References: Delaunay, J., 2007, Blood Rev. 21: 1-20; Hatta, H., K. Tsuneyama, T. Kumada, H. Zheng, C. Cheng, C. Zhengguo, H. Takahashi, K. Nomoto, Y. Murai, and Y. Takano 2006, Pathol. Res. Pract. 202: 439-45; Hite, S.A., and Y.T. Huang 1996, J. Clin. Microbiol. 34: 1819-20; Leong, A.S., and J. Milios 1986, J. Pathol. 148: 183-7.

Protein trap lines of *Drosophila* to demonstrate spatio-temporal localization of proteins in an undergraduate lab.

Puli, Oorvashi Roy[1], and Amit Singh[1,2,3,4]. [1]Department of Biology, University of Dayton, Dayton, OH 45469, USA;   [2]Premedical Program, University of Dayton; [3]Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton; [4]Corresponding Author: Amit Singh; Email: amit.singh@notes.udayton.edu

Abstract

The objective of this teaching note is to generate a laboratory exercise, which allows students to get a hands-on experience of a cell biology technique. The short duration of the laboratory classes is the biggest challenge with the development of a cell biology lab for an undergraduate curriculum. Therefore, it is necessary to design a laboratory exercise that enables the students to carry out cell biological assays in the desired time. This laboratory exercise focuses on tracking protein expression levels along a spatial (space) and temporal (time) axis in developing *Drosophila melanogaster* organ primordium. Here we use the protein trap model developed in *Drosophila* to demonstrate the sub-cellular localization of proteins. The protein trap transgenic flies have Green Fluorescent Protein (GFP) reporter tags to the full-length endogenous proteins that allow observation of their cellular as well as sub-cellular distribution. Since the life cycle of *Drosophila* is short, it is easy to rear them in the lab and also use them as an excellent model for an undergraduate lab curriculum. The goal of this exercise is to train undergraduate students and teach them the use of one such powerful tool which enables the localization of proteins.

Introduction

The present day undergraduate pedagogy puts great emphases on quantitative reasoning and inquiry-based activities in a laboratory experience. It is widely accepted across the teaching community that promotion of intellectual development through habit of enquiry is an important pillar of learning in undergraduate education. We have been introducing new lab exercises in our Cell Biology (Bio-440 Lab) lab curriculum by exploiting the tools available in *Drosophila melanogaster,* a.k.a. fruit fly, model system. The short generation time, ease of handling, high reproductive ability, and wide array of genetic tools make *Drosophila* an excellent choice for demonstration of biological phenomena in the undergraduate labs (Tare and Singh, 2009; Tare *et al.,* 2010).

There are several approaches available to analyze gene expression in the tissues. The commonly used methods are visualizing gene expression by enhancer trapping, epitope tagging,

antibody staining, or gene trapping. These techniques like enhancer trapping and gene trapping essentially involve generation of transgenic animals. Since *Drosophila* has proved to be a versatile organism for transgenics, it can be used for the study of gene expression and protein localization. The protein trap strategy is a modification of gene trapping which allows epitope tagging of the endogenous proteins of interest. The principle of this approach is to tag proteins by an epitope. Protein trapping is achieved by fusing the endogenous messenger of a gene with the DNA sequence encoding the reporter genes like Green Fluorescent Protein (GFP) (Morin *et al.,* 2001; Buszczak *et al.,* 2007; Kelso *et al.,* 2004; Quiñones-Coello *et al.,* 2007). Thus, protein trapping allows spatio-temporal localization of the protein of interest. A transposable artificial exon, which encodes a GFP protein and is flanked by both splice acceptor and donor sites, can get inserted into the endogenous coding region of a gene. It may result in a chimeric protein harboring the GFP reporter, which allows the localization of endogenous proteins. The full-length endogenous proteins resulting from protein trap in *Drosophila* can be seen as GFP fusion proteins from their endogenous promoters. The fusion of reporter serves as an excellent tool to study cellular and sub-cellular localization of the proteins.

