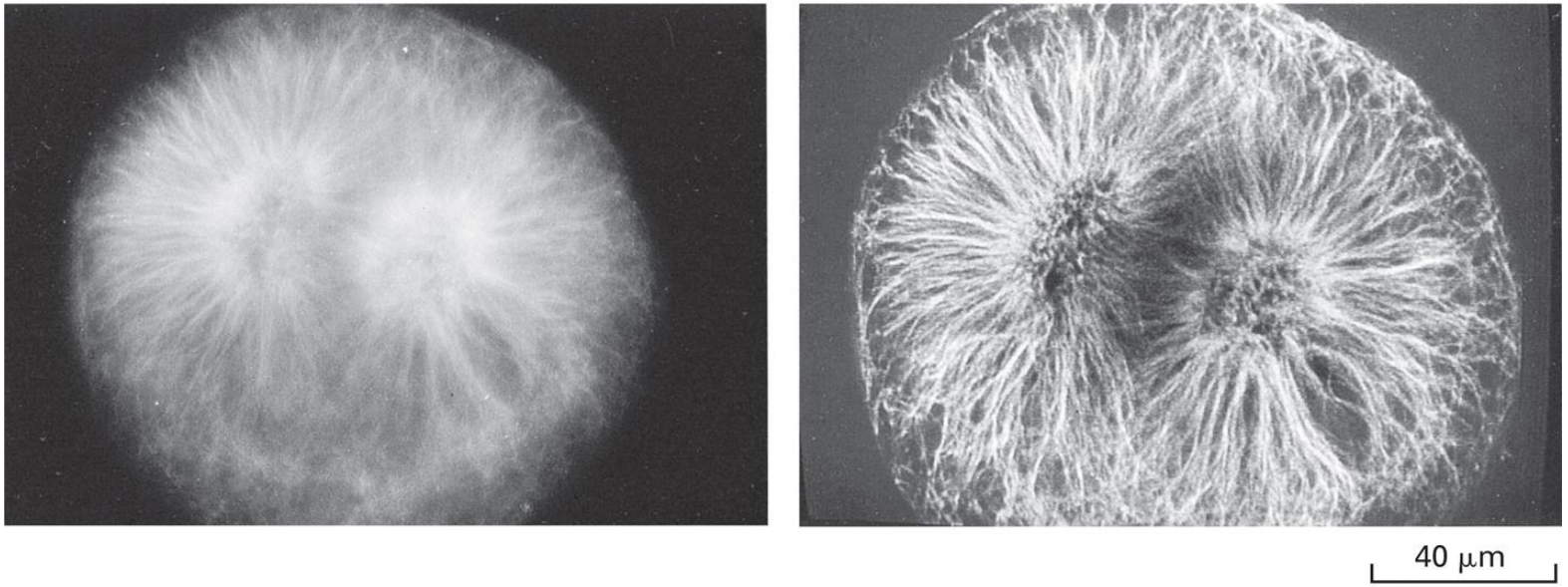
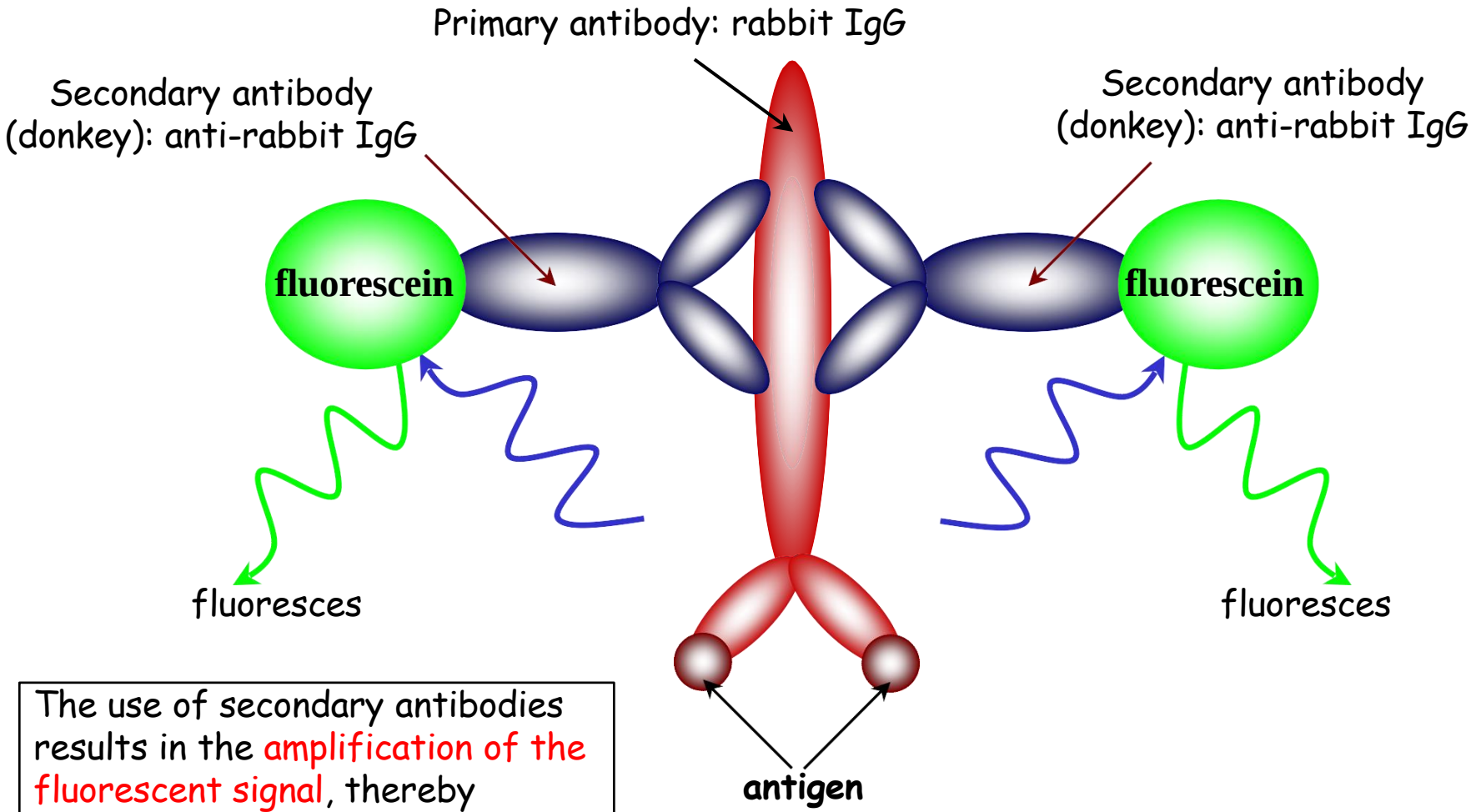


