An accessible digital imaging workflow for multiplexed quantitative analysis of adult eye phenotypes in *Drosophila melanogaster*

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Abstract

The compound eye of *Drosophila melanogaster* has long been a model for studying genetics, development, neurodegeneration, and heterochromatin. Imaging and morphometry of adult *Drosophila* and other insects is hampered by the low throughput, narrow focal plane, and small image sensors typical of stereomicroscope cameras. In educational environments, where data collection may be distributed among many individuals over extended time periods, these limitations are compounded by inter-operator variability in lighting, sample positioning, focus, and post-acquisition processing. Our goal was to develop an affordable and student-friendly method for multiplexed quantitative analysis of adult *Drosophila melanogaster* phenotypes. Here we report a system for efficient data collection and analysis of up to 60 adult flies in a single image with standardized conditions that eliminate inter-operator variability and enable precise quantitative comparison of adult morphological phenotypes. Semi-automated data analysis using ImageJ and R reduces image manipulations, facilitates reproducibility, and can be adapted to emerging automated segmentation methods. This collection of classroom-friendly methods doubles as a hands-on introduction to imaging, data visualization, and statistical analysis for students.

Introduction

The early decades of plant and animal genetics relied on visible morphological traits to reveal the function and inheritance of genes – in peas, flies, maize, and mice, traits defined by size, shape, and color illuminated for the first time the existence and behavior of genes, chromosomes, transposons and more. Tools to analyze more directly the underlying molecular processes are now abundant and powerful, but the importance of physical traits persists, as phenotypes *per se* and also as indirect reporters for genetic interactions. In contrast to the explosive pace of change in molecular techniques, whole-animal imaging has changed slowly and is hampered by a lack of scale: most microscope image sensors have small fields of view and narrow focal planes that photograph a small number of mostly out-of-focus animals. Small working distances on compound microscopes and inconsistent lighting on stereomicroscopes are additional barriers to successful whole animal analysis.

Focus stacking or z-stacking can generate high quality images of 3 dimensional samples for entomology collections (Droege and Gutierrez 2024), and for cataloging of phenotypes (Holtzman and Kaufman 2013). Many Drosophilists' first act as PI is to hang the *Learning to Fly* poster (Childress, Behringer, and Halder 2005) in their fly rooms, but for most labs creating such images is out of reach.

Since Thomas Hunt Morgan isolated the first white-eyed mutant of *Drosophila melanogaster* (Morgan 1910) and Hermann J. Muller generated heterochromatin-silenced chromosomal inversions (Muller 1930), studies of the fly eye have made foundational contributions to our

understanding of gene expression and development. Among these is a century of work to understand the formation of heterochromatin and its role in gene regulation (Elgin and Reuter 2013). Fly eye phenotypes are powerful tools for studying neurodegeneration (McGurk, Berson, and Bonini 2015) and for identifying causal mutations in (and possible treatments of) human disease (Dalton et al. 2022; Manivannan et al. 2022). Spectrophotometric measurement of eye pigment from homogenized flies is quantitative (Huisinga et al. 2016) but collapses inter- and intra-individual variation into a single value. Photographic measurement of eye color is quantitative and captures variation in patterns and levels (Kelsey and Clark 2017; Swenson et al. 2016; Iyer et al. 2016; Diez-Hermano et al. 2020; 2015) but has limited throughput.

