Toxicology 501 (2024) 153690

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Further development of CometChip technology to measure DNA damage in vitro and in vivo: Comparison with the 2 gels/slide format of the standard and enzyme-modified comet assay

Miguel Collia [a], Peter Møller [b], Sabine A.S. Langie [c], Ariane Vettorazzi [a], Amaya Azqueta [a,*]

[a] Department of Pharmaceutical Sciences, School of Pharmacy and Nutrition, University of Navarra, Pamplona, Spain
[b] Department of Public Health, Section of Environmental Health, University of Copenhagen, Copenhagen, Denmark
[c] Department of Pharmacology & Toxicology, School for Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University, Maastricht, the Netherlands

ARTICLE INFO

Handling editor: Dr. Mathieu Vinken

Keywords:
Comet assay
CometChip
In vivo
In vitro
Enzyme
High-throughput

ABSTRACT

DNA damage plays a pivotal role in carcinogenesis and other diseases. The comet assay has been used for more than three decades to measure DNA damages. The 1–2 gels/slide format is the most used version of the assay. In 2010, a high throughput 96 macrowell format with a spatially encoded array of microwells patterned in agarose was developed, called the CometChip. The commercial version (CometChip®) has been used for the in vitro standard version of the comet assay (following the manufacturer's protocol), although it has not been compared directly with the 2 gels/slide format. The aim of this work is to developed new protocols to allow use of DNA repair enzymes as well as the analysis of in vivo frozen tissue samples in the CometChip®, to increase the throughput, and to compare its performance with the classic 2 gels/slide format. We adapted the manufacturer's protocol to allow the use of snap frozen tissue samples, using male Wistar rats orally dosed with methyl methanesulfonate (MMS, 200 mg/kg b.w.), and to detect altered nucleobases using DNA repair enzymes, with TK6 cells treated with potassium bromate ($KBrO_3$, 0–4 mM, 3 h) and formamidopyrimidine DNA glycosylase (Fpg) as the enzyme. Regarding the standard version of the comet, we performed thee comparison of the 2 gel/slide and CometChip® format (using the the manufacturer's protocol), using TK6 cells with MMS (100–800 μM, 1 h) and hydrogen peroxide (H_2O_2, 7.7–122.5 μM, 5 min) as testing compounds. In all cases the CometChip® was performed along with the 2 gels/slide format. Results obtained were comparable and the CometChip® is a good alternative to the 2 gels/slide format when a higher throughput is required.

1. Introduction

It has been estimated that tens of thousands of DNA lesions are generated in human cells per day (Lindahl and Barnes, 2000; Tubbs and Nussenzweig, 2017). DNA damage can occur by endogenous and exogenous agents and can be caused through different mechanisms (Barnes et al., 2018; Phillips and Arlt, 2009). It is vital to repair the DNA lesions correctly (Jackson and Bartek, 2009), otherwise DNA lesions could lead to mutations and, depending on the genes affected by those mutations, promote cancer. Moreover, DNA damage is linked to ageing (Schumacher et al., 2008), neurodegenerative disorders (Jaye Bix et al., 2019; Kulkarni and Wilson, 2008; Rass et al., 2007), and cardiovascular disease (Mercer et al., 2007). In 2019, the annual incidence rate (new cases per 100.000) worldwide for neoplasms was 3943 (accounted for 17.83% of total deaths), 10,406 for neurological disorders, including neurodegenerative disorders (3.93% of total deaths), and 717 for cardiovascular diseases (32.84% of total deaths) (Institute for Health Metrics and Evaluation (IHME), 2020). Therefore, it is important to develop reliable tools for studying and measuring DNA damage and repair.

The single-cell gel electrophoresis assay or comet assay was introduced in 1984 (Ostling and Johanson, 1984) and modified to its more common version (Singh et al., 1988) a few years later. It is a well-established technique for measuring DNA lesions in eukaryotic cells (Azqueta and Collins, 2013; Collins et al., 2023; Møller, 2018). To carry out the assay, cells are embedded in agarose and lysed to release negatively supercoiled DNA loops attached at intervals to a scaffold, the so-called nucleoids (Cook et al., 1976). In the presence of DNA strand breaks, supercoiling is relaxed, and DNA loops migrate towards the

* Corresponding author.
E-mail address: amazqueta@unav.es (A. Azqueta).

https://doi.org/10.1016/j.tox.2023.153690
Received 5 October 2023; Received in revised form 17 November 2023; Accepted 27 November 2023
Available online 29 November 2023

anode when electrophoresis is run. The amount of DNA that is able to migrate can be quantified and reflects the frequency of DNA breaks (Azqueta and Collins, 2013). It is worth mentioning that before the electrophoresis and during it, the nucleoids are exposed to a high pH that converts alkali-labile sites (ALS) into DNA strand breaks. Thus, not only DNA breaks are detected, but also ALS. The comet assay has also been modified to detect cross-links, altered nucleobases through lesion-specific DNA repair enzymes, bulky adducts and even DNA methylation status (Collins et al., 2023; Muruzabal et al., 2021b, 2021a; Ngo et al., 2021; Townsend et al., 2017). Moreover, the comet assay is used to measure DNA repair (Azqueta et al., 2019b, 2014).

The comet assay is relatively simple and versatile and has been used in many animal species, in human biomonitoring and in genotoxicity testing, both in vitro and in vivo (Azqueta et al., 2020; Collins et al., 2023; Gajski et al., 2019b, 2019a; Milić et al., 2021). However, it has some limitations, being the most serious one the high inter-laboratory variation that is partly due to the use of different assay conditions (Ersson et al., 2013; Forchhammer et al., 2012, 2010; Gedik et al., 2004; Johansson et al., 2010; Møller et al., 2023a). Adopting standard protocols should ameliorate this limitation and several efforts have been made in this direction such the development of an Organisation for Economic Co-operation and Development (OECD) test guideline for the in vivo mammalian alkaline comet assay (Organisation for Economic Co-operation and Development (OECD), 2016), or the recent publication of a compendium of protocols covering different sample types and comet assay modifications, prepared by the collaboration of nearly 80 authors from different countries (Collins et al., 2023). Moreover, technical recommendations to perform the comet assay (Azqueta et al., 2019c), and recommendations on the level of details to be reported on the procedure descriptions and results in order to interpret comet assay data across laboratories (Møller et al., 2020a), have been recently published. Protocols to measure DNA repair via a modified version of the comet assay have also been published (Vodenkova et al., 2020).

Another important focus point is the comet assay's throughput. Many laboratories use microscope slides as a support for one or two agarose gels containing the cells/nucleoids. The number of samples that can be analysed using this format of the assay depends on the size of the electrophoresis tank, the use of the standard or a modified-come assay (i.e, if enzymes are going to be used for the detection of some altered nucleobases, several gels have to be prepared per sample) and the use of more or less technical replicates per sample. The throughput can be increased using 12 minigels per slide (Shaposhnikov et al., 2010; Shaposhnikov and Collins, 2017) or even 96 minigels on a GelBond® film (Gutzkow et al., 2013). In the case of the 12 minigels, each of the gels can be incubated separately by using a device that isolate them; this is convenient when using different enzymes to detect altered nucleobases (Muruzabal et al., 2021a, 2021b). In 2010, the use of a single cell gel array, consisting of spatially separated microwells patterned on agarose with the aid of a stamp, to carry out the comet assay was reported (Wood et al., 2010). This version was named the CometChip platform in 2013 (Weingeist et al., 2013) and was some years later made commercially available as CometChip® (Trevigen). The composition of the commercial CometChip® (Trevigen) is unknown.

The new technology is based on a 96-well format by clamping a bottomless 96-well plate or using the commercial CometChip® System (containing a macrowell former of 96 wells, also bottomless, in a 96-wells pattern) on top of the chip (Ge et al., 2015, 2014). In this way, each of the 96 macrowells contains around 400 microwells inside, and each microwell is designed to contain a cell. Thus, this format reduces overlapping of comets, and the nucleoids/comets share the same focal plane. That can, in addition to increase the number of samples, facilitate the scoring process and thus, speed it up.

The protocol to perform the comet assay when using the homemade CometChip is similar to the traditional protocol once cells are loaded in the microwells. However, when using the commercial CometChip®, the protocol provided by the manufacturer should be followed. The main difference is the long electrophoresis time.

The homemade CometChip has been applied to the in vitro standard and enzyme-modified comet assay (Chao et al., 2020; Ge et al., 2021, 2013; Ngo et al., 2021; Rosenthal et al., 2023; Townsend et al., 2017; Watson et al., 2014; Xiong et al., 2021). Very recently, it has also been applied to the in vivo comet assay (Owiti et al., 2022). However, the commercial CometChip® has only been applied to the in vitro standard comet assay (Boyadzhiev et al., 2022; Seo et al., 2022; Sykora et al., 2018) which is expected since the manufacturer's protocol does not cover the use of DNA repair enzymes or in vivo samples.

The aim of this work is to develop new protocols to allow use of DNA repair enzymes as well as the analysis of in vivo frozen tissue samples in the CometChip®. Results obtained using the commercial CometChip® were compared with those obtained with a regular protocol for the classic 2 gels/slide version of the alkaline comet assay (Collins et al., 2023).

2. Materials and methods

2.1. Chemicals and reagents

Low melting point agarose, standard agarose, Hank's balanced salt solution containing Ca^{2+} and Mg^{2+} and phenol red-free (HBSS), sodium hydroxide (NaOH), Na_2EDTA, Triton X-100, Tris base, HEPES, bovine serum albumin (BSA), potassium chloride (KCl), potassium bromate ($KBrO_3$, CAS no. 7758–01–2, >99% purity), hydrogen peroxide (H_2O_2, CAS no. 7722–84–1, 30% w/w), methyl methanesulfonate (MMS, CAS no. 66–27–3, 99% purity), and 4′,6- diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Dulbecco's phosphate-buffered saline (DPBS) 1x and 10x were purchased from Gibco. Dimethyl sulfoxide (DMSO) was purchased from PanReac AppliChem. All cell culture reagents were purchased from Gibco. Formamidopyrimidine DNA glycosylase (Fpg) was obtained from NorGenoTech AS (Oslo, Norway). The 30-micron CometChips® and the CometChip® System were obtained from Bio-Techne.

