Methods for studying metabolism in *Drosophila*

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Abstract

Recent research using*Drosophila melanogaster* has seen a resurgencein studies of metabolism and physiology. This review focuses on major methods used to conduct this work. These include protocols for dietary interventions, measurements of triglycerides, glucose, trehalose, and glycogen, stains for lipid detection, and the use of gas chromatography/mass spectrometry (GC/MS) to detect major polar metabolites. It is our hope that this will provide a useful framework for both new and current researchers in the field.

1. Introduction

Metabolism and physiology provided a major focus for *Drosophila* research in the middle of the last century[1]. This work included fundamental studies ofbiochemical genetics, starting with classic work on eye pigment biosynthesis and the one gene-one enzyme hypothesis[2], as well as detailed characterization of basic energy physiology[e.g. ref. 3, 4, 5]. Although the advent of recombinant DNA technology shifted the *Drosophila* field toward studies of developmental biology, recent efforts have refocused on metabolism, with a particular emphasis on the mechanisms that maintain energy homeostasis and the use of *Drosophila* as a model for studies of diabetes and obesity[6-10]. In this review, we highlight methods that have been developed to conduct current genetic studies of metabolism in *Drosophila*, with a focus on energy homeostasis and physiology. We begin with an overall discussion of the challenges facing researchers in the field, given the contributions of both environmental and genetic factors onmetabolic control. We then move on to review protocols for dietary intervention in*Drosophila*, followed by widely used assays to quantify basic metabolites in the animal. We end with protocols for metabolomic profiling by gas chromatography-mass spectrometry (GC/MS). Our goal is not to be comprehensive in our coverage of metabolic protocols, since that is beyond the scope of one article, but rather to focus on some major methods currently being used, with the hope that this will provide a useful framework for both new and current researchers in the field.

2. Experimental design

The profound interplay between environment and genetics requires that metabolic studies be undertaken with carefulattention to genetic background, diet, stock maintenance, and statistical

analysis of data. Particular concern should be directed toward the selection of control strains and the establishment of an appropriately matched genetic background in the mutant being studied. Outcrossing mutants to the control strain provides a good way to achieve this goal, as well as confirming results with more than one control and/or mutant line. Matching the genetic background becomes more difficult when GAL4 drivers and UAS transgenes are included in the genotype; however, a uniform *white* mutant background and controls with the driver and/or responder alone can be included. Tissue-specific RNAi provides an invaluable and widely used tool for functional studies of metabolism. In addition to the standard concerns with off-target effects, however, RNAi can have nonspecific effects on physiology[e.g. ref. 11]. Finally, some care should be taken when using commercial kits for quantifying specific metabolites. Although these kits may be widely used in mammalian research, most have not been validated in *Drosophila*. As discussed below for quantifying simple metabolites such as glucose, glycogen, and triglycerides, protocols need to be adapted to the system. A good way to achieve this for a new assay is to use known mutants in the pathway being studied, and score for the predicted changes in metabolite levels.Alternatively, methods such as GC/MS can be used to confirm the changes in metabolite levels detected by an enzymatic kit. In general, the more labile the metabolite (such as ATP), the more care that is needed for its quantification.

3. Starvation and dietary paradigms

The metabolic state of an animal is intimately linked with its diet. Accordingly, dietary conditions must be carefully controlled when performing metabolic studies. Often this will require the lab making special batches of media to maintain both breeding and experimental stocks. This point becomes particularly important when considering the recent evidence that parental diet can influence the metabolic state of offspring, as has been welldocumented in mammals[12, 13]. While carefully controlled dietary conditions are essential, dietary manipulation also provides one of the most useful approaches for studies of metabolism and animal physiology. For example, enrichment or restriction of particular nutrients can often evoke robust metabolic phenotypes in mutants that may otherwise appear normal under idealdietary conditions.

Starvationprovides an importantdietary stress that tests the animal's ability to mobilize stored nutrients for survival. This can be achieved by transferring larvae or flies to either water

or PBS. Although moist filter paper is employed for larvae, studies of adult starvation should use 1% agar as a substrate, as it provides a more uniform moist environment for long-term fasting (overnight) or starvation (>12 hours) studies.

