Chapter 14. Photosensitizers and PDT

Rinaldo Cubeddu,[1,2] Paola Taroni[1,2] and Gianluca Valentini[1]

[1]Dipartimento di Fisica, Politecnico di Milano, piazza Leonardo da Vinci 32, 20133 Milan, Italy

[2]Istituto di Fotonica e Nanotecnologie, Consiglio Nazionale delle Ricerche, piazza Leonardo da Vinci 32, 20133 Milan, Italy

14.1. Photosensitization and photosensitizers

14.1.1 What is photosensitization and how it proceeds

Photosensitization is a response to light that is mediated by a light-absorbing molecule, called a 'photosensitizer', which is not the final target and is not destroyed during the process. When it absorbs a photon, the photosensitizer alters another molecule, called 'substrate' or 'acceptor', that can be part of a chemical system or a living organism. Both photosensitizer and light are required for photosensitization to occur, while generally light levels and sensitizer nature and concentration are such that individually they have no main effect on the system or organism.

Insert Fig. 1 here

The first step of the process is absorption of light by a photosensitizer P to produce an excited state (P*). In the presence of oxygen, P* undergoes internal reactions that ultimately

result in the chemical alteration of the substrate S. As shown in **Fig. 14.1**, P* can either react directly with the substrate (***Type I reaction***) or with oxygen (***Type II reaction***).

Type I reactions result in either hydrogen atom or electron transfer, yielding radicals or radical ions, with the excited sensitizer generally acting as an oxidant. The radical species that are formed are often highly reactive, such as superoxide and hydroxyl radicals.

Type II reactions mainly lead to excited state singlet oxygen ($^1O_2$). Singlet oxygen then reacts with the substrate to generate oxidized products. For most photosensitizers, the excited singlet state has very short lifetime and rapidly undergoes intersystem crossing, that is a transition to a slightly lower energy level involving an electron spin flip. The spin flip leads to a triplet energy state. Transitions between triplet and singlet energy states are forbidden. Consequently the excited triplet state generally has a longer lifetime than an excited singlet state, and hence is more likely to enter into an energy transfer reaction. Singlet oxygen is a highly reactive oxygen species that has an excited state lifetime of a few microseconds in most biological environments.

Type II (singlet oxygen) processes are favored over Type I (radical) processes by lower substrate concentration and higher oxygen concentration. Most important photosensitization reactions, either spontaneous or induced ones, occur via Type II processes.

It is also worth noting that oxygen is an important reactant in most photosensitization reactions, whether it is part of the initial reaction with the excited photosensitizer (Type II), or gets involved later, transforming the initial radicals into oxidized products (Type I).

The ***PhotoDynamic Therapy (PDT)*** is a form of treatment that requires oxygen for its therapeutic effect. This implies that in PDT a photosensitizer, light and oxygen are all required. The Type II reaction pathway is expected to be the main one, but Type I reactions

involving oxygen may also be implied. PDT has been investigated and developed mostly for the treatment of cancer and precancereous lesions (actinic keratosis). However, various other applications are under investigation (*e.g.* the treatment of age-related macular degeneration). More details can be found in the following (Subsection 14.1.5).

14.1.2   *Basic properties of photosensitizers*

Let us now examine more in detail excitation and de-excitation processes of a generic molecule to highlight properties that are key for an efficient photosensitizer.

Insert Fig. 2 here

Molecules that absorb light and are excited to the first singlet state subsequently lose the acquired energy through either radiative or radiationless decay (Fig. 14.2). When the higher vibrational levels of the lower electronic state overlap the lower vibrational levels of the higher electronic state (i.e. the nuclear configurations and energies of the two electronic states are identical), vibrational coupling occurs, and crossover from a higher to a lower excited singlet state is possible. This is a very fast process (10-12 s), known as internal conversion.

If the vibrational levels of the two electronic states are separated, a radiative transition (*fluorescence*) may also occur from the lowest vibrational level of the higher electronic state to any vibrational levels of the lower electronic state. Emission takes place from thermally equilibrated excited states, namely from the lowest vibrational level of the lowest excited singlet state, because relaxation from the excited vibrational levels (through emission of infrared quanta or as kinetic energy lost during collisions) is much faster than emission (10^{-14}–10^{-12} s *vs* $\approx 10^{-9}$-10^{-8} s). The transition may lead to any vibrational levels of the ground state because, even though the lifetime of the fluorescent molecule is of the order of 10^{-9}-10^{-8} s, the actual electronic transition occurs much faster (10^{-15} s), so that vibrational relaxation

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cannot take place during the transition. Thus, the emission spectrum generally consists in a broad band, not a narrow line. Moreover, for biomolecules, the emitted photons have most often frequency in the ultraviolet (UV) or visible range. Following radiative transition, the molecule undergoes vibrational relaxation to the lowest vibrational level (10^{-14}–10^{-12} s). Which one of the two deactivation mechanisms is favored depends on the number of vibrational levels and the difference in energy between the vibrational levels of the two electronic states. The closer they are, the more likely internal conversion will occur. This is the reason why molecules with no rigid skeleton, such as aliphatic molecules, rarely exhibit fluorescence, while aromatic molecules, with their rigid ring structures are usually characterized by strong fluorescent emission.

For the same reason, fluorescence occurs almost always only as a deactivation mechanism between the first excited singlet state and the ground state. Higher excited states are generally much closer to each other. Hence, internal conversion is favored and precludes the possibility of fluorescence emission. Consequently, different from the absorption spectrum, the emission line shape is made of just one band. The presence of more than one band in the spectrum of isolated molecules indicates that more than one species is fluorescing. This does not necessarily mean that the emitting molecules have different chemical structure. Actually, when the emitting molecules interact with a complex environment or aggregate, distinct fluorescence bands can be observed, corresponding to different binding sites, states of aggregation, etc.

A feature that is commonly used to characterize each deactivation process is its **_quantum yield_**. Let us for example consider deactivation by fluorescent emission. The _quantum yield of fluorescence_ ϕ_f is the fraction of excited molecules that fluoresce. It can be expressed as:

$$\phi_f = \frac{k_f}{k_f + \sum k_d} = \frac{\tau_f}{\tau_f^o}$$

(1)

where: k_f is the *rate constant for fluorescence* (*i.e.* the probability that the excited molecule will fluoresce); $\sum k_d$ is the sum of the rate constants for all radiationless deactivation mechanisms of the first excited singlet state; $\tau_f = (k_f + \sum k_d)^{-1}$ is the **lifetime** of the first excited singlet state, that is the average time the molecule spends in the excited state; $\tau_f^o = (k_f)^{-1}$ is the *radiative lifetime*, that is the average time the molecule would spend in the excited state if fluorescence were the only deactivation mechanism.