2.2. Cell culture and treatment

TK6 cells (human-derived lymphoblastoid cell line) originally from the American Type Culture Collection (ATCC) were thawed under standard procedures. They were grown as a suspension culture (0.2–1 $\times 10^6$ cells/ml) in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% foetal bovine serum, 100 UI/ml penicillin and 0.1 mg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C and for no longer than 60 days.

For the standard comet assay, TK6 cells treated with four concentrations of MMS (100–800 μM) for 1 h at 37 °C, or five concentrations of H_2O_2 (7.7–122.5 μM) for 5 min on ice were used. Solvents were used as negative controls, DMSO for MMS, and PBS for H_2O_2. After treatment, cells were washed by centrifugation (250 g, 5 min, 4 °C), resuspended in PBS to a concentration of 1×10^6 cells/ml, and kept on ice until the standard comet assay was performed.

For the Fpg-modified version of the assay, we used $KBrO_3$ as genotoxic agent because this chemical at low concentrations generates mainly Fpg-sensitive sites without concomitant generation of DNA strand breaks. In addition, $KBrO_3$ has recently been introduced as a reliable assay/positive control for the Fpg-modified comet assay (Møller et al., 2023b, 2020b). TK6 cells treated with four concentrations of $KBrO_3$ (0.5–4 mM) for 3 h at 37 °C were employed. Milli-Q water was used as solvent for $KBrO_3$ and as negative control. After treatment, the cells were washed in PBS by centrifugation (250 g, 5 min, 4 °C), resuspended in freezing medium (i.e., cell growing medium supplemented with 1% DMSO), and frozen to − 80 °C using Nalgene© Mr. Frosty. Several aliquots of cells treated with the different $KBrO_3$ concentrations or the solvent were stored at − 80 °C until the Fpg-modified comet assay was performed.

2.3. In vivo samples

The objective of the in vivo study was to collect negative and positive samples (i.e., tissues) to study the applicability of the CometChip® to this type of samples, and to compare results obtained with this technology with the ones obtained with the commonly used 2 gels/slide format.

All procedures were approved by the Ethical Committee for Animal Experimentation of the University of Navarra and carried out in accordance with the ethical protocol CEEA 032–21 under the EU Directive 2010/63/EU for animal experiments (transposed to the Spanish Royal Decree 53/2013).

The reporting of the in vivo study has been performed following the 10 essential items of the ARRIVE guidelines (du Sert et al., 2020). Statistical analysis and results are shown in the respective sections.

2.3.1. Experimental-animals

Six male Wistar rats (8 weeks old) were purchased from ENVIGO.

2.3.2. Inclusion and exclusion criteria

Weight variation did not exceed ± 20% at arrival. Other inclusion and exclusion criteria were not applied.

2.3.3. Randomisation

The rats were randomly distributed in two groups (3 animals each). Briefly, the animals were randomly taken, labeled according to the study groups (i.e., negative and positive controls), and then weighed.

2.3.4. Study design

For the collection of negative control tissues, a group of animals were administered with saline. For the positive control tissues, a group of animals received MMS. All animals were sacrificed 3 h after the administration of saline or MMS. One animal of each group was administered per day. Only one piece of tissue from each animal was used for each experiment and therefore was considered to be the experimental unit.

2.3.5. Sample size

Three animals per treatment group and two groups were used. We selected a small sample size because, as mentioned before, the objective of this study was not to elucidate the genotoxicity of a test compounds but to study the applicability of a high throughput version of the comet assay to analyses cells from negative and positive tissues, and to compare the results with the commonly used 2 gels/slide format.

2.3.6. Experimental-procedures

The rats were weighed the day of the arrival and the acclimation period was 5 days. The environmental conditions were 12 h day/night cycle, temperature 22 °C, relative humidity 55 ± 20%, standard diet, and water ad libitum. After the acclimatation period, an overnight fasting period was carried out before the administration. Three animals receive a single dose of saline by oral gavage and the other three animals receive a single dose of 200 mg/kg b.w. of MMS by the same route of administration. As mentioned before, one animal of each group was administered per day. The average weight of the 6 animals was 264.7 ± 21.9 g and a dosing volume of 1 ml/100 g b.w. was taken from a solution of 20 mg/ml MMS or saline. Three hours after the administration, animals were sacrificed via asphyxia in a CO_2 cabin. Samples of liver, kidney (containing cortex and medulla) and duodenum were obtained immediately after sacrifice. The samples were processed into aliquots (sections of approximately 2 × 2 × 2 mm, 2 × 3 × 5 mm and 1.5 cm of liver, kidney and duodenum, respectively), transferred to cryotubes, and snap-frozen in liquid nitrogen. The samples were stored at − 80 °C for 9 months before the analysis.

2.3.7. Blinding/Masking

None of the animal procedures were done blind.

2.3.8. Outcome-measures

The standard comet assay was performed in an aliquot of each sample obtained as described in the following section. The rest of the samples were used for potential future determinations/experiments.

2.4. The standard comet assay

Two different formats were used simultaneously, the CometChip® and the classic 2 gels/slide version of the alkaline comet assay.

2.4.1. In vitro samples

When the 2 gels/slide format was used, the protocol applied was as follows. Thirty μL of MMS-or H_2O_2-treated cell suspensions (1×10^6 cells/ml) was mixed with 140 μL of 1% LMP agarose in PBS at 37 °C (final agarose concentration of 0.82%), and two drops of 70 μL were placed into an agarose precoated glass microscope slide (previously dried). After that, 20 × 20 mm coverslips were used to extend the drops, and slides were placed on a cold metal plate for around 2 min until the gels were solid and the coverslips could be removed. Slides were then placed into a Coplin jar with cold lysis solution (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris, pH 10.0; 1% Triton X-100 added before use) for 1 h at 4 °C. After lysis, slides were moved to an electrophoresis tank inside a cold room (4 °C) and 1 L of alkaline solution (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) was poured in it fully covering the slides for 40 min. Afterwards, electrophoresis was run at 1.2 V/cm (measured as voltage drop across the platform) for 20 min. Then, neutralization was done for 10 min in PBS and 10 min in demineralized water (dH_2O). Gels were then air dried overnight.

The CometChip® was used following the manufacturer instructions. First, the chip was left in PBS for 30 min at room temperature. After that, it was inserted into the CometChip® System (Bio-Techne) and excess PBS was carefully aspired from all wells. Cells were adjusted to 2×10^5 cells/ml and loaded into the CometChip® using a volume of 100 μL per well. Four wells were loaded per condition. The whole system (containing the chip with cells) with the clear lid was then placed into an incubator at 37 °C. After 10 min, the system was rocked N-S and E-W to facilitate cell loading into the micropores and placed back into the incubator for another 10 min.

After cell loading, the CometChip® was removed from the system and washed with around 5 ml of PBS, pipetting in a continuous fashion while having the chip at an angle of around 45° and making sure that all the wells were washed. A quality check was performed under an inverted microscope to ensure that enough cells were loaded into the micropores. Subsequently, the CometChip® was placed on a flat surface. Approximately 5 ml of 1% low melting point (LMP) agarose in PBS at 37 °C was poured in a single continuous serpentine fashion between rows and avoiding wells, evenly covering the chip surface. The chip was kept at room temperature for 3 min and at 4 °C for 12 min to allow agarose gelation. The CometChip® was then placed in a horizontal position in 100 ml of lysis solution 1 h at 4 °C. After lysis, alkaline unwinding was performed at 4 °C for 40 min by immersing it in the alkaline solution. Electrophoresis was run at 1 V/cm for 50 min (the voltage was applied and measured across the platform). Neutralization was done for 2 × 15 min in PBS and 30 min in dH_2O.

2.4.2. In vivo samples

The CometChip® has not been used before with tissue samples, either fresh or frozen. Thus, the manufacturer's protocol was initially followed, modifying only the way the cell suspension was obtained.

Frozen aliquots of liver, kidney or duodenum from non-treated or MMS-treated rats were placed inside a chamber of a dry ice-chilled metal pulveriser and a single sharp hammer impact was given to a piston above the sample. After the compression of the aliquots, the plain dishes

obtained were disaggregated and homogenized in 2 ml of mincing solution (HBSS supplemented with 20 mM Na_2EDTA and adjusted to pH 7.5; just prior use 10% of DMSO was added) with a Pasteur pipette, obtaining a cellular suspension. The cell suspension was diluted 1:5 in cold mincing solution to, immediately after, pipette 100 μL per well using 4 wells per condition into the CometChip® inserted into the CometChip® System. Cells were loaded for 20 min at 37 °C. During cell loading, agarose embedding of the 2 gels/slide format was done, taking cells from the non-diluted cell suspension. The rest of the procedure was as described in in vitro standard comet assay section.

In subsequent attempts, some parts of the CometChip® protocol were modified as follows: all parts of the CometChip® System (the carrier base and the macrowell former) were precooled to 4 °C the day before. Before starting the experiment, the CometChip® was left in PBS for 30 min at 4 °C. The assembled CometChip® System with the chip within was kept on ice during the cell pipetting and inside the fridge for the 20 min cell loading.

2.5. The Fpg-modified comet assay

As in the case of the standard comet assay, two different formats were used simultaneously, the CometChip® and the classic 2 gels/slide version.