Alteration of dietary sugar levels is a useful method for investigating potential defects in carbohydrate homeostasis. This can be easily achievedby using an 8% yeast and 1% agarmediumto serve as the dietary foundation for manipulatingsugar concentrations. Arange from 3-15% dietary sugar (ratio of 2:1 glucose to sucrose, by weight) representsthelow-to-high spectrum found withina relatively normal diet for *Drosophila*(Table 1). In our studies, altering the sugar concentration of this media has proven to be an effective tool forboth enhancingand suppressingdiabetic phenotypes.For example, several *Drosophila* mutants that are sensitive to a sugar diet can maintain euglycemia on a 3% sugar diet but display increasingly severehyperglycemia as dietary sugar is increased(W. Barry, D. Bricker, and R. Somer, unpublished results).

Very high sugar diets have been developed for studies of type 2 diabetes in *Drosophila*. These diets consist ofeither 1% agar, 3.4% yeast, 8.3% cornmeal, and 30% sucrose[15] or 1% agar, 8% yeast, 2% yeast extract, 2% peptone, and 34.2% sucrose[16] (Table 1). These diets lead to hallmarks of type 2 diabetes in wild-type *Drosophila*, including hyperglycemia, insulin resistance, obesity, and cardiac dysfunction[15-17]. High lipid diets have also been developed for studies of diet-induced obesity in *Drosophila*. These can be achieved by supplementation of standard growth media with either soy lipids[18] or coconut oil[19, 20], which has been most widely used for this purpose.

Although the above media allow the researcher to crudely manipulate the overall levels of carbohydrates and lipids in the diet, precise addition or removal of dietary components is often desirable. Fortunately, two labs have recently developed chemically defined diets that support *Drosophila* development and growth (although at a reduced rate), and are well suited for the study of adult physiology and aging[21, 22]. The advent of these defined diets provides a level of control not possible in past work, and should greatly facilitate nutritional and metabolic studies in *Drosophila*.

3.1 Measuring feeding rate:

It is often important to assess whether changes in feeding rate contribute to an observed metabolic phenotype. The use of dietary dyes, for example, can provide a crude yet simple method to visually assess food consumption[23]. This approach is particularly useful when analyzing responses to an acute feeding bout, by ensuring that only animals that have consumed the food are analyzed. Dietary dyes are not well suited for quantitative analysis, however, and cannot be used to accurately assess feeding rate over extended periods of time. In contrast, adding radioactive material to the food provides a more quantitative measurement of consumption based on ingested radiation after a fixed period of time[24]. This can be achieved for individual flies by adding 10^8 counts/min/ml (final volume) α-^{32}P dCTP (or any other nucleotide) to the diet and permitting the animals to feed for 2 hours. Lower levels of radiation can be used if multiple flies are measured. Labeled animals are transferred to unlabeled food for several hours to allow them to clear radioactivity that is nonspecifically bound to the cuticle. They are then subjected to Cerenkov counting in a scintillation counter to quantify food uptake[25, 26]. Scintillation fluid can be used if the animals are sacrificed and lower levels of radiation are employed in the diet.

The Capillary Feeder (CAFE) assay has been developed as a means to directly quantify food consumption by making use of a capillary feeder containing a liquid medium[27]. While this technique allows for direct and precise analysis of ingested food quantities, some researchers have raised concerns that the diet and its method of delivery might not represent a natural feeding scenario[27, 28]. This led to an alternate technique that measures the number of times an animal extends its proboscis to feed in a fixed time period[28]. While labor intensive, this method appears to be accurate and has little effect on feeding paradigms used in studies of dietary restriction and aging.A more detailed description of these techniques can be found in a recent review on *Drosophila* feeding behavior[29].

4. Methods to measure basic metabolites: lipids

Lipids are stored as triglycerides (TAG) in *Drosophila* and, like in other animals, provide a critical source of stored energy. Most lipids reside in the fat body, although some are also present in the gut and oenocytes of both larvae and adults. Two different types of assays can be used to detect lipids – either quantification of lipid levels from whole animals or dissected tissues, or the

use of lipophilic dyes to visualize neutral lipids within cells.Taken together, these provide a valuable indication of the lipid distribution within the animal.

