Biomicroscopy I - Solutions Exercise Sheet 12

December 2, 2024

1 Fluorescence Microscopy

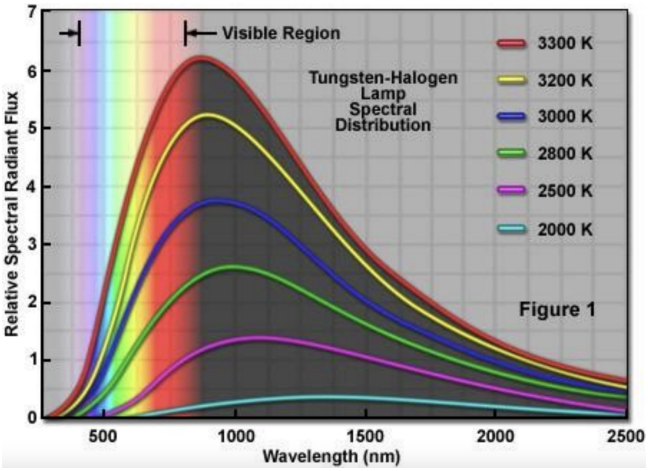

Figure 1: Spectrum of a Quartz tungsten-halogen lamp

A. The spectrum of a quartz tungsten-halogen lamp becomes more "blue" with increasing voltage, and more "red" as voltage goes down (see Figure 1, note that temperature of the lamp increases with the increasing applied voltage). For decreasing the output intensity of the illumination source but not affecting the spectral characteristics one can use neutral density with optical density 1 (ND 1 filter).

B. Long-pass, short-pass and band-pass filters.

C. The *transmission efficiency* T of neutral density filters is related to the *optical density* OD of a filter as

$$\mathrm{OD} = \log_{10} \frac{1}{T} \iff T = 10^{-\mathrm{OD}}$$

Therefore,

- $\mathrm{OD} = 0.3 \implies T = 0.5$ (around 50% of light is transmitted and 50% is blocked).
- $\mathrm{OD} = 0.6 \implies T = 0.25$ (around 25% of light is transmitted and 75% is blocked).
- $\mathrm{OD} = 0.9 \implies T = 0.126$
- $\mathrm{OD} = 1.3 \implies T = 0.05$
- $\mathrm{OD} = 2.3 \implies T = 0.005$

D. See Figure 2:

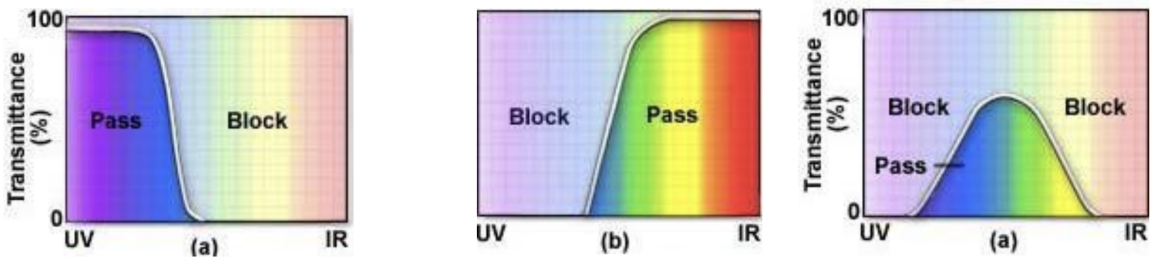

Figure 2: Left: short-pass filter. Center: long-pass filter. Right: band-pass filter.

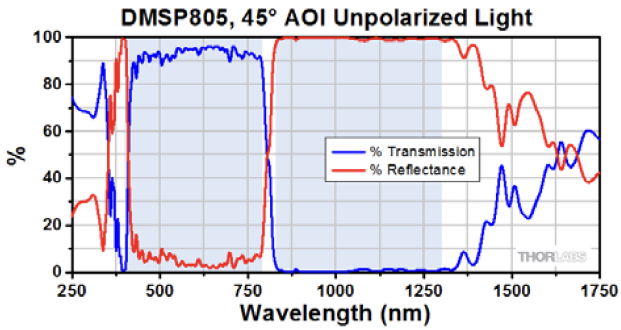

Figure 3: Short-pass dichoic mirror.

