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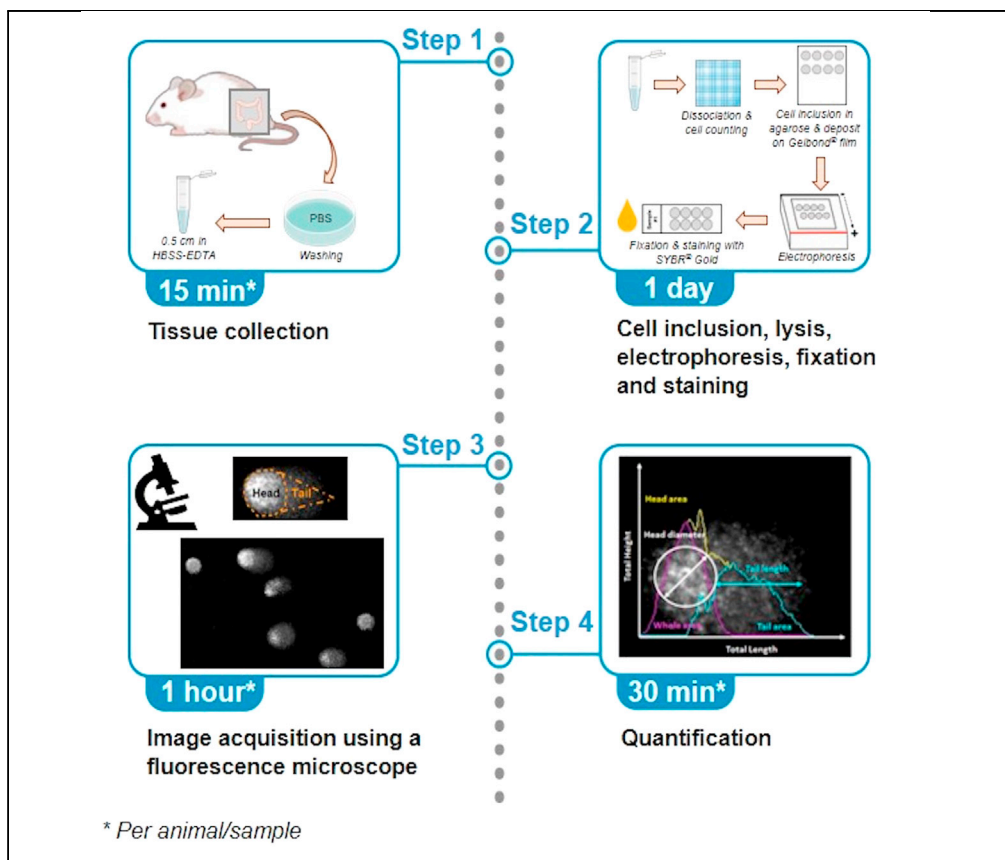
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## Protocol

# Detection of DNA damage by alkaline comet assay in mouse colonic mucosa



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### Highlights

Collection of mice  
colon mucosa for  
comet assay

Detailed description  
and optimization of  
alkaline comet assay

Detection of single  
and double DNA  
strand breaks

Assessment of DNA  
damage extent using  
the percentage of  
DNA in the comet tail

