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TITLE:

A *Drosophila* model to study wound-induced polyploidization

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SUMMARY:

Wound-induced polyploidization is a conserved tissue repair strategy where cells grow in size instead of dividing to compensate for cell loss. Here is a detailed protocol on how to use the fruit fly as a model to measure ploidy and its genetic regulation in epithelial wound repair.

ABSTRACT:

Polyploidy is a frequent phenomenon whose impact on organismal health and disease is still poorly understood. A cell is defined as polyploid if it contains more than the diploid copy of its chromosomes, which is a result of endoreplication or cell fusion. In tissue repair, wound-induced polyploidization (WIP) has been found to be a conserved healing strategy from fruit flies to vertebrates. WIP has several advantages over cell proliferation, including resistance to oncogenic growth and genotoxic stress. The challenge has been to identify whether polyploid cells arise and how these unique cells function. Provided is a detailed protocol to study WIP in the adult fruit fly epithelium where polyploid cells are generated within 2 days after a puncture wound. Taking advantage of *D. melanogaster's* extensive genetic tool kit, the genes required to initiate and regulate WIP, including Myc, have begun to be identified. Continued studies using this method can reveal how other genetic and physiological variables including sex, diet, and age regulate and influence WIP's function.

INTRODUCTION:

Drosophila melanogaster is an attractive model system to study the cellular and molecular mechanisms of epithelial wound repair. As in mammals, the tissue repair mechanisms used depends on both the tissue and its developmental stage. Scarless wound healing occurs in the fruit fly embryo where an actomyosin "purse string" forms at the epithelial leading edge enabling the wound to seamlessly close[1,2]. Post-embryonic wound healing in larvae, pupae, and adult fruit flies results in extracellular matrix remodeling, melanin scar formation, and epithelial cell growth[3-6]. The epithelial cells increase in size by cell fusion and the endocycle, an incomplete cell cycle that bypasses mitosis[3,4,7,8]. As result, cell loss is compensated by polyploid cell growth instead of cell division. The adult fly hindgut, midgut, and follicular epithelium also rely on polyploid cell growth to compensate for cell loss after tissue damage[9-11].

Polyploidy is a well-known aspect of organismal development in plants and insects, but in the last few years it has become more apparent that polyploidy is a conserved tissue repair strategy in vertebrates[12]. The zebrafish, which has the capacity to regenerate its heart, relies on polyploid cell growth to heal damaged epicardium[13]. Polyploidy also contributes to mammalian liver regeneration and kidney tubule epithelium repair after acute injury[14,15]. In these examples, polyploid cells are generated by endoreplication via either endocycle or endomitosis, which results in a binucleated cell due to a block in cytokinesis[12]. The enigma is why polyploid cells arise during wound repair and how polyploidy affects tissue function. Recent studies have provided new insight into the question of whether polyploidy offers a healing advantage or disadvantage. In zebrafish epicardium, polyploidy enhanced the speed of wound healing[13]. In *D. melanogaster* hindgut and mammalian liver, polyploidy was found to be protective against oncogenic growth[11,14]. In adult fly epithelium, it was recently found that polyploidy enables wound repair in the presence of genotoxic stress[16]. Endoreplication is resistant to DNA damage, allowing wound healing when cell proliferation would otherwise be compromised[17]. For cardiomyocytes in mouse and zebrafish hearts, however, polyploidy slows healing, resulting in enhanced scar formation[18,19]. Therefore, depending on the organ and/or cell type, polyploidy can be a beneficial or detrimental tissue repair strategy. The accessibility of *D. melanogaster* genetics coupled with analysis of the wound-induced polyploidization (WIP) response make it an ideal model system for elucidating the molecular and cellular mechanisms that guide this wound healing strategy.

Here, we present a protocol for analyzing WIP in the adult *D. melanogaster* epithelium. Included are instructions for fruit fly injury, dissection, immunostaining, mounting, imaging, and analysis of re-epithelialization, cell fusion, and endoreplication (ploidy). The imaging and ploidy analysis can also be adapted to other models to test whether WIP occurs. It should be noted that with an increase in nuclear DNA content there is often a corresponding increase in nuclear size. However, there are many examples in biology where nuclear size does not reflect a corresponding change in ploidy[20]. Even more caution should be taken when interpreting nuclear size in the context of a wound environment where cells will often spread or stretch to cover the wound site. Therefore, the only definitive proof of change in ploidy is to measure DNA content by this method (or others, such as whole genome sequencing)[21]. This method

increases the suitability of the adult *D. melanogaster* abdominal epithelium as a model to study the role and regulation of polyploidy in wound repair.

PROTOCOL:

1. Staging and wounding of adult fruit flies

1.1. Select *D. melanogaster* strain of choice (i.e., epi-Gal4/ UAS strain, see **Table of Materials**).

NOTE: Here, the Gal4/UAS system is used to enable epithelial specific gene expression (epi-Gal4) of a gene or RNAi encoded downstream of UAS. This study uses fluorescent membrane protein (UAS-Cd8.mRFP), mitotic inducer (UAS-fzr^{RNAi}, UAS-stg), and WIP inhibitor (UAS-E2F1^{RNAi}; UAS-Rac^{DN}).

1.2. Collect two vials of 10–15 newly enclosed female fruit flies each and age on fresh food vials at 25 °C until 3–5 days old. One vial will serve as the uninjured control and the other vial will be wounded as described below. The female flies should be maintained with males (~5/vial).

1.3. To wound the flies, assemble several pin holders each with a single 0.10 mm stainless steel pin. Ensure the sharp end of the pin is facing out. Pins can easily bend or chip after puncturing the fly and hooked or damaged pins should be discarded.

1.4. Anesthetize aged female fruit flies on a CO_2-fly pad under a stereomicroscope and align them into a row using a paint brush. Wearing safety glasses, holding the pin holder in one hand and forceps in the other, use forceps to position a fly with its ventral abdomen facing up.

1.5. Puncture the adult female flies within the epithelial pleurite region of tergite A4 on either side of the ventral midline sternites (**Figure 1A**). Puncturing this ventral region provides optimal space away from the dissection sites where tissue edges will be torn by mechanical processing.

1.6. Return wounded flies to the food vial and age to desired day post injury (dpi). Epithelial wound healing starts at 1 dpi and ends by 3 dpi. Endoreplication peaks at 2 dpi, which is ideal for the EdU assay (section 4, **Figure 2**).

2. Fly abdominal dissection

NOTE: During this step, it is important to avoid touching the ventral abdominal tissue with the dissection tools because it will compromise the integrity of the epithelium.

2.1. Obtain all required materials for dissection: Grace's solution, forceps, Vanna's Spring Scissors, 0.10 mm pins, dissecting plates, 9 well glass dissection dish, fixative solution (4% paraformaldehyde in 1x PBS), 1x PBS, wipes, pipettes and tips for 30 µm, and gloves (see **Table of Materials**).

2.2. Confirm that flies were successfully injured by anesthetizing wounded flies on a CO_2-fly pad

under a stereomicroscope and checking for the presence of the wound scar (i.e., a melanin spot on abdomen, see **Figure 1B**). Discard any flies from the experimental group that were not successfully wounded.

2.3. To start dissection, fill one well of a 9 well glass dissection dish with Grace's solution. Use a pair of forceps to grasp a wounded female fly by the dorsal side of the thorax and submerge the fly in the well containing Grace's solution.

2.4. Using forceps in the opposite hand without releasing the thorax, puncture the dorsal cuticle below tergite A6 and pull the cuticle off the rear end of the fruit fly. The internal organs (ovary and intestine) will usually come out at this step. If not, gently push on the dorsal side of the abdomen with the forceps to squeeze out the remaining organs and discard into an empty well.

2.5. Snap off the full abdomen at the thorax junction above tergite A2 using the forceps and transfer the abdomen to an empty well containing ~100 µL of Grace's solution.