Some protein traps lack the initiation codon and are fused to the N-terminal region of endogenous proteins and are flanked by acceptor and donor sites, which are inserted into an intron separating the exons coding for a chimeric protein in which the GFP is fused with the amino and carboxyl terminal of the trapped protein. So the reporter is expressed only if it gets integrated into the region of the target gene causing GFP expression, hence called Protein Trapped lines (Morin *et al.,* 2001; Buszczak *et al.,* 2007; Kelso *et al.,* 2004; Quiñones-Coello *et al.,* 2007). These protein trap lines have been generated by various labs and are available on request.

The potential of this technology has been extensively documented in yeast, as evident from large collections of protein trap strains generated by using transposable elements or by homologous recombination. Protein trapping has also been employed in cultured embryonic stem (ES) cells.

Protocol

Protein-trap transgenic flies were procured from *Carnegie* protein trap library (http://flytrap.med.yale.edu/index.html). These flies were reared on yeast-cornmeal-agar fly medium at room temperature. Fly food rearing medium is also commercially available (http://lab-express.com/). *Drosophila* life cycle comprises of 24 hours of embryonic development, followed by three larval instar stages. The larva develops into pupa, and adults emerge from pupa upon metamorphosis. *Drosophila* is a holometabolous insect. In *Drosophila*, the precursors of adult organs are housed inside the larva as a monolayer epithelium called imaginal disc. The imaginal disc is a favored model system to study patterning, growth, and differentiation. Imaginal discs are monolayer epithelium comprising of the groups of epithelial cells housed inside the larva that will give rise to adult appendages and cuticle after metamorphosis. We have used the eye-antennal imaginal disc, wing imaginal disc, and leg imaginal disc for this study. The eye-antennal imaginal disc gives rise to eye and the antenna in the adult fly, the wing imaginal disc forms the wing, and the leg imaginal disc forms the leg in the adult fly.

The third instar larvae were selected based on their size and were dissected in Phosphate Buffered Saline (PBS, containing 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4; Dulbecco and Vogt, 1954) using sharp Dumostar forceps (Electron Microscopy Sciences Cat. No. # 72707-01). The salivary glands and imaginal discs attached with mouth parts and brain were dissected. We isolated the leg, wing, and eye-antennal imaginal discs to study the protein trap expression.

Sample preparation (staining)

