Looking at life through molecular vibrations: biomedical applications of CARS spectro-microscopy
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The accumulation of lipids in the body is one of the major factors determining the occurrence of health diseases such as type II diabetes. In particular, the ectopic storage of lipids in skeletal muscles is believed to be related to the regulation of insulin sensitivity. Despite extensive efforts to understand the chemical composition of lipid droplets in tissues, as well their dynamic processes, progress in the field has been hampered by the lack of an experimental technique able to provide simultaneously the visualization of the biological components of the tissue, and information on their chemical composition. We present here the application of CARS spectro-microscopy to study cells in tissues in which one protein is overexpressed, which is known to be important for lipid metabolism. These cells have high density of lipid droplets. Evaluation of the CARS data with multivariate analysis methods allows us the identification of lipid droplets and possibly mitochondria in the tissue, based solely on their vibrational response. Our preliminary results show that CARS spectro-microscopy can complement standard immunohistochemistry techniques to retrieve local chemical information on individual biological components of the muscles.

7.1 Introduction

Obesity\(^a\) is one of the major health risks in western countries, with an increasing prevalence of 1.4 billion adults worldwide [153]. Among other health threats, such as cardiovascular diseases, type II diabetes is one of the best-described resulting pathologic outcomes of chronic obesity. Type II diabetes is defined as the general resistance of an organism to its own insulin. Insulin, whose role in the organism is to trigger the uptake of glucose into cells, will consequently be secreted at higher levels from the pancreas compensating for excess glucose in the blood stream. The manifestation of diabetes occurs when the pancreas is not capable of compensating for this excess of glucose, resulting in relative insulin deficiency and hyperglycaemia. Complications of type II diabetes include

\(^a\)Obesity can be indicatively defined using the oversimplified body mass index (BMI). The index is calculated as the body mass in kg divided by the square of height in meters. Individuals with BMI values of 25 and higher are considered as overweight, 30 and higher as obese.
hypertonia, retinopathy, neuropathy, and nephropathy.

While the cause of type II diabetes is not entirely understood, strong evidence has been presented for imbalanced energy uptake and expenditure as the origin of the pathology (reviewed by Lee et al. [120]). One hypothesis sees lipid overflow as the major contributor to the onset of insulin resistance. Lipid overflow occurs when adipose tissue becomes incapable of storing lipids efficiently. Subsequently, lipids start to accumulate in other tissues of the organism: skeletal muscles, cardiac muscle or the liver. Among these, skeletal muscles take up the highest total amount of glucose via insulin signaling.

The accumulation of lipids in the skeletal muscles, also named intramyocellular lipids (abbreviated hereafter as IMCL), strongly correlates to large values of the body mass index (cf. note a), and has been reported to impair insulin sensitivity in various in vitro and in vivo investigations [3, 88]. On the other hand, diet-induced reduction of IMCL has also been found to reduce insulin resistance [105]. Efforts to understand the impact of IMCL levels on insulin resistance are further complicated by the finding of the so-called athletes paradox. Endurance trained athletes, who are highly insulin sensitive, show similar or even higher IMCL levels when compared to obese diabetics [87]. Furthermore, physical training improves insulin sensitivity in obese subjects, while at the same time elevating the IMCL level [136]. These apparently contradictory findings are currently explained by the occurrence of toxic lipid intermediates, which have been shown to decrease insulin sensitivity. Diacylglycerides (DAG) and ceramides in particular have been shown to interfere with insulin signaling. However, the exact mechanisms responsible for the correlation between IMCL level and insulin resistance remain to be clarified (reviewed by Bosma et al. [12]).

In order to understand ectopic lipid accumulation, the way lipids are stored has to be taken into account. Lipid droplets (LDs), the main site of neutral lipids storage in cells, are nowadays recognized as active organelles as opposed to mere oil droplets. LDs are composed of a phospholipid monolayer and a protein coating, enclosing a core of di- and triacylglycerides (DAGs, TAGs), cholesterol esters and free fatty acids. Proteins coating lipid droplets have been shown to be highly involved in lipid synthesis and lipolysis, thus presenting an important interface for lipid metabolism. The best-characterized group of LD coating proteins are the perilipins (PLIN1-5). Different members of this protein family are expressed differentially depending on the specific tissue. PLIN1 is primarily found in the adipose tissue, PLIN2 and PLIN5 are instead expressed in cardiac and skeletal muscles. Research in LD dynamics and interaction, while gaining momentum, is still a young field of study where several questions remain to be answered. For example, relatively little is known about the composition of LDs in cells, their size distribution and core protein content. Also, the biogenesis of LDs in mammalian cells is still to be clarified, with different studies proposing contradicting hypotheses regarding the mechanisms of LDs formation (reviewed by Suzuki et al. [194]).