2.6. Repeat steps 2.3–2.5 until all fly abdomens are dissected.

2.7. Reduce the volume of Grace's solution to 30 µL in the well containing pooled, dissected abdomens.

2.8. Fillet the abdomens open by positioning the abdomen on the dorsal side with the forceps in one hand and then inserting the bottom blade of Vanna's spring scissors into the abdominal cavity with the other hand. Cut along the dorsal midline until the abdomen is fully opened, which can require up to three cuts (**Figure 1C**,**1D**).

2.9. Set up a dry dissecting plate with four 0.10 mm pins per abdominal mounting area. Each 35 mm dissecting plate can fit up to seven mounting areas. Pipette 30 µL of Grace's solution on each mounting area and transfer one filleted abdomen to each droplet.

2.10. Pin the filleted abdomens to the dish on the four dorsal corners (**Figure 1E**). Ensure that the tissue lies flat without tearing or overstretching the abdominal tissue.

2.11. To fix the tissue, pipette off the Grace's solution and add 30 µL of the fix solution to the pinned abdomen.

CAUTION: Wear gloves while handling the fix solutions, as paraformaldehyde is toxic.

2.12. Repeat steps 2.10–2.11 until all filleted abdomens are pinned onto the dissecting plate.

2.13. Place a tape label on the bottom of each dish to mark each control and experimental group. Fix samples for 30–60 min at room temperature (RT).

2.14. Wash off fix solution by pipetting on 1.5 mL of 1x PBS to each plate. Dispose of fix solution and plastics in appropriate liquid or dry chemical waste containers according to institutional guidelines.

2.15. Wash plates 2x with 1.5 mL of 1x PBS and store fixed tissue covered in 1.5 mL of 1x PBS in a plastic container with a lid. Add a layer of damp paper towel to the bottom of the container and store samples at 4 °C until ready to immunostain within at least 1 week of dissection.

3. Immunofluorescence

3.1. Freshly prepare reagents (see **Table of Materials**): wash buffer solution (0.3% Triton X 100, 0.3% BSA in 1x PBS). Leftover wash buffer can be saved at 4 °C and used for the duration of the 2 day staining protocol. Prepare enough primary antibody solution per assay (**Figure 2**) using Anti-FasIII (1:50 mouse anti-Fasciclin-III) in wash buffer with either anti-Grh (1:300 affinity purified rabbit anti-Grainyhead[8]) or anti-RFP (1:1,000 rabbit anti-RFP). Primary antibody solutions can be saved at 4 °C and reused multiple times until signal is significantly reduced.

3.2. Permeabilize tissue by pipetting off 1x PBS, adding 1.5 mL of wash buffer, and incubating for at least 30 min on an orbital shaker (80 rpm) at RT.

3.3. Remove wash buffer and stain tissue overnight with 1.5 mL of primary antibody solution, incubating on an orbital shaker (80 rpm) at 4 °C. Collect the primary antibody solution and save in a tube at 4 °C for future experiments.

3.4. First rinse the sample quickly with 1x PBS and then wash 3x with 1.5 mL of wash buffer. For each wash, incubate samples at RT on an orbital shaker for at least 30 min.

3.5. During the final wash, prepare the secondary antibody solution: 1:1,000 donkey anti-rabbit Alexa 488 or 568 and 1:1,000 goat anti-mouse Alexa 488 or 568 (or fluorophores of choice) in wash buffer.

3.6. Remove wash buffer and stain tissues with 1.5 mL of secondary antibody solution. Cover samples with aluminum foil and incubate on an orbital shaker at RT for 3 h. Alternatively, samples can be incubated overnight at 4 °C on an orbital shaker.

3.7. Wash samples by first discarding secondary antibody solution and then rinse sample quickly with 1x PBS followed by three washes with 1.5 mL of wash buffer. For each wash, incubate samples at RT on an orbital shaker for at least 30 min.

3.8. Prepare DAPI solution by diluting DAPI to 10 µg/mL in wash buffer. After the final wash, stain samples with 1.5 mL DAPI solution incubating at RT for 30 min.

3.9. Discard DAPI solution and rinse samples 2x in 1.5 mL of 1x PBS. Store stained tissue in 1.5 mL of 1x PBS in the dark, covered with aluminum foil, at 4 °C until ready to mount on a glass

slide with coverslip. The mounting step should be performed within 1 week.

4. Cell cycle activity (EdU Assay)

4.1. Make up a 10 mM EdU stock solution from Click-iT kit (see **Table of Materials**) by dissolving EdU powder in dH_20 and mixing for ~15 min until completely dissolved. The stock solution can be aliquoted (250 µL per tube) and stored at -80 °C.

4.2. Feed flies EdU by first diluting EdU stock to 5 mM in dH_2O. Add dry yeast until the solution is cloudy and briefly vortex to mix. Cut off a 0.5 mL tube cap and place it at the bottom of the fly food vial. Push the cap into the food, so it is stable.

4.3. Anesthetize the flies and transfer 3–5 day old flies into the vial. Tap the flies to one edge so none are stuck in the cap.

4.4. Pipette 75 µL of yeast-EdU solution into the cap. Flies should be fed fresh yeast-EdU solution every day and transferred to a fresh food vial with a cap every other day to ensure that the flies do not get stuck in the bottom of the vial.

4.5. To transfer flies, flip to a new vial with a cap, put flies to sleep, tap flies to one side, and add fresh yeast-EdU solution.

4.6. On the third day, injure the flies and continue to feed yeast-EdU until dissection at 2 dpi (**Figure 4A**). See protocol section 2 for dissection and fixation methods.

4.7. Prepare EdU staining reagents: wash buffer (0.3% Triton X 100, 0.3% BSA in 1x PBS), permeabilization buffer (0.5% Triton X 100 in 1x PBS), blocking buffer (3% BSA in 1x PBS), and prepare reagents from the EdU assay kit (see **Table of Materials**), including 1x reaction buffer and 1x reaction buffer additive as directed by the manufacturer.

4.8. Wash samples for 1 h, mixing at RT in 1.5 mL of wash buffer.

4.9. Add 1.5 mL of permeabilization buffer and incubate samples for 20 min.

NOTE: Thaw and prepare reaction cocktail solution using a volume of 500 µL/plate.

4.10. Wash samples 1x quickly with 1x PBS and then 3x quickly with 1 mL of blocking buffer.

4.11. Pipette off all remaining blocking buffer and add 500 µL of reaction cocktail solution per plate. Swirl plates to ensure tissues are completely covered. Incubate in a drawer in the dark for 1 h at RT.

4.12. Wash samples 1x quickly with 1.5 mL of blocking buffer.

4.13. Stain with 1.5 mL of DAPI solution at 1:5,000 in wash buffer for 30 min.

4.14. Wash 2x with 1x PBS quickly, wrap in foil, and store in the dark at 4 °C until ready to mount samples within 3 days.

5. Mount stained tissue

5.1. Obtain all required materials for mounting: glass slides, glass coverslips, clear nail polish, mounting media, a pair of forceps, and wipes.

5.2. To mount stained fly tissue, unpin abdomens from the dissecting plate using forceps under the stereomicroscope. Transfer tissue to ~30 µL of mounting media on a glass coverslip by gently grabbing the tissue with forceps by its dorsal flanks, taking care to avoid touching the ventral area with forceps.

5.3. Under the stereomicroscope orient the abdominal tissue so that the inside is facing down toward the coverslip (i.e., the external cuticle/bristles are facing up). Pull the oriented abdomens to the edge of the media droplet using the forceps. Surface tension will help keep the tissue flat (**Figure 1F**).

NOTE: It is helpful for imaging to organize the abdomens in a column or row at this stage.

5.4. Label a glass slide (i.e., control or experimental) and pick up the coverslip by slowly bringing the slide closer to the coverslip. Flip slide over and gently blot with a wipe to remove excess mounting media.

5.5. Seal the edges of the coverslip with clear nail polish and repeat for all remaining experimental groups. Store slides in a slide box at 4 °C until ready to image.

