# MICRO-561

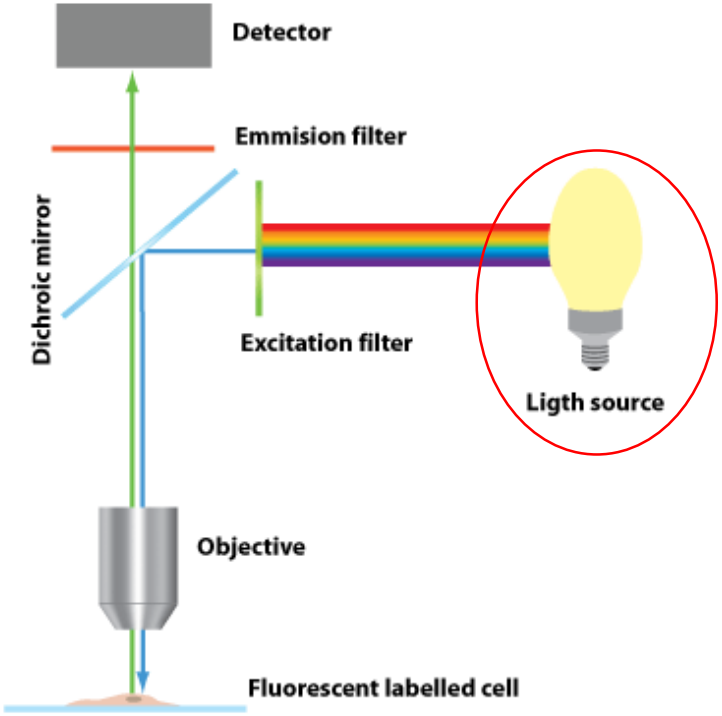
Biomicroscopy I

# 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 | Resolution-4, Contrast-1 |
| Lecture 10 | Contrast-2, Fluorescence-1 |
| Lecture 11 | Fluorescence-2, Sources -1 |
| Lecture 12 | Sources-2 & 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

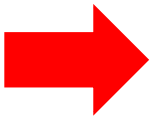
- To obtain optimal imaging performance, the specimen must be properly illuminated.
- This requires:
  - proper selection of the wavelength and the intensity of the illumination source
  - correct alignment & focusing of the source (i.e. recall Kohler illumination)

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

- Incandescent lamps
  Quartz tungsten-halogen

- Ion arc lamps
  Mercury, Xenon

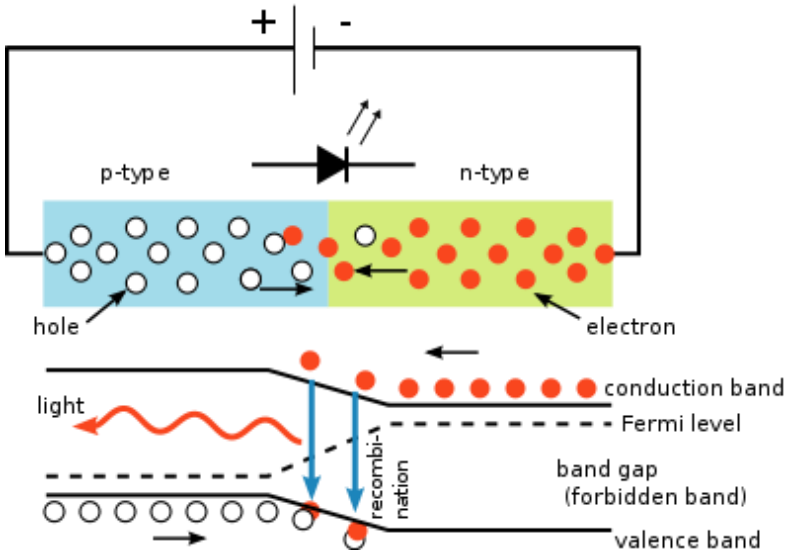
- Metal halides

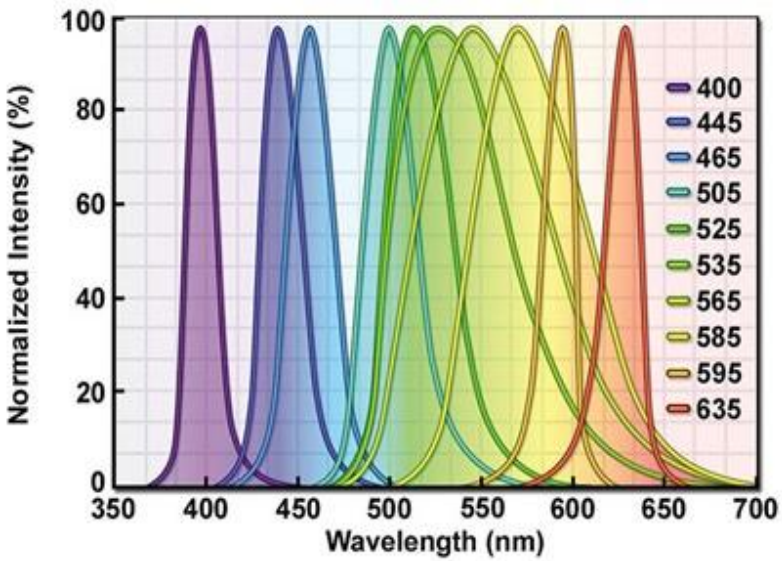


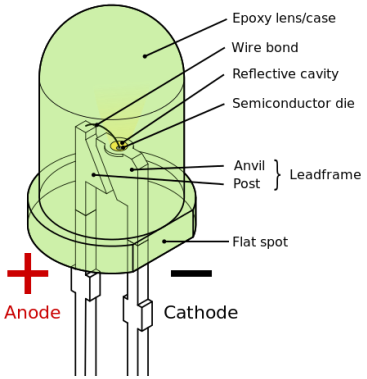
- Solid-state sources:
  LEDs & Lasers

# Light Emitting Diodes (LEDs)

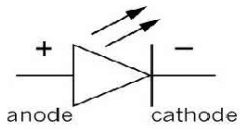
- LED is an **semiconductor p-n junction device**
- Applied electric bias results in current and the **recombination of circulating electrons and holes in the depletion zone** leads to the light generation
- The emitted light has a **relatively narrow 20-50 nm bandwidth**
- **The wavelength (color) of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




Circuit symbol of LED




# Light Emitting Diodes (LEDs)

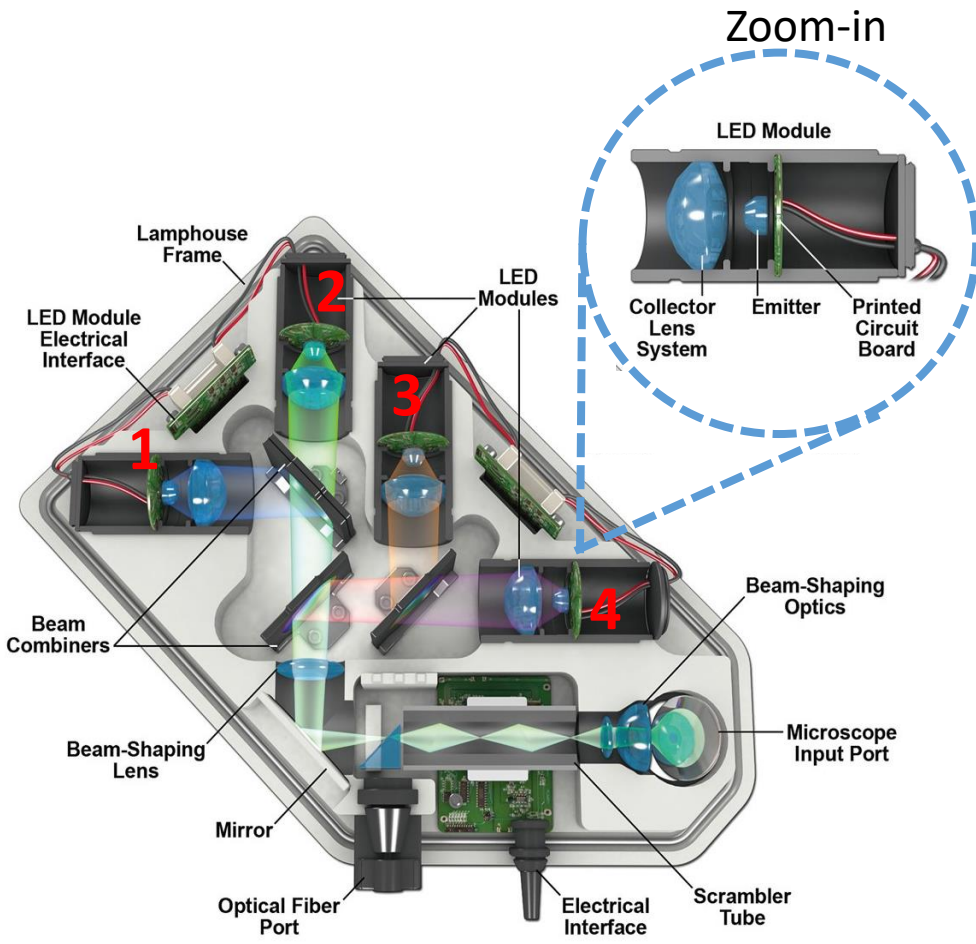
LEDs have several unique advantages:

- 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 L**ight **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

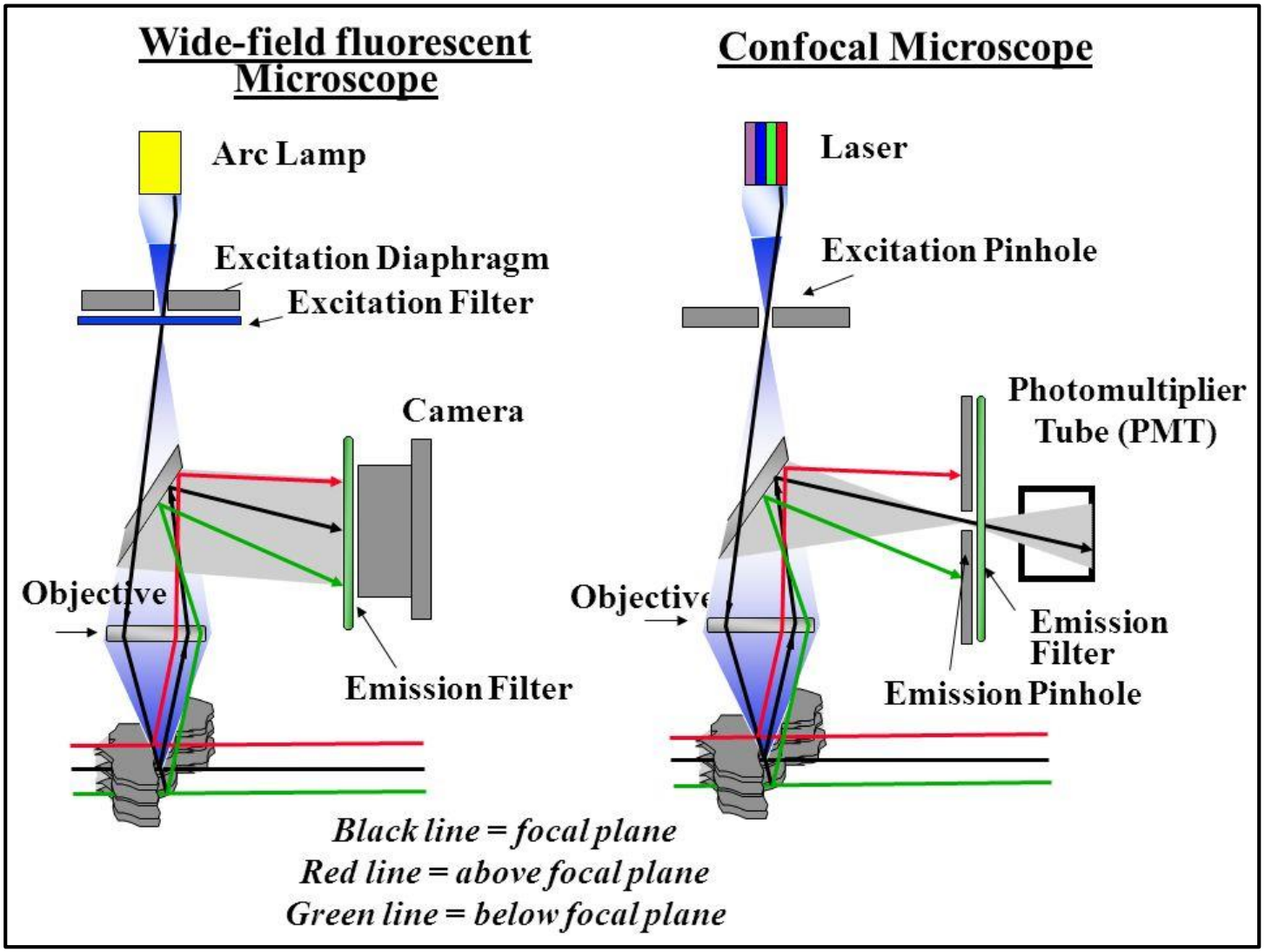
- Continous wave operation
- Pulsed operation
- Ultrafast Lasers (mode-locking & Q-switching)