The protein Perilipin 5 (PLIN5, sometimes referred to in literature as OX-PAT) has been identified as one of the LD coating proteins predominantly ex-
pressed in tissues with high fat-oxidative capacity, such as the heart muscle, type I fibers of skeletal muscles and brown adipose tissue. While it largely localizes to LDs in these tissues, it can also be found in the cytosol of cells, and has recently been reported to be present in myocellular mitochondria [13]. The function of PLIN5 is partly still to be explained; overexpression in vitro leads to increased TAG storage and formation of larger LDs in the cytoplasm, while at the same time increasing fatty acid oxidation and mitochondrial mRNA levels [210]. When overexpressed locally in vivo by electroporation, PLIN5 induces formation of large LDs when compared to empty vector controls, as was observed by immunofluorescence and transmission electron microscopy. Interestingly, muscle tissue in which PLIN5 was overexpressed, showed increased oxidative capacity in the entire muscle homogenates, whereas isolated mitochondria from the same tissue did not show increased oxidative capacity when challenged with free fatty acid. Thus, PLIN5 seems to play a role in the LDs mitochondria interaction, making fatty acids available for mitochondrial β-oxidation.

The major bottleneck in the study of metabolic processes involving lipids in tissues is the lack of suitable techniques capable of simultaneously addressing the localization and the chemical composition of LDs and of the other biological components present in the samples. Thin (5-20 µm) sections of muscle tissue can be routinely investigated by immunohistochemistry approaches, the most employed of which is immunofluorescence. In this technique, specific components of the tissues such as membranes, nuclei, or specific proteins are labeled with an antibody chemically linked to a fluorescent molecule. Direct labeling is achieved when the antibody recognizes the target molecule and binds to it, and the fluorophore it carries can be detected by fluorescence microscopy. Indirect labeling is instead mediated by an unlabeled primary antibody that binds to the molecule of interest. Here, the secondary antibody, which carries the fluorophore, selectively recognizes the primary antibody and binds to it. For the analysis of LDs, usually lipophilic dyes such as Oil red O, Nile red or Bodipy are used. Using a confocal fluorescent microscope, labeled components within the tissue can be visualized with sub-micron resolution. Due to the characteristics of the technique, immunohistochemistry can be employed in the study of tissues only for the visualization of components for which a suitable labeling antibody is available, and only a limited number of structures can be imaged simultaneously. This is particularly problematic for the study of LDs, where information on the local lipid composition would be desirable. The application of immunohistochemistry in the study of biological tissues is reviewed by e.g. Brandtzaeg [19]. The chemical composition of the fatty acids in muscle tissues can instead be obtained by gas chromatography (GC), where the amount of individual chemical species present in the sample is quantified [182]. Hence, GC serves the purpose of identifying the composition of lipids in the investigated tissue section, although the information it provides is global. A satisfactory compromise to obtain the local distribution of chemicals in the tissue is the use of spatially resolved mass spectrometry techniques such as MALDI-Imaging Mass Spectrometry (MALDI-IMS). This technique enables the detection with high sensitivity (femto to attomolar) in a small region of the sample of molecules
in a large range of masses (from small molecules of $\sim 100$ Da to large proteins, bigger than $300$ kDa). Incidentally, the spatial resolution of the technique is limited to tens of micrometers, preventing direct access to the chemical composition on smaller length scales [91]. It is then clear that the understanding of the biological mechanisms involving lipids in muscles, such as the ectopic lipid accumulation, would benefit from the use of a technique capable of determining the local chemical composition of the tissue without the need of exogenous labels.

In this chapter we present the application of broadband CARS spectroscopy to study muscle tissues where the protein PLIN5 was selectively overexpressed. Using CARS in combination with multivariate analysis tools we were able to address the composition of the tissue on the micrometer scale and to distinguish the biological constituents based on their chemical signature, as confirmed by immunofluorescence imaging of the sample.

