EMPA Materials Science & Technology

# Alkaline single cell gel electrophoresis in THP-1 cells

*Detecting DNA damage in THP-1 cells using the Comet Assay*

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# 1 Introduction

DNA damage can be manifold ranging from DNA strand breaks to chromosomal aberrations or whole chromosome loss. Resulting mutations or genetic instability may subsequently cause different diseases including cancer or may even lead to death. Assessing DNA breakage is thus of significant interest. One of the most common methods to detect DNA strand breaks is the alkaline single cell gel electrophoresis or comet assay firstly described by Singh et al. 1988.

# 2 Principle of the Method

A small number of cells are embedded in a thin agarose layer, lysed, subjected to electrophoresis and stained with a fluorescent DNA-intercalating dye (e.g. Ethidium bromide). DNA fragments resulting from DNA damage migrate faster than undamaged DNA. A comet-like structure with a head (undamaged DNA) and a tail (DNA fragments) is formed. For a detailed review see e.g. Collins, 2004 and Brendler-Schwaab et al., 2005.

Different variations of the comet assay have been described. The most frequently used form described here uses alkaline lysis and detects (mainly) single-strand breaks, double-strand breaks and alkalilabile sites. In contrast neutral lysis conditions are chosen to monitor double strand breaks only.

# 3 Applicability and Limitations

The main advantages of the comet assay (as described here) lie in the simple performance, the sensitivity, the possibility to analyze DNA damage in single cells and the need for only low numbers of cells. Furthermore, the comet assay works well for almost any eukaryotic cell population. For a review compare Speit et al. 1999. However, due to the only semi-automated microscopic analysis the assay is fairly time consuming and prone to operator bias as soon as NM agglomerates are visible. Moreover nanomaterial (NM) interference can only be monitored but not eliminated and constitutes a main issue in operator bias.

DNA fragmentation during cell death is also a kind of DNA breakage that can be detected in the comet assay. However this fragmentation is not due to genotoxicity. To avoid such false positive results sublethal concentrations of the stimuli of interest (i.e. NMs) are tested. These sublethal concentrations have to be determined using a cytotoxicity assay (e.g. the MTS assay) prior to genotoxicity testing. As different NMs induce cytotoxicity at different concentrations we are not able to suggest definite concentrations for groups of NMs here. Instead we describe the analysis of ZnO NMs as an **example**. See chapter 6 “Procedure”.

**Note:** The dilution procedure and possibly also the solvent will vary with the type of NM and its specific cytotoxicity.

# 4 Related Documents

**Table 1:** Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

| Document ID | Document Title |
|---|---|
| cell culture_THP-1 | *Culturing THP-1 cells* |
| | *Blinding Sheet* |
| | *Comet Assay IV software manual* |
| M_NM suspension_metal oxides | *Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions* |
| M_NM suspension_ carbon based | *Suspending and diluting Nanomaterials – Carbon based nanomaterials* |

# 5 Equipment and Reagents

## 5.1 Equipment

- 6-well cell culture plates
- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Cover slips 21 x 60 mm
- Electrophoresis chamber (horizontal system for standard microscope slides e.g. Biostep)
- Fluorescence microscope and respective hardware to assess Ethidium bromide fluorescence connected to a computer equipped with Comet Assay IV software (Perceptive instruments)
- Glass cuvette (with space for 16 microscope slides)
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Magnetic stirrer
- Microcope slides SuperFrost (e.g. Roth)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Microwave oven
- Vortex®
- Water bath

## 5.2 Reagents

For cell culturing and differentiation:

- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin[1)]
- Penicillin[1)]
- Phorbol 12-myristate 13-acetate (PMA) *[CAS number: 16561-29-8]*
  **Note: Carcinogenic! Handle with special care! Special waste removal** (see chapter 8)
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin[1)]
- Trypsin-EDTA (0.05%)

[1)] bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

For the comet assay procedure in general and waste treatment:

- Agarose *[CAS number: 9012-36-6]*
- Agarose Low Melt (LMP agarose) *[CAS number: 39346-81-1]*
- Dimethyl sulfoxide (DMSO) *[CAS number: 67-68-5]*
- Ethanol puriss. p.a. *[CAS number: 64-17-5]*
- Ethidium bromide *[CAS number: 1239-45-8]*
  **Note: Potentially mutagenic! Handle with special care! Special waste removal** (see chapter 8)
- Ethyl methanesulfonate (EMS) *[CAS number: 62-50-0]*
  **Note: Toxic! Handle with special care!** (see chapter 8)
- Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2$-EDTA·2 $H_2O$ = EDTA) *[CAS number: 6381-92-6]*
- Hydrochloric acid (HCl) *[CAS number: 7647-01-0]*
  **Note: Corrosive and Irritating! Handle with special care!** (see chapter 8)
- *N*-Lauroylsarcosine sodium salt *[CAS number: 137-16-6]*
  **Note: Irritating! Handle with special care!** (see chapter 8)
- Sodium chloride (NaCl) *[CAS number: 7647-14-5]*
- Sodium hydroxide (NaOH) *[CAS number: 1310-73-2]*
  **Note: Corrosive! Handle with special care!** (see chapter 8)
- Tris(hydroxymethyl)aminomethane buffer substance (TRIS; e.g. Sigma 93367)
- Triton™ X-100 *[CAS number: 9002-93-1]*
  **Note: Irritating! Handle with special care!** (see chapter 8)

## 5.3 Reagent Preparation

### 5.3.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
  - 50 µg/ml Penicillin
  - 50 µg/ml Streptomycin
  - 100 µg/ml Neomycin
- 0.2 mg/ml L-glutamine

### 5.3.2 PMA stock solution

Prepare a 1 mM stock of PMA in DMSO. Therefore resuspend the 1 mg (standard packaging size) PMA powder in 1.62 ml DMSO. Aliquote and freeze at -20°C. Can be stored for years.

