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Immunofluorescent staining for visualization of heterochromatin associated proteins in Drosophila salivary glands

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TITLE:

Immunofluorescent Staining for Visualization of Heterochromatin Associated Proteins in *Drosophila* Salivary Glands

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KEYWORDS:

Drosophila melanogaster, heterochromatin, salivary glands, chromatin, immunostaining, HP1a

SUMMARY:

This protocol aims to visualize heterochromatin aggregates in *Drosophila* polytene cells.

ABSTRACT:

Visualization of heterochromatin aggregates by immunostaining can be challenging. Many mammalian components of chromatin are conserved in *Drosophila melanogaster.* Therefore, it is an excellent model to study heterochromatin formation and maintenance. Polytenized cells, such as the ones found in salivary glands of third instar *D. melanogaster* larvae, provide an excellent tool to observe the chromatin amplified nearly a thousand times and have allowed researchers to study changes in the distribution of heterochromatin in the nucleus. Although the observation of heterochromatin components can be carried out directly in polytene chromosome preparations, the localization of some proteins can be altered by the severity of the treatment. Therefore, the direct visualization of heterochromatin in cells complements this type of study. In this protocol, we describe the immunostaining techniques used for this tissue, the use of secondary fluorescent antibodies, and confocal microscopy to observe these heterochromatin aggregates with greater precision and detail.

INTRODUCTION:

Since the early studies of Emil Heitz[1], heterochromatin has been considered an important regulator of cellular processes such as gene expression, meiotic and mitotic separation of chromosomes, and the maintenance of genome stability[2–4].

Heterochromatin is mainly divided into two types: constitutive heterochromatin that characteristically defines repetitive sequences, and transposable elements that are present at specific chromosome sites such as the telomeres and centromeres. This type of heterochromatin is mainly defined epigenetically by specific histone marks such as the di or tri-methylation of lysine 9 of histone H3 (H3K9me3) and the binding of the Heterochromatin protein 1a (HP1a)[5,6].

On the other hand, facultative heterochromatin localizes through the chromosome's arms and consists mainly of developmentally silenced genes[7,8]. Immunostaining of heterochromatin blocks in metaphase cells, or the observation of heterochromatin aggregates in interphase cells, has unveiled much light in the understanding of the formation and function of heterochromatic regions[9].

The use of *Drosophila* as a model system has allowed the development of essential tools to study heterochromatin without the use of electron microscopy[10]. Since the description of position effect variegation and the discovery of heterochromatin-associated proteins, such as HP1a, and histone post-translational modifications, many groups have developed several immunohistochemical techniques that allow visualization of these heterochromatic regions[10,11].

These techniques are based on the use of specific antibodies that recognize heterochromatin-associated proteins or histone marks. For every cell type and antibody, the fixation and permeabilization conditions must be determined empirically. Also, conditions may vary if additional mechanical processes such as squashing techniques are used. In this protocol, we describe the use of *Drosophila* salivary glands to study heterochromatic foci. Salivary glands have polytenized cells that contain more than 1,000 copies of the genome, thus providing an amplified view of most of the chromatin features, with the exception of satellite DNA and some heterochromatic regions which are under replicated. Nevertheless, heterochromatin regions are easily visualized in polytene chromosome preparations, but the squashing techniques may sometimes disrupt characteristic chromatin-bound complexes or the chromatin architecture. Therefore, immunolocalization of proteins in whole salivary gland tissue can surpass these undesired effects. We have used this protocol to detect several chromatin bound proteins, and we have demonstrated that this protocol combined with mutant *Drosophila* stocks can be used to study heterochromatin disruption[12].

PROTOCOL:

1. Third instar larvae culture

1.1. Prepare 1 liter of standard media by adding 100 g of yeast, 100 g of unrefined whole cane sugar, 16 g of agar, 10 mL of propionic acid and 14 g of gelatin. Dissolve all ingredients except the yeast in 800 mL of tap water and then dissolve the yeast. Autoclave immediately for 30 minutes.

1.1.1. Afterward, let the media cool down to 60 °C and add propionic acid to a final concentration 0.01%. Let the bottle stand until the gelatin is formed.

1.2. To optimize the 3° instar larvae culture, first collect 5-to-10-day old adults and place 50 (25 males and 25 females) in a broad neck bottle of standard media.

1.3. Place the bottle with the flies in a controlled temperature incubator at 25 °C until the number of eggs laid is 50 (approximately 12 hours for the wild-type strain).

1.4. After the incubation time is over, remove the adults and transfer them to a new bottle to repeat the procedure. Let the embryos grow at 18 °C for 72 hours

NOTE: For more about *Drosophila* stock maintenance conditions, see Tennessen & Thymmel[13].

2. Larvae collection

2.1. For larvae collection choose the wandering larvae that do not have everted spiracles. After the eversion of the spiracles, the larva enters the prepupal stage, while retaining excellent polytene chromosomes suitable for analysis. Only after 12 hours do the cells of the salivary gland begin to prepare for programmed cell death[14,15].

2.2. Take fifteen 3° instar larvae and put them in a watch glass to wash them. Then transfer them to an ice-cold saline solution or PBS (1x PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, adjust pH to 7.4).

2.3. Dissect 15 to 30 pairs of salivary glands (or as many as possible in 30 minutes) in cold PBS with protease inhibitors under the stereoscopic microscope. Transfer the salivary glands to a 1.5 mL tube with ice-cold PBS.

2.4. Wash once with 1 mL of PBS plus protease inhibitors. Wait for the tissue to reach the bottom of the tube.

2.5. After the wash, remove the PBS with a 1000 µL pipette. Take care not to touch the tissue.

2.5.1. Alternatively, dissect the salivary glands in 5 mL of PBS to eliminate the need for this washing step and proceed to step 3 by transferring the salivary glands to 0.5 mL of the Ruvkun fixing buffer described below.

3. Salivary gland tissue fixation

3.1. After removing the PBS from the last step, directly add 0.5 mL of 1x Ruvkun fixing buffer, with 50% methanol (add 0.5 mL of methanol) and 2% formaldehyde.

NOTE: 2x Ruvkun solution is 160 mM KCl, 40 mM NaCl, 20 mM EGTA, 30 mM PIPES at pH 7.4.

3.2. Incubate for 2 hours at 4 °C with mild rotation.

4. Salivary gland tissue wash

4.1. Carry out one 5-minute rotation wash with 1 mL of Tris/Triton buffer (100 mM Tris pH 7.4, 1% Triton X-100 and 1 mM EDTA).

