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Stable Isotope Labeling with Amino Acids in *Drosophila* for Quantifying Proteins and Modifications

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SUMMARY

Drosophila melanogaster is a common animal model for genetics studies, and quantitative proteomics studies of the fly are emerging. Here we present in detail the development of a procedure to incorporate stable isotope labeled amino acids into the fly proteome. In the method of Stable Isotope Labeling with Amino acids in *Drosophila melanogaster* (SILAC fly), flies were fed with SILAC labeled yeast grown with modified media, enabling near complete labeling in a single generation. Biological variation in proteome among individual flies was evaluated in a series of null experiments. We further applied the SILAC fly method to profile proteins from a model of fragile X syndrome, the most common cause of inherited mental retardation in human. The analysis identified a number of altered proteins in the disease model, including actin-binding protein profilin and microtubulin-associated protein futsch. The change of both proteins was validated by immunoblotting analysis. Moreover, we extended the SILAC fly strategy to study the dynamics of protein ubiquitination during the fly life span (from day 1 to day 30), by measuring the level of ubiquitin along with two major polyubiquitin chains (K48 and K63 linkages). The results show that the abundance of protein ubiquitination and the two major linkages do not change significantly within the measured age range. Together, the data demonstrate the application of the SILAC principle in *Drosophila melanogaster*, facilitating the integration of powerful fly genomics with emerging proteomics.

Keywords

SILAC; *Drosophila melanogaster*; proteomics; mass spectrometry; fragile X syndrome; ubiquitin

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INTRODUCTION

The fruit fly *Drosophila melanogaster* is a small insect with a short life cycle. Since Morgan's use of *D. melanogaster* for genetics studies in 1910, this species has become one of the most widely used model organisms for the studies of genetics, physiology, development and evolution¹. The fly model has multiple alluring characteristics. Not only can this organism be reared with basic and cheap foods, it rapidly reproduces and matures, and can be easily used to generate large populations for global studies. Morphological diversity during its life cycle is beneficial for genetic research¹. As primary biological pathways between this invertebrate and human beings are usually conserved², the fly continuously serves as an effective model for dissecting the function of human disease genes by genetics and genomics approaches.

While genomic studies in the fly provide important clues on how specific gene alternation affects physiological function or disease development, the underlying molecular mechanisms are often explored by analyzing gene expression, protein localization and post-translational modifications, and protein-protein interaction by the approaches of cell biology, biochemistry and proteomics. Current proteomics technologies are increasingly used to analyze protein compositions in cells, tissues or whole organisms^{3,4}. More recently, quantitative proteomics analyses in the fly have been reported⁵⁻¹⁸. For example, Brunner *et al.* used the isotope-coded affinity tag (ICAT) technique to analyze *Drosophila* proteome⁸, and Xun *et al.* investigated proteomic changes in several fly models of Parkinsonism^{11,16}. In addition to the *in vitro* labeling method used, metabolic labeling *in vivo* with stable isotope may provide a better strategy to minimize variation in sample processing¹⁹. Krijgsveld *et al.* introduced metabolic labeling strategies to the whole fly organism^{5,15}. By feeding flies with ¹⁵N-labeled yeast (*S. cerevisiae*), the fly proteome can be almost completely labeled in one generation. However, even with highly purified ¹⁵N-labeled reagents, complex peptide isotopologues are observed because of numerous partially labeled peaks lower than the monoisotopic peaks, confounding the accuracy of quantification and increasing the chance of peptide mismatching and overlapping, although some algorithms were developed to alleviate the problems¹³.

Stable isotope labeling with amino acids in cell culture (SILAC) overcomes the isotopologue broadening problem associated global ¹⁵N-labeling and gains popularity for quantitative proteomics¹⁹. Mann's group used the SILAC approach to label *Drosophila* SL2 cell line, discovering the correlation between mRNA and protein from significantly changed genes¹². Although SILAC was initially viewed as a technology only applicable to cultured cells, such as *E.coli*²⁰, yeast²¹, and cultured mammalian cells^{22,23}, recent achievement of SILAC mice²⁴ opens a door for its adaption to multicellular organisms. In addition, partial SILAC labeling was performed with success in chicken skeletal muscle²⁵, amphibian tails²⁶, and even human cerebrospinal fluid *in vivo*²⁷. Thus, we decided to develop a SILAC-based method for labeling *Drosophila melanogaster*. During the preparation of this manuscript, Sury *et al.* reported a similar labeling method for the fly and applied the method to identify sex-specific protein expression²⁸. The results from an independent group further demonstrate the feasibility of the SILAC fly approach. However, simply replacing standard fly food with stabled isotope labeled yeast led to low survival rate of larvae, significant retardation in growth and incomplete labeling. We performed a series of experiments to optimize the culture condition in order to obtain essentially complete labeled fly. Using these labeled flies as internal standards, we profiled proteins in a disease model of Fragile X syndrome²⁹ and analyzed protein ubiquitination during fly aging.

EXPERIMENTAL PROCEDURES

SILAC labeling of *S. cerevisiae*

Yeast strain JMP024 (*MATa lys2-810 leu2-3,-112 ura3-52 his3-Δ200 trp1-1[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2, arg4-Δ::Ura3, [pUB221] [pUB100]*) was used in this study³⁰. The *Lys2* gene was deleted in the strain to enable stable isotope labeling with lysine in cell culture. A fresh yeast colony was inoculated in YPD liquid medium, and grown over night as seeding culture. The cells were spun down, washed once with water, and reseeded into light or heavy SC media (0.7% Difco yeast nitrogen base, 2% dextrose, supplemented with adenine, uracil, and amino acids containing 164.3 μM of light lysine or heavy [¹³C₆¹⁵N₂] lysine (+8.0142 Da, Cambridge Isotope Laboratories, Andover, MA)³¹. The cells were then cultured in a shaker (250 rpm and 30°C) for about 8 generations to reach A₆₀₀ of 1.2, harvested and stored at -80°C.