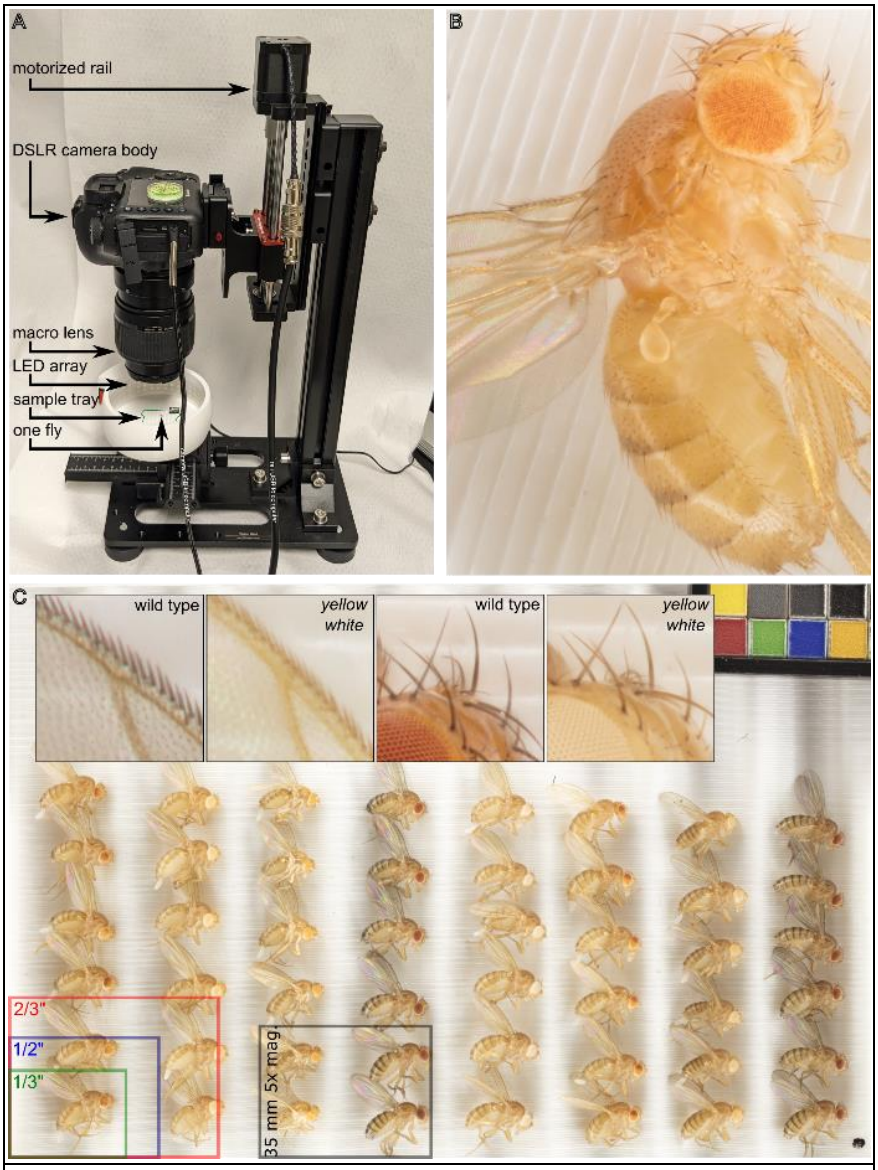

Figure 1. A digital photography workflow for multiplexed analysis of Drosophila melanogaster *phenotypes. (**A**) Canon EOS 5Ds camera body and macro lens mounted to motorized rail and mounted on a vertical stand. Camera and rail are independently connected to and remote-controlled from a Windows computer by USB cables. Flies are positioned on a 3D printed grooved sample tray (outlined in green) and illuminated by 360° LED lighting system on a manually adjustable XY stage. A single fly, colored in purple, is pictured to illustrate scale. (**B**) Focus-stacked 5x magnification image of Drosophila melanogaster with PEV phenotype. (**C**) Full frame image of 48 adult animals of various genotypes as well as a color-checker to ensure consistency. Top inset, from left to right, 5x magnification images of wild type and yellow wing edge, wild type and yellow white ocellus and head bristles. Bottom overlay, areas of common microscope camera image sensor formats and of our system at 5x magnification.*

Here we describe a new protocol for cost-effective reproducible multiplexed photography and quantitative analysis of up to 60 adult *Drosophila* in a single image using a full frame digital camera and macro lens mounted on a motorized rail. Focus stacking (also called z-stacking) combines the most focused pixels of each image into a single composite. Semi-automated data extraction and analysis using ImageJ and R facilitate code sharing and reduce intermediary data products. Inter-operator variability among students with a wide range of experience was between 2% and 6%. This classroom-friendly method supports undergraduate learning outcomes in scientific imaging, data visualization, and statistics.