NOTE: Wait for the tissue to reach the bottom of the tube.

5. Permeabilization

5.1. Incubate the salivary glands in 1 mL of Tris/Triton X-100 (the same as above). For some proteins it might be necessary to add 1% β-mercaptoethanol.

5.2. Incubate for 2 hours at 37 ° C with mild shaking (300 rpm).

6. Preservation step (optional)

NOTE: If not proceeding immediately to the incubation with the antibody, preserve the tissue as follows.

6.1. Wash with 1 mL of BO_3 buffer (0.01 M H_3BO_3 pH 9.2 + 0.01 M NaOH) and then incubate in BO_3/10 mM DTT at 37 ° C with mild shaking (300 rpm) for 15 minutes.

6.2. At the end of the incubation period, perform a wash with 1 mL of BO_3 buffer alone.

NOTE: Wait for the tissue to reach the bottom of the tube.

6.3. Add 1 mL of PBS. Preserve the tissue in this solution at 4 °C for up to 72 hours and then proceed with the next step. This step is particularly helpful when working with different mutant strains that may present a delayed life cycle, so the immunodetection can be performed at the same time along with the controls.

7. Tissue blocking

7.1. Incubate the salivary glands in 1 mL of Buffer B (PBS + 0.1% BSA + 0.5% Triton X-100 + 1 mM EDTA) for 2 hours at room temperature with rotation.

8. Immunostaining

8.1. Remove all buffer B and add buffer A (PBS + 0.1% BSA) plus antibody of interest.

NOTE: We use the HP1a C1A9c (concentrated antibody) from Hybridoma Bank up to 1:3000. When using the C1A9s (supernatant) we have tried from 1:100 to a 1:500 dilution and any dilution between this rank works well) overnight at 4 °C with rotation. At this point it is important that the shaking does not raise bubbles which might damage the antibody.

9. Immunostaining washing

9.1. Perform 3 x 15-minute washes with buffer B under stirring at room temperature using 1 mL each time.

9.2. Transfer the glands to buffer B together with the secondary antibody coupled to a fluorophore for 2 hours under rotation at 4 °C (secondary antibody Alexa fluor 568 Invitrogen were used 1:3000).

9.2.1. Cover the tube with aluminum paper foil to protect the secondary antibody from the light.

9.3. Carry out 2 x 15-minute washes at room temperature while rotation with 1 mL of Buffer B.

9.4. Incubate with a DNA marker such as Sytox (take 2 µL of 5 mM stock and dissolve in 1 mL of Buffer B) or Hoechst (take 1 µL of 10 mg/mL stock and dissolve in 1 mL of Buffer B) for 10 minutes at room temperature with rotation.

9.5. Carry out one wash with Buffer B and once with PBS, each wash lasting 10 minutes while rotating at room temperature.

NOTE: Remember to protect it from the light.

10. Imaging

10.1. Mount the salivary glands on a slide, making a pool with a coverslip.

10.2. Put the salivary glands in the middle of the pool and cover with AF1 citifluor to avoid the formation of bubbles extending the viscous liquid all over the place. Then seal all the sides with clear nail polish.

10.3. Observe under a fluorescence or confocal microscope. If the sample is not going to be observed on the same day, store away from light at 4 °C.

10.4. Use GraphPad Prism 6 to generate all graphs and statistical analyses.

10.5. Analyze the data from HP1a distribution in salivary glands using the Kruskal-Wallis test. Statistical significance was set at ($p < 0.05$*, < 0.01 **, < 0.001***, < 0.0001****).

REPRESENTATIVE RESULTS:

Representative results of HP1a immunostaining in *Drosophila* salivary glands are shown in **Figure 1**. A positive result is to observe one focal point (**Figure 1a**) (heterochromatic aggregate or condensate). A negative result is no signal or a dispersed signal. Sometimes a double signal can be observed, that is, with a double point (**Figure 1c**), but it usually occurs in smaller quantities.

Data analysis can be represented as bar graphs, comparing the distribution of HP1a within different mutant backgrounds. For example, in **Figure 2** we can see that 98% of the wild type nuclei present a distribution of one focal point and 2% of the nuclei present two foci, whereas in the mutant, the proportion changes, and the presence of two foci increases to 40%.

Figure 3 shows representative H3k9me3 immunostaining results in *Drosophila* salivary glands. We can observe one focal point (**Figure 3b**) that resembles the HP1a immunostaining, (heterochromatic aggregate or condensate). A double or triple signal (**Figure 3c**) can be seen on rare occasions in the wild type strains.

FIGURE AND TABLE LEGENDS:

Figure 1. Representative confocal microscopy image from salivary gland immunostaining with HP1a antibody from wild type (wt). a) DNA (cyan signal), HP1a (magenta signal), and merge scale bar 100 µm. In immunostaining for HP1a, a nucleus with a focal point is marked with a white arrow and a nucleus with two foci with a dotted line box. The right column shows a magnified image of a single nucleus with a scale bar of 5 µm. **b**) focal distribution. **c**) two foci distribution. Both nuclei are marked with a white dashed line.

Figure 2. Examples result from counting nuclei foci distribution ofHP1a immunostaining. The first bar represents the counting of the wild-type nuclei (wt), as in **Figure 1**. The second bar represents a mutant strain that affects this distribution.

Figure 3. Representative confocal microscopy image from salivary gland immunostaining with H3K9me3 antibody from wild type (wt). a) DNA (cyan signal), H3K9me3 (magenta signal) and merge scale bar 100 µm. In immunostaining for H3K9me3.The right column shows a magnified image of a single nucleus with a scale bar of 5 µm. **b**) a nucleus with a focal distribution. **c**) three foci distribution. Both nuclei are marked with a white dashed line.

DISCUSSION:

The cellular function of eukaryotic organisms can define the 3D structure within the nucleus, which is supported by interactions between different proteins with chromatin and various molecules including RNA. In the last three years, the biological condensates that have had relevance, including heterochromatin, have taken a fundamental role in the determination of the phase separation promoting the distinct nuclear spatial organization of active and repressive chromatin [16–18].

