Fluorescence microscopy

Fluorescence methods in life sciences

Ctirad Hofr

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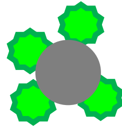
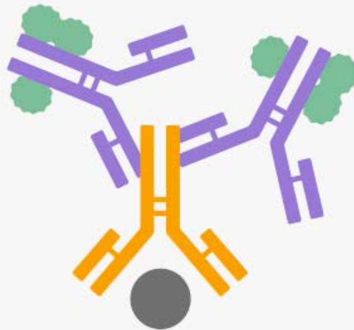
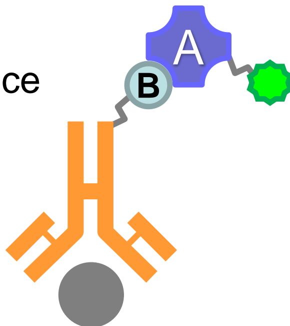
Specimen labeling direct fluorescence and immunofluorescence

Direct fluorescence





Biotin-avidin
immunofluorescence



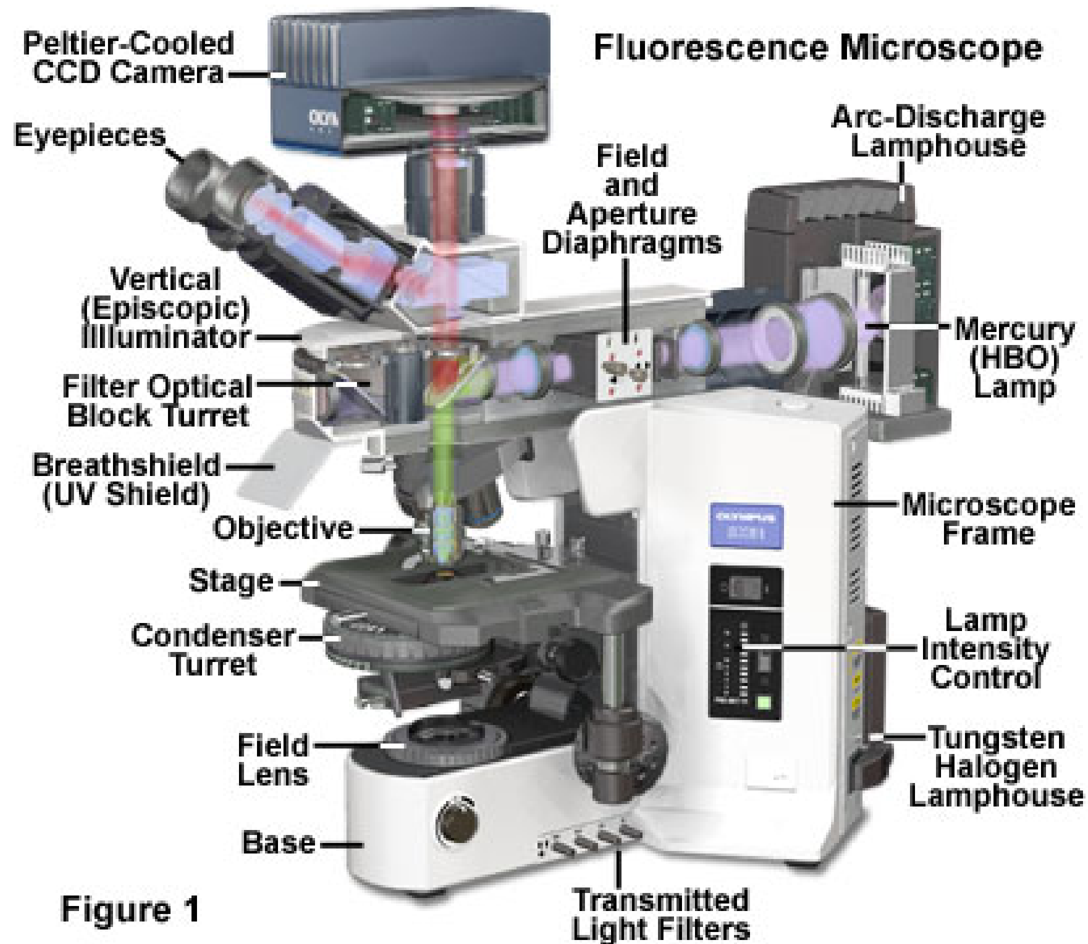
12 https://www.thermofisher.com/cz/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/labeling-your-samples/immunolabeling.html

Direct fluorescence - fluorophore binds directly biomolecule of our interests (BOI), typically DNA (DAPI, ethidium bromide, Hoechst). **Immunofluorescence** is used for visualization of most molecules and structures in cell.

