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**Combining ToF-SIMS imaging mass spectrometry and CARS  
microspectroscopy reveals lipid patterns reminiscent of gene expression  
patterns in the wing imaginal disc of *Drosophila melanogaster***

Marty, Florian ; Rago, Gianluca ; Smith, Donald F ; Gao, Xiaoli ; Eijkel, Gert B ; MacAleese, Luke ;  
Bonn, Mischa ; Brunner, Erich ; Basler, Konrad ; Heeren, Ron M A

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## Combining ToF-SIMS imaging mass spectrometry and CARS microspectroscopy reveals lipid patterns reminiscent of gene expression patterns in the wing imaginal disc of *Drosophila melanogaster*

Florian Marty, Gianluca Rago, Donald F. Smith, Xiaoli Gao, Gert B. Eijkel, Luke MacAleese, Mischa Bonn, Erich Brunner, Konrad Basler, and Ron M.A. Heeren

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4 **1 Combining ToF-SIMS imaging mass spectrometry and CARS microspectroscopy**  
5 **2 reveals lipid patterns reminiscent of gene expression patterns in the wing imaginal disc**  
6 **3 of *Drosophila melanogaster***  
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60**1 Abstract:**

2 Using label-free ToF-SIMS imaging mass spectrometry, we generated a map of small  
3 molecules differentially expressed in the *Drosophila* wing imaginal disc. The distributions of  
4 these moieties were in line with gene expression patterns observed during wing imaginal disc  
5 development. Combining ToF-SIMS imaging and coherent anti-Stokes Raman spectroscopy  
6 (CARS) microspectroscopy allowed us to locally identify acylglycerols as the main  
7 constituents of the pattern differentiating the future body wall tissue from the wing blade  
8 tissue. The findings presented herein clearly demonstrate that lipids localization patterns are  
9 strongly correlated with a developmental gene expression. From this correlation we  
10 hypothesize that lipids play a so far unrecognized role in organ development.

**11 One Sentence Summary:**

12 Spatially confined acylglycerols on the surface of the *Drosophila* wing imaginal disc reveal  
13 known gene expression patterns important for proper development.

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## 1 Introduction

2  
3 Tissue and organ development is governed by a multitude of signaling processes, patterning-  
4 and growth events. Model organisms such as *Drosophila melanogaster* are often used to  
5 study these developmental events and gain fundamental insight of organ development. The  
6 majority of pathways governing the development of *Drosophila* are evolutionarily  
7 conserved[1]. Therefore, knowledge gained by studying this model organism's development  
8 can be transferred to other species such as humans.

9 The appendages of the adult fly such as wings, legs or antenna develop from organ primordia  
10 called imaginal discs[2, 3]. The discs are subdivided into different compartments (e.g. dorsal,  
11 ventral, anterior, posterior compartments). The compartments are made of cells with distinct  
12 identities that do not mix[4-6]. Compartmentalization is established in the early embryo,  
13 when the major body-axes are determined. The compartment boundaries act as organizing  
14 centers that govern growth, patterning and development of these discs. The boundaries define  
15 the expression of so-called morphogens, proteins which act either locally or diffuse over  
16 short and/or long ranges to pattern the growing organ[7-11]. The identification of the  
17 molecules (classically proteins and RNAs) are often assigned to defined developmental  
18 processes due to their expression patterns or localizations to specific areas of the developing  
19 tissue. Genes with similar restricted expression patterns are thought to play related roles. In  
20 contrast to the wealth of knowledge that has been obtained about the spatio-temporal  
21 restriction of gene expression, as well as that of the corresponding RNAs and proteoforms,  
22 the organization and distribution of other molecules, such as carbohydrates and lipids, is not  
23 well understood. This lack of knowledge can be traced back to the challenges associated with  
24 studying the localization of small molecules, e.g. lipids, with high spatial resolution. Recent  
25 work in the model system *Drosophila melanogaster* have described and quantified lipids over  
26 the course of its life cycle [12, 13]. These studies demonstrated that different tissues exhibit  
27 distinct phospholipid compositions but did not report the intra-organ distribution of these  
28 small molecules. Such spatial information is crucial to relate specific molecular moieties to  
29 specific biological processes in genetic, disease or developmental models. In order to identify  
30 novel small molecules, such as lipids and carbohydrates, that are crucial for a developmental  
31 process, we need to look for distribution patterns that resembled those of already known  
32 components.

33 In this work, we determined the distribution of small molecules in the *Drosophila* wing  
34 imaginal disc, using a combination of imaging time-of-flight secondary ion mass

1 spectrometry (ToF-SIMS) and coherent anti-Stokes Raman spectroscopy (CARS). These two  
2 complementary, label-free techniques make use of two inherent properties of small  
3 molecules, their mass and vibrational properties. We demonstrate that small molecules show  
4 well-defined distributions within this tissue. Strikingly, these distributions mimic genetically  
5 established patterns known to be essential for wing development and growth.

