Fluorescence in biological media

Laboratory Exercise

Medical Optics

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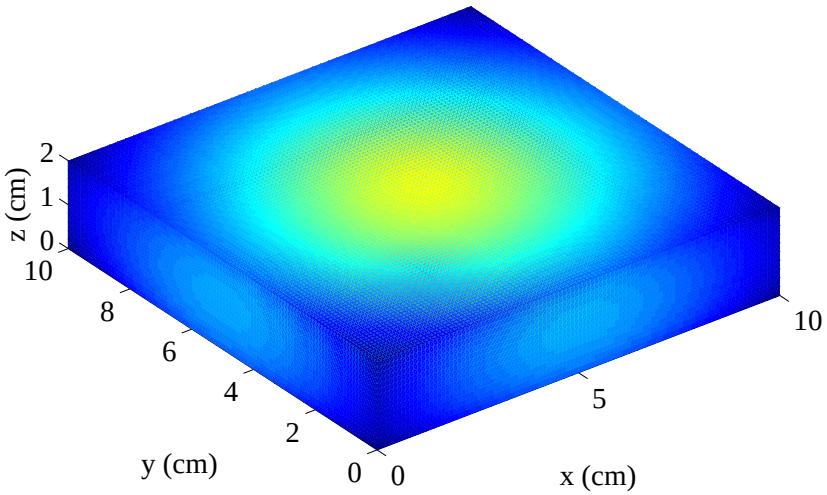

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1 Introduction

In recent years, macroscopic fluorescence imaging and tomgraphy have retreived a great deal of interest. The aim using such approaches is to localize and potentially quantify a fluorescent marker located inside human tissues and small animals. The potential use is within longitudinal studies, i.e. where a biological phenomena is investigated *in vivo* during a long period of time.

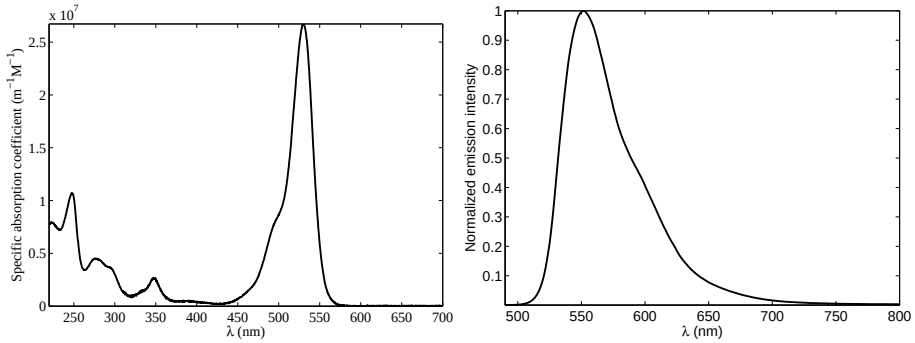
1.1 Fluorescence Molecular Imaging

The research is triggered by the use of different microscopic techniques utilizing fluorescence for imaging biomedical phenomena in cells. The fluorophores can be categorized into active probes and activatable probes. The active probes are non-specific fluorophores that are attached to an affinity ligand specific for the target. These ligands can be antibodies, peptides and labeled small molecules. The active probe emits fluorescence upon excitation even if it is not attached to the target ligand. This results in background fluorescence which is non-specific, i.e. no information about the target to be imaged. The activatable probes are more specific since these only emit fluorescence when "switched on". This can be achieved by either arranging the fluorophores in close proximity to a quencher, or by placing several fluorophore together, causing a self-quenching effect. This arrangement is possible due to an enzyme-specific peptide sequence. In the presence of an enzyme, the peptide sequence can be cleaved, thus the fluorophores are free to emit light, no quenching.