Direct immunofluorescence uses antibody raised against BOI. The antibody is labelled fluorescently by a fluorophore.

Indirect immunofluorescence uses two antibodies. We raise a primary antibody against BOI in rabbit or other species. The primary antibody is allowed to bind in our specimen to BOI. After rinsing and washing out the excess of the primary antibody, the fluorescently labeled secondary antibody(raised in e.g. mouse against rabbit antibodies) is allowed to bind to primary antibody. Secondary antibody label primary antibody and thus BOI. Naturally, indirect immunofluoresce shows lower specificity then direct methods of labeling. **Biotin-avidin immunofluorescence** employs the strong affinity between biotin and basic glycoprotein avidin. Primary antibody is biotinylated. Fluorophore linked with avidin labels primary antibody.

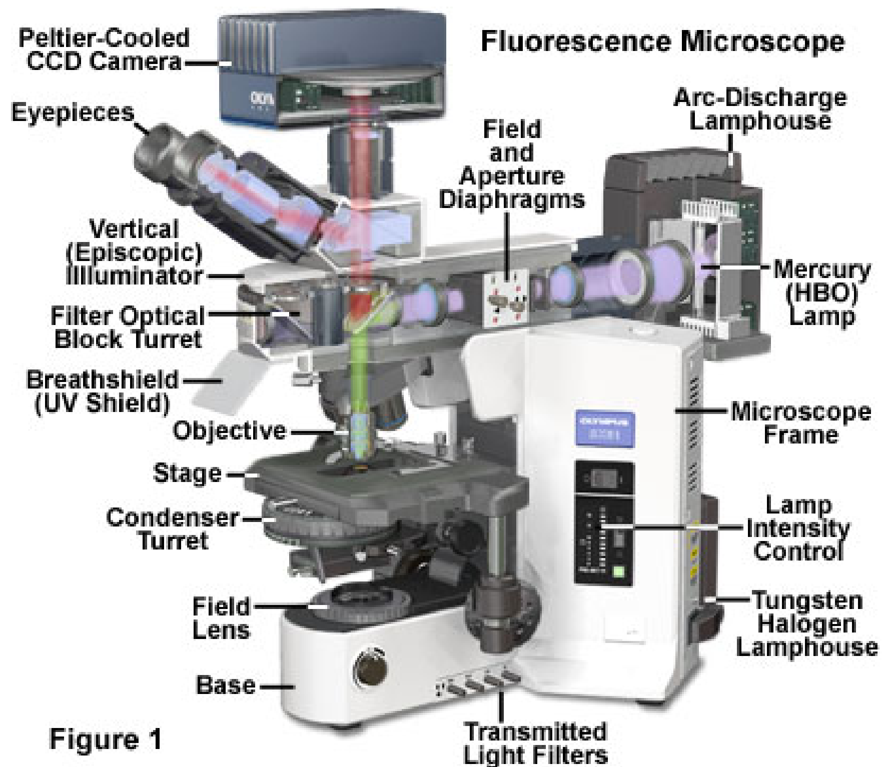
Fluorescence microscope



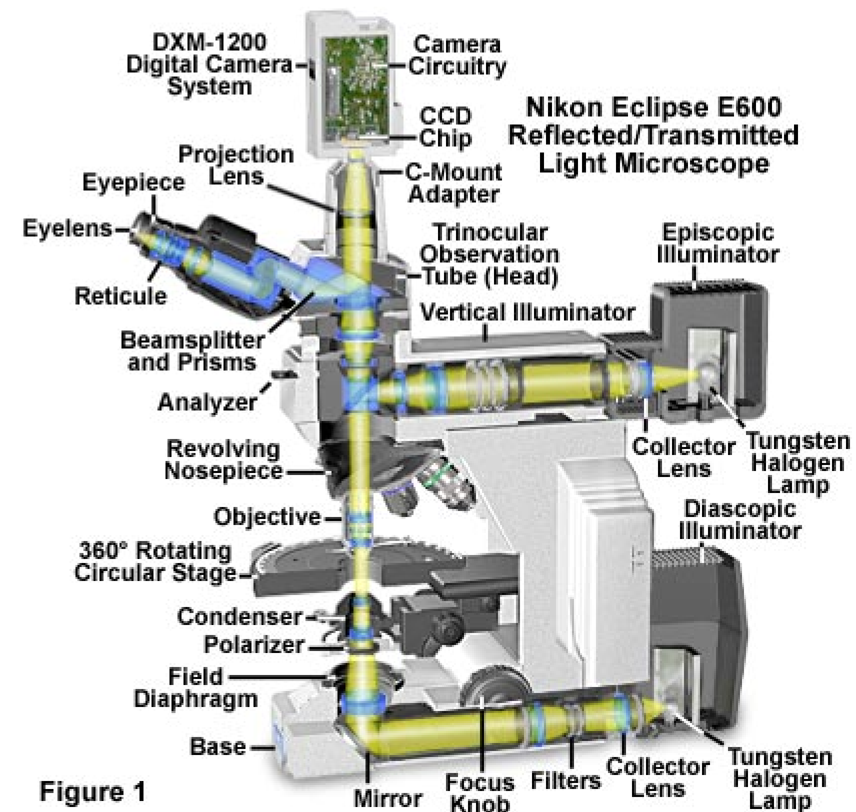
Possible arrangements

Epi fluorescence microscopy

95% of all fluorescence microscopes



Transmitted light microscopy



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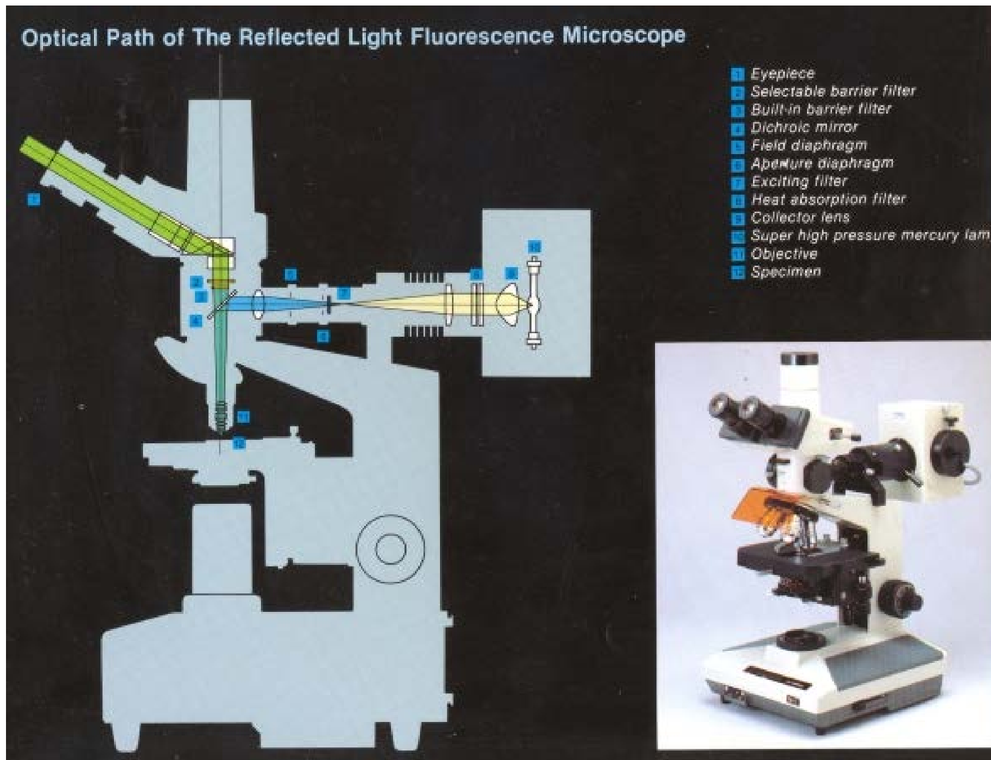
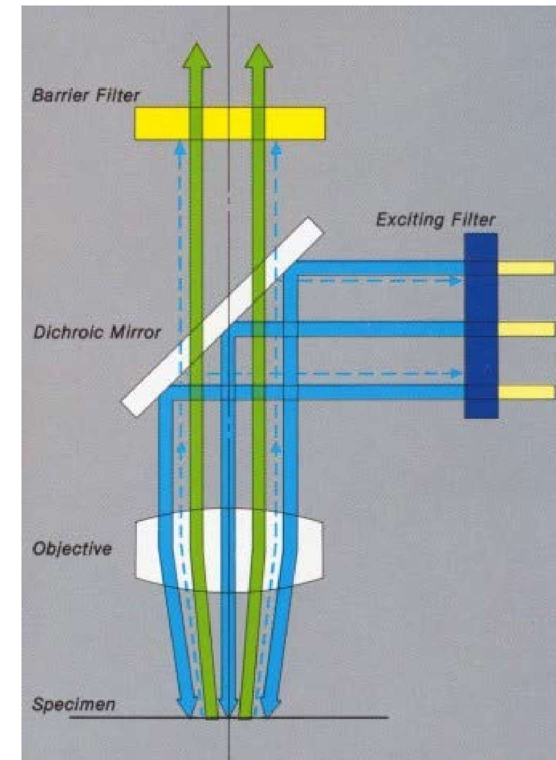
Epi-fluorescence microscopy

