Fluorescence Spectroscopy for Medical and Environmental Diagnostics

Jonas Johansson
Errata

to "Fluorescence Spectroscopy for Medical and Environmental Diagnostics" by J. Johansson

Page 9, line 7: less than a micron, should read: about 10 and 1 microns, respectively.

Page 12, line 10: roughly anisotropic, should read: roughly isotropic.

Page 65, 3:rd paragraph, line 4: the absorption of two photons at 532 nm, should read: a single absorption event at 532 nm.

Page 66, 3:rd paragraph, line 4: immediately after PDT should read: simultaneously with PDT.

Page 66, 3:rd paragraph, line 16: strongly time dependent, should read: strongly temperature dependent.

Paper 6, page 186, line 10: indicating four characteristic wavelength regions; this is not done in the figure.
Paper 6, page 186, line 16: shown in Fig. 4c, should read: shown in Fig. 4d.

Paper 9, page 13, figure: the two photographs should change places
Fluorescence spectroscopy can be used for diagnostics in medical and environmental applications. The many aspects of fluorescence emission are utilized to enhance the accuracy of the diagnosis. A fluorescence detection system, based on nitrogen laser or dye laser excitation and optical multichannel detection, was constructed, and fluorescence spectra from human malignant tumours of various origins, were recorded. Tumour demarcation was observed using exogenous chromophores as well as the endogenous tissue fluorescence. In particular, 8-amino levulinic acid was found to provide very good tumour demarcation. A multi-colour imaging system capable of simultaneous recording of four fluorescence images at selected wavelengths, was developed. Examples of processed images, based on the four sub-images, are shown for malignant tumours. In addition, data from photodynamic treatment of human malignant tumours are presented.

Autofluorescence spectra from excised pieces of human atherosclerotic aorta and atherosclerotic coronary segments were found to be different from those of non-diseased vessels. Furthermore, fluorescence decay curves from atherosclerotic samples were found to differ from those of non-diseased samples. It is concluded that both spectral and temporal information should be utilized to enhance the demarcation. Methods for obtaining fluorescence data free from interference from blood, with applications to in vivo laser angioplasty of atherosclerosis, are discussed.

The optical multichannel system and the multi-colour imaging system were integrated with a remote sensing system, originally used for environmental measurements, to obtain fluorescence spectra as well as fluorescence images of plants at a distance of up to 100 m. The fluorescence data from plants subject to environmental stress or senescent plants were found to differ from those obtained from healthy vegetation.

Key words: Fluorescence spectroscopy, fluorescence imaging, time-resolved spectroscopy, fluorescence detection, remote sensing, atherosclerosis, hematoporphyrin
Fluorescence Spectroscopy for Medical and Environmental Diagnostics

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Papers
Abstract

Fluorescence spectroscopy can be used for diagnostics in medical and environmental applications. The many aspects of fluorescence emission are utilized to enhance the accuracy of the diagnosis. A fluorescence detection system, based on nitrogen laser or dye laser excitation and optical multichannel detection, was constructed, and fluorescence spectra from human malignant tumours of various origins were recorded. Tumour demarcation was observed using exogenous chromophores as well as the endogenous tissue fluorescence. In particular, δ-amino levulinic acid was found to provide very good tumour demarcation. A multi-colour imaging system capable of simultaneous recording of four fluorescence images at selected wavelengths, was developed. Examples of processed images, based on the four sub-images, are shown for malignant tumours. In addition, data from photodynamic treatment of human malignant tumours are presented.

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List of Papers


Authors associated with the Atomic Physics Division are listed in alphabetical order.
In addition to the above Papers, original material is presented in:


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1. Introduction

Following the invention of the laser in 1960, applications in various fields of science appeared rapidly. One of the very first of these applications, was in the field of ophthalmology. The many advantages of lasers over other light sources come to good use in many areas of medicine. The special characteristics of lasers; high intensity, coherence, monochromaticity and short pulse duration, make them useful for both therapy and diagnostics. Due to their coherence, CO\textsubscript{2} and Ar-ion lasers can be focused to a spot size of less than a micron, suitable for precise surgical applications in, for example, ophthalmology. If the output power is lowered, tissue welding with minimal scarring may instead be achieved. Lasers which emit series of very short but intense pulses are available. Excimer lasers are pulsed lasers which emit in the UV range, and their interaction with tissue produces ablation of very thin tissue layers. The non-thermal action of these lasers is useful, for example, for photoablation of atherosclerosis in blood vessels and for reshaping the cornea of the eye. Low-intensity lasers can be utilized to induce hyperthermia and in phototherapy. The latter includes photodynamic therapy (PDT), which is the therapeutic action of a tumour-selective drug mediated by light. This method has important advantages in the selective effect on thin tumours, provides the possibility of repeated sessions and has no major side-effects. Lasers are particularly useful for this application because of their high power per wavelength interval and low divergence.

Lasers are widely used for diagnostic applications in medicine utilizing different spectroscopic techniques. Perhaps the most widespread application of low light level lasers for diagnostics is in flow cytometry, where individual structures, such as chromosomes, can be characterized. Due to the sharp focus of the laser beam, the fluorescence and scattered laser light can be measured within an interaction cross section of a few µm. The laser Doppler technique utilizes the small frequency shift of monochromatic light scattered by moving blood cells. This technique permits the measurement and imaging of superficial blood flow to monitor, for example, wound healing. Laser-induced fluorescence is utilized in scanning laser confocal microscopy. With this technique, a small pinhole diaphragm is inserted in front of the detector to ensure that the detected light originates from a very thin layer, which results in a greatly enhanced contrast of the microscopic image. Furthermore, laser-induced fluorescence is a very sensitive technique for the detection of tissue chromophores \textit{in vitro} and \textit{in vivo}. With the aid of optical fibres, fluorescence can easily be detected through endoscopes in body cavities or interstitially inside solid organs. By choosing suitable light parameters, such as wavelength, selectivity for certain molecules can be achieved. In cases where such selectivity is not possible, there is the possibility of injecting exogenous, tissue-specific substances. This technique has found many applications, the most important ones being in tumour diagnostics. Fluorescence detection has an important advantage over other diagnostic techniques. X-ray tomography measures the interaction of X-ray photons with electrons, the magnetic resonance technique gives a measure of hydrogen atom concentration, an ultrasound signal is proportional to the acoustic impedance (dependent on tissue density and velocity of sound) of the tissue and radio-labelling techniques show the distribution of an exogenous compound in tissue,
while fluorescence detection, on the other hand, gives the concentration of whole molecules or tissue structures directly, which provides a basis for better tumour selectivity.

In this thesis, fluorescence spectroscopy as a tool for monitoring various diseases in medicine will be discussed. The interaction of light with tissue, including fluorescence from endogenous as well as exogenous fluorophores, will be described briefly. An overview of the most useful tumour-selective chromophores with applications in fluorescence detection and photodynamic interaction with tissue, will be presented. The most important of such chromophores has so far proven to be haematoporphyrin and its derivatives. Consequently, the main part of the discussion on this topic will be focused on the uptake and action of this drug. The main part of this thesis will deal with fluorescence spectroscopy in vivo and in vitro. Fluorescence recordings were performed with spectral as well as temporal resolution, to extract as much information as possible from the fluorescence. The various aspects of fluorescence are treated separately but are also combined in an attempt to present the outlines of an improved diagnostic system for in vivo use. Almost all the work was done utilizing a laser as an excitation source, although some results presented here were obtained at the synchrotron radiation facility, MAX, in Lund. The tumour-selective property used for tumour detection can also be utilized for photodynamic therapy (PDT) of malignant tumours. The important aspects of PDT focusing on the interplay with fluorescence detection, will be discussed. Fluorescence spectroscopy may find an important application in the field of laser angioplasty, as it may be used as a diagnostic tool during ablation of atherosclerotic plaque. Fluorescence spectra as well as fluorescence decay curves provide the basis for diagnosis and will be presented in the discussion. The last part of this work concerns a non-medical application, namely the use of fluorescence spectroscopy as a tool for monitoring damage to vegetation. It will be shown that most of the fluorescence characteristics as well as detection systems are basically the same as for medical applications. Finally, 17 papers covering the various topics discussed are reprinted.
2. The interaction of light with tissue

2.1 Light propagation in tissue

The interaction of light with tissue is well understood on a microscopic level but is very difficult to describe accurately on a macroscopic level. A number of different processes may result from the interaction of light with tissue. When light falls onto a tissue surface some of the light is, on a macroscopic scale, reflected off the surface, while the rest enters into the tissue. The amount of reflected light is dependent on the propagation angle at the surface, the polarization and the refractive index, as governed by the Fresnel relations. If the tissue sample is thick the light is sooner or later absorbed in the tissue, except for the portion of light that escapes back out of the surface due to scattering. For a thin sample, on the other hand, some light may also be transmitted through the tissue sample. The amount of transmitted light, as well as reflected and absorbed light, is strongly wavelength dependent. It is also different for different tissue types, structures and other tissue parameters, such as blood perfusion. Once the light is absorbed by the tissue, a number of excited state reactions and deexcitation processes can result. This will be described in Chapter 3, but at this point it should be noted that the major part of the absorbed light energy is re-distributed among the molecules as heat in intermolecular and intramolecular vibronic interactions.

Light dosimetry in tissue has attracted much attention and has resulted in a large number of publications [1-7]. Modelling of the light distribution in tissue is of paramount importance for an accurate prediction of the outcome of PDT, and is also important for the understanding of in vivo fluorescence spectra. The most fruitful approach to modelling light propagation in tissue is to use transport theory [8]. In this model, light is considered to be photons which are subject to scattering and absorption within the tissue. Radiant energy re-emitted by, for example fluorescence or Raman scattering, is not considered to contribute to the total photon flux. The transport equation is expressed as:

\[ s \cdot \nabla L(r,s) = -\mu_t L(r,s) + \int_{4\pi} \mu_s(s',s)L(r,s')d\omega + S(r,s) \quad (2.1) \]

where \( L \) is the radiance at a location \( r \) and in the direction \( s \) in the tissue, \( \mu_t \) and \( \mu_s \) are the total attenuation and scattering coefficients, respectively, and \( d\omega \) is the differential of the solid angle in the direction of \( s' \). \( P \) is the phase function and \( S \) is the source function. \( \mu_t \) is the sum of the attenuation due to scattering and absorption: \( \mu_t = \mu_s + \mu_a \). The phase function describes the distribution of photon redirections by scattering from \( s' \) to \( s \). The source function, \( S \), describes the spatial distribution of any light source, internal or external, contributing light at \( r \) in direction \( s \). The transport equation may be understood as the change of radiance at the point \( r \) in the direction \( s \), which is equal to the sum of the total attenuation due to scattering and absorption, the re-direction of photons into the direction \( s \) and the source of photons. With this theory, using \( \mu_a \), \( \mu_t \), and \( P \), the light propagation in tissue may be accurately modelled.
Unfortunately, the radiative transport equation in its general form is impossible to solve. However, with adequate approximations there exist a number of situations, where rather good results can be obtained. One possibility is to use the diffusion approximation:

\[ D \frac{\partial \phi(r)}{\partial r} - \mu_r \phi(r) = S'(r) \quad (2.2) \]

where \( \phi(r) \) is the flux at location \( r \) and

\[ D = \frac{1}{3(\mu_r + \mu_s(1 - g))} \quad (2.3) \]

is the diffusion constant, and \( S'(r) \) the modified source term. The diffusion approximation is valid far from sources and boundaries under the assumption that the absorption coefficient is small in comparison with the reduced scattering coefficient and that the total flux is roughly anisotropic. The latter assumption means that the scattered light is mathematically expressed as an infinite sum of Legendre polynomials, which is truncated to an isotropic and a moderately forward scattered term. Most biological tissues are actually considerably forward-peaked in scattering. However, since in the red/near-IR region, scattering is strongly dominant over absorption, the diffusion approximation gives a fairly good result at these longer wavelengths. A useful parameter in this context, is the effective attenuation coefficient, \( \mu_{\text{eff}} \):

\[ \mu_{\text{eff}} = \sqrt{3\mu_r(\mu_s + \mu_r(1 - g))} \quad (2.4) \]

The inverse of \( \mu_{\text{eff}} \) is called the penetration depth and is used to describe the depth at which the light has been attenuated by a factor of \( e \), that is to 37%.

Another and simpler approach is the Kubelka-Munk theory [9,10] according to which the radiance is considered as an ingoing and an outgoing component and the isotropic slab is irradiated by a diffuse source. The Kubelka-Munk equations are:

\[ R = \frac{\sinh(Syt)}{x \cosh(Syt) + y \sinh(Syt)} \quad (2.5) \]

\[ T = \frac{y}{x \cosh(Syt) + y \sinh(Syt)} \quad (2.6) \]

where \( R \) and \( T \) are macroscopic reflection and transmission constants, \( S \) the Kubelka-Munk scattering coefficient and \( x \) and \( y \) are obtained from measurements of \( R \) and \( T \). An advantage of the Kubelka-Munk theory is that the scattering \( (S) \) and absorption \( (A = (x - 1)S) \) coefficients can be obtained directly from measurements of \( R \) and \( T \). The results are, however, poor and often not suitable for modelling laser light propagation in tissue.

A quite different approach is the Monte Carlo technique [11] of computer simulation of the trajectories of the individual photons propagating in tissue. The length of the light path is randomly chosen from an exponential distribution around \( \mu_r \). Similarly, the scattering angle is randomly chosen from the phase function. The propagation of photons is simulated until good enough statistics have been obtained. Monte Carlo simulations seem to yield the best results of the presently available models. The computing times, on the
other hand, are long even on powerful computers. Other light propagation models exist, such as the discrete ordinate method [112], but these will not be discussed here.

Most of the work on light propagation in tissue is applied to the irradiation of a thick tissue sample, as is the case in PDT. Much less work has been done on modelling the distribution and propagation of fluorescence produced in tissue. This is an even more difficult task, since light at one wavelength is distributed in the sample to produce fluorescence at different wavelengths which will be further distributed in the tissue. Fortunately, there are a few cases for which this analysis, at least qualitatively, is less difficult. One such case is when a UV source is used to induce fluorescence from red-emitting fluorophores such as haematoporphyrins. In the UV region, the strong absorption prevents multiple scattering and the light is attenuated according to the Beer-Lambert law. The effective penetration of UVA light is comparatively short (≈14 μm), but much longer (≈2-5 mm) at 600-700 nm. Thus, fluorescence is produced only in a very shallow surface region. Since fluorescence is emitted isotropically (see Chapter 3) this situation is equivalent to a diffuse source irradiating a highly scattering medium (cf. Kubelka-Munk). The fluorescence detected at the surface will then be dominated by the fluorescence that has travelled a short distance in the tissue. The effect of reabsorption of fluorescence at certain wavelengths will therefore be small in this case. A further discussion on this topic can be found in Chapter 5.

2.2 Light absorption in tissue

In the previous section the light propagation in tissue prior to absorption was considered. The light was found to be approximately distributed according to the Beer-Lambert law and diffusion theory in the UV/blue and red/near-IR regions, respectively. In this Chapter the interaction mechanisms resulting from the absorption of light in tissue will be described. To obtain a specific biological response at the tissue target area, there are certain light and tissue characteristics which must be well defined. The change in damage to the tissue is determined by the light energy density (J/cm²) and the pulse duration (sec). The energy density is proportional to the number of photons falling onto the tissue surface, and knowing the pulse duration, the temperature within the interaction volume may be estimated. An important factor here is the thermal relaxation time, τ, of the tissue at the wavelength in question, which describes the rate at which the produced heat is dissipated to the surrounding tissue. Obviously, if τ is short in comparison with the pulse duration, a steady-state heat distribution will develop during the light pulse. The degree to which the tissue is changed is then determined by the thermal diffusion length. If, on the other hand, the pulse length is shorter than τ, less energy can be transferred to the surrounding tissue. This results in a higher temperature in the interaction area and less damage to adjacent tissue during tissue removal processes. Another important parameter is the wavelength (λ) of the light. Since the absorption coefficient, μₐ, is wavelength dependent, the effect on the tissue will depend on λ. In the UV/blue region, all proteins absorb strongly. Haemoglobin is an important absorber which exhibits absorption maxima at 420, 540 and 580 nm. Melanin is a tissue chromophore found mainly in the skin, for which μₐ decreases smoothly with increasing wavelength. This is also the case for intact tissue in total. For longer wavelengths, the tissue absorption rises again, mainly due to vibronic transitions in water e.g. at 2 μm and 3 μm. There is, however, a region near
Fig. 2.1 Various effects of light absorption in tissue. Some examples of applications are included. The examples given are found at light doses between 1 and $10^4$ J/cm$^2$.

1000 nm where the tissue light penetration is maximal. This is usually referred to as the therapeutical window. Of importance here is of course also the scattering coefficient as discussed in the previous Chapter.

The processes resulting from light absorption in tissue can be divided into different interaction modes [13]. These are photochemical effects, thermal effects, ablation and electromechanical effects. This is illustrated in Fig. 2.1, where the power density is plotted as a function of pulse duration for different tissue effects. As can be seen, the different processes follow a streak represented by the energy density $1-10^4$ J/cm$^2$. To the lower right, one finds the CW or quasi-CW applications, and in the upper left corner the short-pulse applications are found. The pulse duration appears to distinguish the different interaction modes from each other.

In a photochemical reaction the light is utilized to induce a biochemical reaction only, involving no heat. The photon energy is absorbed by a target molecule and is utilized either for excited-state reaction or transferred to another molecule involved in the chemical reactions. In photochemical reactions the wavelength is a critical parameter, not only in achieving the correct tissue penetration, but also when matching the wavelength-dependent absorption of the target molecules. The most important of these reactions is photodynamic therapy of malignant tumours [14-16]. This is facilitated by the injection of a tumour-selective chromophore, which absorbs light strongly at the irradiation wavelength, preferably in the red region. A power density of 100 mW/cm$^2$ and an
irradiation time of 10 minutes are typical. This methodology is explained in detail in Chapter 6. Another application is the treatment of psoriasis and vitiligo by psoralen UVA (PUVA) therapy [17,18]. A more controversial treatment is biostimulation by extremely low light levels, such as that from HeNe lasers (< mW/cm$^2$) [19,20]. Reports have been published which show stimulation in wound healing and pain relief using, in particular, coherent radiation. Other reports, however, have shown that coherence was not a requirement for biostimulation, but wavelength has been reported to be. Obviously, much well defined research is required in order to clarify the effectiveness of biostimulation.

Nd:YAG lasers and CO$_2$ lasers, as well as other light sources, are used to produce thermal effects in tissue. The low temperature limit at which an irreversible effect is achieved is around 45°C, where cell membrane permeability changes and the macromolecular structure is altered. This leads subsequently to a disruption of blood flow and is utilized as light-induced hyperthermia. Portwine stains are treated by irradiation with an Ar-ion laser, or preferably a pulsed dye laser tuned to the 580 nm absorption maximum of haemoglobin [21]. Tissue welding can be produced by protein linkage between cells. At about 60°C, the proteins are denatured resulting in a coagulation of the tissue. Tissue carbonization occurs at about 80°C, and at 100°C vaporization of water commences and the tissue starts to dry. CW or quasi-CW lasers are used in this region and above for tissue removal in laser surgery. The power density used is in the range of a few tens of W/cm$^2$ up to about $10^5$ W/cm$^2$. A distinct advantage of laser surgery over conventional surgery is the instant closure of ruptured blood vessels by thermal coagulation effects.

Moving to very short pulses with peak powers of MW-GW/cm$^2$, tissue ablation may be achieved. When these high power densities are applied to a tissue surface, a very fast rise in pressure will result. This micro-explosion will launch atoms, molecules and tissue fragments from the centre of the explosion, leaving a crater in the tissue. The action of ablation is different for different wavelengths. In the IR region, where water absorbs strongly, very smooth cuts and shallow ablation depths can be achieved. Furthermore, the temperature rise in adjacent tissue is low since most of the energy is utilized for the evaporation of water. In the visible region, where tissue absorption is lower, the adjacent tissue is affected to a higher extent and thermal damage can result unless the pulse duration is suitably short. In the UV region proteins absorb strongly, whereas water is a poor absorber above 250 nm. In addition, a different mechanism may occur as the result of direct chemical bond breaking facilitated by the high photon energies of several eV. This is useful for ablation of tissues such as the cornea, in which the collagen fibres can be sharply cut by UV lasers.

In the region of very high peak power densities between GW and TW/cm$^2$, extreme processes may occur. This region is classified as the electromechanical region. At the very high power densities generated within the interaction volume, an electric field strength of the same order as the intramolecular forces keeping the atoms and molecules together, is generated. Thus, massive amounts of free electrons and ions are produced which create a hot plasma at 10,000 °C or more. The ensuing rise in pressure results in a micro-explosion confined to a small volume. The plasma cools off rapidly leaving very sharp and clean cuts in the tissue. The electromechanical effect differs from photo-ablation by nanosecond pulses in the collective effect of the electrons. The electrons produced by ionization can interact with incoming photons, gain kinetic energy and produce secondary ionization.
electrons. Hence, the high power density leads to an avalanche process within the pulse, which explains the extreme temperatures. The use of ultrashort laser pulses has proven useful, in particular, for ophthalmological applications [22].
3. Fluorescence of biomolecules

In this chapter the basic principles of fluorescence, with the emphasis on molecules for fluorescence diagnostics of malignant tumours, will be discussed. While most molecules exhibit low or no fluorescence at all, small alterations can make a molecule strongly fluorescent. The local environment also has a strong influence on a fluorophore, as the fluorescence may be quenched or shifted in wavelength by environmental interactions.

Consider a photon, or an electromagnetic wave package, falling within the interaction distance of a molecule in the ground state. If there exists an excited state, which corresponds to the energy of the incoming photon, the photon will interact with the molecule. After an extremely short time, about $10^{-15}$ seconds, the photon may have changed its direction and polarization but may have the same energy and the molecule will remain in its ground state. The photon has then been scattered by the molecule. If, on the

Fig. 3.1 Jablonski diagram showing the various decay paths from an excited state of a molecule. In the lower part of the figure, a fluorescence spectrum from haematoporphyrin in ethanol, is shown. The abbreviations are: $S_n$, singlet states; $T_n$, triplet states; Abs, absorption; Sc, scattering; IC, internal conversion; F, fluorescence; IX, intersystem crossing; P, phosphorescence; A, transfer to other molecules.
other hand, the molecule has an excited state corresponding in energy to that of the photon, the photon may be absorbed by the molecule and the molecule will find itself in an excited state. Excited states are unstable and the molecule must to return to the ground state. This may follow a number of different pathways, as illustrated in the Jablonski diagram in Fig. 3.1. The labelled levels are electronic levels, corresponding to the energy levels of atoms. \( S_0, S_1 \ldots \) are singlet states for which the sum of the electron spin quantum numbers is zero, while \( T_0, T_1 \ldots \) are triplet states for which the spin of one electron has changed sign. In contrast to the atomic case, each electronic level is split into several vibronic states, which are further split into rotational states. For large molecules the intervals between the levels are very small and the states overlap due to molecular interactions. When a molecule is excited to a high energy level, a rapid relaxation to the lowest rotational-vibrational state of \( S_1 \) will follow. The short time scale \( (= 1 \text{ ps}) \) of the relaxation is due to the high density of rotational vibrational levels. The absorption will excite the molecule not necessarily to the lowest vibrational level in the excited electronic level, but more likely to a higher vibronic state. This is a result of the Franck-Condon principle stating that during the rapid \( (= 10^{15} \text{ s}) \) absorption process, the atoms do not change their location in the vibronic motion. From \( S_1 \) the molecules may proceed to the state \( S_0 \) through radiationless kinetic interactions. This is called internal conversion (IC). Alternatively, the deexcitation may result in the emission of a photon and this process is called fluorescence. Since the transition may be terminated in any of the rotational-vibrational states of \( S_0 \), the energy of the photons will not have a distinct value, but rather a broad distribution. Thus, a fluorescence spectrum from a molecule will be broad, most often without any significant structures. The form of the spectrum will reflect the probability of transitions to the lower levels \( (S_0) \). In the lower part of Fig. 3.1 the fluorescence spectrum of haematoporphyrin is shown. The two peaks have been attributed to the 0-0 vibronics of the \( Q_x \) and \( Q_y \) states of haematoporphyrin [23] or to the 0-0 and 0-1 vibronics of the \( Q_z \) state [24,25].

The average time a molecule stays in \( S_1 \) is termed fluorescence lifetime, \( \tau \), and depends on the decay rates:

\[
\frac{1}{\tau} = \frac{1}{\tau_0} + \Sigma k
\]

(3.1)

where \( \Sigma k \) is the sum of all deexcitation processes competing with fluorescence and \( \tau_0 \) is the intrinsic lifetime, which is the fluorescence lifetime when fluorescence is the only decay process. Once the pathway absorption-IC-fluorescence is completed, the molecule is back in its original state and configuration. Hence, the fluorescence process is reversible, which is an advantage in medical diagnostics.

Although spin forbidden, a transition to the triplet system may occur. Also in the triplet system a rapid internal conversion to the lowest excited state will occur. Since a transition to \( S_0 \) is spin forbidden, this will proceed at a much lower rate \( (\tau = 10^{9-1} \text{ s}) \) than the transition \( S_1 \rightarrow S_0 \). This process is called phosphorescence and is less often observed at room temperature.

