**1. Light-tissue interaction (20%)**

Download the data for the molar extinction coefficients of oxy- and deoxyhemoglobin as a function of wavelength from the Website (https://omlc.org/spectra/hemoglobin/), convert the molar extinction coefficients to absorption coefficients of oxy- and deoxyhemoglobin, and plot the two curves in MATLAB (or similar programming platform).

**2. Optical biosensor (20%)**

What is FRET? Explain how it works.

Ans:

FRET is a kind of dipole-to-dipole interaction between proteins that occurs at a very small distance, typically less than 10 nm. The application of this interaction is to reveal if proteins are interacting with each other. The basic idea is to attach(fuse) fluorophores to a protein. As an example, let us say that we have two proteins, protein X and protein Y, and that to protein X we have attached a blue fluorescent protein and to protein Y we have attached a green fluorescent protein. Now, let us also say that if we emit a violet light excitation that the blue fluorescent protein will emit blue light, but that the green fluorescent will only emit green light if excited with blue light. Now with the violet light exciting the blue fluorescent, if the green fluorescent is not in proximity of protein X it will not emit any light. However, if protein X is very close to protein Y, then when the violet light is emitted, the blue fluorescent protein, attached to protein X will emit and cause the green fluorescent protein, attached to protein Y to emit, thereby proving indirectly that protein X and protein Y are near each other.

**3. Microscopy basics (20%)**

Describe and compare polarized light microscopy, phase contrast microscopy and differential interference contrast microscopy (Basic principles, system configurations and advantages).

Ans:

Polarized microscopy is a type of microscope that really depends on some properties of the sample. The specimen to be magnified must contain the property of birefringence, otherwise the contrast of polarization will not yield good results. Basically, the system works as follows. A light source is polarized so that the EM wave is linearly polarized. The sample, usually a crystal or other substance that has the property of birefringence. Then, when the polarized light strikes the specimen, the light is split into two EM waves that are 90 out of phase with each other. These waves are then filtered thru the analyzer, wherever the component of the wave does not pass the image is seen as dark. The main two system configurations of polarized light microscopy are the polarizer and the analyze, with eyepieces, camera extensions and other components added to enhance the image. Also, when configuring a sample with a polarized microscope the preparation is important. The choice of the sample, meaning the thickness and density will affect the image, but unlike other imaging techniques, also the orientation will give different results. In other words, even with the same specimen, to fully analyze the sample, experimentation with orientation is needed to yield good results. One advantage of polarized microscopy is the compact size of the system. Also for crystals a polarized microscope is a quick and convenient method. However, a disadvantage of a polarized microscope is that the sample needs to contain the property of birefringence, without this property results will not offer enough contrast for good viewing.

Phase contrast first works on the principle of what our eye can discern, which is differences in intensity. So light that is slowed down by the thickness of a specimen, also diffracted, lags at a quarter wavelength with the light that was not affected by the specimen. So, if the undisturbed light is sped up by a quarter wavelength there is a destructive interference at the eyepiece that would cause darkness that our eyes are sensitive to and would be able to see. One system configuration of phase contrast microscopes is like a darkfield microscope but has the addition of a phase plate. So, then a system configuration of a phase contract microscopy would be from top to bottom: an ocular eyepiece, phase plate, objective lens, the specimen, a condenser annulus, and the light source. Some of the advantages of the phase contrasting method are the ability to observe without staining the specimen. This is an advantage as this cut down on any toxicity and allows for the possibility of in-vivo imaging. Closely related to the above advantage of phase contrast is the ability to contrast objects that don’t necessarily absorb light.