Materials and Methods

Stocks Used

Name	*Genotype*	*Source*
Dorsal eye	y[1] w[*]; wg[Sp-1]/CyO; P{w[+mW.hs]=GawB}mirr[DE]/TM3, Sb[1]	BDSC 29650 (Morrison and Halder 2010)
lacO 010	*yw;* P{y+ lacO hsp70-white}	P element mobilization of 1198-lacO from the Elgin lab
lacO 025	*yw;* P{y+ lacO hsp70-white}	P element mobilization of 1198-lacO from the Elgin lab
wm4	*y In(1) w^{m4}*	Kind gift of Elgin lab (Muller 1930)
wt 1	MGWT	Isolated in Heidi J.J. Pipkin's kitchen, Maple Grove, MN
wt 2	RAL365	The Drosophila Genetic Reference Panel (Mackay et al. 2012)
eya composite-GAL4	PBac{y+-attP-3B}VK00033	Kind gift of Justin Kumar (Weasner et al. 2016)
mini-white	*yw*; ; eyGal4*	Kind gift of Justin Kumar (Weasner et al. 2016)
White eraser	w[*]; P{y[+t7.7] GFP[3xP3.cUa]=white-eraser}attP2	BDSC 90371 (Liu et al. 2020)
HP1 RNAi 1	y[1] sc[*] v[1] sev[21]; ;P{y[+t7.7] v[+t1.8]=TRiP.HMS00278}attP2	BDSC 33400 (Zirin et al. 2020)
HP1 RNAi 2	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL00531}attP40/CyO	BDSC 36792 (Zirin et al. 2020)

Fly Husbandry and Crosses

Almost all GAL4 stocks use a dominant *white* transgene as a positive selection marker for the presence of GAL4, preventing measurement of *white* reporter gene expression. We used "white eraser" flies expressing Cas9 and guide RNAs (Liu et al. 2020) to eliminate mini-white expression. Female "white eraser" flies were crossed with males carrying GAL4 driven by a composite enhancer constructed from regulatory elements of the *eyes absent (eya)* gene (Weasner et al. 2016) on the third chromosome. Male flies with *eya composite-GAL4* (hereafter referred to as *eya-GAL4*) and *P{white eraser}* were crossed to third chromosome balancer stocks, and individual *white* males with *eya-GAL4* but without the fluorescent markers indicating the presence of *P{white eraser}* were used to establish new white eyed *eya-GAL4* stocks. These stocks were then crossed to *yellow* flies carrying the X ray-induced w^{m4} inversion (Muller 1930) to generate the driver-reporter stock *y* w^{m4}*; ;eya-GAL4*$^{w-}$ (hereafter abbreviated w^{m4}; *eya-GAL4*).

All crosses were incubated on standard Bloomington media (Nutri-Fly BF, Genesee Scientific) at 25°C and at least 60% relative humidity. For generation of eye color variation, 2-4 males of each eye pigment stock were crossed to 6-10 unmated *yw* females in 3 independent vials. For RNAi knockdown experiments, unmated females of the driver-reporter stock (w^{m4}; *eya-GAL4*) were crossed with two different fly stocks created by the Transgenic RNAi Project (Zirin et al. 2020) targeting *Su(var)205,* aka HP1 (James and Elgin 1986). For all crosses, 2-4 day old adult progeny were collected and quickly frozen at -20°C for later analysis.

Image Acquisition

Frozen flies were thawed and arranged on a custom designed 3D-printed sample tray with grooves to facilitate spatial arrangement and a color control to ensure consistency between images (Matte ColorGauge Pico, #87-414, Edmund Optics). The sample tray was then placed in a 3D printed 360° lighting system with 120 white LEDs (color temperature 6000K) on an adjustable XY stage centered under the image sensor (Figure 1). A translucent white mylar strip was taped over the LEDs to act as a diffuser. A parts list and all design files for lighting and mounting can be found at https://www.thingiverse.com/thin g:4688444.

We used a Canon MP-E 65mm f/2.8 1-5x Macro lens attached to a Canon EOS 5Ds camera with 50 megapixel 36 x 24 mm CMOS sensor, pixel pitch of 4.13 µm and pixel area of 17.06

µm². The camera and a vertical rail (WeMacro 100 mm rail #WM001 and vertical stand #WVH01) were controlled from a computer running Windows 10 and Helicon Remote version 3.9.11. Images were acquired in RAW format with exposure of 1/25 s, f/2.8, and either ISO 100 (for 1x magnification) or ISO 500 (for 5x magnification). Rail travel from top to bottom is 2750 µm made up of 55 steps at 50 µm each. The 56-image stack was automatically exported from Helicon Remote to Helicon Focus version 7.7.5 and a composite TIF image combining the most-focused pixels of each individual image was generated using the "C, smoothing 4" setting. All original images described here are publicly available (Arsham 2024).