SILAC labeling of *D. melanogaster*

Wild type Oregon R fly (*D. melanogaster* w¹¹¹⁸) and Fmr1 null allele *Fmr1*^{Δ113M/TM6B} were used in this study³². Flies were maintained with standard methods: 25°C in 50–70% humidity on modified lab food medium (10% cornmeal, 10% molasses, 2% baker's yeast, 1.5% agar, 1.5% tegosept [10% methyl-p-hydrobenzoate in 95% ethanol], 0.3% propionic acid)³³. During labeling, 100 female flies and 100 male flies were mixed in an inverted nursery flask with the bottom removed and covered with four layers of gauze. The inverted mouth of the nursery flask was covered with a 35 mm × 10 mm cell culture dish (Corning Inc., Corning, NY) containing 5 ml of 0.8% glucose-LMT agarose medium (10% glucose, 0.8% Bio-Rad low melt temperature agarose [Bio-Rad Hercules, CA], 0.002% methyl paraben dissolved in ethanol and 0.8% ethyl acetate). About 10 mg of labeled yeast cells were spread on the top of agarose medium to serve as fly food. The female and male flies were mated and laid eggs on the medium for 3 h. The eggs were transferred to a fresh fly vial containing 15 ml of the same 0.8% glucose-LMT-agarose medium and one piece of autoclaved 3 MM chromatography paper (5 cm long, folded in undulation shape). Each vial contained approximately 100 eggs and were kept in the incubator until the eggs hatched into larvae, developed into pupa and finally into flies.

Protein extraction from *S. cerevisiae* and *D. melanogaster*

Yeast cells were lysed in a 1.5 ml eppendorf tube with a lysis buffer (10 mM Tris, pH 8.0, 0.1 M NaH₂PO₄, 8 M urea, 0.02% SDS and 10 mM β-mercaptoethanol) with 0.5 mm glass beads (Biospec Products Inc., Bartlesville, OK)³⁰. The tube was vortexed at the highest speed for 30 sec with 1 min interval on ice for 20 times. The lysate was clarified by centrifugation at 14,000 rpm for 5 min. Proteins were extracted from fly tissues in a similar method with the exception that the fly tissues were first cut by scissors prior to vortexing.

Protein digestion and peptide purification

Total cell lysate (50–100 μg) was reduced with 10 mM DTT at 37°C for 30 min and alkylated with 50 mM iodoacetamide in the dark at room temperature for 30 min. Proteins in the samples were resolved on a 10% SDS gel, and stained with Coomassie blue G250. The entire gel lane was then excised into 13 bands followed by in-gel digestion with 10 ng/μl of Lys-C (Wako, Osaka, Japan) at 37°C overnight according to the manufacturer's protocol. The resulting peptides were dried and reconstituted in 5% acetonitrile (AcN) and 0.5% acetic acid, and desalted by a small plug of C₁₈ material (3M, St. Paul, MN) in a 200 μl pipette tip (Corning Inc., Corning, NY) as previously reported³⁴. The eluted peptides were dried again and dissolved with sample loading buffer (6% acetic acid, 0.005% heptafluorobutyric acid, 0.1% TFA, and 5% AcN) for MS analysis.

Protein analysis by LC–MS/MS

The digested peptides were analyzed by reverse phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, San Jose, CA). The instrument was equipped with an Agilent 1100 Series binary HPLC pump (Agilent Technologies, Palo Alto, CA), a Famos autosampler (LC Packings, San Francisco, CA), and a 100 μm i.d. $\times$ 10 cm fused-silica capillary column packed with C₁₈ reversed-phase 2.7 μm HALO resin (Michrom Bioresources, Auburn). The samples were loaded onto the column by the autosampler, and then eluted with a 2 h gradient with 6%–35% of buffer B (buffer A: 0.4% acetic acid, 0.005% HFBA, 5% acetonitrile; buffer B, the same as buffer A except 95% acetonitrile, flow rate: ~300 nl/min). Eluted peptides were ionized under high voltage (2.5 kV) and detected by the Orbitrap mass spectrometer in a survey scan (350–1500 m/z ; 1×10^6 AGC target; resolution 60,000 at m/z 400; maximum ion accumulation time, 1000 ms) followed by 10 data depending scans in the LTQ MS (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 150 ms maximum ion time, dynamic exclusion range of 30 sec).

