MICRO-561

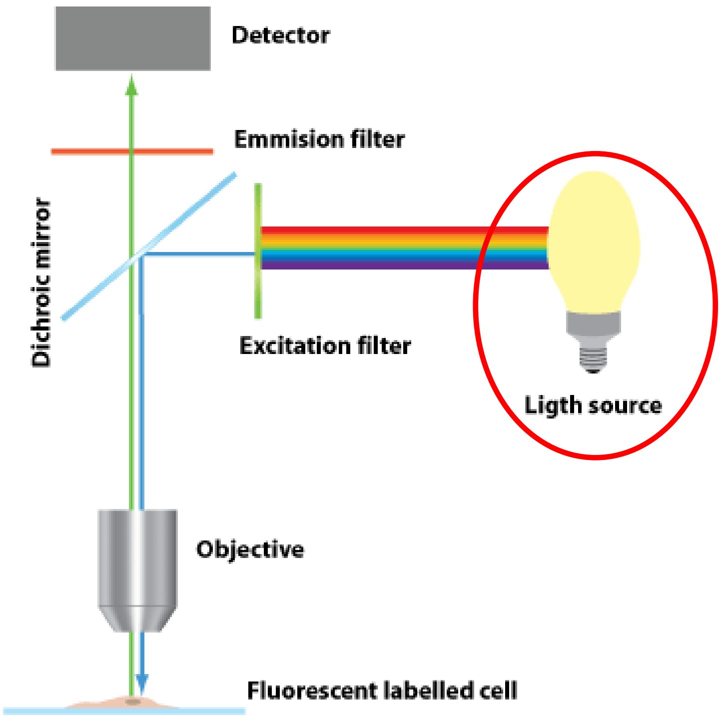
Fundamentals of Biomicroscopy

Syllabus (tentative)

Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Contrast & Fluorescence -1
Lecture 10	Fluorescence-2
Lecture 11	Sources
Lecture 12	Filters
Lecture 13	Detectors
Lecture 14	Bio-application Examples

Outline

- To understand fluorescence microscopy we need to be familiar with:
  - Basic principles of fluorescence
  - Properties of fluorescent dyes
  - Different kinds of fluorescence markers
  - The important optical components
    - Illumination sources
    - Filters and filter sets
    - Detectors
    - Their proper positioning in the optical train of the microscope



Illumination in Microscopy

- To obtain optimal imaging performance, the specimen must be properly illuminated.
- This requires:
  - proper selection of the wavelength and the intensity of the illumination source
  - correct alignment & focusing of the source (i.e. recall Kohler illumination)

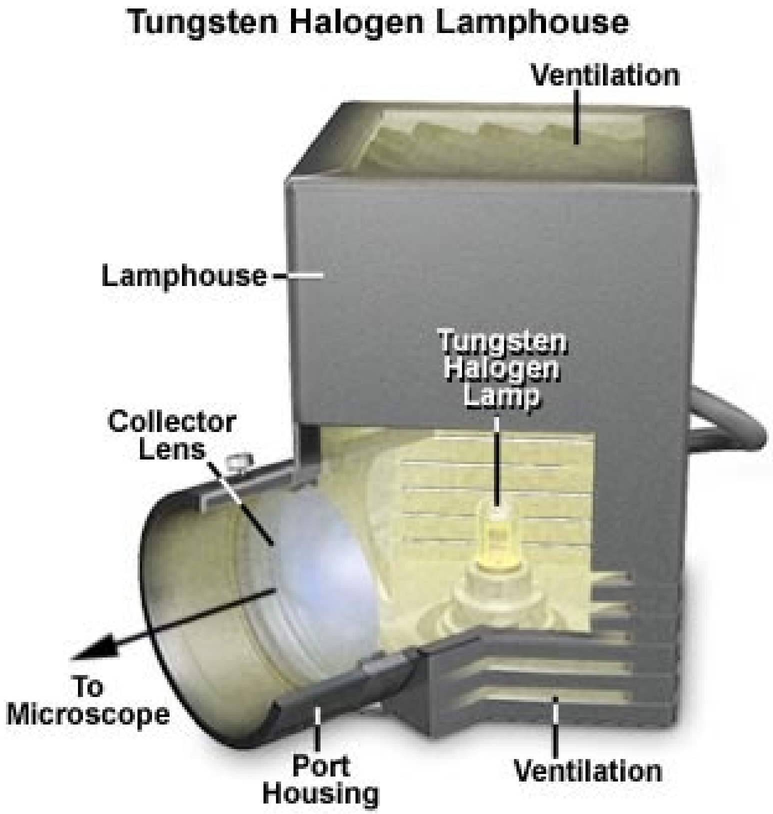
Commonly used illuminators in optical microscopy (bright-field & fluorescence):

- Incandescent lamps
  Quartz tungsten-halogen
- Ion arc lamps
  Mercury, Xenon
- Metal halides
- Solid-state sources:
  LEDs & Lasers

Incandancent Lamps

- **Incandescent lamps** with wire filaments and inert gases are frequently used in microscopy (especially in bright-field microscopy), because they are:
  - Cheap & easy to use
  - Provide sufficiently bright & even illumination when used properly (e.g. with a ground glass filter).

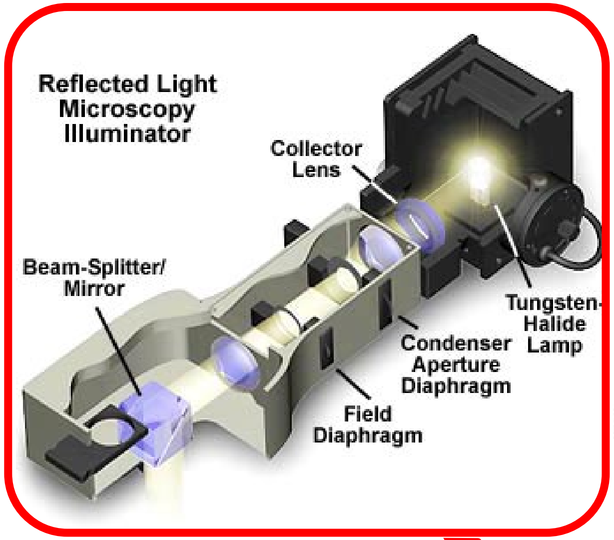
- Quartz tungsten-halogen is a commonly used incandescent lamp.
- Operation:
- It is based on a wire filament (such as **tungsten**) that is heated to a high temperature by passing an electric current through it until it glows with visible light (**incandescent)**.
- The bulb consist of an air-tight glass enclosure. The bulb is typically made of fused silica (quartz) since it is very strong, the gas pressure can be higher.
- The bulb is filled with an inert gas for reducing evaporation of the filament and preventing its oxidation.
- **Halogen** (such as iodine or bromine) increases the lifetime of the bulb and prevents its darkening.

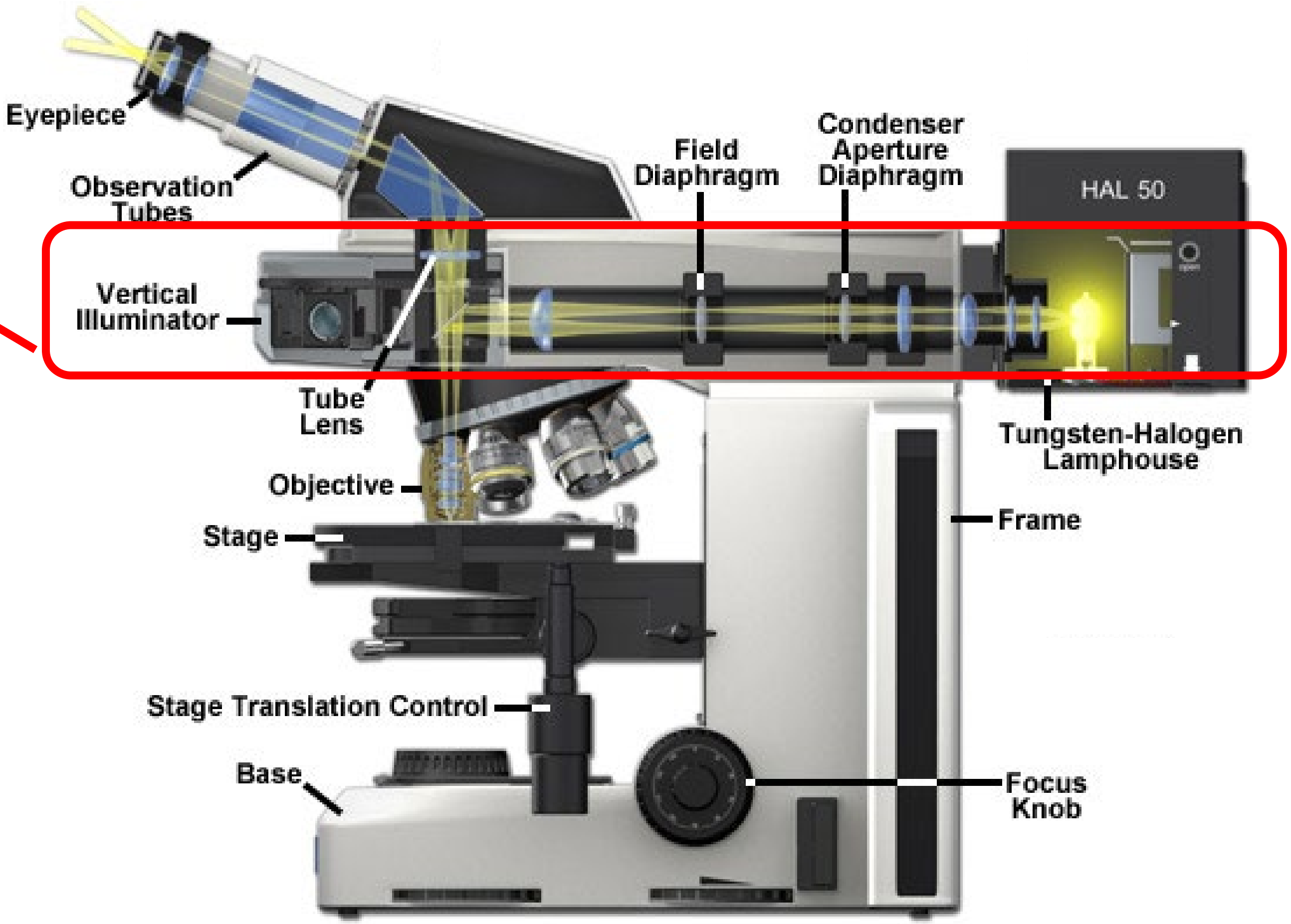


Example: Use of incandescent lamp

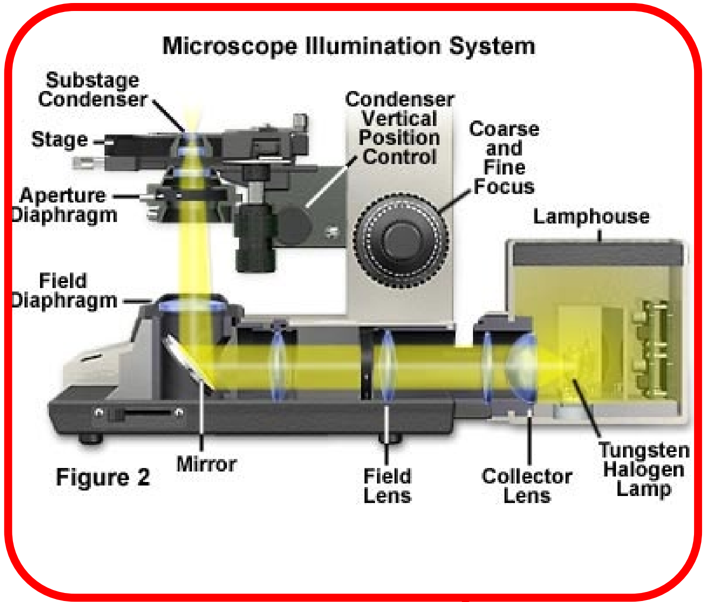
Anatomy of an Upright **– Reflected** Light Microscopy:

- Objective lens is on top of the sample stage.
- Light sources is delivered above the sample stage.



