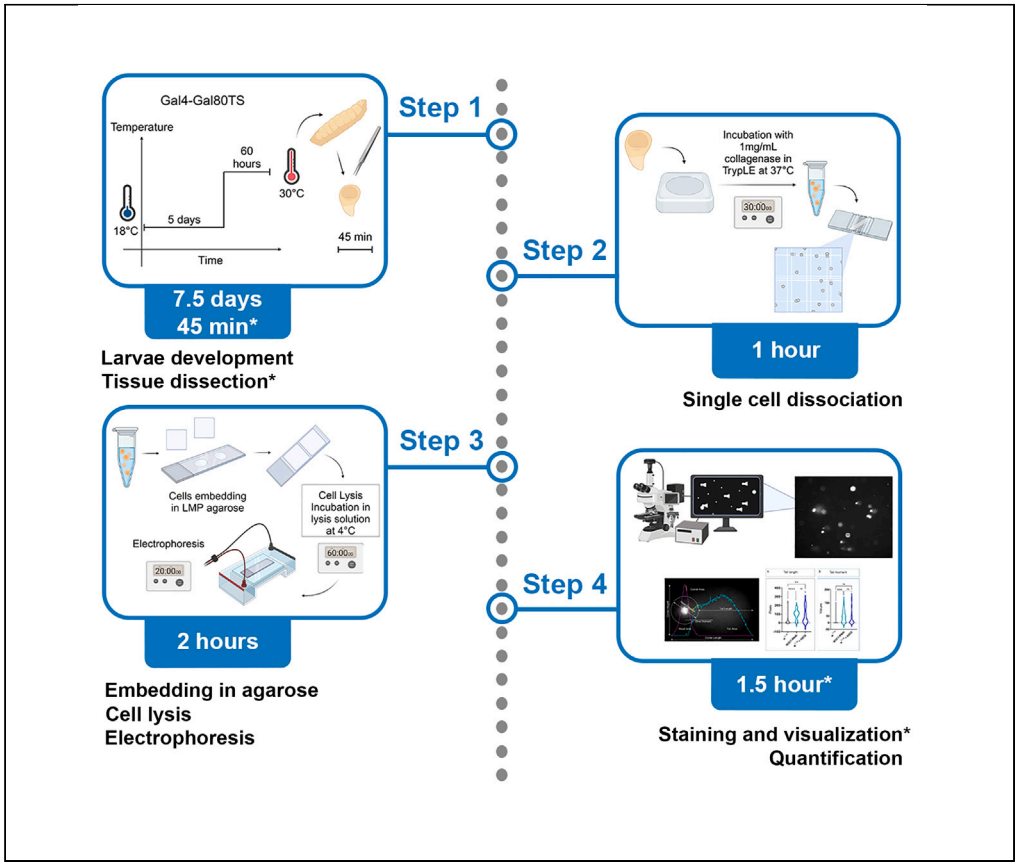
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Optimized protocol for single-cell isolation and alkaline comet assay to detect DNA damage in cells of *Drosophila* wing imaginal discs



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Assessment of DNA damage in a single cell

Isolation of single cells from *Drosophila* imaginal wing discs

Optimization of comet assay conditions for *Drosophila* tissues

Application to highlight the role of genes in DNA damage

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Optimized protocol for single-cell isolation and alkaline comet assay to detect DNA damage in cells of *Drosophila* wing imaginal discs

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SUMMARY

Reduced expression of nucleolar genes induces stress and DNA damage. Here, we present a protocol to analyze DNA fragmentation at the single-cell level in *Drosophila* imaginal discs using an optimized alkaline comet assay. We describe steps for larvae development, tissue disaggregation, and single-cell dissociation. We then detail procedures for cell lysis, electrophoresis, and DNA visualization. This approach provides insights into the molecular consequences of nucleolar stress induction and its role in the DNA damage response pathways *in vivo*.

BEFORE YOU BEGIN

The protocol below describes the specific steps for using the Comet Assay on the cells of the wing imaginal discs of *Drosophila*. This protocol can also be adapted for other cell types present in *Drosophila* larvae by adjusting timings and enzyme concentrations in the disaggregation steps.

Before proceeding with the protocol, you need to prepare the different solutions, the low melting point (LMP) agarose aliquots, and the slides pre-coated with Normal Melting Point (NMP) agarose – all the material can be prepared one or two days before the application of the comet assay. You also need to manage the timing of fly husbandry and development properly.

Solution preparation

Timing: 2 h

1. Prepare the 10x Ringer's Solution and store it at 4°C – it should be stable for at least 3 months.
2. Prepare the Lysis Solution and store it at 4°C – it should be stable for 6 months.

⚠ CRITICAL: Add 10% Triton X-100 to the Lysis Solution to 1% final concentration and mix well before using.

3. Prepare 10x Electrophoresis Stock Solution and store it at 4°C.
4. Prepare the Neutralizing Solution and store it at room temperature (RT) (20°C–25°C).

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Agarose preparation and pre-coating of slides

Timing: 30 min

5. Prepare the agarose:
   a. Prepare Low Melting Point (LMP) agarose 1.5% in 1x PBS.
      i. Prepare aliquots of 1.5 mL and store them at 4°C.

   Note: They will remain stable for at least 6 months.

      ii. When ready to use it, melt the agarose in the Eppendorf using a Thermoblock at 65°C, then cool it down to 37°C.
   b. Prepare Normal Melting Point (NMP) 1% agarose solution in distilled water.
      i. Melt the agarose in boiling water (100°C).
      ii. Let the agarose cool down to 50°C–60°C in a water bath.
      iii. Use the agarose to pre-coat the slides. About 1 mL is sufficient to coat 1 slide.

CAUTION: Be mindful of the high temperatures when boiling water and handling the melted agarose.