TK6 cells treated with $KBrO_3$ and the negative control were thawed. In the case of the 2 gels/slide format, gels were prepared as described in Section 2.4.1 (In vitro samples) and two slides were prepared per condition. Similarly, cells were loaded in the CometChip® as explained in the same section; eight wells were loaded per condition.

In the case of the 2 gels/slide format and after lysis, the slides were washed with enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0) in a Coplin jar for 3 × 5 min. After that, slides were placed on a metal plate and 45 μL of Fpg (1:30000 dilution) or 45 μL of enzyme reaction buffer were added on top of each gel, and covered with a 22 × 22 mm coverslip. The two gels on one of the slides prepared per condition were incubated with the enzyme reaction buffer, while the gels on the other slides prepare per condition were incubated with Fpg. Finally, the slides were transferred to a moist box and incubated for 1 h at 37 °C. After enzyme incubation, coverslips were removed.

The use of the CometChip® with DNA repair enzymes is novel. The manufacturer's protocol does not include the use of this modification of the assay. In the first attempt, the enzyme reaction buffer washing and the enzyme incubation procedures were simply included in the manufacturer's protocol after the lysis step. Briefly, after lysis, the CometChip® was washed 3 times with enzyme reaction buffer (5 min each). Then, it was introduced into the CometChip® System and a volume of 100 μL of 1:30000 Fpg or enzyme reaction buffer was pipetted into each well. The CometChip® System was covered with a lid and incubated for 1 h at 37 °C. Four wells per conditions were incubated with Fpg and the other 4 with the enzyme reaction buffer. Alkaline treatment, electrophoresis, neutralization and washing were performed as described in the Section 2.4.1 (In vitro samples).

In subsequent attempts, the following modifications were made: 1) two different concentrations of agarose were used for the chip overlay: 1% LMP and 0.6% LMP agarose; 2) the duration of enzyme reaction buffer washings was extended to 3 × 15 min; and 3) two different Fpg concentrations were tested (1:5000 and 1:30000).

2.6. Staining and scoring comets

Slides were stained with a drop of 30 μL of 1 μg/ml DAPI per gel, covered with 22 × 22 mm coverslips, and incubated for at least 30 min at room temperature. The CometChip® was immersed in a 1 μg/ml DAPI solution, covered with aluminum foil, and kept at 2–8 °C until next day. Prior to scoring, the chip was distained in Milli-Q water for 1 h.

Comet Assay IV (previously Perceptive Instruments, currently Instem) was the software employed for analysis and % DNA in tail was the parameter chosen to measure DNA damage. A hundred randomly selected comets were analysed per condition; they were scored from two duplicated gels/wells (50 comets each). In the case of the CometChip®, 50 comets were scored per well and only two wells were analysed per condition. The other two wells were prepared just in case not enough cells/comets were found in two wells. Median of 100 comets were calculated per condition. For the enzyme-modified comet assay, net Fpg-sensitive sites were obtained by subtracting % DNA in tail obtained in the enzyme reaction buffer from that measured in Fpg gels/wells. Non-quantifiable hedgehog comets were assigned a value of 100% DNA in tail.

2.7. Statistical analysis

At least three independent experiments were performed and mean and standard deviation (SD) are reported. Results on genotoxicity in cells are assessed by analysis of covariates (ANCOVA) test, which tests the difference in slopes between regression lines. Post-hoc estimation of slopes, standard error (SE) and 95% confidence interval (CI) are based on linear regressions in strata of each condition. Coefficient of determination (r^2-values) of the relationship between concentrations of the compounds and the %DNA in tail obtained were calculated. The full dataset on DNA strand breaks by H_2O_2 exposure has a non-linear concentration-response relationship because of saturation of the comet assay at the high concentrations (i.e. the %Tail DNA comet descriptor cannot be higher than 100%). This deviation from linearity is seen in analyses where the maximal concentration of H_2O_2 are omitted (i.e. the r^2-value increases from 0.74 to 0.87 by excluding 122 μM). Thus, the highest H_2O_2 concentrations are not included in the statistical analysis, although the results are shown in the paper. The full concentration range of MMS in cell culture experiments is included in the statistical analysis because it has a high coefficient of determination ($r^2 = 0.95$). The analyses of Fpg-sensitive sites generated by $KBrO_3$ exposure in cells did not include the highest concentration of $KBrO_3$ because a ceiling effect was obtained at 2 mM $KBrO_3$ (i.e. approximately 90% Tail DNA). Analyses of the effect of agarose density (1% vs 0.6%) and Fpg dilution (1:30,000 vs 1:5000) was assessed by ANCOVA test using $KBrO_3$ concentration as continuous variable and full factorial model with interaction between agarose density and Fpg concentration as independent (categorical) factors. In addition, Student's t-tests have been used to compare differences in DNA migration values by the standard and CometChip® format in different concentration/dose groups and the correlations between the two formats were studied (these analyses were performed with the entire set of data). Results on DNA damage levels in animal tissues have been analyzed by full factorial ANOVA with dose of chemical and detection format (standard vs CometChip®) as independent factors. Statistical analyses were done in Stata 15 (StataCorp LCC, College Station, TX, USA).

3. Results

3.1. DNA strand breaks in cells by exposure to H_2O_2 or MMS

Fig. 1 shows the results obtained in H_2O_2 and MMS-treated cells analysed by the standard comet assay and using the 2 gels/slide format and the CometChip®. Both compounds generated DNA strand breaks in a concentration-dependent manner. Some significant differences in level of migration were seen at some of the concentrations tested. The MMS dataset (Fig. 1A) showed a higher slope in the 2-gel condition (0.11 ± 0.01; %Tail DNA units per μM of MMS) as compared to the CometChip® (0.08 ± 0.01; %Tail DNA units per μM of MMS) (slope ± SE; $P < 0.05$ for differences in slopes). The same effect was seen in the H_2O_2 dataset (Fig. 1B), where the concentration-response relationship was steeper for samples in the 2-gel assay (1.28 ± 0.15; %Tail DNA units per μM of H_2O_2) as compared to the CometChip® (0.89 ± 0.09; %Tail DNA units

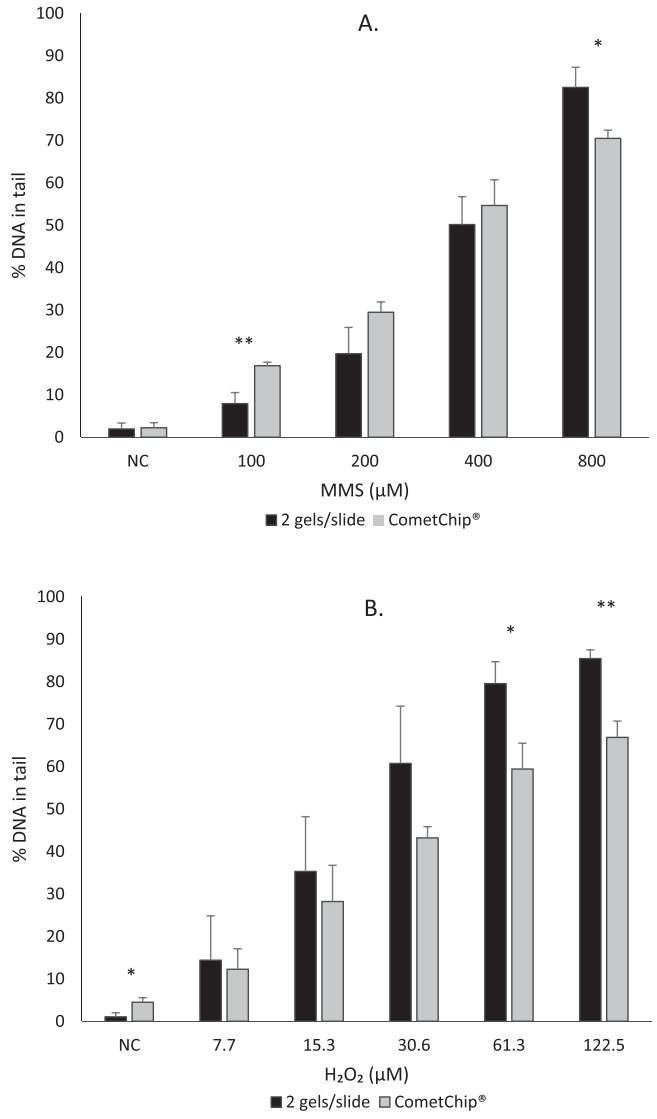

Fig. 1. In vitro standard comet assay. A comparison of the 2 gels/slide (black) with the CometChip® (grey) formats in TK6 cells treated with MMS (A) or H_2O_2 (B). Mean and SD of three independent experiments are represented. Student's t-tests were used to compare formats at each concentration (* $P < 0.05$, ** $P < 0.01$). NC: Negative control.