6. Imaging and processing

6.1. Image the fly abdominal wound area by first locating the melanin scar with a confocal microscope (**Figure 1B**), either a point scanner or a structured illumination (ApoTome) with a 40x oil or dry objective.

6.2. Check the exposure on each channel, ensuring that the signal is below saturation. The imaging settings should be based on the brightest sample group. This is particularly important for ploidy analysis as the DAPI channel needs to stay in the linear range to accurately measure DNA content.

6.3. Take a full z-stack image in all three channels with an optimal distance of at least 0.50 µm between slices. Save captured images and open the file in the image analysis program Fiji (also known as ImageJ).

6.4. For each image, create a z-stack projection using the sum of slices option for all channels.

6.5. Rotate the images as necessary to ensure the nuclei are lined up horizontally across images (**Figure 3A** and **Figure 4E**).

6.6. Crop all the images to a rectangular selection of 300 µm x 300 µm centered around the wound site or center of uninjured control. Identify the area by drawing a rectangle and selecting **Edit**| **Selection** | **Specify**. Check that scaled units are in microns to ensure the same sized box is used for all images to be analyzed.

7. Endoreplication (ploidy) analysis

7.1. Using Fiji, select the **Grh channel window** and duplicate the image. Then use the **threshold tool** to create a mask. Manually adjust the threshold by sliding the top bar to minimize background without causing the nuclei to shrink drastically (**Figure 4D**).

7.2. If any nuclei in the Grh channel are touching in the threshold image, use the **paintbrush tool** (2 pixel width) in the same color as the background, to draw a line between the nuclei. Click to apply 1x when finished to generate the final mask.

7.3. Generate a region of interest (ROI) map by using the **Analyze Particles** function: set size to 5 µm–60 µm in order to capture most of the nuclei without including the background.

7.4. Manually adjust the ROI map as needed in the **ROI manager**. Delete any selections that are not nuclei and add any nuclei to the list that were not identified by outlining the nucleus with the freehand selection tool and adding it to the **ROI manager** (**Figure 4D**).

7.5. Select the **DAPI channel** then click **Show All** in the **ROI manager** to apply the generated ROI map in the Grh channel onto the DAPI channel.

7.6. Delete any selections where the epithelial nuclei outline overlaps with nonepithelial nuclei (e.g., nuclei from muscle or fat) from the ROI map. Grainyhead only stains epithelial nuclei, whereas DAPI stains all nuclei. Check that each outlined selection contains only one nucleus and delete or edit any selections with more than one nucleus. Save edited ROI list.

7.7. Measure the area and integrated density of each epithelial nuclei in the ROI map using the analysis tools in Fiji. Export the values into a spreadsheet program.

7.8. Measure the average image background using the circular selection tool. Draw three circles that do not overlap with any nuclei in different areas of the DAPI image. Add the area and integrated density of each of the circles to a spreadsheet program to establish the background image brightness.

7.9. Start by calculating the average background per unit area for each image by dividing each

background integrated density value by its corresponding area. Then average the three integrated density per area measurements for the image in order to obtain the average background per unit area.

7.10. Next, calculate the total background of each DAPI nucleus by multiplying the area of the nucleus by the average background per unit area. The normalized DAPI intensity for each nucleus measured can then be calculated by subtracting the total background of each nucleus from its measured integrated density.

7.11. Average all the normalized DAPI intensity values from the uninjured epithelial control. The uninjured epithelial nuclei were previously calculated to have a ploidy value of 2C and can serve as a reference for calculating ploidy in the epithelial nuclei from the experimental conditions[8].

7.12. Calculate the ploidy of each nucleus by dividing the normalized DAPI intensity of each nucleus by the normalized value from reference uninjured epithelial control (2C), then multiply the value by 2 to equal the normalized ploidy (C-value):

(Nuclear Integrated Density – Background Nuclear Integrated Density)/Average Nuclear Integrated Density (uninjured epithelial nuclei = 2C) x 2 = epithelial nuclear ploidy (C)

7.13. Graph nuclei with ploidy values as a dot plot, histogram, or grouped into a bar graph accordingly (i.e., 2C [0.6–2.9C], 4C [3.0–5.9C], 8C [6.0–12.9C], 16C [13.0–24.9C], and >32C [>25.0C] (**Figure 3F**).

REPRESENTATIVE RESULTS:

A detailed protocol is provided to use *D. melanogaster* as a model to study wound-induced polyploidization (WIP). This wound healing model provides many advantages over mammalian and other fly models of WIP. Polyploidy was easily induced by a mechanical puncture with an insect pin and polyploid cells were generated within a short period of time (2–3 dpi) (**Figure 1A**,**1B**)[4]. The major challenge is the dissection of intact abdominal tissue without any perturbations to the epithelium. The *D. melanogaster* epithelium is easily accidentally bumped or scratched with the sharp dissection tools. Therefore, the steps of this protocol should be practiced prior to use and analysis.

First, the injury was restricted to the ventral female abdomen, which provides a large, flat opaque tissue area ideal for imaging. The puncture wounds were inflicted in the pleurite epithelium, which lies on either side of the ventral midline sternites and targeted between tergite (T) segments T4–T5 (**Figure 1A**-**C**). This wound placement provides a large visible area that is not disrupted by the dissection. Challenging steps include the abdominal spring scissor cut and pinning steps (**Figure 1D**,**1E**). The spring cutting step worked best when the abdomens were cut in a reduced volume of Grace's solution (~30 µL) to decrease tissue movement. A well-centered cut along the dorsal midline was necessary to provide sufficient area on the abdominal dorsal flaps to pin open on the dissection plate (**Figure 1C**). The abdomen must be gently pinned on the four corners without excessive force (**Figure 1E**). A pin push that is too

hard will distort the abdominal tissue and could even push the tissue into the dissection plate. If this happens, the tissue must be discarded. Once the abdominal tissue was fixed, it remained on the dissection plate until immunofluorescence staining was complete and the abdomens were mounted on a glass coverslip for imaging (**Figure 1F**).

Wound healing requires a continuous epithelial sheet to form, which is dependent on endoreplication and cell fusion[4,16]. The septate junction protein FasIII, which labels cell-cell junctions, provided an indicator for whether any processing perturbations occurred during preparation (**Figure 1G**,**1H**). Abdomens with large scratches (unstained area) that perturb the wound area must be discarded and were not used for further analysis (**Figure 1H**).

The next step was to analyze intact samples for any defects in WIP. This protocol includes distinct assays to detect different aspects of the WIP response (**Figure 2**). Wound repair was complete when a central, large, multinucleated cell covered the wound scab (**Figure 3A**). Here cell fusion was detected by staining for FasIII/Grh and quantifying the number of Grh^{+} epithelial nuclei encompassed in the FasIII outlined area[4]. Defects in wound closure or re-epithelialization were detected when gaps of >10 µm in the epithelial sheet were observed (**Figure 3B**, red arrow). This was the case, for example, when WIP was inhibited by the activation of mitotic cycle via expression of *stg, fzr*^{RNAi}, as recently reported[16]. In this genetic condition, 52% of the wounds were not able to form a continuous epithelial sheet over the wound scab (**Figure 3B**,**3C**).

Another method to measure wound repair in this model was by visualizing the epithelial membrane with epi-Gal4 expression of UAS-mCD8-ChRFP[4] (**Figure 2**, **Figure 3D**). In the control, 91% of epithelial wounds closed completely by 3 dpi, but inhibiting WIP by blocking endoreplication (*E2f1*^{RNAi}) and cell fusion (*RacDN*) simultaneously, as previously reported, caused 92% of epithelial wounds to remain completely open (**Figure 3D**,**3E**)[8,16]. The activation of mitotic cell cycle by expression of *stg, fzr*^{RNAi} also resulted in an epithelial wound closure defect. However, by visualizing the epithelial cell membrane, the extent of re-epithelialization defect could be determined. The WIP mutant (*E2f1*^{RNAi}*, RacDN*) fly wounds were more open than the *stg, fzr*^{RNAi} wounds (**Figure 3D**, dashed red outline)[16]. This membrane wound healing assay provided more information on the extent of the wound repair defect. As a result, re-epithelialization defects could be grouped as either completely open, partially closed (i.e., >10 µm gaps), or completely closed (**Figure 3D**,**3E**).