4.1 Colorimetric quantification:

Overall quantification of TAG is often afirst step in a metabolic study, along with measurements of basic carbohydrates and protein. An accurate, although insensitive means of measuring lipids is to use Bligh and Dyer lipid extraction followed by TLC[30, 31]. This allows fractionation and detection of total fatty acids, diacylglycerol (the major circulating form of lipid in insects), and TAG. The most widely used assay for detecting lipids, however, is a coupled colorimetric assay that detects free glycerol levels after cleaving stored TAG with a lipase[30, 32, 33]. Samples are collected on ice in 1.5 ml tubes, homogenized in 100 µl of cold PBS + 0.005% Tween 20, and immediately heat treated at 70ºC for 5 minutes. 20 µl of the resulting homogenate is incubated at 37ºC for 30 minutes with 20 µl of the lipase reagent (Sigma; T2449). To ensure that the free glycerol measured in this assay is specific to TAG cleavage and does not originate from another cellular source, a negative control containing 20 µl of homogenate is incubated with 20 µl of PBS + 0.005% Tween 20 and processed in parallel with the samples that contain the TAG lipase. Samples are then centrifuged at 20,000 x g for 5 minutes, and 30 µl of supernatantis pipetted into individual wells of a clear 96 well plate (Corning; 9017). In order to make quantitative measurements, a dilution series of glycerol standards (Sigma; G7793)are pipetted into the first row of the plate (0, 0.1, 0.2, 0.4, 0.8 mg/ml). 100 µl of free glycerol reagent (Sigma; F6428) is added to each well, and the plate is incubated at 37ºC for 5 minutes.Glycerol concentration is quantified in a plate reader set to measure absorbance at 540 nm.

A few studies have suggested that thisapproach does not provide an accurate assessment of stored fat in insects[34, 35],however, both TLC analysis and the colorimetric assays have been demonstrated to produce similar results[30]. A caveat remains that this assay also releases glycerol from mono- and diacylglycerides[36] and, therefore, results generated by this method should be validated byeither TLC or qualitative assessment using a lipophilic dye (see below). The presence of eye pigment in adult samples could also interfere with accurate absorbance measurements at certain wavelengths, however, assay kits designed to measure absorbance at 540 nm do not detect eye pigment[30].

As with any metabolic measurement, TAG levels must be considered in the larger context of animal growth and physiology. Mutations that delay developmental progression and alter adult body size couldproduce proportional changes in TAG in that do not reflect metabolic defects. Similarly, wild-type adult flies do not feed for a prolonged period after eclosion, and need to be aged several days to achieve maturity[37]. Thus to prevent inaccurate results, all samplesshould be developmentally staged and normalized to an internal parameter such as soluble protein (although the effects of a mutation on protein levels may require another method, such as body weight, for normalization). A simple method for measuring protein concentration is to remove 10 µl of homogenized sample prior to heat treatment, dilute 1:10 in cold PBS, and use 10 µl of the homogenate to conduct a Bradford assay[38].If protein concentration is not measured immediately, samples should be stored at -80ºC.

4.2 Stains

While colorimetric assays provide an accurate quantification of total stored TAG, staining of neutral lipids in the intestine, fat body, and oenocytes provide a valuable cell based assay. If relative quantification is a goal, all samples must bepreparedsimultaneously and analyzed to ensure that the final results are not influenced by variations in temperature, dye concentration, incubation time, or microscopesettings. Ideally, experimental and control slides should be randomized and scored blindly to eliminate confirmation bias. Many dyes are available for lipid detection, with Oil Red O, Sudan Black, and Nile Red being the most commonly used.

4.2a Oil Red O

The dye Oil Red O provides a reliable means to visually assess neutral lipids in fixed tissues[39-42]. Animals are dissected and fixed using 4% formaldehyde in PBS for 30 minutes, washed twice in PBS and twice in 100% propylene glycol. Fixed tissuesare then stained in a solution of 0.5% Oil Red O(Sigma; O0625)dissolved in propylene glycol, which has been filtered through Whatman #1 filter paper and preheated to 60ºC.Allow samples to incubate for 1 hour at 60ºC, then wash twice with 85% propylene glycol and twice with PBS at room temperature. Stained samples are mounted on microscope slides using glycerol. The use of this dye in *Drosophila* is well documented and provides a sensitive means to assay neutral lipids in the oenocytes and

intestine. The density of TAG in the fat body, however, results in an Oil Red O stain that is too intense for identifying modest defects in fat storage.

