



Review Article

Polytene chromosome squash methods for studying transcription and epigenetic chromatin modification in *Drosophila* using antibodies

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ABSTRACT

The giant polytene chromosomes from *Drosophila* third instar larval salivary glands provide an important model system for studying the architectural changes in chromatin morphology associated with the process of transcription initiation and elongation. Especially, analysis of the heat shock response has proved useful in correlating chromatin structure remodeling with transcriptional activity. An important tool for such studies is the labeling of polytene chromosome squash preparations with antibodies to the enzymes, transcription factors, or histone modifications of interest. However, in any immunohistochemical experiment there will be advantages and disadvantages to different methods of fixation and sample preparation, the relative merits of which must be balanced. Here we provide detailed protocols for polytene chromosome squash preparation and discuss their relative pros and cons in terms of suitability for reliable antibody labeling and preservation of high resolution chromatin structure.

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1. Introduction

Drosophila has long been a favorite model system for studying the relationship between chromatin structure and transcription due to the cytological advantages provided by the giant salivary gland polytene chromosomes of third instar larvae. In this tissue the chromosomes undergo many rounds of replication in the absence of cell division giving rise to approximately 1000 copies. The DNA remains aligned after each replicative cycle resulting in greatly enlarged chromosomes. Using either phase contrast imaging or fluorescent microscopy of Hoechst-stained preparations, the more densely packed chromatin appears as bands whereas a more dispersed packing appears as interbands. The interband-specific localization of RNA polymerase II (Pol II) and associated transcription factors indicate that active genes tend to reside in interbands [1–5]. Further association of decondensed chromatin morphology with gene activity has been provided by studies of developmental- or stress-induced genes that show a “puffing” phenotype of the chromatin that correlates with high gene expression levels [6–9]. The increased decondensation, however, is not a direct consequence of gene expression as it can be uncoupled from transcription by chemical or promoter mutation methods [10,11]. Indeed, dramatic remodeling of nucleosome architecture has been recently found to precede transcriptional activation after heat shock at the *Hsp70* locus [12]. Thus, polytene chromosomes

provide a unique opportunity to examine both architectural changes in chromatin morphology as well as recruitment of specific enzymes and transcription factors involved in the process of transcription initiation and elongation.

Recent results have also underscored the complex choreography of different posttranslational histone modifications associated with regulation of transcription [reviewed in 13,14]. Consequently, there has been a high level of interest in defining the modifications present at different genes and at different stages of the transcription process. An important tool for such studies is the labeling of polytene chromosomes with antibodies to the enzyme, transcription factor, or histone modification of interest. Here we provide various protocols for polytene chromosome squash preparation and discuss their relative pros and cons (summarized in Table 1) in terms of suitability for reliable antibody labeling and preservation of high resolution chromatin structure.

2. Selection of fixation method

2.1. Principles underlying different fixation techniques

In any immunohistochemical experiment there will be advantages and disadvantages to different methods of fixation and sample preparation [15–18], the relative merits of which must be balanced. Thus, for any new antigen of interest, it is necessary to optimize the chosen fixative and fixation conditions. In the case of studies directed towards RNA polymerase II elongation control, the challenge is to identify conditions that suitably preserve the

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Table 1
Polytene chromosome squash techniques.

Smush preparation	Conventional acid-fixation squash protocol	Acid-free squash protocol	High resolution squash protocol
<i>Pros</i>			
Simple and rapid	Provides high quality, well-spread polytene chromosome preparations with well resolved band/interband regions	Provides good quality polytene squash preparations that can be readily labeled by most antibodies. Preserves the native fluorescence of transgenically expressed GFP-tagged proteins	Provides ultra-high resolution of band/interband regions and chromatin structure at near EM levels
Acid-free fixation at physiological pH compatible with labeling of chromatin by most antibodies			
<i>Cons</i>			
Provides only very low resolution of chromatin structure	The acid fixation step prevents reliable immunolabeling by some antibodies and suppresses the native fluorescence of transgenically expressed GFP-tagged proteins	The chromosomes are more difficult to spread well and chromatin morphology is less well preserved in comparison to the conventional technique	Because of the flatness of the chromosomes it can sometimes be difficult to obtain high-quality antibody labeling

chromatin structure, allowing accessibility to antibodies without stripping away key proteins or blocking critical epitopes. Formaldehyde is a standard non-coagulative fixative choice that fixes tissues primarily by cross-linking, principally via lysine residues. Advantages include moderate (or efficient) penetration of tissues, partly due to the fairly slow kinetics of cross-linking, and provides stable covalent linkages [19]. A disadvantage is that chromatin structure is not particularly well preserved in polytene squash preparations. Acetic acid is another popular non-coagulative fixative component known for its swelling affect on tissues [15] that in the case of polytene chromosome squashes helps to accommodate stretching of the chromatin in the interband regions [20]. However acetic acid has a serious disadvantage in that it is prone to extract histones from the tissues [15,21] and can “harden” the chromosomes, inhibiting their spreading. A solution to this problem was the inclusion of lactic acid in the fixative, which helped keep the glands “softer” and allowed for better unfolding of the chromosome arms [22,23]. Still, many methods adopt a strategy of combining different fixatives with varying treatment times in an attempt to exploit the advantages afforded by a given agent while minimizing any inherent disadvantages [24].

2.2. The “smush” preparation

Many studies of transcription and histone modifications rely on commercially available antibodies that often are poorly characterized and of variable quality. For these reasons Wang et al. [25] developed a “smush” technique based on a modified whole-mount staining procedure for *Drosophila* third instar salivary gland nuclei

that provides for a rapid and sensitive screening procedure. In this preparation nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH that preserves the antigenicity of most epitopes. In a test case Cai et al. [26] recently used this technique to assess the suitability of different commercially available H3S10ph antibodies for immunohistochemistry on polytene chromosomes at interphase. The results showed that several of the tested antibodies from three different manufacturers were unreliable and that different lots of the same antibody had, in some cases, different properties [26]. Although in the study of Cai et al. [26] the smush approach was used to identify reliable antibody lots, the ease and convenience of this approach would make it a powerful method to determine the suitability of different fixatives. In addition, for experiments in which it is important to preserve nuclear organization in its native state, for example to visualize the nuclear lamina, interchromosomal proteins, or the relative three-dimensional position of nuclear components, the “smush” technique would be the method of choice.

2.3. Acid versus acid-free techniques for chromosome squashes

Although the resolution provided in smush preparations is sufficient to identify general patterns of chromatin proteins on chromosomes (Fig. 1), the characteristic band/interband pattern of polytene chromosomes is not particularly well-resolved. This contrasts with conventional “squash” procedures where the strongly Hoechst-staining bands, consisting of more condensed chromatin regions, are distinct from the comparatively less-stained interband

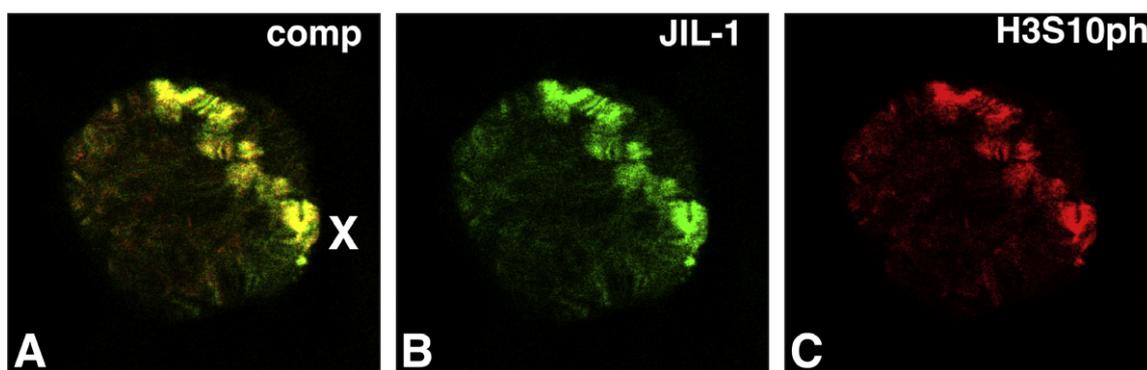


Fig. 1. The smush preparation can serve as a rapid method to screen for proper labeling of antibodies against chromatin proteins. Confocal images of a whole-mount preparation of salivary gland polytene nuclei from a male third instar larva double labeled with antibody against the JIL-1 histone H3S10 kinase (B) and phosphorylated histone H3S10 (C) show co-localization, as indicated in the composite image (A) by the predominantly yellow color. Both JIL-1 and H3S10ph levels are enhanced on the male X chromosome (labeled X in panel A).