(Reflected light fluorescence microscope)





- excitační světlo prochází objektivem, dopadá na preparát a emisní světlo se vrací zpět do objektivu
- nutno použít zvláštní typ zrcadla, které odráží excitační světlo do objektivu a propouští emisní světlo do okuláru
- používá se dichroické zrcadlo, které propouští a odráží světlo podle toho, jakou má vlnovou délku. Používá se tedy vždy takový typ zrcadla, který maximum excitačního světla odráží a maximum emisního světa propouští.
- epifluorescenční typ mikroskopu je v současnosti více oblíbený než transmisní typ

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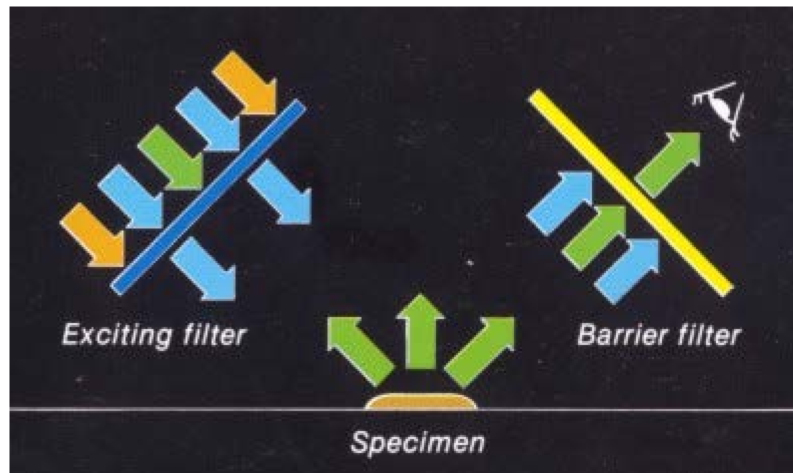
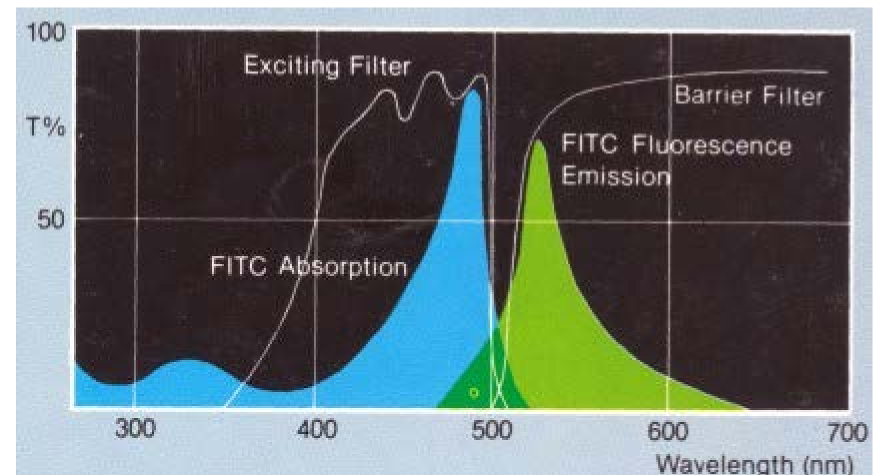
Excitation and barrier (emission) filter