As described previously, when molecules are in the lowest vibrational state of the first excited singlet state, de-excitation may also occur via *intersystem crossing*, which is a transition to a slightly lower energy level involving an electron spin flip. The spin flip leads to a triplet energy state. Transitions between triplet and singlet energy states are forbidden, and consequently the excited triplet state generally has a longer lifetime than an excited singlet state, and hence is more likely to enter into an energy transfer reaction. However, de-excitation of the triplet state may also occur through long-living ($\geq 10^{-3}$ s) photon emission (**phosphorescence**).

Photosensitizers are molecules that efficiently transfer an electron to or from another molecule, or transfer their excitation energy to other molecules (typically molecular oxygen). In most cases, the reason why some molecules are so effective at electron transfer and/or energy transfer is that they very efficiently populate their excited triplet states. The relatively longer lifetime of the triplet state allows more time for energy and/or electron transfer to occur. So, most highly effective photosensitizers are characterized by high quantum yields for

the production of their excited triplet state, which is a competing mechanism against radiative decay (fluorescence).

The ***action spectrum*** is often used to quantify the efficiency of a photosensitizer to produce a specific effect (*e.g.*, cell killing). An action spectrum shows the dependence of the measured effect on the excitation wavelength. As an example, the action spectrum for the photochemical inactivation of cells can be found by measuring the dose of light necessary for inactivating the same cell fraction at different wavelengths, and plotting the results as required dose *vs* excitation wavelength.

14.1.3 *Photosensitizer interactions with biological tissues*

Ground-state molecular oxygen has low reactivity and diffuses rapidly through most biological environments, including cell membranes, while singlet oxygen reacts with several biological substrates. Efficient interaction occurs with certain amino acids in proteins (*e.g.*, tryptophan, tyrosine, histidine), DNA and RNA (guanine bases), as well as a variety of unsaturated lipids (including cholesterol and unsaturated fatty acids). On the contrary, no significant effect is produced on carbohydrates. This reactivity limits the ability of singlet oxygen to diffuse great distances. Thus, Type II photosensitization can affect biological substrates only at a moderate distance, up to fractions of micrometers, from the photosensitizer itself.

In turn, hydroxyl radicals generated by Type I reactions, are so reactive that they typically interact with the first molecule they get close to, limiting their effects to the site where they are generated.

Therefore, for both types of reactions, the photosensitizer localization is of key importance to determine and control biological effects. Some selectivity towards the environment can

inherently be provided by the sensitizer itself. For example, if it is highly soluble in lipids, it will likely tend to localize in the cell membrane rather than in its cytoplasm. However, stronger and more selective targeting can be achieved attaching the sensitizer to a target-specific molecule.

The heat released during photosensitization processes may also be used to cause selective damage, in a process that is named photothermal sensitization.

14.1.4 Types of photosensitizers

Photosensitizers can either be naturally present in living systems (*i.e.*, endogenous) or generated by an external source and administered to the living system (*i.e.*, exogenous).

Endogenous photosensitizers include molecules such as porphyrins or chlorophyll. Under native conditions, their potential photosensitizing effects are not apparent, either because their concentrations are too low, or because they form complexes that inhibit photosensitization reactions. *Exogenous photosensitizers* include many varieties of dyes and biomolecules. Some are natural products (*e.g.*, from plants), while others are synthesized for different applications (*e.g.*, medical or agricultural ones).

A third interesting type of photosensitizers also exists, namely *exogenously induced-endogenous photosensitizers*. These photosensitizers are intermediate between endogenous and exogenous ones. A precursor is administered, which is not active. The metabolic activity of the receiving organism then transforms it into a fully functional photosensitizer.

Porphyrin-based photosensitizers

Hundreds of different compounds act as photosensitizers for biological systems. They are often classified based on their chemical structure.

Porphyrin-based photosensitizers deserve particular attention as they include important endogenous sensitizers, as well as exogenously induced endogenous sensitizers and exogenous sensitizers that are approved for clinical PDT and/or for fluorescence-based cancer detection (Berg et al 2005, Sternberg and Dolphin 1998).

Insert Fig. 3 here

Porphyrins contain four pyrrole subunits linked by methine bridges (**Fig. 14.3**). Tetrapyrroles are naturally occurring pigments, involved in many biological processes. They include the metallopigments heme (the prosthetic group of proteins like hemoglobin), vitamin B12, and chlorophyll. All these compounds allow coordination of different metals at the ring centre. While the presence of the metal prevents photosensitization, its removal yields efficient photosensitizers. Most efficient porphyrin-based photosensitizers generally lack coordinated metal ions. On the other hand, several metallophotosensitizers for clinical purposes have been developed along the years, balancing opposing needs. In most cases, they are less effective than they would be in the absence of metal ions; however they have other properties of basic importance for clinical applications, like improved solubility and stability.

Except for methylene blue, all clinically-approved photosensitizers used in PDT are porphyrin-based sensitizers, with substituents in the peripheral positions of the pyrrole rings, on the methine bridges that link the pyrrols, and/or with coordinated metals. These derivates are synthesized to influence the water/lipid solubility, amphiphilicity, and stability of the compounds, which in turn determine their biodistribution and pharmacokinetics.

Hematoporphyrin was the first photosensitizer applied in humans. In the early 1960s, attempts to purify it led to Hematoporphyrin Derivative (HpD), a mixture of monomers and oligomers. A more purified version (Photofrin®) was then produced and commercialized. The idea was to enrich the solution in oligomers that were recognized as the tumor-localizing

fraction of the porphyrin mixture. HpD and Photofrin are far from being ideal photosensitizers for use in PDT. Their composition is not accurately reproducible. They have long half-life in the body, leading to undesired skin and eye photosensitivity for 4-6 weeks after injection. Even more important limitations are due to their non-ideal spectral properties. Their absorption spectrum consists of a strong absorption Soret band in the UV (350-400nm) and four much weaker Q-bands in the visible range. Tissue attenuation is strong at short wavelengths (UV and blue-green), mostly due to melanin and hemoglobin. Thus, the longest wavelength (red) Q-band is exploited for PDT when deep tissues need to be reached with enough light for an effective treatment, even though this provides weak photosensitizer absorption. Notwithstanding all these limitations, porphyrin derivatives are approved for a wide range of clinical applications and a large number of patients have been treated up to now.

It is interesting to note that, for diagnostic purposes, shorter wavelengths (within the Soret band) have often been applied, favoring efficient excitation at the expenses of deep tissue penetration. This choice is particularly suitable when surface targets are investigated, as in the case of skin pathologies or mucosal lesions that can be reached endoscopically.

Increased absorption in the red-infrared range can be achieved by reducing one (chlorines) or two (bacteriochlorins) double bonds in the conjugated ring structure. Specifically, meso-tetra(hydroxyphenyl)chlorin (m-THPC, Temoporfin, Foscan®) is a chlorine that has been approved for the clinical treatment of head and neck cancer.

