Please cite this article as: Clementi, E. *et al.* (2021). Measurement of DNA Damage Using the Neutral Comet Assay in Cultured Cells. *Bio-protocol* 11(22): e4226. DOI: 10.21769/BioProtoc.4226.

bio-protocol www.bio-protocol.org/e4226 Bio-protocol 11(22): e4226. DOI:10.21769/BioProtoc.4226

Measurement of DNA Damage Using the Neutral Comet Assay in Cultured Cells

Elena Clementi, Zuzana Garajova and Enni Markkanen*

Institute of Veterinary Pharmacology and Toxicology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

*For correspondence: enni.markkanen@vetpharm.uzh.ch

[Abstract] Maintenance of DNA integrity is of pivotal importance for cells to circumvent detrimental processes that can ultimately lead to the development of various diseases. In the face of a plethora of endogenous and exogenous DNA damaging agents, cells have evolved a variety of DNA repair mechanisms that are responsible for safeguarding genetic integrity. Given the relevance of DNA damage and its repair for disease pathogenesis, measuring them is of considerable interest, and the comet assay is a widely used method for this. Cells treated with DNA damaging agents are embedded into a thin layer of agarose on top of a microscope slide. Subsequent lysis removes all protein and lipid components to leave 'nucleoids' consisting of naked DNA remaining in the agarose. These nucleoids are then subjected to electrophoresis, whereby the negatively charged DNA migrates towards the anode depending on its degree of fragmentation, creating shapes resembling comets, which can be visualized and analysed by fluorescence microscopy. The comet assay can be adapted to assess a wide variety of genotoxins and repair kinetics, and both DNA single-strand and double-strand breaks. In this protocol, we describe in detail how to perform the neutral comet assay to assess double-strand breaks and their repair using cultured human cell lines. We describe the workflow for assessing the amount of DNA damage generated by ionizing radiation or present endogenously in the cells, and how to assess the repair kinetics after such an insult. The procedure described herein is easy to follow and cost-effective.

Keywords: DNA damage, DNA repair, DNA repair kinetics, Genotoxic agents, Oxidative stress, Reactive oxygen species

[Background] Maintaining DNA integrity is a pivotal prerequisite for cells to ensure that all physiological processes function immaculately. Cells are constantly exposed to a plethora of exogenous and endogenous agents that can damage their DNA. Examples of exogenous noxious agents include ultraviolet light, ionizing radiation, and reactive chemical compounds, while endogenous damage can arise for example due to reactive oxygen- and nitrogen species derived from cellular metabolism (van Loon *et al.*, 2010). If left unrepaired, damage to DNA can lead to mutations, which can, in turn, alter the functionality of the affected DNA. This potentially results in altered protein abundance or activity, thereby giving rise to processes such as cancer, neurodegeneration, and ageing (Hoeijmakers, 2009; Markkanen, 2017). To counteract the deleterious effects of DNA damage, cells have evolved a series of intricate DNA repair mechanisms that detect and repair such insults (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). Given the relevance of DNA damage for disease, it is of considerable interest to be able to measure levels of DNA damage that are induced by exposure of cells to particular agents, or to

assess whether the repair kinetics of such damage is altered through specific treatments or genetic backgrounds. Methods to measure DNA damage and repair range from PCR-based methods and enzyme-linked immunosorbent assays, all the way to more elaborate next-generation sequencing based methods (Li and Sancar, 2020). Among this multitude of assays, the comet assay is a widely used method to measure both the amount of DNA damage as well as its repair in cells (Collins, 2014; Olive, 2009). For this, cells are treated with DNA damaging agents and embedded into a thin layer of agarose on top of a microscope slide. Subsequent lysis removes all protein and lipid components to leave the so-called 'nucleoids,' which consist of naked DNA remaining in the agarose. These nucleoids are then subjected to electrophoresis, whereby the negatively charged DNA migrates towards the anode. The migrating DNA, depending on its degree of fragmentation, creates shapes its degree of fragmentation and creates shapes resembling comets, which can subsequently be visualized and analysed by fluorescence microscopy. The comet assay can be adapted to assess a wide variety of genotoxins and repair kinetics, and both single-strand DNA breaks as well as double-strand breaks. In this protocol, we describe in detail how to perform the neutral comet assay to assess double-strand breaks and their repair in cultured human cell lines. This exact protocol was used in our recent publication (Clementi *et al.*, 2020). For a detailed description of the alkaline comet assay that can be used to detect single-strand DNA breaks, please refer to the separate protocol that is published in Bio-protocol (Clementi *et al.*, 2021).

