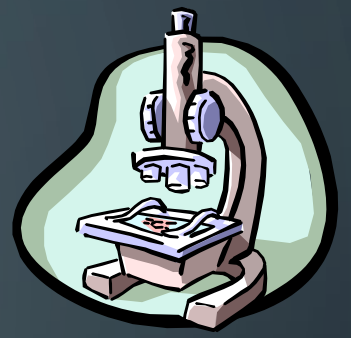
2021/2022 - Light microscopy in Cellular Biology



Fluorescence Microscopy

part1

Gabriele Baj
gbaj@units.it

CONTRASTING TECHNIQUES – A REMINDER…

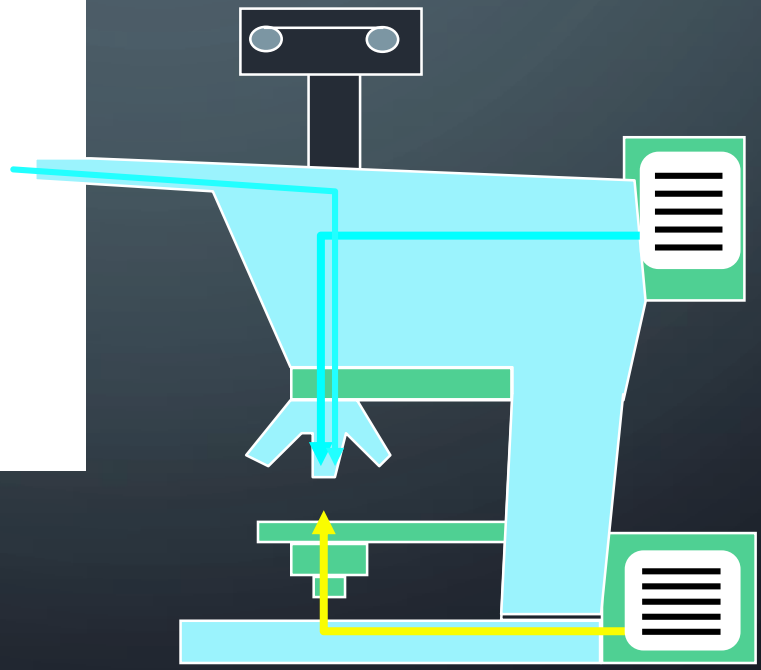
- Brightfield -absorption
- Darkfield -scattering
- Phase Contrast -phase interference
- Differential Interference Contrast (DIC) - polarization + phase interference

- Fluorescence Contrast

UPRIGHT SCOPE



Image from Nikon promotional materials



Epi-illumination Source

Brightfield Source

11:36 AM

INVERTED MICROSCOPE

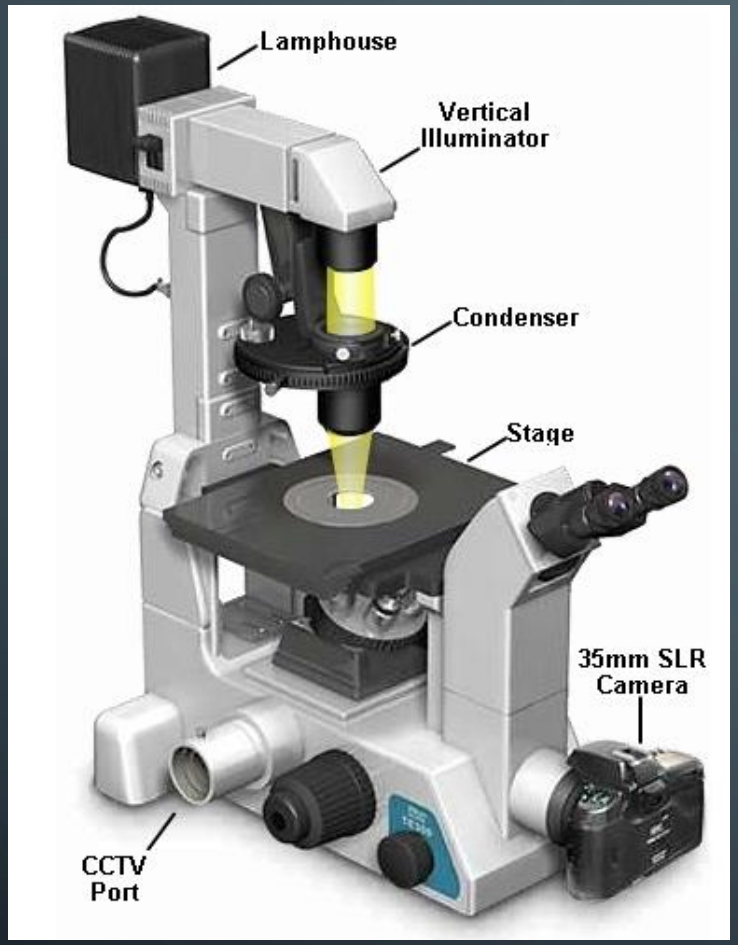
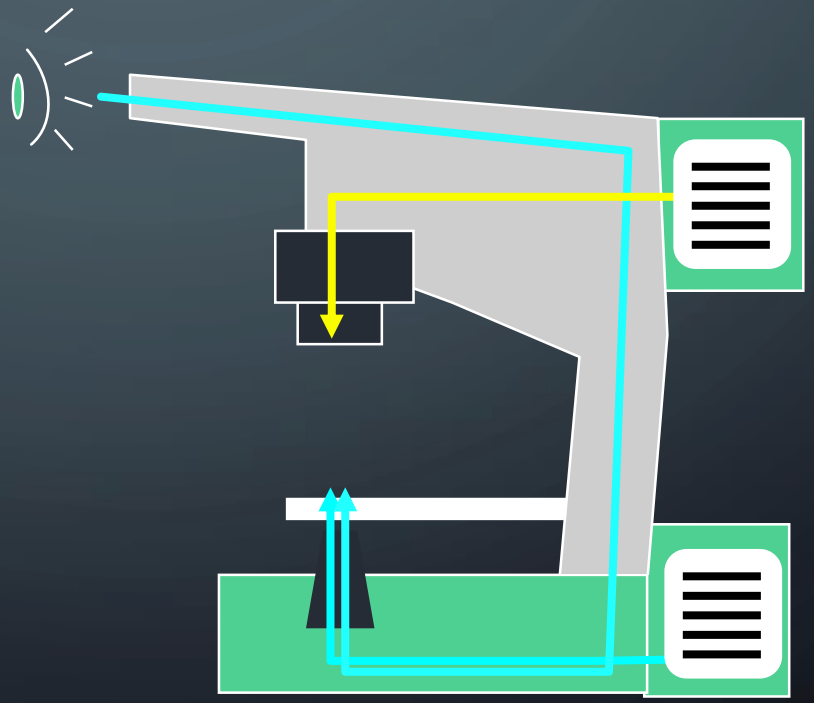


Image from Nikon promotional materials

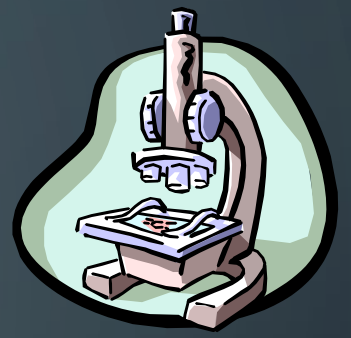


11:36 AM

WHY FLUORESCENCE MICROSCOPY?

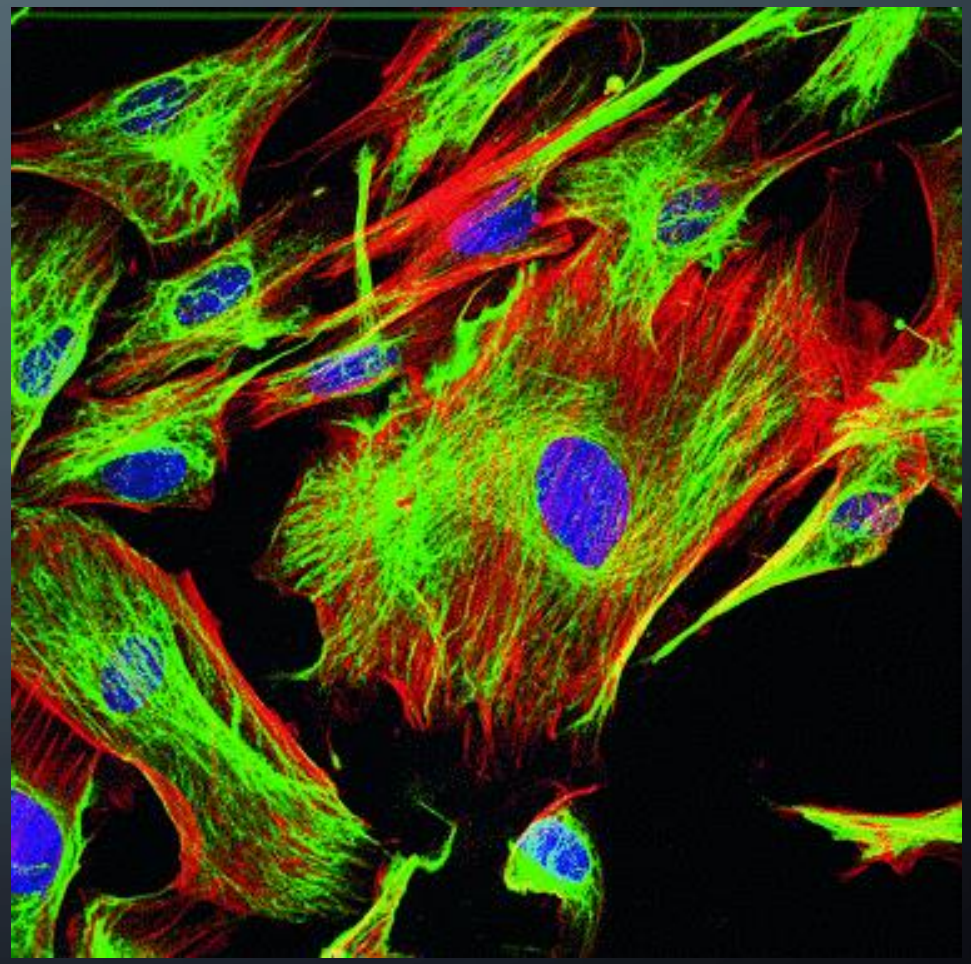
- In all types of microscopes, cell constituents are not distinguishable, although staining dose, but not totally.
- In fluorescent microscopy, various fluorescent dyes are used which gives property of fluorescence to only specific part of the cell and hence it can be focused.
- Fluorescent microscopy depends upon illumination of a substance with a specific wavelength which then emits light at a longer wavelength .

Why fluorescence?

