We recently characterized the association between DNA damage and immunoresponse in vivo in colonic mucosa of mice infected with a Salmonella Typhimurium strain expressing a genotoxin, known as typhoid toxin. In this protocol, we describe the specific steps for assessing DNA damage by the alkaline comet assay of colonic mucosal samples. The description of the comet assay protocol follows the international guidelines (Minimum Information for Reporting on the Comet Assay (Moller et al., 2020)).
Protocol
Detection of DNA damage by alkaline comet assay in mouse colonic mucosa

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SUMMARY
We recently characterized the association between DNA damage and immunoresponse in vivo in colonic mucosa of mice infected with a Salmonella Typhimurium strain expressing a genotoxin, known as typhoid toxin. In this protocol, we describe the specific steps for assessing DNA damage by the alkaline comet assay of colonic mucosal samples. The description of the comet assay protocol follows the international guidelines (Minimum Information for Reporting on the Comet Assay [Moller et al., 2020]). For complete details on the use and execution of this protocol, please refer to Martin et al. (2021).

BEFORE YOU BEGIN
Different solutions have to be prepared the day before or the day of the comet assay.

The day before

© Timing: 1 h

1. Prepare the lysis solution (cf. recipe below) and store it at +4°C
2. Prepare the electrophoresis solution (cf. recipe below) and store it at +4°C
3. Prepare SYBR Gold 1/10 by dilution in Dimethyl sulfoxide (DMSO), aliquot in 20 µL and store at −20°C

The day of the comet assay

© Timing: 2 h and 30 min

4. Prepare agarose
   a. Prepare Low Melting Point (LMP) agarose 0.8% in 1× PBS. For example, for 30 mice dilute 80 mg of Low Melting Point (LMP) agarose in 10 mL of 1× PBS in a 50 mL borosilicate glass bottle.
CRITICAL: Put the agarose in the bottle before adding the PBS. Do not shake the bottle before melting to avoid agarose powder deposit on the bottle sides.

b. Heat 5 min at 100°C using a water bath
c. Incubate at 37°C for at least 2 h

5. Add Triton X-100 and DMSO to the lysis solution (cf. recipe below) and store it at +4°C

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Absolute ethanol | Fisher Scientific | Cat# E/0650DF/21 |
| Ca2+ and Mg2+-free Hanks’ Balanced Salt solution (HBSS) | Sigma-Aldrich | Cat# H6648 |
| Ca2+ and Mg2+-free phosphate-buffered saline (PBS) 10× | EuropeMedex | Cat# ET330-A |
| Dimethyl sulfoxide (DMSO) | EuropeMedex | Cat# UD8050-05C |
| Ethylenediaminetetraacetic acid (EDTA) disodium salt 0.5M | EuropeMedex | Cat# EU0084-B |
| Ethylenediaminetetraacetic acid (EDTA) powder | EuropeMedex | Cat# EU0007-B |
| Gelbond® film 124 × 258 mm cut in 3 pieces of 124 × 86 mm | Cityy | Cat# 80-1129-32 |
| Low Melting Point (LMP) Agarose | Sigma-Aldrich | Cat# A9414 |
| NaCl | EuropeMedex | Cat# 1112-A |
| NaOH pellet | EuropeMedex | Cat# 2020-A |
| SYBR Gold | Invitrogen | Cat# S11494 |
| TE buffer 10× | Fisher Scientific | Cat# BP2477-500 |
| Tris base | EuropeMedex | Cat# 26-128-3094-B |
| Triton X-100 | EuropeMedex | Cat# 2000-B |
| Trypan blue | Gibco | Cat# 15250061 |
| **Software and algorithms** | | |
| NIS Elements software for image acquisition (F-package version no. 4) | Nikon | n/a |
| CometScore 2.0 | rexhoover.com | http://rexhoover.com/index.php?id=cometscore|
| **Other** | | |
| 1.5 mL Tubes | WWR | Cat# 211-2130 |
| Cover slides 22×60 mm | Knittel Glass | Cat# 100267 |
| Cyaocrylate glue | Many convenience stores | n/a |
| Freezing Container, Nalgene® Mr. Frosty | Sigma-Aldrich | Cat# C1562-1EA |
| Microscope slides 76×50×1 mm | Knittel Glass | Cat# 100008 |
| Scalpel blade N°10 | WWR | Cat# 233-5472 |
| Centrifuge | Eppendorf | Cat# 5418R |
| Dounce tissue grinder with the loose pestle | WWR | Cat# BELC1984-10007 |
| Electrophoresis tank with a platform of 20×20 cm and a distance of 35 cm between the 2 platinum electrodes | Econo-Submarine SGE 0220-02 | C.B.S. Scientific, USA |
| Fluorescence microscope with camera | Nikon microscope equipped with a Luca S camera Source of fluorescence: Nikon C-HGFIE HG Fiber illuminator INTENSILIGHT | Eclipse 50i |
| Hemocytometer | WWR | Cat# HEC40453702 |
| pH meter | Mettler Toledo | Cat# SevenCompact pH meter S220 |

