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In vivo stable isotope labeling by amino acids in D. melanogaster

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Running head: SILAC fly

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Summary

The fruit fly *Drosophila melanogaster* is one of the most widely used and well studied model organism in biology and therefore a promising tool for quantitative proteomics. Here, we describe a method to label *D. melanogaster* with stable isotope labeled amino acids *in vivo*. Feeding flies with heavy lysine labeled yeast cells leads to virtually complete heavy labeling already in the first filial generation. The approach is simple, fast, and cost-effective, which makes SILAC flies an attractive model system for the emerging field of *in vivo* quantitative proteomics.

1. Introduction

Studies in *D. melanogaster* have so far mainly focused on genetic aspects. However, recent advances in mass spectrometry have led to an increased interest in quantitative proteomic analysis of *D. melanogaster* (1). SILAC in the fly system can be performed with cell lines grown in culture (2, 3). While highly useful, these cell culture models cannot appropriately reflect all relevant regulatory mechanisms of multi-cellular eukaryotes *in vivo*. As an alternative, we therefore labeled entire flies by feeding larvae on a diet of SILAC labeled yeast (4). SILAC flies generated in this way enable *in vivo* proteome-wide quantification with higher precision than label free methods. Our data shows that feeding flies with heavy lysine labeled yeast leads to almost complete labeling in the first filial generation and can be used to distinguish sex-specific proteins *in vivo* (Fig. 1). To avoid potential arginine to proline conversion, labeling with heavy lysine was chosen. Metabolic labeling by heavy lysine of *D. melanogaster* is simple, fast and cost-effective. Together with other animals in the SILAC zoo (5-8) the fly is an attractive model system to gain new insights into biological processes *in vivo*. 
2. Materials

2.1. D. melanogaster standard culture

1. D. melanogaster standard medium: 8.5 g/l agar, 76.6 g/l cornmeal, 81.6 g/l malt extract, 40.8 g/l molasses, 18.0 g/l brewer’s yeast, 10.0 g/l soy flour, 0.45% (v/v) propionic acid, 0.16% (v/v) methyl 4-hydroxybenzoate (nipagin) in 95% EtOH (see Notes 1 and 2).

2. Overhead stirrer

3. Peristaltic dispenser

4. D. melanogaster culture tubes: polystyrene flat bottom tubes (68 ml), ceaprene foam plugs

2.2. S. cerevisiae SILAC labeling

1. Yeast drop out minimal medium for SILAC labeling (9): 1.7 g/l yeast nitrogen base (without amino acids, without ammonium sulfate), 20 g/l D-glucose, 5 g/l ammonium sulfate, 200 mg/l adenine hemisulfate, 20 mg/l uracil, 100 mg/l Tyr, 10 mg/l His, 60 mg/l Leu, 10 mg/l Met, 60 mg/l Phe, 40 mg/l Trp, 100 mg/l Arg, 30 mg/l Lys\textsuperscript{12C\textsubscript{6}14N\textsubscript{2}} (Lys-0), or 30 mg/l Lys\textsuperscript{13C\textsubscript{6}15N\textsubscript{2}} (Lys-8) (see Note 3)

2. Lysine auxotrophic yeast strain (10): SUB62/DF5 (MATalpha lys2-801 leu2-3/112 ura3-52 his3-delta200 trp1-1) (see Note 4)

3. Orbital shaker

4. Spectrophotometer

2.3. Embryo collection

1. Apple juice agar solution: 22.5 g/l agar, 25% (v/v) apple juice, 25 g/l sucrose, 0.16% (v/v) nipagin in 95% EtOH (see Note 5)
2. Petri dishes (100 mm x 15 mm)

3. Yeast from *Saccharomyces cerevisiae* Type 2 (Sigma-Aldrich, St. Louis, MO, USA)

4. Embryo collection container (Fig. 2 A)

5. Embryo wash unit (Fig. 2 B)

6. CO$_2$ blowgun and CO$_2$ flypad (Genesee Scientific, San Diego, CA, USA)

7. Paint brush

8. Plastic wrap

### 2.4. *D. melanogaster* labeling

1. *Drosophila* labeling medium: 60% (w/v) labeled SUB62 yeast (wet mass), 320 mM sucrose, 0.3 mM ampicillin, 0.1% (v/v) nipagin in 95% EtOH, 0.5‰ propionic acid and 2.5 ‰ phosphoric acid

2. Labeling container (Fig. 3)

3. Petri dish

4. medical grade cotton wool

5. disinfected tissue paper

6. toothpick

7. plastic cup

8. Paint brush

9. Plastic wrap
2.5. **Protein extraction**

1. Modified radioimmunoprecipitation assay (RIPA) buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS and 1x protease inhibitor cocktail complete

2. Microcentrifuge tubes

3. Tissue homogenizer (Ultra-Turrax, Staufen, Germany)

4. Ultrasonic bath

5. Coomassie Plus (Bradford) Protein Assay

3. **Methods**

3.1. **D. melanogaster standard culture**

1. To prepare 1 l of culture medium, boil agar in 800 ml water until agar is dissolved, then add cornmeal by constantly stirring.