Heterochromatin is essential to preserve cell functions and identity. Previously it was thought that these dense areas were not transcribed. However, now that we have more powerful technologies, we can see that the heterochromatin is not only transcribed but also a fundamental process to maintain the scaffold of the nucleus and is sensitive to developmental or pathological processes[12,19]. Besides, certain genes embedded in pericentric heterochromatin need a heterochromatic environment to function properly. HP1a mutations reduce the expression of the *light* and *rolled* genes, which were the first to be discovered[19]. These genes are essential for the organism's survival and are found in heterochromatin blocks. As a result, despite its ability to induce silencing, this peculiar genome component has the potential to be very dynamic[20]. In a complex balance between chromatin-bound and diffuse types that can be controlled by various biological contexts, heterochromatin-associated proteins such as HP1a also exist. It was also recently suggested that phase-separation properties are shown by the assembly of

heterochromatin condensates[21,22].

There are a number of papers in which the authors carried out whole-mount immunostaining of *Drosophila* salivary gland nuclei using different and sometimes simpler protocols[23,24]. In this case we adapted a protocol first described in *C. elegans*[25], and subsequently used in *Drosophila* salivary glands by several groups[26–29] and combined it with the use of confocal microscopy and mutant organisms. This protocol also allows visualization of different types of proteins, including transcription factors such as XPD, XPB and TBP[27], but also heterochromatin bound proteins such as HP1a and histone marks such as H3K9me3, which positions it as a protocol for broad use in this tissue. It also has the advantage that the tissue can be stored at an intermediate step without affecting polytene chromosome banding.

This protocol is reliable and cost-effective due to the use of a specific antibody to view the HP1a protein. The critical step in this protocol is to avoid losing the glands during washes and waiting for the tissue to bottom out. The advantage of using salivary glands is that a 3D view of the nucleus and its conformation can be obtained easily, in contrast to the polytene chromosome technique that requires a mechanical disruption of the cell and can damage the chromatin. While performing this protocol, special care should be taken during the washing steps. If not carefully performed, the tissue will break, and it would not be possible to obtain high quality images.

To evaluate the importance of the lack of binding of RNA to the regions or proteins that are being observed, it is necessary to add a wash with Buffer C (Buffer B without EDTA) and add 100 µM of RNase. This wash should be carried out for 1 h at 37 °C as previously described. Washing should be done before the step where molecules are added to observe the DNA (between steps 9.3 and 9.4).

Confocal microscopy may not seem like a very new methodology to address questions of heterochromatin condensates[25,30], but it has been extremely useful to identify delocalization of the HP1a protein in *Drosophila* nuclei, which suggests severe problems in chromatin structure that can be evaluated with other techniques more thoroughly. Despite its limitation, it can be used in combination with high-resolution microscopy as a first approach to apply novel techniques to clarify the biological activity that modulates heterochromatin condensate assembly, control, and functions[31]. Some of these new methodologies that focus on the molecular and biophysical interactions between heterochromatin, RNA, and heterochromatin-associated proteins are gathered from this set of methods to test heterochromatin condensates.

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DISCLOSURES:
The authors declare that they have no competing interests.

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Figure 1

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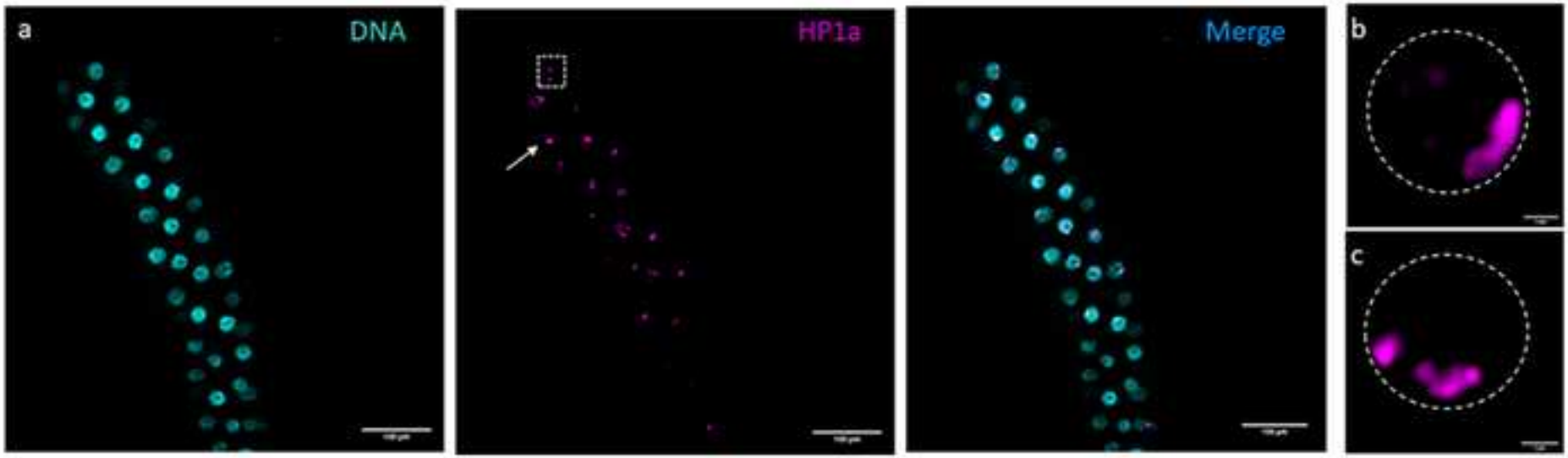