**Note: Carcinogenic! Handle with special care! Special waste removal** (see chapter 8)

### 5.3.3 Agarose

Prepare a 1.5% agarose solution in PBS. Can be stored up to one month at 4°C.

- Dissolve 1.5 g agarose in 100 ml PBS.
- Boil in the microwave oven until completely dissolved.

### 5.3.4 Agarose Low Melt (LMP agarose)

Prepare a 0.5% agarose low melt solution in PBS. Can be stored up to one month at 4°C.

- Dissolve 0.5 g agarose in 100 ml PBS.
- Boil in the microwave oven until completely dissolved.

### 5.3.5 NaOH

Prepare a 5 M NaOH solution. Can be stored for several months at RT.

- Dissolve 200 g NaOH pellets in 1 l dd$H_2O$.

**Note: Be careful, exothermic reaction, gets HOT. NaOH is corrosive, wear protective clothing (especially eye protection).**

### 5.3.6 EDTA

Prepare a 0.2 M $Na_2$-EDTA·2 $H_2O$. Adjust to pH10 with NaOH. Can be stored for several months at RT.

- Dissolve 18.612 g $Na_2$-EDTA·2 $H_2O$ in 250 ml dd$H_2O$.

### 5.3.7 Lysis buffer

2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% N-Laurylsarcosine sodium salt

This buffer is the main component of the "Lysis solution" which always has to be prepared freshly before usage (see chapter 6 "Procedure"). In contrast the lysis buffer itself can be stored up to one month at RT.

| | | |
|---|---|---|
| (1) | 146.1 g/l | NaCl |
| (2) | 37.22 g/l | $Na_2$-EDTA·2 $H_2O$ |
| (3) | 1.21 g/l | TRIS |
| (4) | 10 g/l | *N*-Lauroylsarcosine sodium salt (**Irritating! Handle with special care!**) |

- Dissolve components (1) to (3) in 900 ml dd$H_2O$.
- Adjust pH to 10 with NaOH.
- Add 10 g *N*-Lauroylsarcosine sodium salt (component (4)).
- Add up to 1 l with dd$H_2O$.
- Stir until the solution gets clear. **Note:** this can take several hours.

### 5.3.8 TRIS neutralization buffer

Prepare a 0.4 M TRIS solution in dd$H_2O$. Adjust to pH7.5 with HCl. Can be stored for several months at RT.

- Dissolve 48.452 g TRIS in 1 l dd$H_2O$.
- Adjust pH to 7.5 with NaOH.

### 5.3.9 Ethidium bromide staining solution

Prepare a 20 µg/ml Ethidium bromide solution in dd$H_2O$. Can be stored for several months at RT in the dark.

- Dissolve 1 mg Ethidium bromide in 50 ml dd$H_2O$.

**Note: Be careful, ethidium bromide is a potential mutagen. Wear protective clothing (nitrile gloves), avoid exposure.**

### 5.3.10 Lysis solution

**Note: to be prepared freshly on the day of usage!**

Prepare 75 ml lysis solution directly in the glass cuvette. This volume is enough for a maximum of 16 micrsocope slides. Wrap with aluminum foil. **Cool to 4°C.**

| | |
|---|---|
| 66.75 ml | lysis buffer (see 5.3.7) |
| 7.50 ml | DMSO |
| 0.75 ml | Triton X-100* |

*Triton X-100 is highly viscous. Cut the front tip of the pipette to enlarge the hole. Pipet slowly and carefully. Weighing the liquid would be an alternative. With a density of 1.07 g/$cm^3$ you need 0.8 g.

#### 5.3.11 Electrophoresis buffer

**Note: to be prepared freshly on the day of usage!**

**Note: Be careful, 5 M NaOH is corrosive, wear protective clothing (especially eye protection).**

Prepare 1.25 l electrophoresis buffer. This volume is enough to fill the electrophoresis chamber once and with that to run a maximum of 20 microscope slides in parallel. **Cool to 4°C.**

| | |
|---|---|
| 75.00 ml | 5 M NaOH (see 5.3.5) |
| 6.25 ml | 0.2 M EDTA (see 5.3.6) |

Add up to 1.25 l with dd$H_2O$ (1.17 l).

# 6 Procedure

## 6.1 To get started

All volumes given are enough for a maximum of 16 samples. However, working with fewer samples requires the same volumes of lysis solution and electrophoresis buffer as the same vessels have to be filled.

