Chapter 19

Rapid Detection of γ-H2Av Foci in Ex Vivo MMS-Treated Drosophila Imaginal Discs

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Abstract

In *Drosophila melanogaster*, DNA double-strand breaks (DSBs) created by exposure to gamma or X-ray radiation can be quantified by immunofluorescent detection of phosphorylated histone H2Av (γ-H2Av) foci in imaginal disc tissues. This technique has been less useful for studying DSBs in imaginal discs exposed to DSB-inducing chemicals, since standard protocols require raising larvae in food treated with liquid chemical suspensions. These protocols typically take 3–4 days to complete and result in heterogeneous responses that do not provide information about the kinetics of DSB formation and repair. Here, we describe a novel and rapid method to quantify DSBs in imaginal discs cultured ex vivo with methyl methanesulfonate (MMS) or other DSB-inducing chemicals. The described method requires less than 24 h and provides precise control over MMS concentration and exposure time, enabling reproducible detection of transient DSBs. Furthermore, this technique can be used for nearly any chemical treatment and can be modified and adapted for several different experimental setups and downstream molecular analyses.

Key words Drosophila, Imaginal disc, Mutagen, Cell cycle, DNA damage response, Sensitivity assay, Apoptosis

1 Introduction

DNA damage tolerance is the process by which replication forks bypass DNA lesions during S-phase through the use of specialized translesion synthesis (TLS) polymerases and or homologous recombination [1]. Mutants defective in DNA damage tolerance are susceptible to prolonged replication fork stalls and fork collapse following exposure to DNA-damaging agents, resulting in DNA double-strand breaks (DSBs) and genome instability [2]. To study damage tolerance, organisms can be treated with a number of mutagens, one of which is the DNA alkylating agent, methyl methanesulfonate (MMS). While MMS does not directly cause DNA DSBs, the resulting 7-methylguanine and 3-methyladenine DNA lesions can stall and collapse replication forks in the

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absence of functional damage tolerance responses, generating DSBs and eventually leading to cell death [3–6].

In *Drosophila melanogaster*, various genotoxic chemicals have been used to identify proteins that function in the cellular responses to DNA damage [7, 8]. Many of the pathways and proteins that act in these responses are conserved between Drosophila and humans, making Drosophila an attractive model organism to study metazoan damage tolerance and repair in the context of a whole organism. Well-established mutagen sensitivity assays that involve treatment of larvae with alkylating or crosslinking agents have been used to elucidate responses to various DNA-damaging agents [9]. However, these sensitivity assays utilize adult viability as a quantifiable endpoint and take an average of 20 days. Furthermore, they cannot elucidate the intermediate molecular effects of DNA damage.

To characterize the molecular responses to DNA damage in Drosophila, as well as to determine the underlying causes of lethality, investigators frequently utilize third instar larval imaginal discs. Imaginal discs are tissues consisting of diploid precursor cells fated to develop into adult appendages during metamorphosis; the growth and patterning of which are highly regulated [10]. Patterned early in embryogenesis, imaginal discs of third instar larvae are relatively large, easily accessible by dissection, and thin, making them highly suitable for fixing and immunostaining. Immunofluorescent studies of imaginal discs provide spatial resolution of protein modification and localization within cells and the tissue as a whole [11].

Apoptosis, checkpoint activation, cell cycle progression, protein foci, and DNA DSBs have all been studied in imaginal discs of larvae exposed to γ-ray irradiation (IR) [12–14]. These studies are possible because IR can penetrate the larval cuticle, providing accurate control of exposure and dosage. Unfortunately, imaginal disc studies have not been readily adapted for other treatments. This is largely because most techniques used to treat larvae with DNA-damaging agents require larval consumption of mutagen-dosed food over several hours to days. While this method of drug treatment is suitable for sensitivity assays, it does not provide the fine temporal control necessary to observe transient cellular responses to DNA damage prior to repair or apoptosis.