# **LASERs** as a light source for fluorescence microscopy

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

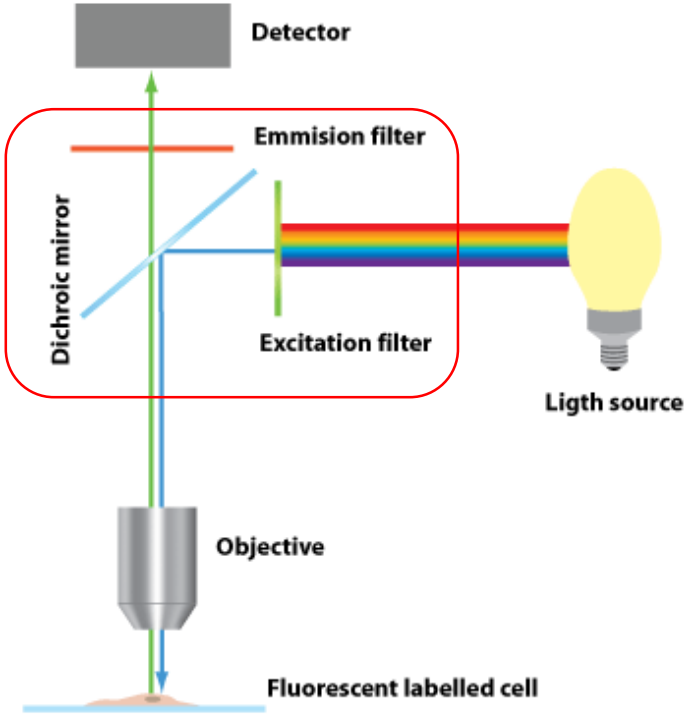
# **LASER** as a light source for fluorescence microscopy

**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.*

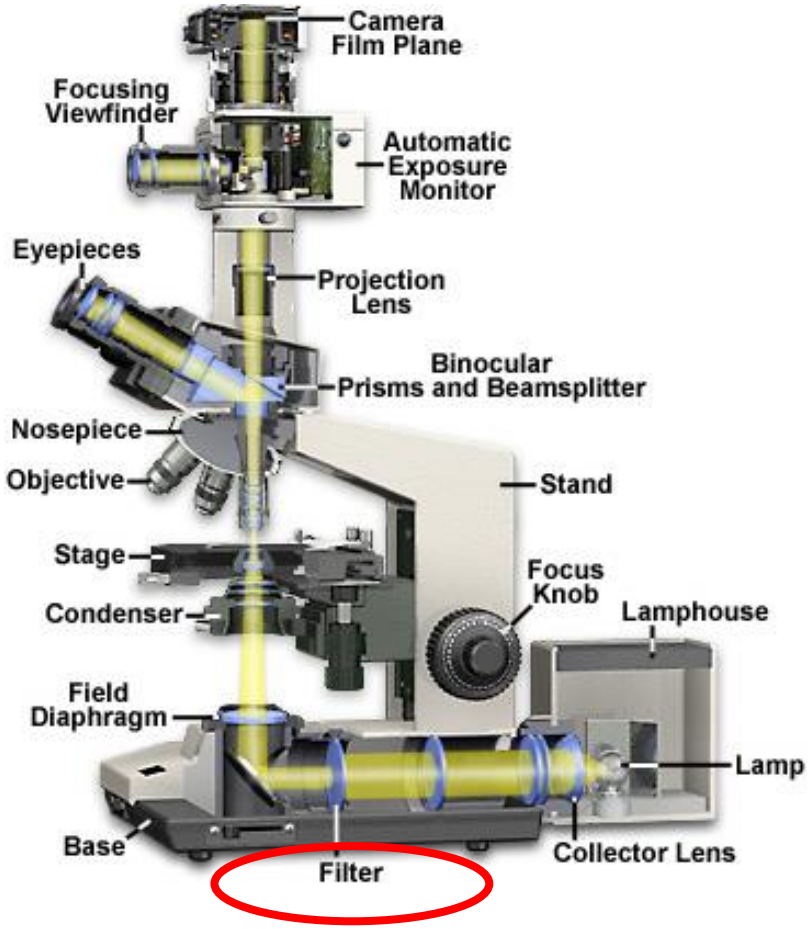
**FILTER TYPES:**

**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.



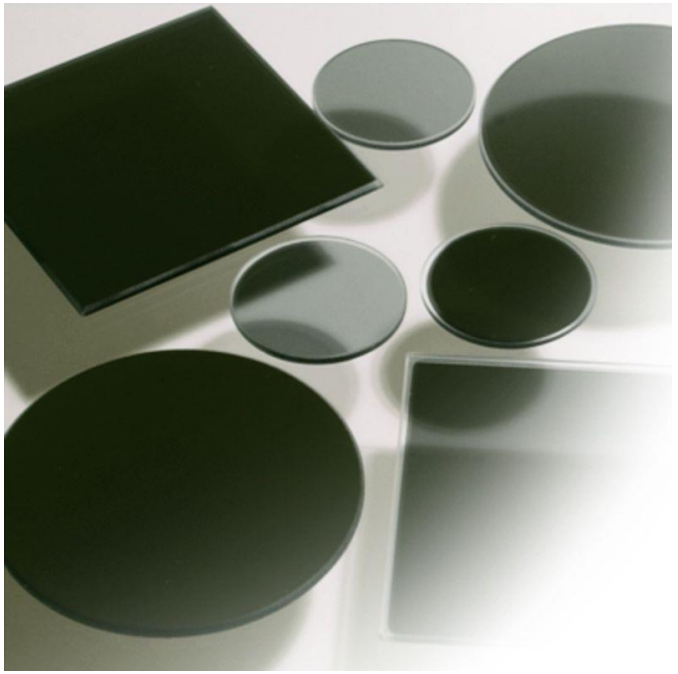
# ND filters can be either absorbing or reflecting

**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

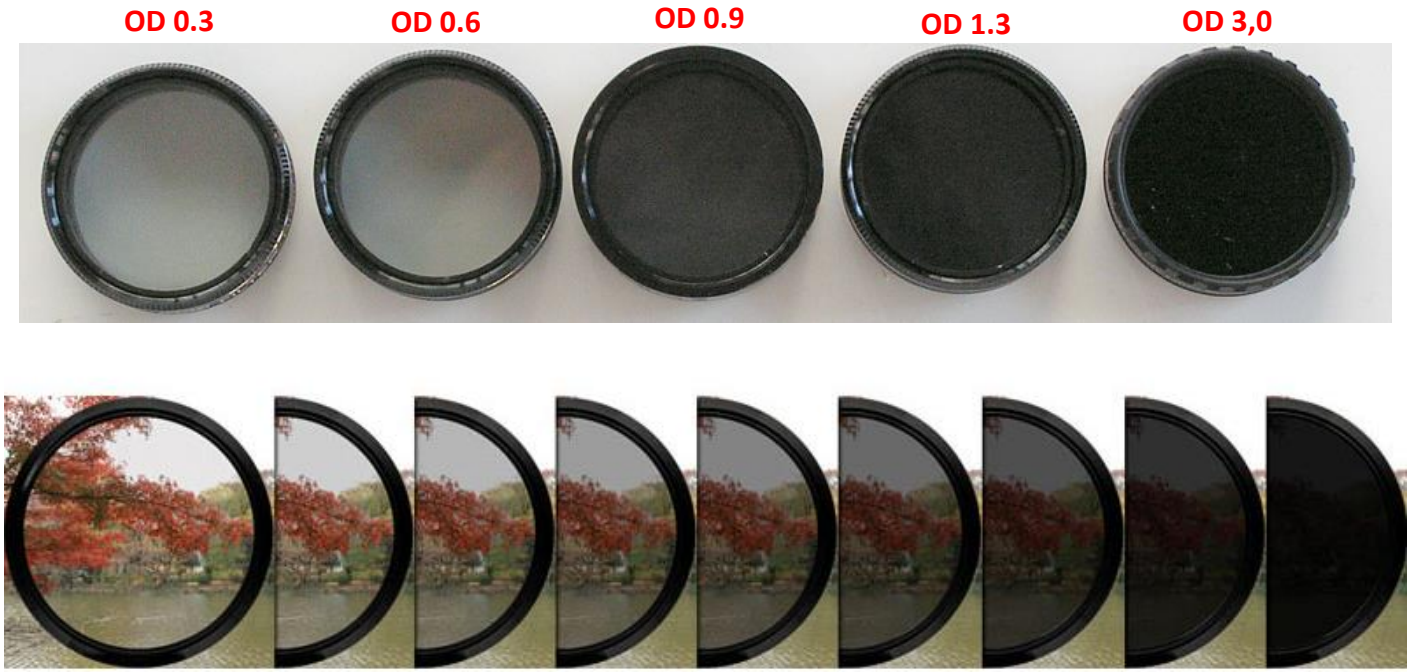


# Optical Density (OD) of ND Filters

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

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

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




**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

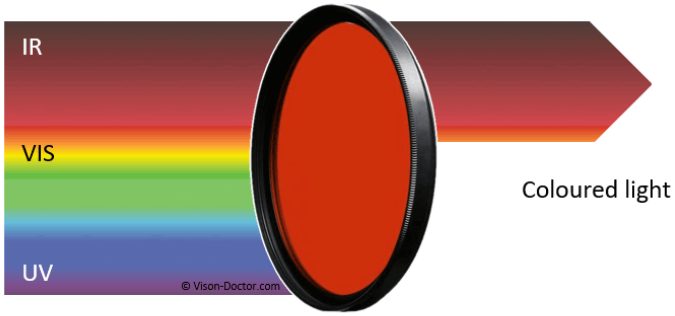
# ND Filters are Used in Microscopy & Photography


Filters can significantly affect image quality:

- not only useful in microscopy but also in photography

# Spectral Filters in Microscopy

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

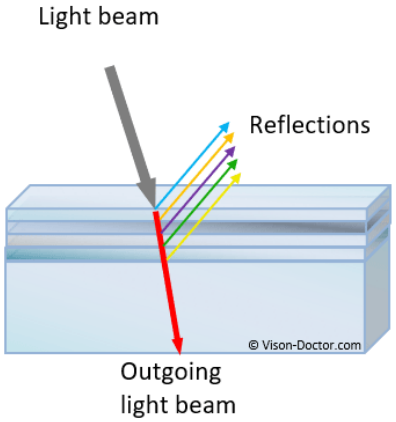



**Colored-Glass Filters:**

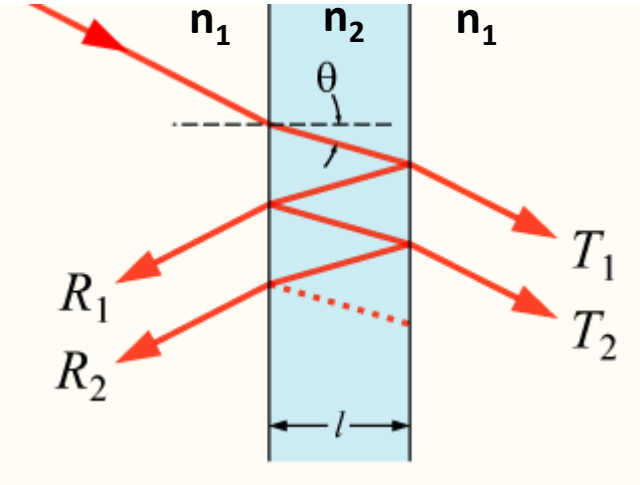
- Contain rare earth transition elements, which absorb non-transmitted wavelengths.
- Suitable for applications that don't require precise definition of transmitted wavelength.
- They are commonly used in wide-field microscopy to remove unnecessary UV or IR photons or give color to the images.
- As they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