We use two filters to observe fluorescence emission whose intensity is 100-fold lower than the intensity of excitation light. Without filters our eyes would not be able to see the florescence.

Excitation filter transmits only light wavelengths that are best for excitation of our fluorophore. Additionally, EF blocks other light wavelengths that could cause intrinsic fluorescence of specimen and increase background fluorescence.

Barrier (emission) filter transmits only light wavelengths of fluorescence emission coming from molecule of our interest. BF blocks excitation light that might be reflected to the detectors.





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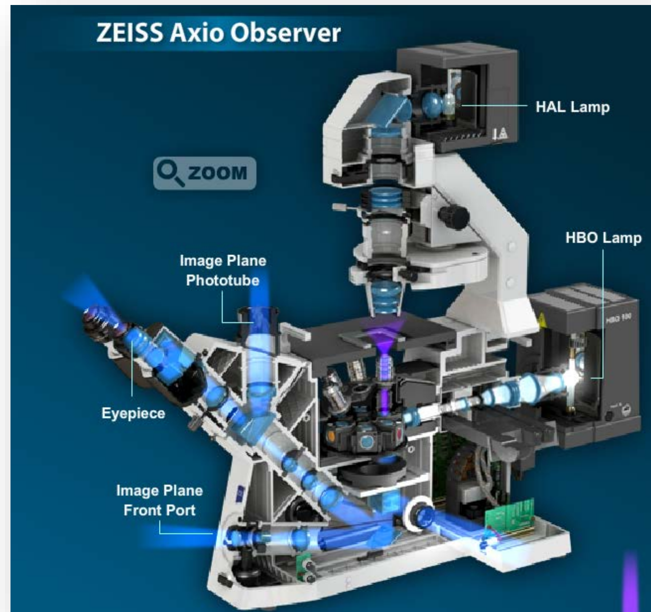
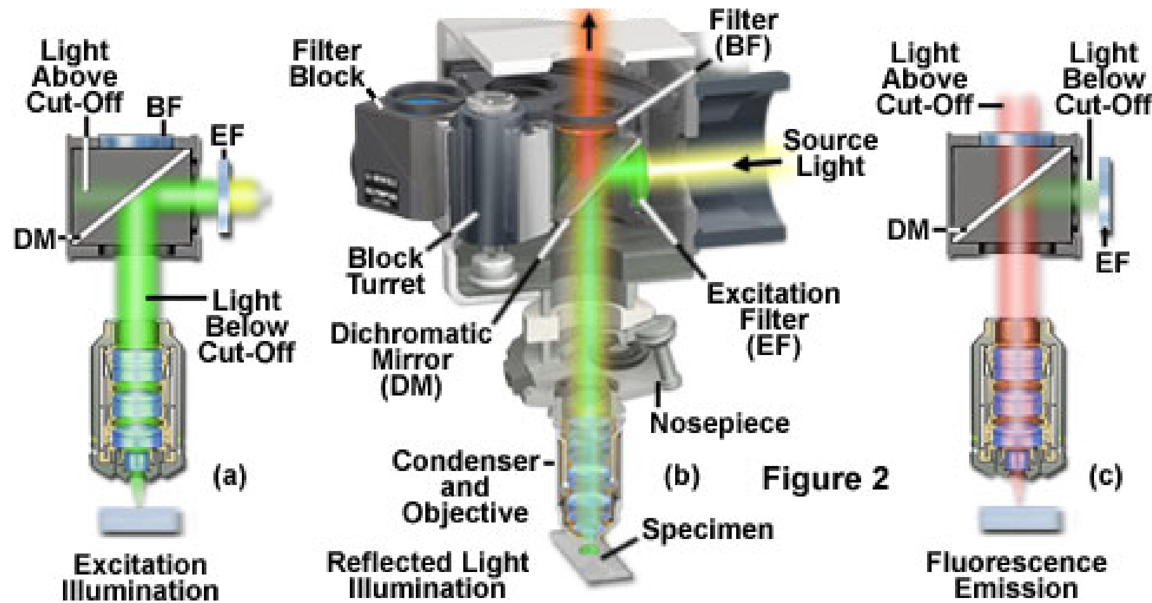
http://micro.magnet.fsu.edu/primer/java/fluorescence/opticalpaths/index.html

