Lab exercise 3: Fluorescence microscopy

Furkan Kaya and Bahareh Nikparvar

1. Question 1

Fluorescence:
Fluorescence occurs by the absorption of a photon of high energy (often from the UV or blue end of the visible spectrum). This excites an electron into one of the many higher energy vibrational states. Some energy is transferred into vibrational energy and then the excited electron relaxes into the ground state, releasing a photon of lower energy than the photon initially absorbed. Fluorescence characterizes emission of a photon under permitted electronic de-excitation ($\Delta J = 0$).

Phosphorescence:
Phosphorescence is a specific type of photoluminescence related to fluorescence. Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs. Excitation of electrons to a higher state is accompanied with the change of a spin state . Once in a different spin state, electrons cannot relax into the ground state quickly because the re-emission involves quantum mechanically forbidden energy state transitions ($\Delta J \neq 0$). As these transitions occur very slowly in certain materials, absorbed radiation may be re-emitted at a lower intensity for up to several hours after the original excitation.

Franck-Condon principle: The Franck-Condon Principle describes the intensities of vibronic transitions, or the absorption or emission of a photon. Absorption of light and promotion of an electron from one state to another happens rapidly. Because of the short time it takes for the electronic transition, there is little or no geometry change in the molecular system. As result, it can be said that electronic transitions occur vertically on a potential energy diagram.

Stokes shift: Stokes shift describes the difference in wavelength between the maximum of the excitation spectrum (shorter wavelength, higher energy), and the maximum of the emission spectrum (longer wavelength, lower energy)

Auto-fluorescence: Auto-fluorescence means that some intracellular molecules are naturally fluorescent. It occurs when biological components like mitochondria absorbs light and is used to distinguish the light that comes from artificially added fluorescent markers.

Dichroic mirror: Dichroic mirrors are coated with a special coating that transmit a certain wavelength and reflect the other wavelength. They are required for different purposes like for example harmonic separators. In fluorescence microscopy a dichroic mirror can be used for separating the fluorescence light from the pump light. Low transmission efficiency and misalignment of dichroic mirrors are severe drawbacks of dichroic mirrors.

Epi-illumination: Epi-illumination means that illumination and the detection of light is done at the same side of the sample. Certain samples that are not transparent, like colorimetric blots, can only be imaged using epi-illumination.

2. Question 2

Pinhole: The purpose of the pinhole in confocal laser scanning microscopy is to improve the contrast in the image. This is due to the fact that traditional fluorescence microscopy has limited axial resolution.
If the image of the object is thicker than the axial resolution, then it affects the contrast. Thus, to avoid that a field diaphragm, or a pinhole as it is called in layman terms, is placed in front of the detector in a conjugate plane. This leads to that only photons from the focal plane will reach the detector. The pinhole's diameter can be adjusted.

3. Question 3

a) Regarding the filter cubes properties and absorption and emission spectra, it seems that the I3 filter is the most appropriate one for Alexa488. That is because the bands

of both I3 excitation and emission filters suit the spectra of Alexa488 better than other filter cubes. In the case of Propidium Iodide, N2.1 is the most suitable one and the reason is just the same: The pass bands of the excitation filter and emission filter of this cube cover the spectrum of Propidium Iodide better than other filter cubes. (it is also good to note that the maximum wavelength for transmission of excitation light should allow excitation within the main absorption peak, but not extend into the emission band.)

b) It seems that none of the two fluorophores will give us a fluorescence signal if we use filter cube A. That is because the emission filter of this cube will pass wavelengths longer than 425 nm, and this range will cover both excitation and emission spectra of both fluorophores. however, an appropriate emission filter has to cover only the emission spectrum.