In addition to cell fusion, epithelial cells grow in size by endoreplication, an incomplete cell cycle that doubles the nuclear DNA content. Endoreplication was assayed by both cell cycle activity and direct nuclear DNA ploidy measurements (**Figure 2** and **Figure 4**). Here, cell cycle activity was detected by incorporation of the thymidine analog, EdU (**Figure 4A**,**4B**). *D. melanogaster* epithelial cells were found to enter the endocycle, an incomplete cell cycle that oscillates between the S and G phases without an intervening M phase[4,12]. The adult *D. melanogaster* diet was supplemented with EdU^{+} food prior to injury and the flies were maintained on an EdU^{+} diet until dissection at 2 dpi (**Figure 4A**). The EdU was then detected using the manufacturer's Click-iT protocol. This EdU assay was used to determine where, when,

and how many nuclei were triggered to enter S phase in response to a wound. Using the Gal4/UAS system, it was recently found that epithelial specific expression of *myc* can either block (*myc*^{RNAi}) or exacerbate (Myc overexpression) the competence of epithelial cells to enter S phase. As result, it has been shown that *Myc* is sufficient to induce endoreplication in postmitotic cells, even without injury[16,22].

Next, epithelial ploidy was determined by directly measuring nuclear DNA content. Epithelial nuclei were identified by immunofluorescence staining for the epithelial specific marker, Grh (**Figure 4D**). In the Fiji imaging software, epithelial nuclei were systemically identified and then thresholded using Grh nuclear stain. Nuclei were then separated and the ROIs overlaid on to the SUM of stacks DAPI image (**Figure 4D**, green arrow). Any overlapping nuclei were manually deleted before the Integrated Density of the selected nuclei was measured (**Figure 4D**, red arrows). This semiautomated method allows one to quantify the distribution and ploidy of most nuclei throughout the uninjured and repaired fly abdominal epithelium[8]. As recently reported, the epithelial nuclei surrounding the wound were composed of 44% polyploid nuclei with DNA content more than 3C at 3 dpi (**Figure 4E**,**4F**)[16]. As expected from the EdU results, knockdown of *myc* led to a significant block in endoreplication, as only 9% of epithelial nuclei were polyploid, whereas overexpression of *Myc* resulted in 100% polyploid epithelial nuclei around the wound site (**Figure 4F**)[16]. Epithelial nuclear size was also visibly affected by *myc* expression with either reduced or enlarged nuclei present. However, nuclear area is not an accurate measure of ploidy and physiological effects, because factors such as cell stretching can also influence nuclear size without affecting nuclear DNA content[20].

FIGURE AND TABLE LEGENDS:

Figure 1: Adult fruit fly abdominal wounding, dissecting, and tissue mounting. (**A**) Diagram of the adult abdominal wounding assay. Flies should be injured on either side of the abdomen at tergite 4 (T4). (**B**) Adult female fruit fly 3 dpi with melanin scab formed from wound healing (white box). Scale bar = 50 µm. (**C**) Dissected adult abdomen, dorsal view, with tergites labeled. Abdomens were filleted down the midline of the dorsal side (white dashed line). Scale bar = 50 µm. (**D**) Dissected and filleted adult abdomens prior to pinning. Scale bar = 50 µm. (**E**) Pinned adult abdomen on a dissecting plate. A pin was placed in each of the four corners of the abdomen on the dorsal side. The tissue was gently opened but not stretched, to avoid tearing. (**F**) Adult abdomens were mounted and placed on a glass coverslip with the inside of the abdomen facing down towards the coverslip and cuticle oriented toward the glass slide. (**G**) FasIII staining of intact wound area with no processing perturbation and a central syncytium (dashed yellow line). Scale bar = 50 µm. (**H**) Image of a scratched wound area (*) with an unstained FasIII region that disrupts the syncytium.

Figure 2: WIP analysis workflow. The flowchart depicts the three assays described in this study and overlapping and distinct steps to detect and measure the WIP response. The EdU assay measures cell cycle activity (blue boxes), ploidy and re-epithelialization are detected by Grh/FasIII immunostaining (green boxes), and expression of membrane RFP allows measurement of the extent of epithelial wound closure (pink boxes). Common steps are in gray boxes and *D. melanogaster* strain genotypes are listed above.

Figure 3: Methods to detect re-epithelialization during WIP. Re-epithelialization was perturbed when WIP was genetically inhibited. Immunofluorescent images of control (**A**) and *stg, fzr^{RNAi}* (**B**) at 3 dpi. Epithelial nuclei and septate junctions were stained with Grh (green) and FasIII (magenta), respectively. (A' and B') FasIII staining alone showed that re-epithelialization was impaired (red arrow) in *stg, fzr^{RNAi}* epithelium. Scale bar = 50 µm. (**C**) Quantification of re-epithelialization defects (%) at 3 dpi (gray): control (n = 8), *stg, fzr^{RNAi}* (n = 6). Error bars indicate standard error; statistical significance was measured via Student's T-test, **P < 0.01. Re-epithelialization during wound repair could also be detected by expression of a membrane linked RFP using epi-Gal4, UAS-mCD8-RFP. (**D**) Immunofluorescent images of control, *E2F1^{RNAi}, RacDN*, and *stg, fzr^{RNAi}* at 3 dpi. Scale bar = 20 µm. Wound scab (yellow outline) and open epithelial wound area (red outline). (**E**) Quantification of wound closure in ctrl (n=11), *E2F1^{RNAi}, RacDN* (n = 13), and *stg, fzr^{RNAi}* (n = 13). Adapted from Grendler et al.[16].

Figure 4: Methods to detect endoreplication during WIP. (**A**) Timeline of EdU assay: adult *Drosophila* were fed 75 µL of 5 mM yeast-EdU every day 2 days prior to injury and continued until 2 dpi. (**B**) Immunofluorescent images of EdU label in fly strains expressed with epi-Gal4/ UAS system at 2 dpi. Wound scab (W). Scale bar = 50 µm. (**C**) Average number of EdU+ epithelial nuclei per fly at 2 dpi: ctrl (n = 37), *myc$^{RNAi\#1}$* (n = 10), and *Myc* (n = 8). Error bars indicate standard error; statistical significance was measured via Student's T-test, *P < 0.05, **P < 0.01. (**D**) Schematic of detection and measurement of epithelial nuclear ploidy. Epithelial nuclei were identified and thresholded by the anti-Grh stain in Fiji. Overlapping epithelial nuclei were separated (green arrowheads) or removed (red arrowheads) if overlaid by nonepithelial nuclei. The Integrated Density and nuclear area of corresponding DAPI stained nuclei image was measured. (**E**) Epithelial nuclear size (Grh) was altered by *myc* expression at 3 dpi. (**F**) Epithelial nuclear ploidy (%) at 3 dpi: ctrl (n = 4), *myc$^{RNAi\#1}$* (n = 6), and *Myc* (n = 3). Adapted from Grendler et al.[16].

DISCUSSION:

Presented is a detailed protocol on how to dissect and use the adult *D. melanogaster* abdominal epithelium to study how genes regulate WIP by altering re-epithelialization and endoreplication during wound repair[16]. Using this method, the proto-oncogene Myc was recently identified as a key regulator of WIP. Myc is required for epithelial cells to endoreplicate post injury and is sufficient for quiescent epithelial cells to endocycle both in adult fly epithelium and accessory glands[16,22]. It was also found that switching epithelial cells to a mitotic cell cycle by expression of *stg, fzr^{RNAi}* is detrimental to wound repair. Continued studies using this method will identify other genes required to regulate re-epithelialization and endoreplication during WIP, revealing both similarities and differences to how polyploidy is regulated and functions in a variety of tissues.