Figure 2

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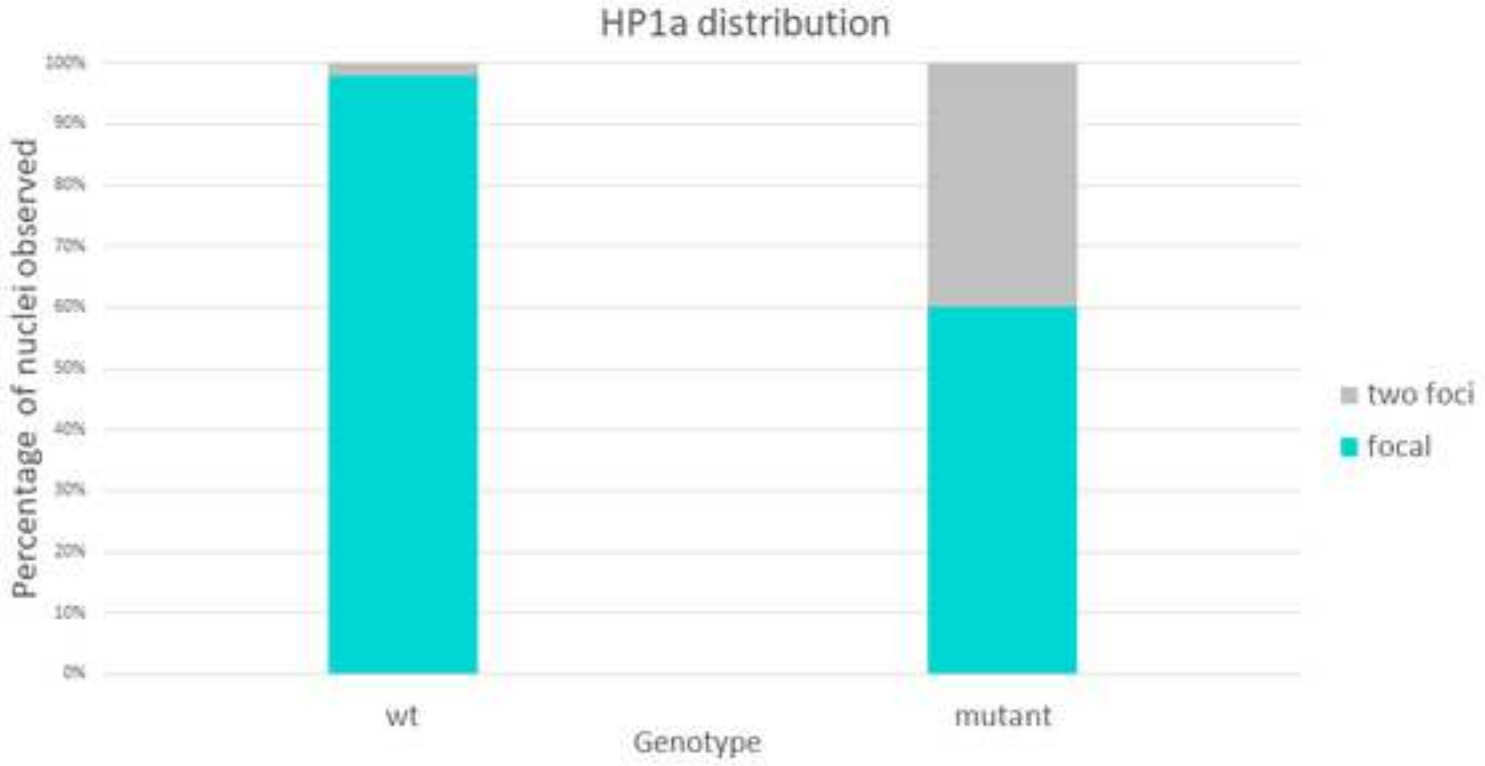

Figure 3

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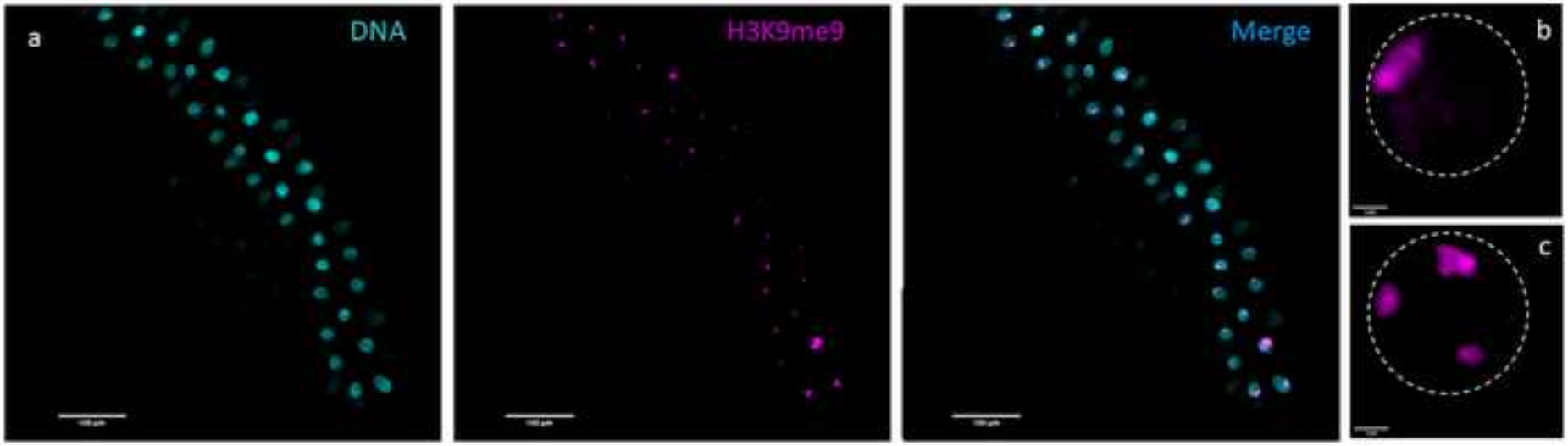

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tubes	Axygen MCT-150-C	11351904	brand not critical
16% formaldehyde	Thermo Scientific	28908	
AF1 Citifluor	Ted pella	19470	25 mL
BSA, Molecular Biology Grade	Roche	10735078001	brand not critical
Complete, protease inhibitors Ultra EDTA-free protease inhibitors	Merck	5892953001	
Coverslip	Corning	CLS285022-200EA	22x22, brand not critical
DTT	Sigma	d9779	brand not critical
EDTA	Sigma	E5134	brand not critical
EGTA			brand not critical
Glass slide	Gold seal	3011	brand not critical
H_3BO_3	Baker	0084-01	brand not critical
H3K9me3	Abcam	8889	
HP1a	Hybridoma Bank	C1A9	Product Form Concentrate
KCl	Baker	3040-01	brand not critical
Methanol	Baker	9070-03	brand not critical
NaCl	Sigma	71376	brand not critical
NaOH			brand not critical
PIPES			brand not critical
Rotator	Thermo Scientific	13-687-12Q	Labquake Tube Shaker
Thermo Mixer C	Eppendorf	13527550	SmartBlock 1.5 mL
Tris	Milipore	648311	brand not critical
Triton X-100	Sigma	T8787	100 mL, brand not critical
β-mercaptoethanol	Bio-Rad	1610710	25 mL, brand not critical

0.1 mL

Rebuttal Letter
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Cuernavaca, Morelos April 5,2021

Dr. Nam Nguyen

Managing editor

JoVE,

Thank you for the opportunity to resubmit our manuscript entitled “Fluorescent Visualization of Heterochromatin in Drosophila Salivary Glands”. We have addressed all of the referees concerns and integrated their suggestions to the revised manuscript. Below you will find a detailed response to each of the referees concerns and our revised manuscript.

Sincerely,

Dr. Viviana Valadez Graham

Investigador Titular A de T.C.,

Insituto de Biotecnología. UNAM. Campus Morelos.

