Comparison between fresh and fixed human biopsies using spectral and lifetime measurements: Fluorescence analysis using one and two photon excitations

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Comparison Between Fresh and Fixed Human Biopsies Using Spectral and Lifetime Measurements: Fluorescence Analysis Using One and Two Photon Excitations

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***Abstract*—The purpose of this study is to make a comparison between the fluorescence emissions of fresh extracted human biopsies and fixed human biopsies, in order to evaluate the impact of fixation on autofluoresence signal. Our group is developing an endo-microscope to image brain tissues in-vivo, however to date, in order to validate our technology the easiest type of samples we can access are fixed samples. However, the fixation is still challenging. For that, we aim through this study to determine whether we should pursue to work on fixed samples or we should shift to work on fresh biopsies. Data were collected on spectroscopic, lifetime measurement and fluorescence imaging set-ups with visible and two-photon excitations wavelengths. Five fresh and five fixed samples are involved in the experiment. Endogenous fluorescence of fixed biopsies were calculated. Experimental results reveal that at 405 nm and 810 nm, the fresh samples have an intensity of fluorescence two times higher than that of fixed samples. However, for each fluorophore and each excitation wavelength, the lifetime for fresh samples is shorter than that for fixed samples. Still, further studies and investigations involving the comparison between different samples are required to strengthen our findings.**

***Index Terms*—Spectroscopic analysis, lifetime domain measurements, human biopsies, fixed tissues, fresh tissues.**

I. Introduction

A major cause of variation in the endogenous fluorescence tissue emission, between fresh and fixed tissue, is related to the tissue fixation, and to a lesser degree, to the tissue processing. The most important problem is the inadequate tissue dehydration prior to paraffin embedding [1]. Researchers have encountered changes in autofluorescence induced by fixation, and evaluated the changes. Thereby, the use of ex-vivo fixed samples as adequate control samples is not adopted. In this study, one and two photons spectroscopy and fluorescence lifetime signals were recorded sequentially from the collected specimen on two set-ups: the first one situated at the Sainte Anne Hospital (Paris, France) and the second one available at PIMPA platform, at the IMNC Laboratory (Orsay, France). The purpose of this study is to compare multimodal optical analysis made on a fresh and fixed samples, in order to determine the variations in autofluorescence induced by the fixation process.

II. Materials and Methods

A. Samples

Five Fresh biopsies were first analyzed with the fibered spectroscopic and lifetime set-up at the Sainte Anne Hospital, then an accredited professional transporter brought tissues to PIMPA platform. Two photon Fluorescence (TPF), Second Harmonic Generation (SHG), spectral signal and Fluorescence Lifetime Imaging Microscopy (FLIM) signal were recorded sequentially from the collected biopsies on a classical bench-top 2PEF microscope. Correlations between point-to-point optical indexes, imaging abnormalities and pathological examination of biopsy samples were performed. An illustration of the multimodal analysis on human biopsies is illustrated in Fig. 1.

After optical analysis on fresh samples, all tissue specimens were fixed according to Sainte Anne Hospital Neuropathology Department's protocol (formalin 4%) [2], studied after an overnight fixation on the PIMPA platform and were transported back to Sainte Anne Hospital. Fixed biopsies have benefited from a second analysis on the optical set-up and undergone pathological analysis.

B. Set-ups

1) Spectroscopic and lifetime measurements: An optical set-up was settled at the Sainte-Anne Hospital in order to measure the autofluorescence of human brain samples as close as possible to the in-vivo conditions. It was a bi-fiber set-up, one fiber to excite the fluorophore at 345nm and 405nm and the other one to collect the intensity of fluorescence and send it to the spectrometer or to the Photo Multiplier Tube for lifetime measurement analysis. This set-up has been previously published [3].