## 6 7 8 **Material and Methods**

### 9 **Sample preparation**

10 *Drosophila melanogaster* were grown under standard growth conditions at 22°C. Adult flies  
11 were transferred to new food every two days. Wing imaginal discs were manually dissected  
12 from wild-type yellow white (*yw*) third instar larvae (day 6 after egg laying) for CARS and  
13 ToF-SIMS analyses unless stated otherwise. For ToF-SIMS analysis (results shown in  
14 Figures 1A and 2A, B, C), genetically modified *Drosophila* lines with the genotype *yw,hsp-*  
15 *flp; UAS-mCD8::GFP/CyO; hh-Gal4/TM6b* were used<sup>32</sup>. The *mCD8::GFP* is a fusion  
16 protein between mouse lymphocyte marker CD8 and the green fluorescence protein which is  
17 expressed under *hh* control in this study. Wing imaginal discs were manually dissected in ice-  
18 cold phosphate-buffered saline (PBS) and dehydrated in a 10 × PBS solution (Sigma-Aldrich,  
19 Zwijndrecht, NL) for 30 min. For CARS, the discs were then mounted on conventional  
20 microscopy cover slides, washed with MS-grade water (Sigma-Aldrich, Zwijndrecht, NL)  
21 three times to remove additional salts and then analyzed.

22 For ToF-SIMS, discs were transferred to a conductive ITO slides with 4–8 Ω resistance,  
23 Delta Technologies, Stillwater, MN). The discs were washed with MS grade water (Sigma-  
24 Aldrich, Zwijndrecht, NL) three times to remove additional salts and then air dried. The discs  
25 were then covered with a 2-nm gold layer using a sputter coater (Quorums Technologies  
26 SC7640, Newhaven, UK) equipped with a FT7607 quartz crystal microbalance stage and a  
27 FT7690 film thickness monitor . For LCMS/MS analysis a Bligh and Dyer extract was  
28 prepared from the wingdiscs prior to analysis [14].

### 29 30 **Microscopy**

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2  
3 1 Discs were imaged with a Leica DMRX (Leica, Wetzlar, Germany) microscope equipped  
4 with an OSRAM HBO 50W/L2 short arc mercury lamp (Osram AG, München, Germany) at  
5  
6 3 10× magnification.  
7

#### 8 **Data acquisition**

##### 9 **ToF-SIMS:**

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12 6 Secondary Ion Mass Spectrometry was performed using a Physical Electronics (PHI) TRIPLE  
13 focusing ToF ion (TRIFT-II) instrument equipped with a gold liquid metal ion gun (Physical  
14 Electronics, Chanhassan, MN, USA). Twenty-two keV Au<sup>+</sup> primary ions were “micro”  
15  
16 8 focused on the sample surface. The total primary ion dose density amount was kept well  
17  
18 9 below the static limit. Positive or negative secondary ions were extracted to the mass analyzer  
19  
20 10 with a static voltage of ±3.5 kV and post-accelerated in front of the ion detector (dual-stage  
21  
22 11 microchannel plate) by an additional 10 kV. Signal from ions in the *m/z* range 1–1500 *m/z*  
23  
24 12 was recorded. Full wing discs were imaged step-by-step in a mosaic formed by 8 × 8  
25  
26 14 individual tiles between which the stage moved [15, 16]. Each tile of about 80–95 μm in  
27  
28 15 width was probed by the primary ion beam in a 256 × 256 pixel raster for a duration of 30 sec  
29  
30 16 to ensure that the total ion dose was below the static limit of SIMS. The size of each pixel  
31  
32 17 was approximately 0.35 μm. The resulting image was saved as a RAW file for further data  
33  
34 18 processing. The pulse width of the primary ion beam was 1 ns.

##### 19 **CARS microspectroscopy:**

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21  
22 20 A dual-output laser source (Leukos-CARS, Leukos, Limoges, France) provided the pump and  
23  
24 21 Stokes beams to enable CARS analysis. The laser source was a passively Q-switched 1064-  
25  
26 22 nm microchip laser, delivering <1-ns pulses at 32 kHz repetition rate and ~300 mW average  
27  
28 23 power. The laser beam was equally divided into two separate beams with a 50/50 beam  
29  
30 24 splitter. One beam was sent through a bandpass filter (FL1064-10, Thorlabs, USA) and used  
31  
32 25 directly as the pump beam. The other beam was introduced into a photonic crystal fiber that  
33  
34 26 creates super continuum emission of 420–2400 nm at the fiber output, with >100 μW nm<sup>-1</sup>  
35  
36 27 spectral power density from 1.05 μm to 1.6 μm. The supercontinuum was coupled out of the  
37  
38 28 fiber with a reflective collimator (RC04APC-P01, Thorlabs, USA) and passed through 700-  
39  
40 29 nm (FEL0700, Thorlabs, USA) and 830-nm (LP02-830RS-25, Semrock, USA) long-pass  
41  
42 30 filters. The Stokes and pump beams overlapped at a dichroic mirror (LP02-1064RU-25,  
43  
44 31 Semrock, USA) and introduced into a modified inverted microscope (Eclipse Ti-U, Nikon,  
45  
46 32 Japan). The pump and Stokes pulses were tightly focused onto the sample with a near IR  
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1 objective (PE IR Plan Apo 100X, NA 0.75, Seiwa, Japan). The sample was mounted on  
2 nested stepper-motor-driven (Microstage, Mad City Labs, USA) and piezo-driven stages  
3 (Nano-PDQ 375 HS, Mad City Labs, USA) that together provide 25-mm travel range with  
4 <1-nm resolution. The CARS signal generated by the sample was collected in the forward  
5 direction by another objective (M-20X, NA 0.4, Newport, USA) and sent through notch  
6 (NF03-532/1064E-25, Semrock), USA and short-pass filters (FES1000, Thorlabs, USA) to  
7 remove the pump and Stokes beams. The filtered CARS beam was dispersed by a  
8 spectrometer (Shamrock 303i, 300 lines mm<sup>-1</sup>, 1000-nm blaze, Andor, UK) and detected on a  
9 deep-depletion CCD (Newton DU920P-BR-DD, Andor, UK). The sample was raster-scanned  
10 across the focal volume with steps of 1 μm in-plane and 2 μm axially. Large three-  
11 dimensional images were reconstructed from adjacent tiles with in-plane dimensions of 75 ×  
12 75 μm or 50 × 50 μm and axial dimension determined by the thickness of the wing disc due  
13 to the large dimension of the wing disc. For each position in the sample, a CARS spectrum in  
14 the range between -3400 and -600 cm<sup>-1</sup> was acquired. CARS images were acquired with pixel  
15 dwell times of 50 ms.

