CHAPTER

The *Drosophila* model system to study tau action

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CHAPTER OUTLINE

Abstract

The fruit fly, *Drosophila melanogaster*, is a powerful model system for applying molecular, cellular, and genetic approaches to understanding human tauopathies, including Alzheimer's disease. Here, we provide an introduction to using *Drosophila* as a tauopathy model system and describe several protocols that we use to analyze human tau protein expressed in flies. Methods to detect tau expression include light and scanning electron microscopy in the fly eye, confocal microscopy of primary neuronal cultures, and preparation of tissue homogenates for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis with analysis by Western blotting.

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1 INTRODUCTION TO USING *Drosophila* AS A MODEL SYSTEM

The fruit fly, *Drosophila melanogaster*, has been an invaluable genetic and cell biological model for the study of human disease for over 100 years, particularly in the field of neuroscience. Studies in flies helped elucidate the key components in nervous system development, including identifying genes involved in neuronal patterning, neurogenesis, neuronal migration, growth cone guidance, and differentiation of neural stem cells. In addition, developmental genetic studies indicate that insect and human brain patterning and development are highly conserved (Reichert, 2005). *Drosophila* has also been essential for dissecting the molecular basis of complex behaviors including circadian rhythms and learning and memory (Bellen, Tong, & Tsuda, 2010). *Drosophila* was among the major model organisms that were sequenced as part of the Human Genome Project (Adams et al., 2000), and subsequent gene analysis revealed that 77% of human disease-associated genes share homology with genes in *Drosophila* (Chien, Reiter, Bier, & Gribskov, 2002; Reiter, Potocki, Chien, Gribskov, & Bier, 2001). Finally, since flies are known to perform important posttranslational modifications implicated in disease (phosphorylation, proteolytic cleavage, acetylation, ubiquitination, glycosylation, glycation, advanced glycation end products, sumoylation, oxidation), they hold great potential for future studies to understand how these processes contribute to disease pathology (Chen, David, Ferrari, & Gotz, 2004; Martin, Latypova, & Terro, 2011).

From a working perspective, *Drosophila* has several advantages over vertebrate model systems, summarized here and described in greater detail in this review. Flies develop from egg to adult in only 10 days, are inexpensive and easy to grow, and a single cross can yield hundreds of offspring. Several companies provide *Drosophila* embryo injection services, simplifying the creation of new transgenic lines. In addition, many established fly strains, stock collections, DNA clones/vectors, and cell lines are publically available. Flies are amenable to genetic screens, including chemical- or transposon-based genome mutagenesis and genome-wide RNAi screens (Kaya-Copur & Schnorrer, 2016), as well as pharmaceutical screens used for drug discovery (Pandey & Nichols, 2011). Molecular tools in flies provide precise temporal and spatial control of gene expression in cell- or tissue-specific patterns (Brand & Perrimon, 1993; Caygill & Brand, 2016). Collectively, these features make *Drosophila* an excellent system to study tauopathy.

2 FlyBase AND STOCK CENTERS

FlyBase (www.flybase.org) was launched in 1992 and serves as a free online database of *Drosophila* genes and genomes. For recent reviews describing the features and functions of FlyBase resources, see Drysdale and FlyBase (2008) and Marygold, Crosby, Goodman, and FlyBase (2016). For any given gene, FlyBase provides a wealth of information including genomic location, expression data, orthologs, available mutant alleles, tissue-specific phenotypes, protein interaction data, and reagents

available (including stocks, strains, cell lines, and cDNAs). FlyBase also organizes and integrates related data into collections, including a collection of human disease model reports that provide information on orthology between a human disease-related gene and the related *Drosophila* gene(s) (Marygold et al., 2016). For investigators who are new to flies, there is a resource page that includes topics such as designing a mating scheme for crosses and finding fly-related equipment, tools, and materials. The website provides FlyBase Guides (pamphlets, Powerpoints, and posters compiled from recent research conferences, meetings, and courses) as well as video tutorials. FlyBase also helps researchers to stay connected with resources for meetings, courses, community advocacy, and e-mail distribution lists.

From the FlyBase home page, investigators can search by keyword to find information about the availability of *Drosophila* stocks. FlyBase provides hyperlinks to the appropriate stock center to facilitate ordering. There are seven stock centers available to purchase *Drosophila* (http://flybase.org/wiki/FlyBase:Stocks):

- Bloomington *Drosophila* Stock Center (Indiana University, IN, USA)
- Kyoto Stock Center (Kyoto Institute of Technology, Kyoto, Japan)
- Exelixis (Harvard Medical School, Boston, MA, USA)
- FlyORF (University of Zurich, Zurich Switzerland)
- NIG-FLY (National Institute of Genetics, Mishima, Japan)
- Tsinghua Fly Center (Tsinghua University, Beijing, China)
- Vienna *Drosophila* RNAi Center (Vienna, Austria)

In addition to purchasing flies through the stock centers, many researchers request stocks used in published articles by writing to the corresponding author.

3 EXPRESSING TAU IN *Drosophila*

In 1993, Brand and Perrimon described the development of the UAS-GAL4 system in *Drosophila* to target the expression of any cloned gene in a spatially and temporally controlled fashion. This system uses the yeast transcriptional activator protein GAL4, which potently activates transcription of genes that possess GAL4-binding sites. Since *Drosophila* has no endogenous GAL4 targets, gene expression can be controlled by cloning a gene-of-interest downstream of GAL4-binding sites called upstream activation sequences (UAS). The original pUAST vector described by Brand and Perrimon consists of five tandemly arrayed, optimized GAL4-binding sites (UAS), the hsp70 promoter, a polylinker with unique restriction sites for *EcoR*I, *Bgl*II, *Not*I, *Xho*I, *Kpn*I, and *Xba*I, and the SV40 terminator (Brand & Perrimon, 1993). The UAS-GAL4 vector has been modified by several labs; pUAST and many other UAS-GAL4 vectors can be purchased from the *Drosophila* Genomics Resource Center (https://dgrc.bio.indiana.edu/vectors/Catalog?product).

The UAS-GAL4 method is a two-part system that involves the creation of two independent transgenic lines: one that expresses GAL4 but has no target gene to activate (GAL4-driver line) and a second that possesses the target gene that is not