## 7.2 Materials and methods

### 7.2.1 Electroporation

A total of eighteen 8-week-old male Wistar rats were purchased from Charles River (Wilmington, Massachusetts, USA). Rats were housed individually on a 12:12 h lightdark cycle (light from 7:00 am to 7:00 pm), at room temperature (21$\pm$2°C) with ad libitum access to tap water. Rats were fed a high fat diet (45% energy from fat, D01060502, Research Diets, New Brunswick, NJ, USA) for the duration of the 3-week intervention. The Animal Care and Use Committee of Maastricht University approved the experiments (approval number 2010-036) and the study complied with the principles of laboratory animal care. During the experiments, all efforts were made to minimize suffering of the animals. Two weeks after the start of the diet, overexpression of Plin5 in either the right or left tibialis anterior (TA) muscle of the rat was accomplished by an in vivo DNA electroporation technique to obtain overexpression of mouse perilipin 5 in one leg; the contralateral TA served as a sham-electroporated internal control. Plin5 was electroporated randomly in the left or the right TA. DNA electroporation was performed under isoflurane anaesthesia. TA muscles were transcutaneously injected with either 150 g (2 g/l) pcDNA3.1-CMV-Plin5 or pcDNA3.1-empty vector in 0.9% sterile NaCl. Within 15 s after the last injection 5 electric pulses were applied by two stainless steel plate electrodes placed at the ventral and dorsal side of the leg. One high voltage pulse of 800 V/cm and four low voltage pulses of 80 V/cm at 1 Hz were generated by an ECM 830 electroporator (BTX, San Diego, CA, USA) as described previously (Bruce et al. 2007, 2009). Rats were sacrificed 8 days post electroporation. TA muscles were excised and rapidly frozen in melting isopentane for histology and protein isolations.
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7.2.2 Bodipy staining and immunofluorescence

Frozen TA muscles were transferred to a cryostat and cut transversally into serial sections (5 \( \mu m \) and 20 \( \mu m \)). After mounting on uncoated glass slides, sections were allowed to dry for 30 min at room temperature and then stored at -20°C until processing. Five consecutive sections were cut from each sample of muscle tissue. The first section of 20 \( \mu m \) was used for CARS measurement, followed by four 5 \( \mu m \) sections, the first two of which were used for CARS and immunofluorescence respectively. The remaining two sections were kept for backup experiments.

One of the 5 \( \mu m \) sections from each sample was stained for lipid droplets, OX-PAT expression, mitochondria localization as well as sarcolemmal cell borders in order to be investigated by immunofluorescence. All staining procedures were carried out at room temperature. Sections were fixed for 1h (4% PFA in 1xPBS, pH 7.4) and washed three times for 5 min in PBS prior to blocking for 1h in blocking buffer containing 2% BSA. The sections were then incubated with primary antibodies diluted in blocking buffer against OXPAT (dilution 1:20; GP31, Progen Biotechnik, Heidelberg, Germany), OXPHOS (dilution 1:20; MitoProfile, Abcam, Cambridge, UK) and Laminin (L9393, Sigma Aldrich, St. Louis, USA) for 1h in a humidified chamber. After washing three times with PBS, sections were incubated with appropriate secondary antibodies (Alexa Fluor 350, 546 and 647, all from Invitrogen, Darmstadt, Germany), DAPI, and Bodipy 493/503 diluted in blocking buffer. Following three washing steps, sections were mounted in fluorescence mounting medium (Dako, Glostrup, Denmark) and imaged on an Olympus IX70 inverted microscope (Olympus, Hamburg, Germany) using Cell F imaging software. Images were processed and overlays created using ImageJ.

7.2.3 CARS experimental procedures

For CARS experiments, 5 and 20 \( \mu m \) consecutive sections of the muscle tissue were used without further preparation. Broadband CARS measurements were performed on the tissues using the experimental setup described in Paragraph 3.2. The sections were initially visualized with standard brightfield imaging to allow for correct identification of the area of interest. CARS imaging was performed in two dimension by measuring adjacent tiles in the sample consecutively until the desired region of the sample was measured. Each individual tile has dimension of 20 \( \times \) 20 \( \mu m^2 \) (corresponding to 81 \( \times \) 81 pixels). Acquisition time per voxel was set to 200 ms in order to obtain good signal to noise ratio without compromising the integrity of the sample.

7.2.4 Data analysis

Raw CARS spectra were analyzed with the program Igor Pro. Pre-treatment of the data with the MEM algorithm [202] retrieves the imaginary component of the third order susceptibility (see Paragraph 2.2), referred to as retrieved CARS
spectra in this thesis. Advanced image treatment made use of the software ImageJ.