6. Pre-coat the agarose slides:
   a. Quickly dip the microscope slides into a beaker containing the agarose solution, kept at 50°C–60°C.
   b. Wipe clean the back of the slide and mark the front to indicate where the coating is – it should be a few mm thick.
   c. Dry the slides overnight (16–18h) at RT (20°C–25°C).

   Note: The dried coating can be seen against the light as a slightly opaque patina. To make the coating more uniform, ensure the slides are clean and do not dip them in agar for too long. Coated slides can be stored at RT for at least 12 months (20°C–25°C).

Drosophila expression system

In *Drosophila*, ubiquitous or tissue-specific expression is achieved with the UAS-Gal4 system, where a promoter drives Gal4 to activate the gene of interest (GOI).[1]

Reducing the expression of essential genes like NOC1 ubiquitously can impair animal growth due to the triggering of cell death.[2] This phenomenon is evident in the wing imaginal disc cells, where NOC1 was reduced using the *rotund* promoter (Figure 1A), leading to apoptosis visualized by the Caspase 3 staining (Figures 1B and 1C). To avoid developmental lethality, we generate animals in which the ubiquitous reduction of NOC1 is temporally regulated. In this protocol, we controlled the timing of the expression of NOC1-RNAi using the Gal4-Gal80 ^{ts} system, where Gal80 inhibits Gal4 at the permissive temperature of 18°C. When the temperature is raised to 30°C, Gal80 becomes inactivated, allowing temporal expression of Gal4.[3] With this approach, we bypassed the developmental lethality of *Actin>NOC1-RNAi* animals,[2] and we temporarily reduced NOC1 in all tissues at 5 days after eggs laying (AEL), when the animals reached approximately the second instar larval stage. Gal80 is inactivated for 60 h at 30°C, allowing Gal4 to be active and to induce NOC1-RNAi.

Note: Using a ubiquitous promoter such as *actin-Gal4* allows for a thorough evaluation of DNA damage across all cells in the imaginal discs. In contrast, *rotund-Gal4* restricts the expression of the *NOC1-RNAi* transgene to the pouch region of the disc. This localized expression is unsuitable for the comet assay, as dissociating the entire disc will include wild-type cells not expressing the transgene (Figures 1B and 1C).

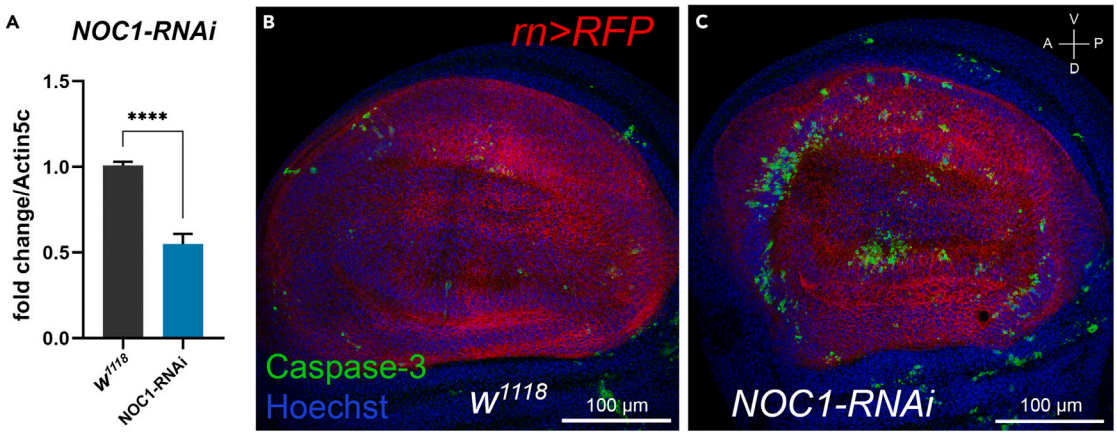


Figure 1. Apoptosis in NOC1 downregulated cells of the wing imaginal discs

(A) RT-qPCR showing NOC1 downregulation in wing imaginal discs. The *p*-value was calculated from the Student *t*-test (****$p < 0.0001$).

(B and C) Photographs of the wing pouch showing apoptosis using anti-cleaved caspase 3 antibody (GFP). The rotund compartment is marked by RFP expression. Nuclei are stained with Hoechst.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-cleaved caspase-3, 1:400	Cell Signaling Technology	Cat# 9661
Anti-rabbit Alexa 555, 1:1,000	Invitrogen	Cat# 21430
Chemicals, peptides, and recombinant proteins		
NaCl	Sigma-Aldrich	Cat# S9888
KCl	Carlo Erba	Cat# 471177
$CaCl_2$	Sigma-Aldrich	Cat# C1016
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Cat# ED2SS
Tris(hydroxymethyl)aminomethane (Tris base)	Fisher BioReagents	Cat# 10376743
Triton X-100	Sigma Life Sciences	Cat# T8532
NaOH pellets	Thermo Scientific Acros	Cat# 10502731
Na_2EDTA	Chem-Lab	Cat# CL00.0503
HCl	Carlo Erba	Cat# 403872
Tris-acetate-EDTA (TAE) 10x	Fisher BioReagents	Cat# 10041223
ddH_2O	N/A	N/A
PBS: Ca^{2+} and Mg^{2+}-free phosphate-buffered saline 10x	Gibco	Cat# 70011044
Hoechst 33342	Life Technologies	Cat# C10338
Trypan blue 0.4%	Corning	Cat# 15393661
Normal melting point (NMP) agarose	Fisher BioReagents	Cat# 10366603
Low melting point (LMP) agarose	Bio-Rad	Cat# 1613113
Collagenase type 4, powder	Cell Signaling Technology	Cat# 44204
TrypLE 1X	Gibco	Cat# 12604013
Fetal bovine serum (FBS)	SAFC Biosciences	Cat# 12103C
Hydrogen peroxide (H_2O_2) 30%	Fisher Chemical	Cat# 10687022
Agar	B&V	https://www.agar.com/it/insectagar
Yellow corn flour	Any brand	N/A
Sugar/Sucrose	Any brand	N/A
Yeast extract	Genesee	Cat# 789126
Fresh yeast	Lievital	Cat# BP033
Molasses	El Granero Integral	900 g
Nipagine/Tegosept	Genesee	Cat# 789063

