Chapter 25

Analysis of DNA Double-Stranded Breaks Using the Comet Assay in Planarians

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Abstract

Comet assay provides the opportunity to detect and characterize DNA strand breaks. Cellular lysing followed by embedding in agarose slide is used to visualize under an electrical current migration patterns corresponding to DNA fragments of different sizes. Here we describe the process of detecting and characterizing DNA damage by Comet assay on planarians, which is a model organism commonly used to understand the process of whole-body regeneration, stem cell regulation, and adult tissue maintenance.

Key words Alkaline Comet Assay, Planarian, Double-stranded breaks, DNA damage

1 Introduction

Single-cell gel electrophoresis or Comet assay is an attractive tool to assess the integrity of DNA molecules [1–4]. DNA maintains its structure through its negatively charged supercoils around the histone core. DNA strand breaks may result from endogenous and exogenous sources (e.g., ROS and ionizing radiation), leading to the disruption of DNA integrity [5]. The Comet assay takes advantage of the relaxation of DNA supercoils to assess the levels of DNA strand breaks. Briefly, cell suspensions are embedded in agarose-coated slides and are lysed with detergent (i.e., Triton-X 100) to remove nuclear membranes and DNA histone structures, resulting in gel-embedded nucleoid bodies [4]. Increases in DNA breaks and subsequent relaxation in its loops can be exposed when an electric field is applied, and a comet tail-like structure is formed in relation to the amount of damaged DNA. Higher amounts of DNA strand breaks yield more prominent comet tail-like structures [3, 4, 6–8]. After the initial comet protocols were established in 1980s [1, 2], many variations to the protocols have been established (e.g., Comet-FISH, Comet-BrdU) [6–10]. However, the

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Comet assay under alkaline conditions (i.e., pH >13) has remained the most widely used method by converting all types of DNA damage (i.e., crosslinks, strand breaks, adducts, etc.) to double-stranded DNA breaks [2–4, 11].

Detection of DNA damage and its repair can be studied in a variety of ways in planarian flatworms. These include immunohistochemistry and Western blot techniques to assess the expression of markers associated with the DNA damage response (i.e., RAD51, H2AX, and PARP) [12–15]. Planarian stem cells known as neoblasts are the only cells with replicative capacity in planarians. Techniques to assess neoblast chromosomal stability and telomeric maintenance have been established [12, 16, 17]. Recent research implemented the use of Comet assay in planarians to characterize the extent of DNA strand breaks [12, 14–16]. The procedure can be guided toward specific cell types by using flow cytometry to sort cells (e.g., neoblasts) or may involve evaluation of different cell types obtained by the dissociation of whole animals. Future adaptations of the Comet assay may also involve double labeling with immunostaining, gene expression probes, and BrdU, which altogether may facilitate characterization of DNA damage and repair on specific cell types.

The Comet assay not only provides a qualitative representation of the extent of DNA strand breaks but can also be used to obtain a precise quantification between different degrees of damage and repair. Here we demonstrate that Comet assay can be used to efficiently detect the extent of DNA strand breaks in a variety of conditions using the highly regenerative planarian model. This includes exposure to gamma irradiation, knockdown of genes, and pharmacological treatments with genotoxic compounds.

2 Materials

2.1 Handling Equipment

1. Electronic pipette.
2. Cell strainer, 70 μm.
3. Carbon steel blade.
4. Anti-static wipes.
5. Dissecting microscope.
6. Black round filter paper.
7. Superfrost microscope slides.
8. Scintillation vials.
9. DNA electrophoresis chamber.
10. Nucleic acid gel stain.
11. Fluorescent microscope.

12. Camera.
13. Imaging software.
14. Data processing software.

2.2 Comet Assay Solutions

All the solutions are prepared with Nanopure water unless otherwise stated.

