Lipidome-wide $^{13}$C flux analysis: a novel tool to estimate the turnover of lipids in organisms and cultures

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ABSTRACT

Lipid metabolism plays an important role in the regulation of cellular homeostasis. However, since it is difficult to measure the actual rates of synthesis and degradation of individual lipid species, lipid compositions are used often as a surrogate to evaluate lipid metabolism even though they provide only static snapshots of the lipodome. Here, we designed a simple method to determine the turnover rate of phospholipid and acylglycerol species based on the incorporation of $^{13}$C$_6$-glucose combined with LC-MS/MS. We labeled adult *Drosophila melanogaster* with $^{13}$C$_6$-glucose that incorporates into the entire lipidome, derived kinetic parameters from mass spectra, and studied effects of deletion of CG6718, the fly homologue of the calcium-independent phospholipase A2β, on lipid metabolism. Although $^{13}$C$_6$-glucose gave rise to a complex pattern of $^{13}$C incorporation, we were able to identify discrete isotopomers in which $^{13}$C atoms were confined to the glycerol group. With these isotopomers, we calculated turnover rate constants, half-life times, and fluxes of the glycerol backbone of multiple lipid species. To perform these calculations, we estimated the fraction of labeled molecules in glycerol-3-phosphate, the lipid precursor, by mass isotopomer distribution analysis of the spectra of phosphatidylglycerol. When we applied this method to *D. melanogaster*, we found a range of lipid half-lives from 2 to 200 days, demonstrated tissue-specific fluxes of individual lipid species, and identified a novel function of CG6718 in triacylglycerol metabolism. This method provides fluxomics-type data with significant potential to improve the understanding of complex lipid regulation in a variety of research models.

KEYWORDS

Genes in lipid dysfunction, lipid metabolism, mass spectrometry, phospholipids/metabolism, stable isotope tracers
INTRODUCTION

Measurements of lipid compositions provide static snapshots of the lipidome but do not capture dynamic features, such as the rates of synthesis, inter-conversion, and degradation. In order to obtain such information, stable isotope experiments are necessary (1-3). A variety of different precursors have been applied, including $^2$H$_3$-methionine, $^2$H$_{13}$-choline (4), $^{15}$N-serine, $^{15}$N-choline (5), $^{13}$C$_5$-glutamine (6), labeled glucose (7, 8), labeled glycerol (9), labeled fatty acids (9-11), and $^2$H$_2$O (12). Most of these precursors target specific lipid classes, but some may gain access to the entire lipidome because their metabolites label a spectrum of building blocks used for lipid synthesis.

However, the latter can make data analysis very complex. Lipids are heterogeneous molecules, in which different moieties turn over independently of each other. For instance in phospholipids, fatty acids have a faster turnover than the glycerol backbone and that again is different from the turnover of the head group. If a single isotope-labeled precursor is incorporated into multiple moieties, the isotopic label will be exposed to more than one turnover mechanism. Thus, it is not surprising that most previous studies have taken a reductionist approach by either targeting specific types of lipids, such as glycosphingolipids (13), or a specific moiety, such as phosphorylcholine (4).

Despite of that, first attempts have been made to establish a “fluxomics”-type analysis of lipid metabolism. For instance, non-targeted isotopomer filtering and matching has been applied to identify 692 new isotopomers in primary muscle cultures labeled with $^{13}$C$_{16}$-palmitic acid (11). Furthermore, the advent of new ultra-high resolution mass spectrometers has enabled the separation of $^{15}$N-isotopomers from $^{13}$C-isotopomers, which allowed the simultaneous measurement of the abundance and the labeling of many nitrogen-containing lipids (5).

Here, we labeled adult fruit flies with $^{13}$C$_6$-glucose, a precursor that is incorporated into the entire lipidome, but focused the data analysis on backbone-labeled isotopomers of glycerol-containing species. By doing so we were able to derive rigorous kinetic parameters for a large segment of the lipidome. Fruit
flies are ideally suited for this type of work because (i) they do not change their body mass after reaching maturity, which eliminates growth as a source of $^{13}$C incorporation, (ii) they are small, which limits the cost for stable isotope reagents, and (iii) they can be genetically modified, which makes them excellent tools to study the effect of various genes on lipid metabolism.

