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Supplementary information

Measuring DNA modifications with the comet assay: a compendium of protocols

In the format provided by the authors and unedited

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Supplementary Protocol 1: Detection of DNA inter-strand crosslinks (ICL-modified alkaline comet assay).

Additional reagents & equipment

- Hydrogen peroxide (H_2O_2) (Millipore, cat. no. 107298)
  ! CAUTION Causes severe skin burns and eye damage.
- Low linear energy transfer ionizing radiation (i.e. X-rays and gamma-rays)
  ! CAUTION Ionizing radiation is harmful to all cells in the human body

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Treatment of the cells

∆ CRITICAL These steps are performed before Stage 1 of the main Procedure.
1) Treat the cells with test compound and positive control (i.e. an agent that induces ICL).
2) Treat the cells subsequently with 0 (control) or 50 µM H_2O_2 for 30 min (the latter is sufficient to induce a requisite number of SB to create a DNA comet tail of suitable size). Ionizing radiation (2 to 5 Gy) is the ideal agent for induction of strand breaks because the comet-to-comet variability in DNA migration is more heterogeneous for hydrogen peroxide-treated cells than for cells exposed to X-rays or gamma-rays [1].

Stage 1: Preparation of cells from fresh or frozen samples

1) Pellet the cells (7600 × *g* for 5 min, 4 °C), and wash three times with 1 mL PBS. Process further starting from Stage 2A of the main Procedure. Alternatively, the exposure to the DNA strand breaking agent can be carried out on gel-embedded cells by submerging slides into H_2O_2 solution or by irradiation of slides.

Anticipated results

The level of DNA inter-strand cross-linking can be expressed as percentage decrease in TI compared to the DNA strand breaking agent (i.e., H_2O_2, or radiation) treated controls, according to the following formula:

$$\text{Percent decrease in TI} = [1-((TIdi - TIcu)/(TIci - TIcu))] \times 100$$

Where TIdi = TI of crosslinking agent-treated and DNA strand breaking agent treated sample;
TIcu = TI of control untreated with crosslinking agent, untreated with DNA strand breaking agent;
TIci = TI of control untreated with crosslinking agent, but treated with DNA strand breaking agent.

Supplementary Protocol 2. Application of HU/AraC repair inhibitors to detect bulky adducts

∆ **CRITICAL** The concentrations of the repair enzyme inhibitors given below are suggestions from using HepaRG^{TM} cell lines. HU and AraC lead to a slight increase in TI. Therefore, dose-dependent experiments with HU and AraC should be performed for each cell line to ensure that the background level of DNA damage is kept low and cell viability is greater than or equal to 80%.

Additional reagents

- Hydroxyurea (HU) (Merck KGaA, cat. no. H8627)
  ! CAUTION Mutagenic
- 1-β-D-arabinofuranosyl cytosine (araC) (Merck KGaA, cat. no. C1768)
  ! CAUTION Potential mutagen

Procedure

∆ **CRITICAL** These steps should be performed in place of the indicated steps of the main Procedure.

Treatment of the cells (Before Stage 1):

1) Incubate the cells with HU and AraC to reveal the presence of UV-induced adducts following option A, or follow option B to study adducts induced by metabolic activation of test compounds such as B[a]P or aflatoxin B1 (AFB1).

(A) ***Detection of UV-induced adducts***

(i) Pre-incubate the cells with a solution made of 1 mM HU and 10 µM AraC in culture medium supplemented with 10 mM glutathione for 40 min before UV irradiation.

∆ **CRITICAL STEP** Some cell types – in particular mononuclear cells (MNCs) – have lower levels of nucleotides and therefore much less HU is needed to block DNA replication. Hence, the protocol for repair inhibition is cell type specific and should be optimized for each cell type.

(ii) Irradiate cells with UVC at 4 °C in the dark at a desired dose range and time.

∆ **CRITICAL STEP** The specific time and dose of the UVC irradiation will depend on the cell type and experimental setup, and therefore need to be tested in each lab. As an example, HepaRG cells irradiated with 5 J/m^2 UVC and allowed to repair in the presence of HU and AraC for one hour after UV exposure reached a steady state of ~80% tail in DNA.

(iii) Post UV irradiation, incubate the cells additionally in medium containing HU and AraC for the desired time points.

(B) ***Detection of adducts induced after metabolic activation***

(i) Incubate the cells with test chemicals together with repair inhibitors for 24 h (or the desired treatment time).

2) In parallel to HU and AraC incubated cells exposed to test chemicals/UV irradiation, prepare untreated cells that have only been exposed to HU and AraC.

∆ **CRITICAL STEP** The inclusion of HU and AraC repair inhibitors in the comet assay cannot be combined with the enzyme-modified comet assay.

Supplementary Protocol 3. High throughput comet assay: Application of mini-gels on a GelBond® film

The high throughput system has been validated using ionizing radiation or different chemicals in combination with enzyme treatment [2,3,4 5].

Additional equipment

- 48- and 96-well frame for moulding the gels in GelBond® films (NorGenoTech)
- 48- and 96-well frame for handling the GelBond® films (NorGenoTech)
- 12-Gel Comet Assay Unit (NorGenoTech)

Equipment set up

The GelBond® film is used as a support for agarose gels in this protocol. The film has two sides, one hydrophobic and one hydrophilic. Make sure to use the hydrophilic side. The film is cut to the size of a standard microtiter plate format (85×125 mm) with holes in each corner and a cut corner down to the right for correct orientation. The film is attached to a plastic frame at all stages of the comet assay for easy handling and to protect the gels. The GelBond® film is versatile as it can be used to process as many mini-gels as desired, typically 12, 48 or 96 gels with different volumes ranging from 4-7 µL respectively. Due to the shape and size of mini-gels, no coverslip is needed.

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 2A: Processing gels for the standard alkaline comet assay

Prepare materials: modification of step 6.

1) Prepare lysis solution and store at 4 °C until use (50 mL per GelBond® film is needed)

Prepare materials: Modification of steps 7-8.