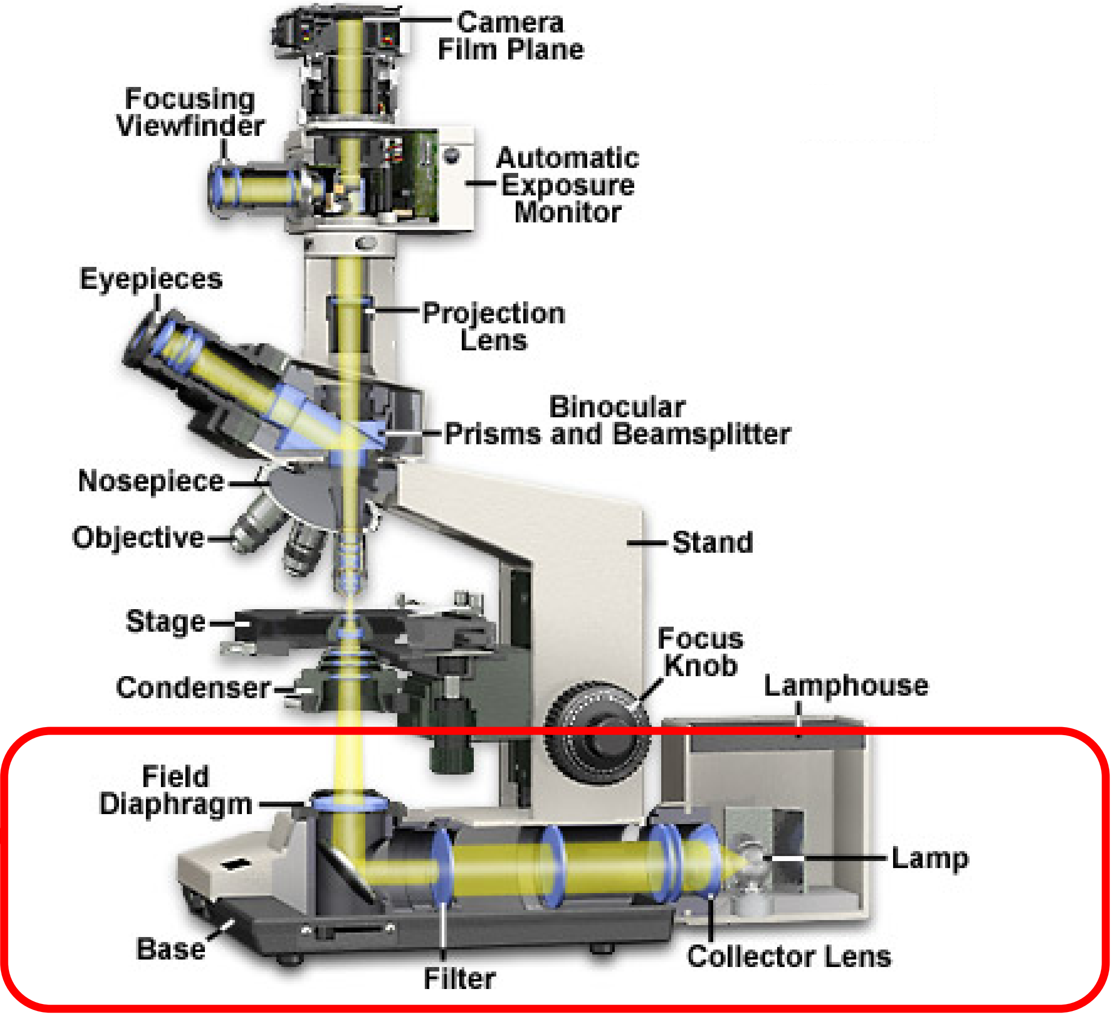


Example: Use of incandescent lamp



Anatomy of an Upright –**Transmitted** Light Microscopy:

- Objective lens is on top of the sample stage.
- Light sources is delivered below the sample stage.

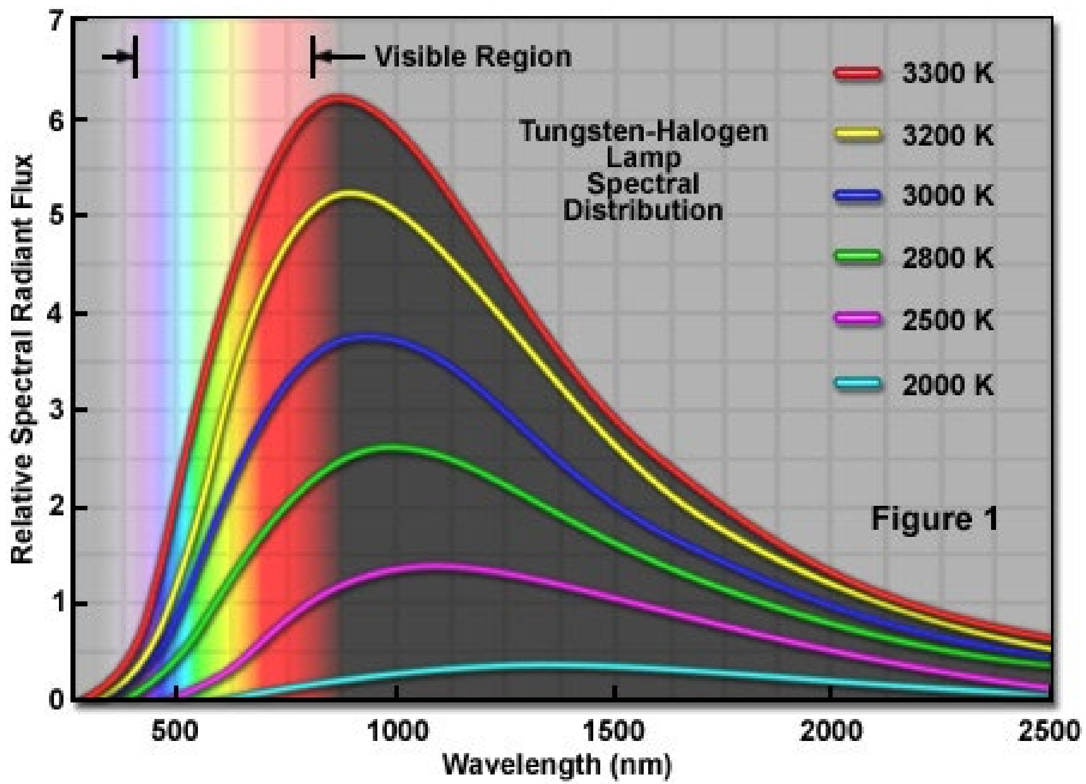


Spectrum of Incandescent lamps

- Quartz halogen tungsten filament lamp has black-body radiation spectrum:
  → it has "continuous spectrum" (and also broad spectrum)
- Figure shows lamp spectrum at different temperature → it has significant power in the infrared!

- The peak wavelength depends on the body temperature.
- With increasing voltage (thus temperature), brightness increases but also the spectrum shifts to shorter wavelengths.

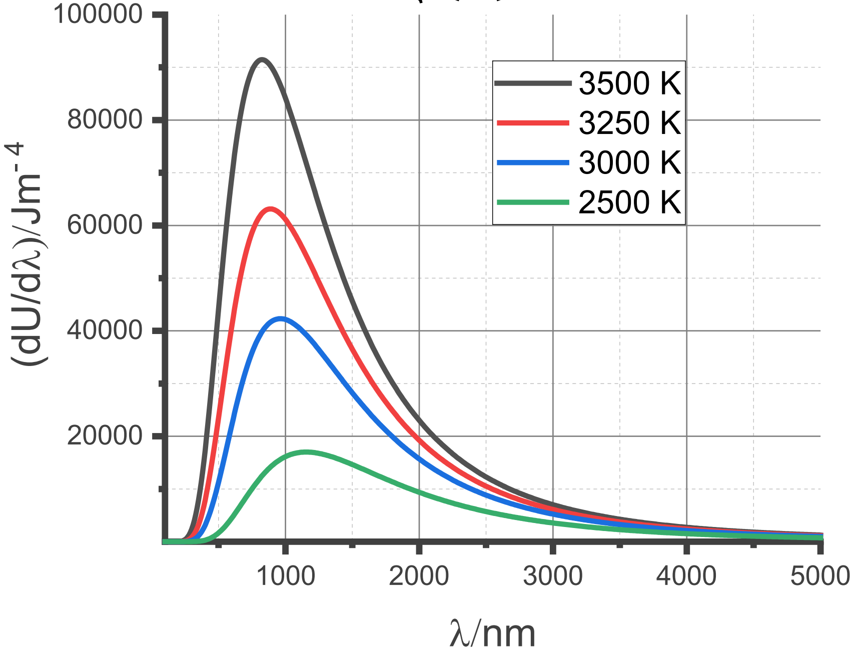
→ In microscopy application, it is better to fix the voltage and adjust the intensity with an absorbing filter.