Image of a mitotic, fertilized sea urchin egg stained for tubulin

Revealing specific proteins in fixed cells - Immunofluorescence microscopy

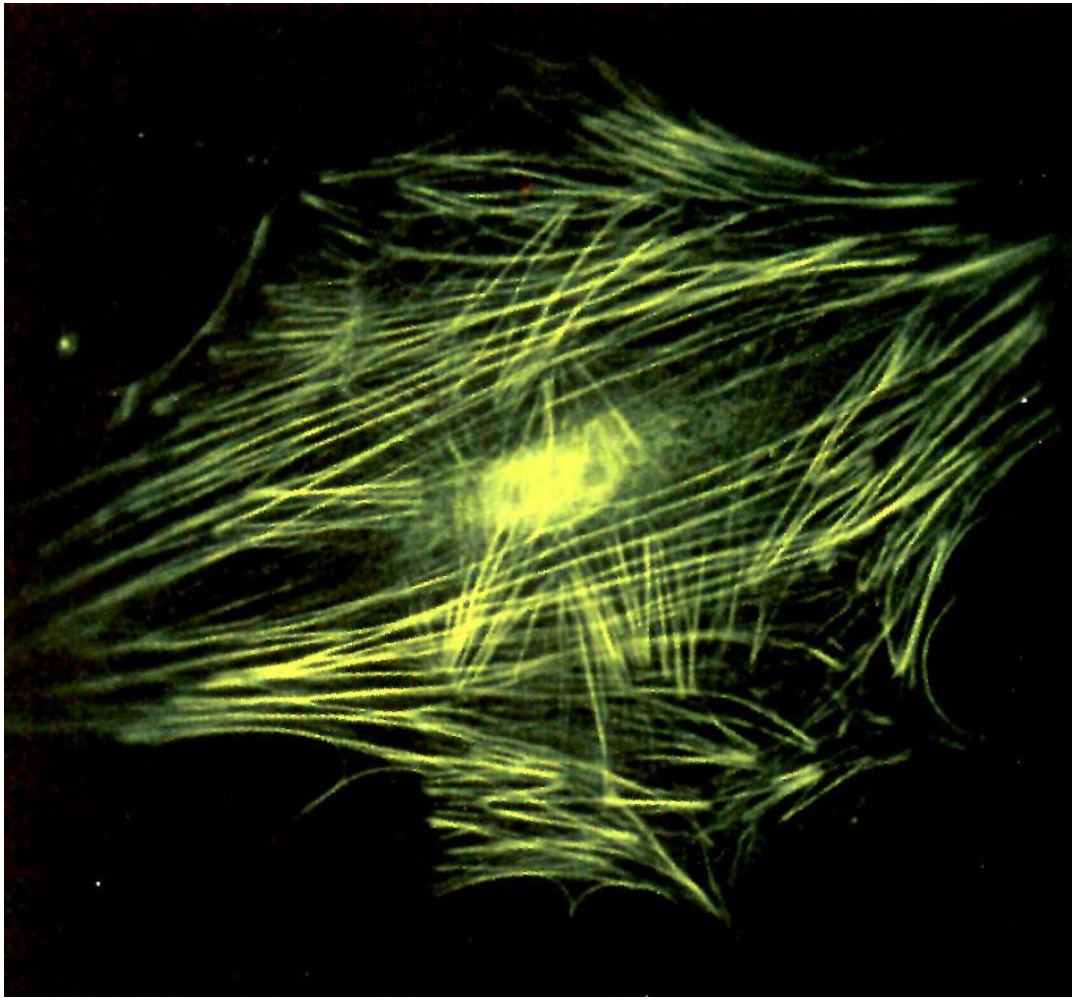


The use of secondary antibodies results in the amplification of the fluorescent signal, thereby increasing the sensitivity of immunofluorescence microscopy

Fixation of a sample "locks" the proteins in place while preserving cell architecture. There are two main types of fixation:

1. Cross-linking reagents such as paraformaldehyde or glutaraldehyde. Glutaraldehyde autofluoresces (green emission) and may interfere with fluorescence signal.
2. Precipitation using a cold organic solutions such as acetone or methanol. Dehydrates the sample and can result in changes to cell architecture.