1) Turn on the heating block (37 °C) and place 0.2 mL-tubes or 8-well strips in the heating block (for a 96-format).
2) Prepare a cooling block for 0.2 mL-tubes or 8-well strips for the required number of samples that fits an 8-channel pipette. Alternatively, a pipette with adjustable tip spacing can be used.
3) Mark the GelBond® films with the sample code/name, date and film number; use a diamond pen to mark the plastic. Make sure that the cut corner of the film is on the lower right.
4) Attach each film to a plastic frame (see **Figure 5**), and mark the frame using tape and pencil (resistant to EtOH).

Embedding cells in LMP agarose and cell lysis and application of cells and agarose to GelBond® film: Modification of step 10.

1) Mix the cell suspension carefully with LMP agarose (37 °C) to a final concentration of 0.5-1×10^5 cells/mL.

∆ CRITICAL STEP If you are using a multichannel pipette, make sure (by visual inspection) that you have equal amounts of agarose/cell mixture in each pipette tip. You should also make sure you have more agarose/cell mixture in the tip than the volume that will be applied to the GelBond® film as viscosity can prevent complete ejection.

Embedding cells in LMP agarose and cell lysis and application of cells and agarose to GelBond® film: Modification of steps 11-14.

1) Add gels quickly while the films are resting on a cooling plate. The agarose settles within seconds and a film can be soaked in lysis solution without any delay.

∆ **CRITICAL STEP** If the films are left for too long on top of the cooling block prior to adding the samples, there is a risk of water condensation (particularly in hot climates). This may result in the agarose-suspension floating out and may cause the gels to mix with their neighbouring sample.

2) Adapt the volume of the gels to the different formats:

- For a 96 gel format: add 4 µL/gel (c.a. 400 cells)
- For a 48 gel format: add 7 µL/gel (c.a. 700 cells) when the distance between the tips is large
- For a 12 gel format: add 5 µL/gel (c.a. 500 cells)

∆ **CRITICAL STEP** The gels can be applied with or without formats, wells, or separating surfaces[2,4]. The agarose/cell samples can easily be applied with a multi-channel pipette.

2) Immerse the films into cold lysis solution immediately after applying the gels.

Stage 2B: Processing gels for the enzyme-modified comet assay

Prepare materials.

1) Omit steps 15-16.

Detection of specific DNA lesions: Modification of steps 20-25.

1) After the lysis, rinse the GelBond® films quickly in cold distilled water

2) Immerse the films in cold enzyme reaction buffer for 50 min at 4 °C

∆ **CRITICAL STEP** Washing can be done using Buffer B without BSA, but you need to add BSA for the incubation step.

3) Add the enzyme(s) of interest to the pre-warmed enzyme reaction buffer with BSA (37 °C). Mix thoroughly before adding the buffer to the film in a suitable sized tray or dish. In parallel, prepare a bath just with reaction buffer for the control incubation.

∆ **CRITICAL STEP** It is essential that the rather viscous protein is diluted into a homogenous solution. If incubating more than one film, prepare the enzyme solution (with enzyme) for all films in one beaker to ensure that the enzyme concentration is the same for all film.

4) Incubate the films at 37 °C in enzyme buffer (+/-enzyme) for 1 h, with manual mild shaking every 10-15 min.

∆ **CRITICAL STEP** A detailed protocol on the use of the 12-gel chamber unit (**Figure 4**) was recently published by Vodenkova et al. (Box 2 in Vodenkova et al. [6]). The assembly of the 12-gel unit is demonstrated in this tutorial video: https://youtu.be/NE2U8f5gwc8

∆ **CRITICAL STEP** The optimum enzyme concentration must be determined by titration. The concentration used with this protocol (i.e., for immersing films in the buffer +/- enzyme) is lower (approximately 5x) than that used when applying droplets to individual gels. Please note that the optimum concentration also depends on both the amount of DNA damage and the type of lesion(s) to be recognized by the enzyme.

Stage 3: Comet formation

Neutralization & Washing: Modification of step 32.

1) After electrophoresis (which is performed preferably with buffer circulation), neutralization and rinsing, fix the gels in ethanol and dry them prior to staining.

Stage 4: Comet visualization & Analysis

Comet visualization: Modification of step 33.

1) Stain gels with SYBR® Gold. Δ **CRITICAL STEP** DAPI is not compatible with plastic films.

Comet analysis: Modification of step 35-36.

1) Score comets either with a semi-automated system or a fully automated system using a microscope equipped with a fitted stage for the film or the film may be cut to sizes fitting a glass slide.

? TROUBLESHOOTING

Supplementary Protocol 4. CometChip procedure

The CometChip's ability to decrease total assay time and decrease sample variation makes the platform a versatile tool for high throughput analysis of DNA damage and DNA repair. CometChip experiments are performed using standard comet materials and methods. However, cells are organized in a micron scale grid, which prevents overlap, decreases imaging time (because all cells are on the same focal plane), and the analysis can be done in seconds using an automated software program. The method requires a new step, which is cell loading to trap cells into microwells that form a grid in agarose (**Figure 6a-e** – CometChip:

Additional equipment

- Optional: pre-made CometChip array (Trevigen, cat. no. 4260-096-01)
- Alternatively for self-made array: bottomless 96-well plate, GelBond® and clamps
- CometChip analysis software is available through Trevigen (cat. no. 4260-000-CS), or MATLAB CometChip analysis program is also available upon request from the Massachusetts Institute of Technology.

∆ CRITICAL: If using pre-made CometChip array from Trevigen, follow the manufacturer's instructions. In this case, to avoid gels detaching from the glass, handle the CometChip array gently throughout.

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 2A: Processing gels for the standard alkaline comet assay

Prepare materials: Modification of steps 4, 7-8.

1) Pour an agarose CometChip in-house or use a pre-made microwells array from Trevigen. See Wood et al. [7] or Chao and Engelward [8] for details on how to create in-house agarose arrays from reusable polydimethylsiloxane stamps.

2) Clamp a bottomless 96-well plate over the agarose slab that has an array of microwells to create 96 separate environments (defined in this text as macrowells) for sample loading. As a result, at the base of each macrowell are ~300 microwells, and each microwell ultimately becomes a comet.

