Fluorescence imaging with medical applications

This lab session will deal with multi-spectral fluorescence imaging with applications in medical and environmental diagnostics. We will use an imaging system for real time measurements. In parallel, a point-monitoring spectrally resolving system will be used facilitating the interpretations.

Background

Laser-induced fluorescence (LIF) diagnostics is a method where spectroscopic measurements on tissue can yield information on the biochemistry and morphology, and indirectly on the pathology of the tissue. The main motivations are that LIF measurements are safe and non-invasive and can be performed in real time during a clinical examination.

Fluorescence measurements can either be performed by point measurements or in an imaging geometry. In point monitoring mode, the entire fluorescence spectrum is obtained from one small tissue spot. In imaging mode, a larger area is monitored, but usually the fluorescence is detected with a much lower spectral resolution. In addition, LIF on tissue can either use the differences in autofluorescence (the natural fluorescence from the tissue itself) between normal and diseased tissue, or the fluorescence from a tumour marker, a drug that is accumulated in the tumours and that has a very strong and specific fluorescence. These topics will be discussed in the following sections.

The basics of fluorescence

The process of light emission from a fluorescing molecule (fluorophore) can be described in a Jablonski diagram, see Fig. 1. If an incoming photon has an energy that corresponds to the gap between two energy bands in the molecule, it can be absorbed. The photon energy will thereby be used for excitation of the molecule to the higher energy band. Excited states are unstable and the molecule will return to the ground state. The de-excitation may follow a number of different pathways, as illustrated in Fig. 1. The labelled levels are electronic levels, corresponding to the energy levels of atoms. S0, S1... are singlet states for which the sum of the electron spin quantum numbers is zero, while T0, T1... are triplet states for which the spin of one electron has changed sign. For large molecules the intervals between the levels are very small and the states overlap due to molecular interactions. When a photon is absorbed by a molecule it will not necessarily excite the molecule to the lowest vibrational level in the excited electronic level, but more likely to a higher vibrational state. This is a result of the Franck-Condon principle stating that during the rapid ($\approx 10^{-15}$ s) absorption process, the atoms do not change their location in the vibrational motion. When a molecule is excited to a high energy level, a rapid relaxation to the lowest rotational-vibrational state of S1 will follow. The short time scale ($\approx 10^{-12}$ s) of this relaxation is due to the high density of rotational vibrational levels. From S1 the molecules can proceed to the state S0 through radiationless kinetic interactions. This is called internal conversion (IC).
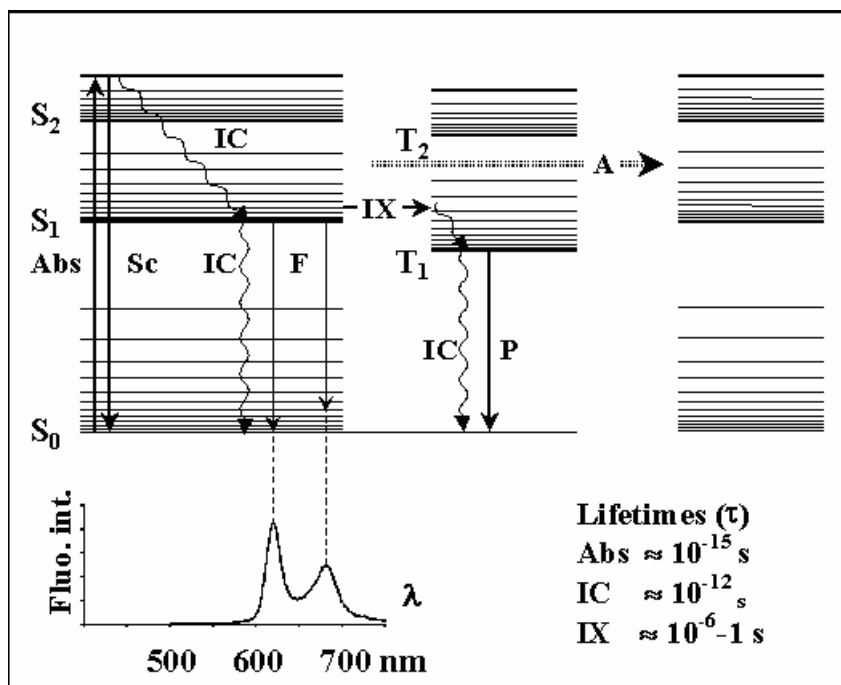
Figure 1: Jablonski diagram showing the various decay paths from an excited state of a molecule. In the lower part of the figure, a fluorescence spectrum from haematoporphyrin in ethanol is shown. The abbreviations are: Sn, singlet states; Tn, triplet states; Abs, absorption; Sc, scattering; IC, internal conversion; F, fluorescence; IX, intersystem crossing; P, phosphorescence; A, transfer to other molecules. Also the approximate time-scale for some processes is shown down right.

Alternatively, the de-excitation may result in the emission of a photon and this process is called fluorescence. Since the transition may be terminated in any of the rotational-vibrational states of S0, the energy of the different photons will not have a distinct value, but rather a broad distribution. Thus, a fluorescence spectrum from a molecule will be broad, most often without any significant structures. The form of the spectrum will reflect the probability of transitions to the lower levels (S0). In the lower part of Fig. 1 the fluorescence spectrum of haematoporphyrin (a sort of tumour marker, or photosensitizer, as will be discussed later on) is shown.

