Fluorescence spectroscopy in tissue for identification and temperature control of embedded lesions

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2005

Link to publication

Citation for published version (APA):
Fluorescence spectroscopy in tissue for identification and temperature control of embedded lesions

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Licentiate Thesis
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LRAP-340

May 2005
Abstract

Fluorescence spectroscopy in two different biomedical applications have been studied. One field has been to explore whether fluorescence spectroscopy can be used to determine the depth of a fluorescent object inside a tissue-like medium. Recorded fluorescence spectra depend on the localization of the fluorescent inclusion and may provide additional information for optical tomography. The second main project has involved temperature measurements, based on the fact that specific crystals change their fluorescence spectrum when the temperature is altered. This technique could be applied during interstitial photodynamic therapy of malignant tumours, by attaching crystals to the fibre tips inserted into the tumour during treatment. The temperature is an important parameter for treatment outcome and any bleeding at the fibre tip could also be detected, which could reduce the treatment efficiency.
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Chapter 1

Introduction

This licentiate thesis is gathered around the concept of fluorescence spectroscopy in medical physics. Fluorescence spectroscopy has been used in several decades, for example for tumour detection and diagnosis. A great advantage with fluorescence monitoring is that it is a non-invasive method and can be performed in real-time. Fluorescence can be used to help physicians in finding areas with cancer that may not be visualized during white-light examination. How is fluorescence induced in tissue then? To be able to detect fluorescence light, the tissue needs to be illuminated with light of a specific wavelength. Different molecules in the tissue can absorb the excitation light, and after a short time period the molecules will emit new light, called fluorescence. Characteristics of fluorescence are that the emitted light is a broad wavelength band and that the emission consists of longer wavelengths than the incoming light.

In this thesis fluorescence have mainly been used in two different applications. The first to be mentioned is within a multidisciplinary field called molecular imaging. My part within this project has been to develop a method to determine the depth of a fluorescent object embedded in tissue (or rather a tissue-like phantom), by studying the emitted fluorescence. The optical properties of tissue affect the fluorescence light as it propagates through tissue, and depending on the depth at which the fluorescence is induced the light is affected to a lower or higher degree.

The second part of my work has been to study temperature increases during simulations of a cancer treatment called interstitial photodynamic therapy. The method to measure the temperature has been to use certain crystals attached to optical fibres. The special feature with these crystals is that the spectral shape of the emitted fluorescence changes when the temperature in the crystals increases or decreases. By studying the spectral changes the temperature can be determined within an object where the crystal is located.

This thesis has four main chapters. Chapter 2 describes the interaction of light with tissue and Chapter 3 explains fluorescence and different detection techniques of fluorescence light. The field of molecular imaging is introduced in Chapter 4. Chapter 5 describes the background of optical temperature measurements.
CHAPTER 2. LIGHT INTERACTION WITH TISSUE

Incoming photons

Photons scattered in to the s-direction

Photons leaving the boundaries

Photon scattered in to another direction

Figure 2.3: The different terms in the radiative transfer equation.

approach is a statistical method where Monte Carlo simulations are performed. Both methods will be described in the following sections.

2.4 Diffusion approximation

Radiation through a medium, where scattering is much larger than the absorption, can be described with the diffusion approximation.[1] In order for the diffusion equation to be valid it is necessary that the light has become diffuse as it hits a detector, therefore the source and detector must be separated. In tissue the light is assumed to be diffuse after approximately ten scattering events. The concept of the diffusion approximation is that the radiance is expanded into spherical harmonics. The diffusion approximation has the advantage that it can be solved analytically for simple geometries. The diffusion approximation is described by the diffusion equation, see formula 2.4:

\[ \frac{1}{c} \frac{\partial \phi(r,t)}{\partial t} - D \nabla^2 \phi(r,t) + \mu_a \phi(r,t) = q(r,t), \]

(2.4)

where \(c\) is the velocity of light in tissue, \(\phi(r,t)\) is the light fluence [W/m²], and \(D\) is the diffusion coefficient, described with formula 2.5:

\[ D = \frac{1}{3(\mu_s + \mu_t^*)}. \]

(2.5)

2.5 Monte Carlo

A statistical method to approximate the radiative transport equation is to perform Monte Carlo simulations of photons in a medium.[5, 6] The light distribution is simulated with a random walk of photons (or photon packages) inside the tissue. By repeating this random walk for a large number of photons, good statistics can be achieved. Different outputs can be obtained from the simulations: absorption, reflection, transmittance and light fluence. The three main input parameters to a Monte Carlo program are the optical properties of the medium, \((\mu_s, \mu_a, g)\), the refractive index and the geometry of the tissue. The principle of a Monte Carlo simulation is that a photon package is launched into the medium. At the boundary the photon package can either be transmitted or reflected, if reflection occurs this event is saved as reflection and a new photon package is launched. If the photon package enters the tissue, the step size of the propagation and the scattering angles are drawn from the probability distributions.
determined by the input parameters, and the photon package is moved to its new coordinates. At each interaction site part of the package can be absorbed, and the absorbed part is saved at that specific location. The random determination of scattering and absorption of the photon package is repeated until the photon package leaves the boundaries of the medium or until the package is too small to survive.

2.5.1 Fluorescence Monte Carlo

It is also of interest to study fluorescence light with Monte Carlo simulations. There exist different Monte Carlo codes to simulate fluorescence. A layered accelerated fluorescence Monte Carlo code has been developed by Swartling et al. This code has been used to simulate fluorescence in tissue phantoms to determine the depth of a fluorescent layer inside a medium in Paper I and Paper III. To be able to simulate fluorescence light several Monte Carlo simulations need to be performed. First a simulation is performed to achieve the absorption of the excitation light by the fluorophores inside a layered medium. Then another simulation is performed to obtain the distribution of the fluorescence light, assuming optical properties for the wavelength of the fluorescence light in the medium. The two matrices, absorption of excitation light and emission of fluorescence light, are convolved in the radial direction to produce the probability of transmitted or reflected fluorescence light as a function of radius on the surface. To simulate fluorescence light at one wavelength a reciprocity theorem is used, making the simulation faster. Only one simulation is required for a specific fluorescence wavelength, assuming the source on the surface and not inside the phantom.

2.6 Determination of optical properties

The optical properties of tissue are often not known, therefore some measurements need to be performed to obtain these properties. There are at least four different methods to obtain the optical properties: spatially resolved measurements, time-resolved measurements, frequency domain measurements, and integrating sphere measurements. All methods, except the last one, give only two of the optical properties $(\mu_a, \mu_s)$. To obtain all three parameters, $(\mu_a, \mu_s, g)$, an integrating sphere set-up can be used. The inner surface of an integrating sphere consists of a highly reflecting material, which makes it possible to collect all light entering the sphere. An additional measurement with a collimated light beam measures the sum of the absorption and scattering coefficients. After these three measurements have been performed, the optical properties of the tissue can be calculated. The method has one big drawback as the measurements cannot be performed in vivo.

2.6.1 Inverse adding doubling

From integrating sphere measurements usually three different measurements are performed, and the measured values are total reflection and transmission, and unscattered transmission. A way to determine the optical properties of the sample from the measured quantities with the integrating sphere is to use
the method inverse adding-doubling (IAD).\cite{17} It is a numerical solution of the radiative transport equation and has the big advantage, compared to e.g. the diffusion approximation, that it requires no limits in the optical properties. IAD is often used to determine the optical properties of biological samples compressed between two glass slides. Using IAD the reflection and the transmission are calculated from a guess of the optical properties. If the calculated and the measured values agree, then the optical properties of the sample have been determined.
Chapter 3

Fluorescence diagnostics

This chapter gives an introduction to fluorescence spectroscopy, including the basic theory of fluorescence and a brief description of tissue fluorescence. The geometry used for illumination and detection also plays an important role in the detected fluorescence light. Point- and imaging detection techniques will be discussed briefly in the last part of this chapter.

3.1 Fluorescence

Just like atoms, energy diagrams of molecules consist of electronic energy levels, but molecules can also rotate and vibrate, introducing a splitting of the electronic energy levels into many other levels. If the energy of an incoming photon matches the energy difference between two levels in the molecule, the photon can be absorbed. From here different processes can take place as illustrated in Figure 3.1. All these processes result in that the molecule releases its excess energy gained by absorbing the photon. If the photon interacts with the molecule during a very short time the photon can be emitted again in a process called scattering. If the energy is absorbed internal conversion (IC) can occur, where the molecule is relaxed to a lower vibrational level within the excited state, without emission of a photon. The remaining excess energy of the molecule can be released as a photon when the molecule returns to any vibrational level in the ground state. This process is called fluorescence. The fluorescence photon will have a longer wavelength than the incoming photon, as the molecule losses some energy in the non-radiative relaxation. The generated fluorescence contains light of a broad wavelength range and not a sharp peak since the relaxation process can be carried out to any vibrational level in the ground state. A transition of the molecule into a triplet state is also possible. In this case it takes a long time for the molecule to return to the ground state as the transition between a triplet to a singlet state is forbidden. This phenomenon is called phosphorescence. There is another alternative relaxation path where the absorbed energy is transferred to and exciting a neighboring molecule. This path is not marked in the figure.
3.2 Autofluorescence and tumour markers

Fluorescence from different fluorophores can be detected when illuminating tissue with UV or blue light. The fluorophores can e.g. be NADH, tryptophan, elastin and collagen. Nicotinamide adenine dinucleotide is an electron acceptor in the energy metabolism of the cell. NADH is the reduced form of the molecule, which fluoresces under violet light illumination. The amount of the reduced form is dependent on the cellular metabolism, therefore in tumours the amount of NADH is lower than in normal tissues. The contributing fluorescence from NADH is thus lower in tumours than in normal tissue. Tryptophan is an amino acid that fluoresces strongly when excited with light with a wavelength shorter than 300 nm. Tryptophan can be used for cancer detection in the epithelial cells of the cervix. Elastin and collagen are proteins that exist in supportive tissue, and the content of these two can be altered in tumour tissue. The left graph in Figure 3.2 shows the autofluorescence, i.e. the fluorescence from the above mentioned fluorophores that are naturally present in tissue. The acquired fluorescence spectrum from tissue shows a broad emission, corresponding to the superposition of the fluorescence emitted from several fluorophores. The shape of the autofluorescence depends on tissue type. In precancerous tissue the fluorescence intensity normally decreases when using near UV excitation and the peak of the emission is shifted towards the red. The spectral shape of the fluorescence light can also be changed e.g. if a large amount of haemoglobin is present, due to the fact that haemoglobin can reabsorb the emitted fluorescence light due to its three strong absorption lines in the visible region (see Figure 2.2).

The autofluorescence is usually less intense in tumours than in normal tissue due to different mechanisms. An increased blood flow in tumours can absorb a large amount of light and therefore not much fluorescence light is induced. Another reason is differences in concentration of certain molecules in tumours, changing the fluorescence.

Sometimes it can be difficult to diagnose a tumour by only studying the autofluorescence from the tissue. As mentioned before, the autofluorescence can change in different ways and this makes it difficult to diagnose the tissue by only studying the autofluorescence. A way to increase the sensitivity of tumour detection, is to administer a fluorescent tumour marker, which accumulates in
3.3. LASER-INDUCED FLUORESCENCE

Figure 3.2: Left graph shows the emitted autofluorescence for tissue fluorophores after excitation with 337 nm. [21] The graph to the right shows the fluorescence from the tumour marker PpIX after excitation with 405 nm for normal and tumour tissue.

tumour tissue to a higher degree than in normal tissue. These tumour markers usually emit fluorescence light in the red or near infrared region, making it easy to separate the fluorescence of the tumour marker from the blue-green autofluorescence. An example of a tumour marker is 6-aminolevulinic acid (ALA), that is a naturally occurring molecule in the body. ALA is part of the haemoglobin cycle and after a few steps in the cycle, ALA is converted into the fluorescent molecule Protoporphyrin IX (PpIX). Normally PpIX is present in a very low concentration in the body. After administration of ALA the concentration of PpIX increases drastically to detectable levels. For different reasons, PpIX accumulates to a higher degree in tumour tissue compared to normal tissue, and can therefore be used as a tumour marker. [22] The fluorescence from PpIX can be seen in the right part of Figure 3.2. The graph shows the measured fluorescence following excitation with 405 nm from normal and premalignant tissue of the vocal cords.

3.3 Laser-induced fluorescence

Laser-induced fluorescence (LIF) spectroscopy is a method developed for tissue diagnostics. [23, 24] The method is safe and non-invasive, and can be performed in real-time. The method makes it possible to investigate inner hollow organs when using optical fibres compatible with endoscopes. The tissue is irradiated with a laser of a specific wavelength to induce fluorescence from the present fluorophores. To study tissue autofluorescence, UV or blue light is primarily used as excitation light. Only superficial lesions can be detected following excitation in the violet to the blue wavelength region, as the penetration depth is very shallow. By using longer wavelengths, the light can penetrate several millimeters inside the tissue and deeper lying lesions can be detected. The fluorescence light will be attenuated as it propagates through tissue, especially for light with long path lengths. It is not only the wavelength though that can influence the recorded fluorescence spectrum, but also the illumination and detection geometries used. The two detection principles, point- and imaging detection, will be discussed in the following sections.
CHAPTER 3. FLUORESCENCE DIAGNOSTICS

Figure 3.3: Different illumination and detection geometries and the corresponding measured fluorescence spectra are shown for fluorescence spectroscopy [21].

Using LIF as a guide, more precise biopsies could be taken. The goal in using LIF as a guiding tool is to avoid taking unnecessary biopsies and only taking a biopsy where LIF finds something suspicious. With LIF it is possible to differentiate between inflammation and early non-invasive carcinoma or precancer.

3.4 Fluorescence detection techniques

It is not only the optical properties of tissue that can affect the spectral shape of the detected fluorescence, but also the detection geometry. Figure 3.3 shows three different detection and illumination geometries [21]. All three recordings were obtained on the same sample, using 405 nm excitation light. Figure 3.3a shows the geometry for a point monitoring detection system. In Figure 3.3b a broad illumination geometry is used, but still point detection in contact with the sample. The detected light corresponds mostly to light that has travelled a relatively long distance in tissue thus being affected to a high degree by the haemoglobin absorption. The resulting fluorescence spectrum is characterized by a relatively high intensity in the red spectral region. In Figure 3.3c the detection fibre is not in contact with the sample any longer and light that has travelled both long and short paths can reach the fibre [21].

Another important factor for LIF is what light source is used for the excitation of the fluorophores. If a continuous wave (CW) source is used superficial measurements need to be performed in a dark room, due to the fact that the induced fluorescence light is much lower in intensity than the background light from the examination lamps. A way to overcome this is to use a pulsed light source, allowing the use of gated detection. This means that the detection system is opened only for a short time period when the induced fluorescence light is assumed to reach the detector. Even though the average fluorescence light intensity is much lower than the scattered background light, it can be much stronger during the short periods when the detector is open [23].

3.4.1 Point monitoring

Point monitoring detection of fluorescence is usually based on a fibre-coupled laser system [25, 26]. The excitation light is often guided through a fibre and
3.4.1 Fluorescence Detection Techniques

The fluorescence is detected through the same or another fibre. The fluorescence light is guided to a spectrometer making it possible to study the entire fluorescence emission spectrum. This is of great importance when learning how the characteristic fluorescence spectra of different tissues appear. A point monitoring system may use a pulsed light source and gated detection. There are some disadvantages, one is that the detection is performed only in one spot in the tissue, making it difficult to find or delineate tumours. Another drawback when optical fibres are used is that they can damage the tissue and start a bleeding. The blood can then reabsorb the fluorescence light and change the spectral shape in a non wanted manner.

3.4.2 Imaging

To be able to delineate tumours a fluorescence imaging technique is preferable. Different techniques are employed relying on the number of detected wavelength bands used; single-band, dual-band or multi-color imaging. A review of fluorescence imaging will appear in a book about biomedical imaging. When working with imaging it is often difficult to detect fluorescence from many wavelengths at the same time in several detection points. Therefore a selection has to be done to choose a few wavelengths that give interesting information for diagnostics. Point detection is needed to find these interesting features in the fluorescence spectrum. Other imaging modalities are time-gated imaging, where the fluorescence lifetimes for different fluorophores are studied.

An interesting application is the delineation of skin tumours, for example basal cell carcinomas (BCC). Sometimes it can be difficult to see the borders of tumours with the naked eye, and in those cases fluorescence imaging can give additional information. A lot of research is conducted within this field. Some of the imaging systems that are developed use both the fluorescence from endogenous molecules and exogenous administered compounds to increase the sensitivity. The fluorescence intensity is studied in two to three different wavelength bands, and the intensities in these bands are compared to provide information on which pixels that correspond to tumour tissue. A system using an acousto-optic tunable filter has been used to be able to detect fluorescence in many different wavelength bands.

Another field where fluorescence imaging is used is in tumour detection in lungs and larynx, often referred to as Laser-induced fluorescence endoscopy (LIFE). To visualize these kinds of tissue, the imaging modality needs to be compatible with endoscopes used to reach the organs. There are commercial imaging systems available. Often only autofluorescence from the tissue is studied for diagnosis, but sometimes also a fluorescent tumour marker is used. These systems are important because they can detect and localize tumours in early stages. When working with vocal cords, for example, the delineation of tumours are important as the doctor does not want to remove too much of the tissue, because that could affect the voice in a negative way.

A third field where fluorescence imaging is applied is the detection of cancer and precancers in the cervix. An instrument, called Hyper-Spectral Diagnostic Imaging (HSDI®), developed by the company Science and Technology International (USA), is used to detect early cervical malignancies. The instrument uses excitation light at 365 nm and detects autofluorescence from the cervix in the range 420-760 nm.
CHAPTER 3. FLUORESCENCE DIAGNOSTICS
Chapter 4

Molecular imaging

Molecular imaging is a multidisciplinary field that visualizes and quantifies biological processes at cellular and subcellular levels in living animals. The goal with molecular imaging is to be able to monitor specific molecules and cellular processes in vivo that give information about what happens in the cells. This can involve gene expression, multiple molecular events and interactions between molecules of interest for drug development. In drug development, the effect the drug has on the cells can be studied. Specific probes that only emit signals when attached to the molecules of interest in the cells, are highly desirable to monitor cellular processes. An important task in the development of powerful molecular imaging techniques is the development of such probes. Probes used in fluorescence measurements can be more or less complicated, ranging from common fluorophores to fluorescent proteins that can be produced by gene-manipulated cells and to the newest developed probes based on quantum dots. Probes emitting fluorescence in the red or NIR region are more favorable. The signal is easier to detect because the tissue autofluorescence is rather weak in this wavelength region and light with longer wavelengths penetrates deeper into the tissue. A detection technique is needed to measure the emitted signal from the probe and there are different detection techniques under development. These will be discussed in the following sections.

The detection techniques in combination with the evaluation algorithms can be used to find the spatial location of fluorescence-marked tumours inside the tissue. Fluorescence tomography is a technique that can be used to image tissue. A goal would be to detect a malignant tumour in an early stage to be able to treat the tumour before the disease spreads with metastatic lesions. In Paper I and II changes in recorded fluorescence spectra have been utilized to determine the depth of a fluorescent inclusion.

4.1 Molecular probes

As mentioned in the introduction, a molecular probe is necessary in the cell or animal that is about to be imaged. These probes often consist of two different parts. One part is an affinity component that interacts with the target, which can be a cell, protein or some other molecule. The second component is often a fluorochrome that emits a detectable signal. The different molecular probes...
4.1.1 Non-specific probes

Non-specific probes do not have a specific target and can therefore not be used to study molecular or cellular processes. These non-specific probes are instead used in the study of physiological processes, like changes in blood flow and blood perfusion as illustrated in Figure 4.1.[42] Fluorochromes that are good to use in fluorescence studies are Fluorescein and Cyanine dyes, because they have a high extinction coefficient and absorb and fluoresce in the range from visible to the NIR region. One example of a non-specific probe is the fluorochrome indocyanine green, which emits in the NIR region.

4.1.2 Specific probes

The Cyanine dyes can be made more specific to attach to certain targets in the cells. A specific probe consists of a ligand, antibody or another substrate that can interact with specific parts inside the cell or attach to specific cells, see Figure 4.2. This interaction allows specific biological structures to be imaged.[44] One disadvantage with the specific probes is that the probes fluoresce even though they have not reached their target. This makes it more difficult to detect the molecules that are actually attached to their targets, due to a decreased signal-to-background ratio.

