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TITLE: Measuring DNA damage with the comet assay: a compendium of protocols.

Main authors:

Andrew Collins*, Department of Nutrition, University of Oslo, Oslo, Norway, a.r.collins@medisin.uio.no,

Peter Møller*, Department of Public Health, Section of Environmental Health, University of Copenhagen, Copenhagen, Denmark, pemo@sund.ku.dk,

Goran Gajski*, Mutagenesis Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia, ggajski@imi.hr,

Sona Vodenkova*, Department of Molecular Biology of Cancer, Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech Republic; and Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Prague, Czech Republic, sona.vodenkova@iem.cas.cz,

Sabine A.S. Langie[$] Department of Pharmacology & Toxicology, School for Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University, The Netherlands, s.langie@maastrichtuniversity.nl,

Amaya Azqueta Oscoz[$#], Department of Pharmacology and Toxicology, University of Navarra, Pamplona, Spain; and IdiSNA, Navarra Institute for Health Research, Pamplona, Spain, amazqueta@unav.es

This paper has been written by 79 co-authors. Details of all co-authors are included in the submission platform.

*These authors contributed equally: Andrew Collins, Peter Møller, Goran Gajski, Sona Vodenkova

[$] These authors contributed equally: Sabine A.S. Langie, Amaya Azqueta

[#]Corresponding author: Amaya Azqueta

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Abstract

The comet assay is a versatile method to detect nuclear DNA damage in individual eukaryotic cells, from yeast to human. The types of damage detected encompass DNA strand breaks and alkali-labile sites (e.g., apurinic/apyrimidinic sites), alkylated and oxidised nucleobases, DNA-DNA crosslinks, UV-induced cyclobutane pyrimidine dimers and some chemically-induced DNA adducts. Depending on the specimen type, there are important modifications to the comet assay protocol to avoid the formation of additional DNA damage during the processing of samples and to ensure sufficient sensitivity to detect differences in damage levels between sample groups. Various applications of the comet assay have been validated by research groups in academia, industry and regulatory agencies, and its strengths are highlighted by the adoption of the comet assay as an *in vivo* test for genotoxicity in animal organs by the Organization for Economic Cooperation and Development (OECD). The present document describes a series of consensus protocols which describe the application of the comet assay to a wide variety of cell types, species, and types of DNA damage, thereby demonstrating its versatility.

1. Introduction

The alkaline comet assay (single-cell gel electrophoresis) is a sensitive method that detects DNA strand breaks (SBs) and alkali-labile sites (ALS) in the nucleus of virtually all types of eukaryotic cells. Alkali-labile sites are not well defined but, as the name suggests, are essentially any DNA modification that becomes a strand break under alkaline conditions e.g., apurinic/apyrimidinic sites. The principle of the comet assay relies on the spatial organisation of DNA in the nucleus; namely, loops of DNA formed by attachment of the linear molecule at intervals to the nuclear matrix, and additional winding of the double helix around protein cores to form nucleosomes. This organisation means that when the proteins are removed during the lysis step of the assay, the DNA remains in a compact supercoiled state. However, if a DNA strand break is present, the supercoiling of the loop relaxes. As a result of this relaxation, these loops, which are still attached to the nuclear matrix, are drawn towards the anode, forming the characteristic 'comet tail', seen under fluorescence microscopy. The relative amount of total DNA in the tail reflects the frequency of breaks. The name 'comet assay' was introduced in 1990 [1] and was adopted as a Medical Subject Heading in PubMed in 2000.

The comet assay is used worldwide as a standard method for the detection of DNA damage in genotoxicity testing and human biomonitoring studies [2]. It is also a popular tool in the field of ecotoxicology and environmental monitoring, studying different animal and plant species [3–5].

The first multi-laboratory, collaborative review on the use of the comet assay, including information about the development of the assay, principles, applications and protocols, was published in 1993 [6]. However, the first initiative to develop a guideline for the comet assay in genetic toxicology, including *in vitro* and *in vivo* studies, was published in 2000 [7]. A formal validation study was performed during 2006-2012, culminating in the adoption of the *in vivo* mammalian, alkaline comet assay as the Organisation for Economic Co-operation and Development (OECD) test guideline no. 489 in 2014 (updated in 2016) [8]. Despite the substantial importance of an OECD guideline, some limitations remain. For instance, it does not include species other than mammals, and lesions other than SBs and ALS are not considered; nor is the measurement of DNA repair, or the application to biomonitoring. Indeed, it was the application of the comet assay to human biomonitoring which led the research community to collaborate and develop standardized procedures, in order to achieve congruent baseline levels of DNA damage, and consistent reporting of procedures. These issues have been addressed through a number of multi-laboratory validation studies, specifically the European Standards Committee on Oxidative DNA Damage (ESCODD) [9–11], the European Comet assay Validation Group (ECVAG) [12–18], and the COST Action hCOMET (CA15132) (The comet assay as a human biomonitoring tool) [19]. Additionally, and in the framework of hCOMET, technical recommendations have been developed for the application of the comet assay to human samples [20,21]. Most recently, a protocol for the comet-based DNA repair assay [22], and recommendations for Minimum Information for Reporting Comet Assay (MIRCA) procedures and results [23], have been published also under the auspices of hCOMET.

The comet assay protocols described in the Nature Protocols article by Olive and Banáth [24] are limited to the neutral comet assay and a specific version of the alkaline assay. Here, we extend this knowledge to cover the most widely used alkaline method, and its various modifications, and we also provide protocols applicable to different sample types, from various eukaryotic species, including yeast, non-mammalian species, mammals, and plants. Prior to describing the comet assay protocol, we provide details of appropriate methods for isolating cells from different specimens, as this is key to avoiding artefactual formation of DNA damage and hence to achieving maximal specificity of the assay.

1.1. The development of the alkaline comet assay

The comet assay was first described in 1984, as a method for the detection of radiation-induced DNA breaks in single mammalian cells [25]. The method was modified a few years later by increasing the pH of the electrophoresis solution, and this is essentially the alkaline comet assay most widely used today [26]. Since the early 1990s, the comet assay has replaced the previously most popular methods for detection of SBs and ALS, namely alkaline elution and alkaline unwinding [27].

The alkaline comet assay measures both single and double SBs (as well as ALS); it is referred to in this paper as the standard comet assay. In the case of other methods for measuring DNA breaks, namely alkaline unwinding and alkaline elution, the alkaline conditions are crucial, as the methods require DNA denaturation. This is not the case for the comet assay [25], as migration of the DNA depends on relaxation of supercoils, which occurs at both neutral and alkaline pH. This explanation is not universally accepted, and the neutral version of the assay is employed in the belief, by some, that it detects only double SBs. Even after 35 years this issue is still controversial, and experiments to decide definitively between the alternative explanations are needed. The neutral comet assay protocol developed by Olive et al.[28] to measure double SBs involves lysis in sodium dodecyl sulphate and incubation for 4 h at 50 °C with proteinase K - conditions sufficiently different from the standard comet assay protocol that separation of DNA from the matrix is likely to occur, so that true double-stranded DNA fragments are released, migrating towards the anode. Protocols described in this article are limited to the alkaline comet assay.

Recent advances in the comet assay have led to high throughput versions of the assay, many of which utilise multiple gels, instead of the conventional one or two per slide; for example, 12 agarose mini-gels on one microscope slide [29], or 48 or 96 mini-gels on a GelBond® film [30], or a 'microarray' of cells, in a 96 well plate pattern (CometChip) [31]. In addition, the spectrum of DNA lesions detected is increased by the inclusion of lesion-specific enzymes capable of converting damaged nucleobases to DNA SBs; for instance, bacterial endonuclease III (EndoIII), catalysing the excision of oxidised pyrimidines, or formamidopyrimidine-DNA glycosylase (Fpg), and human 8-oxoguanine DNA glycosylase 1 (hOGG1), catalysing the excision of oxidised purines [32–34]. Apart from DNA base oxidation, the comet assay is also used for the evaluation of DNA lesions induced by crosslinking agents, such as cisplatin [35–37]. Additionally, the combination of the comet assay and fluorescence *in situ* hybridization (comet-FISH) allows the investigation of gene region-specific DNA damage and repair [38–41]. One of the newest variants of the comet assay includes its adaptation to detect global methylation levels, through treatment with specific restriction enzymes [42,43].

Overview of the protocol for the alkaline comet assay

A single cell suspension is necessary to perform the comet assay. In some cases, the sample is already a cell suspension, but when working with adherent cells, spheroids, whole organisms or tissues, mechanical and/or enzymic processing in specific buffers is required. In some cases, such as yeast, the cell wall also needs to be lysed. All these procedures are described in detail in the protocols below. The possibility of freezing cell suspensions, blood or solid tissues for later analysis, is also discussed; this has logistical advantages for *in vivo* animal experiments and human biomonitoring where samples cannot be analysed immediately.

After isolation of the cells of interest, the comet assay protocol is divided into four main stages, as described below and presented in **Figure 1**, although the precise conditions employed in these stages may vary depending on the type of specimen used (**Table 1**). The protocol is accompanied by tutorial

videos to illustrate the various steps of the protocol (Overview: https://youtu.be/KkuAj_COOR8); we believe that by following these steps, results will become more reproducible and comparable between individual laboratories and research groups.

Stage 1: Preparation of cell suspension from fresh or frozen samples.

The first stage is the isolation of cells from whole organisms, animal or plant tissues, biopsies, blood samples, spheroids or cell culture. Blood cells are most convenient in human biomonitoring studies as they are already a single cell suspension. Likewise, cells growing in suspension cultures can be used directly in the comet assay, whereas adherent cells must be detached from the cell culture plate, and resuspended. Spheroids, tissues, biopsies or whole organisms are homogenised prior to processing in the comet assay. The current protocol will specify these cell-processing steps for a wide variety of organisms and biomatrices. Tutorial videos for certain sample types can be found in this playlist;
https://youtube.com/playlist?list=PLEVxCdaQpbj1LBaBPneAZVaCpwzETlJ65

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

In the second stage (tutorial video: https://youtu.be/FXSTSCtgo-k), cells are suspended in low melting point agarose at 37 °C, and placed on microscope slides, or plastic (GelBond®) films, and the agarose allowed to solidify on a cold plate, or in a fridge. (Normal agarose is not suitable, as the higher temperature required to remain liquid would likely damage the cells' DNA.) The gel-embedded cells are then lysed to remove membranes, and other cytoplasmic material, resulting in protein-depleted nuclei with supercoiled DNA attached to the nuclear matrix - structures known as nucleoids. Modification of the lysis procedure is necessary for specific biomatrices, such as buccal cells, sperm and yeast. In the case of plants, nuclei are released mechanically rather than through lysis.

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

The enzyme-modified comet assay includes an additional step after lysis (tutorial video: https://youtu.be/x0Xt84R6Bho). The gel-embedded nucleoids are incubated with bacterial, bacteriophage or human DNA repair enzymes that recognize the DNA lesions and lead to the creation of additional SBs. The cells are embedded as described in Stage 2A, but 2 sets of duplicate slides need to be prepared: 1 set to incubate with reaction buffer and 1 set to incubate with the enzyme.

Stage 3: Comet formation.

After lysis (and optional enzyme digestion), the samples are transferred to an alkaline solution (tutorial video: https://youtu.be/s52tkqVNTUA). “Comets” are formed during subsequent electrophoresis in this solution. DNA loops containing SBs, with supercoiling relaxed, migrate towards the anode (as DNA is negatively charged) forming the tail of the comet, whereas the DNA without SBs does not move. The proportion of total DNA in the comet tail is a quantitative indicator of the frequency of DNA breaks in the cell. Following electrophoresis, neutralization (i.e., removal of the alkaline solution from the gels), and washing of the slides take place.

Stage 4: Comet visualisation & Analysis.

The final stage in the comet assay is the staining of the DNA, visualization of the comets, and quantification (tutorial video: https://youtu.be/5wIUI4OFwlc). It is possible to store dried, unstained or stained slides for prolonged periods. Comets are visualized by fluorescence microscopy, and analysed using free, or commercially available, semi-automated or fully-automated scoring software, or by visual scoring.

1.2. Technical modifications

Various modifications have been made to the standard comet assay, to allow the measurement of DNA modifications other than SBs and ALS or to examine damage in specific genomic regions. In addition, the throughput of the assay has been increased using different approaches. These changes, which improve the versatility and performance of the assay, are discussed in the following sub-sections.

1.2.1. Enzyme-modified comet assay: measurement of specific DNA lesions

DNA SBs can be regarded as a generic form of DNA damage. They are caused by a variety of chemicals, as well as ionising radiation, and they even arise as transient intermediates during DNA repair. SBs (at least, single strand breaks, SSBs) are quickly rejoined, and so they are unlikely to lead to mutations, and generally do not represent a great threat to genome stability [44,45]. However, as they are unlikely to occur in isolation, they can be indicative of a greater cellular burden of damage, and hence are important to measure. With regard to genotoxicity and carcinogenesis, modification of DNA nucleobases, such as oxidation or alkylation, is more significant. Base lesions are repaired more slowly than SSBs, and can lead to mutations, if they are present in the DNA during replication. For example, 8-oxo-7,8-dihydroxyguanine, a product of oxidative stress, can pair with adenine rather than cytosine, causing mutations [46]. It is therefore important to modify the assay to detect these nucleobase alterations, and this is achieved by using enzymes with the ability to convert the lesions into breaks. The bacterial DNA repair enzyme EndoIII, which recognises oxidised pyrimidines, was the first to be applied [47], followed by bacterial Fpg and human hOGG1 for oxidised purines [48–50]; these are probably the most widely used, although others have been employed (reviewed by Muruzabal et al. [34]).

Incubation of the nucleoids with the repair enzyme takes place following lysis, and washing of the slides in an enzyme-specific reaction buffer. During this step, depending on the enzyme, the DNA is incised at sites of the lesions, or the modified nucleobase is removed leaving an AP site. Under alkaline conditions, AP sites are converted to SSBs. In parallel with the enzyme incubation, a duplicate set of gels is incubated with the enzyme reaction buffer alone. Prior to its experimental use, it is important first to titrate the enzyme using cells containing the lesions of interest, to determine the optimum combination of enzyme concentration, and incubation time [51]. 'Net enzyme-sensitive sites' are calculated as the difference in comet DNA migration (tail intensity) between the enzyme-incubated and reaction buffer-incubated samples.

The bacterial enzymes 3-methyladenine DNA glycosylase (AlkD), and 3-methyladenine DNA glycosylase II (AlkA) have been used in the comet assay to detect alkylated nucleobases [52,53]. However, the use of these enzymes is limited since they are not commercially available. More recently, the comet assay has been combined with human alkyladenine DNA glycosylase (hAAG), a commercially available enzyme, for the detection of alkylated nucleobases [54]. hAAG detects 3-methyladenine, 7-methylguanine, 1-methylguanine and the ring-opened purines derived from N7-methylguanines [55,56]. The hAAG-modified comet assay may also detect ethenoadenines and hypoxanthine [54]. The Fpg-

modified comet assay, normally used for the detection of oxidized nucleobases, also detects alkylated lesions (by virtue of the ring-opened purines derived from 7-methylguanine) [49,54,57–59]. However, oxidatively damaged nucleobases are considered to be the predominant lesions detected, in cells which have not been treated deliberately with alkylating agents.

1.2.2. Detection of DNA inter-strand cross-links

Certain types of DNA-damaging agents form covalent links between two nucleobases, either in the same DNA strand (intra-strand crosslinks), or in opposite DNA strands (inter-strand crosslinks, ICLs) [60]. Chemotherapy is the main, clinical source of ICL-inducing agents (e.g., cisplatin), but there are also environmental agents which cause ICLs, such as a high lipid diet [61], alcohol, natural psoralens [e.g., derived from the diet [62]], oestrogens [63], and ionizing radiation [64]. Clearly the assessment of ICLs is important, and there exists a variant of the comet assay to evaluate this class of DNA lesions [65].

The principle of the ICL-modified comet assay is that the presence of ICLs in DNA will retard the electrophoretic migration of the DNA loops which form the comet tail (**Figure 2**). As part of the assay, SBs are induced *via* exposure to certain genotoxic agents (e.g., H_2O_2 or ionizing radiation). In the absence of ICLs, these SBs will result in a significant comet tail. However, the greater the number of ICLs present in the sample, the shorter the tail will be, owing to ICL-induced retardation of migration, compared with a sample not treated with the crosslinking agent (**Figure 3**). See **Box 1** for a detailed protocol.

1.2.3. Detection of UV-induced cyclobutane pyrimidine dimers, and bulky DNA-adducts

UV-induced cyclobutane pyrimidine dimers, predominantly thymine-thymine dimers, can be detected using the DNA repair enzyme T4 endonuclease V, as a variant of the enzyme-modified comet assay [66]. An alternative to this approach is to exploit the transient SSBs that occur when nucleotide excision repair (NER) enzymes act on UV-induced cyclobutane pyrimidine dimers, and other bulky lesions, in mammalian cells. They accumulate to a measurable level if an inhibitor of DNA synthesis is present, blocking resynthesis at the damage site and preventing ligation [67,68]. Originally, hydroxyurea (HU, which blocks DNA precursor synthesis) and 1-β-D-arabinofuranosyl cytosine (araC, a cytosine structural analogue and chain terminator) were used; later, aphidicolin (an inhibitor of B-family DNA polymerases, comprising Pol α, Pol δ, Pol ϵ and Pol ζ, which are involved in NER [69–71]) was found to be effective. See **Box 2** for a detailed protocol.

Recently this approach was applied to the detection of benzo(a)pyrene diolepoxide (BPDE)-induced adducts, which are also repaired by NER, using the comet assay [72,73]. BPDE-treated cells were incubated with aphidicolin, and the accumulated breaks were easily measured with the standard comet assay. Most recently, Ngo et al. [74] used HU and araC to detect bulky adducts using the CometChip technology, and HepaRG cells. Further work needs to be performed to demonstrate the potential of this DNA synthesis inhibitor approach as a component of genotoxicity testing regimes.

1.2.4. High throughput versions

Most laboratories use standard glass microscope slides as the support substrate for one or two agarose gel samples per slide. In this case, with a standard electrophoresis tank holding around 20 slides, the assay has a low throughput, and sample manipulation can be time-consuming. However, the throughput can be improved by increasing the number of slides in the tank, or by applying mini-gels on glass slides or plastic film, or by precisely locating cells in a micro-array format.

12-gel comet assay: A higher throughput approach has been developed by setting 12 mini-gels on a microscope slide [29]. To incubate each gel independently with various solutions, a gasket with holes over the gel positions can be used (NorGenoTech AS, cat. no. 1201), allowing differential treatment with chemicals, insoluble materials (*e.g.*, nanomaterials), reagents, or enzymes (**Figure 4**). Twenty slides can be run in a single experiment, resulting in a total of 240 gels. A benefit of the mini-gel approach is that it requires fewer cells and smaller volumes of test solutions compared with the conventional assay. The results obtained with the 12-gel comet assay format compare well with the traditional technology [75]. The various steps are suitable for further automation, and the formats can be adapted to fully automated scoring. The procedures save time at all stages as fewer slides are handled. A variant of this approach is the use of 8 mini-gels on a microscope slide [76,77]. A step-by-step protocol to use the 12-gel comet assay was published in Vodenkova et al. [22].

96-well format: In addition to the 12-gel system, the comet assay technology has also been developed to accommodate up to 96 mini-gels, in a 96-well format, on one GelBond® film [30,78] (**Figure 5**). GelBond® film is a thin unbreakable film used generally as a support for agarose gels. It was first applied to the comet assay by McNamee et al.[79]. The cell-containing agarose samples are applied with a multi-channel pipette. The film, previously cut to the size of a standard microtiter plate, with holes in each corner, is at all stages of the comet assay attached to a plastic frame for ease of manipulation, and to protect the gels (see **Figure 5**). It is possible to process almost 400 gels in one electrophoresis tank, holding four films. Processing (per sample) takes in total (but excluding scoring) 5–10 x less time than with glass slides [30]. However, the rate-limiting step is often the sample preparation prior to processing the gels. The use of GelBond® film has two main advantages over the use of glass slides: increased throughput, as it can be used to process as many gels as required up to 96 gels, with volumes ranging from 4-15 µL; and the plastic hydrophilic material reduces the likelihood of the gels detaching. See **Box 3** for a detailed protocol.

Using either the 48- or 96-well format and an electronic 8-channel pipette to apply samples helps to achieve precise positioning of the samples, facilitating automated scoring. This mini-gel system is amenable to full automation of all steps, including addition of samples, and processing of films. It has been validated using ionizing radiation, and a variety of chemicals, together with the enzyme-modified variant [30,75,80,81].

CometChip: This is a high throughput comet assay method that utilizes microfabrication techniques to pattern cells into an array (see **Box 4** for a detailed protocol) [82–84]. Cells are trapped for analysis within agarose microwells that are ~30-50 µm in diameter and spaced ~240 µm apart (**Figure 6**). This results in a regularly spaced grid of comets that appears as a 96-well plate format, allowing for dozens of samples to be analysed in parallel within a single chip, and reducing sample-to-sample variation that may be introduced by running slides across multiple electrophoresis tanks. In addition, arraying the cells (rather than dispersing them in agarose) decreases the likelihood of overlapping comets, and ensures that all comets are within the same focal plane. This allows for automated imaging, and comet scoring, which significantly reduces assay labour, improves assay throughput by at least an order of magnitude, and removes operator bias from the analysis process.

The CometChip has been used to study DNA damage and DNA repair in a wide range of cell types and chemicals. For example, studies of oxidation and alkylation damage have been performed with H_2O_2, and methyl methanesulfonate (MMS) [84–87]. It is also possible to apply the CometChip to detect DNA damage that requires metabolic activation by using metabolically competent cells, such as HepaRG™ [86]. Note that while to date most experiments have been performed with cultured cells, it is

also possible to use the CometChip to analyse cells harvested from minced tissues that have been frozen. Recently, the CometChip protocol has been modified to detect bulky adducts using NER-inhibitors in BPDE-treated cells [74], and it has also been applied in hepatocyte spheroids [88]. A list of CometChip applications can be found in Chao and Engelward [89].

***High Throughput comet assay system**:* Karbaschi and Cooke developed, and patented, a system whereby all the sample work-up steps, electrophoresis, and post-electrophoresis steps are performed with the comet slides held vertically, rather than horizontally, which is the convention [90] (**Figure 7**). A detailed protocol is described in **Box 5**. Holding slides vertically in racks (up to 25 per rack, 100 gels per electrophoresis run, in a novel tank design) allows batch processing - decreasing risk of damage to/loss of gels, and increasing throughput; the footprint of the tank is decreased significantly (allowing tanks to be 'multiplexed' from the same powerpack); and cooling is integrated in the system.