The possibility to develop new photosensitizers with higher absorption at long wavelengths is actively being explored. However, it has to be taken into account that an upper wavelength limit exists around 850-900 nm, due to the minimum energy photons need to absorb to induce singlet oxygen formation. Moreover, due to water absorption, tissue attenuation above 900

nm raises considerably. Thus, the therapeutic window for effective *in vivo* treatments extends approximately from 600 to 800 nm.

14.1.4.1. Non porphyrin-based photosensitizers

A variety of endogenous and exogenous sensitizers with non porphyrin structure exists (Wainwright 1996). Just few examples are mentioned here, as they are much less investigated and used for PDT and fluorescence detection of pathologic lesions than porphyrins and related compounds.

Cyanines are synthetic dyes, originally developed to extend the sensitivity range of photographic emulsions. *Phthalocyanines*, in particular, are macrocyclic compounds, featuring four pyrrole-like subunits, similar to porphyrins (**Fig. 14.3**). They have intense blue-green color (corresponding to strong absorption in the red) and are widely used in dyeing. Phthalocyanines form coordination complexes with most elements. These complexes are also intensely colored and used as dyes or pigments (*e.g.*, in paints). Substitution can increase solubility and shift the absorption to longer (near-infrared wavelengths), with advantage for *in vivo* applications.

Hypericin is isolated from the plant known as St. John's wort (Hypericum perforatum) and is possibly the most powerful photosensitizer present in nature, with quantum yields of singlet oxygen formation up to 0.8. It acts through both Type I and Type II reactions and its spectral properties are not too unfavorable, with peak absorption in the red (around 595 nm). It has been tested clinically for several cancer indications, and also for the detection of bladder cancer. Furthermore, Hypericin has antibiotic and antiviral properties.

14.1.4.2.Precursor-induced photosensitizers

The most common couple of precursor/sensitizer is δ-aminolevulinic acid/Protoporphyrin IX (Collaud et al 2004, Berg 2005).

Protoporphyrin IX (PpIX) is an intermediate in biosynthetic pathways to produce cytochromes, hemoglobin (the oxygen-binding protein in red blood cells), and myoglobin (which binds oxygen in muscles). Under normal conditions, free PpIX is present at too low concentration to produce photosensitization reactions. However, if excess δ-aminolevulinic acid (ALA) is provided (either systemically or topically, depending on application), PpIX may accumulate, causing photosensitization reactions. The administration of ALA-hexyl ester is approved for the therapy of bladder cancer, while ALA-methyl ester is approved for the treatment of Bowen's disease, actinic keratosis and basal cell carcinoma.

PpIX is also characterized by intense red fluorescence, when excited by violet light. Thus, it is also effectively applied for the detection of lesions to be treated with PDT, and for the identification or surgical resection margins.

14.1.5 Why is photosensitization important?

Photosensitization reactions affect our lives in many different ways. For example, they are used in synthetic chemistry to produce products that would be much more difficult or expensive to produce by other means. However, in the following we will focus mostly on therapeutic applications.

14.1.5.1.PDT and detection of tumors

In the last decades photosensitizers and light have extensively been tested and applied to treat malignant tumors (*e.g.*, in the bladder, prostate, lung, brain) and to remove through PDT

other unwanted tissue (*e.g.*, in the precancerous condition represented by Barrett's esophagus) (Hamblin and Mroz 2008, MacDonald and Dougherty 1999, O'Connor et al 2009).

The sensitizer is most often administered systemically and reaches both healthy and diseased tissues. The possibility to perform an efficient photodynamic therapy relies on the fact that the sensitizer tends to accumulate more and is retained longer in the tumor than in the surrounding healthy tissue, due to concurrent factors that differentiate normal from pathologic tissue (metabolism, angiogenesis, etc.). This makes the latter more prone to the photodynamic action, when irradiated with light of suitable wavelength. Obviously, also selective irradiation of the area of interest can contribute to limit undesired photodynamic actions in healthy tissue.

When ALA/PpIX PDT is performed, therapeutic benefit comes also from the fact that the production of PpIX occurs more efficiently in lesions than in surrounding normal tissue.

One of the most interesting phenomena observed in the PDT of cancer is also an advantage over other therapeutic modalities: PDT has the potential to induce strong and long lasting anti-tumor immune response (Canti et al 1994, Korbelik et al 1999).

Besides therapeutic purposes, the fluorescence properties of photosensitizers may be effectively exploited for the detection of cancers. For example, intravesical instillation of ALA-hexyl ester (Hexvix®) is approved for the detection of bladder cancer, in particular for carcinoma *in situ*, which is generally difficult to detect. PPIX fluorescence guided resection of bladder cancer has also shown promise for treatment purposes. Similarly, systemic administration of ALA (Gliolan®) is approved for intra-operative fluorescence guided detection and resection of malignant glioma.

14.1.5.2.PDT in ophthalmology

PDT was originally introduced as a modality for cancer treatment, but one of its most successful applications is certainly the treatment of choroidal neovasculature associated with age-related macular degeneration (AMD). PDT with Verteporfin (Visudyne®), a benzoporphyrin derivative, was the first therapy approved for subfoveal lesions. Since 2000, it spared hundreds of thousands of eyes from blindness and is now a standard treatment for AMD (Lim 2002, Bessler 2004). The drug is injected intravenously and, 15 minutes after the start of the infusion, the retina is irradiated with 690 nm light (50 J/cm^2). At the time of irradiation, the drug is still mostly localized in the blood vessels of the pathologic choroidal neovasculature and causes their occlusion.

14.1.5.3.PDT in dermatology

Dermatologic pathologies are especially suitable for PDT, as they are exposed. So local administration of the photosensitizer is often applied and light can easily be conveyed to the treatment site.

One of the main applications of PDT in dermatology is the treatment of non-melanoma skin cancer and its precursors, such as actinic keratosis. However, several other pathologic conditions have been successfully treated with PDT, including inflammatory and immune diseases (e.g., psoriasis) as well as infections (e.g., human papilloma virus) (Choudhary et al 2009, Nestor et al 2006). ALA-PDT seems to be a safe and a suitable alternative for a variety of conditions encountered in dermatology. The technique is effective in patients of all ages and typically results in better clinical and cosmetic outcomes than conventional surgery, which is an important benefit, when exposed lesions are treated, especially on the head and neck.

14.1.5.4. PDT of cardiovascular diseases

Atherosclerosis and its complications are a leading cause of morbidity and mortality in industrialized countries. In cardiovascular medicine, PDT has been applied to treat atherosclerosis and to inhibit the restenosis due to intimal hyperplasia that often occurs after vascular interventions.

Phase I trials were performed in the last decade, suggesting that good therapeutic response can be achieved and that the therapy is well tolerated (Rockson et al 2000, Kereiakes et al 2003).