Several other paths are possible for the excited molecule, such as energy transfer to other molecules, electron transfer, excimer formation and excitation to repulsive states leading to molecular dissociation. These processes are indicated with an \( A \) in Fig. 3.1. Fluorescent
molecules have one important feature in common, that is the unbroken chain of conjugated double bonds, *i.e.* every second bond is a double bond. These bonds correspond to $\pi\pi^*$ states of the molecules. The structure of haematoporphyrin is shown in Fig. 3.2. This is a fluorescent molecule used for fluorescence diagnostics and photodynamic therapy of tumours. A rough estimate of the Q-band absorption wavelength is given by the number of conjugated double bonds in a row in the molecular structure. In porphyrins the number of conjugated double bonds is quite large, and consequently their long-wavelength absorption bands are located in the red region.

With the knowledge of the fluorescence properties of important tissue fluorophores, a fluorescence recording of an unknown sample will yield the relative contribution of each fluorophore. If the fluorescence characteristics are the same as for the isolated fluorophores, the concentration of the fluorophores can be estimated. This is, however, not always the case. Rather, the fluorescence properties are dependent on environmental factors such as polarity and pH. A phenomenon that influences the fluorescence is that of quenching. By very efficient coupling to certain molecules, the fluorescence efficiency may be significantly lowered. Several processes may lead to quenching, such as complex formation and energy transfer, while the most important is collisional quenching. This is expressed by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q[Q],$$  \hspace{1cm} (3.2)

where $F$ and $F_0$ are the fluorescence intensities with and without quenching molecules, $k_q$ the quenching constant and $[Q]$ is the concentration of quenching molecules. Alternatively, this may be expressed in terms of fluorescence lifetimes:

$$\frac{\tau_0}{\tau} = 1 + k_q[Q]$$  \hspace{1cm} (3.3)

Hence, both the fluorescence yield and the lifetime are decreased by quenching. The quenching constant is dependent on the diffusion constant, which in turn is dependent on temperature. Thus, fluorescence efficiency is temperature dependent. The spectral shape is not influenced by quenching unless the fluorescence population is heterogeneous with different fluorescence peak wavelengths for the two populations. Quenching may provide important information such as fluorophore partition in membranes inaccessible to quenchers or diffusion of quenching molecules over membranes. The most important quencher in biological systems is the oxygen molecule, which is a very efficient quencher of most fluorophores.
Another important aspect of fluorescence is the rapid relaxation in the excited state and in the ground state. The molecule loses some of its excitation energy by relaxation. Furthermore, redistribution of solvent dipoles around the fluorophore and specific interactions, such as hydrogen bonding, contribute to this relaxation procedure. Thus, the energy of the fluorescence photons is lower than that of the excitation, or the fluorescence wavelength is longer than the excitation wavelength. This is called Stokes shift and is different for different molecular environments. The part of the Stokes shift that arises from solvent dipole reorientation is governed by the Lippert equation:

$$
\Delta \nu = \frac{2}{hc} \left( \frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \right) \left( \frac{\mu^* - \mu}{a} \right). 
$$

where $\epsilon$ is the dielectric constant of the solvent, $n$ the refractive index, $\mu^*$ and $\mu$ dipole moments of the excited state and ground state and $a$ is the effective molecular radius. A fluorophore exhibiting a Stokes shift of a few nm in a non polar solution, may show a shift of more than 100 nm in a polar solution such as H$_2$O. Hence, a general knowledge of the molecular environment is required for an adequate fluorescence diagnosis.
4. Tumour-selective substances

4.1 History

By far the most important agent used for PDT and LIF detection of tumours is haematoporphyrin and related compounds. Porphyrins constitute a group of molecules similar in structure to the haeme group of haemoglobin, with a ring of four pyrrole rings bound together with nitrogen atoms, however, with the central iron atom missing. The history of haematoporphyrin goes back to the middle of the nineteenth century when Scherer [26] in 1841 was able to wash blood free of iron by treating dried blood with sulphuric acid. The importance of porphyrins for tumour diagnostics was, however, not discovered until 1924 when Policard found fluorescence similar to that of haematoporphyrin in animal tumours exposed to a Woods lamp [27]. He came to the conclusion that porphyrins were spontaneously accumulated in tumours. In 1942 Auler and Banzer showed the uptake and selective retention of haematoporphyrin in animal tumours upon injection [28]. They investigated and found selective retention in a number of different tumours including human tumours. Similar experiments were performed by Figge et al. who in 1948 reported on haematoporphyrin retention in vivo [29]. They also discussed the use of haematoporphyrin for tumour diagnostics. In the experiments performed thus far crude haematoporphyrin with its relatively poor tumour localizing properties was used. In 1955, however, Schwartz et al. reported on the chemical nature of crude haematoporphyrin which they found to be a mixture of several different porphyrins [30]. Surprisingly, purification of the mixture showed that the tumour localizing ability was associated with the impurities of the mixture rather than the pure haematoporphyrin. In the following years Schwartz et al. continued their work on the purification of crude haematoporphyrin, trying to find the nature of the porphyrin responsible for accumulation in tumours. In 1960 Winkelman and Rasmussen-Taxdal performed a quantitative study on the porphyrins accumulated in tumours by chemical extraction from tissue [31].

A major breakthrough was reported in 1960 when Lipson and Baltes at the Mayo Clinic working along the lines set up by Schwartz et al. presented a derivative of haematoporphyrin with enhanced localization properties and which was much more potent in photodynamic therapy [32,33]. This haematoporphyrin derivative (HpD) was prepared by treating haematoporphyrin with a mixture of sulphuric acid and acetic acid followed by an alkaline hydrolysis using NaOH. The solution was neutralized using HCl and made isotonic before injection. This new derivative was used in several laboratories and clinics for tumour diagnostics and therapy (see Chapters 5 and 6). By the end of the 1970's research had intensified in attempts to find the tumour localizing component of HpD and, if possible, to isolate it. HpD prepared according to Lipson was separated into several components using high-pressure liquid chromatography (HPLC) and other chromatographic methods [34,35]. The four major components were investigated regarding absorption and fluorescence spectra, fluorescence quantum yield, singlet oxygen yield and biological activity. The major components were identified as monomeric haematoporphyrin (Hp), hydroxyethyl vinyldeuteroporphyrin (HVD) (2 isomers) and a broad peak containing, among several components, protoporphyrin (PP). An incomplete
hydrolysis also results in monoacetates and diacetates. Its was found that the hydrophilic haematoporphyrin monomer possessed the highest fluorescence quantum yield as well as singlet oxygen yield \([36,37]\), whereas the more hydrophobic broad component had the best localizing properties \([38,39]\). Similar results were obtained using thin-layer chromatography \([40]\). Two chromatogram of HpD together with the corresponding fluorescence spectra, are shown in Fig. 4.1.

4.2 Composition of HpD

The fact that HpD is a very complex and unstable mixture of different porphyrins is a severe drawback in its use in photodynamic therapy and fluorescence detection. Not only does it contain different porphyrins such as haematoporphyrin and protoporphyrin but they
have also a tendency to aggregate. Thus, in aqueous solution almost all porphyrins are aggregated, whereas in hydrophobic solution or bound to cell membranes they appear as monomers. Furthermore, besides monomers of Hp, HVD, PP etc. dimers and oligomers are found in HpD. The localizing fraction of HpD was suggested to be covalently linked dimers or oligomers of Hp. Three different dimeric structures were proposed: an ether linkage, an ester linkage or a C-C bond [35]. The ether linkage was favoured in a study where aqueous gel-exclusion chromatography was utilized [41]. This component was termed dihaematoporphyrin ether (DHE). This study was complicated by the fact that all porphyrins tend to aggregate in an aqueous phase. In this case impurities of aggregates of Hp, HVD and PP might be present. A different method was used to show that, on the other hand, an ester linkage was the more probable [42]. This investigation was based on non-aqueous phase chromatography. This was done in such a way that ether formation was not possible and indicated a dimer or trimer bound together by an ester bond. Although the tumour localizing fraction has been essentially identified, nobody has so far succeeded in producing a stable preparation of this chromophore without impurities. When this fraction is further separated by chromatographic methods, the chromatogram still reveals Hp, HVD and PP although at lower levels. This purified mixture with a higher content of DHE has been commercially available several years under the name Photofrin II and later PhotoFrin. Later investigations have shown that freshly prepared HpD contains mainly ester-linked oligomers [14]. However, after purification to PhotoFrin II, ethers are instead formed. Moreover, synthesized dihaematoporphyrin ether was reported not to be active in an experimental tumour [43]. In fact, it was found that the localizing fraction of HpD was likely to contain dimers and trimers of porphyrin containing one or more vinyl groups. Since these oligomers are very unstable during analysis, it seems likely that conversion to ether takes place after injection into animals or humans. In any case, it does not seem possible to completely purify HpD to obtain only a tumour-localizing preparation without impurities. The instability of HpD/PhotoFrin II is an important reason for searching for other tumour-localizing drugs for tumour detection and PDT.

4.3. Porphyrin retention in tumours

The selective uptake and retention of porphyrins in neoplastic tissue has been demonstrated in many investigations. Consequently, scientists were very optimistic when HpD started to be used clinically at the end of the seventies. However, reports soon appeared that showed a higher accumulation in non-tumorous tissue than malignant tumours. In the first larger study on the uptake of haematoporphyrin in tissue apart from tumour and the surrounding tissue, liver, kidney, spleen and lung of mice were screened [44]. Radioactively labelled haematoporphyrin was administered to the animals prior to the investigation and the amount of porphyrin was quantitatively determined in tissue samples. The results showed a two-fold higher amount of haematoporphyrin in tumour compared with adjacent skin. Very high amounts of haematoporphyrin were detected in kidney, liver and spleen. The different fractions of HpD was prepared and isolated by HPLC and injected into mice bearing Lewis lung carcinoma in a study of the tumour retention of the different HpD fractions [45]. It was found that the hydrophobic, poorly resolved fractions of HpD showed a higher tumour accumulation than HpD. Furthermore, the uptake in liver was even higher whereas the uptake in kidney was less for this component. The substantial accumulation in liver was attributed to pinocytosis. A high number of LDL receptors has also been proposed as an explanation. Similar results have been found using fluorescence
probing [Papers I.II. 31,34,46,47] as well as radio-labelling [45]. It must be pointed out, though, that the results were quite different for different tumour models. In a study from 1989, no tumour selectivity was achieved over normal surrounding tissue [48]. This was partly explained by the poor vascular supply in that fast growing tumour. On the other hand, other tumours have shown a remarkable uptake ratio. The best example is brain tumours where tumour to normal ratios of porphyrin concentration of up to 80:1 have been reported [49-51]. A similarly high ratio was reported for selective uptake of Al-phthalocyanine in gliomas [52]. The main reason for the favourable uptake in cerebral tumour is probably due to the role of the blood brain barrier (BBB) in tumorous brain tissue. The intact BBB is an effective shield for larger exogenous molecules such as porphyrins. In brain tumours, however, the BBB is partly or completely destroyed, which results in an open route for porphyrins [53,54]. Furthermore, light at 630 nm used for PDT shows a very low attenuation in brain tissue. Therefore, the brain seems to be an ideal target for fluorescence detection as well as PDT.

4.4. Mechanisms for the selective uptake of porphyrins in tumours

The large number of reports on the selective accumulation of porphyrins in tumour tissue has shown different selectivities for different tumour models. Different behaviour has also been found for in vivo and in vitro tumour models. Consequently, a large variety of explanations of the mechanisms behind the porphyrin accumulation, or the lack of accumulation, have appeared. In some uptake models the selective retention of porphyrin is due to the transformation of some of the HpD fractions rather than the accumulation of a certain component. In a study of Sarcoma 180 in Swiss mice the tumours were found to contain mainly monomeric haematoporphyrin [40]. However, Hp is known not to accumulate in tumours when injected into animals. It was then proposed that the hydrophobic fractions of HpD, which are known to localize in tumours, when taken up in the cellular membrane were converted to monomers intracellularly. Once inside the cells these hydrophilic monomers are not able to pass through the membrane and are thus trapped inside the cells. The monomers are, as noted above, highly fluorescent and photodynamically active. In contrast to this hypothesis, porphyrins extracted from tumours in another investigation [45] were reported to be aggregates. These were suggested to enter the cells through diffusion and pinocytosis. This discrepancy might be explained by different times between injection and extraction which would allow such a conversion. It should be noted that porphyrins are accumulated not only in the cells but to a high degree in the stroma.

One important aspect that was reported very early is the fact that haematoporphyrin is actually accumulated in tissue during wound healing [29] and in areas of inflammation [55]. This might be a problem for fluorescence detection of tumours. One way to possibly circumvent this problem would be to include the autofluorescence in the demarcation criterion. A possible reason for the accumulation of haematoporphyrin in tumours is the elevated number of low density lipoprotein (LDL) receptors in regenerating tissue. In another study, 24 patients were injected with 0.35-0.5 mg/kg body weight (mg/kg b.w.) of Photofrin II to reveal bladder tumours prior to Nd:YAG laser ablation [Paper IV]. This study showed a higher porphyrin fluorescence in bladder carcinoma and cancer in situ compared with inflammatory sites, which in turn retained somewhat higher amounts of Photofrin II than non-affected tissue.
In the porphyrin uptake model described above [40] the porphyrins were able to accumulate and to be selectively retained in tumours due to a different equilibrium between different fractions of HpD inside the tumour cell and outside. In a similar fashion, the uptake of porphyrins can also be attributed to the variation of pH in tissue [56-58]. The pH of most normal tissue is about 7.5, while for tumours the pH has been shown to be about one to half a pH unit lower. This is a consequence of the less efficient microvasculature and reduced blood flow inside tumours. The anaerobic conditions will lead to an elevated production of lactic acid and thus to a lower pH. Furthermore, a number of different ionic species of Hp exist in equilibrium. Thus, the nitrogens may attach one proton each. Secondly, the two carboxylic side-chains may loose one proton. In the pH region from tumour to normal tissue, one ionic Hp species is dominant for normal tissue while another dominates in tumours. Assuming different solubility for the different ionic species, the porphyrin retention will be different in normal compared with tumorous tissue. In particular, the solubility of the neutral Hp species is lower than that of charged species. Since the neutral species is found at a much higher level at pH=6, this will be the species found and retained for a longer time in tumours. One advantage with this model is that the tissue structure does not have to be considered.

Another important factor regarding the higher amounts of porphyrins in tumours seems to be the larger interstitial space of tumours compared with normal tissue [59]. When injected into the blood stream, porphyrins are bound to proteins. Because of the increased vascular permeability of tumours, porphyrins bound to proteins will accumulate in the interstitial space, where they will bind to collagen, elastin and fibrin [60-61]. Since the interstitial space of tumours has been shown to occupy about 50% of the total tumour volume, or about double that of normal tissue, a tumour to normal ratio of 2 might be expected disregarding other causes of accumulation. Hp, which is known to be selectively retained in tumours was accordingly found to show a tumour to normal ratio of 2 for a colon experimental tumour in Wistar/Furth rats [Paper I]. Even tritiated water showed the same uptake in a Lewis lung carcinoma in mice [45]. Hence, an uptake better than for tritiated water was proposed by the authors to be a definition of a "true" tumour localizer.

Porphyrin has been found to bind to plasma proteins such as albumin and lipoproteins [62]. Most serum proteins seem to inhibit the uptake of porphyrins. However, for low density lipoproteins (LDL) this might not be true. The number of LDL receptors on the surface of the cells is elevated for fast growing tissue, in particular for malignant tumours. LDL enters the cells primarily by receptor-mediated endocytosis. It has been suggested that binding of porphyrins to LDL might be an important delivery path for haematoporphyrin into malignant cells. Support for LDL-mediated uptake of porphyrins has been reported by several investigators [63-66]. The role of LDL in the cellular uptake of porphyrins is clearly still a matter of debate, as there have also been reports showing that LDL inhibits the uptake of HpD [67]. Although different properties of tumour cells have been proposed to explain the selective uptake of porphyrins in tumours, there are indications that the structure of tumorous tissue rather than tumour cell properties is the main factor. Support for this reasoning lies in the fact that in vitro tumour cell lines do not show a higher porphyrin uptake than normal cells [68,69]. Instead, leaky vasculature and poor lymphatic drainage are properties of tumour tissue suggested to be primary causes of porphyrin retention [59].
Fig. 4.2 Structure of some molecules used for fluorescence detection and photodynamic therapy. The molecules are: haematoporphyrin (Hp), protoporphyrin (Pp), tetra sulphonated phthalocyanine (TSPC), δ-amino levulinic acid (ALA), mono-aspartyl chlorin e₆ (MACE) and benzoporphyrin derivative (BPD).
Lastly, it should be pointed out that the tissue uptake can depend on the means of administration. This was shown in a comparison between intravenous (i.v.) and intraperitoneal (i.p.) injection showing higher uptake in liver, kidney and pancreas after i.v. injection of Photofrin II [70]. The kinetics of drug elimination was also different from tumour tissue, however the sensitivity to PDT was similar for the two routes of administration.

4.5 2nd generation photosensitizers

Clinical work on LIF tumour detection and PDT of tumours has so far almost exclusively been carried out using HpD or Photofrin. However, these preparations have, as already pointed out, several unwanted effects. Hence, in recent years several alternative photosensitizers, more favourable for clinical work, have been presented. The structures of some 2nd generation photosensitizers described below as well as haematoporphyrin, are shown in Fig. 4.2. The main drawback of HpD is its high retention in the skin causing skin photosensitivity [44,63]. For clinical use it is therefore recommended that the patient stay out of direct sunlight for about six weeks. Secondly, HpD and Photofrin have proven to be unstable preparations containing several fractions partly in equilibrium with each other, where only some of these localize in tumours. As a consequence it has not been possible to purify HpD in order to obtain a drug free from non-localizing impurities. Thirdly, the main limiting factor for PDT is the shallow light penetration in tissue at 630 nm. It would be desirable to find a photosensitizer with a major absorption peak further out in the red region, where most tissues have a better light penetration. It should be borne in mind, though, that the mechanism of PDT involves energy transfer from the drug molecule to oxygen and possibly other targets. The action spectrum for this mechanism sets a limit towards the red. Furthermore, for some thin tumours, such as bladder carcinoma in situ, green light (514 nm) has been proven to be as effective in PDT as red light, but with lesser side-effects [71]. Also for LIF tumour detection, shorter wavelengths yielding less tissue penetration are, as a rule, advantageous. The lack of multiple scattering of UV/blue light means better spatial resolution in fluorescence images.

4.5.1 Phthalocyanines

The most investigated new sensitizer is phthalocyanine (Pc). Originally used as printing ink, dye and passive Q-switch material for ruby lasers [72], the phthalocyanines received increasing attention recently when their tumour localizing and sensitizing properties were investigated [73-76]. The early work with phthalocyanine was reviewed in 1986 by Spikes [77]. Phthalocyanines possess, at least to some extent, the attractive qualities discussed above. The absorption spectrum reveals a single peak (Q-band) in the red region at about 670 to 690 nm which is much stronger than the corresponding peak at 630 nm for HpD. The low UV and blue absorption results in a lower cutaneous photosensitivity. The fluorescence spectrum shows a single peak centred at about 700 nm.

The structure of Pc, which is similar to that of Hp, is shown in Fig. 4.2. There are two ways of changing the properties of phthalocyanine, as has been shown by numerous investigators. Firstly, a large variety of metal atoms can be chelated into the centre position inside the porphyrin ring structure. This has a large effect on the spectroscopic properties of the molecule. Thus, by choosing different metal substituents, the fluorescence quantum yield and triplet transfer and lifetime are affected. As an example,
AlPc is highly fluorescent and shows a high photosensitizing ability, whereas CuPc does not fluoresce and is photodynamically inactive. Secondly, the chemical properties of Pc can be changed by substitution of different residues at the peripheral aromatic rings. Crude Pc is insoluble in water while substitution of four sulphonate SO$_3^-$ groups results in water-soluble tetrasulphonated Pc. Two different routes for the synthesis of sulphonated Pc, condensation and direct sulphonation by treatment with sulphuric acid, have been used. The two methods were compared and the condensation method being found to be more reproducible [78]. Chloro aluminium phthalocyanine (ClAlPc) was used to demonstrate Pc sensitization of a Chinese hamster cell line [73]. ClAlPc was dissolved in ethanol and added to the cell culture at concentrations of 0.4-1 µM. A similar experiment showed DNA single strand breaks and DNA protein cross links by ClAlPc photosensitization [79]. Seventeen different water soluble and insoluble phthalocyanines were screened for their photosensitizing abilities on murine embryonic fibroblasts or fibrosarcoma cells [74]. Base-free Pc, MgPc and CIAIPc were reported to have a high (0.6-0.7) fluorescence quantum yield. ZnPc has a fluorescence quantum yield of 0.3, while FePc, CoPc, NiPc and CuPc exhibit very low fluorescence. All sulphonated Pc's were found to be water soluble, whereas the non-sulphonated ones were insoluble in water and formed complex aggregates in PBS. Most of the studied Pc's showed some photosensitizing ability to either of the cell lines. However, only CIAIPc, ClAlPc and Cl$_2$SnPc were active under red light illumination but inactive under room lighting condition. Base-free tetrasulphonated phthalocyanine (TSPc) was found to be selectively retained in colon adenocarcinoma of Wistar/Furth rats as measured in situ using laser-induced fluorescence [Paper I]. The tumour to normal ratio was about 3:1. Most water soluble Pc's are present as aggregates in water solution. One exception is AlPc. The tumour to normal ratio was found to be 2:1 for AISnPc in a colon tumour in Wistar rats [76] using spectrophotometry of extracts. The fractions of AISnPc were isolated and tested for photosensitivity in vitro and in vivo [80]. AIS$_2$Pc was found to be better for the cell culture, while AIS$_3$Pc-AIS$_4$Pc were preferable for the in vivo colorectal carcinoma. The higher activity of the less hydrophilic AIS$_3$Pc in vitro was explained by its higher uptake in cell membranes. The more sulphonated AlPc's are more hydrophilic and localize to a high extent in the tumour stroma of the in vivo tumour. It is possible that they bind to sites more sensitive to PDT. ZnPc was compared with AlCISnPc and GaCISnPc and found to be superior with respect to photosensitization of Chinese hamster cells [75]. The disulphonated Pc's were also more efficient sensitizers compared with the tri- and tetra sulphonated Pc's. The tumour localizing properties of AIS$_4$Pc have been compared with those of Photofrin II. A mammary carcinoma in WAG/Rij rats showed a two-fold better tumour selectivity for AIS$_3$Pc [81]. The same drug in human LOX melanoma in BALB nude mice showed a tumour selectivity of 10:1 compared with 2:1 for Photofrin II [82]. An interesting approach for enhancing the uptake of hydrophobic photosensitizers has recently been proposed. By dissolving Pc in non-polar solutions, they can be incorporated into micelles or vesicles of amphiphilic phospholipids. A considerable uptake of ZnPc incorporated into liposomes of dipalmitoyl phosphatidyl choline (DPPC) was shown in tumour-bearing BALB/c mice [83]. The selective uptake was explained by association with lipoproteins facilitating a receptor-mediated endocytosis. AISnPc incorporated into vesicles was, however, reported to produce a more unpredictable result in the bladder of Wistar rats due to patchy drug distribution, measured by fluorescence microscopy [84]. An alternative to liposomes as vehicles for drugs is nanospheres and nanocapsules. Their main advantages are a high drug encapsulation efficiency, high biodegradability and that the preparations
are stable and thus easy to store. The preparation of Pc-containing nanospheres has been described and a binding efficiency of 80% reported [85].

To sum up, phthalocyanines seem to accumulate in cellular sites more sensitive to photodynamic action. Furthermore, ZnPc has a red absorption peak which is 48 times stronger than the corresponding peak of HpD [64] and the wavelength shift of the red absorption peak to 670 nm for ZnPc compared with 630 nm for haematoporphyrin yields a 20% better tissue penetration. With these factors in mind it is somewhat surprising that Pc's have not been used more. However, the many favourable properties of phthalocyanines make them likely candidates as replacements for Photofrin in the future.

4.5.2 Chlorin e₆

An interesting alternative photosensitizer is mono-aspartyl chlorin e₆, a chlorophyll derivative. It is prepared by extraction from algae followed by alkaline degradation and hydrolysis. The structure of chlorin e₆ is shown in Fig. 4.2. The absorption spectrum of chlorin e₆ is similar to that of Hp with a Soret band at 400 nm and three or four Q-bands ranging from 500 nm to 670 nm. The peak at 670 nm is, however, stronger than the corresponding Hp peak at 630 nm, which is an advantage for PDT. The fluorescence spectrum contains only one peak centred at about 670 nm. Chlorin e₆ was reported to exhibit a higher fluorescence yield than Photofrin II [86]. Chlorin e₆ might therefore be a candidate for use in LIF detection of tumours. Mono-aspartyl chlorin e₆ (MACE) and di-aspartyl chlorin e₆ (DACE) were compared for their photosensitizing capabilities using rat kangaroo and a Chinese hamster cell culture [86]. MACE was found to be a little more effective than DACE but not as good as Photofrin II. The main site of drug accumulation was found to be the lysosomes, as determined by fluorescence microscopy. MACE was further found to be an effective sensitizer for a sarcoma in BALB/c mice [87] using 8 mg/kg b.w. MACE and a light dose of 100 J/cm². A lack of skin photosensitivity was also confirmed. The photophysical properties of a dimer synthesized from chlorin e₆ and haematoporphyrin was studied for different surfactant solutions [88]. The properties of the synthesized dimer were concluded to be a combination of those of chlorin and haematoporphyrin with, in particular, a strong absorption peak at 660 nm. Chlorin e₆ incorporated into microspheres of polystyrene was found to exhibit a 50-fold higher uptake in MGH-U1 human bladder carcinoma cells than free chlorin e₆ [89]. The microspheres were suggested to be taken up by the lysosomes by phagocytosis.