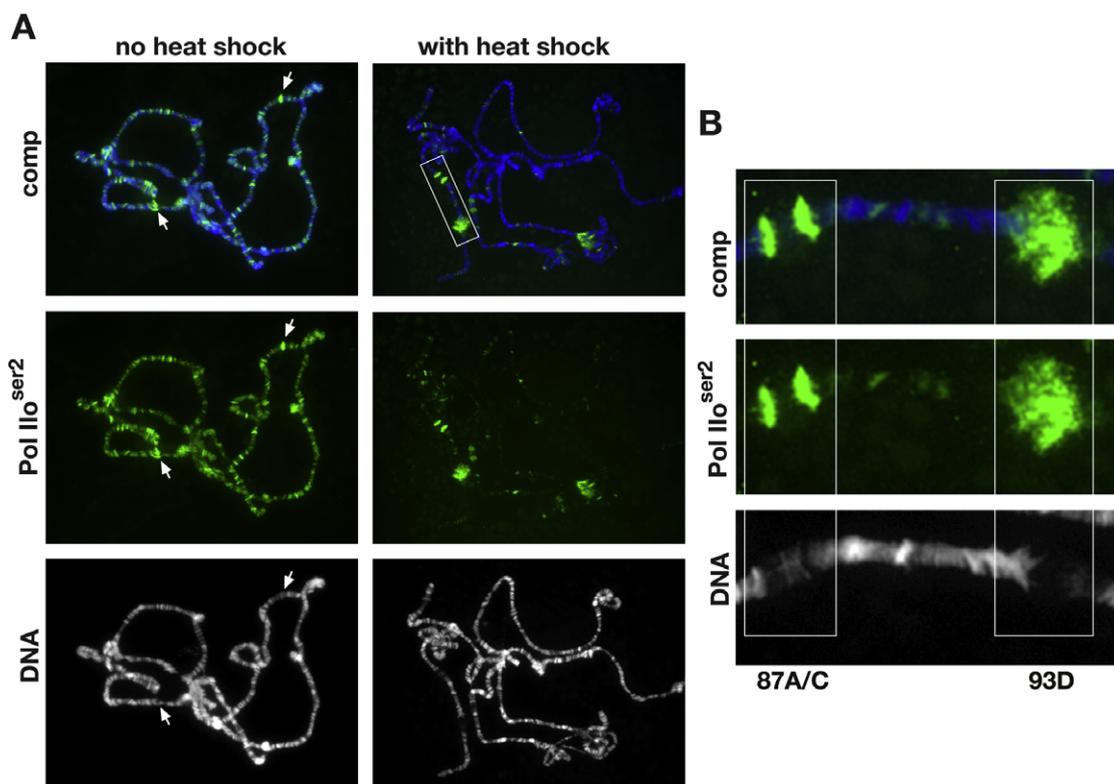


Fig. 2. Visualization of actively transcribing chromatin regions in a conventional acid-fixed polytene squash preparation. (A) Polytene squash preparations double labeled with Pol Ilo^{ser2} antibody (green) and Hoechst (DNA, gray/blue) with (right column) and without (left column) heat shock treatment. Under non-heat shock conditions, many sites show strong labeling of the actively transcribing form of Pol II with particularly high levels observed at developmental puffs (arrows). After heat shock treatment, Pol Ilo^{ser2} labeling was reduced at most sites but dramatically upregulated at heat shock-induced puffs (boxed area). (B) Higher magnification of the boxed area from A shows high levels of the actively-transcribing form of Pol II accumulates at the 87A/C and 93D heat shocks puffs (boxed). For induction of the heat shock response, wandering third instar larvae were placed in 1.5 ml ependorf tubes with punctured lids, incubated at 37 °C for 20 min in a water bath, and salivary glands were dissected and fixed immediately upon removal from the water bath to prevent recovery from heat shock.

regions comprised of more “open” chromatin (Fig. 2). The reproducibility of the polytene chromosome banding pattern allows chromosomal proteins such as polymerases and transcription factors to be localized to specific genomic regions. This presents a unique opportunity to study the molecular requirements for transcriptional regulation [1,27–28, reviewed in 29]. Analysis of the heat shock response has been a particularly good model system since it provides a well-characterized example of the induction of high levels of gene expression at specific “heat shock loci” with a concomitant downregulation of gene expression from other loci [9,30,31]. Antibody generated against RNA polymerase II that is phosphorylated at serine 2 in the C-terminal domain (Pol Ilo^{ser2}) serves as a marker for active transcription [3,32,33]. Using this antibody the broad distribution of Pol Ilo^{ser2}-labeled interbands visible under non-stressed conditions can be observed to change to a highly restricted pattern largely confined to the “heat shock puffs” after exposure to elevated temperatures (Fig. 2). By immunostaining polytene chromosomes, recruitment and redistribution of a number of different proteins involved in the initiation/elongation process has been well characterized [5,34–37]. However, such studies depend on suitable antibodies and fixation conditions that allow for the specific detection of these proteins.

The inclusion of acetic and lactic acids in the conventional squash fixation protocol facilitates both interband resolution and chromosomal arm spreading but unfortunately some epitopes do not survive this treatment. An example of such an epitope is H3S10ph (Fig. 3A and B; also see [26]) which in contrast is preserved after formaldehyde fixation in the smush preparation (Fig. 1). Since acid treatment also has the disadvantage that it

quenches the inherent fluorescence of GFP-tagged proteins, DiMario et al. [38] recently developed a formaldehyde-based “acid-free squash technique” that allowed for direct visualization of GFP-fusion protein on polytene chromosomes without GFP-antibody labeling. In this case salivary glands were dissected directly in a dilute formaldehyde solution, soaked in 50% glycerol, and squashed in 50% glycerol in order to mimic the viscosity of the nucleus to help preserve chromosomal morphology during spreading. Preparation of the chromosomes using this technique allows the chromosomal arms to become extended during the squashing procedure and thus preserves the advantages of improved band/interband resolution of the squash technique. This is illustrated in Fig. 4 where polytene chromosomes from a transgenic female larva expressing a JIL-1-GFP-fusion protein [39] show robust GFP fluorescence that localizes to interband regions (Fig. 4, top left pane) in the same pattern as has been previously observed using antibody-detection methods in female preparations [25,39]. However, when squashes from the same transgenic line are prepared using a conventional squash protocol that includes acetic acid in the fixation buffer, all GFP fluorescence is lost (Fig. 4, bottom left panel.) Furthermore, “acid-free” preparations also showed consistent localization of H3S10ph antibody labeling to interband regions with increased levels on the male X chromosome as expected (Fig. 3C) in contrast to acid-treated preparations where labeling was severely attenuated and there was no detectable upregulation on the male X chromosome (Fig. 3A; [26]).