Once the pathway absorption-IC-fluorescence is completed, the molecule is back in its original state and configuration. Hence, the fluorescence process is non-destructive and reversible, which is an advantage in, for instance, medical diagnostics.

Although spin forbidden, a transition to the triplet system may occur. Also in the triplet system a rapid internal conversion to the lowest excited state will occur. Since a transition to S0 is spin forbidden, this will proceed at a much lower rate (t » 10-6-1 s) than the transition S1 ® S0. This process is called phosphorescence and is less often observed at room temperature.

Several other paths are possible for the excited molecule, such as energy transfer to other molecules, electron transfer, excimer formation and excitation to repulsive states leading to molecular dissociation. These processes are indicated with an A in Fig. 1. Many fluorescent molecules have one important feature in common, that is the unbroken chain of conjugated double bonds, i.e. every second bond is a double bond. The structure of

haematoporphyrin is shown in Fig. 2. This is a fluorescent molecule used for fluorescence diagnostics and photodynamic therapy of tumours.
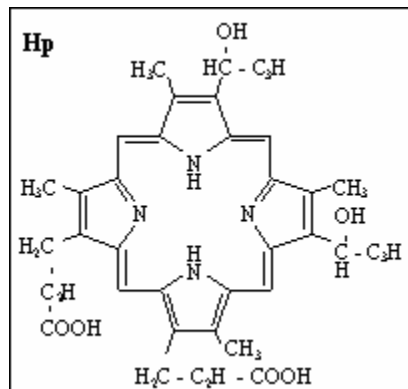

Figure 2, Structure of haematoporphyrin (Hp).

With the knowledge of the fluorescence properties of important tissue fluorophores, a fluorescence recording of an unknown sample will yield the relative contribution of each fluorophore. If the fluorescence characteristics are the same as for the isolated fluorophores, the concentration of the fluorophores can be estimated. This is, however, not always the case. Rather, the fluorescence properties are dependent on environmental factors such as polarity and pH.

Another important aspect of fluorescence is the rapid relaxation in the excited as well as in the ground state. The molecule looses some of its excitation energy by relaxation. Also, redistribution of solvent dipoles around the fluorophore and specific interactions, such as hydrogen bonding, contribute to this relaxation procedure. Thus, the energy of the fluorescence photons is lower than that of the excitation, or in other words, the fluorescence wavelength is longer than the excitation wavelength. This is called Stokes shift and is different for different molecular environments. Hence, a general knowledge of the molecular environment is required for an adequate fluorescence diagnosis.

Medical Applications
In this laboratory exercise we will study some tumour phantoms and see how fluorescence can be used for medical tumour diagnostics. Fluorescence spectroscopy is a very sensitive technique for qualitative as well as quantitative measurements of tissue constituents. Traditionally, fluorescence emission spectra have been used almost exclusively, although there are many other aspects of fluorescence that can be used for diagnostics, e.g. differences in peak excitation wavelength, the fluorescence lifetime and the polarisation of the fluorescence. Among many advantages of fluorescence diagnostics, the most important one is that it can easily be used for in vivo investigations. By using optical fibres, inner organs, such as the lung or urinary bladder, can be accessed. Furthermore, fluorescence spectroscopy may be performed utilizing imaging techniques, making fluorescence investigations less time consuming especially when screening larger areas. The only side effect that has been proposed thus far, the risk of carcinogenic effect of UV light, can be overcome by adjustments of the excitation light wavelength. The one major limitation of fluorescence diagnostics is the limited light

penetration in tissue, less than 1 mm for most tissues. This, however, is also an advantage since the shorter the penetration depth, the better the spatial resolution in fluorescence imaging that can be achieved. The most important applications of fluorescence spectroscopy are fluorescence microscopy and cancer diagnostics.

Autofluorescence

The autofluorescence, or the tissue's own endogenous fluorescence, is caused by several different fluorophores. Some of the common tissue fluorophores are collagen and elastin present in connective fibres, tryptophan present in most proteins and flavins and nicotinamid adenine dinucleotide active in the digestion of cells, see Fig. 3. The spectra are also influenced by the optical properties of the tissue. Strong absorbers, such as haemoglobin, can absorb fluorescence light at certain wavelengths and thus change the appearance of the fluorescence spectrum, creating false dips and peaks. Haemoglobin may also decrease the overall intensity of the fluorescence spectrum, without changing its shape, by absorbing the excitation light.