E. Short-pass dichoic mirrors reflect long wavelengths and transmit short wavelengths (see Figure 3):

   Long-pass dichroic mirrors reflect short wavelengths and transmit long wavelengths (see Figure 4):

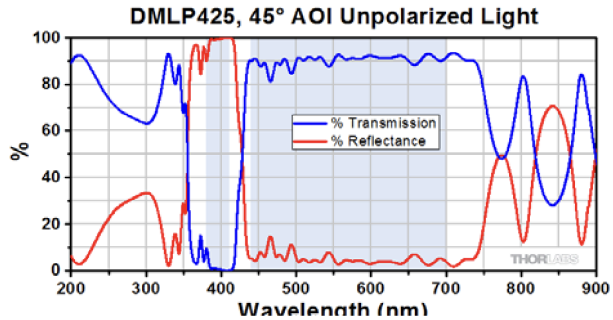

Figure 4: Long-pass dichoic mirror.

2 Quantum dots

A. The yellow and brown curves are the absorption spectra of QD_1 and QD_2, respectively.

B. The green and red curves are the emission spectra of QD_1 and QD_2, respectively.

C. QD_2 is larger in size than QD_1. As the quantum dot size gets smaller, the required energy to confine electrons is higher. So, quantum dots with smaller sizes have emission at shorter wavelengths (higher energy). Therefore, QD_1 is smaller than QD_2.

D. The absorption spectrum of QD_1 ends at ~550nm, meaning the light sources of wavelengths up to 550nm can excite QD_1. But since this quantum dot will be used in fluorescence microscopy, the excitation light source spectrum should ideally not mix with the

emission spectrum of the quantum dot. Therefore, the maximum wavelength suitable for exciting QD_1 is ∼475nm.

E. Applying the same reasoning as in D., the maximum wavelength suitable for exciting QD_2 is ∼580nm.

F. For the excitation of both QD_1 and QD_2, the maximum suitable wavelength is limited to the maximum excitation wavelength of QD_1, which is ∼475nm. In the case of excitation of both quantum dots, the output will have both emissions of QD_1 and QD_2. In order to separate the emission signals of the quantum dots, filters with cutoff wavelength ∼550nm can be used at the output.

3 Jablonski diagram

We can use a pair of band-pass filters as excitation and emission filter set. The excitation/emission profile calculated for Rhodamine green is 497/529 nm, while for CY5 is 647/681 nm.

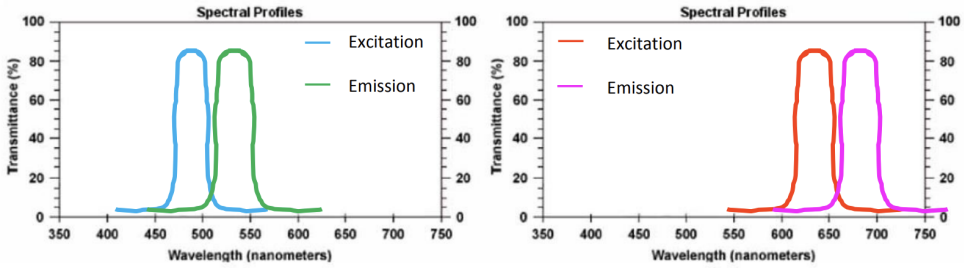

Figure 5: Sketch of the excitation and emmision spectra for Rhodamine and Cyanine (CY5)

4 Filter sets for fluorescence microscopy

A.
- The excitation filter is a band-pass (BP) filter at 475nm with a bandwidth of 50nm: BP475/50.
- The emission filter is a BP530/50.
- The dichoic mirror is a long-pass filter with cut-on 500nm: LP500.