Comparing Hoechst staining of acid and acid-free fixed preparations reveals another contrast between the two procedures: inclusion of acetic and lactic acids in the conventional squash

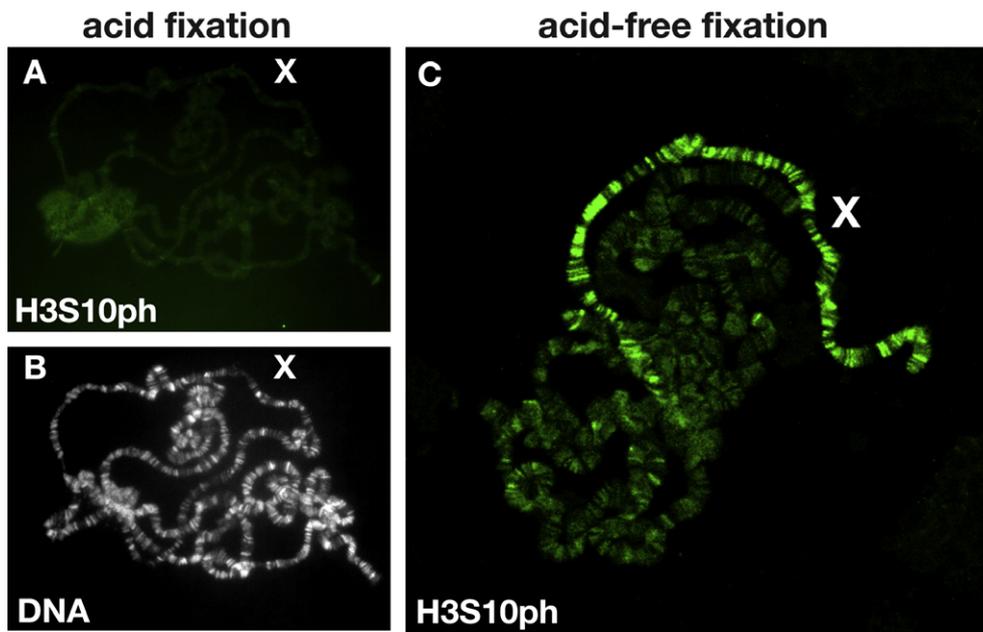


Fig. 3. Fixation protocols can strongly affect antibody performance. The acetic acid-based fixative used in conventional squash protocols, though highly effective in resolving bands from interbands as labeled by Hoechst (B), can eliminate labeling by some chromatin antibodies as is observed for anti-H3S10ph antibody (A). In contrast, the same anti-H3S10ph antibody used for labeling in (A) shows robust signal on a preparation fixed using a paraformaldehyde-based acid-free fixation protocol (C). The squash in (C) is from a male larva and demonstrates the upregulation of H3S10ph on the male X chromosome (X).

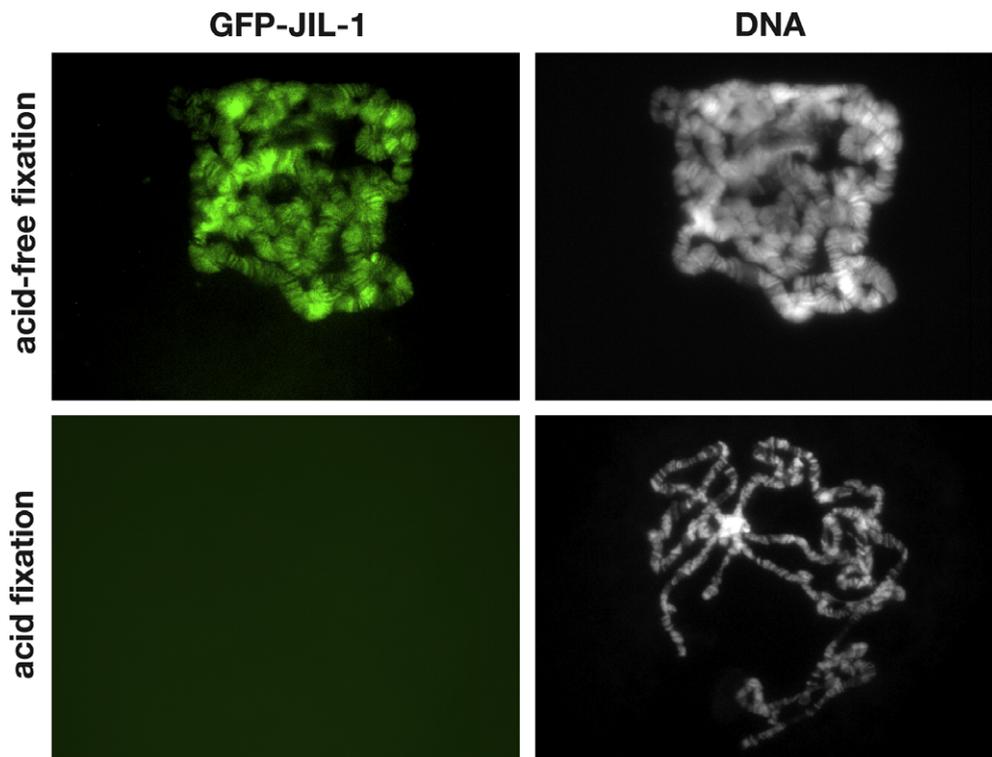


Fig. 4. Fixation protocols can affect the fluorescence properties of GFP-fusion proteins. The inherent fluorescence of the GFP-JIL-1 fusion protein in a JIL-1-GFP transgenic line is retained in squash preparations of female third instar larval polytene chromosomes when fixed using the acid-free protocol (top left panel) but is lost when fixed using the conventional acetic acid-based protocol (bottom left panel). The DNA is labeled by Hoechst in the two right panels.

technique enhances chromosomal spreading and interband resolution. Without the spreading advantages afforded by acid inclusion and since the cross-linking activity of formaldehyde tends to restrict chromosome arm spreading, it is essential to empirically optimize both formaldehyde concentrations and

fixation times. As shown in Fig. 3C, suitable conditions can be established such that comparably spread preparations can be obtained using the acid-free technique, but it is likely to take more squash attempts to obtain suitable samples than in the case of the conventional technique.

2.4. High resolution analysis

In some cases the resolution of the localization of chromatin proteins afforded by conventional squash protocols is still not sufficient to ascertain whether proteins co-localize and potentially interact. Recent developments utilizing different color variants of GFP-tagged proteins to image transcription dynamics on polytene chromosomes in live tissues along with potential applications such as FRET (fluorescence resonance energy transfer) to evaluate proximity of these proteins to each other [40] promises to provide a more detailed picture. However, higher resolution analysis extended to fixed preparations would be an extremely valuable tool in order to utilize many of the excellent antibodies that are already available. Recently a modified squash protocol featuring high pressure treatment that can yield chromosome spreads with resolution similar or equal to that of electron microscopy preparations has been developed [41]. Furthermore, the use of a precision vise for the squashing step in this protocol not only allows increased pressure to be applied to the sample, but also significantly facilitates application of only vertical pressure during the squashing step, since any horizontal pressure at this stage is prone to shear the chromosome arms. This procedure significantly enhances reproducibility since squashing pressure is mechanically controlled and produces exceptionally flat chromosomes, accounting for the ultra-high resolution level. However, in some cases the thinness of the preparation also results in reduced antibody signal. In this case the investigator can adjust the pressure to be applied to optimize squash conditions for the desired purpose. As illustrated in Fig. 5 this approach provided further confirmation of the finding that JIL-1 histone H3 kinase is not associated with Pol II elongation [26], as the enhanced level of resolution afforded by this technique reveals minimal overlap between JIL-1 and Pol Ilo^{ser2} antibody labeling.