Embedding cells in LMP agarose and cell lysis: Modification of steps 10-14.

1) Load cells into the agarose microwells by pipetting the cell suspensions into the CometChip macrowells. For a more detailed discussion of the technical considerations involved in the cell loading step, see Chao and Engelward [8].

2) Allow the cells to settle into the microwells at the base of each 96-well by gravity.

3) Once cells have loaded into the microwells, remove the bottomless 96-well plate and rinse the excess cells from the surface of the CometChip.

∆ CRITICAL STEP It is good to check cell loading efficacy before proceeding. If there are very few cells in the microwells, rinse less vigorously. Only ~10,000 cells per microwell are needed. An option is to load part of a sample, check loading efficacy, and reload if necessary.

∆ CRITICAL STEP Large cells may not load effectively into the microwells, which are ~40 µm in diameter. Larger diameter microwells may be needed. Note that buccal cells do not load efficiently due to their morphology.

4) Trap the cells within the microwells by adding a thin layer of low melting point agarose over the CometChip.
5) If treating cells directly on the CometChip, place a bottomless 96-well plate back over the chip and perform chemical dosing.
6) Lyse the cells in the CometChip by submerging the agarose chip in comet assay lysis buffer.

Stage 3: Comet formation

Alkaline treatment & Electrophoresis: Modification of step 28.

1) Following the lysis step, secure the CometChip into an electrophoresis tank using double sided tape and denature the DNA by submerging the CometChip in comet assay alkaline unwinding buffer.

Stage 4: Comet visualization & Analysis

Comet visualization: Modification of step 33.

1) Submerge the CometChip in SybrGold to stain the DNA for fluorescent imaging.

Comet analysis: Modification of steps 35-36.

1) Capture images of the comet array within each macrowell using a fluorescent microscope. Since the CometChip is set up in a 96-well plate format, automated scanning functions on microscopes may be used to improve imaging throughput. Use CometChip analysis to quantify comets. One to two images per 96 well (each containing dozens of comets) suffices for robust results.

? TROUBLESHOOTING

Supplementary Protocol 5: High Throughput comet assay system: Application of Fisherbrand™ COMPAC-50™ HTP Comet Assay Tank with microscope slides

Karbaschi and Cooke [9] demonstrated that electrophoresis could be performed successfully with the comet slides held vertically, rather than using the conventional horizontal slide arrangement. This led to a number of significant improvements on the processing of slides for the comet assay, via specialized equipment.

Additional equipment
Fisherbrand™ COMPAC-50™ HTP Comet Assay Tank (ThermoFisher Scientific, cat. no. 15381347)

Procedure
∆ **CRITICAL** These steps should be performed in place of the indicated steps of the main Procedure.

Stage 2A: Processing gels for the standard alkaline comet assay
Embedding cells in LMP agarose and cell lysis: Modification of step 14.
1) Place microscope slides vertically, along their longest edge, in polyoxymethylene slide racks (Cleaver Scientific Ltd), and keep them like this for the remainder of the assay steps (**Figure 7A**). Each rack can accommodate up to 25 slides. This batch processing decreases the risk of damage to/loss of gels, and increases throughput.
∆ **CRITICAL STEP** Ensure that all slides have the same orientation in the rack, are level, and are positioned correctly for the electrophoresis step.
2) Assay solutions are contained within dedicated chambers. Transfer the slides between the solutions using the slide racks (**Figure 7B**). The lysis (step 14), washing, neutralization (steps 31-32), staining, and the final washing steps (Stage 4, step 33) are all performed in these chambers. The chambers have integrated lids to minimize exposure to ambient light (Cleaver Scientific Ltd).

Stage 3: Comet formation
Alkaline treatment & Electrophoresis: Modification of steps 28-30.
1) Perform unwinding and electrophoresis in a specialized electrophoresis tank (**Figure 7C**), which can accommodate two racks at the same time. With the slides held vertically, the footprint of the electrophoresis tank is decreased significantly, and with integrated cooling, the need for wet ice is removed.

Supplementary Protocol 6: DNA methylation-sensitive comet assay using two isoschizomeric restriction enzymes.

HpaII and *MspI*, which recognize the same tetranucleotide sequence (5′ CCGG 3′) but display differential sensitivity to DNA methylation, are used. *HpaII* is inhibited when any of the two cytosines are methylated, while *MspI* is not (**Figure 8**).

Additional reagents

- FastDigest HpaII (ThermoFisher Scientific, cat. no. FD0514)
- FastDigest MspI (ThermoFisher Scientific, cat. no. FD0544)
- FastDigest Buffer (10X) (ThermoFisher Scientific, cat. no. B64)
- DL-Dithiothreitol (DTT) (Merck KGaA, cat. no. D9163)
- Proteinase K (Merck KGaA, cat. no. 70663)

Procedure 1: two gels/slide format

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 1: Preparation of cells from fresh or frozen samples

1) When working with attached cells: incubate harvested cells for 1-2 h at 37°C in an orbital shaker at 200 rpm to allow recuperation of cells.

∆ CRITICAL STEP Certain cultured cell types, such as HepG2, do sustain DNA damage when harvested with trypsin, which might negatively influence the integrity of the DNA and lead to incorrect enzyme digestion resulting in unreliable percentage methylation calculations. The optimal recovery time for each cell line should be determined based on the percentage tail DNA (lowest % tail DNA compared to freshly harvested cells (T_0)). This cellular repair step has a minimum effect on DNA methylation (CpG methylation unaffected) as observed from cells treated with 5-Aza-dcR [10].

Stage 2B: Processing gels for the enzyme-modified comet assay

Detection of specific DNA lesions: Modification of step 20.

1) Wash slides in restriction enzyme reaction buffer for 10 min at 37 °C.

Detection of specific DNA lesions: Modification of steps 22-23.

1) Apply 100 µL of the enzyme mixture (1.5 U/100 µL of *HpaII* or *MspI* in enzyme reaction buffer). Include a control group with only enzyme reaction buffer without enzyme. Cover application area with a glass coverslip.