Here, we describe a method to rapidly culture and chemically treat third instar imaginal discs ex vivo for downstream immunofluorescent detection of DSBs. Compared to currently published protocols, which involve treating early-stage larvae and then quantifying DSBs in dissected imaginal discs 3–4 days later, our method takes less than 24 h. Partially dissected third instar larvae imaginal discs are cultured for 5 h in a simple culture media [15], with and without MMS or other DSB-inducing chemicals. They are then fixed, probed for γ-H2Av (phosphorylated histone H2Av, which is

analogous to γ-H2AX in vertebrates and serves as a marker of DSBs) [16], and imaged using fluorescence. During the 5 h incubation period, approximately 90% of all wing imaginal disc cells should have entered S-phase [17], allowing for DSB formation in most genetic backgrounds. This technique allows for accurate temporal exposure to precise concentrations of any chemical, enabling detection of transient cell signals, protein modifications, and foci formation in imaginal discs. Possible applications of the technique include the study of checkpoint activation (Chk1 phosphorylation), cell division (histone H3 phosphorylation), apoptosis (cleaved Caspase-3), and DNA repair (RAD51 focus formation).

2 Materials

Reagents and materials listed below provide us with the results described here. Use of alternative reagents and materials may still work; however, modifications to the method may be necessary.

2.1 Larvae Imaginal Disc Dissection and Ex Vivo MMS Treatment

1. Dissection forceps, tip size D (#5 of biology) × 2.
2. Glass dissection dish or Sylgard® dish.
3. Dissecting microscope.
4. Petri dish.
5. 1.5 mL tube.
6. 96-well plate (flat-bottom).
7. 0.7% NaCl.
8. Culture media: 0.7% NaCl, 20% fetal bovine serum (FBS), 0.1% dimethyl sulfoxide (DMSO) [15].
9. Methane methylsulfonate (MMS), diluted to appropriate concentration in water and mixed well.

2.2 Wing Imaginal Disc Fixing and Antibody Staining

10. 37% formaldehyde.
11. Phosphate-buffered saline (PBS, 1×): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
12. PBS containing 0.1% Tween 20 (PBST).
13. PBS containing 0.3% Triton X-100 (PBSTx).
14. Blocking solution: 5% bovine serum albumin in PBSTx.
15. Primary antibody solution: 1:500 anti-γH2Av antibody (Rockland Inc.) in blocking solution.
16. Secondary antibody solution: 1:1000 goat anti-Rabbit IgG Rhodamine Red conjugated (Invitrogen), 500 μg/mL DAPI in blocking solution.
17. VECTASHEILD® Mounting Media (Vector Laboratories) (*see* **Note 1**).

18. Fluorescent microscope with Z-stack function.
19. Fiji image processing program with extended depth of field plugin [18, 19].

3 Methods

Culture, incubation, and wash times as well as reagent concentrations presented here have been extensively tested in our laboratory, and provide reproducible results if followed closely. Alternative incubation times and reagent concentrations can be adjusted to fit specific experimental demands, but have not been tested by our laboratory. For best results, use antibodies of high quality and purity.