4.2b Sudan Black

Similar to Oil Red O, the dye Sudan Black allows for an accurate assessment of neutral lipid stores in fixed tissues[40, 43]. Tissues that have been fixed with 4% formaldehyde are rinsed twice with PBS, once with 50% ethanol, and then stained for 2 minutes at room temperature with prefiltered 0.5% Sudan Black (Sigma; 199664) dissolved in 75% ethanol. Once staining is completed, samples are sequentially rinsed with 50% ethanol, 25% ethanol, and PBS before mounting on a microscope slide with glycerol. In general, Sudan Black results in less intense tissue stains than Oil Red O and thus is better suited for visualizing the high levels of TAG in the fat body.

4.2c Nile Red

Unlike Oil Red O and Sudan Black, Nile Red can be used to visualize neutral lipids in unfixed tissues[44]. Animals should be quickly dissected in a solution of 0.00002% Nile Red (Sigma;19123) and 75% glycerol, and visualized using confocal microscopy. Although relatively straightforward, the use of Nile Red is somewhat limited and can be employed improperly[45-47]. While this dye allows for the qualitative assessment of lipid droplet size and shape, it should not be used toquantitatively measurefat reserves.

5. Methods to measure basic metabolites: carbohydrates

Proper regulation of carbohydrate homeostasis is critical for maintaining normal physiology. The two primary forms of circulating carbohydrates in *Drosophila* are glucose and trehalose (a disaccharide of glucose). While trehalose is most abundant in larval hemolymph, circulating glucose and trehalosecan be readily detected in the adult fly. These sugars serve a variety of purposes, including providing an essential energy source through glycolysis, substrates forbiosynthetic reactions in growing animals, and energy storage in the form of glycogen.

5.1 Enzymatic Assays

A number of enzymatic assays are commercially available to quantifylevels of specific sugars and related metabolic intermediates. Although initially developed for use in mammalian systems, many of these assays have been successfully co-opted for usein *Drosophila.* Care should be taken, however, to properly validate each enzymatic assay for a particular experimental system and/ordevelopmental stage.

5.1a Glucose

Two separate enzymatic-based methods have been widely used to measure free glucose levels in flies. The first of these utilizes the enzyme Hexokinase to phosphorylate glucose, producing glucose-6-phosphate[48]. Subsequent oxidation of glucose-6-phosphate by Glucose-6-Phosphate Dehydrogenase (G6PD) produces 6-phosphogluconate and NADH. Spectrophotometricreadings at 340 nm can then be used to assess the amount of NADH produced, as it naturally absorbs ultraviolet light at this wavelength. With this method, NADH production is directly proportional to the starting glucose concentration.Alternatively, free glucose levels can be measured through a colorimetric-based enzymatic assay. This method employs Glucose Oxidase (GO) activity to catalyze the oxidation of glucose to hydrogen peroxide and gluconic acid[49]. Subsequently, the hydrogen peroxide and an added compound, o-dianisidine, react to produce an oxidized form of o-dianisidine (orange color) in the presence of peroxidase. Sulfuric acid is then added to stop the reaction and convert the color to a pink hue, which can be quantified at an absorbance of 540 nm.

In our hands, the GO method is more limited in scope when compared with the HK-based assay. For example, we have found that the GO assay cannot be used to accuratelyquantify carbohydrates inpupae; rather, the HK protocol should be followed when studying this stage in development (W. Barry, unpublished results). This finding emphasizes the need to carefully validate the use of metaboliteassays in new experimental contexts prior to their use.

Free glucose levels are nearly undetectable in larvaeand thus trehalose is measured at this stage (see trehalose assay below). In order to measure free glucose in adult flies by either the GO or HK assays,fivemales are placed in a 1.5 ml tube, homogenized in 100 µl of cold PBS, and immediately heated for 10 min at 70°C. Alternatively, the flies are frozen in liquid nitrogen and stored at -80°C until all samples are collected for the assay. The frozen tubes are then kept on dry ice, rapidly homogenized, and heat treated. In either case, it is important to keep the samples cold and to move rapidly prior to heat inactivation, which is essential to prevent the enzymatic

breakdown of glycogen and trehalose into glucose by endogenous enzymes in the extract. Similar to the TAG assay described above, 10µl of homogenate should be removed prior to heat inactivation and reserved for Bradford quantification of protein levels, providing a means to internally normalize glucose levels (although, as discussed above, effects of a mutation on protein levels may require another method for normalization). The resulting homogenate is centrifuged for 3 minutes at 5,000 x g to pellet the debris, and the supernatant is then diluted in PBS for glucose quantification. The appropriate dilution may range from 1:4 to 1:8, depending on genetic background, dietary conditions, developmental stage, number of animals used and animal size. Determining the appropriate dilution is critical, as both the HK and GO assay possess a limited linear range.