**Note:** The glue can be found in many convenience stores, the most important is to use cyanoacrylate glue. As an example we are using Loctite Super Glue-3.

**Alternatives:** Freezing containers can be replaced by multi-compartment polystyrene boxes such as those containing spectrophotometer cuvettes. Any type of fluorescence microscope, with a FITC filter (Emission wavelength 475 +/- 14 nm, transmission Band: 500-550), can be used for the acquisition of comet assay images.
MATERIALS AND EQUIPMENT

Solutions for comet assay
All solutions have to be stored at +4°C.

Lysis solution (pH 10)

| Reagent            | Final concentration | Amount    |
|--------------------|---------------------|-----------|
| NaCl               | 2.5 M               | 146.2 g   |
| EDTA               | 0.1 M               | 37.2 g    |
| Tris base          | 10 mM               | 1.2 g     |
| NaOH pellet        | n/a                 | 7.6 g     |
| ddH2O              | n/a                 | Adjust to 1 L |
| **Total**          | n/a                 | 1L        |

After completion, adjust pH with NaOH (either pellet or SN solution) to pH 10. This solution can be stored for 1 month.

Electrophoresis solution

| Reagent (stock) | Final concentration | Amount |
|-----------------|---------------------|--------|
| EDTA 0.5M       | 1 mM                | 5 mL   |
| NaOH 5N         | 0.3 M               | 150 mL |
| ddH2O           | n/a                 | 2,345 mL |
| **Total**       | n/a                 | 2.5 L  |

This solution has to be prepared the day before the comet assay.

Lysis solution supplemented with Triton X-100 and DMSO

| Reagent            | Final concentration | Amount |
|--------------------|---------------------|--------|
| Triton X-100       | 1%                  | 5 mL   |
| DMSO               | 10%                 | 50 mL  |
| Lysis buffer       | n/a                 | 445 mL |
| **Total**          | n/a                 | 500 mL |

This solution has to be prepared the day of the experiment.

HBSS - EDTA

| Reagent (stock) | Final concentration | Amount |
|-----------------|---------------------|--------|
| HBSS            | n/a                 | 96 mL  |
| EDTA 0.5 M      | 0.02 M              | 4 mL   |
| **Total**       | n/a                 | 100 mL |

The volume to be prepared depends on the number of animals. For 10 mice, we recommend to prepare at least 25 mL of HBSS-EDTA solution.

△ CRITICAL:

Absolute ethanol is a highly flammable liquid and vapor (H225) and causes serious eye irritation (H319).

Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA) disodium salt and sodium chloride (NaCl) cause serious eye damage (H319).

Tris base causes skin irritation (H315), serious eye damage (H319) and may cause respiratory irritation (H335).

TritonX-100 is considered as harmful if swallowed (H302), causes skin irritation (H315) and causes serious eye damage (H319).
Trypan blue may cause cancer (H350) and is suspected to damaging fertility or the unborn child (H361).

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

No data are available addressing the mutagenicity or toxicity of SYBR® Gold nucleic acid gel stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate caution.

For all harmful reagents cited above, it is recommended to wear appropriate recommended 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 for assessing DNA damage by comet assay of colonic mucosal samples. However, other types of cells can be used but will need specific validation and setting up, as shown for human peripheral blood mononuclear cells in Perdry et al. (2018).

**Collection of murine colonic mucosa cells**

© Timing: 15 min/mouse

The following steps aim to collect colonic epithelial cells from mouse mucosa.