∆ CRITICAL STEP Fast Digest versions of the restriction enzymes should be used. Alternatively, a 1.0-1.5 mM solution of proteinase K can be used to unwind nuclear DNA prior to enzyme digestion. This step contributes to making the enzyme recognition sites more accessible for *MspI* and *HpaII*.

Stage 3: Comet formation

Alkaline treatment & Electrophoresis: Modification of step 30.

1) Perform electrophoresis of 45 min at ~0.9 V/cm according to the published protocol by Wentzel et al. [10].

2) Calculate the percentage CpG methylation according to the following formula:

$$\%\, CpG\ methylation = [\left(100 - \frac{HpaII}{MspI} x100\right) - control]$$

Procedure 2: medium-throughput comet assay

Δ **CRITICAL** This is an alternative procedure that performs all the steps using the 12-gel chamber unit. See **Figure 4** for an overview of the unit [11]**.** Enzyme digestion is more effective when done in wells.

Δ **CRITICAL** These steps should be performed in place of the indicated steps of the main Procedure.

Stage 2A: Processing gels for the standard alkaline comet assay

Embedding cells in LMP agarose and cell lysis: Modification of steps 10-14.

1) Place the pre-coated microscope slide in the 12-gel chamber unit (including the silicone gasket).

2) Add a volume of 20 µL of the LMP agarose cell mixture to each well and place the aluminium base on ice for 5 min to allow the LMP agarose to set.

3) Add 150 µL of lysis solution directly to each well and incubate at 4 ^{0}C for 1-16 h.

Stage 2B: Processing gels for the enzyme-modified comet assay

Detection of specific DNA lesions: Modification of steps 20-26.

1) Following lysis, wash each well with cold PBS.

2) Add 50 µL of the enzyme mixture (1.5 U/100 µL of *HpaII* or *MspI* in enzyme reaction buffer) to each well, include a control group with only enzyme reaction buffer and seal wells with silicone caps.

3) Incubate the 12-well unit at 37 ^{0}C for 30 min.

4) After incubation, discard the enzyme mixture and rinse each well with cold PBS.

5) Remove the glass slide from the gasket and proceed with Stage 3.

Δ **CRITICAL STEP** Caution should be used when removing the glass slide from the gasket so as not to disrupt the individual LMP agarose gel spots.

Supplementary Protocol 7: DNA methylation-sensitive comet assay using the EpiComet-Chip with the *McrBC* restriction enzyme.

The EpiComet technology has been merged with the previously described platform, CometChip (**Box 4**, **Figure 6**) [12,13]. *McrBC* specifically recognizes DNA sites of the form 5'- (G/A)mC-3' and cuts DNA at methylated cytosines. Comet analysis will provide a measurement of the relative global DNA methylation status

Additional reagents

- *McrBC* enzyme (New England Biolabs, cat. no. M0272)
- Control treatment buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9, 100 µg/mL BSA, 1 mM GTP)
- Methylation-specific buffer: control treatment buffer plus 0.035 U/µL *McrBC* enzyme.

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 2B: Processing gels for the enzyme-modified comet assay
Detection of specific DNA lesions: Modification of step 22.

1) Incubate gels at 37 ^{o}C in a preheated damp chamber for 105 min by layering the following on the respective samples in parallel:

- Control treatment buffer
- Methylation-specific buffer

Supplementary Protocol 8: Detection of chromosomal breaks in yeast

∆ **CRITICAL** The following procedure is suitable for experiments with yeast cells such as *Saccharomyces* sp. and *Candida* sp.

Additional reagents

- BIORAD CHEF Yeast Genomic DNA Plug Kit (Biorad, cat. no. 1703593)
- Pulsed Field Certified Agarose (Biorad. cat. no. 1620137)
- 10x Tris/Boric Acid/EDTA (TBE) (Biorad, cat. no. 1610733)
  ! CAUTION Exposure to boric acid causes damage to the reproductive system. Use safety glasses and gloves when handling boric acid solution.
- 50x Tris/Acetic Acid/EDTA (TAE) (Biorad, cat. no. 1610743)
- Ethidium Bromide Solution (Biorad, cat. no. 1610433)
  ! CAUTION Mutagenic
- SeaKem® LE Agarose NMP (Lonza, cat. no. 50002)
- SeaPlaqueTM Agarose LMP (Lonza, cat. no. 50100)
- Spermine (Merck KGaA, cat. no. S3256)• YOYO-1 iodide (ThermoFisher Scientific, cat. no. Y3601)
  ! CAUTION Potential mutagen
- Proteinase K (Merck KGaA, cat. no. 70663)

Additional reagent set-up

- Electrophoresis solution: 30 mM NaOH, 1 mM Na_2EDTA; pH>13. Store at 4 °C for up to one week. Another option is to prepare it by diluting stocks of NaOH and Na_2-EDTA in 2L of cold distilled water.

Special equipment

- CHEF-DR®III Pulsed Field Electrophoresis System (Biorad, cat. no. 170-3690)
- UV transilluminator, UVT-14 SE, 254 nm (Carl Roth, cat. no. NK03.1)
- Poly-L-lysine coated glass slides (Merck KGaA, cat. no. P0425)

Procedure

∆ **CRITICAL** These steps should be performed in place of the indicated steps of the main Procedure.

Modifications of Stages 1-3

1) Harvest yeast cells from exponentially growing culture with approximately $6x10^8$ cells as a total cell yield by centrifugation (10 min at 5000 × *g*, room temperature) and then wash the cells with ice-chilled phosphate buffered saline (PBS) containing 0.1 % glucose and 0.5 mM EDTA.
2) Obtain an agarose embedded yeast DNA with BIORAD CHEF Yeast Genomic DNA Plug Kit using a standard protocol with lyticase and proteinase K treatments according to the manufacturer's instructions.
3) Pulsed-field gel electrophoresis (PFGE) separation: Separate the budding yeast chromosomes using 1.0 % pulsed-field certified agarose gel in 0.5 x TBE with a recirculation at 14 °C using CHEF-DR®III Pulsed Field Electrophoresis System for 24 h at 6 V/cm with a 60 to 120 s switch time ramp.

