Endogenous Fluorescence Analysis under Deep UV Excitation to Discriminate Human Brain Tumor Tissue

Difference between Glioblastoma and Healthy Control Tissue

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Abstract: In order to build a multimodal nonlinear endomicroscope to image brain border during operation, our group is building an optical database on brain biopsy tissues analysis collected with excitation panning from deep UV to near infrared. This paper focuses on the results from deep UV excitation of endogenous fluorescence from glioblastoma and control human brain samples. The samples were imaged and spectrally analysed. The excitation wavelengths were tuned from 275 nm to 340 nm. Two promising indicators to discriminate tumorous tissue from the control were found. A preliminary correspondence between fluorescence images and histological H&E staining open a huge door to confirm results with a medical expertise.

1 INTRODUCTION

Cancer is one of the major causes of death worldwide, in 2012, there were 14 million new cases and 8.2 million cancer-related deaths("Cancer Statistics," n.d.). Brain tumour may only be the 17th most common cancer in the world, recent studies in the US showed that it is one of the most dangerous one, inducing the most important number of cancer related death in the population aged between 15 to 39 years ("Brain Tumor Statistics | American Brain Tumor Association," n.d., "Worldwide data | World Cancer Research Fund International," n.d.). Improving survival rate and recovery from such tumours is a constant key challenge for the medical community. One of the major issues to tackle in brain surgery is the extent of resection. Indeed, most of the brain tumours tend to infiltrate quickly the surrounding healthy area, and if actual technologies, such as scanner and IRM give a clear margin around necrosis, they do not give any information on the rate of infiltration from the surrounding. The surgeons today follow the rule of maximum possible resection; stopping before touching any vital functional part of the brain(Sanai and Berger, 2008). The aim of our project is to give new optical contrast and information on the tissue during the operation in order to identify this infiltrated region. To succeed in such project we are building a new endomicroscopic tool combining different imaging modalities to give the more precise answer to the surgeon. In parallel to the instrumental development, it was crucial to discriminate tissue nature in order to confirm the power of multimodal optical analysis. For that purpose, we are creating a database of optical signature from the different tumour tissue and their corresponding control using different optical contrast. This database will help to give a discriminatory answer on the nature of the tissue and to define different indicators. This database groups different optical response: (i) fluorescence imaging, (ii) spectroscopy, (iii) lifetime fluorescence analysis and (iv) second harmonic generation imaging. These data were acquired using an excitation ranging from the deep UV to the near infrared.

In this article we will focus on the results from the deep UV excitation of tissues. Under this excitation window we were able to make full field imaging and

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precise spectral measurements. Emission-collection matrixes of different endogenous molecules were measured and analysed. Some molecules of interest were spectrally followed using different excitation wavelength. Fluorescence images were compared to the hemotoxylin and eosin (H&E) staining standard of the Sainte-Anne hospital. Interesting structures were identified in optical images and matched finely with anatomopathologist diagnostic indicator.

2 MATERIELS AND METHOD

2.1 Imaging and Spectroscopic Measurements

The data have been acquired on the DISCO beamline at the Synchrotron SOLEIL. Two microscopic set-ups are available on the beamline and were used in this study.

The samples are excited with the continuous emittance from the DISCO beamline bending magnet at 275nm and between 310 and 340 nm. First the samples were analysed under a full-field microscope (Zeiss Axio-observer Z-1) with a x40 glycerine immersion objective (Zeiss Ultrafluar, NA 0.6). Emitted fluorescence was collected via a PIXIS 1024 BUV camera (Princeton, USA) through four bandpass filters: 307-323nm, 323-357nm, 408-438nm and 435-455nm (Semrock), with an integration time of 10s. This set-up is completely controlled by an open source microscopy software Micro-Manager (Edelstein et al., 2014). Spectral measurements were recorded with an inverted Olympus IX71 microscope stand with homemade DUV lenses. Light detection was collected through a DUV lens and an adjustable pinhole. Then, the fluorescence emission spectrum was projected onto a −70◦C peltier-cooled iDus CCD detector (Andor) of 1024 × 256 pixels with a 26 × 26 µm pixel size and a 26.6 × 6.7 mm detector size.(Jamme et al., 2013)