The larvae were dissected in PBS, and imaginal discs were fixed for twenty minutes with 4% paraformaldehyde (EMS Cat. No. # 15710) in PBS. The fixation is required to preserve and maintain the morphology of the tissue. After fixation, the fixative was removed by rinsing the tissue with ice cold PBS, which was followed by three washes (of ten minutes each) with PBST [PBS+ 0.2% Triton X-100 (Sigma Aldrich Cat No. # T100)]. The PBST washes allow the permeabilization of the tissue. The tissue was then incubated with dyes in PBS for twenty minutes at room temperature in the dark. The dyes used in this study are – Phalloidin Texas Red (Molecular Probes, Invitrogen, Cat. No. # T7471) and DAPI (1, 4', 6-diamidino-2-phenylindole; Molecular Probes, Invitrogen, Cat. No. # D 1306). Phalloidin specifically marks the actin filament meshwork (Small *et al*., 1999). The DAPI stains the nuclear material within the cell by binding to AT clusters in the minor groove of double stranded DNA (Mikael *et al*., 1987). Tissue after treatment with dye was washed in PBST. Each wash was ten minutes long on a nutator and finally the tissue was mounted on glass slides using Vectashield mountant (Vector labs, Cat. No. # H-1000). Vectashield is a glycerol based mountant that does not solidify. It serves as an antifade agent and prevents rapid loss of fluorescence during examination of the sample under microscope. Thus, Vectashield allows anti-fading ability for long term storage.
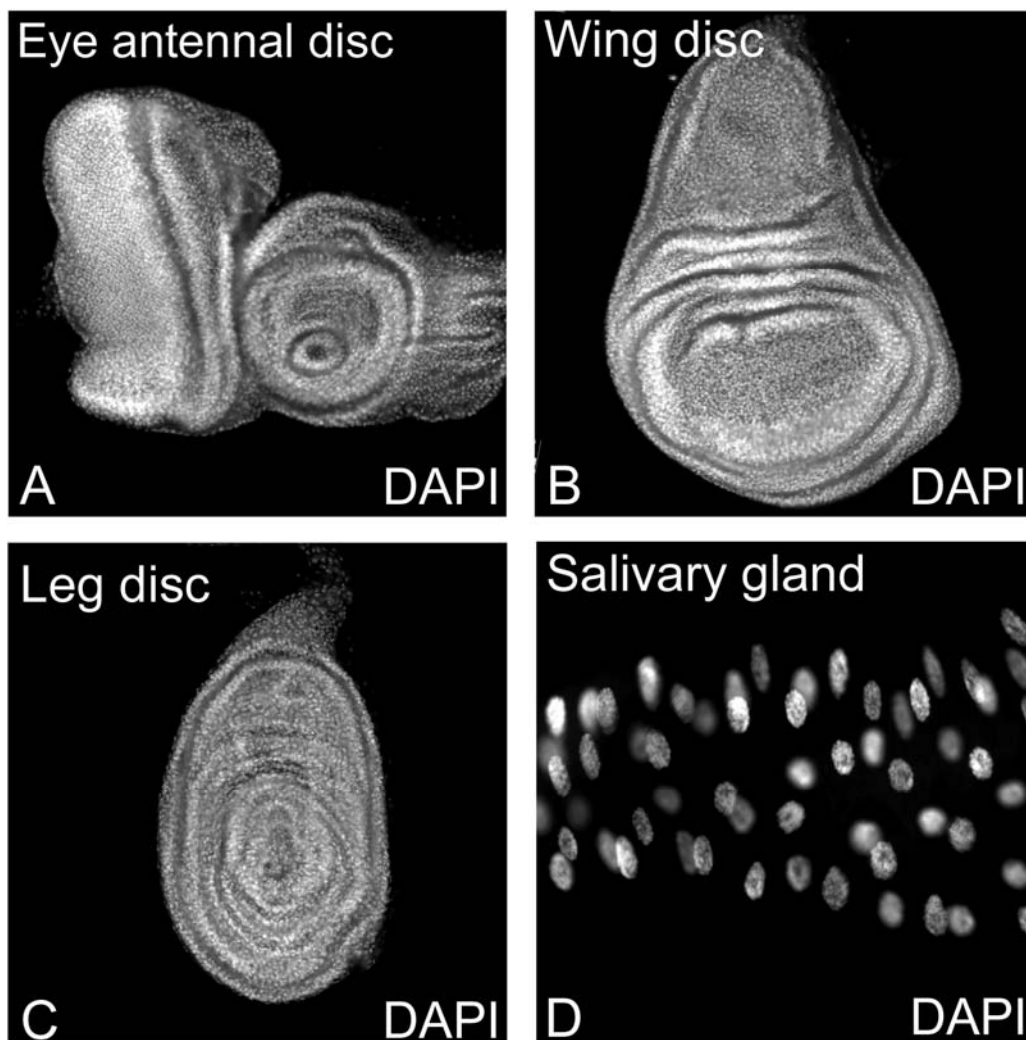

