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### Title

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## Morphometric Analysis of Huntington's Disease Neurodegeneration in *Drosophila*

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### Abstract

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder. The HD gene encodes the huntingtin protein (HTT) that contains polyglutamine tracts of variable length. Expansions of the CAG repeat near the amino terminus to encode 40 or more glutamines (polyQ) lead to disease. At least eight other expanded polyQ diseases have been described [1]. HD can be faithfully modeled in *Drosophila* with the key features of the disease such as late onset, slowly progressing degeneration, formation of abnormal protein aggregates and the dependence on polyQ length being evident. Such invertebrate model organisms provide powerful platforms to explore neurodegenerative mechanisms and to productively speed the identification of targets and agents that are likely to be effective at treating diseases in humans. Here we describe an optical pseudopupil method that can be readily quantified to provide a fast and sensitive assay for assessing the degree of HD neurodegeneration *in vivo*. We discuss detailed crossing schemes as well as factors including different drivers, various constructs, the number of UAS sites, genetic background, and temperature that can influence the result of pseudopupil measurements.

**Key words** Huntington's disease, *Drosophila* model, Neurodegeneration, Polyglutamine disease, Pseudopupil assay, Ommatidium, Photoreceptor cell death

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### 1 Introduction

Huntington's disease (HD) is an inherited genetic disorder, characterized by a combination of chorea, cognitive impairment, and affective changes. The behavioral symptoms of HD are precipitated by progressive neurodegeneration that is particularly acute in the striatum but also involves other regions, primarily the cerebral cortex. HD is the most prevalent autosomal dominant neurodegenerative disorder associated with the expansion of unstable CAG tracts [2]. The product of the HD gene, *huntingtin* (*Htt*), is a 350 kDa cytoplasmic protein with 67 exons [3]. The amino terminus

of the HTT protein contains a CAG repeat beginning at codon 17 that encodes a glutamine repeat (polyQ) followed by two short stretches of prolines within exon 1. The CAG repeat gives rise to proteins containing polyglutamine tracts of varying size. Normal alleles have 35 or fewer glutamines while disease alleles range from 40 to over 150 glutamines [4]. The length of the CAG/polyglutamine repeat sequence is inversely correlated with the age of disease onset [5]. Despite the discovery of the HD gene almost 20 years ago, the pathogenic mechanisms of HD are still unknown. To understand degenerative mechanisms and discover methods of suppression, this dominant gain-of-function disease has been modeled by expressing mutant human huntingtin (*mHtt*) in *Drosophila melanogaster*. Multiple transgenic *Drosophila* models of HD disease have been generated [6–15]. Fly models recapitulate the core phenotypes observed in HD patients, including late onset, progressive cellular pathology as a function of polyQ repeat length, motor dysfunction, protein inclusions, transcriptional dysregulation, mitochondrial dysfunction, and shortened adult lifespan; hence, they provide powerful genetic systems for dissecting the neuronal degeneration induced by glutamine repeat-containing proteins.

As a measure of neurodegeneration, pseudopupil analysis detailed in this chapter allows characterization of the photoreceptor neurons by visualizing rhabdomeres (the light gathering organ of photoreceptor neurons) in the ommatidia (the individual eyes) of the compound eye [16]. Neurotoxicity in HD can be readily monitored by measuring the loss of visible photoreceptor neurons in the eye [11] and photoreceptor loss can be used as a quantitative marker for the *in vivo* assessment of neuronal loss [17].

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## 2 Materials

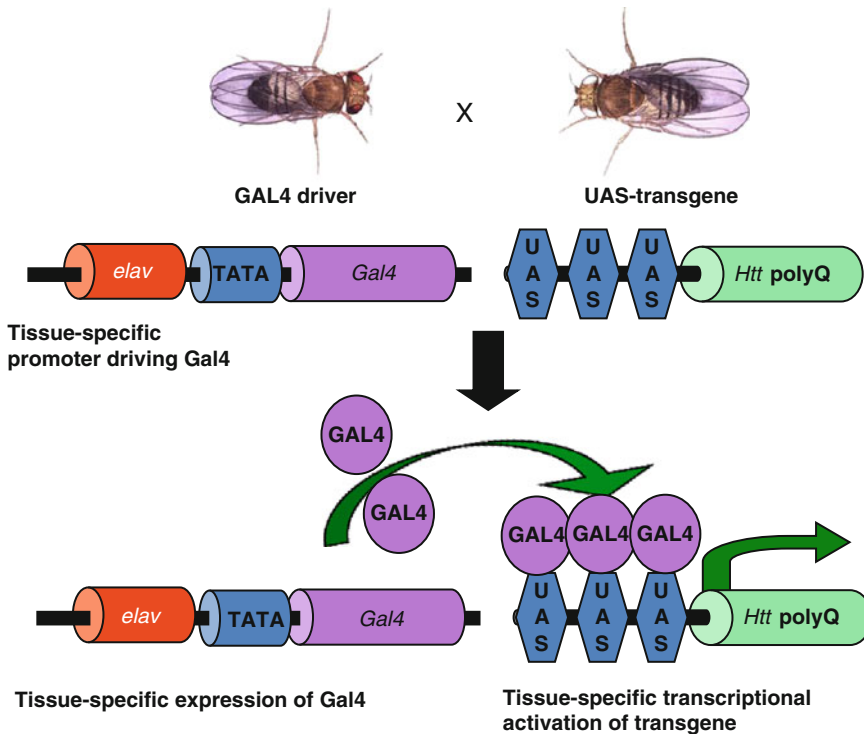
1. *Drosophila* stocks.
2. Standard *Drosophila* food.
3. Incubators.
4. Glass vials.
5. Microscope slides.
6. CO<sub>2</sub> gas supply.
7. Coverslips.
8. Nail polish (clear).
9. Tweezers.
10. 26 G × 5/8" hypodermic needle.
11. 1 cm<sup>3</sup> syringe.