Fluorescent proteins is a special class of fluorescent probes. They are produced by gene-manipulated cells. The gene coding for a fluorescent protein can be linked to the genes of the proteins of interest. When the proteins of interest
are expressed they become fluorescent. An example concerning gene therapy will be given next. The gene coding for a fluorescent protein have been linked to a gene used in therapy, and the whole complex has been given to cells that are supposed to be treated. If the genes are transferred in a correct manner, both the fluorescent protein and the therapy gene will be expressed. A fluorescent signal is emitted indicating that the linked therapy gene is working. The therapy gene needs to be expressed to allow the fluorescent protein gene to be expressed. A commonly used fluorescent protein is the green fluorescent protein (GFP). GFP has been found naturally in different species, for example the jelly fish Aequorea victoria. Other varieties of fluorescent proteins have been found or modified from GFP to change the spectral region of the fluorescent proteins. There are many different varieties, where cyan-shifted proteins (CFPs) and yellow-shifted proteins (YFPs) could be mentioned. A red-shifted fluorescent protein called DsRed has been isolated from corals. Most of the fluorescent proteins emit in the visible wavelength region, providing a low penetration depth of light and a strong influence of tissue fluorescence.

Other fluorescent probes used for molecular imaging are the quantum dots. Quantum dots are semiconductor crystals with sizes of a few nanometers. The size of the quantum dots can be varied as the size affects the absorption and emission properties of a quantum dot. Today the existing dots fluoresce from the visible region to the NIR region. There are some advantages with the quantum dots compared to other fluorescent probes. They often have a broad absorption spectrum, but the emission is in a narrow wavelength region. Another advantage is that the dot is rather photostable, making it possible to use it to study processes during a longer time period. They are also very bright and can be tagged to target molecules. The toxicity of quantum dots needs to be studied in more detail though before they can be used in human applications.

4.1.3 Activatable probes
The activatable probes are also called smart probes or beacons. The great advantage of these smart probes is that a signal is not emitted until the probe finds and is attached to its target, see Figure 4.3. The smart probe often consists of one fluorophore and one quencher at each end of a single DNA strand. As the probe has not found its target, the DNA strand is formed like a hairpin, which means that the fluorophore and the quencher are very close to each other. The quencher prohibit the fluorophore to fluoresce. When the probe reaches its target, a specific enzyme unfolds the DNA strand. The quencher and the fluorophore are then separated, and the fluorophore emits light that can be detected.

4.2 Molecular imaging techniques
There are several different imaging techniques that are used to study the molecular processes within molecular imaging. Techniques to be mentioned are positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT), magnetic resonance imaging (MRI) and different optical imaging techniques. A brief overview of the
different techniques follow in this section. Optical imaging techniques, which are closer to the work in this thesis, are described in more detail in Section 4.3.

PET is a technique where biological molecules have been marked with isotopes that emit positrons. Examples of isotopes are $^{11}$C, $^{15}$O and $^{18}$F. These isotopes emit positrons as they decay. Subsequently a positron and an electron collide and two $\gamma$-rays at 511 keV are emitted in opposite directions and can then be detected. By studying the lines produced by the $\gamma$-rays in a detector utilizing specific algorithms, the exact location of the positron emission can be reconstructed.

SPECT is also a technique where $\gamma$-rays are detected. A rotating gamma camera is used to detect emitted $\gamma$-rays in multiple directions. The radionuclides used are for example $^{123}$I and $^{99m}$Tc, each emitting one $\gamma$-ray.

In CT detection of transmitted X-rays from a narrow beam can be used to image a thin cross-section of a body. The X-ray source and detector are rotated to detect X-rays in several projections to reconstruct the 3D internal structure of the tissue. An imaging contrast can be administered to be able to detect tumours, this because soft tissue can be difficult to study with CT. In molecular imaging microCT are used for animal imaging, yielding a good resolution.

MRI is an imaging technique that separates among various tissue types, due to different environments of protons in the tissue. Protons, which are the nuclei in hydrogen atoms, normally have random nuclear spin orientations. In the presence of a strong magnetic field, the protons become aligned to the magnetic field. A brief radio signal flips the nuclear spins and they emit signals when they reorient parallel to the field. These signals are picked up by sensitive detectors. The difference in magnetic shielding of protons in various molecules permits reconstruction of an image of the tissue types present. It is also possible to image the relaxation time instead of imaging the amount of protons in the tissue, as the relaxation time can be different in tumours and normal tissue.

4.3 Optical imaging techniques

Three different imaging techniques based on detection of light will be mentioned in this section: bioluminescence imaging, fluorescence imaging and optical to­mography.
4.3.1 Bioluminescence imaging

Bioluminescence is a phenomenon where light is emitted from certain species, for example fire-flies and certain bacteria. The light can be emitted as a communication between bacteria or as an attraction to other species. Luciferin is the name of light emitting pigments which are found in the above mentioned species. When luciferin is oxidized by a certain enzyme, a luciferase, photons are emitted. The emission of light occurs when the reaction takes place and no external excitation light source is needed. Genes that express a luciferase can be inserted into tumour cells in organisms. When a luciferin is present light will be emitted if luciferase has been expressed and the chemical reaction has taken place. Bioluminescence has been used to study tumour cell growth. The emitted light is often blue-green, allowing imaging of only superficial tissue. The advantage with bioluminescence is that there is no background tissue fluorescence that can disturb the studied signal.\[43, 44\]

4.3.2 Fluorescence imaging

In contrast to bioluminescence imaging an external light source is needed to induce fluorescence light. The emitted fluorescence light is usually detected with a CCD camera.\[50\] Both reflected and transmitted fluorescence can be detected, but reflectance measurements are more common because the thickness and the optical properties of the sample influence the amount of light that can be transmitted. Figure 4.4 shows an example of the detected fluorescence from a mouse with an implanted tumour marked with a fluorescent probe.

Tissue autofluorescence is usually very strong in the blue-green wavelength region, and the much weaker signal from a fluorescent probe can be drown by the autofluorescence if they fluoresce in the same region. A better alternative is to use NIR fluorescent probes. In this wavelength region tissue yields very weak autofluorescence. Another drawback with fluorescence imaging is that the fluorophores can be bleached. This problem can be minimized by using quantum dots as fluorescent probes.

4.3.3 Optical tomography

Optical tomography is a non-invasive reconstruction method to image objects inside tissues from optical measurements in several projections. The light intensity
detected in the different projections depend on the optical properties of the tissue. Healthy and diseased tissue can be distinguished from each other by using optical tomography. Reconstruction of objects, obtained with optical tomography, is a more difficult problem compared to X-ray methods like CT. X-rays follow a straight line through the sample, and the attenuation due to absorption is measured in several projections. Light of optical frequencies is not only absorbed but also scattered in the tissue. To be able to reconstruct the objects inside the tissue the optical properties of the tissue need to be determined. The problem can be divided into two parts. The first part is the forward problem, where a theoretical model is used to describe the light propagation in tissue with known optical properties. The second part is the inverse problem, where the forward model and the measured projections are used to reconstruct the correct optical properties inside the tissue.

Figure 4.5 shows a schematic diagram of how the reconstruction algorithm in optical tomography is functioning. The reconstruction is started with a guess of the optical properties inside the tissue. With the use of a forward model the light distribution is modelled inside the tissue and the detector readings for the projections are predicted. These predicted values and the values obtained from the real measurements are compared. If there is no match, the optical properties of tissue are updated with some optimization algorithm, which often involves trying to calculate the derivative of the simulated values when small changes in the optical properties are applied. The inverse problem is to find the optimal choice of the changes in optical properties. The procedure is repeated until the difference between the predicted and measured values is sufficiently small, then the reconstruction of the tissue has been accomplished. An often used model to determine the light penetration is the diffusion approximation, described in Section 2.4, because the light becomes diffuse as it propagates through a turbid medium like tissue.

Optical tomography imaging can be performed with three different concepts. Measurements can be performed in the time-domain, see Figure 4.6a, where a short pulse (ps) is launched into the tissue. The pulse is broadened as it propagates through tissue because it is affected by the optical properties of the tissue. Figure 4.5. The diagram shows the algorithm used to solve the non-linear inverse problem in optical tomography.
4.3. OPTICAL IMAGING TECHNIQUES

Figure 4.6: Three different concepts of optical tomography. (a) Time-domain, (b) frequency-domain and (c) steady-state measurements.\[51]\n
tissue. The time, when the maximum of the pulse is detected, corresponds to the scattering coefficient and the slope of the detected pulse is influenced by the absorption coefficient. The second method to be mentioned is the frequency domain, see Figure 4.6b, where sinusoidally amplitude-modulated light is used. The detected light has been phase-shifted and demodulated, which can give information about the optical properties. The third method involves a continuous light source and measurements of the attenuated light (Figure 4.6c).\[51]\n
It should be mentioned that the image reconstruction is an ill-conditioned problem, because different combinations of optical properties can yield very similar values in the projections. Therefore some kind of a priori information is desirable to find the best solution.\[51, 52]\ In Paper II and III we suggest that the spectral information in the measured fluorescence spectra can provide such a priori information. The spectral shape of the measured fluorescence can provide information about the depth of an embedded fluorescent inclusion.

It is not only differences in optical properties that are utilized in the reconstruction in optical tomography. Fluorescence optical tomography is used to find fluorescent inclusions inside tissue. There are different approaches in reconstructing the image by studying the fluorescence.\[53, 54]\ It usually demands two measurements, one with the detection of the transmitted excitation light, and one measurement where the fluorescence light is detected. Fluorescence tomography has for example been used to reconstruct objects inside tissue phantoms of breasts by studying the fluorescence from administered fluorophores.\[55, 56]\n
A special case of optical tomography is fluorescence-mediated molecular tomography (FMT), where in vivo molecular processes in tissue can be studied, resolving and quantifying very low concentrations of fluorophores in animals.\[57, 50]\ FMT can be used for deeper-lying targets that can be difficult to study with ordinary fluorescence imaging.
Chapter 5

Temperature measurements

One of the main interests in our research group is to develop interstitial photodynamic therapy with optical measurements to provide feedback in order to optimize the treatment. The need for thermal measurements during therapy will thus be motivated to a great extent from this perspective, whereas the usefulness of thermal measurements may be very straightforward for other treatment modalities, based on thermal effects, as also indicated briefly below.

Tissue responds in different manners to an increase of temperature. Tissue temperatures ranging from 41 to 47°C are defined as hyperthermia. In this range the enzymatic reactions in tissue are slightly altered. Heat can also change the structure of proteins and inhibit the synthesis of DNA, and thereby damaging the cells. In the range from 42 to 44°C tumour cells are believed to be more sensitive to heat than normal cells in the tissue, and thus hyperthermia can be utilized in a way to cause tumour cell death.[58] Coagulation of tissue occurs above 50°C, and this is used in thermotherapy where higher temperatures than hyperthermia are used. Thermotherapy is not tumour selective and therefore it is important to control that only the diseased tissue is exposed to high temperatures. The coagulation process, where cellular proteins and other molecules are irreversible altered, turns the tissue whitish and can clearly be seen by the eye. When the temperature is increased to 100°C tissue water starts to boil and vapour bubbles are formed. When the temperature reaches 400°C, carbonization of the tissue takes place.[59]

Tissue is in these respects characterized by some thermal properties. These depend on the type of tissue. There are especially three mechanisms to be mentioned: transport of heat by thermal conduction, storage of heat and transfer of heat due to perfusion.[60] For longer time scales the thermal distribution in the tissue is determined by the thermal properties in tissue more than only the optical properties. All these factors, as well as an understanding of what happens with the tissue at different temperatures, are necessary to perform an efficient treatment.

An example where temperature monitoring may be of great importance is in one type of brain disorder therapy, for example a movement disorder. A radio-frequency current is here delivered through an electrode to the brain tissue in order to heat and create a lesion of tissue by coagulation. The lesion destroy the abnormal brain activity in this specific part of the brain. During the heating process it is important to monitor the temperature in order to create a very
CHAPTER 5. TEMPERATURE MEASUREMENTS

Light ~ Photosensitiser

Oxygen

Figure 5.1: Three components are needed to perform PDT: photosensitising drug, light and oxygen.

confined lesion while sparing the normal surrounding tissue.\[61\]

5.1 Interstitial Photodynamic Therapy

Photodynamic therapy (PDT) is a method for treating tumours located superficially in tissue.\[62, 63\] Three components are needed to induce damage to the tumour cells: a photosensitising drug, delivery of light and oxygen present in the tissue, see Figure 5.1.

The photosensitising drug is administered systemically or topically to the patient. After some time the drug has accumulated in tumour tissue to a higher degree than in normal tissue. The reason for the tumour selectivity is not fully understood for all photosensitizers. It depends among other things on enzyme activity and the environment of the tumour. The size of the tumour is then illuminated with light that matches an absorption peak of the photosensitizer. The light excites the photosensitiser molecules, whereby different reactions can take place. Type I reactions lead to electron transfer where radicals, which often involves oxygen, are formed. An example of a radical is hydrogen peroxide. In Type II reactions, energy is transferred from the excited photosensitiser to oxygen, which is excited to the very reactive singlet state. Singlet oxygen reacts with amino acids and nucleic acids and damage the function and structure of the cell.\[64, 59\] During PDT the tumour vasculature can also be damaged and the oxygen supply by the blood is destroyed, which also leads to secondary damage of the tumour.\[63\]

A commonly used compound administered to the patient is δ-aminolevulinic acid (ALA). This compound exists naturally in the body, where it is a constituent in the first part of the haem synthesis pathway. After a few steps in the haem pathway ALA is converted into the fluorescent compound Protoporphyrin IX (PpIX). PpIX will be accumulated in the tumour cells partly due to a decreased amount of the enzyme (Ferrochelatase) that converts PpIX to the final molecule haem. Another reason for the build-up of PpIX in superficial skin tumours following topical application of ALA is that there is an increased penetration of ALA through the damaged epidermis above the tumour, compared to the epidermis above normal tissue.\[65\]

Only superficial and thin tumours can be treated with PDT, because the light penetration is limited to a few millimeters for red light. A way to treat deeper-lying and thicker tumours is to insert a number of optical fibres into
5.1 INTERSTITIAL PHOTODYNAMIC THERAPY

Figure 5.2: The concept of IPDT, where optical fibres are inserted into the tumour.

5.1.1 Why is it important to measure temperature during photodynamic treatments?

When delivering light during a photodynamic treatment session tissue may be heated sufficiently much to cause thermal damage. It is thus of importance to monitor the temperature during the treatment to understand whether the damage is of photodynamic or thermal character.

Measurements of the temperature during the IPDT treatment can also to some extent be used to monitor the progress of the treatment. The primary goal with IPDT is to treat the tumour in a photochemical way and not with thermotherapy. During an IPDT treatment the performance of the treatment is optimized by measuring the light fluence and concentration of the photosensitizing drug. The optical properties of tissue and the penetration depth in tissue may change when the tissue temperature changes, and this will affect the light distribution within the tissue. It is known that the reduced scattering coefficient is increased for temperatures high enough to cause protein breakdown.[67] The absorption coefficient behaves more ambivalent, often it decreases with increasing temperature, but an increase in absorption has also been detected.[67, 68, 59] These alterations result in a decrease in optical penetration depth when higher temperatures are reached. This change in optical properties and penetration depth occurs mainly between 50-65°C. Above 65°C no significant changes are seen.[67]

Another temperature dependent mechanism of importance for PDT is that the chemical conversion of ALA into the photosensitizer PpIX is a temperature dependent reaction. It has been shown that the formation of PpIX in human skin was increased with about 50% when the temperature was raised from 31°C to 36°C, leading to a more efficient treatment.[69] Again it may be essential to be able to measure local temperature with relatively high accuracy.

Another application where temperature control could be of interest is when PDT of tumours is performed in combination with hyperthermia. The combination of these modalities has shown to improve the treatment outcome.[70, 71]
During the treatment the temperature in the tumour is raised to 43-44°C. The hyperthermia can be applied simultaneously or directly after the PDT treatment. The temperature needs to be monitored during the entire PDT treatment to ensure that hyperthermia is present.\[71\]

An abrupt temperature increase during an IPDT session could be an indication of a bleeding in the proximity of the tip of the fibre used in the treatment. A bleeding means a large amount of blood gathered around the fibre tip. This blood absorb the light very locally, due to its strong absorption yielding a local temperature rise. If the light is absorbed, it will not be distributed within the tumour as planned, and this reduces the efficiency of the treatment. By monitoring the temperature a bleeding can be detected providing a possibility to rearrange the fibre.

5.2 Techniques to measure temperature in tissue

Tissue responds to temperature increases in different ways. During different types of treatments, it is as described above important to monitor the temperature in order to control the treatment. There are different techniques to measure the temperature in tissue.

There exist small temperature devices that can be inserted into tissue during treatments. Thermistors are one example of such devices. These are based on semiconductors, of which the resistance is temperature dependent.\[59\] Another probe to be mentioned is the thermocouple, which is a thermolectric sensor, where electrical charges are repositioned in a bimetallic junction as a function of temperature.

Alternatively, crystals, doped with some specific ions that emit temperature dependent fluorescence, can be used to measure the temperature optically. A spectral shape of the emitted fluorescence and/or a fluorescence lifetime change can be used to extract the temperature of a probe at a fibre tip. Crystals doped with rare-earth ions are commonly used for temperature measurements.\[72\] Also a molecular thermometer made of an organic semiconductor material, platinum octaethyl porphyrin, has been demonstrated.\[73\] Fibre optic thermometers that use the principle of detecting the fluorescence decay time of phosphorescent materials are commercially available.\[74\]

Other examples of crystals used for temperature monitoring are crystals doped with Cr$^{3+}$-ions as they show a great variety in temperature dependence. These crystals will be discussed in more detail in the next section.

5.3 Doped crystals

The optical technique, used in Papers IV and V, is based on the method where the fluorescence from crystals doped with the Cr$^{3+}$-ion shows a temperature behavior. Examples of these crystals are Alexandrite, Cr:YAG, Ruby and Cr:LiSAF. All these crystals have different temperature sensitivity in different temperature regions, and therefore it is important to chose the right crystal for each application.\[75, 76\]
5.3. DOPED CRYSTALS

Cr³⁺-doped crystals are a good choice in the case of monitoring temperature during IPDT, because the Cr³⁺-doped crystals can be excited at 635 nm. This particular wavelength is used for the treatment light during IPDT treatments when PpIX is used as a photosensitizer. The temperature can be measured in a spot where the fluence is high if a small piece of the crystal is attached to the fibre tip where the treatment light is delivered. This means that no other objects than the IPDT treatment fibres have to be inserted into the tissue. Another benefit is that the temperature is measured directly at the fibre tip, compared to a thermistor that would measure the temperature at a small distance from the region with the highest light fluence.

There are two alternative ways to determine the temperature using Cr³⁺-doped crystals. One is to study the spectral changes in the acquired fluorescence spectrum and the second is to measure changes in the fluorescence lifetime.[75] In Paper V lifetime measurements were performed in order to measure the temperature of three different Cr³⁺-doped crystals: Alexandrite, Cr:YAG and Cr:LiSAF. In Paper IV the spectral behavior of the fluorescence were used, and the temperature measurements were also incorporated in the IPDT system mentioned in Ref. [66].

5.3.1 Fluorescence spectra measurements

The number of atoms in a certain electronic state, is described by the Boltzmann’s distribution, see formula 5.1.

\[ \frac{N_1}{N_2} = e^{\frac{\Delta \varepsilon}{kT}} \]  

where \( N_1 \) and \( N_2 \) are the number of atoms in the two states, \( \Delta \varepsilon \) is the energy difference between the two states, \( T \) is the temperature in Kelvin and \( k \) is the Boltzmann constant. In room temperature most atoms are in the ground state, but as the temperature increases more atoms will populate higher states.

When the temperature in the tissue changes, the shape of the fluorescence emitted from the crystals is affected, see Figure 5.3. Fluorescence spectra are shown for three different temperatures where a piece of a Cr³⁺-doped Alexandrite crystal has been attached to the tip of a fibre.