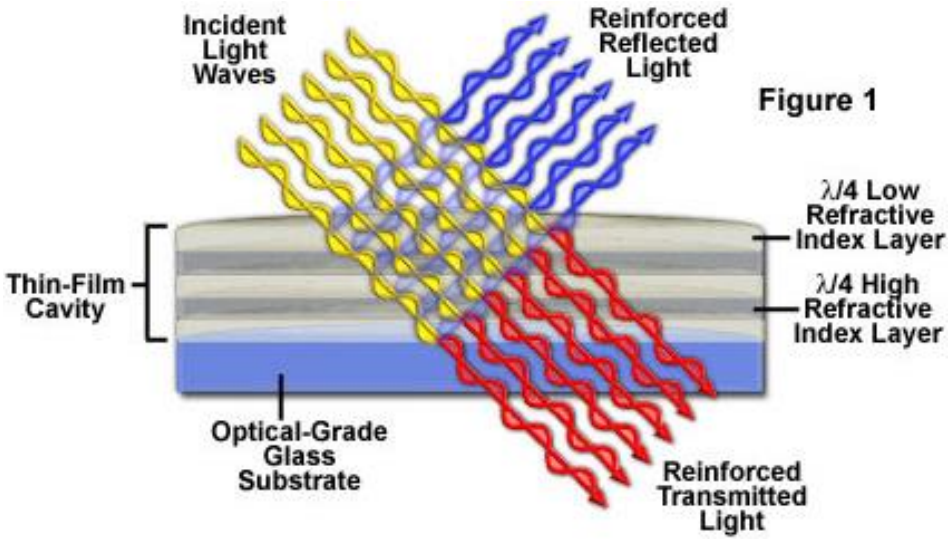
**Interference Filters:**

- 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

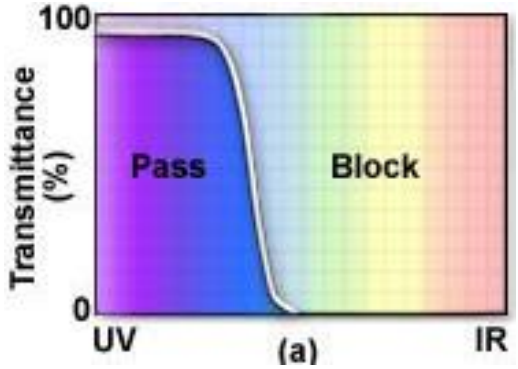


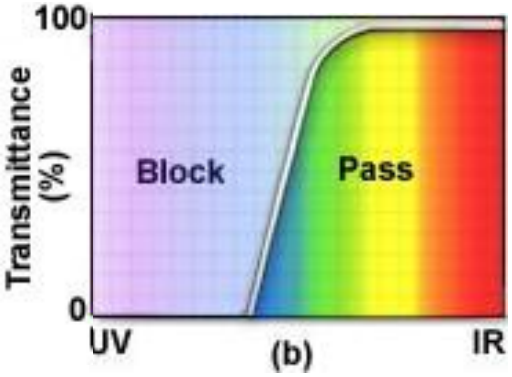

- The interface between two materials of different refractive index ($n_i$) partially reflects incident light backward ($R_i$) & forward ($T_i$).
- Interference filters operate by selectively reinforcing and blocking the transmission/reflection of specific wavelengths through constructive & destructive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

# Spectral Filters in Microscopy

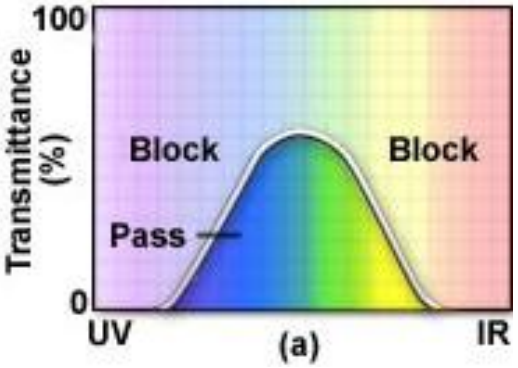
Spectral filters **isolate specific colors or bands of wavelengths.**




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




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

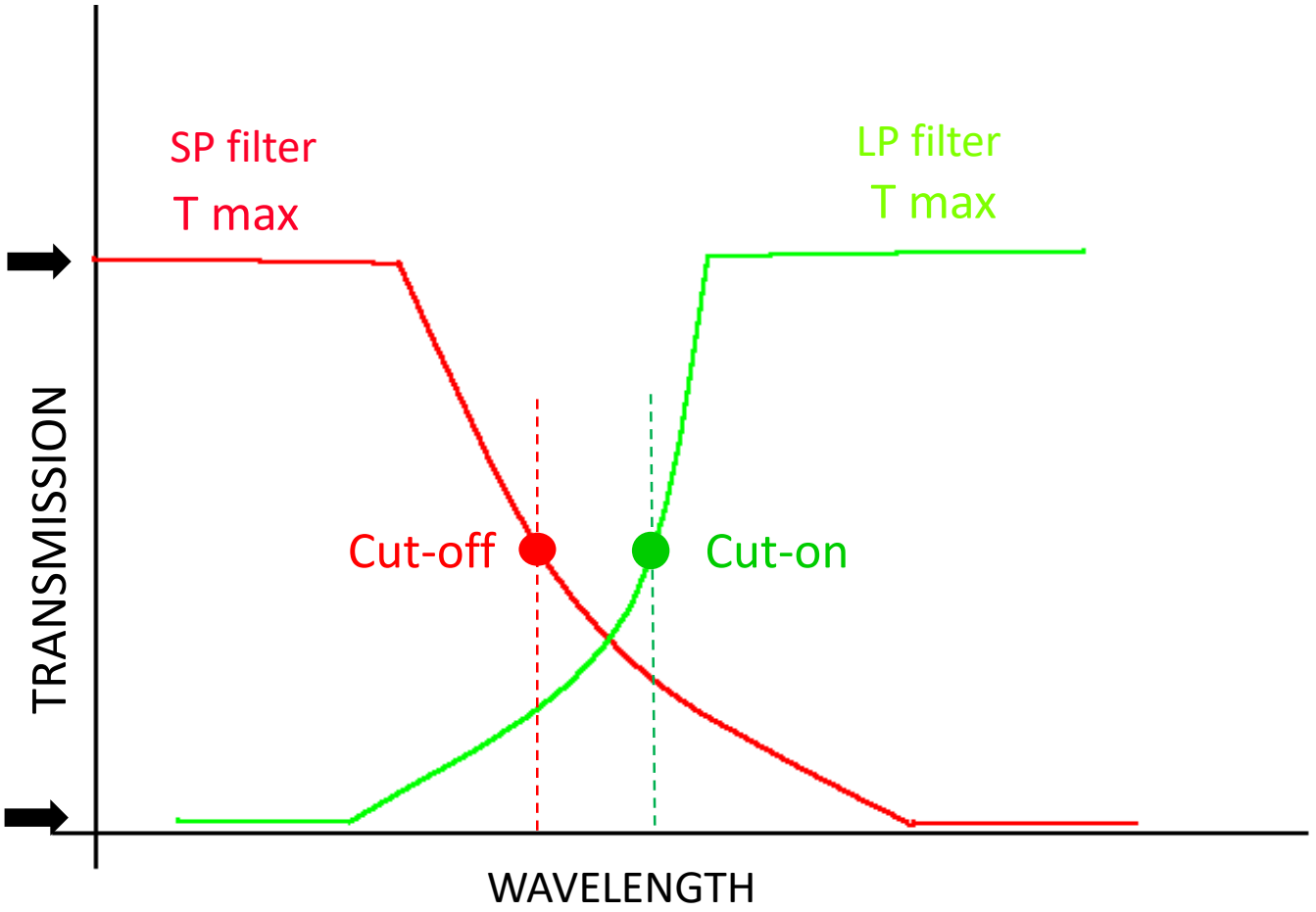



**3) Band-Pass Filters:**
Transmits a band of wavelength while blocking the wavelengths above and below the specified range of transmission.

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

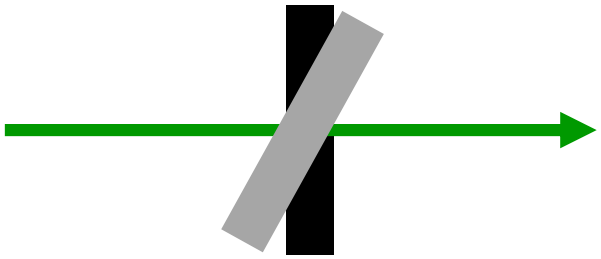
- Optical performance is defined in terms of
  - **Efficiency of transmission and blockage** (i.e. in terms of % of transmission, $T_{max}$)
  - **Steepness of the cut on/off boundary** between the adjacent transmitted and blocked domains.

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

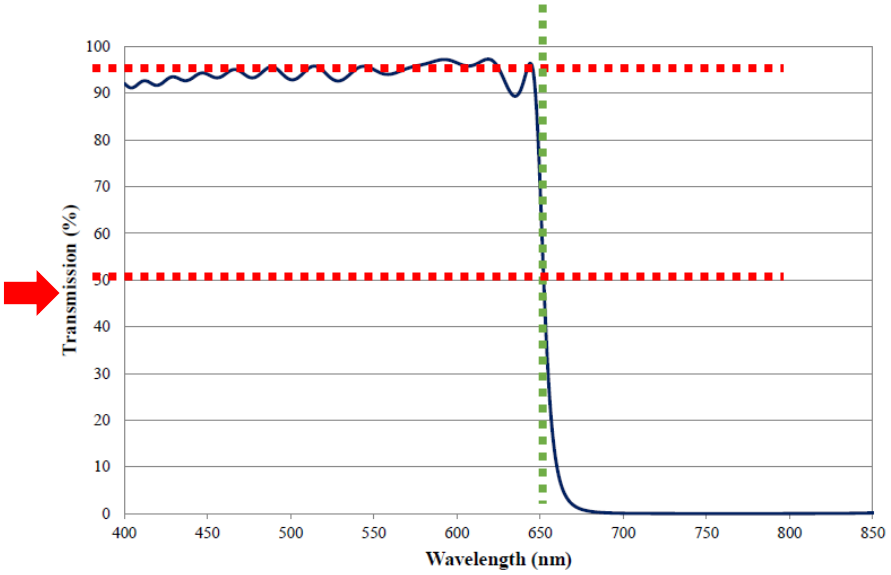


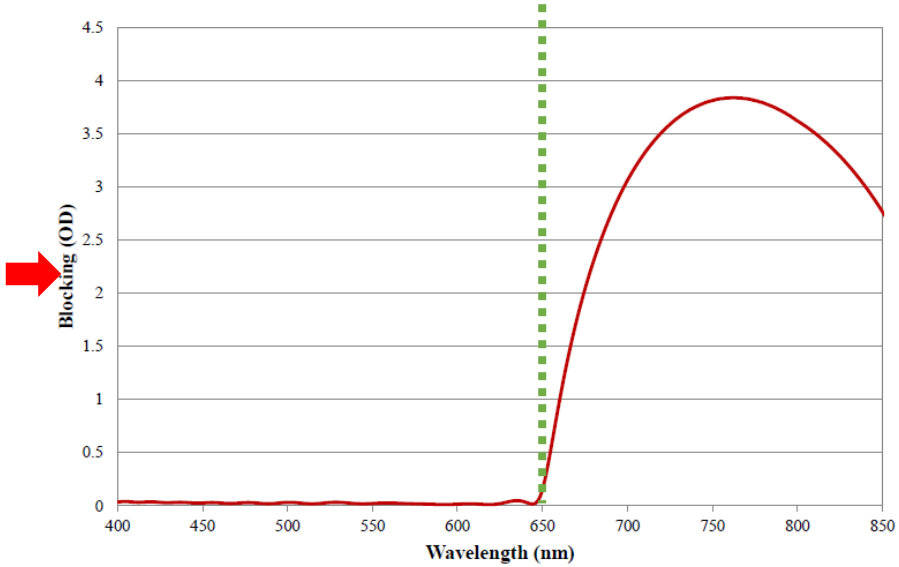
# Example Spectrum for Short-Pass Filters