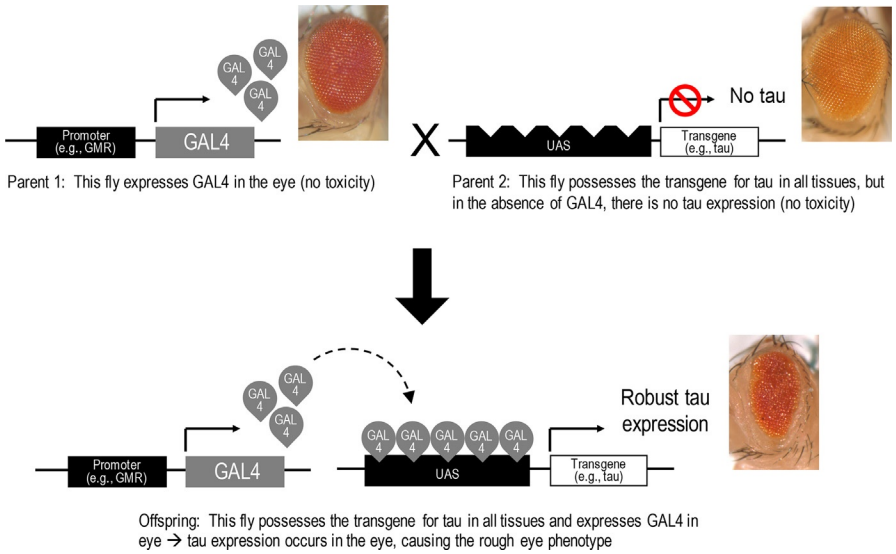

FIG. 1

Example of using the UAS-GAL4 system to express tau in the *Drosophila* eye. A typical mating scheme is illustrated in which a driver line (*top*, parent 1, genotype is *GMR-GAL4*) is crossed to a UAS-target gene line (*top*, parent 2, genotype is *UAS-tau^{WT}*) to produce progeny (*bottom*, offspring, genotype is *GMR-GAL4*; *UAS-tau^{WT}*) that express the gene of interest in a tissue-specific pattern. Parent 1 drives expression of the yeast transcription factor GAL4 in a predominantly eye-specific pattern using the glass multiple reporter (*GMR*) promoter element (GMR-GAL4 fly). Parent 2 has the transgene for tau (UAS-tau^{WT} fly), but since there is no endogenous GAL4 in flies, tau is not expressed. The offspring from these parents receive both the GMR-GAL4 element and the UAS-tau^{WT} gene, resulting in expression of human wild-type tau in the eye.

Modified from Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development, 118, *401–415.*

expressed in the absence of its activator (UAS-target gene line). Progeny that result from mating these two transgenic lines together will express the gene of interest in the temporal and spatial pattern of GAL4 expression (see Fig. 1).

Thousands of GAL4-driver lines with characterized expression patterns exist and can be purchased from stock centers. Of particular interest to neurobiologists, the collection of 6650 transgenic lines created by Jenett and colleagues drives GAL4 expression in a small subset of the ~150,000 neurons in the fly central nervous system (CNS) (Jenett et al., 2012). These flies have been made publically available through the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/Browse/gal4/gal4_main.htm) and are cataloged together with other GAL4 insertion lines, including Rab-GAL4, Trojan-GAL4, Split-GAL4, Ligand sensors,

Geneswitch, Chemosensory, and CoinFLP (for GAL4 and lexA clones). Another important set of techniques that utilizes GAL4 involves the creation and analysis of gene expression in clones. Clonal analysis is a powerful technique pioneered in *Drosophila* that utilizes GAL4 and FLP recombinase (a yeast enzyme) to precisely modulate the timing and duration of gene expression in specific cells. A comprehensive review of the GAL4 system, including methods for refining gene expression and performing clonal analysis, can be found here (Caygill & Brand, 2016).

Creation of the UAS-target gene line involves cloning a gene of interest into pUAST (or another UAS-GAL4 vector) using the unique restriction sites in the polylinker sequence. A summary of tau constructs used previously to create transgenic *Drosophila* lines can be found in Gistelinck, Lambert, Callaerts, Dermaut, and Dourlen (2012). Commercial kits (e.g., QuikChange™, Agilent Genomics) can be used to introduce point mutations, insertions, and deletions in the target gene sequence. After sequencing a newly-created UAS-GAL4 construct to validate its authenticity, the purified DNA can be sent to a company that offers transgenic production services. There are several commercial vendors who provide *Drosophila* embryo injection services, including:

- BestGene, Inc. (https://www.thebestgene.com)
- Genetic Services, Inc. (http://www.geneticservices.com)
- Rainbow Transgenic Flies, Inc. (http://www.rainbowgene.com/default.html)
- WellGenetics (https://wellgenetics.com/microinjection.html)
- GenetiVision (http://www.genetivision.com/ServiceFly.html)
- Centro De Biologia Molecular Severo Ochoa (http://www.cbm.uam.es/joomla-rl/index.php/en/services/scientific-services/drosophila-transgenesis-service)

Investigators choose from a selection of standard strains or can provide their own strain for microinjection. In addition, many companies provide molecular biology services including DNA preparation for microinjection, PCR confirmation of recombinant sites, and RNA synthesis for CRISPR injection, among many others.

4 *Drosophila* AS A MODEL SYSTEM TO STUDY TAU

To study the role of tau in neurodegeneration, several tauopathy models in *Drosophila* have been developed (for reviews, see Bouleau & Tricoire, 2015; Cowan, Chee, Shepherd, & Mudher, 2010; Fernandez-Funez, de Mena, & Rincon-Limas, 2015; Gistelinck et al., 2012; Iijima-Ando & Iijima, 2010; Khurana, 2008; Prussing, Voigt, & Schulz, 2013; Sun & Chen, 2015). Many models drive expression of tau in photoreceptor neurons in the *Drosophila* compound eye. Since the fly eye is not required for viability, it is a desirable tissue to direct the expression of potentially neurotoxic proteins such as tau. The *Drosophila* eye is a simple nervous system that consists of ~800 units called ommatidia, each of which contains eight photoreceptor neurons, four lens secreting cone cells, and two primary pigment cells. Neighboring

ommatidia share additional pigment cells and mechanosensory bristle complexes (Kumar, 2012; Ready, Hanson, & Benzer, 1976). Nontransgenic fly eyes possess an ordered ommatidial morphology characterized by a regular array of bristles and lenses (Fig. 2A and C). Expression of human tau in the retina causes a moderate rough eye phenotype characterized by disorder of the ommatidial array and a modest decrease in the size of the eye (Fig. 2B and D). The tau-induced rough eye phenotype is an excellent system to screen for genetic modifiers and pharmacological agents that may enhance or suppress the tau rough eye (Billmann & Boutros, 2016; Dourlen, 2017; Hannan, Drager, Rasse, Voigt, & Jahn, 2016; Kaya-Copur & Schnorrer, 2016; Lenz, Karsten, Schulz, & Voigt, 2013; Pandey & Nichols, 2011,

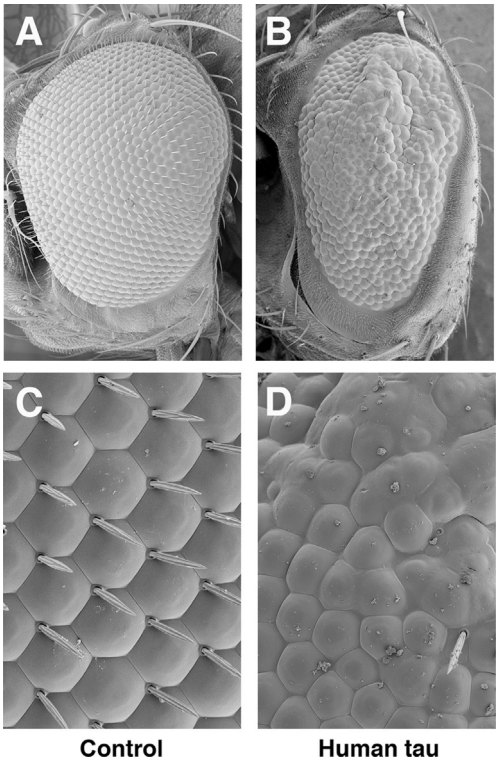

FIG. 2

Expression of human tau in *Drosophila* retinal cells causes the tau-induced rough eye phenotype. As shown using scanning electron microscopy, nontransgenic control flies (Canton S, A and C) have an ordered ommatidial morphology, whereas expression of wild-type human tau (B and D) causes a moderate rough eye phenotype characteristic of tau toxicity in the eye. At higher magnification, the hexagonal geometry of individual ommatidia and the presence of mechanosensory bristles is immediately apparent in control flies (C), but is significantly disrupted with the expression of human tau (D).

and reviewed later). Scoring modification of the rough eye phenotype can be conducted quickly and objectively as a single-blind analysis requiring only basic microscopy, making it simple to identify modifiers from a large number of flies.

