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405 nm versus 633 nm for protoporphyrin IX excitation in fluorescence-guided stereotactic biopsy of brain tumors

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ABSTRACT

Fluorescence diagnosis may be used to improve the safety and reliability of stereotactic brain tumor biopsies using biopsy needles with integrated fiber optics. Based on 5-aminolevulinic-acid-induced protoporphyrin IX (PpIX) fluorescence, vital tumor tissue can be localized in-vivo during the excision procedure to reduce the number of necessary samples for a reliable diagnosis.

In this study, the practical suitability of two different PpIX excitation wavelengths (405 nm, 633 nm) was investigated on optical phantoms. Violet excitation at 405 nm provides a 50-fold higher sensitivity for the bulk tumor; this factor increases up to 100 with decreasing fluorescent volume as shown by ray tracing simulations. Red excitation at 633 nm, however, is noticeably superior with regard to blood layers obscuring the fluorescence. Experimental results on the signal attenuation through blood layers of well-defined thicknesses could be confirmed by ray tracing simulations. Typical interstitial fiber probe measurements were mimicked on agarose-gel phantoms. Even in direct contact, blood layers of 20 – 40 µm between probe and tissue must be expected, obscuring 405-nm-excited PpIX fluorescence almost completely, but reducing the 633-nm-excited signal only by 25.5%. Thus, 633 nm seems to be the wavelength of choice for PpIX-assisted detection of high-grade gliomas in stereotactic biopsy.

Key words: 5-aminolevulinic acid, protoporphyrin IX, stereotactic biopsy, fluorescence spectroscopy, glioblastoma multiforme

1. INTRODUCTION

Gliomas account for about 80% of all primary malignant brain tumors. [1] Among these, glioblastoma multiforme (GBM) constitutes the most frequent and malignant histologic type with a very poor prognosis. It is classified as WHO grade IV and has a 5-year survival rate of less than 3%. [2] The median survival ranges from 6-9 months for patients older than 50 years to 18-21 months for patients aged between 18 and 30. [3] Gliomas generally tend to infiltrate adjacent normal brain in a diffuse way, which prevents a clear demarcation of the tumor and represents a major factor in therapeutic failure. [4, 5]

For patients with a methylated *O*⁶-methylguanine-DNA methyltransferase (MGMT) gene promoter, a chemotherapy based on temozolomide is promising and recommended as part of the therapeutic concept. [6] MGMT is a key enzyme for DNA repair and the methylation of its gene promoter indicates an increased sensitivity of the tumor to alkylating agents such as temozolomide. To assess the patient's MGMT status, biopsies with an approximate volume of 1 mm³ are taken. [7] The clinical benefit of this intervention is based on its potential to support personalized medicine concepts where the MGMT

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status is one of a panel of biomarkers [8, 9]: In specific cases of MGMT-positive patients where a resection is supposed to involve a significant risk, for instance, alkylating agents could replace surgery as first-line treatment. Other biomarkers that can be extracted from biopsies and may help to substantiate the diagnosis comprise the proliferative index Ki-67, mutations on the isocitrate dehydrogenase genes IDH1/2 and LOH (loss of heterozygosity) on chromosomes 1p and 19q. [7] Stereotactic biopsy, illustrated in Figure 1, is a standard procedure for obtaining a tissue sample of the brain tumor. While the complication rate of this neurosurgical procedure is rather low, which becomes manifest in mortality and significant morbidity below 1% and 5%, respectively [10], incidents usually involve severe consequences. With occurrence rates ranging up to 5% [11], the most prominent complications are hemorrhages, which can lead to severe functional deficits or even the patient's death. Sampling of a non-diagnostic tissue specimen represents another risk. Due to deficits of conventional imaging techniques like CT or MRI and the inherent diffuse growth of gliomas, the distal tip of the biopsy needle may be wrongly positioned outside the region of vital tumor. False negative findings could be the consequence, potentially leading to inappropriate further treatment. [12] Actually, the reported diagnostic yield (percentage of biopsy procedures leading to conclusive histopathological diagnosis) of stereotactic biopsy ranges from 76% to 99%. [13-20] These numbers indicate that in many cases additional support for the neurosurgeon beyond the conventional imaging techniques would be desirable. This is all the more true as usually several biopsy samples are taken to permit a diagnosis with acceptable reliability [7, 13]. Especially for deep-seated tumors, however, a minimum of biopsy samples should be aspired to prevent neurological deficits. [21]

Figure 1 Stereotactic biopsy of brain tumors. A biopsy needle is attached to the stereotactic frame and inserted into the brain along a predefined trajectory until the distal needle tip is positioned in an area of vital tumor tissue.

Currently, a mechano-optical instrument for stereotactic biopsy is developed, which consists of a standard biopsy needle with integrated fiber optics. It is capable of overcoming the aforementioned problems by means of fluorescence spectroscopy. As already shown by various groups, the administration of 5-aminolevulinic acid (5-ALA) leads to a highly selective accumulation of protoporphyrin IX (PpIX) in high-grade malignant gliomas. [22-24] A tumor-related deficient blood brain barrier and an altered activity level of several enzymes including ferrochelatase, which catalyzes the conversion of PpIX to heme, are central reasons for this phenomenon. [25] Tumor recognition with 5-ALA-induced PpIX is already effectively used for fluorescence-guided resection of malignant gliomas using surgical microscopes [22, 23, 26] or fiber optical probes [27, 28]. It leads to a significantly increased complete resection rate of these diffusely growing tumors and to a significantly enhanced progression-free survival rate after 6 months, translating into a progression-free survival prolongation of 1.5 months as compared to conventional white light resection. [23] The method has also been tested on excised tissue samples during stereotactic biopsy using surgical microscopes [21, 29-31]. In the latter case, the diagnostic yield of the fluorescent samples was always 100% (total $n = 98$). Regarding the specificity to malignant tissue and the sensitivity to GBM of 5-ALA-induced PpIX fluorescence in stereotactic biopsy, Widhalm et al. [29] report values of 100% and 98% ($n = 150$ and $n = 53$), respectively. Nevertheless, for the stereotactic biopsy procedure, the technique is even more beneficial if the detection is performed in situ before the tissue excision as already tested with an endoscopic system by Eigenbrod et al. [7] to further reduce the number of samples that have to be taken and thus minimize the risk of complications.

Figure 2 Excitation spectrum of PpIX in tissue (emission intensity measured at 710 nm). PpIX can be excited in different spectral regions, ranging from violet (around 405 nm) to red (around 633 nm) light.

