Looking at life through molecular vibrations: biomedical applications of CARS spectro-microscopy
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6 Imaging of cell compartments of the wing disc

A key question in developmental biology is the understanding of how the development of organs is regulated during growth. Evidence has been presented of a strong connection in embryogenesis between the appearance of boundaries between specific cell types and the proliferation of such cell clusters in the growth of a tissue. Despite extensive efforts to understand how the fate of cells is determined during organs growth, little is known on the role that development of compartment boundaries and variations in the chemical composition of cells from different compartments play in the proliferation process.

We present here the application of broadband CARS hyperspectral microscopy to study the local chemical composition of cells in the developing imaginal wing disc of *drosophila melanogaster* larvae. While the occurrence of compartmentalization of the wing disc into the anterior/posterior and the dorsal/ventral regions has been known for decades, insights into variations in the local chemical composition have been lacking. Here, the chemical information contained in the CARS spectra is evaluated using Principal Component Analysis (PCA), allowing us to directly visualize the occurrence of cell clusters in the developing wing that each exhibit common chemical features.

Our results demonstrate that CARS microscopy is a powerful label-free alternative to fluorescence-based assays routinely used in developmental biology for the visualization of known and unknown compartments in developing organs.

6.1 Introduction

The development of a multi-cellular organism is one of the most fascinating issues of biology. Perfectly controlled and highly complex mechanisms are required for embryogenesis, as cells not only have to proliferate and differentiate, but they also have to sort into function-specific tissues. Whereas for most proliferating tissues, cells can move freely and occupy any position, in some cases the cells and their descendants are forced to remain in restricted areas of the tissue, called compartments. Between adjacent compartments, boundaries are created,
associated with a cell-segregation mechanism preventing cells of the compartments to migrate from one to the other. The establishment and maintenance of these sharp borders, called compartment boundaries, between cell populations is a crucial step in ensuring that the position of the regulation centers remains unmodified during development. Such regulation centers, also named organizers, are responsible for the entire signaling pathways at the base of tissue growth [21]. Transcription factors activate the organizer cells at the edge of a compartment, promoting the production of long-range organizing molecules (morphogenes) that orchestrate further growth and patterning of the tissue. The functioning of compartment boundaries is described in detail in Paragraph 6.6.

![Figure 6.1](image)

**Figure 6.1.** (A) Wing imaginal disc (brightfield microscopy image) of third instar larvae of *drosophila melanogaster*. The disc is divided into compartments determined by lineage restriction (classical compartment boundaries) and non-classical compartments. The main classical boundary follows the *decapentaplegic* (*dpp*) expression stripe and discriminates the anterior and posterior compartments (A/P, blue line). The second classical boundary develops along the *wingless* (*wg*) expression stripe separating the dorsal from the ventral cells (V/D, red). The separation between pouch (orange) and non-pouch region represents an example of non-classical compartment boundary. (B) Example of the visualization of the anterior/posterior compartment boundary in the wing disc by fluorescent marking. The fluorescence microscopy image refers to the wing disc of a *hh*Gal4-UAS-CD8-GFP fly. In this system the GFP is localized to the posterior compartment via the tagging to the Gal4 element that is controlled by the transcription factor *hedgehog* (see Paragraph 6.6).

Cell compartments have been known for decades to play an important role in the development of insects and invertebrates [79], but they are also found
in vertebrates [117]. The Drosophila melanogaster is one of the best studied systems as it nicely exemplifies the role of compartments during development of the adult organism. The divisions between anterior/posterior and dorsal/ventral compartment boundaries appear in the early stages of the embryogenesis of the limbs of the drosophila. The adult tissues of the animal develop from clusters of undifferentiated cells, termed imaginal discs. The wing for example is derived from the wing imaginal disc by invagination of the embryonic epidermis along the intersection of the dorsal/ventral and anterior/posterior boundaries depicted in Fig. 6.1 (A). Like all other imaginal discs of the fly, the wing disc is made up of a columnar epithelium (disc proper) that grades into a squamous epithelium on the other side (peripodial membrane). The disc proper is the cell layer which differentiates into wing, hinge and notum (see Fig. 6.2) [74].