B. In principle, other filters could be used, *e.g.* a SP500 filter for excitation (but depending on the spectrum of the light source - strong radiation in UV may damage the sample).

C. See B.

D. If the emission filter is not used, we will observe not only the emission of the fluorophore within the emission window suggested, but also radiation at lower and higher wavelengths that will be allowed by the dichoic mirror. Though since dichroic mirror does not have spectral overlap with excitation window, the microscope will still function.

E. With the same reasoning as in D., if we use an LP500 we would observe not only the emission of the fluorophore but also radiation at lower and higher wavelengths but the microscope will still function.

F. If a blue LED is used, then the excitation source fits by itself within the excitation window and we do not need to perform additional filtering.

5 Commonly used optical microscopes in biology

A. The left one is an upright microscope and the one on the right is an inverted one.

B. Bright-field microscopy works in **transmission** configuration for both microscopes

Upright microscope for bright-field microscopy in transmission configuration: Illumination starts from the Tungsten Halogen Lamp, passes through multiple transmitted light filters, field lens and condenser, and then reaches the sample on the stage. Transmitted light is collected by the objective above the sample, and then to your eyes through the eyepieces or to the CCD camera.

Inverted microscope for bright-field microscopy in transmission configuration: Illumination starts from the Tungsten Halogen Lamp on the very top, passes through filter tray, apertures and condenser, and then reaches the specimen on the stage. Transmitted light is also collected by the objective under the sample, reflected by the mirror and then goes to your eyes through the eyepieces or to the CCD camera.

C. Fluorescence microscopy works in **reflection** configuration for both microscopes

Upright microscope for fluorescence microscopy in reflection configuration: Illumination starts from the Mercury Lamp, passes through field and aperture diaphragms and enters the filter cube situated inside the filter block turret. Light in the excitation wavelength window is then reflected by the dichroic, passes through the objective and reaches the sample on the stage. Emission light is collected again by the objective above the sample, filtered by the emission filter inside the filter cube and goes to your eyes through the eyepieces or to the CCD camera.

Inverted microscope for fluorescence microscopy in reflection configuration: Illumination starts from the Mercury/Xenon Arc Lamp, passes through multiple lenses and enters the filter cube situated inside the filter block turret under the objective nosepiece. Light in the excitation wavelength window is then reflected by the dichroic, passes through the objective and reaches the on the stage. Emission light is collected again by the objective below the sample, filtered by the emission filter inside the filter cube, reflected by the mirror and then goes to your eyes through the eyepieces or to the CCD camera.

6 Selecting filters and illumination for fluorescence microscopy

A. The Stokes shift is $\sim$25nm.

B. Alexa Fluor has a narrow band excitation spectrum, thus can be excited by a single wavelength Dynamic Laser (e.g. LS300K2). The tail of excitation spectrum of WtGFP overlaps with the spectrum of the LS300A laser and therefore can be used as a light source. The excitation spectrum of EGFP is mainly in the UV region, and thus can be excited by LED370E Ultra Bright Deep Violet LED.

C. For EGFP we can use:

- MF390-18 for excitation
- MD416 for the dichroic mirror
- MF460-60 for emission

For WtGFP we can use:

- MF390-18 or MF434-17 for excitation (MF445-45 will be less efficient as dye absorbance in this region is lower).
- MD416 could be used if MF390-18 is used for excitation.
- MD480 is most suitable when MF434-17 is used for excitation.
- MF525-39 has the best overlap with the emission spectrum.

For Alexa Fluor 594 we can use:

- The combination of: MF565-24 (Excitation), MD588 (dichroic mirror) and MF-620-52 (Emission)
- MF 559-34 (Excitation), MD588 (dichroic mirror) and MF630-69 (Emission)