Two-peaked 5-ALA-induced PpIX fluorescence emission spectrum distinguishes glioblastomas from low grade gliomas and infiltrative component of glioblastomas

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Abstract: 5-ALA-induced protoporphyrin IX (PpIX) fluorescence enables to guiding in intra-operative surgical glioma resection. However at present, it has yet to be shown that this method is able to identify infiltrative component of glioma. In extracted tumor tissues we measured a two-peaked emission in low grade gliomas and in the infiltrative component of glioblastomas due to multiple photochemical states of PpIX. The second emission peak appearing at 620 nm (shifted by 14 nm from the main peak at 634 nm) limits the sensibility of current methods to measured PpIX concentration. We propose new measured parameters, by taking into consideration the two-peaked emission, to overcome these limitations in sensitivity. These parameters clearly distinguish the solid component of glioblastomas from low grade gliomas and infiltrative component of glioblastomas.

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1. Introduction

Fluorescence measurements are of considerable interest in oncology [1] and 5-ALA-induced protoporphyrin IX (PpIX) fluorescence is a useful guide during the surgical resection of glioma. However the accuracy of this method remains limited. This is due to a sensitivity which is still too low to evaluate the infiltrative component of high grade glioma (HGG) and low grade glioma (LGG) [2–4]. Current methods are based on intraoperative surgical fluorescence microscopy [5] or optical fiber systems that allow a local emission spectrum of tissue to be predominant in HGG [2,3,6,7]. However some results indicate that the emission spectrum...
can be more complicated [10–12] due to either a shift in the position of the emission maximum towards shorter wavelength or the presence of photoproducts. In the former case, PpIX fluorescence emission is the result of the two PpIX states which are simultaneously present. By integrating the fluorescence emission with a large bandwidth containing both peaks, one cannot measure $[\text{PpIX}]$ because the quantum yields of both states are very different [9]. It is therefore necessary to fit the measured emission spectrum so as to estimate the contribution of the two PpIX states. We introduce here a new measured parameter, the ratio of emitted fluorescence for both PpIX states ($\text{ratio}_{620/634}$) obtained from the fitting procedure of the emission spectrum. We demonstrate both theoretically and experimentally (through in vitro experiments) that the $\text{ratio}_{620/634}$ can be used as a marker of non-uniqueness of the fitted $[\text{PpIX}]$ with current methods. Results on extracted tumor tissues clearly demonstrate that $\text{ratio}_{620/634}$ tends towards 0 in the solid component of glioblastomas (GBM), whereas it is higher than 1 in LGG and in the infiltrative component of GBM.

2. Materials and methods

2.1 The $\text{ratio}_{620/634}$ as a marker of non-uniqueness

Current methods assume the presence of only one state, emitting at 634 nm. Considering the presence of two states for PpIX in vivo, $[\text{PpIX}]$ cannot be retrieved only from the emission intensity ($I_{634}^{em}$) at 634 nm. Indeed, this concentration is the sum of the concentration of both states: $[\text{PpIX}] = [\text{PpIX}_{620}] + [\text{PpIX}_{634}]$ and the emission intensity at a wavelength $\lambda$ is:

$$I_{\lambda}^{em} = \alpha \left( [\text{PpIX}_{620}] \eta_{620} I_{620}^{\lambda} + [\text{PpIX}_{634}] \eta_{634} I_{634}^{\lambda} \right)$$

$$= \alpha \left( [\text{PpIX}_{634}] \eta_{634} I_{634}^{\lambda} \right) \left( 1 + \frac{I_{620}^{\lambda}}{I_{634}^{\lambda}} \text{ratio}_{620/634} \right)$$ (1)