3.1 Third Instar Larvae Imaginal Disc Dissection and Ex Vivo MMS Treatment

1. Collect third instar wandering larvae and place them in a small petri dish with 0.7% NaCl (*see* **Note 2**).
2. Transfer larvae one at a time to a glass-dissecting dish (or Sylgard® dish) containing 0.7% NaCl for dissection. In this protocol, "forceps 1" will designate the forceps held in the dominant hand, and forceps 2 will designate the forceps held in the nondominant hand.
   (a) Grasp the larvae two thirds (2/3) of the way from the head with forceps 1, and with forceps 2 grab the last one third (1/3) of the body (posterior) firmly, and rip it off. Place the posterior portion in a separate part of the dissecting dish to decrease debris.
   (b) Take the 2/3 anterior portion left behind and slip the cuticle, inside out, over the larva's head. The easiest way to do this is to place forceps 1 (closed) at the head of the larva and use forceps 2 to roll the cuticle over the head onto forceps 1. Then use forceps 2 to slide the inside-out cuticle off of forceps 1.
   (c) Holding the posterior portion of the cuticle with forceps 2, carefully remove the gut and fat tissue with forceps 1.
   (d) Look for the wing imaginal discs (WID) (the large, flat tissues that look like the continent of Africa). If you can see at least one, tease the gut and excess tissue from the cuticle. You will be left with the head with the discs attached to a piece of cuticle (Fig. 1) (*see* **Notes 3** and **4**).
3. Transfer cuticles to a 96-well plate with 97.5 μL of culture media. Place up to 8 cuticles per well (*see* **Note 5**).
4. In a chemical hood add 2.5 μL of freshly prepared 0.1% MMS to the cuticles in the culture media with a final concentration of 0.0025% MMS (*see* **Note 6**).

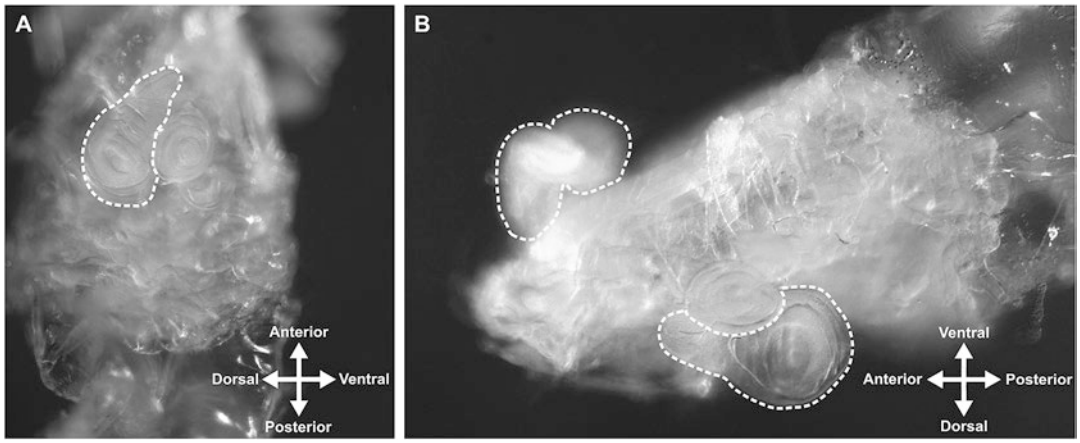

Fig. 1 Dissected cuticle of a third instar larva cleaned of all tissues except for the wing imaginal discs along with the adjacent leg and haltere discs. The wing imaginal discs are outlined with a dotted line. (**a**) A side view of the dissected cuticle presenting the right wing imaginal disc. (**b**) A top down view of the dissected cuticle with both wing imaginal discs visible

5. Incubate the cuticles for 5 h at 25 °C (the ideal temperature for Drosophila culture) with gentle shaking (35 rpm) protected from light (*see* **Notes 7** and **8**).

3.2 Imaginal Disc Fixing and Antibody Staining

6. Remove and dispose of the culture media containing MMS (*see* **Note 9**).
7. Rinse the cuticles by adding and subsequently removing 200 μL of cold PBST to the wells.
8. Add 200 μL of cold PBST to the wells and transfer the cuticles from the 96-well plate to 1.7 mL tubes. At this point you may pool together multiple wells of cuticles of the same treatment and discernable genotypes (*see* **Note 10**).
9. Remove the PBST transferred with the cuticles, and add 480 μL of cold PBST to the cuticles (*see* **Note 11**).
10. Fix the tissues by adding 20 μL of 37% formaldehyde to the PBST (final concentration is 1.48%) in the fume hood. Incubate for 30 min at room temperature while rocking on a nutating platform (*see* **Note 12**).
11. Centrifuge the samples for 10 s at 3000 × *g* to collect the cuticles and solution at the bottom of the tube. Remove formaldehyde mixture and dispose of it appropriately.
12. Wash four times for 5 min with 500 μL PBSTx at room temperature with rocking.
13. Block 30 min in 500 μL blocking solution at room temperature with rocking.
14. Centrifuge for 10 s at 3000 × *g* and remove blocking solution.