12. Dissecting microscope.
13. Light microscope, e.g., Nikon Optiphot-2, with a DPlan 50 0.09 oil lens, Labophot-2 with Plan50 0.85 oil lens, or a Zeiss Axioskop 2 Plus with a Plan100 1.40 oil lens. Important features are to have an oil objective with good depth of field and the ability to adjust the condenser diaphragm to the size of a pinhole.

### 3 Methods

#### 3.1 Theory: Modeling HD Neurodegeneration in *Drosophila*

Nearly all of the current fly models of neurodegenerative diseases have been made using the GAL4/UAS system [18] which allows the ectopic expression of any transgene in a specific tissue or cell type (Fig. 1). In this system, two transgenic fly lines are used.



**Fig. 1** GAL4-UAS system used to model Huntington's and other neurodegenerative diseases in *Drosophila melanogaster*. In this example, transgenic female flies that have a human *Htt* polyQ transgene inserted downstream of a yeast upstream activator sequence (UAS) are crossed with male transgenic flies containing the transcriptional activator, GAL4, under control of the *elav* promoter (*elav-GAL4/Y*; +; + (males) and *w*; +; UAS-*Htt* ex1p-93Q females). In the resulting female progeny, the *elav* promoter induces expression of GAL4 specifically in neuronal cells of the nervous system. GAL4 then binds to the UAS sequences upstream of the *Htt* polyQ gene, activating transcription of the *Htt* polyQ gene in these cells. Male progeny will not have the *elav* driver on the X chromosome and thus will not express the *Htt* polyQ transgene

In one, the human disease-related gene is placed downstream of a yeast upstream activating sequence (UAS) that typically contains several GAL4-binding sites (“UAS-transgene”). GAL4 is a yeast transcriptional activator. In the absence of ectopically expressed GAL4, the transgene is inactive. To activate the disease gene, the UAS-transgene flies are crossed to a “GAL4 driver” line that expresses GAL4 under control of a specific promoter. A wide array of “driver” lines that express in a variety of cell types is available at the Drosophila stock centers (e.g., <http://flystocks.bio.indiana.edu/>) and other sources (<http://flybase.org/>). Drivers that are particularly useful for neurodegeneration studies include the pan-neural driver *elav* (embryonic lethal, abnormal vision) or the eye-specific promoter *GMR* (*Glass Multimer Reporter*). In the progeny of a cross, the transgene will be activated in a specific cell or tissue type, depending on the “driver”. This is especially important in studying neurodegenerative diseases, as questions regarding cell-type-specific death can be investigated.

### **3.2 Crossing Schemes to Analyze Neurodegeneration in Htt Challenged Flies**

Flies expressing mutant human *Htt* exon 1 pathogenic fragments with the pan-neuronal *elav* driver replicate key HD features such as late onset and progressive neuronal dysfunction and degeneration, leading to a decline in motor performance and premature death [11, 19]. A convenient measure of neuronal degeneration is the pseudopupil method. In this protocol, an *elav*-GAL4 driver (the strongest is the one located on the X chromosome, C155, [20]) is used to drive expression of a UAS-*Htt* transgene, i.e., UAS-*Htt* ex1p-93Q with 93Qs contained in exon 1 of the *Htt* gene [11]. Generally, a cross is made between *elav*-GAL4/Y; +; + (males) and +; +; UAS-*Htt* ex1p-93Q (virgin females) to produce progeny that include non-expressing males +/Y; +/+; UAS-*Htt* ex1p-93Q/+ and Htt-expressing *elav*-GAL4/+; +/+; UAS-*Htt* ex1p-93Q/+ females. The males, that do not express the transgene, serve as control animals. Another method is to use a line with the transgene over a marked chromosome such as a balancer and compare the transgene-expressing *vs.* non-expressing flies.

With the most toxic *Htt* fragments (e.g., *Htt* ex1p-93Q) there is considerable lethality in the larval and pupal stages when driven with *elav*. To ensure that enough adult flies are recovered for analysis, crosses can be made at reduced temperature (e.g., 22.5 °C). Virgin females (*w*; +; *Htt* ex1p-93Q) are crossed to males (*elav*-GAL4/Y; +; +) in vials containing fresh Drosophila food and the vials are kept at 22.5 °C until they eclose (about 12 days at this temperature). After eclosion at 22.5 °C, the Htt-expressing virgins and non-expressing male control flies are separated, placed at 25 °C and transferred to fresh food daily, especially if drug containing food is being used. The progeny larvae or adult flies can be used for a variety of analyses including assessing degeneration of photoreceptor neurons by the pseudopupil method [19], survival of

neurons in the larval ventral nerve cord [21], viability/eclosion rate (*see* **Note 1**), motor function by using the climbing assay, survival/longevity assay, etc.