2.2 Samples

A strong collaboration has been established with the departments of anatomopathology and neurology of the Saint-Anne Hospital (Paris, France). The result of this collaboration was the access to a large cohort of human biopsy samples. The protocol of experimentation was approved by the Institutional Review Board of Sainte Anne Hospital (Ref CPP S.C.3227). For this specific study, ten samples have been selected, five of them were from glioblastoma tumour and the other five were control sample from epileptic surgery. The DISCO beamline microscopic set-up requiring 10 microns slices of each sample, a very strict process was put in place. The biopsies were fresh samples conserved at -80°C. A specialized transport service (360°, France) brought the selected cohort to the IMNC lab. Samples were then conserved at -80°C. These samples were kept under -20° freezer 24 hours before being cut with a cryostat (Leica CM 1950). Then 10 microns from samples were deposited on quartz coverslips. Serial 10 microns slices were cut for different colorations. Once on the coverslips or glass slides the samples were fixed at hundred percent alcohol solution. The quartz coverslips were brought to the Synchrotron in a box dedicated to microscopic slides transportation

2.3 Histological Staining

One of the slices was used to perform the gold standard H&E staining. Staining protocol was provided by Sainte-Anne hospital anatomopathological staff. The sample has to go through hydratation, hematoxylin and Eosin coloration, deshydratation and toluene fixation. The stained samples were then imaged in a slide scanner. A comparison between UV results and H&E staining was possible and performed thanks to the expertize of the Sainte-Anne anatomopathologists.

2.4 Data Analysis

Spectral data were acquired with the Labspec software, on each image a square of 12 by 12 points with 5 microns between each points was selected, a spectrum was acquired for every point with a 10s integration time. Preliminary treatments were applied to suppress noise and detector dead pixel using a Matlab script developed by the DISCO team. The treated data are then fitted with a homemade Matlab script. This script was already used and published in the visible (Haidar et al., 2015) and near infrared range and readapted here for the deep UV spectral analysis. Four molecules fluorescence emission were fitted: Tyrosine, tryptophan, collagen and NADH. These molecules were chosen through a complete review of the literature and the expertise of the DISCO scientists (Croce and Bottiroli, 2014; Jamme et al., 2013). The first three components were fitted by a Gaussian curve, the NADH component was fitted by an experimental curve established during previous studies of the group (Haidar et al., 2015).

3 RESULTS

The deep UV excitation range excites four different molecules: Tyrosine, Tryptophan, Collagen and the NADH (Nicotinamide adenine dinucleotide) the acquired emission spectra were fitted in regards of these four molecules. An example of a fitted spectrum is given in the figure 1a. It shows that the main contributing fluorescent molecule in the deep UV range is the tryptophan, its response is two times higher than the other identified molecules. The mean of the fitted curve in each group (glioblastoma and control) were calculated and plotted on the figure 1b, in order to compare the two types of sample. The first thing we observe is that the intensity is 20% lower in Glioma tissue, this phenomenon seems to be in adequacy with some of the literature (Butte et al., 2005; Palmer et al., 2003).

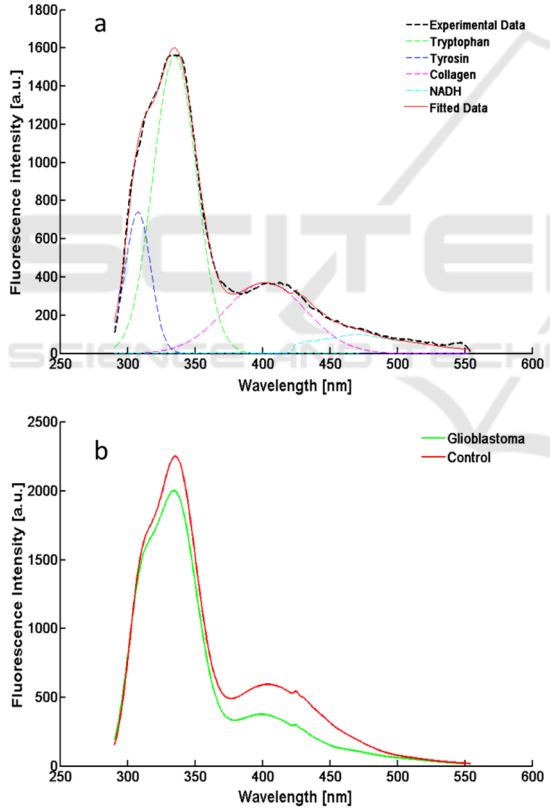

Figure 1: (a) Example of experimental data fitted with our homemade script. (b) Comparison of the mean spectra for the two groups: Glioblastoma and Control samples.