5 *Drosophila* TAUOPATHY SCREENS

Drosophila is particularly useful for performing unbiased, large-scale genetic screens to identify genes that contribute to tau toxicity in vivo (see Hannan et al., 2016; Lenz et al., 2013 for reviews). *Drosophila* is amenable to transposon-based genomic mutagenesis, including the use of EP transposable elements that possess both an *e*nhancer region (with GAL4-binding sites) immediately upstream of a *p*romoter sequence, such that GAL4 activation should produce a transcript initiated within the element (Rorth, 1996).

Shulman and Feany conducted the first large-scale genetic modifier screen in a *Drosophila* model of tauopathy in 2003 (Shulman & Feany, 2003). Using an F_1 screen, they examined a collection of 2276 EP transposable elements to identify progeny with enhancement or suppression of the tau rough eye phenotype using the human disease-associated mutation tau^{V337M}. The largest functional class of modifiers identified in their screen were kinases and phosphatases, including the *Drosophila* homologs of MARK serine/threonine kinase (*par-1*), PP1 phosphatase (CG9238), and PP2A phosphatase (CG5643), all shown previously to modulate phosphorylation of tau. They demonstrated that known tau kinases such as CDK5, PKA, and JNK pathway components also enhanced tau toxicity in their screening system. In addition, they identified genes modulating apoptosis and the neuronal cytoskeleton, suggesting that these pathways may play an integral role in mediating tau toxicity in vivo.

In 2007, Blard and colleagues used the same tau fly construct as Shulman and Feany (tau^{V337M}; Shulman & Feany, 2003) to screen a different P element collection that contained 1250 insertion lines searching for modifiers of the tau rough eye phenotype. Their screen identified 30 genes with an emphasis on components of the cytoskeletal network (three *Drosophila* homologs of actin-binding proteins and a focal adhesion-associated protein) and molecular chaperone proteins (homologs of HSP40/HDJ-1 and heat-shock cognate protein Hsc70Cb). The authors suggest that cytoskeletal components that function in presynaptic nerve terminals (particularly components of the actin network) and molecular chaperone proteins are key modulators of tau-induced neurodegeneration (Blard et al., 2007).

The Jackson lab used the rough eye-inducing *gl*-tau fly model, in which human wild-type tau is fused to the eye-specific promoter *glass* (*gl*), to perform both loss-of-function (LOF) and gain-of-function (GOF) screens. Their LOF screen utilized a collection of ~1000 P-lethal stocks in which *LacZ*-containing transposable P elements are inserted throughout the fly genome; their GOF screen employed a collection of ~900 "EY" stocks in which "empty" UAS elements are inserted throughout the genome that either enhance or suppress gene expression, depending on the orientation of the UAS insertion. Of the ~1900 lines screened, 37 genes were found to

be strong modifiers of tau toxicity including kinases (homologs of GSK-3β, MARK1, CAMK1D, and MAP3K1), motor and cytoskeletal proteins (dynein and kinesin-like homologs), as well as genes related to autophagy, cell cycle, trafficking, and RNA processing, stressing that mechanisms independent of tau phosphorylation can alter toxicity (Ambegaokar & Jackson, 2011). The Jackson lab also used their *gl*-tau fly model in a cross-species functional genomics approach in which they identified modifiers of tau toxicity in mouse brain and validated their candidate modifiers in *Drosophila*. Using this scheme, they found that puromycin-sensitive aminopeptidase degrades tau in vivo, suggesting that it may be important for inhibiting tau-induced neurodegeneration in human disease (Karsten et al., 2006).

Most recently, an RNAi-based screen that silenced more than 7000 genes (including 50% of protein-coding fly genes and 90% of fly genes with human orthologs) identified 62 specific modifiers of tau toxicity using the human disease-associated mutation tau^{R406W}. Among the major classes of modifiers identified (protein anabolism and catabolism, transport, kinases, and phosphatases), the authors found several genes involved in the dynein/dynactin complex, implicating dysfunctional retrograde transport in tau-induced toxicity. Additional studies showed that silencing components of the dynein/dynactin complex enhanced tau toxicity, suggesting that impaired retrograde axonal transport precedes synaptic defects in tau-induced neurodegeneration (Butzlaff et al., 2015).

In addition to these unbiased, large-scale modifier screens, another approach uses human genome-wide association studies to identify genes associated with Alzheimer's disease susceptibility, determine the corresponding fly orthologs, and test these genes in modifier screens. Two studies by Shulman and colleagues tested genes from distinct genomic regions with an association with Alzheimer's pathology and found 15 genes with potential roles in Alzheimer's disease that also modified tau toxicity in vivo. The tau modifying genes have been implicated in glucose transport, cellular adhesion, integrin signaling, and actin cytoskeleton dynamics, suggesting a role for the cytoskeleton in tau-mediated toxicity (Shulman et al., 2011, 2014). Together, these data highlight the utility of *Drosophila* as a model system for identifying genes with important implications for understanding the molecular basis of tau-induced neurodegeneration in human disease.

6 PROTOCOLS FOR ANALYZING TAU EXPRESSION IN *Drosophila*

A detailed protocol for expression of tau in *Drosophila* using the UAS-GAL4 system has been recently described (Dourlen, 2017). In our lab, we routinely (i) analyze tau expression in adult flies using light microscopy and scanning electron microscopy (SEM), (ii) use confocal analysis for primary cultures prepared from third instar larvae, (iii) and prepare tissue homogenates from flies expressing tau that we resolve by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyze by Western blotting (WB). These procedures are described in this section.

6.1 LIGHT MICROSCOPY

Light microscopy is often used for documenting changes in the external appearance of the *Drosophila* compound eye and is particularly useful when screening for modifiers of the tau rough eye phenotype or for hypothesis testing. Other researchers have described techniques for capturing fly eye images, including eye reconstruction from a series of *z*-stack images (Dourlen, 2017) and the use of an extended depth of focus algorithm that renders a three-dimensional reconstruction of the eye yielding quantitative data on eye volume (Ambegaokar & Jackson, 2011). One main advantage of using simple light microscopy is that it can be performed quickly and easily using basic microscopy techniques to produce a publication-quality image (described here). Greater detail and higher-resolution imaging is obtained using SEM, which is described later. The protocol described here is for analysis of eye phenotypes; however, with modification, it could be used to examine other external body features as well.