Once the sample is fixed, the membranes will need to be permeabilized to allow for entry of the antibodies. This is accomplished by organic solutions (precipitation fixation accomplishes this simultaneously) or by treatment with detergent such as Triton X-100.



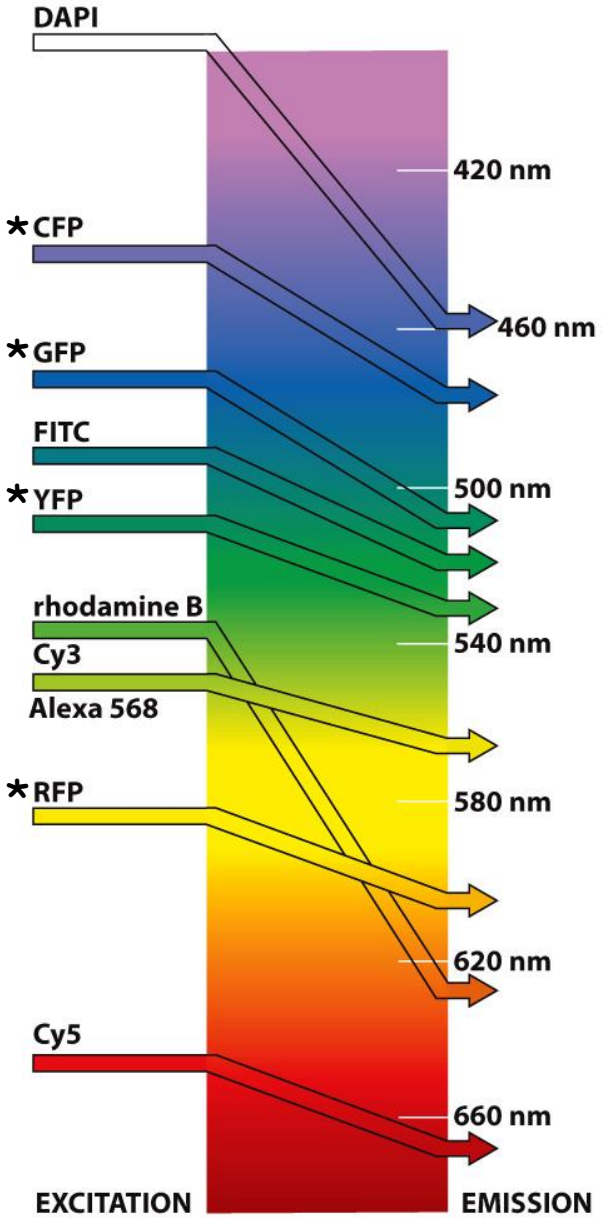
Fluorescence micrograph showing the distribution of long actin fibers in a cultured fibroblast cell:

Primary antibody:
rabbit anti-actin IgG

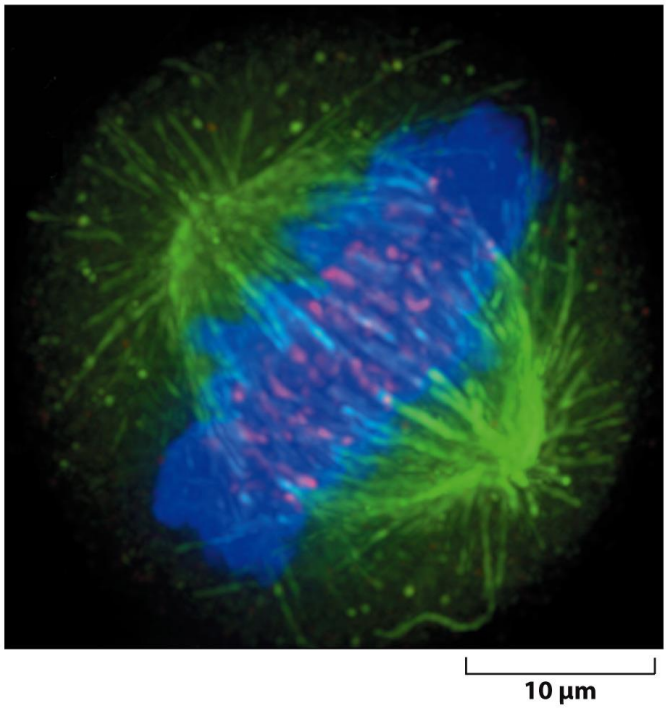
Secondary antibody:
fluorescein-conjugated donkey anti-rabbit IgG