**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 |

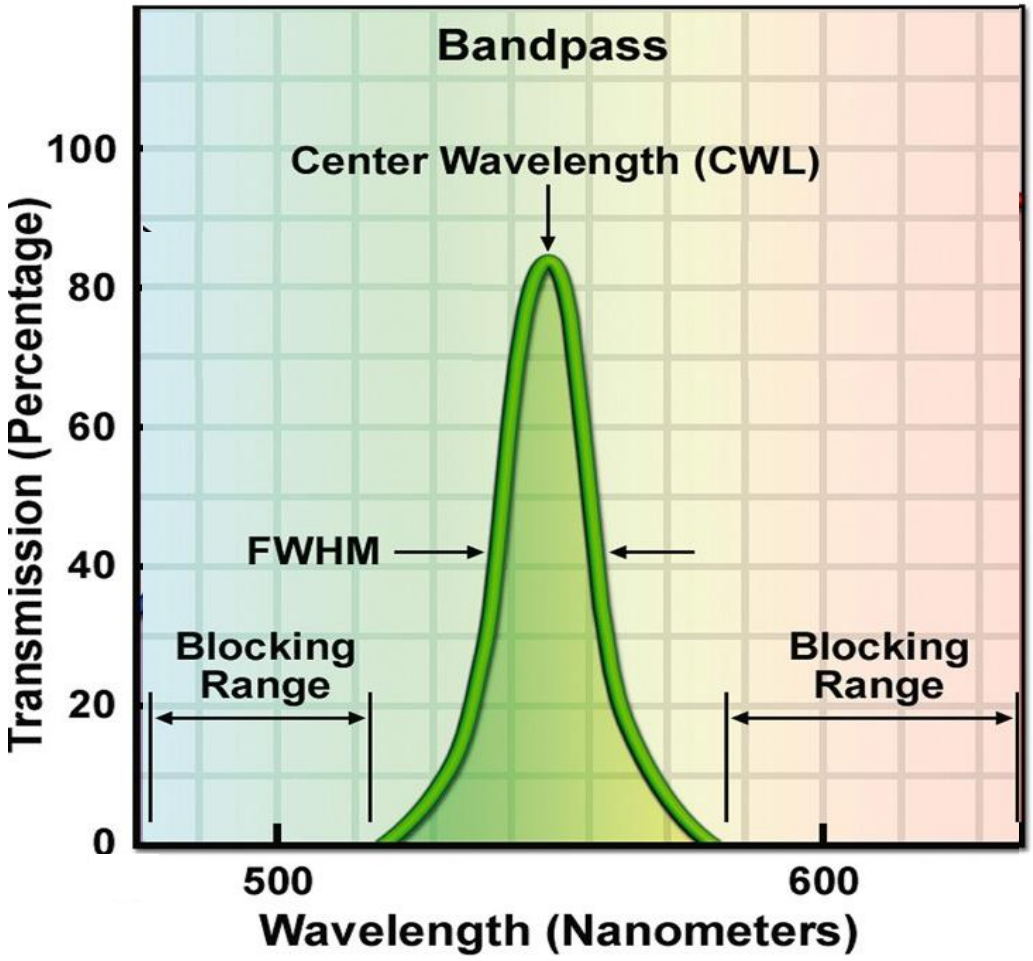


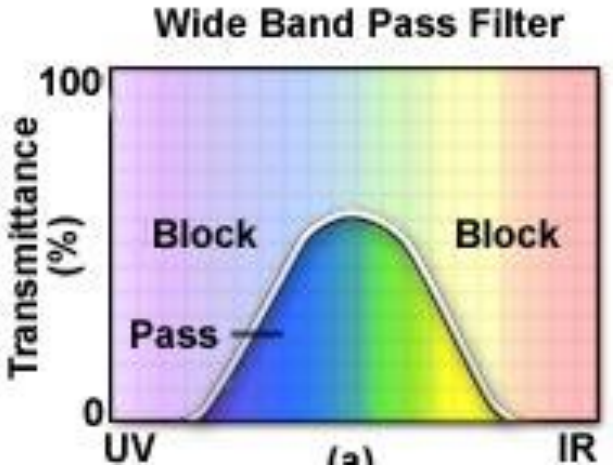



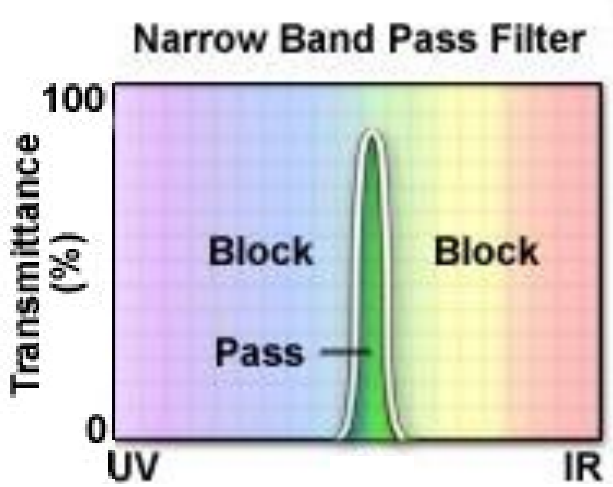
# Band-Pass (BP) Filters

**BP filters are described by referring to the:**

- Peak center wavelength (CWL)
- Full-width-half-maximum (FWHM), which is the range between the edges of the bandpass peak where the transmission is 50% of its maximum value



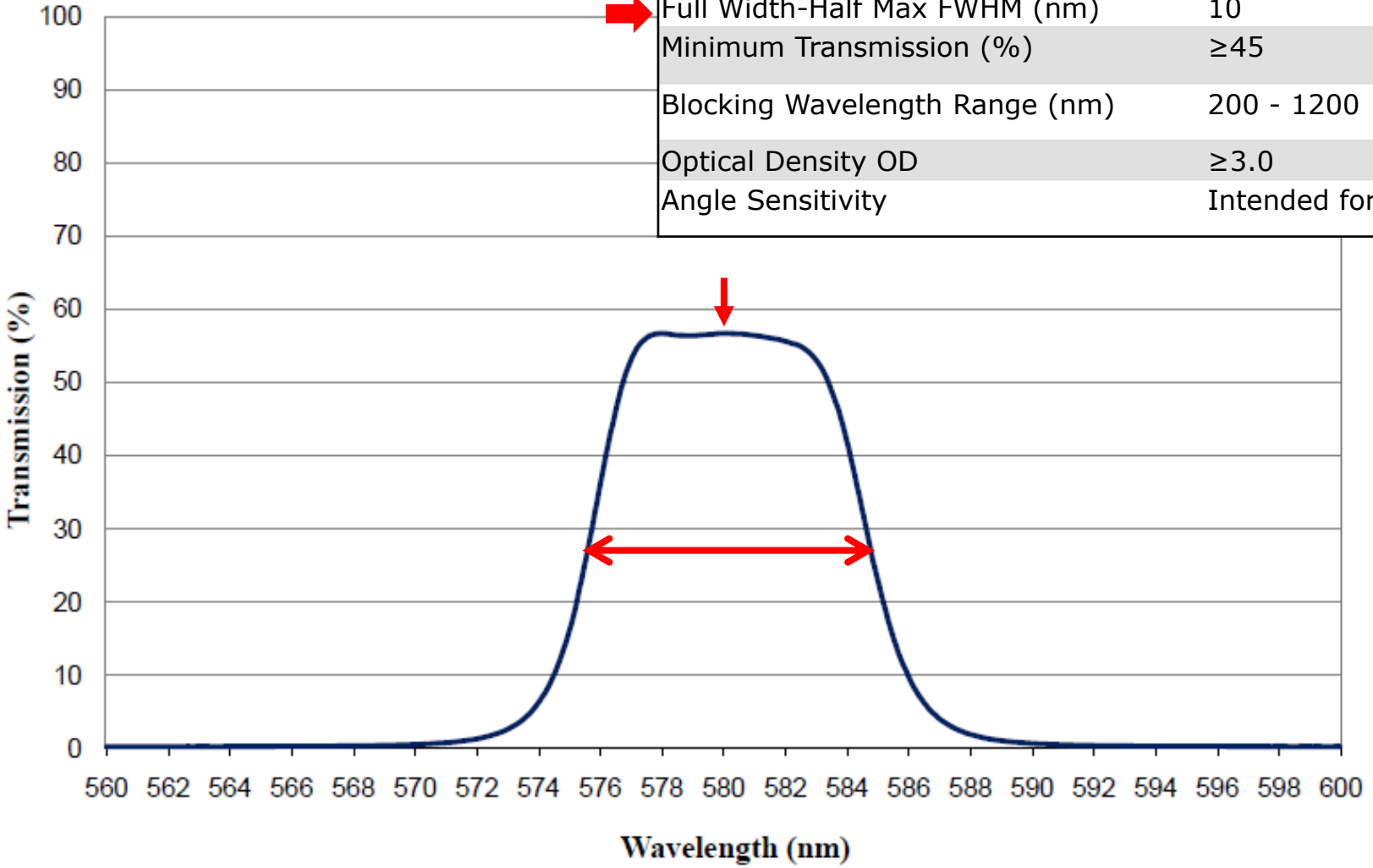



# Example Spectrum for BP Filter

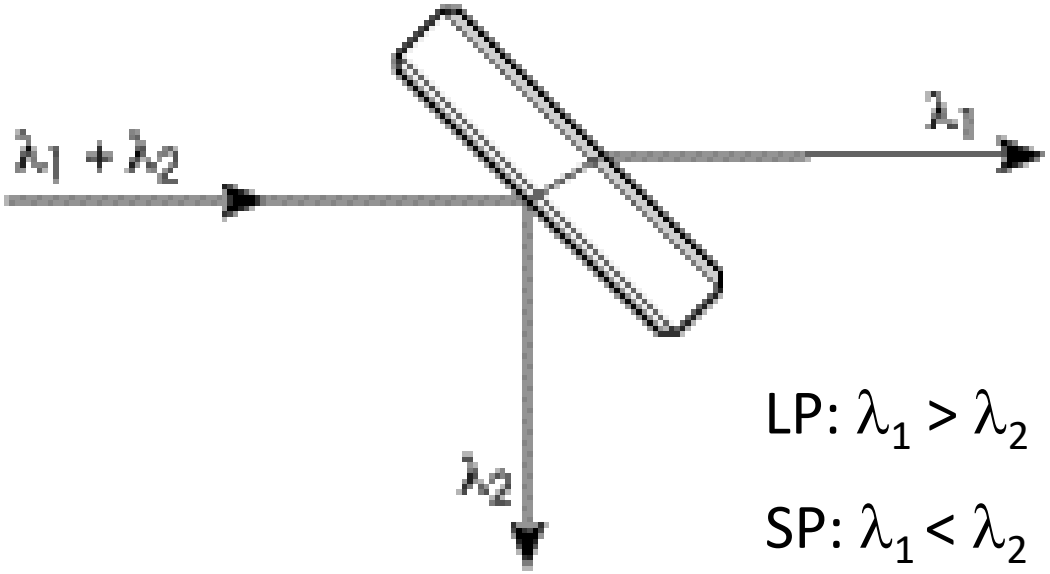
Specs and curves of BP580nm filter from Edmund Optics

| Type | Bandpass Filter |
|---|---|
| Center Wavelength CWL (nm) | 580 |
| Mount Thickness (mm) | 9.6 |
| Full Width-Half Max FWHM (nm) | 10 |
| Minimum Transmission (%) | ≥45 |
| Blocking Wavelength Range (nm) | 200 - 1200 |
| Optical Density OD | ≥3.0 |
| Angle Sensitivity | Intended for Collimated Inpu |



# Dichroic Mirror (DM)

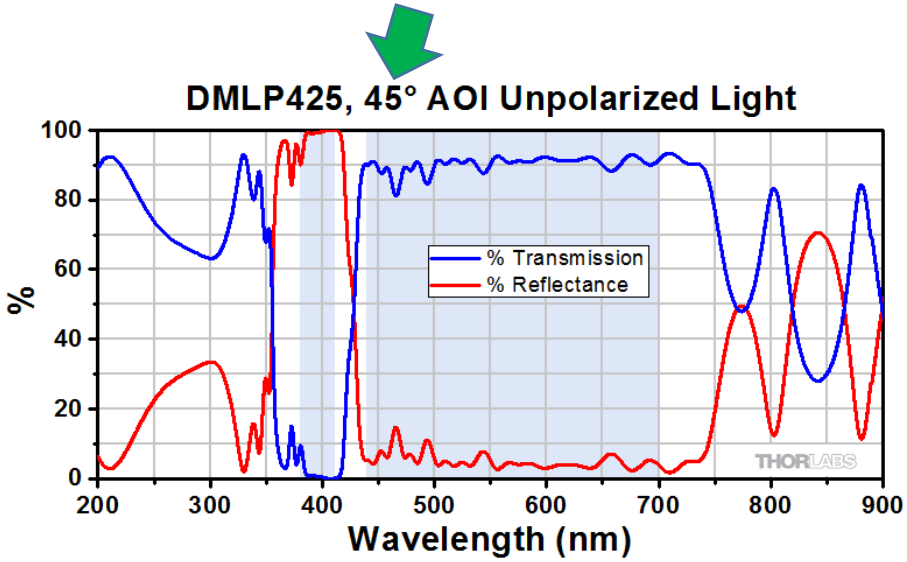
- *Di-* is Greek for two, and *-chroic* is Greek for color ➔ "**bicolored** "
- Spectrally separates (or combines) light by transmitting and reflecting photons as a function of wavelength
- DM is a LP or SP interference filter that is designed to be used at **45º**



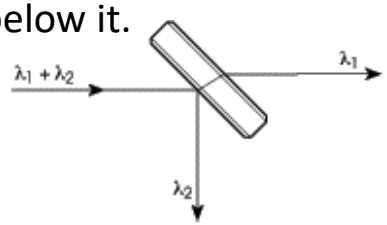

- **Long-pass (LP) dichroic mirrors** dichroic mirrors are highly **transmissive** above the cutoff wavelength and highly reflective below it.
- **Short-pass (SP) dichroic mirrors** are also available which are highly **reflective** above the cutoff wavelength and highly transmissive below it.

# Example Spectra for "Dichroic" Mirror

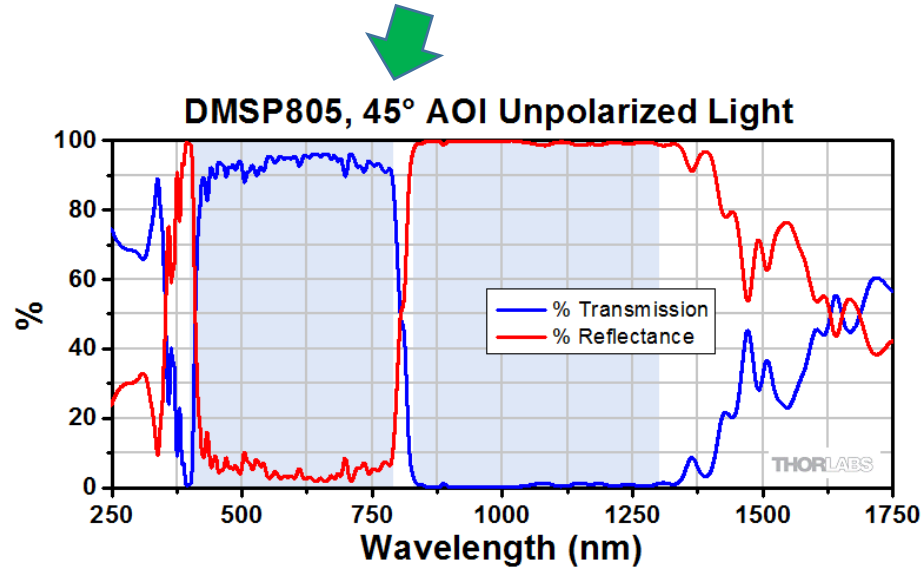
- **Data show additional transmission/reflection bands & sharp oscillations at the spectrum edges. Such features are typical for interference filters.**




- **LP dichroic mirrors (DMLP)** are highly **transmissive above the cutoff** wavelength and highly reflective below it.