The use of activatable probes has been demonstrated for identification of proteases *in vivo*. The activatable probes are sometimes referred to as smart probes or optical beacons since they are only able to emit light upon excitation when the target molecule is present. Fluorescent probes are usually engineered to target a specific molecule or a specific biological event, effectively enabling functional imaging. This is in contrast to other non-targeting fluorescent dyes, e.g. indocyanine green, which is used to visualize vascularization and permeability.

Another way of increasing the contrast is to use probes that are genetically encoded. A transgene (reporter gene) is inserted into a cell. The transgene encodes for a fluorescent protein (FP) which upon transcription will be produced intrinsically inside the animal. The probes can be detected using optical techniques and this modality is called indirect fluorescence imaging, since the fluorescence emitted visualizes the presence of gene regulation or gene expression. Cells can be transfected with a reporter gene and cell trafficking can be imaged. Fusing the FP to a gene of interest makes it possible to image almost any protein *in vivo*. The FPs in indirect fluorescence imaging provides interesting imaging capabilities e.g. protein-protein interactions due to the fact that the protein of interest might be unaffected while the FP emits fluorescence.

There exist several types of fluorescent proteins but the main family is based on green fluorescent proteins (GFP). The probe development is pushing forward to develop GFP emitting and absorbing in the NIR region. Today no NIR FPs is present but yellow and red fluorescent proteins have been reported (YFP and RFP). The contrast is dependent on the fluorophore concentration and the fluorophore position. The contrast is also controlled by so called active probes. If the fluorophore is not active, no fluorescence will be emitted. An ever-present



(a) (b)

Figure 1: (a) The specific absorption coefficient for Rhodamine 6G. (b) Normalized emission spectrum of Rhodamine 6G when excited at 480 nm.

problem using fluorescence diagnostics in biological media is autofluorescence and the background fluorescence. Autofluorescence is the fluorescence emitted by endogenous chromophores, while the background fluorescence is fluorescence originating from fluorescent probes outside the region-of-interest. Ways of theoretically subtract the autofluorescence and the background fluorescence has been reported. Nontheless, the presence of non-specific fluorescence effectively reduces the contrast.

1.2 The role of Tissue Optics in Fluorescece Molecular Imaging

By analyzing the fluorescence spectrum emitted from a fluorophore, it is evident that we can conclude what type of fluorophore it is. The intensity of the fluorescence is dependent on two factors – the excitation light intensity and the concentration of the fluorophore. The coupling between excitation and emission can be described using the fluorescent yield,

$$\eta_{\mathrm{fl}} = \gamma \mu_{\mathrm{a,f}}, \tag{1}$$

where γ is the quantum yield and $\mu_{\mathrm{a,f}} = \epsilon_x c_{\mathrm{fl}}$ is the absorption coefficient due to the fluorophore. The concentration is normally small so that the absorption coefficient is linearly dependent on concentration, ϵ_x is the extinction coefficient at the excitation wavelength for the specific fluorophore. In this laboratory exercise, fluorescent inclusions will be simulated using tubes filled with Rhodamine 6G. Figure 1 shows the specific absorption coefficient and the normalized emission of the dye under 480 nm excitation as a function of wavelength. When a fluorophore is deeply embedded in a scattering volume the bulk tissue will affect the fluorescence spectrum. The intention with this laboratory exercise is

- to retrieve initial experience with a research instrument comprising abilities to image fluorophores in scattering media.
- to investigate what factors will affect the fluorescent spectrum emitted from a deeply embedded fluorophore.
- to conclude how these factors perturb the spectrum.

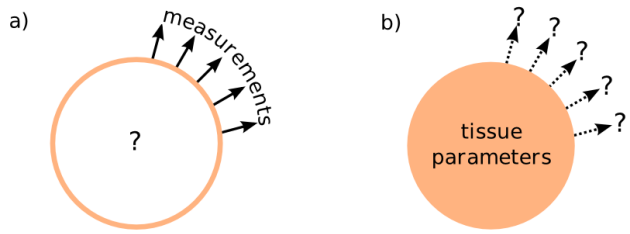


Figure 2: Schematic description of a) the inverse problem, and b) the forward problem.