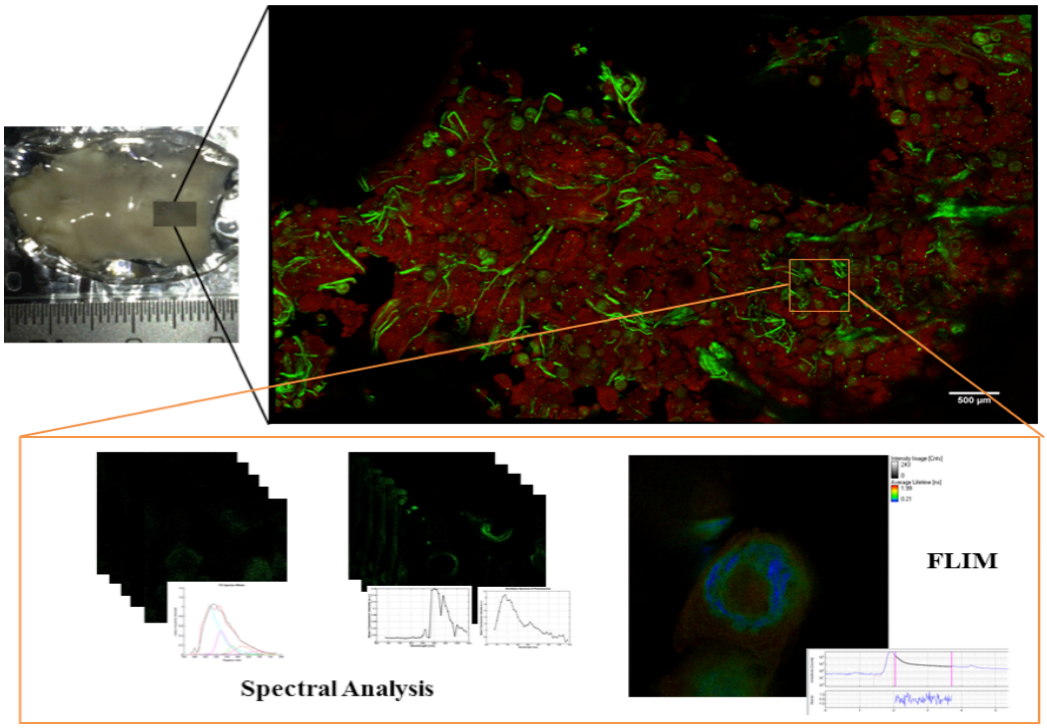

Fig. 1: Our set-up and different imaging modalities.

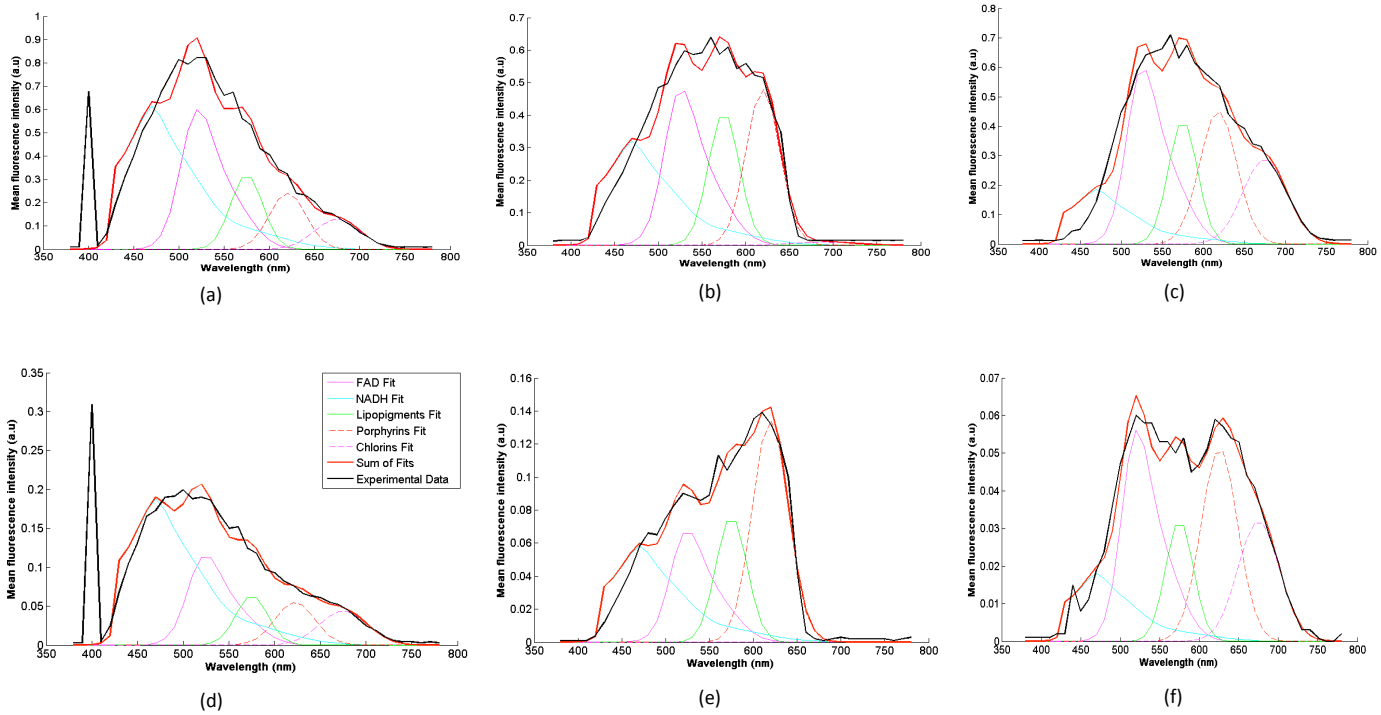

Fig. 2: Representation of the mean fluorescence intensity versus the wavelengths. (a,b,c) fits on the fresh sample. (d,e,f) fits on the fixed sample at 405 nm (a,d), 810 nm (b,e) and 890 nm (c,f).