Naturally, the shape of the spectrum also depends on the excitation wavelength, since this will determine which energy transitions in the fluorophores are possible. Most often, excitation in the ultraviolet or blue wavelength region is used for LIF studies of tissue. Clinical measurements have been performed on many different tissue types. As an example, Fig. 4a shows the fluorescence, following 337 nm excitation with a N2 laser, from normal and adenomatous tissue in vivo in the colon (large intestine). Adenomas are lesions (Sw. lesioner, förändringar i vävnaden, skador) that can be pre-malignant (Sw. malignant= malign, elakartad), and a certain percentage of them will develop into tumours if left untreated.
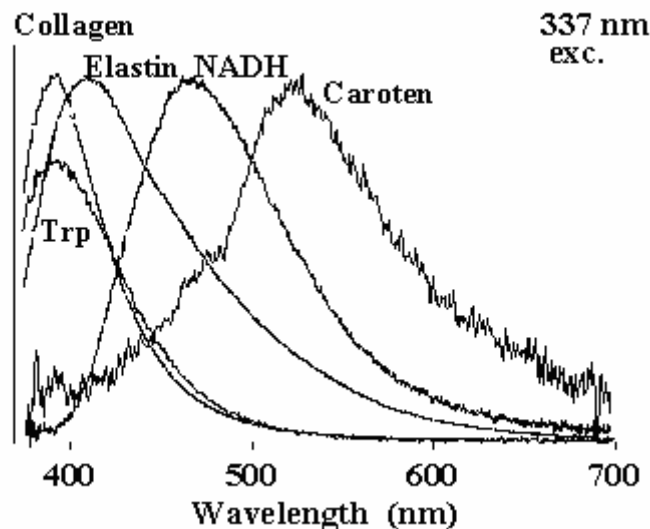

Figure 3, Fluorescence spectra from some tissue fluorophores after 337 nm excitation.

It can be seen in the figure that the fluorescence from healthy tissue has its highest peak around 390 nm, close to the fluorescence peak of collagen. However, in adenomatous tissue, the highest peak is at a higher wavelength, around 460 nm, where NADH has its fluorescence peak. Thus, a biochemical feature in the different tissues can be detected by fluorescence measurements, and these can serve as indicators of cancerous or pre-

cancerous lesions. When moving towards longer excitation wavelengths, e.g. 405 nm as seen in Fig. 4b, one can see a decrease in autofluorescence from adenomas as compared to healthy tissue, probably because of haemoglobin absorption of the excitation light. The high peak at 635 nm in the adenoma spectrum of Fig. 4b is due to a tumour marker, which will be discussed in the next section.
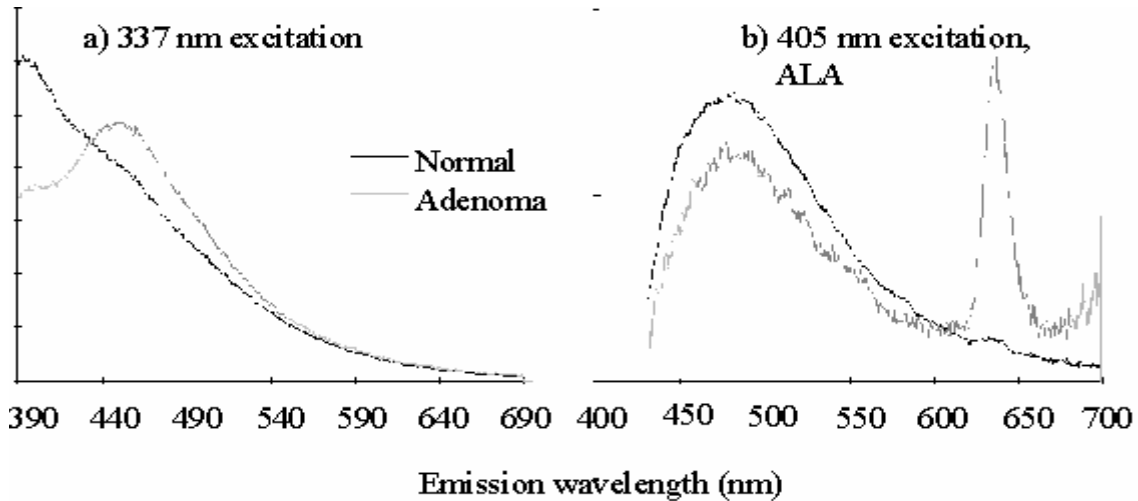

Figure 4, Fluorescence spectra from normal and adenomatous tissue. a) at 337 nm excitation and b) at 405 nm excitation with ALA present

Fluorescent tumour marking drugs

In some cases, the ability to detect lesions by LIF can be further enhanced by the use of fluorescent tumour markers, so called photosensitizers. These are drugs that can be given to the patient (e.g. orally, as an injection or as a cream for skin tumours) before the examination and that are accumulated in tumours or pre-cancerous lesions to a higher degree than in healthy tissue. They are fluorescent, and will add features to the fluorescence spectra from diseased tissue that will help distinguish diseased from healthy tissue.

We have previously mentioned hematoporphyrin, see Figure 2. This can be used to make HpD, hematoporphyrin derivative, which is a well known tumour marker that has been used for LIF detection and photodynamic therapy in many studies throughout the years. The substance has a strong absorption band (the Soret band) near 400 nm and characteristic fluorescence peaks in the red at about 630 and 690 nm. There are, however, some problems related with the use of HpD. The rate of clearance from the body is rather slow, and following an injection of a normal dose of HpD, the patient will remain sensitive to sunlight for four weeks or more. Therefore the search for other photosensitizers has been a topic of intense research.