1.3 Introduction to the inverse problem

The knowledge that will be retrieved within the scope of this laboratory exercise is of ample importance when solving the inverse problem in fluorescence imaging, somtimes referred to as fluorescence tomography. Here the plain 2D image on the surface is used to quantify and localize the fluorophore deeply situated in a scattering body. A schematic picture showing the inverse problem is seen in Figure 2. The reconstruction is a minimization problem where the difference between the measurements (e.g. planar fluorescence images) and a forward model (e.g. the diffusion equation) is minimized. The optimal solution will be the fluorophore distribution that best corresponds to the measured data. In practice the fluorophore distribution is reconstructed using the following scheme:

- Evaluate the optical properties of the volume under study.
- Acquire images (possibly at several spectral bands) for a large number of source positions on one boundary of the scattering body, see Figure 3.
- Extract the shape of the body, as well as the source positions.
- Discretize the body into a finite element mesh.
- Assign optical properties for excitation light and emission light.
- Assign initial fluorophore distribution, usually the same concentration in all nodes.
- Solve the forward model $\leftarrow$ Initial distribution
  $D \leftarrow$ Calculate the difference between model and measurements (regularization methods apply)
  $N \leftarrow$ Update fluorohpore disctribution
  while $D \geq$ predefined limit **do**
    Solve the forward model $\leftarrow N$
    $D \leftarrow$ Recalculate difference between model and measurements
    $N \leftarrow$ Update fluorohpore distribution
  end while
- Done!

The final result is a three-dimensional mesh with a specific fluorophore conncentration in each node – optimally corresponding to the true setting. During the laboratory exercise the full inverse problem will not be solved but the coupling between the measurements and a forward model will be investigated.

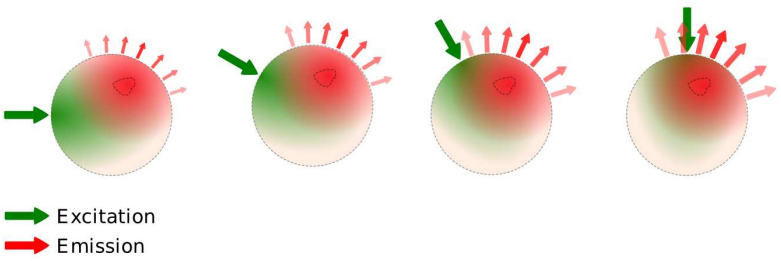


Figure 3: Schematic figure of the fluorescence tomography problem. A light source is scanned over an object of interest and the fluorescence emitted for each source position is recorded.

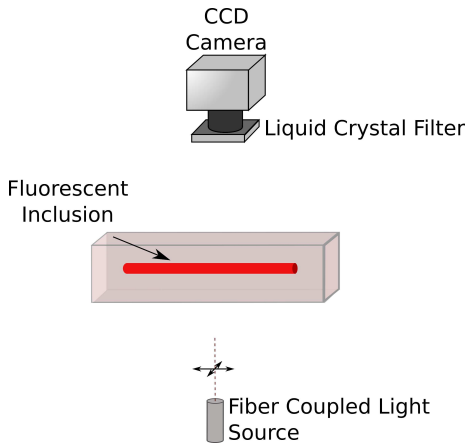


Figure 4: The imaging setup that will be used in this exercise. The light source is fiber coupled and can be easily exchanged. If needed, step motors can also be activated and scanned over the bottom of an object of interest.

2 Home assignment

Read the guidelines in the document carrefully. Look att the Comsol Multiphysics computer exercise dealing with fluorescecne emitted from a spherical inclusion inside a FEM-model mimicing a mouse.