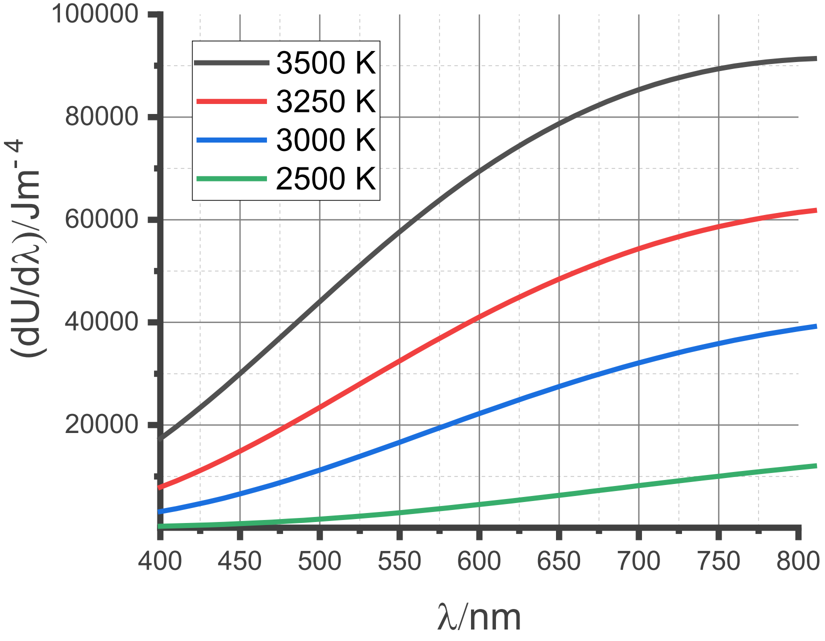


Spectrum of blackbody radiation

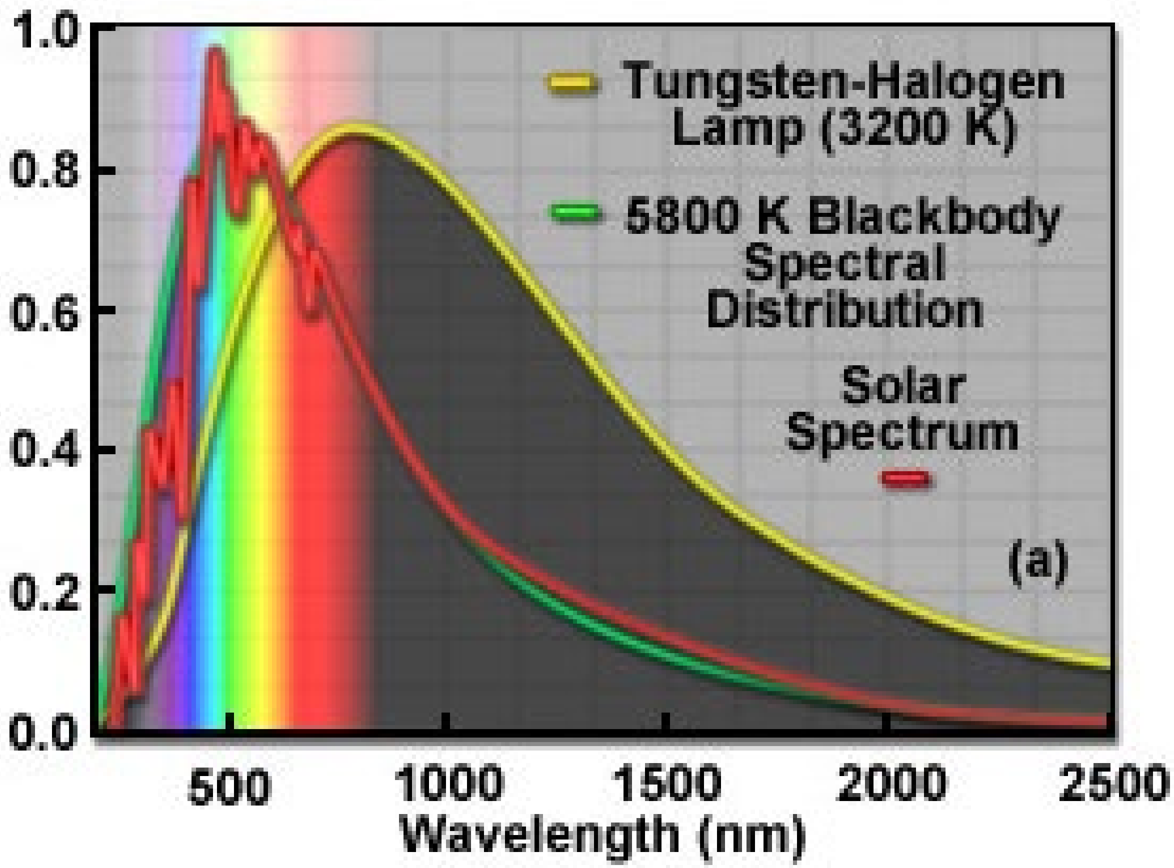
$$\varrho(\lambda) = \frac{8\,\pi\,h\,c}{\lambda^5}\left\{\frac{e^{-\frac{hc}{\lambda kT}}}{1 - e^{-\frac{hc}{\lambda kT}}}\right\}$$

$$d\mathcal{U} = \varrho(\lambda)d\lambda$$



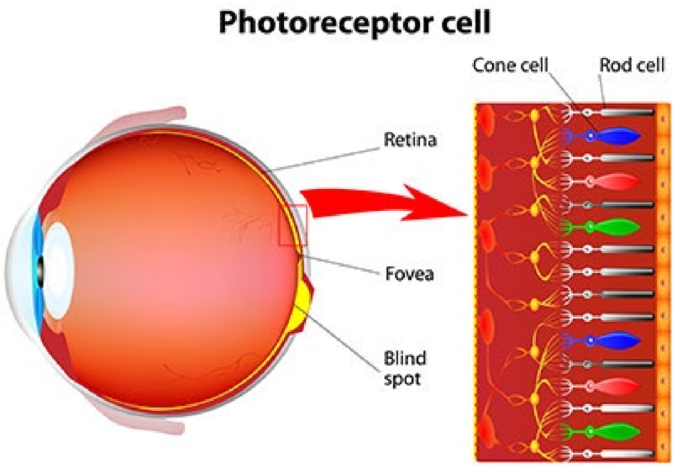


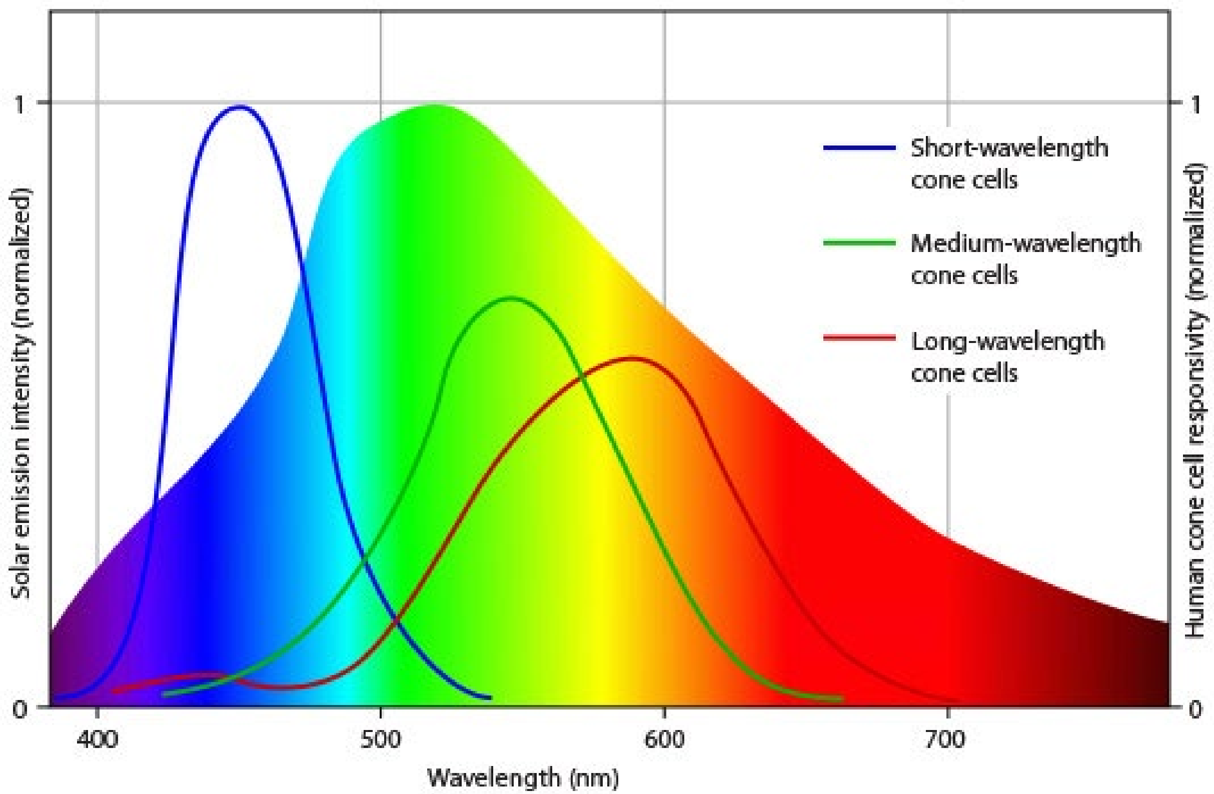
Blackbody radiation: Incandescent lamp & sun



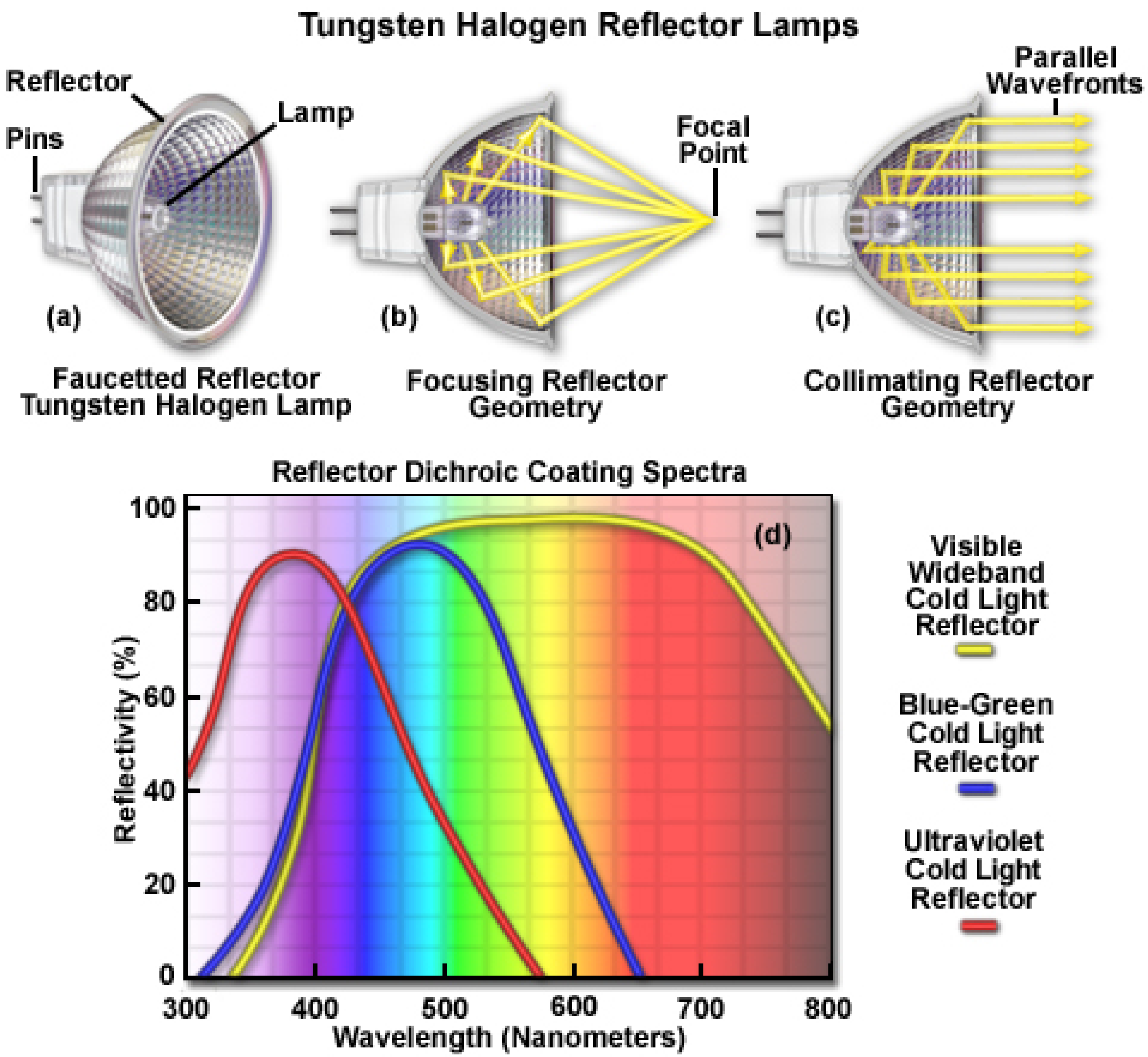
Example:
Sun, with an effective temperature of **~5800 K**, is an approximately black body with an emission spectrum peaked in the central, yellow-green part of the visible spectrum, but with significant power in the ultraviolet as well.