4.5.3 meso-Tetraphenyl porphines

Sulphonated meso-tetraphenyl porphines (TPPSₙ) have significant tumour localizing properties. The absorption and fluorescence spectra of TPPSₙ are similar to those of Hp with a fluorescence maximum at about 640 nm. TPPS₄ was studied first but, in spite of a high singlet oxygen yield, was found to be a poor sensitizer in vitro as well as in vivo. A comparison of TPPSₙ with different degrees of sulphonization revealed, however, a different localization pattern for the different drugs. Thus, TPPS₁ and TPPS₂ₐ were concluded to localize mainly in the tumour cells of LOX tumours in BALB/c nude mice, while the more hydrophilic TPPS₁ and TPPS₄ were found in the tumour stroma [90]. In a human cervix carcinoma cell line (NHIK cells) TPPS₂ and TPPS₄ were mainly found in the lysosomes, particularly at lower drug concentrations [91]. The variation in localization pattern was concluded to be responsible for the difference in photosensitizing efficiency.
TPPS with a lower degree of sulphonization was later reported to show an enhanced photosensitizing capability [92].

4.5.4 Benzoporphyrin
Benzoporphyrin derivative (BPD) is a porphyrin-structured agent synthesized from protoporphyrin. It exists in four analogues with a cyclohexadiene ring at the A or B pyrrole ring and one or two acidic COOH terminated residues, as shown in Fig. 4.2. BPD-MA exhibits a strong absorption peak at 690 nm and a single fluorescence peak at about 700 nm [93]. The biodistribution of all four analogues, BPD-MA, BPD-MB, BPD-DA and BPD-DB is similar to that of Photofrin II with a tumour to normal ratio of 2:1 to 3:1, as determined by ^1H-labelled BPD in tumour-bearing mice [94,95]. The clearance rate of the drugs was, however, much faster (=24 h) for BPD thus eliminating the problem of skin photosensitization. Of the four analogues, BPD-MA appeared to have the highest tumour uptake for in vitro leukaemic cells, as observed by fluorescence [96]. The suitability of BPD-MA for LIF detection of tumours was found to be not as good as that for Photofrin II. In situ fluorescence measurements showed a tumour to normal ratio of 2.5:1 at short times for colon adenocarcinoma in Wistar/Furth rats, while for Photofrin II the ratio was 5:1 [Paper II]. The photosensitizing efficiency for leukaemia L1210 cells was correlated with membrane damage [93]. The high membrane affinity of the lipophilic BPD-MA was furthermore proposed to be the important factor that makes BPD-MA an efficient photosensitizer.

4.5.5 Rhodamine
Several rhodamine dyes, Rh123, Rh3G and Rh6G have been proposed as photosensitizers. Rh3G, Rh6G and to a lesser extent Rh123 were shown to be selectively retained in various in vitro cell cultures and possess a photosensitizing capacity [97]. The binding of these dyes was partly attributed to the positive charge of the rhodamines leading to attraction to transformed cells. The absorption of these dyes peaks at 500 nm to 520 nm, which limits their applications as sensitizers for PDT. For tumour detection, however, the shallow light penetration in tissue yields a high spatial resolution of fluorescence suitable for very small and early tumours.

4.5.6 δ-Amino levulinic acid
Very promising work on a new photosensitizing agent, amino levulinic acid (ALA) was presented recently [98]. ALA, shown in Fig. 4.2, is an early precursor of haeme in the endogenous haeme cycle. The conversion of ALA to haeme proceeds via the semi-stable protoporphyrin IX (PP). PP can be an efficient photosensitizer as has been observed in patients suffering from porphyria. This is a disease where an uncontrolled accumulation of PP in the liver and skin leads to a photosensitization of the skin of the patients. On the other hand, PP has been reported to be inactive as a photosensitizer when administered intravenously [99,100]. The uptake of PP in sarcoma-180 tumours was found to be very low, as determined by fluorescence detection [101]. However, the use of ALA as a delivery system of PP into the cells seems very valuable. ALA was found to produce PP fluorescence in the epidermis but not the dermis of the skin [98]. Similarly, the urothelium of urinary bladder showed ALA accumulation, whereas the underlying layers did not. When applied topically ALA is capable of photosensitizing damaged parts of human skin. A clinical trial was started using topical application of ALA on basal cell carcinomas. A cure rate of 90% for the first 80 patients was reported. Furthermore, the cosmetic results
were found to be good. By 1992 about 300 lesions of basal cell carcinoma had been treated by the same investigators, reporting a complete response of 79% at 3 months [102]. In another report, 80 basal cell carcinoma lesions in 21 patients were treated using ALA and a YAG-laser-pumped dye laser, with a success rate of 100% for superficial lesions and 64% for nodular lesions after one session and 100% after a second treatment [Paper IX]. Ten Mb Bowen lesions in 3 patients were treated with a success rate of 90%, while 2 out of 4 T-cell lymphomas showed complete response. ALA was also used against recurrent breast carcinoma, however, with poor results [98,103]. It was concluded that ALA was not capable of penetrating deep enough to allow a successful eradication of the tumours. Other investigators have reported similarly good results for basal cell carcinoma.

ALA was used for LIF detection of basal cell carcinoma and showed a tumour/normal fluorescence ratio of about 12, while only 2 for Photofrin [103]. When the tissue autofluorescence was included in the demarcation criterion, the tumour/normal ratio was as high as 60. In addition, recurrent breast carcinoma showed a tumour/normal ratio of 6 for ALA, while for Photofrin it was about 2.

4.5.7 Monoclonal antibody conjugates

A major disadvantage with Photofrin II is its poor tumour specificity. One possible way to enhance the tumour specificity is to couple a photosensitizer to a monoclonal antibody (MoAb). This strategy has the advantage that the tumour selectivity can be facilitated by the choice of MoAb alone, so that a photosensitizer with optimum spectroscopic properties for LIF detection or PDT can be selected without the demand for tumour selectivity of the photosensitizer. Furthermore, the mechanism governing the tumour affinity of MoAb's is better understood than that for photosensitizers like Photofrin II. The first efforts were focused on the use of MoAb-sensitizer conjugates for PDT. HpD was coupled to MoAb's against M1 myosarcoma in mice and found to inhibit tumour growth [104]. A significant difficulty with the MoAb-sensitizer conjugate strategy lies in achieving a ratio of sensitizer:MoAb which is high enough for PDT. A method of circumventing this problem is to couple the sensitizer to a carrier which in turn is coupled to the MoAb. A ratio of 30:1 of chlorin e₆ per MoAb was achieved using a dextran polymer intermediate [105]. In this way selective eradication of human T leukaemia cells was accomplished. Using a modified polyvinyl alcohol as a carrier BPD-MA was coupled to a squamous cell carcinoma MoAb. The BPD-MA-MoAb conjugate was found to be about 15 times more efficient than BPD-MA alone for that in vitro cell line [106]. In many cases the sensitizer concentration in the tumours is too low for PDT as the sensitizer-MoAb ratio is too low. Therefore, the main application of the MoAb-sensitizer conjugate is perhaps not PDT but rather fluorescence detection. Using sensitive detection systems the sensitizer concentration can be kept much lower than for PDT, instead taking advantage of the very high tumour to surrounding tissue selectivity. The tumour detection ability of a MoAb-fluorescein conjugate was investigated for a human colon carcinoma in nude mice [107]. At 48 h post-injection a tumour concentration ten times higher than any other tissue was found. It was proposed that the problem caused by autofluorescence interference could be eliminated by fluorescence background subtraction. In conclusion, the MoAb conjugates seem to be the most promising drug preparation for fluorescence detection. The possibility of freely choosing the dye should be utilized to optimize the detectability for each clinical application, taking the optical properties of each tissue type into consideration.
Even though the new photosensitizers proposed above have several properties that make them superior to Photofrin, one has to bear in mind that so far HpD and Photofrin are the only drugs which have been injected into larger groups of patients. With the vast amount of work required for a newly developed drug to reach full clinical approval for clinical use, at a project cost of perhaps 50 million dollars, we will probably have to continue to use Photofrin for at least another five years. Topically applied photosensitizers, such as ALA for tumour diagnostics and PDT appear to be very attractive alternatives for the near future.
5. Fluorescence diagnostics of tumours

Fluorescence spectroscopy is a very sensitive technique for qualitative as well as quantitative measurements of tissue constituents [6,24,108-112]. Among many advantages, the most important one is that it can easily be used for in vivo investigations. By using optical fibres, inner organs, such as the lung or urinary bladder, can be accessed. Traditionally, fluorescence emission spectra have been used almost exclusively, although there are many other aspects of fluorescence that can be used for diagnostics, e.g. differences in peak excitation wavelength, fluorescence lifetime and polarization of fluorescence. Furthermore, fluorescence spectroscopy may be performed utilizing imaging techniques, making fluorescence investigations less time consuming especially for screening of larger areas. The only side effect that has been proposed thus far, the risk of carcinogenic effect of UV light, can be overcome by adjustments of the excitation light wavelength. The one major limitation of fluorescence diagnostics, is the limited light penetration in tissue, less than 1 cm for most tissues even at the optimal wavelength. This, however, is also an advantage since the shorter the penetration depth, the better the spatial resolution in fluorescence imaging that can be achieved. At the moment, fluorescence investigations are limited to small numbers of patients. This will hopefully change in the future as the results become easier to interpret and the equipment becomes easier to use.

5.1 Background

The phenomenon of fluorescence was discovered in 1852 by Stokes [113], who observed fluorescence in fluorite. He found that when such samples are held in front of a UV source, radiation at a longer wavelength was observed. The discovery of fluorescence was for a long time not considered to be of any importance, and it was not until well into the 20th century that fluorescence was applied in medical research. Among the first to study fluorescence from animal tissue was Stiibel who, in 1911, reported that all animal tissues exhibited fluorescence when exposed to UV light [114]. In these early investigations fluorescence was observed with the unaided eye and the fluorescence was classified as bluish-white for bone, dark brown for muscle and yellow for fat, to mention a few examples [115]. At the same time, fluorescence from porphyrins was found in several animal tissues [116]. An important conclusion was made by Policard who found red fluorescence in animal tumours exposed to a Woods lamp, which he suggested to be the result of the accumulation of endogenous porphyrins [27]. In 1942, Auler and Banzer used the fluorescence technique to show that injected haematoporphyrin was taken up and selectively retained in animal tumours [28]. They performed a series of investigations showing selective haematoporphyrin retention in various animal tumours, and they also performed some preliminary studies involving humans. The selective accumulation of haematoporphyrin was confirmed by others [117,118].

With the arrival of HpD in 1960 [32] a much improved tumour selectivity was achieved. At the same time, modern techniques began to be employed for fluorescence detection. Using fluorescence spectroscopy, Winkelmann et al. were able to quantify the selective uptake of haematoporphyrin hydrochloride in a carcinoma-sarcoma experimental tumour.
in Wistar rats by means of chemical extraction [31]. Lipson et al. used their newly developed HpD for fluorescence detection of human bronchial, oesophageal and cervical tumours. In six patients with oesophageal tumours and nine patients with bronchial tumours, all tumours accessible with an endoscope exhibited porphyrin fluorescence [119]. In 51 patients with cervical tumours, 29 out of 31 malignant tumours and 20 out of 20 benign tumours were detected [120]. Gregorie et al. used a high-pressure mercury arc lamp and fluorescence photography for fluorescence detection in 107 patients involving 26 different malignancies [121]. Porphyrin fluorescence was observed in 77% of the tumours. This investigation was later extended to include 226 patients in which 76% of the lesions showed porphyrin fluorescence after i.v. injection of HpD [122]. Notably, the drug dose was 1000 mg (10-20 mg/kg b.w.). Leonard et al. reported porphyrin fluorescence in 29 out of 29 patients with malignant tumours in the oral cavity, pharynx, hypopharynx and larynx [123]. The remaining 11 patients with benign tumours showed no porphyrin fluorescence. Kelly and Snell used fluorescence photography for the investigation of 11 patients with bladder carcinoma and found significant haematoporphyrin fluorescence originating from the tumour areas [124].

5.2 Detection principles

It soon became obvious, that early small tumours could not be detected without sensitive detection systems. For this purpose several new approaches were developed utilizing fluorescence spectroscopy for in vivo diagnostics. The detection systems can be divided into several groups according to measurement principles and measured parameters. First, point monitoring systems require comparatively simple equipment but are nevertheless very sensitive. Imaging systems, on the other hand, are required for screening of large areas and may reveal tumours before being identified by the naked eye. Secondly, the fluorescence investigation can be based on the excitation spectrum or the emission spectrum, the former employing dual excitation, the latter a spectrometer or two or more detection paths. Thirdly, the fluorescence can be recorded as intensity versus wavelength or as intensity versus time on a nanosecond scale. These two methods may provide complementary information and can be used simultaneously. In some situations, one of the two may fail, while the other provides useful selectivity. The optimum combination of parameters, point measurement or imaging, spectral or temporal recording, depends on the given situation.

5.2.1 Optical filter systems utilizing spectral features

A very simple method of enhancing the faint fluorescence from small tumours is to connect an image intensifier at the output of a standard bronchoscope. This was described in 1977 in a feasibility study [125], showing that a lung carcinoma in an HpD-injected patient could easily be identified, although some red fluorescence was also observed from the surrounding tissue. This equipment was further developed by replacing the electrostatic focus image intensifier by a micro-channel plate image intensifier and the lamp by a Kr-ion laser [126]. Four patients were investigated and found to have malignant lung tumours, one of which was roentgenographically occult [127]. The same approach has been utilized by others [128]. During these investigations, an HpD dose of 2 mg/kg b.w. was used. One problem encountered with this system was that the white light used for bronchoscopy had to be switched off during fluorescence measurements in order not to destroy the image intensifier. This problem was addressed by adding a periscope path for
the white light image [129] controlled by a macro switch at the head of the bronchoscope, to by-pass the image intensifier during the white light examination. Furthermore, with this technique, spectroscopy is limited to the use of a passband filter in front of the image intensifier. However, only part of the fluorescence at 630 nm originates from the injected HpD, the rest coming from the tissue autofluorescence. Imaging methods to overcome this problem will be presented in Section 5.2.3, where more sophisticated detection systems for fluorescence imaging are described.

A point monitoring system utilizing lock-in amplifier detection was reported from the Mayo Clinic in 1978 [130-132]. This system utilizes a 405 nm bandpass filter in the excitation path, a bandpass filter centred at 660 nm in the detection path and a low-pass 600 nm filter at the white light source to prevent the white light from reaching the photodetector. Furthermore, lock-in amplification removes the remaining portion of the white light to such an extent that the system can operate completely without interference from the white light source. This system was later equipped with a rotating filter wheel to separate the white light illumination and fluorescence excitation in time. The same Hg lamp was used for white light illumination and fluorescence excitation, the latter employing a 405 nm filter. The signal from the single-channel detector was converted to an audio signal, the frequency of which is proportional to the amount of red fluorescence detected. The detection limit was reported to be a factor of 100 lower than that of a typical HpD containing tumour (without regard to the autofluorescence). A problem with this equipment is that the measured signal is strongly dependent on target distance and angle, among other parameters. The same system was also used to locate carcinoma in situ in human urinary bladder [133]. Another system based on phase-sensitive detection was developed for bladder tumour detection. This was tested according to the formula, \( S = X^2 (R - B) \), where \( X \) is the distance to the target, \( R \) is the fluorescence at 630 nm and \( B \) is the background fluorescence, and the signal was found to be linear with the porphyrin concentration [134]. In an attempt to solve the problem of the distance dependence, a two-channel system based on photomultipliers was developed [135]. One channel was used to measure the red fluorescence and the other to measure the scattered excitation light from the lung tissue. The measured ratio was presented as an audio tone in the same way as described above. The distance and angular dependence of the scattered light and the fluorescence light was theoretically modelled and found to cancel when using the ratio of the fluorescence and the scattered light. This is true only for diffusely, rather than specularly scattered light. Thus, specular reflections will result in a substantial increase in the channel of the scattered light, as was recognized by the authors. Furthermore, the reflectance spectrum may be substantially different for different tissues.

To improve the system, instead of detecting the scattered excitation light, the fluorescence in the green region was used as a reference signal in forming the ratio [136,137]. The signals from the two photomultipliers equipped with optical filters centred at 690 nm and 562 nm, respectively, were sent to an analogue divider circuit. The resulting ratio was confirmed to be independent of distance to the target. A similar point monitoring probe was reported to be further improved by introducing a third channel for fluorescence detection [Paper IV, 138,139]. The calculated ratio \( \frac{(I(630)-I(600))}{I(470)} \) was displayed versus time on a computer monitor. Furthermore, it has been shown that the autofluorescence at 470 nm is actually lower for tumour than for surrounding tissue [Paper III,IV, 46]. Hence, not only independence from distance and angle, but also an
increased contrast is achieved using this procedure. In order to maximize the probing depth [140,141], a fluorescence detection system was developed to utilize the 630 nm output from an Ar-ion-pumped dye laser as excitation source. The fluorescence at 690 nm was detected using a photo diode detector and reported to be linearly dependent on tissue porphyrin concentration. The system was found to be capable of detecting a tumour of less than 100 cells in a study involving seven patients with metastatic breast tumours [141]. In one point monitoring system, the audio tone, proportional to the measured signal, was replaced by "words" (one, two, etc.) to indicate the signal strength to the operator [142]. This system utilized a rotating filter wheel and two photomultipliers to form the diagnostic ratio \((F-b_p)/(R-b_R)\), where \(F\) is the fluorescence at 690 nm, \(R\) is diffusely reflected excitation light and \(b_F\) and \(b_R\) are the dark current signals of the detector, also measured in every detection cycle.

5.2.2 Multichannel systems

All the point monitoring systems described above have one problem in common, i.e. difficulty in reliably subtracting the tissue autofluorescence from the porphyrin signal. Some systems utilize a stored background reading, measured on normal tissue, for subtraction, while others measure the fluorescence at a wavelength close to the porphyrin peak. In either case, it is assumed that the autofluorescence spectra do not change from one location to another. It is, however, well known that this is not the case for many tissue types, especially where blood affects the fluorescence spectrum. For a more reliable detection of \(in\ vivo\) porphyrin fluorescence, optical multichannel analyser (OMA) systems have been employed for clinical use. Instead of presenting a single diagnostic parameter, OMA systems give the full fluorescence spectrum, which clearly displays the contribution from the autofluorescence. An advantage of recording the full spectrum is that the shape of the spectrum is independent of target distance [143].

An OMA system consists of the following parts: a spectrometer, preferably an image intensifier, a multichannel detector, an optical coupling device and a computer [108]. An image intensifier [144] converts a faint image, in this case the output spectrum of the spectrometer, into electrons on a photocathode plate and the electrons are accelerated in a high-voltage field in microchannels, thus starting an electron avalanche in the respective microchannels. The electron showers hit a phosphor screen and photons are emitted in proportion to the number of electrons incident on the photocathode. An amplification factor of up to 40,000 can be achieved. The amplified spectrum is imaged on the detector with either a lens system or by a fibre-optic coupling plate. An array of photo diodes, typically 1024 diodes, or a CCD camera, is used as detector. CCD cameras are often preferred since the CCD detector elements can be "binned" together when the signal is low and can be used for 2D fluorescence imaging. Both diode arrays and CCD detectors are usually cooled to a temperature below 0°C to reduce the dark current and the thermal noise.

A point monitoring system equipped with an image-intensified diode array OMA system has been utilized for displaying \(in\ vivo\) fluorescence spectra in the lungs [136]. The light delivery system was a \(K\)-ion laser coupled through an optical fibre inserted in the biopsy channel of the bronchoscope. Although this was primarily not developed for clinical use, fluorescence spectra from tumours and normal tissue were reported. A system developed for various endoscopic applications utilized a modified endoscope in which a beam splitter
Fig. 5.1 Fluorescence spectra from a lung carcinoma and from the surrounding normal tissue. The patient was injected with 1 mg/kg b.w. of Photofrin. The excitation wavelength was 405 nm.

The fluorescence spectrum can be used to generate a curve representing the contribution from the autofluorescence at wavelengths where porphyrins fluoresce. In Fig. 5.1 the in vivo fluorescence spectra of a lung tumour and the surrounding normal epithelium are shown. The dotted curve is the calculated autofluorescence based on an exponential fit using wavelengths where no porphyrin fluorescence is present. The spectra shown in Fig. 5.1 were recorded using a N₂-laser-based OMA system developed for clinical use [Paper III]. One advantage with this system is that the excitation and fluorescence light is guided through the same optical fibre. This approach results in an automatic overlap of the excitation path and the detection path. The OMA detector is an image-intensified diode array detector which can be gated down to 100 ns. In combination with the short-pulse (3 ns) laser, the use of such a narrow detector gate width completely eliminates any contribution from the white light source, which can thus be used at the same time. The N₂ laser was also used to pump a dye laser to reach 405 nm excitation. Results have been reported from in vivo investigations of lung, oral cavity, brain and urinary bladder [Papers III-V, VIII]. Several other OMA systems have been constructed which are capable of in vivo operation [148-153].

5.2.3 Imaging systems

The use of image intensifiers as detectors for fluorescence detection of tumours was briefly described in the previous section. In early observations, however, the intensified fluorescence was observed by the naked eye only. The addition of a sensitive camera enables a quantitative analysis, means of easy storage of the images and the possibility of performing advanced image processing and rectifying. Apart from being very sensitive, CCD cameras have a fairly linear response over up to 14 bits dynamic range, which is
important in quantitative analysis. In changing from point monitoring to 2D imaging, one has, unfortunately, to give up the possibility of spectrally resolved recordings. However, utilizing image processing it is still possible to include some spectral information in the fluorescence images.

The most straightforward approach for acquiring fluorescence images is to simply use an intensified CCD camera or another 2D camera to detect the fluorescence image for later image processing [154]. By employing various contrast-enhancement techniques, such as edge enhancing, the extent of tumour growth can be visualised. However, detection at a single wavelength has, as previously explained, several disadvantages such as distance and angular dependence of the measured fluorescence and loss of contrast due to background fluorescence. The problem of the background fluorescence can be solved by detecting the fluorescence both at the porphyrin peak and at a nearby wavelength. This can be done by spatial or temporal separation of the two images. This was demonstrated using a filter slide for the detection of a red and a green fluorescence image separated in time by 0.2 seconds [155]. The green fluorescence image was multiplied by a constant factor to simulate the background fluorescence at 690 nm, and was subtracted from the 690 nm image which was displayed on a monitor. A different approach is to simultaneously, or with a short delay, record a fluorescence image and a fluorescence spectrum from a certain area. In this way, the imaging capability is used to screen an area and the spectral recording indicates the amount of porphyrin fluorescence at any location showing bright fluorescence. This technique was used and a white light image was overlaid on the fluorescence image in a system using an excimer-laser-pumped dye laser [156].

To overcome the dependence on distance and measurement angle a reference signal must be included in the processing. Two possibilities have been described for point monitoring systems. Either the diffusely scattered excitation light or the fluorescence in the blue/green region can be utilized. The latter was shown not only to be the more reliable approach, but also to enhance tumour contrast. Moreover, the background fluorescence, i.e. the tissue autofluorescence close to 630 nm (e.g. at 600 nm) should be included for an accurate analysis, as described above. Using computers, this multiplexing of fluorescence images can be done at high speed. The required fluorescence images can be recorded in a sequence, one after the other by means of a filter slide [155], or using a rotating filter wheel [157], or spatially using a split mirror telescope for simultaneous recording of the images [158]. An interesting alternative is to display the fluorescence images recorded through a filter wheel on a monitor using the R,G,B monitor input connections. If the filter wheel is synchronized to the video readout synchronization pulse, the porphyrin fluorescence image can be displayed in red and the autofluorescence as green on the monitor [157]. However, the filter wheel procedure induces image blurring due to movement of the object. This blurring is particularly serious since it is of a chromatic nature, inducing completely erroneous function values close to borders between areas with different spectral characteristics (e.g. tumour and normal tissue). Consequently, a spatial separation of the fluorescence images is the preferable procedure. This can be accomplished by spectral separation of the fluorescence images using dichroic beam-splitters and two or more CCD cameras. Such a scheme has been reported for endoscopic use, capable of real-time multiplexing of images in two wavelength regions [159]. This system also included a white light image overlay onto the processed fluorescence image.
Fig. 5.2 Multi-colour fluorescence imaging of an RG2 tumour in a Fisher rat. Interference filters at 470, 600 and 630 nm were used. In the fourth image quadrant, a processed image, $|I(630)-I(600)|/I(470)$, is shown.