Materials and Reagents

1. Superfrost microscopy slides (Superfrost Plus; ThermoScientic, Menzel-Gläser, catalog number: J1800AMNZ), store at room temperature
2. Square cover slips (Coverslips 22 × 50 mm; ThermoScientic, Menzel-Gläser, catalog number: MA062210), store at room temperature
3. 24-well cell culture plates (TPP, catalog number: 92024), store at room temperature
4. 15 ml Falcon tubes (Greiner ,188271 Zentrifugenröhrchen 15 ml, Producer: Huberlab AG, 7.188 271), store at room temperature
5. Normal melting point agarose (Standard Agarose-Type LE; BioConcept, catalog number: 7-01P02-R), store at room temperature
6. Low melting point agarose (Low Melt Agarose 100 g; Bio & Sell, catalog number: BS20.47.100), store at room temperature
7. Trypsin 10× stock solution (Gibco, catalog number: 15090-046), store stock solution at -20°C. Dilute 1:10 in 1× PBS for working solution, which is stable at 4°C for several weeks.
8. Potassium chloride (KCl) (Merck, catalog number: 1.04936.1000), store at room temperature
9. $Na_2HPO_4 \cdot 7H_2O$ (Sigma-Aldrich, catalog number: S9390-1Kg), store at room temperature
10. KH_2PO_4 (Sigma, catalog number: 602187), store at room temperature
11. NaCl (Sigma, catalog number: 71380-1KG), store at room temperature

12. EDTA disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: 03685-1KG), store at room temperature
13. Tris base (Sigma, Life science, catalog number: T1503-500G), store at room temperature
14. Sodium hydroxide pellets (NaOH) (Merck, catalog number: 1.06498.100), store at room temperature
15. DMSO (Sigma-Aldrich, catalog number: D5879-1L), store at room temperature
16. Triton X-100 (MP-Biomedicals, catalog number: 807426), store at room temperature
17. SYBR Gold nucleic acid gel stain (Life Technologies, catalog number: S11494), aliquot and store at -20°C protected from light
18. N-Lauroylsarcosine (Sigma, catalog number: L5125), store at room temperature
19. Sodium acetate ($C_2H_3NaO_2$) (Fluka, Biochemika, catalog number: 71183), store at room temperature
20. Lysis buffer (see Recipes)
21. Electrophoresis buffer (see Recipes)
22. Comet staining solution (see Recipes)
23. PBS (phosphate buffered saline) (see Recipes)

Equipment

1. Trevigen CometAssay ESII apparatus (Trevigen, catalog number: 4250-050-ES)
2. Fluorescent microscope capable of excitation between 470 and 530 nm to image SYBR-Gold (excitation maximum around 495 nm, emission approx. 537 nm)
3. Water bath able to keep a constant temperature of 37°C and large enough to hold a 250 ml glass bottle
4. Big styrofoam box or similar, large enough to hold a glass plate for 10-12 comet slides on ice
5. Small mechanic's level
6. Coplin jars capable of holding 5 or 10 slides
7. Faxitron Cabinet X-ray system, Model RX-650 (faxitron.com)

Software

1. Fiji image processing package (https://imagej.net/Fiji)
2. OpenComet plugin for Fiji (http://www.cometbio.org/) (Gyori *et al.*, 2014)

Procedure

A. Preparation of agarose-coated slides (the day before the assay or earlier)
   1. Completely dissolve 1 g of normal melting point agarose in 100 ml of dH_2O (1% solution) by heating in a glass bottle in the microwave. Be sure to place the lid on the bottle only loosely, and

be mindful of boiling retardation.

