Metabolite localization in living drosophila using High Resolution Magic Angle Spinning NMR

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We have developed new methods enabling in vivo localization and identification of metabolites through their 1H NMR signatures, in a drosophila. Metabolic profiles in localized regions were obtained using HR-MAS Slice Localized Spectroscopy and Chemical Shift Imaging at high magnetic fields. These methods enabled measurement of metabolic contents in anatomic regions of the fly, demonstrated by a decrease in β-alanine signals in the thorax of flies showing muscle degeneration.

High-resolution magic angle spinning (HR-MAS) proton nuclear magnetic resonance (1H NMR) spectroscopy is a very efficient non-invasive analytical tool for investigating the metabolic profiles of a variety of biological systems1-14. This technique, which removes line broadenings due to magnetic susceptibility effects and residual anisotropic interactions in heterogeneous semi-solid samples, provides 1H high resolution NMR spectra revealing the metabolite contents of unprocessed tissue and also of living cells and small organisms. For example, it has been used to study intact tumors6-8, brain tissue affected by neurodegenerative disorders9, and to obtain in vivo metabolic profiles of bacteria10, C. elegans worms11,12, or Drosophila melanogaster flies13,14. For such small organisms, 1H HR-MAS NMR offers a direct access to metabolic phenotypes. However, the one-dimensional (1D) spectra of whole organisms remain complex and, while several metabolites can be directly identified and quantified, some 1H peaks cannot be assigned to specific molecules due to spectral overlap. This limitation is usually overcome using appropriate two-dimensional (2D) homonuclear (1H-1H) and/or heteronuclear (1H-13C) correlation experiments which provide enhanced spectral resolution allowing distinct overlapping signals to be discriminated and assigned1-3,8,13,14.

Although these HR-MAS methods enable metabolic profiling in small organisms, they do not enable localizing metabolites in different parts of the body or specific organs. Adding spatial resolution provides important additional information, as it allows assigning metabolites to specific regions of the organism and even evidencing subtle change of metabolite concentrations in these regions. However, in vivo localized NMR measurements in small organisms require much higher spatial resolution than that needed to study larger animal models. In this study, we introduce two new 1H HR-MAS NMR methods to spatially localize metabolites in a small living organism and demonstrate their promising applications in in vivo metabolic profiling in the case of drosophila, a widely used genetic model.

Results and Discussion

The methods developed, 1H HR-MAS Slice Localized Spectroscopy (SLS) and HR-MAS Chemical Shift Imaging (CSI), provide direct information about the spatial distribution of metabolites along the anteroposterior body axis of the specimen studied. As mentioned above, localization in small organisms such as drosophila requires increased spatial resolution and thus high sensitivity. The experiments therefore greatly benefit from the use of a high magnetic field (17.6 T) which enhances both sensitivity and spectral resolution. Performing in vivo HR-MAS measurements in drosophila also requires a viable environment minimizing the centrifugal forces induced by rapid sample spinning. For this purpose, the anteroposterior axis of the fly is aligned along the spinning axis by positioning the insect in a shaped insert in the center of the rotor. This allows the use of spinning frequencies up to 2.7 kHz while keeping the fly alive (without observable damage). Under these experimental conditions, spatial localization along the anteroposterior body axis of the living fly is obtained simply using pulsed magnetic field
gradients along the MAS axis corresponding to the usual orientation of gradient coils in HR-MAS NMR probes. With HR-MAS SLS, a localized 1D 1H spectrum is obtained by selecting a transverse slice of the fly body with a defined thickness (Fig. 1a), while HR-MAS CSI provides a 2D map of spatially arrayed 1H spectra along the whole fly body (Fig. 2). Regarding sensitivity, these two methods are complementary since the former allows recording the spectrum of a specific region of interest with a high signal-to-noise ratio, while the latter gives complete spatial distribution of metabolites at the cost of experimental time.