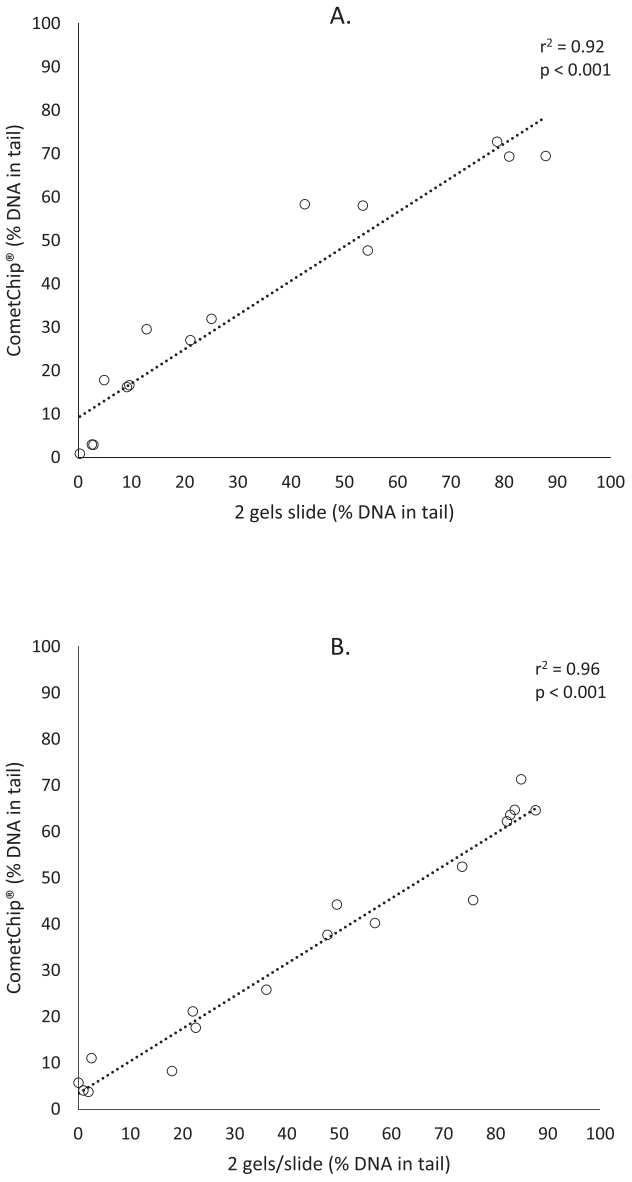

Fig. 2. In vitro standard comet assay. Linear correlation between 2 gels/slide and the CometChip®. Each symbol is one independent experiment. The results are from 3 independent experiments experiments using TK6 cells treated with MMS (A) or H_2O_2 (B) is represented. Determination coefficients (r^2) and p-values are also shown.

per μM of H_2O_2) (slope ± SE; $P < 0.05$ for differences in slopes). The r^2 values for the MMS dataset were 0.97 for the 2 gels/slide and 0.91 for the CometChip®, with $p < 0.001$ in both cases. In the case of the H_2O_2, the r^2 values were 0.86 for the 2 gels/slide and 0.91 for the CometChip® format, with also $p < 0.001$ in both cases.

Linear correlation between the formats were high both for MMS ($r^2 = 0.92$, $P < 0.001$,) and H_2O_2 ($r^2 = 0.96$, $P < 0.001$) treatments (Fig. 2).

3.2. *DNA strand breaks in frozen tissue samples from animals after exposure to MMS*

The first attempt to analyse tissue samples in the CometChip® format resulted in high backgound levels of DNA migration (close to 80% DNA in tail) in frozen liver, kidney and duodenum samples of male Wistar rats treated with 200 mg/kg b.w. MMS (data not shown). This makes it impossible to observe further increase in the DNA migration level in samples from MMS-treated rats.

This prompted us to modify the chip conditions to reduce the background level of DNA migration. The CometChip® System was precooled from the day before use, cell suspension was pipetted into the macrowells with the whole system on ice, and cells were allowed to settle down within the microwells at 4 °C instead of at 37 °C. The genotoxic effect of MMS exposure in animal tissue samples after modifying the CometChip® protocol is shown in Fig. 3. Background levels are low (< 10% tail DNA in all cases) in the three organs tested independently of the format used. There is not statistical significance of the interaction term between MMS exposure and the type of comet assay format (standard vs CometChip®) for DNA strand breaks in the liver ($P = 0.19$), kidney ($P = 0.21$) and duodenum ($P = 0.36$). However, there are single-factor effects of the MMS exposure in all of the three organs ($P < 0.001$). In addition, there are statistically significantly higher DNA migration values in liver ($P < 0.05$) and kidney ($P < 0.05$) tissues when measured by the CometChip® format as compared to the standard assay, whereas the differences were not statistically significant in duodenum tissue ($P = 0.10$). However, no statistically significant differences were obtained between formats except in the liver of the negative control when a

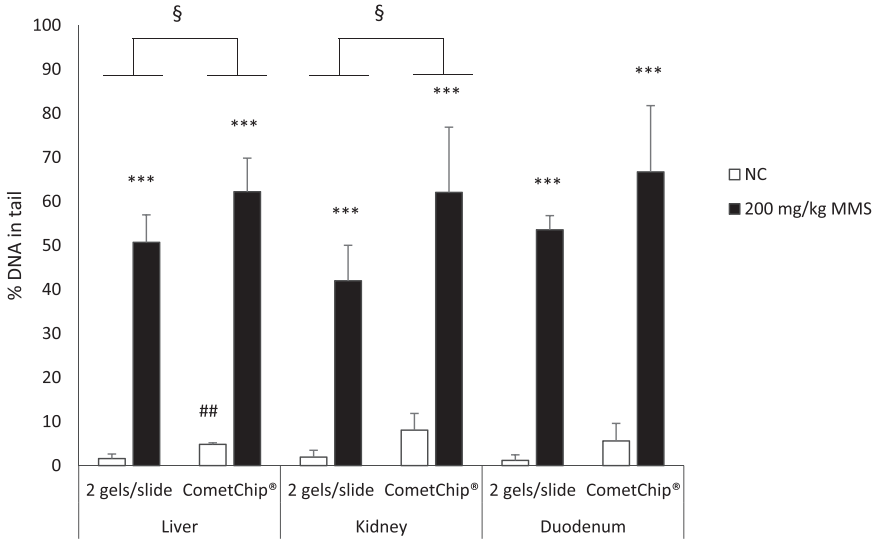

Fig. 3. In vivo standard comet assay. A comparison of DNA strand break and ALS values in frozen samples obtained from untreated (white bars) and MMS-treated (black bars) by the 2 gels/slide (black) with the CometChip® (grey) formats. Mean and SD of three independent experiments are represented. ##P < 0.01 (difference in level of DNA migration, Student's t-test), §P < 0.05 (higher level of DNA migration in samples analysed by CometChip®, single-factor effect), and ***P < 0.001 (difference between MMS and untreated, single-factor effect)." NC: Negative control.

Student's t-test was applied (Fig. 3).

3.3. *Fpg-sensitive sites in cells by exposure to $KBrO_3$*

Adopting the protocol for the 2 gels/slide enzyme-modified comet assay commonly used in our laboratory to the CometChip® resulted in a lack of detection of $KBrO_3$ induced lesions (data not shown).

The effect of reducing the LMP agarose overlay concentration and increasing Fpg concentration, in addition to longer enzyme reaction buffer washings (3 ×15 min), when using the CometChip®, was compared to the 2 gel/slide format by means of a $KBrO_3$ concentration-response curve and the detected Fpg-sensitive sites (Fig. 4). Table 1 shows the results from linear regression analyses called condition 1–5. As it can be seen, the assay condition has a strong effect on the detection of concentration-response relationships (P < 0.001 for difference concentration-response relationships between different assay conditions). The concentration-response relationships (slopes) were not statistically significantly different between assay conditions 1, 3 and 5. However, these were different from assay conditions 2 and 4. A

Table 1
Relationship between $KBrO_3$ concentration and level of Fpg-sensitive sites in TK6 cells.

Condition	Agarose	Fpg	Slope (95% CI)[a]	r^2-value
(1) Standard (2 gels/slide)	1%	1:30000	37.4 (30.8, 44.1)	0.91 ***
(2) CometChip®	1%	1:30000	4.4 (0.4, 8.4)	0.28 *
(3) CometChip®	1%	1:5000	28.7 (18.7, 38.7)	0.72 ***
(4) CometChip®	0.6%	1:30000	7.7 (2.4, 13.0)	0.42 **
(5) CometChip®	0.6%	1:5000	40.2 (28.6, 51.8)	0.80 ***

[a] Slopes are based on linear regression (i.e. increase in %Tail DNA per mM of $KBrO_3$; concentration range 0–2 mM). These are statistically different a 5% level when the 95% confidence intervals (95% CI) do not overlap.

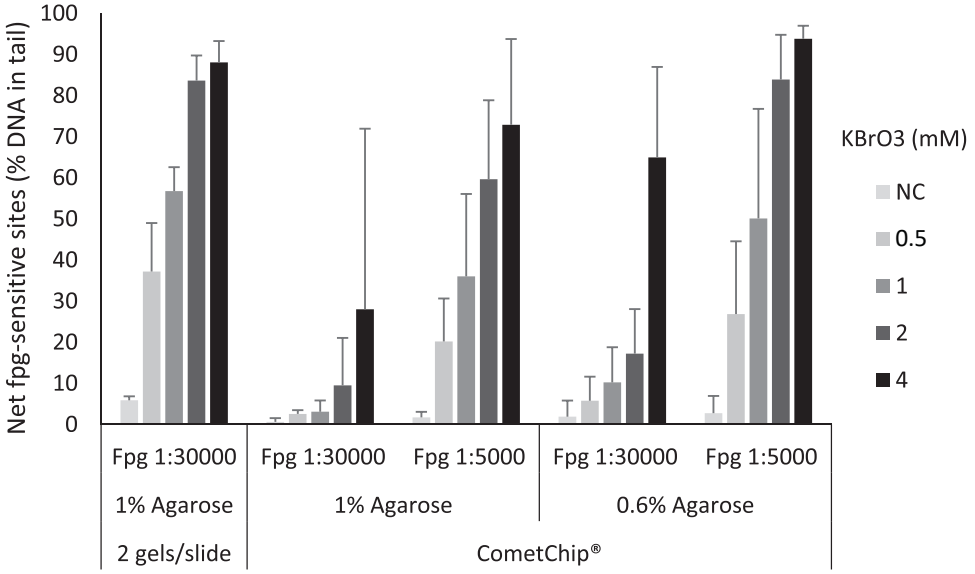

Fig. 4. In vitro Fpg-modified comet assay. Results obtained treating TK6 cells at different concentrations of $KBrO_3$ and modifying agarose and Fpg concentrations in the case of the CometChip®. Mean and SD of four independent experiments are represented. NC: Negative control.

statistical analysis indicates additive effects of the amount of Fpg and agarose (i.e. $P = 0.40$ for interaction between Fpg and agarose), where the amount of Fpg ($P < 0.001$) is a stronger predictor that the density of agarose ($P < 0.05$) for the level of Fpg-sensitive sites.

