



## Novel method for determining chromosome compaction and DNA content of salivary gland nuclei in *Drosophila*.

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### Introduction

For years the endoreplicating nuclei of *D. melanogaster* salivary glands has been a useful tool for researchers interested in investigating the mechanisms of chromosome condensation and DNA replication. These nuclei complete ten successive rounds of replication by the time they are third instar larvae reaching an average ploidy of 1024n (Edgar, 2001). In addition to endoreplication, salivary gland nuclei achieve their distinctive banding pattern by aligning multiple copies of sister chromatids along their lengths and adopting a level of condensation similar to interphase chromosomes (Lee, 2009). The ability to quantitate easily the level of chromosome compaction in normal and polyploid tissues is lacking in the literature. Techniques developed previously to examine chromosome compaction include fluorophore hybridization to chromosome arms (Dej, 1999) or simply categorizing defects by level of severity (Pflumm, 2001). These methods, while useful, are labor intensive and potentially ambiguous.

Described in the following is a novel technique capable of quantitating the compaction ratio of salivary gland polytene chromosomes. Using the Qubit<sup>®</sup> dsDNA assay kit in combination with our novel method for determining the volume of DNA, we are able to establish the quantity of DNA per unit volume ( $\text{pg}/\mu\text{m}^3$ ) – compaction ratio. This technique could be useful for molecular biologists in the quest to understand the dynamics of DNA replication and chromatin formation.

### Materials and Methods

#### *Drosophila Stocks*

The  $w^{1118}$  line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326). The PCNA (Proliferating Cell Nuclear Antigen) mutant strain *mus209<sup>2448</sup>/CyO* was also obtained from the Bloomington Stock Center (Flybase ID: FBgn0005655). Stocks were maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat # D9600-07B).

#### *Tissue Acquisition*

Wandering third instar larvae were collected and placed in a 16 well dissecting dish containing 100  $\mu\text{l}$  of 1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ ). Salivary glands were isolated using No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA). Once salivary glands were dissected, they were transferred to a separate holding well containing 100  $\mu\text{l}$  1× PBS.

#### *Fixing Tissue and DAPI Staining*

After acquiring the desired number of salivary glands, glands were transferred into a new well containing 100  $\mu\text{l}$  of 4% formaldehyde in 1× PBX (1× PBS with 1% Triton X-100) and allowed to incubate for 20 minutes at room temperature. After 20 minutes, salivary glands were moved into another well containing fresh 1  $\mu\text{g}/\text{ml}$  4',6-diamidino-2-phenylindole (DAPI) solution (diluted from 3

$\mu\text{g/ml}$  100 $\times$  DAPI stock with 1 $\times$  PBS) for 5 minutes in order to stain the DNA. After 5 minute incubation, salivary glands were removed, washed twice immediately in 1 $\times$  PBX for 5 minutes, followed by one 45 minute wash, and one 10 minute wash at room temperature. During the final 10 minute wash, prepare slide as directed below:

#### *Slide Preparation and Tissue Mounting*

Using a 20cc syringe equipped with a 22 gauge blunt fill needle filled with Vaseline<sup>®</sup>, two lines of Vaseline<sup>®</sup> were dispensed the width of the slide (Fisherbrand<sup>®</sup> 25  $\times$  75  $\times$  1.0 mm, Cat. No. 22-034-486) about an inch and a half apart. In the space between the two lines of Vaseline<sup>®</sup>, 30  $\mu\text{l}$  of Vectashield<sup>®</sup> Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA) were dispensed along the length of the slide. When the final 10 minute wash was complete, the salivary glands were transferred to the Vectashield<sup>®</sup>. A coverslip (Fisherfinest<sup>®</sup>, 22 $\times$ 50-1, Cat. No. 12-548-5E) was gently placed on top of the slide being careful to avoid air bubbles. With the coverslip on the slide, the two lines of Vaseline<sup>®</sup> were gently tapped to lower the coverslip making sure the entire area between the two lines of Vaseline<sup>®</sup> was taken up by Vectashield<sup>®</sup>.