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

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

Author Response: The text has been proofread and corrected when needed.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

Author Response: The text has been proofread and now the protocol section of the manuscript is in the imperative tense.

3. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Author Response: We have made these changes throughout the manuscript.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Author Response: We have now corrected the numbering of the Protocol.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author Response: Thank you very much for the suggestion, in this version we have avoided the use or personal pronouns.

6. Please do not abbreviate journal titles in the references.

Author Response: Corrections to the references now include full journal Titles.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, the narration is a word for word reading of the written protocol in the imperative tense.

Author Response: We have now homogenized the video and the Protocol.

2. Video edits

• 01:10 - consider removing the quick dip to black here

• 01:54 - consider removing the quick dip to black here

• 02:58 - consider adding a dissolve to fade in this clip

• 03:25 - consider adding a dissolve to fade out this clip

• 04:23 - consider adding a dissolve to fade out this clip

• 05:00 - consider adding a dissolve to fade out this clip

• 05:18 - consider adding a dissolve to fade out this clip

• 07:29 - jump cut, consider adding a dissolve here

Author Response: Thank you very much for the suggestion, the changes were made throughout the video.

3. Audio edits

• Reduce overall audio gain by 3db, and reduce section 8 audio by additional 3db

• Conclusion audio is much quieter compared to rest of video, try to balance the volume from section to section

• 02:44 - edit out mouth clicking sound

• 03:00 - edit out mouth clicking sound

• 04:28 - considering starting VO a few seconds earlier

• 04:49 - edit out mouth clicking sound

• 06:00 - edit out knocking sound

• 06:27 - edit out mouth clicking sound

Author Response: We have addressed and corrected these in the final audio.

4. On-screen text edits

• Title card - remove ""and"" from author list

• Sections 3 and 4 have the same chapter title. Check to ensure accuracy of the chapter titles.

• 02:34 - change ""minutes"" to ""minute""""

Author Response: We have addressed all the issues and made the appropriate changes to the video.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe an HP1a immunostaining protocol for Drosophila salivary gland cells.

Major Concerns:

This is a regular immunostaining protocol. If the authors believe there is anything unique for staining salivary gland cells, they should emphasize the difference and contrast with standard immunostaining protocols.

The images provided are of poor resolution and quality. Please consult John Lis lab (Cornell) publications for better confocal images for whole-mount polytene chromosome immunostaing in Drosophila salivary gland cells

Authors' response: We thank the referee for these observations. To respond to the first part, we have now added a paragraph in the discussion section in which we emphasize the use of this protocol and the advantages versus other similar protocols described in the literature. The paragraph reads as follows:

"There are several papers in which the authors carried out whole-mount immunostaining of Drosophila salivary gland nuclei using different and sometimes simpler protocols23,24. In this case we adapted a protocol first described in C. elegans 25, and subsequently used in Drosophila salivary glands by several groups 26–29 and combined it with the use of confocal microscopy and mutant organisms. This protocol also allows to visualize different types of proteins, including transcription factors such as XPD, XPB and TBP27, and also heterochromatin bound proteins such as HP1a and histone marks such as H3K9me3, which positions it as a protocol for broad use in this tissue. It also presents the advantage that the tissue can be stored at an intermediate step without affecting polytene chromosome banding."

Regarding the quality of the images presented, we did not uploaded high resolution images for the first revision because we uploaded pdf quality images as stated in the instructions for the authors by JoVE. We have now corrected this and have uploaded the high resolution images.

Minor Concerns:

The title should be more specific - for example, Immunofluorescent staining for visualization of….

Authors' response: We have now changed the Tiltle to: "Immunofluorescent staining for visualization of heterochromatin associated proteins in *Drosophila* salivary glands".

The authors mentioned HP1a, the more important marker is H3K9me3. They should also show H3K9me3 staining.

Authors' response: We have now added confocal images of salivary glands using an H3K9me3 antibody. The images are shown in Figure 3.

Should provide information for antibodies (HP1a, H3K9me3) in the Table of materials.

Authors' Response: We have now included this information in the table of the materials section.

The concentration for HP1a (C1A9 at 1:3000) seems too low. Other people use 1:200 for immunostaining. Please check for accuracy, or discuss the advantage of using 1:3000 over 1:200.

Authors' Response: We thank the referee for pointing this out. We have now included a sentence in the protocol section in step number 8, in which we detail the antibody concentrations we have used and that work with this protocol.

The sentence reads as follows: "..we use the HP1a C1A9c (concentrated antibody) from Hybridoma Bank up to 1:3000, when using the C1A9s (supernatant) we have tried from 1:100 to a 1:500 dilution and any dilution between this rank works well)."

Should emphasize the critical steps in salivary gland immunostaining - what make them different from staining other tissues or cells; what steps might cause problems if not carefully followed.

Author Response:, the following paragraph was extended, and the points mentioned by the reviewer were added.

"This protocol is reliable and cost-effective due to the use of a specific antibody to view the HP1a protein. The critical step in this protocol is to avoid losing the glands during washes and waiting for the tissue to bottom out. The advantage of using salivary glands is that you easily get a 3D view of the nucleus and its conformation, in contrast to the polytene chromosome technique which requires a mechanical disruption of the cell and can damage the chromatin. While performing this protocol, you should take special care during the washing steps, if not carefully performed, the tissue will break and you will not be able to obtain high quality images. "

Should cite more recent papers that used Drosophila salivary glands for visualization of heterochromatin.

Author Response: We have now added the following references:

20. Marsano RM, Giordano E, Messina G, Dimitri P. A New Portrait of Constitutive Heterochromatin: Lessons from Drosophila melanogaster. Trends Genet. Published online 2019. doi:10.1016/j.tig.2019.06.002

23. Dialynas G, Delabaere L, Chiolo I. Arp2/3 and Unc45 maintain heterochromatin stability in Drosophila polytene chromosomes. Exp Biol Med. 2019;244(15):1362-1371. doi:10.1177/1535370219862282

24. Kolesnikova TD, Semeshin VF, Andreyeva EN, et al. Induced decondensation of heterochromatin in drosophila melanogaster polytene chromosomes under condition of ectopic expression of the supressor of underreplication gene. Fly (Austin). 2011;5(3):181-190. doi:10.4161/fly.5.3.16729

25. Bettinger JC, Lee K, Rougvie AE. Stage-specific accumulation of the terminal differentiation factor LIN-29 during Caenorhabditis elegans development. Development. 1996;122(8):2517-2527.