Where $\text{ratio}_{620/634} = [\text{PpIX}_{620}] \eta_{620} / [\text{PpIX}_{634}] \eta_{634}$; $\eta_{620}$ and $\eta_{634}$ are respectively the molar quantum yield, $I_{620}^{\lambda}$ and $I_{634}^{\lambda}$ the normalized emission intensity at wavelength $\lambda$ of both PpIX states with maxima at 620 nm and 634 nm; $\alpha$ includes other known contributions to fluorescence intensity: the incident light intensity, the optical path-length and the molar extinction coefficients. $\alpha$ is not quantified in this work as opposed to other studies [7]. As a consequence $[\text{PpIX}_{620}] \eta_{620}$ and $[\text{PpIX}_{634}] \eta_{634}$ are not quantified; they are given in arbitrary units (a.u.) in our study since the emission spectra of both PpIX states are normalized ($\sum I_{634}^{\lambda} = \sum I_{620}^{\lambda} = 1$). Therefore current methods present somehow a non-uniqueness that can be overcome by fitting each measured spectrum with simulated ones: $I_{634}^{em}$, $I_{634}^{em}$, $\eta_{620}$ and $\eta_{634}$ are respectively the molar quantum yield, $I_{620}^{\lambda}$ and $I_{634}^{\lambda}$ the normalized emission intensity at wavelength $\lambda$ of both PpIX states with maxima at 620 nm and 634 nm; $\alpha$ includes other known contributions to fluorescence intensity: the incident light intensity, the optical path-length and the molar extinction coefficients. $\alpha$ is not quantified in this work as opposed to other studies [7]. As a consequence $[\text{PpIX}_{620}] \eta_{620}$ and $[\text{PpIX}_{634}] \eta_{634}$ are not quantified; they are given in arbitrary units (a.u.) in our study since the emission spectra of both PpIX states are normalized ($\sum I_{634}^{\lambda} = \sum I_{620}^{\lambda} = 1$). Therefore current methods present somehow a non-uniqueness that can be overcome by fitting each measured spectrum with simulated ones: $I_{634}^{em}$, $\eta_{620}$, $\eta_{634}$ and $I_{634}^{em} / I_{634}^{em} = 0.26$ as evaluated with in vitro experiments. Then the solution of the problem is unique only for the case $\text{ratio}_{620/634} \rightarrow 0$ and in this case $[\text{PpIX}_{634}] = \eta_{634}^{-1} I_{634}^{em} / \alpha (\eta_{634}^{-1} \eta_{620}^{-1} I_{620}^{em})$. It should be pointed out that because $\eta_{620} \ll \eta_{634}$ [9] the solution can be unique even if $[\text{PpIX}_{620}] = [\text{PpIX}_{634}]$. In this particular case the PpIX concentration measured is not strictly the total concentration of PpIX. For the other cases ($\text{ratio}_{620/634} \rightarrow 0$) the non-uniqueness of the problem occurs. Indeed assuming a known solution $[\text{PpIX}_{620}]^{sol}$; $[\text{PpIX}_{634}]^{sol}$ for a given $I_{634}^{em}$ with a total concentration $[\text{PpIX}]^{sol}$, one can define the following family of solutions: $\{ \alpha ([\text{PpIX}_{620}]^{sol} + \varepsilon); \alpha ([\text{PpIX}_{634}]^{sol} - \varepsilon) \}$ with $\varepsilon = 1 - \frac{\alpha}{\eta_{620}^{-1} \eta_{634}^{-1} \eta_{620}^{-1} \eta_{634}^{-1} I_{620}^{em} I_{634}^{em}} I_{634}^{em}$ which have a total concentration $\alpha [\text{PpIX}]^{sol}$. Because $\alpha \in \mathbb{R}^+$, a given emission intensity $I_{634}^{em}$ can correspond to any $[\text{PpIX}]$. Three experimental parameters are obtained: $[\text{PpIX}_{620}] \eta_{620}$, $[\text{PpIX}_{634}] \eta_{634}$, and also $\text{ratio}_{620/634}$ which enables identification of non-uniqueness cases.
2.2 Instrumentation