1. Prepare flies for analysis either by freezing adults in a 1.5-mL microfuge tube at −20°C overnight or by immobilizing live flies by placement on a cold surface (such as a Tissue-Tek cold plate, VWR catalog #25608-942) or using CO_2 as an anesthetizing agent on a white flypad (Genesee Scientific catalog #59-114).
2. Manually calibrate the light microscope (e.g., Nikon SMZ1500) with the imaging software (e.g., SPOT Advanced Imaging software, Diagnostic Instruments, Inc.) before each session. It is particularly important to calibrate the white balance value. Use sufficient light to effectively illuminate the stage area, but not so bright that it will scorch the fly tissue while capturing the image.
3. Use lab tape (VWR catalog #10141-866) to adhere the fly to white paper (e.g., 7×10 cm blotting paper, VWR catalog #28298-030) or directly to a cold plate or flypad. Place the tape posterior to the fly head, ensuring that legs and wings are underneath the tape (see Fig. 3) so they do not obstruct the view of the eye.

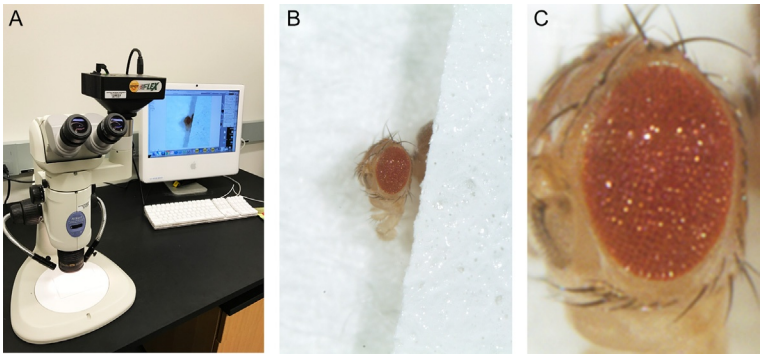

FIG. 3

Laboratory setup for light microscopy. A Nikon SMZ1500 light microscope connected to a SPOT digital camera (A). Using standard lab tape, the fly is adhered to a white piece of paper (B). Photo-editing software is used to rotate the canvas and crop the image (C).

4. Manipulate the immobilized fly until the eye is centered in the field of view of the microscope and imaging software. Use the bristles of a fine-detail paint brush to make adjustments if the head is tilted.
5. Once in focus, capture the image and use photo editing and manipulation software (e.g., Adobe Photoshop) to crop, adjust brightness/contrast, rotate, or resize the image. Image analysis can also be performed using a freeware program called ImageJ (https://imagej.nih.gov/ij/), a public domain open source Java image processing program that can calculate area and pixel value statistics using TIFF, GIF, JPEG, BMP, DICOM, and FITS image formats.

6.2 SCANNING ELECTRON MICROSCOPY

Compared to using light microscopy for analysis of fly eyes, SEM produces images with greater resolution, higher magnification, and added information about the three-dimensional topography of the external surface of the eye (see Fig. 2). Although SEM analysis requires a sophisticated microscope and more time to prepare the flies, the added resolution and magnification help to accurately document the external morphology of the eye, which is particularly useful when examining modifiers of the tau rough eye phenotype. The protocol below employs a Hitachi 3400N-II scanning electron microscope for image acquisition.

1. Fix adult *D. melanogaster* (fresh or frozen) in 1.25% gluteraldehyde in 0.1 *M* phosphate buffer pH 7.2 at room temperature for 2 h (or overnight at 4°C) using a 1.5-mL microfuge tube with a ratio of 10:1 of fixative to fly tissue for each genotype group. If the flies float on the surface of the fixative, add a few drops of 2.5% Triton X-100 to ensure that they are completely submerged.

   Note: Do not remove the fly head from the body, but instead fix the entire adult fly (in order to use the body as the point of attachment of the fly to the mounting stub in step 4).
2. Wash flies with 1 mL of 0.1 *M* phosphate buffer pH 7.2 three times at room temperature for 10 min each in a 1.5-mL microfuge tube (rotation of the sample is not required). Dehydrate using a graded ethanol series, starting at 10% and following with 25%, 50%, 75%, 80%, 95%, ending with 100%—each graded step is performed for 10 min in triplicate using a volume of 1 mL in a 1.5-mL microfuge tube.
3. Dry flies using critical point drying (CPD) or *t*-butyl alcohol with vacuum sublimation. We use an Anatech Hummer 6 critical point dryer; however, we have found that the *t*-butyl alcohol procedure (step *b* below) works equally as well and does not require a CPD dryer. Other labs have reported success using hexamethyldisilazane (HMDS) as a rapid, inexpensive alternative to CPD for insect and other tissues (Heegaard, Jensen, & Prause, 1986; Nation, 1983; Wolff, 2011).
   a. For the CPD method, quickly transfer flies from the final 100% ethanol step into a modified BEEM capsule (see Fig. 4) that is in a petri dish filled

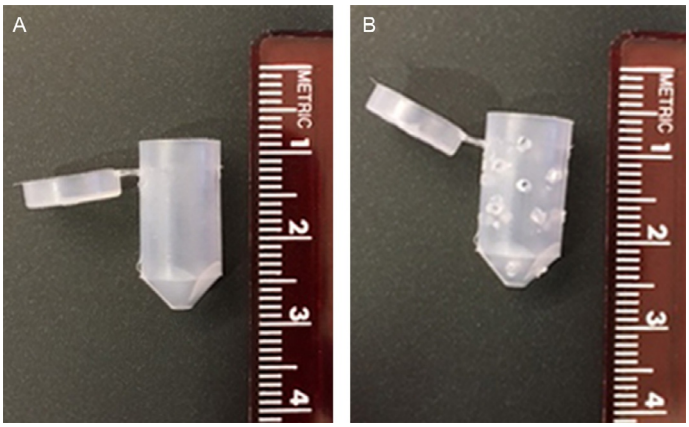

FIG. 4

BEEM capsules modified for use in critical point drying. A normal BEEM capsule with attached cap (A) is used to make a modified BEEM capsule that has holes to allow the transfer of liquid CO_2 while retaining flies inside (B).

with 100% ethanol. BEEM modification (Fig. 4B) allows liquid CO_2 to permeate the capsule without losing any flies. Move the modified BEEM capsule to the CPD chamber filled with 100% ethanol. Inside the CPD chamber, the ethanol is replaced with liquid CO_2 using five 10-min soak and flush cycles. Once infiltrated with CO_2, the temperature and pressure of the CPD chamber are raised through the critical point (31.1°C and 1072 psi), transitioning the liquid CO_2 to gas. This transition dries with minimal surface tension effects. Dried flies can be immediately sputter coated (see step 4) or stored in a desiccator indefinitely. An excellent description of the principles of CPD can be found here: (https://www.emsdiasum.com/microscopy/technical/datasheet/critical_drying.aspx).

b. For the *t*-butyl alcohol with vacuum sublimation method, quickly transfer flies from the final 100% ethanol step into a 50/50 mixture of 100% ethanol and *t*-butyl alcohol (in a microfuge tube or glass scintillation vial) for 15 min. Infiltrate sample with 100% *t*-butyl alcohol three times at 15 min each at 37°C (to ensure that the *t*-butyl alcohol does not freeze). Place the flies in just enough fresh *t*-butyl alcohol to cover them and incubate at 4°C for 10 min to allow the *t*-butyl alcohol to freeze. Once frozen in *t*-butyl alcohol, place flies in a vacuum desiccator with gel ice packs and apply vacuum to the desiccator to sublimate the alcohol. Keep the samples under vacuum for one additional hour after all the alcohol has completely sublimated (~3 h) to ensure that the flies are completely dry. Dried flies can be immediately sputter coated (see step 4) or stored in a desiccator indefinitely (Inoue & Osatake, 1988).