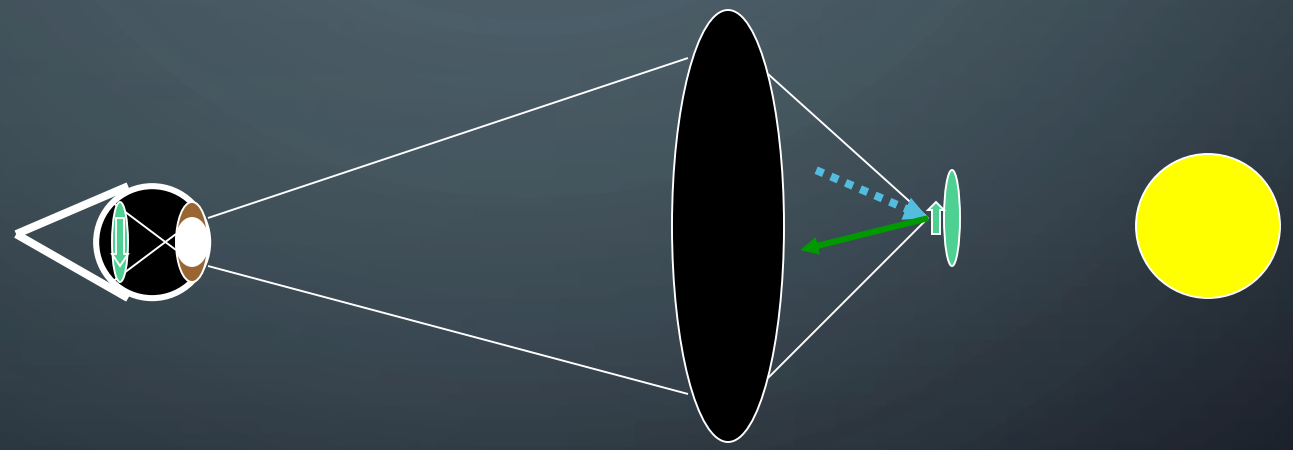


High resolution

High contrast
High specificity
Quantitative
Live Cell Imaging

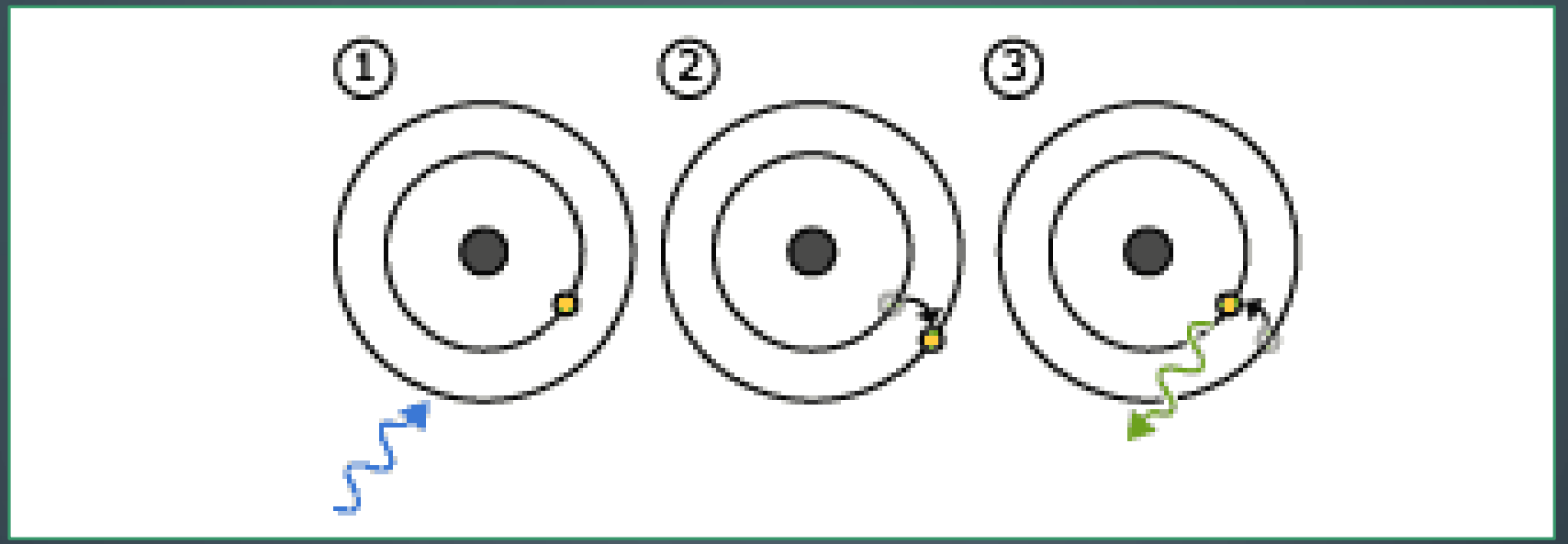


TRANSMITTANCE IS SUBTRACTIVE WHILE FLUORESCENCE IS ADDITIVE



FLUORESCENCE PRINCIPLE

- When certain compounds are illuminated with high energy light, they then emit light of a different, lower frequency. This effect is known as **fluorescence**.
- Often specimens show their own characteristic autofluorescence image, based on their chemical makeup.
- The key feature of fluorescence microscopy is that it employs reflected rather than transmitted light, which means transmitted light techniques such as **phase contrast** and **DIC** can be combined with fluorescence microscopy.

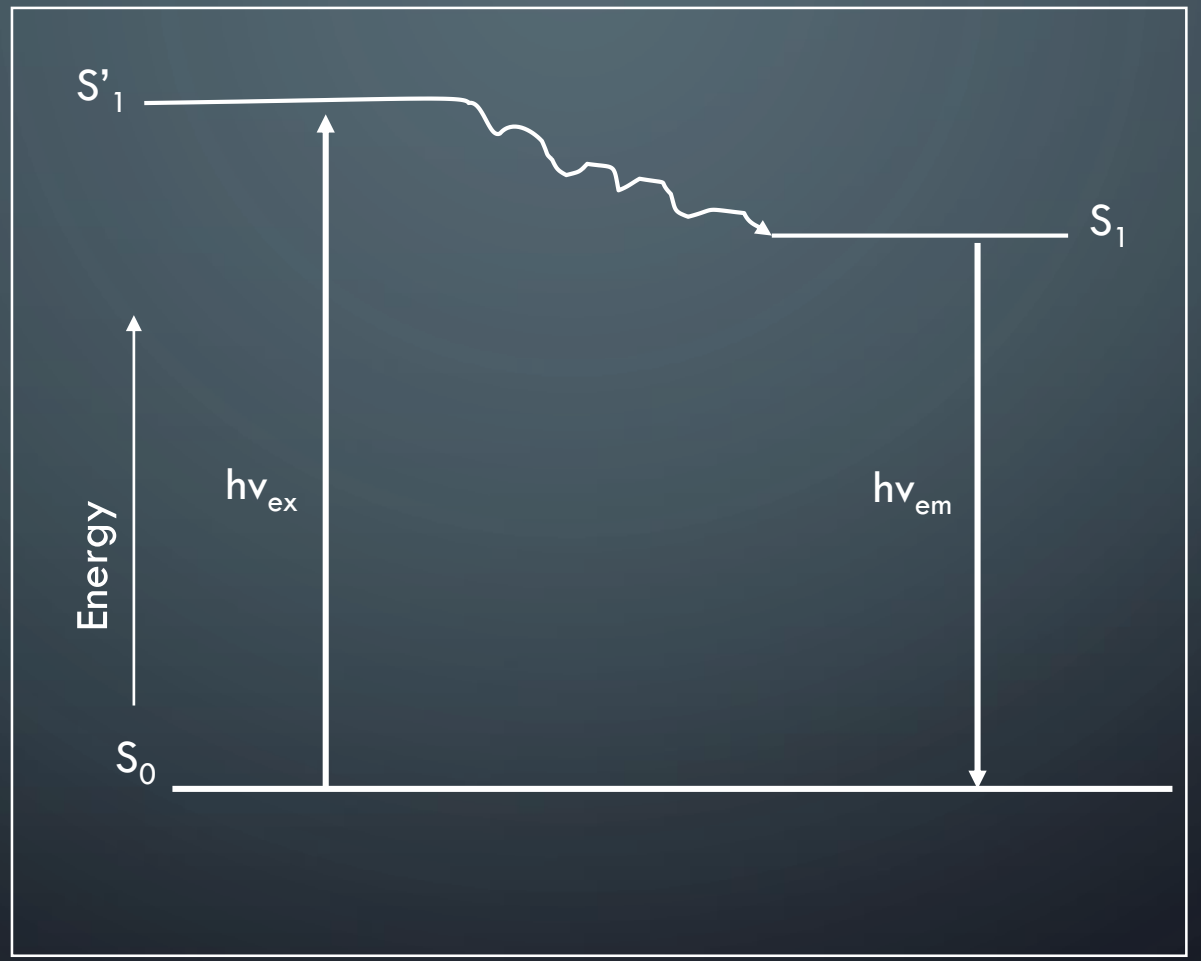


The radiation collides with the atoms in the specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light.

Principle of Fluorescence

1. Energy is absorbed by the atom which becomes excited.
2. The electron jumps to a higher energy level.
3. Soon, the electron drops back to the ground state, emitting a photon (or a packet of light) - the atom is fluorescing.

SIMPLIFIED *JABLONSKI DIAGRAM*



FLUORESCENCE PRINCIPLE

- Fluorescence and phosphorescence are both types of luminescence.
- In fluorescence the emission of light occurs extremely rapidly after the absorption of excitation light.
- phosphorescence emission continues for milliseconds to minutes after the energy source has been removed.

FLUORESCENCE V/S PHOSPHORESCENCE

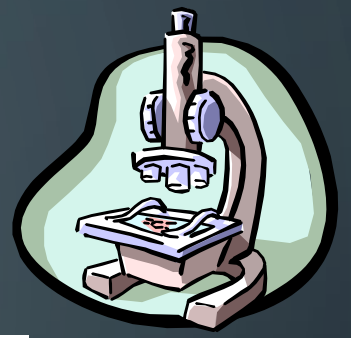
- If the luminescence is caused by absorption of some form of radiant energy, such as ultraviolet radiation or X rays (or by some other form of energy, such as mechanical pressure), **and ceases as soon as** (or very shortly after) **the radiation causing it ceases, then it is known as fluorescence.**

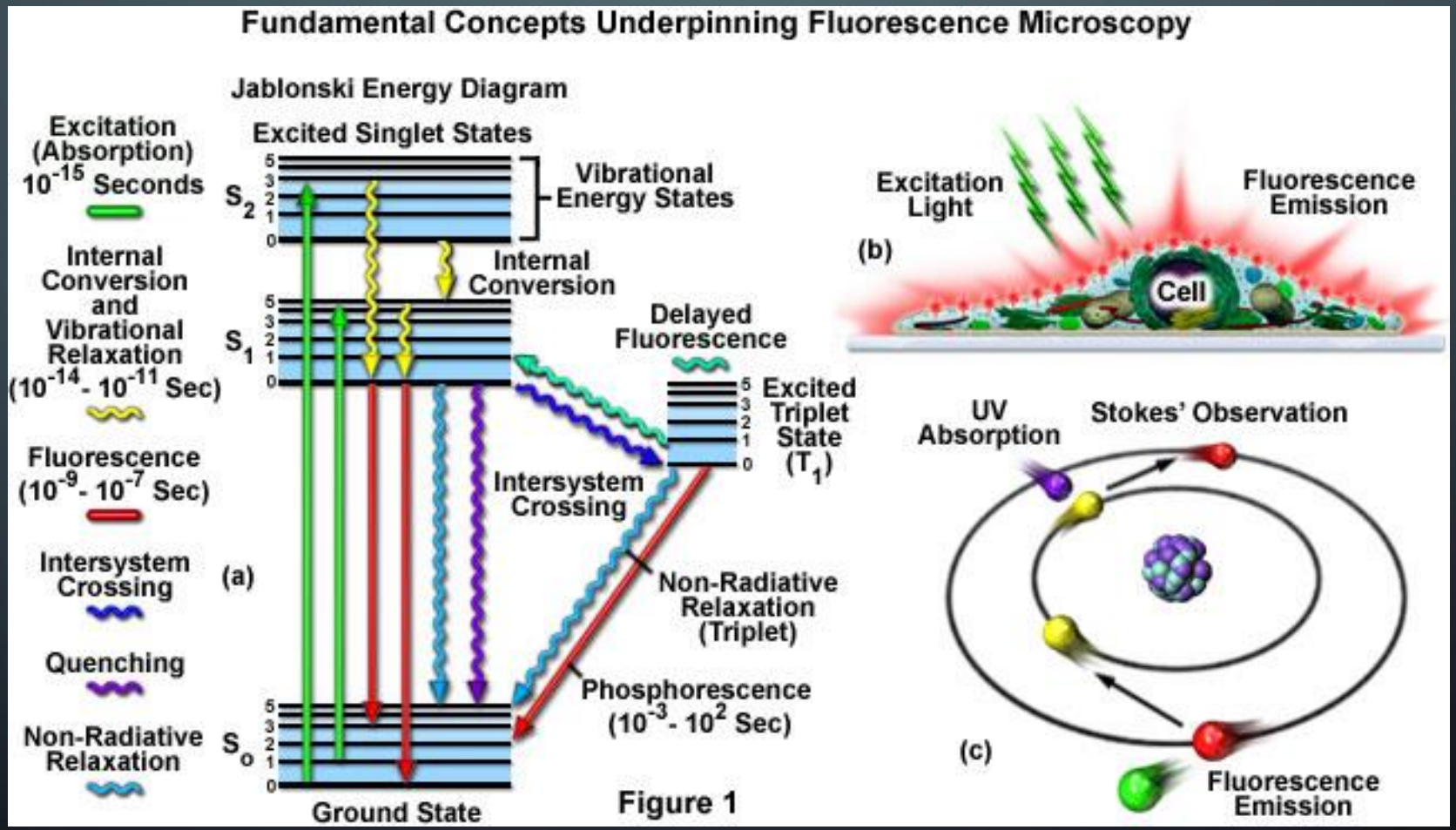
- If the luminescence **continues after the radiation causing it has stopped,** then it is known as phosphorescence..