Excitation/emission spectra of common fluorescent probes

*proteins



Different fluorescent probes can be visualized in the same cell

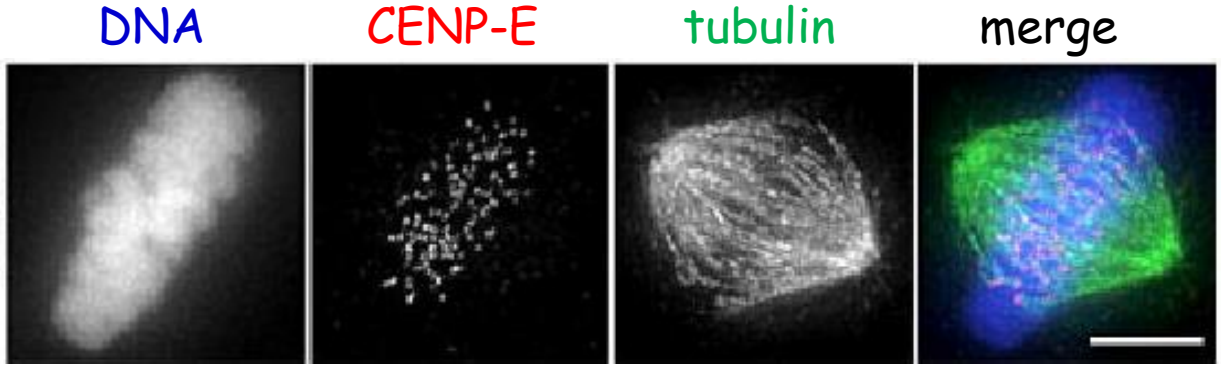


A mitotic cell stained for three cellular components. Three different filter sets were used to acquire three separate images. The images were then overlayed to give the composite image.

Microtubules – revealed with a green fluorescent antibody

Centromeres – revealed with a red fluorescent antibody

DNA – revealed with a stain called DAPI



Considerations when using multiple primary antibodies for immunofluorescence microscopy

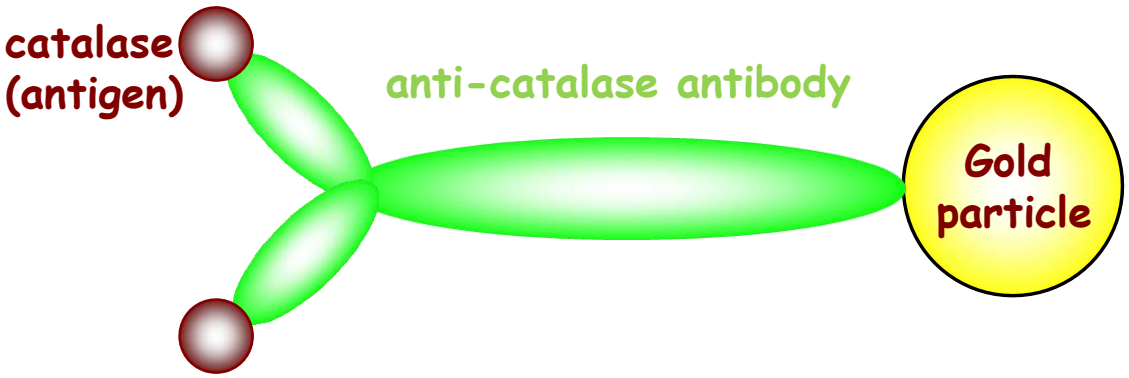
1. Make sure the host species (species in which the antibodies are raised – rabbit, mouse, human) for the primary antibodies are different. Otherwise, the secondary antibodies will bind to both primary antibodies and you will not be able to distinguish your proteins of interest.

2. Make sure the fluorescent molecules or proteins on the secondary antibodies emit at a wavelength that is sufficiently different so that you can distinguish the signals.