This model and method offer unique advantages, including the easy induction of polyploidy with a mechanical puncture and the fact that polyploid cells are generated within days[4]. The tissue dissection and preparation protocols are based on larval dissection techniques[23], but the adult fly abdomen is more rigid and therefore easily perturbed. As a result, this protocol

requires practice and precision to isolate an intact tissue to study WIP. Once dissected, however, the epithelium is clearly visible and easily imaged, yielding a snapshot of the wound healing process. This method provides a wealth of information on the adult fly's epithelial organization, cell and syncytium size, and the ploidy of cells and individual nuclei. While live imaging is not yet possible within the intact fruit fly due to its opaque cuticle, this protocol could be adapted to include currently available ex vivo culture conditions used in *D. melanogaster* to perform short-term live imaging studies[24].

In the future, this model will be ideal to study cell-to-cell cross talk and the contribution of other cell types to WIP by regulating gene expression with the Gal4/UAS system in other cell types of interest. Similar questions can also be answered using a variety of genetic and mutant backgrounds. The dissected adult fly abdomen contains a variety of cell types that can be easily visualized using this method, including fat body and oenocytes, lateral muscle fibers, sensory neurons, trachea, and macrophage-like hemocytes. In addition, this model will allow researchers to investigate how physiological variables influence WIP, including sex, diet, infection, age, and environmental stressors. While the protocol uses the adult female fly due to its larger size, WIP also occurs in the male fruit fly (Gjelsvik and Losick, unpublished). Polyploid cells have been found to arise during aging and age-associated disease in the mammalian liver, brain, eye, and heart[12]. The fruit fly model will enable researchers to study polyploidization in physiological and disease contexts, because human disease-related genes are highly conserved.

ACKNOWLEDGMENTS:

At Boston College, we would like to thank Dr. Eric Folker for use of his lab's camera and stereoscope microscope setup for imaging and the Bret Judson at Boston College Imaging Core for infrastructure and support. We also like to thank the fly community resources: Bloomington Drosophila Stock Center (NIH P40OD018537), Vienna Drosophila Resource Center, and TRiP Center at Harvard Medical School (NIH/NIGMS R01-GM084947) for providing transgenic stocks used in this study. The mouse FasIII antibody were obtained from Developmental Studies Hybridoma Bank supported by NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R35GM124691. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES:

None.