#### *Imaging Salivary Gland Whole Mounts*

Slides were imaged using 20 $\times$  magnification on an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal equipped with Slidebook<sup>™</sup> software (Slidebook<sup>™</sup> V4.2). Salivary gland nuclei are three dimensional structures and because of this, it was necessary to create a three dimensional image using the Z-stack feature of the microscope. The Z-stack images were created using 18-20 1.5  $\mu\text{m}$  steps depending on the thickness of the salivary gland. Salivary glands are also rather large and take up multiple fields of view requiring a montage to accommodate the entire gland in one image. Each image was acquired using epi-fluorescence with a DAPI filter. Each field of view was then aligned using Slidebook<sup>™</sup> software and a maximum projection image was created. Images were saved as .tiff files for analysis using Adobe<sup>®</sup> Photoshop<sup>®</sup> elements CS5.

#### *Salivary Gland Nuclei Size Analysis*

The first step to determining salivary gland nuclei volume is to set the appropriate parameters in Photoshop<sup>®</sup>. To account for the difference in pixel length between Slidebook<sup>™</sup> and Photoshop<sup>®</sup>, the measurement scale in Photoshop<sup>®</sup> was adjusted. In the measurement scale setting pixel length was set to 1 and the logical length was set to .8 to give a scale factor of 1.25. The wand tool sensitivity (used to select individual salivary gland nuclei) was set to 80. With the parameters set, individual nuclei in a gland were selected and the measurements recorded. Measurements were exported as .txt files and transferred into Excel<sup>®</sup> spreadsheets.

#### *Determining Average Volume of Salivary Gland Nuclei*

Statistical analysis was performed using Minitab<sup>®</sup> 14 Statistical Software. Area and circularity measurements for each genotype were imported into Minitab<sup>®</sup> and the mean, standard deviation, and N were recorded. To control for false points resulting from the wand tool analysis, data points lying more than one standard deviation above and below the mean area were removed. With respect to circularity, any data points lying more than one standard deviation below the mean were removed. This middle 68% of the data is a representation of the true mean area and circularity. The mean, standard deviation, and N of the data was then recorded and used to determine DNA volumes. Using the area of a circle equation ( $A = \pi r^2$ ) the area of each data point was converted into a radius. Next, using the volume of a sphere formula ( $V = 4/3 \pi r^3$ ) values for each radius were

converted into volumes. Volume measurements were then transferred into Minitab<sup>®</sup> where the mean, standard deviation, and N were calculated.

#### *Salivary Gland Nuclei Counts*

Using the counter tool available in Photoshop<sup>®</sup>, nuclei counts were taken from ten individual salivary glands. The number of nuclei for each gland was then averaged.

#### *Salivary Gland Digestion and DNA Extraction*

The salivary glands of third instar wandering larva were dissected in 150  $\mu$ l of HyQ<sup>®</sup> Graces's Unsupplemented Insect Cell Culture Medium (Cat. No. 30610.01, HyClone, Logan, UT) and transferred to a holding well also containing 150  $\mu$ l of Grace's. Once the desired number of salivary glands were acquired in the holding well, glands were transferred to PCR tubes (Fisherbrand<sup>®</sup>, Cat. No. 14230225) prefilled with 3-5  $\times$  1 mm glass beads (BioSpec Products, Inc., Cat. No. 11079110) along with 300 micron glass beads (Sigma<sup>®</sup>, 212-300microns Unwashed, Lot. No. 033K1546) and 25  $\mu$ l of squishing buffer (20  $\mu$ g/ml proteinase K, 10 mM Tris-base, 25 mM NaCl, and 1 mM EDTA). Each tube received one pair of glands. PCR tubes were then vortexed at max speed for 15 seconds and spun to collect liquids. PCR tubes were then placed in a thermocycler (C1000<sup>™</sup> Thermo Cycler, Biorad<sup>®</sup>) and incubated at 37°C for 30 minutes then heated to 85°C for 10 minutes. After incubation, the PCR tubes were vortexed for 15 seconds and centrifuged at 12,000 rpm for 2 minutes.

Note: At this point extracts can be frozen at -20°C until desired number of digestions has been completed.