14.1.5.5. Antimicrobial PDT

From the beginning of the 20th century onwards, many reports were published on the photodynamic inactivation of various species of bacteria, fungi, and viruses.

More recently, fundamental differences in susceptibility to PDT were observed between Gram (+) and Gram (-) bacteria. In general, neutral or anionic photosensitizes are efficiently bound to and allow the photodynamic inactivation of Gram (+) bacteria (Malik et al 1992, Merchat et al 1996), but they are unable to photoinactivate Gram (-) bacteria. The latter result can be achieved in various ways. The permeability of the cell outer membrane can be increased administering substances, such as EDTA, together with the photosensitizer. Alternatively one can use a cationic photosensitizer molecule with an intrinsic positive charge, or polycationic sensitizer conjugates formed from polymers such as polylysine.

The demonstration of efficient photoinactivation of several classes of microorganisms, the fact that antibiotic resistant bacteria are as susceptible to photodynamic inactivation as their naive counterparts (Wainwright et al 1998), together with the increasing appearance of

antibiotic resistance amongst pathogenic bacteria, suggest that PDT may be a useful tool to treat infectious diseases (Hamblin et al 2004) and will likely be a growing application of PDT.

14.1.5.6. Photo-activated pesticides

Photoresponsive systems are widespread in nature, and life processes such as photosynthesis and vision are linked with structural changes of molecules caused by sunlight. Similar photochemical transformations can be employed to make synthetic agrochemicals and drugs active, or to control their bioavailability at the site of action. Photo-activated pesticides have been used as insecticides, fungicides and herbicides. Their activity relies on preferential accumulation of photosensitizers (*e.g.*, porphyrins and xanthenes) in target organisms and on phototoxicity elicited by sunlight illumination (Ben Amor and Jori 2000, Heitz and Downum 1987).

Photo-activated pesticides are known as green pesticides as they are environment friendly: they exert their toxic action selectively on the target where they preferentially localize, degrade themselves and cause no contamination of the environment.

14.2.   Fluorescence imaging of photosensitizers in small animals

In section 14.1, it was pointed out that the activation of photosensitizers requires radiation of proper wavelength. Hence, the design of a PDT treatment should consider the absorption spectrum of the drug and the optical properties of biological tissues at the activation wavelength. A wealth of papers deals with PDT dosimetry, *i.e.* mathematical models that take into account the scattering (μ'_s) and the absorption (μ_a) properties of the tissue in order to predict the amount of light absorbed by the photosensitizer. The scattering μ'_s, in fact, modulates the power density distribution in the tissue, while the absorption μ_a, beyond

contributing to determine the light pattern, indicates unspecific absorption of photons, which does not induce any photochemical effect. It is worth noting that most of the dosimetry studies assume that the absorption spectrum of the photosensitizer is the one measured from injectable solutions or in simple biological models, like micelles or similar systems. Nevertheless, it has been found that the interaction of photosensitizers with the biological substrate can lead to an *in vivo* spectrum that cannot be predicted on the basis of *in vitro* studies. The following section deals with a time resolved technique that can be used to measure the actual absorption spectrum of sensitizers upon intravenous injection in mice.

14.2.1   Absorption spectra of photosensitizers measured in vivo

The optical properties of tissues and exogenous substances administered for diagnostic or therapeutic purposes may be influenced by functional state, metabolism, blood perfusion, and so on. Therefore *in vitro* measurements could lead to an inaccurate spectrum. This problem can be avoided by means of time-resolved reflectance, which allows one to determine the optical properties (absorption and scattering coefficients) of tissues *in vivo* (Patterson et al. 1989, Cubeddu et al. 1994a). This can be easily done in anesthetized mice, but could be theoretically performed in humans as well, since the procedure is completely non invasive. Hereafter, only measurements made on mice are reported.

To this purpose, short (picoseconds) light pulses generated by a laser tuneable over a wide spectral range (*e.g.* 600–700 nm) are delivered to an anesthetized mouse through an optical fiber. The light travelling inside the animal, and partially remitted by its surface, is collected by a second fiber placed few millimeters apart (*e.g.*, 1 cm). The distal end of the fiber is coupled to a fast Photomultiplier Tube (PMT) whose output is processed by a Time Correlated Single Photon Counting (TCSPC) apparatus.

Photons undergo multiple scattering and absorption inside the tissue. This results in broadening and shaping of the laser pulses in such a way that the remitted light carries information about the absorption (μ_a) and scattering (μ_s) coefficients of the part of the tissue that is traversed by photons. Assuming an ideally short input pulse (Dirac delta function), the output pulse, measured by the TCSPC apparatus, gives the Temporal Point Spread Function (TPSF) of the overall system, including the response of the experimental equipment and the properties of the analyzed sample. It can be demonstrated that, by fitting the TPSF with a proper mathematical model for photon propagation in turbid media, convoluted with the response of the experimental system, the dependencies of the equipment can be removed and the optical parameters of the sample (μ_a and μ_s) can be recovered. In most cases, the mathematical model used to predict the photon migration inside the tissue relies on the diffusion equation, with proper boundary conditions. This assumes that photons lose information about their original direction after traveling a distance (about 1 mm or less) that corresponds to few mean free paths. Thereafter, they behave like heat diffusing in a homogeneous medium.

Coming back to the problem of estimating the absorption properties of photosensitizers *in vivo,* a differential measurement is performed (Cubeddu et al. 1994b). Actually, by scanning the laser wavelength all over the spectral range of interest, the absorption coefficient μ_a of anesthetized animals is measured twice, as a function of the wavelength, before and after the injection of a therapeutic dose of the sensitizer. The true absorption spectrum of the sensitizer is then recovered through a simple difference between the acquired spectra, in such a way that the absorption properties of tissue are removed.

Insert Fig. 4 here

Experiments performed on mice after the injection of 25 mg/kg body weight (b.w.) of HpD showed that the absorption spectrum of HpD measured in the range 600-640 nm (**Fig. 14.4**) has a peak close to 620 nm. This is in good agreement with the spectrum measured in a solution of HpD bounded to Low Density Lipoproteins (LDL), which simulated the biological environment. This result demonstrates that the absorption properties of HpD are not remarkably modified, at least in the 600-640 nm range (which is of interest for PDT), by interactions with the biological substrate that may occur only *in vivo*. It is worth noting that the described method, besides providing the true spectrum of the sensitizer *in vivo*, also estimates the increase in the absorption caused by the accumulation of the drug in the tissue. This resulted to be close to 20% for the specific case considered in this study. The simultaneous measurement of scattering and absorption properties leads to the knowledge of the penetration depth of light in tissue. This allows one to determine the optimal light dose for therapeutic purposes, while the measurement of the absorption lineshape yields the optimal irradiation wavelength.

Different from what reported for HpD, measurements performed following the same protocol on the absorption spectrum of disulphonated Aluminum phthalocyanine (AlS$_2$Pc) showed a marked difference with respect to the absorption spectrum measured in aqueous solution.