15. Add 500 μL of anti-γH2Av primary antibody solution and incubate overnight at 4 °C with rocking (*see* **Note 13**).
16. Centrifuge for 10 s at 3000 × *g* and remove primary antibody solution.
17. Wash four times for 5 min with 500 μL PBST at RT with rocking. On the final wash centrifuge for 10 s at 3000 × *g* and remove the PBST.
18. Add 500 μL of the secondary antibody solution and incubate for 2 h at RT, with rocking, protected from light.
19. Wash four times for 5 min with 500 μL PBST.
20. Transfer cuticles from the PBST onto a Sylgard® dish.
21. In a separate part of the Sylgard® dish, take an individual cuticle from the PBST droplet and place into a droplet of PBS. Using forceps, tease apart the WIDs from the rest of the cuticle and place the WID into a 30 μL VECTASHEILD® droplet on a microscope slide. Repeat for the rest of the cuticles (*see* **Note 14**).
22. Gently place a coverslip on the top of the VECTASHEILD® allowing for the VECTASHEILD® to spread completely under the coverslip. Seal the edges with clear nail polish and allow 10–15 min for the nail polish to dry protected from light (*see* **Note 15**).
23. Analyze samples for γ-H2Av foci at 10–20× magnification using a Z-stacking microscope and with filter sets compatible with DAPI and Rhodamine. γ-H2Av foci will appear bright red and fill the entire nucleus (Fig. 2) (*see* **Note 16**).

4 Notes

1. If another mounting media is used, ensure that it is compatible with DAPI and the fluorophore conjugated to the secondary antibody.
2. A slightly wet wooden probe works well to remove the larvae from the sides of bottles and vials. These larvae can be obtained from any source, such as bottles, vials, and grape plates. Heterozygotes and homozygotes can be distinguished by using a fluorescently marked balancer chromosome, such as one marked with a green fluorescent protein.
3. It is important to remove as much excess tissue as possible to ensure that the wing discs are not obstructed to allow uniform exposure of the tissues to the treatment. Removing the mandible and brain helps to decrease obstruction. Also removing the anterior spiracles (hand-like structures near the head) helps to expose the wing discs and prevents the cuticle from