MATERIALS AND METHODS

**Drosophila strains and labeling with stable isotope precursors**

The fly strain, y[1] w[67c23]; P[w[mC]y[mDint2] EPgy2]CG6718[EY05103], that carries a P element insertion (EY05103) in the gene CG6718, was obtained from the Bloomington Drosophila Stock Center. We have previously shown that CG6718, the homologue of the mammalian iPLA2β (iPLA2-VIA), is inactivated in these flies (14). Adult male flies of either the wild-type or the mutant were chosen for all labeling experiments. The animals were kept in vials that contained 0.2 mL of the labeling solution placed on a piece of filter paper (0.5 x 0.5 inch) as the only nutrient. The vials were placed in wet chambers at 25°C for up to 2 weeks. The labeling solutions contained 1M $^{13}$C$_6$-glucose, 1M $^2$H$_5$-glycerol, or 1M $^2$H$_9$-choline plus 1M unlabeled glucose in water. They were replenished at least every 4 days. Five flies were collected for each time point. In some experiments, heads, thoraces, and abdomens were dissected under the microscope. In that case, 15 flies were collected per time point. Whole flies or their body parts were stored at -80°C before analysis.

Lipidomics data acquisition by LC-MS/MS

Fly samples were homogenized in water and lipids were extracted with methanol and chloroform (15). Internal standards were added immediately after the initial application of chloroform and methanol. They consisted of 100μl of 4-fold diluted SPLASH LipidoMIX and 50 μl of 4-fold diluted CL standard mix.
(both from Avanti Polar Lipids). The lipid extracts were dried under nitrogen and re-dissolved in 100 μl chloroform/methanol (1:1) except for the fly head samples, which were re-dissolved in 25 μl chloroform/methanol (1:1). Lipid extracts were analyzed by a QExactive HF-X mass spectrometer (ThermoScientific) coupled to an Agilent 1100 high-performance liquid chromatograph (Agilent Technologies) equipped with a Restek Ultra C18 reversed-phase column (particle size: 3μm, dimensions: 100 x 2.1 mm) operated at room temperature, using similar conditions as described by others (16). An aliquot of 7 μl of the extract was injected and chromatographed at a flow rate of 0.25 ml/min. Solvent A contained 600 ml acetonitrile, 399 ml water, 1 ml formic acid, and 0.631 g ammonium formate. Solvent B contained 900 ml 2-propanol, 99 ml acetonitrile, 1 ml formic acid, and 0.631 g ammonium formate. The chromatographic run time was 30 minutes, changing the proportion of solvent B in a non-linear gradient from 30 to 35% (0-2 minutes), from 35 to 67% (2-5 minutes), from 67 to 83% (5-8 minutes), from 83 to 91% (8-11 minutes), from 91 to 95% (11-14 minutes), from 95 to 97% (14-17 minutes), from 97 to 98% (17-20 minutes), from 98 to 100% (20-25 minutes), and from 100 to 30% (25-26 minutes). For the remainder of the run time the proportion of solvent B stayed at 30% (26-30 minutes). The mass spectrometer was operated alternatively in negative or in positive ion mode. Triacylglycerol data were extracted from the positive ion spectra and all other data were extracted from the negative ion spectra. The spray voltage was set to 4 kV and the capillary temperature was set to 350°C. MS1 scans were acquired at a resolution of 120,000, an AGC target of $10^6$, a maximal injection time of 65 ms, and a scan range of 300-2000 m/z. MS2 scans were acquired at a resolution of 30,000, an AGC target of $3\times10^6$, a maximal injection time of 75 ms, a loop count of 11, and an isolation window of 1.7 m/z. The normalized collision energy was set to 30 and the dynamic exclusion time to 13 s.

**Data analysis**
First, mass spectral data were extracted and processed for lipid identification, lipid quantitation, and isotopomer analysis. All analyzed species and their shorthand notations are listed in Table 1. Next, kinetic parameters were estimated, including the labeled fraction of tissue glycerol-3-phosphate (p), fractional syntheses of lipid species (q), as well as their turnover rate constants (k), half-life times (t_h), and flux rates (j).

1.1. **Lipid identification.** Lipids were identified using the software LipidSearch 4.1 SP1 (ThermoScientific). The general database was searched with a precursor tolerance of 2 ppm, a product tolerance of 0.2 Da, an intensity threshold of 1.0%, and an m-score threshold of 5. In negative ion mode, [M+HCO₂] adducts were identified for PC and [M-H] ions for all other lipids. In positive ion mode, [M+NH₄] adducts were identified for TG.