2. Dissolve malt extract in 100 ml water.

3. Warm up molasses in 100 ml water and mix with dissolved malt extract, soy flour and yeast. Add this mixture to the heated agar-cornmeal solution and return to a boil.

4. Cool down to 60°C and then add nipagin and propionic acid. Stir for additional 15 min.

5. Dispense 25 ml culture medium per culture tube by using a peristaltic dispenser. Store tubes at 4°C until use (see Note 6).

6. Keep flies in standard culture tubes at 18°C, ~60% relative humidity (see Note 7) and in a 12 h light--dark cycle. Passage flies every 8 weeks.
### 3.2. *S. cerevisiae* SILAC labeling

1. Transfer a small clone of the unlabeled lysine auxotrophic SUB62 strain to a 100 ml Erlenmeyer flask containing 5 ml of yeast labeling medium.
2. Incubate at 30°C overnight with orbital shaking at 250 rpm.
3. Transfer 10 µl of the liquid culture to another Erlenmeyer flask containing 5 ml of yeast labeling medium. Culture the pre-culture as described in step 2.
4. Transfer 125 µl of the second pre-culture into 125 ml of yeast labeling medium in a 1-liter baffled Erlenmeyer flask.
5. Incubate at 30°C for ~24 h with orbital shaking at 250 rpm. Dilute 100 µl of the liquid culture 1:10 in water. Measure the optical density (OD) of the diluted culture at 600 nm with a spectrophotometer.
6. When the OD600 of the diluted liquid culture reaches 0.7–0.8, harvest the SILAC yeasts by centrifuging at 1900 x g for 10 min at RT.
7. Remove the supernatant and store the SILAC yeast pellet at -20°C (see Note 8).

### 3.3. Embryo collection

1. To prepare 1 l of apple juice agar solution, dissolve 22.5 g agar in 750 ml water.
2. Autoclave the agar solution.
3. When the autoclaved agar solution has cooled down to ~60°C, keep it in a water bath at 60°C.
4. Pre-warm 250 ml of apple juice to 60°C and add sucrose and nipagin.
5. Thoroughly mix the apple juice into the autoclaved agar solution.
6. Dispense the apple juice agar solution into the petri dishes (10 ml/dish).
7. Once the apple juice agar plates have cooled and hardened, store them at 4°C until use.

8. Add a thick paste (~2 g) of non-labeled yeast in the center of the apple juice agar plate. The yeast serves as a food source during egg deposition (see Note 9).

9. To anesthetize the flies, pierce the blowgun into the culture tube and carefully blow CO₂ into the tube. Wait until the flies do not move anymore. Transfer the flies to a CO₂ flypad and sort them according to their sex. Transfer 40--50 male and female flies to an embryo collection container. Seal container with plastic wrap and incubate at 25°C overnight (see Notes 10 and 11).

10. Pierce the blowgun into the embryo collection container and anesthetize the flies with CO₂. To collect the embryos, remove the apple juice agar plate and rinse embryos extensively with water. By using a paint brush, filter the embryos through the embryo wash unit to get rid of the yeast (see Note 12).

11. After working with embryos, freeze all devices over night at -20°C to avoid uncontrolled hatching of *D. melanogaster*.

3.4. *D. melanogaster* labeling

1. Place a thin layer of medical grade cotton wool onto a 100 mm × 15 mm petri dish.

2. Dispense 10 ml of the *Drosophila* labeling medium evenly onto the cotton wool. Cover the cotton with a thin piece of disinfected tissue paper. Make small holes through the paper using a toothpick. This allows the larvae to have easy access to the labeling medium (see Notes 13 and 14)

3. Transfer the embryos onto the tissue paper. Couple the dish to a plastic cup using plastic wrap. Incubate at 25°C until hatching of adult flies (see Note 15).
4. If depleted, additional *Drosophila* labeling medium should be added during larval stage.

5. Transfer hatched flies to a new embryo collection container attached to an apple juice agar plate. Provide ~6 g/day SILAC labeled yeast (wet mass) until the flies reach the desired age for collection.

6. At this stage, hatched adult flies are almost completely labeled. However, they can be kept in an embryo collection container to collect fully labeled embryos.

7. For further processing, snap freeze flies or embryos in dry ice or liquid nitrogen and store at -80°C.

### 3.5. Protein extraction

1. Transfer whole flies or embryos to a 2-ml microcentrifuge tube containing ice-cold modified RIPA buffer (150 µl of modified RIPA buffer per ~10 flies).