Adaptation of human eye to the sun





Reflector can be used to control the light from an incandescent lamp



Illumination in Microscopy

Commonly used illuminators:

- Incandescent lamps
  - Quartz tungsten-halogen
- Ion arc lamps
  - Mercury, Xenon
- Metal halides
- Solid-state sources:
  - LEDs & Lasers

Ion Arc Lamps

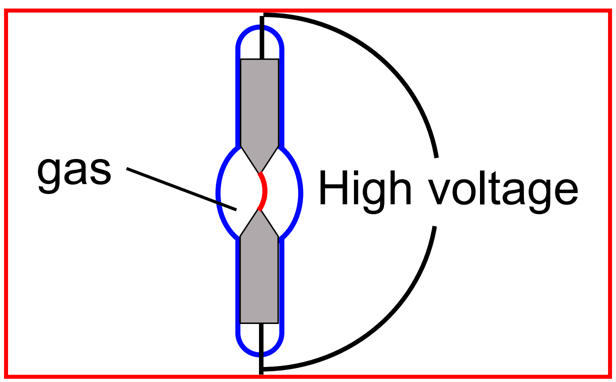
Operation:

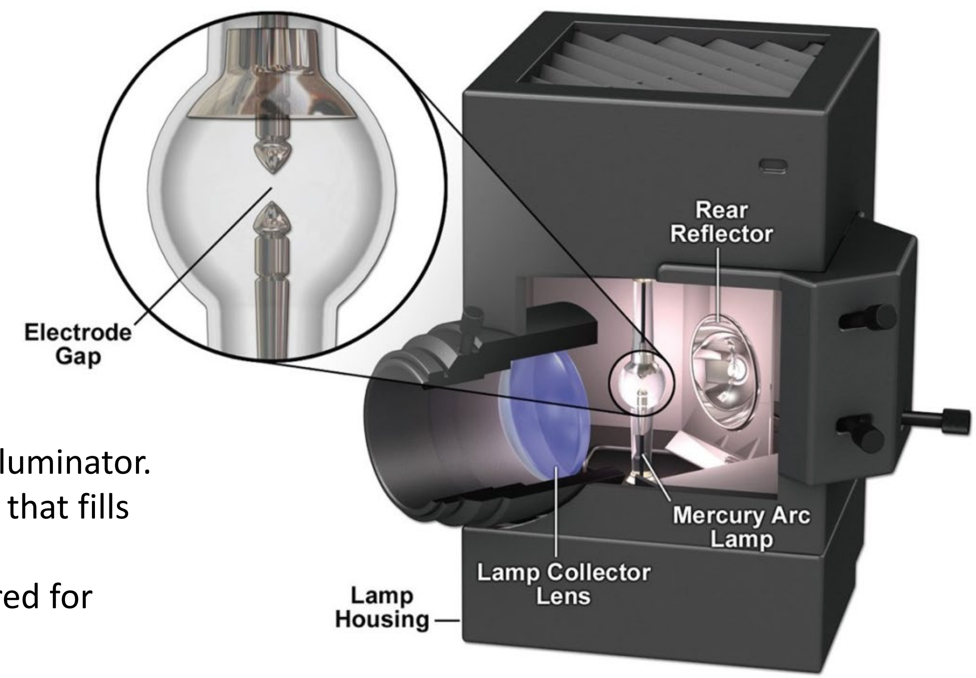
- The gap between the two electrodes contains a bright ionized plasma discharge.
- A high voltage is pulsed across the lamp to ignite the arc at the gap between the two electrodes. After ignitation, discharge can be maintained at a lower voltage.
- Arc lamp produces light by the electric arc, which is a plasma discharge that occurs when a gas is ionized.

Features:

- 10-100X brighter than incandescent lamps
- provide brilliant monochromatic illumination when combined with an filter
- But, they are expensive & have shorter lifetime

Commonly used ones are mercury arc lamp & xenon arc lamp:





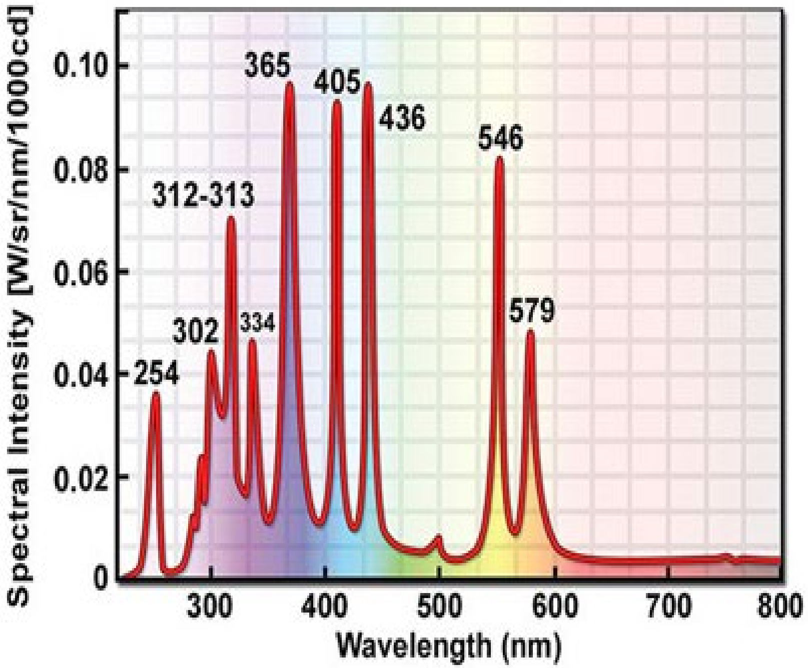
- A rear reflector to increase the output efficiency of the illuminator.
- The collector lens produces a magnified image of the arc that fills the rear aperture of the objective.
- Uniform coverage of the objective rear aperture is required for optimal resolution and illumination of the specimen.

Commonly used Ion Arc Lamps: Mercury & Xenon

- Both produces "continuous spectra" across the entire visible range, extending into the UV and IR.
- Only ~1/5 of the output spectrum is in visible, remainder is in the UV & IR.

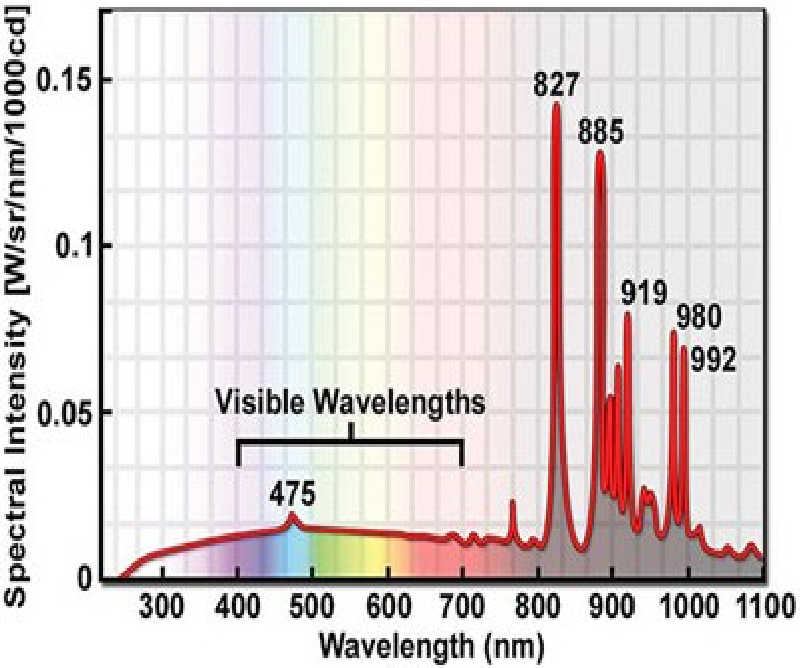
→ this demands to use special blocking filters when examining living cells, which are sensitive to UV & IR radiation

mercury arc lamp



(a)

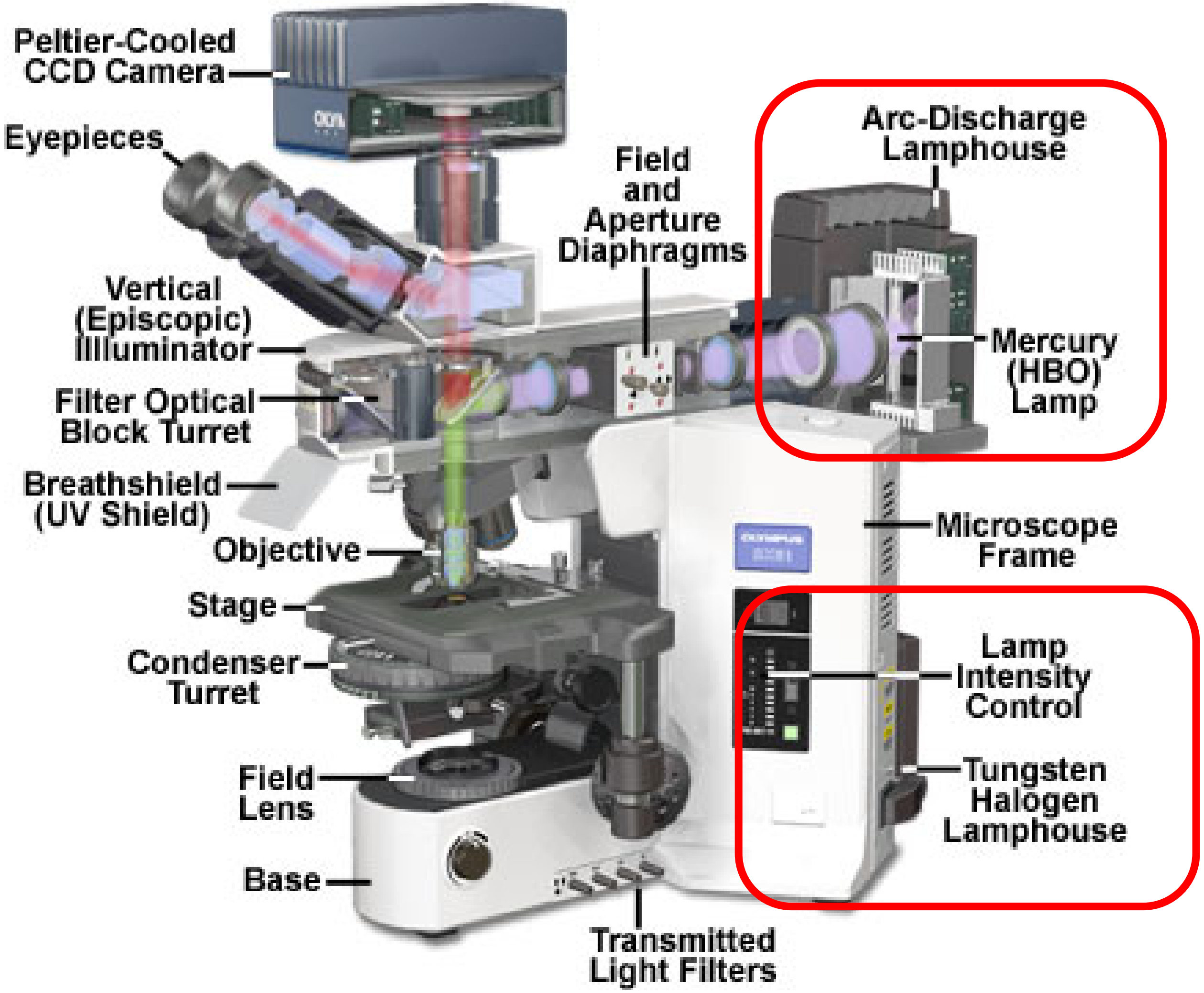
Xe arc lamp



(b)