Revealing specific proteins in fixed cells - Immunogold electron microscopy

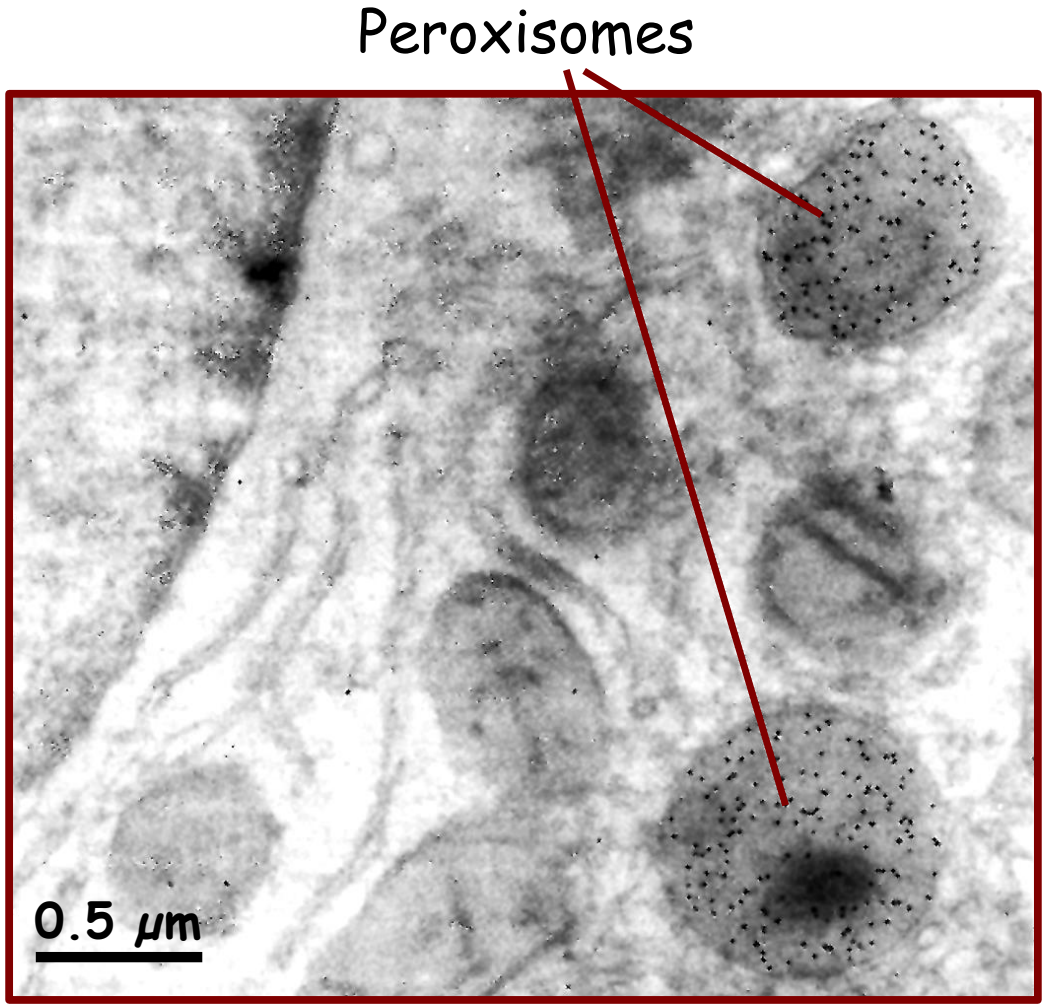
Attach antibodies directed against a specific protein, catalase, to electron-dense colloidal gold particles (5-20 nm in diameter)

These antibodies interact only with their specific antigen (e.g. catalase)



Treat thin sections of glutaraldehyde-fixed cells or tissues with these gold-labeled anti-catalase antibodies

Determine the subcellular location of catalase in the electron microscope



The gold particles (black dots), indicating the presence of catalase, are located exclusively in the peroxisome.

The subcellular location of catalase in a rat liver cell

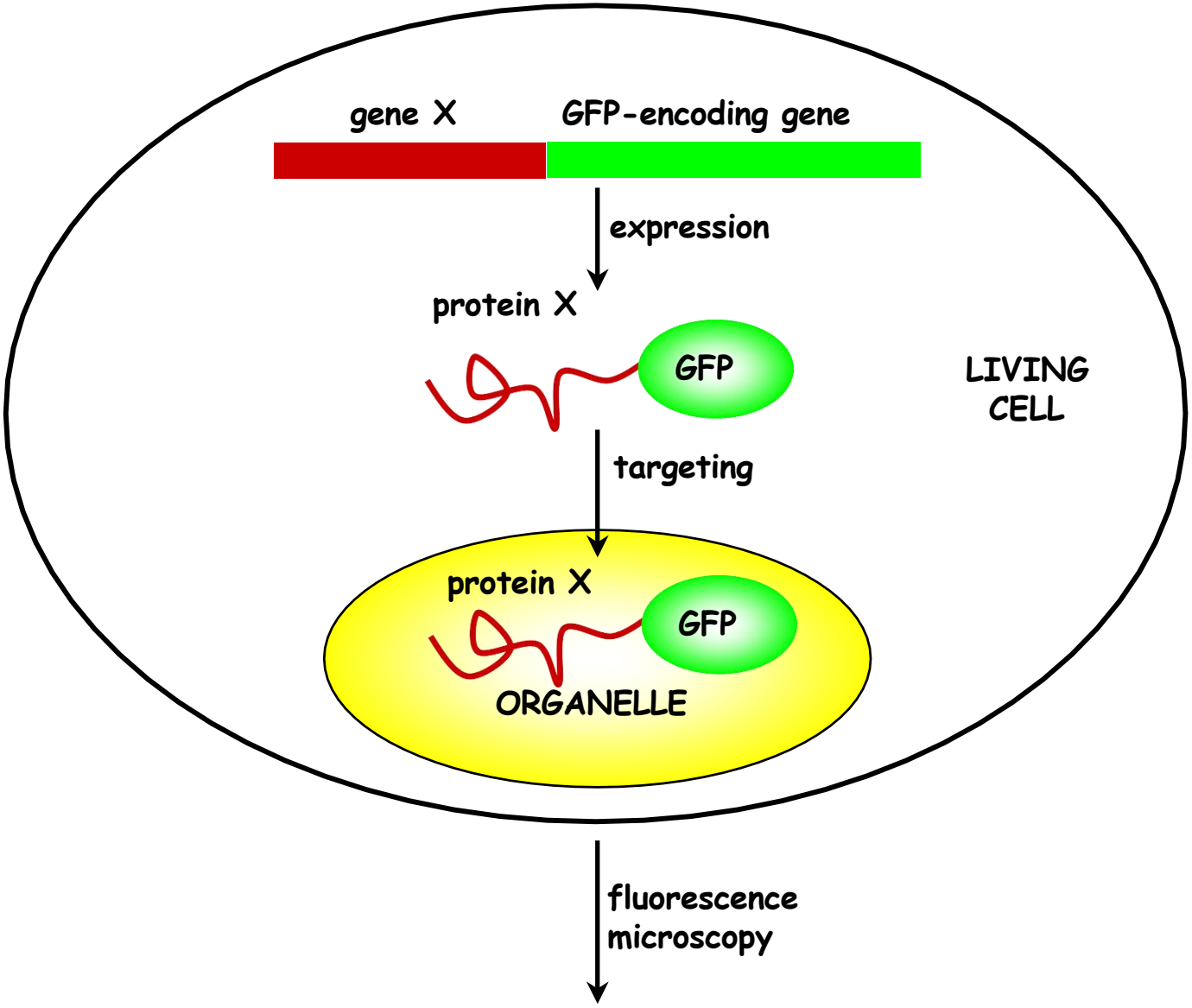
Revealing specific proteins in living cells