2. Homogenize the whole flies vigorously for ~20 s on ice using a tissue homogenizer.

3. Put the fly homogenate for 3 min in an ultrasonic bath filled with ice-cold water.

4. Centrifuge at 16,000 × g for 10 min at 4°C. Transfer the supernatant to a new microcentrifuge tube.

5. Determine the protein concentration by using the Bradford protein assay.

6. Store protein extracts at -80°C until use.

7. Check labeling efficiency by mass spectrometry.
4. Notes

1. To prepare a 16% (w/v) nipagin solution, dissolve 16 g methylparaben in 100 ml EtOH (95%). To get a final concentration of 0.16%, dilute 10 ml nipagin solution (16%) in 990 ml D. melanogaster food.

2. Other Drosophila standard medium recipes can be found on the website of the Bloomington Drosophila Stock Center at Indiana University (http://flystocks.bio.indiana.edu).

3. Met (10 g/l), Lys (146 g/l) and Arg (84 g/l) stock solutions are prepared in PBS, Tyr (50 g/l), His (10 g/l) and Trp (40 g/l) stock solutions are prepared in 0.5 M NaOH, Leu (30 g/l) and Phe (30 g/l) stock solutions are prepared in 0.5 M HCl.

4. The SUB62 yeast strain, also called DF5 (Dan Finley 5), is available at the American Type Culture Collection (ATCC), order number ATCC 200912. However, in principle any other lysine auxotrophic strain can be used.

5. Other juices such as grape juice may also be used.

6. To avoid desiccation, coat the culture tubes with moistened tissue papers and store the tubes in a sealed plastic bag at 4°C.

7. Excessive humidity increases the risk of fungal growth. On the other hand, too low humidity leads to desiccation of the culture medium.

8. Labeling efficiency of SILAC yeast should be almost 100%. Therefore, check labeling efficiency of SUB62 yeast strain by mass spectrometry before continuing with D. melanogaster labeling. Labeling efficiency can be checked by calculating the heavy to light ratio of the 100 most intense proteins [4], for example.

9. Mix the yeast with a small amount of water until it forms a sticky paste.
10. Pay attention that the CO₂ blow is not too strong, otherwise the flies will stick to the culture medium.

11. Female imagines are bigger than males. Furthermore, male flies have a rounded abdomen and strongly pigmented last abdominal tergites.

12. A larger amount of embryos can be collected by using a filter unit connected to a vacuum pump (11).

13. According to our experience, 10 ml of Drosophila labeling medium is enough to breed ~150 flies.

14. The tissue paper prevents embryos from drowning in the Drosophila labeling medium.

15. It takes ~10 days at 25°C until the flies reach their adult stage.
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References


Figure Legends

**Figure 1.** Workflow of stable isotope labeling with amino acids in *D. melanogaster*. Embryos from a mixed male and female *D. melanogaster* population are collected. Larvae are fed with “light” $^{12}$C$_6$$^{14}$N$_2$ L-lysine (Lys-0) or “heavy” $^{13}$C$_6$$^{15}$N$_2$ L-lysine (Lys-8) labeled *S. cerevisiae*. Adult F1 subpopulations are mixed and analyzed by LC-MS/MS. Pairs of identical peptides with different stable-isotope compositions can be distinguished by the mass spectrometer based on their mass difference of 8 Da.

**Figure 2.** (A) Setup of the embryo collection container. A petri dish containing 10 ml apple juice agar and a layer of non-labeled yeast is capped with a plastic cup. For optimal aeration, the bottom of the cup is replaced with a fine mesh. The cup is attached to the petri dish via plastic wrap. (B) Setup of the embryo wash unit. To collect embryos from the apple juice agar plate, embryos are filtered through a 150 µm metal mesh placed between a glass cylinder and a plastic holder.

**Figure 3.** Setup of the labeling container. Cotton wool soaked with labeling medium is placed in a petri dish and covered with a perforated tissue paper. Embryos are dispersed with a paint brush on the tissue paper. The petri dish is capped with a plastic cup with its bottom replaced with a fine mesh. The labeling container is sealed with plastic wrap.
Figure 1

S.cerevisiae

♀ ♂

X

♀ ♂

Lys-0 "light"

S.cerevisiae

Lys-8 "heavy"

S.cerevisiae

♀ ♂

F₁
Figure 2

A

- mesh
- plastic cup
- non-labeled yeast
- apple juice - agar plate

B

- glass cylinder
- mesh
- holder
Figure 3

- Mesh
- Plastic cup
- Perforated tissue paper
- Cotton wool soaked with labeling medium
- Petri dish