Figure 1. DAPI, a nuclear dye, marks the nuclei in (A) Eye antennal-, (B) Wing-, (C) Leg-imaginal discs, and (D) Salivary gland.
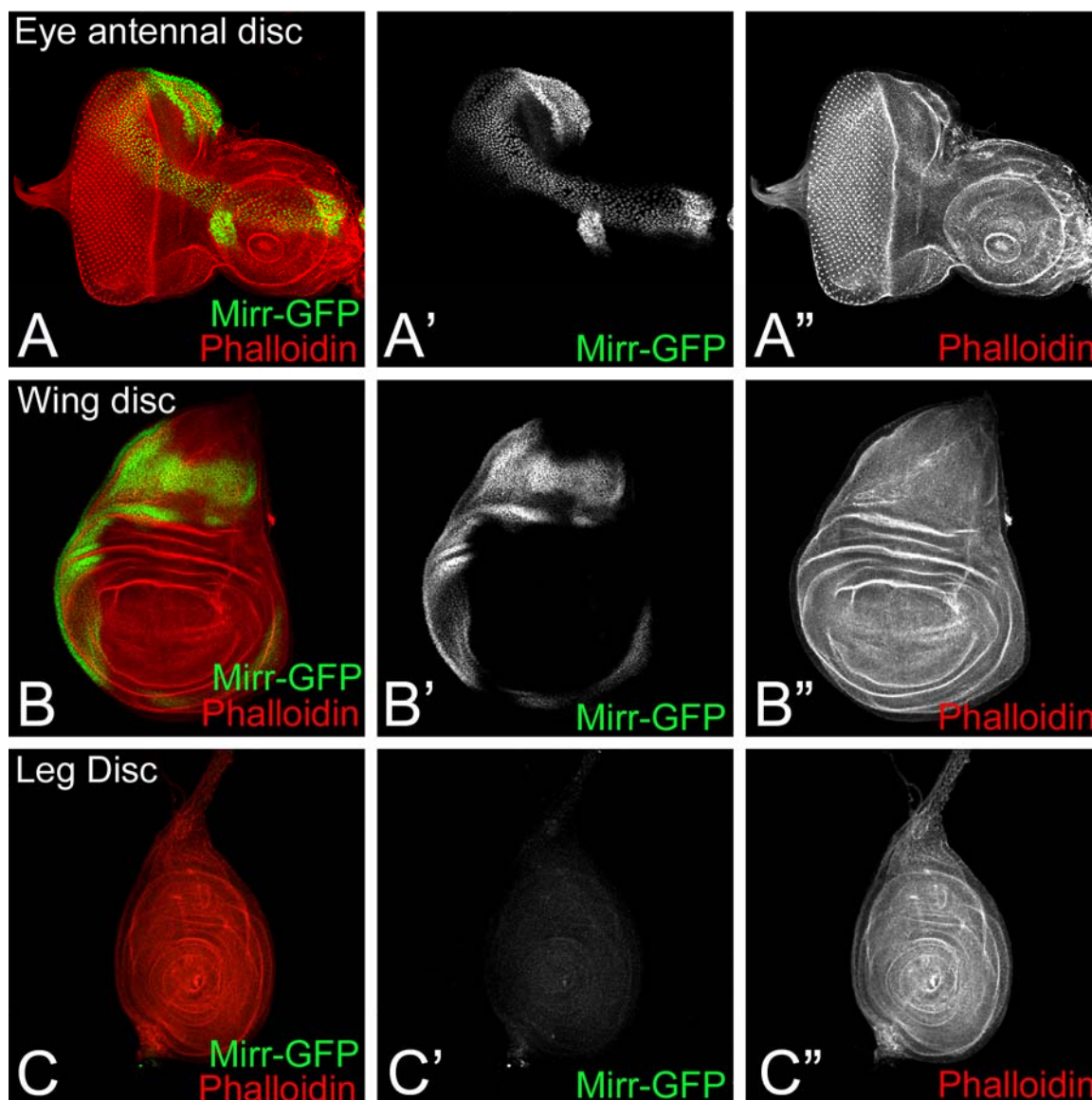
Figure 2. Expression of Green Fluorescent Protein (GFP) reporter marks the localization of Mirror (Mirr) and GFP chimeric protein using the protein trap line. Localization of Mirr and GFP chimeric protein in (A) Eye-antennal-, (B) Wing-, and (C) Leg-imaginal disc. Note that phalloidin (red) marks the actin cytoskeleton and provides the outline of the disc. Both Mirr-GFP (Figures 2A', B', C') expression and phalloidin (Figures 2A", B", C") are also shown in separate channels.