1. Stock lysing solution: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–NaOH in 700 mL H_2O. Stir mixture. Add ~8 g of pelletized NaOH, allow the mixture to go into solution (~30 min). Adjust the pH to 10.0 with NaOH, bring to 900 mL using H_2O, filter (*see* **Note 1**). Store at room temperature for up to 6 months.
2. Final lysing solution: 36 mL stock lysing solution, 4 mL DMSO, 0.4 mL Triton x-100 (*see* **Note 2**). Refrigerate the solution at 4 °C for 60 minutes, allowing the solution to go from opaque to clear.
3. 1× TE Buffer: 200 mL H_2O, 0.395 g Tris–HCl, 0.0925 g EDTA, pH 7.5. Bring volume to 250 mL with H_2O. Store at room temperature.
4. 200 mM EDTA solution: 150 mL H_2O, 14.89 g EDTA, stir, adjust pH to 10 by using NaOH pellets (*see* **Note 3**). Adjust final volume to 200 mL (*see* **Note 4**). Refrigerate the solution at 4 °C.
5. 10 N NaOH solution: 450 mL H_2O, 200 g NaOH, stir and bring the volume to 500 mL (*see* **Notes 4** and **5**). Store at room temperature.
6. 1× electrophoresis buffer: 10 mL 10 N NaOH solution, 5 mL 200 mM EDTA solution in 700 mL H_2O. Adjust pH to more than 13 using NaOH dropwise, bring volume to 1 L (*see* **Note 6**). Refrigerate solution at 4 °C.
7. 0.4 M Tris neutralization buffer: 48.5 g Tris–HCl in 800 mL H_2O, pH 7.5. Adjust final volume to 1 L, store at 4 °C (*see* **Note 7**). Refrigerate solution at 4 °C.
8. Calcium- and magnesium-free (CMF) medium: 15 mL H_2O, 0.0100 g NaH_2PO_4, 0.0200 g NaCl, 0.0300 g KCl, 0.0200 g $NaHCO_3$, 0.0060 g Dextrose, 0.2500 g BSA, 0.0890 g HEPES-HCl, pH 7.3. Bring final volume to 25 mL. Filter the medium with a 0.22-μm filter and store at 4 °C for up to 7 days.
9. 10,000× nucleic acid gel stain: Add the stain (e.g., SYBR Gold Nucleic Acid Gel Stain, Invitrogen) according to the manufacturer's instructions to reach 10,000× of the recommended final concentration. Make aliquots in dark tubes, store at −20 °C. Use within 24 h after thawing.

10. 10× commercially available phosphate-buffered saline (PBS).
11. 0.5% low melting point agarose (LMPA): 0.25 g LMPA in 50 mL 10× PBS (*see* **Note 8**). Aliquot into scintillation vials and refrigerate solution at 4 °C.
12. 1% normal melting point agarose (NMPA): 0.5 g normal melting point agarose, 50 mL 10× PBS, boil until agarose is dissolved. Allow agarose to equilibrate in a 65 °C bath 30 minutes prior to coating slides (*see* **Note 9**). Make fresh when needed.

3 Methods

3.1 Agarose Slide Preparation

1. Pour dissolved and equilibrated 1% NMPA into a 50-mL conical tube.
2. While still hot, slowly dip in 3/4 of the superfrosted plus slide twice (5 mm thick) into the agarose.
3. With a tissue, remove excess agarose from the back of the slide.
4. Lay slides in a flat surface to air dry overnight or use a slide warmer (Fig. 1a) (*see* **Note 10**).
5. Once dry, store slides within a slide box at room temperature in dry low humidity.

3.2 Planarian Dissociation and Cell Recovery

1. Place 10 large planarians (i.e., control or experimental) onto a chilled 10 mm polystyrene petri dish lid on a Peltier cooler (alternatively, ice wrapped in plastic would work), located under a dissecting microscope (Fig. 1b).
2. Remove all excess liquid using anti-static wipes.
3. Finely cut the pool of worms with a carbon steel razor blade by applying rapid movements until tissues look like a homogenous paste of macerated tissues (Fig. 1c) (*see* **Note 11**).
4. Rinse homogenate, razorblade, and petri dish with ice-cold CMF medium.
5. Place suspension into a 15-mL conical tube.
6. Repeat washes until conical tube is filled with 10 mL of suspension.
7. Rock cell suspension at 4 °C for 25–45 min (*see* **Note 12**).
8. Filter cells using a 70-μm mesh.
9. Centrifuge suspension at 2230 rcf for 5 min at 4 °C.
10. Resuspend pellet in 2 mL of CMF media.
11. Calculate a cell density of 50,000 cells/mL using a hemocytometer; mix a 1:1 (5 μL:5 μL) ratio of Trypan Blue and cell suspension.