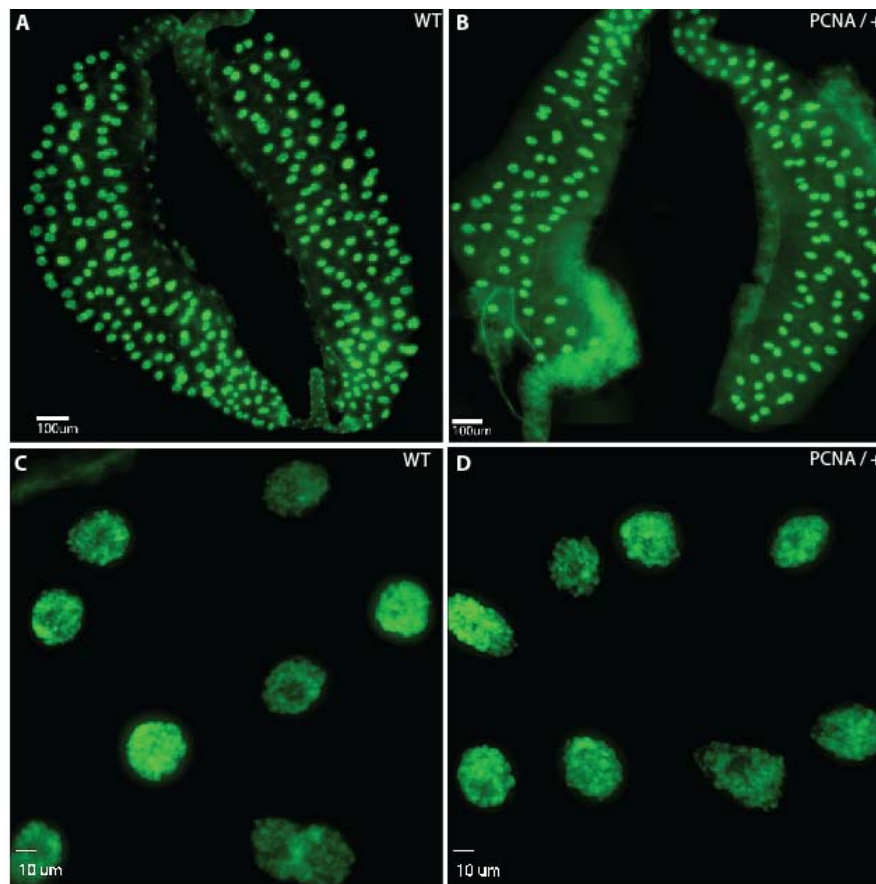
#### *Salivary Gland DNA Quantitation*

DNA content values were determined using the Qubit<sup>®</sup> dsDNA HS Kit (Qubit<sup>®</sup> dsDNA HS Assay Kit, Invitrogen<sup>™</sup>, Cat. No. Q32854) along with the Qubit 2.0 Fluorometer<sup>™</sup> (Invitrogen<sup>™</sup>, Cat. No. Q32866). Qubit<sup>®</sup> working solution was prepared by diluting the Qubit<sup>®</sup> reagent 1:200 in Qubit<sup>®</sup> buffer. 190  $\mu$ l of Qubit<sup>®</sup> working solution was transferred to Qubit<sup>®</sup> assay tubes (Invitrogen<sup>™</sup>, Cat. No. Q32856) along with 10  $\mu$ l of salivary gland DNA extract. After Qubit<sup>®</sup> working solution and salivary gland DNA extract was loaded, each tube was gently vortexed to mix and spun for 10 seconds to collect liquid at the bottom of each tube. DNA content values were determined using the Qubit<sup>®</sup> 2.0 Fluorometer. The Qubit<sup>®</sup> 2.0 Fluorometer was standardized using two standard solutions provided in the Quant-iT<sup>™</sup> dsDNA HS Kit.