Green fluorescent protein (GFP)

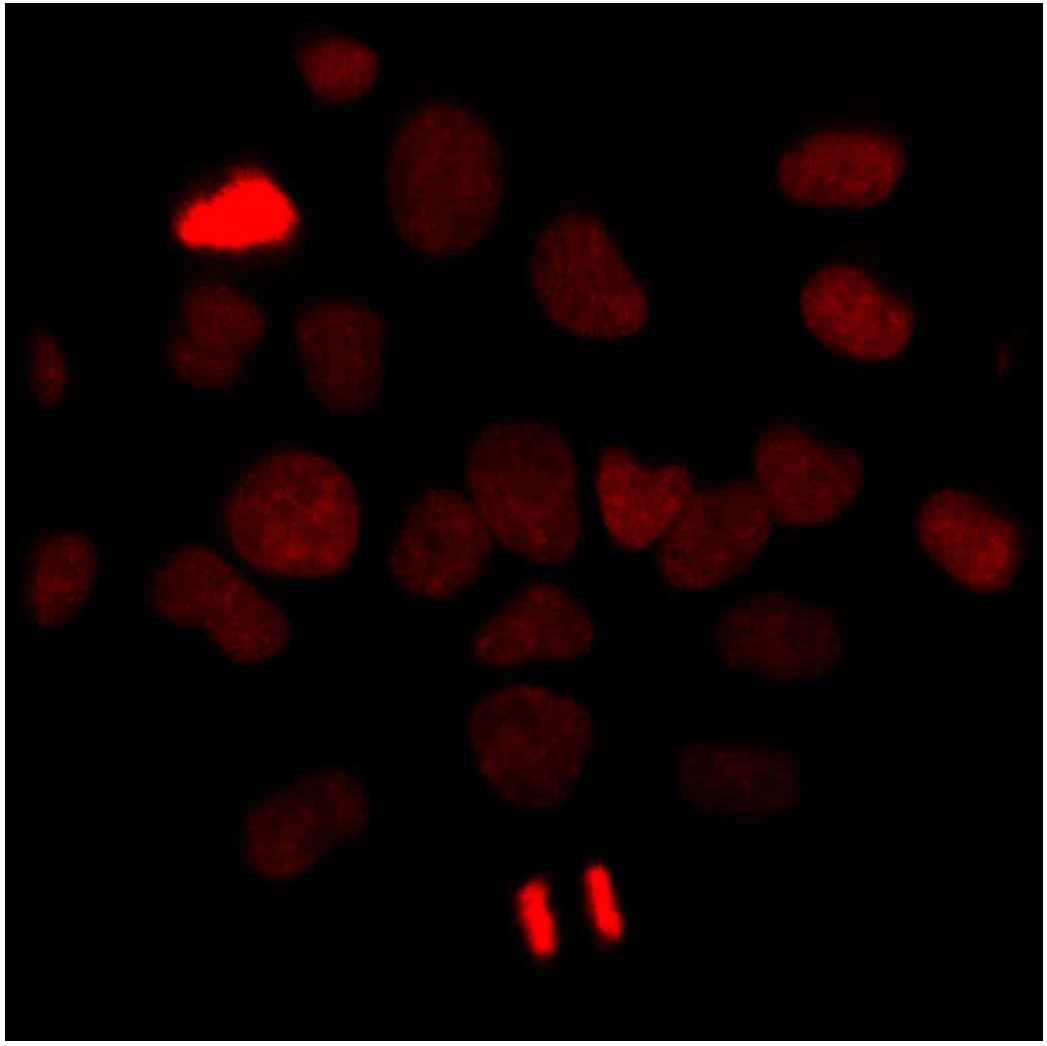
A naturally fluorescent protein from jellyfish.

Emits a green fluorescence when exposed to light of the exciting wavelength.