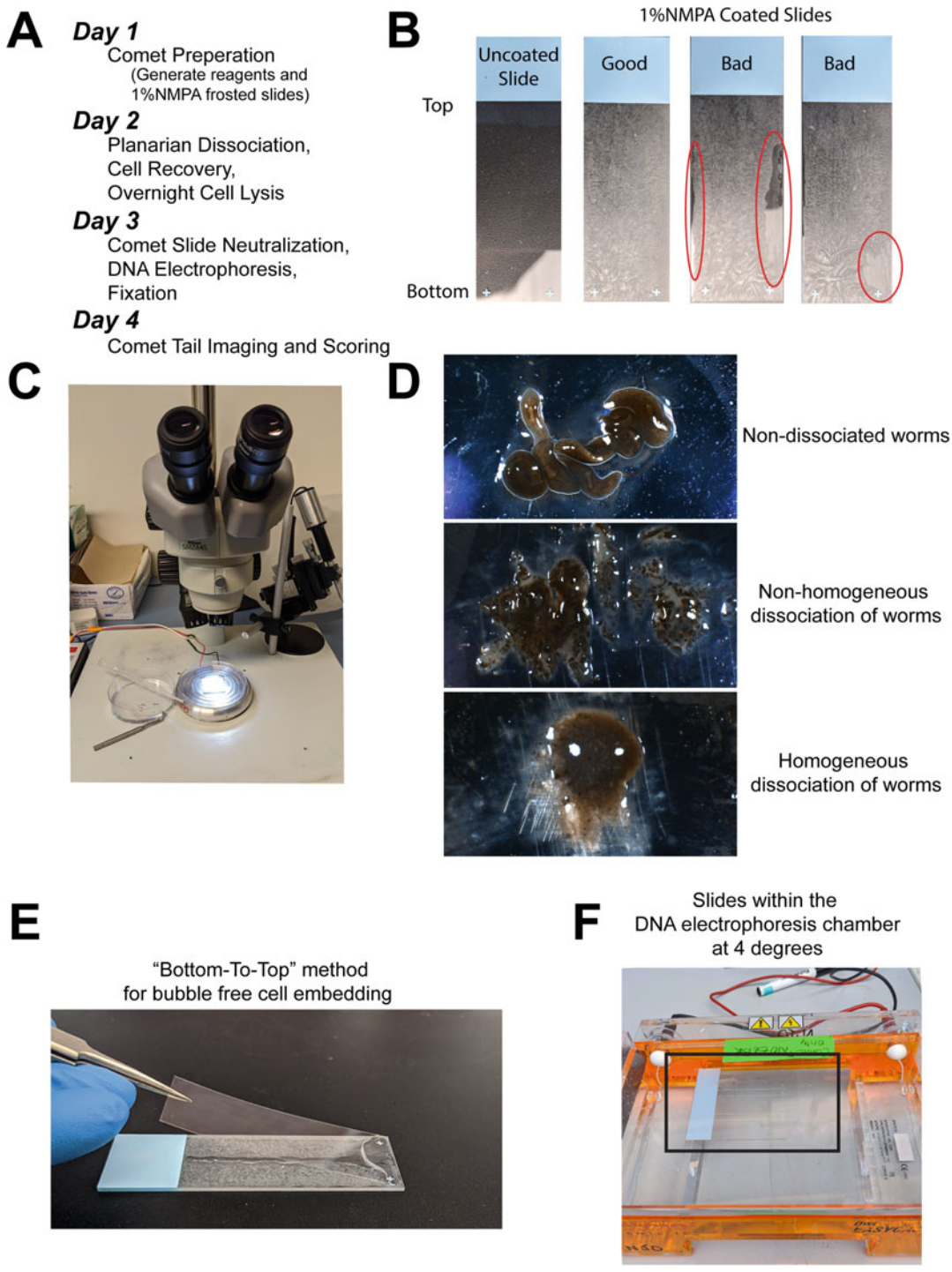

Fig. 1 Comet protocol preparation and visual protocol. (**a**) Comet workflow and timeline. (**b**) Representative images of unfrosted and 1% NMPA-coated slides. A good slide to conduct the Comet protocol with alongside coated slides that are not usable due to voids in agarose (i.e., red circles). (**c**) Setup for planarian dissociation. Needed a dissecting microscope, Peltier cooler, petri dish, and tweezer with a razor blade. (**d**) Representative images of steps in worm dissociation. Note that by the end of the dissociation process, the end product should look homogenous void of remaining tissue structures. (**e**) Image showing the "bottom-to-top" method to

12. Aliquot 50,000 cells/mL into 1.5 mL snap cap tubes; volume determined by cell density (*see* **Note 13**).
13. Allow cells to recover at 37 °C for 2 h (*see* **Note 14**).

3.3 Slide Preparation

1. Microwave and equilibrate 30 mL 0.5% LMPA to 37 °C for 30–60 min.
2. Centrifuge the recovered cells at 3725 rcf for 2 min.
3. Aspirate supernatant leaving ~40 μL of CMF medium on the pellet.
4. Dissolve the pellet by adding 100 μL of warm 0.5% LMPA.
5. Immediately, pipette the suspension to the coated 1% NMPA slide (*see* **Note 15**).
6. Immediately place a coverslip (24 × 50 mm) on top of solution and avoid air bubbles by starting at the bottom of the slide and slowly allow the coverslip to fall toward the top (i.e., plus sign to label of slide, respectively) (Fig. 1d).
7. Allow agarose to solidify in refrigerator at 4 °C for at least 15–20 min (*see* **Note 16**).

3.4 Cell Lysis