Prior to Principal Component Analysis, CARS spectra were exported from the analysis software as text files. The data was converted to Matlab/ChemomeTricks data format using the in-house developed CARS2Tricks software. Individual CARS measurement tiles were stitched together using the in-house developed StitchCARS software. The ChemomeTricks software was then used to normalize the spectra and extract the principal components and associated scores maps.

### 7.3 Results and Discussion

#### 7.3.1 Brightfield and Immunofluorescence Imaging of the Tissues

In order to investigate muscle tissues where the protein PLIN5 was selectively overexpressed, preliminary measurements were performed with brightfield and immunofluorescence microscopy to study the morphology of the system. Fig. 7.1 shows two brightfield images of corresponding areas in two consecutive sections with thickness of 5 (A) and 20 μm (B), cut from the same muscle tissue. As the measurements are performed on consecutive sections of the muscle which have different thickness, some differences in morphology can be noted in the images, although the corresponding cells are easily identified in the two panels. Also, owing to the difference in thickness of the tissues, higher contrast is obtained in the brightfield image of the 20 μm section.

As the sections are obtained as transversal cuts of the muscles, the cells, which are composed of myofibrils, appear as nearly round structures contoured by thick black features corresponding to the sarcolemmal borders. Through visual inspection of the images, the cells can be indicatively divided in two groups according to the different morphology. For the cells of the first group, e.g. the large cell in the center of the figures, the brightfield response appears featureless, indicating a large degree of homogeneity in the local composition. The cells in the second group show instead a large number of dark features, indicating the intracellular presence of biological components with different density. Preliminary experiments with immunostaining of the protein PLIN5 (data not shown) highlighted that this second group corresponds to the cells where the protein was efficiently overexpressed, and large lipid droplets were present in the cells as a result of the modification. The difference in the expression of PLIN5 in neighboring cells in a tissue globally treated with electroporation is related to two factors: (i) the electroporation process is not effective in all the cells in the tissue [155]; (ii) the protein PLIN5 was found to be more efficiently expressed in type I oxidative (slow) cells in the muscles, compared to type II (fast) cells.

Although brightfield microscopy is instructive for the investigation of the morphology of the sample and for tentative identification of the cells expressing PLIN5, it provides limited insights into the intracellular chemical structure of
Figure 7.1. Brightfield images of the corresponding areas of two consecutive sections of muscle tissue of 5 (A) and 20 microns (B) thickness, respectively. The muscle was previously treated to selectively induce the overexpression of the protein PLIN5. Scale bar 20 microns.
Figure 7.2. Immunofluorescence microscopy image of a 5 microns section of the muscle tissue. Three labels were used for the identification of different components: DAPI (blue), Bodipy (green), and laminin (red) bind to nuclei, lipid bodies, and sarcolemmal cell borders, respectively. The image shows the corresponding region of the tissue as the one presented in the brightfield images in Fig. 7.1.

The tissue. The immunofluorescence microscopy image of the corresponding area of the tissue is presented in Fig. 7.2. The measurement was performed on a 5 $\mu$m thick section of the tissue stained for three different components. DAPI (blue) was used to label cell nuclei, Bodipy (green) for lipid bodies, and laminin (red) for sarcolemmal borders. The edges of the cells are neatly identified by the red lines marking the cell membranes. Nuclei are visible as blue features in the proximity of the membrane. In muscle cells the nuclei present elongated shape oriented along the stretching direction of the muscle. Interestingly, the cells showing intracellular structures in Fig. 7.1, which are expected to overexpress the protein PLIN5, also feature the most interesting lipid distribution in the fluorescence image. Several round green features are present in each of these cells, pointing to the presence of intracellular lipid aggregates. The bodipy response in the cells not expected to show protein overexpression is instead reduced to a homogeneous low intensity background, and no distinctive features are visible.
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7.3.2 Hyperspectral imaging of the muscle

Hyperspectral CARS datasets were obtained in two dimensions in the disc in order to image regions of interest in the sections of the muscle tissue. At each position, the CARS spectrum was retrieved in the spectral region between -3200 and -900 cm\(^{-1}\). The intrinsic resolution of the instrument with the employed experimental configuration was independently measured to be \(\sim 1 \times 1 \times 2 \, \mu m^3\). The CARS response measured at an arbitrary position in the tissue is presented in Fig. 7.4 (B) and Fig. 7.3 (B) for the 5 and 20 \(\mu m\) thick sections, respectively. These spectra were measured with 1 sec integration time.