At least one day prior to experimental start:

**Dip-coat SuperFrost microscope slides with 1.5% agarose in PBS:**

- Submerge SuperFrost microscope slides in dd$H_2O$ and autoclave. This procedure improves agarose coating quality and efficiency.
- Use SuperFrost microscope slides while they are still warm but dry them before dip-coating in agarose.
- Boil 1.5% agarose solution in PBS (using a microwave oven). Make sure agarose is dissolved completely.
- Pour agarose into a 50 ml conical tube.
  **Note:** To avoid agarose curing keep temperature up. This can be accomplished by wrapping the tube into tissues and putting it into a Styrofoam box.
- Dip SuperFrost microscope slides into agarose, avoid bubble formation. Repeat dipping process at least three times. Check visually that slides are covered homogenously with agarose.
- Clean the rear side of the slides with a tissue. Place horizontally on a clean table.
- Let slides air dry overnight (ON) at RT.

**Note:** Agarose coated slides can be stored protected from dust at RT up to one month.

At the day of assay performance:

- Pre-warm complete cell culture medium, PBS and Trypsin-EDTA to 37°C.
  *Necessary for cell harvest.*
- Prepare 75 ml lysis solution in a glass cuvette and cool to 4°C
- Prepare electrophoresis buffer and cool to 4°C.
  **Note: 5 M NaOH is corrosive. Wear appropriate protective clothing.**
- Heat LMP agarose in the microwave oven. Make sure agarose is dissolved completely. Temper to 37°C in a water bath.

## 6.2 Flow chart

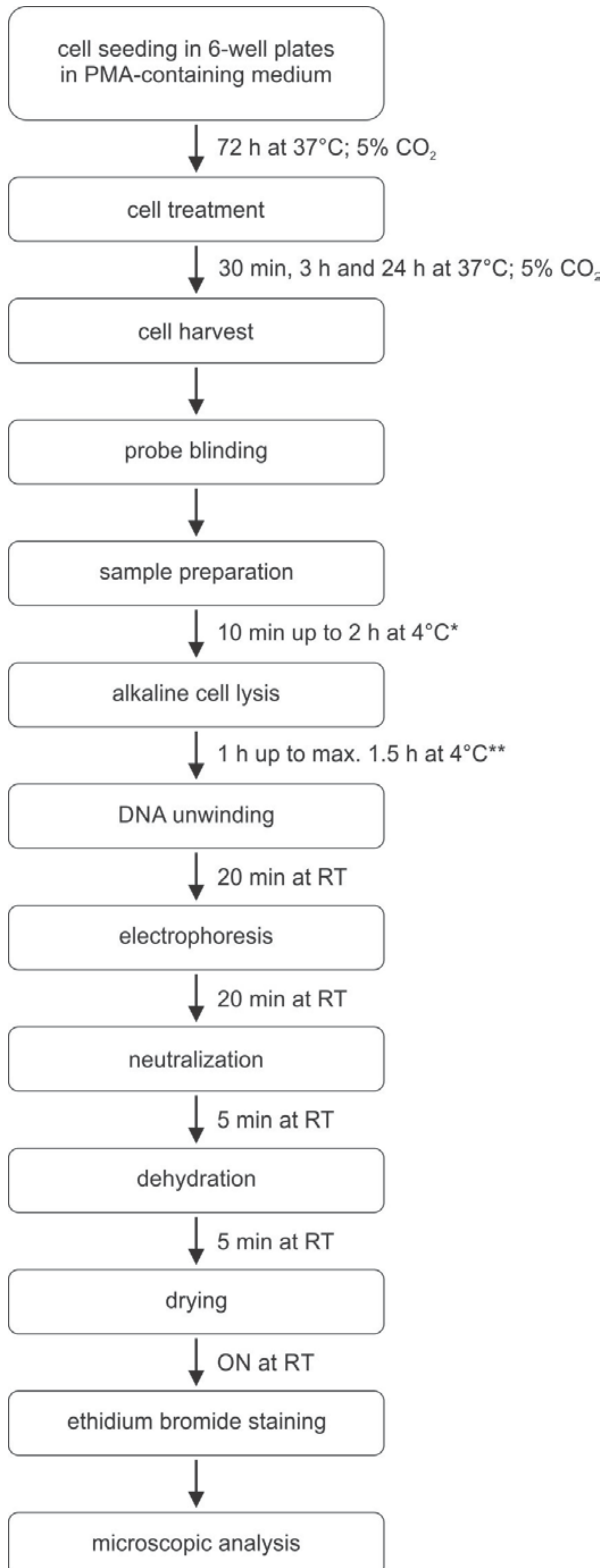


**Figure 1:** Brief outline of the workflow; from cell seeding to analysis. Samples should be processed without delay up to sample preparation.

*Agarose will cure within 10 min. If a break is necessary the curing process can be extended to up to 2 h.

**Alkaline cell lysis: time for cell lysis should be kept constant from one experiment to the next. Up to 30 min difference is acceptable.

## 6.3 Cell seeding

### 6.3.1 Cell culture

THP-1 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% $CO_2$ in humidified air in an incubator (standard growth conditions according to SOP "Culturing and differentiating THP-1 cells").