3. Characterization of antibodies

3.1. Specificity of primary antibody

In a recent study analyzing H3S10ph staining of interphase chromosomes in smush preparation stainings, Cai et al. [26] found

varying results for different commercial antibodies and even between different lots of the same commercial antibody [26]. Thus it is essential to evaluate each individual antibody lot to determine its specificity and suitability for the intended uses. For example, after heat treatment, the high level of transcription of heat shock genes at 87A/C correlates with strong labeling by the actively transcribing Pol Ilo^{ser2} (Figs. 2 and 6). Anti-H3S10ph antibody from two different commercial sources (Epitomics and Cell Signaling Technology) show no labeling of the heat shock puffs (Fig. 6A and B) [26]. However, in contrast a third commercially available antibody (Upstate Biotechnology) showed strong labeling of the heat shock puffs (Fig. 6C). In addition, strong labeling of heat shock puffs by the Upstate antibody was also observed in polytene chromosome squashes from *JIL-1* null mutant larvae [26], which normally have undetectable levels of interphase histone H3S10 phosphorylation. Consequently, it is likely that the labeling of transcriptionally active heat shock puffs by the H3S10ph antibody (Upstate Biotechnology) is due to non-specific cross-reactivity, possibly with proteins involved in the heat shock response [26]. These results underscore the importance of fully characterizing antibody specificity to ensure adequate performance.

“Antibody suitability” can also be strongly influenced by the experimental conditions selected and for that reason it is advisable to heed the manufacturer’s protocols for commercially available antibodies to optimize specificity for use for the preparation of interest. For example, the manufacturer’s instructions for immunoblot applications that accompany a commercially available antibody that detects Pol Ilo^{ser2} (Covance) specifically recommends against use of the popular blocking agent Blotto (5% nonfat dry milk) and instead specifies blocking with 5% BSA (bovine serum albumin). Fig. 7 shows the results from immunoblots of larval salivary gland protein lysate fractionated by SDS-PAGE, electroblotted, and blocked using either 5% BSA or Blotto before probing with Covance anti-Pol Ilo^{ser2} antibody. Whereas antibody detection of the immunoblot that had been blocked in BSA as prescribed revealed a single band migrating at the appropriate molecular weight for Pol Ilo^{ser2}, the immunoblot that had been blocked with Blotto showed, in addition to the Pol Ilo^{ser2} band, cross-reactivity with multiple different-sized bands.

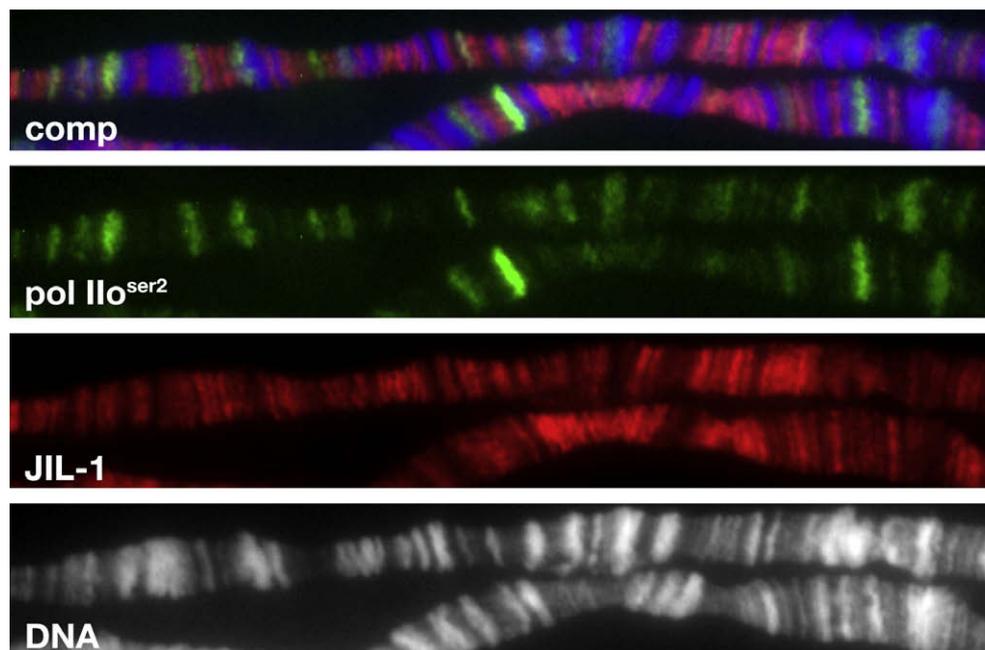


Fig. 5. The high pressure squash technique provides enhanced resolution of interband-localized Pol Ilo^{ser2} and JIL-1 chromatin proteins. Polytene chromosomes prepared using a high-pressure precision vise that achieves thinner squash preparations reveal minimal overlap between actively transcribing Pol Ilo^{ser2} (green) and JIL-1 (red) as revealed by minimal yellow signal in the composite (top) panel. Hoechst labeling of the DNA (lower panel) shows the banding pattern of the chromosomes.

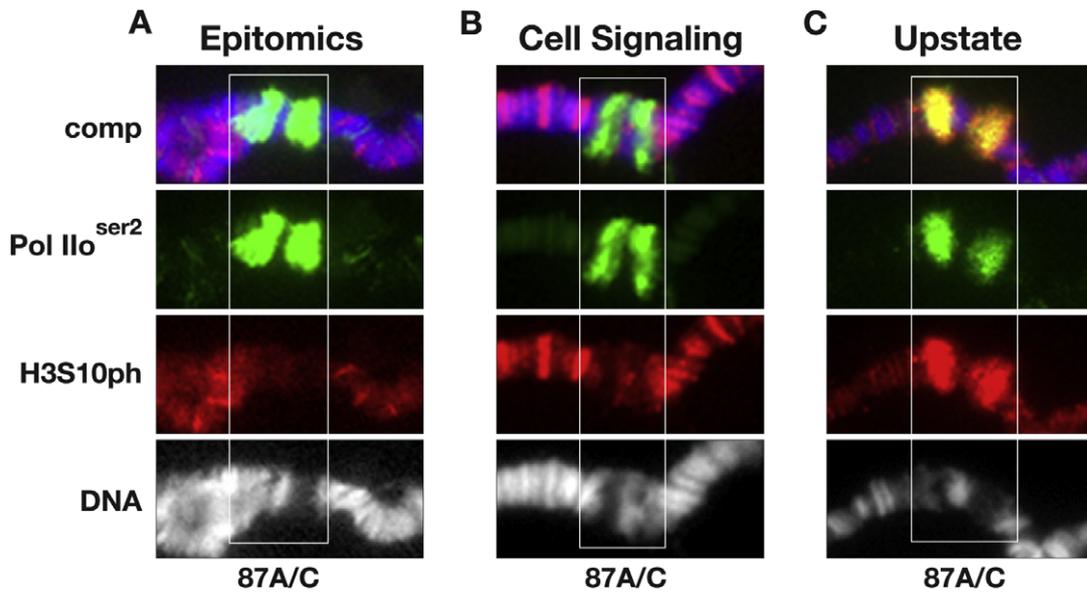


Fig. 6. Different commercial H3S10ph antibodies show different labeling of heat shock puffs. Acid-free polytene chromosome squashes prepared after heat shock treatment were triple-labeled with antibodies to Pol Ilo^{ser2} (green), H3S10ph (red), and with Hoechst (DNA, blue/gray). H3S10ph antibodies were from Epitomics (A), Cell Signaling (B), or Upstate Biotechnology (C). The 87A/C heat shock puffs were strongly labeled by the actively transcribing form of Pol Ilo in all cases (green labeling, boxed region, A–C). As indicated by lack of yellow signal in the upper composite (comp) panel, neither the Epitomics nor Cell Signaling anti-H3S10ph antibody showed labeling of the puffs (boxed region, A,B). In contrast the Upstate Biotechnology anti-H3S10ph antibody showed robust colocalization with Pol Ilo^{ser2} as indicated by the predominant yellow signal in the upper composite (comp) panel in (C).