PpIX fluorescence can be excited in different spectral regions (see Figure 2), each offering advantages and disadvantages especially in terms of fluorescence efficiency and tissue optical properties. Commonly, violet light is used for PpIX excitation in fluorescence-guided resection. Light in this spectral range, however, is highly absorbed by blood, hence blood-covered tumor tissue may easily be missed. During open brain surgery, the blood often but not always can be removed by rinsing the probe or the surgical site; yet, even small amounts of blood will affect proper interpretation of the signals. [32-34] During stereotactic biopsy, however, rinsing appears to be even more challenging because the (diluted) blood cannot drain off and suctioning through the needle would draw even more blood towards the probe tip, at least for conventional needle designs. Even if no blood vessel disruption occurs, the micro-vessels in the tissue right next to the probe do sometimes obstruct the fluorescence signals. Using red light, which is already applied for fluorescence monitoring of PpIX concentrations during photodynamic therapy [35], seems to be a promising alternative as it is considerably less attenuated by blood. In this study, the potentials of the excitation wavelengths $\lambda_{\text{exc}} = 405 \text{ nm}$ and $\lambda_{\text{exc}} = 633 \text{ nm}$ are comparatively investigated with regard to their clinical applicability in stereotactic biopsy.

2. MATERIALS AND METHODS

2.1 Production of PpIX phantoms

Both liquid and agarose-gel phantoms were mixed to model the optical properties of brain tumors, using Lipovenös® (Fresenius Kabi GmbH, Bad Homburg, Germany) and ink (“brilliant black 4001”, Pelikan GmbH, Hannover, Germany) for scattering and absorption, respectively. Separate phantoms were produced for the excitation wavelengths $\lambda_{\text{exc}} = 405 \text{ nm}$ and $\lambda_{\text{exc}} = 633 \text{ nm}$. For this purpose, values referring to brain tumor tissue given by Gebhart et al. (determined ex vivo on $n = 39$ samples) [36] were taken as reference, which are in good accordance with the astrocytoma data published by Yaroslavsky et al. (ex vivo, $n = 4$) [37]. More recent publications from Pitzschke et al. concerning the optical properties of human (ex vivo, $n = 1$) [38] and rabbit (in vivo, $n = 7$) [39] brain tissue were additionally consulted for the red excitation. A comparison with the values of Gebhart et al. showed a reasonable accordance for the absorption coefficient and indicated that the reduced scattering coefficient of brain tumor tissue lies between those of human gray and white matter, a fact that had already been reported by Gebhart et al. The required concentrations of the phantom ingredients have been determined via literature data [40] (Lipovenös®) and integrating sphere measurements (ink), assuming a linear behavior and non-interacting particles. In Table 1, the used concentrations and simulated optical properties of the liquid phantoms are listed. In the following, μ_a denotes the absorption coefficient and $\mu_s' = \mu_s(1-g)$ the reduced scattering coefficient where μ_s and g are the scattering coefficient and anisotropy factor, respectively.

Table 1 Liquid phantoms: Lipovenös® and ink concentrations as well as simulated μ_a and μ_s' .

Phantom (exc. wavelength)	ink concentration (vol/vol) [%]	μ_a [mm ⁻¹]	Lipovenös® concentration (mass/vol soybean oil) [%]	μ_s' [mm ⁻¹]
$\lambda_{\text{exc}} = 405$ nm	0.9	1.4	2.3	3.9
$\lambda_{\text{exc}} = 633$ nm	0.1	0.09	2.0	2.2

Agarose-gel phantoms were made following the protocol of Cubeddu et al. [41] To simulate the mechanical properties of brain tissue, 0.6% (m/v) agarose powder (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used. [42-44] As the addition of agarose decreases the reduced scattering coefficient of Lipovenös® (reduction by 20% at 0.6% agar) [41], slightly different Lipovenös® concentrations were used for the agar phantoms (see Table 2).

Table 2 Agar phantoms: Lipovenös® and ink concentrations as well as simulated μ_a and μ_s' .

Phantom (exc. wavelength)	ink concentration (vol/vol) [%]	μ_a [mm ⁻¹]	Lipovenös® concentration (mass/vol soybean oil) [%]	μ_s' [mm ⁻¹]
$\lambda_{\text{exc}} = 405$ nm	0.9	1.4	2.9	3.9
$\lambda_{\text{exc}} = 633$ nm	0.1	0.09	2.5	2.2

PpIX powder (Sigma-Aldrich Chemie GmbH, Munich, Germany) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemie GmbH, Munich, Germany) and added as fluorescence agent to both liquid and agar phantoms at varying concentrations.

2.2 Measurement setup

As shown in Figure 3, a dual-mode experimental setup was designed for two different excitation wavelengths (mode 1: $\lambda_{\text{exc}} = 405$ nm, mode 2: $\lambda_{\text{exc}} = 633$ nm). Light of both wavelengths was coupled into a single bare fiber with a numerical aperture (NA) of 0.22 and a core diameter of 200 μm (UM22-200, Thorlabs GmbH, Dachau, Germany) and directed onto a PpIX containing liquid or agar phantom. The respective excitation light power at the distal end of the fiber (next to the irradiated medium) was determined as 0.9 mW ($\lambda_{\text{exc}} = 405$ nm) and 5.5 mW ($\lambda_{\text{exc}} = 633$ nm) using a standard power meter (PM100D, sensing head: S130C, Thorlabs GmbH, Dachau, Germany). The fluorescence light was collected with the same fiber and transmitted to a detection spectrometer (USB2000+, Ocean Optics GmbH, Ostfildern, Germany), which was recorded using OOIBase32 (Ocean Optics GmbH, Ostfildern, Germany). The integration time ranged between 15 ms and 500 ms. Data processing was performed with MATLAB (The MathWorks, Inc., Natick, MA, USA) and SigmaPlot (Systat Software GmbH, Erkrath, Germany); for data smoothing, the MATLAB function *filter* was used (calculating a moving average over 10 data points, corresponding to $\Delta\lambda \approx 4$ nm).

In mode 1, violet light from a 405 nm laser diode was reflected towards the fiber by dichroic mirror 1 (cut-off at 440 nm). Dichroic mirror 2 (cut-off at 665 nm) was removed to let fluorescence light between 600 nm and 750 nm pass to the spectrometer. In mode 2, dichroic mirror 2 was required to couple the beam of a 633 nm HeNe laser into the light path. Fluorescence light around 700 nm was guided through both dichroic mirrors towards the spectrometer. Switching between the two modes was enabled by removing (mode 1) or adding (mode 2) dichroic mirror 2 and by introducing the corresponding long pass filter. Cut-on wavelengths of 470 nm and 660 nm were used for $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{exc}} = 633$ nm, respectively.