Microscope anatomy



http://zeiss-campus.magnet.fsu.edu/tutorials/axioobserver/index.html

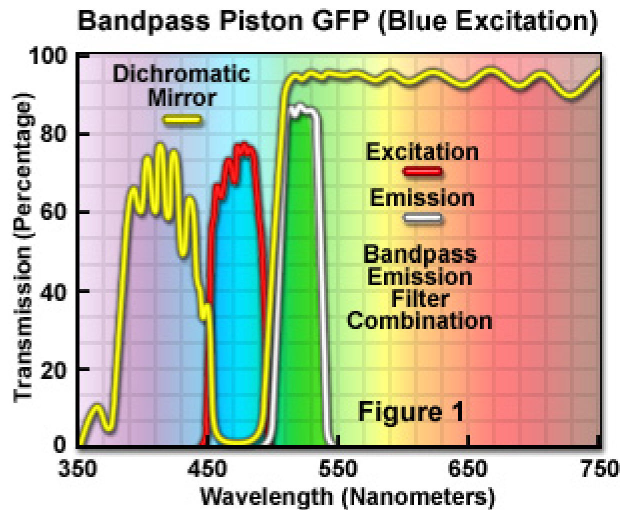
Mysterious dichroic mirror

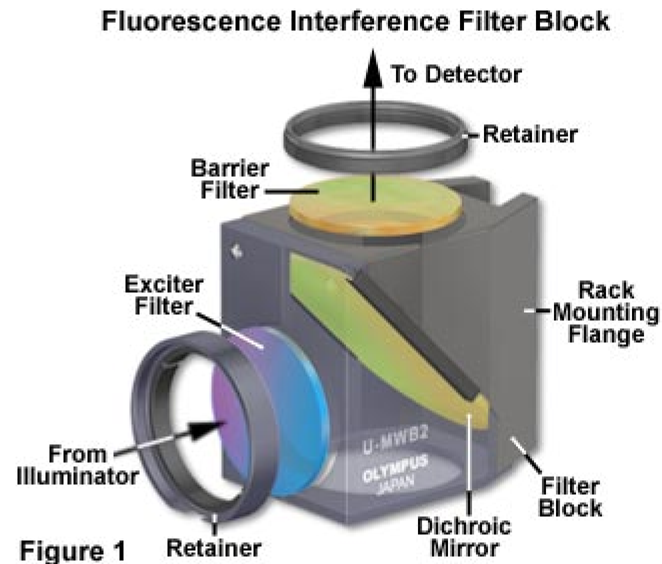


- Dichroic mirror reflect light with lower wavelengths but transmit longer wavelengths. Thus, dichroic mirror reflects maximum of excitation light let go through maximum of emission light used for a particular fluorophore.