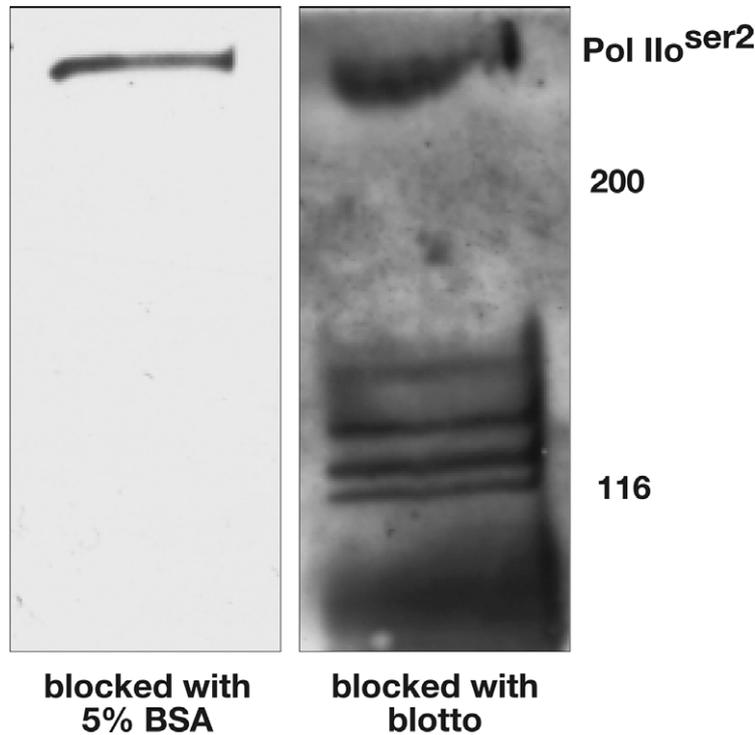


Fig. 7. The influence of blocking conditions on antibody labeling of immunoblots. Salivary gland protein extracts were fractionated by SDS-PAGE, immunoblotted and blocked in either 5% BSA (A) or 5% Blotto (B) before incubation with anti-Pol Ilo^{ser2} antibody (Covance) and subsequent immunodetection. Immunoblots blocked in 5% BSA as recommended by the manufacturer revealed a single band migrating at the size predicted for Pol Ilo^{ser2} while immunoblots blocked in 5% Blotto showed numerous cross-reactive, lower-sized bands in addition to the Pol Ilo^{ser2} band.

3.2. Suitability of secondary antibody

Equally important, the specificity of the chosen secondary antibody should also be ascertained. For double labeling experiments the two separate primary antibodies used must be derived from

different animal species or be of different isotypes in order to allow selective recognition by differentially-labeled secondary antibodies (e.g., one secondary antibody might be fluorescein-tagged while the other might be rhodamine-tagged). The secondary antibodies should be screened to ensure that they recognize only the relevant

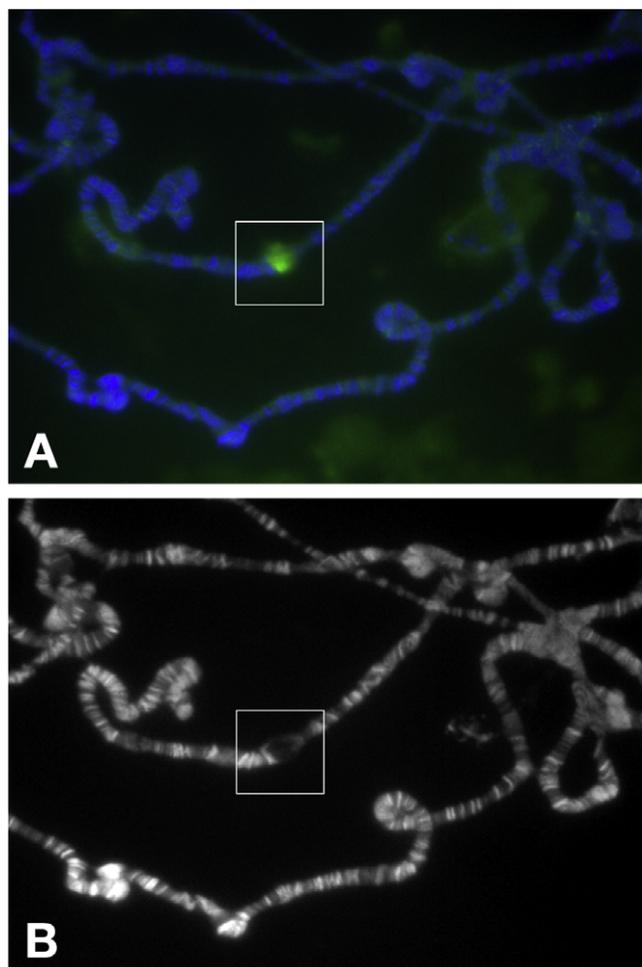


Fig. 8. Cross-reactivity of commercially available secondary antibodies with *Drosophila* heat shock-induced puffs. Polytene chromosome squash preparations from heat shocked animals were double-labeled with a commercially available FITC-conjugated anti-mouse antibody (A, in green) and Hoechst staining to label the DNA (A, blue; B gray). Despite that no primary antibody had been applied, a robust signal on heat shock puffs (A, boxed region) was observed.

primary antibody and show no cross-reactivity to the other primary antibody. However, an additional concern is whether the secondary antibody might exhibit an unexpected cross-reactivity to antigen(s) present in the preparation. For example, we have encountered one case of a secondary antibody that shows a robust staining of transcriptionally active heat shock puffs on its own without addition of any primary antibody (Fig. 8). Therefore control stainings using secondary antibody alone should always be performed to confirm absence of any such cross-reactivity. In some cases low levels of background reactivity can be eliminated by incubating the secondary antibody diluted 1:10 with fixed, devitelinized embryos in order to remove antibodies that might cross-react with *Drosophila* proteins. However, in most cases it is possible to screen different commercially available affinity purified antibodies to find one that does not show such cross-reactivity.

4. Sample preparation protocols

4.1. Culturing of third instar larvae

Drosophila are raised according to standard protocols [42]. In order to obtain optimal polytene chromosomes for any of the techniques described below, uncrowded culturing conditions are

essential (e.g., place around 20 egg-laying female flies in a bottle and change to new bottle each day). Select the fattest individuals from the first crop of climbing 3rd instar larvae while they are still wandering but just prior to pupation. We routinely culture at 21 °C but 18 °C will yield fatter chromosomes that may be more suitable for some purposes.

4.1.1. Materials

Agar
Baker's yeast
Sugar
Dextrose
Corn meal
Propionic acid
Phosphoric acid
Lexgard-M (*p*-hydroxybenzoic acid methyl ester)
Ethanol

4.1.2. Recipes

"Acid A" Preservative (mold inhibitor): 418 ml propionic acid, 82 ml distilled water, 42 ml phosphoric acid, 458 ml distilled water [Note: mix in the order listed and store in a dark bottle at room temperature].