26. Messina G, Damia E, Fanti L, et al. Yeti, an essential Drosophila melanogaster gene, encodes a protein required for chromatin organization. J Cell Sci. 2014;127(11):2577-2588. doi:10.1242/jcs.150243

27. Aguilar-Fuentes J, Fregoso M, Herrera M, et al. p8/TTDA overexpression enhances UV-irradiation resistance and suppresses TFIIH mutations in a Drosophila trichothiodystrophy model. PLoS Genet. 2008;4(11):1-9. doi:10.1371/journal.pgen.1000253

28. Reynaud E, Lomeli H, Vazquez M, Zurita M. The Drosophila melanogaster Homologue of the Xeroderma Pigmentosum D Gene Product Is Located in Euchromatic Regions and Has a Dynamic Response to UV Light-induced Lesions in Polytene Chromosomes. Mol Biol Cell. 1999;10(4):1191-1203. doi:10.1091/mbc.10.4.1191

29. Farkaš R, Mechler BM. The timing of Drosophila salivary gland apoptosis displays an l(2)gl-dose response. Cell Death Differ. 2000;7(1):89-101. doi:10.1038/sj.cdd.4400621

Reviewer #2:

Manuscript Summary:

The visualization of proteins in whole-mount Drosophila salivary gland nuclei is a very urgent task. A good protocol that gives clear, specific staining, devoid of background, and thus preserves good chromosome structure, would be very useful.

In the manuscript under review the authors present a protocol they used in the paper by Meyer-Nava et al., 2020. I have no doubt that this protocol works. Moreover, it is clear that this protocol fully satisfies the tasks set by the authors in the article. Therefore, I believe that this protocol worth publishing.

However, before being published, authors should clarify a number of important issues.

Major Concerns:

The authors should more correctly describe the limitations associated with the use of polytene chromosomes as a model object for studying the three-dimensional organization of the chromocenter. These limitations are associated with a very strong underreplication of heterochromatic regions in polytene chromosomes. For example, it is known that the most important role in the organization of the chromocenter in diploid cells is played by satellite DNA, and this satellite DNA is not at all polytenized in the polytene chromosomes of wild-type larvae.

Authors' response: We thank the reviewer for this observation, and in this revised version we have modified the last paragraph of the introduction which specifies the limitations of the use of polytene chromosomes to study some heterochromatic regions. The last paragraph in the introduction section now reads as follows:

"In this protocol, we describe the use of *Drosophila* salivary glands to study heterochromatic foci, salivary glands have polytenized cells that contain more than 1000 copies of the genome thus providing an amplified view of most of the chromatin features, with the exception of

satellite DNA and some heterochromatic regions which are underreplicated, nevertheless, heterochromatin regions are easily visualized in polytene chromosome preparations, but sometimes the squashing techniques may disrupt characteristic chromatin-bound……"

There are many papers in which the authors carried out whole-mount immunostaining of drosophila salivary gland nuclei. For example, in Dialynas et al., 2019 (Experimental Biology and Medicine 2019; 244: 1362-1371), the immunostaining results look no worse than those obtained by the authors, but the protocol is much simpler. A more complex protocol is presented in the article by Kolesnikova et al., 2011 (Fly (Austin) 2011;5(3):181-90), where the complication of the protocol was justified by the good preservation of the banding pattern of the chromosomes, combined with high-quality and pure immunostaining.

Therefore, it is necessary to give a detailed justification of the need for all the steps taken by the authors.

Authors' Response: We have added a new paragraph in the discussion section which reads as follows:

"There are a number of papers in which the authors carried out whole-mount immunostaining of *Drosophila* salivary gland nuclei using different and sometimes simpler protocols[23,24]. In this case we adapted a protocol first described in *C. elegans* [25], and subsequently used in *Drosophila* salivary glands by several groups [26–29] and combined it with the use of confocal microscopy and mutant organisms. This protocol also allows to visualize different types of proteins, including transcription factors such as XPD, XPB and TBP[27], but also heterochromatin bound proteins such as HP1a and histone marks such as H3K9me3, which positions it as a protocol for broad use in this tissue. It also presents the advantage that the tissue can be stored at an intermediate step without affecting polytene chromosome banding.

Minor Concerns:

The issues to be addressed:

" In this protocol, we describe the use of Drosophila salivary glands to study heterochromatic foci, salivary glands have polytenized cells that contain more than 1000 copies of the genome thus providing an amplified view of chromatin features. …"

This statement is not true for heterochromatin sequences.

Authors' Response: We have changed this paragraph in order to include that some heterochromatic sequences might be under-represented. The paragraph now reads:

"In this protocol, we describe the use of *Drosophila* salivary glands to study heterochromatic foci, salivary glands have polytenized cells that contain more than 1000 copies of the genome thus providing an amplified view of most of the chromatin features, with the exception of satellite DNA and some heterochromatic regions which are underreplicated, nevertheless, heterochromatin regions are easily visualized in polytene chromosome preparations, but sometimes the squashing techniques may disrupt characteristic chromatin-bound complexes or the chromatin architecture, therefore, immunolocalization of proteins in whole salivary gland tissue can surpass these undesired effects. We have used this protocol to detect several chromatin bound proteins, and we have demonstrated that using this protocol combined with mutant *Drosophila* stocks can be useful to study heterochromatin disruption [12]."

It is important to elucidate the question of how applicable the results obtained on the nuclei of the salivary glands are to diploid cells. For example, will the disruption seen in salivary gland cells be replicated in diploid cells?

Authors' response: We have used this immunostaining protocol also to visualize diploid cells of different imaginal discs, but we did not attempt to visualize the chromocenter or heterochromatin disruption by this method. For diploid cells we always use a molecular approach, like chromatin immunoprecipitation using different histone modifications antibodies, MNase digestion assays or ATAC-seq assays which allows us to observe the changes with further detail. This is one of the advantages of making these types of studies in the salivary glands in which we can get an amplified picture of what is happening with the chromatin organization in these cells and how the different mutants affect the chromatin structure; however, we always complement these observations with other molecular assays to confirm them and so far, the results we have obtained in the salivary glands reflect what is happening at other tissues (data not shown). Having said this, we did try to look into our files for amplified images of these tissues but we did not have images with the correct amplification and we could not make a new experiment because our institute is currently on lockdown and the facilities are working with reduced personnel due to the COVID pandemic and we do not have a clear date for resuming normal activities.