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Propionic acid	Thermo Scientific Chemicals	Cat# 149300010
Experimental models: Organisms/strains		
Drosophila strain: *rotund-Gal4*	Bellosta's lab	N/A
Drosophila strain: *actin-Gal4, UAS-GFP/GlaBla; tubulin-Gal80^{TS}*	Bellosta's lab	N/A
Drosophila strain: *w^{1118} (wild type)*	Vienna Drosophila Resource Center (VDRC)	v60000
Drosophila strain: *UAS-NOC1^{2}*	Bloomington Drosophila Stock Center (BDRC)	B25992
Software and algorithms		
CometScore 2.0	rexhoover.com	http://rexhoover.com/index.php?id=cometscore
ImageJ/FiJi	ImageJ	https://imagej.nih.gov/ij/
Prism 10	GraphPad	https://www.graphpad.com/
Other		
Fine-tipped forceps INOX	Electron Microscopy Sciences (EMS)	Dumont #5
Optical stereomicroscope (for larvae dissection)	Nikon optical microscope + light source	Nikon SMZ745 + Photonic Optics PL3000
Frosted microscope slides 76 × 26 mm	Excell	Cat# 59175
Square coverslips 22 × 22 mm	Kristal	Cat# 59134W
Long coverslips 24 × 50 mm	Kristal	Cat# 59139W
Embryo dishes 40 × 40 mm	Electron Microscopy Sciences (EMS)	Cat# 70543-45
Thermoblock	Labnet International	Accublock digital dry bath
Water bath	Biobase	Cat# SY-1L6H
pH meter	Fisherbrand	Accumet AB150 Cat# 12880633
Bürker chamber	HBG Germany	N/A
4°C centrifuge	Eppendorf	Cat# 5424R
40 µm nylon mesh	Falcon	Cat# 352340
Horizonal Electrophoresis System + power supply	Bio-Rad	Cat# 1704403 Cat# 1645050
Optical microscope (for cell counting)	Leica optical microscope with DFC420 C camera	Leica DMIL LED
Fluorescence microscope with camera	X-CITE 120 fluorescence lamp and Zeiss Axiocam 305 monochromatic camera	Zeiss Axio Imager M2
Parafilm	Merck	Cat# HS234526B

Alternatives: Other fluorescent dyes, such as ethidium bromide, DAPI, and SYBR Green, can stain DNA in the comet assay. Any optical or fluorescence microscope with the appropriate excitation filter can be used for imaging. Other software, such as ImageJ plugin OpenComet or Comet Analyser software, can be used to detect and quantify the comets.

CAUTION: Ethidium bromide is a mutagen and potential carcinogen and, as well as DAPI, requires careful handling.

MATERIALS AND EQUIPMENT

Ringer's solution 10x

Reagent	Final concentration	Amount
NaCl	1.3 M	18.993 g
KCl	350 mM	6.523 g
$CaCl_2$	20 mM	554.9 g
NaOH	N/A	N/A
HCl	N/A	N/A
ddH_2O	N/A	N/A
Total	N/A	**250 mL**

Dissolve the salts using autoclaved ddH_2O, then adjust the pH to 7.4 using NaOH (1 M) and HCl (1 M); this solution should remain stable for at least 3 months when stored at 4°C. Dilute the 10x Ringer's solution at the working final concentration 1x using ddH_2O.

Lysis solution		
Reagent	Final concentration	Amount
NaCl	2.5 M	73.03 g
EDTA 0.5 M, pH 8.0	100 mM	100 mL
Tris Base 1 M, pH 10	10 mM	5 mL
Triton X-100, 10% in water	1%	50 mL
ddH_2O	–	N/A
Total	–	**500 mL**

This solution is stable for at least 6 months without Triton X-100 when stored at 4°C.

⚠ CRITICAL: Add Triton X-100 to 1% final concentration before using the solution and mix well for about 1 min until the solution is homogeneous.

CAUTION: NaCl and EDTA cause eye damage. Tris Base causes skin irritation and severe eye damage and may cause respiratory irritation. Triton X-100 is harmful when swallowed and causes skin irritation and severe eye damage.

Alkaline electrophoresis running solution		
Reagent	Final concentration	Amount
NaOH pellets	0.3 M	11.999 g
Na_2EDTA	1 mM	372.24 mg
ddH_2O	N/A	N/A
Total	N/A	**1 L**

The electrophoresis solution should be prepared fresh and stable for 7 days at 4°C. Alternatively, you can prepare 10x NaOH and 10x Na_2EDTA stock solutions using autoclaved ddH_2O, to be stored at 4°C and dilute to 1x concentration on the day of the experiment. Check that the solution has pH > 13 and use it cold.

CAUTION: Be careful when preparing NaOH solutions, as heat is released. NaOH causes severe skin burns and eye damage. Na_2EDTA causes severe eye damage.

Single-cell disaggregation solution		
Reagent	Final concentration	Amount
Collagenase, 50 mg/mL in Ringer's solution	1 mg/mL	20 µL
TrypLE 1x	N/A	980 µL
Total	N/A	**1 mL**

Collagenase solution can be prepared and stored at −20°C for up to 1 year. Prepare the final solution immediately before using it. We recommend preparing about 200 µL for each sample.