The term *phosphorescence* is often incorrectly considered synonymous with *luminescence*

2021/2022 - Light microscopy in Cellular Biology

What is Fluorescence ?

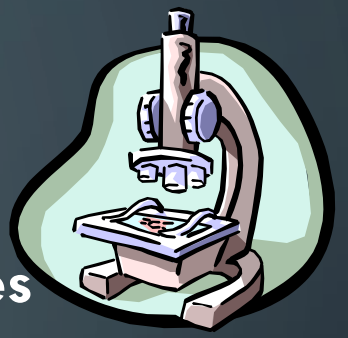




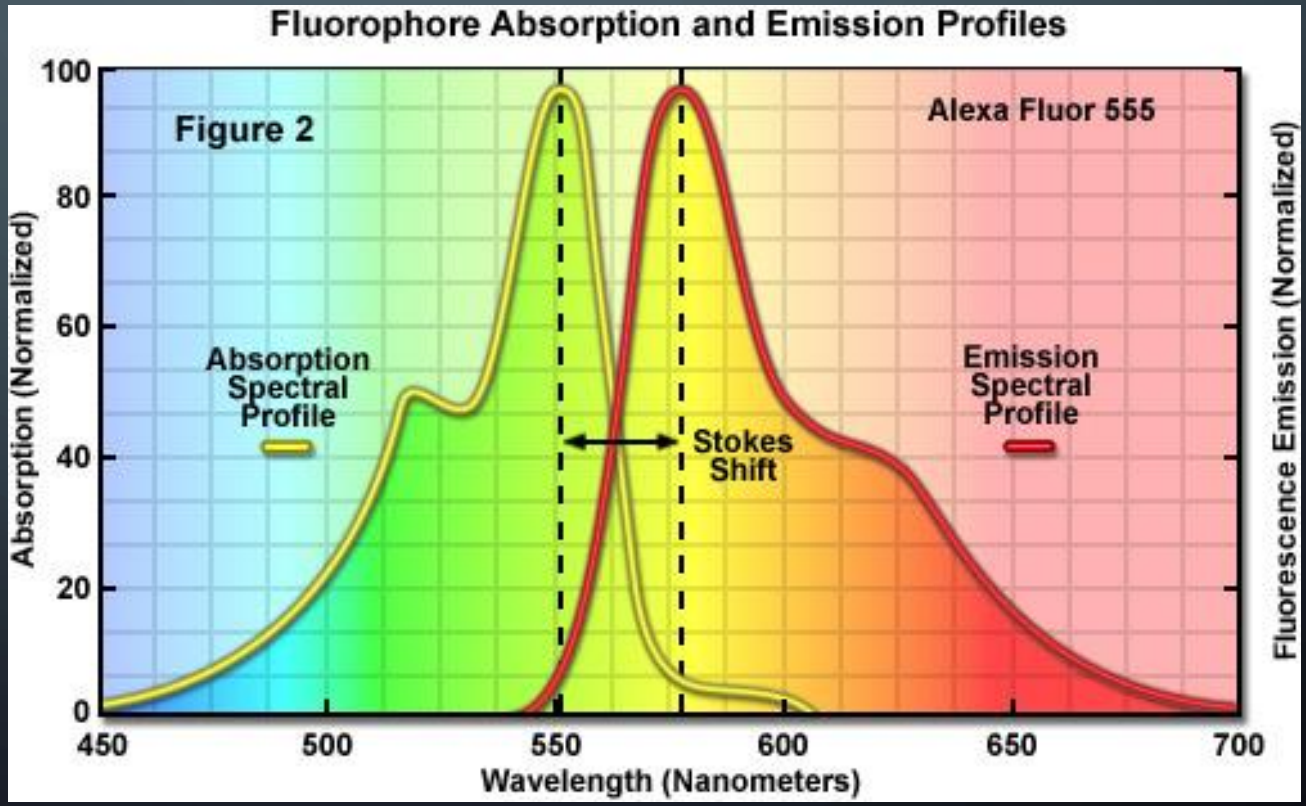
FUNCTIONING

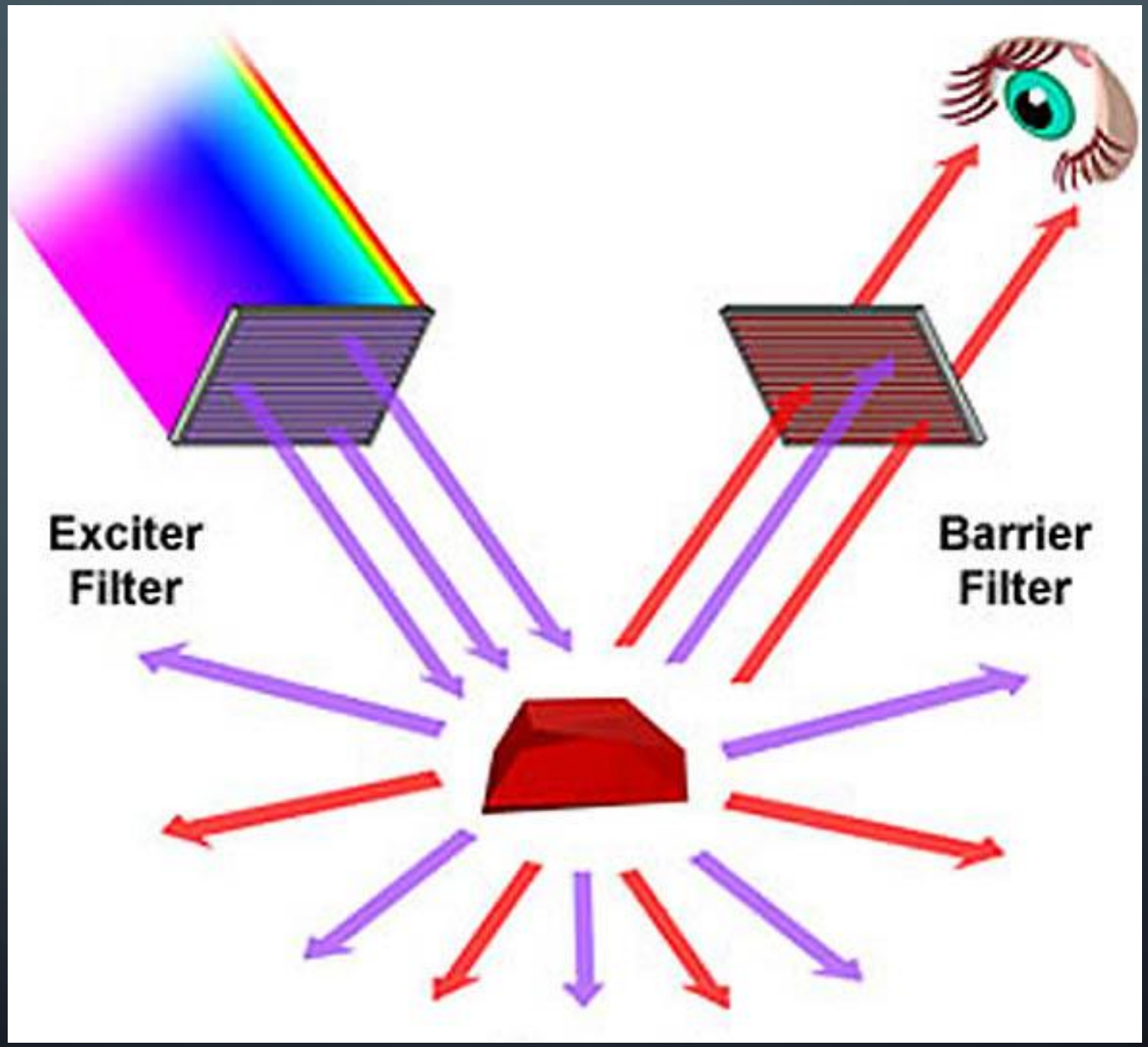
- A component of interest in the specimen is specifically labeled with a fluorescent molecule called a **fluorophore.**
- The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light).

Fluorescence



Molecules absorbing the energy of electromagnetic radiation will jump to a higher energy level. When certain excited molecules return to the ground state they emit radiation. This phenomenon is known as fluorescence. Fluorescent molecules are known as fluorochromes or fluorophores.

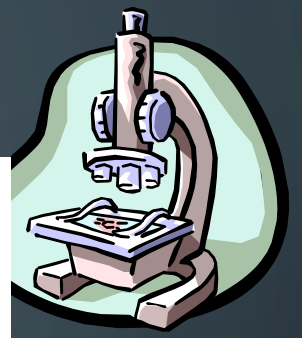



Exciter
Filter
Barrier
Filter

COMPONENTS

- Typical components of a fluorescence microscope are:
- the light source
  - (xenon arc lamp / mercury-vapor lamp / metal *halid*e lamp / LED)
- the excitation filter,
- the dichroic mirror and
- the emission filter.