1.2. **Lipid quantitation.** Lipids were quantified by their MS1 signal intensities relative to that of internal standards added at the beginning of the sample extraction. Specifically, in this paper we used the internal standards PC 15:0/18:1(d7) (5003 pmol), PE 15:0/18:1(d7) (186 pmol), and TG 15:0/18:1(d7)/15:0 (1626 pmol).

1.3. **Isotopomer analysis.** In order to read the signal intensities of isotopomers, spectra were imported into Xcalibur 4.0. The intensities were averaged over a 0.1-min interval centered at the peak retention time of the species to be analyzed. For most lipids (1 glycerol group), we read the intensities of the isotopomers with 0 and 3^{13}C atoms. For PG species (2 glycerol groups), we read the intensities of the isotopomers with 0, 3, and 6^{13}C atoms. For CL species (3 glycerol groups), we read the intensities of the isotopomers with 0, 3, 6, and 9^{13}C atoms.

2.1. **Estimation of p and q.** The labeled fraction of glycerol-3-phosphate (p) was determined from the isotopomer pattern of the two most abundant PG species, PG 16:0/16:1 and PG 16:0/18:2.
according to binomial distribution theory (17). Since PG contains 2 glycerol groups, its isotopomer pattern at time $t$ equals:

$$
\begin{pmatrix}
m_0 \\
m_1 \\
m_2
\end{pmatrix}_t = q \begin{pmatrix}
(1-p)^2 \\
2(1-p)p \\
p^2
\end{pmatrix}_t + (1-q) \begin{pmatrix}
m_0 \\
m_1 \\
m_2
\end{pmatrix}_{t=0}
$$

(equ 1)

In equation (1), $m_0$, $m_1$, $m_2$ are the normalized intensities of the isotopomers with 0, 1, or 2 labeled glycerol groups ($m_0+m_1+m_2=1$), $p$ is the labeled fraction of glycerol-3-phosphate, and $q$ is the fractional synthesis of PG. First, $p$ and $q$ values were calculated by equation (1). Second, the $p$ values were substituted into equation (2) in order to calculate fractional syntheses ($q$) of lipids with one glycerol group:

$$
\begin{pmatrix}
m_0 \\
m_1
\end{pmatrix}_t = q \begin{pmatrix}
1-p \\
p
\end{pmatrix}_t + (1-q) \begin{pmatrix}
m_0
\end{pmatrix}_{t=0}
$$

(equ 2)

In equation (2), $m_0$ and $m_1$ are the normalized intensities of the isotopomers with 0 and 1 labeled glycerol group ($m_0+m_1=1$). Finally, $p$ values obtained from equation (1) were substituted into equation (3) in order to calculate fractional syntheses ($q$) of CL species:

$$
\begin{pmatrix}
m_0 \\
m_1 \\
m_2 \\
m_3
\end{pmatrix}_t = q \begin{pmatrix}
(1-p)^3 \\
3p(1-p)^2 \\
3(1-p)p^2 \\
p^3
\end{pmatrix}_t + (1-q) \begin{pmatrix}
m_0 \\
m_1 \\
m_2 \\
m_3
\end{pmatrix}_{t=0}
$$

(equ 3)

In equation (3), $m_0$, $m_1$, $m_2$, $m_3$ are the normalized intensities of the isotopomers with 0, 1, 2, or 3 labeled glycerol groups ($m_0+m_1+m_2+m_3=1$).

2.2. **Estimation of $k$, $t_n$, and $j$.** Turnover rate constants ($k$) were estimated from serial $q$ values by non-linear regression to equation (4):
½ = 1 − 𝑒−𝑘𝑡  (equation 4)

Half-life times (𝑡ℎ) were calculated from the turnover rate constants:

\[ t_h = \frac{\ln 2}{k} \]  (equation 5)

Synthetic fluxes (𝑗) were calculated by multiplying the turnover rate constant of a lipid species with its concentration (𝑐) as determined in step 1.2.