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 immunor-  
esponse *in vivo* in colonic mucosa of mice infected with a *Salmonella* Typhimu-  
rium 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
<b>Chemicals, peptides, and recombinant proteins</b>		
Absolute ethanol	Fisher Scientific	Cat# E/0650DF/21
Ca <sup>2+</sup> and Mg <sup>2+</sup> -free Hanks' Balanced Salt solution (HBSS)	Sigma-Aldrich	Cat# H6648
Ca <sup>2+</sup> and Mg <sup>2+</sup> -free phosphate-buffered saline (PBS) 10×	Euromedex	Cat# ET 330-A
Dimethyl sulfoxide (DMSO)	Euromedex	Cat# UD8050-05C
Ethylenediaminetetraacetic acid (EDTA) disodium salt 0.5M	Euromedex	Cat# EU0084-B
Ethylenediaminetetraacetic acid (EDTA) powder	Euromedex	Cat# EU0007-B
Gelbond® film 124 × 258 mm cut in 3 pieces of 124 × 86 mm	Cityva	Cat# 80-1129-32
Low Melting Point (LMP) Agarose	Sigma-Aldrich	Cat# A9414
NaCl	Euromedex	Cat# 1112-A
NaOH pellet	Euromedex	Cat# 2020-A
SYBR Gold	Invitrogen	Cat# S11494
TE buffer 10×	Fisher Scientific	Cat# BP2477-500
Tris base	Euromedex	Cat# 26-128-3094-B
Triton X-100	Euromedex	Cat# 2000-B
Trypan blue	Gibco	Cat# 15250061
<b>Software and algorithms</b>		
NIS Elements software for image acquisition (F-package version no. 4)	Nikon	n/a
CometScore 2.0	<a href="http://rexhoover.com">rexhoover.com</a>	<a href="http://rexhoover.com/index.php?id=cometscore">http://rexhoover.com/index.php?id=cometscore</a>
<b>Other</b>		
1.5 mL Tubes	VWR	Cat# 211-2130
Cover slides 22×60 mm	Knittel Glass	Cat# 100267
Cyanoacrylate 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	VWR	Cat# 233-5472
Centrifuge	Eppendorf	Cat# 5418R
Dounce tissue grinder with the loose pestle	VWR	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	VWR	Cat# HECH40453702
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
ddH <sub>2</sub> O	n/a	Adjust to 1 L
<b>Total</b>	<b>n/a</b>	<b>1L</b>

After completion, adjust pH with NaOH (either pellet or 5N 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
ddH <sub>2</sub> O	n/a	2,345 mL
<b>Total</b>	<b>n/a</b>	<b>2.5 L</b>

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
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

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
<b>Total</b>	<b>n/a</b>	<b>100 mL</b>

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  $\mu$ L 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  $\times$  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  $\mu$ L 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  $-80^{\circ}\text{C}$  (Mister frosty or polystyrene box)
11. Wait at least 24 h before transferring the tubes to a classical  $-80^{\circ}\text{C}$  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  $\mu$ L of cold HBSS-EDTA buffer to the tissue and transfer the buffer containing the tissue to the Dounce tissue grinder
  - e. Add 750  $\mu$ L 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  $\mu$ L of sample and dilute it with 15  $\mu$ L 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 cautious 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  $\mu$ M 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, 2x20 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



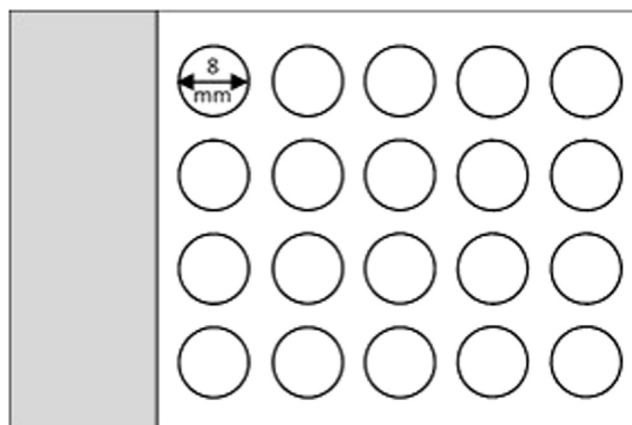
**Figure 1. Sample deposit**

**△ 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  $\mu$ L of LMP agarose
- e. Mix gently using a pipette and load 15  $\mu$ L of samples/deposit.
- f. Be sure to deposit each sample in duplicate or triplicate.