Linear correlation of the results obtained with the 2 gels/slide format and the best CometChip® condition (i.e. condition 1 vs 5 in Table 1) is presented in Fig. 5. A highly statistically significant correlation was found ($r^2 = 0.81$, $p < 0.001$). No significant differences were found after comparing the net-Fpg sensitive sites found in each of the concentrations tested with the two formats when applying a Student's t-test (data not shown).

4. Discussion

4.1. General findings

In this study, we show that analysis of tissue samples and oxidatively damaged DNA (i.e. Fpg-modified comet assay) in the commercial CometChip® format is possible after modifications of the original protocol. In principle, there is not a systematic trend toward a difference in DNA migration levels between the 2 gel/slide and CometChip® formats as DNA migration levels tended to be higher in TK6 cells treated with H_2O_2 and MMS in the 2 gels/slide format, whereas there were higher DNA migration levels by the CometChip® format in animal tissues, and levels of Fpg-sensitive sites in TK6 cells treated with $KBrO_3$ were similar between the 2 gel/slide and CometChip® formats. Nevertheless, it should be kept in mind that DNA migration (i.e. measurement of the percentage of fluorescence in comet tails) is not identical to the number of DNA lesions because it depends on the assay conditions. Concentration of the agarose, and the duration and strength of the electrophoresis influence the DNA migration (Azqueta et al., 2019c, 2011; Ersson and Möller, 2011). Thus, changing these parameters in any of the format used we can modulate the migration of the DNA and thus the results obtained, especially in cells that contains a certain level of lesions (in control cells the effect is limited unless very extreme conditions are used). Cell irradiated with ionizing radiation can be included in the comet assay protocol for the purpose of calibrating the assay and transform the primary comet descriptor to number of lesions per unaltered base pair (Brunborg et al., 2023). This option is very important when results in inter-laboratory trials are going to be compared.

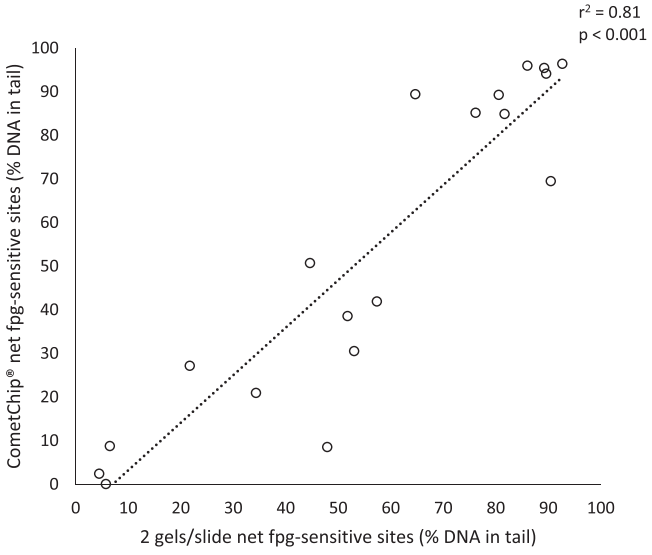

Fig. 5. Fpg-modified comet assay. Linear correlation between 2 gels/slide and the CometChip®. Fpg-sensitive sites (% DNA in tail) from three independent experiments using TK6 cells treated with $KBrO_3$ are represented. Each symbol is one result. Determination coefficient (r^2) is shown. * ** $P < 0.001$.

4.2. In vitro standard comet assay

Concerning the in vitro standard comet assay, the CometChip® manufacturer's protocol was followed step by step, only modifying when the treatment was performed (we selected an off-Chip chemical exposure) and the fluorescent dye (DAPI instead of SYBR Gold). The V/cm applied according to the manufacturer's protocol was estimated to be around 1 V/cm using our tank. The steps of the protocol to perform the comet assay using the chips are similar to the traditional one, but the CometChip® format has some particularities that are discussed below. In addition, our experimental work to amend protocols for the CometChip® setup on tissues and oxidatively damaged DNA led to certain technical issues (see Sections 4.3 and 4.4), which are worthwhile mentioning to researchers who intend on expanding the technique beyond in vitro experiments.

To load the cells in the CometChip® System, suspended cells are added to the macrowells to settle down by gravity into the microwells. Some of the factors affecting this step are cell type, cell size, cell density, microwell size and loading time. Actually, there are CometChips® with different size of microwells diameter (i.e. 20, 30 and 40 μm). After cell loading, excess off-grid cells must be rinsed out, being this the most technically challenging step because shear force is difficult to control. A lack of washing can lead out to off-grid cells while an excess of it can eventually remove them from the microwells. To control that, it is advised to check microwell filling under the microscope before continuing with the experiment (Chao and Engelward, 2020). Cell embedding in agarose is also different than in classic formats. In this case a low melting point agarose overlay is pipetted above the microwell array that has the desired normal melting point agarose concentration in the case of the homemade CometChip, but an unknown composition in the CometChip®.

Another important aspect is that the exposure to compounds of interest can occur before or after loading cells onto the chip. It has been published that differences between on-Chip and off-Chip chemical exposure could be significant, being lower in the off-Chip exposure (Chao et al., 2020). A possible explanation is the interference of DNA repair in the results obtained. Cell loading at 4 °C when off-Chip exposure is selected could be an alternative to try to diminish that influence of DNA repair processes and may be applied also when using in vitro comet assay. DNA repair can be a factor why we observe some differences between the two formats, especially when fresh TK6 (i.e., cultured cells) and the standard version of the assay were used. For loading the cells, they were incubated at 37 °C for 20 min, enough for repairing part of the single strand breaks induced by the MMS and H_2O_2. Frozen cells or tissues do not have the same ability to repair the induced lesions and it is generally avoided to thaw frozen samples to room temperature or above because it may activate endogenous nucleases as well as DNA repair enzymes (Møller et al., 2021).

Moreover, adherent cell lines usually require attachment for normal behavior, which questions the possibility of an on-Chip exposure because agarose may interfere with that behavior and, in addition, interact with the studied compound. Preliminary results in our laboratory indicate that transfer of treated cells between standard 96-well plates and the CometChip® can be done with a multichannel pipette, saving in time and labor.

4.3. In vivo comet assay

When performing an in vivo comet assay, preparation and processing length should be as short and constant as possible especially when the number of animals, studied specimens or conditions is high. To overcome that it is a good alternative to snap freeze samples, and a high throughput comet assay format makes it possible to analyze all samples under the same experimental condition. The thawing process is of crucial importance to ensure that the tissue's DNA is intact (Azqueta et al., 2019a) and samples should be maintained cold (Guérard et al.,

2014) to avoid the interference of DNA repair or an excess of DNA damage. For that reason, the cell loading step should be performed at 4 °C to avoid high background DNA damage in frozen samples, however, keeping samples cold during cell loading could be less relevant when fresh tissues are employed. In case of using fresh tissues the manufacturer protocol to apply the CometChip® may work.

4.4. The enzyme-modified comet assay

We modified three variables in the enzyme-modified comet assay: enzyme buffer washings length, overlay agarose and enzyme concentrations. We increased the enzyme reaction buffer washings to 3 × 15 min instead of 3 × 5 min prior to the incubation with Fpg. A possible explanation is that the CometChip® has a larger surface and the microwell array (with unknown composition) attached to the glass is thicker than in other platforms. We also decreased the low melting point agarose concentration of the layer pipetted above the microwell array, from 1% to 0.6%, to facilitate the enzyme diffusion. Moreover, we used a higher enzyme concentration than the one used in the 2 gels/slide format. As mentioned above, results obtained using these conditions were similar than the ones obtained when using the 2 gels/slide format. Using different DNA repair enzymes, with different specificities, on one sample under the same experimental conditions can provide more mechanistic information about the studied compound. Hence, setting up the CometChip® for its use in the enzyme-modified comet assay can be an optimal tool in this regard.

4.5. Recommendations

The recommendations to use the CometChip®, as discussed in the previous paragraphs (sections 4.2–4.4), are summarized in Table 2. These recommendations should be carried out in combination with the manufacturer's protocol.

4.6. The potential use of the CometChip®

Genotoxicity testing high throughput is always a desirable feature. The approach followed for increasing the throughput of the comet assay has been to optimize the critical steps (Bivehed et al., 2020; Bivehed and Hellman, 2020; Enciso et al., 2018, 2015), to increase the number of samples per slide or Gelbond® or even to expand the electrophoresis tank capacity while diminishing slide manipulation (Karbaschi et al., 2019; Karbaschi and Cooke, 2014). With the CometChip format, the number of samples that can be analyzed per run increases in comparison with the most commonly used formats since three CometChips of 96 macrowells each can fit in a standard electrophoresis tank. Moreover, having arrayed comets can also diminish scoring time, even if no automated scoring is used since less user adjustments using the scoring software are needed and less overlapped comets are found.

CometChip throughput is similar to placing 96 minigels, following a pattern of a 96 well plate, in a Gelbond® film (Gutzkow et al., 2013). Using the CometChip instead of the mentioned version using Gelbond® film has some advantages and disadvantages. The array of cells in the CometChip facilitates the scoring of the results, and using the macrowell format, it is possible to incubate each of the wells separately. In the case of the Gelbond®, the incubations should be done in a bath and all the gels are incubated with the same chemical or enzyme. On the other hand, the CometChip® is by far more expensive than the use of Gelbond® films. Depending on the design of the study, one or the other can be selected.