Imaging

Olympus BX51 epifluorescence microscope was used to take DAPI images, whereas GFP and Phalloidin staining images were taken using Olympus Fluoview 1000 Laser Scanning Confocal Microscope and edited using Adobe Photoshop 5.5 software.

The discs stained for nuclear marker DAPI (Kapuscinski, 1995) can be seen using DAPI filter that has an excitation wavelength of 358 nm and emission wavelength of 461 nm. A strong nuclear

staining was observed using Olympus BX51 Olympus epifluorescence microscope in eye-antennal (Figure 1A), wing- (Figure 1B), leg-imaginal disc (Figure 1C), and salivary gland (Figure 1D).
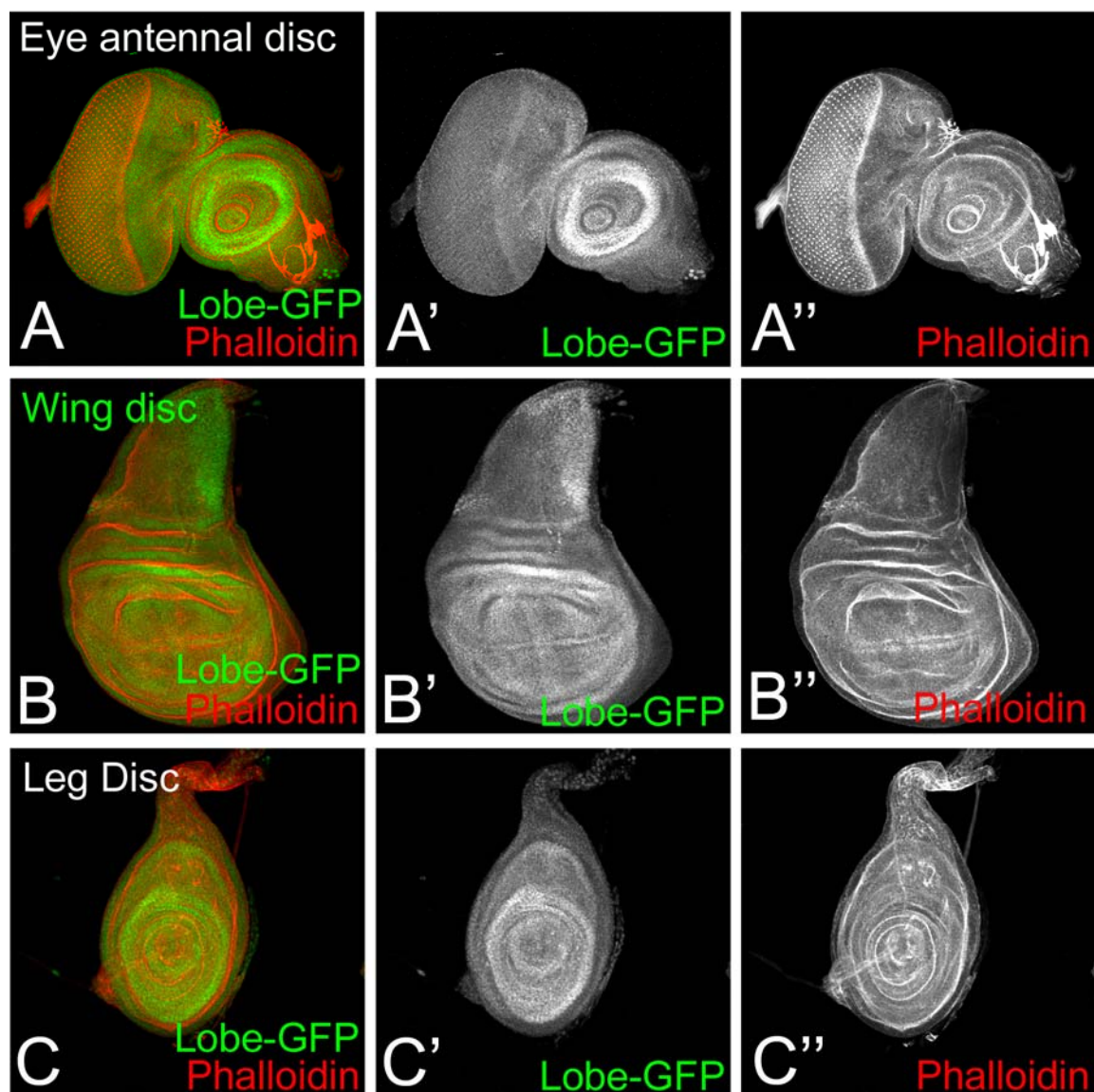