If a third or fourth channel is included for the utilization of other spectral features, several image detectors are involved and the set-up becomes both complicated and expensive. A solution to this problem is to project the spatially separated fluorescence images onto the quadrants of a single CCD camera. In this case the CCD image frame is stored in the computer divided into four segments for later digital operations on the sub-images. This method was realized using a Cassegrainian mirror telescope, where the primary mirror was cut into four parts, which could be individually adjusted to separate four identical images of one and the same object [Papers VI, VII, 158]. By using optical filters in front of each mirror segment, sub-images at different wavelengths were obtained. An example of such fluorescence images is shown in Fig. 5.2, including an image in the function $|I(630)-I(600)|/I(470)$. 
5.2.4. Selective excitation

In the above mentioned systems, the tumour contrast was enhanced by separate determination of porphyrin fluorescence and autofluorescence at 630 nm. Alternatively, the fluorescence may be measured at 630 nm for two excitation wavelengths, one of which excites porphyrin efficiently, while the other does not. If the autofluorescence can be assumed to be the same or only scaled by a constant factor, for the two excitation wavelengths, this method can be used to separate the fluorescence contribution from porphyrins and other fluorophores. Such a scheme has a distinct advantage, especially for imaging applications, in the requirement of a single detection channel only. Thus, neither filter wheels, split telescopes nor multiple detectors are needed. On the other hand, an excitation source capable of rapid switching between light at two wavelengths is required. In one case, this was solved by using a modified Kr-ion laser with two cavities in a collinear arrangement, which allows switching every 40 ms [160,161]. Using this technique, fluorescence images from bladder tumours were presented [162]. Another way of realizing dual excitation is to use a filter wheel or filter shutter in front of a lamp. The latter approach was used in combination with a Xe lamp to measure the fluorescence from a pH-sensitive probe molecule in leukaemic mice [163].

5.3 Light sources

Light sources suitable for fluorescence detection systems can be divided into two groups: lamps and lasers. Lasers have, of course, several advantages over lamps, such as higher output power within a narrow wavelength band, high spatial coherence allowing easy coupling to optical fibres and they are available both in continuous and short-pulsed versions. Lamps, on the other hand, give a high output power over broad spectral regions, are cheap and easy to handle. In the early days, Woods lamps were used [27], and were later replaced by high-pressure Hg lamps [131] and Xe lamps [152]. Hg lamps have their advantage in a high output power at a few suitable wavelengths, particularly at 405 nm coinciding with the Soret band peak of porphyrins. Xe lamps exhibit a broad continuum in the near-UV region and are thus suitable for operation in connection with an excitation monochromator. The multitude of suitable lasers, however, leaves the spectroscopist with a number of attractive alternatives. Because of their size and inconvenient operation, excimer lasers have been used mainly when a high output power is required, such as in fluorescence imaging [147]. Used in connection with dye lasers they span over the entire wavelength region used for fluorescence excitation. Frequency-tripled Nd:YAG or Nd:glass lasers find the same applications, but offer the additional possibility of modelocked operation for time-resolved experiments [164]. Kr-ion lasers have several lines close to 400 nm and have been utilized in several detection systems [165]. Among their advantages are a high coherence and a high output power. The above lasers are comparatively large, require high electric powers and are very expensive. Hence, other lasers are more frequently used today in compact clinical detection systems. The pulsed N₂ laser at 327 nm is a small and very reliable laser with a sufficient pulse energy for imaging small areas [Paper VI]. The short pulse width (3 ns) is an advantage when used together with gated detectors, facilitating fluorescence detection during daylight conditions. Due to short pulses and low power, it can easily be coupled to very compact dye lasers for operation in the visible region. HeCd lasers are available in the UV/blue region and have been used for the excitation of fluorescence [148]. Diode lasers would be
the perfect choice for a compact in vivo system but are not available in the UV/blue spectral region because of the low energy (1-2 eV) band gap of semiconductors. One method of reaching the UV region is to frequency double the output of diode lasers. The output power, however, is much too low in most cases. The development of diode lasers at shorter wavelengths is stimulated by the need for such e.g. data storage and might in the future reach wavelengths useful for fluorescence excitation. Another possibility would be to use a matrix of diode lasers yielding several watts of power to be used for frequency doubling into the UV region.

5.4 Autofluorescence

In the majority of investigations making use of fluorescence detection, an exogenous fluorophore is injected into the blood stream or otherwise administered to the body. Most often, this fluorophore is a porphyrin, utilized as a photosensitizer for PDT after completed fluorescence detection. The selective retention of the fluorophore in tumours results in an improved diagnosis and PDT treatment, but also results in skin photosensitivity, which is the main disadvantage of the use of HpD and Photofrin. The usual drug dose for PDT is about 2 mg/kg b.w. This is, however, well above what is required for successful fluorescence detection [166]. Consequently, much attention has been paid recently to low-dose injection of Photofrin prior to fluorescence detection in patients which are not scheduled for PDT. By lowering the injected dose to 0.25-0.5 mg/kg b.w. the skin photosensitivity is minimized while still allowing successful tumour detection. The applicability of low-dose Photofrin fluorescence detection has been demonstrated for lung carcinoma (0.25 mg/kg b.w.) [167], urinary bladder tumours (0.35-0.5 mg/kg b.w.) [Paper IV] and recurrent breast carcinoma (0.5 mg/kg b.w.) [141] and is currently an accepted method of fluorescence detection. In addition to the lack of skin photosensitivity, surprisingly high tumour to normal ratios have been achieved. A higher uptake in normal tissue rather than in tumorous tissue when increasing the drug dose above 0.5 mg/kg b.w. was suggested as an explanation [167].

Even though the use of low-dose injection for fluorescence detection has greatly reduced skin photosensitivity, it would be advantageous to perform the detection with no drugs and to rely completely on the fluorescence from the endogenous fluorophores, the so-called autofluorescence. A fluorescence spectrum from biological tissue appears at first sight to be dull and without any significant features. The spectrum is built up of the sum of a large number of fluorophores, but is also influenced by non-fluorescing absorbers. The fluorescence spectra from different tissues are the result a variation in fluorophore concentration as well as the different characteristics of the fluorophores in different environments. Furthermore, the fluorescence spectra strongly depend on excitation wavelength. For excitation below 300 nm the spectra are dominated by tryptophan-containing proteins, while for excitation in the blue/green region other fluorophores, such as porphyrin, are found.

It is well known that endogenous porphyrins are found in some tissues [Paper III, 137,168,169]. Their characteristic dual-peaked fluorescence is easy to distinguish from that of other fluorophores. It has been proposed that the fluorescence from endogenous porphyrins be utilized in the diagnosis of malignant tumours. The pattern of endogenous porphyrin occurrence is, however, quite complicated as they are not always confined to
tumours. Endogenous porphyrins were found in the white matter of human astrocytoma patients surrounding the tumours and in the border zone but not in the tumours themselves [Paper III]. Endogenous porphyrins were found at high levels in a tonsil tumour and also in the secretion from the tumour [Paper III]. The endogenous porphyrins in the oral cavity were proposed to be the result of microbial synthesis of protoporphyrin in necrotic areas of the tumours and confined mainly to the secretion from the tumours [170]. Endogenous porphyrins have also been found in colonic tumours [171].

The fluorescence in the blue region is very complex and difficult to interpret. The fluorescence spectra of some common tissue chromophores are shown in Fig. 5.3 for 337 nm excitation. Tryptophan is present in most proteins and completely dominates the fluorescence spectra for excitation below 300 nm, but as the excitation wavelength is increased the contribution to the total fluorescence spectrum decreases drastically. This is illustrated in Fig. 5.4 for an implanted colon tumour on a Wistar/Furth rat. It should be noted that tryptophan has its fluorescence peak at 350 nm when excited below 300 nm. Tryptophan is mainly located in proteins in the mitochondria and the concentration of tryptophan may be higher in tumours than in surrounding tissue. Collagen and elastin are present in supporting tissue, such as tendons and tissue stroma and fluoresce strongly at 390 nm and 410 nm, respectively. Collagen fluorescence is an indicator of atherosclerotic plaque in blood vessels (see Chapter 7) and a lack of collagen fluorescence may be a possible indicator of colonic and bladder tumours [Paper IV, 171]. For N₂ laser excitation at 337 nm, reduced nicotin amide adenine dinucleotide (NADH) dominates the fluorescence spectrum with a peak at 470 nm [172-175]. NADH is present in the cytoplasma and particularly (80%) in the mitochondria of cells where they function as co-enzymes in the citric acid cycle. Thus, malignant tissue with an elevated growth rate may exhibit a different amount of NADH fluorescence. Moreover, tumour pH is different from the pH of normal tissue resulting in a different redox balance of NADH/NAD⁺. Consequently, a lowered blue fluorescence, by about a factor of two, has been observed for a number of in vivo tumours [Papers III,IX, 176]. β-carotene is accumulated in atherosclerotic plaque and shows a weak fluorescence peak at 520 nm [Paper XIV]. At the same wavelength, we also find flavin adenine dinucleotide (FAD) [168,177,178] and keratin [168]. In an extensive fluorescence investigation of in vitro samples of colon tumours, the excitation as well as the emission wavelengths were scanned in the visible and near-UV region [171,179]. The results were presented in excitation/emission maps, which clearly illustrated the contribution from several important tissue fluorophores. Recordings of excitation/emission maps are time consuming, but are powerful and provide

**Fig. 5.3** Fluorescence spectra of some tissue fluorophores obtained with 337 nm excitation. The collagen and elastin were in the form of a powder, the nicotinamide adenine dinucleotide (NADH) and tryptophan (Trp) a buffered water solution and the β-carotene in ethanol.
Fig. 5.4 Fluorescence spectra of a rat tumour for the excitation wavelengths 287, 295, 320, 337 and 405 nm, respectively. The tumour was a colon adenocarcinoma inoculated on the hind leg of a Wistar/Furth rat. The animal was injected with 15 mg/kg b.w. of Photofrin II, 48 h prior to the investigation.

important information on which excitation and fluorescence wavelengths should be used for optimal tumour contrast. Several other fluorophores not listed here may contribute to the very complex fluorescence spectra of tumorous and normal tissue.

The fluorophores listed above, as well as others, all contribute to form the total fluorescence spectrum. If transfer processes between the fluorophores are ruled out, the total fluorescence spectrum will be the trivial sum of the fluorescence spectra of the individual fluorophores. However, one also has to take into consideration non-fluorescing, but strongly absorbing molecules. The most important in this group of molecules is the haeme group of haemoglobin, which has a structured absorption spectrum in the visible region complicating spectral analysis. The transmission profile of a thin layer of arterial blood is shown in Fig. 5.5. The Soret absorption band is located at about 420 nm. Arterial blood exhibits two minor absorption bands (Q-bands) at 540 nm and 580 nm, while for venous blood these two bands are not resolved, resulting in a single band at

Fig. 5.5 Transmission spectrum of a 0.2 mm thick sample of 5 times diluted, arterial blood.
560 nm. These bands are very often present as dips in fluorescence spectra from animal and human tissue. The influence of haemoglobin lowering tissue fluorescence has been known since the beginning of this century [114,116] but only recently recognized to be of importance for in vivo fluorescence diagnostics [Papers XI, XIII, 171, 179-181].

The presence of blood in tissue changes the fluorescence spectra in two ways. First, the overall fluorescence intensity may be altered. This results if a considerable proportion of the excitation light is absorbed by haemoglobin, leaving less excitation light for the excitation of fluorophores. This will not affect the shape of the fluorescence spectrum but only lower its magnitude. Light at 420 nm is to a high degree absorbed by haemoglobin and the penetration depth will be shallow, resulting in fluorescence only from the top layer. Fluorescence, on the other hand, is due to the Stokes shift found at a longer wavelength, where tissue absorption is much lower. Thus, in this case the spectral shape of the fluorescence will not be affected by haemoglobin reabsorption of fluorescence and the spectrum will be a "true" sum of the fluorophore contributions. 405 nm excitation light is often used for tissue diagnostics. The substantial decrease in fluorescence intensity of tumours [Papers IV, 150] may be the result of haemoglobin absorption of the excitation light.

Secondly, the tissue fluorescence spectrum may be influenced by haemoglobin in terms of reabsorption of fluorescence. Since the absorption profile of haemoglobin is very structured, this effect will affect only parts of the spectrum and therefore alter its shape. In order to see this effect, an excitation wavelength allowing a comparatively large penetration depth must be chosen. The greater the penetration depth of the excitation light, the stronger this inner filter effect of reabsorption. In an investigation of the autofluorescence of human lung carcinoma the fluorescence spectra of tumours and normal tissue were compared for 405 nm, 442 nm and 488 nm excitation [150]. For 405 nm and 442 nm excitation the tumour spectra were similar in shape but exhibited a much lower overall intensity compared with normal tissue. For 488 nm excitation, however, the shape of the spectra were significantly different. This might be explained by haemoglobin absorption. The difference in spectral shape is observed only for 488 nm excitation, for which the tissue penetration is comparable to that at 540 nm-580 nm.

The tumour to normal contrast in some cases is especially high, in fact much higher than that expected from the Photofrin injection. One should, however, be careful in utilizing this characteristic for in vivo tumour detection. Lung tumours are very easily damaged by, e.g., an optical fibre tip, resulting in bleeding, which would inevitably result in erroneous fluorescence readings at the tumour site. Furthermore, the fluorescence reabsorption by haemoglobin resulting in a dip at 580 nm may be mistaken for a porphyrin fluorescence peak at 630 nm. Porphyrin fluorescence is situated on a fluorescence background, which is often subtracted for enhanced contrast. If this autofluorescence baseline is lowered by haemoglobin reabsorption, an apparent but erroneous increase in the porphyrin fluorescence criterion will result.

5.5 Multivariate linear regression analysis

The very complex fluorescence of tissue is often difficult to interpret accurately. Haemoglobin reabsorption is only one example of a chromophore, which may lead to false
diagnosis. An approach that includes all possible spectral variations of fluorescence is multivariate linear regression analysis. In this method, the fluorescence intensity is analysed at a multitude of wavelengths and the result is multiplied by weighting factors. A large number of measurements should be used as a learning group from which the weighting factors are determined for each type of tissue. The advantage of this procedure is that only the relevant spectral characteristics survive the analysis. Hence, this method should be particularly useful for diagnostics of tumours, for which the autofluorescence may look very different at different sites, e.g. for brain tumours and colonic tumours. Several reports have been published recently on the use of multivariate analysis of colonic tumours [148,178,182-184]. In these reports adenomatous as well as dysplastic tissue was distinguished with a precision of about 90%, although some results were in contradiction to others. A very large amount of data is probably needed for an accurate multivariate analysis.

5.6 Picosecond spectroscopy

In the preceding sections analysis of steady-state fluorescence spectra was discussed as a highly sensitive technique, although the spectra are generally more or less without structure. In addition to the spectral shape of fluorescence, the lifetime of the excited state of the fluorophores may contribute to the characterization of tissues. In particular, the fluorescence decay time has been shown to be very sensitive to environmental parameters in solution and in tissue. For example, the fluorescence decay time of tryptophan, a major tissue fluorophore, changes by a factor of ten when going from acidic to basic water solution [185]. Furthermore, the fluorescence decay kinetics can be used to probe quenching of fluorescence by molecules such as O$_2$, thereby offering the possibility of investigating the diffusion of such molecules over cell membranes, and protein location and orientation in membranes. The decay kinetics may reveal excited-state reactions, energy transfer or excimer and exiplex formation [186]. By observing the anisotropy of fluorescence decay, rotational diffusion correlation times can be measured which may be used for the estimation of segmental motion or the revolution of whole proteins on a nanosecond time scale [187,188]. Thus, fluorescence decay kinetics offer many opportunities to probe the fundamental properties of molecules. The use of time-resolved techniques has recently also found applications in in vivo investigations. By utilizing the different decay times of fluorophores, the fluorescence contribution from molecules relevant for diagnosis can be selected by gated detection [Papers XI,XIII]. This may be important for future systems for tumour diagnostics in several medical applications.

5.6.1 Kinetics of fluorescence

The process of absorption of a photon by a molecule and the subsequent emission of fluorescence was described in Chapter 3, where it was pointed out that fluorescence is but one of several competing decay paths from an excited state. These competing processes all tend to lower the fluorescence quantum yield and to shorten the fluorescence lifetime. This is summarized by the following rate equation:

$$\tau = \frac{1}{k_F + k_u}$$

where $\tau$ is the fluorescence lifetime of the fluorophore in the given environment, $k_F$ is the rate constant of fluorescence and $k_u$, the sum of the rate constants of all other competing
Fig. 5.6 Fluorescence decay curves from a rat tumour and from surrounding normal muscle tissue. The tumour was a colon adenocarcinoma inoculated on the hind leg of a Wistar/Furth rat. The animal was injected with 15 mg/kg b.w. of Photofrin II, 48 h. prior to the investigation. The decay data were fitted to a multi-exponential decay:

\[ I(t) = 0.52 \exp(-t/14.9) + 0.48 \exp(-t/15.0); \quad I(t) = 0.13 \exp(-t/6.4) + 0.15 \exp(-t/2.88) + 0.72 \exp(-t/0.43) \]

The excitation wavelength was 337 nm and the detection wavelength was 630 nm. The inserts show the weighted residuals for the respective decay curves.

The intrinsic fluorescence lifetime, \( \tau_0 \), is the lifetime that would result in the absence of competing processes. To give the fluorescence lifetime a meaning, consider a population of fluorescing molecules, \( M \), in an excited state after an abrupt excitation. The change in the number of molecules in the excited state per time unit is at all times proportional to the number of excited molecules:

\[
\frac{d[M]}{dt} = -k[M]
\]  

(5.2)

The solution to this equation is:

\[
[M] = [M_0] \exp(-kt) = [M_0] \exp\left(-t/\tau\right).
\]  

(5.3)

where \([M_0]\) is the concentration of excited molecules at \( t = 0 \). Thus, at \( t = \tau \), 37% of the initially excited molecules remain in the excited state. Furthermore, the average time a molecule remains in an excited state is equal to the lifetime, \( <t> = \tau \). The resulting fluorescence intensity is expressed by:
Equation 5.1 shows that when the rate of non-radiating processes increases, such as in quenching, the fluorescence lifetime decreases correspondingly. An example of this is the aggregation of haematoporphyrin to loosely bonded aggregates, for which the fluorescence lifetime has been reported to be much shorter than for haematoporphyrin monomers (189). Note that the decrease in \( \tau \) by fluorescence quenching is accompanied by a corresponding decrease in fluorescence quantum yield.

The fluorescence decay expressed by Eq. 5.4 is valid for a single fluorophore in a homogeneous environment. Biological tissue, however, contains several intense fluorophores which all have different fluorescence lifetimes. Furthermore, each species may exhibit a different lifetime depending on its environment \( e.g. \) hydrophobicity. Hence the decay law of Eq. 5.4 must be modified to fit the fluorescence decay of tissue samples. The total decay of a heterogeneously fluorescing sample is simply the sum of the individual fluorescence components:

\[
I(t) = \sum_{i=1}^{N} A_i \exp \left( -\frac{t}{\tau_i} \right)
\]  

(5.5)

By utilizing computer processing it is possible to reconstruct a complicated decay of at least four components from the measured data. As the number of fitted components increases, the computing time increases dramatically. Furthermore, it is very difficult to separate two components with similar lifetimes. Consequently, decay data fitted to four or more decay components occur only rarely in the literature. The fluorescence decay curves from a colon adenocarcinoma rat tumour and surrounding muscle tissue are shown in Fig. 5.6.

### 5.6.2 Fluorescence anisotropy

Picosecond and femtosecond lasers are used almost exclusively for time-resolved studies due to their superior characteristics regarding pulse duration and monochromacity. Since lasers emit polarized light, an inhomogeneous distribution of excited-state dipole orientation within the excited volume will result. The incoming polarized excitation light will preferentially excite molecules to excited states with a dipole orientation parallel to the polarization of the excitation light. Thus, the fluorophores will emit fluorescence with a corresponding degree of polarization. For fixed molecules this fluorescence anisotropy will persist during the entire decay of the excited state. The anisotropy is usually expressed by the formula:

\[
r = \frac{I_{||} - I_{\perp}}{I_{||} - 2I_{\perp}}
\]  

(5.6)

where \( I_{||} \) and \( I_{\perp} \) are the fluorescence intensities parallel to and perpendicular to the excitation light polarization, respectively. However, even very large proteins may rotate at a surprisingly fast rate comparable to the fluorescence lifetime. In this case the anisotropy induced by the prompt excitation will gradually be destroyed, resulting in a faster apparent decay of the fluorescence. In other words, for observations parallel to the excitation, the apparent decay is the sum of the "true" fluorescence decay and the rate of the fluorophore rotating out of alignment with the detection polarizer. This can be expressed as [190]:

\[
P(t) = P(t) + \frac{1}{\tau_{\text{rot}}}
\]
\[ I(t) = I_0 \sum_{i=1}^{n} A_i \exp \left( -\frac{t}{\tau_i} \right) + I_r \left(3 \cos^2 \psi - 1\right) \sum_{i=1}^{n} \exp \left( -\frac{t}{\tau_i} \right). \]  \hspace{1cm} (5.7)

where \( \psi \) is the polarization angle between the excitation and fluorescence light, \( r \), the initial anisotropies given by Eq. 5.6 and \( \phi \), the rotational correlation times for each degree of freedom for the rotating molecule. As can be seen from Eq. 5.7, the second term, describing the anisotropy decay, can be set to zero by a suitable choice of the detection angle \( \psi \). Thus, for a detection angle of 54.7° (the magic angle), the anisotropy term disappears and the measured decay is the true decay of the fluorescence. Three examples of fluorescence decay curves obtained at different polarization orientations are shown in Fig. 5.7. A plot of the calculated anisotropy is included in the figure. It should be mentioned here, that other decay laws exist, such as non-exponential decay due to fluorescence quenching. The general rule is to fit the decay data to a physically relevant model for the given situation.

5.6.3 Light sources

A variety of short-pulsed lasers exist which provide pulse durations comparable to or shorter than the fluorescence decay of most interesting biomolecules. The use of dye lasers in combination with non-linear crystals, provides wavelengths over the entire UV and visible regions. The most-utilized pulsed excitation source is the dye laser, synchronously pumped by an Ar-ion laser or a Nd:YAG laser. The pulse width of an Ar-ion-laser-pumped dye laser is of the order of a few ps, which is well below the time jitter of available detection systems. The principle of mode locking of a laser is based on the modulation of the losses of a CW laser in such a way that all modes in the cavity are locked to each other and interfere at a given time and location to produce a giant pulse with a short duration. The theory of mode-locking may be understood as follows. The intracavity electromagnetic field, \( E(t) \), may be expressed as [191]:

\[ E(t) = \sum_{n} E_n \exp \left[ i((\omega_n + n\omega)t + \phi_n) \right] \]  \hspace{1cm} (5.8)

If the phases \( \phi_n \) of the \( n \) different modes are fixed, e.g. set to zero, Eq. 5.8 may be rearranged to:

\[ I(t) = \frac{\sin^2 \left( \frac{N\omega t}{2} \right)}{\sin^2 \left( \frac{\omega t}{2} \right)}, \]  \hspace{1cm} (5.9)

where it should be recognized that \( I(t) \) is proportional to \( E(t)E^*(t) \). The solution of Eq. 5.9 consist of a series of principal maxima separated by N-2 secondary
maxima. As the number of modes, $N$, increases the height and sharpness of the principal maxima increase. Thus, the larger the number of active laser modes, the shorter the pulse width: $\Delta t \propto 1/\Delta \omega$. In the frequency domain the active laser modes are represented by a number of sharp peaks within the gain profile of the laser medium. The laser modes are separated by $\Delta \omega = 2\pi\nu/(2L)$, where $L$ is the length of the cavity. The amplitude modulation of the light will create sidebands around the mode $\omega_n$ separated from the mode by the mode-locking frequency $\Omega$. If $\Omega$ is selected to be equal to $\Delta \omega$, the modes interact and a large number of modes with the same phase will result.

Nd:YAG and Nd:glass lasers are other examples of lasers that can be mode locked. They provide higher pulse energies but broader pulse widths, of the order of a few tens of ps. These lasers can also be simultaneously Q-switched and mode-locked, resulting in much higher pulse energy. The output from such lasers consists of a train of mode-locked pulses within the envelope of the Q-switch. The high peak powers and low repetition rate make these lasers suitable for operation with streak cameras [164]. Recently, the pulsed radiation from synchrotrons has come into use as a light source in time-resolved fluorescence measurements [192]. Synchrotron radiation has the advantage of covering the region from X-rays up to the IR, where the maximum output depends on the particle energy in the ring. For electrons moving at low velocities the space distribution of emitted light is symmetric around the acceleration vector, pointing towards the centre of the ring. The intensity is also vanishingly low. As the electrons are accelerated to relativistic energies, the distribution of emitted light will be extremely elongated, pointing in the direction of electron velocity. The more energy given to the electrons, the higher the light intensity and photon energy. If bunches of electrons are accelerated in the ring, corresponding short light pulses will result. Hence, by using a monochromator, a train of short pulses at the desired wavelength can be obtained. This source is very useful for single-photon counting or phase modulation measurements.

5.6.4 Time-resolved detection techniques

Single-photon counting.