The division between the anterior and posterior compartments of the wing is the first to appear in the early stages of the growth, under the influence of the transcription factor engrailed which becomes selectively expressed in the posterior compartment. A more complex boundary perpendicular to the first is formed in the second larval instar, separating the prospective dorsal and ventral wing surfaces. The selector gene aterous expressed in the dorsal compartment is responsible for this further division of the imaginal disc [39]. Remarkably, at the end of the larval development the number of cells in the wing imaginal disc will be thousand-fold, but the activity and the position of these boundaries will be preserved during the growth and into the adulthood of the organism. Along-
side to compartments where cell segregation mechanisms are present, other non-classical boundaries also exist. The boundaries between the pouch and non-pouch areas and between hinge and notum are examples of borders that can be crossed by cells, consequently changing their fate.

Although the genes involved in the establishment of individual compartment boundaries have been known for decades, the phenomena underlying cell segregation are still largely unknown. Cell segregation might be based on variations in the strength of the cell adhesion in opposite compartments, as the result of variations in the activity of the signaling factor [80]. Yet, the identity of the specific molecules responsible for compartment formation and maintenance remains unknown.

The majority of the information relative to compartment boundaries has been obtained by marking groups of founder cells and following the position and shape of the descendent cells (clones). Clones of cells cannot cross the compartment boundary, but will instead remain physically restricted to either one of the two sides of the boundary, and form a straight line where they contact it. The marking procedure involves either genetic means such as somatic recombination, or the injection of intracellular dyes which persist through multiple rounds of cell division [73]. Fluorescent labeling of proteins with *e.g.* GFP (see Fig. 6.1 (B)), and the use of antibody staining has provided extensive information on the role of specific proteins in boundary formation [103]. The drawback of this approach is that the labeling procedure can lead to mislocalization or hindered functionality of the modified molecules, and it is only applicable to a limited range of molecular classes, mainly proteins and lipids [113]. Moreover, fluorescence tagging is only feasible when the target molecule is known, and therefore it is not suited for the screening of potential new candidate molecules. As compartment boundaries rarely follow morphological landmarks and they can only be visualized by lineage markers, it is conceivable that, even for well-studied systems such as the wing imaginal disc, not all boundaries have yet been identified.

In this chapter we present the application of broadband CARS to visualize cell compartments of the imaginal wing disc of *drosophila melanogaster* by determining the local chemical composition in the disc. In order to identify the characteristic chemical features of the system, PCA is used to analyze the CARS data. The application of multivariate statistical analysis to vibrational spectroscopy allows accounting for the small pixel-to-pixel spectral variations in hyperspectral datasets. This has provided a mean to distinguish between normal and diseased states in tissues and isolated cells [54], as well as to identify the individual components of cells [139] and functional units in mice meibomian glands [125]. Recently, PCA was used for processing CARS microscopy data to distinguish the biochemical components of a sample of completely unknown composition [161]. Nonlinear optical techniques have been used in the past to study drosophila. Two-photon fluorescence was used in combination with third harmonic generation to monitor the development of a whole embryo [193], and in combination with second harmonic generation to look at internal organs [124]. Single frequency CARS microscopy was used to obtain information on the struc-
ture of internal organs and fat body [30]. All these studies though, were focused on the study of the entire embryo and were not aimed to obtain information on cell compartments and their boundaries. Also, they did not attempt to retrieve the chemical composition of the system.

6.2 Materials and methods

6.2.1 Sample preparation

Wild type $yw$ third instar larvae or genetically modified flies were used, all presenting the following genotype: $yw\ UAS-mCD8::GFP\ hsp-flp;\ hh-Gal4/TM6b$ [162].

Wing imaginal discs were manually dissected and dehydrated in a 10xPBS solution (Sigma-Aldrich, Zwijndrecht NL) for 30 minutes. The discs were then mounted on conventional microscopy cover slides. Mounted discs were washed with MS grade water (Sigma-Aldrich, Zwijndrecht NL) three times to remove additional salts. For all CARS experiments, the discs were directly analyzed without further sample preparation.

Preliminary investigation of sample integrity was performed by visual inspection using a Leica DMRX microscope (Leica, Wetzlar, Germany). The microscope, equipped with an Osram HBO 50W/L2 short arc mercury lamp (Osram AG, Munchen, Germany), was also used to produce the fluorescence images of the wing discs.

6.2.2 CARS experimental procedures

Broadband CARS measurements were performed on the imaginal wing disc with the experimental setup described in Paragraph 3.2. The wing was initially visualized with standard brightfield imaging to allow for correct identification of the area of interest. The wing disc normally has dimensions of $\sim 300 \times 400 \ \mu m^2$ in plane. The actual size of individual wing discs varies depending on the exact stage of growth of the animal at the time of dissection. CARS imaging was performed in three dimension by measuring adjacent tiles in the sample consecutively until the entire region of the sample was measured. Each individual tile has dimension of $75 \times 75 \times 26 \ \mu m^3$ (corresponding to $76 \times 76 \times 14$ pixels). Acquisition time per voxel was set to 50 millisec in order to obtain good signal to noise ratio without compromising the integrity of the sample.