1. Remove coverslip using a tweezer to gently push toward one slide, the remaining steps in Subheading 3.3 must be conducted on ice.
2. Place 40 mL of cooled transparent final lysing solution in a Coplin jar.
3. Gently insert slides in the filled Coplin jar.
4. Protect slides from light by wrapping Coplin jar with aluminum foil.
5. Place Coplin jars in the 4 °C refrigerator overnight (*see* **Note 17**).

3.5 Comet Slide Electrophoresis

1. Refrigerate 500 mL of 1× electrophoresis buffer at 4 °C for 30 min.
2. Place the Coplin jar on ice.
3. Gently discard the lysing solution from the Coplin jar.
4. Remove the remaining solution by wadding a wipe and securing slides with it before inverting the Coplin jar.
5. Neutralize the slides with 40 mL cooled 0.4 M Tris neutralization buffer for 5 min maximum at 4 °C.

Fig. 1 (continued) prepare cell embedded slides without the generation of bubbles. (**f**) Electrophoresis setup within the 4 °C fridge connected to a voltage power source. The slides are aligned tightly side-by-side on the cathode side of the box

6. Gently discard neutralization buffer from the Coplin jar.
7. Invert the Coplin jar over a wipe.
8. Add 40 mL of 4 °C chilled 1× electrophoresis buffer into the Coplin jar.
9. Allow slides to equilibrate in buffer for 30 min at 4 °C (*see* **Note 18**).
10. With tweezer to gently place equilibrated slides in a precooled electrophoresis chamber (Fig. 1e).
11. Fill chamber with enough 4 °C chilled 1× electrophoresis buffer to cover slide by 3–5 mm (~800 mL) (*see* **Note 19**).
12. Run electrophoresis chamber for 30 min within the fridge. Set current to 12 V and 300 mA.