Figure 3 Dual-mode experimental setup for comparing PpIX excitation wavelengths 405 nm and 633 nm. In mode 1, only a 405 nm laser diode is switched on, the long pass filter 470 (cut-on wavelength: 470 nm) is selected and dichroic mirror 2 is hinged aside. In mode 2, only a 633 nm HeNe laser is switched on, the long pass filter 660 (cut-on wavelength: 660 nm) is selected and both dichroic mirrors are in the light path.

Three different types of measurements were performed with this setup, pertaining to the determination of PpIX detection limits (Section 2.3.1), the attenuation of PpIX signals through blood layers of well-defined thicknesses (Section 2.4.1) and the effectively expectable blood layer thickness in the clinical measurement situation (Section 2.5).

2.3 Determination of PpIX detection limits

2.3.1 Measurements on semi-infinite phantoms

PpIX detection limits for the above-mentioned detection fiber were determined on agar phantoms. For stereotactic biopsies, especially the sensitivity to the bulk tumor is interesting, thus the phantoms were made virtually infinitely large (cylindrical shape, diameter: 8.5 cm, height: 1.5 cm) as compared to the optical penetration depth (0.21 mm for $\lambda_{\text{exc}} = 405$ nm and 1.3 mm for $\lambda_{\text{exc}} = 633$ nm, respectively) and the fiber core diameter (200 μm) to simulate the detection of large tumor volumes. To analyze the sensitivity to smaller tumor parts, ray tracing simulations were performed (see Section 2.3.2). For both excitation wavelengths, four agar phantoms with varying PpIX concentrations and one reference phantom (without PpIX) were mixed. The concentration values, given in nM (nanomoles per liter), can be found in Table 3. Due to the expected difference in sensitivity between the two excitation wavelengths, ten times higher concentration values were used for 633 nm as compared to 405 nm.

Table 3 PpIX concentrations used for the determination of PpIX detection limits.

$\lambda_{\text{exc}} = 405$ nm	50 nM	25 nM	12.5 nM	6.25 nM	0 nM (reference)
$\lambda_{\text{exc}} = 633$ nm	500 nM	250 nM	125 nM	62.5 nM	0 nM (reference)

Bringing the distal fiber end in contact with the agar phantom, the fluorescence of each phantom was measured four times at different sites to minimize the effect of photobleaching. To correct for background fluorescence, the signal of the reference phantoms was measured under the same conditions, normalized to the signal of the PpIX phantoms at 560 nm ($\lambda_{\text{exc}} = 405$ nm) and 750 nm ($\lambda_{\text{exc}} = 633$ nm), respectively, and subtracted from the raw spectra of the PpIX phantoms. Afterwards, the four single spectra of each concentration were averaged and divided by the integration time. The detection threshold was set to the threefold of the background noise level. To examine the proportionality between PpIX concentration and fluorescence signal, a linear regression of the peak intensities (averaged between 632 nm and 634 nm for $\lambda_{\text{exc}} = 405$ nm and between 701 nm and 703 nm for $\lambda_{\text{exc}} = 633$ nm) as a function of the concentration was performed. The detection limits were determined by equating the measured fluorescence intensity per concentration with the detection threshold.

2.3.2 Ray tracing simulations with variable tumor size

To investigate the sensitivity to smaller tumor parts (e.g. isolated tumor cell nests in the infiltration zone of the lesion), ray tracing simulations were performed using the Monte Carlo-based software TracePro (Lambda Research Corporation, Littleton, MA, USA). A tumor cube (fluorescent cube with optical properties of brain tumor taken from [36] and a constant PpIX concentration of 2 μM) with variable edge length between 0.04 mm and 15 mm (the latter one considered infinitely large as compared to fiber diameter and light penetration depth) was embedded in a brain cube (non-fluorescent cube with optical properties of gray matter compiled from [36], [37] and [45]) with an edge length of 30 mm. As the simulation was intended to mimic the experimental setup, one surface of the tumor cube was in contact with the excitation/detection fiber (core diameter 200 μm , NA = 0.22). For both excitation wavelengths, the detected fluorescence signals for each tumor size were normalized to the signal of the biggest tumor cube (15 mm edge length). More detailed information on model and parameters used for the simulations is provided in the Supporting Information (Part 1).

2.4 Attenuation through blood layers of well-defined thicknesses

2.4.1 Measurements

In Figure 4, the experimental setting of the blood layer measurements is illustrated, outlining the phantom-part of Figure 3, in this case to determine the attenuation of PpIX fluorescence signals caused by particular blood layers between fiber and fluorescence phantom. The 200 μm fiber was directed onto a 2 μM liquid PpIX phantom, which was filled into one well of a 96-well plate. Blood and water layer systems were alternatively placed on top of the phantom, leaving a small gap of about 2 mm in between. This gap was experimentally necessary to avoid contaminating the layer system with phantom liquid and thus enable unimpaired measurements on reference phantoms (see below) with the identical blood or water layer systems. Additionally, a small gap of about 1 mm was left between the layer system and the distal fiber end to prevent fiber damage. The layer systems were prepared by attaching distance pieces of defined thicknesses on an object slide (1.0 mm thickness), leaving free space in between. A droplet of blood or water (for reference) was then dripped into this space and spread by putting a cover slide (0.17 mm thickness) on top of it.

Figure 4 Illustration of blood layer measurements. The 200 μm fiber (NA = 0.22) of the measurement setup (see Figure 3) is used to excite and detect fluorescence in a 2 μM liquid PpIX phantom, which is filled into one well of a 96-well plate. Layer systems consisting of an object slide, spacers (tape strips or PDMS layers with indentation), a liquid droplet (blood or water) and a cover slide are placed on top of the liquid phantom.