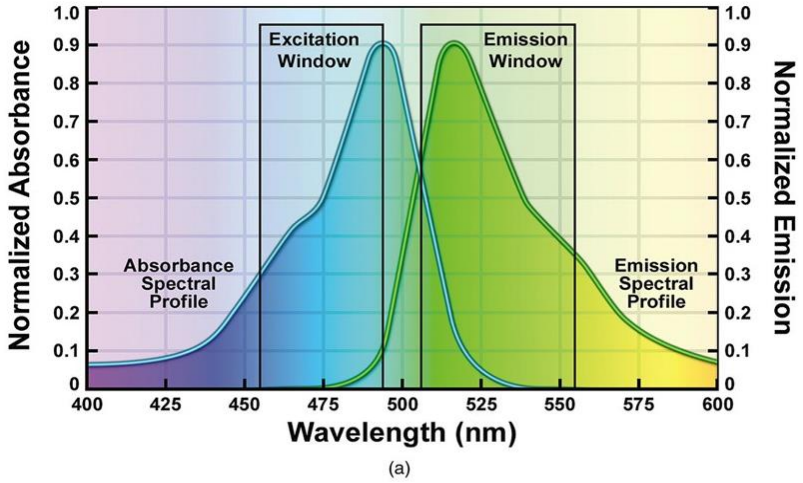

- It operates at **45°**
- **For DMLP425, cut-off wavelength is 425 nm**



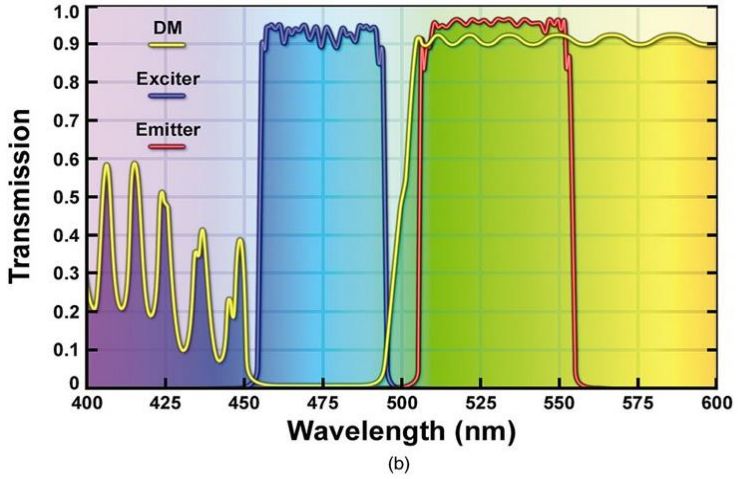

- **SP dichroic mirrors (DMSP)** are highly **reflective above the cutoff** wavelength and highly transmissive below it.
- It operates at **45°**
- **For DMSP805, cut-off wavelength is 805 nm**

# An Example for Choosing a Filter Set

**(a) Fluorescein absorption & emission spectra:**




**(b) Fluorescein filter set spectra:**




**Notes:**

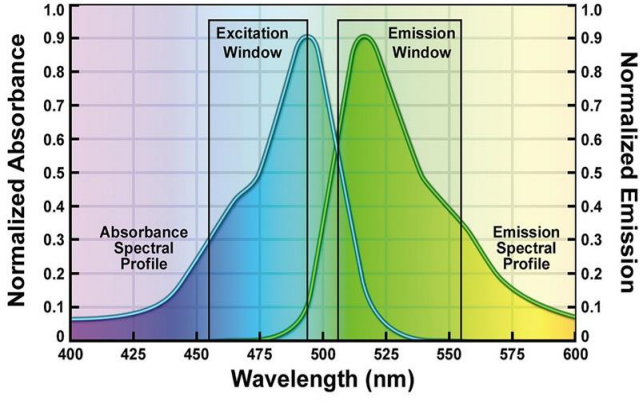
- Fluorescein absorption & emission spectra overlap. Therefore, to maintain a distinct separation, the transmission profiles of the filters are chosen not to exactly centered at the excitation & emission maxima.
- Boundaries between the transmitted & reflected wavelengths are designed to be as steep as possible with the filters.
- Rapid spikes (termed as “ringing”) on the sides are typical for dichroic mirrors – this is due to the thin film interference effect.

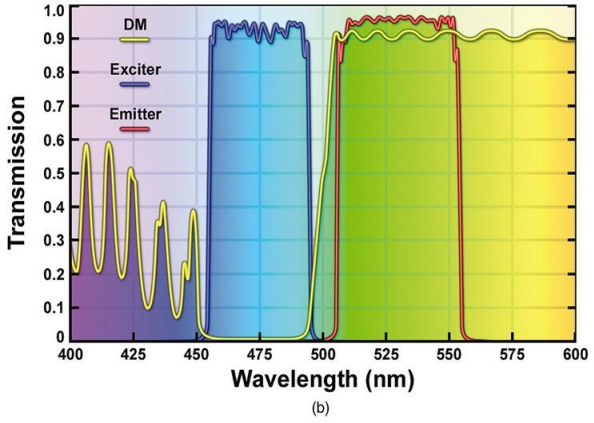
**1) Exciter - BP Filter:**
- Transmits mainly the absorption region

**2) Emitter - BP Filter:**
- Transmits mainly the emission region

**3) Dichroic Mirror - DM**
- **Transmits** most of the emission region
- **Reflects** most of the excitation region

# An Example for Choosing a Filter Set

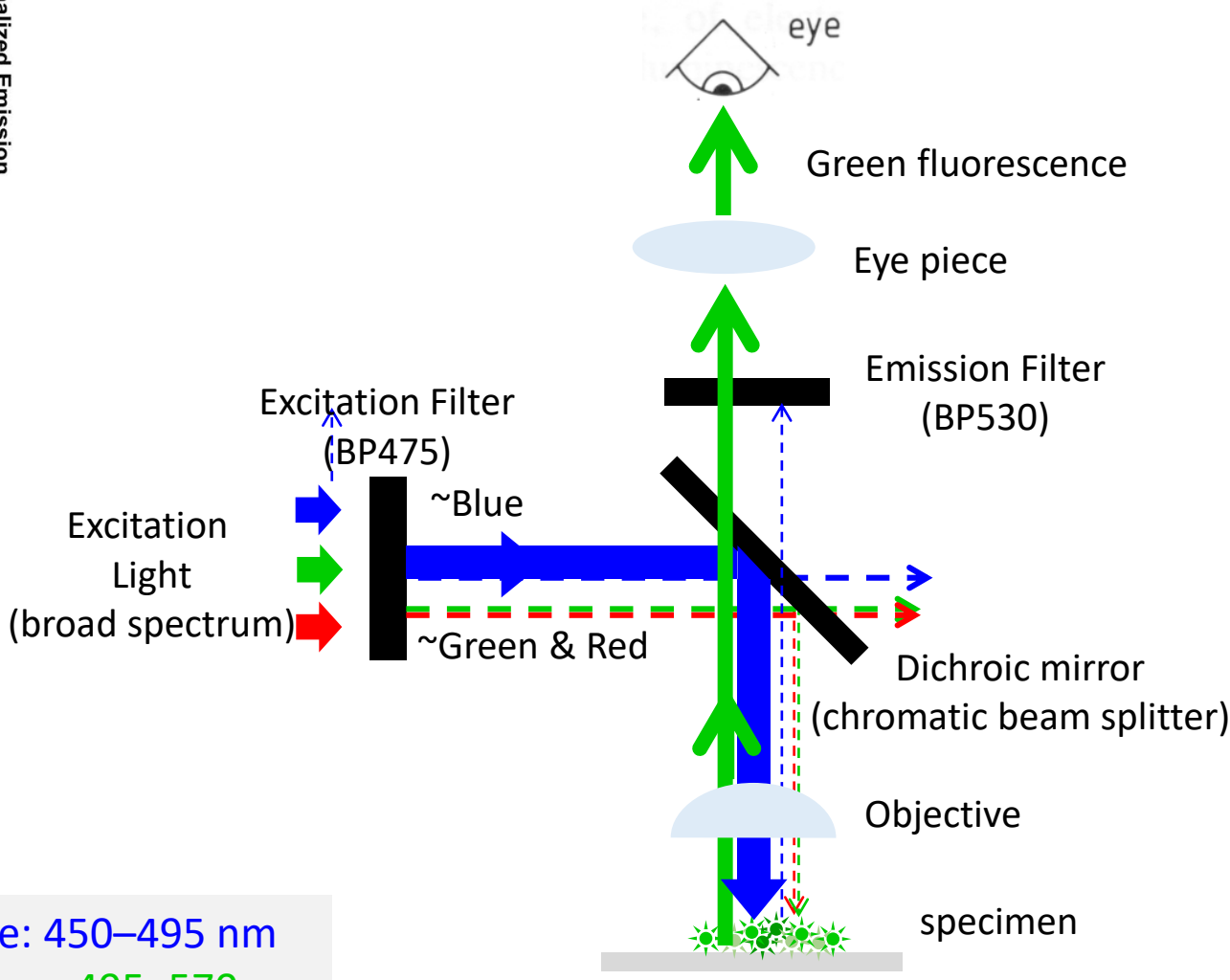



**1) Excitation - BP Filter:**
BP475, ~40nm FWHM

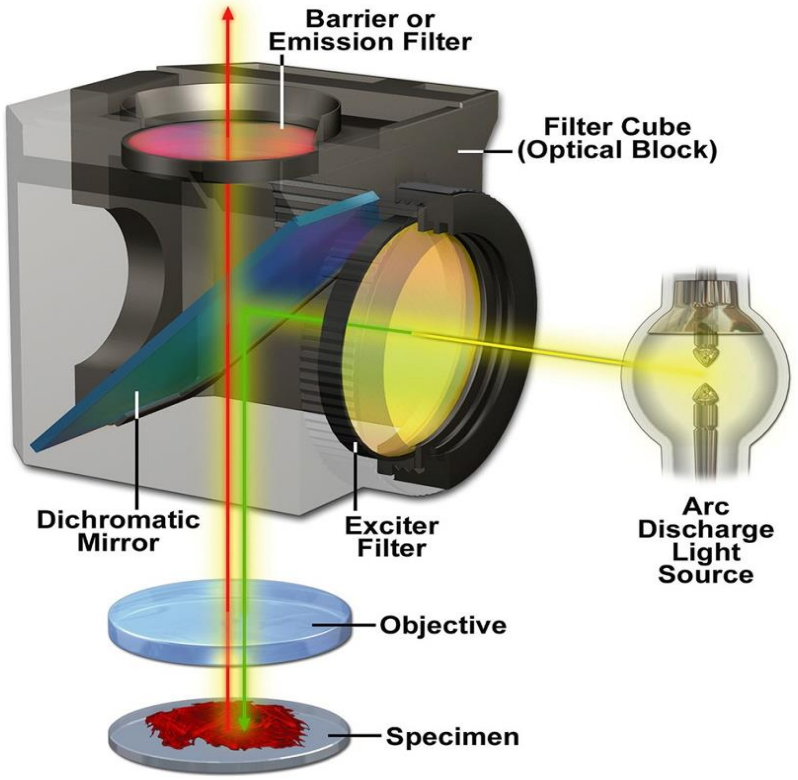
**2) Emission - BP Filter:**
BP530, ~50nm FWHM

