

Neutral Comet Assay

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[Abstract] The Comet assay (or Single Cell Gel Electrophoresis assay) is a sensitive technique to detect DNA damage at the level of an individual cell. This technique is based on micro-electrophoresis of cells DNA content. Briefly, cells are embedded in agarose, lysed and submitted to an electric field, before the staining step with a fluorescent DNA binding dye. Damaged DNA (charged DNA) migrates in this field, forming the tail of a “comet”, while undamaged DNA remained in the head of the “comet”. The following document describes the protocol to realize a neutral comet assay. This assay can be applied to different cell types and has been useful for numerous applications in fields of toxicology or DNA damage and repair.

Materials and Reagents

1. Cells to analyze
2. Low Melting Point (LMP) Agarose (Sigma-Aldrich, catalog number: A9414)
3. Seakem[®] Agarose (Ozyme, catalog number: LON50004)
4. PBS (Ca²⁺ and Mg²⁺-free phosphate-buffered saline)
5. 5 N NaOH
6. 0.5 M EDTA disodium salt solution (pH 8)
7. Trisma base
8. Triton X-100
9. N-Lauroylsarcosine (Sigma-Aldrich, catalog number: L5125)
10. Dimethylsulphoxide (DMSO)
11. Absolute ethanol
12. Ethidium bromide (10 mg/ml)
13. Trypsin/EDTA
14. Sodium acetate
15. Lysis solution (see Recipes)
16. Electrophoresis solution (see Recipes)

Equipment

1. Microscope Super Frost plus glass slides
2. Malassez chamber
3. Microscope coverslips (22 x 22 mm)
4. Microscope coverslips (24 x 32 mm)
5. Centrifuges
6. Electrophoresis tank: Econo-Submarine (20 cm x 30 cm) (C.B.S. Scientific, USA)
7. Fluorescence microscope, camera and software (e.g. Nikon Eclipse 50i microscope equipped with a Luca S camera and Komet 6.0 software)

Software

1. Komet 6.0 software (Andor Technology)

Procedure

1. Prepare agarose solution and slides.
 - a. At least 24 h before the experiments:
 - i. Prepare 0.8% solution of Seakem® Agarose in PBS.
 - ii. Pre-coat Super Frost slides by dipping in a vertical jar containing melted agarose, stirred with a magnetic stirrer and kept at 100 °C (Figure 1).

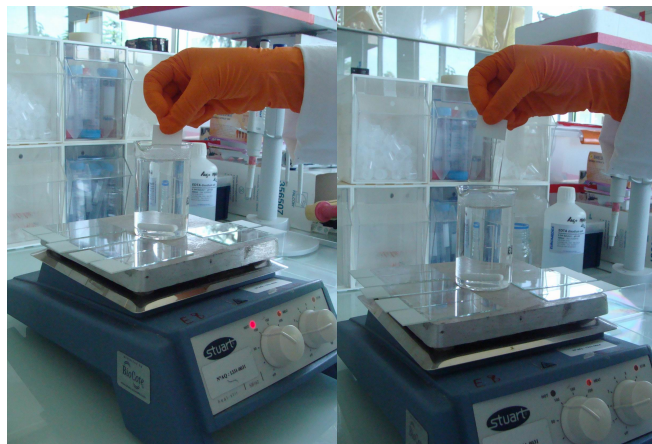


Figure 1. Coating of the slides with agarose

- iii. Drain off the agarose in excess by wiping the back of the slides (Figure 2).



Figure 2. Slides coating with agarose. Removing of the agarose on the back of the slides.

- iv. Let the slides dry and then store at room temperature until use.
 - b. At least 2 h before the experiments: prepare 0.7% solution of LMP agarose in PBS and place it at 37 °C in a water-bath until use.
2. Prepare cells
- The number of harvested cells can be adjusted according to the size of the cells. Cells could be numbered either by an automated cell counter or a counting chamber (e.g. Malassez chamber).
- If cells used are adherent, cells must be carefully detached with trypsin/EDTA and isolated before centrifugation and further use.
3. Embed cells in LMP agarose (in a dark room):
- a. After cell centrifugation, discard the supernatant and resuspend the pellet of cells (150,000 to 200,000 cells) by gently pipetting with 200 μ l of 0.7% LMP agarose.
 - b. Lay 65 μ l of agarose containing the cells on each pre-coated glass slide.
 - c. Immediately cover with a 24 x 32 mm coverslip.
 - d. Put the slide on an ice-pack for solidification during 5-10 minutes.
 - e. Slide off the coverslip to remove it.
 - f. Finally cover with 80 μ l of LMP agarose (top agarose layer) and cover again with a 24 x 32 mm coverslip.
 - g. Put the slide again on an ice-pack for solidification during 5-10 minutes.
 - h. Remove the coverslip.
4. Lysis and electrophoresis (in a dark room):
- a. Place the slides in lysis solution for at least 1 h at 4 °C.
 - b. Wash three times for 5 min with the electrophoresis buffer.
 - c. Transfer the slides in the electrophoresis tank filled with electrophoresis solution (Figure 3).