6.2.3 Data analysis

Raw CARS spectra were analyzed with the program Igor Pro. Pretreatment 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. Spatial binning of the data was necessary to increase the signal to noise ratio and to reduce computer memory requirements. The text 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.

6.3 Results

6.3.1 Hyperspectral imaging of the wing disc

Broadband CARS measurements were performed on the entire imaginal wing disc in three dimensions in order to obtain a hyperspectral image of the system. For each voxel within the disc, the retrieved CARS spectrum was obtained across the spectral region between -3200 and -900 cm\(^{-1}\). The size of each voxel was independently measured to be \(\sim 1 \times 1 \times 2 \ \mu m^3\) determined by the intrinsic confocality of the technique with the employed experimental configuration.

The CARS response measured at an arbitrary position within the disc is presented in Fig. 6.3, where the spectral region between -2700 and -1800 cm\(^{-1}\) has been excluded as vibrations eventually present in this range produce an intensity below the detection limit. This spectral interval characterized by low response is often referred to as the silent region of the Raman spectrum of biological matter [17]. The integration time for this spectrum is 1 second.

Two regions of interest can be distinguished in the spectrum, the fingerprint and the C-H stretching region. The fingerprint region between -1800 and -900 cm\(^{-1}\) presents several overlapping peaks whose chemical nature has been extensively studied and the assignment can be found in literature [144]. Part of the vibrations in this region can be assigned to individual classes of molecules. The peaks between -1290 to -1400 cm\(^{-1}\) (CH bending and deformation), between -1400 to -1480 cm\(^{-1}\) (CH\(_2\) scissoring and bending, CH\(_3\) bending), and at -1650 cm\(^{-1}\) (C=C stretching) are assigned to lipids. The peaks at -1002 cm\(^{-1}\) (phenylalanine), between -1200 and -1300 cm\(^{-1}\) (amide III) and at -1650 cm\(^{-1}\) (amide I) are instead assigned to proteins. Finally, the peak between -1060 and -1095 cm\(^{-1}\) (PO\(_2^−\) stretching) is assigned to nucleic acids.

The C-H stretching region between -3150 and -2750 cm\(^{-1}\) contains overlapping vibrations arising from CH, CH\(_2\), and CH\(_3\) groups abundant in most biological species. Interestingly, 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]. Also, peaks specific of amino acids have been assigned in the high wavenumbers region above -2930 cm\(^{-1}\) [102].

Owing to the large response obtained in the C-H stretching region, unspecific maps of the morphology of the wing disc can be obtained by integrating
6.3 Imaging of cell compartments of the wing disc

Figure 6.3. Retrieved CARS spectrum at an arbitrary position within the wing disc. The region of the spectrum at high wavenumbers (C-H stretching region) 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.

The intensity of the retrieved CARS signal between -3020 and -2850 cm$^{-1}$. As already mentioned in the previous chapters, the intrinsic confocality of CARS provides vertical sectioning capabilities of the wing disc along the axial direction. Hence, individual layers across the sample can be imaged independently. Fig. 6.4 (A) and (B) show the morphology of two individual sections of the disc, recorded at distance of approximately 10 and 2 microns from the glass support respectively. The first layer (A), located further from the glass support, presents a largely inhomogeneous response highlighting the wiggly conformation of the columnar epithelium layer [57]. The Y-shaped trachea is clearly outlined in the right side of the image. Here, the low intensity of the signal is possibly due to the dilution of the nutrients contained in the trachea tube. The second layer (B) is located very close to the glass support, where a flattening of the disc is to be expected. The edges of the disc are clearly distinguishable, and the signal inside the disc is homogeneous, with dark areas visible in correspondence with the folds of the epithelium. Here the trachea is only barely visible, indicating that this component of the disc is not located in this optical layer.