### 6.3.2 Cell seeding into 6-well plates

- Three days (72 h) prior to experimental start harvest and count cells as described in SOP "Culturing and differentiating THP-1 cells".
- Seed $5x10^5$ cells in 2.5 ml complete cell culture medium containing 200 nM PMA per well into a 6-well cell culture plate.
- For one time point 8 wells are needed as shown in Figure 2. Therefore $4.5x10^6$ cells are suspended in 22.5 ml complete cell culture medium ($2x10^5$ cells/ml). To assess 3 time points (30 min, 3 h and 24 h), 24 wells are necessary ($1.35x10^7$ cells in 67.5 ml).

  **Note:** PMA is diluted 1:5000 from the 1 mM stock (13.5 µl/67.5 ml medium).

- Using a 5 ml pipette 2.5 ml of this cell suspension are distributed into each of the green wells depicted in Figure 2.
- Cells are differentiated in a humidified incubator at standard growth conditions for three days (72 h).

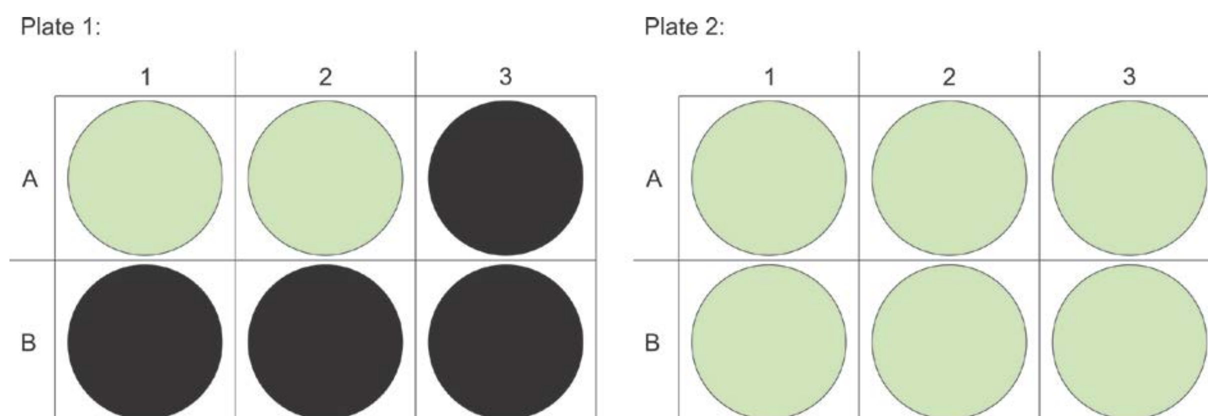


**Figure 2: Cell seeding into 6-well plates.** Depicted are 2 plates (8 wells in green) necessary to analyze one time point. Multiply for each additional time point. Cells are seeded at a density of $5x10^5$ cells per well in 2.5 ml complete cell culture medium into each of the green wells. Black wells remain empty.

## 6.4 Cell treatment

### 6.4.1 Dilution of nanomaterials

As described in chapter 3 "Applicability and limitations" DNA disintegrates during cell death and therefore yields false positive results. Real genotoxic events can only be assessed using sublethal concentrations of the stimuli of interest (i.e. NMs). ZnO NMs show no cytotoxicity up to 12 µg/ml – not even after 72 h of treatment. Here we exemplarily describe the assessment of ZnO NM genotoxicity. We use 4 sublethal concentrations to assess genotoxicity and additionally check for

DNA disintegration at a toxic concentration (100 µg/ml). Three time points are analyzed: 30 min, 3 h and 24 h.

**Note:** The dilution procedure and possibly also the solvent will vary with the type of NM and its specific cytotoxicity.

Volumes given in the following dilution schemes are enough for three time points were cells are seeded in 6-well plates as shown in Figure 2.

**Note:** "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in dd$H_2O$:

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
- Add 2.25 ml dd$H_2O$ to tube no. 2.
- Add 750 µl dd$H_2O$ to tube no. 3.
- Add 1.2 ml dd$H_2O$ to tubes no. 4 to 6

1. ZnO NM stock suspension in dd$H_2O$ → 1 mg/ml (1)
2. 250 µl of the 1 mg/ml suspension (1) are mixed with 2.25 ml dd$H_2O$ → 100 µg/ml (2)
3. 750 µl of 100 µg/ml (2) are mixed with 750 µl dd$H_2O$ → 50 µg/ml (3)
4. 300 µl of 50 µg/ml (3) are mixed with 1.2 ml dd$H_2O$ → 10 µg/ml (4)
5. 300 µl of 10 µg/ml (4) are mixed with 1.2 ml dd$H_2O$ → 2 µg/ml (5)
6. 1.2 ml dd$H_2O$ → solvent control (6)

Preparation of final dilutions:

- Label six conical tubes (15 ml total volume) as follows:
    1. 100 µg/ml
    2. 10 µg/ml
    3. 5 µg/ml
    4. 1 µg/ml
    5. 0.2 µg/ml
    6. Solvent control
- Add 9 ml complete cell culture medium to each tube.
- Mix on the Vortex with 1 ml of the respective NM sub-dilutions or the solvent (dd$H_2O$):
    1. 1 ml of the stock suspension (1 mg/ml) are mixed with 9 ml medium → 100 µg/ml (1)
    2. 1 ml of the 100 µg/ml sub-dilution are mixed with 9 ml medium → 10 µg/ml (2)
    3. 1 ml of the 50 µg/ml sub-dilution are mixed with 9 ml medium → 5 µg/ml (3)
    4. 1 ml of the 10 µg/ml sub-dilution are mixed with 9 ml medium → 1 µg/ml (4)
    5. 1 ml of the 2 µg/ml sub-dilution are mixed with 9 ml medium → 0.2 µg/ml (5)
    6. 1 ml of dd$H_2O$ (solvent) are mixed with 9 ml medium → solvent control (6)

### 6.4.2 Dilution of EMS (chemical positive control)

Prepare a 1 M sub-dilution of the EMS stock (9.7 M) in $ddH_2O$ **right before application (hence, volumes are given for one time point only)**. Make sure to mix well (vortex!) and apply directly after mixing.

- Mix 17.4 µl $ddH_2O$ with 2 µl of the EMS stock.

Apply 12.5 µl of the 1 M sub-dilution directly to the 2.5 ml complete cell culture medium in the 6-well. This results in a final concentration of 5 mM EMS in medium.

### 6.4.3 Application of stimuli

**Note:** All NM dilutions have to be vortexed directly before application to the cells.

Three days (72 hours) after cell seeding:

- Remove PMA-containing complete cell culture medium. ***Toxic! Discard this supernatant separately! See Chapter 8.***
- Wash cells twice with 1 ml of pre-warmed PBS per well.
- Add 2.5 ml per well of complete cell culture medium containing the corresponding NM concentrations or the solvent control (0 µg/ml) according to the pipetting scheme shown in Figure 3.
- Add 2.5 ml complete cell culture medium to untreated as well as EMS samples.
- Culture cells for appropriate time points (30 min, 3 h, 24 h) under standard growth conditions.
- 30 min prior to the end of the NM incubation time add 37.5 µl 1 M EMS directly to the EMS sample. Mix by gently swirling the plate.
- Incubate the remaining 30 min under standard growth conditions.

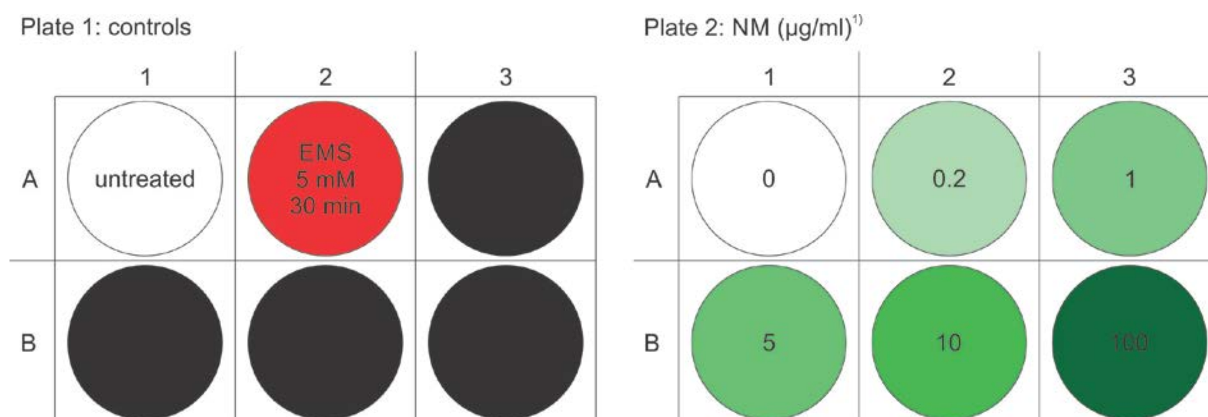


**Figure 3 Application of stimuli**. NMs as well as the solvent (0 µg/ml = $ddH_2O$) are applied in 2.5 ml complete cell culture medium per well. Untreated samples (plate 1 well A1) receive complete cell culture medium only. Black wells on plate 1 remain empty.

[1)] NM concentrations given here refer to ZnO NMs. 100 µg/ml is a lethal dose whereas all the other concentrations are sublethal.

**Note**: EMS is applied at a final concentration of 5 mM 30 min prior to the end of the NM incubation time. All samples of one time point are harvested together.

## 6.5 Cell harvest and actual comet assay procedure

### 6.5.1 To be set before cell harvest

- Ice box to cool down samples after harvest.
- Prepare a set of 1.5 ml microreaction tubes: 1 tube per sample, labeled as follows, to be used for detached cells in suspension:
    - 100 µg/ml
    - 10 µg/ml
    - 5 µg/ml
    - 1 µg/ml
    - 0.2 µg/ml
    - Solvent control
    - EMS
    - Untreated
- Prepare a second set of 1.5 microreaction tubes: 1 per sample, NOT labeled, to be used for LMP agarose.
- Prepare self-sticking labels; numbered (1 to 8) plus time point of harvest (30 min, 3 h, 24 h) to code the samples.
  **Note:** blinding of samples before analysis prevents biased scoring (at least as long as NM agglomerates are not directly visible).
- Prepare a sheet that allows decoding the blinding after analysis. This has to be filled out by a neutral person. (see related documents "blinding sheet")
- Prepare agarose pre-coated SuperFrost microscope slides. 1 per sample. Numbered (1 to 8) plus time point of harvest (30 min, 3 h, 24 h). This labeling relates to the self-sticking labels and is recorded on the blinding sheet.
  **Note:** Use a **pencil** for labeling the slides. Ink of other types of pens dissolve under alkaline conditions during cell lysis later on!