**Measurement of the isotopic labeling of water-soluble metabolites**

In order to measure the \(^{13}\text{C}\) abundance of water-soluble lipid precursors, samples from the same cohort analyzed by lipidomics, were processed for metabolomics. Flies (5 animals per time point) were homogenized in 100 μl methanol/water (8+2) and kept at -80°C overnight. Homogenates were spun for 5 minutes at 15,000 g in Eppendorf tubes. The supernatants were analyzed on a QExactive HF-X mass spectrometer directly coupled to a Vanquish UHPLC system (ThermoFisher Scientific, Waltham, MA, USA). A 5-μL aliquot of the supernatant was injected onto a 5 μm C18 Acclaim 120 column (4.6 x 100 mm, ThermoScientific). Metabolites were eluted with a 5-50% methanol gradient in water containing 0.1% formic acid and 0.2g/l ammonium acetate over 50 minutes at a flow rate of 1ml/min. The mass spectrometer was operated in positive ion mode and data-dependent mode with survey scans acquired at a resolution of 120,000 over a scan range of 100-1000 m/z. Up to five of the most abundant precursors from the survey scan were selected with an isolation window of 1.7Th and fragmented by higher-energy collisional dissociation with normalized collision energy of 30. The maximum ion injection time for the survey and MS/MS scans was 75ms and the ion target value for MS and MS/MS scans were set at 3e6 and 1e5, respectively. The labeling of water-soluble metabolites was determined in MS1 scans acquired from 0.97 to 1.07 minutes. To measure the \(^{13}\text{C}\) labeling of glycerol-3-phosphate, the intensities of the \(^{13}\text{C}_0\) isotopomer (m/z=173.021) and the \(^{13}\text{C}_3\) isotopomer (m/z=176.0311) of the [M+H]\(^+\) ion of glycerol-3-phosphate were determined. To measure the \(^2\text{H}\) labeling of glycerol-3-phosphate, the intensities of the \(^2\text{H}_0\)
isotopomer (m/z=173.021) and the $^{2}$H$_{5}$ isotopomer (m/z=178.0524) of the [M+H]$^+$ ion of glycerol-3-phosphate were determined. To measure the $^2$H labeling of phosphoryl-choline, the intensities of the $^{2}$H$_{6}$ isotopomer (m/z=184.0739) and the $^{2}$H$_{9}$ isotopomer (m/z=193.1304) of the [M+H]$^+$ ion of phosphoryl-choline were determined.

RESULTS AND DISCUSSION

Overview of the methodology

We incubated adult *Drosophila melanogaster* with $^{13}$C$_{6}$-glucose in order to observe the incorporation of $^{13}$C atoms into various lipid species. Flies were separated into 3 body parts, including head, thorax, and abdomen, which roughly represent the nervous system, the indirect flight muscles, and the digestive/reproductive tract, respectively. Lipids were extracted from these tissues or from whole flies and analyzed by LC-MS/MS. The mass spectral data were processed in 3 steps. First, lipids were identified by the mass-to-charge (m/z) ratios of intact molecules and their daughter ions, using the commercial software LipidSearch (ThermoScientific, version 4.1 SP1). Second, lipids were quantified by comparing their MS1 intensity to that of structurally related internal standards. Third, for each lipid species, the distribution of intensities among its isotopomers was determined in MS1 scans acquired within ±0.05 min of its peak retention time. The isotopomer patterns were transformed into normalized vectors $M$, which can take different forms depending on the number of glycerol groups in the lipid molecule (see Methods). The evolution of $M$ from the unlabeled state ($M_0$) to progressively labeled states ($M_t$) formed the data set from which the fraction of labeled glycerol-3-phosphate (labeling of the lipid precursor) and the fractional syntheses of lipids (newly formed molecules/total molecules) were calculated. Those data in turn were used to calculate turnover rate constants, half-life times, and fluxes (Fig. 1).
Intramolecular distribution of $^{13}$C atoms

Since $^{13}$C$_6$-glucose is metabolized by the glycolytic pathway and the Krebs cycle, $^{13}$C atoms gain access to the entire metabolic network and ultimately to all lipid moieties, including fatty acids, glycerol groups, and head groups. As a result, multiple new isotopologues have been observed in cell cultures exposed to $^{13}$C$_6$-glucose (3, 7, 8). However, among the many isotopologues, molecules with 3 $^{13}$C atoms are particularly abundant (7, 8). We confirmed this observation in fruit flies fed with $^{13}$C$_6$-glucose. For example, we found $^{13}$C$_3$ molecules to be most abundant among the isotopologues of PC 16:1/16:1 (Fig. 2a). Collision-induced dissociation of $^{13}$C$_3$-PC 16:1/16:1 produced the expected fragments LPC16:1, the fatty acid FA16:1, phosphoryl-choline, and glycerol phosphate. $^{13}$C atoms were only present in fragment ions that carried glycerol, such as LPC 16:1 and glycerol-phosphate (Fig. 2b). These data demonstrate that the $^{13}$C atoms of $^{13}$C$_3$-PC 16:1/16:1 were confined to the glycerol moiety, a finding we confirmed for other phospholipids, among them PG.

