Spectral and Lifetime Measurements of the Endogenous Fluorescence Variation of Freshly Resected Human Samples over Time

Measuring Endogenous Fluorescence Changes at Different Moment after Tumor or Epileptic Cortex Excision

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Abstract: Analysis of human tissue endogenous fluorescence is becoming a new modality of medical imaging. Its capacities represent the missing link between macroscopic radiological tools such as MRI and CT-scan and the surgeon view during surgical procedures. However, numerous aspects of this signal are not well known. Time dependence is one of these aspects. The aim of this work is to investigate the autofluorescence changes with time. Five ex vivo human samples were studied. Spectral and lifetime measurements were acquired each hour. Fluorescence intensity decreased slightly with time. This decrease existed for healthy and tumoral samples and did not affect the differences between them: higher fluorescence intensity for control samples compared to tumor samples. Lifetime values showed a slight decrease too for both type of tissue. This work is the first report of fresh human brain samples multimodal autofluorescence analysis with time.

SCIENCE AND TECHNOLOGY PUBLICATIONS

1 INTRODUCTION

Progress in medical imaging change the everyday life of doctors. Since the 1950's, doctors had to deliver care based only on clinical examination. If they developed an expertise that tends to disappear nowadays, this approach had some major drawbacks: for instance, appendicitis diagnosis based on clinical symptoms resulted in more than 40% of false positive diagnosis (Raman, 2008).

Neurosurgery has benefited greatly over the last years from technologies such as computed tomography scan, magnetic resonance imaging or ultrasonography (Fontana, 2014). However, it is still impossible for a neurosurgeon to know in real time and with certitude if he performs a gross total tumor resection. This limit can be overcome by modern optical imaging basing on fluorescence contrast. Analysis of the human tissue endogenous fluorescence emission gives information on the tissue microenvironment in real time without any exogenous dye. If promising results were reported (Liu, 2014), some aspects of the autofluorescence signal are not precisely known. For instance variation of this signal in function of time has yet to be investigated. Two photon-imaging microscopes are only present in laboratories nowadays. Consequently, there is a significant time lapse between tumor resection and optical analysis due to the selection and transport of the sample between operating room and microscopy laboratory. Even if we used confocal endomicroscope during the neurosurgical procedure, this time delay still exists: neurooncology interventions last often several hours and boundaries appear at the end of the intervention.

The aim of this preliminary work is to record spectral and lifetime components of the endogenous fluorescence on human samples and to monitor its variation with time after excision.

2 MATERIALS AND METHODS

2.1 Optical Setup

Description of the set-up has been previously

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published (Abi Haidar, 2015), and it is represented in figure 1. Briefly, The excitation source is composed by two separated laser diodes from PicoQuant coupled with a shutter and emitting pulses of 70 ps centered at 405 nm (LDH-P-C-405B) and 375 nm (LDH-P-C-375B) with a repetition rate of 40 MHz. The laser beam is coupled into an optical fiber from SEDI ATI Fibres optiques (HCG M0200T) specifically dedicated to the excitation source. It is a multimode fiber with 200 µm of core diameter and a numerical aperture (NA) equal to 0.22, preceded by an injector coupled with a band pass filter centered at 375 or 407 nm. The average power is less than 1 mW at the fiber output. The spatial resolution was of 500 µm (Leh, 2012). The fluorescence is collected by a second multimode fiber (HCG M0365T) with a core diameter of 365 µm and a NA equal to 0.22, separated from the first fiber and with a collimator at its proximal output, coupled with a high pass filter. A beam splitter separates the collected fluorescence into two detectors. For spectral measurements a cooled spectrometer (QP600-1-UV-VIS, Ocean Optics) was used. For time resolved measurements, the collected fluorescence was guided to a Photomultiplier Tube (PMT) (PMA-182 NM, PicoQuant GmbH, Berlin, Germany). Temporal resolution of the PMT was 220 ps. The synchronization output signal from the diode driver and the start signal from the PMT were connected to their respective channels on the data acquisition board Time-Correlated Single Photon Counting (TCSPC) (TimeHarp 200, PicoQuant GmbH, Berlin, Germany). Motorized filter wheel (FW102C, Thorlabs, Newton, USA) was placed in front of the PMT allowing the selection of spectral emission band. With the 405 nm excitation wavelength, we used five filters (Semrock, New York, USA): 450 ± 10 nm, 520 ± 10 nm, 550 ± 30 nm, 620± 10 nm and 680 ± 10 nm corresponding to five endogenous fluorophores: reduced Nicotinamide adenine dinucleotide (NADH), flavin (FAD), lipopigments (Lip), porphyrin (Porph) and chlorin, respectively. With the 375nm excitation wavelength, we worked only with two of the filters, the 450 ± 10 nm and 520 ± 10 nm filters. Lifetime and spectroscopic measurements were acquired on the same set up and two seconds are required to measure each fluorophore lifetime.

