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

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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 oxidized 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 Organisation for Economic Co-operation and Development. The present document includes a series of consensus protocols that describe the application of the comet assay to a wide variety of cell types, species and types of DNA damage, thereby demonstrating its versatility.

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. ALS are not well defined but, as the name suggests, are essentially any DNA modification that becomes an SB under alkaline conditions, e.g., apurinic/apyrimidinic (AP) sites. The principle of the comet assay relies on the spatial organization 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 organization means that, when the proteins are removed during

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the lysis step of the assay, the DNA remains in a compact supercoiled state. However, if a DNA SB is present, the supercoiling of the loops 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 a fluorescence microscope. The relative amount of total DNA in the tail reflects the frequency of breaks. The name 'comet assay' was introduced in 1990 (ref. [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 for studying different animal and plant species[3–5].

The first multilaboratory, 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 (ref. [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 (ref. [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 importance of an OECD guideline, some limitations remain. For instance, this guideline 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 that led the research community to collaborate and develop standardized procedures, to achieve congruent baseline levels of DNA damage and consistent reporting of procedures. These issues have been addressed through a number of multilaboratory 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.

A previous *Nature Protocols* article described the neutral comet assay and a specific alkaline version of the comet assay[24]. 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. Before describing the comet assay protocol, we provide details of appropriate methods for isolating cells from different specimens, as this is key to avoiding artifactual formation of DNA damage and hence to achieving maximal specificity of the assay.

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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, resulting in 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 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 sulfate 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 nuclear matrix is likely to occur, so that true double-stranded DNA fragments are released, migrating towards the anode. Protocols described in this article are restricted to the alkaline comet assay.

Recent advances in the comet assay have led to high-throughput versions of the assay, many of which utilize 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 (e.g., 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), catalyzing the excision of oxidized pyrimidines, or formamidopyrimidine-DNA glycosylase (Fpg), and human 8-oxoguanine DNA glycosylase 1 (hOGG1), catalyzing the excision of oxidized purines[32–34]. Apart from DNA nucleobase 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 samples, 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,

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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 analyzed immediately.

After isolation of the cells of interest, the comet assay protocol is divided into four main stages, as described below and shown in Fig. 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 (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 cells 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 in a suitable buffer. Spheroids, tissues, biopsies or whole organisms are homogenized before processing in the comet assay. The current protocol describes 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 (LMP) agarose at 37 °C, and placed on microscope slides, or plastic (GelBond) films, and the agarose is allowed to solidify on a cold plate, or in a fridge. (Normal agarose is not suitable, as the higher temperature required to maintain it in a liquid state would probably damage the cells' DNA.) The gelembedded 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 specific DNA lesions and lead to the creation of additional SBs. The cells are embedded as described in Stage 2A, but slides need to be prepared in duplicate: one slide to incubate with reaction buffer and one slide 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

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subsequent electro-phoresis 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 visualization and 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 and unstained slides indefinitely, while stained slides can be stored in dark conditions for a limited time depending on the dyes used. Comets are visualized by fluorescence microscopy, and analyzed using free, or commercially available, semi-automated or fully automated scoring software, or by visual scoring.

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 subsections.

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 ionizing radiation, and 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 considered to have a greater implication than SSBs. Nucleobase 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 advantageous 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 recognizes oxidized pyrimidines, was the first to be applied[47], followed by bacterial Fpg and human hOGG1 for oxidized 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. 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.

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Before 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, TI) 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 that have not been treated deliberately with alkylating agents.

Detection of DNA interstrand crosslinks

Certain types of DNA-damaging agents form covalent links between two nucleobases, either in the same DNA strand (intrastrand crosslinks), or in opposite DNA strands (interstrand crosslinks, ICLs)[60]. Chemotherapy is the main clinical source of ICL-inducing agents (e.g., cisplatin), but there are also environmental agents that cause ICLs, such as a high-lipid diet[61], alcohol, natural psoralens (e.g., derived from the diet[62]), estrogens[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 that form the comet tail (Fig. 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 notable 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 (Fig. 3). For a detailed protocol, see Supplementary Protocol 1.

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. These transient SSBs 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 (which

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blocks DNA precursor synthesis) and 1-β-D-arabinofuranosyl cytosine (araC, a cytosine structural analog 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. For a detailed protocol, see Supplementary Protocol 2.

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 hydroxyurea 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.

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 ~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 microarray 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 (Fig. 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 eight 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] (Fig. 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 (Fig. 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× less time than with glass slides[30]. However, the rate-limiting step is often the sample preparation before

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processing the gels. Apart from being cheaper, the use of GelBond film has two additional 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 to 15 μL; and the hydrophilic plastic material reduces the likelihood of the gels detaching. For a detailed protocol, see Supplementary Protocol 3.

Using the 96-well (or the related 48-well) format and an electronic eight-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 genotoxic chemicals, together with the enzyme-modified variant of the comet assay[30,75,80,81].

CometChip.—This is a high-throughput comet assay method that utilizes microfabrication techniques to pattern cells into an array (for a detailed protocol, see Supplementary Protocol 4)[82–84]. Cells are trapped for the duration of the assay within agarose microwells that are ~30–50 μm in diameter and spaced ~240 μm apart (Fig. 6). This results in a regularly spaced grid of comets arranged as in a 96-well plate format, allowing for dozens of samples to be analyzed 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 substantially reduces assay labor, 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[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 so far most experiments have been performed with cultured cells, it is also possible to use the CometChip to analyze 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 a report by Chao and Engelward[89].

High-throughput comet assay system.—Karbaschi and Cooke developed and patented a system whereby all the sample workup steps, electrophoresis and post-electrophoresis steps are performed with the comet slides held vertically, rather than horizontally, which is the convention[90] (Fig. 7). A detailed protocol is described in Supplementary Protocol 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 the risk of damage to/loss of gels and increasing throughput; the footprint of the tank is decreased substantially (allowing tanks to be ‘multiplexed’ from the same powerpack), and cooling is integrated in the system.

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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 *MspI* was exploited in a modification of the comet assay to measure global DNA methylation levels in individual cells (Supplementary Protocol 6)[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* digests nonmethylated 5′-CCGG-3′ sequences and is inactive when the second cytosine in the recognition sequence is methylated (5′-C^{m}CGG-3′). In contrast, *MspI* cuts nonmethylated 5′-CCGG-3′ and 5′-C^{m}CGG-3′ sequences, but not 5′-^{m}CCGG-3′. The global 5′-CCGG-3′methylation can be assessed by calculating the *HpaII*/*MspI* ratio (Fig. 8).

The newly developed modified comet assay, EpiComet-Chip (Fig. 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-$_{40–3000}$) Pu^{m}C…3’). As *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-Chip assay involves some modifications of the procedure steps, as described in Supplementary Protocol 7. It is worth mentioning that these methods have been applied by a few laboratories so far. Methylation is tissue- and cell-type-specific and the assay should be optimized for the cell/tissue of interest.

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, coated with LMP agarose between two layers of normal-melting-point (NMP) agarose, and then subjected to standard alkaline DNA electrophoresis (for detailed protocol, see Supplementary Protocol 8)[98]. The single chromosome comet assay is a useful approach for studying replication aberrations and replication stress as an alternative to traditional 2D gel analysis[99]. This method has been applied by a few laboratories so far.

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BrdU comet assay: measurement of cell-cycle-specific comet formation

Incorporation of the thymidine analog 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 immuno-fluorescent staining of incorporated BrdU, and the alkaline comet assay (for a detailed protocol, see Supplementary Protocol 9)[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 (undergoing DNA synthesis/replication), which contain a physiological level of DNA discontinuities or gaps (detected as DNA breaks in the comet assay), as a result of ongoing semiconservative replication. Since cells progressing through S phase form comet tails in the alkaline comet assay, this approach helps to distinguish replicating cells among the total population of cells forming comet tails (Fig. 9). Pulse labeling 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-labeled DNA in comet tail. This method has been applied by a few laboratories so far.

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 FISH, using labeled probes targeting particular DNA sequences, allows the study of DNA damage at a gene level (reviewed in Shaposhnikov et al.[38]). In Supplementary Protocol 10, 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 (Fig. 10), including various repetitive elements; chromosome arm- or band-specific probes; whole-chromosome probes; DNA fragments cloned in artificial chromosomes; ‘padlock probes’, which are able to ‘lock’ around the target DNA sequence to allow circularized 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 provided information about rates of DNA repair of different genes, in relation to nuclear structure[40,105,106].

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 (Fig. 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 a large number of animal species, the comet assay has also been performed on a variety of cell types and tissues, including white blood cells, bone marrow, liver, kidney, brain, bladder, lung, stomach, gill, hemolymph, digestive gland, embryo cells, ovary and 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 plants is increasing.

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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 beyond the scope of this paper, and so we provide only key publications, and recent modifications for each of the models and biomatrices.

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, in particular, hepatic cells, have been the most frequently used for genotoxicity testing with the comet assay[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 monocultures 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 monocultures. 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].

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]. Three-dimensional skin models have now reached an advanced state of validation following over 10 years of development, while liver- and lung-based models show promise but are under development[140]. The 3D skin comet assay is now undergoing independent peer review by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM), followed by the development of an OECD Test Guideline[141–145]. The use of liver spheroids with the comet assay is a novel approach[146,147], which has so far been used to assess the genotoxicity of nanoparticles and chemicals[148,149]. 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[140,150].

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

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compliant with the European Union Directive 2010/63/EU[151,152]. The embryos have many advantages; being sensitive to toxic stressors, 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., intercellular signaling, intact organism and functional homeostatic feedback mechanisms) and convenience of cell culture (e.g., small quantities of test item, cost and time efficient, and 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 research on embryos, they are still not frequently used with the comet assay. Most studies have been conducted with adult fish and during the embryo–larval stage. Only a small number of studies have been performed on embryos (Canedo and Rocha[132]; more information is in 'The use of non-mammalian samples' section).

Yeast and filamentous fungi.—The yeast comet assay has been in use for >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 the 'Technical modifications' section, a modified comet assay protocol has been developed to examine damage in single yeast chromosomes[97].

Plants

Application of the comet assay to 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 (ref. [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 and 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]).

Non-mammalian samples

This and the following section ('Non-human mammalian samples') 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.

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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 to perform DNA damage assessments with the comet assay[159–161]. In these species, DNA damage is measured in cells from the hemolymph, 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 (hemolymph, 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 analyze the influence of protein overexpression on genome integrity in vivo[172,173]. Hemocytes 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 hemocytes as a cell target for DNA damage detection started in 2011 (ref. [174]). Augustyniak et al.[175] published a review on the use of the comet assay in insects.

Mollusks.—Marine and freshwater bivalve mollusks 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 favor bioaccumulation of contaminants[176]. A variety of mollusk 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 mollusks was initially developed for hemolymph 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].

Planarians.—Planarians are free-living flatworms (Platyhelminthes) with 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

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of copper sulfate[185]. Since then, planarians have 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 noninvasive extrusion of coelomocytes from earthworms (*Eisenia foetida*) published by Eyambe et al.[190], there have been only a few modifications to the protocol for collecting cells from these worms. Verschaeve and Gilles[191] pioneered 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 (ref. [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 Gajski et al.[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].

Non-human mammalian samples

In vivo comet assay experiments with mammalian samples normally utilize laboratory animals such as mice and rats, which are generally regarded as the standard experimental animal models for genetic toxicology studies. 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[8,203–208].

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[209]. An OECD guideline (TG 489) 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[210,211]. Despite the extensive use of the

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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 (for more details, see 'Technical modifications'). In general, rodent tissues can be used for genotoxicity testing of chemicals present in consumer products, diets, and 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[212] and physical agents such as radiation[213].

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 and the majority used several breeds of both cats and dogs for the evaluation of different chemical and/or physical agents on the extent of DNA damage in blood and bone marrow cells as well as spermatozoa[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 before 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].

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]. The effects of nutritional and therapeutic interventions on DNA damage have also been studied[214–219]. In addition, DNA damage has been assessed in connection with aging and high-prevalence diseases[219,220]. The technique has also been applied to umbilical cord blood cells[221–223] and placenta[224–226]. 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 specimens 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[227]. 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[228]. 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 and nutrition, and so-called seasonal effects[20,27,33,216,229–237]. The comet assay has also been applied to assess DNA damage as a factor in diseases[238,239] and also as a tool in diagnostic and medical treatment procedures[19,240,241]. A recent pooled (meta)analysis of a database of comet assay results from almost 20,000 individuals

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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 and environmental exposure to a variety of genotoxic agents had very significant effects[242]. It is possible to use isolated polymorphonuclear (PMN) cells in the comet assay[243]. PMN cells such as neutrophils[244–247] and granulocytes[248,249] have been used to assess DNA damage 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.[228] and Marino et al.[250]); 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[251], 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[233,252].

Mononuclear cells (MNCs) can be isolated from cord blood, and used in the comet assay[253–255]. The comet assay has been applied to these cells to study DNA damage in preterm infants[253–255], and the correlation between maternal blood glucose levels of women with diabetes or mild gestational hyperglycemia and the DNA damage levels in the MNCs from the offspring[256].

Leukocytes from saliva.—Isolation of leukocytes from saliva (as an alternative to, or to complement, blood samples) represents a potential strategy for noninvasive, human biomonitoring studies using the comet assay[257–259]. These samples 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, patients with dementia, 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[260,261]. Buccal cells have been used since 1996, with at least 50 articles reporting their use[260,262,263]; 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[264–271] to assess the potential antioxidant effects of several compounds[272], and to assess oxidatively damaged DNA[273]. Concerning ocular cells, lens epithelial cells have been used to study age-related cataract[274], and tear duct and corneal cells have been used to test the effect of environmental pollutants, principally ozone[275].

Sperm.—The comet assay has been used extensively to study sperm in the context of the effects of environmental substances on fertility[276,277], with the diagnosis of male infertility[278], and in medically assisted human reproduction[279,280]. 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 to contribute significantly to a decrease in live births in assisted reproduction[281,282]. 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 oxidized DNA bases in sperm (for example, Simon et al.[283], and Sipinen et al.[277]), and a high-throughput method has been described for the sperm comet assay[284].

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Placenta.—Placental cells have been used for the evaluation of prenatal exposure-induced developmental toxicity[285]. In humans, the placenta is a useful biomatrix that is obtained noninvasively[286]. There are a few published studies analyzing DNA damage using the comet assay in cells isolated from human placentas, either for cell characterization[224] or for genotoxicity testing[225].

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 sublethal 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[287]. In the alkaline unwinding method[288], cells are lysed in alkali for a certain time and then neutralized 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 its simplicity, the number of samples that can be processed in a single experiment and the ability to visualize damage at the single-cell level.

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 oxidized 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 HPLC with electrochemical detection, gas chromatography–mass spectrometry, and HPLC with tandem mass spectrometry. By conducting controlled ring studies, an estimate of background levels of oxidatively damaged DNA in human lymphocytes was 4.2 8-oxoguanines per 10^6 guanines, obtained with chromatographic methods, compared with 0.3 8-oxoguanine per 10^6 guanines when employing Fpg[11]. Evidence[289,290] points to adventitious oxidation occurring during the relatively drastic sample workup for chromatographic analyses, compared with the mild procedures employed 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 with tandem mass spectrometry.