"Place the bottle with the flies in a controlled temperature incubator at 25 Celsius degrees for 12 hours. After the incubation time is over, remove the adults and transfer them to a new bottle to repeat the procedure. "

The number of eggs laid at the same time may depend on the genotype, so it is more convenient to indicate how many eggs should be laid per tube.

Authors' Response: We thank the referee for this observation, we have changed the sentence and it now reads:

"..until the number of eggs laid is 50 (approximately 12 hours for the wild-type strain).

"Larvae collection: For larvae collection make sure you choose the wandering larvae which do not have everted spiracles, this will ensure that the chromosomes are fully polytenized and the cells of the salivary glands are not undergoing programmed cell death".

After the eversion of the spiracles, the larva enters the prepupal stage, while retaining excellent polytene chromosomes suitable for analysis. Only after 12 hours do the cells of the salivary gland begin to prepare for programmed cell death.

Author Response: Thank you very much, we have added the suggested sentence to the protocol to reinforce this issue. The paragraph now reads as follows:

"Larvae collection: For larvae collection makes sure choose the wandering larvae which do not have everted spiracles. After the eversion of the spiracles, the larva enters the prepupal stage, while retaining excellent polytene chromosomes suitable for analysis. Only after 12 hours do the cells of the salivary gland begin to prepare for programmed cell death[14,15].

"Take fifteen 3°instar larvae and put them in a watch glass to wash them, then transfer them to an ice-cold saline solution or PBS (PBS 1L 1x: 137 mM NaCl, 2.7 mM KCl, 10mM Na2HPO4, 2 mM

KH2PO4, adjust pH 7.4). Dissect 15 to 30 pairs of salivary glands (NOTE: or as much as you can in a 30-minute period) in cold PBS with protease inhibitors under the stereoscopic microscope."

Do the authors have data on the need to add a protease inhibitor? I would suggest that for the vast majority of proteins, there is no need to add a protease inhibitor during salivary gland dissection, since the glands continue to function normally in PBS for several hours. It is often used for in vitro replication and transcription studies.

Author Response: Thank you very much for the suggestion, some of the proteins associated with heterochromatin which we have worked with are quite permissive to degrade, so we set up the protocols with the use of inhibitors to prevent the degradation effects of the proteins of interest and to ensure that the phenotypes we observe are not due to the degradation of these proteins during the immunostaining protocol.

"Transfer the salivary glands to a 1.5ml eppendorf tube with ice cold PBS. Wash once with 1 ml PBS plus protease inhibitors (NOTE: wait for the tissue to reach the bottom of the tube). After the wash remove the PBS with a 1000ul pipette taking care not to touch the tissue. "

Considering the volume of the salivary gland and the volume of fluid in the eppendorf, the advisability of this washing is not obvious. If the glands were removed in a PBS solution and, before being placed in the Eppendorf, they were cleared of the fatty body and other residues, then they are already well washed. If the glands remain dirty due to the fact that they were isolated on a watch glass, I recommend that the author isolate the glands in a glass petri dish in a volume of at least 5 ml. This will completely eliminate the need for additional washes.

Author Response: We agree with the referee, we have now included this suggestion in the protocol in step 2.3. We did not remove the other part of the protocol as we could not make another shot to change the step in the video, however, the suggestion has been added to the manuscript in this step. The paragraph now reads as follows:

“2.3 Wash once with 1 ml PBS plus protease inhibitors (NOTE: wait for the tissue to reach the bottom of the tube). After the wash removes the PBS with a 1000ul pipette taking care not to touch the tissue. Alternatively, dissect the salivary glands in 5ml of PBS to eliminate the need for this washing step and proceed to step number 3 by transferring the salivary glands to 0.5ml of the Ruvkun fixing buffer described below.”

"3. Salivary gland tissue fixation: After removing the PBS from the last step, add directly 0.5 ml of 1X Ruvkun fixing buffer, plus 50% methanol (add 0.5 ml of methanol) plus 2% formaldehyde and incubate for 2 hours at 4 Celsius degrees with mild rotation. (Ruvkun 2x solution: 160mM KCl, 40mM NaCl, 20mM EGTA, 30mM PIPES pH 7.4) "

It is not obvious why EGTA should be used in this step and EDTA in the next steps. At all steps, the need for EGTA and EDTA after fixation is not clear, since all the enzymes that were in the cell had to be inactivated during fixation.

Author Response: Thank you very much for your concern, EGTA is used at the beginning of the procedure to bind as a chelating agent for calcium ions, also EGTA can be used as a buffer to resemble the pH of a living cell.

EDTA is used in subsequent steps as a chelating agent in case any inactive enzyme remains, It scavenges for trace amounts of metal ions, mainly magnesium, and prevents them from

catalyzing air oxidation in the samples. We have not tried the protocol without these chelating agents and we do not know if it could work without them, at present we cannot make any experiments because our Institute is currently on lockdown and we do not know when we will be able to resume normal activities.

"5. Incubate the salivary glands in 1 ml Tris/Triton X-100 (the same as above) + 1% β mercaptoethanol for 2 hours at 37 ° C with mild shaking (300 rpm)."

Is β-mercaptoethanol (and DTT) required for all proteins? HP1 shown in the example is usually well detectable without it. Perhaps, it makes sense for the authors to cite as an additional example some more difficult-to-detect protein that requires such a complex protocol.

Authors' response: The referee is right, we have tried this protocol in the past for HP1a without these reducing agents and the results are comparable, we have changed the paragraph and also have corrected that this is the permeabilization step, now the paragraph reads as follows:

"5. Permeabilization step: Incubate the salivary glands in 1 ml Tris/Triton X-100 (the same as above). For some proteins it might be necessary to add 1% β-mercaptoethanol. Incubate for 2 hours at 37 ° C with mild shaking (300 rpm)."

"6. Permeabilization step: Wash with 1 ml BO3 buffer (0.01 M H3BO3 pH 9.2 + 0.01 M NaOH) and then incubate in BO3 / 10 mM DTT at 37 ° C with mild shaking (300 rpm) for 15 minutes. At the end of the incubation period, perform a wash with 1 ml of BO3 buffer alone (NOTE: wait for the tissue to reach the bottom of the tube). "Such processing should lead to a significant loss of chromosome structure. How necessary and justified is it? What is it used for? I would like to get a more detailed explanation.

Author response: We thank the reviewer for pointing this out to us. This step is added to preserve the tissue in case you do not proceed with the protocol on the same day. The tissue can be preserved up to 72 hours in PBS at 4 Celsius degrees following these washes. This solution avoids the possible contamination of the sample.