- **△ CRITICAL:** Light might damage cell DNA and induce a bias in the comet assay protocol. Therefore, we recommend to avoid natural light and to work in an environment with no direct source of artificial light.

1. Place 1.5 mL labeled tubes with 500 μL of HBSS-EDTA (1 tube per animal) on ice
2. Longitudinally open the mice abdomen
3. Collect the colon from the caecum to the rectum
4. Wash the colon in a Petri dish full of 1× PBS
5. Open the colon following a lengthwise axis
6. Collect 0.5 cm of the distal part for comet assay (the remaining part of the colon can be used for other types of analysis)
7. Place the colon specimen on a microscope slide and scratch the mucosa using a scalpel blade

- **△ CRITICAL:** It is important to scratch the mucosa profoundly in order to collect all the cells.

8. Transfer the mucosal material in the tube containing 500 μL of HBSS-EDTA
9. Place the tube back on ice

- **△ CRITICAL:** After collection, it is important to freeze the samples within an hour to avoid sample degradation leading to DNA breaks.

10. Let the tube slowly freeze at −80°C (Mister frosty or polystyrene box)
11. Wait at least 24 h before transferring the tubes to a classical −80°C box

**Note:** The freezing step is optional and comet assay can be performed just after collection of mucosal sample in case that there is less than one hour between sampling and the first step of the comet assay. However, make sure to always follow a consistent protocol (with or without freezing) within the same project.
Inclusion of cells in gels, lysis, electrophoresis, neutralization, and fixation

Timing: 1 day or 2 half-days in case of overnight (16 h) lysis step

These steps aim to perform the comet assay per se from the cell isolation to the fixation.

12. Isolation of cells and preparation of single-cell suspension
   a. Allow the samples to defrost approximately 2 h at +4°C
   b. Keep the tubes on ice
   c. Remove the HBSS-EDTA buffer gently (no centrifugation is needed)
   d. Add 750 μL of cold HBSS-EDTA buffer to the tissue and transfer the buffer containing the tissue to the Dounce tissue grinder
   e. Add 750 μL of cold HBSS-EDTA to the grinder for a final volume of 1.5 mL.
   f. Mechanically dissociate the cells (40 times up and down with the pestle)

   ∆ CRITICAL: As any kind of manipulation could induce DNA damage and therefore a bias in the comet assay, we recommend to manipulate the cells as little as possible (do not filter cells and avoid too many up and downs with the pestle).

   g. Collect 15 μL of sample and dilute it with 15 μL of Trypan blue
   h. Determine the number of living cells (for example using hemocytometer)

   ∆ CRITICAL: From animal experiment, it is expected to get at least 75% of alive cells. We recommend to don’t perform the assay if the number of live cells is low or to be precautionous with the interpretation.

   Note: If the number of cells is too high to be accurately counted, an intermediate dilution (for example 1:5) in HBSS-EDTA is recommended. The use of a hemocytometer and Trypan blue allow to us check that the suspension is made mainly of alive single cells.

   i. Collect a total (live + dead) of 90,000 cells/condition
   j. Centrifuge 10 min at 200 g (slow brake around 2, +4°C)

   ∆ CRITICAL: Be sure to prepare in the same way positive and negative samples for your experiment. For example, it is possible to use cryopreserved aliquots from a single batch of peripheral blood mononuclear cells or mammalian cell lines that have been exposed or not to a DNA damaging agent. Cells treated with Methyl methanesulfonate (MMS) or ethyl methanesulfonate (EMS), which are DNA damaging agent, can be used as positive control. The drug concentration is depending on the cell type, we would suggest performing pilot experiment with 500 μM to 1 mM of MMS or 5–10 mM of EMS for 2 h at 37°C.

13. Cell inclusion in agarose and deposit on Gelbond® film
   a. Lay the metal plate (Figure 1) on ice, and place deposit pattern on the top: for example, 2×20 deposits/Gelbond® (Figure 2)
   b. Put the Gelbond® films in the holder (optional), annotate them and position them on their patterns on the appropriate face.

   Note: Gelbond® is a polyester film with a treated surface that binds to the agarose gel on the hydrophilic side (it can be checked using water droplets that spread on the hydrophilic side but bead up on the hydrophobic side).

c. Discard the supernatant of the cells
CRITICAL: It is recommended to be very careful at this step and discard gently the supernatant to avoid detachment and loss of cell pellet.

d. Add 300 μL of LMP agarose
e. Mix gently using a pipette and load 15 μL of samples/deposit.
f. Be sure to deposit each sample in duplicate or triplicate.