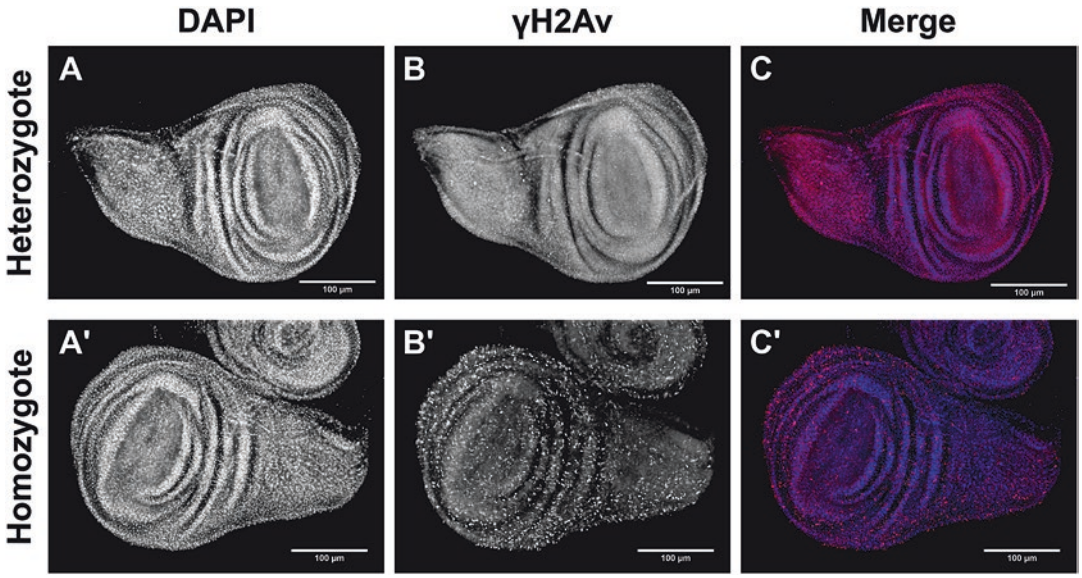

Fig. 2 Representative immunofluorescent results of wing imaginal discs from a MMS-sensitive mutant background. Heterozygotes (**a, b, c**) and homozygotes (**a′, b′, c′**) were treated, fixed, and stained as described in this protocol. The discs were visualized at 20× magnification. Z-stack images were deconvolved using the Zen 2 (Zeiss) program, then a composite image was generated through extended depth of field analysis. (**a, a′**) Nuclei are visible through DAPI staining. (**b**) A disperse fluorescent background is observed in heterozygous mutant discs, with a small number of γ-H2Av foci present. (**b′**) Distinct γ-H2Av foci are prevalent above background in homozygous MMS-sensitive mutants. (**c, c′**) A merge of both channels shows the clear presence of foci in the homozygous mutant compared to the heterozygote

scrunching up. Because the fine dissection results in less tissue to protect the WIDs, some discs may be lost during the procedure. Therefore, it is prudent to dissect a few more larvae than desired to ensure enough WID make it through the protocol.

4. WID are used in this protocol because they are the largest. Other imaginal discs can be used as well, however, culture time may need to be adjusted to correlate with the length of S-phase in these tissues [17].
5. Too many larvae per well will increase the probability that the cuticles may contact each other and obstruct WID exposure to culture media and treatment. 96-well plates are used to decrease the volume of MMS necessary. Larger incubation dishes and volumes may be utilized.
6. The concentration of MMS in the culture media is approximately 5–10 fold lower than what is necessary to observe lethality in a classic mutagen sensitivity assay. If larger volumes of chemicals are required, they should first be diluted in culture media to not drastically affect the concentrations of

the components within the culture media. If the chemical is dissolved in DMSO, adjust the culture media to have a final DMSO concentration of 0.1%.

7. As a safety precaution place the 96-well plate in a secondary container on top of bench paper on the orbital shaker. Incubation times can be adjusted for individual treatments as necessary.
8. Within 5 h 90% of WID cells should have entered S-phase [17]. Decreased incubation times will result in fewer of the WID cells entering and completing S-phase. Increased incubation times may result in increased apoptosis or a decrease in mitotically active cells.
9. To prevent the cuticle from being sucked up during pipetting, place the 96-well plate at angle allowing for the cuticles to gently settle toward one edge of the well. Using a p200 pipette begin pipetting the solution by placing the tip opposite to the cuticle. As the volume of solution within the wells decreases, gently move the tip along the edge of the well until you push the cuticles out of the way at the "bottom" of the well.
10. The easiest way to transfer the cuticles is to use a p1000 pipette and a pipette tip which has had the tip cut using a razor blade, creating a larger hole.
11. The best way to remove solutions without losing or harming the cuticles is to pipette using a p1000 pipette. Remove some of the solution, leaving about 100–200 μL within the tube. Gently pipette mix the remaining solution to lift the cuticles from the bottom, then place the pipette tip at the very bottom of the 1.7 mL tube and pipette the remainder of the solution.
12. Increasing the final formaldehyde concentration to 4% may allow for a decrease in fixing time.
13. The overnight incubation time of the primary antibody allows for complete diffusion of the antibody within the discs. To significantly shorten the protocol, an incubation for 90 min at room temperature may be used [11] or the antibody concentration can be increased.
14. Heterozygotes and homozygotes can be dissected and imaged on the same slide so long as there is a discernable fluorescent marker such as a balancer with GFP. Otherwise, genotypes should be treated, dissected, and imaged separately.
15. Slides can be kept at 4 °C for a few months, but the fluorescent signal may decrease over time. It is recommended to analyze the samples shortly after mounting.
16. To obtain the best images, discs should be imaged multiple times along the Z-axis (Z-stack), processed by deconvolution, and compressed into one image by extended depth of field algorithms.