The source was a TEM$_{00}$ continuous wave laser at 405 nm (LD-WL206; Changchun New Industries Optoelectronics Tech. Co. LTD, Changchun, China) which corresponds to the maximum of the excitation spectrum of PpIX [8,9]. The light was injected into an optical fiber of 550 µm core diameter (HCG M0550T; SEDI Fibres Optiques, Courcouronnes, France). The laser illuminated tissue onto a spot of 0.9 mm in diameter. Its output power was 2 mW; this led to an irradiance of 0.3 W/cm$^2$. The detection was done by an optical fiber (HCS M0600T; SEDI Fibres Optiques, Courcouronnes, France) of 600 µm core diameter. The reflected laser radiation and a part of the auto-fluorescence of the optical fibers were filtered by a high pass filter (HQ435LP; CHROMA, Bellows Falls, USA) with 435 nm cutoff wavelength. The filtered light was then injected into a spectrometer (USB2000; Ocean Optics, Dunedin, USA). The software (Labview, National Instruments, Austin, USA) controlled the spectrometer; the acquisition was synchronized with a shutter (D845H P, Newport, Irvine, USA) placed in front of the laser. This enabled sequential measurement of the emitted spectrum (laser on) and the background spectrum (laser off). The fluorescence emission spectrum was obtained by subtracting the background spectrum to the emitted spectrum. This point is particularly crucial since the system is meant to be used in operating rooms where ambient light cannot be completely shut off and is unstable during the whole operation. The optimum CCD integration time in the spectrometer is a compromise between the stability of the background light during a measurement and the increasing CCD read out noise with the number of accumulation. For in vitro experiments, the optical fibers ends were placed inside the solution. For in vivo experiments, the optical fibers ends were placed above the tissue without contact. The ends of the optical fibers were distant by 1 mm (measured at the center of their cores) and had an angle between their axes of 30°. As a consequence the “mean optical path”, i.e. the geometrical path between the two fibers ends when passing by the intersection of their axes, was 4 mm. The tissue biopsy was placed at this intersection of the optical fibers axes, i.e. at a vertical distance of 2 mm.

2.3 PpIX in solution experiments

6.5 mg of Protoporphyrin IX (Sigma Aldrich, Saint Louis, USA) were dissolved in 10 mL of ethanol giving a stock solution at $1.2 \times 10^{-3}$ mol/L. One mL of stock solution was dissolved in 99 mL of distilled water giving 100mL of aqueous solution at $1.2 \times 10^{-5}$ mol/L which is close to in vivo PpIX concentration [4,13] in high grade glioma. Semi-skimmed milk (containing lipid micelles) was added in concentrations ranging from 0.43 g/L to 12.02 g/L. The same solution was used for all the measurements by increasing the concentration of semi-skimmed milk. The pH (HI9125 pH/ORP Meter; Hanna Instruments, Tanneries, France) of the solution was adjusted by minute addition of HCl and NaOH (Sigma Aldrich). The minute variations in volume due to the addition of HCl, NaOH and semi-skimmed milk did not lead to significant variations of PpIX concentration. Semi-skimmed milk has absorption ($\mu_a$) and reduced scattering ($\mu_s'$) coefficients almost constant on the relevant emission spectrum range [14] (600 to 700 nm). Indeed $\mu_a$ goes from 1.3 cm$^{-1}$ to 1 cm$^{-1}$ and $\mu_s'$ goes from 40 cm$^{-1}$ to 30 cm$^{-1}$, these represent maximum variations below 25%. In the worst case, at 600 nm and for the 12.02 g/L of semi-skimmed milk solution, $\mu_a = 1.5 \times 10^{-2}$ cm$^{-1}$ and $\mu_s' = 0.47$ cm$^{-1}$. In this case the reduced mean free path was $1/\mu_s' = 2.1$ cm which was 5 times higher than the mean optical path of the experimental setup which was 4 mm. Thus light scattering by semi-skimmed milk had a negligible effect. For absorption, in the same worst case, it corresponded to a decrease in the light intensity of 0.6% along the 4 mm of the mean optical path. This is very low and comparable to the decrease due to water at 600 nm (0.1%). As a consequence it can be assumed that the optical properties of the different solutions were similar and did not affect significantly the fluorescence emission spectrum.
2.4 Fitting and relative quantification method