3.6 Comet Slide Neutralization and Fixation

1. Return slides from the electrophoresis chamber to an empty Coplin jar.
2. Invert the Coplin jar over a wipe.
3. Neutralize the slides with 40 mL cooled 0.4 M Tris neutralization buffer for 5 minutes maximum at 4 °C.
4. Gently discard neutralization buffer from the Coplin jar.
5. Invert the Coplin jar over a wipe.
6. Add 40 mL pre-cooled (−20 °C) 100% ethanol into the Coplin jar.
7. Store it in the −20 °C refrigerator for 5 min.
8. Remove slides from Coplin jar and drain excess ethanol by dabbing the bottom of each slide with a wipe.
9. Place the slides with agarose facing upward on a paper towel and allow to dry overnight in low humidity, at room temperature (*see* **Note 20**).

3.7 Comet Slide Staining and Visualization

1. Thaw 10,000× Nucleic Acid Gel Stain working solution.
2. Add 130–200 μL of Nucleic Acid Gel Stain solution on top of each dried slide.
3. Immediately place a coverslip (24 × 50 mm) on top of solution and avoid air bubbles by slowly placing the coverslip onto the slide with a top-down approach.
4. Place slides in a dark slide box.
5. Image each slide using a fluorescent microscope which contains optics recommended by the DNA stain's manufacturer.
6. The total amount of images taken should account for 200–500 randomly selected individual nuclei (i.e., comets) per slide. For statistical purposes, image a large sample size of comets and