3 Lab assignments

3.1 Setup

For this laboration exercise, we will use the imaging setup shown schematically in Figure 4. As you will see during the lab, the setup is very versatile and can be used for multispectral-transillumination imaging from two direction as well as multispectral-epi-fluorescence imaging. The setup is fully controlled by custom-made software. The lab-instructor will give you a brief introduction to the software. When images are acquired use ImageJ to look at them.

3.2 Estimate μ_{eff} of the optical tissue phantom

The Green's function for a point source located at $\mathbf{r}'$ in an infinite homogeneous scattering material under steady state is given by

$$\Phi(\xi) = \frac{1}{4\pi\kappa\xi} e^{-\mu_{\text{eff}}\xi}, \tag{2}$$

where $|\xi = \mathbf{r} - \mathbf{r}'|$, $\kappa = 1/(3(\mu_s' + \mu_a))$ and $\mu_{\text{eff}} = \sqrt{\mu_a/\kappa}$. Using (2), it is also possible to derive the Green's function for a semi-infinite medium, a slab and a sphere using the technique of mirrored sources. Even though realistic tissue is inhomoeneous and does not have some of the simple geometries mentioned above, the analytical Green's function is a very useful tool to visualize light propagation in scattering media.

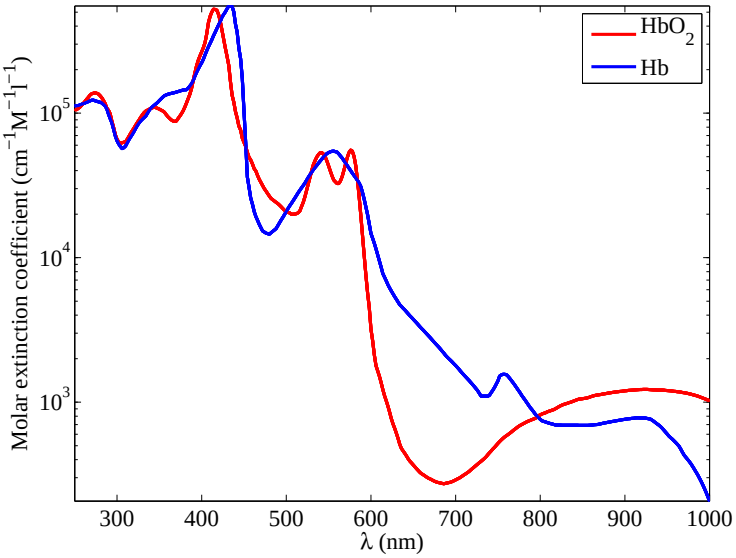


Figure 5: Molar extinction coefficient for oxynated and deoxynated hemoglobin.

In this assignment, we will prepare a liquid phantom (water, intralipid and bovine blood) and estimate μ_{eff} with the help of a white-light source and a CCD camera. μ_{eff} can be used to evaluate μ_a given that μ_s' is known (your lab instructor will provide you with the value of μ_s'). Figure 5 shows the molar extinction coefficient for HbO_2 and Hb.[1]

- Familiarize yourself with the Macroscope control software, take an image, change the filter, etc.
- Start by pouring the phantom fluid (water and intralipid) into the container giving a depth of approximately 1.5 cm − 2 cm.
- Add some small amounts of bovine blood into the phantom. A reasonable volume could be in the range of hundreds of μl.
- Couple the light from the white-light source to the bottom of the phantom.
- Using Macroscope, position the light source in the middle of the phantom and ensure that the field of view of the camera is also centered around the same spot.
- Using Macroscope, take a set of images by looking at the transmission for different wavelengths ranging from 470 to 650 nm, a good number of wavelengths could be 5. Here, it is a good idea to include both the 470 nm and 532 nm wavelengths since they are our excitation sources for the assignments below.
- Measure the size of the field.

[1] The spectra for the water and intralipid have been omitted since they are very dull in our wavelength region of interest

- Using the data collected, how can we estimate μ_{eff} for the different wavelengths?