1.2.5. Detection of global DNA methylation

Apart from detecting SBs, and specific types of DNA damage in single cells, the comet assay has been utilized to evaluate the global DNA methylation status at the single cell level. DNA methylation is tissue-specific, and the comet assay, in combination with methylation-sensitive restriction endonucleases, can be used to measure changes in DNA methylation patterns of a variety of cells under different physiological conditions.

Originally, the difference in the methylation sensitivity of the restriction endonucleases *HpaII* and *Msp*I was exploited in a modification of the comet assay to measure global DNA methylation levels in individual cells (see **Box 6** – Option 1 for the protocol) [91,92]. These two isoschizomeric restriction enzymes recognize the same tetranucleotide sequence (5'-CCGG-3'), but display different sensitivities to DNA methylation, and have been employed in other techniques, such as the cytosine extension assay and the luminometric assay [93,94]. *HpaII* is inactive when either of the two cytosines is methylated, but it digests the hemi-methylated sequences at a lower rate compared with the unmethylated sequences. In contrast, *MspI* cuts unmethylated 5'-CCGG-3' and 5'-C^{m}CGG-3' sequences, but not 5'-^{m}CCGG-3'. The global 5'-CCGG-3'methylation can be calculated by the *HpaII*/*MspI* ratio (**Figure 8**).

The newly developed modified comet assay, the EpiComet-Chip (**Figure 6c**) allows single platform evaluation of genotoxicity (DNA damage), and global DNA methylation [specifically 5-methylcytosine (5-mCyt)] status, of populations of single cells under user-defined conditions [42]. *McrBC* specifically recognizes DNA sites of the form 5'- (G/A)^{m}C-3' and cuts DNA at methylated Cyt, thus forming comets. *McrBC*, unlike other restriction enzymes, cleaves DNA containing 5-methylcytosine, 5-hydroxymethylcytosine or N4-methylcytosine on one, or both, strands [95,96]. *McrBC* recognizes two half-sites on DNA of the form (G/A)^{m}C; these two halves of the recognition site can be separated by up to 3 kb, but the optimal separation is 55-103 bp (recognition site is 5'...Pu^{m}C (N-$_{40\text{-}3000}$) Pu^{m}C…3'). Because *McrBC* has a very short consensus sequence (Pu^{m}C), it potentially can recognize and cut a large proportion of the methylcytosines present in DNA. The EpiComet assay involves some modifications of the procedure steps as described in **Box 6** – Option 2.

1.2.6. Detection of chromosomal breaks in yeast

The chromosome comet assay evaluates chromosomal DNA breaks, and the occurrence of replication intermediates during clonal yeast culture, which may be a sign of replication stress as a consequence of DNA re-replication and/or R-loop formation [97]. Briefly, the yeast chromosomes are

obtained using standard pulsed-field gel electrophoresis. The chromosomes are then cut from the gel, and coated with low melting point agarose between two layers of normal melting point agarose, and then subjected to standard alkaline DNA electrophoresis (see detailed protocol in **Box 7**) [98]. The single chromosome comet assay may be considered as a useful approach for studying replication aberrations and replication stress as an alternative to traditional 2D gel analysis [99].

1.2.7. Bromodeoxyuridine (BrdU) comet assay: measurement of cell cycle specific comet formation

Incorporation of the thymidine analogue 5′-bromo-2′-deoxyuridine (BrdU) is a popular method for determining cell proliferation rates in a wide variety of organisms, ranging from plants to mammalian cells [100,101]. The BrdU comet assay represents a combination of the immunofluorescent staining of incorporated BrdU, and the alkaline comet assay analysis (see **Box 8** for a detailed protocol) [102–104]. This modification of the comet assay can be used for the measurement of DNA damage in cell populations that are unsynchronized, i.e., in different phases of the cell cycle. The advantage of this assay is that it allows discrimination between cells with induced DNA damage, and cells in the S-phase of the cell cycle (DNA synthesis/replication), which contain a physiological level of DNA discontinuities or gaps (detected as DNA breaks in the comet assay), as a results of ongoing semiconservative replication. Since cells progressing through S-phase form comet tails in alkaline comet assay, this approach helps to distinguish replicating cells among the total population of cells forming comet tails (**Figure 9**). Pulse labelling of cells with BrdU can also be used to test post-replication recovery after DNA damage where cells with compromised post-replication repair machinery show marked increase in the amount of BrdU labelled DNA in comet tail.

1.2.8. Comet-FISH assay: measurement of damage in specific DNA sequences

While the comet assay enables the researcher to study DNA damage at the level of single cells, combination of this with fluorescence *in situ* hybridization (FISH), using labelled probes targeting particular DNA sequences, allows the study of DNA damage at a gene-level (reviewed in [38]). In **Box 9** a step-by-step protocol is described. Depending on which target sequences are to be detected, different DNA probes have been applied in comet-FISH techniques (**Figure 10**), including various repetitive elements, chromosome arm- or band-specific probes, whole-chromosome probes, DNA fragments cloned in artificial chromosomes, as well as 'padlock probes' which are able to 'lock' around the target DNA sequence to allow circularised amplification, and peptide nucleic acid probes, in which the nucleobases are attached *via* methylene carbonyl bonds to repeating units of N-(2-aminoethyl) glycine. The application of this technique has given information about rates of repair of different genes, in relation to nuclear structure [40,105,106].

1.3. Applications of the method to different species, tissues and cell types

The comet assay can be applied to virtually any cell type derived from different organs and tissues of eukaryotic organisms (**Figure 11**). Although it is mainly applied to human cells, the assay also has applications for the evaluation of DNA damage in cells in culture, yeast, plant, and animal cells [3–5,107–111]. The assay can be performed on samples from across all invertebrate and vertebrate species [111]. Besides the large number of animal species, the assay has also been performed on a variety of cell types, including white blood cells, bone marrow, liver, kidney, brain, bladder, lung, stomach, gill, haemolymph, digestive gland, embryo cells, ovary, testis but also germ cells (oocytes and sperm) and

even embryos [3–5,110]. Regarding plants, the comet assay can be performed on cells from leaves and roots [109,112,113], and its use in higher terrestrial plant is increasing.

The following sections illustrate the various applications of the *in vitro* and *in vivo* comet assay with different materials. Performing an exhaustive review of the literature is out of the scope of this paper, and so we provide only key publications, and recent modifications for each of the models and biomatrices.

1.3.1. In vitro models

***Cell lines*:** The comet assay has been performed with numerous different cell types, either primary or immortalized cells, of human or animal origin, and from different organs and tissues [114]. Owing to their availability, immortalized cells have been the most frequently used for genotoxicity testing with the comet assay, in particular, hepatic cells [115–119]. Among other tissue-derived cells, neural cells seem to be a reliable alternative to *ex vivo* primary cell culture, since access to brain tissue is challenging [120]. The liver, skin, lungs and intestines are among the main sites for exposure to environmental agents, and therefore established cell-lines from such origins have been used in the comet assay [121–124]. These are just a few examples since the comet assay has been performed in mono-cultures of many different cell lines. Another interesting application of the comet assay is in co-culture experiments with combinations of different cell types, which provide physiologically more relevant culture conditions than mono-cultures. Examples include co-culture of Caco-2 and HT29 cells, as a model of the intestinal barrier [125,126]; co-culture of lung epithelial A549 and THP1 cells [127–129]; and a co-culture model of hepatocarcinoma HepG2 cells and endothelial cells (HUVEC) [130]. Fish cells have been used successfully for the detection of genotoxic effects, and can serve as an alternative to *in vivo* experiments in preliminary (eco-)genotoxicity studies [131–133]. The comet assay has also been used with stem cells from different species, including human mesenchymal stem cells [134], human adipose tissue-derived mesenchymal stem cells [135], and murine bone marrow mesenchymal stem cells [136].

***Three-dimensional (3D) models*:** Cellular organization and function are simulated more accurately in advanced 3D mini-tissue and mini-organ models, compared with traditional two-dimensional cultures with cells growing in monolayer. Utilizing cells of human origin in advanced *in vitro* models may also better reflect human biology compared with *in vivo* rodent models [137–139]. 3D skin models have now reached an advanced state of validation following over 10 years of development, while liver and airway (lung) model-based genotoxicity assays show promise but are at an early stage of development [140]. The 3D skin comet assay is now undergoing independent peer review by EURL-ECVAM, followed by the development of an OECD Test Guideline [141–144]. The use of liver spheroids with the comet assay is a novel approach [145,146] which has so far been used to assess the genotoxicity of nanoparticles and chemicals [147,148]. A protocol for applying the comet assay to 3D lung models was established using two commercially available human reconstructed 3D lung models, and one model developed in-house [149,150].

***Zebrafish embryos*:** The zebrafish embryo, a widely used vertebrate model in (eco)toxicology, is regarded as an *in vitro* system until 120 hours post fertilization (hpf). This allows stressful or invasive procedures to be performed on embryos, as they are not subjected to ethical regulation; only after 120 hpf must research on zebrafish be compliant with the European Union Directive 2010/63/EU [151,152]. The embryos have many advantages; being sensitive, inexpensive, optically transparent, with rapid *ex utero* embryonic development. Thus, the zebrafish embryo has been considered as a powerful alternative model for traditional *in vivo* (geno)toxicity screening, with advantages of whole-

animal investigations (e.g., intercellullar signalling, intact organism, functional homeostatic feedback mechanisms) and convenience of cell cultures (e.g., small quantities of test item, cost- and time-efficient, minimal infrastructure). In 2006, the first comet assay study with zebrafish embryos was conducted in which authors systematically evaluated different protocols for generating a suspension of single cells from treated embryos in terms of cell viability, cell yield and genotoxic damage [153]. Despite the benefits of the research on embryos, they are still not frequently used with the comet assay. Most studies have been conducted with adult fish and during embryo-larval stage. With embryos only a small number of studies have been performed (Canedo and Rocha [132], more information is in section 1.3.3).

Yeast and filamentous fungi: The yeast comet assay has been in use for over 20 years. The ease of cultivation and preparation of yeast cells for the comet assay makes their use promising for the assessment of genotoxicity of environmental pollutants and natural products, and for elucidating mechanisms of action. A particular advantage is that mutants with different signaling pathways, and DNA repair activities, are available. Different yeast and filamentous fungi strains and species have been used for the assessment of spontaneous or agent-induced DNA damage [107,108]. In addition, they have been used to study the mechanisms of DNA damage and DNA repair at the level of individual cells [154]. As described in section 1.2.6, a modified comet assay protocol has been developed to examine damage in single yeast chromosomes [97].

1.3.2. Plants

Use of the comet assay for plants has been focused on a few model species, such as *Allium cepa*, *Nicotiana tabacum*, *Vicia faba* or *Arabidopsis thaliana*, but its use in higher terrestrial plants is increasing (reviewed in: Ghosh et al. [112]; Lanier et al. [113]; Santos et al. [109]). The neutral comet assay was used for the first time with plant tissues in 1993 [155]; the alkaline version was modified and applied to broad bean (*Vicia faba*) a few years later [156]. Application of the comet assay to plants has mostly consisted of testing for genotoxicity of metals, pesticides and other organic pollutants, phytocompounds, nanomaterials, contaminated matrices (water, soils, sediments, air) and radiation; investigating the genotoxic mechanism of chemicals; and studying plant DNA repair [157]. The assay has also been used as a biomonitoring tool to assess environmental pollution, and to evaluate the potential of some plants for the phytoremediation of contaminated soils, sediments or waters (reviewed in: Gichner et al. [158]; Lanier et al. [113]; Santos et al.[109]).

1.3.3. The use of non-mammalian samples

This and the following subchapter (1.3.4) are brief summaries of the most commonly used models for the *in vivo* comet assay. Recently published reviews by Gajski et al.[4,5] provide a comprehensive overview of all animal models that have been used for the comet assay.

Crustaceans - Daphnia magna, Ceriodaphnia dubia: The comet assay has been applied to several freshwater and marine species. Crustaceans are suitable models for both genetic toxicology, and environmental biomonitoring on a large scale [4]. Several freshwater zooplanktonic species are used for DNA damage assessments, using the comet assay [159–161]. In these species, DNA damage is measured in cells from the haemolymph, or in cell preparations from whole animals, exposed to various physical and chemical agents [4,162,163].

***Insects*:** Insects could partially replace vertebrates in toxicological studies, avoiding certain ethical issues. *Drosophila melanogaster* is a valuable model organism for genetic studies, and also for studying the DNA damage response; the comet assay is performed mainly *in vivo* using different

larval cell types (haemolymph, brain and midgut) [164–166]. In 2002, the first paper in which the comet assay was applied to brain ganglia cells of *Drosophila* was published [167]. Since then, other larval cell types have been used, such as midgut cells, alone or in combination with brain cells [168–170]. The comet assay has been applied to *Drosophila* neuroblasts in genotoxicity assessment studies [164,168,169,171]. It has also been used to study the antigenotoxic effect of macroalgae [166], and to analyse the influence of protein overexpression on genome integrity *in vivo* [172,173]. Haemocytes of *Drosophila*, the equivalent of mammalian lymphocytes, represent a general cell model in which to evaluate the genotoxic risk associated with specific exposures. The application of the comet assay to haemocytes as a cell target for DNA damage detection started in 2011 [174]. Augustyniak et al.[175] published a review on the use of the comet assay in insects.

Molluscs: Marine and freshwater bivalve molluscs have been used for many years as sentinel organisms for monitoring environmental pollution, in particular in coastal areas. Their filter-feeding activity, and low metabolic rate favour bioaccumulation of contaminants [176]. A range of mollusc species have been used with the comet assay, including bivalves, gastropods and cephalopods although the majority of studies have been performed on mussels and clams (bivalves), starting in the late 1990s. Several modifications have been introduced to the initial approach [177,178]. The comet assay using bivalve molluscs was initially developed for haemolymph cells from the oyster *Crasostrea virginica* [179], and from the marine mussel *Mytilus edulis* [180], in gill cells from *M. edulis* [181], and with digestive gland cells from the same species [182]. Since then, this assay has been routinely applied for a variety of purposes under laboratory and field conditions; the most commonly used species are described in review articles [3,4,183].

Platyhelminthes: Free-living planarians or flatworms (Platyhelminthes) have a long history of use in regeneration and stem cell biology as a unique *in vivo* model to study stem cell dynamics in various contexts [184]. An important application is the determination of DNA damage during developmental and regenerative processes, or following experimental treatment. Planarians are increasingly used for risk assessment and toxicity screenings as well as to investigate environmentally-induced genotoxicity or drug-related carcinogenicity [185,186]. The comet assay can be applied on whole organisms or on an isolated stem cell cell-enriched fraction (obtained *via* a dissociation protocol). The first use of the comet assay with planarians, in *Dugesia schubarti*, was to identify the genotoxic potential of copper sulphate [185]. Since then, this organism has been used to address various research questions in toxicology screening, as well as for mechanistic stem cell research in relation to the DNA damage response. Moreover, it has been used for dissecting molecular mechanisms in relation to stem cell processes, and regeneration [187–189].

***Annelids*:** Since a study concerning non-invasive extrusion of coelomocytes from earthworms (*Eisenia fetida*) published by Eyambe et al.[190], there have been few modifications to the protocol for collecting cells from these worms. Verschaeve and Gilles [191] were pioneers in the use of the comet assay on coelomocytes from earthworms for the detection of genotoxic compounds in environmentally contaminated samples. Since then, numerous scientific studies have been published using the same method to monitor environmental contamination to reveal the genotoxic effects of xenobiotics, or to allocate ecotoxicological endpoints [192–198].

Amphibians: There are a large number of studies on amphibians for the evaluation of environmental pollution using the comet assay, either following environmental exposures, or under laboratory conditions [5], the first study dating back to 1996 [199]. The most frequently used amphibians are frogs and toads, with the comet assay having been conducted on both tadpoles and fully developed, adult specimens [3,4,199,200]. In both larval and adult stages, different cell types, such as

blood (erythrocytes), liver and sperm, have been sampled. Most studies have been performed with environmental stressors, such as agrochemicals and heavy metals, to which amphibians are very sensitive - (reviewed in [5]).

Fish: Fish (both marine and freshwater) are among the most widely used organisms in ecotoxicology [3], and among the first animal models to which the comet assay was applied as a biomonitoring tool [201]. Studies are performed with several specimens, though most frequently on blood, followed by liver, gills, gonads and sperm [5]. The comet assay has also been used for the evaluation of the genoprotective properties of functional feeds with a combined nutritional-genetic approach [202].

1.3.4. The use of non-human mammalian samples

In vivo mammalian comet assay experiments normally make use of laboratory animals such as mice and rats, the standard experimental animal models for genetic toxicology studies generally. Multiple organs from mice and rats such as blood, liver, kidney, brain, lungs and bone marrow have been used for the genotoxicity testing of a large range of chemicals. Studies with laboratory rodents have been extensively reviewed [203–209].

Rodents: The alkaline comet assay was first used in rats in 1993 for the quantification of DNA SBs to assess the genotoxic effects of lindane in mucosal cells from the nasal cavity, stomach and colon [210]. An OECD guideline (TG 487) for the *in vivo* comet assay to detect DNA SBs was published in 2014, and updated in 2016. However, procedures for the detection of other DNA modifications in rodents, for example, oxidatively damaged DNA were already published in the early 2000s [211,212]. Despite the extensive use of the comet assay to test for genotoxicity in solid tissues from rodents, there are no standardized procedures to collect, store and homogenize samples. The OECD guideline does not address the use of frozen tissue/cell suspensions (see the "technical modifications" section for more details). In general, rodent tissues can be used for genotoxicity testing of chemicals present in consumer products, diets, environmental and occupational settings. Interestingly, the comet assay has been used in studies of complex mixtures such as 'air pollution' [123], as well as nanoparticles [213] and physical agents such as radiation [214].

Domestic and wild mammals: Animals kept as pets (e.g., cats and dogs) may be considered as sentinels for environmental factors to which humans are exposed. Therefore, they can be used as a surrogate for human exposure. Although this is an interesting application, there are few reports [5]. Apart from pets, the comet assay has been applied to several other domestic species, such as horses, donkeys, bulls, goats, sheep and boars, generally performed on sperm to test the semen quality after cryopreservation, and prior to artificial insemination, and this represents a broad field of research (reviewed by Gajski et al.[5]). A variety of wild species have been used to study pollution, and environmental conservation in both marine (e.g., dolphins) and terrestrial environments (mainly rodents and various large wildlife mammals). In addition, the comet assay was used for the evaluation of sperm DNA integrity of several metatherian species and rhinos [3,5].

1.3.5. The use of human samples

The comet assay has been extensively used in human biomonitoring studies, mainly applied to white blood cells, for the purpose of assessing the effect of environmental, and occupational exposures [20]. Also effects of nutritional and therapeutic interventions on DNA damage have been studied [215–220]. In addition, DNA damage has been assessed in connection with aging and high-prevalence diseases

[220,221]. The technique has also been applied to umbilical cord blood cells [222–224] and placenta [225–227]. The use of these samples is a suitable approach to assess exposure and genotoxicity during early life.

White blood cells: Blood is one of the most suitable and widely used specimen in biomonitoring. Blood cells circulate in the body, and the cellular, nuclear, and metabolic state of the blood cells may reflect the overall extent of body exposure [228]. Advantages and limitations of using whole blood, leukocytes, buffy coat (whole blood enriched with leukocytes), and isolated peripheral blood mononuclear cells (PBMCs) have recently been described [229]. The comet assay has been used for three decades in human biomonitoring studies; PBMCs are the most common sample material, though whole blood has also been widely used. Topics investigated include occupational or environmental exposure to air pollution and other genotoxic agents, dietary and lifestyle habits, the effects of oxidative stress related to exercise, nutrition as well as so-called seasonal effects [20,27,33,217,230–238]. The comet assay has also been applied to assess DNA damage as a factor in diseases [239,240] and also as a tool in diagnostic and medical treatment procedures [19,241,242]. A recent pooled (meta)analysis of a database of comet assay results from almost 20,000 individuals found that there was little effect of age on SBs, and no difference in SBs between males and females. Smoking had no effect, while occupational/environmental exposure to a variety of genotoxic agents had very significant effects [243]. It is possible to use isolated polymorphonuclear (PMN) cells in the comet assay [244]. PMN cells such as neutrophils [245–248] and granulocytes [249,250] were used in relation to certain diseases and occupational exposures.

Cryopreservation of blood samples has been used in biomonitoring studies for many years (reviewed by Møller et al. [229] and Marino et al. [251]); biobanks may contain samples of PBMCs, but more often whole blood or buffy coat was stored. The finding that the comet assay can be carried out with frozen whole blood [252], or frozen leukocytes isolated from blood, makes it possible to carry out nested case-control studies to investigate associations between disease incidence (or mortality) and DNA damage measured decades earlier [234,253].

Mononuclear cells (MNCs) can be isolated from cord blood, and used in the comet assay [254–256]. The comet assay has been applied to these cells to study DNA damage in preterm infants [254–256], and the correlation between maternal blood glucose levels of woman with diabetes or mild gestational hyperglycemia and the DNA damage levels in the MNCs from the offspring [257], as examples.

***Leukocytes from saliva*:** Isolation of leukocytes from saliva (as alternative to, or to complement blood samples) represents a potential strategy for non-invasive, human biomonitoring studies using the comet assay [258–260]. They are of particular interest when the main route of exposure is by inhalation or ingestion, or when blood samples are difficult to collect (from children, dementia patients, subjects with vein problems, etc).

Epithelial cells: The comet assay has been applied to epithelial cells of the buccal mucosa, nasal epithelium, and ocular cells including lens epithelium, cornea and tear duct [261,262]. Buccal cells have been used since 1996, with at least 50 articles reporting their use [261,263,264]; they are particularly appropriate for biomonitoring in children. A number of studies have used the comet assay on nasal cells in biomonitoring studies of environmental and occupational exposures [265–272], to assess the potential antioxidant effects of several compounds [273], and to assess oxidatively damaged DNA [274]. Concerning ocular cells, lens epithelial cells were used to study age-related cataract [275], and tear duct and corneal cells have been used to test the effect of environmental pollutants, principally ozone [276].