To illustrate the efficiency of the 1H HR-MAS Slice Localized Spectroscopy experiment, we recorded spatially-resolved spectra for the three main anatomical parts of the fly. As shown in Figure 1b, the 1D localized spectra of the head, thorax (340 μm slice thickness each) and abdomen (680 μm slice thickness) clearly evidence significant differences in 1H signal intensities reflecting variations in metabolite contents, with some appearing mainly localized in a specific region of the body. In drosophila, β-alanine is required in hard tissues for cuticle melanization, and also for vision and inactivation of biogenic amines in the nervous system15,16. 1H HR-MAS SLS measurements reveal here that the largest amount of β-alanine observed in soft tissues was specifically localized in the thorax of the fly (Fig. 1b). This in vivo observation was further confirmed by recording conventional 1H HR-MAS spectra of dissected thorax (Supplementary Fig. 1). Localized 1H HR-MAS experiments were also performed for a drosophila model of muscle degeneration. A mutant in upheld gene (up101) showing degenerative muscle hypercontraction of indirect flight muscles was used17,18. To highlight changes in metabolic profiles resulting from muscle degeneration, the 1H HR-MAS SLS spectrum of the thorax was recorded in vivo. A significant decrease in the β-alanine concentration in the thorax (about four times less) of the mutant with respect to Oregon-R was observed (Fig. 1c). Experiments were performed for 10 up101 mutants (males or females) and this result was found to be highly reproducible (Fig. 1d). Therefore, the marked decrease in the β-alanine content in the thorax of mutants, evidenced by the localized measurements, appeared as a clear metabolic signature of muscle degeneration in the model used.

Spatial localization of tissue metabolites in a living drosophila was also achieved using 1H HR-MAS Chemical Shift Imaging. As shown in Figure 2, HR-MAS CSI spectra directly reflect the metabolite distribution along the fly’s anteroposterior body axis (spatial resolution of 189 μm). In these 2D maps, the 1H NMR spectroscopic signature along the horizontal axis is correlated with the spatial position...
along the vertical axis. The $^1$H peaks associated with the same molecule thus appear on the same ordinate, in the same way as the $^1$H signals of different molecules localized in the same region. The three main anatomic parts of the drosophila (head, thorax and abdomen) are easily distinguishable in the 2D CSI spectra confirming that some metabolites are predominantly localized in one of these specific regions (Fig. 2a). For example, the spectra of both male and female Oregon-R flies show that $\beta$-alanine and taurine are mainly localized in the thorax, while glycerol is mostly found in the abdomen and to a less extend in the head. These measurements also reveal that significant concentrations of phosphoethanolamine (PE), acetate (or acetyl) group (Ac), as well as several $^1$H peaks assigned to a galactoside (Gal), are specifically localized in male but not in female abdomens suggesting that they correspond to metabolites characteristic of the male reproductive tract. Moreover, the HR-MAS CSI spectrum of the male fly for the two specific regions corresponding to the middle (black) and posterior (blue) parts of the abdomen. Ac, acetate (or acetyl); Gal, galactoside; $^{13}$CH$_2$C$\equiv$; $^{15}$CH$_2$:CO; PC, phosphocholine; PE, phosphoethanolamine; Tre, trehalose. All spectra were recorded at a magnetic field of 17.6 T. The spinning frequency was 2630 Hz for in vivo experiments and 4000 Hz for dissected organs.

In summary, these spatially-resolved $^1$H HR-MAS NMR methods localize metabolites in a living drosophila efficiently. The 1D HR-MAS SLS experiment provides the signature of the most abundant metabolites localized in a single slice of the fly and 2D HR-MAS CSI enables complete mapping of metabolites in the whole individual. These methods, using spatial encoding along the anteroposterior axis of the fly body, have been demonstrated to be sensitive enough to evidence a metabolic fingerprint of a specific organ. Using a conventional gradient HR-MAS probe or a standard MAS probe in a

Figure 2 | HR-MAS CSI experiments. (a) In vivo $^1$H 2D HR-MAS CSI spectra of female (left, body length of $\sim 3$ mm) and male (right, body length of $\sim 2.5$ mm) Oregon-R drosophila. In the 2D maps, the regions corresponding to the head (H), thorax (T) and abdomen (A) are separated by the dashed lines. Sixteen contour levels are plotted with a top contour of 5% of the maximum intensity ([C$_2$H$_5$] resonance at 1.3 ppm) and a dividing factor of 1.22. $\beta$-ala: $\beta$-alanine; Tau: Taurine; Gly: glycerol. (b) Left: $^1$H 1D HR-MAS spectra of dissected organs of 2 to 5 Oregon-R males. Green, orange and brown spectra correspond to testis, paragonia and penis, respectively. Middle: Scheme of the male reproductive system (dorso-lateral view oriented anterior top, from reference 24). Te: testis, Pa: paragonia, Pe: penis apparatus. Right: 1D sum along the spatial dimension (vertical) of the 2D $^1$H HR-MAS CSI spectrum of the male fly for the two specific regions corresponding to the middle (black) and posterior (blue) parts of the abdomen. Ac, acetate (or acetyl); Gal, galactoside; $^{13}$CH$_2$:C$\equiv$; $^{15}$CH$_2$:CO; PC, phosphocholine; PE, phosphoethanolamine; Tre, trehalose. All spectra were recorded at a magnetic field of 17.6 T. The spinning frequency was 2630 Hz for in vivo experiments and 4000 Hz for dissected organs.
three-axis field gradient system, these methods offer a unique opportunity to highlight variation of metabolite contents in specific regions of living small animal models. Further applications in the study of drosophila models of human diseases are in progress.