For $\lambda_{\text{exc}} = 405 \text{ nm}$, layers of 10 μm , 20 μm and 50 μm were applied, for $\lambda_{\text{exc}} = 633 \text{ nm}$ 10 μm , 20 μm , 50 μm , 100 μm , 150 μm , 200 μm and 400 μm . Several layers of normal (thickness: 50 $\mu\text{m} \pm 5 \mu\text{m}$) and double adhesive (thickness: 200 $\mu\text{m} \pm 20 \mu\text{m}$) tape strips were used to create distances of 50 μm and more between the object slide and the cover slide. The tape strip thicknesses were measured with a Vernier caliper, putting at least five layers on top of each other to improve precision. To provide smaller distances, transparent polydimethylsiloxane (PDMS) layers were fabricated using SU-8 negative photoresist coated on silicon wafers. The thicknesses of the PDMS layers were 10.0 $\mu\text{m} \pm 0.1 \mu\text{m}$ and

18.7 $\mu\text{m} \pm 0.1 \mu\text{m}$ as was measured on the silicon wafers with a stylus profilometer (Veeco Dektak 6M, Veeco Instruments Inc., Plainview, NY, USA). Since the preparation of the final blood and water layer systems could not be performed with equivalent precision, layer thicknesses with larger uncertainties, i.e. 10 $\mu\text{m} \pm 5 \mu\text{m}$ and 20 $\mu\text{m} \pm 5 \mu\text{m}$, were assumed. The fluorescence signals were obtained letting both excitation and emission light pass through the blood layers. These signals, corrected for background fluorescence by using reference phantoms as described in Section 2.3.1, were then divided by the likewise corrected signals obtained when replacing the blood layers with water layers of the same thickness. These water signals were acquired under identical conditions, especially the distance between fiber end and phantom surface was kept constant. The hereby obtained relative intensities were finally evaluated at the peak wavelengths of the emission spectra as described in Section 2.3.1. This measurement and evaluation procedure was repeated three times for each layer and excitation wavelength. For each thickness and excitation wavelength, three sets of blood/water layers were prepared and measured.

It has to be stated that the details of the excitation and fluorescence capture geometry realized here have an effect on the absolute fluorescence signals. The excitation beam, for instance, is expanded due to the presence of object and cover slides as well as the mentioned air gaps. In these experiments, however, only the ratio of the fluorescence signals measured through blood versus through water was of interest and for each single measurement pair (including blood and water layers of the same thickness), a constant excitation and fluorescence capture geometry could be assured. Thus, the effect of the experimental geometry on the results can be neglected.

2.4.2 Ray tracing simulations

To affirm the results of these blood layer measurements, ray tracing simulations reproducing the experimental settings described in Section 2.4.1 were performed using the Monte Carlo-based software TracePro (Lambda Research Corporation, Littleton, MA, USA). More detailed information on model and parameters used for the simulations is provided in the Supporting Information (Part 2).

2.5 Contact measurements on agar phantoms

With these experiments, minor bleedings during stereotactic biopsy should be mimicked. The blood layer thickness expectable when driving the needle forward through the blood until getting in touch with the tissue was determined with contact measurements on agar phantoms (PpIX concentration: 5 μM) as depicted in Figure 5.

Figure 5 Illustration of contact measurements on agar phantoms. (a) A 200 μm fiber (NA = 0.22) with SMA connector at the distal end is in contact with the agar phantom (reference measurement). (b) After putting a blood droplet on the phantom, the contact position of the fiber is restored and the signal reduction as compared to the reference measurement is recorded.

A 200 μm fiber (NA = 0.22) with SMA connector at the distal end, installed at the measurement setup described in Section 2.2, was used to simulate the blood extrusion of a biopsy needle with one integrated excitation and detection fiber when getting in contact with brain tissue. For this purpose, the distal fiber tip was attached to a LabVIEW-controlled stepping motor. Exact contact positions could be identified exploiting the jump in the remission or fluorescence signal that arose

when the fiber tip touched the phantom and refraction index mismatches (glass – air and air – phantom) disappeared. By means of the stepping motor, these positions could be reproduced with micrometer precision.

After identifying the vertical contact position, the fiber was driven upwards and a blood droplet (about 50 μ l) was dripped onto the phantom surface. Then the contact position of the fiber was restored. The detectable signal through blood (corrected for background fluorescence as described in Section 2.3.1) was divided by the corrected signal without blood and evaluated at the peak emission wavelength as described in Section 2.3.1. This measurement scheme was performed five times for each excitation wavelength with moving the phantom a few millimeters horizontally after each measurement to account for photobleaching.

3. RESULTS AND DISCUSSION

3.1 PpIX detection limits

3.1.1 Measurements on semi-infinite phantoms

Exemplary PpIX fluorescence spectra for both excitation wavelengths are displayed in Figure 6a. Note that even for the lowest of the used PpIX concentrations, spectra with reasonable signal quality could be recorded. In Figure 6b, the peak intensities of all PpIX spectra divided by the excitation power are plotted against the concentration.

Figure 6 (a) Exemplary averaged and smoothed PpIX fluorescence spectra ($n = 4$) for 405 nm (0.9 mW excitation power) and 633 nm (5.5 mW excitation power) excitation. For both excitation wavelengths, the spectra with lowest PpIX concentration are shown. (b) Fluorescence intensity per excitation power as a function of the PpIX concentration. For both excitation wavelengths, a linear relation was observed. The slopes are given in counts/(ms*mW*nM).

The results of the linear regression (R^2 values of 1.000 and 0.994 for $\lambda_{exc} = 405\text{ nm}$ and $\lambda_{exc} = 633\text{ nm}$, respectively) show that the assumed proportionality between fluorophore concentration and fluorescence signal is valid in the investigated concentration range. Consequently, a signal intensity per concentration and excitation power can be calculated. Defining the lowest detectable signal as the threefold of the background noise level, which is about one count per millisecond, this leads to detection limits of 4.4 nM ($\lambda_{exc} = 405\text{ nm}$, 0.9 mW excitation power) and 38 nM ($\lambda_{exc} = 633\text{ nm}$, 5.5 mW excitation power). Considering the respective excitation power, these numbers may be converted to detection limits in terms of fluorophore concentration times the excitation power, which indicate the lowest detectable concentrations at a given laser power. These detection limits were determined as 4.0 nM·mW for $\lambda_{exc} = 405\text{ nm}$ and 200 nM·mW for $\lambda_{exc} = 633\text{ nm}$.

Considering only the fluorescence properties of pure PpIX, which were extracted from measurements in a clear PpIX/DMSO solution (see Supplementary Table S2), the fluorescence signal excited at $\lambda_{exc} = 633\text{ nm}$ and detected at $\lambda_{em} = 702\text{ nm}$ should be about 100 times weaker than for $\lambda_{exc} = 405\text{ nm}$ and $\lambda_{em} = 633\text{ nm}$. Due to the different optical

properties of brain tumor tissue for these wavelengths, however, this factor reduces to 50 in case of the brain tissue equivalent optical phantoms investigated here.