As already discussed for the spectrum from the imaginal wing disc (cfr. Fig. 6.3), two regions of interest can be distinguished in the spectrum, the fingerprint and the C-H stretching region, separated by a silent region between -2700 and -1800 cm\(^{-1}\). As already outlined in Paragraph 6.3.1, the fingerprint region between -1800 and -900 cm\(^{-1}\) presents several peaks assigned in literature to the vibrations of specific molecular bonds [144]: CH bending and deformation between -1290 and -1400 cm\(^{-1}\), CH\(_2\) scissoring and bending, CH\(_3\) bending between -1400 and -1480 cm\(^{-1}\), and C=C stretching at -1650 cm\(^{-1}\) are assigned to lipids. The phenylalanine peak at -1002 cm\(^{-1}\), the amide III between -1200 and -1300 cm\(^{-1}\) and the amide I peak at -1650 cm\(^{-1}\) are instead assigned to proteins. Finally, the PO\(_2^−\) stretching peak between -1060 and -1095 cm\(^{-1}\) is assigned to nucleic acids. The C-H stretching region between -3150 and -2750 cm\(^{-1}\) contains instead overlapping vibrations arising from CH, CH\(_2\), and CH\(_3\) groups abundant in most biological compounds. We note here that, although most of these peaks are indistinctive of the class of molecules, vibrations specific to lipids are found in the lower wavenumbers spectral region below -2875 cm\(^{-1}\) [144], and peaks specific of amino acids have been assigned in the high wavenumbers region above -2930 cm\(^{-1}\) [102].

The spectra retrieved from the sections of different thickness were qualitatively similar, although the intensity of the collected signal was consistently lower for the thinner sections. This prevented the accurate determination of the spectrum in the fingerprint region for the 5 \(\mu m\) thick samples due to the small signal to noise ratio. Hence, for these samples, only the C-H stretching part of the spectrum was taken into account for the following analysis. As the axial resolution of the instrument is smaller than the thickness of the samples, the same response should be expected from both the 5 and 20 \(\mu m\) sections. We assume that the difference is caused by experimental factors. Specifically, the vertical position of the focus in the center of the tissue is determined with respect with the surface of the glass support, identified by the disappearance of the non-resonant response while moving the position of the focus. The accuracy of this procedure is also affected by the vertical resolution of the instrument, inducing a potential error in focusing the beams in the middle of the tissue. Also, CARS measurements are performed on the sections of the tissues kept at room temperature after letting the sample reach thermal equilibrium for one hour. As the tissue is not kept hydrated during this procedure, it is likely that, due to dehydration, the effective thickness of the sections is smaller than its
The large response obtained in the C-H stretching region of the retrieved CARS spectra can be used to obtain unspecific maps of the morphology of the tissue by integrating the response between -3020 and -2850 cm\(^{-1}\). Fig. 7.3 (A) and Fig. 7.4 (A) show the CARS map of corresponding areas in the 20 and 5 \(\mu m\) sections of the muscle, respectively. In the image relative to the 20 \(\mu m\) sample, the individual cells are visible, their shape perfectly identical to the corresponding brightfield image (Fig. 7.1 (B)). The cells expected to present overexpression of PLIN5 (modified) and the ones unaffected by the electroporation procedure (unmodified) appear different also in terms of the features of the distribution of CH groups in the sample. The response of the unmodified group of cells is homogeneous, without any characteristic element. The response of the modified cells instead presents large spatial variations. Both high and low intensity features are visible inside the cells, with either round or elongated shape. The sarcolemmal edges appear as hypointense lines around the cells, also providing the contour to intercellular areas giving low CARS response. Inside the cell at the center of the image, two large dark features are present. We believe the sample was damaged at this location by laser illumination. Similar considerations hold for the description of the CARS map obtained from the 5 \(\mu m\) section. Here, some high intensity nearly-circular features are also visible in the unmodified cells, possibly related to the subdivision of the cell in myofibrils.

The CARS maps allow us to obtain the morphology of the sample in terms of local concentration of CH-rich molecules in the tissue. In order to find an actual correspondence with specific molecular classes, further analysis of the hyperspectral dataset is required.