Phthalocyanines have been considered as possible second generation photosensitizers for PDT (MacDonald and Dougherty 1999). AlS$_2$Pc shows an absorption spectrum peaked at 672 nm or red-shifted of no more than 4 nm in various solution environments. Nevertheless, experiments on the action spectrum for PDT in tumor-bearing mice resulted in a good therapeutic efficacy for wavelengths greater than 670 nm and up to 710 nm, in a range where the absorption measured in solution decreases rapidly (Canti et al. 1992).

Actually, the absorption spectrum of AlS_2Pc measured by time-resolved reflectance in the 650-695 nm range in tumor-bearing mice (dose = 2.5 mg/ml b.w.) peaks at 685 nm instead of 672 nm (**Fig. 14.5**) (Cubeddu et al. 1996). This red-shift with respect to the absorption maximum in solution is consistent with the therapeutic efficacy in the treatment of tumor models that proved to be significantly better at 685 nm than at 672 nm.

The spectral change seems to indicate a strong interaction between the sensitizer and the biological substrate. A modification of the chemical structure can perhaps be speculated, since, as above-mentioned, various solvents and environments (*e.g.* micelles) could never lead to such a remarkable red-shift of the AlS_2Pc absorption spectrum.

This result highlights the importance of the *in vivo* measurement of the optical properties of drugs whenever the interaction with light is a key element for activation, as it is the case of PDT.

14.2.2   *Time-gated fluorescence imaging of photosensitizers in tumor-bearing mice*

14.2.2.1. *In vivo* studies

Fluorescence spectroscopy and imaging offer effective opportunities for cancer diagnosis, which are being carefully investigated since long ago (Wagnières et al. 1998). Fluorescence techniques are minimally invasive, relatively inexpensive with respect to other diagnostic methodologies, and can be easily applied to any part of the human body that can be reached by light, either directly or by means of an endoscope. In diagnostic procedures, fluorescence can in principle provide the clues for the detection of several disorders, and in particular tumors. Yet, unfortunately, a broadband emission is a general characteristic of both cancerous and non-cancerous tissues. Therefore, the mere presence of a fluorescence signal often

provides only a limited diagnostic aid. In order to increase the specificity for tumor detection, the exogenous emission of suitable markers can be considered. To this purpose, great attention has been devoted to photosensitizers originally developed to treat tumors with PDT. Some photosensitizers are also promising for diagnosis, since they accumulate in cancerous tissues with a good selectivity, are fluorescent and, last but not least, have already been approved for human administration (as described in Section 14.1).

To exploit exogenous fluorescence for tumor diagnosis, a selectivity criterion is needed to discriminate the emission of the fluorophore of interest from the background signal, which is mainly due to tissue autofluorescence. The discrimination can be obtained in the spectral domain by selecting two suitable excitation or observation wavelengths (Andersson-Engels et al. 1991). The subtraction between the images acquired at the two wavelengths leads to an image that mainly contains the contribution of the fluorophore of interest. However, this technique requires a non-trivial normalization; moreover the fluorescence spectra of organic compounds often overlap, reducing the selectivity of the spectral approach. Using a pulsed excitation, various events characterized by a different time scale, take place. Such events, like scatted light and emission of different fluorophores, may be discriminated with a properly gated acquisition.

Most intensified CCD-cameras allow very fast electronic gating, almost equivalent to a fast shutter with an exposure time of few nanoseconds or even less. Using such a device, it is possible to acquire only the fluorescence light that falls within a definite time window, properly delayed with respect to the excitation pulses. Taking into account the (multi-)exponential behavior of the fluorescence decays, a suitable combination of gate width and delay provides an effective discrimination amongst fluorophores having lifetimes that differ at least by few nanoseconds. To apply this time-gated fluorescence imaging technique no

narrowband filters are required. Moreover, the images show the fluorophore localization without any image processing, being the selectivity intrinsic in a single acquisition process. This feature led to the development of a tumor detection technique capable of providing good selectivity and real-time operation at a time when the computer performances were several orders of magnitude worse than now.

Time-gated fluorescence imaging was devoted, since the beginning, to the detection of cancer through the labeling of tissues with photosensitizing drugs. Hereafter, a synthetic review is reported of time-gated imaging performed on tumor-bearing mice sensitized with different drugs.

It has been already observed (Section 14.1) that all clinically-approved photosensitizers used in PDT are porphyrin-based drugs. The detection of tumors on the basis of the Hematoporphyrin Derivative (HpD) fluorescence dates back to the Sixties (Lipson et al. 1961). In fact, HpD, when excited at 405 nm (Soret band), emits a strong fluorescence with a multi-component relaxation dynamics, resulting in an average lifetime of about 15 ns, whereas tissue autofluorescence mainly extinguishes within 3-6 ns. This large difference in the decay time of the two emission components is well suited for the application of the time-gated approach. Assuming a preferred localization of HpD in the tumor, the neoplasia can be easily singled out by acquiring the fluorescence of the suspected portion of tissue after a delay greater than 15 ns with respect to the excitation pulses. In fact, such an image will record almost exclusively the long living HpD emission, with a negligible contribution from autofluorescence.

Insert Fig. 6 here

This paradigm was tested on mice bearing experimental tumors implanted intraderma (Cubeddu et al. 1993). Mice received an intravenous injection with different doses of HpD (5-

25 mg/kg b.w.) and underwent a fluorescent measurement after an uptake time of 12 hrs, required for the drug to preferentially localize in the tumor. **Figure 14.6a** shows a time-gated image acquired after a delay of 20 ns with respect to 1 ns long excitation pulses at 405 nm. The gate width was 50 ns, long enough to collect most of the HpD fluorescent emission. For comparison, **Fig. 14.6b** shows the same field of view observed synchronously with the excitation pulses. This arrangement simulates a CW acquisition. The excitation light was removed in both cases with a spectral filter. The tumor, which corresponds to the bright spot, can be distinguished more easily in the delayed image, as expected due to the selective acquisition of the exogenous signal and the effective rejection of the background fluorescence with the delayed gate. It is worth noting that the tumor was clearly detected in spite of the screen action of the skin against light penetration.

Insert Fig. 7 here

A second set of experiments was performed on mice injected with a second generation photosensitizer, *i.e.* AlS$_2$Pc (Cubeddu et al. 1997a). In that case, the excitation light was set to 650 nm, while the detection was at 670 nm, through a high pass spectral filter. The red excitation light gave two advantages with respect to the blue/violet light used to excite HpD: a deeper penetration in tissues and a strong reduction of the autofluorescence. However, being the excitation wavelength not far from the detection one, a simple high pass filter revealed to be insufficient to eliminate the strong laser light scattered by the sample, which is orders of magnitude greater than the fluorescence signal. In this case, the acquisition delay was set to ≈1-2 ns in order to remove the scattered laser light, which was by far the greatest source of noise, since the endogenous fluorescence excited in the red was almost negligible. **Figure 14.7a** shows the tumor area of a mouse treated with Phthalocyanine acquired with delay of

2 ns. For comparison, **Fig. 14.7b** was acquired at zero delay. The improvement in contrast is evident, thus demonstrating the superior performance of the gated acquisition over the CW one.