**Note:** As the volume of agarose is low (15  $\mu$ L) 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)



**Figure 2. Deposit pattern (actual size, printable)**



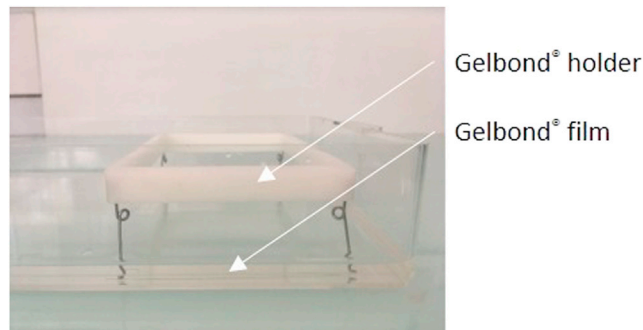


Figure 3. Lysis steps

**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 ( $=35 \times 0.8$ ). 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

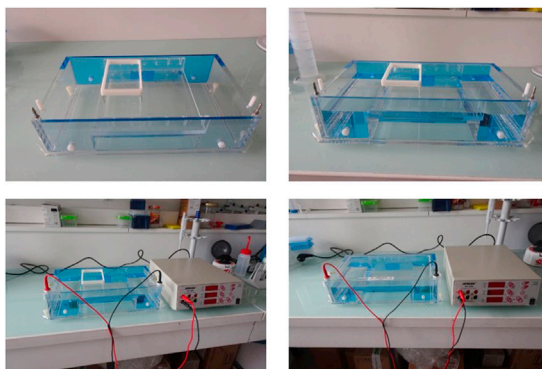
**Note:** After fixation, the Gelbond® film can be stored several months before dyeing step in dried conditions, at room temperature (20°C–25°C), avoiding accumulation of dust on it.

### Dyeing

⌚ Timing: 25 minutes/Gelbond®

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

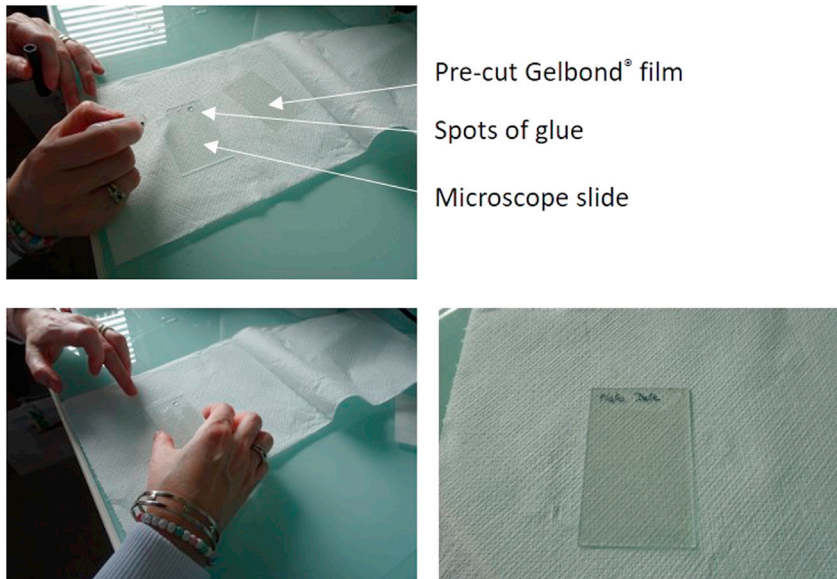
18. Cut the Gelbond® to adjust the size to large microscope glass slide (one Gelbond® corresponds to 2 large microscope glass slides)
19. Glue the Gelbond® on a microscope glass slide (Figure 5)



Setting up Gelbond® films in the tank followed by addition of electrophoresis solution

Electrophoresis of samples with Gelbond® holder (on the left, lid of the tank can be cut if the Gelbond® holder is too high) or without Gelbond® holder (on the right)

Figure 4. Electrophoresis step



**Figure 5. Gluing the Gelbond® film on microscope slides**

20. Add 20  $\mu$ L of SYBR Gold 1/10 in 20 mL of 1  $\times$  TE buffer (obtained by dilution of 10 $\times$  TE Buffer in ddH<sub>2</sub>O)
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 $\times$ 8 cm) 20 mL of SYBR is needed), at room temperature (20°C–25°C).
22. Rinse the slide with ddH<sub>2</sub>O
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 $\times$  magnification.

