SYNCHROTRON DEEP UV FLUORESCENCE MICROSPECTROSCOPY FOR FIBERS CHARACTERIZATION.

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Abstract –Skeletal muscles are composed of 3 to 4 muscle fiber types characterized by the combination of their metabolic pathway (glycolytic or oxydative) and contraction speed (slow or fast). Muscle fiber types are usually identified by or histoenzymological or immunohistochemical techniques. We have implemented a technique of Deep UltraViolet Fluorescence (DUV) microspectroscopy to characterize in situ different types of muscle fibers previously identified by immunohistofluorescence. Multivariate analyzes showed a discrimination of oxidative fibers (I, IIA) from glycolytic fibers (IIB, IIX) in the EDL muscle. In the soleus muscle which is composed of only oxidative fibers, fast and slow (I and IIA respectively), we observed a very good separation of these two populations. In the short term, the different fiber types could be identified by direct analysis of a section of muscle without any probe or dyes.

Key Words – Skeletal muscle, Fiber typing, Fiber composition, Microscopy, Histology, Deep UV Fluorescence microspectroscopy, Synchrotron radiation

I. INTRODUCTION

Skeletal muscles are composed of four types of muscle fibers respectively identified I, IIA, IIB and IIX, characterized by specific isoforms of myosin heavy chain [1]. Intracellular composition varies depending on cell type [2] which differ in their contraction speed (slow type I fibers and fast fibers type II) and energy metabolism (for oxidative fibers I and IIA or IIX fibers to glycolytic and IIB). Muscles are composed of these fiber types in varying proportions according to their anatomical location and physiological function. The technological, nutritional and sensory meat depends on fiber type [3] [4] [5] which give their characterization necessary to explain some of the variability of quality meat products. The most common histological techniques are based on the acid-base resistance ATPases myosin heavy chains or the use of monoclonal antibodies against different myosin isoforms [6] [1].

All these histological methods are cumbersome to implement, require most of the time making serial sections of which are performed enzymology staining and/or immunolabelling colorations complementary to each other. All the data must then be taken into account to accurately identify the type of fiber. Some authors have developed methods of co-staining on the same tissue section to reduce the number of histological sections and therefore the duration of characterization [7]. Others developed image analysis algorithms to automate the identification of cell types from digital images of muscle sections labeled for different myosin heavy chain isoforms [8].

But despite these improvements, the techniques are quite time consuming, expensive and require a specific qualification of laboratory personnel.

Differences in biochemical composition of each type of fiber has led us to explore the use of fluorescence microspectroscopy to discriminate fiber types without any coloration .

II. MATERIALS AND METHODS

A 5 months old male Wistar rat was sacrificed by decapitation with a guillotine. Extensor Digitorum Longus (EDL) and Soleus (So)

muscles were taken from tendon to tendon, and cryofixed in -160°C isopentane. Serial cross-sections (10 µm thick) were collected on glass and silicium slides for histological and DUV microspectroscopy fluorescence analysis.

Fiber typing by immunohistofluorescence

Fiber typing was done according to Astruc et al. 2012 [9]. Briefly, slow and fast myosin heavy chains isoforms (MyHC) were identified using mouse monoclonal antibodies specific to MyHC isoforms BA-D5 (MyHC-I), SC-71 (MyHC-IIA) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and BF-F3 which reveals MyHC-IIB (American Type Culture Collection, USA). The different primary MyHC antibodies were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen).
Observations and images acquisitions were performed using a photonic microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software. Immunohistochemistry images were acquired in fluorescence mode (Cyanine 3: 550/570 nm; Alexa Fluor 488: 495/519 nm). Myofibre subtypes I, IIA, IIB, IIX and hybrid IIB-X were deducted according to their response to the different antibodies.

Deep UV microspectroscopy analysis

Myofibres types, identified by immunohistochemistry, were localised on the unstained serial sections intended for DUV microspectroscopy measurements. In this area, each myofibre characterised on its cell type was identified by a number. Ten cells of each type were selected to make measurement on DUV microspectroscopy.Synchrotron DUV microspectroscopy was performed on the DISCO beamline of the SOLEIL synchrotron radiation facility (Saint-Aubin, France). DUV monochromatized light (typically between 270 and 330 nm) was used to excite tissue sections through a 40x ultrafluar immersion objective (Zeiss, Germany). The emission spectra were acquired from 290 to 540 nm and recorded with a 0.1 nm spectral resolution. The fluorescence spectrum arising from each excited pixel was recorded. On each selected cell, identified by its type by immunohistofluorescence, 20 acquisitions with 1 µm spatial resolution with 20 s acquisition time per spectrum were made in the intracellular space. Among these 20 acquisitions, 10 were acquired in the central part of the cells and 10 were acquired on cell periphery, to target the mitochondria, at several micrometers of the plasma membrane to avoid an overflow into the extracellular space. The excitation wavelength of 275 nm was selected to excite both NADH, tyrosine and tryptophan.
Autofluorescence spectra were spike and noise filtered using an in-house program written in MATLAB version 7.3 (The MathWorks, Natick, MA) and images of each individual fluorescent component were produced. The Unscrambler software (v9.8, Camo Software AS, Norway) was used to perform a baseline adjustment to zero and to apply a unit vector normalization.