***Sperm*:** The comet assay has been extensively used to study sperm in the context of the effects of environmental substances on fertility [277,278], with the diagnosis of male infertility [279], and medically assisted human reproduction [280,281]. The proportion of sperm with highly damaged DNA, assessed by

the comet assay, has been shown to have a predictive value for male infertility and a significant impact on decreased number of live births [282,283]. The latter authors proposed the use of novel comet assay parameters (High damage Comet Score, and Low damage Comet Score), and introduced threshold levels for the proportion of damaged cells. Only a few papers describe the use of enzymes to detect oxidised DNA bases in sperm (for example, Simon et al. [284,], and Sipinen et al.[278]), and a high throughput method has been described for the sperm comet assay [285].

***Placenta*:** Placental cells have been used for the evaluation of prenatal exposure-induced developmental toxicity [286]. In humans, the placenta is a useful biomatrix that is obtained non-invasively [287]. There are a few published studies analysing DNA damage using the comet assay in cells isolated from human placentas, either for cell characterization [225], or for genotoxicity testing [226].

1.4. Comparisons with other methods for assessing DNA damage

The alkaline comet assay, alkaline elution and alkaline unwinding are comparable in terms of ability to detect low levels of DNA breaks, in the sub-lethal range for mammalian cells, and all three have been employed in biomonitoring, genotoxicity testing and ecotoxicology as well as basic research. The principle of alkaline elution is that when cells are lysed on a microporous filter and then an alkaline solution is gently pumped through the filter, the single-stranded DNA molecules (denatured by the high pH) elute through the filter at a rate inversely related to their size [288]. In the alkaline unwinding method [289], cells are lysed in alkali for a certain time and then neutralised and sonicated, resulting in a mixture of single- and double-stranded fragments; these are separated by hydroxyapatite chromatography, and the proportion of single-stranded DNA is related to the break frequency. The main advantages of the comet assay are the number of samples that can be processed in a single experiment, the ability to visualise damage at the single cell level, and the existence of various modifications for detection of specific DNA damage.

These three methods were among the methods examined in the ESCODD project [11], which aimed to resolve discrepancies in estimates of the background level of 8-oxoguanine found in human cells. Methods based on detection of the oxidised nucleobase with Fpg - including alkaline elution and alkaline unwinding as well as the comet assay - routinely came up with estimates an order of magnitude, or more, lower than the concentrations determined by analytical methods such as high-performance liquid chromatography with electrochemical detection (HPLC-EC), gas chromatography-mass spectrometry (GC-MS), and also HPLC with tandem mass spectrometry (HPLC-MS/MS). By means of controlled ring studies with human lymphocytes, the best estimate of background levels of oxidatively damaged DNA was 4.2 8-oxoguanines per 10^6 guanines with chromatographic methods, compared with 0.3 8-oxoguanine per 10^6 guanines when employing Fpg. Evidence [290,291] points to adventitious oxidation occurring during the sample workup for the chromatographic analyses, typically more drastic than the preparation for the enzyme-based assays. The results of ESCODD led to the development of improved DNA extraction methodology, and lower levels of damage detected by methods such as HPLC-MS/MS.

The comet assay adapted to assess DNA methylation status relies on the use of methylation-sensitive and insensitive restriction endonucleases. The first version by Wentzel et al.[92] employed the most commonly used isoschizomer pair *HpaII* and *MspI*, and showed highly comparable results to those obtained with the well-established cytosine extension assay. This cytosine extension assay involves DNA digestion by *HpaII*/*MspI*, followed by single nucleotide extension using either radio-labelled [^{3}H]dCTP [93] or biotinylated dCTP [292]. More recently, the EpiComet-Chip was developed,

involving the restriction enzyme *McrBC*. This EpiComet-Chip showed high validity when compared to the MethylFlash Methylated DNA Quantification Assay (using capture and detection antibodies, followed by fluorometric quantification): single sample hypermethylation (≥1.5-fold) was correctly identified at 87% (20/23), hypomethylation (≥1.25-fold) at 100% (9/9), with a 4% (2/54) false negative rate (FNR) and 10% (4/40) false positive rate (FPR) [42].

DNA-DNA cross-links have been measured by both the comet assay and alkaline elution, and both assays reply upon the ability of cross-links to retard the migration or elution of DNA; however, there is apparently no report in the literature of a direct comparison of the two approaches, nor a comparison of either with an approach which can provide absolute quantification, such as mass spectrometry.

1.5. Limitations, and attempts to overcome them

Although there are many advantages, the comet assay does have limitations, related to the challenges of obtaining absolute quantification, and unequivocal identification of the damage. Other limitations include differences in results between laboratories, because of different ways to measure the DNA migration and differences in comet assay procedures [230,293].

The scoring of comets is the major technical limitation in the comet assay. The level of DNA damage is inferred from the extent of DNA migration. After staining, comets can be scored by either image analysis or visual assessment. In the case of image analysis, there is a choice of descriptors; tail length, tail intensity (TI, also referred as % DNA in tail) and tail moment (TM). They give rise to results expressed in different units, which cannot easily be compared [294,295]. The tail length is proportional to the extent of DNA damage but reaches its maximum at a relatively low level of damage, which is why it is not recommended for biomonitoring purposes [296]. TI is expressed as % of total DNA fluorescence in the tail of the comet. TM is calculated as the product of the tail length and the fraction of total DNA in the comet tail. The TI is currently recommended by the OECD as the best descriptor for DNA break frequencies since it uses a quantitative measure of damage (from 0 to 100 %) [204]. However, several researchers still tend to use TM, since it takes into account both the length and DNA content of the comet tail. TM has the disadvantage of not having standard units, and given a particular TM, it is impossible to visualise the level of damage being described [295–301]. Each of these primary comet descriptors can be transformed to a break frequency, such as breaks per million normal nucleotides or base pairs, using calibration with ionizing radiation that has a known relationship between the dose and induction of DNA SBs [288,289,302]. Such a transformation produces comet assay results that are much easier to understand than the primary comet assay descriptors [295]. However, lack of access to sources of X- or gamma-rays has limited the adoption of transformation of comet assay results to 'real' break frequencies.

Inter-laboratory variation in the reported levels of DNA damage has been recognized as a limitation of the comet assay, dating back to the early 2000s [303]. It results from differences in technique between labs and variation in scoring [19]. Inter-laboratory variation is especially recognized as a limitation in human biomonitoring studies as the apparent heterogeneity between DNA damage levels in different populations might in fact be due to varied technical procedures being used in the laboratories involved[17].

Attempts to standardize the comet assay protocol in validation trials have been partly successful in the sense that the inter-laboratory variation is decreased by using standardized protocols [14]. This issue of lab-to-lab heterogeneity in reported levels of DNA damage is probably the most serious limitation of the comet assay; resolving it will depend on the introduction and adoption of better

protocols, and the rigorous application of assay controls; it follows that publications should include a detailed description of the protocol used [21,23,232].

While there are no published data demonstrating that DNA damage levels measured by the comet assay can predict the development of cancer or other diseases, a recent analysis of prospective studies has shown that high levels of DNA SBs are significantly associated with higher overall mortality in a healthy human population [304]. Patients with the most prevalent non-communicable diseases have elevated levels of DNA damage in PBMCs, but this association may be due to reverse-causality as the observations stem from cross-sectional studies of patients and healthy controls [221] There is evidence demonstrating that many genotoxic carcinogens cause DNA damage, measured by the comet assay, in animal organs and cell cultures [208,305]. Certainly, the comet assay is not expected to be a stand-alone test with power to accurately predict individual risk of disease such as cancer but it is likely to be of value at the population level. The comet assay is typically combined with tests for clastogenic effects and mutations in animal models to characterise carcinogens with different genotoxic mechanisms of action [306,307]. This is not standard practice in biomonitoring studies of humans or sentinel species and further research is needed to obtain information on the optimal combinations of biomarkers.

A potential limitation of the comet assay, particularly in biomonitoring studies, is the logistical difficulty of processing large numbers of samples and analysing them on the same day. However, for many years it has been standard practice with isolated PBMCs to suspend them in freezing medium (e.g., culture medium with 10 % foetal bovine serum and 10 % dimethyl sulfoxide (DMSO)) and freeze them slowly to −80 °C. This avoids the risk of adventitious damage to the DNA through the formation of ice crystals. An important advance is the finding that whole blood can be snap-frozen, in small volumes, and successfully analysed - even after storage for 5 years [229,240,252,308–312]. The implication is that such samples could be used in large-scale human biomonitoring and long-term epidemiological studies. The risk of adventitious generation of DNA damage by freezing and thawing may have limited the use of tissue biopsies in the comet assay. However, it is possible to snap freeze the tissue, store it at -80 °C and process it in such a way that the tissue remains frozen until the cells are in suspension - thus ensuring reliable comet assay results [313].

1.6. Experimental design

It is recommended that comet assay experiments should be designed to include specimens from different exposure groups in the same experiment - especially in the case of biomonitoring studies and low-dose toxicology studies used for risk assessment, which look for small increases in DNA damage levels that are easily obscured by inter-assay variation. Studies where specimens are analysed *ad hoc* should incorporate cryopreserved assay control samples in the experimental design; these can be used to standardize the results, if needed, to adjust for the variations between experiments, over time, or between laboratories [232].

1.6.1. Controls

If possible, comet assay experiments should have negative and positive controls. Negative controls are vehicle-exposed cells and animals, and placebo or unexposed humans. For positive controls, the OECD recommends a number of direct-acting alkylating agents for the standard comet assay in animal organs (OECD TG 489), which can be used as positive controls for *in vitro* studies too. Ionizing radiation is by far the best positive control for the standard comet assay because it is

applicable to all species and cells, but it can be difficult to get access to X-ray equipment, or gamma-sources. Hydrogen peroxide is a reasonable alternative as a positive control in cell culture experiments, but is not suitable for *in vivo* studies. Unfortunately, there are no positive controls that can be used for all versions of the comet assay. A positive control agent for the enzyme-modified comet assay should generate DNA lesions that are excised by the relevant enzyme, but should not give rise to SBs. The photosensitizer Ro19-8022 has been the most widely used control for the Fpg- and hOGG1-modified comet assay, although 4-nitroquinoline-1-oxide and potassium bromate are also good candidates [314]. Potassium bromate has been tested in a multi-laboratory ring-trial, and shows consistent results in cell culture experiments from different laboratories [23]. It has also been used as a positive control by oral administration to animals for the hOGG1-modified comet assay in the liver and kidney [315].

In certain cases, it is not possible to include a positive control group. For instance, a positive control group is not possible in human biomonitoring studies, because it is unethical to expose human beings to potentially carcinogenic compounds. The solution is to use positive assay controls, which are cells that have been exposed to DNA-damaging compounds and cryopreserved. Cryopreserved unexposed cells serve as negative assay controls. The assay controls thus serve the purpose of checking the quality of the comet assay experiment, and also allow comparison of results from different laboratories, if each laboratory has access to the same control samples.

1.6.2. Optimization

The relationship between the actual number of DNA SBs and a comet assay end point descriptor resembles a sigmoid curve. There is a flat section at the bottom of the curve because a minimum number of DNA SBs are required before the DNA will migrate, and form a comet tail. At the upper part of the curve, there is a flattening of the curve because the assay reaches saturation, with virtually all the DNA in the tail, so that additional breaks will not cause further DNA migration. The middle part of the curve shows a linear relationship between the number of DNA SBs and the comet descriptor. This part of the curve determines the dynamic range of the comet assay (and therefore the upper limit of concentration or dose that can be analysed). In optimization, there is a trade-off between detection of low levels of DNA SBs (i.e., the sensitivity of the assay) and width of the dynamic range. Conditions that favour high sensitivity tend to narrow the dynamic range. Thus, the optimal comet assay protocol entails a reasonable sensitivity of the assay, together with a wide dynamic range. The optimization of the comet assay focuses on the best conditions for the specific specimen that is to be investigated. In the standard assay, DNA migration is affected by the percentage of agarose in which the cells are embedded, and the electrophoresis conditions (mainly the duration and strength of the electric field). For the enzyme-modified comet assay, it is important to optimize the enzyme concentration, and incubation time.

Optimization of the number of cells

The number of cells in each gel should be optimized to have a sufficient number of comets to score, but to avoid the likelihood of cells overlapping. Optimization should take into account that the presence of breaks will produce comet tails that can overlap with other comets. Overlapping comets are not scorable when using an image analysis system, although they may be scored when using visual scoring. Long comets are the result of highly damaged DNA, and are more likely to overlap, and so if they are not scored there is a risk of underestimating the damage.

Optimization of percentage of agarose

The optimal concentration of agarose ranges between 0.5% and 1.5% with most laboratories using a final agarose concentration of about 0.7% [21]. A high percentage of agarose impedes the migration of DNA in the gel, whereas a low percentage increases the fluidity of the gel, and risks detachment of the gels from the slides. In between these extremes, the optimization of the agarose concentration depends on the type of specimen (i.e., specimens with high basal levels of DNA damage may require a higher percentage of agarose), and the substrate used (such as glass slides, plastic GelBond® films and mini-gel formats).

Titration of enzyme concentration in the enzyme-modified comet assay

The enzyme-modified comet assay is based on the principle that treatment of gel-embedded nucleoids with an added DNA repair enzyme produces additional SBs because of the excision of specific lesions in DNA. This procedure is especially useful for studying DNA lesions that are not converted to SBs by the alkali treatment. It has been observed that the same enzyme from different producers may show substantial differences in activity and specificity [314]. Thus, it is paramount to titrate the enzyme and vary the incubation period, prior to analysis of test samples. The titration experiment aims at detecting all lesions that are recognized by the enzyme while avoiding non-specific incisions of the DNA [51]. **Figure 12** depicts an idealized two-step titration experiment with cells that have been treated with a genotoxic agent. First, gel-embedded nucleoids are incubated for a specific period with different concentrations of the enzyme. The optimal concentration of enzyme is obtained in the middle part of the titration curve where a plateau is reached. The subsequent step uses this concentration to determine the incubation time where all lesions are recognized, which is observed as a plateau in the comet score.

Optimization of electrophoresis conditions

The electrophoresis conditions are critically important because they determine the extent of DNA migration. Careful control of the electrophoresis step decreases assay variation, and increases sensitivity. There are proportional relationships between DNA migration levels, and both the electrophoretic field strength (i.e., voltage drop in the electrophoresis tank), and the duration of electrophoresis. These factors should be optimized to make it possible to score all comets in the sample, including comets with long tails. For instance, it is not advisable to use electrophoresis conditions that favour the formation of very long comets because it will create a problem with overlapping comets that are difficult or impossible to score in image software systems. As most comet assay researchers use image software systems to score comets, the practical solution is to use an electrophoresis condition that produces comets that can be captured as single isolated structures with the image analysis system. However, there are also other optimizations to consider, including achieving a homogeneous electrophoretic field and constant temperature during the electrophoresis. There is a proportional relationship between the temperature of the electrophoresis solution and the comet tail length [6,316,317]. Thus, care should be taken to avoid temperature differences in the electrophoresis tank because it lays the foundation for intra-assay variation. This source of intra-assay variation can be avoided by using homogeneous chilling across the tank or by recirculating the electrophoresis solution [30,318,319]. If recirculation of the solution is not possible, it is recommended to check the voltage at different positions in the electrophoresis tank, using a voltmeter, or making an experiment with identical samples of cells at all positions in the electrophoresis tank to assess the spatial variation in DNA damage.

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2. MATERIALS

Biological materials

Table 1 summarises the various experimental models, and sample types, that can be used with the procedures described in this paper. For a full list of animal species evaluated by the comet assay, see the following review papers (for invertebrates Gajski et al.[4], and for vertebrates Gajski et al.[5]).

A) *In vitro* models

A1. 2D Cell culture: For suspension cells, the most commonly used are leukaemia cells (e.g., TK6 and THP-1 cells), while for monolayer cells these are hepatic HepG2 or cervical HeLa cancer cell lines. However, almost, if not all animal and human-derived cell lines can be used. Primary cell cultures have also been used successfully [122].

! CAUTION The cell lines used in research should be regularly checked to ensure that they are authentic, and are not infected with Mycoplasma, or any other organism, as this may have an effect on the results, in particular the DNA damage response [320].

A2. 3D cell models

- Human reconstructed full-thickness skin tissues: e.g., Phenion® full-thickness (FT) skin (www.phenion.com), or EpiDerm FT skin tissue (www.mattek.com).
- Human reconstructed 3D airway models: MucilAir™ produced by Epithelix Sàrl, Switzerland (https://www.epithelix.com/products/mucilair) and EpiAirway™ produced by MatTek Corporation, US (https://www.mattek.com/products/epiairway/), or investigator-established ALI airway epithelial cell cultures sources [321].

A3. Zebrafish embryos: Embryos are collected after spawning and only freshly fertilized eggs (2 hpf) are used for the experiments with a duration of exposure up to 96 hpf [153,322]. Additionally, it is also possible to freeze (-80 ºC) freshly harvested cells isolated from embryos in physiological buffer containing 10% v/v DMSO, without a significant increase of DNA damage up to two weeks [323].

A4. Yeast and fungi. When working with *Saccharomyces cerevisiae, S. paradoxus, S. kudriavzevii, S. bayanus, Candida albicans, Cryptococcus neoformans, Schizosaccharomyces pombe*, it is highly recommended to use yeast cells isolated from a single cell colony transferred to liquid cell culture in the logarithmic phase of growth. The filamentous fungus *Ashbya* is usually cultivated on solidified *Ashbya* Full Medium (AFM).

B) *In vivo* models

B1. Plants. Collect roots and leaves from plants preferably fresh to get the best results with low background damage. Previously published studies reported the use of snap frozen leaves [324,325], but this remains to be optimized and validated with lab-to-lab comparisons.

B2. Invertebrate samples

- Haemolymph cells, coelomocytes, neuroblasts, and cells from other tissues can be used depending on the species (see Table 2). Heparinized haemolymph is normally used.
- The most frequently used organs from molluscs are digestive glands, and gills.
- For very small animals, such as some crustaceans and insects, whole body squashing can be performed to yield a generalised population of cells.

B3. Non-human vertebrate samples

The most frequently used tissues are blood (or isolated MNCs), liver, gills, and gonads, though other tissues have also been used (e.g., kidney, spleen, heart, duodenum, glandular stomach, jejunum, colon, brain, bladder, adrenals, hypothalamus, thyroid, pituitary, pineal gland, pancreas, ovary, prostate, mammary gland, uterus, testis, etc). Tumour samples can also be used. Whole blood is collected with an anti-coagulant such as citrate, EDTA or heparin.

Rodents could be anesthetized and exanguinated before taking the tissue samples. Immediately after removal of the tissue, excess blood and debris are flushed from the tissue with mincing buffer, or ice-cold Merchant's buffer before collecting a ~1 cm^3 portion and submerging in 0.5 mL mincing buffer on ice. Anesthetization and exsanguination steps should be very brief (<3 min), and consistent between animals with sample collection immediately afterwards, to minimize sample degradation and variability. Alternatively, tissues from non-exsanguinated animals should be thoroughly washed to remove blood by performing several washes in mincing buffer or ice-cold Merchant's buffer. Snap-frozen rodent solid tissues can also be used; the comet assay has been successfully applied to frozen tissues, such as liver, kidney, lung, brain and spleen (see examples of studies in Azqueta et al.[313]). In fact, the OECD test guideline 489 recognises that tissues can be frozen for later analysis but currently there is no agreement on the best way to freeze and thaw tissues [8]. Azqueta et al.[313] have described a protocol to freeze and thaw rodent liver, kidney and lung tissues prior to performing the standard and the Fpg-modified comet assay. The protocol is based on the study of Jackson et al.[326]. Freezing the whole tissue may not be convenient for some tissues such as the glandular stomach in which scraped epithelial cells are used for the comet assay analysis. In this case, freezing the cell suspension may be a better option.

Regarding fish, zebrafish, mosquitofish (*Gambuzia holbrooki*), gilthead seabream (*Sparus aurata*), Senegalese sole (*Solea soleganensis*) and European eel (*Anguilla anguilla*) are the most frequently used species, while blood, liver, gills, and gonads are the most often used biological matrices. The storage of snap-frozen fish tissues in liquid nitrogen is reported to lead to an increase in DNA breakage [327]; however, further investigation is required to confirm and/or ameliorate this effect. The use of snap-frozen amphibian solid tissue has not yet been reported in the literature.

! CAUTION All experiments involving animals must be approved by the relevant animal care and use committee, and adhere to local and national regulations.

Δ CRITICAL During any painful or stressful procedure, anaesthetization is recommended by ethical principles and regulation. However, the impact of chemical anaesthetics on the DNA integrity should be considered as some studies have shown the time-dependent induction of SBs in some tissues [328].

B4. Human samples:

- Whole blood: collect blood into an anticoagulant, such as Na_2EDTA or heparin, by venipuncture or lancet; only if the blood sample is to be used immediately after obtaining *via* a lancet, may

the anticoagulant be omitted. Choice of anticoagulant should be kept consistent within one study.

! CAUTION Do not use needles with very small diameter as this will cause a greater shearing effect, and may increase background DNA damage levels. It is recommended to use 20G (0.9 mm diameter) or 22G (0.7 mm diameter) needles.

- MNCs: Mononuclear cells can be obtained from cord, or peripheral blood after centrifugation by density gradient (https://youtu.be/tgNHWVqF52I). PBMCs can also be isolated from blood collected *via* lancet, as a finger prick (https://youtu.be/drbMxbFf3TM).
- PMN cells: After density gradient isolation of PBMCs, resuspend the remaining PMN-red cell mixture and isolate PMN cells by adding erythrocyte lysis buffer (https://youtu.be/tgNHWVqF52I) or polygelin solution [244] (see Procedure: Stage 1, step 1A).
- BMCs from saliva: Saliva samples are collected by performing four consecutive mouth rinses with 10 mL of 0.9% NaCl sterile solution for 1 min each. The rinses are combined in sterile 50 mL tubes. No changes in the oral hygiene habits are required, but consuming anything but water is prohibited for the hour before sampling. The oral rinses are centrifuged, and washed with cold PBS, and the cell pellet is re-suspended in RPMI 1640 cell culture medium. Leucocytes are isolated from the cell suspension by standard density gradient centrifugation [329,330].
- Buccal cells: before sampling, the subject should perform two consecutive rinses with water (room temperature). The sample is collected with a cytobrush or toothbrush.

Δ CRITICAL The initial collection/scraping of both cheeks (using separate brushes) is discarded. The superficial layer of the buccal mucosa is mainly composed by cells in early or late apoptotic phase (cells with condensed chromatin or in karyorrhexis) or necrosis (pycnotic or karyolytic cells). To collect viable buccal cells for use in the comet assay, scrape with new brush in circular movements of 10-15 circles on the same place on each cheek [261,263].