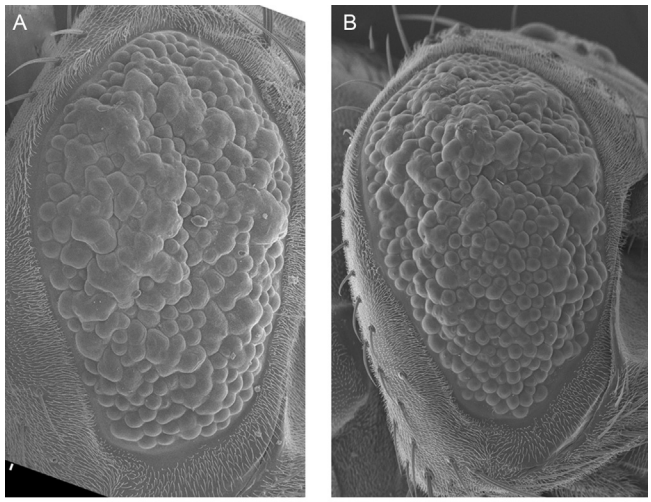

FIG. 5

Comparison of critical point drying (CPD) and vacuum sublimation from *t*-butyl alcohol as drying methods for *Drosophila*. There is no difference in the integrity of the tissue or the quality of the final image captured when using CPD (A) or *t*-butyl alcohol (B) as the drying method for the analysis of wild-type human tau expression in retinal tissues in *Drosophila* (both flies are *GMR-GAL4*; tau^{WT}).

4. Regardless of drying method, place dried flies on SEM specimen mounts (Electron Microscopy Sciences catalog #75220-12) and sputter coat with gold–palladium (Ted Pella, Inc. catalog #91212) on top for 60 s and on each side for 30 s to produce a 25-nm-thick coating using a sputter coater such as the Denton Desk II (or equivalent). Image flies with a scanning electron microscope (e.g., Hitachi 3400N-II). A comparison of tau flies dried using CPD and *t*-butyl alcohol is shown in Fig. 5.

6.3 PRIMARY CULTURE OF *Drosophila* NEURONS

Primary cell culture experiments are valuable for complimenting in vivo studies and are useful for both genetic and biochemical experiments including pharmacological or RNAi screens (Egger, van Giesen, Moraru, & Sprecher, 2013; Reinecke et al., 2011). Primary culture is particularly useful for studying tauopathy since neuronal cultures can be created from flies with any genetic background, including flies expressing genes under control of the UAS-GAL4 system. This protocol describes how to culture neurons from third instar larvae.

1. Sterilize 5 mm glass coverslips (Electron Microscopy Sciences catalog #72296-05) with 70% ethanol and allow to air-dry. Place each coverslip into a single well of a sterile 96-well plate and UV-sterilize the plate with coverslips for 30 min.

Following UV sterilization, replace the plate lid or cover with self-adhesive plate sealing film.

2. Coat coverslips (250 μL/well) with 167 μg/mL concanavalin A (Sigma catalog #C5275) and 1.67 μg/mL mouse-laminin (Fisher catalog #23-017-015) and incubate for 2 h at 37°C (Kraft, Levine, & Restifo, 1998). Remove coating solution and replace with Plating Media [Schneider's *Drosophila* media (Fisher catalog #21-720-024) with 50 μg/mL insulin (Sigma catalog #I0516) and 10% heat-inactivated bovine serum (Fisher catalog #10-082-139)].
3. Use Dumont #5 forceps to dissect the CNS from surface sterilized (1 min in 70% ethanol) wandering third instar larvae in sterile calcium-free buffer (137 m*M* NaCl, 2.7 m*M* KCl, 0.4 m*M* NaH_2PO_4, 11.9 m*M* $NaHCO_3$, 5.6 m*M* glucose). There are several good videos demonstrating how to perform the dissections on YouTube.com: (https://www.youtube.com/watch?v=Y8py_dPMtao and https://www.youtube.com/watch?v=9WlIoxxFuy0).
4. Place dissected CNS in a solution containing 0.2 mg/mL collagenase (Sigma catalog #C9722) diluted in calcium-free buffer for 30 min at 37°C.
5. Wash tissue by transferring CNS to a 1.5-mL microfuge tube containing 1 mL of Plating Media and centrifuge for 1 min at 1000 rpm. Remove media and replace with fresh Plating Media and centrifuge for 1 min at 1000 rpm. Repeat wash step 1 additional time. After final spin, remove media and add 200 μL of Plating Media to tube containing washed CNS.
6. Disperse CNS into single cells by repeated passage through a pipet tip (~100 times). Remove the media in the wells from step 2 and plate cells on the coverslips.
7. Allow cells to adhere to the coverslip for 24 h at 25°C then remove the media and replace with fresh Plating Media. Grow cells at 25°C for 2–3 additional days.

6.4 IMMUNOFLUORESCENCE AND CONFOCAL ANALYSIS OF PRIMARY LARVAL CNS

1. Remove Plating Media and rinse cells twice in phosphate-buffered saline (PBS).
2. Fix cells in 100 μL of 4% paraformaldehyde (Electron Microscopy Sciences catalog #157-4) for 10 min at room temperature.
3. Wash cells three times in 100 μL of PBSTx (PBS + 0.3% Triton X-100).
4. Incubate cells in 100 μL of blocking solution [PBSTx + 0.2% bovine serum albumin (Sigma catalog #B4287) and 5% normal goat serum (VWR catalog #RLB304)] for 30 min at room temperature.
5. Dilute the primary antibody in blocking solution (see Table 1). Remove blocking solution from cells and incubate cells in primary antibody solution overnight at 4°C in the dark.
6. Wash cells three times (10 min) in PBSTx.
7. Dilute the appropriate fluoro-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) in blocking solution (typical dilution is 1:200). Remove the final PBSTx wash from cells and incubate in secondary antibody solution for 1 h at room temperature in the dark.