Three dimensional rendering of the entire wing disc can be obtained by combining the optical sections. From the rendering view of the disc it can be concluded that the overall chemical integrity of the sample is well preserved and
Figure 6.4. CARS images of individual layers of the wing disc. Layer (A) and (B) refer to an axial position of approximately 10 and 2 microns away from the glass support respectively. The intensity values in the image are obtained by integrating the CARS response in the C-H stretching region of the spectrum at each position in the sample. Scale bar: 50 microns
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6.3.2 Chemical differences in the CARS spectra

Unspecific maps of the wing disc only make use of a small portion of the information contained in the CARS spectra of the system. In particular, the quantitative chemical information has so far been left practically unused. As an initial method to identify chemical differences at different locations within the disc, visual inspection of the spectra was employed. Fig. 6.5 (A) presents the map of the C-H CARS intensity of an area of $75 \times 75$ $\mu$m$^2$ located in a region of the disc where the boundaries between the hinge/notum and the anterior/posterior compartments are expected to be visible (purple square in the brightfield image at the top of Fig. 6.5 (A)). The image refers to an optical layer located $\sim 8$ $\mu$m above the glass support. The CARS map resembles closely the features visible in the brightfield image, with a low intensity region cutting across the area in correspondence with a fold of the epithelium. The most intense response (yellow areas) is found in the inner part of the sample. The simple observation of the CARS map provides no elements allowing the identification of compartment
boundaries, although the trend of the intensity of the signal vaguely reminds of the configuration expected for the notum/hinge division (cfr. Fig. 6.2). Five locations are marked on the map, and the corresponding spectra in the C-H and fingerprint region are shown in Fig. 6.5 (B) and (C) respectively. In the C-H region, the main differences in the spectra are the relative intensities of the three major peaks at ∼-2930, ∼-2880 and ∼-2845 cm\(^{-1}\). The spectra at position 1, 2, and 3 are qualitatively similar, although they differ in the intensity of the signal. The spectrum at position 4 differs from the others by the lower relative intensity of the two peaks at lower wavenumbers, whereas the spectrum of position 5 presents higher relative intensity of these peaks and a blueshift of the position of the -2880 cm\(^{-1}\) vibration.

In the fingerprint region of the spectrum, the reduced signal to noise ratio makes the identification of distinctive vibrational features more challenging. Again, we found a similar ratio (∼1.3) of the peaks at -1650 and -1450 cm\(^{-1}\) for the spectra 1, 2, and 3. This ratio becomes close to one for spectrum 4 and in the case of spectrum 5 the -1450 cm\(^{-1}\) peak becomes larger than the -1650 cm\(^{-1}\) peak.

Inspection of the spectra of Fig. 6.5 thus clearly indicates that the vibrational response of the wing disc varies with the position. On the other hand, even if quantitative parameters could be defined using peak fitting, the enormous amount of data points in the disc makes the visual inspection of the spectra an impractical approach to identify the chemical differences between cell compartments.

### 6.3.3 PCA of the Wing Disc CARS Data

The retrieved CARS signal from individual optical sections isolated from the three-dimensional dataset were investigated with Principal Component Analysis

<table>
<thead>
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<th>EV</th>
<th>% Variance</th>
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<td>15.73</td>
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<tr>
<td>2</td>
<td>26.71</td>
<td>6.482</td>
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<tr>
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<td>3.408</td>
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<td>12.61</td>
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<tr>
<td>10</td>
<td>3.290</td>
<td>0.798</td>
</tr>
</tbody>
</table>

Table I. Eigenvalues and variance percentage associated with the first ten principal components. The PCs are calculated from the hyperspectral dataset relative to an individual optical layer inside the wing disc.
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to identify recurring features in the spectra. The eigenvalues and the associate percentage of variance for the first ten principal components (PCs) are listed in Table I.

The function associated to the first PC is presented in its statistically normalized form in Fig. 6.6 (A). Statistically normalized refers here to rescaling the intensity values at different frequencies in order to give to each frequency the same statistical weight. This component features a collection of a large number of peaks across the entire spectral window, with a broad peak centered at \(-2915 \text{ cm}^{-1}\) with two shoulders at \(-2880 \text{ and } -2850 \text{ cm}^{-1}\) corresponding to undifferentiated CH stretches. Also the negative peaks in the fingerprint region at \(-1650 \text{ and } -1450 \text{ cm}^{-1}\) are insufficiently specific to relate the first component to a distinct molecular class. The non-normalized function representing the first PC is also shown in Fig. 6.6 (B) to provide an expression of the component directly comparable with the measured CARS spectra.

The scores maps and the false color map relative to the first PC are shown in Fig. 6.7. As described in Paragraph 2.3, the scores map quantify spatially the importance of the features present in the associate \(n^{th}\)-PC. At each position, the score value is in fact the coefficient of the development of the original spectrum along the \(n^{th}\) dimension of the PCs basis. Positive and negative score intensity values are generally represented in two separate maps. Interestingly, the scores maps in figure show a division indicative of the anterior/posterior differentiation, with the posterior compartment highlighted in the negative map and the anterior compartment highlighted in the positive map.