The spectral measurements were processed using homemade Matlab software and fluorescence lifetime data were collected and analysed via the Symphotime software (PicoQuant, GmbH, Berlin, Germany). A specific mechanical support was mounted on a motorized micro translator stage (Thorlabs, Newton, USA) for XY scanning. The X-dimension scanning velocity was 100 µm/s and the acquisition time during X-line scanning was 3 sec per fluorescence spectrum.

To be as close as possible to the in vivo conditions, this optical set-up was placed in the Neuropathology Department of Sainte Anne Hospital (Paris, France).

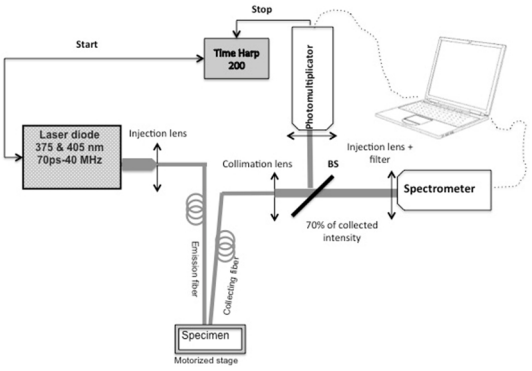

Figure 1: The optical setup for spectral and lifetime fluorescence measurements.

2.2 Samples

Samples were provided from adult patients operated in the Sainte Anne Hospital Neurosurgery Department (Paris, France). The protocol was approved by the Institutional Review Board of Sainte Anne Hospital (Ref CPP S.C.3227). Five samples had been analyzed: three metastasis samples (tumor samples) and two epilepsy surgery samples (control sample). A senior pathologist selected each sample on a fresh resected specimen. A sample was analyzed if: 1) there was enough material for gold-standard histopathology; 2) resected specimen was representative of a tumor or a healthy tissue.

2.3 Protocol and Data Acquisition

Autofluorescence measurements have been made at different times starting from T_0: time of the reception of the sample in the Neuropathology Department. The same region on the sample has been measured every 60 minutes, between T_0 and T_0 plus five hours. The first four samples (Metastasis 1 and 2; Cortex 1 and 2) were studied during three hours: at T_0; and every 60 minutes, respectively T_1, T_2 and T_3. The fifth sample (Metastasis 3) was studied at T_0, T_1, T_2, T_3 and also after four and five hours, respectively T_4 and T_5.

Table 1: Mean maximum fluorescence intensity of the five endogenous fluorophores at different times with 405 nm excitation wavelength: after excision (T0), one hour after excision (T1), two hours after excision (T2) and three hours after excision (T3).

Excitation wavelength (nm)	405				
Spectra fitting: Fluorophore	NADH	FAD	Lip	Porph	Clorin
Sample Time after selection	Fluorescence intensity (a.u.)				
Metastasis 1					
T0	2.29	8.00	7.81	7.95	16.50
T1	0.38	9.89	8.58	9.47	16.65
T2	0.59	7.70	8.30	7.69	15.47
T3	0.77	6.90	6.13	5.71	11.39
Metastasis 2					
T0	0.08	12.2	9.61	7.82	8.01
T1	0.41	11.6	7.66	8.00	8.92
T2	3.07	13.3	7.13	12.56	9.48
T3	0.14	7.85	9.30	6.34	8.95
Metastasis 3					
T0	6.25	26.1	23.1	39.5	46.18
T1	1.36	19.1	18.8	35.2	41.7
T2	1.68	17.9	15.9	25.5	32.9
T3	5.51	20.2	12.9	22	32.7
Cortex 1					
T0	5.47	20.4	13.1	29.7	30.06
T1	5.08	11.6	10.5	15.3	16.34
T2	4.21	11.5	9.02	14.4	14.85
T3	4.53	13.3	9.61	18.7	16.67
Cortex 2					
T0	3.99	21.1	14.0	9.92	16.80
T1	7.31	16.3	13.4	14.3	16.93
T2	1.44	7.96	4.24	8.50	6.87
T3	3.19	17.2	12.4	10.6	16.64

Fluorescence lifetime acquisitions were made on a selected Region of Interest (ROI) of the sample.