14.2.2.2.*Ex vivo* studies

The application of the time-gated imaging to the detection of tumors in locations other than the dermis represents a natural extension of this technique. A prerequisite for reliable tumor detection is the knowledge of the fluorescence properties, after porphyrin sensitization, of healthy organs, which may represent the peritumoral tissue.

Measurements of the fluorescence of selected tissues (skin, bone, bowel, brain, muscle, lymph node) in tumor-bearing mice were performed after the administration of HpD or Photofrin II (Cubeddu et al. 1995). The emissions from different tissue types were compared to that of the tumor using the time-gated approach (30 ns delay) for image acquisition. The animals were sacrificed and dissected; the tumor with the surrounding skin was removed and its fluorescence was measured from the dermal side. Tissue samples of muscle, fat, brain, lymph nodes, bowel, and bone were excised and their fluorescence was measured as well.

Insert Fig. 8 here

The average intensity measured in different organs, as a function of the drug dose, is shown in **Fig. 14.8**. The strongest fluorescence was observed from the tumor through the inner mucosa (dermal side). The fluorescence of lymph node, fat, and muscle only differ in intensity, while presenting a similar dependence on the drug dose. The most fluorescent tissue in this group is lymph node, whose intensity is 0.4 times that of the tumor. The fluorescence signal of the fat is intermediate, whereas the signal recorded from the muscle is appreciably lower (< 0.2 times that of the tumor). The fluorescence of the bowel partially depends on the

mouse feed and deceases with uptake time (data not shown). Hence, the contrast with respect to the tumor is rather high after 12 hrs.

The fluorescence of the bone in control animals is by far higher than that of other tissues and it is more than half the fluorescence of bone in sensitized animals. Finally the fluorescence of the brain is negligible for all the examined animals due to the blood-brain barrier that prevents drug uptake.

The signal detected in the tumor is very high, yet also the fluorescence of the derma, which in this model is the peritumoral tissue, is remarkable. Better contrast could be expected with peritumoral tissues other than skin.

Time resolved spectra taken in all the examined tissues confirmed that the only fluorophore with a long lifetime is the sensitizer.

From the diagnostic standpoint, the rather high fluorescence observed in adipose tissues and in bones could be misinterpreted and cause false positives. This is especially true for bones due to the long lifetime emission that is naturally present in the tissue. As a consequence of this endogenous emission, the fluorescence intensity of bones can compete with that of the tumor, mainly at low dose of sensitizer. The awareness of this possible mistake should suggest particular care to avoid any wrong or uncertain diagnosis.

In summary, it can be affirmed that, except for bones, the endogenous fluorescence does not impair the tumor detection by the time-gated imaging technique, whose reliability is strictly related to the selectivity of the drug.

As conclusive remarks, it is worth noting that the time-gated imaging technique provides several interesting features for cancer detection after sensitization with exogenous compounds, mainly porphyrin based ones. Amongst them: real time operation, complete removal of laser excitation light and operation under ambient light, since the synchronous acquisition

effectively removes any CW illumination. On the other hand, it requires a pulsed laser and a rather expensive gated camera.

14.2.3   *Fluorescence lifetime imaging of photosensitizers in tumor-bearing mice*

In the previous Section, we observed that the concentration of HpD in the peritumoral tissue is often not negligible. The ratio of fluorescence intensity in the tumor to the intensity in the nearby tissues is dose-dependent and sets a lower limit to the drug dose that is required for reliable tumor detection using porphyrins.

It is well known that the time-resolved emission of HpD presents three main components, having different lifetimes and amplitudes. Moreover, the biological environment influences the fluorescence properties of the drug. This observation suggests one to study the spatial distribution of the HpD fluorescence decay time in sensitized mice, with the aim to discriminate tumor from healthy tissue.

Fluorescence lifetime imaging (FLIM), which is the natural evolution of time-gated imaging, is the preferred option for cancer detection through exogenous sensitization. To fully understand its effectiveness, the fluorescence lifetime maps of the HpD fluorescence was measured in tumor-bearing mice as a function of the drug dose (Cubeddu et al. 1997b). Mice bearing a fibrosarcoma (MS-2, diameter of ~6 mm) on the back were injected intra-peritoneally with the following HpD doses: 0.1, 0.25, 1, 2.5, and 10 mg/kg b.w.. The highest dose was considered for comparison with time-gated imaging (see previous Section). A very simple approach, consisting in the acquisition of only two images delayed by 10 and 30 ns with respect to the excitation pulses, allows one to measure the map of the effective lifetime in the field of view, in a very short time. In fact, the acquisition of the images take a fraction of a second and the image processing is also very fast since only algebraic functions are

required to recover the lifetime. Moreover, since the first delay is longer than the typical decay time of the endogenous fluorescence, the lifetime map is minimally affected by the endogenous emission of the tissues.

Figure 14.9a, shows the fluorescence lifetime map of the back of a mouse sensitized with 0.25 mg/kg body weight of HpD. The tumor boundaries are clearly outlined in the time domain map, since the neoplasia corresponds to a fluorescence lifetime longer than that of the healthy tissue. On the contrary, an intensity based fluorescence image, which is shown in **Fig. 14.9b** for comparison, is much less effective for tumor detection. It is worth noting that **Fig. 14.9b** displays only the exogenous fluorescence since it has been acquired 20 ns after the excitation. Fluorescence images taken at zero delay are even worse, since the tissue natural fluorescence completely masks the drug signal, leading to useless images. A similar result was achieved with all the drug doses, but the highest one. In fact, even though the FLIM technique still works at high dose, delayed intensity images also allow a reliable detection of the neoplasia, as already observed in the previous Section. As an example, **Figs. 14.10a and 14.10b** show a decay time image and an intensity image respectively, of the back of a mouse sensitized with 10 mg/kg b.w. of HpD.

It is worth noting that the lifetime measured by means of this technique does not represent any true physical parameter. However, it gives a diagnostic index resulting from a weighted average of the lifetimes of the different HpD fractions that localize in tumor and healthy tissues.

As a matter of fact, the imaging study indicates that the HpD fluorescence emission is characterized by a longer lifetime when incorporated into neoplastic tissues. This is in

agreement with the dependence on the environment typically observed for various photophysical properties of HpD.. In particular, as described in Subsection 14.1.4.1, HpD and its commercial version Photofrin are mixtures of porphyrin monomers and different types of oligomers. Monomers and oligomers have different fluorescence properties for what concerns both the emission spectrum and the corresponding lifetimes. The tumor-localizing fraction consists mostly in oligomers, thus different fluorescence properties are expected from tumors as compared to healthy tissues.