16

### 17 **Lipid analysis by HPLC-ESI-MS/MS**

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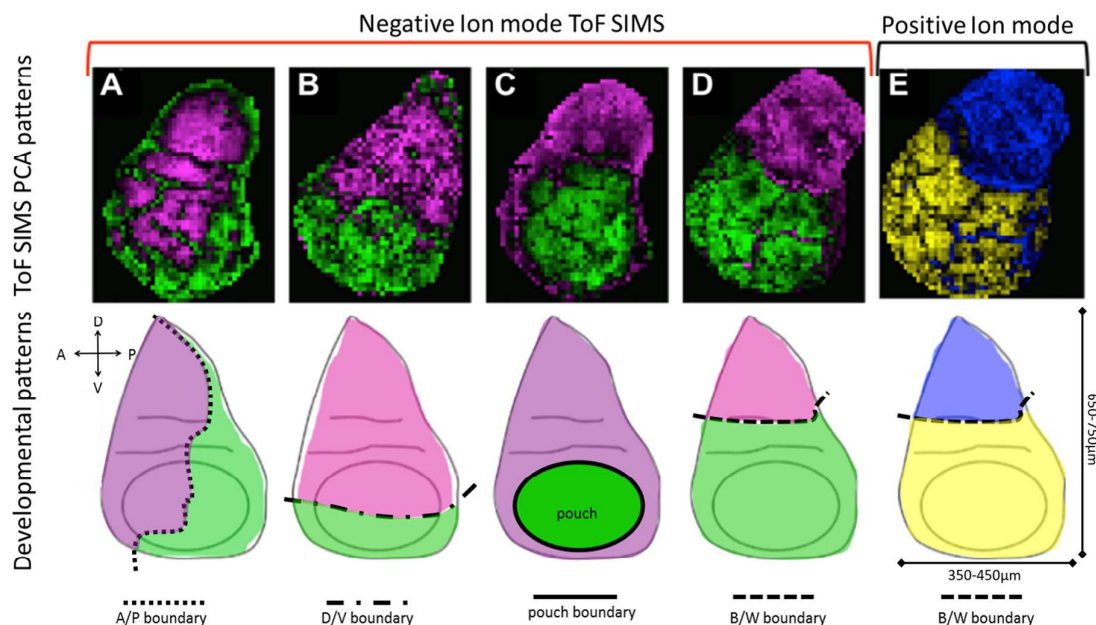


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3 1 Third instar wing imaginal discs were collected using the mass isolation approach developed  
4 in-house as described in [16]. Approximately 1000 individual discs were used per replicate.  
5  
6 2 Lipids were extracted using a modified Bligh and Dyer method [14]. The extracts were  
7 removed, dried *in vacuo* and reconstituted in isopropanol. HPLC-ESI-MS/MS analyses were  
8  
9 3 conducted on a Thermo Fisher Q Exactive mass spectrometer (San Jose, CA) with on-line  
10 separation using a Thermo Fisher/Dionex RSLC nano HPLC. HPLC conditions were:  
11  
12 4 Atlantis dC18, 3  $\mu\text{m}$ , 300- $\mu\text{m}$   $\times$  150-mm column (Waters Corporation, Massachusetts);  
13 mobile phase A, acetonitrile/water (40:60) containing 10 mM ammonium acetate; mobile  
14  
15 5 phase B, acetonitrile/isopropanol (10:90) containing 10 mM ammonium acetate; flow rate, 6  
16  $\mu\text{l}/\text{min}$ ; gradient, 10% B to 60% B over 5 min, 60% B to 99% B over 35 min and held at 99%  
17  
18 6 B for 10 min. Data-dependent MS/MS scans were performed using one full MS scan [ $m/z$   
19 200–2000; 70,000 resolution ( $m/z$  300)] followed by fragmentation in the HCD collision cell  
20  
21 7 of the six most abundant ions in the precursor scan using a normalized collision energy of 35  
22  
23 8 arbitrary units and mass analysis in the Orbitrap at 17,500 resolution. Separate analyses were  
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25 9 conducted using positive and negative ions detections.  
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18 Data analysis details for all experimental techniques have been provided in the supplemental  
19 information.  
20