The most-utilized technique for measuring time-resolved fluorescence is the time-correlated single-photon counting (TCSPC) technique [193]. This is a method where, in principle, the time between an excitation pulse and a fluorescence photon is registered for single photons. This procedure is repeated at a repetition rate of a few kHz and within minutes the distribution in time of the fluorescence decay kinetics is acquired. The instrumentation may consist of an excitation source, a non-linear crystal for frequency doubling, a sample holder, focusing lenses, a monochromator, a fast micro-channel plate photomultiplier and detection electronics. Some of the excitation light is split off to a photo diode to provide a zero time reference for the timing electronics. The pulses from the detector and the photo diode are fed to a constant fraction discriminator for more accurate determination of the arrival times and the output signal is sent to a time-to-amplitude converter (TAC). The photo diode pulses are used as start pulses and the photomultiplier pulses as stop pulses to the TAC, or preferably the other way around to avoid dead time in the electronics. The amplitude of the TAC signal is proportional to the difference in arrival time between the start and stop pulses. The TAC signal is integrated to produce a decay curve in a multichannel analyser operating in pulse height mode. The decay curve is sent to a computer where the decay constants are calculated using an
iterative evaluation procedure. Most often, the least square procedure is used to describe the deviation of the fitted curve from the measured curve. In this method the sum of the residuals:

$$\chi^2 = \sum_{i=1}^{n} \frac{1}{w_i} (f_i - F_i)^2$$  \hspace{1cm} (5.10)

is minimized by variation of the fitting parameters. $f_i$ are the measured data points in time channels $k$, $F_i$ the calculated intensities and $w_i$ the weighting factors. Several iterative methods for solving this minimization problem are available. Newton’s method is one of the fastest but requires reasonable starting values. If Poisson statistics are assumed, the proper weighting factors are equal to $F_i$. If the fitted parameters correctly describe the decay kinetics, $\chi^2/(n-p)$ should be close to 1, where $n$ is the number of data points and $p$ the number of fitted parameters. Furthermore, by plotting the weighted residuals the correct number of decay components can be estimated.

For the evaluation of fluorescence decay times comparable with the time response of the detection system, a deconvolution procedure must be employed. The recorded fluorescence is the convolution of the response function and the fluorescence decay:

$$f(t) = \int f(t) g(t-\tau) d\tau ,$$  \hspace{1cm} (5.11)

where $f(t)$ is the response function, often called the apparatus function and, $g(t)$ is the true fluorescence decay. In practice, $g(t)$ is not deconvoluted from the measured decay but a forward convolution procedure is employed, in which the above convolution is calculated after Fourier transformation:

$$F(\omega) = I(\omega)G(\omega)$$  \hspace{1cm} (5.12)

After returning to the time domain $f(t)$ is compared with $F(t)$ according to the least square procedure. $f(t)$ is best measured by recording elastically scattered light from the sample. The width of $f(t)$ can be estimated from:

$$sf = \sqrt{st^2 + s\Delta t^2} ,$$  \hspace{1cm} (5.13)

where $st$ is the width of the laser pulse, $s\Delta t$ the time jitter of the detector and $st$ the time jitter of the electronics. The dominating factor here is $s\Delta t$, which is about 40 ps for currently available micro-channel plate photomultipliers.

Several aspects of the fluorescence must be considered in decay measurements in order not to end up with erroneous results. One such parameter is the polarization angle of the fluorescence light. As was described in Section 4.7.2, the fluorescence must be recorded at a polarization angle of 54.7° to the excitation light to avoid mixing of the fluorescence decay and anisotropy kinetics. This is usually accomplished by inserting a polarizer before the monochromator. For measurements of fluorescence anisotropy one must also correct for the different throughput of the monochromator for parallel and perpendicularly polarized light. Hence, a more elegant method is to introduce a polarization rotator in the excitation path [Paper XII]. In this way the entire optical detection path is indifferent for all polarization directions. Furthermore, maximum throughput of the monochromator can be employed for every polarization direction with this configuration. In Fig. 5.7 the
fluorescence decay for NADH in phosphate buffered water solution is shown for different polarization angles. Note the slower onset of fluorescence for the perpendicular detection angle.

Furthermore, corrections must be made for the intrinsic anisotropy. When a certain angle, e.g. 54.7°, is selected between the detection and excitation polarizers, this is the angle between the favoured absorption dipole direction of the molecules and the detector polarizer. If, however, after absorption to the initial highly excited state the molecules relax to a lower state before emission which has a different dipole direction, this angle must be added to the observation angle. This is often the case when excitation occurs to a state with substantially higher energy than the one from which the emission occurs. Hence, the energy level dipole orientations of the molecules to be studied must be known or the angles must be found empirically. The latter can be accomplished by calculating the anisotropy for a number of pairs of parallel and perpendicular observation angles, for which different zero angles are assumed. The angles which yield the maximal anisotropy are the actual parallel and perpendicular observation angles. If the detection polarizer is rotated 54.7° from the parallel direction, the true fluorescence decay will be observed.

The study of fluorescence decay from biological tissue requires special care regarding light propagation in tissue. Especially in the red spectral region, scattering dominates strongly over absorption, resulting in very long effective photon paths. This may affect the decay curve seriously by prolonging the lifetime, especially if the fluorescence decay is short. One way to reduce this effect is to choose an excitation wavelength which has a very poor penetration in tissue, such as wavelengths below 400 nm. The red fluorescence photons may then still be multiply scattered before detection, but less scattered photons will dominate in the region of the illuminated area. The contribution to the decay curve from scattered excitation light can be eliminated by recording the apparatus function using scattered excitation light from the tissue sample. The contribution from scattering of fluorescence light can not be completely eliminated and one should always be aware of this problem. For blue/green fluorescence photons this problem is less serious due to a much higher absorption coefficient.

Time-resolved fluorescence has been studied for different haematoporphyrin preparations in solution under different conditions using the TCSPC technique. In water solution, up to three decay components are necessary to fit the decay curves [194-197]. The slow decay component (=15 ns) is attributed to monomeric species or unfolded oligomers, while the two faster ones (=3 ns and 0.5 ns) may originate from more aggregated species. When Hp or HpD is dissolved in methanol, only the slow decay is observed, indicating a monomerization of the porphyrins [194,195]. Photofrin II in cetyltrimethylammonium bromide (CTAB) was studied for different CTAB concentrations below and above the critical micelle concentration (CMC) [196]. For low CTAB concentrations the fast decay component was smaller and the decay time was also shorter. For higher CTAB concentrations, again the fluorescence decay became longer. Furthermore, time-resolved fluorescence has been recorded from in vitro cell cultures and animals [Paper XII, 198-201]. It was concluded that the porphyrins were taken up primarily by the hydrophobic compartments of the tissue, as was shown by fluorescence decay time studies and time-gated spectra. Using a fluorescence microscope, time-resolved fluorescence was recorded for different cellular compartments [201]. Lower amounts of aggregates in the
nucleus and a faster wash-out of aggregates were attributed to an intracellular monomerization of the aggregates.

The TCSPC technique was further utilized to measure fluorescence anisotropy from cell cultures incubated with protoporphyrin or Photosan (similar to Photofrin II) [202]. Using polarization diagrams, it was possible to distinguish monomer fluorescence from the fluorescence from aggregates.

**Pulse sampling.**

In single-photon counting the time between an excitation pulse and a single fluorescence photon is registered. In this method it is important to operate at a low count rate to avoid pile-up at the detector. Another method, which is referred to as pulse sampling, involves the detection of many fluorescence photons within a short time interval. Such time windows can be recorded sequentially for different laser pulses, as with photomultipliers and boxcar integrators or the complete decay curve is registered for each laser pulse as with streak cameras. The latter have the advantage of a time resolution of 1 ps, but have then a very low dynamic range. Streak cameras are often operated with short-pulsed Nd:YAG lasers in Q-switched and mode-locked operation.

Fluorescence decay curves were measured for HpD solution [203] and HpD- or Photofrin II-incubated cell cultures and animal tissue [204,205]. A fluorescence band at 660 nm with a decay time of 100 ps was revealed and attributed to haematoporphyrin dimers. Decay curves were also recorded at 600 nm from excised pieces of human cancerous and non-cancerous breast and lung tissue. In both cases the decay time for tumorous tissue was reported to be shorter than for normal tissue [206]. Streak cameras have also been utilized to study the fluorescence anisotropy of tumorous and normal rat tissue [164]. Different wavelength dependences of anisotropy were reported for tumour and normal tissue. The pulse sampling method can be used to good advantage for time-resolved imaging at ns time resolution. This was done with a short-pulse N\textsubscript{2} laser and an image-intensified CCD camera for the study of tumour-bearing mice [207]. The conclusion was that the 15 ns decay component provided the best tumour contrast.

**Phase modulation.**

Phase modulation is a technique that was developed before TCSPC but has only recently come into widespread use [186,190,208-210]. The phase modulation technique is based on the detection of the fluorescence decay in the frequency domain rather than in the time domain. If the excitation source is intensity-modulated:

\[
I(t) = I_0 (1 + a \sin(\omega t))
\]  

the resulting fluorescence:

\[
F(t) = \gamma I_0 (1 + b \sin(\omega t - \phi))
\]  

will be modulated at the same frequency \(\omega\) but will be shifted in phase due to the delay of the fluorescence. The fluorescence will also possess a lower degree of modulation. It can be shown that the phase shift and modulation factor can be obtained from two equations:

\[
\tan \phi = \omega \tau
\]  

and

\[
\tan \phi = \omega \tau
\]
Either the phase or the modulation factor can be used to determine the fluorescence lifetime. Most often, the phase shift is utilized since phase measurements are more accurate than measurements of small intensity modulations. The reconstruction of a complicated decay is best accomplished by performing several measurements at different modulation frequencies.

It is not clear which of the different time-resolved methods gives the most accurate results. One advantage of the phase modulation technique is that it can be performed with comparatively simple equipment [208]. Furthermore, the mean power of a modulated light source is higher than that of a mode-locked laser which would result in shorter acquisition times. One has to be careful, though not to cause photo-bleaching of the samples at high light powers. Probably both phase modulation and time domain spectroscopy have advantages in different situations.

5.7 Fluorescence detection utilizing a combination of techniques

It is interesting to see if there is an optimal fluorescence detection system for use in in vivo investigations, and what such a system would look like. Or perhaps we should rephrase the question: with our current knowledge of different detection techniques, how do we best combine these into one system that is not too complicated or expensive and that is convenient enough to operate in a clinical situation?

To answer this question we have, first of all, to try to find out if any of these methods are replaceable or if all of them give complementary information. The excitation wavelength, for instance, determines which chromophores are excited and what the probing depth in the tissue will be. By varying the excitation wavelength, different fluorophores can be probed. Thus, by selecting one or more excitation wavelengths the fluorophores that provide information about a certain tissue abnormality, can be selectively investigated.

Next, let us consider emission spectroscopy where the detection wavelengths are varied. This can be done with a monochromator or with a suitable set of wavelength-selective filters. Different fluorophores emit at different wavelengths so that the interesting fluorophores can be preferentially detected. This is also possible with selective excitation spectroscopy, however, the difference between the two methods is that the probing depth in excitation spectroscopy is different for different molecules (i.e. excitation wavelengths), whereas in the case of emission spectroscopy it is not. Hence, we can conclude that these methods provide, at least to some extent, complementary information. Furthermore, the fluorescence yield is optimized if both optimal excitation and emission wavelengths are chosen for the individual fluorophores.

Another possibility is to utilize time-resolved spectroscopy. The basis of these techniques lies in the difference in the temporal behaviour of the fluorophores. Once again we have to address the question of whether the time resolution gives information that is not provided by emission spectroscopy. One reason why we do obtain complementary information from the two techniques is that we have to consider not only the fluorescence from the relevant fluorophores but also the background fluorescence. For example, the emission profiles of
the relevant fluorophores and the background fluorophores might be nearly identical in a certain situation, while the fluorescence decay times of the relevant fluorophores and background fluorophores are substantially different. In another situation or tissue compartment, we might have the reverse behaviour and the contrast is provided by the emission profile. Thus, by including spectral as well as temporal information, a more accurate diagnosis is possible [Paper XIII]. Furthermore, the fluorescence decay time can be very sensitive to tissue environmental parameters such as pH, while a fluorescence spectrum is sensitive to the amount of non-fluorescing absorbators, e.g. haemoglobin, which the decay time is not. The choice of time-resolved method is probably not important for the result and we may choose the least complicated one.

The last of the techniques described above is polarization spectroscopy. The degree of polarization of fluorescence and its time development is a sensitive tool, which reveals information primarily about fluorophore kinetics and their dependence on neighbouring molecules. This is information that the other methods do not give and polarization spectroscopy should therefore give additional information.

The methods described here can, to varying extents and varying degrees of ease be incorporated into a clinical system. The combination of excitation and emission spectroscopy has been demonstrated [138] and both these methods have been utilized for imaging systems [Papers VI, VII, 161]. It is somewhat more difficult to incorporate time resolution into the system. High-time-resolution techniques such as TCSPC are too complicated for clinical instruments and cannot be performed in an imaging mode. With the use of image-intensified CCD cameras a very sensitive system with gating times of down to 5 ns can be realized utilizing the pulse sampling technique [207]. Using multi-colour imaging telescopes [Papers VI, VII], where several images are monitored simultaneously at selected wavelengths, spectral information can be included in the tissue discrimination function. A very interesting characteristic of image intensifiers is that the gain can be modulated at high frequency facilitating phase modulation detection as an alternative to gated detection. However, with this technique the background ambient light is difficult to suppress.

The use of time-resolved polarization spectroscopy appears to be more difficult since this requires a much higher time resolution and the resulting signals may be too weak. Furthermore, the multiple scattering of light by tissue will effectively destroy any information about polarization except for a very shallow probing layer. For each scattering event the degree of polarization has been reported to decrease by a factor of 0.7 [211]. Consequently, very few reports have appeared in the literature on polarization spectroscopy of intact tissue samples [see e.g. ref. 164].

To sum up, the various aspects of fluorescence can be combined in order to enhance the tissue discrimination. Examples of spectrally and temporally resolved recordings were given in Papers XI-XIV (see also Fig. 7.3). In particular, the system employing a N₂ laser and boxcar integrators described in Paper XIII, may be very useful for clinical point measurements. The system presented in Paper VII provides imaging with spectral resolution and may also be operated with a short time gating for additional temporal resolution.
A photodynamic reaction is a chemical reaction which is facilitated by the presence of light. This type of reaction is utilized in photodynamic therapy (PDT) where an exogenous chromophore exhibiting photodynamic behaviour, is in some way incorporated into tissue. The area is then illuminated with light of a suitable wavelength and intensity. If the photosensitizer is selectively retained in the tissue to be treated, a tumour for example, the light may be applied without any knowledge of the exact spatial limits of the target tissue. Among the advantages of this treatment modality are:

* selectivity of certain cells or tissue structures
* smooth healing of the treated area
* an unlimited number of treatment sessions can be applied
* anaesthesia is not requirement
* the cost of the treatment is moderate
* no hazardous effects for patients or medical personnel

Of course there are some disadvantages, the most important being an elevated skin sensitivity to ambient light for a few weeks post-treatment. PDT is also limited by light penetration in living tissue, which makes PDT suitable mainly for superficial lesions on the skin and in body cavities such as the urinary bladder.

6.1 History

Phototherapy was employed already in ancient times B.C. by the Egyptians and in the Asian cultures. They made use of the actinic action of the sun on skin without any special photosensitizers. Exposed to the sun, vitiligo, rickets, psoriasis and skin cancer were treated. The Greeks used whole-body exposure in the sun, which they termed heliotherapy. In the 19th century these ancient methods of phototherapy were rediscovered. One of the more important researchers was Niels Finsen, who at the end of the last century used a carbon arc for phototherapy of lupus vulgaris [212]. The use of photosensitizers to enhance the effectiveness of phototherapy was also known very early by, for example the Indians, who used psoralens obtained from the seeds of *Psoralea corylifolia* for repigmentation of vitiligious skin. A very important modern discovery was made in 1897 by Raab, who was a student of von Tappeiner, when studying the toxicity of acridine on paramecia [213]. He obtained completely different results for two experiments performed under identical conditions except for the ambient light level. Raab therefore came to the conclusion that the toxicity of acridine was strongly influenced by light intensity. Raab's work was continued by von Tappeiner, who in later experiments, used eosin as a photosensitizer. von Tappeiner reported that photosensitizers were active only in the presence of oxygen [214]. This action of photosensitizers was termed photodynamic therapy by von Tappeiner. Furthermore, he was the first scientist to use PDT on patients and treated nine cases of skin tumours using eosin as a photosensitizer [215]. On this first occasion the tumours were irradiated by sunlight or a lamp over a period of a few weeks. In the years which followed, several other sensitizers, e.g.
chlorophylls, were investigated for their photosensitizing capability. In 1908 Hausman discovered the photosensitizing properties of haematoporphyrin [216], and five years later, Meyer-Betz injected himself intravenously with haematoporphyrin [217]. As a result he developed a marked photosensitivity, which persisted for about two months. The next important discovery was made by Auler and Banzer, who found that tumours became photosensitized by haematoporphyrin [28]. They studied the selectivity of haematoporphyrin uptake in animals and also performed some studies on humans. Further work on haematoporphyrin accumulation in tumours was performed by Figge et al. who investigated the possibility of using haematoporphyrin as an aid in tumour diagnostics [29].

Due to the poor therapeutic effect of crude haematoporphyrin there was no further development of the technique and it was not until Schwartz et al. and later Lipson et al. succeeded in refining the more effective haematoporphyrin derivative (HpD), that progress once again was made. The first report of the therapeutic use of HpD came from Lipson et al. in 1966 when they reported moderate response to PDT on a recurrent breast carcinoma [218]. Furthermore, Diamond et al. used HpD to successfully treat an experimental glioma in 1972 [219]. Kelly and Snell studied the selective uptake of HpD in human bladder tumours and they also reported on one case treated with PDT using HpD. The modern history of PDT started in 1978 when Dougherty et al. presented the first large study of PDT involving 25 patients with various tumours [220]. By that time Dougherty had already been working for several years on animals with different photosensitizing drugs. Their work triggered a rapid expansion of the field and shortly after, tumour diagnostics and PDT using HpD had been reported on basalioma, malignant melanoma, bladder tumours, lung tumours, colon tumours, prostate tumours and several other types of tumours. References to the above described work can be found in reviews on the history of phototherapy [221,222]. See also references 15,16,223-227 for more recent clinical achievements.

6.2 Mechanisms of PDT

The treatment of tumours by photodynamic therapy involves a photosensitizer which is selectively retained in the tumour after injection, and the subsequent exposure of the tumour to light at a wavelength matching the absorption spectrum of the photosensitizer. Another important factor is the availability of oxygen in the tumour region. It has been shown in numerous investigations that the photodynamic action can not occur in the absence of oxygen supply [228]. The induced reaction is of a purely photochemical nature and does not rely on any heat that might be produced by the light source. However, if the irradiation is intense enough to create heat, a synergistic hyperthermal reaction may take place, as will be discussed below. The various stages of the photodynamic process are shown in Fig. 6.1. A photon is absorbed by the photosensitizer which is excited to a singlet state. The major absorption peak corresponds to the higher singlet states where the energy level density is high, termed the Soret band. At this wavelength, roughly 400 nm, the light penetration is, however, very poor in tissue. Therefore, the light source is usually tuned to the Q-band, at the lowest energy, 630 nm for porphyrins, where the light penetration in tissue is an order of magnitude better. Thus, the photosensitizer is found in the lowest vibrational level of the first excited singlet state (S1). Inter-system crossing, although spin forbidden, can proceed with a high quantum yield, leaving the
photosensitizer in the lowest triplet state (T₁) after rapid internal relaxation. Since the energy of T₁ is lower than S₁, this path cannot be reversed. The production of triplet-state sensitizer can be expressed in terms of the rate equation:

$$\Phi_{T} = \frac{k_{\Delta}}{k_{\gamma} + k_{\chi}}$$

where \(k_{\Delta}, k_{\gamma}, k_{\chi}\) are the rate constants for radiative decay, non-radiative decay and intersystem crossing, respectively. Radiative decay to the ground state, which is a singlet state, is spin forbidden and exhibits a low probability. Thus, the lifetime of T₁ is comparatively long (~ ns) and the interaction with surrounding molecules is highly probable. In the case of PDT, two kinds of reactions can occur: an electron transfer to a target molecule (Type I reaction) and an energy transfer mainly to oxygen leading to the production of excited-state oxygen (Type II reaction). In competition with these processes, phosphorescence and quenching by other target molecules which do not lead to a photodynamic effect, may take place. In Type I reactions, several radicals, often involving oxygen, are formed [229], such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radicals (OH⁻). The most important Type II reaction is the promotion of ground state \(\Sigma_g^*\) oxygen to one of the two excited singlet states \(\Delta_g\) and \(\Sigma_u^*\), of which \(\Delta_g\) is the active one in PDT. Singlet oxygen acts as a very aggressive oxidant to many target molecules in tissue, such as proteins, nucleic acids and phospholipids. The rate equation for singlet oxygen production is then:

$$\Phi_{\Delta} = \Phi_{O} \frac{k_{\Delta}(O)}{k_{\Delta}(O) + k_{\alpha}(O) + k_{\beta}[A] + k_{\gamma}}$$

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where \(k_A\), \(k_{ox}\), \(k_Q\) and \(k_p\) are the rate constants for \(O_2(^1D_g)\) production, other oxygen quenching processes, quenching by other molecules and photosensitizer phosphorescence. Of the different photochemical reactions taking place in PDT, type II singlet oxygen production has so far been considered as the most important. Evidence for this hypothesis is the observation that PDT action is dependent on the oxygen concentration. It should, however, be remembered that oxygen is also a major mediator in type I reactions. Thus, the presence of oxygen in tissue seems to be an important factor for effective PDT. A set of photographs of a human basalioma treated with PDT is shown in Fig. 6.2. The photographs were taken before, six days after and two months after PDT, respectively. The tumour was topically treated with ALA and a light dose of 60 J/cm\(^2\).

**6.3. Techniques for quantifying photodynamic action**

A major problem with PDT has always been the difficulty in predicting the PDT effect on tumorous and adjacent tissue. The PDT effect is clearly a function of injected dose and applied light dose. However, other factors, which are much more difficult to control also play an important role. These include optical parameters of the tissue including surface reflectance, the actual photosensitizer uptake and retention in different tissue compartments and the quantum yield for singlet oxygen production, \(\Phi_A\), determined by the factors in Eq. 6.2, which is different for different tissue compartments. One way to improve the prediction of the outcome of PDT is to monitor the fluorescence from the photosensitizer [103]. The fluorescence is approximately proportional to photosensitizer concentration (see however ref. 44) but does not take into account the variations in \(\Phi_A\) or optical parameters at the therapeutic wavelength. A more direct approach is to measure
the faint phosphorescence from $^{1}\text{O}_2$, which is roughly proportional to the $^{1}\text{O}_2$ available for the oxidation of target molecules. The first report on the successful measurement of in vivo singlet oxygen showed a very weak emission peaking at 1260 nm [230]. A sensitive lock-in technique together with a modulated Ar-ion-laser-pumped dye laser was used to study subcutaneous murine tumours injected i.p. with as much as 50 mg/kg b.w. of Photofrin II. It was found to be necessary to correct for the emission from the photosensitizer, to be able to detect the weak $^{1}\text{O}_2$ emission. Another successful investigation showed $^{1}\text{O}_2$ emission from a leukaemia cell culture incubated with polyphosphoryl [231]. The $^{1}\text{O}_2$ emission was separated from the photosensitizer emission by its longer excited state lifetime, about 50 μs for $^{1}\text{O}_2$ in cell membranes. Several investigators have reported failure to detect singlet oxygen both in vivo and in vitro [232-234]. The reason for these failures was suggested to be the short $^{1}\text{O}_2$ lifetime within the cells hiding the $^{1}\text{O}_2$ phosphorescence in the stronger photosensitizer fluorescence. There are two ways to circumvent this problem: the first is to have a good temporal resolution in the detection system and the second, for in vitro studies, is to use D₂O instead of water [231]. The singlet oxygen lifetime in D₂O is about 60 μs while in water it is only 4 μs. These lifetimes correspond, however, to $^{1}\text{O}_2$ produced in the cell membrane which diffuses out into the H₂O/D₂O buffer. The intracellular lifetime is at least two orders of magnitude shorter. A higher limit for the $^{1}\text{O}_2$ lifetime was estimated to be about 0.5 μs. [232,234]. Based on the assumption that the $^{1}\text{O}_2$ diffusion distance is of the order of 0.01 μm, the $^{1}\text{O}_2$ lifetime was estimated to be about 0.01-0.04 μs [235].

An alternative approach for the direct measurement of the $^{1}\text{O}_2$ phosphorescence is to measure the action of $^{1}\text{O}_2$. This can be accomplished by the extensive photobleaching of photosensitizers during PDT [236]. This photobleaching is not the result of direct photodestruction of the photo-sensitizers, but rather a photooxidation process [237]. The $^{1}\text{O}_2$ produced by the therapeutic light not only oxidizes target molecules in the tissue, such as nucleic acids and proteins, but also leads to a photo-oxidation of the photosensitizer molecules. The photoproduction is a porphyrin-like molecule with an additional fluorescence band at 660 to 670 nm observed during and after PDT [103]. The effect of photobleaching of ALA in human basalioma is illustrated in Fig. 6.3, showing fluorescence spectra before

![Fluorescence spectra from a human basalioma obtained before (left) and after (right) PDT. The tumour was locally treated with ALA 4 h prior to PDT. In the right diagram, an enlargement (x8) of the red part of the spectrum is shown. The excitation wavelength was 405 nm.](image-url)
Fig. 6.4 Diagram showing photobleaching of ALA-induced protoporphyrin during PDT. The logarithm of the ratio of the fluorescence at 630 nm after PDT and the fluorescence before PDT (open squares) and the logarithm of the ratio of the fluorescence at 670 nm and the fluorescence at 630 nm, are shown.