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

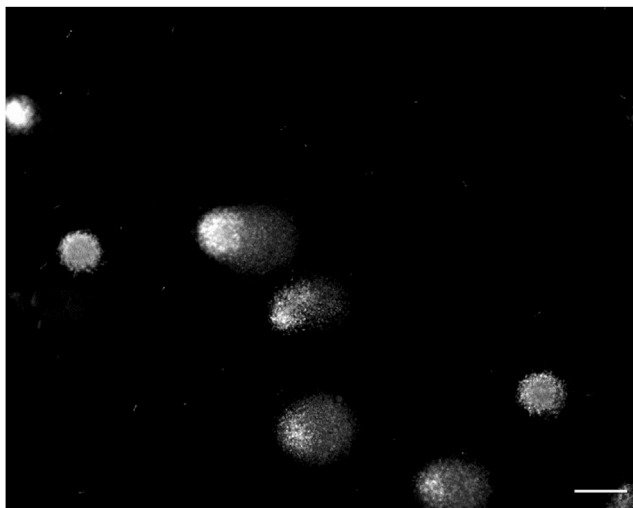
⏸ Pause point:

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.



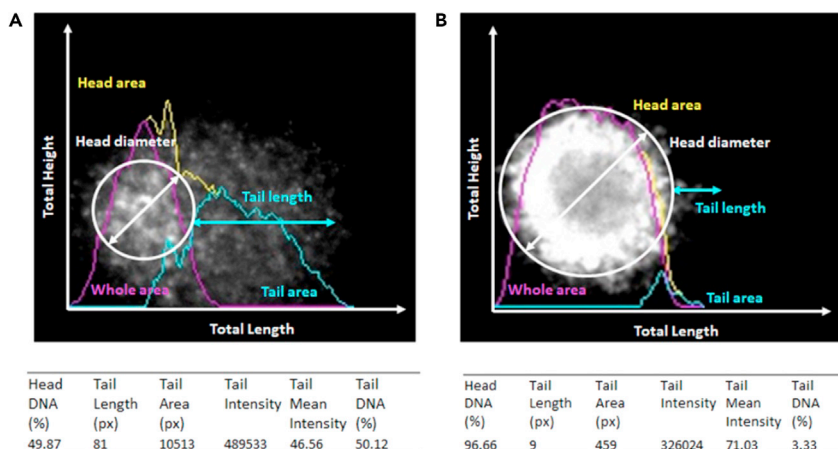
**Figure 6.** Example of DNA comets image (Source: Martin et al., 2021)  
Scale bar represents 10  $\mu$ m.

## 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 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.

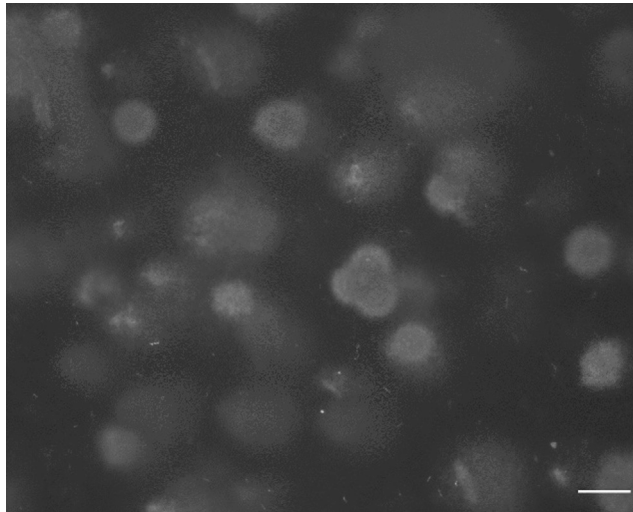
### Problem 2

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



**Figure 8. Example of a field with many doublet cells**  
Scale bar represents 10  $\mu\text{m}$ .

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, mechanic 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](mailto: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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