The function associated with the second PC is presented in its statistically normalized form in Fig. 6.8 (A). This function is characterized by a pronounced dispersive feature in the C-H region of the spectrum. The negative part of the feature extends from \(-3020 \text{ to } -2920 \text{ cm}^{-1}\), whereas the positive part continues till \(-2830 \text{ cm}^{-1}\). The shape of the function hints to the possibility that the negative score points are associated to a larger presence of proteins features in the original spectra, whereas the positive points are more lipid related [102,144]. This explanation is supported by the presence of two positive peaks in the fingerprint region at \(-1430 \text{ and } -1300 \text{ cm}^{-1}\) assigned to the CH\(_2\) bending and CH\(_2\) deformation of lipids in tissues respectively [132]. The non-normalized form of the second PC is also presented in Fig. 6.8 (B).

The scores maps and false color maps relative to the second PC show a differentiation reminiscent of the hinge/notum compartments. Large positive scores value are visible in the central part of the disc, where the notum is located (cfr Fig. 6.2). The negative scores reach lower absolute values, and they are localized in the area corresponding to the hinge.

It is important to underline that PCA results, both in terms of score maps and principal components, were consistent for all the sections of the four wing discs independently investigated. Fig. 6.10 shows the first two components (blue) retrieved from the analysis of the measurement performed on the wing disc of another fly. The PCs obtained are compared with the results previously shown (red). Blue and red functions are compatible, and differ, at most, in their intensities. The score maps associated with the new measurement are
Figure 6.6. Statistically normalized (A) and non normalized (B) spectra of the first Principal Component retrieved from the analysis of the CARS hyperspectral image of the wing disc also shown. Further spatial binning of the data prior to PCA analysis was also tested with compatible results. This excludes the influence of the spatial inhomogeneities over the micrometer lengthscale on the results obtained.

The results obtained for the third principal component, although not yet conclusive, should still be mentioned. The most interesting spectral features of the third PC are found in the C-H spectral region, shown in Fig. 6.11 (A) together with the score maps (B) and (C). The function has the same dispersive shape found for the second PC, with a negative peak from -3020 to -2920 cm$^{-1}$, followed by a positive part that continues till -2830 cm$^{-1}$. The scores maps on the other hand, do not resemble the hinge/notum compartments found for the second component. The signal instead is rather homogeneous both for the positive and negative map. Closer inspection of the data shows round features in the maps, with spatial correlation across consecutive layers. The third com-
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Figure 6.7. (A) negative, (B) positive, and (C) false color scores maps of the first Principal Component retrieved from the analysis of the CARS hyperspectral image. The scores maps show a division compatible with the anterior/posterior boundaries in the disc.
6.4 Discussion

The unspecific maps of the wing disc obtained by visualizing the integrated signal in the C-H region of the spectrum at each position within the sample provide clear visualization of the morphology of the wing disc. Albeit accurate, the use of hyperspectral CARS images to address the morphology of the system is possibly a suboptimal choice. The main drawback of broadband CARS is the duration of the experiments required to obtain good signal to noise ratio across the entire spectral window measured. The imaging process of the entire wing

Figure 6.8. Statistically normalized (A) and non normalized (B) spectra of the second Principal Component retrieved from the analysis of the CARS hyperspectral image

ponent is therefore tentatively attributed to identify the outside membranes of the cells. In order to confirm this hypothesis, additional measurements with higher resolution would be required.
Figure 6.9. (A) negative, (B) positive, and (C) false color scores maps of the second Principal Component retrieved from the analysis of the CARS hyperspectral image. The scores maps show a division compatible with the hinge/notum boundaries in the disc.
Figure 6.10. (A) and (B) functions of the first and second Principal Components respectively (red) calculated from an independent CARS measurement performed on the wing disc of a different fly, and the associated scores maps. In blue the functions of the components calculated for the first wing disc (Fig. 6.6 and Fig. 6.8).
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Figure 6.11. (A) C-H region of the spectrum of the third PC. A dispersive lineshape centred at $\sim -2920$ cm$^{-1}$ is present, similarly to the case of the first PC. (B) and (C) are the associated scores maps. Closer inspection reveals features reminiscent of the cell membranes.

disc in three dimensions can take as long as three to five days depending on the size of the disc. Such long measuring time becomes worthwhile only thanks to the chemical information contained in the vibrational spectra. For simple morphology studies, single frequency CARS experiments are instead recommended. As shown in the images presented in Paragraph 6.7, single frequency CARS can provide as well the maps of the concentration of CH moieties in the disc, with higher contrast and spectral resolution, and reduced measurement time compared to broadband CARS.