A ratio can be calculated by dividing the intensities measured in two different wavelength regions, where the temperature has influenced the emitted fluorescence. The ratio, \( \gamma \), is then calculated for several temperatures using formula 5.2 to build a calibration curve.

\[ \gamma(\text{temperature}) = \frac{I(\lambda_1)}{I(\lambda_2)} \]  

where \( I(\lambda_1) \) and \( I(\lambda_2) \) are the fluorescence intensities in the two wavelength bands. The concept of forming an intensity ratio is often used when working with rare-earth-doped fibres.[75] Figure 5.4 shows an example of a calibration curve, where the dimensionless ratio as a function of the corresponding temperature is shown. Each fibre with an attached crystal needs to be treated individually and is required to be calibrated once. After the calibration, the fibre can be used to determine the temperature by calculate the ratio from a fluorescence measurement, and compare
Figure 5.3: The fluorescence spectrum changes when the temperature changes.

Figure 5.4: A calibration curve to determine the temperature where the dimensionless ratio as a function of corresponding temperature is shown (Paper IV).
Figure 5.5: Modulated light source for lifetime measurements of the induced fluorescence. A phase shift, \( \phi \), appears between the incoming light and the fluorescence light.

the calculated ratio with the calibration curve. An advantage of using fluorescence intensity measurements is that the instrumentation is relatively simple, in principle only consisting of a spectrometer. Using a dimensionless ratio has the advantage that not well-defined parameters are cancelled out. I.e. if different powers are used for different treatments this will not affect the measured ratio since the dependence of the laser power is cancelled out. One disadvantage is that background fluorescence from other fluorophores in the tissue can influence the temperature measurements, if it is emitted in the same wavelength region as the fluorescence from the crystal.

5.3.2 Fluorescence lifetime measurements

To be able to measure the fluorescence lifetime, the light source used to induce fluorescence needs to be modulated. One option is to use a sinusoidally modulated light source as illustrated in 5.5. The emitted fluorescence light will also be modulated, but lagging the excitation light, and thereby introducing a phase-shift. The fluorescence lifetime is calculated with formula 5.3.

\[
\tan \phi = \frac{\omega}{\tau}
\]

where \( \phi \) is the measured phase difference between the laser source and the induced fluorescence light, \( \omega \) is the angular frequency of the modulated signal and \( \tau \) is the fluorescence lifetime.[7, 77] The fluorescence lifetime is calculated for several temperatures and a calibration curve can be constructed in the same way as for the intensity measurements explained in the previous section. The limitation when measuring the fluorescence lifetime is the need for more complicated instrumentation, but has the advantage that the temperature dependence is more robust due to a decreased sensitivity to background signals, for example room light, that interfere with the measurements. The lifetime of the autofluorescence is in the order of ns, while the fluorescence lifetime of the doped crystals are in the range of \( \mu s - ms \). The difference in lifetime will prevent autofluorescence to disturb the measurements of the fluorescence lifetimes of the crystal.
Chapter 6

Summary of Papers

Paper I
Spectral changes of the fluorescence caused by the optical properties of tissue can be used to determine the depth of a fluorescent object inside the tissue. A ratio can be calculated from the intensities at two wavelengths. This ratio is dependent on the depth of the object. Both experimental work and simulations were performed to investigate the depth dependent ratio. The effect of the detection geometry on the measured fluorescence was also studied with Monte Carlo simulations and experiments on a resin phantom.
I performed the work concerning the effect of the detection geometry on the measured fluorescence spectrum.

Paper II
Experiments were performed to study the fluorescence emitted from an embedded object inside different phantoms, e.g. tissue. The fluorescence were detected in two wavelength bands, where the ratio of the intensities gave information about the depth of the object.
I performed the experimental work and the evaluation. I also wrote most parts of the manuscript.

Paper III
Fluorescence Monte Carlo simulations of a layered geometry were performed to study the ratio of two fluorescence wavelengths to find the depth of a fluorescent inclusion. The study was focused on the fluorescence contrast influences on the determination of the depth. Simulations were also performed to investigate the depth determination when there are uncertainties in the optical properties of the tissue.
I performed the simulations, the evaluation and wrote the manuscript.

Paper IV
An Alexandrite crystal was used to measure the temperature during simulated PDT treatments. The crystal was attached to the tip of an optical fibre used in an interstitial photodynamic therapy system. The temperature was determined by studying the spectral changes in the fluorescence. Simulated treatments were performed both superficially on skin and interstitially in meat.
I took part in the experimental work and also wrote the manuscript.
Paper V
In this project an optical technique to measure temperature was evaluated. Crystals doped with Cr$^{3+}$-ions were used to measure the temperature, using the fact that the fluorescence lifetime of the crystals changes when the temperature changes. A piece of a crystal was attached to the tip of an optical fibre and the lifetime was measured as a function of temperature. Lifetime measurements were performed with three different crystals.

I took part in the experimental work, evaluation and wrote the manuscript.
Acknowledgements

I would like to express my gratitude to many people who have supported me throughout this work, and among them I would like to especially thank:

My supervisor, Stefan Andersson-Engels, for encouragement and support during my three years at the Department of Atomic Physics.

My two co-supervisors, Katarina Svanberg and Sune Svanberg, for their support regarding my research projects.

Present and former members of “Medicin-gruppen” for interesting discussions and nice collaboration. I would also like to thank you for the great times outside work.

Ann, for being such a fun travel companion around the world. Christoffer, for our morning discussions about anything (mellan himmel och jord), and Florian, for all valuable help with experimental set-ups and interesting discussions.

The EU Integrated Project “Molecular imaging”, LSHG-CT-2003-503259, for financial support.

My parents, Karin and Gunnar, my sister, Malin, and Peter, for all your support and for always believing in me during the years.

Markus, for being who you are.

Jenny
Bibliography


Fluorescence spectra provide information on the depth of fluorescent lesions in tissue

Johannes Swartling, Jenny Svensson, Daniel Bengtsson, Khaled Terike, and Stefan Andersson-Engels

The fluorescence spectrum measured from a fluorophore in tissue is affected by the absorption and scattering properties of the tissue, as well as by the measurement geometry. We analyze this effect with Monte Carlo simulations and by measurements on phantoms. The spectral changes can be used to estimate the depth of a fluorescent lesion embedded in the tissue by measurement of the fluorescence signal in different wavelength bands. By taking the ratio between the signals at two wavelengths, we show that it is possible to determine the depth of the lesion. Simulations were performed and validated by measurements on a phantom in the wavelength range 815-930 nm. The depth of a fluorescing layer could be determined with 0.6-mm accuracy down to at least a depth of 10 mm. Monte Carlo simulations were also performed for different tissue types of various composition. The results indicate that depth estimation of a lesion should be possible with 2-3-mm accuracy, with no assumptions made about the optical properties, for a wide range of tissues. © 2005 Optical Society of America

OCIS codes: 170.3660, 170.3880, 170.6280, 170.7050.

1. Introduction
There is a rapidly growing interest in fluorescence measurements of embedded structures for tissue diagnostics. The principle is based on noninvasive or low-invasive techniques in which the tissue is irradiated with light and the remitted fluorescence signal is detected on the surface. The measured signal contains information about the concentration and distribution of the fluorophore. This type of measurement has the potential to discriminate diseased regions inside the tissue (e.g., tumors), provided that there is some mechanism for selective uptake of the fluorophore.

By use of long excitation wavelengths, approximately within the 600-900-nm range, it is possible to reach deep into the tissue, partly because of lower scattering, but mainly owing to the lower absorption in this region. A field of intense research is cancer diagnostics that uses fluorophores, which have long excitation wavelengths in the red or near-infrared (NIR) region. This would enable fluorescence emission from deep structures, of the order of several centimeters. A specific application that has been suggested is detection of sentinel nodes in cancer patients. Tumor spreading is routinely investigated by lymphoscintigraphy of the lymph nodes to which the lymphatic channels drain the tumor bed. A radioisotope is injected into the tumor volume, and the clinician searches for gamma decay in the nearby lymph nodes. Fluorescence-based detection could be a simpler and safer alternative to this procedure. Another application that has attracted interest is measurement of the fluorescence from photosensitizers for photodynamic therapy, with the aim of optimizing treatment parameters and monitoring photobleaching of the drug.

Several reconstruction algorithms have been developed that attempt to recover the distribution of a fluorophore in a tissue volume given a set of measurements on the tissue surface. Fully three-dimensional reconstruction methods are being developed by some groups. They are commonly based on use of diffusion theory as the forward model, but an algorithm based on the discrete-ordinates solution of the transport equation has also been proposed. Many combinations of light source and detection points are needed for full reconstruction, which implies a high complexity of both the instrumentation and the reconstruction algorithm. A sim-
pler approach has been suggested by some authors, based on the approximation that the tissue can be regarded as a semi-infinite volume with fluorescing lesions at some depth beneath the surface. Slanica et al. presented a method based on the diffusion equation for a two-layer medium and spatially resolved measurements of both diffuse reflectance and fluorescence. This method was successful in recovering the absorption and reduced scattering coefficients ($\mu_a$ and $\mu_s'$, respectively) of both layers, as well as the fluorophore concentration and layer depth. However, recovery of all parameters was limited to a depth of $\sim 3$ mm, and determination of the depth itself proved to be difficult for larger depths owing to model coupling among depth, fluorophore concentration, and tissue absorption. Edsath et al. have described a method based on image fluorometry. Using a random-walk model, they demonstrated good accuracy of the determined depth for pointlike fluorescent lesions.

None of the methods described in the literature seem to make specific use of the spectral properties of the fluorescence signal. The measured emission spectrum following irradiation by excitation light at the surface is a function of several parameters, because the fluorescence light has to pass through tissue with characteristic scattering and absorption properties. Thus the intrinsic fluorescence emission spectrum will be altered in a way determined by the tissue optical properties, by the depth of the fluorophore, and also by the geometry of the light irradiation and the detection system. These effects have been noted by several authors in conjunction with fluorescence from shallow layers (less than $\sim 1$ mm) and with ultraviolet or blue excitation light.\(^1\)\(^-\)\(^20\)

In the first part of this paper we investigate the effects of the optical properties of the tissue and the measurement geometry on the recorded fluorescence spectra. We use Monte Carlo simulations to compute fluorescence spectra from a turbid medium. The Monte Carlo code was developed earlier and utilizes several techniques to reduce the number of photons necessary in the simulation to make the computation time reasonable even for entire fluorescence spectra and for large depths.\(^21\)\(^-\)\(^22\) The computed spectra are compared with experimental results from a tissue phantom for different irradiation-detection geometries. The optical properties of the phantom, which are necessary for the simulations, are measured independently with an integrating sphere. The results serve as a verification of this method and demonstrate the effects on the measured fluorescence spectra.

Next we turn to the problem of determining the depth of fluorescent lesions in tissue. We recognize that the shape of the fluorescence spectrum from an embedded lesion is influenced by the depth of the lesion, owing to the fact that the fluorescence light is filtered when it migrates through the tissue to the surface. Thus we investigate the potential of using changes in the fluorescence spectra to estimate the depth, $d$, of a fluorescing lesion in a semi-infinite volume.

Our method relies on the fact that the absorption coefficient of the tissue is nonuniform over the spectral region of fluorescence emission. This means that relative spectral intensities change for different wavelength bands as the fluorescence light migrates to the surface. These changes can serve as a measure of the depth of the fluorophore. A simple approach is to form the ratio between the measured fluorescence signals at two wavelengths, $\lambda_1$ and $\lambda_2$.

$$\gamma = \frac{\Gamma(\lambda_2)}{\Gamma(\lambda_1)}$$

where $\Gamma$ represents the probability of detecting a fluorescence photon and then evaluating the dependence of $\gamma$ on $d$. An advantage of this approach is that by forming a ratio there is no need for absolute measurements of the fluorescence light, and some uncontrollable parameters cancel out. To prove the principle of this approach, we performed Monte Carlo simulations of excitation and fluorescence light. The simulation results were directly compared with the results from measurements performed on a tissue phantom to provide experimental verification of the method. We also performed a series of Monte Carlo simulations with realistic tissue optical properties, for various tissue types, to assess the robustness of the method with respect to biological variability.

2. Materials and Methods

A. Monte Carlo Simulations

The Monte Carlo method for simulating fluorescence from layered tissues has been described in depth in a previous study.\(^21\) Briefly, our method takes advantage of the symmetry aspects to reduce the computation time. We also split the simulation of the excitation light and the emission light into two separate problems. The resulting data sets are convolved to provide the final answer. To increase the efficiency further, we apply a reciprocity theorem for the calculation of the emission light. This effectively reverses the photon paths of the fluorescence light so that only one simulation is needed, with the source of photons placed at the surface. It was shown that for certain applications, the computation time could be reduced by 2 orders of magnitude or more by use of these techniques, compared with the conventional method for simulating fluorescence.\(^21\) The code is time resolved, which permits studies of the effects of fluorophore lifetimes and photon migration time dispersion.

For experimental validation, we performed Monte Carlo simulations with optical properties similar to those of phantoms, which were measured independently with an integrating-sphere method (Subsections 2.B-2.D below).

We also performed simulations with optical properties similar to those in real tissue. The absorption spectra for tissue were modeled based on data from previous measurements of breast tissue, in which the dominant absorbers in the red and NIR regions...
are water, fat, and deoxygenated and oxygenated hemoglobin. The spectra of absorption and the reduced scattering that were used in the simulations are shown in Fig. 1. The tissue types ranged from water rich (62% water, 16% lipid for type 1) to lipid rich (12% water, 68% lipid for type 6) and thus represent a very wide span in terms of different tissue types.

B. Resin Phantom

We constructed a homogeneous solid phantom made of epoxy resin, following the guidelines in Ref. 23. We used TiO₂ particles at a concentration of 1 mg/g (T-8141; Sigma-Aldrich, St. Louis, Missouri) as a scatterer, and we used Amaranth dye at a concentration of 0.8 mg/g (12,056-1; Aldrich Chemical Company, St. Louis, Missouri) as an absorber. Small amounts of the Coumarin 30 dye were used to provide a fluorescence peak in the green region, 450-500 nm, and Sulforhodamin provided a peak in the red region, 550-650 nm, of the spectrum. The fluorescence spectrum of the phantom, following excitation at 407 nm, mimicked that of real tissue. It exhibited a broad peak in the green region that corresponded to tissue autofluorescence, and a red peak similar to the fluorescence from a fluorescent tumor marker.

C. Intralipid Phantom

To show the differences in the fluorescence spectrum for a lesion at different depths, we needed a phantom with an embedded inclusion at a variable depth d. To this end, a phantom was prepared from 1 part Intralipid-20% (Fresenius Kabi, Sweden) and 21 parts water. The phantom consisted of three layers, as depicted in Fig. 2. The upper and lower layers had the same optical properties, and black ink was added to provide background absorption (2.3 μl/l). The middle layer, 1-mm thick, was separated by thin plastic foil, and to this layer a fluorescent dye (IR-140; Exciton, Dayton, Ohio) was added. The depth of the upper layer, d, could easily be varied by the addition or removal of the liquid.

D. Integrating Sphere Measurements

The optical properties of the phantoms were determined by use of an integrating sphere. In the case of the solid phantom, a small amount of the resin was sandwiched between two glass slides while still not hardened. To measure the optical properties of the Intralipid phantom, we used a cuvette made of glass slides. In both cases the thickness of the samples was 1.00 mm and the lateral dimensions were 3 cm × 3 cm. The integrating-sphere setup was used to measure the total transmission, total reflectance, and collimated transmittance. In the case of the Intralipid phantom, the optical properties—μₐ, μₛ, and the scattering anisotropy factor g—were evaluated with Monte Carlo look-up tables. For the solid phantom, the collimated measurement could not be performed because of high attenuation; instead, data for the g factor were taken from previous measurements on resin phantoms. The data for the resin phantom were evaluated by use of the inverse adding-doubling method, which was more convenient than the Monte Carlo look-up tables owing to the large differences in the optical properties at different wavelengths for this phantom.

E. Fluorescence Measurements

For the measurements on the resin phantom, we used a compact fluorescence point monitoring system to record the fluorescence spectra. The fluorescence light from the sample was guided through a 600-μm-core-diameter step-index fiber (N.A. = 0.22) to a spectrometer and a cooled CCD camera (DH501-25U-01, Andor Technology, North-
ern Ireland). The tip of the fiber was in contact with the sample. For the excitation light source, we used a krypton-ion laser emitting at 407 nm with an output power of 20 mW. Light from the laser was focused on a 400-\(\mu\)m fiber, and the distal end of the fiber was imaged with a lens on the surface of the phantom. This 1:1 imaging arrangement gave a 400-\(\mu\)m top-hat excitation distribution on the surface. Fluorescence spectra were acquired for distances of 0.5–7 mm, in steps of 0.5 mm, from the excitation laser spot, over the spectral range 540–700 nm. The acquired fluorescence spectra were white-light calibrated and deconvolved from the spectral system response function.

We used a Ti:sapphire laser at 780 nm as the excitation source for the Intralipid phantom with a fluorescent layer. The power was limited to ~10 mW. The light was guided to the phantom surface by a 400-\(\mu\)m-core-diameter step-index fiber (N.A. = 0.22), and a similar fiber collected the fluorescence light and guided it to a spectrometer (HoloSpec/1.8i; Kaiser Optical Systems, Ann Arbor, Michigan). The laser wavelength was removed with a long-pass filter. A cooled CCD camera (LN/CCD-1024-EEQ/1; Princeton Instruments, Trenton, New Jersey) was used for detection. Spectra were acquired over the spectral range 815–930 nm.

**Fig. 3.** Optical properties of the resin phantom measured with the integrating-sphere setup. Also shown is the intrinsic fluorescence spectrum (Fluo.) following excitation at 407 nm (arbitrary units).

**Fig. 4.** Experimentally measured and calculated fluorescence spectra for the resin phantom. Results from different distances between the excitation spot and the detection fiber are shown: (a) 0.5 mm, (b) 1 mm, (c) 3 mm, and (d) 5 mm. Int., intensity; norm., normalized.
3. Results

A. Resin Phantom and Monte Carlo Simulations

The optical properties of the resin phantom, as determined with the integrating-sphere method, are presented in Fig. 3, together with the intrinsic fluorescence spectra of the fluorophores. These data were subsequently used in the Monte Carlo simulations. The scattering anisotropy factor \( g \) was around 0.7 over the wavelength range. \(^{23}\) The results of the simulations are presented in Fig. 4, together with the measurement results for the excitation wavelength of 407 nm. Four different fiber distances are shown: 0.5, 1, 3, and 5 mm. We observed a significant spectral shift as the fiber distance increased. Clearly, a good agreement between simulation and measurement was achieved.

B. Intralipid Phantom and Monte Carlo Simulations

The optical properties of the Intralipid phantom, as determined by the integrating-sphere method (shown in Fig. 5), were used as input for the Monte Carlo simulations. In this case \( g \) was around 0.65 over the wavelength range (data not shown). The measured fluorescence spectra at different depths are presented in Fig. 6, which shows the shift of the spectrum as the depth increases. In the Monte Carlo simulations the highest value of the ratio \( \gamma \) was obtained with \( \lambda_1 = 815 \) nm and \( \lambda_2 = 960 \) nm. However, owing to the low fluorescence signal at long wavelengths, we had to use a lower \( \lambda_2 \) to obtain a good signal-to-noise ratio from the measurements. Owing to the influence of autofluorescence when the excitation and detection fibers were close together, we used a fiber distance of 5 mm. Therefore the measurement was limited to depths larger than \(-1\) mm. In Fig. 7 we present \( \gamma \) (normalized to the value at \( d = 2 \) mm) as a function of the depth \( d \) for \( \lambda_1 = 886 \) nm and \( \lambda_2 = 922 \) nm.

By using the simulation results as a calibration curve, we also attempted to predict the depth of the fluorophore from the experimental values of \( \gamma \). We then calculated the differences between the predicted and the true values of \( d \), which resulted in a standard deviation in the predicted values of 0.6 mm.