## 1 Results:

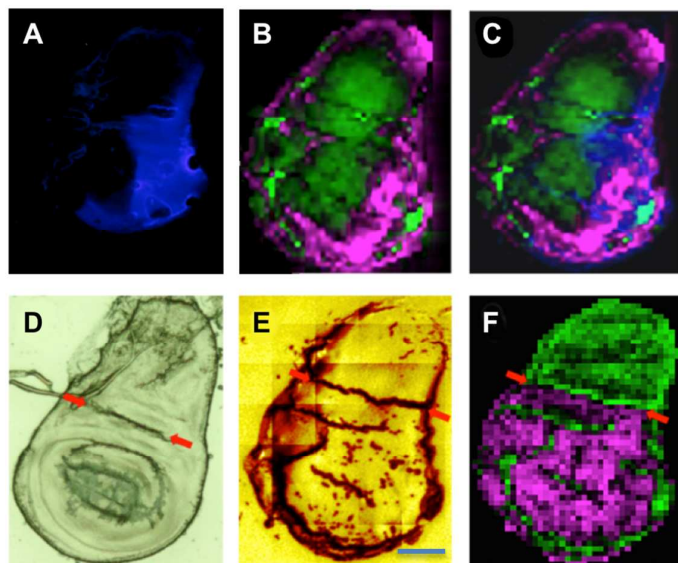
2 Third instar wing imaginal discs were analyzed with ToF-SIMS imaging mass spectrometry  
 3 in both negative and positive ion mode. Principal component analysis (PCA) was used to  
 4 unravel the complexity of the ToF-SIMS datasets. Inspection of the principal components  
 5 (PC) from the negative ion mode measurements revealed four PCs reflecting molecular  
 6 distributions (**Figure 1**, top row), which co-localized with known genetically predicted tissue  
 7 sub types such as anterior vs. posterior compartmentalization (A/P) (**Fig. 1A**) and dorsal from  
 8 ventral tissue (D/V) (**Fig. 1B**). Additionally, patterns reminiscent of the pouch/non-pouch  
 9 (**Fig. 1C**) and the body wall/wing blade (B/W) (**Fig. 1D**) differentiation were observed. All  
 10 these compartments are known to play an important role in *Drosophila melanogaster* wing  
 11 development and are associated with specific gene expression patterns [6, 9, 17-21]. Positive  
 12 ion mode experiments repeatedly confirmed the B/W distribution and it was therefore chosen  
 13 to follow up (**Fig. 1E** and **Suppl. Fig.1**).



14

15 **Fig. 1. Small molecule distributions revealed by ToF-SIMS on the wing imaginal disc of *Drosophila***  
 16 ***melanogaster* (top) and corresponding developmental patterns (bottom (References[22, 23] ).** PCA of ToF-  
 17 SIMS PCA data (top row) revealed patterns reminiscent of developmental patterns (bottom row). The patterns  
 18 anterior/posterior (A/P) (**Fig. 1A**), dorsal/ventral (D/V) (**Fig.1B**), pouch/non-pouch (N/NP) (**Fig.1C**) and  
 19 body wall/wing blade (B/W) (**Fig.1D**) were detected. The A/P, D/V and P/NP compartments were only observed in  
 20 negative ion mode. The B/W subdivision was observed in both negative and positive ion modes (**Fig.1E**).  
 21 A=anterior, P=posterior, D=dorsal, V=ventral, B=body wall, W=wing blade. Wing imaginal discs dimensions  
 22 indicated.