2021/2022 - Light microscopy in Cellular Biology



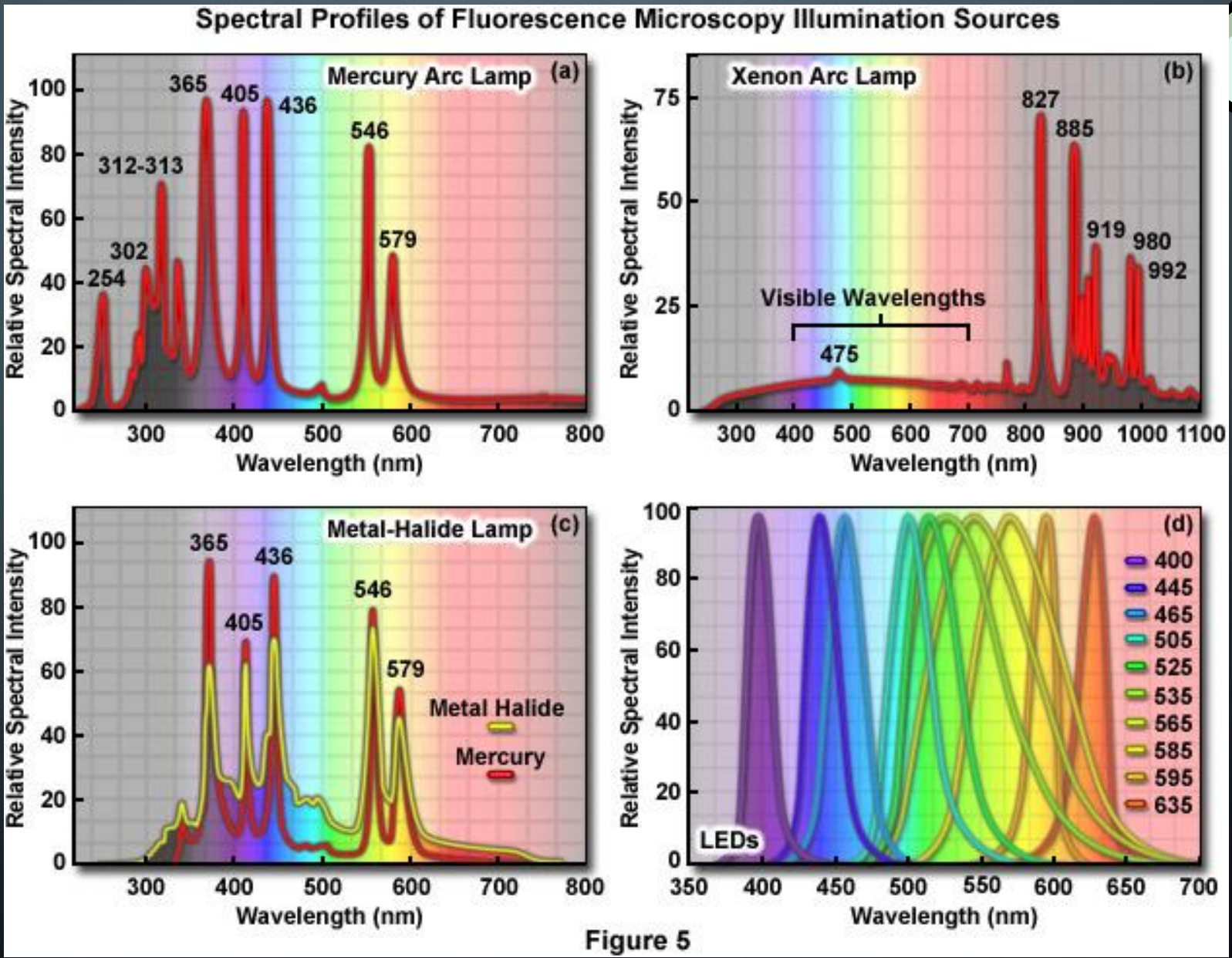


Figure 5

A fluorescence microscope is basically a conventional light microscope with added features and components that extend its capabilities.

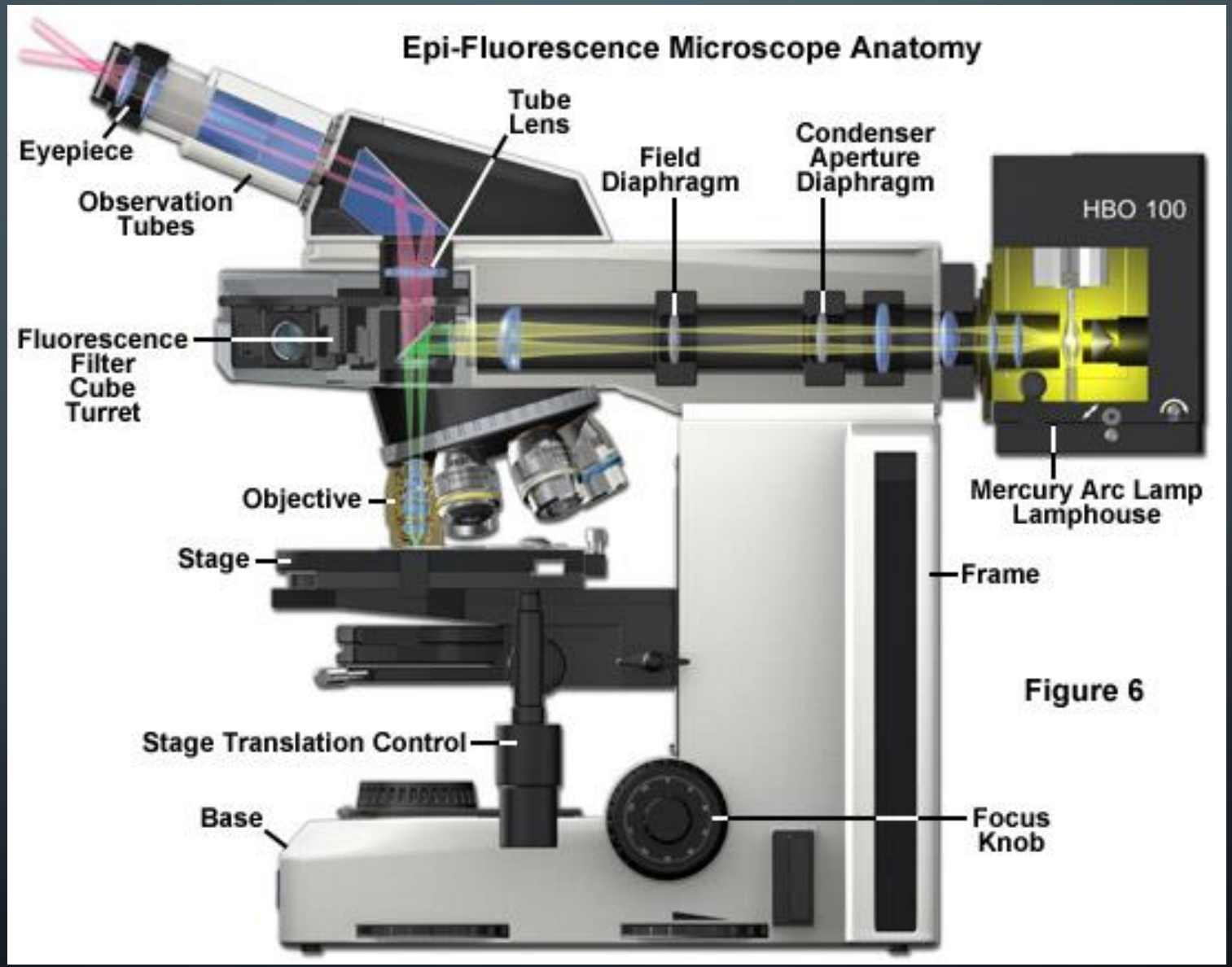
conventional microscope

- uses light to illuminate the sample and produce a magnified image of the sample.

fluorescence microscope

- uses a much higher intensity light to illuminate the sample
- This light excites fluorescence species in the sample, which then emit light of a longer wavelength.
- A fluorescent microscope also produces a magnified image of the sample, but the image is based on the second light source

2021/2022 - Light microscopy in Cellular Biology



WORKING IN GREATER DETAIL

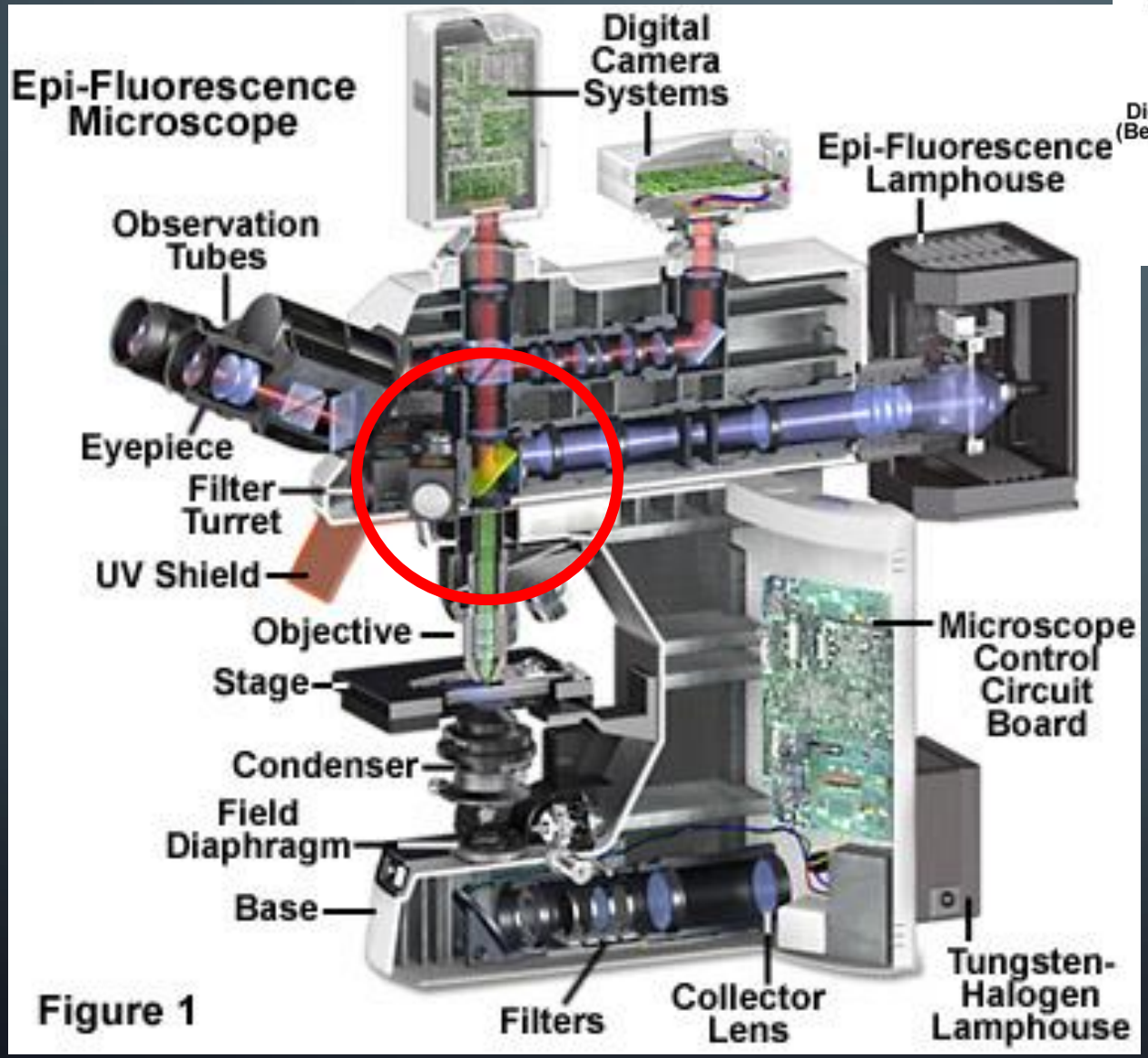


Figure 1

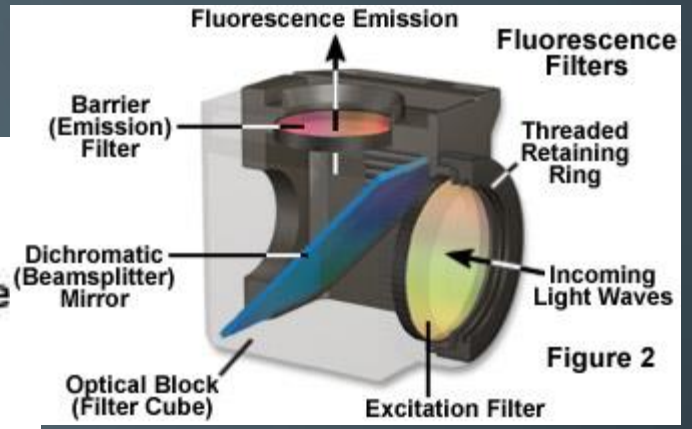


Figure 2

1. Excitation light travels along the illuminator perpendicular to the optical axis of the microscope

2. The light then impinges upon the excitation filter where selection of the desired band and blockage of unwanted wavelength occurs.