**3) Dichroic Mirror: LP500**




Blue: 450–495 nm
Green: 495–570 nm
Red: 620-750

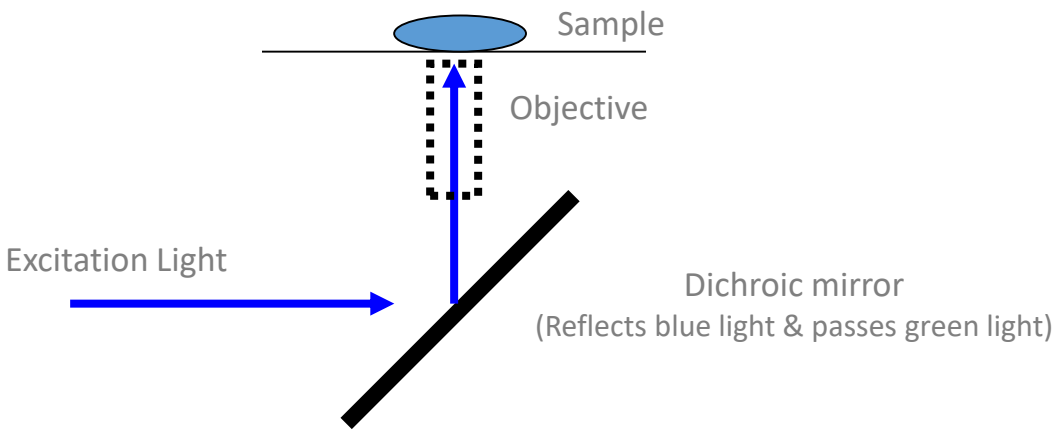
# Arrangement of a Filter Cube

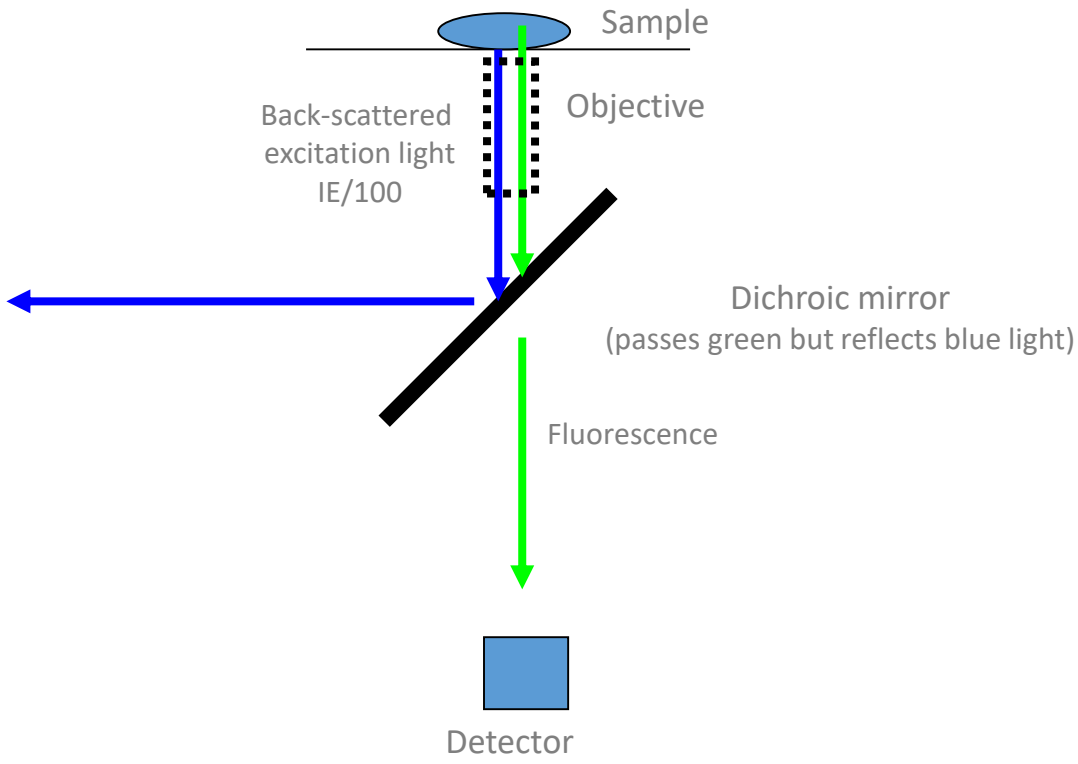

**Arrangement of filters in a fluorescence filter cube.**

- The diagram shows the orientation of filters for an epi-illuminator for an upright microscope.
- The excitation beam (**indicated by yellow line**) passes through exciter filter, reflected by the dichroic mirror and directed towards specimen (**green line**).
- Excitation light back-reflected or scattered at the specimen are again reflected by the dichroic mirror back towards the light source. Additionally, any excitation light that has managed to pass through the dichroic mirror is further blocked by the emission (barrier) filter.
- The emitted fluorescence signal **(red line**) passes through the dichroic mirror and emission filter to the eye or camera.