2. Pipet 1 ml of the agarose solution onto a superfrost slide, overlay with a cover slip to spread it evenly across the slide, and let the agarose set at room temperature. The thickness of the agarose coating will be around 1 mm, and overlaying with the cover slip aids to distribute the agarose evenly across the slide and to ensure an even surface.
3. When the agarose has set, gently remove the coverslip by sliding it towards the short end of the slide and air-dry the slides overnight.
4. When the slides have dried completely, they can directly be used or stored for several months in a dry, cool space.

   Note: Pre-coating the slides with normal melting point agarose increases the adhesion of the agarose layer containing the cells for comet analysis.

B. Culturing cells of interest

   Ensure that your cells of interest are in culture and growing exponentially (*i.e.*, approximately between 60% and 80% confluence at the time of the assay). Split your cells of interest 1-3 days before running the assay according to your established cell culture procedure in the medium of choice.

   Note: The comet assay described here can be used to determine DNA damage in a wide variety of cultured cells, provided they can be brought into single cell solution. As the amount of DNA damage induced by a particular setting and the repair kinetics will depend on the cell line used, ideal settings for treatment and repair need to be experimentally determined.

C. Preparation of material on the day of the assay

1. Prepare appropriate amounts of neutral comet lysis buffer and cool to 4°C. Be sure to have enough cold dH_2O to freshly prepare the cold electrophoresis buffer. The amount of lysis buffer required depends on the volume and number of the coplin jars that will be used. Typically, a medium-sized coplin jar can hold 10 slides and requires around 150 ml of buffer. The amount of the electrophoresis buffer needed depends on the apparatus that is used. For the CometAssay ESII apparatus that is used here, calculate approximately 850 ml. This amount does not depend on the number of slides since empty spaces for slides will be filled up with dummy slides.
2. Place the comet electrophoresis apparatus (including cooling pack) at 4°C to cool down.
3. Warm the water bath to 37°C.
4. Completely dissolve 0.5 g of low melting point agarose (LMP) in 50 ml of 1× PBS (for this 1% solution, a volume of 1 ml per sample plus some extra will be required, so increase the volume if more samples will be processed) by heating in a glass bottle in the microwave. Be sure to place the lid on the bottle only loosely and mind the boiling retardation. Once completely dissolved, place the bottle in the 37°C water bath to equilibrate to 37°C.

   Note: Keeping the correct temperature of the water bath is essential – if too cold, the agarose will solidify; if used too hot, this will cause additional damage to the cells.

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5. Label the agarose-coated slides using a pencil. Be sure to prepare at least duplicates, or better, triplicate slides for each assay condition (*e.g.*, time-point or amount of DNA damaging agent).
6. Prepare your workplace (see Figure 1):

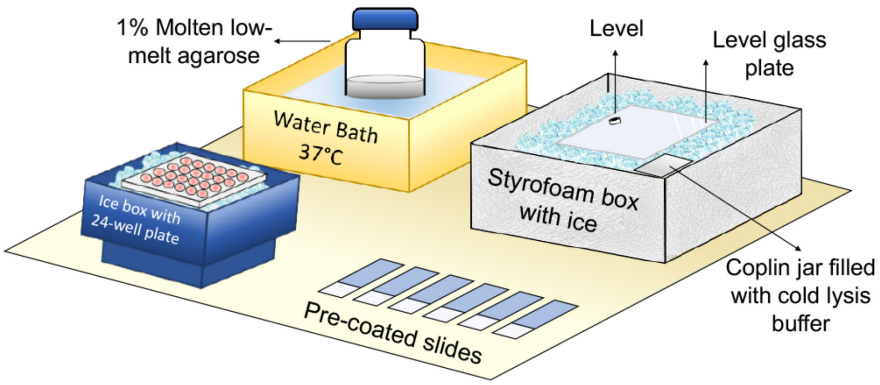

Figure 1. Proposed layout of a workplace to perform the comet assay.
An optimal arrangement of the materials as discussed in the text is shown.