3.3 Fluorescence emission as a function of concentration

We will now study the influence of the fluorophore concentration in a fluorescent inclusion. To do this, two fluorescent tubes with different concentrations of Rhodamine 6G will be prepared and lowered into the phantom sequentially to the same position. To excite the fluorophores, a frequency-doubled-Nd:YAG laser, emitting at 532 nm, will be used.

- If we double the concentration of a fluorescent inclusion and study the fluorescence, how much do you expect the detected intensity will change with?
- Fill one tube with Rhodamine 6G ($c = 5$ μM) and secure it onto the fluorophore holder. Ensure that it is not leaking!
- Lower the tube into the phantom to a depth corresponding to approximately half the thickness of the phantom and measure the emission using Macroscope. Make sure that you have remembered the depth of the tube so that it can be reproduced using a second tube.
- Fill the second tube with Rhodamine 6G ($c = 10$ μM) and measure the emission from it by lowering the tube to the same position as the first tube.

For the remaining lab assignments only one concentration will be used. Select one concentration and, if needed, prepare it to be used.

3.4 Fluorescence emission as a function of depth

This part of the exercise aims to develop an understanding for how the emission from a fluorescent inclusion changes with different depths.

- Decide upon four depths and measure the emission using Macroscope.
- Analyze and compare the images at the four different depths for different wavelengths. Acquire images at spectral bands ranging from 550-650 nm (use 5 or more spectral bands), effectively covering the emission spectrum for Rhodamine 6G. Do the intensities between different wavelengths differ? How does the depth influence on the differences? If you find it hard to compare all the images you can extract one, or several, point intensities from the images using a Matlab-routine `extractpoint.m`. In this way you can create a low resolution spectrum in one point based on the images. Why is it low resolution?
- Using the knowledge gained from the exercise so far, can you explain why a separation of depth and concentration distribution of a fluorescent inclusion can be ambiguous?

3.5 Fluorescence emission as a function of excitation source position

So far, the source has been fixed to a position straight under the fluorophore. In this assignment, we will investigate how the emission changes as we start to sweep the source in a line perpendicular to the direction of the inclusion.

- Using Macroscope, setup a sweep along a line with 8 or more acquisition points.
- Analyze and compare the images at the different source positions. Have we actually gained any new information concerning the location and/or the concentration of the fluorescent inclusion?
- Can you think of any circumstances when it would be advantageous to translate the source, within the scope of fluorescence imaging?

3.6 Fluorescence emission as a function of excitation source position and wavelength

We will now change the excitation source from 532 nm to 470 nm, and perform the same sweep as described in the previous assignment.

- What do you expect to happen when we change the excitation wavelength to a shorter wavelength?
- Couple the 470 nm laser into the fiber.
- Perform the sweep.
- Analyze and compare the images with the images obtained from section 3.5.
- Which parameters have been affected when we changed the source?
- Disregarding the differences in light propagation, how would you expect the detected intensity to change?

3.7 Introduction to the inverse problem

We now want to use the experimental data in order to see if we can assess the depths of the fluorophore in assignment 3.4. Even though the emitted fluorescence changes with the depth, it is still hard to explicitly assess the depth. For this purpose we need a forward model. The forward model, named `fluorescenceslab.mph`, can be downloaded from the course homepage. The assignment is to input the optical properites that you have evaluated for excitation light and two emission bands, into the forward model. In Comsol, position the cylinder at several depths and model the excitation as well as the emission bands. Create a measurable quantity that is independent of concentration and illumination/detection angles (see Comsol Multiphysics computer exercise for guidance). Look at the measurable quantity as a function of cylinder depth, we can call this the calibration curve. Use this calibration curve to determine the true depths by creating the same measurable quantity using the experimental data. Do you get the true depths?

4 Report

Briefly summarize the factors that affect the fluorescence as you have investigated during the lab. Plot the measurable quantity (both experimental and modelled) as a function of fluorescent inclusion depth.