- Nasal cells: These samples are taken with a nylon brush or cytobrush. The participant must stand up, while the person taking the sample will hold their head to prevent it from moving during the sampling. The brush will be introduced slowly into either nostril, following the course of the nasal cavity vertically towards the superior turbinate and meatus; a delicate turn is made in the lower part of the cavity and the brush is carefully removed [331].
- Lachrymal cells: In parallel to collecting nasal cells, tears containing lachrymal duct and corneal cells can also be collected [276]. Once the brush is removed, given the stimulation of the olfactory bulb, reflex tearing occurs. To collect the tears, a capillary tube with a capacity of 10 - 30 µL is placed on the bridge of the nose in the direction of the tearing eye, and by capillarity the tear is introduced into the tube. The sample is maintained in the capillary tubes at room temperature prior to performing the comet procedure. The capillary will be placed in a microcentrifuge tube to subsequently elute the tears using a rubber bulb.
- Semen samples are obtained after three days of ejaculatory abstinence by ejaculation directly into sterile specimen beakers made of nontoxic plasticware. These need to be delivered to the laboratory, and analysis begun, within one hour of collection.
- Placental tissue: Collect a tissue section (5 x 5 x 3 cm) from the parenchyma villous of the fetal side, at least 4 cm from the cord insertion; discard the tissue immediately below the fetal membrane (~ 1 cm). Keep the sample in NaCl 0.9% at 4 °C until further processing.
- Biopsies: biopsies from different human tissues have also been used, such as eye lens [332], colon [104], and testis [333].

! CAUTION All experiments involving human tissues must be approved by the relevant institutional ethical committee and adhere to local and national regulations.

Reagents

For all the reagents mentioned below, an example of commonly used supplier is mentioned, although reagents of the same quality, purchased from other providers, should perform equally well.

General reagents

- Agarose, normal melting point (NMP) (Merck KGaA, cat. no. A4718)
- Agarose, low melting point (LMP) (Merck KGaA, cat. no. A9414)
- Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Merck KGaA, cat. no. P4417)
- Triton® X-100 (Merck KGaA, cat. no. X100)
- Dimethyl sulfoxide (DMSO) (Merck KGaA, cat. no. 41639)

! CAUTION DMSO readily penetrates skin and may carry other dissolved chemicals into the body, so wear protective gloves.

- Glycerol (Merck KGaA, cat. no. G5516)
- 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (Merck KGaA, cat. no. H3375)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2.$2H_20$) (Merck KGaA, cat. no. E5134)
-
- Trizma® base (Merck KGaA, cat. no. T1503)
- Tris Hydrochloride (Tris-HCl) (Merck KGaA, cat. no. 648317)
- Potassium chloride (KCl) (Merck KGaA, cat. no. P3911)
- Sodium chloride (NaCl) (Merck KGaA, cat. no. S9888)
- Potassium hydroxide (KOH) (Merck KGaA, cat. no. P5958)

! CAUTION KOH is caustic, so wear protective gloves.

- Sodium hydroxide (NaOH) (Merck KGaA, cat. no. 795429)

! CAUTION NaOH is caustic, so wear protective gloves.

- Bovine Serum Albumin (BSA) (Merck KGaA, cat. no. A2153)
- Ethanol (EtOH) 96% (Merck Millipore, cat. no. 159010)
- Liquid nitrogen (e.g., Linde Gas or Nippon Gases)
- Isopropanol (Merck KGaA, cat. no. I9516)
- N-lauroylsarcosine sodium salt (Merck KGaA, cat. no. L9150)
- Hydrochloric Acid (HCl) (Merck, cat. no. 1090571003)

! CAUTION HCl is a strong acid, so wear protective gloves.

Specific reagents

Cell lines and 3D models:

- Cell culture medium. Media may be specific for each cell type, or 3D tissue model, and should be chosen according to the advice given by the manufacturer, or literature recommendations.
- Trypsin-EDTA 0.05% (Gibco, ThermoFisher Scientific, cat. no. 25300062)
- Trypsin-EDTA 0.25% (Gibco, ThermoFisher Scientific, cat. no. 11560626)

- TrypLE™ without phenol red (Gibco, ThermoFisher Scientific, cat. no. 12604013)
- Hank's Balanced Salt solution, phenol red-free and with Ca^{2+} and Mg^{2+} ions (HBSS) (Merck KGaA, cat. no. 55037C)

***Platyhelminthes* (Planarians):**

- Papain (Merck KGaA, cat. no. P4762)
- L-cystein-hydrochloride monohydrate (Applichem, cat. no. A3665)
- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) (Merck KGaA, cat. no. 106346)
- Sodium Bicarbonate ($NaHCO_3$) (Acros Organics, cat. no. 123360010)
- Glucose (ThermoFisher Scientific, cat. no. G/0450/53)

Annelids:

- PBS (Merck KGaA, cat. no. 806544)
- EtOH (Merck KGaA, cat. no. 51976)
- EDTA (Merck KGaA, cat. no. E9884)
- Guaiacol glycerol ether (Merck KGaA, cat. no. G5627)

Molluscs:

- Glucose (Merck KGaA, cat. no. G7021)
- Sodium citrate ($Na_3C_6H_5O_7$) (Supelco, cat. no. 106448)
- di-Potassium Hydrogen Phosphate anhydrous (K_2HPO_4) (PanReac-AppliChem, cat. no. 131512)
- Sodium bicarbonate ($NaHCO_3$) (Merck KGaA, cat. no. S5761)
- HBSS (Merck KGaA, cat. no. 55037C)

Amphibians:

- PBS (Merck KGaA, cat. no. 806544)

Fish:

- MS-222 (ethyl meta-aminobenzoate or methanesulfonate salt) (Merck KGaA, cat. no. E10521)
- HBSS (Merck KGaA, cat. no. 55037C)

Rodent tissues:

- HBSS (Merck KGaA, cat. no. 55037C)

Human samples

- Polygelin solution normally used as plasma expander (Emagel®, Hoechst, Germany)
- Proteinase K (Merck KGaA, cat. no. 70663)

Enzymes for enzyme-modified comet assay

- *E. coli* Endonuclease III (Endo III) detects damaged pyrimidines, including thymine glycol and 5, 6-dihydroxythymine (New England Biolabs, cat. no. M0268S)
- *E. coli* Formamidopyrimidine DNA glycosylase (Fpg) detects 8-oxo-7,8-dihydroguanine and open ring forms of 7-methylguanine, formamidopyrimidines (FaPy), 5-hydroxycytosine and 5-hydroxyuracil (New England Biolabs, cat. no. M0240S; Norgenotech AS, cat. no. E0103-10)
- Human 8-oxoguanine DNA glycosylase (hOGG1) catalyses the removal of 8-oxoguanine and formamidopyrimidine moieties in double stranded DNA, followed by cleavage of the resulting AP site (Trevigen, cat. no. 4130-100-EB).
- T4 Endonuclease V (T4endoV) detects cis-syn cyclobutane pyrimidine dimers, including T<>T, T<>C and C<>C, (New England BioLabs, cat. no. M0308S)
- Human Alkyl Adenine DNA Glycosylase (hAAG) detects a wide variety of alkylated and oxidized purines, including 3-methyladenine, 7-methylguanine, 1,N6-ethenoadenine, and hypoxanthine as major substrates (New England Biolabs, cat. no. M0313S)
- Uracil DNA glycosylase (UDG) detects misincorporated uracil in DNA followed by cleavage of the resulting AP site by alkaline treatment (Merck KGaA, cat. no. 1144464001)

Some enzymes can be produced 'in-house' as crude extracts from transfected *E.coli*.

Reagents for comet visualization

Several fluorescent DNA dyes are suitable; the most commonly used are listed below.

- SYBR® Gold (ThermoFisher, cat. no. S11494)

! CAUTION Potential mutagen; wear protective gloves and dispose of waste in proper containers.

- SYBR® Green (ThermoFisher, cat. no. S7567)

! CAUTION Potential mutagen; wear protective gloves and dispose of waste in proper containers.

- Ethidium Bromide (EtBr) (ThermoFisher, cat. no. 17898)

! CAUTION Mutagenic; wear protective gloves and dispose of waste in proper containers.

- DAPI (ThermoFisher, cat. no. D1306)

! CAUTION Mutagenic; wear protective gloves and dispose of waste in proper containers.

Other newly developed "safer-to-use" dyes can be used as well:

- GelRed® (Biotium cat. no. 41003; Merck KGaA, cat. no. SCT123) is an ultra-sensitive, very stable replacement for EtBr DNA/RNA gel stain, safe for humans and the environment, shown to be non-mutagenic and non-cytotoxic.

Equipment

Special equipment and consumables needed for the comet assay can be procured from a variety of providers, unless otherwise specified. Although certain providers may be recommended, the protocol works with various standard laboratory equipment.

General laboratory equipment and consumables

- Microwave oven
- Freezers

- Refrigerator
- pH meter
- Cooled centrifuge
- Plastic tubes, well plates, Petri dishes, etc.
- Vortex mixer
- Plastic tips
- Pipettors
- Plastic Pasteur-pipettes
- Micropipettes

Equipment and consumables to perform cell culture

- Cell culture laminar flow cabinet
- Cell culture incubator with CO_2
- Cell counter
- Culture flasks and dishes
- Visible light inverted microscope

Equipment and consumables to use other sources of cells

- For 3D-models and planarians: cell strainer with 35-70 µm pores
- For molluscs: hypodermic syringe, dissection scissors and tweezers
- For solid tissues: cylindrical stainless steel metal sieve (NorGenoTech AS, cat. no. 1202)

Special equipment and consumables needed for the comet assay

- Microscope slides - standard microscope slides with frosted end are used (VWR, cat. no. HECH42406020, slides are also available as part of the TREVIGEN Kit, cat. no. 3950-075-02). Alternatively, fully frosted slides can also be used (Surgipath® Fully Frosted Slides, cat. no. 3800280).

Δ **CRITICAL** Fully frosted slides do not need to be coated with NMP agarose but they present some background when viewed under a fluorescence microscope.

- GelBond® films (Lonza, cat. no. 53734) can be used as support for the gels instead of microscope slides. These polyester films may be cut to the size of standard glass slides; technology has been developed so that larger films can accommodate up to 96 mini-gels on one GelBond® film in a 96-well format. The GelBond® film is versatile as it can be used to process as many mini-gels as desired. A major advantage is that the agarose gels stick very firmly to the plastic, and seldom fall off, which is sometimes experienced with glass slides.
- 20×20 mm, 21x26 mm or 22×22 mm coverslips to form gels
- 24×60 mm coverslips
- Water bath or thermoblock
- Staining (Coplin) jars - for cell lysis and slide washing
- For 3D skin model: 40 µm cell strainers (BD Biosciences)
- Metal trays or plates – to keep slides cold and prevent enzyme reaction from starting (a convenient example is the Slide Chilling Plate from Cleaver Scientific Ltd)
- Incubator and humidified box **–** for the enzyme-modified comet assay (an alternative is a heating plate or 'slide moat', for example those available from Boekel Scientific)

- Large-bed horizontal gel electrophoresis chamber (for horizontal slide electrophoresis)
- Power supply. It is advised to use one that can reach 1-2 Amp at 20-50 V, i.e., at voltage which is sufficient to give 1 V/cm in the electrophoresis chamber. The amperage increases with the width of the tank and the volume depth; the latter should always be more than a few millimetres. Consort (BE) is an example of a suitable brand (EV2000, EV3000)
- External peristaltic pump to recirculate the electrophoresis solution, such as those used in aquariums (optional). Alternatively, a gel system with built-in recirculation may be purchased (Fisher Scientific). The stabilization of conditions allows more precise measurement of the electric potential.
- Recirculating chiller or metal coil in ice bath, to cool the platform of the electrophoresis tank (optional). Alternatively, the electrophoresis tank can be put in a cold room, dedicated fridge, or even put on ice (see **Figure 7**).
- Optional: Slide warmer for drying slides
- Epifluorescence microscope and appropriate filter blocks optimized for the fluorochrome, charge-coupled device (CCD) camera (8-bit black-and-white camera is adequate); high sensitivity and high pixel density are preferred

Software

- For scoring comets, the use of a computer-assisted image analysis system is recommended, using commercially available software, which gives the most reproducible results. Examples of scoring software include Comet assay IV (Instem), Comet Analysis software (Trevigen), Lucia Comet Assay™ software (Laboratory Imaging), Metafer (MetaSystems), KOMET 6 (Andor).
- Several free scoring programs are available, such as Casplab or CometScore, among others.

Reagents setup

General solutions

- 1% (w/v) NMP agarose in distilled water (for pre-coating slides): microwave to dissolve the agarose and cool to about 50-60 °C in a water bath before use. Approximately 100 mL are sufficient to coat 75-100 microscope slides. 1% NMP agarose is usually made up fresh, but can be reheated once, or twice, with the lid placed loosely on top to minimize evaporation.
- 1% (w/v) LMP agarose in PBS (for embedding cells in agarose): microwave to dissolve the agarose (or put in a 100 °C water bath for 5 min). It is advisable to make aliquots of 2-5 mL and store at 4 °C. Before use, microwave or immerse the aliquot in boiled water to melt the agarose, and then cool to 37 °C (in a water bath or thermoblock).

Δ **CRITICAL** It is best not to reheat LMP agarose aliquots (as evaporation can cause a significant increase in concentration).

Δ **CRITICAL** A lower percentage of LMP agarose can be used to increase sensitivity. The final concentration of the LMP agarose gel, after mixing with the cells, is normally 0.7-0.8% (w/v). Higher concentrations decrease the sensitivity of the assay (in some cases, a reduced sensitivity is intended, as with human sperm, and therefore higher concentrations are acceptable). Do not use percentages below 0.5% as this will increase the risk of gels detaching, or breaking, especially during the enzyme-modified comet assay.

- Lysis solution: 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Trizma® base, pH 10 (with 10 M NaOH). Stable for at least 6 months when stored at 4 °C. Before use, add 1 mL of Triton® X-100 per 100 mL.

Δ **CRITICAL** Lysis solution can be freshly supplemented with 10% DMSO and 1% N-lauroylsarcosine sodium salt. The addition of 10% DMSO to the lysis solution may be useful to prevent potential radical-induced DNA damage associated with the iron released during lysis from erythrocytes present in blood, and tissue samples. The addition of 1% N-lauroylsarcosine is optional but considered redundant for most purposes.

- Electrophoresis solution: 0.3 M NaOH, 1 mM Na_2EDTA. Store at 4 °C for up to one week. Another option is to prepare stock concentrated solutions and mix them on the day.
- Neutralising solution: 1×PBS. Store at 4 °C, or according to manufacturer's instructions.
- Tris-HCl: 0.4 M Tris (Trizma® base) in 1 L of redistilled H_2O (adjust pH to 7.5 using HCl)

■ **PAUSE POINT** For neutralisation step both PBS and Tris-HCl work equally well. For PBS use single wash for 10 min while for Tris-HCl use three washes, 5 min each (15 min in total).

- TE buffer (for staining with SYBR® Gold and SYBR® Green): 10 mM Trizma® base, and 1 mM EDTA-Na. Store at room temperature (approx. 22 °C). Stable for at least up to 6 months. Alternatively, it is possible to use TBE or TAE buffer as recommended by the provider.

Reagents for enzyme-modified comet assay

- Buffer B (post-lysis washing buffer, and enzyme reaction buffer for Fpg, hOGG1, EndoIII, Udg, and hAAG): 40 mM HEPES, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, 0.1 M KCl, pH 7.6-8 (with 10 M KOH). We advise preparing 500 mL of 10× concentrated stock solution of buffer B and freezing (-20°C) in 50 mL tubes (to use for washing slides after lysis) and in 1 mL aliquots (to use as incubation reaction buffer). Washing can also be done using Buffer B without BSA, but you need to add BSA for the incubation step. Stable for at least 6 months. Dilute 10× in distilled water on the day of use. Note: the diluted buffer B can be stored at 4 °C for use in a second assay within the same week.
- Buffer N (washing buffer after lysis and incubation reaction buffer for T4EndoV): 45 mM HEPES, 0.25 mM Na_2EDTA, 0.3 mg/mL BSA, 2% (v/v) glycerol, pH 7.8 (with 10 M KOH). We advise preparing 500 mL of 10× concentrated stock and freezing (-20 °C) in 50 mL tubes (to use for washing slides after lysis) and in 1 mL aliquots (to use as incubation reaction buffer). Stable for at least 6 months. Dilute 10× in distilled water on the day of use. Note: The diluted buffer N could be stored at 4 °C for usage in a second assay within the same week.

Note: The names of the buffers (buffer B and buffer N) are kept consistent with the nomenclature used in the paper on the comet-based *in vitro* DNA repair assay [22].

- Prepare the enzymes according to manufacturer's instructions, and titrate them to optimize the enzyme concentration and incubation time before use. See Table 2 for guidelines for your own titrations. Keep the same experimental conditions within one series of experiments. Muruzabal et al.[51] describe how to perform the titration using the enzymes in combination with the comet assay. Normally, incubation times for 30 to 60 min are used. Buffer B and Buffer N work with the corresponding enzymes (see the preparation of buffers, above), though other buffers suggested by the manufacturers can also be used.

Specific reagents for cell cultivation, preparing cell suspension and freezing

Cell lines and 3D models:

- Cell culture medium for growing cells: Some cell culture media must be supplemented with different substances such as serum or non-essential amino acids to grow cells. Check with the cell line provider the medium needed to grow the cells, or the 3D tissues.
- Cell freezing medium (for freezing cells): DMEM, 10% (v/v) FBS, 10% (v/v) DMSO. Mix 8 mL of DMEM, with 1 mL FBS and 1 mL DMSO. Prepare fresh on the day of use. The proportion of FBS in the freezing medium will depend on the cell type used. If needed, the freezing medium can be stored at 4 °C for up to 24 h.
- For 3D skin models: Thermolysin (0.5 mg/mL in buffer containing 10 mM HEPES, pH 7.2–7.5; 33 mM KCl, 50 mM NaCl and 7 mM $CaCl_2$) to aid cell dissociation. Freezing of the skin models has not yet been attempted.
- For 3D skin models: mincing buffer (20 mM EDTA in HBSS without Ca^{2+}/Mg^{2+}, 10% DMSO added freshly, pH 7.0–7.5)

***Platyhelminthes* (Planarians):**

- 10X CMF (Ca^{2+}/Mg^{2+} free buffer): 25.6 mM $NaH_2PO_4.H_2O$, 142.8 mM NaCl, 102.1 mM KCl, 94.2 mM $NaHCO_3$ in distilled water (pH 7). Store at 4°C.
- CMFH: 0.1% BSA, 0.5% glucose, 15 mM Hepes in 1X CMF (pH 7). Prepare fresh on the day of use.
- Papain solution: 30 units papain/mL, plus 2 mM L-cysteine-HCl prepared in CMFH. Prepare fresh on the day of use. Stock solution of 0.2M L-cysteine-HCl prepared in distilled water can be kept in aliquots at -20 °C.
- 2% L-cysteine-HCl in distilled water (pH 7). Prepare fresh on the day of use. Adjust pH using NaOH.

Drosophila

- Ringer's solution: prepare 250 mL containing 130 mM NaCl, 35 mM KCl, 2 mM $CaCl_2$. Adjust the pH to 6.5 with NaOH, and sterilize by autoclaving. Stable for at least up to 3 months, at 4 °C.

Annelids:

- Extrusion buffer: 5% EtOH, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glycerol ether in PBS; pH 7.3

Mollusks:

- Alsever´s anticoagulant solution: 382 mM NaCl, 115 mM glucose, 27 mM sodium citrate, 11.5 mM EDTA. Store at room temperature (approx. 22 °C). Stable for at least up to 1 month.
- Ca^{2+}/Mg^{2+}-free saline solution (CMFS): 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA. Store at room temperature (approx. 22 °C). Stable for at least up to 1 month.
- Kenny's salt solution (KSS): 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4,2 mM $NaHCO_3$. Store at room temperature (approx. 22 °C). Stable for at least up to 1 month.

Rodent tissues:

- Mincing buffer: HBSS, 20 mM Na_2EDTA, pH 7.5 (adjusted with NaOH). Add 10% DMSO just before using.

- Merchant's buffer (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 10 mM Na_2EDTA; pH 7.4). Stable for at least up to 1 month.

Human samples

- For blood: Erythrocyte lysis buffer (8.29 g NH_4Cl (155mM), 1.0 g $KHCO_3$ (10 mM), 0.372 g EDTA (1.0 mM), dissolved in 1000 mL H_2O; pH 7.4, sterile filtered.
- For saliva: (1) For sample collection (mouth rinses): NaCl (0.9%) in distilled water and sterilize the solution; (2) For freezing samples: freezing medium containing FBS (50%), RPMI 1640 (40%) and DMSO (10%) at a concentration of $2.5x10^6$ cells/mL (prepared in 0.5mL aliquots by mixing 250 µL of FBS, with 200 µL RPMI 1640 and 50 µL DMSO). Prepare fresh on the day of use. Stored at -80 °C.
- Buccal cell buffer: 0.1 M Tris-HCl, 0.1 M Na4EDTA, 0.02 M NaCl; pH 7.0 (by adding HCl). Autoclave it at 121°C for 15 minutes. When cold, store the buffer at 4°C.
- Buccal lysis solution 1: 2.5 M NaCl, 100 mM EDTA tetrasodium, 10 mM Tris-HCl, 1% Na L-sarcosinate pH 10. Before use, add 1% Triton X-100 and 10% DMSO.
- Buccal lysis solution 2: 2.5 M NaCl, 100 mM EDTA tetrasodium, 10 mM Tris-HCl, 1% Na L-sarcosinate. Before use, add 1% Triton X-100 and 10% DMSO. Adjust pH to 7, optimum for Proteinase K activity and warm to 37 °C.
- For placenta tissue: Mincing solution: PBS without Ca^{2+} and Mg^{2+}, 20 mM Na_2EDTA. Store at 4 °C; stable for at least two months.

Equipment setup

Δ CRITICAL Most of the equipment does not require any special setup, apart from those mentioned below. These setups are also demonstrated in the associated video protocols, which are available here: https://youtu.be/23IcSCZ-kuQ; https://youtu.be/NE2U8f5gwc8; https://youtu.be/s52tkqVNTUA.