Table 1 Antibodies Used to Analyze Tau Phosphorylation Patterns in *Drosophila* Models of Tauopathy

Antibody	Epitope	Application	Concentration	References
12E8	pS262 and/or S356	WB	1:2000–1:4000	Chatterjee, Sang, Lawless, and Jackson (2009), Huang et al. (2014), Mudher et al. (2004), Nishimura, Yang, and Lu (2004), Park et al. (2012), Wang, Imai, and Lu (2007), and Xiong et al. (2013)*
		IF	1:3000–1:4000	Nishimura et al. (2004) and Xiong et al. (2013)
5A6	aa 19–46	WB	1:2000–1:6000	Blard, Frebourg, Campion, and Lecourtois (2006), Chanu and Sarkar (2017), and Huang et al. (2014)
Alz50	Conformation specific (epitope within aa 312–322)	WB	1:100	Papanikolopoulou and Skoulakis (2015)
		IF	1:100	Steinhilb, Dias-Santagata, Fulga, Felch, and Feany (2007) and Steinhilb, Dias-Santagata, Mulkearns, et al. (2007)
AT8	pS202, pT205	WB	1:200–1:100,000	Ali, Ruan, and Zhai (2012), Ambegaokar and Jackson (2010), Blard et al. (2006), Chanu and Sarkar (2017), Chatterjee et al. (2009), Chau, Chan, Shaw, and Chan (2006), Cowan et al. (2015), Dias-Santagata, Fulga, Duttaroy, and Feany (2007), Folwell et al. (2010), Fulga et al. (2007)*, Gorsky, Burnouf, Dols, Mandelkow, and Partridge (2016), Grammenoudi, Kosmidis, and Skoulakis (2006), Huang et al. (2014), Iijima, Gatt, and Iijima-Ando (2010), Khurana et al. (2010, 2006), Kosmidis, Grammenoudi, Papanikolopoulou, and Skoulakis (2010), Lin, Tsai, Wu, and Chien (2010), Nishimura et al. (2004), Papanikolopoulou and Skoulakis (2015), Povellato, Tuxworth, Hanger, and Tear (2014), Quraishe, Cowan, and Mudher (2013), Steinhilb, Dias-Santagata, Fulga, et al. (2007), Steinhilb, Dias-Santagata, Mulkearns, et al. (2007), Wang et al. (2007), and Xiong et al. (2013)*
		IF	1:1000	Chanu and Sarkar (2017), Falzone, Gunawardena, McCleary, Reis, and Goldstein (2010), and Khurana et al. (2006)

AT100	pT212, pS214	WB	1:200–1:2000 or 0.2–10 μg/mL	Chatterjee et al. (2009), Dias-Santagata et al. (2007), Folwell et al. (2010), Gorsky et al. (2016), Grammenoudi et al. (2006), Jackson et al. (2002), Kosmidis et al. (2010), Lin et al. (2010), Nishimura et al. (2004), Papanikolopoulou and Skoulakis (2015), Povellato et al. (2014), and Steinhilb, Dias-Santagata, Fulga, et al. (2007)
		IF	1:100–1:1000 and 10 μg/mL	Chau et al. (2006), Jackson et al. (2002), and Nishimura et al. (2004)
		ELISA	—	Steinhilb, Dias-Santagata, Mulkearns, et al. (2007)
AT180	Around pT231	WB	1:100–1:2000	Ali et al. (2012), Ando, Maruko-Otake, et al. (2016), Chanu and Sarkar (2017), Chatterjee et al. (2009), Chau et al. (2006), Cowan et al. (2015), Dias-Santagata et al. (2007), Folwell et al. (2010), Grammenoudi et al. (2006), Huang et al. (2014), Huang, Wu, and Zhou (2015), Iijima et al. (2010), Iijima-Ando et al. (2012), Iijima-Ando, Zhao, Gatt, Shenton, and Iijima (2010), Khurana et al. (2010, 2006), Nishimura et al. (2004), Park et al. (2012), Quraishe et al. (2013), Steinhilb, Dias-Santagata, Mulkearns, et al. (2007), and Wang et al. (2007)
		IF	1:500–1:1000	Chanu and Sarkar (2017), Fulga et al. (2007), and Khurana et al. (2006)
AT270	Around pT181	WB	1:400–1:100,000	Chanu and Sarkar (2017), Chatterjee et al. (2009)*, Chau et al. (2006), Huang et al. (2014), Khurana et al. (2010), Lin et al. (2010), Nishimura et al. (2004), Povellato et al. (2014), and Steinhilb, Dias-Santagata, Fulga, et al. (2007)
		IF	1:1000	Chanu and Sarkar (2017) and Lin et al. (2010)
Clone 46.1	aa 315–352	WB	1:1000	Geng, Xia, Li, and Dou (2015)
CP13	pS202	WB	1:500–1:2000	Ando, Maruko-Otake, et al. (2016), Ando, Oka, et al. (2016), Falzone et al. (2010), Huang et al. (2014, 2015), Iijima-Ando et al. (2012, 2010), Nishimura et al. (2004), and Park et al. (2012)
		IF	np	Falzone et al. (2010)

Continued

Table 1 Antibodies Used to Analyze Tau Phosphorylation Patterns in *Drosophila* Models of Tauopathy—cont'd

Antibody	Epitope	Application	Concentration	References
Dako polyclonal anti-C-terminal	aa 243–441	WB	1:1000–1:10^6	Ali et al. (2012), Chau et al. (2006), Cowan, Bossing, Page, Shepherd, and Mudher (2010), Cowan et al. (2015), Dias-Santagata et al. (2007), Feuillette, Miguel, Frebourg, Campion, and Lecourtois (2010), Folwell et al. (2010), Fulga et al. (2007), Gorsky et al. (2016), Khurana et al. (2010, 2006), Mershin et al. (2004), Mudher et al. (2004), Povellato et al. (2014), Quraishe et al. (2013), Sinadinos et al. (2013), Steinhilb, Dias-Santagata, Fulga, et al. (2007), Steinhilb, Dias-Santagata, Mulkearns, et al. (2007), Talmat-Amar et al. (2011), Yeh, Chang, et al. (2010), and Yeh, Chien, et al. (2010)
		IF	1:100–1:200	Ali et al. (2012), Chau et al. (2006), and Yeh, Chang, et al. (2010)
HT7	aa 159–163	WB	1:2000	Gorsky et al. (2016)
MC1	Conformation specific (epitope within aa 2–10 and 312–342)	WB	1:100–1:200	Blard et al. (2006), Cowan et al. (2015), and Papanikolopoulou and Skoulakis (2015)
		IF	1:100	Steinhilb, Dias-Santagata, Fulga, et al. (2007), Steinhilb, Dias-Santagata, Mulkearns, et al. (2007), and Wittmann et al. (2001)
PHF-1	pS396, pS404	WB	1:100–1:100,000	Ando, Maruko-Otake, et al. (2016), Blard et al. (2006), Chatterjee et al. (2009), Cowan, Bossing, et al. (2010), Cowan et al. (2015), Dias-Santagata et al. (2007), Falzone et al. (2010), Folwell et al. (2010), Fulga et al. (2007)*, Huang et al. (2014, 2015), Khurana et al. (2010, 2006), Kosmidis et al. (2010), Mudher et al. (2004), Nishimura et al. (2004), Park et al. (2012), Povellato et al. (2014), Quraishe et al. (2013), Sinadinos et al. (2013), Steinhilb, Dias-Santagata, Fulga, et al. (2007), and Steinhilb, Dias-Santagata, Mulkearns, et al. (2007)
		IF	1:200	Fulga et al. (2007) and Khurana et al. (2006)