The potential impact of photobleaching had been tested in two ways. First it was verified that the total signal loss due to photobleaching during the selected integration times (between 15 ms and 500 ms) was in any case below 10%. Repeated measurements were performed on different sites of the phantom. To estimate the effect of photobleaching in the clinical situation, photobleaching measurements on excised GBM tissue after 5-ALA administration were conducted with the experimental setup used in this study. The induced photobleaching lifetimes τ (time periods after which the fluorescence signal has decreased to $1/e$) were determined as $\tau_{405} = (9 \pm 2)$ s and $\tau_{633} = (21 \pm 2)$ s for $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{exc}} = 633$ nm, respectively. Assuming that the actual measurement period (when the fiber is in contact with the tumor tissue) does not have to be longer than 1 s to get a sufficient signal, photobleaching should not be a limiting factor, even if needle approach and repeated light exposure are considered as well.

3.1.2 Ray tracing simulations with variable tumor size

In Figure 7a, the dependency of the detected PpIX signal on the tumor size is shown. For both excitation wavelengths, the fluorescence intensity increases with rising tumor size up to a saturation level, thereby following a sigmoidal curve shape in a semi-logarithmic plot. In the investigated tumor size range, the relative intensity (as compared to infinitely large tumors) is always higher for $\lambda_{\text{exc}} = 405$ nm than for $\lambda_{\text{exc}} = 633$ nm with a maximum ratio of about 2 for infinitesimally small tumors (see Figure 7b). The edge lengths of the tumor cube at which the intensity has reached 95% of the maximum (referred to as critical length scales) are 0.55 mm and 1.8 mm for $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{exc}} = 633$ nm, respectively.

Figure 7 (a) Simulated fluorescence intensity as a function of tumor cube edge length. For each excitation wavelength, the intensities were normalized to the signal of the tumor cube with the largest edge length (15 mm). Error bars indicate standard errors of the means obtained via three simulations with different random number seeds.

(b) Ratio of normalized intensities 405 nm / 633 nm.

Above these thresholds, the detected PpIX signal does not depend on the tumor size and the detection limits presented in Section 3.1.1 are valid. However, for smaller tumor parts below the critical length scales, the detected intensity per PpIX concentration diminishes, leading to higher detection limits than determined above. This intensity loss is stronger for $\lambda_{\text{exc}} = 633$ nm than for $\lambda_{\text{exc}} = 405$ nm, which can be explained by the higher penetration depth of red as compared to violet light. According to the presented simulations, the sensitivity ratio rises from 50 to 100 for infinitesimally small tumors where the mean free path length of the excitation or fluorescence light is large compared to the tumor size. This increase is in accordance with the determined fluorescence properties of pure PpIX (see Section 3.1.1 and Supplementary Table S2).

Considering only tumor parts larger than 1 mm^3 , which is the biopsy volume usually required for molecular diagnosis, detection limits stay constant for $\lambda_{\text{exc}} = 405$ nm and are only weakly increased for $\lambda_{\text{exc}} = 633$ nm: Given a tumor size of 1 mm^3 where the relative fluorescence intensities are 0.99 and 0.89, the limits are $4.0 \text{ nM} \cdot \text{mW}$ and $220 \text{ nM} \cdot \text{mW}$ for $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{exc}} = 633$ nm, respectively. Sampling of smaller tumor parts would be subject to a declined sensitivity, but is not desirable because the specimen would necessarily contain both tumor and healthy tissue. Apart from stereotactic biopsies where the planned target is usually the vital margin of the bulk tumor, these results are also particularly relevant

for open brain surgery – fluorescence-guided resection and surgical biopsies – where it is fundamental to achieve a total resection and to gain histopathological information on different tumor parts.

3.2 Attenuation through blood layers of well-defined thicknesses

The attenuation of PpIX signals caused by blood layers between fiber and phantom is extremely different for the two excitation wavelengths. This is illustrated in Figure 8a where the spectra recorded through 10 μm layers of blood and water are shown exemplarily. In Figure 8b, all measured relative intensities, plotted against the blood layer thickness, are compared with the results obtained from ray tracing simulations.

Figure 8 (a) Averaged and smoothed PpIX fluorescence spectra ($n = 3$) detected through 10 μm layers of water and blood, respectively. For each excitation wavelength, the spectra were normalized to the peak intensity of the spectrum recorded through water. The layer systems were freshly assembled for each measurement.

(b) Relative peak intensity as a function of the blood layer thickness. Measurement (meas.) and simulation (sim.) results are shown for both excitation wavelengths. Vertical error bars indicate standard errors of the means obtained via three independent measurements with freshly assembled layer systems or three simulations with different random number seeds, respectively. Horizontal error bars reflect the thickness uncertainties of the layer systems. Single and double ($\lambda_{\text{exc}} = 633 \text{ nm}$) or only single ($\lambda_{\text{exc}} = 405 \text{ nm}$) exponential functions were fit to the simulation results.

While for $\lambda_{\text{exc}} = 405 \text{ nm}$, the fluorescence is already blocked by a 50 μm blood layer, even layers of 400 μm still allow relative signal amplitudes (blood versus water) of about 5% for $\lambda_{\text{exc}} = 633 \text{ nm}$. The intensities detected through thinner blood layers drop exponentially with the layer thickness. It can be seen that experimental and simulated results are in good accordance. Considering the uncertainties given by standard errors of the mean ($n = 3$), only two outliers can be identified, one of which (at 10 μm for $\lambda_{\text{exc}} = 405 \text{ nm}$) is located in a range of very steep decrease. The simulation results for $\lambda_{\text{exc}} = 633 \text{ nm}$ could be best described with a double-exponential fit

$$I_r = a \cdot \exp(-b \cdot d) + (1 - a) \cdot \exp(-c \cdot d) \quad (1)$$

where I_r and d denote the relative peak intensity and the blood layer thickness, respectively, and $a = 0.59 \pm 0.20$, $b = (17.8 \pm 5.1) \text{ mm}^{-1}$ and $c = (5.2 \pm 1.8) \text{ mm}^{-1}$ are the fit parameters. The indicated uncertainties denote standard errors. A single-exponential fit

$$I_r = \exp(-b \cdot d) \quad (2)$$

with $b = (10.5 \pm 0.7) \text{ mm}^{-1}$ was also acceptable for thinner layers ($d < 200 \text{ }\mu\text{m}$), but overestimated the signal attenuation through thicker layers ($d > 200 \text{ }\mu\text{m}$). For $\lambda_{\text{exc}} = 405 \text{ nm}$, only three sampling points were given so that only a single-exponential fit as in Equation (2) was possible, yielding $b = (179 \pm 3) \text{ mm}^{-1}$. A theoretical interpretation of the single-exponential fit parameter b can be found in the Supporting Information (Part 3).