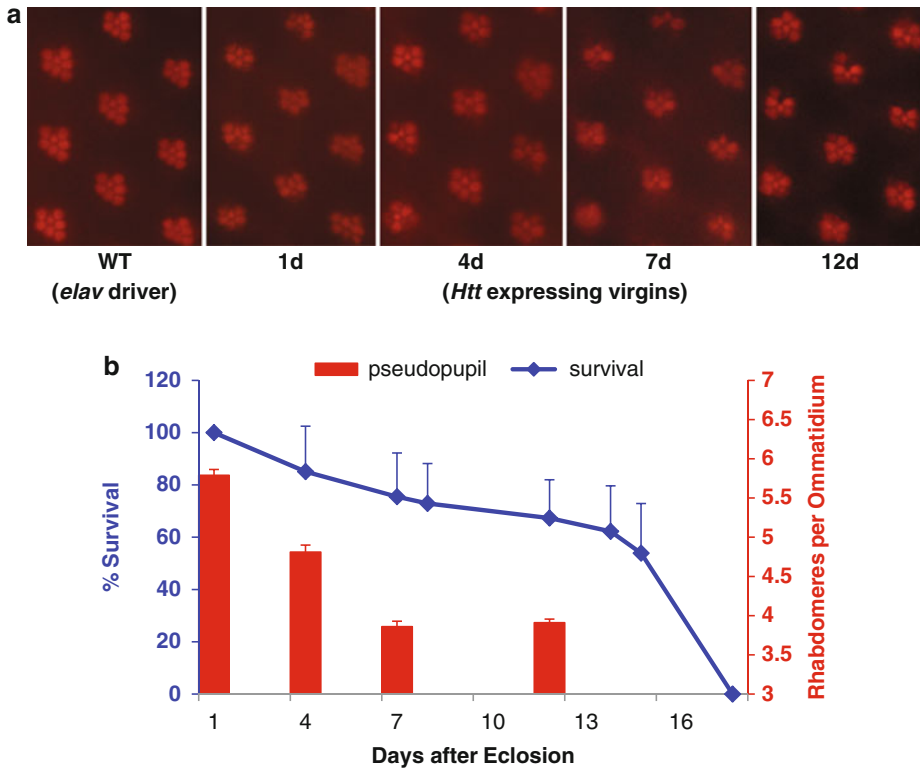
The adult compound eye of *Drosophila* consists of a unit structure, the ommatidium, that is repeated nearly 800 times in a regular symmetrical array. Each hexagonally shaped ommatidium consists of approximately 20 cells: 8 photoreceptors and 12 non-neuronal accessory cells. Each of the photoreceptor neurons forms a highly fenestrated membrane structure, the rhabdomere (subcellular light-gathering structure), and these are arranged in a regular trapezoidal array [22]. The rhabdomeres of photoreceptors 7 and 8 are stacked one above the other such that only seven rhabdomeres are seen at any given plane of focus. By measuring the loss of visible photoreceptor neurons, one can readily monitor the degree of neurotoxicity caused by a particular mutant *Htt* [11]. Adult flies expressing mutant human *Htt* ex1p-93Q exhibit progressive degeneration of the photoreceptor neurons and this can be measured by counting the number of intact rhabdomeres (*see* Fig. 2 and **Note 2**). Since the neuropathology is progressive and the number of rhabdomeres in the eyes decreases as the flies age [7, 19], freshly eclosed animals typically show mild levels of degeneration (because they have just emerged from 5 days in the pupal case where degeneration was ongoing) while the level of degeneration is more extensive and lethality is more aggravated with increasing time after eclosion (Fig. 2). For a single time point comparison, one can perform the pseudopupil assay on the 7th day post-eclosion when the degeneration has sufficiently progressed and yet death of the flies has not occurred.

### **3.3 Factors Influencing the Neurodegeneration Analysis**

Various factors, such as drivers, constructs, number of UAS transgenes, genetic background, and temperature, can influence the level of neurodegeneration. It is helpful to consider the potential advantages and challenges these factors may pose before carrying out the crosses.

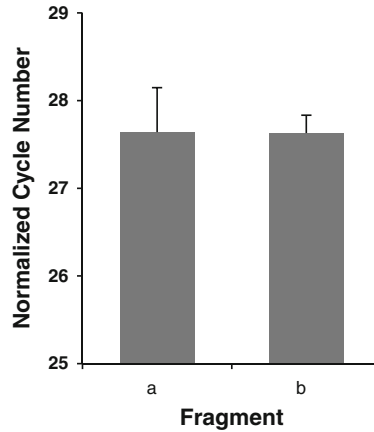
#### **3.3.1 Different Transgenes and Chromosomal Position Effects**

Different methods of creating transgenic flies have distinct advantages and disadvantages depending on the experimental objectives. In some cases, the experimental goal is to create an “allelic” series of transgenes with varied levels or patterns of expression, while in other cases the objective is to compare the pathophysiology of two different transgenes (e.g., a mutant *Htt* ex1p *vs.* one where a putative phosphorylation site has been altered (e.g., S>A)). To generate an allelic series of a particular transgene, it is convenient to generate transgenes by random, traditional P-element mediated transformation [23]. Depending on where the transgene is inserted in the chromosome and the resulting chromosomal environment, the expression level and/or patterns may be classified as strong, medium, or weak in phenotype (e.g., ref. 9).