Neutralizing solution		
Reagent	Final concentration	Amount
Tris Base 1 M, pH 10	0.4 M	200 mL
ddH_2O	N/A	300 mL
Total	**0.4 M**	**500 mL**

Before bringing it to volume, adjust the pH to 7.5 using 1 M HCl 1 M. Store at 4°C and use when still cold.

CAUTION: Tris Base and HCl can cause skin irritation and severe eye damage and may cause respiratory irritation.

DNA staining solution		
Reagent	Final concentration	Amount
Hoechst 33342, 20 mg/mL	1 μg/mL	2.5 μL
TAE Buffer 1x	N/A	47.5 mL
Total	N/A	**50 mL**

Prepare fresh before using. We recommend preparing about 200 μL for each sample.

CAUTION: Hoechst should be treated as a possible mutagen.

Fly food		
Reagent	Final concentration	Amount
Agar	8 g/L	8 g
Corn flour	75 g/L	75 g
Sugar	50 g/L	50 g
Yeast extract	30 g/L	30 g
Fresh yeast	50 g/L	50 g
Molasses	30 mL/L	30 mL
Nipagine, 10% in ethanol	10 mL/L	10 mL
Propionic acid	10 mL/L	10 mL
ddH_2O	N/A	1 L
Total	N/A	**1 L**

Dissolve the ingredients in ddH_2O and bring to a boil. Cook at 90°C for 1 h, then cool down to 65°C. Add Nipagine and propionic acid and pour into vials or bottles. 1 L can fill 100 vials or about 50 bottles.

⚠ CRITICAL: When preparing fly food, ensure that both Nipagine and propionic acid are added after the temperature of the food has cooled down to avoid inactivation of these anti-mold components.

Ethylenediaminetetraacetic acid (EDTA), disodium salt, sodium chloride (NaCl), and hydrochloric acid (HCl) cause severe eye damage (H319).

Sodium hydroxide (NaOH) and H_2O_2 cause severe skin burns and eye damage (H314).

TRIS Base causes skin irritation (H315), severe eye damage (H319), and may cause respiratory irritation (H335).

Triton X-100 is considered harmful if swallowed (H302), causes skin irritation (H315), and causes severe eye damage (H319).

Trypan blue may cause cancer (H350) and is suspected to impair fertility or harm development (H361).

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Hoechst binds to nucleic acids and should be treated as a potential mutagen.

For handling the harmful reagents cited above, it is recommended to wear appropriate PPE (personal protection equipment), such as skin and eye/face protection, and to handle them under a chemical hood.

STEP-BY-STEP METHOD DETAILS

The protocol below describes the specific steps to assess and quantify the effect of DNA damage by comet assay on cells of the wing imaginal discs of *Drosophila*'s third instar larvae, caused by genes that induce nucleolar stress and stress reagents such as H_2O_2.[4]

Larvae development

Timing: 7.5 days

1. Egg deposition (1 day):
   a. Cross 20 females with 10 males of the genotype of interest or control.
   b. Leave parents to lay eggs for 24 h at 18°C and then transfer into a new vial daily.
2. Larval development (5 days):
   a. Leave animals to develop at 18°C for 5 days to reach the second instar.

   Note: At this temperature, Gal80 is active and blocks the expression of Gal4.

3. Gal4 induction (60 h):
   a. Transfer the vials containing the larvae to the restrictive temperature of 30°C for 60 h.

   Note: This allows Gal80 to be degraded and Gal4 to express the GOI. At this point, larvae reach the third instar, and the wing imaginal discs can be dissected.

Larvae dissection and H_2O_2 treatment

Timing: at least 30 min for genotype

4. Larvae collection:
   a. Add 20–30 mL of RT (20°C–25°C) ddH_2O into the vial and pour the contents into a petri dish.

   Note: Wash the larvae extensively to clean them from the food.

   b. Transfer at least 20 larvae in a glass embryo dish filled with cold (4°C) 1x Ringer's Solution.

   Note: The glass dish can be substituted with a small 3.5 mL plastic petri dish.

5. Larvae dissection:
   a. Under an optical microscope, dissect the larvae. Collect at least 30 wing imaginal discs (see Video S1).

   Note: 30–40 discs are the optimal condition.

6. H_2O_2 treatment (positive control):
   a. Incubate wild-type discs in 200 μL of H_2O_2 (100 μM) in PBS[5] for 15 min, then proceed with the disaggregation.

Video S1 shows how to dissect Drosophila third instar larvae to expose the imaginal discs.