Pre-coating microscope slides

Δ CRITICAL When using GelBond® films, pre-coating is not needed. The films can simply be cut to the desired size/shape, and LMP agarose (including the cells) can be applied directly to the hydrophilic side. Generally, for use in the comet assay, the films are cut to the size of a microscope slide to fit 2 or 12 gels, but bigger formats can be used (Box 3).

Δ CRITICAL Various methods exist to coat slides, of which the 2 most common ones are described step-by-step below (tutorial video: https://youtu.be/23IcSCZ-kuQ). Additional steps to improve gel adherence, if needed, have been described before [24]. Alternatively, fully frosted slides can be first covered with 1% NMP agarose, and after solidification, the gel is scraped from the slides to improve adherence.

1) Prepare 1% NMP agarose solution in H_2O, and dissolve in the microwave (see Reagents setup) and keep at 50-60 °C in water bath. For the 3D airway model, a 1.5% NMP agarose solution is used.
   Δ CRITICAL STEP To prevent boiling, you can put the microwave at the lowest power for a longer time, until you see bubbles. At that point, you can give the agarose a stir, and put it back. Repeat this until all the agarose has dissolved. To minimize evaporation, put a loose lid on top.

2) Dip the slides into the gel briefly until only the frosted part remains unsubmerged. Wipe the back of the slide clean. Alternatively, pipette ~100 µL of NMP agarose on the slide and cover with a coverslip, or spread agarose over the slide with a clean fingertip.
3) Put the slide flat to dry on a heating plate/slide warmer (about 40-50 °C), or overnight on the bench. Remember to indicate with a mark on the frosted part, which side of the slide is coated.

   Δ **CRITICAL STEP** slides coated with NMP agarose should be dried, and maintained at <60% relative humidity to minimize the risk of gels coming off during, or immediately after electrophoresis.
4) Store coated slides in slide boxes at room temperature (after removing coverslips in case of using the alternative method). They can be kept for at least up to 12 months.

? TROUBLESHOOTING

Δ **CRITICAL** At higher relative humidity (above about 60%), the LMP agarose solution may absorb atmospheric moisture over time reducing the LMP concentration leading to variable DNA migration. At lower relative humidity (below about 30%) the LMP agarose solution might lose atmospheric moisture increasing the LMP agarose concentration, and thus decreasing DNA migration.

Electrophoresis setup

Δ **CRITICAL** As the duration of electrophoresis (Stage 3, step 29), and the electric potential (voltage drop across the electrophoresis tank platform) are the most important drivers of DNA migration, these parameters should be measured, and standardized for all experiments. Video instructions are available here: https://youtu.be/s52tkqVNTUA

1) Ensure that the tank is flat using a spirit level
2) Measure the distance between the electrodes in the electrophoresis tank.
3) Add enough electrophoresis solution to cover the microscope slides with at least 5 mm of excess liquid.
4) Switch on the power supply and measure the voltage over the platform using a voltmeter (holding an electrode at each edge of the platform). Alternatively, an approximate measure is obtained by dividing the applied electrode voltage by the distance between the electrodes, but it is more accurate to use a voltmeter.

   Δ **CRITICAL** Ensure that the power supply can provide the output current at the constant voltage, and with sufficient volume of liquid (a power supply that reaches 1-2 Amperes should suffice for most tanks but higher currents may be needed for larger tanks). The depth above the samples should not be made too shallow (i.e., < 5 mm) to accommodate a power supply with low capacity.
5) The electrophoresis conditions normally used are ~1 V/cm (on the platform of the tank) and ~20 min.

   Δ **CRITICAL** The electrophoresis conditions can differ depending on the biological samples used; exceptions are mentioned in the text/boxes. Other electrophoresis conditions can also work.

   Δ **CRITICAL** The same electrophoresis conditions should be used for all experiments within the same study

   Δ **CRITICAL** The Electric Potential * Time (EPT) value (dimension: (V/cm)*min) can be calculated and designates a specific assay sensitivity. This value allows the comparison of the electrophoresis conditions between labs. EPT ~20 is advised for most biological samples; exceptions are indicated in the procedure and boxes.

1400
1401

3. PROCEDURE

Δ **CRITICAL** Stage 1 can be performed on the day of the comet assay (i.e., Stages 2.1., 2.2., and 3), alternatively cell suspensions can be frozen and stored until later analysis. Before starting the enzyme-modified comet assay, it is essential to have optimized the concentration of the lesion-specific enzymes, and to determine their suitable incubation time with gel-embedded nucleoids ('Experimental design' section).

Δ **CRITICAL** In all cell handling: never vortex cells, avoid rapid pipetting (especially through narrow-bore tips), and keep cells on ice after harvesting. Use as short a time as possible from harvesting of the samples until lysis.

Δ **CRITICAL** Stages 1-4 are identical for all specimens, except for yeast, plant and sperm cells, which require modified protocols as specified in Boxes 9-11.

Stage 1: Preparation of cell suspension from fresh or frozen samples – day 0 or 1, ● Timing 0.5 – 3 h (depending on the cell type and the number of samples)

1) Prepare a cell suspension containing the desired number of cells. In some cases, the sample obtained is already a cell suspension (e.g., cultured cells in suspension, blood or saliva; option A), but when working with other *in vitro* models (options B-D), invertebrates as whole organisms or tissues (options E-J), vertebrate tissues (options K-M), or human tissue samples (options N-O), a mechanical and/or enzymatic processing in specific buffers is required.

(A) Preparation of cells in suspension from (co-)cultures, blood, or saliva

(i) Collect the required number of cells:

i.i. Grow the desired cell line in suspension according to the provider's instructions. Collect an aliquot from the cell suspension.

i.ii. MNCs are routinely isolated from venous blood [229] or saliva [330] using a standard density gradient centrifugation method.

i.iii. To isolate PMN cells, after density gradient isolation of MNCs, resuspend the remaining PMN-erythrocyte mixture and add erythrocyte lysis buffer (https://youtu.be/tgNHWVqF52I). Using this procedure, about $2.5x10^7$ PMN cells are typically isolated from 10 mL of blood, viability >95%. Alternatively, dilute the PMN-erythrocyte mixture 1:5 with PBS and mix with an equal volume of a 3.5% polygelin solution for about 45 min at room temperature, to obtain the separation of the red cells in a lower layer and PMN cells in the upper layer (containing mainly neutrophils) [244].

(ii) Count the number of cells in the sample of the cell suspension.

(iii) Centrifuge cells at about 150-300 × g, for 5 min, at 4 °C.

(iv) Wash cells with ice-cold PBS, and centrifuge again.

Δ **CRITICAL STEP** Whole blood or buffy coat can be mixed directly with LMP agarose (stage 2.1).

(B) Prepare the cell suspension from adherent cells (co-)cultures, and 3D liver spheroids

(i) Grow cells in a flask or dish in culture medium to near confluence. For 3D liver spheroids: grow hepatocellular carcinoma cells (such as HepaRG, HepG2, Huh6 or C3A) in a 96-well ultra-low attachment plate at a density of 2,000 cells/well, change medium after 2-3 days and use spheroids at specific age (depending on cell line and application) (NOTE: The spheroids grown in static conditions can develop a necrotic core after 10 days).

(ii) Remove medium, wash cells with PBS, and dissociate cells.

i.i. For adherent (co-)cultures: trypsinize according to standard cell subculturing procedure using 0.25% trypsin-EDTA.

i.ii. For spheroids obtained with HepaRG: pool 11 spheroids in a 1.5 mL microtubes, and dissociate by adding 200 µL of TrypLE™ for 40 min at 37 °C.

i.iii. For liver spheroids obtained from non-quiescent cells such as HepG2, Huh6, etc., add 50 µL 0.25% trypsin-EDTA, or TrypLE™, and incubate for 10 min at 37 °C.

Δ CRITICAL STEP Avoid long trypsin treatment as this can increase background levels of damage. Scraping off the cells can be an option in some cases.

(iii) Neutralize trypsin with cell culture medium, containing 10% serum.

(iv) Transfer the cells to appropriate tubes and centrifuge for 5 min at 4 °C at 150-300 x *g* (depending on cell line).

(C) Prepare cell suspension from 3D airway models

(i) Culture the MucilAir™ models on 12- or 24-well Transwell™ culture supports at the air-liquid interface (ALI).

(ii) Following exposure, wash the airway model with 800 µL saline (add 600 µL to the well and 200 µL on the insert (24-well plate)) and keep it for 2 minutes at room temperature.

(ii) Transfer the insert to a new 24-well culture plate filled with 600 µL 0.05% Trypsin-EDTA and add another 200 µL 0.05% Trypsin-EDTA to the insert.

(iv) Following a 10-minute incubation at 37 °C, resuspend the cells and transfer the cell suspension to 15 mL centrifuge tubes that are filled with 2 mL 10% FBS.

(v) Harvest the cells by centrifugation (5 min, 200 x *g*).

(vi) Decant the medium, and resuspend the pellet in 150 µL 0.5% LMP agarose.

(D) Prepare cell suspension from 3D skin models

(i) When using the Phenion®FT skin model, after exposure, wash the tissue with 1 mL PBS.

(ii) Place the Phenion®FT tissue in 300 µL thermolysin in a 12-well plate and incubate 2 h at 4 °C.

(iii) Separate the dermis and epidermis using forceps.

(iv) Transfer each layer separately to 1 mL of cold mincing buffer, cut into small pieces with scissors and leave to incubate on ice for 5 min.

(v) Resuspend by pipetting, and filter through 40 µm cell strainers.

(vi) Harvest the mixture of cells and nuclei by centrifugation (5 min, 250-300 × g).

(vii) Decant the medium, and resuspend the pellet in the residual supernatant (about 200 µL).

(E) Preparation of zebrafish embryos

(i) *Whole body squashing (for embryos at the age of maximum 48 hpf)*: After the treatment, expose embryo with minimal volume of medium to Pronase E (2 g/L) for 4 min to soften the chorion. Pronase E is diluted with fresh medium, and embryos rinsed at least once in the medium. Place the embryos directly in a drop of LMP, cover with a coverslip and gently squash to gain single cells. The cells spread all over the microscope slide, remaining embedded in the agarose. Optionally, another layer of 1% LMP agarose (80 µL) can be added on the top of the squashed embryo.

Δ CRITICAL STEP Ensure that the embryos are gently squashed in LMP agarose.

(ii) *Whole body cell isolation using a mechanical isolation procedure* (for embryos at the age of up to 96 hpf): gently disintegrate the embryos (usually pool of 8-10, depending on required single cell yield) in 2 mL cold PBS using a tissue grinder (glass-glass homogenizer), or only using scissor followed by gentle pipetting. Filter the cell suspension through a gauze/mesh with 70 µm pores, and then centrifuge the suspension (200 x *g* for 10 min at 4˚C). Resuspend the pellet with cold PBS and repeat

centrifugation (180 x *g* for 7 min at 4˚C). Finally, resuspend the pellet in ice-cold PBS (or Leibowith L-15 medium). Before performing the comet assay, assess viability.

(F) Preparation of cells from invertebrates: Crustaceans (*Daphnia magna* and *Ceriodaphnia dubia*)

(i) After exposure to the test compound, transfer the organisms in tubes.
(ii) Add 1 mL of lysis solution (1 mL PBS containing 20 mM EDTA and 10% DMSO) to dissociate the exoskeleton.
(iii) Isolate cells by repeated, light pipetting for 5 min.
(iv) Centrifuge (10 min, 2292 x *g*, 4 °C); discard supernatant and resuspend the pellet in LMP agarose (0.7%).

(G) Preparation of cells from invertebrates: Planarians

(i) Using a plastic Pasteur pipette, transfer the worm(s) to a Petri dish with 2% L-cysteine-HCl to remove mucus. Incubate for 2 min under soft shaking. You can pool multiple worms per biological sample to increase yield.
(ii) Transfer worm(s) to a Petri dish with CMFH for rinsing.
(iii) Transfer worm(s) to a glass slide; remove CMFH as much as possible and cut into small pieces using a scalpel. Regularly wipe the scalpel to avoid mucus accumulation.
(iv) Transfer the pieces to a 1.5 mL tube using CMFH (125 µL for 1 worm, 250 µL if using multiple worms per sample).
(v) Add an equal volume of papain solution to the tube and incubate for 1 h at 26°C without shaking (e.g., in a heat block).
(vi) Add 700 µL CMFH, vigorously pipette up and down repeatedly to further macerate the fragments and filter using a 35 µm strainer, into a plastic centrifuge tube. Keep samples on ice.
(vii) Centrifuge (5 min, 350 x *g*); discard the supernatant and resuspend the pellet in 4 mL CMFH. Keep sample on ice.
(viii) Optional: additional filtration with a cell strainer with smaller mesh size. Mesh size can be adjusted based on the cell types under investigation.
(ix) Centrifuge (5 min, 350 x *g*); discard supernatant and resuspend the pellet in 1 mL CMFH. Keep sample on ice.
(x) Transfer the sample to a 1.5 mL tube and centrifuge for 5 min, 350 x *g*. Keep sample on ice.
(xi) Aspirate the supernatant and keep the sample on ice until embedding in LMP.

(H) Preparation of cells from invertebrates: *Drosophila*

(i) Collect the tissue of interest (e.g., brain ganglia, anterior region of the midgut, or haemocytes) and pool from 5-50 larvae.
(ii) Transfer solid tissues to washing solutions (Poels' salt solution, Ringer's solution, or PBS containing phenylthiourea may be used): 100 µL/tissue from 5 larvae. Haemocytes are mixed with PBS plus 0.07 % phenylthiourea.
(iii) Treat solid tissues with collagenase or disaggregate them physically, and pass the tissues through nylon mesh to prepare a single cell suspension.
(iv) Centrifuge at 300 x *g* for 20 min at 4 ºC.

(I) Preparation of cell suspension from *Chironomus riparius* larvae

(i) Whole-body squashing: Use a pool of at least 10 fourth-instar larvae to ensure that a sufficient number of cells are obtained in the final cell suspension. If larvae are from earlier stages, more will be needed.
(ii) Place the larvae on a fine mesh strainer (0.3 mm mesh) laid over a mortar containing 3 mL of ice-cold 1X PBS.

Δ **CRITICAL STEP** Keep the sample on the strainer immersed in cold PBS until step (v) to avoid artificial DNA damage caused by oxidation.

(iii) Make several transverse cuts in the larval bodies with a scalpel to facilitate cell extraction, as larvae have a hard exoskeleton.

(iv) Use a pestle to gently grind up the sample (mechanical mincing) to get the cell suspension. Avoid as much as possible the presence of cuticle debris.

(v) Homogenize the sample by pipetting and transfer to 1.5 mL tubes (on ice).

(vi) Centrifuge cells at about 150-300 x *g* for 5 min at 4 °C.

(vii) Discard the supernatants and resuspend the pellets in 250 µL of ice-cold 1X PBS to obtain the final cell suspension.

Δ **CRITICAL STEP** Keep the tubes on ice until embedding the cells in the LMP agarose gel.

(J) Preparation of cells from invertebrates: Annelids (*Oligochaetes*, earthworms)

(i) Collect the earthworms from experimental soil, and rinse in cold PBS at 4 °C.

(ii) Place each earthworm on moist paper with PBS and massage half of its posterior length to expel the contents from the lower gut to reduce faecal contamination of the extrusion fluid.

(iii) Place each worm in a tube containing 3 mL of the extrusion buffer during 3 min at room temperature.

(iv) Collect the extruded coelomic fluid containing coelomocytes by centrifugation at 150 x *g* for 10 min and wash the resulting pellet in 3 mL of PBS 3 times.

(v) Transfer coelomocytes into a tube with 1 mL of cold PBS.

Δ **CRITICAL** An alternative method to extract coelomocytes involves stimulating electrically twice for 1 s with 4.5 V, which results in extrusion of coelomocytes through the dorsal pores.

(K) Preparation of cells from invertebrates: Molluscs (*Bivalves*)

(i) Haemolymph cells:

i.i. Make an incision in the mollusc shell and withdraw ~1.5 mL haemolymph from the posterior adductor muscle with a sterilized hypodermic syringe containing precooled modified Alsever´s anticoagulant solution [1:5 (v/v), haemolymph:Alsever).

i.ii. Keep the samples on ice until centrifugation for 5 min at 250 × g.

(ii) Solid tissue (gills and digestive glands):

ii.i. Dissect and slice the tissue into small pieces using dissection scissors and tweezers.

ii.ii. Place excised tissues in tubes containing 3 mL of CMFS, and leave for 1 h with gentle, horizontal shaking.

ii.iii. Place the tubes in a vertical position for 5 min to allow the fragments of tissue to settle.

ii.iv. Collect the supernatant containing the suspended cells with a pipette, transfer to another clean tube and centrifuge at 500 × g for 5 min at 4°C.

ii.v. Remove the supernatant and wash cells twice in 1.5 mL Kenny's salt solution (KSS) with centrifugations of 3 min at 1000 × *g*.

ii.vi. Alternatively, if not enough single cells are obtained, dispase II digestion can be conducted: after rinsing dissected tissues with HBSS, add 1 mL of 1.6 mg/mL dispase II solution freshly prepared in HBSS and incubate for 30 min at 37 °C in the dark, shaken every 10 min. After digestion, spin samples at 160 × *g* for 5 min. Collect the supernatant containing the cells in suspension and centrifuge again at 775 x *g* for 2 min.

(L) Preparation of cells from vertebrates: Amphibians

(i) Blood cells from tadpoles:

i.i. Section tadpoles in the ventral position at the level of the operculum.

i.ii. The blood sample is obtained by soaking tadpole and dripping blood in PBS and subsequent centrifugation at 160 x *g* for 9 min. Up to 5 µL of blood can be obtained from a single tadpole.

(ii) Blood cells from fully developed specimens:

ii.i. Draw blood through heart puncture using heparinized syringes/collection tubes, collect in individual microtubes and, refrigerate at 4 °C until slides preparation.

ii.ii. Whole blood cells are directly embedded in LMP agarose.

(M) Preparation of cells from vertebrates: Fish:

(i) Blood cells:

i.i. Collect blood using a method such as caudal puncture, which is easily applicable to specimens weighing more than 200 g. Alternatively, adopt more invasive methods such as caudal peduncle transection (e.g., *D. rerio*), decapitation and sampling with heparinized capillary tubes in the cardiac region (recommended for very small fish, such as *G. holbrooki*, and larval stages), or puncture on posterior cardinal vein or heart (most species). Even if a large amount of blood is collected (e.g., *S. aurata*, *S. soleganensis* and *A. anguilla*), only 2 µL is required. This is diluted in 1 mL of PBS to obtain a cell suspension; 20 µL of this suspension are directly mixed with 70 µL 1% LMP.

Δ CRITICAL To avoid obtaining an insufficient cell number, mix the sampled blood with an adequate smaller volume of ice-cold PBS, to obtain the cell suspension.

(ii) Organs (liver, gills and gonads):

ii.i. Collect organs (ensuring a proper fish exsanguination) and place (and rinse) them immediately in ice-cold PBS, to remove blood cells.

ii.ii. Obtain the cell suspension by briefly homogenizing/mincing in PBS a small portion of the tissue into small pieces, using scissors, tweezers, or a scalpel, promoting cell dissociation. Moreover, a soft mechanical dissociation (pipetting up and down) is helpful.

ii.iii. The digestion with trypsin (and/or collagenase) can increase the cells' dispersion (10-15 min depending on the enzyme concentration and temperature of incubation). To get rid of larger tissue pieces, filter the cell suspension, using a sterile mesh (usually with 50-100 µm pores). If necessary, centrifuge the cell suspension (200 x *g*, 4 °C, 5-10 min), discard the supernatant, and resuspend the pellet in 1 mL of ice-cold PBS. Repeat the centrifugation/washing step (usually twice).

ii.iv. Resuspend the pellet in 20 µL (adjusted according to the desired final cell density) of ice-cold PBS (or DMEM/F12 medium), and place on ice, or immediately mix with 70 µL of 1% LMP agarose. Place the cell suspensions in LMP agarose onto precoated slides (with 1% NMP agarose).

(N) Preparation of cells from vertebrates: Rodents

(i) From fresh tissue

i.i. Rinse the tissue using cold PBS (Ca^{2+} and Mg^{2+} free, 20 mM EDTA), mincing buffer or Merchant's buffer.

Δ CRITICAL The buffer should be ice-cold (4 °C) to avoid the risk of artifactual generation of DNA damage.

i.ii. Add 200 µL of the preferred cold buffer (see (i)) to approximately 5 mg wet tissue (approximately 15 mm^3). Some recommendations about the size of the different organs can be seen in table 3.

i.iii. Mince the tissue using scissors or surgical blade, using a 1 mL syringe (13 x 0.45 mm – without a needle) and move the suspension back and forth 5-10 times, or with a mechanical strainer using a plastic plunger from a 1 mL syringe and a cylindrical

stainless-steel metal sieve (NorGenoTech), in all three cases to obtain a cloudy suspension.

i.iv. Collect cell suspension after large tissue debris has settled (5 min) or filter the suspension through a 100 µm nylon mesh.

(ii) From frozen tissue

ii.i. Place the cryotube containing the sample on dry ice.

ii.ii. Add a drop of Merchant's medium or mincing buffer on top of the sample to create a protective ice cap.

ii.iii. Transfer the deep-frozen tissue, using dry ice-cold tweezers, into a cylindrical stainless-steel metal sieve (NorGenoTech) previously immersed in Merchant's medium or mincing buffer on ice.

ii.iv. Homogenize the tissue by moving a plastic plunger from a 1 mL syringe up and down several times (forcing the tissue to pass through the sieve).

ii.v. Collect the homogenised samples in 3 mL Merchant's medium or mincing buffer (kept on ice).

Alternatively, frozen tissues can be pulverized by giving a single sharp impact with a dry ice-cooled hammer after placing the tissue in a dry, ice-chilled metal pulverizer. The powder is then resuspended in 3 mL Merchant's medium or mincing buffer (kept on ice).

Δ **CRITICAL** To prepare the cell suspension from frozen tissues, the sample should still be frozen when starting the homogenisation.

(O) Human samples: Preparation cell suspension from placenta

(i) Wash the fresh placenta piece using cold PBS (Ca2+ and Mg2+ free, 20 mM Na_2EDTA).

(ii) Add 5 mL of cold PBS and mince the tissue using scissors.

(iii) Recover 2 mL of cell suspension avoiding transfer of debris, and run it slowly through a 23 G needle.

(iv) Add 5 mL of PBS and centrifuge twice (350 x *g* for 15 min; 4°C).

(P) Preparation cell suspension from epithelial cells (buccal, nasal, tears)

Δ **CRITICAL** Tears can be mixed directly with LMP agarose.