### 6.5.2 Cell harvest

- After appropriate time points (30 min, 3 h, 24 h) remove complete cell culture medium.
- Wash cells twice with pre-warmed (37°C) PBS.
- To detach cells add 0.2 ml Trypsin-EDTA per well and incubate for approximately 5-10 min at 37°C.
  **Note**: Differentiated THP-1 cells stick strongly to the cell culture plate. Detachment might take even longer than 10 min. However, 15 min of Trypsin-EDTA incubation should not be exceeded.
- Add 0.3 ml complete cell culture medium to inhibit Trypsin and stop the detachment reaction. Remove cells by rinsing the bottom of the wells several times.
- Transfer cell suspension to the labeled 1.5 ml microreaction tubes and put directly on ice.

### 6.5.3 Probe blinding – to be done by a neutral person!

- Randomly put the self-sticking labels (numbered 1 to 8 plus time point of harvest) on to the labeled 1.5 ml microreation tubes (containing the cell suspension, staying on ice). Original label (on the microreaction tubes) shall not be visible any more.
- Record which sample received which number on the blinding sheet.

- Sort samples numerically (1 to 8) so the operator cannot remember the initial order (and content) of the samples.
- Samples and prepared agarose pre-coated SuperFrost microscope slides are now labeled identically. During sample preparation put each sample on to its respective SuperFrost microscope slide.

### 6.5.4 Sample preparation and cell lysis

- Use non labeled 1.5 ml microreaction tubes to mix 160 µl LMP agarose with 40 µl cell suspension.
- Mix by carefully pipetting up and down. Avoid bubble formation!
- Pipet 180 µl of the LMP agarose-cell mixture on the respective agarose pre-coated SuperFrost microscope slide.
  **Note:** To assure single cell suspensions (rather than cell agglomerates) and homogenous distribution of the cells in the agarose it is recommended to use a yellow (200 µl) pipette tip for this step.
- Cover with a 21 x 60 mm cover slip.

**Note:** LMP agarose will cure quite fast. Therefore do not pipet LMP agarose for all samples in advance. Rather prepare one sample after the other: LMP agarose first, cell suspension second, put mixture onto microscope slide, cover with cover slip, proceed to next sample.

- Let agarose cure at 4°C (in the fridge) for approximately 10 min.
  **Note:** This step can be extended to up to 2 h.
- Remove cover slip by gently turning it off the slide.
- Put slides back-to-back into the pre-cooled (4°C) lysis solution in the glass cuvette.
- Lyse cells for 1 h (to maximally 1.5 h) at 4°C.

### 6.5.5 DNA unwinding and electrophoresis

- Prepare the electrophoresis chamber:
  - Ensure that the chamber is leveled (in case of doubt use a bubble level).
  - Fill the chamber with electrophoresis buffer.
    **Note: Be careful, buffer is corrosive; wear protective clothing (especially eye protection).**
  - Fix the voltage at the power supply to 24 V.
  - Check the current and adjust it to 300 mA by adapting the buffer volume (current above 300 mA: remove buffer from the chamber; current below 300 mA: add buffer to the chamber).
- After 1 h of cell lysis take slides out of the lysis solution.
- Drain slides on a tissue. Be careful not to remove the agarose layers – only remove excess drops of buffer!
- Put slides into the prepared electrophoresis chamber.
  **Note:** DNA migrates towards the positively charged anode. Make sure the labeling of the slides faces the cathode (negative pole) as shown in Figure 4 A
- Slides should be completely covered with electrophoresis buffer.
  **Note:** Do not change buffer volume any more since current has already been adjusted.

- Allow DNA unwinding during 20 min **without** current.
- Perform electrophoresis during additional 20 min.

### 6.5.6 Neutralization, dehydration and ethidium bromide staining

- After electrophoresis take slides out of the chamber, drain them on a tissue and put them (back-to-back) into a glass cuvette filled with TRIS neutralization buffer.
- Incubate for 5 min at RT.
- Rinse slides with $ddH_2O$ by gently shaking the slides in a beaker filled with water.
- For dehydration put slides (back-to-back) into a glass cuvette filled with ethanol (absolute).
- Incubate for 5 min at RT.
- Take slides out, dry ON leaning upright at RT.
  **Note:** Avoid dust to deposit on the slides. Drying in a fume cupboard may help.

- Add 60 µl of a 20 µg/ml ethidium bromide solution per slide.
  **Note: Potentially mutagenic! Handle with special care! Special waste removal.**
- Cover with a 21 x 60 mm cover slip.

- Analyze at a fluorescence microscope using Comet Assay IV software. For software details refer to the manual (related documents). Procedure in brief below.