Spectral acquisitions were made on a line for the first four samples. This longitudinal acquisition allowed us to investigate a large part of the sample. For the fifth sample (Metastasis 3), spectral acquisitions were made on a single ROI during five hours.

Samples were conserved in physiological solution during the procedure to be as close as possible to the brain during neurosurgery.

2.4 Results

2.4.1 Spectral Emission

Fluorescence intensity showed a slight decrease within time. A strong decrease (>50%) of the fluorescence intensity was observed for the longest time intervals (T4 and T5). For the majority of fitted spectra, maximum fluorescence intensity of the five investigated endogenous fluorophores was lower after three hours (T3) than at the initial measurement (T0). Note that this decrease was not always observed and a large variability existed on the data set. The mean fluorescence intensity of each explored fluorophore is detailed on Table 1 at at 405 nm excitation wavelength and for every sample. Figure 2 summarizes the results at 375 nm excitation wavelength.

At 405 nm excitation wavelength, cortex samples showed stronger fluorescence intensity values than metastasis and this remained true during all the protocol. At 375 nm excitation wavelength, the opposite situation seemed to exist (figure 2) even if partial results did not allow any conclusion.

To go further and to explore change in the spectra shape, we recorded the last metastasis sample until the fifth hour and on the same ROI. Results are presented in figure 3 and figure 4.

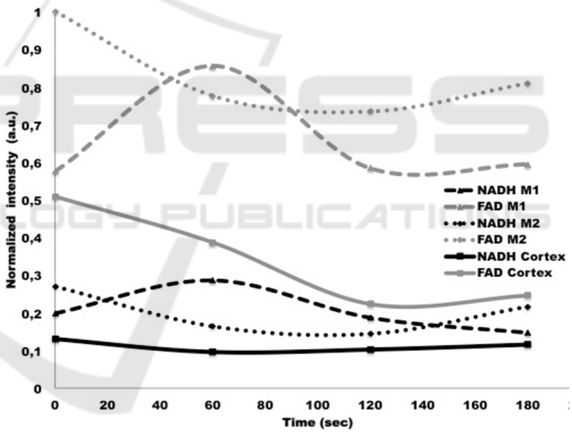

Figure 2: Time variation of the mean maximum fluorescence intensity emission of NADH and FAD for different samples: Metastasis 1 (M1), Metastasis 2 (M2) and Cortex 1 (cortex) at 375 nm excitation wavelength.

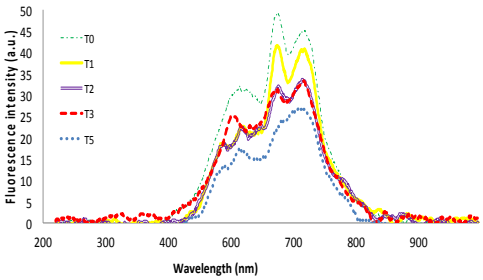

Figure 3: The spectral shape of the metastasis 3 sample fluorescence emission until the fifth hour and on the same ROI using 405 nm excitation wavelength.

Spectral shape was also affected with time: at T0, three peaks existed around 600 nm, 680 and 700 nm with 405 nm excitation wavelength and the first two first peaks (600 nm and 680 nm) were also present at 375 nm, whereas after five hours (T5), no peak was visible with both 375 nm and 405 nm excitation wavelengths. These changes could be related to tissue oxygenation.

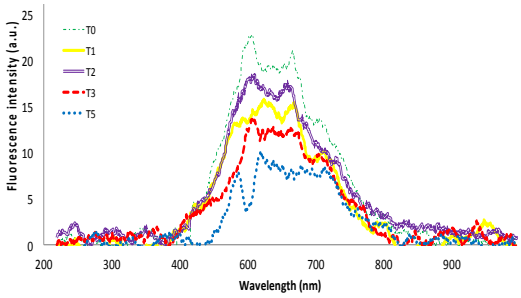

Figure 4: the spectral shape of the metastasis 3 sample fluorescence emission until the fifth hour and on the same ROI using 375 nm excitation wavelength.

2.4.2 Lifetime Measurements

Table 2 summarizes the lifetime fluorescence measurements overtime for the three-metastasis samples and the two samples of healthy human cortex using 405 nm excitation wavelength.