### 7.3.3 PCA of the muscle tissue CARS data

Hyperspectral CARS datasets measured in the sections of the muscle tissue were investigated with Principal Component Analysis in order to evaluate the chemical signatures contained in the vibrational spectra. We present separately the results obtained from the measurements performed on sections of the tissue with different thickness.

**Muscle section of 20 \(\mu m\) thickness.** The first three positive and negative scores maps calculated by PCA of the 20 \(\mu m\) section of the tissue are presented in Fig. 7.5. The positive scores map corresponding to the first principal component (+1) shows a uniformly intense image of the tissue with dark areas present at the edges of the cells and, locally, inside the modified cells. These dark areas appear bright in the complementary negative map (-1) allowing us to better identify their position in the sample. Comparison with the C-H CARS map in Fig. 7.3 (A) shows that the -1 map highlights the position of the sample where the overall intensity of the measured CARS signal was lower. This normalizing character of the first component is sometime found as the outcome of PCA when large differences in the intensity of the response are present in the original data. In this case, the first component is proportional to the mean spectrum of
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Figure 7.3. (A) CARS image of a region of $120 \times 120 \ \mu m^2$ of a 20 $\mu m$ section of the muscle tissue. The intensity values in the image are obtained by integrating the CARS response between -3050 and -2850 cm$^{-1}$ at each position in the sample. Scale bar: 20 microns. (B) Retrieved CARS spectrum at an arbitrary position within the muscle tissue. The C-H stretching region at high wavenumbers contains overlapping vibrations from the CH, CH$_2$, and CH$_3$ groups abundant both in lipids and proteins. The region at low wavenumbers (fingerprint region) contains a large number of peaks that have been assigned to specific vibrations of lipids, proteins and nucleic acids. The intermediate region of the spectrum is omitted due to the absence of vibrations specific to biological compounds.
Figure 7.4. (A) CARS image of a region of $120 \times 100 \ \mu m^2$ of a 5 $\mu m$ section of the muscle tissue. The intensity values in the image are obtained by integrating the CARS response between -3020 and -2850 cm$^{-1}$ at each position in the sample. Scale bar: 20 microns. (B) C-H stretching region of the retrieved CARS spectrum at an arbitrary position in the sample.
Figure 7.5. Positive and negative scores maps of the first three principal components calculated with PCA from the CARS hyperspectral dataset of the 20 µm thick section of the tissue.
the sample, and the second component effectively represents the larger variance due to differences in composition \[54\]. The scores maps corresponding to the second and third PCs qualitatively give the same results. The positive maps (+2 and +3) highlight again the spaces between adjacent cells, together with the intracellular region of the modified cells. Here, a collection of many individual round features is found. The scores values inside the unmodified cells are instead uniform and small in value. The negatives maps (-2 and -3) highlight instead homogeneously the region inside the unmodified cells, and elongated shapes of high intensity are found in the modified cells next to a low intensity background. The main difference in the maps of the second and the third PCs is the contrast in the images, found to be larger for the third component.

As the analytic form of the first three components (data not shown) provides insufficient indications to give a chemical interpretation of the scores maps, we can only provide a tentative interpretation based on the preliminary knowledge on muscles tissues in presence of local overexpression of the protein PLIN5 \[13\]. We suggest that the round features in the positive second and third scores maps represents the numerous lipid droplets present in the modified cells as a result of the overexpression of PLIN5. This is plausible as also the lipid-rich sarcolemmal edges of the cells are visible in the maps. Large lipid droplets are not known to be formed in the unmodified cells that then appear dark in the images. The negative maps are complementary and mutually exclusive with the positive scores maps. Hence, at this stage we can assume that the lipid-poor sarcoplasm is identified by negative scores values. However, the presence of high intensity features in the proximity of the cell membranes and inside the modified cells cannot be justified at this point simply by the absence of lipid aggregations. In order to find a preliminary explanation to these features, further analysis of the PCA results was required.