3.3 Contact measurements on agar phantoms

The relative PpIX peak intensities detected after immersing the distal fiber end in the blood droplet until getting in contact with the agar phantom surface were quite reproducible: Averaging of five single measurements for each excitation wavelength performed at different positions of the phantom yielded values of $(0.3 \pm 0.3)\%$ for $\lambda_{\text{exc}} = 405 \text{ nm}$ and $(74.5 \pm 1.5)\%$ for $\lambda_{\text{exc}} = 633 \text{ nm}$, the indicated uncertainties denoting standard errors of the mean. The respective averaged spectra are shown in Figure 9. When driving the fiber even further into the phantom, no signal recovery was visible until 1 mm below the phantom surface.

Figure 9 Averaged and smoothed PpIX fluorescence spectra ($n = 5$) detected in contact with the agar surface before and after dripping a blood droplet onto the phantom. **(a)** Excitation at 405 nm. **(b)** Excitation at 633 nm.

By means of Equations (1) ($\lambda_{\text{exc}} = 633 \text{ nm}$) and (2) ($\lambda_{\text{exc}} = 405 \text{ nm}$) and the corresponding fit results, the ratio of the peak intensities with and without blood layer can be converted to thicknesses of $32 \text{ }\mu\text{m}$ ($\lambda_{\text{exc}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 633 \text{ nm}$) and $24 \text{ }\mu\text{m}$ ($\lambda_{\text{exc}} = 633 \text{ nm}$, $\lambda_{\text{em}} = 702 \text{ nm}$). Hence, in case of minor bleedings during stereotactic biopsy, fluorescence obscuring blood layers of about $20 \text{ }\mu\text{m}$ to $40 \text{ }\mu\text{m}$ should be reckoned with when advancing the biopsy needle until getting in contact with the investigated brain tissue.

4. CONCLUSION

With regard to their suitability for fiber-based PpIX detection in brain tumor tissue, both examined excitation wavelengths feature notable advantages and drawbacks:

On the one hand, violet excitation at the Soret peak (around 405 nm), which represents the maximum of the PpIX excitation spectrum, allows for a considerably higher sensitivity: Realistic PpIX detection measurements in contact with bulk tumor mimicking phantoms showed an enhancement by a factor of 50 for equal excitation powers as compared to the red excitation at 633 nm (see Figure 6b). For smaller tumor parts, this factor is elevated up to a maximum of 100. This yields not only better signal to noise ratios, but also enables the detection of tumor entities with a fainter fluorescence as for example grade III glioma or peripheral regions of the tumor. Additionally, a broad and characteristic emission spectrum is induced, allowing the extraction of valuable spectral information. [46, 47] Moreover, the use of a 405 nm light source also

leads to the excitation of tissue autofluorescence, which could be used as an additional parameter for tissue discrimination [48-50] or serve as reference for the normalization of PpIX signals [27].

On the other hand, red excitation at 633 nm offers an increased tissue penetration, which extenuates the effect of the comparably low fluorescence excitation efficiency of this wavelength, at least for tumors that are thick enough to cover the whole penetration depth. Based on the bulk-tumor detection limit of 200 nM·mW for $\lambda_{\text{exc}} = 633$ nm (see Section 3.1.1), vital GBM tissue, which typically exhibits PpIX concentrations of a few μM after 5-ALA administration at 20 mg per kg body weight [24, 33, 51], should well be detectable with a 200 μm diameter fiber ($\text{NA} = 0.22$) in contact with the tissue. As shown in Section 3.1.2, the detection limit for smaller tumor parts that are still big enough to be sampled during stereotactic biopsy is only slightly increased. As a consequence, the currently approved and conventionally applied 5-ALA dose (20 mg per kg body weight) is assumed to be sufficient also for 633-nm-excited PpIX fluorescence; higher doses with a higher risk of producing side effects such as skin photosensitivity or nausea and vomiting [52] are not necessary.

Another advantage of the increased penetration depth of 633 nm (1.3 mm instead of 0.21 mm for 405 nm) is that for conventional biopsy needles, a better congruence between mechanical (usually a few mm^3) and optically probed volume can be achieved. For example, a small layer containing PpIX, which is on top of normal brain tissue, would deliver a strong signal using 405 nm excitation. Thus, a biopsy would possibly be taken at this site, which would largely consist of normal tissue, though. In contrast, the risk that tumor tissue which is located outside the needle's coverage and excited with 633 nm delivers a signal amplitude that induces the surgeon to take a biopsy is rather low.

Finally, the results of Sections 3.2 and 3.3 clearly show that 633 nm excitation is unambiguously superior in case of blood-covered tumor tissue. Even though the actual numbers depend on the exact excitation and detection geometry, the qualitative result is generally valid and entails important consequences as when inserting a needle into human brain, minor blood vessel ruptures followed by blood moistening of the analyzed tissue can never be completely prevented. The presented contact measurements on agar phantoms demonstrate that one has to reckon with remaining blood layers between 20 μm and 40 μm even when the biopsy needle is driven through the blood until the distal fiber end is in contact with the tissue. This refers to the range where the difference in relative signal magnitudes between $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{exc}} = 633$ nm is particularly large (see Figure 8b) and the risk of not detecting blood-covered tumor tissue is considerably high for $\lambda_{\text{exc}} = 405$ nm. Beyond stereotactic biopsies, these results on the practical implications of blood interference are also very relevant for fluorescence guidance in open brain surgery, e.g. when examining the resection cavity with a fiber optical probe as described in [27, 28].

The results of this preclinical experimental evaluation indicate that $\lambda_{\text{exc}} = 633$ nm seems to be the wavelength of choice for PpIX detection during stereotactic biopsies of high-grade gliomas. Despite the reduced sensitivity as compared to $\lambda_{\text{exc}} = 405$ nm, at least grade IV glioma (GBM) should well be detectable with single-fiber probes, and in case of unavoidable bleeding, $\lambda_{\text{exc}} = 633$ nm seems to be significantly better suited. Nevertheless, devices where both excitation wavelengths are used simultaneously might be of even greater benefit because the advantages of both excitation modes could be combined. Finally, it has to be emphasized that the presented results, especially the potentials of the red excitation in case of blood interference, are not only interesting for the stereotactic biopsy procedure, but also for other applications such as photodynamic therapy or fluorescence-guided resection.