Figure 3. Expression of Green Fluorescent Protein (GFP) reporter marks the localization of Lobe (L) and GFP chimeric protein using the protein trap line. Localization of L and GFP chimeric protein in (A) Eye-antennal-, (B) Wing-, and (C) Leg-imaginal disc. Note that phalloidin (red) marks the actin cytoskeleton and provides the outline of the disc. Both L-GFP expression (Figures 3A', B', C') and phalloidin (3A'', B'', C'') are also shown in separate channels.

The protein trap transgenic lines used in this study are Mirror (Mirr) and Lobe (L), which are part of the protein trap lines collection. The database of these protein trap lines is called Flytrap (http://flytrap.med.yale.edu/index.html) (Kelso *et al*., 2004). Mirror is a homeodomain transcription factor which belongs to the Pbx (pre-B cell leukemia homeobox) class of genes, which is expressed in the dorsal half of the eye (Figure 2). Lobe, a PRAS40 homolog in fly, is expressed uniformly in

the developing imaginal discs (Figure 3). The protein trap approach employs the use of Green Fluorescent Protein (GFP) reporter (Chalfie *et al.*, 1994). GFP can be seen using <u>fluorescein isothiocyanate</u> (FITC) filter having an absorption maximum at 495 nm and excitation wavelength of 488 nm. The GFP reporter expression marks Mirror and Lobe protein expression in eye-antennal (Figures 2A, 3A), wing (Figures 2B, 3B), and leg imaginal discs (Figures 2C, 3C). Note that split channels in Figure 2 and Figures 3 show the expression of GFP and phalloidin separately.

Advantages

1. The protein trap approach can be used to study the sub-cellular localization of proteins.

2. The protein traps are useful to analyze protein localization in the live tissue and can reveal dynamic processes within a cell or multi-cellular organ/ organism.

3. Antibody staining provides more specificity but is more time consuming when compared to dyes. Since antibody staining procedure requires two days to finish, it is profitable to use the protein trap lines and dyes to demonstrate reporter assays and protein localization within the allocated time of a single lab session.

4. These laboratory techniques will allow instructors to depend less on the expensive experimental demonstration kits that are supplied commercially.

5. The variation of markers used and protein trap lines will allow instructors to generate a database of expression profiles, which can be used as a teaching tool for Cell Biology lectures.

Conclusion

Images are considered a powerful means of presenting and communicating scientific results. A high-resolution image can validate an experimental result more effectively than a statement. However, a poor resolution image may raise doubts on any result or conclusion. The majority of the laboratory exercises taught in the undergraduate laboratories are either from commercially developed expensive kits or other conventional experimental labs that do not emphasize communication skills through the use of images. Our laboratory exercises (Tare and Singh, 2009; Tare *et al.,* 2010) including this one, address this problem and are designed to expose students to basic lab skill sets (a) involving epifluorescence microscopy and Laser Scanning Confocal Microscopy, (b) imaging, (c) processing and analyzing the images.