Each well of a clear 96 well plate is loaded with 30µl of diluted sample, with a dilution series of free glucose standards (0, 0.01, 0.02, 0.04, 0.08 and 0.16 mg/ml) loaded in the first row to generate a standard curve. The assay is initiated by adding 100µl of HK (Sigma; GAHK20) or GO reagent (Sigma; GAGO20) with a multichannel pipette. The HK assay is incubated for 15 min at room temperature and the absorbance of each sample is measured at 340 nm. For the GO assay, incubate the reactions at 37ºC for 30 minutes, add 100µl of 12N sulfuric acid, and measure absorbance at 540 nm.

Glucose transporters are bidirectional, and thus free glucose willtravel freely between intracellular and extracellular compartments. As a result, most free glucose is found in the circulating fluid rather than within cells. Measurements of free glucose from whole animal homogenates thereforeprovide an indirect assessment of circulating glucose levels. As always, however, accurate measurements of circulating trehalose or glucose can only be made by using hemolymph samples (see below).

5.1b Trehalose

Trehalose representsamajor circulating sugar in *Drosophila* and, together with glucose, provides essential information about the metabolic state of an animal. Trehalose is quantified by an assay that is based on the protocol for measuring glucose levels, described above[50, 51].The difference is that trehalase is added to the extract to digest the trehalose into free glucose, which can then be quantified by standard assays and compared to the background level of glucose present in the original sample. Although the number of animals required for this analysis varies

with developmental age and genotype, we have found that 25 mid-second instar larvaeor fiveadult male flies are normally sufficient for a single sample. Animals are homogenized in 100µl of trehalase buffer (5mM Tris pH 6.6, 13mM NaCl, 2.7mM KCl). Note that trehalase activity is exquisitely sensitive to buffer conditions and will not work in PBS.Once the samples have been homogenized, heat-treated, and centrifuged at 5,000 x g, 30µl of the supernatant is diluted 1:1 in a 1.5 ml tube with trehalase buffer containing trehalase[3µl of porcine trehalase (Sigma; T8778-1UN) per 1 ml of buffer]. An additional 30µl of supernatant diluted 1:1 in a separate tube with trehalase buffer (no enzyme) will serve as a negative control for the reaction. Finally, a trehalose standard is diluted in buffer containing trehalase to generate a standard curve (0, 0.01, 0.02, 0.04, 0.08 and 0.16 mg/ml). All reactions are incubated at 37ºC for 18-24 hours.

Once the enzymatic reaction is complete, all trehalose should be broken down into glucose, which can then be measured using either the HK or GO method described above. In orderto calculate thetrehalose levels in a given sample, the background absorbance from the reaction without enzyme must be subtracted from the reaction that containstrehalase, as the amount of glucose in the former samplerepresents the background free glucose that was present in the original extract.

5.1c Hemolymph

While analysis of whole animal homogenates provides a relatively easy and high throughput approach for quantifyingglucose and trehalose, this method does not distinguish between circulating and stored sugars, and can be complicated by the presence of food within the gut. Therefore, hemolymph samples are used to directly assay circulating sugars.Several alternate methods for hemolymph collection have been described for both larvae and adults[52-55]. Protocols based onfiltration bycentrifugation and capillary-based collection of hemolymph aremost widely used. The centrifugation approach requires approximately 30-50 adult females (adult males contain less hemolymph per fly and thus require larger numbers) carefully punctured in the thorax using a tungsten needle. Punctured flies are placed in a 0.5 ml microfugetube that contains a hole at the bottom of the tube, which is packed with glass wool. This tube is then placed within a 1.5 ml collection tube and centrifuged at 9,000 x g for 5 minutes at 4ºC, yielding approximately 1.5µl of hemolymph. Longer spin times, higher speeds, and more flies can increase yield, but may also increase cellular or intestinal contamination. One

way to detect possible contamination from intestinal contents is to addfood coloring to the medium andcheck the collected hemolymph for theabsence of the dye. Additionally, hemolymph canbe analyzed under a dissection scope for the absence of cellular debris.