## **Results**

#### *Epi-fluorescence imaging and Photoshop<sup>®</sup> analysis is sufficient to determining nuclear DNA volume and total salivary gland nuclei*

In order to establish the compaction ratio of polytene chromosomes, it was first necessary to determine the volume of DNA present in each nucleus. Epi-fluorescence images of WT and PCNA/+ salivary gland whole mounts stained with 4',6-diamidino-2-phenylindole (DAPI) were acquired using z-stack and montage features available in Slidebook<sup>™</sup> (Figure 1A and B). Representative images of the two dimensional projection image used for size analysis can be seen in Figure 1C and D. Using the wand tool feature available in Photoshop<sup>®</sup>, the area of DNA for each nucleus was recorded (data not shown). As described in the materials and methods, the volume of nuclear DNA was derived using the area of a circle equation to determine the radius followed by the volume of a sphere equation. Using this method, we were able to determine that there is an 81.21% reduction in the average volume of PCNA/+ nuclear DNA compared to WT (Figure 1F). Volumes for PCNA/+ and



E

	Genotype	
	WT	PCNA / +
Volume of Nuclei	13116 $\mu\text{m}^3 \pm 209$	10652 $\mu\text{m}^3 \pm 152$
# of Nuclei Per Gland	116.4 $\pm 5.34$	117.6 $\pm 4.23$
DNA Content (ng / gland Pair)	22.04 $\pm 2.23$	14.24 $\pm 1.10$
DNA / Nuclei (pg)	94.6 pg	60.05 pg
Packing Ratio ( $\text{pg}/\mu\text{m}^3$ )	0.0072	0.0056

F

	Volume of Nuclei ( $\mu\text{m}^3$ )	# of Nuclei Per Gland	DNA Content (ng/gland pair)	DNA (pg) Per Nuclei	Packing Ratio ( $\text{pg}/\mu\text{m}^3$ )
PCNA / + relative to WT	81.21% ( $p < .000$ )	101% ( $P = .852$ )	64.6% ( $P = .003$ )	63.50%	78%

Figure 1. Data derived from salivary gland polytene chromosome size analysis and DNA content quantitation. Panels A and B: representative images of salivary gland montages. Panels C & D: two dimensional maximum projection images of nuclei used for size analysis. Panel E (top): average volume of nuclei DNA for each genotype ( $T = 9.53$ ,  $P < 0.000$ ,  $N = 356$  WT and  $357$  PCNA/+). Panel E (second from top): the total number of nuclei in each gland averaged from glands ( $T = -.18$ ,  $P = 0.862$ ,  $N = 10$ ). Panel E (third from top): DNA content (pg) per nucleus. Panel E (bottom): compaction ratio of polytene chromosomes. Panel F: percents relative to WT ( $p$ -values depict significance).

WT were  $10,653 \mu\text{m}^3$  and  $13,116 \mu\text{m}^3$ , respectively (Figure 1B upper panel). The results of a two-sample t-test confirmed the difference in volume to be significant with  $T = 9.53$  and  $P < 0.000$ . The  $N$  for WT was 356 and PCNA/+ 357. Total nuclei counts were completed using the montages as well (Figure 1B, second panel from top). There was no significant difference in total number of nuclei between the two genotypes ( $T = -0.18$ ,  $P = 0.852$ ).

#### *Determining salivary gland DNA content*

Spectroscopic analysis using A260/A280 is usually sufficient in determining the quantity of DNA in a sample. Unfortunately, the results of this method are easily influenced by the presences of interfering molecules like proteins. As an alternative to performing chloroform DNA extractions on each pair on salivary glands (which would be both costly and time consuming), we used the Qubit<sup>®</sup> dsDNA HA kit. This kit is selective for dsDNA over RNA and is not influenced by salts, free nucleotides, or protein that remained in the sample after extraction. Our results showed PCNA/+ mutants have an average of 14.24 ng of DNA per gland pair compared to 22.04 ng seen in WT; a 63.5% reduction (Figure 1E, third panel from top). T-tests show the reduction in DNA content to be significant:  $T = 3.12$  and a  $P = 0.003$  ( $N = 49$  for both genotypes). Having established the average number of nuclei per gland and the DNA content per gland pair, we were able to infer the average DNA content per nucleus. On average, PCNA/+ nuclei contain 60.05 pg of DNA per nucleus, while WT contain 94.6 pg, a reduction of 63.5% (Figure 1E and F). Using volumes derived from the size analysis in conjunction with DNA content data, we are able to deduce the compaction ratio of the polytene chromosomes. The packing ratios of PCNA/+ and WT polytene chromosomes were determined to be  $0.0056 \text{ pg}/\mu\text{m}^3$  and  $0.0078 \text{ pg}/\mu\text{m}^3$ , respectively, a 78% reduction.