**Fig. 2** Neurodegeneration is progressive. (a) Pseudopupil images of females from an *elav* driver line (“WT”) and representative virgin females expressing *Htt* ex1p-93Q under control of the neuron-specific driver *elav*-GAL4 aged for 1, 4, 7, and 12 days after eclosion at 25 °C. Images were taken with a Zeiss microscope with 100× oil objective at the same settings. (b) Percent survival (blue line, left vertical axis) and number of rhabdomeres per ommatidia (red columns, right vertical axis) in 1, 4, 7, 12 day old Huntingtin-challenged flies. The percent survival is based on the loss of *Htt*-expressing virgins over time ( $n=15\sim25$  each in six vials). The different numbers of rhabdomeres per ommatidium in WT, *Htt*-expressing flies aged for 1, 4 and 7 days are significant ( $p<0.01$ , *t*-test) while those between *Htt*-expressing flies aged for 7 and 12 days are not

On the other hand, if the intent is to compare the impact of two different transgenes, it is desirable that the two transgenes have the same level and pattern of expression and are in the same genetic background. For this purpose it is useful to utilize the targeted gene insertion approach that employs the phiC31 insertion system [24]. In these cases, the transgenes are all inserted into the same chromosomal location in the identical orientation and have similar levels of expression (barring expression differences occasioned by the design of the transgene itself). For example, two different *Htt* fragments of variable lengths inserted in the same chromosomal location (i.e., 51D) produce the same levels of RNA (Fig. 3).



**Fig. 3** Targeted inserts give similar expression levels. Expression of different lengths of human Huntingtin (represented by “a” and “b”) inserted at cytological region 51D in *Drosophila*. RNA expression level is quantified by qRT-PCR and represented by the cycle number normalized to that of *rp49* ( $n=3$ )

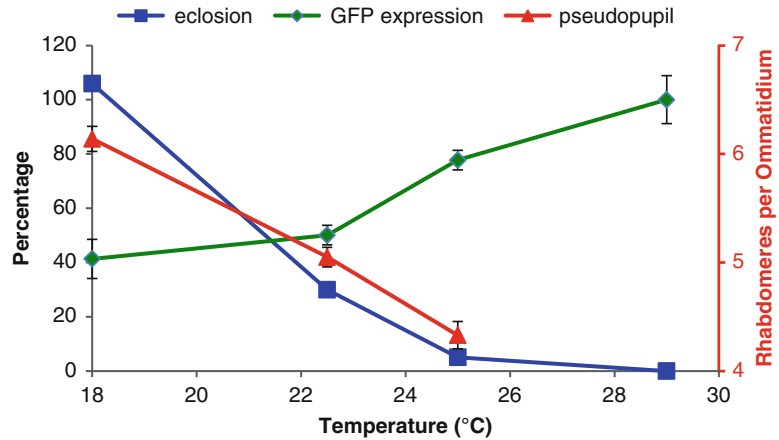
### 3.3.2 Temperature Effects

Induced expression of the transgene by the GAL4/UAS system is particularly temperature dependent. By rearing flies at temperatures ranging from 18 to 29 °C, a range of expression levels of any responder can be achieved [25]. We compared the temperature dependence of the expression of a GFP transgene and neurodegeneration in *Htt*-expressing flies at a set of temperatures ranging from 18 to 29 °C (Fig. 4). The GFP expression increases from  $72.6 \pm 12.7$  (Mean  $\pm$  SD) arbitrary units (at 18 °C) to  $175.7 \pm 15.5$  at 29 °C, an  $\sim 2.4$ -fold difference. Concurrently, neurodegeneration becomes more severe with the increase of temperature, reflecting the augmented transgene protein production. The number of rhabdomeres per ommatidium in 7-day-old flies dropped from  $6.1 \pm 0.1$  (at 18 °C) to  $4.3 \pm 0.1$  (at 25 °C). Note also that higher temperature results in greater lethality (106 % eclosion rate at 18 °C vs. 0 % eclosion rate at 29 °C and 5 % at 25 °C).

### 3.3.3 Inducible GAL4

In some cases, the experimental objective is to monitor the progression of events from a defined starting time or in a particular developmental stage. Several inducible GAL4 systems are available for this purpose. One method, the TARGET technique, utilizes a temperature sensitive mutant of the yeast GAL80 protein (GAL80<sup>ts</sup>) that specifically binds the transactivation domain of GAL4 to prevent transcription at low temperatures (i.e., 18 °C) while allowing expression of the transgene to be activated by shifting the flies to a higher temperature (i.e., 22.5 °C or greater) [26–28]. Another method uses temperature to activate a heat shock inducible FLP recombinase. The recombinase can then remove a terminator cassette flanked by FLP recognition target sequences that is placed in