Vegetation fluorescence

In this section, chlorophyll and its relation to vegetation fluorescence are described. The first steps in the photosynthesis include the absorption of a photon by a pigment molecule, for example chlorophyll a or b. Upon irradiation of light, the excitation energy is transferred to a reaction centre where the chemical reactions constituting the photosynthesis take place. When excited around 405 nm the fluorescence from

chlorophyll a consists of two prominent peaks, positioned at 685 and 735 nm. Excitation of chlorophyll b does not result in any characteristic peak since the absorbed energy is being transferred to chlorophyll a very quickly.

The strong absorption from chlorophyll masks less intense colours from other substances. In the autumn when the chlorophyll decays and the valuable magnesium is withdrawn from the leaves, these substances, including carotene and quercetin, show as the typical yellow, orange and red colours of senescent leaves.

Instrumentation

During the lab, two systems will be used. One is a point monitoring fluorescensor, which is not an imaging system but which will be used to explain the spectral information in the images made with the other instruments. The imaging system is a CCD camera with a tunable filter to select wanted detection wavelengths.

Point-monitoring fluorosensor

The general lay-out of the point monitoring fluorosensor is demonstrated in Figure 5. As a light source for inducing fluorescence we use a violet diode laser with a nominal operating wavelength of 375 nm. We note that below 400 nm the eye safety regulations are relaxed by a factor of about 1000 since the cornea no longer transmits the radiation. Thus it is particularly convenient to be able to operate at these sufficiently short excitation wavelengths. The diode laser is placed in a tube together with a collimating lens.
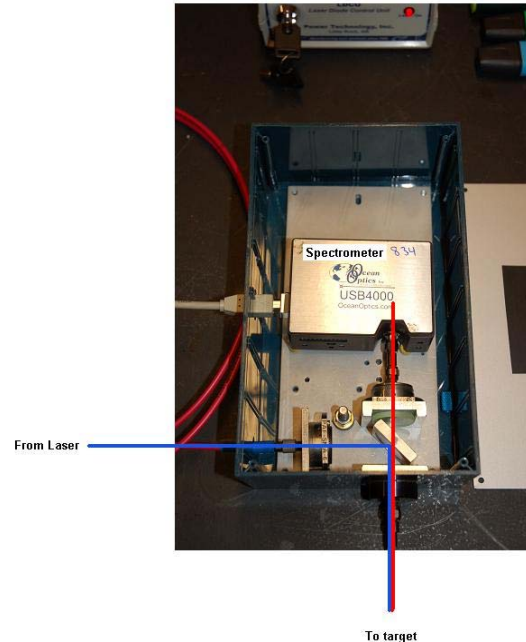

Figure 5, Point-monitoring fluorosensor

The output light is cleaned up for broadband spontaneous emission using a narrow-band interference filter and focused into a 600 mm diameter optical fiber by a fiber-port lens assembly. Before entering the fiber the light beam is reflected off an appropriate dichroic

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beamsplitter. Fluorescence is induced in an object placed in contact with the distal tip of the fiber, where about 1.2 mW of laser power is available, and Stokes-shifted fluorescence light is guided back through the fiber. Since the spectrometer is fiber-coupled, the fluorescence light is focused by a fiber port into a short fiber which is connected to the spectrometer. The spectrometer is equipped with a 100 μm slit. Elastically backscattered diode laser light is effectively blocked by a colored glass cut-off filter placed behind the dichroic. The grating (600 lines/mm) disperses the light and a spectral region of about 350 - 1000 nm is captured on the 3648 element CCD detector. A spectral resolution of about 5 nm is obtained, which is fully adequate for environmental and medical monitoring. Since the laser source is not pulsed and the detection is not gated in contrast to the situation in more complex systems, there is clearly a risk that ambient light would add an undesired spectrum on top of the fluorescence spectrum. However, since the fiber is put in contact with the specimen under study, the ambient light is effectively shadowed away by the fiber itself in most cases. Under special circumstances (observing weak fluorescence) a reduction of the ambient light or a shadowing by the measuring site could be required. In indoor work with luminescent lamp illumination the presence of sharp mercury line peaks in a recorded spectrum would alert on problems due to ambient light.

CCD camera with tunable wavelength filter for multicolor imaging

The imaging system that will be presented during the exercise consists of a CCD camera and a liquid crystal tunable filter (LCTF) attached to the camera, see figure 6. With this tunable filter the wanted detection wavelengths can be easily selected.
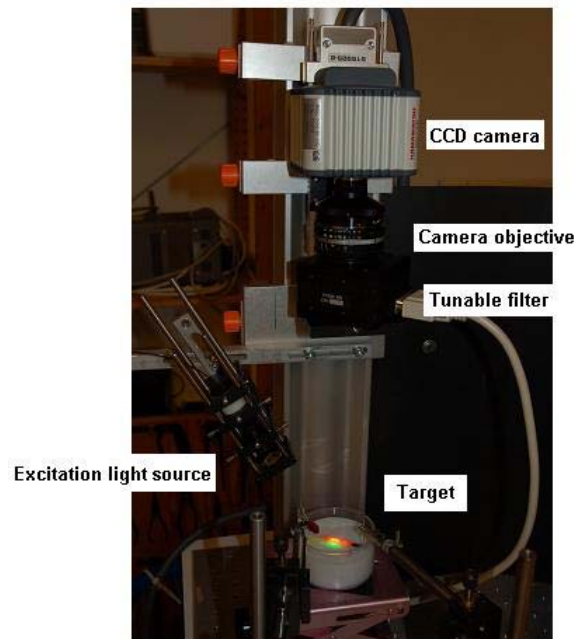