Tegosept Preservative (mold inhibitor): 106 g. Lexgard-M, 1L 95% ethanol. [Store at 4 °C].

Culturing medium: 8.2 g agar, 19 g baker's yeast, 30 g sugar, 55 g dextrose, 90 g cornmeal, 1L water, 10 ml "Acid A" and 10 ml Tegosept [Note: Mix all dry ingredients except agar and corn meal with water, shaking in a closed container until there are no lumps. Add solution to agar in a steam kettle and stir until it comes to a simmer. Simmer for 30 s, add cornmeal, simmer for 3 min while stirring. Turn steam off, cool for 2 min, add "Acid A" and Tegosept, stir thoroughly, and dispense into vials or bottles].

4.2. Smush technique

4.2.1. Smush materials

Poly-lysine-coated microscope slides (Fisher No. 12-544-3)
22 × 40 mm No. 15 coverslips (Fisher No. 12-530B)
Petri dish (60 × 15 mm)
Drummond dissection forceps (2)
PBS
0.2% PBST (PBS with 0.2% Triton X-100)
0.4% PBST (PBS with 0.4% Triton X-100)
4% paraformaldehyde (prepare freshly in 0.1% PBST)
Parafilm strips (cut to match dimension of a microscope slide)
Kim-wipes
Coplin jar
Normal goat serum (NGS) [or other serum; see note in step 9 below].
Blocking buffer (0.4% PBST + 1% NGS) [see note in step 9 below].
Acrodisc syringe filter (Fisher No. 09-730-218)
1° antibody (as determined experimentally)
2° antibody (for detection of the selected 1° antibody) [Note: optimal dilution should be empirically determined for each antibody lot. Select only affinity purified antibodies and test for cross-reactivity in the absence of primary antibodies. Filtering the diluted antibody through a syringe filter will remove fluorescent aggregates that may otherwise appear as speckles in the epifluorescent images].

Hoechst 33258 (Molecular Probes)

Hoechst Solution (Hoechst 33258 0.2 µg/ml in PBS) (store in dark bottle at 4 °C) [Note: This concentration of Hoechst is less than recommended in most standard protocols, but in our experience results in superior labeling and resolution of band/interband regions].

Nail polish for sealing coverslips [*Note: use brightly colored nail polish instead of clear in order to observe whether the edges are fully sealed.*].

4.2.2. Smush protocol

1. Rinse larvae with water and transfer to PBS in a tissue culture dish for dissection.

2. Grasp the tip of the mouth hooks with one pair of forceps, hold the body about 2/3 of the way down with the other pair, and pull on the mouth hooks so the salivary glands are exposed. Separate the salivary glands from the brain and eye-antennal discs (if they separated out with the salivary glands), and dissect away the fat body and any other associated tissues from the glands.

3. Transfer one to three pairs of salivary glands to a drop of 25 μ l of 0.2% PBST on a poly-lysine-coated slide.

4. Gently lay a clean 22 \times 40 mm coverslip on the drop. For wild-type preparations the tissue often immediately appears cloudy, which is a good indication that the cells have separated. If this is not observed, carefully grasp the coverslip on one edge and gently move the coverslip slightly back and forth, trying to minimize any vertical pressure on the tissue (minimize any “squashing” forces). Repeat this procedure until most of the tissue looks cloudy.

5. Gently remove the coverslip. Most of the tissue will have adhered to the poly-lysine-coated slide but some cells will be on the solution remaining on the coverslip. Gently touch the edge of the coverslip to the slide to let the remaining solution flow back onto the slide. Tilt the slide to facilitate dispersion of the solution to better spread cells out.

6. Add 50–100 μ l of 4% paraformaldehyde onto the slide and cover with a piece of Parafilm cut to fit the slide. Incubate in a covered box for 20–25 min at room temperature.

7. Gently remove the parafilm and touch the edge of the slide to a Kim-wipe to dry it.

8. Incubate the slides in a Coplin jar with 0.4% PBST for 30–60 min.

9. Block the slides in Blocking Buffer (0.4% PBST + 1% NGS) at room temperature for 30 min [*Note: the blocking buffer will typically contain 1% normal serum from the species that was the source of the secondary antibody; i.e., if the primary antibody is from goat, a different source of serum must be selected for the blocking buffer.*].

10. Incubate slides in primary antibody diluted appropriately in Blocking Buffer overnight at 4 °C.

11. Wash 3 \times 10 min in 0.4% PBST.

12. Dilute the appropriate secondary antibody into Blocking Buffer and incubate 3 h at room temperature.

13. Wash 3 \times 5 min in 0.4% PBST in the dark.

14. Wash 2 \times 5 min in PBS in the dark.

15. Incubate 10 min in Hoechst solution.

16. Wash 2 \times 5 min in PBS in the dark.

17. Touch the edge of the slide to a Kim-wipe to drain away excess PBS. Add several drops of glycerol containing 5% *n*-propyl gallate to the region of the slide containing the sample preparation.

18. Gently place a 22 \times 40 mm No. 15 coverslip over the sample, taking care not to trap bubbles. Seal the edges with nail polish.

19. Image the chromosomes using a fluorescent microscope or store in slide boxes at –20 °C in the dark until imaging can be performed.

4.3. Conventional squash technique [*modified from 43*]

4.3.1. Conventional squash materials

Drummond dissection forceps (2)

Petri dishes (60 \times 15 mm)

Frosted microscope slides (Fisher No. 12-544-3) (poly-lysine-coated)

22 \times 22 mm No. 15 cover slips (Fisher No. 12-520B) (coated with Sigmacote; Sigma #SL2)

22 \times 40 mm No. 15 cover slips (Fisher No. 12-530B)

Kim-wipes

Phase contrast microscope with 20 \times objective

Small Dewar (e.g., vacuum flask or thermos bottle)

Long forceps

Razor blades

Coplin Jar

Rubber-Maid or Tupperware tray (or equivalent sealable tray)

Parafilm (cut into 22 mm squares)

Five times formaldehyde stock (0.74 g. paraformaldehyde/4.0 ml dH_2O /28 μ l of 1 N KOH; warm to 65 °C to dissolve, then store on ice. Make fresh daily)

Fix #1 (0.5 ml 10 \times PBS/50 μ l Triton X-100/3.45 ml dH_2O /1.0 ml of 5 \times formaldehyde stock. Warm to disperse the Triton X-100. Prepare fresh and use within 1 h)

Fix #2 (1.5 ml dH_2O /2.5 ml glacial acetic acid/1.0 ml of 5 \times formaldehyde stock. Prepare fresh and use within 1 h)

Lactoacetic acid solution (1 ml lactic acid/2 ml dH_2O /3 ml acetic acid)

Liquid nitrogen

95% ethanol

PBS

PBST (PBS + 0.4% Triton X-100)

Blocking Buffer (PBST + 0.2% BSA + 5% goat serum)

Primary antibody solution (1 $^\circ$ antibody diluted as appropriate in Blocking Buffer)

Secondary antibody solution (2 $^\circ$ antibody diluted as appropriate in Blocking Buffer)

Hoechst 33258 (Molecular Probes)

Hoechst Solution (Hoechst 33258 0.2 μ g/ml in PBS) [store in dark bottle at 4 °C]

Mounting solution (80% glycerol in 10 mM Tris, pH 9.0 + 5% *n*-propyl gallate)

Nail polish for sealing coverslips [*Note: use brightly colored nail polish instead of clear in order to observe whether the edges are fully sealed.*].