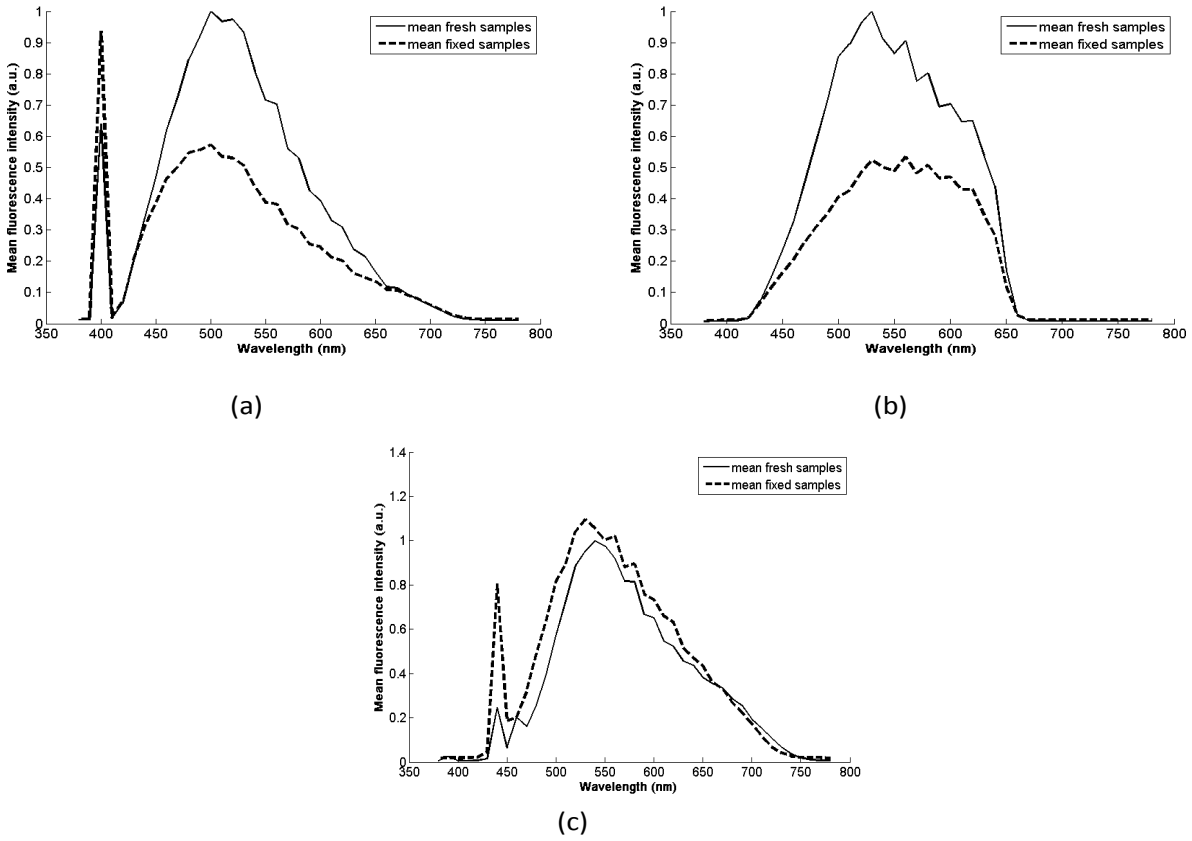

Fig. 3: Mean on five fresh and fixed samples at (Top) 405 nm, (Middle) 810 nm and (Bottom) 890 nm.

2) Multimodal wide-field excitation imaging: one and two-photons: At the IMNC laboratory, samples were imaged using a Leica TCS SP8-FLIM microscope on PIMPA platform. The confocal spectral detection, using 405 nm excitation wavelength, was used to make an emission spectrum. A Mai Tai DeepSee Ti: Sapphire oscillator with automated dispersion compensation was used to produce non-linear excitations. The Mai Tai DeepSee laser has over 2.4 W of average power and 350 nm (690-1040 nm) in usable tuning range. Respectively, 810 and 890 nm excitation wavelengths are used to perform multimodal imaging of fluorescence, SHG, spectral signal and FLIM.