∆ **CRITICAL STEP** Separation of *C. albicans* chromosomes should be performed using 1% agarose gel in 0.8 × TBE using CHEF-DR®III Pulsed Field Electrophoresis System and the following conditions should be considered: 60 to 120 s switch, 6 V/cm, 120 angle for 36 h, followed by 120 to 300 s switch, 4.5 V/cm, 120 angle for 12 h. If chromosomes migrate as a single band instead of separate bands, use TAE buffer instead of TBE buffer, lower concentration of TBE buffer and/or decrease the value of V/cm (lower than 6 V/cm at the first step).

4) Stain the gel using 1 µg/mL EtBr for 30 min with gentle shaking in the dark.

5) Collect a single band containing a single chromosome under UV light (302 nm) using a razor blade and keep agarose blocks with single chromosomes in a test tube. Work fast as EtBr is light-sensitive. If the EtBr staining of the band is fading, re-stain the gel using EtBr staining solution. Protect your eyes by wearing protective goggles.

6) Prepare poly-L-lysine-coated microscope slides with two layers of agarose, namely 0.8% NMP agarose (bottom) and 0.6% LMP agarose (top) and cover agarose-coated slides with a coverslip.

7) Create holes in the LMP agarose layer and put carefully the agarose blocks containing single chromosomes after PFGE separation into the holes and cover with a coverslip.

8) Add the third layer of agarose, 0.5% NMP agarose) Place the slides with agarose blocks containing separate chromosomes after PFGE separation in an electrophoresis solution at room temperature for 10 min.

10) Start the electrophoresis at 0.5 V/cm at room temperature for 10 min in the same electrophoresis solution.

11) Immediately after the electrophoresis, transfer the slides into a neutralizing solution (50% ethanol, 20 mM Tris-HCl and 1 mg/mL spermine) at room temperature for 20 min in the dark. Repeat this step twice.

Stage 4: Comet visualization & Analysis

Modification of steps 33-36.

1) Stain DNA with 2.5 µM YOYO-1 iodide in 2.5% DMSO and 0.5% sucrose for 10 min at room temperature and cover with a coverslip

2) Capture at least fifty images per biological replicate for each chromosome. Consider performing the experiment in biological triplicates.

3) Analyse the structures of DNA chromosomes including DNA breaks and replication intermediates (RIs). Consider the following categories of RIs: simple replication intermediates (type A, Y-shaped, bubbles, double Y, bubbles with Y), unusual replication intermediates (type B, branched intermediates that may be a result of forced termination of replication or re-replication) and replication intermediates with DNA breaks that may promote chromosomal DNA breaks (type C) according to Adamczyk et al. [14].

Supplementary Protocol 9. Bromodeoxyuridine (BrdU) comet assay

In this method, BrdU is incorporated into newly synthesized DNA by cells entering and progressing through the S-phase (DNA synthesis) of the cell cycle. The incorporated BrdU is then revealed in the two-step staining. The first step involves incubation with primary anti-BrdU antibody conjugated with biotin. In the second step, streptavidin-Cy3 conjugate is used for fluorescent labelling of DNA incorporated BrdU. Filtering out the BrdU-positive comets during the comet scoring will allow quantification of induced DNA damage only and not in combination with physiological DNA discontinuities or gaps presented in replicating cells. This corrects false-positive increase in the final DNA damage levels.

Additional reagents

- BrdU (e.g., from Roche, cat. no. 10280879001)
  ! CAUTION Mutagenic
- BSA (biotin-free, e.g., from ROTH, cat. no. 0163.2)
- Anti-BrdU monoclonal antibody conjugated with biotin (Abcam, cat. no. ab2284)
- Streptavidin conjugated with Cy3 (ThermoFisher Scientific, cat. no. 434315)
- Tween®-20 (Merck KGaA, cat. no. P1379)
- Vectashield Antifade Mounting medium (Vector Laboratories, cat. no. H-1000)
- YOYO™-1 Iodide - 1mM solution in DMSO (ThermoFisher Scientific, cat. no. Y3601)
  ! CAUTION Potential mutagen

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 1: Preparation of cells from fresh or frozen samples

Modification of step 1 (A, B) - in vitro labeling of cultured cells with BrdU.

∆ CRITICAL A wide variety of human and mouse cell lines and normal cell populations can be incubated with BrdU. Use cells from the same population that are not BrdU-labeled as a negative staining control for this assay. This allows you to determine background staining levels for the anti-BrdU monoclonal antibody.

1) Remove cell culture medium from cells and replace it with fresh cell culture medium containing 10 µM of BrdU (follow manufacturer's instructions).

2) Incubate the treated cells for the desired length of time (usually 15 - 45 min) at 37 °C [15,16].

∆ CRITICAL STEP For each different cell line or cell population within a particular experimental system, a different length of time is required for incubation. Therefore, it is recommended to optimize this before you begin the experiment.

∆ CRITICAL STEP Avoid disturbing the cells in any way (e.g., centrifugation steps or temperature changes) that may disrupt the normal cell cycling patterns. The cell culture density should not exceed 2×10^6 cells/mL.

3) Remove labelling solution and wash cells two times with PBS for about 5 s per wash.

4) Wash cells three more times with PBS for 2 min each, process them according to the cell type and proceed to Stage 2A.

Modification of step 1 (N) - in vivo labelling of mouse thymocytes with BrdU.

1) Inject mice intraperitoneally with 1 mg BrdU (i.e., 100 µL of BrdU solution in PBS at a concentration of 10 mg/mL)

2) After 2.5 h, sacrifice the mice according to standard protocols and isolate the thymus.
3) Gently homogenize the thymus of each mouse separately in a dounce homogenizer in a 1 mL solution of 1% BSA (biotin-free) in PBS. Alternatively, thymus can be homogenized by pushing it through the cell strainer with pore size 100µm.
4) (Optional) If a particular thymic subpopulation will be analyzed, stain cell suspension with fluorescent primary antibodies and sort thymocytes based on the expression of their surface markers [17].
5) Collect cell suspensions of 300,000 cells into 2mL tubes and centrifuge for 5 min, 500 × *g*, 4 °C.
6) Remove the supernatant and resuspend the pellet in 0.5% LMP agarose at the concentration of 300,000 cells in 1.5 mL of LMP agarose (~14,000 cells per 70 µL gel) and proceed to Step 2A.