The photobleaching during PDT may be used as an internal dosimeter for the photodynamic action in vivo. Hence, only when the sensitizer is being significantly bleached, does the PDT have an effect on the tissue, and when all the sensitizer molecules have been bleached there is no point in continuing the therapy. An advantage with utilizing the photobleaching for the prediction of the outcome of PDT is that it by-passes variations in quantum yield of the tissue, at least to a first approximation. It must be remembered, though, that the fluorescence technique probes only a very thin tissue layer. There are two ways of measuring the bleaching. First, the fluorescence intensity at 630 nm, or preferably the ratio \(I(630)/I(470)\), can be monitored. The 630 nm signal may decrease by about one order of magnitude during PDT depending on the light dose. Secondly, the additional peak at 670 nm can be utilized to monitor the fluorescence ratio \(I(670)/I(630)\), which increases by about 50% during PDT [103]. These two criteria are shown in Fig. 6.4 for human basalioma treated with ALA and varying light doses. The decrease of the porphyrin fluorescence is expected to follow an exponential decay. The slight deviation from an exponential decay may be explained by the fact, that fluorophores far away from blood vessels are not accessible to singlet oxygen and can, thus, not be destroyed by PDT. Finally, it should be noted that it is not clear that the 670 nm peak is a new fluorophore produced during PDT. A possible alternative is that the 670 nm fluorophore is always present (look more closely at Fig. 6.3) and is observable only when enough of the 630 and 690 nm sensitizer has been bleached away.

6.4 Tissue targets for PDT

The targets of photodynamic action have been studied on a microscopic level both in vitro and in vivo. In the first studies, different cellular sites, mainly membranes were identified as being important primary targets for PDT. However, as more sophisticated in vivo models are being developed using fluorescence microscopy techniques, vascular compartments appear to be more important.

The cellular membrane consists of a bilayer of phospholipids with proteins in the lipophilic membrane interior. The photodynamic action results in a peroxidation of the phospholipids but also, and perhaps more important, in direct damage to the proteins facilitated by singlet oxygen oxidation of mainly tryptophan, which may lead to peptide crosslinking. Membrane damage can be observed by microscopy as a swelling of the cells and as bleb formation on the membrane [238]. Depolarization of mouse myeloma cell membranes was measured during PDT using a fluorescence indicator, and was observed to be an
immediate response to PDT [239]. A dependence of light dose and photosensitizer concentration on the effect of PDT was observed. Alternatively, membrane damage can be detected indirectly by increased leakage of potassium across the membranes.

The mitochondria have been shown to be a site of HpD accumulation as well as a target for PDT [240]. After systemic administration of HpD or Photofrin II into Fisher rats bearing a transplanted mammary tumour, high porphyrin concentrations were found in the mitochondrial membranes. Mitochondrial enzyme cytochrome C oxidase was found to be very sensitive to photodynamic treatment.

DNA has been suggested as a target for photodynamic therapy. The rate of DNA synthesis has been reported to decrease as a result of PDT [241]. After a few hours, the DNA synthesis rate increases again, indicating a repairable damage to the DNA. Changes in chromosomes were also reported as a result of PDT. The affected parts of the chromosomes appear to be those closest to the nuclear membranes, due to the high porphyrin accumulation in membranes, in contrast to the nuclear interior. Although there are some effects on the cell mitosis apparatus, these effects are not thought to be very important in comparison with other effects of PDT.

Recently, damage of the micro-vasculature, as a major contribution to photodynamic action in tissue, has been considered more important than direct cellular damage [228,240,242]. Several initiators of vascular breakdown have been suggested, among which the release of eicosanoids seems to be one of the most important. The result of initiator release is vascular occlusion, leading to a decreased flow of nutritional substrates to neoplastic cells and a reduced oxygen supply, which leads to hypoxia and anoxia. There have been a few, more or less successful, attempts to study vascular changes during and immediately after PDT. One problem encountered was the difficulty in developing an animal model that allows in vivo studies. Success was reported for window chambers which were surgically placed on animals [243]. However, this model had the disadvantage of high mortality and significant surgical difficulties in placing the chambers on the animal. More recently, these drawbacks seem to have been overcome. In a comparative study [244] of DHE and CIAISP the former drug showed a rapid rupture of the vasculature immediately after PDT whereas the latter only showed vasoconstriction and spasm, even though the overall outcome of PDT was the same for the two. An interesting pattern of tumour growth with an alternating increase in neovascularization in the tumour area and tumour growth during a decrease in vascularity was also found.

Different photosensitizers thus appear to exhibit different effects on tumours during PDT. In the future, it would probably be an advantage to find a photosensitizer with a direct effect on tumour cells rather than an effect linked to vascularity. Such behaviour would probably be more specific against malignant tumours.

6.5 Light sources

The light sources used for PDT can be divided into two main groups: lamp sources and lasers. Lamps have some advantages but have now more or less been replaced by various lasers. Other light sources have been used historically. For instance, filtered sunlight was
used by von Tappeiner to treat skin tumours using the photosensitizer eosin [221]. With this technique only a few mW/cm² of therapeutic light can be achieved.

The main advantages of filtered lamps in PDT are the low cost and the high reliability. For gas discharge lamps, e.g. Xe lamps, power levels of a few kW can be obtained, yielding several watts of useful red light after wavelength selection. This power level is certainly enough for the treatment of tumours of some five cm in diameter. The lamps are placed in a housing with a reflecting mirror, preferably elliptical, to focus the light onto the tumour, thereby achieving the power densities required for PDT [245,246]. The size of the discharge, however, places a limit on the minimum focus, introducing unacceptable losses for coupling into optical fibres. Therefore, the main application of lamps is in the use of PDT of skin tumours. An alternative use of lamps is far-red/IR light delivery for controlled hyperthermia in connection with PDT in the treatment of thicker tumours e.g. recurrent breast tumours [Paper VIII].

The most frequently used light source in PDT is the Ar-ion-laser-pumped dye laser. This laser has an output of 3-4 watts in the CW mode of operation with a beam diameter of a few mm and a very low beam divergence, and is therefore ideal for coupling to optical fibres. The use of a dye laser also provides flexibility in terms of choosing a suitable wavelength from the whole region of interest from 630 nm up to 750 nm. Thus, with the use of new photosensitizers, this will still be a useful light source. The main disadvantage is the difficulty in maintaining a stable output power from the dye laser. For this, qualified personnel are required. Furthermore, the dye must be changed after every 100 hours of operation, the Ar-ion laser requires installations for water cooling and the cost of this laser is comparatively high.

An alternative to the Ar-ion laser is the metal vapour laser [245]. Especially the gold vapour laser is a good choice since the wavelength, 628 nm, matches the absorption peak of haematoporphyrin. This has the important advantage that a dye laser is not needed. Otherwise, the cost and requirements are about the same as for the Ar-ion laser. The gold vapour laser is a pulsed laser, in contrast to the Ar-ion laser, with a mean power of about 3 watts. This laser is of course only useful for PDT utilizing haematoporphyrin as photosensitizer. However, it can be converted to a copper vapour laser by changing the tube to obtain a pumping source for a dye laser.

Historically, the HeNe laser was used for PDT only because of its operating wavelength, 633 nm. The output power, however, is far from useful for conventional PDT. Nevertheless, it has been used for post-surgical treatment of the tumour bed of brain tumours [247].

An attractive alternative now available for PDT is the Nd:YAG laser. The output of the Nd:YAG laser at 1064 nm can be frequency doubled in a KDP or BBO crystal to achieve a wavelength of 532 nm, which can be used to pump a dye laser. For efficient frequency doubling a pulsed laser must be used. However, the conventional Q-switched Nd:YAG laser operates at a 10 Hz repetition rate, which is too slow to give a suitable mean output power at 532 nm. Therefore, a medical Nd:YAG laser has been developed, which is a compromise between high peak power and high mean power with a repetition rate of a few kHz and 100 ns pulse duration [Paper IX, 103]. The peak power is sufficient for
efficient frequency doubling and the mean output power is about 20 watts at 532 nm, yielding about 2 W mean power at 630 nm. The advantage of this laser is the more stable operation of the dye laser, which does not require an open dye jet. Furthermore, with this system 532 nm and 1064 nm light is available for laser surgery. The Nd:YAG-pumped dye laser is of course still a large system and also very expensive.

The laser which has the highest potential for future PDT is without doubt the diode laser. Diode lasers were originally available only in the IR region but have now been developed to reach 630-640 nm, however, they do not yet have enough power for PDT. Because of their size and low cost, the diode lasers can be used in large arrays to increase the power to several watts. Large arrays can be used because of the extremely high quantum efficiency of diode lasers, up to 70%. Which means that the amount of heat that must be removed is relatively small. Furthermore, diode lasers are operated at a very low voltage, and do therefore not require large power supplies. One of the disadvantages is the high divergence, which makes coupling to optical fibres somewhat difficult, especially for diode arrays. This problem can, to some extent, be dealt with by attaching the fibres directly to the output window of the lasers. So far there have been no reports on PDT utilizing diode lasers, but there is little doubt that this will soon come.

6.6 Light delivery systems

To achieve flexibility and convenience, the light from the lamp or the laser should be applied to the patient by means of some optical delivery system. Optical waveguides and mirror systems with 3D flexibility developed for CO₂ laser use can be utilized for easily accessible tumours, such as skin tumours or tumours in the oral cavity. The obvious choice and the only possibility for endoscopic operation, however, is optical fibres. For surface illumination optical fibres are usually used together with a micro-lens or a microscope objective. The Gaussian far-field profile of an optical fibre is not appropriate for PDT. A microscope objective, however, creates an image of the fibre tip at the tissue, facilitating a top-hat intensity distribution. Typically a light dose of 30-60 J/cm² is used for treatment of skin tumours, with excellent results especially for basal cell carcinoma. Complete response can be obtained in almost 100% of the cases after a single, or sometimes two treatment sessions [Paper IX, 248]. Less favourable results have been obtained for recurrent breast cancer due to multiple metastases and the thickness of the tumours. Some work has been done on PDT of malignant melanoma, however, with poor results.

For interstitial PDT one or more bare fibres are usually used. The multiple scattering of the red light in tissue creates a symmetric light distribution around the fibre tip so no additional optics are necessary. An interesting prospect is the utilization of a 3D array of optical fibres to facilitate PDT of large tumours. Such a device might be useful for PDT of tumours in inner organs such as the liver and pancreas.

PDT can be used to good advantage in body cavities such as the lungs or urinary bladder. In this case the light should be delivered unidirectionally at a solid angle covering the tumours, or preferably, at a space angle of 4π as in the case of whole bladder illumination. This can be realised using a diffusive fibre tip; the fibre end being covered with a non-absorbing, highly scattering medium, usually about 2 mm in diameter. A special
consideration in PDT of hollow organs is the light dosimetry. In contrast to flat surface PDT, reflected light will be absorbed inside the cavity, so all the light delivered can be absorbed by an air-filled organ. Since tissue reflectivity for tissues such as the bladder is of the order of 0.6-0.8, the light dose per unit area should be 3-5 times lower than that used for skin tumours (10-30 J/cm$^2$) [249]. If the bladder volume is measured, the total bladder area can be calculated assuming spherical geometry. An alternative to a diffusive fibre tip is to fill the bladder with a non-absorbing highly scattering medium to achieve even illumination [250]. This approach may be advantageous for highly asymmetric bladders. For lung applications the diffusive tip used is usually cylindrical due to the cylindrical geometry of the bronchi. The light dose is then calculated as delivered light energy per unit length of fibre tip, usually 200 J/cm. The cylindrical tip, usually about 25 mm in length, placed in the centre of a bronchus, provides an even illumination of the bronchus surface.

6.7 Light delivery considerations

6.7.1 Dose rate effects
Photodynamic therapy is the selective treatment of tumours requiring light of the correct wavelength, tumour uptake of an injected photosensitizer and the availability of oxygen in the treatment areas. As explained earlier, oxygen is consumed during PDT. For intercapillary distances larger than 200 μm the oxygen concentration decreases with distance from the capillaries in such a way that the outcome of PDT will no longer be proportional to the light dose and the in vivo sensitizer concentration [251]. Thus, for optimal treatment oxygen must be transported to the tumour at the same rate as it is consumed during PDT. This is, however, usually not possible when the light is delivered continuously at a dose rate of 100 mW/cm$^2$ or more. To improve tumour response the treatment can be divided into two or more sessions. This was done on R3230AC tumour tumours where two 15 min irradiation periods were separated by a 1 hr dark period [252]. A significant delay in tumour growth was observed compared with tumours exposed to the same light dose without an intermediate dark period. This was explained by a very low ATP level at the beginning of the second treatment period, less vascular damage from the first light period with vascular repair during the dark period and diffusion of oxygen from the treated surface layers into deeper layers not sufficiently treated during the first period. Similar results were obtained with alternating 30 sec. light and dark periods for a total irradiation period of 2 hours [253]. A theoretical model showed that the distance from an isolated capillary to oxygenated cells should decrease during continuous PDT, supporting the results described above.

A second way of enhancing the PDT efficiency is to lower the light dose rate. This was demonstrated by comparing dose rates between 50 and 200 mW/cm$^2$ [252]. As a possible explanation of the enhanced effects the authors suggested less vascular damage and less photobleaching for lower dose rates. However, a similar investigation using 5 to 125 mW/cm$^2$ did not show this effect [254].

The Nd:YAG laser-pumped dye laser is a convenient light source for PDT. A question that arises, however, is whether the high repetition rate, short-pulsed light produces the same effect as a continuous light source. The time constants of the processes involved in PDT are certainly long when compared with the μs light pulses. However, the high peak power of the light pulses might induce tissue effects that will not be obtained with CW
irradiation. A comparison of a CW Ar-ion-laser-pumped dye laser and a 50 ns 10 kHz repetition rate gold vapour laser showed no difference for either in vitro cell lines or transplanted nude mice tumors [255]. These findings were supported by the outcome of PDT of 18 patients utilizing a gold vapour laser [255]. This seems also to be true also for a 100 ns 4 kHz repetition rate Nd:YAG laser pumped dye laser, although no direct comparisons with a CW laser were made [103]. In conflict with the above results, a 1.6 μs, 4 Hz pulsed flash-lamp-pumped dye laser was reported to be significantly less efficient in PDT when compared with an Ar-ion-laser-pumped dye laser [256]. In this latter investigation, however, a very high pulse energy, up to 0.25 J/cm² was applied, resulting in significantly shorter irradiation times. The results were explained by saturation effects of the absorbing molecules due to the high peak powers. A more likely explanation may be the high light dose rates for the pulsed sources causing immediate vascular rupture and oxygen depletion in the tumours hampering further PDT.

6.7.2 Wavelength dependence of PDT

The light wavelength utilized for PDT has in most cases been 630 nm, corresponding to an absorption peak of haematoporphyrin. The absorption maximum in the visible region is, however, situated at 405 nm with a 30-fold higher light absorption than at 630 nm [257]. PDT would therefore be much more efficient using 405 nm light. The reason for using red light for PDT is that the total light attenuation by the tissue decreases with increasing wavelength over the visible region. Thus, by using the haematoporphyrin peak furthest towards the red, the PDT is more efficient at deeper tissue layers. On the other hand, for thin tumours, such as basaloma, the higher light absorption at 405 nm or 520 nm would be advantageous. The higher cell killing efficiency of blue light was demonstrated for two in vitro cell cultures treated with HpD [246]. Three broad spectral bands (blue, green and red) were filtered out of a Xe lamp and applied at a dose rate of 10-20 mW/cm². The same behaviour was found for an in vivo rhabdomyosarcoma tumour in WAG-Rij rats [257]. A linear relationship was found between the depth of necrosis and the logarithm of the light dose. 405 nm light was concluded to be the most efficient for tumours thinner than 0.2 mm, 630 nm most efficient for tumours thicker than 1.2 mm and 514 nm irradiation most efficient for tumour thicknesses between 0.2 and 1.2 mm. Similar results were obtained in a comparison between 514 nm and 630 nm for an in vivo bladder tumour in C3H mice [71]. It was also concluded that the deeper muscle layers of the normal bladder were less affected after 514 nm irradiation, a very important factor in PDT in the urinary bladder which would prevent bladder shrinkage post PDT.

A different approach for increasing the light penetration in tissue during PDT has been suggested based on two-photon absorption of the photosensitizer molecules [258,259]. Utilizing a 1064 nm Nd:YAG laser, a two-photon process would correspond to the absorption of two photons at 532 nm. The high penetration of 1064 nm photons would thus be used in connection with the high photosensitizer absorption at 532 nm. This principle was demonstrated for a rat glioma using i.p. injection of pheophorbide a, a chlorophyll derivative with an absorption peak close to 532 nm. Good tumour response was achieved although no comparison was made with conventional PDT using HpD and 630 nm light. There are, however, some arguments against this treatment strategy. There is a clear advantage in light penetration in tissue for 1064 nm photons compared with 630 nm, at best a factor of two [2]. Two-photon absorption, however, has a quadratic dependence on light intensity. Therefore, at a tissue depth where the light intensity has
decreased to $1/e=0.37$ of the initial intensity, the probability of two-photon absorption has decreased by a factor of $e^2$ to a level of 0.14 of the initial intensity. At the same depth light at a wavelength with half the penetration depth (630 nm), the light level would be $(1/e)^2 = 0.14$. Thus in the above case, all that is gained in light penetration is lost in decreased probability for two-photon processes. Furthermore, the absorption of tissue is higher at 532 nm compared with at 630 nm, which leads to a heating of the tissue surface.

6.7.3 Photobleaching during PDT
Animal experiments and clinical trials have shown a reciprocal relationship between tumour drug concentration (proportional to injected dose) and delivered light dose [260-262]. This means that the photodynamic effect will be the same whether the light dose is doubled or the drug dose is doubled. However, there are lower thresholds for the photodynamic effect: about 1.2 mg/kg b.w. for drug dose and 15 J/cm² for light dose [262]. These values are, of course, different for different tissues, but are valid at least approximately for most tissues. The drug dose threshold can be used in PDT as a method of increasing the therapeutic ratio between tumour and surrounding tissue. As was described in Section 6.3, HpD fluorescence (roughly proportional to drug concentration) follows first order kinetics, and is decreased during PDT by bleaching with an exponential decay. This means that for a given drug dose the maximum achievable photodynamic effect will be obtained when almost all the drug has been destroyed by photobleaching. Beyond this, no further photodynamic effect can result. Thus, if the drug dose is low enough, the photodynamic effect is determined by the tissue uptake and not by the light dose. Consequently, the injected drug dose can be chosen such that the level of drug taken up by normal tissue is below the threshold for tissue photodynamic destruction, while the drug level in tumour is above that threshold. This strategy has the advantage that, since the normal tissue is not affected by the light, the dose can be increased to such a level that deeper tumour layers receive enough light to achieve tumour necrosis. The concept of photobleaching has been demonstrated clinically with good results [261].

6.7.4 Combined PDT and hyperthermia
Hyperthermia can be combined with PDT to treat thicker tumours. This was demonstrated for a mammary carcinoma in mice to which heat was applied immediately before or after PDT by means of a microwave transducer [263]. Hyperthermia was also induced in a human recurrent breast cancer immediately after PDT [paper VIII]. The 4-10 mm thick tumours were, in this case, treated successfully. The heat profile was forced to peak a few mm deep in the tissue using surface cooling in connection with the heating filtered projector lamp. The temperature was measured at several depths during the hyperthermia. A synergistic effect of hyperthermia induced post PDT has been suggested, whereas hyperthermia applied pre PDT seems to have a protective influence against PDT [264]. It was suggested that the synergy was due to the shut-off of capillaries as an immediate response to PDT, preventing heat transport from the tumour area during hyperthermia. Furthermore, PDT is not effective against hypoxic cells, whereas such cells are very sensitive to heat. When, on the other hand, hyperthermia is administered before PDT, the vascular activity is impaired by the hyperthermia, a vascularity that is an absolute requirement for PDT. The different type II PDT reactions were studied and reported to be strongly time dependent [265]. Thus, when hyperthermia is applied simultaneously with PDT the reaction rates increased, which was proposed to be an explanation of the synergism.
7. Fluorescence diagnostics of atherosclerotic plaque

Much attention has been paid lately to laser angioplasty as a treatment modality for vascular diseases. High-energy laser pulses are used to remove atherosclerotic plaque by ablation and molecular dissociation processes. Various lasers as well as non-laser diagnostic methods have been suggested to provide a safe recanalization procedure for coronary arteries. A method with great potential is laser-induced fluorescence spectroscopy, as it offers a high selectivity for most types of plaque. In this section, spectrally and temporally resolved fluorescence spectroscopy of atherosclerotic plaque will be discussed.

7.1. Background

Currently, the standard methods of treatment of coronary occlusion involve by-pass surgery and percutaneous transluminal coronary angioplasty (PTCA) [266]. The latter makes use of a catheter, terminated with a balloon, which is inserted into the arteries. The balloon is then expanded at the location of the plaque. This leads to a compression of the plaque material and a mild rupture of intimal fibres. After repeated expansions, the lumen has increased in diameter. However, the residual surface is damaged, which leads to early restenosis. Furthermore, the success rate in totally occluded arteries has been found to be very low. The use of endoluminal stents to secure an open lumen, has been suggested, but this seems to cause thrombus formation and to induce restenosis [267]. Mechanical devices, such as scrapers and drills [268,269] have been used, as well as ultrasound recanalization [270] of peripheral arteries, all with moderate success.

As an alternative to by-pass surgery and PTCA, laser angioplasty was introduced in the early 1980's [271,272]. Initially, the results were not acceptable, mainly due to accidental perforations and dissections and to thermal damage by CW lasers [273,274]. Various probes were developed to circumvent the mechanical perforations, such as metal cap or hot-tip fibre probes [275]. These do not make use of the direct action of the laser irradiation, but are based on pure heating of the plaque. Sapphire tips were also developed, which increase the size of the ablation crater and lower the incidence of perforations [276]. The use of thermal probes, however, can damage to the coronaries and they are not effective against calcified plaque. To avoid thermal damages, excimer lasers were introduced for plaque ablation. The action mechanism of excimer lasers is different from that of CW Nd:YAG lasers, due to the short pulse width and high tissue absorption [277,278]. These properties lead to an instant ablation and possibly a direct bond-breaking of tissue molecules, as was explained in Chapter 2. Clinical success of excimer laser angioplasty has been reported by several authors, especially when performed in combination with PTCA [279-284]. In a multi-centre study involving 685 patients, the success rates of laser angioplasty and laser angioplasty plus PTCA were reported to be 83% and 93%, respectively [279]. Recently, the Ho:YAG laser and the HF laser were presented as alternatives to excimer lasers, yielding sharp cuts due to high water absorption [285,286]. However, some thermal damage has been reported using a Ho:YAG laser [287].
In addition to the above mentioned methods of treatment, a lipid-lowering diet to decrease stenosis rate and to restore the coronary blood flow, would of course be the preferable choice. In fact, regression of coronary stenosis has been reported to be, to some extent, a consequence of LDL-lowering medical treatment [288, 289]. In practice, a lipid-lowering diet should be advised in conjunction with laser angioplasty, or whatever the method of choice is.

### 7.2 Fluorescence detection of atherosclerotic plaque

Although the fibre probes and excimer lasers of today provide smooth ablation, an accurate guidance system during plaque ablation is still required. In particular, the heterogeneity of plaque lesions and the sharp curvature of coronary arteries constitute a diagnostic problem. The use of an ablation wavelength, at which plaque exhibits a higher light absorption compared with normal vessel wall, was suggested as offering a safe recanalization procedure [290]. The wavelength region of high plaque absorption was found to be about 500-550 nm which, however, is not ideal regarding the ablation mechanism. Furthermore, the preferential light absorption does not include calcified plaque and the effect is absent in a blood-filled lumen [291].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Excitation region</th>
<th>Detection</th>
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<tbody>
<tr>
<td>Autofluorescence</td>
<td>UV</td>
<td>380/435nm and 520/490 nm</td>
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<tr>
<td></td>
<td>Blue</td>
<td>Overall intensity</td>
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<td></td>
<td></td>
<td>600/580 nm</td>
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<tr>
<td>Time-resolved fluorescence</td>
<td>UV</td>
<td>Decay time at 380 nm</td>
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<td>Fluorescence marking</td>
<td>UV</td>
<td>Tetracycline fluorescence at 550 nm</td>
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<td></td>
<td>Blue</td>
<td>Haematoporphyrin at 630 nm</td>
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<tr>
<td>Raman scattering</td>
<td>Red/Near IR</td>
<td>Calcification at 980 cm&lt;sup&gt;-1&lt;/sup&gt; and cholesterol at 701 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ablation plasma</td>
<td>UV</td>
<td>Ca-lines at 397, 447, 560 nm</td>
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**Table 7.1 Different spectroscopic techniques for detection of atherosclerotic plaque.**

Various spectroscopic methods have so far provided the most successful diagnostic procedures during plaque ablation. A number of spectroscopic techniques and diagnostic criteria have been proposed, such as fluorescence spectroscopy, Raman scattering and plasma detection. These aspects of plaque detection are summarized in Table 7.1. In most cases the tissue autofluorescence is utilized, but exogenous chromophores, such as tetracycline [292, 293] and the tumour photosensitizers haematoporphyrin [294] and phthalocyanine [295] have also been utilized. Autofluorescence spectroscopy was introduced in 1985 [296] and found to be sufficient to distinguish plaque from normal tissue in in vitro samples. A structured fluorescence was found for normal vessel wall, in contrast to plaque, for excitation wavelengths around 480 nm [296], and the demarcation criterion I(600)/I(580) was proposed for the detection of plaque. Detection probes based on these findings, utilizing a single [297] as well as multiple [298, 299] detection fibres were developed. In the latter case, a quasi imaging technique is employed to map out the vessels. Another method of plaque detection is to utilize the difference in fluorescence
peak intensity. It was shown, that the peak intensity was lower for plaque than for normal vessel, utilizing a scanning system [300] or as SIT camera [301]. This method is, however, not suited for \textit{in vivo} detection since the intensity is dependent on target distance as well as interference from intra-luminal blood.