3 filters make a filter **cube**

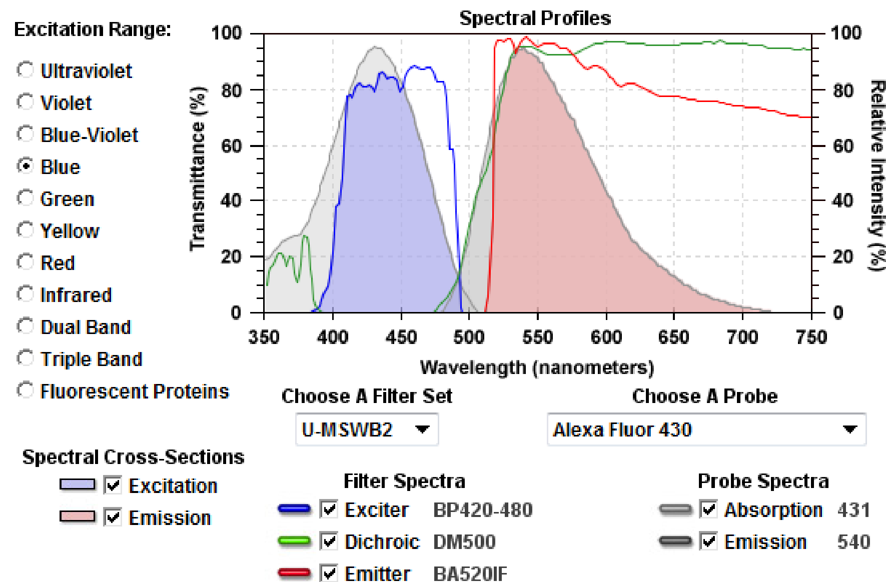
A proper combination of dichroic mirror, excitation and emission filter for a given fluorophore makes a filter block called as a filter **cube**. Two sides are filters and diagonal is dichroic mirror. Cubes are in a carousel and are exchanged by carousel rotation.





http://micro.magnet.fsu.edu/primer/java/lightpaths/fluorescence/index.html

Selection of a proper filter set (cube) for your fluorophore



http://www.olympusmicro.com/primer/java/fluorescence/matchingfilters/index.html

http://www.microscopyu.com/tutorials/flash/spectralprofiles/index.html

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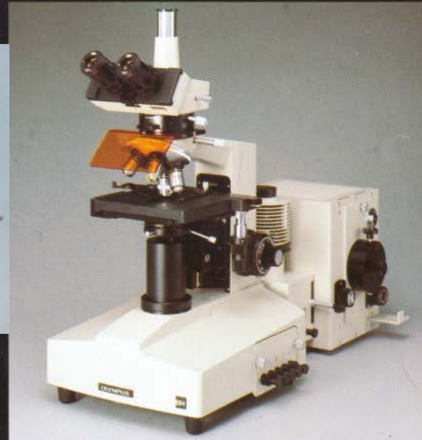
Transmitted light fluorescence microscope



Excitation light goes through excitation filter and comes from below as in case of a classical wide-field microscope. The most important part here is **darkfield condenser** that adjusts excitation light path so the light illuminate specimen from sides. Thus, only fluorescence emission goes in objective and is observed through objective. Excitation light is reflected so does not enter objective.

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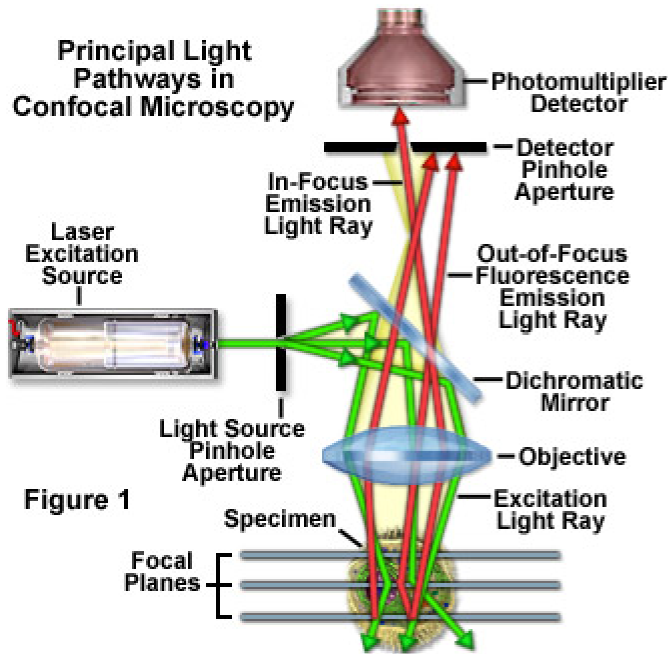
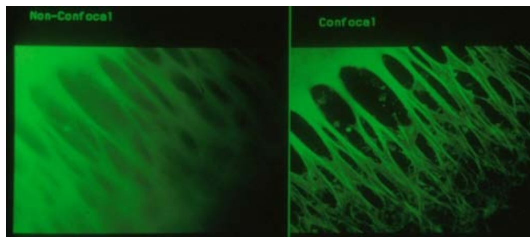
Confocal microscopy