References

1. Ghosal G, Chen J (2013) DNA damage tolerance: a double-edged sword guarding the genome. Trans Cancer Res 2:107–129. doi:10.3978/j.issn.2218-676X.2013.04.01
2. Branzei D, Foiani M (2010) Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol 11:208–219. doi:10.1038/nrm2852
3. Lundin C, North M, Erixon K et al (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. Nucleic Acids Res 33:3799–3811. doi:10.1093/nar/gki681
4. Beranek DT (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 231:11–30
5. Tercero JA, Diffley JF (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature 412:553–557. doi:10.1038/35087607
6. Fu D, Calvo JA, Samson LD (2012) Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat Rev Cancer 12:104–120. doi:10.1038/nrc3185
7. Eeken JC, Romeijn RJ, de Jong AW, Pastink A, Lohman PH (2001) Isolation and genetic characterisation of the Drosophila homologue of (SCE)REV3, encoding the catalytic subunit of DNA polymerase zeta. Mutat Res 485:237–253. doi:10.1016/S0921-8777(01)00062-3
8. Ravi D, Wiles AM, Bhavani S, Ruan J, Leder P, Bishop AJR (2009) A network of conserved damage survival pathways revealed by a genomic RNAi screen. PLoS Genet 5:e1000527. doi:10.1371/journal.pgen.1000527
9. Henderson DS, Grigliatti TA (1992) A rapid somatic Genotoxicity assay in Drosophila-Melanogaster using multiple mutant mutagen-sensitive (Mus) strains. Mutagenesis 7:399–405. doi:10.1093/mutage/7.6.399
10. Cohen SM (1993) Imaginal disc development. In: Bate M, Martinez Arias A (eds) The development of *Drosophila melanogaster*, vol 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 747–841
11. Klein T (2008) Immunolabeling of imaginal discs. Methods Mol Biol 420:253–263. doi:10.1007/978-1-59745-583-1_15
12. Brodsky MH, Sekelsky JJ, Tsang G et al (2000) mus304 encodes a novel DNA damage checkpoint protein required during Drosophila development. Genes Dev 14:666–678
13. Bilak A, Uyetake L, TT S (2014) Dying cells protect survivors from radiation-induced cell death in Drosophila. PLoS Genet 10:e1004220. doi:10.1371/journal.pgen.1004220
14. Wells BS, Johnston LA (2012) Maintenance of imaginal disc plasticity and regenerative potential in Drosophila by p53. Dev Biol 361:263–276. doi:10.1016/j.ydbio.2011.10.012
15. Gatti M, Santini G, Pimpinelli S, Olivieri G (1979) Lack of spontaneous sister chromatid exchanges in somatic cells of *Drosophila melanogaster*. Genetics 91:255–274
16. Madigan JP, Chotkowski HL, Glaser RL (2002) DNA double-strand break-induced phosphorylation of Drosophila histone variant H2Av helps prevent radiation-induced apoptosis. Nucleic Acids Res 30:3698–3705
17. Adler PN, MacQueen M (1984) Cell proliferation and DNA replication in the imaginal wing disc of *Drosophila melanogaster*. Dev Biol 103:28–37
18. Schindelin J, Arganda-Carreras I, Frise E et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. doi:10.1038/nmeth.2019
19. Forster B, Van De Ville D, Berent J, Sage D, Unser M (2004) Complex wavelets for extended depth-of-field: a new method for the fusion of multichannel microscopy images. Microsc Res Tech 65:33–42. doi:10.1002/jemt.20092