Another possibility is to use a UV excitation source to produce the fluorescence spectra. It has been shown that a fluorescence peak at 390 nm is specific for the presence of atherosclerotic plaque [Paper X, 302-309] although a lack of demarcation has been reported for calcified plaque [310]. In an investigation utilizing excitation/emission maps, it was concluded that UV excitation provide a better discrimination compared with blue light excitation [306]. In Fig. 7.1, the spectra from plaque and a normal sample utilizing 337 nm excitation, are shown. UV excitation was also utilized to produce multi-colour fluorescence images of atherosclerotic plaque [Paper VI, 158]. The ratio of fluorescence images at 390 nm and

\begin{align*}
\text{Plaque:} & \quad I(t) = 0.21 \exp(-t/6.75) + 0.38 \exp(-t/2.20) + 0.41 \exp(-t/0.30); \\
\text{Normal:} & \quad I(t) = 0.10 \exp(-t/5.47) + 0.29 \exp(-t/1.41)+0.61 \exp(-t/0.14). 
\end{align*}

The inserts show the weighted residuals for the respective decay curves.

\[\text{Plaque} \quad \text{Normal}\]

\[\text{Plaque} \quad \text{Normal}\]
450 nm was employed to distinguish plaque from normal vessel. The characteristic fluorescence at 390 nm is clearly confined to the plaque lesion, as was shown in investigations of the fluorescence during laser ablation of plaque [Paper XIV, 305]. As the plaque was ablated from the vessel wall, the shape of the fluorescence spectrum changed to the shape of that of a normal vessel. In addition, a peak at 520 nm was observed in lipid-rich plaque [Paper XIV] as well as a broad structure peaking at 450-500 nm, extending out into the red region, mainly in calcified plaque [302,311]. Moreover, time-resolved methods using UV excitation can be employed to distinguish plaque from normal vessels. This was done using the single-photon counting technique, showing that plaque exhibits a slower fluorescence decay than normal vessel [Papers XI,XII]. In Fig. 7.2, the decay curves of fibrotic plaque and a normal aortic sample, are shown. For diagnostic use, a simpler approach was suggested, employing a pulsed N\textsubscript{2} laser, photomultipliers and a boxcar integrator. By detecting the delayed photons and the prompt photons at 380 nm in separate channels, the ratio of the two can be shown to be an indicator of plaque. Furthermore, the detection of plaque can be improved by combining the spectral and the temporal demarcation criteria for UV excitation [Papers XI,XII]. In Fig. 7.3, examples of 6-ns-gated fluorescence spectra of an atherosclerotic sample, are shown. The peak at 390 nm is most obvious in the delayed spectrum from the sample.

Other spectroscopic methods for the detection of atherosclerotic plaque include Raman detection and plasma detection. Calcified plaque contains hydroxyapatite, which exhibits strong Raman bands at e.g. 980 cm\textsuperscript{-1} and 1082 cm\textsuperscript{-1}. These bands have also been detected in samples of calcified plaque [310,312-314]. Cholesterol is another indicator of plaque, mainly found in lipid-rich plaque, with Raman peaks at e.g. 701, 882 and 959 cm\textsuperscript{-1} [315,316]. The advantage of the Raman technique is the sharp peaks obtained. The signals are, however, very weak in comparison with fluorescence signals. Plasma detection of plaque utilizes the emission lines from Ca atoms and ions produced in the hot plasma during plaque ablation. These lines are also very sharp and give an accurate diagnosis of calcified plaque [Paper X, 317]. A major disadvantage is, however, that the diagnosis occurs immediately after the ablation pulse.

**Fig. 7.3** Time-resolved fluorescence spectra from an atherosclerotic plaque. The excitation wavelength was 337 nm. The gate width was approximately 6 ns and the delay relative to the laser pulse was adjusted to record the prompt, the intermediate and the delayed fluorescence, respectively.
7.3 Origin of plaque fluorescence

The origin of plaque fluorescence has been the subject of debate lately. The complex structure of atherosclerotic lesions and necrotic molecular transformations together with the lack of sharp features in fluorescence spectra, makes identification a difficult task. In particular, the difference in fluorophore concentration between plaque and normal vessel is an important feature for in vivo fluorescence diagnostics.

Fluorescence spectra of some fluorophores found in atherosclerotic vessels can be found in Fig. 5.3. At excitation wavelengths below 308 nm tryptophan dominates the spectrum [318] with a fluorescence maximum at 350 nm, while at longer excitation wavelengths the fluorescence yield decreases and the peak shifts towards longer wavelengths, as shown in Fig. 5.3. Collagen and elastin are proteins which exhibit strong fluorescence peaking at 390 nm and 410 nm, respectively. Especially elastin has a very broad excitation spectrum and also fluoresces strongly for excitation wavelengths in the blue/green spectral region. Other examples of potential fluorophores are NADH and cholesterol for UV excitation and carotene, ceroids and vitamin A for longer excitation wavelengths.

Using UV excitation, the main difference between fibrotic plaque and a normal vessel is the fluorescence peak at 390 nm, which is much stronger for plaque than for normal tissue, as shown in Fig. 7.1. The origin of this peak has been shown to be fluorescence from collagen fibres in the plaque [Paper XIV, 319-322]. In fact, the balance between collagen and elastin determines the peak fluorescence wavelength, which is 390 nm for plaque as well as for purified collagen powder. For excitation wavelengths close to 300 nm and below, elastin fluorescence becomes weak and tryptophan fluorescence becomes more prominent. The tryptophan fluorescence may also be used as a reference for the plaque-related collagen fluorescence and at some wavelength close to 300 nm, the contrast of collagen fluorescence against other fluorophores is optimal. In Fig. 7.4, the contrast ratio of plaque to normal tissue, using the fluorescence decay criterion delayed/prompt fluorescence at 380 nm, is plotted for a number of wavelengths. The data is obtained from fluorescence decay curves recorded at the MAX synchrotron in Lund. The diagram does not include measurements further to the visible region due to a decrease in the fluorescence yield at 380 nm for longer excitation wavelengths. The maximum contrast seems, however, to be located close to 337 nm. A similar plot may be obtained for spectral criteria, such as $I(390)/I(450)$ which may be used to find an optimal excitation wavelength.

Another spectral feature observed with for 337 nm excitation, is the fluorescence peak.
at 520 nm, reported for fatty plaque. This fluorescence contribution was confirmed to arise from the necrotic, lipid-rich core of the plaque by dissection of fatty plaques [Paper XIV]. This peak has been suggested to originate from β-carotene, which is known to accumulate in plaque [323,290]. The fluorescence quantum yield of β-carotene has been reported to be quite low in solutions, although clearly detectable [324,325], and to peak at 520 nm, which is in good agreement with what is found for fatty plaque. Furthermore, β-carotene exhibits a distinct hydrophobicity, a property which would also favour an accumulation in fatty plaque. The broad spectral feature in the green/yellow region found in fatty plaque and, in particular, in calcified plaque, has been reported to originate from ceroid deposits [311,321,322,326,]. The fluorescence from ceroid deposits was measured using fluorescence microscopy at 515 nm and 610 nm and simultaneous spectral recordings.

In the first fluorescence investigations of atherosclerotic vessels, Ar-ion lasers were used as excitation sources. The ratio $I(600)/I(580)$ was proposed as a demarcation criterion for plaque. Later, this structure was confirmed to originate from fluorescence reabsorption by haemoglobin in the tissue [327-329], present in normal vessels, but absent in plaque. The Soret absorption band of haemoglobin is found at 420 nm and the two Q-band absorption peaks at about 540 nm and 580 nm, as shown in Fig. 5.5. These absorption peaks correlate well with the dips in the spectrum of normal vessel wall shown in Fig. 7.1. A demarcation criterion, which is based on the amount of blood in the tissue might be difficult to use in an in vivo situation, since the intra-luminal blood will also affect the fluorescence spectra. Even if the fibre probe is held close against the vessel wall, which is difficult, there is always some blood present, unless the vessel is shut off completely. Two ways of obtaining fluorescence data free from blood interference were recently suggested [Paper XIII]. First, a pair of wavelengths can be chosen, such that the blood absorption is the same at the two wavelengths, while the fluorescence from plaque and normal tissue differs. By forming a ratio of the two fluorescence intensities, a plaque demarcation criterion insensitive to blood, is achieved. One such ratio is $I(380)/I(437)$, which displays the collagen fluorescence of the tissue. Another is $I(520)/I(489)$, which is an indicator of fatty plaque, possibly due to the accumulation of β-carotene, and perhaps also of calcified plaque due to ceroid accumulation. Secondly, fluorescence decay curves can be utilized as an indicator of plaque. The ratio of delayed to prompt fluorescence, which is high for collagen-containing plaque, is not sensitive to the absorbing but non-fluorescent haemoglobin in the vessels. The use of the time-resolved plaque criterion was proven successful in the presence of blood, in an investigation of plaque fluorescence [Paper XIII]. The demarcation ratio was found to be independent of the thickness of a blood layer between the probe and the plaque. The measured fluorescence decay is only independent of the amount of haemoglobin in tissue to a first approximation, since highly absorbing haemoglobin will change the light propagation properties of the tissue, and a long propagation path will be recorded as a prolonged fluorescence lifetime by the detector. Fortunately, the absorption coefficient of the tissue is very high in the UV region. The fluorescence probing depth at 325 nm excitation and 430 nm emission has been estimated to be about 150 μm [330]. Thus, the influence of haemoglobin on light propagation, may affect the fluorescence decay time in the ps region only, while in the ns region, where plaque demarcation in obtained, no effect from light propagation will be observed.
There are other methods, which are more complicated, but which yield results free from interference from blood reabsorption. By modelling an exponential attenuation of the excitation light and the induced fluorescence in tissue, the contribution from collagen/elastin fluorescence and haemoglobin reabsorption could be separated for 476 nm excitation [331]. An important observation was, that when interference by haemoglobin reabsorption was corrected for, the spread in the data decreased.

In conclusion, optical spectroscopy offers several criteria for in vivo atherosclerotic plaque detection. Fibrotic plaque is best diagnosed using UV excitation and spectral or temporal fluorescence detection of the 380 nm peak. Fatty plaque may be demarcated utilizing either UV excitation and 520 nm fluorescence detection, or blue light excitation and 580 nm detection. Alternatively, Raman scattering from cholesterol at 701 cm\(^{-1}\) may be employed. Lastly, calcified plaque may be demarcated by UV excitation of the 380 nm structure, by the lack of haemoglobin reabsorption at 580 nm, by Raman scattering of hydroxyapatite at 980 cm\(^{-1}\) or by plasma detection of Ca emission during ablation. Whether the best procedure is to utilize one of the above criteria, such as the blood-independent \(I(380)/I(437)\) ratio, or to use a combination of them, is not yet clear.
8. Laser-induced fluorescence of vegetation

In the final chapter of this thesis, remote fluorescence detection of vegetation will be discussed. At first sight this topic may seem to be very different from the medical applications constituting the main part of this thesis. However, the same techniques can be employed, only differing in size of optics and target distance. Furthermore, the biological systems exhibit similarities with several fluorophores in common.

Measurements of reflected sunlight over land provide information about terrestrial vegetation cover and differentiation between plant species. In some cases the spectral information contained in reflectance measurements has also revealed stress due to water shortage or air and soil pollutants. Passive recordings of plant reflectance using sunlight as a light source has its obvious advantages and good results have been obtained with satellite systems such as the LANDSAT satellites [332]. Data from various systems for reflectance measurements have been used for several years to map the growth rate and decline in spruce trees, beech and other species in Europe and North America. However, the applications of these systems are limited in the important field of detecting forest decline.

8.1 Background

It has been known for more than a hundred years [333] that green plants exhibit fluorescence upon light excitation. The mechanism of chlorophyll fluorescence was studied in more detail by Kautsky who discovered that the fluorescence behaviour showed good correlation with the rate of photosynthesis [334,335]. Since then many reports have been published on this topic [336-339]. Using laser-induced fluorescence detailed knowledge about the physiology of green plants can be obtained. Besides basic studies, LIF finds applications in remote sensing of the status of green plants. In the early seventies systems for measurement of chlorophyll fluorescence using lasers were discussed by Hickman and Moore [340]. Early tests of laser-based systems for marine fluorescence recordings were performed by Hoge and Swift [341]. They found that chlorophyll fluorescence from phytoplankton was detectable from aircraft. The more complex fluorescence from terrestrial plants and grass has also been recorded by airborne systems [342,343]. Parallel to the development of airborne systems, the physiological significance of the fluorescence signals was demonstrated for various stress factors. Using a system based on the Fraunhofer line depth principle, increased chlorophyll fluorescence was found for lemon trees subject to water stress [344].

Several authors have established an inverse relationship between chlorophyll fluorescence and CO\(_2\) assimilation rate [345-347]. Moreover, variations in chlorophyll fluorescence have been reported due to elevated levels of SO\(_2\), NO, NO\(_2\), and O\(_3\) [346]. In some cases, SO\(_2\) was shown to stimulate plant growth resulting in a lowered fluorescence, whereas in other cases SO\(_2\) was found to inhibit plant growth leading to an increase in fluorescence. A natural variation in chlorophyll fluorescence due to leaf structure and growth site has also been described. Fluorescence dependence on various stress factors was presented in
an extensive review article [347]. Among natural fluorescence variation factors, autumnal senescence is the most obvious [348]. The fluorescence dependence on chlorophyll concentration has also been described [349]. Many authors have reported changes in other plant fluorophores, mainly in the blue spectral region, due to different stress factors [348,350-353]. The main fluorescence peak in the blue region was attributed to carotenoids, riboflavin, cinnamic acids, NADH or NADPH among several other substances. Also, the blue fluorescence has shown small variations for plants exposed to water stress, potassium deficiency or the herbicide DCMU [351,352].

The fluorescence lifetime for excited states of chlorophyll varies depending on the photosynthetic state. This research field was recently reviewed [354], and it was concluded that up to four decay components were necessary to describe the fluorescence decay. The fast and the slow components were attributed to pigments in the two photosystems. Different time dependences are reported for dark-adapted and light-exposed plants. Time-resolved spectroscopy was used in the blue spectral region to determine the origin of the blue and green fluorescence using synchrotron radiation as the excitation source [351]. The principle of differentiating stressed from healthy leaves using fluorescence decay measurements has so far not been reported for remote sensing systems.

8.2 Principles of plant fluorescence

Photosynthesis in green plants is based on a light-harvesting system that converts solar energy into chemical energy. After the absorption of a photon by a pigment molecule, the excitation energy is transferred in a series of Förster type energy transfer processes to a reaction centre where chemical reactions take over.

8.2.1 Structure of photosynthesis apparatus

On a sunny day the light flux reaches values corresponding to an excitation rate of less than 1 absorbed photon per second and per pigment molecule. The rate of the photochemical reactions and the following processes, on the other hand, is several orders of magnitude faster. In order to optimize the photosynthetic efficiency, the pigment molecules are arranged in antennas of several hundreds of pigment molecules connected to each reaction centre. The main photosynthetic pigments are chlorophyll-carotenoid complexes incorporated in the double layered internal membranes, the thylakoids, of special plant cell organelles. These organelles which possess a light-harvesting capacity are the so-called chloroplasts. The structure and function of the chloroplasts are different at different locations within the leaf and for different ambient light conditions. The chloroplasts exposed to high light levels, the sun-type chloroplasts, possess a highly efficient photosynthetic apparatus and a lower amount of light-harvesting pigments. The thylakoids are fewer and not so densely packed. The shade-type chloroplasts, on the other hand, have a higher level of pigments, they are organized in large antenna systems and the process of photosynthesis is less employed.

The chlorophyll-carotene proteins in the chloroplasts are the primary target for the photons. The major pigment is the chlorophyll molecule, a porphine ring structure with a long hydrocarbon residue and a magnesium atom at the centre position of the ring structure. The chain of conjugated π bonds roughly decides the absorption and fluorescence properties of the chlorophyll. In higher green plants there are two types of
chlorophyll, chlorophyll a and chlorophyll b. The absorption spectra of chlorophyll a and b are similar in shape with two major peaks, one in the red part and one in the blue part of the spectrum. For chlorophyll b the long-wavelength peak is slightly shifted towards shorter wavelengths. The fluorescence spectrum of chlorophyll a and b are also quite similar, chlorophyll a exhibiting a double peak structure at about 690 nm and 735 nm, while that for chlorophyll b is shifted about 20 nm further to the blue. In isolated chlorophyll this can easily be verified. For intact chloroplasts or whole leaves, however, only the chlorophyll a fluorescence can be detected. This is the result of the highly efficient energy transfer from chlorophyll b to chlorophyll a due to the overlap of the chlorophyll b fluorescence peak and the chlorophyll a absorption peak in addition to the organized structure of the pigments. The so-called accessory pigments, mainly the carotenoids, which absorb in the 400 to 500 nm region are also important for the light-harvesting process. The main carotenoids are β-carotene, lutein, violaxanthin and neoxanthin.

The light-harvesting chlorophyll/carotenoid complexes are bound to large protein structures called LHCP₁, LHCP₂ and LHCP, found in the thylakoids. Together they form the light-absorbing antennas which transport the excitons to the reaction centres by energy transfer. In order to function well the different pigments must have specific positions in the antennas, separated by a well defined distance and with a certain orientation in relation to each other. When this is the case, an efficient Förster type energy transfer can occur between neighbouring pigments. For each transfer event, however, some of the available energy is lost by competing processes in the form of heat. The pigments for the reaction centres are accordingly shifted somewhat towards longer wavelengths. There are two types of photochemical reaction centres, namely photosystem I (PS I) and photosystem II (PS II). The two photosystems act as catalysts for the electron transport from the splitting of a water molecule to the synthesis of NADPH which is used mainly for CO₂ assimilation and the formation of sugar.

8.2.2 Light reaction kinetics

If green plants are kept in darkness for time periods of more than 15 minutes, a sudden exposure to light will result in a time-dependent fluorescence behaviour termed Kautsky kinetics. In dark-adapted leaves the fluorescence upon low light excitation is thought to originate from the chlorophyll a proteins of the LHCP's, and from the pigments of PS II. At this stage, the electron acceptors of PS II, QA and QB, are fully oxidized. All reaction centres are then defined as open. As light is switched on the absorbed energy can start to migrate through the reaction centres, rapidly altering the balance between the competing processes. The total fluorescence can be expressed in terms of a rate equation:

\[
F = J \cdot \frac{k_F \cdot A}{k_f + k_r + k_T + k_p} + J \cdot \frac{k_F \cdot (1 - A)}{k_f + k_r + k_T},
\]

where \(k_F\), \(k_r\), \(k_T\) and \(k_p\) are the rate constants for radiative and non-radiative deexcitation, energy transfer and photochemical deexcitation, respectively. \(A\) is the fraction of open reaction centres and \(J\) is the absorption factor. The available photochemical energy will immediately lead to a reduction in the primary acceptor, QA, mediated by pheophytin a. Returning the excited state energy to the pigments of PS II when the reaction centres are closed, will result in an increase in fluorescence. The electrons are further transported via a secondary acceptor, QB, to plastoquinone pool (PQ). When QA, QB and PQ are completely reduced the fluorescence is at its maximum after approximately 0.5 s. As the PS I reaction
slowly starts to re-oxidize the acceptors, the fluorescence intensity will decrease to a constant level which reflects the photochemical equilibrium of the photosynthetically active chloroplasts. This equilibrium fluorescence level is higher than the fluorescence intensity of dark-adapted leaves but lower than the initial maximum.

8.3 Various aspects of chlorophyll fluorescence

8.3.1 Chlorophyll-dependent fluorescence ratio

For very low concentrations of chlorophyll pigments in green plants, the fluorescence intensity will be roughly proportional to the amount of chlorophyll in the leaves. However, for higher concentrations, corresponding to intact green leaves, a substantial proportion of the fluorescence will be reabsorbed by the pigments in the leaves. The most important absorbing pigment in the red region is the chlorophyll itself. Due to the partial overlap of the red absorption peak and the 690 nm fluorescence peak, the reabsorption of the 690 nm fluorescence becomes more pronounced with increasing amounts of chlorophyll in the leaves. The 735 nm fluorescence peak, on the other hand, is affected to a much less extent by chlorophyll reabsorption. Thus, the shape of the fluorescence spectrum will change with the amount of chlorophyll in the plants. This is shown in Fig. 8.1. for a green leaf and a senescent, low-chlorophyll-containing, beech leaf (Fagus Sylvatica). The amount of fluorescence emitted by the chlorophyll molecules in the chloroplasts is also dependent on the status of the photosynthesis. For chloroplasts with a reduced electron transport chain, more reaction centres will be closed during high light level conditions, resulting in an increased fluorescence. Together, this forms the basis of a very direct method of measuring the health status of green plants. If the fluorescence intensities at 690 nm and 735 nm, corresponding to the two chlorophyll peaks, are measured, the ratio of the two will be a measure of the chlorophyll concentration. Furthermore, being a dimensionless quantity, this ratio will be largely independent of target distance variations and variations in excitation energy, thus making $I(690)/I(735)$ suitable for remote sensing of forest decline. However, the $I(690)/I(735)$ ratio changes not only with the health status of the plant, but also with a number of naturally occurring conditions, as will be explained below. The absolute values of this ratio is therefore valid for specific measuring situations only, but can not be compared, for example, for different plant species.
8.3.2 Dependence of chlorophyll fluorescence on excitation wavelength

In a given situation, a suitable excitation wavelength must be chosen, taking into consideration the species to be analysed. If excitation wavelengths in the region around 450 nm or 650 nm are being used, the chlorophyll pigments are effectively excited. Around 450 nm the chlorophyll $a$ molecules themselves exhibit high absorption. For longer wavelengths the accessory pigments provide an effective absorption followed by energy transfer to chlorophyll $a$. In this way, most of the visible part of the spectrum is covered. However, the blue/green fluorophores are not excited by these long wavelengths. If, on the other hand, the UV and violet region is used for excitation, the blue fluorescence becomes stronger, and the information about the photosynthesis apparatus possibly obtained from the blue fluorescence can be utilized. Unfortunately, this will be at the expense of a much weaker chlorophyll fluorescence. Thus, the choice of excitation wavelength is a trade-off in order to gather as much information as possible from the full fluorescence spectra. The dependence on excitation wavelength is shown in Fig. 8.2 for $I_{685}/I_{530}$ and $I_{685}/I_{735}$ for beech leaves (Fagus Sylvatica). Note that the reduction in chlorophyll fluorescence ($I_{685}/I_{530}$) for short wavelengths is associated with an increase in the chlorophyll fluorescence ratio ($I_{685}/I_{735}$). The reason for this is probably that longer wavelengths penetrate deeper into the leaves thus increasing the importance of fluorescence reabsorption at 685 nm.

Another aspect which must be taken into consideration if the fluorescence equipment is to be used in an airborne system for forest decline research, is laser safety. The transparency of the human eye falls dramatically for wavelengths below 400 nm. For this reason the safety distance for lasers is about a factor of 30 times longer for wavelengths above 400 nm (or laser pulse energies 1000 times lower for equal distance). Bearing this in mind excitation wavelengths above 400 nm should not be used if laser safety regulations are to be followed.

8.3.3 Dependence of chlorophyll fluorescence on chloroplast location

As already mentioned above, there are two types of chloroplasts, sun-type and shade-type chloroplasts, the names indicating the position of the corresponding leaves in a tree. The structure of the sun leaves is different from that of the shade leaves in that the parenchyma
cells on the upper side of the sun leaves are greater in number and more densely packed. This will result in a lowered chlorophyll fluorescence. The higher amount of chlorophyll and the more effective photosynthesis of the sun leaves will also lead to a lower fluorescence and an decreased I690/I735 ratio. This effect is more or less obvious in different species, being more pronounced for e.g. *Fagus Sylvatica*.

Another aspect of the leaf structure is the difference between the upper side and the lower side of most leaves. The parenchyma cells of the upper side are densely packed in a palisade structure with a high number of chloroplasts, while the parenchyma cells of the lower side form a spongy structure with a small number of chloroplasts. In accordance with the previous discussion, this will result in a stronger fluorescence and a higher I690/I735 ratio for the lower side compared with the upper side.

Another important effect to bear in mind in field measurements is that of ambient light conditions. At a low light level, all reaction centres are open so most of the photon energy is used in the photosynthetic apparatus resulting in a low fluorescence yield. As more light falls on the leaves, more reaction centres are closed and the fluorescence will increase. At a certain light level a steady state is reached where the fluorescence is independent of the amount of light. This level is, of course, different for different plants but roughly corresponds to full sunlight during the midday hours. Thus, for the remote sensing of forest damage the steady-state condition gives the best information on the plant condition.