Hierarchical Cluster Analysis (HCA, see Paragraph 2.3) was performed on the hyperspectral dataset to cluster together the CARS spectral points with larger similarities. HCA found six spectral clusters in the data of the 20 µm section of the tissue. Scores maps were calculated for each of the clusters, representing the importance of the cluster at the different locations in the sample. Interestingly, these spectral clusters could be further associated together in two groups when the corresponding scores maps were found to be identical. The scores map representative of the first group is presented in Fig. 7.6 (A). The image resembles closely the positive scores map of the second and third PCs of Fig. 7.5. Round high intensity features are visible in the modified cells with higher definition compared to the PCA maps, and the sarcolemmal edges are again visible. A number of round features with lower intensity compared to the features found inside the modified cells are also present in the unmodified cells. In order to understand the chemical meaning of this map, we report the spectral intervals identified by the three spectral clusters belonging to this group: from -2896 to -2863 cm\(^{-1}\), from -2863 to -2834 cm\(^{-1}\), and finally from -1660 to -1613 and from -1491 to -1403 cm\(^{-1}\). The spectral region identified by the first two clusters contains the CH\(_2\) and CH\(_3\) symmetric stretches, and the third region contains the CH\(_2\) scissoring and bending, CH\(_3\) bending, and C=C stretching
Figure 7.6. HCA scores maps calculated from the CARS hyperspectral dataset of the 20 $\mu$m thick section of the tissue. (A) Scores maps representing three spectral clusters: -2896 to -2863 cm$^{-1}$, -2863 to -2834 cm$^{-1}$, -1660 to -1613 and -1491 to -1403 cm$^{-1}$. (B) Scores maps representing three spectral clusters: -3042 to -3005 cm$^{-1}$, -1356 to -1136 cm$^{-1}$, and -1003 to -1000 cm$^{-1}$.
vibrations. Hence, all the bands found in the first group of clusters are assigned to lipids. We can then conclude that the scores map in panel (A) is likely to image the lipid aggregations in the tissue.

The scores map representative of the second group of spectral clusters is shown in Fig. 7.6 (B). Here, the response is uniform in the sarcoplasm of all cells, irrespective of their modified or unmodified status. Modified cells present additional intracellular features of high intensity with elongated shape. The spectral clusters of this group contain the phenylalanine peak at -1002 cm\(^{-1}\), the amide III peak between -1200 and -1300 cm\(^{-1}\) and the part of the C-H spectrum assigned to proteins. Part of the spectral region identified by the clusters cannot be directly related to proteins, hence we cannot provide a conclusive description of the chemical composition of the areas of the tissues visualized in the image. Based on previous results on this tissue, it is known that large mitochondria are present in the cells overexpressing PLIN5 in the vicinity of LDs. The high intensity features in the figure could then represent the mitochondria. In order to confirm this hypothesis, immunofluorescence experiments providing the localization of mitochondria in the same tissue section used for the CARS experiments would be required. Unfortunately, at present we have obtained reliable immunostaining results only from separate sections of the tissue, preventing direct validation of the results.

**Muscle section of 5 \(\mu m\) thickness.** The first three positive and negative scores maps calculated by PCA of the 5 \(\mu m\) section of the tissue are presented in Fig. 7.7, and the corresponding analytical form of the components is shown in Fig. 7.8. The first positive scores map (+1) highlights the sarcolemmal edges and distinct intracellular features inside the modified cells. Several of these high intensity elements present a round shape compatible with their identification with lipid droplets. The unmodified cells that appeared dark in the positive map show instead high response in the negative map, with nearly uniform scores values. Inside the modified cells, high intensity lines are instead present on a low intensity background. The corresponding fist PC in Fig. 7.8 (A) presents two positive peaks at -2855 and -2895 cm\(^{-1}\), with an intermediate relative dip at -2871 cm\(^{-1}\). The negative peaks are instead centered at -2946, -2984 and -3060 cm\(^{-1}\). The positive peaks identify vibrations specific of lipids as discussed in Paragraph 7.3.2, supporting the interpretation of the +1 scores maps to visualize LDs in the modified cells. The negative peaks in the region of the C-H part of the spectrum have also been discussed previously in this thesis to be predominantly related to the presence of proteins.