The method used was as follows: (i) Measurements consisted in \( N \) series of 400 ms duration constituted by 200 ms emitted spectrum and 200 ms background. \( N \) was chosen (typically \( N = 30 \)) during the experiment so as to obtain a sufficient signal to noise ratio. (ii) The emission spectrum was obtained by subtracting the background from the emitted spectrum and by accumulating the \( N \) series. (iii) Then the auto-fluorescence of tissues, mainly NADH, was removed from the measured emission spectrum. This was done by fitting (“fit”; Matlab R2011b, The Mathworks Inc., Natick, USA) its decay in the 520-530 nm and 900-1000 nm ranges with an exponential decay as proposed elsewhere [10]. (iv) The simulated spectrum \( I_{em}^\lambda \) was fitted (“lsqcurvefit”; Matlab R2011b) to the measured emission spectrum in the 520-1000 nm range knowing the normalized emission spectra \( I_{620}^\lambda \) and \( I_{634}^\lambda \). This procedure yielded the 2 parameters \( [PpIX_{620} \eta_{620}] \) and \( [PpIX_{634} \eta_{634}] \) enabling the calculation of \( \text{ratio}_{620/634} \). (v) \( [PpIX_{620} \eta_{620}] \) and \( [PpIX_{634} \eta_{634}] \) were then normalized by the auto-fluorescence intensity of each tissue sample. This intensity was obtained by subtracting the fitted \( I_{em}^\lambda \) from the measured emission spectrum and by integrating along the entire wavelength range. The emission spectra \( I_{620}^\lambda \) and \( I_{634}^\lambda \) (Fig. 1(a)) used for fitting were measured \textit{in vitro} with the PpIX solution described in the preceding section. To obtain the PpIX spectrum with a maximum at 620 nm the solution was fixed to pH = 9.77 and 0.43 g/L of semi-skimmed milk. For the PpIX with a maximum at 634 nm the solution was fixed to pH = 4.54 and 12.02 g/L of semi-skimmed milk. The emission spectrum of PpIX photoproducts was obtained by subtracting from the emission spectrum at 634 nm the same spectrum after a continuous irradiation at a power of 36 mW during 1 min. The photoproducts spectrum [15] was not measured for the emission spectrum with a maximum at 620 nm because no photo-bleaching was noticeable.

2.5 Patients selection

All experiments were approved by the local ethics committee of Lyon University Hospitals (France). All participating patients signed written consent. Inclusion criteria were preoperative diagnosis of low grade glioma (no enhancement contrast MRI) or high grade glioma (enhancement contrast MRI); tumor judged suitable for open cranial resection; age equal to or older than 18 years; and patient ability to provide written consent. Exclusion criteria included pregnancy or breastfeeding; history of cutaneous photo sensitivity or hypersensitivity to porphyrins; photodermatosis, exfoliative dermatitis, or porphyria; history of liver disease within the last 12 months; alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, or bilirubin levels greater than 2.5 times the normal limit at any time during the previous 2 months; plasma creatinine in excess of 180 µmol/L; patient inability to comply with the photosensitivity precautions associated with the study; and serious associated psychiatric illness. Preoperative, high-resolution contrast-enhanced T1- and/ or T2-weighted axial magnetic resonance images were acquired and used for navigational guidance.