Working in greater detail

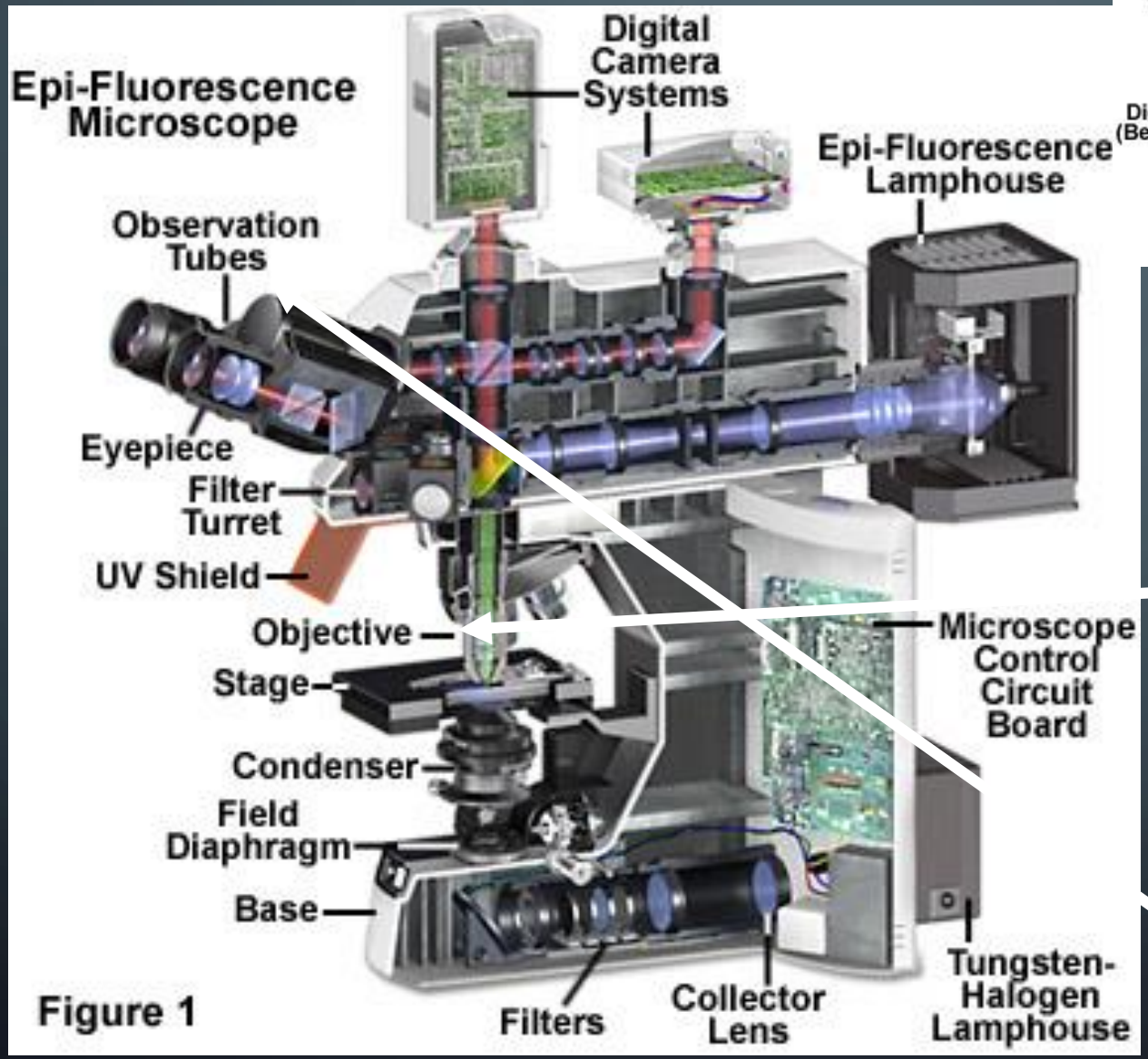


Figure 1

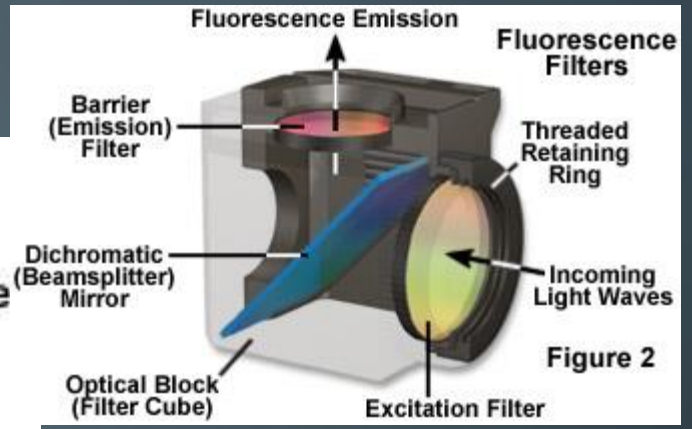
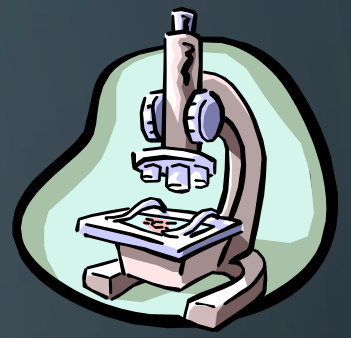


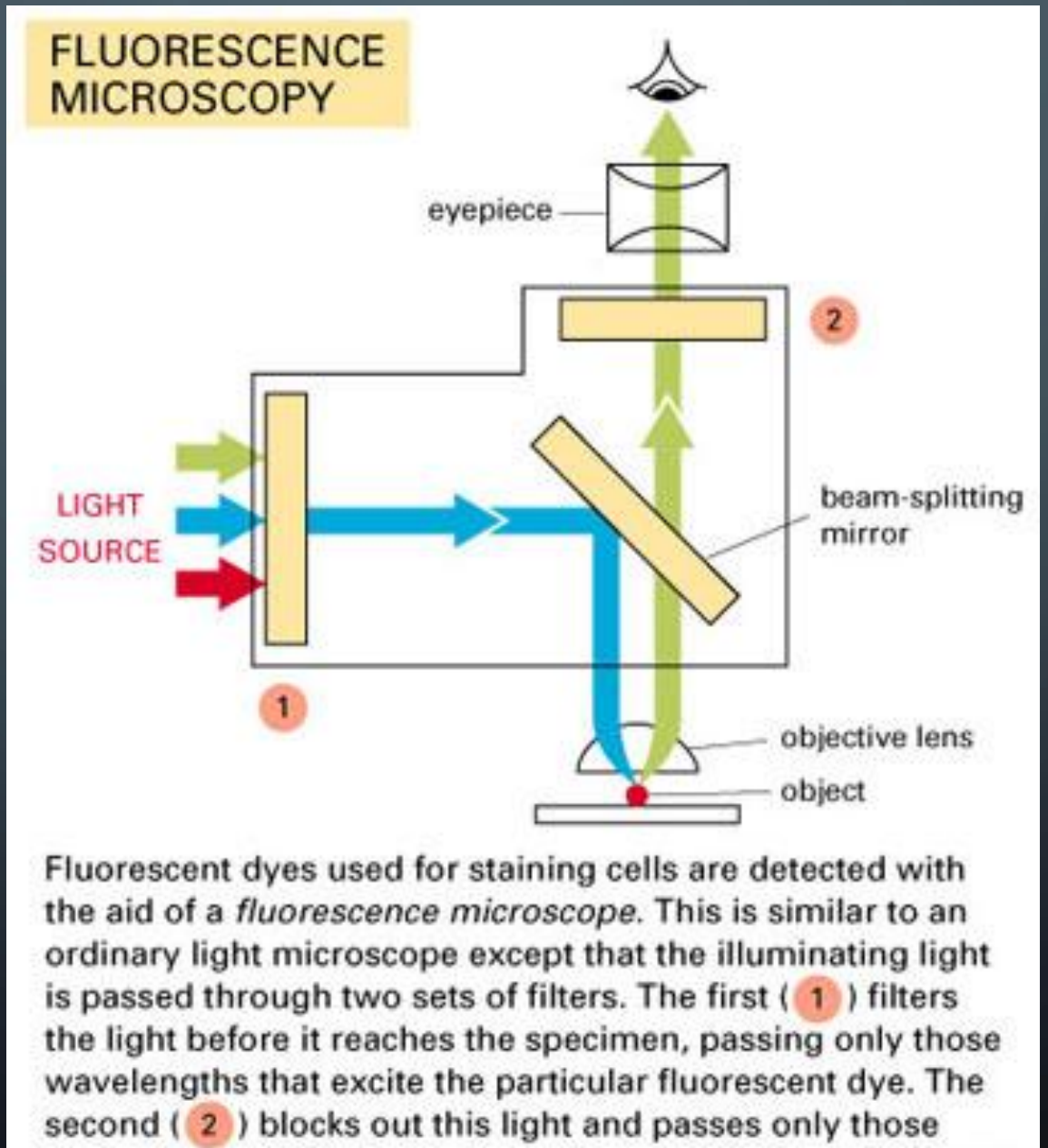
Figure 2

3. Fluorescence emission produced by the illuminated specimen is gathered by the objective

4. Because the emitted light consists of longer wavelengths than the excitation illumination, it is able to pass through the dichromatic mirror and upward to the observation tubes or electronic detector.

2021/2022 - Light microscopy in Cellular Biology





Fluorescent dyes used for staining cells are detected with the aid of a *fluorescence microscope*. This is similar to an ordinary light microscope except that the illuminating light is passed through two sets of filters. The first (1) filters the light before it reaches the specimen, passing only those wavelengths that excite the particular fluorescent dye. The second (2) blocks out this light and passes only those

2021/2022 - Light microscopy in Cellular Biology

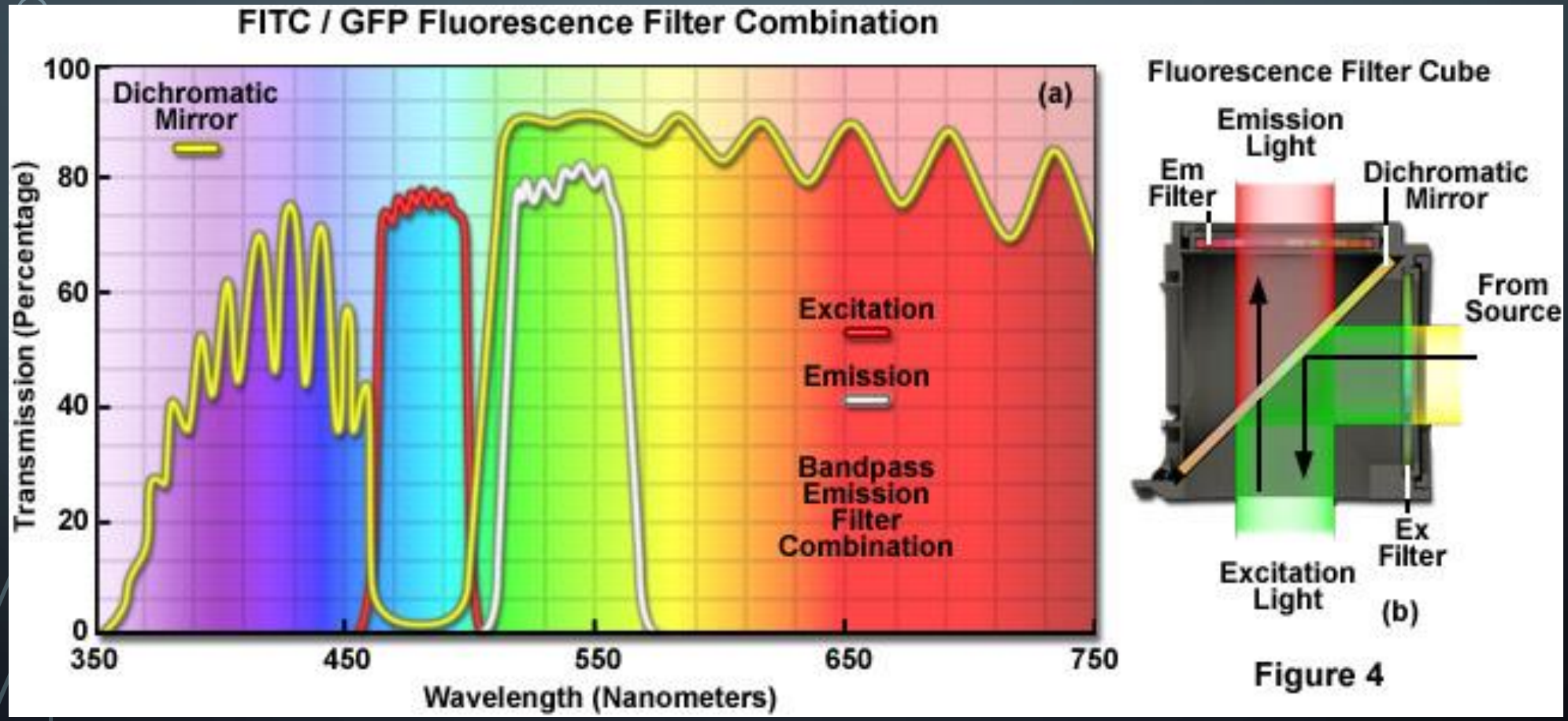


Figure 4

The Dichroic Mirror

dichroic, two color

- Each dichroic mirror has a set wavelength value -- called the **transition wavelength value** -- which is the wavelength of 50% transmission.