**Note:** As the volume of agarose is low (15 μL) and the Gelbond® is laid on the cold metal plate, the agarose solidifies immediately and there is no need to wait after the last deposit to transfer the Gelbond® in the lysis solution.

14. Lysis step
   a. Transfer the holder with Gelbond® film in a container containing enough cold lysis solution to cover all the deposits during at least 1 h at +4°C (this step can be prolonged to overnight, 16 h) *(Figure 3)*

15. Unwinding and electrophoresis step *(Figure 4)*
   a. Place the holder with Gelbond® for 40 min in a box containing the electrophoresis solution at +4°C
   b. Transfer them to the electrophoresis tank
   c. Cover with fresh and cold electrophoresis solution (+4°C)
   d. Run the samples for 24 min at 28 V (corresponding to 0.8 V/cm, see Note)
Note: The voltage used depends on the length of the electrophoresis tank. As the distance between the 2 electrodes of our tank is 35 cm, the voltage used is 28 V (=35 × 0.8). If there are doubts about electrophoresis, a pilot experiment with positive and negative controls should be performed in order to check the presence of DNA in comet tails only in positive controls as shown in Figure 7.

16. Neutralization step
   a. Put the Gelbond® in cold PBS (+4°C, Enough volume to cover the Gelbond® depending on the size of the container, for example for a container 16 × 25 cm, 200 mL is needed) for 5 min
   b. Repeat previous step (16 a.) one more time

17. Fixation step
   a. Soak the Gelbond® in cold absolute ethanol for 1.5 h at +4°C

Note: After fixation, the Gelbond® film can be stored several months before dyeing step in dried conditions, at room temperature (20°C–25°C), avoiding accumulation of dust on it.

Dyeing

© Timing: 25 minutes/Gelbond®

These steps aim to stain the cell DNA before the analysis.

18. Cut the Gelbond® to adjust the size to large microscope glass slide (one Gelbond® corresponds to 2 large microscope glass slides)
19. Glue the Gelbond® on a microscope glass slide (Figure 5)
20. Add 20 µL of SYBR Gold 1/10 in 20 mL of 1× TE buffer (obtained by dilution of 10× TE Buffer in ddH2O).
21. Incubate the slide with the glued Gelbond® film in SYBR Gold solution sufficient volume to cover the slide for 20 min in darkness (for example in small black boxes (10×8 cm) 20 mL of SYBR is needed), at room temperature (20°C–25°C).
22. Rinse the slide with ddH2O.
23. Cover with two rectangle cover slides.

Analysis

⏰ Timing: 2 h for 1 slide with 20 deposits

This step will allow taking pictures of cells that will be then quantified.

Acquire images using a fluorescence microscope the same day (or after 1 night if kept in a humidified chamber at +4°C) with a FITC filter (Emission wavelength 475 +/- 14 nm, transmission Band: 500–550). Slides can be scanned using the Nikon NiS software at 20× magnification.

Note: This step can be optimized by using a motorized microscope.

ชะ Time point:

Several pause points are possible but it is important to ensure that pause points are similar between different experiments inside the same project.

- Lysis step overnight (16 h)
- After fixation but before dyeing, Gelbond® can be stored for several months
- After dyeing and before analysis

EXPECTED OUTCOMES

The scan step above will allow to get a picture such as the one presented in Figure 6 where six cells will be quantified.

Figure 5. Gluing the Gelbond® film on microscope slides
QUANTIFICATION AND STATISTICAL ANALYSIS

This section of the protocol offers indications for analyzing DNA content in comet tails (Figure 7).

Use the following steps to analyze comet assay images using CometScore 2.0, free software that has the ability to automatically score comets.

1. Launch CometScore 2.0 and import an image to analyze.
2. Determine the cutoff to correct the background. Comet assay images often do not have uniform background illumination. Using a cutoff is useful for finding comet shapes but will not affect the calculation of measured parameters.
3. Score comets using one out of the three techniques (manual scoring, semi-automated scoring or completely automated scoring) according to the basal level of damages in the tissue of interest.