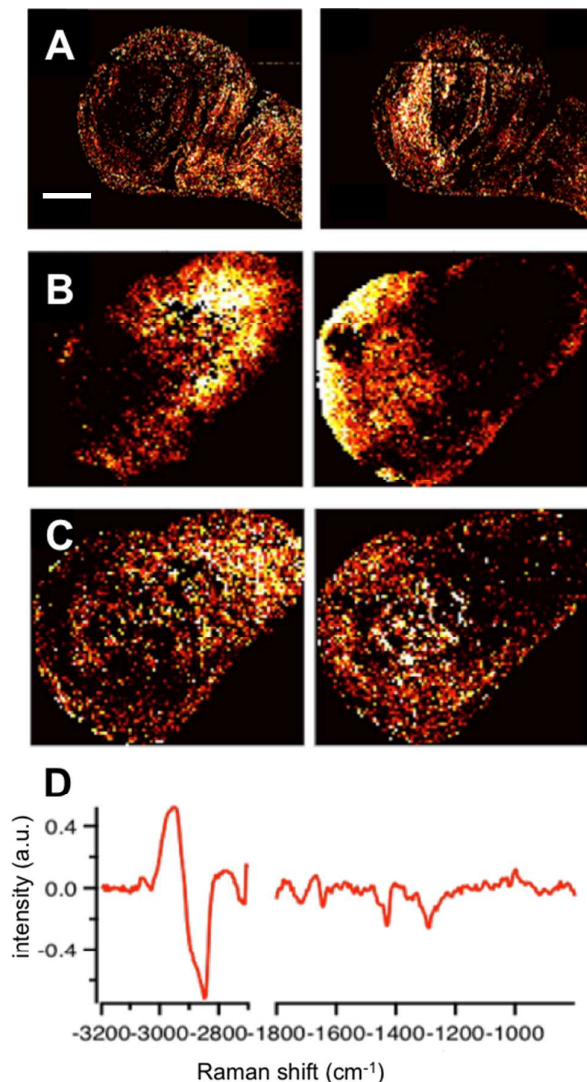
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3 1 Next, we determined whether two compartment boundaries (A/P and B/W) observed by ToF-  
4 SIMS matched with known developmental boundary markers. For the A/P subdivision, we  
5 2 used the classical compartment boundary established by differential expression of the  
6 3 morphogen *hedgehog* (*hh*), which is responsible for the establishment and maintenance of the  
7 4 A/P compartment [24]. Fluorescent images of third instar imaginal discs expressing GFP  
8 5 under the control of *hh-Gal4* promoter were generated and co-registered with the ToF-SIMS  
9 6 PCA obtained from the same imaginal discs (**Fig. 2A and B**). Clearly, the subdivisions  
10 7 identified by ToF-SIMS were related to the compartmentalization established by the known  
11 8 genetic circuits (**Fig. 2C**).  
12 9  
13 10 The B/W compartment boundary (**Fig. 2D**, arrows) is normally delineated by a tissue fold  
14 11 appearing in late third instar[20]. Comparing the total ion image with the PCA image of the  
15 12 same imaginal disc, the fold leading to a crack in the ToF SIMS instrument provides a  
16 13 landmark to determine the expression pattern of small molecules observed by ToF-SIMS in  
17 14 the PCA (**Fig. 2E and F**).



15  
16 **Fig. 2. Small molecule patterns identified by ToF-SIMS mimic known developmental patterns.** *Upper*  
17 *panel:* Imaginal disc microscopy images of GFP-labeled *hedgehog* expression patterns that occur exclusively in  
18 the posterior compartment (A) and ToF-SIMS PCA showing the anterior compartment (green) and posterior  
19 compartment (purple)(B) of the same imaginal disc were superimposed (C). *Lower panel:* The fold appearing in  
20 late third instar (D, E, F, red arrows) was used as a landmark for the non-classical body wall/wing blade (B/W)  
21 pattern. In the high vacuum of the instrument, this fold expands to a crack (E, red arrows). The expression of  
22 small molecules, determined by ToF-SIMS PCA, changes exactly at this crack boundary (F, red arrows). Thus,  
23 both boundaries revealed by PCA coincide with established compartment boundaries in *Drosophila*  
24 development. The scale bar in E indicates 150 micrometer.