Stage 2A: Processing gels for the standard alkaline comet assay
Additional step after step 14.
1) After lysis, wash slides briefly in cold (4 °C) PBS.

Stage 4: Comet visualization & Analysis
Comet visualization: Modification of step 33.
1) Wash the slides into 50 mL PBS for 30 min at room temperature in a Coplin jar.
2) To block non-specific antibody binding, transfer slides into 50 mL solution of 1% BSA (biotin-free) in PBST (0.02% Tween-20 solution in PBS) in a Coplin jar and incubate for 30 min at room temperature.
3) After incubation, wipe the bottom of the slide with cellulose wadding and drain the excess solution from the slides.
∆ CRITICAL STEP This step and any steps which involve the addition of antibodies must always be done slide by slide.
∆ CRITICAL STEP The slides must not be allowed dry out from this point on.
4) Transfer the slides in a humid chamber in a horizontal position and apply 100 µL of a 1:250 dilution (i.e. 4 µg/ml) of anti-BrdU-biotin antibody solution to the slides. Antibody is diluted in 1% BSA/PBST solution.
5) Cover each slide with a coverslip, transfer the humid chamber to the fridge and leave it there overnight.
6) The next day, remove the coverslips from the slides by vertical immersion of slides in PBS solution in a Coplin jar.
7) Wash the slides twice for 3 min in PBST solution and then for 15 min in 1% BSA / PBST in a Coplin jar.
8) Place slides in a humid chamber in a horizontal position and apply 100 µL of streptavidin-Cy3 solution diluted 1: 400 (1% BSA / PBST) to each slide, cover each slide with a coverslip and incubate for 1 h at room temperature.
9) Remove the coverslips from the slides by vertical immersion of slides in PBS solution in a Coplin jar.
10) Wash the slides twice for 3 min in PBST solution and twice for 3 min in PBS in a Coplin jar.
11) Transfer the slides in a humid chamber and incubate with 100 µL of a 100 nM solution of YOYO™-1 (10,000X diluted in PBS) for 30 min at room temperature (it is no longer necessary to cover with coverslips).
∆ CRITICAL STEP YOYO™-1 is dissolved in DMSO; therefore, it is advantageous to dilute the stock solution 10 times in DMSO to a 100 µM working concentration.

12) After incubation, wash the YOYO™-1 solution from the slides twice for 10 min in PBS in a Coplin jar.
13) Finally, drain the excess of PBS from the slides and apply 11 µL of Vectashield mounting medium to the slides, cover them with coverslips and incubate for 10 min at room temperature in a horizontal position.
Δ **CRITICAL STEP** Slides must not be allowed to dry before they are covered with coverslips.
14) Remove excess medium from the samples by tilting the slides so that their long edge touches the cellulose wadding laid on the bench.
15) The slides thus prepared can be used for scoring comets (stained with YOYO™-1) and BrdU-positive comets or can be stored in the fridge horizontally in a humid chamber for later scoring.
Δ **CRITICAL STEP** Before scoring, it is always necessary to dry the slides so that the coverslip does not float on the slide.

Comet visualization: Modification of step 34.
1) Visualize comets with a fluorescent microscope using two filters - FITC filter (for YOYO™-1 staining) and TRITC (for BrdU staining). If the cells are visible only in the FITC filter, it means that they only contain DNA damage. On the other hand, if they are visible in both filters, they contain physiological DNA repair intermediates detected as SBs (**Figure 9**).

Supplementary Protocol 10: Overview of the most common comet-FISH steps

FISH can be combined with the comet assay to investigate the structure of the chromatin within comet preparations and to study specific DNA sequences within comets.

Additional reagents

- There is a wide variety of suitable FISH probes: Repeats, fragments of chromosomes, whole-chromosomes, DNA fragments cloned in plasmids, 'padlock probes', peptide nucleic acid probes. Many of the probes can be prepared in the lab: Fragments of DNA can be cloned in P1 artificial chromosomes (PACs); Bacterial Artificial Chromosomes (BACs) from Roswell Park Comprehensive Cancer Center can be used after labelling with biotin-14-dCTP (Life Technologies) or digoxigenin-11-dUTP (Roche Applied Sciences) by conventional nick translation. Any commercial probes used for classic FISH can be used after optimisation with the comet assay.
- Hybridisation buffer: saline-sodium citrate (SCC) (20xSCC, Merck)
- Washing solutions and detection reagents are available as various kits from different providers. Examples of detection reagents that have been successfully used in the comet-FISH include: Cy3–conjugated streptavidin (Jackson Immuno Research Laboratories), biotinylated anti-avidin D (Vector Laboratories), fluorescent antibody enhancer set for digoxigenin detection (Roche Applied Science).

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 3: Comet formation

Neutralization & Washing: Extra steps to be added instead of or just after step 32.

1) Denature comet assay slides and hybridisation probes: place slides for 25 min in 0.5 M NaOH and dehydrate in a series of increasing ethanol concentrations; denature probes/hybridisation mix for 10 min at 70oC. Commercial probes should be denatured following manufacturer instructions.

2) Hybridisation start: apply the probes on the slides, seal with coverslips and incubate over night at 37 °C.

3) Post-hybridisation wash: remove the seal and incubate the slides in a set of washing solutions: three times 5 min in 50% formamide/ 2×SSC at 42 °C and twice in 2×SSC at 42 °C for 10 min.

4) Signal detection: place the slides in a blocking solution (4×SSC, 0.05% Tween-20, 5% non-fat milk powder) for 10 min. To detect the signal apply sequential layers of antibodies using Cy3–conjugated streptavidin and biotinylated anti-avidin D for biotinylated probes, and fluorescent antibody enhancer set for detection of digoxigenin.