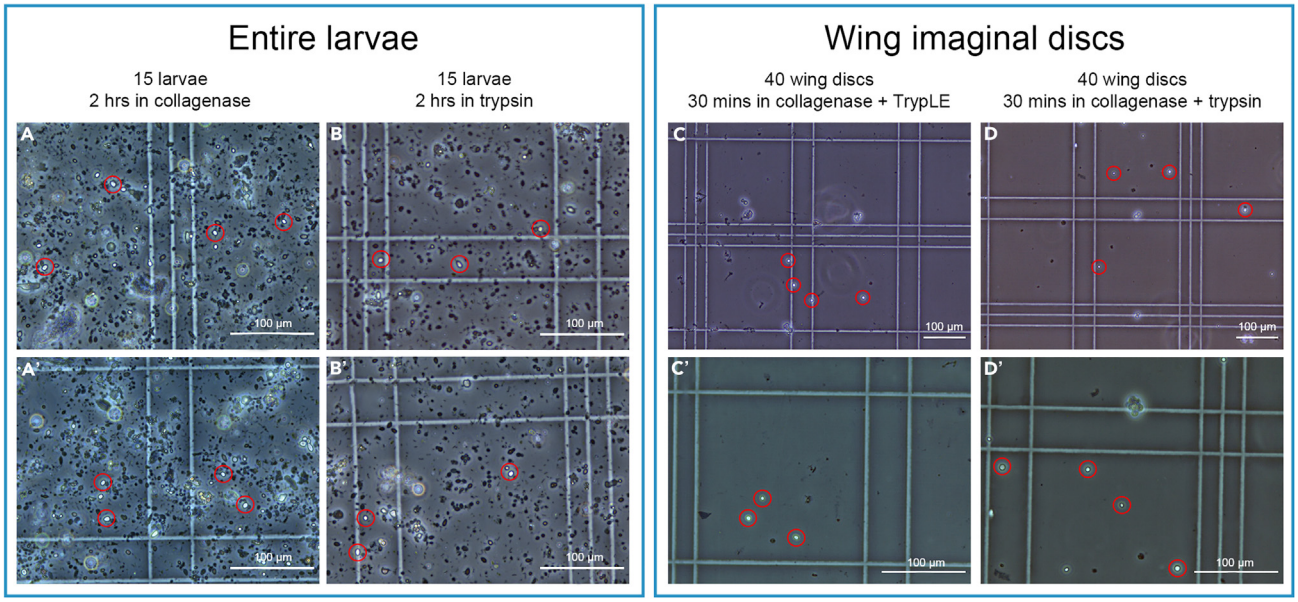


Figure 2. Optimization of the disaggregation process

Photos of cell suspension after enzymatic disaggregation stained with trypan blue.

(A and B) Cells from whole larvae, treated with 1 mg/mL collagenase (A-A′) or 0.25% Trypsin EDTA (B-B′). The suspension presents many debris and dead cells, rendering them unsuitable for the comet assay.

(C and D) Cells from wing imaginal discs, treated with 1 mg/mL collagenase in 1x TrypLE (C-C′) or 1 mg/mL collagenase in 0.25% Trypsin EDTA (D-D′). Since less debris and death cells are present, the 1 mg/mL collagenase 1x TrypLE solution is ideal for the comet assay. Cells are highlighted with red circles. Scale bars: 100 µm (C and D were taken at a lower magnification).

Enzymatic disaggregation and cell count

Timing: 1 h

During this step, we proceed with the enzymatic disaggregation of the collected tissues to obtain a single-cell suspension (Figure 2). We also execute a rapid cell count to establish the vital/dead cells ratio using the Bürker chamber and Trypan blue staining.

7. Enzymatic disaggregation of the tissues (45 min):
   a. From the embryo dish containing the wing imaginal discs, carefully remove the Ringer's Solution with a pipette.
   b. Add 200 µL of 1 mg/mL collagenase in 1x TrypLE to start the enzymatic disaggregation process.

⚠ CRITICAL: Wing discs should not be moved from the dish to reduce cell loss. To ensure an adequate number of discs and avoid aspirating them, perform this step under the microscope.

   c. Cover the embryo dishes with parafilm and incubate them in a water bath at 37°C. Shake them gently every 5 min for 30 min.
   d. Gently pipette up and down the tissues for 10 times with a 1000 µL pipette to favor the disaggregation process.
   e. Add 20 µL of stock solution FBS (final concentration 10%) to neutralize the enzymes.

⚠ CRITICAL: Perform this step under the microscope to ensure that tissues are completely disaggregated.

f. Filter the obtained suspension through a 40 μm nylon mesh into a 1.5 mL Eppendorf.
g. Centrifuge at 4 °C at 500g/rcf for 4 min.
h. Discard the supernatant and gently resuspend the pellet in 200 μL of 1X PBS.
i. Centrifugate at 4 °C at 500g/rcf for 4 min
j. Discard the supernatant and resuspend the pellet in 85 μL 1X PBS.

8. Cell count using the Bürker chamber (15 min):
   a. In an Eppendorf, mix 5 μL of the cellular suspension with 5 μL of Trypan blue (1:1 ratio).
   b. Add 2 μL of the mix in a Bürker chamber.
   c. Under a light microscope, assess cell vitality by counting the number of cells stained with Trypan Blue, which indicates dying or dead cells, and compare it to the total number of cells.
   d. Estimate the vital/dead cell ratio, which should be at least > 80% to continue.

⚠ CRITICAL: Cell number and viability can be influenced by several factors during the dissection and isolation steps. Be sure to pipette gently, handle the suspension with care, and keep it cold during the whole process. More details are specified in the troubleshooting section.

Embedding of the cells in agar on slides, lysis, and electrophoresis run

Timing: 2 h

In this step, we embed the single cells in the LMP agarose. The embedded cells are then lysed and subjected to electrophoresis run using the precoated slides.

Note: For this step, remember to melt one of the LMP agarose aliquots prepared previously and then let it cool in a Thermoblock at 37°C. At this point, we need to have the pre-coated slides ready to use. Remember to label the slides with the respective genotype/condition. Work quickly to avoid cell damage or loss.

9. Embedding of the cells in LMP agarose on the pre-coated slides:
   a. Mix 80 μL of the cell suspension with 80 μL of the melted 1.5% LMP agarose (1:1 ratio).

Note: At this stage, the suspension should contain about 3,000-7,000 live cells, but lower numbers are still feasible, provided that you can visualize and score at least 50-100 cells at the end of the protocol.

   b. On a pre-coated slide, spot two 70 μL drops of the mix and rapidly cover both with square coverslips – in this way, we have two replicates of the same genotype on a single slide.
   c. Keep the slides horizontally at 4°C for at least 15–20 min to let the agar solidify completely.
   d. Carefully remove the coverslips from the solidified agar by gently sliding them and discarding them (see Video S2).

⚠ CRITICAL: If the agar is not completely solidified and coverslips do not easily slide off, keep the slides at 4°C for another 5–10 min. If the agar gets damaged in some spots or at the edges the slide can still be analyzed, provided that the number of embedded cells is high enough.