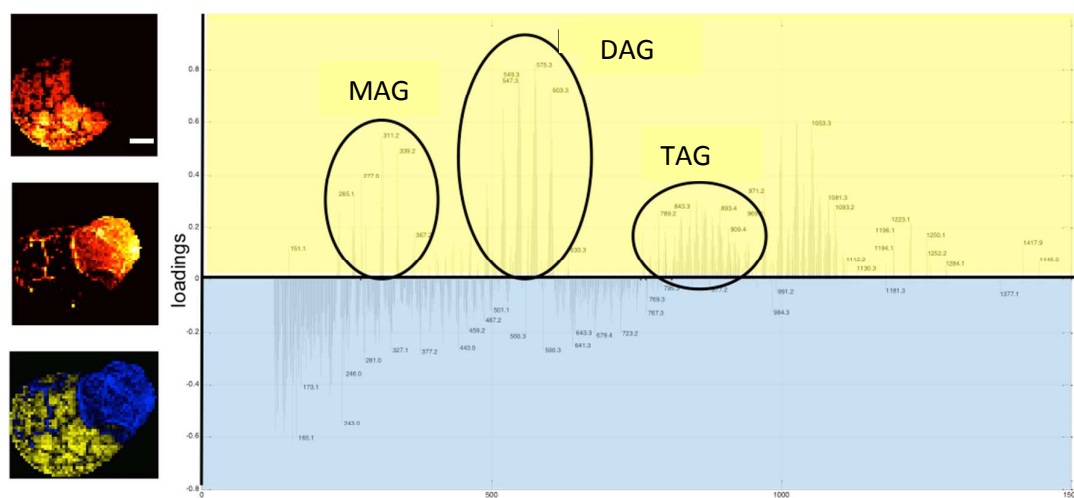
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5 2 The analytical limitation of ToF-SIMS restrict the molecular identification of the PC patterns  
6 3 to small molecules [25]. We employed CARS to identify the main small molecule classes  
7 4 represented in the ToF-SIMS PCA expression patterns. CARS has the chemical specificity to  
8 5 allow the assignment of molecular classes. In that respect the CARS data allows us to  
9 6 determine the nature of majority of peaks and provided guidance in the selection of the  
10 7 patterns to investigate further. CARS provides image contrast based on molecular vibrations  
11 8 that are distinct for different molecules. Using CARS, we acquired the vibrational signatures  
12 9 of the molecules (in the frequency range corresponding to Raman shifts from  $-3200\text{ cm}^{-1}$  to  $-$   
13 10  $900\text{ cm}^{-1}$ ) from late third instar wing imaginal discs. In the fingerprinting region ( $-1800\text{ cm}^{-1}$   
14 11 to  $-900\text{ cm}^{-1}$ ), the chemical moieties giving rise to specific peaks is largely known and can be  
15 12 found in the literature [26]. Multivariate statistical analysis (PCA) was applied to screen for  
16 13 patterns in the CARS dataset, similar to that performed for the ToF-SIMS datasets. A clear  
17 14 B/W pattern similar to the one found by ToF-SIMS (**Fig. 3A, B and C**) is observed. No other  
18 15 CARS PCA pattern was observed that resembled any of the previously described patterns.  
19 16 Canonical correlation analysis (CCA) revealed a positive correlation score between the  
20 17 CARS and ToF-SIMS datasets for the B/W patterns (**Fig. 3C and Supplementary Table 1**).  
21 18 Next, we investigated the spectral region of the CARS measurement from the correlating  
22 19 value 2 (CV2) to further identify the molecular components of the B/W pattern. In the CARS  
23 20 spectra, the dispersive C-H feature and the phenylalanine peak at  $-1000\text{ cm}^{-1}$  suggested a  
24 21 protein dominance for the body wall, whereas the spectral composition indicated a  
25 22 dominance of lipids in the wing blade section (**Fig. 3 D**). Therefore, we conclude that the  
26 23 components of the B/W patterns observed in ToF-SIMS are lipids. From the ToF-SIMS  
27 24 spectra of the wing blade section, we observed a highly reproducible pattern of peaks. Based  
28 25 on previously assigned  $m/z$  values, 22 lipids were assigned to the classes of monoacyl-  
29 26 (MAGs), diacyl- (DAGs) and triacylglycerols (TAGs) (**Fig. 4**) with DAG(34:2/  $375.3m/z$ )  
30 27 being the most abundant peak in the positive part of the PC loadings spectra. The principal  
31 28 component loading spectra show little correlation between the mono- and di-acylglycerols  
32 29 and the intact phospholipids. This indicates that these peak most likely do not originate from  
33 30 SIMS induced lipid fragmentation. The correlation of the MAG's and DAG's with the TAG's  
34 31 indicates that the acylglycerols in general play an important role in maintaining cellular  
35 32 organization in the wing discs. Supporting this identification is the observation of the  
36 33 corresponding  $[M+NH_4]^+$  DAG ions in the LC-MS/MS experiment is an indication that at

1 least part of the overserved ions are endogenous. However, we cannot exclude that the MAG  
2 and DAG related peaks in the SIMS experiments are fragments from TAG's present on the  
3 cellular surface. The analysis conditions were optimized to minimize the fragmentation of  
4 intact phospholipids and TAG's.



5  
6 **Fig. 3. CARS revealed a lipid origin for the B/W pattern:** CARS was applied to test if patterns observed by  
7 ToF-SIMS can be reproduced and to gain further knowledge about the molecules involved. PCA of CARS data  
8 demonstrated a similar B/W pattern (A) to that observed in ToF-SIMS (B). CCA was applied to visualize the  
9 correlation of the observed patterns from ToF-SIMS and CARS (C; quantification listed in **Supplementary**  
10 **Table 1**). Investigation of the normalized CARS spectra in arbitrary units (a.u.) revealed a higher content of  
11 lipids in the wing blade region compared to the body wall (D). The scale bar in A indicates 200 micrometer. The  
12 left and right images in all panels A-D represent principal components in the 3200-2700 cm<sup>-1</sup> and the 1800-800  
13 cm<sup>-1</sup> wavenumber range respectively.

1 To confirm this identification of the ToF-SIMS peaks, we performed orthogonal validation  
2 using high-performance liquid chromatography, electrospray ionization tandem mass  
3 spectrometry (HPLC-ESI-MS/MS, hereafter referred to as MS/MS for simplicity) of lipid  
4 extracts of whole wing imaginal disc [27]. This dataset was utilized as a high-mass-accuracy  
5 MS reference data set for tissue-specific lipids. We identified 156 lipid species (for details,  
6 see *Materials and Methods* section) containing most of the major lipid classes previously  
7 assigned [12, 13]. Of the 156 lipid species, 5 were MAGs, 22 DAGs and 10 TAGs. Seven  
8 species overlapped between the differential patterns observed by ToF-SIMS and MS/MS  
9 methods (**Table 1**). Among these lipids was also the previously reported DAG(34:2). Its  
10 identity in the ToF-SIMS data could further be confirmed using the loadings spectra from the  
11 negative ion mode ToF-SIMS data (**Suppl. Fig 2**).