The comet assay for determining 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 produced results that were consistent with 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 radiolabeled [^{3}H]dCTP[93] or biotinylated dCTP[291]. More recently, the EpiComet-Chip was developed, involving the restriction enzyme *McrBC*. This EpiComet-Chip showed high validity compared with the MethylFlash Methylated DNA Quantification Assay (using capture and detection antibodies, followed by fluorometric

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quantification): single-sample hypermethylation (≥1.5-fold) was correctly identified at 87% (20/23) and hypomethylation (≥1.25-fold) at 100% (9/9), with a 4% (2/54) false negative rate and 10% (4/40) false positive rate[42].

DNA–DNA crosslinks have been measured by both the comet assay and alkaline elution, and both assays rely on the ability of crosslinks to retard the migration or elution of DNA; however, there are apparently no reports in the literature of a direct comparison of the two approaches, nor a comparison of either with an approach that can provide absolute quantification of crosslinks, such as mass spectrometry.

Limitations, and attempts to overcome them

Despite its many advantages, the comet assay has 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 DNA migration and differences in comet assay procedures[229,292].

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 (semi-)automated image analysis or visual assessment. In the case of image analysis, there is a choice of descriptors; tail length, TI (also referred to as percentage of DNA in tail) and tail moment (TM). They give rise to results expressed in different units, which cannot be easily compared[293,294]. 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[295]. TI is expressed as percentage 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%)[286]. 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 visualize the level of damage being described[294–300]. 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[287,288,301]. Such a transformation produces comet assay results that are much easier to understand than the primary comet assay descriptors[294]. 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.

Interlaboratory variation in the reported levels of DNA damage has been recognized as a limitation of the comet assay, dating back to the early 2000s[302]. It results from differences in technique between labs and variation in scoring[19]. Interlaboratory 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 variations in the technical procedures used in the laboratories involved[17].

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Attempts to standardize the comet assay protocol in validation trials have been partly successful in the sense that the interlaboratory variation is decreased by using standardized protocols[14]. The lab-to-lab variations in reported levels of DNA damage are 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[21,23,231].

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[303]. Patients with the most prevalent noncommunicable 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[220] There is evidence demonstrating that many genotoxic carcinogens cause DNA damage, measured by the comet assay, in animal organs and cell cultures[207,304]. Certainly, the comet assay is not expected to be a stand-alone test with the power to accurately predict individual risk of diseases 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 characterize carcinogens with different genotoxic mechanisms of action[305,306]. 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 of genome stability.

A potential limitation of the comet assay, particularly in biomonitoring studies, is the logistical difficulty of processing large numbers of samples and analyzing 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% fetal bovine serum (FBS) 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 analyzed with the comet assay upon thawing, even after storage for 5 years[228,239,251,307–311]. 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[312].

Experimental design

It is recommended that comet assay experiments 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 interassay variation. Studies where specimens are analyzed ad hoc should incorporate cryopreserved assay control samples in the experimental design; these control samples can be used to standardize the

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results, if needed, to adjust for the variations between experiments, over time or between laboratories[231].

Controls

If possible, comet assay experiments should have negative and positive controls. Negative controls are vehicle-exposed cells and animals, and human samples from placebo or unexposed groups. 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[313]. Potassium bromate has been tested in a multilaboratory 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[314].

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. This also applies to domestic and wild animals. 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.

Optimization

The relationship between the actual number of DNA SBs and a comet assay endpoint 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 of genotoxic agent that can be analyzed). In optimization, there is a tradeoff between detection of low levels of DNA SBs (i.e., the sensitivity of the assay) and width of the dynamic range. Conditions that favor 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

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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 cannot be scored with an image analysis system, but they may be scored visually. 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 the percentage of agarose

The optimal concentration of agarose ranges between 0.5% and 1.5% (wt/vol), with most laboratories using a final agarose concentration of ~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[313]. Thus, it is of paramount importance to titrate the enzyme and vary the incubation period before analysis of test samples. The titration experiment aims at detecting all lesions that are recognized by the enzyme while avoiding nonspecific 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)

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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 favor the formation of very long comets because this will result in 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 by 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[6,315,316]. Thus, care should be taken to avoid temperature differences in the electrophoresis tank because this can lead to 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[30,317,318]. 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 to perform an experiment with identical samples of cells at all positions in the electrophoresis tank to assess the spatial variation in DNA damage.

Materials

Biological materials

▲CRITICAL Table 1 summarizes the various experimental models, and sample types that can be used with the procedures described in this protocol. For a full list of animal species in which DNA damage has been evaluated by the comet assay, see the reviews by Gajski et al.[4] for invertebrates and Gajski et al.[5] for vertebrates.

2D cell culture

The most commonly used suspension cells are leukemia cells (e.g., TK6 and THP-1 cells), while hepatic HepG2 or cervical HeLa cancer cell lines are the most commonly used cells grown in a monolayer. 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 on the DNA damage response[319].

3D cell models

- Human reconstructed full-thickness (FT) skin tissues: e.g., Phenion FT skin (www.phenion.com) or EpiDerm FT skin tissue (www.mattek.com). A video showing how to perform the comet assay using the Phenion FT skin model can be found here: https://www.phenion.com/information-center
- Human reconstructed 3D airway models: MucilAir produced by Epithelix Sàrl (https://www.epithelix.com/products/mucilair) and EpiAirway produced by MatTek Corporation (https://www.mattek.com/products/epiairway/), or

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investigator-established air–liquid interface airway epithelial cell cultures sources[320]

- Liver minitissue models: hepatocellular carcinoma cells such as HepaRG, HepG2, Huh6 or C3A can be used to obtain 3D spheroids[146–149]

Zebrafish embryos

Embryos should be collected after spawning, and only freshly fertilized eggs (2 hpf) should be used for the experiments with a duration of exposure up to 96 hpf (refs. [153,321]). It is also possible to freeze (at −80 °C) up to 2 weeks freshly harvested cells isolated from embryos in physiological buffer containing 10% (vol/vol) DMSO, without a significant increase of DNA damage[322].

Yeast and fungi

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

Plants

Collect (preferably fresh) roots and leaves from plants to get the best results with low background DNA damage. Previously published studies reported the use of snap-frozen leaves[323,324], but this remains to be optimized and validated with lab-to-lab comparisons.

Invertebrate samples

- Collected hemolymph cells, coelomocytes, neuroblasts and cells from other tissues can be used depending on the species (Table 1). Heparinized hemolymph is normally used
- The most frequently used organs from mollusks are digestive glands, and gills
- For very small animals, such as some crustaceans and insects, whole body squashing can be performed to yield a generalized population of cells

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). Tumor samples can also be used. Whole blood is collected with an anticoagulant such as citrate, EDTA or heparin
- Rodents should be anesthetized and exanguinated before obtaining 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

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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. Snapfrozen 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 (for examples of studies, see Azqueta et al.[312]). In fact, the OECD test guideline 489 recognizes 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.[312] have described a protocol to freeze and thaw rodent liver, kidney and lung tissues before performing the standard and the Fpg-modified comet assay. The protocol is based on the study of Jackson et al.[325]. Freezing the whole tissue may not be convenient for some tissues such as the glandular stomach as scraped epithelial cells from this tissue 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[326]; however, further investigation is required to confirm and/or ameliorate this effect. The use of snapfrozen 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. ▲CRITICAL During any painful or stressful procedure, anesthetization is recommended by ethical principles and regulation. However, the impact of chemical anesthetics on the DNA integrity should be considered as some studies have shown the time-dependent induction of SBs in some tissues[327].

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 between 20 G (0.9 mm diameter) and 22 G (0.7 mm diameter) needles.
- MNCs: MNCs 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 from 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

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(https://youtu.be/tgNHWVqF52I) or polygelin solution[243] ('Procedure': Stage 1, Step 1A)

- Blood mononuclear cells (BMCs) from saliva: collect saliva samples by performing four consecutive mouth rinses with 10 mL of 0.9% (wt/vol) NaCl sterile solution for 1 min each. Combine the rinses 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. Centrifuge the oral rinses (15 min, 1,100*g*, at 4 °C), wash the cell pellet with cold PBS and resuspend in RPMI 1640 cell culture medium. Leukocytes are isolated from the cell suspension by standard density gradient centrifugation[328,329]
- Buccal cells: before sampling, the subject should perform two consecutive rinses with water (room temperature (RT), ~22 °C). The sample is collected with a cytobrush or toothbrush ▲CRITICAL The initial collection/scraping of both cheeks (using separate brushes) is discarded. The superficial layer of the buccal mucosa is mainly composed of 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[260,262].
- 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[330]
- Lachrymal cells: in parallel to collecting nasal cells, tears containing lachrymal duct and corneal cells can also be collected[275]. 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 RT before performing the comet procedure. The capillary should be placed in a microcentrifuge tube to subsequently elute the tears using a rubber bulb
- Semen samples are obtained after 3 d 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 1 h of collection
- Placental tissue: collect a tissue section (5 × 5 × 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[331], colon[104] and testis[332] ! CAUTION All experiments involving human

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tissues must be approved by the relevant institutional ethical committee and adhere to local and national regulations, including the requirement for subjects to give written consent.

Reagents

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

General reagents

- Agarose, NMP (Merck KGaA, cat. no. A4718)
- Agarose, LMP (Merck KGaA, cat. no. A9414)
- PBS without Ca^{2+} and Mg^{2+} (Merck KGaA, cat. no. P4417)
- Triton X-100 (Merck KGaA, cat. no. X100)
- DMSO (Merck KGaA, cat. no. 41639) ! CAUTION DMSO readily penetrates the 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.$2H_2O$; 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 KGaA, 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 KGaA, cat. no. 1090571003)

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- Trypan blue (Merck KGaA, cat. no. T8154) ! CAUTION HCl is a strong acid, so wear protective gloves.

Cell lines and 3D models

- Cell culture medium. Medium may be specific for each cell type, or 3D tissue model, and should be chosen and supplemented according to the advice given by the manufacturer, or literature recommendations
- FBS (Merck KGaA, cat. no. F2442)
- Trypsin–EDTA 0.05% (Gibco, Thermo Fisher Scientific, cat. no. 25300062)
- Trypsin–EDTA 0.25% (Gibco, Thermo Fisher Scientific, cat. no. 11560626)
- TrypLE without phenol red (Gibco, Thermo Fisher 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)
- Saline solution (B. Braun, cat. no. 10258674)
- Thermolysin (Merck KGaA, cat. no. T7902)

Zebrafish embryos

- Pronase E (Merck KGaA, cat. no. 1.07433)
- Leibowith L-15 medium (ATCC, cat. no. 30–2008)

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 (Thermo Fisher Scientific, cat. no. G/0450/53)

Drosophila

- Collagenase type IV (Merck KGaA cat. no. C4-BIOC)
- *N*-Phenyltiourea (Merck KGaA cat. no. P7629)

Annelids

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

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Mollusks

- 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)
- Dispase II (Merck KGaA cat. no. D4693)

Fish

- Ethyl meta-aminobenzoate or methanesulfonate salt (MS-222; 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)
- Proteinase K (Merck KGaA, cat. no. 70663)

Enzymes for enzyme-modified comet assay

▲CRITICAL Some enzymes can be produced ‘in-house’ as crude extracts from *E. coli* transformed with the corresponding expression vector.

- *Escherichia 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 1 (hOGG1) catalyzes the removal of 8-oxoguanine and formamidopyrimidine moieties in double-stranded DNA, followed by cleavage of the resulting AP site ▲CRITICAL Previously, hOGG1 from Trevigen (cat. no. 4130-100-EB) and New England Biolabs (cat. no. M0241) was used in the comet assay; however, it was recently discontinued. Alternative suppliers could be Prospec (cat. no. ENZ-253) or Abbexa (cat. no. abx073274), but these sources of hOGG1 still need to be tested for their enzyme activity in the comet assay.

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- T4 endonuclease V (T4endoV) detects *cis*-syn cyclobutane pyrimidine dimers, including T<>T, T<>C and C<>C, (New England BioLabs, cat. no. M0308S)
- 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)

Reagents for comet visualization

▲CRITICAL Several fluorescent DNA dyes are suitable; the most commonly used are listed below. Other newly developed 'safer-to-use' dyes can be used as well. ! CAUTION These dyes are known or potential mutagens; wear protective gloves, and dispose of waste in proper containers.

- SYBR Gold (Thermo Fisher Scientific, cat. no. S11494) ! CAUTION Potential mutagen.
- SYBR Green (Thermo Fisher Scientific, cat. no. S7567) ! CAUTION Potential mutagen.
- Ethidium bromide (EtBr; Thermo Fisher Scientific, cat. no. 17898) ! CAUTION Mutagenic.
- DAPI (Thermo Fisher Scientific, cat. no. D1306) ! CAUTION Mutagenic.
- GelRed (Biotium cat. no. 41003; Merck KGaA, cat. no. SCT123) is an ultrasensitive, very stable replacement for EtBr DNA/RNA gel stain, safe for humans and the environment, shown to be nonmutagenic and noncytotoxic

Equipment

▲CRITICAL 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 should work with standard laboratory equipment of any brand.

General laboratory equipment and consumables

- Microwave oven
- Freezers
- Refrigerator
- pH meter
- Cooled centrifuge
- Automatic cell counter
- Plastic tubes, well plates, Petri dishes, etc.
- Vortex mixer

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- Plastic tips
- Pipettors
- Plastic Pasteur pipettes
- Micropipettes
- Hemocytometer
- Mr. Frosty container (or cell freezing container)

Equipment and consumables for cell culture

- Cell culture laminar flow cabinet
- Cell culture incubator with CO_2
- Cell counter
- Culture flasks and dishes
- Visible light inverted microscope
- 12- or 24-well Transwell

Equipment and consumables for other sources of cells

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

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) ▲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. The reader should note that, each time the protocol refers to slides, it also applies to GelBond film

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- 20 × 20 mm, 21 × 26 mm or 22 × 22 mm glass coverslips to form gels
- 24 × 60 mm glass coverslips
- Water bath or thermoblock
- Staining (Coplin) jars, for cell lysis and slide washing
- For 3D skin model: 40 μm cell strainers (Corning, cat. no. 352340)
- Metal trays or plates, to keep slides cold and prevent enzyme reactions 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 tank (for horizontal slide electrophoresis)
- Power supply. It is advised to use one that can reach 1–2 A at 20–50 V, i.e., at a voltage that is sufficient to give 1 V/cm on the platform of an electrophoresis tank. The amperage increases with the width of the tank and the depth of the electrophoresis solution over the platform; the latter should always be more than a few millimeters. Consort (BE) is an example of a suitable brand (cat. nos. EV2000 and 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 or dedicated fridge, or even put on ice (Fig. 7).
- Optional: slide warmer/incubator for drying slides
- Epifluorescence microscope and appropriate filter blocks optimized for the fluorochrome, charge-coupled device camera (8-bit black-and-white camera is adequate); high sensitivity and high pixel density are preferred

Software

- For scoring comets, using commercially available software for image analysis is recommended, as it 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) and KOMET 6 (Andor)

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- Several free scoring programs are available, such as Casplab (https://casplab.com) or CometScore (http://rexhoover.com/index.php?id=cometscore), among others

Reagent setup

General solutions

1% (wt/vol) NMP agarose in distilled water (for precoating slides).: Microwave to dissolve the agarose and cool to ~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% (wt/vol) 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 that can be stored at 4 °C and are stable for at least 6 months. 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).▲CRITICAL It is best not to reheat LMP agarose aliquots (as evaporation can cause a considerable increase in concentration). ▲CRITICAL A lower percentage of LMP agarose can be used to increase sensitivity. The final agarose concentration, after mixing with the cells, is normally 0.7–0.8% (wt/vol). 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. ▲CRITICAL At higher relative humidity (>60%), the LMP agarose solution may absorb atmospheric moisture over time, reducing the LMP concentration and leading to variable DNA migration. At lower relative humidity (<30%) the LMP agarose solution might lose atmospheric moisture, increasing the LMP agarose concentration and thus decreasing DNA migration.