MS data processing

The MS/MS spectra from the fly samples were searched with the Sequest-Sorcerer algorithm (version 2.0) on a Sorcerer 2 IDA (Sage-N-Research, Inc., Milpitas, CA)³⁵ against a composite target/decoy database to estimate false discovery rate^{36,37}. The target proteins were derived from a combination of *D. melanogaster* (<http://flybase.org>, release 5.13) and common contaminants, such as porcine trypsin and human keratins. The decoy proteins were derived from pseudo-reversed sequences of the target proteins³⁷. Searching parameters consisted of semitryptic restriction, fixed modification of Cys (+57.0215 Da, alkylation by iodoacetamide), and dynamic modifications of oxidized Met (+15.9949 Da) and Lys (+8.01420 Da), and maximal 5 modifications in a single peptide. Mass tolerance was set to 50 ppm for precursor ions and 0.5 Da for product ions. Only *b* and *y* ions were considered during the database match. Peptide matches were filtered by a minimal peptide length of 6 amino acids³⁷, then grouped by trypticity (only fully and partially tryptic peptides were accepted) and charge states³⁶. In each group, the peptide matches were further filtered by maximal modification sites of 4 (including SILAC-labeling), maximal miscleavage of 2, no partial labeling of Lys (e.g. assigned peptides containing both heavy and light isotopes), and by dynamically increasing XCorr and ΔCn cutoffs until all decoy matches were discarded. Only proteins with at least two peptide matches were accepted to further minimize false discoveries. The MS/MS data from yeast were searched with the same methods described above with the exception that the database used was that of *S. cerevisiae* (www.stanford.edu/saccharomyces). When matching peptides to proteins, we assigned the proteins sharing the same peptide(s) in one group, and used the proteins with highest peptides matches to represent the group. Protein quantification was performed with an in-house program³⁰, in which peptide ion peaks in survey scans were first defined to include m/z , retention time, peak intensity and signal-to-noise ratio. The heavy and light ion peaks were matched using predicted m/z (± 6 ppm) and the same retention time.

Quantification of ubiquitin and polyubiquitin linkages by antibody enrichment and LC-SRM

The analysis used the SILAC labeled animals as an internal standards based on a previously reported protocol with modifications³⁰. Labeled animals were mixed with wild type flies collected at different ages. The total proteins were extracted and digested by trypsin. The resulting K-GG peptides were enriched by a monoclonal K-GG antibody based on the manufacturer's protocol (Cell Signaling Technology, Inc)³⁸, and then analyzed by reverse phase liquid chromatography followed by LC-SRM on an LTQ-Orbitrap Velos (Thermo Electron). During the analysis, ion pairs of ubiquitin, K48 and K63 linkage-specific peptides

were selected for fragmentation and quantified by intensity ratio of coeluting, related product ion pairs, a process termed selected reaction monitoring (SRM).

Western blot analysis

Drosophila heads were homogenized in a buffer [150 mM NaCl, 20 mM Tris, pH 8.0, 20 mM EDTA, 0.1 mM MgCl₂, and 1× complete protease inhibitor (Roche Applied Science, Indianapolis, IN)] for western analysis. The used antibodies included anti-dFmr1 (Sigma, St. Louis, MO), anti-profilin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IO), anti-Futsch (Developmental Studies Hybridoma Bank at University of Iowa) and anti-β-actin (Abcam, Cambridge, MA) and anti-ubiquitin (Millipore, Billerica, MA).

Microscopy

Whole flies were stored at −20°C for 30 min before pictures were taken. Adult eyes were photographed under a Zeiss Stemi SV6 dissecting microscope. Wings were mounted in Euparal, and photographed with a Leica DFC500 digital camera mounted on a Leica dissecting microscope.

RESULTS

Development of the SILAC labeling procedure

The labeling procedure for the SILAC approach involves two stages (Fig. 1A). In the first stage, a yeast strain *S. cerevisiae* was cultured in the presence of heavy stable isotope labeled lysine. Complete labeling of the yeast proteome was achieved because the *lys2* gene was deleted in the strain³⁰. The labeling efficiency of the yeast was examined by MS and no residual light lysine containing peptides were detected (data not shown). In the second stage, the flies were labeled by feeding with the labeled yeast. As the flies are usually fed with the Lab Food (LF) medium consisting of 10% cornmeal, 10% molasses, 2% baker's yeast, 1.5% tegosept, 0.3% propionic acid and 1.5% agar, regular lysine may be present in most of these impure compounds. To eliminate the light version of regular lysine from the medium, we replaced 1.5% agar with the same concentration of pure agarose and used 10% glucose as the only carbon source. During testing experiments, the light lysine labeled yeast was used as the nitrogen source to mimic heavy labeled yeast to reduce experimental cost (Table 1). In this case, the flies grew slower and developed poorly (6 day pupa stage, 64±4% of hatchability rate, 8±2% pupation rate, and 25±7% eclosion rate) when compared to the flies grown with regular LF medium (5 day pupa stage, 86±2% hatchability rate, 97±3% pupation rate, and 94±3% eclosion rate). The results suggested that the modified agarose medium was not ideal for healthy fly development. This may be caused by the solidity of medium with 1.5% agarose because larvae prefer to drill and eat soft sugar-containing medium. To test this possibility, we substituted 1.5% regular agarose with 0.8% low melt temperature (LMT) agarose, and found dramatic improvement in growth (89±1% hatchability rate, 86±3% pupation rate, and 90±1% eclosion rate). However, under this condition, mature male flies weighed 611±17 μg and female flies weighed 767±28 μg, which was still 16% and 28% lower than their counterparts grown under regular condition, respectively. To further improve the growth condition, we performed a number of other trials (data not shown) and found that the flies appeared to grow well when a piece of filter paper was inserted into the vial to avoid water accumulation on the top of the LMT agarose medium. In addition, the paper may also provide extra space for larva movement (Supplemental Fig. S1). Under this optimized condition, the flies grew almost equally as well as those under regular medium (Table 1). Thus, we used this novel SILAC medium with the filter paper for all of the following experiments.