**Methods**

**Sample preparation.** *Drosophila melanogaster* strain Oregon-R and mutant strain sp313 were obtained from Bloomington Drosophila Stock Center (Indiana University 1001 E. Third St., Bloomington, IN 47405-7005 USA). Fly stocks were maintained in our laboratory by mass culture on standard medium at 22 °C (362g cornmeal, 200g dry yeast, 60g agar and 150mL of a 10% solution of methyl-4-hydroxybezoate in ethanol, water qsp 4 L). Experiments were performed on adult flies (7 to 10 days old).

To perform all *in vivo* HR-MAS experiments, a fly was placed in a cylindrical PTFE insert (outer diameter of 2.6 mm, external length of 7 mm, inner diameter of 1.2 mm and internal length of 4 mm) located in the center of the rotor, such that the anteroposterior axis of the fly was aligned along the spinning axis. A petri dish, filled with ice was covered with aluminum foil and the fly was put on this foil to cool for anesthesia purposes. The aluminum foil was perfectly dry to avoid the fly sticking to it. The anesthetized drosophila was then placed in the insert with small tweezers under a magnifying glass. The *H* NMR experiments were performed at 3 °C to keep the fly anesthetized and alive18. After the experiments, the insert was opened to check that the fly was alive without observable damage.

To study the dissected parts of drosophila, flies (2 to 5) were first dissected in saline solution (9g/L NaCl in D2O) under a stereomicroscope. The organs, heads or thorax were then placed in a 4 mm HR-MAS rotor containing the same saline solution. *H* HR-MAS experiments were performed at 3 °C.

**NMR experiments.** All *H* HR-MAS NMR experiments were carried out on a Bruker AVANCE III spectrometer operating at a magnetic field of 17.6 T (corresponding to *H* Larmor frequency of 750.13 MHz). *In vivo* measurements were performed using a Bruker microimaging probe (3 axis, 2.0 mm spacing, 3.2 mm double resonance MAS microimaging probe. Proton-free commercial rotors and caps. The fly were recorded at a spinning rate of 4000 Hz using a Bruker 4.0 mm double resonance MAS probe and commercial HR-MAS rotors and caps.

For all experiments, the spectral width was set to 10000 Hz and the 90° and 180° pulse durations were 25 μs and 50 μs, respectively (rf-field strength of 10 KHz). *H* chemical shifts were referenced using the resonance of the (CH2)n of drosophila fatty acids at 1.3 ppm as an internal reference. Water suppression (WS) was achieved using a selective presaturation of the H2O resonance (low-power pulse duration of 1 s). Data were processed with zero filling of twice the number of real points. Exponential apodization with a 2 Hz line broadening was applied prior Fourier transform. Uncertainties on isotropic chemical shift measurements are 0.02 ppm (i.e. ±0.02 ppm).

The *H* 1D HR-MAS spectra of living drosophila and of the dissected parts of the fly were recorded using a spin echo sequence (echo time TE = 0.19 ms). 512 transients were acquired with a recycle delay of 2 s, corresponding to an experimental time of ~20 min. Assignment of the *H* resonances was performed using 2D through-bond 1H homonuclear correlation HR-MAS spectra and data from the literature13,19,21.

Supplementary Figure 3d. The 1D profiles of the slices centered on the head and the head, thorax and abdomen of the fly (Supplementary Fig. 4a). 1D water density images of the selected spatial region were acquired with the sequence shown in Supplementary Figure 3d. The 1D profiles of the slices centered on the head and thorax (340 μm thickness each) and on the abdomen (680 μm thickness) are shown in Supplementary Figure 4b. Images of the 1D HR-MAS SLS spectra of these slices are shown in Supplementary Figure 4c. 2D HR-MAS spectra of a single dissected thorax is shown in Supplementary Figure 1 (wild type male fly).

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Author contributions
V.S.K. and F.F. developed the experiments. V.S.K., N.J, F.L., M.Y., F.S, M.D., J.C.B performed the NMR experiments. M.D. performed the laboratory growth of the drosophila and dissection for the ex vivo analyses S.M. and D.M. contributed to analysis of results and discussion. V.S.K., F.F., M.D, J.C.B wrote the manuscript.

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