The use of the CometChip® is quite limited nowadays, probably due to the relatively high cost per chip. However, it is a great tool when a lot of samples need to be analyzed at the same time, as in a human biomonitoring study or in an in vivo experiment. It may be a great tool to perform the in vitro repair assay in which substrate cells containing specific lesions are incubated with cell extracts from different donors or treatments (Vodenkova et al., 2020). Moreover, it may be very interesting to explore if the use of the CometChip® decreases the quite high inter-laboratory variation reported for the comet assay.

Table 2
Recommendations to use the CometChip® with cells cultured in vitro, frozen tissues or in combination with Fpg.

Assay	Recommendation
In vitro comet assay	- Check the loading of the cells under the microscope (Chao and Engelward, 2020). - Cell loading can be performed 4 °C when off-Chip exposure is selected to diminish the influence of DNA repair
In vivo comet assay with frozen animal tissues	- Perform the cell loading at 4 °C
Enzyme-modified comet assay	- After loading the cells on the chip add a layer of 0.6% low melting point agarose in PBS at 37 °C - After lysis wash the chip with the corresponding enzyme buffer 3 × 15 min - After washing the chip with the enzyme buffer, inserted it into the CometChip® System and add 100 μL of enzyme. In the case of the Fpg the concentration should be 6 x the used in the 2 gels/slide format.

5. Conclusions

In summary, the pursuit of a high throughput comet assay is a popular topic among comet assay's users. The CometChip technology is a promising tool in this respect. The commercial CometChip® protocol only covers the standard in vitro version of the assay and to try to leverage this tool some modifications were made to adapt it to allow the use of DNA repair enzymes and in vivo frozen samples, versions that may significantly benefit from the increase of throughput of the assay.

Funding

This work has been supported by the Grant PID2020–115348RB-I00 funded by MCIN/AEI/10.13039/501100011033 and the European Partnership for the Assessment of Risks from Chemicals (PARC) under the Horizon Europe Research and Innovation Programme, Grant Agreement No. 101057014. MC was supported by a predoctoral grant (PRE2021-096942) funded by MCIN/AEI/10.13039/501100011033 and the FSE+ .

CRediT authorship contribution statement

Collia Miguel: Formal analysis, Investigation, Methodology, Writing – original draft. **Møller Peter:** Data curation, Formal analysis, Writing – review & editing. **Sabine A.S. Langie:** Methodology, Writing – review & editing. **Vettorazzi Ariane:** Conceptualization, Writing – review & editing. **Azqueta Amaya:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Amaya Azqueta reports financial support was provided by Spanish Ministry of Science and Innovation. Miguel Collia reports financial support was provided by Spanish Ministry of Science and Innovation. Amaya Azqueta reports financial support was provided by European Commission.

Data Availability

Data will be made available on request.

References

Azqueta, A., Collins, A.R., 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch. Toxicol. 87, 949–968. https://doi.org/10.1007/s00204-013-1070-0.

Azqueta, A., Enciso, J.M., Pastor, L., López de Cerain, A., Vettorazzi, A., 2019a. Applying the comet assay to fresh vs frozen animal solid tissues: A technical approach. Food Chem. Toxicol. 132, 110671 https://doi.org/10.1016/j.fct.2019.110671.

Azqueta, A., Gutzkow, K.B., Brunborg, G., Collins, A.R., 2011. Towards a more reliable comet assay: optimising agarose concentration, unwinding time and electrophoresis conditions. Mutat. Res-Gen. Tox En. 724, 41–45. https://doi.org/10.1016/j.mrgentox.2011.05.010.

Azqueta, A., Ladeira, C., Giovannelli, L., Boutet-Robinet, E., Bonassi, S., Neri, M., Gajski, G., Duthie, S., Del Bo', K., C., Riso, P., Koppen, G., Basaran, N., Collins, A., Møller, P., 2020. Application of the comet assay in human biomonitoring: An hCOMET perspective. Mutat. Res 783, 108288. https://doi.org/10.1016/j.mrrev.2019.108288.

Azqueta, A., Langie, S.A.S., Boutet-Robinet, E., Duthie, S., Ladeira, C., Møller, P., Collins, A., Godschalk, R., 2019b. DNA repair as a human biomonitoring tool: Comet assay approaches. Mutat. Res Rev. Mutat. Res 781, 71–87. https://doi.org/10.1016/j.mrrev.2019.03.002.

Azqueta, A., Muruzabal, D., Boutet-Robinet, E., Milic, M., Dusinska, M., Brunborg, G., Møller, P., Collins, A.R., 2019c. Technical recommendations to perform the alkaline standard and enzyme-modified comet assay in human biomonitoring studies. Mutat. Res Gen. Tox En. 843, 24–32. https://doi.org/10.1016/J.MRGENTOX.2019.04.007.

Azqueta, A., Slyskova, J., Langie, S.A.S., Gaivão, I.O.N., Collins, A., 2014. Comet assay to measure DNA repair: approach and applications. Front. Genet. 5, 288. https://doi.org/10.3389/fgene.2014.00288.

Barnes, J.L., Zubair, M., John, K., Poirier, M.C., Martin, F.L., 2018. Carcinogens and DNA damage. Biochem Soc. Trans. 46, 1213–1224. https://doi.org/10.1042/BST20180519.

Bivehed, E., Hellman, B., 2020. Flash-comet assay. MethodsX 7, 101161. https://doi.org/10.1016/J.MEX.2020.101161.

Bivehed, E., Söderberg, O., Hellman, B., 2020. Flash-comet: Significantly improved speed and sensitivity of the comet assay through the introduction of lithium-based solutions and a more gentle lysis, 503240 Mutat. Res Genet Toxicol. Environ. Mutagen 858–860. https://doi.org/10.1016/J.MRGENTOX.2020.503240.

Boyadzhiev, A., Solorio-Rodriguez, S.A., Wu, D., Avramescu, M.L., Rasmussen, P., Halappanavar, S., 2022. The high-throughput in vitro cometchip assay for the analysis of metal oxide nanomaterial induced DNA damage. Nanomaterials 12, 1844. https://doi.org/10.3390/nano12111844.

Brunborg, G., Eide, D.M., Graupner, A., Gutzkow, K., Shaposhnikov, S., Kruszewski, M., Sirota, N., Jones, George D.D., Koppen, G., Vanhavere, F., Møller, P., Stetina, R., Dahl, H., Collins, A., Jones, G.D.D., 2023. Calibration of the comet assay using ionising radiation. Mutat. Res. -Genet. Toxicol. Environ. Mutagen. 885, 503560 https://doi.org/10.1016/j.mrgentox.2022.503560.

Chao, C., Engelward, B.P., 2020. Applications of CometChip for Environmental Health Studies. Chem. Res. Toxicol. 33, 1528–1538. https://doi.org/10.1021/acs.chemrestox.9b00393.

Chao, C., Ngo, L.P., Engelward, B.P., 2020. SpheroidChip: patterned agarose microwell compartments harboring hepg2 spheroids are compatible with genotoxicity testing. ACS Biomater. Sci. Eng. 6, 2427–2439. https://doi.org/10.1021/acsbiomaterials.9b01951.

Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G., Chao, C., Cooke, M.S., Costa, C., Costa, S., Dhawan, A., de Lapuente, J., Bo', C.Del, Dubus, J., Dusinska, M., Duthie, S.J., Yamani, N.El, Engelward, B., Gaivão, I., Giovannelli, L., Godschalk, R., Guilherme, S., Gutzkow, K.B., Habas, K., Hernández, A., Herrero, O., Isidori, M., Jha, A.N., Knasmüller, S., Kooter, I.M., Koppen, G., Kruszewski, M., Ladeira, C., Laffon, B., Larramendy, M., Hégarat, L.Le, Lewies, A., Lewinska, A., Liwszyc, G.E., de Cerain, A.L., Manjanatha, M., Marcos, R., Milić, M., de Andrade, V.M., Moretti, M., Muruzabal, D., Novak, M., Oliveira, R., Olsen, A.-K., Owiti, N., Pacheco, M., Pandey, A.K., Pfuhler, S., Pourrut, B., Reisinger, K., Rojas, E., Rundén-Pran, E., Sanz-Serrano, J., Shaposhnikov, S., Sipinen, V., Smeets, K., Stopper, H., Teixeira, J.P., Valdiglesias, V., Valverde, M., van Acker, F., van Schooten, F.-J., Vasquez, M., Wentzel, J.F., Wnuk, M., Wouters, A., Žegura, B., Zikmund, T., Langie, S.A.S., Azqueta, A., 2023. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat. Protoc. 18, 929–989. https://doi.org/10.1038/s41596-022-00754-y.

Cook, P.R., Brazell, I.A., Jost, E., 1976. Characterization of nuclear structures containing superhelical DNA. J. Cell Sci. 22, 303–324. https://doi.org/10.1242/jcs.22.2.303.

du Sert, N.P., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The arrive guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 18 https://doi.org/10.1371/journal.pbio.3000410.

Enciso, J.M., Gutzkow, K.B., Brunborg, G., Olsen, A.K., López De Cerain, A., Azqueta, A., 2018. Standardisation of the in vitro comet assay: Influence of lysis time and lysis solution composition on the detection of DNA damage induced by X-rays. Mutagenesis 33, 25–30. https://doi.org/10.1093/mutage/gex039.

Enciso, J.M., Sánchez, O., López de Cerain, A., Azqueta, A., 2015. Does the duration of lysis affect the sensitivity of the in vitro alkaline comet assay? Mutagenesis 30, 21–28. https://doi.org/10.1093/mutage/geu047.

Ersson, C., Möller, L., 2011. The effects on DNA migration of altering parameters in the comet assay protocol such as agarose density, electrophoresis conditions and durations of the enzyme or the alkaline treatments. Mutagenesis 26, 689–695. https://doi.org/10.1093/mutage/ger034.