2.6 Surgical procedure

Patients were given an oral dose of 20 mg/kg of body weight of 5 amino-levulinic acid (Gliolan; Medac GmBH) dissolved in 100 ml of water approximately 3 hours prior to the induction of anesthesia. The patient’s head was prepared and registered using a StealthStation Treon image-guidance system (Medtronic) following standard practice. A Zeiss OPMI Pentero surgical microscope (Carl Zeiss Surgical GmbH) modified for fluorescence guidance with a 400-nm wavelength source for excitation and a 620-710 nm bandpass filter to record fluorescence emissions on a sensitive 3-chip CCD camera was also coregistered with the surgical field. At various points during resection, the surgeon switched from white to blue light exposure to visualize fluorescence. Biopsy specimens were collected at the beginning to
confirm the diagnosis and also during the whole resection procedure. Specimens were collected from fluorescing and non-fluorescing regions within the preoperatively planned resection volume. The sites were identified with navigational guidance as corresponding to either the center or the edge of tumors. Digital images under white and blue light and coordinates were recorded for each biopsy acquisition. The site was assigned a fluorescence score from 0 to 2 (0: no fluorescence; 1: minimal fluorescence; 2: high fluorescence) based on the appreciation of the surgeon (blinded to the spectroscopic measurements) prior to the tissue removal. However only 2 samples showed a score higher than 0. Thus no statistical comparison with the different parameters was possible. Biopsy specimens were separated into 2 equal parts: one part was analyzed with the spectroscopic set up, one part was conditioned to histopathological analysis. Resection was continued until the surgeon judged that no more malignant tissue that could be safely removed was present.

2.7 Histopathology

Histopathological analysis was performed on formalinfixed paraffin embedded biopsy tissue specimens processed for H & E staining. Each H & E stained tissue section was assessed for the presence of tumor cells, necrosis, mitotic activity, nuclear atypia, microvascular proliferation and reactive astrocytosis, and subsequently classified as normal or abnormal tissue (tumor) based on WHO histopathological criteria. Biopsy specimens were classified into 5 categories: normal tissue, Ginf (Glioma, infiltration; WHO Grade II), Gst (Glioma, solid tumor; WHO Grade II), GBMinf (GBM, infiltration; WHO Grade IV), GBMst (GBM, solid tumor; WHO Grade IV).

2.8 Statistical treatments

Statistical significance was investigated with a two samples Kolmogorov-Smirnov hypothesis test (“kstest2” Matlab R2011b). It is a non-parametric method well suited for our relatively small samples. We tested the hypothesis that the distributions of $\text{ratio}_{620/634}$ were different from one another for each group. The same test was applied for $[\text{PpIX}_{620}]_{620}$ and $[\text{PpIX}_{634}]_{634}$. Three levels of statistical significance were used: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3. Results

3.1 PpIX in solution

The method was validated in vitro with aqueous solutions at constant [PpIX]. The $\text{ratio}_{620/634}$ was altered by controlling the pH and the macromolecule concentration. The measured spectra were fitted by $I^k_{em}$ after the contribution of photoproducts of PpIX was added due to its presence in vitro (the solution was not deoxygenated). As a consequence the emission spectrum of PpIX was a linear combination of these three spectra (Fig. 1(a)) and the resulting spectrum showed two maxima separated by a dip (Fig. 1(b)) or a shift in central wavelength (Fig. 1(c)) depending on $\text{ratio}_{620/634}$. We obtained $\text{ratio}_{620/634}$ from 0.1 to 1.8 by variation of the macromolecule concentration at constant $[\text{PpIX}] = 1.2 \times 10^{-5}$ mol/L (Fig. 2(a)) which was in the in vivo $[\text{PpIX}]$ range in HGG [4,12]. Results, not shown, demonstrated the same effect due to pH variation (the $\text{ratio}_{620/634}$ increases from acid to alkaline pH for given $[\text{PpIX}]$ and macromolecule concentration). The method allowed the fitting of the spectra along the entire $\text{ratio}_{620/634}$ range (Figs. 1(b) and 1(c)). The fitted photoproducts contributions were low and constant with the $\text{ratio}_{620/634}$ (Fig. 2(b)). Fitted $[\text{PpIX}_{620}]_{620}$ was also rather stable along the $\text{ratio}_{620/634}$ range, whereas $[\text{PpIX}_{634}]_{634}$ increased steeply and was 28 times higher at $\text{ratio}_{620/634} = 0.1$ than at 1.8 (Fig. 2(b)).
Fig. 1. Simulated and in vitro calibration experiments. (a) PpIX emission spectra measured in vitro for the two physicochemical states with peak wavelength at 634 nm (blue dotted line) and 620 nm (red dashed line) and for photoproducts (brown dashed and dotted line). (b, c) Two examples of fitting of PpIX emission spectra in vitro (solutions at ratio<sub>620/634</sub> = 0.98 (b) and ratio<sub>620/634</sub> = 0.38 (c)), measured (x signs), simulated (green solid line), fitted PpIX<sub>634</sub> (blue dotted line), fitted PpIX<sub>620</sub> (red dashed line) and fitted photoproducts (brown dashed and dotted line).