Hemolymph can also be collected by immobilizing an adult fly wing-side down on a piece of double-sided tape and carefully puncturing the head cuticle with a tungsten needle[55]. Gentle pressure is applied to the thorax with a blunt object, and a capillary tube or pipette tip isused tocollect the small drop of hemolymph that is forced out of the puncture site. While this method is labor intensive and not well suited for the collection of large numbers of samples, it minimizes potential hemolymph contamination.

Regardlessof which method is used, a 1µlaliquot ofthe collected hemolymph isdiluted in 99 µl of trehalase buffer (1:100), followed by heat treatment for 5 minutes at 70°C to inactivate endogenous trehalase. After heat treatment, the sample is split into two 50 µlaliquots, one of which is dilutedfurther with an equal volume of trehalase buffer alone, and the other with trehalase buffer plus trehalase [3 µl of porcine trehalase (Sigma; T8778-1UN) per 1 ml of buffer].The resulting sample will have a final dilution of 1:200. These diluted samples are then placed at 37˚C for 18-24 hours to allow for breakdown trehalose into free glucose. A 30 µl aliquot of each sample (+/- trehalase) is then loaded onto a 96 well plate to measure the concentration of hemolymph trehalose and glucose using the enzymatic assays described above. As previously mentioned, the ideal dilution will depend on dietary conditions, genetic background and developmental stage.

5.1d Glycogen

Most carbohydrate in the fly is stored in the form of glycogen, which provides a large and accessible energy source during times of fasting and intense activity. Most glycogen in the adult fly is stored in the fat body, flight muscle, halteres, and gut, while larval glycogen is primarily located in the body wall muscle[3, 56]. Although periodic acid/Schiff staining can be used to visually assess glycogen deposits, the easiest and most quantitative method uses an enzymatic assay that breaks down glycogen into molecules of free glucose, which is then quantified using the GO or HK assays described above[57]. Samples are homogenized in 100 µl of PBS, after which10 µl of the homogenate is removed to measure soluble protein andthe sample is transferred to70°C for heat inactivation followed by centrifugation.Samples must be kept cold

andmoved rapidly to the heat treatment step to prevent the breakdown of glycogen into free glucose by endogenous enzymes present in the extract. Due to the high level of glycogen in the samples, the resulting supernatant must be diluted between 1:5 and 1:20 in PBS.The appropriate dilution will vary depending on diet, genotype, and developmental stage, and special care must be taken to ensure that the concentrations of all samples are within the linear range of the assay. If the concentration of any sample exceeds that of the 0.16 mg/ml glycogen standard, then the assay should be repeated using more dilute samples.

The protocol to generate free glucose from glycogen depends on the preferred method for measuring glucose. If using the GO kit,two 30 µlaliquotsof each diluted sample and each diluted glycogen standard (0, 0.01, 0.02, 0.04, 0.08, 0.16 mg/ml)are loaded into two wells of a 96 well plate. The first well is used to measure free glucose in the original samplewhile the second well is used to determine glycogen plus free glucose levels.Add 100 µl of glucose reagent to the free glucose sample and 100 µl of glucose reagent plus amyloglucosidase to the glycogen sample [1 µl of amyloglucosidase (Sigma; A1602-25MG) per ml of glucose reagent]. Cover the plate with parafilm, incubate at 37ºC for 30 minutes, and follow the protocol described above for measuring glucose.

If using the Sigma HK kit, aliquots of samples and standards should be split into two 1.5 ml tubes and appropriately diluted with PBS or PBS plus amyloglucosidase (final concentration after dilution of 1 mg/ml enzyme) to a final volume of greater than 30 µl.Samples are incubated at 37°C for 30 minutes, centrifuged, and 30 µl are loaded into a single well of a 96 well plate at room temperature.Glycogen-derived glucose is measured by adding 100 µl of HK reagent and incubating the plate for 15 minutes at room temperature. As described above, absorbance at 340 nm is used to calculate free glucose versus glycogen-derived glucose.