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GRAPHICAL ABSTRACT

Abstract figure PpIX signal attenuation through clinically relevant blood layers for 405 nm (violet) and 633 nm (red) excitation.

The safety and reliability of stereotactic brain tumor biopsies can be improved through fluorescence-based tumor detection: After administering 5-aminolevulinic acid, the endogenous fluorophore protoporphyrin IX (PpIX) is accumulated in vital tumor tissue. Here, two different PpIX excitation wavelengths are investigated with regard to their clinical practicability. While 405 nm allows for a higher sensitivity in general, blood covered tumor tissue only produces a non-vanishing signal when excited with 633 nm.

SUPPORTING INFORMATION FOR

405 nm versus 633 nm for protoporphyrin IX excitation in fluorescence-guided stereotactic biopsy of brain tumors

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Part 1: Ray tracing simulations of PpIX detection for varying tumor size

To analyze the effect of the tumor size on the detected PpIX signal – and thus on the detection limits –, ray tracing simulations for the three wavelengths 405 nm (excitation), 633 nm (excitation and emission) and 702 nm (emission) were performed using the Monte Carlo-based software TracePro (Lambda Research Corporation, Littleton, MA, USA). The applied model is illustrated in Supplementary Figure S1.

Supplementary Figure S1 Simulation model used to analyze the effect of the tumor size on the detected PpIX signal. A fluorescent tumor cube with varying edge length between 0.04 mm and (as shown here) 15 mm is embedded in a non-fluorescent brain cube. The tumor cube is in contact with the lower end of the excitation and detection fiber.

The model basically consisted of three parts: a non-fluorescent brain cube with an edge length of 30 mm, a fluorescent tumor cube with variable edge length, which was embedded into the brain cube, and a fiber. In the x-y-plane, the tumor cube was centrally arranged within the brain cube; in z-direction, the tumor cube was bound to the upper surface of the brain cube (at $z = 10$ mm). The fiber, composed of core, cladding and coating, enclosed the z-axis and its lower end was in contact with the tumor cube. A circular light source with 10^5 emitted rays restricted to a cone with a half-angle of 12.7° (corresponding to a numerical aperture of 0.22) was positioned in the fiber core at $z = 0.5$ mm. The upper surface of the fiber core ($z = 0$) served as detector.

For each tumor cube edge length and excitation wavelength, three simulations with different random number seeds were performed. To realize tumor cubes with different edge lengths, a macro was written that inserted the tumor cube with the required edge length into the predefined model containing only fiber and brain cube. Edge lengths between 0.04 mm and 15 mm were used. As this maximum value was much larger than the fiber core diameter (200 μm) and the penetration depths of all considered wavelengths (0.21 mm, 1.3 mm and 1.6 mm for 405 nm, 633 nm and 702 nm, respectively), it could be regarded as infinitely large. The same applied to the brain cube exhibiting an edge length of 30 mm. Hence, the excitation and detection geometry for the largest tumor cube could be identified with the measurements on semi-infinite phantoms described in the main article, Section 2.3.1. For both excitation wavelengths, the detected fluorescence signal for each tumor size was thus normalized to the signal of the tumor cube with 15 mm edge length.

Supplementary Table S1 Optical parameters used for the tumor size simulations.

Parameter	n	μ_a [mm ⁻¹]				μ_s' [mm ⁻¹]		
Wavelength [nm]	500	405	633	702	405	633	702	
Core	1.46	-	-	-	-	-	-	
Cladding	1.44	-	-	-	-	-	-	
Coating	1.66	-	-	-	-	-	-	
Tumor cube	1.37	1.4	0.09	0.07	3.9	2.2	1.8	
Brain cube	1.37	0.6	0.05	0.04	2.0	1.0	0.9	

In Supplementary Table S1, the refraction indices (n) and optical properties of the simulation objects are listed. The optical properties of the tumor cube (simulating brain tumor tissue) were taken from Gebhart et al. [1]; the optical properties of the brain cube (simulating gray matter) were compiled from Gebhart et al. [1], Yaroslavsky et al [2] and Tuchin [3]. Fluorescence properties, listed in Supplementary Table S2, were extracted from PpIX measurements in dimethyl sulfoxide (DMSO) performed with commercial fluorescence (FluoroMax-2®, Horiba Jobin Yvon GmbH, Unterhaching, Germany) and absorption (Lambda 40, Perkin Elmer GmbH, Überlingen, Germany) spectrometers.

Supplementary Table S2 Fluorescence properties of PpIX applied to the tumor cube.

Wavelength [nm]	rel. absorption	rel. excitation	rel. emission
405	0.99	0.99	-
633	0.03	0.03	1
702	-	-	0.31
Peak Molar Extinction [$\text{l}/(\text{mole}\cdot\text{cm})$]		1.77e5	
Concentration [μM]		2.0	

Part 2: Ray tracing simulations of light attenuation through blood layers

To affirm the results of the blood layer measurements described in Section 2.4.1, ray tracing simulations for the three wavelengths 405 nm (excitation), 633 nm (excitation and emission) and 702 nm (emission) were performed using the Monte Carlo-based software TracePro (Lambda Research Corporation, Littleton, MA, USA). The applied model, which reflected the geometrical dimensions of the measurements, is illustrated in Supplementary Figure S2.

Supplementary Figure S2 Simulation model used to affirm the results of the blood layer measurements. The object lengths in z direction are indicated. Except the fiber, exhibiting the dimensions of the type UM22-200 (Thorlabs GmbH, Dachau, Germany), all cylindrical objects have a diameter of 6.8 mm corresponding to the wells of a 96-well plate.

The fiber (including core, cladding and coating) was adopted from the model used for the simulations with varying tumor size (see Supporting Information (Part 1)). A circular light source with 10^6 emitted rays restricted to a cone with a half-angle of 12.7° (corresponding to a numerical aperture of 0.22) was positioned in the fiber core at $z = 0.5$ mm. The upper surface of the fiber core ($z = 0$) served as detector. In Supplementary Table S3, the refraction indices (n) and optical properties of the objects are listed.