(i) Collect cells with a spatula or cyto/toothbrush as mentioned under the section "Biological Materials".

(ii) Immerse the cytobrush or spatula in 1 mL of cold (4 °C) buccal cell buffer or PBS (Ca^{2+} and Mg^{2+} free), gently shaking to collect as many of the cells as possible, while keeping the tube on ice. Discard the brush.

Δ **CRITICAL STEP** PBS can be used if you process cells immediately and buccal cell solution is advised to be used in case cells need to be stored/transported a bit longer (as might happen during human biomonitoring).

(iii) Centrifuge at 250 × g at 4 °C for 5-10 min.

Δ **CRITICAL** Keep the tubes on ice during all subsequent steps until the embedding of the cells in LMP agarose, or until freezing of cell suspension, to avoid repair of DNA lesions.

? TROUBLESHOOTING

2) To use the cells directly/fresh for embedding in LMP agarose (Stage 2.1), remove supernatant and resuspend the cell pellet, unless specified otherwise, and mix the cells/nuclei well with LMP agarose as suggested in Table 3.

3) When desired, freeze cell suspensions for later use. This is an optional step. In case freezing is not required, proceed directly to Stage 2.1.

(A) Freezing cells from cultures, blood (PBMCs, leukocytes) or saliva BMCs using freezing medium

(i) Resuspend the cell pellet in cold freezing medium at ~1×10^6 cells/mL.
Δ **CRITICAL STEP** Cell suspension of placental tissues can be cryopreserved using 90% FBS, 10% DMSO as freezing medium.

(ii) Prepare aliquots, for instance 0.5 mL (containing approximately 500,000 cells) in 1.5 mL microtubes. Each aliquot will have enough cells for 20 gels in 2 gels/slide format (Stage 2.1). Larger aliquots can be prepared in case you plan to run more gels or slides per assay. When using the high throughput formats with mini-gels (Box 3-4), smaller aliquots can be frozen.

(iii) Cryopreserve at -80 °C (the vials can be slowly frozen using Mr Frosty® containers with isopropanol or in a thick-walled polystyrene box).

(B) Freezing whole blood with cryopreservative

(i) Centrifuge 100 µL whole blood at 1000 x *g* for 1 min and remove the excess plasma

(ii) Add 100 µL ice-cold freezing medium (i.e., 70% RPMI 1640 cell culture medium, 20% FBS and 10% DMSO).

(iii) Cryopreserve at -80 °C (the vials can be slowly frozen using Mr Frosty® containers with isopropanol or in a thick-walled polystyrene box).

(C) Freezing whole blood or buffy coat without cryopreservative

(i) Prepare small aliquots (~250 µL) of whole blood or buffy coat samples.

(ii) Simply place them at -80 °C without the need to add freezing medium [229,252].

(D) Freezing harvested cells from zebrafish embryo:

(i) After the treatment (48 hpf), place the embryos (n=4) in 200 µL of 10 % v/v DMSO in PBS (pH 7.4) and gently mince with a scissor and soft pipetting.

(ii) Centrifuge the suspension (250 x *g* for 2 min at 4 ˚C).

(iii) Collect the supernatant to a new tube.

(iv) Store supernatant at -80˚C up to 2 weeks.

(v) Mix 20 µL of supernatant with 180 µL 1 % LMP agarose and add to the precoated slide.

Δ **CRITICAL** If the freezing procedure for a specific species/sample type is not described above, this means it has not been tested yet.

■ **PAUSE POINT** In case samples can be frozen, the next stages can be performed later on; ensure that samples are stable during storage (this needs to be tested for each type of sample; as an example of a stability study, check Azqueta et al.[313]).

Stage 2A: Processing gels for the standard alkaline comet assay – day 1, ● Timing ~2-24 h (depending on the number of samples and the lysis time used)

Prepare materials

4) Immerse the required number of LMP agarose aliquots in boiling water to melt the agarose, and then cool to 37 °C (in water bath or thermoblock).
   Δ **CRITICAL STEP** Agarose should be mixed with cells at physiological temperature (i.e., around 37 °C) to prevent the induction of any additional DNA damage.
5) Pre-cool the centrifuge to 4 °C.
6) Prepare standard lysis solution according to option (A), or option (B) for fish samples (blood, liver, gills) and option (C) for human buccal cells (100 mL are needed for a Coplin jar that can contain 16 slides):
   (A) Standard lysis solution: to 99 mL of lysis stock solution (4 °C) add 1 mL of Triton® X-100, and mix, put into a Coplin jar, store at 4 °C until use.
   (B) Lysis solution for fish samples and 3D skin models: to 89 mL of lysis stock solution (4 °C) freshly add 10 mL of DMSO and 1 mL of Triton® X-100 and mix.
   (C) Lysis solution for human buccal cells:
      (i) Buccal lysis solution 1: add 10% DMSO and 1% Triton® X-100 to buccal lysis solution and keep at 4 °C.
      (ii) Buccal lysis solution 2: add 10% DMSO and 1% Triton® X-100 to buccal lysis solution and adjust pH to 7 (optimal condition for the activity of Proteinase K); pre-warm to reach 37 °C. Just prior to transferring the slides add Proteinase K to a final concentration of 30 µg/mL.

   Δ **CRITICAL** When working with whole blood, buffy coat, tissues or similar samples that may still contain haemoglobin, add 10% DMSO to the lysis solution to prevent artifactual DNA damage associated with the iron released during lysis from erythrocytes present in blood. The addition of 10% DMSO is also recommended for 3D skin tissues.

7) Place a metal chilling plate on ice in a box, or use a commercially available slide chilling plate.
8) Label the slides on the frosted end using a pencil, or diamond pen.

Embedding cells in LMP agarose and cell lysis

9) Prepare the cells for embedding in agarose
   (A) Continue directly with the cell suspension prepared in Stage 1, step 2.
   (B) From an aliquot of frozen cells
      (i) Thaw the aliquot of cells quickly at 37 °C (in water bath or thermoblock).
      (ii) As soon as the aliquot is thawed, add 1 mL of cold (4 °C) PBS to the 1.5 mL microtube and centrifuge at 150-300 × g for 5 min at 4 °C to wash cells.
      (ii) Suspend cell pellets in cold PBS and spin again.
      (iii) Resuspend the cells in cold PBS to ~1×10^6 cells/mL, or as specified in Table 3.
10) Either mix the cell suspension directly with LMP agarose (option A) or spin down the cells and add LMP agarose to the cell pellet (option B).
   (A) Embedding a suspension of cells: Mix LMP agarose with the cell suspension by pipetting gently up and down while avoiding the introduction of air bubbles, according to instructions in Table 3. For example, for cultured cells, remove 45 µL of the cell suspension, and mix with 105 µL of 1% LMP agarose at 37 °C, resulting in a final concentration of 0.7% LMP agarose. This

option is often used when working with a large number of samples, so that cells can be kept on ice until use.

(B) Embedding a cell pellet: Remove the supernatant, disperse the pelleted cells by mixing by pipetting up and down (or tapping the bottom of the tube vigorously) with the required volume of LMP agarose at 37 °C to reach a concentration of 2×10^5 cells/mL, or to the concentration specified in Table 3.

Δ **CRITICAL STEP** See modifications for using high throughput formats with mini-gels in Box 3-4.

11) From each LMP agarose-cell suspension, transfer two 40-75 µL drops to each pre-coated microscope slide. In the case of amphibians, 250 µL drop are used. See Table 3 for specifications per sample type.

12) Cover gels with 20×20mm coverslips.

Δ **CRITICAL STEP** It is important to work fast, to avoid gels solidifying before the coverslip is put on. When covering the gels with coverslips it is important to avoid bubble formation.

13) Keep for 5-10 min at 4 °C or place on a metal plate, which is on ice, for about 5 min.

Δ **CRITICAL STEP** Sometimes an extra layer of LMP agarose is applied to achieve a flatter gel, and rectify bubbles that may have occurred accidentally in the first layer. In the case of whole-body squashing of zebrafish embryo also additional LMP agarose is applied to fixate the squashed embryo. However, this should not be included when planning to perform an enzyme incubation step, as it will limit the movement of the enzymes through the gel to reach the nucleoids.

14) Carefully remove the coverslips and perform standard lysis according to option A, or use option B for lysis of human buccal cells.

(A) Place slides in standard lysis solution for at least 1 h in a Coplin jar at 4 °C in the dark.

(B) Human buccal cells require two lysis steps:

(i) First lyse at 4 °C in a dark jar, containing buccal lysis solution 1, for at least 1 h.

(ii) After this first lysis step, add Proteinase K (final concentration 10 mg/mL) to the pre-warmed (37 °C) buccal lysis solution 2.

(iii) Transfer the slides to the second buccal lysis solution and incubate for 1.5 h, maintaining a temperature of 37 °C.

Δ **CRITICAL STEP** When working with whole blood, especially fresh, 24 h is advised to ensure lysis of all the erythrocytes, resulting in slides with much cleaner gels than after only 1 h lysis. To split experiments over two days, the specimens can stay in lysis overnight, with no detriment to their integrity. 3D skin models require overnight lysis.

? TROUBLESHOOTING

Δ **CRITICAL** After lysis, any excess lysis solution can be removed by gently placing the longer edge of the slides against a paper towel, or the slides can be washed briefly using cold (4 °C) PBS before alkaline treatment. Washing of the slides after lysis is necessary in the case of subsequent incubation of nucleoids with the enzymes (enzyme-modified comet assay - Stage 2.2., step 20), where the presence of lysis solution could interfere with enzyme activity.

■ **PAUSE POINT** Slides can be left in lysis solution for a period between 1 and 48 h. Longer lysis periods can be applied, but it is advised to leave them no more than one week. The duration of lysis should be kept identical within a set of experiments.

Stage 2B: Processing gels for the enzyme-modified comet assay – day 1, ● Timing ~2 h

Prepare materials

15) Prepare four gels per sample, experimental controls, or assay controls (when applicable) by following the steps in Stage 2A, until cell lysis. Specifically, prepare 2 sets of duplicate slides: 1 set to incubate with reaction buffer and 1 set to incubate with the enzyme. If different enzymes/buffers will be used extra slides should be prepared.
16) Place a metal tray or plate on a box of ice.
17) Prepare a humidified chamber/box in a 37 °C incubator, containing suitable racks above water to ensure humidity, without the slides getting wet. Alternatively, use a slide moat at 37 °C.
18) Thaw aliquots of working solutions of the lesion-specific enzymes of interest on ice.
19) Dilute an aliquot of the 10× reaction buffer B or N in water to 1× working solution. Alternatively, thaw or prepare the reaction buffer specific for the enzyme that will be used.

Detection of specific DNA lesions

20) Wash slides in buffer B or N or another reaction buffer, 3 times for 5 min at 4 °C (using Coplin jar or another container).
21) Place slides on a metal plate on ice to prevent premature incision activity when the enzyme is added.
22) Prepare enzyme solutions, according to the titration experiments, and control solutions for the incubation reaction. For a 2 gels/slide format, it is advised to prepare at least 250 µL of enzyme mixed with incubation reaction buffer. If using Fpg, hOGG1, EndoIII or hAAG, follow option A. If applying enzyme T4endoV, follow option B. Table 2 provides recommendations on final enzyme concentrations that can be applied for the incubation.
    (A) To detect Fpg-, hOGG1-, EndoIII-, Udg and hAAG-sensitive lesions
        (i) Mix an aliquot of the enzyme with the required volume of reaction buffer B, to achieve the final concentrations based on your own titration experiments.
        (ii) Prepare a control solution (i.e., buffer B or a buffer provided with the enzyme), respecting the glycerol concentration.
    (B) To detect T4EndoV-sensitive sites
        (i) Mix an aliquot of the enzyme with the required volume of reaction buffer N, to achieve the final concentrations based on your own titration experiments.
        (ii) Prepare a control solution composed of buffer N respecting the glycerol concentration
        Δ **CRITICAL STEP** Keep enzyme, and control solutions, on ice during steps 18-23.
        Δ **CRITICAL STEP** Enzyme reaction buffers provided by enzyme suppliers can also be used.
23) Add 50 µL of the enzyme or control solution to each gel (containing nucleoids of samples, experimental controls or assay controls; see **Figure 1**). Incubate duplicate aliquots of each sample (i.e., two gels incubated with enzyme and two gels with control solution).
24) Cover gels with coverslips (22 × 22 mm for each gel or 24 × 60 mm to cover both gels).
25) Incubate at 37 °C in a humidified chamber/box in the incubator or slide moat for the required time. The incubation time is generally 30 min but needs to be tested/optimized (see "Experimental Design" and "Reagent setup" sections). For incubation reactions using 12 gels/slide or other high throughput formats, see Box 3-4.
    Δ **CRITICAL STEP** It is important to keep the slides moist during the incubation to prevent gels from drying out. Alternatively, enzyme incubations can be performed in a bath, where

microscope slides are fully immersed in an enzyme solution, and a second set in the control solution.

26) After the incubation of the gel-embedded nucleoids with the enzyme(s)/control solution(s), place slides immediately on ice to stop the reactions.

27) Keep on ice and carefully remove the coverslips just before alkaline treatment.

? TROUBLESHOOTING

Stage 3: Comet formation – day 1, ● Timing ~3 h (including washing steps)

Alkaline treatment & Electrophoresis

28) Transfer the microscope slides directly to the electrophoresis tank containing electrophoresis solution. Avoid direct light.

29) Incubate in cold (4°C) electrophoresis solution for 20-40 min at 4 °C in the dark, in the tank while keeping the power supply switched off; or the alkaline treatment can be done in a separate Coplin jar, the slides then being placed in the tank just before electrophoresis.

Δ CRITICAL STEP 4 °C conditions can be obtained in several ways. By putting the system in the fridge at 4 °C, by placing the tank on ice, by working in a cold room or by having a tank with a cooling system. If doing alkaline treatment in a Coplin jar (or other container), this can be placed at 4 °C. Variation in the temperature may occur between labs; the temperature should be kept constant for all experiments and not higher than 10 °C.

30) Electrophorese at ~1V/cm for ~20 min at 4 °C (EPT ~20).

Δ CRITICAL STEP Cells from 3D lung models require an EPT=30 (1V/cm for 30 min). See Box 10 and Box 11 for instructions for yeast and plant cells, respectively.

Δ CRITICAL STEP To ensure an accurate measure of the voltage gradient, it should be measured using a voltmeter. Alternatively, an approximate measure is obtained by dividing the applied electrode voltage by the distance between the electrodes. Please see the equipment setup.

Δ CRITICAL STEP When possible, samples from the same experiment together with corresponding controls (negative, solvent, positive) should undergo the same electrophoresis run. When a high number of samples need to be analysed, use inter-assay controls in each electrophoresis run.

Neutralization & Washing

31) Neutralise gels by washing slides in the neutralising solution for 10 min with cold PBS or 3 times for 5 min with cold, 400 mM Tris-HCl (pH 7.5). Afterwards, wash slides (optional) for 10 min in cold (4 °C) dH_2O (use Coplin jar, or lay slides flat in a dish).

32) Allow gels to air dry overnight, or dehydrate them by immersing them in 70% and subsequently 96-100% EtOH for 5-15 min and then, let them air dry. Alternatively, EtOH can be gently added on top of the gels by using a Pasteur pipette. Before each EtOH addition, remove previous EtOH by slowly leaning the tray with slides to one side.

Δ CRITICAL It is advisable to wash the slides with dH_2O after the neutralization when gels are going to be dried.

■ **PAUSE POINT** Dried gels/slides can be stored in the dark at room temperature for years. Usually, slides are stained and scored immediately. Alternatively, they can be stored unstained in dark until analysis for months. Stained slides can also be stored and re-stained prior to scoring, or rescoring.

Stage 4: Comet visualization & Analysis – day 2, ● Timing ~2 h – several days (depending on the number of samples)

Comet visualization

33) Stain slides with DNA fluorescent dye. When using dyes that allow direct visualisation, follow option A. For dyes that require a longer incubation time, follow option B.

Δ **CRITICAL** All the following steps should be performed away from direct light, since the DNA fluorescent dyes are light sensitive.

(A) Use of dyes for direct visualization

(i) For staining with EtBr (10 µg/mL in water), or DAPI (1 µg/mL in water), add 20-40 µL of staining solution to each gel, and cover with a coverslip.

Δ **CRITICAL** It is advisable to wash the excess of EtBr by immersing the slides in Tris-HCl (0.4 M Tris–HCl, pH 7.5) before covering them with coverslips.

Δ **CRITICAL** It is advisable to incubate the gels for 20 min at room temperature when DAPI is used. DAPI cannot be used with GelBond® films, due to autofluorescence of the GelBond® at the wavelengths used to detect DAPI.

(ii) Dilute the GelRed® stock (10,000 x in water) 1:3,333 in water, add 20-40 µL to each gel, and cover with a coverslip.

(B) Use of dyes requiring longer incubation times

(i) For staining with SYBR® Gold or SYBR® Green, which give intense fluorescence, it is recommended to immerse slides in a bath of the dye at a dilution of 1:10,000 in TE buffer for 20 min, followed by two 10 min washes with dH_2O. Alternatively, SYBR® Gold can also be added as 50 µL of the 1:10,000 dilution on top of each gel and subsequently covered with a coverslip (in this case skip step (ii)).

(ii) Allow slides to dry (up to overnight), and for viewing, add 20 µL of dH_2O to each gel and cover with a coverslip.

! CAUTION All dyes may be mutagenic, carcinogenic, and/or teratogenic, apart from GelRed®. Wear protective gloves when using them, and dispose waste in containers labelled for hazardous chemicals.

34) Subsequently, visualize comets with a fluorescence microscope using appropriate filters.

■ **PAUSE POINT** Stained gels can be stored overnight in the dark at room temperature and hydrated before scoring them the following morning.

Comet analysis

35) Score at least 50 comets per gel, i.e., 100 comets per sample when working in duplicates (or 100 comets if using only one gel). The OECD guideline for the in vivo comet assay advises to score 150 comets per sample.

36) Assess the level of DNA damage by means of image analysis software (option A) or visual scoring (option B).

(A) Using image analysis software

Obtain the TI (i.e., % DNA in tail) values per sample using the image analysis system by first calculating the median TI for each gel over the scored comets (i.e., the 50 comets in each gel) and then the mean TI over the replicate gels. Alternatively, the median of the 100 comets can also be used.

Δ **CRITICAL** It is possible to use other central estimates of non-normal distribution of comets, or arithmetic mean. All estimates are highly correlated and it has little practical implication to use one or the other because the statistical inference is based on differences between samples and not individual comets in the same sample. However, the same type of central estimate should be used for all samples in the same experiment.

(B) Using visual scoring

Compute DNA damage from comets by discriminating between the degrees of damage according to comet appearance (**Figure 13**). There are 5 classes, from 0 (no tail) to 4 (almost all DNA in tail) that give sufficient resolution [230]. If 100 comets are scored, and each comet is assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 and 400 "arbitrary units."

Δ **CRITICAL** All slides, including those of the negative/positive and assay controls, should be independently coded before microscopic analysis and scored without knowledge of the code. Within one study, one set of experiments, or a trial, all comets should be scored by the same person to minimize inter-operator variations using the same software for the entire experiment/trial. Score the comets in gel in a logical, and methodical way. The usual start point is in the top left of the gel, then score across the gel to the top right, adjust the stage so you are viewing comets slightly below the ones you just scored, staying on the right side of the gel. Journey back across the gel to the left side. Then, continue moving back and forth across the slide, getting further and further towards the bottom of the gel. Continue until you have scored the required number of comets. This helps to avoid scoring a single comet multiple times. Comets near the edges of the gel should not be scored as they may appear distorted (this could be due to the drying effect on the gel on the microscope slide). The same advice should be followed if you have any other imperfections in the gel, such as cracks or bubbles.

? TROUBLESHOOTING

4. TROUBLESHOOTING

Step	Problem	Possibly reason	Solution
Experimental design	High inter-assay variation.	Alterations in room temperature, equipment performance, reagent lots, etc.	Use internal controls and create own historical data to control/identify variability.
Equipment setup: Pre-coating microscope slides	Agarose does not attach to the slides.	The presence of grease and dust on the slides. Agarose is not mixed well.	Degrease the slides by washing them with EtOH. Leave them to dry at room temperature or pass the slide through the flame of a Bunsen burner.
1	Too many or too few MNCs in the gel.	Too low MNCs density following density gradient separation might be due to the temperature of blood (too warm) or blood is older than 6 h or too cold Histopaque/Ficoll or long waiting time after laying the blood on density gradient.	Process samples before 6 h. Histopaque/Ficoll needs to be at room temperature.
9-14	Loss of gels while removing the coverslip.	Gels do not set properly because of condensation in rooms with high temperature and/or humidity. Use of slides with charge. Agarose concentration is too high, not well mixed or too thin gels.	Mix agarose well. Cool the working room, ideally to ~20°C. Embedding cells in gels in an air-conditioned room is a good option. Direct airflow form heating fan over the slides. Use recommended slides.
25-27	Loss of gels during the enzyme incubation at 37°C.	Gels may be weakened by being at 37°C and they detach when removing the coverslips for the next step.	Cool the slides very quickly before removing the coverslips after enzyme incubation. Consider increasing the agarose concentration.
22-27/35-36	No increase in DNA migration in the enzyme-incubated positive control cells compared to buffer incubated cells.	Enzyme used after expiration date or subjected to multiple variations in storage temperature.	Check the expiration date, or use a cooler block when the enzyme is out of the freezer. Aliquot enzyme in appropriate concentration to prevent multiple freezing-thawing cycles.

35-36	High background on slides, comets cannot be scored.	The presence of dust or other impurities in agarose. Contamination of agarose with moulds. Re-used slides.	Prepare/use new agarose and/or slides.
30/35-36	Low DNA damage detection in positive controls.	Problems with electrophoresis. Miscalibration of the image analysis software-fluorescence microscope.	Check the power supply. Calibrate the software according to the manufacturer's instructions.
Box 3	Comet tails are going in all directions in the edge of mini-gels.	Uneven drying of the mini-gels.	Take care to dry the gels using EtOH immediately after the neutralization. Dehydration is crucial to avoid this edge effect.
Box 4	Few cells loaded into the microwells of the CometChip.	Excessive rinsing of unloaded cells might lead to loss of cells embedded in the microwells.	Reduce the intensity of the PBS rinse step by tilting the chip and slowly pipetting 5 mL of PBS across the top macrowells. Use vacuum around the macrowells to remove excess cells.
Box 10	Variability in the levels of DNA damage among cells.	Incomplete cell lysis.	Lyse and digest samples on slides with proteinase K (0.5 mg/mL) and reduced glutathione (2 mg/mL) for 15 min at room temperature.