We have changed the paragraph to explain with further detail this step. The paragraph now reads:

6. Preservation step (optional): In case you do not proceed immediately to the incubation with the antibody, the tissue can be preserved as follows: Wash with 1 ml BO_3 buffer (0.01 M H_3BO_3 pH 9.2 + 0.01 M NaOH) and then incubate in BO_3 / 10 mM DTT at 37 ° C with mild shaking (300 rpm) for 15 minutes. At the end of the incubation period, perform a wash with 1 ml of BO_3 buffer alone (NOTE: wait for the tissue to reach the bottom of the tube). Add 1 ml of PBS. You can preserve the tissue in this solution at 4 degrees Celsius for up to 72 hours and then proceed with the next step. This step is particularly helpful when you are working with different mutant strains which may present a delayed life cycle, so you can perform the immunodetection at the same time along with the controls.

"10. Carry out 2 x 15-minute wash at room temperature while rotation with 1ml of Buffer C (Buffer B without EDTA).

11. Incubate with a DNA marker such as Sytox (take 2µl of stock 5 mM and dissolved in 1 ml of Buffer B) or Hoechst (take 1µl of stock 10 mg/mL and dissolved in 1 ml of Buffer B) for 10 minutes at room temperature with rotation.

12. Carry out one wash with Buffer B and once with PBS, each wash lasting 10 minutes while rotating at room temperature (NOTE: remember to protect it from the light). "

I don't understand why the authors use a buffer of a different composition at each stage. For example, at this stage it is not obvious why we need to wash first with a buffer without EDTA, then with a buffer with EDTA. If this is important, please comment on why.

Author Response: At this point in the protocol, there is probably no difference in washing the tissue with buffer B without EDTA or with EDTA. After analyzing it and reading it in several articles, we decided to eliminate the step with buffer C and keep all the washes with buffer B that contains the chelating agent and then one final wash with PBS.

"13. Finally, mount the salivary glands on a slide, making a pool with a coverslip. Put the salivary glands in the middle of the pool and cover with citifluor to avoid the formation of bubbles extending the viscous liquid all over the place, then seal all the sides with clear nail polish. "

At this stage, I would like to clarify in which fluid the authors place the glands for microscopic analysis. What antifade the authors use.

Author's response: We usually use AF1 Citifluor (Ted Pella) for all our preparations. We pointed our this in the paragraph and also in the table of materials.

"REPRESENTATIVE RESULTS: Representative results of HP1a immunostaining in Drosophila salivary glands are shown in Figure 1. A positive result is to observe one focal point (a) (heterochromatic aggregate or condensate). A negative result is no signal or a dispersed signal. Sometimes a double signal can be observed, that is, with a double point (b), but it usually occurs in smaller quantities. "

It is not clear how a photograph is analyzed: whether only one optical section being analyzed, or all? It is not clear how the authors check whether the double signal is a consequence of the corresponding optical cut through a single chromocenter of a complex structure.

Author's Response: The analyzed images come from ten confocal Z-sections each one with 50 to 60 microns depth. These sections are first obtained and then the Z-stack projection is analyzed. The projection represents the depth of the entire sample. The images taken this way allow a better definition of the 3D final image and the chromatin organization within the nucleus and prevent misinterpretation of the "split" signals observed in the mutants.

"To analyze the data, they can be represented as bar graphs, comparing the distribution of HP1a with different mutant backgrounds. For example, in Figure 2 we can see that 98% of the nuclei present a distribution of one point, 2% of two foci in wt. In the mutant, the proportion change and the presence of two foci increase to 40%."

If the authors provide a graph with the results of the calculation of a particular experiment, it would be logical to provide the corresponding statistical processing, which is given in the original article.

Authors' response: Thank you very much for the observation, we added step 14 in the protocol to explain the statistics. It reads as follows:

"14. GraphPad Prism 6 was used to generate all graphs and statistical analyses. The data from HP1a distribution in salivary glands was analyzed using the Kruskal-Wallis test. Statistical significance was set at ($p < 0.05$*, < 0.01 **, < 0.001***, < 0.0001****)."

Reviewer #3:

Manuscript Summary:

The manuscript titled "Fluorescent Visualization of Heterochromatin in Drosophila Salivary Glands" represents an interesting protocol to immunostain heterochromatin in Drosophila polytene chromosomes.

Major Concerns:

A very similar protocol was used in another publication. It would be fair to includ it in the literature list:

Messina G, Damia E, Fanti L, Atterrato MT, Celauro E, Mariotti FR, Accardo MC, Walter M, Vernì F, Picchioni D, Moschetti R, Caizzi R, Piacentini L, Cenci G, Giordano E, Dimitri P. Yeti, an essential Drosophila melanogaster gene, encodes a protein required for chromosome organization. J Cell Sci, 2014. 127(Pt 11): p. 2577-88.

Author Response: Thank you very much for the suggestion, the literature was added in the discussion and in the reference section.

Minor Concerns:

First, there are some mistakes when scrolling through the manuscript including punctuation, please rectify properly.

Author response: Thank you very much for the comments, we have made grammar and punctuation changes through the manuscript.

Second, It would be appreciate if authors add some evidence about the presence of quite a few essential genes in heterochromatin in introduction and/or discussion, arguing the greater importance of the protocol for studing them too.

Accordingly, please include the following references in the literature list:

1. Hoskins RA, Carlson JW, Wan KH, Park S, Mendez I, Galle SE, Booth B, Pfeiffer BD, George RA, Svirskas R, Krzywinski M, Schein J, Accardo MC, Damia E, Messina G, Demakova OV, Andreyeva EN, Lidiya Boldyreva V, Moore R, Marra M, Carvalho AB, Villasante A, Dimitri P, Zhimulev IF, Rubin GM, Karpen GH, Celniker SE. The Release 6 Drosophila melanogaster reference genome. Genome Res, 2015. Jan 14. pii: gr.185579.114

2. Marsano RM, Giordano E, Messina G, and Dimitri P. A New Portrait of Constitutive Heterochromatin: Lessons from Drosophila melanogaster. Trends Genet 2019, 35, 615-631

Author response: Thank you very much for the suggestion, a paragraph was added in discussion and the required citation.

Besides, certain genes embedded in pericentric heterochromatin need a heterochromatic environment to function properly. HP1a mutations reduce the expression of the light and rolled genes, which were the first to be discovered [19]. These genes are essential for the organism's survival and are found in heterochromatin blocks. As a result, despite its ability to induce silencing, this peculiar genome component has the potential to be very dynamic. [20]