To evaluate any effect of isotopic labels or yeast sources on growth and development of the flies, we compared the flies derived from three different yeast sources: regular light yeast from rich YPD medium, light yeast from SC medium, and heavy yeast from SC medium. No significant difference was found with respect to hatchability, pupation, and eclosion and body weights (Table 1). Close examination under microscope did not reveal abnormalities in pupa shape, fly body size, eye configuration, wing structure from matured flies (Fig. 1). Since the fly eye and wing phenotypes are highly sensitive to perturbation, the results suggest that our developed SILAC medium and apparatus are suitable for proper fly growth and development.

Measurement of labeling efficiency of heavy stable isotope labeled *Drosophila*

As complete labeling of the fly proteome is a prerequisite for accurate quantitation of proteome, we examined labeling efficiency by collecting samples from four main developmental stages. The proteins were extracted and in-solution digested with Lys-C for mass spectrometric analysis. After quantifying over 100 proteins in the samples, we used the heavy over light ratio as an index for incorporation rate (Fig. 2). The results showed that the heavy lysine incorporated rapidly, and a majority of proteins from the L1 stage had lysine replacement. For instance, one peptide (DNEIAIIGFFK) from disulfide isomerase A in the L1 sample displayed about 20% of light version and 80% of heavy one (Fig. 2A). The light portion of this peptide decreased to less than 5% in the L2 stage, became barely detectable in the L3 stage, and was not found in the mature fly. We further analyzed global incorporation of heavy lysine into the fly proteome. By fitting all datasets to a Gaussian distribution, we were able to model a trend for isotope labeling. The heavy labeled portion occupied about 80%, 95%, 96%, 98% for L1, L2, L3 and adult fly respectively (Fig. 2B). The labeling efficiency in the adult fly could be even higher, because only 2% (4/217) identified proteins were matched by weakly detectable light peptides and were used in the calculation (Fig. 2C). Further labeling to F2 generation was not necessary (data not shown). The data strongly support that essentially complete labeling can be achieved within one generation of *D. melanogaster*, consistent with the recent SILAC fly report²⁸.

Evaluation of biological variation in individual flies in quantitative proteomics

Individual variation of animals is a confounding factor in biological studies. The fully labeled *D. melanogaster* provides a useful tool to analyze the individual difference with the same genetic background. As shown in Fig. 3A, light and heavy yeast cells were cultured and fed to the wild type flies, producing two populations of flies differing in lysine isotope. Theoretically, no protein difference should be anticipated among the two populations of flies. However, when analyzing the equal mix of a single light fly with a single heavy fly, the distribution of the log2 ratios of quantified proteins displayed a broad curve (Fig. 3B). Fitting to a Gaussian distribution led to a standard deviation (SD) of 0.54, which is much larger than the SD (~0.3) in our previous SILAC studies in yeast and human cells³⁰, indicating a large variation in the individual flies. To reduce this variation, we mixed three light flies with the same number of heavy ones, and the SD was reduced to 0.46. Further analysis of mixing 10 light and 10 heavy flies had only minor effect, and the SD was slightly lowered to 0.44 (Fig. 3B). This phenomenon was more obvious when we plotted the fly number against their corresponding SD values (Fig. 3C). These results suggested that the pooling strategy of samples for quantitative proteomics could reduce individual variation.

Application of the SILAC flies to protein profiling in a disease model

To test the feasibility of the SILAC method, we designed a strategy to profile proteins in a disease model of Fragile X syndrome²⁹ (Fig. 4A). Fragile X syndrome is the most common inherited mental retardation disorder, majorly caused by loss-of-function of *fragile X mental retardation 1* (Fmr1) gene. Fmr1 protein regulates the translation of numerous mRNA

species during synapse formation and plasticity. The patients have lower expression level of Fmr1 than healthy individuals. *Drosophila* Fmr1 is the ortholog of human Fmr1 and its deletion in the fly partially recapitulates phenotypes of Fragile X syndrome. Therefore the fly Fmr1 mutant is commonly used as a model for studying Fmr1 function³⁹.

In this analysis (Fig. 4A), we used a heterozygous strain with deletion of one copy of Fmr1 gene, which resulted in a partial loss of Fmr1 function. Heavy stable isotope labeled wild type fly heads were used as an internal standard to compare the proteome of wild type and the Fmr1^{Δ113M/+} mutant strain, both of which were cultured with regular lab food medium. Protein extracted from the fly heads were resolved by a SDS gel, separated into 13 gel bands, digested with Lys-C, and analyzed by LC-MS/MS. After stringently filtering to reduce false discovery rate to nearly zero by discarding all decoy matches, we further increased the threshold by accepting proteins identified by at least two peptides. Proteins with shared peptides were classified into one group that was represented by one top protein with the highest number of identified peptides. In the comparison of wild type and the internal standard, we identified and quantified 1,903 proteins with at least two matched peptides (Fig. 4B, supplemental Table S1, S2).

The distribution of quantified log2 ratios was symmetrical and could be fitted to a Gaussian curve with a SD of 0.61. Similarly, 1,617 proteins were analyzed in the comparison of the Fmr1^{Δ113M/+} mutant and the internal standard (supplemental Table S3, S4). The distribution of quantified data showed a SD of 0.74 (Fig. 4C), indicating that more proteins were altered in the mutant. By crossing out the wild-type data from the Fmr1^{Δ113M/+}, we obtained protein ratios of Fmr1^{Δ113M/+} over wild type (supplemental Table S5). It is clear that more proteins fall outside the Gaussian distribution curve (SD = 0.39) (Fig. 4D), representing differentially expressed proteins in the mutant compared to wild-type.