The -2 and +3 scores maps show similar, featureless images of the cells that present low scores values. The +2 and -3 scores maps also look similar, with high intensity lines present inside the modified cells at the same locations as in the -1 map. However, here the unmodified cells are characterized by low signal and features in the intracellular areas appear bright. The bright elements in the vicinity of the sarcolemmal borders present some mislocalization between the +2 and -3 maps, their shape and localization in the -3 map being closely reminiscent of the cell nuclei (cfr. Fig. 7.2). Immunostaining experiments
Figure 7.7. Positive and negative scores maps of the first three principal components calculated with PCA from the CARS hyperspectral dataset of the 5 μm thick section of the tissue.
Figure 7.8. Spectra of first (A), second (B) and third (C) principal component calculated with PCA from the CARS hyperspectral dataset of the 5 µm thick section of the tissue.
on the same section of the tissue would also in this case be helpful to confirm this interpretation. The corresponding second and third PCs are presented in Fig. 7.8 (B) and (C), respectively. The second PC presents positive peaks at -2850, -2880, -3030, and -3080 cm$^{-1}$, and negative peaks at -2871, and -2930 cm$^{-1}$ with a shoulder at -2971 cm$^{-1}$. The third component is more noisy than the other two, and contains only positive peaks centred at -2834, -2871, -2920, and -3050. The considerations discussed above on the lipid and protein origin of the peaks located in the low and high wavenumbers region of the C-H spectrum, respectively, were not sufficient to provide a meaningful explanation of the maps obtained by PCA for the second and third components. Further analysis of the PCA results was hence necessary also for better interpreting the CARS results from the 5 µm section of the tissue.

HSA was performed on the CARS data measured in the 5 µm thick section of the muscle tissue. The analysis found six spectral clusters, the first five of which ranging over the entire spectral region between -2846 to -2970 cm$^{-1}$. The scores maps calculated for the first five clusters are presented in Fig. 7.9. The scores maps of the first two clusters (A and B) contain the spectral region from -2846 to -2863 cm$^{-1}$ and from -2880 to -2905 cm$^{-1}$. This region of the spectrum contains the CH vibrations specific of lipids. In the two maps the modified and unmodified cells can be distinguished based on the difference in intensity in the sarcoplasm. Perfectly defined round elements are present in the modified cells, indicating the presence of LDs. The sarcolemmal edges are also clearly visible in the figures. The score map of the third cluster is shown in panel (C). This cluster contains the vibrations from -2913 to -2917 cm$^{-1}$, intermediate between the portions of the spectrum specific to lipids and proteins. The last two scores maps (D and E) contain the spectral region from -2921 to -2942 cm$^{-1}$ (D), and from -2946 to -2970 (E), respectively, indicating a dominant protein character of the resonances. The intensity in the unmodified cells is large and uniform. The intensity drops slightly in the sarcoplasm of the modified cells, where dark round features are also visible. These features colocalize with the high intensity elements found in the scores maps of the first two clusters (A and B) identifying the LDs.

The scores map of the last spectral cluster (-3013 to -3046 cm$^{-1}$) is shown in Fig. 7.10. The scores in the sarcoplasms of the modified and unmodified cells present similar values. Bright elongated elements are present in the modified cells, similar to the features found in the scores maps calculated by PCA and hypothesized to identify mitochondria. Similarly, also the features in the proximity of the sarcolemma visible in the -3 scores map are as well present in the last cluster map. It was previously discussed that these features might correspond to the nuclei of the cells. Although mitochondria and cell nuclei present very different chemical composition, we note that mitochondria also contain some DNA. Also in this case, validating experiments with immunohistochemistry are required to confirm this preliminary finding.
Figure 7.9. HCA scores maps calculated from the CARS hyperspectral dataset of the 20 /mum thick section of the tissue. Scores maps representing the spectral cluster from -2846 to -2863 cm\(^{-1}\) (A), from -2880 to -2905 cm\(^{-1}\) (B), from -2913 to -2917 cm\(^{-1}\) (C), from -2921 to -2942 cm\(^{-1}\) (D), and from -2946 to -2970 (E).
7.4 Imaging of biological components in muscles

Figure 7.10. HCA scores map (CARS dataset of the 20 /mum thick section of the tissue) representing the spectral cluster from -3013 to -3046 cm$^{-1}$.

7.4 Conclusions

In this chapter we have presented the application of CARS spectro-microscopy to provide label free identification of biological components in muscle tissues with chemical specificity. We used CARS to study muscles were genetic modification induces locally the overexpression of the LD coating protein PLIN5. The overexpression of the protein is known to increase the storage of intra myocellular lipids.

We found that the interpretation of the CARS hyperspectral dataset using multivariate analysis methods (PCA and HCA) allowed the unambiguous identification of lipid droplets in sections of the muscle tissue with different thickness. These results could be used as a starting point for follow up studies where the vibrational signature can be employed to determine e.g. the degree of order and saturation of individual LDs. Also, preliminary results indicate that CARS might be able to visualize mitochondria in the tissue. This result, requiring validation with immunohistochemistry, is, to the best of our knowledge, the first example of label free visualization of mitochondria in tissues.