## **Discussion**

Endoreplicating tissues provide a unique system to study defects in DNA replication. In endoreplicating tissues (such as the salivary glands in *D. melanogaster*), cells undergo successive rounds of DNA replication to reach an average ploidy of  $1024n$  (Edgar, 2001). Using this ploidy, we were able to calculate the theoretical quantity of DNA present in salivary gland nuclei. The *Drosophila* genome ( $n$ ) is estimated to be around 180Mb. Of this 180Mb, 70% is euchromatin that reaches an average ploidy of  $1024n$  and 30% is tightly packed heterochromatin that remains at  $2n$  (Gall, 1973). Using these ploidy values and multiplying by the average atomic mass of a nucleotide (330Da), we determined the theoretical mass of salivary gland nuclei to be  $1.2 \times 10^{11}$  Daltons or 135.3 pg. This number probably represents an overestimate as it has been demonstrated that within a particular gland there are different levels of DNA content. Nuclei near the distal tip tend to contain more DNA as compared to those near the duct (Park, 2008). Our estimation of 94.6 pg of DNA/nucleus in WT is in good agreement (30% different) with the theoretical estimates of DNA content of 135.3 pg per nucleus. Put another way our estimates put the average ploidy of WT nuclei at  $\approx 716n$  which is right in between the  $512n$  and  $1024n$  that would compromise the last two rounds of endoreplication.

Techniques previously described for measuring DNA content involved the A260/A280 ratio derived from spectroscopic analysis (Yanjuan Xu *et al.*, 2009). In our analysis, using the A260/A280 ratio resulted in inflated DNA content values due to the presence of interfering proteins (our unpublished data). As an alternative to labor intensive and costly chloroform purification to remove proteins, we found a method for quantitating DNA concentrations not influenced by the presence of common contaminants. The Qubit<sup>®</sup> dsDNA HS kit uses a fluorophore that binds selectively to

dsDNA eliminating interference from free nucleotides, salts, and proteins. Using this method to quantitate the amount of DNA per gland pair then dividing by the total number of nuclei, we determined that on average wild type nuclei contain 94.6 pg of DNA. Testing the validity of our methods, we chose to perform the analysis on *Mus209*<sup>2448</sup>, a PCNA mutant.

PCNA is a protein clamp that functions as a processivity factor allowing DNA polymerases  $\delta$  and  $\epsilon$  to move with the replication fork (Scalfini, 2007). Using BrdU incorporation assays, recent research has shown *Mus209* to be defective for DNA replication (Pflumm, 2001). Using the methods described, we have been able to further the findings of Pflumm *et al.* and quantitate the reduction in DNA synthesis in endoreplicating salivary gland nuclei attributed to the PCNA/+ mutant. We show PCNA/+ mutants to have a 63.5% reduction in DNA content per nucleus and an 81.21% reduction in average volume relative to wild type.

It is generally accepted that organisms can grow either by increasing their cell size or cell number. In the terminal tissues of *D. melanogaster* salivary glands, the short term need for copious amount of saliva has been met by increasing the ploidy of nuclei. Research in plant cells has shown a direct link between ploidy levels and cell size (Kondorosi, 2000). Here we show our technique is sensitive enough to detect minor differences in both DNA content and volume. We confirm and extend the findings of Pflumm *et al.* where they analyzed homozygous *Mus209* mutant salivary glands and showed a decreased DNA synthesis, by showing that salivary glands from heterozygous larvae are also defective for endoreplication.

The overall reduction in nuclei size observed in the PCNA/+ larvae is not solely due to a reduction in DNA content. If it were the case, then we would expect that the compaction ratio would be unchanged compared to WT. Instead we observe a lowered compaction ratio that would be indicative of condensation defects. It is interesting to note that in addition to its role as a processivity factor, PCNA has been shown to be involved in chromatin restoration and *de novo* histone deposition on newly synthesized DNA (Groth, 2007). As part of the histone restoration process, PCNA directly interacts with Caf-1 which recruits histone methyltransferases (HMTase) responsible for the methylation of histones tails, which are usually a determinant in the formation of transcriptionally inactive euchromatin and heterochromatin (Weaver, 2008). The reduced compaction ratio seen in the *Mus209* mutant is possibly a consequence of the failed recruitment of HMTase's resulting in improperly condensed chromatin.