Lysis solution—2.5 M NaCl, 0.1 M Na_2EDTA and 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. ▲CRITICAL Lysis solution can be freshly supplemented with 10% (vol/vol) DMSO and/or 1% (wt/vol) *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% (wt/vol) *N*-lauroylsarcosine is optional but considered redundant for most purposes, except for the use of buccal cells.

Electrophoresis solution—0.3 M NaOH and 1 mM Na_2EDTA. Store at 4 °C for up to 1 week. Another option is to prepare concentrated stock solutions and mix them on the day.

Neutralizing solutions—PBS (Store at 4 °C, or according to manufacturer's instructions); or Tris–HCl: 0.4 M Tris (Trizma base) in 1 L of distilled H_2O (adjust pH to 7.5 using HCl). ▲CRITICAL For the neutralization step, both PBS and Tris–HCl work

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equally well. If using PBS, perform a single wash for 10 min; if using Tris–HCl, perform 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 RT. Stable for at least 6 months. Alternatively, it is possible to use TBE or TAE buffer as recommended by the manufacturer of the staining dye.

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 Na2EDTA, 0.2 mg/mL BSA, 0.1 M KCl, pH 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. 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 and 2% (vol/vol) 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. The diluted buffer N could be stored at 4 °C for usage in a second assay within the same week. ▲CRITICAL 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 the manufacturer's instructions, and titrate them to optimize the enzyme concentration and incubation time before use. For guidelines for your own titrations, see Table 2. 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 of 30–60 min are used. Buffer B and buffer N work with the corresponding enzymes (see the preparation of buffers, above), although other buffers suggested by the manufacturers can also be used.

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 nonessential amino acids. Check with the cell line provider the medium needed to grow the cells, or the 3D tissues.

Cell freezing medium.: DMEM, 10% (vol/vol) FBS and 10% (vol/vol) 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.

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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 dissociation of epidermis and dermis.

For cell dissociation.: Mincing buffer (20 mM EDTA in HBSS without Ca^{2+}/Mg^{2+}, 10% (vol/vol) DMSO added freshly, pH 7.0–7.5). Freezing of the skin models or isolated cells thereof has not yet been attempted.

Planarians

10× CMF (Ca^{2+}/Mg^{2+}-free buffer).: 25.6 mM $NaH_2PO_4.H_2O$, 142.8 mM NaCl, 102.1 mM KCl and 94.2 mM $NaHCO_3$ in distilled water (pH 7). Store at 4 °C.

CMFH: 0.1% BSA (wt/vol), 0.5% glucose (wt/vol) and 15 mM HEPES in 1× 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 for at least 3 months (avoid multiple freeze–thaw cycles).

2% (wt/vol) 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 and 2 mM $CaCl_2$. Adjust the pH to 6.5 with NaOH, and sterilize by autoclaving. Stable for at least 3 months, at 4 °C.

Annelids

Extrusion buffer.: 5% (vol/vol) 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 and 11.5 mM EDTA. Store at RT. Stable for at least 1 month.

Ca^{2+}/Mg^{2+}-free saline solution (CMFS).: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl and 5 mM EDTA. Store at RT. Stable for at least 1 month.

Kenny's salt solution (KSS).: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4 and 2 mM $NaHCO_3$. Store at RT. Stable for at least 1 month.

Rodent tissues

Mincing solution.: HBSS and 20 mM Na_2EDTA, pH 7.5 (adjusted with NaOH). Add 10% (vol/vol) DMSO just before using.

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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 1 month. Stored at 4 °C.

Human samples—*For blood.*

(1) Erythrocyte lysis buffer (8.29 g NH_4Cl (155 mM), 1.0 g $KHCO_3$ (10 mM) and 0.372 g EDTA (1.0 mM), dissolved in 1,000 mL H_2O; pH 7.4, sterile filtered. (2) Freezing medium for blood cells typically contains cell medium, DMSO and FBS, but there is no consensus on the proportion of each ingredient, except that DMSO should be 10% of the final volume.

For saliva.: (1) For sample collection (mouth rinses): dissolve NaCl (0.9% (wt/vol)) in distilled water, and sterilize the solution; (2) for freezing samples: resuspend cells in freezing medium containing FBS (50% (vol/vol)), RPMI 1640 (40% (vol/vol)) and DMSO (10% (vol/vol)) at a concentration of 2.5×10^6 cells/mL (prepare the freezing medium fresh on the day of use in 0.5 mL aliquots by mixing 250 μL of FBS, with 200 μL RPMI 1640 and 50 μL DMSO). Store 0.5 mL aliquots of cells + medium at −80 °C for up to 5 months.

Buccal cell buffer.: 0.1 M Tris–HCl, 0.1 M Na_4EDTA and 0.02 M NaCl; pH 7.0 (by adding HCl). Autoclave at 121 °C for 15 min. When cold, store the buffer at 4 °C.

Buccal lysis solution 1.: 2.5 M NaCl, 100 mM EDTA tetrasodium, 10 mM Tris–HCl and 1% (wt/vol) *N*-lauroylsarcosine sodium salt. Then adjust pH to 10 using NaOH. Before use, add 1% (vol/vol) Triton X-100 and 10% (vol/vol) DMSO.

Buccal lysis solution 2.: 2.5 M NaCl, 100 mM EDTA tetrasodium, 10 mM Tris–HCl and 1% (wt/vol) *N*-lauroylsarcosine sodium salt. Add 1% (vol/vol) Triton X-100 and 10% (vol/vol) DMSO just before use. Then adjust pH to 7 using HCl, which is optimal for proteinase K activity, and warm to 37 °C.

Mincing solution for placenta tissue.: PBS without Ca^{2+} and Mg^{2+}, 20 mM Na_2EDTA. Store at 4 °C; stable for at least 2 months.

Equipment setup

▲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.

Precoating microscope slides—▲CRITICAL When using GelBond films, precoating 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 (Supplementary Protocol 3). ▲CRITICAL Various methods exist to coat slides, of which the most common one (and its variations) 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].

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

2. Dip the slides into the agarose gel briefly and 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. ▲CRITICAL In both cases, make sure to cover in agarose ~4 mm of the frosted part of the slide.

3. Put the slide flat on a heating plate/slide warmer/incubator (~40–50 °C) until dried, or overnight on the bench. Remember to mark the frosted part to indicate which side of the slide is coated. ▲CRITICAL 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 RT (after removing coverslips if used). They can be kept for at least 12 months.

   ? TROUBLESHOOTING

Electrophoresis setup—▲CRITICAL As the duration of electrophoresis (Stage 3, Step 30), 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 liquid covering the gels.

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 can be obtained by dividing the applied electrode voltage by the distance between the electrodes, but it is more accurate to use a voltmeter. ▲CRITICAL Ensure that the power supply can provide the output current at a constant voltage and that the tank is filled with a sufficient volume of liquid (a power supply that reaches 1–2 A should suffice for most tanks, but higher currents may be needed for larger tanks). The samples should be covered with at least 5 mm of liquid. The depth above the samples should not be made too shallow in order to enable the use of 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. ▲CRITICAL The electrophoresis conditions can differ

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depending on the biological samples used; exceptions are mentioned in the Procedure and Supplementary Protocols. Other electrophoresis conditions can also work. ▲CRITICAL The same electrophoresis conditions should be used for all experiments within the same study. ▲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 Supplementary Protocols. As an example, if a tank has a platform that is 25 cm wide and the voltage over the platform is 16.5 V as measured by a voltmeter, the electrophoresis needs to run for 30 min to reach an EPT of 20 (0.66 V/cm × 30 min = 20).

Procedure

▲CRITICAL If the comet assay for genotoxicity testing is used, the treatment of the cells/3D models/ animals should be performed before the collection of the samples for the comet analysis. The same applies when the comet assay is used in human biomonitoring after, for example, a nutritional intervention study. However, lymphocytes (or other cells) from animals or humans can be treated in vitro; in that case, they should be isolated in advance and processed as cells in suspension.

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

▲CRITICAL Stage 1 can be performed on the day of the comet assay (i.e., Stages 2A, 2B and 3). In this case, we advise to prepare the materials described in Steps 4–8 before starting. 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’).

▲CRITICAL In all cell handling: never vortex cells, avoid rapid pipetting (especially through narrow-bore tips) and keep cells on ice after harvesting. Minimize as much as possible the time from harvesting of the samples until lysis. In all cases, information about cell viability can be obtained using the Trypan blue stain.

▲CRITICAL Stages 1–4 are identical for all specimens, except for yeast and filamentous fungi, plant and sperm cells, which require modified protocols as specified in Supplementary Protocols 11–13, respectively.

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

1. I Prepare a cell pellet when possible. In some cases, the sample obtained is 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), whole invertebrate organisms or tissues (options E–K), vertebrate tissues (options L–N) or human

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tissue samples (options O and P), a mechanical and/or enzymatic processing in specific buffers is required, and a cell pellet is not always obtained. Proceed immediately to Step 2 after preparing the cells.

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

i. Collect the required number of cells:

- Grow the desired cell line in suspension according to the provider's instructions. Collect an aliquot from the cell suspension
- MNCs are routinely isolated from venous blood[228] or saliva[329] using a standard density gradient centrifugation method
- 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, ~2.5 × 10^7 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 ~45 min at RT, to separate the red cells in the lower layer and PMN cells in the upper layer (containing mainly neutrophils)[243]

ii. Count the number of cells in the cell suspension using a hemocytometer or an automatic cell counter.

iii. Centrifuge cells at ~150–300*g* for 5 min at 4 °C.

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

▲CRITICAL STEP Whole blood or buffy coat can be mixed directly with LMP agarose (Stage 2A).

B. Preparation of cells from adherent cell (co-)cultures or 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 according to previously published protocols[146,147,149] and use spheroids at specific age (depending on cell line and application).

▲CRITICAL STEP The spheroids grown in static conditions can develop a necrotic core after 10 d.

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

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- For adherent (co-)cultures: trypsinize according to standard procedures using 0.25% trypsin–EDTA.
- For spheroids obtained with HepaRG: pool 11 spheroids in a 1.5 mL microtube, and dissociate by adding 200 μL of TrypLE for 40 min at 37 °C.
- For liver spheroids obtained from nonquiescent cells such as HepG2, Huh6, etc., add 50 μL 0.25% trypsin–EDTA, or TrypLE, and incubate for 10 min at 37 °C.

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

iii. Neutralize trypsin with cell culture medium containing 10% FBS.

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

C. Preparation of cells from 3D airway models

i. Culture the MucilAir models on 12- or 24-well Transwell culture supports at the air–liquid interface.

ii. Following exposure, wash the airway model with 800 μL saline (add 600 μL to each well and 200 μL on the insert (24-well plate)) and incubate for 2 min at RT.

iii. Transfer the inserts to a new 24-well culture plate filled with 600 μL 0.05% trypsin–EDTA per well, and add another 200 μL 0.05% trypsin–EDTA to each insert.

iv. Following a 10 min 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*g*, RT).

D. Preparation of cells 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 on 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.

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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*, 4 °C).

E. Preparation of zebrafish embryos

i. *Whole body squashing (for embryos at the age of maximum 48 hpf)*: only freshly fertilized eggs (2 hpf) should be used for the experiments. After the treatment with genotoxic agent, submerge the embryo in a minimal volume of fresh medium supplemented with pronase E (2 g/L) for 4 min to soften the chorion. Then rinse the embryos with fresh medium (without pronase E). Place the embryos directly in a drop of LMP agarose, cover with a coverslip and gently squash to obtain single cells. The cells will spread all over the microscope slide, remaining embedded in the agarose. Optionally, another layer of 1% LMP agarose (80 μL) can be added on top of the squashed embryo.

▲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 dissociate the embryos into single cells (usually pool of eight to ten, depending on required single-cell yield) in 2 mL cold PBS using a tissue grinder (glass–glass homogenizer), or scissors followed by gentle pipetting. Filter the cell suspension through a gauze/mesh with 70 μm pores, and then centrifuge the suspension (10 min, 200*g*, 4 °C). Resuspend the pellet with cold PBS, and repeat centrifugation (7 min, 180*g*, 4 °C). Finally, resuspend the pellet in ice-cold PBS (or Leibowith L-15 medium). Before proceeding to Stage 2A or 2B, assess viability using a Trypan blue dye assay or similar.

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

i. After exposure to the test compound, transfer the organisms to 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, 2,292*g*, 4 °C).

G. Preparation of cells from invertebrates: planarians

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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 with gentle shaking. You can pool multiple worms per biological sample to increase yield.

ii. Transfer worm(s) to a Petri dish with CMFH to rinse.

iii. Transfer worm(s) to a glass slide; remove as much CMFH as possible, and cut worm(s) 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 thermoblock).

vi. Add 700 μL CMFH, vigorously pipette up and down repeatedly to further macerate the fragments and filter into a plastic centrifuge tube using a 35 μm strainer. Keep samples on ice.

vii. Centrifuge (5 min, 350*g*, 4 °C); discard the supernatant, and resuspend the pellet in 4 mL CMFH. Keep sample on ice.

viii. Optional: perform an additional filtration with a cell strainer with smaller mesh size. Mesh size can be adjusted on the basis of the cell types under investigation.

ix. Centrifuge (5 min, 350*g*, 4 °C); 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 at 350*g* at 4 °C.

H. Preparation of cells from invertebrates: Drosophila

i. Collect the tissue of interest (e.g., brain ganglia, anterior region of the midgut, or hemocytes) and pool from 5–50 larvae.

ii. Transfer solid tissues to washing solution (Ringer's solution or PBS containing phenylthiourea may be used): 100 μL per tissue from five larvae. Hemocytes are mixed with PBS plus 0.07% phenylthiourea.

iii. Treat solid tissues with collagenase for 15 min at 24 ± 1 °C or disaggregate them physically by breaking/tearing/shredding them with tungsten wires, and pass the tissues through nylon mesh to prepare a single-cell suspension.

iv. Centrifuge for 20 min at 300*g* at 4 °C.

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I. Preparation of cells from *Chironomus riparius* larvae

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

▲CRITICAL STEP Keep the sample on the strainer immersed in cold PBS until Step 1(I)(iv) to avoid DNA damage caused by oxidation.

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

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

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

v. Centrifuge cells at ~150–300*g* for 5 min at 4 °C.

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 paper moistened 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 for 3 min at RT.