Data Analysis

Individual users defined a region of interest (ROI) for every eye in an image using the elliptical ROI tool in the FIJI distribution of ImageJ (version 1.53). The BAR plugin (version 1.51 (Ferreira et al. 2017)) was used to apply a standardized colon-delimited naming convention to all ROIs specifying sex, genotype, replicate number, user, and other key experimental variables. A custom ImageJ macro converted the image to RGB, inverted the colors so that higher pigment levels (darker red eyes) correspond to higher RGB values, and converted to 8-bit grayscale using ImageJ's built-in weighted grayscale conversion:

```
row = 0; //resets results row
      roiCount = roiManager("count");
      for (i=0; i<roiCount; i++) {
            run("RGB Color");
            run("Conversions...", "scale weighted");
            run("8-bit");
            roiManager("select", i);{  //start loop
                  Roi.getBounds(rx, ry, width, height);
                        for(y=ry; y<ry+height; y++) {
                  for(x=rx; x<rx+width; x++) {
                  if(Roi.contains(x, y)==1) {
                        setResult("ROI", row, Roi.getName);
                        setResult("pixel", row, getPixel(x, y));
                  row++;
    }
   }
  }
 }
}
```

Each pixel has a single grayscale color value between 0 (white) and 255 (black) that correlates to eye pigmentation. The ImageJ Macro saves the grayscale value of each pixel from each segmented eye into a single CSV file that is saved alongside the original (still unmodified) image file. CSV files are imported into R Studio and data from multiple images concatenated into a single dataframe for all conditions and replicates. The colon-delimited ROI identifiers contain the experimental conditions for each pixel and are separated into factors so that any pixel can be grouped by sex, genotype, replicate, user, etc. All code and data described here are publicly available

Results and discussion

Principles of computational biology

Our goal was to develop an affordable and student-friendly method for multiplexed quantitative analysis of adult *Drosophila melanogaster*. We also wanted to follow sound data management and computational practices by automating as many steps as possible, minimizing manipulations of original photos, and eliminating intermediate data products to create simple clear paths from data to analysis that can be run by any scientist at any time (Royle 2019). To this end, the only manual step after arranging the flies on the sample tray is to use built-in ImageJ tools to label each eye by defining their boundaries on an XY coordinate plane. This process, known as image segmentation, converts human visual pattern recognition into a computer readable list of coordinates.

The system described here, including all hardware, software, and 3D printed parts, can be assembled for less than $5,000 with used camera equipment, substantially less than commercially available “macroscopes.” While proprietary software is used to capture and process the images, all post-acquisition analysis steps use freely available open-source cross-platform software.

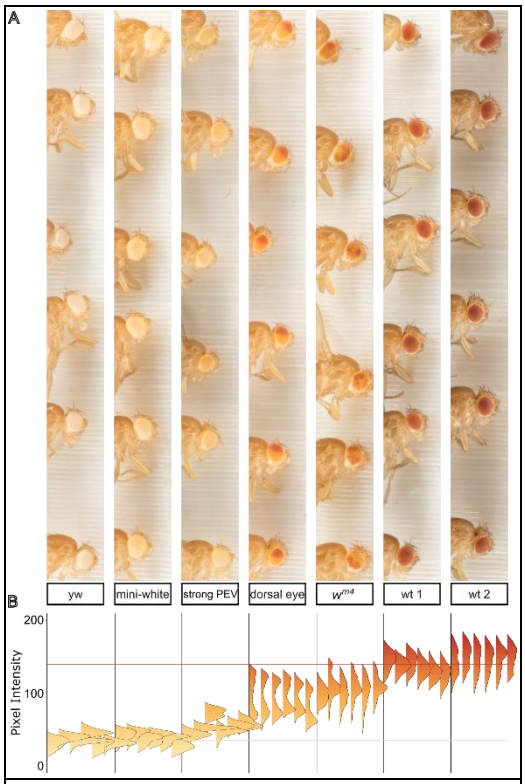

*Figure 2. Pigment analysis of eye color mutants. (**A**) A single unmagnified macro image of flies from various stocks was cropped and arranged in order of pigment expression. Individual eyes were segmented in ImageJ and the intensity value of all pixels of each eye are displayed as a vertical histogram (**B**). The beige horizontal line at y = 35 denotes average pixel intensity value for white eyes; red horizontal line at y = 140 denotes average pixel intensity for wild type.*

Analysis of color variation in *Drosophila* mutants

Microscope cameras are typically optimized for high sensitivity and low noise for light-limited applications like fluorescence or for small flat areas like slide-mounted samples. These trade-offs are poorly suited to applications with abundant illumination but larger three-dimensional objects like whole insects. Our system optimizes for sensor size and resolution under bright light rather than optimizing for signal/noise ratio. The area of a 35 mm image sensor is 8.4 x 10^8 microns (Figure 1 shows the approximate size of common microscope image sensors for comparison) and with no magnification (a 1:1 or true macro image) can capture up to 60 flies, with each individual fly comprising about 0.7 megapixels (Figure 1 and Figure 2). At 5x magnification (the maximum optical zoom of our macro lens) phenotypes of individual ommatidia, ocelli, bristles, and wing cells are clearly visible (Figure 1B).