In the PG spectra, we found that $^{13}$C$_6$-PG isotopologues were most abundant, consistent with the presence of 2 glycerol groups per molecule (Fig. 3a). Fragmentation of $^{13}$C$_6$-PG produced monoisotopic acyl ions and $^{13}$C$_3$-labeled LPA ions, corroborating the positional specificity of $^{13}$C atoms in the glycerol groups (Fig. 3b). In conclusion, we demonstrated the preferential labeling of glycerol moieties and identified specific isotopomers in which all $^{13}$C atoms were confined to the glycerol group. Our data are consistent with previous reports showing glycerol-specific $^{13}$C$_9$-isotopomers of CL, a lipid with 3 glycerol groups (8, 18). Thus, $^{13}$C$_3$-isotopomers (or $^{13}$C$_6$-isotopomers in the case of PG or $^{13}$C$_9$-isotopomers in the case of CL) can be used to monitor selectively the incorporation of $^{13}$C atoms into the glycerol backbone, which provides an opportunity to measure the rate of de novo synthesis of phospholipid and acylglycerol species.

Estimation of precursor labeling
In order to determine the fractional synthesis of lipids, it is necessary to know the abundance of $^{13}$C in the intracellular precursor molecule, which in our case is glycerol-3-phosphate. The labeling of glycerol-3-phosphate can be determined by LC-MS/MS of the *Drosophila* metabolome. However, this requires the analysis of each sample on a metabolomics platform, in addition to the lipidomics analysis. Alternatively, it is possible to calculate the labeling of glycerol-3-phosphate from lipidomics data by mass isotopomer distribution analysis (17). Lipids with more than one glycerol group, such as PG, bis(monoacylglycerol)-phosphate, and CL, are amenable to this technique, which estimates the glycerol-3-phosphate labeling from the isotopomer pattern of lipids harboring multiple glycerol units. CL is the most abundant of these lipids, but its slow turnover prevents any substantial accumulation of $^{13}$C atoms and consequently makes it a poor choice for mass isotopomer distribution analysis (18). Instead, we performed this analysis with PG, measuring the signal intensities of the glycerol-specific isotopomers $^{13}$C$_0$-PG, $^{13}$C$_7$-PG, and $^{13}$C$_6$-PG. We verified that the 3 signals had the same retention times (Fig. 4a) and a reproducible and highly accurate mass difference between each other (Fig. 4b). The high accuracy of the mass difference between isotopomers (<1 ppm) was important because it allowed the unequivocal identification of isotopomers in spectra crowded with different $^{13}$C-labeled species (Fig. 4c).

As expected, the incorporation of $^{13}$C atoms shifted the distribution of abundancies gradually from $^{13}$C$_0$ to $^{13}$C$_6$ isotopomers (Fig. 5a). From that distribution, we calculated the fraction of labeled glycerol-3-phosphate (p) and the fractional synthesis of PG (q) by solving equation 1 for p and q. In order to test the accuracy of this calculation, we measured the fraction of labeled glycerol-3-phosphate by LC-MS/MS. The measured labeling of glycerol-3-phosphate was slightly higher than the calculated labeling but the difference was small and it vanished after reaching saturation at about 2 days of incubation (Fig. 5b). As expected, the fractional synthesis of PG increased from 3-9 days (Fig. 5c). Together these data demonstrate that the labeling of tissue glycerol-3-phosphate can be estimated with sufficient accuracy from the PG spectra, which makes the method solely reliant on lipidomics, circumventing the need for any additional measurement.
Turnover rates of lipid molecular species

Next we determined the fractional syntheses of various lipid species. These values were calculated by equations 2 and 3, using the various isotopomer distributions and the glycerol-3-phosphate labeling data obtained from PG analysis. The rate by which the fractional synthesis increased during the incubation varied tremendously between different lipid classes (Fig. 6a). From the time evolution of the fractional syntheses, we calculated turnover rate constants, which showed a strong head-group specificity (Fig. 6b). In contrast, we found less turnover variations within a given lipid class except for PE, where large differences between species were observed. For instance PE 16:0/16:1 turned over 4 times faster than PE 16:1/18:1 and 10 times faster than the alkyl-acyl species PE 18:0e/18:2 and PE18:0e/18:3 (Fig. 6c). Half-life times calculated from the turnover rates of Drosophila lipids ranged from 2 to 200 days. PC, PI, and PG species had the fastest turnover whereas CL and alkyl-acyl-PE species had the slowest (Fig. 6d). The half-lives of CL and ether-PE exceeded the average life span of the flies, which was about 60 days, underscoring the remarkable stability of these two phospholipids. Furthermore, our data showed a strong dependence of the TG turnover rate on the length of the acyl chains (Fig. 6e).