14.3.   Fluorescence imaging of photosensitizers in humans

14.3.1   Detection of skin lesions by fluorescence imaging

As described in Subsection 14.1.4.3, a very special photosensitizer is δ-aminolevulinic acid (ALA), which is a naturally occurring precursor in the cycle of heme biosynthesis (Kennedy et al. 1990). The exogenous administration of this substance bypasses a physiologic regulation mechanism and gives rise to an excess of the intermediate molecule Protorphyrin IX (PpIX), which is photoactive and strongly fluorescent. Even though this process takes place also in healthy tissue, in cancerous tissue it is more effective due to enhanced uptake of ALA and enzymatic deficiencies occurring in neoplastic cells. This unbalance in the content of PpIX provides a selectivity criterion that can be profitably exploited not only for PDT, but also to detect tumors by means of fluorescence techniques. In fact, PpIX, when excited with light at 400 nm, emits a characteristic red fluorescence (peaking around 635 nm) with lifetime of about 18 ns, *i.e.* much longer than that of the endogenous tissue fluorescence (3-6 ns or shorter).

Topical administration of ALA allows the patient to avoid skin sensitization. It is especially suitable for the detection of exposed pathologies, like skin tumors, even though pigmented lesions, like the very aggressive melanoma, cannot be detected by fluorescence imaging because no appreciable emission can be revealed from these strongly absorbing lesions. This not withstanding, a great interest exists in dermatology for the classification of lesions that present a different degree of malignancy. In addition, a technique capable of showing the actual extent of the pathologic area would allow surgeons to perform conservative excisions, while reducing the recurrence rate. In the following, we will report on the use of fluorescence lifetime imaging to distinguish basal cell carcinomas and squamous cell carcinomas from benign lesions (Cubeddu et al. 1999).

The excitation light is provided by a dye laser pumped by a sub-nanosecond Nitrogen laser. The laser emission peaks around 400 nm with average power of 800 µW. Such a power level does not raise any safety concern and allows one to perform the diagnostic procedure even on the patient's head, close to the eyes. This is very important since most dermatological lesions are on parts of the body exposed to sunlight and in particular on the face. An optical fiber is used to deliver the light to the patient, while the fluorescence signal is collected using a high aperture photographic lens. The system was designed in such a way that even non-technical personnel can operate it for routine clinical examinations.

The diagnostic procedure involves the preparation of an ointment made of 2% ALA powder in a lipid emulsion. The ALA ointment is applied onto the lesion with a margin of at least 10 mm in the visibly normal skin and the lesion is covered with an occlusive dressing. The patient is kept at rest for about 1 hr to allow the metabolism to transform ALA into PpIX. Then, the excess cream is gently removed from the lesion and the fluorescence test is carried

out. A preliminary examination is performed using only two gated images. In this case the map of the fluorescence lifetime is calculated in real time using an algebraic equation and displayed in pseudocolors at few frames per second. Then, for the regions that present a clinical relevance, a more precise measurement is performed by acquiring more images with delays ranging from 0 to 20 ns, relative to the excitation pulses. The images are processed offline immediately after the acquisition to calculate the maps of the average fluorescence lifetime and of its amplitude.

The classification of the lesions from fluorescence images is made according to the following considerations. It is proved that the malignant character of a skin lesion is associated with an excess of exogenous fluorescence and a reduction in natural emission, as compared to healthy tissues. As a consequence, the average lifetime in tumors is longer that in healthy tissues since the amplitude of the long living component (PpIX fluorescence) is higher than that of the short living emission (endogenous fluorescence). The use of the fluorescence lifetime as a diagnostic index, instead of intensity or spectral features, gives additional advantages. In fact, the detection index is based on the relaxation dynamics of the fluorescence emission. Hence, it is almost insensitive to artifacts due to the spatial variation of the fluorescence intensity. In particular, the time domain approach gets rid of an uneven distribution of the excitation light and - most important - of local differences in skin absorption. These may be due to anomalies in pigmentation, vascularization, or blood perfusion, due to an inflammatory status, which might lead to severe artifacts if one looks at the fluorescence intensity. Finally, the diagnostic procedure can be carried out under normal illumination.

Insert Fig. 11 here

Thirty-four patients affected by 48 lesions, either malignant (mainly basal cell carcinomas) or benign ones, were included in the study considered hereafter. For all the patients, the classification of the lesions on the basis of fluorescence lifetime maps was compared to histology, which was routinely performed. A typical fluorescence lifetime image of a malignant lesion is shown in **Fig. 14.11a**, while the map of the CW fluorescence intensity is displayed in **Fig. 14.11b**. For comparison, the color photo of the lesion is also reported in **Fig. 14.11c**. The tumor, which was classified as a basal cell carcinoma, is characterized by a significantly longer lifetime (18 ns) than that of the surrounding healthy tissue ($\approx$10 ns). It is worth noting that the region where the ALA ointment was applied is larger than the lesion itself and can be easily distinguished in **Fig. 14.11a**. The fluorescence amplitude is lower in the lesion, as expected. However, the clinical experience demonstrates that, while the fluorescence amplitude presents a strong variability from patient to patient, the lifetime is much more stable and thus reliable for the classification of the lesions. In the present trial, 27 out of 35 malignant lesions were correctly identified, while only 1 out of 13 benign lesions was misinterpreted. On the basis of these outcomes, the diagnostic procedure showed a sensitivity of 88% and a specificity of 74%. However, it is very important to observe that the test was not conducted according to a strictly blind protocol. Hence, the results are only indicative. Actually, it has been reported that the typical PpIX red fluorescence can be directly observed on the skin lesions of ALA-sensitized patients under CW excitation. This is certainly true only when the marker dose is rather high. When the ALA dose is reduced to few percents in the ointment, as it is required in a minimally invasive diagnostic protocol, the interference of the natural skin fluorescence and the uneven pigmentation prevent reliable

lesion identification by means of an intensity-based approach. In such a condition the lifetime technique exhibits its maximum effectiveness as it has been previously shown.

14.3.2   *Fluorescence imaging of brain tumors*

Brain tumors, either primary or resulting from metastasis, are amongst the most aggressive neoplastic diseases. In particular, gliomas account for a large fraction of all primary brain tumors and are characterized by high morbidity. In fact, in almost all but very few patients, such a tumor leads to inevitable death within a relatively short time after diagnosis. Moreover, treatment of gliomas poses a challenge due to their tendency to infiltrate the surrounding normal brain and to a high rate of recurrence.