Processed spectra were analyzed in principal component analysis (PCA) under the Unscrambler software (v9.8, Camo Software AS, Norway). PCA was applied as an unsupervised approach, in order to handle this new set of data and reveal variances or combination of variables among this large multivariate data set. For this study, the fluorescence spectral domain has been focused on 290-540 nm. The number of possible components was always let sufficiently high (7). After analysis, the family label of each spectrum was revealed and the two first components were plotted. The mean characteristic spectrum of each group was also plotted in order to relate the separation to spectral feature. Score plots were used to show similarity maps allowing comparison of spectra regardless to sample categories. Loading plots derived from list and second principal components X-loading plots were used to reveal and identify characteristics vibrational absorption bands.

III. RESULTS AND DISCUSSION

DUV spectra were acquired for the first time on muscle fibers (Fig.1) previously identified according to their metabolic type determined by immunofluorescence (Fig.2). On EDL muscle, we observed a fairly good separation of oxidative fiber (I and IIA) compared to glycolytic fibers (IIB and IIX) according to PC1 for the 410 nm wavelength. Most of the fibers IIX are separated by PC2 at 306 nm and 360 which may assigned to tyrosine and tryptophan, respectively [10] (Fig.3A). Contrary to our expectations, we did not separate fibers at 460 nm assigned to the NADH fluorescence, suspected to be more concentrated in oxidative fiber. This result is probably due to the fact that our measurements were performed on a muscle fixed just after animal bleeding. Indeed, NADH is very little concentrated in oxygenated cells, unlike the NAD which autofluorescence is not detectable [11].The soleus is composed exclusively of type I fibers and IIA. These two populations are very well separated by the PC2 at 306 nm assigned to tyrosine (Fig.3B). The separation is therefore the criterion "speed of contraction," which suggests differences tyrosine content or environment between slow and fast fibers twith fibers.

IV. CONCLUSION

Deep UV Fluorescence Microspectroscopy could discriminate directly the various types of muscle fibers in histological sections without the use of dyes or probes. Separation is clearer on the soleus than on the EDL muscle which is composed of four fiber types. These results are very encouraging and suggest that typing muscles can be done by acquiring fluorescence mapping of muscle tissue sections. A better understanding of the mechanisms that govern these discrimination will undoubtedly refine the acquisition conditions to improve the discrimination of different cell types.

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Fig 1: Deep UV spectral acquisitions

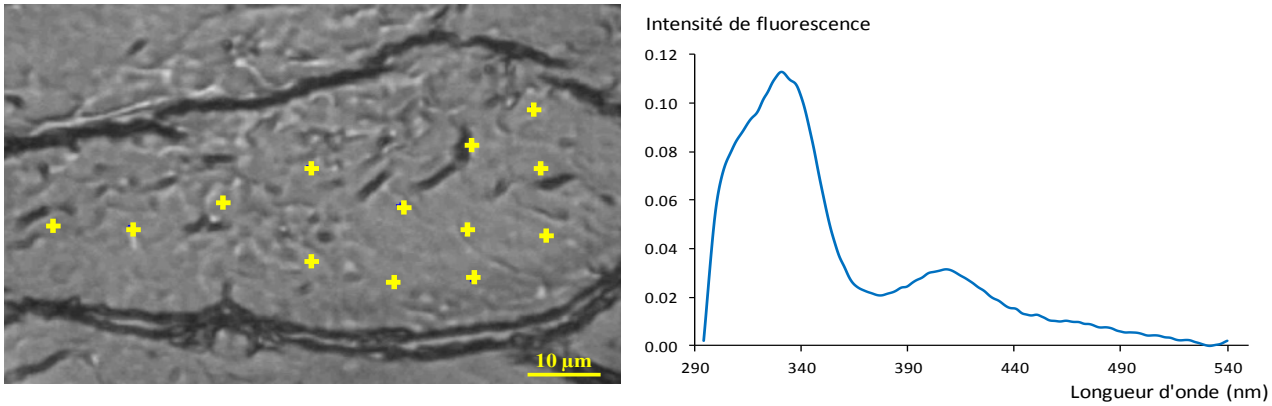

Fig 2: fiber typing by immunohistofluorescence

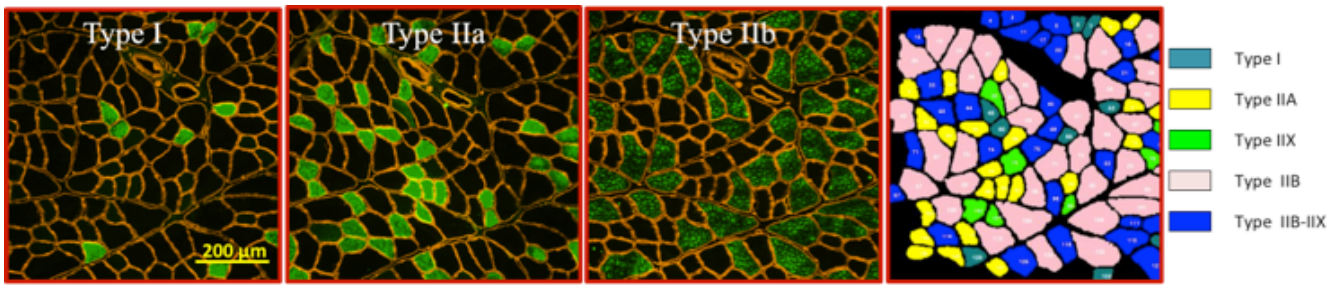

Fig 3: Principal Component analysis