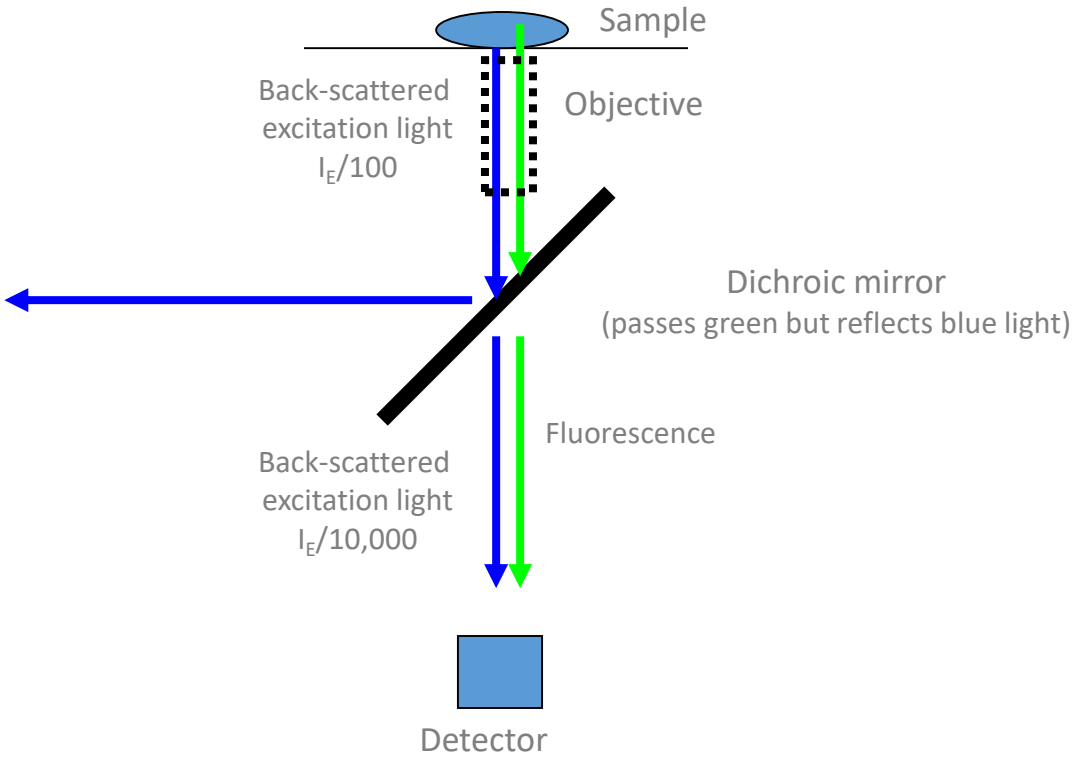
# Epifluorescence Microscopy



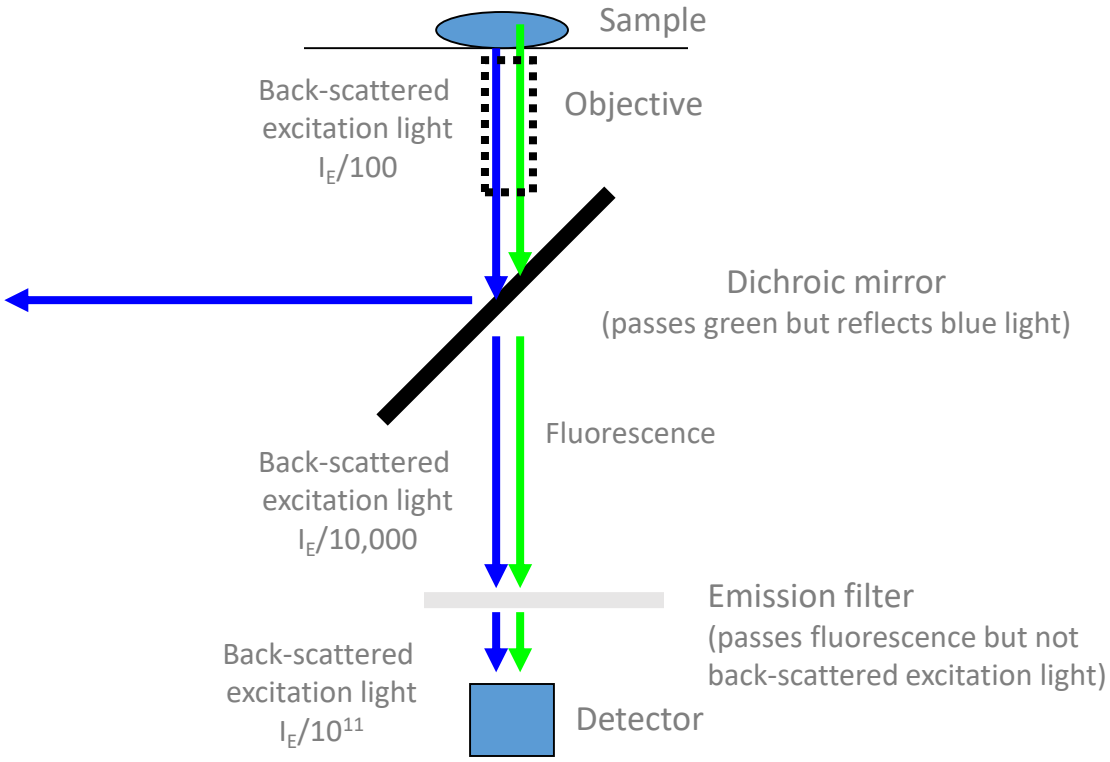
# Epifluorescence Microscopy



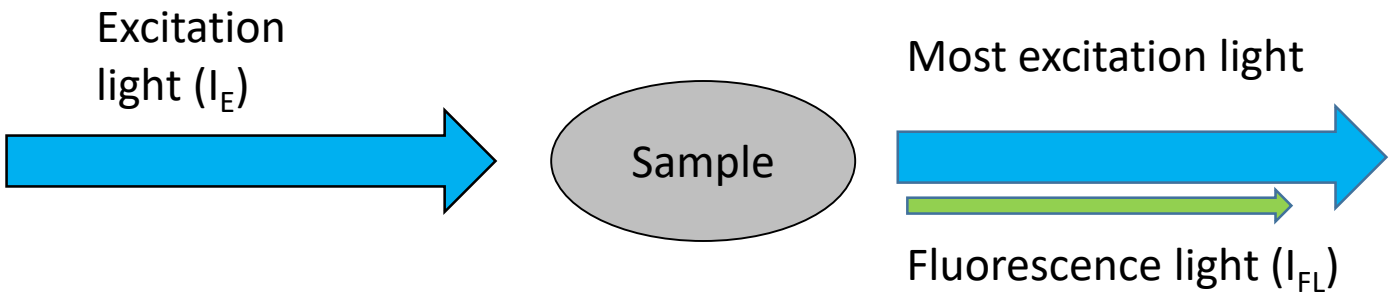
# Epifluorescence Microscopy



# Epifluorescence Microscopy



# Fluorescence Detection

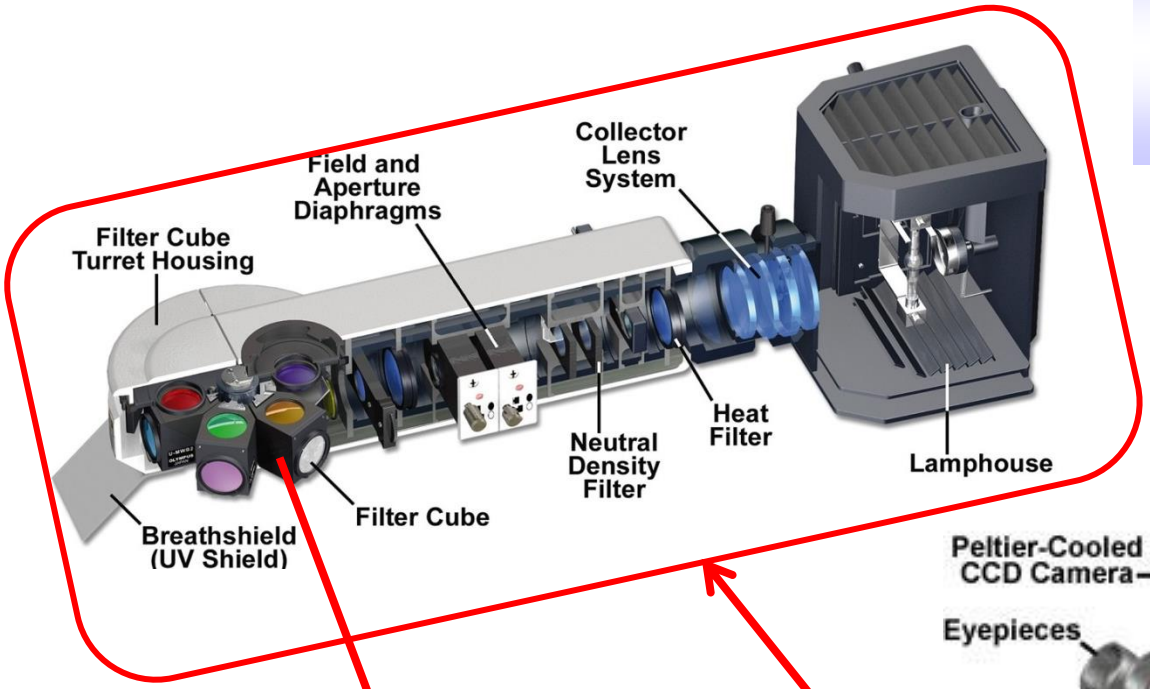


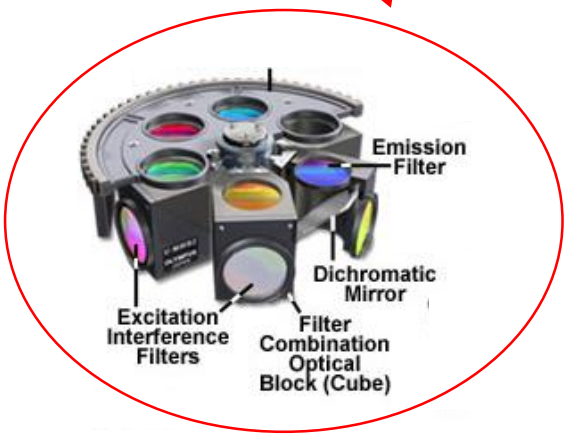

$I_E/I_{FL}$ ~ $10^4$ for strong fluorescence

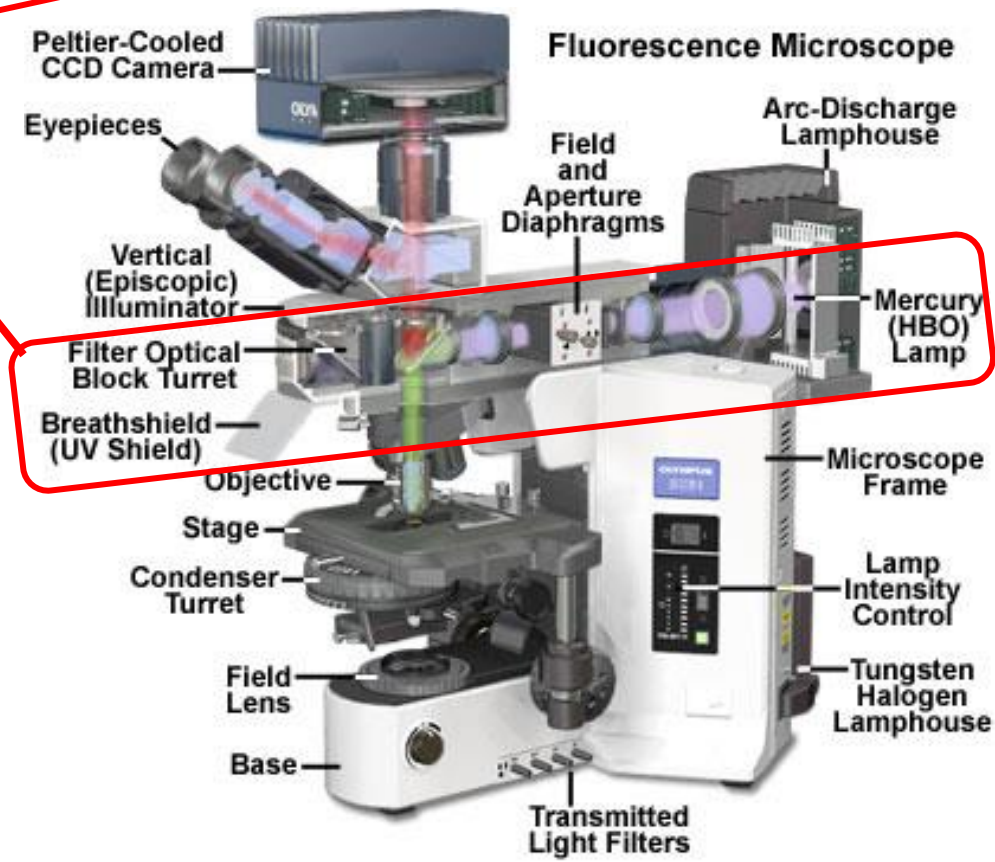
$I_E/I_{FL}$ ~ $10^{10}$ for weak fluorescence (e.g. *in situ* hybrid.)

In order to detect the fluorescence at 10% background
the excitation light must be removed or attenuated by a factor up to ≈ $10^{11}$

# Epi-illuminator: Filter Turret & Cubes



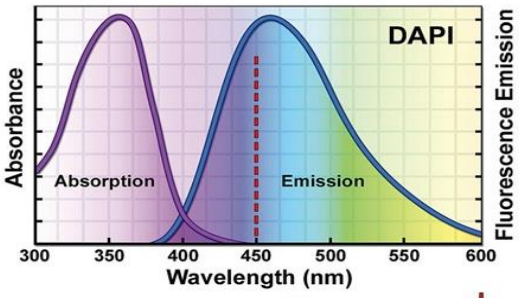
# An Example for Multi-Color Imaging

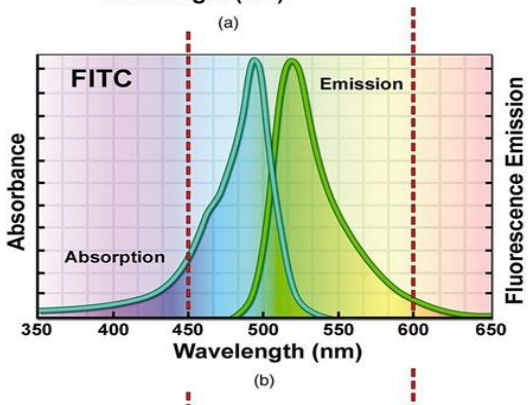
The absorption & emission spectra of:

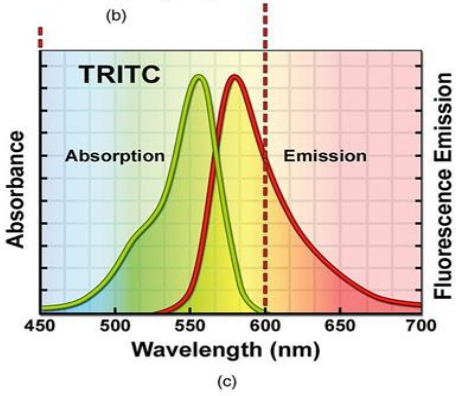
**(a) DAPI**

**(b) Fluorescin** isothiocyanate (FITC)

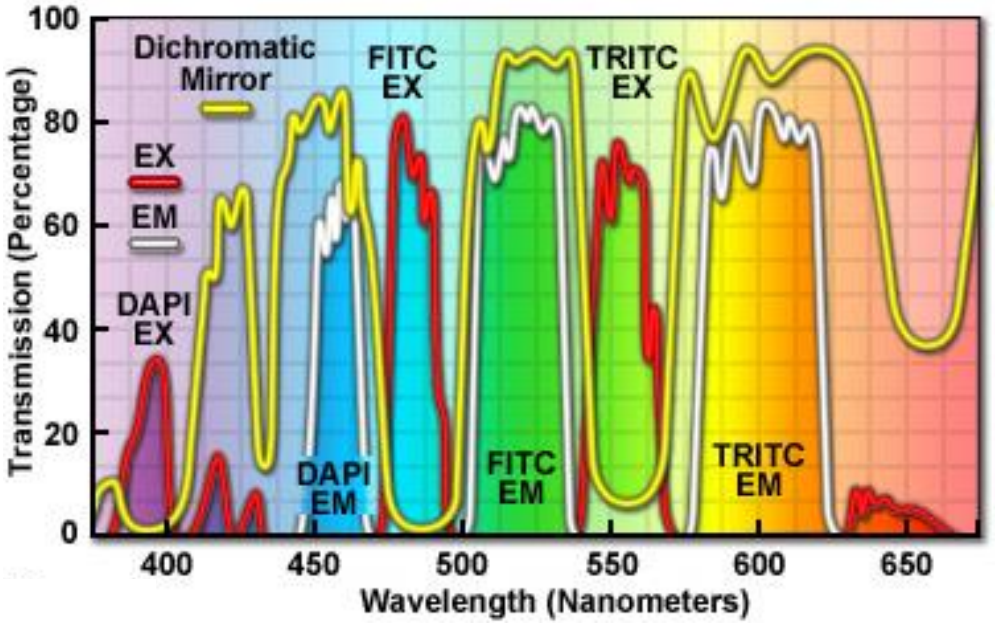
**(c)** Tetramethyl **rhodamine** (TRITC)




- Although the emission spectra of these 3 dyes partially overlap, it is possible to examine each dye separately using suitable filter sets.




**Triple Excitation Filter Block for DAPI-FITC-TRITC from Nikon:**

**Wavelengths of Excitation Filters (EX):** 1) 385-400 nm (**BP** with 393 CWL), 2) 475-490 nm (**BP** with 483 CWL), 3) 545-565 nm (**BP** with 555 CWL)

**Wavelengths of Dichromatic Mirrors:** 1) 435-470 nm (**DM**), 2) 500-540 nm (**DM**), 3) 570-645 nm (**DM**)

**Wavelengths of Emission Filters (EM):** 1) 450-465 nm (**BP** with 458 CWL), 2) 505-535 nm (**BP** with 520 CWL), 3) 580-620 nm (**BP** with 600 CWL)

# Example for multi-color imaging

- Multi-colored fluorescence image of a culture of bovine pulmonary artery endothelial cells obtained by **DAPI-FITC-TRITC bandpass emission (triple band excitation) set.**

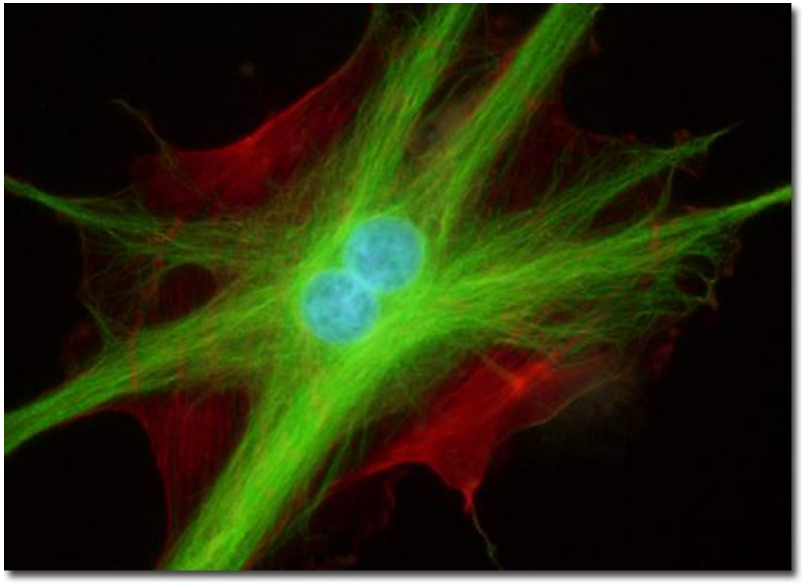


Image and notes are from Nikon web-site.

The labellings are as follows:

- immunofluorescently labeled with primary anti-bovine ***alpha*-tubulin** mouse monoclonal antibodies followed by goat anti-mouse Fab fragments **conjugated to BODIPY FL (green)**.
- simultaneously stained with **Texas Red (red)**, which target the **cytoskeletal actin network**
- simultaneously stained with **DAPI (blue)**, which target **DNA in the nucleus**.

The absorption maximum and the emission maximum occurs at:

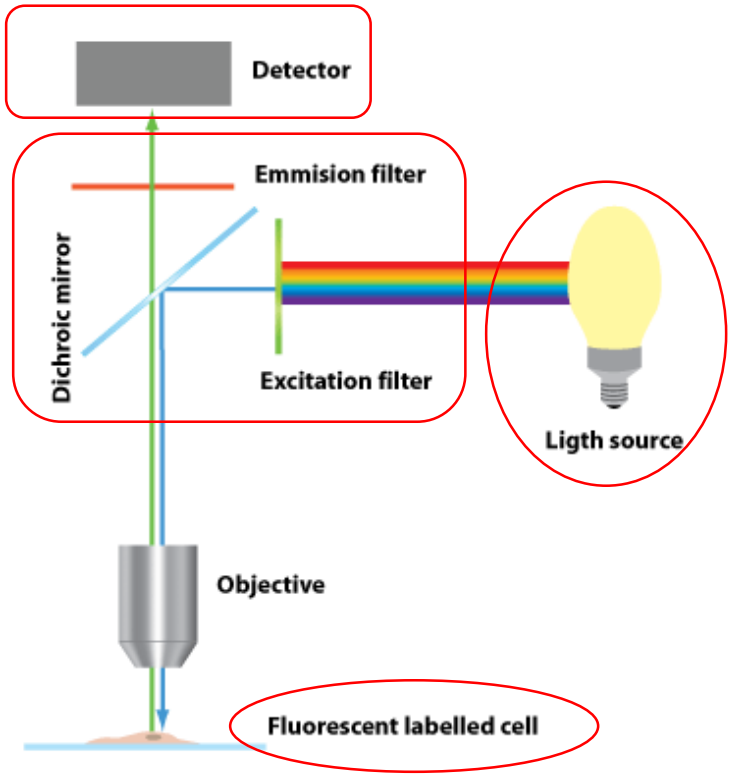
- 505 nm and 513 nm for BODIPY FL (green)
- 595 nm and 620 nm for Texas Red (red)
- 358 nm and 461 nm for DAPI (blue)

- Note the presence of high signal levels from DAPI and BODIPY FL, but the relatively low signal exhibited by Texas Red because this filter combination does not efficiently gather signal at longer red wavelengths).