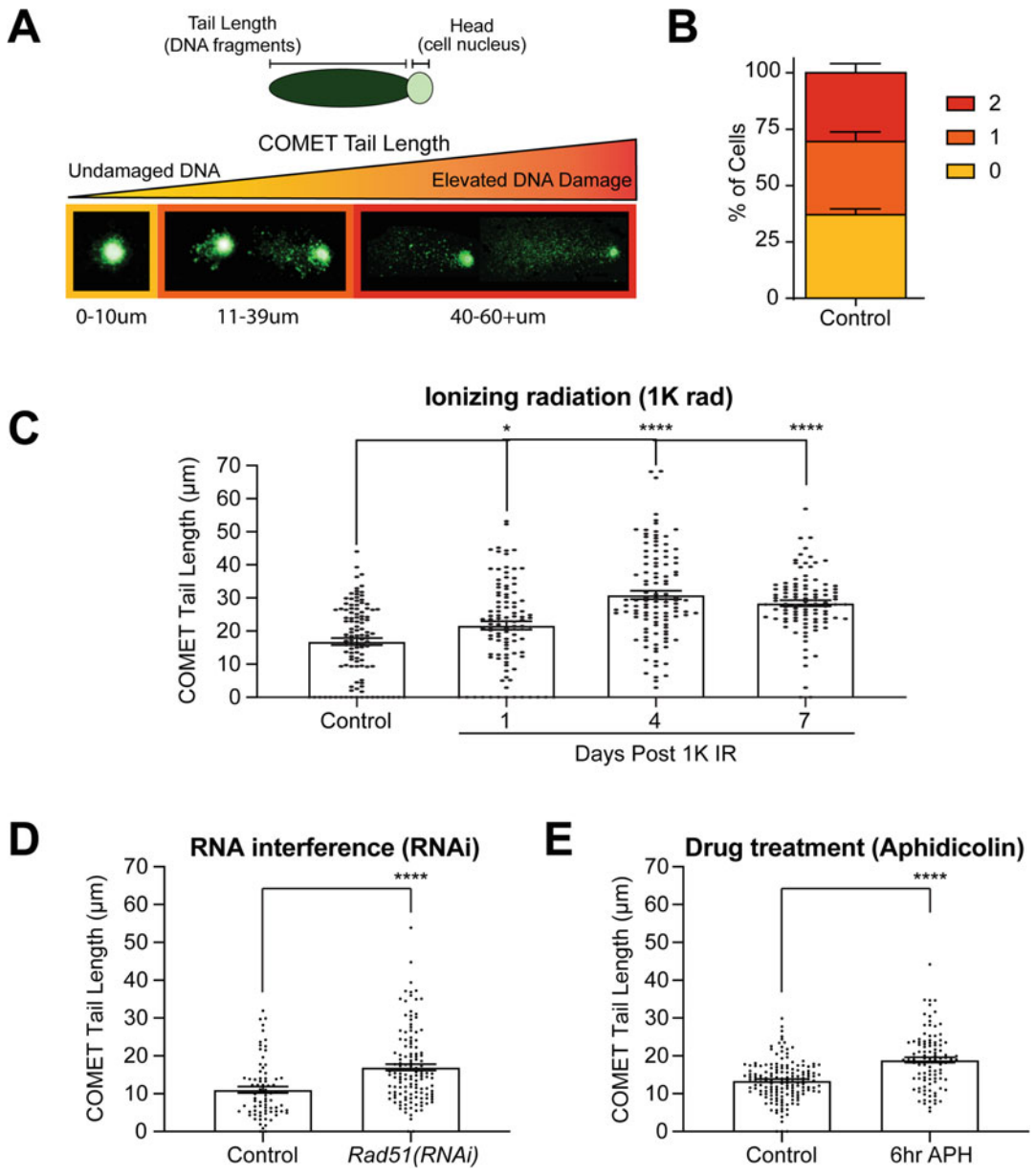

Fig. 2 The effect of various treatments on DNA damage using the Comet assay. (**a**) Visual representation of single nuclei post alkaline Comet electrophoresis and staining. Nuclei are stained with DNA dye (i.e., SYBR green) and imaged using florescent microscopy, revealing the severity of DNA damage per cell. Tail length can be ranked, categorizing cells as undamaged, moderate, and severe damage (e.g., yellow (0), orange [1], and red [2], respectively). (**b**) Quantification of three independent Comet assays using the ranking score method on 7-day starved animals. Approximately 40% of planarian cells contain undamaged DNA (i.e., score of 0) and is consistent with other experimental models [18]. (**c**) Comet-tail length after exposure to 1 K rad gamma irradiation (sub-lethal) in a 7-day time course post treatment. Planarian stem cells are lost by 1–2 days post sub-lethal irradiation due to increased DNA damage. However, by days 4–5 post irradiation, planarian stem cell begin to repopulate, and this is accompanied by an increase in DNA damage and DNA repair proteins as shown previously [12]. By day 7 post radiation, DNA integrity begins to reestablish. (**d**) Increase in DNA damage can be achieved through RNA interference (RNAi). Graph represents comet-tail length of 30-day

repeat each experiment in triplicated form for each independent biological replicate.

7. Measure the comet-tail length from the edge of the comet-head to the edge of the comet-tail using imaging software (e.g., ImageJ). Rank comet-tail lengths from 0 to 2, where a score of 0 shows little DNA damage, 1 moderate, and 2 is a dispersed tail with no nucleus visible (i.e., 0–10 μm, 11–39 μm, and 40–60 μm, respectively) (Fig. 2).

3.8 Comet Slide Storage

1. Remove coverslip.
2. Place in a Coplin jar.
3. Add 40 mL pre-cooled (−20 °C) 100% ethanol.
4. Store the Coplin jar in a − 20 °C refrigerator for 5 min.
5. Remove slides from Coplin jar and drain excess ethanol by dabbing the bottom of each slide with a wipe.
6. Place slide with agarose facing upward on a paper towel.
7. Allow to dry overnight in a region void of humidity, at room temperature.
8. Place slides in a slide box.
9. Store in a dark area with low humidity until the slides are no longer needed.
10. To re-stain the slides, follow Subheading 3.5. This re-stain process can occur multiple times as long as the gel is still present.

4 Notes

1. For the stock lysing solution, the remaining volume (100 mL) will be adjusted to 1 L when the final lysing solution is made as these components must be added fresh and prior to use.
2. Final lysing solution should be made fresh on the day of use and used only once. When generating final lysing solution, prepare