- reflects wavelengths of light below the transition wavelength value (90%)

- transmits wavelengths above this value. (90%)

- Ideally, the wavelength of the dichroic mirror is chosen to be between the wavelengths used for excitation and emission.

THE FILTERS

Excitation Filters

- to select the excitation wavelength, an excitation filter is placed in the excitation path just prior to the dichroic mirror.

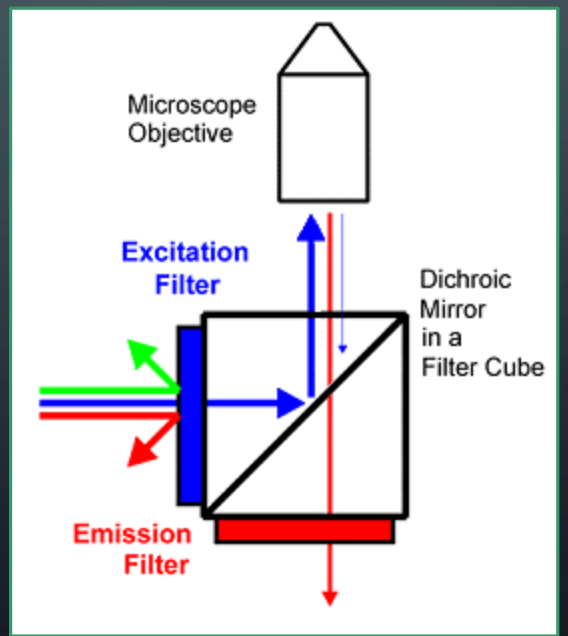


Fig: Light path through the filter cube in a fluorescence microscope.

Emission Filters

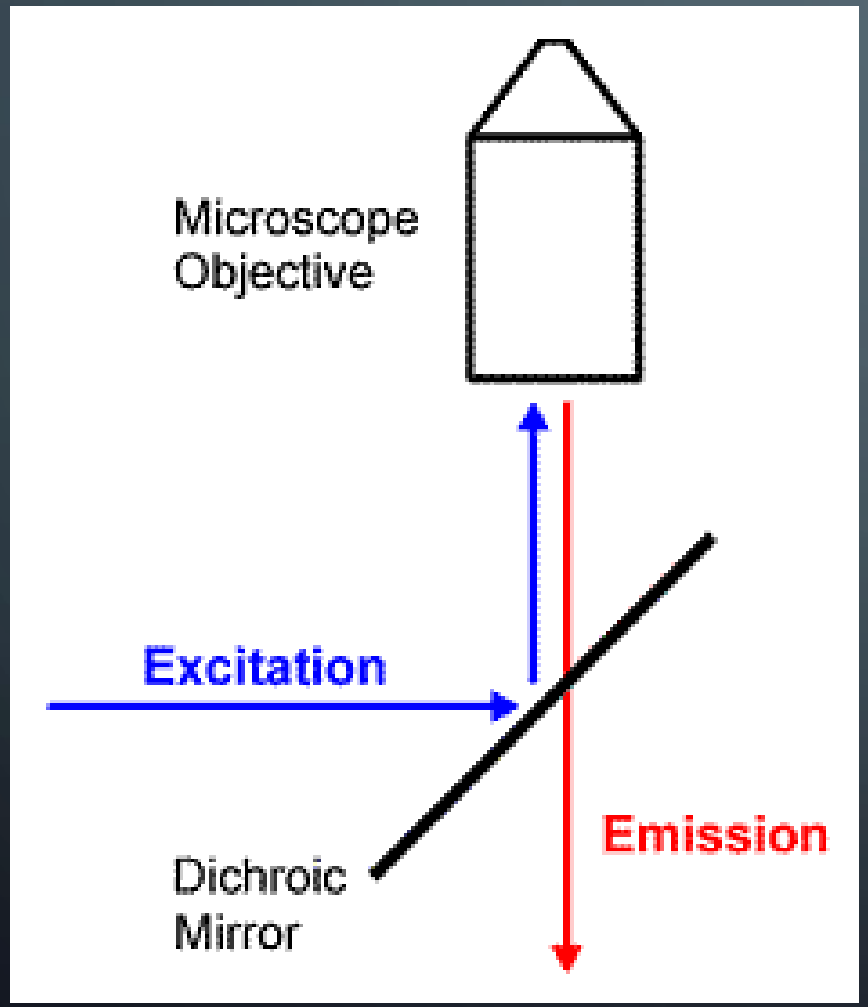
- In order to more specifically select the emission wavelength of the light emitted from the sample and to remove traces of excitation light

BASIC CONCEPTS

- let excitation light radiate the specimen
- then sort out the much weaker emitted light to make up the image.
- the fact that the emitted light is of lower energy and has a longer wavelength is used.
- The fluorescing areas can be observed in the microscope and shine out against a dark background with high contrast

THE DICHROIC MIRROR

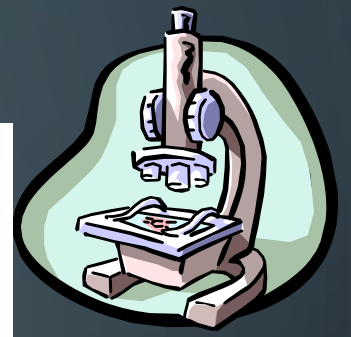
dichroic, two color

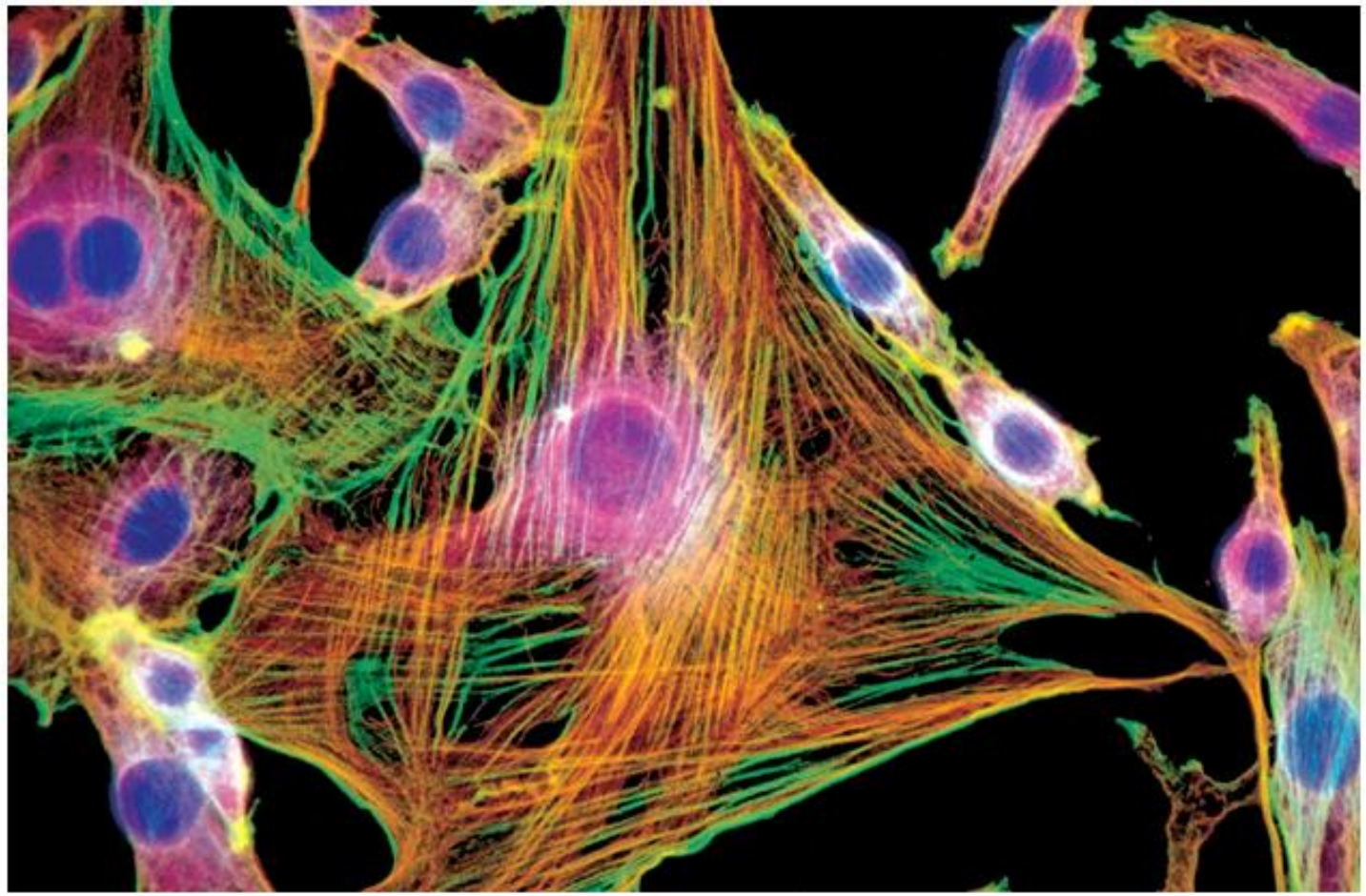


- The **excitation** light reflects off the surface of the dichroic mirror into the objective.

- The fluorescence **emission** passes through the dichroic to the eyepiece or detection system.