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Figure 1

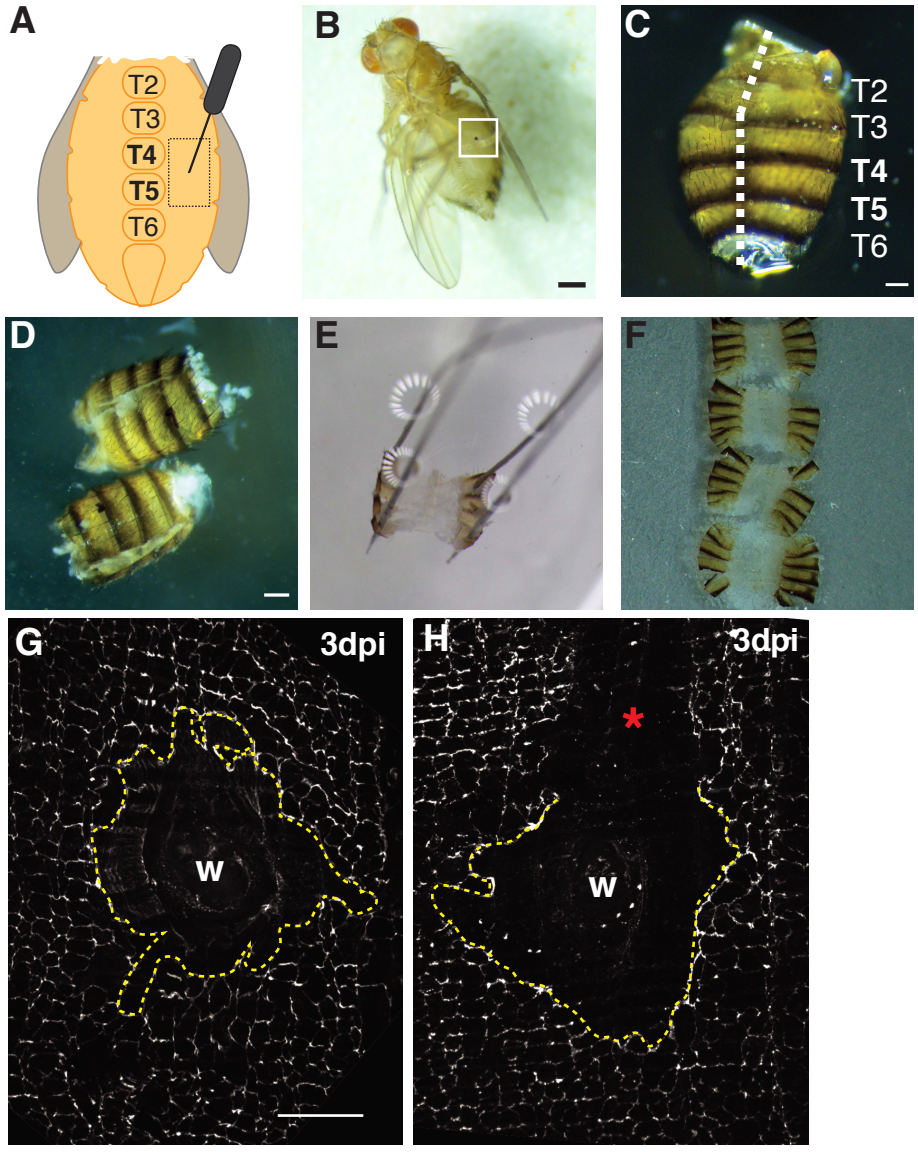


Figure 2

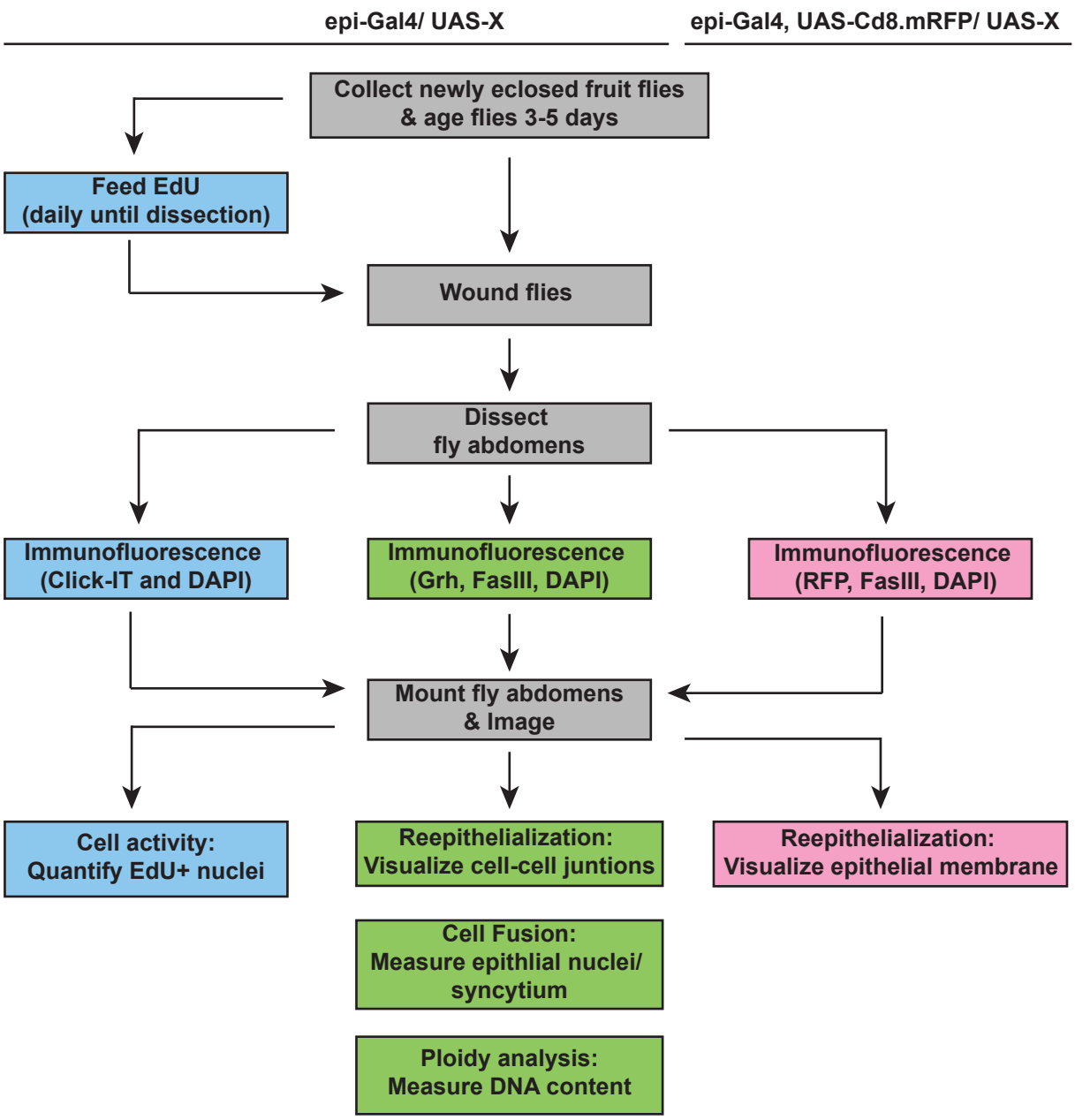
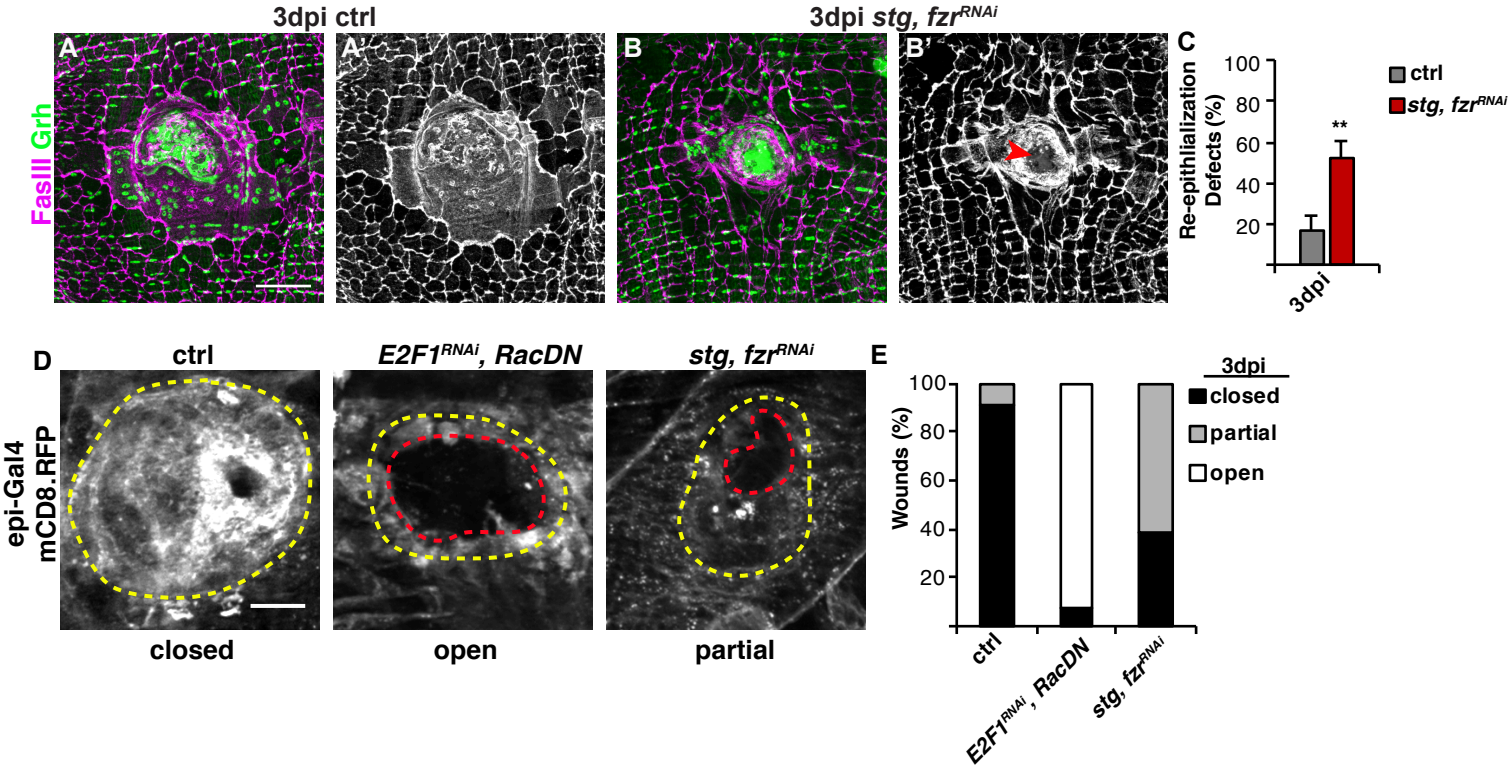
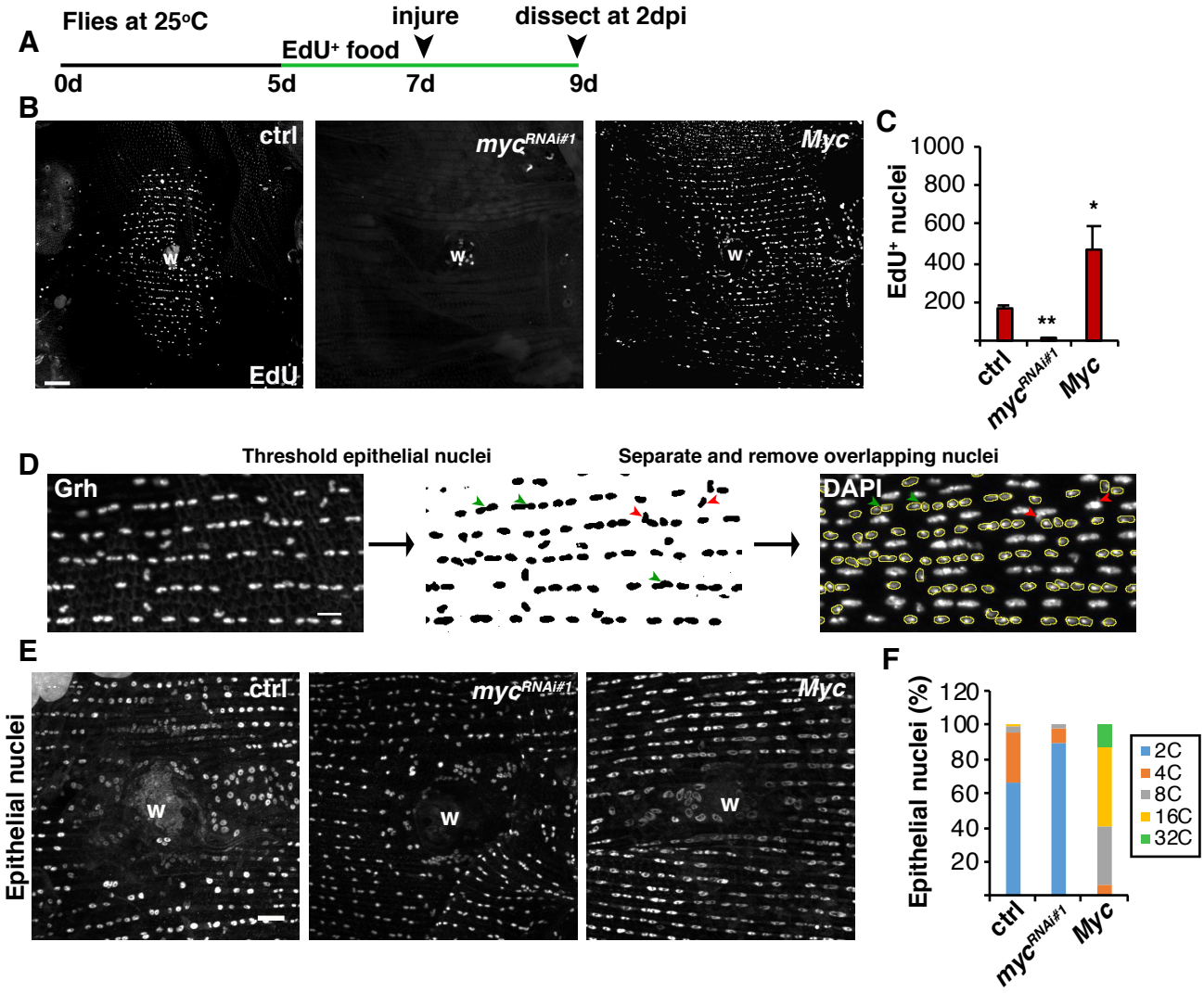