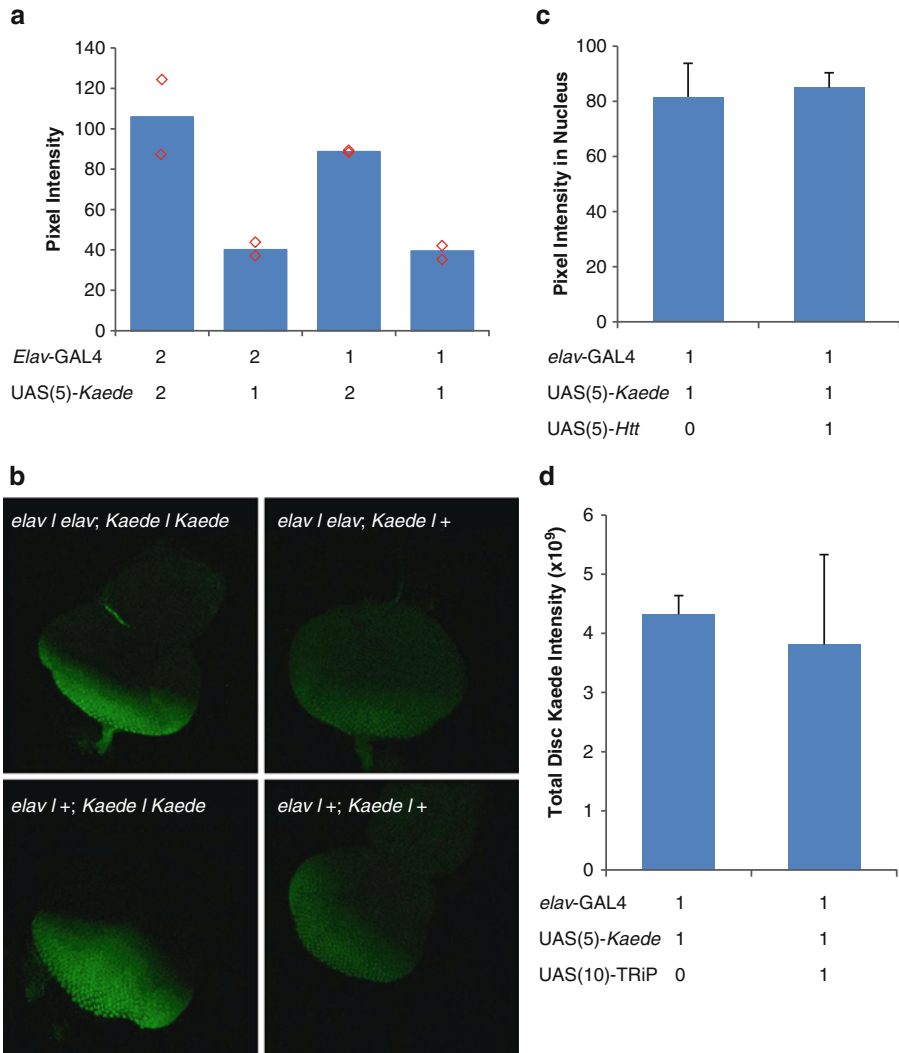


**Fig. 4** Effect of temperature on UAS-GAL4 expression. The eclosion rate (*blue line, left vertical axis*), expression of transgene GFP (*green line, left vertical axis*) and neurodegeneration measured by pseudopupil assay (*red line, right vertical axis*) in flies expressing *Htt* ex1p-93Q and mCD8-GFP (mCD8 is a transmembrane protein and GFP labels the cell surface) under control of the neuron-specific driver *elav*-GAL4 (Bloomington stock #5146) raised at various temperatures. Percent eclosion is represented by the ratio of *Htt*-expressing females to non-expressing males that eclose ( $n=3\sim5$  vials). Percentage of GFP expression in eye discs is expressed relative to the highest expression at 29 °C ( $n=3\sim5$  discs) by measuring intensity across multiple lines drawn across each disc imaged on a Zeiss LSM780. The pseudopupil assay is performed on flies aged for 7 days after they eclose at different temperatures ( $n=5\sim7$ )

front of GAL4, thus allowing expression of GAL4 and activation of the GAL4-UAS controlled transgene [27, 29, 30]. Other inducible drivers are controlled by specific ligands such as mifepristone/RU486 in a dose-dependent manner [27, 31, 32]. Where expression levels are critical, it is important to verify the relative levels in induced vs. uninduced systems.

### 3.3.4 UAS Dependence

A number of UAS-based vectors with various numbers of UAS binding sites are available [18, 33, 34]. In addition, because multiple UAS transgenes are often employed in a given experiment, the possibility of titration of GAL4 with additional UAS binding sites must be considered. In our experience, the effect of introducing a second UAS transgene on the expression of another is generally minimal. For example, in one case, we expressed homozygous or heterozygous Kaede (a fluorescent protein, [35]) with one or two copies of the *elav*-GAL4 driver and compared the Kaede intensities in eye discs (Fig. 5a, b). With one copy of *elav*-GAL4, doubling the UAS-Kaede transgene doubled the level of fluorescent protein while doubling the GAL4 driver with a fixed dose of UAS-Kaede did not change the level of expression (note that each