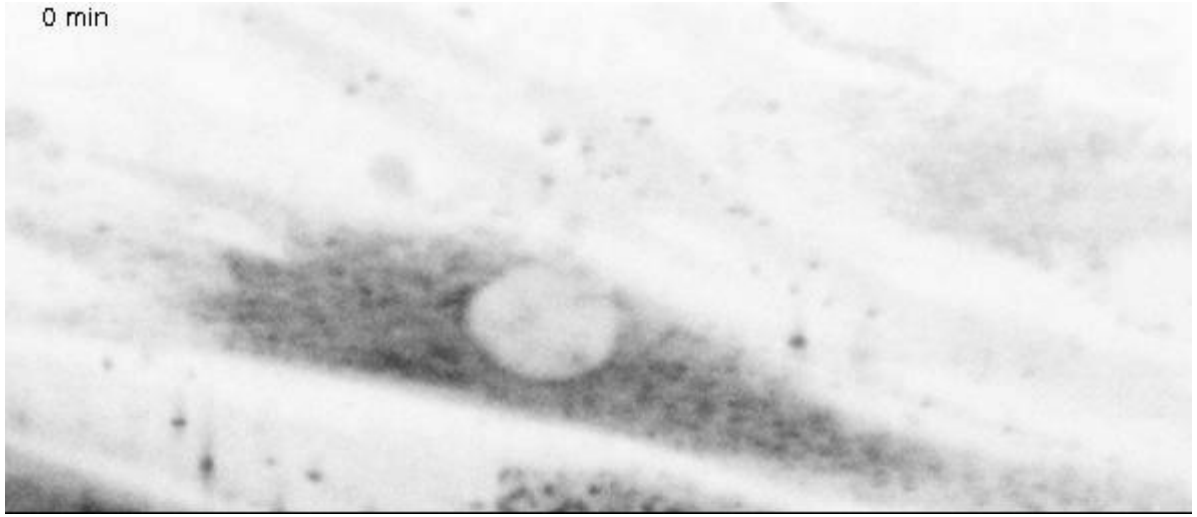
When "linked" to a protein of interest, GFP (or other fluorescent proteins) can be used to follow the localization and movement of the protein of interest in live cells.


gene X
GFP-encoding gene
expression
protein X
GFP
LIVING CELL
targeting
protein X
GFP
ORGANELLE
fluorescence microscopy

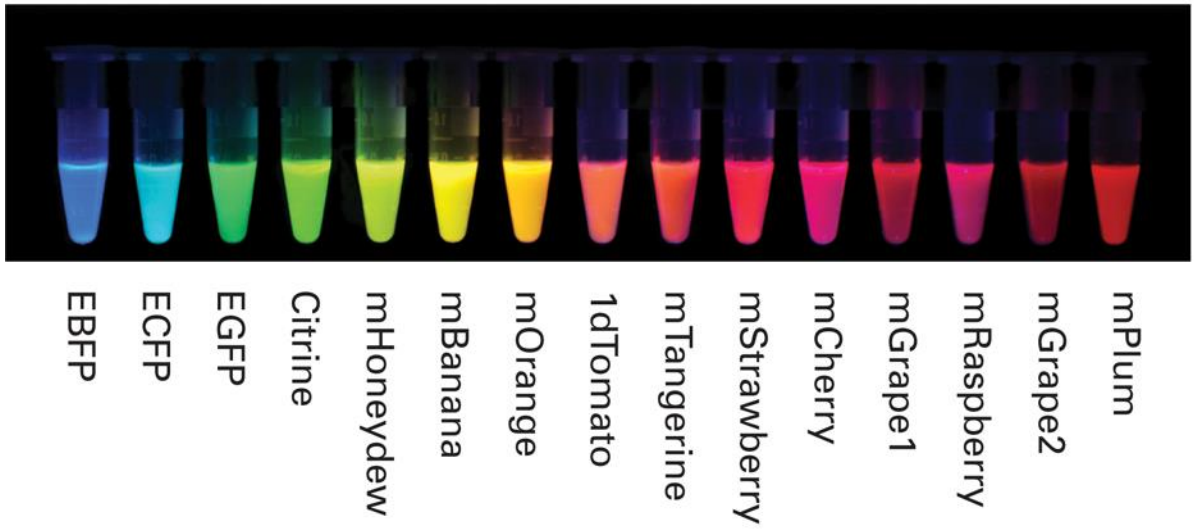
Behaviour of chromosomes during the cell cycle as revealed by RFP-tagged histone H2B



Movement of proteins (GFP-tagged VSVG) from ER to Golgi to cell surface)



There are now many fluorescent proteins, spanning numerous different wavelengths. This allows researchers to investigate several proteins in the same cell.