Ersson, C., Møller, P., Forchhammer, L., Loft, S., Azqueta, A., Godschalk, R.W.L., Van Schooten, F.-J., Jones, G.D.D., Higgins, J.A., Cooke, M.S., Mistry, V., Karbaschi, M., Phillips, D.H., Sozeri, O., Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Stępnik, M., Ferlińska, M., Teixeira, J.P., Costa, S., Corcuera, L.-A., López De Cerain, A., Laffon, B., Valdiglesias, V., Collins, A.R., Möller, L., 2013. An ECVAG inter-laboratory validation study of the comet assay: inter-laboratory and intra-laboratory variations of DNA strand breaks and FPG-sensitive sites in human mononuclear cells. Mutagenesis 28, 279–286. https://doi.org/10.1093/mutage/get001.

Forchhammer, L., Ersson, C., Loft, S., Möller, L., Godschalk, R.W.L., Van Schooten, F.J., Jones, G.D.D., Higgins, J.A., Cooke, M., Mistry, V., Karbaschi, M., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Steëpnik, M., Komorowska, M., Teixeira, J.P., Costa, S., Corcuera, L.A., López De Cerain, A., Laffon, B., Valdiglesias, V., Møller, P., 2012. Inter-laboratory variation in DNA damage using a standard comet assay protocol. Mutagenesis 27, 665–672. https://doi.org/10.1093/mutage/ges032.

Forchhammer, L., Johansson, C., Loft, S., Möller, L., Godschalk, R.W.L., Langie, S.A.S., Jones, G.D.D., Kwok, R.W.L., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Ste, pnik, M., Palus, J., Vogel, U., Wallin, H., Routledge, M.N., Handforth, C., Allione, A., Matullo, G., Teixeira, J.P., Costa, S., Riso, P., Porrini, M., Møller, P., 2010. Variation in the measurement of DNA damage by comet assay measured by the ECVAGy inter-laboratory validation trial. Mutagenesis 25, 113–123. https://doi.org/10.1093/mutage/gep048.

Gajski, G., Žegura, B., Ladeira, C., Novak, M., Sramkova, M., Pourrut, B., Del Bo, C., Milić, M., Gutzkow, K.B., Costa, S., Dusinska, M., Brunborg, G., Collins, A., 2019a. The comet assay in animal models: From bugs to whales - (Part 2 Vertebrates). Mutat. Res Rev. Mutat. Res 781, 130–164. https://doi.org/10.1016/j.mrrev.2019.04.002.

Gajski, G., Žegura, B., Ladeira, C., Pourrut, B., Del Bo, C., Novak, M., Sramkova, M., Milić, M., Gutzkow, K.B., Costa, S., Dusinska, M., Brunborg, G., Collins, A., 2019b. The comet assay in animal models: From bugs to whales - (Part 1 Invertebrates). Mutat. Res Rev. Mutat. Res 779, 82–113. https://doi.org/10.1016/J.MRREV.2019.02.003.

Ge, J., Chow, D.N., Fessler, J.L., Weingeist, D.M., Wood, D.K., Engelward, B.P., 2015. Micropatterned comet assay enables high throughput and sensitive DNA damage quantification. Mutagenesis 30, 11–19. https://doi.org/10.1093/mutage/geu063.

Ge, J., Ngo, L.P., Kaushal, S., Tay, I.J., Thadhani, E., Kay, J.E., Mazzucato, P., Chow, D. N., Fessler, J.L., Weingeist, D.M., Sobol, R.W., Samson, L.D., Floyd, S.R., Engelward, B.P., 2021. CometChip enables parallel analysis of multiple DNA repair activities. DNA Repair (Amst.) 106, 103176. https://doi.org/10.1016/j.dnarep.2021.103176.

Ge, J., Prasongtanakij, S., Wood, D.K., Weingeist, D.M., Fessler, J., Navasummrit, P., Ruchirawat, M., Engelward, B.P., 2014. Cometchip: A high-throughput 96-well platform for measuring DNA damage in microarrayed human cells. J. Vis. Exp. 8–15. https://doi.org/10.3791/50607.

Ge, J., Wood, D.K., Weingeist, D.M., Prasongtanakij, S., Navasumrit, P., Ruchirawat, M., Engelward, B.P., 2013. Standard fluorescent imaging of live cells is highly genotoxic. Cytom. A 83, 552–560. https://doi.org/10.1002/cyto.a.22291.

Gedik, C.M., Collins, A., Dubois, J., Duez, P., Kouegnigan, L., Loft, S., Møller, P., Jensen, A., Poulsen, H., Riis, B., Cadet, J., Douki, T., Ravanat, J.-L., Sauvaigo, S., Dolara, P., Giovannelli, L., Lodovici, M., Guglielmi, F., Olinski, R., Bialkowski, K., Foksinski, M., Gackowski, D., Duračková, Z., Muchová, J., Korytar, P., Sivonová, M., Viña, J., Lloret, A., Sáez, G., Möller, L., Hofer, T., Eriksson, H., 2004. Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. FASEB J. 19, 82–84. https://doi.org/10.1096/fj.04-1767fje.

Guérard, M., Marchand, C., Plappert-Helbig, U., 2014. Influence of experimental conditions on data variability in the liver comet assay. Environ. Mol. Mutagen 55, 114–121. https://doi.org/10.1002/em.21835.

Gutzkow, K.B., Langleite, T.M., Meier, S., Graupner, A., Collins, A.R., Brunborg, G., 2013. High-throughput comet assay using 96 minigels. Mutagenesis 28, 333–340. https://doi.org/10.1093/mutage/get012.

Institute for Health Metrics and Evaluation (IHME), 2020. GBD Compare Data Visualization [WWW Document]. IHME, University of Washington. URL ⟨http://vizhub.healthdata.org/gbd-compare⟩ (accessed 11.13.23).

Jackson, S.P., Bartek, J., 2009. The DNA-damage response in human biology and disease. Nature 461, 1071–1078. https://doi.org/10.1038/nature08467.

Jaye Bix, G., Margarida Ledo, A., Arai, K., Lok, J., Li, P., Li, Y., Xie, L., Huang, T., Zhang, Y., Zhou, J., Qi, B., Wang, X., Chen, Z., 2019. Aging Neurovascular Unit and Potential Role of DNA Damage and Repair in Combating Vascular and Neurodegenerative Disorders. Front Neurosci. 13, 778 https://doi.org/10.3389/fnins.2019.00778.

Johansson, C., Møller, P., Forchhammer, L., Loft, S., Godschalk, R.W.L., Langie, S.A.S., Lumeij, S., Jones, G.D.D., Kwok, R.W.L., Azqueta, A., Phillips, D.H., Sozeri, O., Routledge, M.N., Charlton, A.J., Riso, P., Porrini, M., Allione, A., Matullo, G., Palus, J., Stepnik, M., Collins, A.R., Möller, L., 2010. An ECVAG trial on assessment