The most common treatment of gliomas is surgery followed by chemotherapy and radiation therapy. It has been observed that the extent of tumor resection plays a significant role in patient prognosis and disease progression, resulting in the most important factor for longer survival. Nevertheless, the extent of resection compares with the need to minimize the removal of normal brain, which might result in neurological impairment and affect the patient's quality of life. Although of paramount importance, complete tumor resection is limited by the difficulty in visually detecting differences between normal brain and malignant tissue during surgery. Thus, patients with malignant gliomas often have a subtotal resection.

As a consequence, there is a pressing need to develop new strategies to improve the intraoperative imaging of malignant glioma. To this purpose, fluorescence imaging of brain tumor during surgery has been extensively studied since long ago (Moore et al. 1948). Yet, it was not until the last decades when the technological advancement and the advent of ALA–PpIX sensitization fostered this research field toward a widespread use. This led to multicentre clinical trials, mainly performed in Germany (Stummer et al. 2006), while other

clinical efforts were carried out in Japan focusing on a different class of markers (*i.e.*, fluorescein labeling) (Kuroiwa et al. 1998, Shinoda et al. 2003).

More generally, in a wealth of studies, either experimental or clinical ones, three major labeling strategies exploiting fluorescence emerged for brain tumor imaging, with a different degree of maturity (Pogue et al. 2010): endogenous fluorophores (autofluorescence); exogenous agents that are routinely used in humans (*e.g.*, ALA-PpIX, fluorescein, indocyanine green); exogenous agents developed for first-time use in humans, with molecular targeting potential.

Autofluorescence does not require the administration of any drug. The signal is largely attributed to collagen, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and endogenous porphyrins. Yet, it is usually very dim and imposes a rather long acquisition time, which might hamper surgical procedures. Moreover, fluorescence images can be affected by artifacts cased by blood and scattered light. Different strategies, mainly based on spectral ratios, have been devised to gain specificity for tumor tissue versus normal one. However, the reliability of the autofluorescence approach for tumor demarcation is still critical and site dependent.

The ALA–PpIX system has been extensively studied for PDT and photodiagnosis, including fluorescence-guided resection of brain tumors (Stummer et al. 2000). In fact, a significant accumulation of PpIX has been found in brain tumors, mainly high grade gliomas, even if a still detectable PpIX emission chracterizes also low grade gliomas, meningiomas and brain metastases. (a Valdés et al. 2011).

Other low molecular weight markers, like Sodium Fluorescein or Indocyanine Green, have been studied for tumor labeling, because they are retained within tumor tissue as a result of

slow clearance. In particular, fluorescein labeling of brain tumors dates back to the Forties (Moore et al. 1948) and has been applied even recently in Japan (Kuroiwa et al. 1998).

Finally, functionalized nanoparticles, and other molecular probes providing active tumor targeting are intensively studied for tumor detection, but are still not used in clinical applications due to safety concerns.

From the technological point of view, several devices were proposed for brain tumor demarcation, ranging from point-like spectroscopes, to modified surgical microscopes and commercial systems with built-in fluorescence channels. Laser-induced fluorescence spectroscopy has been used since long time ago for diagnosis of brain cancer. Most of the measurements were performed under steady state excitation. Yet, in the last years time-resolved spectroscopy of several types of brain tumors demonstrated the potential of this strategy on patients. In particular, a recent study (Butte et al. 2011) showed that time-resolved fluorescence holds the potential to diagnose brain tumors intra-operatively and to provide a valuable tool for aiding the neurosurgeon to rapidly distinguish between tumor and normal brain tissue..

Point like spectroscopy proved very effective to detect biochemical differences in tissues leading to diagnostic outcomes; yet, the choice of the measurement site requires a priori information or clear clues of the disease in the operation field. This is not always the case, mainly close to the tumor margins. Time is a precious resource during surgery and imaging devices are certainly preferred over point like instruments, since they can provide an immediate view of critical areas. Moreover, the margins between tumor and healthy tissue could be displayed in real time, thus giving the surgeon an effective guidance. Amongst imaging devices, the ones based on the time-resolved approach, like FLIM, provide extra

valuable features, like good immunity versus unspecific absorbers (*e.g.*, blood) and uneven excitation patterns.

The first attempt to develop a FLIM system for fluorescence-guided brain resection was recently made by Sun and coworkers (Sun et al. 2010), exciting with a nitrogen laser and detecting the endogenous fluorescence with a gated CCD coupled to a fiber optic endoscope. Even if the results of the study were still preliminary, they suggest that a FLIM assisted surgical microscope could be very beneficial for conservative brain surgery with a high degree of selectivity for tumor. Such a device would certainly result in an increase in patient survival time.

As the reader has likely noted from what reported here above, none of the attempts to the fluorescence imaging of the brain made up to now has combined exogenous markers with time domain detection. The advantages of either of the two approaches were demonstrated on patients separately, but they have not been combined yet.

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14.5. Figure captions

Figure 14.1 – Type I *vs* Type II photosensitization reactions. P: Photosensitizer; S: Substrate.

Figure 14.2 – Jablonsky diagram showing excitation and de-excitation transitions. A1 (A2): absorption to the first (second) excited singlet state; F: fluorescence; IC: internal conversion; VR: vibrational relaxation; ISC: intersystem crossing; P: phosphorescence.

Figure 14.3 – Porphyrin (left) and phthalocyanine (right) structure.

Figure 14.4 – Absorption line shape of HpD measured *in vivo* in tumor-bearing mice (25 mg/kg b.w.) (■) and in LDL (10 µM HpD in 0.3 mg/mL LDL) (solid line).

Figure 14.5 – Absorption line shape of 2.5 mg/kg b.w. AlS_2Pc measured *in vivo* in tumor-bearing mice (□) and 10 µg/ml AlS_2Pc in aqueous solution (solid line).

Figure 14.6 – Fibrosarcoma on the back of a mouse treated with 10 mg/kg b.w. of HpD 12 hrs before the experiment: (a) image acquired using a 20-ns delay after the excitation pulses; (b) image acquired synchronously with the excitation.

Figure 14.7 – Fibrosarcoma on the back of a mouse treated with 5 mg/kg b.w. of AlS$_2$Pc 3 hrs before the experiment: (a) image acquired using a 2-ns delay after the excitation pulses; (b) image acquired synchronously with the excitation.

Figure 14.8 – Average fluorescence intensity of tumor, fat, bowel, muscle, lymph node, bone and dermis.

Figure 14.9 – Decay time image (a) and time gated image (b) of a tumor on the back of a mouse sensitized with 0.25 mg/kg b.w. of HpD.

Figure 14.10 – Decay time image (a) and time gated image (b) of a tumor on the back of a mouse sensitized with 10 mg/kg b.w. of HpD.

Figure 14.11 – Fluorescence lifetime map (a) and intensity image (b) of a basal cell carcinoma on the cheek, 1 h after the topical administration of 1% ALA cream. The color image of the lesion is also shown (c).