### 6.5.7 Microscopic analysis

- Put slide into the slide support with the cover slip facing the objective.
- For the software the orientation of the comet is important. In practical terms: put the labeling area of the microscope slide to the right. With that also the comet head will be on the right while the tail extends to the left (according to the migration direction of the DNA during electrophoresis). **Important to know:** the picture you see in the microscope oculars and on the computer screen will be exactly the other way around: comet head on the left and the comet tail extending to the right (see Figure 4 B).
- Get an overview on the homogeneity of each sample by looking around the whole slide. Quality criteria see below (chapter 7).
- Do not score comets at the outermost edges of the slide (see Figure 4 C), these are usually highly damaged.
- Score 100 comets in total. Make sure not to score the same comet twice. Therefore find a suitable direction of working. E.g. start close to the labeling area of the microscope slide and proceed to the opposite edge zigzagways (see Figure 4 C).
- Scoring itself:
  - Focus in the live mode on the computer screen.
  - Click on the center of the comet head (left mouse key) to initiate the measurement.
  - A photograph is taken and the fluorescence intensity of the comet (head and tail) as well as tail length is measured (see Figure 4 D).
  - In Figure 4 D: The blue line represents the comet start, the green line the comet center (usually the brightest spot) and the purple line the comet end. A symmetrical head is modeled from its center and depicted as the green fluorescence intensity curve underneath the comet. The fluorescence intensity of the tail is shown as an

orange curve and resembles the fluorescence between the comet head center and the comet (tail) end minus the fluorescence that is attributed to the head.
    - Start (blue), middle (green) and end (purple) lines can be adjusted and placed manually. Therefore the software button “Edit” is used and the lines are dragged with the mouse. Editing is finished by clicking the right mouse key.
- The program alerts you when you have scored 100 comets. Safe the results of all scorings in an excel file (software simply allows to “safe as” as in almost all windows programs, export to excel is done automatically). The excel-file will contain the following information:
    - Head length
    - Tail length
    - Head intensity
    - Tail intensity
    - Tail moment
    - Total area
    - Mean gray level
    - Total intensity
    - Recording data: year – month – day – hour – minute – second
- The program furthermore safes all pictures automatically in the “Audit” folder, marked by date and time. So every picture can be allocated to the scoring values in the excel file.

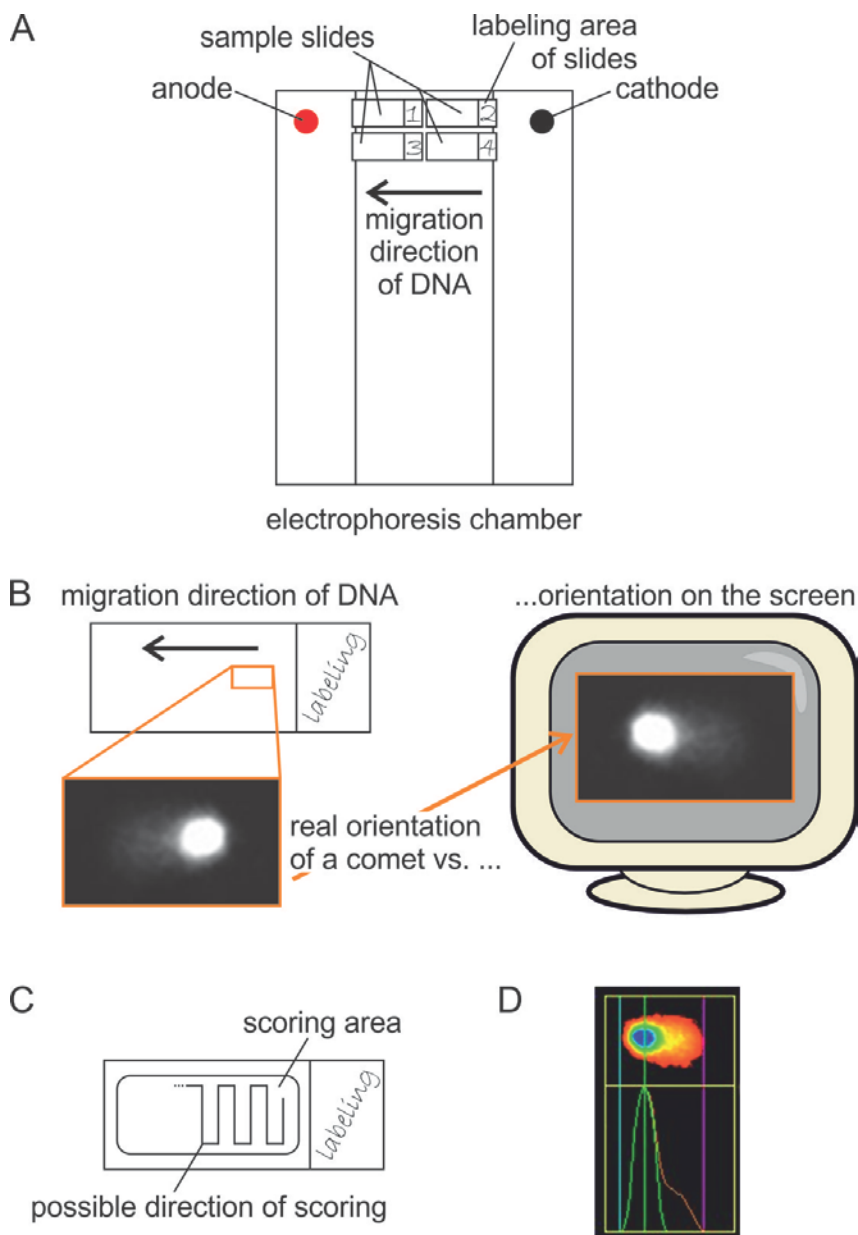


**Figure 4:**
**Practical overview of comet assay performance.**

A) Electrophoresis chamber with orientation of the SuperFrost microscope slides and migration direction of DNA towards the anode.