Figure 6. Example of DNA comets image (Source: Martin et al., 2021)
Scale bar represents 10 µm.

Figure 7. Quantification of DNA damage
Examples of analyzed DNA comets: damaged (A) or undamaged (B) cells. DNA damage induce relaxing of DNA loops that favor DNA migration. In this case, some DNA appears in the tail. The percentage of DNA in the comet tail DNA increases with the DNA damage level. In the absence of DNA damage, the DNA is supercoiled and appears in the head. Some of the different parameters assessed by the software are represented in the tables. The main descriptor is the tail DNA (percentage).
For example, the colon being a tissue with a high background level of damages, we used the manual scoring technique.

**Note:** Comet exclusion criteria: avoid analyzing doublets (cells that are too close) or comets at slide edges. At least 50 cells per deposit should be scored so a minimum of 100 cells scored for each animal if using duplicate deposit.

4. The software then generates the following outputs:
   - Cropped images of each analyzed comet,
   - Output spreadsheet containing comet measurements for all analyzed images.

5. Using the spreadsheet, assess DNA damage using the tail DNA percentage which is the tail intensity as a percentage of whole comet intensity and the most used type of primary comet assay descriptor.

**Note:** Among all cells acquired per animal, the values would not follow a Gaussian distribution, in that case it is recommended for statistical analysis to select the median (and not the mean) of Tail DNA percentage.

**LIMITATIONS**

**Time between sampling and comet assay.** Contrary to lymphocytes or white blood cells (Moller et al., 2021) no study has been done about the impact of the time between collection of colonic samples and the comet assay. Therefore, we recommend short-term storage.

**Fast freezing or defrosting.** Rapid cells freezing or defrosting may cause shattering of the cells and therefore false positive results.

**Cell dissociation.** It is important to have a soft but efficient dissociation of the cells in order to be able to accurately count them. This step may need some setting up depending on the tissue.

**Electrophoresis duration.** Electrophoresis duration may influence results especially because the tail DNA percentage is linearly related to both V/cm and to duration of electrophoresis. Recommendations can be found in (Azqueta et al., 2019).

**TROUBLESHOOTING**

**Problem 1**
It is possible that the Gelbond® film detaches from the glass slide during the dyeing step (Dyeing, step 4).

**Potential solution**
This is most probably due to the fact that not enough glue has been used or that the glue has dried on the slide before gluing the Gelbond® film. In that case, we recommend gluing again the Gelbond® film on the slide before rinsing it.

**Problem 2**
It is possible that many doublet cells are in a field to be accurately analyzed (Figure 8) (Quantification and statistical analysis step).

**Potential solution**
If most of the acquired fields have a lot of doublet cells, a setting up experiment would be necessary and several points have to be checked:

Cells were probably not efficiently dissociated with the Dounce tissue grinder and this point was not checked while counting with the hemocytometer. It could be necessary to increase the number of up
and down with the pestle to separate cells. However, we remind that heavy manipulation could induce cell DNA damage and therefore a bias in the comet assay.

It might be necessary to reduce the number of cells embedded in the agarose.

**Problem 3**

High DNA damage is observed in all cells, even in negative control (Quantification and statistical analysis step).

**Potential solution**

DNA damage may be induced during several steps in the protocol such as freezing, defrosting, mechanical dissociation of cells or during comet assay. In order to identify where the problem came from, we recommend performing different controls in a setting up experiment such as use of fresh samples, use of cells in suspension (e.g., lymphocytes) and/or use of cells from in vitro culture which usually exhibit lower levels of DNA damage than cells from in vivo experiments.

**Problem 4**

There is no or very few cells per acquired field (Quantification and statistical analysis step).

**Potential solution**

This could come from Step 13c where supernatant is discarded before resuspending the pellet in the agarose. It is recommended to pay attention to this step and discard gently the supernatant in order to avoid detachment and loss of cell pellet.

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts Océane C.B. Martin (oceane.martin@u-bordeaux.fr).

**Materials availability**

This study did not generate any specific material/reagent.

**Data and code availability**

Original data for figures are available upon request to the lead contact.
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AUTHOR CONTRIBUTIONS

O.C.B.M. and T.F. designed the experiments. M.M.H., S.H., E.B.-R., and O.C.B.M. performed the experiments. All authors contributed to the protocol writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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