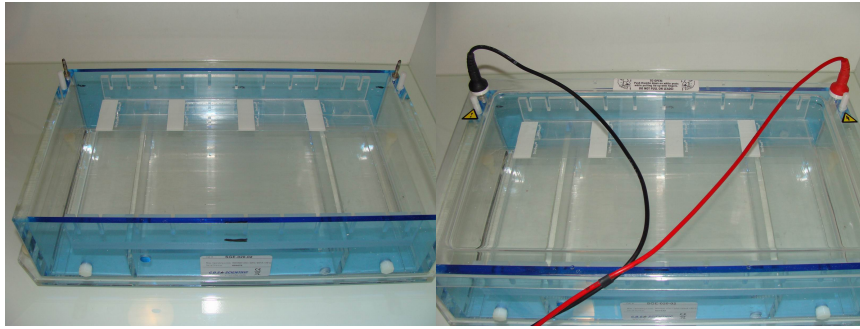


Figure 3. Electrophoresis of the slides. Disposition of the slides in the tank. In this case eight slides are subjected to electrophoresis, four by row.

- d. Proceed to electrophoresis at 18 V (0.5 V/cm) during 1 h.
- e. Wash in PBS for 2 x 5 min.
5. Dehydration, staining and analysis:
 - a. Fix the cells with 2 x 10 min washes in absolute ethanol, air-dry for at least 2 hours at room temperature.
 - b. Add 50 μ l ethidium bromide (2 μ g/ml in water) on the microscope slide and cover with a 22 x 22 mm coverslip for staining.
 - c. Analyze the cells: score 50 cells per slide, 2 slides per condition with the fluorescence microscope equipped with a camera and adapted software (Figures 4 and 5).

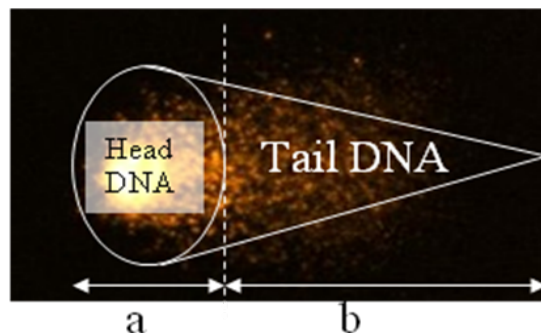


Figure 4. Representing analysis of each cell

a = head length

b = tail length

% tail DNA = fraction of DNA in the tail

Tail moment = % tail DNA x b

Comet tail length can be calculated by different ways depending on the authors. Tail moment is a common parameter used to characterize the comet. For this, the fraction of DNA in the tail is evaluated by the fluorescence in the tail and divided by the total

fluorescence (in the head and in the tail) to be expressed in percentage. Tail moment is the product of % tail DNA and tail length.

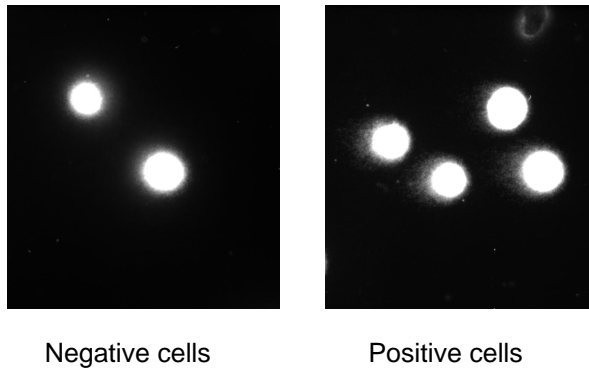


Figure 5. Images representing nucleus of undamaged cells, negative for the presence of comet (left panel) and nucleus of damaged cells presenting comet (right panel)

As a positive control, cells irradiated with ionizing radiations at 20 Gy and examined just after irradiation are a good control as shown in the photographs above.

Recipes

1. Lysis solution
 - 2.5 M NaCl
 - 0.1 M EDTA
 - 10 mM Trizma base (pH 10)
 - 1% N-laurylsarcosine
 - 0.5% Triton X-100
 - 10% DMSO final
 - Keep at 4 °C
2. Electrophoresis solution
 - 300 mM sodium acetate
 - 100 mM Tris-HCl (pH 8.3) at 4 °C

Acknowledgments

This protocol was adapted from previously published papers (Courilleau *et al.*, 2012; Olive *et al.*, 1990; Ostling and Johanson, 1984; Ostling and Johanson, 1987; Wojewodzka *et al.*, 2002).

References

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