$pSer^{199}$	pS199	WB	1:1000	Yeh, Chang, et al. (2010) and Yeh, Chien, et al. (2010)
$pSer^{202}$	pS202	WB	np	Yeh, Chien, et al. (2010)
$pSer^{214}$	pS214	WB	1:40,000	Steinhilb, Dias-Santagata, Fulga, et al. (2007)
		ELISA	—	Steinhilb, Dias-Santagata, Mulkearns, et al. (2007)
$pSer^{262}$	pS262	WB	1:500–1:5000	Ali et al. (2012), Ando, Maruko-Otake, et al. (2016), Ando, Oka, et al. (2016), Blard et al. (2006), Cowan et al. (2015), Feuillette et al. (2010), Gorsky et al. (2016), Grammenoudi et al. (2006), Huang et al. (2015), Iijima et al. (2010), Iijima-Ando et al. (2012, 2010), Kosmidis et al. (2010), Papanikolopoulou and Skoulakis (2015), Quraishe et al. (2013), Sinadinos et al. (2013)*, Sofola et al. (2010), and Wu et al. (2011)
		IF	1:200	Ali et al. (2012)
$pSer^{356}$	pS356	WB	1:500–1:2000	Ando, Oka, et al. (2016), Blard et al. (2006), Huang et al. (2015), Kosmidis et al. (2010), Sofola et al. (2010), and Yeh, Chang, et al. (2010)
$pSer^{396}$	pS396	WB	1:1000–1:2000	Grammenoudi et al. (2006), Papanikolopoulou and Skoulakis (2015), Yeh, Chang, et al. (2010), and Yeh, Chien, et al. (2010)
$pSer^{404}$	pS404	WB	1:1000	Papanikolopoulou and Skoulakis (2015) and Yeh, Chang, et al. (2010)
$pThr^{181}$	pT181	WB	1:200–1:1000	Chatterjee et al. (2009)* and Feuillette et al. (2010)
$pThr^{205}$	pT205	WB	1:1000	Yeh, Chang, et al. (2010) and Yeh, Chien, et al. (2010)
$pThr^{212}$	pT212	WB	1:40,000	Steinhilb, Dias-Santagata, Fulga, et al. (2007)
$pThr^{231}$	pT231	WB	1:1000	Yeh, Chang, et al. (2010) and Yeh, Chien, et al. (2010)
T14	Phosphorylation-independent total tau	WB	1:1000–1:3000	Blard et al. (2006), Jackson et al. (2002), Nishimura et al. (2004), and Wang et al. (2007)
		IF	1 μg/mL	Jackson et al. (2002)
T46	aa 395–432	WB	1:1000–1:3000	Ambegaokar and Jackson (2010), Blard et al. (2006), Chatterjee et al. (2009), Iijima et al. (2010), Iijima-Ando et al. (2010), Kosmidis et al. (2010), Papanikolopoulou and Skoulakis (2015), and Xiong et al. (2013)*
		IF	1:1000	Xiong et al. (2013)

Continued

Table 1 Antibodies Used to Analyze Tau Phosphorylation Patterns in *Drosophila* Models of Tauopathy—cont'd

Antibody	Epitope	Application	Concentration	References
Tau-1	Unphosphorylated aa 189–207	WB	1:1000–1:10,000	Ando, Maruko-Otake, et al. (2016), Ando, Oka, et al. (2016), Blard et al. (2006), Cowan et al. (2015), Dias-Santagata et al. (2007), Feuillette et al. (2010), Fulga et al. (2007)*, Grammenoudi et al. (2006), Lin et al. (2010), Mudher et al. (2004), Nishimura et al. (2004), Povellato et al. (2014), Quraishe et al. (2013), Reinecke et al. (2011), Steinhilb, Dias-Santagata, Fulga, et al. (2007), Steinhilb, Dias-Santagata, Mulkearns, et al. (2007), Wu et al. (2011), Xiong et al. (2013)*, Yeh, Chang, et al. (2010), and Yeh, Chien, et al. (2010)
		IF	1:1000	Fulga et al. (2007)
Tau-5	aa 210–241	WB	1:500–1:2000	Blard et al. (2006), Falzone et al. (2010), Huang et al. (2014, 2015), Kosmidis et al. (2010), Reinecke et al. (2011), Wu et al. (2011), and Yeh, Chien, et al. (2010)
		IF	1:100	Reinecke et al. (2011)
Tau-46	Total tau	WB	np	Ando, Maruko-Otake, et al. (2016), Ando, Oka, et al. (2016), Iijima et al. (2010), and Iijima-Ando et al. (2012)
Tau-C3	aa 412–421 at the C-terminus of tau truncated at Asp421	WB	1:1000	Khurana et al. (2010)
		IF	1:1000	Khurana et al. (2010)
TG3	pT231, pS235	WB	1:100–1:1000	Dias-Santagata et al. (2007), Khurana et al. (2010), Steinhilb, Dias-Santagata, Fulga, et al. (2007), and Steinhilb, Dias-Santagata, Mulkearns, et al. (2007)

aa, *amino acid;* ELISA, *enzyme-linked immunosorbent assay;* IF, *immunofluorescence;* np, *not provided;* WB, *Western blot;* *, *in supplementary information section of the paper cited.*

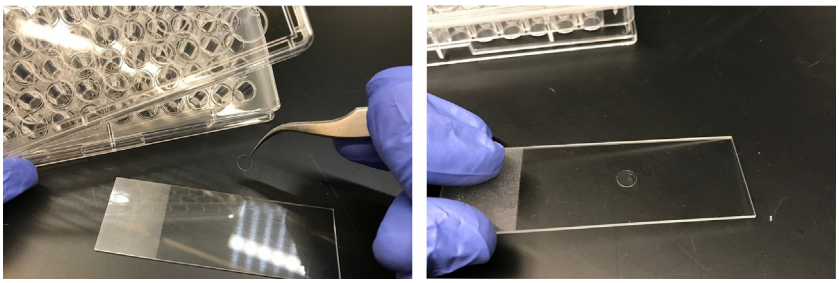

FIG. 6

Transferring a 5-mm glass coverslip to a microscope slide. A coverslip is removed from the well of a 96-well plate (*left*) and transferred, cell side down, to a glass microscope slide on which 0.2 μL of hard-set mounting media has been added (*right*).

8. Wash cells three times (10 min) in PBSTx.
9. Remove coverslip from the 96-well plate and mount in 0.2 μL of Vectashield HardSet mounting medium (Fisher catalog #NC9029228) on a glass slide (see Fig. 6). Store mounted slides at 4°C in the dark.

   Note: If using a nonhardening mounting media formulation, seal the edges of the coverslip with nail polish or a plastic sealant.
10. Visualize cells by confocal microscopy.

6.5 *Drosophila* TISSUE HOMOGENATES

To analyze tau protein expressed in *Drosophila* neurons, tissue homogenates are prepared, proteins are resolved by SDS-PAGE, and specific antibodies are used to visualize tau via WB. One challenge in preparing tissue homogenates is the presence of the cuticle that encapsulates the fly head. Use of a FastPrep high-speed benchtop homogenizer (MP Biomedicals) eliminates the need to dissect the cuticle from the fly head. The protocol below describes both the tissue homogenization and the subsequent analysis by WB.

1. Fill Duratube (Research Products International catalog #163283) with 10 large diameter (2 mm) bashing beads (Zymo Research ZR BashingBead™ Lysis Tubes catalog #S6003). These are designed to be chemically inert and effectively homogenize the fly cuticle.