In order to assess the accuracy of our method, we compared the results to turnover measurements obtained with 2 other precursors, including 2H5-glycerol and 2H9-choline. When we analyzed the glycerol turnover of PC by measuring the relative intensities of 2H5-isotopomers in 2H5-glycerol-labeled flies, we found that the turnover rate constants were remarkably similar to those obtained with 13C6-glucose (Fig. 7). In contrast, a similar approach with 2H9-choline yielded much higher turnover rates, which is consistent with established pathways for head group recycling of PC (19). In summary, these data support the validity of our method. They demonstrate distinct turnover kinetics of Drosophila lipids with half-life times stretching over 2 orders of magnitude and identify CL and ether-PE’s as the most stable phospholipids.
**Tissue-specific lipid fluxes in wild-type and ΔiPLA2β flies**

To determine whether lipid dynamics varies between different tissues, we divided fruit flies into body parts containing the nervous system (head), indirect flight muscles (thorax), and the digestive and reproductive systems (abdomen). Comparison between these tissues identified both similarities and differences. For instance, the half-life of PE 18:1/18:2 was longer in indirect flight muscles (>20 days) than in the head or the abdomen (<10 days) whereas the half-lives of PI 18:1/18:2, PC 18:1/18:2, and dMePE 18:1/18:2 were similar (<10 days) (Fig. 8a). In all tissues, the half-lives of PC species were much shorter than the half-lives of PE species (Fig. 8b). Equal half-lives were observed of TG in different tissues (Fig. 8c). However, when we determined flux rates, we found the turnover of most TG species to be lower in the nervous system than in the muscular or the digestive/reproductive system (Fig. 8d). Furthermore, the TG fluxes were dependent on the number of acyl carbons, peaking at a carbon number of 44. Tissue-specific flux rates were also found for various PE and PC species, with each species having its own pattern of specificity (Fig. 8e, f).

Finally, we applied our method to a *Drosophila* mutant in order to demonstrate its power to identify specific changes in lipid metabolism in response to the deletion of a lipid-metabolizing enzyme. We chose the enzyme iPLA2β (CG6718, iPLA2-VIA), an important phospholipase involved in membrane remodeling and signal transduction (20). Deletion of iPLA2β had no effect on the half-lives of either PC or PE (Fig. 8b) but significantly prolonged the half-life of TG, suggesting that CG6718 is involved either directly or indirectly in TG lipolysis of *Drosophila* (Fig. 8c). Thus, our data reveal tissue-specific fluxes of individual lipid species and suggest a role of iPLA2β in TG metabolism.

**Conclusions**
In summary, we have developed a method to measure the dynamics of phospholipids and acyl glycerol lipids. It yields precursor labeling, fractional syntheses, turnover rate constants, half-life times, and flux rates from a series of mass spectra collected at different time points during the incorporation of $^{13}$C$_6$-glucose. In the steady state, the fluxes represent the rates of de novo synthesis and degradation, which must be distinguished from the recycling rates of acyl chains and head groups. Thus, our method measures specifically the turnover of the glycerol backbone, the most stable part of lipid molecules that does not undergo recycling. It has the distinct advantage that it enables comparison among glycerol containing lipids, a very large section of the lipidome, whereas other methods are limited to lipids with specific fatty acids (9-11) or with specific head groups (4, 5).

We applied our method to Drosophila melanogaster, but it is readily applicable to cell cultures and to other organisms, including mouse models. Interestingly, we observed a wide range of turnover rates among the lipid species of Drosophila and showed that the rates are tissue-specific. CL and ether-PE were the lipids with the longest half-life. Previously, we have already demonstrated the extraordinary stability of CL and its dependence on tafazzin and respiratory enzymes (18, 21). Our data also show that lipid flux analysis in gene knockout models can reveal novel functions of lipid metabolizing enzymes. As a case in point, we identified the unexpected involvement of the fly homologue of the calcium-independent phospholipase A2β in TG metabolism. We therefore believe that the present method is a very useful tool to study lipid metabolism.