Ion Arc Lamps in Microscopy

Anatomy of an upright microscope that can operate both in transmission & reflection mode
→ It is suitable for both bright-field & fluorescence microscopy

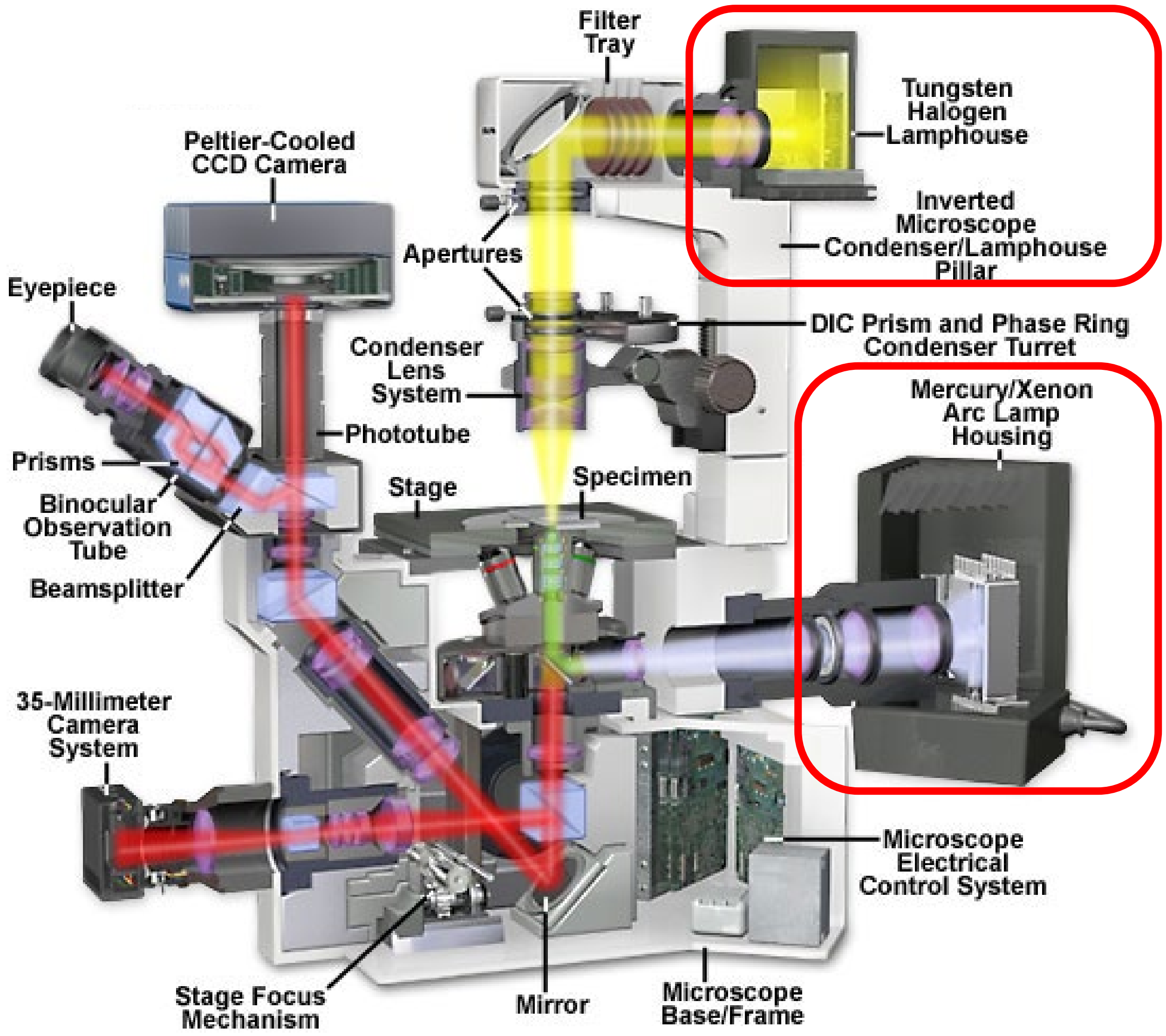


Reflection arm:
with Ion lamp

Transmission arm:
With basic lamp

Ion Arc Lamps in Microscopy

Anatomy of an inverted microscope that can operate both in transmission & reflection mode
→ It is suitable for both bright-field & fluorescence microscopy



Transmission arm:
With basic lamp

Reflection arm:
With Ion arc lamp

Commonly used light sources in microscopy

- Incandescent lamps
  - Quartz tungsten-halogen
- Ion arc lamps
  - Mercury, Xenon
- Metal halides
- Solid-state sources
  - LEDs, lasers

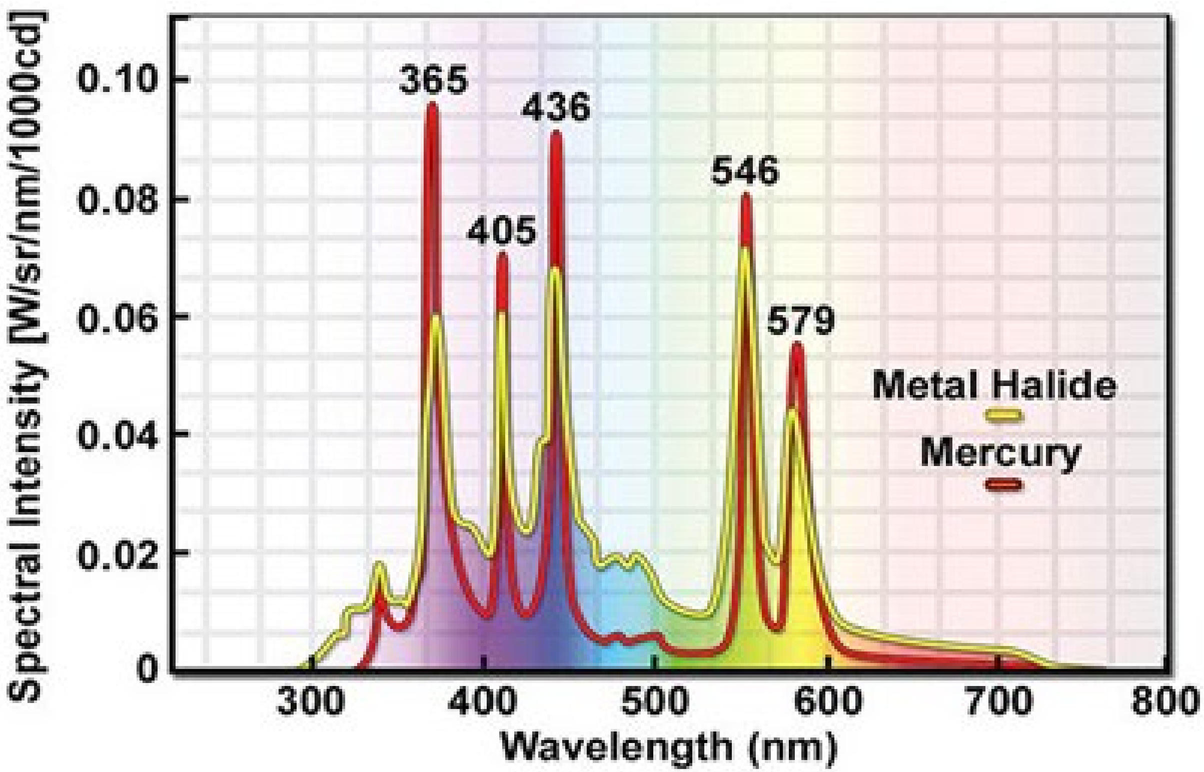
Metal Halide Lamps

Operation:

- A metal-halide lamp produces light by ionizing a mixture of gases in an electric arc.
- The arc tube contains a mixture of mercury, argon or xenon and a variety of metal halides, such as sodium iodide.

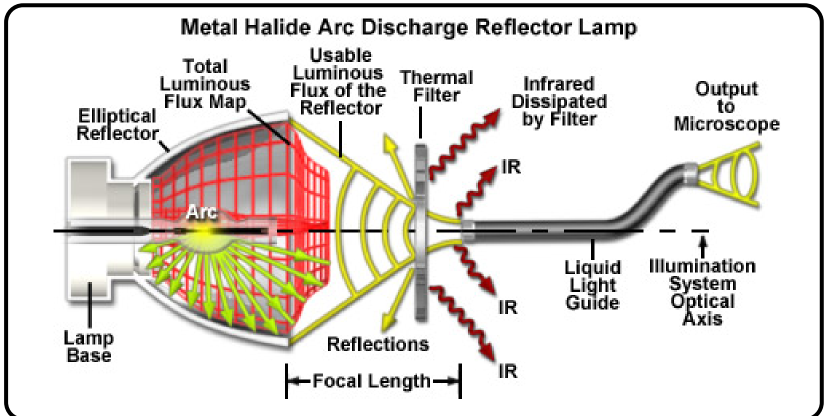
Features:

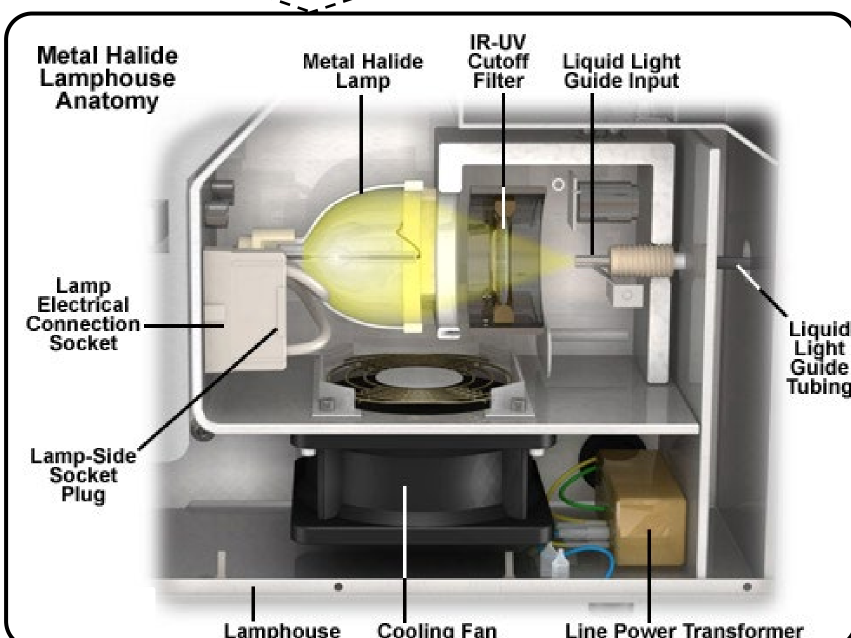
- They produce a spectrum with emission lines very similar to mercury.
- They are **bright,** have **long life (2000 hours)**, and give **homogenous illumination**
- They are more suitable than mercury arc lamp for exciting dyes such as fluorescein and GFP
- But, the bulb & power supply come at **high cost**.