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

▲CRITICAL STEP An alternative method to extract coelomocytes involves stimulating worms 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: mollusks (Bivalvia)

i. Hemolymph cells:

- Make an incision in the mollusk shell, and withdraw ~1.5 mL hemolymph from the posterior adductor muscle with a sterilized hypodermic

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syringe containing precooled modified Alseve's anticoagulant solution (1:5 (vol/vol), hemolymph: Alsever)

- Keep the samples on ice until centrifugation for 5 min at 250*g* at 4 °C.

ii. Solid tissue (gills and digestive glands):

- Solid tissue (gills and digestive glands):
- Dissect and slice the tissue into small pieces using dissection scissors and tweezers
- Place excised tissues in tubes containing 3 mL of CMFS, and incubate for 1 h at RT with gentle, horizontal shaking
- Place the tubes in a vertical position for 5 min to allow the fragments of tissue to settle
- Collect the supernatant containing the suspended cells with a pipette, transfer to another clean tube and centrifuge for 5 min at 500*g* at 4°C
- Remove the supernatant, and wash cells twice in 1.5 mL KSS with centrifugations of 3 min at 1,000*g* at 4 °C
- 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, shaking every 10 min. After digestion, spin samples for 5 min at 160*g* at RT. Collect the supernatant containing the cells in suspension, and centrifuge again for 2 min at 775*g* at RT

L. Preparation of cells from vertebrates: amphibians

i. Blood cells from tadpoles:

- Section tadpoles in the ventral position at the level of the operculum
- Obtain blood samples by soaking the tadpole and dripping blood into PBS, followed by centrifugation for 9 min at 160*g* at RT. Up to 5 μL of blood can be obtained from a single tadpole

ii. Blood cells from fully developed specimens:

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

M. Preparation of cells from vertebrates: fish

i. Blood cells:

- Collect blood using a method such as caudal puncture, which is easily applicable to specimens weighing >200 g
- Alternatively, adopt more invasive methods such as caudal peduncle transection (e.g., *Danio 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

  ▲CRITICAL STEP When <2 μL of blood is available, to avoid obtaining an insufficient cell number in the cell suspension, mix the sampled blood with <1 mL of ice-cold PBS (defined on a case-by-case basis).

ii. Organs (liver, gills and gonads):

- Collect organs (ensuring proper exsanguination of the fish), and place (and rinse) them immediately in ice-cold PBS, to remove blood cells
- Obtain a cell suspension by briefly homogenizing/mincing in PBS a small portion of the tissue into small pieces, using scissors, tweezers or a scalpel. This can be followed by a soft mechanical dissociation (pipetting up and down) to further promote cell dissociation
- Additional 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 (5–10 min, 200*g*, 4

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°C), discard the supernatant and resuspend the pellet in 1 mL of ice-cold PBS. Repeat the centrifugation/washing step (usually twice)

N. Preparation of cells from vertebrates: rodents

i. From fresh tissue:

- Rinse the tissue using cold PBS (Ca^{2+} and Mg^{2+} free, 20 mM EDTA), mincing buffer or Merchant's buffer. The buffer should be ice-cold (4 °C) to avoid the risk of artifactual generation of DNA damage
- Add 200 μL of the preferred cold buffer (i.e., PBS, mincing buffer or Merchant's buffer) to ~5 mg wet tissue (~15 mm^3). Recommendations about the size of the different organs can be seen in Table 3
- Use one of the following methods to obtain a cloudy suspension: (1) mince the tissue using scissors or surgical blade, (2) aspirate tissue in a 1 mL syringe (13 × 0.45 mm, without a needle) and move the suspension back and forth five to ten times, or (3) filter the suspension through a cylindrical stainless-steel metal sieve (NorGenoTech) using a plastic plunger from a 1 mL syringe
- Collect cell suspension after large tissue debris have settled (5 min) or filter the suspension through a 100 μm nylon mesh

ii. From frozen tissue:

- Place the cryotube containing the sample on dry ice
- Add a drop of Merchant's buffer or mincing buffer on top of the sample to create a protective ice cap
- Transfer the deep-frozen tissue, using tweezers chilled on dry ice, into a cylindrical stainless-steel metal sieve (NorGenoTech) previously immersed in ice-cold Merchant's buffer or mincing buffer
- 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)
- Collect the homogenized samples in 3 mL Merchant's buffer or mincing buffer (kept on ice)
- Alternatively, frozen tissues can be pulverized by a single sharp impact with a dry ice-cooled hammer

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after placing the tissue in a dry, ice-chilled metal pulverizer. The powder is then resuspended in 3 mL Merchant's buffer or mincing buffer (kept on ice)

▲CRITICAL STEP To prepare the cell suspension from frozen tissues, the sample should still be frozen when starting the homogenization.

O. Human samples: preparation of cells from placenta

i. Wash the fresh placenta piece using cold PBS (Ca^{2+} and Mg^{2+} free, 20 mM Na_2EDTA).

ii. Add 5 mL of cold (4 °C) 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 (15 min, 350*g*, 4 °C).

P. Human samples: preparation of cells from epithelial cells (buccal, nasal and tears)

▲CRITICAL Tears can be mixed directly with LMP agarose.

i. Collect cells with a spatula or cyto/toothbrush as described in '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.

▲CRITICAL STEP PBS can be used if you are going to process cells immediately, while buccal cell solution should be used in case cells need to be stored or transported (as might happen during human biomonitoring).

iii. Centrifuge for 5–10 min at 250*g* at 4 °C.

2. To use the cells directly for embedding in LMP agarose, remove supernatant and go to Stage 2A (Step 10).

3. Optional) If desired, freeze cell suspensions for later use.

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

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

i. Resuspend the cell pellet in cold freezing medium at ~1×10^6 cells/mL.

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▲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 ~500,000 cells) in 1.5 mL microtubes. Each aliquot will have enough cells for 20 gels in 2 gels/slide format (Stage 2A). 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 (Supplementary Protocols 3 and 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 for 1 min at 1,000*g* at RT, and remove the excess plasma.

ii. Add 100 μL ice-cold (4 °C) 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[228,251].

D. Freezing harvested cells from zebrafish embryo

i. After the treatment (48 hpf), place the embryos ($n = 4$) in 200 μL of 10% (vol/vol) DMSO in PBS (pH 7.4) and gently mince with scissors and gentle pipetting.

ii. Centrifuge the suspension (2 min, 250*g*, 4°C).

iii. Collect the supernatant in 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.

■ 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

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study, check Azqueta et al.[312]). When ready to thaw cells, prepare the materials as explained in Steps 4–8, and follow instructions in Step 9 to embed the cells in LMP agarose.

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).

▲CRITICAL STEP LMP agarose should be mixed with cells at physiological temperature (i.e., ~37 °C) to prevent the artifactual generation of DNA damage.

5. Precool the centrifuge to 4 °C.

6. Prepare standard lysis solution according to option A, or option B for fish samples (blood, liver and gills) and 3D skin models, or option C for human buccal cells (100 mL lysis solution are needed for a Coplin jar that can hold 16 slides):

A. Standard lysis solution:

i. To 99 mL of lysis stock solution (4 °C) add 1 mL of Triton X-100, and mix, put into a Coplin jar and store at 4 °C until use.

B. Lysis solution for fish samples and 3D skin models:

i. 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 before transferring the slides, add proteinase K to a final concentration of 30 μg/mL.

▲CRITICAL STEP When working with whole blood, buffy coat, tissues or similar samples that may still contain hemoglobin, add 10% DMSO to the lysis solution to prevent artifactual DNA damage associated with the iron released during lysis from erythrocytes present in blood.

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 a diamond pen.

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Embedding cells in LMP agarose and cell lysis

4. (Optional) If starting from an aliquot of frozen cells (Step 3):
   - Thaw the aliquot of cells quickly at 37 °C (in water bath or thermoblock)
   - As soon as the aliquot is thawed, add 1 mL of cold (4 °C) PBS to the 1.5 mL microtube and centrifuge for 5 min at 150–300*g* at 4 °C to wash cells
   - Suspend cell pellets in cold PBS, centrifuge again and remove the supernatant before proceeding to Step 10

5. Either resuspend the cells/nuclei in PBS and mix them with LMP agarose as suggested in Table 3 (option A) or mix the cell pellet directly with LMP agarose (option B).

   A. Embedding a suspension of cells:

   i. 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, take 45 μL of the cell suspension (~1×10^6 cells/mL) 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:

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

   ▲CRITICAL STEP See modifications for using high-throughput formats with mini-gels in Supplementary Protocols 3 and 4.

6. From each LMP agarose–cell suspension, transfer two 40–75 μL drops to each precoated microscope slide. In the case of amphibian samples, 250 μL drops are used. For specifications per sample type, see Table 3.

7. Cover gels with 20 × 20 mm coverslips.

   ▲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.

8. Keep for 5–10 min at 4 °C or place on a metal plate on ice for ~5 min.

   ▲CRITICAL STEP Sometimes an extra layer of LMP agarose is applied to achieve a flatter gel and remove bubbles that may have occurred accidentally

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in the first layer. In the case of whole body squashing of zebrafish embryos, additional LMP agarose is applied to fixate the squashed embryo. However, this additional layer 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.

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

A. Standard lysis:

i. Place slides in standard lysis solution for at least 1 h in a Coplin jar or any other container at 4 °C in the dark.

B. Lysis of human buccal cells:

i. Place slides in buccal lysis solution 1 for at least 1 h at 4 °C in the dark.

ii. After this first lysis step, add proteinase K (final concentration 10 mg/mL) to the prewarmed (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.

▲CRITICAL STEP When working with whole blood, especially fresh blood, we advise incubating the slides for 24 h to ensure lysis of all the erythrocytes, resulting in slides with much cleaner gels than after only 1 h lysis. Three-dimensional skin models also require overnight lysis. To split experiments over 2 d, the specimens can stay in lysis solution overnight, with no detriment to their integrity.

▲CRITICAL STEP 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 enzymes (enzyme-modified comet assay; Stage 2B, 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 h and 48 h. Longer lysis periods can be applied, but it is advised to leave them no more than 1 week. The duration of lysis should be kept identical within a set of experiments.

? TROUBLESHOOTING

Stage 2B: processing gels for the enzyme-modified comet assay (day 1) ● Timing ~2 h

Prepare materials

4. Prepare two slides per sample (one slide to incubate with reaction buffer and one slide to incubate with the enzyme), and lyse the cells as outlined in Stage 2A. If different buffers/enzymes will be used, extra slides should be prepared.

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5. Place a metal tray or plate on a box of ice.

6. 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.

7. Thaw aliquots of working solutions of the lesion-specific enzymes of interest on ice.

8. 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

4. Wash slides in buffer B or N or another reaction buffer, three times for 5 min at 4 °C (using a Coplin jar or another container).

5. Place slides on a metal plate on ice to prevent premature incision activity when the enzyme is added.

6. Prepare enzyme solutions, using the optimal enzyme concentration determined by the titration experiments (‘experimental design’), and control solutions for the incubation reaction. For a two gels/slide format, it is advised to prepare at least 250 μL of enzyme mixed with incubation reaction buffer. If using Fpg, hOGG1, EndoIII, Udg 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- or 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).

   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 (i.e., buffer N or a buffer provided with the enzyme).

   ▲CRITICAL STEP Keep enzyme and control solutions on ice during Steps 18–23.

   ▲CRITICAL STEP Enzyme reaction buffers provided by enzyme suppliers can also be used.

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▲CRITICAL STEP In case glycerol is used in the enzyme storage buffer (e.g., buffer B with 10% glycerol), it may be important to match its concentration in the control solution.

7. Add 50 μL of the enzyme or control solution to each gel (containing nucleoids of samples, experimental controls or assay controls; Fig. 1). Incubate duplicate aliquots of each sample (i.e., two gels incubated with enzyme and two gels with control solution).

8. Cover gels with coverslips (22 × 22 mm for each gel or 24 × 60 mm to cover both gels).

9. 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 ('Experimental design' and 'Reagent setup'). For incubation reactions using 12 gels/slide or other high-throughput formats, see Supplementary Protocols 3 and 4.

   ▲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.

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

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

12. ? TROUBLESHOOTING

Stage 3: comet formation (day 1) ● Timing ~3 h (including washing steps)

Alkaline treatment and electrophoresis

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

2. Incubate in cold (4 °C) electrophoresis solution in the tank for 20–40 min at 4 °C in the dark, while keeping the power supply switched off; alternatively, perform the alkaline treatment in a separate Coplin jar, placing the slides in the tank just before electrophoresis.

   ▲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 another 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 should not be >10 °C.

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

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▲CRITICAL STEP Cells from 3D lung models require an EPT = 30 (1 V/cm for 30 min). For instructions for yeast and filamentous fungi and plant cells, respectively, see Supplementary Protocols 11 and 12.

▲CRITICAL STEP To ensure an accurate calculation of the voltage gradient, the voltage across the platform should be measured using a voltmeter. Alternatively, an approximate measure can be obtained by dividing the applied electrode voltage by the distance between the electrodes. Please see 'Equipment setup'.

▲CRITICAL STEP When possible, samples from the same experiment together with corresponding controls (negative, solvent and positive) should undergo the same electrophoresis run. When a large number of samples need to be analyzed, use interassay controls in each electrophoresis run.

Neutralization and washing

4. Neutralize gels by washing slides in the neutralizing solution, in cold (4 °C) PBS for 10 min or cold (4 °C) 400 mM Tris–HCl (pH 7.5) three times for 5 min. Afterwards, wash slides (optional) for 10 min in cold (4 °C) dH_2O (use a Coplin jar, or lay slides flat in a dish).

▲CRITICAL STEP It is advisable to wash the slides with dH_2O after the neutralization (i.e., after washing with PBS/Tris–HCl), before drying the gels (optional).

5. (Optional) 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 using a Pasteur pipette. Before each EtOH addition, remove previous EtOH by slowly leaning the tray with slides to one side.

▲CRITICAL STEP Dry slides facilitate the scoring since comets in dry slides are in the same plane in the gel.

■PAUSE POINT Dried gels/slides can be stored in the dark at RT 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 restained before scoring or rescoring.

Stage 4: comet visualization and analysis (day 2) ● Timing ~2 h to several days (depending on the number of samples)

Comet visualization

4. Stain gels with DNA fluorescent dye ('Reagents'). When using dyes that allow direct visualization, follow option A. For dyes that require a longer incubation time, follow option B.

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

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! CAUTION All dyes may be mutagenic, apart from GelRed. Wear protective gloves when using them, and dispose waste in containers labeled for hazardous chemicals.

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.

▲CRITICAL STEP 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.

▲CRITICAL STEP It is advisable to incubate the gels for 20 min at RT when DAPI is used. DAPI cannot be used with GelBond films owing to autofluorescence of the GelBond at the wavelengths used to detect DAPI.

ii. If using GelRed, dilute the GelRed stock (10,000× 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, 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, dilute SYBR Gold 1:10,000, add 50 μL on top of each gel and cover with a coverslip (in this case, skip Step 33B(ii)).

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

5. Visualize comets with a fluorescence microscope using appropriate filters.

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

Comet analysis

4. Score at least 50 comets per gel, i.e., 100 comets per slide/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.

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

▲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

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trial, all comets should be scored by the same person to minimize interoperator 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 and 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.

A. Using image analysis software

i. Obtain the TI (i.e., percentage of 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.

▲CRITICAL STEP It is possible to use other central estimates of nonnormal distribution of comets, or arithmetic mean. All estimates are highly correlated, and using one or the other has minimal practical implications 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.

▲CRITICAL STEP Comet analysis by using fully automated image analysis systems omits interoperator heterogeneity in scoring. However, bias related to omission of unmeasurable comets is a concern for analysis by fully automated image analysis systems. The risk of biased analysis by automated image analysis systems can be inferred by comparing the ratio of measured/total objects (i.e., a decreased ratio should alert the investigator to the risk of measurement bias).