Supplementary Table S3 Optical parameters used for the blood layer simulations.

Parameter	n	μ_a [mm ⁻¹]				μ_s [mm ⁻¹]			g		
Wavelength [nm]	500	405	633	702	405	633	702	405	633	702	

Core	1.46	-	-	-	-	-	-	-	-	-
Cladding	1.44	-	-	-	-	-	-	-	-	-
Coating	1.66	-	-	-	-	-	-	-	-	-
Cover slide	1.52	-	-	-	-	-	-	-	-	-
Blood	1.39	140.7	0.275	0.15	41.4	89.0	86.3	0.747	0.979	0.980
Water	1.33	2e-5	3e-4	6e-4	-	-	-	-	-	-
Object slide	1.52	-	-	-	-	-	-	-	-	-
Phantom (exc. 405 nm)	1.33	1.4	0.9	-	26	8.1	-	0.85	0.69	-
Phantom (exc. 633 nm)	1.33	-	0.09	0.015	-	7.1	5.6		0.69	0.64

The optical parameters of the phantoms were calculated from the Lipovenös® and ink concentrations used for the liquid phantoms (see main article, Table 1). The absorption coefficients of water were given in the database of the software, scattering in water was neglected; the optical properties of blood are averages of Bosschaart et al. [4] and Prahl [5] for μ_a and of Bosschaart et al. [4] and Tuchin [3] for μ_s and g , respectively. The given data were interpolated to obtain the values at the required wavelengths where necessary. The used fluorescence properties are given in Supplementary Table S2. To simulate all measurement points, a macro was written that inserted water or blood layers of the required thicknesses at the correct position and displaced all objects below correspondingly (see Supplementary Figure S2). For each thickness and excitation wavelength, three simulations with different random number seeds were performed both for water and blood and the average ratio blood/water was calculated.

Part 3: Theoretical interpretation of the single-exponential fit parameter b

Both simulated and experimental results of the light attenuation through blood layers of well-defined thicknesses could be approximately described with a single-exponential fit

$$I_r = \exp(-b \cdot d) \quad (1)$$

where I_r , d and b denote the relative peak intensity (blood versus water), the blood layer thickness and a free fit parameter, respectively. The fit yielded $b = (179 \pm 3) \text{ mm}^{-1}$ for $\lambda_{\text{exc}} = 405 \text{ nm}$ and $b = (10.5 \pm 0.7) \text{ mm}^{-1}$ for $\lambda_{\text{exc}} = 633 \text{ nm}$.

These fit results may be interpreted using a simplified model: If an infinitesimally thin fiber is assumed, which emits a pencil beam with zero divergence, and if multiple scattering is neglected for each transit through the blood layer (excitation and emission), the relative fluorescence intensity (blood versus water) detected with the fiber is determined by the excess absorption (μ_a) and scattering (μ_s) coefficients of blood with respect to water at the considered excitation (exc) and emission (em) wavelengths:

$$I_r = \exp\left[-(\mu_{a,\text{exc}} + \mu_{s,\text{exc}} + \mu_{a,\text{em}} + \mu_{s,\text{em}}) \cdot d\right] \quad (2)$$

In this model, the light attenuation through blood is systematically overestimated as photons that are scattered away from the initial direction do not contribute to the detected signal anymore. In reality, however, due to the finite thickness and acceptance angle of the fiber, slightly deflected excitation and fluorescence photon paths may still lead to fluorescence photons which

reach the fiber end face within the acceptance angle. Since most scattering events occur into directions near the forward direction ($0.75 < g < 1$), this fact is of special importance. By replacing the scattering coefficients μ_s in Equation (2) with the corresponding reduced scattering coefficients μ_s' , the strong forward scattering in blood is taken into account:

$$I_r = \exp\left[-\left(\mu_{a,\text{exc}} + \mu_{s,\text{exc}}' + \mu_{a,\text{em}} + \mu_{s,\text{em}}'\right) \cdot d\right] \quad (3)$$

With the transition from μ_s to μ_s' , the scattered photons are virtually replaced by two groups of photons: exactly forward scattered (relative strength: $\mu_s \cdot g$) and isotropically scattered (relative strength: $\mu_s' = \mu_s \cdot (1-g)$) photons. Thus, in Equation (3), all forward components of the scattered photons are assumed to contribute to the measured fluorescence signal. This assumption is only correct when considering the forward-directed photon flux, but in the experimental situation discussed here, it actually underestimates the number of photons which are lost due to scattering, to an extent depending on the fiber diameter, its numerical aperture and the geometry of the experimental setup. The actual experimental results may therefore be expected to lie in between those derived from Equations (2) and (3).

Comparing Equations (1) and (2), the fit parameter b can be identified with the sum

$$\mu_{a,\text{exc}} + \mu_{s,\text{exc}} + \mu_{a,\text{em}} + \mu_{s,\text{em}} \quad (4)$$

Comparing Equations (1) and (3), the fit parameter b can be identified with the sum

$$\mu_{a,\text{exc}} + \mu_{s,\text{exc}}' + \mu_{a,\text{em}} + \mu_{s,\text{em}}' \quad (5)$$

When inserting the optical properties of blood used for the simulations (see Supplementary Table S3), the sum defined in Equation (4) amounts to 176 mm^{-1} for $\lambda_{\text{exc}} = 633 \text{ nm}$ and to 271 mm^{-1} for $\lambda_{\text{exc}} = 405 \text{ nm}$, the sum defined in Equation (5) to 4.02 mm^{-1} for $\lambda_{\text{exc}} = 633 \text{ nm}$ and to 153 mm^{-1} for $\lambda_{\text{exc}} = 405 \text{ nm}$. A comparison with the single-exponential fitting results for b , i.e. 10.5 mm^{-1} ($\lambda_{\text{exc}} = 633 \text{ nm}$) and 179 mm^{-1} ($\lambda_{\text{exc}} = 405 \text{ nm}$), shows that Equation (2) overestimates the light attenuation through blood by far. Equation (3) provides a much better approximation, even though it slightly underestimates the light attenuation. This is not surprising as even this modified model does still not reflect the experimental excitation and detection geometry correctly. Amongst others, light paths through blood that are longer than the layer thickness d are not considered. However, this model already yields b values of the right order of magnitude and allows interpreting this fit parameter as a sum of absorption and effective scattering coefficients at the considered excitation and emission wavelengths, in which the effective scattering coefficient can be approximated by μ_s' .

SUPPLEMENTARY REFERENCES

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