Fig. 2. Simulated and in vitro calibration experiments. (a) Influence of the microenvironment on ratio<sub>620/634</sub>. (b) Fitted PpIX fluorescence intensities of [PpIX<sub>634</sub>]<sub>[η<sub>634</sub>]</sub> ( + signs), [PpIX<sub>620</sub>]<sub>[η<sub>620</sub>]</sub> (o signs) and photoproducts (* signs) in function of ratio<sub>620/634</sub>.
3.2 Extracted tumor tissues

Extracted tumor tissues from 2 patients with LGG (2 males; 52 and 32 years of age) and 2 patients with GBM (2 males; 66 and 68 years of age) were classified in 4 groups based on histopathology: 7 samples of Ginf (infiltrative component of LGG), 7 samples of Gst (solid tumor of LGG), 16 samples of GBMinf (infiltrative component of GBM), and 5 samples of GBMst (solid component of GBM). There were only 2 samples classified as normal tissue. Therefore no group was constituted for normal tissue because of a too low statistical significance. Measurements were fitted (Figs. 3(a) and 3(b)) on these 35 samples using the method described. The photoproducts spectrum was fitted but did not show any significant contribution. The statistical representations of the results are summarized in Table 1.

Table 1. Statistical Representation of the Extracted Tumor Tissues Experiments

| Group                      | [PpIX634]η<sub>634</sub> | [PpIX620]η<sub>620</sub> | ratio<sub>620/634</sub> |
|----------------------------|--------------------------|--------------------------|--------------------------|
| GBMst: GBM solid tumor     | 8.26 ± 4.80              | 0.05 ± 0.04              | 0.03 ± 0.02              |
| GBMinf: GBM infiltration   | 0.41 ± 0.08              | 0.35 ± 0.06              | 1.04 ± 0.10              |
| Gst: Glioma solid tumor    | 0.19 ± 0.04              | 0.29 ± 0.06              | 1.62 ± 0.16              |
| Ginf: Glioma infiltration  | 0.28 ± 0.06              | 0.35 ± 0.09              | 1.25 ± 0.09              |

For the 4 groups GBMst (n = 5), GBMinf (n = 16), Gst (n = 7) and Ginf (n = 7) we represented the mean and the standard error of the mean for [PpIX634]η<sub>634</sub>, [PpIX620]η<sub>620</sub> and ratio<sub>620/634</sub>. For GBMst the ratio<sub>620/634</sub> (Fig. 4(a)) was close to 0 and very significantly different from ratio<sub>620/634</sub> in the 3 other groups, GBMinf (P = 7.0 x 10<sup>-4</sup>), Gst (P = 1.6 x 10<sup>-3</sup>) and Ginf (P = 1.6 x 10<sup>-3</sup>), where it was higher than 1. The ratio<sub>620/634</sub> was around 1 in GBMinf and higher than 1.2 in Gst and Ginf. However the difference was only statistically significant between GBMinf and Gst (P = 4.7 x 10<sup>-3</sup>). Therefore, based on ratio<sub>620/634</sub>, these results showed a clear distinction between GBMst and the other groups.