C. Tissue Monte Carlo Simulations

For the simulations of realistic tissue, we assumed an excitation wavelength of 615 nm and simulated the fluorescence in the region 625–1005 nm in steps of 10 nm. For each individual simulation (i.e., one wavelength and one set of tissue optical properties), \( 5 \times 10^5 \) photon histories were traced. The fluorescing lesion was simulated as a 1-mm-thick layer. The depth of the upper layer to the lesion was varied from 0 to 10 mm.
The simulations were repeated for six different sets of tissue optical properties to account for typical biological variability of the tissue composition, as presented in Fig. 1. First, we wanted to investigate which ratios are the largest and thus best suited for evaluating the depth of the fluorophore. To determine the optimal choice of wavelengths for the method, we plotted $d\gamma / dd$ for 625 nm $<$ $\lambda_1$ $<$ 1025 nm, 625 nm $<$ $\lambda_2$ $<$ 1025 nm. Since the initial results showed that $\gamma(d)$ was approximately linear, we chose to plot $d\gamma / dd$ as determined by the slope of a regression line, rather than plotting $\gamma(d)$ for various depths. The results are presented in Figs. 8(a) and 8(b) for tissue types 1 and 6, respectively. The average of $d\gamma / dd$ for all six tissue types is shown in Fig. 8(c). The points with the largest values in these plots may be interpreted as corresponding to the best wavelength choices in terms of obtaining a high ratio.

By looking at $d\gamma / dd$, it is also possible to assess how robustly the ratios correspond to a given depth for different tissue types. We accomplished this by searching for the $\lambda_1$-$\lambda_2$ pair that gives the least variation in $d\gamma / dd$ among the six tissue types. With the requirement that we want $d\gamma / dd$ to be relatively high, the best combinations turned out to be $\lambda_2$ = 935 nm, with $\lambda_1$ = 695 nm, $\lambda_1$ = 745-755 nm, or $\lambda_1$ = 875 nm. At ($\lambda_1$, $\lambda_2$) = (695, 935 nm), the mean value was $d\gamma / dd$ = 0.063 mm$^{-1}$, with a standard deviation of 0.005 mm$^{-1}$. At ($\lambda_1$, $\lambda_2$) = (875, 935 nm), the mean value was $d\gamma / dd$ = 0.043 mm$^{-1}$, with a standard deviation of 0.005 mm$^{-1}$. We then applied these values to predict $d$ from the simulation of each of the six tissue types. The result is shown in Fig. 9(a) for ($\lambda_1$, $\lambda_2$) = (695, 935 nm), and in Fig. 9(b) for ($\lambda_1$, $\lambda_2$) = (875, 935 nm). In the first case, most of the predicted values of $d$ are within 1.5 mm of the true value, except for the most water-rich tissue type (1), which consistently overestimates the depth by approximately 2-2.5 mm. In the second case, the largest errors occur for tissue types 2 and 6, which are underestimated by as much as 2 mm.

4. Discussion

The results from the resin phantom (Fig. 4) clearly reveal that the shape of the recorded emission spectrum changes according to the detection geometry. As the distance between the source and the detector increases, more spectral intensity shifts to longer wavelengths where the absorption is lower. Similar findings have been reported by other authors. 24-26

The other important conclusion from the results of the resin phantom is that there is very good agreement between the Monte Carlo simulations and the measurements. Thus we are confident in the method of measuring the optical properties of the phantom independently by using the integrating spheres and then applying these values in the Monte Carlo simulations.

The results from the Monte Carlo simulations for a fluorescing layer show that the ratio $\gamma(\lambda_1, \lambda_2)$ is indeed a useful indicator of the depth of a fluorescing lesion for the right combinations of wavelengths $\lambda_1$ and $\lambda_2$. The results from the simulations corroborate the simulations and show that the method is practically feasible with a relatively simple experimental setup. In the case of the Intralipid phantom, we utilize the difference in water absorption at the slope of the vibrational overtone band that

\[ \gamma(\lambda_1, \lambda_2) = \frac{\int_{\lambda_1}^{\lambda_2} I(\lambda) \, d\lambda}{\int_{\lambda_1}^{\lambda_2} \alpha(\lambda) \, d\lambda} \]

where $I(\lambda)$ is the recorded intensity and $\alpha(\lambda)$ is the measured absorption coefficient.

...
the best choice is also governed by the requirement that $\gamma$ be insensitive to biological variability. At $970$ nm the water absorption band yields large differences between water-rich and lipid-rich tissue types, which means that there will be a large variation in $d\gamma/dd$ if $\lambda_2 = 975$ nm is chosen. Instead, using $\lambda_2 = 930$ nm is useful because this is a quasi-isosbestic point at which the variation between various soft tissues is minimal, owing to the fact that the absorption spectra of lipid and water cross at this wavelength.\(^2\) Considering this, we found that the most robust combination of wavelengths was $\lambda_1 = 695$ nm and $\lambda_2 = 935$ nm. With this combination we could recover the depth of the layer with an error of less than $1.5$ mm for all tissue types except the most water rich (type 1). Considering the very large differences in tissue composition and thus optical properties between the six tissue types, such a good prediction accuracy is quite encouraging. The use of the wavelength pair $(\lambda_1, \lambda_2) = (665, 935)$ nm may be problematic in practice, owing to difficulties in finding a suitable fluorophore with such a wide emission spectrum. Using the wavelengths $(\lambda_1, \lambda_2) = (975, 935)$ nm is a more realistic alternative, and the results in Fig. 9(b) show that the depth prediction accuracy for these wavelengths is similar.

It should also be noted that we found the relation between $\gamma$ and $d$ to be close to linear in all the cases we investigated, meaning that a single value of $d\gamma/dd$ is all that is needed to estimate the depth of a lesion for any measurement. However, the linear dependence of $\gamma$ on $d$ was only true when small distances between the excitation and the detection fibers were used. For distances above $5$ mm, the relation deviated from linear when the depth was smaller than $1$ or $2$ mm. This could be of practical concern in some instances, since it may not be possible to place the excitation source and the detector close to each other because of the influence of autofluorescence in the tissue close to the excitation source. In such cases it may be necessary to have a longer distance and to use nonlinear calibration curves.

It may be possible to improve the accuracy of the depth measurement by incorporating methods to estimate the optical properties of the tissue, e.g., by measuring the diffuse white-light reflectance of the tissue. This could provide some a priori information about the absorption and scattering properties in a manner related to the methods described by Stasie et al.\(^2\) and Muller et al.\(^7\)

We have also considered using temporal information to estimate the depth. The Monte Carlo simulations show that this may be possible, but there are several difficulties. Apart from the increased complexity in the instrumentation, decoupling the fluorophore lifetime and the photon migration time of flight is a nontrivial problem, especially since fluorophore lifetimes are typically an order of magnitude longer than the time of flight. The intrinsic fluorophore lifetime may change depending on the chemical environment, making this procedure increasingly difficult. For our method, we are analogously dependent on the

![Fig. 9. Values of the depth $d$ predicted from the Monte Carlo simulations for each of the six different tissue types. In all six cases the same calibration was used. (a) Calibration based on the mean value $d\gamma/dd = 0.063$ mm$^{-1}$ at $(\lambda_1, \lambda_2) = (695, 935)$ nm. (b) Calibration based on the mean value $d\gamma/dd = 0.043$ mm$^{-1}$ at $(\lambda_1, \lambda_2) = (875, 935)$ nm.](image)
assumption that the intrinsic emission spectrum of the fluorophore does not change according to the chemical environment in the lesion. However, in our case such variations are likely to be small, whereas in the case of the lifetime it will be the dominating effect.

With the present method, we envision a potential application based on a probe that delivers excitation light and at the same time detects the fluorescence light by means of relatively simple photodetectors and wavelength-selecting filters. The operator would manually scan the probe across the tissue, and the information would be presented in the form of an indicator of fluorescence intensity and the estimated depth of the fluorophore. We also consider our method to be quite promising in terms of providing the depth information for image fluorometry and molecular imaging as a means for resolving the fluorophore distribution in three dimensions.26

The authors want to thank Gabriel Somesfalean, Ann Johansson, and Christoffer Abrahamsson. This study was supported by the European Commission grant LSHG-CT-2003-502268 “Molecular Imaging.” The authors also acknowledge financial support by the Swedish Research Council. J. Swartling acknowledges support from the Swedish Foundation for International Cooperation in Research and Higher Education and from the Swedish Research Council.

References
Paper II
Fluorescence spectroscopy in tissue phantoms for improved depth resolution in tissue imaging

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Abstract
One way of determining the depth of a fluorescent inclusion is to detect fluorescent light that has propagated through the medium at two different wavelength bands, in which case a ratio can be calculated between the corresponding intensities. The wavelength regions should be chosen such that there is a difference in the absorption in the medium. The method could be used to determine the depth of deep-lying tumors marked with a fluorescent compound. This spectral information could be used as a complement in other methods, for example in tomography, due to its straightforward implementation. In this study we have performed phantom measurements to determine the depth of a fluorescent object, filled with fluorophores. The transmission of yellow and red fluorescence was measured and a ratio of yellow to red fluorescence was calculated for four different depths in a 2.5 cm thick liquid phantom. The ratio showed a clear dependence on the depth of the object. In order to study the performance of the method in a realistic situation, measurements were also performed in tissue where autofluorescence is expected, obtaining encouraging results.

Keywords: fluorescence spectroscopy, molecular imaging, fluorescence tomography, light propagation
1. Introduction

The position of small fluorescing volumes embedded in highly scattering media can be extracted using optical tomography. One important potential application of this is in the development of fluorescence mammography to find tumors in the breast at an early stage. To be able to find an inclusion using illumination in the near-infrared (NIR) region, a fluorescent contrast agent can be used to enhance the contrast. \(^1\) Indocyanine green is a dye that has been used as an absorption-based contrast agent to find embedded tumors in breasts with diffuse optical tomographic imaging. \(^2\) Three-dimensional images of breast phantoms have also been successfully reconstructed using time-resolved fluorescence measurements and a finite-element reconstruction technique. \(^2\) Several different tomographic techniques have been evaluated to reconstruct the spatial distribution of fluorescent objects in tissue, where the diffusion approximation is often used in this context to describe the light propagation. \(^4\) - \(^8\)

It is also of interest to visualize fluorescent volumes in the field called molecular imaging, in which molecular pathways and events occurring at the molecular level can be monitored by means of fluorescent reporters. \(^9\) - \(^10\) To be able to monitor the molecular interactions in vivo, specific molecular probes are needed, which tag specific target molecules of interest. These probes may be designed to emit fluorescent light upon excitation and to be activated only in the presence of specific enzymes, \(^11\) yielding information on processes occurring at the molecular level. Fluorescence can then be used to determine both the location and concentration of the probe molecules in tissue. Several different optical techniques have been developed for light emission-based molecular imaging: fluorescence reflectance imaging (FRI), \(^10\) - \(^12\) bioluminescence imaging (BLI) \(^10\) and fluorescence-mediated molecular tomography (FMT). \(^11\) - \(^13\) - \(^15\) FMT is a technique that reconstructs three-dimensional images of the distribution of a fluorescent molecular probe in tissue. In FMT, tissue is illuminated with excitation light at several projections, followed by detection of the fluorescence that has propagated through the tissue. \(^14\) FMT has been shown to be able to resolve and quantify very low concentrations of molecular probes in the range of pico- to femtomoles in whole animals. \(^11\) In a recent study it has been shown that the tumor response to chemotherapy could be monitored with the use of FMT and a specific fluorescent probe. \(^15\) The use of FMT has also been discussed in drug therapies to study e.g. if a drug reaches its target or if a drug has a disease-modifying effect. \(^11\) - \(^16\)

An inherent complication of fluorescence tomography in turbid media is that the tomographic algorithm is often relatively ill conditioned, making it difficult to accurately and independently predict the depth, size and concentration of the fluorescent inclusion. This is especially true in the NIR wavelength region where light penetrates well and is highly scattered, due to the relatively low absorption. In these cases it is of interest to use as much a priori knowledge as possible. One approach is to employ the spectral information to improve the robustness in the evaluation.

An independent way of determining the depth of an embedded inclusion labeled with a fluorescent probe is to study the spectral changes in the fluorescence as it propagates through tissue. \(^17\) - \(^18\) The idea of determining the depth of an embedded object has been investigated previously by studying the propagation of photon-density waves. These waves are affected by the optical properties of the medium, and it has been shown that by changing the modulation frequency of the incident light, inclusions can be detected at different depths. \(^19\) The changes in the fluorescence spectral shape depend on the wavelength dependence of the optical properties in tissue, the depth of the fluorescent inclusion and the detection geometry. \(^20\) - \(^23\)
Tissue exhibits a wavelength-dependent absorption coefficient in the region from the visible to the NIR region. The absorption will attenuate the different wavelength components of the fluorescent light differently as it propagates through the tissue. Therefore the deeper a fluorescent lesion is located in the tissue, the greater the change in intensity between different fluorescent wavelengths. Additional information about the depth of the fluorescent inclusion can thus be provided by forming the ratio of the fluorescence signals at two wavelengths. In a previous study, the concept of forming a dimensionless ratio between two fluorescence wavelengths was demonstrated with both Monte Carlo simulations and experiments on a liquid phantom using a fluorophore in the 800–900 nm range. The depth of a fluorescent layer could be determined with an accuracy of 0.6 mm down to a depth of 10 mm. One goal for this method is now to optimize the wavelengths used for such a ratio, to obtain a good depth resolution. In this development it would be favorable if the ratio would be more or less independent of the type of tissue. Monte Carlo simulations were performed by Swartling et al. for six tissue types, ranging from water-rich to lipid-rich. Using the data obtained from the simulations the depth of a lesion could be determined directly from the ratio with an accuracy of 2 mm, without a priori knowledge of the composition of the tissue.

In the NIR region, light can penetrate deep into tissue, providing the possibility to study inclusions located deeply in the tissue. A group of molecular probes are based on the specific genes that can control the production of fluorescent proteins in cells. The green fluorescent protein (GFP) is an example of a fluorescent protein. The GFP gene can be linked to the genes of the protein of interest. When the protein of interest is expressed GFP will also be expressed. GFP fluoresces in the green wavelength region, while there exist a variety of GFP-like proteins that fluoresce in different parts of the visible spectrum. Investigations utilizing these probes are of great importance, since they can be used, for example to identify tumors in experimental studies. Studies with these fluorescent proteins however, have to deal with the limitation that they emit light in the visible wavelength region where high absorption yields a low penetration of light and there is strong autofluorescence from tissue. These effects have so far limited their use to small animals or to low absorbing tissues. A benefit of the high absorption is that the tomographic algorithm is less ill conditioned, as light in a more narrow path between excitation and detection positions is favored.

The aim of this study was to investigate whether the fluorescence ratio concept could also provide useful information in the visible region with shallow light penetration and relatively low fluorescence contrast between the inclusion and the bulk tissue. Such information could complement the information obtained by fluorescence tomography measurements to make these tomographic reconstructions more robust. Fluorescence measurements were performed on two different phantoms with an object inside containing a fluorescent liquid. The object used was a layer or a glass tube. The object was placed at different depths in tissue phantoms and fluorescence images, in two different wavelength bands, were acquired in order to calculate a depth-dependent ratio.

This paper is structured as follows: In section 2, the experimental set up and the measurement procedure is presented, in section 3 the results of our experiments both in a liquid phantom and in tissue are demonstrated and a discussion is followed in section 4. The article is ended with a conclusion.
2. Material and Methods

The experimental setup used in the study is illustrated in Figure 1. A laser beam illuminates one side of the phantom to be probed. The light excites the fluorophores in the sample. Some of the induced fluorescence propagates through the phantom and can be detected at the other side. A CCD camera is used to detect the transmitted fluorescence from a specific area of the phantom. By sequentially using two different band pass filters in front of the CCD, yellow and red fluorescence images can be acquired.

![Figure 1. Schematic illustration of the experimental setup used for the two experiments. The fluorescent object was either a 1.7 mm thick layer or a thin glass tube, containing fluorophores.](image)

Two different phantoms were used: (1) a liquid phantom containing a thin fluorescent layer and (2) an excised tissue sample consisting of porcine muscle in vitro with a thin glass tube containing the fluorophores. The layer was placed at the depths: 5, 10, 12.5 and 15 mm in the liquid phantom. The tube was placed at the depths: 1, 3, 6, 8, 11, 13, 17 and 19 mm in the tissue.

2.1 Experimental equipment

The fluorescent layer in the liquid phantom

The excitation light was produced by a multiline argon-ion laser (LaserPhysics, Reliant 1000m, West Jordan, UT, USA) emitting at several wavelengths the strongest laser lines being at 488 and 514 nm. The light was guided through an optical fiber to a beam collimator forming a collimated beam with a diameter of 5 mm. A 488 nm interference filter was placed in front of the collimated laser beam, so that only excitation light of 488 nm entered the liquid phantom. The phantom was placed behind the cooled CCD camera (ANDOR Corp., DV 434, Belfast, Northern Ireland) equipped with a 50 mm f/2.8 macro objective lens (Sigma, Tokyo, Japan). The back surface of the phantom was placed in focus of the CCD camera. Different anti-reflection-coated emission band pass filters (Chroma Technology Corp., Bellows Falls, VT, USA) were placed in front of the objective lens of the CCD camera to select either yellow (525-570 nm) or red (570-670 nm) light.

The fluorescent tube in the tissue phantom

A multiline argon-ion laser (Coherent, Innova400, Santa Clara, CA 95054 USA) emitting at several wavelengths, strongest laser lines at 488 and 514 nm, was used as an excitation source. The beam diameter was 6 mm. The phantom was placed between the laser and a cooled ICCD camera (ANDOR Corp., DH734-1BF-73, Belfast, Northern Ireland) equipped
with a 50 mm f/4 macro standard objective camera lens. A 3 mm thick cut-off filter OG550 (Schott, Bromma, Sweden) was placed in front of the objective. The back surface of the phantom was placed in focus of the CCD camera. Different band pass filters (ESCO, Oak Ridge, New Jersey, USA) were placed in front of the objective lens of the CCD camera to select either yellow (550 ± 10 nm) or red (630 ± 10 nm) light.

2.2 Preparation of phantoms

The fluorescent layer in the liquid phantom

The fluorophores used in the experiment consisted of a mixture of the two compounds CFSE (Sigma Aldrich, 21888) and SNARF (Molecular probes, S2280). These two compounds were used instead of GFP and DsRed for practical reasons. The two fluorophores used exhibit similar fluorescence emission spectra to GFP and DsRed. CFSE and SNARF were dissolved in a phosphate buffer solution, at a concentration of 2 μM. This mixture was used in the layer. As an absorber in the phantom, a specific dye (PRO-JET 900NP, Avecia) was used. The dye was dissolved in 20 ml ethanol. As scattering medium, 0.80 g TiO₂ was mixed with 280 ml water. The dye solution was poured into the TiO₂/water mixture. The liquid was poured into a vessel 25 mm thick, 100 mm wide and 100 mm high. The front and back were made of 1.7 mm thick glass plates. A magnet was placed in the bottom of the vessel so that the liquid could be gently stirred to prevent TiO₂ from sedimenting. The absorbance of the dye solution was measured independently with a spectrometer. The reduced scattering coefficient of TiO₂ was calculated using Mie scattering theory. The absorption coefficient, \( \mu_a \), and the reduced scattering coefficient, \( \mu_s' \), of the liquid phantom for the three wavelength regions are given in Table 1. The fluorescent layer inserted into the liquid phantom in the 25 mm thick vessel, was a homemade cuvette consisting of two transparent 0.1 mm thick plastic films glued along three sides onto 1.7 mm thick pieces of glass. This cuvette was filled with the fluorescent solution.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>( \mu_s' / \text{cm}^2 )</th>
<th>( \mu_s / \text{cm}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>1.57</td>
<td>19</td>
</tr>
<tr>
<td>540</td>
<td>1.27</td>
<td>18</td>
</tr>
<tr>
<td>615</td>
<td>1.03</td>
<td>17</td>
</tr>
</tbody>
</table>

The fluorescent tube in the tissue phantom

For experiments with tissue, a piece of porcine muscle was placed in a similar glass vessel as in the previous experiment, with a thickness of 20 mm as relevant for small animal imaging studies. The fluorophores used in the tissue experiments was a 50/50 mixture of Rhodamine 110 (Lambda Physik, LC5700, Goettingen, Germany) (2mg/ml) and Rhodamine 101 (Lambda Physik, LC6400, Goettingen, Germany) (1mg/ml) solved in 95% ethanol. A glass tube with an inner diameter of 1.50 mm and an outer diameter of 2.3 mm was filled with the fluorophore mixture and placed at different positions inside the meat.