   Note: The Zymo Bashing Beads come with ~30 beads (0.7 mL dry volume) per 2 mL tube. To maximize recovery of small volumes of fly homogenate, remove all of the beads from the lysis tubes and store them in a sterile 15-mL Falcon tube. Separately purchase Duratubes (to use after depleting the Zymo tubes) and add only 10 beads per Duratube to perform each homogenization.
2. Add Laemmli buffer (Sigma-Aldrich catalog #S3401, or another suitable lysis buffer) including protease inhibitors (Sigma-Aldrich catalog #11836170001) into

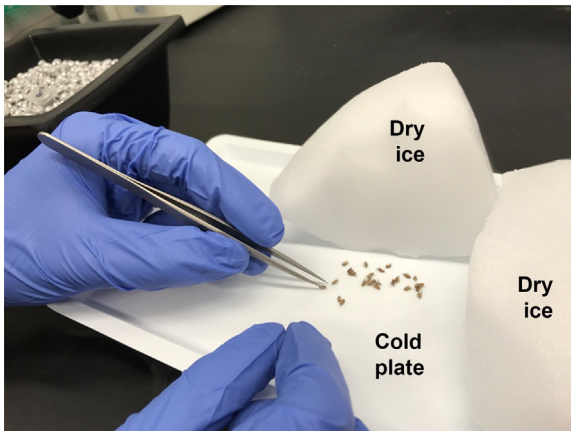

FIG. 7

Laboratory setup for preparing *Drosophila* heads for tissue homogenization. A Tissue-Tek cold plate on which large pieces of dry ice are placed is effective at keeping the working surface cold. Flies will remain frozen when placed on the surface, which limits protease activity and enhances dissociation of the head from the thorax.

the Duratube with the bashing beads. The amount of buffer depends on number of fly heads—use the equation: volume = 40 μL + (heads)(1 μL), so 5 heads would require 45 μL of buffer, 25 heads would require 65 μL of buffer, etc.

3. Decapitate flies by separating the head from the thorax and immediately put heads into a Duratube filled with bashing beads and lysis buffer on ice.

   Note: It is critical to keep the fly tissue as cold as possible at all times. Use a Tissue-Tek cold plate (VWR catalog #25608-942) and large pieces of dry ice to keep the working surface very cold (see Fig. 7).

4. Place tubes into a FastPrep FP120 (or an equivalent high-speed benchtop homogenizer) and run on speed 4.0 (slowest) for 45 s. Remove tubes and place on ice for 1 min to cool the samples. Repeat step 4 then move the samples to ice.

   Note: Alternatively, fly heads can be homogenized manually using either by a Dounce tissue homogenizer (such as VWR catalog #71000-514 with "tight" pestle) or by flaming the tip of a 5-in. Pasteur pipet to form a "bulb" at the end of the pipet and manually crushing each fly head with the bulb.

5. Centrifuge samples for 1 min at 13,000 × *g* in a microcentrifuge. The centrifugation step effectively collapses any foam that may form inside the tube from FastPrep agitation (especially if using Laemmli or another lysis buffer with detergent) and also pellets any small cuticle particles away from the tissue homogenate.

6. Remove the liquid homogenate and transfer to a new 1.5-mL microfuge tube. Gel-loading pipet tips (VWR catalog #37001-152) work well since they can fit between the bashing beads to capture the most homogenate.

Note: Adding additional fresh lysis buffer (same volume as used during homogenization) back to the "used" bashing beads, vortexing (not FastPrep), and recovering this "wash step" buffer will yield additional protein that was left behind during the initial buffer removal (trapped in the interstitial spaces between the beads).

7. Boil tubes containing fly head homogenate at 100°C for 10 min. If proceeding directly to SDS-PAGE, load homogenate on gel immediately, otherwise store samples at −20°C for up to 3 months.

6.6 SDS-PAGE AND WESTERN BLOT

1. Resolve proteins in *Drosophila* head homogenates by separation on a 12% Mini-PROTEAN TGX precast protein gel (Bio-Rad catalog #4561043) for 1 h at 100 V using Tris/glycine/SDS running buffer (Bio-Rad catalog #1610732) in the Mini-PROTEAN tetra cell (Bio-Rad catalog #1658001).

   Note: The detection limit by Western blot using the conditions detailed here is 1 fly head (∼5 ng) per lane, but typically 3–5 fly heads per lane ensures a robust signal.
2. Transfer proteins in the gel to a polyvinylidene difluoride (PVDF) membrane (GenHunter Corporation catalog #B301) using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad catalog #1703930) and ice-cold transfer buffer (25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol, pH 8.3) for 1 h at 100 V.
3. Block the membrane in Rapidblock™ solution (Amresco catalog #M325) for 5 min at room temperature with gentle agitation on a rocking platform shaker.

   Note: 5% milk in PBSTw (PBS with 0.1% Tween20) is a suitable alternative for blocking and antibody dilution, particularly if the primary or secondary antibody exhibits high background.
4. Dilute the primary antibody (see Table 1) in Antibody Signal Enhancer (Amresco catalog #M336) at the optimal working concentration and incubate with gentle agitation for 2 h at room temperature (or overnight, or several days, at 4°C).
5. Wash the membrane three times for 10 min each in PBS with 0.1% Tween20 (PBSTw).
6. Dilute the appropriate secondary antibody in Antibody Signal Enhancer and incubate with gentle agitation for 1 h at room temperature.

   Note: Horseradish peroxidase (HRP)-conjugated secondary antibodies work well for enhanced chemiluminescence (ECL) detection, but colorimetric and fluorescence detection chemistries are also widely used. Typical secondary antibodies include HRP-conjugated goat–anti-mouse (ThermoFisher catalog #31430) or HRP-conjugated goat–anti-rabbit (ThermoFisher catalog #31460) used at a dilution of 1:20,000 in Antibody Signal Enhancer.
7. Wash the membrane three times for 10 min each in PBSTw.
8. Prepare the ECL working solution by mixing equal volumes (1:1 ratio) of the two substrate solutions (ThermoFisher catalog #34083). Remove the membrane from

the final wash step and incubate in ECL working solution for 2–5 min at room temperature.

9. Drain excess ECL solution and image the membrane using a ChemiDoc Imaging System (Bio-Rad catalog #17001402) or other equivalent digital imaging platform.

Note: Some imaging systems provide relative and/or absolute (using known standards to create a calibration curve) quantitation using their proprietary software package; however, densitometry can also be performed using ImageJ.

6.7 ANTIBODIES FOR DETECTING HUMAN TAU IN *Drosophila* MODELS OF TAUOPATHY

Many labs have described the use of tau-specific antibodies for Western blot (WB), immunofluorescence (IF), and enzyme-linked immunosorbent assays (ELISA) in *Drosophila* tissues. Table 1 consolidates the details from these experiments by describing the specific target to which each antibody binds (epitope) and providing the working concentration used for each antibody application (WB, IF, ELISA). For a comprehensive list of commercial, academic, and private source antibodies relevant to Alzheimer's disease and related neurodegenerative disorders, visit the AlzForum antibody database at (http://www.alzforum.org/antibodies). AlzForum categorizes antibodies by topic (the tau group contains 572 antibody listings), and the entire database is searchable by name, source, or epitope as well.

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