In summary, we have created a novel technique capable of quantitating minute differences in the DNA content and volume of individual nuclei. These two values in combination allow us to ascertain the compaction ratio of chromosomes. This technique provides an easy, highly reproducible, and cost effective alternative to similar methods for quantitating levels of condensation. Moreover, this technique can be easily adapted to other research models where tissues with a finite number of nuclei can be dissected.

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**References:** Dej, K.J., and A.S. Spradling 1999, *Development* 126: 293-303; Edgar, B.A., and T.L. Orr-Weaver 2001, *Cell* 105: 297-306; Gall, B.B., 1973, *PNAS* 70(5): 1359-1363; Groth, A.R., 2007, *Cell* 128: 721-733; Kondorosi, E., F. Roudier, and E. Gendreau 2000, *Curr. Opin. Plant Biol.* 3: 488-492; Lee, H.D., 2009, *Genes and Development* 23: 2461-2477; Park, S.Y., 2008, *PNAS* 105: 12343-12348; Pflumm, M.F., and M.R. Botchan 2001, *Development* 128: 1697-1707; Scalfini, R.H., 2007, *Annual Review of Genetics* 41: 237-280; Weaver, R.F., 2008, *Molecular Biology*, 4th edition. New York: McGraw-Hill; Yanjuan Xu, *et al.*, 2009, *PLoS ONE* 4(7): 1-13.

## Protocol: Salivary Gland Whole Mounts with Size Analysis

### Reagents

1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>)  
4% formaldehyde in 1× PBX (1× PBS with 1% Triton X-100)  
1 µg/ml DAPI in 1× PBS

### Equipment

Slides (Fisherbrand<sup>®</sup> 25 × 75 × 1.0 mm, Cat. No. 22-034-486)  
Vectashield<sup>®</sup> Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)  
Coverslip (Fisherfinest<sup>®</sup>, 22×50-1, Cat. No. 12-548-5E)

### Dissecting Salivary Glands and DAPI Staining

1. Using a 16 well dissecting dish or two well depression slide, dissect 3rd instar wandering larva salivary glands in 100 µl of 1× PBS.

2. Dissect five pairs of salivary glands for a total of 10× glands. Try to extract salivary glands as a pair – keeping them attached to each other.

3. After dissections, move salivary glands into a second well filled with a 4% formaldehyde solutions in PBX (1× PBS with 1% Triton X-100) for 20 minutes (make fresh).

*\*Helpful Hint: Be careful. After fixation the salivary glands become very rigid and will break easily when transferring from well to well from this point on.*

4. After 20 minutes, move salivary glands into another well with 1 µg/ml DAPI solution for 5 minutes (make fresh).

5. After 5 minute DAPI incubation, move salivary glands into a well with 1× PBX and wash twice for 5 minutes each wash.

6. After two immediate 5 minute washes, move salivary glands into a well with 1× PBX and wash for 45 minutes followed by a final 10 minute wash.

7. During final 10 minute wash, prepare slide as follows:

a. Use a syringe to dispense two lines of Vaseline<sup>®</sup> the width of the slide, about an inch and a half apart.

b. In the middle of the space between the two lines of Vaseline<sup>®</sup>, dispense 30 µl of Vectashield<sup>®</sup> mounting media along the length of the slide.

8. When the final 10 minute wash is complete, transfer the salivary glands into the Vectashield<sup>®</sup>.

9. Gently place coverslip on the slide and tap the two lines of Vaseline until the Vectashield takes up the entire area between the two lines of Vaseline.

10. Seal the coverslip around the edges with nail polish to prevent Vectashield<sup>®</sup> from evaporating.

11. Image, using procedure below.

### Imaging Salivary Gland

1. Salivary gland preps were imaged on an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal.

2. Locate salivary glands using 10× objectives and DAPI eyes filter.

3. Once the first gland you are attempting to image is in the field of view, move to the 20× objective and focus on a nucleus.