12  
13 **Fig. 4. Assignment of peaks from ToF-SIMS measurements:** The images shows the positive and negative  
14 part of the PC distinguishing the B/W compartments (left top and middle panel) from the same imaginal disc as  
15 used in Figure 1. The loading spectrum on the right shows the positive loadings corresponding to W with a  
16 yellow background and negative loadings corresponding to B with a lightblue background The lower left panel  
17 is a color coded overlay (yellow=W, blue=B). To assign the potential lipid species, we used a recently published  
18 m/z catalogue with assigned lipids [12]. Loading spectra show that monoacyl- (MAG), diacyl- (DAG) and  
19 triacylglycerols (TAG) are overrepresented in the wing blade section (yellow). The white scale bar in the top left  
20 image indicates 150 micrometer.

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Lipid class (C:DB)	Passarelli et al. m/z	Adduct	ToF-SIMS						LC MS/MS m/z	Adduct	Monoisotopic mass
			Rep. 1 m/z	Rep. 2 m/z	Rep. 3 m/z	Rep. 4 m/z	Rep. 5 m/z	Rep. 6 m/z			
MAG(16:1)	311.3	[M+H-H <sub>2</sub> O] <sup>+</sup>	311.2	311.1	311.1	311.1	311.1	311.1	n.d.	-	328.2614
MAG(16:0)	313.3	[M+H-H <sub>2</sub> O] <sup>+</sup>	313.2	313.1	313.1	n.d.	n.d.	313.1	348.3092	[M+NH <sub>4</sub> ] <sup>+</sup>	330.2270
MAG(18:1)	339.3	[M+H-H <sub>2</sub> O] <sup>+</sup>	339.2	339.2	339.2	339.2	339.2	339.2	n.d.	-	356.2927
DAG(30:2)	519	[M+H-H <sub>2</sub> O] <sup>+</sup>	519.3	519.3	519.3	519.3	519.3	519.3	n.d.	-	536.4441
DAG(30:1)	521	[M+H-H <sub>2</sub> O] <sup>+</sup>	521.3	521.3	521.2	521.3	521.2	521.2	n.d.	-	538.4597
DAG(30:0)	523	[M+H-H <sub>2</sub> O] <sup>+</sup>	523.3	523.3	523.3	523.3	523.3	523.3	n.d.	-	540.4754
DAG(32:2)	547	[M+H-H <sub>2</sub> O] <sup>+</sup>	547.3	547.3	547.2	547.3	547.2	547.2	n.d.	-	564.4754
DAG(32:1)	549	[M+H-H <sub>2</sub> O] <sup>+</sup>	575.3	575.3	575.3	575.3	575.3	575.3	567.4964	[M+H] <sup>+</sup>	566.4910
DAG(32:0)	551	[M+H-H <sub>2</sub> O] <sup>+</sup>	551.3	551.3	551.3	551.3	551.3	551.3	586.5389	[M+NH <sub>4</sub> ] <sup>+</sup>	568.5067
DAG(34:3)	573	[M+H-H <sub>2</sub> O] <sup>+</sup>	573.3	573.3	573.2	573.3	573.3	573.2	608.5209	[M+NH <sub>4</sub> ] <sup>+</sup>	590.4910
DAG(34:2)	575	[M+H-H <sub>2</sub> O] <sup>+</sup>	575.3	575.3	575.3	575.3	575.3	575.3	593.5110	[M+H] <sup>+</sup>	592.5067
DAG(34:1)	577	[M+H-H <sub>2</sub> O] <sup>+</sup>	577.3	577.3	577.3	577.3	577.3	577.3	612.5544	[M+NH <sub>4</sub> ] <sup>+</sup>	594.5223
DAG(34:0)	579	[M+H-H <sub>2</sub> O] <sup>+</sup>	579.3	579.3	579.2	n.d.	n.d.	579.2	n.d.	-	596.5380
DAG(36:4)	599	[M+H-H <sub>2</sub> O] <sup>+</sup>	599.3	599.2	599.2	599.2	599.2	599.2	634.5365	[M+NH <sub>4</sub> ] <sup>+</sup>	616.5067
DAG(36:3)	601	[M+H-H <sub>2</sub> O] <sup>+</sup>	601.3	601.3	601.2	601.2	601.2	601.2	n.d.	-	618.5223
DAG(36:2)	603	[M+H-H <sub>2</sub> O] <sup>+</sup>	603.3	603.3	603.3	603.3	603.3	603.2	n.d.	-	620.5380
TAG(50:3)	851	[M+Na] <sup>+</sup>	n.d.	n.d.	n.d.	n.d.	851.3	n.d.	n.d.	-	828.7207
TAG(50:2)	853	[M+Na] <sup>+</sup>	853.4	n.d.	853.3	853.3	853.3	853.3	n.d.	-	830.7363
TAG(50:1)	855	[M+Na] <sup>+</sup>	855.4	n.d.	n.d.	n.d.	855.3	n.d.	n.d.	-	832.7520
TAG(52:3)	879	[M+Na] <sup>+</sup>	n.d.	n.d.	n.d.	n.d.	879.3	n.d.	n.d.	-	856.7520
TAG(52:2)	881	[M+Na] <sup>+</sup>	n.d.	n.d.	n.d.	n.d.	881.3	n.d.	n.d.	-	858.7676
TAG(52:1)	883	[M+Na] <sup>+</sup>	n.d.	n.d.	n.d.	883.3	n.d.	n.d.	n.d.	-	860.7833