B. Using visual scoring

i. Assess DNA damage from comets by discriminating between the degrees of damage according to comet appearance (Fig. 13). Scoring comets using the classification system composed of five classes, from 0 (no tail) to 4 (almost all DNA in tail) results in sufficient resolution[229]. If 100 comets are scored,

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and each comet is assigned a value of 0–4 according to its class, the total score for the sample gel will be between 0 and 400 'arbitrary units'.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 4.

Timing

Day 0 or 1

Steps 1–3, Stage 1: preparation of cells from frozen or fresh samples: 0.5–3 h (depending on the cell type and the number of samples)

Day 1

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

Steps 15–27, Stage 2B: optional extra steps for enzyme-modified comet assay: ~2 h Steps 28–32, Stage 3: comet formation: ~3 h (including washing steps)

Day 2

Steps 33–36, Stage 4: comet visualization and analysis: ~2 h to several days (depending on the number of samples)

Anticipated results

The comet assay can detect between ~50 and ~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 percentage of 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 nongenotoxic detergents produced comets with >90% tail DNA and shapes of comets that are commonly described as 'hedgehogs', 'clouds' or 'ghosts'[333]. However, the effect of cell death (or apoptosis) decreases with less severe exposure conditions. It has been shown that the presence of >25% dead cells, assessed by the Trypan blue assay, results in an increase of the mean level of DNA migration in the comet assay[334]. Thresholds of cytotoxicity and cell death reported in the literature are usually between 20% and 30%. 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 reducing biases due to cell death[7]. The effect of cytotoxicity on comet assay endpoints should be assessed by a case-by-case approach rather

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than by adopting a predetermined threshold; cytotoxicity assays may be test system specific, and they measure different types and severity of the toxicity endpoints. In addition, it should be noted that 'hedgehogs', 'clouds' or 'ghosts' do not necessarily represent apoptotic or dead cells[333]. Thus, omission of such comets is not recommended as a way of avoiding biases due to cell death.

Detection of DNA crosslinks

DNA crosslinking may appear to be nongenotoxic in the standard comet assay. If a compound is suspected to cause DNA crosslinks, 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 SB levels by a direct DNA strand breaking agent are lowered when cells are treated with the suspected crosslinking agent as compared with the control exposure with the DNA strand breaking agent only[36,335].

DNA SBs formed by repair processes

Certain agents (e.g., UVC) do not produce ALS and SBs, but SBs are generated by excision repair enzymes in the cells[68,336]. 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 (Fig. 15).

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 (assuming migration is measured as percentage of tail DNA):

$$\text{'Enzyme-sensitive sites'} = \%\ \text{tailDNA}_{\text{Enzyme}} - \%\text{tailDNA}_{\text{Buffer}}$$

The measurement of enzyme-sensitive sites and global methylation requires an additional step in the comet assay protocol that affects the level of DNA migration. 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 should be 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 from four different samples. The

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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 values of 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 two gels) can by chance alone result in 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 has 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.

Variation in DNA damage levels

The variation in DNA damage in different samples stems from interindividual, intraindividual 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 intraindividual 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 intragroup coefficient of variation in DNA SBs in leukocytes of 36% (95% confidence interval (CI) 27%, 46%) in cross-sectional studies on healthy humans[337]. 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[313].

It should be anticipated that the variation in enzyme-sensitive sites is similar to or higher than the variation in DNA SBs because the variances are additive. It should also be anticipated that assay control samples display some interday variation. This is illustrated in Fig. 17, using results from assay controls from a human biomonitoring study[338]. The mean and standard deviations of the samples are 0.29 ± 0.14, 0.85 ± 0.35 and 1.43 ± 0.26 lesions per 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 with the buffer-treated sample.

Lastly, it should be expected that exposure to a genotoxic agent increases both the level of DNA damage and the intragroup variation in biomonitoring, animal and cell culture studies. This is illustrated by the example in Fig. 18 that depicts levels of Fpg-sensitive sites in cells

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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 Fig. 18). It is common to obtain a larger standard deviation in treated specimens than in unexposed specimens irrespective of whether the specimens originate from cell cultures, animals or biomonitoring studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The majority of the data shown here as examples or anticipated results are available in the original papers. Figures 12 and 14–16 are theoretical results, which are inspired by unpublished work from the authors' laboratories. Other supporting data are available upon reasonable request to the corresponding author.

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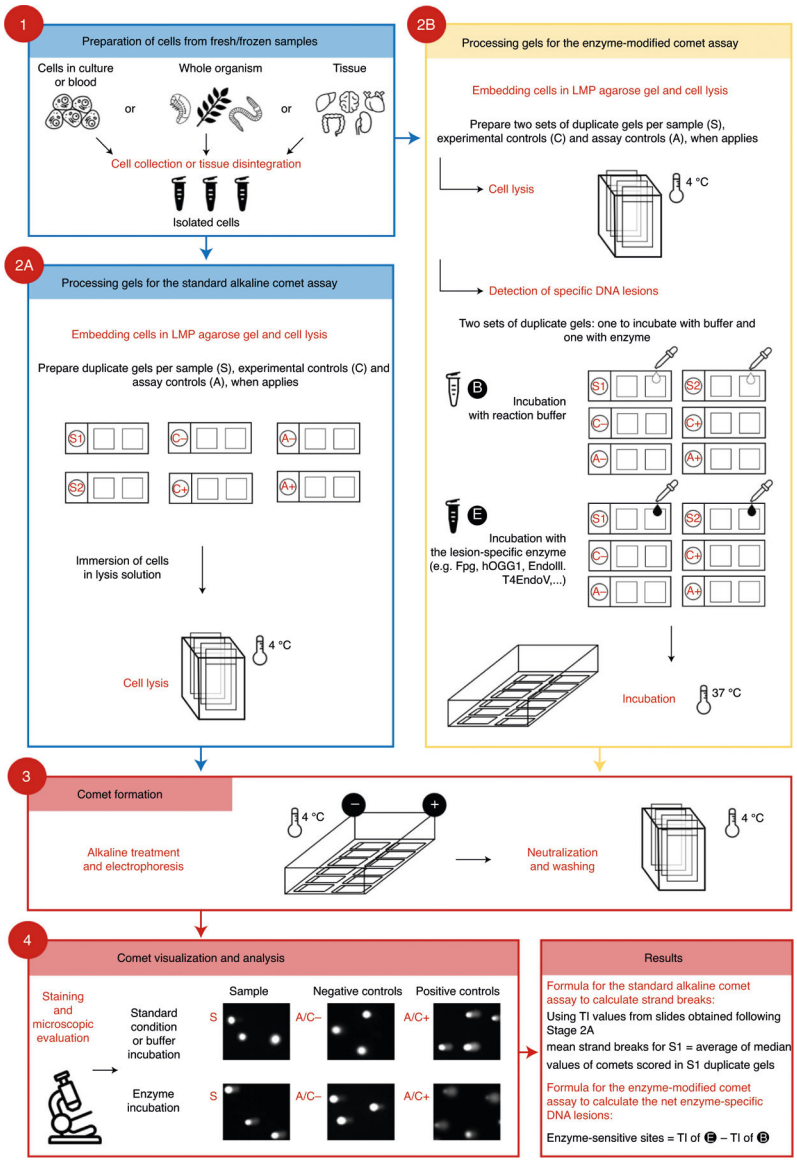

Fig. 1 |. Overview of the standard and the enzyme-modified comet assay protocols.
Stage 1 involves the isolation of single cells, which are processed in either the standard (Stage 2A) or enzyme-modified (Stage 2B) comet assay. In the second stage of the standard comet assay, nucleoids are embedded in 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 step, electrophoresis and subsequent neutralization of the slides. Stage 4 is the visualization 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 SBs, 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.

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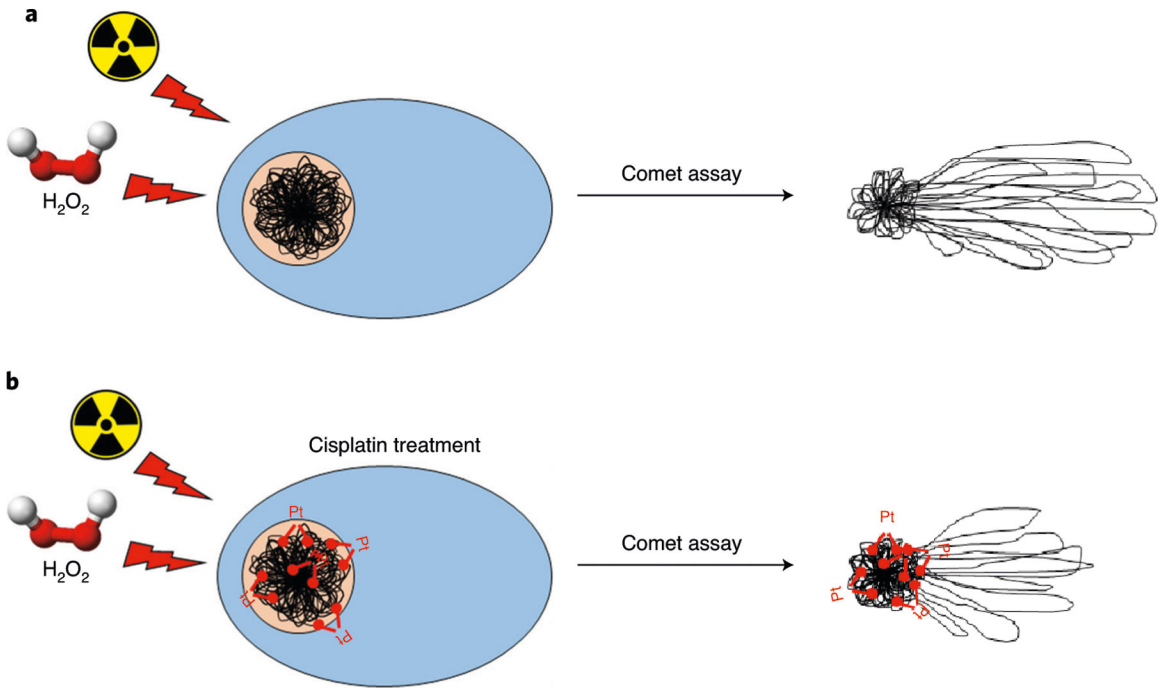

Fig. 2 |. A schematic representation of interstrand crosslinks (ICLs) formation by cisplatin and detection with a variant of the alkaline comet assay.

a, In the absence of cisplatin treatment, relaxed DNA loops migrate towards the anode forming the comet tail. **b**, In the presence of cisplatin, and with exposure to a strand-breaking agent such as ionizing radiation or H_2O_2, migration of the DNA is inhibited by the ICLs—the more ICLs, the less the migration of the DNA.

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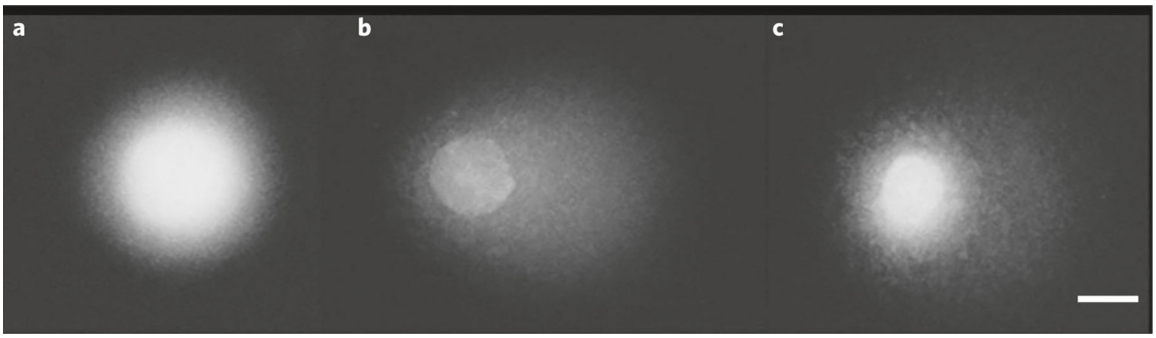

Fig. 3 |. Representative images of three comets illustrating interstrand crosslinks (ICLs) detection following cisplatin treatment.

a–c, Cells from an ovarian cancer cell line (SKOV-3) were first treated with 0 μM or 200 μM cisplatin. SBs were then induced using H_2O_2 (50 μM). The presence of cisplatin-induced crosslinks resulted in a decrease in tail moment (TM) after DNA damage induced by H_2O_2 (50 μM), compared with the H_2O_2 treatment control, in the absence of cisplatin. Control cells without any treatment (**a**); cells treated with H_2O_2 (50 μM) only (**b**); cells treated with cisplatin (200 μM) and subsequently H_2O_2 (50 μM) (**c**). Scale bar, 10 μm.

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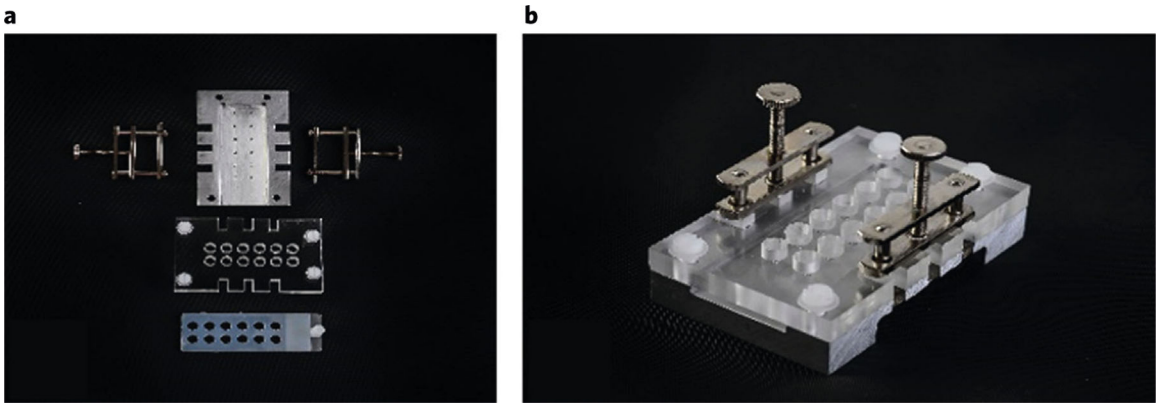

Fig. 4 |. Component parts of the 12-gel chamber unit.

a, Top view, showing metal base with marks for positioning gels on slide, silicone rubber gasket, plastic top-plate with wells, and silicone rubber seal. **b**, Assembled unit.