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| Shorthand notation | Lipid species                                      |
|--------------------|---------------------------------------------------|
| **CL**             | **Cardiolipin**                                   |
| CL 18:1/(18:2)₃    | Oleoyl-trilinoleoyl-cardiolipin                   |
| CL (18:2)₄         | Tetralinoleoyl-cardiolipin                        |
| **dMePE**          | **Phosphatidyl(dimethyl-ethanolamine)**           |
| dMePE 18:1/18:2    | Oleoyl-linoleoyl-phosphatidyl(dimethyl-ethanolamine) |
| **FA**             | **Fatty acid**                                    |
| FA 16:1            | Palmitoleic acid                                  |
| **LPA**            | **Lysophosphatidic acid**                         |
| **LPC**            | **Lysophosphatidylycerol**                        |
| LPC 16:1           | Lyso-palmitoleoyl-phosphatidylycerol              |
| **PA**             | **Phosphatidic acid**                             |
| **PE**             | **Phosphatidylethanolamine**                      |
| PE 16:0/16:1       | Palmitoyl-palmitoleoyl-phosphatidylethanolamine   |
| PE 16:1/16:1       | Dipalmitoleoyl-phosphatidylethanolamine           |
| PE 16:0/18:1       | Palmitoyl-oleoyl-phosphatidylethanolamine         |
| PE 16:1/18:1       | Palmitoleoyl-oleoyl-phosphatidylethanolamine      |
| PE 16:1/18:2       | Palmitoleoyl-linoleoyl-phosphatidylethanolamine   |
| PE 18:0/18:1       | Stearoyl-oleoyl-phosphatidylethanolamine          |
| PE 18:1/18:1       | Di oleoyl-phosphatidylethanolamine                |
| PE 18:1/18:2       | Oleoyl-linoleoyl-phosphatidylethanolamine         |
| PE 18:2/18:2       | Dilinoleoyl-phosphatidylethanolamine              |
| PE 18:0e/18:2      | Octadecyl-linoleoyl-phosphatidylethanolamine      |
| PE 18:0e/18:3      | Octadecyl-linolenoyl-phosphatidylethanolamine     |
| **PG**             | **Phosphatidylglycerol**                          |
| PG 16:0/16:1       | Palmitoyl-palmitoleoyl-phosphatidylglycerol       |
| PG 16:0/18:2       | Palmitoyl-linoleoyl-phosphatidylglycerol          |
| **PGP**            | **Phosphatidylglycerolphosphate**                 |
| **PI**             | **Phosphatidylinositol**                          |
| PI 18:1/18:1       | Di oleoyl-phosphatidylinositol                    |
| PI 18:1/18:2       | Oleoyl-linoleoyl-phosphatidylinositol             |
| **PS**             | **Phosphatidylserine**                            |
| PS 18:1/18:1       | Di oleoyl-phosphatidylserine                     |
| PS 18:1/18:2       | Oleoyl-linoleoyl-phosphatidylserine               |
| **TG**             | **Triacylglycerol**                               |
| TG 36:0            | Triacylglycerol with 36 acyl carbons and 0 double bond |
| TG 38:0            | Triacylglycerol with 38 acyl carbons and 0 double bond |
| TG 42:1            | Triacylglycerol with 42 acyl carbons and 1 double bond |
| TG 44:1            | Triacylglycerol with 44 acyl carbons and 1 double bond |
| TG 46:1            | Triacylglycerol with 46 acyl carbons and 1 double bond |
| TG 48:1            | Triacylglycerol with 48 acyl carbons and 1 double bond |
| TG 50:1            | Triacylglycerol with 50 acyl carbons and 1 double bond |
Figure 1. Flowchart of the methodology. Adult flies were labeled with $^{13}$C$_6$-glucose, harvested at different time points, and dissected. Lipid extracts of the individual body parts were analyzed by LC-MS/MS. The MS data were processed in order to identify and quantify lipid species and to determine their isotopomer patterns (green box). The isotopomer patterns were used to estimate kinetic parameters (blue box).
Figure 2. Glycerol-specific labeling of $^{13}$C$_3$-PC 16:1/16:1. Adult wild-type flies were labeled with $^{13}$C$_6$-glucose for 9 days. Lipids were extracted from whole flies and analyzed by LC-MS/MS. 

**a:** The relative abundances of PC 16:1/16:1 isotopologues were determined from MS1 spectra. The isotopologue with 3 $^{13}$C atoms was most abundant (Mean values ± standard error, N=7).