We further confirmed the SILAC data by an independent western blotting method. First, we analyzed the Fmr1 expression level in the mutant. As expected, we found that residual amount of Fmr1 in the heterozygous mutant is approximately 50% of that in wild type (Fig. 4E, 4F). Manual examination of a peptide ion (SGVFRIK) of Fmr1 also identified about two-fold difference (Fig. 4G). These consistent results strongly support high accuracy of the SILAC approach. Additionally, we validated two other changed proteins by western blotting (Fig. 5). Profilin is up regulated in an Fmr1 deletion strain⁴⁰ and also increased ~3 fold in the heterozygous strain (Fig. 5A, 5B). Futsch is the fly homolog of the microtubule binding protein MAP1B and is upregulated the Fmr1^{Δ113M} mutant line³². Similar results were revealed in the heterozygous mutant (Fig. 5C, 5D).

Application of the SILAC flies to protein modification

In addition to the large-scale discovery proteomic analysis, SILAC fly can be used as internal standards to perform targeted proteomic quantification. As an example, we quantified protein ubiquitination during the fly aging process. Ubiquitin (Ub) is a versatile signaling molecule conjugated to amine groups of substrates to form monoUb or polyUb chains (M1, K6, K11, K27, K29, K33, K48 and K63)^{30,41,42}. Conventional K48-linked polyUb chains mediate substrate degradation by the 26S proteasome, whereas K63-linked chains function in a variety of proteasome-independent processes⁴². Although Ub signaling has been long proposed to contribute to neurodegeneration and aging⁴³, the regulation of protein ubiquitination during these processes remains unclear. In this analysis, we measured the level of ubiquitin and two main polyUb chains (K48 and K63) in fly heads of different ages (1, 3, 10, and 30 days old, Fig. 6A), using the established liquid chromatography coupled with selective reaction monitoring (LC-SRM) method^{44,45}. To improve accuracy of the measurement, we enriched the chain specific peptides from total peptide mixtures by a newly developed antibody³⁸ and used housekeeping proteins (e.g. histone 2B) to correct

systematic errors during sample mixing (Fig. 6B). No significant changes in total Ub, K48 or K63 linkages were detected (Fig. 6C). The total Ub levels in these samples were further measured by western blotting (Fig. 6D), showing consistent results. In a separate aging study⁴⁶, the assembly of 26S proteasome was investigated and no alternation was observed in the flies from day 1 to day 32. Together, these data support that the ubiquitin proteasome system is not significantly altered during the measured life span (day 1–30).

DISCUSSION

Drosophila melanogaster is a common model organism with a known complete genome sequence⁴⁷ and other invaluable resources, such as Flybase and a full collection of deletion strains. We developed a simple and robust procedure for stable isotope labeling of amino acid in *D. melanogaster* (SILAC). The SILAC flies can be used to accurately identify and quantify proteins and protein posttranslational modifications. The SILAC medium contains glucose as the only carbon source, labeled yeast as a nitrogen source, and low melting temperature agarose as a solidifying agent. In order to improve growth environment, we supplemented folded filter paper to increase larva mobility in and out of the SILAC culture media. The medium components and apparatus are easily accessible and attainable for any fly lab. Unlike the SILAC fly labeling recently reported by another group²⁸, we used a different setting for fly culture, described the optimization process in detail, analyzed proteome variation among individual flies, and applied such a method to address different biological questions.

Labeling efficiency and effect of labeling reagents are major factors to evaluate metabolic labeling methods in quantitative proteomics. In our procedure, we found that heavy labeled lysine in yeast proteome could be incorporated into fly proteome efficiently in a single generation. The whole process takes only about 10 days starting from egg to labeled mature fly. The fast incorporation and high labeling efficiency may be related to high turnover rate of cells and proteins during fly development and remodeling. As some genes function only in specific developmental stages, the procedure could also be used to produce fully labeled proteome at multiple developmental stages. In addition, the heavy isotope appears to have no effect on fly development, consistent with the similar conclusion obtained from SILAC-labeled mice²⁴.

The analysis of biological samples is influenced by biological variation among individuals. This issue is attracting more attention in genetics studies because, even under the same background, variation still exists, due to environmental or epigenetic influences. The phenomenon remains to be explored in the proteomics field. We used two chemically identical SILAC labeled populations of flies, distinguishable only by stable isotope, to search for efficient sampling strategies in quantitative proteomics. We found that pooling three or more flies could significantly reduce the individual variation. The strategy is beneficial for other quantitative analysis, although the pooling size of other model organisms may need to be adjusted depending on genetic variation.

The sensitivity and true discovery rate are heavily dependent on measurement accuracy in large scale proteomics studies. We applied this SILAC method to evaluate the sensitivity and accuracy of quantitative MS measurement using a partial loss-of-function model of Fragile X syndrome. The heterozygous fly strain lacks one copy of the Fmr1 gene in the genome, which served as a restricted model for sensitivity assay. MS and western blot analysis confirmed that Fmr1 protein in the heterozygous was ~50% of that in wild type. We validated the dosage effect of Fmr1 on the upregulation of a series of proteins, such as Futsch and profilin^{40,48}. We also provided a list of changed proteins that may be related to Fmr1 signal pathway (supplemental Table S5). Furthermore, we successfully used the

SILAC flies for monitoring protein ubiquitination during aging of adult fly. Recently, a ubiquitin tagging strategy was developed to isolate neuronal-specific ubiquitinated proteins from the fly⁴⁹. It would be highly possible to combine the SILAC and the Ub tagging strategy for mapping protein ubiquitination in vivo. Based on these results, the SILAC strategy presented here provides a highly sensitive and accurate global quantification method.