Tissue culture

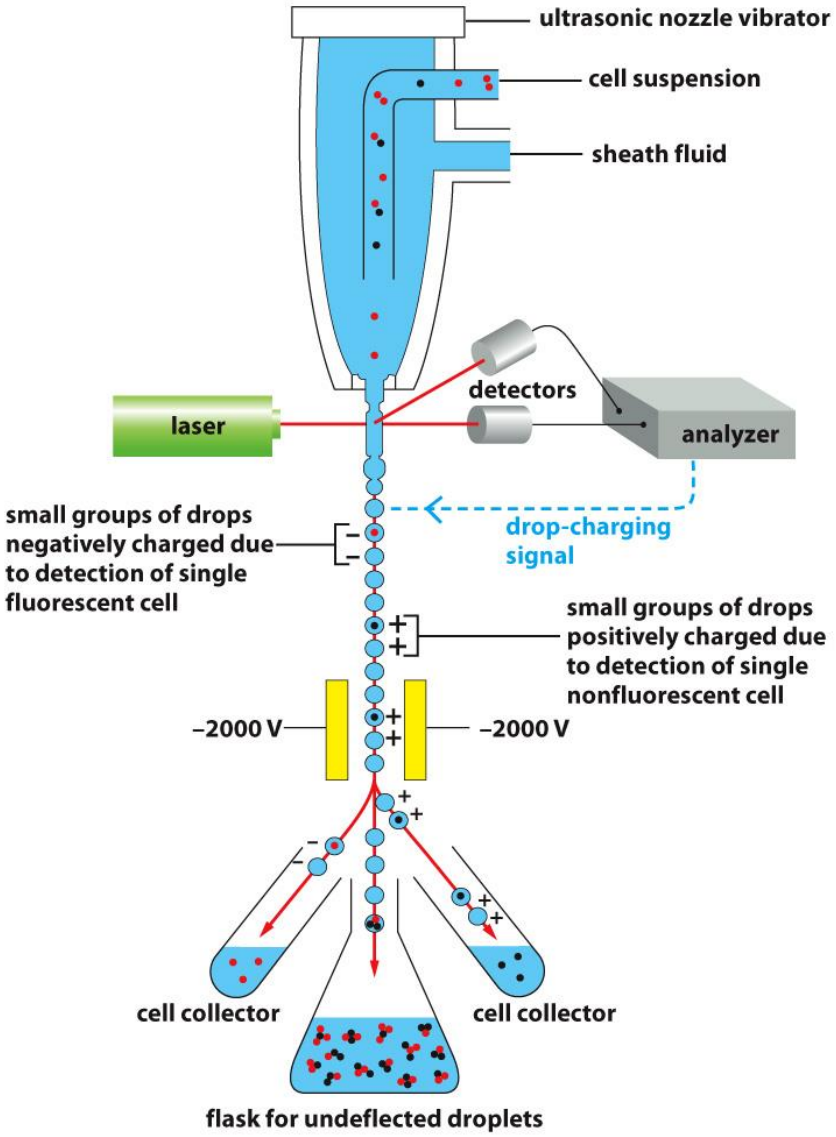
Cells can be isolated from tissues

1. Disrupt cell-cell contacts with a protease such as trypsin or collagenase, or with EDTA which chelates Ca^{2+} (required for cell-cell adhesion)

2. Plate the cells in a plastic dish. Some cells require a layer of collagen to adhere to the plates, others can adhere to the plastic (adherent cells) while others are will not adhere to the plastic or to an extracellular component (non-adherent cells).

3. If there is a mixed population of cells, fluorescence-activated cell sorting (FACS) can be used to separate the population.

Sorting cell types by FACS



An antibody recognizing a protein facing the outside surface of the cell is coupled to a fluorescent dye and suspended in a fluid.

Droplets with single cells pass by a laser to excite the fluorescent dye. If the detector registers fluorescence, the droplet is immediately negatively charged. Otherwise, the drops are not charged.

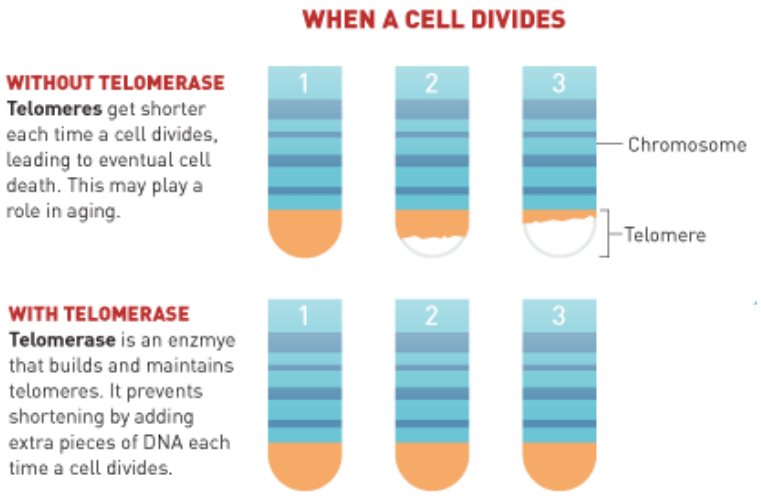
The droplets are then deflected in an electric field and collected. They are then put into culture on plastic dishes.

As the cells grow, they occupy more space on the dish. Once they reach 100% confluency (a measure of the surface area occupied by the cells), they must be passaged. They are removed from the dish with trypsin or EDTA and plated onto a new dish.

Cells that are derived from a tissue are called primary cells. These cells can only be passaged 25-40 times and then they stop dividing (they become senescent). They do not express telomerase.

Primary cells can be immortalized by adding DNA that expresses telomerase. The cells are referred to as a cell line.

Some cancer cells have the ability to grow indefinitely and are referred to as transformed cell line.



Common cell lines:
HeLa (human epithelial), 293 (human kidney), CHO (hamster ovary), MDCK (dog epithelial), COS (monkey kidney)

The growth media for cultured cells contains:

- 9 essential amino acids (Phe, Val, Thr, Trp, Ile, Met, Leu, Lys, His)
- Glutamine – non-essential amino acid but used as a nitrogen source
- Vitamins
- Fatty acids
- Glucose
- Serum (non-cellular portion of clotted blood)
  - Hormones (e.g. insulin)
  - Transferrin (iron transport)
  - Growth factors