Stage 4: Comet visualization & Analysis

1) Stain gels with 20 µL of DAPI prepared in Vectashield (Vector Laboratories).
Alternative stains: Propidium iodide (2.5 µg/mL), Hoechst 33258 (0.5 µg/mL), SYBR Gold (0.1 µl/mL) or ethidium bromide (20 µg/mL).

2) Visualize and record the signals using appropriate filters.

Δ CRITICAL STEP Comets (and thus signals) are organized in three-dimensional space. Comet-FISH experiments allow investigating DNA damage in the context of the three-dimensional organization of chromatin in living cells.

Supplementary Protocol 11: Comet assay with yeast and filamentous fungi

Additional reagents

- Yeast Extract (ThermoFisher Scientific, cat. no. 210929)
- Bacto-Peptone (ThermoFisher Scientific, cat. no. 211820)
- SC minimal medium/Yeast Nitrogen Base (YNB) (ThermoFisher Scientific, cat. no Q30007)
- Tryptone (Merck KGaA, cat. no. T7293)
- Glucose (Merck KGaA, cat. no. G7021)
- Myo-inositol (Merck KGaA, cat. no. 57570)
- Sorbitol, (Merck KGaA, cat. no. S6021)
- KH_2PO_4 (Merck KGaA, cat. no. P9791)
- Zymolyase 20T (MP Biomedicals, LLC, cat. no. 08320921)
- Lysing Enzymes from *Trichoderma harzianum* (Merck KGaA, cat. no. L1412)
- $MgSO_4$ (Merck KGaA, cat. no. M7506)
- Paraffin oil light (Applichem, cat. no. A2135)

Reagent setup

- Lysis solution: 30 mM NaOH, 1 M NaCl, 0.05% (w/v) N-laurylsarcosine sodium salt, 50 mM EDTA and 10 mM Tris-HCl, pH 10

For yeast:

Δ CRITICAL Use these reagents with *Saccharomyces cerevisiae, S. paradoxus, S. kudriavzevii, S. bayanus, Candida albicans, Cryptococcus neoformans, Schizosaccharomyces pombe*.

- YPG medium (1 % w/v Yeast Extract, 2 % w/v Bacto-Peptone and 2%)
- YPD (1 % w/v Yeast Extract, 2 % w/ Bacto-Peptone, 2 % w/v glucose)
- Medium for treatment: YPD/YPG/YNB or PBS containing 0.1 % glucose
- S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Alternatively, use a buffer composed of 5 mM MOPS–NaOH, pH 7.2, 1.3 M sorbitol and 1 mM EDTA. All S buffers must be supplemented with 2 mg/mL zymolyase (20T; 20,000 U/g)

For filamentous fungi Ashbya:

Δ CRITICAL Use these reagents with *Ashbya gossypii* (filamentous fungus).

- Solidified AFM (1% w/v Tryptone, 1% w/v Yeast Extract, 2% w/v glucose, 0.1% w/v myo-inositol).
- Solution A (1.2 M $MgSO_4$, 10 mM Na-phosphate buffer, pH 5.8) with Lysing Enzymes from *Trichoderma harzianum* (5 mg/mL; ≥10 U/g)
- Solution B (1 M sorbitol, 10 mM Tris-HCl, pH 7.5)

Procedure

Δ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 1: Preparation of yeast cell suspension

Modification of step 1.

1) Harvest approximately 10^6 yeast cells from an exponentially-growing culture (~200 µL) by centrifugation (10 min at 5,000 × *g*, room temperature). Next, wash with the same volume of ice-cold deionized water. After a second spin resuspend yeast cells in 200 µL S buffer

supplemented with 2 mg/mL zymolyase (20T; 20 000 U/g). Incubate for 30 min at 30 °C before mixing with LMP agarose.
2) For experiments with *Ashbya gossypii*: Collect mycelium from the edges of a colony and suspend in solution A with 5 mg/mL Glucanex® (≥10 U/g). Incubate for 1 h at 30 °C, and centrifuge at 4,000 × *g* for 10 min at 4 °C. Next wash the pellet with solution B and resuspend in solution B.
Δ **CRITICAL STEP** It is common to have spores contaminating the protoplast suspension. To remove the spores from the final suspension, add 1 mL paraffin oil light, mix and leave until the two phases separate. Collect the protoplast-rich lower phase and dilute 1:4 in solution B.

Stage 2A: Processing gels for the standard alkaline comet assay
Embedding cells in LMP agarose and cell lysis: Modification of steps 10-11.
1) Mix the cell suspension carefully with LMP agarose to achieve 5×10^4 cells per 50 µL gel of 0.7% LMP agarose. Δ **CRITICAL STEP** LMP agarose containing 2 mg/mL zymolyase 20T can be used.
2) Mix cells with LMP agarose to achieve 5×10^4 cells per 50 µL gel of 1.5% LMP agarose. This higher LMP concentration results in sharper comets for cells with low DNA content and less condensed chromatin.

Embedding cells in LMP agarose and cell lysis: Modification of step 14.
5) Treat the cells for 2 h in the dark with cold lysis solution.
? TROUBLESHOOTING
Δ **CRITICAL STEP** For *Schizosaccharomyces pombe* use the following lysis solution: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1%, Triton® X-100, 10% DMSO, pH 10. Perform lysis overnight at 4 °C in the dark.
6) For *Ashbya gossypii*: Treat the cells for 2 h in the dark with cold lysis solution.
Δ **CRITICAL STEP** Very good results are also obtained without washing of the slides between lysis and alkaline treatment.

Stage 3: Comet formation
Δ **CRITICAL** Use 30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10 as electrophoresis solution. Alternatively, the standard electrophoresis solution can be used (see Materials section) or an electrophoresis solution containing 30 mM NaOH and 10 mM EDTA or 1mM EDTA, 0.2% DMSO, 300 mM NaOH, pH > 12. The supplementation of the electrophoresis solution with DMSO may protect against artifactual generation of DNA damage by secondary ROS during prolonged electrophoresis and the use of lower voltage/cm conditions.