5. TIMING

Day 0 or 1:

Stage 1: Preparation of cell suspension: Steps 1-3, 0.5 – 3 h (depending on the cell type and the number of samples)

Day 1:

Stage 2A: Embedding cells in LMP agarose & cell lysis: Steps 4-14, ~2-24 h (depending on the number of samples and the lysis time used)

Stage 2B: Optional extra steps for enzyme-modified comet assay: Steps 15-27, ~2 h

Stage 3: Comet formation: Steps 28-32, ~3 h (including washing steps)

Day 2:

Stage 4: Comet visualization & Analysis: Steps 33-36, ~2 h – several days (depending on the number of samples)

6. ANTICIPATED RESULT

The comet assay can detect above ~50 lesions per cell, whereas the assay cannot detect more than approximately 10,000 lesions per cell [24]. It should be emphasized that the primary comet assay descriptors are merely proxy-measures of the true level of DNA damage; therefore the actual %tail DNA depends on the assay conditions, in addition to the amount of damage present. As a rule of thumb, the level of SBs should not exceed 10% tail DNA (or TI) in unexposed cells, and tissues.

Cell death is a problem in all genotoxic assays because it is associated with degradation of DNA and so adds to the DNA damage caused directly by the genotoxic exposure. It has been demonstrated that cell death after exposure to non-genotoxic detergents produced comets with >90% tail DNA and shapes of comets that are commonly described as “hedgehogs”, “clouds” or “ghosts” [334]. However, the effect of cell death (or apoptosis) becomes smaller as the exposure condition is less severe. It has been shown that >25% dead cells, assessed by the trypan blue assay, increase the mean level of DNA migration in the comet assay [335]. Thresholds related to cytotoxicity and cell death are usually between 20% and 30% in publications. However, there are no gold standard method(s) that can be recommended for the evaluation of cytotoxicity and there is considerable uncertainty about the validity of a threshold of viability for considering comet assay results biased due to cell death [7]. The effect of cytotoxicity on comet assay endpoints should be assessed by a case-by-case approach rather than by adopting a predetermined threshold; cytotoxicity assays may be test system specific and the assays measure different types and severity of the toxicity endpoints. In addition, it should be noted that apoptotic or dead cells cannot be distinguished as “hedgehogs”, “clouds” or “ghosts” [334]. Thus, omission of such comets is not recommended as a way of avoiding indirect genotoxicity by toxicity to the cells.

6.1. Detection of DNA cross-links

DNA cross-linking may appear to be non-genotoxic in the standard comet assay. If a compound is suspected to cause DNA cross-links, it is advisable to confirm this by testing in the DNA-crosslink variant of the comet assay. **Figure 14** illustrates the anticipated results from a confirmatory experiment where the increased DNA strand break levels by a direct DNA strand breaking agent are lowered when cells are treated with the suspected cross-linking agent as compared to the control exposure with the DNA strand breaking agent only [36,336]

6.2. DNA SBs formed by repair processes

Certain agents (e.g., UV-C) do not produce ALS and SBs, but SBs are generated by excision repair enzymes in the cells [68,337]. To study such a case, it is advisable to incubate the cells with DNA repair inhibitors that blocks DNA polymerases or other enzymes in the late stage of the excision repair process (e.g., aphidicolin or hydroxyurea/Ara-C). DNA SBs will then accumulate as incomplete repair sites as the cells are incubated with the test compound and DNA repair inhibitors (**Figure 15**).

6.3. Enzyme-sensitive sites

Results from enzyme-modified comet assays should be reported as levels of DNA migration with the corresponding, background (no enzyme) subtracted, using the following formula:

$$\text{"Enzyme-sensitive sites"} = DNA\ migration_{\text{Enzyme}} - DNA\ migration_{\text{Buffer}}$$

The measurement of enzyme-sensitive sites, and global methylation, require an additional step in the comet assay protocol. The step affects the level of DNA migration. However, the variability in DNA damage levels between samples is also increased because the experimental variation in the extra step is added to the variation in the standard comet assay; this can be checked by comparing the standard deviations of the standard DNA SBs and those as a result of enzyme-sensitive sites. As a rule of thumb, there are at least as many oxidatively damaged DNA lesions as DNA SBs in cells/tissues that have not been exposed to a genotoxic agent. The background level of DNA SBs and enzyme-sensitive sites should not be too different, unless there are special circumstances such as cells or tissues from DNA repair knockout variants. However, chemical agents have different mechanisms of action and it is therefore possible that certain agents cause mainly DNA SBs, while other agents produce mainly enzyme-sensitive sites.

It is very important to understand that the anticipated results from the enzyme-modified comet assay are substantially different from DNA SBs. **Figure 16** illustrates the anticipated results of enzyme-sensitive sites, using theoretical data in four different samples. The first two samples are measurements where the level of DNA SBs (i.e., "buffer") differs, whereas the levels of enzyme-sensitive sites are identical. Thus, it is misleading to conclude that the enzyme-modified comet assay shows that sample 2 has a higher level of DNA damage than sample 1 when in fact it only has a higher level of DNA SBs. Samples 3 and 4 illustrate situations where negative enzyme-sensitive sites are obtained. It is not biologically meaningful to measure fewer than zero DNA lesions; thus, it is not an option to use enzyme-sensitive sites with negative values. Sample 3 represents a situation where the DNA has no enzyme-sensitive sites; thus, the buffer and enzyme treatment should have had the same level of DNA migration. The experimental uncertainty in the scoring of comets (i.e., results are usually based on analysis of 50-100 images in 2 gels) will by chance alone give rise to lower values in enzyme-treated slides than the buffer treated slides. In this case, it is advisable to set the enzyme-sensitive sites to zero. Sample 4 also gives rise to a negative value of enzyme-sensitive sites, but in this example, it is due to a high level of DNA SBs. As the comet assay has a ceiling of 100% tail DNA, there is increasingly less DNA migration left for the determination of enzyme-sensitive sites. In this case, the enzyme-modified comet assay cannot be applied, although reducing the concentration of DNA-damaging agent, if possible, might solve the problem.

6.4. Variation in DNA damage levels

The variation in DNA damage in different samples stems from inter-individual, intra-individual and technical (assay) variation. The contribution of these sources to the overall variation depends on the type of study. For instance, biomonitoring studies encompass all sources of variation, whereas the latter two are only relevant for cell culture studies (i.e., the variation in different passages of cell cultures is equivalent to intra-individual variation in a biomonitoring study).

In general, a relatively large variation in DNA damage levels by the comet assay should be anticipated. For instance, a systematic review has shown a mean intra-group coefficient of variation in DNA SBs in leukocytes of 36% (95% CI: 27%, 46%) in cross-sectional studies on healthy humans [338]. Likewise, a systematic review obtained a coefficient of variation of 66% (95% CI: 51%, 82%) for Fpg-sensitive sites and 103% (95% CI: 56%, 151%) for hOGG1-sensitive sites in leukocytes from healthy humans in cross-sectional studies [314]

It should be anticipated that the variation in enzyme-sensitive sites is similar or higher variation than the variation in DNA SBs because the variances are additive. Moreover, it should also be anticipated that assay control samples display some inter-day variation. This is illustrated in **Figure 17**, using results from assay controls from a human biomonitoring study [339]. The mean and standard deviations of the samples are 0.29 ± 0.14, 0.85 ± 0.35 and 1.43 ± 0.26 lesions/10^6 bp DNA SBs in samples that were incubated with buffer, hOGG1 and Fpg, respectively. Note the larger standard deviation in the enzyme-treated samples as compared to the buffer-treated sample.

Lastly, it should be expected that exposure to a genotoxic agent increases both the level of DNA damage and the intra-group variation in biomonitoring, animal and cell culture studies. This is illustrated by the example in **Figure 18** that depicts levels of Fpg-sensitive sites in cells after exposure to a genotoxic agent (i.e., diesel exhaust particles). As can be seen, the DNA damage level increases as the concentration of the diesel exhaust particles increases. The standard deviation also increases as the level of exposure increases (seen as wider error bars in **Figure 18**). It is common to obtain a larger standard deviation in treated specimens than unexposed specimens irrespective of whether the specimens originate from cell cultures, animals or biomonitoring studies.

Reporting Summary

Further information on research design and ethical approvals is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the majority of the data shown here as examples or anticipated results are available in original papers. **Figures 12, 14, 15 and 16** are theoretical results, which are inspired by unpublished work in the authors' laboratories. Other supporting data are available upon reasonable request to the corresponding author. For instance, **Figures 3, 9 and 13** are based on unpublished data.

FIGURE LEGENDS

Figure 1. **Overview of the standard, and the enzyme-modified comet assay protocols.** Stage 1 involves the isolation of single cells, which are processed in either the standard (2A) or enzyme-modified (2B) comet assay. In the second stage of the standard comet assay, nucleoids are embedded in agarose and lysed. The enzyme-modified comet assay contains an additional step where the nucleoids are incubated with DNA repair enzymes such as formamidopyrimidine DNA glycosylase (Fpg), human 8-oxoguanine DNA glycosylase 1 (hOGG1), endonuclease III (EndoIII), or T4 Endonuclease V (T4endoV). Stage 3 entails a DNA unwinding period, electrophoresis and subsequent neutralisation of the slides. Stage 4 is the visualisation and microscopic evaluation of comets in the samples (S) as well as negative (A/C-) and positive (A/C+) assay controls. Finally, the results are expressed as e.g., tail intensity (TI) for DNA strand breaks, or in the case of enzyme-sensitive sites as net TI by subtracting TI for the buffer-treated slides Ⓑ from TI for the enzyme-treated slides Ⓔ.

Figure 2. A schematic representation of inter-strand crosslinks (ICLs) formation by cisplatin and detection with a variant of the alkaline comet assay. a, In the absence of cisplatin treatment, relaxed DNA loops migrate towards the anode forming the comet tail. **b,** In the presence of the cisplatin, and with exposure to a strand-breaking agent such as ionising radiation or H_2O_2, migration of the DNA is inhibited by the ICLs – the more ICLs, the less the migration of the DNA.

Figure 3. Representative images of comets illustrating ICL detection following cisplatin treatment. Cells from an ovarian cancer cell line (SKOV-3) were first treated with 200 µM or 0 µM cisplatin. Strand breaks were then induced using H_2O_2 (50 µM). Cisplatin-induced crosslinks resulted in a decrease in tail moment after DNA damage induced by H_2O_2 (50 µM), compared to the H_2O_2 treatment control, in the absence of cisplatin.. **a**, Control cells without any treatment; **b,** Cells treated with H_2O_2 (50 µM) only; **c**, Cells treated with cisplatin (200 µM) and subsequently H_2O_2 (50 µM). Scale bar represents 10 µm.

Figure 4. Component parts of the 12-gel chamber unit, including metal base with marks for positioning gels on slide, silicone rubber gasket, plastic top-plate with wells, and silicone rubber seal.

Figure 5. **Images illustrating the 96-gel format using GelBond® film** (Adapted from Gutzkow et al.[30])

Figure 6. The CometChip Platform. a, Cells in medium or PBS are loaded by gravity into a microwell array in agarose that was created using a mould with pegs approximately the diameter of a single cell [82,89]. Excess cells are removed by shear force, leaving behind an array of cells. Cells are retained with a layer of low melting point agarose (not shown). **b**, An agarose slab with thousands of microwells is created with the dimensions of a 96-well plate. A bottomless 96 well plate is pressed into the agarose, creating 96 compartments, each with more than 100 microwells. After cell loading, rinsing, capping, and treatment, the agarose slab is processed using standard comet assay protocol conditions. Cells can be either pre-treated or treated on-chip. Each of the 96 wells substitutes for a single glass slide using the

traditional comet assay. Scale bar is 100 µm. **c**, For the EpiCometChip (see section 1.2.6. Detection of global DNA methylation), immediately after lysis, the agarose slab is rinsed and incubated with *McrBC* prior to processing using standard comet analysis conditions (Adapted from Townsend et al.[42]).

Figure 7. The vertical comet system, comprising: **a**, racks to hold slides vertically (up to 25 slides per rack); **b**, treatment chambers which accommodate the slide-containing racks; **c,** electrophoresis tank (possesses integrated cooling) holding two racks; **d**, improvement in size of the high throughput tank (left), over the standard comet assay tank (right) is seen clearly.

Figure 8. Principle of the DNA methylation-sensitive comet assay, using the methylation sensitive *HpaII* (the activity of the enzyme is inhibited by the presence of a methyl group on either C) and methylation insensitive *MspI* restriction enzymes. Scale bar is 10 µm.

Figure 9. **Visualization of comets and BrdU-positive comets with fluorescence microscope, using two filters.** With the FITC filter (image on the left), comets stained with YOYO-1 for detection of DNA breaks are visualised. With the TRITC filter (middle image), BrdU-positive comets formed by cells in the S-phase of the cell cycle are visualised. The image on the right shows both BrdU-positive and -negative comets. Scale bar is 20 µm.

Figure 10. Example pictures of different types of signals seen in comet-FISH experiments after alkaline electrophoresis using U-2 OS cells. a, Probe RPCI-1 213H19 labelled with two colours (digoxigenin as green dots and biotin as red dots), in comets from cells irradiated with UVC at 0.2 Jm^{-2} ; **b**, Probe RPCI1 213H19 labelled with biotin (red dots), in comets from cells treated with 0.1 mM H_2O_2; **c**, Probes RPCI-1 213H19 and RPCI-6 32H24 labelled with digoxigenin (green) and biotin (red), respectively, in comets from cells irradiated with UVC at 0.2 Jm^{-2}. Bars represent 20 µm. (Adapted from Shaposhnikov et al.[340]).

Figure 11. **Overview of various species, and different sample types that have been used in the (enzyme-modified) comet assay**. *So far only roots from Monocots and Eudicots have been used for the comet assay, but there is no reason why roots from other plants could be used as well.

Figure 12. Titration steps in the enzyme-modified comet assay. **a,** The graph illustrates the titration curve that is usually obtained when the optimal concentration of enzymes is found. Cells with a known level of DNA damage (e.g., potassium bromate-treated cells) are incubated with different dilutions of the enzyme for a specific period (e.g., 30 min). The plateau represents a range of concentrations over which the enzyme has excised all available lesions (i.e., specific incisions), and the subsequent increase in comet score is attributed to non-specific incisions. **b,** The graph illustrates the time-curve from a comet assay experiment, where the optimal incubation time is selected to be on the plateau where all lesions are recognized by the enzyme.

Figure 13. **Representative images of comets classified in 5 different classes for visual scoring:** 0 (no tail), 1, 2, 3 and 4 (almost all DNA in tail; sometimes described as a hedgehog). Scale bar is 20 µm.

Figure 14. **Detection of DNA cross-links in a theoretical cell culture study.** Experiments are first carried out to find a suitable level of DNA strand breaks, using an agent that directly causes breaks in DNA such as H_2O_2 or ionizing radiation (left figure). Subsequently, experiments are done where cells are exposed to the test agent (compound) and ionizing radiation. The presence of cross-links in DNA is concluded if the irradiated samples plus the tested compound have less DNA migration as compared to the irradiated samples without the tested compounds (black bars compared with grey bars).

Figure 15. Assessment of DNA lesions by inhibition of late-stage excision repair processes in a theoretical cell culture study. The cells are incubated with the test agent (compound, **C**) and inhibitor (**I**) (bold letter in the left graph refers to the presence of compound or inhibitor; in case of incubations with I/**C** and I/C the lines overlap). The effect of DNA repair on the determination of genotoxicity is inferred by the higher level of DNA migration in samples that have been exposed to both the compound and repair inhibitors (right graph).

Figure 16. Examples of data output of the enzyme-modified comet assay in theoretical samples. Sample 1 and 2 exemplify two different samples where the levels of DNA strand breaks differ, whereas the levels of enzyme-sensitive sites are identical. The total level of DNA damage (i.e., "enzyme" treatment) is higher in sample 2 than the level in sample 1, but interpreting that as a higher level of DNA damage in the enzyme-modified comet assay is misleading. Samples 3 and 4 exemplify two different samples that have few enzyme-sensitive sites, but low or high levels of DNA strand breaks, respectively. In these samples, the DNA damage level measured by the "buffer" and "enzyme" treatments are identical. However, negative values of enzyme-sensitive sites will occur in some sample because of experimental variation in the scoring of comet assay slides. Sample 3 represents a situation with a valid measurement of few enzyme-sensitive sites because the level of total DNA damage is relatively low (i.e., close to 10% tail DNA). Sample 4 is a different situation; the level of DNA strand breaks is so high that the comet assay is saturated (i.e. DNA migration is close to 100% tail DNA). Therefore, it is not possible to increase the DNA migration by the enzyme-treatment and so enzyme-sensitive levels are under-estimated.

Figure 17. Levels of DNA migration in assay control samples from a biomonitoring study, encompassing 11 days of comet assay experiments. Peripheral blood mononuclear cells were exposed to 1 µM Ro-19-8022 and irradiated for 4 min with white light, and subsequently cryopreserved. The DNA migration is depicted as lesions/10^6 bp in samples treated with buffer (i.e., DNA strand breaks, SB), formamidopyrimidine glycosylase (Fpg) or human oxoguanine DNA glycosylase (hOGG1). Original results published by Jensen et al.[339].

Figure 18. Example of results from a cell culture study on Fpg-sensitive sites after exposure to diesel exhaust particles in human HepG2 cells. Symbols and whiskers are mean value and standard deviation of six experiments (number in brackets is standard deviation). Original results published by Vesterdal et al.[341].

TABLES

Table 1: Experimental models and sample types that can be used with the described procedure

In vitro	**Types**
Cell lines and primary culture	Single culture and co-culture
3D cell models	Liver spheroids and skin (dermis and epidermis) and lungs
Zebra fish	Embryos and larvae
Yeast	Single culture of different strain and species
Plants	**Organs**
Bryophyta, Pinophyta, Ginkgophyta, Monocots, Eudicots	Roots, leaves
***In vivo* – non mammalian**	**Organs/samples**
Crustaceans: *Daphnia magna, Ceriodaphnia dubia*	Whole organism
Platyhelminthes: planarians	Whole organism
Insects: *Drosophila melanogaster*	Hemocytes and neuroblasts
Insects: *Chironomus riparius*	Larvae, whole organism
Annelids: earthworm, *Eisenia foetida*	Coelomocytes
Molluscs: *Bivalves*	Haemolymph, gills, digestive glands
Amphibians	Blood from anuran amphibians at premetamorphic stages
Fish: Zebrafish (*Danio rerio*), Mosquitofish (*Gambuzia holbrooki*), gilthead seabream (*Sparus aurata*), Senegalese sole (*Solea soleganensis*) and European eel (*Anguilla anguilla*)	Blood, liver, gills, gonads and sperm
***In vivo* –mammalian**	**Organs/samples**
Rodents	Blood, bone marrow, liver, kidney, lung, spleen, brain (hippocampus, prefrontal cortex), glandular stomach, duodenum, jejunum, ileum, colon, skeletal muscle, heart, aorta, bladder, adrenals, hypothalamus, thyroid, pituitary, pineal gland, pancreas, epidermal cells, ovary, prostate, mammary gland, uterus, testis, germ cells and sperm
Humans (for biomonitoring studies)	Blood and derived cells (including buffy coat); buccal mononuclear cells; buccal, nasal, lachrymal and conjunctival epithelial cells; sperm; and placental cells

253 **Table 2: Suggested enzyme concentration, as a guide for own titration experiments.**

Enzyme	Format	Final enzyme concentration	Duration of incubation
Fpg (NorGenoTech)	2 gels (70 µL of gel; 20 x 20 mm coverslip) 45-50 µL enzyme/gel (22 x 22 mm coverslip)	0.16 ng/µL	30 min
Fpg (New England Biolabs)	12 mini-gels (5 µL of gel) 30 µL enzyme/gel using the 12-well chamber unit	0.026 U/mL	1 h
Endo III (New England Biolabs)	12 mini-gels (5 µL of gel) 30 µL enzyme/gel using the 12-well chamber unit	33.3 U/mL	1 h
hOGG1 (Trevigen)	2 gels (80 µL of gel; 20 x 20 mm coverslip) 50 µL enzyme/gel (22 x 22 mm coverslip)	1.6 U/mL	10 min
hOGG1 (Trevigen)	12 mini-gels (5 µL of gel) 30 µL enzyme/gel using the 12-well chamber unit	6.66 U/mL	1 h
T4endoV (New England Biolabs)	2 gels (70 µL of gel; 20 x 20 mm coverslip) 45-50 µL enzyme/gel (22 x 22 mm coverslip) Incubation in slide moat	3.33 U/µL	30 min

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256 **Table 3: Recommended cell suspension processing and embedding in LMP agarose, as a starting guide for own optimizations.**

Species/cell type	Cell suspension	Dilution in LMP agarose	Final cell density (Final LMP agarose %)*
In vitro models			
Cell (co-)cultures	Resuspend the cell pellet to ~1×10^6 cells/mL using cold (4 °C) PBS	Mix 3:7 with 1% LMP agarose	~2.1×10^4 per 70 µL gel (0.7% LMP agarose)
Liver spheroids prepared from HepaRG cells	20 000 cells/mL	Mix cell suspension pellet with 100 µL of 0.5% LMP agarose	150 000 cells/mL (0.5% LMP agarose)
Liver spheroids prepared from HepG2 cells	130 000 cells/mL; resuspend pellet in 70 µL cell culture medium	Mix 50 µL of the cell suspension 1:3 with 0.8 % LMP agarose	~3.2×10^4 per 70 µL gel 0.6% LMP agarose
3D airway model	Resuspend in LMP agarose	Add 150 µL of 0.5% LMP agarose	Not determined, but a good comet density for scoring is achieved (0.5% LMP agarose)
3D skin model	Resuspend the cell pellet in remaining buffer (about 200 µL)	Add 300 µL of 0.75% LMP agarose	3 -6 x 10^4 per 75 µL gel (~0.5% LMP agarose)
Zebrafish embryos	Whole-body squashing (1 embryo/slide) or Whole-body cell isolation (pool of more embryos (up to 8, depending on single cells yield, 5-6 x 10^6 cells/mL)	Whole-body squashing; 1 embryo directly in 1.5% LMP agarose Whole-body cell isolation; 20 µL of cell suspension in 180 µL of 1% LMP agarose	Whole-body squashing; 1 embryo in 60 µL (1.5% LMP agarose) Whole body cell isolation; up to 5-6 x 10^6 cells/mL (0.9% LMP agarose)
Non-mammalian models			
Crustaceans	~1.0 x 10^5 cells/140 µL	Resuspend cells in 0.7% LMP agarose	~5×10^4 per 70 µL gel 0.7% LMP agarose
Platyhelminthes – Planarians	Lyse entire animal + filter with cell strainer to obtain cell suspension. Cells are generally not counted	Resuspend the cell pellet directly in 160-180 µL 0.8% LMP agarose	One sample can be one or multiple worms. This sample is then divided, 70 µL per gel (2 technical duplicates)