10. Incubate the slides in the Lysis Solution for 1 h at 4°C, horizontally in a small plastic box, or vertically in a Coplin jar.

⚠ CRITICAL: Before use, add the Triton X-100 to the Lysis Solution.

11. Electrophoresis run:

⚠ CRITICAL: To preserve DNA from degradation/damage and to obtain a better image quality, perform the next steps at 4°C and avoid direct ambient light; for example, work in a cold room with dim light.

a. Fill the chamber with the Electrophoresis Solution, then submerge the slides for 10 min.
b. Start the electrophoresis run at 0.7 V/cm (300 mA/25 V) for 20 min.
c. Remove the slides from the electrophoresis chamber and place them horizontally in a small plastic box, or vertically in a Coplin jar.
d. To neutralize the gel on the slides, execute 3 washes of 5 min each, using cold (4°C) Neutralizing Solution.

Pause point: Leave the slides to dry overnight (16–18 h) at 4°C, placed horizontally in a covered plastic box to avoid direct light.

Video S2 shows how to embed cells in agarose slides before electrophoresis.

Staining, visualization, and quantification

Timing: 1.5 h

During this step, we proceed with staining the slides and visualizing the results.

12. Staining:
    a. Prepare fresh DNA staining solution and incubate the slides for 30 min, horizontally in a small plastic box, or vertically in a Coplin jar.
    b. Execute 3 washes of 2 min each with distilled water to remove the excess dye.
    c. Cover the gel on the slides using a long coverslip.

Note: Performing this step immediately after the final wash prevents the gel from drying, resulting in fewer air bubbles and a better outcome during the acquisition of the images.

13. Visualization and image acquisition:
    a. Take 30-50 pictures for each genotype (or the appropriate number to perform a statistic analysis).

Note: We use a Zeiss Axio Imager M2 with a Zeiss Axiocam 305 monochromatic camera, and take pictures at 2464 × 2056 pixels of resolution. Any microscope with the appropriate filters and resolution is feasible for the visualization.

EXPECTED OUTCOMES

Comet visualization allows the observation of stained DNA either as dots (representing intact DNA) or as comets (indicating damaged DNA), as shown in Figure 3.

QUANTIFICATION AND STATISTICAL ANALYSIS

Using the Software CometScore 2.0 analyze the images acquired previously following these steps.

1. Launch the software CometScore 2.0 and select the folder containing the images to be analyzed – these can be in .bmp, .jpeg, .jpg or .png format.

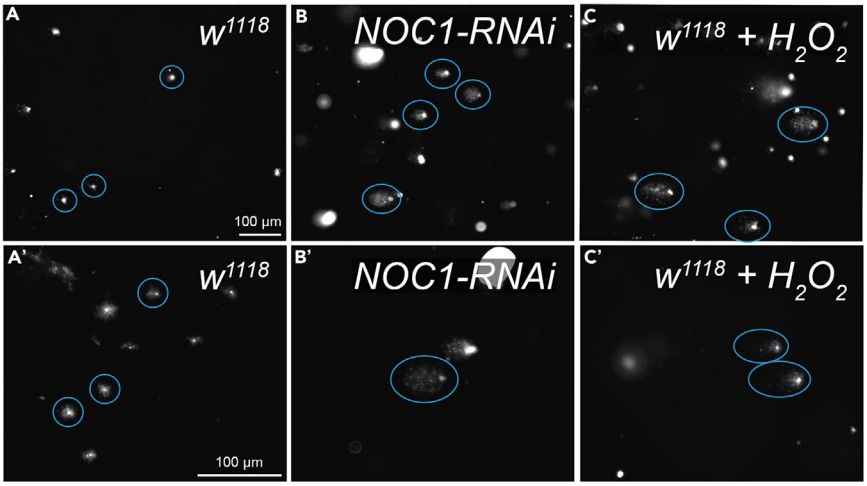


Figure 3. Comets visualization

Photos of DNA from cells after electrophoresis were acquired with a Zeiss Axio Imager fluorescent microscope with 20X (A–C) or 40X (A′–C′) objectives. Representative cells are highlighted with blue circles. (A): Wild-type (negative control). (B): *NOC1-RNAi*. (C): Wild-type cells treated with 100 µm of H_2O_2 (positive control). DNA in wild-type cells (A) is visible as a dot, and in (B) and (C), it is visible as comets, indicating DNA breaks. Scale bars: 100 µm.

Note: Other file formats can be converted in one of the above supported formats using Fiji/ImageJ.

2. Follow the instructions in the program and first select the appropriate cutoff to correct the background signal – this allows you to visualize better and select the single cells with comets.
3. Select the single comets using 3 different methods:
   a. Manual scoring – the one we used.
   b. Semi-automated scoring.
   c. Automated scoring.

Note: After automated scoring, manually eliminate the overlapping cells or duplicates from different images from the raw data.

4. The software produces different outputs (Figure 4):
   a. Spreadsheets containing the raw data and histograms of the measurements of all the selected comets. From this file, it is possible to analyze the DNA percentage in the heads and in the tails of the comets (which is the intensity of the signal in each of these regions as a percentage of the intensity of the whole comet), the tail length of the comets, and the tail moment. The tail moment is calculated as the product between the tail length and the percentage of DNA in the tail.
   b. Cropped images of every analyzed comet were analyzed with a graphical visualization of comet intensity and length.
5. Open the spreadsheets of the raw data with Excel and select the following data: DNA percentage in the heads and the tails of the comets, the tail length of the comets, and the tail moment.
6. Proceed with the statistical analysis in Excel or transfer the data to other software.

Note: We used GraphPad Prism 10 and presented data using violin graphs.