Pixel intensity values within or across experiments can be grouped or compared using any experimental variables, and histograms of pigment intensity in individual eyes can reveal bimodal or other non-normal pigment distributions (Figure 2B). To sample and compare multiple populations of flies we generated a single mean pixel intensity value for each eye and a population mean from all the eyes. To visually orient the viewer and provide internal landmarks for comparison we established average mean eye color values for *white* (pixel intensity = 35)

and wild type (pixel intensity = 140) flies and plot standard lines on each graph: a beige line to represent white eyes and a red line for wild type.

This system compares favorably in throughput and precision with other computational approaches to eye color (Kelsey and Clark 2017; Swenson et al. 2016), and we tested it on several phenotypically distinct populations of flies. These included flies with no eye pigmentation (*yw),* a low expression mini-white transgene (mini-white) producing very light yellow eye color, a stock from our lab that expresses a mini-white transgene that is stochastically silenced (strong PEV), the "dorsal eye" stock in which pigmentation is spatially and spectrally bimodal (Morrison and Halder 2010), the w^{m4} inversion generated by Hermann J. Muller in his studies of the effect of X-rays on inherited phenotypes (Muller 1930), and two wild-type stocks, one isolated locally and one obtained from the *Drosophila melanogaster* Genetic Reference Panel (Mackay et al. 2012), labelled wt 1 and wt 2 respectively. In each case we crossed the indicated stock with *yw* so that all groups were heterozygous for whichever form of the reporter gene they carried. Three independent crosses were set up for each genotype and adult female progeny were collected, frozen, and photographed.

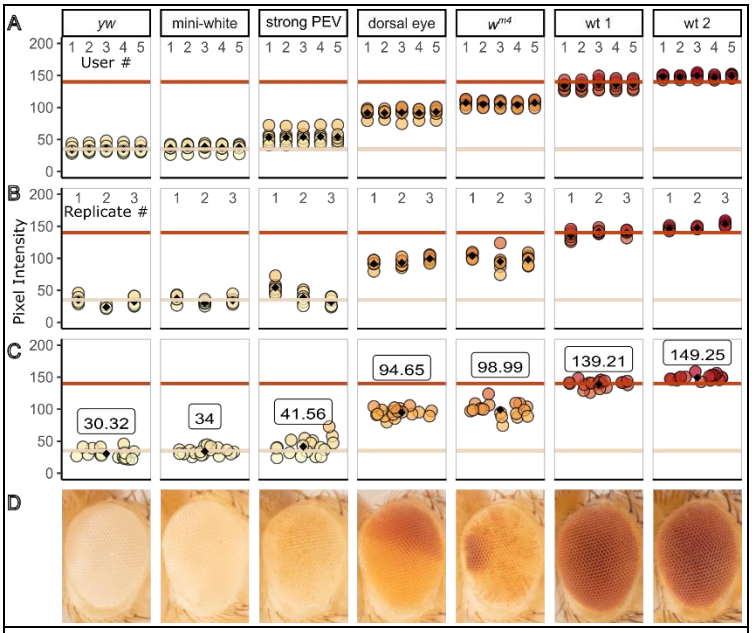

Figure 3. Morphometric analysis of eye pigment data demonstrates precision, accuracy, and reproducibility. Seven fly stocks with different eye color phenotypes were crossed to yw *and three independent biological replicates were collected for analysis. A single experimental image was independently segmented by five undergraduate students with lab experience ranging from a month to four years (**A**); a single experienced student analyzed three independent biological replicates (**B**); and all three replicates from (**B**) were pooled for quantitative analysis in (**C**). Beige horizontal line at y = 35 denotes the average pixel intensity value for a* white- *mutant; red horizontal line at y = 140 denotes the average pixel intensity for a wild type eye. For visual reference, representative images from the same experimental groups taken at 5x magnification are shown in (**D**).*

A single photo containing 6 flies from each genotype (such as that shown in Figure 2A) was given to 5 undergraduate students ranging in lab experience from a few weeks to a few years and each student independently segmented the image and extracted the pixel intensity data. Figure 3A shows that there is no substantial difference in performance between the students despite their varying lab experience. Variation between the highest and lowest student averages was 5.8% for the lightest eyes and 4% or below for all other stocks. We then photographed 3 independent biological replicates in three separate images for analysis by a single experienced research student. Biological variation is evident across the three replicates but even modest inter-group differences are easily detectable (Figure 3B). Finally, flies from all three single-user biological replicates in Figure 3B are combined into a single graph to compare the overall group means (Figure 3C) as in a typical experiment comparing genotypes or treatments.