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Insects – *Drosophila melanogaster*	**Resuspend about 1000 cell/µL in Poel's salt solution, Ringer solution, or PBS containing phenylthiourea**	**Mix 2:8 with 1% LMP agarose**	**50-100 cells/µL gel (0.8% LMP agarose)**
Insects –*Chironomus riparius*	Resuspend the cell pellet to ~1×10^4 cells/mL using cold (4 °C) PBS	Mix 10 µL of the cell suspension with 100 µL of 1% LMP agarose	~300 cells per 75 µL gel (0.91% LMP agarose)
Annelids – earthworm	Resuspend the cell pellet to ~1.5×10^4 cells/mL using cold (4 °C) PBS	Mix 1:1 with 1% agarose	~450 cells in 60 µL 0.5% LMP agarose
Molluscs – mussels	Gills and digestive glands: Resuspend the cell pellet to ~$5x10^5$ cells/mL in KSS Haemolymph: dilute haemolymph from one animal in modified Alsever (1:5)	Resuspend the cell pellet in 75 µL 0.5-0.85% LMP agarose	$2.5x10^3$ cells/µL (0.45-0.75% LMP agarose)
Amphibians	Resuspend the blood cell pellet in 50 µL cold (4 °C) PBS (~1.0×10^6 ± 0.3 cells/mL)	Mix 3:7 with 0.5% LMP agarose	4×10^4 cells per 250 µL gel (0.5% LMP agarose)
Fish (considering gilthead seabream Senegalese sole and European eel)	Blood: 2 µL peripheral blood mixed with 1 mL PBS Liver and gills: resuspend the minced (and washed) small part of the tissue in 1 mL PBS	Blood: mix 20 µL of the cell suspension with 70 µL (1%) LMP agarose Live and gills: mix 20 µL of the cell suspension with 70 µL (1%) LMP agarose	Blood: ~ 2×10^4 cells in 70 µL gel (0.8% LMP agarose) Liver and gills: ~ 2×10^4 cells in 70 µL gel (0.8% LMP agarose)

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Zebrafish (small fish)	Blood: 10 µL peripheral blood mix with 90 µL PBS without Ca2+/Mg2+ Liver, gills and gonads: resuspend the minced (and washed) small part of the tissue in 1 mL PBS supplemented with 0.02% EDTA Sperm: resuspend cells in HBSS	Blood: mix 10 µL of peripheral blood cells in PBS with 70 µL (1 % LMP) Liver: 10 µL of cell suspension in PBS mix with 70 µL (1 % LMP) Gills and gonads: 25 µL of cell suspension mix with 75 µL 1% LMP Sperm: 10 µL of sperm cells mix with 180 µL HBSS (0.5 % LMP)	Blood: ~1.5 × 10^3 cells in 70 µL gel (0.9% LMP agarose) Liver: ~1.5-3.0 x 10^3 in 70 µL gel (0.9% LMP agarose) Gills and gonads: ~2.5 × 10^4 cells in 70 µL gel (0.75 % LMP agarose) Sperm cells: ~2-5 x 10^3 cells in 70 µL gel (0.5% LMP agarose)
Mammalian models			
Rodent tissues	Liver: 3x3x3 mm Kidney: 2x3x5 mm Lung: 5x5x5 Spleen: 1x1x1 mm Brain: 2x3x5 mm Duodenum, yeyuno, yleon, colon: segment 1.5 cm (Cells from the gastrointestinal tract can also be obtained by scraping off the inner part of the organ.) Add 1.5 mL (mice) or 2 mL (rat) of cold PBS (Ca^{2+} and Mg^{2+} free, 20 mM EDTA), mincing buffer or Merchant's buffer to the minced tissues. Cells are generally not counted	Mix 30 µL of cell suspension with 140 µL 1% LMP agarose	(0.82% LMP agarose)

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Whole blood	Use 5-20 µL WB directly Alternatively, mix 10 µL WB with 40 µL PBS	Mix 20 µL of WB in 480 µL 0.8% LMP agarose; Alternatively, add 160 µL of 1% LMP agarose to the WB/PBS mixture	50-125 cells/µL gel (0.5-0.7% LMP agarose)
Buffy coat	Use 5 µL BC directly	Mix 5 µL of BC in 200 µL 0.8% LMP agarose	Enough number of cells to carry out the assay (~0.8% LMP agarose)
Leukocytes, PBMCs	*Idem as cell (co)cultures*		
Salivary BMCs	~2×10^5 cells/160 µL	Resuspend the cell pellet in 0.71% LMP agarose	~1×10^5 per 80 µL gel (0.71% LMP agarose)
Buccal cells	100,000 – 500,000 cells/ 1mL PBS	Resuspend the cell pellet in 0.5% LMP agarose	10,000 - 50,000 cells/75 µL gel (0.5% LMP agarose)
Nasal cells	50,000 cells/50 µL of PBS	Resuspend the cell pellet in 0.5% LMP agarose	50,000 - 100,000 cells/75 µL gel (0.5% LMP agarose)
Tears (lachrymal duct and cornea cells)	Use tear directly	Mix the tear (10-30 µL) with 30 µL LMP agarose	100 – 1,000 cells (0.5% LMP agarose)
Placenta	Centrifuge a cell suspension of ~2.5×10^4 cells/mL (in PBS)	Add 200 µL 0.6% LMP agarose to cell pellet	~500 cells/5 µL gel (0.6% LMP agarose) (12-gel format)

** This is the most commonly used percentage of LMP agarose for the specific assays, but other concentrations between 0.5-0.8% may work as well (also see section 1.6.2. Optimization of percentage of LMP agarose). For other species/cell type, see corresponding Boxes 10-12.*

BOXES

Box 1: The detection of DNA inter-strand crosslinks (ICL-modified alkaline comet assay).

Additional reagents & equipment

- Hydrogen peroxide (H_2O_2) (Millipore, cat. no. 107298)
- UVB-lamp - Blak-Ray Lamp, model LW-BLB (Spectrum Chemical Corp., cat. no. 371-10811)

Procedure

Stage 1: Preparation of cell suspension from fresh or frozen samples

Additional steps after Stage 1, step 1.

1) Treat the cells with 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 [342].

3) The cells are then pelleted (7600 x *g* for 5 min), and washed three times with 1 mL PBS and further processed starting from Stage 2A. 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

Previous literature on ICL so far seems to use comet tail moment as the descriptor. However, identical results will be obtained by use of any primary comet assay descriptor. In keeping with the recommended use of tail intensity (TI, percentage of tail DNA) as primary comet assay descriptor, the level of DNA interstrand 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:

Percent decrease in TI = [1− ((TIdi − TIcu)/(TIci − TIcu))] x100

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.

Plotting the per cent-decrease TI against the concentration of crosslinking agent produces a line with a negative slope.

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

Δ **CRITICAL** The concentrations of the repair enzyme inhibitors given below are suggestions from using HepaRG™ cell lines. HU and AraC lead to a slight increase in TI. Therefore, dose-dependent experiments with HU/AraC should be performed for each cell line used 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)
- 1-β-D-arabinofuranosyl cytosine (araC) (Merck KGaA, cat. no. C1768)

Procedure

Stage 1: Preparation of cell suspension from fresh or frozen samples

Additional steps after Stage 1, step 1.

1) Incubate the cells with HU/AraC to reveal the presence of UV-induced adducts following option A, or 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 HU/AraC 1 mM HU and 10 µM AraC prepared in culture medium supplemented with 10 mM glutathione for 40 min before UV irradiation.

Δ **CRITICAL STEP** Some cell types – in particular mononuclear cells (MNCs) – have lower levels of nucleotides and therefore much less HU is needed to block synthesis. 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.

Δ **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/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/AraC for desired time points.

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

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

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

3) For subsequent comet assay steps follow Stage 2A to reveal the amount of damage induced.

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

Box 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 [30,75,78,80].

Additional special equipment

- 49- and 96-well frame for moulding the gels in GelBond® films (NorGenoTech)
- 49- 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 (85x125 mm) with holes in each corner and a cut corner down to the right for correct orientation. It is at all stages of the comet assay attached to a plastic frame 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

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

Prepare materials

Modification of step 6.

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

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 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 into the plastic. Make sure you have the cut corner of the film to its 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 - Application of cells and agarose to GelBond® film

Modification of step 10.

Mix the cell suspension carefully with LMP agarose (37 °C) to a final concentration of 0.5-1x10^5 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.

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.

Δ **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:

(A) For a 96 gel format: add 4 µL/gel (c.a. 400 cells)

(B) For a 48 gel format: add 7 µL/gel (c.a. 700 cells) when the distance between the tips is large

(C) For a 12 gel format: add 5 µL/gel (c.a. 500 cells)

Δ **CRITICAL STEP** The gels can be applied with or without formats, wells, or separating surfaces [30,78]. The agarose/cell samples can easily be applied with a multi-channel pipette.

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

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

Prepare materials

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

Δ **CRITICAL STEP** Washing can be done using Buffer B without BSA, but you need to add it 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.

Δ **CRITICAL STEP** It is essential that the rather viscous protein is allowed to be 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.

Δ **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.[22]). The assembly of the 12-gel unit is demonstrated in this tutorial video: https://youtu.be/NE2U8f5gwc8

Δ **CRITICAL** The optimum enzyme concentration must be determined by titration. The concentration used with this protocol (i.e., 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.

After electrophoresis (which is performed preferably with buffer circulation), neutralisation and rinsing, the gels must be fixed in ethanol and dried prior to staining.

Stage 4: Comet visualization & Analysis

Comet visualization

Modification of step 33.

It is advised to stain with SYBR® Gold. Δ **CRITICAL** DAPI is not compatible with plastic films.

Comet analysis

Modification of step 35-36.

Scoring is performed 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

Box 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, to trap cells into the agarose grid for analysis, the CometChip introduces a cell loading step to the comet assay protocol (Figure 6 – CometChip (A-E):

Additional special 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.

Δ **CRITICAL:** If pre-made CometChip array from Tevingen is used, follow the manufacture instructions to use it.

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 array from Trevigen. See Wood et al.[82] or Chao and Engelward [89] for details on how to create in-house agarose arrays from reusable polydimethylsiloxane stamps.

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

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 [89].

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.

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.

Following the lysis step, secure the CometChip into an electrophoresis chamber 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.

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

Comet analysis

Modification of steps 35-36.

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.

? TROUBLESHOOTING

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

Karbaschi and Cooke [90] demonstrated that electrophoresis could be performed successfully with the comet slides held vertically, rather than horizontally, which is the convention. This led to a number of developments that represent significant improvements on the processing of slides for the comet assay, via specialized equipment.

Additional special equipment

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

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.

ΔCRITICAL STEP Ensure that all slides have the same orientation in the rack, are placed level, and are positioned correctly for the electrophoresis step.

2) Transfer slide racks are between dedicated chambers (Figure 7B), with integrated lids to minimize exposure to ambient light (Cleaver Scientific Ltd), in which the lysis (step 14), washing, neutralization (steps 31-32), staining, and the final washing steps (Stage 4, step 33) are performed.

Stage 3: Comet formation

Alkaline treatment & Electrophoresis

Modification of steps 28-30.

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.

Box 6: DNA methylation-sensitive comet assay

Option 1: Using two isoschizomeric restriction enzymes, *HpaII* and *MspI*, which recognize the same tetranucleotide sequence (5' CCGG 3') but display differential sensitivity to DNA methylation. *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

Stage 1: Preparation of cell suspension from fresh or frozen samples

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

Δ **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 of DNA methylation (CpG methylation unaffected) as observed from cells treated with 5-Aza-dcR.

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

Detection of specific DNA lesions

Modification of step 20.

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

Modification of steps 22-23.

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.

Δ **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 the nucleus 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.

According to the published protocol by Wentzel et al.[92], an electrophoresis of 45 min at ~0.9 V/cm is advised.

Anticipated results

The percentage CpG methylation is calculated according to the following formula:

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

Alternative procedure using the 12-gel chamber unit (Figure 4) as medium-throughput comet assay [91]**.**

Δ **CRITICAL** Enzyme digestion is more effective when done in wells.

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 into the 12-gell chamber unit, including the 12-well 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 ^{o}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 cap.
3) Incubate the 12-well unit at 37 ^{o}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 as not to disrupt the individual LMP agarose gel spots.

Option 2: 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) [83,84]. *McrBC* specifically recognizes DNA sites of the form 5'- (G/A)mC-3' and cuts DNA at methylated Cyt. 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 0.035U/µL

Procedure

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

Detection of specific DNA lesions

Modification of step 22.

Incubate gels at 37 °C in a preheated damp chamber for 105 min by layering either of the following in parallel:

(A) Control treatment buffer;

(B) Methylation-specific buffer

Box 7: Detection of chromosomal breaks in yeast

Δ **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)
- 50x Tris/Acetic Acid/EDTA (TAE) (Biorad, cat. no. 1610743)
- Ethidium Bromide Solution (Biorad, cat. no. 1610433)
- 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)
- 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

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 according to the manufacturer's instructions with lyticase and proteinase K treatments.

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 using 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.

Δ **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. Please note that if chromosomes will 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 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 fade-sensitive. If band is bleaching, 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 the holes in the LMP agarose layer and put carefully into the holes the agarose blocks containing single chromosomes after PFGE separation and cover with a coverslip.

8) Add the third layer of agarose, namely 0.5% NMP agarose.

9) 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 buffer.

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 for each chromosome. Consider three biological replicates.

3) Analyze 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.[97]

Box 8. 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, will allow quantification of induced DNA damage and not in combination with physiological DNA repair intermediates, which may then false-positively increase DNA damage levels.

Additional reagents

- BrdU (e.g., from Roche, cat. no. 10280879001)
- Bovine serum albumin (BSA) (biotin-free, e.g., from ROTH, cat. no. 0163.2)
- Anti-BrdUmonoclonal 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)

Procedure

Stage 1: Preparation of cell suspensions from fresh or frozen samples

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

A wide variety of human and mouse cell lines and normal cell populations can be used for incubation 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 manufactures instructions).

2) Incubate the treated cells for the desired length of time (usually 15 - 45 min) at 37°C [102,103].

Δ **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.

Δ **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 x 10^6 cells/mL.

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

4) Wash cells three more times with PBS for 2 minutes each and proceed to Stage 2A.

Modification of step 1 (M) - 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 of incubation, 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) 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.
5) Collect cell suspensions of 300,000 cells into 2mL tubes and spin for 5 min, 500xg, 4 °C.
6) Remove the supernatant and suspend 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 3 times for 5 min in cold 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 into50 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.

Δ **CRITICAL STEP** This step and steps of the addition of antibodies must always be done slide by slide.

Δ **CRITICAL STEP** The slides must not dry up 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 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) Similarly, as in the case of the primary antibody, 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 2 times for 3min 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).

Δ **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 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 slides.

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**).

Box 9: 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 huge variety of probes and number of options: Repeats, fragments of chromosomes, whole-chromosomes, DNA fragments cloned in plasmids, 'padlock probes', peptide nucleic acid probes. They can be prepared in the lab: Fragments of DNA cloned in P1 artificial chromosomes (PACs) or 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 optimised/used with the comet assay.
- Hybridisation buffer: saline-sodium citrate (SCC) (20xSCC, Merck)
- Washing solutions and detection reagents are available as various types of 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

Stage 3: Comet formation

Neutralization & Washing

Extra steps to be added instead of or just after step 32.

Δ **CRITICAL** 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 70°C. Commercial probes should be denatured following manufacturer instructions.

1) Hybridisation start: apply the probes on the slides, seal and incubate over night at 37 °C.
2) Post-hybridisation wash: remove the seal and incubate the slides in a set of washing solutions: three times 5 min in 50% formamide/ 2xSSC at 42°C and twice in 2xSSC at 42°C for 10 min.
3) Signal detection: place the slides in a blocking solution (4xSSC, 0.05% Tween-20, 5% non-fat milk powder) for 10 min. Develop the signals with 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

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).
Visualize and record the signals using appropriate filters; overlay the recorded images.

Note: Comets (and thus signals) are organized in three-dimensional space. This allows investigating the real organization of chromatin in living cells.

Box 10: Yeast and filamentous fungi comet assay

Options A in the protocol steps can be applied to experiments with *Saccharomyces cerevisiae, S. paradoxus, S. kudriavzevii, S. bayanus, Candida albicans, Cryptococcus neoformans, Schizosaccharomyces pombe*. For *Ashbya gossypii* (filamentous fungus) follow options B.

Additional reagents

Extra specific chemicals needed:

- 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 (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, 50 mM EDTA and 10 mM Tris-HCl, pH 10

(A) For yeast:

- YPG medium (1 % w/v Yeast Extract, 2 % w/v Bacto-Peptone and 2% glycerol)
- 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 buffer (5 mM MOPS–NaOH, pH 7.2, containing 1.3 M sorbitol and 1 mM EDTA). All S buffers must be supplemented with 2 mg/mL zymolyase (20T; 20,000 U/g)

(B) For filamentous fungi *Ashbya*:

- 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

Stage 1: Preparation of cell suspension from fresh or frozen samples for yeast colonies

Modification of step 1.

1) Harvest approximately 10^6 yeast cells from exponentially-growing cultures by centrifugation (10 min at 5,000 × *g*, room temperature). Next, wash with ice-cold deionized water. After a second spin

resuspend yeast cells in 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 x *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 resting for the separation of the two phases. 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) 5×10^4 cells per 50 µL gel of 1.5% LMP agarose. The LMP concentration provides sharper comets for cells with low content and less condensed chromatin.

Modification of step 14.

1) Treat the cells for 2 h in the dark with cold lysis solution.

? TROUBLESHOOTING

Δ CRITICAL STEP *Schizosaccharomyces pombe* the following lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1%, Triton® X-100, 10% DMSO, pH 10) should be used and lysis overnight at 4 °C in the dark is recommended.

2) For *Ashbya gossypii*: Treat the cells for 2 h in the dark with cold lysis solution.

Δ CRITICAL Very good results are also obtained without washing of the slides between lysis and alkaline treatment.

Stage 3: Comet formation

Alkaline treatment & Electrophoresis

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 some protocols recommend also electrophoresis solutions 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.

Modification of steps 29-30. 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

Box 11: Plant comet assay

Δ **CRITICAL** All steps should be performed in the dark under inactinic lamp.

Additional reagents set up

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

Additional material

- Sharp razorblades

Procedure

Stage 1: Preparation of cell suspension from fresh or frozen sample - preparation of plant nuclei suspension

Δ **CRITICAL** Due to the absence of free cells in plants and the presence of a cell wall, which is a barrier to cell lysis, plant nuclei need to be extracted mechanically from roots or leaves [343]. It is important to determine the optimal extraction time leading to high nucleus extraction yield without causing DNA damage. This parameter should be adjusted according to plant species but also to specific organs (e.g., lignified tissues).

Modification of step 1.

Use a sharp razorblade for the mechanical extraction of nuclei from plant roots or leaves by chopping/slicing the plant material into cold plant extraction buffer on ice. Thus, nuclei are directly released into the extraction buffer.

Δ **CRITICAL STEP** Nuclei extraction needs to be performed in the dark under inactinic lamp

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. Δ **CRITICAL STEP** This will result in approx. 100 nuclei per gel, but it depends on the amount of extraction buffer used, the amount of plant material, and the duration and intensity of material chopping/slicing.

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

Δ **CRITICAL** A lysis step is not necessary as plant nuclei are directly extracted by mechanical extraction.

Stage 3: Comet formation

Alkaline treatment & Electrophoresis

Modification of step 29

Incubate in cold electrophoresis solution for 15 min at 4 °C in the dark.

Modification of step 30.

Electrophorese at ~0.7V/cm for 5 min at 4 °C in the dark (EPT=3.6).

Box 12: Sperm comet assay

Δ **CRITICAL** Sperm are different from somatic cells in the structure of chromatin. For human sperm, about 85% of the DNA is packed with protamines in a laminar structure; the remaining 15% contains histone. The protamines contain disulfide bonds and DTT is used to break such bonds. 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 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

Stage 1: Preparation of cell suspension from fresh or frozen samples - preparation of sperm cell suspension

Modification of step 1.

Perform seminal liquefaction to obtain a usable cell suspension

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

Modification of step 3.

Aliquots of ~25 µL can be flash frozen in liquid nitrogen and stored at -80°C until use.

Δ **CRITICAL STEP** 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.

Before processing for the comet assay, the tubes are thawed in a 37°C water bath for 10 sec and then immediately processed for the comet assay.

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

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

Modification of step 14.

The lysis protocols vary and may comprise 1-3 three steps.

Two examples: Protocol A:

(i) Standard lysis solution with 10mM DTT for 1 h at 4 °C;

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

Protocol B:

(i) Standard lysis solution overnight at 4 °C;
(ii) Standard lysis solution with 10 mM DTT for 1 h at 4°C followed by the addition of 4 mM (final concentration) lithium diiodosalicylate for 1.5 h at room temperature;
(iii) Standard lysis solution with 10 mM DTT for 1 h at 4°C followed by 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.

Incubation of 45-60min at 37 °C is advised.

∆ **CRITICAL** Positive controls are in general inefficient. E.g., compared to somatic cells, 20x higher doses of X-rays are needed to induce sizable levels of damage in sperm, and the expected number of induced breaks per base pair as calculated in somatic cells is not found. 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.

AUTHOR CONTRIBUTIONS STATEMENTS:

P.M., S.V., S.L., K.G., M.C., B.E., J.W., and S.S. designed/provided figures; P.M. provided anticipated results; A.C., G.G., P.M., S.V., S.L., and A.A. drafted the paper and revised the manuscript; all other co-authors contributed to the Methods and Procedure sections; A.L-C., E.B-R., F.S., M.C., S.C. and S.K. thoroughly reviewed the manuscript before submission; S.L. and A.A. managed the manuscript preparation; All authors read and approved the final manuscript.

ORCID FOR CORRESPONDING AUTHORS:

ORCID: 0000-0003-4857-9931

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The authors declare no competing interests.

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