Metal Halide Lamps

- Unlike ion arc lamps, they are **housed in an external unit** that is coupled to microscope using a liquid light guide and a collimating lens that spreads the illumination to fill the objective back aperture.





Commonly used light sources in microscopy

- Incandescent lamps
  Quartz tungsten-halogen
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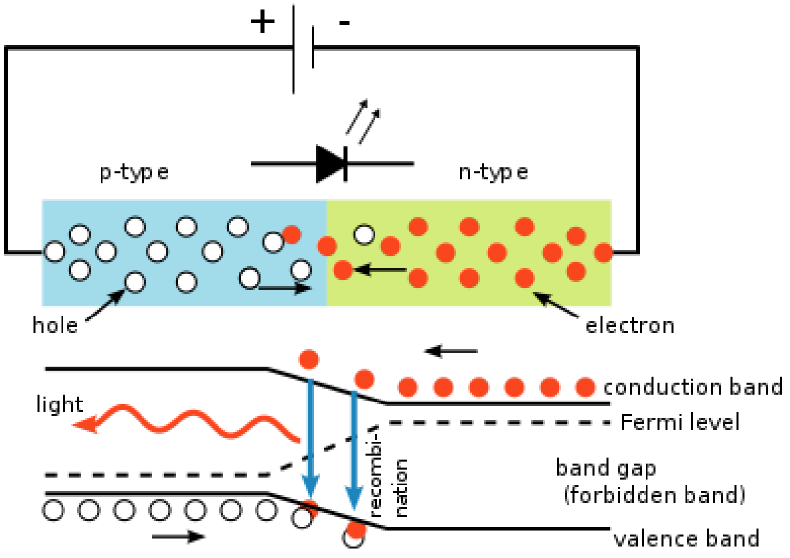
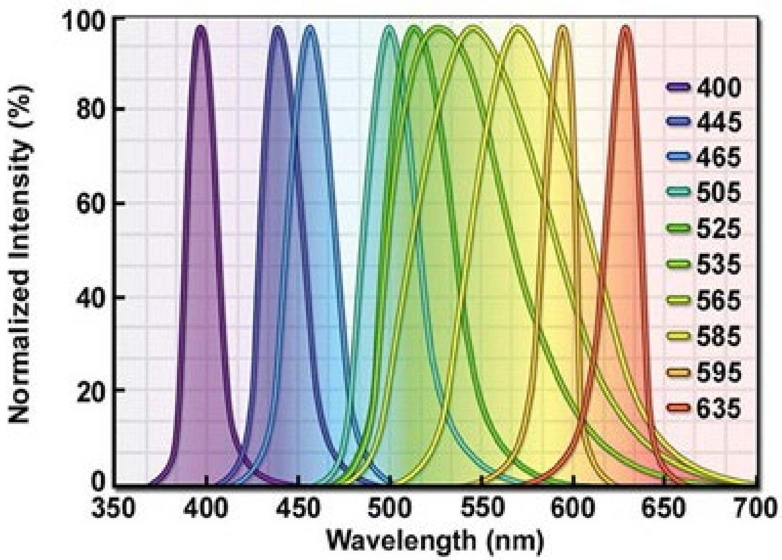
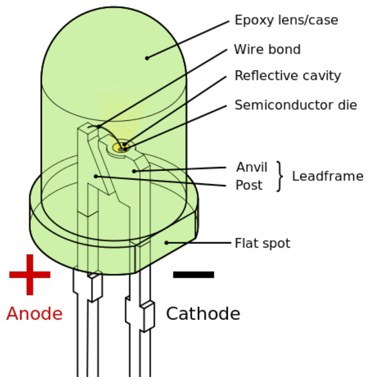
  Mercury, Xenon
- Metal halides
- Solid-state sources
  LEDs, lasers

Light Emitting Diodes (LEDs)

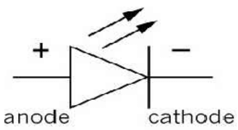
- LED is an **semiconductor p-n junction device**
- Applied electric bias results in current and the **recombination of circulating electrons and holes in the depletion zone** leads to the light generation
- The emitted light has a **relatively narrow 20-50 nm bandwidth**
- **The wavelength (color) of the light emission is dependent on the energy band-gap size (at the p-n junction)**







Circuit symbol of LED



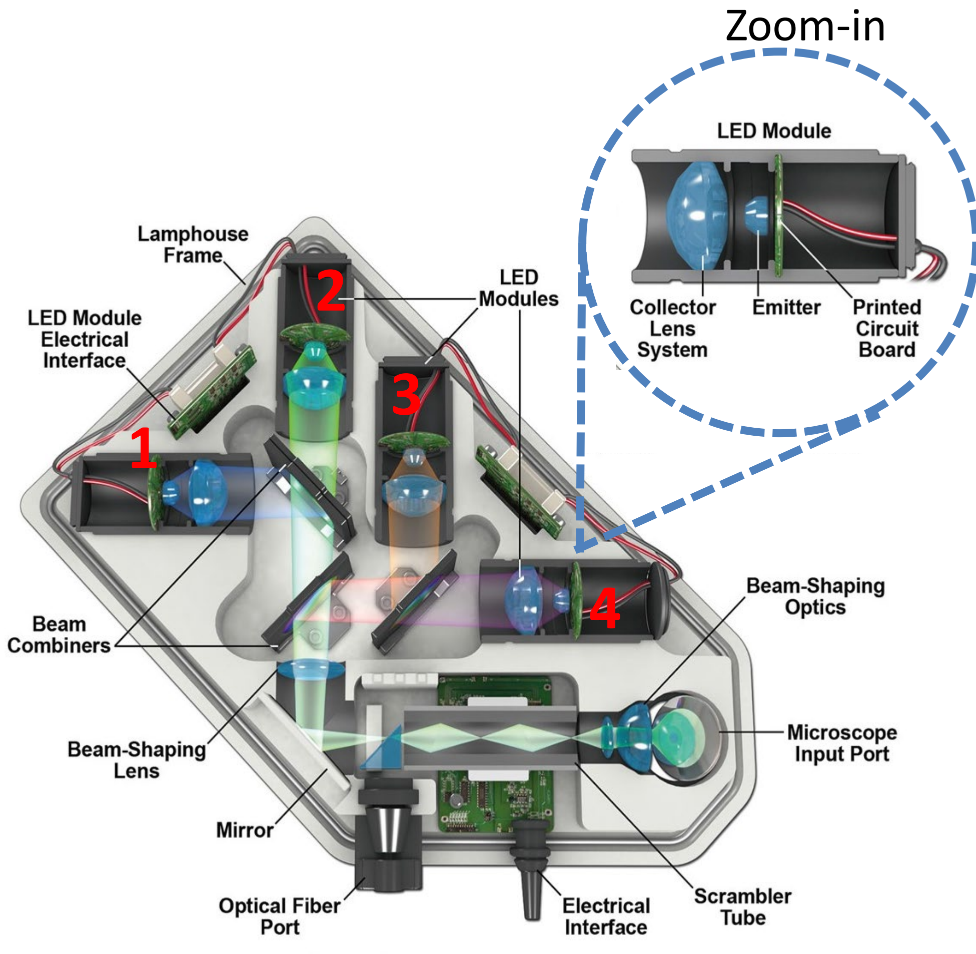
Light Emitting Diodes (LEDs)

LEDs have several unique advantages:

- Spectrum does not contain unwanted UV or IR radiation
- Efficient, cold (no heating & unwanted energy loss) and compact
- Can be turned on or off in millisecond (semiconductor chip-based technology)
- Controlled by inexpensive power supply
- Can last thousands of hours

✓ At 10-50W, LEDs have sufficient power for fluorescence excitation, and also examination of fixed cells, tissues & live cell imaging.

✓ LED sources can provide spectrally tailored UV, visible and near-IR wavelengths for optimum illumination of fluorescence labels.



- Figure on right shows Zeiss Colibri which can hold up to 4 swappable LEDs
- Three dichroic mirrors allow new wavelength addition within a few minutes.

LED illuminator with six LED modules and folded optical pathway

LASER as a light source for fluorescence microscopy

LASER stands for Light **A**mplification by **S**timulated **E**mission of **R**adiation

Properties of light from lasers:

- High intensity
- Uniform wavelength, phase, polarity
- Can be tightly focused

Laser types:

- Gas lasers
- Dye lasers
- Free-electron lasers
- Fiber lasers
- Solid-state lasers
- Semiconductor lasers

- Continous wave operation
- Pulsed operation
- Ultrafast Lasers (mode-locking & Q-switching)

LASERs as a light source for fluorescence microscopy

We can have a broad spectral coverage with different types of lasers:

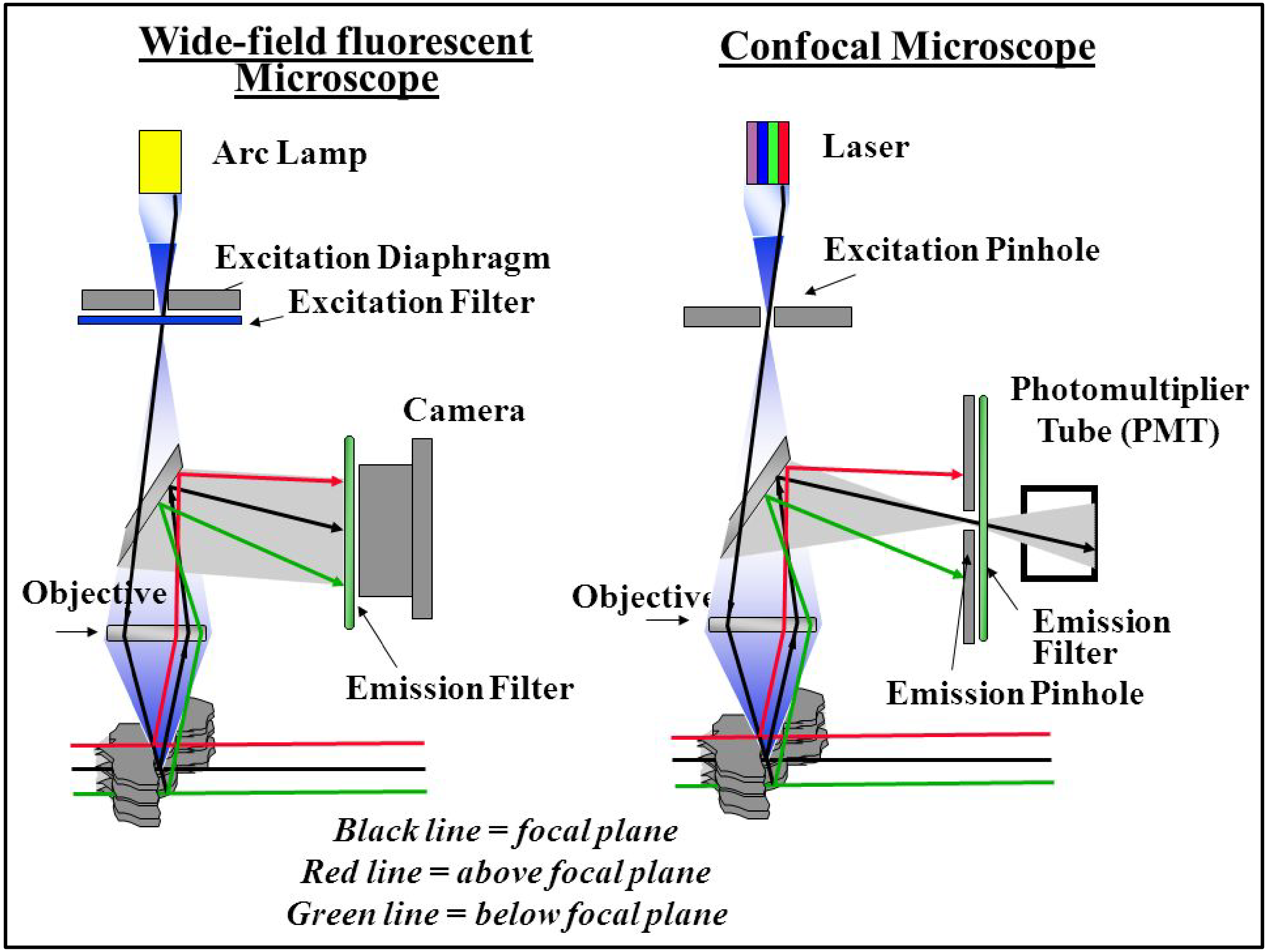
<u>Laser types</u>

UV  **IR**

Argon	**351 364 457 477 488 514**
Blue diode	**405 440**
Helium-Cadmium	**354 442**
Krypton-Argon	**488 569 647**
Green Helium-Neon	**543**
Yellow Helium-Neon	**594**
Orange Helium-Neon	**612**
Red Helium-Neon	**633**
Red diode	**635 650**
Ti:Sapphire	**720-980**

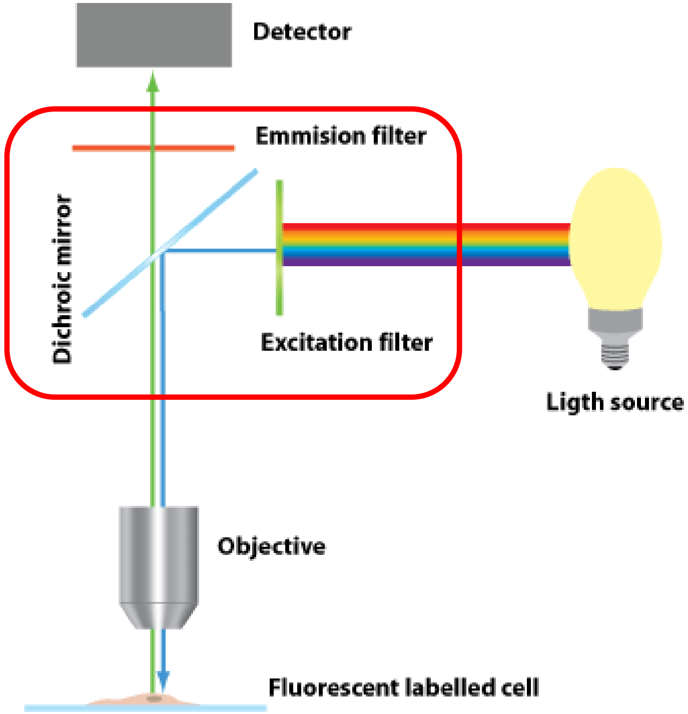
LASER as a light source for fluorescence microscopy

LASERS are especially used in high-end fluorescence microscopy techniques such as confocal, FRAP, FRET, FLIM, super-resolution etc.



Fluorescence Microscopy

- To understand fluorescence microscopy we need to be familiar with:
  - Basic principles of fluorescence
  - Properties of fluorescent dyes
  - Different kinds of fluorescence markers
  - **Important optical components**
    - Illumination sources
    - **Filters and filter sets**
    - Detectors
    - Their proper positioning in the optical train of the microscope



Filters in Microscopy

- Properties of the incident light on the sample are important for microscopy.
  - The power (intensity) of the incident light.
    Example: high power can **photo-damage** delicate live cells
  - The color (energy) of photons.
    Example: **UV photons** cells can kill live cells

- Properties of the incident light depend on the used illumination source.
- In practice, the color and the intensity of the light on the sample are controlled by external filters that are attached to the microscope (… in the optical train, filters are after the illumination source).

To properly use the filters in microscopy, it is crucial to understand their properties:

- Interpreting their spectra
- Selecting the best filter combination/set

➔ *This is particularly important for fluorescence microscopy because the spectra of the filters must MATCH to the excitation & emission spectra of the fluorescent markers.*

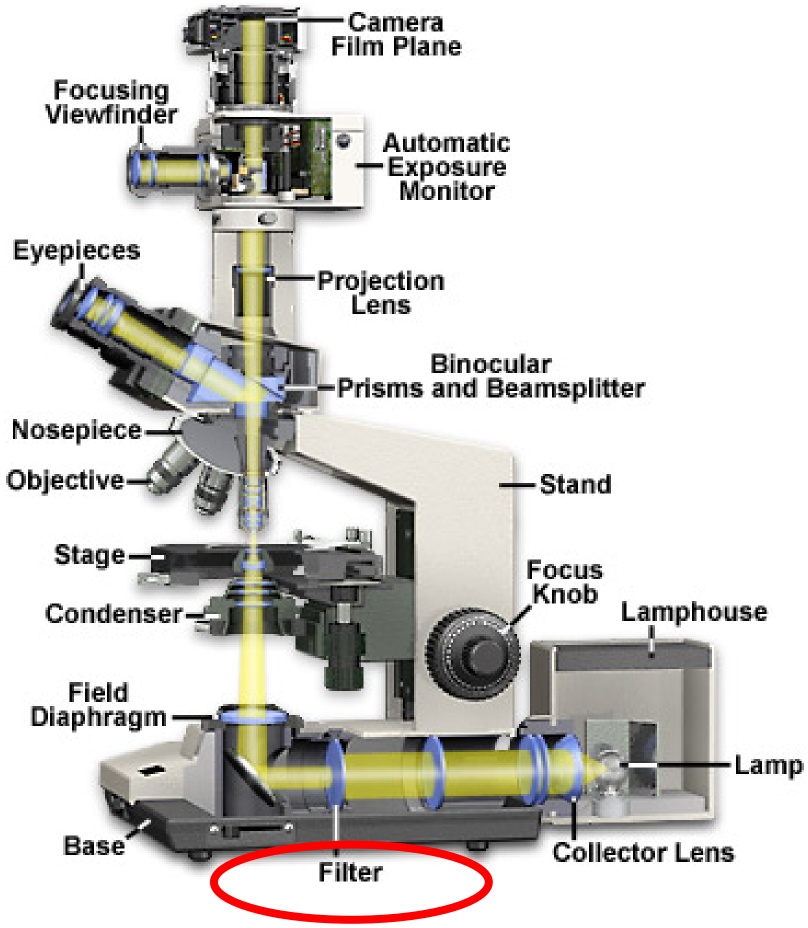
<u>Two main filter types are commonly used in microscopy:</u>

➔ **1) Neutral density filters**

2) Spectral filters

Neutral Density (ND) Filters in Microscopy

- **ND attenuates uniformly the light intensity over the entire visible spectrum**
- They are especially useful with high intensity sources (i.e. arc lamps) that cannot be regulated with an adjustable power supply.



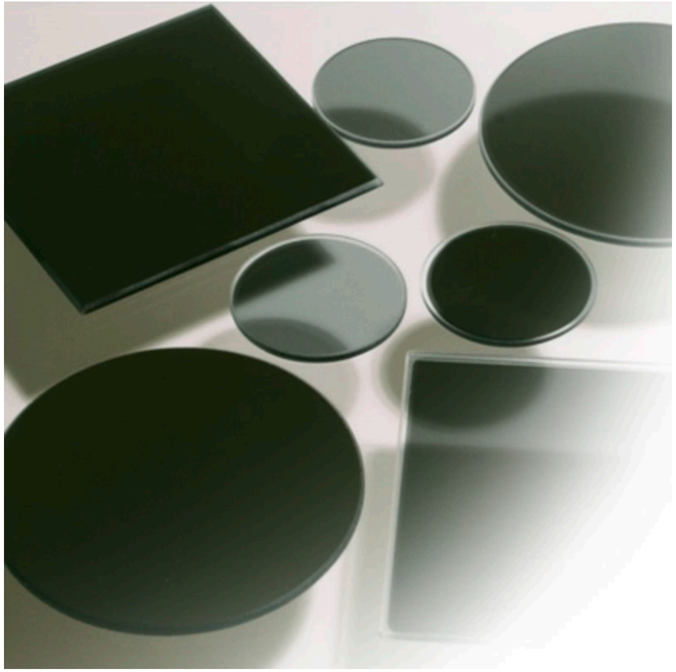
ND filters can be either absorbing or reflecting

Reflective NDs:

- Contain an evaporated metal coating on one of the surfaces
- Care must be taken not to scratch their surface
- Coated side must face to the light source

Absorbing NDs:

- Contain rare earth elements
- Less sensitive to scratches & their orientation is not critical
- More expensive & thicker than reflective NDs



Optical Density (OD) of ND Filters

ND filters are calibrated in units of absorbance or optical density (OD) where:

$$OD = log_{10}^{1/T}$$

Here, T is the transmittance: $T = \frac{I_{transmitted}}{I_{incident}}$

OD 0.3 OD 0.6 OD 0.9 OD 1.3 OD 3,0

Example 1: An ND filter with 0.1 OD gives ~79% transmission & 21% blockage of the incident light

Example 2: When stacking multiple filters, total OD of the stack is equal to the SUM of individual NDs

ND Filters are Used in Microscopy & Photography

Filters can significantly affect image quality:

- not only useful in microscopy but also in photography

Filters in Microscopy

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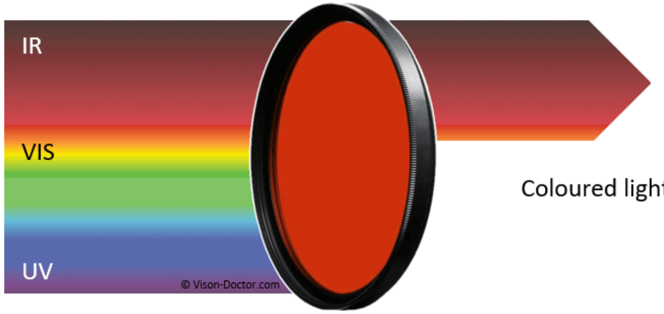
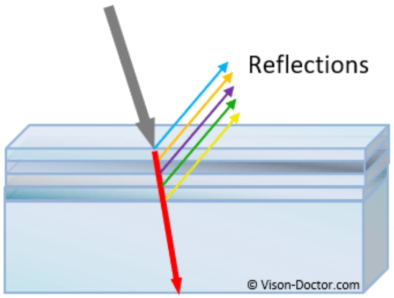
Spectral Filters in Microscopy

Spectral filters can isolate specific colors or color (wavelength) bands



Colored-Glass Filters:

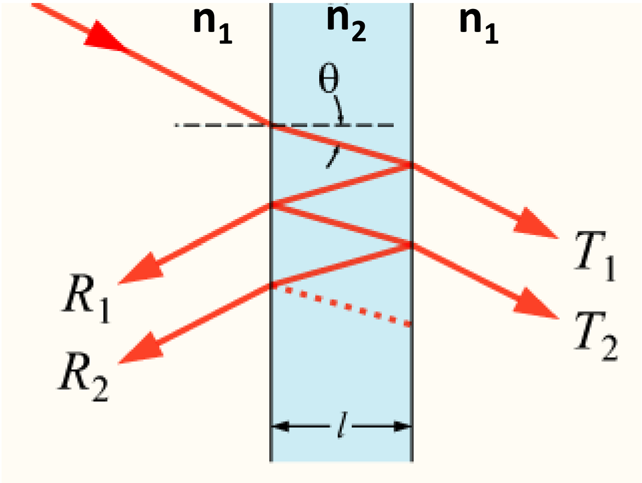
- Contain rare earth transition elements, which absorb non-transmitted wavelengths.
- Suitable for applications that don't require precise definition of transmitted wavelength.
- They are commonly used in wide-field microscopy to remove unnecessary UV or IR photons or give color to the images.
- As they are based on "absorption" they can get heated up & subject to alteration and even damage after prolonged use.