In the figure1b, we can hint that the ratio between tryptophan and collagen change with the type of tissue. To precise the tryptophan-collagen ratio, for each type of tissue, we measured it in all the spectra recorded for the five samples. The results were then presented in a boxplot using the software R, the ratio was calculated from the max of emission obtained in the Matlab fit and rearranged in boxplot, see figure 2b. A significant difference appeared between the mean of the two boxplots, to confirm this hypothesis a Mann-Whitney analysis was performed on the ratio data. The hypothesis of identical groups, gave a p value of $3.6.10^{-4}$ which is lower than 5.10^{-2} and rejected the hypothesis. This statistical result gives more confidence to confirm that the collagen-tryptophan ratio can discriminate glioblastoma from control samples.

A spectral analysis was accomplished using different excitation wavelengths. This study was performed from 310 to 340 nm by step of 5 nm. We could follow the behavior of different endogenous molecules through the excitation and go further in the exploration of the characteristics of the tissue. From the spectra, we obtained a closer look to the maximum emission of NADH component to look for the most appropriate excitation wavelength. Results are shown in Figure 3.

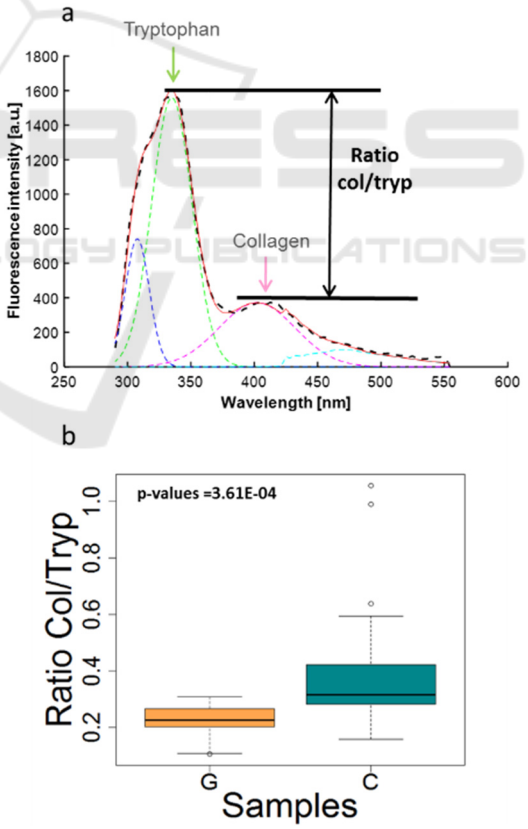

Figure 2: (a) Illustrate the chosen ratio for a statistical analysis of the tissues. (b) Box plots of the ratio.

This graph shows that the NADH component increases with the wavelength. This results is

explained by an optimal excitation wavelength at 345nm(Jamme et al., 2013), nevertheless here in tumorous tissue, the maximum of emission is at an excitation at 325nm, this shift could be a new indicator of change in cancerous cells.

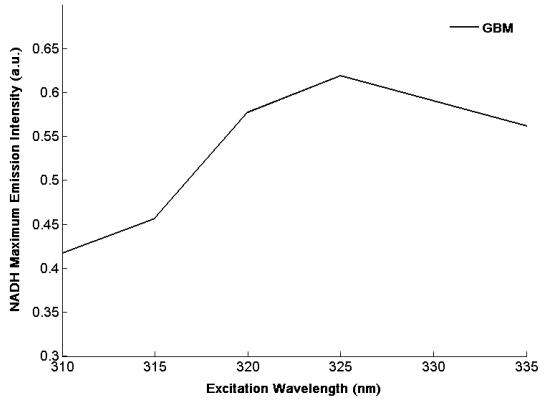

Figure 3: Variation of NADH maximum emissions as a function of the excitation wavelength.

Our collaboration with the Sainte-Anne hospital gives us access to a histological analysis. The deep UV wide-field images have been compared to the H&E staining, figure 4. Deep UV fluorescence images as presented in the figure 4 are the combination of four channels: Tyrosine for red channel, Tryptophan for Green channel, Collagen for Blue channel and the NADH for yellow channel, all combined with an identical coefficient. While pointing out specific structures in each type of tissue is difficult, we were still able to extract some information; indeed the area showing high density of cells on the H&E images appears darker on the deep UV images. The tryptophan being the main component (green images) these darker areas could correspond to a loss of tryptophan, but also to an increase of blue channel, corresponding to collagen.