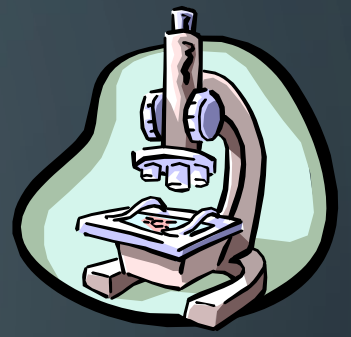
2021/2022 - Light microscopy in Cellular Biology

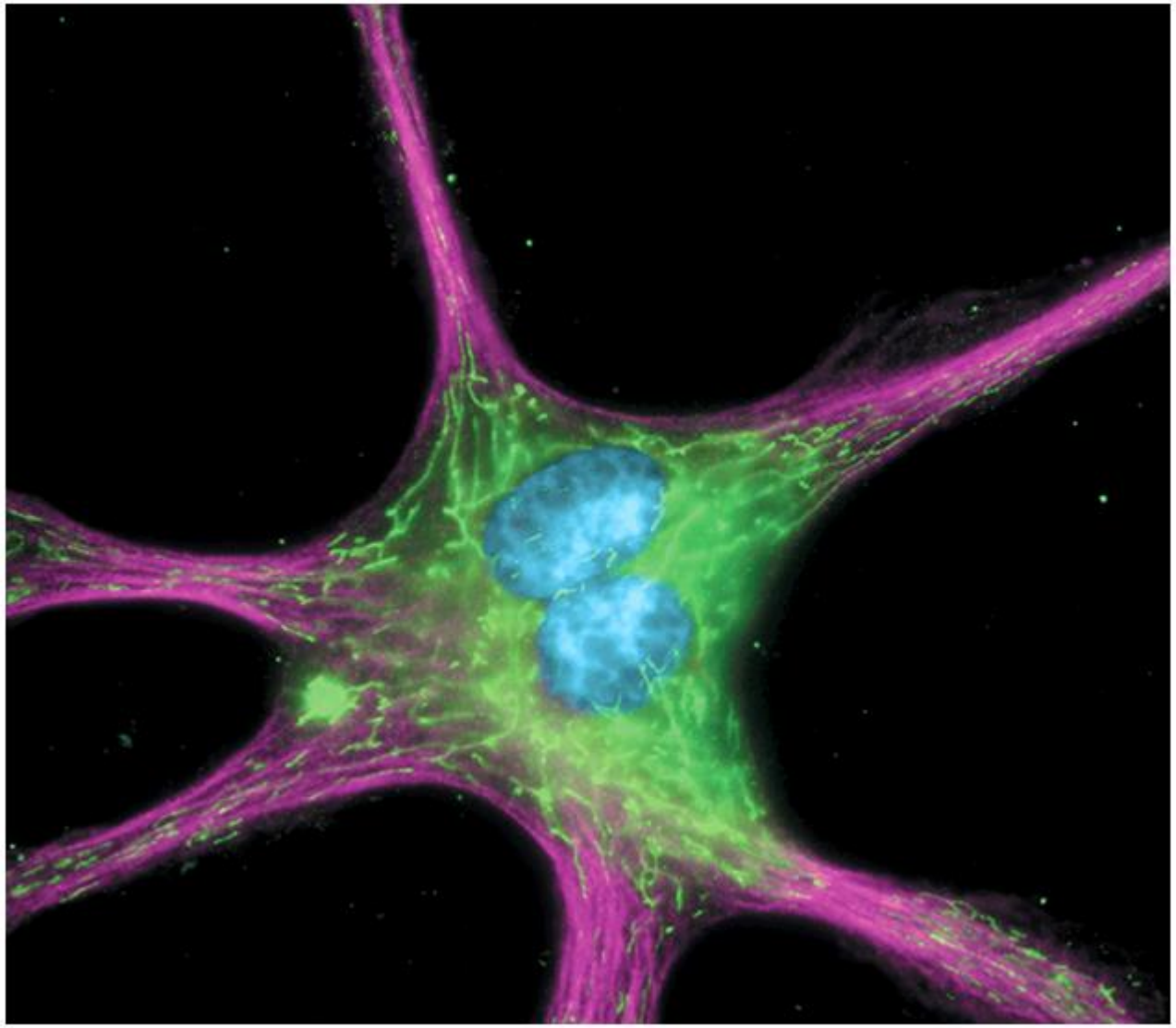




Photomicrograph of mouse fibroblasts that have been formaldehyde-fixed, acetone-permeabilized and triple-stained with the F-actin–specific probe BODIPY FL phallacidin (B607), with mouse monoclonal anti– -tubulin antibody in conjunction with Texas Red goat anti–mouse IgG antibody (T862) and with DAPI (D1306, D3571, D21490). The image was obtained by taking multiple exposures through bandpass optical filter sets appropriate for fluorescein, Texas Red dye and DAPI using a 100X Plan Apochromat objective.

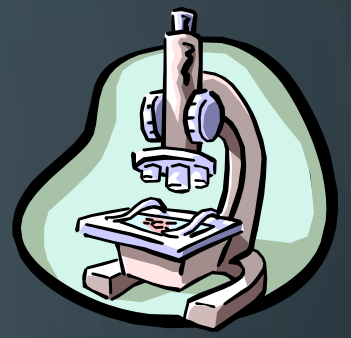
2021/2022 - Light microscopy in Cellular

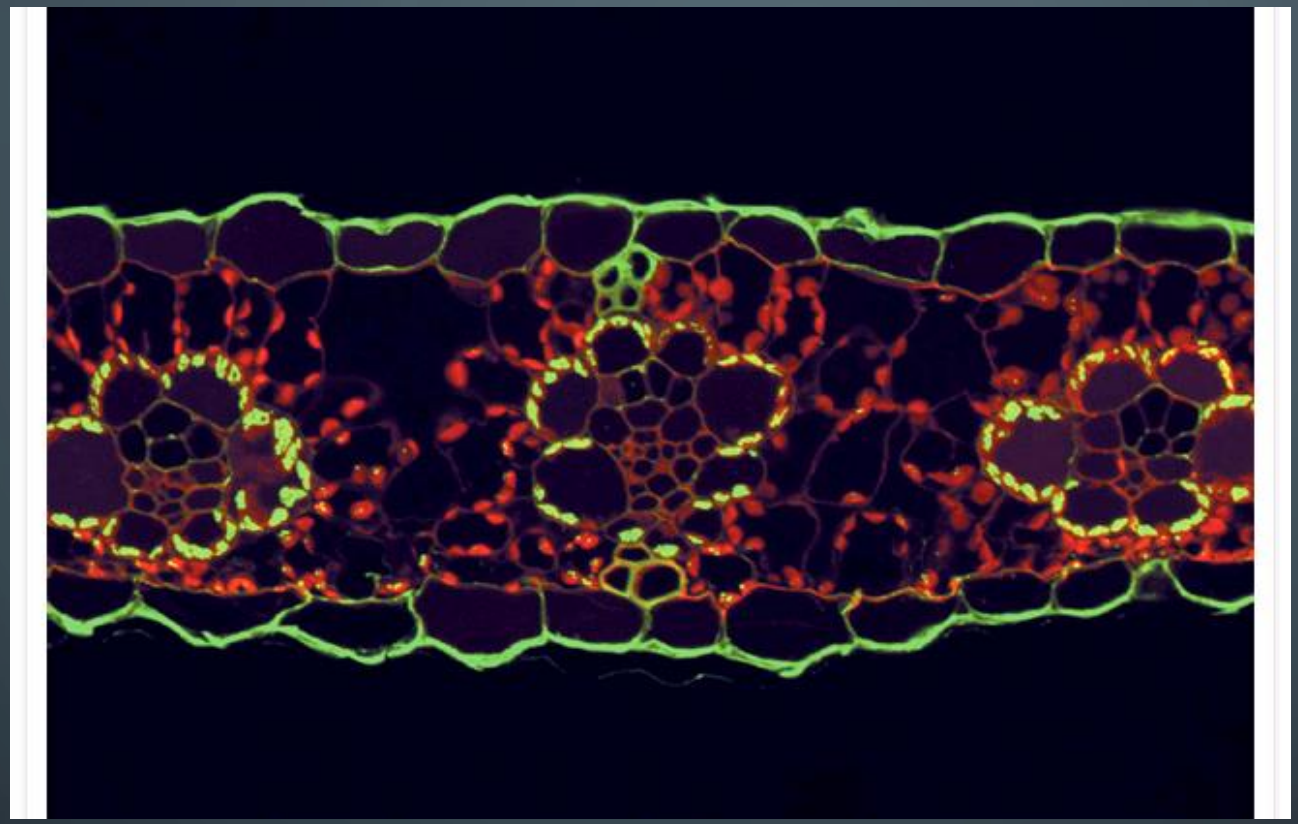




mouse monoclonal anti– -tubulin antibody (A11126), visualized with Alexa Fluor 647 goat anti–mouse IgG antibody (A21235) and pseudocolored magenta. Endogenous biotin in the mitochondria was labeled with green-fluorescent Alexa Fluor 488 streptavidin (S11223) and DNA was stained with blue-fluorescent DAPI (D1306, D3571, D21490).

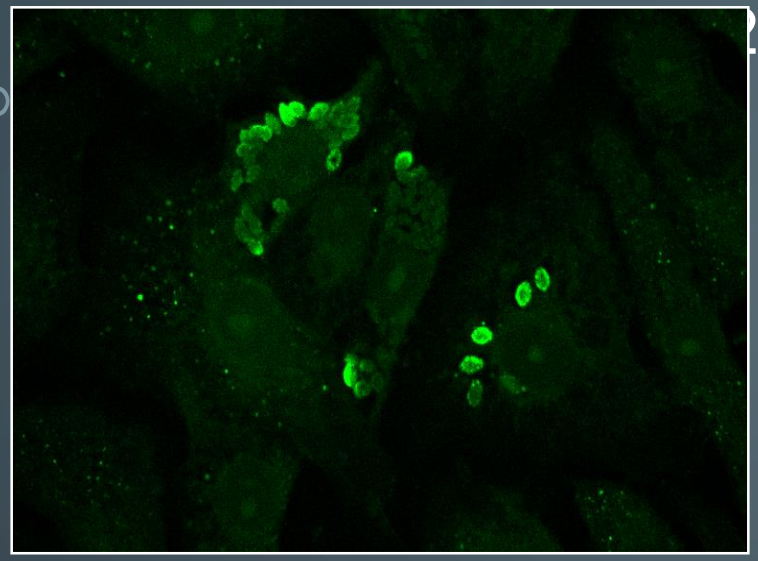
2021/2022 - Light microscopy in Cellular Biology