2.3 Measurement procedure

The fluorescent layer in the liquid phantom

Six different measurements were performed at each depth of the layer. All measurements were recorded in darkness. First a measurement with no filter in front of the CCD camera was performed. This provided an intensity map of light transmission through the sample. Then a background image was acquired, with the laser turned off. The next measurement was performed with the yellow filter in front of the CCD. The exposure time was adjusted to
ensure the dynamic range of the CCD camera was exploited to its maximum, and an image was acquired. A new background image was acquired with this filter with no laser light. The yellow filter was replaced with the red filter in the last measurements, following the same procedure as with the yellow filter. The fluorescent layer was positioned at the depths 5, 10, 12.5 and 15 mm in the vessel, measured from the face where the laser entered the vessel. The power of the excitation light used for the fluorescence recordings was approximately 14.6 mW. When no filter was placed in front of the CCD, a power of 2.5 mW was used. The exposure times and optical density filters used for each measurement are listed in Table 2.

![Table 2: Exposure times and optical density filters used with the fluorescent layer in the liquid phantom.](image)

<table>
<thead>
<tr>
<th>Depth / mm</th>
<th>Exp. time / s (yellow)</th>
<th>Exp. time / s (red)</th>
<th>Exp. time / s (no filter)</th>
</tr>
</thead>
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<tr>
<td>5</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>5</td>
<td>0.01 (OD3.0+OD1.0)</td>
</tr>
<tr>
<td>12.5</td>
<td>5</td>
<td>5</td>
<td>0.01 (OD3.0+OD1.0)</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>5</td>
<td>0.01 (OD3.0+OD1.0)</td>
</tr>
<tr>
<td>No layer</td>
<td>5</td>
<td>5</td>
<td>0.01 (OD3.0+OD1.0)</td>
</tr>
</tbody>
</table>

The fluorescent tube in the tissue phantom

The total power for the tissue measurements was approximately 0.5 W for the excitation light. Different exposure times were used for the yellow and the red filters, as can be seen in Table 3. The depths of the glass tube were 1, 3, 6, 8, 11, 13, 17 and 19 mm. An image was acquired with each of the filters.

![Table 3: Exposure times used with the tube in the tissue phantom.](image)

<table>
<thead>
<tr>
<th>Depth / mm</th>
<th>Exp. time / s (yellow)</th>
<th>Exp. time / s (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
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</tr>
<tr>
<td>6</td>
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<td>8</td>
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</tr>
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</tr>
<tr>
<td>13</td>
<td>5</td>
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</tr>
<tr>
<td>17</td>
<td>3</td>
<td>0.300</td>
</tr>
<tr>
<td>19</td>
<td>1.5</td>
<td>0.300</td>
</tr>
</tbody>
</table>

2.4 Evaluation of the data

In the data evaluation, the backgrounds were first subtracted from the data images recorded. The intensities in the acquired images were also normalized with respect to the exposure time. When the optical density filter had been used in front of the CCD camera, its attenuation was also taken into account in preparing the images for analysis. A region of interest in the images was selected. This region was chosen to be the same for all images from the same phantom. Then a depth-dependent ratio was calculated in two different ways. First, a ratio was calculated for each pixel in the selected region of interest by dividing the value in the yellow image by corresponding value in the red image. A new image was then formed as a pixel-by-pixel ratio. The other method involved calculating a mean value over the entire region of interest for the yellow and red images separately. The mean value for the yellow image was then divided by the mean value for the red image, resulting in a value corresponding to a certain depth.
3. Results

In previous studies,\textsuperscript{17,18} Monte Carlo simulations have been performed to demonstrate the method of using spectral information to determine the depth of a fluorescent object inside a tissue phantom. In this study, the focus is on imaging recordings where the medium is inhomogeneous and some level of autofluorescence is expected.

The fluorescent layer in the liquid phantom

Figure 2 shows the normalized images acquired for two different depths of the layer in the liquid phantom together with a radial profile. The depths of the layer for the images shown are 10 mm (left column) and 15 mm (right column). The upper curves show the radial profile for the yellow images for the two depths. The second row shows the images acquired with the yellow filter in front of the CCD camera. The images in the third row were acquired with the red filter. The images in the last row show the calculated ratio for each pixel. The images correspond to an area of about 5x5 mm. In this experiment the background fluorescence from the liquid phantom was minimal; therefore the intensity shown in the images corresponds almost entirely to the induced fluorescence in the layer. It should be noted that no fluorescence could be detected from the liquid phantom in this experiment without a fluorescent layer.

![Image of Figure 2](image_url)

Figure 2. The spectra at the top of the figure show the radial profile of the intensity for the images acquired with the yellow filter, for a layer located at depths of 10 and 15 mm in the liquid phantom. The images in the second row show the images acquired with the yellow filter and those in the third row show the corresponding red images. The images in the lower row show the yellow/red ratio for the two depths.

The graph in Figure 3 shows the ratio calculated from the mean values of the yellow and red fluorescence, as a function of the depth of the inserted layer in the liquid phantom. A distinct increase in the calculated ratio can be seen as the layer is located closer to the detection side. Although there is only a small difference in absorption in the liquid phantom between the yellow and the red wavelengths (see Table 1), clear changes in the ratio can be observed.
Figure 3. The ratio between the mean intensity of the yellow light and the mean intensity of the red light as a function of the depth of a fluorescing layer in the liquid phantom.

The fluorescent tube in the tissue phantom
The method used to determine the depth by calculating a ratio between the intensities at two fluorescence wavelengths was also applied to tissue. Data was acquired on porcine muscle in vitro with an inserted glass tube containing the fluorophores. In the image a region of interest of 33x33 mm was selected in the evaluation to calculate a mean yellow/red ratio for each depth of the tube. Figure 4 shows the yellow/red ratio as a function of depth for a tube filled with fluorophores. These results indicate that the method of determining the depth of a fluorescent object also works in tissue.

Figure 4. The yellow/red ratio for a fluorescent tube located at different depths in a tissue phantom.

4. Discussion

Today there is a great interest in finding an embedded inclusion using optical tomography. One application is to find a tumor employing a tumor seeking fluorescent compound. The fluorescence from the compound can then be detected in several projections to reconstruct the location of the tumor.\textsuperscript{12,25} The reconstruction problem becomes, however, ill conditioned when scattering in the medium is high compared to the absorption. It is in those cases that it would be of great aid to utilize all available \textit{a priori} information to be able to perform the
reconstruction in a robust manner. This a priori information could include the spectral information obtained by measuring the fluorescence intensity at several wavelengths. In this study, the fluorescence intensities in two wavelength bands were measured from fluorophores located at different depths in tissue phantoms. The surrounding medium exhibited different values for the absorption coefficient for the two fluorescence wavelengths. This difference affected the spectral shape of the recorded fluorescence. By forming a ratio of the intensities in the two wavelength regions, a value was obtained that provides information on the depth of the fluorescent object.

By evaluating a ratio of two intensities, no absolute measurements are necessary. This yields certain benefits, because unknown parameters can be canceled out, for example instrument-dependent factors that may not be known in detail. The concept used in this study is to utilize differences in the absorption at the two wavelengths studied. The idea is adopted from earlier work, where a fluorescence ratio was evaluated in such a way that it became insensitive to tissue reabsorption. That was accomplished by selecting two wavelengths that were equally attenuated in the tissue. In the mentioned study, atherosclerotic lesions were studied in wavelength regions where no difference in absorption of hemoglobin was present.

Figure 2 shows the calculated yellow/red ratio for a thin fluorescent layer inside a non-fluorescent liquid phantom at two depths, 10 and 15 mm. The ratio is higher when the layer is located closer to the detector. Not only the spot in the middle of the image showed a higher ratio, but the entire region of interest selected. One can, however, observe that the ratio decreases with radial distance. This can all be explained by variations in the effective path length of the light from the position of the fluorescence emission.

Today, most of the fluorescent probes used emit in the visible region, where strong background fluorescence from tissue is present. This provides a very limited signal-to-background ratio, where it is difficult to detect the weak fluorescence signal from the probe. Commonly used probes for studying molecular interactions in cells are the fluorescent proteins emitting in the visible region. When measuring visible fluorescence from a small inclusion in tissue, there will thus always be background fluorescence from the tissue itself. This was studied in a tissue specimen. Figure 4 shows the yellow/red ratio for a tube in tissue with a relatively large fluorescence contrast. To be able to detect the fluorescence from the fluorophore, a relatively high concentration is needed so that it is not overwhelmed by tissue autofluorescence. This becomes increasingly important for smaller inclusions, since the relative volume becomes small compared to the bulk tissue. The relative signal of the fluorophore needs thus to be much higher in the visible region compared to the NIR region. The depth-dependent ratio, with different degrees of fluorescence contrast has been investigated with Monte Carlo simulations in a recent study. The results show that the depth-resolving technique works, despite some autofluorescence of the phantom. However, autofluorescence will lead to poorer depth resolution due to a reduction in contrast. This problem is expected to be reduced if a tomographic algorithm is employed, as the fluorescence contribution from much smaller volumes can be evaluated.

The magnitude of the difference in absorption between the two detection wavelengths influences the depth resolution of this technique. A larger difference in absorption provides a better depth resolution, but if one of the absorption coefficients is too high, this will prevent the light from penetrating deep into the tissue limiting the largest depth possible to measure. In tissue the difference in absorption can be relatively large due to the absorption of hemoglobin. In comparison of the two cases, tissue and an absorbing dye, in this study, it can
be seen in Figure 3 and 4 that the dynamic in the ratio for the tissue is much larger than for the absorbing dye used in the liquid phantom.

In the preparation of the tissue phantoms some problems were observed. We originally planned to use Intralipid as the scattering material in the liquid phantom, but during the initial experiment it was seen that Intralipid fluoresces quite strongly in the visible range when excited at 488 nm, mainly due to the lipid compounds contained. Intralipid has previously been used for fluorescence experiments in the NIR region, but no disturbance in these measurements was detected. Instead of Intralipid, TiO$_2$ was used as a scattering compound in the phantoms. The TiO$_2$ did not fluoresce when illuminated, but had a tendency to settle somewhat, even though the liquid was stirred during the experiments. Sedimentation of TiO$_2$ would change the optical properties of the phantom. We believe however that this was controlled and that the optical properties of the liquid phantom were maintained homogeneous.

All these concerns need to be taken into account in selecting the optimal wavelength region for fluorescence tomography. Another aspect of interest is how the depth-dependent ratio is affected when the optical properties of the tissue are not fully known. Such uncertainty arises from inhomogenous tissue and intersubject variations (lipid and water content may for instance vary between subjects). The amount of blood that light encounters on its propagation towards the detector is usually position dependent. If the properties change drastically, the ratio will be affected and decrease the depth resolution consequently. Alteration of a few tenths percent has been shown to be handled relatively well.$^{17,18}$

**Conclusion**

In conclusion, the results showed that the ratio of the fluorescence in two wavelength regions, where the rest of the tissue phantom exhibits different absorption properties, can provide information on the depth of a fluorescent object. This suggests that spectral information can be used to provide additional useful information to improve fluorescence tomography of tissue in terms of accuracy and robustness.

**Acknowledgements**

The authors would like to thank Lotta Gustafsson. This research was supported by the EU Integrated Project “Molecular Imaging” LSHG-CT-2003-503259. H. Meyer acknowledges support from the EU MOLEC-IMAG Early Stage Training site MEST-CT-2004-007643.

**References**


Paper III
Modeling of spectral changes for depth localization of fluorescent inclusion
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Abstract: We have performed modeling of fluorescence signals from inclusions inside turbid media to investigate the influence of a limited fluorescence contrast and how accurately the depth can be determined by using the spectral information. The depth was determined by forming a ratio of simulated fluorescence intensities at two wavelengths. The results show that it is important to consider the background autofluorescence in determining the depth of a fluorescent inclusion. It is also necessary to know the optical properties of the tissue to obtain the depth. A 20% error in absorption or scattering coefficients yields an error in the determined depth of approximately 2-3 mm (relative error of 10-15%) in a 20 mm thick tissue slab.

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OCIS codes: (170.5280) Photon migration; (170.3660) Light propagation in tissues; (170.6510) Spectroscopy, tissue diagnostics

References and links
tissue. In the NIR region, deeply located inclusions could then also be monitored in highly vascularized tissue [5]. Molecular probes emitting fluorescence in the NIR region are to be found is molecular imaging, where biological processes can be studied at cellular and molecular levels [1-3]. Biological processes can be monitored by using specific molecular probes, which seek out target molecules. These probes are frequently designed to provide characteristic fluorescence. To collect the induced fluorescence light, an optical imaging technique is employed.

Detection of fluorescence light emitted from the molecular probes can be used to determine the spatial location of specific cells inside the tissue and the concentration of the probe. Several optical imaging techniques can be used to detect tissue luminescence: fluorescence reflectance imaging (FRI), bioluminescence imaging (BLI) and fluorescence-mediated molecular tomography (FMT) [2-4].

One aspect of molecular imaging is the development of molecular probes emitting in the NIR region, where tissue exhibits low absorption, allowing deep penetration of light into the tissue. In the NIR region, deeply located inclusions could then be monitored in highly vascularized tissue [3]. Molecular probes emitting fluorescence in the NIR region are...
desirable and under development [5-10]. Presently most fluorescence detection is conducted with probes fluorescing in the visible, mainly by fluorescent proteins. Tissue autofluorescence is relatively high in this wavelength region, leading to a relatively low fluorescence contrast between the fluorescence from the probe at low concentrations and tissue autofluorescence [5]. Fluorescence contrast is defined as the ratio of the induced fluorescence in the fluorescent probe and the background fluorescence. Visible light also has low penetration in tissue, and deeply located inclusions, especially in tissue with a high hemoglobin concentration, are difficult to detect.

In a previous study, we demonstrated a method to determine the depth of an embedded inclusion marked with a fluorescent probe [11]. As the depth, size of inclusion, and concentration are parameters that can be difficult to evaluate independently with fluorescence imaging [4], independent information on any of these parameters will make the determination and localization of the object more accurate. The demonstrated method relies on spectral changes in the fluorescence as it propagates through the tissue. The characteristics of the detected fluorescence light depend on many different factors, for example, the optical properties of the tissue, the depth of the fluorescent inclusion, and the detection geometry [12-18]. Studies to determine the depth of a luminescent source embedded in a liquid phantom have been performed previously [19,20]. The concept of forming a ratio of acquired data to determine the depth of a fluorescent inclusion has been reported using two different excitation beam diameters [19]. An expression of diffusion theory for the emittance from a luminescent source has been fitted to spatially resolved measurements of the diffuse reflectance, providing an accuracy of the depth of an isotropic point source of 1 mm [20]. Also Patterson et al. used fluorescence to study the depth of light transport in tissue [21,22].

The absorption coefficient in tissue is strongly wavelength-dependent in the region from the visible to the NIR region. The dependence of tissue absorption and scattering on wavelength leads to a spectral change in the fluorescence light as it propagates through the tissue, especially the dependence of tissue absorption has been discussed in [18]. The deeper a fluorescent lesion is located in the tissue, the greater the change in intensity between different fluorescence wavelengths. In our previous study [11], the depth of a fluorescent layer was determined with both Monte Carlo simulations and experiments on a liquid phantom, by studying the fluorescence emitted in the 800–900 nm range in reflection geometry. The depth of a fluorescent layer could be determined with an accuracy of 0.6 mm down to a depth of 10 mm.

The aim of this study was to further investigate the usefulness of a depth-resolving technique based on spectral information in the visible wavelength region. Two fluorescence wavelengths (540 nm and 615 nm) were chosen where difference in tissue absorption is large. The wavelengths could in principle be chosen according to a difference in tissue scattering, but as the differences in scattering in general are smaller, the wavelengths were selected based on absorption changes. Limited fluorescence contrast and uncertainties in the optical properties are two important issues in the technique investigated here, especially of importance for detection in the visible region. The effect on a calculated ratio between the fluorescence intensities at two wavelengths as a function of depth was investigated when the optical properties were changed. Low fluorescence contrast is also a realistic problem when working in the visible wavelength region, because of the tissue autofluorescence emitted upon illumination with blue-green light. When working in the NIR region the problem with low fluorescence contrast is much smaller, because tissue autofluorescence is very weak in this wavelength region. The fluorescence contrast will be low when working with fluorescent proteins, because most of them fluoresce in the visible region.
2. Material and Methods

2.1 Geometry of the modeled tissue

In Fig. 1, a schematic of the simulation geometry is shown. A layered structure is assumed. The model of tissue is divided into 60 elements in the radial direction, each element being 0.05 mm wide. The z-direction is divided into 80 or 120 elements depending on the thickness of the model, each element being 0.025 mm long. A fluorescent layer with a thickness of 1 mm can be inserted at any depth in the model.

A pencil beam is assumed to enter the phantom at the point \((r,z) = (0,0)\), as illustrated in Fig. 1. The transmitted fluorescence light is detected as a function of the radius, \(r\). This corresponds to illumination with a thin laser beam and imaging detection of the transmitted fluorescence.

2.2 Fluorescence Monte Carlo simulations

An accelerated fluorescence Monte Carlo method, described in detail previously [23], was used to simulate the fluorescence generated inside an object and its transport to the back surface, where it could be detected. In this code, a Monte Carlo simulation is first performed using optical properties specified for the modeled tissue at the excitation wavelength. This simulation provides an absorption map of the fluorophore. Another simulation is then performed at the fluorescence emission wavelength, yielding a probability distribution of the generated fluorescence inside the volume to be detected at position \((0, z)\). The matrices for the excitation and emission wavelengths are then convolved to yield the probability of detecting a transmitted fluorescent photon \((T)\) as a function of radial distance. Additional inputs to the Monte Carlo simulations, apart from the optical properties of the different layers and the geometry, are the fluorescence contrast and the absorption coefficient of the fluorophores in the fluorescent layer and surrounding tissue. The simulations are accelerated by using a reciprocity theorem. With this concept only one simulation is required for each emission wavelength, rather than one per wavelength for each volume element in the model [23].

2.3 Evaluation of the data

For each wavelength a vector with the probability of detecting a transmitted fluorescence photon as a function of radial distance was calculated. The ratio of the vectors obtained from simulations at two different emission wavelengths was calculated according to Eq. (1):
\[ \gamma = \frac{\Gamma(540)}{\Gamma(615)} \]  

(1)

Data from a point \((0, 0)\) was selected for the illustrations, in order to easily compare the results of the ratio as a function of depth for different parameters in the same graph.

2.4 Optical properties of the tissue model

The optical properties used in the simulations were extracted from the results presented in Ref. [24]. In that study, the measurements were performed on \textit{in vivo} muscle tissue with relatively low blood content. The relations in Eqs (2) and (3) were employed to obtain optical properties at the wavelengths used, where hemoglobin and oxyhemoglobin are assumed to be the dominant absorbers in the visible wavelength region [24]. The absorption is given by

\[ \mu_a(\lambda) = \sum c_i e_i(\lambda) \]  

(2)

where \(c_i\) is the concentration and \(e_i\) is the extinction coefficient of the absorbing molecule, while the reduced scattering can be expressed as

\[ \mu_s(\lambda) = a \lambda^b \]  

(3)

The extinction coefficients for hemoglobin and oxyhemoglobin at 488 and 540 nm were obtained from Ref [25] and the concentrations of hemoglobin and oxyhemoglobin were obtained from Ref [24]. The optical properties obtained for the phantom at the three wavelengths are given in Table 1. The fluorescence yields were assumed to be the same at both wavelengths.

<table>
<thead>
<tr>
<th>Wavelength / nm</th>
<th>(\mu_a / \text{cm}^{-1})</th>
<th>(\mu_s / \text{cm}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>0.09</td>
<td>18</td>
</tr>
<tr>
<td>540</td>
<td>2.13</td>
<td>16</td>
</tr>
<tr>
<td>615</td>
<td>0.14</td>
<td>14</td>
</tr>
</tbody>
</table>

2.5 Simulation of a 3 cm thick tissue model

The number of photons used in each simulation was 70 million, with a total thickness of the phantom of 3 cm. The optical properties used are given in Table 1. One simulation was performed for the excitation wavelength and for each of the two fluorescence emission wavelengths. The matrix obtained for the excitation light was multiplied, pixel-by-pixel, by the matrix giving the fluorescence yield. One such multiplication was performed for each depth of the fluorescence layer, yielding an excitation matrix for each depth of the layer. Finally, a convolution was performed between the excitation matrix and each of the two fluorescence matrices. The convolution was performed for each depth of the fluorescent layer. The depths of the layer in the model were: 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28 mm from the excitation surface.