of oxidative damage to DNA measured by the comet assay. Mutagenesis 25, 125–132. https://doi.org/10.1093/mutage/gep055.
Karbaschi, M., Cooke, M.S., 2014. Novel method for the high-throughput processing of slides for the comet assay. Sci. Rep. 4, 7200 https://doi.org/10.1038/srep07200.
Karbaschi, M., Ji, Y., Abdulwahed, A.M.S., Alohaly, A., Bedoya, J.F., Burke, S.L., Boulos, T.M., Tempest, H.G., Cooke, M.S., 2019. Evaluation of the major steps in the conventional protocol for the Alkaline comet assay. Int J. Mol. Sci. 20, 6072. https://doi.org/10.3390/IJMS20236072.
Kulkarni, A., Wilson, D.M., 2008. The Involvement of DNA-damage and -repair defects in neurological dysfunction. Am. J. Hum. Genet 82, 539–566. https://doi.org/10.1016/J.AJHG.2008.01.009.
Lindahl, T., Barnes, D.E., 2000. Repair of endogenous DNA damage. Cold Spring Harb. Symp. Quant. Biol. 65, 127–133. https://doi.org/10.1101/SQB.2000.65.127.
Mercer, J., Mahmoudi, M., Bennett, M., 2007. DNA damage, p53, apoptosis and vascular disease. Mutat. Res Fund. Mol. Mech. Mutagen 621, 75–86. https://doi.org/10.1016/J.MRFMMM.2007.02.011.
Milić, M., Ceppi, M., Bruzzone, M., Azqueta, A., Brunborg, G., Godschalk, R., Koppen, G., Langie, S., Møller, P., Teixeira, J.P., Alija, A., Anderson, D., Andrade, V., Andreoli, C., Asllani, F., Bangkoglu, E.E., Barančoková, M., Basaran, N., Boutet-Robinet, E., Buschini, A., Cavallo, D., Costa Pereira, C., Costa, C., Costa, S., Da Silva, J., Del Bo´, C., Dimitrijević Srećković, V., Djelić, N., Dobrzyńska, M., Duračková, Z., Dvořáková, M., Gajski, G., Galati, S., García Lima, O., Giovannelli, L., Goroshinskaya, I.A., Grindel, A., Gutzkow, K.B., Hernández, A., Hernández, C., Holven, K.B., Ibero-Baraibar, I., Ottestad, I., Kadioglu, E., Kažimirová, A., Kuznetsova, E., Ladeira, C., Laffon, B., Lamonaca, P., Lebailly, P., Louro, H., Mandina Cardoso, T., Marcon, F., Marcos, R., Moretti, M., Moretti, S., Najafzadeh, M., Nemeth, Z., Neri, M., Novotna, B., Orlow, I., Paduchova, Z., Pastor, S., Perdry, H., Spremo-Potparević, B., Ramadhani, D., Riso, P., Rohr, P., Rojas, E., Rossner, P., Safar, A., Sardas, S., Silva, M.J., Sirota, N., Smolkova, B., Staruchova, M., Stetina, R., Stopper, H., Surikova, E.I., Ulven, S.M., Ursini, C.L., Valdiglesias, V., Valverde, M., Vodicka, P., Volkovova, K., Wagner, K.H., Živković, L., Dušinská, M., Collins, A.R., Bonassi, S., 2021. The hCOMET project: International database comparison of results with the comet assay in human biomonitoring. Baseline frequency of DNA damage and effect of main confounders. Mutat. Res Rev. Mutat. Res 787, 108371. https://doi.org/10.1016/J.MRREV.2021.108371.
Møller, P., 2018. The comet assay: ready for 30 more years. Mutagenesis 33, 1–7. https://doi.org/10.1093/mutage/gex046.
Møller, P., Azqueta, A., Boutet-Robinet, E., Koppen, G., Bonassi, S., Gajski, G., Costa, S., Teixeira, Paulo, Costa Pereira, J., Dusinska, C., Godschalk, M., Brunborg, R., Langie, S.A.S, G., 2020a. Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results. Nat. Protoc. 15, 3817–3826. https://doi.org/10.1038/s41596-020-0398-1.
Møller, P., Azqueta, A., Collia, M., Bakuradze, T., Richling, E., Bankoglu, E.E., Stopper, H., Bastos, V.C., Langie, S.A.S., Jensen, A., Ristori, S., Scavone, F., Giovannelli, L., Wojewódzka, M., Kruszewski, M., Valdiglesias, V., Laffon, B., Costa, C., Costa, S., Teixeira, J.P., Marino, M., Del Bo, C., Riso, P., Zhang, C., Shaposhnikov, S., Collins, A., 2023a. Inter-laboratory variation in measurement of DNA damage by the alkaline comet assay in the hCOMET ring trial. Mutagenesis. https://doi.org/10.1093/MUTAGE/GEAD014.
Møller, P., Azqueta, A., Rodriguez-Garraus, A., Bakuradze, T., Richling, E., Bankoglu, E. E., Stopper, H., Bastos, V.C., Langie, S.A.S., Jensen, A., Ristori, S., Scavone, F., Giovannelli, L., Wojewódzka, M., 2023b. Long-term cryopreservation of potassium bromate positive assay controls for measurement of oxidatively damaged DNA by the Fpg-modified comet assay: results from the hCOMET ring trial. Mutagen. XX, 1–9. https://doi.org/10.1093/mutage/gead020.
Møller, P., Eyluel Bankoglu, E., Stopper, H., Giovannelli, L., Ladeira, C., Koppen, G., Gajski, G., Collins, A., Valdiglesias, V., Laffon, B., Boutet-Robinet, E., Perdry, H., Del Bo', 15, C., Langie, S.A.S., Dusinska, M., Azqueta, A., 2021. Collection and storage of human white blood cells for analysis of DNA damage and repair activity using the comet assay in molecular epidemiology studies. Mutagenesis 36, 193–211. https://doi.org/10.1093/mutage/geab012.
Møller, P., Muruzabal, D., Bakuradze, T., Richling, E., Bankoglu, E.E., Stopper, H., Langie, S.A.S., Azqueta, A., Jensen, A., Scavone, F., Giovannelli, L., Wojewódzka, M., Kruszewski, M., Valdiglesias, V., Laffon, B., Costa, C., Costa, S., Teixeira, J.P., Marino, M., Del Bo' 15, C., Riso, P., Shaposhnikov, S., Collins, A., 2020b. Potassium bromate as positive assay control for the Fpg-modified comet assay. Mutagenesis 35, 341–347. https://doi.org/10.1093/mutage/geaa011.
Muruzabal, D., Collins, A., Azqueta, A., 2021a. The enzyme-modified comet assay: Past, present and future. Food Chem. Toxicol. 147, 111865 https://doi.org/10.1016/J.FCT.2020.111865.
Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., Olsen, A.K., López de Cerain, A., Vettorazzi, A., Azqueta, A., 2021b. Validation of the in vitro comet assay for DNA cross-links and altered bases detection. Arch. Toxicol. 95, 2825–2838. https://doi.org/10.1007/S00204-021-03102-3.
Ngo, L.P., Owiti, N.A., Swartz, C., Winters, J., Su, Y., Ge, J., Xiong, A., Han, J., Recio, L., Samson, L.D., Engelward, B.P., 2021. Sensitive Comet Chip assay for screening potentially carcinogenic DNA adducts by trapping DNA repair intermediates. Nucleic Acids Res 48, e13. https://doi.org/10.1093/NAR/GKZ1077.
Organisation for Economic Co-operation and Development (OECD), 2016. Test No. 489: In Vivo Mammalian Alkaline Comet Assay, OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264264885-en.
Ostling, O., Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem. Biophys. Res. Commun. 123, 291–298. https://doi.org/10.1016/0006-291X(84)90411-X.
Owiti, N.A., Corrigan, J.J., Pribyl, L.J., Kay, J.E., Engelward, B.P., 2022. Novel In Vivo CometChip Reveals NDMA-Induced DNA Damage and Repair in Multiple Mouse Tissues. Int J. Mol. Sci. 23, 11776. https://doi.org/10.3390/ijms231911776.
Phillips, D.H., Arlt, V.M., 2009. Genotoxicity: damage to DNA and its consequences. Mol. Clin. Environ. Toxicol. 1, 87–110. https://doi.org/10.1007/978-3-7643-8336-7_4.
Rass, U., Ahel, I., West, S.C., 2007. Defective DNA repair and neurodegenerative disease. Cell 130, 991–1004. https://doi.org/10.1016/J.CELL.2007.08.043.
Rosenthal, D.S., Kuo, L.-W., Seagrave, S.L., Soni, V., Islam, N., Minsky, G., Dussan-Cuellar, L., Ell, B., Simbulan-Rosenthal, C.M., Sykora, P., 2023. Skin Immuno-CometChip in 3D vs. 2D Cultures to Screen Topical Toxins and Skin-Specific Cytochrome Inducers. Genes (Basel) 14, 630. https://doi.org/10.3390/GENES14030630.
Schumacher, B., Garinis, G.A., Hoeijmakers, J.H.J., 2008. Age to survive: DNA damage and aging. Trends Genet 24, 77–85. https://doi.org/10.1016/J.TIG.2007.11.004.
Seo, J.E., He, X., Muskhelishvili, L., Malhi, P., Mei, N., Manjanatha, M., Bryant, M., Zhou, T., Robison, T., Guo, X., 2022. Evaluation of an in vitro three-dimensional HepaRG spheroid model for genotoxicity testing using the high-throughput CometChip platform. ALTEX 39, 583–604. https://doi.org/10.14573/altex.2201121.
Shaposhnikov, S., Azqueta, A., Henriksson, S., Meier, S., Gaivão, I., Huskisson, N.H., Smart, A., Brunborg, G., Nilsson, M., Collins, A.R., 2010. Twelve-gel slide format optimised for comet assay and fluorescent in situ hybridisation. Toxicol. Lett. 195, 31–34. https://doi.org/10.1016/J.TOXLET.2010.02.017.
Shaposhnikov, S., Collins, A., 2017. Twelve-gel comet assay format for quick examination of DNA damage and repair. Methods Mol. Biol. 1644, 181–186. https://doi.org/10.1007/978-1-4939-7187-9_16.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191. https://doi.org/10.1016/0014-4827(88)90265-0.
Sykora, P., Witt, K.L., Revanna, P., Smith-Roe, S.L., Dismukes, J., Lloyd, D.G., Engelward, B.P., Sobol, R.W., 2018. Next generation high throughput DNA damage detection platform for genotoxic compound screening. Sci. Rep. 8, 2771 https://doi.org/10.1038/s41598-018-20995-w.
Townsend, T.A., Parrish, M.C., Engelward, B.P., Manjanatha, M.G., 2017. The Development and Validation of EpiComet-Chip, a Modified High-Throughput Comet Assay for the Assessment of DNA Methylation Status. Environ. Mol. Mutagen 58, 508–521. https://doi.org/10.1002/em.22101.
Tubbs, A., Nussenzweig, A., 2017. Endogenous DNA Damage as a Source of Genomic Instability in Cancer. Cells 168, 644–656. https://doi.org/10.1016/J.CELL.2017.01.002.
Vodenkova, S., Azqueta, A., Collins, A., Dusinska, M., Gaivão, I., Møller, P., Opattova, A., Vodicka, P., Godschalk, R.W.L., Langie, S.A.S., 2020. An optimized comet-based in vitro DNA repair assay to assess base and nucleotide excision repair activity. Nat. Protoc. 15, 3844–3878. https://doi.org/10.1038/s41596-020-0401-x.
Watson, C., Ge, J., Cohen, J., Pyrgiotakis, G., Engelward, B.P., Demokritou, P., 2014. High-throughput screening platform for engineered nanoparticle-mediated genotoxicity using cometchip technology. ACS Nano 8, 2118–2133. https://doi.org/10.1021/nn404871p.
Weingeist, D.M., Ge, J., Wood, D.K., Mutamba, J.T., Huang, Q., Rowland, E.A., Yaffe, M. B., Floyd, S., Engelward, B.P., 2013. Single-cell microarray enables high-throughput evaluation of DNA double-strand breaks and DNA repair inhibitors. Cell Cycle 12, 907–915. https://doi.org/10.4161/cc.23880.
Wood, D.K., Weingeist, D.M., Bhatia, S.N., Engelward, B.P., 2010. Single cell trapping and DNA damage analysis using microwell arrays. Proc. Natl. Acad. Sci. USA 107, 10008–10013. https://doi.org/10.1073/pnas.1004056107.
Xiong, A., Kaushal, S., Tay, I.J., Engelward, B.P., Han, J., Preiser, P.R., 2021. MalariaCometChip for high-throughput quantification of DNA damage in Plasmodium falciparum. STAR Protoc. 2, 100797 https://doi.org/10.1016/j.xpro.2021.100797.