6. ATP

ATP measurements represent a direct readout of cellular energy levels and thus can provide important insights into metabolic phenotypes. This analysis can be conducted with a luciferase-based assay kit that uses endogenous ATP to generate light (Molecular Probes; A22066)[58]. Because ATP is so unstable, a chaotropic buffer is used to preserve as much of the intact metabolite as possible. Five adult male flies or 25 mid-second instar larvae are washed in cold PBS and homogenized in 100 µl of homogenization buffer [6 M guanidine HCL, 100 mM Tris

(pH 7.8), 4 mM EDTA]. Samples of 10 µl of the homogenate are removed to measure soluble protein (see above) and the remaining samplesare boiled for 5 minutes, centrifuged at 5,000 x g, and the supernatant is diluted 1:750 with a solution of 25 mM Tris (pH 7.8) and 100 µM EDTA.The diluted homogenated is centrifuged at 20,000 x g, and 10 µl of the supernatant is transferred to individual wells of a white, opaque 96 well plate (Corning; 3362). Additionally, 10 µl of ATP standards (0, 0.01, 0.05, 0.1, 0.5, 1 µM) should be placed in the first row of the plate to provide a standard curve. Start the assay by adding 100 µl of the luciferase reaction mix with a multichannel pipette and immediately begin measuring luminescence with a plate reader. A minimum of three measurements should be made for each assay, and the results should be averaged.

7. Metabolomics

The emerging field of metabolomics provides an unprecedented opportunity to simultaneouslymeasure hundreds of metabolic compounds in animal extracts. This technique has proven to be a powerful tool for conducting studies inthe fly[59-63]. In addition, when combined with classical genetic analysis, metabolomics can be used to precisely identifymetabolicreactions that are affected by a mutation, providing key insights into gene function. The field of metabolomics is too large to summarize in a single review because a variety of methods have been developed to assayseveral classesof metabolites, ranging from small,charged molecules to large, fatty acids. Rather, we provide here a relatively simple protocol that allows users to quantify ~100 small, polar molecules, whichincludes the basic amino acids, sugars, and intermediates in glycolysis and the TCA cycle. This range of compounds is sufficient to provide insights into the metabolic state of the corresponding animal.

7.1 Genetic background

Metabolomic analysis is particularly sensitive to genetic background effects. An example of this is the use of*rosy*mutant strains as a control for transgenic lines that were established by scoring for rescue of the eye phenotype. *ry*mutationsrender animalsunable to synthesize uric acid, which not only results in elevated levels of purine-related intermediates[64], but also induces significant changes in a diverse group of metabolites, including tryptophan, kynurenine, and related compounds[65].Studies of transgenic lines in which the *ry* background is not controlled

would therefore inaccurately identifywidespread metabolic defects. Similarly, *yellow* mutants exhibit defects in lysine metabolism[66], andother widely-used mutant lines, such as *white* or *vermillion*,may produce similar metabolomic artifacts. Therefore, the same genetic background should be used for all samples, ideally using multiple control genotypes for internal confirmation.

7.2 Sample Collection

The amount of material required for a successful metabolomics experiment varies depending on the developmental stage and type of analysis. For a basic survey of small, polar metabolites using GC/MS, samples should contain at least 300 embryos, 25 second instar larvae, and 20 mature adult males.All samples, except for larvae, are collected in screw-cap tubes that contain 1.4 mm ceramic beads (MoBio; 13113-50) and flash frozen in liquid nitrogen. Embryos do not need to be dechorionated prior to collection, but should be washed gently with a paintbrush on a piece of Whatman filter paper soaked in PBS. Larvae are collected in a 1.5 ml tube, repeatedly washed with ice-cold PBS, and flash frozen in liquid nitrogen. The frozen pellet is then dislodged by gently flicking the tube and transferred into a pre-chilled screw cap tube with ceramic beads.