2.6 Changes in optical properties

The optical properties of the tissue model were altered slightly to evaluate the sensitivity of the fluorescence emission ratio, \(\gamma\), to such variations. Five different simulations were performed, changing the absorption or reduced scattering coefficient at all wavelengths by \(\pm 20\%\). When not altered, the optical properties given in Table 1 were used. The properties
changed are listed in Table 2. The total thickness of the model was 20 mm, and the number of photons for each simulation was 10 million. In all other aspects, the simulations were identical to those described in Section 2.5.

Table 2. The optical properties used to simulate the effects of changes in optical properties on the fluorescence ratio.

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>μa (cm⁻¹)</th>
<th>μ′a (cm⁻¹)</th>
<th>μa (cm⁻¹)</th>
<th>μ′a (cm⁻¹)</th>
<th>μa (cm⁻¹)</th>
<th>μ′a (cm⁻¹)</th>
<th>μa (cm⁻¹)</th>
<th>μ′a (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>540</td>
<td>1.7</td>
<td>16</td>
<td>2.56</td>
<td>16</td>
<td>2.13</td>
<td>12.8</td>
<td>2.13</td>
<td>19.2</td>
</tr>
<tr>
<td>615</td>
<td>1.1</td>
<td>14</td>
<td>0.17</td>
<td>14</td>
<td>0.14</td>
<td>11.2</td>
<td>0.14</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Simulations were also performed to model how the ratio is affected when the relative attenuation of the two fluorescence wavelengths is changed. The absorption coefficient was varied for the fluorescence wavelength at 540 nm according to Table 3. The depths of the layer in the model were: 3, 7, 10, 13 and 19 mm from the excitation surface.

Table 3. Absorption coefficient of the tissue model with different attenuation at the fluorescence wavelengths

<table>
<thead>
<tr>
<th>p (540 nm) (cm⁻¹)</th>
<th>p (615 nm) (cm⁻¹)</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.28</td>
<td>0.14</td>
<td>2</td>
</tr>
<tr>
<td>0.7</td>
<td>0.14</td>
<td>5</td>
</tr>
<tr>
<td>2.4</td>
<td>0.14</td>
<td>10</td>
</tr>
<tr>
<td>21.3</td>
<td>0.14</td>
<td>100</td>
</tr>
</tbody>
</table>

2.7 Contrast in fluorescent phantoms

In the last part of this study, the influence of fluorescence contrast in the tissue model was studied. Here we simulated the case where the bulk tissue surrounding the layer also exhibited fluorescence, and the contrast between the bulk tissue and the fluorescent layer was varied. The fluorescence contrast of the tissue model was assumed to be 1, 10, 25, 50, 100 and infinity. Fluorescence contrast of 1 corresponds to the situation of having only autofluorescence in the entire model and a contrast of infinity corresponds to fluorescence induced in the fluorescent layer only. The different values of the fluorescence contrast were chosen to study the trend of the intensity ratio as a function of depth when changing the contrast value. The investigated depths of the layer were 4, 10, 16, 19, 25 and 28 mm. Otherwise the input data in Section 2.5 were used.

3. Results

Figure 2 shows the simulated radial profiles in a logarithmic scale of fluorescence emitted from the surface of a tissue model containing a fluorescent layer at three different depths 13, 16 and 19 mm. The graph to the left shows the radial profiles for the transmitted fluorescence at 540 nm, while the graph in the middle shows the corresponding fluorescence at 615 nm. The graph to the right shows the radial profile of the ratio between 540 and 615 nm. These results are given for an ideal case with infinite fluorescence contrast where fluorescence is induced only in the thin fluorescent layer.
Fig. 2. Left: the radial profile for the yellow fluorescent wavelength for depths of 13, 16 and 19 mm. Middle: the radial profile for the red fluorescence at the same depths, and right: the ratio between the yellow and the red fluorescence at the same depths.

The image in the upper left corner of Fig. 3 shows the simulated distribution of the excitation light on a logarithmic scale in a tissue model, assuming the optical properties listed in Table 1 in the entire volume. The image to the lower left describes the fluorescence emitted in a layer at $z = 4$ mm. The images in the center show the probability of detecting fluorescence at $r = 0$ in the model, emitted from various positions in the model at two fluorescence wavelengths (upper row 540 nm and lower row 615 nm). These maps were modeled using a reverse Monte Carlo simulation from the backside of the model.

Fig. 3. Upper row: left, the distribution of the excitation light where the light can be absorbed; middle, the probability of fluorescence emitted at 540 nm to be detected at position $r = 0$ and $z = 30$; and right, the result of multiplying the excitation and emission images. Lower row: corresponding images for fluorescence emission light at 615 nm. The image in the lower left corner shows the distribution of the fluorescence emission inside the model with a fluorescence layer at $z = 4$ mm and an infinite fluorescence contrast.
To the right in Fig. 3, the results of a multiplication of the excitation by the fluorescence light is shown, assuming that the entire model has a uniform fluorescence efficiency of unity, i.e. using the map in the upper left corner for the excitation. This multiplication provides maps of the areas contributing to the fluorescence emitted at \( r = 0 \), and are calculated as an overlap between the excitation and the fluorescence light. Such a map is of interest when evaluating fluorescence at different wavelengths escaping the model versus the position of the fluorescent layer.

In Fig. 4 the calculated yellow/red ratio as a function of depth of the fluorescent layer is presented in logarithmic scale for various optical properties (see Table 2). The absorption or scattering coefficients for the three wavelengths were changed by ±20% at the same time. Figure 5 shows the logarithmic value of the ratio as a function of depth when the relative attenuation of the two wavelengths is changed. The absorption coefficient at 540 nm was changed to achieve a relative difference of the two wavelengths by the factors 2, 5, 10 and 15.

The results below show how the detected fluorescence is altered by autofluorescence from the surrounding bulk tissue. Figure 6 shows the detected yellow and red intensities as a function of depth of the fluorescent layer for different contrast between the layer and the rest of the tissue model and also the yellow/red ratio. As can be seen the slope of the curve decreases as the contrast is reduced, leading to a less sensitive determination of the depth of the object. For a low contrast it becomes difficult to determine the depth, especially in the center of the model.
4. Discussion

Monte Carlo simulations were performed to determine the depth of a fluorescent layer inside a homogeneous tissue-like medium, assuming excitation with blue light and detection of yellow and red fluorescence wavelengths in transmission geometry. A calculated yellow/red ratio is depth-dependent when the tissue model exhibits difference in absorption at the two detection wavelengths, as the spectral shape changes as the light propagates through the medium [18].

To determine the depth, we utilized the concept of a dimensionless ratio. In real measurements there are always advantages in using a dimensionless ratio, because some of the experimental parameters will be canceled out. Examples of these parameters are fluctuations in the illumination source and object distance [26]. By forming a ratio, we limit the evaluation to two wavelengths. This automatically leads to the question of which wavelengths should be used to yield the best depth resolution and robustness. The choice of wavelengths depends on both the availability of a suitable fluorophore as well as the optical properties of the tissue and the presence of tissue autofluorescence. Below we discuss the choice of wavelength range in terms of light penetration, uncertainties in optical properties, and the presence of tissue autofluorescence.

Light obviously penetrates better, the lower the attenuation coefficient. This means that it is easier to detect fluorescence embedded in tissue in the NIR region between 600 and 900 nm, as at these wavelengths the absorption by hemoglobin, lipids and water is low. This is especially important for thick tissues containing large amounts of hemoglobin. Higher absorption will, however, preferentially attenuate light with a long path length in the tissue, yielding a less diffuse image. Increased absorption thus means a lower signal, but higher spatial resolution. This has previously been explored by Yoo et al. [27].

One should also choose a pair of wavelengths for which the relative attenuation is more or less independent of the exact composition of the tissue. This is of great importance when the tissue composition varies in the volume studied, or is unknown because of large interindividual variations, for example, due to lipid and water content. If two wavelengths are identified, for which different tissue types exhibit almost the same optical properties, the depth of a fluorescent inclusion can be determined much more robustly. In a previous simulation study [11], the depth resolution for NIR fluorescence was investigated for six tissue types,
ranging from water-rich to lipid-rich. Using the data obtained from those simulations, the depth of a lesion could be determined with an accuracy of 2 mm, without knowing the tissue composition. It thus seems to be possible to find such a wavelength pair around 930 nm, where water and lipids are the major absorbers, and absorb to similar degree. Variations in tissue composition are, from the absorption point of view, due to the relative amounts of lipids and water. In the visible region, where hemoglobin is the major absorber, it is not possible to find such wavelength regions, where the absorption is equal for tissue with various amount of hemoglobin. As the absorption of both wavelengths used scale linearly with the hemoglobin concentration, the relative absorption will, however, remain the same and a proper wavelength pair can thus be selected. Alterations of ±20% correspond to the variation in hemoglobin content that we found to be realistic for intersubject variation [28]. As hemoglobin is the main absorber for the excitation wavelength, as well as for both detection wavelengths in the visible region, all absorption coefficients were varied simultaneously in the simulations. The results show that a change of 20% in either the absorption or scattering coefficient still causes a considerable difference in the ratio at positions far from the detector (small depths), yielding a depth variation determined from the ratio of approximately 2–3 mm, see Fig. 4. This corresponds to a relative error in the determination of the depth of 10–15%. For layers closer to the detector the ratio is more or less independent of the optical properties, and the depth variation is only 0.5–1 mm. It is interesting to note in Fig. 4 that a change in \( \mu_a \) of ±20% and a change in \( \mu_s \) of ±20% cause almost the same effect on the ratio. If diffusion of light can be assumed in the medium, scattering effects are described with the reduced scattering coefficient \( \mu_s' \), and the remaining light fluence rate as a function of distance from a source is described by the exponential law \( \exp(-\mu_{eff} d) \), where \( \mu_{eff} \) is the effective attenuation coefficient, see Eq. (4), and \( d \) is the distance.

\[
\mu_{eff} = \sqrt{\frac{2}{3}} \mu_s \left( \mu_a + \mu_s' \right)
\]  

(4)

This explains why the change in \( \mu_a \) and \( \mu_s' \) provides similar results. A ±20% change in the absorption or scattering coefficient results in an approximately ±10% difference in \( \mu_{eff} \). If diffusion is assumed, the logarithmic ratio between the fluorescence intensities at two wavelengths as a function of depth will thus be linear with a slope determined by the differences in \( \mu_{eff} \). Figures 4 and 5 show linear relations of the logarithmic ratio as a function of depth, which suggests diffusion being valid. It should, however, be noted that diffusion is not always valid and non-linear shapes of the logarithmic ratio may occur.

This leads to the question of how great a difference in absorption between the two detection wavelengths is needed to achieve sufficient depth resolution. It is not always ideal to have as large difference as possible. A larger difference in absorption provides better depth resolution, but too high absorption prevents the light from penetrating deeply into the tissue. This is clearly illustrated in Fig. 3. Only inclusions close to the detector would be detected with a high absorption. Here the overlap of excitation light and emitted fluorescence light can be seen for the two fluorescence wavelengths involved. If there is no overlap, no fluorescence will be observed at the detector surface. As can be seen, no value can be obtained for the ratio for layers located at depths less than 0.5 cm when using the optical properties given in Table 1. This would be the case for the layer at the depth of 4 mm where the fluorescence would only be induced in this layer, which is indicated in the lower left image in Fig. 3. The absorption at 540 nm is so large that the light cannot reach the detector side. This can be compared to the situation when measuring in reflection geometry, then the method would be more sensitive to fluorescence from depths closer to the excitation side. These images show how important it is to select the fluorescence wavelengths carefully. If a wavelength with too high an absorption is chosen then the detectable depths will be limited to a smaller region. Another interesting remark is that the trend of the yellow fluorescence curve differs from that of the red fluorescence curve, as can be seen in Figs. 2 and 6. The trend of the yellow
fluorescence curve indicates an increase in fluorescence; this is because of the higher absorption of the yellow light compared to the absorption of the excitation light. This can also be seen in the images in the upper row in Fig. 3. For the red wavelength it is the opposite relation providing a decrease in fluorescence instead, as can also be seen in the images in the lower row in Fig. 3. In Fig. 5 it is also illustrated how important it is to know the relative attenuation of the two wavelengths used in order to determine the depth of an inclusion, as the slope of the ratio as a function of depth alters significantly with changes in the relative attenuation.

The concept of using a ratio to evaluate the spectral changes may be improved by using the statistical approach of multivariate analysis. These techniques have been used to analyze tissue in order to distinguish between malignant and non-malignant tissue and to delineate tumors in various types of tissue [29,30]. Utilizing this concept, information obtained from many wavelengths could be used to increase the depth-resolution. More spectral information should make the analysis more robust when the optical properties of the medium are not fully known. It would also reduce some of the work involved in finding the wavelength pair that provides the best depth resolution. This is something we plan to investigate in the near future.

When illuminating tissue with blue or green light, it will always emit autofluorescence. This autofluorescence is often strong compared to the weak signals from the fluorescence probe, as the volume of the surrounding tissue is so much larger than that of the embedded inclusion. Many fluorescent probes used today, for example the fluorescent proteins, emit in the visible wavelength region, where tissue autofluorescence is much stronger than in the NIR range. It is thus of importance to investigate the more realistic case when the rest of the phantom also fluoresces and not only the embedded object. The results of our Monte Carlo simulations show that a low fluorescence contrast makes it difficult to investigate the fluorescent inclusion, including determining its depth. When the fluorescence contrast is 10, the fluorescence detected from the inclusion is strongly influenced by the autofluorescence, making it difficult to determine the depth of the inclusion as illustrated in Fig. 6. It can also be seen in Fig. 6 that a tissue model containing autofluorescence only, meaning that the contrast is 1, gives a constant value of the ratio. This is a rather trivial result, as the fluorescence should be independent of the position of the layer, in this case when the layer has identical fluorescence properties as the rest of the model. The autofluorescence will contribute to a background fluorescence level. If the detectable fluorescence from the layer is not stronger than this background fluorescence, it will fall into the background noise. This is clearly shown for both wavelengths in Fig. 6. Only in the case with infinite fluorescence contrast, when the surrounding tissue has zero autofluorescence, no such effect is seen. It is interesting to note that it is in the central part of the model where it is difficult to evaluate the depth. This is due to that the 540 nm light is more absorbed than light at the excitation wavelength, yielding a positive slope of the detected fluorescence as a function of depth, while the corresponding curves for 615 nm exhibit a negative slope, as fluorescence emission at this wavelength is less attenuated than the excitation light. This yields a flat region in the central part of the model for the ratio curves. If other wavelengths with a different relation of the optical properties had been chosen, the flat region could have been at either end of the tissue model. The contribution from the autofluorescence thus clearly needs to be considered when the depth is determined by the ratio. The ongoing development of probes in the NIR region is therefore of great importance, so that imaging can be performed in the region where the tissue autofluorescence is minimized and also greater depths can be investigated. Both quantum dots and fluorescent proteins are under development in the NIR region [10,31].

In this study we focused on a homogeneous layered structure, but of course it needs to be investigated what happens for other geometries. There is also a need to study the ratio as a function of depth for a fluorescent object with a different shape. It should be noted that a large object close to the detector has the same radial profile as a small object far from the detector.
It is also important to investigate how this method would work in tissues with other optical properties. The spectral information to provide depth information of an inclusion may be shown to be useful as independent a priori information in tomographic evaluation algorithms. In the NIR region where scattering dominates over absorption the reconstruction of the medium becomes ill conditioned, as discussed for absorption tomography by e.g. Arridge [32]. It can thus become difficult to determine the spatial location of the object and also its size. Additional information may help to refine the reconstruction algorithm.

5. Conclusions

In this study, we have shown that the depth of a fluorescent inclusion can be determined by calculating the ratio of the intensities of two fluorescent wavelengths (yellow and red). We have especially investigated the effect on the ratio when the optical properties are changed, and with different fluorescence contrast.

Acknowledgments

The authors would like to thank Johannes Swartling and Christoffer Abrahamsson. This research was supported by the EU Integrated Project "Molecular Imaging" LSHG-CT-2003-503259.
Paper IV
Tissue temperature monitoring during Interstitial Photodynamic Therapy

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ABSTRACT

During 5-aminolevulinic acid (ALA) based Interstitial Photodynamic Therapy (IPDT) a high light fluence rate is present close to the source fibers. This might induce an unintentional tissue temperature increase of importance for the treatment outcome. In a previous study, we have observed, that the absorption in the tissue increases during the treatment. A system to measure the local tissue temperature at the source fibers during IPDT on tissue phantoms is presented.

The temperature was measured by acquiring the fluorescence from small Cr$^{3+}$-doped crystals attached to the tip of the illumination fiber used in an IPDT-system. The fluorescence of the Alexandrite crystal used is temperature dependent. A ratio of the intensity of the fluorescence was formed between two different wavelength bands in the red region. The system was calibrated by immersing the fibers in an Intralipid solution placed in a temperature controlled oven. Measurements were then performed by placing the fibers interstitially in a pork chop as a tissue phantom. Measurements were also performed superficially on skin on a volunteer. A treatment was conducted for 10 minutes, and the fluorescence was measured each minute during the illumination. The fluorescence yielded the temperature at the fiber tip through the calibration curve. The measurements indicate a temperature increase of a few degrees during the simulated treatment.

Keywords: interstitial photodynamic therapy, temperature-dependent fluorescence, Cr$^{3+}$-ions

1. INTRODUCTION

The aim of this study is to present results of an optical method to monitor tissue temperature behaviour during simulated Photodynamic Therapy (PDT) treatments superficially on skin, but also interstitial PDT illumination in a piece of meat.

1.1 Interstitial Photodynamic Therapy (IPDT)

Photodynamic therapy, PDT, is a method for treating locally situated tumours. The patient is given a photosensitising drug a certain time period before the treatment. The photosensitizer can be administered in different ways. After some time the photosensitizer has selectively accumulated in the tumour and the treatment is initiated. By illuminating the tumour, with light of a wavelength matching an absorption peak of the photosensitizer, the photosensitizer is excited. The photosensitizer can interact with other molecules and an energy transfer is therefore possible. If the energy is transferred to an oxygen molecule, this molecule can be excited from a triplet ground state to a first singlet excited state. This singlet state is very reactive and can oxidise proteins and cell constituents, which can lead to cell necrosis or apoptosis in the tumour.

The main advantages of PDT are that the treatment is selective, can be repeated and that the treated areas heal fast. A major disadvantage is that only superficial tumours can be treated and with tumour thickness only up to a few millimetres. A possible solution to this problem is to use interstitial photodynamic therapy, IPDT, where optical fibers are inserted into the tumour mass. This will give the advantage to enable treatment of more solid tumours, either primary tumours or e.g. liver metastases.
1.2 Why is it important to monitor the temperature locally?

During an IPDT treatment procedure a high light fluence rate is present close to the source fibers, especially when using bare end cut fibers. This might induce an unintentional tissue temperature increase of importance for the treatment outcome. In addition, an increase in the measured absorption coefficient has been seen during IPDT treatments, in a recent study. The increase in the absorption coefficient could be an effect of tissue deoxygenation, changes in blood flow, local bleeding or temperature increase close to the fiber tips. The goal of an IPDT treatment is to not have a hyperthermal, but a photochemical effect, on the lesion. If a small bleeding occurs at the fiber tip, the absorption coefficient will increase drastically and this could lead to an abrupt temperature increase. Such bleeding would reduce the efficacy of the treatment, since most of the light would be absorbed in the blood right at the fiber tip.

In an earlier published study, the blood perfusion and the temperature were measured during superficial PDT, both on patients with lesions and on skin on voluntes. The temperature was studied with an infrared camera and an increase in temperature 1-4 °C could be seen during the treatment. This small temperature increase shows that there is no hypothermal effect during the treatment when an initial temperature was about 35 °C. The infrared camera only monitor the really superficial layer of the skin and in deeper lying tissue the temperature could actually be higher. With a thermometer based on an optical technique, the temperature could actually be measured at other deeper locations.