4. Change scope setting to either confocal mode or epifluorescence mode with a DAPI filter and put the central most part of the salivary gland in the field of view.

5. Since the salivary gland is a three dimensional image, it is necessary to take an image from multiple focal planes. This type of image is usually referred to as a z-stack.

*\*Helpful Hint:* Defining the step of your z-stack between 1-1.5  $\mu\text{m}$ , is generally a good idea. Steps sizes lower than 1  $\mu\text{m}$  result in too large of a file for some imaging software.

6. With the three dimensional parameters of the z-stack set, it is necessary to utilize the montage feature of your imaging software, since salivary glands take up multiple fields of view.

7. After the montage parameters are sent, move the field of view back to central most portion of the salivary gland.

8. Set the ND filter and Intensity to settings that allow for low exposure times.

*\*Helpful Hint:* When imaging several different slides, make sure you use the same ND filter setting and Intensity settings.

9. Adjust the exposure time and take the picture.

10. Save each montage as its own file for analysis later.

### Analyzing Salivary Gland Pictures Using Adobe® Photoshop®

1. Use the wand tool to select individual nuclei. You want to make sure there are no gaps around the parameter of the nuclei. Not having a good fit on every nucleus will artificially inflate the area of your nuclei resulting in larger volumes.

*\*Helpful Hint:* In our attempts, we find that a wand tool setting of 80 provides the best results.

2. With all of the nuclei of one salivary gland selected, record measurements and export data.

a. Data for each measurement will appear. You will need to delete the first data point each time as it is a total of all the data points.

b. I recommend setting up a folder dedicated solely to exported files. Photoshop® will export both a data folder and .txt file.

*\*Attention:* Be sure to adjust the measurement scale in Photoshop® to account for the pixel length used by your imaging software.

### Determining Average Area and Volume for Nuclei

1. Compile the data points for area and circularity into one spreadsheet.

2. Determine the mean, standard deviation, and N for both area and circularity.

3. With the parameters above defined, remove points that are beyond one standard deviation away from the mean area; remove points that lie one standard deviation below the mean circularity.

- This will allow us to select the middle 68% of the data each time in addition to eliminating false points that can be acquired during the size acquisition.

4. After you have eliminated points for each of the genotypes, again determine the mean, standard deviation, and N.

5. With the area of each nucleus determined, use the area of a circle formula ( $A = \pi r^2$ ) to turn the area into a radius for each point. With a radius for each point, you can use the volume of a sphere formula ( $V = 4/3 \pi r^3$ ) to determine the volume of each nucleus.



## Protocol: Salivary Gland DNA Extraction and Quantitation

### Reagents

100 ml Squishing buffer (- Proteinase K) (10 mM Tris-Base, 25 mM NaCl, 1 mM EDTA, dH<sub>2</sub>O to 100 ml)

Proteinase K Stock solution (200 µg/ml dissolved in dH<sub>2</sub>O)

HyQ<sup>®</sup> Graces's Unsupplemented Insect Cell Culture Medium (Cat No. 30610.01, HyClone, Logan, UT)

Qubit<sup>®</sup> dsDNA HS Kit (Invitrogen<sup>™</sup>, Cat. No. Q32854)

### Equipment

0.2 ml PCR tubes (Fisherbrand<sup>®</sup>, Cat. No. 14230225)

1 mm glass beads (BioSpec Products, Inc., Cat. No. 11079110)

212-300 micron glass beads (Sigma<sup>®</sup>, 212-300microns Unwashed, Lot. No. 033K1546)

Thermocycler (C1000<sup>™</sup> Thermo Cycler, Biorad<sup>®</sup>)

Qubit<sup>®</sup> assay tubes (Invitrogen<sup>™</sup>, Cat. No. Q32856)

Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen<sup>™</sup>, Cat. No. Q32866)

### DNA Extraction

#### Preparation

1. Obtain desired number of 3rd instar larvae and wash them in a 16 well dissecting dish with dH<sub>2</sub>O to remove food particles.