**b:** Collision-induced dissociation of $^{13}$C$_3$-PC 16:1/16:1 produced $^{13}$C$_3$ fragments of LPC 16:1 and glycerophosphate and $^{13}$C$_0$ fragments of the acyl groups and the phosphorylcholine group.
Figure 3. Glycerol-specific labeling of $^{13}$C$_6$-PG 16:0/18:2. Adult wild-type flies were labeled with $^{13}$C$_6$-glucose for 3 days. Lipids were extracted from whole flies and analyzed by LC-MS/MS. a: The relative abundances of PG 16:0/18:2 isotopologues were determined from MS1 spectra. The isotopologue with 6 $^{13}$C atoms was most abundant (Mean values ± standard error, N=5). b: Collision-induced dissociation of $^{13}$C$_6$-PC 16:1/16:1 produced $^{13}$C$_0$ fragments of the fatty acids and $^{13}$C$_3$ fragments of LPA.
Figure 4. Isotopomers of PG 16:0/18:2. Adult wild-type flies were labeled with $^{13}$C$_6$-glucose for 6 days. Lipids were extracted from whole flies and analyzed by LC-MS/MS to determine the spectrum of PG 16:0/18:2. a: Isotopomer signal intensities were plotted versus the retention time. b: The deviation of the observed from the predicted value of the monoisotopic mass ($^{13}$C$_0$) and the mass differences between isotopomers ($^{13}$C$_3$-$^{13}$C$_0$, $^{13}$C$_6$-$^{13}$C$_0$) are shown. Mean values (±standard deviation) were compared by $t$-test. c: Isotopomers and their mass differences are indicated in a MS1 spectrum.
Figure 5. Estimation of precursor labeling (p) and fractional synthesis (q) of PG. Adult wild-type flies were labeled with $^{13}$C$_6$-glucose for 0-9 days. Lipids and water-soluble metabolites were extracted from whole flies. Lipids were analyzed to determine the spectra of PG species. Water-soluble metabolites were analyzed to determine the $^{13}$C-labeling of glycerol-3-phosphate. 

a: Relative abundances of PG isotopomers were measured after different periods of exposure to $^{13}$C$_6$-glucose. 

b: The labeling of glycerol-3-phosphate (p) was either measured by LC-MS/MS or calculated from the relative abundancies of PG isotopomers. 

c: The fractional syntheses of PG species (q) were calculated from the relative abundancies of PG isotopomers. Bar graphs represent mean values with standard deviation (N=10).
Figure 6. Turnover rates and half-life times of *Drosophila* lipids. Adult wild-type flies were labeled with $^{13}$C$_6$-glucose and harvested at different time points. Lipids were extracted from whole flies and analyzed by LC-MS/MS. 

**a:** Fractional syntheses of different lipid species were determined from MS1 data. 

**b-e:** Rate constants were estimated from the fractional syntheses by non-linear regression. Half-life times were calculated from the rate constants. Bar graphs represent mean values and standard errors of the regression analysis to 4 different time points.
Figure 7. PC turnover measured with different isotope-labeled precursors. Adult wild-type flies were labeled with the indicated precursor and harvested at different time points. Lipids were extracted from whole flies and analyzed by LC-MS/MS in order to determine the isotopomer pattern of different PC species. Water-soluble metabolites were extracted and analyzed separately in order to determine the relative abundance of labeled molecules in the lipid precursors ($^{13}$C$_6$-glycerol-3-phosphate, $^2$H$_5$-glycerol-3-phosphate, or $^2$H$_9$-phosphorylcholine). Fractional syntheses were calculated for each time point. Rate constants were estimated from the fractional syntheses by non-linear regression. Bar graphs represent mean values and standard errors of the regression analysis to 4 different time points.
Figure 8. Lipid half-lives and fluxes in different tissues of wild-type (WT) and iPLA2β-deletion (ΔiPLA2β) flies. Adult flies were labeled with $^{13}$C₆-glucose and harvested at different time points. Body parts were dissected and lipids were extracted and analyzed by LC-MS/MS with internal standards. Lipid quantities and fractional syntheses were determined from the MS data. Rate constants were estimated from the fractional syntheses by non-linear regression analysis. Half-life times and fluxes were calculated from the rate constants and the lipid quantities. Bar graphs represent mean values and standard errors. a: Regression analysis to 8 data points; b: Analysis of 16:1/16:1, 16:0/18:1, and 18:1/18:2 molecular species (N=3); c: Comparison of mean values by t-test (N=7); d-f: Regression analysis to 4 data points.