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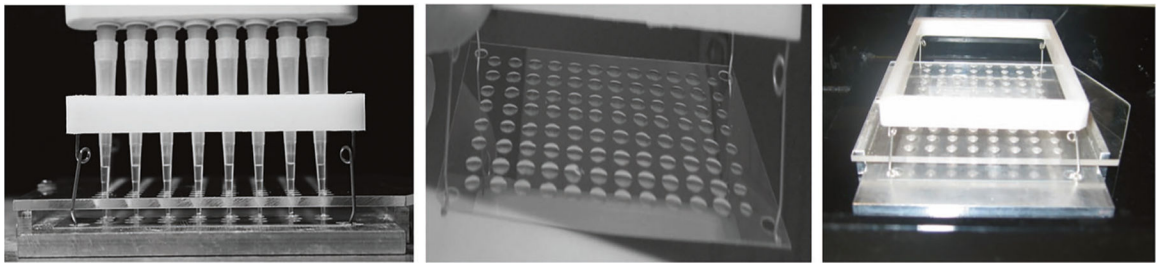

Fig. 5 |. Images illustrating the 96-gel format using GelBond film.
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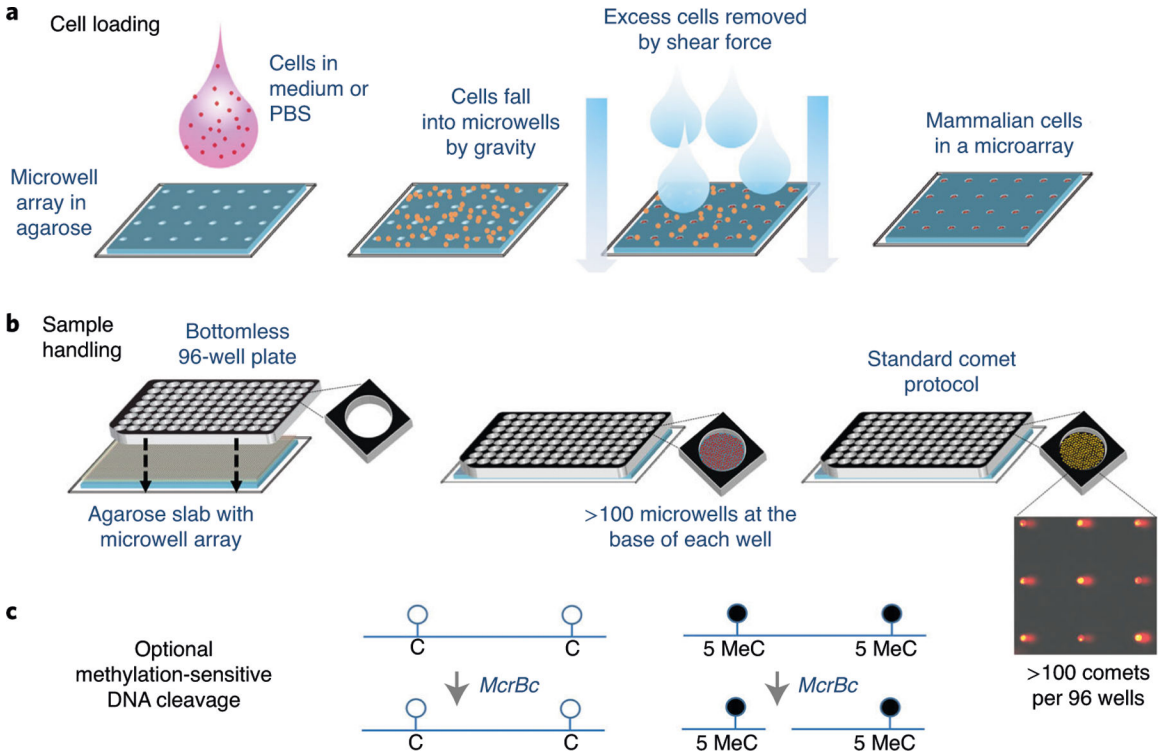

Fig. 6 |. The CometChip platform.

a, Cells in medium or PBS are loaded by gravity into a microwell array in agarose that was created using a mold 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 LMP 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 >100 microwells. After cell loading, rinsing, capping and treatment, the agarose slab is processed using standard comet assay protocol conditions. Cells can be either pretreated or treated on-chip. Each of the 96 wells substitutes for a single glass slide used in the traditional comet assay. **c**, For the EpiCometChip (see 'Detection of global DNA methylation'), immediately after lysis, the agarose slab is rinsed and incubated with *McrBC* before processing using standard comet analysis conditions. C, nonmethylated cytosine; 5MeC, 5-methylcytosine. Panels **b** and **c** adapted with permission from ref. [42], Wiley.

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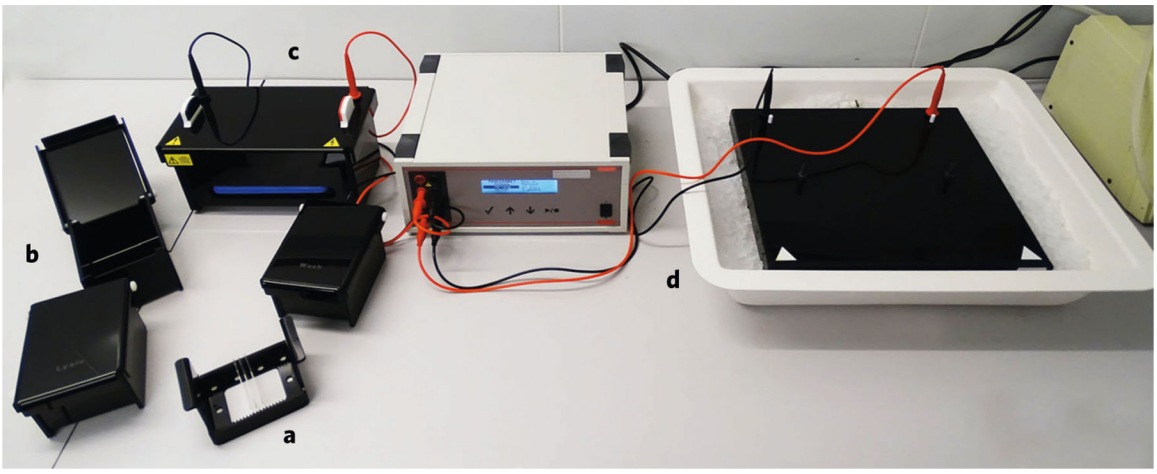

Fig. 7 |. The vertical comet system.

a, Racks hold slides vertically (up to 25 slides per rack). **b**, Treatment chambers that accommodate the slide-containing racks. **c**, High-throughput electrophoresis tank (possesses integrated cooling, so no wet ice needed) holding two racks. **d**, Standard comet assay tank in tray of wet ice; the improvement in size of the high-throughput tank (**c**) over the standard comet assay tank is clearly seen.

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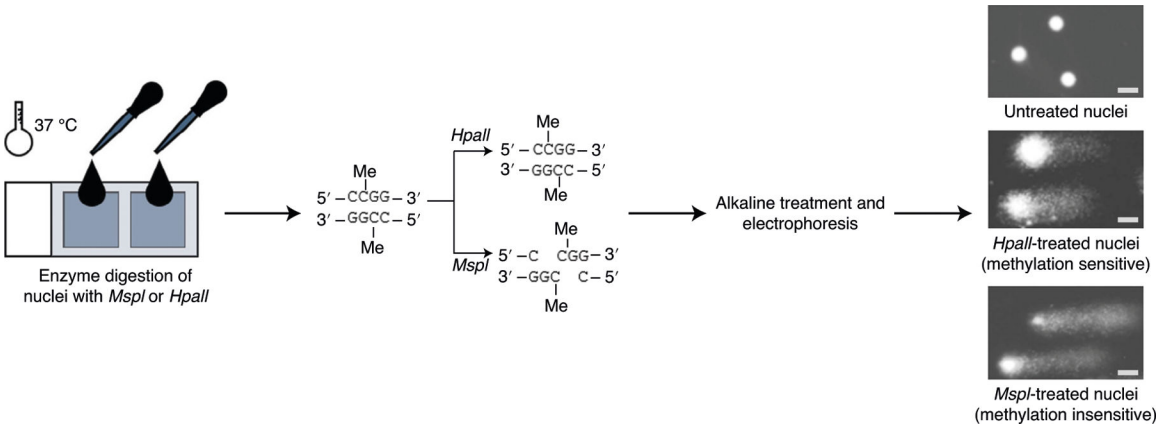

Fig. 8 |. Principle of the DNA methylation-sensitive comet assay.

This assay uses two isoschizomeric restriction enzymes that recognize the same tetranucleotide sequence (5′-CCGG -3′), but display different sensitivities to DNA methylation; *HpaII* is inhibited by the presence of a methyl group on the second cytosine in the recognition sequence (it is able to recognize unmethylated sequences), while *MspI* is able to cut both methylated and unmethylated sequences. The global methylation can be assessed by calculating the *HpaII*/*MspI* ratio. Scale bars, 10 μm.

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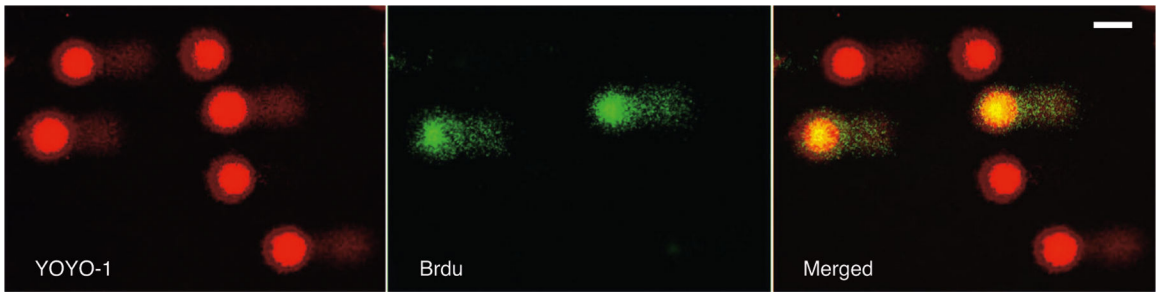

Fig. 9 |. Visualization of all comets and BrdU-positive comets only by fluorescence microscopy, using two filters.

With the FITC filter (left), comets stained with YOYO-1 for detection of DNA breaks are visualized. With the TRITC filter (middle), BrdU-positive comets formed by cells in the S phase of the cell cycle are visualized. The image on the right shows both BrdU-positive and BrdU-negative comets. Scale bar, 40 μm.

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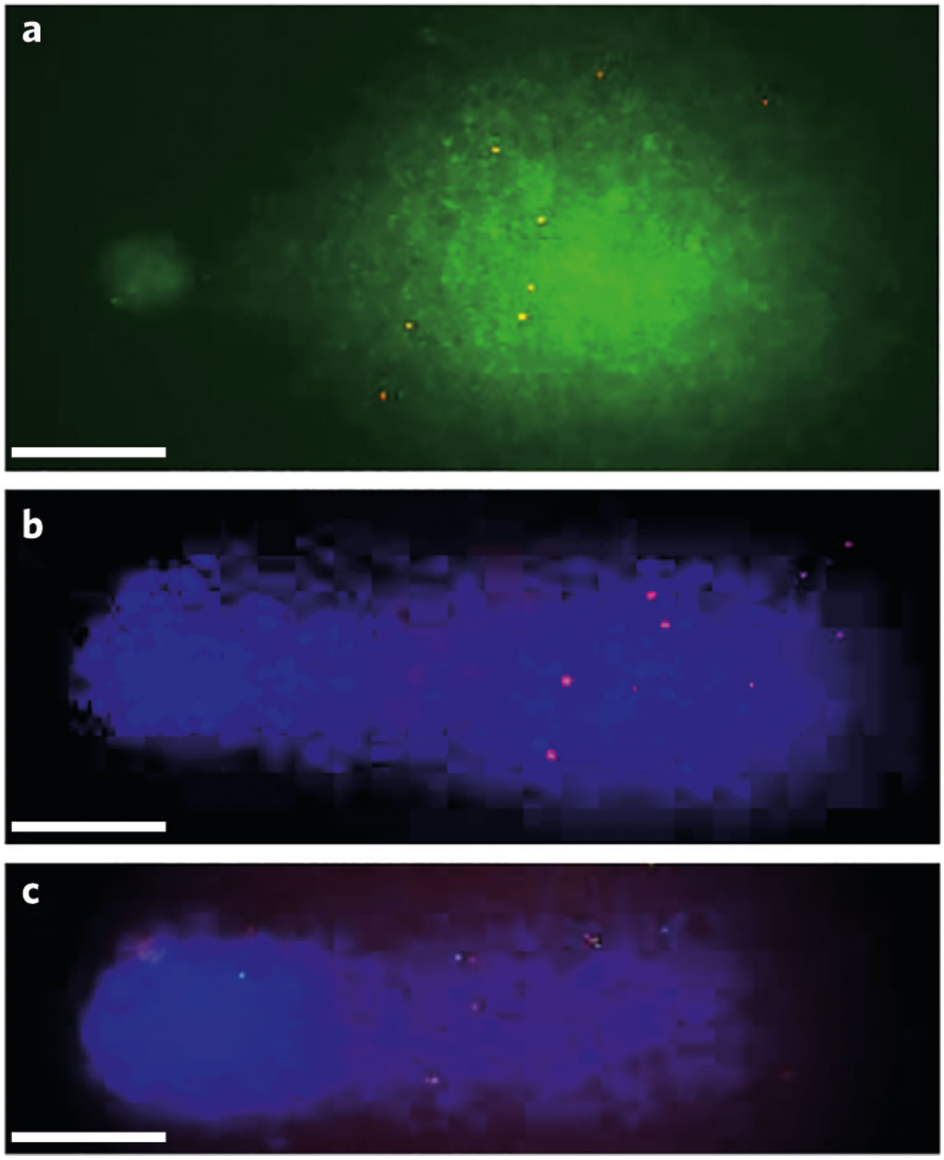

Fig. 10 |. Example pictures of different types of signals seen in comet–FISH experiments after alkaline electrophoresis using U-2 OS cells.

a, Probe RPCI-1 213H19 labeled with two colors (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 labeled 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 labeled with digoxigenin (green) and biotin (red), respectively, in comets from cells irradiated with UVC at 0.2 Jm^{-2}. Scale bars, 20 μm. Figure adapted with permission from ref. [339], Wiley.

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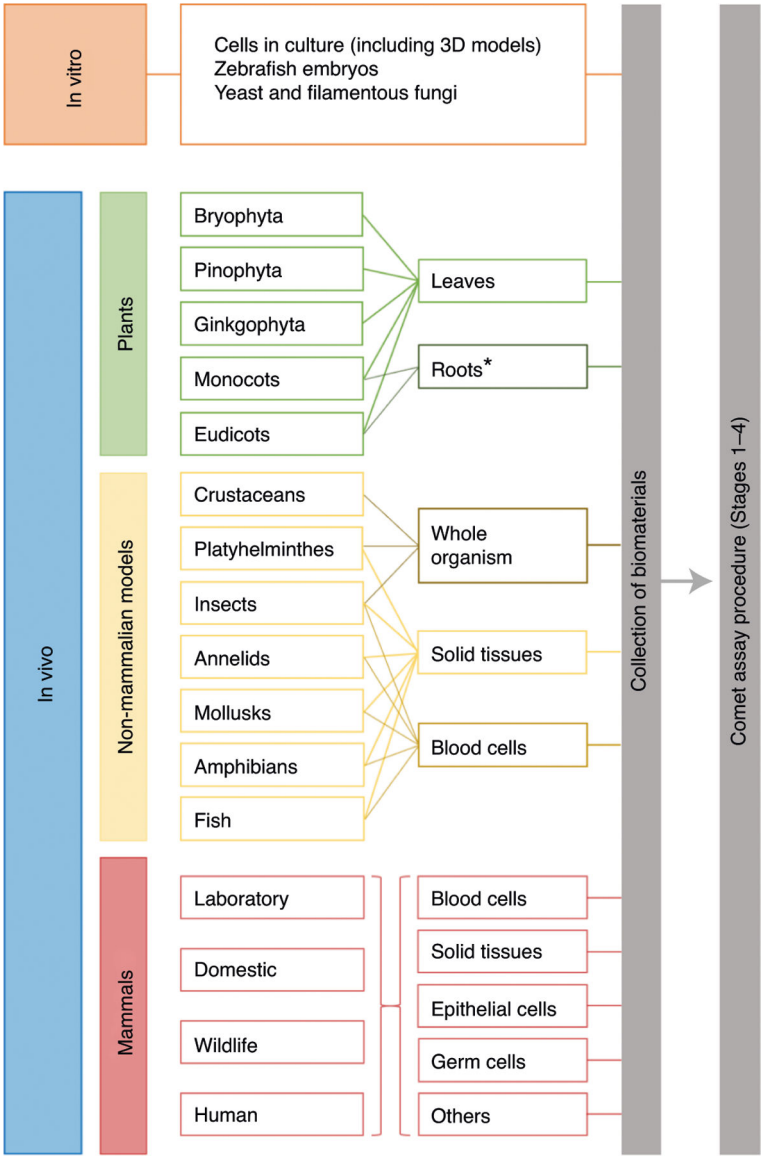

Fig. 11 |. Overview of various species and different sample types that have been used in the comet assay.