In summary, the procedure and the data described here revealed that the SILAC procedure is a simple and effective strategy for quantitative proteomics of *Drosophila melanogaster*. We expect that this method could be highly instructive and broadly applicable for other proteomics analysis in *Drosophila melanogaster*. The additional pooling strategy provides a useful solution to reduce individual variation. The development of the SILAC should accelerate the exploration of novel gene function in the fly and in human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

SILAC	stable isotope labeling with amino acids in cell culture
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LMT	low melt temperature

REFERENCES

1. Beller M, Oliver B. Chromosome Res. 2006; 14:349–362. [PubMed: 16821132]
2. Chien S, Reiter LT, Bier E, Gribskov M. Nucleic Acids Res. 2002; 30:149–151. [PubMed: 11752278]
3. Aebersold R, Mann M. Nature. 2003; 422:198–207. [PubMed: 12634793]
4. Cravatt BF, Simon GM, Yates JR 3rd. Nature. 2007; 450:991–1000. [PubMed: 18075578]
5. Krijgsvelde J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH, Heck AJ. Nat. Biotechnol. 2003; 21:927–931. [PubMed: 12858183]
6. Alonso J, Santaren JF. Proteomics. 2005; 5:474–489. [PubMed: 15751120]
7. Bodenmiller B, Mueller LN, Pedrioli PG, Pflieger D, Junger MA, Eng JK, Aebersold R, Tao WA. Mol. Biosyst. 2007; 3:275–286. [PubMed: 17372656]
8. Brunner E, Ahrens CH, Mohanty S, Baetschmann H, Loevenich S, Potthast F, Deutsch EW, Panse C, de Lichtenberg U, Rinner O, Lee H, Pedrioli PG, Malmstrom J, Koehler K, Schimpf S, Krijgsvelde J, Kregenow F, Heck AJ, Hafen E, Schlapbach R, Aebersold R. Nat. Biotechnol. 2007; 25:576–583. [PubMed: 17450130]
9. Pflieger D, Junger MA, Muller M, Rinner O, Lee H, Gehrig PM, Gstaiger M, Aebersold R. Mol. Cell. Proteomics. 2008; 7:326–346. [PubMed: 17956857]
10. Pinkse MW, Mohammed S, Gouw JW, van Breukelen B, Vos HR, Heck AJ. J. Proteome Res. 2008; 7:687–697. [PubMed: 18034456]

11. Xun Z, Sowell RA, Kaufman TC, Clemmer DE. *Mol. Cell. Proteomics*. 2008; 7:1191–1203. [PubMed: 18353766]
12. Bonaldi T, Straub T, Cox J, Kumar C, Becker PB, Mann M. *Mol. Cell*. 2008; 31:762–772. [PubMed: 18775334]
13. Gouw JW, Tops BB, Mortensen P, Heck AJ, Krijgsveld J. *Anal. Chem*. 2008; 80:7796–7803. [PubMed: 18808151]
14. Loevenich SN, Brunner E, King NL, Deutsch EW, Stein SE, Aebersold R, Hafen E. *BMC bioinformatics*. 2009; 10:59. [PubMed: 19210778]
15. Gouw JW, Pinkse MW, Vos HR, Moshkin Y, Verrijzer CP, Heck AJ, Krijgsveld J. *Mol. Cell. Proteomics*. 2009; 8:1566–1578. [PubMed: 19321433]
16. Xun Z, Kaufman TC, Clemmer DE. *J. Proteome Res*. 2009; 8:4500–4510. [PubMed: 19705877]
17. Pedersen KS, Codrea MC, Vermeulen CJ, Loeschcke V, Bendixen E. *Heredity*. 2010; 104:125–134. [PubMed: 19812620]
18. Gouw JW, Krijgsveld J, Heck AJ. *Mol. Cell. Proteomics*. 2010; 9:11–24. [PubMed: 19955089]
19. Ong SE, Mann M. *Nat. Chem. Biol*. 2005; 1:252–262. [PubMed: 16408053]
20. Neher SB, Villen J, Oakes EC, Bakalarski CE, Sauer RT, Gygi SP, Baker TA. *Mol. Cell*. 2006; 22:193–204. [PubMed: 16630889]
21. Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON. *Mol. Cell. Proteomics*. 2005; 4:310–327. [PubMed: 15665377]
22. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol. Cell. Proteomics*. 2002; 1:376–386. [PubMed: 12118079]
23. Ong SE, Mann M. *Nat. Protoc*. 2006; 1:2650–2660. [PubMed: 17406521]
24. Kruger M, Moser M, Ussar S, Thievensen I, Luber CA, Forner F, Schmidt S, Zanivan S, Fassler R, Mann M. *Cell*. 2008; 134:353–364. [PubMed: 18662549]
25. Hayter JR, Doherty MK, Whitehead C, McCormack H, Gaskell SJ, Beynon RJ. *Mol. Cell. Proteomics*. 2005; 4:1370–1381. [PubMed: 15965267]
26. Looso M, Borchardt T, Kruger M, Braun T. *Mol. Cell. Proteomics*. 2010; 9:1157–1166. [PubMed: 20139370]
27. Bateman RJ, Munsell LY, Morris JC, Swarm R, Yarasheski KE, Holtzman DM. *Nat. Med*. 2006; 12:856–861. [PubMed: 16799555]
28. Sury MD, Chen JX, Selbach M. *Mol. Cell. Proteomics*. 2010; 9:2173–2183. [PubMed: 20525996]
29. Penagarikano O, Mulle JG, Warren ST. *Annu. Rev. Genomics Hum. Genet*. 2007; 8:109–129. [PubMed: 17477822]
30. Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, Rush J, Hochstrasser M, Finley D, Peng J. *Cell*. 2009; 137:133–145. [PubMed: 19345192]
31. Guerrero C, Tagwerker C, Kaiser P, Huang L. *Mol. Cell. Proteomics*. 2006; 5:366–378. [PubMed: 16284124]
32. Zhang YQ, Bailey AM, Matthies HJ, Renden RB, Smith MA, Speese SD, Rubin GM, Broadie K. *Cell*. 2001; 107:591–603. [PubMed: 11733059]
33. Chang S, Bray SM, Li Z, Zarnescu DC, He C, Jin P, Warren ST. *Nat. Chem. Biol*. 2008; 4:256–263. [PubMed: 18327252]
34. Rappsilber J, Mann M, Ishihama Y. *Nat. Protoc*. 2007; 2:1896–1906. [PubMed: 17703201]
35. Eng J, McCormack AL, Yates JR 3rd. *J. Am. Soc. Mass Spectrom*. 1994; 5:976–989.
36. Peng J, Elias JE, Thoreen CC, Licklider LJ, Gygi SP. *J. Proteome Res*. 2003; 2:43–50. [PubMed: 12643542]
37. Elias JE, Gygi SP. *Nat. Methods*. 2007; 4:207–214. [PubMed: 17327847]
38. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP. *Mol. Cell*. 2011; 44:325–340. [PubMed: 21906983]
39. Zarnescu DC, Shan G, Warren ST, Jin P. *Genes Brain Behav*. 2005; 4:385–392. [PubMed: 16098136]
40. Reeve SP, Bassetto L, Genova GK, Kleyner Y, Leyssen M, Jackson FR, Hassan BA. *Curr. Biol*. 2005; 15:1156–1163. [PubMed: 15964283]

41. Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP. Nat. Biotechnol. 2003; 21:921–926. [PubMed: 12872131]
42. Pickart CM, Fushman D. Curr. Opin. Chem. Biol. 2004; 8:610–616. [PubMed: 15556404]
43. Ciechanover A, Brundin P. Neuron. 2003; 40:427–446. [PubMed: 14556719]
44. Xu P, Cheng D, Duong DM, Rush J, Roelofs J, Finley D, Peng J, Israel J. Chem. 2006; 46:171–182.
45. Kirkpatrick DS, Hathaway NA, Hanna J, Elsasser S, Rush J, Finley D, King RW, Gygi SP. Nat. Cell Biol. 2006; 8:700–710. [PubMed: 16799550]
46. Vernace VA, Arnaud L, Schmidt-Glenewinkel T, Figueiredo-Pereira ME. FASEB J. 2007; 21:2672–2682. [PubMed: 17413001]
47. Kornberg TB, Krasnow MA. Science. 2000; 287:2218–2220. [PubMed: 10731136]
48. Bettencourt da Cruz A, Schwarzel M, Schulze S, Niyyati M, Heisenberg M, Kretschmar D. Mol. Biol. Cell. 2005; 16:2433–2442. [PubMed: 15772149]
49. Franco M, Seyfried NT, Brand AH, Peng J, Mayor U. Mol. Cell. Proteomics. 2011; 10 M110 002188.

Figure 1.

Procedure of stable isotope labeling of amino acid in *Drosophila melanogaster* (SILAC).

(A) SILAC flow chart to obtain F1 and F2 flies fed with SILAC labeled yeast.

(B) Morphological comparison of pupa fed with three different yeast cells. The cells were cultured in yeast YPD media, light lysine or heavy lysine containing yeast SC media.

(C–E) No significant difference in morphology was found in flies fed with the three different yeast cells. The morphologies of the entire fly body, and the structure of eyes and wings were evaluated.

A **B** **C**

Protein labeling efficiency by heavy isotope in the fly

Developmental stage	Number of proteins identified	#protein with paired light/heavy peptides	Average log ₂ (Heavy/Light)	Labeling efficiency (%)
Embryo	205	0	NA	0
Larva 1	106	49	2.0	79.5±2.7
Larva 2	244	105	4.2	94.8±0.7
Larva 3	168	19	4.7	96.3±2.2
Adult fly	217	4	5.4	97.7

Figure 2.

Analysis of labeling efficiency of stable isotope in the SILAC process.

(A) Labeling of a representative peptide in various developmental stages of the fly. Fly proteins were extracted from whole animal body, digested and analyzed by LC-MS/MS to examine the level of labeling.

(B) Histograms of quantified proteins as to the labeling efficiency that is indicated by log transformed ratio of heavy versus light proteins.

(C) Detailed information on the results of LC-MS/MS runs. The labeling efficiency was shown as the mean and standard error of the mean, which were calculated from protein data based on matched light and heavy peptides.

Figure 3.
 Effect of pooling sample number in SILAC evaluated by null experiments.
 (A) Flow chart for a null experiment. Although the light and heavy flies were mixed together at 1:1 ratio, different pool size was used (e.g. 1, 3, or 10 flies from each pool).
 (B) Histograms of log₂ ratios of quantified proteins in three null experiments, in which varied fly numbers were pooled.
 (C) The increase of pooling sample number decreased the variation reflected by standard deviation.

Figure 4.

Protein profiling of wild type (WT) and *Drosophila* Fmr1 heterozygous flies by the SILAC approach.