# Outline: Detectors in 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

➔ **Different detector types used in microscopy**



# Detector Types

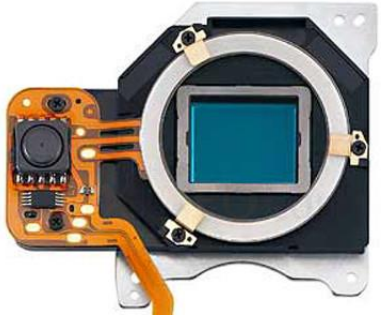
- **Eye**

- **Photodiode**
  - single element → no spatial information
  - Limited sensitivity & time resolution

- **PMT: PhotoMultiplier Tube**
  - single element → no spatial information
  - very high time resolution
  - used for laser scanning confocal microscopy

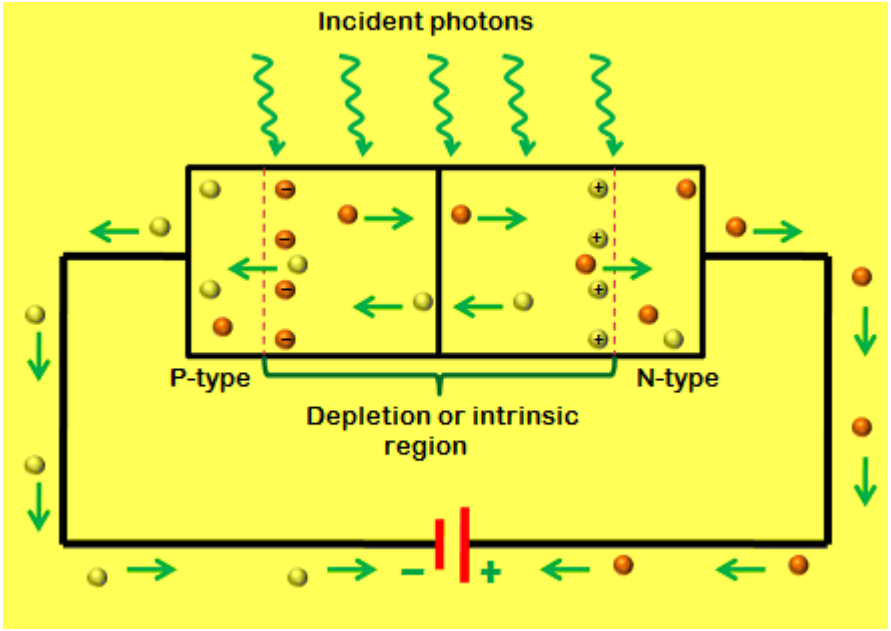
- **APD: Avalanche PhotoDiode**
  - very sensitive to low intensity lights
  - pixels can be arranged in 2D arrays → it offers spatial information & can be used in 2D imaging

- **CCD, EMCCD & CMOS cameras**
  - 2D pixelated → offers spatial information (2D imaging)
  - Limited time resolution
  - Sensitive with high quantum efficiency

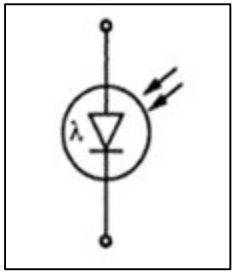


→ *most commonly used in biomicroscopy*

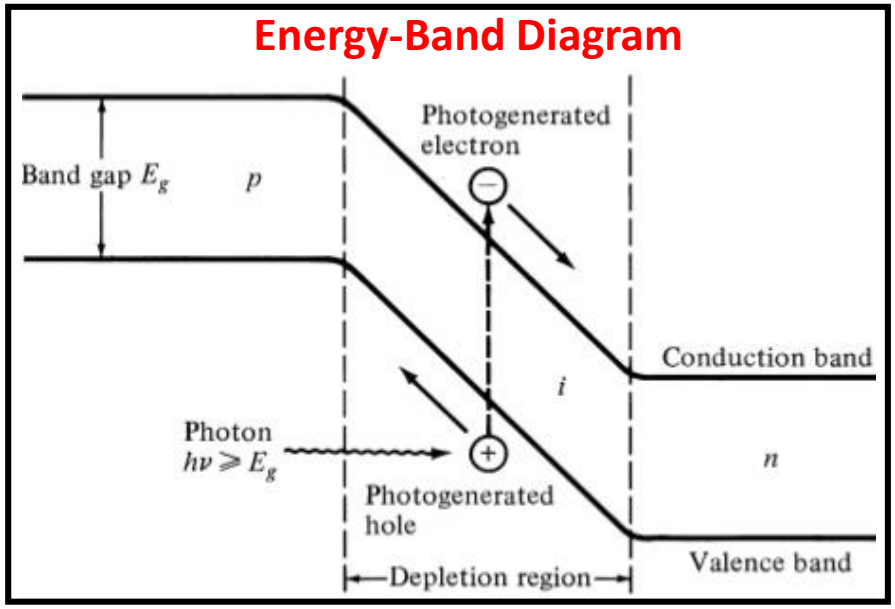
# Photo-diode & Photo-current




- Photo-diode is an semiconductor p-n junction device




Circuit symbol of photodiode

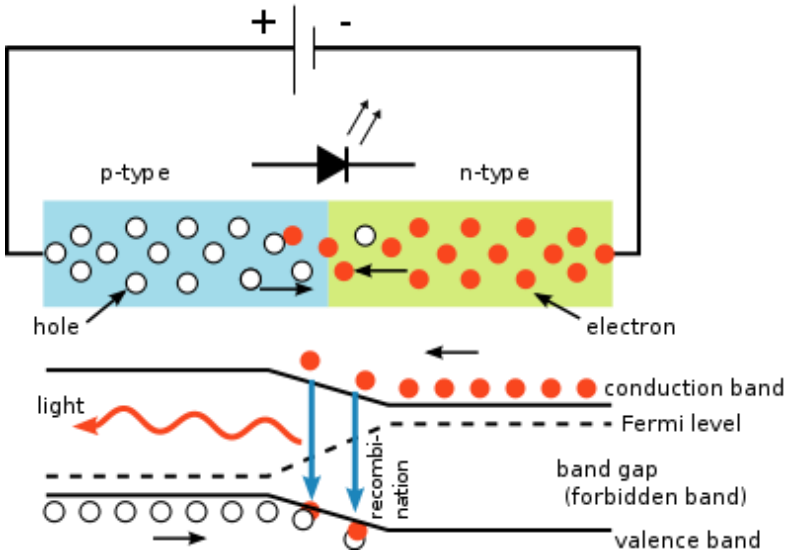


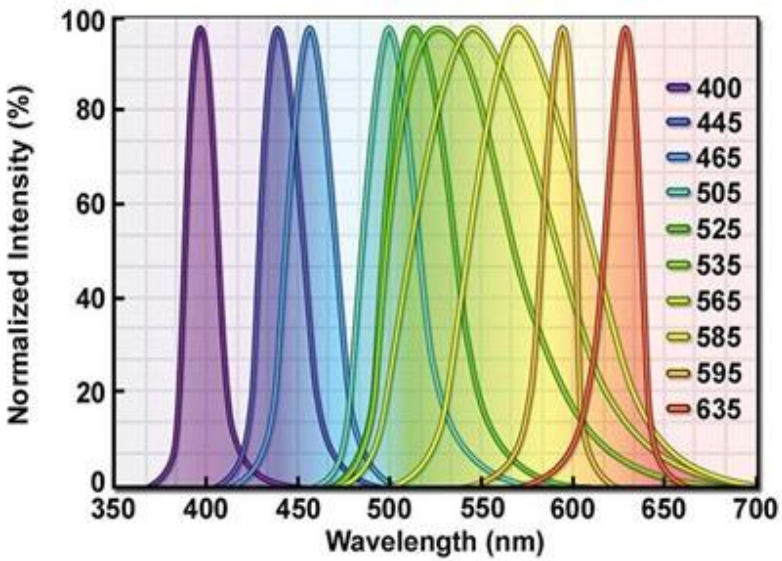

- Photo-current is generated when the incident photon energy is above the band-gap.
- Cut-off wavelength ($\lambda_c$) depends on the band gap energy of the pn-junction material.

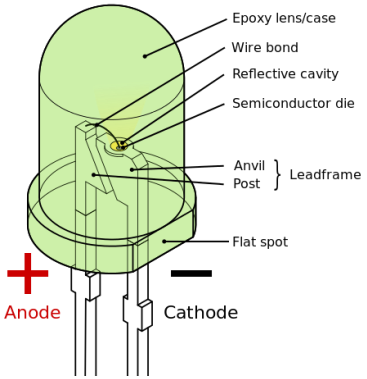
$$\Rightarrow \quad \lambda_c = \frac{hc}{E_g} = \frac{1.24}{E_g(eV)} \ \mu m$$

# Recall: Light Emitting Diodes (LEDs)

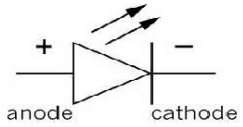
- LED is an **semiconductor p-n junction device**
- Applied electric bias results in current and the **recombination of circulating electrons and holes in the depletion zone** leads to the light generation
- The emitted light has a **relatively narrow 20-50 nm bandwidth**
- **The wavelength (color) of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




Circuit symbol of LED

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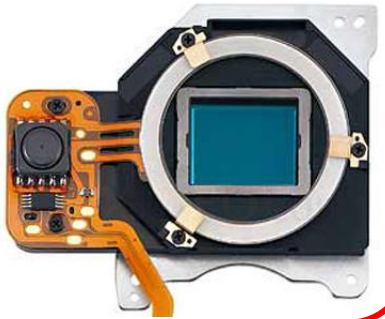


→ *most commonly used in biomicroscopy*

# Digital Imaging System


(a)

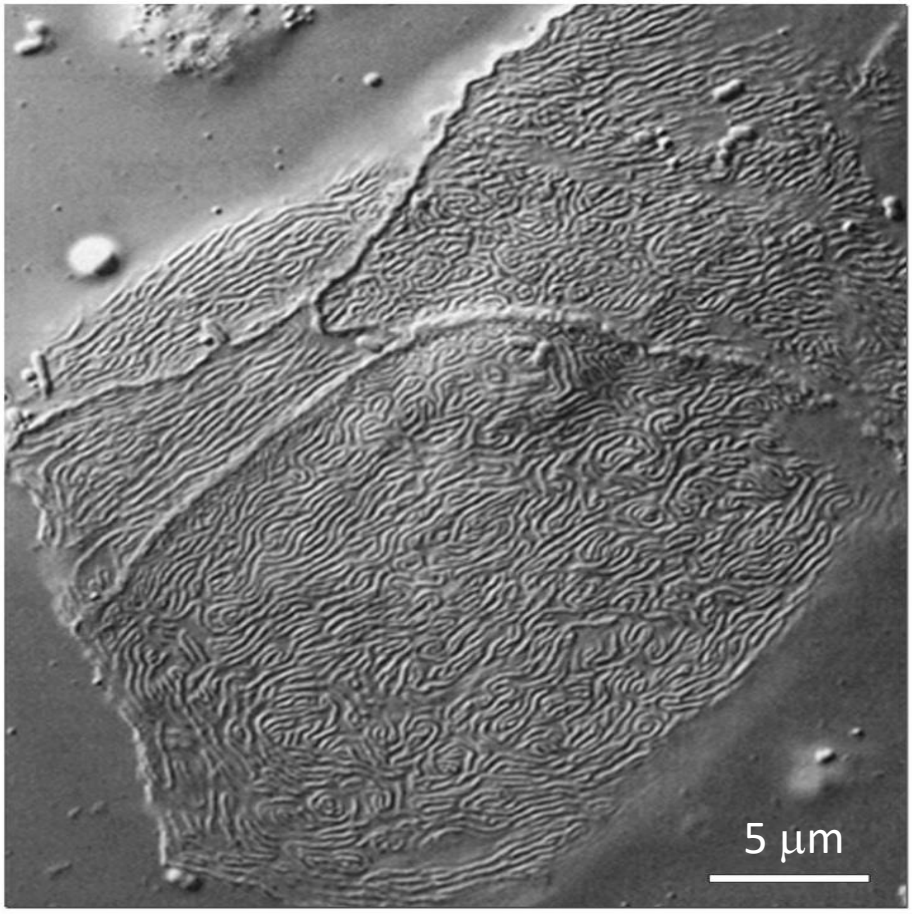

(b)

The combination of microscope and digital camera, together with a computer, camera control unit & imaging software, defines what is called a **digital imaging system**:

(a) Camera is mounted on the microscope. Camera control unit connected to the camera communicates with a host computer.

(b) Multiple options available ... cooled CCD, color camera, EMCCDs, scientific CMOS camera

# Imaging in Microscopy




- DIC (differential interference contrast) microscope image of the surface of an epithelial cell **recorded with a 1.4 megapixel CCD having a pixel size of 6.8 μm at 100x magnification with 1.3 NA objective.**
- Scale bar is 5 μm.
- The spacing between the ridges is ~400 nm.

➔ At an illumination peak wavelength of ~550 nm, diffraction limited spot at the camera is ~25 μm. Therefore the full optical resolution is retained with this camera choice.