2. Mix enough squishing buffer with Proteinase K to aliquot 25 µl to each of the dissected salivary glands.

3. Store squishing buffer at 4°C until ready to use.

*\*Attention:* Squishing buffer should be stored in the 4°C refrigerator without Proteinase K. Proteinase K stock solution should be stored at -20°C. Dilute stock solution of Proteinase K to 20 µg/ml with squishing buffer.

4. Place ~3-5 1 mm glass beads along with 300 micron glass beads (just enough to cover the bottom) in a PCR tube.

#### Dissection and Salivary Gland Digestion

5. Transfer larva from the washing well to a new well containing 150 µl Grace's dissecting medium.

6. Fill a separate "holding well" with 100 µl of Grace's.

7. Dissect out the salivary glands from each larva and transfer to the "holding well."

8. Once you have dissected out the desired number of salivary glands, aliquot 25 µl of squishing buffer into each PCR tube containing glass beads.

*\*Helpful Hint:* Limit the amount of time from the start of the dissection of the salivary glands to their placement into PCR tubes to the number of salivary glands that can be dissected in 25 minutes.

9. Place one set of salivary glands into each PCR tube.

*\*Helpful Hint:* Make sure that the salivary glands come off of the tip of the tweezers. If salivary glands sit in Grace's for too long they tend to become sticky.

10. Vortex the PCR tubes at max speed for ~10 seconds (pulse).

11. Briefly spin PCR tubes to collect the entire sample in the bottom.

12. Place the PCR tubes in thermocycle and run cycle (heat block will also work).
  - a. Incubate at 37°C for 30 minutes followed by 10 minute incubation at 85°C.
13. Remove PCR tubes from thermocycler and vortex again for ~10sec (pulse w/ vortex); spin to collect sample.
14. At this point you can freeze samples at -20°C until desired number of digestions have been completed.
15. After thawing, or if you continue from Step 12, centrifuge samples for 2 min. at 12,000 rpm.

### DNA Concentration Quantitation using Qubit<sup>®</sup> dsDNA HS Kit

1. Prepare the proper amount of Qubit<sup>®</sup> working solution by diluting the Qubit<sup>®</sup> reagent 1:200 in Qubit<sup>®</sup> buffer.
  - a. 190 µl of working solution is required for each sample and standard.
  - b.  $1 \mu\text{l} \times N \# \text{ of samples} = X \mu\text{l of Qubit}^{\text{®}} \text{ Reagent}$
  - c.  $199 \mu\text{l Qubit}^{\text{®}} \text{ buffer} \times N \# \text{ of samples} = X \mu\text{l of Qubit}^{\text{®}} \text{ Buffer}$
2. Aliquot 190 µl of Qubit<sup>®</sup> working solution into Qubit<sup>®</sup> ultra-clear assay tubes.
3. Dilute DNA extract 1:20 in Qubit<sup>®</sup> working solution.
  - a. 10 µl of DNA extract into 190 µl of Qubit<sup>®</sup> working solution.

**\*Attention:** *Be sure to make samples with Standard #1 and Standard #2 to standardize the Qubit<sup>®</sup> Fluorometer each time.*

1. Vortex all tubes for 2-3 seconds to mix and spin to collect sample at the bottom of the tube.
2. Incubate the tubes for ~2 minutes at room temperature.
3. Select proper program, dsDNA HS, on Qubit<sup>®</sup> Fluorometer and standardize machine.
4. Read tubes in the Qubit<sup>®</sup> Fluorometer.

**\*Helpful Hint:** *You can automatically calculate the concentration of your original sample by selecting "calculate sample concentration" → GO → 10µl → GO.*

### The Citric-Arabic-glycerated gum: An alternative to Fourè gum.



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### Introduction

The conservation of biological material for analysis of results in different areas of biology is a process of great importance. Making a microscopic preparation involves enclosing the material of study between two sheets of glass: the slide serving as a support, and the coverslip, used to cover the material.

The material can be mounted in air or in a liquid medium. In the first case material is solidified by cooling or by evaporation. Another side, when the material is put in liquid medium, the preparation has to be locked to avoid mounting fluid evaporation and subsequent drying of the material. In all cases, the object must be placed between the slide and coverslip so that the preparation remains uniformly flat and the front lens of high magnification objectives do not touch