Preparation of cells from different sample types is described in Stage 1 of the Procedure. *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 not be used as well.

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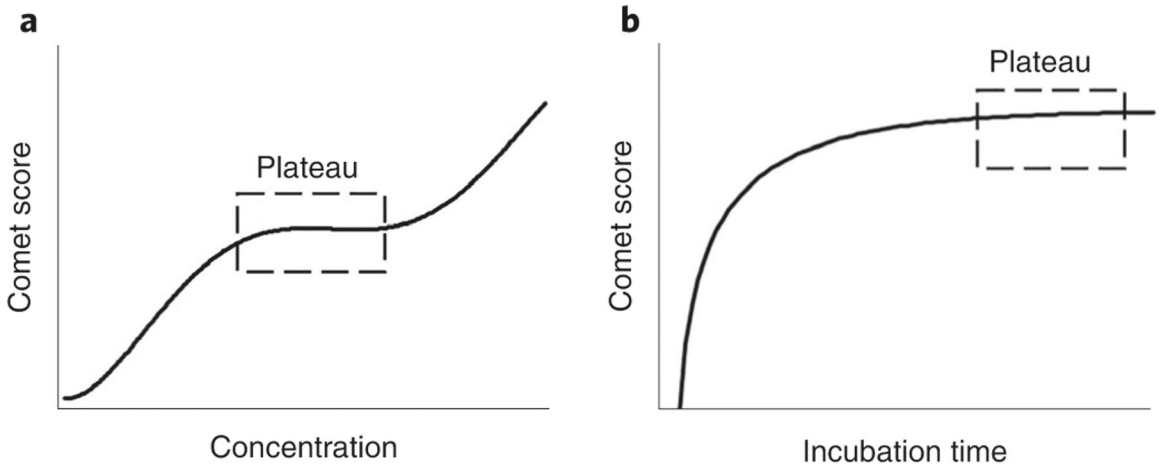

Fig. 12 |. Titration steps in the enzyme-modified comet assay.

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 nonspecific 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.

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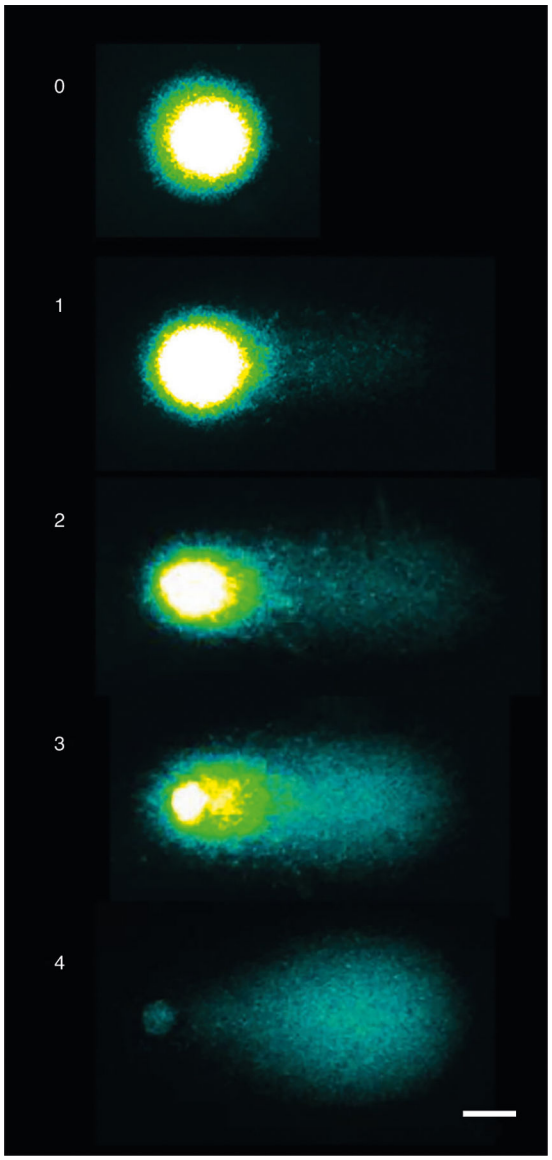

Fig. 13 |. Representative images of comets classified in five different classes for visual scoring. 0 (no tail), 1, 2, 3 and 4 (almost all DNA in tail; sometimes described as a hedgehog). The colorectal cancer cell line HCT116 was used to obtain the images. Scale bar, 20 μm.

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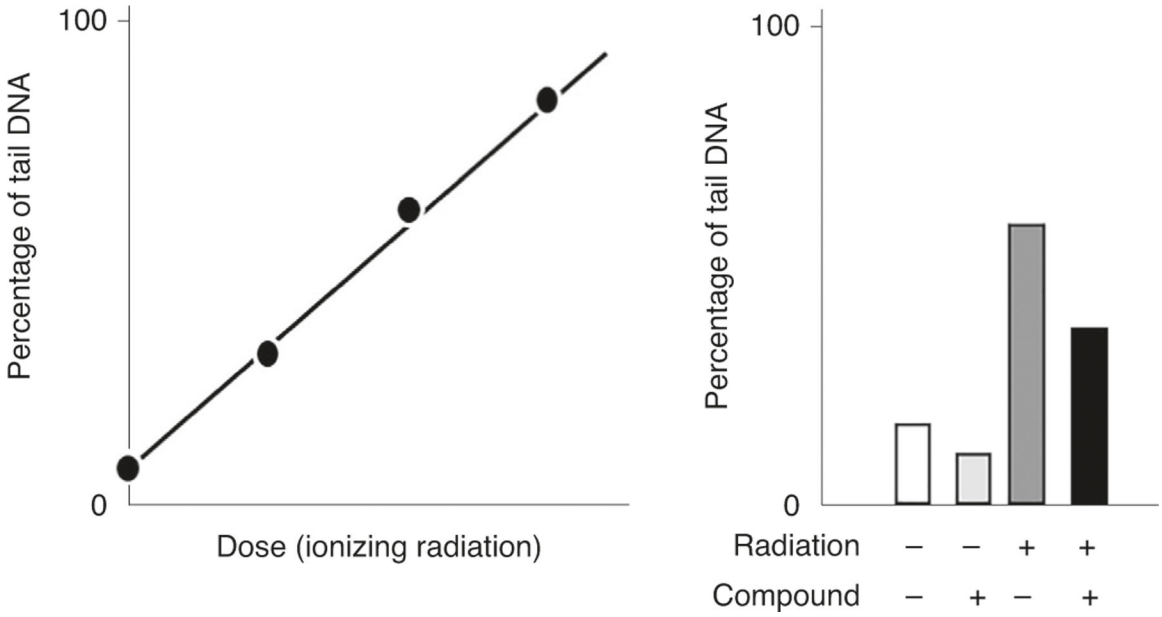

Fig. 14 |. Detection of DNA crosslinks in a theoretical cell culture study.

Experiments are first carried out to find a suitable level of DNA SBs, using an agent that directly causes breaks in DNA such as H_2O_2 or ionizing radiation (left). Subsequently, experiments are done where cells are exposed to the test agent (compound) and ionizing radiation. The presence of crosslinks in DNA is concluded if the irradiated samples plus the tested compound have less DNA migration as compared with the irradiated samples without the tested compounds (black bars compared with gray bars).

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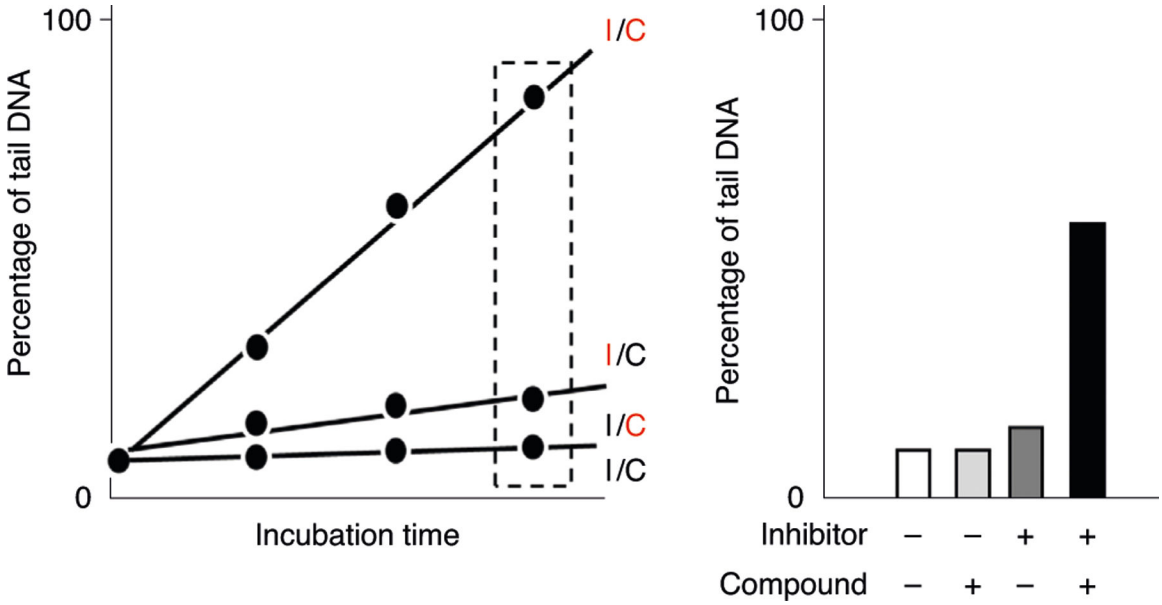

Fig. 15 |. Assessment of DNA lesions by inhibition of late-stage excision repair processes in a theoretical cell culture study.

The cells are incubated with the test agent (compound, C) and inhibitor (I) (red letter in the left graph refers to the presence of compound or inhibitor; in case of incubations with I/C-red and I/C the lines overlap). The data included in the dashed box are represented in the bar graph on the right. 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).

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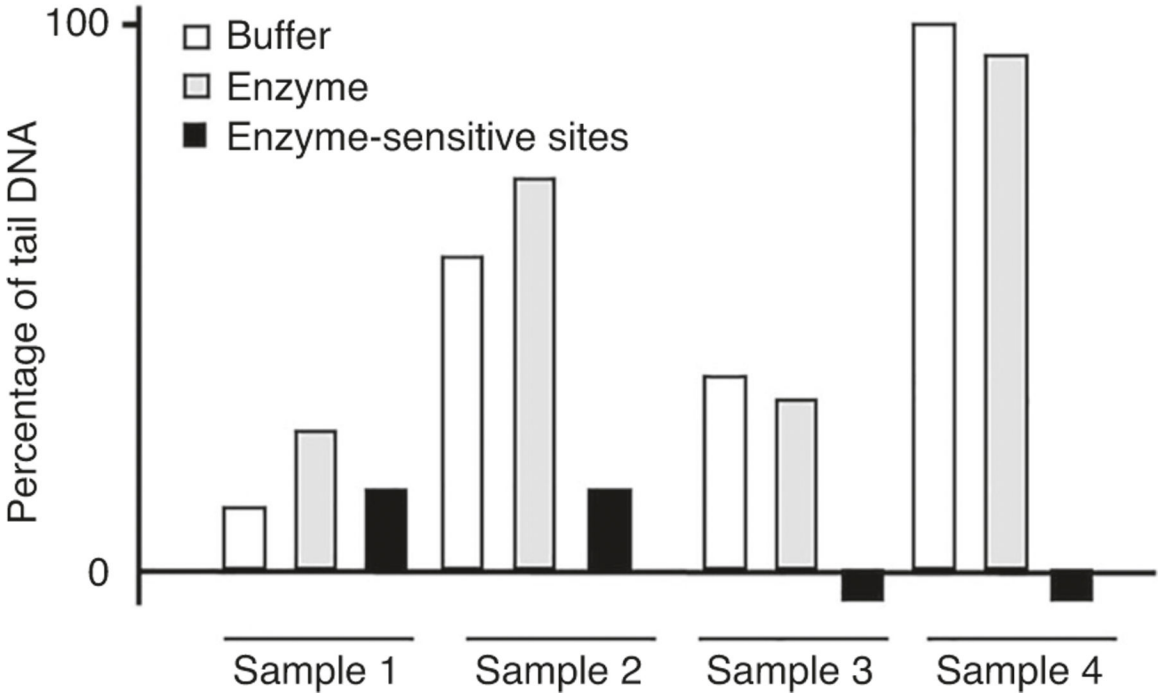

Fig. 16 |. Examples of data output of the enzyme-modified comet assay in theoretical samples. Samples 1 and 2 exemplify two different samples where the levels of DNA SBs differ, whereas the levels of enzyme-sensitive sites are identical. The total level of DNA damage (i.e., 'enzyme' treatment) is higher in sample 2 than 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 SBs, respectively. In these samples, the DNA damage level measured by the 'buffer' and 'enzyme' treatments is identical. Negative values of enzyme-sensitive sites will occur in some samples 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). In sample 4, the level of DNA SBs is so high that the comet assay is saturated (i.e., DNA migration is close to 100% tail DNA). Therefore, it is not possible for the enzyme treatment to increase the DNA migration, and so enzyme-sensitive levels are underestimated.