Alkaline treatment & Electrophoresis: Modification of steps 29-30.
7) After 20 min of unwinding, perform electrophoresis at 0.7 V/cm for 10 min at 4 °C in the dark (EPT=7).
Δ **CRITICAL STEP** For *Schizosaccharomyces pombe* 0.86 cm/V for 20 min is recommended.

Supplementary Protocol 12: Plant comet assay

Additional Reagent setup

- Extraction buffer: freshly prepare a mix of 1:9 v/v EDTA 200 mM pH 10 and PBS pH 7.

Additional materials

- Sharp razorblades

Procedure

∆ **CRITICAL** These steps should be performed in place of the indicated steps of the main Procedure.

∆ **CRITICAL** All steps should be performed in a darkroom under a non-actinic lamp on ice.

Stage 1: Preparation of plant nuclei suspension

∆ **CRITICAL** Due to the plant cell wall, which is a barrier to cell lysis, plant nuclei need to be extracted mechanically from roots or leaves [18]. It is important to determine the optimal extraction time leading to high nucleus extraction yield without causing DNA damage [18]. This parameter should be adjusted for different plant species but also for specific organs (e.g., lignified tissues).

Modification of step 1.

1) Use a sharp razor blade for the mechanical extraction of nuclei from plant roots or leaves. Chopping or slicing the plant material into cold plant extraction buffer on ice to release nuclei.

Stage 2A: Processing gels for the standard alkaline comet assay

Embedding cells in LMP agarose and cell lysis: Modification of steps 10 - 11.

1) Mix gently 225 µL of nuclei suspension with 150 µL LMP agarose (2%) to reach a concentration of 0.8 % LMP agarose.

∆ **CRITICAL STEP** This will result in approx. 100 nuclei per gel, but the exact number of nuclei depends on the amount of extraction buffer used, the amount of plant material, and the duration and intensity of material chopping/slicing [18].

2) Add 70 µL cell-LMP agarose mixture per slide for the 2 gels/slide format and cover with a coverslip. Alternatively, add 10 µL for the 12 gels/slide format.

∆ **CRITICAL STEP** A lysis step is not necessary as plant nuclei are directly extracted by mechanical extraction. No filtration to remove plant debris should be performed as it induces additional DNA damage [18].

Stage 3: Comet formation

Alkaline treatment & Electrophoresis: Modification of step 29.

1) Incubate gels in cold electrophoresis solution for 15 min at 4 °C in the dark. This time could be optimised depending on the plant material [19].

Alkaline treatment & Electrophoresis: Modification of step 30.

1) Electrophorese at ~0.7V/cm at 4 °C in the dark for 5 to 20 min depending on plants.

Supplementary Protocol 13: Sperm comet assay

∆ CRITICAL Sperm differ from somatic cells in the structure of their chromatin. For human sperm, about 85% of the DNA is packed with protamines in a laminar structure; the remaining 15% contains histones. The protamines contain disulfide bonds and DTT is used to break them. However, sperm chromatin is notoriously difficult to deproteinize, and high levels of DNA breaks in controls may represent heterogenous breakage of differently packed DNA. Background levels of DNA damage vary significantly depending on methodology (in particular, on lysis conditions). Some protocols employ proteinase K in the lysis step to remove protamines that otherwise impede DNA migration through the agarose. These peculiarities probably limit the sensitivity of the assay for biomonitoring studies.

Additional reagents

- Lithium diiodosalicylate (Merck KGaA, cat. no. D3635)
- DL-Dithiothreitol (DTT) (Merck KGaA, cat. no. D9163)
- Proteinase K (Merck KGaA, cat. no. 70663)

Procedure

∆ CRITICAL These steps should be performed in place of the indicated steps of the main Procedure.

Stage 1: Preparation of sperm cell suspension

Modification of step 1.

1) Perform seminal liquefaction to obtain a usable cell suspension.

∆ CRITICAL STEP Collect semen samples into sterile specimen beakers made of nontoxic plastic ware.

Modification of step 3.

1) Flash freeze aliquots of ~25 µL in liquid nitrogen and store at -80 °C until use.

∆ CRITICAL Cryostorage of human sperm could generate DNA damage.

Stage 2A: Processing gels for the standard alkaline comet assay

Embedding cells in LMP agarose and cell lysis: Modification of steps 9-11.

1)Thaw the tubes in a 37 °C water bath for 10 s and then immediately process samples for the comet assay.

2)Prepare a mix of 50,000 cells in 200 µL LMP agarose (1-2%). Add 70 µL per slide for the 2-gel/slide format.

∆ CRITICAL STEP The optimal LMP agarose concentration could be adjusted based on the background levels of DNA damage. Too high agarose concentrations could lead to limited DNA migration and reduced assay sensitivity.

Embedding cells in LMP agarose and cell lysis: Modification of step 14.

1) Perform lysis according to either Option A or Option B. The latter can be chosen when for practical reasons when it is useful to divide the experiment in two days. It is not still clear which option yields better results.

A) Short Lysis

(i) Incubate slides in standard lysis solution with 10 mM DTT for 1 h at 4 °C;

(ii) Incubate slides in standard lysis solution with proteinase K (0.05 mg/mL) for 1 h at 4 °C.

B) Overnight Lysis

(i) Incubate slides in standard lysis solution overnight at 4 °C;

(ii) Incubate slides in standard lysis solution with 10 mM DTT for 1 h at 4 °C;

(iii) Incubate slides in 100 mM Tris buffer with 4 mM lithium diiodosalicylate pH 7.6 for 1.5 h at room temperature.

Stage 2B: Processing gels for the enzyme-modified comet assay

Detection of specific DNA lesions: Modification of step 25.

1) Incubate for 45-60 min at 37 °C.

∆ CRITICAL STEP Positive controls are in general inefficient, e.g., compared to somatic cells, 20× higher doses of X-rays are needed to induce sizable levels of damage in sperm. UVC light may be used but requires enzymatic cleavage of dimers (e.g., by T4endoV). A few chemicals seem to induce oxidatively modified lesions (e.g., BPDE, glycidamide) detectable with Fpg, hOGG1, EndoIII.

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