Differential Interference contrast (DIC) microscopy is an improvement on the original phase contrast by Zernike’s original idea. The basics of DIC are that a prism splits our polarized light into two orthogonal rays of light. The light then passes thru the sample and then a second prism recombines and translates those beams of light into amplitudes that can be seen. The main characteristic of the DIC is that contrast in directional and that there is a maximum in one direction and a minimum in another direction. Comparing this to polarized microscopy the contrast is highlighted at the edges. Delving a bit more into the basic details, there are two overlapping airy disks in the image where one is brighter, and the other is darker than the background. Thus, the direction of the airy disk separation is the shear direction and direction of maximum contrast. In a system configuration the condensers above the light source can include multiple stages with internal lenses, aperture diaphragm and a Nomarski prism to help create a kind of double dual beam interferometer. The advantages of DIC are in building on the foundations of phase contrasting, providing a sharper resolution image than the birefringence of polarization.

**4. Fluorescence microscopy (20%)**

Describe and compare widefield fluorescence microscopy, confocal laser scanning microscopy and two-photon excitation microscopy (Basic principles, system configurations and advantages).

Ans:

Widefield fluorescence microscopy basically works on the principle of fluorescence which immediately emits light after photon absorption. A typical scenario will see a molecule, fluorophore, attach itself to a molecule or protein of interest and then respond to a stimulus within a prescribed localized biological region. A basic system configuration would see a lamp, lens, aperture, and diaphragm direct light at an excitation filter which would then pass thru typically a dichroic mirror to an objective lens and ultimately to the specimen. This excitation light would then stimulate the specimen which in turn would respond with a weaker emission light passing back thru our emission filter for capture and processing. Some of the advantages of a fluorescent microscope our: development of hundreds of fluorochromes, availability of different contrasting techniques (DIC as an example), highly sensitive and simple to implement.

The basic principle of Confocal laser scanning is that a laser scans across a sample to reconstruct. One system configuration would use a motorized mirror to scan across the sample, while another would use a spinning disc of micro lenses. Also, a camera would not work as well for detection, so instead the use of photomultiplier tubes would be used to collect and amplify the light. After the PMT the signal would be digitized so that then the image can be reconstructed. An advantage of laser scanning is that you can get clear images in thin optical sections from thick samples. However, a disadvantage of confocal laser scanning is that it is slower than other optical techniques and requires deconvolution of the light

Two-photon microscopy is like confocal in that it focuses on a very small area, this differentiates both two-photon and confocal from wide-field fluorescence which has a wider focus area. So, while it focuses on a small area it is much brighter and sharper than confocal that has to use a pin hole as part of its’ focusing mechanism. The principle and system configuration of two-photon microscopy is that the fluorophore that has attached to the specimen that we wish to excite has to receive and absorb both photons to properly excite our system and have re-emission of another photon for detection. This is a great advantage of two-photon over normal one-photon fluorescence, the focal plane is smaller, so there is a sharper image, and since the area has to capture both photons, extraneous fluorophore emissions, and background signals are not permitted to excite because they do not receive both photons.

**5. Super-resolution microscopy (20%)**

What is the resolution limit of an optical imaging system? Explain why there’s such limit and describe at least one method to break the resolution limit.

Ans:

Part 1: The resolution limit of optical imaging systems is the Abbe diffraction limit which is about

200 nm.

Part 2: The reason that there is a diffraction limit is that the focus size of an object has a limit, this limit is the point spread function (PSF). Now, if the distance between PSF is greater than 200 nm then we can resolve individual PSFs, but if not then the two light objects appear as one and we can no longer resolve.

Now, there are two schools of thought on now to break this diffraction limit, one relies on shrinking the PSF, by pattern illumination. Here methods such as Stimulated emission depletion (STED) and RESOLFT microscopy use this idea of a smaller PSF to get sub-diffraction imaging.

Another method to get beyond the diffraction limit is by the method of stochastic switching of single molecules that is popular. Here the basic idea is to apply a widefield excitation of different fluorophores in the specimen. The switching of light will cause a weak activation in a small subset of all the possible fluorophores. Now their will be a stochastic behavior of which local fluorophores are activated and an accumulation of the images can be used to discern sub-diffraction structures, like organelles. A biomedical imaging technique that uses this method is called Stochastic Optical Reconstruction Microscopy or STORM.