The three-metastasis samples showed the same trend: lifetime values shortened slightly with time at 405 excitation wavelengths. Longer lifetime values were observed at 375 nm excitation wavelength for the first two filters (450 ±10 nm and 520 ±10 nm) with regard to 405 nm excitation wavelength values. Lifetime values ranged from 1.69 ns to 5.63 ns. Interestingly, at 405 nm, the values for the last two filters (620 ±10 nm and 680 ±10 nm) seemed longer for metastasis samples than for cortex samples. The variation of the fluorescence lifetime could be related to the viability of the tissue. Indeed after three hours the cellular structure changed as we can easily notice on histological analysis done right after the surgery or after T3. Consequently the microenvironement of the tissue changes too, which

Table 2: Lifetime measurements of the five endogenous fluorophores at different times with 405 nm excitation wavelength: after excision (T0), one hour after excision (T1), two hours after excision (T2) and three hours after excision (T3).

Excitation wavelength (nm)	405				
Filter wavelength (nm)	450 ± 10	520 ± 10	550 ± 30	620 ± 10	680 ± 10
Sample Time after selection	Lifetime value (ns)	Lifetime value (ns)	Lifetime value (ns)	Lifetime value (ns)	Lifetime value (ns)
Metastasis 1					
T0	2.98	3.28	3.99	2.66	1.93
T1	2.40	2.27	3.48	2.19	1.80
T2	2.60	2.46	3.73	2.09	1.81
T3	3.04	3.01	3.94	2.47	2.15
Metastasis 2					
T0	2.70	2.94	3.70	2.69	1.86
T1	2.35	2.64	3.52	2.62	2.01
T2	2.33	2.97	3.40	2.63	1.85
T3	2.17	2.52	3.47	2.33	1.69
Metastasis 3					
T0	2.20	2.07	3.74	5.63	4.32
T1	2.64	2.93	3.93	3.96	3.74
T2	2.81	2.81	3.83	4.21	3.48
T3	2.59	3.05	3.81	3.62	3.19
Cortex 1					
T0	2.24	2.37	3.45	1.88	1.76
Cortex 2					
T0	3.14	2.94	4.13	2.03	1.75

could affect lifetime measurements. In general, three hour after tissue excision, no (remarkable) clear difference was observed on fluorescence lifetime measurements between all studied fluorophores on these metastasis samples.

Healthy cortex samples seemed to present shorter lifetimes values than metastasis samples especially for the last two filters (620 ±10 nm and 680 ±10 nm) corresponding to porphyrin and chlorin, at 405 nm excitation wavelength.

3 DISCUSSIONS AND CONCLUSION

Our work, the first study of fresh human brain samples autofluorescence over time, led to four main conclusions 1) fluorescence intensity decreased slightly with time, 2) spectral shape was considerably modified over time, 3) fluorescence lifetime measurements decreased marginally with time too, and 4) concerning ex vivo studies, autofluorescence measurements should be acquired in less than three hours after excision.

This investigation is crucial to validate the use of endogenous fluorescence as a new imaging tool. More than 70% of intracranial surgery last longer than two hours and duration of surgery is a risk factor for extracranial complications (Golebiowski, 2015). Prior to develop a device able to help neurosurgeon during his interventions, it is necessary to take into account the potential variation of autofluorescence over time.

Our work revealed that autofluorescence decreased with time after extraction but that this decrease was highly variable for fluorescence intensity and not strong for both fluorescence intensity and lifetime measurements. This is in accordance with the only reference on autofluorescence variation with time (Groce, 2003). Metastasis and control samples (cortex providing from epilepsy surgery) showed the same trend to a slight decrease in fluorescence intensity and lifetime values with time. Higher fluorescence intensity values at 405 nm excitation wavelength for the control samples compared to metastasis samples were found during all the protocol. If multimodality is the clue to overcome previous limits of autofluorescence per operative use (Marcu, 2012) (Groce, 2014), it is not possible to distinguish healthy boundaries and tumor with the spectra shape: spectra did not show any recognizable peak five hours after extraction.

These results are preliminary and need to be confirmed and specified. Larger cohort with more ROI for lifetime measurements is required.

Our work underlines the necessity to take into account clinical issue to develop and calibrate an adequate and precise tool to help neurosurgeon performing gross total resection. Close collaboration between clinical and scientific teams is required to investigate brain autofluorescence.

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