Figure 6, CCD imaging setup

The filter can be tuned from 400-700 nm and each detection wavelength is approximately 20 nm wide. The LCTF works basically as a multiple Lyot filter. A Lyot filter consists of a birefringent crystal and two crossed polarizers, where the wavelength-dependent

polarization rotation leads to wavelength-dependent loss at the polarizers. By changing the applied voltage across the crystal the transmitted wavelength can be tuned. According to the Lyot design, the thickness of each crystal is half the thickness of the previous one. In this way one can combine a good wavelength resolution with a large period of the transmission peaks.

Preparatory exercises

Read in the course literature chapter 4.1-4.5 and in chapter 7 (note!) the sections 6.1-6.3. Make sure you and your partner for this practical exercise bring one passport, a credit card and some bills.

Exercises during the lab

During the laboratory exercise you will study fluorescence from a number of samples e.g. leaf, fluorescent dyes and fluorescent pens (sv. överstrykningspennor). As a tumour-tissue phantom you will be given a piece of wood with HpD applied onto it. The fluorescence measurements will be performed using the point-monitoring fluorosensor as well as the fluorescence imaging system.

1) Point-monitoring instrument

Use the point-monitoring instrument (guidance of how to use it is given last in this document). The laser excitation source can and should be changed. You can choose between lasers emitting at 375 nm, 473 nm and 532 nm. Investigate the samples given below and try to answer the questions. When using the point-monitoring instrument be sure to acquire spectra from several spot on the target.

Measurement procedure
1. Acquire a background spectrum, i.e. with no laser irradiating the target.
2. Click the "background subtraction option (dark lamp)" in the SpectraSuite software. The software will now subtract the background from your spectra. It is important to know that the background spectra stored is only representative for the present conditions. Hence you should acquire a new background whenever the ambient light conditions change or you change the exposure time.
3. Put the fiber in contact with the target and acquire aspectrum, remember to save the spectrum (in correct file format!).
4. When you want to change the laser excitation source **BE CAREFUL**, remember that **LASER SAFETY** always apply! First block the laser and then uncouple the optical fiber.

Samples to investigate
- fluorescent pens on black piece of paper; blue, green, yellow and orange
- wood with and without HpD-staining
- croton leaf; green and red parts
- fluorescent dyes on an optical phantom

Answer the following:
Why does the supervisor's sweater appear red?
Why does a healthy leaf appear green?
Does the blue pen fluoresce?
Why does the "autofluorescence" from the stained piece of wood decrease?
By studying the fluorescence spectra obtained from different parts of the leaf, would it be possible to determine the concentration of chlorophyll in the different parts?
What happens when you change the excitation source?

2) CCD camera with tunable wavelength filter for multicolor imaging

Here you will work with a imaging setup. First you will study the same objects as before and compare the differences between the two instruments.

Measurement procedure (using VsGui and Wasabi)
1. Place the target under the CCD-camera setup
2. Adjust the lens to get a sharp image, alternatively adjust the distance to target.
3. Make sure the excitation spot is irradiating the region-of-interest.
4. Adjust the filter
5. Adjust the exposure time, the camera system has a 12 bit A/D converter hence the saturation level is approximately 2^{12}=4096 counts. Do not saturate the camera!
6. Acquire image.

What samples to investigate:
- fluorescent pens on black piece of paper; blue, green, yellow and orange
- wood with and without HpD-staining
- croton leaf; green and red parts
- fluorescent dyes on an optical phantom

Answer the following:
At what wavelengths do you get a strong signal from the croton leaf?
Can you correlate the signals to the acquired point measurements?
Does the signal differ at the different parts of the leaf? Why?
What happens when the excitation light is not uniformly irradiating the sample?
How can one compensate for the non-uniform irradiation?

3) Tumour delineation

The imaging system, and its predecessors, has been used in clinic for skin tumour delineation. The purpose is then to acquire images of the skin in different wavelength bands and use these to make a contrast function based on the spectroscopic properties of the fluorescence that is high wherever the tumor is and low where the skin is normal.

In this laboratory exercise we will make use of some common fluorescent dyes. It is important to mention that these are not for medical use. Despite this the dyes are good targets to study in fluorescence imaging applications.