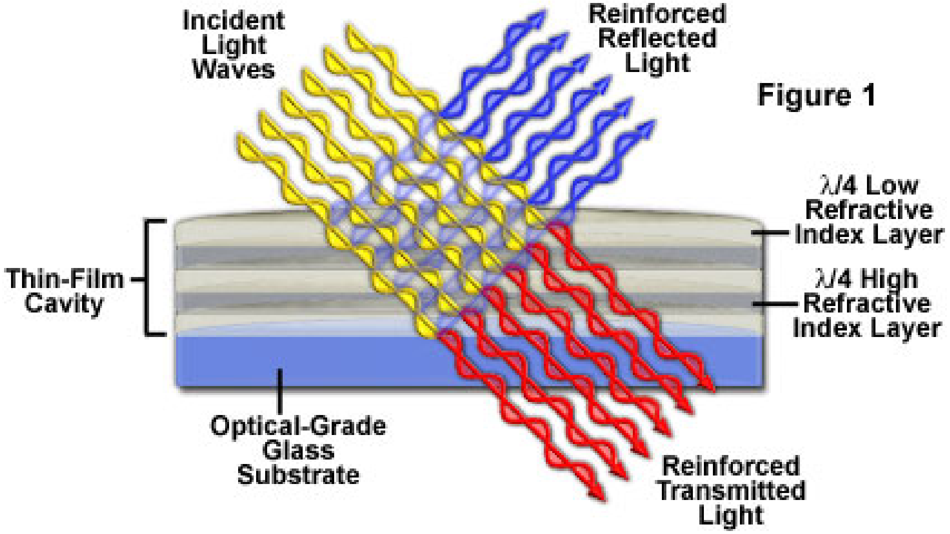


Interference Filters:

- Based on interference effects from optically transparent multi-layer dielectric stacks.
- Offer steeper transmission boundaries & cut-on & cut-off wavelengths → They offer precise definition of the transmitted spectrum.
- Frequently used in fluorescence microscopy.

Interference Filters

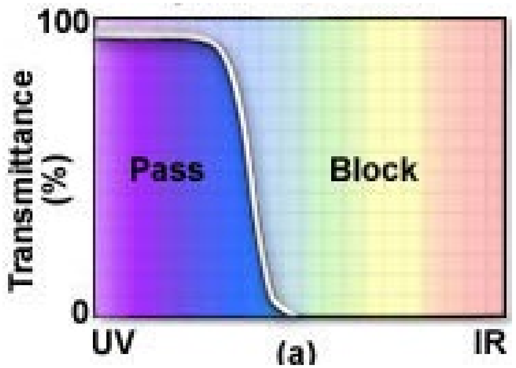




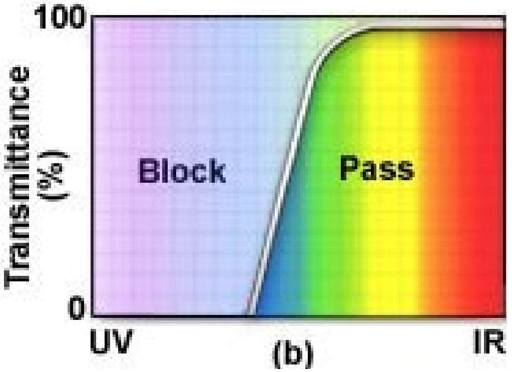
- The interface between two materials of different refractive index (n_i) partially reflects incident light backward (R_i) & forward (T_i).
- Interference filters operate by selectively reinforcing and blocking the transmission/reflection of specific wavelengths through constructive & destructive interference.
- Total transmission and transmitted/reflected wavelengths depend on the thickness, refractive index and the design (stacking) of the layers.

Spectral Filters in Microscopy

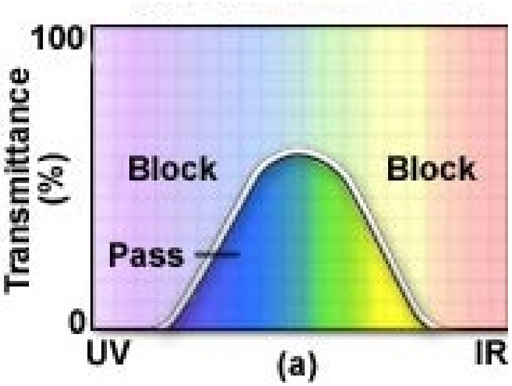
Spectral filters **isolate specific colors or bands of wavelengths.**



1) Short Pass Filters:
Transmits short wavelengths and block long ones



2) Long Pass Filters:
Transmits long wavelengths and block short ones

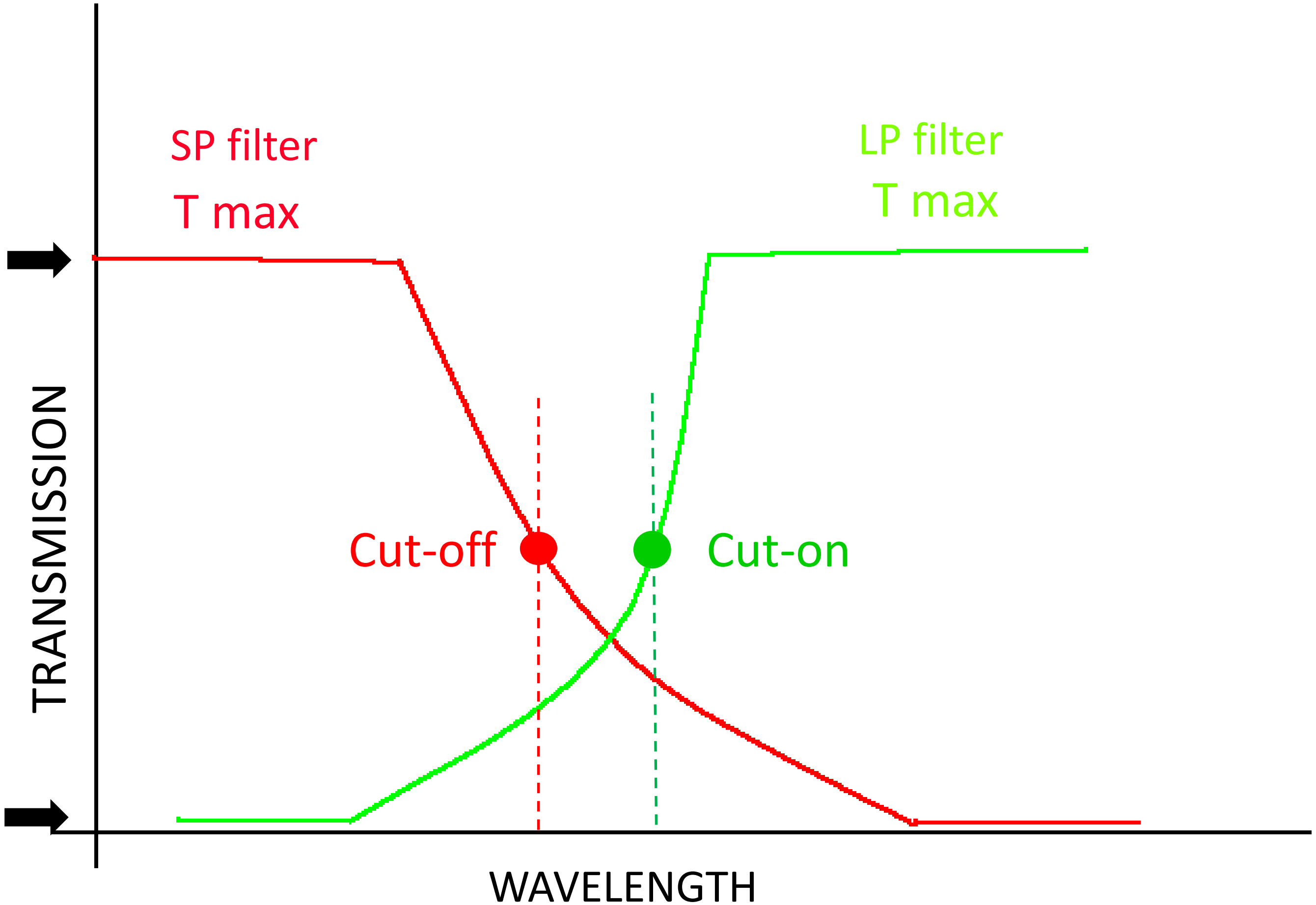


3) Band-Pass Filters:
Transmits a band of wavelength while blocking the wavelengths above and below the specified range of transmission.

Short-Pass (SP) Filter & Long-Pass (LP) Filter

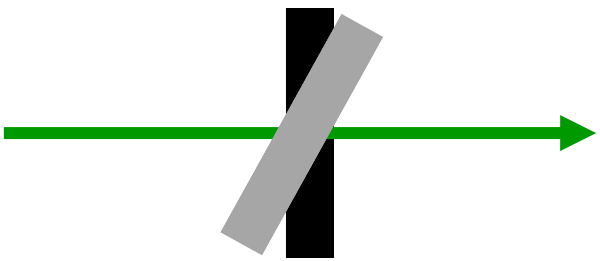
- Optical performance is defined in terms of
  - **Efficiency of transmission and blockage** (i.e. in terms of % of transmission, T_{max})
  - **Steepness of the cut on/off boundary** between the adjacent transmitted and blocked domains.

→ Here, cut on/off wavelength is defined by 50% of peak transmission.



Example Spectrum for Short-Pass Filters

Specs and curves of SP650 from Edmund Optics



Type	**Short-pass Filter**
Wavelength Range (nm)	400 - 850
Transmission Wavelength (nm)	400 - 630
Transmission (%)	>85
Cut-Off Wavelength (nm)	650
Rejection Wavelength (nm)	675 - 850
Optical Density OD	≥2.0
Angle of Incidence (°)	0