Note: selecting between 50 and 100 comets per genotype will provide a robust sample size for meaningful statistical analysis. This range should capture variability within each genotype while ensuring that any observed differences between genotypes are statistically significant.

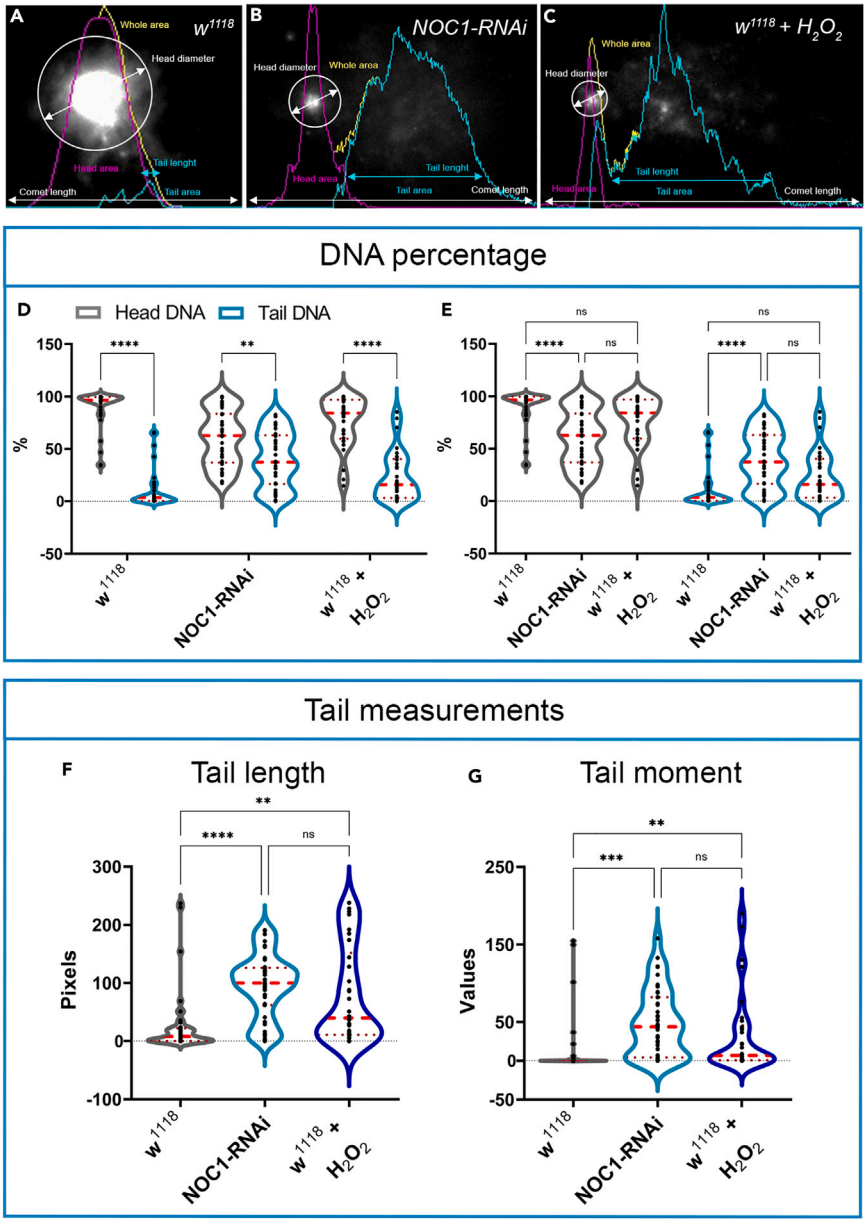


Figure 4. Comet assay quantification

(A–C) Output photos of single comets analyzed with the CometScore 2.0 software, which calculates the length and area of the head and tail of the comet and the fluorescence intensity of these regions to estimate the amount of DNA present.

(D and E) Graphs reporting the quantification of DNA percentage detected in heads or tails. The two graphs report the same results but are distributed in two ways to highlight better the different comparisons that can be obtained. Statistical analysis: two-way ANOVA ($n > 35$). (D): DNA percentage differences between heads and tails in the same sample. While control cells have most of their DNA in the heads, in both *NOC1-RNAi* cells and cells treated with H_2O_2 the DNA is more homogeneously distributed among heads and tails. (E): Difference between DNA percentage in heads and tails among different samples. *NOC1-RNAi* cells and cells treated with H_2O_2 present less DNA amount in the heads and more DNA in the tails compared to the control. These data indicate that *NOC1-RNAi* and H_2O_2-treated cells have more DNA migration, so damage is present. DNA from cells treated with H_2O_2 seems overall less damaged than *NOC1-RNAi* cells; this could derive from a less precise quantification due to the high toxicity of H_2O_2, leading to a higher percentage of dead cells and a less effective DNA staining.

(F and G) Graphs reporting the quantification of tail parameters. Statistical analysis: Kruskal-Wallis non-parametric test ($n > 35$). (F): Tail length, reported in pixels. *NOC1-RNAi* and H_2O_2-treated cells have longer tails than the control,

Figure 4. *Continued*
indicating a higher DNA migration and, therefore, more damage. (G): Tail moment is the product between tail length and DNA percentage in cells. This parameter allows us to evaluate the overall "weight" of tails since it combines the length and the amount of DNA present. *NOC1-RNAi* and H_2O_2-treated cells have a higher tail moment than control cells, indicating that their tails are more significant than the control ones and again suggesting the presence of DNA damage. Graphs were produced and analyzed with the GraphPad Prism 2.0 software. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The number of dots represents the number of comets analyzed in each sample. Quantification was performed with CometScore 2.0, plotted, and analyzed with GraphPad Prism 10. Violin plots allow for more accurate visualization of a large sample size distribution. Overall, the data indicates that both NOC1 downregulation and treatment with H_2O_2 induce comets with longer tails and increased DNA percentage in the tail compared to the control, suggesting the presence of DNA damage.

