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Experimental Decorrelation Requirement for Robust Estimation of Fluorophores using Multiple-Wavelength Excitation Fluorescence Spectroscopy

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ABSTRACT

Protoporphyrin IX (PpIX) is a fluorophore being currently used to localize tumoral tissues. The tissue is usually excited at one wavelength, e.g., 405 nm, and the fluorescence signal is used to estimate the amount of PpIX during surgery. However, other fluorophores (baseline) whose emission spectra are close to the one of PpIX impair the quantification of PpIX and consequently the tissue pathological status classification. An efficient multi-excitation wavelengths method, free from any *a priori* on the baseline shape, has been proposed to cope with this issue. This method requires decorrelated measurements in the range of PpIX emission at multiple excitation wavelengths. We investigated the influence of the source bandwidth on this decorrelation by comparing two experimental setups using either LED or laser diode sources. The experimental setup using laser diodes for excitation increases the decorrelation by 35.3 % compared to the one using LEDs in the spectral range of PpIX emission.

Keywords: 5-ALA, fluorescence spectroscopy, glioma, Protoporphyrin IX, multispectral excitation, neurosurgery

1. INTRODUCTION

Still hardly curable today, diffuse gliomas account for more than fifty percent of primitive brain tumors. Most studies commonly consider two separate groups having different biological, molecular and tissue properties: High Grade Gliomas (HGG) are mainly malignant tumors and Low Grade Gliomas (LGG) are benign tumors. All subtypes of gliomas share the same highly infiltrative behavior of individual tumor cells. However surrounding infiltrated tissue often resemble normal tissues. Pre-operative MRI combined with neuro-navigation is currently used to localize surgical tools and tumour cells in the operating theater but it shows strong limitations.

As a complementary method to pre-operative MRI, fluorescence microscopy has shown its relevance in neuro-oncology¹⁻⁵ and 5-aminolevulinic acid (5-ALA) induced fluorescence of protoporphyrin IX (PpIX) is currently used through surgical microscopes.⁶ This technique is the current clinical standard for PpIX-based surgical assistance. However, its sensitivity is still limited when applied to low density infiltrative parts of HGG or to LGG. Thus, many 5-ALA induced PpIX spectroscopy methods arise to tackle this issue.⁷ The accuracy of this fluorescence spectroscopy methods remains limited by the presence of other non 5-ALA induced fluorophores such as NADH, FAD, flavins or lipopigments. These fluorophores and their high variability can lead to important crosstalks with PpIX.^{8,9} To reduce crosstalk, the overall approach is to model the baseline with everything

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that is not due to 5-ALA-induced PpIX. Existing approaches are effective when the emission spectral band of the baseline is far from the one of the PpIX.^{8,10–12} The baseline is modeled by a Gaussian function,^{13,14} or expert dependent weighted sum, which contain a finite number of fluorophores whose fluorescence spectral shape is assumed known.^{8,15} However, fluorophores that overlap with PpIX spectral emission band are difficult to consider. These important crosstalks largely impact specificity. In a previous work,¹⁶ we introduce a novel method to estimate the PpIX fluorescence-related biomarkers contribution. As opposed to state of the art, several excitation wavelengths are used. This additional input enables to be free from any *a priori* on the baseline shape. We applied our method in the case of the two forms of PpIX (PpIX₆₂₀, PpIX₆₃₄) using a numerical phantom calibrated on real data.^{13,14,17} Our method improves the robustness of the estimation of PpIX contributions when the measurements are decorrelated in the PpIX emission spectral range.

In this work, we are interested in the comparison of spectra acquired by two experimental systems. Each of the two experimental systems allows to acquire the spectra for two different excitation wavelengths. Assuming that the spectral width of the source influences the decorrelation required for the proper functioning of the method, we compare the spectra acquired on ex-vivo low-grade glioma tissues by two experimental systems.

The results indicate that the experimental system using the most monochromatic excitation sources reduces the correlation of the two fluorescence spectra in the emission wavelength range of the PpIX by 35.3 %.

2. MATERIALS AND METHOD

One of the custom acquisition devices employed in this study is described in Ref¹⁴ and will be called SystemA hereinafter. It contains different LEDs for fluorescence excitation including one centered at 405 nm with a 16 nm Full-Width Half Maximum (M405F1, *Thorlabs*) and another one center 385 nm with a 14 nm Full-Width Half Maximum (M385F1, *Thorlabs*). A second custom acquisition device, called SystemB hereinafter, is employed in this study. SystemB is composed of a probe that is set on ex-vivo brain tissues, a box containing electrical and optical components and a laptop (processor: Intel Core i5-8350U, 1.70GHz 4, ram: 15.9GiB) offering a Python interface to drive the system. Inside the box, the fluorescence excitation is performed through two laser diodes. One is centered at 408.2 nm with a 2.1 nm Full-Width (LBX-405-900-HPE-PPA, *Oxxius*), and the other is centered at 379 nm with a 3.2 nm Full-Width (LBX-375-200-HPE-PPA, *Oxxius*). Light is transmitted through an optical fiber coupler (TM105R5S1A, *Thorlabs*) to a probe developed for this study by IDIL Company, France.