7. Fill a large styrofoam bucket with ice, place a glass plate on top of the ice horizontally, and check using a level to ensure that cells in agarose will be evenly spread across the entire slide.
8. Place coplin jar filled with cold lysis buffer into the same bucket (or a different ice-containing bucket next to it).
9. Place an empty 24-well plate into a second ice bucket ready to receive the cell suspension.
10. Prepare the following materials to be ready and close at hand next to the 37°C water bath: a P100 or P200 pipet plus tips, a P1000 pipet plus tips, molten low-melting point agarose equilibrated at 37°C, labelled microscope slides, cover slips, and timer.

D. Treatment of cells and embedding into agarose

Depending on the research question, the neutral comet assay can be used to assess the amount of DNA damage caused by a specific amount of a fast and direct acting DNA damaging agent such as ionizing radiation (see D1). Moreover, the comet assay can be exploited to assess the repair kinetics after exposure to a certain amount of a DNA damaging agent (see D2) and also the levels of endogenous DNA damage present in cells (*e.g.*, due to a genetic ablation of a DNA repair pathway) (see D3). Be sure to include appropriate positive and negative controls when running the assay.

D1) Assessment of DNA damage caused by treatment of cells with ionising radiation

1. Irradiate exponentially growing cells by placing the dish in the faxitron cabinet and following the manufacturer's detailed instructions to deliver the planned irradiation dose. As positive control, use a dose between 2 and 4 Gy.
2. Trypsinize cells, neutralize trypsin by addition of complete medium, and count cells and dilute to 2 × 10^5 cells/ml in cold complete medium of choice in 15 ml Falcon tubes.

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3. Place Falcon tubes on ice to transfer them from the cell culture lab to the work bench.
4. To ensure an even suspension of cells, carefully invert the Falcon tube a few times (avoid foam formation or vigorous shaking to avoid damaging the cells); then, pipet 250 µl of cell suspension into the wells of the 24-well plate (1 well per slide/sample) that is kept on ice.
5. Ready the ice bucket with the glass plate, ensure it is still level, get the P1000 and the cover slips, and open the low-melting point agarose bottle (still keeping it in the 37°C water bath).
6. Add 1 ml of LMP-Agarose into the well, pipetting carefully to avoid strong bubble formation, aspirate the mixture, and transfer 1 ml of the cell-agarose mix onto the appropriate agarose-coated microscopy slide. Immediately overlay with a cover slip and, paying attention to keep the slide as level as possible, transfer the slide onto the cold glass plate on ice for the agarose to gel. Repeat this for every well until all cell samples have been transferred onto slides. This procedure is visualised in Video 1.

Video 1. Transfer of cells in LMP-Agarose onto microscopy slide

7. When all samples have been transferred onto the slides, gently remove the cover slips by sliding them along the longitudinal axis (starting with the first sample) and place the slides into the coplin jar to immerse them in the lysis buffer. Keep the samples in cold (on ice) lysis buffer protected from light for at least 1 h.
   Note: This step can be prolonged to an overnight incubation provided that the samples are constantly kept at 4°C maximum and protected from light. Protection from light aims at reducing additional DNA damage that could be generated through, e.g., UV light or other sources.

D2) Assessment of the repair kinetics after ionising radiation

1. Prepare one dish of exponentially growing cells per time-point.
2. Irradiate exponentially growing cells (*i.e.*, approximately 60-80% confluence) by placing the dish in the faxitron cabinet and following the manufacturer's detailed instructions. As positive control use a dose between 2 and 4 Gy. The ideal dose of irradiation depends on your research question

and the cell line used and should be experimentally determined.

3. Start the timer to keep track of the post-irradiation time-points.
4. Place the cell dishes that will be collected at later time-points back into the incubator.
5. Trypsinize and embed the cells that are ready to be collected as outlined in section D, subsection D1, steps 2-7.
6. Proceed with harvesting and embedding the cells of the remaining time-points accordingly.
7. When all slides are in the lysis buffer, incubate the samples for at least 1 h before continuing with the electrophoresis.
   Note: This step can be prolonged to an overnight incubation provided that the samples are constantly kept at 4°C maximum and protected from light.