4.3.2. Conventional squash protocol

1. Rinse larvae with water and transfer to PBS in a tissue culture dish for dissection.

2. Grasp the tip of the mouth hooks with one pair of forceps, hold the body about 2/3 of the way down with the other pair, and pull on the mouth hooks so the salivary glands are exposed. Separate the salivary glands from the brain and eye-antennal discs (if they separated out with the salivary glands), and dissect away the fat body and any other associated tissues from the glands.

3. Transfer salivary glands to 200 μ l of Fix #1 in a depression slide for 1–2 min (some epitopes, such as for most of the histone modifications, require 5-min fixation; empirically determine conditions for the antigen of interest). Any additional extraneous tissue such as the fat bodies adhering to the glands can be removed at this point.

4. Remove Fix #1 with a drawn-out Pasteur pipette, being careful not to aspirate the salivary glands. Immediately add 200–300 μ l Fix #2 for two minutes (alternatively use a double-well depression slide and simply transfer glands to a fresh well with 200–300 μ l Fix #2). Continue to remove any remaining fat body tissue but note that tissue is very fragile in Fix #2, so try to achieve most of the trimming work during step 3.

5. Transfer the glands to 10–30 μ l of lactoacetic acid solution on a clean Sigmacoted coverslip (find the minimal volume that promotes sufficient spreading of chromosomes without generating excessive streaming forces during the squashing step that can wash chromosome arms away). Gently lower a poly-lysine-coated

microscope slide onto the coverslip and, without applying vertical pressure, pick up the coverslip so the glands are between the slide and the coverslip. *Immediately* facilitate cell lysis and chromosomal spreading by carefully grasping the coverslip with forceps on one edge and gently moving it slightly back and forth, trying to minimize any vertical pressure on the tissue. Alternatively use the eraser side of a pencil or even a fingertip to gently move the coverslip back and forth. Choose whichever approach best achieves the primary goal of gently moving the coverslip to lyse the cells and spread the chromosomes while avoiding any vertical pressure on the coverslip [also note that any delay in moving the coverslip back and forth is likely to diminish spreading of the chromosomal arms as they will tend to become more rigid upon exposure to the lactoacetic acid solution]. Clouding of the solution is often a good indication of cell separation. Gently tapping the coverslip obliquely (to avoid vertical pressure) with the eraser side of a pencil a few times may also assist in chromosomal spreading.

6. Immediately examine the tissue under a phase contrast microscope with the 20× objective. If many of the chromosomes appear well-spread, go to step 8. If not, go to step 7.

7. Many protocols recommend tapping the coverslip with a pencil or the back of a forceps to encourage further chromosomal spreading and this approach can give very nice preparations. However, in our experience it can be difficult to control the strength and extent of tapping needed and the vertical pressure applied with this technique can cause chromosomes to adhere to the poly-lysine-coated slide and thus become refractory to further spreading. Therefore we prefer to use the eraser side of a pencil or a fingertip to gently move the coverslip back and forth a few additional times, as described in step 5. Check again under the microscope and repeat one or more additional times, if necessary.

8. When the chromosomal spreading is sufficient, set the slide with the coverslip side down on a stack of clean Kim-wipes. Place a second Kim-wipe on top and flatten chromosomes by placing your thumb over where the coverslip is positioned and pressing firmly, avoiding any horizontal movement of the coverslip that would shear the chromosomes.

9. Examine the slide again under the microscope to determine if the preparation is suitable for the intended purpose. If chromosomes have moved significantly upon squashing, use less volume of lactoacetic acid solution in your subsequent preparations. Repeat steps 1–9 until a sufficient number of suitable slides have been obtained.

10. Fill a small Dewar (e.g., thermos bottle) with liquid nitrogen. Using a long forceps, dip slide into liquid N₂ until the boiling stops, remove slide, and immediately use the edge of a clean razor blade at one corner to flip off the coverslip. If slide will be used immediately, place in a Coplin jar with PBS at 4 °C. Otherwise collect the slide into a Coplin jar filled with 95% ethanol. Examine the coverslip once the frost has sublimed away to confirm that the tissue adhered to the slide and did not remain with the coverslip. Repeat with the remainder of the slides until all slides are stored in either PBS (for use within several hours) or ethanol (for longer term storage) [Note: ethanol is a precipitative fixative and some antibody performance may be sensitive to this step].

11. If slides will be used immediately, go to step 13. If slides will not be processed within a few hours, store slides in ethanol at –20 °C and then go to step 12.

12. Rehydrate slides for 30 min at room temperature in a Coplin jar filled with PBST.

13. Transfer slides to a fresh Coplin jar filled with PBST for 30 min at room temperature.

14. Remove a slide from PBST and touch its edge to a Kim-wipe for a few seconds to remove excess solution but do not let it dry out. Place slide tissue side up in a humid chamber (e.g., line a Rubber-Maid tray with moistened Kim-wipes or filter paper). Put

50 µl of Blocking Buffer on tissue and place a 20 mm square of parafilm over the Blocking Buffer so it covers all of the tissue. Repeat for all of the slides being prepared.

15. Cover humidity chamber and allow slides to block for 30–120 min.

16. Prepare 20 × 44 mm coverslips with a narrow 1 mm strip of scotch tape at each end, one coverslip for each slide, and line them up on a paper towel with the taped side up.

17. Dilute the 1° antibody(ies) appropriately in Blocking Buffer and place 50 µl in the center of one of the coverslips prepared in 16. Remove the parafilm square from one of the slides being blocked in step 15, drain the blocking buffer by touching the slide edge to a Kim-wipe without allowing it to dry out, and pick up the 20 × 44 mm coverslip by gently touching the tissue region of the slide to the drop on the coverslip. Turn the slide over so the coverslip side is up but do not seal the edges. Any bubbles can usually be dislodged by tilting the slide slightly. Return the slide to the humid chamber. Repeat for all of the slides, seal the chamber, and incubate overnight at 4 °C.

18. Remove the coverslips and wash slides 3 × 10 min in a Coplin jar with PBT.

19. Prepare the appropriately diluted 2° antibody(ies) in Blocking Buffer and place 50 µl on raised 20 × 40 mm coverslips, applying to slides as described in step 17. Return slides to a humid chamber and incubate for 2 h at room temperature in the dark (weaker antibodies may be incubated 3–4 h).

20. Remove coverslips and wash 3 × 10 min in PBST in the dark.

21. If DNA staining is desired, wash slides 2 × 5 min in PBS, incubate for 10 min in Hoechst solution in the dark, and wash 2 × 5 min in PBS in the dark.

22. Drain slide, touch the edge to a Kim-wipe for a few seconds and wipe the back dry. Place approximately 2–3 drops of mounting solution on the tissue region of the slide and apply a clean 22 × 40 mm coverslip so as not to trap bubbles. Seal the edge of the coverslip with nail polish.

23. Best results are achieved if samples can be viewed immediately after preparation. However, if they must be stored, store at –20 °C and protect from any exposure to the light.