7.3 Sample Processing for GC-MS

All samples should be stored at -80ºC until processing, at which point they are transferred to an enzyme-type carrier caddy (Nunc Lab-Top cooler) that has been chilled to -20ºC.Add 800 µl of prechilled 90% methanol containing 1.25 µg/ml succinic-*d*4 acid (Sigma-Aldrich; 293075) and 6.25 µg/ml U-13C, U-15N amino acid mix (Cambridge Isotope; CDNLM-6784)to each tube with a positive displacement pipette. These stable-isotope labeled internal standards provide a means to normalize samples, provide quality control, and allow for the monitoring of instrument efficiency across batches. Additionally, negative controls that contain no fly tissue should be prepared to detect chemical contamination and false-positive peaks during the subsequent GC/MS analysis.

The hard cuticle of the fly is difficult to homogenize and requires a strong physical disruption to efficiency release metabolites. We have found that theOmni Bead Ruptor 24 homogenizer(Omni International)is ideally suited to rapidly and efficiently disrupt fly tissue,

with samples homogenized for 30 seconds at 6.5 m/sec.While a variety of bead-filled tubes can be used for this purpose, screw-cap tubes containing 1.4 mm ceramic beads are optimal for removingextraction solvent after processing. Homogenized samples are incubated at -20ºC for one hour to enhance protein precipitation and centrifuged at 20,000 x g for 5 minutes at4ºC to remove the resulting precipitate. The supernatant is transferred to a 1.5 ml microfuge tube and the solvent removed with a Speed-Vac (Genevac).

7.4 GC-MS analysis

All GC-MS analysis is performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples are suspended in 40 µl of 40 mg/ml O-methoxylamine hydrochloride (MOX)(MP Biomedicals; 155405**)** in pyridine solution(EMDMillipore;PX2012-7)and incubated for one hour at 30°C. Samples are then centrifuged for 5 minutes at 20,000 x g to remove particulate matter, and 25 µl of the supernatant is placed in an autosampler vial (Agilent; 8010-0172 and 5181-1215) with a 250 µl deactivated glass microvolume liner(Agilent**;**5183-2086**)**. Forty microliters of N-methyl-N-trimethylsilyltrifluoracetamide containing 1% TMCS(MSTFA) (ThermoScientific;TS-48915**)** is added automatically via a Gerstal autosampler and the samples are incubated for 60 minutes at 37°C with shaking. Following incubation, 3 µl of a fatty acid methyl ester standard solution(FAMES; Table 2) is added via the autosampler and 1 µl of the prepared sample is injected to the gas chromatograph inlet at a 10:1 split ratio with the inlet temperature held at 250°C.Fatty acid methyl esters do not occur naturally, but will elute in a highly reproducible manner across the entire chromatogram. The retention time curve of FAMES solution, therefore, allows for the building of reliable retention time libraries of metabolites. Furthermore, this solution is also used to assess column quality because a degraded column will exhibit peak tailings of the FAMES standards.

The gas chromatograph is set to an initial temperature of 95°C for one minute followed by a 40°C/min ramp to 110°C and a hold time of 2 minutes. This is followed by a 5°C/min ramp to 250°C and a third ramp to 330°C with a final hold time of 3 minutes. A 30 meter Phenomex ZB5-5 MSi column with a 5 meter long guard column is employed for chromatographic separation. For each analysis, instrument performance is assessed by analyzing the negative control samples,whichonly contain the internal standard. Experimental samples are only

processed if the instrument passes a preset sensitivity and peak shape criteria. Finally, samples are run in a randomized order, except for quality control samples, which are analyzed every nine samples.

7.5 Data Analysis

An initial dataset is prepared using a targeted approach to identify known metabolites. Chromatograms are analyzed using the MassLynx utility QuanLynx, and metabolites are identified based on known retention times and mass fragmentation patterns.The peak area for each metabolite is recorded, and the data is exported to Excel. For our analyses, metabolite identity in QuanLynx isexperimentally established using pure, purchased standards, and in limited cases by the commercially available NIST library (National Institute of Standards and Technolgy; version 11). MarkerLynx is used for peak identification during a second, non-targeted analysis of the chromatograms, and the formatted data is transferred to SIMCA-P+ for principle component analysis (PCA) and partial least squares-discriminate (PLS-DA) analysis. If the PCA analysisidentifies significant separation between the experimental groups, PLS-DA analysis is employed to detectsignificantly altered metabolites. The peaks of unknown metabolites are quantified and the resulting data is exported to Excel.Statistical analysis can be performed with any number of software packages that are available for uni- and multivariate analysis, including JMP and Statistica.

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