On the other hand, hyperspectral CARS microscopy provides direct access to the chemical composition of the compartments in developing organs. Retrieving and quantifying this information though is complicated primarily due to the large variations in the composition of the system on the length scale probed by CARS. The typical spectrum measured inside the disc presented in Fig. 6.3 contains the overlapped spectral signatures of several compounds, often belonging to different chemical classes, e.g. proteins, lipids, and nucleic acids. Direct deconvolution of the vibrational spectrum is non-trivial even in the case of a limited number of known constituents, but becomes unfeasible in the case of biological tissues where the constituents are unknown. Visual inspection of the spectra at individual spatial position within the disc provides some basic knowledge on the chemical differences as shown in Fig. 6.5. In particular, the ratios between the amplitude of selected peaks could in principle provide local information on the degree of saturation of the organic compounds and the order of their arrangement [179]. This approach, that is very successful for the study of cells composition in vitro, produces less exciting results in the case of a
system like the wing disc of the drosophila. In fact, tissues are in general much more compact than cell cultures in vitro. As a result, it becomes very difficult to distinguish components such as lipid droplets or any other intracellular structure.

Moreover, the compartmentalization of the disc intrinsically induces another complication. If we simplify the functional divisions of the disc by assuming that only two compartment boundaries are present, e.g. the A/P and hinge/notum boundaries, and we also assume that complementary compartments are characterized by some chemical difference, we immediately notice that each cell of the disc must simultaneously belong to two compartments. Hence, if a given cell is for example part of the anterior compartment and of the ventral compartment, it will be characterized by the chemical signature of both.

In order to visualize individual compartment boundaries and access the underlying chemical differences, Principal Component Analysis provides the ideal approach for spectral decomposition. In PCA, the original spectra are projected along a new basis in the vibrational space calculated by the maximization of the variance contained in the CARS dataset (cfr Paragraph 2.3). This unsupervised approach is likely to serve the purpose of deconvoluting the chemical differences relative to non complementary compartments. This concept is more easily explained using the previous example where only two boundaries are present in the disc. In this case the successful application of PCA separates the chemical information underlying each of the two compartmentalization. The chemical features characterizing the A/P division are then entirely contained in one of the principal components. Specifically, in our experiments we found that the A/P division was expressed by the first PC as shown in Fig. 6.6 and Fig. 6.7. Along the spectral direction identified by the PC, the two compartments are associated to scores values of opposite sign, comparable to the on- and off-states of the underlying chemical feature. The hinge/notum division was instead identified along the second principal component, shown in Fig. 6.8 and Fig. 6.9.

Although these results were consistent in terms of spatial distribution and spectral signature of the compartments for all the investigated layers from several wing disc samples, only two compartmentalizations were found in the CARS data. PCA could not identify other spectral directions immediately related to known compartmentalizations of the disc. It is possible that the information relative to other compartments is also contained in the CARS data, although presenting lower variance, undetectable by PCA. Alternatively, the chemical differences in other complementary compartments might be beyond the detection capabilities of CARS. As the Raman contrast arises from vibrations of chemical bonds, molecules belonging to the same chemical class are hardly distinguishable, restricting in fact the capability of CARS to distinguish compartments associated with chemically macroscopic differences in composition.

The results presented in Table I show that part of the information contained in the CARS data is not efficiently detected by PCA. The first two PCs are characterized by the larger values of associated percentage of variance, although \( \sim 70 \% \) of the variance is still contained in the remaining components. All the first ten PCs express enough variance to be considered statistically significant,
although they were not found to be associated with recognizable compartmentalizations. It is hence possible that other information on cell boundaries is still contained in the other PCs.

As mentioned above, the first PC was found to identify the A/P division in the disc. The spectrum representing the component shows no vibrational features allowing the identification of the molecular classes intervening in the process. The scores maps in Fig. 6.7, outline clearly the two compartments. Comparison with fluorescent images of the posterior compartment (Fig. 6.1) shows a clear compatibility of the results. Direct comparison of the results is not possible as hyperspectral CARS experiments were unsuccessful on fluorescently marked wing discs, due to the partial absorption of the light from the dye. The presence of fluorescent markers in the sample is also likely to contaminate the CARS spectra, inducing possible artefacts in the results. Also, differences in the shape of the compartments can be explained by the intrinsic differences between the CARS and fluorescence measurement. Due to the intrinsic confocality, CARS measures the signal from optical sections within the disc, whereas the fluorescence system used in our experiments measures the response integrated over the entire thickness of the sample. The compartments found from the CARS data should be best compared with the results obtained with a confocal fluorescence microscope.