A tumor-sensing drug has been administered to the simulated patient (in this case an optical phantom with similar optical properties as human skin). What we expect is that the tumor will express high levels of the fluorescent marker while surrounding regions will not. The absorption (extinction coefficient) and intrinsic fluorescence spectrum of the (fictitious) fluorescent tumour marker is shown below.
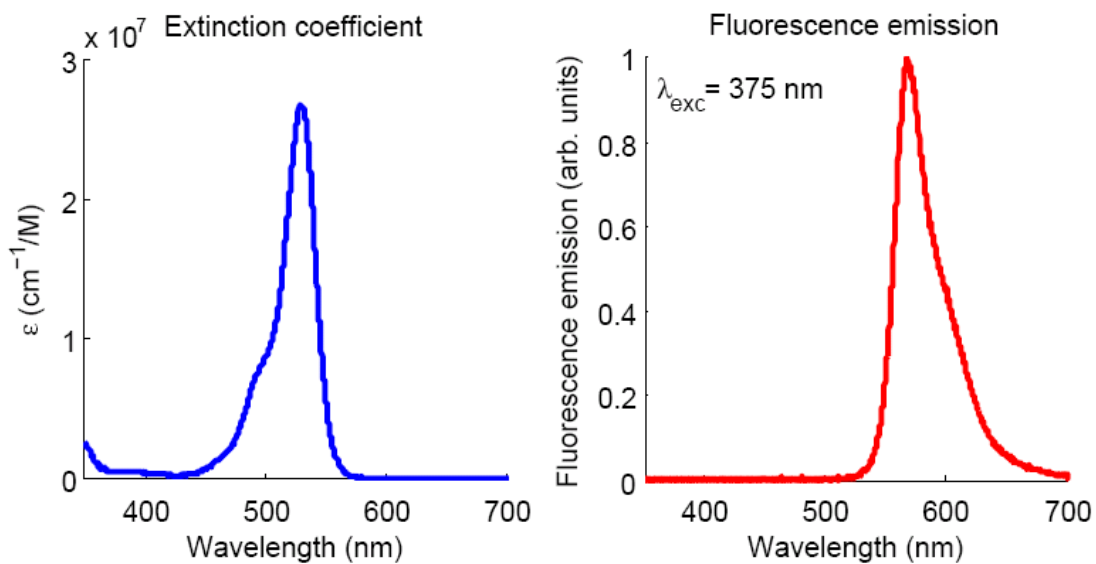
The intrinsic absorption and fluorescence spectrum of the "Fluorescent tumour marker" used in the laboratory exercise. The fluorescence tumour marker is in fact not a real tumour marker but instead Rhodamine 6G is used.

The optical phantom is in addition to the tumour marker emitting autofluorescence, as is human tissue. There might be other fluorescent substances in the tumor.

Your task is to delineate the regions where the tumour marker is present, i.e. the tumour. Do this by constructing a contrast function that show high contrast for the tumour and low contrast where no tumour is present.

Use the previously acquired point-monitoring spectra to discuss how to make the image acquisition of the multispectral dataset and how to construct the contrast function. Remember that you can whenever you like change the excitation source. Use the point-monitoring instrument to investigate suspicious regions whenever in doubt.

Measurement procedure
Here you will use the LabView program "Select_filter.vi". This program facilitates the acquisition since you will acquire several images in different spectral bands (multispectral dataset).
The measurement procedure is similar to what was mentioned above with some differences:

1. In Select_filter.vi you need to set the specific exposure time for each spectral band. Investigate proper exposure times using Wasabi and put them into Select_filter.vi.
2. Acquire the multispectral dataset (remember to input the file name root).
3. After the image acquisition block the laser and acquire a background dataset – again remember to change the file name root!
4. The images will be processed using Matlab – see guide in the end of this document.

Answer/Do the following
Discuss why the point-monitoring and the imaging spectra does not look exactly the same.
What are the differences, in the acquired images, when changing the excitation source?
Based on your experience with the images above – construct a "contrast function" that has high contrast for the fictitious tumour marker.

What does the program-template evalImages do (answer this question before you insert your script)?

Report

The report should contain an introduction to fluorescence multi-spectral imaging, explain the experimental steps and the results achieved with an overall discussion about the results and answers to all of the questions addressed above. Include a comparison chart summarizing the benefits and disadvantages of the different systems compared to each other.
Further present the contrast function that you constructed with a motivation of why this function produces high contrast for the target-of-interest.

Please hand in the report to the tutor one week after the exercise (preferably via e-mail).

Guide to hardware and evaluation-software

Point-monitoring system

How to acquire and save a spectrum:

Open SpectraSuite

- Acquire a background by holding the fiber into the air by pressing the button "play", see figure 7 below. This step needs only be done once.
- Acquire a fluorescence spectrum by gently pressing the fiber end onto the sample and press the button "play". The integration time can be changed when the screen appears as seen in the figure below.
- Save the spectrum by pressing the "Save" button and chose the correct catalog; My Documents\FI_2007 and enter a filename. Choose desired spectrum as "Processed spectrum" and File type as "Tab delimited".
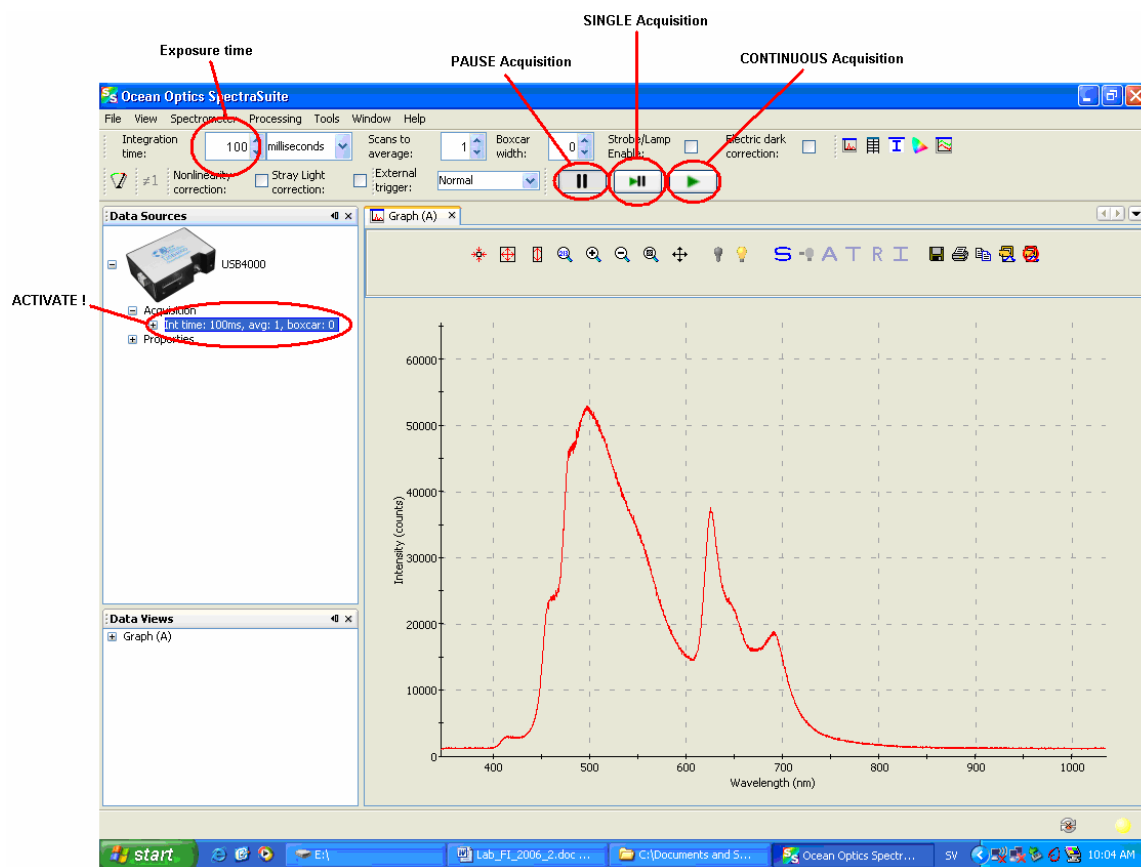

Figure 7 SpectraSuite for point-monitoring acquisitions.

How to evaluate the spectra:
Open Matlab, use the m-file "plotUSB4000" to both read the fluorescence spectra and to plot them. Take printouts of the spectra since you will make use of those further on.

CCD camera with tunable wavelength filter for multicolor imaging

How to select filter (spectral-band):

Open VsGui

A simple program to control the tunable filter is VsGui.exe, placed on the desktop. The program simplifies the change of transmitted wavelength band. By setting the program to e.g. 550 nm, the filter will transmit light between 540-560 nm.
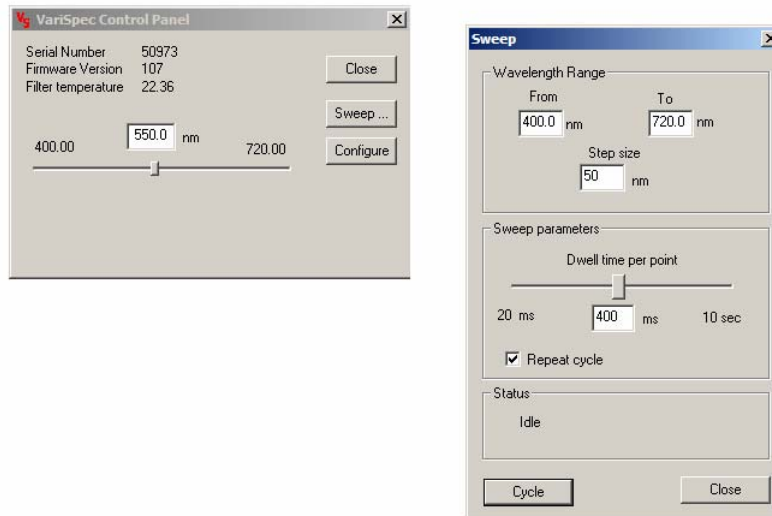

Figure ,8 VsGui for controlling the tunable filter.

By clicking the "Sweep"-button the right hand window in figure 8 pops up.
The sweep-mode lets the user specify a wavelength interval within the filter that is sequentially swept with some wavelength step size and some dwell time per wavelength band.