III. Results

A. Two photon spectral analysis

Using visible and Infra-Red (IR) excitations, we were able to excite five endogenous fluorophore: reduced Nicotinamide adenine dinucleotide (NADH), Flavins (FAD), Lipopigments, Porphyrins and Chlorins. We could also measure SHG at 445 nm using 890 nm excitation wavelength. We developed a Matlab script to fit these different fluorophores [3], we will use it here to fit the spectra and observe the change in the mean intensity of fluorescence between fresh and fixed samples.

Fig. 2, a and d showcase the variation of the intensity of fluorescence as a function of the wavelengths using FAD, NADH, Lipopigments, porphyrins, chlorins, sum of fits and experimental data at 405 nm, for fresh and fixed tissues, respectively. In the former figure the maximum mean intensity is obtained using NADH for both fresh and fixed tissues.

In fresh tissues, the flavins contribute to the the increase of 25 % in the mean fluorescence compared to the fixed tissues. Moreover, Lipopigments contribute to 10 % the increase in the mean fluorescence compared to fixed tissues. In Fig. 2, b and e, there is a significant shift in the mean intensity of fluorescence; in the (b) fresh samples, FAD is the dominant fluorophore, whereas, in the (e) fixed samples Porphyrin is the dominant one.

At 890 nm, Fig. 2 c and f showcase that in fresh samples, FAD fit contributes to the highest intensity of fluorescence for both fresh and fixed tissues. Moreover, porphyrins contribute to 75 % the maximum intensity of fluorescence for the fixed samples.

In a fixed biopsy both FAD and porphyrins give a peak at the maximum intensity of the fluorescence signal. Moreover, using SHG, the peak at 445 nm, there is a 20 % increase in the intensity from fresh to fixed samples.

Fig. 3 showcases the mean spectrum at 405, 810 and 890

nm, respectively, for fresh and fixed samples. At 405 nm and 810 nm, the fresh samples have an intensity of fluorescence two times higher than that of fixed samples, however, at 890 nm there was no change in the mean of the intensity of fluorescence between the fixed and fresh samples.

B. One photon spectral and lifetime analysis

At 375 nm and 405 nm we measured the fluorescence lifetime on a PMT linked to a PicoQuant system. In front of the PMT, a 5 band-pass filters were placed to select each fluorophore. The measurements have been done on the 5 samples in the fresh and fixed conditions. Table I reports the mean results. For each fluorophore and each excitation wavelength, the lifetime for fresh samples is shorter than that for fixed samples.

IV. Discussion and Conclusion

Studies have shown that the endogenous fluorescence of human tissues decreases with the time afterwhich the biopsy is taken out [4], then the endogenous fluorescence stabilizes at a value lower than that in in-vivo measurements. The lower endogenous fluorescence value in fixed tissues could be due to the delay between the removal of the biopsy and the fixation of the sample.

TABLE I: Fluorescence Lifetime.

		Fresh Samples	Fixed Samples
375 nm	NADH	3.44 ± 0.35	4.30 ± 0.26
	FAD	3.66 ± 0.08	3.89 ± 0.03
405 nm	NADH	3.09 ± 0.32	3.57 ± 0.11
	FAD	2.78 ± 0.22	3.46 ± 0.20
	Lipopigments	4.21 ± 0.09	9.05 ± 0.30
	Prophyrins	1.80 ± 0.13	2.37 ± 0.06
	Chlorins	1.59 ± 0.12	1.89 ± 0.04

The impact of fixation on lifetime measurement is not yet a popular subject, and our literature review found only few existing studies. Ganguly et al. and Joosen et al. published work tackling this issue [5], [6]. They investigated the effect of cells fixation on the fluorescence lifetime. In both studies, the fixation by the formaldehyde has reduced the fluorescence lifetime. The difference between these results and our work could be explained by the use of different samples: several previous work involved proteins in fixed cells, while, we worked on the endogenous fluorescence of fixed biopsies. The lifetime value of each molecule and of the surrounding environment is specific [7] . Probably, the formaldehyde does not have the same effect on proteins in single cells compared to biopsies of human brain tumor.

Results reveal that at 405 nm and 810 nm, the fresh samples have an intensity of fluorescence two times higher than that of fixed samples. However, for each fluorophore and each excitation wavelength, the lifetime for fresh samples is shorter than that for fixed samples.

Still, further studies and investigations involving the comparison between different media and samples are required to strengthen the former conclusion.

Acknowledgements

This work is supported by a Plan Cancer with Physicancer program grant MEVO, a Dfi instrumental program grant from CNRS.

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