D3) Assessment of endogenous DNA damage in cultured cells

1. Trypsinize exponentially growing cells (control versus, *e.g.*, KO cells), neutralize the trypsin by addition of complete medium, count cells and dilute them to 2×10^5 cells/ml in complete medium of choice in 15 ml Falcon tubes.
2. To embed the cells, follow the procedure as outlined in section D, subsection D1, steps 2-7.
   Important note: Be sure to include samples with appropriate positive and negative controls (e.g., ionizing radiation and no-treatment control) to ensure that the assay has worked!

E. Electrophoresis and staining of comet slides

1. Prepare an appropriate amount of cold neutral comet electrophoresis buffer and store at 4°C until required.
2. Slowly drain the lysis buffer from the coplin jars and replace with fresh cold electrophoresis buffer. Then drain the electrophoresis buffer and replace with fresh electrophoresis buffer. Repeat once more. Finally, incubate the slides in electrophoresis buffer for 60 min on ice, protecting from light.
3. Fill the comet buffer tank with fresh cold electrophoresis buffer to the required level. Transfer the comet slides to the comet assay apparatus, paying attention to the orientation of the slides. Ensure that all slides are fully immersed in buffer and fill up the remaining space with dummy slides.
4. Close the lid of the comet apparatus and run at 21 V for 60 min (this will result in *ca*. 120 mA current).
5. Fill the coplin jar with dH_2O.
6. After electrophoresis, remove the slides from the tank and transfer them to the coplin jar containing dH_2O.
7. Incubate for 5 min, then drain the dH_2O and carefully replace with fresh dH_2O. Repeat once.
8. In the meantime, prepare sufficient comet staining solution (approximately 600 µl per slide plus a bit extra). Keep protected from light.

9. Transfer the slides to a tray lined with paper towels, paying attention to lay them down as level as possible.
10. Cover each slide with approximately 600 µl of staining solution (ensure that the entire agarose part is covered with the solution), cover the tray with foil or a lid to protect from light, and incubate for 20 min at room temperature.
11. Decant the staining solution and shortly dip each slide into fresh dH_2O.
12. Let the slides air dry overnight, protected from light.

F. Imaging and analysis of comet slides
1. Image the slides on the fluorescent microscope, taking pictures at 10× or 20× magnification from different fields randomly picked across the entire slide. Be sure to use exposure and light intensities that allow clear visualization of your comets from the background, and try to focus as well as possible. Image an absolute minimum of 50 cells per condition, but preferentially at least 100 cells or more. Keep separate image folders for every single slide, containing all separate images of the different fields. Save all individual images as tiff files in one folder per slide.
2. Analyse the images using the OpenComet plugin available for FIJI as follows.
3. Open the OpenComet plugin.
4. Choose the input file folder (all images for one slide can be selected and analysed at once) and an output file directory.
5. Using the 'auto' head finding mode, click run. Results of this analysis will be summarized in an Excel file that is saved in the output file directory chosen previously.
6. Check that all the comets have been correctly detected anf deselect the ones that clearly need to be excluded/are incorrect (*e.g.*, artefacts and debris that are not comets that have been scored, overlays of multiple nucleoids, comets in which the head has been wrongly defined, or similar) by clicking on them. OpenComet will outline every regularly detected comet in red (with a red circle around the head and a red line to delineate the tail, see Figure 2A and 2B). In addition to this, there is a green line that shows the intensity distribution of the signal detected as the head, and a blue line for the signal detected as the tail. Sometimes the software is unsure whether something is a correctly detected comet or not (*e.g.*, unusual head). In that case, OpenComet will outline these 'outliers' in yellow (Figure 2C and 2D). However, it is also possible that shapes are incorrectly scored, as in Figure 2E. Here, the very low intensity signal was incorrectly scored as having a minuscule head and most of the signal being in the tail. Inclusion of such a shape would strongly influence the result, especially if this happens more than once or if only a few cells are assessed per condition. If OpenComet can't analyse the shapes (*e.g.*, if two or more comets are too close to each other or overlying), the shapes will be outlined in grey (Figure 2F). The same will happen to those comets that you have deselected/excluded by clicking on them. It is very important to visually double-check every comet, as incorrectly assessed ones can strongly influence the result.