The hinge/notum compartment was instead identified by the second principal component. The spectrum of the component in Fig. 6.8 (A) features a dispersive line shape centered at $-2920$ cm$^{-1}$. The negative peak in the higher wavenumber region could be an indication of a large presence of proteins in the notum compartment visible in Fig. 6.8 (A). The positive peak in the lower wavenumber region could instead indicate a large presence of lipids in the hinge compartment, Fig. 6.8 (B). The lipidic origin of the positive features is also supported by the presence of the two peaks in the fingerprint region at $-1430$ and $-1300$ cm$^{-1}$. As already mentioned, these peaks have been assigned to the CH$_2$ bending and CH$_2$ deformation of lipids in tissues respectively [132].

An alternative explanation refers instead to the order of the molecules in the system. In fact, Raman measurements on lipid samples at varying temperatures have been used to demonstrate that the variation in the peak intensities of the $-2850$ cm$^{-1}$ (CH$_2$ symmetric stretch), $-2880$ cm$^{-1}$ (CH$_2$ asymmetric stretch), $-2935$ cm$^{-1}$ (CH$_3$ symmetric stretch), and $-2960$ cm$^{-1}$ (CH$_3$ asymmetric stretch) can be used as probes for investigating the conformational arrangement of lipids. In particular, an increase in the ratio of the intensities of the $-2935$ and $-2850$ cm$^{-1}$ peaks reflects chain melting and an increment in trans-gauche isomerization in the system [24]. In our results, this would be reflected in a high level of local disorder associated with the negative features of the second principal component, hence in the notum compartment on the disc.

The same arguments also apply to the description of the third component. The scores maps presented in Fig. 6.11 contain features arguably representing the cell membranes. If this hypothesis were to be demonstrated, an explanation of the spectral features in terms of lipid order would be of great interest.
6.5 Conclusions

The establishment of compartment boundaries and maintenance during embryogenesis are very well studied phenomena. In *Drosophila melanogaster* a multitude of compartments are described in literature [45, 103]. Although the signaling pathways regulating the growth of the tissue are often known, the chemical differences between complementary compartments are yet to be clarified. In order to overcome the limitation of the techniques currently available based on genetic markers, we have tested broadband CARS microscopy as a tool to investigate compartments in the wing disc of *Drosophila melanogaster*, based on the chemical signature of the molecules present in the system. The intrinsic vibrations of molecules directly provide label free access to the composition of the systems. The interpretation of the CARS hyperspectral dataset using a multivariate analysis method (PCA) allowed identifying two independent compartmentalizations in the disc, the anterior/posterior and the hinge/notum compartments. The hinge/notum division was interpreted by a different composition in terms of lipids and proteins in the two regions. The A/P division instead, although clearly visible in the scores map, could not be interpreted in terms of chemical differences. Additional measurement with other chemically specific hyperspectral techniques such as secondary ion mass spectrometry (SIMS) could provide a complementary mean to help identifying the molecules involved in the formation and maintenance of compartment boundaries in developing organs.

6.6 Appendix I: Compartment Boundaries

Although the establishment and maintenance of cell compartments and their boundaries during cell proliferation is a phenomenon that depends greatly on the chemical species involved in the specific process, the underlying biochemistry remains mostly unchanged. We will briefly introduce this process, using the anterior/posterior (A/P) division in the imaginal wing disc as typical example [118]. The structure of the typical compartment boundary is shown in figure Fig. 6.12. At the beginning of the compartmentalization, two groups of cells physically located in the vicinity of the appearing boundary line are selected in the embryo. The two groups will be forced by the patterning mechanism to differ in the expression of a selector gene, which in the case of A/P division is the gene *engrailed*. This gene is expressed by the posterior cells (selector gene on) and it is not expressed by the anterior cells (selector gene off). This is the only step of the process where an external mechanism is involved, as the state of the expression of the selector gene becomes a hereditary factor for the cells involved. The two initial groups of cells are consequently compartmentalized according to their capability to express the selector gene. An important ingredient for the success of boundary formation is the concurring cell segregation system that acts by sorting the two cell groups apart, in order to create a defined edge of the compartments (represented by the dashed line in figure) taking the name of
Figure 6.12. Representation of the organization of cells and signaling expression across a compartment boundary. A tissue is subdivided into two cell population that different in the expression of a selector gene. A cell segregation mechanism provides a sharp separation of the two different cell groups that identifies the compartment boundary (dashed line). The cells on the left (grey and blue) and on the right (red) of the line are characterized by the on and off state of expression of the selector gene with short signaling range. The selector gene activates the expression of a long range organizing molecule (morphogen, blue dots) in the cells of the opposite compartment located in the vicinity of the boundary (blue). As the morphogen diffuses in the tissue, it regulates the growth and patterning of both compartments.