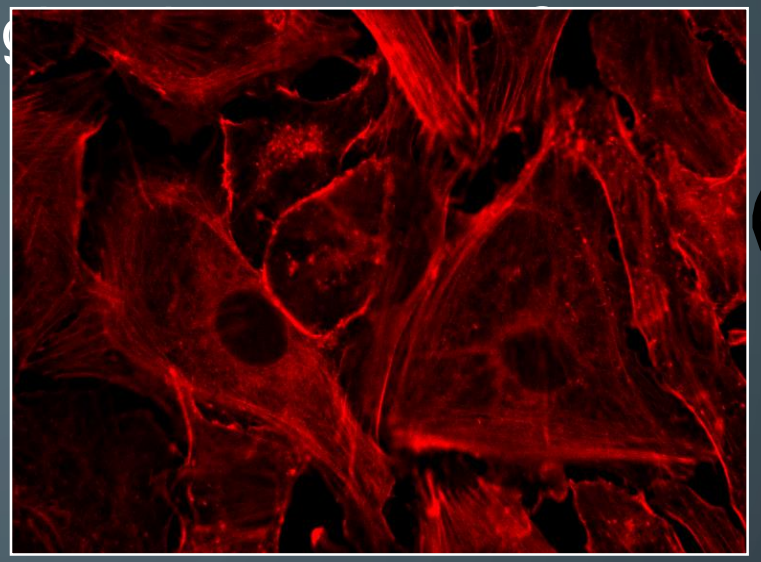


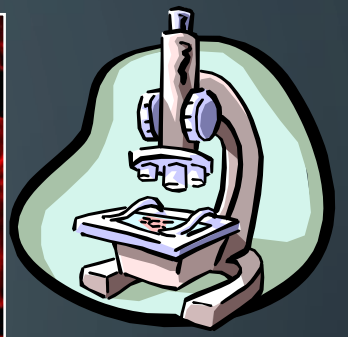


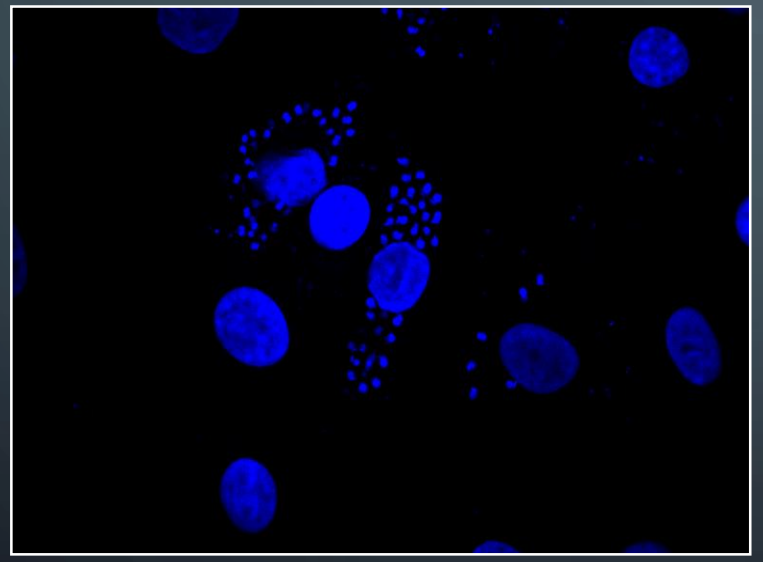
A 2.0 µm maize leaf section illustrating the immunolocalization of the enzyme ribulose bisphosphate carboxylase (rubisco) in the chloroplasts of the bundle sheath cells surrounding the vascular bundles. Maize is a C4 plant and, as a result, spatially segregates components of the photosynthetic process between the leaf mesophyll and the bundle sheath. Rubisco was localized using a rabbit anti-rubisco antibody and visualized using the highly cross-adsorbed Alexa Fluor 488 goat anti–rabbit IgG antibody (A11034). The remaining fluorescence is due to the autofluorescence of chlorophyll, which appears red and is localized to the mesophyll plastids; lignin, which appears dull green and is localized to the xylem of the vascular bundle; and cutin, which appears bright green and is localized to the cuticle outside the epidermis. Image contributed by Todd Jones, DuPont.

2 - Li



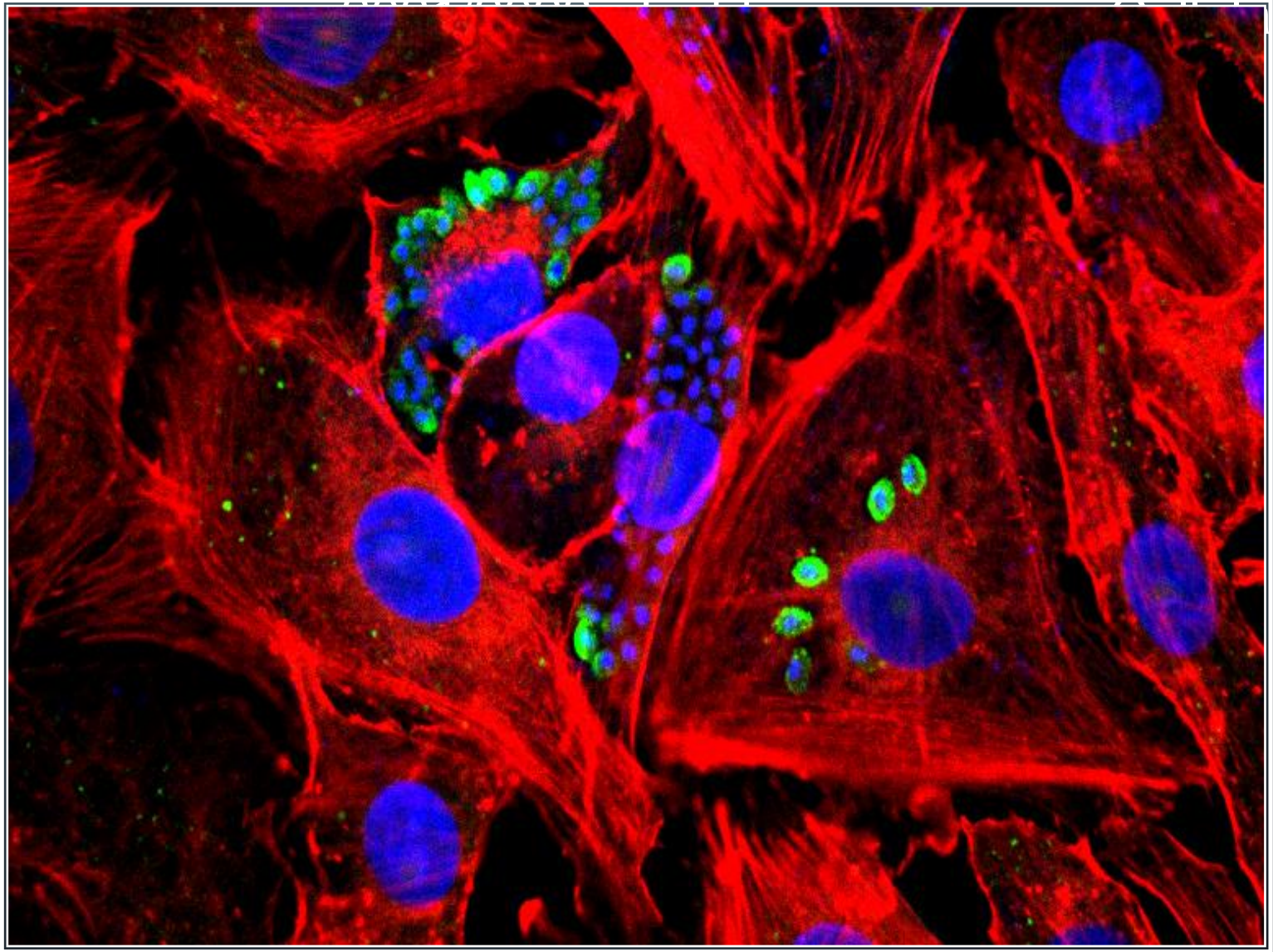


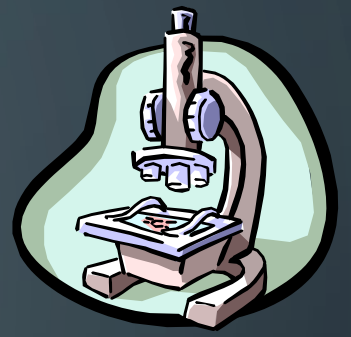






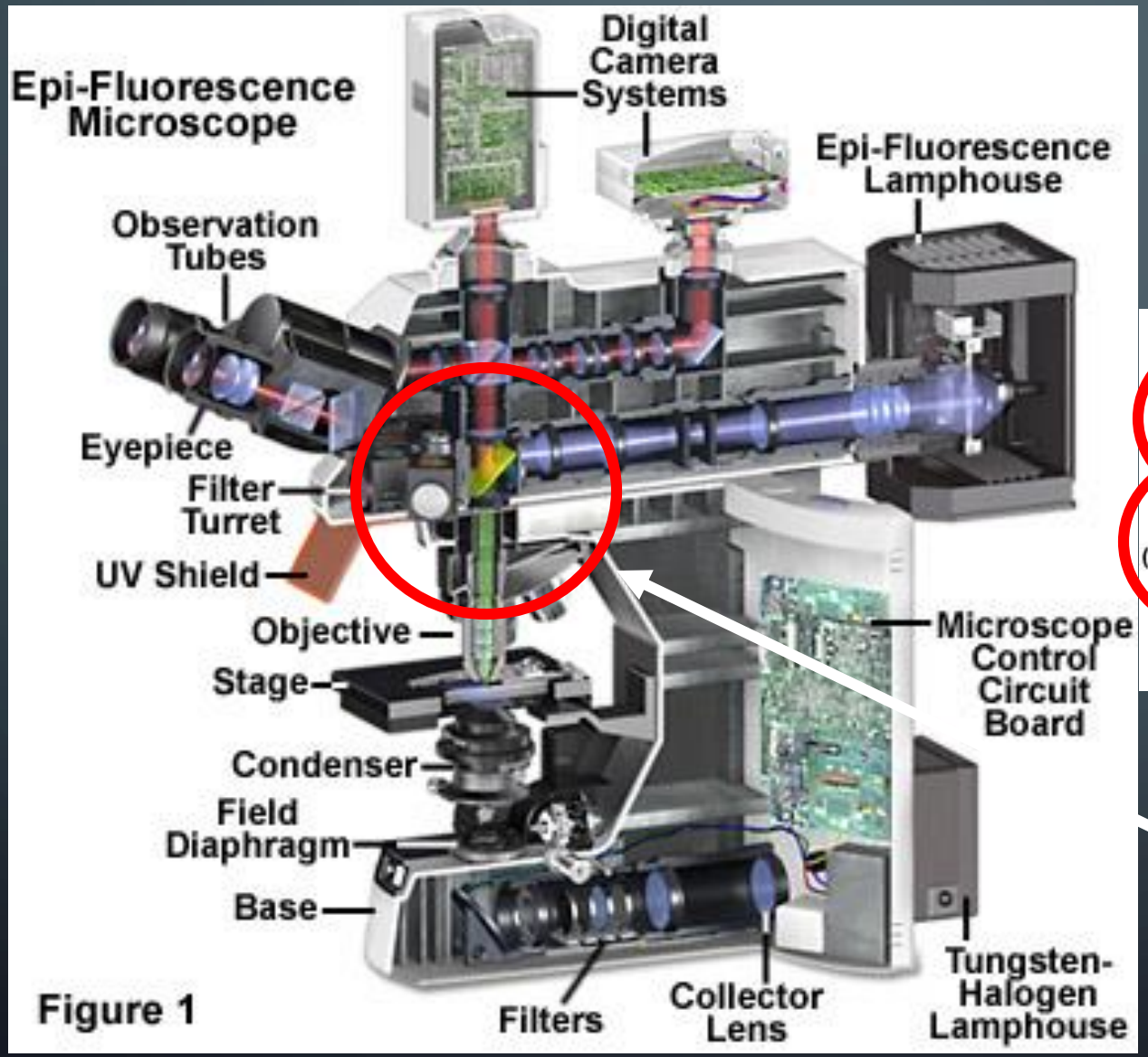
Actin - Rhodamine-phalloidin
Antibody to *T.cruzi* - FITC
DNA - Dapi

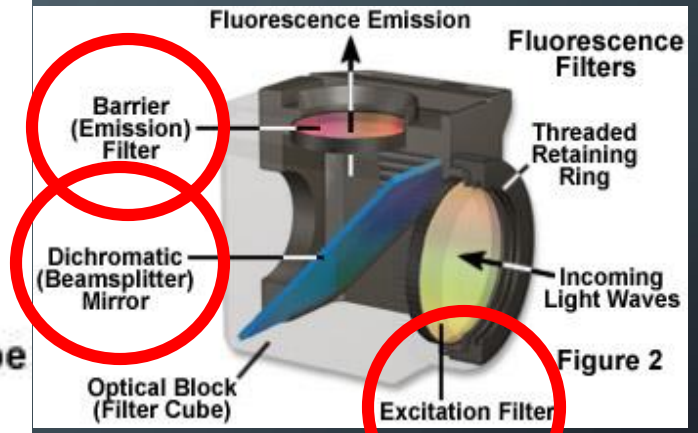




Actin - Rhodamine-phalloidin
Antibody to *T.cruzi* - FITC
DNA - Dapi

Imaged using an MRC 1000
Confocal Microscope, 40 x 1.3 NA Fluor


Epi-Fluorescence Microscope
Digital Camera Systems
Epi-Fluorescence Lamphouse
Observation Tubes
Eyepiece
Filter Turret
UV Shield
Objective
Stage
Condenser
Field Diaphragm
Base
Microscope Control Circuit Board
Tungsten-Halogen Lamphouse
Collector Lens
Filters
Figure 1


Fluorescence Emission
Fluorescence Filters
Barrier (Emission) Filter
Threaded Retaining Ring
Dichromatic (Beamsplitter) Mirror
Incoming Light Waves
Optical Block (Filter Cube)
Excitation Filter
Figure 2

The "cube"