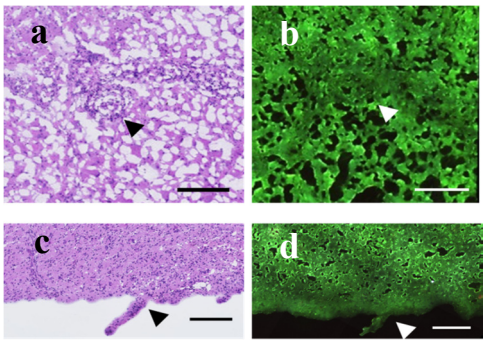

Figure 4: Comparison between H&E staining images, (a,c) and wide-field fluorescence (b,d) in control tissue (a,b) and in glioblastoma (c,d). The scales are 250 (a), 200 (b,c), 100 nm (d).

4 DISCUSSION

This study was accomplished thanks to the straightforward collaboration between physicist, neurosurgeon and anatomopathologists team of Sainte-Anne hospital and DISCO beamline Staff of Synchrotron SOLEIL.

In this paper we performed a preliminary study on two groups of biopsy samples: a healthy control group and glioblastoma tissues. For this preliminary study, we choose a tumorous group in an advanced stage that shall present major differences compared to the control. This choice shall allow us to find indicators of discrimination more easily. Thereafter we will be able to analyze infiltrated tissues, which have a lower density of cancerous cells, with analysis tools strengthened by this preliminary work. Tissues were analyzed under deep UV excitation, through imaging and spectroscopic techniques. Deep UV is a well-known energy range to look at endogenous fluorescence molecules of interest to discriminate the nature of tissue. However, the literature lack of analysis on human tissues and the different types of tumor. It will be completed by an analysis at visible and near-infrared excitation to cover the whole spectrum and establish relationships between the endogenous molecules.

In the paper we were able to highlight two indicators that discriminate the nature of tissue: the fluorescence intensity and the ratio collagen-tryptophan.

The fluorescence intensity was already found useful in our previous study on the grade of meningioma in the visible and near infrared (Zanello et al., 2016) and appears so as one of the promising way to find an optical signature on tumorous brain tissues. However, the spectral response is controlled by numerous environmental and experimental factors such as molecules concentration or laser power, creating a huge experimental uncertainty and needing as much parameters as possible into consideration to make a realistic comparison between signals intensities. In the literature a lot of groups have already started to work with a spectral response of endogenous fluorescence(Chorvatova and Chorvat, 2014; Croce and Bottiroli, 2014), looking for changes in the quantity of molecules present between types of tissues. In this paper and our future works we want to correlate the spectral data to other modalities such as lifetime measurement or second harmonic generation to build a matrix of optical characteristics for each type of tissue.

The second indicator presented in this article is a ratio on two major components of the tissue, the use of a ratio eliminates all the previous bias enumerated before and will bring more reliable results. Finding a change in Tryptophan-Collagen ratio seems realistic in tumorous tissues, knowing that tryptophan is linked to vascular region and the tumorous tissue present increased vascularity. Necrosis tissues in glioblastoma are poor in collagen such as control tissues and the change in tryptophan can be highlighted through this ratio, however in other tumorous tissues such as metastasis, a collagen matrix spreads to organize cells migration inducing both an increase of tryptophan and collagen, therefore this ratio will not change as significantly as in glioblastoma, giving false negative. It will be interesting to look at other ratio and increase the number of tumor types in the cohort.

The data showed that in this excitation range we were able to fit the NADH component, this molecule plays an essential role in metabolism, as a coenzyme in redox reactions. And appears in the literature as a major indicator in endogenous fluorescence. Its behavior in tissues under visible and two photon excitation has been well documented (Huang et al., 2002; Skala et al., 2007). Articles looked at its cross section over the excitation range or the redox ratio. Knowing the role of this component we followed it in deep UV, with a study over the excitation wavelength from 310 to 340nm. The maximum in excitation for the NADH in solution is at 345nm, so we should get a curve increasing with the wavelength. In our result we noticed a decrease at 340nm in the glioblastoma tissue. It could be either a new indicator for cancerous tissue or just an experimental artifact, due to the fact that this measurement has been done on a very small number of sample. Increasing the statistic of this analysis could give significance to this result and highlight an important phenomenon in tumorous metabolism.

Wide field images of endogenous fluorescence allow us to correlate an area on each sample to the H&E staining, the gold standard in histology to validate the tumorous nature of a sample. This correlation was possible thanks to the help of anatomopathologists from Sainte-Anne hospital. Correlation allowed us to demonstrate that high cells density area in H&E images correspond to darker area in wide-field images. Green channel in images represent Tryptophan filter, these areas could correspond to a loss of tryptophan or an increase if the other channels, especially the collagen one. Correlation with two-photon imaging could be an interesting way to find more information.

All this promising resulfts encourage to increase the cohort in order to have a better statistic on the results.

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