### 8.3.4 Dependence of chlorophyll fluorescence on environmental stress factors

The water content of green plants is a balance between the uptake of the root system and the transpiration through the pores of the leaves, the stomata. The opening of the stomata is regulated by the ambient light conditions, CO$_2$ partial pressure and water content within the cells. When the transpiration, for some reason, exceeds the water uptake a water stress condition will occur. In a low-stress situation the electron transport chain may be affected, resulting in an increase in the fluorescence. As the water stress is prolonged, the chloroplasts become more densely packed and the fluorescence will decrease again, however with a higher I690/I735 ratio. Therefore, the I690/I735 ratio seems to be an adequate indicator of water stress. This was shown, for example, by Lichtenthaler and Rinderle (1988) for *Fagus Sylvatica* and *Nicotiana* [347].

Other factors affecting the fluorescence spectrum of green plants have been reported, such as soil composition (Mg$^{2+}$, N$_2$, NaCl and others) [347,355] as well as air pollutants (O$_3$, SO$_2$) [346]. Generally, those environmental factors which inhibits plant growth also result in an increased fluorescence and an increase in the ratio I690/I735.

### 8.4 Blue fluorescence from plants

Apart from the red fluorescence from chlorophyll, the broad fluorescence of green plants can also be used for vegetation studies. Provided that the excitation wavelength is correctly chosen, both the blue and the red fluorescence can be recorded simultaneously. By using both red and blue fluorescence a dimensionless quantity may be formed. Such a fluorescence ratio is insensitive to variations in, for instance, excitation energy. Furthermore, the blue part of the spectrum may contain useful information on the photosynthetic status of green plants.
The origin of the blue fluorescence is still quite unclear, since most biomolecules exhibit some fluorescence in the blue spectral region. Among several fluorophores, carotenoids, riboflavin, cinnamic acids, coumarin and NADPH have been mentioned [348,350-353]. The carotenoids act mainly as accessory pigments, transferring absorbed photon energy to the chlorophyll. They can be divided into two groups, carotenes and xanthophylls. The carotenes exists in an α form and a β form. The fluorescence properties of β-carotene are not quite clear. Some groups have claimed that β-carotene exhibits some fluorescence in the region from 400 nm to 500 nm [Paper XIV, 324,325], whereas others claim that β-carotene is completely free from fluorescence in the visible region. The major xanthophylls in green plants are violaxanthin, lutein and neoxanthin. The role of the xanthophylls is probably to act as accessory pigments. β-carotene acts as an accessory pigment as well as protecting chlorophyll b from photo-oxidation.

In 1957 Duysens et al. reported on an emission band at 440 nm in photosynthetic cells, which they attributed to NADPH [350]. This was further supported by others through excitation and emission studies [352], as well as time-resolved fluorescence measurements [351]. NADP is an acceptor for the energy absorbed in chlorophyll a and may therefore be sensitive to the functioning of the electron transport chain. Thus, the fluorescence at 440 nm may provide useful information about the photosynthetic apparatus. This was shown in a study reporting a good correlation between the rate of photosynthesis and the blue fluorescence [352]. Riboflavin is another compound that have been considered as a major fluorophore in green plants [352]. During autumnal senescence the peak at 530 nm increases. The fluorescence peak of riboflavin is found at about 530 nm, where a fluorescence peak is observed for most green plants. Even brown leaves from late autumn give rise to a substantial fluorescence with a peak at about 450 nm [Paper XVI]. This may indicate that the cell wall, which contains cellulose, is important for the blue fluorescence [356].

In conclusion, it should be possible to use the blue/green fluorescence information in addition to red chlorophyll fluorescence in order to be able to better distinguish between different stress factors for green plants. The very complex variation in plant fluorescence with a number of environmental factors, as well as natural variations, will probably necessitate a more complex fluorescence criterion based on both red and blue fluorescence.

8.5 Kautsky kinetics

The onset of photosynthesis when a dark-adapted plant is suddenly exposed to light, is not an abrupt process but follows a complicated scheme referred to as the Kautsky kinetics. This behaviour of the fluorescence was first presented in 1931 by Kautsky [334]. The various features of the kinetic curve provide information on the vitality of the stages along the energy transport pathway. Therefore, measurement of the Kautsky kinetics provides a useful alternative to the fluorescence ratio I690/I735 in forest decline research.

8.5.1 Mechanisms

As light is switched on, there is an immediate rise in the chlorophyll fluorescence up to a level O or F0. This level is usually called constant or initial fluorescence and is thought to
originate mainly from pigments of PS II. The $F_o$ level is independent of photochemical events. There follows a fast rise proceeded by a slower decay of the fluorescence to a steady-state level. In contrast to $F_o$, this variable fluorescence, $F_v$, is dependent on the functioning of the photosynthetic apparatus. The rise from $F_o$ via an inflection point and a dip up to the maximum level, $P$, takes 0.3 s. From $P$ there is a decrease to a plateau, possibly a second maximum, down to the terminal level after about 3 minutes. The second maximum may sometimes be missing, or there may be more than one second maximum. The biochemical interpretation of the rise from $F_o$ to $P$ can be understood as the state of the electron acceptors $Q_A$ and $Q_B$. Provided that the leaf is dark-adapted, which means that $Q_A$ and $Q_B$ are fully oxidized, the photon energy is used for the reduction of $Q_A$, $Q_B$ and possibly the PQ pool. As the PQ pool becomes completely reduced the reaction centres close, corresponding to a fluorescence maximum $P$. Gradually, the activity of PS I increases, causing a re-oxidation of the PQ pool and $Q_A$ and $Q_B$. This will result in a decline of the fluorescence level down to the terminal level where an equilibrium is reached. The fluorescence is now partially quenched by the re-oxidation of $Q_A$ and a build-up of a pH gradient across the thylakoid membrane.

8.5.2 Analysis of Kautsky kinetics
The Kautsky kinetics of green plants can be analysed in several different ways. First, the variable fluorescence, $F_v$, can be measured utilizing the fast fluorescence rise. This is often used to form a ratio $F_v/F_o$ indicating the relative amount of fluorescence arising from the photosynthetically active part of the plant. High values of $F_v/F_o$ indicate that photosynthesis is functioning well. This method has the advantage of being fast although it is sometimes difficult to identify the level of $F_o$. Secondly, the slow fluorescence decrease after the maximum $P$ can be used to form the ratio $Rfd = f_d/f_s$, where $f_d$ is the fluorescence decrease from $P$ to the terminal level $f_s$. The $Rfd$ value is sometimes termed the vitality index as it is a measure of the vitality of the photosynthesis. Generally, $Rfd$ values of about 3 are typical for healthy plants while lower values are found for damaged plants. In contrast to the fluorescence rise ratio, the $Rfd$ values include the equilibrium state of the electron transport, which is dependent on the consecutive stages in the photosynthesis, such as CO$_2$ assimilation. It may therefore be advantageous, compared with $F_v/F_o$. On the other hand, $Rfd$ measurements require at least 3 minutes time which is longer than the time required for $F_v/F_o$ measurements. There are other evaluation methods, such as the adaption index, which is based on $Rfd$ values at 690 nm and 735 nm.

8.5.3 Applications in the field
As in the case of the fluorescence ratio 1690/1735, the above described methods are influenced by environmental stress factors as well as variations due to measurement topology. Thus, the $F_v/F_o$ and $Rfd$ values are higher for sun leaves compared with shade leaves. Autumnal senescence will result in lower values of $F_v/F_o$ and $Rfd$ ratios. Water stress also gives lower values of $F_v/F_o$ and $Rfd$. The $F_v/F_o$ and $Rfd$ values also show variations depending on the naturally varying light conditions during the a daily cycle. In general, stress factors which tend to slow down the rate of photosynthesis also show lower $F_v/F_o$ and $Rfd$ values.

When discussing the applicability of the fluorescence criteria based on Kautsky kinetics, one must remember that these methods require seconds or minutes to execute. This may limit the applicability of such methods. One very important application of plant
fluorescence is the use of airborne systems for forest decline research. It would be very difficult to perform airborne recordings of the Kautsky kinetics since the recording time will be very long especially in the case of Rfd values. The ratio I690/I735 is probably a much better choice for airborne systems since it can be recorded in one laser shot (~10 ns) also in an imaging mode. However, the Kautsky kinetics are of great value for ground truth measurements as a fast alternative to direct measurements of e.g. CO₂ assimilation.

8.6 Fluorescence lifetimes of chlorophyll systems

8.6.1 Fluorescence decay
If the light used for the excitation of fluorescence in green plants is a very short pulse or train of pulses, the rate of the fluorescence decay may be recorded. The fluorescence decay time, τ, for chlorophyll is in the range of a few ns in solution but much faster in vivo. The research in this field has been very intense for a number of years, aimed at a basic understanding of the various steps in photosynthesis, rather than direct applications in field measurements. It should be noted that the fluorescence decay discussed here means the lifetime of the excited state of the chlorophyll molecules, and has nothing to do with Kautsky kinetics, although they are both connected with the electron transport mechanism. The fluorescence decay is not homogeneous but consists of at least three components [354]. When trying to assign the different decay components to the various pigment pools one must consider the two cases of open and closed reaction centres. In the case of open reaction centres at least four decay components (70-100 ps, 180-300 ps, 500-600 ps and 2-3 ns) are found. For closed reaction centres three decay components (70-100 ps, 1.2-1.4 ns and 2.2-2.4 ns) appear to be enough to describe the fluorescence decay. There is agreement between researchers that the fast component may be attributed to PS I and the slower ones to PS II.

8.6.2 Fluorescence lifetime measurements for remote sensing
The sensitivity of fluorescence decay to environmental stress factors has been discussed regarding use in the remote sensing of green plants. It is clear that this is a very complicated problem. Firstly, measurements in the picosecond region require sophisticated equipment (described in Section 5.6). It is doubtful that such equipment can be operated accurately in an airborne system. Secondly, until now such techniques have only been available for point measurements. Therefore, scanning techniques must be utilized for the recording of full images. This is, of course both time-consuming and difficult. Furthermore, there is a problem of a more fundamental character in the mixing of true decay time and light propagation in a canopy. In other words, a slower fluorescence decay may always be replaced by a longer effective light path within the target. This problem has been addressed by Niemann et al. that have modelled the time behaviour for elastic scattering in a canopy. If the time response of a short light pulse scattered in the canopy is known, then the fluorescence decay may be calculated. The procedure is in close analogy with the case of fluorescence decay recordings from human tissue, where the apparatus function must be recorded using the scattered light (see Section 5.6). In this case, decay times of a factor of ten times shorter compared with the apparatus may optimally be detected. Since the time scale for light scattering in a whole tree would be in the region of tens of ns, a time resolution of fluorescence decay of a few ns may be achieved at best. However, this is not accurate enough for fluorescence decay measurements of chlorophyll in vivo which makes this concept appear impractical. For smaller plants, such as maize
plants, the temporal behaviour of the light scattering is less complicated and the technique may work.

8.7 Instrumentation

8.7.1 Laboratory equipment
Some of the experimental arrangements described in Chapter 5 are very well suited for measurements of laser-induced fluorescence from green plants. Photomultipliers equipped with interference filters or attached to scanning spectrometers provide very sensitive systems at a low cost. In combination with boxcar integrators or transient digitizers, a time resolution sufficient for time-resolved spectroscopy can be obtained. Diode arrays and one- and two-dimensional CCD detectors have the advantage of recording the whole fluorescence spectrum in a single laser shot. This provides the possibility of studying fluorescence kinetics simultaneously over the whole spectral region. For time-resolved spectroscopy, synchrotron radiation utilizing the single-photon counting technique has been employed. Similar measurements have been performed with picosecond lasers and similar detection units. Also, streak cameras with ps time resolution can be used in connection with a powerful YAG laser-pumped dye laser. In the latter case, however, the high peak powers might induce photosynthetic reactions in the vegetation being studied.

8.7.2 Portable field systems
Portable systems for fluorescence recording have the restriction that they must not be heavy, but need to be equipped with a long-lasting battery. For these reasons HeNe or N\textsubscript{2} lasers are useful alternatives. In the near future, diode lasers operating near 600 nm seem very promising. Two similar systems based on a HeNe laser have been used recently and useful results reported. One system employs two photomultipliers with interference filters transmitting at 685 nm and 730 nm [357]. Dichroic mirrors are used to increase the efficiency of the system. A fibre probe for transmission and reception through a single fibre has been constructed. It consists of a chamber with a window which transmits actinic ambient light in the blue-green region while red ambient light is blocked out in order not to interfere with the induced fluorescence. The signals are recorded in a micro-processor and are stored in a RAM. Upon completion of the measurements, the data are transferred through a serial interface to a 386 lap-top computer for post-processing. The spectral ratios 1685/1730 as well as Kautsky kinetics can be displayed on a LC display. A second system utilizes two photo diodes with cut-off filters and interference filters matching the two peaks at 690 nm and 730 nm [347]. The fibre probe is a split-fibre system with separate branches for the excitation light and the two fluorescence channels. Both spectral ratios and kinetics may also be presented with this system. In yet another system called the PAM fluorometer, [358], the characteristics of Kautsky kinetics are easily obtained. The \( F_0 \) ground fluorescence is measured using 1 \( \mu \)s excitation pulses at 1.6 kHz well below the threshold for inducing photosynthetic reactions. When \( F_0 \) is recorded a 1 \( \mu \)s saturation pulse is applied, which results in the \( F_{max} \) fluorescence peak. After relaxation to \( F_0 \), actinic light is switched on and saturation pulses are delivered every 10 seconds. This will result in a Kautsky kinetics fluorescence curve with fluorescence peaks overlaid on the curve corresponding to the saturation pulses. The height of these modulation peaks is proportional to the amount of photochemical quenching, while the non-photochemical quenching can be obtained from the \( F_{max} \) level in relation to the height of the modulation
peaks. Hence, the PAM fluorometer has the advantage of identifying the photochemical fluorescence quenching that is related to the photosynthetic capacity.

8.7.3 Mobile remote sensing systems
The important features of fluorosensor systems designed for larger measuring distances are a powerful light source, a large-diameter receiving telescope and a sensitive detection system. Excimer lasers have been used to pump dye lasers to produce high light powers at wavelengths near 470 nm where chlorophyll exhibits high absorption. By using other dyes, a large optical range can be covered. Furthermore, excimer lasers are pulsed with pulse lengths of about 10 ns, which makes gated detection possible. Pulsed lasers and gated detection are definitely required for remote sensing in order to suppress the strong sunlight interfering with fluorescence measurements. The construction of a small sealed-off 100 ml excimer laser system emitting light at 308 nm suitable for airborne operation has been described [359]. This excimer laser can run without gas exchange for several hours. The excimer laser was used to pump a dye laser to induce fluorescence in remote beech trees. The fluorescence was collected with a Newtonian telescope and focused onto the tip of an optical fibre connected to a spectrometer. The detector was the same OMA system as that described in Chapter 5 including an image-intensified diode array detector.

In another system, a frequency-tripled Nd:YAG laser was used to produce 10 ns light pulses at up to 200 mJ at 355 nm [Paper XV]. The 355 nm light was used directly or Raman shifted in a 1 m Raman cell containing deuterium. At the output window, a Pellin Broca prism was used to separate the first Stokes component at 397 nm from the fundamental. Alternatively, second, third or fourth Stokes or first anti-Stokes components could be chosen. In this way, a large wavelength region was covered. This system employs a Newtonian telescope with a receiving mirror on the roof of the system van. The telescope is computer-controlled and has a TV monitor for target selection. An optical fibre was placed in the focal plane of the telescope to direct the fluorescence light onto the entrance slit of a 27 cm spectrometer. Scattered laser light was blocked out by cut-off filters. This system employs the same OMA detection unit as the system described above. Spectra are stored on floppy disks and are corrected for the optical sensitivity profile of the detection system. The detection system was recently modified to include imaging capability. A second, Cassegrainian telescope was placed with its object plane coinciding with the image plane of the receiving Newtonian telescope. The first mirror of the Cassegrainian telescope was split into four segments producing four identical images. Different interference filters or coloured glass filters were placed in front of each segment matching the chlorophyll fluorescence peaks as well as the blue and green fluorescence. In this way, four similar images at different wavelengths were produced. The images were recorded with an image-intensified CCD detector and a computer-processed image could be calculated corresponding to, for instance, the ratio I(685)/I(740), i.e. an image in false colour showing the spatial distribution of the ratio of the two chlorophyll peaks for a target area. By inserting a flip-in mirror the image could instead be formed on a white screen with a hole where the optical fibre to the OMA system was placed. In this way fluorescence spectra were recorded for different locations in the images for comparison of the values of the fluorescence ratio. Examples of fluorescence images of two maple leaves are shown in Fig. 8.3. Below, the fluorescence spectra from the two leaves obtained with the OMA system, are included.
Fig. 8.3 Remote multi-colour fluorescence imaging of two leaves, a fully green (large leaf) and a slightly senescent leaf (small leaf) from a maple (Acer Platanoides) recorded at 690, 740 and 450 nm. A processed image displaying the ratio $I(690)/I(740)$ is shown in the fourth image quadrant. Remote fluorescence spectra from the two leaves are shown below. The excitation wavelength was 355 nm and the target distance was about 50 m.
Another approach that has been used for a number of years, is to use both the reflected sun-light and the sun-light-induced fluorescence at the same time. This is called the Fraunhofer line-depth principle and is based on the fact that the Fraunhofer Hα line at 656.3 nm is partially filled in by fluorescence from chlorophyll. Commercial systems are available which utilize this technique. In short, a detection system is placed on a steel frame above the ground facing an object. The diffuse reflected sunlight from a white screen as well as the object plant is recorded in two optical bands. Both bands are centred at 656.28 nm but one is narrow and the other is broader (=1 nm). The fluorescence can be calculated according to the formula: \[ F = d - \frac{a(d-c)}{a-b}, \] where \( a \) and \( d \) are broadband emission and \( b \) and \( c \) are narrowband emission from the screen and target, respectively. This method has the disadvantage that the fluorescence is measured at 656 nm only while the chlorophyll-concentration-dependent ratio of the two chlorophyll peaks can not be displayed.

8.7.4 Airborne systems

Marine airborne systems have been in operation for several years for the detection of chlorophyll fluorescence from marine phytoplankton. The spectral shape of the fluorescence from marine phytoplankton is similar to that from terrestrial plants and also originates from chlorophyll \( \alpha \) molecules. Additionally, a marine fluorescence spectrum contains water fluorescence, peaking at about 450 nm, and composite fluorescence from several organic compounds found in water referred to as "Gelbstoff". Apart from fluorescence the spectra also contain a sharp Raman peak corresponding to a Stokes shift of the excitation laser with the energy of the OH stretch vibration of water molecules. Since the Raman signal is proportional to the probed water volume, it can be used as a reference for the chlorophyll fluorescence.

In the 1980's, equipment for marine monitoring was modified for use on terrestrial vegetation. One of these systems was based on an excimer-laser-pumped dye laser emitting at 450 nm for the highest chlorophyll fluorescence yield [343]. The telescope was a 40 cm diameter Schmidt-Cassegrainian telescope. Dichroic mirrors and interference filters with bandwidths of 10 nm were selected to match the fluorescence peaks of green plants at 690 nm, 735 nm as well as the scattered laser light at 450 nm. Three fast photomultipliers with gating electronics were used as detectors and the respective signals were connected to a transient digitizer. The time resolution of the system was set by the laser pulse width to about 10 ns. With this system fluorescence signals from grass and trees were recorded at each laser shot for a height of 700 feet.

Several other groups are presently constructing airborne systems based on the various equipment used in remote fluorescence measurements on the ground. One important aspect which has to be considered when adapting ground systems to airborne systems is the necessity of live recordings. When flying over vegetation, data integration over several laser pulses for each point is impossible, due to the movement of the aircraft. Also using a helicopter, integration for a particular spot would be very difficult and would require some kind of target identification system. Therefore, much work will have to be done on evaluation techniques for weak signals. Especially for imaging systems, very weak signals can be expected and the use of different smoothing and averaging profiles necessary. Imaging techniques may, however, have an advantage over scanning techniques. If a false colour image showing, for instance, the ratio \( \frac{I(685)}{I(740)} \) is updated on a computer...
screen at a rate of several Hz, the human brain is capable of performing a time averaging. The human brain has the excellent capability of recognising a faint object within a noisy image and following its movements over the screen, something that even the best pattern recognition programs can not yet do. The advantage of time averaging may be more important than the signal-to-noise advantage of point detectors, such as photomultipliers of scanning systems.
9. Summary of papers

Papers I-VII deal with laser-induced fluorescence for malignant-tumour diagnosis. In the experiments described in Papers I and II, fluorescence spectra were recorded from various tumour-selective chromophores in solution and from injected animals. Paper II was prepared four years after Paper I and contains a comparison with the earlier results. Photofrin II was concluded to exhibit the highest tumour-to-normal contrast ratio of the chromophores studied. The system utilizing recordings of laser-induced fluorescence spectra in all seventeen papers, is presented in Paper III. The system was designed for clinical use with a plexiglass covered small nitrogen-laser-pumped dye laser as an excitation source, optics and a multichannel detection system, fitted onto a trolley for high system mobility. The image-intensified diode array detector was sensitive enough for all the measurements. Fluorescence spectra were recorded from lung tumours during bronchoscopy, from astrocytomas during brain surgery and from human tonsil tumours. High flexibility was obtained by utilizing a single optical fibre for both excitation and fluorescence light. In addition, the system was used for the detection of urinary bladder carcinoma during cystoscopy following low dose injection of Photofrin, as described in Paper IV. A surprisingly high tumour demarcation using pure autofluorescence, was found. In Paper V, a larger study on fluorescence detection of astrocytoma, is presented. The autofluorescence was considered not to be sufficient for accurate tumour demarcation. Paper VI and VII report on a multi-colour imaging system. The system contains a split-mirror Cassegrainian telescope with optical filters and an image-intensified CCD camera. Four simultaneous fluorescence images of an object can be recorded at selected wavelengths and processed to generate one false-colour image showing pixel values calculated according to a tumour demarcation criterion. The simultaneous recording at several wavelengths facilitates an enhanced demarcation without image blur from movements of the objects.

Papers VIII & IX report on photodynamic therapy of malignant tumours. In the study reported on in Paper VIII, Photofrin II was used to treat a case of basal cell carcinoma and a case of recurrent breast carcinoma. In the latter case light-induced hyperthermia was administered in addition to the photodynamic treatment to achieve a deeper therapeutic action. This work constitutes the first PDT in Scandinavia. In Paper IX, ALA was utilized on 94 skin tumours of different types in 27 patients. The excellent results were mainly explained by the high selectivity of ALA to malignant skin tumours.

Papers X-XIV describe results from measurements on excised pieces of atherosclerotic blood vessel using UV excitation. A classification scheme of four degrees of atherosclerotic plaque is presented in Paper X. The main difference between plaque and normal tissue seems to be an enhanced fluorescence at wavelengths below 400 nm relative to the fluorescence at about 450 nm. In Papers XI-XIII time-resolved fluorescence is introduced for plaque diagnosis. The first report on this topic, Paper XI, reveals that the fluorescence decay for plaque is longer than that for normal vessels. In Paper XII the system used for these recordings, which utilizes the single-photon counting technique, is presented. Furthermore, decay curves are analysed and separated into the various decay
components assuming a multi-exponential decay. Fluorescence data, including mean values and standard deviations, are given for samples from the different classification groups. It was concluded that the main difference between plaque and normal tissue is the higher proportion of the slow decay component in plaque fluorescence. In addition, time-resolved data from tumour-bearing animals injected with Photofrin II, are presented. Paper XIII is an invited review paper, which, however contains some original material. The importance of interference with blood in the spectroscopic data is discussed and two methods of circumventing this problem are presented, including measurements utilizing photomultipliers and a boxcar integrator. In Paper XIV both spectral and temporal methods are utilized in an investigation on pure fluorophores as well as excised pieces of blood vessels. It could be shown, that the spectral as well as temporal differences between plaque and normal tissue could be correlated with a higher amount of collagen fibres in atherosclerotic plaque. Furthermore, a fluorescence peak at 520 nm may be attributed to the weakly fluorescent β-carotene.

Papers XV-XVII deal with forest decline research utilizing remote sensing fluorescence techniques, which at first sight seems to be a very different topic from the main part of this thesis. However, conceptually and technically the two fields have very much in common, the main difference being the size of the lasers and the optics. Paper XV gives a description of for the integrated set-up, which includes the medical point-monitoring and imaging systems and a remote sensing LIDAR system for atmospheric measurements. The resulting system is very powerful utilizing a Nd:YAG laser excitation source and a 40 cm diameter telescope, capable of multi-colour fluorescence imaging of 1 m diameter plants at a distance of up to about 100 m. Fluorescence spectra can be obtained from selected areas within the imaging area. The system is hosted in a van for field measurements. Papers XVI and XVII preceded Paper XV and contain spectral data only, recorded during a field campaign in Tuscany, Italy. Paper XVI reports on different trees, while Paper XVII deals with remote fluorescence/Raman measurements on the water of the Arno river.

* * *

The list of authors of the papers presented is alphabetical and does not reflect the contribution of each individual. My own contributions to the papers are as follows:

Contribution to laboratory work, including set-up of equipment and measurements, in Papers I-XVII. Evaluation of data in Papers I,V,XI,XII and XIV-XVII and partly in Paper VII. Preparation of figures and tables in Papers I,II,IV,V,XI,XII and XIV-XVII and partly in Papers VII and XIII. Writing of manuscript for Papers I,XII,XIV,XV and partly for Paper XI.
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