2. MEASUREMENT PRINCIPLES

One way to measure the temperature with an optical technique is to use crystals, which fluorescence is temperature dependent. To be able to measure the temperature locally in the tissue where the treatment fibers are located, a small crystal can be attached to the fiber tip. In this study a crystal doped with Cr3+-ions was used. Cr3+-ions in ionic crystals interact strongly with the crystal field and the lattice vibrations. The crystal field arises because of the influence on the Cr3+-ion from the neighbouring ions. The interaction between the Cr3+-ions and the crystal field arises due to the fact that there are no outer shells to shield the three valence electrons. As a result Cr3+-activated materials are characterised by a wide absorption spectrum, from UV to infrared. This has two advantages, the possibility to choose excitation source and that a small drift in the excitation source will not cause a significant change in the fluorescence intensity. Because of the strong crystal field interaction, the energy gaps of the electronic levels of Cr3+ can vary from one host crystal to another. The temperature dependence of the fluorescence lifetime varies with the energy gap and will thus differ for different Cr3+-doped materials. Different Cr3+-doped materials show different temperature dependence in the fluorescence signal. Among the different crystals, Alexandrite seems to show good temperature sensitivity in the interesting temperature range (15 - 100 °C) in this case.

A Jablonsky diagram of Alexandrite is shown in Figure 1. The ground state in Cr3+ is always A2, independent of the strength of the crystal field. Two excited energy levels are involved, following excitation at 635 nm: T1 and E. The energy splitting between these two low-lying states is denoted ΔE = E(T1) - E(E). ΔE varies strongly with the strength of the crystal field and can be both negative and positive. The emission spectrum of Cr3+ consists of two different features, a broad spectral band and two sharp peaks, so called R-lines. The broad band originates from the vibrational transitions, T1 → A2, where ions in the T1 state decay to the empty vibrational levels of the A2 state. The R-lines appear because of a further split of the E state into two levels, E and EA, separated by a small energy gap. The R1-line is the transition E → A2 and the R2-line comes from the transition 2A2 → A2. Lattice vibrations in the crystal interact with the electronic levels of the Cr3+-ion. The effects of this are the initiation of vibrational transitions, radiationless transitions and phonon scattering. The first of these effects produces broad bands in the spectra, the second effect leads to a temperature dependent decrease of the fluorescence lifetimes of the R-lines and both the second and third effect can cause a thermal broadening of the R-lines.

In Alexandrite, the lowest excited state of the Cr3+-ions is the E state (Figure 1). At low temperatures the emission is dominated by the transition E → A2 (the R-lines), yielding an effective long fluorescence lifetime, since the transition is parity and spin forbidden. The T1 state has a much shorter lifetime than the E state. When the temperature increases, a higher percentage of the Cr3+-ions will populate the T1 state according to the Boltzmann distribution. Consequently more ions will decay through the T1 → A2 path, resulting in a decrease of the fluorescence lifetime. Thus, at low temperatures the thermally activated populations of the E and T1 states determine the fluorescence properties.
This phenomenon can also be seen in the intensity of the fluorescence. The fluorescence, corresponding to the R-lines, will decrease when less ions will follow the path $E_2 \rightarrow A_2$. This can be used as a measure of the temperature by forming a dimensionless ratio for the intensities from the R-lines divided by the intensity at longer wavelengths.

![Energy levels as a function of nearest neighbour distance for a crystal with a high crystal field strength](image)

An evaluation of the method measuring the temperature with fluorescence from crystals has been reported earlier. In that study fluorescence lifetime measurements were performed. In this study we go one step further and use an integrated system developed for IPDT to measure the fluorescence and calculate an intensity ratio of the fluorescence to give a value of the actual temperature. There are commercially available fiber based thermometers using optical techniques to measure temperature dependent fluorescence, which can be used for temperature monitoring. The concept we use in this study is that the crystal is located on the fiber tip of the fiber delivering the treatment light during a real IPDT treatment.

3. MATERIAL AND METHODS

3.1 Material
In this section the Interstitial Photodynamic Therapy system used will be described and also a short description of how the crystal was attached to a bare end fiber tip will be given.

3.1.1 IPDT system
An instrument for interstitial photodynamic therapy has been developed by the company SpectraCure AB (Ideon Research Park, Lund, Sweden). A general schematic drawing of the instrument is shown in Figure 2. The instrument uses a maximum of six bare end optical fibers that are used to deliver the therapeutic light into the tumor mass. The same fibers can also be used in order to perform diagnostic measurements during the treatment session. The therapeutic light unit consists of six diode lasers emitting at 635 nm with an individual maximum output power of 200 mW. While in treatment mode, light from the therapeutic light unit is guided into the light distribution module and further coupled into the six 400 μm diameter fibers, which are inserted in the tumor.

![A schematic description of the interstitial photodynamic therapy system used for the experiments](image)
In the measurement mode, light from the diagnostic light unit is coupled into one of the "patient fibers" via the light distribution module. After interacting with the sample the fluorescence light is collected by the other fibers and coupled into an imaging spectrometer covering the spectral range between 620 and 810 nm. A cut-off filter (Schott RG665) is used to attenuate the intense laser light at 635 nm from the laser light source. Wavelength calibration of the spectrometer is carried out using an HgAr lamp to determine the relation between wavelength and pixel number in the horizontal direction of the CCD chip.

3.1.2 Attachment of crystal to fiber tip
For the measurements, an end cut fiber with a core diameter of 400 μm (Fiberguide Industries, USA), was used. A small Alexandrite crystal was glued to the clear cut fiber tip, using the glue (Nordland Optical Adhesive 68, Norland Products) which needed to cure in UV-light for 30 minutes.

3.2 Calibration of fiber
The set-up used for temperature calibration is shown in Figure 3. The calibration was performed in the temperature interval 15 - 50 °C. Two fibers were immersed in an Intralipid solution of 1.28 % in a vessel. The distance between the fibers was approximately 12 mm. Initial tests were performed to investigate the influence of different scattering coefficients, by changing the Intralipid concentration. Different distances were also tested to study the effect of the distance between the two fibers. One fiber was used only for delivering the excitation light and the other one, with an attached piece of crystal, was used for detection of induced fluorescence light. The vessel and the fibers were placed inside a temperature controlled oven (FN300 Nive Microprocessor). To monitor the actual temperature in the Intralipid, two thermistors were also placed in the vessel, close to the fibers. After approximately 45 minutes the temperature had stabilized in the solution, the laser in the PDT system was turned on and was illuminating through fiber 1. The induced fluorescence light from the crystal was detected through fiber 2 in Figure 3, and an image was acquired with the detection unit. For each temperature, two different powers (90 mW and 130 mW) of the laser light were tested, in order to monitor the influences of the power in the fluorescence light. Then the temperature of the oven was increased and the procedure was repeated.

![Figure 3](image-url)

**Figure 3.** The set-up used for calibration. The fiber used for illumination of the laser light (denoted 1) and the fiber with a piece of crystal (marked 2) are shown.

3.3 Evaluation of a fluorescence spectrum
The evaluation of the temperature was performed by using the concept of forming a dimensionless ratio. A summation was made for the intensities in two different wavelength bands, marked with Area 1 and Area 2 in Figure 4. A ratio was then formed between the two values from Area 1 and Area 2 for each measured temperature.
3.4 Experiment

For the experiments performed in this study, two different arrangements of the two fibers were used. In arrangement A, see Figure 5a, the end cut fiber, denoted with 1 in the figure, was used for illumination during the simulated treatment. The other fiber (denoted 2), with a crystal attached to the fiber tip, was used to monitor the temperature. The treatment light was used to induce fluorescence in the crystal. The fluorescence light was guided through the fiber into the detection unit in the IPDT system. With this arrangement the temperature was monitored in the spot where the doped fiber was placed, which means a small distance from the treatment fiber.

In fiber arrangement B, see Figure 5b, the fiber with a piece of crystal attached to the tip (denoted 2), was used for illumination during the treatment. This fiber was illuminating the whole time except during the temperature measurements, when it was used for detection of fluorescence light. The other fiber, denoted 1 in Figure 5b, was placed approximately 2 mm apart from the first fiber. Fiber 1 was only illuminating during the temperature measurements to induce the fluorescence in the crystal at the other fiber tip. With this set-up the temperature is monitored where the illumination fiber is located and not at a small distance as in arrangement A.

3.4.1 Simulation of a superficial treatment on skin

Both the fiber arrangements were used for the investigation on skin. With arrangement A, (Figure 5a), the two fibers were placed in close contact with the skin on the arm of a healthy volunteer. In the first measurement, a power of 130
mW through the illumination fiber was used, and detection of fluorescence light, with the doped fiber, was performed each 10 seconds during 2 minutes. This was done to see how the temperature increases in the beginning of the illumination period. In the following three measurements the distance between the two fibers was changed (see distance d in Figure 5a) with the values of 1 and 3 mm. The power from the illumination fiber was measured to 130 mW and the illumination was continued during 10 minutes. A fluorescence measurement was acquired each minute. The temperature of a larger area of the skin was monitored using an infrared camera (uncooled, AGEMA 570 Elite, Flir Systems Inc), which was saving a temperature image every minute.

In the experiment using fiber arrangement B, two different powers, 75 mW and 110 mW, were used for illumination in order to monitor how the temperature changed as a result of different treatment power. One temperature measurement was performed before the treatment, to get information about the initial temperature. The treatment started by illuminating with the doped fiber (denoted 2 in Figure 5b). After 1 minute the other fiber (denoted 1 in Figure 5b) started to illuminate. Detection of induced fluorescence light from the crystal was performed through fiber 2. The illumination was continued as before and a temperature measurement was recorded each minute during the treatment. After 10 minutes the treatment was interrupted, but the measurements continued for a few minutes to study the temperature change without any illumination.

3.4.2 Simulation of an interstitial treatment in meat
In the final experiments, regarding interstitial treatment, measurements were performed using a pork-chop as a tissue phantom. The two fibers were placed next to each other inside the meat. By using fiber arrangement A, a 10 minutes treatment was performed. In this case an output power from the illumination fiber was 130 mW and the depth of the fibers in the meat was approximately 12 mm. Two experiments were also conducted with arrangement B. In the first experiment the depth of the fibers was 18 mm and a power of 110 mW. The treatment continued during 20 minutes. In the last experiment the fibers were placed at a depth of 10 mm and a power of 110 mW, but only for 10 minutes. A temperature measurement was performed each minute.

4. RESULTS AND DISCUSSION

4.1 Calibration curve for fiber with attached crystal
A calibration curve is shown in Figure 6, with the dimensionless ratio as a function of the corresponding temperature. For the calibration curve different fluorescence excitation powers were used. It can be seen in the figure that the influence of the power is very weak. The reason for that might be the formation of a dimensionless ratio, with the main advantage of cancelling out environmental effects. Tests were also performed when changing the distance between the fibers and different scattering in the Intralipid. The results, not presented here, show that the distance, between the illumination and detection fiber, does not affect too much, more when the distance is too big so the detected fluorescence signal becomes too weak, and noise disturbs the measurements. A different scattering power of the Intralipid phantom does not affect the ratio either. A quadratic polynomial was fitted to the acquired data points. This curve was later used as a calibration curve for determining the temperature measured during the tissue measurements.
Figure 6. A calibration curve for the fiber with an attached crystal is shown. Measured values (crosses) and the fitted calibration curve (solid line) to these values are shown.

4.2 Superficial measurements on skin

The absolute temperature as a function of time can be seen at a small distance from the illumination fiber during a two minute long session on skin (Figure 7). A quadratic polynomial has been fitted to the measured values. As can be seen the temperature is increasing during the first two minutes and it is stabilizing at later times.

Figure 7. The temperature increase during the first two minutes of superficial illumination on skin is shown. Measured temperatures (crosses) and a fitted line to the measured values are shown.

The temperature increase can be seen as a function of time when changing the distance between the illumination and the doped detection fiber (denoted d in Figure 5A) for two different simulated treatment sessions, in Figure 8. When changing the distance between the two fibers, the temperature in different spots from the illumination spot will be monitored. As can be seen the temperature is increasing the closer to the illumination fiber the detection fiber is located.
An infrared image is shown after 5 minutes of a superficial treatment on skin (Figure 9). The same figure presents a graph of the temperature increase as a function of distance in the infrared image. As can be seen the increase is about 4 °C close to the middle, which can be related to the same increase seen in Figure 8 for the distance 1 mm. Further out the increase is not of that high value. This shows how important it is to monitor the temperature in the right spot where the illumination is.

It is interesting to see the temperature increase when using two different powers, as shown in Figure 10. In this figure the illumination fiber is the same as the doped fiber, (see fiber arrangement in Figure 5b), which means that it is the local temperature where the illumination fiber is located that is measured. As can be seen, the temperature increase is larger when using the power of 110 mW compared to a power of 75 mW. This is not an unexpected behavior when more energy is delivered to the tissue with a higher power. Another interesting feature is that the temperature increase is stabilized after 3 minutes for the power of 75 mW and after 4 minutes with the power of 110 mW, and for the rest of the treatment the temperature is rather constant. This can be explained by a balance between energy deposition due to light absorption and energy dissipation due to the blood flow where the illumination fiber is located. If the increase, as can be seen in the graph, is about 2-4 °C, the blood perfusion will increase to the area of the fiber to remove some of the extra heat. This might take about 3-4 minutes. The laser illumination was stopped after 10 minutes, but a few extra
temperature measurements were performed to see how the temperature was decreasing. It seems like the temperature decreased even below the initial temperature. An explanation to this can be the increased blood perfusion in the area stimulated by the procedure. Only a few minutes after the treatment was interrupted, it seems like the temperature is stabilized again and probably will reach the initial temperature after a short while. A change in the blood perfusion to the initial perfusion will probably take a few minutes.

The reason that the temperature increase is shown instead of an absolute temperature is that there is a difference in the initial temperatures on different locations of the skin.

4.3 Interstitial measurements in meat
The temperature increase during an interstitial treatment in a piece of meat is shown in Figure 11. Three different curves are shown, corresponding to three different treatment sessions. Two of the treatments were conducted for 10 minutes and one for 20 minutes. As can be seen in the graph, the temperature is increasing during the whole session. This can be compared to the superficial measurements on skin where the temperature increase is stabilized after a while as the blood perfusion most probably removes the heat. In interstitial treatment in meat there is no convection or blood perfusion which can stabilize the temperature, instead there will be an increase during the whole treatment. The measurements on meat symbolize thus an entirely realistic scenario because of the lack of tissue perfusion.
5. CONCLUSIONS

In this study we have shown that tissue temperature can be measured with an optical technique based on the detection of temperature dependent fluorescence light from the Alexandrite crystal. Simulations of superficial treatments on skin, using a bare cut fiber delivering 110 mW light power at 635 nm, showed that the temperature increase is about 2-4 °C for a treatment of 10 minutes. After 3-4 minutes the temperature remains almost constant most likely due to an increased blood perfusion to the area where the illumination fiber is located.

Simulations of interstitial treatment in meat were also conducted, showing the similar temperature increase, but continuing through the entire treatment. In these cases an increase was shown for the whole treatment as no blood flow is existent in the meat.

In the study we worked with two different fiber arrangements used for treatment. In the first arrangement the illumination fiber was not the same as the doped fiber. With this arrangement the temperature is not measured right at the illumination fiber where the light fluence rate is high. With the second arrangement, where the illumination fiber is also the crystal doped fiber, the temperature will be monitored exactly at the fiber tip where a high fluence rate is located. A big temperature increase will only occur in a distance of a few hundred micrometers from the tip of the illumination fiber. This means that the fiber arrangement B (see Figure 3B) is the best for monitoring the local temperature at the fiber tip.

Future work in the project involves more temperature measurements on simulated superficial treatments on volunteers to get a better statistics. Also more interstitial measurements in meat will be conducted. The robustness of the attachment of the crystal to the fiber tip is also an urgent question to be solved. So far, we have seen that only using glue to attach the crystal is not enough. The fiber tip becomes very fragile.

ACKNOWLEDGEMENTS

The authors would like to thank Nina Reistad, for supplying an infrared camera, and Lars Wallman, for supplying us with an oven used for calibration of fiber. The financial support by the company SpectraCure AB and the Swedish Strategic Research Foundation (SSF) are gratefully acknowledged.
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Paper V
Tissue temperature measurements during interstitial laser therapy using Cr\textsuperscript{3+}-doped crystals at the fiber tip

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ABSTRACT
In this project a technique to optically measure the temperature is evaluated. The measurement is to be performed through optical fibres during photodynamic laser treatments or laser thermo therapy of malignant tumours. For this technique Cr\textsuperscript{3+}-doped crystals were used. The lifetime of the ions' fluorescence were measured, since the fluorescence is strongly temperature dependent. A piece of a crystal was attached to the tip of an optical fibre. The crystal was excited at 635 nm, which is the wavelength most frequently used for photodynamic treatment.

An accuracy in the temperature measurement of ± 0.3 °C was obtained for Cr:LiSAF in the region 20 – 70 °C. This is well within the requirements for this application. Alexandrite and Cr:YAG were also evaluated in this study, also yielding a very good accuracy. A laser treatment was simulated using pork chop as tissue phantom and the temperature was measured.

Keywords: Temperature-dependent fluorescence, Cr\textsuperscript{3+}-ions

1. INTRODUCTION
The aim of this project was to evaluate an optical technique to measure the temperature in the tissue, during interstitial photodynamic therapy (IPDT) treatment\textsuperscript{1,2} or laser thermo therapy of malignant tumours.\textsuperscript{3} During an IPDT treatment, fibres are inserted into the tumour. Laser light guided through these fibres induces tumour cell death together with a tumour marker, e.g. Protoporphyrin IX (PpIX). If a local bleeding occurs close to the fibre tip, the temperature will increase due to the higher absorption of light in blood. The high absorption will reduce the efficiency of the treatment. Therefore, it would be favourable if the temperature could be monitored, so the treatment can be optimised and the outcome accurately predicted.\textsuperscript{4,5}

Preferably one would like to be able to measure the temperature with the same fibres that delivers the light during the treatment. A possible way to do this is to attach a crystal, whose fluorescence is temperature dependent, to the fibre tip. Some requirements are that it should be possible to excite the crystal at the treatment wavelength, 635 nm, and that the fluorescence induced in the crystal is sensitive to temperatures in the region 20 – 70 °C, because this temperature interval is interesting for the applications. When doing experiments with tissue phantoms, it is necessary to be able to measure down to room temperature and in laser thermo therapy temperatures up to at least 70 °C must be measurable. The accuracy required is approximately 1 °C in order to predict treatment results. An advantage would be if the fluorescence spectrum from the crystal easily could be separated from tissue autofluorescence. It is also desirable that the crystal should not absorb light around 405 nm, to prevent interference with the PpIX fluorescence measurements during an IPDT treatment.