**Table 1. Identified MAGs, DAGs, and TAGs by ToF-SIMS and MS/MS:** The columns show the lipid class, mass value ( $m/z$ ), species as well as the monoisotopic mass of each lipid identified in the six replicate ToF-SIMS measurements (Fig. S1). The lipids identified by both ToF-SIMS and MS/MS are highlighted in yellow. The most abundant peak of the ToF-SIMS loading spectra ( $m/z$  573.3) also identified by MS/MS is shown in blue text. n.d indicates a non-detected species in that measurement. The asterisk in the last column indicates that the listed SIMS  $m/z$  values could also be explained as the sodium adduct of an ion that contains 2 carbon atoms and 3 double bonds less. For example [M+H-H<sub>2</sub>O]DAG36:4 is nominally equivalent with [M+Na-H<sub>2</sub>O]DAG34:1.

## Discussion

In this study, we applied ToF-SIMS to analyze the distribution of small molecules in the wing imaginal disc of third instar *Drosophila melanogaster* larvae. We found that their distributions separated into two PCs with distinct subdivisions (**Fig. 1**). Intrigued by these ToF-SIMS patterns, we compared them to known genetic expression patterns at the same developmental time-point and found striking similarities. These pattern similarities were in part confirmed by co-registration of images of GFP fluorescence under *hedgehog* promoter control, a classical marker of the A/P compartment boundary. Additionally the B/W pattern is defined and confirmed by a well-known visible margin that correlates with the ToF-SIMS images (**Fig. 2**). By investigating CARS PCA spectra (**Fig. 3**) and tentative assignments from ToF-SIMS peaks (**Fig. 4**), using a previously published catalogue, we identified the predominant small molecules species in the wing blade region of the B/W pattern as lipids.

1 The possible role of these lipids in pattern formation and maintenance must be evaluated in  
2 light of their crucial metabolic and biochemical properties. Recent lipidomic studies revealed  
3 that DAGs and TAGs levels increase in the wing imaginal disc during larval development,  
4 reaching a peak at late third instar [12, 13]. While TAGs are mainly considered storage lipids  
5 from which energy can be gained via  $\beta$ -oxidation [28, 29], they are not used for energy  
6 production during pupation and metamorphosis [12]. Whether TAGs assume a different  
7 function in imaginal discs remains to be elucidated. In contrast to TAGs, DAGs show  
8 different kinetics based on their fatty acid (FA) chain length. Medium-FA-chain DAGs  
9 drastically decrease at the transition from third larval instar to the pupa stage whereas long-  
10 chain DAGs continue to increase, which suggests that different DAGs assume distinct  
11 metabolic functions during *Drosophila* development; these functions also remain to be  
12 determined. We identified one DAG, termed DAG(34:2), by both ToF-SIMS and MS/MS,  
13 and found intermediate chain C16:1 and C18:1 as the two most abundant FA in this DAG.  
14 This, and other intermediate-chain DAGs, may be used as building blocks for the rapid  
15 synthesis of new phospholipids.

16 Several roles of DAGs have been described. Some DAGs serve as second messengers in  
17 several receptor signal transductions via the hydrolysis of membrane lipids into DAGs and  
18 inositol-3,4,5-triphosphate (IP<sub>3</sub>) by the enzyme phospholipase C. The generation of IP<sub>3</sub> leads  
19 to a release of Ca<sup>2+</sup> from the endoplasmic reticulum. Ca<sup>2+</sup> together with the DAG signaling  
20 pathway governs processes like cell division and differentiation [30]. Additionally,  
21 phospholipase D activity is known to regulate cell growth and proliferation using Raf and  
22 mTOR as mediators [31]. DAGs remodel the membrane in response to effector signals,  
23 which might contribute to compartment boundary formation and maintenance. In the immune  
24 system, for example, successful phagocytosis requires actin remodeling by DAG generation  
25 [32]. Other mechanisms leading to similar membrane remodeling and polarization are  
26 reviewed in [33]. It has been demonstrated elsewhere that DAGs in combination with other  
27 membrane lipids in distinct proportions have an important function in determining the  
28 structure and dynamics of biological membranes [34]. For example, the membrane  
29 composition of a cell is important for the curvature of the membrane itself on a single cell  
30 level [35]. Likewise, it can be presumed that the membrane composition of a sub-tissue  
31 region, such as a compartment, may influence the curvature of the tissue. Such a phenomenon  
32 might be responsible for the generation of tissue folds, and our data seem to be consistent  
33 with this notion (**Figs. 1 and 2**). Alternatively, an overrepresentation of DAGs in one cell



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2  
3 1 population might increase the cells' affinity to each other and thereby contribute to the  
4 2 segregation of cells of different populations. We hypothesize that DAGs might also be  
5 3 differentially distributed in the two leaflets of the lipid bilayer as a consequence of the  
6 4 increasing tension generated during metamorphosis. As the organism undergoes tremendous  
7 5 change during pupariation, it is likely that an enormous amount of membranes must be  
8 6 generated in a very short time. The lipid patterns observed from our studies offer important  
9 7 insights for the potential role(s) of lipids during *Drosophila* wing development and cellular  
10 8 organization.  
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