          References: Buszczak, M., S. Paterno, D. Lighthouse, J. Bachman, J. Plank, S. Owen, A. Skora, T. Nystul, B. Ohlstein, A. Allen, J. Wilhelm, T. Murphy, R. Levis, E. Matunis, N. Srivali, R. Hoskins, and A.C. Spradling 2007, Genetics 175(3): 1505-1531; Chalfie, M., Y. Tu, G. Euskirchen,

W.W. Ward, and D.C. Prasher 1994, Science 263: 802–805; Dulbecco, R., and M. Vogt 1954, J. Exp. Med. 99(2): 167-182; Kapuscinski, J., 1995, Biotech. Histochem. Sep; 70(5): 220-33; Kelso, R.J., M. Buszczak, A.T. Quinones, C. Castiblanco, S. Mazzalupo, and L. Cooley 2004, Nucleic Acids Research 32: D418-420; Mikael, K., B. Aakerman, and B. Norden 1987, Biochemistry 26: 4545–4553; Morin, X., R. Daneman, M. Zavortink, and W. Chia 2001, Proc. Natl. Acad. Sci. USA 98: 15050-15055; Quiñones-Coello, A.T., L.N. Petrella, K. Ayers, A. Melillo, S. Mazzalupo, A.M. Hudson, S. Wang, C. Castiblanco, M. Buszczak, R.A. Hoskins, and L. Cooley 2007, Genetics 175(3): 1089-1104; Small, J., K. Rottner, P. Hahne, and K.I. Anderson 1999, Microsc. Res. Tech. Oct 1; 47(1): 3-17; Tare, M., and A. Singh 2009, Dros. Inf. Serv. 91: 160-164; Tare, M., G. Puli, O. Roy, M.S. Oros, and A. Singh 2009, Dros. Inf. Serv. 92: 174-180.

Effect of *hsp*83 activation on cell death as quantified using phenotypic variation of Bar eye in *Drosophila melanogaster*.

Thompson, James N., jr., Clayton N. Hallman, Frank Blixt, Laura Bock, Nam Do, Greg Emde, Daniel Mach, Kimberly May, Valerie Miller, Diana Nguyen, Kayla Owens, Lucas Toho, Lane Donaldson, Joseph Lykins, Barbara Safiejko-Mroczka, and Jenna J. Hellack. Department of Zoology, University of Oklahoma, Norman, OK 73019.

Quantifying the phenotypic variation in a trait is a sensitive way to assess the role a genetic or environmental factor has on a targeted developmental process. Thompson *et al*. (2009) used this approach to evaluate heat shock effects on cell death. Scanning electron micrographs allowed us to measure phenotypic changes very precisely. The current study draws upon that same experimental design to measure the influence of heat shock protein 83 (*hsp*83) on the expression of cell death in the *Drosophila* eye.

The Experimental Genetics and Cell Biology Lab (ZOO 4970) course used this genetic system to study the effect of an *hsp*-defective allele on cell death under normal room temperature (control) or heat shock (37°C) conditions. The hypothesis was that a significant increase in cell death would occur in the *hsp*-defective genotype when stressed by elevated temperature. The normal heat shock system, represented by a dominantly-marked balancer chromosome, would have significantly reduced cell death.

The *hsp*83 mutation was balanced over the dominant Tubby, which causes a shortening of the body (Bloomington Stock Center #5696, w*; *Hsp*83^{e6D}/TM6B, *Tb*^{1}). By crossing Bar females with males from this stock, F1 flies that are either *hsp*83 or Tubby are easily distinguishable as 3rd instar larvae. There were four treatments: third instar larvae (*hsp*83 or Tubby) treated for 40 minutes at 37°C and the same two genotypes raised at room temperature. The 37°C exposure activates the heat shock activity, although it is defective in the *hsp*83 strain.

Treatment was done by selecting F1 larvae of each genotype and placing them in 1.5 ml microfuge tubes containing 0.5 ml of yeast-glucose medium. These were then placed in baggies and either submerged in a 37°C water bath for 40 minutes or left on the lab bench for the same period. Tubes were then uncapped and inserted into a normal food tube, where the flies were allowed to pupate and eclose. Heads were removed from Bar-eyed males eclosing from each of the four conditions. In addition to touring the Electron Microscope Facility and developing this experimental plan, the students benefitted from the microdissection practice needed for successful mounting of