compartment boundary. To the left and the right of the dashed line in Fig. 6.12 the cells belonging to the anterior and posterior compartments respectively are present.

In the following step, the cells in the posterior compartment (red cells in Fig. 6.12) begin to synthesize the short-range signaling molecule hedgehog ($hh$). This process is directed by the selector gene, that also makes the posterior cells immune to the interaction with the $hh$ itself. On the contrary, the cells in the anterior compartment are prone to respond to the interaction with the $hh$. Incidentally, due to the short range of the signaling molecule, only the anterior cells close to the compartment boundary will be involved in the interaction (blue cells). These cells take the name of organizer.

Finally, the anterior cells reached by the $hh$ will respond to the interaction by emitting a morphogen, a long range organizing molecule. The morphogen, Decapentaplegic ($DPP$) for the A/P division, diffuses away from the organizer cells giving rise to a graded distribution (blue dots). The growth and the patterning of the whole tissue are then regulated by the morphogen via the induced expression of target genes in a concentration-dependent manner.

A complete treatment of the development of compartment boundaries in developing organs can be found in [21].
6.7 Appendix II: single frequency CARS art

In this paragraph, single frequency CARS microscopy images of the imaginal wing disc are shown. These measurements are not part of the study on the chemical composition of the system, but belong instead to a set of preliminary measurements aimed to produce high resolution, label free images of the shape and arrangement of the cells in the disc across compartment boundaries. Changes in the arrangement of the cells are in fact believed to be of relevance for elucidating the role of cell adhesion in pattern formation (cfr. Paragraph 6.1). Single frequency CARS tuned to match the -2845 cm$^{-1}$ vibration of CH bonds allows for accurate visualization of the edges of the cells in the tissue, although at this stage it cannot be ruled out the possibility that other components of the tissue produce high contrast in the CARS images. Conclusive results obtained with this approach would require to be validated by the comparison with standard immunostaining measurements where cell membranes can be labeled with a fluorescent tag.

For these experiments, the sample preparation was the same as described in Paragraph 6.2.1, and the experimental setup is described in Paragraph 3.1. The images depict consecutive layers within the wing disc separated of 1 µm covering an area of 50 × 50 µm$^2$ (corresponding to 256 × 256 pixels). Acquisition time for each layer is 20 s, with signal intensity normalized by dividing the response by the non-resonant signal measured in the glass support. The panels in the image are numbered consecutively according to the vertical position, starting above the disc and moving toward the glass support.

Fig. 6.13 and Fig. 6.15 refer to two regions where the boundary of the notum/hinge is expected to be present. It should be noted how the contours of the cells, visible as high intensity lines, change in shape across the layers. We should remind here that, at this stage of the growth, the wing disc consists of two layers of epithelium tissue only, the thick columnar epithelium and the thin peripodial membrane. The enlargements of the previous figures shown in Fig. 6.14 and Fig. 6.16 also highlight that the alignment and shape of the cells changes sharply in the images. We believe that these transition lines might colocalize with the compartment boundaries. The validation of this hypothesis is non-trivial as CARS measurements on the wing disc in presence of a fluorescent marker for a specific compartment are hindered by absorption effects of the signal due to the large concentration of the dye. CARS images then appear clear in the non-labeled compartment, but the intensity of the response drops considerably in the complementary compartment where the marker is present.

In Fig. 6.17 compartment boundaries are not expected to be present. The figure gives instead a clear representation of the three dimensional structure of the trachea inside the disc. High resolution images of the trachea in vivo are, at the top of our knowledge, not accessible with standard fluorescent techniques.
6.7 Imaging of cell compartments of the wing disc

Figure 6.13.
Figure 6.14.
6.7 Imaging of cell compartments of the wing disc

Figure 6.15.
Figure 6.16.
Figure 6.17.