(A) Flow chart for identifying protein changes between WT and *Drosophila* Fmr1^{Δ113M/+} heterozygous flies (1 day old). The heavy isotope labeled wild type fly heads were used as an internal standard (IS) to equally mix with either WT or Fmr1^{Δ113M/+} mutant fly heads ($n = 10$ for each), followed by protein extraction, SDS gel analysis and LC-MS/MS.

(B–C) Histograms of log₂ ratios of quantified proteins in two experimental comparisons, WT versus the internal standard, and Fmr1^{Δ113M/+} versus the internal standard.

(D) Histograms log₂ ratios of proteins in WT versus Fmr1^{Δ113M/+}. The results were computed from the two experimental datasets.

(E) Fmr1 protein level was decreased ~50% in the Fmr1^{Δ113M/+} heterozygous strain when compared to the WT strain. Total tissue lysate from both strains were loaded as indicated level on a SDS gel followed by western blotting with Fmr1 and actin antibodies. The loading amount of the WT lysate was titrated down for quantification.

(F) Quantification of Fmr1 protein in the mutant fly using a working curve of Fmr1 from the WT lysate during western blotting.

(G) SILAC analysis of FMR1 abundance in WT and Fmr1^{Δ113M/+}. MS spectra and monoisotopic m/z values of a detected peptide from FMR1 protein were shown.

Figure 5.

Validation of two altered proteins in *Drosophila* Fmr1 heterozygous flies by western analysis.

(A) The protein level of profilin was increased in the Fmr1^{Δ113M/+} strain, shown by western blotting.

(B) MS spectra in SILAC data showing the profilin difference in WT and Fmr1^{Δ113M/+}, both of which were compared to the internal standard (IS) strain.

(C) Western blotting analysis of Futsch in the lysates of WT and Fmr1^{Δ113M/+}.

(D) MS spectra in SILAC data showing the Futsch difference in WT and Fmr1^{Δ113M/+}.

Figure 6.

The analysis of total ubiquitin and two main polyUb linkages (K48 and K63) during the aging process of the fly.

(A) Flow chart for the analysis. The insect heads with different ages (1, 3, 10 or 30 days, 15 heads at each stage) were harvested and mixed with stable isotope labeled animals as an internal standard. Then the protein samples were extracted and digested by trypsin. The Ub GG peptides were purified by an antibody affinity method and analyzed by LC-MS for determining the abundance of Ub and the two primary polyUb chains.

(B) Extracted ion currents of targeted peptides for quantification. The light and heavy peptides shared the same retention time (RT). Although we quantified the total levels of light and heavy proteins prior to mixing the samples equally, experimental errors were still observed. So we further used the levels of other housekeeping proteins to correct the mixing errors, assuming that these housekeeping proteins are not changed during the life span. For example, one of the histone 2B peptides (LLPGELAK, doubly charged) was shown as a control. The total Ub level was represented by the level of a Ub peptide (TLSDYNIQK, doubly charged). Although this Ub quantification is affected by possible modifications of the peptide (e.g. phosphorylation on S57 and ubiquitination on K63), we have found that these modifications exist at a much lower level and thus do not influence this measurement significantly.

(C) The measurement of total Ub and two chains during aging. The results (averaged from two biological replicates) were normalized by setting data points of the day 1 flies to 1.

(D) The immunoblotting analysis to validate that the total Ub was not altered in fly heads from day 1 to day 30. The Ponceau S staining of the blot showed equal loading. The running dye (bromophenol Blue) was shown in the gel front. The strongest pink protein band in each lane was generated by compressing all proteins below ~40 kDa on an 8% SDS gel.

Table 1

The effect of culture conditions on fly proliferation (in three repeated experiments)

Culture conditions	Regular ¹	Agarose ²	LMT agarose ³	LMT & filter paper ⁴	LMT & filter paper	LMT & filter paper
Medium	Molasses	Glucose-agarose	Glucose-agarose	Glucose-agarose	Glucose-agarose	Glucose-agarose
Gelling agent	1.5% agar	1.5% agarose	0.8% LMT agarose	0.8% LMT agarose	0.8% LMT agarose	0.8% LMT agarose
Feeding yeast ⁵	baker's yeast	YPD/light	YPD/light	YPD/light	SC/light	SC/heavy
Hatchability rate (larvae/egg, %)	86±2	64±4	89±1	83±5	88±2	91±3
Length of pupa stage (day)	5	6	6	6	5.5	6
Pupation rate (pupae/larvae, %)	97±3	8±2	86±3	92±5	89±5	81±3
Ecdlosion rate (imago/pupae, %)	94±3	25±7	90±1	90±2	88±1	91±2
Male imago weight (µg)	729±9	ND ^{&}	611±17	702±21	722±7	655±10
Female imago weight (µg)	1064±14	ND ^{&}	767±28	878±23	962±27	834±8

¹One liter containing 14.8 g agar, 41.2 g baker's yeast, 100 ml molasses, 100 ml commeal, 22.5 ml tegosept and 8 ml propionic acid (Chang *et al. Nat Chem Biol* 4, 256-263).

²One liter including 10% glucose, agarose as indicated, 0.2 ml 10% 22.5 ml tegosept (10% methyl-p-hydrobenzoate in 95% ethanol), and 8 ml ethyl acetate.

³One liter including low-melting-temperature agarose as indicated and other components as in condition 2.

⁴The condition used filter paper and other reagents in condition 3.

⁵Other than baker's yeast, yeast strain JMP024 was cultured in either standard YPD or synthetic complete (SC) media containing light or heavy stable isotope labeled lysine.