B) Magnification of the microscope slide and an example of a comet. On the left: real orientation how it appears on the microscope slide. On the right: orientation seen on the computer screen.

C) Do not score comets on the edges of the microscope slide. A fictional scoring area is shown. To avoid scoring the same comet twice a possible zigzag way of scoring is drawn.

D) Automated scoring by the software. Blue line: start of the comet. Green line: center of the comet head. Purple line: end of the comet. Green curve underneath the comet: fluorescence intensity of the head. Orange curve underneath the comet: fluorescence intensity of the tail.

## 6.6 Data evaluation

Tail intensity or the tail moment are the two most common measures of DNA breakage. Tail intensity equals the percentage of DNA present in the tail of the comet. The tail moment further takes the tail length into account. It is defined as the product of the tail length and the fraction of total DNA in the tail.

In both cases the mean of the 100 scoring values and their standard deviations are calculated. At least three independent experiments have to be performed and the mean and standard deviation of the three experiments are calculated.

Additionally a frequency distribution of the tail intensities (or tail moments) can be shown for each sample.

# 7 Quality Control, Quality Assurance, Acceptance Criteria

Pay attention to the following parameters:

- Is the size of the comet tail homogeneous? Different sizes ranging from no tails (basically only completely round comet heads) to large comet tails may point to apoptosis induction.
- Are so called “ghosts” present? Comets consisting of only a tail having nearly no more head are called “ghosts”. They represent most likely apoptotic cells. Ghosts usually appear at the edges of the microscope slide and are not scored. In untreated control samples no more than 5% of all comets should appear like “ghosts”.
- The mean tail intensity in untreated control samples (THP-1 cells) should not exceed 8%.

Measurements (by the software) can be erroneous. Find some examples and solutions to the problems below:

- The brightest spot is NOT in the center of the comet head.
  In this case fluorescence intensities are not assigned correctly to head and tail regions respectively. Adjust the center line (green) manually.
- NMs or dust may hide parts of the comet.
  Do not score comets which are associated with foreign objects!
- Two comets are very close to each other or even overlapping.
  Do not score these ones. Only score single comets.

# 8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards).

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

Handle all the following chemicals with special care. **Wear suitable protective clothing** (gloves, lab coat, respiratory protection, eye protection):

- Ethidium bromide is a **potential mutagen.**
  It is irritating to eyes, the respiratory system and the skin. Therefore do not breathe in dust, avoid contact with skin and eyes and avoid exposure in general. Furthermore, it may cause heritable genetic damage.
  All materials used to handle Ethidium bromide (including pipet tips, gloves, slides) are to be disposed separately.
- EMS is **toxic**.
  It is harmful when swallowed and may cause heritable genetic damage. Avoid exposure, do not breathe in gas/fumes/vapor/spray.
- NaOH is **corrosive**.
  It causes severe burns. Wear especially eye/face protection.
  Dissolution of NaOH is an exothermic reaction, the solution will get fairly hot – be careful! It

is strongly recommended to wear eye protection when handling 5 M NaOH as well as the electrophoresis buffer.

- *N*-Lauroylsarcosine sodium salt is **irritating**.
  It is irritating to eyes, the respiratory system and skin. Therefore do not breathe in dust, avoid contact with skin and eyes and avoid exposure in general.
- HCl is **corrosive and irritant**.
  It is very hazardous in case of skin contact, of eye contact and of ingestion. It is slightly hazardous in case of inhalation. Therefore avoid inhalation as well as contact with skin and eyes and avoid exposure in general.
- Triton X-100 is **irritant**.
  It is hazardous in case of eye contact, ingestion and inhalation and slightly hazardous in case of skin contact. Therefore avoid inhalation as well as contact with skin and eyes and avoid exposure in general.
- **PMA waste treatment:** use a separate exhaust extraction system with a collecting flask containing already 20 ml 5 M NaOH to neutralize PMA. The resulting non-toxic solution is very alkaline and has to be neutralized using HCl before final disposal in the sink.

# 9 Abbreviations

| | |
|---|---|
| dd$H_2O$ | double-distilled water |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid disodium salt dihydrate |
| EMS | ethylmethanesulfonate |
| FCS | fetal calf serum |
| g | constant of gravitation |
| LMP | Low Melting Point |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-cyrboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| NM | nanomaterial |
| ON | overnight |
| PMA | phorbol 12-myristate 13-acetate |
| PSN | Penicillin, Streptomycin, Neomycin |
| RPMI | Roswell Park Memorial Institute medium |
| RT | room temperature |
| TRIS | Tris(hydroxymethyl)aminomethane buffer substance |

## 10 References

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