Figure 3

Click here to access/download;Figure;Figure 3. revision.ai





Name
35 mm Petri dishes
50 mL Conical Centrifuge Tubes
AxioImager M2 with Apotome
Blowgun mini
Bovine Serum Albumin, 30%
Carbon dioxide tank
Click-iT EdU 594 Kit
Coverslips
DAPI
Dissecting Plates (use Sylgard 184 Sil Elastic Kit)
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568 conjugate
Drosophila tubing and fittings
Dumont #5 Forceps
epi-Gal4
epi-Gal4, UAS-mCD8.RFP
Excel
Fiji/ImageJ (image analysis software)
Fly food
Flystuff Flypad
Glass dissecting dish
Glass slides
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 568 conjugate
Grace's Insect Medium, unsupplemented
Insect pins
Mouse anti-Fasciclin III (Drosophila) Primary Antibody
Mouting media
Nail polish
Ortibal shaker
Phosphate Buffered Saline, PH 7.4
Pin holders
Rabbit anti-Grainyhead Primary Antibody
Rabbit anti-RFP Primary Antibody
Stereomicroscope
Triton X-100
UAS-E2F RNAi, UAS-RacDN
UAS-fzr RNAi, UAS-Stg
UAS-Myc
UAS-myc RNAi
Vannas Spring Scissors

Company	Catalog Number
Fisher Scientific	FB0875713
Fisher Scientific	14-432-22
Zeiss	NA
Genesee Scientific	54-104M
Sigma	A7284-500ML
various distributors	N/A
Thermofisher	C10339
Thermofisher	3406
Sigma	D9542-10MG
Ellsworth Adhesives	184SIL
Thermofisher	A21206
Thermofisher	A10042
Genesee Scientific	59-124C, 59-123, 59-140
Fine Science Tools	11252-20
Bloomington Drosophila Stock Center (b)	b38793
Bloomington Drosophila Stock Center (b)	b38793, b27392
Microsoft	
NIH	https://imagej.nih.gov/ij
Archon Scientific	N/A
Genesee Scientific	59-114
Fisher Scientific	13-748B
Fisher Scientific	12-518-104C
Thermofisher	A11001
Thermofisher	A11031
Thermofisher	11595030
Fine Science Tools	26002-10
Developmental Studies Hybridoma Bank	7G10
Vector Laboratories	H-1000
Electron Microscopy Sciences	72180
Fisher Scientific	02-217-988
Sigma	P3813-10PAK
Fine Science Tools	91606-07
N/A	N/A
MBL	PM005
Olympus	SZ51
Sigma	10789704001
VDRC (v) and Bloomington Drosophila Stock	v108837, b6292
VDRC (v) and Bloomington Drosophila Stock	v25550, b56562
Bloomington Drosophila Stock Center (b)	b9674
Bloomington Drosophila Stock Center (b)	b36123
Fine Science Tools	15000-00

Notes

For creating plates to dissect in
For preparing staining reagents in
For imaging samples
For anethesizing D. melanogaster strains
For immuostaining
For anethesizing D. melanogaster strains
For EdU assay
For mounting
For immuostaining
For creating plates to dissect in. Mix epoxy as directed, let dry overnight
For secondary immuostaining
For secondary immuostaining
For anethesizing D. melanogaster strains
For dissecting
Losick et al. Current Biology, 2013
Losick et al. Current Biology, 2013
For performing ploidy calculations
For image analysis
Corn Syrup/Soy food
For anethesizing D. melanogaster strains
For performing dissections in
For mounting
For secondary immuostaining
For secondary immuostaining
For dissecting in
For wounding and pinning fly abdomens flat
For immunostaining epithelial cell-cell junctions
Anti-fade mounting media to prevent photo bleaching during imaging
For sealing slides
For immuostaining
For staining
For wounding
For immunostaining epithelial nuclei. Protocol to make antibody can be found (Ref. #4 and 8)
For immunostaining mCD8-RFP fly epithelium
For dissecting and mounting fly tissue
For immuostaining
Losick et al. Current Biology, 2013
Grendler et al. Development, 2019
Grendler et al. Development, 2019
Grendler et al. Development, 2019
For dissecting

Response to Reviewers:
We are grateful to the Editor and Reviewers for their time and helpful comments. We have addressed all the comments and added a flow chart (Figure 2) for clarity on the different methods used as suggested by the Reviewers. We feel the paper has been improved and hope you will consider this revision for publication in JoVE. A point-by-point description of our response to the reviewers is listed below.

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Done.

2. Please include all authors' emails in the manuscript itself.
Done.

3. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
Done.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sylgard, Tupperware, Eppendorf
Fixed.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
Done.

Figures:
1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.
Development reprint permission available at https://dev.biologists.org/content/rights-permissions

2. Figure 1G: What is the length of the scale bar here?

Added to legend 50μm.

3. Figure 2C, 3C: What do '*' and '**' mean, and what statistical test was used? Also, what do the error bars measure?
Added T-test p value significance (or **) and mean standard error to legends where included in Figures.*

References:
1. Please do not abbreviate journal titles.
Corrected.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
The Table of Materials is in excel format, but somehow got converted to a word document splitting up the columns. We doubled checked that table formatting is not altered by this resubmission.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The Losick lab, which has been pioneering research of wound-induced polyploidization in adult flies, demonstrates detailed methods to induce wounds, dissect tissues, immunostain, mount samples and quantify data. Explanation of the protocol is detailed and will be helpful for the researchers in the field. I recommend publication of this paper.
Thank you.

Reviewer #2:
Manuscript Summary:
In this methods paper the Losick group describes methodologies for wounding, mounting, and staining the adult Drosophila epidermis with a variety of markers (antibodies, EdU, DAPI) so as to measure epithelial morphology/wound closure and epithelial ploidy. Some suggestions are made below as to modifications to the protocols that will hopefully make it easier to follow for most readers/users.

Major Concerns:
1. There are actually several protocols in here, reflected in the different sections: Staging/Wounding; Dissection; Immunofluorescence; EdU; mounting; Imaging and processing; Ploidy analysis. I suggest a reordering of the sections so it is easier for the reader. If I understand correctly ploidy (measuring DAPI/DNA content) and EdU (measuring DNA synthesis) are done on separate samples. I suggest the incorportation of a flow chart that diagrams what procedures measure what, which ones can be done in parallel/concurrently, etc. I would also move the mounting up after the staining. Changing the section titles so they include not just the assay but what is being measured will help.