When testing the impact of genetic or environmental variation on eye pigmentation we designate a control and a treatment group for basic null-hypothesis testing, comparing the treated and untreated populations using Student's t test. To test the system in a real-world scenario, we crossed a reporter-driver stock with variegating eye color and eye-specific GAL4 expression (*w^{m4}; eya-GAL4*) with flies carrying a UAS-driven RNAi against heterochromatin protein 1 (James and Elgin 1986). Heterochromatin silencing of the translocated *white* gene in flies with the *w^{m4}* inversion causes most ommatidia to have very light pigmentation. Disruption of heterochromatin formation in the eye by RNAi knockdown of HP1 more than doubled the average pigment intensity ($p<2 \times 10^{-16}$; Figure 4A) – while some variegation is still visible in the RNAi knockdown eye, the average pigmentation levels are similar to wild type flies. Representative images are shown below each group (Figure 4B).

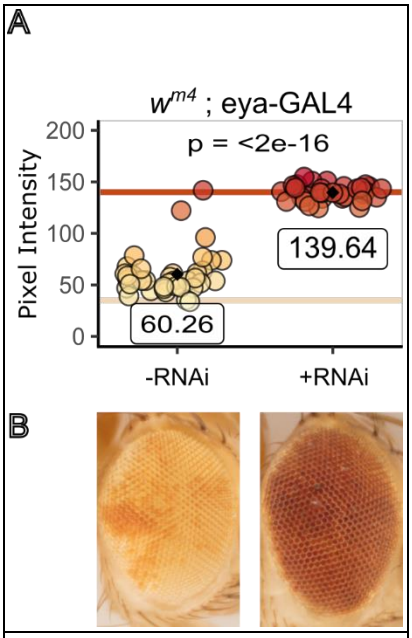

*Figure 4. RNAi knockdown of Su(var)205 (HP1a) results in a substantial and significant suppression of variegation (**A**). For visual reference, representative images from the same experimental groups taken at 5x magnification are shown in (**B**).*

Other applications and future directions

The workflow described here could be useful to biologists seeking to collect consistent morphometric data on shape, size, and color of features as small as 10 µm. For example, studies of pupal size (Sriskanthadevan-Pirahas et al. 2022), wing vein patterning (Alba et al. 2021), photoreceptor neurodegeneration (Dalton et al. 2022), or eye mosaic or clonal analysis (Merkle et al. 2023) could be accelerated by the capacity to image and measure hundreds of samples in a single image. Photographic analysis of pigmentation also preserves population variation, allowing for a variety of different quantitative measurement or qualitative scoring systems to be applied. This could facilitate studies of traits like pigment gene regulation (Akiyama et al. 2022). It could also be used for genome wide association and QTL mapping, such as in recent studies of the genetic architecture of abdominal pigmentation across populations of *D. melanogaster* (Dembeck et al. 2015) or the *Drosophila* genus (Ng et al. 2008; Signor et al. 2016). In many of the above examples sample preparation involves a labor-intensive combination of immersion, dissection, or mounting, which are dramatically simplified here.

A variety of computational methods have been developed to automatically segment fly eyes or individual ommatidia (Currea et al. 2023) within an eye, and to reproducibly measure photoreceptor neurodegeneration (Iyer et al. 2016; Diez-Hermano et al. 2015; 2020). But using eye morphology as a readout for gene expression and interaction means that in any given experiment the eyes can have irregular phenotypes, confounding automated image segmentation algorithms which rely on consistent size, shape, color, or position. Where powerful computers with large training datasets struggle, a student can quickly and reliably identify a fly's eye under the microscope on their first day in lab, even if that eye is a non-standard shape, size, or color. While Figure 3A demonstrates that students always identify and segment fly eyes with a high level of precision, this step is labor-intensive and perhaps can soon be automated (Kirillov et al. 2023; Ma and Wang 2023). Focus stacked macro images like the ones described here could then be fed into computational pipelines like those used to screen pharmaceutical

candidate compounds (Stirling et al. 2021).

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Declaration of interest statement

The authors declare no conflict of interest.

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