1.1 Cr\textsuperscript{3+}-doped materials
Cr\textsuperscript{3+}-ions in ionic crystals interact strongly with the crystal field and the lattice vibrations. The crystal field arises because of the influence on the Cr\textsuperscript{3+}-ion from the neighbouring ions. The interaction between the Cr\textsuperscript{3+}-ions and the crystal field arises due to the fact that there are no outer shells to shield the three valence electrons. As a result Cr\textsuperscript{3+}-activated materials are characterised by a wide absorption spectrum, from UV to infrared. This has two advantages, the possibility to choose excitation source and that a small drift in the excitation source will not cause a significant change in fluorescence intensity. Because of the strong crystal field interaction, the energy gaps of the electronic levels of Cr\textsuperscript{3+} can vary from one host crystal to another. The temperature dependence of the fluorescence lifetime varies with the energy
gap and will thus differ for different Cr\(^{3+}\)-doped materials. To measure the temperature using the fluorescence signal from a Cr\(^{3+}\)-doped crystal, the type of crystal should thus be selected with respect to the temperature interval of interest.\(^6\)

The ground state in Cr\(^{3+}\) is always \(^4A_2\), independent of the strength of the crystal field. Two excited energy levels are involved, following excitation at 633 nm: \(^4T_2\) and \(^2E\). The energy splitting between these two low-lying states is denoted \(\Delta E = E(^4T_2) - E(^2E)\). \(\Delta E\) varies strongly with the strength of the crystal field and can be both negative and positive. At high crystal field strength, for example in ruby, Cr:YAG and alexandrite, \(\Delta E\) is positive and the emission of the Cr\(^{3+}\) is dominated by the transition, \(^2E\) \(\rightarrow\) \(^4A_2\). In low crystal field strength, for example in Cr:LiSAF, the dominating transition is \(^4T_2\) \(\rightarrow\) \(^4A_2\).\(^6\)

The emission spectrum of Cr\(^{3+}\) consists of two different features, a broad spectral band and two sharp peaks, so called R-lines. The broad band originates from the vibronic transitions, \(^4T_2\) \(\rightarrow\) \(^4A_2\), where ions in the \(^4T_2\) state decay to the empty vibrational levels of the \(^4A_2\) state. The R-lines appear because of the further split of the \(^2E\) state into two levels, \(^2E\) and \(2A\), separated by a small energy gap. The R\(_1\)-line is the transition \(^2E\) \(\rightarrow\) \(^4A_2\) and the R\(_2\)-line comes from the transition \(2A\) \(\rightarrow\) \(^4A_2\).\(^6\)

Lattice vibrations in the crystal interact with the electronic levels of the Cr\(^{3+}\)-ion. The effects of this are the initiation of vibronic transitions, radiationless transitions and phonon scattering. The first of these effects produces broad bands in the spectra, the second effect leads to a temperature dependent decrease of the fluorescence lifetimes of the R-lines and both the second and third effect can cause a thermal broadening of the R-lines.\(^7\)

1.1.1 Low crystal field strength

In low crystal field strength, the transition \(^4T_2\) \(\rightarrow\) \(^4A_2\) occurs through two processes, one is the radiative transition initiated from I seen in Figure 1. The other is a nonradiative process due to thermal quenching of the Cr\(^{3+}\)-ions. Some ions will be thermally elevated to \(Q\), which is the energy crossing between the excited state and the ground state. These ions will decay to the ground state and will undergo stepwise transitions to the lowest level of the ground state through nonradiative relaxation. These two processes compete with each other constantly.\(^6\)

When the temperature increases (above 275 K), more excited ions will be elevated to \(Q\), due to the larger neighbour distance, and the nonradiative process, called quenching, will become stronger. Normally, the nonradiative transitions have a shorter lifetime resulting in a decrease in the observed fluorescence lifetime with increasing temperature. The fluorescence intensity will decrease with increasing temperature due to an increase in the fraction of ions decaying through nonradiative transitions.\(^6\)

The impact of the \(^2E\) state on the fluorescence lifetime is negligible. This is partly due to the low population of the \(^2E\) state according to the Boltzmann distribution and partly due to that the transition \(^2E\) \(\rightarrow\) \(^A_2\) is forbidden both by parity and spin. The transition rate is thus one or two orders of magnitude lower than the transition \(^4T_2\) \(\rightarrow\) \(^4A_2\), and only a very
small fraction of the excited ions will decay through this path. This explains why there are no visible R-lines in the emission spectra from Cr$^{3+}$-ions in crystals with low crystal field strength.\(^6\)

### 1.1.2 High crystal field strength

In high crystal field strength, the lowest excited state of the Cr$^{3+}$-ions is the $^{2}E$ state, see Figure 2. At low temperatures the emission is dominated by the transition $^{2}E \rightarrow ^{4}A_2$ (R-lines), yielding an effective long fluorescence lifetime, since the transition is parity and spin forbidden. The $^{4}T_2$ state has a much shorter lifetime than the $^{2}E$ state. When the temperature increases, a higher percentage of the Cr$^{3+}$-ions will populate the $^{4}T_2$ state according to the Boltzmann distribution. Consequently more ions will decay through the $^{4}T_2 \rightarrow ^{4}A_2$ path, resulting in a decrease of the fluorescence lifetime. Thus, at low temperatures the thermally activated populations of the $^{2}E$ and $^{4}T_2$ states determine the fluorescence properties. At high temperatures, above 600 K, more ions will be elevated to $Q$ and nonradiative transitions become more significant and will speed up the decay even further.\(^6\)

![Figure 2. The energy levels as a function of nearest neighbor distance for a crystal with a high crystal field strength](image)

### 1.1.3 Temperature sensitivity

Different Cr$^{3+}$-doped crystals have different temperature dependence of the fluorescence. This leads to a higher sensitivity for temperature changes in some temperature regions. In Figure 3 the temperature dependence on the fluorescence lifetime can be seen for alexandrite, Cr$:$LiSAF and ruby.\(^6\) As can be seen in the figure, ruby has a very low sensitivity in the temperature region 300 – 400 K. Due to this, ruby was not a strong candidate for this project.

![Figure 3. Temperature dependences of the fluorescence lifetime for Cr$^{3+}$-ions in different host materials; 1 LiSAF, 2 alexandrite and 3 ruby](image)
1.2 Fluorescence lifetime

The fluorescence lifetime can be measured by using an excitation source whose intensity is modulated by a sinusoidal signal, $\nu_m$. The fluorescence signal will then be forced to follow the same modulation frequency as the excitation source, but lagging with a phase shift, illustrated in Figure 4.9.

$$\nu_f = \nu_m \sin(\omega t - \phi).$$

where $\nu_f$ is the amplitude of the fluorescence response signal, $\omega$ is the angular frequency of the modulation signal, $t$ is the elapsed time and $\phi$ is the phase lag with respect to $\nu_m$. The fluorescence lifetime, $\tau$, is given by (2)\textsuperscript{6,8}

$$\tan \phi = \omega \tau.$$  

2. MATERIAL AND METHODS

In the beginning fluorescence lifetime measurements were performed on small crystals of three different types. Two of the crystals were chosen for further experiments. The crystals were pulverised and attached to a fibre tip with glue. The same experiment was performed again with this configuration. One of these two crystals was selected to make a calibration curve with a more powerful laser used for photodynamic therapy. An experiment was also performed on pork chop as a tissue phantom.

2.1 Fluorescence lifetime measurements of three Cr$^{3+}$-doped crystals

Three different Cr$^{3+}$-doped crystals were found to be suitable for the purpose, namely alexandrite, Cr:YAG and Cr:LiSAF. These crystals have sufficient temperature sensitivity in the region 20 - 70 °C and can be excited by light at 635 nm.

2.1.1 Experimental set-up

The measurements were made with the set-up illustrated in Figure 5. The laser used was a He-Ne laser, Latronix, Sweden, emitting light at 632.8 nm with an output power of 2.3 mW. The laser light was focused into a 600 μm core diameter optical quartz fibre (Fiberguide Industries, USA) with the help of a 50 mm focal length lens with a diameter of 25 mm. In the beam path there was a chopper wheel, model SR540 from Stanford Research Systems. The distal end of the fibre was led into an oven, model FN300 Niöve Microprocessor, where the fibre tip was held in contact with the crystal with the use of a specially designed holder. Here the laser light excited the crystal. The holder was designed to fasten the crystal and to hold up to three fibres in their desired positions.
One detection fibre was fixed in the designed holder, opposite to the fibre from the light source. It collected both the fluorescence light from the crystal and some of the scattered laser light, and guided the light to a photomultiplier tube, model R928 Hamamatsu. Between the fibre tip and the photomultiplier tube there was a holder for optical filters. The holder has two different positions. In one position the filters only let the laser light pass and in the other position only the fluorescence light passes through. The signal from the photomultiplier tube was led to the input channel of a lock-in amplifier, model SR830 from Stanford Research Systems. The photomultiplier tube generated a current signal captured by the lock-in amplifier in the voltage mode as the current passed through a resistance of 10 kΩ. A reference signal to the lock-in amplifier was obtained from the chopper wheel. The lock-in amplifier measured the phase difference between the input signal and the reference signal. Another detection fibre was positioned next to the first one. It collected some of the fluorescence induced in the crystal and guided it to an optical multichannel analyzer (OMA) system. This system recorded the fluorescence emission spectrum.

Two thermistors were used inside the oven to monitor the temperature changes. The resistance of a semiconductor material is the temperature dependent parameter in a thermistor. The signal from the thermistors was analyzed by a computer program, which made it possible to monitor the temperature on a computer screen in real time during the experiments.

The chopper frequency, \( f \), was chosen close to

\[
 f \sim \frac{1}{2\pi \tau},
\]

in order to get a good accuracy in the measurement of the fluorescence lifetime.

2.1.2 Experimental description

The crystal was fixed in the holder, which was placed inside the oven. The excitation and detection fibres were inserted into the oven through a hole. The fibres were placed in the holder. The two thermistors were placed in contact with the crystal. The laser was turned on and the oven was heated to the desired temperature. The temperature was monitored on
the computer screen and when it had stabilized a measurement was made. First the fluorescence light was detected and the phase difference between the fluorescence signal and the reference signal was displayed on the lock-in amplifier. Then the filters were shifted so that only scattered laser light could be detected, and the phase difference between the laser signal and the reference signal was displayed. After this the temperature was raised to the next temperature and the procedure was repeated. Measurements were performed until temperatures of about 70 °C were reached. The data recorded at each temperature were the fluorescence emission spectrum, the two phase differences, the both thermistors’ temperatures and the chopper frequency. Four separate series of measurements were conducted sequentially for each crystal, to study the reproducibility.

2.1.3 Calculation of fluorescence lifetime
To calculate the fluorescence lifetime, a program in MATLAB was written for each crystal. The two phase differences, the frequency and the temperatures from the thermistors were the input data to the program. The first computation was to calculate the phase difference between the fluorescence and the laser light, with the formula (4):

$$\Delta \varphi = \varphi_{\text{fluorescence}} - \varphi_{\text{laser}}$$  \hspace{1cm} (4)

The lifetime could then be calculated as (5):

$$\tau = -\frac{\tan \Delta \varphi}{2\pi f}$$  \hspace{1cm} (5)

A graph was drawn with the lifetime as a function of temperature including all four series of measurements. The temperature was chosen as a mean value of the two thermistors’ temperatures. A polynomial of degree two \((ax^2 + bx + c)\) was fitted to the data from the four measurements, with the least squares method. In a new graph a polynomial of degree two was fitted to all the datapoints from the four series. The standard deviation was also calculated for the difference between the data points. The relative standard deviation was calculated as the standard deviation divided by the mean value of the lifetime.

2.2 Characterization of crystal attached to fibre tip
After the initial experiments alexandrite and Cr:LiSAF were chosen for the continuing experiments. The crystals were pulverised in a mortar. One grain of the crystal was glued to the fibre tip using a non-fluorescent epoxy. After curing, a second layer of epoxy was applied around the crystal to minimise the influence of the environment on the fluorescence of the crystal.

2.2.1 Experimental set-up
The experimental set-up was almost the same as in the previous section 2.1.1. The difference was that the crystal was much smaller and glued onto the tip of the fibre, coming from the laser. The fibre used was 400 \(\mu\)m in diameter. A bandpass filter, 630 ± 5 nm, was now placed in front of the laser to filter out unwanted emission of the other wavelengths.

2.2.2 Experimental description
The fibres were inserted into the oven and fastened in the holder and the thermistors were placed as close to the small crystal as possible. The experiment was conducted in the same way as in section 2.1.2. Only two measurement series for each crystal were performed. Calculations were conducted in the same way as in section 2.1.3.

2.3 Fluorescence lifetime measurements on crystals attached to fibre tip with a PDT -laser
After selecting the best suited crystal for our purpose, Cr:LiSAF, the fibre was tested with a more powerful laser, a high power laser normally used for PDT. A new fibre had to be prepared since the first one was broken. In this experiment both the excitation and fluorescence light were guided through the same fibre. With this new set-up a calibration curve was made.
2.3.1 Experimental set-up
The experiment was performed with the set-up seen in Figure 6. The laser was a Cerasla™ PD7635, CeramOptec, diode laser. Since the laser exhibited a broad emission profile from 630 - 700 nm with a peak at 635 nm, a bandpass filter 635 ± 5 nm was inserted in front of the laser, so that no laser light, except 635 nm, could influence the fluorescence measurements. The laser was focused into the fibre with a lens system, which produced a 1:1 image of the object.

![Figure 6. The experimental set-up to make the calibration curve](image)

To be able to excite the crystal and detect the fluorescence light through the same fibre, a small box containing a beamsplitter (50/50) and three fibre connections was used. From one of the connections the laser light is partly transmitted straight through the box into the probe fibre, which leads the excitation light to the crystal. The fluorescence light from the crystal is collected by the same probe fibre and the light is guided back to the filter box. Here the light is partly reflected by the beamsplitter into the third fibre, which leads to the photomultiplier tube.

2.3.2 Experimental description
The lifetime was measured for several temperatures in the same way as in previous experiments, see section 2.1.2. The laser power was adjusted so that 7 mW came out of the fibre with the crystal. The set-up was however optimised for high throughput. After three series of measurements a calibration curve for the fibre was made. The calculations were conducted in the same way as in section 2.1.3.

2.4 Characterization of fibre sensor in in vitro measurements of pork chop
To see if it was possible to measure the temperature with the same accuracy in tissue as in air, an experiment was made on pork chop as a tissue phantom.

2.4.1 Experimental set-up
The set-up is the same as in Figure 6. The fibre tip with the crystal was now placed between two pork chops inside the oven. The thermistors were placed on either side of the fibre, thermistor 1 placed 1 - 2 mm from the fibre and thermistor 2, 5 - 6 mm from the fibre.
2.4.2 Experimental description
The temperature in the oven was raised and when the temperatures measured by the thermistors had stabilized a
fluorescence lifetime measurement was performed in the same way as in section 2.1.2. This was repeated until the pork
chop reached the temperature of 59 °C. The lifetime was calculated for the different temperatures and was drawn in the
same figure as the calibration curve. The lifetime was plotted as a function of temperature of the closest thermistor.

3. RESULTS

3.1 Results of the lifetime measurements of whole crystals of alexandrite, Cr:YAG and Cr:LiSAF
In Figure 7 the lifetime for alexandrite is plotted as a function of temperature. Four different series of measurements are
included and the graph shows a good reproducibility.

The curve fitted to the four measurements and the standard deviation is included in Figure 8. The standard deviation was
determined to $2.5 \times 10^{-7}$ s and the relative standard deviation was 0.12 %. Only a fraction of the curve is shown in the
graph to make it more readable. The precision in the temperature measurements can be estimated as the standard
deviations of specific lifetime readings. The temperature precision for alexandrite can be estimated to ± 0.2 °C in the
studied interval.
The measured lifetime for Cr:YAG as a function of temperature is shown in Figure 9. Four different measurements are included and this graph illustrates also a good reproducibility.

![Figure 9. The lifetime as a function of temperature for Cr:YAG. The reproducibility is almost as good as for alexandrite](image)

The curve fitted to the four measurements and the standard deviation, \(2.7 \times 10^{-6}\) s are seen in Figure 10. The relative standard deviation was determined to 0.20 %. Only a part of the curve is shown in the graph. The temperature precision of Cr:YAG is approximately \(\pm 0.25\) °C.

In Figure 11 the lifetime for Cr:LiSAF is plotted as a function of temperature. Four different measurements are included illustrating the reproducibility.

![Figure 11. The lifetime as a function of temperature for Cr:LiSAF. It also shows the reproducibility](image)

The curve fitted to the last three measurements and the standard deviation, \(1.4 \times 10^{-7}\) s is seen in Figure 12. The first measurement was omitted due to its deviation from the others. The relative standard deviation was 0.35 %. Only a part of the curve is shown in the graph. The temperature precision for Cr:LiSAF is estimated to \(\pm 0.3\) °C.
3.2 Characterization of crystal attached to fibre tip
The experiments were repeated for alexandrite and Cr:LiSAF, this time with the crystal fragments glued to fibre tip. The same precision for both crystals was obtained as in section 3.1. The only difference was that there was a slight difference in the calculated values of the lifetimes compared to the ones in section 3.1.

3.3 Fluorescence lifetime measurements on crystals attached to fibre tip with a PDT-laser
The calibration curve for Cr:LiSAF can be seen in Figure 13. The lifetime is plotted versus the temperature, indicating also the standard deviation, $3.2 \times 10^{-7}$ s. The relative standard deviation was determined to 0.85 %. The corresponding temperature precision is approximately ± 0.5 °C.

![Figure 13. The calibration curve, solid line, for Cr:LiSAF and standard deviation, dashed line, measured values.](image)

3.4 Characterization of fibre sensor in in vitro measurements of pork chop
Figure 14 shows the calibration curve together with the measurements on the pork chop.

![Figure 14. Calibration curve, solid line, and standard deviation, dashed line, together with the measurements on pork chop.](image)
4. DISCUSSION AND CONCLUSION

4.1 Fluorescence lifetime measurements of three Cr\textsuperscript{3+}-doped crystals

When comparing the three crystals they all provide a relatively high accuracy, within the estimated limit ± 0.5 °C. Cr:LiSAF exhibit not as high slope between 20 – 25 °C as the other two crystals. However since the temperature during an IPDT treatment is over 30 °C, this has less relevance for some applications. All the crystals have longer lifetimes, μs – ms, than PpiX, which has a lifetime in the nanosecond region. This makes it possible to separate the fluorescence from the crystals from the fluorescence from PpiX in time. For further experiments Cr:YAG was not selected because of the strong fluorescence in the red region, making it impossible to detect other fluorophores, for example PpiX, when monitoring the fluorescence spectrum during an IPDT treatment.

4.2 Characterization of crystal attached to fibre tip

One can see that there is a difference in measured lifetimes, when comparing the results from the previous measurements on a whole crystal with the ones obtained when a small crystal was glued to the fibre tip. This can be due to that the crystal can be warmer than the two thermistors, when they are not in the exact same position as the crystal. The equipment is also very sensitive to the amount of fluorescence intensity. If the intensity is too low, the system becomes unstable, thus showing a too short lifetime. It is important that no laser light is detected together with the fluorescence light, since this will shorten the lifetime obtained. Even a very small amount of laser light can ruin a measurement. It is therefore important to choose optical filters that efficiently block the laser light.

The method to glue the crystal to the fibre is not a good choice. This is due to the difficulty to place the crystal in a position on the fibre tip so that the detected fluorescence intensity is as high as possible. As stated before, only a little decrease in the fluorescence can effect the fluorescence lifetime measurements. Another problem is that the glue does not attach so hard to the fibre, resulting in that the crystal very easily falls off when subjected to mechanical stress. The amount of glue used can also affect the results. Large amount of glue between the fibre and crystal can decrease the amount of fluorescence induced and detected. A better alternative would be to dope the fibre tip with the crystal.

4.3 Fluorescence lifetime measurements on crystals attached to fibre tip with a PDT-laser

The calibration curve was done with a different fibre than the one used in section 4.2, explaining why the calibration curve has different lifetimes. This measurement did not give the same accuracy as our previous results, but it still fulfils the requirements defined for this project. An explanation to the larger deviations can be that the crystal is further away from the fibre tip, resulting in a decrease in fluorescence intensity. This indicates that each fibre made in this way needs to be treated individually and a calibration curve versus known temperatures has to be measured for each fibre. The most important thing is to know which temperature a specific lifetime corresponds to for a specific fibre probe. If this is in agreement with theoretical data is of less importance.

4.4 Characterization of fibre sensor in in vitro measurements of pork chop

In Figure 14 one can see that the lifetime measurements on pork chop give a shorter fluorescence lifetime than expected for lower temperatures. The results for higher temperatures follow the calibration curve better. This can be interpreted as, for lower temperatures the laser light also heats the crystal, but at higher temperatures this heating process becomes negligible.

4.5 Future developments

This preliminary study indicates that this type of fibre probes could be used for measurements of local tissue temperatures in connection with laser therapy. Since the intensity of the fluorescence has been sufficient in these preliminary and unoptimized measurements using low laser power, it should not be any problem to use much smaller crystals than has been used here. Our suggestion is therefore to "dope" the fibre ends with miniature crystals. It is important that such fibre tip will not absorb too much of the treatment light, or it will decrease the therapeutic effect and act as a local heat source. One could further consider the concept suggested by Lilge et al. to use a single fibre probe measuring at several positions along the fibre.
REFERENCES