The mean value of [PpIX634]η<sub>634</sub> for GBMst (Fig. 4(b)) was around 8.5 a.u. and was significantly different from the values in the 3 other groups, GBMinf (P = 2.3 x 10<sup>-4</sup>), Gst (P = 1.6 x 10<sup>-3</sup>) and Ginf (P = 1.6 x 10<sup>-3</sup>), where it was less than 0.5 a.u.. Therefore these results showed also a differentiation of the extracted tissues, based on [PpIX634]η<sub>634</sub>, into two categories: GBMst and the other groups.
The mean value of $\text{[PpIX}_{620}\eta_{620}$ for GBMst (Fig. 4(c)) was close to 0 a.u. whereas it was around 0.35 a.u. and therefore significantly different for GBMinf ($P = 6.1 \times 10^{-3}$) and Ginf ($P = 2.7 \times 10^{-3}$). $\text{[PpIX}_{620}\eta_{620}$ in Gst was lower (0.29 ± 0.06 a.u.) but still significantly different ($P = 2.4 \times 10^{-2}$). However the mean value of $\text{[PpIX}_{620}\eta_{620}$ in Gst was not statistically different from the mean values in GBMinf and Ginf.

4. Discussions

4.1 Ratio$_{620/634}$

*In vitro* results showed (Fig. 2(b)) that at a constant $\text{[PpIX]}$ and in the case of a decreasing ratio$_{620/634}$, $\text{[PpIX}_{634}\eta_{634}$ increases very steeply while $\text{[PpIX}_{620}\eta_{620}$ is rather stable. The high sensitivity of $\text{[PpIX}_{634}\eta_{634}$ to a small variation of the ratio$_{620/634}$ is due to the fact that $\eta_{620} \ll \eta_{634}$ [9]. Therefore the relationship between $\text{[PpIX}_{634}\eta_{634} and [PpIX]$ should be considered carefully even if the ratio$_{620/634}$ is small. Furthermore when the measured ratio$_{620/634}$ is not close to 0 the contribution of $\text{[PpIX}_{620}\eta_{620}$ has to be taken into account, particularly when it is higher than 1.

Focusing on $\text{[PpIX}_{634}\eta_{634}$ and $\text{[PpIX}_{620}\eta_{620}$ both are able to distinguish the solid component of GBM from the infiltrative component of GBM and LGG. But the preponderance of $\text{[PpIX}_{634}\eta_{634}$ in the solid component of GBM is inverted in the infiltrative component of GBM and LGG, where $\text{[PpIX}_{620}\eta_{620}$ is preponderant. Thus, taking the ratio$_{620/634}$ enhances this difference making ratio$_{620/634}$ a very significant marker in identifying the solid component of GBM.

In GBM the ratio$_{620/634}$ was very close to 0 in the solid component meaning that current methods can lead to a unique solution. This is in accordance with results [4,11,13] showing that these methods are able to identify HGG. However the ratio$_{620/634}$ was close to 1 in the
infiltrative component of GBM, meaning that non-uniqueness of [PpIX] was critical. In LGG the ratio_{620/634} was higher than 1 whatever the histopathological classification. These results might suggest the limitation of current methods to measure [PpIX] in LGG. Nevertheless, the results in this study are preliminary on a small number of samples and as such future work will further validate these results.

These results show that current methods used to evaluate the infiltrative component of GBM and LGG do not only suffer from limitations in sensitivity [2–4]. It seems that in the infiltrative component of LGG and GBM, the PpIX states peaking at 620 nm is the main contributor meaning that it has to be taken into account when fitting the emission spectrum and evaluating [PpIX]. Further investigations are needed so as to evaluate the relevance of the ratio_{620/634} to identify the infiltrative component of LGG and HGG from normal tissue, and to overcome the limitations in sensitivity of current methods.

4.2 A marker of infiltrative component?

It should be pointed out that [PpIX_{620}]_{620} was lower in the solid component of GBM than in the other groups. This result goes towards the assumption that the high fluorescence intensity in the solid component of HGG is more linked to increased tissues acidity than to increased [PpIX], but this is only an indication.

The results indicate a general trend that in the solid component of LGG the mean concentrations of both states of PpIX are lower and the ratio is higher compared to the infiltrative component of GBM. However this trend should be considered very carefully because it is only partly significant.