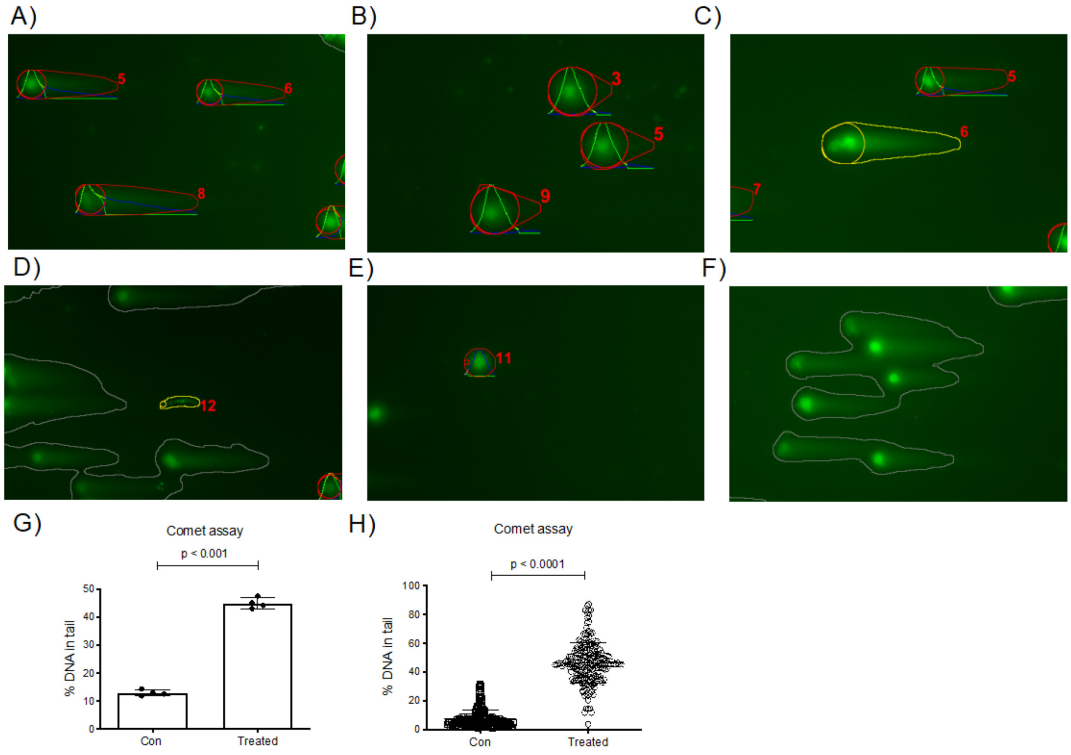

Figure 2. Example of images of comets analysed using the OpenComet plugin running the «auto» head finding mode and possible result plots.

These data were obtained using Tig-1 human primary fibroblasts after irradiation with 4 Gy. A) OpenComet outlines every regularly detected comet in red with a red circle around the head and a red line to delineate the tail. The green line shows the intensity distribution of the head signal, and the blue line shows the tail signal. Red numbers indicate the individual comets as listed in the quantification sheet. B) Representative picture showing control cells. Clearly, these cells show much less DNA in the tail than the cells in A), which were treated with ionizing radiation. C) and D) A yellow shape indicates that the software is unsure about the correct detection of comets. Be sure to visually inspect these comets and exclude/include as appropriate. E) Example of an incorrect score of a comet shape. The very low intensity signal was incorrectly scored to have a minuscule head and most of the signal in the tail. Inclusion of such a shape would strongly influence the result. F) A grey shape indicates that OpenComet is not able to outline comets, *e.g.*, because cells are too close together. G-H) The results obtained in the Excel file can be plotted (G) as a mean value of the duplicates or triplicates that were analysed as percentage of DNA in tail, tail moment, or olive moment or (H) as individual data points (*e.g.*, of 50 cells that were quantified).