Fig. 2 (continued) starved animals for both the control and experimental group *Rad51(RNAi). Rad51(RNAi)* animals contain cells that harbor increased DNA damage and chromosomal abnormalities [13]. RAD51 is a key component in DSB repair within the planarian during homeostasis and pore-radiation stem cell repopulation. Chromosomal abnormalities. (**e**) Treatment with pharmacological agents such as Aphidicolin can result in increased DNA damage within the planarian model system. Aphidicolin (APH) is an inhibitor of DNA replication, blocking DNA polymerase Alpha and Delta during S-phase of the cell cycle. Comet assay was performed on animals exposed to DMSO and APH [0.5 mM] for 6 hours (i.e., control and experimental group, respectively). It is evident that APH treatment increases DNA DSBs within the planarian. (**c**–**e**) Each dot represents an individual planarian cell's comet-tail length. (**b**–**e**) All graphs represent mean ± s.e.m. Statistics were obtained by two-way ANOVA; * <0.05 and **** < 0.0001

a volume of 40 mL or an adequate volume to fill one Coplin jar. Increase volume depending on the number of Coplin jars required to hold all slides. Furthermore, this solution will turn opaque and requires cooling at 4 °C to turn clear. Solution must be clear prior to use.

3. NaOH pellets help increase pH and allow EDTA dissolve.
4. Both the 200 mM EDTA and 10 N NaOH solutions should be stored at 4 °C as it will increase their shelf life of 1 year. If left at room temperature, solutions will have a shelf life of 2 weeks.
5. 10 N NaOH solution is commercially available or can be made in the lab.
6. 1× electrophoresis buffer must be made fresh and cooled to 4 °C prior to use. It is important to have a pH greater or equal to 13. The high pH allows for proper alkaline Comet assay to occur by converting all types of DNA damage to double-stranded DNA strand breaks.
7. 1 M neutralization buffer is also commercially available and can be diluted into a 0.4 M solution.
8. Aliquot liquid 0.5% LMPA into scintillation vials and refrigerate at 4 °C until needed.
9. Microwave in small increments of time 25 s. Swirl the solution in between to aid in the dissolving process. Repeat until the mixture is fully dissolved.
10. The slides will look milky when dried, and this is normal (Fig. 1a). Avoid slides that look like they have pits, swirls, and gaps at the edges, resulting in an edge effect and distorting the shape of the comet when visualizing. To account for this during the coating process, dip ~50 slides to generate ~20 useable ones. Store the coated slides at room temperature until needed but avoid humidity to preserve the quality of the slides.
11. Make sure that animal dissociation results in a fine paste-like solution, lacking clumps of visible tissues (Fig. 1c). This process should be completed quickly (i.e., no longer than 10–15 min) to avoid excessive cellular lost due to the adverse conditions in which the process is performed (e.g., oxygen, pressure, media nutrients).
12. The time rocking cells post maceration depends on the general condition of the experimental group. Some RNA interference regimens or drug treatments are extremely harsh on animals. This could lead to friable tissues that can deteriorate prior to the start of the Comet assay. Therefore, reducing stress by limiting rocking time is advisable.

13. Generate 3–6 tubes per sample. This will account for the generation of at least two comet slides per group and handling error in losing cell pellet in subsequent steps. You will not need to generate all but two slides, the 3–6 tubes per sample are in place for backup just in case the pellet is lost during the subsequent steps.
14. Allowing cellular resting time post maceration and centrifugation enhances cellular viability and is crucial for the quality of the assay.
15. Use one 0.5% LMPA vial at a time. Once the agarose can no longer be pipetted with ease, use the second vial and allow the initial vial to go back into solution at 37 °C.
16. To make sure that 0.5% LMPA plus cell mixture has solidified check resistance of slide by gently rotating the coverslip from side-to-side. If there are still bubbles present after coverslip has been removed, add an additional layer of 0.5% LMPA (~100 μL) and repeat the solidification step by placing a coverslip again.
17. Overnight lysis step works best but a minimum of 4 hours is possible. Furthermore, slides can remain in lysis solution for up to 2 weeks. However, the slides become more sensitive the longer they are in the refrigerator, and the slides must be handled with caution during the subsequent steps.
18. Incubate slides in the alkaline 1× electrophoresis buffer to allow DNA unwinding and the expression of alkali-labile damage.
19. Slides must be submerged no more than 3–5 mm with 1× electrophoresis buffer. Electrophoresis chambers normally include labels for a fill line, but only filling in 3–5 mm over the slides will suffice.
20. Once slides are fixed, this can be a stopping point. Store slides until they are ready for staining in an environment with low humidity. Slides may be stored for years.

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