How to acquire images:

Open Wasabi

The program to control the camera acquisition is called Wasabi and is placed on the desktop. The GUI is shown in figure 9.
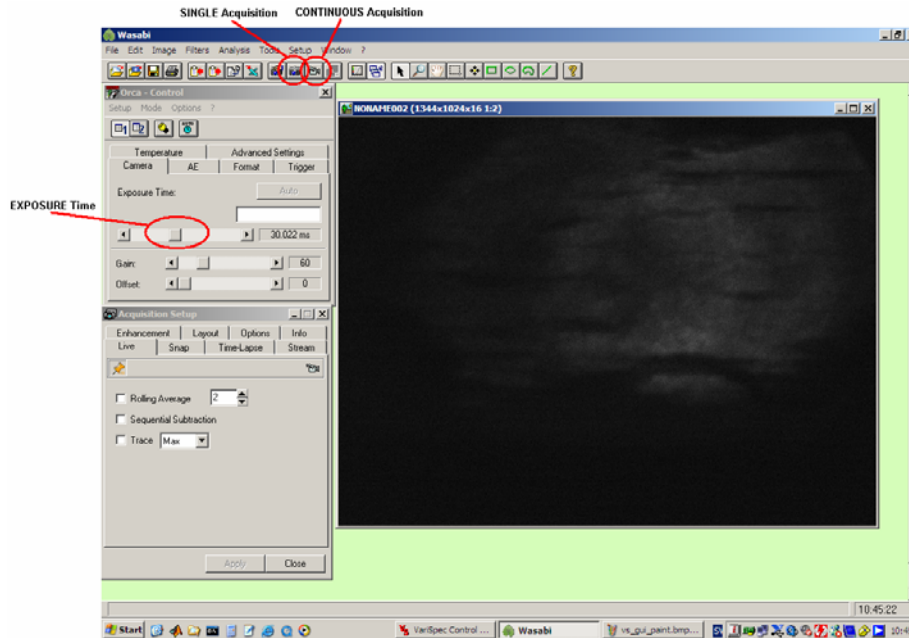
Figure 9 Wasabi for image acquisition

When the filter is adjusted, or put in sweep mode, images can be acquired by clicking the buttons, indicated in figure 9.

How to acquire a multispectral dataset
To make the acquisition a bit more automated a program has been developed in LabView that controls the filter and the camera. The program is called "Select_filter.vi"

Open Select_filter
The program combines the features of both VsGui and Wasabi is seen in figure 10.
- Start the program, using the arrow pointing towards right in the top-left corner.
- Specify the directory (directory is placed in C:\imaging\output\"*directory*")
- Specify the filename (filename is placed in directory and is assigned additional numbers to identify the wavelength.)
- Write the wavelength bands in the green square (Specify wavelength bands).
- Define the exposure times to be used – make sure that you don't saturate the detector (typically 50 ms might be good, but try this).
- Define the number of filter positions, i.e. the number of wavelength bands.
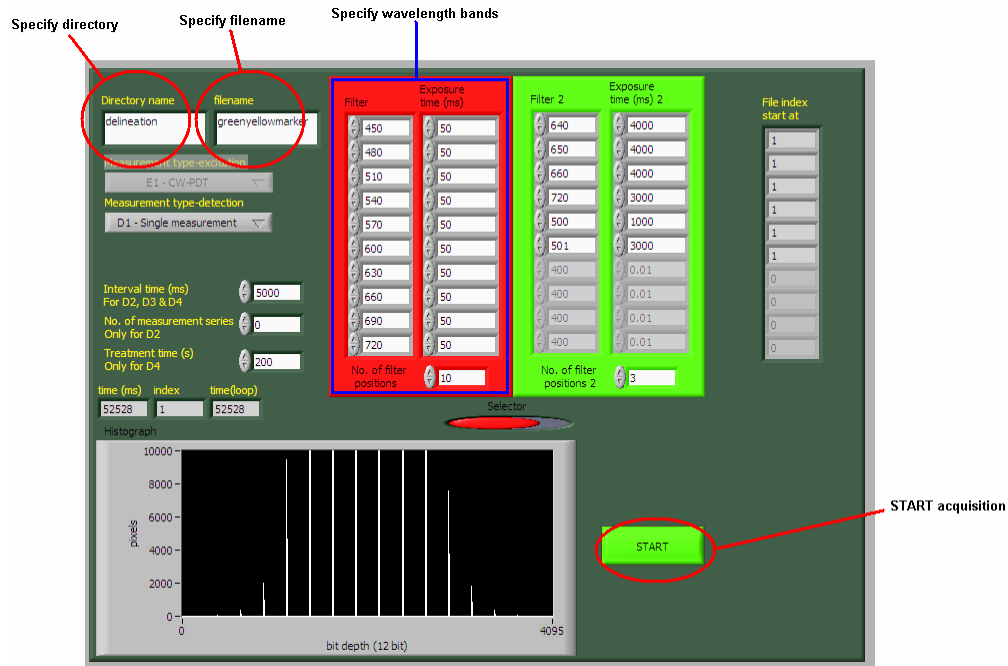- Press START
- Press Start Measurements

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Figure 10 Select_filter for multispectral image acquisition

How to evaluate images (after acquisition with Select_filter.vi):
The multispectral dataset can be treated as an image stack. The task is to find out how to create a spectrum from the multispectral image stack.

Look at the images and select one or several pixel (x- and y-coordinates), in each coloured area, in which you want to extract the spectrum. Use the point-monitoring fluorosensor to acquire a spectrum from some of the pixels, selected above.

Use the Matlab-script "evalImages.m" in the folder C:\imaging\evaluation\ and insert a script that extracts the spectrum in a pixel, based on the multispectral dataset.