7. After double-checking all comets on all slides, click ‘update’. This will generate a second Excel sheet with the suffix ‘_update’ that takes into account the comets that have been excluded.
8. This excel sheet contains all the values for all comet images of the slide that was analysed. In the last few rows are values for mean, median, standard deviation, minimum, and maximum for

all detected comets, divided into either only all normal comets or the normal and outlier comets. The choice of which of these values to use depends on whether you are fine with including the values of the outliers or not. Normally, both these values are very similar to one another.

9. There are two ways to plot the results: first, you can calculate a mean value of the duplicates or triplicates that were analysed as percentage of DNA in tail, tail moment, or olive moment, *e.g.,* using a bar graph. Repeat the entire assay at least two more times to obtain three or more independent values for each data point (Figure 2G). Second, you can also plot all the individual data points (*e.g.,* of 50 cells that were quantified) of at least three repeats of the entire assay (Figure 2H).

   Note: The tail moment describes the product of multiplying the length of the comet tail with the percentage of DNA in the tail, while the olive moment is the product of the total percentage of DNA in the tail and the distance between the centres of the masses of both head and tail regions. The olive moment is particularly useful to describe heterogeneity within a cell population, as the olive moment picks up variations in how the DNA is distributed in the tail. Of all the three options, the percentage of DNA in the tail is the measurement that seems the most intuitive to most researchers.
10. To test whether the groups differ significantly from each other, you can use the Student's *t*-test when only two groups are compared. When comparing three or more groups, use one-way ANOVA followed by, *e.g.*, Bonferroni's multiple comparison test.
11. When publishing comet assay data, it's strongly advisable to adhere to the 'minimum information for reporting on the comet assay (MIRCA)' guidelines that were just recently published in an attempt to ensure better interpretation, verification and reproducibility of results across laboratories (Moller *et al.*, 2020).

Recipes

1. Lysis buffer for neutral comet assay
   a. Prepare 'premix' by dissolving 146.1 g of NaCl (final: 2.5 M), 37.2 g of EDTA disodium salt ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, final: 100 mM), 1.2 g of Tris base (final: 10 mM) and 10 g of N-Lauroylsarcosine (final: 1%) in 800 ml of dH_2O.
   b. Once all solids have dissolved, adjust pH to 9.5 by dropwise addition of 5 M of NaOH. Adjust volume to 1 L by adding dH_2O, store at 4°C.
   c. To prepare a 'complete solution' of lysis buffer just prior to use, add 1 ml of DMSO and 0.5 ml of Triton X-100 to 98.5 ml of cold lysis buffer.
2. Electrophoresis buffer for neutral comet assay
   a. Prepare separate stock solutions: 1 M Sodium-Acetate (82.03 g of $C_2H_3NaO_2$ in 1 L dH_2O), 1 M Tris-HCl pH 8.3 (121.14 g of Tris base in 1 L dH_2O; adjust pH dropwise using HCl to 8.3).
   b. To prepare electrophoresis buffer (final composition: 300 mM Sodium-Acetate ($C_2H_3NaO_2$),

100 mM Tris-HCl pH 8.3), add 300 ml of 1 M Na-Acetate and 100 ml of 1 M Tris-HCl pH 8.3 to 600 ml of cold dH_2O; store at 4°C until required.

3. Comet staining solution
   Dilute SYBR Gold 1:10,000 in dH_2O just prior to use. Protect from light.
4. PBS (phosphate buffered saline)
   a. To make a 10× PBS stock solution, dissolve 80 g of NaCl, 2 g of KCl, 26.8 g of $Na_2HPO_4 \cdot 7H_2O$, and 2.4 g of KH_2PO_4 in 800 ml of dH_2O. Adjust pH to 7.4 using HCl, add dH_2O until 1 L, autoclave to store.
   b. To make 1× PBS working solution, dilute 100 ml of 10× PBS stock solution with 900 ml of dH_2O.

Acknowledgments

The authors wish to thank the following funding bodies for supporting research in the group of EM: Swiss National Science Foundation, Promedica Stiftung Chur, the Sassella Stiftung, and the Kurt und Senta Herrmann Stiftung.

Competing interests

The authors declare that they have no conflicts of interest.

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