**Fig. 5** GAL4 is not the limiting factor with multiple UASs. **(a)** Comparison of relative Kaede expression in eye discs of flies containing various numbers of *elav-GAL4* and 5×*UAS-Kaede* constructs (*diamonds* represent high and low values from different discs). Pixel intensity is obtained by measuring intensity across multiple lines drawn across each disc ( $n=2\sim3$ ). **(b)** Representative compiled 3D confocal Z-stack images of fluorescent eye discs dissected from third instar larvae imaged on a Zeiss LSM780. **(c)** Comparison of relative Kaede expression in nuclei of salivary glands of *elav-Kaede* flies with or without the *UAS-Htt* transgene (containing 5×UASs). Pixel intensity is obtained by measuring intensity across the nucleus in each cell ( $n=5$  cells). **(d)** Comparison of Kaede expression in discs of *elav-Kaede* flies with or without another transgene (TRiP line containing 10×UASs). Total intensity of Kaede is obtained by summing up the pixel intensity of each Z stack slice of a disc ( $n=4\sim5$ )

transgene has 5×UAS binding sites). In another experiment, we monitored the level of Kaede expression in salivary glands in the presence and absence of a *UAS-Htt* transgene and could detect no influence of the extra *UAS-Htt* on Kaede expression (Fig. 5c). In a third case, the levels of UAS-Kaede expression in discs with and

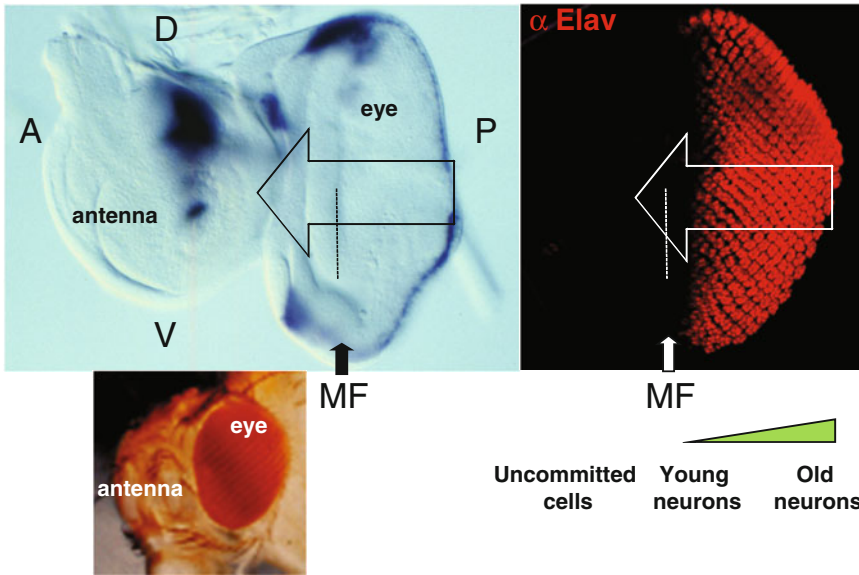
without an unrelated TRiP RNAi construct [36] that has 10×UAS sites was monitored (Fig. 5d). Again, no major change in levels was observed although an increase in the variance from animal to animal was seen. Another independent study indicates that the level of expression from a single UAS-transgene is influenced by the number of GAL4 DNA-binding sites (e.g., vectors with 10 vs. 5×UAS binding sites boost GFP levels more than twofold) [37]. These data indicate that GAL4 is in excess and that levels of expression of a transgene depend primarily on the number of transgenes and UAS/GAL4 binding sites driving their expression rather than the number of GAL4 drivers.

### 3.3.5 Drivers: *elav*, *GMR*, *Rh1*, *OK107*, *Da*, *Act*, *Arm*

For many studies, and for neurodegenerative studies in particular, it is often advantageous to test the cell type specificity of an effect. Among the GAL4 drivers available for such studies, the *elav*-GAL4 construct is particularly useful for monitoring neurodegeneration by the pseudopupil method because the *elav* promoter is expressed in all neurons of the peripheral and central nervous system beginning in embryonic stages and continuing into adult life. Importantly, its expression in the eye is limited to neuronal cells only and it is not expressed in the support cells. In addition, expression is initiated only as the morphogenetic furrow passes by and commits previously uncommitted cells to a neuronal lineage [38]. In the case of the eye imaginal disc, this provides a powerful temporal gradient of exposure of neurons to the transgene in question (Fig. 6). In the disc, there are rows of developing photoreceptor neuron clusters with each row being ~2 h older and thus having been exposed to transgene expression ~2 h longer than the row anterior to it. This driver provides chronic expression of expanded polyQs in neurons. There are several *elav*-GAL4 drivers. The X chromosome *elav* driver is the strongest and is an enhancer trap insert into the endogenous *elav* locus while the other *elav* drivers (e.g., on chr II) are fusions of the *elav* promoter and GAL4, and are weaker [39].

The GMR driver is often used to express *Htt* and other degenerative genes in the eyes [9, 19, 40] but some considerations should be noted. Because the GMR promoter expresses GAL4 in all cells of the developing eye [41], and development of the eye involves extensive cell signaling [42], the *GMR*-GAL4 driver alone exhibits a weak rough eye phenotype indicative of disturbed eye development. Expression of various peptides containing an expanded polyQ tract in all cell types of the retina with the *GMR*-GAL4 driver results in progressive pigment cell degeneration, but the level of neuronal degeneration as distinct from support cell degeneration is more difficult to assess, although the overall severity is evident (e.g., ref. 7–9).