- High **signal:noise** ratio
- Light source is **laser**,
- Two **pinholes** in light path. Light goes through the **source pinhole**. The beam of light is focused into a single point.
- On the light path from specimen to detector, there is the **detector pinhole**.
- Only light from focus plane is detected => sharp image
- One point in time is detected => scanning microscopy

http://www.olympusconfocal.com/java/confocalsimulator/index.html

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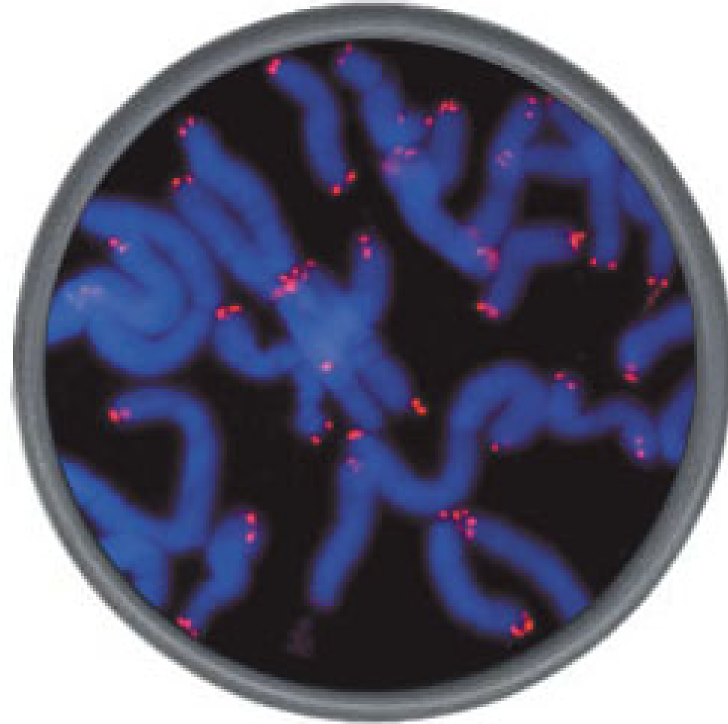
Virtual microscopy



http://micro.magnet.fsu.edu/primer/virtual/fluorescence/index.html

http://www.microscopyu.com/tutorials/java/kohler/index.html

Practical demonstration of fluorescence microscopy

Olympus BX 51 Transmitted and Reflected Light Microscope Equipped For Epi-Fluorescence Illumination

Camera Black Box
Film Plane
Electrical Contacts
Focusing Telescope
Automatic Exposure Monitor
Shutter
Light Sensor
Rubber Eyecup
Extension Tube
Projection Eyepiece
Lenses
Eyepiece (Ocular)
Trinocular Head
Prisms
Vertical Illuminator
Shutter
Cooling Vents
Episcopic Lamphouse
Beamsplitters
Filter Cube Turret
Dichroic Filters
Breathshield
Apertures
Nose-piece
Objective
Collector Lens
Lamp
Fan
Mechanical Stage
Microscope Control Circuit Board
Condenser
Field Diaphragm
Base
Diascopic Lamphouse
Illumination Intensity Adjustment
Line Cord
Mirror
Filters
Focus Knob
Collector Lens
Transformer