Be also sure to select the appropriate statistical test based on the distribution of your samples and the number of conditions you are analyzing. For example, two-way ANOVA can be used to evaluate how the percentage of DNA intensity changes based on the genotype and the localization (head or tail) (Figures 4D and 4E). Instead, student's t-test or one-way ANOVA (or corresponding non-parametrical tests) can be used to compare the difference in tail length and tail moment between two or more conditions (Figures 4F and 4G). Increasing the sample size can reduce the error margin, which helps draw more reliable conclusions.

Advantages

The comet assay is an established and widely used protocol for analyzing the presence of double-strand breaks in single cells; however, its use in *Drosophila* tissues is still uncommon, and there are no systematic protocols describing its application in specific larval tissues; moreover, methods requiring the isolation of single cells from larval tissues are not often described or easy to apply. In this work, we illustrate the steps to perform this assay in cells extracted from the wing imaginal discs, dissected from *Drosophila* larvae. The wing disc is a widely used tissue for the study of development, proliferation, stress and regeneration. This work, therefore, provides an additional assay for investigating the effects of irradiation and other damages - such as nucleolar stress - on DNA integrity in these cells, allowing these results to complement and integrate other established methods of DNA damage investigation, such as the visualization of apoptosis or of histone γH2Av phosphorylation. By adapting the concentration and the timings of enzymatic digestion, this protocol has also the potential to be applied to other larval tissues and imaginal discs, expanding its utility for other types of *Drosophila* studies.

LIMITATIONS

Tissue collection and disaggregation is the most delicate and challenging step. We experimented with several protocols using different enzymes and varying disaggregation times.[6] Mechanical disaggregation was also attempted but did not yield optimal results. Initially, we tested the protocols for disaggregation on whole larvae and then used it on the wing imaginal discs (Figure 2).

When dissecting multiple genotypes, collecting wing imaginal discs can become a bottleneck due to the time-intensive process. Having more people dissect simultaneously can help reduce the interval between dissection and enzymatic disaggregation, minimizing the risk of tissue deterioration. Furthermore, the tissues must be kept fresh and cannot be frozen since they are highly susceptible to deterioration.

A known limitation of the comet assay technique is that dead cells in the cell suspension can also produce comets, which may falsely indicate DNA damage. So, some of the final comets could derive from apoptotic/dead cells rather than comet DNA damage. To address this, limiting the proportion of dead cells in the single-cell suspension to no more than 20% is important, as previous literature recommends.[7,8]

Optimizing image acquisition conditions is also crucial for obtaining accurate results. Individual cells should be clearly distinguishable to prevent the quantification of false positives or cell aggregates. Ensuring sharp focus and proper contrast will help achieve reliable measurements in the analysis. Maintain consistency while moving the microscope field of view and z-plane to avoid capturing comets more than once. During quantification, be mindful of excluding doublets and unclear/background signals.

TROUBLESHOOTING

Problem 1

High levels of DNA damage can be observed in all cells, even in the negative control (quantification and statistical analysis step).

Potential solution

During the various steps of the protocol, the cells can be subjected to the induction of DNA damage, i.e., from the collection of the wing imaginal discs during dissections or disaggregation (steps 5 and 7). These steps, including pipetting and centrifuging, must be performed gently to avoid damaging the cells. Do not freeze or subject the discs to a temperature higher than 37°C; immediately proceed with the disaggregation step after collection from the larvae. Avoid exceeding the incubation time with the enzymes and neutralize their activity by adding FBS.

Problem 2

Too few or no live cells are present in the cell suspension ([enzymatic disaggregation and cell count] step) or in the imaging field (quantification and statistical analysis step), which does not allow quantification.

Potential solution

Overall, cell numbers can be increased by using more imaginal discs (steps 4 and 5). Dissections should be performed quickly to minimize tissue exposure to external damage. The extracted cells can be easily damaged or lost during the successive steps of the protocol, in particular when preparing the cell suspension (step 7). Be sure to work quickly, handle the cell suspension gently, and maintain it on ice. After the centrifuges, when it's necessary to discard the supernatant and maintain the pellet (steps 7h and 7j), be very careful not to lift or discard the pellet, which could lead to lose all the extracted cells. During the filtration step (step 7f), some of the cells could be lost due to the mesh static charge – to avoid this, it could be useful to coat the filter with 1% BSA, which can create a film and prevent the cells from sticking to the mesh.

Problem 3

High background signal on slides, rendering comet evaluation difficult (quantification and statistical analysis step).

Potential solution

High background could be caused by dirt on slides or in the agarose. In this case, clean microscope slides before pre-coating and ensure that agarose aliquots are clean and well-melted before using them (step 6 during preparation). If the background is caused by tissue debris, filter the dissociated cells (step 7f) to eliminate tissue clumps and cell aggregates.

Problem 4

Low fluorescence signal, rendering cell visualization a difficult field ([staining, visualization, and quantification] step).

Potential solution

Hoechst concentration and incubation timing can be increased to improve the fluorescent signal (step 12). Other dyes for DNA visualization can be used, such as SYBR Safe and similar.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paola Bellosta (paola.bellosta@unitn.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Valeria Manara (valeria.manara@unitn.it).

Materials availability

This study did not generate any specific material/reagent.

Data and code availability

This study did not generate any specific material/reagent.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.M., M.P., A.V., and E.B.; investigation, V.M., M.P., A.V., E.B., and A.V.C.; writing – original draft, V.M., M.P., and P.B.; writing – review and editing, V.M., M.P., and P.B.; funding acquisition, P.B.; supervision, V.M. and P.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used ChapGPT to enhance the clarity and fluency of the English text. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the publication's content.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103590.

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