A rhodopsin driver (*Rh1*-GAL4) induces transgene expression during the last half of pupal development in the outer six (R1–R6) photoreceptor neurons of each ommatidium in adult flies [43, 44];



**Fig. 6** The fly eye imaginal disc provides a temporal gradient of Htt challenge. In eye imaginal discs, a wave of differentiation (morphogenetic furrow, MF) passes over a field of ~30,000 uncommitted cells from posterior (P) towards anterior (A) (indicated by *open arrow*). The wave (*solid arrow* and *vertical dashed line* indicate the leading edge of the wave) consists of a line of morphogen expression. As this wave of differentiation passes, cells are “born” as neurons and expression of *elav* is initiated (*red stain*), as is expression of the *elav*-GAL4 and with it, the *Htt* transgene. Each row of cells counting from leading edge of the furrow toward posterior has been expressing the *Htt* transgene for 2 h longer than the one anterior to it

thus, not affecting neuronal development but only mature neuron survival. Expression of *Htt* ex1p-Q93 using this driver results in later onset of progressive degeneration with intact rhabdomeres present until approximately day 8 but mild degeneration with the number of rhabdomeres per ommatidium decreasing by day 12 [45].

OK107 drives transgene expression in the mushroom body and in other well-defined subsets of CNS neurons [17, 46]. Flies expressing *Htt* ex1p-93Q under the OK107 driver exhibit a 25 % loss of total mushroom body volume and show specific changes to the structure of the various mushroom body lobes and Kenyon cell bodies [17].

Interestingly, based on our observations, there is no pseudopupil phenotype with tissue-general drivers, such as *Actin* (*Act*), *Armadillo* (*Arm*), and *Daughterless* (*Da*). Typically, when driving the expression of an expanded polyQ Htt peptide, these drivers kill the animals well before eclosion and thus must be reared at lowered temperature to allow survival to adulthood. However, animals reared at low temperatures and shifted to higher temperatures after eclosion do not typically exhibit photoreceptor degeneration, although they do exhibit reduced lifespan. It is worth noting that the relative levels of expression in photoreceptor neurons by these drivers remain to be determined.

### 3.3.6 Marker and Balancer Effects

Genetic markers and balancers used for genetic stabilization and screening in some crosses may also affect phenotypes of Htt-challenged flies. For example, *Scutoid* (*Sco*), a genetic marker on the second chromosome, has a negative effect on eye phenotype in Htt-challenged flies. Likewise, *Ultrabithorax* (*Ubx*), a marker on several third chromosome balancers, also has a negative effect and thus these markers should be avoided in crosses when analyzing the pseudopupil phenotype. We have not observed any untoward pseudopupil effects with *Stubble* (*Sb*), *Curly* (*Cy*), *Humeral* (*Hu*), *Sternopleural* (*Sp*), Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) when used for tracking chromosomes in crosses.

## 3.4 Pseudopupil Method of Monitoring Photoreceptor Degeneration

The steps involved in performing a pseudopupil assay and the details for analysis of pseudopupil data are described.

### 1. Prepare a microscope slide

Clear fingernail polish is used to mount the fly head to a microscope slide. If the fingernail polish is not very tacky, place a dot of nail polish on the slide before decapitation to allow time to dry. If the nail polish is already tacky, then it can be placed on the slide after decapitation (*see Note 3*).

### 2. Decapitate the fly

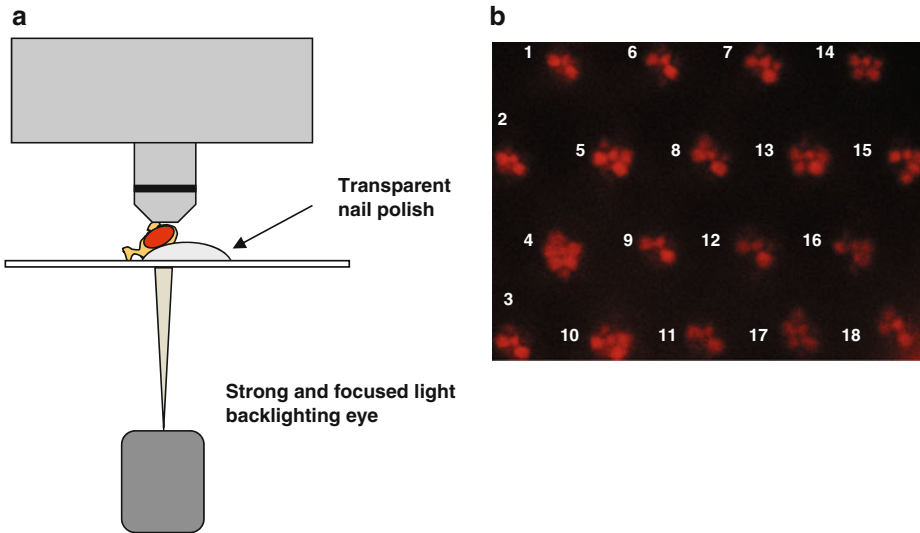
The fly should be alive before decapitation and it is necessary to work quickly through this assay to minimize the amount of cellular degeneration that will occur before the rhabdomeres can be counted. We put the fly to sleep using CO<sub>2</sub> and decapitate the fly with a hypodermic needle attached to a syringe (*see Note 4*).