Thank you for this helpful organizational comment. We have added a flow chart (Figure 2) as, yes, EdU and ploidy are measurements cannot be performed on the same sample.

2. The paper is written as if groups will directly repeat prior Losick lab experiments. In reality this is unlikely (all those results are published) as what other groups may want to do is analyze these responses in different mutant backgrounds or environmental conditions than what has been done previously. Some indication of how this could be done might be helpful. Similarly, the relation of the "sample data" to the protocol is not entirely clear- especially the emphasis on reepithelialization which is not mentioned earlier. This should be clarified. Fig. 2 is example of X. Fig. 3 is example of Y.
The flowchart (Figure 2) illustrates how Grh/FasIII stained fly epithelium reveals multiple aspects of the WIP response: wound closure (re-epithelialization, cell fusion (nuclei/ syncytium), and endoreplication (ploidy). Other permeations on this central assay allow extent of cell cycle activity (EdU assay) and extent of wound closure (epithelial > membrane RFP) to be quantified as well.

3. The protocol will likely be a little easier to follow if there is a strict adherence to one action item per line.
We checked that each step meets the journals requirements with 2-3 actions and not more than 4 sentences per step.

Minor Concerns:
1. Section 1.1 mentions Gal4/UAS here with little context for non-fly researchers.
Added. See line 90-94.

2. Section 1.2- why would virgins be preferred?
This has been removed from the protocol, but is still included in discussion as other researchers may be interested to determine whether mating status affects the WIP response.

3. Section 1.4- Stereomicroscope
Corrected.

4. Section 2: Sylgard plates. These are not a standard reagent. Is there a catalog source where they can be bought or another JoVE protocol pointing to making them?
We added a brief description of how to make these plates per manufacturers instructions in the Tables on Materials.

5. Section 3- immunofluorescence. Maybe have a table here of the antibodies/labels that could be used for different purposes? Anti-fasc III for epidermal membranes. Anti-Grh for epidermal nuclei, etc. Nuclei could also be labeled genetically, no? Would take away the need for an antibody…
We included this in the flow chart. See Figure 2.

6. Section 4: EdU assay. Needed a little clarification here- this can or cannot be performed on the same samples as the DAPI/ploidy?
These are separate measurements and cannot be performed on same sample. This is clarified in flow chart (see Figure 2).

7. Section 7.6- How are non-epithelial nuclei excluded? They stain with DAPI but not Grh? Say this explicitly.
Clarified this important point in protocol line 319.

8. Line 311- ventral female abdomen.
Corrected.

9. Line 326- septate
Corrected.

10. line 343. Defect is singular
Corrected.

11. Figure 1- add dotted line on where to cut the abdomen.
Added to Figure 1C and figure legend.

Reviewer #3:
Overall this is a well written and useful protocol for those researchers who wish to study wound induced polyploidization in the adult fly cuticle epithelium.

Few comments:

"A cell is defined as polyploid if it contains more than the diploid copy of its chromosomes, which occurs by endoreplication or cell fusion." Polyploidy can also arise from endomitosis, as well as through cell fusion and endoreduplication. In fact, most polyploidy in vertebrates arises from endomitosis. This should be noted in the manuscript whenever relevant.
Endoreplication encompasses both endocycle and endomitosis and we defined this in the introduction that zebrafish epicardial cells and liver hepatocytes arise by both endocycle and endomitosis, whereas kidney epithelial arise by endocycle during tissue repair. See line 58-60.

The section on regents is problematic:
More detailed information needs to be provided regarding reagents and the information should be next to the reagent. Catalog numbers, Bloomington numbers, etc…
All information is included in the supplemental Table of Materials.

Listing a company followed by a company is inappropriate (page 19-20). One should not have to look up the catalog number to see what reagent the authors are referring to.
Fiji/ImageJ is a publicly available NIH image analysis software and depending on the

version it is named differently (i.e. newer software is called Fiji and older software is called ImageJ), hence why we refer to it as Fiji/ImageJ.

Page 21 makes no sense to me. It's just a list of "For …" statements. Maybe the formatting messed up in the download?
This is our supplemental Table of Materials, but the submission converted the excel file into a word document causing the list of material columns to be split over multiple pages.

Page 21-22 has a list of references. But they are not linked to any section.
Again this is part of Table of Materials and will discuss with editor how to ensure excel file formatting is not disrupted upon submission.

Reviewer #4:
Manuscript Summary:
The manuscript provides great insights into an injury and dissection protocol in adult drosophila wound healing studies. A more detailed description of the how re-epithelialization and wound closure are analyzed would improve the understanding of the importance of these distinct phases in wound healing. The methods of ploidy analysis provide insight into an interesting process, but maybe beyond the capability of most research labs that try to repeat the protocol. Overall, the manuscript is clear and will be a valuable resource to the scientific community.

Minor Concerns:
Are the primary antibodies (Grh and FasIII) commercially available?
These are included in the Table of Materials. FasIII is commercially available from DSHB, but Grh is homemade and references are provided.

It is not clear what the distinction is between how re-epithelialization and wound closure are measured/analyzed. For example, in figure 2C - 52% of the wounds are not able to form a continuous epithelial sheet over the wound scab in stg, fzrRNAi and in figure 2E - 62% of stg, fzrRNAi wounds had re-epithelialization defects. The results of staining with FasIII and Grh vs. epi-Gal4 expression of UAS-mCD8-ChRFP4 can be more elaborated on how the two methods measure distinct processes in wound healing.
Thank you for this helpful comment. We have elaborated on this important distinction in results section as two assays reveal differences in the reepithelialization process. FasIII highlights the epithelial cell junctions and used to determine whether there is a reepithelization defect. Whereas, epi-Gal4 expression of UAS-CD8-mRFP is used to determine extent of wound closure defect (open, partial, or closed).

The methods to analyze endoreplication appear to use a high level of manual image processing and calculations. It is not clear if these techniques would be easily reproduced within a laboratory with average imaging capabilities. This section may be beyond a reasonable interest of the scientific community and could benefit from a more elaborate description of the methods in a separate manuscript.

This is an important part of this paper's method as it details how ploidy is measured using publicly available ImageJ software. Papers have been published that erroneously use nuclear area and not ploidy as validation of endoreplication (Lee CW et al. Mol Bio Cell, 2019). Here we provide a protocol that can be adapted to other cells/tissues to analyze and measure ploidy.

Reviewer #5:

Manuscript Summary:

This manuscript from the Losick lab provides methods and protocols for the use of adult Drosophila as a model system to study wound-induced polyploidization (WIP), an emerging field of study pioneered by the senior author. In addition to describing detailed protocols for wounding, mounting, and analyzing WIP in the abdominal epithelium the paper illustrates the use of the protocols with some representative results, and discusses their potential broader applications. Overall the paper is good and the protocols well-articulated. There are a few minor issues however that need attention before it is suitable for publication, as noted below.

Major Concerns:

None.

Minor Concerns:

(1) In protocol 4 (EdU Assay) wash times should be provided for steps 4.10, 4.12, and 4.14.

Added.

(2) In protocol 7: Shouldn't 7.7 read "export the values into an excel spreadsheet"? And in 7.12, "IntDen" should be defined (Integrated Density).

Fixed.

(3) Representative Results, paragraph 4: "Figure 2E" on line 345 refers to Fig. 2D. On line 348 "Figure 2C" refers to Fig. 2E. Also, it might be helpful to add labels to Fig. 2D indicating that the three panels provide examples of wounds that are respectively closed, open, and partial.

Fixed and Added.

(4) Last paragraph of Representative Results: There red and green arrows referred to in the main text don't seem to correspond to the ones shown on the Figure. They all appear to point to overlapping nuclei, so the sentence on lines 366-367 referring to the "green arrow" doesn't make sense. On line375, "Figure 3E" should be Figure 3F.

Added.

(5) Legend to Fig. 3: what the red and green arrows are pointing to should be described, and "IntDen" should be defined (Integrated Density).

Clarified and Added.