4.4. Acid-free squash technique [modified from 38]

4.4.1. Materials

Drummond dissection forceps (2)

Petri dishes (60 × 15 mm)

Frosted microscope slides (Fisher No. 12-544-3) (poly-lysine-coated)

22 × 22 mm No. 15 cover slips (Fisher No. 12-520B) (coated with Sigmacote; Sigma #SL2)

22 × 40 mm No. 15 cover slips (Fisher 12-530B)

Kim-wipes

Phase contrast microscope with 20× objective

Small Dewar (e.g., vacuum flask or thermos bottle)

Long forceps

Razor blades

Coplin Jar

Rubber-Maid or Tupperware tray (or equivalent sealable tray)

Parafilm (cut into 22 mm squares)

Brower's Fixation Buffer (see Blair, 2000): 0.15 M PIPES/3 mM MgSO₄/1.5 mM EGTA/1.5% NP40; adjust pH to 6.9)

Formaldehyde Fixation Buffer (add formaldehyde to Brower's Fixation Buffer to a final concentration of 2% just before use)

Liquid nitrogen

95% ethanol

PBS

PBST (PBS + 0.2% Triton X-100)

Blocking Buffer (PBST + 0.2% BSA + 5% goat serum)

Primary antibody solution (1° antibody diluted as appropriate in Blocking Buffer)

Secondary antibody solution (2° antibody diluted as appropriate in Blocking Buffer)

Hoechst 33258 (Molecular Probes)

Hoechst Solution (Hoechst 33258 0.2 µg/ml in PBS) [store in dark bottle at 4 °C]

Mounting solution (80% glycerol in 10 mM Tris, pH 9.0 + 5% *n*-propyl gallate)

Nail polish for sealing coverslips [Note: use brightly colored nail polish instead of clear in order to observe whether the edges are fully sealed].

4.4.4. Methods

1. Rinse larvae with water and transfer to freshly made Formaldehyde Fixation Buffer in a tissue culture dish and dissect immediately as described in step 2.

2. Grasp the tip of the mouth hooks with one pair of forceps, hold the body about 2/3 of the way down with the other pair, and pull on the mouth hooks so the salivary glands are exposed. Separate the salivary glands from the brain and eye-antennal discs (if they separated out with the salivary glands), and dissect away the fat body and any other associated tissues from the glands. Complete the dissection and fixation within 2–3 min.

3. Transfer the glands to PBST and incubate 2–3 min.

4. Transfer the glands to 50% glycerol and allow to soak for ~5 min.

5. Transfer the glands to 10–30 µl of 50% glycerol on a clean Sigmacoted coverslip. Gently lower a poly-lysine-coated microscope slide onto the coverslip and spread chromosomes and process preparations for immunostaining as described in steps 5–23 of Section 4.3.2 [Note: if chromosomes will not be immunostained but only imaged for GFP-fluorescence, proceed from step 11 to step 22 or, if Hoechst staining is also desired, proceed from step 11 to step 21].

4.5. High resolution squash technique [modified from 41]

4.5.1. Materials

Drummond dissection forceps (2)

Petri dishes (60 × 15 mm)

Frosted microscope slides (Fisher No. 12-544-3) (poly-lysine-coated)

22 × 22 mm No. 15 cover slips (Fisher No. 12-520B) (coated with Sigmacote; Sigma #SL2)

22 × 40 mm No. 15 cover slips (Fisher No. 12-530B)

Kim-wipes

Dremel 200

Dremel 200 Flex-Shaft attachment with hand-made head device (see Novikov et al., 2007 for instructions and pictures)

Avenger Gold toolmaker, tin coated precision MTC-300-1 vise (Penn Tool: <http://www.penntoolco.com/catalog/products/products.cfm?categoryID=3945>)

Phase contrast microscope with 20× objective

Small Dewar (e.g., vacuum flask or thermos bottle)

Long forceps

Razor blades

Coplin Jar

Rubber-Maid or Tupperware tray (or equivalent sealable tray)

Parafilm (cut into 22 mm squares)

Five times formaldehyde stock (0.74 g. paraformaldehyde/4.0 ml dH₂O/28 µl 1 N KOH; warm to 65 °C to dissolve, then store on ice. Make fresh daily.)

Fix #1 (0.5 ml 10× PBS/50 µl Triton X-100/3.45 ml dH₂O/1.0 ml 5× formaldehyde stock. Warm to disperse the Triton X-100. Prepare fresh and use within 1 h)

Fix #2 (1.5 ml dH₂O/2.5 ml glacial acetic acid/1.0 ml 5× formaldehyde stock. Prepare fresh and use within 1 h)

Lactoacetic acid solution (1 ml lactic acid/2 ml dH₂O/3 ml acetic acid)

Liquid nitrogen

95% ethanol

PBS

PBST (PBS + 0.2% Triton X-100)

Blocking Buffer (PBST + 0.2% BSA + 5% goat serum)

Primary antibody solution (1° antibody diluted as appropriate in Blocking Buffer)

Secondary antibody solution (2° antibody diluted as appropriate in Blocking Buffer)

Hoechst 33258 (Molecular Probes)

Hoechst Solution (Hoechst 33258 0.2 µg/ml in PBS) [store in dark bottle at 4 °C]

Mounting solution (80% glycerol in 10 mM Tris, pH 9.0 + 5% *n*-propyl gallate)

Nail polish for sealing coverslips [Note: use brightly colored nail polish instead of clear in order to observe whether the edges are fully sealed].

4.5.2. Methods

1. Follow steps 1–4 of the conventional squash procedure.

5. Remove Fix #2 from the depression slide with a drawn-out pipette tip and replace with 200 µl lactoacetic acid solution (alternatively simply transfer glands to a fresh well with 200 µl lactoacetic acid solution). After 1 min transfer the glands in 13–20 µl of lactoacetic acid solution to the middle of a clean (but not coated) glass slide.

6. Cover the drop with a clean, Sigmacoted 22 mm² coverslip.

7. Place the glass slide on the bench surface, cover with a strip of filter paper and then overlay with a sheet of clear transparency film.

8. Spread the chromosomes by gently applying the tip of a 200 rpm Dremel that is equipped with a modified knob attachment (see Materials) in circular, spiral movements over the coverslip.

9. Remove filter and transparency paper and monitor chromosomal spreading using a 20× objective by phase contrast microscopy. Repeat vibration procedure as necessary, typically for 3–10 min total time, until chromosomes appear suitably spread.

10. Place a filter paper over the slide and layer another slide on top. Set glass slide sandwich in the center of an Avenger Gold toolmaker, tin coated precision MTC-300-1 vise and tighten with a wrench with an extended handle as smoothly as possible (keep moving surfaces and tightening threads moderately oiled for best operation). First press the preparation lightly for 2 min to force out excess fixative and stabilize the preparation.

11. After 2 min, gradually increase vise to its maximum pressure for 1 min. If properly centered, the slides will rarely crack.

12. Gradually release pressure and transfer slide to a Dewar with liquid N₂ for 1–2 min.

13. Remove the slide and flip the top, Sigmacoted coverslip off with a razor blade. Glass slides can be used immediately or can be stored for up to one week in 100% ethanol at –20 °C.

14. Wash slides in 3 × 15 min in PBS (if they have been stored at –20 °C, allow them to warm to room temperature first).

15. Wash in PBST 3 × 10 min.

16. Block in Blocking Buffer for 1 h.

17. Incubate with 1° antibody(ies) appropriately diluted in Blocking Buffer overnight at 4 °C in a humidified chamber.

18. Wash 3 × 10 min in PBST.

19. Incubate with 2° antibody(ies) appropriately diluted in Blocking Buffer 2–3 h at room temperature in a humidified chamber in the dark.

20. Wash 3 × 10 min in PBST in the dark.

21. Wash 2 × 10 min in PBS in the dark.
22. Incubate in Hoechst Solution 10 min at room temperature in the dark.
23. Wash 2 × 5 min in PBS in the dark.
24. Add 2–3 drops mounting medium to region on the glass slide where the tissue is, gently apply a clean 22 × 40 mm coverslip, allowing mounting medium to spread without trapping bubbles, and seal coverslip with nail polish.
25. Best results are achieved if samples can be viewed immediately after preparation. However, if they must be stored, store at –20 °C and protect from any exposure to the light.

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