### 3. Mount the head

Using forceps to grip the proboscis, place the head onto the tacky drop of nail polish in a position so that it is resting at an angle with the tangent of the eye as close as possible to parallel with the surface of the slide (Fig. 7a).

### 4. Visualization of rhabdomeres

The general strategy for visualization is to create a situation where transmitted light is shining on one side of the head such that the majority of light that one sees on the other side must come through the rhabdomeres (Fig. 7a). For example, an upright Nikon Optiphot-2, with a DPlan 50 0.09 oil lens can be used to visualize the rhabdomeres in each ommatidium. The lighting must be adjusted correctly to see the pattern clearly. Make sure that all filters are removed to maximize the amount of light getting through. Focus the condenser. With a low power objective, find and focus on the head, then move the objective aside, add a drop of oil, and swing the oil objective into place (*see Note 5*). Close the iris diaphragm to a diameter approximating the size of the head so that all light must go through the object (*see Note 6*).



**Fig. 7** Schematic of the microscope setup for pseudopupil analysis. **(a)** The fly head is immobilized on a slide with transparent nail polish and the eye is positioned for optimal visualization with a narrow beam of light illuminating the eye from below. **(b)** Example of a counting pattern for the pseudopupil assay. Start at the top of the eye and count downward until ommatidia are no longer clearly visible, then count up the next row in an S-like pattern. Continually adjust the focus of the microscope for optimal visibility while progressing from one ommatidium to another. Avoid the two rows of ommatidia on the edges of the eye where there is a greater amount of degeneration. Any rhabdomere that is not an intact circle is regarded as degenerated. For example, with the caveat that final scoring involves focusing up and down, one might score the second column (ommatidia #3–6) as, 3, 5, 4, 3

#### 5. Count the rhabdomeres

Start at the top of the eye and count down until you are no longer able to see clearly defined ommatidia, and then count back up the next row (as shown in Fig. 7b, also *see Note 7*). Any rhabdomere that is not an intact circle is regarded as degenerated. Be consistent in counting each eye. In an HD fly, the number of visible intact rhabdomeres varies from 0 to 7, and the structure of rhabdomeres in the ommatidium becomes disorganized (Fig. 7b). A given experiment should typically be scored by one individual, and it is best to score the eyes blind. Record at least 30–40 ommatidia per head and score at least five heads for each genotype/treatment.

#### 6. Data analysis

Sum the total number of ommatidia with different numbers of rhabdomeres (0–7) and obtain the average. Two sets of pseudopupil data from different genotypes or drug groups can be compared for statistical significance using the Student's *t*-test (*see Note 8*). It is sometimes useful to graph the distribution of the number of photoreceptors per ommatidium for comparison.

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## 4 Notes

1. For analyzing viability and assessing eclosion rate, the set up of the crosses will be different from the one presented here for pseudopupil analysis. We typically set up at least ten vials of crosses with five pairs of virgins and males in each vial and keep the parents for 4–5 days before emptying the vials. We then count the number of flies of each genotype each day as they eclose and express the eclosion rate as the ratio of *Htt*-expressing flies *vs.* non-expressing controls.
2. One's ability to see the rhabdomeres depends on the level of pigment in the eye. In flies with very little pigment, the rhabdomeres can be impossible to see by this technique; thus, it is better to use a driver that has a strong mini *w+* or cross a wild-type *w+* gene into your *w-* transgenic flies. If pigmented eyes are not possible, another measure of retinal degeneration is to fix and section the eye and measure the retinal thickness [21].
3. We have successfully used Sally Hansen's "Advanced Hard as Nails" clear nail polish. It is best to use an older bottle of fingernail polish in which the polish is slightly tacky. Older nail polish can be mixed with newer nail polish to produce an ideal moderately tacky consistency. This helps keep the eye in its upright position. As an alternative, a glob of Vaseline (petroleum jelly) can be used but is less stable than fingernail polish.
4. Within 15 min after decapitation, the pseudopupil image will begin to fade. To decapitate flies, we use forceps to hold the fly body with its ventral side down or on its side on a microscope slide under a dissecting scope and use a 26 G×5/8" needle attached to a 1 cm<sup>3</sup> syringe to cut through the neck, taking care not to touch the eyes at any point during the dissection. Some methods of manipulation cause the proboscis to extend more than others. The proboscis can then be used as a handle to move the head into position without damaging the eyes.
5. It is better to lower the stage a bit by turning the coarse adjustment knob about a quarter turn before changing from a low magnification objective to the oil objective to avoid the objective touching or damaging the head.
6. By closing or opening the diaphragm you can increase or decrease the contrast in the ommatida.
7. It is usually necessary to refocus and re-center the eye in the field of view for optimal visualization. Focus just below the highest point of the curved surface of the eye. Choose a plane of focus where you can clearly see and count the rhabdomeres in ≥30 ommatidia. Continually adjust the focus of the microscope for



optimal visibility as you progress from one ommatidium to another, avoiding the two rows of ommatidia on the edges of the eye where there is a greater amount of variation.

8. A two-tailed unpaired *t*-test is appropriate if one is comparing the effect of a drug or genetic manipulation on flies that already have some level of degeneration (e.g., flies expressing the mutant *Huntingtin* exon 1 peptide where the average number of rhabdomeres per ommatidium is about 4.0). A paired *t*-test would be used in a case where the same fly is tested before and after a specific treatment.

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