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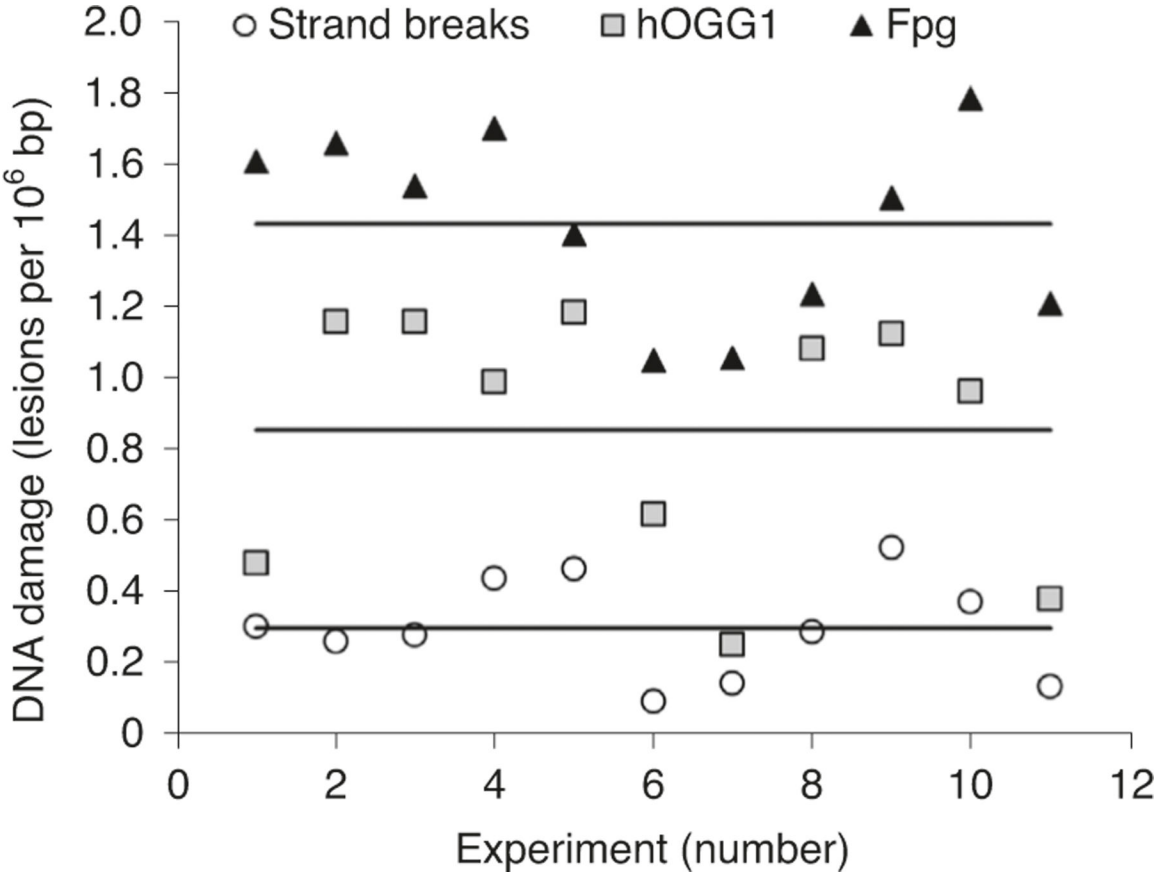

Fig. 17 |. Levels of DNA migration in assay control samples from a biomonitoring study, encompassing 11 d of comet assay experiments.

PBMCs 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 per 10^6 bp in samples treated with buffer (i.e., DNA SBs), formamidopyrimidine glycosylase (Fpg) or human oxoguanine DNA glycosylase (hOGG1). Figure adapted with permission from ref. [338], Elsevier.

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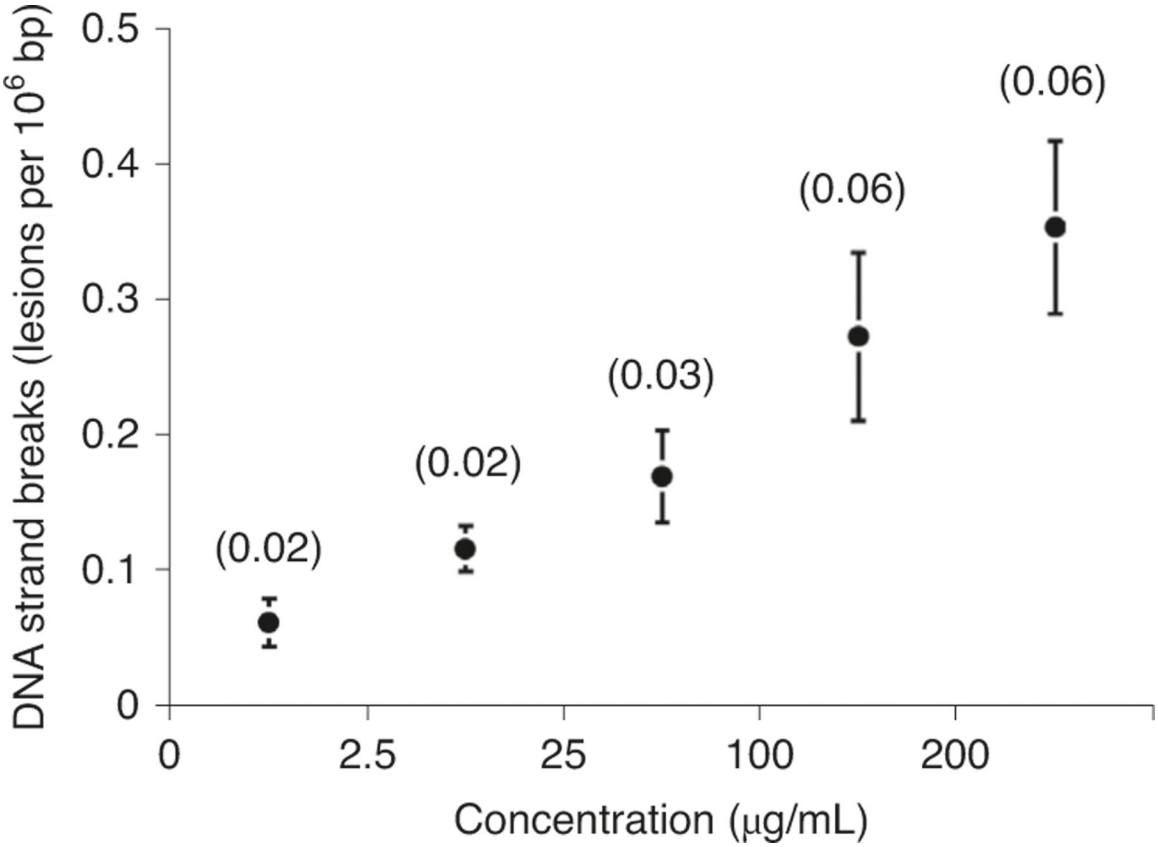

Fig. 18 |. Example results from a study of Fpg-sensitive sites after exposure to diesel exhaust particles in cultured human HepG2 cells.

Filled circles and whiskers are mean value and standard deviation, respectively, of six experiments (numbers in brackets are standard deviation). The concentration of diesel exhaust particles is shown on the *x* axis. Figure adapted with permission from ref. [340], Elsevier.

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Table 1 |

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

Experimental models	Sample types
In vitro	
Cell lines and primary culture	Single cell culture and co-culture
3D cell models	Liver spheroids, reconstructed human full-thickness (FT) skin tissues (dermis and epidermis) and reconstructed airway/lung tissues
Zebrafish	Embryos and larvae
Yeast	Single culture of different strains and species
Plants	**Organs**
Bryophyta, Pinophyta, Ginkgophyta, monocots, eudicots	Roots, leaves
In vivo—non-mammalian	**Organs/samples**
Crustaceans: *Daphnia magna*, *Ceriodaphnia dubia*	Whole organism
Planarians: *Schmidtea mediterranea*, *Dugesia japonica*	Whole organism
Insects: *Drosophila melanogaster*	Hemocytes and neuroblasts
Insects: *Chironomus riparius*	Larvae, whole organism
Annelids: earthworm, *Eisenia foetida*	Coelomocytes
Mollusks: *Bivalvia*	Hemolymph, 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 (MNCs); buccal, nasal, lachrymal and conjunctival epithelial cells; sperm; and placental cells

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Table 2 |

Suggested enzyme concentration based on titration experiments

Enzyme	Format	Final enzyme concentration	Duration of incubation at 37 °C
Fpg (NorGenoTech)	2 gels (70 μL of gel; 20 × 20 mm coverslip) 45–50 μL enzyme per gel (22 × 22 mm coverslip)	0.16 ng/μL	30 min
Fpg (New England Biolabs)	12 mini-gels (5 μL of gel) 30 μL enzyme per 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 per gel using the 12-well chamber unit	33.3 U/mL	1 h
hOGG1 (Trevigen)[a]	2 gels (80 μL of gel; 20 × 20 mm coverslip) 50 μL enzyme per gel (22 × 22 mm coverslip)	1.6 U/mL	10 min
hOGG1 (Trevigen)[a]	12 mini-gels (5 μL of gel) 30 μL enzyme per gel using the 12-well chamber unit	6.66 U/mL	1 h
T4endoV (New England Biolabs)	2 gels (70 μL of gel; 20 × 20 mm coverslip) 45–50 μL enzyme per gel (22 × 22 mm coverslip) Incubation in slide moat	3.33 U/mL	30 min

[a]Discontinued from sale. See potential alternatives in the reagents list.

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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 %)[a]
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 (~200 μL)	Add 300 μL of 0.75% LMP agarose	3–6 × 10^4 per 75 μL gel (~0.5% LMP agarose)
Zebrafish embryos	Whole-body squashing (one embryo per slide)	1 embryo directly in 60 μL of 1.5% LMP agarose	1.5% LMP agarose
	Whole-body cell isolation (from a pool of up to 8 embryos, depending on single cells yield, 5–6 × 10^6 cells/mL)	20 μL of cell suspension in 180 μL of 1% LMP agarose	Up to 5–6× 10^6 cells/mL (0.9% LMP agarose)
Non-mammalian models			
Crustaceans	~1.0 × 10^5 cells per 140 μL	Resuspend cells in 0.7% LMP agarose	~5 × 10^4 per 70 μL gel (0.7% LMP agarose)
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 (two technical duplicates)
Insects—*Drosophila melanogaster*	Resuspend the obtained cells (~1,000 cells/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 (if the pellet contains cells from 10 fourth-instar larvae, ~250 μL should be added)	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 (1 mL of PBS is normally used per earthworm)	Mix 1:1 with 1% agarose	~450 cells in 60 μL (0.5% LMP agarose)
Mollusks—mussels	Gills and digestive glands: resuspend the cell pellet to ~5 × 10^5 cells/mL in KSS Hemolymph: dilute hemolymph from one animal in modified Alsever (1:5)	Resuspend the cell pellet in 75 μL 0.5–0.85% LMP agarose	2.5 × 10^3 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)
Large fish (e.g., Gilthead seabream, Senegalese sole and European eel)	Blood: 2 μL peripheral blood mixed with 1 mL PBS Liver and gills: after mincing, to complete cell dissociation, resuspend the small pieces of tissue in 1 mL PBS by pipetting up and down	Mix 20 μL of the cell suspension with 70 μL (1%) LMP agarose Mix 20 μL of the cell suspension with 70 μL (1%) LMP agarose	~2 × 10^4 cells in 70 μL gel (0.8% LMP agarose) ~2 × 10^4 cells in 70 μL gel (0.8% LMP agarose)
Small fish (zebrafish)	Blood: mix 10 μL peripheral blood with 90 μL PBS without Ca^{2+}/Mg^{2+}	Mix 10 μL of peripheral blood cells in PBS with 70 μL 1% LMP)	~1.5 × 10^3 cells in 70 μL gel (0.9% LMP agarose)

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Species/cell type	Cell suspension	Dilution in LMP agarose	Final cell density (final LMP agarose %)[a]
	Liver, gills and gonads: resuspend the minced (and washed) small portion of the tissue in 1 mL PBS supplemented with 0.02% EDTA	Liver: mix 10 μL of cell suspension in PBS with 70 μL 1% LMP Gills and gonads: mix 25 μL of cell suspension with 75 μL 1% LMP	Liver: ~1.5–3.0 × 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)
Mammalian models			
Rodent tissues	Liver: 3 × 3 × 3 mm Kidney: 2 × 3 × 5 mm Lung: 5 × 5 × 5 mm Spleen: 1 × 1 × 1 mm Brain: 2 × 3 × 5 mm Duodenum, jejunum, ileum, colon: 1.5 cm segments (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)
Whole blood	Use 5–20 μL whole blood directly. Alternatively, mix 10 μL whole blood with 40 μL PBS	Mix 20 μL of whole blood with 480 μL 0.8% LMP agarose. Alternatively, add 160 μL of 1% LMP agarose to the whole blood/PBS mixture	50–125 cells/μL gel (0.5–0.7% LMP agarose)
Buffy coat	Use 5 μL buffy coat directly	Mix 5 μL of buffy coat with 200 μL 0.8% LMP agarose	Sufficient number of cells to carry out the assay (~0.8% LMP agarose)
Leukocytes, PBMCs	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)
Salivary BMCs	~2 × 10^5 cells per 160 μL	Resuspend the cell pellet in 0.7% LMP agarose	~1 × 10^5 per 80 μL gel (0.7% LMP agarose)
Buccal cells	100,000–500,000 cells per 1 mL PBS	Resuspend the cell pellet in 0.5% LMP agarose	10,000–50,000 cells per 75 μL gel (0.5% LMP agarose)
Nasal cells	50,000 cells per 50 μL of PBS	Resuspend the cell pellet in 0.5% LMP agarose	50,000–100,000 cells per 75 μL gel (0.5% LMP agarose)
Tears (lachrymal duct and cornea cells)	Use tear directly	Mix the tears (30 μL) with 30 μL 1% 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 per 5 μL gel (0.6% LMP agarose) (12-gel format)

[a]This is the most commonly used percentage of LMP agarose for each sample type, but other concentrations between 0.5 and 1.5% may work as well (see also 'Optimization of percentage of LMP agarose'). For other species/cell types, see Supplementary Protocols 11–13.

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Table 4 |

Troubleshooting table

Step	Problem	Possible reason	Solution
Experimental design	High interassay variation	Alterations in RT, equipment performance, reagent lots, etc	Use internal controls and create own historical data to identify and control variability
Equipment setup: precoating microscope slides	Agarose does not attach to the slides	Presence of grease and dust on the slides	Degrease the slides by washing them with EtOH. Leave them to dry at RT or pass the slides through the flame of a Bunsen burner
		Agarose is not mixed well	Ensure agarose is fully dissolved before coating slides (see instructions in 'Equipment setup')
14	Loss of gels while removing the coverslip	Gels may not set properly because of condensation in rooms with high temperature and/or humidity	Cool the working room, ideally to ~20 °C. Embedding cells in gels in an air-conditioned room is a good option. You can also provide direct airflow from a heating fan over the slides
		Use of slides with charge	Use recommended slides ('Equipment')
		Agarose concentration is too high, not well mixed or gels are too thin	Mix agarose well
27	Loss of gels during the enzyme incubation at 37 °C	Gels may be weakened by being at 37 °C, causing them to detach when the coverslips are removed for the next step	Cool the slides very quickly before removing the coverslips after enzyme incubation. Consider increasing the agarose concentration
34	Too many or too few cells in the gel	It can be due to several reasons depending on the biological material use Cells in suspension: wrong counting or bad isolation (e.g., MNCs) Organoids or solid tissues: incorrect size of the portion used to obtain the cell suspension	Optimization in the number of cells, isolation process or size of the solid tissue to use is recommended before starting the experiments ('Experimental design')
	No increase in DNA migration in the enzyme-incubated positive control cells compared with buffer-incubated cells	Enzyme used after expiration date or subjected to 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 freeze–thaw cycles
	Comets cannot be scored owing to high background on slides	The presence of dust or other impurities in agarose	Prepare new agarose solution and/or slides
		Contamination of agarose solution with mold	
		Reused slides	
	Low levels of DNA damage in positive controls	Problems with electrophoresis Improper setting of the image analysis software and/or low intensity of fluorescence in the microscope	Check the power supply Adjust the software according to the manufacturer's instructions. Change the bulb in the microscope
Supplementary Protocol 3	Comet tails are oriented in all directions at 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
Supplementary Protocol 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
Supplementary Protocol 11	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 RT