[PpIX] is known to be low in normal tissues [2,4]. Thus it is likely that [PpIX_{620}]_{620} should also be low in normal tissues. Therefore considering the high values of [PpIX_{620}]_{620} in the infiltrative component of GBM and LGG, it will be interesting to explore its relevance to distinguish the infiltrative component of gliomas from normal tissues.

4.3 In vivo fitting discordance

The photoproducts spectrum was fitted but did not show any significant contribution. This is not in accordance with previous studies [11] which revealed significant levels of photoproducts. This could be explained by lower laser irradiance in our study (0.3W/cm^2). Furthermore because the fluorescence as seen by the surgical microscope were low in our study (only two scores higher than 0), the surgeon did not use the 400-nm source as much as he usually does. Another point concerns photochemical pathways in tissues which are probably different before and after extraction, particularly because of the different conditions of oxygenation. In addition in our study, the fitting procedure showed a small discordance in the 650-680 nm range. This could be due to different photochemical pathways in vitro as compared to in vivo. It should be also mentioned that results, not shown, indicated significant levels of photoproducts when we increased the irradiance. Furthermore there was also a more marked discordance in the range below 615 nm that could come from complex tissues optics phenomena, due to tissues absorption and scattering [16] or from other minority intrinsic fluorophores [17]. Lipofuscin concentration is known to be age related and localized around the nucleus of neuronal cells. However the histopathology revealed no correlation between the neurons proportion and the discordance in this range. A link between this discordance and the presence of microcystic changes or edema in the sample was suspected, but the data did not allow us to conclude on this point.

4.4 Towards a tumor pH-sensitive method?

PpIX in solution results showed that the presence of both PpIX states, and as a consequence the shape of the emission spectrum, is highly linked to the microenvironment. The ratio_{620/634} could be useful not only as an indicator of non-uniqueness; the pH of tumor tissues strongly influences its value. However Fig. 2(a) showed that, in vitro, a given ratio_{620/634} does not
correspond to a given pH of the microenvironment since it also depends on the concentration of macromolecules. It is therefore impossible to differentiate the effects of pH from the concentration of macromolecules based only on the emission spectrum. On the other hand it is interesting to emphasize that ratio$_{620/634}$ is close to 0 for the solid component of HGG and close to 1 for LGG. This can be related to higher acidity in HGG than in LGG since it is known that the tumor tissues acidity is correlated to the grade of glioma because of high glycolytic activity [18]. With these results we think that the ratio$_{620/634}$ would seem to be a possible way to identify low pH associated to HGG. However to realize a quantitative pH-sensitive measurement one needs a bijective relation between the ratio$_{620/634}$ and the pH. Such a relation should be calibrated in vivo for the pH. It should also be calibrated for the other microenvironment parameters which influence the PpIX states, like the concentration of macromolecules. Furthermore PpIX distribution is mainly intra-mitochondrial but some results indicate [19–21] that in ALA-induced and tumor conditions PpIX could diffuse outside the mitochondria and be partly sensitive to cytosolic microenvironment. The relation between pH and ratio$_{620/634}$ is probably very complex and further investigations are required to address the feasibility of a quantitative pH-sensitive measurement.

5. Conclusion

A new method has been proposed enabling retrieval of a unique $[\text{PpIX}]$ from fluorescence measurements even when ratio$_{620/634} \gg 0$. The ratio$_{620/634}$ and $[\text{PpIX}]_{\eta_{620}}$ can significantly distinguish the solid component of GBM from the infiltrative component of GBM and LGG. This improvement can be easily implemented in quantification methods [2,7] and it can bring methodological and technical improvements for intra-operative 5-ALA-induced fluorescence microscopy. Further clinical studies will be conducted in order to investigate the relevance of the method in identifying the infiltrative component of LGG and HGG from normal tissue. Fluorescent dyes are promising for intracellular pH measurement [22], and since the ratio$_{620/634}$ is correlated to the pH, this method could give a new in vivo pH-sensitive indicator for tumor tissues.

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