The fluorescence emission spectrum used in this study relies on multiple excitation wavelengths. At each excitation wavelength λ_e , the fluorescence spectrum $m_{\lambda_e}(\lambda)$ can be written :

$$m_{\lambda_e}(\lambda) = \alpha_1 \eta_1(\lambda_e) S_1(\lambda) + \alpha_2 \eta_2(\lambda_e) S_2(\lambda) + \gamma(\lambda_e) b(\lambda), \quad (1)$$

where all quantities indexed by 1, *resp.* 2, refer to PpIX₆₂₀, *resp.* PpIX₆₃₄, with S_i the normalized emission spectrum, η_i the quantum yield at excitation wavelength, α_i the contribution of the given PpIX form, and b , named baseline, represents other endogenous fluorophore emission spectra. If we consider two wavelengths λ_e, λ'_e , we can then write:

$$\begin{aligned} m &= \alpha_1 \eta_1 S_1 + \alpha_2 \eta_2 S_2 + b \\ m' &= \alpha_1 \eta'_1 S_1 + \alpha_2 \eta'_2 S_2 + \gamma b. \end{aligned} \quad (2)$$

where $m, m', S_1, S_2, \eta_1, \eta_2, \eta'_1$ and η'_2 are given.

In order for the two equations of 2 to be jointly exploitable, as in the method described in the article,¹⁶ the measurements m and m' must contain complementary information, i.e. they must be as orthogonal as possible. For example, if they are totally correlated then $m \propto m'$, and we finally have only one equation.

We therefore consider that the criterion of decorrelation of m and m' is a prerequisite for the use of the multiple wavelength excitation fluorescence spectral unmixing method.¹⁶ This quantitative criterion will be used as a metric to compare the quality of the measurements generated by SystemA and SystemB and then used by our multiple wavelength excitation fluorescence spectral unmixing method.

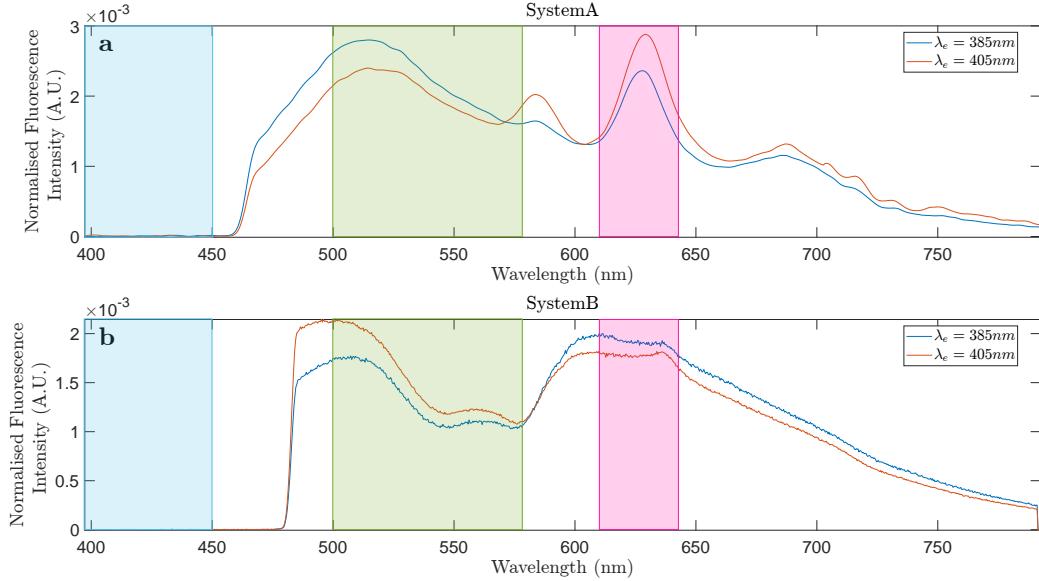


Figure 1. Fluorescence spectrum acquired with the SystemA, *resp.* SystemB, in **a**, *resp.* in **b** for two different excitation wavelengths : $\lambda_e = 385\text{nm}$ in blue and $\lambda_e = 405\text{nm}$ in orange. The noise, *resp.* NADH and PpIX, regions of Table 1 are represented by the light blue, *resp.* the green and the purple rectangles.

3. RESULTS AND DISCUSSION

In Fig. 1 is displayed an example of spectrum acquired by each system. Fig 1-a, *resp.* 1-b, contains the measurements m and m' acquired with the SystemA, *resp.* SystemB, for excitations at 385 nm and 405 nm. We focus on the correlation between the two measurements m and m' acquired with the same system in three different spectral bands: one corresponding to noise (λ between 397 nm and 450 nm), another corresponding to the baseline (λ between 500 nm and 580 nm corresponding to NADH) and the last corresponding to PpIX (λ between 615 nm and 640 nm).

	Noise ($\lambda \in [397, 450]$)	NADH ($\lambda \in [500, 580]$)	PpIX ($\lambda \in [615, 640]$)
SystemA (LEDs)	-0.147 ± 0.255	0.680 ± 0.373	0.861 ± 0.305
SystemB (Laser Diodes)	0.043 ± 0.099	0.896 ± 0.315	0.508 ± 0.400
Numerical Phantom ¹⁶	0.022 ± 0.114	0.622 ± 0.389	0.029 ± 0.122

Table 1. Mean and standard deviation of correlation coefficient for different spectral bands computed over 10 measurements for the experimental setup SystemA, *resp.* SystemB, which contains LED for fluorescence excitation, *resp.* contains Laser Diodes for fluorescence excitation.

In Table 1, one notice that the correlation coefficient of the spectral range corresponding to PpIX is lower for SystemB than SystemA, i.e. the measurements are more decorrelated, by 35.3 %. The correlation coefficient of the NADH spectral range is higher for SystemB than SystemA by 21.6 %. Finally, as expected, the noise is well decorrelated for both SystemA and SystemB, i.e. the correlation coefficient of the corresponding spectral range tends to 0.

To conclude, this work emphasises that the spectral width of the fluorescence excitation source influences the decorrelation of measurements required to quantify PpIX fluorescence. We have shown that SystemB, containing laser diodes for fluorescence excitation, allows a better decorrelation of the PpIX spectral band from measurements for different excitation wavelengths. These results could be strengthened by a complementary study on phantoms.

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